STUDIES WITH PLANT CELL CULTURES OF <u>TRIPTERYGIUM WILFORDII</u>.

ISOLATION OF METABOLITES AND BIOTRANSFORMATION STUDIES

by

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Abstract

This thesis is concerned with the use of plant cell cultures in combination with organic synthesis to provide efficient routes for the synthesis of novel diterpene analogs of triptolide (Tl) and tripdiolide (Td), the two most active principles of the Chinese medicinal plant *Tripterygium wilfordii*. The present study is also devoted to providing a better understanding with regard to the biosynthesis of related diterpenes in the plant cell culture line of *T. wilfordii* designated as TRP4a.

The initial phases of the research involved isolation and identification of diterpene metabolites from TRP4a cell cultures. Twenty nine compounds (28 diterpenes) were isolated, of which 26 (including 14 new natural products) were isolated for the first time from this cell culture line. Based on the structural relationships revealed within this series, a biosynthetic pathway from dehydroabietane to Tl and Td is proposed.

The synthetic studies involved initial synthesis of isodehydroabietenolide from the readily available dehydroabietic acid and subsequent ring C functionalization to provide a series of ring C "activated" analogs such as isotriptophenolide, triptophenolide and demethyl isoneotriptophenolide. A new, efficient sequence from isodehydroabietenolide to triptophenolide was developed and a synthesis of demethyl isoneotriptophenolide was completed.

The later stages of the present studies were concerned with biotransformation of the various synthetic precursors in both TRP4a whole cell systems and crude enzyme preparations (cell free extract, or CFE). Incubation of isotriptophenolide in TRP4a cell cultures produced two novel epoxy dienones, $(7,8)\beta$-epoxy-19-hydroxy-12-oxo-18$(4\rightarrow3)abeo$-abieta-3,9(11),13-trien-18-oic acid lactone, and $(7,8)\alpha$-epoxy-19-hydroxy-12-oxo-18$(4\rightarrow3)abeo$-abieta-3,9(11),13-trien-18-oic acid lactone, along with additional hydroxylated compounds. This is the first example in which a "*para*-alkylated" phenol is enzymatically converted to the corresponding *para*-epoxy dienone in one step. Older cell cultures (21 day) tend to give higher

yields of these epoxides. Further biotransformation results indicated a possible involvement of a quinone methide intermediate in the formation of these epoxides. Biotransformation of isotriptophenolide with CFE preparations from TRP4a cell cultures gave comparable results as the whole cell system except that the biotransformation proceeded much faster. Certain factors in relation to the yield of the epoxides were also studied.

Incubation of triptophenolide in whole cell cultures and CFEs showed only a low level of biotransformation in comparison with isotriptophenolide. A large scale experiment did allow the isolation of some hydroxylated products in low yields. These compounds are structurally comparable with those obtained from biotransformation of isotriptophenolide.

Biotransformation of demethyl isoneotriptophenolide in whole cell cultures showed rapid consumption of the precursor. The major product isolated was the hydroxy quinone, 12,19-Dihydroxy-11,14-dioxo-18(4→3)*abeo*-abieta-3,8,12-trien-18-oic acid lactone.

Biotransformation of the above ring C "activated" precursors has demonstrated that the position and number of the hydroxy groups in ring C are critical to the fate of the biotransformation.

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List of Abbreviations

$[\alpha]_D^t$	specific rotation recorded at t°C using sodium D-line
AB_q	AB quartet
Ac	acetyl
ATCC	American Type Culture Collection
atm	atmosphere
B-5	standard cell culture medium developed by Gamborg and Eveleigh
br	broad
brine	saturated sodium chloride solution
Bu	butyl
c	concentration (g/100 mL)
°C	degree Celsius
c. c.	column chromatography
CFE	cell free extract
CH	cell homogenate
cm^{-1}	wave number
COSY	^{1}H-^{1}H 2-dimensional COrrelated NMR SpectroscopY
δ	chemical shift
d	doublet
dd	doublet of doublets
ddd	doublet of doublet of doublets
dddd	doublet of doublet of doublet of doublets
dec	decomposition
Δ	heat
Δν	chemical shift difference
DDQ	2,3-dichloro-5,6-dicyano-1,4-benzoquinone
DINTP	demethyl isoneotriptophenolide
DMF	dimethylformamide
DMP	3,5-dimethylpyrazole
DMSO	dimethylsulphoxide
Et	ethyl
ED_{50}	median effective dose
FMN	flavin mononucleotide
Fr.	Fraction
g	gram

g	gravitation constant
GTW	a multi-glycoside extract from the plant *Tripterygium wilfordii*
h	hour
[H]	reduction
hexanes	generally a mixture of several isomers of hexane (C_6H_{14}), predominantly n-hexane, and methylcyclopentane (C_6H_{12})
HMBC	Heteronuclear Multiple Bond Connectivity
HMPA	hexamethylphosphoramide
HMQC	Heteronuclear Multiple-Quantum Coherence
hv	light radiation
HPLC	high pressure (performance) liquid chromatography
HRMS	high resolution mass spectrum
Hz	hertz
IR	infrared
J	coupling constant
KB	a tissue culture cell line derived from human carcinoma of the nasopharynx
λ	wavelength
L	liter
L-1210	a tissue culture cell line derived from mouse leukemia
LAH	lithium aluminum hydride
LD_{50}	median lethal dose
LDA	lithium diisopropyl amide
log ε	the log of extinction coefficient
LTA	lead tetraacetate
LRMS (or MS)	low resolution mass spectrum
m	multiplet
M	molar
M^+	molecular ion
m-CPBA	*meta*-chloroperbenzoic acid
Me	methyl
mg	milligram
MHz	megahertz
min	minute
mL	milliliter
mmol	millimole

mp	melting point
$MSNA_{0.5}K_{0.5}$	MS medium of Murashige and Skoog supplemented with naphthaleneacetic acid (NA, 0.5 mg/L) and kinetin (K, 0.5 mg/L)
μ	micro (10^{-6})
m/z	mass to charge ratio
ν	frequency
NBS	N-bromosuccinimide
n_D	refractive index
nm	nanometre
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
[O]	oxidation
P-388	a tissue culture cell line derived from mouse leukemia
PCC	pyridinium chlorochromate
Ph	phenyl
ppm	parts per million
PRD_2Co_{100}	PRL-4 medium of Gamborg and Eveleigh supplemented with 2,4-dichlorophenoxyacetic acid (D, 2 mg/L) and coconut milk (Co, 100 mL/L)
PRI_2Co_{100}	PRL-4 medium of Gamborg and Eveleigh supplemented with indole-3-acetic acid (I, 2 mg/L) and coconut milk (Co, 100 mL/L)
PRL-4	standard cell culture medium developed by Gamborg and Eveleigh
RCF	relative centrifugation force
RCP	resuspended cell pellet
rpm	revolutions per minute
r.t.	room temperature
s	singlet
sept	septet
sept d	septet of doublets
SINEPT	selective insensitive nucleus enhancement by polarization transfer
t	triplet
Td	tripdiolide
TFA	trifluoroacetic acid
TFAA	trifluoroacetic anhydride
THF	tetrahydrofuran
Tl	triptolide

TLC	thin layer chromatography
TMS	tetramethylsilane
Tosyl (Ts)	*para*-toluenesulfonyl (the abbreviation tosyl is employed in the text while Ts is employed in structures)
Tosylate	*para*-toluenesulfonate
TRP4a	a cell line of plant cell culture developed from *Tripterygium wilfordii*
UV	ultraviolet

Notes

The numbering system used throughout this thesis is that used by contemporary natural products chemists and is illustrated below.
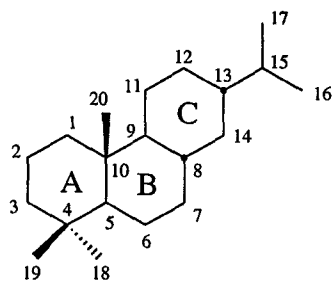
abietane

The abietane skeleton is designated as 18-nor if C18 is absent or if a double bond is present at C3 or C4. The rearranged abietane skeleton is designated as 18(4→3)*abeo*.
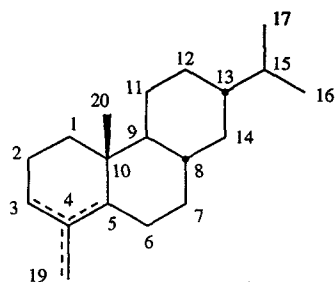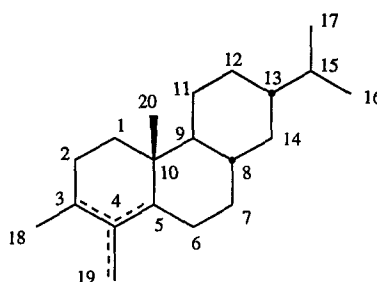
18-nor   18(4→3)*abeo*

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CHAPTER 1   GENERAL INTRODUCTION

1.1   The Plant *Tripterygium wilfordii* and Related Species

Tripterygium wilfordii Hook. f. (Hooker filius) belongs to the genus *Tripterygium*, a group of woody vines and shrubs of the Celastraceae family. *Tripterygium* has four species in China:[1-3] *Tripterygium wilfordii* Hook. f., *Tripterygium hypoglaucum* (Level.) Hutch, *Tripterygium forrestii* Dicls, and *Tripterygium regelii* Sprague et Takeda.

T. wilfordii is usually found in large mountainous areas of south-eastern and southern China, but mainly in south-eastern China.[4,5] As a wild plant, it grows in the shady, humid woods and bushes on the hills, in the valleys and near the streams. It is a perennial twining vine of about 2-3 meters in height. The reddish brown twigs have longitudinal ridges and are covered with fine, brown hairs. Its egg-shaped leaves are alternately grown with small serrated edges. The plant blooms in May and June. The small white flowers have five petals, five stamens and a triangular ovary which later develops into a seed that has three jutting longitudinal wings.[4] The plant *T. wilfordii* is named Lei Gong Teng (Thunder God vine) in traditional Chinese medicine. It is also called Mang Cao (Rank grass), San Leng Hua (Three-Wing flower) or Huang Teng (Yellow vine) among the local people, apparently based on the physical appearance of this plant.

T. hypoglaucum appears in the same areas as *T. wilfordii*, but mainly in southern and south-western China. It is distinguished from *T. wilfordii* by virtue of larger leaves and flowers, and the leaves have white powder on the back.[5] *T. forrestii* also grows in south-western China,[3] but this species was not recorded in the early botanical books.[5,6] *T. regelii*, is mainly found in the mountainous areas or near the forests in the north-eastern part of China and Japan. It is also called Dong Bei Lei Gong Teng (North-Eastern China Thunder God vine) or Hei Man (Black vine).

Due to its vast geographical distribution and potent pharmacological activities, *T. wilfordii* is the predominant species of *Tripterygium* that is utilized in Chinese herbal medicine, and of the four species it is also the most studied. *T. hypoglaucum* is also commonly used in Chinese folk medicine for treatment of some diseases.

1.2 *T. wilfordii* as Traditional Chinese Medicine: History, Current Clinical Applications and Interests

The traditional Chinese medicine, or Chinese materia medica, classifies medicinal substances into three general groups: those which originate from either botanical, zoological or mineral sources. Because those obtained from botanical sources constitute the majority of the traditional Chinese medicine, traditional Chinese medicine is also referred to as Chinese herbal medicine.

The use of herbal medicine in China to treat illness and disease can be traced back thousands of years. Some recordings of herbal medicines and their medicinal properties have been obtained from inscriptions found on unearthed bones and tortoise shells that date back to about 16th-11th century B.C.. However, little is known about the practice of medicine prior to the writing of a book *Huang Di Nei Jing* (*Yellow Emperor's Inner Classic*, also known as *Inner Classic*), compiled by unknown authors between 200 B.C. and 100 A.D..[7,8] This book is the oldest major Chinese medical text extant, which laid the theoretical and philosophical foundations for traditional Chinese medicine. In addition to its emphasis on theory and philosophy, this book also includes 12 prescriptions, with a total of 28 medicinal substances noted. However, from the perspective of herbal medicine, the *Inner Classic* is not a particularly important document. A more specific, systematic and inclusive work, though still quite primitive from today's point of view, is known as *Shen Nong Ben Cao Jing* (*Divine Husbandman's Classic of the Materia Medica*, or *The Saint Peasant's Scripture of Materia Medica*). This work was compiled by unknown authors in the Later Han dynasty (25-220

A.D.). Legend ascribes the authorship of this book to the mythical figure Shen Nong (Divine Husbandman, or Saint Peasant), who, in addition to introducing agriculture and animal husbandry, was also believed to have tasted "the hundred herbs" himself and thus, is viewed as the founder of Chinese herbal medicine. The writing of this book is generally regarded as the start of the historical tradition of Chinese herbal knowledge. In this book, there are 364 entries of medicinal substances of botanical (252 entries), zoological (67 entries) and mineral (45 entries) origins.[8]

One of the herbs recorded in *Shen Nong Ben Cao Jing* is *T. wilfordii* (as Rank grass). It was noted that *T. wilfordii* could be used for the treatment of fever, chills, edema and carbuncle. In the early times, Chinese farmers used the powdered root of this plant (the most toxic part) to protect their crops from chewing insects, which earned the plant a name as Cai Chong Yao, meaning a pesticide for vegetable insects. There are still studies on its application as a natural means of pest control in recent years.[9,10] It was also used as folk medicine for the treatment of cancer and inflammatory diseases.[11]

Since the late 1960's, the Chinese medical community has increasingly used this plant's extracts to treat rheumatoid arthritis, chronic hepatitis, chronic nephritis, ankylosing spondylitis and various skin disorders with quite encouraging results.[12-14]

In Chinese folk medicine the plant is used directly with little preparation. For example, in the treatment of rheumatoid arthritis or skin rashes, the fresh roots and leaves are simply smashed and ground up, and the resulting paste is then applied to troubled areas. In clinical treatments of rheumatoid arthritis and lepra reactions, a decoction is used. Plants are usually collected in summer or early autumn. Once dried, the root xylem is obtained after removal of two layers of cortices which are suspected to contain most of the plant's toxic substances. Isolated xylem is then cut into small pieces and gently boiled in water for several hours. The water extract, or decoction, may then be administered orally for a certain period of time, and the symptoms are usually soon alleviated.[4]

Since the late 1970's, the most common form of *T. wilfordii* preparations used in the clinical treatment of rheumatoid arthritis and some skin disorders are tablets derived from the refined extract or the so-called total multi-glycosides of *T. wilfordii* (GTW). The dried root xylem is cut into small pieces and extracted with water. The water extract is then extracted with chloroform, and the resulting chloroform solution is concentrated, and then column chromatographed to produce the so-called GTW. About 25 g of xylem yields 1 mg of GTW and each tablet contains 10 mg of the GTW. The term "multi-glycosides" may not be a technically correct term for this preparation. It only implies that the prepared form contains a number of glycosides in addition to other constituents, and does not mean that the active principle(s) must necessarily be a glycoside(s).[11]

Although some American agricultural researchers came across this plant while searching for insecticidal alkaloids in the 1950's (*vide infra*), Western interests in this plant were not aroused until 1972. In the study of the antileukemic activity of maytansine, an active principle from *Maytenus ovatus*, a plant of the same Celastraceae family, Kupchan and co-workers isolated diterpenes triptolide (Tl, **1**) and tripdiolide (Td, **2**) from an extract of *T. wilfordii*. Triptolide and tripdiolide showed strong antileukemia and antitumor activities in pharmacological study.[15] Since then, broader efforts have been made in research related to this plant.
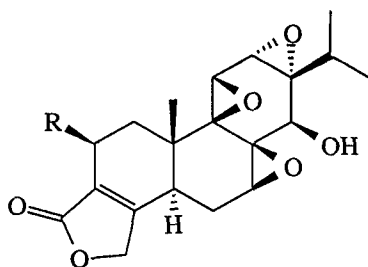
R = H,   triptolide (**1**)

R = OH, tripdiolide (**2**)

In recent years in the course of treatment of rheumatoid arthritis, dermatoses and during the ongoing evaluation of its general toxicity, GTW and some extracts of *T. wilfordii* have been found to have immunosuppressive activity.[12] Additionally, reversible antifertility activity in

male rats and in men have been observed after oral administration at a dose level that shows no apparent side effects or signs of toxicity.[11] This finding, though still under study, may provide an alternative for male contraception, and thus has triggered enormous research interests in China. Most recently, some constituents isolated from this plant have been found to show anti-HIV activity.[16,17]

T. wilfordii, a medicinal plant used as an ancient remedy for thousands of years in China is now attracting greater attention not only in China, but also in other parts of the world.

1.3   Phytochemistry of *T. wilfordii* and Related Species

As a result of the wide pharmacological activities *Tripterygium* plants have shown, isolation of the active constituents from these plants started in the early 1930's in China. Chou and Mei isolated a red triterpene, named as tripterine, from the petroleum ether extract of the root bark.[18] The red triterpene later proved to be identical with celastrol isolated from *Celastrus scandens* L by the American scientist Gisvold.[19,20] In searching for the insecticidal components from the root of *T. wilfordii*, Acree and Haller reported the isolation of an alkaloid, wilfordine, in 1950.[21] This alkaloid was soon proved to be a mixture by Beroza,[22] and he isolated several more in the following years (*vide infra*). The most interesting discovery was made by Kupchan and co-workers in 1972, when the antileukemia and antitumor diterpenes, Tl (1) and Td (2), were isolated. In recent years, the attention paid to the immunosuppressive and antifertility activities of *Tripterygium* plants has generated even greater interest, especially in China, in the isolation of active components from these plants. So far, about 140 natural products, most of them alkaloids, sesquiterpenoids, triterpenoids and diterpenoids, have been isolated from *Tripterygium* plants.

Alkaloids

In 1951 and 1952, Beroza isolated five insecticidal alkaloids, wilforine, wilfordine, wilforgine, wilfortrine and wilforzine, from the root of *T. wilfordii*.[23-25] Since then, a total of fourteen macrocyclic lactone alkaloids (Table 1.1) and three spermidine alkaloids have been isolated from *Tripterygium* plants (Table 1.2).
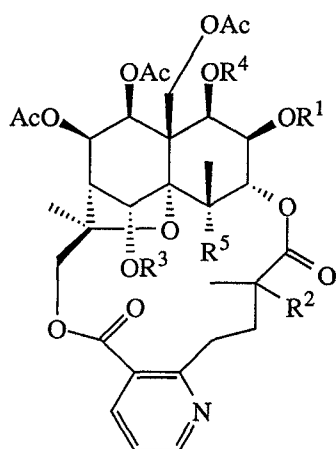
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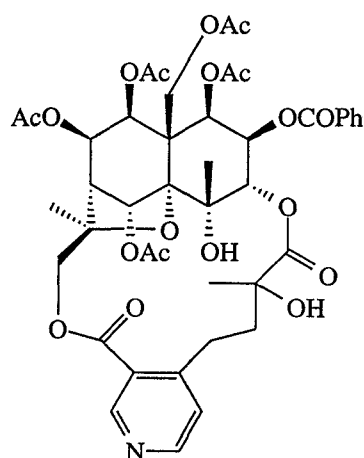
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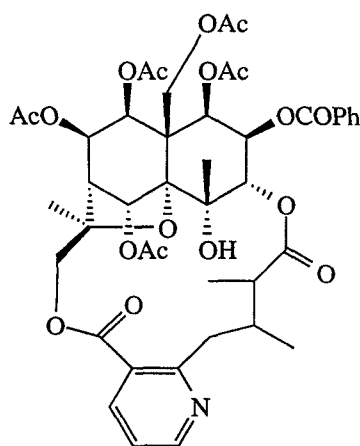
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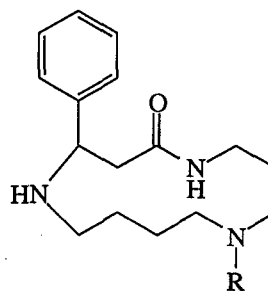
17   R = *trans* -PhCH=CHO

18   R = β-furanoyl

19   R = PhCO

Table 1.1   Macrocyclic Lactone Alkaloids from *Tripterygium* Species

	Compound	R^1	R^2	R^3	R^4	R^5	Mol. formula	Species
3	wilfordine	benzoyl	OH	Ac	Ac	OH	$C_{43}H_{49}NO_{19}$	TW,[23] TH[26]
4	wilforine	benzoyl	H	Ac	Ac	OH	$C_{43}H_{49}NO_{18}$	TW,[23] TH[27]
5	wilforgine	β-furanoyl	H	Ac	Ac	OH	$C_{41}H_{47}NO_{19}$	TW,[24] TH[26]
6	wilfortrine	β-furanoyl	OH	Ac	Ac	OH	$C_{41}H_{47}NO_{20}$	TW,[24] TH[26]
7	wilforzine	benzoyl	H	H	Ac	OH	$C_{41}H_{47}NO_{17}$	TW[25]
8	wilforidine	H	OH	Ac	Ac	OH	$C_{36}H_{45}NO_{18}$	TW[28]
9	wilformine (euonine)	Ac	H	Ac	Ac	OH	$C_{38}H_{47}NO_{18}$	TW[29]
10	wilfornine	nicotinoyl	H	Ac	Ac	OH	$C_{42}H_{48}N_2O_{18}$	TW[30]
11	wilforjine	H	H	Ac	Ac	OH	$C_{36}H_{45}NO_{17}$	TW[31]
12	1-desacetyl-wilfordine	benzoyl	OH	Ac	H	OH	$C_{41}H_{47}NO_{18}$	TW[32]
13	1-desacetyl-wilfortrine	β-furanoyl	H	Ac	H	OH	$C_{39}H_{45}NO_{19}$	TW[32]
14	neowilforine	benzoyl	H	Ac	Ac	H	$C_{43}H_{49}NO_{17}$	TW[33]
15	isowilfordine						$C_{43}H_{49}NO_{19}$	TW[34]
16	forrestine						$C_{43}H_{49}NO_{19}$	TF[35]

TW = *T. wilfordii*; TH = *T. hypoglaucum*; TF = *T. forrestii*.

Table 1.2   Spermidine Alkaloids from *Tripterygium* Species

	Compound	R	Mol. formula	Species
17	celacinnine	*trans*-PhCH=CHCO	$C_{25}H_{31}N_3O_2$	TW[36,37]
18	celafurine	β-furanoyl	$C_{21}H_{27}N_3O_3$	TW[36,37]
19	celabenzine	benzoyl	$C_{23}H_{29}N_3O_2$	TW[36,37]

TW = *T. wilfordii*

Sesquiterpenoids

Since the first isolation of sesquiterpene polyesters from *T. wilfordii* in 1987 by Y. Takaishi,[38] more than forty sesquiterpenes of these types have been isolated. Most of them were obtained from *T. wilfordii* var. *regelii* by Takaishi's group. These types of sesquiterpene polyesters contain a dihydroagarofuran skeleton (Table 1.3).
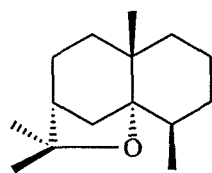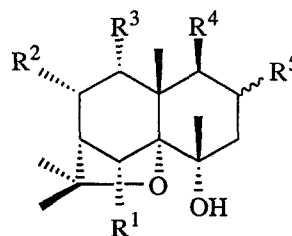
dihydroagarofuran skeleton   **20-24**

Table 1.3   Sesquiterpene Polyesters from *Tripterygium* Species[3]

	Compound	R^1	R^2	R^3	R^4	R^5	Mol. formula	Species
20	triptofordin A	H	H	OCin	Bz	H	$C_{31}H_{36}O_6$	TW v.R[38]
21	triptofordin B	OH	H	OBz	Bz	H	$C_{29}H_{34}O_7$	TW v.R[38]
22	triptofordin C-1	OAc	OBz	OBz	Ac	=O	$C_{33}H_{36}O_{11}$	TW v.R[38]
23	triptofordin C-2	OAc	OBz	OBz	Ac	β-OH	$C_{33}H_{34}O_{11}$	TW v.R[38]
24	regilidine	ONic	H	OBz	Bz	H	$C_{35}H_{37}NO_8$	TR[39]

TW v. R = *T. wilfordii* var. *regelii*; TR = *T. regelii*.

Ac = acetyl; Bz = benzoyl; Cin = *trans*-cinnamoyl; Nic = nicotinoyl.
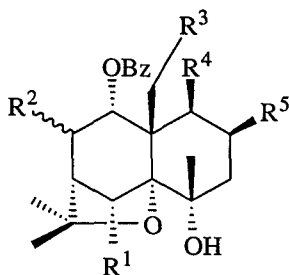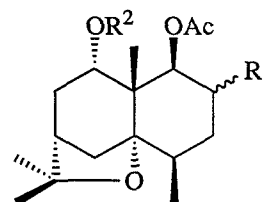
25-33   **34-43**

Table 1.3   Sesquiterpene Polyesters from *Tripterygium* Species (continued)

	Compound	R^1	R^2	R^3	R^4	R^5	Mol. formula	Species
25	triptofordin D-1	OAc	=O	OAc	Cin	H	$C_{35}H_{38}O_{11}$	TW v.R^{40}
26	triptofordin D-2	OAc	β-OAc	OAc	Cin	H	$C_{37}H_{42}O_{12}$	TW v.R^{40}
27	triptofordin E	OAc	=O	OAc	Bz	β-OAc	$C_{35}H_{38}O_{13}$	TW v.R^{40}
28	triptofordin F-1	OH	α-OAc	OAC	Cin	β-OAc	$C_{37}H_{42}O_{13}$	TW v.R^{41}
29	triptofordin F-2	OH	α-OBz	OAc	Ac	β-OAc	$C_{35}H_{40}O_{13}$	TW v.R^{41}
30	triptofordin F-3	OAc	α-OAc	OAc	Bz	β-OAc	$C_{37}H_{42}O_{14}$	TW v.R^{41}
31	triptofordin F-4	OH	α-OH	OAc	Cin	β-OAc	$C_{35}H_{40}O_{12}$	TW v.R^{41}
32	triptofordinine A-1	OAc	β-ONic	OAc	Cin	H	$C_{41}H_{43}O_{12}$	TW v.R^{42}
33	triptofordinine A-2	OAc	β-ONic	OAc	c-Cin	H	$C_{41}H_{43}O_{12}$	TW v.R^{42}

TW v. R = *T. wilfordii* var. *regelii*; TR = *T. regelii*.

Ac = acetyl; Bz = benzoyl; Cin = *trans*-cinnamoyl; Nic = nicotinoyl; c-Cin = *cis*-cinnamoyl.

Table 1.3   Sesquiterpene Polyesters from *Tripterygium* Species (continued)

	Compound	R^1	R^2	Mol. formula	Species
34	triptogelin E-1	β-O(2-MeBu)	Bz	$C_{29}H_{40}O_7$	TW v. R^{43}
35	triptogelin E-2	β-OAc	Bz	$C_{26}H_{34}O_7$	TW v. R^{43}
36	triptogelin E-3	β-O(2-MeBu)	Cin	$C_{31}H_{42}O_7$	TW v. R^{43}
37	triptogelin E-4	=O	Bz	$C_{24}H_{30}O_6$	TW v. R^{43}
38	triptogelin E-5	β-OCO(CH$_2$)$_2$CH$_3$	Bz	$C_{28}H_{38}O_7$	TW v. R^{44}
39	triptogelin E-6	β-OCOCH(CH$_3$)$_2$	Bz	$C_{28}H_{38}O_7$	TW v. R^{44}
40	triptogelin E-7	β-OCO(CH$_2$)$_2$CH$_3$	Cin	$C_{30}H_{40}O_7$	TW v. R^{44}
41	triptogelin E-8	β-OCOCH(CH$_3$)$_2$	Cin	$C_{30}H_{40}O_7$	TW v. R^{44}
42	triptogelin G-1	H	Cin	$C_{26}H_{34}O_5$	TW v. R^{43}
43	triptogelin G-2	H	Bz	$C_{24}H_{32}O_5$	TW v. R^{44}

TW v. R = *T. wilfordii* var. *regelii*.

β-O(2-MeBu) = β-OCOCHCH$_3$CH$_2$CH$_3$; Ac = acetyl; Bz = benzoyl; Cin = *trans*-cinnamoyl.
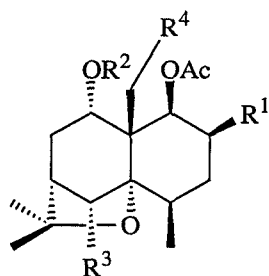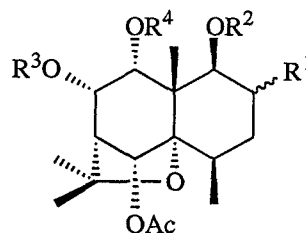
44-50   **51-63**

Table 1.3   Sesquiterpene Polyesters from *Tripterygium* Species (continued)

	Compound	R^1	R^2	R^3	R^4	Mol. formula	Species
44	triptogelin D-1	OAc	Bz	H	OAc	$C_{28}H_{36}O_9$	TW v.R[43]
45	triptogelin F-1	H	Bz	ONic	H	$C_{30}H_{35}NO_7$	TW v.R[44]
46	triptogelin F-2	H	Cin	OAc	H	$C_{28}H_{36}O_7$	TW v.R[44]
47	triptogelin C-1	OAc	Bz	OAc	H	$C_{28}H_{36}O_9$	TW v.R[45]
48	triptogelin C-2	OAc	Bz	ONic	H	$C_{32}H_{37}NO_9$	TW v.R[45]
49	triptogelin C-3	a	Bz	OAc	H	$C_{31}H_{42}O_9$	TW v.R[45]
50	triptogelin C-4	OH	Bz	ONic	H	$C_{30}H_{35}NO_8$	TW v.R[43]
51	triptogelin B-1	H	H	Bz	Bz	$C_{31}H_{36}O_8$	TW v.R[46]
52	triptogelin B-2	H	Nic	Bz	Bz	$C_{37}H_{39}NO_9$	TW v.R[46]
53	triptogelin A-1	β-OBz	Bz	Bz	Bz	$C_{45}H_{44}O_{11}$	TW v R[46]
54	triptogelin A-2	β-OH	Bz	Bz	Bz	$C_{38}H_{40}O_{10}$	TW v R[46]
55	triptogelin A-3	β-OH	H	Bz	Bz	$C_{31}H_{36}O_9$	TW v R[46]
56	triptogelin A-4	=O	H	Bz	Bz	$C_{31}H_{34}O_9$	TW v.R[46]
57	triptogelin A-5	β-OBz	Nic	b	Bz	$C_{42}H_{47}NO_{11}$	TW v.R[47]
58	triptogelin A-6	β-OBz	Bz	Nic	Bz	$C_{44}H_{43}NO_{11}$	TW v.R[47]
59	triptogelin A-7	β-OH	H	Nic	Bz	$C_{30}H_{35}NO_9$	TW v.R[47]
60	triptogelin A-8	β-OH	H	H	Bz	$C_{24}H_{32}O_8$	TW v.R[47]
61	triptogelin A-9	c	Bz	Nic	Bz	$C_{43}H_{49}NO_{11}$	TW v.R[47]
62	triptogelin A-10	β-OBz	Nic	Bz	Bz	$C_{44}H_{43}NO_{11}$	TW v.R[45]
63	triptogelin A-11	β-OBz	Bz	Ac	Bz	$C_{40}H_{42}O_{11}$	TW v.R[45]

TW v. R = *T. wilfordii* var. *regelii*; Ac = acetyl; Bz = benzoyl; Cin = *trans*-cinnamoyl; Nic = nicotinoyl.
[a] 49: R^1 = OCOCHCH₃CH₂CH₃; [b] 57: R^3 = COCHCH₃CH₂CH₃; [c] 61: R^1 = OCO(CH₂)₄CH₃.

Triterpenoids:

Triterpene tripterine, or celastrol, was the first constituent isolated from *T. wilfordii* by Chou and Mei in 1936.[18] Presently, more than thirty triterpenoids have been separated from *Tripterygium* plants (Table 1.4). Except zeorin, these triterpenoids have friedelane, oleanane, or ursane skeletons.

Table 1.4   Triterpenoids from *Tripterygium* Species

	Compound	Mol. formula	Species
64	celastrol (tripterine)	$C_{29}H_{38}O_4$	TW,[18] TR[48]
65	pristimein	$C_{30}H_{40}O_4$	TR[48]
66	3-hydroxy-25-norfriedel-3,1(10)-dien-2-one-30-oic acid	$C_{29}H_{42}O_4$	TR[49]
67	3,24-dioxo-friedelan-29-oic acid	$C_{30}H_{46}O_4$	TW[50]
68	polpunonic acid	$C_{30}H_{48}O3$	TW[51]
69	orthosphenic acid	$C_{30}H_{48}O_5$	TW,[52] TR[53]
70	salaspermic acid	$C_{30}H_{48}O_4$	TW,[54] TR[53]

TW = *T. wilfordii*; TR = *T. regelii*; TH = *T. hypoglaucum*.
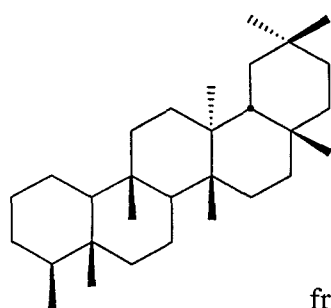
friedelane skeleton
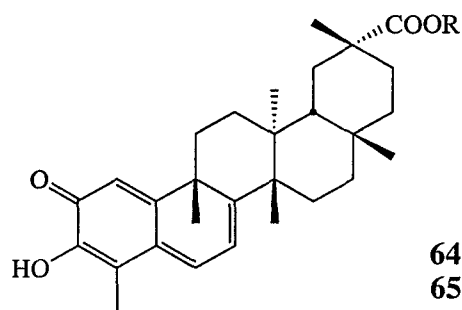
64 R = H
65 R = CH_3
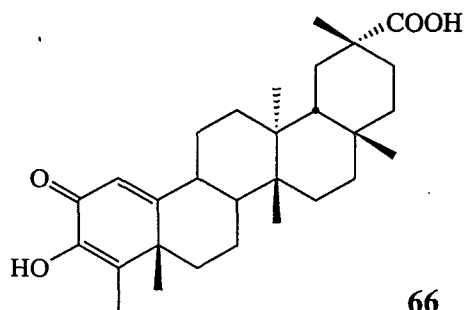
66
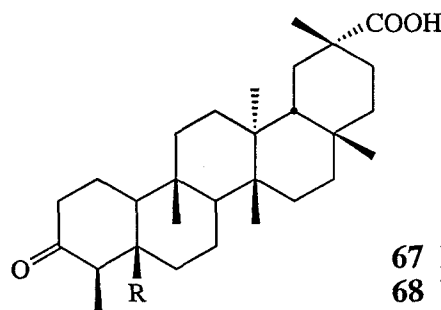
67 R = CHO
68 R = CH_3
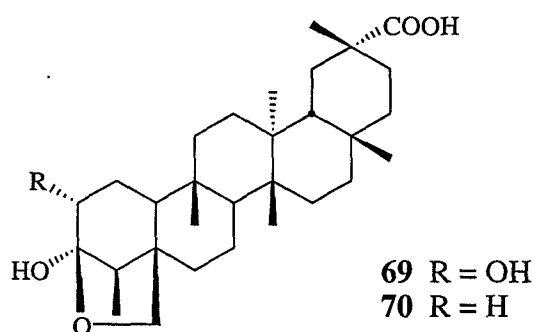
$$\text{COOH}$$

69 R = OH
70 R = H

Table 1.4   Triterpenoids from *Tripterygium* Species (continued)

	Compound	Mol. formula	Species
71	wilforlide A	$C_{30}H_{46}O_3$	TH,[55] TW,[56] TR[57]
72	wilforlide B	$C_{30}H_{44}O_3$	TW,[56] TH,[58] TR[57]
73	regelide	$C_{30}H_{42}O_3$	TR[53]
74	3β-hydroxy-olean-11,13(18)-diene	$C_{30}H_{48}O$	TR[53]
75	triptotriterpenoidal lactone A	$C_{30}H_{46}O_3$	TW[59]
76	3-epikatonic acid	$C_{30}H_{48}O_3$	TW,[54] TR,[53] TH[60]
77	triptotriterpenic acid A	$C_{30}H_{48}O_4$	TW,[61] TH[50]
78	triptotriterpenic acid B	$C_{30}H_{48}O_4$	TW[52,62]
79	triptodihydroxy acid methyl ether	$C_{31}H_{50}O_4$	TW[51]
80	3-acetoxyoleanolic acid	$C_{32}H_{50}O_4$	TH,[55] TR[53]
80-A	oleanolic acid	$C_{30}H_{48}O_3$	TH[58]
80-B	3-oxo-olean-12-en-29-oic acid	$C_{30}H_{46}O_4$	TH[63]
81	regelindiol B	$C_{31}H_{50}O_4$	TR[64]
82	regelin D	$C_{31}H_{48}O_4$	TR[64]
83	hypoglauterpenic acid	$C_{30}H_{46}O_3$	TH[58]
84	3β,15β-dihydroxy-Δ^{12}-oleanen-28-oic acid	$C_{30}H_{48}O_4$	TW[65]

TW = *T. wilfordii*; TR = *T. regelii*; TH = *T. hypoglaucum*.
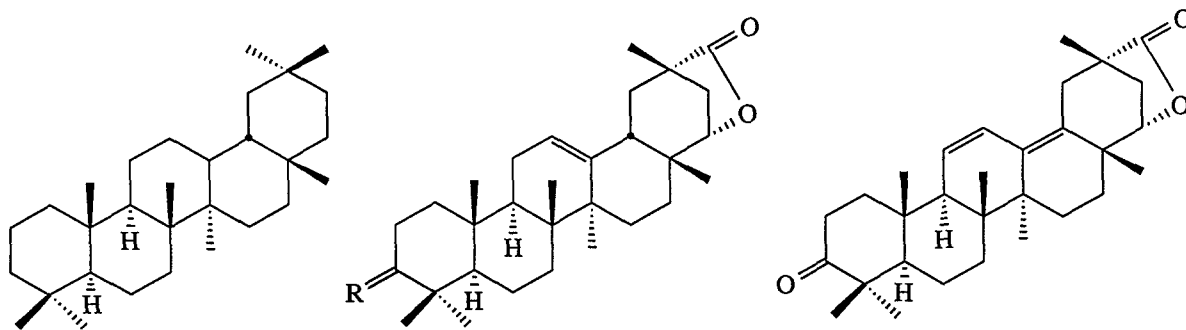
oleanane skeleton

71   R = β-OH, α-H

72   R = O

73

74

75

76   R = H$_2$

77   R = α-OH, β-H

78   R = β-OH, α-H

79
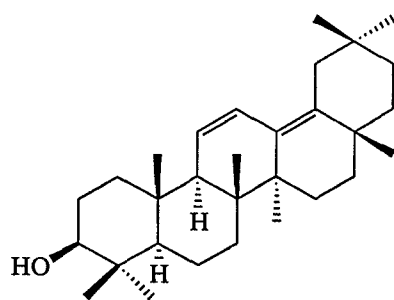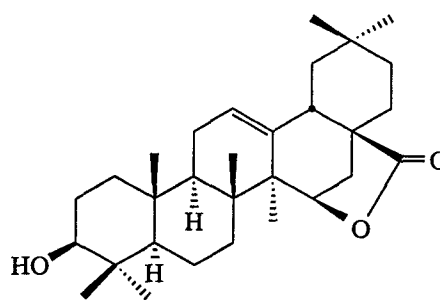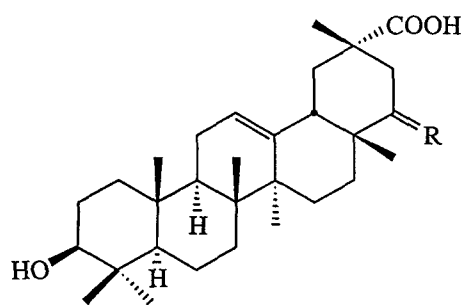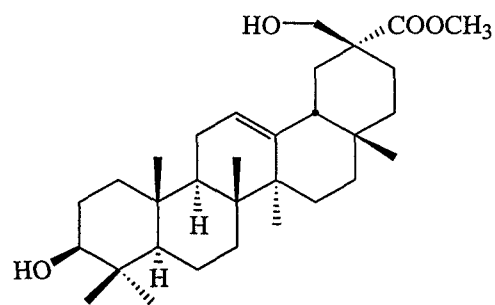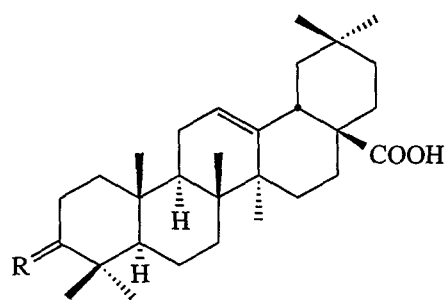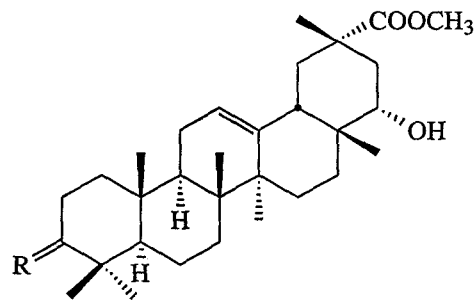
80	R = β-OAc		81	R = β-OH
80-A	R = β-OH		82	R = O
80-B	R = O			
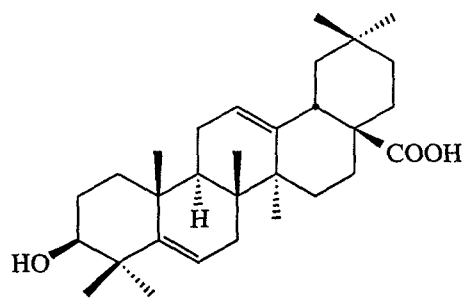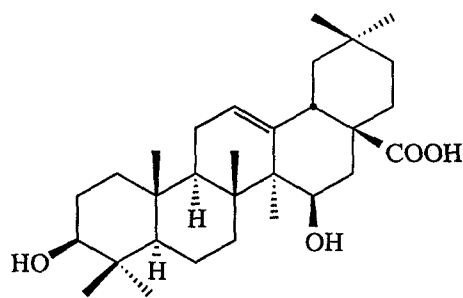
83   84

Table 1.4   Triterpenoids from *Tripterygium* Species (continued)

	Compound	Mol. formula	Species
85	regelindiol A	$C_{31}H_{50}O_4$	TR,[64] TH[60]
86	regelin	$C_{31}H_{48}O_4$	TR,[66] TH[67]
87	triptotriterpenic acid C (tripterygic acid A)	$C_{30}H_{48}O_4$	TW,[62,68] TH[67]
88	regelinol	$C_{31}H_{48}O_5$	TR[66]
89	regelin C	$C_{33}H_{50}O_6$	TR[64]
90	2α,3α,24-trihydroxy-Δ^{12}-ursene-28-oic acid	$C_{30}H_{48}O_5$	TR[49]
91	hypoglaulide	$C_{30}H_{44}O_3$	TH[67]
92	zeorin	$C_{30}H_{52}O_2$	TR[69]
93	glut-5-en-2β, 28-diol	$C_{30}H_{50}O_2$	TW[70]
94	ursan-3β,5α-diol	$C_{30}H_{52}O_2$	TW[70]

TW = *T. wilfordii*; TR = *T. regelii*; TH = *T. hypoglaucum*.
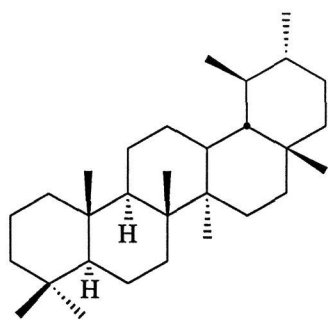
ursane skeleton
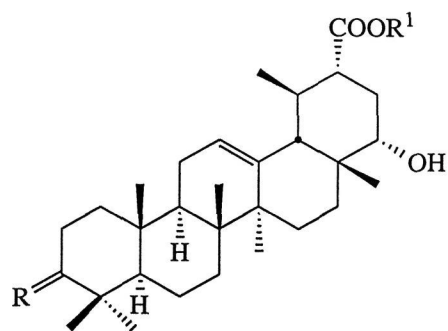
85   R = β-OH, α-H, R^1 = CH$_3$

86   R = O, R^1 = CH$_3$

87   R = β-OH, α-H, R^1 = H
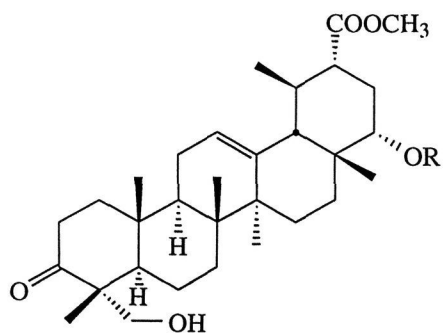
88   R = H

89   R = Ac
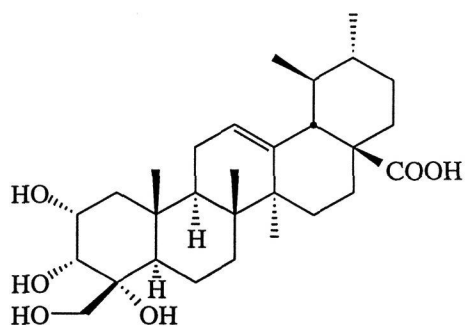
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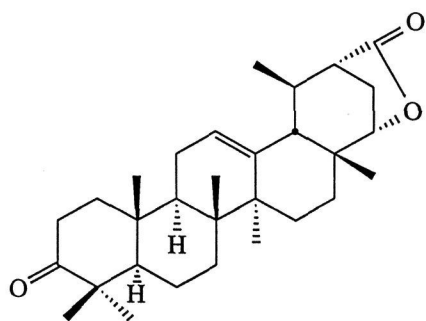
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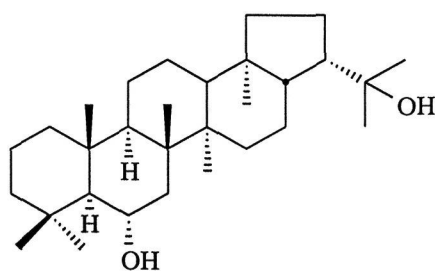
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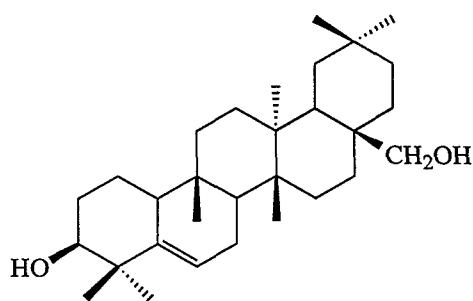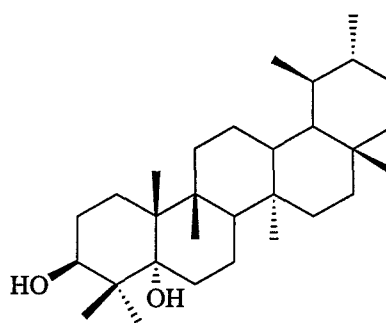
93   **94**

Diterpenoids:

Some of the most interesting compounds from *Tripterygium* belong to the diterpene family. In addition to their strong antileukemic and antitumor activities, Tl (**1**) and Td (**2**) are the first reported natural products containing the 18(4→3) *abeo*-abietane skeleton and the first recognized diterpenoid triepoxides.[15] Both Tl (**1**) and Td (**2**) were isolated from the plant in 0.001% yields. So far 36 diterpenoids have been isolated from *T. wilfordii* and from its related species. These diterpenes all contain the abietane-type skeleton except for tripterifordin (Table 1.5).

It has been shown that triptriolide (**100**) and 12-epitriptriolide (**102**) are not artifacts resulting from the extraction processes, but may have been formed in the plants.[71,72] Tripchlorolide (**103**) was believed to be an artifact formed during the extraction processes.[73,74]

18(4→3)*abeo*-abietane   **95**   **1**   R = H
skeleton   **2**   R = β-OH
                                                96   R = α-OH

Table 1.5   Diterpenoids from *Tripterygium* species

	Compound	Mol. formula	mp, °C	Species
1	triptolide (Tl)	$C_{20}H_{24}O_6$	226-227	TW,[15]TH[75]
2	tripdiolide (Td)	$C_{20}H_{24}O_7$	210-211	TW,[15]TH[63]
95	triptonide	$C_{20}H_{22}O_6$	210-211	TW,[15] TH[75]
96	tripterolide	$C_{20}H_{24}O_7$	225-228	TH[2]
97	triptolidenol	$C_{20}H_{24}O_7$	193-194	TW[76]
98	16-hydroxytriptolide	$C_{20}H_{24}O_7$	232-234	TW[77]
99	triptetraolide	$C_{20}H_{26}O_8$	258-260	TW[31]
100	triptriolide	$C_{20}H_{26}O_7$	260-262	TW[71]
101	isotriptetraolide	$C_{20}H_{26}O_8$	250-252	TW[78]
102	12-epitriptriolide	$C_{20}H_{26}O_7$	268.5±1.0	TW[72]
103	tripchlorolide	$C_{20}H_{25}ClO_6$	256-258	TW[73]
104	13,14-epoxide-9,11,12-trihydroxytriptolide	$C_{20}H_{26}O_7$	268-270	TW[79]
105	tripdioltonide	$C_{20}H_{24}O_6$	222-224	TW[79]

TW = *T. wilfordii*; TH = *T. hypoglaucum*; TR = *T. regelii*.
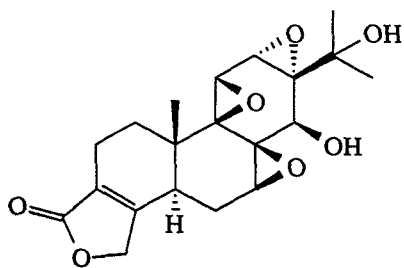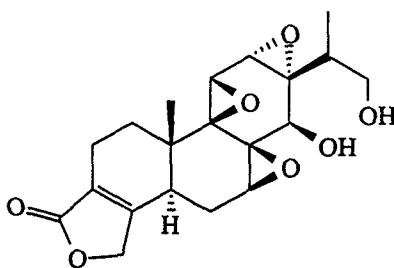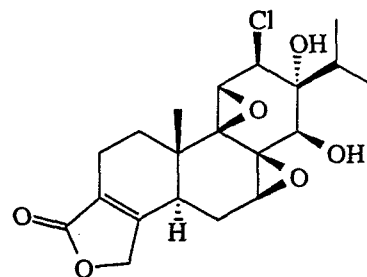
97   **98**   **103**
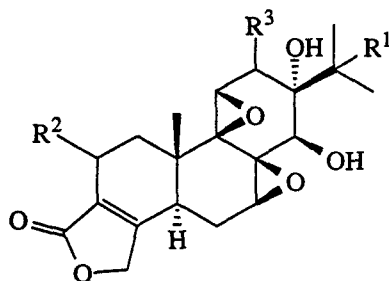
99 R^1 = OH, R^2 = H, R^3 = β–OH
100 R^1 = R^2 = H, R^3 = β–OH
101 R^1 = H, R^2 = R^3 = β–OH
102 R^1 = R^2 = H, R^3 = α–OH
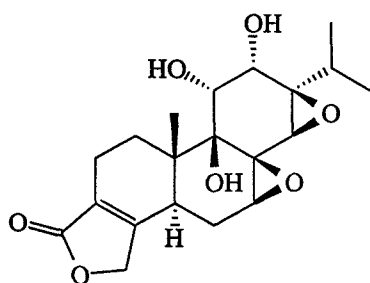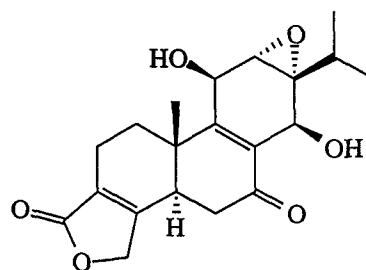
104   **105**

Table 1.5   Diterpenoids from *Tripterygium* species (continued)

	Compound	Mol. formula	mp, °C	Species
106	triptophenolide (hypolide)	$C_{20}H_{24}O_3$	232-234	TW,[80] TH,[2] TR,[2] TF[2]
107	triptophenolide methyl ether	$C_{21}H_{26}O_3$	152-154	TW[80]
108	triptonolide	$C_{20}H_{22}O_4$	214-215	TW[81]
109	neotriptophenolide	$C_{21}H_{26}O_4$	189-191	TW[80]
110	isoneotriptophenolide	$C_{21}H_{26})4$	185-187	TW[82]
111	triptoditerpenic acid	$C_{21}H_{28}O_3$	-	TH[83,84]
112	hypoglic acid	$C_{21}H_{28}O_4$	219-224	TH[85]
113	triptonoditerpenic acid	$C_{21}H_{28}O_4$	189-191	TH[86]
114	triptoquinonoic acid A	$C_{20}H_{24}O_4$	182-183	TR,[87] TH[88]
115	triptoquinonoic acid C	$C_{20}H_{24}O5$	202-203	TR[89]

TW = *T. wilfordii*; TH = *T. hypoglaucum*; TR = *T. regelii*; TF = *T. forrestii*.
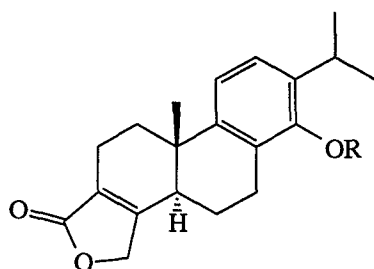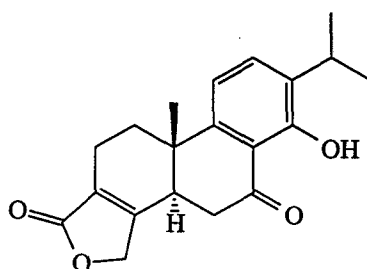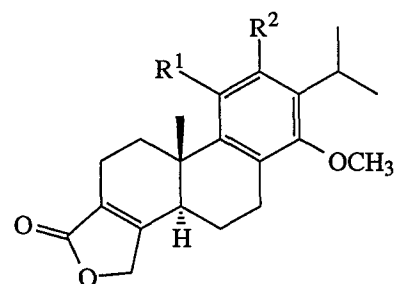
106   R = H   **108**   **109** R^1 = OH, R^2 = H
107   R = CH₃   **110** R^1 = H, R^2 = OH
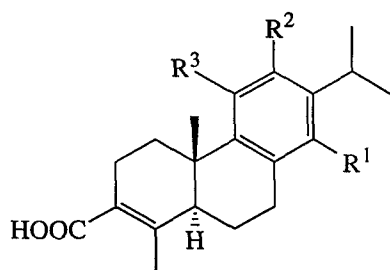
111 $R^1 = OCH_3$, $R^2 = R^3 = H$

112 $R^1 = OCH_3$, $R^2 = H$, $R^3 = OH$

113 $R^1 = OH$, $R^2 = OCH_3$, $R^3 = H$
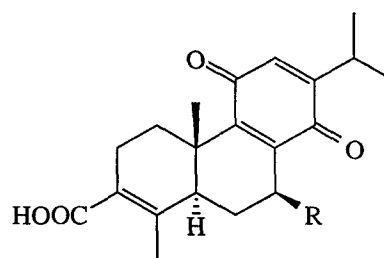
114 $R = H$

115 $R = OH$

Table 1.5   Diterpenoids from *Tripterygium* species (continued)

	Compound	Mol. formula	mp, °C	Species
116	triptonoterpene	$C_{20}H_{28}O_2$	153-155	TW[76]
117	triptonoterpene methyl ether	$C_{21}H_{30}O_3$	209-211	TW,[76] TH[85]
118	neotriptonoterpene	$C_{21}H_{30}O_3$	205-207	TW[90]
119	triptonoterpenol	$C_{21}H_{30}O_4$	197-199	TW,[91] TH[92]
120	11-hydroxy-14-methoxy-dehydroabietane	$C_{21}H_{32}O_2$	194-195	TR[89]
121	triptoquinonoic acid B	$C_{20}H_{26}O_4$	212-213	TR[87]
122	triptoquinonal	$C_{20}H_{26}O_3$	127-128	TR[87]
123	triptoquinone G	$C_{20}H_{26}O_5$	-	TW v. R[93]
124	triptoquinonol	$C_{20}H_{28}O_3$	165-166	TR[87]
125	triptoquinondiol	$C_{20}H_{28}O_4$	183-184	TR[87]
126	3-oxo-triptoquinonol	$C_{20}H_{26}O_4$	135-136	TR[87]
127	tripterifordin	$C_{20}H_{30}O_3$	255-256	TW,[16] TH[84]
128	wilforonide	$C_{13}H_{16}O_3$	187-189	TW[82]

TW = *T. wilfordii*; TR = *T. regelii*; TW v. R = *T. wilfordii* var. *regelii*; TH = *T. hypoglaucum*.

abietane
skeleton
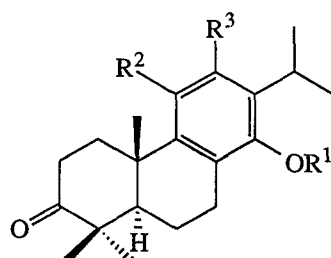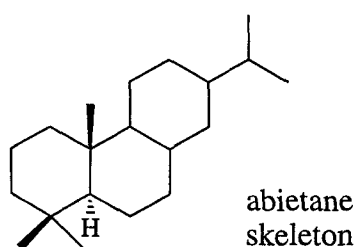
116 $R^1 = R^2 = R^3 = H$

117 $R^1 = CH_3$, $R^2 = OH$, $R^3 = H$

118 $R^1 = CH_3$, $R^2 = H$, $R^3 = OH$

119

120
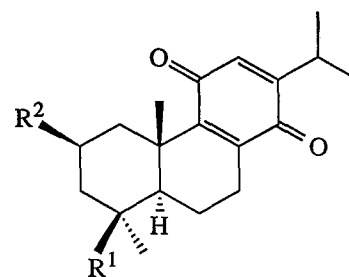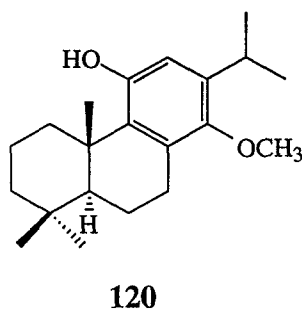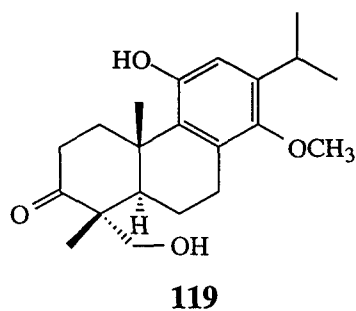
121 R^1 = COOH, R^2 = H

122 R^1 = CHO, R^2 = H

123 R^1 = COOH, R^2 = OH

124 R = H$_2$

125 R = β-OH, α-H

126 R = O

127

128
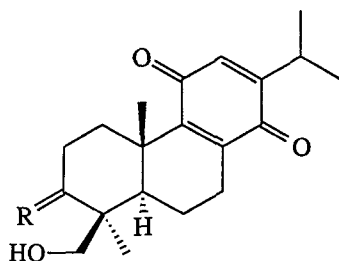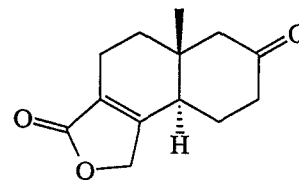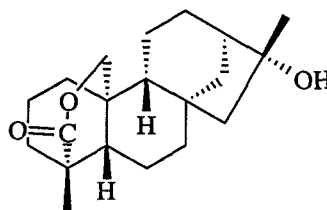
Other Compounds:

Quite a few other types of compound have also been isolated from *Tripterygium* species, including dulcitol,[57,94] euonymine,[26] β-sitosterol, daucosterol, fumaric acid,[50] (-)-syringaresinol,[95] *l*-epicatechin,[94] maytenfolic acid,[88] 1,8-dihydroxy-4-hydroxymethylanthraquinone,[96] fatty acids,[97] and carbohydrates.[98]

1.4 Pharmacological Activities of Extracts and Compounds from *T. wilfordii* and Related Species

Antileukemia and antitumor activities

In search of the antileukemia principles from *T. wilfordii,* Kupchan and co-workers found that the alcoholic extract of *T. wilfordii* exhibited significant *in vivo* activity against the L-1210 and P-388 leukemia in mice as well as *in vitro* activity against cells derived from human carcinoma of the nasopharynx (KB). Guided by the assays against KB, L-1210 and P-388, they finally found that diterpene triepoxides Tl (**1**) and Td (**2**) were responsible for these activities.[15] Tl (**1**) and Td (**2**) showed significant life-prolonging effects in mice afflicted with the L-1210 lymphoid leukemia at 0.1 mg/kg level and cytotoxicity (ED_{50}) against KB cell culture at 10^{-3} μg/mL concentration.[99] Triptolide (**1**) administered intraperitoneally at 0.2 mg/kg per day for six days markedly prolonged the survival time of mice with L-615 leukemia.[100] In addition to its antileukemic activity, Tl (**1**) also showed strong inhibitory effects on the colony formation of several human breast and stomach cancer cell lines (MCF-7, BT-20, MKN-45, MKN-7 and Kato-III) at magnitudes similar to those of the leukemia cell line (HL-60). This may suggest that Tl (**1**) might have a potential therapeutic value for some types of solid tumors.[101] A study with human HeLa cells showed that Tl (**1**) is an agent which is non-specific to cell cycle phases (a character shown by alkylating drugs, *vide infra*), but more sensitive to S phase cells.[102]

Triptolide (**1**) and tripdiolide (**2**) may be classified as alkylating anti-neoplastic agents based on their mechanism of action[103] as shown by a number of studies conducted so far.[99,104] Both the epoxide functionality and the α,β-unsaturated butenolide moiety have been shown to be important for the tumor-inhibitory activity. It was postulated that the 9,11-epoxy-14β-hydroxy system is necessary for the antileukemia activity of Tl (**1**) and Td (**2**). Biological and chemical data are in support of a mechanism that involves intramolecular catalysis (by a

neighboring hydroxyl group on the opening of an epoxide by nucleophiles).[105] Some plant-derived tumor inhibitors may act via selective alkylation of thiol groups of key enzymes that are responsible for growth regulation. Intramolecular catalysis by the C14-hydroxyl group may assist in selective alkylation of the 9,11-epoxide by biological macromolecules[99] (Figure 1.1, PrSH = propanethiol). Triptonide (**95**), differing from (**1**) solely at C14, by virtue of a ketonic functionality instead of a β oriented hydroxyl group, showed no antileukemic activity in doses up to 0.4 mg/kg. 14-Epitriptolide (**129**), a minor variant of Tl (**1**) with an α oriented C14 hydroxyl group, and the thiol adducts **130** and **131**, also showed no antileukemic activity up to 0.4 mg/kg.

Additional evidence indicates that nucleophilic addition of thiols to the α,β-unsaturated system may also be involved in the tumor inhibitory properties of some plant-derived compounds.[99,106] G. A. Berchtold *et al.* reported that an analog of Tl (**1**), **132**, which did not have the α,β-unsaturated butenolide moiety, failed to show antileukemic activity against P-388 lymphocytic leukemia.[107]

A more recent study showed that the epoxide ring opening by attack of nucleophiles took place at the less hindered 12,13-epoxide rather than at the more hindered 9,11-epoxide, as previously postulated. X-ray analysis of the product from the reaction of Tl (**1**) with propanethiol under identical conditions as those reported earlier confirmed that the thiol exclusively attacked at C12 from the convex side, thus opening the 12, 13-epoxide.[104]

Aside from diterpenes such as Tl (**1**) and Td (**2**), some triterpenes (regelin (**86**), regelinol (**88**) and wilforlide A (**71**)) have been shown to have antitumor activities.[66] Some sesquiterpenes, particularly triptofordin F-2 (**29**) and triptogelin A-1(**32**), have recently been found to exhibit remarkable antitumor promoting activities both *in vitro*[108] and *in vivo*.[109]
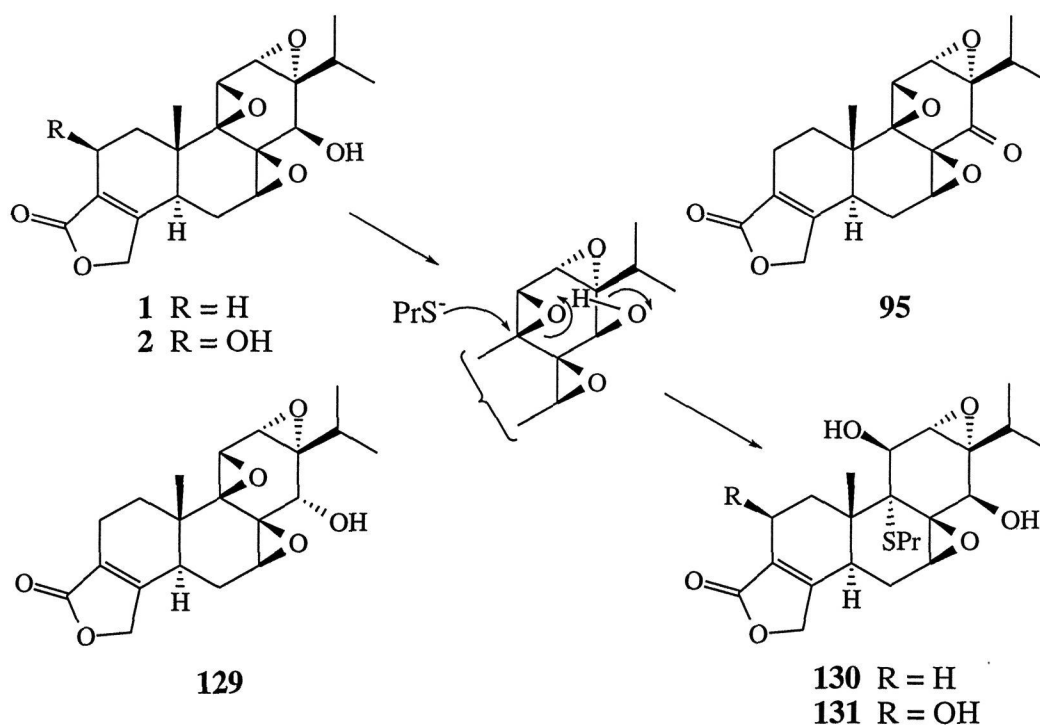
Figure 1.1   Alkylation of Thiols by the Diterpene Triepoxides via Hydroxyl-assisted
Epoxide Ring Opening

132
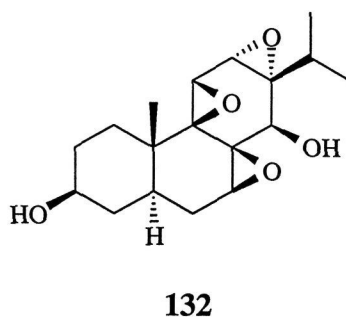
Immunosuppressive and Anti-inflammatory Activities

One of the activities of some alkylating drugs is their immunosuppressive action.[103] Rheumatoid arthritis and some skin diseases are autoimmune disorders in mechanism and constitute one type of inflammation. The major clinical applications of *T. wilfordii* as a Chinese herbal medicine are for the treatment of these two diseases. However, which particular

compound(s) is the main active principle is still under extensive study. The drug for most of the clinical trials is in a form of extract, such as GTW, and most of the pharmacological studies are also conducted on the extracts.

There have been numerous reports of successful clinical treatments of rheumatoid arthritis and skin disorders since 1969, when *T. wilfordii* started to be rather widely used in China.[110] An early clinical trial with 133 consecutive cases of rheumatoid arthritis and ankylosing spondylitis patients showed that treatment with a tincture of *T. wilfordii* had remarkably relieved cardinal signs of joint inflammation, pain, swelling, and improved joint function.[12] Similar successful results were also shown in the treatment of a wide variety of skin diseases, including allergic diseases and diseases with a mechanism possibly related to allergies.[14] Several studies showed that the advantageous therapeutic effects of *T. wilfordii* included relatively rapid relief of the symptoms with high effective treatment rates.[13,111] The drug was more potent than the conventional non-steroid antirheumatic agents such as salicylates, indomethacin, phenylbutazone, etc.[12] and can be used as a substitute for corticosteroids in the treatment of some skin diseases for patients who are steroid-dependent or steroid-resistant, or who have contraindications to steroids.[14]

In addition to clinical application of GTW in the treatment of rheumatoid arthritis and skin disorders, there were also reports of satisfactory treatment of patients with asthma.[112]

The mechanism of action of *T. wilfordii* , though still not clear, has received more attention in recent years.[113-116] Its anti-inflammatory effect was demonstrated by its significant inhibition of acute agar-induced edema and histamine-induced capillary hyperpermeability in animals.[117] Additional inflammatory mediators such as prostaglandin E_2 (PGE_2), which are secreted by human peripheral blood mononuclear cells, were shown to be reduced by this drug in *in vitro* experiments.[118] The immunosuppressive activity of the drug was shown by its inhibitory effects on both the humoral and cell mediated immunities as monitored by the hemolysin assay, the splenic cell immuno-specific rosette assay and the active rosette assay.[14,117] More detailed studies indicated that *in vitro* GTW inhibited antigen and mitogen

stimulated proliferation of human T (thymus-derived) and B (bone marrow derived) cells, inhibited T cell responses and interleukin-2 (IL-2) production by T cells, and suppressed immunoglobulin (Ig) production by B cells at the 0.08-1 μg/mL level. At these concentrations, GTW did not affect IL-2R expression by T cells or IL-1 production and antigen presentation by monocytes.[119] The immunosuppressive effect of GTW was further exhibited by its ability to inhibit the activation of T cells. The suppression of T cell proliferation is due to this drug's inhibitory effects on key facets in the cell cycle of the T cells.[113,114] The above experiments also demonstrated that the immunosuppressive effects were reversible after cessation of exposure to the drug. As an immunosuppressive agent, GTW has significantly prolonged the mean survival time of cardiac allografts in rats, comparable with the well-known anti-rejection agent cyclosporine A.[120]

The compounds which have been shown to be responsible for the anti-inflammatory and immunosuppressive activity include alkaloids, triterpenoids and diterpenoids. Isolated compounds and their pharmacological activities are summarized in Table 1.6. Celastrol (64) was shown to inhibit the proliferation of lymph cells,[121] to inhibit the activities of IL-1, IL-2, the release of PGE_2 and the antibody response.[122,123] Tl (1) and Td (2) have both anti-inflammatory and immunosuppressive activities. In animal tests, Tl (1) decreased the effect of the humoral-mediated immunity as monitored by the hemolysin test, but not the cell-mediated immunity as assayed by the graft versus host reaction and the tumor-concomitant immunity.[100]

Antifertility Activity

During the evaluation of the general toxicity of GTW, it was discovered that in hybrid mice, when both the male and female animals were simultaneously fed a laboratory chow containing GTW for 4.5 months, both the body weights and the birth rates decreased. In clinic practices that utilize crude extracts of *T. wilfordii* for the treatment of rheumatoid arthritis and other skin disorders, clinicians have observed the fact that a decrease in the testicular volume

might take place in a few patients after long term exposure to the drug. In some cases, necrospermia or azoospermia occurred, but libido and potency were normal in all subjects, and the seminal indices were found normal three months after cessation of the treatment.[11] Detailed animal tests showed that male rats given GTW by gastric gavage at a dose of 10 mg/kg daily, 6 days a week for 8 weeks, all became infertile. The body weight gain, histology of various organs, serum testosterone level and the mating behavior were found normal. Significant decreases in the density and particularly the motility of the spermatozoa from the cauda epididymis were observed. Interestingly, the fertility of the treated rats began to recover 4 weeks after withdrawal of GTW and was fully restored one more week later. Similar results were also obtained from a comparative retrospective study on male patients with rheumatoid arthritis treated with GTW. These results suggested that the antifertility activity of the drug is very likely reversible. The effective dosage for antifertility is only about one third of that for treatment of arthritis and skin disorders, therefore the side effects could be lower. As to the action of the drug, it was believed that at the dose level, GTW mainly causes damage to the epididymal spermatozoa and to a lesser extent the spermatogenic cells.[11]

Table 1.6   Isolated Compounds and Their Pharmacological Activities

Compound	Pharmacological activities				
	anti tumor	anti-inflamm.	immuno. suppres.	anti fertility	anti-HIV
4 wilforine[124]			+		
6 wilfortrine[125]			+		
9 euonine (wilformine)[125]			+		
29 triptofordin F-2[108]	+a				
32 triptogelin A-1[109]	+b				
86 regelin[66]	+				
88 regelinol[66]	+				
71 wilforlide A[66]	+				
77 triptotriterpenic acid A[61]		+			
87 triptotriterpenic acid C (tripterygic acid)[126]		+			
84 $3\beta,15\beta$-dihydroxy-Δ^{12}-oleanen-28-oic acid[65]		+			
78 triptotriterpenic acid B[49,126]		+			
76 3-epikatonic acid[53]				+	
69 orthosphenic acid[126]		+			
70 salaspermic acid[17]					+

anti-inflamm. = anti-inflammation; immuno suppres. = immunosuppression; +: active, -: not active, blank: data not available. a,b antitumor promotion activity.

The total alkaloids fraction[127] and several isolated diterpene epoxides from the plant also showed antifertility activity.[128] One of the diterpene epoxides, tripchlorolide (**103**), was found to have an antifertility potency 200 times stronger than that of GTW. Unlike GTW or Tl (**1**), which cause relatively broad damage, especially to the sperm head (a potential source of mutation), tripchlorolide (**103**) only causes damage to the spermatozoa in the epididymis without significantly inducing sperm head anomalies or affecting other related organs.[129] This compound is now under more extensive evaluation.

Table 1.6   Isolated Compounds and Their Pharmacological Activities (continued)

Compound	Pharmacological activities				
	anti tumor	anti-inflamm.	immuno suppres.	anti fertility	anti-HIV
1 triptolide[15,130]	+	+	+	+	
2 tripdiolide[15,130]	+	+	+	+	
95 triptonide[130,131]		+	+	+	
97 triptolidenol[130]		+	+	+	
98 16-hydroxytriptolide[130]		+	+	+	
100 triptriolide[130]		+	-	-	
102 12-epitriptriolide[72]		+			
103 tripchlorolide[130]		+	+	+	
106 triptophenolide[132]			+		
114 triptoquinonoic acid A (triptoquinone A)[93,133]			+		
121 triptoquinonoic acid B (triptoquinone F)[93]			+		
122 triptoquinonal (triptoquinone E)[93]			+		
124 triptoquinonol (triptoquinone D)[93]			+		
125 triptoquinondiol (triptoquinone C)[93]			+		
126 3-oxo-triptoquinonol (triptoquinone B)[93,133]			+		
127 tripterifordin[16]					+

Another independent study was carried out by a group of British and Chinese scientists. Guided by bioassay-directed preparative high pressure liquid chromatography (HPLC) sub-fractionation of material extracted from both the GTW tablets and the native plants, this group has found that the active antifertility principles of *T. wilfordii* are Tl (**1**), Td (**2**) and closely related derivatives of this class of diterpene epoxides, including tripchlorolide (**103**), an artifact formed during processing of the plant material.[74]

Anti-HIV Activity

During the growing worldwide campaign against AIDS, people are again turning to nature for a cure. A team of researchers from the United States and China has found that the ethanol extract of the roots of *T. wilfordii* shows significant anti-HIV activity. Bioassay-directed fractionation of the active extract has led to the isolation and characterization of a new anti-HIV component, a kaurane-type lactone tripterifordin (**127**). This compound inhibited HIV replication in H9 lymphocyte cells with an EC_{50}* of 1 µg/mL (6 µM) and did not inhibit uninfected H9 cell growth at 15 µM.[16]

This group also found GTW, the total glycoside of *T. wilfordii*, has significant anti-HIV activity. Bioassay-directed fractionation of GTW has led to isolation and characterization of a triterpene, salaspermic acid (**70**), as the anti-HIV principle from the chloroform-soluble fraction. Salaspermic acid inhibited HIV replication in H9 lymphocytes with an EC_{50} value of 5 µg/mL (10 µM), and it inhibited uninfected H9 cell growth with an IC_{50}** value of 53 µM. This compound also showed an inhibitory effect against HIV-1 recombinant reverse-transcriptase-associated reverse transcriptase activity. Salaspermic acid and its related compounds are now under further anti-HIV evaluation.[17]

Side Effects and Toxicity

In the treatment of rheumatoid arthritis or dermatitis, the major side effects at regular clinical dose levels, *i.e.*, a decoction from 15-25 g of root xylem per day or GTW 60-90 mg/day (1.0-1.5 mg/kg/day), are gastrointestinal disturbances which include nausea, vomiting, anorexia, epigastric burning sensation, xerostomia, diarrhea and constipation. Most of them will subside in the course of treatment and discontinuation of medication is usually not

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* EC_{50}: medium effective concentration.
** IC_{50}: medium inhibitory concentration.

necessary. Leukopenia or thrombocytopenia may be found in a few patients, but the cell counts recover shortly after withdrawal of the drug. Other side effects include menstrual disturbances, oligospermia, azoospermia and a decrease in the size of the testis.[11]

The single dose toxicity (LD_{50}) of GTW in mice was about 160 mg/kg. The repeated dose toxicity in rats at doses of 30, 60 or 120 mg/kg/day caused lethargy, and lower body weight growth, particularly in the large dose group with longer treatment time. Histological examination revealed damages in the seminiferous tubules, the endometrium and the myometrium. No other significant changes were found in the tested animals. Dogs fed with the drug at a dose of 10-15 mg/kg/day for 14.5 months decreased their food intake, and exhibited a decrease in their white blood cell count.[134] This demonstrated that the pharmacologically active dosage is approximately six times less than a harmful dosage.

A smaller dosage of 20 mg of GTW per day was sufficient to produce reversible infertility in human males. Immunosuppressive side effects were not seen in human subjects at this level while higher dosages (in treatment of arthritis and dermatitis) had produced an increase in secondary infections.[11,12]

Triptolide (1) elicits strong antileukemic activity in mice. Triptolide (1) at a 0.1 mg/kg level in vivo,[15,99] or intraperitoneally at 0.25 mg/kg [100] greatly extended the survival time of mice injected with leukemic cells. The LD_{50} for Tl (1) (intravenous injection of mice) has been determined to be 0.8 mg/kg for a single dose and 0.16 mg/kg/day over seven days with concurrent fatal degeneration of heart tissue and bone marrow.[11] This shows that the toxic dosage is very close to its therapeutic dosage and thus triptolide has not been applied as an antileukemic pharmaceutical for human patients.[104]

The immunosuppressive and antifertility activities seem to overlap with each other in GTW and other isolated compounds from pharmacological studies. However, animal tests showed that the effective antifertility thresholds were 40-60 times lower than LD_{50}, 5-28 times lower than the dosages for anti-inflammatory activity and 5-12 times lower than dosages for

immunosuppressive activity. Therefore, effective antifertility activity could be achieved without necessarily depressing the immune system.[128]

In another attempt to further reduce the toxicity caused by oral administration, researchers have attempted to formulate the extract of *T. wilfordii* into an injection solution for external use in the troubled area to treat rheumatoid arthritis patients.[135]

1.5   Total Syntheses of Triptolide (1)

Due to the strong antileukemic and antitumor activities shown by Tl (**1**) and Td (**2**), and their low yields in the plants (0.001%), numerous efforts have been made in searching for alternatives to obtain these materials in quantities for further pharmacological studies since the isolation of these two compounds in the early 1970's. The two major routes were via synthesis and plant cell culture technology (*vide infra*). The unique abietane-type triepoxide structure imposed a challenge to synthetic chemists and invoked their interests as well. There has been no report on the total synthesis of Td (**2**) (which was successfully obtained by plant cell culture technology, *vide infra*), probably because Tl (**1**) has similar activities and major structural features, or due to some difficulty in adding the extra hydroxyl group.

Although there was some synthetic work on the construction of the butenolide moiety,[136-138] or the triepoxide system in ring C[139,140] (the two major challenges towards the synthesis of Tl (**1**)), respectively, the total synthesis falls into two main classes, the synthesis of racemic Tl from small achiral starting materials and the synthesis of optically pure Tl (**1**) with the same stereochemistry as the isolated *l* -triptolide (**1**).

The racemic synthesis adopted by Berchtold *et al.* was to synthesize the dihydronaphthalenone **133** as a starting material with the B/C ring fragment of the abietane skeleton (Scheme 1.1).[107,141]

Construction of ring A via alkylation and annulation of the naphthelenone provided a suitably functionalized tricyclic intermediate **136** for the construction of the ring C triepoxide

system and the butenolide moiety. Annulation started by the alkylation of **133** with iodobutyrolactone **134**, followed by an opening of the lactone and oxidation to provide the desired intermediate **135**. Aldol condensation then yielded the key tricyclic intermediate **136**. Reduction of the aldehyde, acidic hydrolysis and rearrangement of the double bond completed the synthesis of the butenolide ring. Ring C construction was achieved by hydroxylation at C7 and subsequent conversion to the epoxy dienone **139** by periodate oxidation. Further epoxidation gave racemic triptonide (**95**), which, after reduction by sodium borohydride, yielded a 3:1 mixture of racemic 14-epitriptolide (**129**) and triptolide (**1**).

van Tamelen and co-workers have devised a number of routes to the total synthesis of triptolide (**1**) and triptonide (**95**). A synthesis of racemic Tl (**1**)[142] involving fewer steps, was comprised of a construction of ring C onto an appropriate AB fragment that was derived from decalone **140** and that possessed a hydroxyl functionality at C14 (Scheme 1.2). An efficient Diels-Alder addition to the furan derivative **142**, afforded **143**. Ring A butenolide construction proceeded from the alkene intermediate **144** via introduction of a hydroxyl group at C3, rearrangement with thionyl chloride and conversion of the C19 allylic chloride to the allylic alcohol **145**. Addition of dimethylformamide dimethylacetal to the allylic alcohol **145**, was followed by a carbene [2,3]-sigmatropic rearrangement to **146**. Further elaboration yielded the key intermediate **107**.

A biogenetic-type synthesis of racemic Tl (**1**) consisted of the construction of a geranylgeraniol-type intermediate **153**, and cyclization to the tricyclic skeleton **154**. Appropriate functionalization at C3 and C4 led to facile conversion to the butenolide ring and yielded the key intermediate **107** (Scheme 1.3).[143]
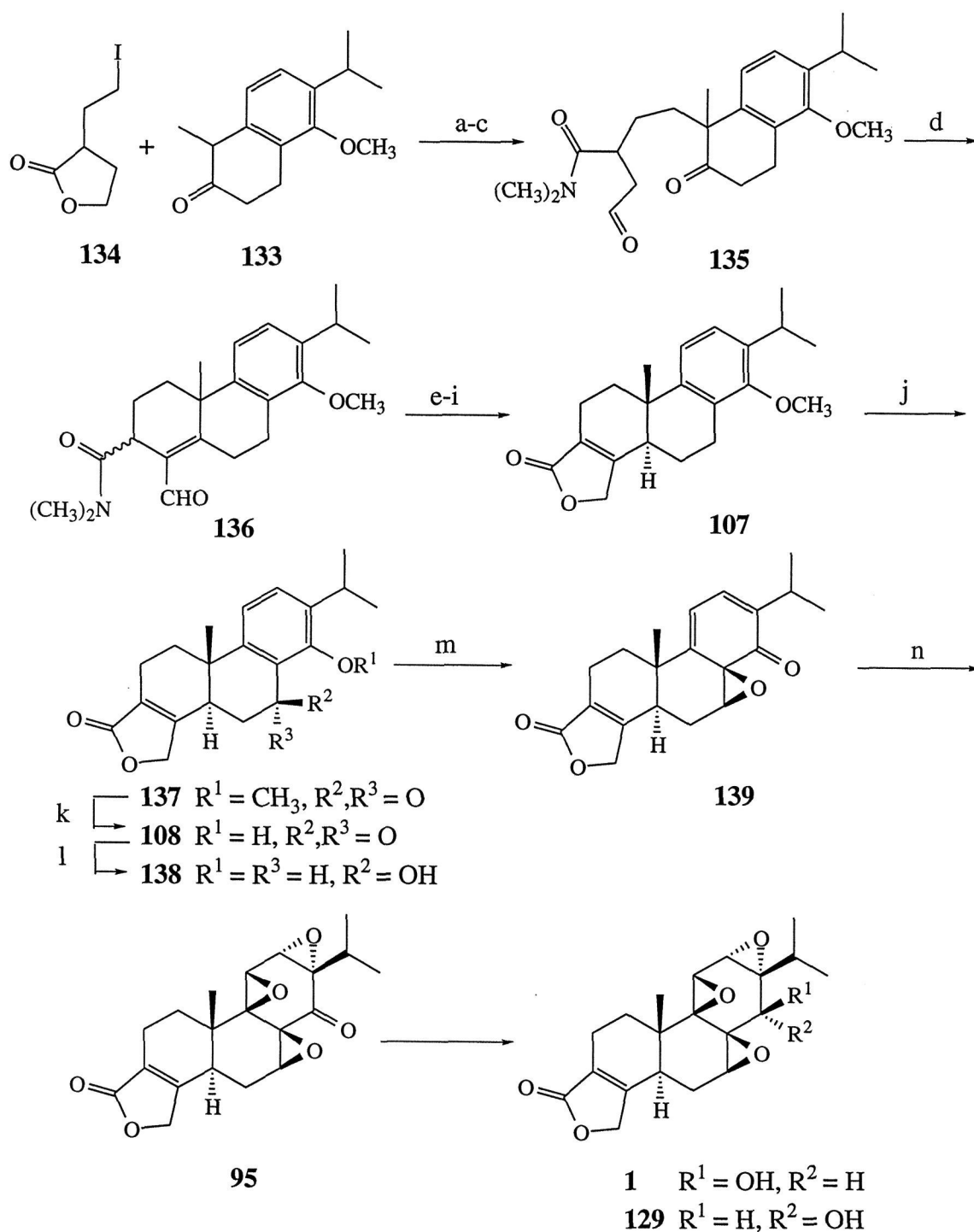
Scheme 1.1   Synthesis of (±) Triptolide (1) via BC→ABC Abietane Construction

a) NaH, DMF; b) Me$_2$NH; c) CrO$_3$, pyridine, CH$_2$Cl$_2$; d)neutral Al$_2$O$_3$, EtOAc; e) NaBH$_4$, EtOH, 2N HCl; f) m-CPBA, CH$_2$Cl$_2$; g) Et$_3$N, CH$_2$Cl$_2$; h) 2,4,6-trimethylpyridine, MeSO$_2$Cl, DMF; i) H$_2$, Pd-C, EtOAc; j) CrO$_3$, HOAc; k) BBr$_3$, CH$_2$Cl$_2$, 0°C; l) NaBH$_4$, EtOH; m) NaIO$_4$, MeOH; n) m-CPBA, CH$_2$Cl$_2$; o) NaBH$_4$, EtOH
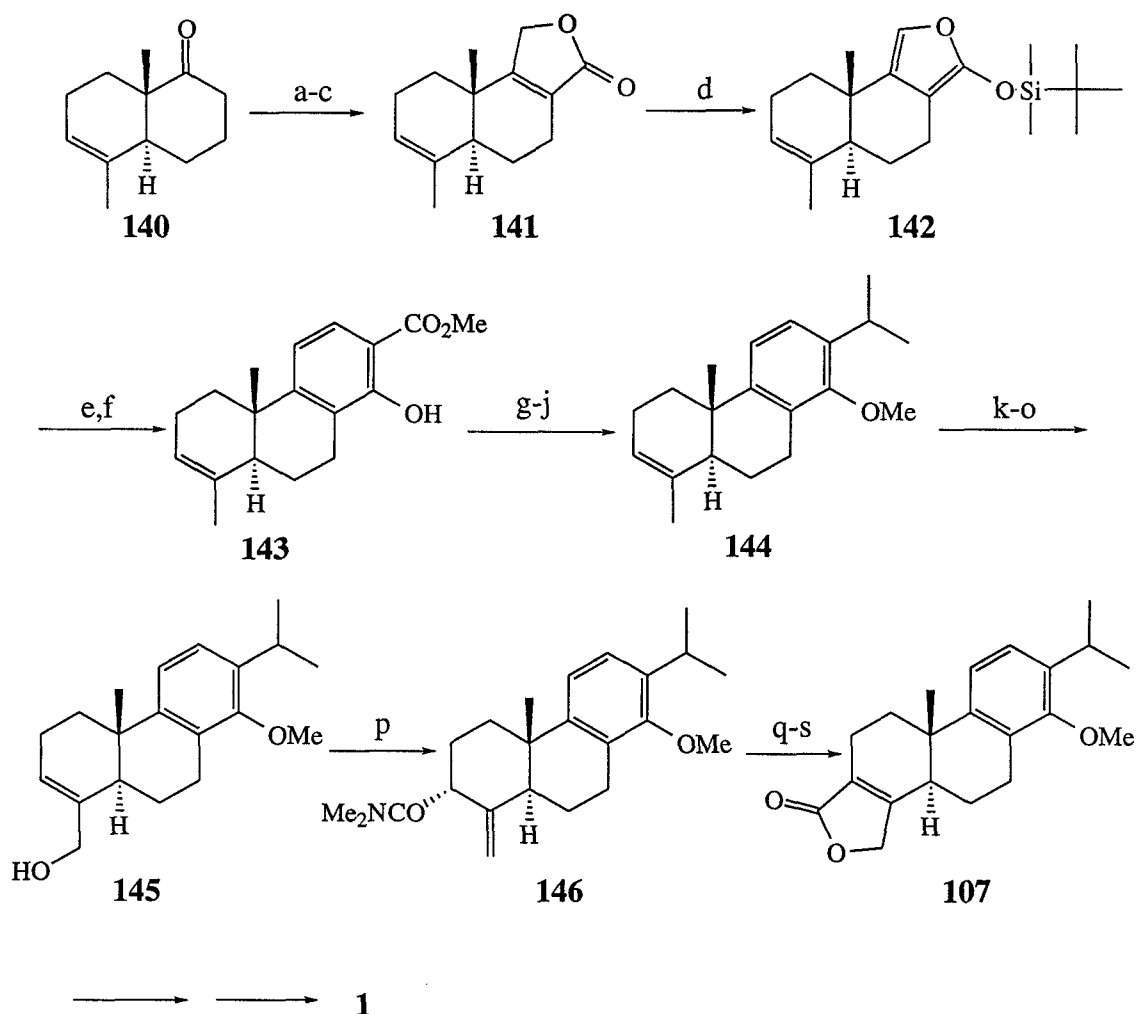
Scheme 1.2   Synthesis of (±) Triptolide (**1**) via AB→ABC Abietane Construction

a) CS2, 2,6-di-*t*-Bu-4-Me-C6H2OLi, THF, MeI; b) (CH3)2=CH2, NaH, DMSO, -10°C; c) HCl (aq)-MeOH; d) LDA, HMPA, THF, TBDMSCl; e) CH2=CCO2Me, PhH, 65-70°C; f) 5:1 MeOH-6M HCl; g) MeI, NaH, THF; h) MeLi, THF, -15°C; i) MeSO2Cl, Et3N, CH2Cl2; j) Li, NH3, THF, -78°C; k) *m*-CPBA, CH2Cl2; l) LDA, THF; m) SOCl2, Et2O, pyridine, 0°C; n) KOAc, DMSO, 75°C; o) NaOMe, NaOH; p) (MeO)2CHNMe2, xylene, Δ, 4Å sieves; q) *m*-CPBA, CH2Cl2; r) (Me3Si)2NLi, THF, 0°C; s) 1M HCl
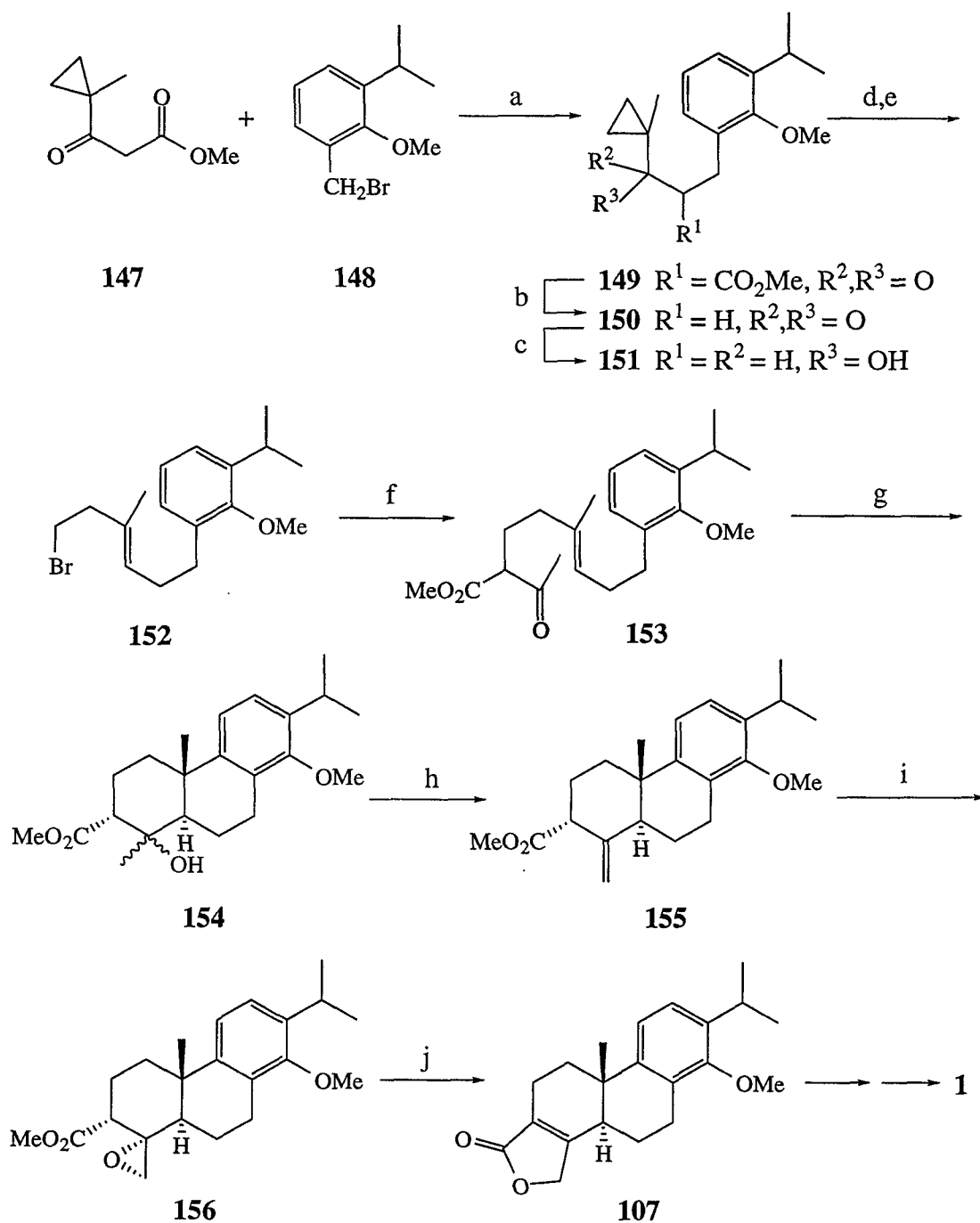
Scheme 1.3 Biogenetic-type Synthesis of Triptolide (**1**)

a) NaH, THF, 0°C; b) Ba(OH)$_2$, H$_2$O-Et$_2$O, 90°C; c) LAH, Et$_2$O, 0°C; d) LiBr, PBr$_3$, collidine, Et$_2$O, -40°C; e) ZnBr$_2$, Et$_2$O, 0°C; f) LiH, CH$_3$COCH$_2$CO$_2$Me, DMF, 75°C; g) SnCl$_4$, CH$_2$Cl$_2$, 0°C; h) MeSO$_2$Cl, Et$_3$N, CH$_2$Cl$_2$, 0°C; i) m-CPBA, CH$_2$Cl$_2$; j) LDA, -78°C

The only non-racemic synthesis of triptonide (**95**) (which could be further elaborated to *l* -triptolide (**1**)) from *l* -dehydroabietic acid (**157**) was also devised by van Tamelen and co-workers,[144] employing a strategy of introducing the C14 hydroxyl group first, and then constructing the A ring butenolide moiety followed by further elaboration of the epoxide system on ring C. Nitration of dehydroabietic acid (**157**) gave the 12,14-dinitro compound **158**, which was then subjected to catalytic hydrogenation to give a 12-amino-14-nitro compound **159**. Subsequent diazotization, and substitution by iodide led to **160**, which was then reduced to the amine **161**. Diazotization in trifluoroacetic acid yielded the trifluoroaceate **162**, which was then used to construct the ring A butenolide moiety. Curtius degradation of **162** provided the isocyanate **163**, which was then reduced to secondary amine with lithium aluminum hydride and converted to tertiary amine **164** by refluxing with formaldehyde and formic acid. Oxidation of **164** with *m*-CPBA gave the *N*-oxide, which underwent a Cope elimination in refluxing chloroform to give olefin **165**. Oxidative cleavage afforded ketone **166**, which, after quite a few steps, gave the important intermediate **171**. Introduction of the hydroxyl group at C7 followed by periodide oxidation led to the epoxy dienone **139**, which was further elaborated to yield *l*-triptonide (**95**). Even though the last step to *l*-triptolide (**1**) was not completed in this case, in view of the reported reconstitution of the triptolide system by sodium borohydride reduction of **95**, the above synthesis can be considered a synthesis of triptolide (**1**) as well. The yield from dehydroabietic acid (**157**)to **162** was reported to be 40%,[145] and the rest of the steps from **162** to **171** were calculated to be in the 0.68% yield range. Thus an overall yield of 0.27% from dehydroabietic acid (**157**) to **171** was achieved. The overall yield to Tl (**1**) could be even less.
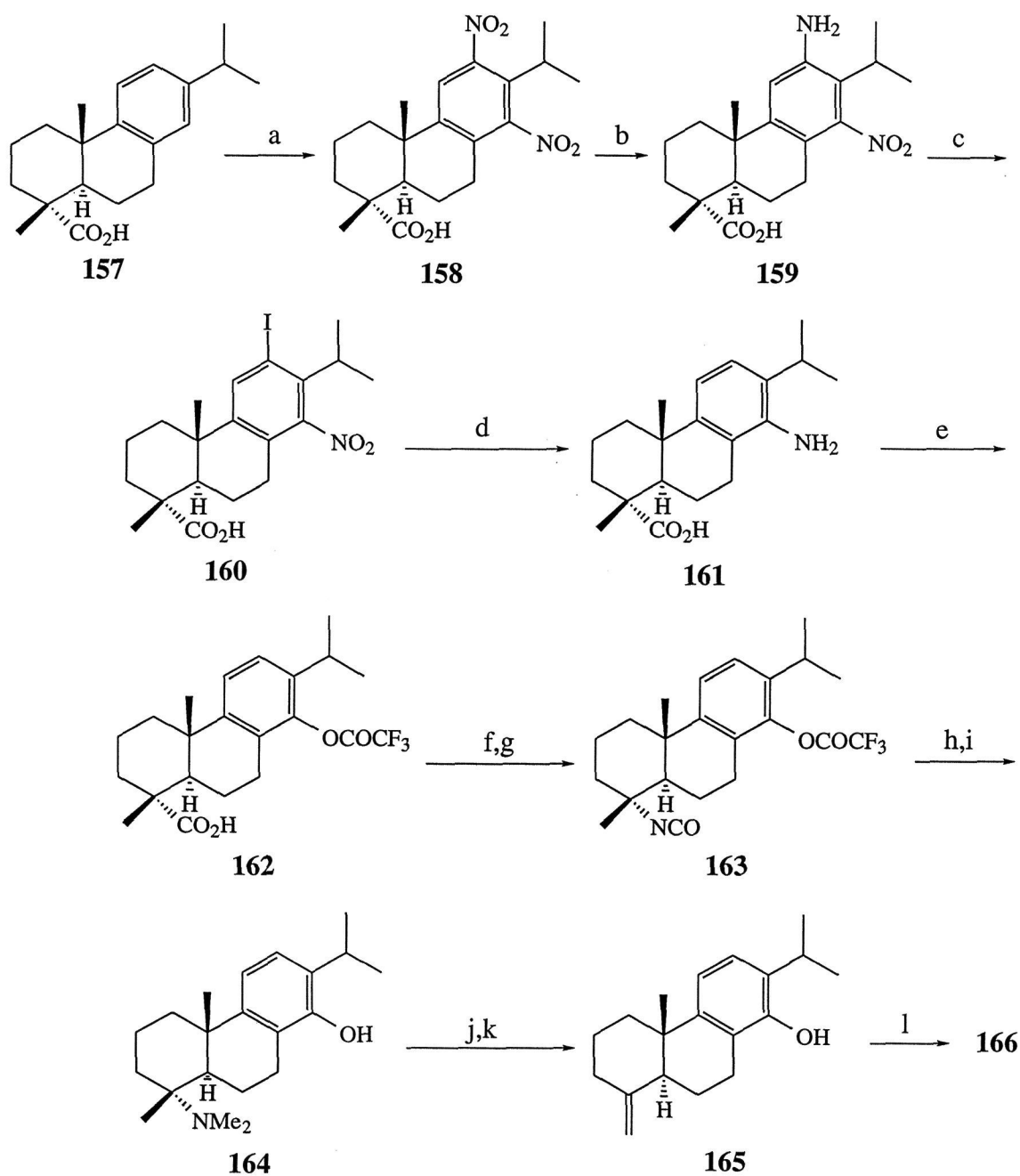
Scheme 1.4   Synthesis of Triptolide (**1**) from Dehydroabietic Acid (**157**)

a) HNO₃-H₂SO₄, HOAc; 60%; b) H₂, Pd/C, HOAc, CF₃CO₂H; 80%; c) NaNO₂, CF₃CO₂H; KI; d) Zn, HOAc, 65-70°C; e) NaNO₂, CF₃CO₂H; 40% from **157**; f) SOCl₂, PhH, DMF; g) NaN₃, acetone, H₂O; PhH, 100°C; 90% from **162**; h) LAH, THF, refluxing; i) HCO₂H-aq HCHO; 50% from **163**; j) m-CPBA, CHCl₃, -20°C; k) CHCl₃, refluxing; 80% from **164**; l) OsO₄, NaIO₄, HOAc-dioxane-H₂O; 30%

166

n-p [
— **167** R^1=H, R^2=CH$_2$OH, X^1,X^2=O
— **168** R^1=H, R^2=CH$_2$OH, X^1=OH, X^2=CH$_2$OCH$_2$Ph
q-s [
— **169** R^1=Ac, R^2=CH$_2$OH, X^1=OH, X^2=CH$_2$OCH$_2$Ph

170

x [
— **171** R=Ac, X^1=X^2=H
— **172** R=Ac, X^1,X^2=O
y,z [
— **138** R=H, X^1=OH, X^2=H
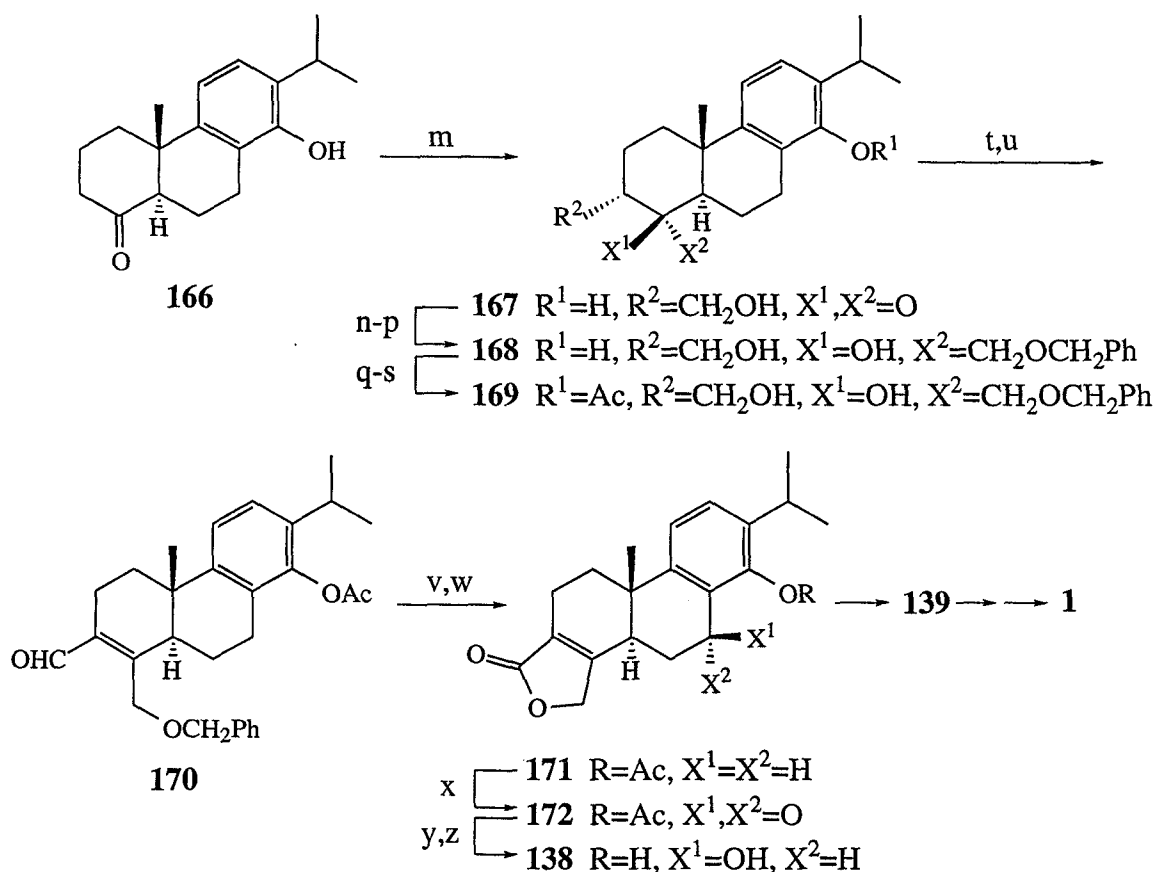
Scheme 1.4   Synthesis of Triptolide (**1**) from Dehydroabietic Acid (**157**) (continued)

m) LDA, HCHO, THF, -78°C; 50%; n) MeOC(CH$_3$)=CH$_2$, HOAc; o) PhCH$_2$OCH$_2$Li, THF, -78°C; p) HCl, THF; 70% from **167**; q) MeOC(CH$_3$)=CH$_2$, HOAc; r) Ac$_2$O, pyridine; s) HCl, MeOH; t) PCC, CH$_2$Cl$_2$; u) o-C$_6$H$_4$(NH$_2$)$_2$, PhCO$_2$H, EtOH, HCl; 18% from **168**; v) NaClO$_2$, HOSO$_2$NH$_2$, dioxane-H$_2$O; w) H$_2$, Pd/C, EtOH; 100% from **170**; x) CrO$_3$, HOAc-H$_2$O, 40°C; 20%; y) KOH, MeOH-H$_2$O; z) NaBH$_4$, EtOH; 95% from **172**

1.6   Plant Cell Culture Biotechnology

As more Tl (**1**) and Td (**2**) are required for detailed pharmacological studies or for potential clinical applications, isolation of these compounds from the plants would not be practical because of their low isolation yields (0.001%). The low isolation yields of Tl (**1**) and Td (**2**) and their interesting pharmacological activities were among the driving forces for the development of *T. wilfordii* cell cultures in the early 1980's by Professor Kutney's research

group. This technology provided a feasible alternative to obtain these valuable natural products in quantities much higher than from the plants.[146,147]

At the present time, there are usually three general methods employed in the production of these biologically active natural products:

1.   Isolation from the intact plant;

2.   Total synthesis or semi-total synthesis;

3.   Isolation from cell cultures of the plant.

Traditionally (and now still in application), isolation from the intact plant has played an important role in the pharmaceutical industry.[148] However, for large scale productions of these biologically active natural products for use as pharmaceuticals, various problems are inherent with the isolation technique. Usually, isolations from plants yield only minute quantities of the desired compounds (such as in the case of Tl (1) and Td (2)), and because of the complexity of the crude extracts, separation from co-occurring materials is often difficult, costly, and time-consuming. Furthermore, the concentration of the desired compound may vary according to the time of plant harvest. Other constraints are incurred by the fact that the plants may grow very slowly, and environmental concerns (a growing factor in recent years) along with geopolitical constraints may make the required plant species actually unavailable.[149] For example, taxol is extracted from the bark of 50-60 year-old Pacific yew trees and as many as 12 trees are required to produce enough taxol to treat one patient. It has been estimated that there are only about 10^6 trees left in nature and current requirements are about 10^5 trees per year.[150] Therefore, if the trees were harvested at the requirements we have now, this natural resource would disappear from our planet in 10 years.

Developing efficient synthetic strategies and methodology has always been the goal for organic chemists in order to eliminate the dependence on the living plant as the source for those natural products, and great progress has been made to date. Efforts to synthesize Tl (1) have been carried out by a number of research groups, though its total synthesis suffers from the disadvantages of multiple steps involved and overall low yields (*vide supra*). Moreover,

tripdiolide (2) has not been synthesized yet. This illustrates another frustration facing synthetic chemists and the pharmaceutical industry in general. The structural complexity inherent in these biologically active natural products demands multi-step syntheses, and thus has generated great interests and accounted for enormous development in organic synthesis to date. However, though many elegant syntheses have been accomplished in the laboratory, presently only some of them are finally applied in the commercial production of these compounds on a large scale.[151]

The methodology of plant cell culture development is not new, but is a rapidly expanding area of research that offers new, unique alternatives in acquiring the desired biologically active natural products.[151,152]

The interest in development of the science of *in vitro* tissue culture had been foreseen prior to the middle of the nineteenth century by promoters of the cell theory. One experimental approach was unsuccessfully attempted during the first years of the twentieth century, but success was only reached in 1912 with animal cells, and not until 22 years later with plant tissues. The technique of cell culture was rapidly exploited with animal cells, while in the case of plant tissue cultures, a long period of stagnation followed the initial establishment of basic methodology. After 30 years of relative indifference, thousands of scientists rediscovered this "new field" of plant biology, which currently is undergoing considerable expansion under the new name of biotechnology.[152]

It should be noted, however, that in the last two or three decades of fast development in this area, a substantial percentage of work has been directed to the use of plant cell cultures to study the more "biological" aspects of such cultures, such as investigating cell growth regulation, cell structure, cytodifferentiation, somatic embryogenesis, morphogenesis, and physiology.[151] Fermentation technology with fungal and bacterial cultures has made dramatic advances particularly in the pharmaceutical industry, but similar technology with plant cells has not yet reached this level of application.[151] Therefore, there has been a great need to explore

the potential of plant cell cultures for the production of plant-derived biologically active compounds.

There are several advantages associated with plant cell culture biotechnology:[151]

1. The desired compounds can be produced year-round under controlled laboratory conditions, assuring a steady supply of the materials without seasonal fluctuation;

2. Cloning provides selected cell lines for optimized production of desired agents, and metabolic processes may be regulated or manipulated to maximize the production of the desired compounds in yields higher than in the wild-type plant;

3. The cell cultures provide an excellent media for biosynthetic studies, biotransformation studies and even enzyme isolation.

These advantages are quite obvious and there are many examples. There have been some successes in the commercial production of secondary metabolites by plant cell cultures such as the red dye shikonin by cell cultures of *Lithospermum erythrorhizon* and the yellow anti-microbial dye, berberine, by a cell culture of *Coptis japonica*. There have also been a few notable successes in the elucidation of the biosynthesis of secondary metabolites by using cell culture technology, such as the discovery of the enzymatic steps required to synthesize berberine.[150]

In 1980, Kutney *et al.* first reported the production of tripdiolide (2) by a cell culture of *T. wilfordii* grown in modified B-5 and PRL-4 suspension media. A yield of 0.003% tripdiolide (2) was reported based on the dry cell weight, and thin layer chromatography (TLC) evidence for the presence of triptolide (1) was also reported.[146] A thorough program by Kutney *et al.*, aimed at maximization of Td (2) yield by way of cell line selection and media optimization, resulted in the development of a callus cell line from a leaf explant of *T. wilfordii*, designated as TRP4a.[147] The callus was initiated on PRI_2Co_{100} agar (PRL-4 medium of Gamborg and Eveleigh[153] (without casein hydrolysate) which was supplemented with indole-3-

acetic acid (I) (2 mg/L) and coconut milk (Co) (100 mL/L)). After initiation, the callus was transferred and maintained on PRD_2Co_{100} agar (PRL-4 medium supplemented with 2,4-dichlorophenoxyacetic acid (D) (2 mg/L) and coconut milk (100 mL/L)). The cell line was selected for further development based on TLC and KB cytotoxicity activity analyses and on growth vigour. Stock suspension cultures of TRP4a were initiated and maintained in PRD_2Co_{100} broth as a growth medium. Maintenance of the stock culture was carried out by subculturing of a 10% inoculum at 3 week intervals into fresh media. An extensive study of the growth parameters revealed that resuspension from the growth medium into $MSNA_{0.5}K_{0.5}$ broth (MS medium of Murashige and Skoog[154] supplemented with naphthaleneacetic acid (NA) (0.5 mg/L) and kinetin (K) (0.5 mg/L)) produced the highest levels of tripdiolide. After 35 days of incubation, tripdiolide (2) levels peaked at 4.0 mg/L, a level 36 times greater than that isolated from the whole plant (based on a dry cell weight of 10 mg/mL). Therefore, the development of plant cell culture technology has provided an additional and more effective route for the production of Tl (1) and Td (2). A study on the levels of various nutrients in the media also showed that Td (2) production was highest in $MSNA_{0.5}K_{0.5}$ medium containing 1650 mg/L of ammonium nitrate, 40 g/L of sucrose, and 880 mg/L of calcium chloride. The method for the production of the TRP4a cell line and growth of cell suspension cultures for Td (2) production is summarized in Figure 1.2. The TRP4a cell line has been a stable cell line since its establishment in the early 1980's and is continuously producing the important diterpene metabolites triptolide (1) and tripdiolide (2). Two other groups have developed suspension cultures of *Tripterygium*, but the maximum yields of Td (2) were relatively low (below 0.01%).[155,156]
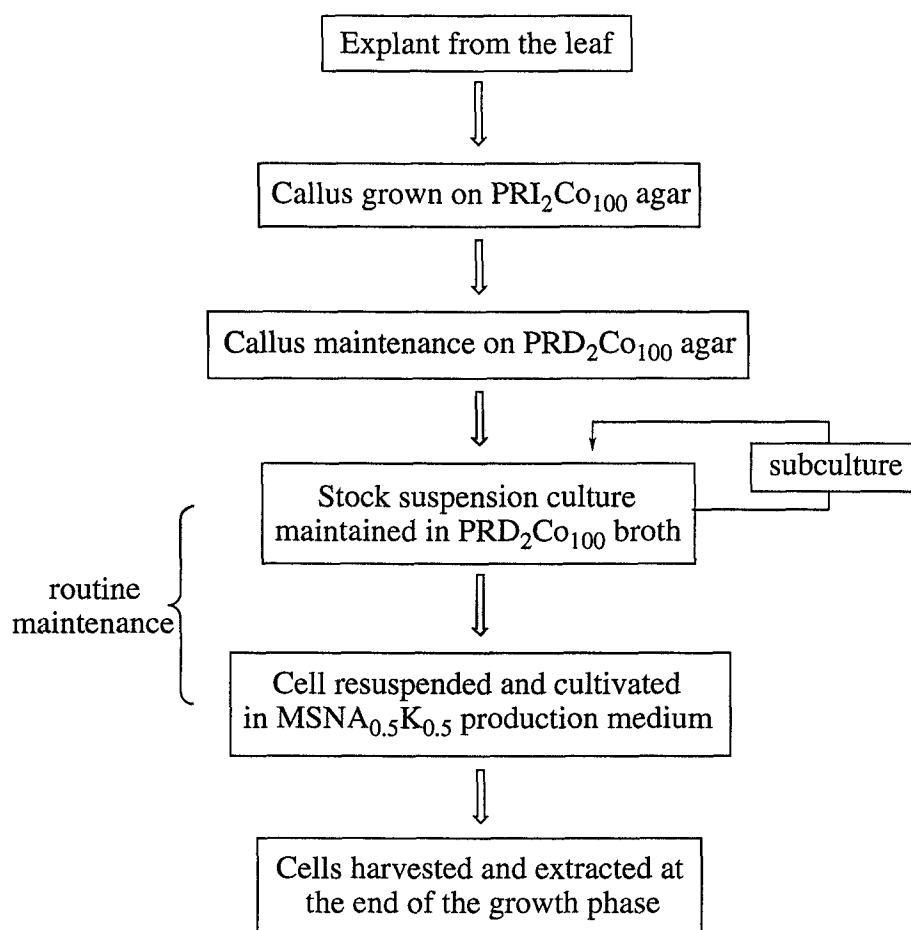
Figure 1.2   Development and Routine Maintenance of the TRP4a Cell Line of *T. wilfordii*

In addition to triptolide (**1**) and tripdiolide (**2**), several other metabolites have been isolated from the TRP4a cell cultures. Most of the metabolites were isolated under the guidance of biological assay. Some were new compounds (**174-176, 183**), while one (**78**) was later found in the *T. wilfordii* plant. Others (**64, 68, 77, 184**) have been isolated from *Tripterygium* plants, but the rest have not yet been isolated from the *Tripterygium* plants.[146,157,158] These compounds include quinone methide-type compounds (**64, 173, 173-A**), friedelene-type triterpenes (**68, 174**), oleanene-type triterpenes (**77, 78, 175-180**), a ursene-type triterpene (**181**), diterpenes (**157, 182, 183**) and phytosterols (**184**). There has not been detailed isolation work on diterpene metabolites from this cell culture.

Compounds isolated from TRP4a cell cultures:
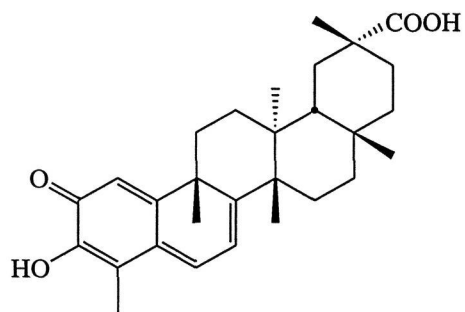
64

173

173-A

68

174

77 R = α-OH
78 R = β-OH
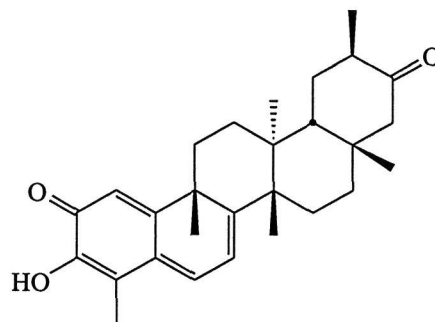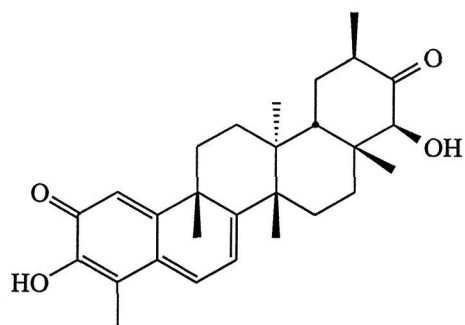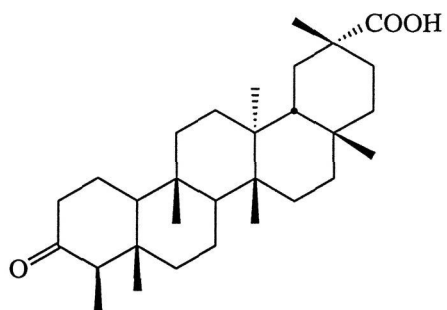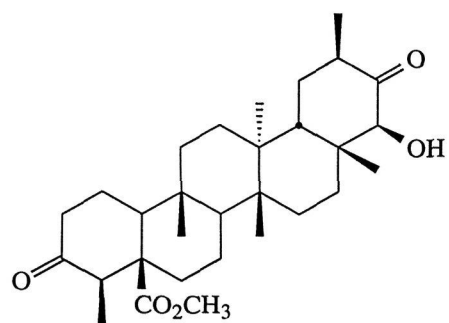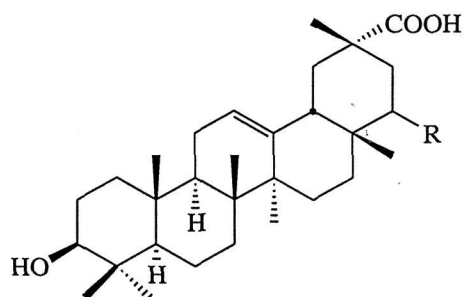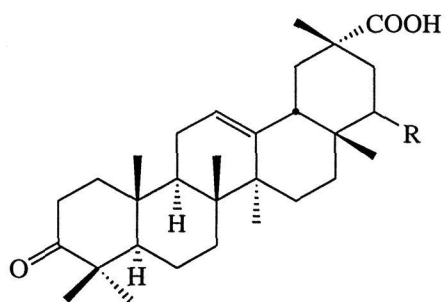
175 R = β-OH
176 R = α-OH
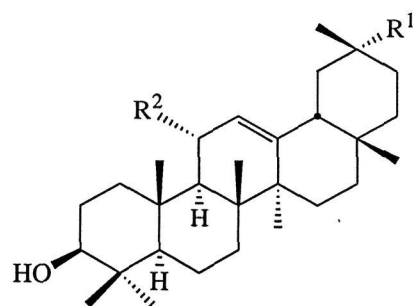
177 R¹ = R² = H
178 R¹ = CH₂OH, R² = H
179 R¹ = H, R² = OH
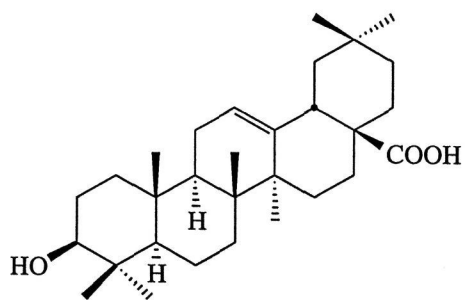
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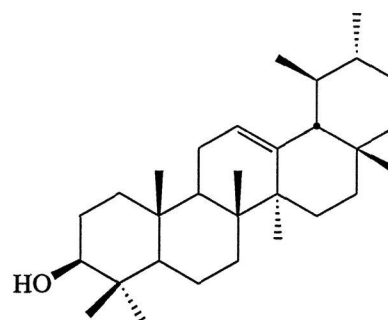
181
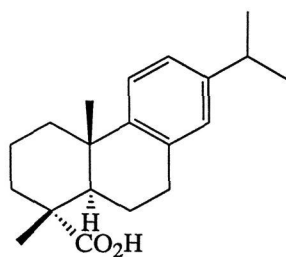
157 dehydroabietic acid
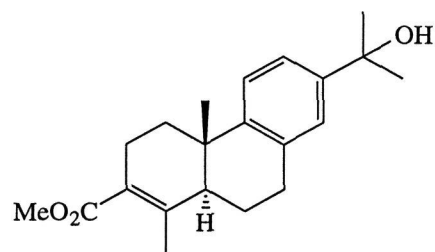
182
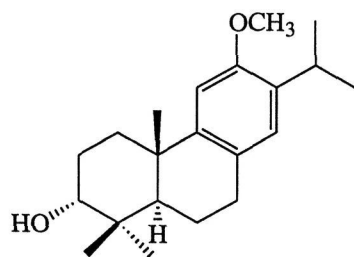
183
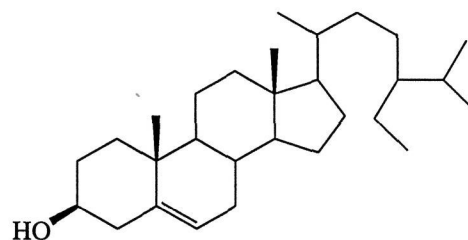
184

Manipulation of cell cultures to obtain higher production yields of desired secondary metabolites not only can be achieved by changing the media nutrients and growth parameters as we have seen in the development of the TRP4a cell line, but also may be accomplished by adding synthesized biosynthetic intermediates, or precursors, through biotransformations, or by elicitation.[150]

Early biotransformation studies with TRP4a cell cultures were focused on the formation of the butenolide moiety of the molecule with mixed results.[159,160] M. Roberts used isodehydroabietenolide (**193**) as a starting material and obtained several products with hydroxyl groups introduced at C7 and/or C2 positions, but no change to the C ring was observed.[160] F. Kuri-Brena continued the work and started to use the C ring "activated" precursor, isotriptophenolide (**194**), for the biotransformation study in an attempt to bring some changes to the C ring. The results showed that only small amounts of the starting material were transformed to its methyl ether while most of the starting material was recovered.[161] The detailed results will be given in Chapter 4 of this thesis.

On another front, M. Samija of this group succeeded in increasing the yield of some triterpene acids, which are among those with biological activities, by elicitation of the cell cultures with fungal preparations.[162]
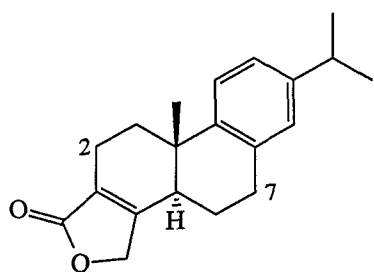
isodehydroabietenolide (**193**)   isotriptophenolide (**194**)
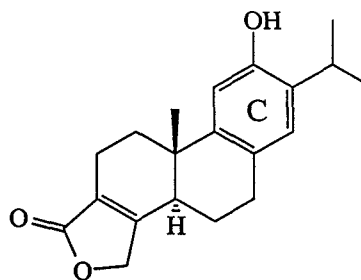
Biotransformation studies of TRP4a cell cultures by other members of this group showed that the cell cultures were capable of hydroxylating or epoxidizing compounds with totally different structures (*e.g.*, tobacco cembranoids).[163]

From our previous work related to the TRP4a cell cultures, it can be seen that we have achieved our primary goal of obtaining triptolide (**1**) and tripdiolide (**2**) by cell culture in greater yields than from the plants, and we also have acquired some results and experience in biotransformation studies. It is important then, to take advantage of what we have achieved and to advance further.

1.7   Challenges and Current Research Trends

As was previously mentioned, the immunosuppressive and antifertility activities from the compounds isolated from *Tripterygium* overlap, and their toxicity is still relatively high. These side effects and toxicity, though not very serious, are definitely not desirable. These potential problems restrict the use of these compounds as a long term measure for male contraception, or for prolonged treatment of patients with rheumatoid arthritis or skin disorders, or as potential anti-rejection agents for organ transplantation. Therefore, looking for close analogs of this class of compound but with more defined pharmacological activity and less toxicity has been the focus of current research efforts in recent years.

Major efforts that have been made include:

1.   Isolation of new compounds from native plants or their extracts, with a focus on Tl (**1**) and Td (**2**) closely related diterpene epoxides;

2.   Structural modifications of relatively readily available diterpene epoxides such as Tl (**1**); and

3.   Combination of organic synthesis with plant cell culture technology to obtain related novel diterpene epoxide analogs.

Several new, related diterpene epoxides have been isolated from extracts of *Tripterygium* plants by Chinese researchers in recent years. These epoxides include triptolidenol (**97**), 16-hydroxytriptolide (**98**), triptetraolide (**99**), triptriolide (**100**), isotetraolide (**101**), 12-epitriptriolide (**102**), tripchlorolide (**103**), 13,14-epoxide-9,11,12-trihydroxytriptolide (**104**) and tripdioltonide (**105**) (structures etc., *vide supra*).

Tripchlorolide (**103**) was isolated from GTW and was suspected to be an artifact since organochlorine compounds usually do not occur naturally in higher plants. This compound may have been produced from Tl (**1**) by the action of HCl at some stage in the extraction process, for instance, during the extraction with chloroform, which usually contains traces of HCl. This hypothesis was confirmed by chemical correlation experiments by different groups.[73,74] Treatment of Tl (**1**) with HCl in acetic acid (0.4 N) at 0-4°C gave tripchlorolide (**103**), which could be converted back to Tl (**1**) by a brief reflux in ethanol under basic conditions.[73]

triptolide (**1**)   tripchlorolide (**103**)

Interestingly, compared with Tl (**1**), tripchlorolide (**103**) exhibited less toxicity without significantly compromising its activities.[128,130] Another isolated compound, triptriolide (**100**), showed very low toxicity (LD$_{50}$>250 mg/kg) but stronger anti-inflammatory activity.[71] These findings have inspired people to modify the triepoxide system in order to find more effective compounds.

Yu *et al.* prepared 9 derivatives (**103, 185-192**) from Tl (**1**) and studied their structure-activity relationship. They found that the chlorohydrin **103** and bromohydrin (**185**) had similar

activities as Tl (**1**), but the chlorohydrin **103** was less toxic than Tl (**1**). However, the rest of the compounds (**186-192**) all showed much decreased activities. In view of the facile elimination of HCl or HBr from **103** or **185** to form Tl (**1**), (note: they obtained 91.8% yield of **103** from Tl (**1**) and 88.0% yield of Tl (**1**) from **103** under mild conditions), they suggested that these two compounds might undergo an identical elimination step to form Tl (**1**) in biological systems, thus showing similar activity as Tl (**1**). Other derivatives that did not revert back to Tl (**1**) easily, or had the C14 hydroxyl group blocked (**191**), showed that their activities were greatly reduced. However, they did not offer any explanations as to why the toxicity of **103** was lower.[104] One fact that is clear from their results is that the C12, C13-epoxide and C14 hydroxyl groups likely play important roles in the biological activities of this class of compounds, and thus closely related analogs could be more effective.
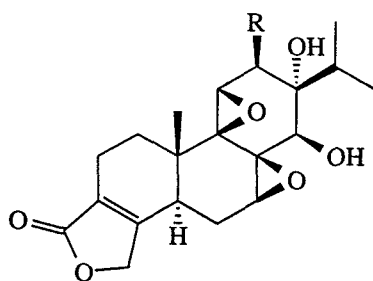
103	R = Cl		**189**	R^1 = H, R^2 = OH
185	R = Br		**190**	R^1 = Cl, R^2 = H
186	R = SPr			
187	R = OAc			
188	R = OCH_3			
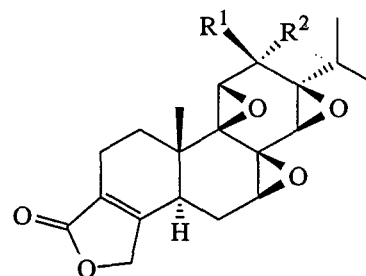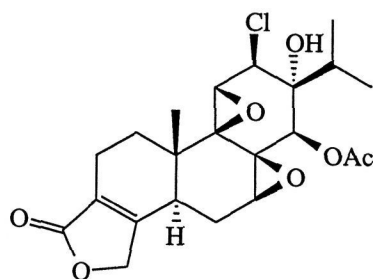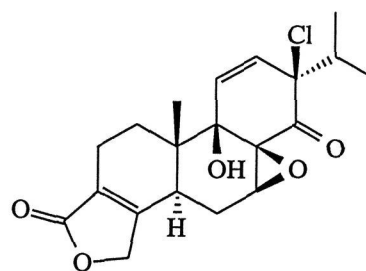
191   **192**

The combined approach of chemistry and plant cell culture biotechnology by Professor Kutney's group has led to a number of results. The most recent and interesting results will be discussed in the following chapters of this thesis.

Other new developments in recent years include the establishment of quantitative HPLC and high resolution gas chromatographic (GC) analyses of Tl (**1**) in prepared drug formulations or plant specimens.[135,164,165] GC-MS analysis of volatile constituents from *Tripterygium* plants have also been investigated.[97]

1.8 Objectives and Strategies of the Present Investigation

In view of the challenges and current interests related to *Tripterygium*, and our advantage of having an established *T. wilfordii* cell culture, the present investigation is aimed at a systematic study in an attempt to:

1. Explore the possibility of increasing Tl (**1**) and Td (**2**) yields by incubating appropriate synthetic precursors;

2. Obtain Tl (**1**) and Td (**2**) analogs by a combination of organic synthesis and biotransformation;

3. Isolate new diterpene metabolites from the cell cultures in order to understand the metabolism of these diterpenes and the possible link to the biosynthesis of Tl (**1**) and Td (**2**).

Previous results have shown that there is a great similarity in the spectrum of metabolites isolated thus far from TRP4a cell cultures to those isolated from the whole plant, suggesting that the diterpenes and triterpenes are metabolized in a similar way as they are in whole plants. Therefore, our strategy was to systematically isolate diterpene metabolites from the cell cultures (Chapter 2), combine the information we would obtain from the isolation and the results from previous biotransformation studies to design and synthesize appropriate precursors (Chapter 3), and then carry out biotransformation studies with these synthetic precursors (Chapter 4).

By systematic isolation of these diterpenes, we could obtain some information about the metabolism of related diterpenes, which probably would give us some ideas about the biosynthesis of Tl (1) and Td (2). This would also help us in choosing appropriate precursors for biotransformation studies, and at the same time provide information as to the diterpenes naturally present in the cell culture in order to differentiate them from possible biotransformation products. In addition, isolated diterpene metabolites may have some biological activities as well.

With respect to biotransformation studies, and at the time when this program was initiated, we were in a position to evaluate ring C "activated" precursors. It was of interest to have access to various ring C "activated" precursors, available by chemical synthesis, since such substances may increase the possibility of enzyme-catalyzed biotransformations to novel ring C functionalized diterpene analogs. These latter compounds may possess interesting pharmacological activities and would provide some information about the biosynthesis of Tl (1) and Td (2).

CHAPTER 2   ISOLATION AND STRUCTURE ELUCIDATION OF DITERPENE METABOLITES FROM PLANT CELL CULTURES OF *T. WILFORDII*

2.1   Introduction

As noted earlier, the unique triepoxide moiety of diterpenoids Tl (1) and Td (2) is the key element to their pharmacological activities. In addition to Tl (1) and Td (2), various other diterpenoids have been isolated from *Tripterygium* plants. Some of them have a close structural relationship with Tl (1) and Td (2), but they are not likely to be the biosynthetic intermediates of Tl (1) and Td (2) (*vide supra*). In previous studies, our group had isolated some triterpenes but only a few diterpenes (including Tl (1) and Td (2)) from the TRP4a cell cultures. A main focus of the earlier studies was directed at isolation of pharmacologically active compounds. A detailed investigation aimed at more extensive isolation of diterpene metabolites, in order to obtain some insight into their metabolic processes, and their possible connections to the biosynthesis of Tl (1) and Td (2), was desirable. Cell culture technology provided us with a very powerful tool to accomplish our goal because it produces large quantities of fresh materials with constant quality, while isolations from whole plants are usually done on dried material, in which some components may have been lost or undergone some irreversible changes.

As discussed previously, the diterpenoids isolated from *T. wilfordii* plant sources or the cell cultures are basically abietane or *abeo*-abietane type diterpenes. Most of them have various functionalizations on rings A and C, while some of them have functional groups on ring B as well. Characteristic signals of these functionalities, observable in ^{1}H NMR spectra, are signals of an AM or AMX system* in the aromatic region, a resonance between δ 4.5-5.0

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* A and M are much more strongly coupled with each other than with X for all relevant compounds discussed in this thesis. In some cases, the spin system may be classified as an AB or an ABX system; however, for the sake of convenience, the term AM or AMX system will still be used in those situations.

from methylene protons of the butenolide moiety, signals from protons on epoxide rings, and the readily recognizable isopropyl group signals. These distinct resonances provided a guide to locating these diterpenoid compounds during isolation studies. Extracts from the cell cultures were column chromatographed and all eluates were pooled into fractions based on their major spots on TLC plates. These fractions were briefly analyzed by ^{1}H NMR, and those which showed relevant signals were further purified.

2.2   Results and Discussion

T. wilfordii Plant Cell Cultures (TRP4a): Growth and Production

The *T. wilfordii* plant cell culture was first initiated in the late 1970's, and the TRP4a cell line was selected in the early 1980's, based on its ability to maximize the production of Td (**2**) and Tl (**1**). Since then, the culture has been grown and maintained according to the published procedure outlined in *Plant Medica* (1983).[147] A minor change made in the past few years to the original protocol was that the subculturing interval was shortened to around 2 weeks from 3 weeks, because no significant changes in the growth and metabolism of the cell cultures had been observed with the shorter subculturing interval. Thus, the cells are maintained in PRD_2Co_{100} medium (growth medium) with subculturing to new growth medium at 14 day intervals. At the time of subculturing, an inoculum (10%, v/v) of the cells are transferred to $MSNA_{0.5}K_{0.5}$ medium (production medium) for the production of Tl (**1**) and Td (**2**). All cell suspension cultures grown in conical flasks were incubated without illumination at $27 \pm 1°C$ on a rotary shaker with a 7/8" throw and run at 140 rpm. The cell suspension cultures that were used in this study were grown in $MSNA_{0.5}K_{0.5}$ medium.

Previous results showed that the growth of the TRP4a cells includes a lag phase (first 7-10 days), a growth phase (from 7-10 to 25-35 days) and a stationary phase (after 25-35 days). Td (**2**) and Tl (**1**) started to accumulate rapidly during the growth phase and leveled off when

cell growth reached the stationary phase. With the isolation of biosynthetic intermediates in mind, the cell culture was harvested near its stationary phase and utilized for the isolation study.

```
                         ┌──────────────────────────┐
                         │ cell suspension cultures │
                         └──────────────────────────┘
                                    │
                                    │ filtration through Miracloth™
                         ┌──────────┴──────────┐
                         ▼                     ▼
                     ┌───────┐             ┌───────┐
                     │ broth │             │ cells │
                     └───────┘             └───────┘
                         │                     │
         saturation with NaCl,      homogenization
         extraction with EtOAc      in EtOAc
                         │                     │
         solvent                     filtration,
         removal                     separation
                         │           ┌─────────┴─────────┐
                         ▼           ▼                   ▼
               ┌──────────────┐  ┌─────────────┐   ┌─────────────┐
               │ broth extract│  │ aqeous layer│   │ EtOAc layer │
               └──────────────┘  └─────────────┘   └─────────────┘
                                      │                   │
        saturation with NaCl,         │                   │
        extraction with EtOAc ────────┘                   │
                                       └─────────┬─────────┘
                                                 │ solvent
                                                 │ removal
                                                 ▼
                                         ┌──────────────┐
                                         │ cell extract │
                                         └──────────────┘
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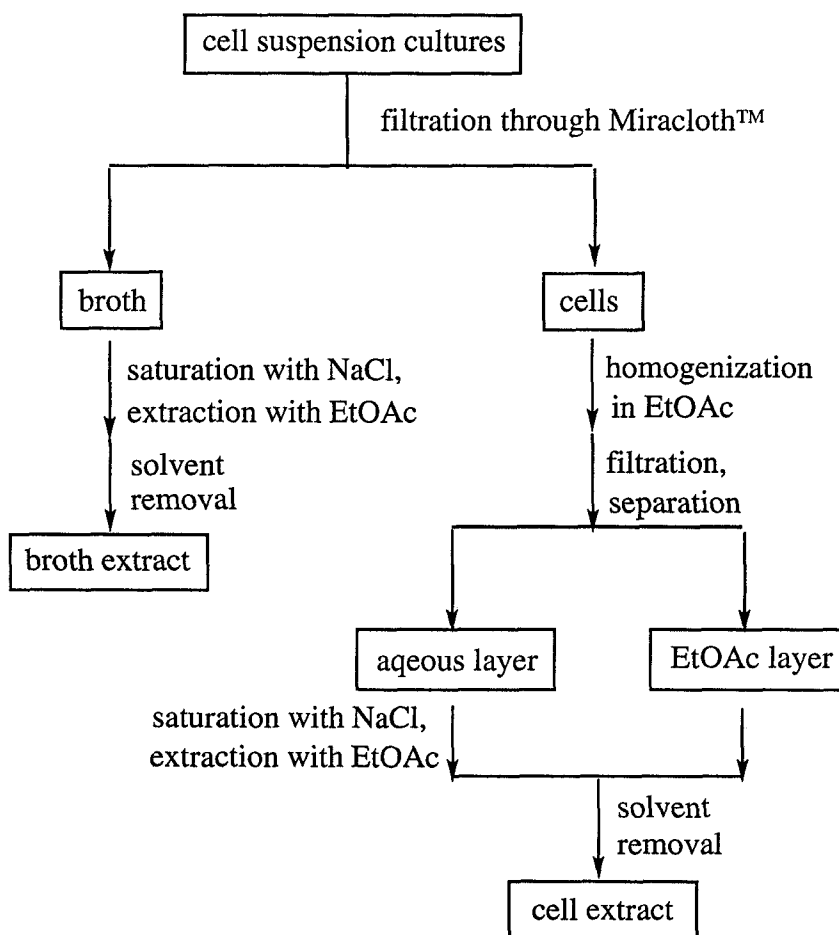
Figure 2.1   General Procedure for Cell Culture Harvesting

The general procedure for harvesting and extracting the cell culture is outlined in Figure 2.1. The cell cultures (5 x 550 mL) were harvested after growth in $MSNA_{0.5}K_{0.5}$ medium for 28 days (n_D = 1.3330, pH = 6.29). The combined cell suspenion was filtered through Miracloth™, a coarse fibrous cloth which separates solid cell substances from liquid broth. The cells (wet weight 299 g) were frozen until the time of extraction. The broth was saturated with sodium chloride and extracted with ethyl acetate. The ethyl acetate solution was dried,

filtered, and the solvent was removed to give a brown colored broth extract (543 mg). The cells were thawed and homogenized in ethyl acetate. The resulting suspension was filtered through Celite and the filtrate was separated into an ethyl acetate layer and an aqueous layer. The aqueous layer was re-extracted with ethyl acetate and the ethyl acetate extracts were combined. Removal of the solvent yielded a dark colored cell extract (1.04 g).

Isolation of Diterpene Metabolites from TRP4a Cell Cultures

TLC analysis (toluene:chloroform:ethyl acetate:formic acid, 105:48:45:3, developed twice) of the broth and cell extracts exhibited similar chromatograms, except that the cell extract showed more of the faster migrating spots (less polar compounds) compared to the broth extract. The broth extract and cell extract were combined and extensive column chromatography on silica gel was performed.

The chromatographic separation of the extract is depicted in Figure 2.2. After repeated column chromatography (c. c.) with various solvent systems, 29 compounds were isolated.

Chromatographic purification of a previously isolated, crude Td fraction (49 mg) provided additional quantities of 209 and a small amount of 212 (0.2 mg). Since 212 was also suspected to be produced by the cell cultures, it is discussed here together with the other isolated compounds.

The compounds isolated from the extract of the TRP4a cell culture are summarized in Table 2.1.

Combined Broth and Cell Extract (1.58 g)

c. c.
i)   hexanes-EtOAc (6:4, 5:5, 3:7, 1:9)
ii) EtOAc
iii) MeOH

I   II   III   IV   V   VI   VII

I (264 mg)

c. c.
CH_2Cl_2-EtOAc (95:5, 90:5, 1:1)

IA   IB   IC   ID   IE   IF   IG

c. c.
hexanes

195
(2.6 mg)

c. c.
i) hexanes
ii) hexanes-EtOAc
(97:3)

c. c.
i) CH_2Cl_2-EtOAc (97:3)
ii) hexanes-acetone (9:1)
iii) CH_2Cl_2-EtOAc (95:5)

108
(0.1 mg)

IB1

c. c.
hexanes

IB2

c. c.
i) CH_2Cl_2-hexanes (3:7, 1:1)
ii) CH_2Cl_2

195   **196**
(8.5 mg)   (17.5 mg)

IB2a

c. c.
hexanes-acetone
(98:2)

IB2b

c. c.
hexanes-
acetone
(98:2)

IB2c

c. c.
hexanes-
acetone
(98:2)

(IB2ai)[a]   IB2aii   IB2aiii

c. c.
hexanes-
acetone
(97:3)

c. c.
hexanes-
acetone
(97:3)

198
(1.5 mg)

199
(1.0 mg)

196   **197**
(1.5 mg)   (1.0 mg)

---

[a] discarded

Figure 2.2   Column Chromatographic Separation of Extract from TRP4a Cell Cultures

IC (17.1 mg)

c. c.
hexanes-EtOAc
(95:5, 9:1)

200 **201** **202** **116**
(3.5 mg) (0.9 mg) (1.2 mg) (4.1 mg)

IE (14.9 mg)

c. c.
hexanes-
EtOAc
(85:15, 80:20)

IE1 IE2 IE3
118 **108**
(1.5 mg) (5.3 mg)

c. c.
CH_2Cl_2-
EtOAc
(99:1)

205
(1.2 mg)

ID (49.2 mg)

c. c.
hexanes-EtOAc
(9:1, 8:2, 7:3)

ID1 ID2 ID3 ID4 ID5 ID6 ID7
117

c. c. (12.3 mg) c. c. c. c. c. c. c. c.
CH_2Cl_2- hexanes- CH_2Cl_2- CH_2Cl_2- hexanes-
EtOAc EtOAc EtOAc EtOAc EtOAc
(98:2) c. c. (85:15) (99:1) (99:1) (1:1)
hexanes-
EtOAc
202 (9:1) **117** **193** **107** **107**
(2.3 mg) (9.2 mg) (1.4 mg) (2.1 mg) (1.4 mg)

ID2a ID2b ID2c
193
(1.4 mg) c. c. c. c.
CH_2Cl_2- CH_2Cl_2-
EtOAc EtOAc
(95:5) (95:5)

IG (8.8 mg)

c. c.
hexanes-EtOAc
(8:2)

203 **204** **109**
(1.1 mg) (2.1 mg) (3.0 mg)

Figure 2.2   Column Chromatographic Separation of Extract from TRP4a Cell Cultures
(continued)

II (125 mg)   III (38.8 mg)

c. c.   c. c.
CH₂Cl₂-EtOAc   CH₂Cl₂-EtOAc
(97:3, 3:7, 9:1, 8:2, 6:4, 1:1)   (95:5, 9:1, 8:2,
                                          2:1, 1:2)

IIA   IIB   IIC   IID

c. c.   **106** (2.1 mg) **109**   **206**   **106**
hexanes-   **108** (2.3 mg)(4.0 mg) (2.9 mg)   (2.7 mg)
EtOAc
(6:4)

108   **106**
(1.1 mg)   (6.3 mg)

IV (128 mg)   V (85.1 mg)

c. c.   c. c.
CH₂Cl₂-EtOAc   CH₂Cl₂-EtOAc
(9:1, 8:2, 6:4,   (95:5, 9:1, 8:2,
4:6, 1:4)   2:1, 1:2)

IVA   IVB   IVC   IVB (11.9 mg)   VA   VB
106   **138**   Tl (**1**)
(1.0 mg)   (2.4 mg)   (0.6 mg)

                                                    c. c.
                                                    CH₂Cl₂-
                                                    EtOAc
                                                    (9:1)

                                            207
                                           (0.8 mg)

Figure 2.2   Column Chromatographic Separation of Extract from TRP4a Cell Culture
              (continued)

VI (119 mg)

c. c.
i) CH$_2$Cl$_2$-EtOAc
(8:2, 6:4,
4:6, 1:4)
ii) EtOAc

VIA
Tl (1)
(0.6 mg)

VIB

c. c.
CH$_2$Cl$_2$-EtOAc
(8:2, 2:1),

VIB1
Tl (1)
(0.6 mg)

VIB2
Td (2)
(54.8 mg)

VIB3

c. c.
hexanes-
EtOAc
(3:7)

VIB3i

c. c.
hexanes-
acetone
(7:3)

208
(0.6 mg)

VIB3ii
209
(1.0 mg)

VII (599 mg)

c. c.
i) hexanes-CHCl$_3$-
EtOAc-HCO$_3$H
(110:50:45:3,
110:50:184:3)
ii) CHCl$_3$-EtOAc-HCO$_2$H
(50:150:3)

VIIA
210
(2.9 mg)

VIIB

c. c.
hexanes-CHCl$_3$-
EtOAc-HCO$_3$H
(150:50:45:3,
200:50:45:3)

211
(0.7 mg)

Figure 2.2   Column Chromatographic Separation of Extract from TRP4a Cell Culture
(continued)

Table 2.1   Compounds Isolated from TRP4a Cell Cultures (5 x 550 mL)

Compound	mg	Compound	mg	Compound	mg	Compound	mg
106	12.1	**138a**	2.4	**200**	3.5	**207**	0.8
107	3.5	**193**	2.8	**201**	0.9	**208**	0.6
108	8.8	**195**	11.1	**202**	3.5	**209**	1.0
109	7.0	**196**	19.0	**203**	1.1	**210**	2.9
116	4.1	**197**	1.0	**204**	2.1	**211**	0.7
117	21.5	**198**	1.5	**205**	1.2	**212**	0.2
118	1.5	**199**	1.0	**206**	2.9	**Tl (1)**	7.3
						Td (2)	54.8
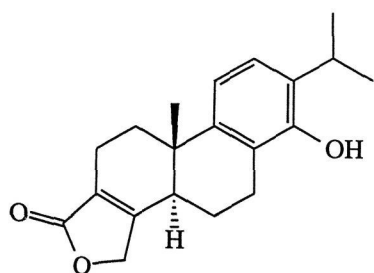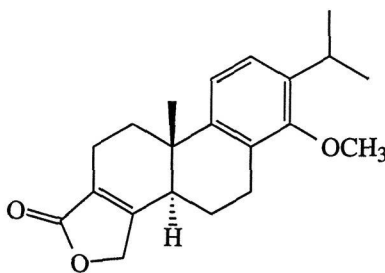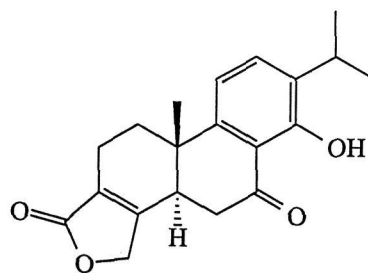
106   **107**   **108**
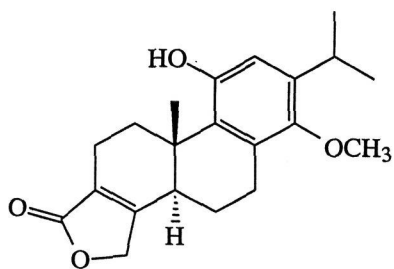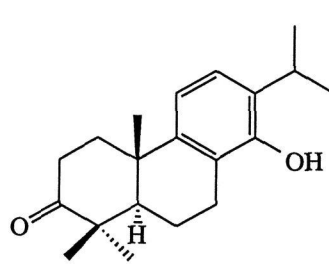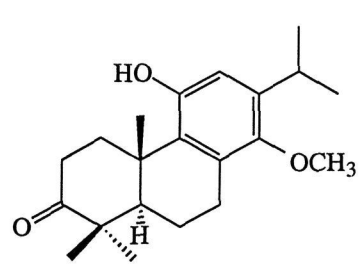
109   **116**   **117**
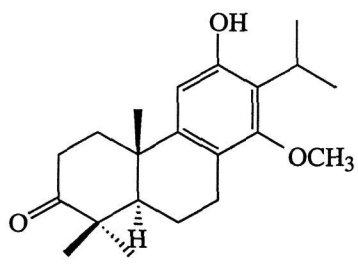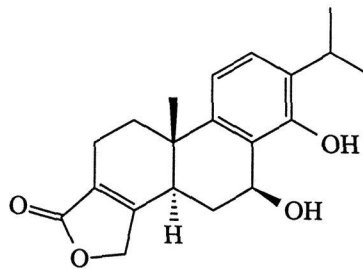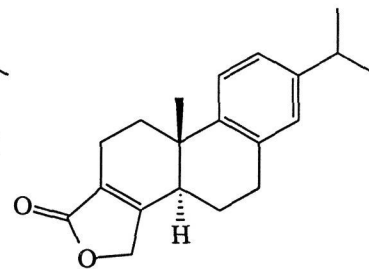
118   **138a**   **193**
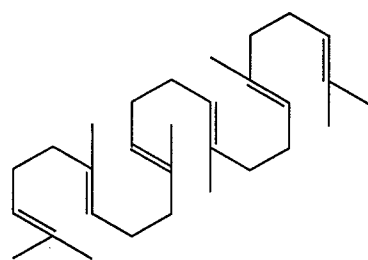
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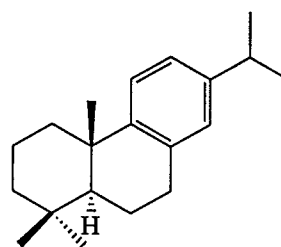
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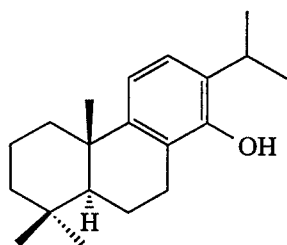
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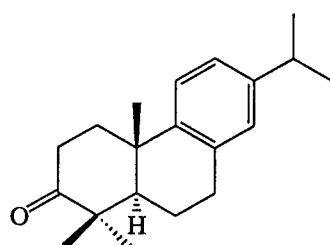
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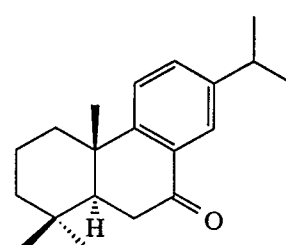
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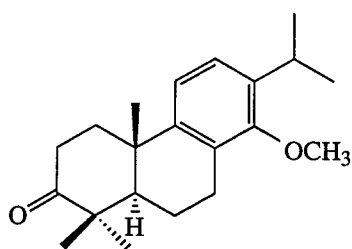
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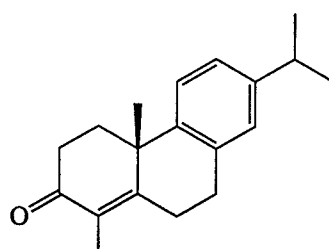
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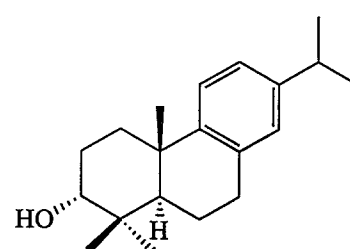
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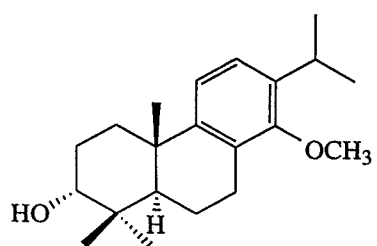
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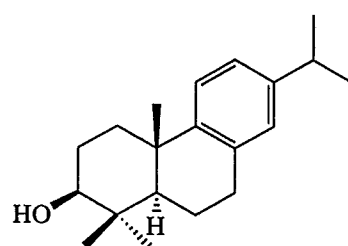
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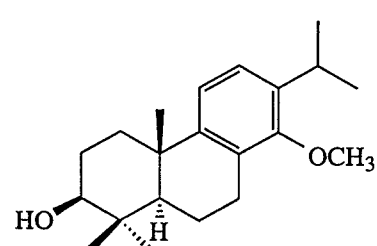
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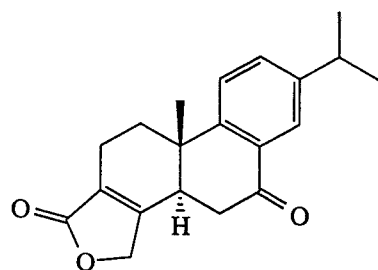
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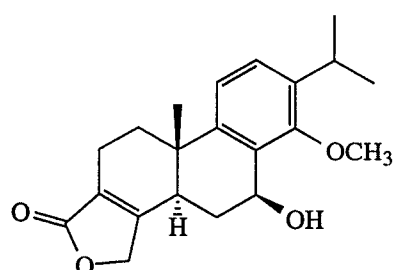
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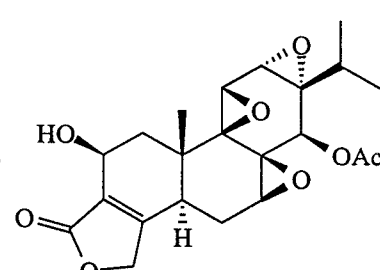
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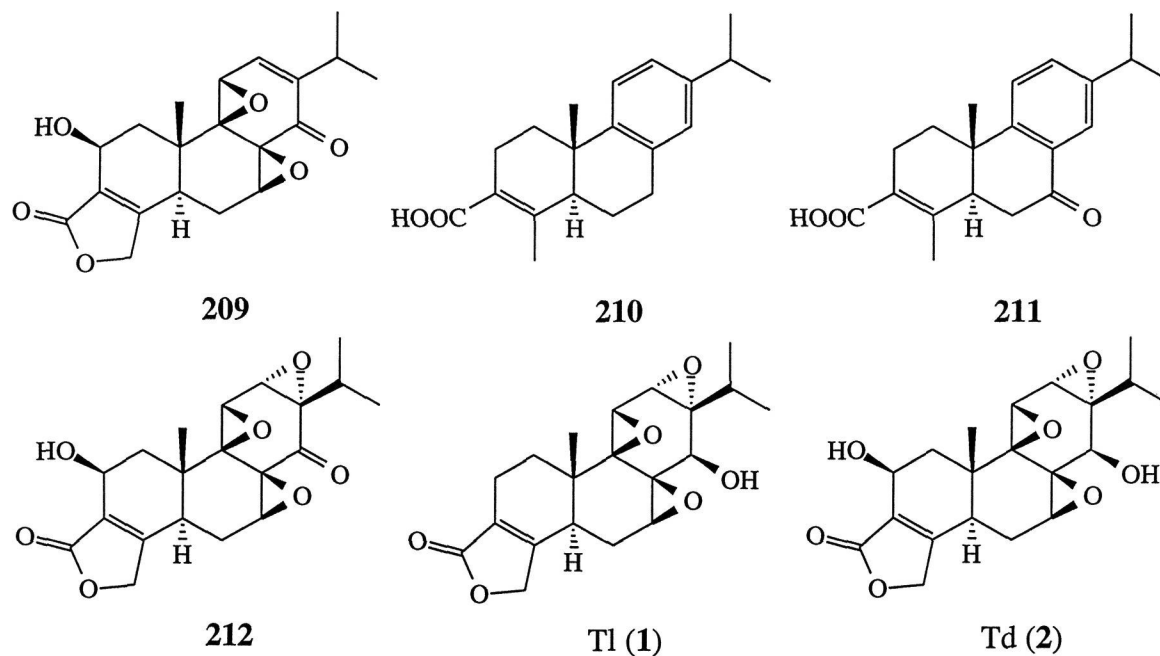
209

210

211

212

Tl (1)

Td (2)

Structure Elucidation of Diterpene Metabolites Isolated from TRP4a Cell Cultures

Compound **196** was isolated as an optically active ($[\alpha]_D^{20}$ +49.2°), colorless oil with a molecular weight of 270 (M$^+$). Its IR spectrum indicated aromatic (3070, 1600, 1480 cm^{-1}) and saturated hydrocarbon moieties (2975, 1460 cm^{-1}) in the molecule. High resolution mass spectrometry gave a molecular mass which corresponds to a molecular formula of $C_{20}H_{30}$.
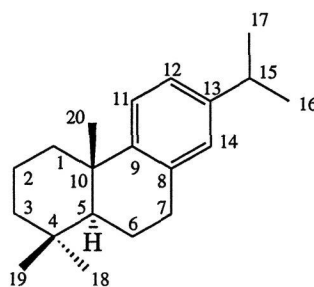
196

The ^{1}H NMR spectrum of **196** showed signals of an AMX system in the aromatic region (δ 6.88, 1H, d, J = 1.6 Hz; δ 6.97, 1H, dd, J = 8.2, 1.6 Hz; δ 7.16, 1H, d, J = 8.2 Hz), indicating an 1, 2, 4 tri-substituted benzene ring. Three tertiary methyl signals were found at δ 0.92, 0.93, 1.17, and two secondary methyl signals were found at 1.21 (6H, d, J = 6.9 Hz), respectively. The 2D ^{1}H-^{1}H COrrelated SpectroscopY (COSY) spectrum showed that the secondary methyl signal at δ 1.21 was coupled with a signal at δ 2.83 (1H, sept, J = 6.9 Hz), indicating that an isopropyl group was attached to the aromatic ring. The multiplet (2H) at δ 2.89, which was coupled with signals at δ 1.64-1.81 (1H) and δ1.86 (1H), was apparently due to benzylic methylene protons (H7). The remaining carbon attached to the benzene ring was therefore a tertiary carbon since no other benzylic proton signals were found in the spectrum. All the spectral information suggested that **196** had a dehydroabietane type skeleton. In view of the fact that dehydroabietic acid (**157**) had been previously isolated from TRP4a cell cultures by this group, and that this compound has only carbon and hydrogen in the molecule, **196** was tentatively assigned as dehydroabietane. The COSY and Nuclear Overhauser Effect (NOE) results were supportive of such a structure, and the proton signals were assigned accordingly. Comparison of all the spectral data with the literature,[166] and finally a synthesis of **196** from dehydroabietic acid (**157**) (*vide infra*) confirmed the identity of **196** as (+)-dehydroabietane. Dehydroabietane has been detected from several other cell cultures capable of producing diterpenes with an abietane skeleton.[167] Its isolation, however, has never been reported from *Tripterygium*, and it was also first time that dehydroabietane was identified in TRP4a cell cultures.

Compound **204** was isolated as colorless needles (mp 98-100°C) with a molecular formula of $C_{20}H_{30}O$. It was optically active ($[\alpha]_D^{20}$ +45.6°) and its IR spectrum indicated the presence of a hydroxyl group (3600, 1040, 1020 cm^{-1}) and a benzene ring (3025, 1600, 1500 cm^{-1}).
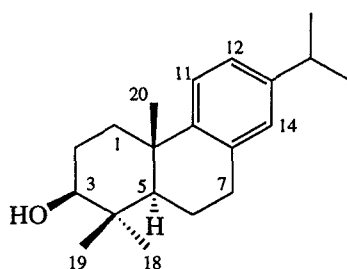
204

The ^{1}H NMR spectrum of **204** showed typical dehydroabietane-type AMX system signals at δ 6.88 (1H, br s, H14), 6.97 (1H, br d, J = 8.2 Hz, H12), 7.13 (1H, d, J = 8.2Hz, H11), three tertiary methyl signals at δ 0.88, 1.05, 1.17 and two isopropyl methyl signals at 1.20 (6H, d, J = 6.9 Hz). A signal at δ 3.27 (1H, dd, J = 10.8, 5.2 Hz) indicated a proton geminal to a hydroxyl group. The above spectral information was indicative of a hydroxylated dehydroabietane structure. Signals at δ 2.84 and 2.93 were due to geminally coupled benzylic protons, and assigned to the two C7 protons. The protons at C7 were also coupled with C6 protons in the four-proton multiplet between δ 1.64 and 1.92 (H2 and H6).

The proton signal at δ 2.28 (br ddd, J = 13.0, 3.3, 3.3 Hz) showed a strong NOE with the H11 resonance (Figure 2.3). The signal at δ 2.28 was enhanced by 8% when H11 was irradiated, and the H11 signal was increased by 20% upon saturation of the signal at δ 2.28. By inspection of a model of the molecule, it is obvious that only H1β has the proximity to H11, and because of its nearly co-planar relationship to the benzene ring (ring C), the anisotropic deshielding influence of the benzene ring has resulted in a downfield shift of the H1β signal as compared with that of its geminal partner, H1α (δ 1.52).

This particular spatial relationship between H1β and H11 has aided to facilitate the structure elucidation of these types of compounds, since, by locating the H1β signal (through NOE with H11), assignment of other ring A proton signals would be relatively easy. On the other hand, benzylic proton signals (H7), recognizable by their relatively low field chemical shifts and in some cases through NOEs with the C14 proton, provided a starting point to locate other ring B proton signals in the ^{1}H NMR spectra.
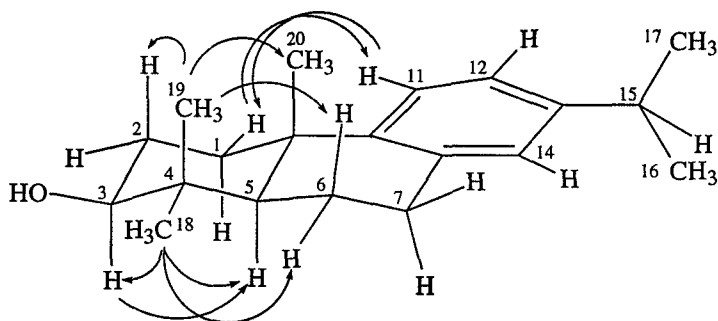
Figure 2.3   Major NOEs Observed in NOE Difference Spectra of Compound **204**

The proton signal of H1β showed strong cross peaks to its geminally coupled H1α signal (δ 1.52) in the COSY spectrum, and both were correlated with the H2 signals in the four-proton multiplet between δ 1.64-1.92 (H2, H6). The proton signal at δ 1.31 (dd, J = 12.1, 2.1 Hz) showed cross peaks to the same group of signals as did the H7 signals and thus was assigned to H5. Therefore, the resonance at δ 3.27, which was due to a proton geminal to the hydroxyl group, must arise from the proton at C3. This signal was a doublet of doublets (with cross peaks to H2) with coupling constants of 10.8 and 5.2 Hz, indicating that it was an axial proton.[168] On this basis, the hydroxyl group at C3 must be in a β orientation.

The results of NOE difference experiments were also consistent with the stereochemistry of this molecule (see Figure 2.3). Irradiation of the H3α proton at δ 3.27 showed an enhancement of the H5 signal (9%) due to their 1,3-diaxial relationship. Saturation of the methyl resonance at δ 1.05 increased the intensity of signals at δ 3.27 (H3α), 1.83-1.89 (H6α) and 1.31 (H5), suggesting that this methyl signal was due to the C18 methyl group. Since the environment of the C20 methyl group is similar to that of the C16 and C17 methyl groups, their chemical shifts are not expected to vary significantly and should be slightly shifted downfield (deshielded by the benzene ring) compared with the signals of the C18 and C19 methyl groups. Therefore, the signal at δ 0.88 was attributed to the C19 methyl group, and the signal at δ 1.17 was assigned to the C20 methyl group. Irradiation of the C19 methyl resonance showed NOEs to the C20 methyl signal (1,3-diaxial), and H2β, H6β resonances at δ

1.70-1.80. The mass spectrum of **204** showed the expected fragmentation[169] at m/z 271 (M - CH$_3$), m/z 253 (M - CH$_3$ - H$_2$O, base peak), m/z 185 and 159 (Scheme 2.1).

m/z 271   **204** m/z 286   m/z 271

- CH$_3$

-H$_2$O, CH$_3$

m/z 253

m/z 271

m/z 159   m/z 227   m/z 185
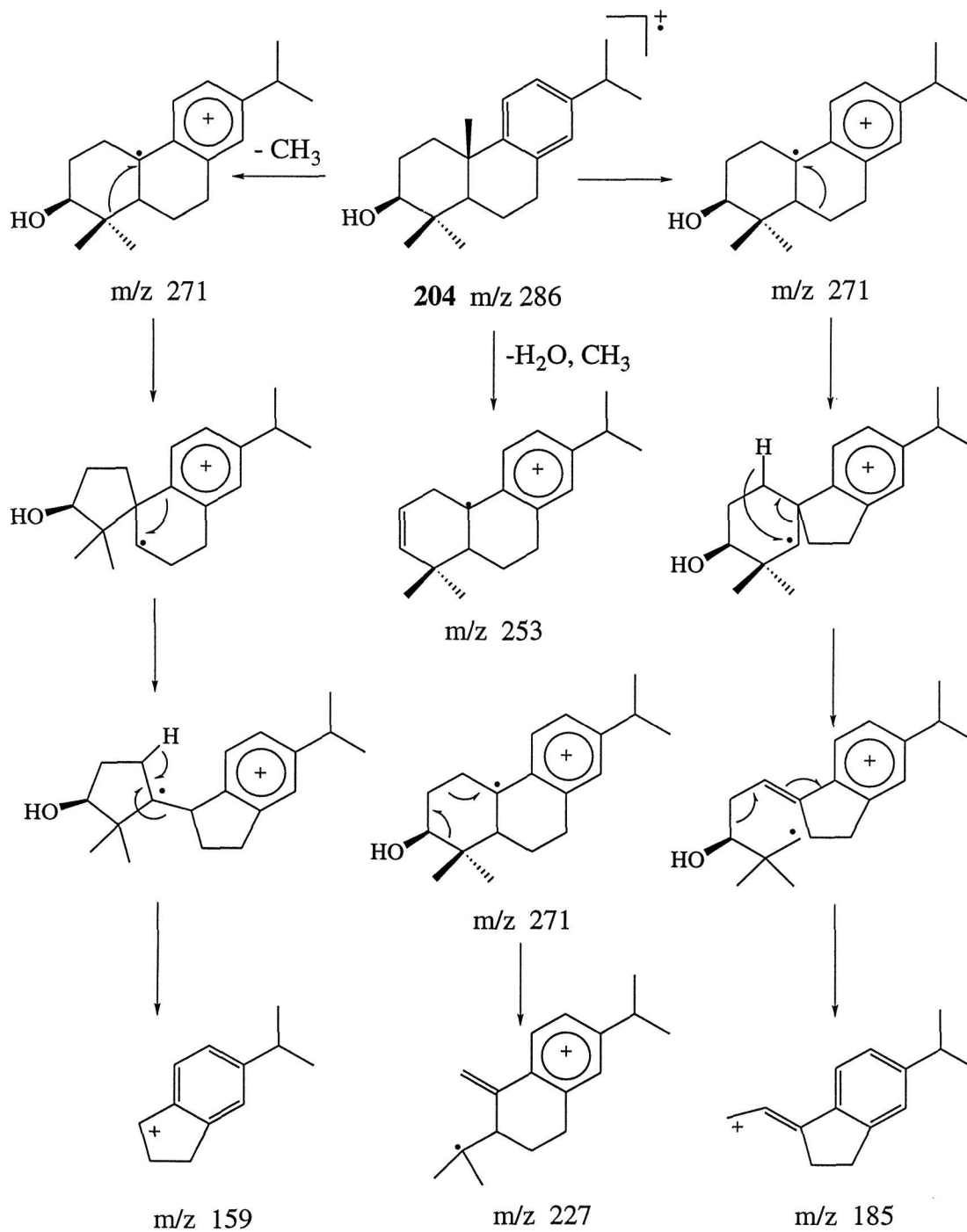
Scheme 2.1   Proposed Mass Spectral Fragmentation of **204**

J. G. Urones *et al.* reported the first isolation of compound **204** as a natural product from *Nepeta tuberrosa* subsp. *reticulata* in 1988.[170] It has also been synthesized by T. Matsumoto *et al.* from dehydroabietic acid (**157**).[171] This compound has never been reported from *Tripterygium*, and the present work documents the initial separation and identification of **204** in TRP4a cell cultures. The IR, [1]H and [13]C NMR spectral data obtained were identical with those reported in the literature.
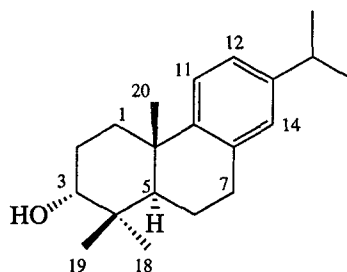
202

Compound **202** was obtained as a colorless, wax-like solid (mp 102-104°C). This optically active compound ($[\alpha]_D^{20}$ +18.2°) revealed a molecular formula of $C_{20}H_{30}O$ by high resolution mass spectrometry. Its IR spectrum showed bands for the hydroxyl group (3628, 1057 cm^{-1}) and the benzene ring (3004, 1498 cm^{-1}).

The [1]H NMR spectrum of **202** showed comparable features with that of **204**, suggesting that **202** was likely to be a similar hydroxylated dehydroabietane compound. The multiplets at δ 2.89 (2H) were due to benzylic C7 protons, which, in a COSY experiment, showed cross peaks (Figure 2.4, d) to a four-proton multiplet between δ 1.65-1.80, suggesting the presence of two C6 protons. Irradiation of the proton at δ 7.15 (H11) caused an 8% enhancement of the signal at δ 2.01 (1H, br ddd, J = 13.7, 3.5, 3.5 Hz) in NOE difference experiments (Figure 2.5), indicating that the enhanced signal was due to H1β. The H1β proton showed a strong geminal coupling with the H1α proton at δ 1.85 (1H, br ddd, J = 13.7, 13.7, 3.5 Hz) (Figure 2.4, i), and both protons exhibited correlations with signals at δ 2.09 (1H, dddd, J = 13.7, 13.7, 3.5, 2.5 Hz) (Figure 2.4, f, g) and δ 1.65-1.80 (4H, two of which were H6, *vide supra*) (Figure 2.4, h, j).

Thus, the signal at δ 2.09 must be from one of the C2 protons and the other C2 proton signal must be in the multiplet at δ 1.65-1.80.
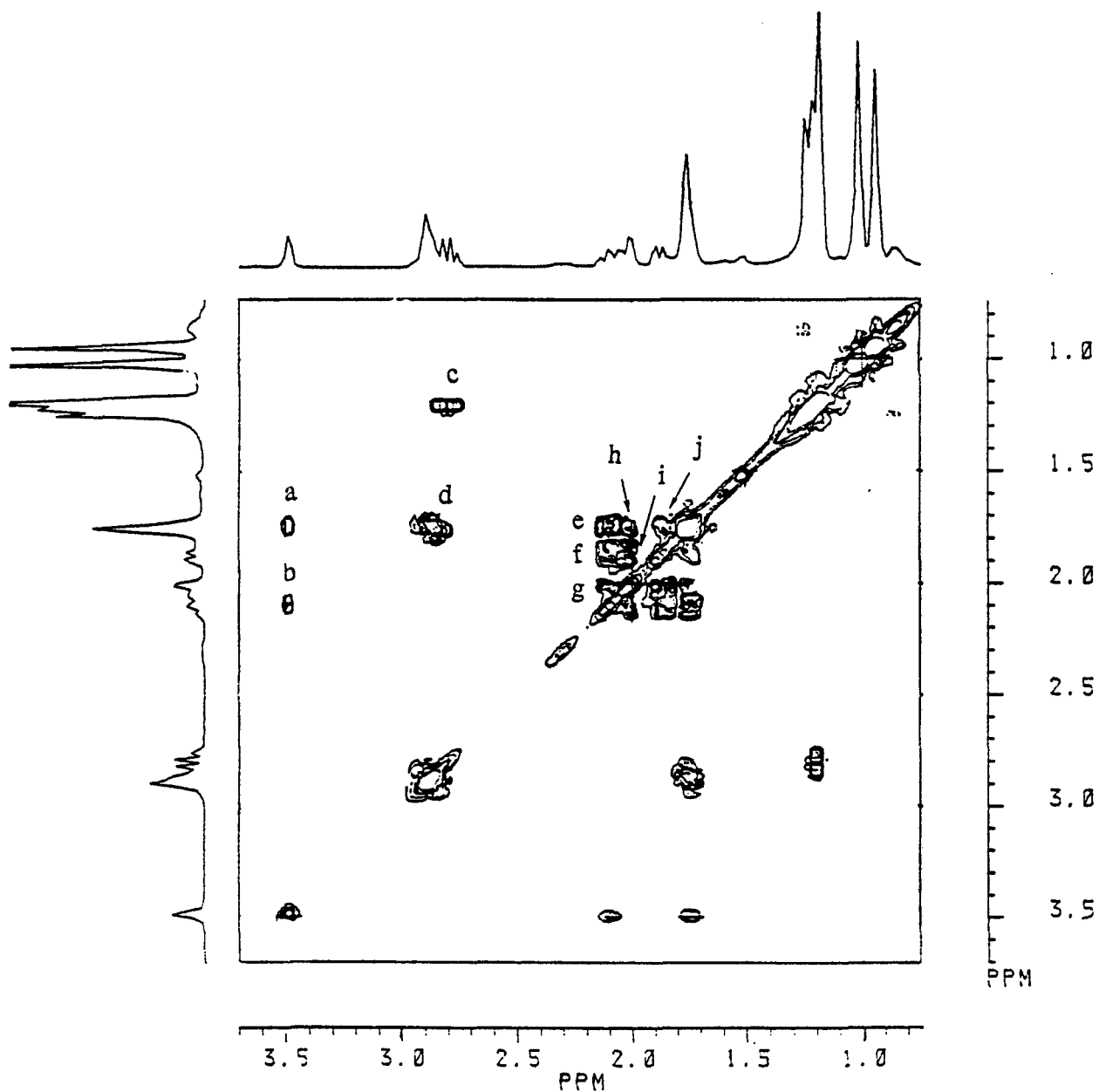
a, H3β/H2α; b, H3β/H2β; c, H15/H16,H17; d, H7/H6; e, H2β/H2α; f, H2β/H1α; g, H2β/H1β;
h, H1β/H2α; i, H1β/H1α; j, H1α/H2α

Figure 2.4   Expanded COSY Spectrum of Compound **202**

The proton geminal to the hydroxyl group appeared at δ 3.48 (br dd, J = 2.5, 2.5 Hz) and was coupled to both C2 protons (Figure 2.4, a, b); therefore, it was identified as the proton at C3. The small coupling constants suggested that this proton was in an equatorial position and the hydroxyl group must be α-axially situated, i.e., this compound was an α alcohol.

Because of the axial hydroxyl group at C3, the H1α signal was shifted downfield from δ 1.52 in **204** to 1.85 in **202** (1,3-diaxial interaction), and the H5 resonance also shifted downfield from δ 1.31 in **204** to δ 1.65-1.80 (H5 was assigned to the only remaining proton in that multiplet). A comparison of the ^{1}H NMR data is provided in Table 2.2 (vide infra).

Irradiation of H3β caused NOEs to both C2 protons, and the methyl groups at δ 0.94 and 1.01 (C18 and C19 methyl groups). Compared with **196** and **204**, the methyl signal at δ 1.18 in compound **202** can only arise from the C20 methyl group. Saturation of the C20 methyl resonance increased the intensity of the H2 signal at δ 2.09 by 3%, and that of the H1β signal by a similar percentage, indicating that the H2 signal at δ 2.09 was due to H2β. The coupling constants of this proton (dddd, J = 13.7,13.7, 3.5, 2.5 Hz) also supported this assignment (geminal coupling constant J = 13.7 Hz; H2β-H1α coupling constant J = 13.7 Hz). Irradiation of the C20 methyl group also enhanced the methyl resonance at δ 0.94, so the latter signal can only be due to the C19 methyl group (1,3-diaxial relationship with the C20 methyl). The remaining methyl resonance at δ 1.01 was assigned to the C18 methyl group.
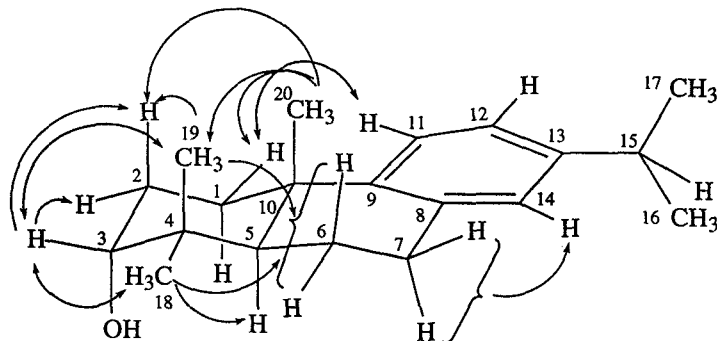
Figure 2.5   Major NOEs Observed in NOE Difference Spectra of Compound **202**

The ^{13}C NMR spectrum of **202** revealed the chemical shift of C3 at δ 75.8, while the chemical shift of C3 in **204** was at δ 78.8. This also provided strong evidence that the hydroxyl group was α (axial) in this compound but β (equatorial) in **204**. It is well known that steric interactions (γ gauche butane-like effect) shift upfield not only the signal of a carbon with a γ substituent, but also the signals of the carbons that are in between ("internal" gauche butane effect).[172,173] Since the steric interactions are stronger in the α isomer **202**, the chemical shift of the C3 carbon in the α isomer should be at a higher field than that in the β isomer.

γ gauche effect

202   **204**
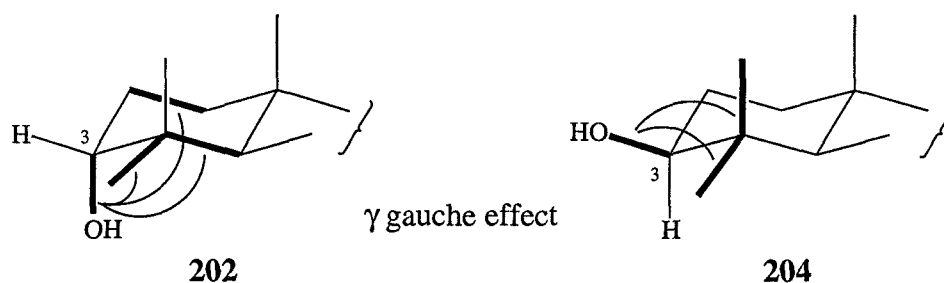
The mass spectrum of **202** exhibited a similar fragmentation pattern as **204**, but the molecular ion and other fragments were relatively weak when compared with the base peak at m/z 253 (M - CH_3 - H_2O) for **202**. Compound **202** is a new compound, its isolation also constitutes its first isolation from *T. wilfordii* plant cell cultures.
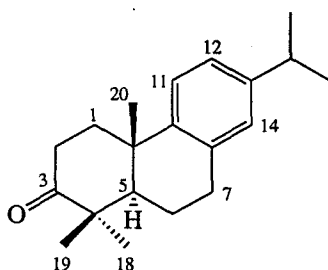
198

Compound **198** was isolated as an optically active compound ($[\alpha]_D^{20}$ +31.1°) with a molecular formula of $C_{20}H_{28}O$. Its IR spectrum displayed absorption bands for a carbonyl (1708 cm^{-1}) and a benzene ring (3010, 1500 cm^{-1}).

The ¹H NMR spectrum showed the typical AMX system pattern in the aromatic region, and the protons were assigned accordingly. The two-proton multiplet centered at δ 2.88 was assigned to the benzylic protons (H7), on the basis of their NOEs observed with H14. The methylene protons at C6 were found at δ 1.79 by their cross peaks to H7. A signal at δ 1.90 (1H, dd, J = 11.3, 3.2 Hz) also showed a cross peak to the H6 signal, indicating that this signal must be due to H5. Irradiation of H11 caused an enhancement (11%) of the H1β signal at δ 2.46 (1H, ddd, J = 13.3, 7.5, 4.2 Hz). The COSY spectrum revealed a strong geminal coupling of H1β with the proton at δ 1.94 (1H, m, H1α), and both showed correlations with protons at δ 2.55 (1H, ddd, J = 15.7, 7.6, 4.2 Hz) and 2.67 (1H, ddd, J = 15.7, 10.0, 7.5 Hz). It meant that the signals at δ 2.55 and 2.67 must belong to the two C2 protons, and the coupling constants of these two protons indicated that the one at δ 2.67 was the axial proton (H2β), which was confirmed by a NOE enhancement upon irradiation of the C20 methyl group. The downfield shift of the H2 signals compared with their counterparts in **196** suggested that the carbonyl was at C3. Irradiation of the methyl group at δ 1.27 (H20) showed NOEs to H11 and the methyl group at δ 1.12 (H19). The remaining methyl signal at δ 1.14 was assigned to the C18 methyl group. Therefore, compound **198** was assigned as abieta-8,11,13-trien-3-one.

Ketone **198** has been isolated from *Juniperus sabina*[174] and *Salvia wiedemannii*.[175] It has never been isolated from *Tripterygium* plants, and here we report its initial isolation from TRP4a cell cultures.

Compound **201** was isolated as a pale yellowish oil and showed a specific rotation of +136.9° ($[\alpha]_D^{23}$, c =0.0132, MeOH). High resolution mass spectrometry revealed its molecular formula as $C_{19}H_{24}O$, one carbon less than normal diterpenes. The IR absorptions indicated the presence of benzene (3025, 1500 cm⁻¹) and α,β-unsaturated ketone (1660 (strong), 1620 cm⁻¹) functionalities. Strong UV absorptions at 219.0 nm (log ε = 3.93) and 243.3 nm (log ε = 4.03) also supported the presence of an α,β-unsaturated ketone system in this molecule.
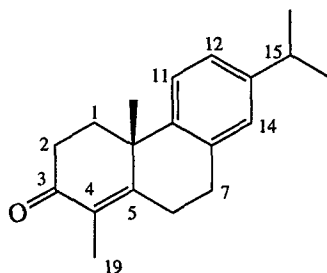
201

The [1]H NMR spectrum exhibited the familiar AMX system signals in the aromatic region. Only four methyl group signals were observed, which indicated that loss of a methyl group was probably attributed to the missing carbon in the molecular formula. One methyl signal was shifted downfield to δ 1.82 (3H, br s), suggesting that this methyl group was very likely attached to an unsaturated carbon.[168] The isopropyl methyl signals were found at δ 1.22 (6H, d, J = 7.0 Hz), and the H15 signal was found in the three-proton multiplet between δ 2.76-2.91.

Irradiation of H11 enhanced H1β signal at δ 2.35 (1H, ddd, J = 13.2, 5.1, 2.3 Hz) (Figure 2.6). The COSY spectrum showed correlations between H1β and protons at δ 2.04 (1H, br ddd, J = 14.8, 13.2, 4.9 Hz), 2.52 (2H, m), and 2.71 (1H,ddd, J = 17.7, 14.8, 5.1 Hz) (Figure 2.7, k, j, f). Saturation of the H1β resonance increased the intensities of the signal at δ 2.04 by 22% and another one at δ 2.71 by 7%. This suggested that the signal at δ 2.04 was due to proton H1α (spatially nearer to H1β). As it was expected, irradiation of H1α enhanced the signal of H1β by 14%, and also resulted in a moderate enhancement of the signal at δ 2.52 (about 3%). Based on these observations, it was certain that the proton at δ 2.71 was H2β, while the other in the two-proton multiplet at δ 2.52 was H2α. The chemical shifts of these C2 protons indicated that the ketone group was at C3.

Irradiation of H14 showed NOE enhancements of the signal at δ 2.98 (1H, m), and some proton signals in the three-proton multiplet between δ 2.76 and 2.91 (one of them was H15, *vide supra*). This means that the proton at δ 2.98 should be one of the C7 protons, and the

other C7 proton must be in that multiplet (the enhancement should include H7 and also H15 because of their similar relationships with H14). The proton signal at δ 2.98 showed correlations to this multiplet (Figure 2.7, b) and another multiplet at δ 2.52 (2H, one was H2α, *vide supra*) (Figure 2.7, a), and these two multiplets were also mutually correlated (Figure 2.7, d). It was not difficult to conclude that one of the remaining protons in the multiplet at δ 2.52 must be a C6 proton, and accordingly the only proton left in the multiplet between δ 2.76 and 2.91 must be the other C6 proton. The H7 signal at δ 2.98 was assigned to H7β because, by inspection of a model, it can be seen that H7β is in a quasi-equatorial orientation, thus more deshielded than H7α by the nearby benzene ring.

Irradiation of the methyl group at δ 1.50 (C20 methyl group) increased the signal intensity of H11 at δ 7.20. The downfield shift of this methyl signal compared with its counterpart in **198** (from δ 1.27 to 1.50), and even greater downfield shift of the C6 protons (from δ 1.79 in **198** to 2.52 and δ 2.76-2.91 in this compound) indicated that there must be a double bond between C4 and C5, thus forming the α,β-unsaturated ketone with the carbonyl at C3.
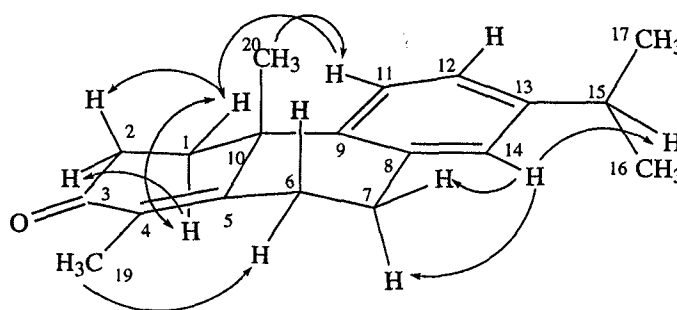
Figure 2.6   Major NOEs Observed in NOE Difference Spectra of Compound **201**

The methyl group at δ 1.82 can only be attached to C4 and this was confirmed by a weak COSY correlation (Figure 2.7, h; only showing slight broadening in ¹H NMR spectrum) between this methyl group and the H6 in the multiplet at δ 2.52 (homoallylic coupling[168]). This also revealed that the H6 resonance at δ 2.52 was due to the H6β proton, because H6β is

nearly perpendicular to the C4, C5 double bond plane while the other H6 proton at δ 2.76-2.91

(therefore, must be the H6α) is lying in the plane, thus being more deshielded by C4, C5

double bond. Furthermore, this assignment was justified by the enhancement of H6α (11%)

upon irradiation of the methyl signal at δ 1.82 (H19).
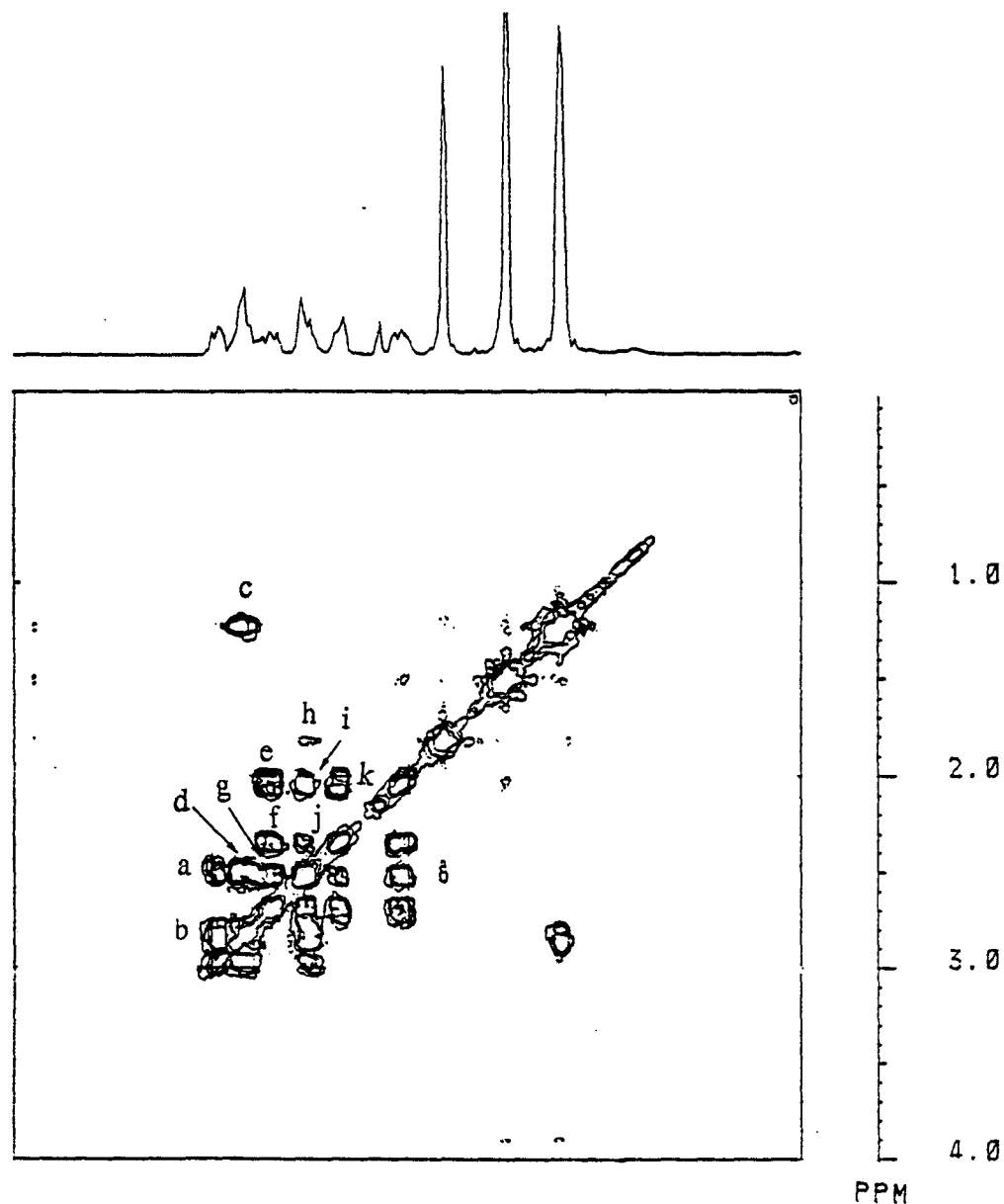
a, H7β/H6β; b, H7β/H6α,H7α; c, H15/H16,H17; d, H6α,H7α/H6β: e, H2β/H1α; f, H2β/H1β;

g, H2β/H2α; h, H6β/H19; i, H2α/H1α; j, H2α/H1β; k, H1β/H1α

Figure 2.7   Expanded COSY Spectrum of Compound **201**
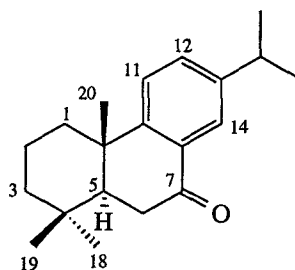
199

Compound **199** had a molecular formula of $C_{20}H_{28}O$. An IR absorption at 1660 cm⁻¹ suggested the presence of a carbonyl group adjacent to an aromatic ring. The latter was supported by a strong UV absorption at 254.0 nm (log ε = 4.03). The ¹H NMR spectrum displayed some similarity to that of dehydroabietane (**196**), thus this compound was probably the 7-oxo analog of **196**. The aromatic signals were shifted to lower field. The H14 signal was found at δ 7.85 (1H, d, J = 2.1 Hz), which was about 1 ppm lower than its counterpart in compound **196**, apparently due to the deshielding effect of a nearby keto group at C7. The two H6 signals, which were also shifted downfield, were found at δ 2.62 (1H, dd, J = 18.1, 13.5 Hz) and δ 2.71 (1H, dd, J = 18.1, 4.4 Hz), respectively. The COSY spectrum indicated that the two C6 protons were only coupled with H5 at δ 1.86 (1H, dd, J = 13.5, 4.4 Hz). The remaining proton signals were assigned as based on the NOE and COSY spectra. All the spectral data were in agreement with the structure assigned to **199**, and its identity was confirmed by a synthesis from dehydroabietane (**196**, *vide infra*). A comparison of the ¹H NMR data of **196**, **204**, **202**, **198**, **201** and **199** is given in Table 2.2.

This compound is a known compound,[176,177] but it is the first isolation from TRP4a cell cultures. No isolation from *Tripterygium* plants has been reported.

Table 2.2 ^{1}H NMR Spectral Data of Abietane Diterpenes **196**, **204**, **202**, **198**, **201** and **199** (400 MHz in CDCl$_3$, δ in ppm, *J* in Hz in parentheses)

	196	204	202	198	201	199
1α	1.39, ddd (13.1, 12.7, 3.6)	1.52, m	1.85, ddd (13.7, 13.7, 3.5)	1.94, m	2.04, ddd (14.8, 13.2, 4.9)	1.52, m
1β	2.26, d (12.7)	2.28, ddd (13.0, 3.3, 3.3)	2.01, ddd (13.7, 3.5, 3.5)	2.46, ddd (13.3, 7.5, 4.2)	2.35, ddd (13.2, 5.1, 2.3)	2.31, d (12.6)
2α	1.59, m	1.64-1.92, m	1.65-1.80, m	2.55, ddd (15.7, 7.6, 4.2)	2.52, m	1.66, m
2β	1.64-1.81, m	1.64-1.92, m	2.09, dddd (13.7, 13.7, 3.5, 2.5)	2.67, ddd (15.7, 10.0, 7.5)	2.71, ddd, (17.7, 14.8, 5.1)	1.75, dddd, (13.7, 13.7, 3.3, 3.3)
3α	1.21, m	3.27, dd (10.8, 5.2)	-	-	-	1.27, m
3β	1.46, d (13.2)	-	3.48, dd (2.5, 2.5)	-	-	1.52, m
5	1.34, dd (12.4, 2.4)	1.31, dd (12.1, 2.1)	1.65-1.80, m	1.90, dd (11.3, 3.3)	-	1.86, dd (13.5, 4.4)
6α	1.86, dddd (13.2, 7.1, 2.4, 2.4)	1.64-1.92, m	1.65-1.80, m	1.79, m	2.76-2.91, m	2.71, dd (18.1, 4.4)
6β	1.64-1.81, m	1.64-1.92, m	1.65-1.80, m	1.79, m	2.52, m	2.62, dd (18.1, 13.5)
7α	2.89, m	2.84, m	2.89, m	2.88, m	2.76-2.91, m	-
7β	2.89, m	2.93, dd (17.2, 6.0)	2.89, m	2.88, m	2.98, m	-
11	7.16, d (8.2)	7.13, d (8.2)	7.15, d (8.2)	7.15, d (8.2)	7.20, d (8.1)	7.27, d (8.2)
12	6.97, dd (8.2, 1.6)	6.97, d (8.2)	6.96, d(8.2)	7.00, d (8.2)	7.08, dd (8.1, 1.5)	7.37, dd (8.2, 2.1)
14	6.88, d (1.6)	6.88, s	6.86, s	6.90, s	6.95, d (1.5)	7.85, d (2.1)
15	2.83, sept (6.9)	2.80, sept (6.9)	2.80, sept (7.0)	2.81, sept (6.9)	2.76-2.91, m	2.90, sept (6.9)
16, 17	1.21, d (6.9)	1.20, d (6.9)	1.20, d (7.0)	1.21, d (6.9)	1.22, d (7.0)	1.225, 1.227, d (6.9)
18	0.93, s	1.05, s	1.01, s	1.14, s	-	0.98, s
19	0.92, s	0.88, s	0.94, s	1.12, s	1.82, s	0.91, s
20	1.17, s	1.17, s	1.18, s	1.27, s	1.50, s	1.215, s
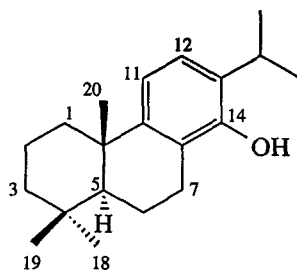
197

Compound **197** had a molecular formula of $C_{20}H_{30}O$. The IR spectrum showed an absorption band for a hydroxyl group at 3620 cm^{-1}. The sharp appearance of this absorption indicated that this hydroxyl group was very likely to be a phenolic hydroxyl group, because this IR spectral feature was frequently observed with phenolic compounds during the course of the present investigation.

The ^{1}H NMR spectrum showed AM system signals in the aromatic region, suggesting that the phenolic hydroxyl group was attached to C14. The downfield shift of the H15 signal (δ 3.15, sept, J = 6.9 Hz) compared with that (δ 2.83) in **196** supported this assumption. The proton resonance at δ 4.59 (1H, br s) was due to a hydroxyl group because irradiation of this signal caused the water peak of the solvent to become negative in NOE difference spectra (saturation transfer[178]). This irradiation also enhanced the signals of H15, and the protons at δ 2.81 (1H, br dd, J = 16.5, 6.6 Hz) and 2.61 (1H, ddd, J = 16.5, 11.3, 7.9 Hz), which were assigned to C7 protons accordingly. Therefore, the hydroxyl group was situated at C14, *i.e.*, **197** was a C14-hydroxyl analog of compound **196**. The rest of the proton signals were assigned according to the data from COSY and NOE spectra.

The structures of **196, 197** and **199** were finally confirmed by chemical synthesis from dehydroabietic acid (**157**). Reduction of **157** by lithium aluminum hydride (LAH)[179] gave the alcohol **196(i)**, which was then converted to the tosylate[180] and subsequently reduced to give dehydroabietane (**196**)[181] (Scheme 2.2).
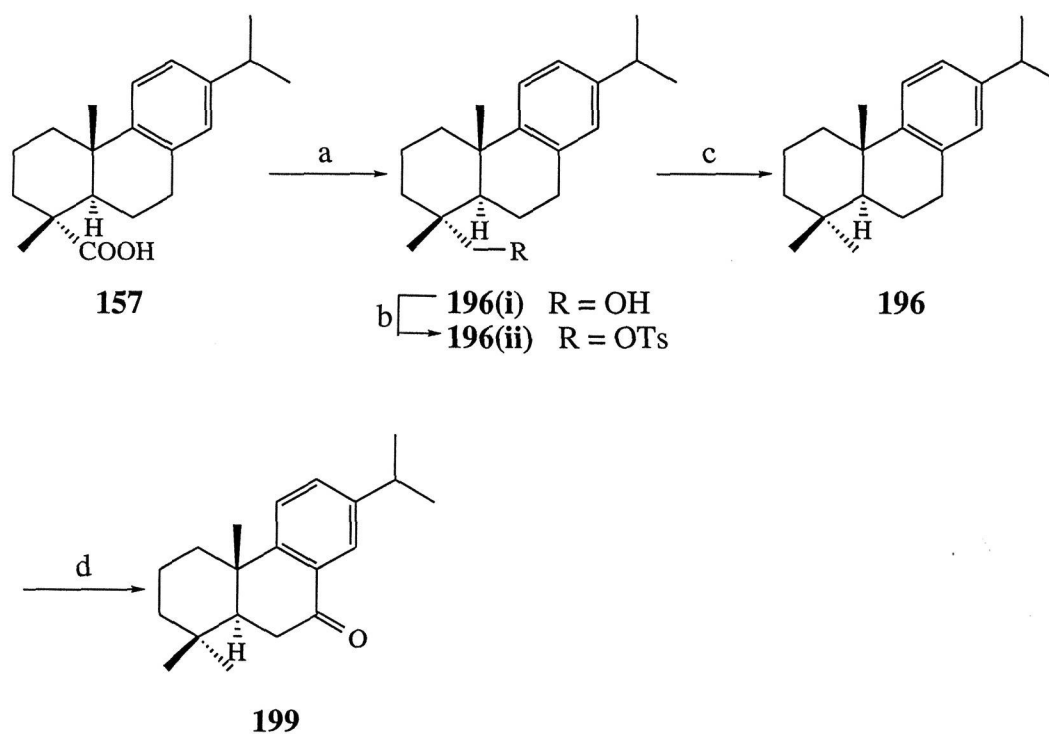
Scheme 2.2   Syntheses of 196 and 199 from 157

a) LAH, ether; b) *p*-toluenesulfonyl chloride, pyridine; c) NaI, Zn, HMPA, Δ; d) CrO₃
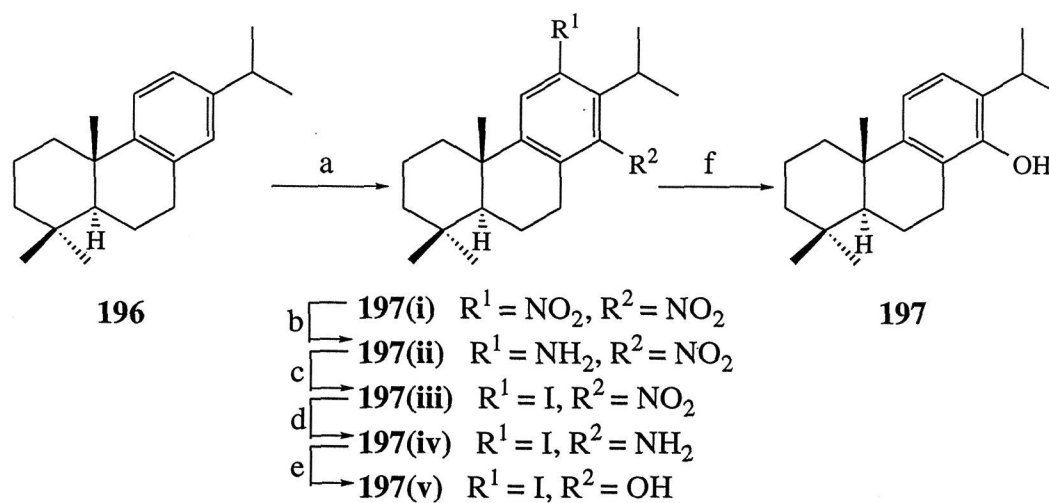
Scheme 2.3   Synthesis of 197 from 196

a) HNO₃, H₂SO₄, MeNO₂; b) H₂, Pt/C, HOAc-EtOH-benzene; c) NaNO₂, KI, TFA-HOAc-H₂O; d) Na₂S₂O₄, EtOH, Δ; e) NaNO₂, TFA; HCl, MeOH; f) H₂, Pd/C, Na₂CO₃, MeOH-benzene

The synthetic sequences leading to **197** and **199** from **196** were based on the results from the synthetic studies of biotransformation precursors (see Chapter 3 for details). Oxidation of **196** with chromium (VI) oxide (CrO₃) yielded the 7-oxo compound **199** (Scheme 2.2). A multistep synthesis from dehydroabietane (**196**) afforded compound **197** (Scheme 2.3). The spectral data of the synthetic compounds were identical to those of the corresponding isolated compounds. Compound **197** has been synthesized before,[182] but its isolation as a natural product has not been found in the literature.
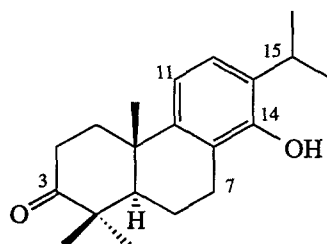
116

Compound **116** was isolated as an optically active ($[\alpha]_D^{23}$ +117.4°, c = 0.0145, MeOH) white powder (mp 138-140°C) with a molecular formula of $C_{20}H_{28}O_2$. Its IR spectrum revealed the presence of hydroxyl (3620 cm⁻¹), carbonyl (1700 cm⁻¹) and benzene ring (3025, 1580, 1500 cm⁻¹) functionalities. The similarity between this compound and **197** in the aromatic region (AM system) and the broad singlet at δ 4.65 in the ¹H NMR spectrum suggested that this compound also had a hydroxyl group at C14. The signals of the methyl groups and most of the aliphatic protons showed a comparable pattern with that of **198**, indicating the location of the carbonyl group at C3.

Irradiation of the proton at δ 4.65 (1H, br s) caused signal increases of H15 and the proton at δ 2.90 (1H, br dd, J = 16.6, 5.9 Hz, H7β), but depressed the signal from traces of water in the solvent, thereby confirming the presence of a hydroxyl group at C14. All the spectral information suggested that compound **116** was 14-hydroxyl-abieta-8,11,13-trien-3-one.

This compound has been previously isolated from *Tripterygium* plants, and our ^{1}H NMR data were identical to those reported.[89]
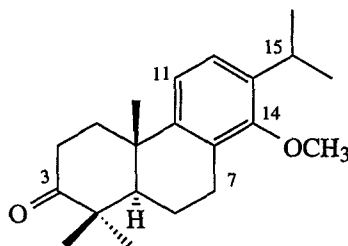
200

Compound **200** ($C_{21}H_{30}O_2$) was isolated as colorless needles (mp 134-136°C) with a specific rotation of +84.9° ($[\alpha]_D^{23}$, c = 0.0171, MeOH). The IR absorptions established the presence of a ketone (1700 cm^{-1}), a benzene ring (3025, 1500 cm^{-1}) and possibly an aryl alkyl ether (1260, 1040 cm^{-1}).

The ^{1}H NMR spectrum of **200** showed close resemblance with that of **116**. The H11 signal (1H, d, J = 8.3 Hz) was shifted downfield to δ 7.00 (δ 6.83 in **116**) while H12 (1H, d, J = 8.3 Hz) was less affected at δ 7.06. Meanwhile, the phenolic hydroxyl proton was not observed but a methoxy signal was found at δ 3.71 (3H, s). Therefore, compound **200** was very likely to be the methyl ether of **116**. The downfield shifts of resonances due to H15 (δ 3.27, septet, J = 6.9 Hz), H7β (δ 3.10, br dd, J = 17.4, 5.5 Hz) and H7α (in the two-proton multiplet at δ 2.66) from δ 3.10, 2.90 and near δ 2.60, respectively, compared with their counterparts in compound **116**, provided strong supporting evidence for the presence of a methoxy group at C14. Irradiation of the methoxy group at δ 3.71 showed NOE effects with H15 (6%), H7β (4%), and H7α (4%), thus confirming this assignment. Other individual protons were assigned according to coupling constants, and the data obtained from COSY and NOE experiments. This compound has not been reported from the *Tripterygium* plants.
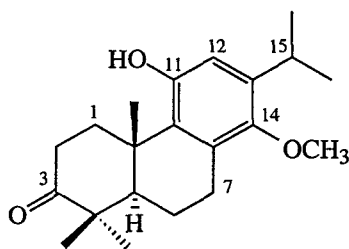
117

Compound **117** was isolated as colorless needles (mp 184-186°C) with an optical activity of +190.2° ($[\alpha]_D^{23}$, c = 0.00736, MeOH). Its molecular formula was determined as $C_{21}H_{30}O_3$. The IR spectrum displayed bands for hydroxyl (3600 cm^{-1}), carbonyl (1700 cm^{-1}), and benzene ring (3025, 1490 cm^{-1}) functionalities.

The ^{1}H NMR spectrum showed a methoxy group at δ 3.66 (3H, s), but only one aromatic proton at δ 6.36 (1H, s). The signal at δ 4.55 (1H, s) changed its shape (from sharp to very broad) as well as its chemical shift position when the concentration of the NMR sample was changed, suggesting that it was a phenolic hydroxyl proton.

Irradiation of the proton at δ 6.36 increased the signal intensities of the hydroxyl proton (2%), H15 (1%), and the C16, C17 methyl protons (1%), while saturation of the hydroxyl proton resonance caused NOE enhancements to the signals at δ 6.36 (18%) and δ 3.05 (1H, ddd, J = 13.8, 8.5, 6.3 Hz) (2%). It was obvious that the signal at δ 6.36 was due to H12, which is close to the C16, C17 methyl groups, H15, and any proton in the group attached to C11 (Figure 2.8). The NOEs observed upon irradiation of the hydroxyl proton at C11 not only confirmed its spatial proximity to H12 but also revealed the location of the H1β signal in the spectrum. The downfield shift of H1β signal (δ 3.05, compared to δ 2.44 in **200**) was another indication of severe crowding caused by introduction of the hydroxyl group at C11.[183] Irradiation of the methoxy group at δ 3.66 produced enhancements of the H15 signal (4%), and the H7 signals at δ 3.10 (1H, ddd, J = 17.1, 4.7, 1.8 Hz) (3%) and 2.56 (1H, ddd, J = 17.1, 13.0, 6.0 Hz) (3%), respectively. Therefore, the methoxy group was situated at C14. The rest of the protons were assigned based on their coupling constants, COSY, and NOE results. This

compound has been isolated from *Tripterygium* plants. Our ^{1}H NMR and specific rotation were consistent with those previously reported.[90]
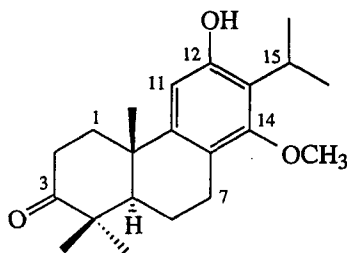
118

Compound **118** was obtained as optically active ($[\alpha]_D^{23}$ +56.6°, c = 0.145, CHCl$_3$) colorless prisms. It melted at 188-190°C and had a molecular formula of C$_{21}$H$_{30}$O$_3$. The IR spectrum showed absorptions of hydroxyl (3600 cm^{-1}), keto (1702 cm^{-1}), and benzene (3029, 1609, 1585 cm^{-1}) groups.

Its ^{1}H NMR spectrum showed an aromatic proton at δ 6.38, and a methoxy group at δ 3.67 (3H, s). A signal at δ 4.47 (1H, s) was likely to be a phenolic proton as compared with **117**. The isopropyl methine resonance (H15, septet, J = 7.2 Hz) was found shifted downfield to δ 3.41. Also shifted downfield were the two isopropyl methyl signals at δ 1.34 and 1.35 (3H each, both d, J = 7.2 Hz).

Saturation of the resonance at δ 6.38 enhanced the signals at both δ 4.47 (6%) and δ 2.33 (1H, ddd, J = 13.1, 7.4, 4.2 Hz) (11%), and slightly increased the methyl resonance at δ 1.25 (3H, s) (Figure 2.8). Upon irradiation of the δ 4.47 signal, NOEs were observed for the proton signal at δ 6.38 (10%) as expected, and the isopropyl methyl signals (1%), but the signal at about δ 1.5 due to traces of water in the solvent was depressed. This indicated that the signal at δ 4.47 was from a hydroxyl proton, and this hydroxyl group was situated at C12, where it was closer to the C16, C17 isopropyl methyl groups, rather than at C11. Accordingly, the resonance at δ 6.38 was due to H11 and the signal at δ 2.33 must arise from H1β. This explained why the H15 and the isopropyl signals were shifted downfield, while the H1β

resonance appeared at a chemical shift similar to the one found in **117**. A position change of the hydroxyl group from C11 to C12 increases steric crowding around the isopropyl group, but in turn releases tension previously directed towards H1β. NOEs were observed between the methoxy group at δ 3.67 and H15, and a signal at δ 2.99 (1H, ddd, J = 17.1, 5.7, 1.6 Hz), as well as one of the multiplet signals between δ 2.50 and 2.71. Therefore, the methoxy group was located at C14, and the proton at δ 2.99 was H7β while the one in the multiplet was H7α.

117   **118**
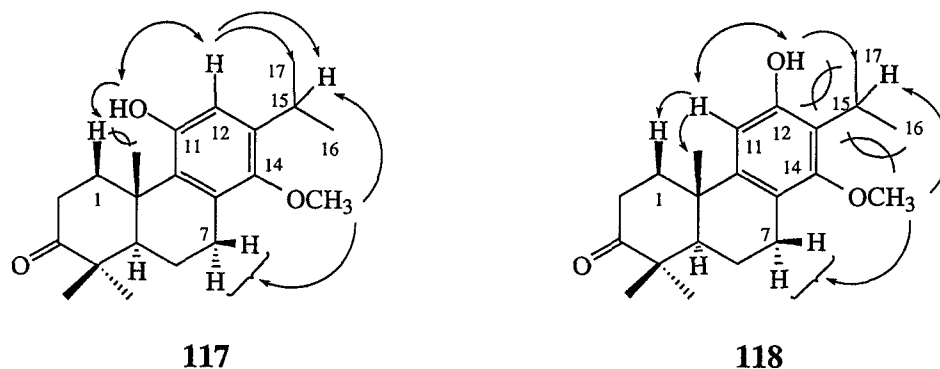
Figure 2.8   A Comparison of NOE Results between **117** and **118**

The H7β proton of **118** coupled intensively with the proton observed at δ 1.68 (1H, ddd, J = 12.5, 12.5, 5.7 Hz), while H7α signal showed cross peaks to both the signals at δ 1.68 and at δ 1.83 (1H, m) with the coupling being stronger to the latter (δ 1.83). Inspections of the molecular model revealed that the required conformation places the C7 and C6 protons in a spatial situation which is in the middle between eclipsed and staggered relationships with each other. Such a relationship existed in virtually all compounds of the dehydroabietane or the *abeo* dehydroabietane type discussed in this thesis if ring A and B were *trans* fused and ring C was a benzene ring.

The dihedral angle between H7β and H6β is around 30°, thus there is a strong coupling between these two protons, but since the angle between H7β and H6α is near 90°, little or no coupling between them is usually observed. The proton signal at δ 1.68 was thus assigned to H6β. On the other hand, H7α is separated by a small dihedral angle with H6α, but an angle

close to 180° with H6β, so it was strongly coupled with both of the C6 protons. The coupling of H7α may be slightly stronger with one of the two C6 protons depending on the individual compound. This coupling pattern between H6 and H7 was helpful in assigning these protons. Thus, the signal at δ 1.83 was assigned to H6α.
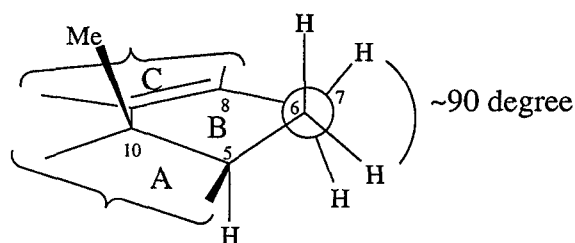
Coupling of H5 to both H6 signals was obscured by diagonal crowding, but the appearance of the H5 resonance as a sharp doublet of doublets at δ 1.84 allowed facile recognition. The remainder of the protons were assigned accordingly by COSY and NOE experiments. This compound has been previously reported by B.-N. Zhou *et al.* from an isolate of *T. wilfordii*. Their ^{1}H NMR data (100 MHz) were comparable with ours.[90]
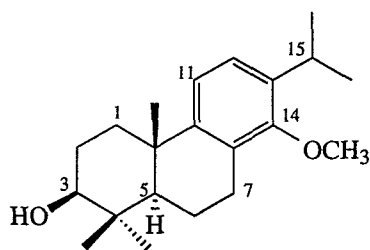
205

The optically active compound **205** ($[\alpha]_D^{23}$ +34.1°C, c = 0.063, CHCl$_3$) was isolated as a wax-like solid with a molecular formula of $C_{21}H_{32}O_2$. The IR absorptions indicated the presence of a hydroxyl group (3614 cm^{-1}), benzene ring (3002, 1480 cm^{-1}), and a possible ether (1030 cm^{-1}) linkage. The ^{1}H NMR spectrum was generally very similar with that of **200**

from δ 3 ppm to the aromatic region, but resembled that of compound **204** from δ 2.5 ppm to the methyl signal region (at about δ 1 ppm).

Irradiation of the signal at δ 6.98 (1H, d, J = 8.4 Hz, H11) produced an enhancement of the signal at δ 2.27 (1H, ddd, J = 13.1, 3.5, 3.5 Hz, H1β) (12%). NOEs were observed for the signal at 3.26 (1H, septet, J = 6.9 Hz, H15) and another one at δ 3.02 (1H, br dd, J = 17.6, 5.3 Hz) upon saturation of the methoxy resonance at δ 3.70 (3H, s). Therefore, the methoxy group was again attached to C14, and the resonance at δ 3.02 was assigned to H7β.

The H1β proton showed strong coupling to H1α at δ 1.52 (1H, m), and both were correlated with a two-proton multiplet at δ 1.77 (H2). The C2 protons exhibited cross peaks to a signal at δ 3.28 (1H, dd, J = 10.9, 4.9 Hz), indicating that this proton, which was geminal to a secondary hydroxyl group, was at C3 and α oriented (axial proton with large coupling constants), i.e., compound **205** was a β alcohol (^{13}C NMR showed C3 at δ 78.8, same as for compound **204**).
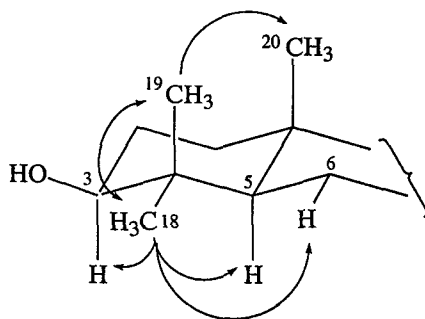
Figure 2.9   Some NOEs observed for **205**

Figure 2.9 portrays some NOEs observed for **205** with regard to the assignment of the tertiary methyl groups. Irradiation of the methyl signal at δ 1.06 enhanced signals of H3α (8%), H6α (10%), H5 (3%), and of a methyl group at δ 0.88 (slightly), indicating that the irradiated methyl group was the C18 methyl group, and the enhanced methyl signal could only be due to the C19 methyl group. Saturation of the C19 methyl resonance caused signal

intensity increases of the C18 methyl (slightly), and another tertiary methyl group at δ 1.17 (2%), which was then assigned to the C20 methyl group. Therefore, the structure of **205** was determined as shown. Compound **205** is a new compound, and isolated for the first time from TRP4a cell cultures.
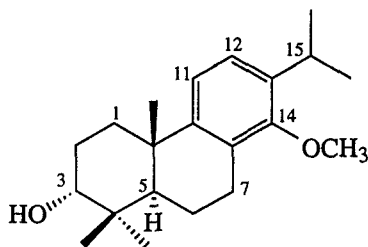
203

Compound **203** ($C_{21}H_{32}O_2$) was obtained as colorless needles with a melting point of 157-159°C. It was optically active ($[\alpha]_D^{23}$+27.1°) and exhibited IR absorptions of hydroxyl (3626 cm^{-1}), benzene ring (3007, 1480 cm^{-1}) and possible ether (1029 cm^{-1}) groups.

The ^{1}H NMR spectrum indicated some similarities of this compound to **205**, but there were some differences. The AM system signals in **205** did not compare favorably with the singlet-like signal (about δ 7, 2H) in aromatic region of **203**. The resonance of the proton geminal to the hydroxyl group was slightly shifted downfield (from δ 3.28 to δ 3.49) and showed smaller coupling constants, indicating its equatorial orientation at C3 (*i.e.*, an α alcohol). The methoxy group, isopropyl methine, and the two C7 proton signals, were virtually identical in chemical shifts and coupling patterns to their counterparts in **205**.

Irradiation of the methoxy group at δ 3.69 enhanced signals of H15 (5%), H7β (2%, assigned in comparison with **205**), and H7α (3%), confirming that this methoxy group was attached to C14 (Figure 2.10b). Irradiation of the singlet-like aromatic protons (2H) centered at δ 7.15 caused enhancements of the isopropyl methyl signals, and a signal at δ 2.00 (1H, br ddd, *J* = 13.1, 3.8, 3.5 Hz), which was thus assigned to H1β (Figure 2.10a). It seemed likely that the singlet-like aromatic proton signals were due to protons H11 and H12.

The COSY spectrum of **203** displayed a cross peak between the H7β signal and a three-proton multiplet from δ 1.62 to 1.77, while H7α resonance showed two cross peaks, one to the above multiplet and the other to a multiplet observed between δ 1.77 and 1.90 (2H). This suggested that H6β was part of the first multiplet and H6α part of the second one.

Proton H1β exhibited strong coupling with part of the multiplet containing H6α (δ 1.77-1.90), indicating that the other proton in this two-proton multiplet must be H1α. Both H1α and H1β signals were also correlated with a signal at δ 2.09 (1H, dddd, J = 14.1, 14.1, 3.8, 2.4 Hz), and with part of the three-proton multiplet containing H6β, indicating that the protons at C2 were in the above locations. The two H2 signals exhibited cross peaks to the signal at δ 3.49 (1H, br dd, J = 3.3, 2.4 Hz), suggesting that this signal, geminal to the hydroxyl group, arose from a β-oriented (equatorial) H3 (thus an α alcohol at C3).

The remaining proton in the three-proton multiplet between δ 1.62 and 1.77 (the other two had been assigned to H2α and H6β, *vide infra*) was assigned to H5 in comparison with **202**, and also because of an enhancement observed for the doublet-like signal (H5) in the multiplet when H7α was irradiated.

Accordingly, the methyl signals at δ 0.94, 1.03 and 1.178 were assigned to the C19, C18 and C20 methyl groups, respectively, in comparison with compound **202**. Irradiation of the C16, C17 and C20 methyl groups (chemical shifts were very close) caused NOEs to H15, H12 (H11), H1β, C19 methyl group, as well as H2 at δ 2.09 (thus assigned as H2β) (not shown in Figure 2.10).

The ^{13}C NMR spectrum showed a C3 signal at δ 75.7, upfield shifted as compared with the chemical shift of C3 (δ 78.8) in **205**, but almost the same as that (δ 75.8) in **202**. Thus, compound **203** was assigned as 14-methoxy-abieta-8,11,13-trien-3α-ol.
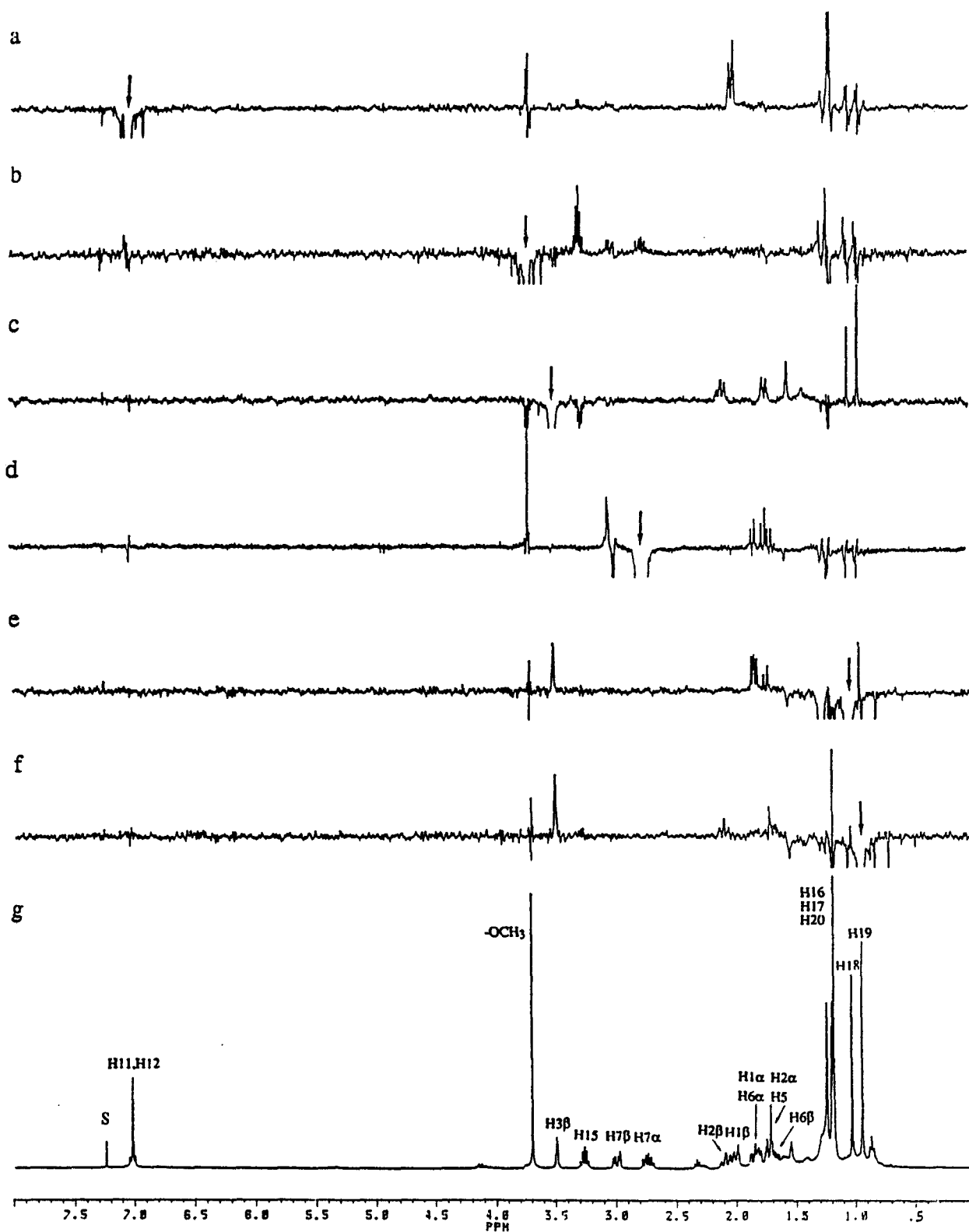
a, {H11,H12}: H1β, H16,H17; b, {C14-OCH3}: H15, H7β, H7α; c, {H3β}: H2β, H2α, OH, H18, H19;

d, {H7α}: C14-OCH3, H6α, H5α, (H6β); e, {H18}: H3β, H6α, H5, H19; f, {H19}: H3β, H2β, H6β,

H20; g, Off-resonance spectrum of 203 (400 MHz, CDCl3). ({irradiated}: enhanced)

Figure 2.10   NOE Difference Spectra of Compound 203
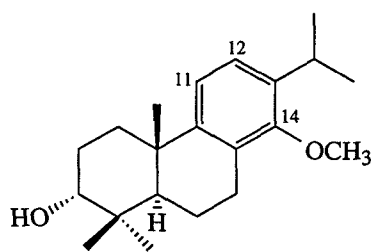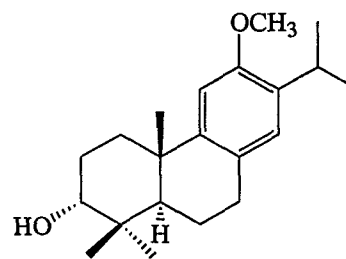
203   183

This assignment was contradictory, in the aspect of the location of the methoxy group, to the compound **183** previously isolated from TRP4a cultures by this group,[158,160] because both compounds had identical ^{1}H NMR spectral data. In order to clarify the assignment, a ^{1}H NMR spectrum was taken on the original sample of **183** dissolved in deuterated benzene (C_6D_6), and the spectrum was compared with that of the same sample measured in deuterated chloroform ($CDCl_3$). The spectrum recorded in C_6D_6 unambiguously exhibited typical AM system signals in the aromatic region, showing one proton at δ 7.06 (d, J = 8.3 Hz) and the other at δ 7.10 (d, J = 8.3 Hz) (Figure 2.11). The large coupling constant demonstrated that these two protons must be in *ortho* positions with respect to each other on the benzene ring, such as H11 and H12, rather than in the *para* positions, such as H11 and H14 as was proposed in the early reports (for benzene, J_{ortho} = 6.0-9.0, J_{meta} = 1.0-3.0, J_{para} = 0.0-1.0 Hz[168]).

The SINEPT (Selective Insensitive Nucleus Enhancement by Polarization Transfer) experimental results described in the early reports indicated that the methoxy group could not be attached to C11, but these SINEPT results cannot differentiate whether the methoxy group is attached to C12 or C14. A close analysis of the spectrum, recorded in $CDCl_3$, revealed that there was an AM system in the aromatic region, but the two doublets were so close to each other that their inside halves almost overlapped, thus appearing as either a broad singlet or two poorly resolved singlets. The outside halves of these two doublets were so weak in intensity that they might be overlooked or mistaken as "spinning sidebands", a phenomenon which may be ruled out by examining the nearby solvent peak which showed no such sidebands. After a careful examination of the spectra, the doublet at δ 7.03 (J = 8.4 Hz, in CD_3Cl) was assigned to

H12 and the other at δ 7.00 (J = 8.4 Hz) was assigned to H11, in comparison with compound 205. The H12 signal in both compounds appeared shorter and broader than did its neighboring H11 signal, perhaps due to a very small, long range, allylic coupling of H12 with H15. Therefore compound 183 should be assigned as 203. This compound has never been isolated from *Tripterygium* plants. A comparison of [1]H NMR data of compounds 197,116, 200, 117, 118, 205 and 203 is provided in Table 2.3.

a)   in CDCl3   b)   in C6D6
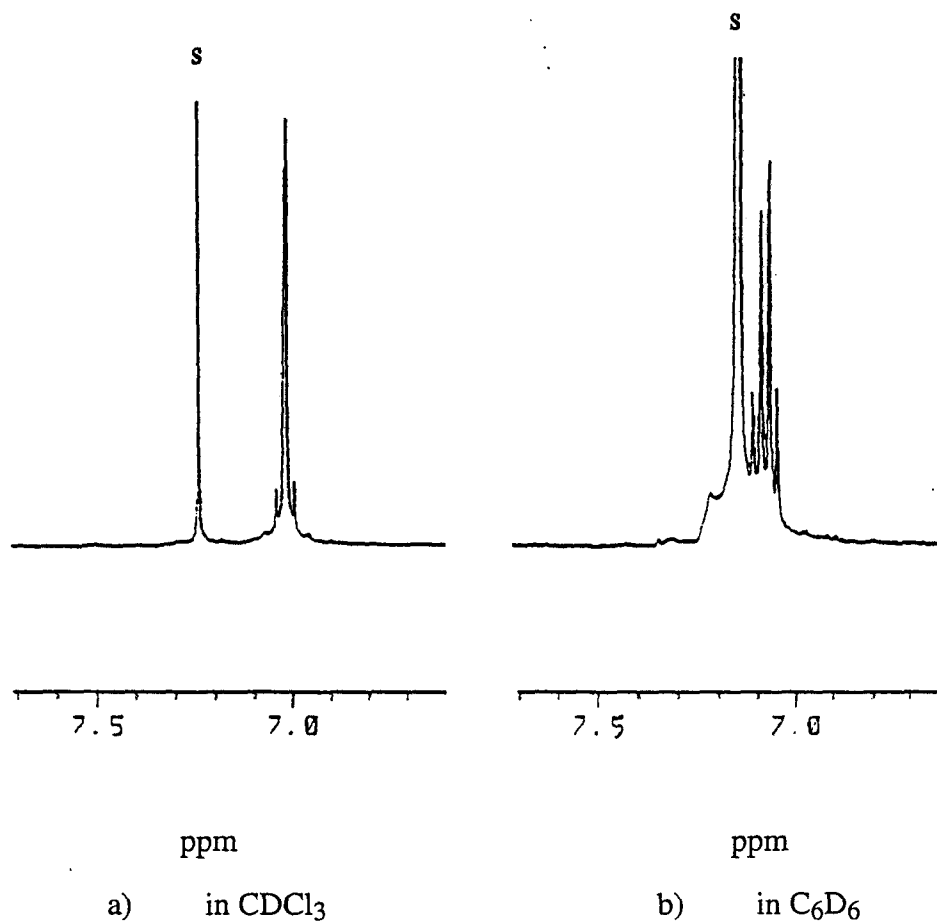
Figure 2.11   [1]H NMR spectra of 203 (400 MHz, aromatic region)

Table 2.3 ^{1}H NMR Spectral Data of Abietane Diterpenes **197, 116, 200, 117, 118, 205 and 203** (400 MHz in CDCl$_3$, δ in ppm, J in Hz in parentheses)

	197	116	200	117	118	205	203
1α	1.39, ddd (12.8, 12.8, 3.7)	1.71-1.96, m	1.92, m	1.94, m	1.91, m	1.52, m	1.77-1.90, m
1β	2.27, d (12.8)	2.41-2.64, m	2.44, ddd (13.2, 7.5, 4.1)	3.05, ddd (13.8, 8.5, 6.3)	2.33, ddd (13.1, 7.4, 4.2)	2.27, ddd (13.1, 3.5, 3.5)	2.00, ddd (13.1, 3.8, 3.5)
2α	1.65-1.81, m	2.41-2.64, m	2.55, ddd (15.8, 7.6, 4.1)	2.66, ddd (15.2, 9.9, 6.3)	2.50-2.71, m	1.77, m	1.62-1.77, m
2β	1.60, m	2.69, ddd (15.7, 10.7, 7.1)	2.66, m	2.43, ddd (15.2, 8.5, 5.9)	2.50-2.71, m	1.77, m	2.09, dddd (14.1, 14.1, 3.8, 2.4)
3α	1.21, m	-	-	-	-	3.28, dd (10.9, 4.9)	-
3β	1.48, d (13.2)	-	-	-	-	-	3.49, dd (3.3, 2.4)
5	1.33, dd (12.7, 2.1)	1.71-1.96, m	1.87, dd (12.6, 1.5)	1.99, dd (12.3, 1.8)	1.84, dd (12.5, 2.1)	1.28, dd (12.5, 2.0)	1.62-1.77, m
6α	1.98, dd (13.3, 7.9)	1.71-1.96, m	1.84, m	1.79, dddd (13.0, 6.0, 1.8, 1.8)	1.83, m	1.91, dd (13.2, 7.5)	1.77-1.90, m
6β	1.65-1.81, m	1.71-1.96, m	1.70, dddd (12.6, 12.6, 12.6, 5.5)	1.54, dddd (13.0, 13.0, 12.3, 4.7)	1.68, ddd (12.5, 12.5, 5.7)	1.68, dddd (13.2, 12.5, 11.6, 5.3)	1.62-1.77, m
7α	2.61, ddd (16.5, 11.3, 7.9)	2.41-2.64, m	2.66, m	2.56, ddd (17.1, 13.0, 6.0)	2.50-2.71, m	2.70, ddd (17.6, 11.6, 7.5)	2.73, ddd (17.8, 10.6, 8.6)
7β	2.81, dd (16.5, 6.6)	2.90, dd (16.6, 5.9)	3.10, dd, (17.4, 5.5)	3.10, ddd (17.1, 4.7, 1.8)	2.99, ddd (17.1, 5.7, 1.6)	3.02, dd (17.6, 5.3)	2.99, dd (17.8, 4.9)
11	6.86, d (8.2)	6.83, d (8.2)	7.00, d (8.3)	OH, 4.55, s	6.38, s	6.98, d (8.4)	7.00, d (8.4)
12	7.02, d (8.2)	7.02, d (8.2)	7.06, d (8.3)	6.36, s	OH, 4.47, s	7.03, d (8.4)	7.03, d (8.4)
14	OH, 4.59, s	OH, 4.65, s	-	-	-	-	-
15	3.15, sept (6.9)	3.10, sept (7.0)	3.27, sept (6.9)	3.23, sept (6.9)	3.41, sept (7.2)	3.26, sept (6.9)	3.26, sept (6.9)
16,17	1.24, 1.26, d (6.9)	1.22, 1.24, d (7.0)	1.19, 1.20, d (6.9)	1.15, 1.165, d (6.9)	1.34, 1.35, d (7.2)	1.18, 1.19, d (6.9)	1.180, 1.189, d (6.9)
18	0.96, s	1.15, s	1.15, s	1.163, s	1.14, s	1.06, s	1.03, s
19	0.94, s	1.12, s	1.12, s	1.12, s	1.11, s	0.88, s	0.94, s
20	1.20, d (0.4)	1.30, s	1.27, s	1.27, s	1.25, s	1.17, s	1.178, s
-OMe	-	-	3.71, s	3.66, s	3.67, s	3.70, s	3.69, s
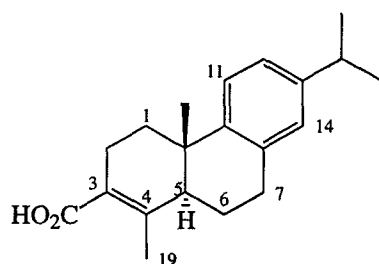
210

Compound **210** (C$_{20}$H$_{26}$O$_2$) was isolated from the polar fraction of the extract (Fr. VII, eluate of methanol; see Figure 2.2). The IR spectrum showed the presence of hydroxyl (3525 cm^{-1}), benzene ring (3034, 1500 cm^{-1}), and possibly α,β-unsaturated carboxyl groups (1685 cm^{-1}).

The ^{1}H NMR spectrum exhibited the AMX system in the aromatic region. Irradiation of proton H14 caused NOE enhancements to signals at δ 2.95 (2H, m, H7) (2%), the septet at δ 2.83 (1H, J = 7.0 Hz, H15) (7%) and the two isopropyl methyl groups at δ 1.22 (6H, d, J = 7.0 Hz, C16, C17 methyls) (1%) (Figure 2.13). The COSY spectrum showed correlations of these two C7 protons to mutually coupled protons (H6) at δ 2.25 (1H, m) and δ 1.71 (1H, ddd, J = 13.1, 10.1, 8.1 Hz). On the basis of the large coupling constants of the δ 1.71 signal, it was logical to assign this signal to the proton H6β (NOE was also observed upon irradiation of the C20 methyl group), and the signal at δ 2.25 to proton H6α.

Saturation of the H11 resonance resulted in NOEs to a methyl signal at δ 1.03 (C20 methyl group) , the H12 signal, and a signal (H1β) in the middle of a two-proton multiplet between δ 2.30 and 2.40. The other proton in this multiplet showed NOE when the two C7 protons were irradiated. Also enhanced were the signals of H14 and the two C6 protons. So the proton signal in the multiplet with H1β must be due to H5, since H7α experiences a nearly 1,3-diaxial relationship with H5.

Proton H1β was strongly coupled with H1α at δ 1.61 (1H, br ddd, J = 12.7, 12.7, 7.5 Hz), and both protons were correlated with the multiplet between δ 2.42 and 2.63 (2H, H2). The chemical shift of the methyl resonance at δ 2.09 (3H, br d, J = 1.2 Hz) indicated that this

methyl group was attached to an unsaturated carbon. The only position left for unsaturated

carbons were C3 and C4. The chemical shifts of H2, H5 and H6 suggested that they were in a

proximity of a double bond, thus there was a likelihood of a double bond between C3 and C4.

Based on the observations of only four methyl group signals in the ^1H NMR spectrum,

two oxygen atoms in the molecular formula (a polar compound), and an IR absorption at 1685

cm^{-1}, the remaining functional group of this compound was probably a carboxyl group. There

are two possible positions that the carboxyl group and a methyl may occupy within the alkene

linkage in this molecule: one is that the carboxyl group is attached to C3 and the methyl to C4

(Figure 2.12a), and the other is just the reverse (Figure 2.12b).

a   b
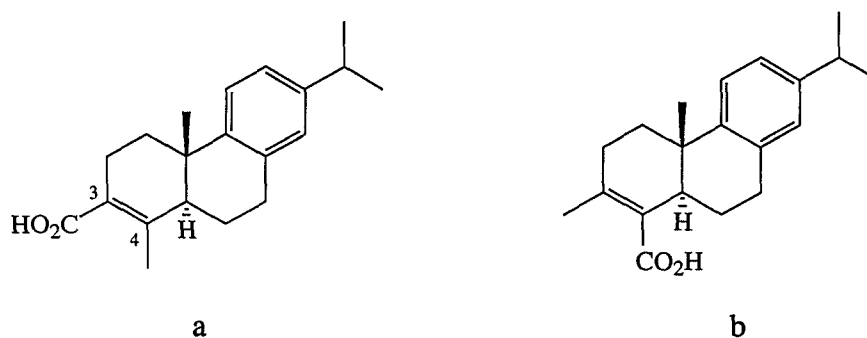
Figure 2.12   Two Possible Structures for **210**

Figure 2.13 illustrates the NOE results in the spectral analyses of **210**. Irradiation of the

multiplet containing H1β and H5 resulted in signal enhancements of H11 (by H1β), H7 (H7α,

by H5), the C20 methyl (by H1β), H1α (by H1β and H5), and of the methyl at δ 2.09 (3% for

the methyl, by H5). Saturation of the H6α resonance showed NOEs to H6β (6%), H7 (4% for

both protons), and to the methyl group at δ 2.09 (5% for the methyl). However, irradiation of

the C2 multiplet did not produce any enhancement of the δ 2.09 methyl signal. These NOE

results demonstrated that the methyl group (C19 methyl) was attached to C4 (close to H5 and

almost co-planar with H6α as well) while the carboxyl group was attached to C3.
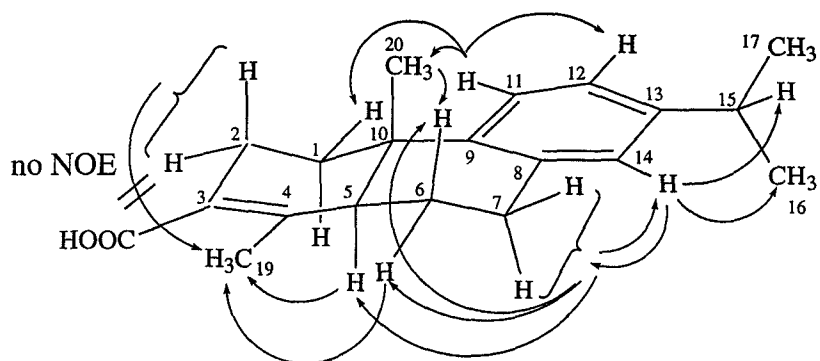
Figure 2.13   NOEs Observed for Compound **210**

The COSY spectrum also displayed small cross peaks of the C19 methyl signal to both the H2 (homoallylic coupling) and H5 (long range coupling) signals, providing additional evidence for a double bond between C3 and C4.

A similar compound with a hydroxyl group at C15 (**182**) was previously isolated from TRP4a cell cultures, and **210** was also obtained as an intermediate in the synthesis of **182** by this group.[157] The spectral data here were comparable with those reported for the synthetic intermediate. Compound **210** is a first time isolate from TRP4a cell cultures.
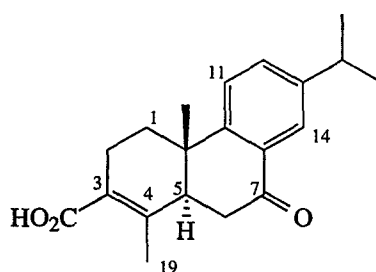
211

Compound **211** ($C_{20}H_{24}O_3$) was also isolated in the polar fraction of the extract. The IR spectrum suggested the presence of hydroxyl (3530 cm^{-1}), benzene ring (3033, 1590 cm^{-1}), and possible α,β-unsaturated carboxyl and aromatic carbonyl (1682 cm^{-1}) functionalities.

Its ^{1}H NMR spectrum showed some similarities between this compound and **210**, but in the aromatic region, the AMX system signals bore a resemblance to compound **199**. The large downfield shift of H14 from δ 6.94 in **210** to 7.90 in this compound suggested that there was a keto group at the C7 position as in compound **199**. Irradiation of H11 at δ 7.35 showed NOE enhancements to a signal at δ 2.44 (1H, br dd, J = 11.2, 6.0 Hz, H1β), and a methyl signal at δ 1.13, which was assigned to the C20 methyl group accordingly.

The COSY spectrum exhibited cross peaks between H1β and H1α at δ 1.71 (1H, m) (strong coupling). Both protons were coupled with C2 protons at δ 2.51 (1H, m) and 2.68 (1H, m), respectively. Irradiation of H1α caused signal enhancements of, not only H1β and H2α but also a proton signal at δ 2.83 (1H, br d, J = 14.2 Hz). This signal was assigned to H5 because of its 1,3-diaxial relationship with H1α. The H5 proton in turn was strongly coupled with two mutually correlated signals at δ 2.59 (1H, dd, J = 18.1, 14.2 Hz) and δ 3.04 (1H, dd, J = 18.1, 4.4 Hz), which were thus assigned to the two C6 protons. No other protons were coupled with these two C6 protons. In consideration of this fact that there were no C7 protons along with the downfield shift of the H6 and H14 signals, it was concluded that C7 contained a keto group.

The rest of the protons were assigned according to NOE and COSY results, as well as in comparison with compounds **210** and **199**. Similarly to compound **210**, the C19 methyl resonance showed small cross peaks to H5 and to both H2 signals. Saturation of the C19 methyl resonance showed NOEs to H6α (7%) and H5 (5%), while irradiation of either H6α or H5 reciprocated the enhancement of the C19 methyl signal. These results provided supporting evidence for the assignment of this compound as a 7-oxo analog of **210**.

Compound **195** was isolated as a colorless oil. It was obtained during the search for compounds that may be present prior to dehydroabietane (**196**) in the metabolic process of *T. wilfordii*. The overwhelming simplicity displayed by its ^{1}H NMR spectrum made us feel somewhat discouraged, yet at the same time very curious. The IR spectrum exhibited virtually only saturated hydrocarbon absorptions at 2950, 1460 and 1390 cm^{-1}. Upon further

examination, a small shoulder at 3050 cm^{-1} and a weak peak at 1670 cm^{-1} were observed, suggesting that there may be some double bonds in the molecule.
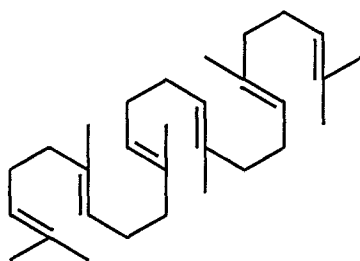
195

The ^{1}H NMR spectrum showed four sets of signals at δ 5.10 (m), 2.03 (m), 1.66 (br s) and 1.58 (br s) in a ratio of 3:10:3:9. The signals at δ 5.10 were likely due to olefinic protons and were correlated with the signals at δ 2.03. These two groups of signals exhibited slight couplings with the other two groups. The mass spectrum showed a molecular ion peak at m/z 410 and other major ion peaks at m/z 367, 341, 81, and 69 (base peak). The base peak suggested a fragment of isopentene and the molecular weight corresponded to squalene. Squalene is a known biosynthetic precursor of triterpenes and steroids.[184] Triterpenoids and phytosterols are two groups of compounds which have been widely isolated from both *Tripterygium* plants and TRP4a cell cultures. High resolution mass spectrometry confirmed its molecular formula as C$_{30}$H$_{50}$ and the spectral data (^{1}H NMR, MS) were in agreement with those in the literature.[185]

Compound **193** was obtained as a solid with a molecular formula of C$_{20}$H$_{24}$O$_3$. Its IR spectrum displayed absorption bands due to a benzene ring (3020, 1600, 1500 cm^{-1}) and an α,β-unsaturated ester or a butenolide (1745, 1670 cm^{-1}). The ^{1}H NMR spectrum exhibited AMX system signals in the aromatic region and an AB quartet centered at δ 4.76. The AB quartet was characteristic of a butenolide functional group, and this functionality has been found in many diterpene metabolites from *Tripterygium* plants, including triptolide (**1**) and

tripdiolide (2). These distinct spectroscopic features drew the attention to isodehydroabietenolide (193), a compound which had been synthesized and extensively studied by our group (see Chapter 3 for the synthesis of this compound). The ^{1}H NMR and mass spectra of isolated 193 were identical with those of the synthetic one.
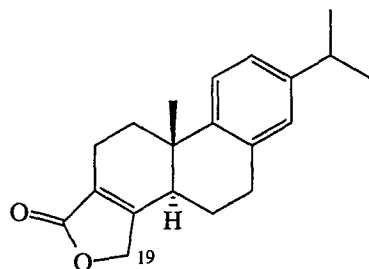
193

(isodehydroabietenolide)

Compound 193 was isolated for the first time from TRP4a cell cultures even though it had been postulated as one of the intermediates in the biosynthetic pathway.[157] It is likely formed by enzymatic hydroxylation of 210 at C19, followed by a facile lactonization to form the butenolide moiety in the molecule. Literature sources make no mention of the isolation of such a compound from *Tripterygium* plants.
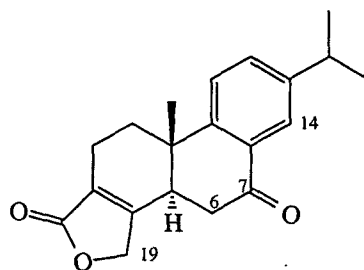
206

Compound 206 was isolated as a solid with a molecular formula of $C_{20}H_{22}O_3$. Its IR spectrum revealed the presence of a carbonyl group conjugated to a benzene ring (3010, 1580, 1550, 1490 cm^{-1}), and of a butenolide (1750, 1670 cm^{-1}) functional group. A strong UV absorption at 255.8 nm (log ε 4.05) also indicated an aromatic ketone.

The ^{1}H NMR spectrum of **206** displayed extremely similar AMX system signals in the aromatic region to those found in compound **211**, indicating a carbonyl group at C7. The aliphatic region of the spectrum exhibited some similarities to that of **193**. The lack of the C7 protons, along with additional spectral information mentioned above suggested that this compound was the 7-oxo derivative of **193**. The H5 resonance was shifted downfield from δ 2.70 in **193** to 3.21, and the H6 resonances from δ 1.91 to about 2.74 in this compound, apparently caused by the presence of a carbonyl group at C7. Comparison of the spectra of isolated **206** with those of synthetic **206** confirmed that they were identical (see Chapter 3 for the synthesis of this compound). This compound has previously been obtained in the biotransformation of isodehydroabietenolide (**193**) in TRP4a cell cultures.[160]
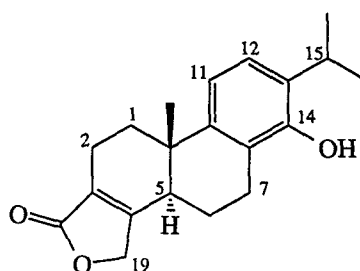
106 (triptophenolide)

Compound **106** was obtained as optically active ($[\alpha]_D^{20}$ +37.6°, c = 0.25, MeOH), colorless prisms (mp 228-230°C, dec.) with a molecular formula of $C_{20}H_{24}O_3$. Its IR absorptions indicated the presence of hydroxyl (3620 cm^{-1}), benzene ring (3025, 1575, 1500 cm^{-1}) and butenolide (1750, 1680 cm^{-1}) functional groups.

The ^{1}H NMR spectrum showed similar AM system signals in the aromatic region as those of **116**, and had some similarities to **193** in the aliphatic region, indicating a 14-hydroxy analog of **193**. The signal at δ 4.71 (1H, br s, D$_2$O exchangeable) was due to the hydroxyl group attached to C14 because of its NOEs with H7 and H15. The remainder of the protons were assigned according to the COSY and NOE results. All the spectral data were identical

with those from an authentic triptophenolide sample obtained from the *T. wilfordii* plant.* This compound has been isolated from the four species of *Tripterygium* plants,[2] but has not previously been isolated from TRP4a cell cultures.
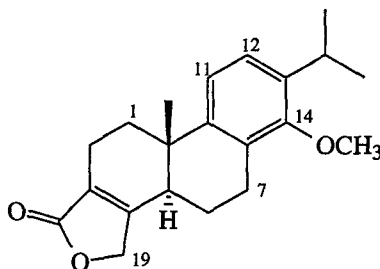
107

Compound **107** was obtained as a solid with a molecular formula of $C_{21}H_{26}O_3$. The IR spectrum showed absorption bands for benzene ring (3050, 1600, 1500 cm^{-1}), butenolide (1740, 1680 cm^{-1}), and possible ether (1280, 1020 cm^{-1}) groups.

The ^{1}H NMR spectrum was almost identical with that of **106** except that the signal in the aromatic region appeared to be a singlet at δ 7.10 (2H) and the hydroxyl proton signal in **106** at about δ 4.7 was now a methoxy signal at δ 3.72. In consideration of the similar situation with **203** (methoxy group at C14 made H11 and H12 appear as a "singlet", *vide supra*), it was evident that this compound was the methyl ether of **106**. Irradiation of the singlet-like aromatic signal (2H) resulted in signal enhancements of H1β (6%), the C16, C17 methyl groups (4% for two methyls) and the C20 methyl group (1% for the methyl), confirming that the aromatic signals actually arose from H11 and H12. Other spectral differences from that of **106** were a small downfield shift of the H15 and H7 resonances. This compound has been isolated from the *Tripterygium* plants, but this is its initial isolation from the TRP4a cell cultures. The spectral data were identical to those in the literature. A comparison of ^{1}H NMR spectral data of the isolated *abeo*-abietane diterpenes **210, 211, 193, 206, 106** and **107** is given in Table 2.4.

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* The authentic sample was generously provided by Professor B.-N. Zhou, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, the People's Republic of China.

Table 2.4 ^{1}H NMR Spectral Data of *abeo*-Abietan Diterpenes **210**, **211**, **193**, **206**, **106** and **107** (400 MHz in CDCl$_3$, δ in ppm, J in Hz in parentheses)

	210	211	193	206	106	107
1α	1.61, ddd (12.7, 12.7, 7.5)	1.71, m	1.69, ddd (12.4, 12.4, 6.5)	1.90, ddd (11.6, 11.6, 6.5)	1.68, ddd (12.3, 12.3, 6.5)	1.69, ddd (12.2, 12.2, 6.3)
1β	2.30-2.40, m	2.44, dd (11.2, 6.0)	2.50, m	2.58, m	2.44-2.57, m	2.44-2.56, m
2α	2.42-2.63, m	2.68, m	2.50, m	2.58, m	2.44-2.57, m	2.44-2.56, m
2β	2.42-2.63, m	2.51, m	2.37, m	2.43, m	2.39, m	2.37, m
5	2.30-2.40, m	2.83, d (14.2)	2.70, m	3.21, m	2.69, d (13.4)	2.67, d (13.1)
6α	2.25, m	3.04, dd (18.1, 4.4)	1.91, m	2.743, d (7.8)	1.99, m	1.80-2.00, m
6β	1.71, ddd (13.1, 10.1, 8.1)	2.59, dd (18.1, 14.2)	1.91, m	2.738, d (11.3)	1.90, m	1.80-2.00, m
7α	2.95, m	-	3.00, m	-	2.73, m	2.93, ddd (18.2, 10.3, 2.0)
7β	2.95, m	-	3.00, m	-	2.73, m	3.05, ddd (18.2, 7.5, 1.9)
11	7.22, d (8.1)	7.35, d (8.2)	7.25, d (8.1)	7.38, d (8.0)	6.93, d (8.2)	7.10, s
12	7.00, d (8.1)	7.42, dd (8.2, 2.0)	7.03, dd (8.1, 1.5)	7.45, dd (8.0, 1.9)	7.05, d (8.2)	7.10, s
14	6.94, s	7.90, d (2.0)	6.96, d (1.5)	7.93, d (1.9)	OH, 4.71, s	-
15	2.83, sept (7.0)	2.93, sept (7.0)	2.82, sept (6.9)	2.95, sept (6.9)	3.08, sept (7.0)	3.28, sept (6.9)
16, 17	1.22, d (7.0)	1.25, d (7.0)	1.22, d (6.9)	1.25, d (6.9)	1.24, 1.26, d (7.0)	1.20, 1.22, d (6.9)
19	2.09, d (1.2)	2.07, s	4.76, ABq (17.2)	4.76, m	4.77, ABq (17.2)	4.77, ABq (17.3)
20	1.03, s	1.13, s	1.01	1.14, s	1.01, s	1.01, s
-OMe	-	-	-	-	-	3.72, s
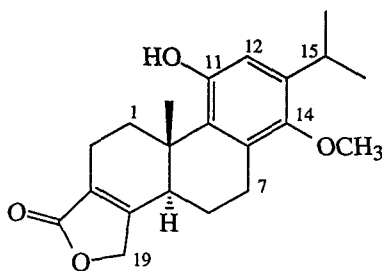
109

Compound **109** was isolated as a pale yellowish powder with a molecular formula of $C_{21}H_{26}O_4$. It was optically active ($[\alpha]_D^{24}$ +113.4°, c = 0.307, MeOH), and the IR spectrum established the presence of hydroxyl (3600 cm^{-1}), butenolide (1745 cm^{-1}), benzene ring (3020 cm^{-1}), and ether (1240, 1030 cm^{-1}) functionalities.

The ^{1}H NMR spectrum exhibited an aromatic signal at δ 6.39 (s), a hydroxyl proton at δ 4.58 (s, D_2O exchangeable) and a methoxy group at δ 3.66, similar to compound **117**. Irradiation of the proton at δ 6.39 showed NOEs to the hydroxyl proton at δ 4.58 and the two isopropyl methyl groups at 1.18 (6H, d, J = 6.9 Hz), suggesting that the irradiated proton was H12 and the hydroxyl group was at C11 as in **117**. Saturation of the methoxy resonance at δ 3.66 resulted in signal enhancements of H15 at δ 3.23 (1H, septet, J = 6.9 Hz) and a proton at δ 3.06 (1H, br dd, J = 17.9, 4.6 Hz), confirming that the methoxy was attached to C14, and the enhanced signal at δ 3.06 was H7β. The downfield-shifted signal at δ 3.51 (1H, ddd, J = 13.5, 5.4, 2.0 Hz) due to the presence of a hydroxyl group at C11 was assigned to H1β. The remainder of the protons were assigned accordingly as based on COSY and NOE results. The structure of this compound was determined as 11-hydroxyl-14-methoxy-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (**108**). This compound has been isolated from *Tripterygium* plants, but its isolation here constitutes the first isolation from TRP4a cell cultures.
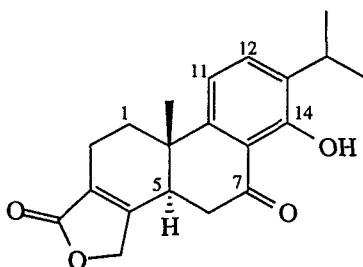
108

Compound **108** was isolated as optically active ($[\alpha]_D^{20}$ -36.6°, c = 1.82, CHCl$_3$), colorless needles (mp 202-204°C) with a molecular formula of $C_{20}H_{22}O_4$. Its UV spectrum showed a strong absorption at 268.4 nm (log ε = 4.02), indicating an aromatic ketone chromophore. The IR spectrum suggested the presence of hydroxyl (3700, 1240 cm^{-1}), butenolide (1750, 1670 cm^{-1}), and aromatic carbonyl (1620 cm^{-1}) functionalities.

The ^{1}H NMR spectrum of **108** revealed some similarities with **106**, such as the AM system signals in the aromatic region. The H7 signals were not found in the spectrum. This suggested that C7 contained a keto group, which was consistent with the UV and IR spectral data. The close proximity of this carbonyl to the C14 hydroxyl proton allowed for the possibility of a hydrogen bonding, thus this active proton (D$_2$O exchangeable) was found at a very low field at δ 13.3. The H6 signals always appear as sharp peaks around δ 2.7-2.8 when there is a keto group at C7 for compounds of the isodehydroabietenolide series. The doublet at δ 2.77 (1H, J = 7.1 Hz) was assigned to H6α while the doublet at δ 2.78 (1H, J = 11.7 Hz) to H6β since irradiation of the C20 methyl group greatly enhanced the doublet at δ 2.78. This compound has previously been isolated from the *Tripterygium* plants, but was obtained for the first time from TRP4a cell cultures. A comparison of the spectral data confirmed its identity.

Compound **138a** (structure on next page) was isolated as a solid with a molecular formula of $C_{20}H_{24}O_4$. The presence of hydroxyl (3600, 3400, 1260, 1040 cm^{-1}), benzene ring (3050, 1630, 1580, 1500 cm^{-1}) and butenolide (1745, 1680 cm^{-1}) groups was indicated by its IR absorptions.
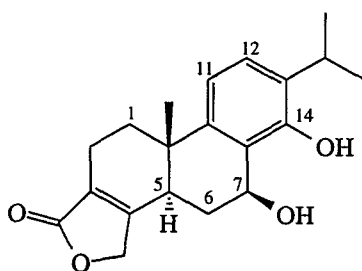
138a

The ^{1}H NMR spectrum of **138a** was similar to that of **106**, but no H7 signals were observed in the normal region around δ 2.8-2.9. However, a new signal, probably due to a benzylic or allylic proton geminal to a hydroxyl group, appeared at δ 5.22 (1H, ddd, J = 10.0, 7.8, 7.8 Hz). Figure 2.14 depicts the major results from COSY and NOE experiments. The signal at δ 5.22 showed cross peaks to signals at δ 3.14 (1H, br d, J = 7.8 Hz), 2.33 (2H, m), and 1.99 (1H, ddd, J = 13.9, 12.7, 10.0 Hz). The H5 resonance, which was easily located once it was compared with the spectrum of **106**, was found at δ 2.76 (1H, br d, J = 13.8 Hz). The strong coupling shown between H5 and the proton at δ 1.99 suggested that this proton was H6β. Both H5 and H6β were also coupled with part of the two-proton multiplet at δ 2.33, indicating that one of the protons in this multiplet was H6α. Because the signal at δ 5.22 was only coupled with two C6 proton, it was assigned to proton at C7.
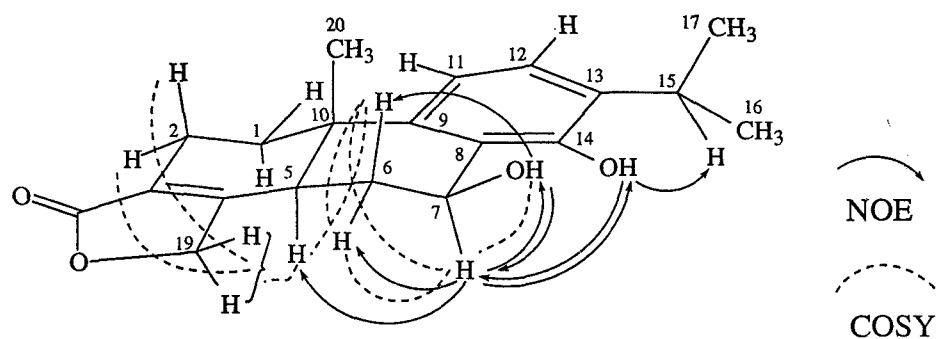
Figure 2.14   Major COSY and NOE Results Observed for **138a**

The signal at δ 3.14, which was also coupled with the H7 signal, was assigned to the proton of the hydroxyl group at C7 since its chemical shift changed with the concentration of the sample. Irradiation of H7 at δ 5.22 resulted in NOEs to H5 (5%) and H6α (6%), indicating that H7 was α-oriented, and thus the compound was a C7 β alcohol. Other signals that were enhanced due to irradiation at δ 5.22 were the 7-hydroxyl proton at δ 3.14 and another one at 8.22, the latter was believed to be the proton of the C14-hydroxyl group. Saturation of the C7 hydroxyl resonance showed NOEs to H7α (11%) and H6β (4%). These results proved that the assignment of the β hydroxyl group at C7 was correct.

The mass spectrum of compound **138a** showed a very weak molecular ion (0.5%),[145] but moderate M - 2 and quite strong M - H₂O and M - H₂O - CH₃ ion peaks. The identity of this compound was finally confirmed by comparing it with the reduction product of **108**. Compound **108**, treated with sodium borohydride in ethanol at room temperature for a couple of hours, gave **138a** (β alcohol[145]) as the major product, and a small amount of mixture containing both 7α (**138b**) and 7β alcohols.

108

NaBH₄

138a 7β-OH
138b 7α-OH
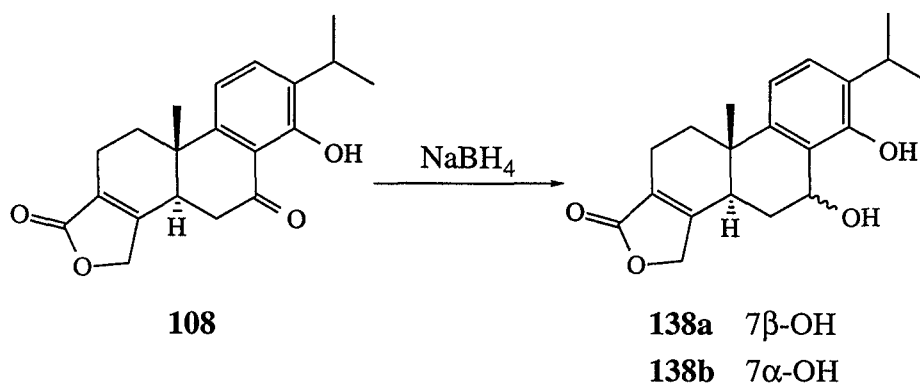
Compound **207** (structure on next page) was obtained as a solid. High resolution mass spectrometry showed a molecular formula of $C_{21}H_{26}O_4$. The [1]H NMR spectrum of **207** exhibited great similarities with **138a**, with the exception of a methoxy group at δ 3.83 and the lack of a C14 hydroxyl proton. Furthermore, the split between AM system signals in the aromatic region was much smaller than in **138** (one signal at δ 7.20 (1H, d, J = 8.4 Hz) and the

other at δ 7.12 (1H, d, *J* = 8.4 Hz)), suggesting a methoxy group at C14, as compared with **107**

and **106**. If the hydroxyl group at C7 was methylated, then the H7 signal was expected to move

upfield by approximately 0.2-0.4 ppm.[168,186] However, the chemical shift of H7 was virtually

unchanged, so this compound was proposed as the C14 hydroxyl methyl ether of **138a** and the

remaining protons were assigned accordingly.
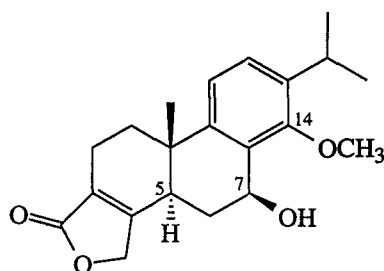
207

The structural assignment of **207** was supported by NOEs to the methoxy group, H5 at δ

2.72 (1H, br d, *J* = 14.0 Hz), H6α at δ 2.32 (1H, m), and the hydroxyl group at δ 1.5 (buried in

the water signal), caused by irradiation of H7α at δ 5.25 (1H, dd, *J* = 9.0, 9.0 Hz). Irradiation

of the methoxy group increased the intensities of H7α and H15 signals as expected. The [1]H

NMR data for **109**, **108**, **138a**, and **207** are summarized in Table 2.5.
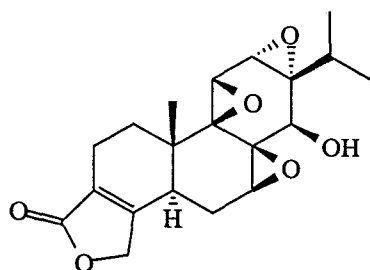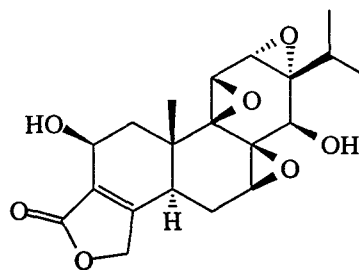
Tl (**1**)   Td (**2**)

Tl (**1**) and Td (**2**) were isolated as colorless crystals and their spectra were identical with

those obtained from authentic samples.

Table 2.5 ^{1}H NMR Spectral Data of *abeo*-Abietane Diterpenes **109**, **108**, **138a** and **207** (400 MHz in CDCl$_3$, δ in ppm, J in Hz in parentheses)

	109	108	138a	207
1α	1.53, m	1.79, ddd (12.4, 12.4, 5.9)	1.64, ddd (12.6, 12.6, 6.6)	1.65, m
1β	3.51, ddd (13.5, 5.4, 2.0)	2.51, dd (12.4, 6.0)	2.44, m	2.27-2.53, m
2α	2.38, m	2.57, m	2.44, m	2.27-2.53, m
2β	2.38, m	2.40, m	2.33, m	2.27-2.53, m
5	2.76, m	3.16, m	2.76, d (13.8)	2.72, d (14.0)
6α	1.77, m	2.77, d (7.1)	2.33, m	2.27-2.53, m
6β	1.77, m	2.78, d (11.7)	1.99, ddd (13.9, 12.7, 10.0)	1.93, ddd (14.0, 13.0, 9.0)
7α	2.76, m	-	5.22, ddd (10.0, 7.8, 7.8)	5.25, dd (9.0, 9.0)
7β	3.06, dd (17.9, 4.6)	-	OH, 3.14, d (7.8)	-
11	OH, 4.58, s	6.86, d (7.9)	6.85, d (8.2)	7.12, d (8.4)
12	6.39, s	7.40, d (7.9)	7.13, d (8.2)	7.20, d (8.4)
14	-	OH, 13.3, s	OH, 8.22, s	-
15	3.23, sept (6.9)	3.33, sept (7.0)	3.28, sept (6.9)	3.27, sept (6.9)
16, 17	1.18, d (6.9)	1.21, 1.23, d (7.0)	1.20, 1.22, d (6.9)	1.18, 1.27, d (6.9)
19	4.77, AB$_q$ (17.2)	4.75, AB$_q$ (17.1)	4.75, AB$_q$ (17.2)	4.77, m
20	1.14, s	1.12, s	1.08, s	1.10, s
-OMe	3.66, s	-	-	3.83, s

Compound **212** (structure on next page) was isolated as a colorless powder with a molecular formula of C$_{20}$H$_{22}$O$_7$. Its IR spectrum indicated the presence of hydroxyl (3270 cm^{-1}), butenolide (1757 cm^{-1}) and keto (1731 cm^{-1}) functional groups.

The ^{1}H NMR spectrum of **212** displayed a very similar spectrum to that of Td (**2**), but the H14 signal was not present and the H11, H12 resonances were shifted downfield by 0.15

and 0.3 ppm, respectively. The H7 and H15 signals were also shifted downfield by 0.05 and 0.15 ppm. This was a clear indication of the presence of a keto group at C14. The other proton signals were found at almost the same positions in the spectrum as those of Td (2). The H2α signal at δ 4.62 (1H, m) was correlated with H1α at δ 1.53, which, in turn, was coupled with H1β at δ 1.88 (1H, br d, J = 14.1 Hz). The H11α at δ 4.06 (1H, d, J = 2.9 Hz) was coupled with H12β at δ 3.81 (1H, d, J = 2.9 Hz). The H7α resonance at δ 3.40 (1H, d, J = 5.4 Hz) showed cross peaks to H6α at δ 2.25 (1H, br ddd, J = 15.0, 5.8, 5.4 Hz), which was coupled with H6β at δ 2.09 (1H, br dd, J = 15.0, 13.4 Hz). Both H6 signals showed correlations with H5 at δ 2.74 (1H, br dd, J = 13.4, 5.8 Hz). The C19 protons appeared at δ 4.76 (2H, m) not as a broad AB quartet as in 106, but as a sharp peak, a feature typical of Td (2) and Tl (1) related compounds.
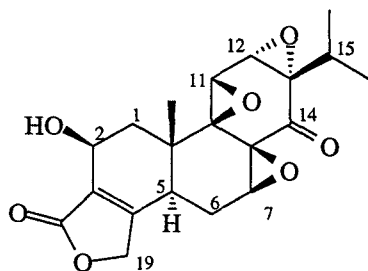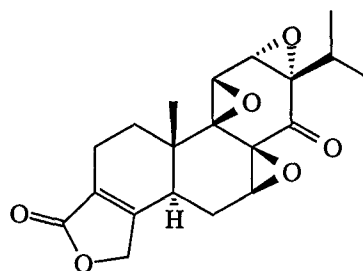
212   triptonide (**95**)

Triptonide (**95**), the "Tl version" of compound **212** which has no hydroxyl group at C2, has been isolated from the *Tripterygium* plants, but compound **212** has never before been isolated from either the *Tripterygium* plants or from TRP4a cell cultures. The [1]H NMR data were comparable with those of triptonide (**95**)[187] except for the H2 related signals. This new compound is named triptolonide.
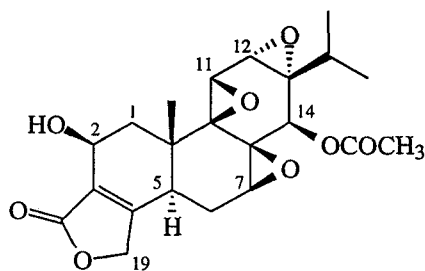
208

Compound **208** was obtained as a colorless solid with a molecular formula of $C_{22}H_{26}O_8$. Its IR spectrum displayed absorptions for hydroxyl (3600 cm⁻¹), butenolide (1752, 1680 cm⁻¹), and possible ester (1752, 1234 cm⁻¹) functionalities.

The ¹H NMR again exhibited a very similar spectrum to that of Td (**2**), except that the H14 resonance was not found around δ 3.4, but a new signal appeared at δ 5.06. An acetate methyl signal was found at δ 2.15. Irradiation of the proton at δ 5.06 (1H, s) resulted in signal enhancements of H7α at δ 3.44 (1H, d, J = 5.6 Hz), of the methyl at δ 2.15 and of the two isopropyl methyl groups at δ 0.82 and 0.94 (3H each, both d, J = 7.0 Hz). NOEs were observed between H7α and the proton at δ 5.06, and H6α at δ 2.20 (1H, ddd, J = 14.7, 5.8, 5.6 Hz). The signal at δ 5.06 was also enhanced (together with H15) upon irradiation of the isopropyl methyl signals. These NOE results along with those from molecular model analysis confirmed that the proton at δ 5.06 was the H14α proton, and it was about 1.7 ppm downfield due to the ester group at C14.[168]
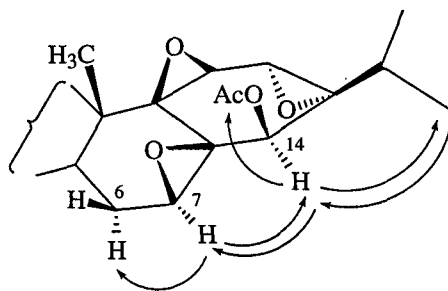
208

The structure elucidation of **208** was further supported by mass spectrometry. The mass spectrum of this compound showed the molecular ion peak at m/z 418 (1.1%) and the M - H_2O ion peak at m/z 400 (4.1%). Cleavage of either CH_3CO or CH_3COO group from the molecular ion or from the M - H_2O fragment was seen at m/z 375 (1.1%), 359 (1.3%) or 357 (1.3%), 341 (2.0%), respectively. The structure of the new compound **208** was thus assigned as the C14-acetate of Td.
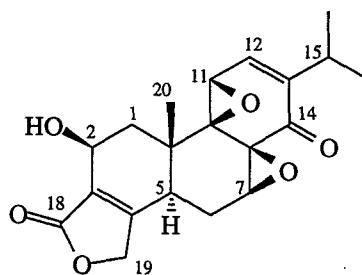
209

Compound **209** was isolated as an optically active ($[\alpha]_D^{24}$ -225.9°, c = 0.251, $CHCl_3$) white powder with a molecular formula of $C_{20}H_{22}O_6$. Its IR spectrum showed presence of hydroxyl (3600 cm⁻¹), butenolide (1755 cm⁻¹) and α,β-unsaturated keto groups. The UV spectrum had a strong absorption at 255.8 nm (log ε = 3.57).

Its ^1H NMR spectrum revealed some similarities with that of Td (**2**). The signals of the protons on ring A and B were little changed. The C19 protons were found at δ 4.76 (2H, m, sharp), and H2α at δ 4.63 (1H, br d, J = 5.9 Hz). Proton H5 appeared at δ 2.66 (1H, br dd, J = 13.1, 5.7 Hz), and the H6α and H6β resonances were located at δ 2.24 (1H, ddd, J = 14.6, 5.7, 5.3 Hz) and 2.13 (1H, br dd, J = 14.6, 13.1 Hz), respectively.

The differences between **209** and Td (**2**) were seen by their ring C proton signals. There were only two doublets present in the region from δ 4.0 to 3.3, whereas four doublets can be found for Td (H7, H11, H12, and H14). The H15 signal was shifted downfield from δ 2.20 to 2.83 (1H, septet d J = 6.8, 1.0 Hz) as compared with Td (**2**). A new signal was found at δ 6.91

(1H, dd, J = 4.8, 1.0 Hz), which was coupled with a proton at δ 3.82 (1H, d, J = 4.8 Hz) as shown by the COSY spectrum (Figure 2.15, a).

a, H12/H11α; b, H2α/H1α; c, H19/H5; d, H7α/H6α; e, H15/H16,H17; f, H5/H6α,H6β;
g, H1β/H1α; h, H6α/H6β
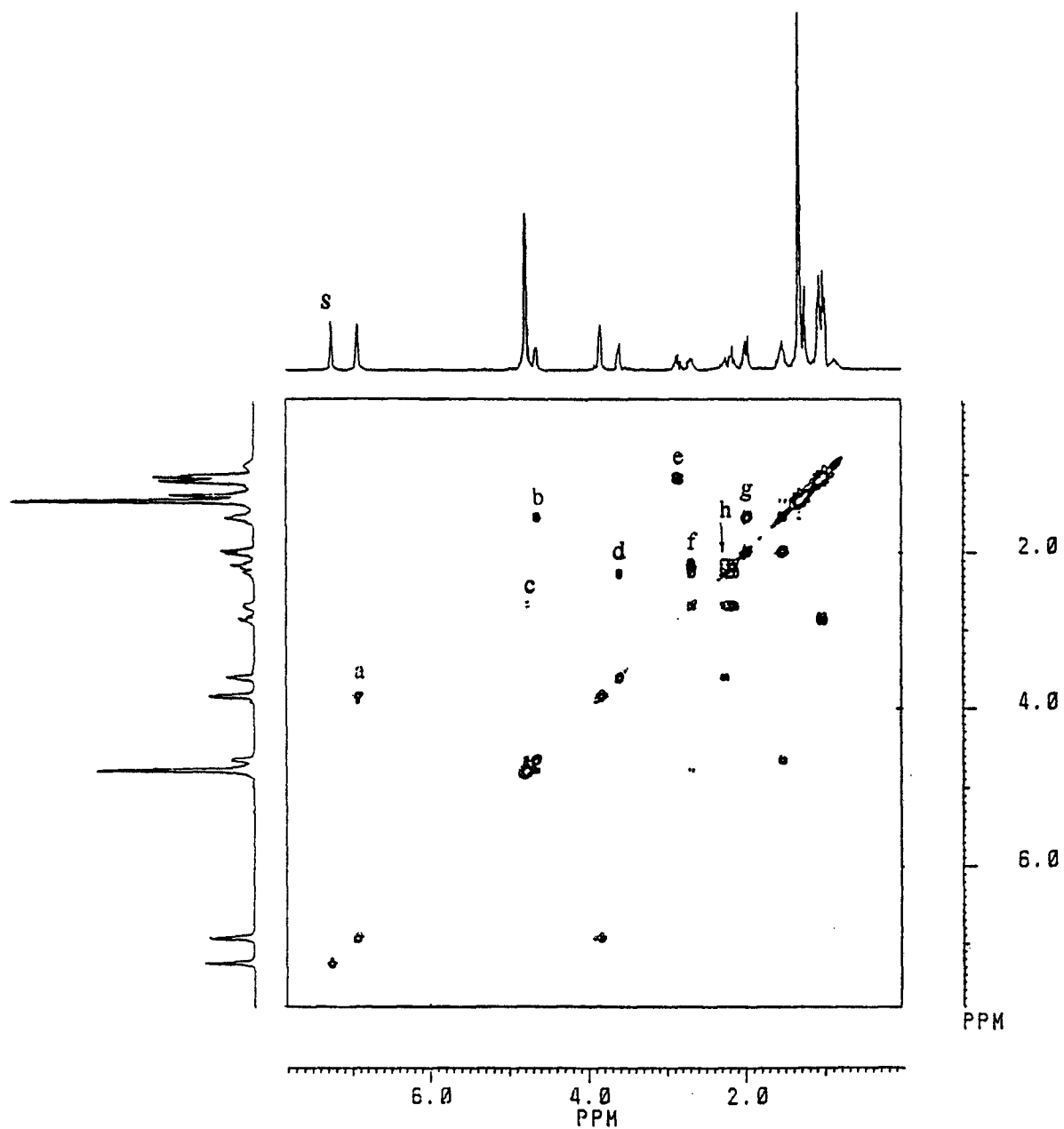
Figure 2.15   COSY Spectrum of Compound **209**

Irradiation of the proton at δ 6.91 resulted in signal enhancements of the proton at δ 3.82 (5%), H15 (2%) and the isopropyl methyl groups (10% for two methyls) (Figure 2.16a). In addition, irradiation of the proton at δ 3.82 showed NOEs not only to the proton at 6.91, but also to H1β (4%) and H1α (8%) (Figure 2.16c). These two irradiations demonstrated that the signal at δ 6.91, which must be from a proton attached to an unsaturated carbon, was due to H12, while the one at δ 3.82 was H11. H11 was believed to be an α proton on the basis of biogenesis, and its orientation was subsequently confirmed by the NOE experiments (H1α was more enhanced than H1β when H11 was irradiated).

Irradiation of the proton at δ 3.59 showed NOEs to H6α (5%) and H6β (1%), indicating that the irradiated proton was the H7α proton (Figure 2.16d). There was no H14 proton in this compound since irradiation of H7α should have enhanced this H14α signal. Furthermore, in conjunction with information provided by IR and UV spectra, along with the fact that H15 and H7α signals were shifted downfield significantly in comparison with Td (2), it was concluded that C14 was a carbonyl group and thus formed an α,β-unsaturated ketone system with a double bond between C12 and C13. The small coupling (1 Hz) that existed between H12 and H15 (allylic coupling, not shown in Figure 2.15) also provided additional support for this assignment.

In order to assign the carbon signals in the ^{13}C NMR spectrum, HMBC (Heteronuclear Multiple Bond Connectivity) and HMQC (Heteronuclear Multiple-Quantum Coherence) experiments were performed on a Bruker AMX-500 (500 MHz) instrument. The HMQC spectrum (Figure 2.17) showed cross peaks of isopropyl methyl proton signals to two carbon signals at δ 21.3 and 21.5, indicating that these two carbon signals arose from C16 and C17 (Figure 2.17, 1). The angular methyl proton signal shared a cross peak with a carbon signal at δ 15.4, suggesting that the carbon signal was due to C20 (Figure 2.17, k). Both H1 proton signals were correlated with a carbon signal at δ 38.6, so this carbon must be C1 (Figure 2.17, i, j). Similarly, C2, C5, C6, C7, C11, C12, C15 and C19 were found at δ 59.43, 41.3, 23.1, 61.9, 52.2, 136.5, 27.1 and 70.1, respectively.

Figure 2.16   NOE Difference Spectra of Compound **209** (continued to the next page)

f, {H1β}: H2α, H11α, (H1α), H20 ; g, {H1α}: H2α, H11α, H5, H1β; h, {H20}: H6β, H1β;
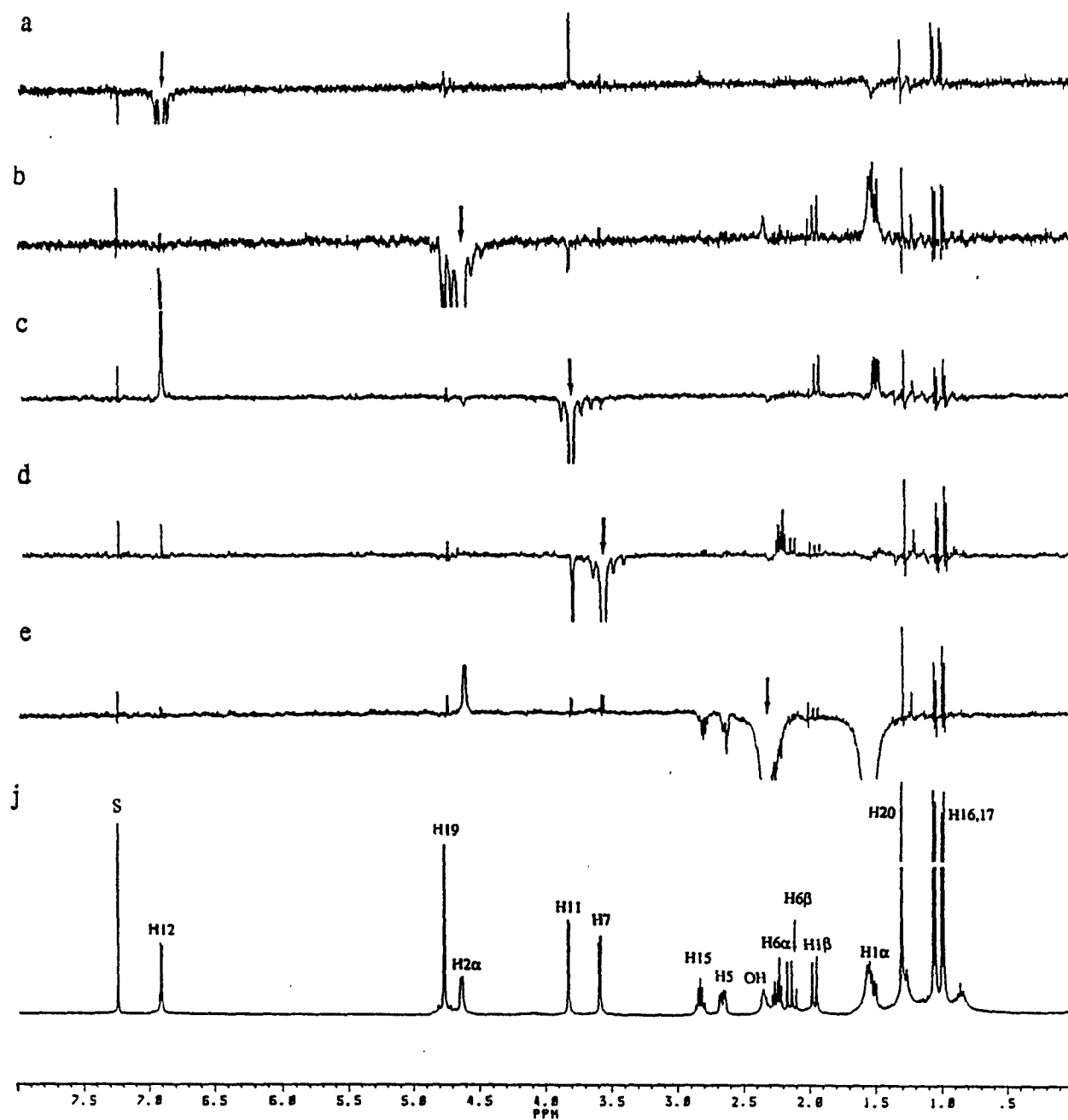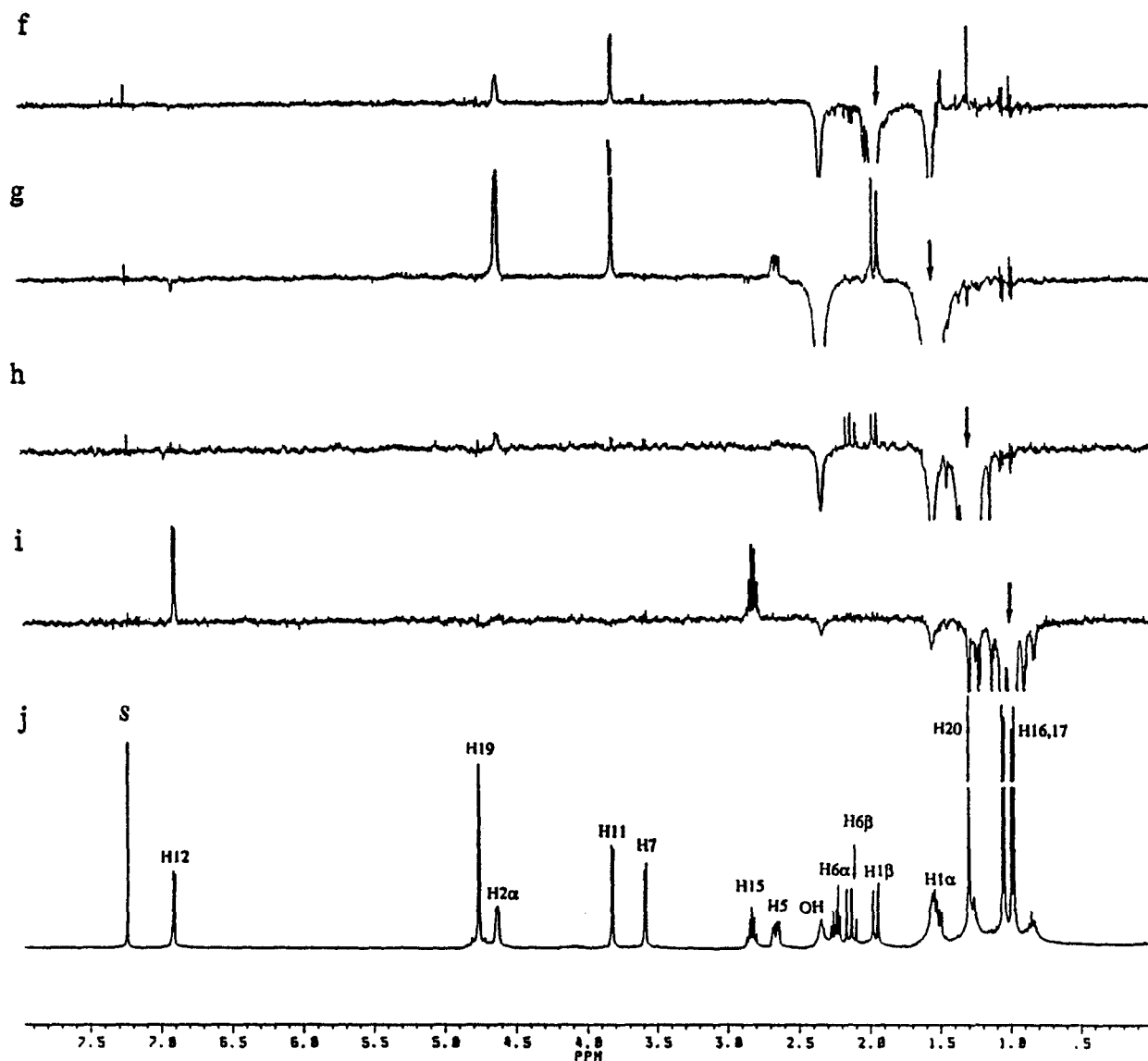
i, {H16,H17}: H12, H15; j, Off-resonance spectrum of **209** (400 MHz, CDCl₃). ({irradiated}:

enhanced)

Figure 2.16   NOE Difference Spectra of Compound **209** (continued)

The HMBC spectrum was helpful in determination of those quaternary carbon signals. Figure 2.18 highlights correlations between the quaternary carbon resonances and the assigned

proton signals. It showed that the signals of angular methyl protons, the two H1, the H6α and H2α protons were all correlated with a carbon signal at δ 35.1 (Figure 2.18, c, i, j, k, l). Since the number of bonds over which the hydrogen-carbon long range coupling can be detected is usually two or three in these experiments, the only carbon that has two or three bonds connections with protons H20, H1 (two bonds) and with the protons H2α and H6α (three bonds) is C10. So the carbon signal at δ 35.1 was due to the carbon C10. Because the geometry between these bonds, i.e., the coupling path, is important for the transfer of this type of long range coupling,[172] not all the protons within two or three bonds exhibit cross peaks to this carbon (for instance, H6β, H5 and H11α in this case), but all the protons showing cross peaks should be within that 2-3 bond range. The carbon signal at δ 63.9 was correlated with H1β, the angular methyl protons, and H12 (Figure 2.18, o, r, a), suggesting that it was the C9 signal. The C3 resonance was found at δ 127.2 because it had correlations with the H1β, H2α and the H19 signals. The carbon signal at δ 172.6 was assigned to C18 since all butenolide compounds of this series show the C18 signal around δ 173 although no cross peaks were observable to any protons in this case. The rest of the carbon signals were assigned readily except for the C8 signal.

Of the 20 carbons, only the C8 signal was not easy to locate in the HMBC spectrum. The C2 carbon signal at δ 59.43 exhibited a cross peak to H1β, which is connected to C2 with two bonds. However, C2 seemed to have another cross peak to H6β that is five bonds away. Upon closer examination of the "cross peak" between C2 and H6β (Figure 2.18, m), it was found that this cross peak corresponded to a carbon signal at a slightly higher field than that of C2, suggesting that this cross peak was actually caused by H6β with another carbon identified to be C8 (three bonds). This signal was so weak and close to the C2 signal that it was hard to find by routine [13]C NMR spectra recorded on instruments with low or moderate magnetic field. A [13]C NMR spectrum run on the AMX-500 spectrometer with expansion of the spectrum finally revealed the C8 signal at δ 59.38, right near the foot of the C2 signal at δ 59.43. Since

C8 is in the center of the rigid epoxide functions and has fewer protons nearby, it probably has a longer relaxation time (T$_1$) and thus results in a very weak signal.
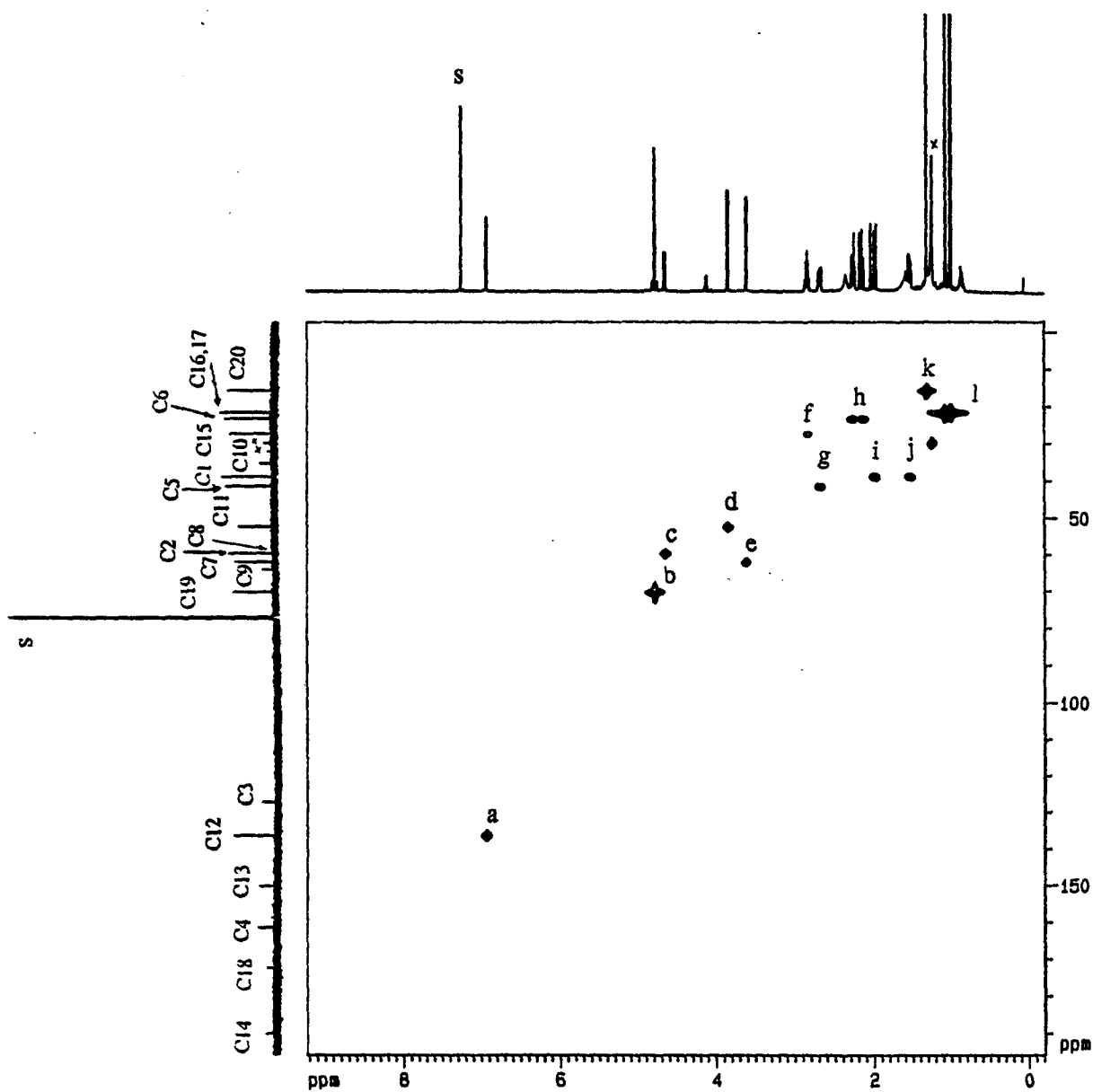
a, C12/H12; b, C19/H19; c, C2/H2α; d, C11/H11; e, C7/H7; f, C15/H15; g, C5/H5; h, C6/H6;
i, C1/H1β; j, C1/H1α; k, C20/H20; l, C16,C16/H16,H17

Figure 2.17   HMQC Spectrum of **209**
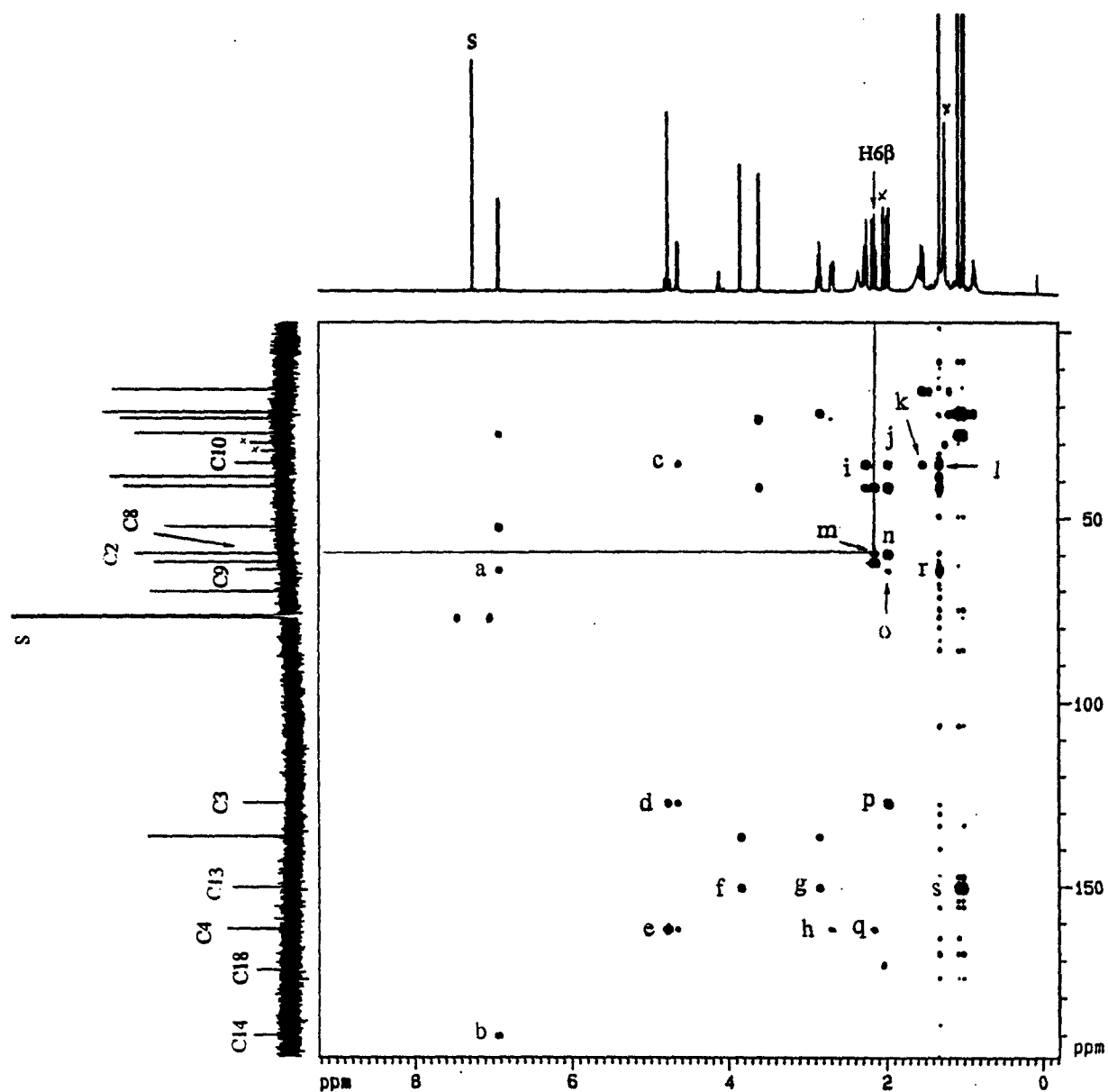
a, C9/H12; b, C14/H12; c, C10/H2α; d, C3/H19,H2α; e, C4/H19,H2α; f, C13/H11α; g, C13/H15;

h, C4/H5; i, C10/H6α; j, C10/H1β; k, C10/H1α; l, C10/H20; m, C8/H6β; n, C2/H1β; o, C9/H1β;

p, C3/H1β; q, C4/H6β; r, C9/H20; s, C13/H16,H17

Figure 2.18   HMBC Spectrum of **209**

Table 2.6 ^{1}H NMR Spectral Data of Epoxides Tl (**1**), Td (**2**), **212**, **208** and **209** (400 MHz in CDCl$_3$, δ in ppm, J in Hz in parentheses)

	Tl (**1**)	Td (**2**)	**212**	**208**	**209**
1α	1.19, ddd (12.4, 12.4, 5.9)	1.41, dd (13.8, 5.6)	1.53, m	1.43, dd (14.1, 5.8)	1.52, dd (13.9, 5.9)
1β	1.53, ddd (12.4, 5.4, 1.2)	1.83, d (13.8)	1.88, d (14.1)	1.862, d (14.1)	1.97, d (13.9)
2α	2.29, d (18.2)	4.58, d (5.6)	4.62, m	4.59, d (5.8)	4.63, d (5.9)
2β	2.11, m	-	-	-	OH, 2.35, s
5	2.67, m	2.62, ddd (13.1, 6.3, 1.5)	2.74, dd (13.4, 5.8)	2.60, dd (13.0, 5.8)	2.66, dd (13.1, 5.7)
6α	2.15, ddd (14.8, 5.9, 5.5)	2.20, m	2.25, ddd (15.0, 5.8, 5.4)	2.20, ddd (14.7, 5.8, 5.6)	2.24, ddd (14.6, 5.7, 5.3)
6β	1.94, dd (14.8, 13.3)	2.05, dd (14.7, 13.1)	2.09, dd (15.0, 13.4)	2.00, dd (14.7, 13.0)	2.13, dd (14.6, 13.1)
7α	3.34, d (5.5)	3.34, d (5.3)	3.40, d (5.4)	3.44, d (5.6)	3.59, d (5.3)
7β	-	-	-	-	-
11α	3.87, d (3.1)	3.90, d (3.2)	4.06, d (2.9)	3.83, d (3.1)	3.82, d (4.8)
12β	3.49, dd (3.1, 0.8)	3.49, dd (3.2, 0.8)	3.81, d (2.9)	3.51, d (3.1)	H12, 6.91, dd (4.8, 1.0)
14α	3.39, d (10.5)	3.38, s	-	5.06, s	-
14β	OH, 2.71, d (10.5)	OH, 2.74, s	-	-	-
15	2.22, sept (7.0)	2.20, m	2.38, sept (6.9)	1.860, sept (7.0)	2.83, sept d (6.8, 1.0)
16, 17	0.86, 0.98, d (7.0)	0.85, 0.98, d (6.9)	0.87, 0.96, d (6.9)	0.82, 0.94, d (7.0)	0.99, 1.05, d (6.8)
19	4.65, s	4.74, s	4.76, m	4.74, s	4.76, m
20	1.09, s	1.29, s	1.26, s	1.24, s	1.30, s
-OAc	-	-	-	2.15, s	-

All the spectral evidence confirmed the structure of **209** as (7,8)β,(9,11)β-bis(epoxy)-2β,19-dihydroxy-14-oxo-18(4→3)*abeo*-abieta-3,12-dien-18-oic acid lactone (named triptolenonide), a new compound that has not been reported in the literature. A comparison of ^{1}H NMR spectral data of isolated epoxides Tl (**1**), Td (**2**), **212**, **208**, and **209** is provided in Table 2.6.

In summary, 29 compounds were isolated from the ethyl acetate extract of the TRP4a cell cultures, 28 of them diterpene metabolites. Of the 29 compounds, 26 were isolated for the first time from the plant cell cultures of *T. wilfordii*, and 20 were not yet isolated from *Tripterygium* species. Eight compounds (**200, 202, 205, 207, 208, 209, 211** and **212**) were new compounds and 6 compounds (**138a, 193, 197, 201, 206** and **210**) were isolated for the first time as natural products.

Plausible Biosynthetic Pathways

For chemists, the motivation for the use of plant cell cultures for the production of biologically active compounds is the understanding of the corresponding secondary metabolic processes, so that one may logically and consistently control and manipulate the relevant parameters.

Secondary metabolites isolated from a plant or from a plant cell culture are not necessarily biosynthetic intermediates, but as more metabolites are isolated, there is an increasing chance of isolating the true intermediates. A biosynthetic pathway has to be ultimately proven by labeling experiments etc., however a proposed biosynthetic pathway based on isolated metabolites often provides a frame or a basis on which detailed labeling studies may be designed or conducted.

As illustrated in the previous section, our efforts in the isolation of diterpene metabolites from cell cultures of *T. wilfordii* have resulted in addition of many new and related compounds to the list of diterpene metabolites from *Tripterygium*. Study of these structurally

related compounds may provide important information about their metabolic relationships, and their possible role in the biosynthesis of the triepoxides, Tl (**1**) and Td (**2**).

geranylgeraniol

labdane-type

abietane-type

pimarane-type

dehydroabietic acid (**157**)

210

Td (**2**)

193
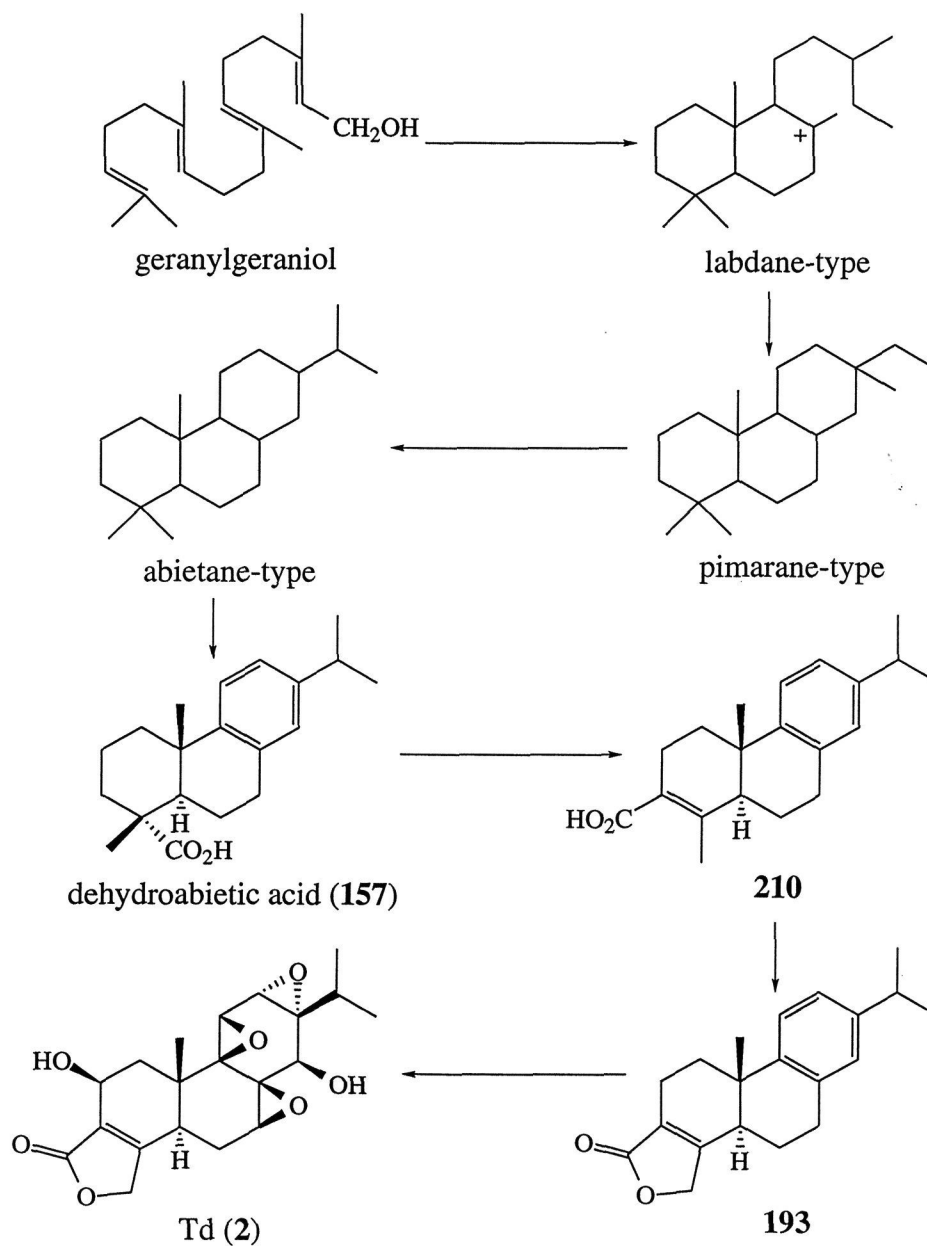
Scheme 2.4   Proposed Biosynthetic Pathway to Td (**2**) via Dehydroabietic Acid (**157**)

The isolation of dehydroabietic acid (**157**) and the hydroxyl ester, **182**, as co-occurring metabolites from TRP4a cell cultures in an earlier study, led to proposals of the biosynthetic

pathway of Tl (**1**).[159] A biosynthetic pathway from geranylgeraniol through dehydroabietic acid (**157**) and the acid **210** was proposed (Scheme 2.4).

Alternatively, a methyl group transfer was proposed from dehydroabietane (**196**) followed by oxidation and butenolide formation, prior to oxidation of ring C to give Tl (**1**) (Scheme 2.5).
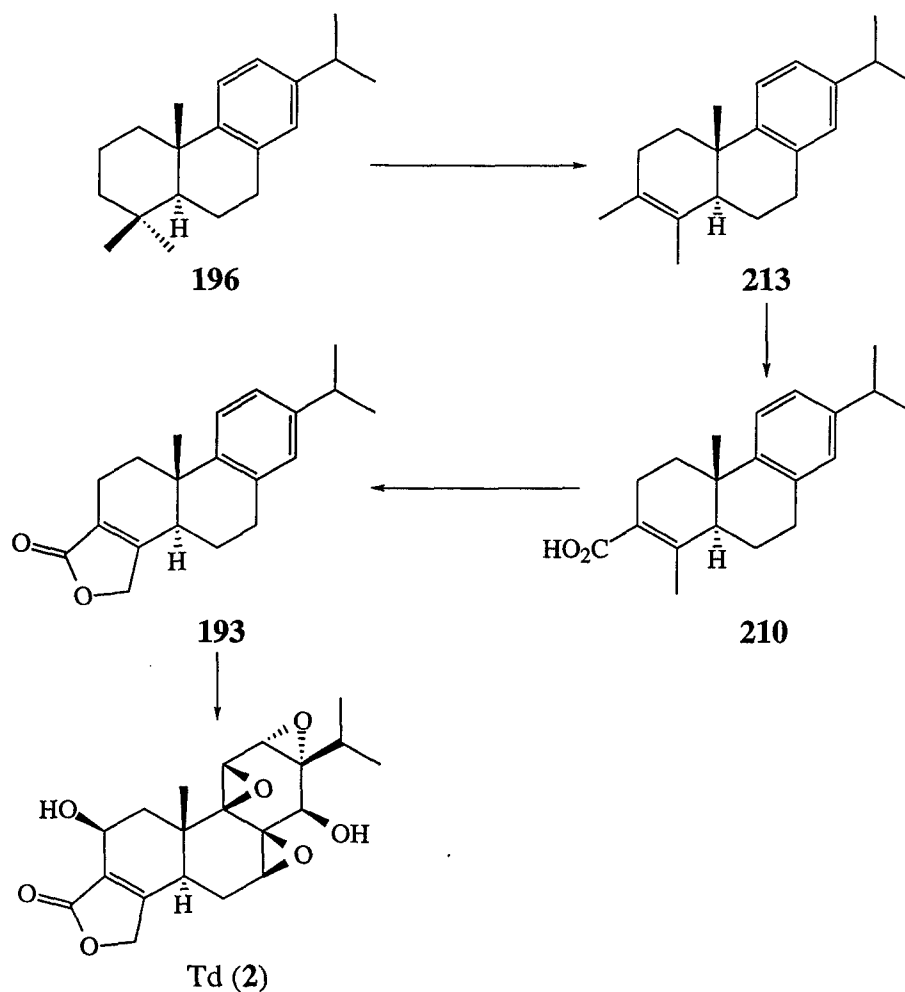
Scheme 2.5   Proposed Biosynthetic Pathway of Td (**2**) via 4→3 Methyl Transfer

The latest biosynthetic pathway was proposed by M. Roberts based on his work in this project (Scheme 2.6).[160] We now have successfully isolated some of these proposed intermediates, such as **210**, **193** in Scheme 2.4, **196**, **210**, **193** in Scheme 2.5, and compounds

197, 106 and **138** in Scheme 2.6. However, with the discovery of additional metabolites, the proposed biosynthetic pathways for Tl (**1**) and Td (**2**) production may be amended and expanded to accommodate all current data.

pimaradiene

197

138

106

139

95

Td (2) ◄──── Tl (1)
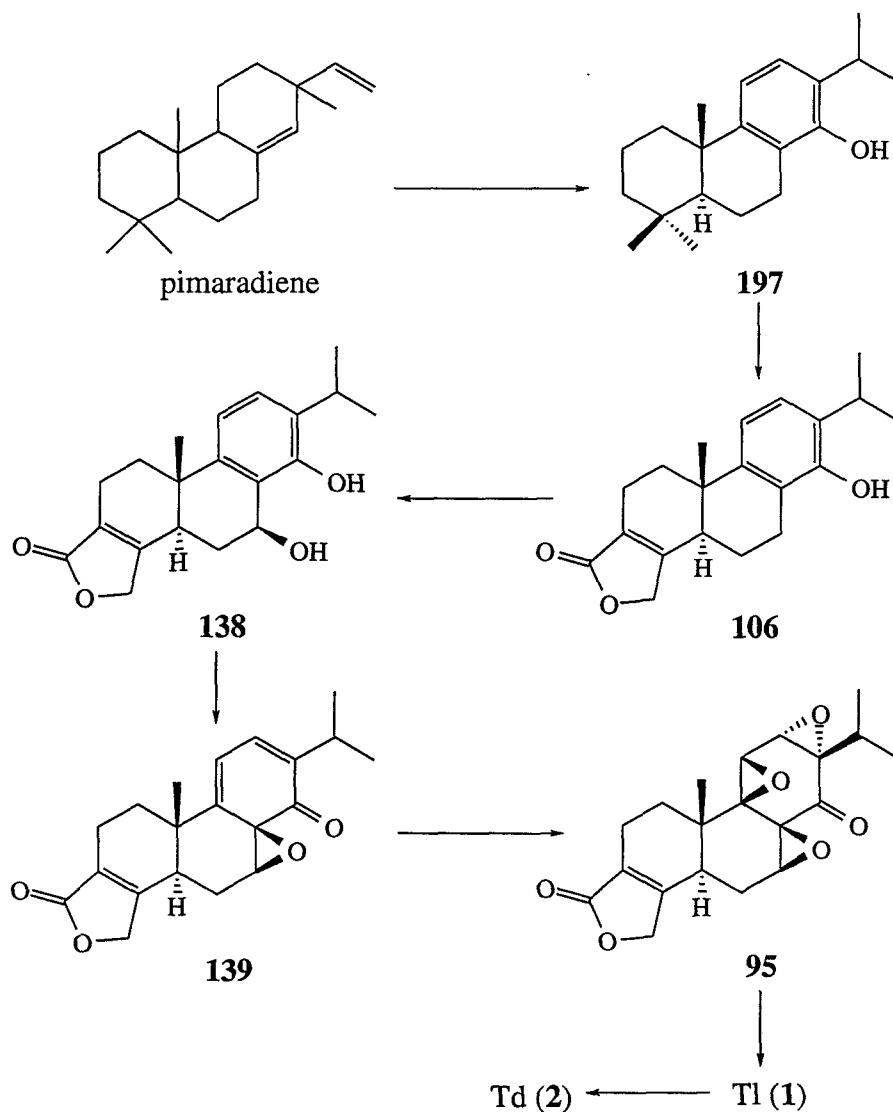
Scheme 2.6   Proposed Biosynthetic Pathway to Tl (**1**) and Td (**2**) by M. Roberts

The diterpenes isolated from the TRP4a cell cultures may be classified into five groups. The first group consists of only one compound, dehydroabietane (**196**), which has no other oxygenated functional groups in any part of the molecule. The second group contains

compounds that have various degrees of functionalization on ring A. Examples are **198, 202, 204, 210** and **193**. The third group includes compounds with functional groups on either ring C or B, such as **197** and **199**. The fourth group is comprised of compounds that are functionalized on rings A and C (some on ring B as well), but exhibit no epoxide functionalities. Examples are **116, 200, 203, 205, 211, 206, 106, 107, 108** and **138a**. The fifth group contains compounds possessing a lactone moiety on ring A and epoxide groups on rings B and C. This group includes compounds like Tl (**1**), Td (**2**), and the newly isolated **208, 209** and **212**.

Examples of Group 2 compounds:

198   **202**   **204**

210   **193**
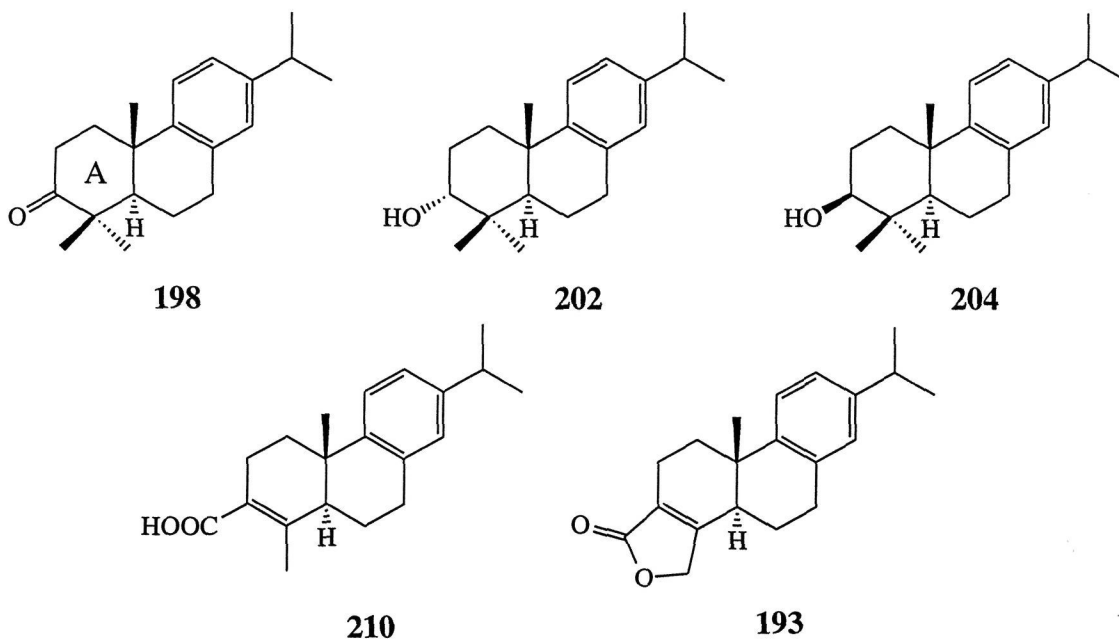
Examples of Group 3 compounds:

197   **199**

123
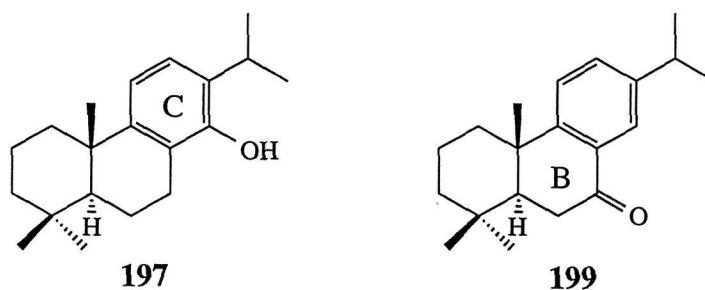
Examples of Group 4 compounds:

116 R = H
200 R = CH₃

203

205

211

206

106 R = H
107 R = CH₃

108

138a
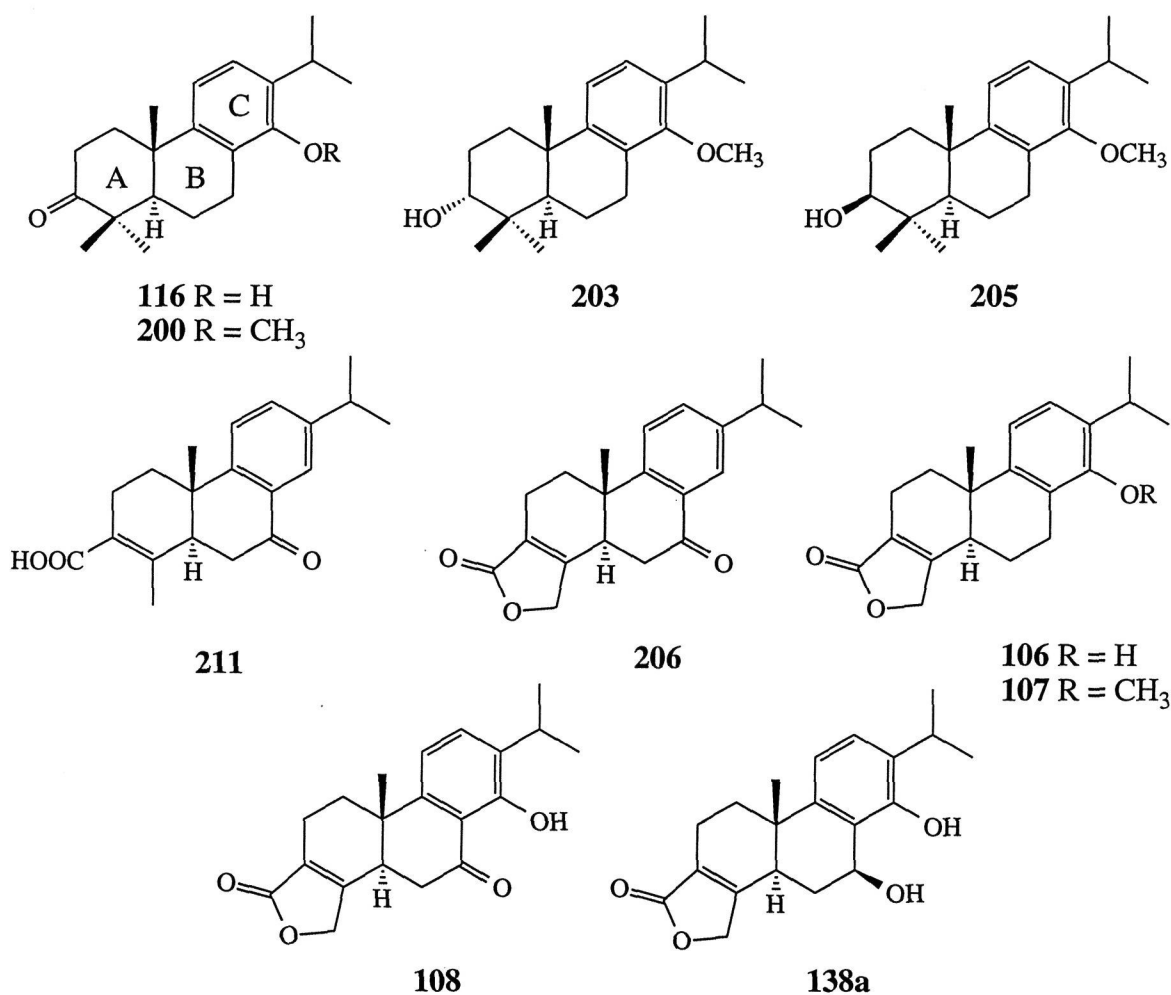
The biosynthesis of dehydroabietane (**196**) from geranylgeraniol was not the focus of our interests, but rather, the biosynthesis from dehydroabietane to Tl (**1**) or Td (**2**) was more appealing to us.

It is both probable and logical that dehydroabietane (**196**) is the common precursor for nearly all the diterpene metabolites in *T. wilfordii*. Dehydroabietic acid (**157**), previously isolated from the TRP4a cell cultures, likely resulted from dehydroabietane (**196**). In consideration of the lactone moiety on ring A of Tl (**1**) and Td (**2**), it is likely that the lactone carbonyl group is derived from the methyl group that has migrated from C4 (as suggested by

Scheme 2.5). This kind of methyl migration is a very common occurrence in natural product biosynthesis.[184] Migration of a carboxy group (such as that from C4 to C3 in dehydroabietic acid, Scheme 2.4) is rarely seen in the literature.

Examples of Group 5 compounds:

1   2   208

209   212
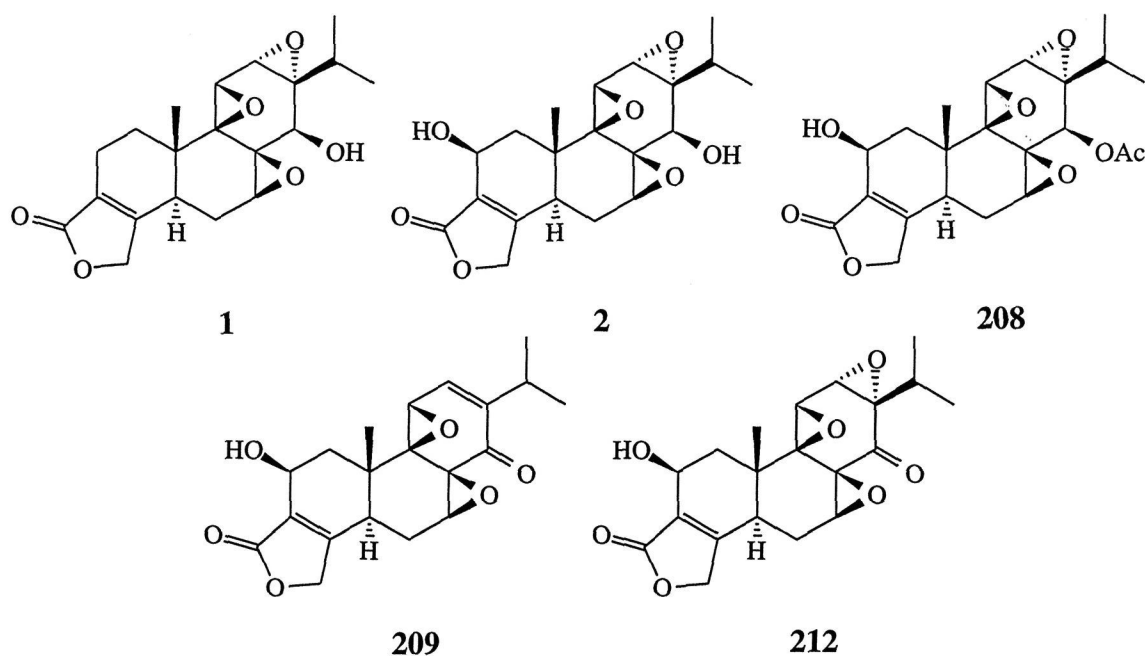
Isolation of Group 2 and Group 3 compounds reveals that the functionalization of ring A or ring C may possibly be the initial step in this biosynthetic pathway, or perhaps the functionalizations of ring A or ring C may actually take place simultaneously. The biosynthesis of Tl (1) and Td (2) at this stage is not a linear process. Isolation of Group 4 compounds gives us evidence that the functionalization of ring A may proceed after the functionalization of ring C and *vice versa*. No major differences are found between ring A functional groups in Group 2 and 4, and also no major differences occur between ring C functional groups in Group 3 and 4. So dehydroabietane (196) may first be functionalized on ring A then C, or first on ring C and then A, both routes producing Group 4 compounds. Some Group 4 compounds may be the intermediates to Group 5 compounds. The lactone moiety on

ring A seems to be necessary for the enzymatic formation of the epoxide functionality on ring C or B, because of all the Group 5 diterpenes isolated thus far from either the plants or from the cell cultures, none of them have an epoxide without already containing the lactone moiety on ring A. Scheme 2.7 outlines the general relationship and transformation between these five groups of compounds.

Group 1   dehydroabietane (**196**)

ring A
functionalized   Group 2   Group 3   ring C
functionalized

Group 4   both ring A and C
functionalized

Group 5   epoxides
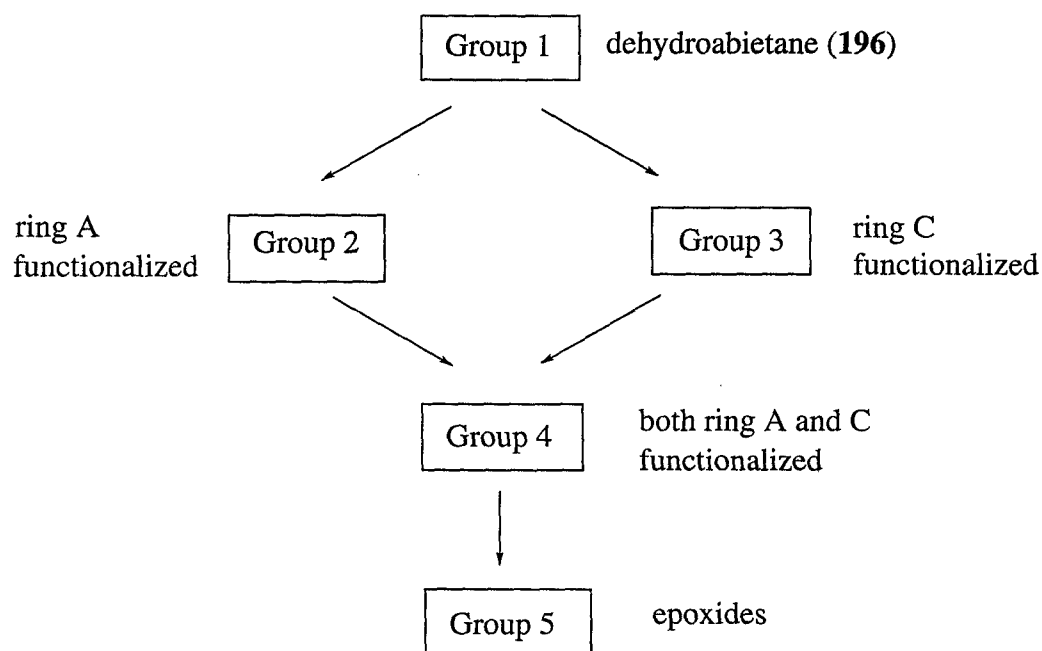
Scheme 2.7   Relationship between Different Groups of Compounds

The enzymatic formation of the lactone can start with a hydroxyl group at C3 position (Scheme 2.8). Loss of the hydroxyl group and migration of the methyl from C4 leads to the alkene **213**. Allylic hydroxylation at C18 generates the alcohol **214**, which is subsequently oxidized to the acid **210**. Another allylic hydroxylation at C19 produces the hydroxyl acid **215**, which readily lactonizes to form the butenolide **193**.
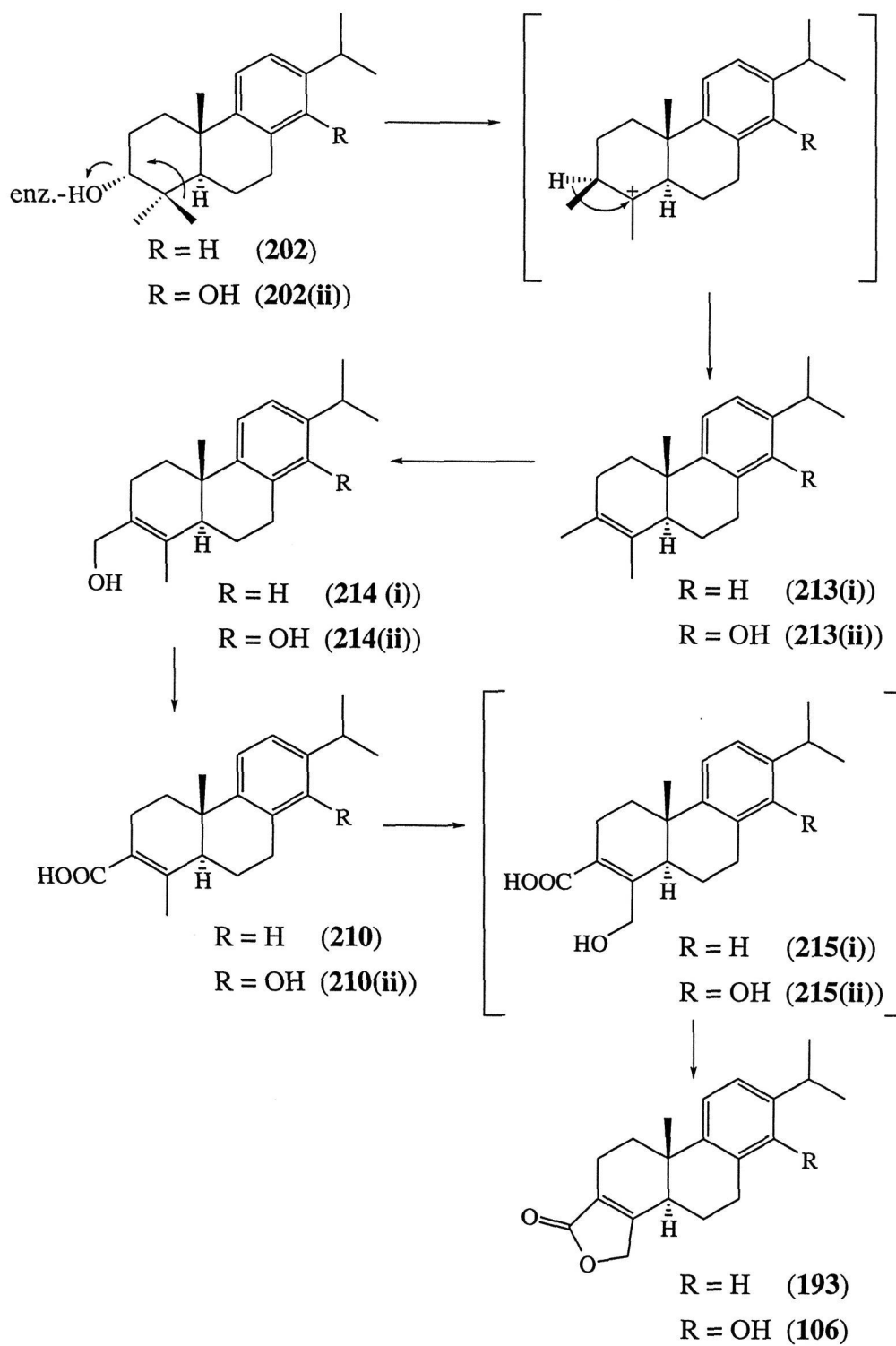
Scheme 2.8   Proposed Mechanism for Enzymatic Formation of the Butenolide

As shown in Scheme 2.8, elimination of the hydroxyl group followed by migration of the methyl from C4 in the first stage may take place in a concerted manner, or in steps. The *trans* diaxial relationship between the leaving group and the migrating group is favored for such a rearrangement from a chemical point of view.[188] Therefore, only the 3α-hydroxyl isomer is a more plausible precursor of the butenolide. Usually enzymatic reactions are stereospecific,[184] therefore the hydroxylation of dehydroabietane should yield only one isomer, such as the 3α hydroxyl isomer. This compound should then be the preferred substrate for the following enzymatic transformation. However, both 3α-alcohols (**202, 203**) and 3β-alcohols (**204, 205**) were isolated from the cell cultures. One possibility for this product distribution is that the formation of the 3-hydroxyl compound is not so specific and the next step is also not specific, so that the enzyme can convert both alcohols to a same intermediate, which is then proceeded by a methyl migration. Another postulation is that the enzyme reaction is stereospecific in hydroxylating dehydroabietane (**196**) to form only the 3α-alcohol **202**, which is then transformed to the butenolide. However, if the rearrangement step is not strictly concerted, it is reasonable that a water molecule from the surroundings can act as a nucleophile and substitute the leaving group, thereby forming the β alcohol (Figure 2.19). On the basis of this hypothesis, the 3β-alcohol is formed as a side product prior to the methyl migration. The "wrong" β alcohol isomer may probably remain there, or be oxidized to a ketone (**198, 200**). The ketone could be a dead end in terms of further biosynthesis, or it could be reduced to the "right" alcohol by certain enzymes in the system and re-enter the biosynthetic process.

Functionalization of ring C is probably via a direct hydroxylation through an arene oxide.[184] All the mono hydroxylation occurs at C14. The second phenolic hydroxyl group could enter at either C11 (mostly) or C12. This suggests that the C14 hydroxyl group is the starting point for further transformations in the C ring. It is interesting to note that in most cases the C14 hydroxyl group is methylated as the methyl ether, and that in most 12,14-dihydroxy compounds, it is the C14 hydroxyl group, not the C12 hydroxyl group, that is methylated. The C14 hydroxyl group is much hindered than the C12 hydroxyl, thus

methylation at that site should not be favored. One possibility could be that the methylation of

the hydroxyl group at C14 may act as a means of a metabolic regulation by the cells. A free

C14 hydroxyl group could be part of the active binding site for the enzyme that is responsible

for further transformations (for example, epoxidation); and such a process may be regulated by

methylation of this group. The methylated compounds may stay "locked" in a pool, and when

needed, cleavage of the methyl provides enzymes with the proper substrate for the next

transformation.
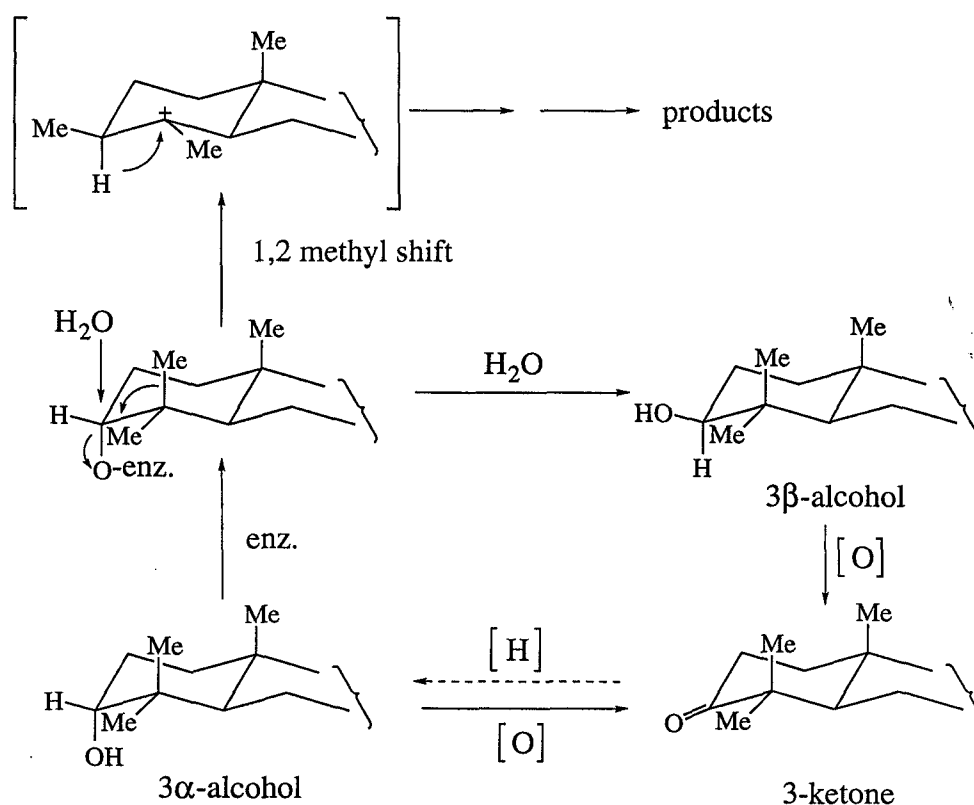
Figure 2.19   Proposed Mechanism for Formation of 3β-alcohol and the Probable Relation
                with the α Isomer

Ring A and ring C functionalization seem to be a combination of convergent and

ramified paths (metabolic grid[184]), leading to the key intermediate triptophenolide (106)

(Scheme 2.9). As mentioned earlier, epoxidation can only take place after the butenolide

moiety on ring A has been constructed. The functionalization on the C ring exclusively involves the introducing of hydroxyl group at C14, indicating that the starting point for epoxidation must be at C14.

Based on the above postulations, compound **106** may likely be the key intermediate in the biosynthesis of Tl (**1**) and Td (**2**) since it has the lactone on ring A and its C14 hydroxyl is free. It has been isolated from all four species of *Tripterygium plants*.[2] The appropriate precursors in Group 2 or Group 3 can only pass through this "gate" to reach their destiny, Tl (**1**) or Td (**2**). The first epoxidation probably occurs on ring B (Scheme 2.10). There are two possible mechanistic pathways to the formation of Tl (**1**) and Td (**2**). One is that a hydroxylation at C7 takes place first, followed by an Adler-type oxidation to form the epoxy dienone **139** (7-hydroxylation path way). The other possible route suggests that the substrate is first oxidized to an *ortho* quinone methide **219** and then epoxidation takes place between C7 and C8 to form **139** (quinone methide pathway). Quinone methides are believed to play a very important role as intermediates in the biosynthesis of secondary metabolites, for example, the lignans.[184,189] They are also transient intermediates in many synthetic reactions.[190,191] Compound **139** has not been isolated from the *Tripterygium* plants or from the cell cultures, but it is likely an intermediate involved in the biosynthesis of Tl (**1**) and Td (**2**). Alternatively, an intermediate such as 7-hydroperoxide of **106** may also be possible in the formation of **139**.

The sequence for the formation of the three epoxide groups in Tl (**1**) and Td (**2**) is still not clear, though they are likely formed in such a sequence as C7, C8 → C9, C11 → C12, C13. The isolation of **209** and **212** provides a supporting evidence for such a postulation. The ketone at C14 is finally reduced to a β hydroxyl group to form Tl (**1**). It is also possible that the C14 ketone is initially reduced to alcohol **220** and followed by epoxidation (Scheme 2.11), but there is insufficient evidence to support this mechanistic postulation at this stage.
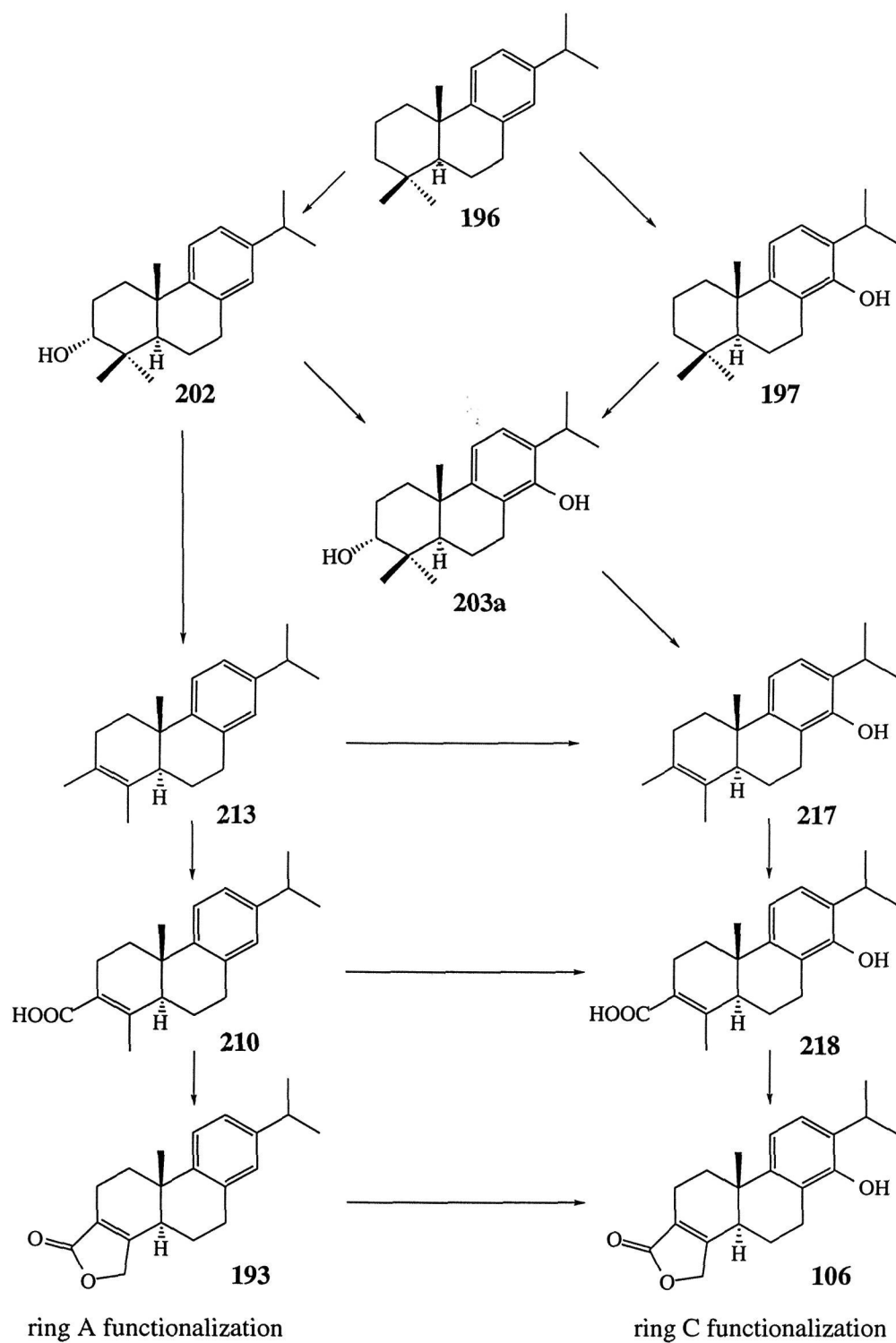
Scheme 2.9   Proposed Biosynthetic Pathway from Dehydroabietane (**196**) to the Key
Intermediate **106**

quinone methide pathway

7-hydroxylation pathway

139 R = H
139(ii) R = OH

209(i) R = H
209 R = OH

95 R = H
212 R = OH

Tl (1) R = H
Td (2) R = OH
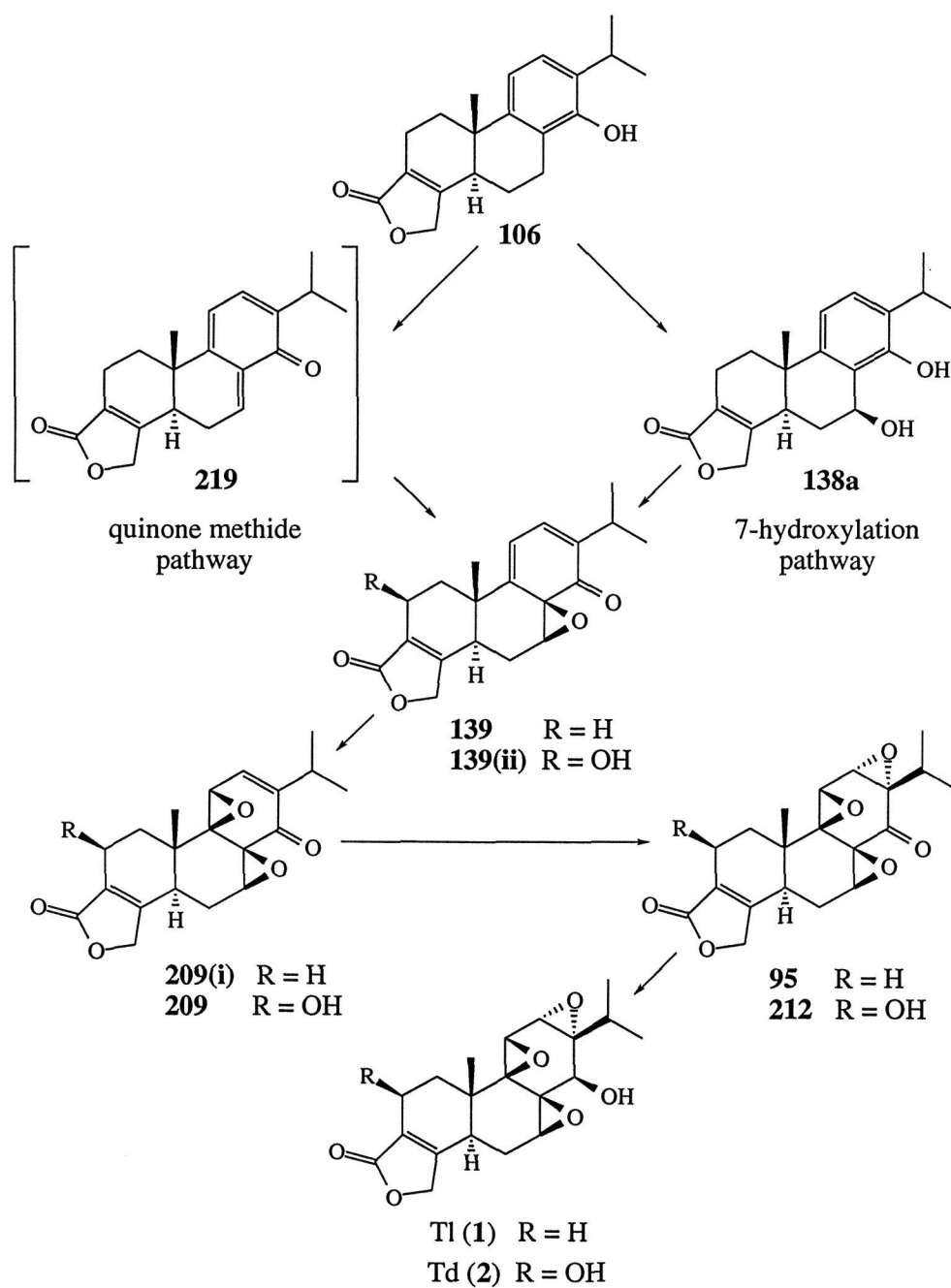
Scheme 2.10   Proposed Biosynthetic Pathway from Triptophenolide (**106**) to Tl (**1**) and Td (**2**)
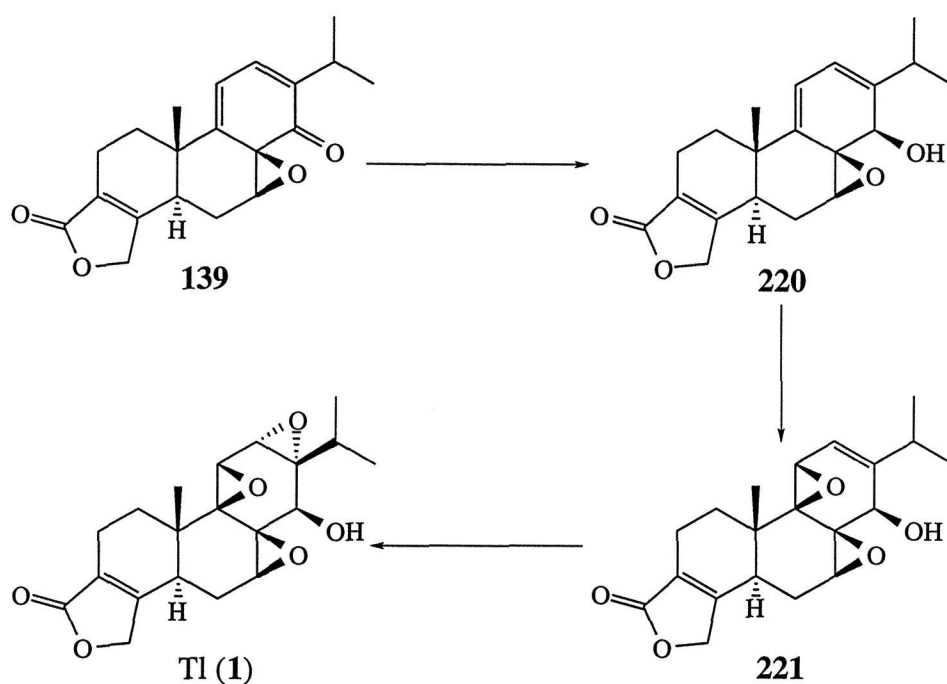
Scheme 2.11   An Alternative Pathway from **139** to Tl (**1**)

Td (**2**) was thought to be the C2 hydroxylation product of Tl (**1**). Isolation of **209** implies that C2 hydroxylation could occur prior to completion of the epoxidation, but since no other compounds with a C2 hydroxyl group were isolated without two or three epoxide groups already attached, hydroxylation at C2 appears to take place only when epoxide groups have been incorporated into the molecule. The completed biosynthetic pathway is proposed as in Scheme 2.9 and Scheme 2.10.

CHAPTER 3   SYNTHESES OF PRECURSORS FOR BIOTRANSFORMATION STUDIES

3.1   Introduction

As shown earlier, the formation of the epoxide system in Tl (**1**) and Td (**2**) likely takes place after the butenolide moiety on ring A, and a hydroxyl group at C14 have been incorporated by the appropriate enzymes. It was considered essential to evaluate appropriate synthetic precursors for the purpose of biotransformation to Tl (**1**) or Td (**2**) in the later stage of the biosynthetic process. It was hoped that such experiments would: (a) increase the yields and reduce the time required for metabolite production, (b) generate some new analogs of Tl (**1**) or Td (**2**) that might also have some biological activities, and (c) afford additional information about the biosynthesis.

Initial biotransformation studies utilizing TRP4a cell cultures focused on the construction of the butenolide on ring A.[159] The various precursors employed in the early studies include dehydroabietic acid (**157**) and some synthetic compounds with abietane-type structures.[159,192]

In recent years, our interest has turned to building the epoxide system in the molecule. Investigation in our laboratories led to the syntheses of several precursors, of which isodehydroabietenolide (**193**) showed some results in biotransformation studies with TRP4a cell culture (*e.g.*, hydroxylations at C2 and/or C7, etc.).[160] However, lack of functionalization on ring C during biotransformation of isodehydroabietenolide (**193**) suggested that ring C should be "activated" by introduction of hydroxyl groups. A synthetic sequence to isotriptophenolide (**194**), a C12 hydroxylated derivative of isodehydroabietenolide (**193**), was then developed.[161] Application of **194** in the biotransformation studies with the TRP4a cell cultures resulted in a small yield of the methyl ether of **194** with much starting material recovered.[161]

193   **194**   **106**
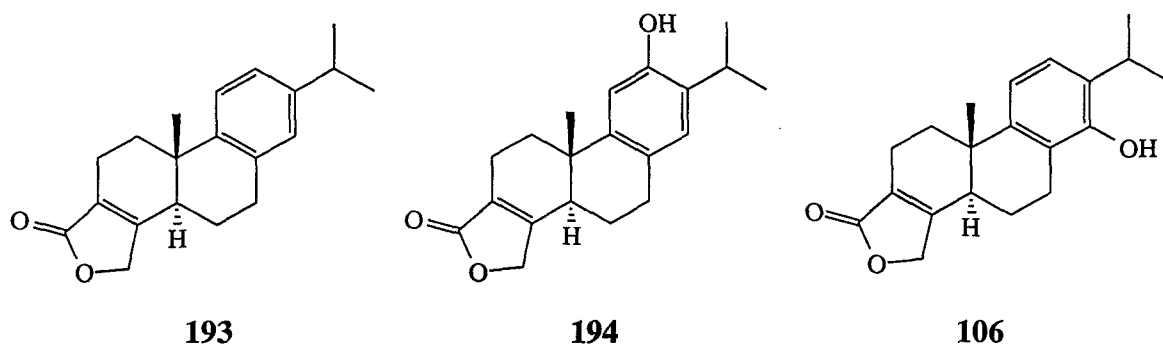
The lack of extensive biotransformation of **194** with TRP4a cell cultures raised a challenge to the idea that ring C "activated" precursors would be transformed by the cells. But there were several ways we might be able to achieve our goals. First, perform the biotransformation of **194** under different experimental conditions. Secondly, synthesize other ring C "activated" precursors and carry out the biotransformation studies. Furthermore, it would also be interesting to synthesize some ring B functionalized, or both ring B and C functionalized precursors, to examine the possibility of biotransformations with the TRP4a cell cultures.

As the synthetic sequences to isodehydroabietenolide (**193**) and isotriptophenolide (**194**) had been fairly well established, the focus of the present work was directed on the development of synthetic sequences for new ring C "activated" precursors, such as triptophenolide (**106**) and 12,14-dihydroxylated isodehydroabietenolide. As a whole, isotriptophenolide (**194**), triptophenolide (**106**), and the 12,14-diol would represent a series of compounds with different positions and degrees of hydroxylation on ring C. This situation would provide an interesting opportunity to develop a systematic approach to our biotransformation studies, and could afford synthetic strategies for chemical functionalization of aromatic rings within this diterpene family.

3.2   Results and Discussion

3.2.1   Syntheses of Isodehydroabietenolide and Related Compounds

Synthesis of Isodehydroabietenolide (193)

The synthetic sequence to isodehydroabietenolide (**193**) from dehydroabietic acid (**157**) was initially developed by M. Roberts.[160] A slightly modified sequence, developed by F. Kuri-Brena,[161] was applied in the synthesis of this compound.

The starting material, dehydroabietic acid (**157**), was purchased as a technical grade, crude product (K & K Laboratories). Dehydroabietic acid incorporates most of the carbon skeleton, and most importantly, the chiral centers at C5 and C10 required for Tl (**1**) and Td (**2**) related molecules. However, the disadvantage to start with dehydroabietic acid is that "the range of synthetic approaches is limited by the chemistry of the starting material rather than by one's imagination".[145,161] This became more apparent when ring C functionalization was attempted (*vide infra*).

The commercial sample of crude dehydroabietic acid was purified before further synthesis. The yield of partially purified dehydroabietic acid (89-92% by gas chromatography) from the commercial product was about 30%. The remaining impurities, which are isomeric resin acids and difficult to remove at this stage, were readily eliminated after the first step in the synthetic sequence.

The partially purified dehydroabietic acid (**157**) was treated with excess thionyl chloride and a catalytic amount of dimethylformamide (DMF) in benzene to give the acid chloride **222** (Scheme 3.1). The crude acid chloride was reacted with sodium azide in acetone and the resulting acyl azide **223** was heated in toluene to effect the Curtius rearrangement to afford the isocyanate **224**. Reduction of the isocyanate with lithium aluminum hydride (LAH) in tetrahydrofuran (THF) under reflux for about 22 h yielded the secondary methyl amine **225**,

which was then refluxed with aqueous formaldehyde and formic acid to give the tertiary dimethyl amine **226** (Eschweiler-Clarke methylation). The tertiary amine was converted to the corresponding *N*-oxide **227** by treatment with *meta*-chloroperbenzoic acid (*m*-CPBA) at -20°C. The reaction mixture was heated at reflux for 30 min to eliminate dimethyl hydroxyamine, giving exo-olefin **228** as the only isomer. At this stage, the crude exo-olefin was readily purified by column chromatography to afford pure **228** as a colorless oil with an overall yield of 71% from dehydroabietic acid (**157**).

Ozonolysis of exo-olefin **228** carried out in a mixture of methanol-dichloromethane (5:1) at -78°C followed by treatment with dimethyl sulfide, generating the ketone **229** as a colorless oil (90% yield) which crystallized upon cooling. A minor by-product in this reaction was the 4,7-diketone **231**. On attempting to perform the reaction on a larger scale (25 g), the reaction time became lengthy and the yield decreased due to an increasing amount of the diketone **231**.
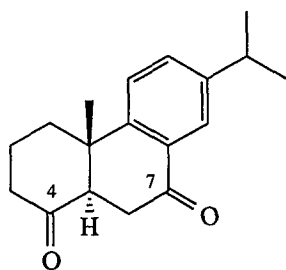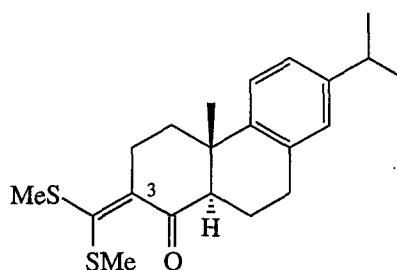
231   **230**

The attachment of a one carbon unit to the C3 position was achieved in nearly quantitative yield by initial deprotonation at C3 with lithium 4-methyl-2,6-di-*t*-butyl phenolate, followed by alkylation with carbon disulfide, and treatment with methyl iodide to form the α-oxoketene dithioacetal (α-dithiomethylene ketone) **230** as yellow crystals.
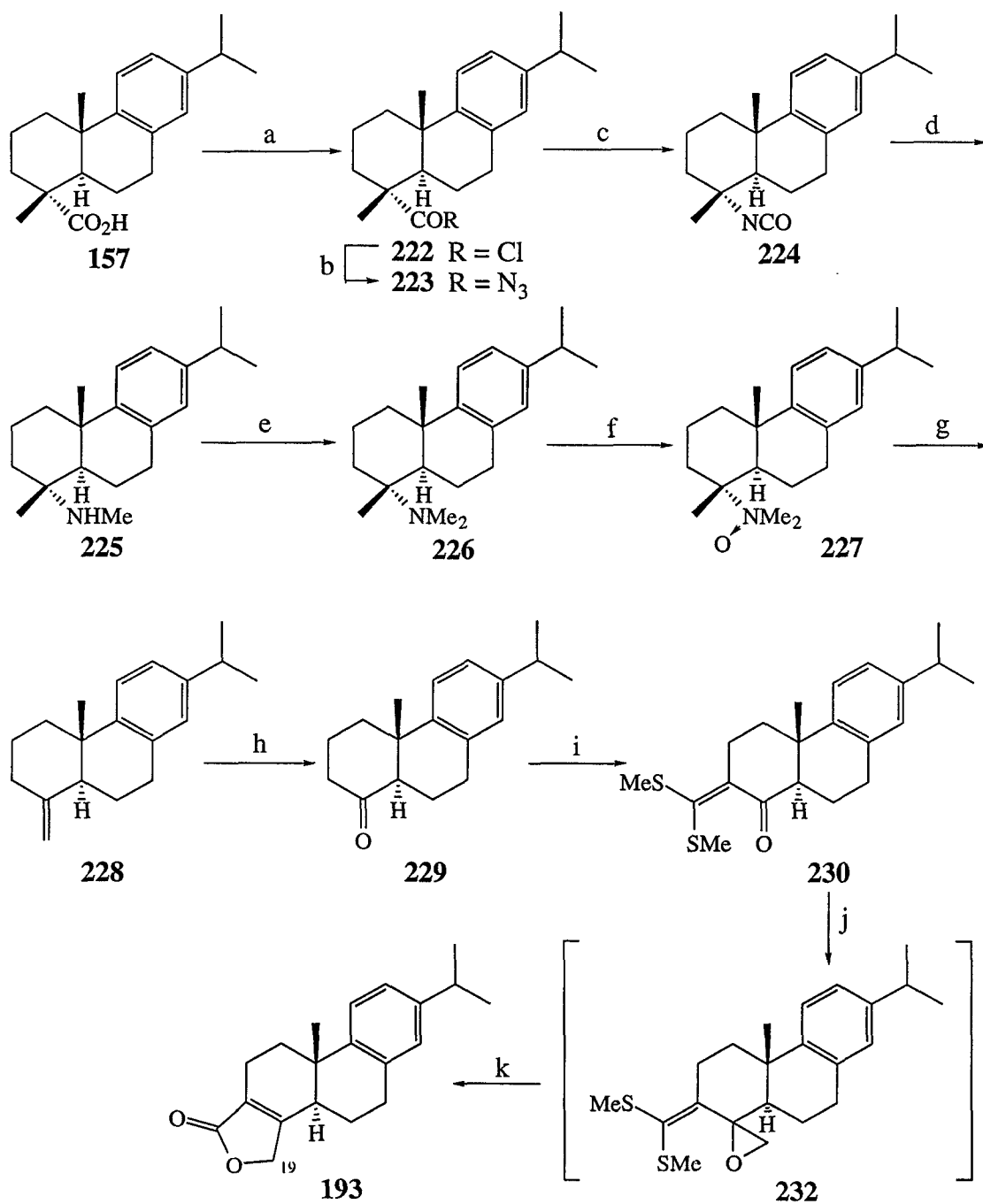
Scheme 3.1   Synthesis of Isodehydroabietenolide (**193**) from Dehydroabietic Acid (**157**)

a) SOCl₂, benzene, DMF; b) NaN₃, acetone; c) Δ, toluene; d) LiAlH₄, THF; e)HCHO, HCO₂H; f) *m*-CPBA; g) Δ; h) O₃, -78°C; Me₂S; i) lithium 4-methyl-2,6-di-*t*-butyl phenolate, THF, CS₂; MeI; j) Me₂S=CH₂, THF, -20°C; k) HCl, MeOH-MeCN

Completion of the synthesis of isodehydroabietenolide (**193**) was accomplished in a two-step, one pot reaction. Trimethylsulfonium iodide was first treated with *n*-butyl lithium in THF at low temperature to generate the dimethylsulfonium methylid. Ketone **230** was then added to the ylid to give, presumably, an epoxide[193] **232** (one isomer or a mixture of both isomers **232a** and **232b**) which was hydrolyzed without isolation to directly yield the butenolide **193**. Recrystallization of the crude product with ethyl acetate and hexanes afforded pure isodehydroabietenolide **193** as colorless needles (mp 98-100°C, 58% yield from **230**; 35% overall yield from dehydroabietic acid (**157**)).

The ^{1}H NMR spectrum of **193** exhibited signals of an AMX system in the aromatic region due to the ring C protons. One of the characteristic features of this compound was the resonances due to protons H19, which appeared at δ 4.76 as a broad AB quartet. In the course of this study, some ^{1}H NMR spectral characteristics of this series of compounds were noted. Changes in chemical shifts or appearance of these H19 signals (*e.g.*, wider split between the AB quartet, etc.) usually reflected the change in stereochemistry or functionalization at C5. Furthermore, the C20 methyl group signal was located at δ 1.01, while the isopropyl methyl resonances were at δ 1.22 (6H, d, J = 6.9 Hz). It has been found that the C20 methyl signal usually appears at higher field than the isopropyl methyl signals when the A/B ring junction is *trans*. If the A/B ring is *cis*, the C20 methyl resonance would shift downfield by about 0.2 to 0.3 ppm,[145] thus often appearing at the low field side of the isopropyl signals. The change in the chemical shifts of these methyl signals usually implicated the change from A/B *trans* to A/B *cis* in the molecule.

Variable yields encountered in the conversion of **230** to **193**[161] provoked the interest to investigate the reaction in more detail. In the reaction of the sulfonium ylid with the dithioacetal **230** (Scheme 3.1, step j), the progress of the reaction was monitored by TLC (hexanes-ethyl acetate, 9:1). Two major intermediates (R_f = 0.58, 0.32, respectively) were observed, and they had been thought to be the two isomeric epoxides (**232a, 232b**) formed in the reaction.

232a   **232b**
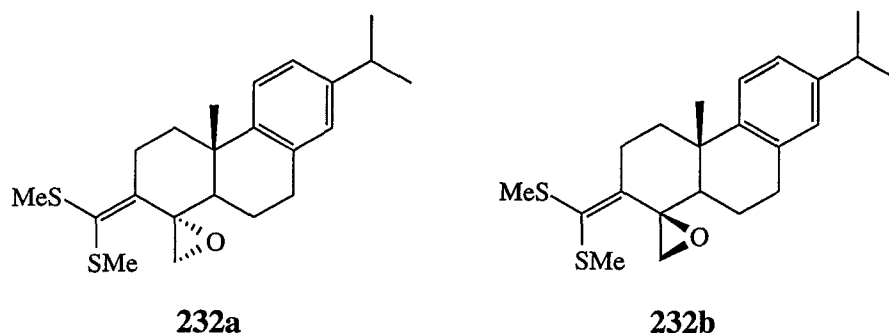
The reaction intermediates were subjected to a rapid column chromatography using dichloromethane-hexanes (2:1) as eluent to remove unreacted starting material and other polar compounds. The intermediates thus obtained were chromatographed again using hexanes-ethyl acetate (9:1) as eluent to separate the two pure intermediates to which structures **233** (R_f = 0.58) and **234** (R_f = 0.32) were assigned. It was noticed that **234** partially converted to **233** during the column chromatography. Both intermediates (**233** and **234**) were able to be converted to **193** upon treatment with concentrated hydrochloric acid under the same conditions (Scheme 3.1, step k).

233
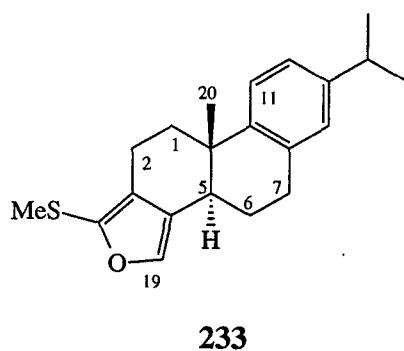
High resolution mass spectrometry of **233** revealed its molecular formula as $C_{21}H_{26}OS$. The ^{1}H NMR spectrum showed some similarities to that of the dithioacetal **230**. In the aromatic region, a new signal was found at δ 7.26 (1H, d, J = 1.8 Hz, H19). Only one methylthio group signal was found at δ 2.33. All the spectral analyses of this compound

(COSY, NOE, [13]C NMR etc.) confirmed its structure as the furan derivative **233**. Similar reactions had been reported in the synthesis of 3- and 3,4-substituted furans.[194]

234
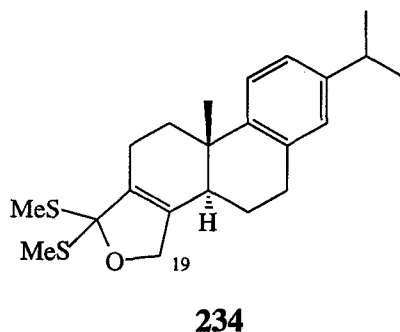
The [1]H NMR spectrum of **234** was measured immediately after isolation (in acetone-d_6). The spectrum showed a multiplet at δ 4.75, but 1.5 h later the intensity of this signal decreased while a new signal at δ 7.47 appeared. Analysis of this sample one day later revealed that the signal at δ 4.75 had already disappeared and the spectrum was identical to that of **233** (Figure 3.1), indicating a facile transformation of **234** to **233**.

The chemical shift and the appearance of the signal at δ 4.75 were very similar to the H19 signals in isodehydroabietenolide (**193**), and the intermediate did show a m/z 374 ion peak in the mass spectrum. On this basis the structure of this intermediate was proposed as **234**, rather than epoxides of **232a** or **232b**, since the chemical shift of protons of these epoxides cannot be at such a low field as δ 4.75.[186]

Therefore, in the reaction of dithioacetal **230** with sulfonium ylid, regardless whether one epoxide isomer is formed predominantly over the other (probably this is the case), this epoxide or the mixture of both must have rearranged rapidly to the dihydrofuran derivative **234**. Subsequent elimination of methanethiol from **234** leads to **233**. Intermediates **233** and **234** are able to be converted to **193** upon hydrolysis. The overall mechanism of butenolide formation is proposed in Scheme 3.2.[194,195]
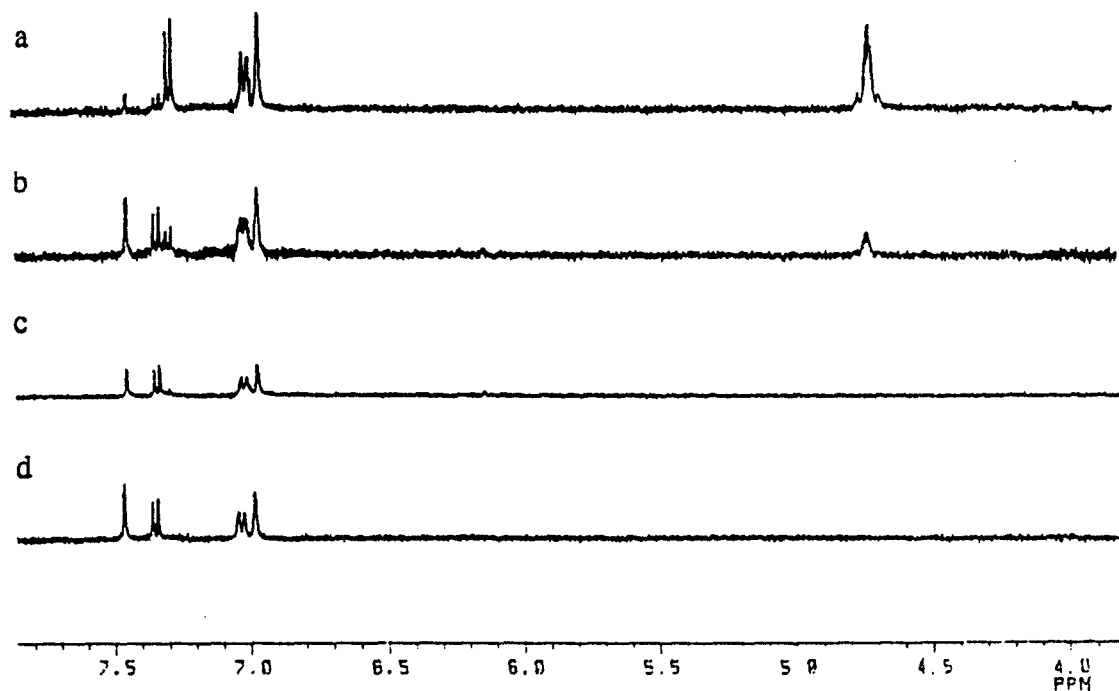
a, [1]H NMR spectrum of **234** taken immediately after separation; b, 1.5 h later; c, 1 day later;

d, spectrum of **233** (400 MHz, acetone-d_6)

Figure 3.1   [1]H NMR Spectra of Intermediates in the Reaction from **230** to **193**

Major by-products isolated from the butenolide formation step were **237**, **238** and **239**. β-Keto ester **237** and β-keto thioester **238** (written in their enolic forms on the basis of [1]H NMR analysis) were likely hydrolysis products of unreacted starting material **230**.[193] The A/B *cis* structure in both compounds was probably due to epimerization at C5 in the hydrolysis step since C5 readily epimerizes under basic or strong acidic conditions.[145]
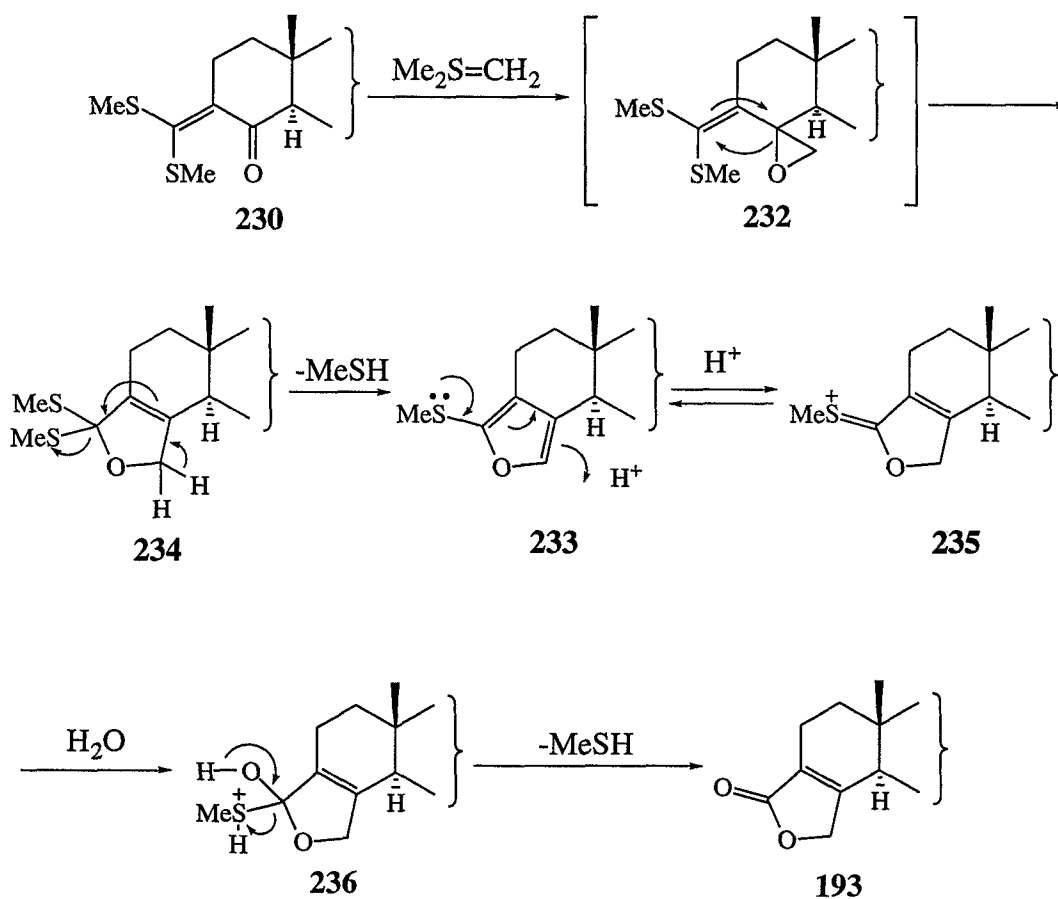
Scheme 3.2   Proposed Reaction Mechanism for the Formation of the Butenolide Moiety
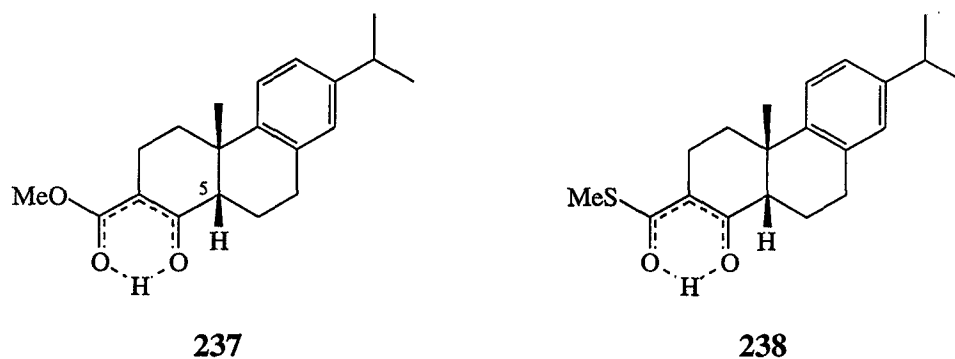
Compound **239** was probably formed from **234** in the hydrolysis step (Scheme 3.3). The thiomethyl group, acting as a nucleophile, substituted the protonated hydroxyl group and finally generated the thiophene moiety in **239**. These results suggested that further

improvement of the yield of this reaction may be achieved by optimizing the reaction conditions to reduce or eliminate these byproducts (*e.g.*, by completely converting **230** to **233** and **234**; and converting **234** to **233** before hydrolysis, etc.).

234   **234(i)**   **234(ii)**

234(iii)   **239**   (H-Nu: H-SMe, etc.)
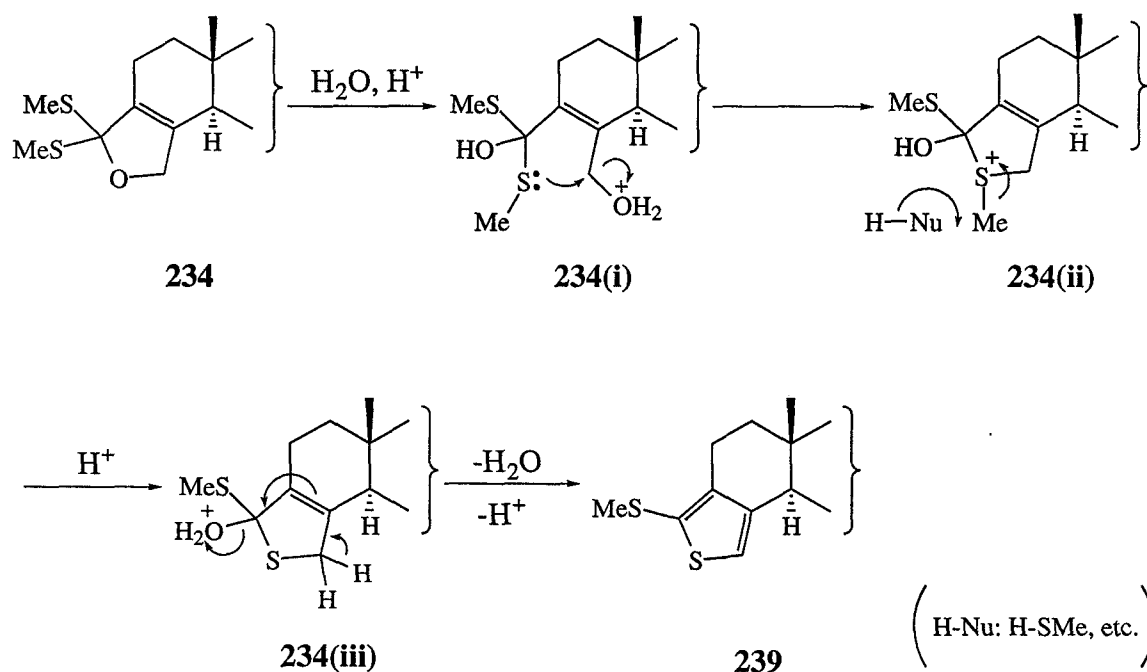
Scheme 3.3   Proposed Mechanism for the Formation of **239**

Syntheses of Isodehydroabietenolide Related Compounds

Previous results showed that biotransformation of isodehydroabietenolide (**193**) in TRP4a cell cultures produced some C7 and C2 hydroxylated compounds.[160] In order to examine whether some of these compounds can be further transformed by the cell culture, 7-oxo-isodehydroabietenolide (**206**) and 7β-hydroxy-isodehydroabietenolide (**240**) were synthesized.

The starting material **193** was treated with CrO_3 to give **206** in 49% yield (based on recovered starting material). Several different conditions and oxidizing agents[196-200] (Jones;

CrO₃-Ac₂O-HOAc-C₆H₆; DMP-CrO₃; NBS-*hv* ; PCC-CH₂Cl₂; PCC-C₆H₆) were attempted with similar or poorer yields.
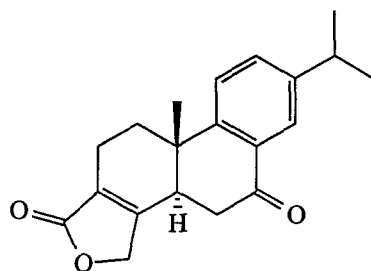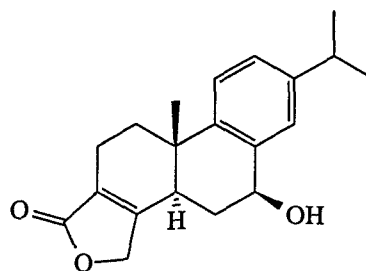
206   **240**

The ketone **206** was treated with sodium borohydride in ethanol at room temperature, stereoselectively affording 7β-hydroxy-isodehydroabietenolide (**240**) in 88% yield. Its IR spectrum showed a hydroxyl band at 3600 cm⁻¹ and the ¹H NMR spectrum exhibited the H7α signal at δ 4.99 (1H, dd, *J* = 8.6, 8.6 Hz), which was enhanced when H5 was irradiated.

3.2.2 Syntheses of Isotriptophenolide and Related Compounds

Synthesis of Isotriptophenolide (194)

Isotriptophenolide (**194**) has not been isolated from *Tripterygium* plants or from the TRP4a cell cultures. It is an "artificial" precursor in terms of trying to incorporate it into the biosynthesis of Tl (**1**) and Td (**2**). However, it can be readily synthesized in good yield, and its close relationship with triptophenolide (**106**) may provide some interesting results in biotransformation studies with TRP4a cell cultures. The synthetic sequence was developed by F. Kuri-Brena[161], and was adopted with a few changes.

The synthesis of isotriptophenolide (**194**) involved Friedel-Crafts acylation of isodehydroabietenolide **193**, followed by Baeyer-Villiger oxidation, and hydrolysis of the resulting ester to the desired phenol (Scheme 3.4). In the acylation reaction, 12-acetyl

isodehydroabietenolide (**241**) was obtained as a white solid. The yields of the crude product (reasonably pure and can be used directly for the next step) ranged from 95% to quantitative. Purification of the crude product with column chromatography afforded the pure product as a white crystalline solid in a yield of 92% from the starting material **193**.

The acylation occurred exclusively on C12 as shown by the ^{1}H NMR spectrum. The H11 signal (1H, s) was shifted downfield to δ 7.44, as compared with δ 7.25 in the starting material, as a result of the introduction of the acetyl group at C12.
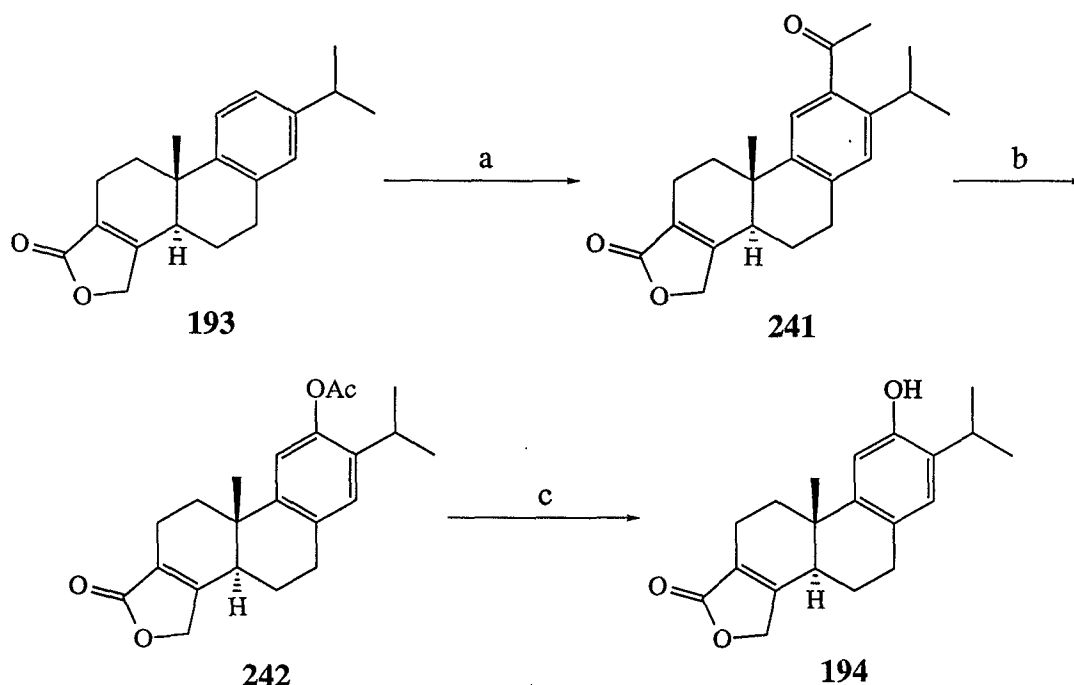
Scheme 3.4   Synthesis of Isotriptophenolide (**194**) from Isodehydroabietenolide (**193**)

a) acetyl chloride, anh. AlCl$_3$, CS$_2$, reflux; b) m-CPBA, CH$_2$Cl$_2$; c) HCl, MeOH

The Baeyer-Villiger oxidation of **241** was carried out by treatment of the starting material **241** in dichloromethane with m-CPBA. The crude 12-acetoxy-isodehydroabietenolide (**242**) was obtained in high yield as a yellowish powder. Purification of the product could be achieved by chromatography on silica gel with isopropyl ether as eluent. However, in the routine preparation of starting material for biotransformations or other purposes, this

purification was omitted since the unreacted starting material (in small amounts) was difficult to separate from the product by column chromatography. Also partial hydrolysis of the 12-acetoxy compound **242** to the phenol **194** on the column complicated the purification. It was found that as long as these intermediates were sufficiently pure by TLC, it was preferable to purify the final product **194** since different retention times between **194** and the impurities allowed an efficient column separation. The ^{1}H NMR spectrum exhibited the H11 signal at δ 6.92 as a singlet.

Hydrolysis of the acetate **242** was performed in methanol with concentrated hydrochloric acid. The crude phenol was purified by column chromatography with isopropyl ether, and recrystallized in ethyl acetate and isopropyl ether. The pure isotriptophenolide (**194**) was obtained as a white crystalline solid and in an overall yield of 84% from isodehydroabietenolide (**193**). The IR spectrum of the phenol showed a hydroxyl absorption at 3400 cm^{-1}, and its ^{1}H NMR spectrum exhibited the hydroxyl proton signal at δ 4.84 (exchangeable with D$_2$O). The H11 resonance was found at δ 6.72 (s) and the H14 signal at δ 6.90 (s), on the basis of NOE results.

The omission of intermediate purification facilitated the routine preparation of compound **194**. However, care should be taken in the work-up stages to make sure that the crude intermediates are free of any inorganic salts or remaining excess reagents. In one case, a by-product was isolated at the end of the synthesis, which was found to be 11-chloro-isotriptophenolide (**243**). The formation of this by-product could be caused by chloration in the last one or two steps due to small amounts of impurities. The mass spectrum of **243** exhibited two molecular ion peaks at m/z 348 (with ^{37}Cl) and 346 (with ^{35}Cl) in a ratio of about 1 to 3, indicating a chlorine atom in the molecule. Its ^{1}H NMR spectrum showed a very similar spectrum to that of **194**, but only one aromatic proton signal was found at δ 6.85 (s), which was assigned to H14 due to its NOE effect with protons at C7. Thus the chloro group must be at C11, and this was confirmed by the dramatic downfield shift of the H1β signal (from δ 2.28-2.54 in **194** to δ 3.64), apparently due to the crowding caused by the chloro group at C11.
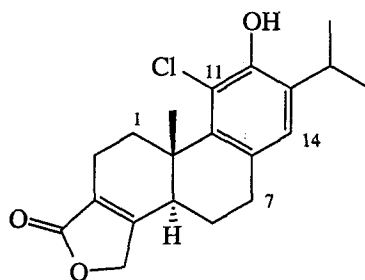
243

Syntheses of 7-Oxo- and 7β-Hydroxy-Isotriptophenolide

The synthesis of 7β-hydroxy-isotriptophenolide was carried out with a similar procedure as in the synthesis of 7β-hydroxy-isodehydroabietenolide (**240**) (section 3.2.1) (Scheme 3.5). Because direct oxidation of isotriptophenolide (**194**) with CrO_3 could result in undesired oxidation of ring C, 7-oxo-isotriptophenolide (**244**) was synthesized by oxidation of 12-acetoxy-isotriptophenolide (**242**) with CrO_3 solution in aqueous acetic acid. The oxidation afforded 12-acetoxy-7-oxo-isotriptophenolide (**244**) as a yellowish powder in a yield of 63% based on recovered starting material.

Compound **244** was then hydrolyzed to 7-oxo-isotriptophenolide (**245**) by treatment with hydrochloric acid in methanol. The resulting product, after recrystallization in methanol and water, was obtained as colorless needles (mp 287-289°C, dec) in a yield of 84%. Reduction of ketone **245** with sodium borohydride in ethanol (Method A) gave the required 7β-hydroxy-isotriptophenolide **246b** in 26% yield. The diol **246b** can also be synthesized from **244** by direct reduction with sodium borohydride in ethanol (Method B). The acetate group was also cleaved during the reduction. Purification of the crude product gave the diol **246b** in 27% yield from **244**. This compound was not very stable, and decomposition was observed during workup and column chromatography. The diol **246b** decomposed to the C6, C7 olefin **247** in the NMR tube when deuterated chloroform was used as the solvent prior to passage through a short column of alumina. The IR spectrum of **246b** showed a band for hydroxyl

groups at 3350 cm^{-1} and its ^{1}H NMR spectrum displayed the H7α signal at δ 4.95 (1H, br dd, J = 8.6, 8.6 Hz). The stereochemistry at C7 was confirmed by observation of NOEs between H7α and H5.
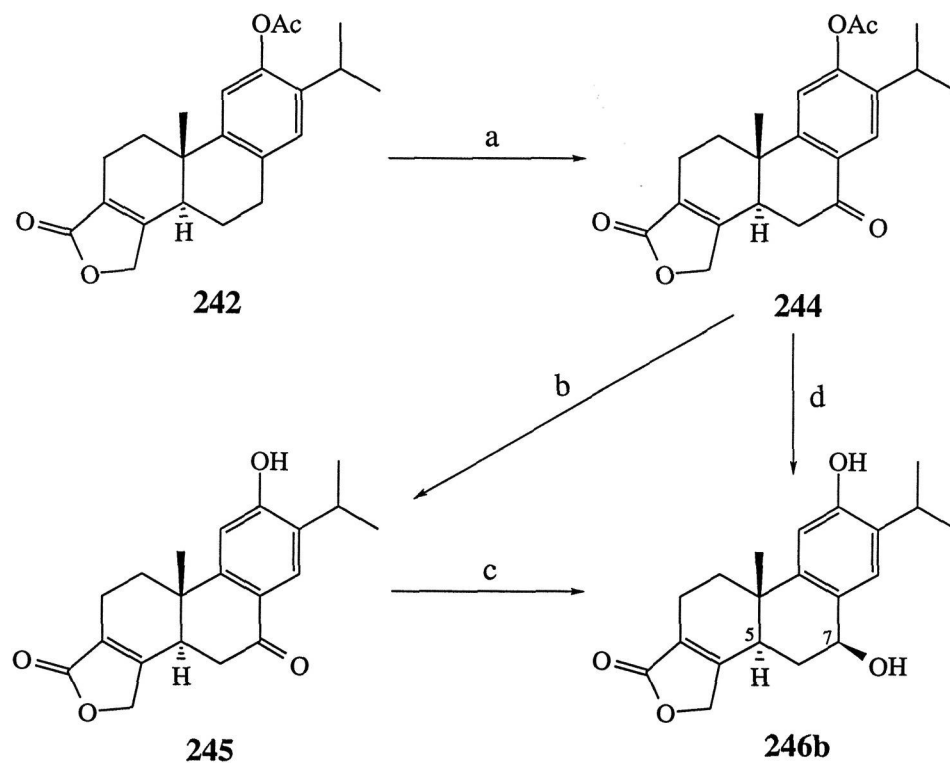
Scheme 3.5   Synthesis of 7β-Hydroxyl-isotriptophenolide (**246b**)

a) CrO$_3$, HOAc; b) HCl, MeOH; c), d) NaBH$_4$, MeOH
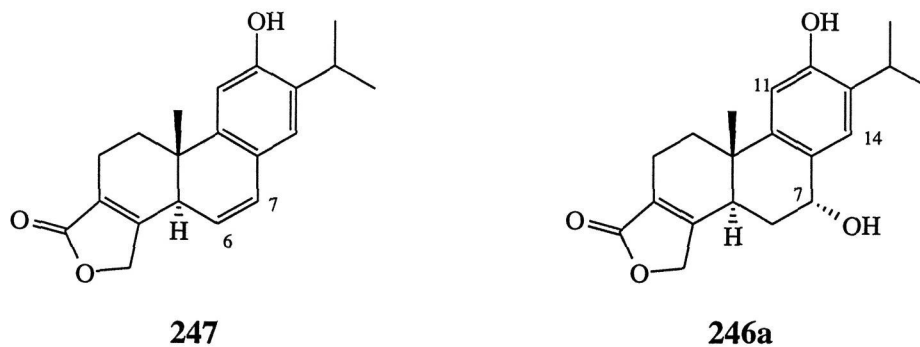
A very small amount of the other isomer, 7α-hydroxy-isotriptophenolide (**246a**), could also be seen from the spectrum.[*] The H14 and H11 signals of the α isomer were located at δ 7.17 (1H, s) and 6.73 (1H, s), respectively. Compared with δ 7.35 (1H, s) and 6.69 (1H, s) for the β isomer, this rather large difference in chemical shift of the H14 resonance (0.18 ppm) between these two isomers reflected the fact that the β-hydroxyl group in **246b** is in a quasi equatorial orientation and in close proximity to proton H14 in space. On the other hand, the 7α-hydroxyl group in **248** is close to an axial orientation, thus removed from proton H14, so the chemical shift of H14 is less affected. Another difference was the chemical shift of H5. The spectrum of the α isomer exhibited a downfield shifted H5 signal at δ 3.15 compared with δ 2.75 in the β isomer, due to its 1,3-diaxial interaction with the α-hydroxyl group at C7. These [1]H NMR spectral features proved very helpful in structure elucidation in the later biotransformation studies.

Attempted Synthesis of 8,12-Quinone Methide from Isotriptophenolide (194)
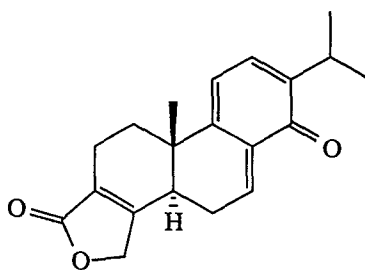
219

One of the proposed mechanisms to form the epoxide group between C7 and C8 is via an *ortho*-quinone methide intermediate **219** (section 2.2). If a synthesis of a *para*-quinone methide from the available isotriptophenolide (**194**) could be achieved and then this precursor was incubated with TRP4a cells, the epoxidation between C7 and C8 might be possible (Scheme 3.6).

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[*] Essentially pure **246a** was obtained later in another chemical reaction and also from a biotransformation experiment.
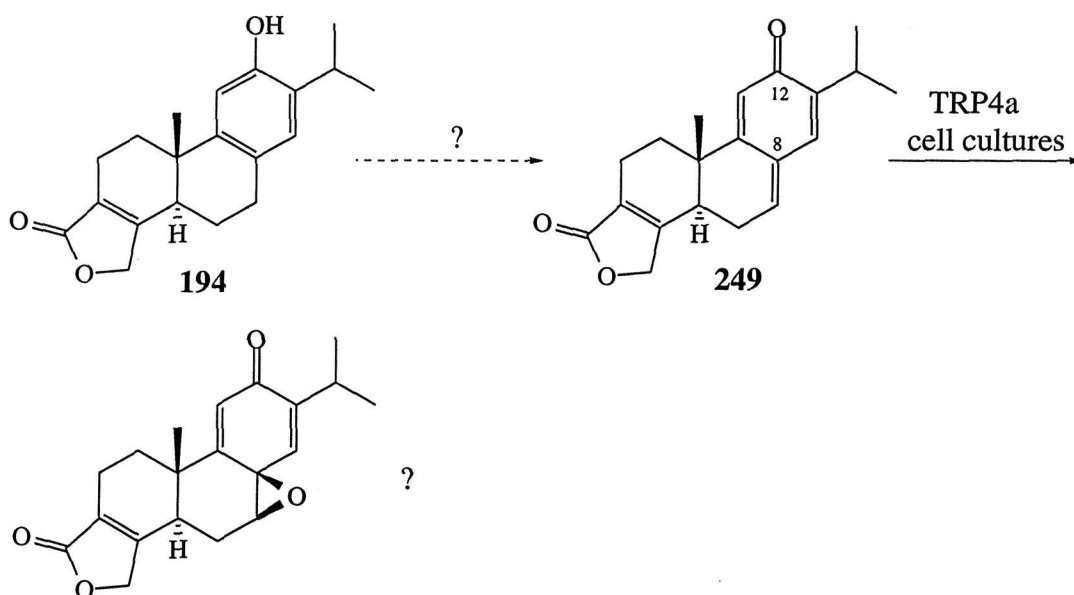
Scheme 3.6   Quinone Methide **249** as a Possible Biotransformation Precursor

Extended quinone methide structures have been found in some natural products and they are believed to be the key function to those compounds' biological activities.[189] Despite the long-lasting interests in synthesis of simple *para-* or *ortho*-quinone methide structures, only a few attempts have been successful and with some limitations such as the requirement of certain groups attached to the ring or the methide carbon.[201-205]
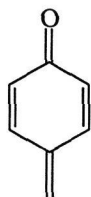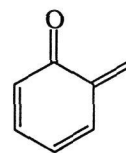
para-quinone methide   *ortho*-quinone methide

However, the simple quinone methides are believed to be the intermediates involved in some biological processes and also in many synthetic reactions.[189,206,207] Several methods

have been developed in making such structures as reaction intermediates, such as oxidation, elimination and so on.[208,209]

As to our particular molecule, oxidation seemed to be the best route to success. Oxidation of isotriptophenolide (**194**) with oxygen and cuprous chloride $(CuCl)^{210}$ led to a complicated mixture. Treatment of **194** with potassium ferricyanide $(K_3Fe(CN)_6)^{211}$ also gave a complicated mixture with some unreacted starting material remaining.

Dichlorodicyanoquinone (DDQ) was reported capable of effecting such an oxidation from a phenol to a quinone methide.[212] Preliminary results showed that the starting material disappeared in about 2-3 h and TLC showed a yellow spot (before spraying) among a moderately complicated mixture. As DDQ seemed more promising than the other reagents, it was subsequently more extensively examined.

Among the solvents tested, THF was better than benzene, dichloromethane and dioxane as based on TLC observations. Different conditions for oxidation of isotriptophenolide (**194**) by DDQ in THF, such as the equivalents of DDQ, temperature and reaction time, showed that 2 equivalents of DDQ in about 2-3 h at room temperature gave better results for the product which showed the yellow spot on TLC.
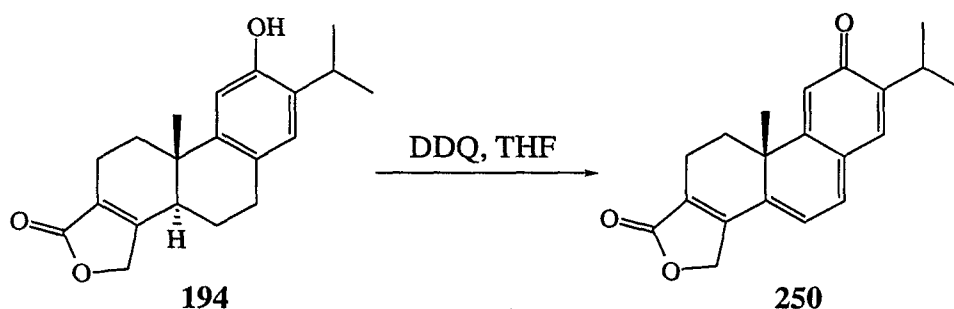
Scheme 3.7   Reaction of Isotriptophenolide (**194**) with DDQ in THF

A typical reaction, for example, was carried out by adding DDQ (291 mg, 2 equiv.) to a stirred solution of isotriptophenolide (**194**, 200 mg, 0.641 mmol) in THF (30 mL), and the mixture was stirred at room temperature for 3 h (Scheme 3.7). The solvent was removed and

the crude product was purified by repeated column chromatography. The compound isolated was a dark orange solid (**250**, 35 mg) in a yield of 18%.

250
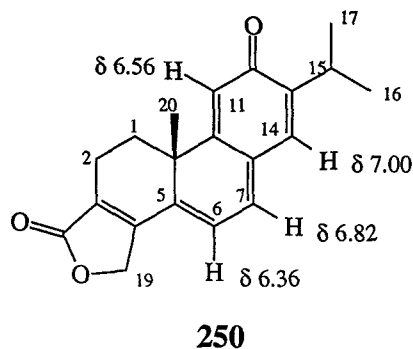
The IR spectrum of the product **250** showed the butenolide carbonyl at 1750 cm^{-1} and typical quinone bands at 1640 and 1600 cm^{-1}. The molecular formula was determined by high resolution mass spectrometry as $C_{20}H_{20}O_3$, two hydrogen atoms less than the anticipated quinone methide **249**. Its ^{1}H NMR spectrum displayed four olefinic proton signals at δ 6.36 (1H, d, J = 7.0 Hz), 6.56 (1H, d, J = 1.6 Hz), 6.82 (1H, dd, J = 7.0, 1.6 Hz), and 7.00 (1H, s). Protons at δ 6.36 and 6.82 were coupled with each other with a coupling constant of 7.0 Hz. In addition, the proton at δ 6.82 was also coupled with the proton at δ 6.56 with a small coupling constant of 1.6 Hz. The H19 signals were located at δ 4.98, at a much lower field than that of the corresponding signals in isotriptophenolide **194** (H19, δ 4.76), and with a wider splitting (Δv = 0.16 ppm) between the AB quartet, suggesting that there must be some reaction at C5. The C20 methyl signal was shifted downfield to δ 1.29, also suggesting some reaction at C5 (for example, double bond formation). Irradiation of H11 at δ 6.56 resulted in an enhancement of the H1β signal at δ 2.33 (1H, br d, J = 13.3 Hz), which was strongly coupled with H1α at δ 1.88 (1H, m). Both protons were also correlated with a multiplet at δ 2.62 (2H), which were then assigned to the C2 protons. The H15 signal was found at δ 3.14 (1H, septet, J = 6.4 Hz), which showed cross peaks to the two isopropyl methyl signals at δ 1.15. These signals accounted for all the aliphatic proton signals in the spectrum; therefore, the four olefinic proton signals must be due to protons at C6, C7, C11 (assigned) and C14. Irradiation of the C19

protons increased the intensity of the signal at δ 6.36, and *vice versa*, indicating the enhanced signal was due to H6. The proton (δ 6.82) which was strongly coupled with H6, therefore, must be H7. Finally, irradiation of the signal at δ 7.00 caused NOE enhancements to H15, two isopropyl methyl groups as well as H7; thus, this proton was assigned to H14. The small coupling between H11 and H7 was a long range coupling (^5J), which has been found in other similar structures.[213] On this basis, the structure of the oxidation product was assigned as **250**, an extended quinone methide. Compounds **247, 251, 252** and **245** were isolated as by-products.

247

251

252

245
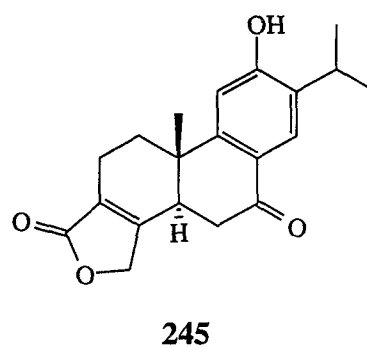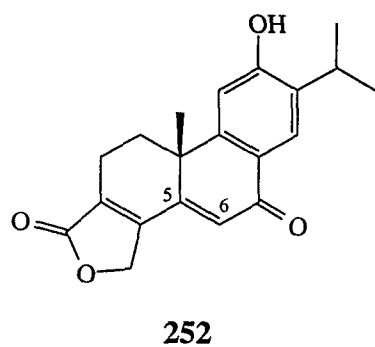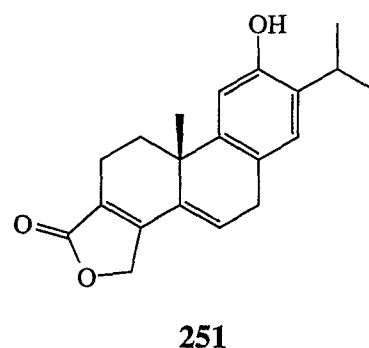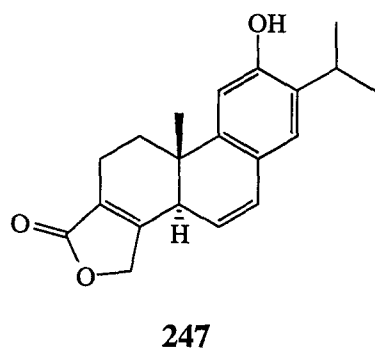
Unfortunately oxidation of isotriptophenolide (**194**) with DDQ yielded the extended quinone methide **250** rather than the desired **249**, regardless whether the reaction was performed at low temperatures or in short time periods. The structure of **250** suggested that the reaction likely went through the intermediate stage of **249**, but this compound may be labile and thus undergo further reactions very rapidly, so it was simply impossible to isolate the

intermediate **249**. This postulation could be further examined if this intermediate could be trapped.

Therefore, an oxidation of isotriptophenolide (**194**, 100 mg) with DDQ was carried out in methanol rather than in THF.[214] After a short reaction time, the crude product was subjected to a rapid column chromatography, yielding four fractions (Frs. 1-4). Fr. 1 (11 mg) was a mixture of unreacted starting material and compound **247**. Fr. 2 (24 mg, 22%) was an 1:1 mixture of 7α and 7β-methoxy-isotriptophenolide (**253**), and Fr. 3 (59 mg) was a complicated mixture containing some **250**. The final fraction (8 mg) was essentially pure **246a**. The [1]H NMR spectrum of Fr. 2 exhibited great similarity to those of 7α and 7β-hydroxy-isotriptophenolide (**246a, 246b**). The H14 signal of the 7β and α-methoxy compounds was located at δ 7.28 and 7.13, respectively, and the corresponding H7 was found at δ 4.64 (dd, J = 8, 8 Hz) and 4.30 (br s), respectively. The methoxy group for both isomers was located about δ 3.47. These methoxylated compounds suggested that the reaction proceeded via the intermediate **249** (Scheme 3.8), and the latter underwent a facile Michael addition type reaction[215] with methanol and water acting as nucleophiles. In the reaction in THF, the intermediate **249** was further oxidized to extended quinone methide **250**.
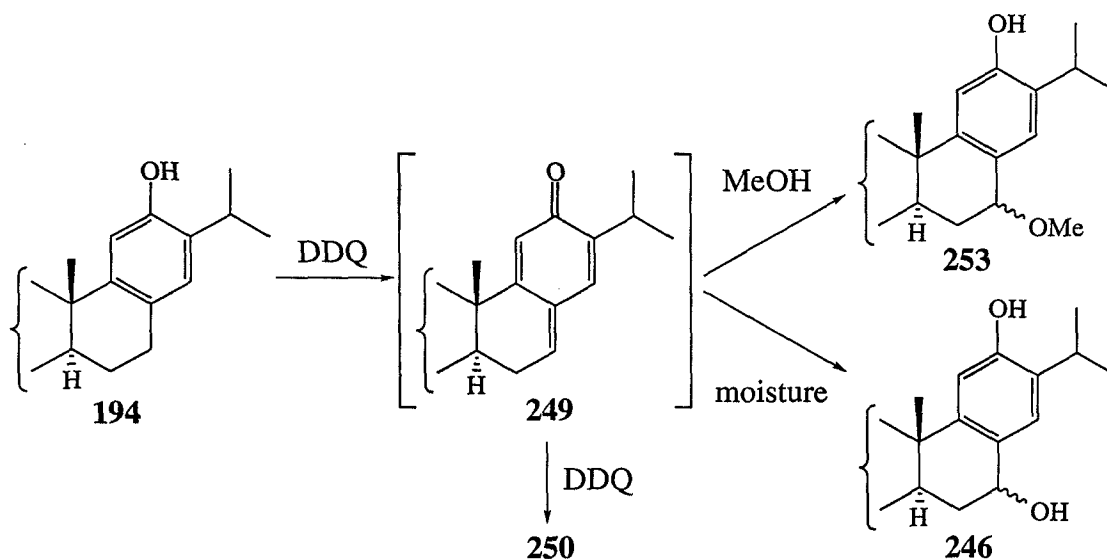
Scheme 3.8   Proposed Mechanism for the Formation of Compounds **253**, **246** and **250**

In conclusion, it appeared difficult to obtain the quinone methide **249** and further investigations were discontinued. However, the conjugated quinone **250** may be of further interest since some natural products with a similar quinone system have shown interesting biological activities.[216]

3.2.3 Synthesis of Triptophenolide (106)

The synthesis of **106** (in most cases as a racemic mixture), as an intermediate towards the synthesis of Tl (**1**), has already been reported.[107,142] The synthetic route of more direct relevance to the present study was developed by E. E. van Tamelen *et al.*, in which they employed dehydroabietic acid (**157**) as a starting material.[144] The strategy of the published sequence was to first functionalize ring C and then elaborate the butenolide ring system in the later stages (Scheme 3.9).[144]
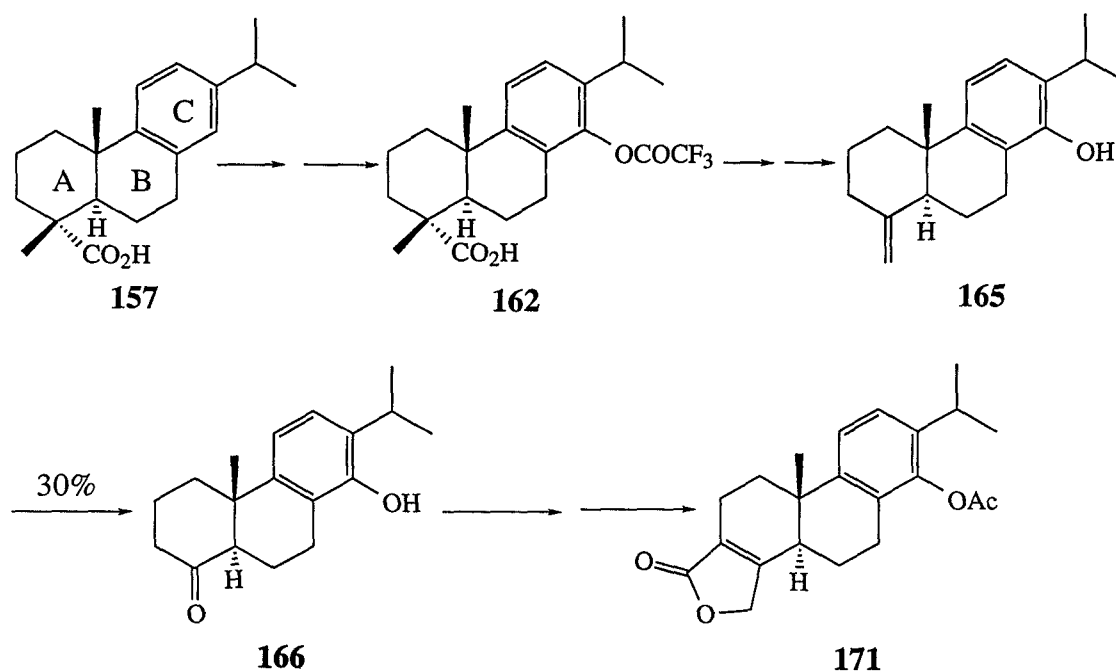
Scheme 3.9   Summary of the Synthesis of Isotriptophenolide Acetate (**171**) via C→A
Functionalization

The problem encountered in this sequence was that ring C was extensively degraded during the oxidative conversion of exo-olefin **165** to ketone **174**, thus lowering the overall yield of the sequence.

In order to avoid such losses, our preliminary experiments were designed to initially synthesize the ketone **229** (or its protected form), and then attempt the introduction of the C14 hydroxyl group by the established procedure.[145] Ketone **229** was readily available as an intermediate in the synthesis of **193**, but it was not a good starting material for the intended sequence because epimerization at C5 occurred in the reactions (probably in the zinc-acetic acid reduction step, according to [1]H NMR results) (Scheme 3.10).
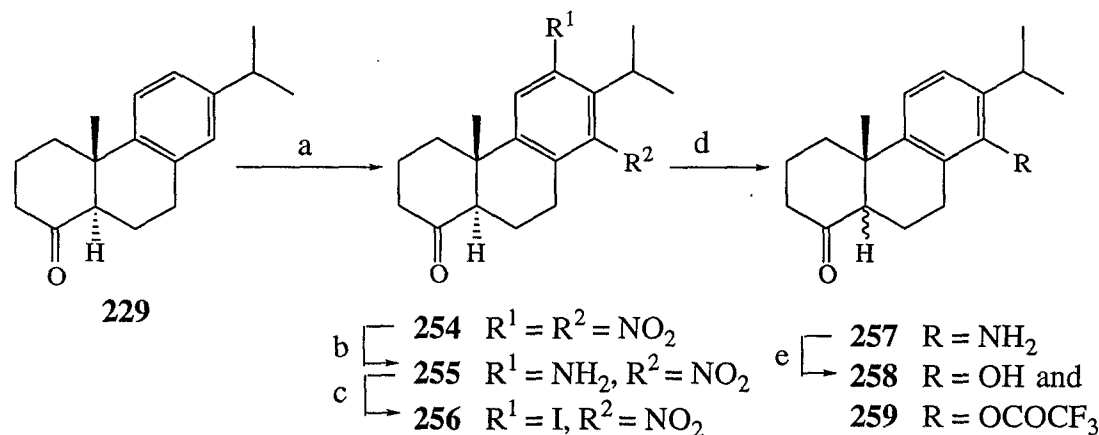
Scheme 3.10 Preliminary Attempt to Synthesize **106** from **229**

a) HNO3, H2SO4, HOAc; b) H2, Pt/C; c) NaNO2, KI; d) Zn, HOAc; e) NaNO2, CF3CO2H, HOAc

In order to avoid epimerization at C5, the ketone **229** was first reduced to the alcohol and then converted to the corresponding acetate (Scheme 3.11). Thus, the ketone **229** was treated with sodium borohydride in ethanol, and the resulting alcohol **260** (β alcohol according to its [1]H NMR) was treated with acetic anhydride in pyridine. The acetate **261** was then nitrated, reduced, diazotized, treated with iodide and finally reduced again to afford the phenol trifluoroacetate **266**. The latter was then hydrolyzed to give the phenol **267**. The phenol was protected as its 14-methyl ether **268** and then the acetate group at C4 was reduced back to

alcohol **269**. The alcohol was oxidized to ketone **270** by titration with Jones reagent and the ketone was then converted to the dithioacetal **271**. Treatment of the dithioacetal **271** with dimethylsulphonium methylid, followed by acid hydrolysis afforded the methyl triptophenolide (**107**) as colorless prisms in an overall yield of 5.7 % from **229**.

229

b [**260** R = OH
  261 R = OAc

d [**262** R^1 = R^2 = NO$_2$
e [**263** R^1 = NH$_2$, R^2 = NO$_2$
f [**264** R^1 = I, R^2 = NO$_2$
g [**265** R^1 = H, R^2 = NH$_2$
h [**266** R^1 = H, R^2 = OCOCF$_3$
i [**267** R^1 = H, R^2 = OH
  268 R^1 = H, R^2 = OMe

k [**269** R = OH/H
  270 R = O

271

n [**107** R = Me
  106 R = H
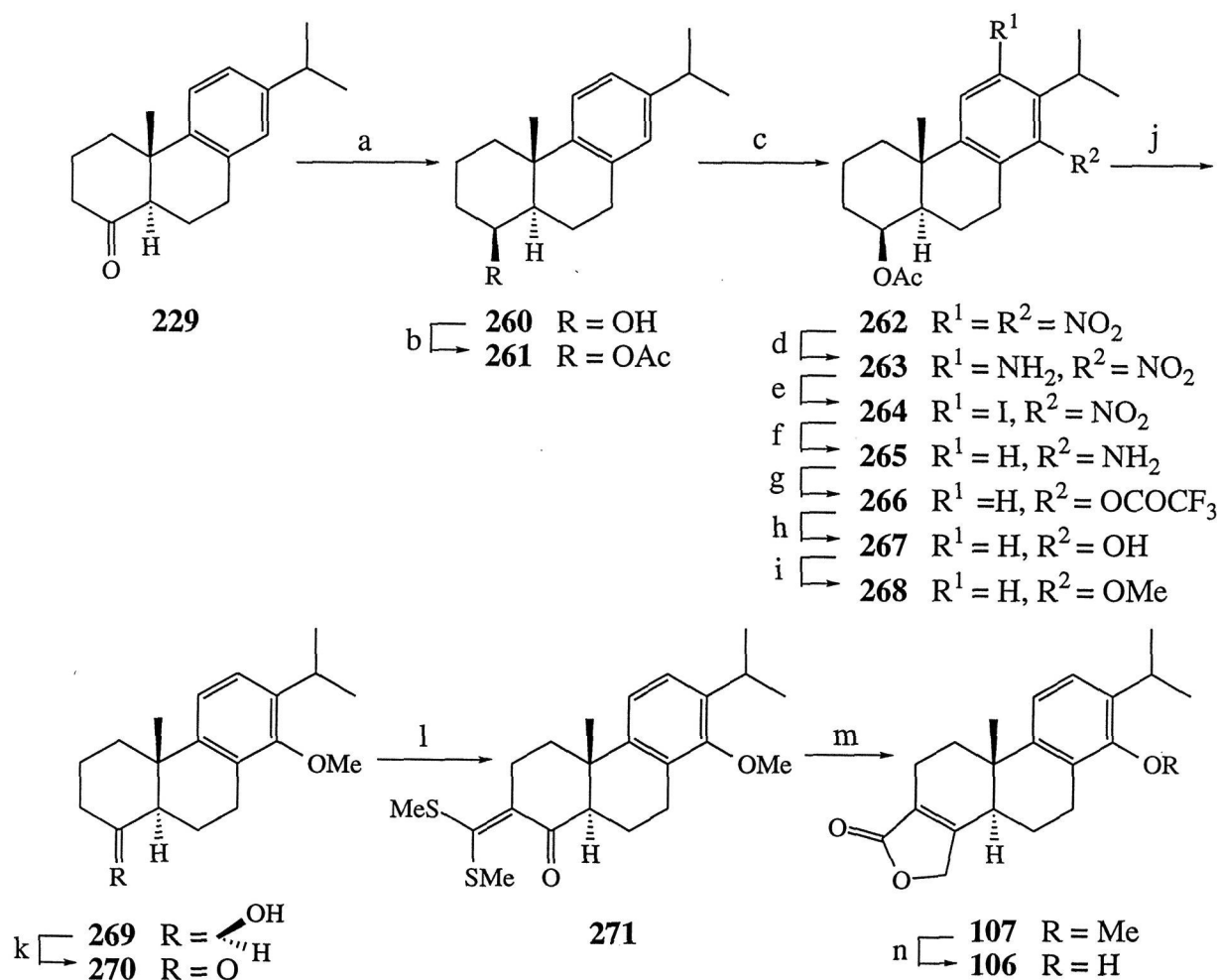
Scheme 3.11   Preliminary Attempt to Synthesize **106** from a Derivative of **229**

a) NaBH$_4$, EtOH; b) Ac$_2$O, pyridine; c)HNO$_3$, H$_2$SO$_4$, HOAc; d)H$_2$, Pt/C; e)NaNO$_2$, KI; f) Zn, HOAc; g) NaNO$_2$, CF$_3$CO$_2$H, HOAc; h) HCl, MeOH; i) n-Bu Li, MeI; j) LAH, THF; k) Jone's reagent; l) potassium 4-methyl-2,6-di-t-butyl phenolate, CS$_2$, MeI; m) Me$_2$S=CH$_2$; HCl; n) HBr, HOAc

Hydrolysis of the methyl ether was achieved by refluxing with hydrobromic acid in acetic acid,[217] giving triptophenolide (**106**) in excellent yield. Ether cleavage by reaction with

boron tribromide was not successful,[107,218,219] probably due to lack of a neighboring carbonyl group.[220] The synthesized triptophenolide (106) was spectrally identical with authentic and isolated samples.

The second sequence was successful in providing triptophenolide (106), but there were still some shortcomings. First, the protection and deprotection of the C4 hydroxyl group and the C14 phenolic hydroxyl group prolonged the already long sequence. Secondly, the overall yield (not optimized), although similar to the reported sequence, was too low for our requirements in terms of preparing larger quantities of 106 for future studies. A possible solution to this problem, and with the syntheses of other ring C activated precursors in mind, was to use a common, "advanced" intermediate, such as the butenolide 193, in which the butenolide ring system has been already elaborated (Scheme 3.12).
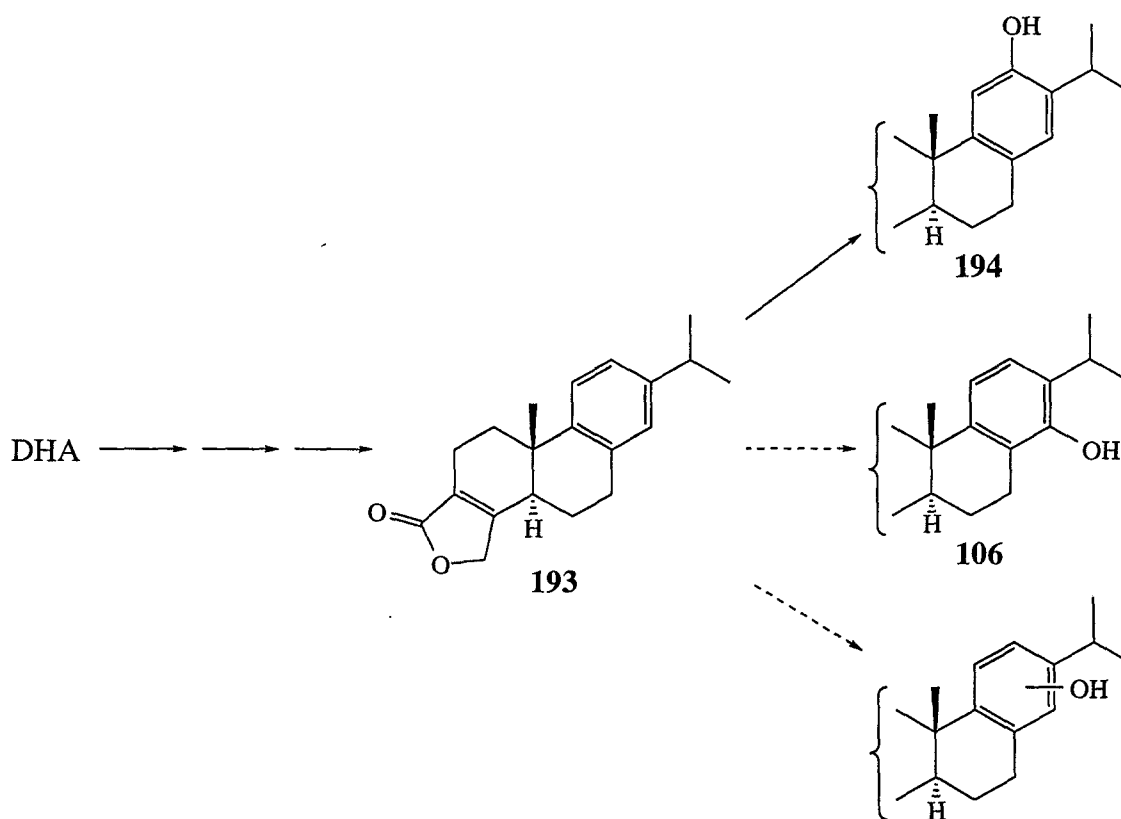
Scheme 3.12 Synthesis of Ring C "Activated" Compounds via the Common Intermediate 193

With the butenolide **193** as the common starting material, one could take advantage of the well established sequence to synthesize **193**, and then use it to further synthesize ring C hydroxylated compounds, such as isotriptophenolide (**194**), triptophenolide (**106**) and others.

There had been some concern about the liability of the butenolide moiety in the subsequent reactions to ring C hydroxylated compounds.[161] However, high yields of acetylation of **193** in the synthesis of isotriptophenolide (**194**), and of the hydrolysis of the methyl ether **107** in the preliminary synthesis of triptophenolide (**106**) were obtained under harsh reaction conditions. These results reflected the fact that the butenolide moiety was much more stable than we had predicted. A series of small scale experiments under some relevant reaction conditions, such as nitration, catalytic hydrogenation, and zinc-acetic acid reduction, confirmed that compound **193** was stable enough under those conditions. Therefore, a sequence using isodehydroabietenolide (**193**) as starting material to synthesize triptophenolide (**106**) was investigated.

Initially, similar conditions as those in the literature were applied.[145] The butenolide **193** was nitrated with a mixture of concentrated nitric acid and sulfuric acid at 0-5°C. The product precipitated as a pale yellow solid. Column chromatography of the crude product gave the 12,14-dinitro compound **272** as a pale yellow solid (mp 112-115°C) in 55% yield and 12-nitro compound **273** in 15% yield.

272   **273**
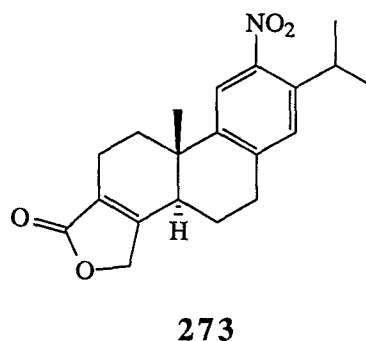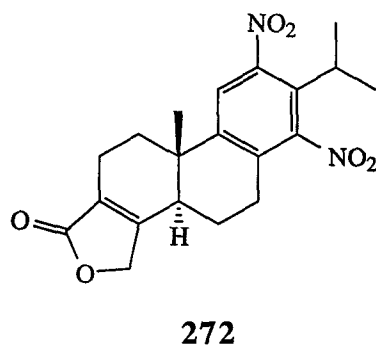
The IR spectrum of **272** showed the butenolide absorptions at 1740 and 1680 cm⁻¹, and strong bands for nitro groups at 1530 and 1370 cm⁻¹. The ¹H NMR spectrum exhibited only one

aromatic proton signal at δ 7.62 (1H, s, H11), which showed an NOE to the H1β at δ 2.46 (1H, dd, J = 12.3, 6.6 Hz). The remainder of the spectrum was similar to that of **193**. The 12-nitro compound **273** was obtained as a pale yellow crystalline solid (mp 176-178°C, dec). Its IR spectrum showed the absorption bands for the nitro group at 1520 and 1370 cm^{-1}. Two aromatic signals were observed as singlets in the ^{1}H NMR spectrum.

Since the desired 12,14-dinitro compound **272** was only obtained in a moderate yield under this condition, further studies to improve the yield were essential. The nitration was repeated using nitromethane[221] as the solvent (the starting material was more soluble in nitromethane). The reaction went well and afforded the dinitro compound **272** in 86% yield. The reaction was repeated a number of times and the average yield was consistently greater than 86%.

The next step was catalytic reduction of the 12-nitro group in **272**. The reaction was carried out under hydrogen (about atmospheric pressure) at room temperature with Pt (10% on carbon) as catalyst. The crude product was recrystallized in ethyl acetate to give 14-nitro-12-amine **274** as yellow needles (mp 227-229°C, dec). The mother liquor was chromatographed to afford more product, bringing the total yield to 83%.
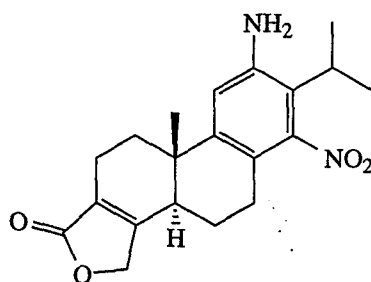
274

The IR spectrum of **274** showed absorption bands for the amino group at 3500 and 3400 cm^{-1}, and the nitro group at 1520, 1350 cm^{-1}, respectively. The H11 signal was found at δ 6.71 in its ^{1}H NMR spectrum. The reduction occurred on the C12 nitro group regioselectively, because the C14 nitro group is much more hindered than the C12 nitro group.

Compound **274** was then diazotized with sodium nitrite (Scheme 3.13), followed by addition of potassium iodide. The crude product **275** was isolated by filtration and used directly for the next reaction. The crude **275** was treated with zinc powder in warm acetic acid (65-70°C) overnight and the resulting crude amine **276** was diazotized again with sodium nitrite in trifluoroacetic acid to form the trifluoroacetate **277**. The latter was then subsequently hydrolyzed to the phenol **106** in 33% overall yield from **274**, and with a yellow by-product in 5% yield.

The spectra of the synthetic triptophenolide (**106**) were identical with those from the authentic sample obtained in the earlier studies. The yellow by-product **278** showed quinone absorption bands at 1680 and 1640 cm^{-1}. Its ^{1}H NMR spectrum exhibited the proton at C12 as a doublet at δ 6.39 (1H, d, J = 1.1 Hz, allylic coupling with H15). All the spectral data indicated that the by-product was the 11,14-quinone **278**.
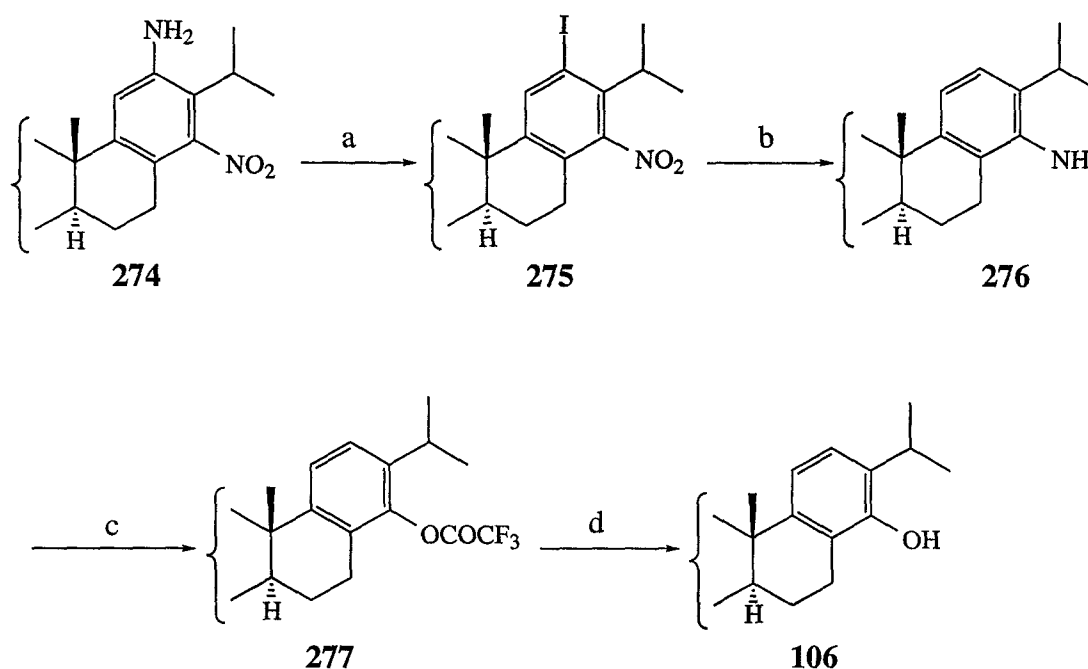
Scheme 3.13 Reactions from **274** to **106**

a) NaNO$_2$, TFA, HOAc, H$_2$O; KI; b) Zn, HOAc, Δ; c) NaNO$_2$, TFA; d) HCl, MeOH
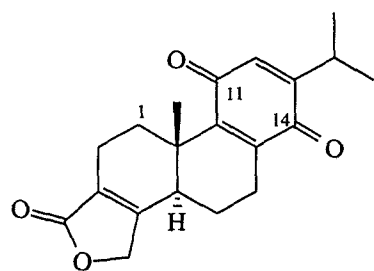
278
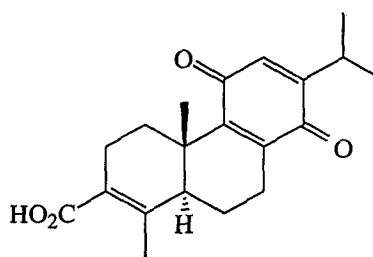
114

Quinone **278** is structurally related to compound **114** isolated from *T. regelii*[89] and may exhibit similar pharmacological activities (inhibition of interleukin-1),[133] but screening data are not yet available. However, in the synthesis of triptophenolide (**106**), formation of this by-product would decrease the yield of **106**, and thus its formation should be decreased. An attempt was made to examine whether lowering the temperature of the reduction with zinc in acetic acid could decrease the formation of the quinone, because once the C14-amine **276** is formed, some of it may be oxidized to the quinone. Therefore, a reaction was carried out in the similar manner but the reduction was conducted at 30-40°C for 14 h and then 65-75°C for only 2 h. Column chromatography of the crude product from the final step gave triptophenolide (**106**) in 23% yield, quinone **278** (10% yield), and another compound **279** (9%).
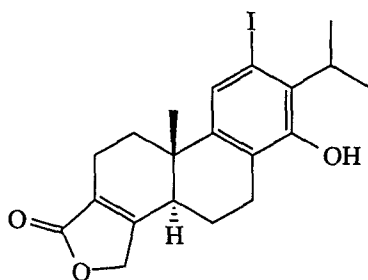
279

The new compound had a molecular formula of $C_{20}H_{23}IO_3$. Its IR spectrum suggested the presence of a hydroxyl group (3625, 1240 cm^{-1}). The ^{1}H NMR spectrum showed a similar pattern to that of triptophenolide (**106**) except that only one aromatic proton signal was found at

δ 7.42 (1H, s), and the H15 resonance was shifted downfield to δ 3.42. Irradiation of the aromatic proton enhanced H1β at δ 2.43, indicating it to be due to H11. Therefore, the new compound was assigned as 12-iodo-triptophenolide (**279**).

It was obvious that lowering the temperature of the reduction step did not decrease the formation of the quinone, instead, incomplete reduction produced another by-product **279**. Since the overall yield from **274** to **106** was not very satisfactory, each step of the reaction sequence was then examined individually.

Therefore, the 14-nitro-12-amino analog **274** was treated with zinc powder in acetic acid at 65-70°C overnight. The resulting crude product was chromatographed to give a mixture of the C14 amine **276** and the 12-iodo-14-amine **280** in a ratio of about 3.5 to 1 by comparing the integrations of their C20 methyl signals in the ^{1}H NMR spectra (δ 1.01 and 0.99, respectively). Compound **281** was also isolated.

280   **281**
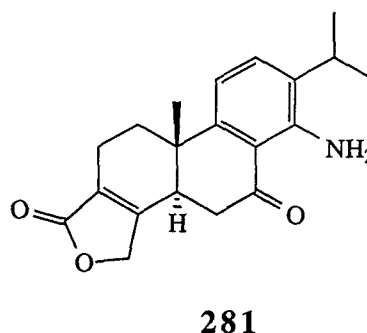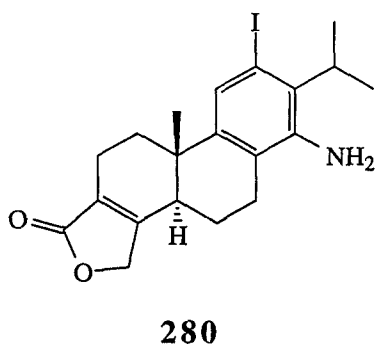
Compound **281** had a molecular formula of $C_{20}H_{23}NO_3$ with the IR spectrum revealing an amino group at 3510 and 3350 cm^{-1}. The ^{1}H NMR spectrum was similar to that of **108**.

Further separation of **280** and **276** was not successful, and thus the mixture (208 mg) was used in the next step to synthesize the phenol. The reactions (diazotization and hydrolysis) were carried out under the same conditions as before, and the final product was chromatographed to give the quinone **278** (18 mg), triptophenolide (**106**, 38 mg) and 12-iodo-triptophenolide (**279**, 49 mg). The ratio of **279** to **106** was about 1 to 1 in the products, although the ratio of their corresponding starting materials was about 1 to 3.5 based on ^{1}H NMR data. In other

words, the 12-iodo-14-amino analog **280** afforded the corresponding phenol **267** in more than 3 times higher yield than the conversion of the C14 amine **276** to **106** (Scheme 3.14).
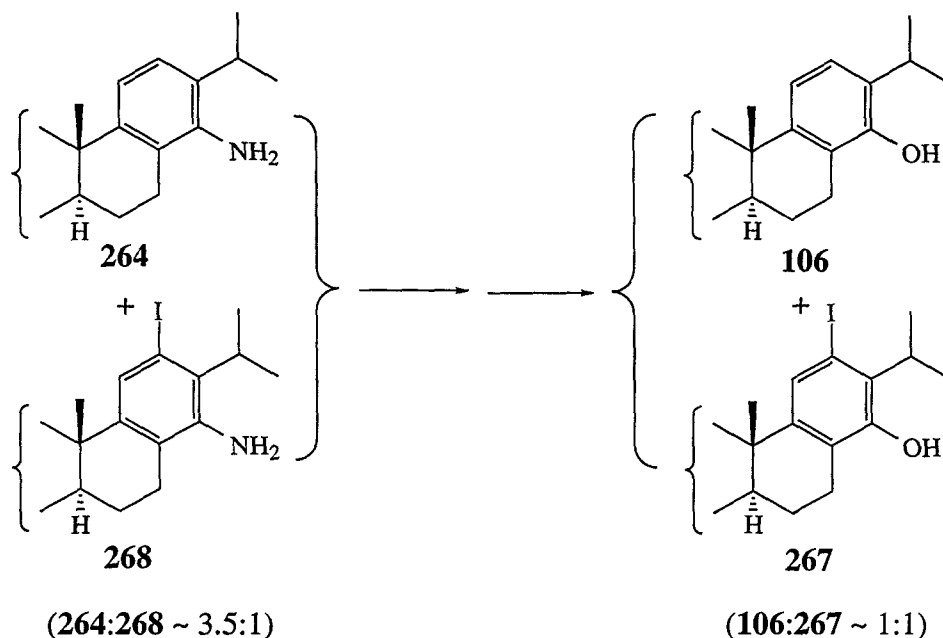
Scheme 3.14   Comparison of Yields of **106** and **267** with respect to **264** and **268**

The iodo group was originally introduced to improve the yield of indirect deamination at C12, because direct deamination with sodium nitrite and hypophosphorous acid was reported to afford poor yields.[145] In the present investigation, the presence of this iodo group at C12 increased the yield of the subsequent conversion of the C14 amino group to a hydroxyl group. This is a new finding. The corresponding quinone (see **278**) but bearing the 12-iodo function was not found in this particular experiment.

In order to use the new finding in the effort to improve the yield of **106**, and also because of the overall unsatisfactory results obtained with zinc-acid reduction, the synthetic strategy should be re-organized. The possible alternatives in obtaining **106** from the various available synthetic intermediates are summarized in Scheme 3.15.
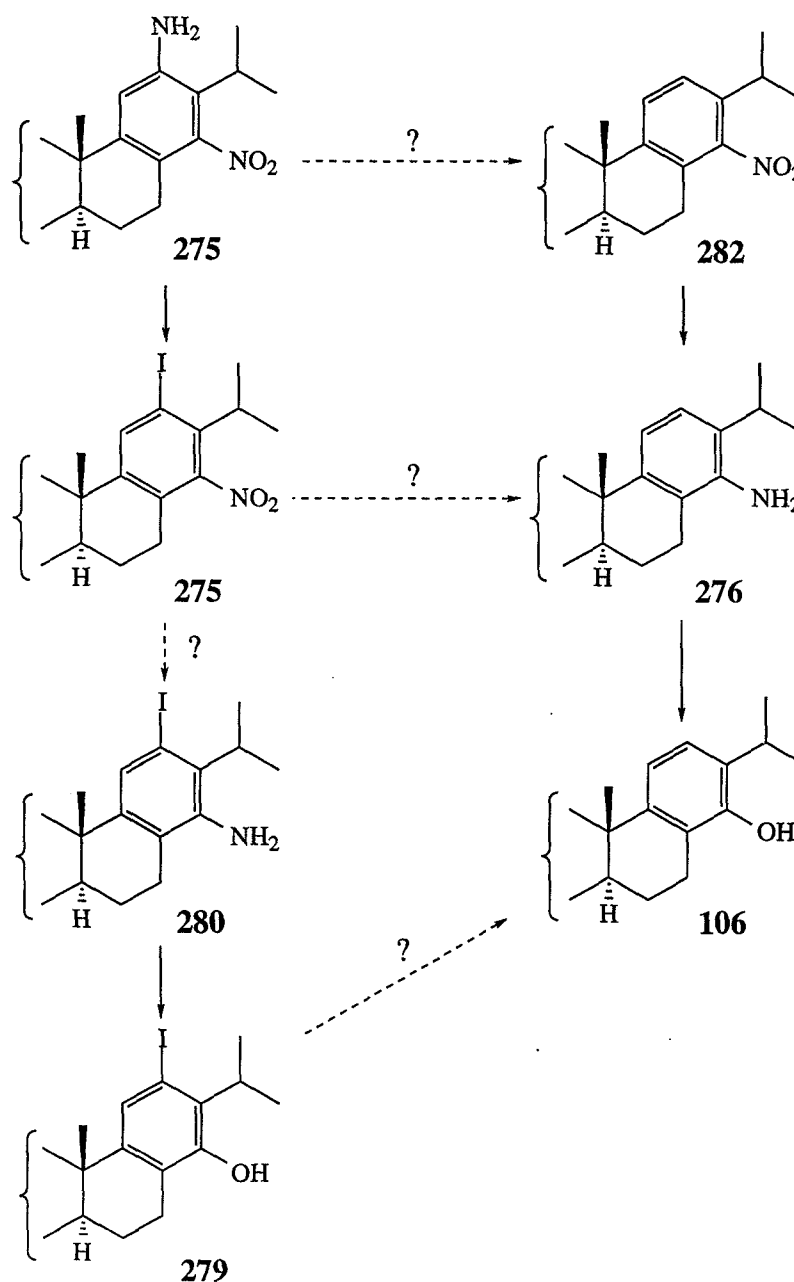
Scheme 3.15 Proposed Sequences for Synthesis of **106**

Common procedures for the replacement of an aromatic primary amino group by hydrogen involve preliminary diazotization of the aromatic amine followed by reductive substitution by a hydrogen donor.[222] Hypophosphorous acid has proven to be a generally effective reducing agent for diazonium salts, and remains the standard reagent for such reactions.

However, as hypophosphorous acid was reported not so effective in a very similar situation,[145] another alternative was sought. A method of reductive deamination of arylamines by alkyl nitrites (pentyl nitrite, isopentyl nitrite, *t*-butyl nitrite etc.) came to our attention. Moderate and high yields were obtained for such *in situ* one-pot conversions of aromatic amines to the corresponding hydrocarbons.[223,224] Thus, a small scale experiment was attempted. A solution of the 14-nitro-12-amino compound **274** in DMF was added dropwise to a stirred solution of isoamyl nitrite in DMF at 65°C (Scheme 3.17). The reaction was continued for a short time and then worked up. Column chromatography of the crude product yielded the 14-nitro-compound **282** in 33% yield. A by-product was also isolated.

274   a   **282**
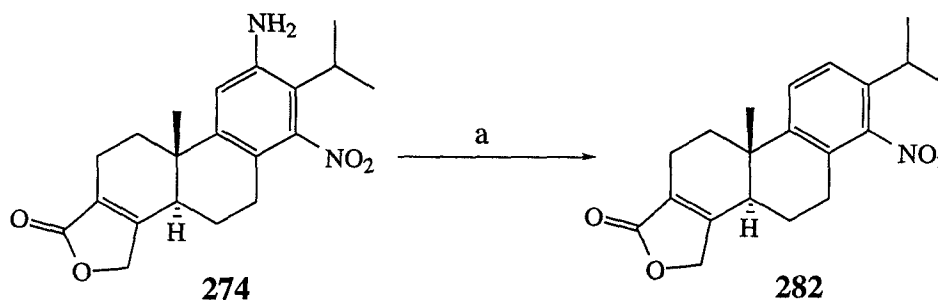
Scheme 3.16   Deamination of **274** by Isoamyl Nitrite

a) isoamyl nitrite, DMF, Δ

Compound **282** was isolated as pale yellow prisms (mp 231-233°C,dec). The ^{1}H NMR spectrum was similar to that of the C14 amine **276**, but the H11 and H12 signals were slightly shifted downfield to δ 7.43 (1H, d, J = 8.3 Hz) and 7.24 (1H, d, J = 8.3 Hz). The by-product had a molecular formula of $C_{20}H_{23}NO_5$. Its IR spectrum suggested the presence of hydroxyl (3700-3600 cm^{-1}) and nitro groups (1560, 1370 cm^{-1}). The ^{1}H NMR spectrum was similar to that of **282**, except that only one H7 signal was found at δ 5.73 (1H, dd, J = 5.5, 1.8 Hz) and that the H5 signal was significantly shifted downfield to δ 3.15 (1H,br d, J = 12.2 Hz). This was an indication of the presence of an α hydroxyl group at C7. On the basis of NMR, IR and MS spectra, this by-product was proposed as **283**.
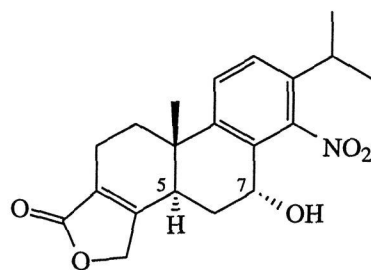
283

The low yield of this direct deamination turned our attention to reactions that could simultaneously reduce the nitro and iodo groups. Most metal borohydrides are generally unable to reduce aromatic nitro compounds under ordinary conditions.[188] However, it has been reported that aromatic nitro compounds can be reduced with sodium borohydride-transition metal salts systems in good yields.[225-230] Interestingly, it was reported that iodo substituents on the aromatic ring were also reduced together with the nitro groups by a potassium borohydride-copper (I) chloride system.[230] Therefore, the 12-iodo-14-nitro compound **275** was treated with cuprous chloride and sodium borohydride in methanol (Scheme 3.17). The crude product was chromatographed to afford the C14 amine **276** in about 60 % yield (not optimized). No 12-iodo-14-amino compound **280** could be detected from the ^{1}H NMR spectrum.

275   **276**
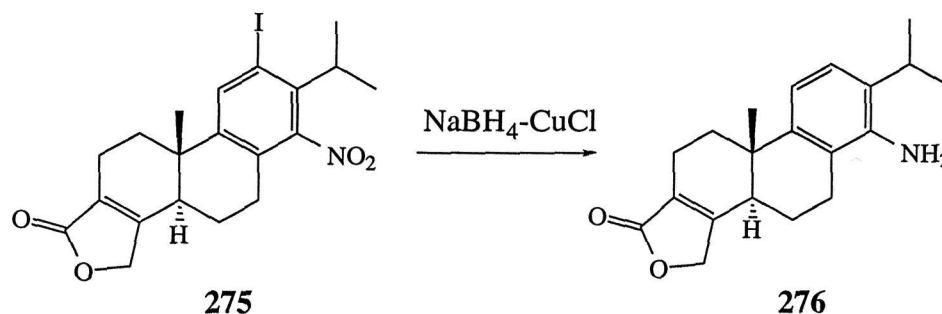
Scheme 3.17   Reduction of Iodo and Nitro Groups with NaBH₄-CuCl System

The amine **276** was isolated as a pale pink crystalline solid (mp 186-188°C). The IR spectrum showed the amine bands at 3550 and 3350 cm^{-1}. The ^{1}H NMR spectrum exhibited the H11 and H12 resonances at δ 6.84 (1H, d, J = 8.2 Hz) and 7.04 (1H, d, J = 8.2 Hz), respectively. The remainder of the spectrum was similar to that of triptophenolide (**106**).

Catalytic hydrogenation has also been used to cleave aromatic halogen groups in synthesis.[47,231] In our case, palladium (10% on carbon) was used in an attempt to cleave the iodo group.[232] The 12-Iodo-14-nitro compound **275** in methanol solution was stirred under hydrogen atmosphere with the palladium catalyst for several hours, giving the 14-nitro compound **282** in 48% yield. In order to determine whether the reaction can further reduce the 14-nitro group, a second reaction was run for a prolonged time. The products, after purification, were **282** (23% yield) and the amine **276** (44% yield). The catalytic hydrogenation for the above reaction was slow and the yield was unsatisfactory. Among the methods tried, sodium borohydride-copper (I) chloride seemed promising.

While a consideration to pursue a more detailed study with the sodium borohydride-cuprous chloride reduction was under way, another experiment with sodium dithionite ($Na_2S_2O_4$)[233,234] showed very encouraging results. Reduction of the 12-iodo-14-nitro compound **275** with sodium dithionite selectively reduced the nitro group without affecting the iodo group, giving a very high yield of the 12-iodo-14-nitro compound **280** (87%, Scheme 3.18).
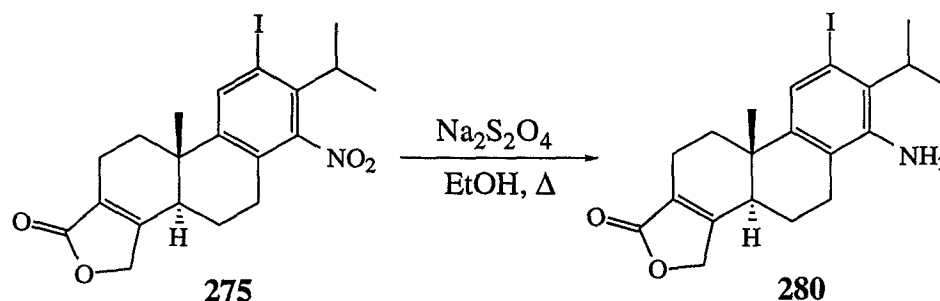
Scheme 3.18   Selective Reduction of Nitro Group with $Na_2S_2O_4$

In consideration of the potential high yield from **280** to the corresponding phenol **279**, this sequence would be very promising if an appropriate condition for the last step, removal of the iodo group from the phenol **279**, could be found.

Catalytic dehalogenation was attempted again to remove the iodo group from **279**. The reaction was carried out in methanol with palladium on carbon as catalyst.[232] An equivalent amount of potassium carbonate was added as the acceptor of hydrogen iodide. The reaction proceeded very well and afforded the desired product in 97% yield. With the excellent yield obtained for the last step, the overall sequence was now efficient. The new sequence is summarized in Scheme 3.19.

With the individual sequences from the 14-nitro-12-amine **274** to triptophenolide (**106**) being established, the formal synthesis of **106** from isodehydroabietenolide (**193**) was carried out. The 12,14-dinitro compound **272** and 14-nitro-12-amine **274** were synthesized following the procedures we have discussed earlier (*vide supra*). Then **274** was treated with sodium nitrite followed by potassium iodide to afford the 12-iodo-14-amine **275** in 65% yield. Although a few attempts were made to improve the yield, no significant improvement was achieved. Compound **275** was then treated with sodium dithionite in refluxing ethanol to give the 12-iodo-14-amine **280**. The average yield of this reaction was 86-88%.

Compound **280** was diazotized in trifluoroacetic acid at -12 to -10°C. The crude product, triptophenolide trifluoroacetate (**282**), was directly hydrolyzed with concentrated hydrochloric acid in methanol. In a preliminary experiment, an attempt was made to purify the trifluoroacetate by column chromatography. However, partial hydrolysis of the ester was observed during the purification, and since we were only interested in the final product **106**, the purification was omitted. The hydrolysis was carried out overnight, and the solid product precipitated out when the reaction was almost completed. Workup of the reaction gave a crude product which, after column chromatography, afforded 12-iodo-triptophenolide (**279**) in 77% yield. The average yield of the reaction from **280** to **279** was between 77 to 79%.
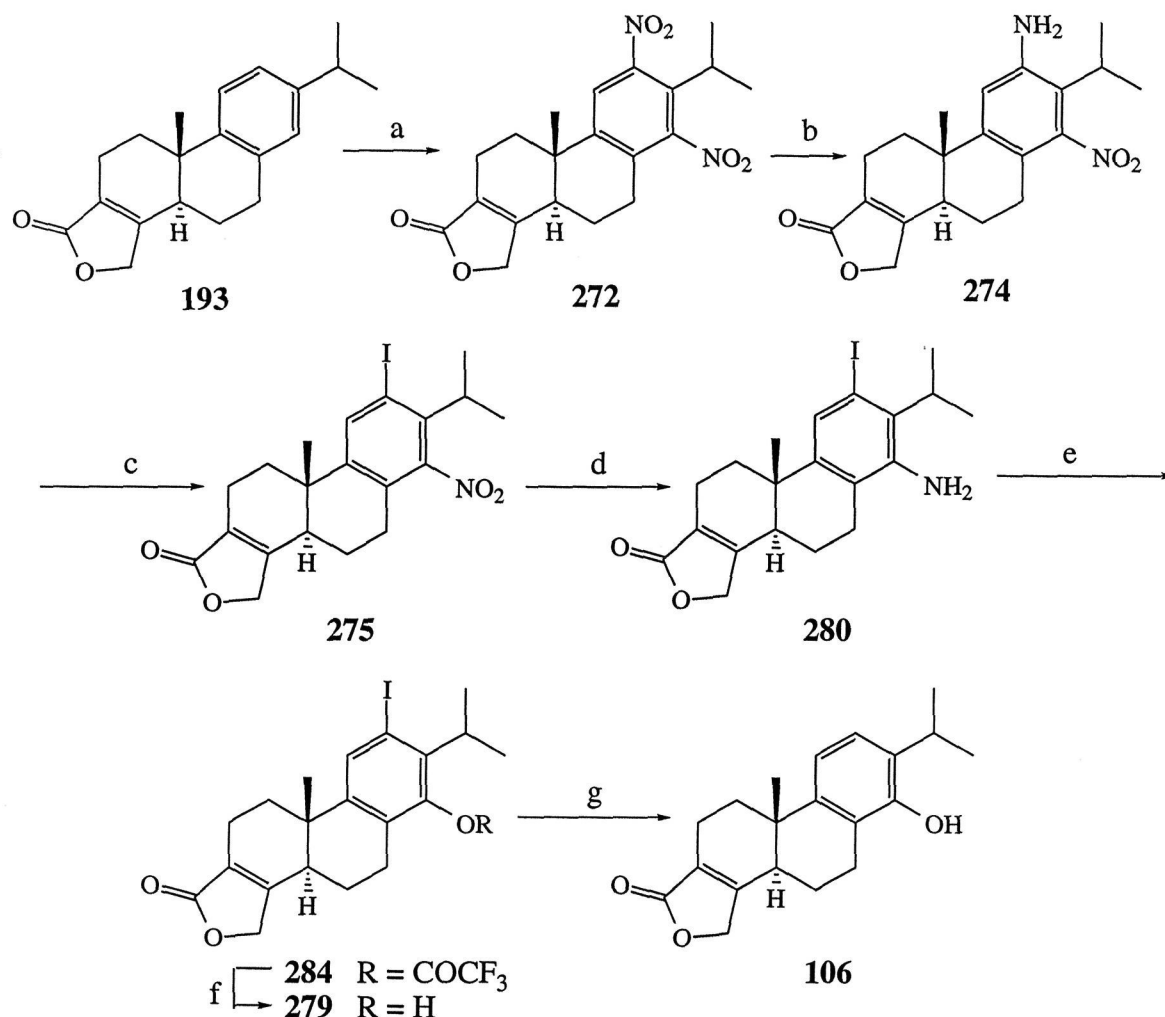
Scheme 3.19 The New Sequence for the Synthesis of Triptophenolide (**106**)

a) HNO3, H2SO4, MeNO2; b) H2, Pt/C, HOAc; c) NaNO2, TFA, HOAc, KI; d) Na2S2O4, EtOH, Δ;
e) NaNO2, TFA; f) HCl, MeOH; g) H2, Pd/C, Na2CO3

As a comparison, two sets of the reaction, with the C14 amines **276** and **280** as the starting materials, respectively, were carried out at the same time and under identical conditions. The results showed that the reaction with **280** constantly afforded 12-iodo-triptophenolide (**279**) in 77-78% isolated yields, while with **276**, the reaction only gave 45-46% of **106** after purification. This clearly demonstrated that the iodo group at C12 greatly increased the yield of converting the C14 amino group to a hydroxyl group via diazotization (Scheme 3.20).

a, b

I: 78%

II: 77%

280

279

a, b

I: 46%

II: 45%

276

106
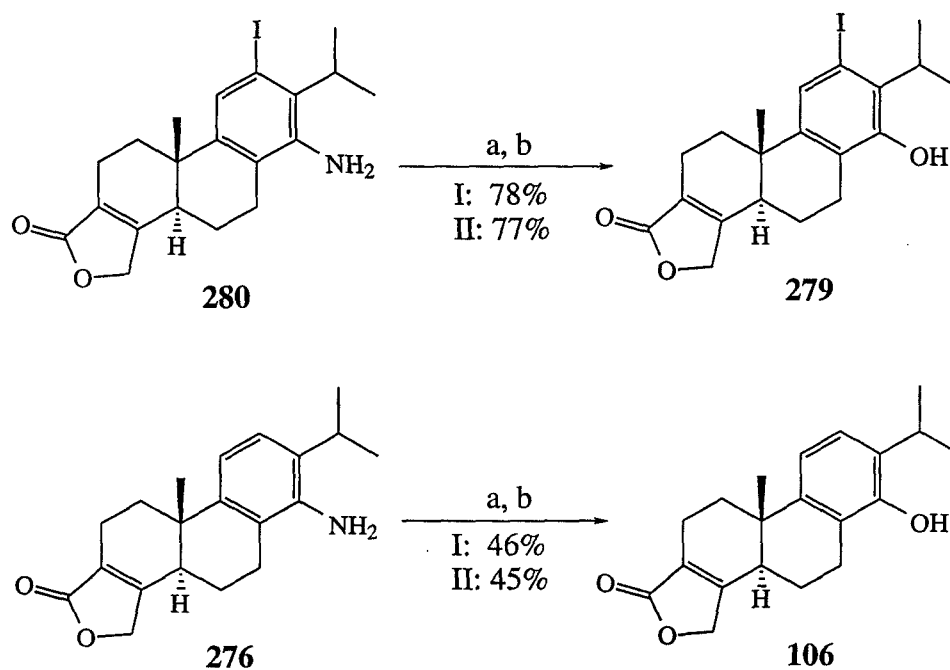
Scheme 3.20   Comparison of Reaction Yields between **268** and **264**

a) NaNO₂, TFA; b) HCl, MeOH

In order to examine whether zinc or iodide salts were catalysts for the last diazotization step,[145] small amounts of both salts were intentionally added to the reaction mixture. No significant differences were observed as compared with those without the salts. Therefore, the reaction is not catalyzed by these inorganic salts.

Another interesting point was that, in contrast to reactions with the amine **276** as starting material, diazotizations with the 12-iodo-14-amine **280** only produced about 1.5% yield of the 12-iodo-quinone **285** as a by-product while the former usually gave about 10% yield of quinone **278**. The other by-products isolated in small quantities from diazotization of **280** were **286** and **287**.

Compound **285** was isolated as orange prisms (mp 186.5-187.5°C, dec). Its IR spectrum showed the quinone absorptions at 1680 and 1650 cm⁻¹. The ¹H NMR spectrum was similar to that of **278**, except for the absence of the H12 signal.
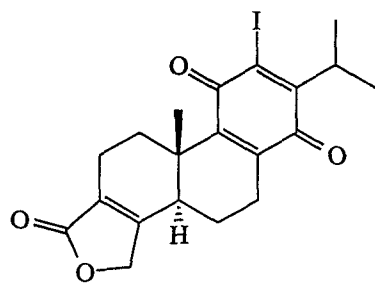
285
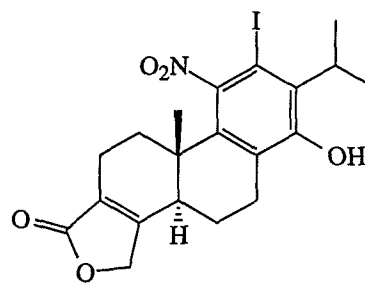
286

Compound **286** was obtained as pale yellow prisms with a molecular formula of $C_{20}H_{22}INO_5$. The IR spectrum indicated the presence of hydroxyl (3400, 1180 cm^{-1}) and nitro (1520, 1360 cm^{-1}) groups. Its ^{1}H NMR spectrum showed no aromatic signals. All the spectral information suggested the structure as assigned to **286**. This compound was probably produced via nitration by small amounts of nitric acid in the reaction mixture.
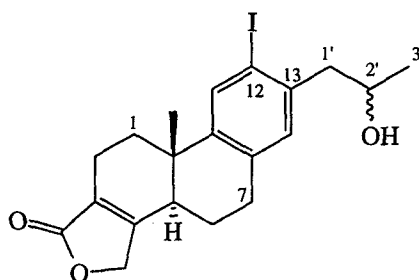
287

Compound **287** was isolated as a white powder with a molecular formula of $C_{20}H_{23}IO_3$. The IR spectrum showed hydroxyl bands at 3400 and 1010 cm^{-1}. Its ^{1}H NMR spectrum displayed two aromatic proton signals at δ 7.75 (1H, s) and 6.99 (1H, s). The pattern of H19, H7, H5, H6, H2, H1 and the C20 methyl signals was very similar to that of isotriptophenolide (**194**). However, one of the two methyl signals was found at δ 1.28 (3H, d, J = 6.2 Hz) where usually the two isopropyl methyl signals were located. The characteristic septet signal of the isopropyl methine proton was also not present. Instead, one multiplet appeared at δ 4.09 (1H) and other two at δ 2.73 (1H) and 2.85 (1H), respectively. The COSY spectrum exhibited cross

peaks between the proton at δ 4.09 and the other two multiplets, and the methyl signal at δ 1.28. However, no cross peaks were found between the multiplets (δ 2.73, 2.85) and the methyl signal. These data showed that the isopropyl group was no longer present.
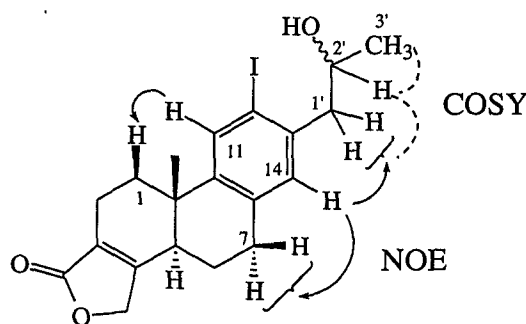
287

The iodo group was probably still at C12 and the proton at δ 4.09 (not in a benzylic position) was likely geminal to a hydroxyl group. Irradiation of the proton at δ 7.75 resulted a signal enhancement of H1β centered at δ 2.45 (doublet of doublets), indicating that the irradiated proton was H11. Irradiation of the proton at δ 6.99 showed NOE enhancements to the C7 protons at δ 2.94 (2H, m) and the two multiplets at δ 2.73 and 2.85, suggesting that the irradiated proton was H14 and the two multiplets were in a position similar to the original "H15" in the molecule (the chemical shifts were also similar). In consideration of the coupling relationships among the proton at δ 4.09, the two multiplets and the methyl group, the structure was assigned as **287**. Because the carbon at C2' was chiral, so the compound was actually a pair of diastereomers, which were supported by the presence of slightly different two sets of signals in [1]H and [13]C NMR spectra. No further separation of these two diastereomers was attempted. Further studies as to the formation of **287** were not undertaken though hydride transfer and methyl group migration might be involved in the process.[235-237]

In summary, the newly developed sequence from isodehydroabietenolide (**193**) to triptophenolide (**106**) significantly increased the yield of triptophenolide (**106**) (30% overall yield from **193**) (Scheme 3.21). The overall yield from dehydroabietic acid (**157**) via **193** to

triptophenolide (**106**) was 11%, which was much higher (about 40 times) than that previously reported (0.27%).[145] Finally, the strategy of the synthesis of ring C activated compounds via isodehydroabietenolide (**193**) was more logical, and the use of triptophenolide (**106**) as starting material for biotransformations or other studies was now realistic.
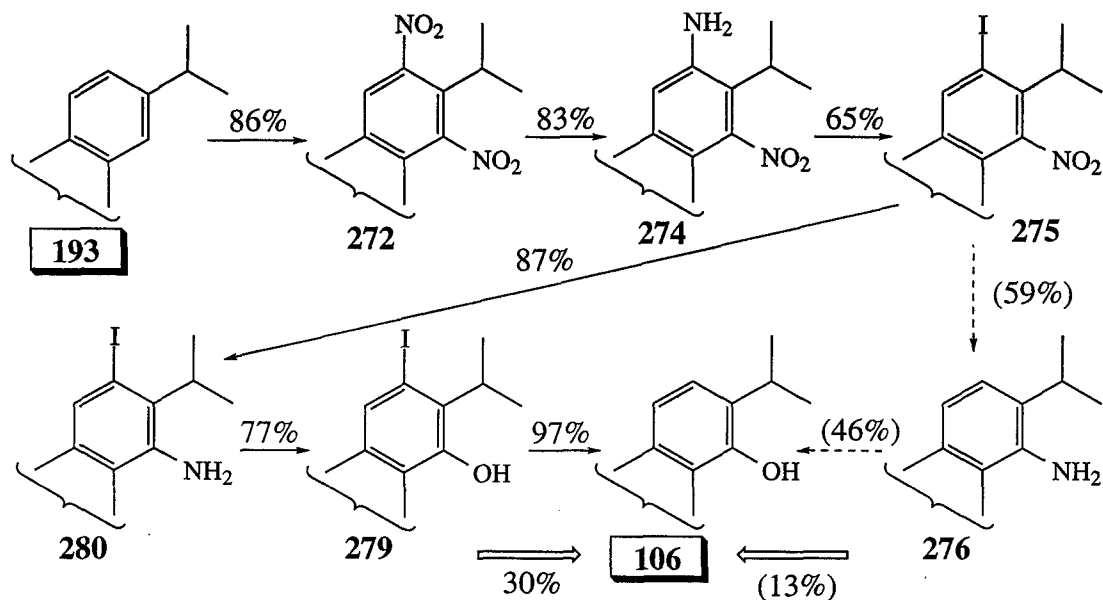
Scheme 3.21   Overall Yields for the Synthesis of **106** from **193**

3.2.4 Synthesis of Demethyl Isoneotriptophenolide (DINTP, 288)
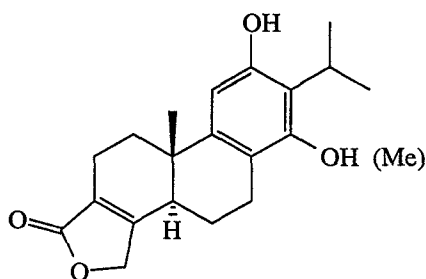
demethyl isoneotriptophenolide (DINTP) (**288**)

In planing the synthetic strategy to **288**, it was noted that the synthesis of methyl 12,14-dihydroxy-dehydroabietate (**290**) by diazotization of the corresponding 12,14-diamine **289** was reported unsuccessful.[238] Consequently, the appropriate 12,14-diamino intermediate which could be made available by reduction of the 12,14-dinitro compound **272**, did not appear attractive.

NH2

diazotization

X

OH

OH

CO2Me

CO2Me

289   **290**
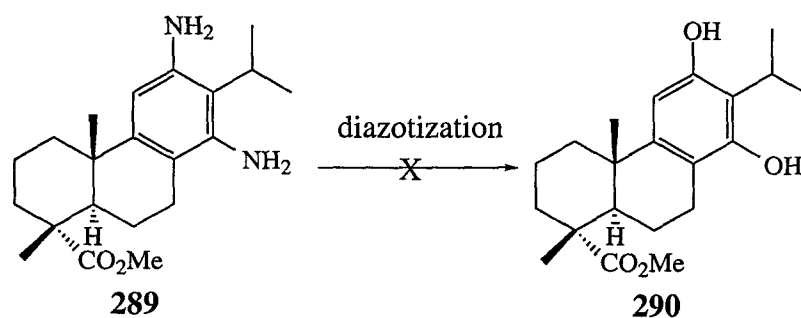
It is known that phenols can be oxidized by lead tetraacetate (LTA) to *ortho*-and *para*-quinol acetates (acetoxycyclohexadienones),[239,240] which then can be converted to resorcinol derivatives by a dienone-phenol rearrangement and subsequent hydrolysis.[241-243] By using this strategy, M. Shimagaki *et al.* synthesized methyl 12,14-dihydroxy-dehydroabietate (**290**) from methyl 12-hydroxy-dehydroabietate (**291**).[238] Preliminary results* showed that this reaction was also applicable to our synthesized isotriptophenolide (**194**), so studies along this route were pursued.

OH

OH

CO2Me

O

O

291   **194**
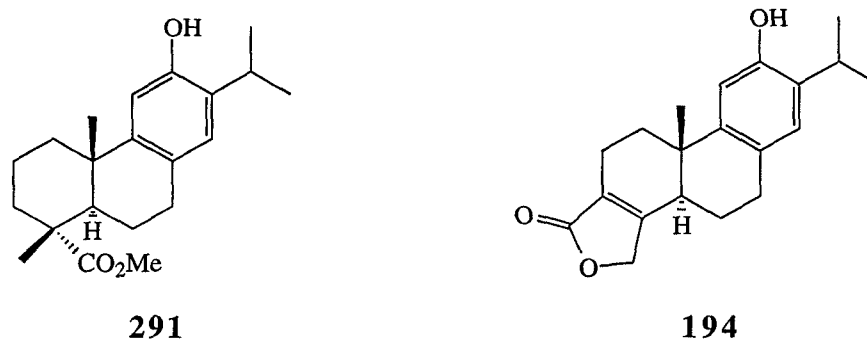
* F. Kuri-Brena of this group had done some preliminary work in reactions of **194** with LTA and subsequent rearrangement.

Isotriptophenolide (**194**) was treated with LTA (1.1 equiv.) in acetic acid solution at room temperature.[239,244] The resulting crude product (*para-* and *ortho*-quinol acetates, **292** and **293**) was not purified since both isomers should rearrange to the same product[238] (Scheme 3.22). Thus the crude product was dissolved in trifluoroacetic acid anhydride (TFAA) and stirred at room temperature for 1 day.[238] The resulting product was chromatographed to give 14-DINTP acetate **294** in 49% yield. Another trial was run under similar conditions but using 2 equivalents of LTA, and the rearrangement step was continued for about 42 h. Chromatography of the crude product afforded **294** in 54% yield. It was apparent that increases in amount of LTA and reaction time provided only a slightly improvement in the yield.
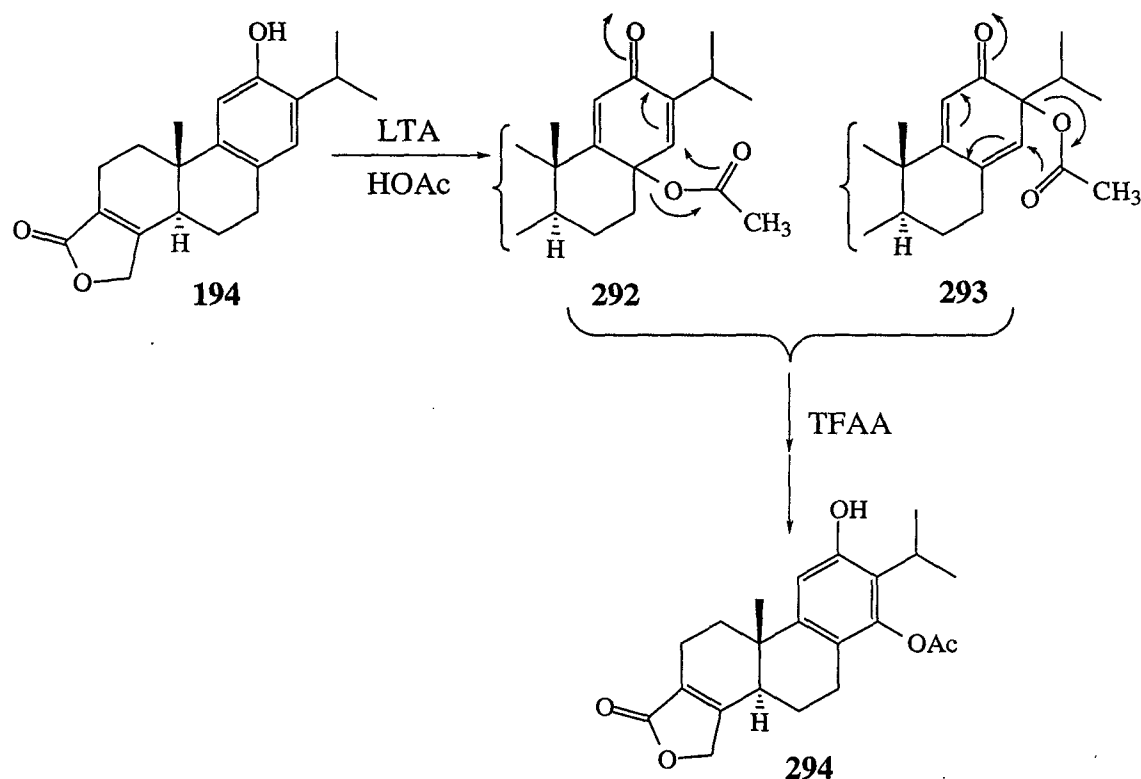
Scheme 3.22   Oxidation of **194** with LTA and Subsequent Dienone-Phenol Rearrangement

The IR spectrum of compound **294** showed a hydroxyl group at 3400 cm⁻¹ and the acetate at 1740 (broad, overlapping with the absorption from the butenolide moiety) and 1220

cm^{-1}. The ^{1}H NMR spectrum displayed the H11 signal at δ 6.66 and the acetate methyl group at δ 2.32.

Hydrolysis of the acetate provided some difficulty. The hydrolysis conditions used in the preliminary experiments[238] failed to achieve removal of the acetate function. Reflux of the acetate **294** with moderate amounts of concentrated sulfuric acid in methanol were similarly unsuccessful. Finally, the hydrolysis was achieved in an aqueous sulfuric acid solution under reflux temperature. The yield of crude DINTP (**288**, one spot on TLC) was essentially quantitative.

DINTP (**288**) was recrystallized in ethyl acetate and dichloromethane to give colorless prisms (mp 198-199°C). Its IR spectrum showed hydroxyl group absorption at 3400 cm^{-1}. The ^{1}H NMR displayed the H11 (NOE effect with H1β) signal at quite high field (δ 6.36), as compared with isotriptophenolide (**194**) and triptophenolide (**106**), but the H15 resonance was remarkably shifted downfield to about δ 3.4 due to the adjacent two hydroxyl groups.
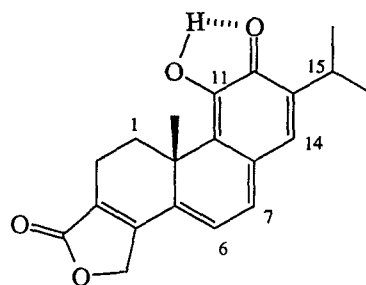
295

One by-product **295** was isolated as a red powder (mp 174-175°C) with a molecular formula of $C_{20}H_{20}O_4$. Its IR spectrum showed a hydroxyl group at 3300 cm^{-1} and a conjugated enone at 1640, 1590 and 1560 cm^{-1}. The ^{1}H NMR spectrum was similar to that of **250**, but the H1β signal was shifted downfield to δ 3.51 (1H, ddd, J = 13.6, 4.5, 2.4 Hz), suggesting that there was a group at C11.[183] At the low field, there were four signals, one proton at δ 7.68 (1H, br s) was exchangeable with D_2O, indicating it was a hydroxyl proton. Irradiation of this proton resulted in an enhancement of the H1β signal, suggesting that the hydroxyl group was at C11.

Irradiation of the signal at δ 6.93 (1H, s, H14) enhanced resonances of H15 (δ 3.14, septet, J = 6.9 Hz), the two isopropyl methyls and a proton at δ 6.69 (1H, d, J = 6.6 Hz, H7). The H7 proton was coupled with a proton at δ 6.29 (1H, d, J = 6.6 Hz, H6). Therefore, the structure of this compound was determined as **295**. This compound has a similar structure as the antimalarial principles **295-A** and **295-B** isolated from an African plant *Hoslundia opposita* Vahl. Our [1]H NMR data were comparable with those relevant in the literature.[245] A possible mechanism for the formation of **295** is presented in Scheme 3.23.

295-A R = benzoyl
295-B R = cinnamoyl
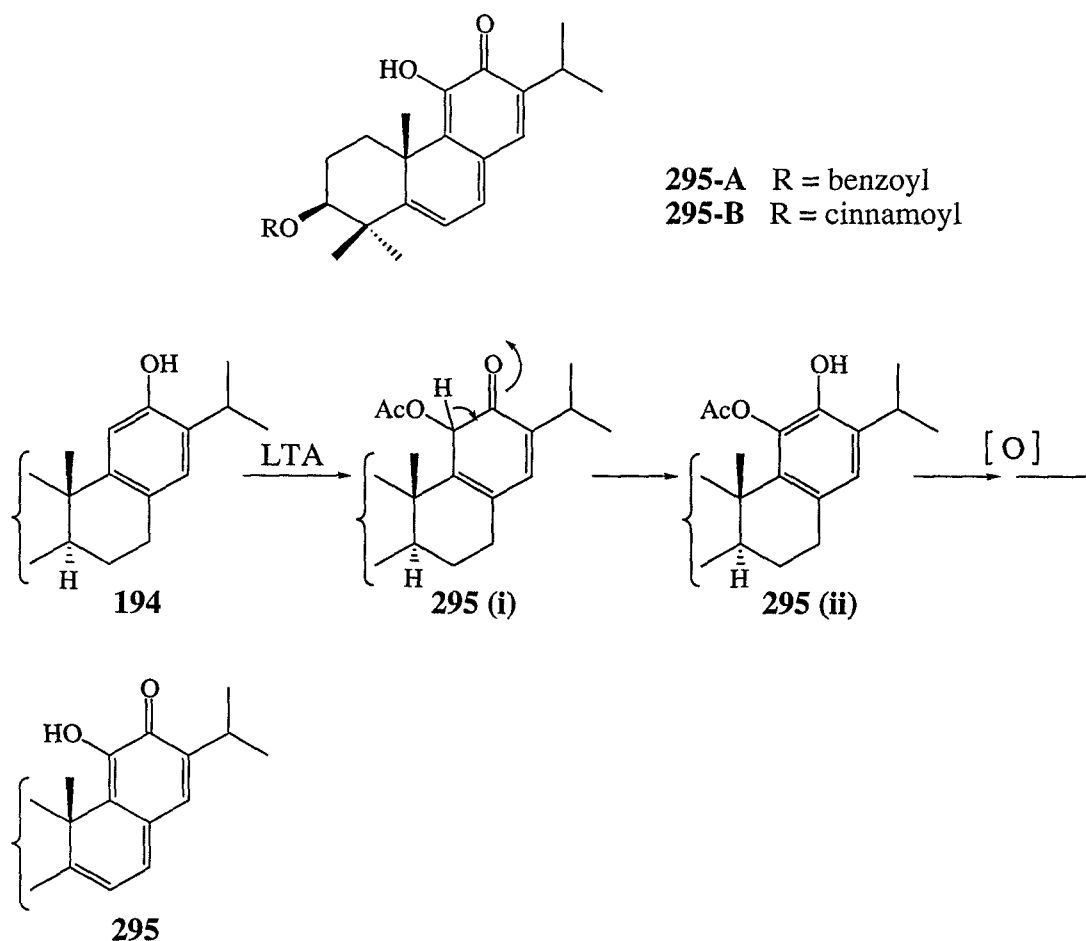
Scheme 3.23   Proposed Mechanism for the Formation of **295**

A series of experiments were conducted to examine whether the yield of DINTP could be improved (Table 3.1). The results showed that both acetic acid and chloroform could be used as

solvents in the oxidation step. The amount of LTA could range from 2.2 to 1.5 equivalents without significantly changing the yield. In the rearrangement step, zinc chloride seemed to have little effect on the yield.[243] Since chloroform can be used at lower temperature, and the workup conditions are less complex, this solvent is preferable to acetic acid.

Table 3.1   Yields for Conversion of **194** to **288**

| entry | Oxidation | | | | Rearrangement | | | Hydrolysis | |
	194 (mg)	solvent (35 mL)	LTA (equiv.)	time (min)	solvent (mL)	$ZnCl_2$ (mg)	time (h)	time (h)	overall yield (%)
1	500	HOAc	2	20	TFAA (8)	-	48	4	53
2	500	HOAc	2	10	TFAA (8)	-	48	4	54
3	500	HOAc	1.5	20	TFAA (6)	50	50.5	4	56
4	500	$CHCl_3$	2.2	30	TFAA (5.6)[a]	50	50	4	56

[a] additional 0.8 mL TFA was added.

Because the overall yield of **288** from isotriptophenolide (**194**) was about 56% (three steps), it was acceptable for our purpose and no further studies were pursued.

CHAPTER 4   BIOTRANSFORMATION STUDIES

4.1   Introduction

In the study of diterpene metabolites from the plant cell cultures of *T. wilfordii*, a biosynthetic pathway from dehydroabietane (**196**) to Tl (**1**) and Td (**2**) was proposed (*vide supra*). In order to provide further information relating to possible biosynthetic intermediates and simultaneously, provide a family of novel diterpene analogs for pharmacological screening, a series of biotransformation experiments with the compounds synthesized in the previous chapter were pursued.

Generally speaking, a biotransformation refers to the conversion of a given substrate into another product by biological means such as the use of micro-organisms, cultures of animal and plant cells, or purified and partially purified enzymes.[246,247] In the context of organic synthesis, biotransformations are reactions which utilize these biological catalysts.[248,249] Substrates are not limited to those compounds which are normally present in the living organism (the biosynthetic intermediates of secondary metabolism) but instead may be "foreign" compounds which could undergo chemical alternations.[248] The role of biotransformation in organic synthesis is one of support rather than supplantation. Biotransformations should be employed to their advantages when a given reaction step is not easily accomplished by "ordinary" chemical methods.[250] The best results are obtained with the combination of these methods.[248]

In initial biotransformation studies with TRP4a cell cultures, interest was focused on the formation of the butenolide moiety in the Tl (**1**) or Td (**2**) skeleton. Preliminary biotransformation studies examined radio-labeled dehydroabietic acid (**157**) and the hydroxy ester **182**,[159] as well as unlabeled synthetic precursors **210**, **213(i)** and **296-300**.[192]
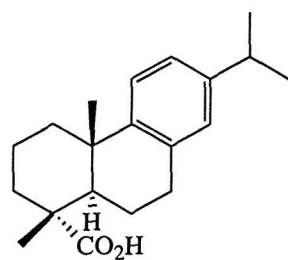
157
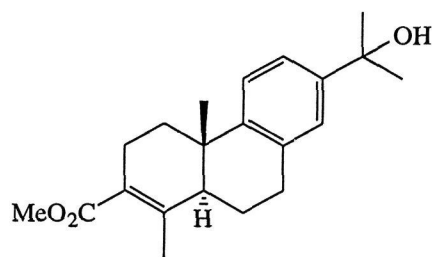
182
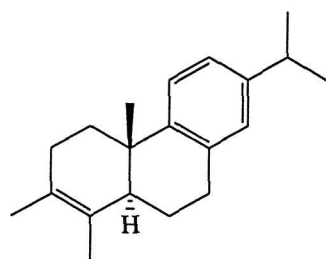
213(i)
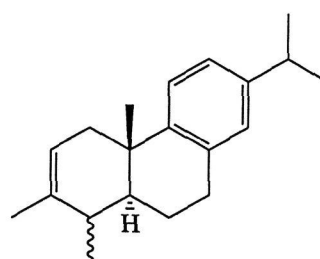
296
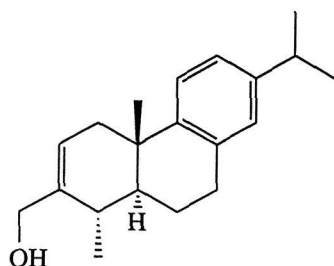
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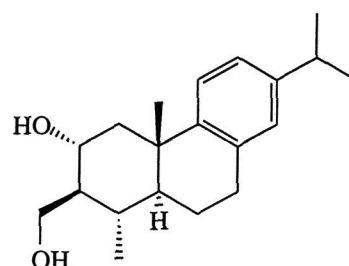
298
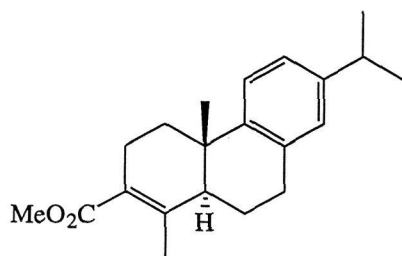
210
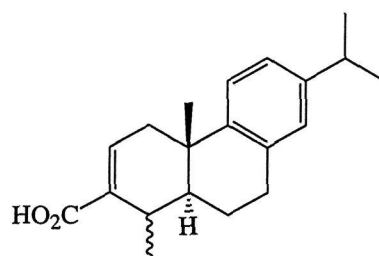
299
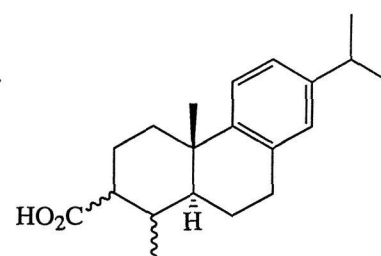
300

Labeled **297**, an allylic alcohol, was shown to be converted to the corresponding aldehyde **301** and acid **299a**[160] (Scheme 4.1). This result is in agreement with the proposed mechanism for the formation of the butenolide moiety in the molecule (section 2.2).
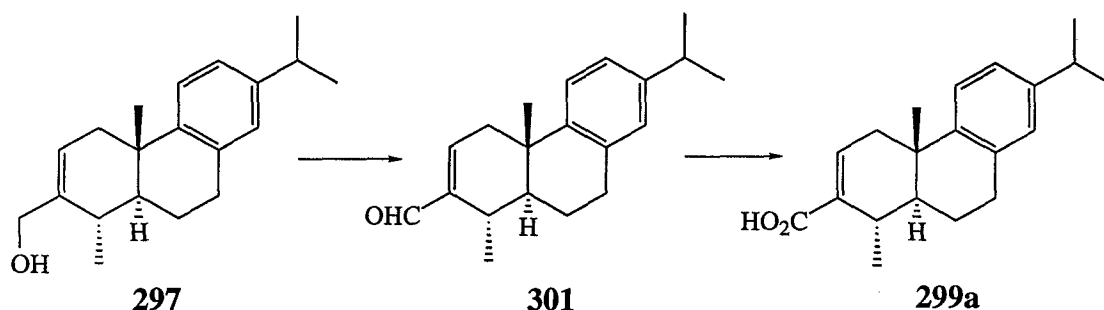
Scheme 4.1   Biotransformation of Allylic Alcohol **297** with TRP4a Cell Cultures

Administration of synthetic isodehydroabietenolide (**193**) as a precursor, one in which the butenolide ring system is intact, showed that TRP4a cell cultures was able to convert this compound to certain hydroxylated compounds; however, no epoxide formation was observed (Scheme 4.2).[160] At the time of the above study, the natural occurrence of **193** and **206** in the cell cultures was unknown. Labeling experiments clearly demonstrated that the hydroxylated products were derived, at least in part, from the exogenous starting material. Biotransformations of the ring C hydroxylated precursor isotriptophenolide (**194**) by TRP4a cell cultures gave only small amounts of one product, the methyl ether of the starting material (Scheme 4.3).[161]

The lack of an obvious biotransformation of **194** contradicted the idea that a ring C activated precursor could be transformed by the cells. However, there were still many opportunities for progress. The work included in this thesis was part of an effort to validate this hypothesis.

In the present biotransformation studies, attention was paid to selection of appropriate experimental conditions and to examination of the relevant precursors. In the studies with the plant cell cultures of *T. wilfordii*, our focus was directed at possible yield improvement of the diterpene triepoxides Tl (**1**) and Td (**2**) by incubation of appropriate synthetic precursors, and also at synthesis of new epoxide analogs (through biotransformations). Therefore, the proposed biosynthetic intermediate, triptophenolide (**106**), and related precursors were used as substrates.

Of the substrates prepared, triptophenolide (**106**) and the 7-oxo-butenolide **206** have been isolated from TRP4a cell cultures, the others are "foreign" precursors.
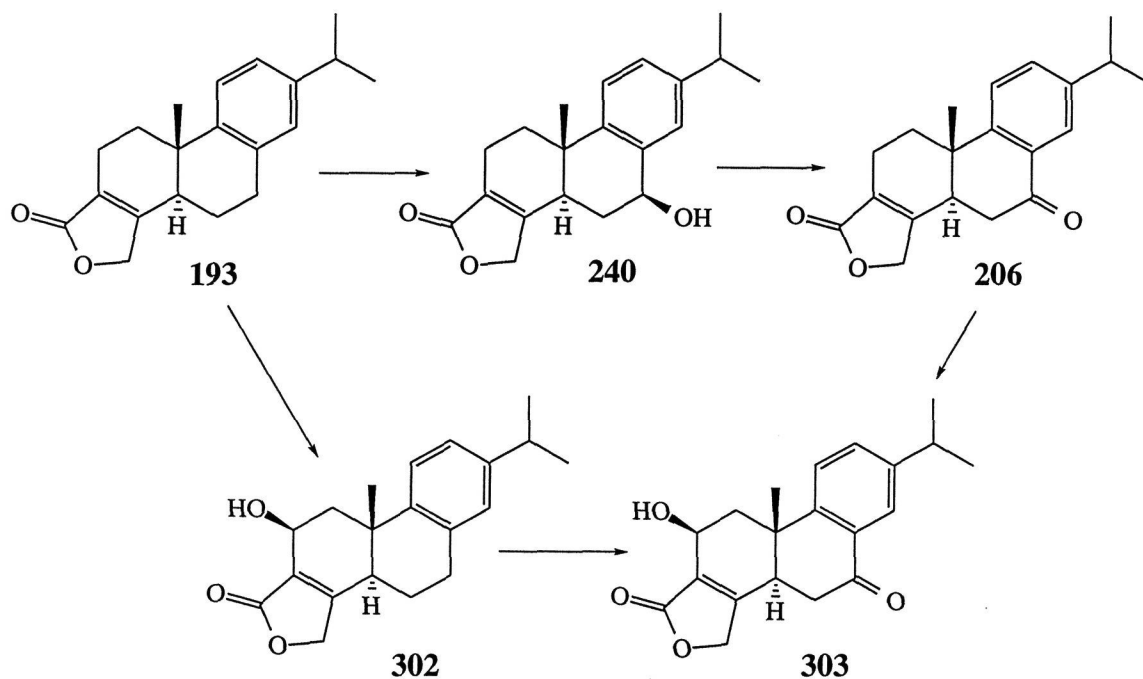
Scheme 4.2   Biotransformation of the Butenolide **193** with TRP4a Cell Cultures
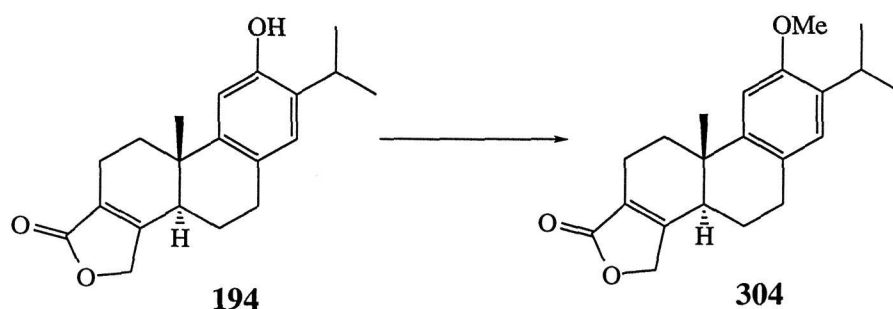
Scheme 4.3   Biotransformation of Isotriptophenolide (**194**) with TRP4a Cell Cultures

Cell cultures were grown and maintained under the conditions described in Chapter 2. Cell cultures grown in $MSNA_{0.5}K_{0.5}$ medium were used in biotransformation studies. In general, the precursor was dissolved in a certain amount of ethanol, added to the culture, and

culture incubation continued under standard conditions. Samples were extracted and subjected to column chromatography to isolate the products. In time course studies, samples were taken at certain intervals and analyzed by TLC and/or high performance liquid chromatography (HPLC).

4.2 Results and Discussion

4.2.1 Biotransformation of 7-Oxo and 7β-Hydroxy-isodehydroabietenolide with TRP4a Cell Cultures

Biotransformation of isodehydroabietenolide (**193**) with TRP4a cell cultures produced hydroxylated compounds and ketones. In order to examine whether some of these compounds could be further transformed, 7-oxo-isodehydroabietenolide (**206**) and 7β-hydroxy-isodehydroabietenolide (**240**) were incubated with TRP4a cell cultures.

The starting material **206** (100 mg) was dissolved in ethanol (8 mL) and added to a 14-day-old culture (2 x 550 mL). A control experiment (4 mL ethanol added in 550 mL culture), and a blank experiment (50 mg starting material in 4 mL ethanol added to 550 mL sterile medium (no cells) were also conducted under the same conditions. Samples were taken after incubation for 1, 4 and 6 days, and the culture was harvested after 7 days. The samples were extracted and then analyzed by TLC. Results showed that there were no significant changes to the starting material as well as Tl (**1**) and Td (**2**) in the test samples. The biotransformation was repeated under the same conditions except that 50 mg of starting material was dissolved in 50 mL of ethanol and incubated in 550 mL of the cultures. Samples taken after incubation for 1, 5, 7 days also showed no reaction.

7β-Hydroxy-isodehydroabietenolide (**240**) was also evaluated. The starting material (80 mg) in ethanol (4 mL) was added to a 14-day-old culture (550 mL). A control and a blank experiments were also run under the same conditions. Samples taken after 3 days of incubation

showed a spot corresponding to **206** on TLC and some remaining starting material. After the culture was harvested at 7 days, the starting material was almost completely oxidized to ketone **206**. The blank sample showed little reaction; therefore, the oxidation of **240** to **206** was caused by the enzymes in the cells, not simply by atmospheric oxygen (Scheme 4.4).
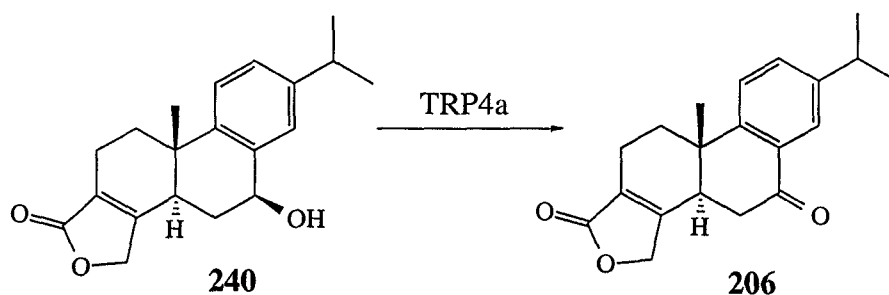
Scheme 4.4   Biotransformation of Alcohol **240** with TRP4a Cell Cultures

The results from biotransformation of **240** and **206** suggested that cell-produced 7-oxo compounds, such as **199, 211, 206** and **108** are likely to be formed in a similar manner, *i.e.*, hydroxylation and further oxidation. However, experiments to confirm this speculation were not performed.

These experiments, as a supplement to the early investigation by M. Roberts,[160] completed the biotransformation of the isodehydroabietenolide (**193**) series. These results demonstrated again the need for the introduction of activating groups in ring C.

4.2.2   Biotransformation of Isotriptophenolide (194) and Related Compounds with TRP4a Cell Cultures

Biotransformation of 194 with Older TRP4a Cell Cultures (Trp#300a)

Previous biotransformation studies with isotriptophenolide (**194**) in TRP4a cell cultures used a 7-day-old culture and a 7 day incubation.[161] Earlier studies had showed that Td (**2**) and

Tl (**1**) were produced during late growth phase.[147] This indicates that the enzymes responsible

for the biosynthesis of Tl (**1**) and Td (**2**) may only be present or active in older cells. Therefore,

biotransformations with older cells were studied.

Isotriptophenolide (**194**, 46.5 mg) dissolved in ethanol (1 mL) was added to a 21-day-

old TRP4a cell culture (550 mL). Samples were taken after incubation for 1, 2 and 4 days,

respectively. The samples were extracted and then analyzed by TLC (toluene-chloroform-ethyl

acetate-formic acid, 105:48:45:3, developed twice). The results showed that the starting

material (R_f 0.53) remained in substantial quantities, although a less polar spot (R_f 0.71)

appeared after 1 day, and a polar spot (R_f 0.24) appeared after 2 days of incubation. No

significant changes to Tl (**1**) and Td (**2**) were observed. The culture was harvested after 5 days

and the cells and broth were extracted. Column chromatography of the cell and broth extracts

gave isotriptophenolide methyl ether (**304**, 3 mg) and the starting material (**194**, 17 mg).

Attempt of isolation of the polar spot failed due to decomposition on the column (very likely

the polar compound was 7-hydroxy-isotriptophenolide, *vide infra*). Again, with the exception

of the possible formation of 7-hydroxy-isotriptophenolide, biotransformation of

isotriptophenolide (**194**) with an older cell culture gave no new products other than the methyl

ether **304**.

304
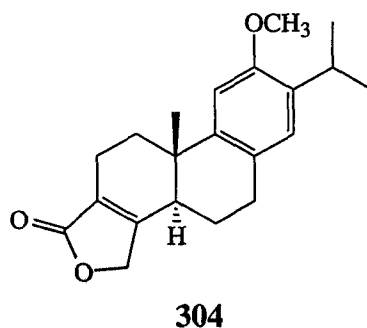
Compound **304** was isolated as a white powder with a molecular formula of $C_{21}H_{26}O_3$.

The [1]H NMR spectrum was identical to that of the authentic compound previously obtained.

Increase of Starting Material (194) Solubility and Isolation of New Products (Trp#301)

There are various factors which may result in a lack of incorporation or biotransformation of exogenous precursors: the specificity of the enzyme system, the impermeability of the cellular membrane towards the exogenous material, or an induced alteration or destruction of the substrate before reaching the active sites.[184] One of the factors which might have played a role in the previous experiments was that the starting material was not highly soluble in the cell culture medium and thus may not pass through the cell membrane.

To increase the solubility of the starting material, the amount of the solvent (ethanol) used to dissolve the starting material was increased from 0.2% to 4.5% with respect to the total volume of the culture. Thus, isotriptophenolide (**194**, 100 mg) was dissolved in ethanol (50 mL) and added evenly to two flasks of TRP4a cell cultures (21 days old, 550 mL each). Samples were taken after incubation for 26 h (Sample 1) and 72 h (Sample 2), and were subsequently extracted with ethyl acetate. TLC (toluene-chloroform-ethyl acetate-formic acid, 105:48:45:3, developed twice) showed that Sample 1 contained a considerable amount of starting material (R_f 0.57), and that the methyl ether was hardly detectable. A new, polar, UV active spot (R_f 0.41) was detected in a small amount by TLC. Another polar spot (R_f 0.22), which had been found in Trp#300a, was also present. Sample 2 showed increased amounts of the polar spots (R_f 0.41 and R_f 0.22) and much residual starting material. No significant changes were observed in the amounts of Tl (**1**) and Td (**2**) as determined by TLC.

The culture was harvested after incubation for 96 h, and the cells and the broth were extracted to yield a cell extract (257.9 mg) and a broth extract (162.4 mg). Both extracts were subjected to repeated column chromatography (Figure 4.1), yielding 8 products along with recovered starting material (Table 4.1). The total recovery of material was about 89%.

Table 4.1   Compounds Isolated from Biotransformation of **194** with TRP4a Cell Cultures (Trp#301)

Compound	194[a]	246	305 + 306[b]	309	245	308	307	304
mg	58.6	8.9	15.2	2.6	1.6	1.6	0.7	0.6
%[c]	-	20	35	6	4	4	1.5	1

[a] Recovered starting material; [b] Mixture of **305** and **306** (7.9 mg) plus **305** (1.0 mg) and **306** (6.3 mg); [c] Based on recovered starting material.

Compound **307** had a molecular formula of $C_{22}H_{28}O_4$. The ^1H NMR spectrum showed that it was actually a mixture of two isomers, 7α-ethoxy (**307a**) and 7β-ethoxy-isotriptophenolide (**307b**), in a ratio of approximately 4 to 1. For the α isomer, the H11 and H14 signals were located at δ 6.70 and 7.11, respectively, whereas for the β isomer, these two signals were at δ 6.68 and 7.29, respectively. As in 7-hydroxy-isodehydroabietenolide (see discussion in the synthesis of **246b**), the H14 resonance of the β isomer was found at lower field than that of the α isomer. The H7β signal of **307a** was located at δ 4.38 (br dd, J = 4.2, 1.6 Hz), at a higher field than that in **307b**. The methylene protons of the ethoxy group appeared around δ 3.53-3.37 and the methyl group at δ 1.24. In agreement with the assignment, irradiation of the 7β proton resulted in signal enhancements of H14, H6 and the methylene protons of the ethoxy group. The two isomers were not readily separable by column chromatography and they decomposed rapidly; therefore, further separation was not attempted

307a   7α-ethoxy

307b   7β-ethoxy

Broth Extract (162.4 mg)

c. c.
hexanes-EtOAc
(6:4); EtOAc

I   II   III   IV   V   VI   VII

c. c.
CH$_2$Cl$_2$-
EtOAc
(9:1)

c. c.
CH$_2$Cl$_2$-
EtOAc
(8:2)

c. c.
CH$_2$Cl$_2$-EtOAc
(8:2)

194   **307**   **309**   c. c.   **305**   c. c.
(2.0 mg)   (0.7 mg)   (2.6 mg)   CH$_2$Cl$_2$-   1.0 mg   hexanes-EtOAc
                                    EtOAc   (6:4)
                                    (90:5)

306   **245**   **308**   **246**
(6.3 mg)   (1.6 mg)   (1.6 mg)   (0.8 mg)

Cell Extract (257.9 mg)

c. c.
hexanes-EtOAc
(6:4); EtOAc

I + VIII   IV + IX   VI + X   VIB + VII

c. c.   c. c.   c. c.   c. c.
CH$_2$Cl$_2$-   CH$_2$Cl$_2$-   hexanes-   hexanes-
EtOAc   EtOAc   EtOAc   EtOAc
(90:5)   (90:5)   (5:5)   (5:5)

304   **194**   **305+306**   **246**   VIB   **246**
(0.6 mg)   (56.6 mg)   (7.9 mg)   (4.5 mg)   (3.6 mg)

Figure 4.1   Column Chromatographic Separation of Extracts from TRP#301
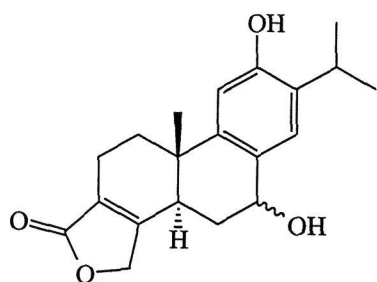
246a 7α-OH
246b 7β-OH
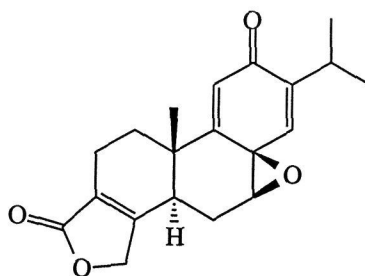
305
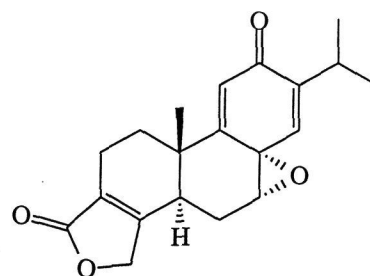
306

309

308

245
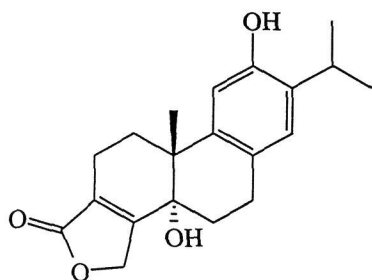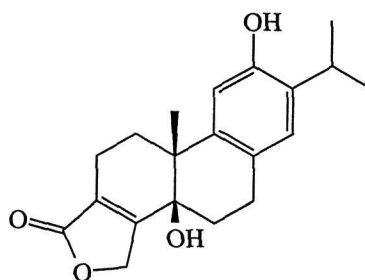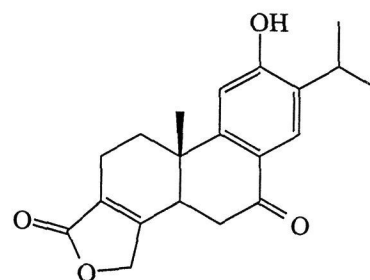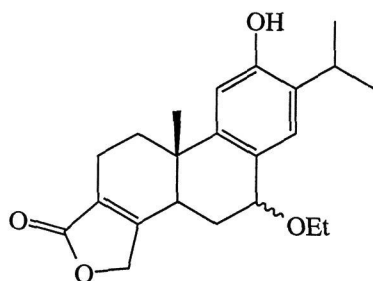
307a 7α-OEt
307b 7β-OEt
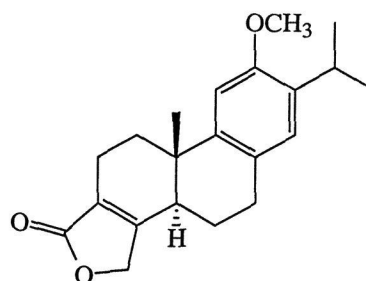
304

Compound **246** was isolated as a mixture of 7α-hydroxy (**246a**) and 7β-hydroxy-isotriptophenolide (**246b**), with **246a** as the major component. The two sets of ¹H NMR signals were identical with those obtained from the synthetic compounds (section 3.2.2). As mentioned earlier, since these compounds were readily decomposed, further separation of the two isomers was not performed.

309
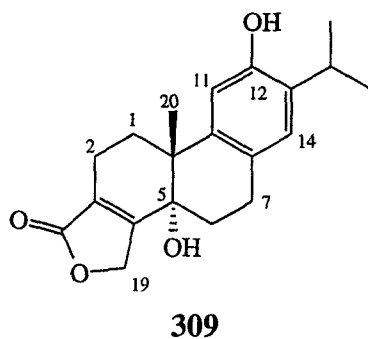
Compound **309** was isolated as an optically active ($[\alpha]_D^{23}$ -37.9°, c = 0.070, CHCl₃),

white crystalline solid (mp 190-192°C) with a molecular formula of $C_{20}H_{24}O_4$. One additional

oxygen atom in the molecule suggested a new hydroxyl group. The IR spectrum showed bands

for the hydroxyl group at 3650 and 1040 cm⁻¹. Its ¹H NMR spectrum displayed some

similarity to that of isotriptophenolide (**194**). The C19 proton signals were slightly shifted

downfield to δ 4.89 (2H, br AB$_q$, Δv = 0.18 ppm, J = 17.1 Hz) with wider splitting (Δv) of the

AB quartet. As mentioned in previous chapter, this usually meant a change at C5, for example,

hydroxylation or formation of a double bond. The phenolic hydroxyl proton signal was found

at δ 4.78, which was exchangeable with D_2O. The C7 protons at δ 3.01 (2H, m) were coupled

with a proton at δ 1.97 (1H, m) and another in the multiplet between δ 2.04 and 2.26 (3H),

indicating they were C6 protons. Protons at δ 2.36 (1H, m) and 2.54 (1H, br d, J = 17.3 Hz)

were coupled with each other, and both had cross peaks with C19 protons, indicating that they

were C2 protons. Irradiation of H11 enhanced signals of the C12 hydroxyl proton at δ 4.78, of

another hydroxyl proton at δ 1.90 (D_2O exchangeable), and of H1β centered at δ 2.19 as part of

the three-proton multiplet (one was H6, *vide supra*) between δ 2.04 and 2.26. The two C2

protons were coupled with H1β, and with the H1α centered at δ 2.12 which was the third

proton in the H1β, H6 multiplet. No C5 proton was found in the spectrum; therefore, the new

hydroxyl group must be at C5. As the C20 methyl protons (δ 1.08) were still at higher field

than the isopropyl methyl groups (about δ 1.23), the A and B rings were believed to be in *trans*

junction, in other words, the hydroxyl group at C5 was α oriented. This assignment was

supported by the downfield shift of the H1α signal to δ 2.12 from 1.68 as compared with its

counterpart in the starting material **194** due to the 1,3-diaxial relation. The ^{13}C NMR spectrum showed the C5 carbon signal at δ 70.1. The mass spectrum exhibited the molecular ion peak at m/z 328, an M - H_2O fragment at 310 and an M - H_2O - CH_3 fragment at 295, respectively. All these data were consistent with the structure assigned to **309**.

308
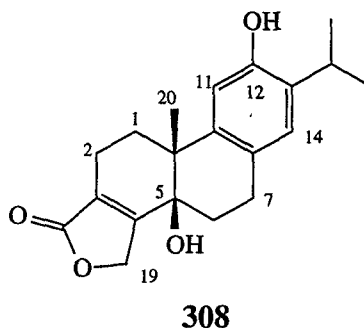
Compound **308** was obtained as an optically active ($[\alpha]_D^{24}$ = +171.9°, c = 0.281, $CHCl_3$), pale yellow powder (mp 92-95°C). The molecular formula was determined as $C_{20}H_{24}O_4$. Its IR spectrum showed absorption bands for hydroxyl groups at 3600 cm^{-1} (sharp, phenolic hydroxyl group) and 3470 cm^{-1} (broad, alkyl hydroxyl group). The ^1H NMR spectrum exhibited the C19 protons at δ 4.90 (2H, br AB_q, Δν = 0.1 ppm, J = 12 Hz), which also showed a wider split between the AB quartet. All the other proton signals were found except the H5 signal. Furthermore, the C20 methyl signal (δ 1.32) was at lower field than the isopropyl methyl signals (δ 1.21). These data suggested that the new hydroxyl group was at C5, but in a β orientation, thus resulting in an A/B cis junction. Irradiation of the hydroxyl proton signal at δ 2.30 resulted in an enhancement of C20 methyl signal and thereby confirmed that the hydroxyl at C5 was β oriented. All the remaining protons were assigned according to COSY and NOE experimental results.

The mixture of **305** and **306** was repeatedly chromatographed on preparative TLC with anhydrous ether to give pure **305** and **306**, respectively.

Compound **306** was isolated as optically active ($[\alpha]_D^{24}$ = +161.5°, c = 0.117, $CHCl_3$), colorless needles (mp 198-200°C, dec) with a molecular formula of $C_{20}H_{22}O_4$. The UV

spectrum displayed absorption maxims at 219.5 nm (log ε = 4.17) and 260.2 nm (log ε = 4.36). Its IR spectrum showed quinone type absorption bands at 1680 and 1650 cm^{-1} and a possible epoxide band at 920 cm^{-1}.
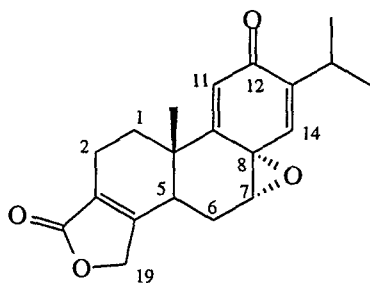
306

The ^{1}H NMR spectrum showed that **306** was still a butenolide type compound but structural changes were apparent. The two proton signals of ring C were shifted upwards to δ 6.48 (1H, s) and 5.99 (1H, s), respectively, and a new signal appeared at δ 3.82 (1H, d, J = 2.4 Hz) coupled with some aliphatic protons (COSY spectrum, Figure 4.2). Irradiation of the signal at δ 6.48 resulted in signal enhancements of H1β at δ 2.19 (1H, br dd, J = 13.3, 6.1 Hz) and of the C20 methyl group at δ 0.95, thereby indicating that the irradiated proton was H11 (Figure 4.3a). Irradiation of the proton at δ 5.99 showed NOEs to H15 at δ 2.95 (1H, septet d, J = 6.9, 0.9 Hz) and the two isopropyl methyl groups at 1.05 and 1.08 (3H each, both d, J = 6.9 Hz). Also enhanced was the new signal at δ 3.82, suggesting that the irradiated proton was H14 and the new signal was due to a proton at C7 (Figure 4.3b).

Compared with the spectra of Tl (**1**) and Td (**2**), the chemical shift and the appearance of the new signal from H7 was very similar to those signals from protons attached to the epoxide carbons of Tl (**1**) and Td (**2**). The H7 of **306** was coupled with a proton at δ 2.02 (1H, br dd, J = 14.4, 12.8 Hz) and a multiplet at δ 2.30 (2H), indicating that the two C6 protons were in these locations (Figure 4.2, d, e). Also coupled with the C6 protons was a signal at δ 3.23 (1H, br d, J = 12.8 Hz), which suggested that it was due to H5 (Figure 4.2, f, g).
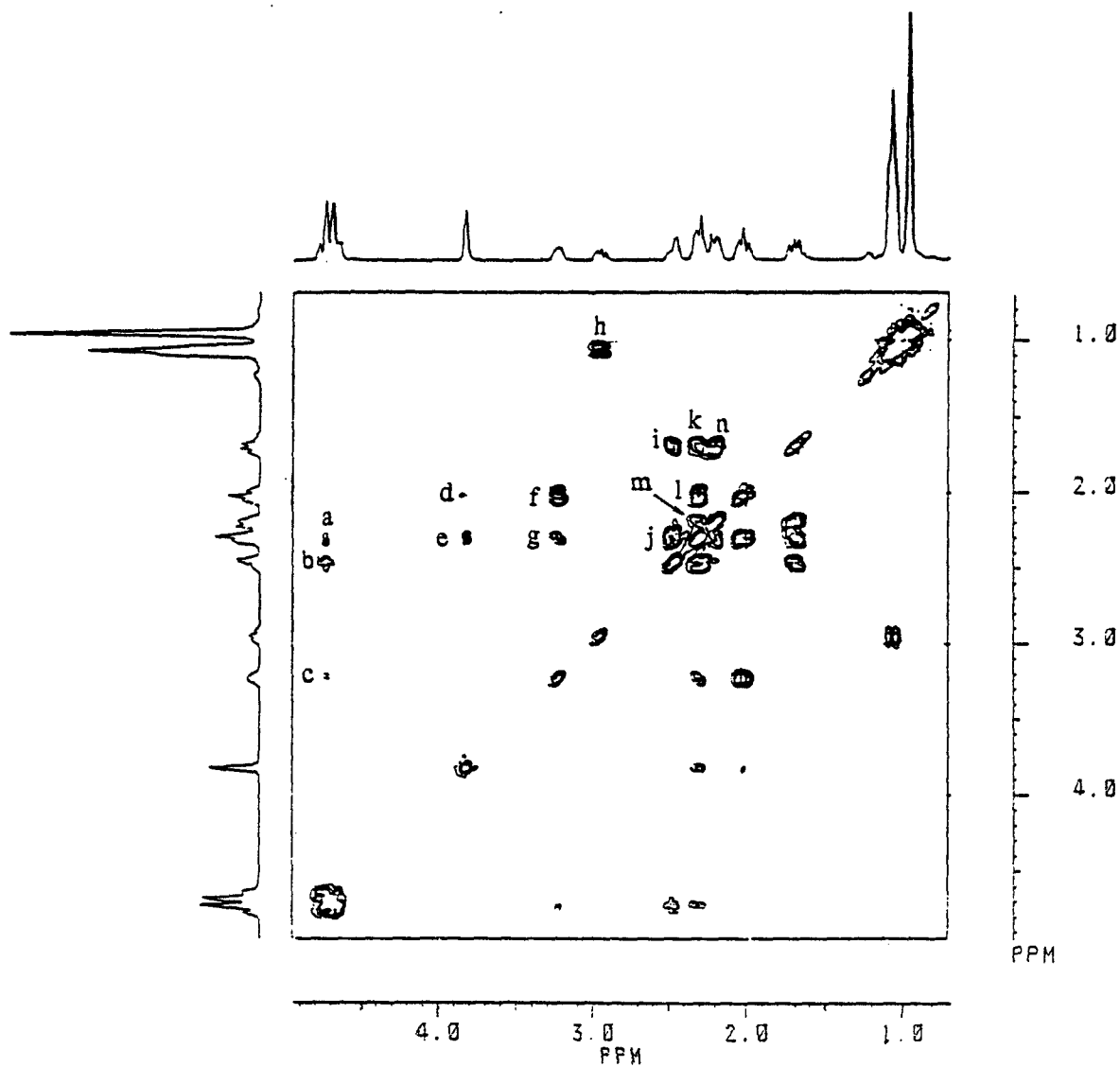
a: H19/H2β; b: H19/H2α; c: H19/H5; d: H7β/H6β; e: H7β/H6α; f: H5/H6β; g: H5/H6α;

h: H15/H16,H17; i: H2α/H1α; j: H2α/H2β, H1β; k: H2β/H1α; l: H6α/H6β; m: H2β/H1β; n: H1β/H1α

Figure 4.2   Expanded COSY Spectrum of Compound **306**
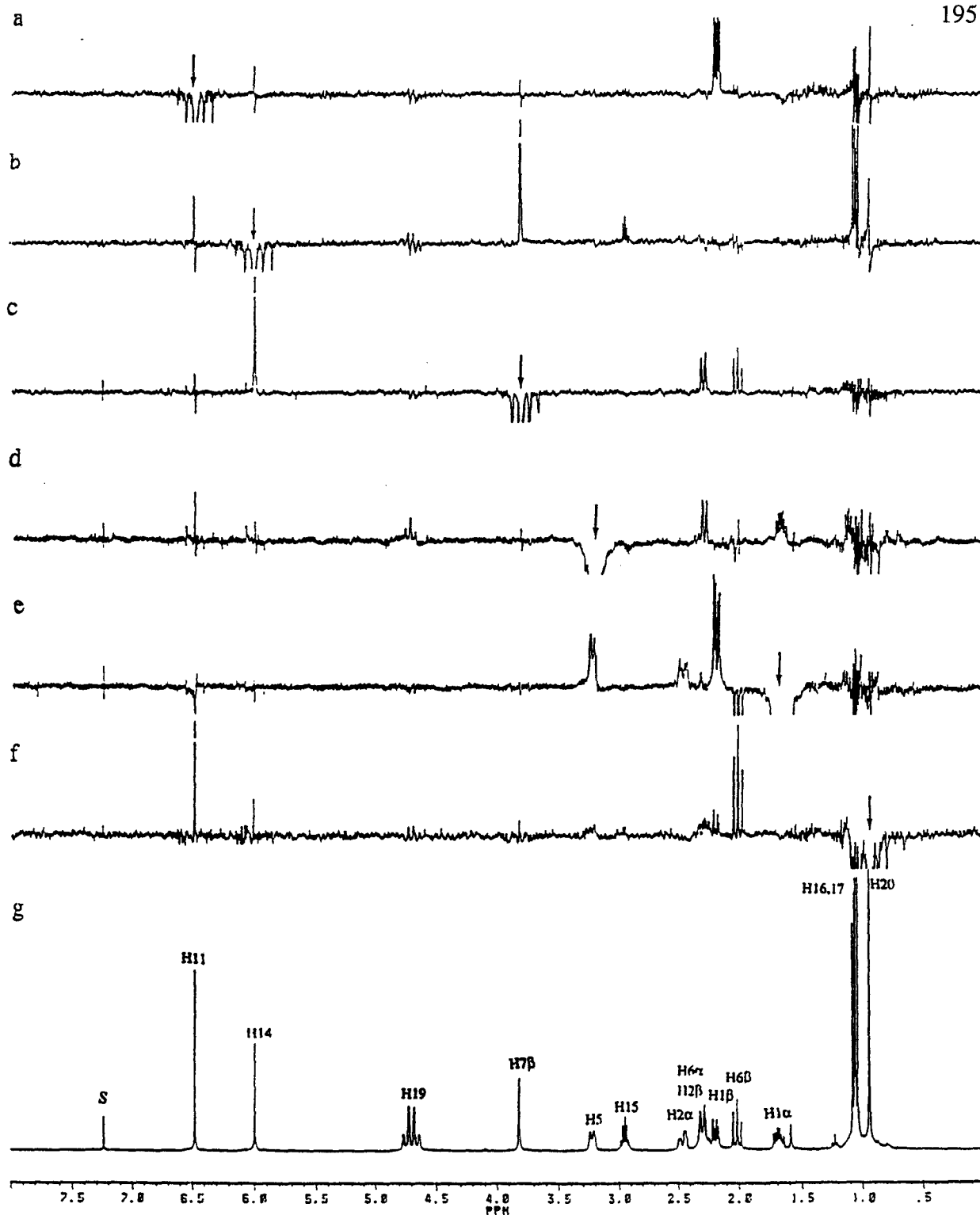
a, {H11}: H1β, H20; b, {H14}: H7β, H15, H16,H17; c, {H7β}: H14, H6α, H6β; d, {H5}: H19, H6α, H1α; e, {H1α}: H5, H2α, H1β; f, {H20}: H11, H2β, H1β, H6β; g, off-resonance spectrum (400 MHz, CDCl₃). ({irradiated}: enhanced)

Figure 4.3   NOE Difference Spectra of Compound **306**

The COSY spectrum also showed correlations between H19 signals (δ 4.70, 2H, br AB_q, Δv = 0.08 ppm, J = 17.1 Hz) and the H5 signal (Figure 4.2, c). Also coupled with H19 was a proton at δ 2.47 (1H, br d, J = 17.9 Hz) and another proton in the multiplet at δ 2.30 (2H, one was H6) (Figure 4.2, b, a). This indicated that the signal at δ 2.47 was one of the C2 protons, whereas the other was in the multiplet at δ 2.30. Irradiation of the C20 methyl group resulted in signal enhancements of H11, H1β, the H6 at δ 2.02 and the H2 in the multiplet at δ 2.30, which showed that the H6 at δ 2.02 and the H2 in the multiplet (δ 2.30) were β oriented (Figure 4.3f). The chemical shift of the C5 proton was at lower field as compared with other related compounds, which indicated that the epoxy group was α oriented and thus had an approximate 1,3-diaxial interaction with H5. Based on all the spectral information, this compound was identified as the (7,8)β-epoxy-dienone **306**.

Examination of the molecular model showed that the dihedral angles between H7β/H6α and H7β/H6β were of a similar magnitude (less than 90°) while that of H7β/H6α was slightly smaller (Figure 4.5). Therefore, H7β was coupled with both C6 protons but more strongly with H6α, which made the H7β signal appear as a broad doublet. Experience suggested that, if the 7,8-epoxy group was β oriented as in Tl (**1**) and Td (**2**), then the C19 protons would appear as a broad singlet. But in the spectrum of **306**, the C19 protons were still evident as an AB quartet, which supported the assignment of the epoxide to an α position.

All the signals of the [13]C NMR were assigned in accord with the results from HMBC and HMQC experiments. The carbon signals of C7 and C8 were found at δ 60.9 and 54.3, respectively. Finally, an X-ray analysis of the single crystals from a large scale experiment (*vide infra*) confirmed that the structural assignment was correct (Figure 4.4).
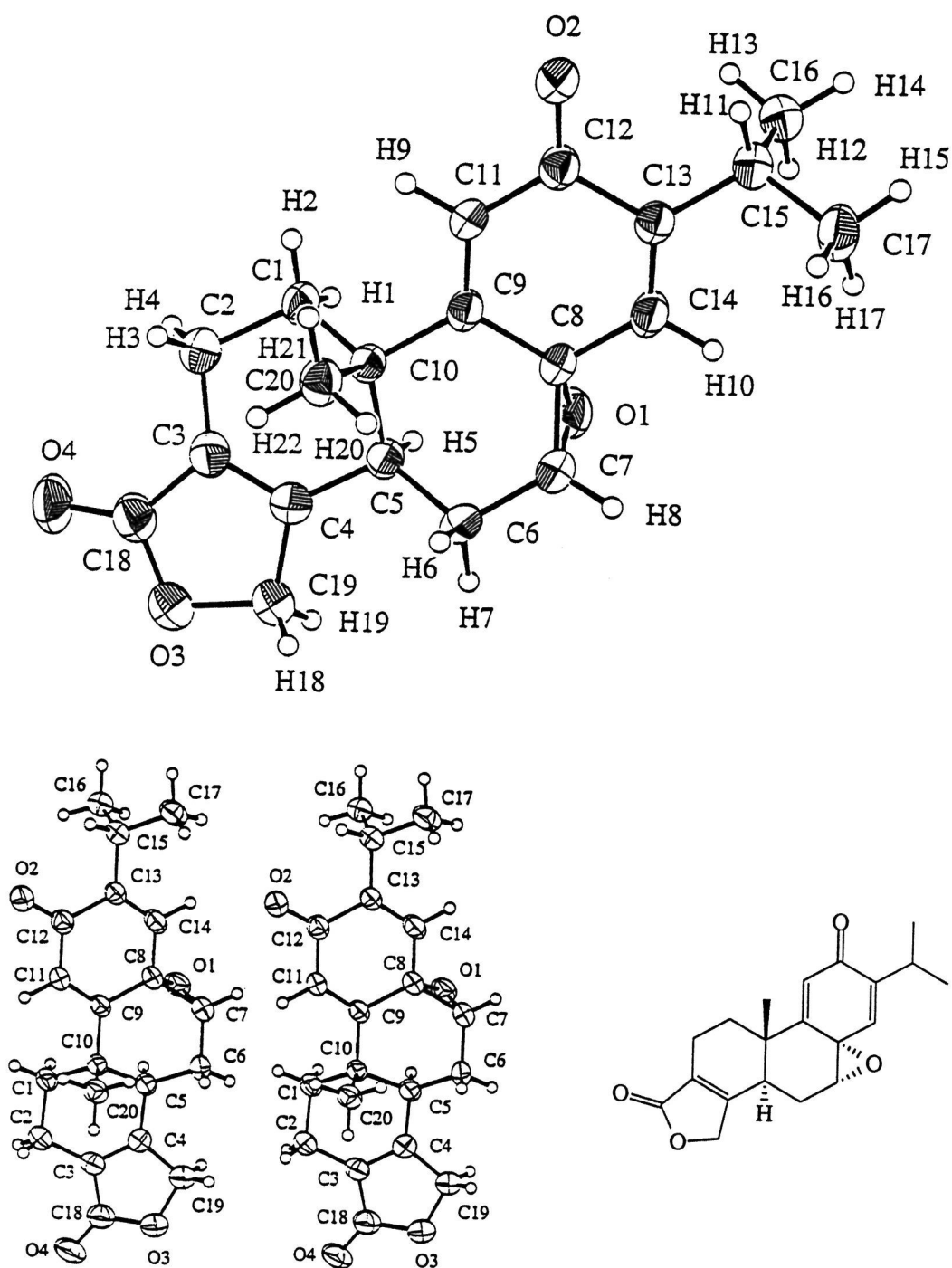
Figure 4.4   X-ray Structure of **306**

The resemblance between **305** and **306** in the ^{1}H NMR spectra suggested that **305** was probably the β isomer. Compound **305** was isolated as colorless plates (mp 230-232°C, dec) with a specific optical rotation of -295.5° ($[\alpha]_D^{24}$, c = 0.0670, CHCl$_3$). The molecular formula was determined as $C_{20}H_{22}O_4$, which was identical to that of **306**. Its IR spectrum also showed the quinone absorption bands at 1660 and 1640 cm^{-1} and an epoxide band at 910 cm^{-1}.
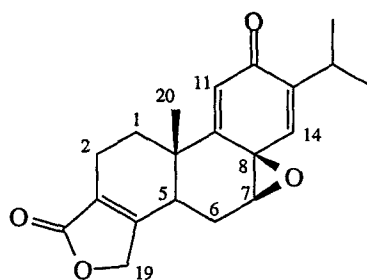
305

The ^{1}H NMR spectrum of **305** displayed the H11 and H14 signals at δ 6.42 (1H, s) and 5.99 (1H, d, J = 1.0 Hz), respectively. The C19 protons appeared as a broad singlet at δ 4.68 (2H). The corresponding H7 was located at δ 3.84 (1H, d, J = 5.8 Hz) and was strongly coupled with a proton at 2.26 (1H, ddd, J = 14.5, 5.8, 5.8 Hz) thereby indicating that it was a C6 proton (Figure 4.6, d). This C6 proton then was coupled with its geminal partner at δ 2.13 (1H, br dd, J = 14.5, 13.4 Hz) (Figure 4.6, m) and both were correlated with H5 at δ 2.58 (1H, br d, J = 13.4 Hz) (Figure 4.6, f, g). Irradiation of H11 at δ 6.42 resulted in signal enhancements of H1β at δ 2.10 (1H, br dd, J = 12.9, 5.4 Hz, 11%) and of H1α at δ 1.62 (1H, br ddd, J = 12.9, 12.4, 6.0 Hz, 3%) (Figure 4.7a). The H19 signals showed cross peaks to H5 (Figure 4.6, c), and the two H2 signals at δ 2.30 (1H, m) and 2.47 (1H, br d, J = 18.5 Hz) (Figure 4.6, a, b), respectively.

Compared with **306**, the relatively higher field of the H5 signal and the lower field of the C20 methyl proton signal (δ 1.17, 3H) indicated that the epoxy group between C7 and C8 was β oriented. Irradiation of the C20 methyl group enhanced the signals of the H2 at δ 2.30 and the H6 at δ 2.13, suggesting that these two signals arose from the corresponding β oriented

protons (Figure 4.7f). Saturation of the H7 resonance caused NOEs to H14 and the other C6 proton (which must be H6α) at δ 2.26 and confirmed that the C7 proton was an α proton, *i.e.*, the epoxy group was β oriented (Figure 4.7c). Examination of the molecular model revealed that the dihedral angle between H7α and H6α was very small but the one between H7α and H6β was around 90° (Figure 4.5). This is the reason why the H7α showed a cross peak only to H6α in the COSY spectrum and appeared as a sharp doublet in the ¹H NMR spectrum, exactly as the H7 in Tl (1) or Td (2).

305   **306**
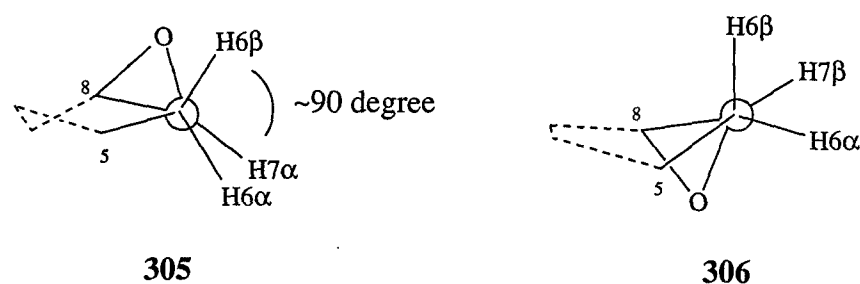
Figure 4.5   Comparison of Dihedral Angles between H7 and H6 in Compounds **305** and **306**

The ¹³C NMR and HMBC, HMQC experiments were performed and all the carbon signals were assigned accordingly. The carbon signals of the epoxy group (C7 and C8) were located at δ 64.0 and 56.0, respectively. Single crystals of **305** obtained from a large scale experiment (*vide infra*) were attempted for X-ray analysis; however, the crystals suffered X-ray induced decomposition, thus no X-ray analysis was obtained. Based on the correlation between **305** and **306** in the ¹H NMR spectra, the confirmation of the structure of **306** was an indirect confirmation of the structure assigned to **305**.
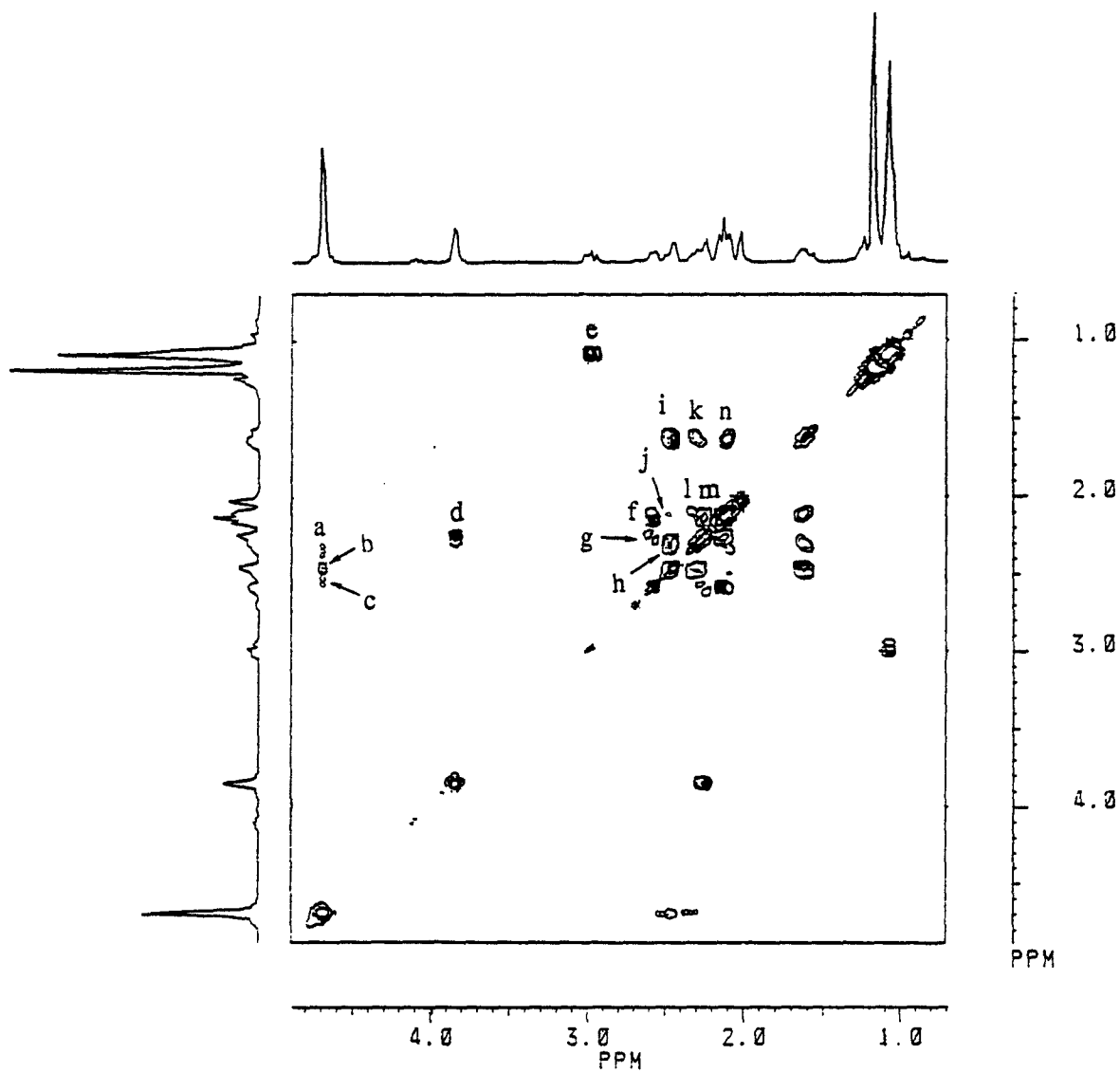
a: H19/H2β; H19/H2α; c: H19/H5; d: H7α/H6α; e: H15/H16,H17; f: H5/H6β; g: H5/H6α;

h: H2α/H2β; i: H2α/H1α; j: H2α/H1β; k: H2β/H1α; l: H2β/H1β; m: H6α/H6β; n: H1β/H1α

Figure 4.6   Expanded COSY Spectrum of Compound **305**
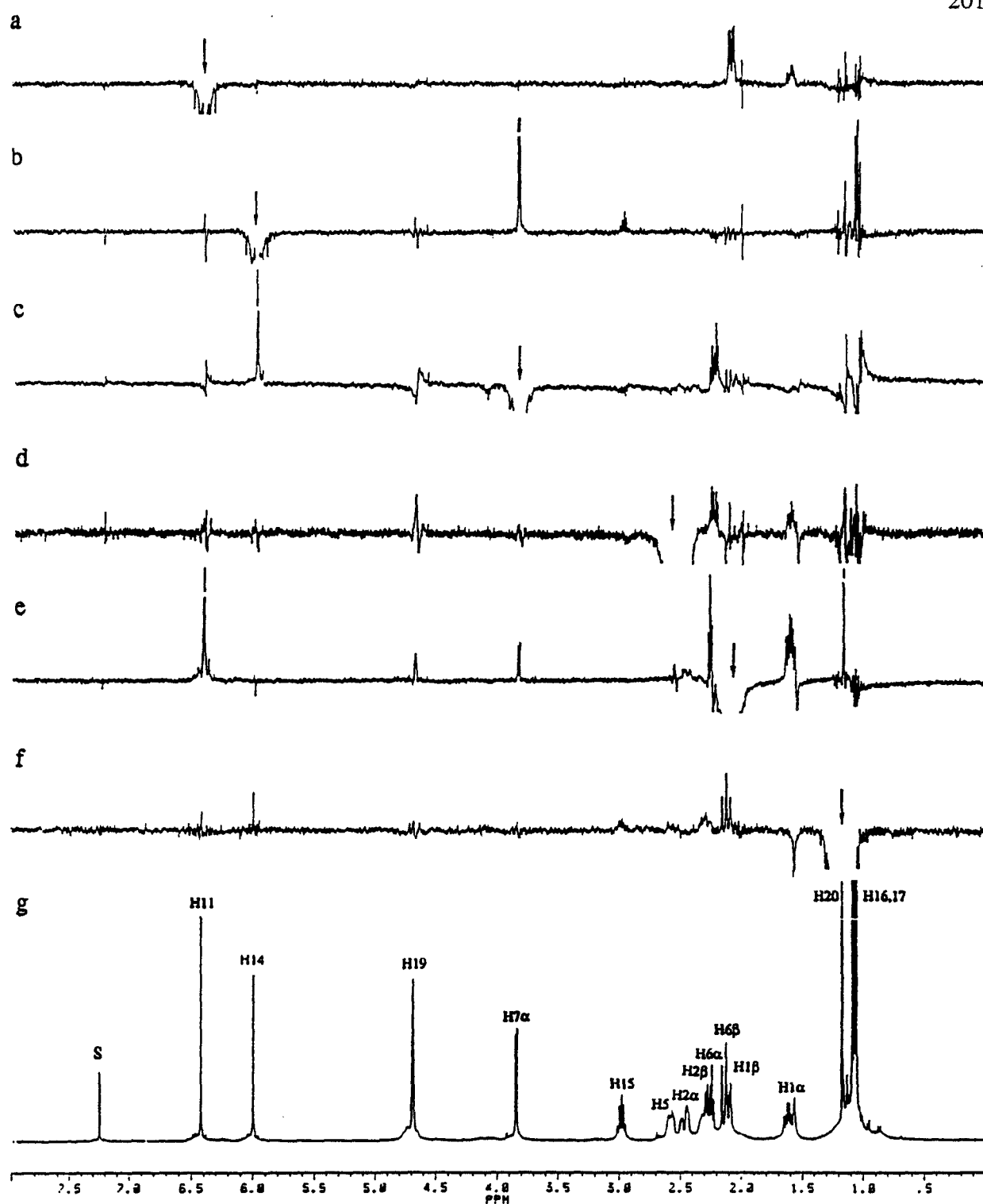
a

b

c

d

e

f

g

H11

H14

H19

H7α

S

H15

H5 H2α

H2β H6α

H6β

H1β

H1α

H20 H16,17

7.5   7.0   6.5   6.0   5.5   5.0   4.5   4.0   3.5   3.0   2.5   2.0   1.5   1.0   .5
                                              PPM

a, {H11}: H1β, H1α; b, {H14}: H7α, H15, H16,H17; c, {H7α}: H14, H6α; d, {H5}: H6α, H1α;

e, {H1β, H6β}: H14, H19, H7α, H2α, H6α, H1α, H20; f, {H20}: H2β, H6β; g, off-resonance

spectrum (400 MHz, CDCl₃). ({irradiated}: enhanced)

Figure 4.7   NOE Difference Spectra of Compound **305**

The isolation of the two novel mono-epoxides, **305** and **306**, from biotransformation of isotriptophenolide (**194**) with TRP4a cell cultures was one of the most interesting results obtained in recent years. It strongly demonstrated that the cell culture of *T. wilfordii* has the ability, as we anticipated, to transform the C ring "activated" precursor to the corresponding epoxide. The combined yield of the epoxides was moderate at 35% based on recovered starting material, but it had potential for improvement. Moreover, this result has expanded our knowledge of the capability of the biological system in this cell culture and has provided a unique venue for the production of such novel diterpene epoxides as **305** and **306**. These two compounds may have some biological activities and may also be used as intermediates to synthesize Tl analogs by chemical means or through biotransformations. Significance in this result also existed as it was the first example in which a "*para*-alkylated" phenol was converted to a *para*-epoxy dienone in a single step through biotransformation.

Biotransformation of 194 with TRP4a Cell Cultures: Time Course Studies

There are many factors which may affect the yield of the epoxides, **305** and **306**. The most important ones are the age of the cells, the duration of the incubation and the starting material-to-culture ratio. A series of biotransformations were carried out in order to investigate these influences and hence to find the appropriate conditions for the optimal formation of the products.

Influence of the Cell Age and Incubation Time

Typically, TRP4a cell cultures grown in $MSNA_{0.5}K_{0.5}$ have a 7 to 10 day lag phase followed by a rapid growth phase that gives way to a stationary phase by 28-35 days. The production of Td (**2**) was found highest in the later growth phase.[147] To examine the influences

of culture age on the yield of the epoxides, three typical ages (7, 15 and 21 days) were chosen for the studies.

Isotriptophenolide (**194**, 200 mg) in ethanol (50 mL) was added to TRP4a cell cultures (1.1 L) of 7, 15 and 21 days old, respectively. The cultures were incubated under standard conditions and samples were taken at regular intervals. The samples were filtered, and the resulting broth and cells were extracted separately with ethyl acetate and analyzed by TLC and HPLC.

An experiment with a 15-day-old cell culture was examined first and was followed by experiments with cell cultures of 7 and 21 days old. Figure 4.8 shows the changes of isotriptophenolide (**194**) and the epoxides **305** and **306** with incubation time in the biotransformation with the 15-day-old TRP4a cell culture (Trp#305). From Figure 4.8a and b, it can be seen that the starting material decreased very rapidly from initial value of 200 mg/L (not shown in the figures) to a total of about 110 mg/L after the first day of incubation, then the decrease slowed down. The epoxides were mainly in the broth and increased significantly during 7 to 9 days of incubation. The starting material was more or less equally distributed between the cells and the broth. The amount "in" the cells may include both the starting material which was in the cells during the biotransformation and that which was only adsorbed on the cell walls or cell debris. The latter may account for most of the starting material "in" the cells because of the relatively large amount and the low solubility of the starting material in the culture medium.

The starting material must have entered into the cells if the corresponding enzymes were inside the cells (this was later proven to be true by cell free extract experiments, *vide infra*) and the products were then excreted by the living cells, or released from the dead cells into the broth. That would explain why the products (epoxides) were mainly in the broth and their amounts in the cells were small and almost constant. This was in accord with the fact that Tl (**1**) and Td (**2**) were also almost exclusively in the broth.
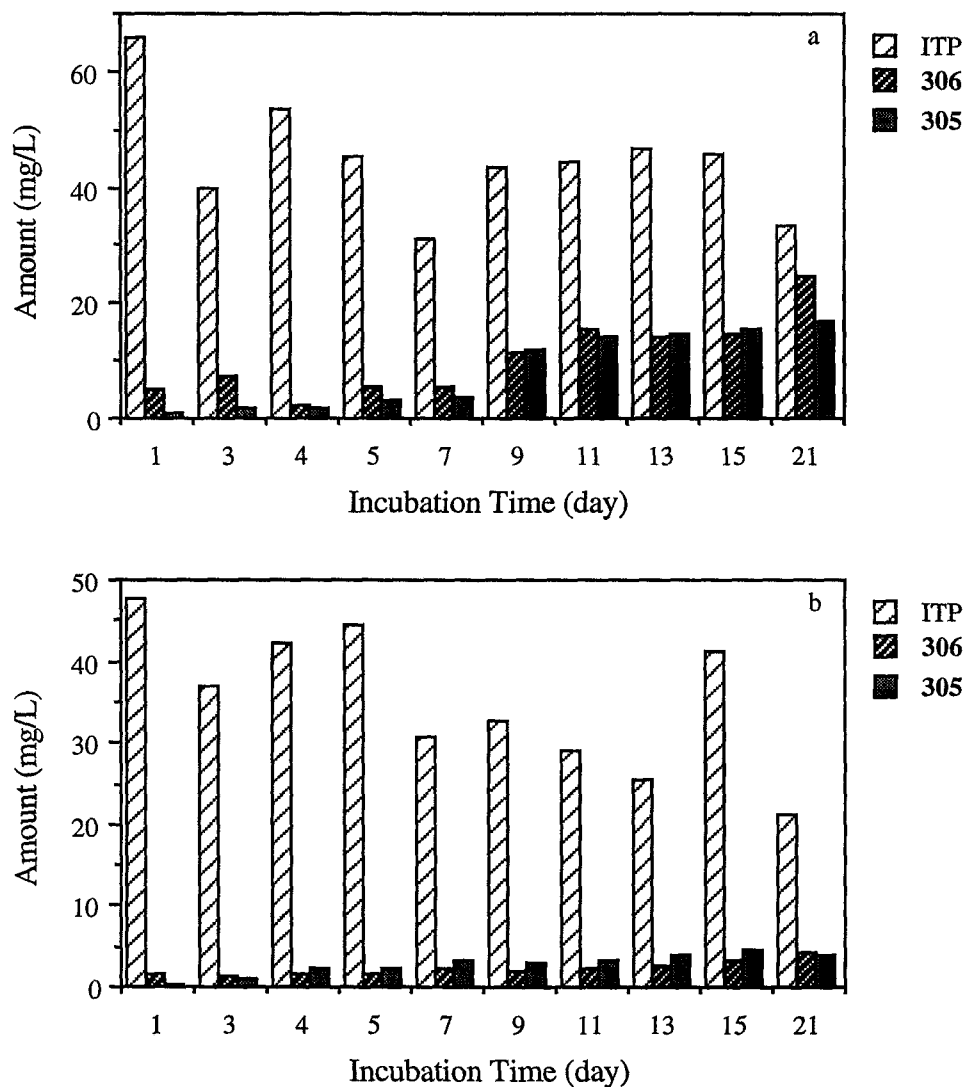
Figure 4.8   Changes in the Amounts of Isotriptophenolide (ITP, **194**), **305** and **306** during the Biotransformation (Trp#305, cell age: 15 day). a, in the broth; b, in the cells

Figure 4.8c illustrates the conversion of the starting material into products in the whole cell culture (broth and cells). The amounts of **305** and **306** were almost equal, and the total amount of products reached the highest level after incubation for 15 days and then remained at that level until 21 days, even though the starting material continued to decrease during that time. The total yield of the epoxides (based on recovered starting material) at day 15 was about 36% (Figure 4.8d), the best yield that could be obtained with a 15-day-old TRP4a cell culture.
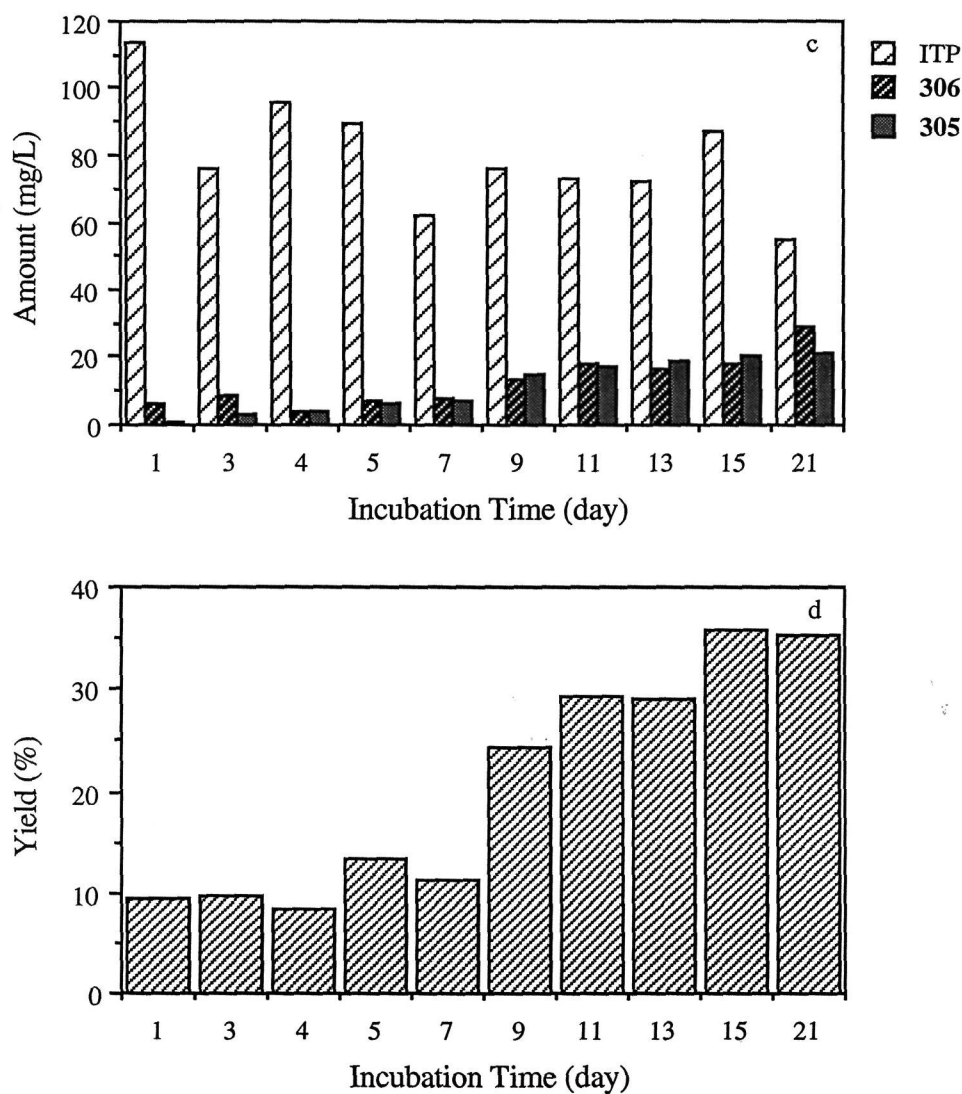
Figure 4.8   Changes in the Amounts of Isotriptophenolide (ITP, **194**), **305** and **306** during the Biotransformation (Trp#305, cell age: 15 day) (continued). c, in the cell culture (broth and cells); d, total yields of the epoxides **305** and **306** (based on recovered starting material)
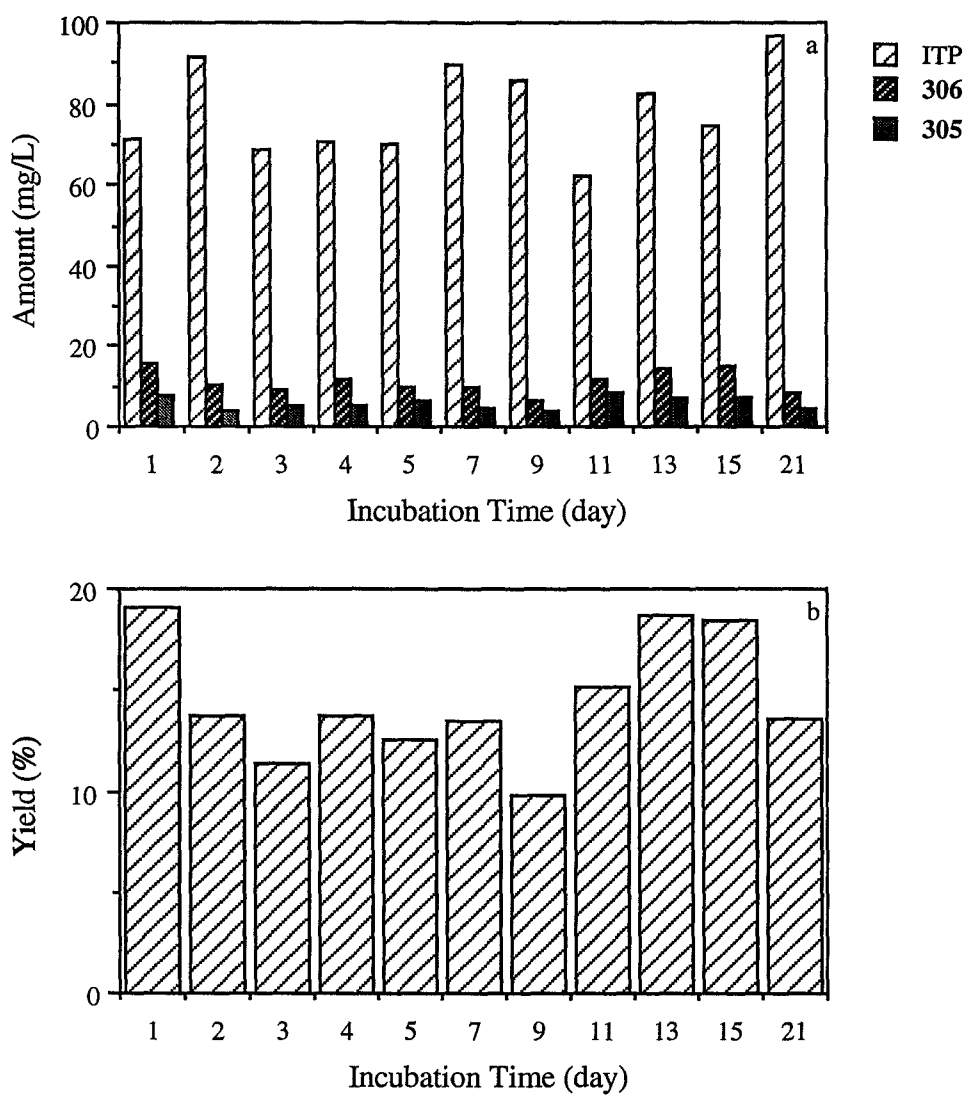
Figure 4.9   Changes in the Amounts of Isotriptophenolide (ITP, **194**), **305** and **306** during
the Biotransformation (Trp#306, cell age: 7day). a, in the cell culture (broth
and cells); b, total yields of epoxides **305** and **306** (based on recovered starting
material)

Figure 4.9 shows the corresponding results from biotransformation of
isotriptophenolide (**194**) in a 7-day-old cell culture (Trp#306). The yields of both epoxides
were quite low and constant during the course of biotransformation (Figure 4.9a). The best
yield obtained from a 7-day-old cell culture was 18% after 13 to 15 days of incubation (Figure
4.9b). This demonstrated that the younger cell cultures were also capable of transformation of

the starting material **194** to the epoxides. The relatively low yield of the epoxides (about half the yield obtained from a 15-day-old culture) suggested that the cells at that age did possess the required enzymatic activity but at a lower level when compared with the 15-day-old culture.

Because separate HPLC analyses of broth and cell samples provided similar data, starting from experiment Trp#306, the broth and cell extracts obtained in the whole cell experiments were combined and then analyzed for total amounts of isotriptophenolide (**194**), **305** and **306**, respectively.

Figure 4.10 portrays the relationship between incubation time and the amounts of isotriptophenolide (**194**), **305** and **306** in the biotransformation of **194** with a 21-day-old cell culture (Trp#307). It can be seen that the yields of both epoxides increased rapidly during the first 7 days of incubation and then remained relatively unchanged in the following 14 days (Figure 4.10a). The best yield from a 21-day-old cell culture was about 54% (based on recovered starting material) after 7 days of incubation (Figure 4.10b), and this was the highest yield among the experiments with the three different age groups. The rapid increase of product levels and the relatively high yield were the major differences between this experiment (using 21-day-old cell culture) and the previous two experiments (using 7-day-old or 15-day-old cell cultures). This suggests that among the three different age groups, 21-day-old cell cultures contain the highest level of the required enzymes. The yield based on recovered starting material dropped after 7 days of incubation while the actual amount of both epoxides was little changed (Figure 4.10b). This indicated that either the epoxides were further converted to other products or there were some other reactions which also consumed the starting material. The α isomer **306** was in greater quantity than **305**.
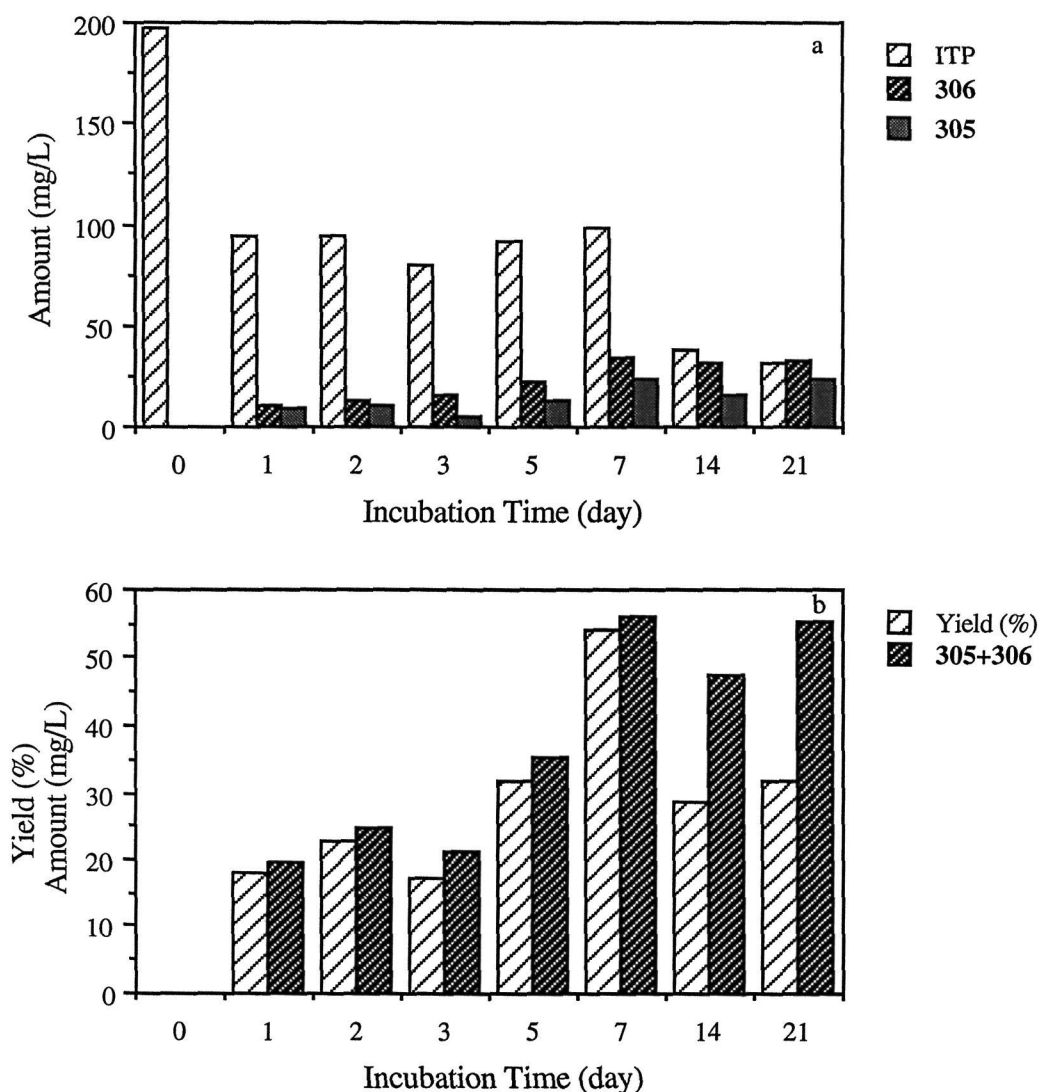
Figure 4.10   Changes in the Amounts of Isotriptophenolide (ITP, **194**), **305** and **306** during
the Biotransformation (Trp#307, cell age: 21 day). a, in the cell culture (broth
and cells); b, total amounts and yields of epoxides **305** and **306** (based on
recovered starting material)

Figure 4.11 compares the actual yields of the epoxides **305** and **306** and the yields based

on recovered starting material among the three different age groups. It was found that

experiments with 7-day-old cell culture gave the lowest yields in both cases.

Biotransformation with 15-day-old cell culture afforded similar yields to the 7-day-old culture

in the early stage during the biotransformation, but the yields increased in the late stage.

Biotransformation with 21-day-old cell culture gave the highest yields throughout the incubation period. The highest yields here were obtained after about 7 days of incubation, but in the late stage, a rapid depletion of the starting material made the yield (based on recovered starting material) lower. Nevertheless, the trend is clear, that the older cell cultures are likely to give higher yields of the epoxides in a relatively short time of incubation.
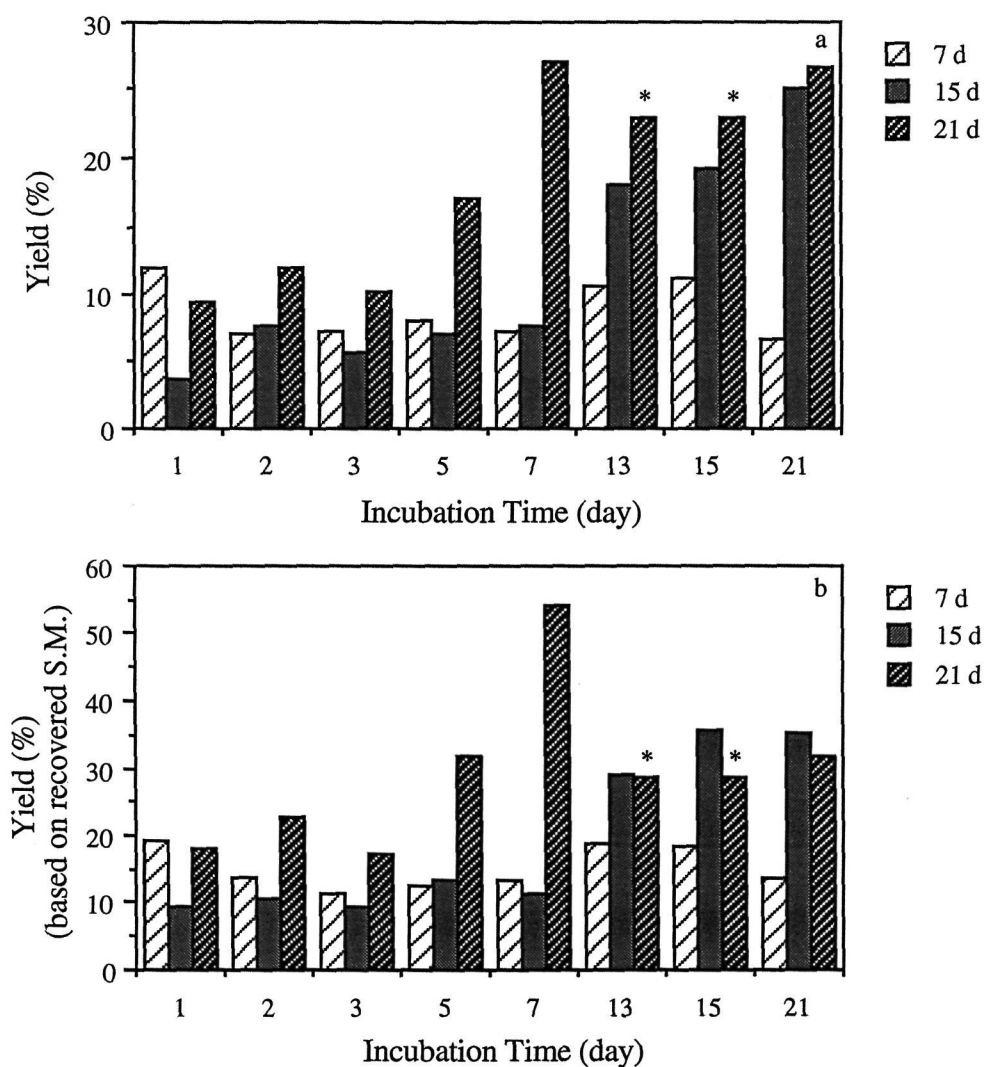
Figure 4.11   Comparison of the Epoxide Yields in Biotransformations of **194** with TRP4a Cell Cultures of Different Ages. a, the actual amounts of the epoxides in the cell cultures; b, the yields based on recovered starting material (* data from samples after 14 days of incubation)

Influence of Starting Material-to-Culture Ratio

One common feature of the above biotransformation results was that the starting material did not react completely, even after 21 days of incubation. This could result from an excess of starting material beyond the enzymatic capacity, or from a deleterious effect of high concentrations of the starting material. Therefore, it was of interest to see whether the amount of added starting material affected the yield of the epoxides.

Three experiments, Trp#307, 308 and 309, were run under the same conditions except that the ratio of starting material-to-culture was 100, 50 and 25 mg to 550 mL of culture (21 days old), respectively. Figure 4.12a shows that the actual yields increased slightly when the amount of starting material added was decreased from 100 mg to 50 mg in 550 mL of culture. The yields for Trp#309 (25 mg starting material) were calculated from the amounts of **306** because the small amounts of **305** were difficult to detect accurately by HPLC. Even though, the yields from Trp#309 were comparable with those from Trp#307.

For certain enzymatic reactions, after the initial concentration of a substrate reaches a certain level, the maximum reaction rate is proportional to the initial concentration of the enzyme but independent of the initial concentration of the substrate.[184] As a result, increasing initial substrate concentration beyond that point would result in a lower percentage yield. The present results suggested that the amounts of the starting material were probably in excess of the amount for the maximum reaction rate; therefore, when the amount of starting material was decreased, the percentage yields increased. However, the differences were not significant, and a low starting material-to-culture ratio would result in low overall productivity.

Figure 4.12c shows another drawback of a low starting material-to-culture ratio. In the early hours of the biotransformation, the yield based on recovered starting material was higher for Trp#309 (lower starting material-to-culture ratio), but later, the trend reversed. It suggested that when the starting material-to-culture ratio was low, the relative amount of starting material

consumed by side reactions increased, making the overall recovery lower. Therefore, based on the above results, the optimal range of the added amount of starting material was 50 to 100 mg per 550 mL of cell culture.
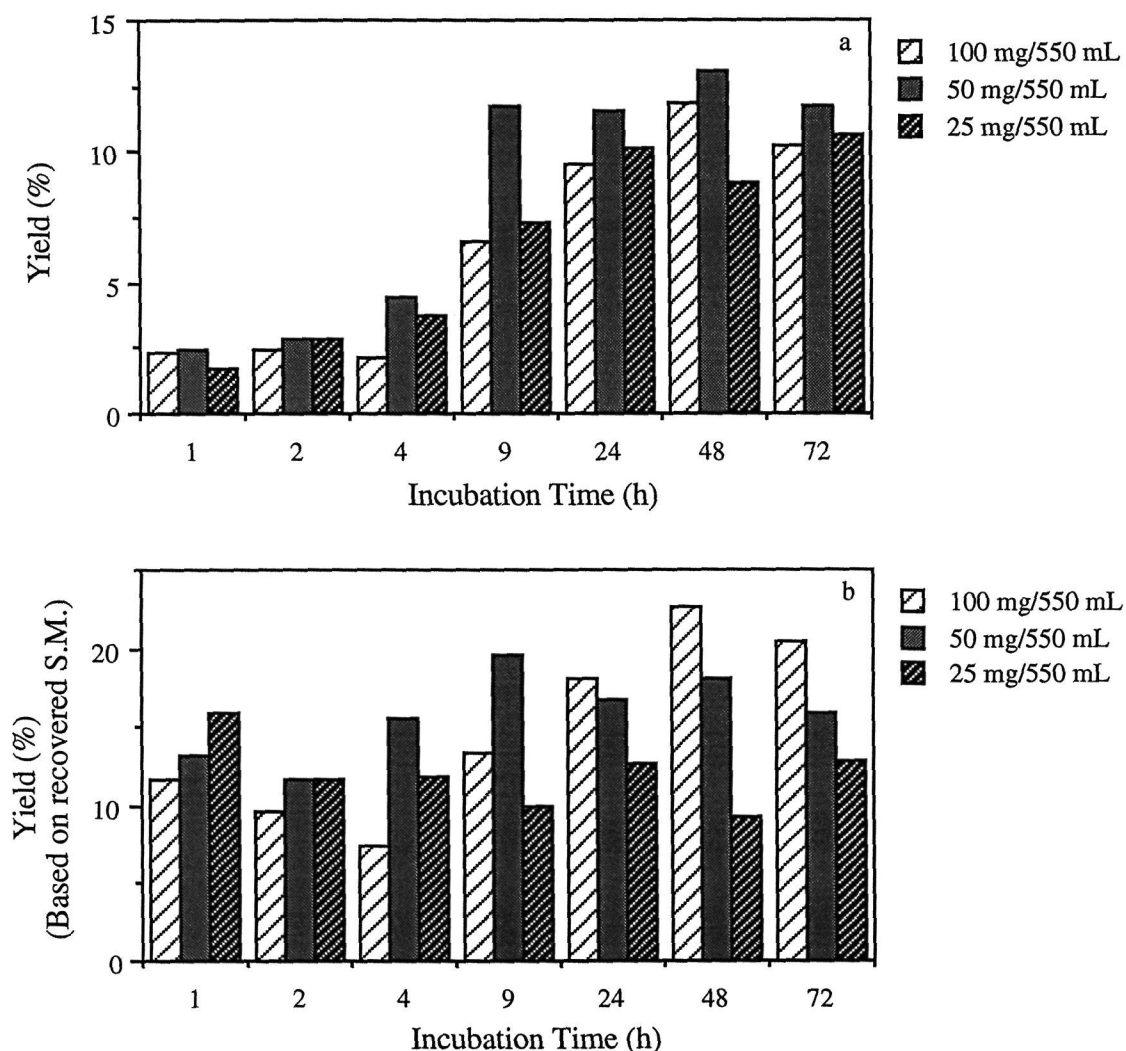
Figure 4.12   Comparison of the Epoxide Yields in Biotransformations of **194** with TRP4a Cell Cultures in Different Starting Material-to-Culture Ratios (Trp#307, 308, 309, cell age: 21 day). a, yields of the epoxides **305** and **306**; b, yields of the epoxides **305** and **306** (based on recovered starting material)

Biotransformation of 194 with Cell Free Extracts (CFE) from TRP4a Cell Cultures

Preliminary Results from CFE Experiments

The ideal biotransformation should provide high selectivity, high yields and flexible reaction conditions. The advantages of the whole cell system are its low cost and the presence of endogenous cofactors.[247] However, there are drawbacks. There are certain factors affecting the permeability of the starting material through the cell membrane; the amount of the starting material must not exceed the level that the cells can tolerate (toxicity); the overall reaction rate may be limited simply by non-enzymatic factors such as the rate of diffusion through the cell membrane; and workup as well as product separation may involve a large volume of material and it may often be tedious.[247] These limitations may be overcome by use of cell free extracts (CFE) or isolated enzymes.

A cell free extract is a crude enzyme preparation obtained by cellular disintegration and subsequent removal of the insoluble cellular material. This is usually accomplished by homogenization of the cells followed by centrifugation. It should be noted that although variation of experimental conditions are still limited to avoid denaturation of the enzymes, they can be varied over a wider range than is possible for a whole cell system. Some enzymes can be used in organic solvents to perform biotransformations.[251] The use of CFE or purified enzymes as catalysts in syntheses of complicated molecules or for a certain reaction with a high requirement for selectivity has broad applications and will have great potential in the near future.[247]

A general procedure for the preparation of CFE from the TRP4a cell culture is shown in Figure 4.13. The cell culture was filtered through Miracloth™ to remove the broth. A sample was routinely taken from the broth to assay nutrient consumption and other parameters. The cells were then washed with distilled water and dried by removal of water *in vacuo*. The cells

were weighed and resuspended in a certain volume of buffer (140 mL buffer per 100 g cells) and homogenized at 0-4°C (3 x 0.5 min). The resulting cell homogenate (CH) was used directly for biotransformation studies in some cases, but usually was centrifuged at 10,000 g (maximum; average 6,600 g) for 30 min at 4°C to separate the cell pellet (cell wall materials and debris) from the supernatant (CFE). The CFE thus obtained was ready for the biotransformation study. In order to determine whether the enzymes responsible for the epoxide formation are membrane bound, several experiments were performed with a resuspended cell pellet (RCP).

```
                    ┌──────────────┐
                    │ cell culture │
                    └──────────────┘
                           │
                           │   1) filtration and washing
                           │   2) homogenization in buffer
                           ↓
                 ┌─────────────────────┐
                 │ cell homogenate (CH) │
                 └─────────────────────┘
                           │
                           │   centrifugation
                           │
              ┌────────────┴────────────┐
              ↓                         ↓
        ┌─────────────┐         ┌──────────────────┐
        │ cell pellet │         │ supernatant (CFE) │
        └─────────────┘         └──────────────────┘
              │
              │  resuspension
              │  in buffer
              ↓
        ┌──────────────────────┐
        │ resuspended cell pellet │
        │        (RCP)          │
        └──────────────────────┘
```
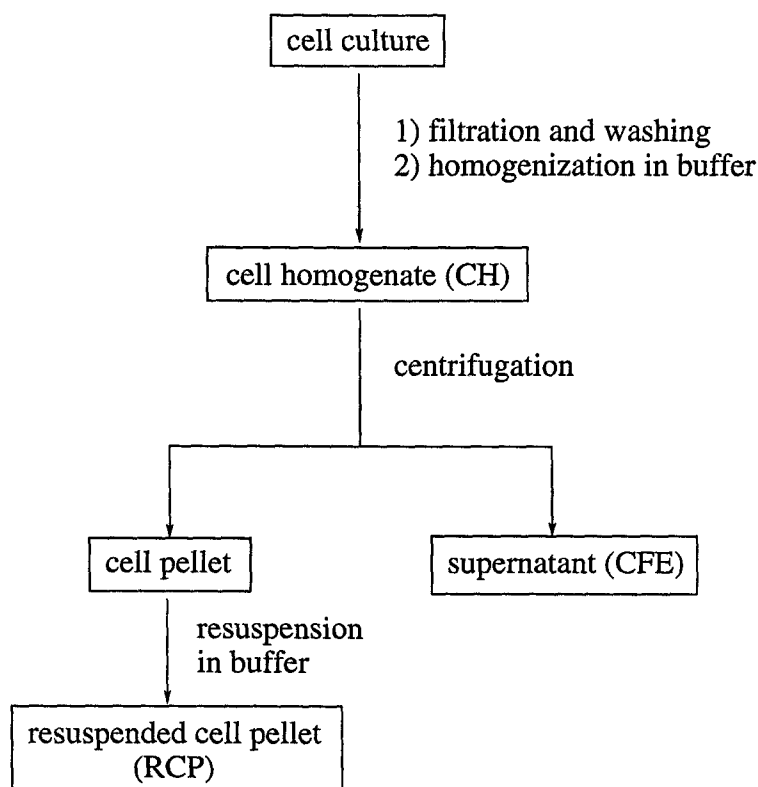
Figure 4.13   A General Procedure for Preparation of CFE, CH and RCP

The procedures used previously by members of Dr. Kutney's group for biotransformations with CFE, CH and RCP from TRP4a cell cultures[163] were used as a starting point for our study with isotriptophenolide (194) as substrate.

An evaluation of the enzyme capability of CFE, CH or RCP was made by measuring peroxidase activity and soluble protein concentration. The real nature of the epoxide synthesizing enzyme or enzymes was not known, so a more specific evaluation of a well defined activity was not possible. However, according to the proposed biosynthetic pathway (section 2.2), a quinone methide intermediate or a 7-hydroxylated compound could be involved in the initial formation of the epoxide group between C7 and C8. This step could be catalyzed by some type of peroxidase; therefore, the values of peroxidase activity and protein concentration were likely relevant to the epoxide synthesis. It was hoped that these parameters would provide, to some extent, a common reference for comparison of the results of separate experiments.

The peroxidase activity of CFE etc. was assayed by the pyrogallol-purpurogallin method and expressed as units/mL of the enzyme preparation.[252] The soluble protein concentration was determined by the Bio-Rad procedure and expressed as mg/mL of enzyme preparation.[253]

A preliminary experiment with CFE was carried out with a 15-day-old TRP4a cell culture (2 x 550 mL). The cell culture was filtered through Miracloth™ and washed with distilled water. The cells obtained (100 g) were suspended in a phosphate buffer (0.1 M, pH 6.6, 140 mL) and homogenized. Centrifugation of the homogenate at 10,000 g for 30 min at 4°C afforded 176 mL of the clear supernatant (CFE) which had a peroxidase activity of 4.05 units/mL and a protein concentration of 0.939 mg/mL.

Because the cells had been disintegrated, in $vivo$ generation of enzyme cofactors was impossible; therefore, certain cofactors were added to the CFE (or CH, RCP). One of the most important cofactors for peroxidases is hydrogen peroxide (H_2O_2).[184,254] Thus, the CFE mixture was prepared for biotransformation by combination of CFE (62 mL, 250 peroxidase units), phosphate buffer (0.1 M, pH 6.6, 350 mL), distilled water (150 mL) and 0.24% hydrogen peroxide solution (10 mL, 2.2 molar equivalent with respect to the starting material to be added). Then the starting material, isotriptophenolide (194, 100 mg), was added as a

solution in ethanol (30 mL) to the stirred CFE mixture at room temperature. A blank experiment was done on a smaller scale under the same conditions, except that the CFE was substituted by an equal amount of the buffer. Samples were taken at 0.5, 2, 6, and 22 h, and the remainder was worked up after 48 h. All the samples were extracted with ethyl acetate and analyzed by TLC. The results showed that there were no epoxides formed in the experiment. The only new spot on TLC, found in both test and blank samples as a very small component, was 7-hydroxy-isotriptophenolide (**246**). This compound was probably formed through benzylic oxidation by the hydrogen peroxide added to both CFE mixture and the blank solution. The lack of biotransformation in this particular case suggested that other cofactors may be needed, and that the pellet removed from the cell homogenate should also be assayed to see whether the enzymes are membrane bound.

Time Course Studies with CFE, Cell Homogenates(CH) and Resuspended Cell Pellet (RCP) from TRP4a Cell Cultures of Different Age

Because 21-day-old cell cultures gave highest yield of the epoxides in the whole cell experiments, it was decided to start with a CFE made from a cell culture of this age. Two additional cofactors, flavin mononucleotide (FMN) and manganese (II) chloride ($MnCl_2$), which were proven to be effective in CFE biotransformation with other compounds,[163] were added to the transformation cocktail. Three different preparations, CFE, CH and RCP, were studied simultaneously in order to assay which preparation, if any, would give the highest yield of the epoxides.

A cell culture of 21 days old (Trp#311, 2 x 550 mL) was used to prepare the CFE, CH and RCP. A typical experiment is discussed presently. Further details are provided in the experimental section. The cells obtained were suspended in a phosphate buffer and homogenized to give a cell homogenate (CH) (293 mL), of which an aliquot was taken and centrifuged to give a CFE and the pellet. The pellet was resuspended in buffer to prepare the

RCP. The peroxidase activities and protein concentrations of the CFE, CH and RCP were measured (Table 4.2). The data show that the CH had the highest peroxidase activity as expected, and the CFE had higher peroxidase activity than the RCP. This suggested that the peroxidase(s) was not membrane bound. The protein concentration was also measured.

Table 4.2   Conditions for Biotransformation of **194** with CFE, CH and RCP Prepared from a 21-day-old TRP4a Cell Culture (Trp#311)

		CFE	CH	RCP
assays	peroxidase activity (unit/mL)	3.57	7.91	1.47
	protein concentration (mg/mL)	0.977	1.110	0.678
composition of biotrans-formation mixture	CFE, CH, RCP volume (mL)	70	31.6	170
	peroxidase (unit)	250	250	250
	distilled water (mL)	75	75	75
	phosphate buffer (0.1 M, pH 6.6) (mL)	175	175	175
	0.24% H_2O_2 (equiv.)	2.2	2.2	2.2
	$MnCl_2$ (equiv.)	0.5	0.5	0.5
	FMN (equiv.)	0.5	0.5	0.5
substrate	isotriptophenolide (**194**) (mg)	50	50	50
solvent	ethanol (mL)	20	20	20

The compositions of each reaction mixture are also given in Table 4.2. The ratio of peroxidase units-to-starting material and the amount of ethanol were increased in this experiment. A solution of isotriptophenolide (**194**, 50 mg) in ethanol (20 mL) was added at room temperature to each stirred mixture, respectively. At the same time, two control experiments for CH and RCP were also run at one tenth of the scale as the corresponding CH and RCP experiments, but with no starting material added. A blank experiment was carried out at one tenth of the scale as the CFE experiment but with the CFE being substituted by an equal amount of the buffer. All the reactions were performed at room temperature. Samples were taken at 0.5, 2, 6 and 24 h, and the remainder was worked up at 48 h. TLC showed that the

samples from CFE and CH experiments taken at 0.5 h already revealed a spot corresponding to the epoxides. This result indicated that with the addition of appropriate cofactors, CFE was able to perform the same biotransformation as the whole cells. The blank samples showed no sign of epoxidation, but only small amounts of 7-hydroxy-isotriptophenolide (**246**) appeared on TLC. The CH and RCP control samples provided HPLC and TLC references for the corresponding CH and RCP biotransformation samples. The ratio among each component in the biotransformation mixture of this experiment (peroxidase units, volumes of the buffer and distilled water, the amounts of cofactors, and starting material) was used as a standard ratio in the rest of the experiments with CFE (CH, RCP) unless otherwise noted.

Similarly, biotransformations of isotriptophenolide (**194**) with CFEs, CHs and RCPs prepared from cell cultures of 13 days old (Trp#312) and 7 days old (Trp#313) were performed. The blank experiment was omitted because the starting material had remained virtually unchanged in the previous blank experiments. Table 4.3 and Table 4.4 show the biotransformation conditions for experiment Trp#312 and Trp#313, respectively.

Figure 4.14 depicts the changes of isotriptophenolide (**194**), epoxides **305** and **306** during the biotransformation with CFE from the 21-day-old cell culture (Trp#311). Concentrations of **305** and **306** peaked at 2 h, with epoxide **306** present in higher concentration than **305**, as in the whole cell experiments. The total yield of **305** and **306** at 2 h was 24.5% (or 45% based on recovered starting material), which was similar to the best yields (27%, or 54% based on recovered starting material) obtained from biotransformation with 21-day-old whole cells after 7 days of incubation (Trp#307).

Biotransformation of **194** was faster with CFE than with the whole cells, which was in agreement with the assumption that in the whole cell experiments, the diffusion of the starting material and the products through the cell membrane may limit the overall biotransformation rate. Alternatively, in the whole cell experiments, the substrate may not reach the active site of the enzyme effectively, if the enzyme is associated with some cell materials inside the cells.

Table 4.3   Conditions for Biotransformation of **194** with CFE, CH and RCP Prepared from a 13-day-old TRP4a Cell Culture (Trp#312)

		CFE	CH	RCP
assays	peroxidase activity (unit/mL)	4.78	7.34	3.32
	protein concentration (mg/mL)	1.13	1.40	0.85
composition	CFE, CH, RCP volume (mL)	52.3	34.1	75.3
of	peroxidase (unit)	250	250	250
biotrans-	distilled water (mL)	75	75	75
formation	phosphate buffer (0.1 M, pH 6.6) (mL)	175	175	175
mixture	0.24% H_2O_2 (equiv.)	2.2	2.2	2.2
	$MnCl_2$ (equiv.)	0.5	0.5	0.5
	FMN (equiv.)	0.5	0.5	0.5
substrate	isotriptophenolide (**194**) (mg)	50	50	50
solvent	ethanol (mL)	20	20	20

Table 4.4   Conditions for Biotransformation of **194** with CFE, CH and RCP Prepared from a 7-day-old TRP4a Cell Culture (Trp#313)

		CFE	CH	RCP
assays	peroxidase activity (unit/mL)	3.57	7.91	1.47
	protein concentration (mg/mL)	0.977	1.110	0.678
composition	CFE, CH, RCP volume (mL)	70	31.6	170
of	peroxidase (unit)	250	250	250
biotrans-	distilled water (mL)	75	75	75
formation	phosphate buffer (0.1 M, pH 6.6) (mL)	175	175	175
mixture	0.24% H_2O_2 (equiv.)	2.2	2.2	2.2
	$MnCl_2$ (equiv.)	0.5	0.5	0.5
	FMN (equiv.)	0.5	0.5	0.5
substrate	isotriptophenolide (**194**) (mg)	50	50	50
solvent	ethanol (mL)	20	20	20

Figure 4.14   Changes in the Amounts of Isotriptophenolide (ITP, **194**), epoxides **305** and **306** in the Biotransformation of **194** with CFE Prepared from a 21-day-old Cell Culture

Although in the CFE experiments, the enzymes were now available for reaction, the yield of epoxides was similar to that of the whole cell experiment. There were several possible explanations. Perhaps the biotransformation proceeded very rapidly and the highest yield could have been reached between samplings, for example, between 0.5 to 2 h, or between 2 to 6 h. Another explanation was that in the CFE experiment, the optimum requirement for additional cofactors to keep the enzymes functioning effectively, or the amount of cofactors, or the optimum pH had not been achieved.

One common feature shared with the whole cell experiment was that only about half of the consumed starting material had been converted to the epoxides by the time that the epoxides reach their highest concentration. Longer incubation times led to an overall decrease in recovered starting material and the yield of the epoxides. This result suggested that the epoxides were likely further transformed to other compounds, or that the starting material underwent alternative conversions with the other enzymes present in the CFE.
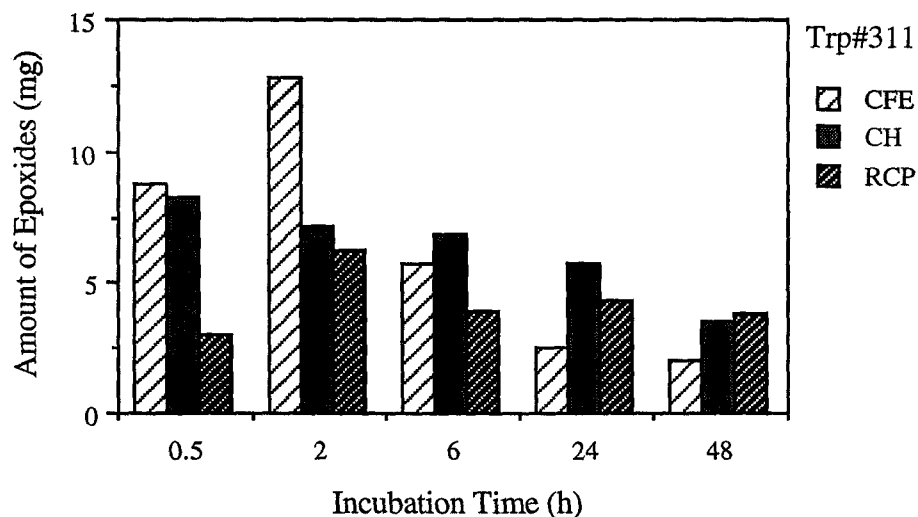
Figure 4.15   Comparison of the Epoxide Yields in Biotransformations of **194** with CFE,
CH and RCP Prepared from a 21-day-old Cell Culture

Figure 4.15 compares yields of the epoxides in biotransformations of **194** with CFE, CH and RCP prepared from a 21-day-old cell culture (Trp#311). With the same amount of peroxidase in each experiment, CFE gave the highest yield of epoxides in the first 2 h and then the yield decreased rapidly. The yield of epoxides in the CH experiment reached its peak at about 0.5 h and then also decreased, but not as rapidly as in the CFE experiment. The yield of epoxides in the RCP experiment proceeded to its peak at about 2 h and then slowly declined. The overall result seemed to indicate that, in terms of yield of the epoxides and biotransformation rate, CFE was enzymatically more active than CH, and CH was more active than RCP.

However, if the epoxidation rate was proportional to the number of peroxidase units, all three preparations, namely, CFE, CH and RCP, should give more or less the same amount of epoxides, but they did not. Although peroxidases may be involved in the formation of the epoxides, the results suggested that peroxidase activity was not a good indicator for the evaluation of the enzymes responsible for the epoxide synthesis.

Although the peroxidase units were the same for the different preparations (CFE, CH and RCP) in the biotransformations, the actual "original cell culture contents" may not be the same, that is, the enzymes responsible for epoxidation may be present in different concentrations in the three preparations. Therefore, the data relating to epoxide yield may be better presented in terms of the <u>volumes</u> of the cell cultures needed to prepare the CFE, CH or RCP that have been actually used in the experiment. If the effect of the total volumes of the biotransformation mixtures on the rate can be neglected, the yields based on "unit" original volume of whole cell culture ("specific yield") would be more accurate and realistic. As we did not have the possibility to determine the activity of the relevant enzymes, the only way to correlate results of the different experiments (such as comparisons among CFE, CH and RCP, or among preparations of different age, etc.) was by their specific yields. These yields would reflect the activities of CFE, CH and RCP prepared from the same batch of cell culture or, the activities of the CFE (or CH, RCP) prepared from cell cultures of different ages. For example, a younger cell culture has less cell mass, therefore, a larger volume of cell culture is needed to prepare the same amount of CFE (or CH, RCP) compared with an older cell culture. Although the resulting CFE from a younger cell culture may have a similar peroxidase activity as a CFE from an old one, the "specific yield" of a given product would be effectively lower in the young culture since a larger volume of culture is utilized to prepare the CFE (or CH, RCP).

Figure 4.16 shows the data processed in such a way. It can be seen that, as expected, the cell homogenate (CH) gave the highest specific yields of the epoxides.

It was noticed that in some cases the yields from the CH experiments were higher than the sum of the yields from the corresponding CFE and RCP studies. This may be attributed to the possibilities that the relevant enzyme activity may have been partially lost during the preparation of CFE and RCP from CH. In comparison of the yields from CFE with those from RCP, it was fairly safe to conclude that the enzyme(s) responsible for the formation of the epoxide functionality from isotriptophenolide (**194**) was mainly present in the CFE, that is, the enzyme(s) was likely not membrane bound.

Trp#311

CFE
CH
RCP

Amount of Epoxides
(mg/mL cell culture)

0.08

0.06

0.04

0.02

0.00

0.5   2   6   24   48

Incubation Time (h)
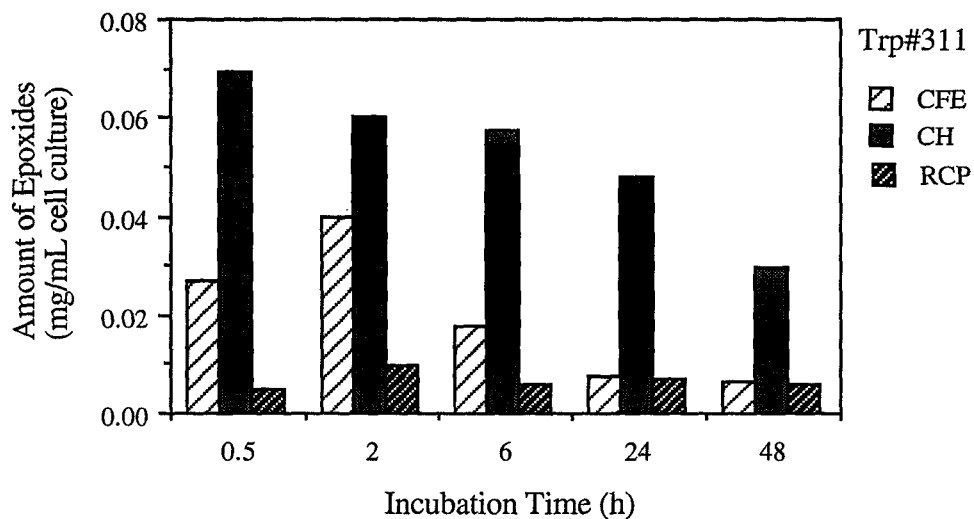
Figure 4.16   Comparison of the Epoxide Yields (Specific Yields) in Biotransformations of
194 with CFE, CH and RCP Prepared from a 21-day-old Cell Culture

Trp#312

CFE
CH
RCP

Amount of Epoxides
(mg/mL cell culture)

0.03

0.02

0.01

0.00

0.5   2   6   24   48

Incubation Time (h)
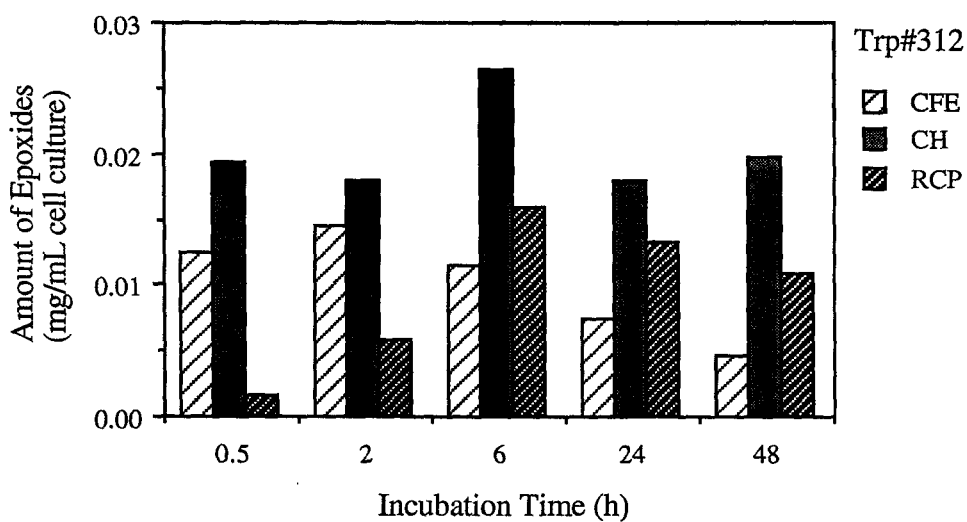
Figure 4.17   Comparison of the Epoxide Yields (Specific Yields) in Biotransformations of
194 with CFE, CH and RCP Prepared from a 13-day-old Cell Culture

Figure 4.17 gives the biotransformation results with CFE, CH and RCP prepared from a
13-day-old cell culture (Trp#312). Again, the cell homogenate gave the highest specific yields

of the epoxides among the three preparations. The CFE gave higher yields than the RCP in the initial hours of incubation but the RCP yields increased with time.

Figure 4-18 displays a similar trend in biotransformations of **194** with CFE, CH and RCP prepared from a 7-day-old cell culture (Trp#313). The RCP gave lowest yields in the initial hours of incubation, but reached its peak several hours later in the biotransformation.

Figure 4-19 compares the specific yields of epoxides in the biotransformations of **194** with CFEs prepared from cell cultures of different ages. The CFE prepared from the 21-day-old cell culture gave the highest yields (up to the sixth hour) compared with those from younger ages (13 and 7 days). The differences in yields between CFEs from 13-day-old and 7-day-old cultures were not significant.
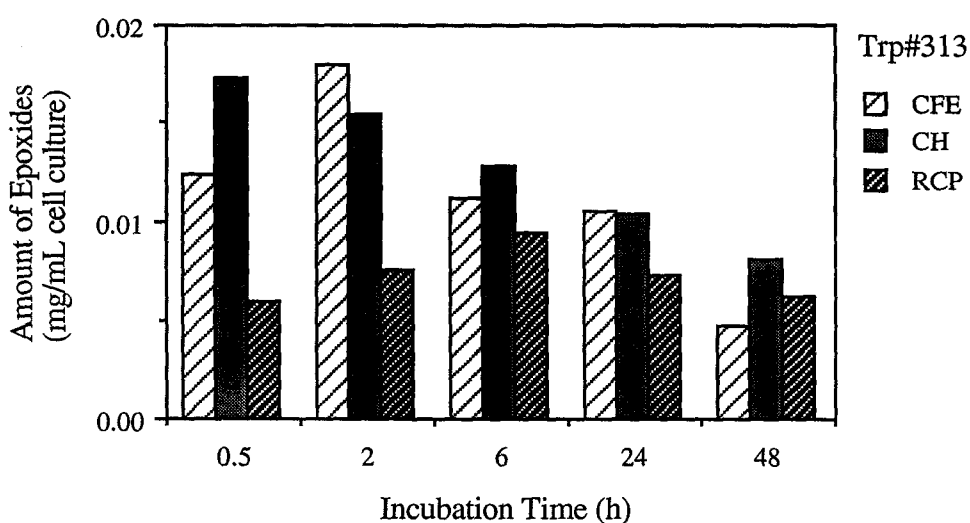
Figure 4.18   Comparison of the Epoxide Yields (Specific Yields) in Biotransformations of **194** with CFE, CH and RCP Prepared from a 7-day-old Cell Culture

Figure 4.20 depicts the results with CHs prepared from cell cultures of different ages. It clearly demonstrates that CH from the 21-day-old cell culture gave significantly higher yields of epoxides than those from 13-day-old and 7-day-old cultures. The differences between 13-day-old and 7-day-old were not significant, with the CH derived from the 13-day-old culture giving slightly higher yields than those derived from the 7-day-old culture.
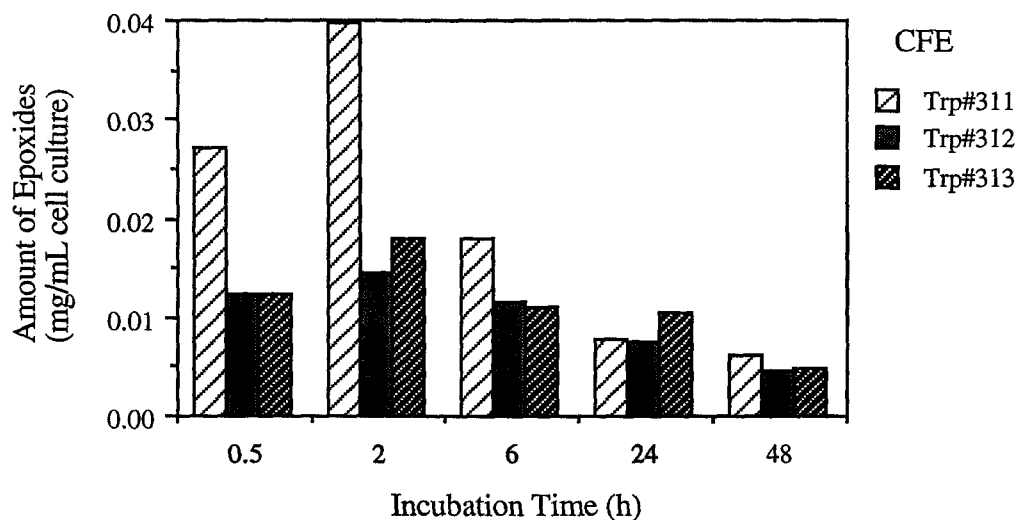
Figure 4.19   Comparison of the Epoxide Yields (Specific Yields) in Biotransformations of
194 with CFEs Prepared from Cell Cultures of Different Ages (Trp#311: 21
day, Trp#312: 13 day, Trp#313: 7 day)
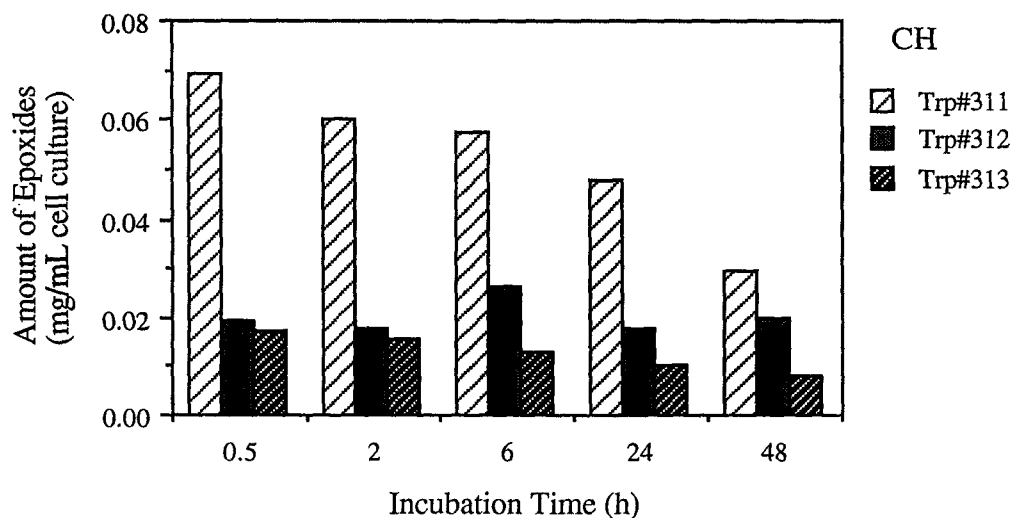
Figure 4.20   Comparison of the Epoxide Yields (Specific Yields) in Biotransformations of
194 with CHs Prepared from Cell Cultures of Different Ages (Trp#311: 21
day, Trp#312: 13 day, Trp#313: 7 day)

Comparison of results from RCPs of different ages, as shown by Figure 4.21, gave
some mixed information. However, it showed that RCPs from young cells gave comparable or

higher yields of epoxides than the RCP from a 21-day-old cell culture after 6 h incubation. Lower yields from the 7-day-old culture were due in part to the lower mass of cells per unit volume in comparison with the 13-day-old culture.
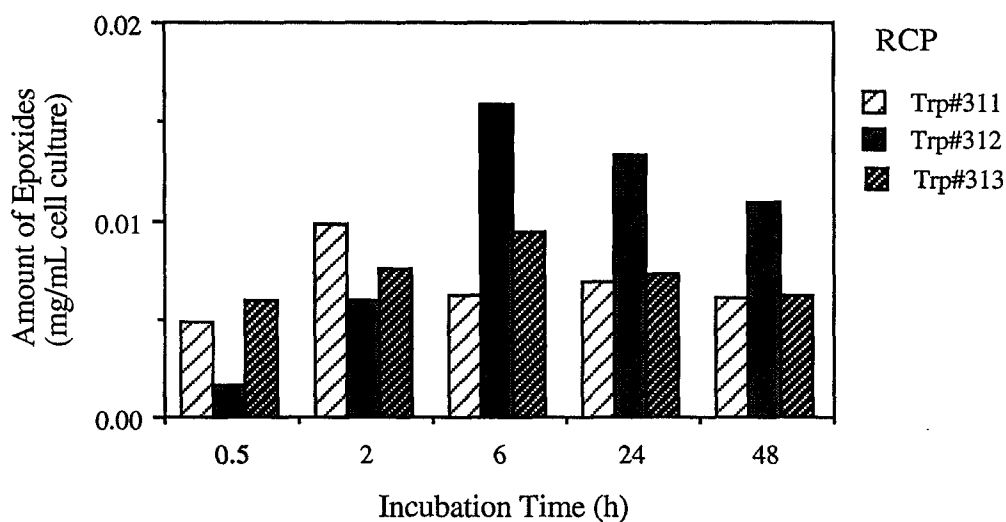
Figure 4.21   Comparison of the Epoxide Yields (Specific Yields) in Biotransformations of **194** with RCPs Prepared from Cell Cultures of Different Ages (Trp#311: 21 day, Trp#312: 13 day, Trp#313: 7 day)

As a summary, Figure 4.22 shows the combined results from CFEs, CHs and RCPs of TRP4a cell cultures of different ages. Both the cell homogenate and the cell free extract from 21-day-old cell cultures would be the best choices to perform biotransformation of isotriptophenolide (**194**) to obtain epoxides **305** and **306**. The optimal incubation time was from 0.5 to 2 h for CFE and 0.5 h or less for CH.
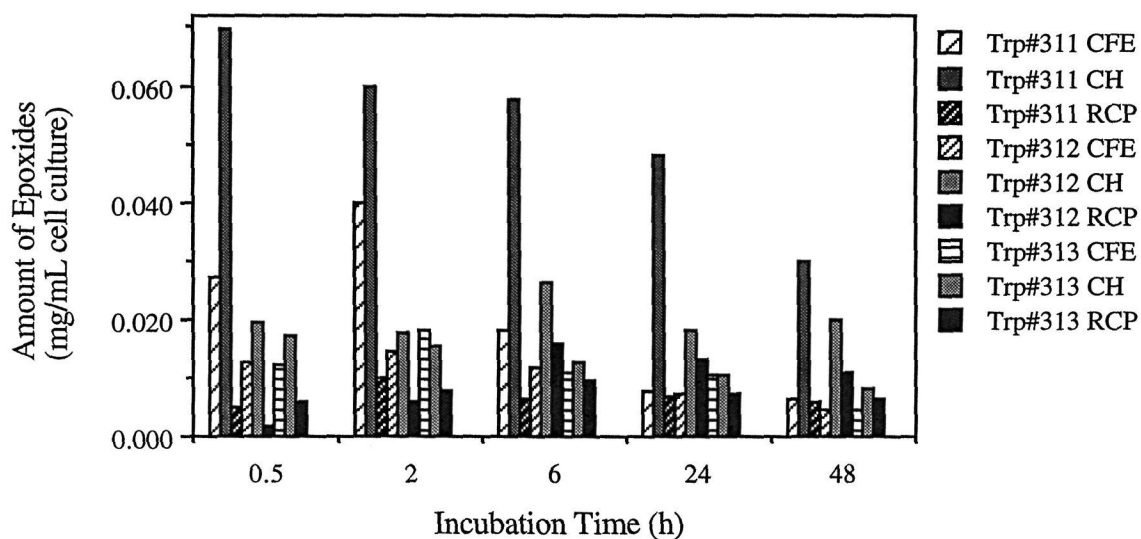
Figure 4.22   Comparison of the Epoxide Yields (Specific Yields) in Biotransformations of **194** with CFEs, CHs and RCPs Prepared from Cell Cultures of Different Ages (Trp#311: 21 day, Trp#312: 13 day, Trp#313: 7 day)

Biotransformation of 194 with CFEs and RCPs Obtained by Varying RCF* of Centrifugation

From the comparison of the yields of epoxides among CFEs, CHs and RCPs (Figure 4.16, 17, 18), it can be seen that the yields of epoxides decreased more rapidly in biotransformation with CFEs. This suggested that CFE may also contain some other enzymes which convert the epoxides to other compounds. If it was possible to partially purify the enzymes of interest, then this would improve the yields by reducing side reactions. Centrifugation is one of the convenient means to separate the enzymes from other cellular materials (which may be associated with other undesired enzymes) according to their masses.[255]

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* RCF: Relative Centrifugation Force.

Cell Homogenate (CH)
(90.5 g cells in 127 mL buffer)

centrifugation
(6,600*g*, 30 min)

Pellet 1
(discard)

CFE1 (~170 mL)

save

centrifugation
(30,000*g*, 30 min)

CFE1 (~50 mL)

Pellet 2

CFE2 (~120 mL)

buffer
(120 mL)

save

RCP2

centrifugation
(85,000*g*, 30 min)

CFE2 (~40 mL)

Pellet 3

CFE3 (~80 mL)

buffer
(80 mL)

RCP3
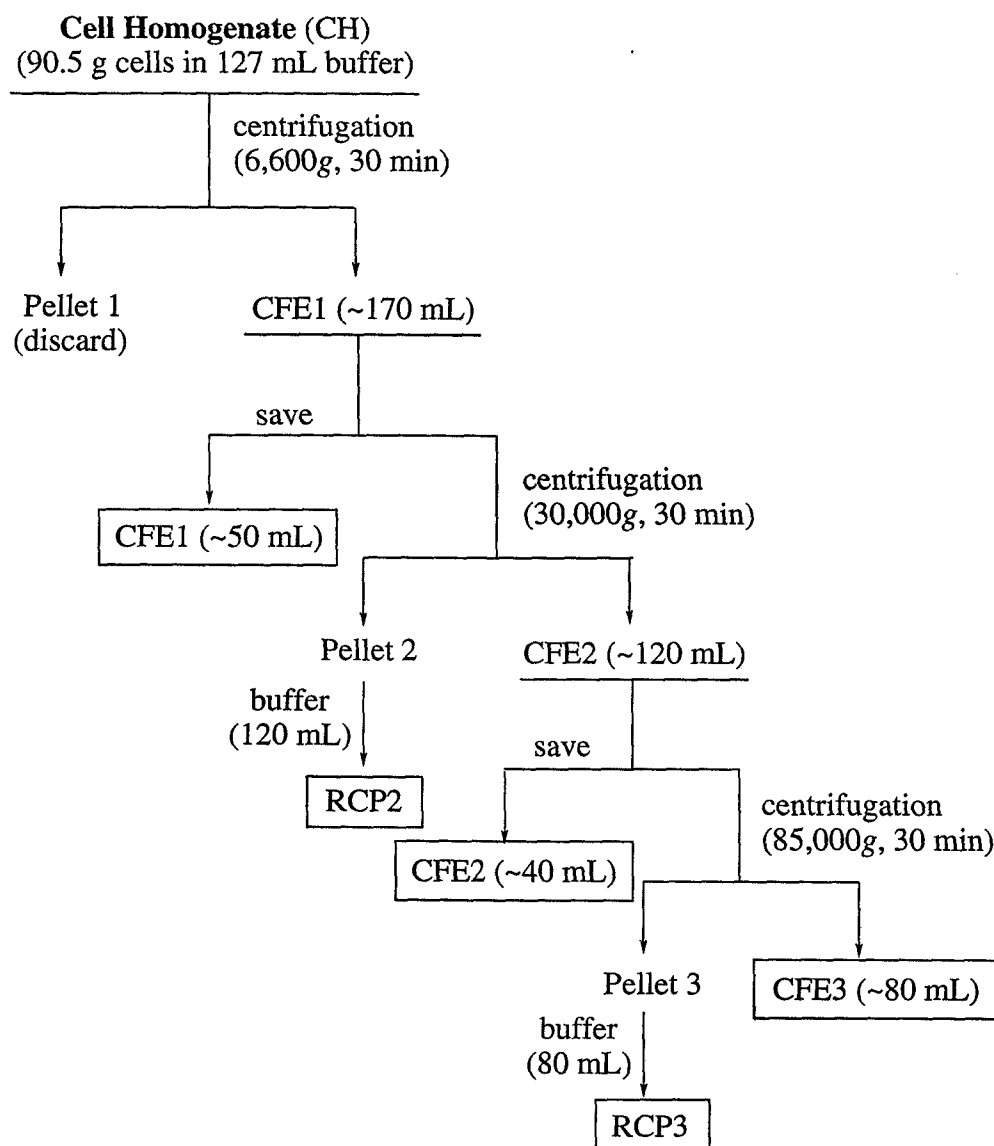
Figure 4.23   Preparation of CFEs and RCPs by Varying RCF of Centrifugation

Figure 4.23 illustrates the procedure for the preparation of CFEs and RCPs from cell homogenate by centrifugation at increasing RCF (Trp#314). A cell culture of 15 days old (2 x 550 mL) was used to prepare the CFEs and RCPs. The cells obtained (90.5 g) were suspended in phosphate buffer (127 mL, 0.1 M, pH 6.6) and homogenized. The cell homogenate was centrifuged at 4°C as usual at 10,000 *g* (maximum; average RCF, 6,600 *g*) for 30 min to give Pellet 1 and CFE1 (~170 mL). Pellet 1 was discarded and CFE1 (120 mL) was centrifuged

again at an average RCF of 30,000 g for 30 min to give CFE2 (~120 mL) and Pellet 2. Pellet 2 was washed with a small amount of buffer and resuspended in the buffer (120 mL). A portion (~80 mL) of the CFE2 obtained was then centrifuged further at an average RCF of 85,000 g for 30 min to yield Pellet 3 and CFE3 (~80 mL). Pellet 3 was washed with a small amount of buffer and resuspended in buffer (80 mL). Table 4.5 summarizes the peroxidase activities and protein concentrations of each fraction and their corresponding biotransformation conditions.

Table 4.5   Conditions for Biotransformation of **194** with CFEs and RCPs Prepared from a 15-day-old TRP4a Cell Culture by Varying RCF of Centrifugation (Trp#314)

		CFE1	CFE2	CFE3	RCP2	RCP3
assays	peroxidase activity (unit/mL)	6.21	5.09	5.55	0.0212	0.0212
	protein concentration (mg/mL)	1.22	1.17	1.15	0.069	0.076
composition of biotrans- formation mixture	CFE, RCP volume (mL)	20.1	24.6	22.5	70[a]	70[a]
	peroxidase (unit)	125	125	125	1.48	1.48
	distilled water (mL)	37.5	37.5	37.5	37.5	37.5
	phosphate buffer (0.1 M, pH 6.6) (mL)	87.5	87.5	87.5	87.5	87.5
	0.24% H_2O_2 (equiv.)	2.2	2.2	2.2	2.2	2.2
	$MnCl_2$ (equiv.)	0.5	0.5	0.5	0.5	0.5
	FMN (equiv.)	0.5	0.5	0.5	0.5	0.5
substrate	ITP (**194**) (mg)	23.8	23.8	23.8	23.8	23.8
solvent	ethanol (mL)	10	10	10	10	10

[a] All the RCP2, RCP3 available.

Figure 4.24 summarizes the results obtained. In general, the activities of RCP2 and RCP3 were lower than the corresponding CFEs, and RCP3 was the lowest. This suggested that the enzymes for epoxidation were not bound to the cell membrane. The reaction with CFE1 followed the usual course, $i.e.$, the yields of epoxides increased rapidly and then decreased

rapidly. The reaction with CFE2, on the other hand, showed a comparable yield at the beginning but showed a general trend of increasing yields throughout the experiment. CFE3 behaved similarly as CFE2, though it showed slightly lower yields than CFE2. This result indicated that CFE obtained by centrifugation with an RCF of 30,000 g (average) could increase the yield of epoxides by removing some substances which may have a negative influence on the yield of epoxides.
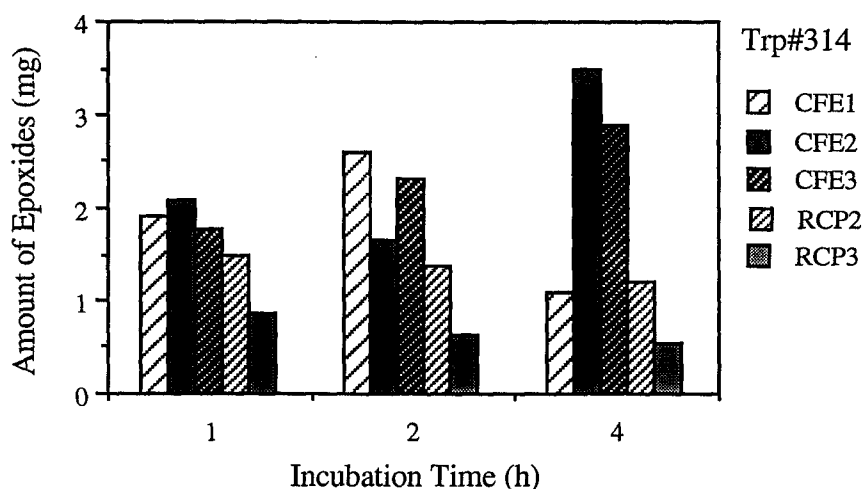
Figure 4.24   Comparison of the Epoxide Yields in Biotransformations of **194** with CFEs and RCPs Obtained by Varying RCF of Centrifugation

Influence of pH on the Yield of the Epoxides (305, 306) in CFE Biotransformations

The previous biotransformations with CFEs, CHs and RCPs were carried out in phosphate buffer at pH 6.6. This pH value was adopted, as a starting point, from other experiments with CFEs from TRP4a cell cultures.[163]

However, this pH may not be appropriate for the enzymes involved in the present biotransformations. The pH values of the broth of the TRP4a cell cultures usually ranged from

4.5 to 6.0. This may not be the pH at which the enzymes function most effectively, but it gave some indication at which pH the cells had adapted and it was reasonable that within this pH range, an optimal pH for the relevant enzymes may exist.

Table 4.6 lists the conditions of biotransformations of **194** with CFEs obtained by using phosphate buffers of different pH (pH 7.6-5.0) (Trp#315). The cells obtained from a 21-day-old culture were divided equally into 6 portions, which were then mixed with phosphate buffers of pH 7.6, 7.0, 6.6, 6.2, 5.8 and 5.0, respectively. Each of the resulting mixtures was homogenized and centrifuged to give 6 CFEs. Their peroxidase activities and protein concentrations were measured accordingly. An equal volume of CFE was used for each of the biotransformation mixtures so that each biotransformation mixture would contain an equal amount of the relevant enzymes, even though the number of peroxidase units varied with pH as indicated by Table 4.6.

The starting material, isotriptophenolide (**194**), was added to each biotransformation mixture. Samples were removed from each flask at 1h and 2h intervals, and the remainder was worked up at 4h. Samples were extracted, and analyzed by TLC and HPLC. Figure 4.25 shows the results from HPLC analysis. It can be seen that, in general, there were no significant differences among the CFEs made from phosphate buffers of pH 7.6 to 5.0, though CFEs of pH about 6.2 appeared to give higher yields in the late stages of the biotransformation.

A second experiment with CFEs prepared from acetate buffers of pH from 5.0 to 4.0 was also performed (Trp#317). The biotransformation conditions are given in Table 4.7 and the HPLC results in Figure 4.26. A CFE made from phosphate buffer (0.1 M, pH 5.0) was used in the biotransformation at the same time as a reference (Entry 1 in Table 4.7)

Table 4.6   Conditions for Biotransformation of **194** with CFEs Prepared from Phosphate Buffers of Different pH (Trp#315)

	pH of the buffer	7.6	7.0	6.6	6.2	5.8	5.0
assays	peroxidase activity (unit/mL)[a]	6.63	6.29	5.82	5.24	4.81	4.64
	protein concentration (mg/mL)[b]	1.13	1.19	1.11	1.12	1.03	0.89
composition of biotrans- formation mixture	CFE volume (mL)	27	27	27	27	27	27
	peroxidase (unit)	179	170	157	142	130	125
	distilled water (mL)	33.5	33.5	33.5	33.5	33.5	33.5
	phosphate buffer (0.1 M) (mL)[c]	87.5	87.5	87.5	87.5	87.5	87.5
	0.24% H_2O_2 (equiv.)	2.2	2.2	2.2	2.2	2.2	2.2
	$MnCl_2$ (equiv.)	0.5	0.5	0.5	0.5	0.5	0.5
	FMN (equiv.)	0.5	0.5	0.5	0.5	0.5	0.5
substrate	ITP (**194**) (mg)	25	25	25	25	25	25
solvent	ethanol (mL)	10	10	10	10	10	10

[a], [b] The peroxidase activity and protein concentration of each CFE were measured with the corresponding buffer, rather than with pH 6.6 phosphate buffer; [c] The buffer of corresponding pH was used to prepare the individual CFE.

Figure 4.26 shows a trend that the yields of epoxides seemed to be higher when the pH was around 4.7, which, coincidentally, was the most common pH value of the broth of 21-day-old cell cultures. This suggested that the enzymes responsible for synthesis of the epoxide system may be more active at approximately this pH. The change from phosphate buffer to acetate buffer may also have some influence on the results, yet both buffers produced an equivalent yield at pH 5 after a 4 h incubation. In this experiment, there appeared to be a correlation between the yields of epoxides and the pH of the buffer systems.

Table 4.7   Conditions for Biotransformation of **194** with CFEs Prepared from Acetate
Buffers of Different pH (Trp#317)

	pH of the buffer	5.0[a]	5.0[b]	4.7[b]	4.4[b]	4.0[b]
assays	peroxidase activity (unit/mL)	4.15	3.09	2.61	2.33	2.25
	protein concentration (mg/mL)	1.09	0.70	0.51	0.38	0.26
composition of biotrans- formation mixture	CFE volume (mL)	23	23	23	23	23
	peroxidase (unit)	95	71	60	54	52
	distilled water (mL)	25.4	25.4	25.4	25.4	25.4
	buffer volume (mL)	63.4	63.4	63.4	63.4	63.4
	0.24% H_2O_2 (equiv.)	2.2	2.2	2.2	2.2	2.2
	$MnCl_2$ (equiv.)	0.5	0.5	0.5	0.5	0.5
	FMN (equiv.)	0.5	0.5	0.5	0.5	0.5
substrate	ITP (**194**) (mg)	18.2	18.2	18.2	18.2	18.2
solvent	ethanol (mL)	7.7	7.7	7.7	7.7	7.7

[a] Phosphate buffer (0.1 M) was used in this entry as a reference; [b] Acetate buffer (0.1 M). In order to compare with Trp#315, the CFE mixture made from phosphate buffer was prepared according to the ratio used in Trp#315. The composition of the remaining CFE mixtures (made from acetate buffers) was then set accordingly.

Figure 4.27 summarizes the yields based on recovered starting material from both experiments (Trp#315 and 317), with an adjustment made as based on the volume of the cell cultures which were used in preparing the CFEs (the specific yield). It shows a positive correlation between lower pH (4-5) and increased yields, especially in the later stages of the biotransformation. However, there may be more than one enzyme involved in the synthesis of the epoxides, and each enzyme may have particular optimal pH. On the other hand, pH may also affect other enzymes in the CFE, for example, those causing side reactions. Therefore, the dependence of epoxide yields on pH may be quite complex, and further investigation is needed.
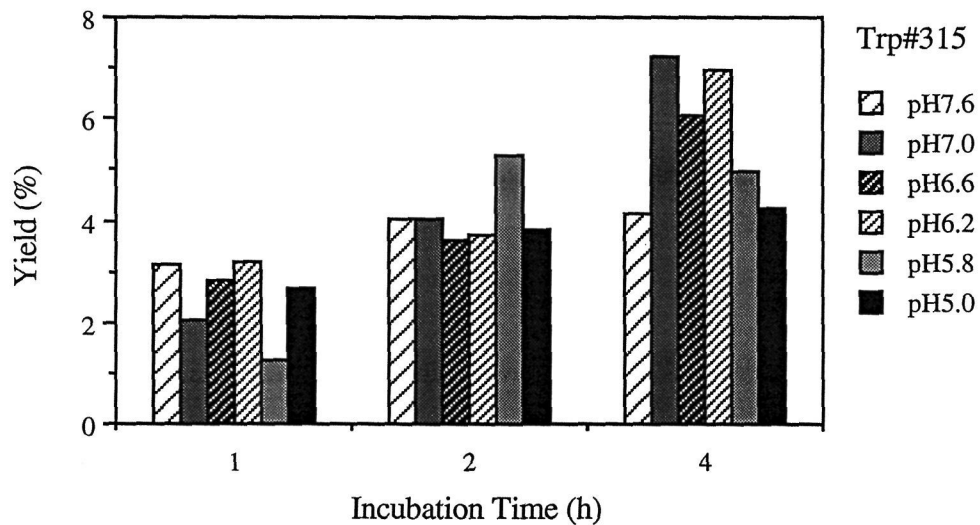
Figure 4.25   Comparison of the Epoxide Yields in Biotransformations of **194** with CFEs
Prepared from Phosphate Buffers of Different pH
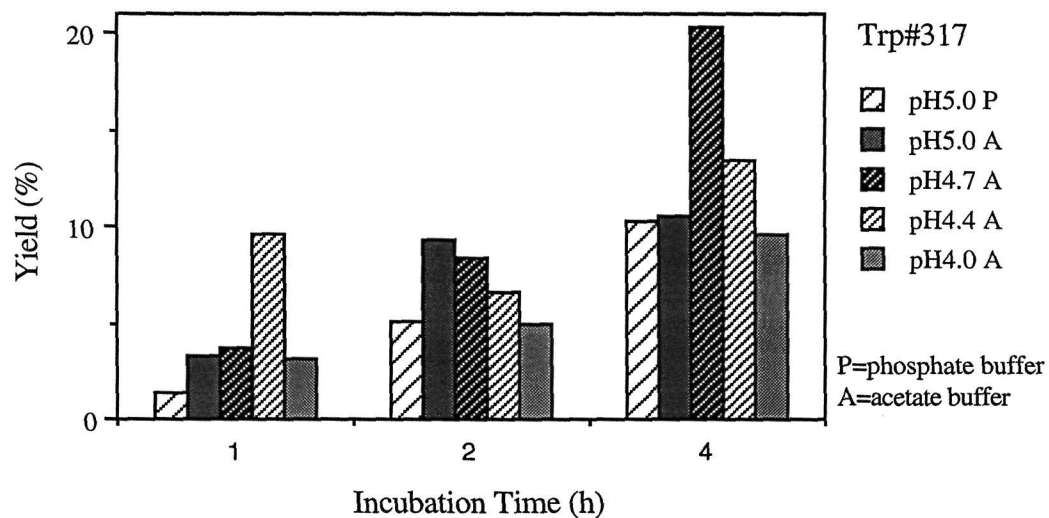
Figure 4.26   Comparison of the Epoxide Yields in Biotransformations of **194** with CFEs
Prepared from Acetate Buffers of Different pH
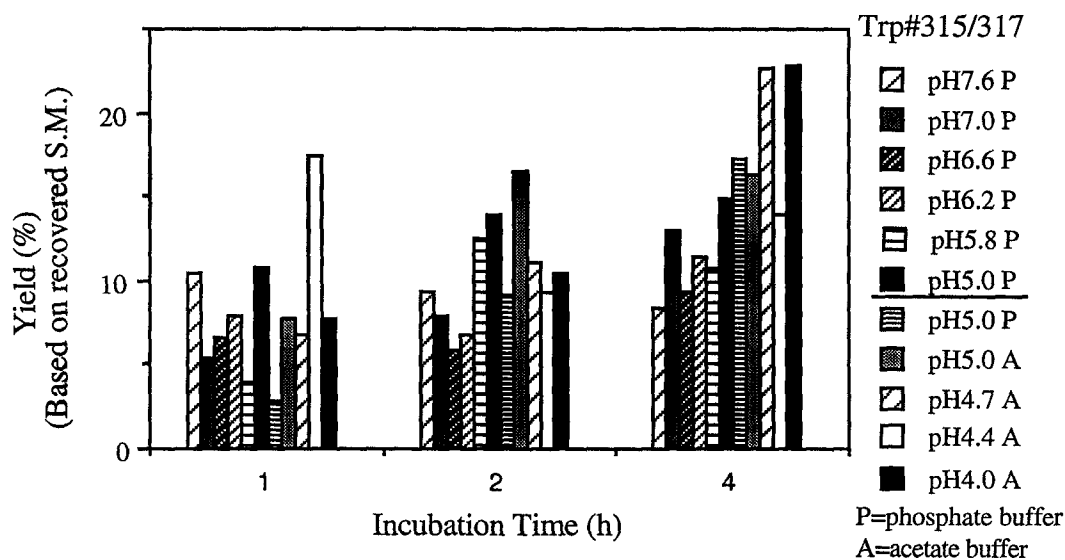
Figure 4.27   Comparison of the Epoxide Yields (based on recovered starting material) in Biotransformations of **194** with CFEs Prepared from Buffers of Different pH

Influence of Starting Material-to-CFE Ratio on the Yield of the Epoxides (305, 306)

The ratio of starting material-to-CFE was another factor which may affect the yield of the epoxides. A 14-day-old cell culture was used to prepare the CFE in a phosphate buffer (0.1 M, pH 5.0). The resulting CFE had a peroxidase activity of 5.82 unit/mL and a protein concentration of 0.83 mg/mL.

Table 4.8 gives the details of the experimental conditions. A volume of 21.5 mL of CFE (125 units of peroxidase) was used as the base amount. Amounts of twice and four times of the base amount were used in the other two flasks, respectively. The differences in volume were eliminated by adding corresponding amounts of buffer to equalize the total volumes in the experiments. The results (Figure 4.28) showed that with increasing amounts of CFE, the yields

did increase significantly, even though not in direct proportion to the amount of CFE used. Experiments with higher CFE-to-starting material ratio were not continued beyond this point, but an optimal ratio could certainly be found with additional studies.

Table 4.8   Conditions for Biotransformation of **194** with Different Starting Material-to-CFE Ratios (Trp#316)

		1	2	3
composition of biotrans-formation mixture	CFE volume (mL)	21.5	43	86
	peroxidase (unit)	125	250	500
	distilled water (mL)	37.5	37.5	37.5
	phosphate buffer (0.1 M, pH 5.0) (mL)	87.5	66	23
	0.24% H_2O_2 (equiv.)	2.2	2.2	2.2
	$MnCl_2$ (equiv.)	0.5	0.5	0.5
	FMN (equiv.)	0.5	0.5	0.5
substrate	ITP (**194**) (mg)	25	25	25
solvent	ethanol (mL)	10	10	10
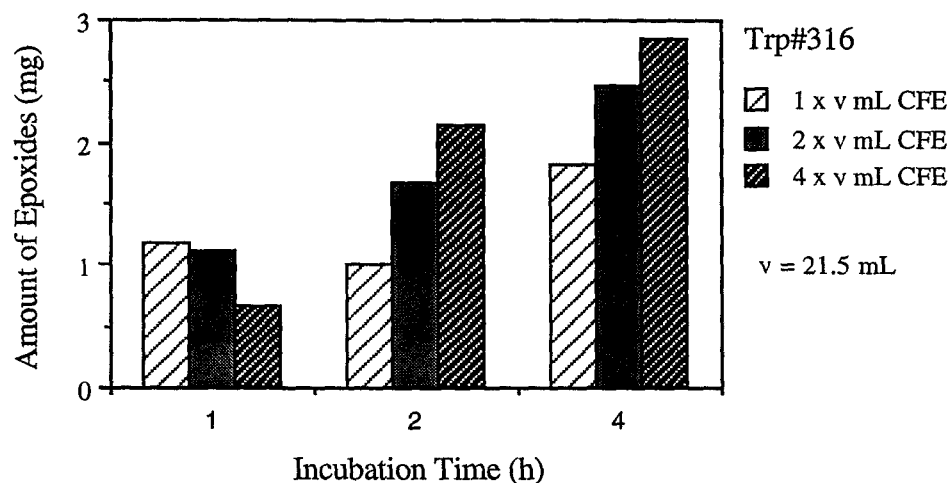
Figure 4.28   Comparison of the Epoxide Yields in Biotransformations of **194** with Different Starting Material-to-CFE Ratios

Influence of Equivalents of Hydrogen Peroxide on the Yield of the Epoxides (305, 306)

Hydrogen peroxide was used in the experiments with CFE (or CH, RCP) on the assumption that peroxidase enzymes, which generally require hydrogen peroxide as a cofactor, might be involved in the biotransformation of the substrates. However, the results with isotriptophenolide (**194**) obtained so far showed no direct relationship of peroxidase activity with the yield of the epoxides. In order to examine whether peroxidases were involved in the epoxide formation, the influence of cofactor hydrogen peroxide was evaluated.

Table 4.9   Conditions for Biotransformation of **194** with Different Equivalents of Hydrogen Peroxide (Trp#318a)

		1	2	3
composition	CFE volume (mL)	24	24	24
of	peroxidase (unit)	100	100	100
biotrans-	distilled water (mL)	32	30	24
formation	acetate buffer (0.1 M, pH 4.7) (mL)	70	70	70
mixture	0.24% H_2O_2 (mL) (equiv.)	0 (0)	2 (2.2)	8 (8.8)
	$MnCl_2$ (equiv.)	0.5	0.5	0.5
	FMN (equiv.)	0.5	0.5	0.5
substrate	ITP (**194**) (mg)	20	20	20
solvent	ethanol (mL)	8	8	8

A 14-day-old cell culture was used to prepare the CFE using acetate buffer (0.1 M, pH 4.7). The resulting CFE had a peroxidase activity of 4.15 unit/mL and a protein concentration of 0.659 mg/mL. The reactions (Trp#318a) were set up in the standard ratio except for the amounts of hydrogen peroxide (Table 4.9). The results are presented in Figure 4.29. Very interestingly, the experiment with no hydrogen peroxide added gave almost the same results as

those with 2.2 or 8.8 equivalents of hydrogen peroxide. There were no significant differences among the three experiments. This result strongly indicated that peroxidases were not the enzymes, or at least not the key enzymes responsible for the epoxide formation in this biotransformation process. However, the addition of hydrogen peroxide was not detrimental to the yields of epoxides.
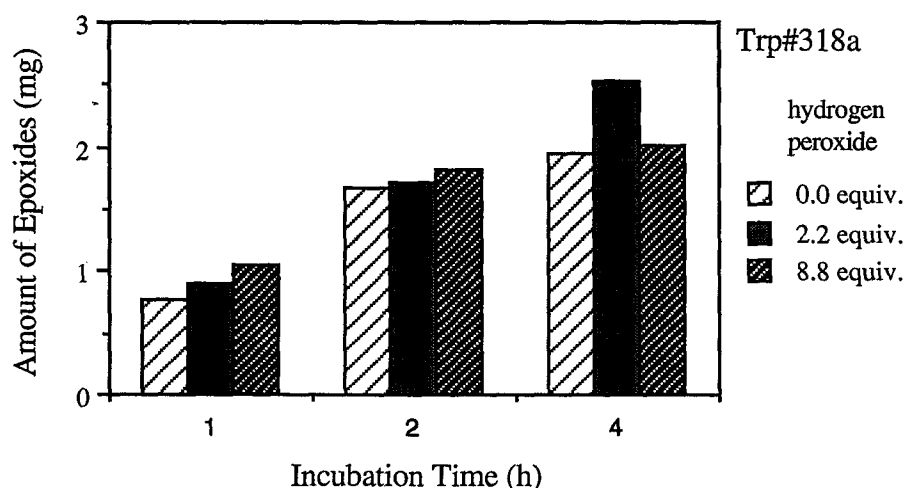
Figure 4.29   Comparison of the Epoxide Yields in Biotransformations of **194** with
                Different Equivalents of Hydrogen Peroxide

Influence of Metal Ions on the Yield of the Epoxides (305, 306)

Metal ions are involved in a wide variety of biological processes. An estimated one-third of all enzymes require a metal ion in one or more phases of the catalytic process.[256] Serving as essential structural components, metal ions participate in oxidative and hydrolytic reactions. Heme groups (Fe^{2+}) and hematin (Fe^{3+}), most frequently involving a complex with protoporphyrin, are the coenzymes of a number of redox enzymes, including catalase and peroxidases. A group of heme proteins known as cytochrome P-450 plays a very important role in oxidative biological processes in animals, plants and microorganisms. Many biological

oxidations, including hydroxylations are catalyzed by cytochrome P-450 enzymes.[257] There are many enzymes which also contain metal ions to maintain their structures and activities.[256]

Metal ions may be lost during the preparation of CFE and this may have an adverse effect on the epoxide yield, if they are part of the epoxide synthesis enzyme system. Therefore, metal ions are usually used as cofactors in such circumstances.

In the previous experiments, manganese (II), as the chloride, was used as a cofactor. The most common metal ions found in enzymes include Mn, Fe, Co, Cu, and Zn.[256] Earlier studies by this group on the cell culture media indicated that the level of micronutrients (e.g., Mn^{2+}, Zn^{2+}, Cu^{2+} and Co^{2+}) was important to the yield of Td (2).[147] In order to examine whether and which metal ions have an impact on the yield of the epoxides, three additional metal ions, cobalt (II), nickel (II) and zinc (II) were tested in comparison with manganese (II). An experiment without a metal ion being added was conducted simultaneously.

Table 4.10   Conditions for Biotransformation of 194 with Different Metal Ions Being Added (Trp#320)

		1	2	3	4	5
composition of biotrans- formation mixture	CFE volume (mL)	45	45	45	45	45
	peroxidase (unit)	125	125	125	125	125
	distilled water (mL)	37.5	37.5	37.5	37.5	37.5
	acetate buffer (0.1 M, pH 4.7) (mL)	87.5	87.5	87.5	87.5	87.5
	0.24% H_2O_2 (equiv.)	2.2	2.2	2.2	2.2	2.2
	metal ions (0.5 equiv.)	none	Mn (II)	Co (II)	Ni (II)	Zn (II)
	FMN (equiv.)	0.5	0.5	0.5	0.5	0.5
substrate	ITP (194) (mg)	25	25	25	25	25
solvent	ethanol (mL)	10	10	10	10	10

A 21-day-old cell culture was used in this experiment. The cells obtained were homogenized in an acetate buffer (0.1 M, pH 4.7). Centrifugation of the resulting cell

homogenate gave a CFE with a peroxidase activity of 2.78 unit/mL and a protein concentration of 0.375 mg/mL. The CFE was divided into aliquots of 45 mL (125 peroxidase unit) and added to five flasks. The experiments were set up as shown in Table 4.10 and the results are given in Figure 4.30.
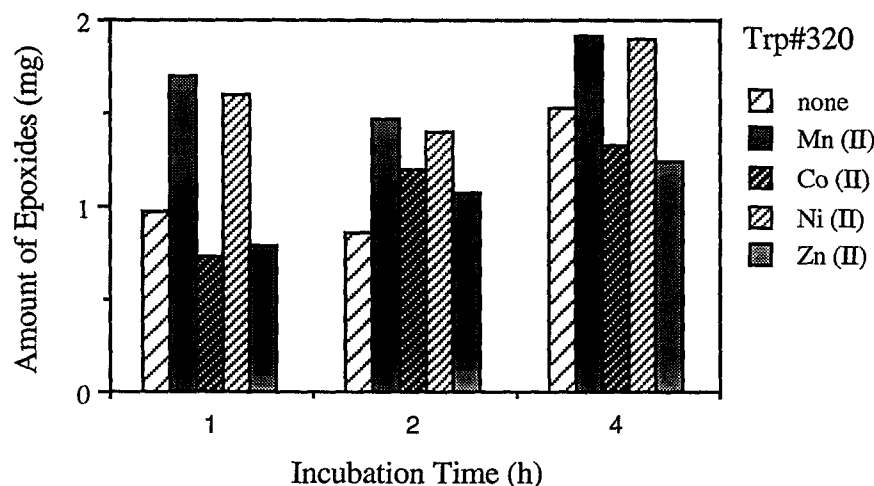
Figure 4.30   Comparison of the Epoxide Yields in Biotransformations of 194 with Different Metal Ions Being added

It can be seen from Figure 4.30 that CFE without addition of metal ions was capable of transforming isotriptophenolide (194) to the epoxides, but addition of manganese (II) and nickel (II) ions appeared to enhance the yield of the epoxides, while cobalt (II) and zinc (II) ions showed little effect.

In summary, the above studies with CFEs etc. from TRP4a cell cultures have provided some interesting results, which have either confirmed some data previously obtained from the whole cell experiments (such as the influences of cell ages, starting material amounts, etc.), or afforded new information and understanding about this cell culture (such as a rapid rate of biotransformation in CFE etc., the association of the relevant enzymes with the cell plasma rather than with cell membranes, the influences of pH, hydrogen peroxide, metal ions, etc.).

Biotransformation of 7-Hydroxy-Isotriptophenolide (246) with TRP4a Cell Cultures

From the experiments of isotriptophenolide (194) with whole cell cultures (*vide supra*), various products, such as the epoxides 305 and 306, 7-hydroxy-isotriptophenolide (246) and others were isolated. It was appropriate to consider the relationship between these products in the hope that some understanding as to their formation could be obtained.

There are two plausible mechanistic pathways for introduction of the C7, C8 epoxide functionality (section 2.2). In the biotransformation of isotriptophenolide (194), the starting material may be first hydroxylated at C7, and then be converted to the epoxides; or it may be first oxidized to the quinone methide intermediate 249 and then undergo epoxidation (Scheme 4.5).

In order to examine whether 7-hydroxy-isotriptophenolide (246) is the intermediate to the epoxides, an experiment was performed using 7-hydroxy-isotriptophenolide (246) as starting material. Though 7β-hydroxy-isotriptophenolide (246b) was available through synthesis, 7α-hydroxy-isotriptophenolide (246a) was chosen as precursor since the α-epoxide 306 was usually the major product obtained in the biotransformation experiments. Fortunately, almost pure 246a isolated from a biotransformation experiment with 194 was available.

A solution of 246a in ethanol was added to a cell culture (21 days old) and incubated under standard conditions (Trp#340). Samples were taken after incubation for 1 and 3 days. TLC analysis showed no sign of epoxides formation. The culture was harvested after 7 days, and the cells and broth were extracted. The extracts were chromatographed and several compounds were isolated (recovery of material was about 61%) (Table 4.11). However, no epoxides were isolated in this experiment (Scheme 4.6).
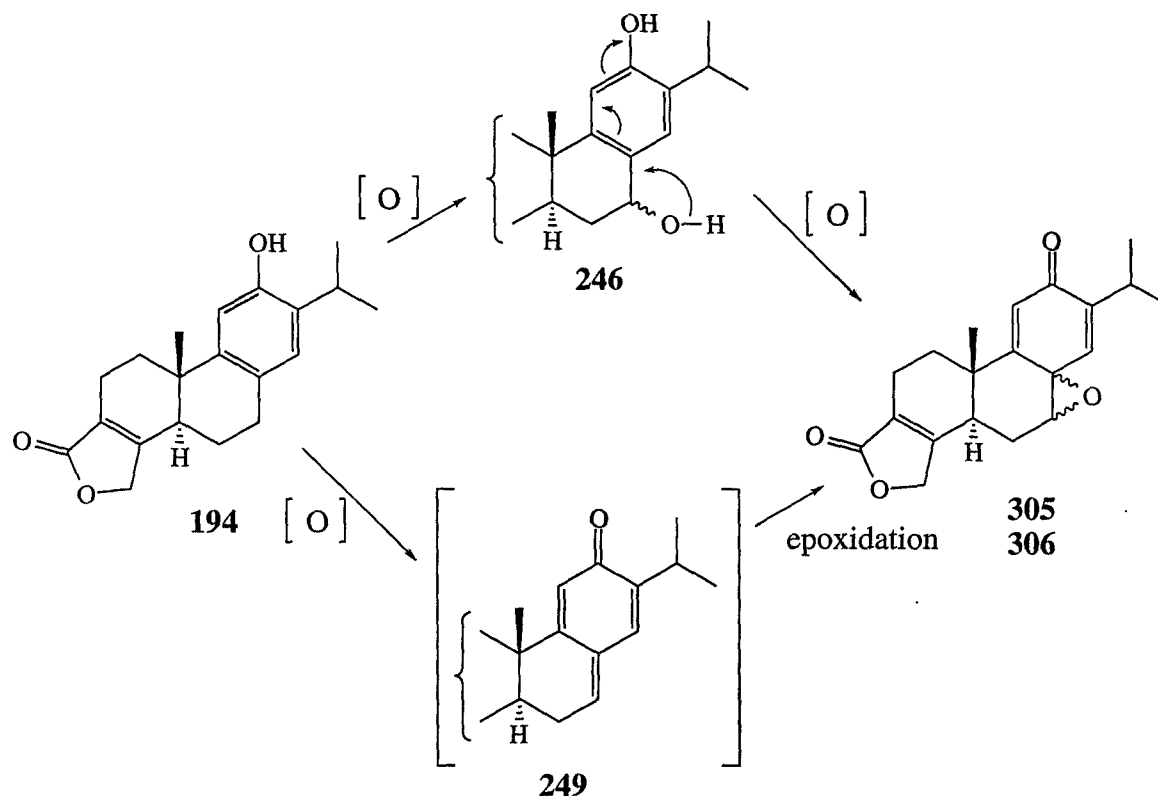
Scheme 4.5   Proposed Mechanism for the Formation of Epoxides **305** and **306**

Table 4.11   Compounds Isolated from Biotransformation of **246** with the TRP4a Cell Culture

Compound	246[a]	245	307	310	252
mg	3.1	4.7	3.3	1.5	0.6

[a] Recovered starting material, in which **246a** was ~70% determined by [1]H NMR.

Compound **310** showed a very similar [1]H NMR spectrum to that of **246a**, but it exhibited a methoxy signal at δ 3.84 in the spectrum, and its H11 and H15 signals were slightly shifted downfield. This was apparently caused by the methoxy group at C12. Methylation at the C12 phenolic hydroxyl group was also found in experiments with **194** so such methylation appeared quite general with the enzymes of TRP4a.
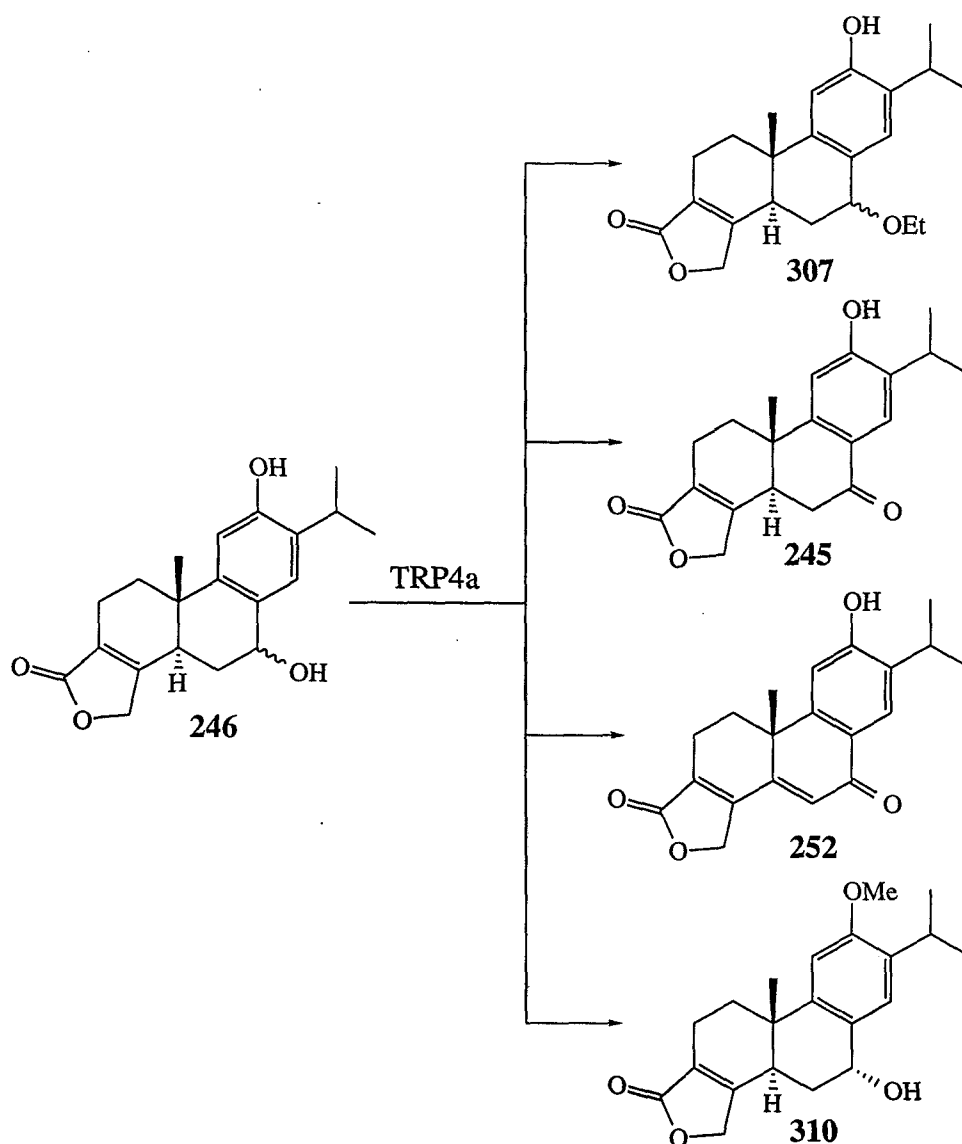
Scheme 4.6   Biotransformation of 7-Hydroxy-isotriptophenolide (246) with the TRP4a Cell Culture

Compounds 245 and 252 were apparently the oxidation products of the starting material 246. There was the possibility that the C7 ether functionality of 295 was formed through enzymatic activation of 246 followed by the addition of ethanol, since the latter was the solvent employed to dissolve the substrate prior to addition. Since the culture medium was slightly acidic (pH 4.5-6.0), ethanol or water acting as a nucleophile may substitute the protonated C7-

hydroxyl group, or attack the carbon cation if it is involved, to form C7 substituted compounds, such as **307**, or **246a** and **246b** (Figure 4.32). The increased percentage of **246b** (7β-isomer) in the recovered 7-hydroxy compounds, as compared with that in the starting material, may also be partially contributed by this type of "exchange" reactions. The above reactions were likely to proceed without enzyme catalysis. This was confirmed by a small scale blank experiment with the starting material being "incubated" in a sterile medium. After 7 days of "incubation", TLC analysis showed spots corresponding to dehydrated compound **247**, the 7-ethoxy compound **307**, the 7-oxo compound **245** and unreacted starting material **246**. ^{1}H NMR analysis of the crude extract from the blank experiment confirmed the presence of the above compounds. The amount of **246** corresponded to about 57% in the mixture and the ratio of α isomer **246a** to the β isomer **246b** was lower than that in the starting material.

The most interesting result from this experiment was that epoxides **305** and **306** were not detected during the biotransformation, or isolated in the final products. It is clear that the formation of the C7, C8 epoxy group to provide the metabolites **305** and **306** does not occur via **246** as an intermediate but rather via the quinone methide intermediate **249**. It is reasonable to assume that a similar epoxidation may occur in the biosynthesis of Tl (**1**) and Td (**2**) via the quinone methide intermediate **219**. However, direct evidence to support this postulation is not available from the present study. The formation of the ketones **245**, **252** may occur via either enzymatic or atmospheric oxidation, but the formation of the 7-ethoxy compound **307** probably occurs as shown in Figure 4.32.

247
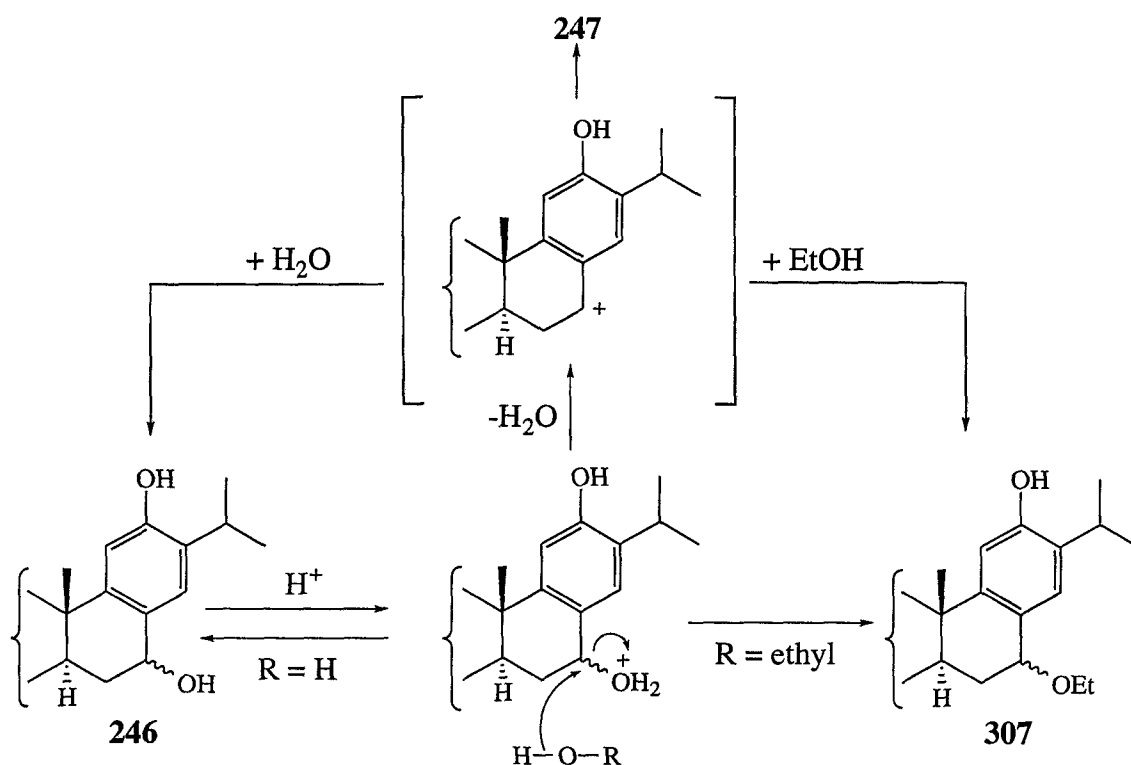
Scheme 4.7   Proposed Mechanism for the Formation of **307** and **247**

Biotransformation of the Epoxides (305, 306) with TRP4a Cell Cultures

In the time course studies of biotransformation of isotriptophenolide (**194**) with TRP4a cell cultures (*vide supra*), it was found that in the late stages of the incubation, the level of the epoxides **305** and **306** remained essentially constant, whereas the starting material continued to decrease. One explanation could be that the epoxides were further transformed to other products. However, we did not isolate any further epoxidized products, such as diepoxides or triepoxides, which were what we expected from a cell line that produces Tl (**1**) and Td (**2**) (note: a small amount of a diepoxide was isolated in a large scale experiment later, *vide infra*). In the biotransformation of **194**, the epoxides were present as relatively major components in the fermentation mixture and remained in the culture throughout the entire incubation.

Unfortunately, the presence of many other products made it difficult to detect their subsequent biotransformation products. Therefore, it was interesting to see what would happen if the epoxides alone were incubated with the cell cultures.

A solution of the epoxides (ratio of **305**:**306** about 1, isolated from biotransformations of **194**) in ethanol was added to a cell culture of 21 days old and incubated under standard conditions (Trp#338). Samples were withdrawn after 48 h of incubation and checked by TLC. It was a surprise to find that a polar spot (R_f 0.19; epoxides R_f 0.33) appeared on the TLC in a rather substantial amount. This spot had the identical R_f, and color after spraying, as 7-hydroxy isotriptophenolide (**246**). The experiment was continued for another 42 h and then the culture was harvested. The cells and broth were extracted with ethyl acetate, respectively and the combined extract was repeatedly chromatographed to yield 4 products along with recovered starting material (Table 4.12) (Scheme 4.8). The total recovery of material was about 61%.

Table 4.12   Compounds Isolated from Biotransformation of **305**, **306** with the TRP4a Cell Culture

Compound	**305**[a]	**245**	**307**	**246**[b]	**252**
mg	8.8	11.6	7.3	4.7	trace

[a] Recovered starting material, almost pure **305**; [b] α isomer **246a** in 65-70% by [1]H NMR.

It was very surprising to find that the epoxides were reduced by the cells to 7-hydroxy isotriptophenolide (**246**). A small scale blank experiment was carried out in a sterile medium. After "incubation" for the same period of time, the starting material was recovered unchanged, while only a small amount of **245** (less than 7%) was detected by [1]H NMR in the crude extract. Therefore, the other products from the biotransformation with the cell culture, such as **307**, **252** and **245**, were as a result of further reactions on **246**, although **245** could also be partially produced directly from the epoxides.
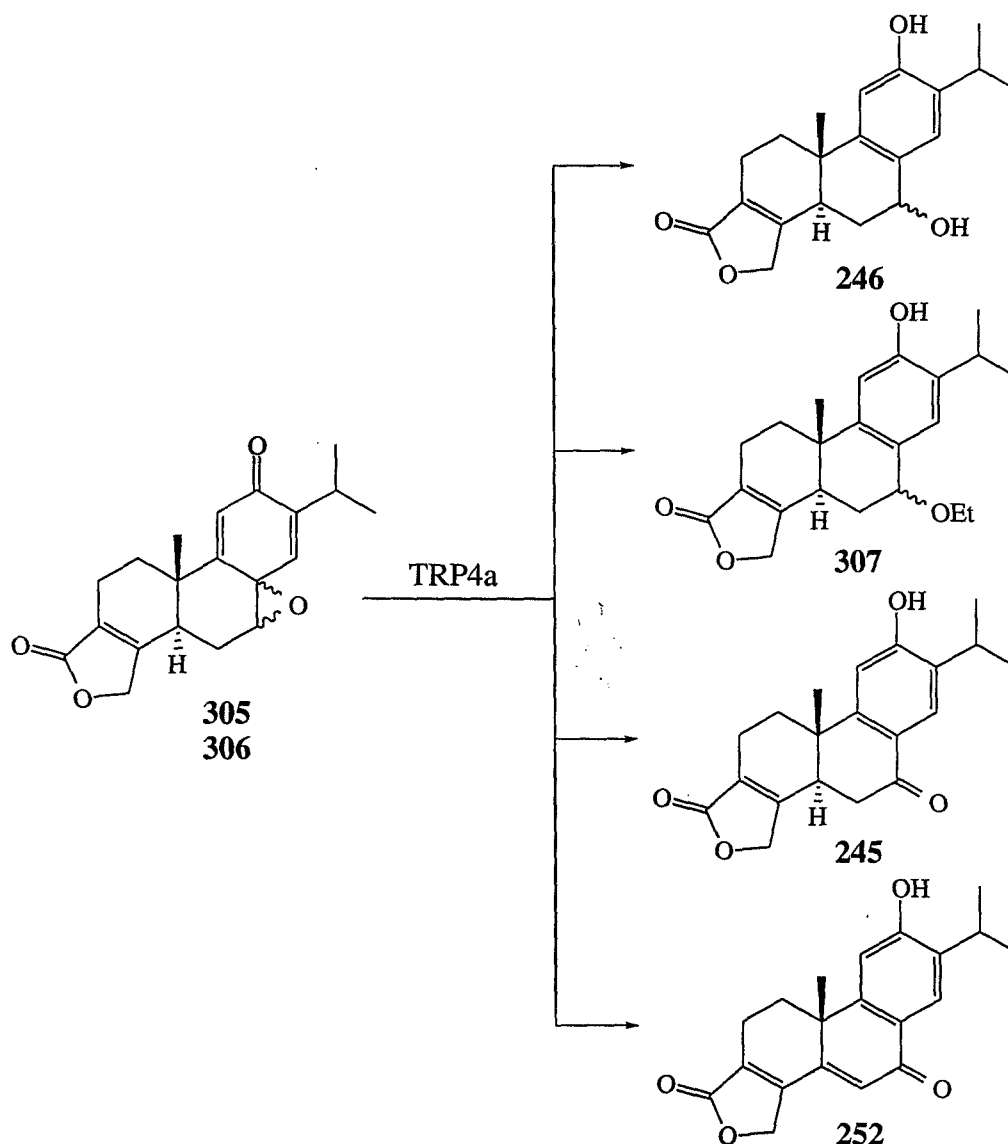
Scheme 4.8   Biotransformation of the Epoxides **305** and **306** with the TRP4a Cell Culture

The conversion of the epoxides **305** and **306** to the 7-hydroxy compound **246** in TRP4a cell cultures provided further evidence that the C7, C8 epoxy group was not derived from the C7-hydroxyl group. Considering this and the result from the biotransformation of 7-hydroxy-isotriptophenolide (**246**), we can conclude that 7-hydroxy-isotriptophenolide is derived from the epoxides, not the opposite (Scheme 4.9).
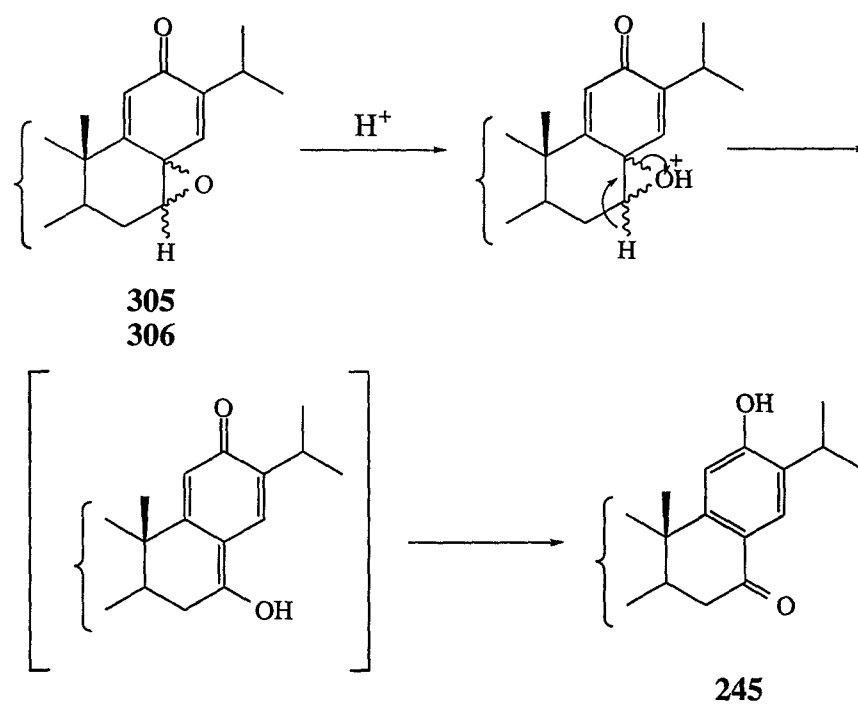
alternative formation of **245** from the epoxides

It seemed quite interesting that oxidation (epoxidation) and reduction could be carried out by the same cells at the same time, though they unlikely proceeded in the same pathway. However, if the biosynthesis of Tl (**1**) and Td (**2**) proceeds as has been proposed (section 2.2), there must be one reduction step in the later stage to reduce the keto group to a hydroxyl group at C14.
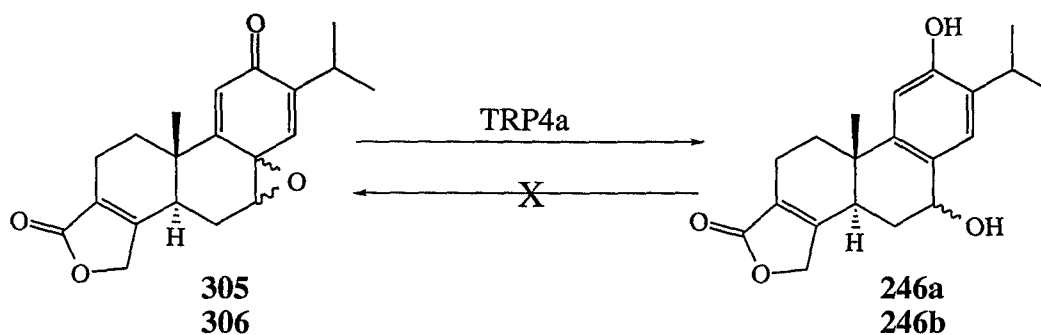
Scheme 4.9   Relationship between the Epoxides (**305, 306**) and 7-Hydroxy-
             isotriptophenolide (**246a, 246b**)

Epoxidation at C7, C8 followed by reduction also explained the results obtained from the time course studies with the whole cell cultures (*vide supra*). The starting material **194** was transformed to the epoxides (**305, 306**) via the quinone methide pathway, and then the epoxides were reduced to 7-hydroxy-isotriptophenolide (**246**). As a result, the starting material kept decreasing, but the concentration of the epoxides was not increased. The resulting 7-hydroxy compounds were then oxidized, most likely by the cells (sections 4.2.1), to **245** and **252**, or converted via nucleophilic substitution by ethanol to afford **307**. Dehydration of 7-hydroxy-isotriptophenolide (**246**) in the cell culture may also provide a path to form the quinone methide intermediate **249**, however no epoxidation was observed.

Based on the above results, the yields of epoxides **305** and **306** could be further improved, either by selecting the optimal conditions for the biotransformation, or by inhibiting the activity of, or removing, the reductive enzymes which converted the epoxides to the 7-hydroxylated compounds .

If the reduction of the epoxides to the corresponding 7-hydroxylated compounds could be decreased, not only the yield of the epoxides but also the overall recovery of material would be improved, because these 7-hydroxylated compounds and their dehydrated products were readily decomposed. In previous studies, the dehydrated compound **247** was found to be unstable. For example, samples remaining in NMR tubes for several days became a complicated mixture which could not be analyzed by NMR. This rapid degradation could perhaps explain some of the material losses in these biotransformation experiments.

Biotransformation of 194 with TRP4a Cell Cultures: Large Scale Experiment (Trp#336)

In order to find out whether any other products which were not isolated in the small scale experiments, and also as a conclusion to the biotransformation studies of isotriptophenolide (**194**) with TRP4a cell cultures, a large scale experiment was carried out.

Isotriptophenolide (**194**, ~1.5 g) in ethanol (250 mL) was divided equally into 10 flasks of TRP4a cell cultures (21 days old, 550 mL each), and was incubated under standard conditions for 7 days (Trp#336). Then the cultures were harvested, and the broth and cells were extracted to give a broth extract (~1.84 g) and a cell extract (~3.25 g). A portion of the broth extract (~1.81 g) and a portion of the cell extract (~3.21 g) were subjected to an extensive column chromatographic separation (Figure 4.31).

Table 4.13 summarizes the results from isolation of biotransformation products from the large scale experiment. The total recovery of material was about 80%, and the major products were the epoxides **305** and **306** (47% of the total products isolated), and the 7-hydroxy compounds, **246a** and **246b** (29% of the total products isolated). The yield of epoxides based on recovered starting material was about 27%. A blank experiment, with **194** being "incubated" in a sterile medium for the same period of time, resulted in almost quantitative recovery of the starting material. Only a very small amount of **246** was observed on TLC.

Table 4.13   Compounds Isolated from Biotransformation of **194** with the TRP4a Cell
Culture (large scale, Trp#336)

Compound	194[a]	305,306	246	308	309	245	307	252	304	311
mg	781.5	199.0	122.9	34.1	23.3	18.9	10.4	7.5	2.9	2.7

[a] Recovered starting material.

Also isolated were some cell-produced metabolites, which included **106** (15.3 mg), **108** (10.6 mg), **117** (30.3 mg), **196** (28.3 mg), **200** (5.3 mg), **201** (2.1 mg), **203** (3.2 mg), **204** (2.4 mg), **278** (1.8 mg), squalene (36.1 mg) and Tl (**1**, 30.7 mg).*

* Fractions containing Td (**2**) were not further chromatographed.

Cell Extract (3.2079 g)

c. c.
i) hexanes-EtOAc
(6:4); ii) EtOAc;
iii) MeOH

I   II   III   IV   V   (VI)[a]

Broth Extract (1.8103 g)

c. c.
i) hexanes-EtOAc
(1:1); ii) EtOAc;
iii) MeOH

VII   VIII   IX   (X)

I

c. c.
hexanes-EtOAc
(9:1; 8:2)

(IA-IE)   IF

c. c.
i) CHCl₃
ii) CH₂Cl₂-
EtOAc
(90:5)

304
(2.9 mg)

IIC1

c. c.
CH₂Cl₂-EtOAc
(90:5; 90:8)

IIC1a   (IIC1b)   IIC1c   IIC1d   (IIC1e)

c. c.
hexanes-EtOAc
(6:4)

IIC1c1   **308**
(0.8 mg)

II

c. c.
hexanes-EtOAc
(90:5)

IIA   IIB   IIC

c. c.
hexanes-
EtOAc
(9:1; 8:2)

c. c.
hexanes-
EtOAc
(1:1)

(IIA1)   IIA2

IIC1 IIC2 IIC3 IIC4

c. c.
hexanes-EtOAc
(6:4)

194
(777.4 mg)

IIC1c1

c. c.
CH₂Cl₂-
EtOAc
(9:1)

307
(10.0 mg)

[a] Fractions in parentheses, for example (VI), contained cell-produced metabolites and their subsequent chromatography is not presented here.

Figure 4.31   Column Chromatographic Separation of Extracts from Biotransformation of **194** in TRP4a Cell Cultures (large scale, Trp#336)

Figure 4.31   Column Chromatographic Separation of Extracts from Biotransformation of **194** in TRP4a Cell Cultures (large scale, Trp#336) (continued)

IIC4

c. c.
CH$_2$Cl$_2$-EtOAc
(8:2)

(IIC4a)   IIC4b   IIC4c

IIC4b:
c. c.
CH$_2$Cl$_2$-
EtOAc
(9:1)

IIC4c:
c. c.
hexanes-EtOAc
(1:1)

311   **308**   **246**
(0.9 mg) (3.1 mg) (6.0 mg)

III

c. c.
CH$_2$Cl$_2$-EtOAc
(90:5; 9:1)

(IIIA)   IIIB   IIIC   (IIID)   IIIE

IIIC:
c. c.
hexanes-EtOAc
(7:3)

IIIC1   IIIC2   IIIC3   **305, 306**
                        (3.8 mg)

IIIC1:
↓ + IIC1a + IIC1d

c. c.
CH$_2$Cl$_2$-
EtOAc
(90:6)

IIIC1a + IIIB1 = **194**
                   (4.1 mg)

IIIE

c.c.
i) hexanes-EtOAc
   (6:4; 5:5)
ii) CH$_2$Cl$_2$-EtOAc
   (9:1; 85:15)

309
(3.4 mg)

IV + VIII

c. c.
CH$_2$Cl$_2$-
EtOAc
(90:5)

(IVA) (IVB)   **305**   (IVD)   IVE   (IVF)
              306
              (195.2 mg)

IVE:
c. c.
hexanes-
EtOAc
(1:1)

IIIB + IVE1   (IVE2)   IVE3   (IVE4)

IIIB + IVE1:
c. c.
CH$_2$Cl$_2$-
EtOAc
(90:6)

IIIB1

IVE3:
c. c.
CH$_2$Cl$_2$-
EtOAc
(90:8)

252
(6.7 mg)

V + IX

c. c.
hexanes-
EtOAc
(1:1)

(VA)   (VB)   VC

VC:
c. c.
CHCl$_3$-
acetone
(9:1)

246
(116.9 mg)
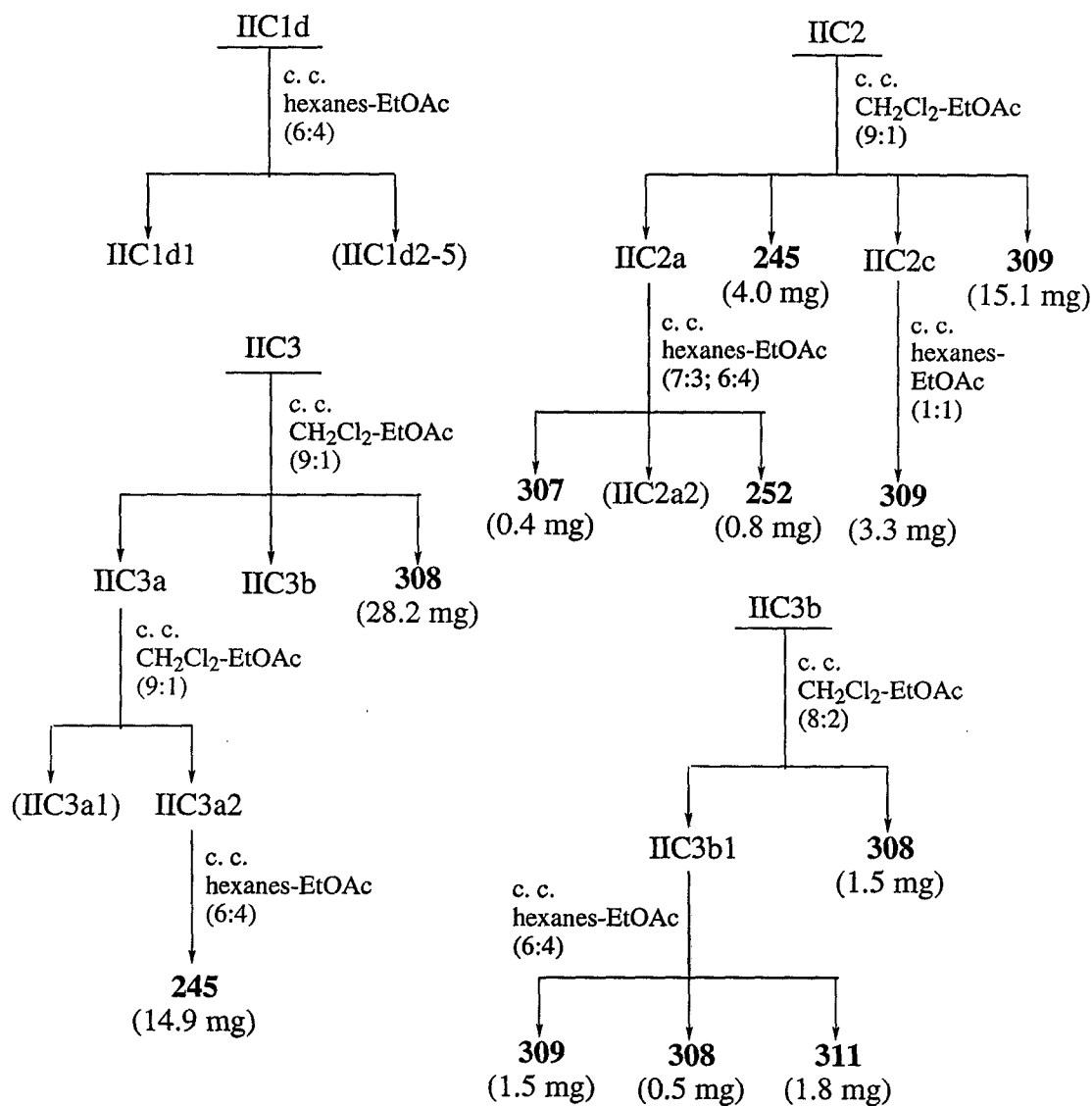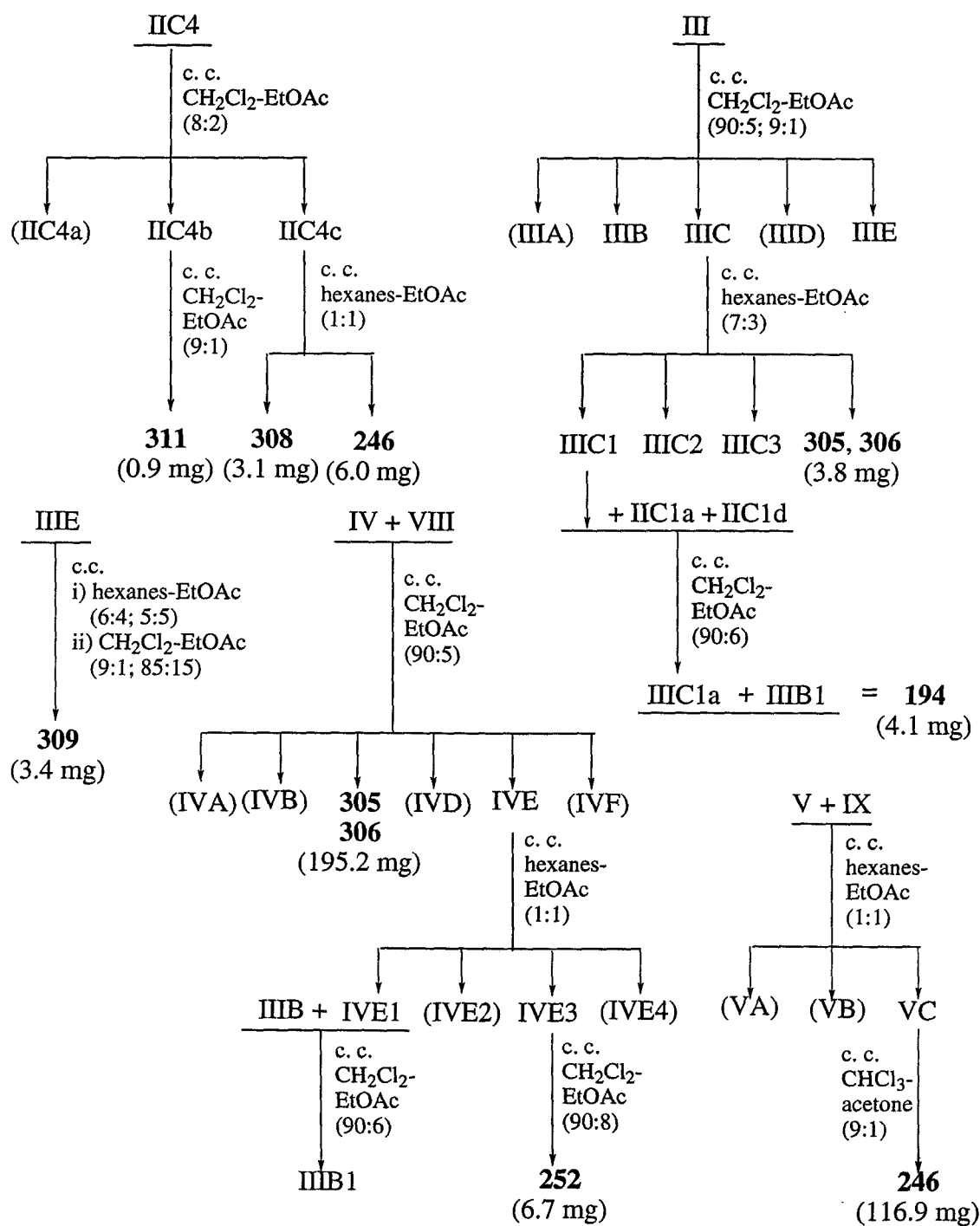
Figure 4.31   Column Chromatographic Separation of Extracts from Biotransformation of **194** in TRP4a Cell Cultures (large scale, Trp#336) (continued)

The mixture of epoxides **305** and **306** was repeatedly chromatographed with anhydrous ethyl ether as eluting solvent, affording pure **305** and **306**. Recrystallization of **305** and **306**, respectively, from ethyl acetate afforded single crystals, which were then analyzed by X-ray crystallography. The X-ray result of **306** is shown in Figure 4.4 (*vide supra*) but the crystals of **305** suffered X-ray induced decomposition, thus no X-ray analysis was obtained.

305 β-epoxide	**246a** 7α-OH	**308** 5β-OH
306 α-epoxide	**246b** 7β-OH	**309** 5α-OH

245   **307**   **252**

304   **311**
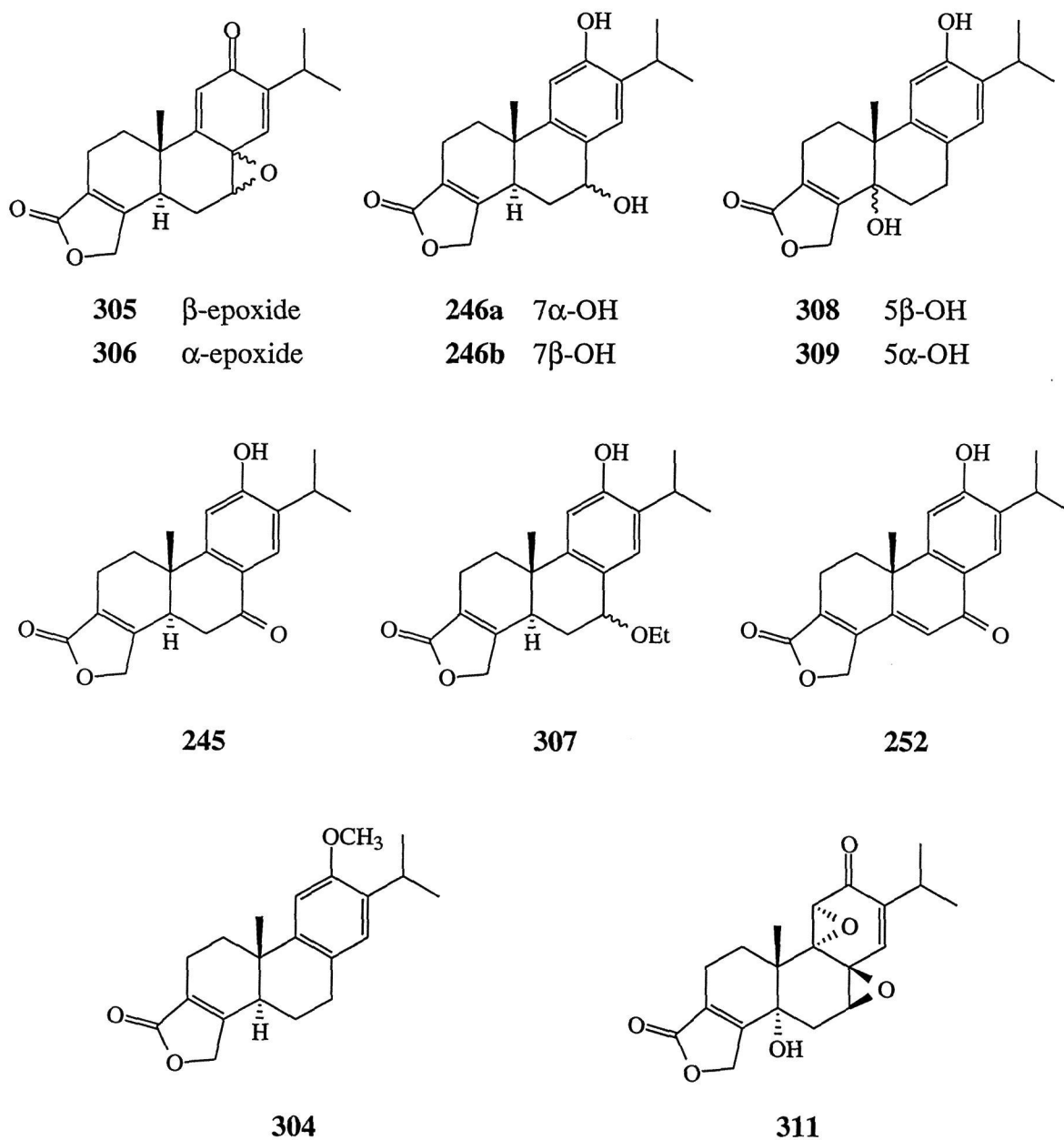
The isolation of compound **311**, even in a small amount, was a very important result. It demonstrated that the enzymes in TRP4a cell culture were capable of further epoxidation on a mono-epoxide substrate.

The diepoxide **311** was obtained as an optically active ($[\alpha]_D^{24}$ -59.1°, c = 0.164, MeOH), colorless powder with a molecular formula of $C_{20}H_{22}O_6$. Its IR spectrum showed absorptions indicative for a hydroxyl group between 3500 and 3600 cm^{-1}, and for an unsaturated ketone band at 1690 cm^{-1}.
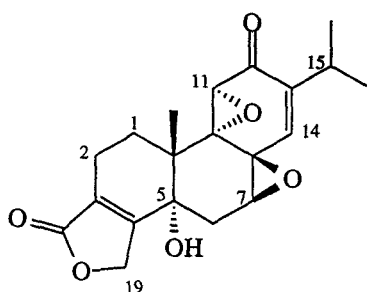
311

The ^{1}H NMR spectrum of **311** revealed some similarities to those of the epoxides **305** and **306**, but exhibited only one olefinic proton at δ 5.77 (1H, d, J = 1.0 Hz), and two epoxy protons at δ 3.65 (1H, d, J = 5.3 Hz) and δ 3.63 (1H, s), respectively. Apparently, one of the double bonds in the epoxides, **305** or **306**, had been epoxidized. The COSY spectrum (Figure 4.32) displayed a cross peak between the olefinic signal at δ 5.77 and the H15 signal at δ 2.84 (1H, sept d, J = 6.9, 1.0 Hz), indicating that the olefinic proton was H14 (allylic coupling). Therefore, the new epoxy group must be between C9 and C11. The singlet at δ 3.63 must be due to H11 and the doublet at δ 3.65 must be attributed to H7.

It was apparent from the spectra of **305** and **306** that if the epoxy group between C7 and C8 was β oriented, the H7 signal would appear as a well-resolved doublet (J = 5.8 Hz); if it was α oriented, the H7 resonance would appear as a broad doublet with a smaller coupling constant (J = 2.4 Hz) and sometimes even as a broad singlet. The H7 resonance in the spectrum of **311** was a doublet with a coupling constant of 5.3 Hz, indicating that the epoxy

group between C7 and C8 was β oriented. This C7 proton was also coupled with a proton at δ 2.39 (1H, dd, J = 15.1, 5.3 Hz) which, in turn, was correlated with another signal at δ 2.30 (1H, d, J = 15.1 Hz). As a result, these two signals were assigned to the two protons at C6, and the proton coupled with H7α was assigned to H6α in comparison with a similar situation in the spectrum of **305**. The two H6 protons were not coupled with any other protons in the molecule, and the H6β signal appeared as a doublet, revealing that the hydroxyl group must be at C5. This was also indicated by the widely split AB quartet of the H19 protons centered at δ 4.84 (2H, br AB$_q$, Δν = 0.24 ppm, J = 17.1 Hz). Generally, the C19 proton signals appeared as a broad singlet when the epoxy group between C7 and C8 was β oriented.
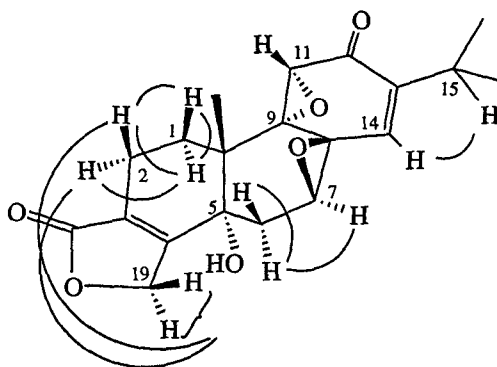
Figure 4.32   Proton Correlations Observed in the COSY Spectrum of **311**

The H19 signals showed cross peaks to the two H2 signals at δ 2.22 (1H, m) and 2.41 (1H, m), and they were assigned to H2β and H2α, respectively, in comparison with the spectrum of **305**. Both H2 signals were correlated with a signal at δ 1.81 (1H, br ddd, J = 12.8, 12.8, 6.4 Hz), and this proton and H2β were coupled with another proton at δ 1.26 (1H, m). This coupling pattern was the same as seen from **305** and thus the signal at δ 1.81 was assigned to H1α and the one at δ 1.26 to H1β.

Irradiation of H14 caused signal enhancements of H7α and the isopropyl methyl groups (Figure 4.33). Saturation of the H7α resonance showed NOEs to H14 (7%), H6α (6%) and H6β (2%). Separate irradiation of the two protons at C6 did not cause any enhancement of the

H19 signals, suggesting that the hydroxyl group at C5 was likely α oriented (that is, A/B *trans*). If the hydroxyl group were β oriented, irradiation of the H6 signals, especially H6α, would have some NOE effect on the C19 protons since they would be closer in space. This assignment was also supported by a downfield shift of the H1α resonance, which was at lower field than either of the H1 signals in Tl (1), apparently due to a 1,3-diaxial interaction with the hydroxyl group at C5.

Irradiation of H1α did not show any NOE effect to H11, but irradiation of H11 showed strong signal enhancements of H1β and C20 methyl group. By inspecting the molecular model, this NOE result clearly indicated that the epoxy group between C9 and C11 was α oriented. Therefore, the structure of this compound was assigned as **311**. A comparison of the ^{1}H NMR data of epoxides **305, 306, 311** and **312** is given in Table 4.14 (compound **312** will be discussed in section 4.2.3).
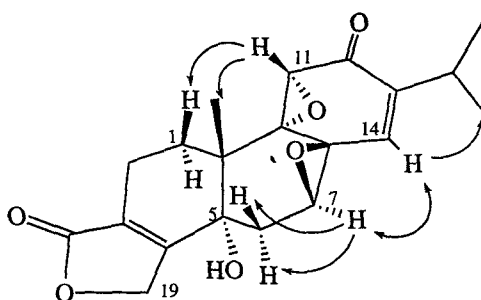
Figure 4.33   Major NOEs Observed in the NOE Difference Spectra of **311**

Compound **252** was isolated as yellowish prisms (mp 289-291°C) with a molecular formula of $C_{20}H_{20}O_4$. Its ^{1}H NMR spectrum showed some resemblance with that of compound **245**, but no signal for H5 was found. A signal for only one C6 proton was found at δ 6.31 (1H, s). The H19 signals were shifted to lower field at δ 5.04 (2H, br AB_q, Δv = 0.17 ppm, J = 16.3 Hz) with a wider split between the AB quartet. Also the C20 methyl signal was shifted downfield to δ 1.36. All these changes indicated that there was a double bond between C5 and C6, and the structure was assigned as **252**.

Table 4.14　^{1}H NMR Spectral Data of Epoxides **305, 306, 311** and **312** (400 MHz in CDCl$_3$, δ in ppm, J in Hz in parentheses)

	305	306	311	312
1α	1.62, ddd (12.9, 12.4, 6.0)	1.69, ddd (13.3, 12.4, 6.4)	1.81, ddd (12.8, 12.8, 6.4)	1.56, m
1β	2.10, dd (12.9, 5.4)	2.19, dd (13.3, 6.1)	1.26, m	2.12, dd (13.3, 5.9)
2α	2.47, d (18.5)	2.47, d (17.9)	2.41, m	2.47, d (18.4)
2β	2.30, m	2.30, m	2.22, m	2.25, m
5	2.58, d (13.4)	3.23, d (12.8)	-	2.87, d (13.1)
6α	2.26, ddd (14.5, 5.8, 5.8)	2.30, ddd (14.4, 3.1, 2.4)	2.39, dd (15.1, 5.3)	2.31, ddd (14.5, 4.1, 2.3)
6β	2.13, dd (14.5, 13.4)	2.02, dd (14.4, 12.8)	2.30, d (15.1)	1.97, ddd (14.5, 13.1, 1.4)
7α	3.84, d (5.8)	-	3.65, d (5.3)	-
7β	-	3.82, d (2.4)	-	3.80, br s (W$_{1/2}$ 4.7)
11	6.42, s	6.48, s	3.63, s	6.16, s
12	-	-	-	-
14	5.99, d (1.0)	5.99, d (0.9)	5.77, d (1.0)	H14α, 3.06, s
15	2.98, sept d (6.9, 1.0)	2.95, sept d (6.9, 0.9)	2.84, sept d (6.9, 1.0)	2.55, sept (6.9)
16, 17	1.06, 1.08, d (6.9)	1.05, 1.08, d (6.9)	1.00, 1.06, d (6.9)	0.937, 0.99, d (6.9)
19	4.68, s	4.70, AB$_q$ (17.1)	4.84, AB$_q$ (17.1)	4.69, m
20	1.17, s	0.95	1.26, s	0.939, s

Despite the substantial reduction of the epoxides **305** and **306** to the 7-hydroxy isotriptophenolide (**246**), further epoxidation of **194** did occur. One interesting point which should be noted was that this diepoxy compound **311** was formed from the precursor with the correct stereochemistry for the epoxy group between C7 and C8 as in the natural products Tl

(1) and Td (2). This meant that the enzymes selectively chose the β isomer to place the second epoxy group. Similarly, as with the diepoxy compound 209 produced by the cells, the second epoxy group in 311 was placed between C9 and C11, but not at the more accessible C13 and C14 double bond, where chemical epoxidation took place (section 4.2.3).

One remaining question is why the epoxy group of the biotransformation products 305 and 306, though regiospecifically placed between C7 and C8, have both α and β orientations, whereas all the Tl (1) and Td (2) related diterpene epoxides isolated from the plants or from the TRP4a cell cultures have only β orientation. It is plausible that since isotriptophenolide (194) and its corresponding quinone methide intermediate 249 are structurally similar to the proposed precursor triptophenolide (106) and its corresponding quinone methide 219, it is feasible that they are both substrates for the same epoxidation enzyme. However, the structural variation in 249 may not allow an effective or correct binding in the active site, which results in the epoxide being formed from both α and β sides of the intermediate; whereas for 106, on the other hand, intermediate 219 probably binds to the active site effectively thus only allowing the epoxidation to proceed from the β side (Figure 4.34).

As a summary for the biotransformation studies of isotriptophenolide (194) with TRP4a cell cultures, the major products and their relationship with one another are illustrated in Scheme 4.10.

The formation of 7-hydroxy compounds 246a and 246b has been shown to result from enzymatic reduction of the corresponding epoxides 305 and 306, but the possibility of their partial origin through direct hydroxylation of 194 cannot be ruled out, because hydroxylation at C7 was observed in the early studies with butenolide 193 in TRP4a cell cultures.
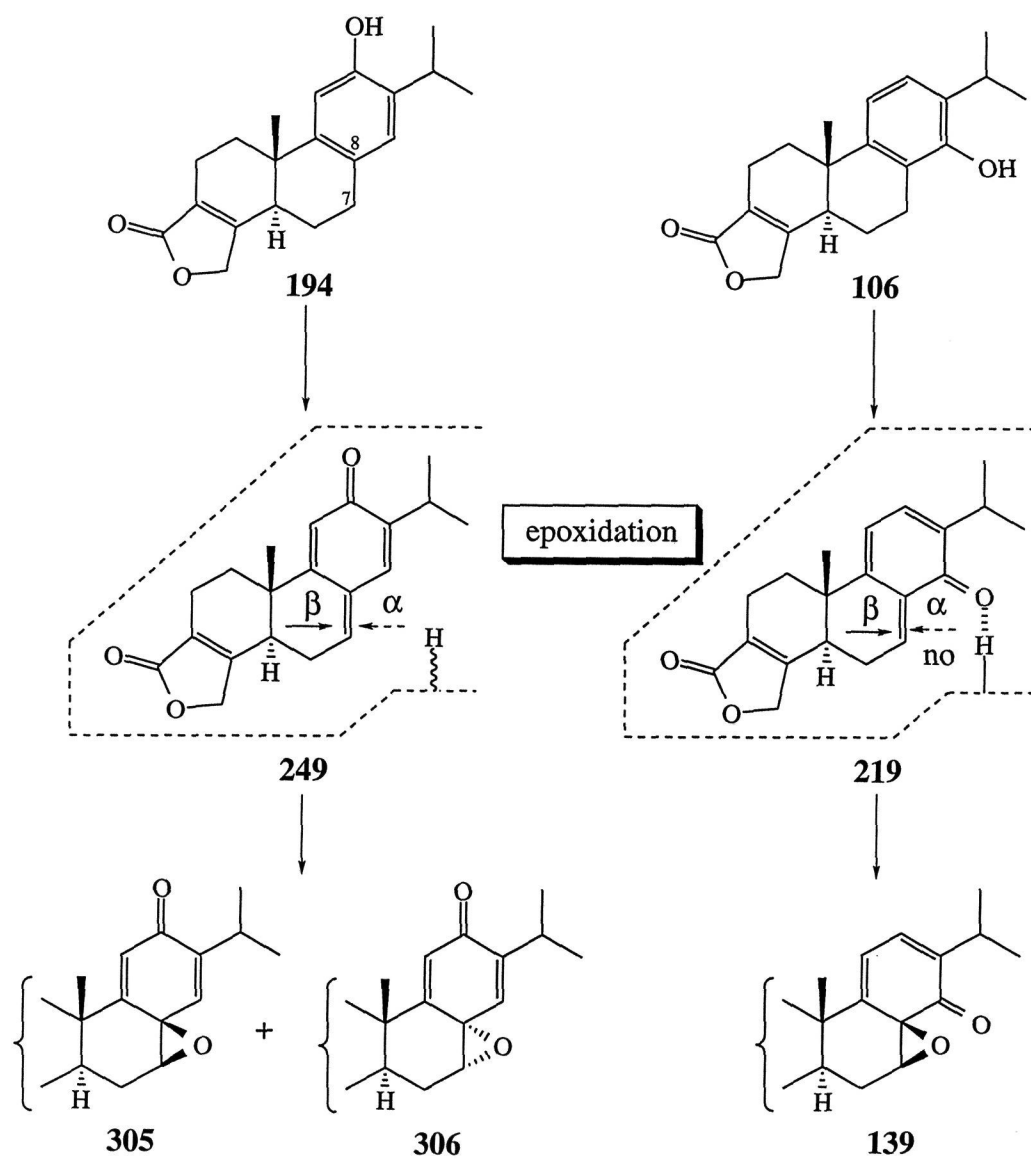
Figure 4.34   Proposed Mechanism for the Formation of the C7, C8 Epoxide Group in **305**, **306** and **139**
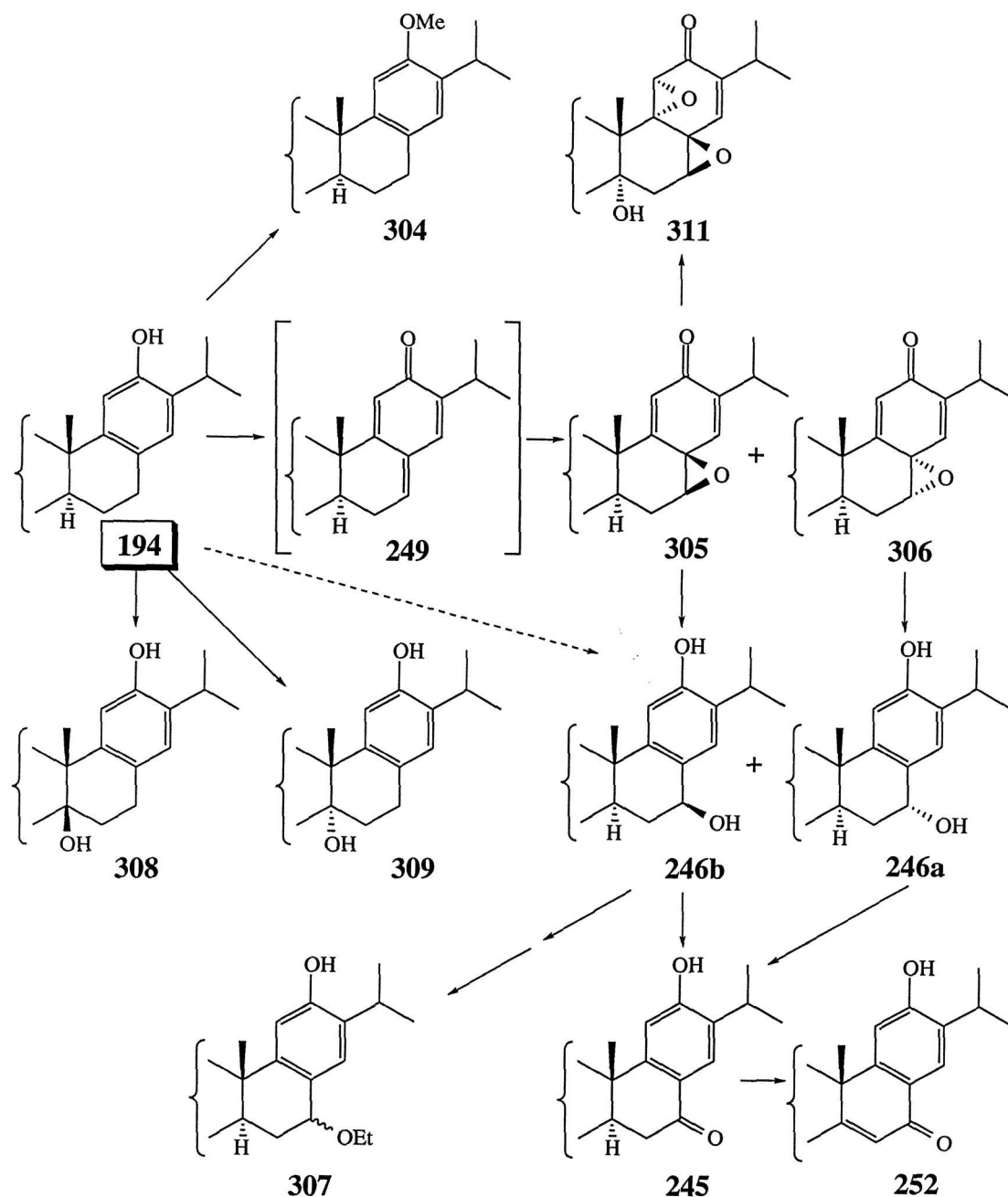
Scheme 4.10   Biotransformation of Isotriptophenolide (**194**) with TRP4a Cell Cultures

4.2.3   Chemical Epoxidation of the Epoxides (305, 306)

With the novel diterpene epoxides **305** and **306** available from the biotransformations, it would be interesting to use them as starting material to synthesize diepoxides or triepoxides as

new analogs of Tl (**1**). These analogs might provide pharmacologically active, but less toxic drugs.

An experiment of epoxidation was carried out with a mixture of the epoxides **305** and **306** (14.7 mg, **305** /**306** 1:1). The starting material in benzene was repeatedly treated with *t*-butyl peroxide and Triton B[145,258] and the reaction was worked up after 29 h. The crude product was column chromatographed to give recovered **305** (0.7 mg), a new product **312** (6.0 mg) and 8.6 mg of unidentified products.
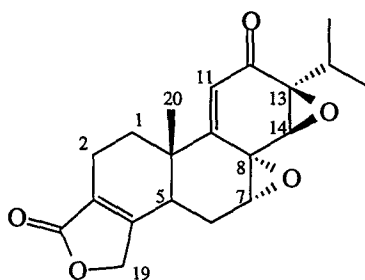
312

Compound **312** was obtained as a colorless, crystalline solid (mp 196-198°C) with a molecular formula of $C_{20}H_{22}O_5$. Its IR spectrum was similar to those of the epoxides **305**, **306** with an unsaturated ketone absorption at 1660 cm[-1]. The [1]H NMR spectrum showed only one olefinic proton at δ 6.16 (1H, s), and two epoxide protons at δ 3.80 (1H, br s) and 3.06 (1H, s), respectively. Obviously, a double bond in the epoxides, **305** or **306**, had been epoxidized. The H15 signal was shifted upfield to δ 2.55 (1H, sept, J = 6.9 Hz), as compared with this corresponding signal seen in the spectrum of **305**, **306** and **311**, and the olefinic proton was at fairly low field. These changes indicated that the epoxidation occurred at the double bond between C13 and C14.

The COSY spectrum displayed cross peaks between the signal at δ 3.80 and two other signals at δ 1.97 (1H, ddd, J = 14.5, 13.1, 1.4 Hz) and 2.31 (1H, ddd, J = 14.5, 4.1, 2.3 Hz), suggesting that the proton at δ 3.80 was H7 and the other two were C6 protons. Both C6 protons were also coupled with H5 at δ 2.87 (1H, br d, J = 13.1 Hz). The appearance of the C7

proton as a broad singlet, and the relatively low field of the H5 signal indicated that the epoxy group between C7 and C8 was α oriented. Therefore, the signal at δ 3.06 must be H14 and its relationship with H7 was confirmed by NOE experiments. The stereochemistry of the epoxy group between C13 and C14 was difficult to determine even by NOE experiments. However, the chemical shift of H14 (δ 3.06) was at significantly higher field, as compared with all the epoxide proton signals (δ 3.3-3.9) of Tl (**1**). By examination of the molecular model, it could be seen that if the C13, C14 epoxy group was β oriented (Figure 4.35a), the proton at C14 is situated directly above the C7, C8 epoxy function and is shielded, that is, it will appear at higher field.[186] On the other hand, if the C13, C14 epoxy group were α oriented (Figure 4.35b), the H14 proton would be directed away from the C7, C8 epoxy ring, and thus subjecting to little shielding, or even being deshielded by the epoxy ring. In other word, H14 signal would appear in the normal region for epoxide protons or even at a lower field. The low chemical shift of H14 indicated that the epoxy group between C13 and C14 was β oriented. In consideration of the reaction mechanism, the β side of the double bond between C13 and C14 is more accessible than the α side; therefore the reagent would attack towards the β face. The COSY and NOE results were in agreement with this assignment. A comparison of the [1]H NMR data with other previously obtained epoxides is given in Table 4.14 (section 4.2.2, *vide supra*).

a   b
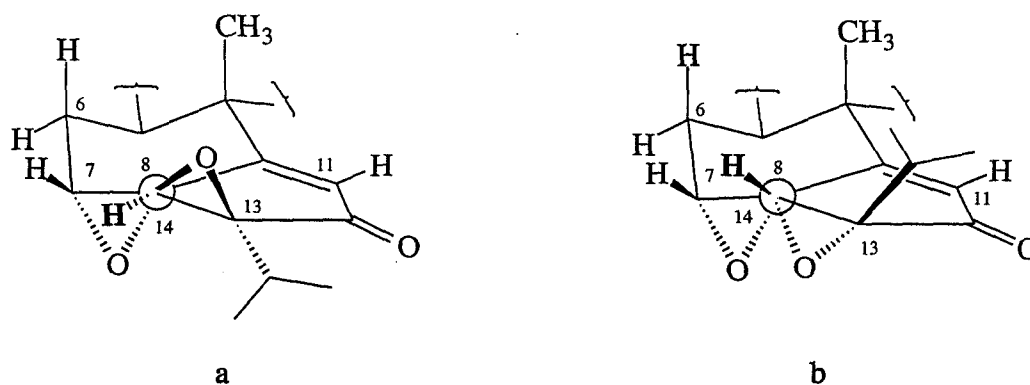
Figure 4.35   Stereochemistry of the Epoxy Group between C13 and C14

The yield of this diepoxide was calculated as 78%, since the starting material was a 1:1 mixture of **305** and **306**, and this product resulted from selective epoxidation of **306**. The mixture of unidentified products may contain isomers of epoxidation products from **305** as indicated by ^{1}H NMR.

4.2.4 Biotransformation of Triptophenolide (106) and Related Compounds

Biotransformation of 106 with TRP4a Cell Cultures: Preliminary Experiments

With the encouraging results from biotransformation studies with isotriptophenolide (**194**), an experiment with triptophenolide (**106**), the proposed precursor for the biosynthesis of Tl (**1**) and Td (**2**), was started with TRP4a whole cell cultures.

A solution of triptophenolide (**106**) in ethanol was added to a cell culture of 14 days old and incubated under standard conditions (Trp#322). Samples were taken after 2 and 4 days, and the culture was harvested after incubation for 7 days. To our surprise, TLC monitoring showed no major new spots from the test samples except for a small spot that was slightly less polar and was UV active. No significant changes to Tl (**1**) and Td (**2**) were observed. The biotransformation samples were combined and subjected to extensive column chromatography. Most of the starting material was recovered, and the two minor compounds isolated were 7-oxo-triptophenolide (**108**), and quinone **278**. It should be noted that these compounds were also present as minor components in the cell culture themselves (*vide supra*).
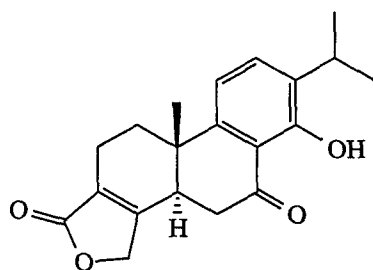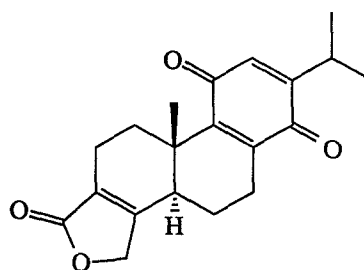
108   **278**

Biotransformation of 106 with TRP4a Cell Cultures of Different Ages

It was shown by the results from the biotransformation of isotriptophenolide (**194**) that the age of the cell culture played a role in the biotransformation. Therefore, a series of experiments were performed with triptophenolide (**106**) with TRP4a cell cultures of different ages.

The starting material **106** was incubated with cell cultures of different ages (0, 8, 14, 21 and 28, respectively). Samples were taken at certain intervals (1, 2, 4 and 7 days), and the cultures were harvested after 15 days of incubation. TLC analysis of these samples showed that little changes had occurred. The samples taken at and after 4 days of incubation (including the final harvested broth samples) revealed some less polar, UV active spots on TLC similar to those exhibited by **108** and **278**, and very small amounts of some polar spots. No significant changes in the amounts of Tl (**1**) or Td (**2**) were observed by TLC. The final cell extracts only showed remaining **106** on TLC. A blank experiment gave total recovery of the starting material. Surprisingly, biotransformation of triptophenolide (**106**) with TRP4a cell cultures, unlike isotriptophenolide (**194**), proceeded very slowly and to much lesser extent. The relatively low solubility of **106**, even in ethanol, as compared with **194**, must be noted. This, among other factors, could contribute to the lack of extensive biotransformation of **106** with the TRP4a cell cultures.

Biotransformation of 106 with CFEs from TRP4a Cell Cultures

The lack of extensive biotransformation of **106** with TRP4a whole cell cultures shifted attention to the CFE experiments. As had been demonstrated in the CFE experiments with isotriptophenolide (**194**), biotransformation with CFE usually proceeds much faster than with whole cells, and the solubility of the starting material would not be a major factor. Another

advantage of experiments with CFE as had been experienced in the biotransformations of isotriptophenolide (**194**) was that the ethyl acetate extracts of the CFE samples were much less complex than those from a whole cell experiment. Clearly, mixtures from CFE experiments do not carry significant quantities of cell-produced metabolites so it should be possible to detect smaller amounts of new compounds more readily.

A 14-day old cell culture was used to prepare the CFE in an acetate buffer (0.1 M, pH 4.7) (Trp#318b). The starting material (**106**) in ethanol was added to a CFE mixture composed in a similar ratio as in the previous CFE experiments with **194**. Samples were taken at 1 and 2 h intervals and the reaction was worked up after 4 h. TLC analysis showed that most of the starting material was unreacted. There were the less polar, UV active compounds and two new polar spots, which were present in small amounts.

Encouraged by these results, an experiment was set up using CFE from an older cell culture, and with an increased peroxidase units-to-starting material ratio. Thus a cell culture of 21 days old was used to prepare the CFE in an acetate buffer. The CFE thus obtained and with a total of 312 peroxidase units (2.5 times the standard ratio) was mixed with an acetate buffer (pH 4.7, 14.3 mL) and the cofactors (hydrogen peroxide, $MnCl_2$ and FMN, all 2.5 times the normal ratio). The starting material (**106**) was added to the CFE mixture, and samples were taken at 1 and 2 h, and the reaction was worked up after 4 h (Trp#319). TLC evaluation of these samples showed that most of the starting material still remained, but the two polar spots noted earlier did increase with the reaction time.

In order to examine the time required for a completed reaction, another experiment was carried out. A cell culture of 15 days old was used to prepare the CFE in an acetate buffer. The resulting CFE, containing a total of 550 peroxidase units (4.4 times the standard ratio), was mixed with distilled water and the cofactors (hydrogen peroxide, $MnCl_2$ and FMN, all 4.4 times the standard ratio). The starting material (**106**) was added to the CFE mixture and samples were taken at 4 and 24 h. The reaction was worked up at 48 h (Trp#321). The samples were analyzed by TLC, which showed that the amount of the two polar spots increased

from 4 h to 24 h, but remained little changed between 24 h and 48 h. This suggested that a reaction time of about 24 h was sufficient to maximize the two compounds observed on TLC.

All the samples from the above experiments (Trp#318b, 319 and 321) were combined and chromatographed, resulting in a major recovery of stating material **106** (~70%), and isolation of minor amounts of quinone **278** (~2%), **108** (~1%) and Tl (**1**, trace amount). Efforts to isolate the two polar compounds failed because of decomposition of these compounds during the column chromatography. TLC analysis of the control samples did not show any **108** or **278**; therefore, they must have been produced from biotransformation of **106** in the CFE experiments. As for Tl (**1**), it was difficult to determine whether the small amounts found came from the biotransformation of **106**, or came with the CFE, even though many early results had shown that Tl (**1**) and Td (**2**) were mostly, if not entirely, present in the broth.[160,259] In other words, CFE, because it was prepared from the cells, should not contain Tl (**1**). This suggested that in the future studies concerning triptophenolide biotransformation, labeled starting material (**106**) should be used in order to determine whether Tl, or other products, originated from the added starting material.

Biotransformation of Quinone 278 with TRP4a Cell Cultures

From a chemical point of view, **106** should be readily oxidized to quinone **278** as had been noted in the chemical synthesis of **106** (section 3.2.3). However, in the TRP4a cell cultures, there was much more **106** than quinone **278**. For example, in the previous large scale experiment of **194** with the whole cell culture (10 x 550 mL, section 4.2.2), only 1.8 mg of **278** was isolated from the cell culture while 15.3 mg of **106** was isolated from the same culture (**106:278** ~ 10:1). Even in the attempted biotransformation of **106** in the whole cell cultures, or in CFE experiments, only small amounts of quinone **278** were isolated, probably from biotransformation of **106**. The apparent lack of enzyme catalyzed oxidation of a phenol to a quinone, a normal facile process, remains an interesting question.

As has been noted from biotransformation of **194** in the whole cell culture, the cell culture did have the ability to epoxidize quinone-like compounds to form products such as **311** (section 4.2.2). It was an interesting question whether the cells could epoxidize the quinone **278**. If they possessed such capability, another series of interesting analogs of Tl (**1**) would become available for pharmacological screening.

A preliminary experiment was carried out by incubation of the quinone **278** in a cell culture of 21 days old (Trp#328). Samples were taken at 1, 3, 7 days, and the culture was harvested after 8 days of incubation. TLC showed that there were no significant changes after one day of incubation, but a new spot was found after 3 days of incubation. The culture was harvested at day 8, and the cells and broth were extracted. Column chromatography of the broth and cell extracts yielded the unreacted quinone **278**, the new product noted on TLC, and compound **313**.

In order to obtain sufficient material for spectroscopic analysis, a larger scale experiment (Trp#332) was set up according to Trp#328. The starting material, **278** (53.2 mg), was incubated in a 21-day-old cell culture (550 mL) (Trp#332). The cell culture was harvested sooner (after 2 days of incubation) for this experiment, since the low recovery of material in the preliminary experiment suggested that oxidative decomposition had probably occurred during the prolonged incubation. The broth and cell extracts were chromatographed repeatedly on column and preparative TLC plates, resulting in recovery of the starting material **278** (40.2 mg), and isolation of **313** (0.9 mg) and the new product (1.7 mg).

The new product had a molecular formula of $C_{21}H_{26}O_4$ and its ^1H NMR spectrum showed an identical spectrum to that of compound **109**, which had been previously isolated from the cell culture itself (section 2.2). However, this compound obviously came from the biotransformation of the quinone **278** in the present experiments, because its appearance and progress were observed and monitored by TLC during the process of the biotransformation, and the quantity of this compound per volume of the cell culture was greater than that isolated from the cell culture itself (2 mg in 250 mL culture in this biotransformation vs. 8 mg obtained from

isolation of 7 x 550 mL of cell cultures, see section 2.2). A blank experiment with the quinone **278** being "incubated" in a sterile medium gave total recovery of the starting material.
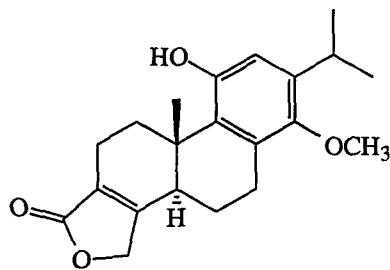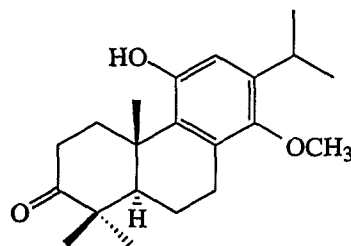
109   **117**

It was very interesting to observe that the cells had reduced the quinone compound to the hydroquinone. It appeared that the TRP4a cell system had a mechanism which either suppressed the formation of the quinone, or once it was formed, reduced it to hydroquinone and then converted the hydroquinone to its 14-methyl ether. The methylation may occur soon after the reduction; otherwise, it should be possible to isolate the 11,14-hydroquinone **314** (*vide infra*). Some other cell-produced diterpenes, such as **117**, may also be formed by such a mechanism.
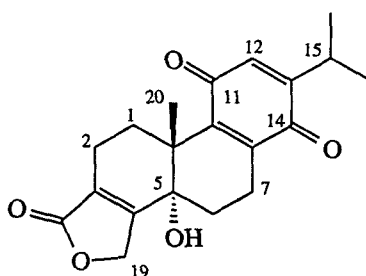
313

Compound **313** was isolated as an optically active ($[\alpha]_D^{24}$ -28.4°, c = 0.095, CHCl$_3$) pale yellow powder (mp 161-163°C) with a molecular formula of $C_{20}H_{22}O_5$. Its IR spectrum indicated the presence of a hydroxyl group (3600 cm^{-1}) and a quinone moiety (1660 cm^{-1}). The ^{1}H NMR spectrum showed some similarities with that of the quinone **278**. The proton at

the quinone double bond was found at δ 6.40 (1H, d, J = 1.0 Hz), and was coupled with H15 at

δ 2.99 (1H, sept d, J = 6.9, 1.0 Hz) (allylic coupling). The H19 signals were shifted downfield

to δ 4.86 (2H, br AB_q, Δv = 0.21 ppm, J = 17.2 Hz) and the AB quartet was more widely split

as compared with its counterpart in **278**. This indicated that the hydroxyl group was situated at

C5. The resonance of H1β, as in **278**, was located at lower field (δ 3.16, 1H, br dd, J = 15.0,

6.0 Hz), and coupled with the C2 protons at δ 2.41 (2H, m) as well as with H1α in the multiplet

between δ 1.85 and 2.06 (3H). The H7α was shifted downfield when compared with that in

278, and both C7 protons appeared together as a multiplet at δ 2.72 (2H, m). The H5 signal

was not found in the spectrum, but a hydroxyl proton was located at δ 1.84 (1H), which was

exchangeable with D_2O. The downfield shifts of H7α and H1α, when compared with **278**,

strongly indicated that the hydroxyl group at C5 was α oriented, thus having 1,3-diaxial

interactions with H1α and H7α. No epoxidation products of quinone **278** were isolated from

the above experiments.

Biotransformation of Triptophenolide (106) with TRP4a Cell Cultures: Large Scale Experiment (Trp#334)

As already noted from the attempted biotransformations of triptophenolide (**106**) with

TRP4a cell cultures, the substrate **106** did not show as extensive a biotransformation as **194**,

and any products formed were very minor components. Therefore, a large scale experiment

was appropriate so as to examine these minor components.

Triptophenolide (**106**, 300 mg) was incubated in a 21-day-old cell culture for 7 days

(Trp#334). The culture was harvested at the end of incubation, and the cells and broth were

extracted to yield a broth extract (~548 mg) and a cell extract (~743 mg), respectively. The

extracts were combined and repeatedly column chromatographed as shown in Figure 4.36.

Combined Broth and Cell Extract (1.29 g)

c. c.
i) hexanes-EtOAc
(6:4; 4:6; 5:5)
ii) EtOAc
iii) EtOAc-MeOH
(1:1)

(I) II III IV V VI (VII)

c. c.
CH_2Cl_2-EtOAc
(98:2; 90:5)

c. c.
CH_2Cl_2-EtOAc
(90:3; 90:5),

IIA IIB IIC **109** **106** IIIB IIIC IIID
(2.6 mg) (192 mg)

c. c.
*

c. c.
hexanes
-EtOAc
(8:2)

c. c.
hexanes-
EtOAc (7:3)

c. c.
hexanes
-EtOAc
(7:3)

c. c.
hexanes
-EtOAc
(7:3)

c. c.
hexanes
-acetone
(8:2)

108
(0.3 mg)
278
(0.3 mg)

108
(0.2 mg)

108
(5.7 mg)

278
(1.7 mg)

108
(1.3 mg)

108
(2.1 mg)

IIID1 IIID2

c. c.
CH_2Cl_2-
acetone
(85:5; 9:1)

c. c.
CH_2Cl_2-
acetone
(9:1)

138a
(6.8 mg)

315
(2.4 mg)

*
i) hexanes-
   EtOAc (8:2)
ii) hexanes-
   acetone (9:1)

IV

c. c.
CH_2Cl_2-
acetone
(8:1; 8:2)

V

c. c.
i) CH_2Cl_2-
   acetone
   (9:1; 8:2)
ii) acetone

VI

c. c.
i) CH_2Cl_2-
   acetone (8:2)
ii) hexanes-
   acetone (7:3)

138b
(2.5 mg)

316
(1.6 mg)

106
(1.4 mg)

316
(4.9 mg)

315
(5.5 mg)

278
(2.8 mg)

Figure 4.36   Column Chromatographic Separation of Biotransformation Products of **106**

Table 4.15 summarizes the products isolated from biotransformation of triptophenolide (**106**) in the TRP4a cell culture. The starting material was recovered in about 65%, which was higher than the recovery yield of isotriptophenolide (**194**) in its biotransformation experiments (50% or lower). The total recovery of material was about 78%. Some of the compounds may have been partially produced by the cell culture *de novo*, rather than from **106**.

Table 4.15   Compounds Isolated from Biotransformation of Triptophenolide (**106**) with the TRP4a Cell Culture (Trp#334)

Compound	106[a]	108	315	138a	138b	316	278	109
mg	193.4	9.6	7.9	6.8	2.5	6.5	4.8	2.6
%[b]	-	9	7	6	2	6	4	2

[a] Recovered starting material; [b] Based on recovered starting material.
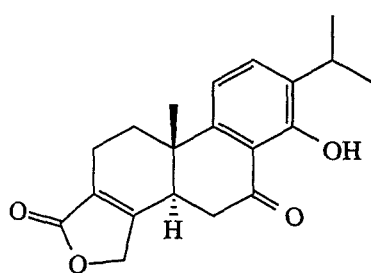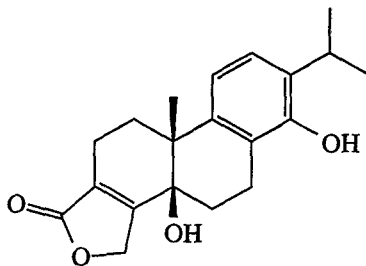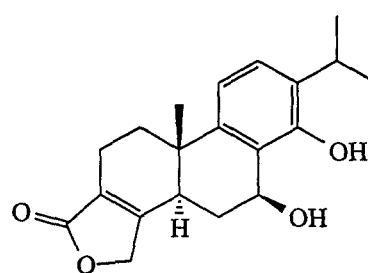
108   **315**   **138a**
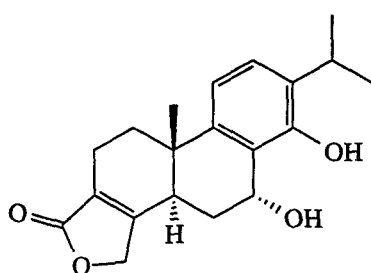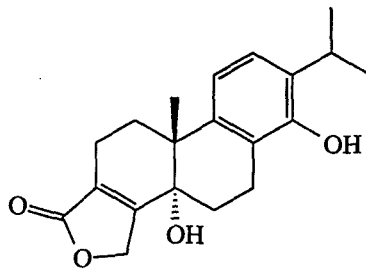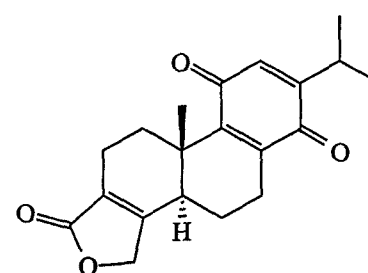
138b   **316**   **278**
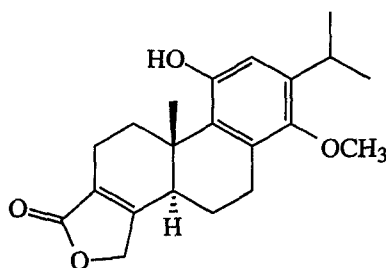
109

Compounds **108**, **138a** and **278** had been previously isolated from the cell cultures, but the quantities obtained from this experiment were higher than from the culture, so some of them may have resulted from the biotransformation of **106**. Compound **109** may be produced from the cells, or from the biotransformation of the quinone **278**.

Compound **138b** had the identical ^{1}H NMR spectrum as the α isomer observed in the reduction of **108** (section 2.2). The major spectral difference in the spectrum of **138b** was that the H7 signal was slightly shifted upfield to δ 5.12, as compared with δ 5.22 in **138a**, and it appeared as a broad multiplet. This indicated that this proton was in an equatorial position, therefore the hydroxyl group must be axial, *i.e.*, α oriented. Another difference was that the H5 signal was shifted downfield from δ 2.76 in **138a** to δ 2.95 in this compound. This reflected a 1,3-diaxial interaction caused by an α hydroxyl group at C7. Because this compound decomposed after the ^{1}H NMR spectrum was measured, no other spectral data were obtained. However, based on its ^{1}H NMR spectrum and chemical correlation with **108**, the assignment of this compound as **138b** was considered to be correct.

Compound **316** was isolated as optically active ($[\alpha]_D^{20}$ -58.8°, c = 0.32, MeOH), colorless prisms (mp 190-191°C). Its molecular formula was determined as $C_{20}H_{24}O_4$, one oxygen more than the starting material. The IR spectrum of this compound showed absorption bands for hydroxyl groups at 3607 cm^{-1} (sharp) and 3560 cm^{-1} (broad).
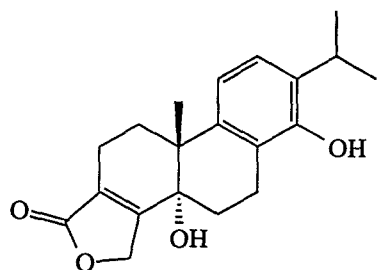
316
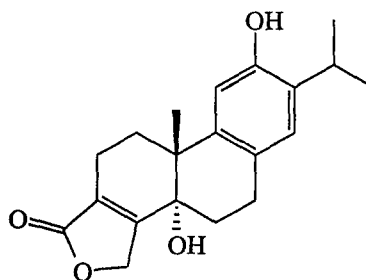
309

The ¹H NMR spectrum of **316** displayed a similar spectrum to that of **106**. However, the H5 signal was not found in the spectrum and a new hydroxyl proton appeared at δ 1.90 (exchangeable with D₂O). The H19 resonances were shifted downfield from δ 4.77 in **106** to δ 4.90 (2H, br ABq, Δν = 0.19 ppm, *J* = 17.1 Hz) and split more widely, thereby indicating that the new hydroxyl group was at C5. The C20 methyl signal was located at δ 1.09 (3H, s), which was at higher field than the isopropyl methyl signals (δ 1.25 and 1.27), indicating an A/B *trans* junction. In comparison of the spectral data with those of **106** and **309**, the hydroxyl group at C5 must be α oriented. The remainder of the protons were assigned based on COSY and NOE results.
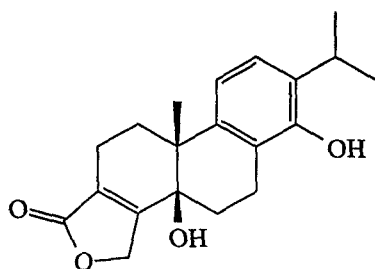
315
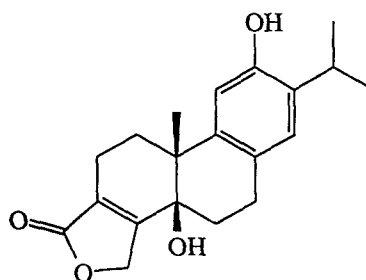
308

Compound **315** was obtained as a pale yellowish powder with a molecular formula of C₂₀H₂₄O₄. Its IR spectrum exhibited absorption bands for hydroxyl groups at 3600 and 3500 cm⁻¹. The ¹H NMR spectrum showed great resemblance to that of **308**, except that the aromatic proton signals were, of course, two doublets rather than two singlets. The slightly

downfield shift and the splitting pattern of the H19 signals at δ 4.91 (2H, br AB_q, $\Delta v = 0.10$ ppm, $J = 16.8$ Hz), as compared with the spectrum of **106**, indicated that this compound was very likely to be a C5 hydroxylated product of **106**.

observed NOE

transfered NOE

315
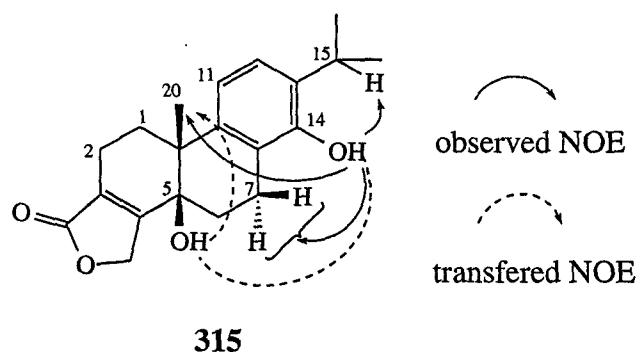
Figure 4.37   Some NOEs Observed in NOE Difference Spectra of **315**

Irradiation of the phenolic hydroxyl proton at δ 4.72 not only showed NOEs to H15 (δ 3.03, sept, $J = 6.8$ Hz) and the two C7 protons as expected, but also to the C20 methyl group (Figure 4.37). This was a phenomenon observed quite frequently with this class of compounds. This phenomenon is known as "transferred NOEs" in NOE experiments. When one of the hydroxyl protons in the molecule is affected (saturation or NOE) others will experience the same effect if the hydroxyl protons exchange slowly.[178,260] If there were two or more hydroxyl groups in the same molecule, when one was irradiated, the others, due to exchanges with the irradiated one (as shown by a dashed line in Figure 4.37), also responded as if they were irradiated as well, and showed corresponding NOE effects with nearby protons (transferred NOEs, as shown by a dashed arrow in Figure 4.37). In this case, it meant that the hydroxyl group at C5 was β oriented. This was also supported by the fact that the C20 methyl signal (δ 1.35) was at lower field than the isopropyl methyl signals (δ 1.24) (*i.e.* A/B *cis*). The H7 signal at δ 2.86 was assigned to H7α because it resonated at lower field than the other H7 signal, caused by the interaction with ring A due to the A/B *cis* junction.

Unfortunately, no epoxides such as **139** and **317** were isolated. The formation of **138a** and **138b** may follow the same mechanism as **246b** and **246a**, that is, via the corresponding epoxides, but direct hydroxylation cannot be ruled out.
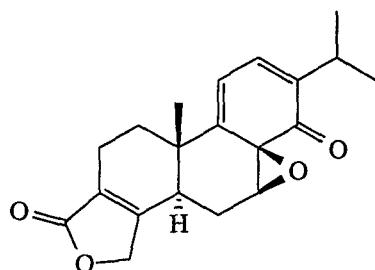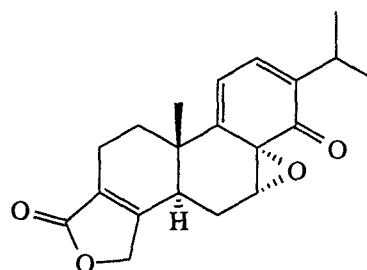
139 (not isolated)   **317** (not isolated)

One very interesting point was that if the biotransformation results of **106** were combined with the related compounds isolated from the cell cultures, and then compared with the biotransformation products of **194**, there was an astonishing similarity between these two groups of compounds (Scheme 4.11).

It was reasonable to postulate that these two groups of compounds were formed via similar mechanisms, and by the same group of enzymes. Since 7-hydroxy-isotriptophenolide (**246**) was not involved in the formation of the epoxides **305** and **306**, it was not likely that 7-hydroxy-triptophenolide (**138**) would be involved in the formation of the corresponding epoxy quinone **139**. Therefore, although we have not obtained direct evidence that the quinone methide **219** was involved, or that epoxide **139** was the initial epoxide formed, all indirect evidence, based on the biotransformation results obtained so far, suggested that these two compounds may be part of the biosynthesis of Tl (**1**). Based on this hypothesis, **106** was believed to be the most plausible intermediate, prior to epoxidation, in the biosynthesis of Tl (**1**) and Td (**2**).

Furthermore, the isolation of the diepoxy compound **311** from the biotransformation of **194** was some support for the proposed biosynthetic pathway due to the demonstration of the ability of the cells to form diepoxides.

106

194

107

304

(139)*

305

138a

246b

138b

246a
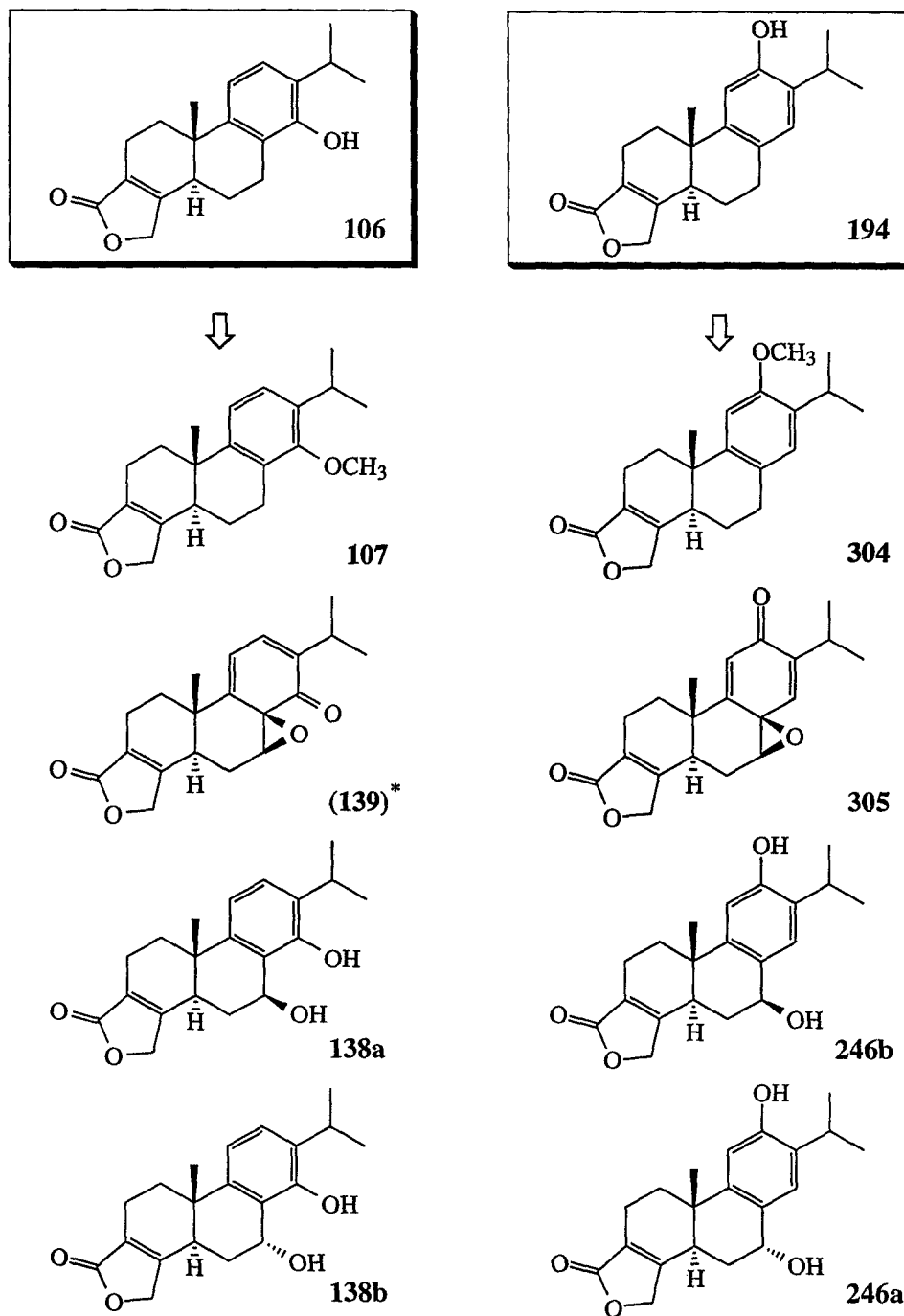
* not isolated

Scheme 4.11 Comparison of **106** and **194** Related Compounds Isolated from Biotransformation and TRP4a Cell Cultures (continued to next page)
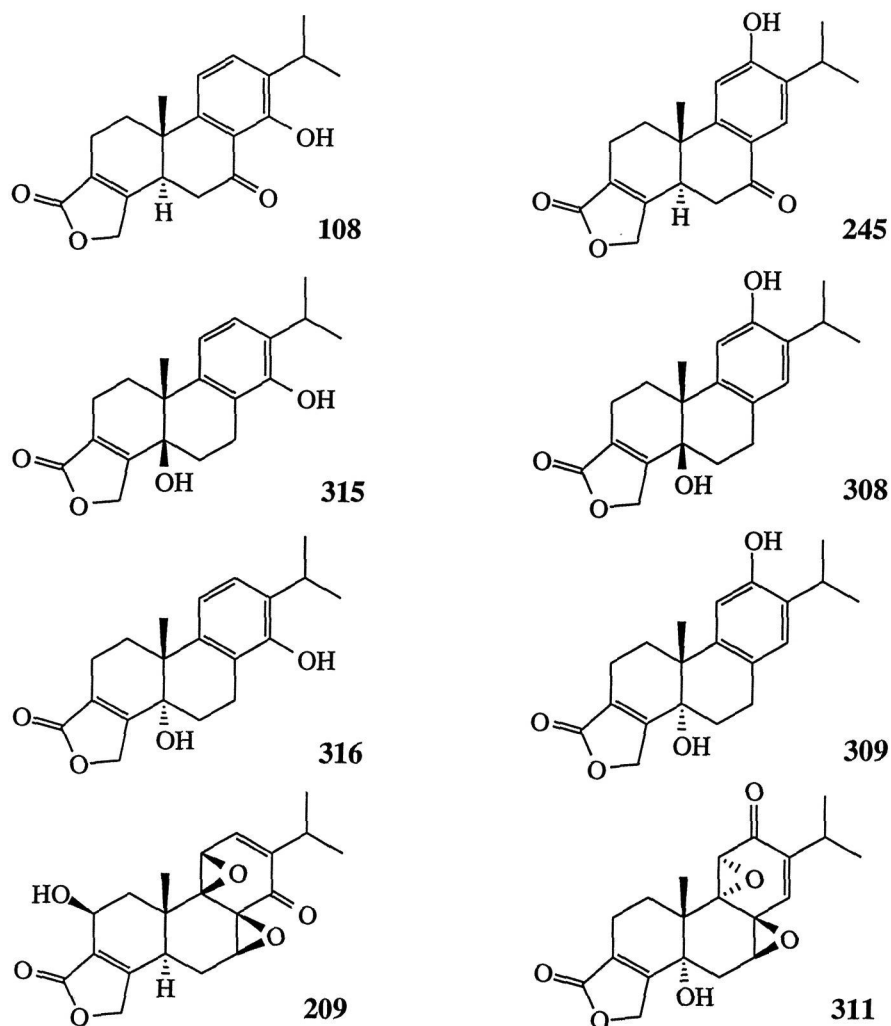
Scheme 4.11   Comparison of **106** and **194** Related Compounds Isolated from
Biotransformation and TRP4a Cell Cultures (continued)

Despite the great similarity between the biotransformations of **106** and **194**, one major difference was that triptophenolide **106** showed biotransformation to a lesser degree than the artificial precursor **194** and that the corresponding epoxy dienone **139** was not isolated from the biotransformation mixtures.

Obviously the way to prove whether **106** is the real precursor is by doing a labeling experiment. [14]C labeled butenolide **193** was successfully synthesized by M. Roberts.[160] By using the modified synthetic sequence to triptophenolide (**106**), we can also readily introduce a

^{2}H or ^{3}H isotope at C12 in the last step and thereby provide enough material for biosynthesis studies.

Biotransformation of 106 with the Fungus *Cunninghamella elegans*

Since significant biotransformation of triptophenolide (**106**) was not observed with TRP4a cell cultures, the attention was turned to other possible routes of biotransformations. Biotransformation of organic compounds by cultures of microorganisms has received increasing attention in recent years. The diversity and selectivity of the reactions that the fungi can perform have made this approach an important adjunct to organic synthesis.

A fungal strain, *Cunninghamella elegans* var. chibaensis Kuwabara *et* Hoshino (ATCC# 20230), was found capable of hydroxylation of isodehydroabietenolide (**193**) at the C7 position.* A C7 hydroxylated **106**, *i.e.*, **138a** would be of interest as a starting material for a biotransformation study with TRP4a cell cultures. Therefore, a biotransformation experiment of **106** with *Cunninghamella elegans* was carried out.

Cunninghamella elegans culture (1 L) in SSBF medium (see Experimental) with 2% glucose was used in the experiment.** After the culture was grown for 44 h, the starting material (**106**, 200 mg) in ethanol (20 mL) was added. The culture was incubated under certain conditions (see Experimental for details), and samples were taken after incubation for 30, 48 and 72 h. Two new spots were detected by TLC after 30 h of incubation, and no substantial changes occurred between 48 and 72 h. The culture was harvested after 76 h of incubation and extracted, yielding a broth extract (~193 mg) and a cell extract (~1.97 g).

Column chromatography of the extracts yielded 5 products along with recovered starting material. The results are summarized in Table 4.16.

Table 4.16   Compounds Isolated from Biotransformation of **106** with *C. elegans*

Compound	**106**[a]	**278**	**314**	**316**	**318**	**313**
mg	4.8	30.9	12.9	10.5	7.7	1.2

[a] Recovered starting material.
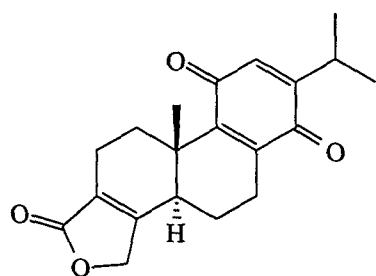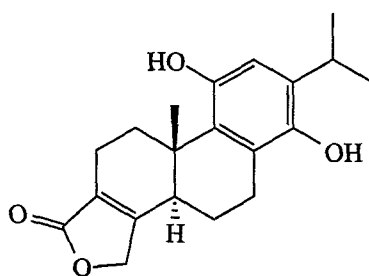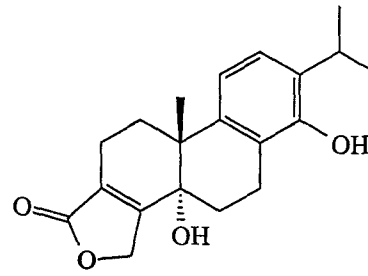
278   **314**   **316**
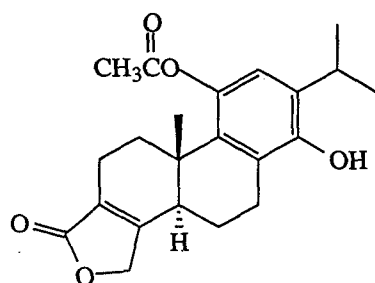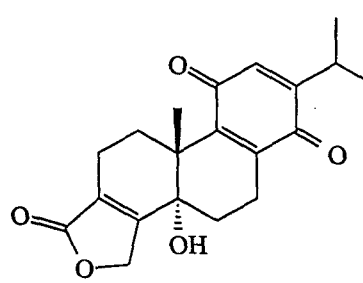
318   **313**

For comparison, compounds **278** and **316** have been isolated in the biotransformation of **106**, and **313** has been obtained in the biotransformation of **278**, with TRP4a cell cultures.

Compound **314** was isolated as a white powder (mp 197-199°C, dec), which was always contaminated with some **278** during the column chromatography, and slowly turned yellow in the NMR tube if $CDCl_3$ was used as the solvent (probably catalyzed by traces of HCl). Its IR spectrum exhibited a hydroxyl group band at 3300 cm[-1], and high resolution mass spectrometry revealed its molecular formula as $C_{20}H_{24}O_4$. It was suspected to be an 11,14-hydroquinone, based on the above experimental observations and the possible mechanism of formation of the quinone **278** from the phenol **106**. In order to examine the relationship of compound **314** with

the quinone **278**, a catalytic hydrogenation was performed. Thus, the quinone **278** (8.2 mg) was stirred with a catalytic amount of palladium (10% on carbon) in methanol under hydrogen (atmospheric pressure) at room temperature. About 15 min later, nearly half of the quinone was converted to **314**, and all the starting material disappeared in about 2 h. Work-up of the reaction yielded almost pure **314** (7.2 mg) with the [1]H NMR spectrum identical to that of the compound isolated from the biotransformation. This result confirmed that **314** was the 11,14-hydroquinone.
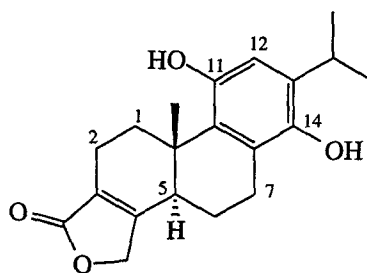
314

It was found that this compound was more stable in methanol than in chloroform, and the [1]H NMR spectrum was then recorded using deuterated methanol (CD$_3$OD) as the solvent. The H12 signal was found at δ 6.47 as a singlet. The H1β resonance, apparently affected by the hydroxyl group at C11, was shifted downfield to δ 3.72 (1H, br d, J = 13.0 Hz), as compared with δ 3.09 in the quinone **278** and about δ 2.5 in the starting material **106**. The COSY spectrum showed that H1β was coupled with H1α at δ 1.47 (1H, m), and a two-proton multiplet at δ 2.29 (H2). The H7β signal was located at δ 2.91 (1H, dd, J = 17.6, 6.0 Hz), and showed cross peaks to H7α in a multiplet with H5 at δ 2.72 (2H, m). Both C7 protons also showed couplings with two other protons at δ 1.76 (1H, dddd, J = 12.6, 12.6, 12.6, 6.0) and δ 1.91 (1H, m), respectively, of which the former was assigned to H6β based on its coupling constants, and the other to H6α.
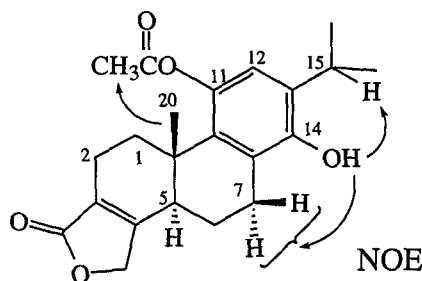
318

Compound **318** was obtained as an optically active ($[\alpha]_D^{20}$ = +75.6°, c = 0.17, CHCl$_3$), white crystalline solid (mp 282-284°C, dec) with a molecular formula of C$_{22}$H$_{26}$O$_5$. Its IR spectrum (KBr) indicated the presence of a hydroxyl group (3425 cm^{-1}), and a new ester group (1750 cm^{-1}). In the ^{1}H NMR spectrum, only one aromatic proton signal was present at δ 6.66 (1H, s), and a new acetate methyl resonance was located at δ 2.32 (3H, s). The remainder of the spectrum was similar to that of **314**. This compound was probably an acetate of **314**. Whether the acetate group was located at C11 or C14 was determined by NOE experiments. Irradiation of the phenolic hydroxyl proton at δ 4.67 (1H, s, D$_2$O exchangeable) resulted in signal enhancements of H15 at δ 3.05 (1H, sept, J = 6.9 Hz), the H7β at δ 2.89 (1H, dd, J = 17.2, 5.5 Hz), as well as the H7α in the multiplet between δ 2.67-2.79 (2H, m, H7α and H5). This clearly indicated that the hydroxyl group was at C14, therefore, the acetate group should be placed at C11. Irradiation of C20 methyl signal caused NOEs to H1β at δ 2.99 (1H, br dd, J = 12.5, 6.2 Hz), H2β at δ 2.34 (1H, m), the acetate methyl and the H6β at δ 1.81 (1H, m), confirming that the location of the acetate group was at C11.

The overall recovery of material was low in this experiment, probably due to prolonged incubation, or because some polar compounds were not recovered. However, no 7-hydroxylated compounds were isolated from the extracts. Of the products isolated in this experiment, the two major ones were quinone **278** and hydroquinone **314**. It was apparent that the enzymes first hydroxylate C11, a position activated by the hydroxyl group at C14, to form **314**, which then undergoes facile oxidation, either by the enzymes or by atmospheric oxygen to form the quinone **278**. Compound **318** was probably formed by enzymatic acetylation of **314**.

The interesting point was that the acetylation took place at the more hindered hydroxyl group in **314**. The formation of **316** could be explained as an enzymatic hydroxylation at C5. Compound **313** probably resulted from either oxidation of **316**, or hydroxylation of the quinone **278**. The overall relationship among the starting material and products is shown in Scheme 4.12.
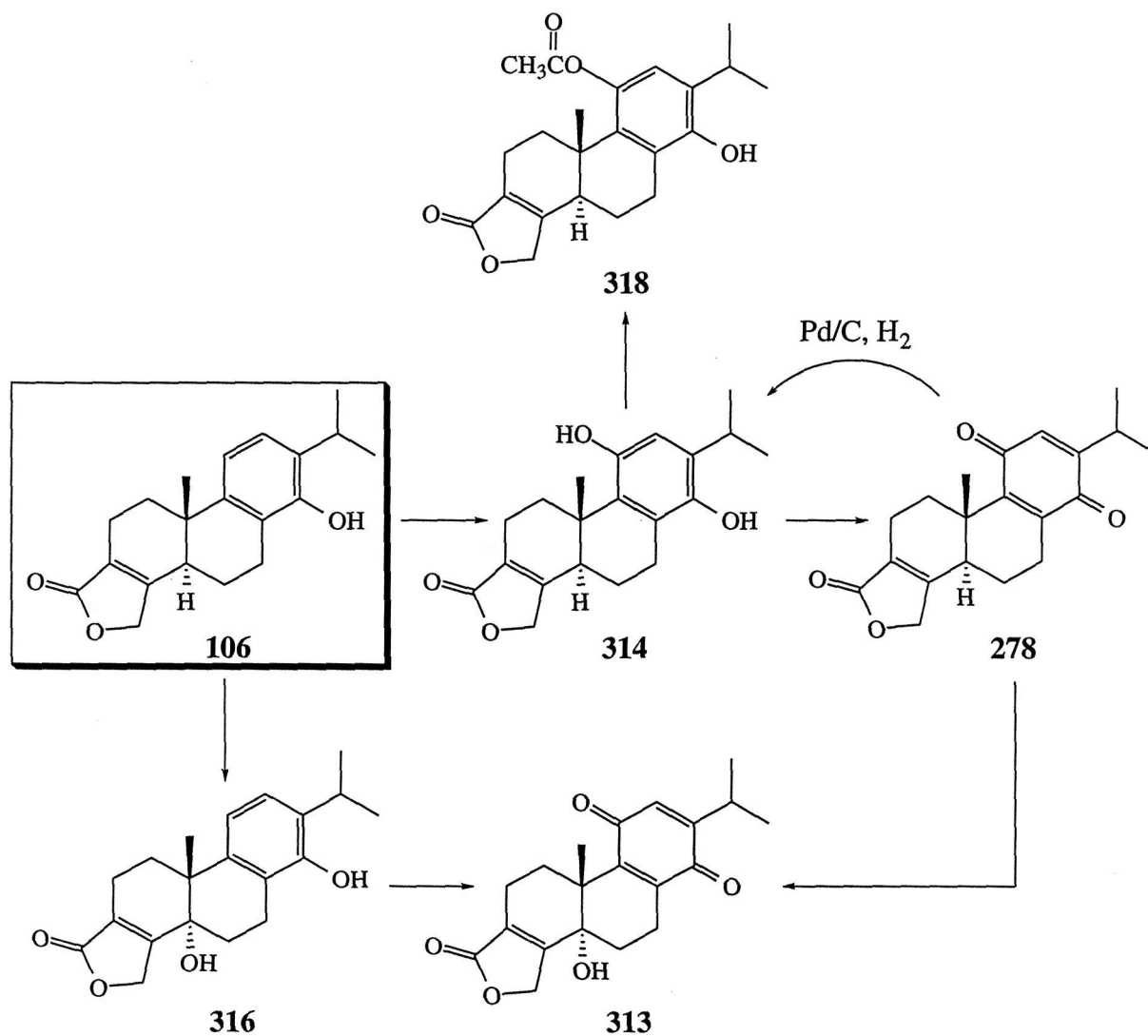
Scheme 4.12 Relationship of Triptophenolide (**106**) with Its Products in the Biotransformation with *C. elegans*

4.2.5 Biotransformation of Demethyl Isoneotriptophenolide (288) with TRP4a Cell Cultures

The different extent of biotransformation achieved with isotriptophenolide (**194**) compared to triptophenolide (**106**) in experiments with TRP4a cell cultures have demonstrated that the location of the hydroxyl group on ring C plays an important role in determining the course of the biotransformations. It would be very interesting to see what would happen if a compound with hydroxyl groups at both positions, such as demethyl isoneotriptophenolide (DINTP, **288**), was incubated with TRP4a cell cultures.
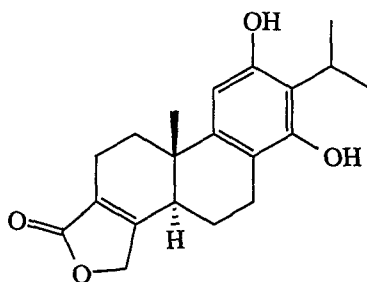
288

A preliminary experiment was carried out using DINTP (**288**) as starting material. A solution of **288** in ethanol was introduced into TRP4a cell culture (21 days old), and the culture was incubated under standard conditions. TLC analysis after 26 h of incubation showed some small amounts of less polar spots, while a substantial amount of starting material remained. However, after 72 h of incubation the starting material had disappeared, while the less polar spots remained and several new polar spots appeared. This result showed that DINTP **288** was more readily "consumed" by the cells than were isotriptophenolide (**194**) and triptophenolide (**106**). In this situation, a shorter incubation time would be appropriate.

In a subsequent large scale experiment, **288** (300 mg) was incubated with a 21-day-old TRP4a cell culture (Trp#335). The cell culture was harvested after 41 h of incubation, and the broth and cells were extracted with ethyl acetate separately, yielding a broth extract (~539 mg)

and a cell extract (~668 mg), respectively. The extracts were then subjected to repeated column chromatography, and several compounds were isolated (Table 4.17).

Table 4.17   Compounds Isolated from Biotransformation of DINTP (**288**) with the TRP4a Cell Culture

Compound	**288**[a]	**321**	**320**	**319**
mg	70.3	14.6	2.9	1.2
%[b]	-	6	1	0.5

[a] Recovered starting material; [b] Based on recovered starting material.

321   **320**   **319**
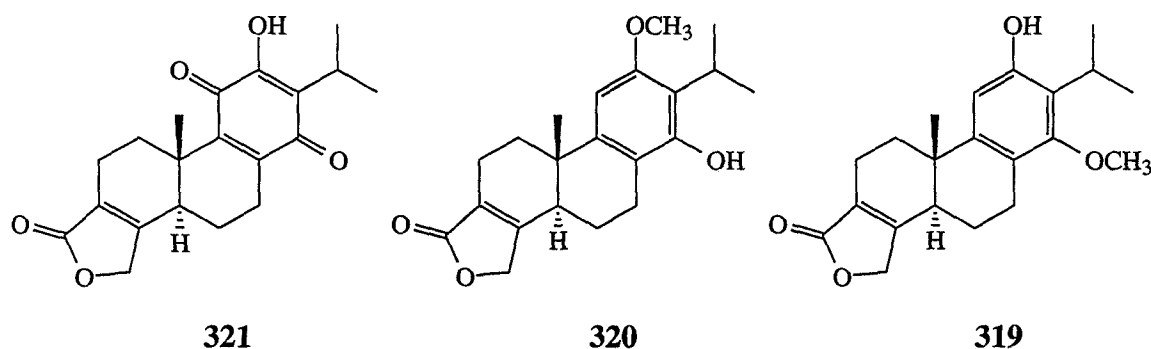
The overall recovery of material was only about 30%, which was the lowest among the biotransformations of the three major starting materials (**194, 106** and **288**). The low recovery suggested that the introduction of the second hydroxyl group increased its susceptibility to various reactions, thus likely leading to the break-down of the molecule.

Compound **320** was isolated as an optically active ($[\alpha]_D^{24}$ +30.9°, c = 0.081, MeOH), colorless powder (mp 206-208°C), with a molecular formula of $C_{21}H_{26}O_4$. Its IR spectrum displayed an absorption band for a hydroxyl group at 3630 cm^{-1}. The ^{1}H NMR spectrum exhibited a very similar spectrum to that of **288**, except for a new methoxy group at δ 3.78 (3H, s). It suggested that this compound was a mono methyl ether of the starting material **288**. The remainder of the protons were assigned accordingly in comparison with the spectrum of **288**. The location of the methoxy group was determined by NOE experiments. Irradiation of H11 at

the methoxy group at δ 3.78, while irradiation of the phenolic hydroxyl group at δ 4.73 showed

NOEs to H15 at δ 3.45 (1H, sept, J = 7.2 Hz), the C7 protons in the multiplet between δ 2.63

and 2.84 (3H, m, H5, H7), and the isopropyl methyl groups at δ 1.32 (6H, d, J = 7.2 Hz).

Accordingly, irradiation of the methoxy group increased the intensity of the H11 signal. These

results indicated that the methoxy group was at C12.
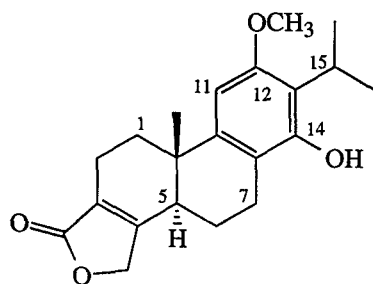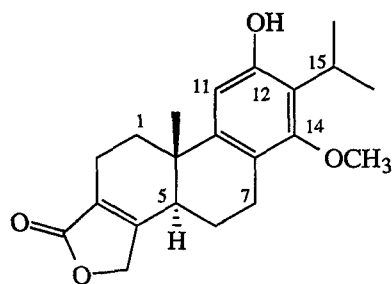
320   319

Compound **319** was obtained as an optically active ($[\alpha]_D^{24}$ +20.0°, c = 0.125, MeOH)

pale yellow powder with a molecular formula of $C_{21}H_{26}O_4$. Its IR spectrum showed a band for

a hydroxyl group at 3600 cm^{-1}. The ^{1}H NMR spectrum exhibited a spectrum comparable with

that of **320**. The C7 protons were slightly shifted downfield, and the methoxy methyl signal

was located at δ 3.68 (3H, s), slightly shifted upfield as compared to **320**. It was logical to

deduce that this compound was the C14 methoxy isomer. Irradiation of the methoxy group

resulted in signal enhancements of H15 at δ 3.41 (1H, sept, J = 7.1 Hz), of the two C7 protons

at δ 2.83 (1H, ddd, J = 17.8, 10.3, 8.2 Hz) and 2.94 (1H, ddd, J = 17.8, 7.4, 1.6 Hz), and of the

isopropyl methyl protons at δ 1.35 and 1.36 (3H each, both d, J = 7.1 Hz), thereby confirming

the methoxy group at C14. The remainder of the protons were assigned as based on their NOE

and COSY results, and by comparison with **288** and **320**.
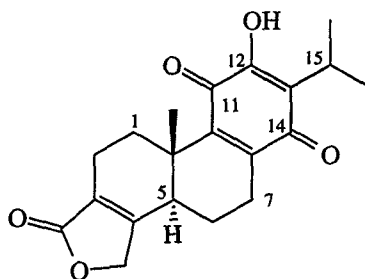
321

Compound **321** was isolated as a yellow powder with the molecular formula $C_{20}H_{22}O_5$. Its IR spectrum showed the presence of a hydroxyl group (3450 cm^{-1}), and a quinone moiety (1640 cm^{-1}). The ^{1}H NMR spectrum exhibited a great similarity to that of the quinone **278**, except for the absence of a quinone olefinic proton signal around δ 6.4. Instead, a hydroxyl proton was found at δ 7.16 (1H, s, exchangeable with D_2O). The H15 resonance was shifted downfield as compared with **278**, an effect which has been observed in the related 12,14-disubstituted compounds, such as **288, 319** and **320**. Therefore, this compound was assigned as 12-hydroxy quinone **321**. The proton signals were assigned accordingly in comparison with quinone **278**.

Several other compounds were also isolated from the biotransformation, but their unstable nature and/or small quantities made their full structural elucidation difficult.

The formation of compounds **319** and **320** were not surprising, as the corresponding methyl ethers of isotriptophenolide **194** and triptophenolide **106** had already been isolated from the biotransformations and from the cell cultures. The two isomeric methyl ethers were probably formed from **288** by the same mechanism. The yield of quinone **321** from **288** was substantially greater than the yield of quinone **278** obtained in the biotransformation of triptophenolide (**106**). A small scale blank experiment with DINP being "incubated" in a sterile medium under the same conditions showed virtually no reaction (TLC, ^{1}H NMR). Therefore, the extensive biotransformation was performed by the cells, not due to oxidation by atmospheric oxygen. The overall low recovery of material was probably due to decomposition

of some biotransformation products. The results suggested that the incubation time should be shorter.

The differences between biotransformations of the three ring C "activated" precursors, namely, isotriptophenolide (**194**), triptophenolide (**106**) and DINTP (**288**), were quite remarkable. It indicated that the position and the number of hydroxyl groups on ring C were critical to the results of the biotransformation with TRP4a cell cultures. A further study may reveal the enzyme specificity and mechanisms involved in these biotransformations and probably give some insight into the biosynthesis of Tl (**1**) and Td (**2**).

CHAPTER 5   CONCLUSION AND OUTLOOK

The extensive investigation of diterpene metabolites in TRP4a cell cultures has resulted

in the isolation of 28 diterpenes (Figure 5.1), of which 25 (including 14 new natural products)

were isolated for the first time from the TRP4a cell cultures. These diterpenes of the abietane

and *abeo*-abietane families constitute a series of metabolites, ranging from the relatively simple

hydrocarbon dehydroabietane (**196**) to the highly oxygenated triptolide (Tl, **1**) and tripdiolide
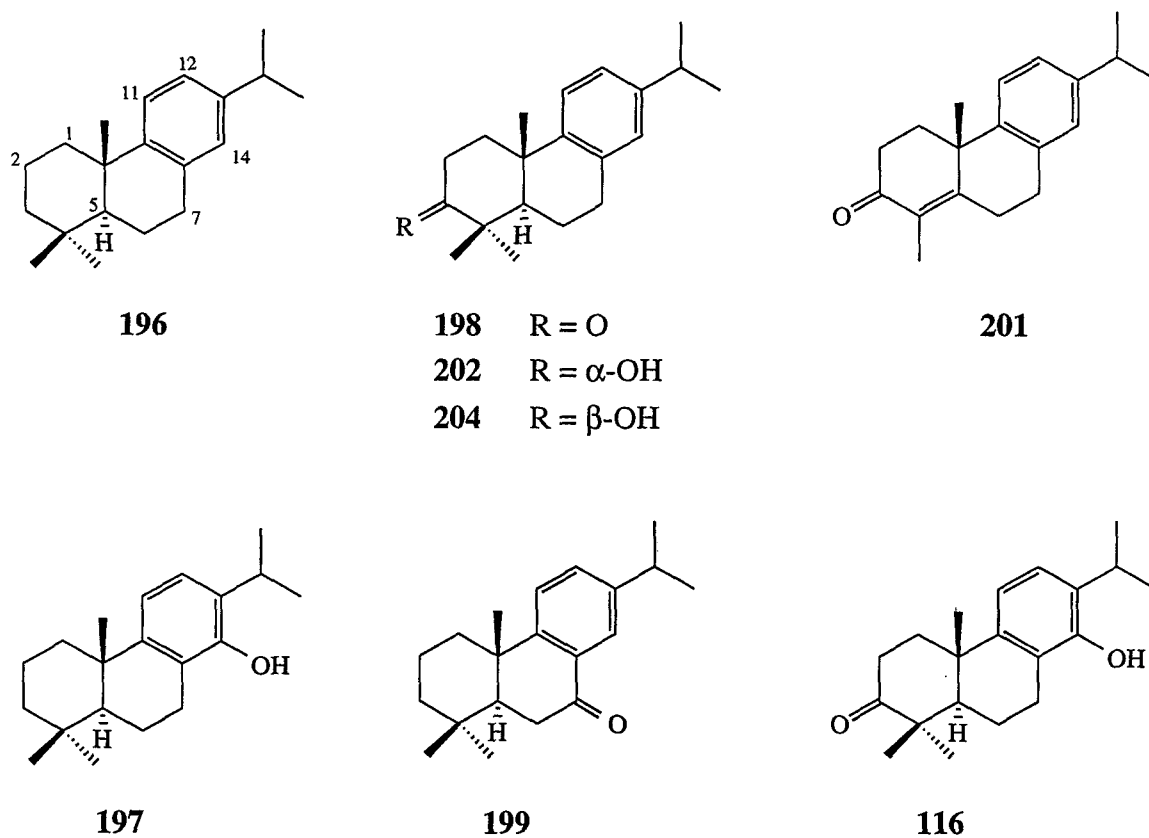
(Td, **2**).

196

198 R = O
202 R = α-OH
204 R = β-OH

201

197

199

116

Figure 5.1   Summary of Diterpene Metabolites Isolated from TRP4a Cell Cultures
(continued to next page)

200	R = O	**117**	R^1 = OH, R^2 = H	**210**	R = H$_2$
203	R = α-OH	**118**	R^1 = H, R^2 = OH	**211**	R = O
205	R = β-OH				

| **193** | R = H$_2$ | **106** | R = H | **108** | R = O |
| **206** | R = O | **107** | R = CH$_3$ | **138a** | R = β-OH |

109

207

Figure 5.1   Summary of Diterpene Metabolites Isolated from TRP4a Cell Cultures
(continued to next page)

212

208

209

1   R = H

2   R = OH
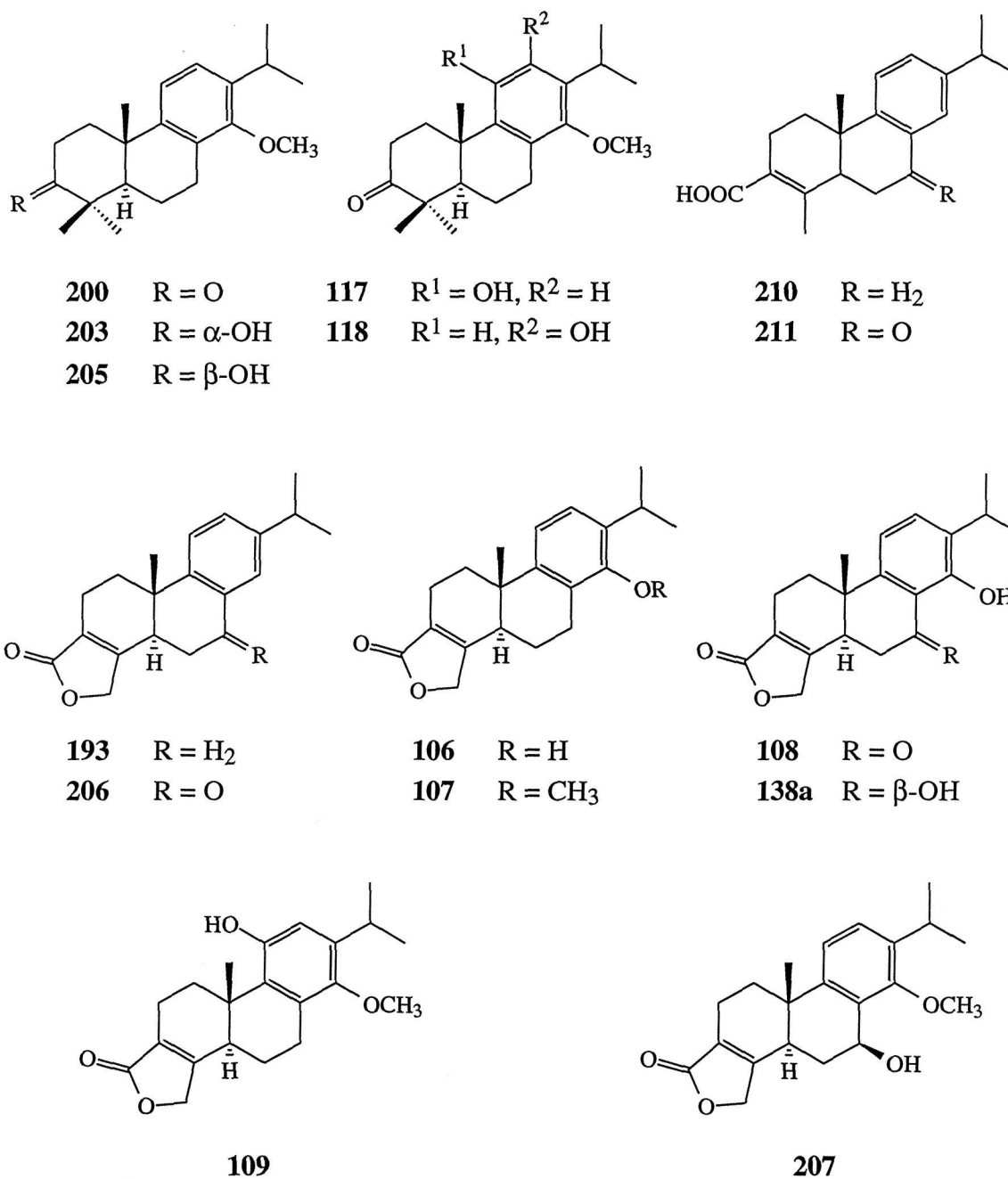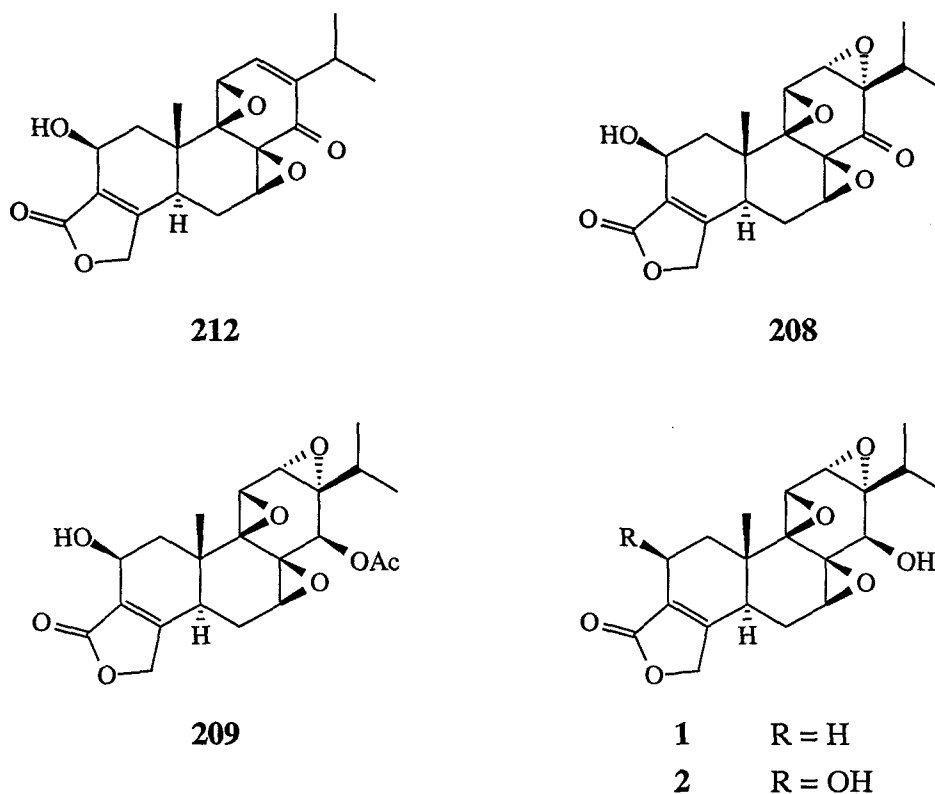
Figure 5.1   Summary of Diterpene Metabolites Isolated from TRP4a Cell Cultures (continued)

This series of metabolites provided some indication about the metabolic process of diterpenes in TRP4a cell cultures. Based on the above results, a plausible biosynthetic pathway from dehydroabietane (**196**), via the key intermediate **106**, to the diterpene triepoxides Tl (**1**) and Td (**2**) has been proposed (Schemes 5.1, 5.2). In order to provide additional quantities of selected diterpene analogs for future biosynthetic experiments, and novel analogs for pharmacological screening, a combination of synthetic chemistry and biotransformation experiments with TRP4a derived enzymes was undertaken.
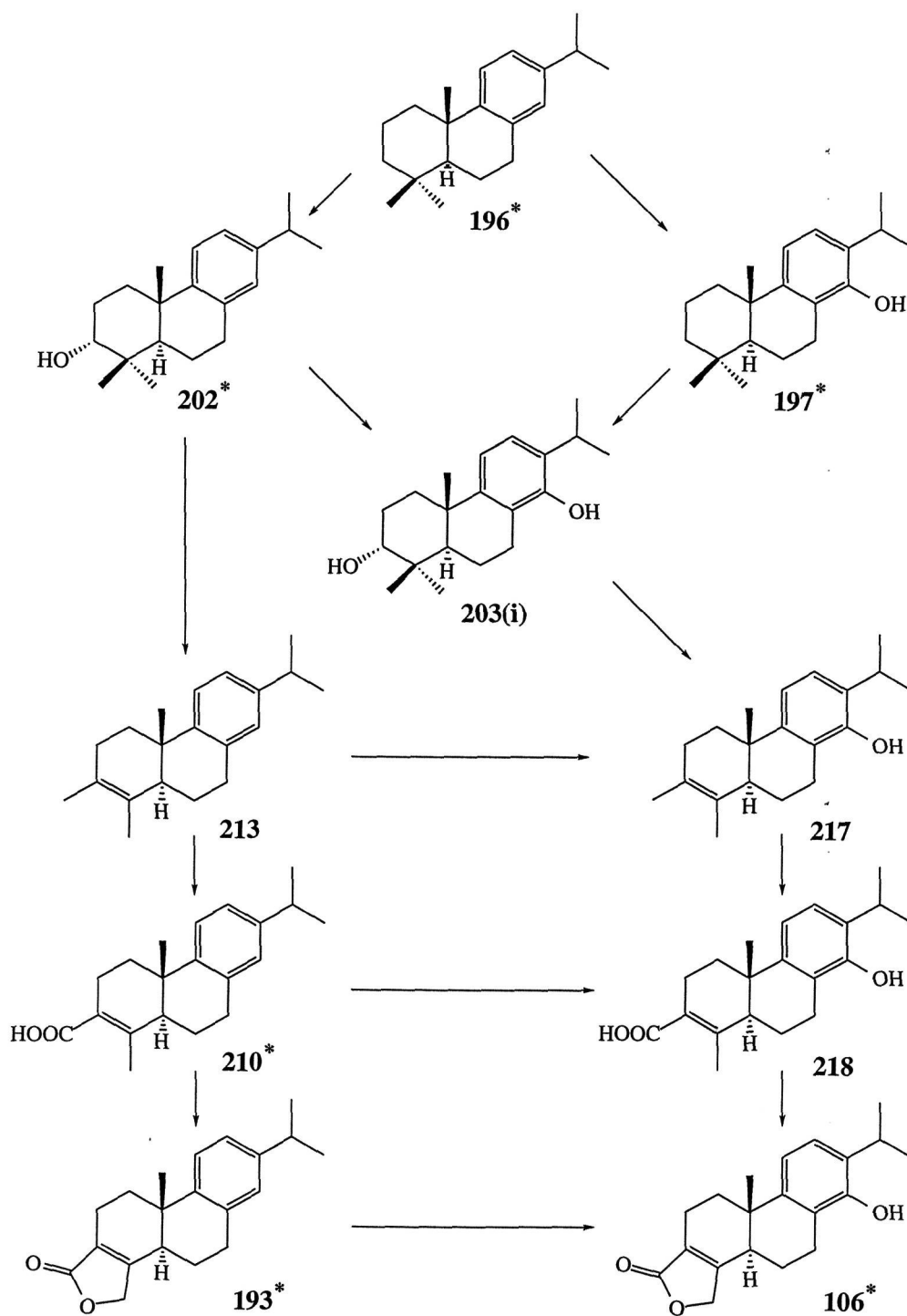
* Compounds isolated

Scheme 5.1   Proposed Biosynthetic Pathway from Dehydroabietane (**196**) to the Key
Intermediate **106**

Scheme 5.2   Proposed Biosynthetic Pathway from Triptophenolide (**106**) to Tl (**1**) and Td (**2**)

The synthetic program involved the syntheses of four "precursors" (**193, 194, 106** and **288**) from dehydroabietic acid (**175**). The previously established synthetic route from **175** to

$193^{160,161}$ was repeated and the latter compound was then used as the starting material for the syntheses of the ring C "activated" analogs **194, 106** and **288**. The sequences from **193** to **194** and partially to **288** developed earlier[161] were followed in order to secure sufficient quantities of relevant compounds for the various biotransformation studies presented. The successful route from **193** to **106** was developed by the present author and is summarized below.
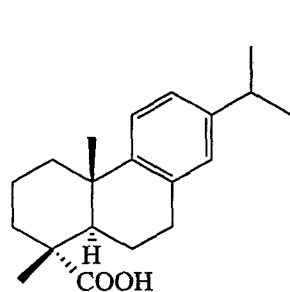
175
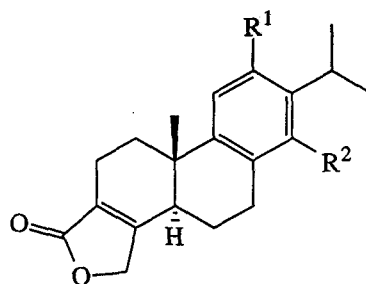
193	$R^1 = R^2 = H$	**272**	$R^1 = R^2 = NO_2$
194	$R^1 = OH, R^2 = H$	**274**	$R^1 = NH_2, R^2 = NO_2$
106	$R^1 = H, R^2 = OH$	**275**	$R^1 = I, R^2 = NO_2$
288	$R^1 = R^2 = OH$	**279**	$R^1 = I, R^2 = OH$
		280	$R^1 = I, R^2 = NH_2$

Synthesis of **106** involved initial nitration of **193**. The resulting dinitro analog **272** was selectively reduced by catalytic hydrogenation to give the 12-amino-14-nitro intermediate **274** which, upon treatment with sodium nitrite followed by potassium iodide, yielded compound **275**. A selective reduction of the nitro group was achieved in high yield by treatment of **275** with sodium dithionite, and the resulting intermediate **280** was diazotized to afford **279**. Final cleavage of the iodo group was accomplished in excellent yield by catalytic hydrogenation. The improved overall yield of **106** from dehydroabietic acid (**175**) was 11%.

Systematic biotransformation studies of the synthetic precursors **106, 194** and **288** with TRP4a cell cultures and cell free extract preparations were then pursued. Detailed experiments were completed with **194** and **106**, and the major results are summarized in Scheme 5.3. Some **106** related compounds which were isolated from the cultures are also included in Scheme 5.3 for a comparison with the biotransformation products of **194**.
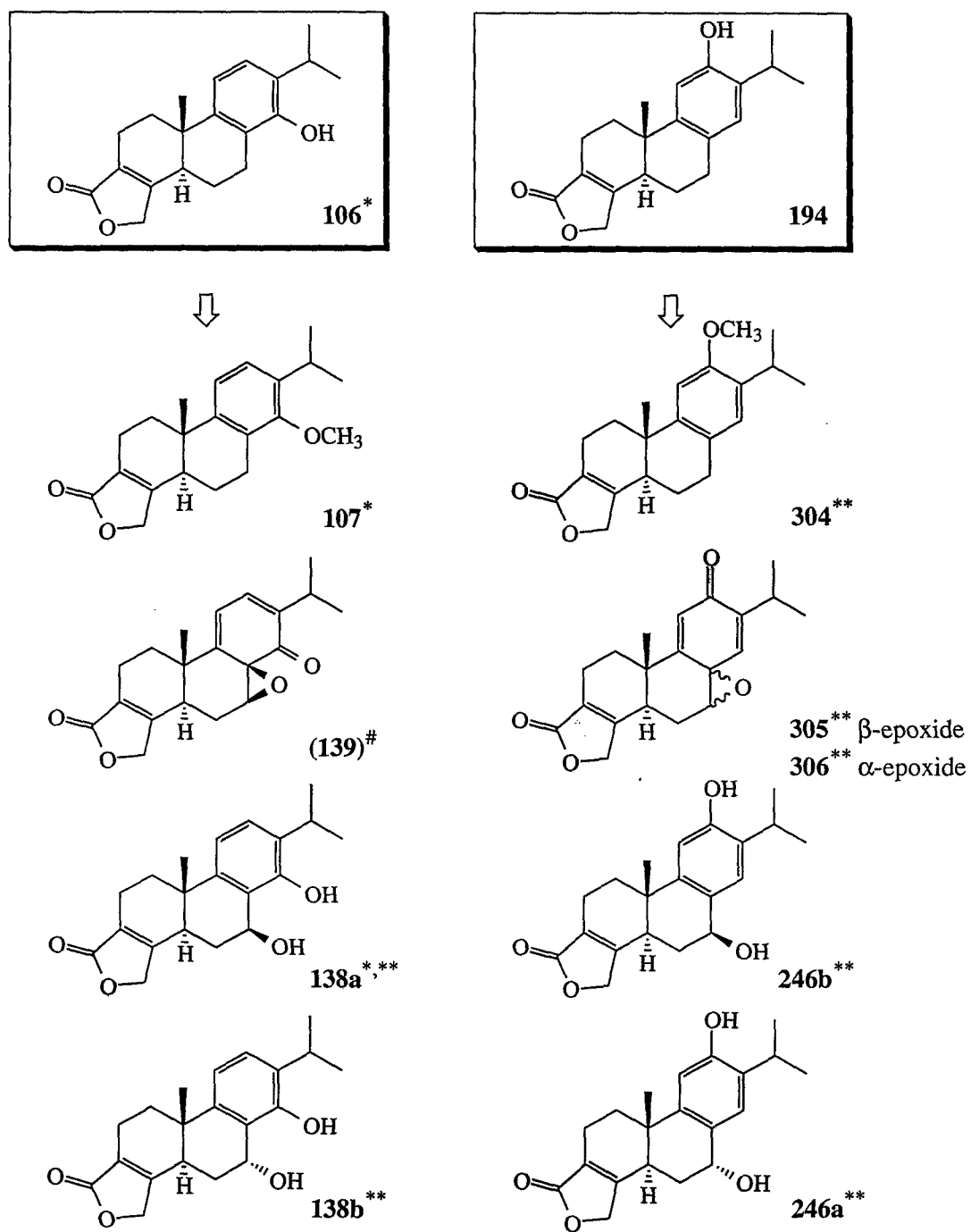
* Isolated from cell cultures. ** Isolated from biotransformation experiments. # Not isolated.

Scheme 5.3   Comparison of 106 and 194 Related Compounds Isolated from
                Biotransformation and TRP4a Cell Cultures (continued to next page)
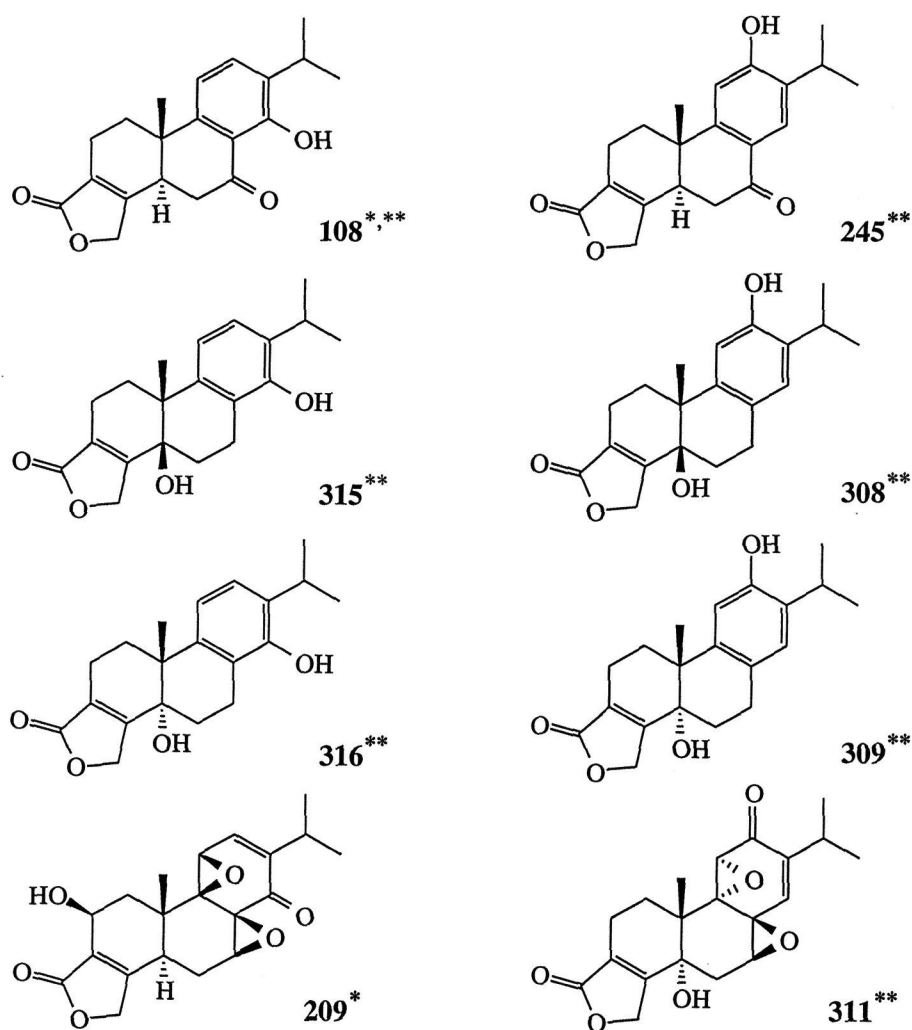
Scheme 5.3   Comparison of **106** and **194** Related Compounds Isolated from Biotransformation and TRP4a Cell Cultures (continued)

Of particular interest was the isolation of the epoxides **305** and **306** in the biotransformation of **194**. This is the first example in which a "*para*-alkylated" phenol is enzymatically converted directly to a *para*-epoxy dienone in a single step. The products obtained may be pharmacologically active, and can also be used as starting materials to synthesize (chemically or biologically) other novel diterpene epoxides for pharmacological studies. Older cell cultures were found to provide a higher yield of the two epoxides. Further biotransformation studies of 7-hydroxy-isotriptophenolide (**246**) and the epoxides (**305, 306**)

indicated that the epoxides were likely produced via a quinone methide intermediate **249**, and not derived from the 7-hydroxy compounds. The formation of epoxides **305**, **306** and **311** from biotransformation of **194** also provided some supporting evidence for the proposed biosynthetic pathway of Tl (**1**) and Td (**2**). Biotransformation of isotriptophenolide (**194**) with TRP4a cell free extracts, cell homogenates and resuspended cell pellets (RCP) showed similar results to those obtained with the whole cell systems. The reaction proceeded much faster in the CFE experiments than in the whole cell cultures. The yield of epoxides was higher with CFE as compared with RCP, which indicated that the enzymes were probably not membrane bound. Hydrogen peroxide as cofactor showed little effect on the yield of the epoxides, while starting material-to-CFE ratio, pH and metal ions showed some influence on the biotransformations.

249   **321**
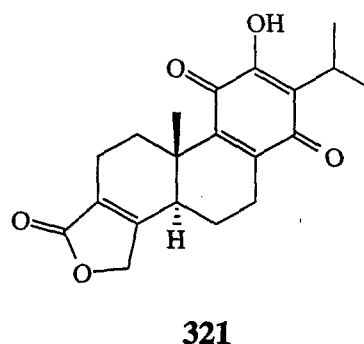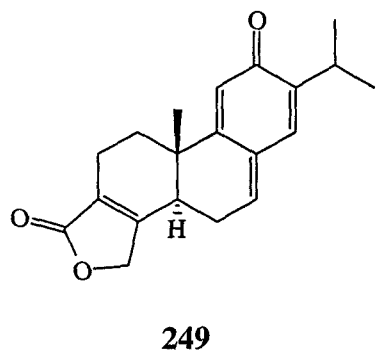
Incubation of **106** with TRP4a cell cultures and CFEs showed a low level of biotransformation as compared with **194**, and no corresponding epoxides were isolated.

Biotransformation of **288** with TRP4a cell cultures showed rapid consumption of the precursor and low recovery of material. The major product isolated was the hydroxy quinone **321**.

Biotransformation of **194**, **106** and **288** has demonstrated that TRP4a cell cultures do have the capability to convert certain ring C "activated" precursors to a series of metabolites including the corresponding epoxides. However, the position and the number of the hydroxyl groups in ring C of the substrate are crucial to the fate of the biotransformations. Though more work is needed to fully understand the enzymatic processes involved in these

biotransformations and in the biosynthesis of Tl (**1**) and Td (**2**), the present study has laid down some background.

The outlook of this project is very promising. The work described in this thesis has opened several new avenues for future work. The yields of epoxides **305** and **306** could be further improved, either by selecting the optimal conditions for the biotransformation, or by inhibiting the activity of, or removing, the reductive enzymes which reduced the epoxides to the hydroxyl compounds. This reduction was a major factor that limited the yield of the epoxides.

Synthesis of diepoxides and triepoxides from **305** and **306**, by further biotransformation or through chemical reactions, would also be an interesting subject which could produce many new diterpene epoxide analogs for pharmacological screening.

A radio labeled triptophenolide (**106**) would be an appropriate starting material to study metabolic pathways in TRP4a cell cultures, since such studies would likely provide a better understanding of the biosynthetic pathway of Tl (**1**) and Td (**2**).

CHAPTER 6   EXPERIMENTAL

6.1   General

Chemicals and solvents used in all synthetic reactions were of reagent grade. Technical grade solvents were used for extractions without purification. Anhydrous Na_2SO_4 or $MgSO_4$ were used as drying agents for organic extracts.

Prior to use, anhydrous THF, ether and benzene were freshly distilled in the presence of sodium and benzophenone under argon. Moisture-sensitive reactions were carried out in oven-dried glassware and under a positive pressure of argon or nitrogen.

Analytical TLC was performed on aluminum-backed, silica gel plates (Merck silica gel 60 F_{254}). Preparative TLC was carried out on glass-backed, pre-coated plates (Merck silica gel 60 F_{254}, 0.25 mm). Developed TLC plates were initially visualized with UV illumination, and then by heat treatment of plates sprayed either with a 5% solution of ammonium molybdate in 10% sulfuric acid, or with a 30% solution of concentrated sulfuric acid in acetic acid followed by a 5% solution of anisaldehyde in isopropanol.

Flash column chromatography on silica gel (Merck silica gel 60, 230-400 mesh) was run under moderate air pressure to maintain a proper eluent flow rate. Reagent grade solvents or distilled technical grade solvents were employed for column chromatography.

Melting points were measured on a Reichert melting point apparatus and are uncorrected.

Optical rotations were recorded on a Perkin-Elmer 141 polarimeter using a quartz cell with a 10 cm path length. The concentration of the samples in units of grams per 100 mL is given in parentheses.

UV spectra were obtained with a Perkin-Elmer Lambda 4B UV/Vis spectrophotometer using quartz cells of 1 cm path length.

IR spectra were recorded either on a Perkin Elmer 710B infrared spectrophotometer or on a Perkin Elmer 1710 infrared Fourier transform spectrophotometer.

^{1}H NMR spectra were recorded on Bruker WH-400, AE-200 or AMX-500 spectrometers. Chemical shifts (δ) are reported in ppm relative to tetramethylsilane (TMS)*. COSY experiments were run on Bruker AE-200 or WH-400 spectrometers. NOE experiments were performed on the Bruker WH-400 spectrometer.

^{13}C NMR spectra were obtained on Bruker AE-200 (at 50.2 MHz), Varian XL-300 (at 75.3 MHz) or Bruker AMX-500 (at 125.8 MHz) spectrometers. Chemical shifts (δ) are cited in ppm relative to TMS. Signals in low intensity are given in parentheses in a few cases. HMBC and HMQC were performed on the AMX-500 spectrometer.

Low resolution mass spectra (LRMS) were determined on Kratos MS 50 or MS 80 mass spectrometers. High resolution mass spectra (HRMS) were recorded on the Kratos MS 50 spectrometer. Electron impact ionization was used unless otherwise stated.

Elemental analyses were performed by Mr. P. Borda, Microanalytical Laboratory, University of British Columbia.

X-ray analyses were carried out by Dr. S. Rettig, Department of Chemistry, University of British Columbia, on a Rigaku AFC6S diffractometer.

Experimental for Chapter 2

6.2 Isolation of Diterpene Metabolites from Plant Cell Cultures of *T. wilfordii* (TRP4a)

Growth Conditions of TRP4a Cell Cultures

Plant cell culture production was carried out in conjunction with Gary Hewitt and David Chen at Biological Services, Department of Chemistry, University of British Columbia. Cultures

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* Residual ^{1}H signals of the deuterated solvents were used as indirect references related to TMS for ^{1}H NMR. Similarly, the carbon signals of the deuterated solvents were used as indirect references to TMS for ^{13}C NMR. The chemical shifts of the solvent signals (^{1}H and ^{13}C) relative to TMS were reported by the supplier of the solvents (Merck Sharp & Dohme Isotopes, Canada Limited). For example, the residual ^{1}H signal of CDCl$_3$ was reported to be at 7.24 ppm for ^{1}H NMR, and the carbon signal of CDCl$_3$ at 77.0 ppm for ^{13}C NMR, respectively.

were grown in conical flasks and incubated without illumination at $27 \pm 1°C$ on a rotary shaker with a 7/8" throw and run at 140 rpm. The cultures were grown in PRD_2CO_{100} growth medium for 14 days and then resuspended as a 10% inoculum in $MSNA_{0.5}K_{0.5}$ production medium. The growth and viability of the cultures were assayed by measuring refractive indices (n_D, Galileo refractometer, $25.0°C$), and monitoring microscopic purity and pH.

General Harvesting and Extraction Procedures

Cells were harvested after growth in $MSNA_{0.5}K_{0.5}$ medium for a certain period of time (usually about 21 days) by filtration through Miracloth™, a coarse fibrous cloth which separates cellular material from liquid broth. The cells were frozen until the time of extraction. The broth (spent medium) was saturated with NaCl and extracted with EtOAc (3 x broth volume). The extracts were combined, dried over anhydrous Na_2SO_4 or $MgSO_4$, and filtered through paper. The solvent was removed by rotary evaporation and the residual extract was dried *in vacuo*.

Frozen cells were thawed and homogenized with a homogenizer (Ultra Turrax, TK-25, 28,000 rpm) in EtOAc (3-5 x cells volume). The homogenized cell suspension in EtOAc was filtered through a pad of Celite, and separated into an organic and an aqueous fraction. The aqueous layer of the filtrate was saturated with NaCl and further extracted with EtOAc (3 x aqueous layer volume). All the EtOAc extracts of the cells were combined, washed with saturated NaCl solution (equal volume) and dried. The EtOAc extract was filtered, the solvent removed by rotary evaporation and the residual extract dried *in vacuo*.

Isolation of Diterpene Metabolites from TRP4a Cell Cultures: A Typical Procedure (Trp #339)

The cells were grown in $MSNA_{0.5}K_{0.5}$ medium under standard conditions. Five flasks (1L Erlenmeyer flask) of cell cultures (550 mL each) were harvested after growth for 28 days (n_D

= 1.3330, pH = 6.29). The cell suspension was filtered through Miracloth™, and the cells (wet weight 298.81 g) were frozen until the time of extraction. The broth was saturated with NaCl and extracted with EtOAc (3 x 1.7 L). The combined EtOAc extract was dried and filtered. The solvent was removed and the residue was dried *in vacuo*, yielding a dark brown extract (543.4 mg).

Thawed cells were homogenized with an Ultra Turrax homogenizer in EtOAc at 28,000 rpm (3 x 1 min). After filtration through Celite, the EtOAc layer was washed with a saturated NaCl solution (3 x 1.7 L), while the aqueous layer was saturated with NaCl and then extracted with EtOAc (3 x 200 mL). All the EtOAc extracts were combined and dried. The solvent was removed and the residue was dried *in vacuo*, providing a dark colored cell extract (1.0358 g).

Both broth and cell extracts were combined and chromatographed (see Figure 2.2 for a graphical presentation of this isolation work) on a silica gel column by consecutive elution with hexanes-EtOAc (6:4, 5:5, 3:7, 1:9), followed by EtOAc and MeOH. Eluates were pooled based on their major spots on TLC (hexanes-EtOAc, 1:1), yielding seven fractions (Frs. I-VII). Fr. VI was derived from EtOAc eluates, while Fr. VII was obtained by resuspending pooled MeOH eluates (after removal of MeOH) in acetone followed by filtration and solvent evaporation.

Fr. I was further chromatographed by stepwise elution with CH_2Cl_2-hexanes (95:5, 90:5, 50:50), yielding seven additional fractions (Frs. IA-IG).

Fr. IA was further purified by chromatography with hexanes to yield **195** (2.6 mg).

Fr. IB was separated into two fractions (Frs. IB1, IB2) by column chromatography with hexanes and hexanes-EtOAc (97:3). Fr. IB1 yielded **196** (17.5 mg) and **195** (8.5 mg) after being chromatographed with hexanes. Fr. IB2 separated into three fractions (Frs. IB2a-IB2c) by chromatography with hexanes-CH_2Cl_2 (7:3, 1:1) and CH_2Cl_2. Fr. IB2a, eluted with hexanes-acetone (98:2), provided three fractions (Frs. IB2ai-IB2aiii). Fr. IB2aii gave **196** (1.5 mg) and Fr. IB2aiii yielded **197** (1.0 mg) after elution with hexanes-CH_2Cl_2 (1:1) and hexanes-acetone (97:3), respectively. Fr. IB2b gave **198** (1.5 mg) with hexanes-acetone (98:2) and Fr. IB2c yielded **199** (1.0 mg) with the same solvent system.

Fr. IC was eluted with hexanes-EtOAc (95:5, 9:1) to give **200** (3.5 mg), **201** (0.9 mg), **202** (1.2 mg) and **116** (4.1 mg).

Fr. ID separated into seven fractions (Frs. ID1-ID7) after being chromatographed with hexanes-EtOAc (9:1, 8:2, 7:3). Fr. ID1 afforded **202** (2.3 mg) by elution with CH_2Cl_2-EtOAc (98:2). Fr. ID2 was chromatographed with hexanes-EtOAc (9:1) to give **193** (1.4 mg) and two other fractions (Frs.ID2b, ID2c). Fr. ID2b yielded **203** (1.1 mg) by elution with CH_2Cl_2-EtOAc (95:5) and Fr. ID2c afforded **204** (2.1 mg) with the same solvent system. Fr. ID3 was **117** (12.3 mg) and Fr. ID4 yielded another 9.2 mg of **117** after chromatography with hexanes-EtOAc (85:15). Fr. ID5 provided **193** (1.4 mg) and Fr. ID6 gave **107** (2.1 mg) by elution with CH_2Cl_2-EtOAc (99:1). Fr. ID7 yielded another 1.4 mg of **107** by column chromatography with hexanes-EtOAc (1:1).

Fr. IE was separated into three fractions (Frs. IE1-IE3) after being chromatographed with hexanes-EtOAc (85:15, 80:20). Fr. IE1 produced **205** (1.2 mg) by elution with CH_2Cl_2-EtOAc (99:1). Fr. IE2 was **118** (1.5 mg) and Fr. IE3 was **108** (5.3 mg).

Fr. IF was eluted with CH_2Cl_2-EtOAc (97:3), hexanes-acetone (9:1) and CH_2Cl_2-EtOAc (95:5) to give **108** (0.1 mg).

Fr. IG was chromatographed with hexanes-EtOAc (8:2) to provide **109** (3.0 mg).

Fr. II was separated into four fractions (Frs. IIA-IID) by chromatography with CH_2Cl_2-EtOAc (97:3, 3:7, 9:1, 8:2, 6:4, 5:5). Fr. IIA afforded **108** (1.1 mg) and **106** (6.3 mg) after elution with hexanes-EtOAc (6:4). Fr. IIB was a mixture of **106** (2.1 mg) and **108** (2.3 mg)[*]. Frs. IIC and IID were **109** (4.0 mg) and **206** (2.9 mg), respectively.

Fr. III was purified by column chromatography with CH_2Cl_2-EtOAc (95:5, 9:1, 8:2, 2:1, 1:2) to yield **106** (2.7 mg).

Fr. IV was chromatographed with CH_2Cl_2-EtOAc (9:1, 8:2, 6:4, 4:6, 1:4) to give three fractions (Frs. IVA-IVC). Fr. IVA was **106** (1.0 mg), Fr. IVB contained **207**, and Fr. IVC was **138** (2.4 mg).

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[*] The relative amounts of **106** and **108** were determined by integration of relevant signals in ^{1}H NMR.

Fr. V separated into two fractions (Frs. VA, VB) by elution with CH$_2$Cl$_2$-EtOAc (9:1, 8:2, 5:5, 3:7). Fr.VA was combined with Fr. IVB and chromatographed with CH$_2$Cl$_2$-EtOAc (9:1) to give **207** (0.8 mg). Fr. VB was Tl (**1**) (6.0 mg).

Fr. VI was eluted with CH$_2$Cl$_2$-EtOAc (8:2, 6:4, 4:6, 1:4) and EtOAc to yield Tl (**1**) (0.6 mg) and a crude fraction VIB which, after chromatography with CH$_2$Cl$_2$-EtOAc (8:2, 2:1), gave small amounts of Tl (**1**) (0.6 mg), Td (**2**) (54.8 mg) and another fraction (Fr. VIB3). This fraction was separated into Frs. VIB3i and VIB3ii by elution with EtOAc-hexanes (7:3). Fr. VIB3i was purified with hexanes-acetone (7:3) to yield **208** (0.6 mg), and Fr.VIB3ii **209** (1.0 mg).

Fr. VII was chromatographed with hexanes-CHCl$_3$-EtOAc-formic acid (110:50:45:3, 110:50:184:3) and CHCl$_3$-EtOAc-formic acid (50:150:3) to give two fractions (Frs. VIIA, VIIB). Fr. VIIA was **210** (2.9 mg), and Fr. VIIB was eluted with hexanes-CHCl$_3$-EtOAc-formic acid (200:50:45:3, 150:50:45:3) to yield **211** (0.7 mg).

In addition, column chromatography purification (CH$_2$Cl$_2$-EtOAc, 6:4, 2:1) of a previously isolated crude Td fraction (49 mg) provided additional quantities of **209** and a small amount of **212** (0.2 mg). Some compounds obtained in small quantities were combined with similar isolates from other experiments with TRP4a cell cultures and then their physical data were measured.

In summary, column chromatography of broth and cells extracts (1.5792 g) of the TRP4a cell culture yielded 29 compounds. Please see Table 2.1 in Chapter 2 for details.

Abieta-8,11,13-triene (196) (dehydroabietane)

Colorless oil; $[\alpha]_D^{20}$: +49.2° (c = 2.02, CHCl$_3$) (lit.: +54° (CHCl$_3$),[261] +49° and +63° (MeOH)[166]); UV λ_{max}^{MeOH} (log ε): 204.9 (4.13), 267.4 (2.63), 275.8 (2.61); IR (neat) v$_{max}$ cm^{-1}: 3070 (aromatic CH), 2975 (CH), 1600 and 1480 (aromatic C=C), 1460, 1370, 890, 810; ^{1}H NMR (400 MHz, CDCl$_3$) δ: 0.92 (3H, s, H19), 0.93 (3H, s, H18), 1.17 (3H, d, J = 0.5 Hz,

H20), 1.21 (6H, d, J = 6.9 Hz, H16, H17), 1.21 (1H, m, H3α, overlapped with H16, H17),

1.34 (1H, dd, J = 12.4, 2.4 Hz, H5), 1.39 (1H, br ddd, J = 13.1, 12.7, 3.6 Hz, H1α), 1.46

(1H, br d, J = 13.2 Hz, H3β), 1.59 (1H, m, H2α), 1.64-1.81 (2H, m, H2β, H6β), 1.86 (1H,

br dddd, J = 13.2, 7.1, 2.4, 2.4 Hz, H6α), 2.26 (1H, br d, J = 12.7 Hz, H1β), 2.83 (1H, sept,

J = 6.9 Hz, H15), 2.89 (2H, m, H7), 6.88 (1H, d, J = 1.6 Hz, H14), 6.97 (1H, dd, J = 8.2,

1.6 Hz, H12), 7.16 (1H, d, J = 8.2 Hz, H11); ^{13}C NMR (50.2 MHz, CDCl$_3$) δ: 19.1, 19.3,

21.6, 24.0 (2C), 24.9, 30.5, 33.3, 33.4 (2C), 37.5, 38.9, 41.8, 50.4, 123.8, 124.3, 126.8,

134.9, 145.4, 147.6; LRMS m/z (rel. intensity): 270 (M$^+$, 38.7), 255 (base peak), 227 (5.8),

199 (10.1), 185 (35.3), 173 (49.3), 159 (53.6), 69 (93.2); HRMS calcd. for C$_{20}$H$_{30}$:

270.2347; found: 270.2345; Anal. calcd. for C$_{20}$H$_{30}$: C, 88.82; H, 11.18; found: C, 88.72;

H, 11.23.

Synthesis of 196 from dehydroabietic acid (157)

To a stirred suspension of LAH (3.11 g) in anhydrous ether (120 mL) was added a

solution of 157 (2.829 g, 95-96%) in ether (60 mL) over a period of 30 min at r.t. under argon.

The mixture was stirred for 4.5 h (TLC, hexanes-EtOAc, 8:2) followed by addition of MeOH (10

mL in 50 mL ether) over 2 h to decompose the excess hydride. Water (5 mL) and 5% H$_2$SO$_4$

(150 mL) were added and the mixture was stirred until two clear layers formed. The aqueous

phase was extracted with ether (3 x 100 mL), and all the ethereal extracts were combined and

washed with a saturated NaHCO$_3$ solution (300 mL), brine (300 mL), and dried. The crude

product was chromatographed with hexanes-EtOAc (8:2) to give the alcohol 196(i) as a thick

colorless oil (1.5881 g, 62%).

Physical data of **196(i)**:

A thick colorless oil; IR (neat) cm^{-1}: 3400 (OH), 2950 (CH), 1060 (C-O); ^{1}H NMR (400 MHz, CDCl$_3$) δ: 0.88 (3H, s, H19), 1.21 (3H, s, H20), 1.21 (6H, d, J = 6.9 Hz, H16, H17), 1.21 (1H, m, aliphatic proton, overlapped with H16, H17), 1.39 (3H, m, aliphatic protons), 1.59-1.85 (4H, m, aliphatic protons), 2.27 (1H, br d, J = 12.9 Hz, H1β), 2.81 (1H, sept, J = 6.9 Hz, H15), 2.88 (2H, m, H7), 3.22, 3.46 (1H each, both d, J = 10.9 Hz, H18), 6.88 (1H, br s, H14), 6.98 (1H, dd, J = 8.2, 1.4 Hz, H12), 7.17 (1H, d, J = 8.2 Hz, H11); ^{13}C NMR (50.2 MHz, CDCl$_3$) δ: 17.4, 18.6, 18.8, 24.0 (2C), 25.2, 30.1, 33.4, 35.1, 37.3, 37.8, 38.4, 43.9, 72.2, 123.8, 124.2, 126.8, 134.7, 145.5, 147.3; LRMS m/z (rel. intensity): 286 (M$^+$, 31.1), 271 (89.9), 253 (base peak), 241 (7.7), 225 (9.5), 211 (30.7), 197 (13.6), 185 (32.6), 173 (73.9), 159 (66.7); HRMS calcd. for C$_{20}$H$_{30}$O: 286.2297; found: 286.2292.

The alcohol **196(i)** (1.54 g, 5.38 mmol) was dissolved in dry pyridine (41 mL) followed by addition of p-toluenesulfonyl chloride (4.11 g, 21.6 mmol). The reaction mixture was stirred at r.t. for about 60 h (TLC, hexanes-EtOAc, 8:2) and then poured into ice water (200 mL) followed by extraction with EtOAc (3 x 150 mL). The EtOAc extracts were combined, washed with 10% HCl (2 x 150 mL), saturated NaHCO$_3$ (2 x 150 mL), brine (2 x 150 mL), then dried, and concentrated. Column chromatography (hexanes-EtOAc, 8:2) of the crude product gave the pure tosylate **196(ii)** as a thick colorless oil (2.3172 g, 97.8% yield).

Physical data of tosylate **196(ii)**:

A thick colorless oil; IR (neat) cm^{-1}: 3050 (aromatic CH), 2975 (CH), 1600 and 1500 (aromatic C=C), 1460, 1360 and 1170 (tosylate), 950, 840; ^{1}H NMR (400 MHz, CDCl$_3$) (partial) δ: 0.87 (3H, s, H19), 1.15 (3H, s, H20), 1.20 (6H, d, J = 6.9 Hz, H16, H17), 2.22 (1H, br d, J = 12.7 Hz, H1β), 3.59, 3.80 (1H each, both d, J = 9.3 Hz, H18), 6.84 (1H, br s,

H14), 6.96 (1H, dd, J = 8.2, 1.6 Hz, H12), 7.12 (1H, d, J = 8.2 Hz, H11); ^{13}C NMR (50.2 MHz, CDCl$_3$) δ: 17.1, 18.3, 18.8, 21.6, 24.0 (2C), 25.2, 29.8, 33.4, 35.0, 37.1, 37.3, 38.0, 43.6, 77.8, 123.9, 124.2, 126.8, 127.9 (2C), 129.8 (2C), 133.0, 134.5, 144.6, 145.7, 146.8; LRMS m/z (rel. intensity): 440 (M$^+$, 6.2), 425 (1.2), 253 (base peak), 211 (18.3); HRMS calcd. for C$_{27}$H$_{36}$SO$_3$: 440.2385; found: 440.2383.

The tosylate **196(ii)** (2.3014 g, 5.23 mmol) was treated with NaI (3.92 g, 26.15 mmol) and Zn powder (17.1 g, 261.5 mmol) in HMPA (55 mL) at 110 ± 5°C for 19.5 h, and then the mixture was filtered through Celite. The filtrate was diluted with water (400 mL) and extracted with hexanes (3 x 300 mL). The hexanes extracts were combined, washed with 5% HCl (300 mL), saturated NaHCO$_3$ (300 mL), brine (2 x 300 mL), and concentrated. Column chromatography of the crude product with hexanes gave dehydroabietane (**196**) as a colorless oil (1.0149 g, 71.9% yield). The spectra of the synthetic **196** were identical to those obtained from the isolated **196**.

Abieta-8,11,13-trien-3β-ol (204)

Colorless wax-like needles (CH$_2$Cl$_2$); mp: 98-100°C (lit.: 109-111°C^{170}; 136.5-138°C^{171}); $[\alpha]_D^{24}$: +45.6° (c = 0.241, MeOH) (lit.: +37.26° (CHCl$_3$)^{170}; +50.4° (CHCl$_3$)^{171}); UV λ_{max}^{MeOH} (log ε): 215.5 (3.84), 267.5 (2.97), 275.6 (2.99); IR (CHCl$_3$) ν_{max} cm^{-1}: 3600 (OH), 3025 (aromatic CH), 2950 (CH), 1600 and 1500 (aromatic C=C), 1470, 1380, 1210 (O-H), 1040 (C-O); ^{1}H NMR (400 MHz, CDCl$_3$) δ: 0.88 (3H, s, H19), 1.05 (3H, s, H18), 1.17 (3H, s, H20), 1.20 (6H, d, J = 6.9 Hz, H16, H17), 1.31 (1H, dd, J = 12.1, 2.1 Hz, H5), 1.52 (1H, m, H1α), 1.64-1.92 (4H, m, H2, H6), 2.28 (1H, br ddd, J = 13.0, 3.3, 3.3 Hz, H1β), 2.80 (1H, sept, J = 6.9 Hz, H15), 2.84 (1H, m, H7α), 2.93 (1H, br dd, J = 17.2, 6.0 Hz, H7β), 3.27 (1H, dd, J = 10.8, 5.2 Hz, H3α), 6.88 (1H, br s, H14), 6.97 (1H, br d, J = 8.2 Hz, H12), 7.13 (1H, br d, J = 8.2 Hz, H11); ^{13}C NMR (50.2 MHz, CDCl$_3$) δ: 15.3, 18.9,

24.0 (2C), 24.9, 28.0, 28.1, 30.7, 33.4, 36.9, 37.3, 39.0, 49.9, 78.8 (C3), 123.9, 124.4, 126.8, 134.7, 145.8, 146.8; LRMS m/z (rel. intensity): 286 (M$^+$, 39.6), 271 (53.3), 253 (base peak), 227 (12.6), 211 (20.2), 199 (25.2), 185 (34.9), 183 (27.2), 159 (43.9); HRMS calcd. for $C_{20}H_{30}O$: 286.2297; found: 286.2299.

Abieta-8,11,13-trien-3α-ol (202)

A colorless wax-like solid (hexanes-EtOAc); mp: 102-104°C; $[\alpha]_D^{24}$: +18.2° (c = 0.190, MeOH); UV λ_{max}^{MeOH} (log ε): 206.9 (3.85), 267.4 (2.93), 275.7 (2.90); IR (CHCl$_3$) v_{max} cm^{-1}: 3628 (OH), 3004 (aromatic CH), 2962 (CH), 1498 (aromatic C=C), 1458 (CH), 1381, 1057 (C-O), 985; ^{1}H NMR (400 MHz, CDCl$_3$) δ: 0.94 (3H, s, H19), 1.01 (3H, s, 18), 1.18 (3H, s, H20), 1.20 (6H, d, J = 7.0 Hz, H16, H17), 1.65-1.80 (4H, m, H2α, H5, H6), 1.85 (1H, br ddd, J = 13.7, 13.7, 3.5 Hz, H1α), 2.01 (1H, br ddd, J = 13.7, 3.5, 3.5 Hz, H1β), 2.09 (1H, dddd, J = 13.7, 13.7, 3.5, 2.5 Hz, H2β), 2.80 (1H, sept, J = 7.0 Hz, H15), 2.89 (2H, m, H7), 3.48 (1H, br dd, J = 2.5, 2.5 Hz, H3β), 6.86 (1H, br s, H14), 6.96 (1H, br d, J = 8.2 Hz, H12), 7.15 (1H, d, J = 8.2 Hz, H11); ^{13}C NMR (50.2 MHz, CDCl$_3$) δ: 18.7, 22.1, 24.0 (2C), 24.7, 25.9, 28.1, 29.7, 30.3, 31.5, 33.5, 37.7, 43.7, 75.8 (C3), 123.8, 124.2, 126.8, 134.8, 145.5, 147.2; LRMS m/z (rel. intensity): 286 (M$^+$, 13.2), 271 (13.9), 253 (base peak), 238 (1.7), 227 (2.4), 211 (5.5), 185 (4.6), 169 (5.1), 159 (8.9), 143 (7.7), 129 (8.5), 117 (7.2); HRMS calcd. for $C_{20}H_{30}O$: 286.2297; found: 286.2298.

Abieta-8,11,13-trien-3-one (198)

A colorless solid; $[\alpha]_D^{23}$: +31.1° (c = 0.188, CHCl$_3$); IR (CHCl$_3$) cm^{-1}: 1310 (aromatic CH), 2930 (CH), 1708 (C=O), 1500 (aromatic C=C), 1462, 1385, 1281; ^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.12 (3H, s, H19), 1.14 (3H, s, H18), 1.21 (6H, d, J = 6.9 Hz, H16, H17), 1.27 (3H, s, H20), 1.79 (2H, m, H6), 1.90 (1H, dd, J = 11.3, 3.3 Hz, H5), 1.94 (1H, m, H1α),

2.46 (1H, ddd, J = 13.3, 7.5, 4.2 Hz, H1β), 2.55 (1H, ddd, J = 15.7, 7.6, 4.2 Hz, H2α),

2.67 (1H, ddd, J = 15.7, 10.0, 7.5 Hz, H2β), 2.81 (1H, sept, J = 6.9 Hz, H15), 2.88 (2H, m,

H7), 6.90 (1H, br s, H14), 7.00 (1H, br d, J = 8.2 Hz, H12), 7.15 (1H, d, J = 8.2 Hz, H11);

^{13}C NMR (50.2 MHz, CDCl$_3$) δ: 20.3, 21.1, 23.9 (2C), 24.7, 26.8, 29.7, 30.9 (2C), 33.5,

34.7, 37.5, 50.7, 124.3, 125.3, 126.8, 128.8, 130.9, 134.8, (217.4); LRMS m/z (rel.

intensity): 284 (M$^+$, 42.6), 269 (84.1), 227 (base peak), 213 (11.4), 199 (10.6), 185 (32.6),

171 (28.7), 159 (18.1), 143 (32.5), 125 (35.9); HRMS calcd. for C$_{20}$H$_{28}$O: 284.2140; found:

284.2142.

18-Norabieta-4,8,11,13-tetraen-3-one   (201)

A pale yellow oil; $[\alpha]_D^{23}$: +136.9° (c = 0.01132, MeOH); UV λ_{max}^{MeOH} (log ε): 219.0

(3.93), 243.3 (4.03); IR (CHCl$_3$) v_{max} cm^{-1}: 3025 (aromatic CH), 2975 (CH), 1660 (C=C-

C=O), 1620 (C=C-C=O), 1500 (aromatic C=C); ^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.22 (6H, d, J

= 7.0 Hz, H16, H17), 1.50 (3H, s, H20), 1.82 (3H, br s, H19), 2.04 (1H, br ddd, J = 14.8,

13.2, 4.9 Hz, H1α), 2.35 (1H, ddd, J = 13.2, 5.1, 2.3 Hz, H1β), 2.52 (2H, m, H2α, H6β),

2.71 (1H, ddd, J = 17.7, 14.8, 5.1 Hz, H2β), 2.76-2.91 (3H, m, H6α, H7α, H15), 2.98 (1H,

m, H7β), 6.95 (1H, d, J = 1.5 Hz, H14), 7.08 (1H, dd, J = 8.1, 1.5 Hz, H12), 7.20 (1H, d, J

= 8.1 Hz, H11); ^{13}C NMR (50.2 MHz, CDCl$_3$) δ: 10.9, 23.9, 24.0, 27.0, 27.5, 30.1, 33.5,

34.2, 36.2, 39.5, 125.0, 125.5, 126.3, 128.7, 135.4, 142.3, 146.5, 162.6, 198.2; LRMS m/z

(rel. intensity): 268 (M$^+$, 28.2), 253 (base peak), 225 (18.0), 211 (4.5), 197 (4.5), 183 (5.2),

165 (6.4), 155 (5.7), 141 (7.3); HRMS calcd. for C$_{19}$H$_{24}$O: 268.1827; found: 268.1826.

Abieta-8,11,13-trien-7-one   (199)

A colorless oil; $[\alpha]_D^{27}$: +12.7° (c = 0.331, CHCl$_3$) (Lit.: +14° (CHCl$_3$)[176]); UV λ_{max}^{MeOH}

(log ε): 215.0 (4.09), 254.0 (4.03), 302.0 (3.33); IR (CHCl$_3$) v_{max} cm^{-1}: 2950 (CH), 1660

(aromatic C=O), 1600, 1480, 1450; ^{1}H NMR (400 MHz, CDCl$_3$) δ: 0.91 (3H, s, H19), 0.98 (3H, s, H18), 1.215 (3H, s, H20), 1.225, 1.227 (3H each, both d, J = 6.9 Hz, H16, H17), 1.27 (1H, m, H3α, partially overlapped with H16, H17), 1.52 (2H, m, H1α, H3β), 1.66 (1H, m, H2α), 1.75 (1H, dddd, J = 13.7, 13.7, 3.3, 3.3 Hz, H2β), 1.86 (1H, dd, J = 13.5, 4.4 Hz, H5), 2.31 (1H, br d, J = 12.6 Hz, H1β), 2.62 (1H, dd, J = 18.1, 13.5 Hz, H6β), 2.71 (1H, dd, J = 18.1, 4.4 Hz, H6α), 2.90 (1H, sept, J = 6.9 Hz, H15), 7.27 (1H, d, J = 8.2 Hz, H11), 7.37 (1H, dd, J = 8.2, 2.1 Hz, H12), 7.85 (1H, d, J = 2.1 Hz, H14); ^{13}C NMR (50.2 MHz, CDCl$_3$) δ: 18.9, 21.3, 23.4, 23.75, 23.82, 32.5, 33.2, 33.6, 36.3, 37.9, 38.0, 41.4, 49.4, 123.7, 124.9, 130.7, 132.4, 146.6, 153.8, 199.8; LRMS m/z (rel. intensity): 284 (M$^+$, 51.5), 269 (base peak), 241 (3.5), 227 (21.9), 201 (38.0), 199 (40.7), 187 (44.0); HRMS calcd. for C$_{20}$H$_{28}$O: 284.2140; found: 284.2140.

Synthesis of 199 from dehydroabietane 196

To a stirred solution of **196** (100 mg, 0.37 mmol) in HOAc (5 mL) was added a freshly prepared CrO$_3$ solution (286 mg, 2.86 mmol) in aqueous HOAc (10 mL HOAc with 1mL H$_2$O) over a period of 1 h. The reaction continued for 1 h and then isopropyl alcohol (2.5 mL) was added. The resulting mixture was stirred for further 10 min and then was diluted with water (150 mL). The aqueous mixture was extracted with EtOAc (3 x 80 mL) and the extracts were combined, washed with saturated NaHCO$_3$ (2 x 80 mL), brine (2 x 80 mL), dried, and concentrated. The crude product thus obtained was purified by column chromatography with hexanes-EtOAc (95:5) to give the ketone **199** as a colorless oil (79.5 mg, 75.6% yield). The spectra of the synthetic **199** are identical to those obtained from the isolated **199**.

Abieta-8,11,13-trien-14-ol (197)

A pale yellow oil; $[\alpha]_D^{27}$: +48.3° (c = 0.721, CHCl$_3$); UV λ_{max}^{MeOH} (log ϵ): 204.0 (3.67),

272.0 (2.39); IR (CHCl$_3$) ν_{max} cm^{-1}: 3620 (sharp, OH), 3050 (aromatic CH), 2980 (CH), 1580

and 1500 (aromatic C=C), 1480, 1430, 1180 (C-O); ^{1}H NMR (400 MHz, CDCl$_3$) δ: 0.94 (3H,

s, H19), 0.96 (3H, s, H18), 1.20 (3H, d, J = 0.4 Hz, H20), 1.21 (1H, m, H3α, overlapped

with H20), 1.24, 1.26 (3H each, both d, J = 6.9 Hz, H16, H17), 1.33 (1H, dd, J = 12.7, 2.1

Hz, H5), 1.39 (1H, ddd, J = 12.8, 12.8, 3.7 Hz, H1α), 1.48 (1H, br d, J = 13.2 Hz, H3β),

1.60 (1H, m, H2β), 1.65-1.81 (2H, m, H2α, H6β), 1.98 (1H, br dd, J = 13.3, 7.9 Hz, H6α),

2.27 (1H, br d, J = 12.8 Hz, H1β), 2.61 (1H, ddd, J = 16.5, 11.3, 7.9 Hz, H7α), 2.81 (1H,

br dd, J = 16.5, 6.6 Hz, H7β), 3.15 (1H, sept, J = 6.9 Hz, H15), 4.59 (1H, br s, C14-OH),

6.86 (1H, d, J = 8.2 Hz, H11), 7.02 (1H, d, J = 8.2 Hz, H12); ^{13}C NMR (50.2 MHz, CDCl$_3$)

δ: 18.4, 19.3, 21.6, 22.5, 22.8, 24.3, 24.8, 26.9, 33.3, 33.4, 37.5, 38.9, 41.6, 49.7, 116.4,

120.6, 123.2, 129.9, 149.1, 150.2; LRMS m/z (rel. intensity): 286 (M$^+$, 88.7), 271 (83.0),

257 (3.5), 201 (38.0); HRMS calcd. for C$_{20}$H$_{30}$O: 286.2297; found: 286.2297.

Synthesis of 197 from dehydroabietane (196)

The following synthetic procedures were based on the results from Chapter 3 (Synthesis

of Precursors) with few changes. The corresponding typical procedures can be found in

experimental section for Chapter 3.

In a dry flask was mixed concentrated HNO$_3$ (1.9 mL) with concentrated H$_2$SO$_4$ (2.8

mL) at 0°C (ice water). A suspension of **196** (465.6 mg, 1.72 mmol) in dry (MgSO$_4$)

nitromethane (2.7 mL) was added dropwise over a period of 17 min while stirring at 0-5°C. The

resulting light orange suspension (products precipitated) was stirred at the bath temperature for

1.5 h, and then poured into crushed ice (30 mL) in beaker with swirling. The flask was washed

with EtOAc (2 x 10 mL) and the washings were also added into the beaker. After all the ice

melted, the suspension was extracted with EtOAc (3 x 80 mL), and then the combined EtOAc was washed with saturated NaHCO$_3$ (100 mL), brine (2 x 100 mL) and dried. Removal of the solvent gave a crude product, which was recrystallized in EtOAc to give 12,14-dinitro dehydroabietane **197(i)** as colorless needles (180.6 mg). Chromatography of the mother liquor with hexanes-EtOAc (95:5) yielded more **197(i)** (102.9 mg), bringing the total amount of **197a** to 283.5 mg (45.7% yield).

Physical data of 12,14-dinitro dehydroabietane (**197(i)**):

Colorless needles (EtOAc); mp: 186-188°C; IR (CHCl$_3$) cm^{-1}: 2975 (CH), 1600 (aromatic C=C), 1560 and 1380 (-NO$_2$); ^{1}H NMR (400 MHz, CDCl$_3$) δ: 0.91 (3H, s, H19), 0.94 (3H, s, H18), 1.18 (3H, d, J = 0.4 Hz, H20), 1.20 (1H, m, H3α, overlapped with H20), 1.26 (1H, dd, J = 12.8, 2.4 Hz, H5, overlapped with H16, H17), 1.296, 1.299 (3H each, both d, J = 7.1 Hz, H16, H17), 1.38 (1H, br ddd, J = 12.4, 12.4, 3.8 Hz, H1α), 1.49 (1H, m, H3β), 1.61-1.80 (3H, m, H2, H6β), 1.93 (1H, br dd, J = 13.9, 7.5 Hz, H6α), 2.20 (1H, br d, J = 12.4 Hz, H1β), 2.72 (2H, m, H7), 3.00 (1H, sept, J = 7.1 Hz, H15), 7.52 (1H, s, H11); LRMS m/z (rel. intensity): 360 (M$^+$, 16.9), 345 (5.2), 344 (15.9), 343 (68.6), 313 (4.2), 301 (11.5), 283 (8.4), 263 (19.9), 69 (base peak); HRMS calcd. for C$_{20}$H$_{28}$N$_2$O$_4$: 360.2049; found: 360.2049.

In a solution of **197(i)** (282 mg, 0.78 mmol) in a mixture of glacial HOAc (5.7 mL), EtOH (2 mL) and benzene (1.5 mL) (the starting material dissolved when benzene was added) was placed 10% Pt/C (12.8 mg), and the mixture was stirred under H$_2$ (1 atm) for 20 h at r.t. (TLC, hexanes-EtOAc, 8:2). The catalyst was removed by filtration through Celite, and the Celite was washed thoroughly with EtOAc (60 mL). The combined filtrate was concentrated, and the resulting crude product **197(ii)** was used directly in the next step without purification.

The crude **197(ii)** was suspended in a mixture of trifluoroacetic acid (TFA, 1.7 mL), HOAc (4.7 mL) and water (1.7 mL). The suspension was cooled to 0°C (ice-water-traces acetone), and $NaNO_2$ (78.7 mg, 1.14 mmol) was added portionwise over 10 min. The resulting mixture was stirred at 0°C for 70 min, and during this period of time the suspension turned into a yellow solution. A solution of KI (247.4 mg, 1.49 mmol) in water (0.6 mL) was added dropwise over 5 min (dark solid precipitated out) and the suspension was stirred for another 15 min until gas evolution ceased. A solution of Na_2SO_3 (63.2 mg, 0.50 mmol) in water (2.9 mL) was added dropwise (the dark color faded), and then the bath was removed. Stirring was continued for 30 min and then water was added (50 mL). The suspension was extracted with EtOAc (3 x 60 mL), and then combined EtOAc was washed with 5% Na_2SO_3 (2 x 60 mL), saturated $NaHCO_3$ (2 x 60 mL), brine (2 x 60 mL), dried, and concentrated to give crude **197(iii)** as a yellow solid.

The crude **197(iii)** was dissolved in EtOH (9.6 mL) and heated to reflux followed by dropwise addition of a sodium dithionite solution ($Na_2S_2O_4$) (78%, 1.41 g) in water (3.7 mL). The reaction mixture was stirred for 1 h under reflux, and during which time excess sodium dithionite was added (TLC, hexanes-EtOAc, 6:4). The mixture was then allowed to cool to r.t. and the solvent was removed. The residue was mixed with EtOAc (50 mL) and brine (50 mL), and the EtOAc layer was separated. The aqueous layer was extracted with EtOAc (3 x 50 mL), and then the combined EtOAc extract was dried and concentrated to give a crude **197(iv)**.

To a stirred solution of the crude **197(iv)** in TFA (5.4 mL) at -12 to -10°C (ice-acetone-water), $NaNO_2$ (107.4 mg, 1.56 mmol) was added portionwise over 5 min. The mixture turned brown and gases evolved. The mixture was stirred at the bath temperature for 30 min and a solution of Na_2SO_3 (49 mg, 0.39 mmol) in water (0.66 mL) was added. The bath was removed and the mixture was stirred for another 30 min. Water (2.7 mL) was added and stirring was continued overnight. The resulting suspension was diluted with water (100 mL) and extracted with EtOAc (3 x 70 mL). The combined EtOAc was washed with saturated $NaHCO_3$ solution (3 x 20 mL), brine (2 x 30 mL) and dried. Removal of the solvent gave a crude intermediate, which

was then dissolved in MeOH (7.2 mL) followed by addition of concentrated HCl (1.1 mL) while stirring at r.t. (solid material precipitated). Benzene (1 mL) was added, and then most of the solid dissolved. The mixture was stirred for 9 h and then was diluted with water (100 mL) followed by extraction with EtOAc (3 x 70 mL). The combined EtOAc was washed with brine (50 mL), dried and evaporated to give a crude **197(v)**.

The crude **197(v)** was dissolved in MeOH (30 mL) and benzene (1 mL) before 10% Pd/C (excess) and K_2CO_3 (excess) were added. The mixture was stirred under H_2 (1 atm) at r.t. for 12 h (TLC, hexanes-EtOAc, 8:2). After the reaction was completed, the mixture was filtered through Celite and the Celite was washed with EtOAc. The filtrates were combined and concentrated. The resulting residue was suspended in 5% NaH_2PO_4 (60 mL) and extracted with EtOAc (3 x 50 mL). The combined EtOAc was washed with brine (100 mL) and dried. Removal of solvent gave a crude product, which was repeatedly chromatographed with hexanes-EtOAc (9:1 and 95:5) to afford **197** as a pale yellow oil (63.4 mg, 28.1% yield from **197(i)**). The spectra of the synthetic **197** were identical with those obtained from the isolated **197**.

14-Hydroxy-abieta-8,11,13-trien-3-one (116) (triptonoterpene)

A white powder; mp: 138-140°C (lit.: 153.5-155.5°C[89]; 135-137°C[187]); $[\alpha]_D^{23}$: +117.4° (c = 0.0145, MeOH) (lit.: +53.2° (CHCl₃)[89]); UV λ_{max}^{MeOH} (log ε): 218.5 (3.78), 278.4 (3.24); IR (CHCl₃) v_{max} cm⁻¹: 3620 (OH), 3025 (aromatic CH), 2990 (CH), 1700 (C=O), 1580 and 1500 (aromatic C=C); ¹H NMR (400 MHz, CDCl₃) δ: 1.12 (3H, s, H19), 1.15 (3H, s, H18), 1.22, 1.24 (3H each, both d, J = 7.0 Hz, H16, H17), 1.30 (3H, s, H20), 1.71-1.96 (4H, m, H1α, H5, H6), 2.41-2.64 (3H, m, H1β, H2α, H7α), 2.69 (1H, ddd, J = 15.7, 10.7, 7.1 Hz, H2β), 2.90 (1H, br dd, J = 16.6, 5.9 Hz, H7β), 3.10 (1H, sept, J = 7.0 Hz, H15), 4.65 (1H, br s, C14-OH), 6.83 (1H, d, J = 8.2 Hz, H11), 7.02 (1H, d, J = 8.2 Hz, H12); ¹³C NMR (50.2 MHz, CDCl₃) δ: 19.5, 21.2, 22.5, 22.7, 24.4, 24.6, 26.6, 26.9, 34.7, 37.1, 37.6, 47.3, 50.1, 117.3, 120.8, 123.6, 130.4, 146.4, 150.1, 217.1; LRMS m/z (rel. intensity): 300

(M$^+$, 97.3), 285 (base peak), 243 (78.8), 213 (34.0), 199 (30.5), 187 (25.5), 175 (17.8), 159 (21.1), 147 (27.7), 125 (46.0); HRMS calcd. for $C_{20}H_{28}O_2$: 300.2089; found: 300.2090.

14-Methoxy-abieta-8,11,13-trien-3-one (200)

Colorless needles (EtOAc-hexanes); mp: 134-136°C; $[\alpha]_D^{23}$: +84.9° (c = 0.0171, MeOH); UV λ_{max}^{MeOH} (log ε): 217.3 (3.71), 253.5 (2.43); IR (CHCl$_3$) v_{max} cm^{-1}: 3025 (aromatic CH), 2990 (CH), 1700 (C=O), 1460, 1340, 1260, 1040; ^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.12 (3H, s, H19), 1.15 (3H, s, H18), 1.19, 1.20 (3H each, both d, J = 6.9 Hz, H16, H17), 1.27 (3H, s, H20), 1.70 (1H, br dddd, J = 12.6, 12.6, 12.6, 5.5 Hz, H6β), 1.84 (1H, m, H6α), 1.87 (1H, dd, J = 12.6, 1.5 Hz, H5), 1.92 (1H, m, H1α), 2.44 (1H, ddd, J = 13.2, 7.5, 4.1 Hz, H1β), 2.55 (1H, ddd, J = 15.8, 7.6, 4.1 Hz, H2α), 2.66 (2H, m, H2β, H7α), 3.10 (1H, br dd, J = 17.4, 5.5 Hz, H7β), 3.27 (1H, sept, J = 6.9 Hz, H15), 3.71 (3H, s, -OCH$_3$), 7.00 (1H, d, J = 8.3 Hz, H11), 7.06 (1H, d, J = 8.3 Hz, H12); ^{13}C NMR (50.2 MHz, CDCl$_3$) δ: 19.8, 21.1, 23.9 (2C), 24.6, 25.3, 26.1, 26.8, 29.7, 34.7, 37.6, 47.3, 50.4, 60.5, 121.3, 124.1, 128.5, 138.5, 146.5, 154.7, 217.3; LRMS m/z (rel. intensity): 314 (M$^+$, base peak), 299 (73.9), 257 (75.6), 229 (13.7), 215 (20.8), 201 (25.8), 189 (17.5), 173 (21.8), 163 (12.9), 149 (44.7), 125 (60.9); HRMS calcd. for $C_{21}H_{30}O_2$: 314.2246; found: 314.2251.

11-Hydroxy-14-methoxy-abieta-8,11,13-trien-3-one (117) (triptonoterpene)

Colorless needles (EtOAc-hexanes). mp: 184-186°C (lit.: 209-211°C[90]); $[\alpha]_D^{23}$: +190.2° (c = 7.36x10^{-3}, MeOH) (lit.: +189.5° (MeOH)[90]); UV λ_{max}^{MeOH} (log ε): 209.8 (4.05), 286.4 (3.39); IR (CHCl$_3$) v_{max} cm^{-1}: 3600 (OH), 3025 (aromatic CH), 2975 (CH), 1700 (C=O), 1670, 1420, 1220; ^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.12 (3H, s, H19), 1.15, 1.165 (3H each, both d, J = 6.9 Hz, H16, H17), 1.163 (3H, s, H18), 1.27 (3H, s, H20), 1.54 (1H, dddd, J = 13.0, 13.0, 12.3, 4.7 Hz, H6β), 1.79 (1H, dddd, J = 13.0, 6.0, 1.8, 1.8 Hz, H6α),

1.94 (1H, m, H1α), 1.99 (1H, dd, J = 12.3, 1.8 Hz, H5), 2.43 (1H, ddd, J = 15.2, 8.5, 5.9 Hz, H2β), 2.56 (1H, ddd, J = 17.1, 13.0, 6.0 Hz, H7α), 2.66 (1H, ddd, J = 15.2, 9.9, 6.3 Hz, H2α), 3.05 (1H, ddd, J = 13.8, 8.5, 6.3 Hz, H1β), 3.10 (1H, ddd, J = 17.1, 4.7, 1.8 Hz, H7β), 3.23 (1H, sept, J = 6.9 Hz, H15), 3.66 (3H, s, -OCH₃), 4.55 (1H, s, C11-OH), 6.36 (1H, s, H12); ^{13}C NMR (50.2 MHz, CDCl₃) δ: 19.9, 20.0, 20.4, 23.7, 23.8, 26.1, 26.5, 28.4, 34.4, 35.5, 38.1, 47.2, 52.0, 60.7, 111.8, 131.0, 131.3, 139.4, 148.5, 150.7, 219.5; LRMS m/z (rel. intensity): 330 (M⁺, base peak), 315 (16.0), 283 (16.7), 273 (52.6), 231 (15.5), 217 (14.5), 205 (23.2), 189 (15.1); HRMS calcd. for C₂₁H₃₀O₃: 330.2195; found: 330.2191.

12-Hydroxy-14-methoxy-abieta-8,11,13-trien-4-one   (118)   (neotriptonoterpene)

Colorless prisms (CH₂Cl₂). mp: 188-190°C; $[\alpha]_D^{23}$: +56.6° (c = 0.145, CHCl₃) (lit.: +95.1° (MeOH)[90]); IR (CHCl₃) ν_max cm⁻¹: 3600 (OH), 3029 (aromatic CH), 2935 (CH), 1702 (C=O), 1609, 1585, 1460, 1412, 1102, 1047; ^{1}H NMR (400 MHz, CDCl₃) δ: 1.11 (3H, s, H19), 1.14 (3H, s, H18), 1.25 (3H, s, H20), 1.34, 1.35 (3H each, both d, J = 7.2 Hz, H16, H17), 1.68 (1H, ddd, J = 12.5, 12.5, 5.7 Hz, H6β), 1.83 (1H, m, H6α), 1.84 (1H, dd, J = 12.5, 2.1 Hz, H5), 1.91 (1H, m, H1α), 2.33 (1H, ddd, J = 13.1, 7.4, 4.2 Hz, H1β), 2.50-2.71 (3H, m, H2, H7α), 2.99 (1H, ddd, J = 17.1, 5.7, 1.6 Hz, H7β), 3.41 (1H, sept, J = 7.2 Hz, H15), 3.67 (3H, s, -OCH₃), 4.47 (1H, s, C12-OH), 6.38 (1H, s, H11); ^{13}C NMR (50.2 MHz, 75.3 MHz, CDCl₃) δ: 19.9, 21.07, 21.14 (2C), 24.5, 24.9, 25.2, 26.9, 34.6, 37.2, 37.6, 47.3, 50.4, 60.7, 109.0, 121.0, 125.0, (135.6), 146.7, 153.4, (217.1); LRMS m/z (rel. intensity): 330 (M⁺, base peak), 315 (61.1), 299 (8.4), 287 (6.7), 273 (28.4), 245 (19.1), 231 (15.5), 229 (17.0), 217 (22.3); HRMS calcd. for C₂₁H₃₀O₃: 330.2195; found: 330.2198.

14-Methoxy-abieta-8,11,13-trien-3β-ol (205)

A white powder; $[\alpha]_D^{23}$: +34.1° (c = 0.063, CHCl$_3$); IR (CHCl$_3$) cm^{-1}: 3614 (OH), 3002 (aromatic CH), 2965 (CH), 1480 (aromatic C=C), 1457, 1030 (C-O); ^{1}H NMR (400 MHz, CDCl$_3$) δ: 0.88 (3H, s, H19), 1.06 (3H, s, H18), 1.17 (3H, s, H20), 1.18, 1.19 (3H each, both d, J = 6.9 Hz, H16, H17), 1.28 (1H, dd, J = 12.5, 2.0 Hz, H5), 1.52 (1H, m, H1α), 1.68 (1H, br dddd, J = 13.2, 12.5, 11.6, 5.3 Hz, H6β), 1.77 (2H, m, H2), 1.91 (1H, br dd, J = 13.2, 7.5 Hz, H6α), 2.27 (1H, ddd, J = 13.1, 3.5, 3.5 Hz, H1β), 2.70 (1H, ddd, J = 17.6, 11.6, 7.5 Hz, H7α), 3.02 (1H, br dd, J = 17.6, 5.3 Hz, H7β), 3.26 (1H, sept, J = 6.9 Hz, H15), 3.28 (1H, dd, J = 10.9, 4.9 Hz, H3α), 3.70 (3H, s, -OCH$_3$), 6.98 (1H, d, J = 8.4 Hz, H11), 7.03 (1H, d, J = 8.4 Hz, H12); ^{13}C NMR (75.3 MHz, CDCl$_3$) δ: 15.4, 18.4, 23.9, 24.0, 24.9, 25.2, 26.1, 28.1, 28.2, 37.1, 37.5, 38.9, 49.5, 60.5, 78.8 (C3), 120.4, 123.8, 128.6, (138.2), 148.5, (152.7); LRMS m/z (rel. intensity): 316 (M$^+$, 54.6), 301 (30.4), 283 (86.5), 259 (6.5), 241 (47.3), 229 (12.1), 189 (32.2); HRMS calcd. for C$_{21}$H$_{32}$O$_2$: 316.2402; found: 316.2402.

14-Methoxy-abieta-8,11,13-trien-3α-ol (203)

Colorless needles or a wax-like solid (EtOAc-hexanes); mp: 157-159°C (lit.: 157-158°C[160]); $[\alpha]_D^{23}$: +27.1° (c = 0.059, CHCl$_3$) (lit.: +44.4° (CHCl$_3$)[160]); IR (CHCl$_3$) ν_{max} cm^{-1}: 3600 (OH), 2950 (CH), 1600, 1490 (aromatic C=C), 1230 (aromatic C-O), 1030 (C-O); ^{1}H NMR (400 MHz, CDCl$_3$) δ: 0.94 (3H, s, H19), 1.03 (3H, s, H18), 1.178 (1H, s, H20), 1.180, 1.189 (3H each, both d, J = 6.9 Hz, H16, H17), 1.62-1.77 (3H, m, H2α, H5, H6β), 1.77-1.90 (2H, m, H1α, H6α), 2.00 (1H, br ddd, J = 13.1, 3.8, 3.5 Hz, H1β), 2.09 (1H, dddd, J = 14.1, 14.1, 3.8, 2.4 Hz, H2β), 2.73 (1H, ddd, J = 17.8, 10.6, 8.6 Hz, H7α), 2.99 (1H, br dd, J = 17.8, 4.9 Hz, H7β), 3.26 (1H, sept, J = 6.9 Hz, H15), 3.49 (1H, br dd, J = 3.3, 2.4 Hz, H3β), 3.69 (3H, s, -OCH$_3$), 7.00 (1H, d, J = 8.4 Hz, H11), 7.03 (1H, d, J = 8.4

Hz, H12); ^{13}C NMR (75.3 MHz, CDCl$_3$) δ: 18.2, 22.1, 23.9, 24.0, 24.7, 24.8, 25.9, 26.0, 28.1, 29.7, 31.7, 37.7, 43.4, 60.5 (-OCH$_3$), 75.7 (C3), 120.3, 123,7, 128.5, 138.0, 148.9, 154.8; LRMS m/z (rel. intensity): 316 (M$^+$, 28.4), 301 (7.2), 283 (base peak), 257 (11.5), 253 (21.4), 241 (6.2), 213 (5.2), 199 (5.7), 189 (9.5), 185 (7.7), 173 (8.2), 159 (8.0), 141 (8.4); HRMS calcd. for C$_{21}$H$_{32}$O$_2$: 316.2402; found: 316.2393.

18(4→3)abeo-Abieta-3,8,11,13-tetraen-18-oic acid (210)

A white powder; [α]$_D^{23}$: +2.6° (c = 0.116, CHCl$_3$); IR (CHCl$_3$) cm^{-1}: 3525 (OH), 3034 (aromatic CH), 2929 (CH), 1685 (carboxy C=O), 1620 (C=C), 1500 (aromatic C=C); ^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.03 (3H, s, H20), 1.22 (6H, d, J = 7.0 Hz, H16, H17), 1.61 (1H, br ddd, J = 12.7, 12.7, 7.5 Hz, H1α), 1.71 (1H, ddd, J = 13.1, 10.1, 8.1 Hz, H6β), 2.09 (3H, br d, J = 1.2 Hz, H19), 2.25 (1H, m, H6α), 2.30-2.40 (2H, m, H1β, H5), 2.42-2.63 (2H, m, H2), 2.83 (1H, sept, J = 7.0 Hz, H15), 2.95 (2H, m, H7), 6.94 (1H, br s, H14), 7.00 (1H, br d, J = 8.1 Hz, H12), 7.22 (1H, d, J = 8.1 Hz, H11); LRMS m/z (rel. intensity): 298 (M$^+$, 85.0), 283 (74.9), 265 (4.0), 253 (7.7), 241 (6.7), 237 (14.3), 199 (base peak), 185 (24.1); HRMS calcd. for C$_{20}$H$_{26}$O$_2$: 298.1933; found: 298.1929.

7-Oxo-18(4→3)abeo-abieta-3,8,11,13-tetraen-18-oic acid (211)

A colorless solid; IR (CHCl$_3$) cm^{-1}: 3530 (OH), 3033 (aromatic CH), 1682 (C=O), 1590 (aromatic C=C), 1462, 1257; ^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.13 (3H, s, H20), 1.25 (6H, d, J = 7.0 Hz, H16, H17), 1.71 (1H, m, H1α), 2.07 (3H, br s, H19), 2.44 (1H, br dd, J = 11.2, 6.0 Hz, H1β), 2.51 (1H, m, H2β), 2.59 (1H, dd, J = 18.1, 14.2 Hz, H6β), 2.68 (1H, m, H2α), 2.83 (1H, br d, J = 14.2 Hz, H5), 2.93 (1H, sept, J = 7.0 Hz, H15), 3.04 (1H, dd, J = 18.1, 4.4 Hz, H6α), 7.35 (1H, d, J = 8.2 Hz, H11), 7.42 (1H, dd, J = 8.2, 2.0 Hz, H12), 7.90 (1H, d, J = 2.0 Hz, H14); LRMS m/z (rel. intensity): 312 (M$^+$, 24.8), 297 (10.3), 283

(5.9), 279 (5.5), 267 (5.4), 251 (8.5), 213 (base peak), 185 (13.2), 171 (21.6); HRMS calcd. for $C_{20}H_{24}O_3$: 312.1725; found: 312.1723.

Squalene (195)

A colorless oil; IR (neat) v_{max} cm^{-1}: 3050 (=CH-), 2950 (CH), 1670 (C=C), 1460 (C=C), 1390; ^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.58 (18H, br s, -CH$_3$), 1.66 (6H, s, -CH$_3$), 2.03 (20H, m, -CH$_2$CH$_2$-), 5.10 (6H, m, =CH-); ^{13}C NMR (50.2 MHz, CDCl$_3$) δ: 15.99 (2C), 16.03 (2C), 17.7 (2C), 25.7 (2C), 26.7 (2C), 26.8 (2C), 28.3 (2C), 39.7 (4C), 124.3 (4C), 124.4 (2C), 131.2 (2C), 134.9 (2C), 135.1 (2C); LRMS m/z (rel. intensity): 410 (M$^+$, 2.8), 367 (1.1), 341 (3.0), 273 (1.1), 149 (9.3), 137 (18.2), 121 (11.5), 109 (11.5), 95 (18.2), 81 (59.8), 69 (base peak); HRMS calcd. for $C_{30}H_{50}$: 410.3912; found: 410.3917.

Compounds **193, 206, 106** and **107**: Spectral data are given in experimental section for Chapter 3.

11,19-Dihydroxy-14-methoxy-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (109) (neotriptophenolide)

A pale yellow powder (EtOAc-hexanes); mp: 98-100°C; $[\alpha]_D^{24}$: +113.4° (c = 0.307, MeOH); UV λ_{max}^{MeOH} (log ε): 221.7 (3.98), 287.4 (3.46); IR (CHCl$_3$) v_{max} cm^{-1}: 3600 (OH), 2950 (CH), 1745 (lactone C=O), 1645, 1020; ^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.14 (3H, s, H20), 1.18 (6H, d, J = 6.9 Hz, H16, H17), 1.53 (1H, m, H1α), 1.77 (2H, m, H6), 2.38 (2H, m, H2), 2.76 (2H, m, H5, H7α), 3.06 (1H, br dd, J = 17.9, 4.6 Hz, H7β), 3.23 (1H, sept, J = 6.9 Hz, H15), 3.51 (1H, ddd, J = 13.5, 5.4, 2.0 Hz, H1β), 3.66 (3H, s, -OCH$_3$), 4.58 (1H, s, C11-OH), 4.77 (2H, br AB$_q$, Δv = 0.10 ppm, J = 17.2 Hz, H19), 6.39 (1H, s, H12); ^{13}C NMR (50.2 MHz, CDCl$_3$) δ: 17.3, 18.6, 19.5, 23.7, 23.8, 25.5, 26.1, 31.1, 37.3, 44.0, 60.7,

70.5, 112.0, 117.3, 125.3, 129.4, 130.8, 140.0, 150.7, 163.2, (173.2); LRMS m/z (rel. intensity): 342 (M^+, 50.7), 327 (25.2), 313 (6.4), 297 (2.4), 285 (4.1), 218 (8.5); HRMS calcd. for $C_{21}H_{26}O_4$: 342.1813; found: 342.1825.

14,19-Dihydroxy-7-oxo-18(4→3)abeo-abieta-3,8,11,13-tetraen-18-oic acid lactone (108) (triptonide)

Colorless needles (EtOAc-hexanes); mp: 202-204°C (lit.: 214-215°C,[81] 197-198°C[82]); $[\alpha]_D^{20}$: -36.6° (c = 1.82, $CHCl_3$); UV λ_{max}^{MeOH} (log ε): 217.0 (4.44), 268.4 (4.02), 343.7 (3.71); IR ($CHCl_3$) ν_{max} cm^{-1}: 3700 (OH), 2975 (CH), 1750 (C=O), 1670, 1620, 1240; ^1H NMR (400 MHz, $CDCl_3$) δ: 1.12 (3H, s, H20), 1.21, 1.23 (3H each, both d, J = 7.0 Hz, H16, H17), 1.79 (1H, br ddd, J = 12.4, 12.4, 5.9 Hz, H1α), 2.40 (1H, m, H2β), 2.51 (1H, br dd, J = 12.4, 6.0 Hz, H1β), 2.57 (1H, m, H2α), 2.77 (1H, d, J = 7.1 Hz, H6α), 2.78 (1H, d, J = 11.7 Hz, H6β), 3.16 (1H, m, H5), 3.33 (1H, sept, J = 7.0 Hz, H15), 4.75 (2H, AB_q, Δν = 0.07 ppm, J = 17.1 Hz, H19), 6.86 (1H, d, J = 7.9 Hz, H11), 7.40 (1H, d, J = 7.9 Hz, H12), 13.3 (1H, s, C14-OH, D_2O exchangeable); ^{13}C NMR (50.2 MHz, $CDCl_3$) δ: 17.8, 21.7, 22.1, 22.2, 26.2, 31.7, 36.4, 36.5, 40.4, 69.9, 113.6, 114.7, 126.1, 133.7, 136.1, 149.1, 159.6, 161.8, 173.3, 202.2; LRMS m/z (rel. intensity): 326 (M^+, 49.6), 311 (77.8), 297 (9.8), 269 (4.8), 161 (17.6); HRMS calcd. for $C_{20}H_{20}O_4$: 326.1518; found: 326.1518.

7β,14,19-Trihydroxy-18(4→3)abeo-abieta-3,8,11,13-tetraen-18-oic acid lactone (138a)

A colorless amorphous solid; $[\alpha]_D^{24}$: +63.79° (c = 1.845, $CHCl_3$); UV λ_{max}^{MeOH} (log ε): 206.5 (4.59), 282.2 (3.54); IR ($CHCl_3$) ν_{max} cm^{-1}: 3600 (OH), 3400 (OH), 3050 (aromatic CH), 2980 CH), 1745 (C=O), 1680 (C=C), 1630, 1580, 1450, 1260, 1040; ^1H NMR (400 MHz, $CDCl_3$) δ: 1.08 (3H, s, H20), 1.20, 1.22 (3H each, both d, J = 6.9 Hz, H16, H17),

1.64 (1H, ddd, J = 12.6, 12.6, 6.6 Hz, H1α), 1.99 (1H, ddd, J = 13.9, 12.7, 10.0 Hz, H6β), 2.22-2.52 (4H, m, H1β, H2, H6α), 2.76 (1H, br d, J = 13.8 Hz, H5), 3.14 (1H, br d, J = 7.8 Hz, C7-OH), 3.28 (1H, sept, J = 6.9 Hz, H15), 4.75 (2H, br AB$_q$, $\Delta\nu$ = 0.085 ppm, J = 17.2 Hz, H19), 5.22 (1H, ddd, J = 10.0, 7.8, 7.8 Hz, H7α), 6.85 (1H, d, J = 8.2 Hz, H11), 7.13 (1H, d, J = 8.2 Hz, H12), 8.22 (1H, s, C14-OH); ^{13}C NMR (50.2 MHz, CDCl$_3$) δ: 18.0, 22.4, 22.6, 23.1, 26.5, 30.4, 32.6, 36.5, 40.4, 69.0, 70.4, 115.5, 121.0, 125.6, 126.1, 134.0, 143.3, 154.3, 161.8, 174.2; m/z (rel. intensity): MS 328 (M$^+$, 0.5), 326 (2.3), 310 (45.7), 295 (65.8), 279 (6.3), 267 (23.0), 253 (base peak), 209 (15.0), 165 (15.1); HRMS calcd. for C$_{20}$H$_{24}$O$_4$: 328.1675; found: 328.1663.

Synthesis of 138a from 108

Compound 108 (37.8 mg) was treated with sodium borohydride (75.6 mg) in ethanol (7 mL) at r.t. for 2 h, followed by evaporation of the solvent. The residue was suspended in 5% NaH$_2$PO$_4$ (120 mL) and extracted with EtOAc (3 x 100 mL). The EtOAc extracts were combined, washed with 5% NaH$_2$PO$_4$, brine, dried, and concentrated. The crude product (28.4 mg) was purified by column chromatography (CH$_2$Cl$_2$-EtOAc, 85:15) to give 16.6 mg of the pure product 138a and a small amount of mixture containing both 7α (138b) and 7β alcohols. The spectra of synthetic 138a were identical with those of the isolated 138a.

7β,19-Dihydroxy-14-methoxy-18(4→3)abeo-abieta-3,8,11,13-tetraen-18-oic acid lactone (207)

^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.10 (3H, s, H20), 1.18, 1.27 (3H each, both d, J = 6.9 Hz, H16, H17), 1.65 (1H, m, H1α), 1.93 (1H, ddd, J = 14.0, 13.0, 9.0 Hz, H6β), 2.27-2.53 (4H, m, H1β, H2, H6α), 2.72 (1H, br d, J = 14.0 Hz, H5), 3.27 (1H, sept, J = 6.9 Hz, H15), 3.83 (3H, s, -OCH$_3$), 4.77 (2H, m, H19), 5.25 (1H, dd, J = 9.0, 9.0 Hz, H7α), 7.12

(1H, d, J = 8.4 Hz, H11), 7.20 (1H, d, J = 8.4 Hz, H12); LRMS m/z (rel. intensity): 342 (M$^+$, 21.6), 324 (18.1), 309 (42.5), 293 (75.7), 279 (14.7), 267 (45.8), 247 (17.8), 229 (14.1); HRMS calcd. for $C_{21}H_{26}O_4$: 342.1831; found: 342.1833.

Triptolide (1)

Colorless prisms (EtOAc); mp: 232-235°C; $[\alpha]_D^{26}$: -151.4° (c = 0.148, MeOH); IR (CHCl$_3$) cm^{-1}: 3510 (OH), 2950 (CH), 1750 (C=O), 1673 (C=C), 1440, 1400, 1344, 1210, 1072, 1033, 974, 918, 865; ^{1}H NMR (400 MHz, CDCl$_3$) δ: 0.86, 0.98 (3H each, both d, J = 7.0 Hz, H16, H17), 1.09 (3H, s, H20), 1.19 (1H, br ddd, J = 12.4, 12.4, 5.9 Hz, H1α), 1.53 (1H, ddd, J = 12.4, 5.4, 1.2 Hz, H1β), 1.94 (1H, dd, J = 14.8, 13.3 Hz, H6β), 2.11 (1H, m, H2β, overlapped with H6α), 2.15 (1H, ddd, J = 14.8, 5.9, 5.5 Hz, H6α), 2.22 (1H, sept, J = 7.0 Hz, H15), 2.29 (1H, br d, J = 18.2 Hz, H2α), 2.67 (1H, m, H5), 2.71 (1H, d, J = 10.5 Hz, C14-OH), 3.34 (1H, d, J = 5.5 Hz, H7α), 3.39 (1H, d, J = 10.5 Hz, H14α), 3.49 (1H, dd, J = 3.1, 0.8 Hz, H12β), 3.87 (1H, d, J = 3.1 Hz, H11α), 4.65 (2H, br s, H19); ^{13}C NMR (50.2 MHz, CDCl$_3$) δ: 13.6, 16.8, 17.0, 17.7, 23.7, 28.2, 29.8, 35.8, 40.4, 54.5, 56.7, 60.0, 60.7, 65.8, 66.2, 69.9, 73.4, 125.6, 159.9, 173.2; LRMS m/z (rel. intensity): 360 (M$^+$, 1.4), 342 (15.5), 327 (18.2), 313 (15.8), 299 (17.2), 285 (14.0), 271 (27.9), 259 (16.3), 241 (32.3), 179 (22.8), 165 (31.2), 151 (42.7); HRMS calcd. for $C_{20}H_{24}O_6$: 360.1573; found: 360.1562. The above spectral data were found to be identical with those of an authentic sample of triptolide.

Tripdiolide (2)

Colorless prisms (EtOAc); mp: 223-225°C; $[\alpha]_D^{26}$: -115.3° (c = 0.183, MeOH); IR (CHCl$_3$) cm^{-1}: 3580 (OH), 3530 (OH), 2910 (CH), 1759 (C=O), 1680 (C=C), 1449, 1415, 1350, 1230, 1079, 1032, 982, 921, 878, 720; ^{1}H NMR (400 MHz, CDCl$_3$) δ: 0.85, 0.98 (3H

each, both d, J = 6.9 Hz, H16, H17), 1.29 (3H, br s, H20), 1.41 (1H, br dd, J = 13.8, 5.6 Hz,

H1α), 1.83 (1H, br d, J = 13.8 Hz, H1β), 2.05 (1H, dd, J = 14.7, 13.1 Hz, H6β), 2.20 (2H,

m, H6α, H15), 2.62 (1H, ddd, J = 13.1, 6.3, 1.5 Hz, H5), 2.74 (1H, br s, C14-OH), 3.34

(1H, d, J = 5.3 Hz, H7α), 3.38 (1H, br s, H14), 3.49 (1H, dd, J = 3.2, 0.8 Hz, H12), 3.90

(1H, d, J = 3.2 Hz, H11), 4.58 (1H, br d, J = 5.6 Hz, H2α), 4.74 (2H, br s, H19); ^{13}C NMR

(50.2 MHz, $CDCl_3$) δ: 15.3, 16.8, 17.7, 23.3, 28.2, 35.9, 38.1, 40.8, 54.5, 57.4, 59.3, 60.0,

60.8, 65.7, 66.3, 70.1, 73.4, 126.9, 162.0, 172.7; LRMS m/z (rel. intensity): 376 (M^+, 0.2),

358 (4.1), 343 (2.9), 331 (3.3), 329 (3.9), 315 (3.5), 311 (3.4), 269 (9.4), 243 (10.9), 239

(13.6), 149 (19.6), 139 (19.1); HRMS calcd. for $C_{20}H_{24}O_7$: 376.1522; found: 376.1525. The

above spectral data were found to be identical with those of an authentic sample of tripdiolide.

(7,8)β,(9,11)β,(12,13)α-Tris(epoxy)-2β,19-dihydroxy-14-oxo-18(4→3)*abeo*-abieta-3-en-18-oic acid lactone (212)

A colorless powder; $[\alpha]_D^{23}$: -25° (c = 0.046, $CHCl_3$); IR ($CHCl_3$) cm^{-1}: 3270 (OH),

2930 (CH), 1757 (C=O), 1731 (C=O), 1143; ^1H NMR (400 MHz, $CDCl_3$) δ: 0.87, 0.96 (3H

each, both d, J = 6.9 Hz, H16, H17), 1.26 (3H, s, H20), 1.53 (1H, m, H1α, overlapped with

water signal), 1.88 (1H, br d, J = 14.1 Hz, H1β), 2.09 (1H, br dd, J = 15.0, 13.4 Hz, H6β),

2.25 (1H, br ddd, J = 15.0, 5.8, 5.4 Hz, H6α), 2.38 (1H, sept, J = 6.9 Hz, H15), 2.74 (1H,

br dd, J = 13.4, 5.8 Hz, H5), 3.40 (1H, d, J = 5.4 Hz, H7α), 3.81 (1H, d, J = 2.9 Hz, H12),

4.06 (1H, d, J = 2.9 Hz, H11), 4.62 (1H, m, H2α), 4.76 (2H, m, H19); LRMS m/z (rel.

intensity): 374 (M^+, 3.7), 358 (24.3), 356 (24.9), 340 (15.4), 338 (11.4), 327 (19.5), 325

(10.3), 309 (15.2), 295 (23.1), 293 (16.6), 279 (20.6); HRMS calcd. for $C_{20}H_{22}O_7$:

374.1365; found: 374.1356.

14β-Acetoxy-(7,8)β,(9,11)β,(12,13)α-tris(epoxy)-2β,19-dihydroxy
18(4→3)abeo-abieta-3-en-18-oic acid lactone (208)

A colorless solid; IR (CHCl₃) νₘₐₓ cm⁻¹: 3600 (OH), 2929 (CH), 1752 (C=O), 1680 (C=C), 1455, 1374, 1234, 1030; ¹H NMR (400 MHz, CDCl₃) δ: 0.82, 0.94 (3H each, both d, *J* = 7.0 Hz, H16, H17), 1.24 (3H, s, H20), 1.43 (1H, br dd, *J* = 14.1, 5.8 Hz, H1α), 1.860 (1H, sept, *J* = 7.0 Hz, H15), 1.862 (1H, br d, *J* = 14.1 Hz, H1β), 2.00 (1H, dd, *J* = 14.7, 13.0 Hz, H6β), 2.15 (3H, s, -COCH₃), 2.20 (1H, ddd, *J* = 14.7, 5.8, 5.6 Hz, H6α), 2.60 (1H, br dd, *J* = 13.0, 5.8 Hz, H5), 3.44 (1H, d, *J* = 5.6 Hz, H7α), 3.51 (1H, d, *J* = 3.1 Hz, H12), 3.83 (1H, d, *J* = 3.1 Hz, H11), 4.59 (1H, br d, *J* = 5.8 Hz, H2α), 4.74 (2H, br s, H19), 5.06 (1H, s, H14); LRMS m/z (rel. intensity): 418 (M⁺, 1.1), 400 (4.1), 329 (11.7), 311 (10.8), 295 (48.6), 287 (10.5), 269 (9.9); HRMS calcd. for C₂₂H₂₆O₈: 418.1628; found: 418.1627.

(7,8)β,(9,11)β-Bis(epoxy)-2β,19-dihydroxy-14-oxo-18(4→3)abeo-abieta-3,12-
dien-18-oic acid lactone (209)

A white powder; mp: 137-139°C; [α]_D²⁴: -225.9° (*c* = 0.251, CHCl₃); UV λₘₐₓ^MeOH (log ε): 219.1 (3.99), 255.8 (3.57); IR (CHCl₃) νₘₐₓ cm⁻¹: 3800 (OH), 2950 (CH), 1760 (C=O), 1680, 1220, 1040; ¹H NMR (400 MHz, CDCl₃) δ: 0.99, 1.05 (3H each, both d, *J* = 6.8 Hz, H16, H17), 1.30 (3H, s, H20), 1.52 (1H, dd, *J* = 13.9, 5.9 Hz, H1α), 1.97 (1H, d, *J* = 13.9 Hz, H1β), 2.13 (1H, br dd, *J* = 14.6, 13.1 Hz, H6β), 2.24 (1H, ddd, *J* = 14.6, 5.7, 5.3 Hz, H6α), 2.35 (1H, br s, C2-OH), 2.66 (1H, br dd, *J* = 13.1, 5.7, Hz, H5), 2.83 (1H, sept d, *J* = 6.8 Hz, 1.0 Hz, H15), 3.59 (1H, d, *J* = 5.3 Hz, H7α), 3.82 (1H, d, *J* = 4.8 Hz, H11α), 4.63 (1H, br d, *J* = 5.9 Hz, H2α), 4.76 (2H, m, H19), 6.91 (1H, dd, *J* = 4.8, 1.0 Hz, H12); ¹³C NMR (125.8 MHz, CDCl₃) δ: 15.4 (C20), 21.3, 21.5 (C16, C17), 23.1 (C6), 27.1 (C15), 35.1 (C10), 38.6 (C1), 41.3 (C5), 52.2 (C11), 59.38 (C8), 59.43 (C2), 61.9 (C7), 63.9 (C9),

70.1 (C19), 127.2 (C3), 136.5 (C12), 150.3 (C13), 161.5 (C4), 172.6 (C18), 190.1 (C14);

LRMS m/z (rel. intensity): 358 (M$^+$, 26.1), 343 (11.0), 342 (18.6), 340 (17.0), 327 (20.6), 325 (12.7), 315 (11.6), 312 (16.6), 311 (15.0), 309 (12.5), 297 (14.9), 279 (17.5), 269 (16.0), 251 (23.2), 219 (26.4), 191 (37.9), 179 (57.2), 163 (36.4), 150 (55.2), 135 (39.3); HRMS calcd. for $C_{20}H_{22}O_6$: 358.1416; found: 358.1409.

Experimental for Chapter 3

6.3 Syntheses of Precursors

Some of the synthetic intermediates (**228-231, 193, 241, 242** and **194**) presented below had also been obtained by previous co-workers in this laboratory. In the present study, additional spectroscopic data were obtained and detailed analyses were made for these compounds (including some corrections for previous assignments), therefore these data are included in the appropriate sections.

6.3.1 Synthesis of Isodehydroabietenolide (193)

Purification of dehydroabietic acid (DHA) (157)

Technical grade dehydroabietic acid (K & K Laboratories, 488 g) was dissolved in warm EtOH (1.2 L, 40-50°C) in a liquid-liquid extraction apparatus with magnetic stirring. After the DHA was completely dissolved, ethanoamine (98 mL) and water (1.2 L) were added. The resulting brown-colored mixture was extracted continuously with petroleum ether (60-80°C) for 20 h while stirring at approximately 50°C. The petroleum ether layer, which contained the neutral materials, was discarded, while the aqueous layer was briefly boiled to drive off the remaining petroleum ether and then stored at 0-5°C overnight. The moist, crude ethanoamine salt of DHA was vacuum filtered, resuspended in aqueous EtOH (50%, 400 mL) at 4°C and vacuum

filtered a second time. The resulting salt was then dissolved in hot EtOH (820 mL) followed by addition of HOAc (100 mL). The solution was heated to boiling and water (400 mL) was added gradually until the solution just turned cloudy. The hot solution was filtered and cooled to r.t.. Crude DHA crystals were obtained by filtration and washing with aqueous EtOH (50%, 200 mL). Recrystallization of the crude acid was carried out by dissolving the product in boiling EtOH (600 mL), followed by the addition of water (250 mL) just to reach the solution's cloud point. The solution was cooled to r.t. and kept overnight at 4°C. The resulting crystals were filtered and dried to give purified DHA (147.5 g, 89-92% by gas chromatography).

18-Norabieta-4(19),8,11,13-tetraene   (228)

In a flask equipped with a thermometer, a magnetic stirrer, a Dean-Stark trap and a condenser with a moisture trap (anhydrous $CaCl_2$) was placed a solution of purified DHA (50 g, 0.167 mol) in benzene (278 mL). The solution was brought to reflux for 1 h and then allowed to cool to r.t.. The Dean-Stark trap was replaced by the condenser, and thionyl chloride (14.4 mL, 0.197 mol) was added to the solution. Following stirring at r.t. for 1 h, the solution was warmed to 70°C ± 2°C and kept at this temperature for 30 min. The solution was then refluxed for 15 min and cooled to r.t.. Removal of solvents by rotary evaporation provided the crude acid chloride **222** (65.24 g) as a thick brown oil (IR: 2950, 1780, 1460 cm^{-1}).

A solution of the acid chloride **222** (65.24 g) in acetone (373 mL) was cooled to -5 to 0°C in an ice-MeOH bath and then a solution of sodium azide (14.8 g, 0.228 mol) in water (49 mL) was added dropwise with vigorous stirring. After the addition was completed the suspension was stirred for 5 min at the bath temperature and then allowed to warm up to r.t.. Toluene (120 mL) was then added and the mixture was stirred vigorously for an additional 10 min. The organic layer was separated, dried, filtered and concentrated to 120 mL by rotary evaporation. Additional toluene (253 mL) was added and the solution was warmed slowly and

refluxed for 30 min. The solvents were removed and the product was dried *in vacuo* to give the crude isocyanate **224** (52.24 g) as a dark oil (IR: 2950, 2250, 1460 cm⁻¹).

A solution of the crude isocyanate **224** (52.24 g) in dry THF (232 mL) was slowly added through an addition funnel to a stirred suspension of LAH (8.68 g, 0.229 mol) in dry THF (405 mL) at -5 to 0°C (ice-MeOH bath) under argon. After the addition, the mixture was allowed to warm to r.t. and stirred for 1 h. The mixture was then warmed slowly and kept refluxing under argon for 22.5 h. After the reaction mixture was cooled down, acetone (11.6 mL) was added slowly. The mixture was stirred for 30 min and water (30 mL), 15% NaOH (7.8 mL) and additional water (21 mL) were added slowly, followed by vigorous stirring for 30 min after each addition. The resulting suspension was filtered through a sintered glass funnel and the inorganic solid was washed with hot THF (226 mL). The filtrate was concentrated, and the resulting residue was redissolved in ether (154 mL), dried, filtered and evaporated to give the crude mono methyl amine **225** (46.6 g) as a thick brown oil (IR: 3300, 2950, 1460 cm⁻¹).

Crude **225** was dissolved in formic acid (124.4 mL) followed by addition of 30% formaldehyde solution (62.2 mL). The mixture was refluxed for 4 h and the solvents were removed after the mixture was cooled to r.t.. The residue was treated with a mixture of ether (330 mL) and 4N NaOH (248.8 mL) until it was dissolved. The organic layer was dried, filtered and evaporated to dryness, yielding a thick oil of crude dimethyl amine **226** (49.5 g) (IR: 3350, 2950, 1460 cm⁻¹).

To a stirred solution of the dimethyl amine **226** (49.5 g) in CHCl₃ (991 mL) at -40°C (dry ice-CCl₄-acetone), *m* -CPBA (80%, 42.3 g, 0.196 mol) was added in small portions over a period of 20 min, and the mixture was then stirred for another 10 min before addition of Et₃N (9.9 mL, 0.071 mol). The mixture was allowed to warm to r.t. and then brought to reflux for 30 min. The mixture was left to cool to r.t. and the solvent was removed. The residue was dissolved in ether (653 mL) and washed consecutively with 10% H₂SO₄ (653 mL), 10% Na₂CO₃ solution (2x 653 mL) and brine (653 mL). The organic layer was dried, filtered and the solvent was evaporated to give the crude exo-olefin as a pale-colored oil (37.9 g). The crude

product was purified by column chromatography with hexanes to give the pure exo-olefin **228** as a colorless oil (27.0 g, 70.8% yield from dehydroabietic acid **157**).

Physical data of 18-norabieta-4(19),8,11,13-tetraen (**228**):

A colorless oil; $[\alpha]_D^{24}$: +217.8° (c = 2.6, CHCl$_3$); UV λ_{max}^{MeOH} (log ε): 217.9 (3.53), 267.4 (2.87), 275.6 (2.90); IR (neat) cm^{-1}: 3070 (aromatic and olefinic CH), 2950 (CH), 1650 (C=C), 1610 and 1500 (aromatic C=C); ^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.01 (3H, s, H20), 1.24 (6H, d, J = 6.9 Hz, H16, H17), 1.57 (1H, ddd, J = 12.8, 12.8, 4.7 Hz, H1α), 1.65-1.90 (4H, m, H2, H6), 2.06 (1H, ddd, J = 13.0, 13.0, 5.8 Hz, H3α), 2.22 (1H, dd, J = 11.9, 1.0 Hz, H5), 2.28 (1H, br d, J = 12.8 Hz, H1β), 2.38 (1H, br d, J = 12.9 Hz, H3β), 2.85 (1H, sept, J = 6.9 Hz, H15), 2.90 (2H, m, H7), 4.60 (1H, d, J = 1.5 Hz, H$_B$), 4.85 (1H, d, J = 1.5 Hz, H$_A$), 6.94 (1H, br s, H14), 7.01 (1H, dd, J = 8.2, 1.6 Hz, H12), 7.23 (1H, d, J = 8.2 Hz, H11); ^{13}C NMR (50.2 MHz, CDCl$_3$) δ: 21.4, 22.8, 23.8, 24.0 (2C), 30.0, 33.5, 36.4, 38.4, 39.2, 47.9, 106.4, 123.9, 125.4, 127.1, 134.9, 144.7, 145.7, 150.7; LRMS m/z (rel. intensity): 254 (M$^+$, 45.1), 239 (83.5), 211 (8.3), 197 (base peak), 183 (6.8), 169 (14.7), 155 (18.7), 141 (23.5), 129 (17.3), 112 (16.2); HRMS calcd. for C$_{19}$H$_{26}$: 254.2034; found: 254.2034; Anal. calcd. for C$_{19}$H$_{26}$: C, 89.70; H, 10.30; found: C, 89.80; H, 10.43.

18,19-Dinorabieta-8,11,13-trien-4-one (229)

A stock solution of exo-olefin **228** (27.0 g, 0.106 mol) in MeOH-CH$_2$Cl$_2$ (5:1, 1216 mL) was divided into 6 portions (about 200 mL each portion) and ozonized. Ozone was generated by a laboratory ozonator (Welsbach, model T-23) set at 2.2 psi of oxygen, 90 volts and a flow of ozone of 0.013-0.014 Ft^3/min. Ozone was passed through the vigorously stirred solution at -78°C (dry ice-acetone) until the solution turned pale blue (about 40 min for each portion; TLC, hexanes-EtOAc, 9:1). The mixture was continuously stirred at the bath

temperature for an additional 30 min and then dimethyl sulfide (1.6 mL for each portion) was added. The mixture was allowed to warm to r.t. and stirred for 20 h. The 6 portions were combined and the solvents removed. The residue was taken into a hexanes-ether mixture (2:1, 912 mL) and washed with water (3 x 300 mL) and brine (300 mL). The aqueous layer was extracted again with ether (600 mL) and the combined organic fractions were dried and concentrated. The crude product was purified by column chromatography with hexanes-EtOAc (9:1, 8:2), yielding pure ketone **229** as a colorless, thick oil (24.4 g, 89.7% yield), which crystallized when stored under refrigeration.

Physical data of 18,19-dinorabieta-8,11,13-trien-4-one (**229**):

Colorless prisms; mp: 36-38°C (lit.: 40-42°C[161]); $[\alpha]_D^{24}$: +171.6° (c = 0.80, $CHCl_3$); UV λ_{max}^{MeOH} (log ε): 205.1 (4.23), 267.5 (3.05), 275.4 (3.08); IR (neat) cm^{-1}: 3050 (aromatic CH), 2950 (CH), 1700 (C=O), 1605 and 1490 (aromatic C=C); ^1H NMR (400 MHz, $CDCl_3$) δ: 1.04 (3H, s, H20), 1.22 (6H, d, J = 6.9 Hz, H16, H17), 1.73-1.93 (2H, m, H1α, H6β), 1.95-2.17 (3H, m, H2, H6α), 2.39 (3H, m, H1β, H3), 2.58 (1H, dd, J = 12.4, 2.4 Hz, H5), 2.83 (3H, m, H7, H15), 6.92 (1H, br s, H14), 7.02 (1H, dd, J = 8.1, 1.6 Hz, H12), 7.20 (1H, d, J = 8.1 Hz, H11); ^{13}C NMR (50.2 MHz, $CDCl_3$) δ: 17.5, 22.6, 23.7, 23.9 (2C), 28.8, 33.5, 37.0, 40.9, 42.3, 55.4, 124.1, 124.9, 127.3, 134.6, 143.0, 146.4, 212.4; LRMS m/z (rel. intensity): 256 (M$^+$, 33.5), 241 (base peak), 223 (20.4), 213 (24.9), 199 (12.4), 181 (29.2), 171 (17.1), 143 (12.6), 129 (19.8); HRMS calcd. for $C_{18}H_{24}O$: 256.1827; found: 256.1820; Anal. calcd. for $C_{18}H_{24}O$: C, 84.32; H, 9.47; found: C, 84.32; H, 9.47.

Physical data of 18,19-dinorabieta-8,11,13-trien-4,7-dione (**231**):

Colorless prisms; mp: 107-109°C (lit.: 107-108°C[161]); UV λ_{max}^{MeOH} (log ε): 211.8 (4.17), 254.3 (3.83), 301.9 (3.21); IR (CHCl$_3$) cm^{-1}: 3020 (aromatic CH), 2970 (CH), 1700

(C=O), 1676 (C=O), 1600 and 1490 (aromatic C=C); ^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.14 (3H, s, H20), 1.24 (6H, d, J = 6.9 Hz, H16, H17), 1.93-2.25 (3H, m, H1α, H2), 2.33-2.53 (3H, m, H1β, H3), 2.73 (1H, dd, J = 18.7, 4.3 Hz, H5), 2.87 (1H, dd, J = 18.7, 13.2 Hz, H6β), 2.93 (1H, sept, J = 6.9 Hz, H15), 3.09 (1H, dd, J = 13.2, 4.3 Hz, H6α), 7.35 (1H, d, J = 8.1 Hz, H11), 7.42 (1H, dd, J = 8.1, 2.0 Hz, H12), 7.91 (1H, d, J = 2.0 Hz, H14); ^{13}C NMR (50.2 MHz, CDCl$_3$) δ: 22.4, 22.5, 23.7, 23.8, 33.6, 34.3, 35.9, 40.7, 42.4, 54.2, 124.2, 125.5, 130.9, 132.6, 147.6, 149.2, 197.1, 209.2; LRMS m/z (rel. intensity): 270 (M$^+$, 68.1), 255 (base peak), 237 (3.3), 227 (12.3), 213 (37.2), 199 (15.1), 185 (22.1), 173 (11.5), 157 (10.8), 141 (13.5), 128 (20.5), 115 (15.5); HRMS calcd. for C$_{18}$H$_{22}$O$_2$: 270.1620; found: 276.1613; Anal. calcd. for C$_{18}$H$_{22}$O$_2$: C,79.95; H, 8.20; found: C, 79.80; H, 8.24.

3-Dimethylthiomethylene-18,19-dinorabieta-8,11,13-trien-4-one (230)

To a stirred solution of 4-methyl-di-2,6-t-butyl phenol (17.7 g, 80.2 mmol) in anhydrous THF (330 mL) n-BuLi (1.6 M in hexanes, 49.8 mL, 79.7 mmol) was added at -5 to 0°C (ice-MeOH) under argon. A few minutes later, carbon disulfide (16.1 mL, 267.7 mmol) was added and the light yellow mixture was then allowed to reach r.t.. A solution of the 4-ketone 229 (8.13 g, 31.7 mmol) in anhydrous THF (83.8 mL) was added, as was the washing of the 4-ketone flask (THF, 10 mL). The brown colored reaction mixture was stirred at r.t. for 48 h until the starting material disappeared (TLC, hexanes-EtOAc, 9:1). Methyl iodide (10.7 mL, 171.9 mmol) was added and the resulting light yellow mixture was stirred for another 20 h, with the flask wrapped in aluminum foil. The solvent was removed, and the residue was dissolved in ether (1.5 L), washed with water (3 x 500 mL), brine (3 x 420 mL), dried, and concentrated. Two additional reactions were carried out at the same time and all the crude products were combined. Column chromatography of the crude product with hexanes-EtOAc (9:1) gave 230 as a thick orange oil (32.0 g, 93.3% yield), which solidified when stored in a refrigerator.

Physical data of 3-dimethylthiomethylene-18,19-dinorabieta-8,11,13-trien-4-one (**230**):

A yellow solid; UV λ_{max}^{MeOH} (log ε): 205.9 (4.28), 319.4 (3.82); IR (neat) cm^{-1}: 3050 (aromatic CH), 2975 (CH), 1670 (C=O); ^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.09 (3H, s, H20), 1.21 (6H, d, J = 7.1 Hz, H16, H17), 1.80 (1H, m, H6β), 1.93 (1H, ddd, J = 12.5, 12.5, 6.1 Hz, H1α), 2.23 (1H, m, H6α), 2.35 (3H, s, -SCH$_3$), 2.37 (3H, s, -SCH$_3$), 2.47 (1H, m, H1β), 2.59 (1H, dd, J = 12.1, 2.5 Hz, H5), 2.69-2.94 (4H, m, H2β, H7, H15), 3.37 (1H, ddd, J = 16.5, 6.1, 2.6 Hz, H2α), 6.92 (1H, br s, H14), 7.02 (1H, dd, J = 8.0, 1.6 Hz, H12), 7.20 (1H, d, J = 8.0 Hz, H11); ^{13}C NMR (50.2 MHz, CDCl$_3$) δ: 17.8, 17.9, 18.1, 23.8, 23.9 (2C), 29.2, 29.7, 33.5, 36.9, 39.9, 56.3, 124.2, 125.1, 127.2, 134.8, 139.5, 142.7, 143.8, 146.4, 201.0; LRMS m/z (rel. intensity): 360 (M$^+$, 57.7), 345 (base peak), 313 (24.4), 297 (3.8), 237 (1.6), 199 (23.3), 179 (13.6), 127 (46.9); HRMS calcd. for C$_{21}$H$_{28}$OS$_2$: 360.1581; found: 360.1576.

19-Hydroxy-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (193)

n-BuLi (1.6 M in hexanes, 18.78 mL, 30.0 mmol) was added to a stirred suspension of trimethyl sulfonium iodide (6.83 g, 33.5 mmol) in anhydrous THF (135 mL) at -70°C (dry ice-acetone) under argon. The mixture was allowed to warm to -10°C and stirred at that temperature for 30 min before being cooled down to -70°C again. A solution of the ketene dithioacetal **230** (8.0 g, 22.2 mmol) in anhydrous THF (50 mL) was slowly added to the above mixture while the temperature was kept between -65 and -70°C. The resulting light orange reaction mixture was stirred for another 30 min at -70°C, allowed to warm to r.t., and stirred an additional 2 h at r.t. until the starting material disappeared (TLC, hexanes-EtOAc (9:1), or CH$_2$Cl$_2$). The solvent was evaporated using a r.t. water bath. The residue was dissolved in ether (718 mL) and washed with water (3 x 70 mL). The organic layer was separated and evaporated to give a residue which was then dissolved in acetonitrile (67 mL), followed by an addition of MeOH (141.5 mL). The

reaction mixture was cooled in an ice-water bath, and concentrated HCl (17 mL) was added slowly with vigorously stirring. The bath was removed and the heterogeneous mixture was stirred at r.t. for 40 h. The solvents were then removed, and the residue was dissolved in ether (530 mL), washed with saturated NaHCO$_3$ (3 x 90 mL), water (2 x 90 mL), dried and concentrated. Four reactions were carried out at the same time and all crude products were combined. The crude product was repeatedly recrystallized in EtOAc and hexanes, and the mother liquor was column chromatographed (hexanes-EtOAc, 7:3) to yield the butenolide **193** as colorless needles (15.3 g, 58.2% yield from **230**, 34.5% overall yield from dehydroabietic acid (**157**)).

Physical data of 19-hydroxy-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid (**193**):

Colorless needles; mp: 98-100°C (lit.: 97-99°C[161]); $[\alpha]_D^{20}$: +39.1° (c = 0.89, MeOH); UV λ_{max}^{MeOH} (log ε): 218.3 (4.21), 267.4 (2.92), 275.6 (2.94); IR (CHCl$_3$) cm^{-1}: 3020 (aromatic CH), 2960 (CH), 1745 (C=O), 1670 (C=C), 1600 and 1500 (aromatic C=C), 1340, 1030 (C-O); ^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.01 (3H, s, H20), 1.22 (6H, d, J = 6.9 Hz, H16, H17), 1.69 (1H, ddd, J = 12.4, 12.4, 6.5 Hz, H1α), 1.91 (2H, m, H6), 2.37 (1H, m, H2β), 2.50 (2H, m, H1β, H2α), 2.70 (1H, m, H5), 2.82 (1H, sept, J = 6.9 Hz, H15), 3.00 (2H, m, H7), 4.76 (2H, br AB$_q$, Δv = 0.06 ppm, J = 17.2 Hz, H19), 6.96 (1H, d, J = 1.5 Hz, H14), 7.03 (1H, dd, J = 8.1, 1.5 Hz, H12), 7.25 (1H, d, J = 8.1 Hz, H11); ^{13}C NMR (50.2 MHz, CDCl$_3$) δ: 18.2, 20.2, 22.3, 24.0 (C16, C17), 28.4, 32.6, 33.6, 36.3, 41.5, 70.5 (C19), 124.1 (C11, C12), 125.0, 127.5, 134.3, 142.5, 146.7, 163.0, 174.2; LRMS m/z (rel. intensity): 296 (M$^+$, 31.7), 281 (base peak), 253 (3.5), 239 (24.6), 221 (7.6), 209 (8.6), 193 (19.0), 186 (40.3); HRMS calcd. for C$_{20}$H$_{24}$O$_2$: 296.1776; found: 296.1780; Anal. calcd. for C$_{20}$H$_{24}$O$_2$: C, 81.05; H, 8.16; found: C, 80.95; H, 8.14.

Isolation of reaction intermediates and by-products in the step from 230 to 193

In the reaction of dimethylsulfonium methylid with the dithioacetal **230** (Scheme 3.1, step j), the progress of the reaction was monitored by TLC, using hexanes-EtOAc (9:1) as eluent. The TLC showed two major orange spots (anisaldehyde spray; R_f = 0.58, 0.32, respectively), and the lower one partially overlapped with the spot from the unreacted starting material **230** (brown color). The crude intermediates were initially separated by column using CH_2Cl_2-hexanes (2:1) as eluting solvent, since in this solvent system the two orange spots had similar R_f values (R_f = 0.70) and thus the unreacted starting material (R_f = 0.24) could be readily separated. Two combined fractions were collected from this column (Frs. I, II): Fr. I was basically the fraction containing the compounds corresponding to the orange spots and Fr. II was mainly the unreacted starting material and some other polar compounds. Fr. I was then chromatographed using hexanes-EtOAc (9:1) to separate the two orange spots. Two fractions were thus obtained (Frs. IA-IB): Fr. IA was the less polar compound **233** and Fr. IB was the polar one **234**. A ratio change between these two compounds was noticed, which indicated a possible transformation from **234** to **233** during the column chromatography. Portions of each fraction were analyzed by spectroscopic means and the rest, together with Fr. II, were subjected to the hydrolysis reaction, respectively. The results showed that Frs. IA and IB gave the butenolide **193** as the major product; however, Fr. II yielded no butenolide **193** but only some less polar brown spots on TLC (hexanes-EtOAc, 6:4).

The combined by-products (mainly those less polar brown spots, same as those from hydrolysis of Fr. II) were chromatographed with hexanes-EtOAc (100:1, or 98:2) to give **237**, **238** and **239**.

Physical data of furan intermediate **233**:

A pale yellow oil; UV λ_{max}^{MeOH} (log ε): 207.0 (4.40), 251.9 (3.52); IR (neat) cm^{-1}: 3075 (aromatic CH), 2970 (CH), 1640, 1610, 1540, 1500, 1450, 1110, 1060, 970, 900, 810, 740; ^{1}H NMR (400 MHz, CDCl$_3$) δ: 0.96 (3H, s, H20), 1.23 (6H, d, J = 6.9 Hz, H16, H17), 1.70 (1H, ddd, J = 12.7, 12.7, 7.1 Hz, H1α), 1.87 (1H, m, H6β), 2.11 (1H, m, H6α), 2.33 (3H, s, -SCH$_3$), 2.49 (1H, dd, J = 12.7, 6.1 Hz, H1β), 2.61-2.84 (3H, m, H2, H5), 2.84 (1H, sept, J = 6.9 Hz, H15), 2.88-3.09 (2H, m, H7), 6.96 (1H, br s, H14), 7.02 (1H, br d, J = 8.1 Hz, H12), 7.26 (1H, d, J = 1.8 Hz, H19), 7.30 (1H, d, J = 8.1 Hz, H11); ^{13}C NMR (50.2 MHz, CDCl$_3$) δ: 17.9, 18.5, 21.8, 21.9, 24.0 (2C), 29.1, 33.5, 34.0, 36.4, 39.1, 123.9, 125.0, 125.2, 127.5, 128.0, 135.1, 139.2, 141.0, 143.1, 146.1; LRMS m/z (rel. intensity): 326 (M$^+$, base peak), 311 (34.5), 279 (8.6), 268 (5.9), 251 (2.7), 155 (18.0); HRMS calcd. for C$_{21}$H$_{26}$OS: 326.1704; found: 326.1704.

Physical data of β-keto ester **237**:

A colorless oil; UV λ_{max}^{MeOH} (log ε): 202.6 (4.63), 258.4 (4.33); IR (CHCl$_3$) cm^{-1}: 3800 (OH), 3020 (aromatic CH), 2975 (CH), 1650 (C=O), 1610 (C=C); ^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.22 (6H, d, J = 6.9 Hz, H16, H17), 1.29 (3H, s, H20), 1.62 (1H, dt, J = 13.5, 5.8 Hz, H1α), 1.88-2.26 (5H, m, H1β, H2, H6), 2.35 (1H, dd, J = 8.8, 2.9 Hz, H5β), 2.73 (2H, t, J = 6.3 Hz, H7), 2.82 (1H, sept, J = 6.9 Hz, H15), 3.72 (3H, s, -OCH$_3$), 6.88 (1H, d, J = 1.9 Hz, H14), 7.02 (1H, dd, J = 8.2, 1.9 Hz, H12), 7.19 (1H, d, J = 8.2 Hz, H11), 12.30 (1H, br s, D$_2$O exchangeable, enolic H); ^{13}C NMR (50.2 MHz, CDCl$_3$) δ: 19.8, 23.8, 23.9, 24.0, 28.77, 28.84, 33.5, 34.2, 36.3, 46.3, 51.4, 97.2, 124.4, 126.5, 126.7, 136.0, 140.6, 145.9, 173.0, 173.2; LRMS m/z (rel. intensity): 314 (M$^+$, base peak), 299 (45.2), 282 (30.3), 267 (79.8), 254 (33.0), 239 (70.6), 211 (48.7), 186 (38.3); HRMS calcd. for C$_{20}$H$_{26}$O$_3$: 314.1882; found: 314.1884.

Physical data of β-keto thioester **238**:

A yellow oil; UV λ_{max}^{MeOH} (log ε): 204.4 (4.20), 286.6 (3.93); IR (CHCl₃) cm⁻¹: 3800 (OH), 2980 (CH), 1620, 1570, 1170; ¹H NMR (400 MHz, CDCl₃) δ: 1.22 (6H, d, J = 6.9 Hz, H16, H17), 1.31 (3H, s, H20), 1.69 (1H, dt, J = 13.5, 5.9 Hz, H1α), 1.91-2.22 (4H, m, H2, H6), 2.30 (3H, s, -SCH₃), 2.33 (1H, m, H1β), 2.39 (1H, dd, J = 8.8, 3.2 Hz, H5), 2.74 (2H, t, J = 6.3 Hz, H7), 2.83 (1H, sept, J = 6.9 Hz, H15), 6.89 (1H, d, J = 1.9 Hz, H14), 7.04 (1H, dd, J = 8.1, 1.9 Hz, H12), 7.20 (1H, d, J = 8.1 Hz, H11), 13.30 (1H, s, D₂O exchangeable, enolic H); ¹³C NMR (50.2 MHz, CDCl₃) δ: 11.1, 20.0, 23.8, 23.9 (2C), 28.8, 28.9, 33.5, 34.2, 35.9, 46.5, 106.2, 124.5, 126.4, 126.8, 135.9, 140.3, 146.0, 171.6, 197.2; LRMS m/z (rel. intensity): 330 (M⁺, 18.1), 315 (1.0), 283 (base peak), 241 (14.2), 141 (16.0); HRMS calcd. for C₂₀H₂₆O₂S: 330.1652; found: 330.1653.

Physical data of thiophene derivative **239**:

A yellow oil; UV λ_{max}^{MeOH} (log ε): 210.3 (4.21), 251.4 (3.87); IR (CHCl₃) cm⁻¹: 3050 (aromatic CH), 2975 (CH), 1610, 1500, 1460, 1440, 1380, 1240, 1060, 820, 730; ¹H NMR (400 MHz, CDCl₃) δ: 0.95 (3H, s, H20), 1.25 (6H, d, J = 6.9 Hz, H16, H17), 1.80 (1H, ddd, J = 12.7, 12.7, 7.1 Hz, H1α), 1.90 (1H, m, H6β), 2.34 (1H, m, H6α), 2.42 (3H, s, -SCH₃), 2.55 (1H, dd, J = 12.7, 6.3 Hz, H1β), 2.70-3.11 (6H, m, H2, H5, H7, H15), 7.00 (2H, m, H14, H19), 7.06 (1H, dd, J = 8.0, 1.8 Hz, H12), 7.34 (1H, d, J = 8.0 Hz, H11); ¹³C NMR (50.2 MHz, CDCl₃) δ: 20.8, 21.8, 21.9, 22.3, 24.0 (2C), 29.5, 33.6, 34.4, 36.6, 43.6, 120.0, 124.0, 125.3, 127.3, 129.5, 135.1, 139.8, 143.0, 143.2, 146.1; LRMS m/z (rel. intensity): 342 (M⁺, base peak), 327 (92.2), 285 (17.6), 284 (16.4), 280 (16.6), 265 (8.6), 209 (16.4); HRMS calcd. for C₂₁H₂₆S₂: 342.1476; found: 342.1478.

6.3.2 Synthesis of 7β-Hydroxy-isodehydroabietenolide (240)

19-Hydroxy-7-oxo-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (206)

To a stirred solution of the butenolide **193** (400 mg, 1.35 mmol) in glacial HOAc (20 mL) a solution of CrO_3 (1.372 g, 13.7 mmol) in aqueous HOAc (90%, 90 mL) was slowly added in 3 portions (25 mL each portion). Each portion was added over a period of 30 min, followed by stirring for 2 h at r.t.. Isopropanol (10 mL) was then added and the mixture was stirred for another 15 min. Water (300 mL) was added and the mixture was extracted with $CHCl_3$ (4 x 80 mL). The $CHCl_3$ extracts were combined and washed with saturated $NaHCO_3$ (3 x 80 mL), brine (2 x 80 mL), dried, and concentrated. The crude product was purified by column chromatography (hexanes-acetone, 7:3) to give **206** as a colorless crystalline solid (198.2 mg, 49.0% yield based on recovered starting material).

Physical data of 19-hydroxy-7-oxo-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (**206**):

A colorless crystalline solid; mp: 139-141°C; UV λ_{max}^{MeOH} (log ε): 212.5 (4.47), 255.8 (4.05), 297.7 (3.74); IR (CHCl$_3$) cm^{-1}: 3010 (aromatic CH), 2975 (CH), 1750 (C=O), 1670 (C=O), 1580, 1550, 1490, 1250,1020; ^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.14 (3H, s, H20), 1.25 (6H, d, J = 6.9 Hz, H16, H17), 1.90 (1H, ddd, J = 11.6, 11.6, 6.5 Hz, H1α), 2.43 (1H, m, H2β), 2.58 (2H, m, H1β, H2α), 2.738 (1H, d, J = 11.3 Hz, H6β), 2.743 (1H, d, J = 7.8 Hz, H6α), 2.95 (1H, sept, J = 6.9 Hz, H15), 3.21 (1H, m, H5), 4.76 (2H, m, H19), 7.38 (1H, d, J = 8.0 Hz, H11), 7.45 (1H, dd, J = 8.0, 1.9 Hz, H12), 7.93 (1H, d, J = 1.9 Hz, H14); ^{13}C NMR (50.2 MHz, CDCl$_3$) δ: 17.8, 21.8, 23.7, 23.8, 31.6, 33.7, 36.4 (2C), 40.8, 70.0, 123.7, 125.9, 126.3, 131.1, 132.8, 147.9, 148.9, 160.2, 173.4, 195.7; LRMS m/z (rel.

intensity): 310 (M$^+$, 72.4), 295 (base peak), 262 (9.8), 253 (21.5), 237 (14.7), 213 (50.0), 209 (29.6), 187 (44.4), 165 (35.0); HRMS calcd. for $C_{20}H_{22}O_3$: 310.1569; found: 310.1573.

7β,19-Dihydroxy-18(4→3)abeo-abieta-3,8,11,13-tetraen-18-oic acid lactone (240)

$NaBH_4$ (233.0 mg, 6.16 mmol) was added in portions to a stirred solution of **206** (198.2 mg, 0.64 mmol) in EtOH (12 mL). The mixture was stirred at r.t. for 3 h, then the solvent was removed. The residue was suspended in a 5% NaH_2PO_4 solution (25 mL) and extracted with ether (3 x 40 mL). The combined ethereal extract was washed with brine (40 mL), dried, filtered and evaporated. Column chromatography of the crude product with hexanes-EtOAc (1:1) yielded **240** as a while powder (174.6 mg, 87.5% yield).

Physical data of 7β,19-dihydroxy-18(4→3)abeo-abieta-3,8,11,13-tetraen-18-oic acid lactone (**240**):

A white powder; mp: 89-91°C; $[\alpha]_D^{22}$: +59.3° (c = 2.309, MeOH); IR (CHCl$_3$) cm^{-1}: 3600 (OH), 3500 (OH), 3040 (aromatic CH), 3000 (CH), 1750 (C=O), 1690 (C=C), 1620, 1580, 1510; ^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.10 (3H, s, H20), 1.24 (6H, d, J = 6.9 Hz, H16, H17), 1.68 (1H, ddd, J = 12.0, 12.0, 6.3 Hz, H1α), 1.92 (1H, m. H6β), 2.26-2.57 (4H, m, H1β, H2, H6α), 2.78 (1H, br d, J = 13.6 Hz, H5), 2.89 (1H, sept, J = 6.9 Hz, H15), 4.77 (2H, br AB$_q$, Δv = 0.08 ppm, J = 17.2 Hz, H19), 4.99 (1H, dd, J = 8.6, 8.6 Hz, H7α), 7.14 (1H, dd, J = 8.2, 1.9 Hz, H12), 7.26 (1H, d, J = 8.2 Hz, H11), 7.41 (1H, br s, H14); ^{13}C NMR (50.2 MHz, CDCl$_3$) δ: 18.1, 23.0, 23.8, 24.0, 30.8, 32.7, 33.7, 36.8, 40.7, 69.4, 70.4, 124.3, 125.4, 126.1, 126.2, 137.4, 142.4, 147.5, 162.0, 174.0; LRMS m/z (rel. intensity): 312 (M$^+$, 52.4), 297 (26.5), 295 (25.5), 279 (37.9), 269 (91.5), 251 (9.9), 237 (base peak), 213 (11.9); HRMS calcd. for $C_{20}H_{24}O_3$: 312.1726; found: 312.1726.

6.3.3 Synthesis of Isotriptophenolide (194)

12-Acetyl-19-hydroxy-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (241)

A solution of **193** (4.0 g, 13.5 mmol) in carbon disulfide (40 mL) and acetyl chloride (6.7 mL, 94.2 mmol) was added to a suspension of anhydrous $AlCl_3$ (6 g, 45.0 mmol) in carbon disulfide (400 mL) with vigorously stirring under nitrogen. The mixture was refluxed overnight (about 19 h). The solvent was removed and iced aqueous HCl (concentrated $HCl:H_2O$, 3:10) was added to the residue. The resulting suspension was stirred in an ice-water bath for 15 min and then at r.t. for 30 min. After all the complex was destroyed, EtOAc (200 mL) was added and the mixture was stirred for another 30 min. The aqueous layer was further extracted with EtOAc (2 x 100 mL), and all the EtOAc extracts were combined, washed with saturated $NaHCO_3$ (3 x 130 mL), brine (3 x 130 mL), dried, and concentrated. The crude product (4.6145 g, quantitative yield) obtained was a pale white solid, which showed only one spot on TLC (hexanes-EtOAc, 6:4) and was thus used directly in the next step. Pure **241** was obtained by column chromatography with hexanes-EtOAc (7:3).

Physical data of 12-acetyl-19-hydroxy-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (**241**):

A white crystalline solid; mp: 153-155°C; UV λ_{max}^{MeOH} (log ε): 217.3 (4.52), 253.6 (4.01); IR ($CHCl_3$) cm^{-1}: 3025 (aromatic CH), 2950 (CH), 1750 (C=O), 1680 (C=O), 1270; ^1H NMR (400 MHz, $CDCl_3$) δ: 1.03 (3H, s, H20), 1.19, 1.21 (3H each, both d, J = 6.9 Hz, H16, H17), 1.72 (1H, ddd, J = 12.5, 12.5, 6.5 Hz, H1α), 1.94 (2H, m, H6), 2.32-2.61 (3H, m, H1β, H2), 2.55 (3H, s, -COCH$_3$), 2.69 (1H, m, H5), 3.03 (2H, m, H7), 3.45 (1H, sept, J

= 6.9 Hz, H15), 4.77 (2H, br AB$_q$, Δv = 0.06 ppm, J = 17.2 Hz, H19), 7.12 (1H, s, H14),

7.44 (1H, s, H11); [13]C NMR (50.2 MHz, CDCl$_3$) δ: 18.1, 20.0, 22.2, 24.1, 24.2, 28.3, 28.8,

30.6, 32.5, 36.3, 41.3, 70.4, 123.9, 125.1, 127.7, 136.7, 138.1, 142.2, 145.7, 162.5, 174.0,

203.2; LRMS m/z (rel. intensity): 338 (M$^+$, 30.3), 323 (86.2), 305 (3.7), 281 (5.5), 265 (1.1),

239 (3.2), 193 (4.2), 43 (base peak); HRMS calcd. for C$_{22}$H$_{26}$O$_3$: 338.1882; found:

338.1875.

12-Acetoxy-19-hydroxy-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (242)

To a solution of **241** (4.6145 g) in CH$_2$Cl$_2$ (45 mL) was added *m*-CPBA (80%, 6.26 g) in one portion. The mixture was cooled to -5 to 0°C and TFA (1.13 mL) was added as a catalyst. The mixture was allowed to warm to r.t. and stirred for 29 h in the dark (TLC, isopropyl ether, developed twice). The reaction mixture was diluted with EtOAc (210 mL), and the solution was washed with 10% Na$_2$SO$_3$ (32 mL), saturated KHCO$_3$ (32 mL), brine (32 mL), dried, filtered and concentrated. The crude product (4.9249 g, a yellow powder) showed one major spot on TLC (isopropyl ether, developed twice) and was used directly in the next step. Samples for the analyses were purified by column chromatography with isopropyl ether.

Physical data of 12-acetoxy-19-hydroxy-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (**242**):

A white powder; mp: 70-72°C; UV λ_{max}^{MeOH} (log ε): 218.9 (4.25), 268.1 (3.27), 276.8

(3.25); IR (CHCl$_3$) cm^{-1}: 3075 (aromatic CH), 2975 (CH), 1730 (C=O), 1680 (C=C), 1500

(C=C), 1380, 1240, 1180, 1040; [1]H NMR (400 MHz, CDCl$_3$) δ: 1.01 (3H, s, H20), 1.17,

1.18 (3H each, both d, J = 6.9 Hz, H16, H17), 1.71 (1H, m, H1α), 1.90 (2H, m, H6), 2.30

(3H, s, -OCOCH$_3$), 2.26-2.54 (3H, m, H1β, H2), 2.69 (1H, m, H5), 2.92 (1H, sept, J = 6.9

Hz, H15), 2.99 (2H, m, H7), 4.76 (2H, br AB_q, Δv = 0.07 ppm, J = 17.2 Hz, H19), 6.92

(1H, s, H11), 7.02 (1H, s, H14); ^{13}C NMR (50.2 MHz, $CDCl_3$) δ: 18.1, 20.1, 20.9, 22.2,

23.0 (2C), 27.2, 27.9, 32.5, 36.4, 41.1, 70.5, 118.0, 125.0, 127.7, 132.4, 137.9, 143.6,

146.2, 162.8, 169.9, 174.2; LRMS m/z (rel. intensity): 354 (M^+, 7.2), 339 (1.0), 312 (base

peak), 284 (5.5), 255 (8.5), 149 (36.6); HRMS calcd. for $C_{22}H_{26}O_4$: 354.1837; found:

354.1832.

12,19-Dihydroxy-18(4→3)$abeo$-3,8,11,13-tetraen-18-oic acid lactone (194) (isotriptophenolide)

To a stirred solution of **242** (4.5203 g) in MeOH (168 mL) concentrated HCl was added

dropwise at r.t.. The mixture was stirred in the dark at r.t. for 7 h (TLC, isopropyl ether,

developed twice). After the starting material disappeared, the mixture was evaporated to remove

MeOH. The residue was then extracted with EtOAc (200 mL), and the EtOAc layer was washed

with saturated $NaHCO_3$ (3 x 50 mL), brine (2 x 50 mL), dried, filtered and concentrated. The

crude product was chromatographed with isopropyl ether and recrystallized from EtOAc and

isopropyl ether to give pure isotriptophenolide (**194**) as a colorless crystalline solid (3.2677 g,

84.4% overall yield from **193**).

Physical data of 12,19-dihydroxy-18(4→3)$abeo$-3,8,11,13-tetraen-18-oic acid lactone (**194**):

A colorless crystalline solid; mp: 198-200°C (dec) (lit.: 194-197°C[161]); $[\alpha]_D^{20}$: +42.0°

(c = 0.37, MeOH); UV λ_{max}^{MeOH} (log ϵ): 220.2 (4.38), 283.0 (3.87); IR ($CHCl_3$) cm^{-1}: 3400

(OH), 3025 (aromatic CH), 2975 (CH), 1740 (C=O), 1670, 1620, 1500 (aromatic C=C), 1030;

^1H NMR (400 MHz, $CDCl_3$) δ: 1.00 (3H, s, H20), 1.22, 1.23 (3H, each, both d, J = 6.9 Hz,

H16, H17), 1.68 (1H, m, H1α), 1.88 (2H, m, H6), 2.28-2.54 (3H, m, H1β, H2), 2.69 (1H,

m, H5), 2.94 (2H, m, H7), 3.13 (1H, sept, J = 6.9 Hz, H15), 4.76 (2H, br AB_q, Δv = 0.07

ppm, J = 17.1 Hz, H19), 4.84 (1H, br s, C12-OH, D_2O exchangeable), 6.72 (1H, s, H11), 6.90 (1H, s, H14); ^{13}C NMR (50.2 MHz, $CDCl_3$) δ: 18.2, 20.3, 22.2, 22.5, 22.7, 26.8, 27.6, 32.6, 36.3, 41.5, 70.6, 111.1, 124.9, 126.3, 127.4, 132.6, 143.4, 151.1, 163.3, 174.4; LRMS m/z (rel. intensity): 312 (M^+, 75.6), 297 (base peak), 277 (9.9), 255 (20.2), 237 (8.6), 220 (4.3), 205 (10.4), 149 (93.3), 134 (22.1); HRMS calcd. for $C_{20}H_{24}O_3$: 312.1725; found: 312.1729; Anal. calcd. for $C_{20}H_{24}O_3$: C, 76.90; H, 7.74; found: C, 76.91; H, 7.72.

Physical data of 11-chloro-12,19-dihydroxy-18(4→3)$abeo$-abieta-3,8,11,13-tetraen-18-oic acid lactone (**243**):

A pale white powder; mp: 83-86°C; UV λ_{max}^{MeOH} (log ε): 221.9 (4.06), 289.9 (3.53); IR (CHCl$_3$) cm^{-1}: 3550 (OH), 3050 (aromatic CH), 2960 (CH), 1750 (C=O), 1690 (C=C), 1620, 1480, 1420, 1190, 1080, 1040; ^1H NMR (400 MHz, $CDCl_3$) δ: 1.20, 1.22 (3H each, both d, J = 6.9 Hz, H16, H17), 1.24 (3H, s, H20), 1.52 (1H, m, H1α), 1.78 (2H, m, H6), 2.42 (2H, m, H2), 2.77 (1H, m, H5), 2.84 (1H, br d, J = 16.7 Hz, H7β), 3.00 (1H, ddd, J = 16.7, 11.1, 8.6 Hz, H7α), 3.24 (1H, sept, J = 6.9 Hz, H15), 3.64 (1H, ddd, J = 13.6, 5.5, 2.1 Hz, H1β), 4.76 (2H, br AB$_q$, Δν = 0.12 ppm, J = 17.2 Hz, H19), 5.90 (1H, s, C12-OH, D_2O exchangeable), 6.85 (1H, s, H14); ^{13}C NMR (50.2 MHz, $CDCl_3$) δ: 16.8, 18.6, 19.7, 22.1, 22.3, 27.7, 30.7, 31.2, 38.9, 45.2, 70.5, 119.2, 125.2, 126.6, 128.5, 134.0, 138.1, 147.8, 163.0, 174.0; LRMS m/z (rel. intensity): 348 (M^+ (^{37}Cl), 1.6), 346 (M^+ (^{35}Cl), 4.3), 333 (0.7), 331 (2.1), 303 (0.5), 289 (1.5), 183 (18.8); HRMS calcd. for $C_{20}H_{23}^{37}ClO_3$ ($C_{20}H_{23}^{35}ClO_3$): 348.1306 (346.1336); found: 348.1302 (346.1344).

6.3.4 Synthesis of 7β-Hydroxy-isotriptophenolide (246b)

12-Acetoxy-19-hydroxy-7-oxo-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (244)

To a stirred solution of 12-acetoxy butenolide (**242**, 495.7 mg, 1.40 mmol) in glacial HOAc (25 mL), a freshly prepared CrO_3 (1.715 g, 17.2 mmol) solution in aqueous HOAc (90%, 112.5 mL) was added slowly in 3 portions (22.5 mL each portion). The reaction mixture was stirred for 2 h after each addition (TLC, hexanes-EtOAc, 1:1), then isopropanol (10 mL) was added followed by stirring for another 15 min. Water (200 mL) was added and the aqueous solution was extracted with $CHCl_3$ (4 x 100 mL). The combined $CHCl_3$ extract was washed with saturated $NaHCO_3$ (3 x 100 mL), brine (2 x 100 mL), dried, and concentrated. The crude product was chromatographed with hexanes-EtOAc (7:3) to provide **244** as a yellowish powder (317.2 mg, 63.1% yield based on recovered starting material).

Physical data of 12-acetoxy-19-hydroxy-7-oxo-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (**244**):

A yellowish powder; mp: 180-182°C; ^1H NMR (400 MHz, $CDCl_3$) δ: 1.16 (3H, s, H20), 1.21, 1.23 (3H each, both d, J = 6.9 Hz, H16, H17), 1.83 (1H, m, H1α), 2.34 (3H, s, -OCOCH₃), 2.42 (1H, m, H2β), 2.47 (1H, dd, J = 12.6, 6.1 Hz, H1β), 2.57 (1H, br d, J = 17.4 Hz, H2α), 2.732 (1H, d, J = 11.9 Hz, H6β), 2.738 (1H, d, J = 7.2 Hz, H6α), 3.01 (1H, sept, J = 6.9 Hz, H15), 3.23 (1H, m, H5), 4.76 (2H, m, H19), 7.10 (1H, s, H11), 8.06 (1H, s, H14); ^{13}C NMR (50.2 MHz, $CDCl_3$) δ: 17.7, 21.0, 21.7, 22.7 (2C), 27.3, 31.6, 36.3, 36.5, 40.7, 70.0, 118.1, 125.9, 127.8, 129.2, 139.6, 150.2, 152.8, 160.0, 169.0, 173.3, 194.6; LRMS m/z (rel. intensity): 368 (M^+, 6.9), 326 (base peak), 311 (99.8), 298 (19.4), 284

(12.4), 229 (58.2), 203 (90.0), 187 (29.1), 163 (97.1); HRMS calcd. for $C_{22}H_{24}O_5$: 368.1624; found: 368.1623.

7β,12,19-Trihydroxy-18(4→3)abeo-abieta-3,8,11,13-tetraen-18-oic acid lactone (246b)

<u>Method A (via reduction of 245)</u>

To a stirred solution of **244** (217 mg, 0.589 mmol) in MeOH (7 mL), concentrated HCl (0.6 mL) was added. The reaction mixture was stirred for 9.5 h (TLC, isopropyl ether, developed twice) and then the solvent was removed. The residue was extracted with EtOAc (50 mL) and washed with saturated NaHCO$_3$ (3 x 20 mL), brine (2 x 20 mL), and dried. Removal of solvent gave the crude product, which was then recrystallized in MeOH and water yielding pure **245** as colorless needles (161.5 mg, 84% yield).

Compound **245** (30 mg, 0.092 mmol) was dissolved in EtOH (7 mL) with drops of EtOAc to increase solubility. NaBH$_4$ (94 mg, 2.48 mmol) was added in small portions and the mixture was stirred for 21 h until the starting material disappeared (TLC, hexanes-EtOAc, 1:1). The solvent was removed and the residue was suspended in water (6 mL), neutralized with dilute HCl and extracted with ether (3 x 10 mL). The combined ethereal extract was washed with brine (2 x 10 mL), dried, filtered and evaporated. The crude product was purified by chromatography with hexanes-EtOAc (1:1) to give the 7β-hydroxy compound **246b** as a colorless crystalline solid (7.7 mg, 25.5% yield, some decomposition occurred during column chromatography).

Physical data of 12,19-dihydroxy-7-oxo-18(4→3)abeo-abieta-3,8,11,13-tetraen-18-oic acid lactone (**245**):

Colorless needles; mp: 287-289°C (dec); UV λ_{max}^{MeOH} (log ε): 208.6 (4.45), 232.0 (4.41), 287.0 (4.19); IR (KBr) cm^{-1}: 3150 (br, OH), 2990 (CH), 1760 (C=O), 1690, 1650,

1580 (C=O), 1500 (aromatic C=C), 1280 (C-O), 1180, 1030; ^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.14 (3H, s, H20), 1.25, 1.27 (3H each, both d, J = 7.0 Hz, H16, H17), 1.79 (1H, m, H1α), 2.43 (2H, m, H1β, H2β), 2.57 (1H, m, H2α), 2.684 (1H, d, J = 11.5 Hz, H6β), 2.689 (1H, d, J = 7.4 Hz, H6α), 3.14 (1H, sept, J = 7.0 Hz, H15), 3.18 (1H, m, H5), 4.75 (2H, m, H19), 5.37 (1H, s, C12-OH, D$_2$O exchangeable), 6.78 (1H, s, H11), 7.97 (1H, s, H14); LRMS m/z (rel. intensity): 310 (M$^+$, 73.5), 295 (base peak), 267 (5.0), 253 (19.6), 225 (13.3), 213 (51.0), 187 (36.8); HRMS calcd. for C$_{20}$H$_{22}$O$_4$: 326.1518; found: 326.1513; Anal. calcd. for C$_{20}$H$_{22}$O$_4$: C, 73.60; H, 6.79; found: C, 73.40; H, 6.63.

Physical data of 7β,12,19-trihydroxy-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (**246b**):

A colorless crystalline solid; mp: 135-137°C (dec); $[\alpha]_D^{23}$: +27.9° (c = 0.034, CHCl$_3$); UV λ_{max}^{MeOH} (log ϵ): 205.7 (4.56), 281.4 (3.72); IR (CHCl$_3$) cm^{-1}: 3350 (OH), 3025 (aromatic CH), 2950 (CH), 1740 (C=O), 1670, 1620, 1500 (aromatic C=C), 1010; ^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.10 (3H, s, H20), 1.24, 1.26 (3H each, both d, J = 6.8 Hz, H16, H17), 1.66 (1H, m, H1α), 1.87 (1H, ddd, J = 13.8, 12.9, 9.2 Hz, H6β), 2.30 (1H, ddd, J = 12.9, 7.7, 2.7 Hz, H6α), 2.34-2.56 (3H, m, H1β, H2), 2.75 (1H, br d, J = 13.8 Hz, H5), 3.14 (1H, sept, J = 6.8 Hz, H15), 4.76 (2H, br AB$_q$, Δv = 0.07 ppm, J = 17.1 Hz, H19), 4.78 (1H, s, C12-OH), 4.95 (1H, br dd, J = 8.6, 8.6 Hz, H7α), 6.69 (1H, s, H11), 7.35 (1H, s, H14); ^{13}C NMR (50.2 MHz, CDCl$_3$) δ: 18.1, 22.5 (2C), 22.8, 27.0, 31.0, 32.7, 36.7, 40.8, 69.1 (C7), 70.3, 110.9, 125.4, 126.6, 130.0, 137.3, 144.0, 156.9, 161.9, 174.9; LRMS m/z (rel. intensity): 328 (M$^+$, 3.3), 313 (3.1), 310 (77.8), 295 (67.8), 279 (42.9), 267 (40.4), 253 (base peak); HRMS calcd. for C$_{20}$H$_{24}$O$_4$: 328.1675; found: 328.1670.

Method B (direct reduction of 244)

To a stirred solution of **244** (100 mg, 0.27 mmol) in EtOH (10 mL) NaBH₄ (120 mg, 3.17 mmol) was added in portions. The mixture was stirred for 8 h at r.t. (TLC, hexanes-EtOAc, 1:1) and then the solvent was removed. The residue was suspended in 5% NaH₂PO₄ solution (12 mL) and extracted with ether (3 x 20 mL). The combined ether extract was washed with brine (2 x 20 mL) and dried. Removal of the solvent gave the crude product, which was chromatographed with hexanes-EtOAc (1:1) to yield the diol **246b** as a colorless, fine crystalline solid (23.9 mg, 26.8% yield).

Physical data of 12,19-dihydroxy-18(4→3)*abeo*-abieta-3,6,8,11,13-pentaen-18-oic acid lactone (**247**):

A reddish powder; IR (CHCl₃) cm⁻¹: 3600 (OH), 3050 (aromatic and olefinic CH), 2990 (CH), 1750 (C=O), 1680, 1630, 1610, 1580, 1500, 1360, 1040; ¹H NMR (400 MHz, DMSO-d_6) δ: 0.94 (3H, s, H20), 1.21, 1.23 (3H each, both d, J = 7.0 Hz, H16, H17), 1.86 (1H, m, H1α), 2.24-2.49 (3H, m, H1β, H2), 3.29 (1H, sept, J = 7.0 Hz, H15), 3.54 (1H, br s, H5), 5.00 (2H, br AB$_q$, Δv = 0.1 ppm, J = 17.3 Hz, H19), 5.85 (1H, dd, J = 9.4, 2.6 Hz, H6), 6.62 (1H, dd, J = 9.4, 3.2 Hz, H7), 6.88 (1H, s, H11), 7.02 (1H, s, H14); ¹³C NMR (50.2 MHz, CDCl₃) δ: 18.2, 18.4, 22.4, 22.6, 26.7, 30.8, 37.0, 42.6, 70.7, 110.2, 120.3, 125.0, 125.8, 126.0, 129.9, 132.1, 143.0, 153.0, 161.1, 173.2; LRMS m/z (rel. intensity): 310 (M⁺, 57.6), 295 (38.6), 293 (29.3), 281 (8.0), 267 (36.0), 253 (base peak), 178 (18.0), 165 (23.0); HRMS calcd. for C₂₀H₂₂O₃: 310.1569; found: 310.1568.

6.3.5 Attempted Synthesis of 8,12-Quinone Methide from Isotriptophenolide (194)

DDQ/THF method (a typical procedure)

DDQ (291 mg, 2 equiv.) was added to a stirred solution of **194** (200 mg, 0.641 mmol) in THF (30 mL). The solution rapidly turned light purple and then reddish. After the mixture was stirred at r.t. for 3 h (TLC, hexanes-EtOAc, 1:1), the solvent was removed. Repeated column chromatography of the crude product with hexanes-EtOAc (65:35) gave the quinone **250** as a red solid (34.9 mg, 17.8% yield), and two additional mixtures: mixture A (**247** and **251**, 27.1 mg) and mixture B (**245** and **252**, 55 mg).

Physical data of compound **251**:

^{1}H NMR (200 MHz, CDCl$_3$) (partial) δ: 1.16 (3H, s, H20), 1.23, 1.26 (3H each, both d, J = 6 Hz, H16, H17), 3.19 (1H, sept, J = 6 Hz, H15), 3.50 (2H, br d, J = 5 Hz, H7), 6.17 (1H, dd, J = 5, 5 Hz, H6).

compound **252**: Spectral data are given in experimental section for Chapter 4.

Physical data of 19-hydroxy-12-oxo-18(4→3)*abeo*-abieta-3,5,7,9(11),13-pentaen-18-oic acid lactone (**250**):

A red powder; UV λ_{max}^{MeOH} (log ε): 207.4 (4.22), 265.2 (4.10), 438.0 (3.13); IR (CHCl$_3$) cm^{-1}: 3050 (olefinic CH), 2975 (CH), 1750 (C=O), 1640 (quinone C=O), 1600 (quinone C=C); ^{1}H NMR (200 MHz, CDCl$_3$) δ: 1.15 (6H, d, J = 6.4 Hz, H16, H17), 1.29 (3H, s, H20), 1.88 (1H, m, H1α), 2.33 (1H, br d, J = 13.3 Hz, H1β), 2.62 (2H, m, H2), 3.14 (1H, sept, J = 6.4 Hz, H15), 4.98 (2H, br AB$_q$, Δv = 0.16 ppm, J = 16.0 Hz, H19), 6.36 (1H,

d, J = 7.0 Hz, H6), 6.56 (1H, d, J = 1.6 Hz, H11), 6.82 (1H, dd, J = 7.0, 1.6 Hz, H7), 7.00 (1H, s, H14); ^{13}C NMR (50.2 MHz, CDCl$_3$) δ: 18.1 (C2), 21.9 (C16, C17), 26.5 (C15), 29.7 (C20), 32.2 (C1), 40.0 (C10), 68.5 (C19), 121.0 (C6), 125.7 (C11), 128.1 (C3), 131.8 (C8), 132.5 (C14), 133.1 (C7), 144.4 (C5), 146.7 (C13), 152.6 (C4), 153.1 (C9), 172.9 (C18), 185.3 (C12); LRMS m/z (rel. intensity): 308 (M$^+$, 25.1), 293 (base peak), 279 (24.5), 265 (35.4), 253 (12.5); HRMS calcd. for C$_{20}$H$_{20}$O$_3$: 308.1412; found: 308.1416.

DDQ/MeOH

DDQ (109 mg, 1.5 equiv.) was added to a stirred solution of **194** (100 mg, 0.32 mmol) in MeOH (15 mL) under argon. After 28 min the solvent was removed and the crude product was chromatographed with hexanes-EtOAc (1:1) to give 7-methoxy-isotriptophenolide (**253**, 24 mg, α:β = 1, 21.9% yield), **246a** (8.8 mg, 7.7% yield) and a mixture consisting of unreacted starting material and **251** (11.3 mg).

Physical data of 7-Methoxy-isotriptophenolide (**253**):

^{1}H NMR (400 MHz, CDCl$_3$) δ (α isomer, partial): 4.30 (br s, H7β), 6.75 (s, H11), 7.13 (s, H14); ^{1}H NMR (400 MHz, CDCl$_3$) δ (β isomer, partial): 4.64 (dd, J = 8, 8 Hz, H7α), 6.72 (s, H11), 7.28 (s, H14); DCIMS (NH$_3$) m/z (rel. intensity): 360 (M + NH$_4$, 15.5%), 343 (M + H, 29.0%), 328 (M - CH$_3$ + H, 9.31), 311 (M - MeOH + H, base peak).

Physical data of 7α,12,19-trihydroxy-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (**246a**):

Colorless crystalline solid; mp: 139-141°C (dec); [α]$_D^{24}$: +23.6° (c = 0.233, CHCl$_3$); UV λ$_{max}^{MeOH}$ (log ε): 222.8 (4.12), 279.9 (3.56); IR (CHCl$_3$) cm^{-1}: 3600 (OH), 3450 (OH), 2990 (CH), 1750 (C=O), 1680 (C=C), 1040; ^{1}H NMR (400 MHz, CDCl$_3$) δ: 0.95 (3H, s, H20), 1.24, 1.26 (3H each, both d, J = 6.8 Hz, H16, H17), 1.72 (1H, m, H1α), 1.97 (1H, br

ddd, J = 13.7, 2.2, 2.2 Hz, H6α), 2.10 (1H, ddd, J = 13.7, 13.7, 4.2 Hz, H6β), 2.32-2.56 (3H, m, H1β, H2), 3.15 (2H, m, H5, H15), 4.78 (2H, br AB$_q$, Δv = 0.12 ppm, J = 17.2 Hz, H19), 4.85 (1H, m, H7β), 4.91 (1H, s, C14-OH, D$_2$O exchangeable), 6.73 (1H, s, H11), 7.17 (1H, s, H14); ^{13}C NMR (50.2 MHz, CDCl$_3$) δ: 18.2, 22.1, 22.4, 22.6, 26.9, 29.4, 32.6, 36.5, 36.6, 66.9 (C7), 70.5, 111.2, 125.3, 128.5, 128.7, 133.5, 143.8, 153.2, 163.2, 174.1; MS m/z (rel. intensity): 328 (M$^+$, 0.3), 310 (46.0), 295 (44.5), 279 (13.0), 267 (42.9), 253 (base peak); HRMS calcd. for C$_{20}$H$_{24}$O$_4$: 328.1674; found: 328.1679.

6.3.6 Synthesis of Triptophenolide (106)

Preliminary Experiments

NaBH$_4$ (0.3 g, 7.94 mmol) was added to a stirred solution of the ketone **229** (2.01 g, 7.85 mmol) in EtOH (100 mL). The mixture was stirred at r.t. for 3 h (TLC, hexanes-EtOAc, 8:2) and then the solvent was removed. The residue was suspended in 5% NaH$_2$PO$_4$ (100 mL) and extracted with EtOAc (3 x 50 mL). The combined EtOAc was washed with 5% NaH$_2$PO$_4$ (2 x 20 mL), brine (2 x 20 mL), dried, filtered and the solvent was removed. The resulting crude alcohol **260** in pyridine (140 mL) was treated with acetic anhydride (35 mL), and the mixture was stirred at r.t. for 45 h (TLC, hexanes-EtOAc, 8:2). The solvents were removed and the residue was dissolved in EtOAc (150 mL), washed with saturated NaHCO$_3$ (2 x 50 mL), 5% HCl (50 mL), saturated NaHCO$_3$ (50 mL), brine (2 x 50 mL), and dried. The solvent was evaporated to give crude acetate **261** (2.3605 g).

A solution of the crude acetate **261** in glacial HOAc (6.8 mL) was added slowly to a stirred mixture of concentrated HNO$_3$ (9 mL) and H$_2$SO$_4$ (11.8 mL) at 0 to 5°C (ice-water) over 20 min. The mixture was stirred for 1.5 h and then poured onto crushed ice (about 3 g). The resulting suspension was diluted to 70 mL with water and the product was isolated by filtration. The moist product was then dissolved in EtOAc (50 mL), washed with saturated NaHCO$_3$ (2 x

50 mL), brine (2 x 50 mL) and dried. Removal of the solvent yielded the dinitro compound **262** as a yellow solid (2.8688 g).

The dinitro compound **262** was selectively reduced by catalytic hydrogenation with 10% Pt/C (151 mg) in HOAc (47.1 mL) under H_2 (1 atm) for 20 h at r.t.. The resulting mixture was diluted with EtOAc (22 mL) and filtered through Celite. The Celite was then washed with EtOAc (3 x 20 mL) and all EtOAc solutions were combined and evaporated to give crude **263** as a yellow solid (2.914 g).

263 was dissolved in a mixture of TFA (15.6 mL), HOAc (15.6 mL) and water (15.6 mL), and was cooled to 0-5°C (ice-water). $NaNO_2$ (766.8 mg) was added in portions to the stirred mixture over 1 h, and stirring continued for another 2 h. A solution of KI (1.974 g) in water (3.2 mL) was added over 15 min. Thirty minutes later, a solution of Na_2SO_3 (610.6 mg in 26.3 mL water) was added and the mixture was stirred for an additional 30 min at r.t.. Water (30 mL) was added and the resulting suspension was filtered to separate the product. The solid product **264** was suspended in HOAc (46.2 mL). Zinc powder (3.578 g) was added at about 10°C (cold water bath) while stirring, and then the mixture was brought to 65-70°C and stirred at that temperature for 23 h. After cooling in a cold water bath, the suspension was filtered through Celite and the zinc salt cake was washed with EtOAc. The combined filtrate was evaporated to give crude **265** as a brown solid (6 g).

The crude **265** was dissolved in TFA (30.6 mL), and $NaNO_2$ (1.88 g) was added in portions over 1 h to the stirred solution at 0°C (ice-water). Thirty minutes later, a solution of Na_2SO_3 (923 mg in 6.4 mL water) was added. Additional Na_2SO_3 was added until the solution did not darken KI-starch paper. Water (26 mL) was added and the mixture was stirred overnight. The resulting mixture was then extracted with EtOAc (3 x 50 mL) and the combined EtOAc extract was washed with saturated $NaHCO_3$ (2 x 80 mL), brine (80 mL) and dried. Rotary evaporation of the solvent provided the crude product **266** (2.409 g). The crude **266** was then treated with $NaBH_4$ (888 mg) in EtOH (80 mL) for 3.5 h at r.t.. Then the solvent was removed, the residue suspended in 5% NaH_2PO_4 (200 mL), and extracted with EtOAc (3 x 120

mL). The combined EtOAc was washed with saturated $NaHCO_3$ (2 x 100 mL), brine (2 x 100 mL), dried and evaporated. The obtained crude product (2.0084 g) was column chromatographed with hexanes-EtOAc (8:2) to give **267** (523.5 mg) and a 4,14-diol (70 mg).

Physical data of compound **267**: ·

[1]H NMR (200 MHz, $CDCl_3$) δ: 1.22, 1.26 (3H each, both d, $J = 6$ Hz, H16, H17), 1.28 (3H, s, H20), 2.07 (3H, s, $-COCH_3$), 1.4-3.3 (11H, m, aliphatic H), 3.12 (1H, sept, $J = 6$ Hz, H15), 4.65 (1H, s, C14-OH), 5.15 (1H, m, H4α), 6.86 (1H, d, $J = 8$ Hz, H11), 7.06 (1H, d, $J = 8$ Hz, H12); LRMS m/z (rel. intensity): 316 (M^+, 39.8), 301 (15.5), 241 (base peak), 199 (14.7).

To a stirred solution of **267** (523.5 mg, 1.66 mmol) in dry THF (17 mL) was added n-BuLi (1.6 M in hexanes, 1.08 mL, 1.73 mmol), and the reaction mixture was stirred at r.t. for 30 min. Methyl iodide (0.108 mL) was added and the solution was stirred overnight. The reaction was not completed until more n-BuLi and CH_3I were added (n-BuLi (0.27 mL) and CH_3I (0.27 mL) at 21 h, n-BuLi (0.2 mL) and CH_3I (0.06 mL) at 30 h and the same amounts again at 48 h). The reaction was finished at 72 h (TLC, hexanes-EtOAc, 8:2) and the solvent was removed. The residue was suspended in 5% NaH_2PO_4 (60 mL) and extracted with EtOAc (3 x 80 mL). The combined EtOAc was washed with water (80 mL), brine (80 mL), dried and evaporated. Crude **268** was dissolved in dry THF (10 mL), treated with LAH under argon and stirred at r.t. overnight. The mixture was then refluxed for 1 h but still little reaction was observed by TLC (hexanes-EtOAc, 8:2). MeOH (10 mL) and KOH (2 pellets) were added and the mixture was refluxed for 21 h. Solvents were removed and the residue was suspended in 5% HCl until the pH was almost neutral. The resulting mixture was extracted with EtOAc (3 x 20 mL) and the combined EtOAc was washed with saturated $NaHCO_3$ (2 x 30 mL), brine (30 mL) and dried. Crude **269**, combined with an additional quantity (about 70 mg) of this same compound

prepared from the 4,14-diol from the previous reaction, was dissolved in acetone (50 mL) and treated with Jones' reagent (8N) until the brown color of the reagent remained for 5 min. Isopropyl alcohol was added to the point where the solution turned green again. The solvent was removed and the residue was suspended in water (40 mL), and extracted with EtOAc (3 x 40 mL). The combined EtOAc was washed with saturated $NaHCO_3$ (2 x 50 mL), brine (50 mL) and dried. The crude product thus obtained (562.8 mg) was chromatographed with hexanes-EtOAc (8:2) to give **270** (360.2 mg) as an off-white solid.

n-BuLi (1.25 M in hexanes, 2.52 mL, 3.15 mmol) was added to a stirred solution of 2,6-di(*t*-butyl)-4-methyl phenol (0.71 g, 3.23 mmol) in anhydrous THF (10 mL) at -5-0°C (ice-MeOH). Carbon disulfide (0.69 mL) was added and the mixture was allowed to reach r.t.. A solution of **270** (360.2 mg, 1.26 mmol) in dry THF (6 mL) was added. The reaction mixture was stirred at r.t. for 48 h, then CH_3I (0.43 mL) was added and the reaction continued for 20 h in the dark. The solvent was removed, and the residue was dissolved in EtOAc (50 mL), washed with 5% NaH_2PO_4 (2 x 20 mL), saturated $NaHCO_3$ (2 x 20 mL), brine (2 x 20 mL), and dried. Column chromatography of the crude product (hexanes-EtOAc, 9:1) gave the corresponding ketene dithioacetal **271** as yellow crystals (413.8 mg, 84% yield).

n-BuLi (1.25 M in hexanes, 1.15 mL, 1.44 mmol) was added to a stirred suspension of trimethyl sulfonium iodide (325.9 mg, 1.60 mmol) in dry THF (6.5 mL) at -70°C (dry ice-acetone) under argon. The mixture was then allowed to warm to -10°C and stirred for 30 min at that temperature before being cooled to -70°C again. A solution of **271** (413.8 mg, 1.06 mmol) in dry THF (4 mL) was added, and the mixture was stirred for 30 min at the bath temperature. The mixture was warmed to r.t. and stirred for 2 h (TLC, hexanes-EtOAc, 7:3). The solvent was removed by rotary evaporation using a r.t. water bath, and the residue was dissolved in ether (67 mL). The ethereal layer was washed with water (3 x 10 mL) and the solvent was removed. The residue was dissolved in CH_3CN (5.2 mL) and MeOH (8.7 mL), and cooled to 0°C. Concentrated HCl (0.81 mL) was added while stirring and the mixture was then stirred at r.t. for 41 h (TLC, hexanes-EtOAc, 7:3). The solvent was removed and the residue was dissolved in

ether (30 mL), washed with saturated $NaHCO_3$ (3 x 30 mL), brine (30 mL), and dried. Solvent removal gave the crude product which was then purified by chromatography (hexanes-EtOAc, 7:3) to give pure **107** (146.1 mg, 42.2% yield) as colorless crystals.

Physical data of 19-hydroxy-14-methoxy-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (**107**):

Colorless prisms; mp: 148-150°C (lit.: 152-154°C[80]); $[\alpha]_D^{20}$: +41° (c = 0.40, $CHCl_3$); UV λ_{max}^{MeOH} (log ε): 212.9 (3.84); IR ($CHCl_3$) cm^{-1}: 3050 (aromatic CH), 2950 (CH), 1740 (C=O), 1680 (C=C), 1600 and 1500 (aromatic C=C), 1280, 1020; ^1H NMR (400 MHz, $CDCl_3$) δ: 1.01 (3H, s, H20), 1.20, 1.22 (3H each, both d, J = 6.9 Hz, H16, H17), 1.69 (1H, ddd, J = 12.2, 12.2, 6.3 Hz, H1α), 1.80-2.00 (2H, m, H6), 2.37 (1H, m, H2β), 2.44-2.56 (2H, m, H1β, H2α), 2.67 (1H, br d, J = 13.1 Hz, H5), 2.93 (1H, ddd, J = 18.2, 10.3, 2.0 Hz, H7α), 3.05 (1H, ddd, J = 18.2, 7.5, 1.9 Hz, H7β), 3.28 (1H, sept, J = 6.9 Hz, H15), 3.72 (3H, s, -OCH_3), 4.77 (2H, br AB_q, Δv = 0.05 ppm, J = 17.3 Hz, H19), 7.10 (2H, s, H11, H12); ^{13}C NMR (50.2 MHz, $CDCl_3$) δ: 18.2, 19.8, 22.3, 22.8, 23.8, 24.0, 26.2, 32.7, 36.4, 41.1, 60.6, 70.5, 120.2, 124.1, 125.0, 128.0, 139.3, 144.1, 155.8, 163.0, 174.3; LRMS m/z (rel. intensity): 326 (M^+, 67.3), 311 (base peak), 295 (1.8), 283 (7.2), 269 (26.2), 251 (6.2), 163 (17.8), 141 (21.7); HRMS calcd. for $C_{21}H_{26}O_3$: 326.1882; found: 326.1884; Anal. calcd. for $C_{21}H_{26}O_3$: C, 77.28; H, 8.03; found: C, 77.03; H, 8.06.

To a stirred solution of **107** (10 mg) in HOAc (0.3 mL) was added HBr (48%, 0.6 mL) and the mixture was heated slowly to reflux for 1 h (TLC, hexanes-EtOAc, 7:3) under nitrogen. The mixture was diluted with EtOAc (30 mL), washed with saturated $NaHCO_3$ (3 x 30 mL), brine (2 x 30 mL), and dried. Removal of the solvent produced crude **106** as pale crystals (8.8 mg, 92% yield), which was pure according to TLC (hexanes-EtOAc, 7:3) (spectral data, *vide infra*).

Synthesis of Triptophenolide (106) from 193

19-Hydroxy-12,14-dinitro-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (272)

<u>Method A</u>

A solution of **193** (500 mg, 1.69 mmol) in HOAc (2.2 mL) was added dropwise to a stirred mixture of concentrated HNO₃ (3.25 mL) and H₂SO₄ (3 mL) at 0 to 5°C (ice-water) over 20 min. The resulting light orange mixture was stirred at the bath temperature for 1.5 h before being poured onto crushed ice (about 6 g) in water (20 mL). The product which precipitated as a pale yellow solid was separated by filtration and then dissolved in CHCl₃ (25 mL). The CHCl₃ solution was washed with water (3 x 10 mL), brine (3 x 10 mL), dried and evaporated. The crude product was column chromatographed with hexanes-EtOAc (7:3) to give 12,14-dinitro butenolide **272** as a pale yellow powder (361 mg, 55.4%), and 12-nitro-butenolide **273** as a pale yellow crystalline solid (84.6 mg, 14.7% yield).

Physical data of 19-hydroxy-12,14-dinitro-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (**272**):

A pale yellow powder; mp: 112-115°C; UV λ_{max}^{MeOH} (log ε): 214.3 (4.41); IR (CHCl₃) cm⁻¹: 3050 (aromatic CH), 2990 (CH), 1740 (C=O), 1680 (C=C), 1530 and 1370 (-NO₂), 1020; ¹H NMR (400 MHz, CDCl₃) δ: 1.05 (3H, s, H20), 1.277, 1.280 (3H each, both d, *J* = 7.1 Hz, H16, H17), 1.74 (1H, br ddd, *J* = 12.3, 12.3, 6.3 Hz, H1α), 1.84-2.05 (2H, m, H6), 2.38 (1H, m, H2β), 2.46 (1H, dd, *J* = 12.3, 6.6 Hz, H1β), 2.53 (1H, m, H2α), 2.69 (1H, br d, *J* = 12.8 Hz, H5), 2.85 (2H, m, H7), 2.99 (1H, sept, *J* =7.1 Hz, H15), 4.75 (2H, br ABq, Δν = 0.75 ppm, *J* = 17.3 Hz, H19), 7.62 (1H, s, H11); ¹³C NMR (50.2 MHz, CDCl₃) δ:

17.9, 18.7, 20.55, 20.57, 22.1, 23.0, 29.1, 32.3, 36.9, 40.0, 70.2, 121.3, 125.4, 129.4, 130.2, 146.7, 149.5, 152.4, 160.7, 173.4; LRMS m/z (rel. intensity): 386 (M$^+$, 15.8), 371 (16.3), 369 (base peak), 353 (6.5), 327 (21.3), 280 (25.8), 165 (49.1), 128 (62.3), 109 (78.8); HRMS calcd. for $C_{20}H_{22}N_2O_6$: 386.1478; found: 386.1471.

Physical data of 19-hydroxy-12-nitro-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (**273**):

A pale yellow crystalline solid; mp: 176-178°C (dec); UV λ_{max}^{MeOH} (log ε): 214.9 (4.34), 270.2 (3.67); IR (CHCl$_3$) cm^{-1}: 3050 (aromatic CH), 2990 (CH), 1760 (C=O), 1680, 1520 and 1370 (-NO$_2$), 1230, 1080, 1040; ^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.01 (3H, s, H20), 1.22, 1.23 (3H, each, both d, J = 6.8 Hz, H16, H17), 1.71 (1H, ddd, J = 12.3, 12.3, 6.5 Hz, H1α), 1.95 (2H, m, H6), 2.36 (1H, m, H2β), 2.48 (2H, m, H1β, H2α), 2.66 (1H, br d, J = 12.0 Hz, H5), 3.05 (2H, m, H7), 3.39 (1H, sept, J = 6.8 Hz, H15), 4.75 (2H, br AB$_q$, Δν = 0.06 ppm, J = 17.2 Hz, H19), 7.16 (1H, s, H14), 7.69 (1H, s, H11); ^{13}C NMR (50.2 MHz, CDCl$_3$) δ: 18.0, 19.7, 22.0, 23.5, 23.6, 28.1 (2C), 32.4, 36.4, 40.9, 70.3, 120.1, 125.2, 128.6, 140.4, 140.5, 144.0, 147.6, 161.9, 173.8; LRMS m/z (rel. intensity): 341 (M$^+$, 4.2), 324 (99.1), 309 (2.7), 296 (10.0), 280 (3.8), 160 (36.0); HRMS calcd. for $C_{20}H_{23}NO_4$: 341.1627; found: 341.1620.

<u>Method B</u>

In a dry flask concentrated HNO$_3$ (7.2 mL) was mixed with concentrated H$_2$SO$_4$ (10.2 mL) at 0°C. A solution of **193** (2g, 6.76 mmol) in dry CH$_3$NO$_2$ (MgSO$_4$) (10 mL) was added dropwise over 17 min while stirring at 0-5°C (ice-water). The resulting light orange mixture was stirred at 0-5°C for 1.5 h, and then poured onto crushed ice (60 mL) in a beaker while swirling. The flask was washed with EtOAc (2 x 10 mL) and the washings were also added. After all the ice melted, the suspension was extracted with EtOAc (3 x 80 mL), and then combined EtOAc was washed with saturated NaHCO$_3$ (2 x 80 mL), brine (80 mL) and dried. Removal of the

solvent gave a crude product which, after chromatography with hexanes-EtOAc (7:3), yielded pure **272** as a pale yellow powder (2.248 g, 86.2% yield).

12-Amino-19-hydroxy-14-nitro-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (274)

To a solution of **272** (1.9759 g, 5.12 mmol) in glacial HOAc (25.6 mL) was added 10% Pt/C (89.6 mg), and the mixture was stirred under H_2 (1 atm) for 20.5 h at r.t. (TLC, hexanes-EtOAc, 6:4). EtOAc (20 mL) was added to dissolve the solid product, while the catalyst was removed by filtration through Celite. The Celite was washed thoroughly with EtOAc (100 mL), and the combined filtrate was concentrated. The crude product was recrystallized in EtOAc to give **274** as yellow crystals (0.9943 g), while the mother liquor was column chromatographed (hexanes-EtOAc, 6:4) to yield more **274** (0.5218 g), bringing the total amount to 1.5161 g (83.2% yield).

Physical data of 12-amino-19-hydroxy-14-nitro-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (**274**):

Yellow needles; mp: 227-229°C (dec); UV λ_{max}^{MeOH} (log ε): 210.2 (4.61), 295.8 (3.69); IR (CHCl$_3$) cm^{-1}: 3500 (NH), 3400 (NH), 2975 (CH), 1750 (C=O), 1670 (C=C), 1620 (NH$_2$), 1520 (-NO$_2$), 1380, 1350 (-NO$_2$), 1030; ^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.01 (3H, s, H20), 1.32, 1.33 (3H each, both d, J = 7.2 Hz, H16, H17), 1.67 (1H, m, H1α), 1.80-1.95 (2H, m, H6), 2.28-2.57 (3H, m, H1β, H2), 2.60-2.80 (3H, m, H5, H7), 2.85 (1H, sept, J = 7.2 Hz, H15), 3.84 (2H, br s, -NH$_2$), 4.75 (2H, br AB$_q$, Δν = 0.075 ppm, J = 12.7 Hz, H19), 6.71 (1H, s, H11); ^{13}C NMR (50.2 MHz, CDCl$_3$) δ: 18.1, 19.3, 19.8 (2C), 22.1, 22.3, 28.2, 32.6, 36.4, 40.6, 70.3, 113.9, 114.5, 120.2, 125.1, 143.5, 145.1, 152.7, 162.1, 173.8; LRMS m/z (rel. intensity): 356 (M$^+$, 59.5), (339 (32.3), 323 (13.5), 311 (13.3), 309 (12.2),

294 (8.1); HRMS calcd. for $C_{20}H_{24}N_2O_4$: 356.1736; found: 356.1740; Anal. calcd. for $C_{20}H_{24}N_2O_4$: C, 67.40; H, 6.79; N, 7.86; found: C, 67.19; H, 6.82; N, 7.78.

Synthesis of 106 from 274 using Zn-HOAc in the reduction step

<u>Method A</u>

In a mixture of TFA (3.8 mL) and HOAc (3.8 mL) was dissolved **274** (756.9 mg, 2.13 mmol), followed by addition of water (3.8 mL). The solution was cooled to 0°C (ice-water-traces acetone), and $NaNO_2$ (192 mg, 2.78 mmol) was added in portions over 10 min. The reaction mixture was stirred at 0°C for 30 min and then an aqueous solution of KI (499 mg, 3.01 mmol in 0.77 mL water) was added over 10 min. The resulting dark brown suspension was stirred further for 15 min until gas evolution ceased. A solution of Na_2SO_3 (154 mg, 1.22 mmol) in water (7.7 mL) was added. The bath was removed and stirring was continued for 30 min. The product was isolated by filtration and washed thoroughly with water. Crude **275** thus obtained was dissolved in HOAc (11.6 mL) and cooled in an ice-water bath. Zn powder (921 mg) was added and the mixture was stirred for 15 min at the bath temperature, and then for 14 h at 30-40°C under nitrogen. TLC (hexanes-EtOAc, 6:4) showed that the reaction was not completed, thus the temperature was raised to 65-75°C and the mixture was stirred for another 2 h. The mixture was cooled in a cold water bath for 2-3 h and then filtered through Celite. The Celite was washed with EtOAc, and all the filtrates were combined and concentrated to give a gum-like crude product (1.3536 g, contained some zinc salts). A portion of the crude product (1.1569 g) was dissolved in TFA (5.2 mL), and the mixture was cooled to 0°C (ice-water-traces acetone). $NaNO_2$ (393.6 mg) was added in portions over 10 min, and the reaction mixture was stirred for another 30 min before addition of a Na_2SO_3 (196.8 mg) solution in water (1.3 mL). The bath was removed and the mixture was stirred for 30 min. Water (6.6 mL) was added and the mixture was stirred at r.t. overnight. The EtOAc extract of the reaction mixture (50 mL) was washed with water (50 mL), 10% Na_2SO_3 (50 mL), saturated $NaHCO_3$ (2 x 50 mL), brine (50

mL), dried and evaporated. The residue was dissolved in MeOH (19.9 mL), and concentrated HCl (1.7 mL) was added slowly with stirring. The mixture was stirred for 7 h at r.t. (TLC, hexanes-EtOAc, 6:4), then the solvent was removed. The residue was dissolved in EtOAc (90 mL), washed with saturated NaHCO$_3$ (2 x 50 mL), brine (50 mL) and dried. Removal of the solvent gave the crude product, which was column chromatographed with hexanes-EtOAc (8:2) to yield **278** (69.1 mg), triptophenolide (**106**) (128.6 mg, 22.7% yield from **274**) and **279** (84.8 mg).

Physical data of 19-hydroxy-11,14-dioxo-18(4→3)*abeo*-abieta-3,8,12-trien-18-oic acid lactone (**278**):

A yellow solid; mp: 58-60°C; $[\alpha]_D^{25}$: +130.7° (c = 0.225, CHCl$_3$); UV λ_{max}^{MeOH} (log ε): 219.2 (4.14), 258.8 (4.15); IR (neat) cm^{-1}: 2950 (CH), 1750 (C=O), 1680 (quinone C=O), 1640 (quinone C=C), 1590, 1480; ^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.09, 1.10 (3H each, both d, J = 6.9 Hz, H16, H17), 1.14 (3H, s, H20), 1.46 (1H, ddd, J = 13.3, 10.8, 7.6 Hz, H1α), 1.68 (1H, dddd, J = 13.3, 13.3, 11.2, 6.2 Hz, H6β), 1.86 (1H, br ddd, J = 13.3, 7.4, 2.6 Hz, H6α), 2.40 (2H, m, H2), 2.49 (1H, ddd, J = 20.5, 11.2, 7.4 Hz, H7α), 2.59 (1H, br d, J = 13.3 Hz, H5), 2.77 (1H, ddd, J = 20.5, 6.2, 0.8 Hz, H7β), 2.99 (1H, sept d, J = 6.9, 1.1 Hz, H15), 3.09 (1H, ddd, J = 13.3, 5.6, 2.2 Hz, H1β), 4.73 (2H, br AB$_q$, Δν = 0.1 ppm, J = 17.2 Hz, H19), 6.39 (1H, d, J = 1.1 Hz, H12); ^{13}C NMR (50.2 MHz, CDCl$_3$) δ: 18.3, 18.4, 18.7, 21.34, 21.30, 24.3, 26.5, 30.8, 36.8, 42.5, 70.2, 125.6, 131.7, 142.6, 147.7, 153.5, 161.2, 173.7, 187.2, 187.5; LRMS m/z (rel. intensity): 326 (M$^+$, 75.2), 311 (28.4), 298 (7.4), 283 (13.0), 267 (13.3), 203 (17.5); HRMS calcd. for C$_{20}$H$_{22}$O$_4$: 326.1518; found: 326.1521.

compound **279**: Spectral data are given under its synthesis

<u>Method B</u>

Compound **274** (500 mg) was treated as described in Method A except for the Zn reduction step, in which the temperature was raised to 65-70°C after initial cooling and kept stirring at that temperature for 15 h under nitrogen. The crude product was chromatographed (hexanes-EtOAc, 6:4) to give **281** (22.8 mg, 8.0% yield) and a mixture of **276** and **280** (258.9 mg) in a ratio of about 3.5 to 1 as determined by ^{1}H NMR. A portion of the mixture (208 mg) was dissolved in TFA (2.7 mL) to which $NaNO_2$ (138 mg) was added portionwise over 25 min while stirring at 0°C (ice-water). A solution of Na_2SO_3 (163.8 mg) in water (1.1 mL) was added 5 min later and the bath was removed. The mixture was stirred for 30 min followed by the addition of water (3.5 mL). Stirring then continued overnight. The mixture was diluted with water (40 mL) and extracted with EtOAc (3 x 40 mL). The combined EtOAc was washed with saturated $NaHCO_3$ (2 x 40 mL), brine (40 mL), dried and concentrated. The residue was dissolved in MeOH (8 mL) and treated with concentrated HCl (0.7 mL). The mixture was stirred at r.t. for 6 h. The solvent was removed and the residue was extracted with EtOAc (2 x 50 mL). The combined EtOAc was washed with saturated $NaHCO_3$ (2 x 50 mL), brine (50 mL), dried and evaporated. The crude mixture was column chromatographed with hexanes-EtOAc (8:2) to give the quinone **278** (18.2 mg), triptophenolide (**106**) (38 mg) and **279** (49.3 mg).

Direct deamination of 274 with isoamyl nitrile

A solution of **274** (75.5 mg) in DMF (1.5 mL) was added dropwise over 15 min to a stirred solution of isoamyl nitrile (0.1 mL) in DMF (0.5 mL) at 65°C. The solution was stirred for 10 min and then cooled to r.t.. The mixture was diluted with water (20 mL) and extracted with EtOAc (3 x 20 mL). The combined EtOAc was washed with brine (30 mL), dried and concentrated. The crude product was column chromatographed (hexanes-EtOAc, 7:3) to afford **282** (23.6 mg, 32.6% yield) and **283** (26.6 mg).

Physical data of 19-hydroxy-14-nitro-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (**282**):

Pale yellow prisms; mp: 231-233°C (dec); UV λ_{max}^{MeOH} (log ε): 215.0 (4.40), 266.0 (3.52); IR (CHCl$_3$) cm^{-1}: 2975 (CH), 1745 (C=O), 1530 (-NO$_2$), 1380 (-NO$_2$); ^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.03 (3H, s, H20), 1.21, 1.24 (3H each, both d, J = 6.8 Hz, H16, H17), 1.70 (1H, ddd, J = 12.6, 12.6, 6.7 Hz, H1α), 1.91 (2H, m, H6), 2.40 (1H, m, H2β), 2.52 (2H, m, H1β, H2α), 2.70 (1H, br d, J = 12.9 Hz, H5), 2.80 (1H, sept, J = 6.8 Hz, H15), 2.86 (2H, m, H7), 4.76 (2H, br AB$_q$, Δν = 0.07 ppm, J = 17.2 Hz, H19), 7.24 (1H, d, J = 8.3 Hz, H12), 7.43 (1H, d, J = 8.3 Hz, H11); ^{13}C NMR (50.2 MHz, CDCl$_3$) δ: 18.1, 19.1, 22.2, 22.9, 23.6, 23.7, 28.8, 32.6, 36.6, 40.6, 70.3, 124.4, 124.8, 125.3, 126.1, 137.3, 144.5, 151.5, 161.7, 173.8; LRMS m/z (rel. intensity): 341 (M$^+$, 7.6), 326 (27.2), 324 (base peak), 308 (16.3), 296 (9.7), 280 (8.5), 266 (8.2); HRMS calcd. for C$_{20}$H$_{23}$NO$_4$: 341.1627; found: 341.1622.

Physical data of compound **283**:

A yellowish solid; IR (CHCl$_3$) cm^{-1}: 3700-3600 (OH), 2975 (CH), 1750 (C=O), 1560, 1530 and 1370 (-NO$_2$), 1130, 1030; ^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.03 (3H, s, H20), 1.25, 1.26 (3H each, both d, J = 6.8 Hz, H16, H17), 1.82 (1H, m, H1α), 2.35-2.64 (5H, m, H1β, H2, H6), 2.84 (1H, sept, J = 6.8 Hz, H15), 3.15 (1H, br d, J = 12.2 Hz, H5), 4.74 (2H, br AB$_q$, Δν = 0.11 ppm, J = 17.3 Hz, H19), 5.73 (1H, dd, J = 5.5, 1.8 Hz, H7β), 7.57 (1H, d, J = 8.5, H12), 7.63 (1H, d, J = 8.5 Hz, H11); ^{13}C NMR (50.2 MHz, CDCl$_3$) δ: 18.2, 22.8, 23.5, 23.6, 26.3, 29.1, 32.2, 36.4, 37.6, 69.9, 80.2, 117.3, 126.3, 127.8, 129.8, 139.3, 145.5, (150.0), 159.4, 173.0; LRMS m/z (rel. intensity): 357 (M$^+$, 0.5), 355 (1.6), 340 (base peak), 324 (33.9), 323 (27.5), 308 (19.8), 306 (26.6); HRMS calcd. for C$_{20}$H$_{23}$NO$_5$: 357.1576; found: 357.1600.

19-Hydroxy-12-iodo-14-nitro-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (275)

In a mixture of TFA (3 mL) and HOAc (3 mL) was dissolved **274** (500 mg, 1.40 mmol), followed by addition of water (3 mL), and the solution was cooled to 0°C (ice-water-traces acetone). $NaNO_2$ (139.4 mg, 2.02 mmol) was added portionwise over 15 min and the resulting brownish mixture was stirred at 0°C for 50 min. A solution of KI (438.2 mg, 2.64 mmol) in water (1 mL) was added dropwise over 10 min (dark solids precipitated out), and the suspension was stirred for another 15 min until gas evolution ceased. A solution of Na_2SO_3 (112 mg, 0.89 mmol) in water (5.1 mL) was added dropwise (the dark color faded), and the bath was removed. Stirring was continued for 30 min and then water was added (100 mL). The suspension was extracted with EtOAc (3 x 70 mL), and the combined EtOAc was washed with 5% Na_2SO_3 (50 mL), saturated $NaHCO_3$ (2 x 50 mL), brine (50 mL), dried and concentrated. Column chromatography of the crude product with petroleum ether-EtOAc (8:2) produced **275** (426.1 mg, 65.0% yield) as colorless prisms.

Physical data of 19-hydroxy-12-iodo-14-nitro-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (**275**):

Colorless prisms; mp: 183-185°C; UV λ_{max}^{MeOH} (log ε): 211.5 (4.56); IR ($CHCl_3$) cm^{-1}: 2980 (CH), 1760 (C=O), 1720, 1540 and 1370 (-NO_2); ^1H NMR (400 MHz, $CDCl_3$) δ: 1.03 (3H, s, H20), 1.27 (6H, br s, H16, H17), 1.70 (1H, ddd, J = 12.0, 12.0, 6.5 Hz, H1α), 1.80-1.99 (2H, m, H6), 2.31-2.59 (3H, m, H1β, H2), 2.64 (1H, br d, J = 13.2 Hz, H5), 2.75 (2H, m, H7), 3.30 (1H, br s, H15), 4.75 (2H, br AB_q, Δν = 0.08 ppm, J = 17.2 Hz, H19), 7.95 (1H, s, H11); LRMS m/z (rel. intensity): 467 (M^+, 55.4), 452 (25.5), 451 (27.8), 450 (66.4),

437 (17.0), 436 (18.9), 435 (29.5), 434 (57.5), 422 (23.5), 324 (70.3), 308 (89.8), 128

(54.7), 109 (54.8); HRMS calcd. for $C_{20}H_{22}INO_4$: 467.0595; found: 467.0592.

Reaction of 275 with NaBH$_4$-CuCl

To a stirred solution of **275** (150 mg, 0.321 mmol) in MeOH (12.5 mL) was added CuCl

(190 mg, 1.92 mmol), followed by NaBH$_4$ (170.8 mg, 4.52 mmol) in portions. The solution

turned brown then black and gases evolved. The mixture was stirred at r.t. for 30 min during

which time two additional portions of NaBH$_4$ (170.8 mg each) were added (TLC, hexanes-

EtOAc, 6:4, or CH_2Cl_2-EtOAc, 90:5). The reaction mixture was filtered, and the filtrate

concentrated. The residue was treated with water (15 mL), and then 10% HCl was added

carefully to adjust the pH between 8-9. The aqueous mixture was extracted with EtOAc (3 x 20

mL), and the combined EtOAc was washed with brine (30 mL), dried and evaporated. The crude

product was purified by column chromatography (hexanes-EtOAc, 6:4) to give **276** as a pale

pink crystalline solid (59.7 mg, 59.8% yield).

Physical data of 14-amino-19-hydroxy-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid

lactone (**276**):

A pale pink crystalline solid; mp: 186-188°C; UV λ_{max}^{MeOH} (log ε): 209.4 (4.65), 286.0

(3.65); IR (CHCl$_3$) cm^{-1}: 3550 (NH), 3050 (aromatic CH), 2980 (CH), 1745 (C=O), 1680

(C=C), 1620 (-NH$_2$), 1350; ^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.01 (3H, s, H20), 1.25, 1.27

(3H each, both d, J = 6.8 Hz, H16, H17), 1.68 (1H, ddd, J = 12.2, 12.2, 6.5 Hz, H1α), 1.87-

2.07 (2H, m, H6), 2.27-2.43 (1H, m, H2β), 2.43-2.57 (2H, m, H1β, H2α), 2.57-2.76 (3H,

m, H5, H7), 2.88 (1H, sept, J = 6.8 Hz, H15), 3.67 (2H, br s, -NH$_2$, D$_2$O exchangeable),

4.77 (2H, AB$_q$, Δν = 0.07 ppm, J = 17.1 Hz, H19), 6.84 (1H, d, J = 8.2 Hz, H11), 7.04 (1H,

d, J = 8.2 Hz, H12); ^{13}C NMR (50.2 MHz, CDCl$_3$) δ: 18.2, 19.9, 22.2, 22.3, 22.4, 24.0,

27.7, 32.6, 36.2, 40.7, 70.5, 114.2, 118.6, 122.8, 125.1, 129.8, 141.2, 143.2, 162.9, 174.2; LRMS m/z (rel. intensity): 311 (M^+, 55.0), 296 (57.2), 268 (1.5), 254 (9.6), 236 (1.9), 148 (15.1); HRMS calcd. for $C_{20}H_{25}NO_2$: 311.1885; found: 311.1888.

Deiodination of 275 by catalytic hydrogenation

To a solution of **275** (88.6 mg, 0.19 mmol) in MeOH (13.2 mL) were added 10% Pd/C (9 mg) and K_2CO_3 (13 mg, 0.094 mmol). The mixture was stirred under H_2 (1 atm) at r.t. for 3 h. TLC (hexanes-EtOAc, 6:4) showed little reaction. Additional quantities of 10% Pd/C (9 mg) and K_2CO_3 (13 mg) were added and the mixture was stirred for another 7.5 h. The mixture was filtered through Celite, which was washed with EtOAc. The filtrates were combined and concentrated. The resulting residue was suspended in water (40 mL) and extracted with EtOAc (3 x 40 mL). The combined EtOAc was washed with brine (50 mL), dried and evaporated. Column chromatography of the crude product gave **282** as pale yellow crystals (31.0 mg, 47.9% yield).

Deiodination and reduction of 275 by catalytic hydrogenation

To a solution of **275** (58.5 mg, 0.127 mmol) in MeOH (6 mL) were added 10% Pd/C (6 mg) and K_2CO_3 (8.8 mg, 0.064 mmol). The mixture was stirred under H_2 (1 atm) at r.t. overnight. TLC (hexanes-EtOAc, 6:4) showed a slightly less polar spot and some polar spots relative to the starting material. HOAc (1 mL) was added and the reaction continued under H_2 overnight. The mixture was filtered through Celite and the filtrate was concentrated. The residue was suspended in water (20 mL), extracted with EtOAc (3 x 20 mL), and the combined extract washed with saturated $NaHCO_3$ (3 x 20 mL), brine (20 mL) and dried. The crude product was chromatographed with hexanes-EtOAc (6:4) to give **282** (9.6 mg, 22.5% yield) and **276** (17.1 mg, 43.9% yield).

14-Amino-19-hydroxy-12-iodo-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (280)

A suspension of **275** (539.8 mg, 1.16 mmol) in EtOH (14.1 mL) was heated to reflux (starting material began to dissolve), followed by dropwise addition of a solution of sodium dithionite ($Na_2S_2O_4$, 78%, 2.0739 g, 9.29 mmol) in water (5.4 mL). The reaction mixture was stirred for 30 min under reflux (TLC, hexanes-EtOAc, 6:4), and then left to cool to r.t.. The solvent was removed and the residue was mixed with EtOAc (100 mL) and brine (100 mL) (small amounts of water could be added to dissolve remaining inorganic salts if necessary). The EtOAc layer was separated, the aqueous layer was extracted with EtOAc (3 x 60 mL), and the combined EtOAc extract was dried and concentrated. The crude product was purified by column chromatography with CH_2Cl_2-EtOAc (90:5) to give **280** as a colorless crystalline solid (439.3 mg, 87.0% yield).

Physical data of 14-amino-19-hydroxy-12-iodo-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (**280**):

A colorless crystalline solid; mp: 215-217°C (dec); $[\alpha]_D^{20}$: +20.6° (c = 0.50, $CHCl_3$); UV λ_{max}^{MeOH} (log ε): 220.2 (4.57), 292.8 (3.53); IR ($CHCl_3$) cm^{-1}: 3525 (NH), 3425 (NH), 3020 (aromatic CH), 2975 (CH), 1743 (C=O), 1680 (C=C), 1620 (-NH$_2$), 1540, 1400 (C-N), 1060, 1040; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.99 (3H, s, H20), 1.34, 1.36 (3H each, both d, J = 7.7 Hz, H16, H17), 1.64 (1H, ddd, J = 12.2, 12.2, 6.2 Hz, H1α), 1.82-2.04 (2H, m, H6), 2.25-2.67 (6H, m, H1β, H2, H5, H7), 3.62 (1H, br sept, J = 7.7 Hz, H15), 4.75 (2H, br AB$_q$, Δν = 0.075 ppm, J = 17.0 Hz, H19), 7.32 (1H, br s, H11); LRMS m/z (rel. intensity): 437 (M$^+$, 35.2), 422 (21.8), 311 (6.4), 296 (8.6); HRMS calcd. for $C_{20}H_{24}INO_2$: 437.0854; found: 437.0853.

14,19-Dihydroxy-12-iodo-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (279)

To a stirred solution of **280** (340 mg, 0.78 mmol) in TFA (5.4 mL) at -10 to -12°C (ice-acetone-water), NaNO$_2$ (107.4 mg, 1.56 mmol) was added in portions over 10 min, resulting in a brown mixture with gas evolution. The mixture was stirred at the bath temperature for 35 min and a solution of Na$_2$SO$_3$ (49 mg, 0.39 mmol) in water (0.66 mL) was added. The bath was removed and the mixture was stirred for 30 min. Water (2.7 mL) was added and stirring was continued overnight. The reaction mixture was diluted with water (100 mL) and extracted with EtOAc (3 x 70 mL). The combined EtOAc was washed with saturated NaHCO$_3$ solution (50 mL), brine (50 mL) and dried. Removal of solvent gave a crude intermediate, which was then dissolved in MeOH (7.2 mL) followed by addition of concentrated HCl (1.1 mL) while stirring. The mixture was stirred overnight and some product precipitated. The suspension was diluted with water (100 mL) and extracted with EtOAc (3 x 70 mL). The combined EtOAc was washed with brine (50 mL), dried and evaporated. Column chromatography of the crude product with hexanes-EtOAc (6:4, 4:6) yielded **279** (262.4 mg, 77.0% yield) as a colorless crystalline solid, **285** (5.3 mg, 1.5%) as orange crystals and a fraction (46.0 mg) containing some polar compounds. A combined polar fraction (172.2 mg) was chromatographed with CH$_2$Cl$_2$-EtOAc (90:5) to give **286** (71.4 mg) and **287** (74.9 mg).

Physical data of 14,19-dihydroxy-12-iodo-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (**279**):

A white crystalline solid; mp: 216-218°C (dec); UV λ_{max}^{MeOH} (log ε): 218.3, (4.45), 277.8 (3.45); IR (CHCl$_3$) cm^{-1}: 3625 (OH), 3040 (aromatic CH), 2950 (CH), 1760 (C=O), 1680 (C=C), 1600, 1550, 1410, 1360, 1240, 1040; ^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.00 (3H,

s, H20), 1.34, 1.35 (3H each, both d, J = 7.3 Hz, H16, H17), 1.66 (1H, ddd, J = 12.1, 12.1, 6.5 Hz, H1α), 1.80-2.05 (2H, m, H6), 2.25-2.57 (3H, m, H1β, H2), 2.57-2.84 (3H, m, H5, H7), 3.42 (1H, sept, J = 7.3 Hz, H15), 4.76 (2H, br AB$_q$, Δν = 0.07 ppm, J = 17.2 Hz, H19), 4.84 (1H, s, C14-OH, D$_2$O exchangeable), 7.42 (1H, s, H11); ^{13}C NMR (125.8 MHz, CDCl$_3$) δ: 18.1, 19.4, 20.2, 20.3, 22.0, 22.2, 32.5, 36.0, 39.2, 40.6, 70.4, 121.9, 125.3, 128.0 (2C), 132.7, 145.8, 152.0, 162.1, 173.9; LRMS m/z (rel. intensity): 438 (M$^+$, base peak), 423 (94.8), 312 (12.5), 297 (30.0), 296 (30.2), 275 (34.6), 197 (28.7), 128 (21.6); HRMS calcd. for C$_{20}$H$_{23}$IO$_3$: 438.0694; found: 438.0694.

Physical data of 19-hydroxy-12-iodo-11,14-dioxo-18(4→3)*abeo*-abieta-3,8,12-trien-18-oic acid lactone (**285**):

Orange prisms; mp: 186.5-187.5°C (dec); UV λ$_{max}^{MeOH}$ (log ε): 205.0 (4.39), 288.8 (3.88); IR (KBr) cm^{-1}: 2950 (CH), 1750 (C=O), 1680 (quinone C=O), 1650 (quinone C=C), 1570; ^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.15 (3H, s, H20), 1.27, 1.29 (3H each, both d, J = 7.0 Hz, H16, H17), 1.45 (1H, ddd, J = 13.2, 10.9, 7.3 Hz, H1α), 1.68 (1H, dddd, J = 13.3, 13.3, 11.2, 6.3 Hz, H6β), 1.88 (1H, ddd, J = 13.3, 7.5, 2.6 Hz, H6α), 2.40 (2H, m, H2), 2.47 (1H, ddd, J = 20.6, 11.2, 7.5 Hz, H7α), 2.59 (1H, br d, J = 13.3 Hz, H5), 2.80 (1H, br dd, J = 20.6, 6.3 Hz, H7β), 2.98 (1H, ddd, J = 13.2, 5.9, 1.8 Hz, H1β), 3.23 (1H, sept, J = 7.0 Hz, H15), 4.72 (2H, br AB$_q$, Δν = 0.11 ppm, J = 17.2 Hz, H19); ^{13}C NMR (50.2 MHz, CDCl$_3$) δ: 18.2, 18.4, 18.7, 20.0, 20.1, 24.1, 30.9, 37.5, 40.9, 42.5, 70.2, 124.1, 125.8, 143.0, 147.2, 158.2, 161.0, 173.6, 180.0, 182.5; LRMS m/z (rel. intensity): 452 (M$^+$, base peak), 437 (13.1), 424 (12.7), 325 (46.4), 310 (14.4), 297 (12.9), 128 (29.6); HRMS calcd. for C$_{20}$H$_{21}$IO$_4$: 452.0486; found: 452.0487; Anal. calcd. for C$_{20}$H$_{21}$IO$_4$: C, 53.11; H, 4.68; I, 28.06; found: C, 53.00; H, 4.71; I, 27.89.

Physical data of 14,19-dihydroxy-12-iodo-11-nitro-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (**286**):

Pale yellow prisms; mp: 246-248°C (dec); UV λ_{max}^{MeOH} (log ε): 217.8 (4.51), 272.1 (3.38); IR (KBr) cm⁻¹: 3400 (OH), 2950 (CH), 1700 (C=O), 1670 (C=C), 1560, 1520 (-NO₂), 1360 (-NO₂), 1180 (aromatic C-O), 1080; ¹H NMR (400 MHz, CDCl₃) δ: 1.17 (3H, s, H20), 1.36, 1.37 (3H each, both d, *J* = 7.2 Hz, H16, H17), 1.66 (1H, m, H1α), 1.84-2.01 (2H, m, H6), 2.18-2.34 (2H, m, H1β, H2β), 2.42 (1H, m, H2α), 2.70 (1H, br d, *J* = 13.3 Hz, H5), 2.80 (2H, m, H7), 3.62 (1H, sept, *J* = 7.2 Hz, H15), 4.74 (2H, m, H19), 5.18 (1H, s, C14-OH, D₂O exchangeable); LRMS m/z (rel. intensity): 483 (M⁺, base peak), 468 (8.0), 466 (22.7), 438 (16.7), 423 (15.2), 356 (10.7), 347 (25.6), 128 (17.7); HRMS calcd. for C₂₀H₂₂INO₅: 483.0545; found: 483.0543; Anal. calcd. for C₂₀H₂₂INO₅: C, 49.70; H, 4.59; N, 2.90; I, 26.26; found: C, 49.80; H, 4.54; N, 2.82; I, 26.40.

Physical data of compound **287**:

A white powder; UV λ_{max}^{MeOH} (log ε): 217.9 (4.41), 274.9 (3.15); IR (KBr) cm⁻¹: 3400 (OH), 2900 (CH), 1720 (C=O), 1660 (C=C), 1340, 1240, 1010; ¹H NMR (400 MHz, CDCl₃) δ: 1.00 (3H, s, H20), 1.28 (3H, d, *J* = 6.2 Hz, H3'), 1.68 (1H, ddd, *J* = 12.3, 12.3, 6.3 Hz, H1α), 1.90 (2H, m, H6), 2.29-2.56 (3H, m, H1β, H2), 2.65 (1H, br d, *J* = 10.8 Hz, H5), 2.73, 2.85 (1H each, both m, H1'), 2.94 (2H, m, H7), 4.09 (1H, m, H2'), 4.75 (2H, br AB_q, Δν = 0.07 ppm, *J* = 17.2 Hz, H19), 6.99 (1H, s, H14), 7.75 (1H, s, H11); ¹³C NMR (50.2 MHz, CDCl₃) δ: 18.1, 19.9, 22.2, 23.1, 27.7, 32.5, 36.2, 41.1, 49.3, 67.6, 70.4, 98.1, 125.1, 131.8, 135.0, 135.5, 139.2, 145.8, 162.3, 173.9; LRMS m/z (rel. intensity): 438 (M⁺, 33.0), 394 (base peak), 379 (94.4), 307 (16.1), 267 (48.8), 252 (36.7), 149 (53.7), 128 (31.0), 127 (11.2), 45 (58.9); HRMS calcd. for C₂₀H₂₃IO₃: 438.0694; found: 438.0693; Anal. calcd. for C₂₀H₂₃IO₃: C, 54.81; H, 5.29; I, 28.95; found: C, 54.97; H, 5.41; I, 28.80.

14,19-Dihydroxy-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (106) (triptophenolide)

To a solution of **279** (1.1743 g, 2.68 mmol) in MeOH (117 mL), 10% Pd/C (117 mg) and K_2CO_3 (185 mg, 1.34 mmol) were added. The mixture was stirred under H_2 (1 atm) at r.t. for 8-9 h (TLC, hexanes-EtOAc, 6:4). After the reaction was completed, the mixture was filtered through Celite and the Celite was washed with EtOAc. The filtrates were combined and concentrated. The resulting residue was suspended in 5% NaH_2PO_4 (100 mL) and extracted with EtOAc (3 x 100 mL). Combined EtOAc was washed with brine (100 mL) and dried. Removal of solvent gave a crude product, which was purified by column chromatography (petroleum ether-EtOAc, 6:4) to afford **106** as colorless prisms (807.5 mg, 96.5% yield; 30.1% overall yield from **193**).

Physical data of 14,19-Dihydroxy-18(4→3)abeo-abieta-3,8,11,13-tetraen-18-oic acid lactone (**106**) (triptophenolide):

Colorless prisms; mp: 228-230°C (dec) (lit.: 232-233°C[80]); $[\alpha]_D^{20}$: +37.6° (c = 0.25, MeOH) (lit.: +36.2° $(CHCl_3)$[89]); UV λ_{max}^{MeOH} (log ε): 220.2 (4.08), 279.2 (3.23); IR $(CHCl_3)$ cm^{-1}: 3620 (OH), 3025 (aromatic CH), 2975 (CH), 1750 (C=O), 1680 (C=C), 1575 and 1500 (aromatic C=C), 1350, 1080; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.01 (3H, s, H20), 1.24, 1.26 (3H each, both d, J = 7.0 Hz, H16, H17), 1.68 (1H, ddd, J = 12.3, 12.3, 6.5 Hz, H1α), 1.90 (1H, m, H6β), 1.99 (1H, m, H6α), 2.39 (1H, m, H2β), 2.44-2.57 (2H, m, H1β, H2α), 2.69 (1H, br d, J = 13.4 Hz, H5), 2.73-2.95 (2H, m, H7), 3.08 (1H, sept, J = 7.0 Hz, H15), 4.71 (1H, br s, C14-OH, D_2O exchangeable), 4.77 (2H, br AB_q, Δv = 0.07 ppm, J = 17.2 Hz, H19), 6.93 (1H, d, J = 8.2 Hz, H11), 7.05 (1H, d, J = 8.2 Hz, H12); ^{13}C NMR (50.2 MHz, $CDCl_3$) δ: 18.2, 19.6, 22.4, 22.5, 22.7, 26.9, 29.7, 32.6, 36.2, 40.9, 70.5, 116.3, 120.5,

123.4, 125.1, 130.9, 143.9, 150.8, 162.8, 174.0; LRMS m/z (rel. intensity): 312 (M$^+$, 43.3), 297 (base peak), 269 (2.9), 255 (24.0), 237 (10.7), 169 (12.3), 165 (14.9), 149 (31.1), 129 (19.7); HRMS calcd. for $C_{20}H_{24}O_3$: 312.1726; found: 312.1726; Anal. calcd. for $C_{20}H_{24}O_3$: C, 76.89; H, 7.74; found: C, 76.94; H, 7.60.

6.3.7 Synthesis of 12,14,19-Trihydroxy-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic Acid Lactone (DINTP, 288)

To a stirred solution of **194** (500 mg, 1.60 mmol) in HOAc (35 mL) was added lead tetraacetate (LTA) (1.42 g, 2 equiv.) in one portion. The mixture was stirred at r.t. for 10 min (TLC, hexanes-EtOAc, 1:1), ethylene glycol (10 drops) was added, and the mixture stirred for another 5 min before being poured into water (200 mL). The water suspension was extracted with EtOAc (2 x 200 mL), and the combined EtOAc extract was washed with saturated NaHCO$_3$ (5 x 150 mL), brine (2 x 150 mL), dried and concentrated to give the acetoxy dienone as a yellowish solid. The crude intermediates were dissolved in trifluoroacetic anhydride (8 mL) and the mixture was stirred under nitrogen at r.t. for 48 h followed by evaporation of the solvent. Crude **294** thus obtained was dissolved in MeOH (38 mL), followed by addition of water (4.7 mL) and concentrated H$_2$SO$_4$ (9.4 mL). The mixture was heated slowly to reflux and kept refluxing under nitrogen for 4 h (TLC, isopropyl ether, developed twice). The cooled mixture was poured into cold water (200 mL) and extracted with EtOAc (2 x 150 mL). The combined EtOAc was washed with saturated NaHCO$_3$ (2 x 150 mL), brine (150 mL), dried and concentrated. The crude product was chromatographed with hexanes-EtOAc, 11:9) to give **288** as a pale pink solid (282.5 mg, 53.7% yield from **194**). The product was further recrystallized in EtOAc and CH$_2$Cl$_2$. Column chromatography also isolated **295**, a by-product from the above reaction.

Physical data of 14-acetoxy-12,19-dihydroxy-18(4→3)abeo-abieta-3,8,11,13-tetraen-18-oic acid lactone (**294**):

A pale pink solid; IR (neat) cm⁻¹: 3400 (OH), 2950 (CH), 1740 (C=O), 1670, 1620, 1420, 1220, 1020; ¹H NMR (200 MHz, CDCl₃) δ: 0.98 (3H, s, H20), 1.28, 1.29 (3H each, both d, J = 7.0 Hz, H16, H17), 1.70 (1H, m, H1α), 1.85 (2H, m, H6), 2.32 (3H, s, -OCOCH₃), 2.32-2.90 (6H, H1β, H2, H5, H7), 3.06 (1H, sept, J = 7.0 Hz, H15), 4.75 (2H, m, H19), 5.34 (1H, br s, C12-OH, D₂O exchangeable), 6.66 (1H, s, H11); LRMS m/z (rel. intensity): 370 (M⁺, 16.4), 328 (91.4), 313 (85.8), 297 (8.9), 285 (4.5), 271 (7.7), 189 (5.1), 165 (base peak); HRMS calcd. for C₂₂H₂₆O₅: 370.1780; found: 370.1782.

Physical data of 12,14,19-trihydroxy-18(4→3)abeo-abieta-3,8,11,13-tetraen-18-oic acid lactone (DINTP, **288**):

Colorless prisms; mp: 198-199°C; $[\alpha]_D^{21}$: +23.1° (c = 0.45, MeOH); UV λ_{max}^{MeOH} (log ε): 219.1 (4.26), 282.7 (3.32); IR (neat) cm⁻¹: 3400 (OH), 2950 (CH), 1730 (C=O), 1660 (C=C), 1610, 1580 and 1500 (all aromatic C=C), 1420, 1250 (aromatic C-O), 1010; ¹H NMR (400 MHz, CDCl₃) δ: 0.99 (3H, s, H20), 1.35 (6H, d, J = 7.1 Hz, H16, H17), 1.65 (1H, m, H1α), 1.88 (1H, m, H6β), 1.97 (1H, m, H6α), 2.35 (2H, m, H1β, H2α), 2.47 (1H, m, H2α), 2.61-2.81 (3H, m, H5, H7), 3.40 (1H, sept, J = 7.1 Hz, H15), 4.76 (2H, br s, C12-OH, C14-OH), 4.77 (2H, br AB_q, Δν = 0.07 ppm, J = 17.2 Hz, H19), 6.36 (1H, s, H11); ¹³C NMR (50.2 MHz, CDCl₃) δ: 18.2, 19.7, 20.8, 20.9, 22.0, 22.3, 24.6, 32.6, 36.1, 40.9, 70.6, 104.2, 112.8, 117.9, 125.0, 143.9, 152.6, 152.7, 163.0, 174.2; LRMS m/z (rel. intensity): 328 (M⁺, 86.8), 313 (base peak), 285 (3.0), 271 (10.4), 179 (11.3), 165 (95.5); HRMS calcd. for C₂₀H₂₄O₄: 328.1674; found: 328.1679.

Physical data of 11,19-dihydroxy-12-oxo-18(4→3)*abeo*-abieta-3,5,7,9(11),13-pentaen-18-oic-acid lactone (**295**):

A dark red powder; mp: 174-175°C; $[\alpha]_D^{21}$: ≈+0° (c = 0.266, $CHCl_3$); UV λ_{max}^{MeOH} (log ε): 205.0 (4.28), 239.8 (4.15), 278.0 (4.10), 432.4 (4.25); IR (neat) cm^{-1}: 3300 (OH), 2950 (CH), 1740 (C=O), 1640, 1600, 1570, 1500, 1340, 1050; ^1H NMR (400 MHz, $CDCl_3$) δ: 1.17, 1.18 (3H each, both d, J = 6.9 Hz, H16, H17), 1.41 (3H, s, H20), 1.75 (1H, ddd, J = 13.6, 10.2, 7.5 Hz, H1α), 2.56 (2H, m, H2), 3.14 (1H, sept, J = 6.9 Hz, H15), 3.51 (1H, ddd, J = 13.6, 4.5, 2.4 Hz, H1β), 4.96 (2H, br AB_q, Δν = 0.19 ppm, J = 15.9 Hz, H19), 6.29 (1H, d, J = 6.6 Hz, H6), 6.69 (1H, d, J = 6.6 Hz, H7), 6.93 (1H, s, H14), 7.68 (1H, br s, C11-OH, D_2O exchangeable); ^{13}C NMR (50.2 MHz, $CDCl_3$) δ: 18.3, 21.6, 21.75, 21.80, 27.1, 28.8, 39.5, 68.6, 120.8, 122.4, 128.6, 132.1, 133.3, 134.7, 143.3, 144.2, 147.5, 153.0, 173.1, 178.7; LRMS m/z (rel. intensity): 324 (M^+, 22.2), 309 (41.9), 281 (17.9), 265 (base peak), 247 (14.1), 237 (9.0), 223 (14.8), 205 (15.9), 178 (18.5), 165 (25.8), 152 (18.9); HRMS calcd. for $C_{20}H_{20}O_4$: 324.1362; found: 324.1366; Anal. calcd. for $C_{20}H_{20}O_4$: C, 74.06; H, 6.21; found: C, 74.40; H, 6.41.

Experimental for Chapter 4

6.4 Biotransformation Studies

6.4.1 General

T. wilfordii cell cultures grown in $MSNA_{0.5}K_{0.5}$ media were used for biotransformation studies unless otherwise noted. The age of the cell culture at the time of biotransformation was calculated from the day when the inoculation from PRD_2Co_{100} media to $MSNA_{0.5}K_{0.5}$ media

was made. The conditions for culture growth and maintenance were identical to those described in the experimental section for Chapter 2.

For the whole cell experiments, the synthetic precursors or other compounds were dissolved in a certain amount of EtOH, and then added to the cell culture under sterile conditions. Incubation was carried out under the same conditions as the intact cell cultures (standard conditions). Samples were taken at specified intervals under sterile conditions and filtered to separate the cells and the broth. Refractive indices (n_D) and pH measurements, as well as microbial contamination determinations were carried out on the broth samples. The cells were kept frozen in the freezer until time of extraction. Subsequent homogenization and extraction of the cells were carried out by the same procedure as described in the experimental section for Chapter 2. In some cases, the cell culture samples (cells with broth) were sonicated with EtOAc (same volume as the sample) for 5 min, and the resulting extracts were used for TLC analysis. Harvesting and sample extractions of the biotransformation experiments were performed according to the general procedure as stated in the experimental section for Chapter 2. Control experiments that were conducted were similar to the corresponding biotransformations (in the same or a smaller scale), except that an equal volume of pure solvent was added instead of the starting material solutions. As references, the control experiments were always carried out simultaneously and under identical conditions unless otherwise stated. To examine whether any change was brought about by the medium and atmospheric oxygen, blank experiments were usually performed once or more with each starting material. The starting material solution was added to the corresponding sterile medium (no cells) and "incubated" under identical conditions as those biotransformation experiments .

For the cell free extract (CFE) and related experiments, the following general procedures were followed.

<u>A general procedure for CFE and related experiments</u>

The CFE was prepared from cell cultures of specified ages. The cell culture suspension was filtered through Miracloth™. A sample of the broth was taken for pH and refractive index

(n_D) measurements as well as microbial contamination determination. The cells were washed with distilled water (about an equal amount (V/W) of the wet cell mass) and vacuum filtered with a water aspirator until no liquid was dripping. The weight of the cells were measured and recorded. During the rest of the procedure, care was taken to keep all the preparation process between 0-4°C until the beginning of the biotransformation. Potassium phosphate (KH_2PO_4-K_2HPO_4) buffer (0.1 M, pH 6.6), unless otherwise stated, was added to the cells in a ratio of 140 mL buffer to 100 g of the cells, and the resulting suspension was homogenized with a homogenizer (Ultra turax, T-25) at 28,000 rpm for three times (30 second each time with a 1 min rest period between each operation). The cell homogenate (CH) thus obtained was used for biotransformations or, in most cases, was centrifuged at 4°C using a Sorvall RC-5B centrifuge with a GSA rotor at 10,000 g (max.) for 30 min. The supernatant was decanted from the cell debris (the cell pellet) and was defined as the cell free extract (CFE). In some cases, the cell pellet was resuspended in buffer and thus named resuspended cell pellet (RCP).

The CFE was analyzed for its peroxidase activity and soluble protein concentration by established procedures.

<u>Measurement of peroxidase activity (Pyrogallol-Purpurogallin Assay)</u>[252]

CFE (1 mL) was added to a 50 mL Erlenmeyer flask containing 5% aqueous pyrogallol solution (2 mL), 0.1 M phosphate buffer (2 mL, pH 6.6), freshly prepared 0.5% hydrogen peroxide (H_2O_2) solution (1 mL) and distilled water (14 mL) at 20°C. This mixture was allowed to stand for 20 seconds at 20°C and then 2M H_2SO_4 (1 mL) was added to quench the reaction, and the solution was then extracted with ether (2 x 25 mL). The absorbance reading of the extract at 420 nm (Bausch & Lomb, model Spectronic 20 UV spectrometer) was measured against a blank solution containing an ethereal extract (2 x 25 mL) of a mixture comprised of 5% pyrogallol solution (2 mL), 0.1 M phosphate buffer (3 mL, pH 6.6), freshly prepared 0.5% H_2O_2 solution (1 mL) and distilled water (14 mL). The peroxidase activity was calculated from a standard curve, which was prepared by measuring the absorbance at 420 nm of a set of standard solutions of purpurogallin (0.5 to 3.5 mg) in ether (50 mL).

<u>Measurement of protein concentration (Bio-Rad Protein Assay)[253]</u>

One part of dye reagent (Bio-Rad Protein Assay Dye Reagent Concentrate) was diluted with four parts of distilled water. This solution (5 mL) was added to a test tube containing CFE (0.1 mL), and the resulting solution was mixed thoroughly. A reference sample, prepared by mixing phosphate buffer (0.1 mL, 0.1 M, pH 6.6) and the diluted dye solution (5 mL), was used to adjust the reading of the UV spectrometer to zero at 595 nm. The absorbance of the CFE testing sample was then measured at the same wavelength. The protein concentration was calculated from a standard curve. The standard curve was obtained by measuring the absorbance of a set of standard solutions at 595 nm. The standard solutions were made by separately dissolving known amounts of bovine serum albumin (BSA) powder in buffer (0.1 mg/mL to 1.0 mg/mL), and then by adding aliquots (0.1 mL) of each solution to a diluted dye solution (5 mL), respectively.

Peroxidase activity and protein concentration of CFEs prepared from phosphate buffers of different pH, or prepared from acetate buffers were determined in the same manner except that the corresponding buffer was also used for the preparation of the samples for measurement. In the experiments with CH and RCP, their peroxidase activity and protein concentration were also measured accordingly.

A typical composition of a CFE (or CH, RCP) mixture for biotransformation experiments is as following:

The CFE (250 units of peroxidase per 50 mg starting material), distilled water (75 mL), buffer (0.1 M, required pH, 175 mL), 0.24% H_2O_2 (5 mL, 2.2 equiv. to the starting material), flavin mononucleotide (FMN) (Sigma, 75%, 0.5 equiv.) and $MnCl_2$ (0.5 equiv.). The starting material (50 mg) was dissolved in EtOH (20 mL) and then added to the CFE mixture.

The experiments with CFE (or CH, RCP) were carried out at r.t.. The starting material was dissolved in EtOH and then added to the stirred CFE (or CH, RCP) mixture. Samples (from CFE or CH experiments) were taken at certain intervals, saturated with NaCl, and extracted with EtOAc (3 x volume of the sample). The combined EtOAc was dried and concentrated. Samples

from RCP experiments were initially mixed with EtOAc (same volume as the sample) and then filtered through Celite. The filtrate was separated and the aqueous layer was saturated with NaCl and extracted with EtOAc again (2 x volume of the sample). The Celite pad was sonicated with EtOAc (about the same volume of the sample) for 5 min, or less if the solution turned warm, and then filtered. The EtOAc from the Celite pad was combined with other EtOAc extracts from that sample, dried and concentrated.

The extracts from biotransformation experiments (with whole cells or CFE etc.) were analyzed by TLC and HPLC. HPLC was performed using a Waters C_{18} Radial Pack liquid chromatography cartridge, a Waters 440 absorbance detector set at 280 nm (recording wavelength) and 254 nm. The eluent was a mixture of MeOH and water with 0.1% HOAc. Samples, which had been filtered through short silica gel columns, were quantitatively dissolved in MeOH prior to HPLC analysis.

6.4.2 Attempted Biotransformation of 7-Oxo-isodehydroabietenolide (206) with TRP4a Cell Cultures

7-Oxo-isodehydroabietenolide (206, 100 mg) in EtOH (8 mL) was equally divided into two flasks containing a 14-day-old culture (550 mL each) and incubated under normal conditions. A control (4 mL EtOH in 550 mL culture) experiment and a blank experiment (50 mg starting material in 4 mL EtOH added to 550 mL $MSNA_{0.5}K_{0.5}$ medium) were carried out under the same conditions. Samples were taken at 1, 4 and 6 days, with the culture harvested after 7 days of incubation. The samples were extracted with EtOAc (sonicated in EtOAc) and analyzed by TLC (hexanes-EtOAc, 1:1). There were no significant changes in the test samples in comparison with control and blank samples.

The biotransformation was repeated under the same conditions except that the ratio of MeOH was increased. Thus the starting material 206 (50 mg) in 50 mL EtOH was incubated in

550 mL of a 14-day-old cell culture (Trp#294). Samples were taken after incubation for 1, 5 and 7 days, extracted with EtOAc and analyzed by TLC. No significant reactions were observed.

6.4.3 Biotransformation of 7β-Hydroxy-isodehydroabietenolide (240) with TRP4a Cell Cultures (Trp#295)

The starting material **240** (80 mg) in EtOH (4 mL) was incubated in a 14-day-old culture (550 mL). A control (4 mL EtOH in 550 mL culture) experiment and a blank experiment (16 mg of **240** in 0.8 mL EtOH added to 100 mL $MSNA_{0.5}K_{0.5}$ medium) were also conducted under identical conditions. Samples were taken after 3 days of incubation, extracted with EtOAc (sonicated with EtOAc), and analyzed by TLC (hexanes-EtOAc, 1:1). The cultures were harvested after 7 days and filtered. The blank and the broth samples from the test and control were extracted with EtOAc according to the general procedure. All samples were analyzed by TLC (hexanes-EtOAc, 1:1 and $MeOH-CH_2Cl_2$, 4:96). Results are given in section 4.2.1.

6.4.4 Biotransformation of Isotriptophenolide (194) and Related Compounds with TRP4a Cell Cultures

Biotransformation of 194 with Older Cell Cultures (Trp#300a)

Isotriptophenolide (**194**, 46.5 mg) in EtOH (1 mL) was incubated in a TRP4a cell culture (21 days old, 550 mL). Samples (5 mL) were taken after 1, 2 and 4 days of incubation. The samples were extracted with EtOAc and analyzed by TLC (toluene-$CHCl_3$-EtOAc-HCO_2H, 105:48:45:3, developed twice). The culture was harvested after 5 days, and the cells and broth were extracted with EtOAc. Column chromatography (hexanes-EtOAc, 6:4; EtOAc) of the cell extract and broth extract gave three fractions (Frs. I-III). Fr. I was purified by elution with CH_2Cl_2-EtOAc (90:5) to yield isotriptophenolide methyl ether (**304**, 2.8 mg). Fr. 2 was

chromatographed with the same solvent system to recover the starting material (**194**, 17.1 mg). Further purification of Fr. III failed due to decomposition of the polar compound on the column.

Physical data of 19-hydroxy-12-methoxy-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (**304**):

A white powder; mp: 54-56°C; $[\alpha]_D^{24}$: +49.4° (c = 0.090, CHCl$_3$); UV λ_{max}^{MeOH} (log ε): 208.8 (4.32), 280.2 (3.55); IR (CHCl$_3$) cm^{-1}: 3025 (aromatic CH), 2950 (CH), 1740 (C=O), 1670 (C=C), 1600 and 1490 (aromatic C=C), 1260 (aromatic C-O), 1040; ^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.03 (3H, s, H20), 1.17, 1.19 (3H each, both d, J = 6.8 Hz, H16, H17), 1.73 (1H, m, H1α), 1.89 (2H, m, H6), 2.32-2.56 (3H, m, H1β, H2), 2.71 (1H, m, H5), 2.94 (2H, m, H7), 3.23 (1H, sept, J = 6.8 Hz, H15), 3.71 (3H, s, -OCH$_3$), 4.76 (2H, br AB$_q$, Δν = 0.06 ppm, J = 17.2 Hz, H19), 6.78 (1H, s, H11), 6.81 (1H, s, H14); ^{13}C NMR (50.2 MHz, CDCl$_3$) δ: 18.2, 20.3, 22.3, 22.6, 22.8, 26.5, 27.7, 32.8, 36.6, 41.6, 55.5, 70.5, 106.3, 124.9, 126.0, 127.1, 135.2, 143.0, 155.1, 163.2, 174.2; LRMS m/z (rel. intensity): 326 (M$^+$, 72.8), 311 (base peak), 297 (6.7), 281 (5.4), 283 (5.3), 269 (12.2), 254 (6.4), 163 (51.6); HRMS calcd. for C$_{21}$H$_{26}$O$_3$: 326.1882; found: 326.1880.

Increase of Starting Material Solubility (Trp#301)

Isotriptophenolide (**194**, 100 mg) in EtOH (50 mL) was evenly divided into two flasks of TRP4a cell cultures (21 days old, 550 mL each) and incubated. A control experiment (25 mL EtOH in 550 mL culture) was set up under the same conditions. Samples (10-15 mL)were taken after incubation for 26 h (Sample 1) and 72 h (Sample 2). TLC (toluene-CHCl$_3$-EtOAc-HCO$_2$H, 105:48:45:3, developed twice) showed that Sample 1 still contained considerable amounts of starting material (R$_f$ 0.57), and the methyl ether was hardly observable. A new spot, which was polar (R$_f$ 0.41) and UV active, was found on the TLC. An additional spot (R$_f$ 0.22), which had

been found in Trp#300a, was also present. Sample 2 showed increasing amounts of the spots at R_f 0.41 and R_f 0.22, but much of the starting material was still present. The culture was harvested after incubation for 96 h, and the cells and the broth were extracted with EtOAc to give a cell extract (257.9 mg) and a broth extract (162.4 mg).

Column chromatography of the broth extract with hexanes-EtOAc (6:4) and EtOAc gave 7 fractions (Frs.I-VII). The cell extract was separated into 3 fractions (Frs. VIII-X) by elution with the same solvent system. Frs. I and VIII were combined and chromatographed with CH_2Cl_2-EtOAc (90:5) to yield isotriptophenolide methyl ether (**304**, 0.6 mg) along with recovered starting material **194** (56.6 mg). Fr. IV was combined with Fr. IX and eluted with CH_2Cl_2-EtOAc (90:5) to give a mixture of **305** and **306** (7.9 mg). Separation of Fr. II on a column with CH_2Cl_2-EtOAc (9:1) yielded **194** (2.0 mg) and **307** (0.7 mg). Fr. III was chromatographed with CH_2Cl_2-EtOAc (8:2) to give **309** (2.6 mg) and a mixture (IIIB), which yielded **306** (6.3 mg) and **245** (1.6 mg) after elution with CH_2Cl_2-EtOAc (90:5). Frs. VI and X were combined and chromatographed with hexanes-EtOAc (1:1) to produce **246** (4.5 mg) and a mixture Fr. VIB which was combined with Fr. VII to yield another 3.6 mg of **246** by elution with the same solvent system. Purification of Fr. V with CH_2Cl_2-EtOAc (8:2) produced **305** (1.0 mg), and another fraction which gave **308** (1.6 mg) and **246** (0.8 mg) after elution with hexanes-EtOAc (6:4). The isolation results are summarized in Table 4.1 (section 4.2.2).

The mixture of **305** and **306** was combined with a similar sample from another small scale biotransformation and chromatographed by preparative TLC. The plate was eluted with anhydrous ether (Fishers) 6 times, yielding **305** (4.3 mg) and **306** (6.0 mg), respectively.

Physical data of 7-ethoxy-12,19-dihydroxy-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (**307**, $\alpha:\beta$ = 4:1):

IR (CHCl₃) cm⁻¹: 3600 (OH), 2950 (CH), 1760 (C=O), 1610, 1170, 1040; ¹H NMR (400 MHz, CDCl₃) (α isomer) δ: 0.95 (3H, s, H20), 1.24 (9H, m, H16, H17, ethoxy CH₃),

1.71 (1H, m, H1α), 1.95 (1H, ddd, J = 13.2, 13.2, 4.3 Hz, H6β), 2.04 (1H, br d, J = 13.2 Hz, H6α), 2.22-2.53 (3H, m, H1β, H2), 3.13 (2H, m, H5, H15), 3.53-3.73 (2H, m, ethoxy CH$_2$), 4.38 (1H, br dd, J = 4.2, 1.6 Hz, H7β), 4.78 (2H, br AB$_q$, Δν = 0.10 ppm, J = 17.2 Hz, H19), 4.94 (1H, s, C12-OH). 6.70 (1H, s, H11), 7.11 (1H, s, H14); ^{13}C NMR (50.2 MHz, CDCl$_3$) (α isomer) δ: 15.8, 18.2, 22.0, 22.4 (2C), 26.0, 27.0, 29.7, 32.6, 37.0, 64.4, 70.4, 74.0, 110.9, 125.2, 126.8, 129.4, 132.8, 143.9, 152.9, 163.4, 174.1; LRMS m/z (rel. intensity): 356 (M$^+$, 5.5), 327 (13.0), 311 (50.9), 310 (80.8), 295 (51.6), 253 (base peak); HRMS calcd. for C$_{22}$H$_{28}$O$_4$: 356.1988; found: 356.1996.

Physical data of 5α,12,19-trihydroxy-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (**309**):

A white crystalline solid; mp: 190-192°C (dec); [α]$_D^{23}$: -37.9° (c = 0.070, CHCl$_3$); UV λ$_{max}^{MeOH}$ (log ε): 205.1 (4.50), 282.5 (3.68); IR (CHCl$_3$) cm^{-1}: 3650 (OH), 3000 (aromatic CH), 1760 (C=O), 1690 (C=C), 1180 (tertiary C-O), 1040; ^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.08 (3H, s, H20), 1.227, 1.234 (3H each, both d, J = 6.9 Hz, H16, H17), 1.90 (1H, s, C5-OH, D$_2$O exchangeable), 1.97 (1H, m, H6α), 2.04-2.26 (3H, m, H1, H6β), 2.36 (1H, m, H2β), 2.54 (1H, br d, J = 17.3 Hz, H2α), 3.01 (2H, m, H7), 3.13 (1H, sept, J = 6.9 Hz, H15), 4.78 (1H, s, C12-OH, D$_2$O exchangeable), 4.89 (2H, br AB$_q$, Δν = 0.18 ppm, J = 17.1 Hz, H19), 6.71 (1H, s, H11), 6.96 (1H, s, H14); ^{13}C NMR (50.2 MHz, CDCl$_3$) δ: 18.0, 22.5, 22.6, 23.0, 25.5, 26.27, 26.34, 26.8, 41.4, 69.3, 70.1, 112.0, 125.5, 127.0, 127.5, 132.9, 140.2, 151.6, 160.9, 173.9; LRMS m/z (rel. intensity): 328 (M$^+$, 3.9), 310 (4.0), 295 (5.5), 267 (9.4), 253 (22.6); HRMS calcd. for C$_{20}$H$_{24}$O$_4$: 328.1674; found: 328.1680.

Physical data of 5β,12,19-trihydroxy-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (**308**):

A pale yellowish powder; mp: 92-95°C; UV λ_{max}^{MeOH} (log ε): 216.9 (4.38), 283.4 (3.85); $[\alpha]_D^{24}$: +171.9°; (c = 0.281, CHCl₃); IR (CHCl₃) cm⁻¹: 3600 (OH), 3470 (OH), 3025 (aromatic CH), 2975 (CH), 1750 (C=O), 1680 (C=C), 1500, 1240, 1170 (tertiary C-O), 1070, 1040, 920; ¹H NMR (400 MHz, CDCl₃) δ: 1.21 (6H, d, J = 6.8 Hz, H16, H17), 1.32 (3H, s, H20), 1.83-2.00 (3H, m, H1α, H2α, H6α), 2.06 (1H, ddd, J = 13.3, 5.1, 5.1 Hz, H1β), 2.15 (2H, m, H2β, H6β), 2.30 (1H, br s, C5-OH, D₂O exchangeable), 2.62 (1H, ddd, J = 16.9, 8.1, 6.0 Hz, H7β), 2.87 (1H, ddd, J = 16.9, 6.3, 6.3 Hz, H7α), 3.09 (1H, sept, J = 6.8 Hz, H15), 4.71 (1H, s, C12-OH, D₂O exchangeable), 4.90 (2H, br ABq, Δv = 0.1 ppm, J = 17.2 Hz, H19), 6.69 (1H, s, H11), 6.82 (1H, s, H14); ¹³C NMR (50.2 MHz, CDCl₃) δ: 17.9, 22.47, 22.54, 24.0, 25.7, 26.8, 30.1, 33.8, 42.0, 69.4, 71.6, 113.3, 125.7, 126.6, 127.9, 133.1, 139.2, 151.8, 162.9, 173.9; LRMS m/z (rel. intensity): 328 (M⁺, 46.7), 310 (49.1), 295 (58.6), 267 (69.5), 253 (base peak), 189 (49.4), 147 (94.3); HRMS calcd. for C₂₀H₂₄O₄: 328.1674; found: 328.1680.

Physical data of (7,8)β-epoxy-19-hydroxy-12-oxo-18(4→3)abeo-abieta-3,9(11),13-trien-18-oic acid lactone (305):

Colorless long plates; mp: 230-232°C (dec); $[\alpha]_D^{24}$: -295.5° (c = 0.0670, CHCl₃); UV λ_{max}^{MeOH} (log ε): 217.7 (4.09), 259.7 (4.26); IR (CHCl₃) cm⁻¹: 3050 (olefinic CH), 3000 (CH), 1750 (C=O), 1730, 1660 (quinone C=O), 1640 (quinone (C=C), 1450, 1380, 1250, 1050, 910; ¹H NMR (400 MHz, CDCl₃) δ: 1.06, 1.08 (3H each, both d, J = 6.9 Hz, H16, H17), 1.17 (3H, s, H20), 1.62 (1H, br ddd, J = 12.9, 12.4, 6.0 Hz, H1α), 2.10 (1H, br dd, J = 12.9, 5.4 Hz, H1β), 2.13 (1H, br dd, J = 14.5, 13.4 Hz, H6β), 2.26 (1H, ddd, J = 14.5, 5.8, 5.8 Hz, H6α), 2.30 (1H, m, H2β), 2.47 (1H, br d, J = 18.5 Hz, H2α), 2.58 (1H, br d, J = 13.4 Hz, H5), 2.98 (1H, sept d, J = 6.9, 1.0 Hz, H15), 3.84 (1H, d, J = 5.8 Hz, H7α), 4.68 (2H, br s, H19), 5.99 (1H, d, J = 1.0 Hz, H14), 6.42 (1H, s, H11); ¹³C NMR (125.8 MHz, CDCl₃) δ: 15.9 (C20), 17.4 (C2), 21.3 (C16*), 21.6 (C17*), 23.9 (C6), 26.3 (C15), 32.6 (C1), 38.1

(C10), 42.0 (C5), 56.0 (C8), 64.0 (C7), 69.9 (C19), 125.6 (C3), 128.5 (C11), 139.0 (C14), 149.8 (C13), 157.9 (C9), 159.4 (C4), 173.1 (C20), 186.1 (C12) (note: * interchangeable); LRMS m/z (rel. intensity): 326 (M$^+$, 72.5), 311 (base peak), 295 (14.7), 283 (7.9), 267 (9.6), 253 (15.8), 241 (8.1), 229 (12.1), 203 (22.8), 163 (31.8), 141 (23.4); HRMS calcd. for C$_{20}$H$_{22}$O$_4$: 326.1518; found: 326.1517; Anal. calcd. for C$_{20}$H$_{22}$O$_4$: C, 73.60; H, 6.79; found: C, 73.42; H, 6.81.

Physical data of 7α,8α-epoxy-19-hydroxy-12-oxo-18(4→3)$abeo$-abieta-3,9(11),13-trien-18-oic acid lactone (**306**):

Colorless needles; mp: 198-200°C (dec); UV λ$_{max}^{MeOH}$ (log ε): 219.2 (4.17), 260.2 (4.36); [α]$_D^{24}$: +161.5° (c = 0.117, CHCl$_3$); IR (CHCl$_3$) cm^{-1}: 3050 (olefinic CH), 3000, 2975 (CH), 1770 (C=O), 1680 (quinone C=O), 1650 (quinone C=C), 1250, 1070, 1050, 920; ^{1}H NMR (400 MHz, CDCl$_3$) δ: 0.95 (3H, s, H20), 1.05, 1.08 (3H each, both d, J = 6.9 Hz, H16, H17), 1.69 (1H, br ddd, J = 13.3, 12.4, 6.4 Hz, H1α), 2.02 (1H, br dd, J = 14.4, 12.8 Hz, H6β), 2.19 (1H, br dd, J = 13.3, 6.1 Hz, H1β), 2.30 (1H, m, H2β, overlapped with H6α), 2.30 (1H, br ddd, J = 14.4, 3.1, 2.4 Hz, H6α), 2.47 (1H, br d, J = 17.9 Hz, H2α), 2.95 (1H, sept d, J = 6.9, 0.9 Hz, H15), 3.23 (1H, br d, J = 12.8 Hz, H5), 3.82 (1H, br d, J = 2.4 Hz, H7β), 4.70 (2H, br AB$_q$, Δν = 0.08 ppm, J = 17.1 Hz, H19), 5.99 (1H, d, J = 0.9 Hz, H14), 6.48 (1H, s, H11); ^{13}C NMR (125.8 MHz, CDCl$_3$) δ: 17.6 (C2), 21.4 (C16*), 21.5 (C17*), 22.1 (C20), 23.6 (C6), 26.4 (C15), 30.9 (C1), 32.4 (C5), 37.3 (C10), 54.3 (C8), 60.9 (C7), 69.9 (C19), 125.4 (C3), 130.4 (C11), 137.7 (C14), 150.0 (C13), 157.3 (C9), 160.8 (C4), 173.2 (C18), 186.0 (C12) (note: * interchangeable); LRMS m/z (rel. intensity): 326 (M$^+$, 30.0), 311 (49.6), 310 (26.7), 295 (27.5), 283 (6.2), 267 (17.2), 253 (26.2), 203 (12.4), 185 (10.4), 167 (10.1), 163 (22.4), 149 (29.5), 141 (17.7); HRMS calcd. for C$_{20}$H$_{22}$O$_4$: 326.1518; found: 326.1512; Anal. calcd. for C$_{20}$H$_{22}$O$_4$: C, 73.60; H, 6.79; found: C, 73.55; H, 6.73.

Influence of Cell Age and Starting Material-to-Culture Ratio: Time Course Studies

<u>15-day-old cell culture (Trp#305)</u>

Isotriptophenolide (**194**, 200 mg) in EtOH (50 mL) was added to a cell culture of 15 days old (1.1 L in a 2 L flask) and incubated under standard conditions. Samples were taken after incubation for different time (1, 2, 3, 4, 5, 7, 9, 11, 13, 15 days) and the culture was harvested at 21 day. The cells and broth from all the samples were extracted with EtOAc and analyzed by HPLC, respectively (for results see section 4.2.2).

<u>7-day-old cell culture (Trp#306)</u>

The experiment was carried out under similar conditions as Trp#305 except that a cell culture of 7 days old was used.

<u>21-day-old cell culture (Trp#307)</u>

The experiment was carried out under similar conditions as Trp#305 except that a cell culture of 21 days old was used and the sampling schedule was slightly different. The samples were taken after incubation for 1 h, 2 h, 4 h, 9 h, 24 h (1 day), 2, 3, 5, 7 and 14 days, and the culture was harvested at 21 day.

Meanwhile, two additional experiments (Trp#308 and Trp#309) using different amounts of starting material (50 mg and 25 mg in 550 mL cell culture, respectively) were also performed with the same batch of 21-day-old cell cultures as Trp#307. Samples were taken after incubation for different time (1 h, 2 h, 4 h, 9 h, 24 h (1 day), 2 days) and the cultures were harvested at 3 day. The cells and broth of all the samples were extracted with EtOAc and analyzed by HPLC, respectively.

Relevant data from Trp#307 are used together with the results from Trp#305 and Trp#306 in the discussion of influences of the cell age on the biotransformation and are also used

along with the results from Trp#308 and Trp#309 in the discussion of influences of starting material/culture ratio on the biotransformation.

Preliminary Biotransformation of 194 with CFE Prepared from TRP4a Cell Cultures (Trp#310)

A TRP4a cell culture (15 days old, 2 x 550 mL) was filtered through Miracloth™, washed with distilled water and dried by suction with a water aspirator. The cells thus obtained (100 g) were suspended in a phosphate buffer (0.1 M, pH 6.6, 140 mL) and homogenized at 0-4°C. Centrifugation of the cell homogenate at 10,000 g (max.) for 30 min at 4°C produced a supernatant (CFE) (176 mL), and some cell pellet. The peroxidase activity and protein concentration of the CFE were measured to be 4.05 units/mL and 0.939 mg/mL, respectively.

The CFE mixture was prepared by mixing together CFE (62 mL, 250 units), phosphate buffer (0.1 M, pH 6.6, 350 mL), distilled water (150 mL) and 0.24% H_2O_2 (10 mL, 2.2 equiv.). The starting material **194** (100 mg) in EtOH (30 mL) was added to the stirred CFE mixture at r.t.. A blank experiment with buffer substituting for CFE was carried out under the same conditions but on a smaller scale (1/10). Samples were taken at different time (0.5, 2, 6, 22 h) and the experiment was worked up at 48 h. All the samples were extracted with EtOAc and analyzed by TLC (toluene-$CHCl_3$-EtOAc-HCO_2H, 105:48:45:3).

Biotransformation of 194 with CFE, CH and RCP from TRP4a Cell Cultures of Different Ages: Time Course Studies

A cell culture of 21 days (2 x 550 mL) was used to prepare the CFE etc. (Trp#311). The cells obtained (122 g) were suspended in phosphate buffer (0.1 M, pH 6.6, 171 mL) and homogenized to give a cell homogenate (CH, 293 mL), of which 200 mL was taken and centrifuged to give a CFE (163 mL) and a pellet. The pellet was resuspended in buffer (0.1 M,

pH 6.6, 163 mL) to make the RCP. The peroxidase activities and protein concentrations of the CFE, CH and RCP were measured (see Table 4.2 in section 4.2.2).

The compositions of each biotransformation mixture are also shown in Table 4.2. An equal amount of isotriptophenolide (**194**, 50 mg) in EtOH (20 mL) was added at r.t. to each stirred mixture. At the same time, two control experiments for CH and RCP were run in one tenth of the scale of the corresponding CH and RCP experiments. A blank experiment was also run in one tenth the scale of the CFE experiment, but with CFE being substituted by an equal amount of buffer. Samples were taken at 0.5, 2, 6 and 24 h, and the reactions were worked up at 48 h. All samples were extracted with EtOAc and analyzed by TLC (toluene-$CHCl_3$-EtOAc-HCO_2H, 105:48:45:3) and HPLC.

Experiments Trp#312, 313 were carried out accordingly and the details of the preparation of CFE etc. are summarized in Table 6.1. The results of these 3 experiments are discussed in section 4.2.2.

Table 6.1   Preparation of CFE, CH and RCP for Trp#311, 312 and 313

	Trp#311	Trp#312	Trp#313
age of the cell culture (day)	21	13	7
volume of the cell culture (mL)	2 x 550	2 x 550	3 x 550
amount of cells obtained (g)	122	98	80
volume of buffer[a] used for CH (mL)	171	138	112
total volume of CH (mL)	293	224	172
volume of CH used for CFE, RCP (mL)	200	154	107
volume of CFE (mL)	163	129	97
volume of buffer[a] used for RCP (mL)	163	129	97
biotransformation mixture composition	Table 4.2	Table 4.3	Table 4.4

[a] Phosphate buffer, 0.1 M, pH 6.6.

Biotransformation of 194 with CFEs and RCPs Obtained by Varying RCF of Centrifugation (Trp#314)

A cell culture of 15 days (2 x 550 mL) was used. The cells obtained (90.5 g) were suspended in 127 mL of phosphate buffer (0.1 M, pH 6.6) and homogenized. The resulting cell homogenate was centrifuged at 4°C as usual at 10,000 g (max.) (Sorvall® Centrifuge, GSA rotor, average RCF, 6,600 g) for 30 min to give Pellet1 and CFE1 (~170 mL). Pellet1 was discarded, while a portion of CFE1 (~120 mL) was centrifuged again at an average RCF of 30,000 g for 30 min at 4°C (Beckman L3-50 Ultracentrifuge, 35 rotor) to give CFE2 (~120 mL) and Pellet2. Pellet2 was washed (buffer 10 mL) and resuspended in 120 mL of buffer. A portion (~80 mL) of CFE2 was then further centrifuged at an average RCF of 85,000 g for 30 min (Beckman L3-50 Ultracentrifuge, 35 rotor) to yield Pellet3 and CFE3 (~80 mL). Pellet3 was washed (buffer 10 mL) and resuspended in 80 mL of buffer. Peroxidase activities and protein concentration were measured and the results are given in Table 4.5 (section 4.2.2).

The composition of each biotransformation mixture is also given in Table 4.5. An equal amount of isotriptophenolide (194, 23.8 mg) in EtOH (10 mL) was added at r.t. to each stirred mixture (125 mg of starting material in 50 mL EtOH was equally divided into 5 flasks, with a residue of 5.9 mg starting material left.). Samples were taken at 0.5, 2, 6 and 24 h, while the reactions were worked up at 48 h. All samples were extracted with EtOAc and analyzed by TLC (toluene-CHCl3-EtOAc-HCO2H, 105:48:45:3) and HPLC (see section 4.2.2 for the results).

Biotransformation of 194 with CFEs Prepared with Buffers of Different pH

pH 7.6-5.0: phosphate buffer (Trp#315)

A cell culture of 21 days (2 x 550 mL) was used to prepare the CFE. The cells (105 g) obtained were divided into 6 portions (16 g each), which were then mixed with phosphate buffer

(0.1 M) of pH 7.6, 7.0, 6.6, 6.2, 5.8 and 5.0, respectively. Each of the resulting mixtures was homogenized and centrifuged to give 6 CFEs. Their peroxidase activities and protein concentrations were measured accordingly (see Table 4.6 in section 4.2.2). Equal amounts of CFE were used for each experiment, with the volume being determined by the requirement for a total of 125 peroxidase units for the CFE which exhibited the lowest measured peroxidase activity (CFE made from pH 5.0 buffer).

The composition of each biotransformation mixture is also given in Table 4.6. An equal amount of isotriptophenolide (**194**, 25 mg) in EtOH (10 mL) was added at r.t. to each stirred mixture. Samples were taken at 1 and 2 h, with the reactions being worked up at 4 h. All the samples were extracted with EtOAc and analyzed by TLC (toluene-$CHCl_3$-EtOAc-HCO_2H, 105:48:45:3) and HPLC (see section 4.2.2 for the results).

pH 5.0-4.0: acetate buffer (Trp#317)

The cells (70.5 g) obtained from a 21-day-old cell culture (2 x 550 mL) were divided into 5 portions (14 g each). One of them was mixed with a phosphate buffer (0.1 M, pH 5.0), while the remaining four were mixed with an acetate buffer (0.1 M) of pH 5.0, 4.7, 4.4 and 4.0, separately. Each of the resulting mixtures was homogenized and centrifuged to give 5 CFEs. Their peroxidase activities and protein concentrations were measured accordingly (see Table 4.7 in section 4.2.2). Equal amounts of CFE were used for each experiment, with the volume being determined by the volume of the CFE prepared with the phosphate buffer (0.1 M, pH 5.0) in order to make comparisons with Trp#315.

The composition of each biotransformation mixture is shown in Table 4.7. An equal amount of isotriptophenolide (**194**, 18.16 mg) in EtOH (7.7 mL) was added at r.t. to each of the stirred mixtures. Samples were taken at 1 and 2 h, with the reactions being worked up at 4 h. All samples were extracted with EtOAc and analyzed by TLC (toluene-$CHCl_3$-EtOAc-HCO_2H, 105:48:45:3) and HPLC (see section 4.2.2 for the results).

Biotransformation of 194 with CFE at Different Starting Material-to-CFE Ratios (Trp#316)

A 14-day-old cell culture (2 x 550 mL) was used to give 81.2 g cells, which were suspended in phosphate buffer (0.1 M, pH 5.0, 113.7 mL) and homogenized. Centrifugation of the resulting suspension gave 155 mL of CFE, and its peroxidase activity and protein concentration were measured (see Table 4.8 in section 4.2.2). Table 4.8 also gives the details of the experiment conditions. A volume of 21.5 mL CFE (125 units of peroxidase) was used as the base amount. Two and four times that volume were used in the other two flasks, respectively. The total volume for each reaction was made equal by adding corresponding amounts of the buffer, respectively. An equal amount of the starting material **194** (25 mg) in EtOH (10 mL) was added to each of the mixtures, and the reaction mixtures were stirred at r.t.. Samples were taken at 1 and 2 h, with the rest being worked up at 4 h. All samples were extracted with EtOAc, and the resulting extracts were analyzed by TLC (toluene-CHCl$_3$-EtOAc-HCO$_2$H, 105:48:45:3) and HPLC (see section 4.2.2 for the results).

Biotransformation of 194 with CFEs Containing Different Equivalents of Hydrogen Peroxide (Trp#318a)

A 14-day-old cell culture (2 x 550 mL) was used to prepare the CFE. The cells obtained (53 g) were suspended in an acetate buffer (0.1 M, pH 4.7, 74.2 mL) and homogenized. Centrifugation of the suspension yielded a CFE (100 mL), and its peroxidase activity and protein concentration were measured (see Table 4.9 in section 4.2.2). Biotransformation reactions were set up with the typical ratio except that the H$_2$O$_2$ volume added was 0, 2 and 8 mL (or 0, 2.2 and 8.8 equivalents), respectively (Table 4.9). An equal amount of the starting material **194** (20 mg) in EtOH (8 mL) was added to each of the three mixtures, respectively. The reactions were stirred at r.t. and samples were taken at 1 and 2 h. The reactions were worked up at 4 h, and all the

samples were extracted with EtOAc and analyzed by TLC (toluene-CHCl3-EtOAc-HCO2H, 105:48:45:3) and HPLC (see section 4.2.2 for the results).

Biotransformation of 194 in CFEs with Different Metal Ions Being Added (Trp#320)

A cell culture of 21 days (2 x 550 mL) was used. The cells obtained (123 g) were added to an acetate buffer (0.1 M, pH 4.7, 173 mL) and homogenized. Centrifugation of the cell homogenate gave 243 mL CFE, and its peroxidase activity and protein concentration were determined (see Table 4.10 in section 4.2.2). The CFE was divided into five flasks with 45 mL (125 peroxidase units) in each flask. The experiments were set up as shown in Table 4.10 (section 4.2.2). One flask was left without any metal ions being added, while the other four were supplemented with 0.5 equivalents of $MnCl_2$, $CoCl_2$, $NiCl_2$ and $Zn(OAc)_2$, respectively. An equal amount of the starting material 194 (25 mg) in EtOH (10 mL) was added to each of the flasks, and the reaction mixtures were stirred at r.t.. Samples were taken at 1 and 2 h, and the reactions were worked up at 4 h. All samples were extracted with EtOAc and analyzed by TLC (toluene-CHCl3-EtOAc-HCO2H, 105:48:45:3) and HPLC (see section 4.2.2 for the results).

Biotransformation of 7-Hydroxy-isotriptophenolide (246) with TRP4a Cell Cultures (Trp#340)

A solution of 246 (21.8 mg, α isomer > 90%) in EtOH (12.5 mL) was added to a cell culture (21 days old, 250 mL in 500 mL flask) and incubated under standard conditions. In a control experiment, only EtOH was added. Samples were taken after 1 and 3 days, and TLC analysis of these samples (toluene-CHCl3-EtOAc-HCO2H, 105:48:45:3) showed no epoxide spots. The culture was harvested after 7 days, and the cells and broth were extracted with EtOAc to give a cell extract (101.9 mg) and a broth extract (79.8 mg). A blank experiment was

performed. The starting material **246** (2.2 mg. α isomer was about 70%) in EtOH (1.25 mL) was "incubated" for 7 days in a sterile $MSNA_{0.5}K_{0.5}$ medium (25 mL). The mixture was extracted with EtOAc and analyzed by TLC (CH_2Cl_2-EtOAc, 90:5) and ^1H NMR.

The cell and broth extracts form the biotransformation were combined and chromatographed with hexanes-EtOAc (1:1) to give 3 fractions (Frs. I-III). Fr. I was eluted with CH_2Cl_2-EtOAc (90:5, 90:10) to yield **307** (3.3 mg). Fr. II was repeatedly chromatographed with CH_2Cl_2-EtOAc (9:1) and hexanes-acetone (65:35), giving pure **245** (3.6 mg), a mixture of **245** and **252** (1.7 mg) (see section 4.2.2 for structure assignments), and a new compound **310** (1.5 mg). Starting material **246** (3.1 mg, about 37% of which was **246a**, the α isomer, as calculated from the ^1H NMR spectrum) was recovered from Fr. III after purification with CH_2Cl_2-EtOAc (8:2). No epoxides were isolated from this experiment. Compounds isolated from experiment Trp#340 are summarized in Table 4.11 (section 4.2.2).

Physical data of 7α,19-dihydroxy-12-methoxy-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (**310**):

^1H NMR (400 MHz, $CDCl_3$) δ: 0.98 (3H, s, H20), 1.18, 1.20 (3H each, both d, $J = 6.7$ Hz, H16, H17), 1.77 (1H, m, H1α), 1.98 (1H, br d, $J = 13.8$ Hz, H6α), 2.10 (1H, ddd, $J = 13.8, 13.8, 4.6$ Hz, H6β), 2.33-2.59 (3H, m, H1β, H2), 3.17 (1H, br d, $J = 13.8$ Hz, H5), 3.25 (1H, sept, $J = 6.7$ Hz, H15), 3.84 (3H, s, -OCH_3), 4.79 (2H, br AB_q, $\Delta v = 0.12$ ppm, $J = 17.3$ Hz, H19), 4.86 (1H, m, H7β), 6.79 (1H, s, H11), 7.17 (1H, s, H14).

Biotransformation of the Epoxides (305, 306) with TRP4a Cell Cultures (Trp#338)

A solution of the epoxides **305** and **306** (52.7 mg, **305**:**306** approximately 1, isolated from biotransformations of **194**) in EtOH (25 mL) was added to a cell culture of 21 days (550

mL) and incubated under usual conditions. Samples were taken after 48 h incubation and checked by TLC (hexanes-EtOAc, 1:1, developed twice). The experiment continued for another 42 h and then the culture was harvested. The cells and broth were extracted with EtOAc, and the extracts were combined. A blank experiment was carried out. The starting material **305** and **306** (2.6 mg, of which **306** was the major component) in EtOH (1.25 mL) was "incubated" in a sterile $MSNA_{0.5}K_{0.5}$ medium (28 mL) for 90 h. The mixture was extracted with EtOAc and analyzed by TLC (CH_2Cl_2-EtOAc, 90:5) and ^1H NMR.

The combined extract from the biotransformation was chromatographed with hexanes-EtOAc (1:1) to give 5 fractions (Frs. I-V). Fr. I was eluted with CH_2Cl_2-EtOAc (90:5, 90:10) to yield **307** (7.0 mg), while Fr. II gave another small amount of **307** (0.3 mg) by elution with the same solvent system. Fr. III was chromatographed with CH_2Cl_2-EtOAc (90:5) to yield **305** (8.8 mg), **245** (11.6 mg), and a trace amount of **252**. Fr. V was purified with CH_2Cl_2-EtOAc (75:25) to give a mixture of **246a** and **246b** (4.7 mg, **246a**: 65-70%). Compounds isolated from experiment Trp#338 are summarized in Table 4.12 (section 4.2.2).

Biotransformation of Isotriptophenolide (194) with TRP4a Cell Cultures: Large Scale Experiment (Trp#336)

Isotriptophenolide (**194**, 1.4953 g) in EtOH (250 mL) was equally divided into 10 flasks of a TRP4a cell culture (21 days old, 550 mL each), and incubated under usual conditions for 7 days. The culture was harvested, and the broth and cells were extracted with EtOAc to give 1.8373 g of broth extract and 3.2540 g of cell extract. A blank experiment was conducted. The starting material **194** (2.5 mg) in EtOH (1.3 mL) was "incubated" in a sterile $MSNA_{0.5}K_{0.5}$ medium (27.5 mL) for 7 days. The mixture was extracted with EtOAc and analyzed by TLC (CH_2Cl_2-EtOAc, 90:5) and ^1H NMR.

Portions of the broth extract (1.8103 g) and the cell extract (3.2079 g) from the biotransformation were subjected to extensive column chromatography. The cell extract (3.2079

g) was chromatographed by stepwise elution with hexanes-EtOAc (6:4), EtOAc and MeOH to give 6 fractions (Frs. I-VI), of which Fr. VI was the MeOH fraction. The broth extract (1.8103 g) was eluted with hexanes-EtOAc (1:1), EtOAc and MeOH to yield 4 fractions (Frs. VII-X), of which Fr. X was the MeOH fraction.

Fr.I was eluted with hexanes-EtOAc (9:1, 8:2) to give 6 fractions (Frs. IA-IF). Fr. IF was purified with CHCl$_3$ and CH$_2$Cl$_2$-EtOAc (90:5) to give **304** (2.9 mg).

Fr. II, combined with Fr. VII, was chromatographed with CH$_2$Cl$_2$-EtOAc (90:5) to yield 3 additional fractions (Frs. IIA-IIC). Fr. IIA was eluted with the same solvent system to give 2 fractions (Frs. IIA1, IIA2), and Fr. IIA2 was combined with Fr. IIB and purified with hexanes-EtOAc (6:4) to recover the starting material **194** (777.4 mg). Fr. IIC was eluted with hexanes-EtOAc (1:1) to produce four fractions (Frs. IIC1-IIC4). Fr. IIC1 was then chromatographed with CH$_2$Cl$_2$-EtOAc (90:5, 90:8), yielding 5 fractions (Frs. IIC1a-IIC1e). Fr. IIC1c, after elution with hexanes-EtOAc (6:4), gave 2 fractions (Frs. IIC1c1, IIC1c2). Fr. IIC1c2 was **308** (0.8 mg), and Fr. IIC1c1 afforded **307** (10.0 mg) after purification with CH$_2$Cl$_2$-EtOAc (9:1). Fr. IIC1d was separated into 5 fractions (Frs. IIC1d1-IIC1d5) by elution with hexanes-EtOAc (6:4). Fr. IIC2 was chromatographed with CH$_2$Cl$_2$-EtOAc (9:1) to yield 4 fractions (Frs. IIC2a-IIC2d). Fr. IIC2b was **245** (4.0 mg) and Fr. IIC2d was **309** (15.1 mg). Fr. IIC2a was then eluted with hexanes-EtOAc (7:3, 6:4) to generate 3 fractions (Frs. IIC2a1-IIC2a3). Fr. IIC2a1 was **307** (0.4 mg) and Fr. IIC2a3 was **252** (0.8 mg). Fr. IIC2c afforded another **309** (3.3 mg) after purification with hexanes-EtOAc (1:1). Fr. IIC3 was chromatographed with CH$_2$Cl$_2$-EtOAc (9:1) to produce 3 fractions (Frs. IIC3a-IIC3c), of which Fr. IIC3c was **308** (28.2 mg). Fr. IIC3a then was separated into 2 fractions (Frs. IIC3a1, IIC3a2) with the same solvent system, and Fr. IIC3a2 was purified with hexanes-EtOAc (6:4) to yield **245** (14.9 mg). Fr. IIC3b was chromatographed with CH$_2$Cl$_2$-EtOAc (8:2) to give 2 fractions (Fr. IIC3b1 and **308** (1.5 mg)). Fr. IIC3b1 was chromatographed with hexanes-EtOAc (6:4) to yield **309** (1.5 mg), **308** (0.5 mg) and **311** (1.8 mg). Fr. IIC4 was eluted with CH$_2$Cl$_2$-EtOAc (8:2) to afford 3 fractions (Frs. IIC4a-IIC4c). Fr. IIC4b was further purified with CH$_2$Cl$_2$-EtOAc (85:15) to give

311 (0.9 mg). Fr. IIC4c was chromatographed with hexanes-EtOAc (1:1) to yield **308** (3.1 mg), and a mixture of **246a** and **246b** (6.0 mg, **246a** about 85%).

Fr. III was chromatographed with CH_2Cl_2-EtOAc (90:5, 9:1) to give 5 fractions (Frs. IIIA-IIIE). Fr. IIIC was then separated into 4 fractions (Frs. IIIC1-IIIC4) with hexanes-EtOAc (7:3), of which Fr. IIIC4 was a mixture of **305** and **306** (3.8 mg). Fr. IIIC1, combined with Fr. IIC1a and Fr. IIC1c1, was purified with CH_2Cl_2-EtOAc (90:6) to recover some **194** (Fr. IIIC1a). Fr. IIIE was chromatographed with hexanes-EtOAc (6:4, 5:5) and CH_2Cl_2-EtOAc (9:1, 85:15) to yield **309** (3.4 mg).

Fr. IV, combined with Fr. VIII, was chromatographed with CH_2Cl_2-EtOAc (90:5) to give 6 fractions (Frs. IVA-IVF), of which Fr. IVC was a mixture of **305** and **306** (195.2 mg). Fr. IVE was separated into 4 fractions (Frs. IVE1-IVE4) by elution with hexanes-EtOAc (1:1). Fr. IVE1 was combined with Fr. IIIB and purified with CH_2Cl_2-EtOAc (90:6) to give some **194** (Fr. IIIB1), which was combined with Fr. IIIC1a to yield **194** (4.1 mg). Fr. IVE3 was purified with CH_2Cl_2-EtOAc (90:8) to afford **252** (6.7 mg).

Fr. V, combined with Fr. IX, was chromatographed with hexanes-EtOAc (1:1) to yield 3 fractions (Frs. VA-VC). Fr. VC was further purified with $CHCl_3$-acetone (9:1) to yield a mixture of **246a** and **246b** (116.9 mg).

The MeOH fractions, which contained Td (**2**) and other polar compounds, were not further chromatographed. Compounds isolated from Trp#336 are summarized in Table 4.13 (section 4.2.2).

Also isolated were some cell-produced compounds, which included **106** (15.3 mg), **108** (10.6 mg), **117** (30.3 mg), **196** (28.3 mg), **200** (5.3 mg), **201** (2.1 mg), **203** (3.2 mg), **204** (2.4 mg), **278** (1.8 mg), squalene (**195**, 36.1 mg) and Tl (**1**, 30.7 mg).

Physical data of (7,8)β,(9,11)α-bis(epoxy)-5α,19-dihydroxy-12-oxo-18(4→3)*abeo*-abieta-3,13-dien-18-oic acid lactone (**311**):

A colorless powder; mp: 114-116°C; $[\alpha]_D^{24}$: -59.1° (c = 0.164, MeOH); UV λ_{max}^{MeOH} (log ε): 216.4 (4.09), 254.3 (3.93), 288.8 (shoulder) (3.55); IR (CHCl$_3$) cm^{-1}: 3500-3600 (OH), 2980 (CH), 1760 (C=O), 1730, 1690 (unsaturated ketone C=O), 1380, 1250, 1050; ^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.00, 1.06 (3H each, both d, J = 6.9 Hz, H16, H17), 1.26 (3H, s, H20), 1.26 (1H, m, H1β, overlapped), 1.81 (1H, br ddd, J = 12.8, 12.8, 6.4 Hz, H1α), 2.22 (1H, m, H2β), 2.30 (1H, d, J = 15.1 Hz, H6β), 2.39 (1H, dd, J = 15.1, 5.3 Hz, H6α), 2.41 (1H, m, H2α), 2.84 (1H, sept d, J = 6.9, 1.0 Hz, H15), 3.63 (1H, s, H11), 3.65 (1H, d, J = 5.3 Hz, H7α), 4.84 (2H, br AB$_q$, Δv = 0.24 ppm, J = 17.1 Hz, H19), 5.77 (1H, d, J = 1.0 Hz, H14); ^{13}C NMR (50.2 MHz, CDCl$_3$) δ: 16.7, 18.4, 20.4, 21.5, 22.8, 27.5, 31.1, 40.4, 54.8, 57.8, 58.6, 65.8, 68.4, 70.9, 126.9, 133.4, (148.6), (159.7), 172.2, (192.2); LRMS m/z (rel. intensity): 358 (M$^+$, 30.1), 340 (41.3), 325 (41.3), 310 (34.5), 297 (50.1), 295 (98.7), 283 (60.8), 281 (34.6), 269 (29.0), 267 (45.1), 219 (86.6), 192 (base peak), 163 (66.4), 149 (52.4); HRMS calcd. for C$_{20}$H$_{22}$O$_6$: 358.1416; found: 358.1407.

Physical data of 12,19-dihydroxy-18(4→3)*abeo*-abieta-3,5,8,11,13-pentaen-7-oxo-18-oic acid lactone (**252**):

Yellowish prisms; mp: 289-291°C; UV λ_{max}^{MeOH} (log ε): 212.7 (4.38), 250.4 (4.09), 285.2 (4.16), 343.7 (3.76); IR (CHCl$_3$) cm^{-1}: 3600 (OH), 2970 (CH), 1745 (C=O), 1650 (unsaturated C=O), 1600 (C=C), 1280, 1030; ^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.29, 1.31 (3H each, both d, J = 6.9 Hz, H16, H17), 1.36 (3H, s, H20), 1.88 (1H, ddd, J = 13.1, 11.1, 6.1 Hz, H1α), 2.50 (1H, m, H1β), 2.60-2.76 (2H, m, H2), 3.18 (1H, sept, J = 6.9 Hz, H15), 5.04 (2H, br AB$_q$, Δv = 0.17 ppm, J = 16.3 Hz, H19), 5.48 (1H, br s, C12-OH), 6.31 (1H, s, H6), 6.90 (1H, s, H11), 8.07 (1H, s, H14); LRMS m/z (rel. intensity): 324 (M$^+$, 45.1), 309

(base peak), 294 (17.5), 281 (8.2), 267 (10.5), 165 (8.7); HRMS calcd. for $C_{20}H_{20}O_4$: 342.1362; found: 324.1364.

6.4.5 Chemical Epoxidation of the Epoxides (305, 306)

A mixture of the epoxides **305** and **306** (14.7 mg, **305:306** approximately 1) was dissolved in benzene (1 mL) followed by addition of t-butyl peroxide (90%, 0.015 mL) and Triton B (0.010 mL) while stirring at r.t.. Two more portions of t-butyl peroxide (0.010 mL each) and Triton B (0.010 mL each) were added at 10.5 h and 15 h, respectively (TLC, hexanes-EtOAc, 1:1, or ether). The reaction was worked up at 29 h, the mixture was diluted with EtOAc (20 mL), and then washed with water (20 mL). The aqueous layer was extracted again with EtOAc (4 x 20 mL) and the EtOAc extracts were combined, washed with brine (60 mL) and dried. The resulting product was repeatedly chromatographed with ether to give recovered **305** (0.7 mg), a new product **312** (6.0 mg) and a mixture of unidentified products (8.5 mg).

Physical data of (7,8)α,(13,14)β-bis(epoxy)-19-hydroxy-12-oxo-18(4→3)*abeo*-abieta-3,9(11)-dien-18-oic acid lactone (**312**):

A colorless crystalline solid; mp: 196-198°C; UV λ_{max}^{MeOH} (log ε): 221.6 (4.52), 250.0 (shoulder) (4.23); IR (CHCl₃) cm⁻¹: 3050 (olefinic CH), 2950 (CH), 1760 (C=O), 1710, 1690 (unsaturated C=O), 1370, 1240, 1050; ¹H NMR (400 MHz, CDCl₃) δ: 0.937, 0.99 (3H each, both d, J = 6.9 Hz, H16, H17), 0.939 (3H, s, H20), 1.56 (1H, m, H1α), 1.97 (1H, ddd, J = 14.5, 13.1, 1.4 Hz, H6β), 2.12 (1H, br dd, J = 13.3, 5.9 Hz, H1β), 2.25 (1H, m, H2β), 2.31 (1H, ddd, J = 14.5, 4.1, 2.3 Hz, H6α), 2.47 (1H, br d, J = 18.4 Hz, H2α), 2.55 (1H, sept, J = 6.9 Hz, H15), 2.87 (1H, br d, J = 13.1 Hz, H5), 3.06 (1H, s, H14), 3.80 (1H, br s, $w_{1/2}$ = 4.7 Hz, H7β), 4.69 (2H, m, H19), 6.16 (1H, s, H11); LRMS m/z (rel. intensity): 342 (M⁺,

2.7), 327 (2.9), 314 (19.3), 299 (base peak), 285 (6.1), 272 (6.7), 257 (5.5), 179 (13.9), 163 (16.6), 151 (19.3); HRMS calcd. for $C_{20}H_{22}O_5$: 342.1467; found: 342.1465.

6.4.6 Biotransformation of Triptophenolide (106) and Related Compounds with TRP4a Cell Cultures

Preliminary Experiments (Trp#322)

A solution of triptophenolide (**106**, 99.3 mg) in EtOH (25 mL) was added to a cell culture of 14 days (550 mL) and incubated under standard conditions. Samples were taken after 2 days (10 mL) and 4 days (22 mL), while the culture was harvested after 7 days. The culture was filtered and the cells and broth were extracted with EtOAc. TLC (toluene-$CHCl_3$-EtOAc-HCO_2H, 105:48:45:5) showed no significant differences between the test samples and control samples, except for a small, slightly less polar, UV active spot in the test samples. The biotransformation samples were combined and subjected to extensive column chromatography (toluene-$CHCl_3$-EtOAc-HCO_2H, 105:48:45:3; hexanes-acetone, 7:3; toluene-$CHCl_3$-EtOAc-HCO_2H, 105:115:45:3; hexanes-EtOAc, 8:2, 7:3). Most of the starting material **106** was recovered (73.8 mg), and two minor compounds (**108**, 0.2 mg; **278**, 0.2 mg) were isolated.

Biotransformation of 106 with TRP4a Cell Cultures of Different Ages (Trp#323-327)

An equal amount of the starting material **106** (25 mg) in EtOH (12.5 mL) was added to the cell cultures (250 mL each in 500 mL flasks) of different ages (0, 8, 14, 21 and 28 days). Control experiments (12.5 mL EtOH in 250 mL culture) were run simultaneously. The cultures were incubated under standard conditions. Samples were taken after incubation for certain periods (1, 2, 4 and 7 days) and extracted with EtOAc (sonicated in EtOAc). The cultures were

harvested after 15 days of incubation and filtered to separate the cells and the broth. The cells and broth were extracted with EtOAc, and all the samples were analyzed by TLC (toluene-$CHCl_3$-EtOAc-HCO_2H, 105:48:45:3).

Biotransformation of 106 with CFEs from TRP4a Cell Cultures (Trp#318b)

The CFE was the same as in experiment Trp#318a (section 6.4.4). The starting material **106** (20 mg) in EtOH (8 mL) was added to a CFE mixture composed of CFE (24 mL, 100 units of peroxidase), acetate buffer (0.1 M, pH 4.7, 70 mL), distilled water (30 mL), 0.24% H_2O_2 (2 mL, 2.2 equiv.), $MnCl_2$ (0.5 equiv.) and FMN (0.5 equiv.). The mixture was stirred at r.t. and samples were taken at 1 and 2 h. The reaction was worked up at 4 h and all samples were extracted with EtOAc and analyzed by TLC (toluene-$CHCl_3$-EtOAc-HCO_2H, 105:48:45:3).

Biotransformation of 106 with CFE from TRP4a Cell Cultures: Increase of CFE-to-Starting Material Ratio (Trp#319)

A cell culture of 21 days (550 mL) was used to prepare the CFE. The cells (68.3 g) were suspended in acetate buffer (0.1 M, pH 4.7, 96 mL) and homogenized. Centrifugation of the resulting suspension gave a CFE (128 mL) with a peroxidase activity of 2.56 units/mL, and a protein concentration of 0.484 mg/mL. The starting material (**106**, 25 mg) in EtOH (10 mL) was added at r.t. to the stirred CFE mixture which was composed of CFE (122 mL, 312 peroxidase units, 2.5 times the normal ratio), acetate buffer (0.1 M, pH 4.7, 14.3 mL; volume adjusted for increased CFE volume), distilled water (33.75 mL; volume adjusted for increased H_2O_2 volume), 0.24% H_2O_2 (6.25 mL, 2.5 x 2.2 equiv.), $MnCl_2$ (2.5 x 0.5 equiv.) and FMN (2.5 x 0.5 equiv.). Samples were taken at 1 and 2 h, and the reaction was worked up at 4 h. All

the samples were extracted with EtOAc and analyzed by TLC (toluene-CHCl$_3$-EtOAc-HCO$_2$H, 105:48:45:3).

Biotransformation of 106 with CFE from TRP4a Cell Cultures:
Increase of Reaction Time (Trp#321)

A cell culture of 15 days (2 x 550 mL) was used to prepare the CFE. The cells obtained (103 g) were suspended in acetate buffer (0.1 M, pH 4.7, 144 mL) and homogenized. Centrifugation of the cell homogenate gave a CFE (190 mL), which had a peroxidase activity of 2.93 units/mL and a protein concentration of 0.446 mg/mL. The CFE mixture was prepared by mixing 188 mL of CFE (4.4 x 125 peroxidase units), distilled water (29 mL; volume adjusted for increased H$_2$O$_2$ volume), 0.24% H$_2$O$_2$ (11 mL, 4.4 x 2.2 equiv.), MnCl$_2$ (4.4 x 0.5 equiv.) and FMN (4.4 x 0.5 equiv.). Buffer was not added because increased CFE volume was more than the required volume of the buffer. The starting material (106, 25 mg) in EtOH (10 mL) was added to the stirred CFE mixture at r.t.. Samples were taken at 4 and 24 h, while the reaction was worked up after 48 h of incubation. All samples were extracted with EtOAc and analyzed by TLC (toluene-CHCl$_3$-EtOAc-HCO$_2$H, 105:48:45:3).

All the samples from above experiments (Trp#318b, 319 and 321) were combined and column chromatographed. Elution with hexanes-EtOAc (6:4), and then EtOAc resulted in 4 fractions (Frs. I-IV). Fr. II was chromatographed with hexanes-EtOAc (8:2) to give recovered starting material 106 (48.5 mg), and quinone 278 (1.7 mg). Fr. III was separated into 2 fractions (Frs. IIIA, IIIB) by elution with hexanes-EtOAc (6:4). Fr. IIIA was purified with hexanes-EtOAc (7:3) to give 108 (0.8 mg), and Fr. IIIB was chromatographed with CH$_2$Cl$_2$-EtOAc-acetic acid (90:5:2.5) to yield Tl (1) (0.3 mg). Efforts to isolate the two polar spots failed because sample decomposition was encountered during column chromatography.

Biotransformation of Quinone 278 with TRP4a Cell Cultures

Preliminary Experiment (Trp#328)

A solution of the quinone **278** (25.8 mg) in EtOH (7.5 mL) was added to a cell culture of 21 days (250 mL in 500 mL flask). The cell culture was incubated under normal conditions. Samples were taken after 1, 3 and 7 days and checked by TLC (toluene-CHCl$_3$-EtOAc-HCO$_2$H, 105:48:45:3). The culture was harvested at day 8, and the cells and broth were extracted with EtOAc.

Column chromatography of the broth extract with hexanes-EtOAc (6:4) and EtOAc gave 3 fractions (Frs. I-III). Fr. I was eluted with CH$_2$Cl$_2$-EtOAc (90:5) to give quinone **278** (3 mg) and a new product (**109**, 1.3 mg). Fr. II was purified with CH$_2$Cl$_2$-acetone (15:1) to yield **313** (0.2 mg). The cell extract was repeatedly chromatographed with CH$_2$Cl$_2$-acetone (15:1, 9:1, 90:3) and hexanes-EtOAc (8:2) to produce recovered **278** (1.6 mg), as well as **313** (0.7 mg).

Larger Scale Experiment (Trp#332)

The starting material **278** (53.2 mg) in EtOH (15 mL) was added to a 21-day-old cell culture (550 mL) and incubated under normal conditions. The cell culture was harvested after 2 days of incubation, and the cells and broth were extracted with EtOAc. a blank experiment was conducted. The starting material **278** (2.6 mg) in EtOH (0.75 mL) was "incubated" in a sterile MSNA$_{0.5}$K$_{0.5}$ medium (25 mL) for 2 days. The mixture was extracted with EtOAc and analyzed by TLC (CH$_2$Cl$_2$-EtOAc, 90:5) and ^{1}H NMR.

The broth extract from the biotransformation was chromatographed consecutively with hexanes-EtOAc (6:4), EtOAc and MeOH to give 5 fractions (Frs. I-V), while the cell extract was eluted with the same system to provide 4 additional fractions (Frs. VI-IX). Fr. II was purified with CH$_2$Cl$_2$-EtOAc (90:3) to give the recovered starting material **278** (18.3 mg) and a new

product (**109**, 1.7 mg). Fr. III was purified by preparative TLC with CH_2Cl_2-acetone (14:1) to give **313** (0.9 mg). Fr. VI was chromatographed with CH_2Cl_2-EtOAc (90:5) to recover more starting material **278**, which was then combined with the recovered starting material from the broth and purified again to yield pure **278** (40.2 mg).

Physical data of 5α,19-dihydroxy-11,14-dioxo-18(4→3)*abeo*-abieta-3,8,11-trien-18-oic acid lactone (**313**):

A pale yellow powder; mp: 161-163°C; $[\alpha]_D^{24}$: -28.4° (c = 0.095, $CHCl_3$); UV λ_{max}^{MeOH} (log ε): 215.5 (3.95), 259.8 (3.98), 341.5 (2.52); IR ($CHCl_3$) cm^{-1}: 3600 (OH), 3010, 2975 (CH), 1765 (C=O), 1725, 1660 (quinone C=O), 1610 (quinone C=C), 1380, 1220, 1060; ^1H NMR (400 MHz, $CDCl_3$) δ: 1.10, 1.11 (3H each, both d, J = 6.9 Hz, H16, H17), 1.21 (3H, s, H20), 1.84 (1H, s, C5-OH, D_2O exchangeable), 1.85-2.06 (3H, m, H1α, H6), 2.41 (2H, m, H2), 2.72 (2H, m, H7), 2.99 (1H, sept d, J = 6.9, 1.0 Hz, H15), 3.16 (1H, br dd, J = 15.0, 6.0 Hz, H1β), 4.86 (2H, br AB_q, Δν = 0.21 ppm, J = 17.2 Hz, H19), 6.40 (1H, d, J = 1.0 Hz, H12); ^{13}C NMR (50.2 MHz, $CDCl_3$) δ: 17.9, 20.2, 21.3, 21.4, 23.4, 23.5, 23.9, 26.5, 41.7, 68.8 (C19), 69.9 (C5), 127.9, 131.9, 142.7, 144.9, 153.4, 160.6, 173.6, 186.8, 188.0; LRMS m/z (rel. intensity): 342 (M^+, 47.5), 326 (17.1), 324 (14.3), 309 (21.0); HRMS calcd. for $C_{20}H_{22}O_5$: 342.1467; found: 342.1459.

Biotransformation of 106 with TRP4a Cell Cultures: Large Scale Experiment (Trp#334)

Triptophenolide (**106**, 300 mg) in EtOH (75 mL) was equally divided into 3 flasks of TRP4a cell cultures (21 days old, 550 mL each) and incubated under standard conditions for 7 days. The culture was harvested at the end of the incubation and filtered through Miracloth™. The resulting cells and broth were extracted with EtOAc to yield 547.7 mg of broth extract and

743.1 mg of cell extract. The extracts were combined and column chromatographed. A blank experiment was conducted. The starting material **106** (2.5 mg) in EtOH (0.6 mL) was "incubated" in a sterile $MSNA_{0.5}K_{0.5}$ medium (13.8 mL) for 7 days. The mixture was extracted with EtOAc and analyzed by TLC (hexanes-EtOAc, 1:1) and ^1H NMR.

Column chromatography of the combined extract from the biotransformation (Trp#334) with hexanes-EtOAc (6:4, 4:6, 5:5), EtOAc and EtOAc-MeOH (1:1) gave 7 fractions (Frs. I-VII). Fr. I was a mixture of lesser polar compounds from the cell culture, and Fr. VII was the combined MeOH eluates.

Fr. II was eluted with CH_2Cl_2-EtOAc (98:2, 90:5) to yield 4 fractions (Frs. IIA-IID), of which Fr. IID was **109** (2.6 mg). Fr. IIA was chromatographed with hexanes-acetone (9:1), hexanes-EtOAc (8:2) and CH_2Cl_2-EtOAc (97:3) to give **108** (0.3 mg) and **278** (0.3 mg). Fr. IIB was purified with hexanes-EtOAc (8:2) to give some **108** (0.2 mg), and Fr. IIC produced more **108** (5.7 mg) and **278** (1.7 mg) after elution with hexanes-EtOAc (7:3).

Fr. III was separated into 4 fractions (Frs. IIIA-IIID) by chromatography with CH_2Cl_2-EtOAc (90:3, 90:5). Fr. IIIA was recovered starting material **106** (192.0 mg) and Fr. IIIB yielded **108** (1.3 mg) after being eluted with hexanes-EtOAc (7:3). Fr. IIIC was chromatographed with hexanes-EtOAc (7:3) to give **108** (2.1 mg). Fr. IIID was eluted with hexanes-acetone (8:2) to give 2 fractions (Frs. IIID1, IIID2). Fr. IIID1 was further purified with CH_2Cl_2-acetone (85:15, 9:1) to provide **138a** (6.8 mg), and Fr. IIID2 was repeatedly chromatographed with CH_2Cl_2-acetone (9:1) to afford **315** (3.2 mg).

Fr. IV was chromatographed with CH_2Cl_2-acetone (8:1, 8:2) to yield **138b** (2.5 mg) and **304** (1.6 mg).

Fr. V was eluted with CH_2Cl_2-acetone (9:1, 8:2) and acetone to give **106** (1.4 mg), **316** (4.9 mg) and **315** (5.5 mg).

Fr. VI was purified with CH_2Cl_2-acetone (8:2), hexanes-acetone (7:3) to afford **278** (2.8 mg).

Compounds isolated from Trp#334 are summarized in Table 4.15 in section 4.2.4.

Physical data of 7α,14,19-trihydroxy-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (**138b**):

^{1}H NMR (400 MHz, CDCl$_3$) δ: 0.97 (3H, s, H20), 1.23, 1.24 (3H each, both d, J = 7.0 Hz, H16, H17), 1.67 (1H, m, H1α), 2.02 (1H, m, H6β), 2.16-2.60 (4H, m, H1β, H2, H6α), 2.95 (1H, br d, J = 12.5 Hz, H5), 3.24 (1H, sept, J = 7.0 Hz, H15), 4.79 (2H, br AB$_q$, Δν = 0.1 ppm, J = 17.2 Hz, H19), 5.12 (1H, m, H7β), 6.72 (1H, s, C14-OH), 6.93 (1H, d, J = 8.2 Hz, H11), 7.17 (1H, d, J = 8.2 Hz, H12).

Physical data of 5β,14,19-trihydroxy-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (**315**):

A pale yellowish powder; mp: 95-98°C; UV λ_{max}^{MeOH} (log ε): 222.0 (3.81), 279.8 (3.32); $[\alpha]_D^{24}$: +124.9° (c = 0.37, CHCl$_3$); IR (CHCl$_3$) cm^{-1}: 3600 (OH), 3500 (OH), 3050 (aromatic CH), 2980 (CH), 1750 (C=O), 1715, 1460, 1240, 1080, 1040; ^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.24 (6H, d, J = 6.8 Hz, H16, H17), 1.35 (3H, s, H20), 1.88-2.06 (3H, m, H1α, H2α, H6β), 2.12 (1H, m, H1β), 2.15-2.27 (2H, m, H2β, H6α), 2.54 (1H, ddd, J = 17.3, 7.4, 7.4 Hz, H7β), 2.86 (1H, ddd, J = 17.3, 6.3, 6.3 Hz, H7α), 3.03 (1H, sept, J = 6.8 Hz, H15), 4.72 (1H, br s, C14-OH), 4.91 (2H, br AB$_q$, Δν = 0.10 ppm, J = 16.8 Hz, H19), 6.91 (1H, d, J = 8.3 Hz, H11), 7.06 (1H, d, J = 8.3 Hz, H12); ^{13}C NMR (50.2 MHz, CDCl$_3$) δ: 18.0, 20.5, 22.5, 22.6, 23.8, 26.9, 29.3, 33.9, 42.0, 69.2, 71.4, 119.2, 120.6, 124.2, 128.1, 130.4, 139.7, 149.8, 162.3, 173.6; LRMS m/z (rel. intensity): 328 (M$^+$, 82.4), 313 (22.9), 310 (36.5), 295 (71.5), 267 (36.0); HRMS calcd. for C$_{20}$H$_{24}$O$_4$: 328.1675; found: 328.1669.

Physical data of 5α,14,19-trihydroxy-18(4→3)abeo-abieta-3,8,11,13-tetraen-18-oic acid lactone (**316**):

Small colorless prisms; mp: 190-191°C; UV λ_{max}^{MeOH} (log ε): 222.7 (3.86), 282.4 (3.55); $[\alpha]_D^{20}$: -58.8° (c = 0.32, MeOH); IR (CHCl₃) cm⁻¹: 3607 (OH), 3560 (OH), 3020 (aromatic CH), 2973 (CH), 1756 (C=O), 1500, 1430, 1340, 1240, 1180; ¹H NMR (400 MHz, CDCl₃) δ: 1.09 (3H, s, H20), 1.25, 1.27 (3H each, both d, J = 6.8 Hz, H16, H17), 1.90 (1H, s, C5-OH, D₂O exchangeable), 1.98-2.15 (2H, m, H1α, H6α), 2.23 (1H, ddd, J = 13.9, 9.1, 9.1 Hz, H6β), 2.31 (1H, dd, J = 12.9, 6.2 Hz, H1β), 2.39 (1H, m, H2β), 2.55 (1H, br d, J = 16.2 Hz, H2α), 2.80-3.02 (2H, m, H7), 3.08 (1H, sept, J = 6.8 Hz, H15), 4.80 (1H, s, C14-OH), 4.90 (2H, br ABq, Δν = 0.19 ppm, J = 17.1 Hz, H19), 6.91 (1H, d, J = 8.2 Hz, H11), 7.08 (1H, d, J = 8.2 Hz, H12); ¹³C NMR (50.2 MHz, CDCl₃) δ: 18.0, 18.3, 22.5, 22.6, 25.4, 26.2, 26.3, 26.9, 41.3, 69.2 (C19), 69.8 (C5), 117.1, 119.7, 124.1, 127.1, 131.4, 140.4, 150.7, 160.9, 173.8; LRMS m/z (rel. intensity): 328 (M⁺, 25.7), 310 (51.0), 295 (89.5), 267 (42.9), 253 (base peak), 165 (25.5), 147 (35.9); HRMS calcd. for C₂₀H₂₄O₄: 328.1674; found: 328.1675.

6.4.7 Biotransformation of Triptophenolide (106) with the Fungus *Cunninghamella elegans*

Ten flasks of *Cunninghamella elegans* var. chibaensis Kuwabara *et* Hoshino (ATCC#20230) cultures (100 mL each flask) grown in SSBF medium with 2% glucose for 44 h were used in the experiment (SSBF medium with 2% glucose (g/L): soybean flour 1% fat (powder), 5.0 g; NaCl, 5.0 g; KH₂PO₄, 5.0 g; yeast extract, 5.0 g; glucose, 20.0 g; pH = 7.0). The starting material **106** (200 mg) was dissolved in EtOH (20 mL) and evenly divided into the 10 flasks. The culture was incubated at 28°C on a rotary shaker (240 rpm). A control experiment (2 mL EtOH added to 100 mL culture) was conducted at the same time. Samples

were taken at 30, 48 and 72 h and checked by TLC (hexanes-EtOAc, 1:1). The culture was harvested after 76 h of incubation by adding EtOAc (100 mL each flask) and mixing thoroughly prior to storage at 4 °C overnight. The mixtures were combined and the EtOAc layer was separated. The aqueous layer was re-extracted with EtOAc (3 x 600 mL), and all EtOAc extracts were combined, dried, and concentrated to give a broth extract (193.1 mg). The cells were homogenized with a homogenizer (Ultra Turrax, TK 25) (28,000 rpm, 3 x 1 min) in EtOAc (1.5 L) and filtered. The EtOAc extract of the cells was dried, filtered and the solvent was removed to yield the cell extract (1.9676 g).

Column chromatography of the broth extract with hexanes-EtOAc (8:2, 7:3, 6:4) and EtOAc yielded 6 fractions (Frs. I-VI). Fr. II was eluted with CH_2Cl_2-EtOAc (95:5) to give quinone **278** (13.1 mg) and recovered starting material **106** (2.5 mg). Fr. III was chromatographed with the same solvent system yielding an additional amount of the quinone **278** (8.2 mg), and a fraction which basically contained **314** (12.9 mg). Fr. IV produced crystals of **318** (3.5 mg) during concentration and the mother liquor was purified with hexanes-EtOAc (1:1) to yield another portion of **318** (3.0 mg). Fr. V was eluted with hexanes-EtOAc (1:1) to afford **313** (1.1 mg) and **316** (10.5 mg), while Fr. VI provided more **318** (1.2 mg) and **313** (0.1 mg) after being chromatographed with hexanes-EtOAc (1:1) and CH_2Cl_2-EtOAc (8:2). Column chromatography of the cell extract with hexanes-EtOAc (6:4, 1:1) and EtOAc gave 4 fractions (Frs. VII-X). Repeated chromatography of Fr. VIII with hexanes-EtOAc (7:3) and CH_2Cl_2-EtOAc (95:5) yielded more quinone **278** (9.6 mg) and the starting material **106** (2.3 mg). The compounds isolated from this experiment are summarized in Table 4.16.

Physical data of 11,14,19-trihydroxy-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (**314**):

A white powder; mp: 197-199°C (dec); IR (KBr) cm^{-1}: 3350 (OH), 2975 (CH), 1730 (C=O), 1670 (C=C), 1405, 1080, 1020; ^{1}H NMR (400 MHz, CD$_3$OD) δ: 1.147 (3H, s, H20),

1.150, 1.17 (3H each, both d, J = 6.8 Hz, H16, H17), 1.47 (1H, m, H1α), 1.76 (1H, dddd, J = 12.6, 12.6, 12.6, 6.0 Hz, H6β), 1.91 (1H, m, H6α), 2.29 (2H, m, H2), 2.72 (2H, m, H5, H7α), 2.91 (1H, dd, J = 17.6, 6.0 Hz, H7β), 3.22 (1H, sept, J = 6.8 Hz, H15), 3.72 (1H, br d, J = 13.0 Hz, H1β), 4.88 (2H, br ABq, Δν = 0.13 ppm, J = 17.5 Hz, H19), 6.47 (1H, s, H12); ^{13}C NMR (50.2 MHz, CD$_3$OD) δ: 17.5, 19.4, 20.5, 23.2, 23.5, 26.8, 27.6, 32.2, 38.2, 45.4, 72.4, 112.1, 125.4, 126.8, 129.8, 135.7, 145.1, 151.0, 167.1, 177.1; LRMS m/z (rel. intensity): 328 (M$^+$, base peak), 313 (67.2), 271 (17.6), 204 (27.3), 149 (10.0); HRMS calcd. for C$_{20}$H$_{24}$O$_4$: 328.1675; found: 328.1671.

Catalytic reduction of quinone 278

The quinone **278** (8.2 mg) was dissolved in MeOH (1.5 mL) and stirred under H$_2$ (1 atm) with a catalytic amount of Pd/C. TLC (CH$_2$Cl$_2$-EtOAc, 90:5) showed that about half amount of the quinone **278** was reduced to **314** in about 15 min and the reaction was completed in 2 h. The reaction mixture was filtered through Celite and the filtrate was concentrated (7.2 mg). ^{1}H NMR analysis showed that the product was almost pure **314**.

Physical data of 11-acetoxy-14,19-dihydroxy-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (**318**):

A small white crystalline solid; mp: 282-284°C (dec); [α]$_D^{20}$: +75.6° (c = 0.17, CHCl$_3$); UV λ$_{max}^{MeOH}$ (log ε): 213.0 (4.18), 278.0 (3.30); IR (KBr) cm^{-1}: 3425 (OH), 2950 (CH), 1750 (acetate C=O), 1720 (lactone C=O), 1670 (C=C), 1210, 1070, 1020; ^{1}H NMR (400 MHz, CDCl$_3$) δ: 1.07 (3H, s, H20), 1.21, 1.24 (3H each, both d, J = 6.9 Hz, H16, H17), 1.64 (1H, ddd, J = 12.5, 12.5, 6.7 Hz, H1α), 1.81 (1H, m, H6β), 1.90 (1H, m, H6α), 2.32 (3H, s, -COCH$_3$), 2.34 (1H, m, H2β), 2.44 (1H, br d, J = 18.0 Hz, H2α), 2.67-2.79 (2H, m, H5, H7α), 2.89 (1H, dd, J = 17.2, 5.5 Hz, H7β), 2.99 (1H, br dd, J = 12.5, 6.2 Hz, H1β), 3.05 (1H, sept, J = 6.9 Hz, H15), 4.67 (1H, s, C14-OH, D$_2$O exchangeable), 4.76 (2H, br ABq, Δν

= 0.11 ppm, J = 17.2 Hz, H19), 6.66 (1H, s, H12); ^{13}C NMR (50.2 MHz, CDCl$_3$) δ: 18.5, 18.9, 19.3, 21.7, 22.2, 22.5, 24.8, 27.0, 31.6, 37.0, 43.0, 70.5, 119.5, 123.1, 125.1, 132.1, 134.1, 142.5, 148.6, 162.4, 170.2, 174.0; LRMS m/z (rel. intensity): 370 (M$^+$, 11.6), 328 (base peak), 313 (41.4), 285 (3.4), 271 (7.2), 204 (9.8), 165 (9.1), 149 (13.3); HRMS calcd. for C$_{22}$H$_{26}$O$_5$: 370.1780; found: 370.1780.

6.4.8 Biotransformation of DINTP (288) with TRP4a Cell Cultures

Preliminary Experiment (Trp#301b)

A solution of DINTP (**288**, 100 mg) in EtOH (50 mL) was equally divided into 2 flasks of a TRP4a cell culture (21 days, 550 mL each) and the culture was incubated under normal conditions. Samples were taken at 26 and 72 h and checked by TLC (toluene-CHCl$_3$-EtOAc-HCO$_2$H, 105:48:45:3). The culture was harvested at 75 h.

Large Scale Experiment

A solution of DINTP (**288**, 300 mg) in EtOH (75 mL) was equally divided and added to 3 flasks of a TRP4a cell culture (21 days, 550 mL each). The cell cultures were incubated under normal conditions for 41 h and then harvested. The broth and cells were extracted with EtOAc separately to give 539.3 mg broth extract and 668.2 mg cell extract. A blank experiment was carried out. The starting material **288** (2.5 mg) in 0.6 mL EtOH was "incubated" in a sterile MSNA$_{0.5}$K$_{0.5}$ medium (13.8 mL) for 41 h. The mixture was extracted with EtOAc and the resulting sample was analyzed by TLC and ^{1}H NMR.

Column chromatography of the broth extract from the biotransformation with hexanes-EtOAc (6:4) and EtOAc yielded 4 fractions (Frs. I-IV), and the cell extract was eluted with the same solvent system to give 5 fractions (Frs. V-IX).

Frs. I and V were combined and chromatographed with CH$_2$Cl$_2$-EtOAc (90:5, 9:1, 8:2) and hexanes-EtOAc (7:3) to yield **319** (0.6 mg).

Fr. II was eluted with CH$_2$Cl$_2$-EtOAc (90:5), EtOAc to give 4 fractions (Frs. IIA-IID). Fr. IIA was chromatographed with CH$_2$Cl$_2$-acetone (15:1), yielding **320** (1.9 mg). Fr. IIB was separated into 3 fractions (Frs. IIB1-IIB3) by elution with CH$_2$Cl$_2$-acetone (20:1). Fr. IIB3 was compound **321** (5.3 mg), and Fr. IIB1 afforded **319** (0.6 mg) after being eluted twice with CH$_2$Cl$_2$-acetone (20:1) on preparative TLC.

Fr. III was separated into 3 fractions (Frs. IIIA-IIIC) with elution of hexanes-EtOAc (6:4, 5:5) and EtOAc. Fr. IIIA yielded **322** (3.3 mg) by chromatographed with CH$_2$Cl$_2$-EtOAc (9:1), and Fr. IIIB gave **322** (3.0 mg) and another fraction (Fr. IIIB2) by elution with the same solvent system. Fr. IIIC, after elution with CH$_2$Cl$_2$-EtOAc (9:1, 8:2), produced some crude **322**, which was combined with Fr. IIIB2 and chromatographed with ether, CHCl$_3$-acetone (95:3) to afford **322** (4.7 mg).

Fr. VI was chromatographed with hexanes-EtOAc-CHCl$_3$-formic acid (7:5:5:0.3) to give 2 fractions (Frs. VIA, VIB). Fr. VIA was eluted with CH$_2$Cl$_2$-acetone (40:1) to yield 2 fractions (Frs. VIA1, VIA2). Fr. VIA2 was chromatographed with hexanes-EtOAc (8:2, 6:4) to yield a mixture of **319** and **320** (0.5 mg).

Fr. VII was separated into 2 fractions (Frs. VIIA, VIIB) by elution with hexanes-EtOAc-CHCl$_3$-formic acid (7:5:5:0.3). Fr. VIIA was chromatographed with CH$_2$Cl$_2$-acetone (40:1) to yield 2 fractions (Frs. VIIA1, VIIA2). Fr. VIIA1 was combined with Fr. VIA1 and chromatographed with hexanes-EtOAc (7:3) to give 2 fractions (Frs. VIA1a, VIA1b), of which Fr. VIA1b was **321** (4.7 mg), and Fr. VIA1a was further purified by chromatography with isopropyl ether, CH$_2$Cl$_2$-EtOAc (90:5) to yield **320** (1.9 mg). Fr. VIIA2 was combined with Fr. IIB2, IID, IIC, VIB and VIIB, and then chromatographed with CH$_2$Cl$_2$-acetone (95:5) to produce 4 fractions (Frs. IIC1-IIC4). Fr. IIC2 was eluted with CHCl$_3$-acetone (95:5) to give recovered starting material **288** (70.3 mg) and another fraction (Fr. IIC2a), which was combined

with Fr. IIC1 and chromatographed with $CHCl_3$-acetone (99:1) to yield **321** (4.6 mg). Fr.IIC3 gave **322** (2 mg) by elution with hexanes-EtOAc (1:1)

Fr. IX was separated into 3 fractions (Frs. IXA-IXC) by elution with CH_2Cl_2-EtOAc (9:1, 8:2, 6:4). Fr. IXB was chromatographed with hexanes-EtOAc (6:4, 5:5) to yield **322** (2.7 mg). Fr. IXC also gave **322** (2.6 mg) after elution with hexanes-EtOAc (1:1).

Compounds isolated from biotransformation of **288** are summarized in Table 4.17 (section 4.2.5). Compound **322** was decomposed thus it is not included.

Physical data of 12,19-dihydroxy-14-methoxy-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (**319**):

A pale yellow powder; $[\alpha]_D^{24}$: +20.0° (c = 0.125, MeOH); UV λ_{max}^{MeOH} (log ε): 212.0 (4.26), 283.4 (3.42); IR ($CHCl_3$) cm^{-1}: 3600 (OH), 2950 (CH), 1750 (C=O), 1680 (C=C), 1610, 1420, 1060; ^1H NMR (400 MHz, $CDCl_3$) δ: 0.99 (3H, s, H20), 1.35, 1.36 (3H each, both d, J = 7.1 Hz, H16, H17), 1.66 (1H, m, H1α), 1.81 (1H, m, H6β), 1.90 (1H, m, H6α), 2.36 (2H, m, H1β, H2β), 2.48 (1H, m, H2α), 2.64 (1H, br d, J = 13.3 Hz, H5), 2.83 (1H, ddd, J = 17.8, 10.3, 8.2 Hz, H7α), 2.94 (1H, ddd, J = 17.8, 7.4, 1.6 Hz, H7β), 3.41 (1H, sept, J = 7.1 Hz, H15), 3.68 (3H, s, C14-OCH_3), 4.64 (1H, br s, C12-OH), 4.75 (2H, br AB_q, Δν = 0.05 ppm, J = 17.1 Hz, H19), 6.50 (1H, s, H11); ^{13}C NMR (50.2 MHz, $CDCl_3$) δ: 18.2, 19.7, 21.0, 21.1, 22.2, 22.4, 25.2, 32.7, 36.3, 41.2, 60.7, 70.5, 108.3, 118.0, 120.3, 124.9, 143.6, 153.5, (156.9), 163.0, 174.2; LRMS m/z (rel. intensity): 342 (M+, 69.7), 327 (39.7), 299 (6.9), 285 (11.0), 271 (4.2), 179 (base peak), 165 (7.4), 149 (18.3); HRMS calcd. for $C_{21}H_{26}O_4$: 342.1831; found: 342.1838.

Physical data of 14,19-dihydroxy-12-methoxy-18(4→3)*abeo*-abieta-3,8,11,13-tetraen-18-oic acid lactone (**320**):

A colorless powder; mp: 206-208°C (dec); UV λ_{max}^{MeOH} (log ε): 206.5 (4.59), 276.9 (3.59); $[\alpha]_D^{24}$: +30.9° (c = 0.081, MeOH); IR (CHCl₃) cm⁻¹: 3630 (OH), 2950 (CH), 1750 (C=O), 1680 (C=C), 1620, 1580, 1070; ¹H NMR (400 MHz, CDCl₃) δ: 1.02 (3H, s, H20), 1.32 (6H, d, J = 7.2 Hz, H16, H17), 1.70 (1H, br ddd, J = 12.6, 12.6, 6.8 Hz, H1α), 1.88 (1H, m, H6β), 1.96 (1H, m, H6α), 2.38 (1H, m, H2β), 2.46 (1H, dd, J = 12.6, 6.4 Hz, H1β), 2.50 (1H, m, H2α, overlapped), 2.63-2.84 (3H, m, H5, H7), 3.45 (1H, sept, J = 7.2 Hz, H15), 3.78 (3H, s, C12-OCH₃), 4.73 (1H, s, C14-OH), 4.77 (2H, br ABq, Δν = 0.07 ppm, J = 17.1 Hz, H19), 6.48 (1H, s, H11); ¹³C NMR (50.2 MHz, CDCl₃) δ: 18.2, 19.7, 20.9, 21.0, 22.1, 22.3, 24.3, 32.7, 36.5, 41.1, 55.7, 70.5, 99.8, 113.5, 119.5, 125.1, 143.7, 152.3, 156.7, 162.9, 174.1; LRMS m/z (rel. intensity): 342 (M⁺, 80.8), 327 (base peak), 299 (1.8), 285 (8.9), 267 (2.6), 241 (2.0), 179 (83.4), 163 (12.9), 149 (29.4); HRMS calcd. for C₂₁H₂₆O₄: 342.1831; found: 342.1827.

Physical data of 12,19-dihydroxy-11,14-dioxo-18(4→3)*abeo*-abieta-3,8,12-trien-18-oic acid lactone (**321**):

A yellow powder; $[\alpha]_D^{24}$: +138.0° (c = 0.487, MeOH); UV λ_{max}^{MeOH} (log ε): 206.6 (4.33), 273.8 (4.31); IR (CHCl₃) cm⁻¹: 3450 (OH), 2950 (CH), 1750 (C=O), 1680 (C=C), 1640 (quinone C=O), 1600 (quinone C=C), 1460, 1400, 1300, 1040; ¹H NMR (400 MHz, CDCl₃) δ: 1.12 (3H, s, H20), 1.20, 1.21 (3H each, both d, J = 7.0 Hz, H16, H17), 1.49 (1H, ddd, J = 13.3, 11.2, 7.3 Hz, H1α), 1.66 (1H, dddd, J = 13.4, 13.4, 11.2, 6.1 Hz, H6β), 1.87 (1H, br ddd, J = 13.4, 7.4, 1.9 Hz, H6α), 2.41 (2H, m, H2), 2.54 (1H, ddd, J = 21.2, 11.2, 7.4 Hz, H7α), 2.59 (1H, br d, J = 13.4 Hz, H5), 2.81 (1H, dd, J = 21.2, 6.1 Hz, H7β), 3.12 (1H, br dd, J = 13.3, 4.8 Hz, H1β), 3.14 (1H, sept, J = 7.0 Hz, H15), 4.74 (2H, br ABq, Δν =

0.11 ppm, J = 17.2 Hz, H19), 7.16 (1H, br s, C12-OH, D_2O exchangeable); ^{13}C NMR (50.2 MHz, $CDCl_3$) δ: 18.3, 18.4, 18.5, 19.8, 19.9, 24.2, 25.0, 30.8, 36.6, 42.5, 70.2, 124.6, 125.6, 143.4, 145.7, 150.6, 161.1, 173.7, 183.1, 186.7; LRMS m/z (rel. intensity): 342 (M^+, base peak), 327 (32.3), 309 (8.3), 299 (7.3), 281 (9.5), 218 (11.7), 179 (98.4), 149 (15.7); HRMS calcd. for $C_{20}H_{22}O_5$: 342.1467; found: 342.1467.

Reference

(1)   X.-Y. Lu, C.-P. Zhang, and Y. Chen. *Jiang Su Yi Yao* **1987**, 640-643.

(2)   D.-G. Wu, X.-C. Sun, and F. Li. *Acta Botanica Yunnanica* **1979**, *1*, 29-35.

(3)   X.-Y. Wu. M. Sc. Thesis, Shanghai Institute of Materia Medica, Chinese Acadmy of Sciences, 1993.

(4)   *An Encyclopedia of Traditional Chinese Medicinal Substances*; 1 st ed.; Jiangsu College of New Medicine, Ed.; Shanghai Publishing House of Science and Technology: Shanghai, 1977; Vol. 2.

(5)   *Iconographia Cormophytorum Sinicorum, Supplementum II*; 1 st ed.; Botany Institute-Chinese Academy of Sciences, Ed.; Science Publishing House: Beijing, 1983, p 266.

(6)   *Iconographia Cormophytorum Sinicorum*; 1 st ed.; Botany Institute-Chinese Academy of Sciences, Ed.; Science Publishing House: Beijing, 1972; Vol. 2, p 686.

(7)   Nanjing College of Traditional Chinese Medicine. *Chinese Materia Medica*; Jiangsu Science and Technology Publishing House: Nanjing, 1978, p 1.

(8)   *Chinese Herbal Medicine-Materia Medica*; revised ed.; D. Bensky, A. Gamble, and T. Kaptchuk, Eds.; Eastland Press, Inc.: Seattle, 1993.

(9)   S.-F. Chiu, B.-Q. Huang, and M.-Y. Hu. *Acta Entomologica Sinica* **1989**, *32*, 158-165.

(10)   S.-F. Chiu and Y.-T. Qiu. *Journal of Applied Entomology* **1993**, *116*, 479-486.

(11)   S.-Z. Qian. *Contraception* **1987**, *36*, 335-345.

(12)   J.-L. Guo, S.-X. Yuan, X.-C. Wang, S.-X. Xu, and D.-D. Li. *Chinese Medical Journal (English Edition)* **1981**, *94*, 405-412.

(13)   Institute of Dermatology-Chinese Academy of Medical Sciences. *Zhong Hua Pi Fu Ke Za Zhi (Chinese Dermatology Journal)* **1982**, *15*, 199-201.

(14)   W.-Y. Xu, J.-R. Zheng, and X.-Y. Lu. *Intl. J. Dermatol.* **1985**, *24*, 152-157.

(15)   S.M. Kupchan, W.A. Court, R.G. Dailey Jr., C.J. Gilmore, and R.F. Bryan. *J. Am. Chem. Soc.* **1972**, *94*, 7194-7195.

(16)   K. Chen, Q. Shi, T. Fujioka, D.-C. Zhang, C.-Q. Hu, J.-Q. Jin, R.E. Kilkuskie, and K.-H. Lee. *J. Nat. Prod.* **1992**, *55*, 88-92.

(17)   K. Chen, Q. Shi, Y. Kashiwada, D.-C. Zhang, C.-Q. Hu, J.-Q. Jin, H. Nozaki, R.E. Kilkuskie, E. Tramontano, Y.-C. Cheng, D.R. McPhail, A.T. McPhail, and K.-H. Lee. *J. Nat. Prod.* **1992**, *55*, 340-346.

(18)   T.-Q. Chou and P.-F. Mei. *Chinese J. Physiol.* **1936**, *10*, 529-534.

(19) O. Gisvold. *J. Am. Pharm. Assoc.* **1939**, *28*, 440-443.

(20) M.S. Schechter and H.L. Haller. *J. Am. Chem. Soc.* **1942**, *64*, 182-183.

(21) J. Acree Fred and H.L. Haller. *J. Am. Chem. Soc.* **1950**, *72*, 1608-1611.

(22) M. Beroza. *Anal. Chem.* **1950**, *22*, 1507-1510.

(23) M. Beroza. *J. Am. Chem. Soc.* **1951**, *73*, 3656-3659.

(24) M. Beroza. *J. Am. Chem. Soc.* **1952**, *74*, 1585-1588.

(25) M. Beroza. *J. Am. Chem. Soc.* **1953**, *75*, 2136-2138.

(26) D.-G. Wu. *Acta Botanica Yunnanica* **1986**, *8*, 343-354.

(27) D.-G. Wu, L. Liu, and K.-C. Chen. *Acta Bontanica Yunnanica* **1981**, *3*, 471-473.

(28) Z.-S. He, S.-H. Hong, Y. Li, H. Sha, and X.-G. Yu. *Acta Chimica Sinica* **1985**, *43*, 593-596.

(29) Z.-S. He, Y. Li, S.-D. Fang, and S.-H. Hong. *Acta Chimica Sinica* **1987**, *45*, 510-513.

(30) F.-X. Deng, J.-H. Cao, Z.-L. Xia, S. Lin, and X.-Y. Wang. *Acta Bontanica Sinica* **1987**, *29*, 523-526.

(31) F.-X. Deng, Z.-L. Xia, R.-Q. Xu, and J.-Y. Chen. *Acta Bontanica Sinica* **1992**, *34*, 618-621.

(32) Y. Li, G.M. Strunz, and L.A. Calhoun. *Can. J. Chem.* **1990**, *68*, 371-374.

(33) Z.-S. He, Y. Li, S.-D. Fang, and S.-H. Hong. *Acta Chimica Sinica* **1989**, *47*, 178-181.

(34) Y. Li, G.M. Strunz, and L.A. Calhoun. *Phytochemistry* **1991**, *30*, 719-721.

(35) C.-Q. Chen, J.-K. Liu, and D.-G. Wu. *Phytochemistry* **1992**, *31*, 4391-4392.

(36) S.M. Kupchan, H.P.J. Hintz, R.M. Smith, A. Karim, M.W. Cass, W.A. Court, and M. Yatagai. *J. Chem. Soc., Chem. Commun.* **1974**, 329-330.

(37) S.M. Kupchan, H.P.J. Hintz, R.M. Smith, A. Karim, M.W. Cass, W.A. Court, and M. Yatagai. *J. Org. Chem.* **1977**, *42*, 3660-3664.

(38) Y. Takaishi, K. Ujita, K. Nakano, K. Murakami, and T. Tomimatsu. *Phytochemistry* **1987**, *26*, 2325-2329.

(39) H. Hori, G.-M. Pang, K. Harimaya, Y. Iitaka, and S. Inayama. *Chem. Pharm. Bull.* **1987**, *35*, 4683-4686.

(40) Y. Takaishi, K. Ujita, K. Kida, M. Shibuya, and T. Tomimatsu. *Phytochemistry* **1987**, *26*, 2581-2584.

(41) Y. Takaishi, K. Ujita, K. Nakano, and T. Tomimatsu. *Chem. Pharm. Bull.* **1988**, *36*, 4275-4283.

(42)   Y. Takaishi, K. Ujita, H. Noguchi, K. Nakano, T. Tomimatsu, S. Kadota, K. Tsubono, and T. Kikuchi. *Chem. Pharm. Bull.* **1987**, *35*, 3534-3537.

(43)   Y. Takaishi, S. Tamai, K. Nakano, K. Murakami, and T. Tomimatsu. *Phytochemistry* **1991**, *30*, 3027-3031.

(44)   Y. Takaishi, F. Aihara, S. Tamai, K. Nakano, and T. Tomimatsu. *Phytochemistry* **1992**, *31*, 3943-3947.

(45)   Y. Takaishi, K. Tokura, S. Tamai, K. Ujita, K. Nakano, and T. Tomimatsu. *Phytochemistry* **1991**, *30*, 1567-1572.

(46)   Y. Takaishi, H. Noguchi, K. Murakami, K. Nakano, and T. Tomimatsu. *Phytochemistry* **1990**, *29*, 3869-3873.

(47)   Y. Takaishi, K. Tokura, H. Noguchi, K. Nakano, K. Murakami, and T. Tomimatsu. *Phytochemistry* **1991**, *30*, 1561-1566.

(48)   K. Nakanishi, Y. Takahashi, and H. Budzikiewicz. *J. Org. Chem.* **1965**, *30*, 1729-1734.

(49)   D.-M. Zhang, D.-Q. Yu, and F.-Z. Xie. *Acta Pharmaceutica Sinica* **1991**, *26*, 341-344.

(50)   L. Ding, Z.-X. Zhang, and D.-K. An. *Journal of China Pharmaceutical University* **1991**, *22*, 175-176.

(51)   F.-X. Deng, J.-H. Cao, Z.-l. Xia, S. Lin, and X.-Y. Wang. *Acta Bontanica Sinica* **1987**, *29*, 73-76.

(52)   C.-P. Zhang, Y.-G. Zhang, X.-Y. Lu, P.-C. Ma, D.-Q. Yu, C.-H. He, F.-L. Sheng, J.-J. Yang, J. Yang, and Q.-T. Zheng. *Acta Academicae Mediceae Sinicae* **1989**, *11*, 322-325.

(53)   J.-H. Shen and B.-N. Zhou. *Acta Bontanica Sinica* **1992**, *34*, 475-479.

(54)   W.-J. Zhang, D.-J. Pan, L.-X. Zhang, and Y.-D. Shao. *Acta Pharmaceutica Sinica* **1986**, *21*, 592-598.

(55)   K.-C. Chen, R.-Z. Yang, and D.-G. Wu. *Acta Botanica Yunnanica* **1983**, *5*, 227-228.

(56)   G.-W. Qin, X.-M. Yang, W.-H. Gu, B.-D. Wang, Z.-X. Chen, R.-X. Guo, and K.-W. Shao. *Acta Chimica Sinica* **1982**, *40*, 637-647.

(57)   Q.-P. Gao, C.-S. Li, and J.-S. Lu. *Bulletin of Chinese Materia Medica* **1987**, *12*, 41-42.

(58)   X.-M. Zhang, D.-G. Wu, J.-W. Zhou, R.-N. Pan, L.-W. Liu, L. Peng, and Y. Luo. *Acta Bontanica Yunnanica* **1993**, *15*, 92-96.

(59)   S.-Z. Li, Z.-X. Zhang, L.-S. Sheng, W.-Y. Liu, P.-C. Ma, and D.-K. An. *Journal of Nanjing College of Pharmacy* **1984**, *15*, 1-7.

(60)   J.-H. Yi, H. Yang, Q.-L. Zhang, T.-R. Pei, Z.-Z. Chen, and Y.-B. Hu. *Zhong Cao Yao* **1993**, *24*, 398-340.

(61) C.-P. Zhang, Y.-G. Zhang, X.-Y. Lu, Y. Chen, P.-C. Ma, Y.-P. Yin, and L.-F. Xu. *Acta Academiae Medicinae Sinicae* **1986**, *8*, 204-206.

(62) C.-P. Zhang, Y.-G. Zhang, Q.-T. Zheng, and C.-H. He. *Acta Pharmaceutica Sinica* **1989**, *24*, 225-228.

(63) Z.-X. Zhang, L. Ding, S.-Z. Qian, and D.-K. An. *Journal of Chinese Pharmaceutical Sciences* **1993**, *2*, 144-147.

(64) G.-M. Pang, C.-J. Zhao, H. Hori, and S. Inayama. *Acta Phamrmaceutica Sinica* **1989**, *24*, 75-79.

(65) C.-P. Zhang, Y.-G. Zhang, X.-Y. Lu, Y. Chen, P.-C. Ma, S.-Z. Li, and Z.-X. Zhang. *Journal of Nanjing College of Pharmacy* **1984**, *15*, 69.

(66) H. Hori, G.-M. Pang, K. Harimaya, Y. Iitaka, and S. Inayama. *Chem. Pharm. Bull.* **1987**, *35*, 2125-2128.

(67) X.-M. Zhang, C.-F. Wang, and D.-G. Wang. *Acta Botanica Yunnanica* **1992**, *14*, 211-214.

(68) D.-M. Zhang and D.-Q. Yu. *Planta Medica* **1990**, *56*, 98-100.

(69) S. Inayama, H. Hori, G.-M. Pang, H. Nagasawa, and H. Ageta. *Chem. Pharm. Bull.* **1989**, *37*, 2836-2837.

(70) Y.-Y. Wang and D.-G. Wang. *Acta Botanica Sinica* **1993**, *15*, 309-310.

(71) P.-C. Ma, X.-Y. Lu, C.-H. He, and Q.-T. Zheng. *Acta Botanica Sinica* **1991**, *33*, 370-377.

(72) P.-C. Ma and C.-L. Yang. *Acta Botanic Sinica* **1993**, *35*, 637-643.

(73) X.-Y. Lu, P.-C. Ma, Y. Chen, C.-P. Zhang, Y.-G. Zhang, Z.-X. Zhang, L.-S. Sheng, S.-Z. Li, D.-K. An, C.-H. He, and Q.-T. Zheng. *Acta Academiae Medicinae Sinicae* **1990**, *12*, 157-161.

(74) S.A. Matlin, A. Belenguer, V.E. Stacey, S.-Z. Qian, Y. Xu, J.-W. Zhang, J.K.M. Sanders, S.R. Amor, and C.M. Pearce. *Contraception* **1993**, *47*, 387-400.

(75) Yunnan Provincial Institute of Botany and Henan Provincial Institute of Medical Sciences. *Ke Xue Tong Bao* **1977**, *22*, 458-460, 436.

(76) F.-X. Deng, S.-Q. Huang, J.-H. Cao, Z.-L. Xia, S. Lin, D.-Y. Zhu, S.-H. Jiang, and Y.-L. Zhu. *Acta Botanica Sinica* **1985**, *27*, 516-519.

(77) P.-C. Ma, X.-Y. Lu, J.-J. Yang, and Q.-T. Zheng. *Acta Pharmaceutica Sinica* **1991**, *26*, 759-763.

(78) S. Lin, F.-X. Deng, N. Sakurai, D. Hong, and Y.-Z. Chen. *Acta Botanica Sinica* **1993**, *35*, 385-389.

(79) C.-P. Zhang, X.-Y. Lu, P.-C. Ma, Y. Chen, Y.-G. Zhang, Z. Yan, G.-F. Chen, Q.-T. Zheng, C.-H. He, and D.-Q. Yu. *Acta Pharmaceutica Sinica* **1993**, *28*, 110-115.

(80) F.-X. Deng, B.-N. Zhou, G.-Q. Song, and C.-Q. Hu. *Acta Pharmaceutica Sinica* **1982**, *17*, 146-150.

(81) F.-X. Deng, S.-Q. Huang, Z.-D. Wang, G.-G. Ma, G.-Q. Song, and Z.-X. Chen. *Acta Pharmaceutica Sinica* **1981**, *16*, 155-157.

(82) K.-C. Chen, R.-Z. Yang, and C.-B. Wang. *Zhong Cao Yao* **1986**, *17*, 242-245.

(83) L. Zhang, Z.-X. Zhang, L.-S. Sheng, and D.-K. An. *Journal of China Pharmaceutical University* **1991**, 22, 132.

(84) L. Zhang, Z.-X. Zhang, L.-S. Sheng, D.-K. An, Y. Lu, Q.-T. Zheng, and S.-C. Wang. *Acta Pharmaceutica Sinica* **1992**, *28*, 32-34.

(85) X.-M. Zhang, C.-F. Wang, and D.-G. Wang. *Acta Botanica Yunnanica* **1992**, *14*, 319-322.

(86) L. Zhang, Z.-X. Zhang, D.-K. An, and C. Kong. *Acta Phamaceutica Sinica* **1991**, *26*, 515-518.

(87) J.-H. Shen and B.-N. Zhou. *Chin. Chem. Lett.* **1992**, *3*, 113-116.

(88) L. Zhang, Z.-X. Zhang, L.-S. Sheng, and D.-K. An. *Zhong Cao Yao* **1992**, *23*, 339-340, 360.

(89) J.-H. Shen. Ph. D. Thesis, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 1990.

(90) B.-N. Zhou, D.-Y. Zhu, F.-X. Deng, C.-G. Huang, J.P. Kutney, and M. Roberts. *Planta Medica* **1988**, *54*, 330-332.

(91) F.-X. Deng, J.-H. Cao, Z.-L. Xia, S. Lin, D.-Y. Zhu, and S.-H. Jiang. *Acta Pharmaceutica Sinica* **1987**, *22*, 377-379.

(92) L. Ding and Z.-X. Zhang. *Journal of China Pharmaceutical University* **1991**, 22, 25-26.

(93) K. Shishido, K. Nakano, N. Wariishi, H. Tateishi, T. Omodani, M. Shibuya, K. Goto, Y. Ono, and Y. Takaishi. *Phytochemistry* **1994**, *35*, 731-737.

(94) N.-N. Fang, Y.-C. Cai, H.-M. Liu, Q.-S. Liang, and R.-S. Xu. *Western China Journal of Pharmaceutical Science* **1987**, *2*, 145-147.

(95) R.F. Bryan and L. Fallon tert. *J. Chem. Soc., Perkin Trans. II* **1976**, 341-345.

(96) Z.-L. Xia, S.-Q. Huang, J.-Y. Chen, F.-X. Deng, and Z.-L. Chen. *China Journal of Chinese Materia Medica* **1989**, *14*, 675-676.

(97) L. Zhang, Z.-X. Zhang, L.-S. Sheng, and D.-K. An. *Journal of China Pharmaceutical University* **1992**, *23*, 301-303.

(98) H.-R. Li, Q.-P. Gao, G.-Z. Du, Y. Lu, and L. Lu. *Zhong Cao Yao* **1992**, *23*, 233-235, 242.

(99) S.M. Kupchan and R.M. Schubert. *Science* **1974**, *185*, 791-792.

(100) T.-M. Zhang, Z.-Y. Chen, and C. Lin. *Acta Pharmacologica Sinica* **1981**, *2*, 128-131.

(101) Y.-S. Wei and I. Adachi. *Acta Pharmacologica Sinica* **1991**, *12*, 406-410.

(102) J.-H. Xu, C.-C. Li, and Z.-Q. Huang. *Acta Pharmacologica Sinica* **1989**, *10*, 550-553.

(103) *Goodman and Gilman's The Pharmacological Basis of Therapeutics*; 8 th ed.;
A.G. Gilman, T.W. Rall, A.S. Nies, and P. Taylor, Eds.; Pergamon Press: Elmsford,
NY, 1990, Section XII.

(104) D.-Q. Yu, D.-M. Zhang, H.-B. Wang, and X.-T. Liang. *Acta Pharmaceutica Sinica*
1992, *27*, 830-836.

(105) B.H. Barton and Y. Houminer. *J. Chem. Soc., Chem. Commun.* **1973**, 839-840.

(106) S.M. Kupchan, D.C. Fessler, M.A. Eakin, and T.J. Giacobbe. *Science* **1970**, *168*,
376-378.

(107) C.K. Lai, R.S. Buckanin, S.J. Chen, D.F. Zimmerman, F.T. Sher, and
G.A. Berchtold. *J. Org. Chem.* **1982**, *47*, 2364-2369.

(108) Y. Takaishi, K. Ujita, H. Tokuda, H. Nishino, A. Iwashima, and T. Fujita. *Cancer Lett.*
1992, *65*, 19-26.

(109) K. Ujita, Y. Takaishi, H. Tokuda, H. Nishino, A. Iwashima, and T. Fujita. *Cancer Lett.*
1993, *68*, 129-133.

(110) X.-Y. Li. *Int. J. Immunotherapy* **1993**, *IX*, 181-187.

(111) X.-L. Tao, Y. Sun, Y. Dong, Y.-L. Xiao, D.-W. Hu, Y.-P. Shi, Q.-L. Zhu, H. Dai,
and N.-Z. Zhang. *Chinese Medical Journal* **1989**, *102*, 327-332.

(112) X.-Q. Zhang and Z.-H. Zhang. *Chinese Journal of Microbiology and Immunology*
1991, *11*, 117-120.

(113) L.-P. Zhu, Y.-Z. Dong, M. Jiang, X. Wang, and C.-L. Yan. *Chinese Journal of
Immunology* **1992**, *8*, 379-382.

(114) M. Jiang, Y.-Z. Dong, F.-C. Zhang, C.-L. Yan, L.-P. Zhu, B. Li, X. Wang, and
Y.-P. Cai. *Zhong Hua Yi Xue Za Zhi* **1992**, *72*, 473-475.

(115) L.-P. Zhu, Y.-Z. Dong, M. Jiang, X. Wang, and C.-L. Yan. *Chinese Journal of
Immunology* **1992**, *8*, 276-282.

(116) F.-G. Wu, L.-P. Zhu, L.-X. Cui, X. Wang, S.-Z. Zhang, K. Qu, and B.-C. Guo.
Chinese Journal of Microbiology and immunology **1993**, *13*, 193-197.

(117) J.-R. Zheng, L.-F. Xu, L. Ma, D.-H. Wang, and J.-W. Gao. *Acta Academiae Medicinae
Sinicae* **1983**, *5*, 1-8.

(118) X.-L. Tao, H. Dai, Y.-P. Shi, N.-Z. Zhang, J.-X. Cheng, F.-Y. Han, and J.-H. Duan.
Acta Academiae Medicinae Sinicae **1989**, *11*, 36-40.

(119) X.-L. Tao, L.S. Davis, and P.E. Lipsky. *Arthritis and Rheumatism* **1991**, *34*, 1274-1281.

(120) X.-F. Yang, L.-T. Xu, H. Ren, Z.-J. Li, C.-F. Sun, and Z.-Y. Zhang. *Chin. Med. Sci. J.* **1992**, *7*, 232-234.

(121) L.-X. Zhang, K.-X. Gu, Z.-F. Cao, D.-J. Pan, and W.-J. Zhang. *Shang Hai Mian Yi Xue Za Zhi* **1986**, *6*, 277-279.

(122) W.-M. Xu, L.-X. Zhang, Z.-H. Cheng, W.-Z. Cai, H.-H. Miao, and D.-J. Pan. *Acta Pharmaceutica Sinca* **1991**, *26*, 641-645.

(123) L.-X. Zhang, F.-K. Yu, Q.-Y. Zheng, Z. Fang, and D.-J. Pan. *Acta Pharmaceutica Sinica* **1990**, *25*, 573-577.

(124) Z.-L. Xia, J.-Y. Chen, R.-Q. Xu, F.-X. Deng, and F.-J. Zhang. *Zhong Guo Yao Xue Za Zhi* **1990**, *25*, 266-267.

(125) Y.-L. Zheng, Y. Xu, and J.-F. Lin. *Acta Pharmaceutica Sinica* **1989**, *24*, 568-572.

(126) J.-R. Zheng, J.-L. Fang, K.-X. Gu, Y.-P. Yi, L.-F. Xu, J.-W. Gao, H.-Z. Guo, Y.-H. Xu, and H.-Z. Sun. *Acta Academiae Medicinae Sinicae* **1987**, *9*, 323-328.

(127) L. Qiao, N. Xu, Y.-G. Zhang, and G.-Z. Huang. *Chinese Journal of Pharmacology and Toxicology* **1990**, *4*, 282-285.

(128) J.-R. Zheng, K.-X. Gu, J.-W. Gao, L.-F. Xu, Y.-H. Yu, and M.-Y. Tang. *Acta Academiae Medicinae Sinicae* **1991**, *13*, 398-403.

(129) W.-S. Ye, Y.-C. Deng, Y.-L. Huang, Y.-H. Han, and S.-P. Xue. *Acta Anatomica Sinica* **1992**, *23*, 290-293.

(130) J.-R. Zheng, K.-X. Gu, L.-F. Xu, J.-W. Gao, Y.-H. Yu, and M.-Y. Tang. *Acta Academiae Medicinae Sinicae* **1991**, *13*, 391-397.

(131) R.-J. Pei, L.-H. Qi, and X.-J. Liu. *Acta Pharmacologica Sinica* **1993**, *14*, 238-242.

(132) D.-F. Yu, B.-H. Hu, G.-P. Chen, C.-X. Yang, J. Yang, J.-Y. Xu, and L.-Z. Li. *Acta Pharmaceutica Sinica* **1990**, *25*, 929-931.

(133) Y. Takaishi, K. Shishido, N. Wariishi, M. Shibuya, K. Goto, M. Kido, M. Takai, and Y. Ono. *Tetrahedron Lett.* **1992**, *33*, 7177-7180.

(134) J.-R. Zheng, J.-H. Liu, L.-F. Xu, J.-W. Gao, and B.-L. Jiang. *Acta Academiae Medicinae Sinicae* **1983**, *5*, 75-78.

(135) C.-P. Zhou, S. Peng, and W.-Z. Liao. *Zhong Cao Yao* **1993**, *24*, 191-192.

(136) E.E. van Tamelen, E.G. Taylor, T.M. Leiden, and A.F. Kreft III. *J. Am. Chem. Soc.* **1979**, *101*, 7423-7424.

(137) H. Koike and T. Tokoroyama. *Chem. Lett.* **1979**, 333-336.

(138) T. Tokoroyama, A. Kondo, and Y. Ezaki. *Chem. Lett.* **1983**, 203-206.

(139) H. Koike and T. Tokoroyama. *Tetrahedron Lett.* **1978**, 4531-4534.

(140) D.M. Frieze, G.A. Berchtold, and J.F. Blount. *Tetrahedron Lett.* **1978**, 4607-4610.

(141) F.T. Sher and G.A. Berchtold. *J. Org. Chem.* **1977**, *42*, 2569-2574.

(142) L.C. Garver and E.E. van Tamelen. *J. Am. Chem. Soc.* **1982**, *104*, 867-869.

(143) E.E. van Tamelen and T.M. Leiden. *J. Am. Chem. Soc.* **1982**, *104*, 1785-1786.

(144) E.E. van Tamelen, J.P. Demers, E.G. Taylor, and K. Koller. *J. Am. Chem. Soc.* **1980**, *102*, 5424-5425.

(145) J.P. Demers. Ph. D. Thesis, Stanford University, 1980.

(146) J.P. Kutney, M.H. Beale, P.J. Salisbury, R.D. Sindelar, K.L. Stuart, B.R. Worth, P.M. Townsley, W.T. Chalmers, D.J. Donnelly, K. Nilsson, and G.G. Jacoli. *Heterocycles* **1980**, *14*, 1465-1467.

(147) J.P. Kutney, L.S.L. Choi, R. Duffin, G. Hewitt, N. Kawamura, T. Kurihara, P. Salisbury, R. Sindelar, K.L. Stuart, P.M. Townsley, W.T. Chalmers, F. Webster, and G.G. Jacoli. *Planta Medica* **1983**, *48*, 158-163.

(148) N.R. Farnsworth and A.S. Bingel. In *First International Congress on Medicinal Plants Research*; Springer-Verlag Berlin Heidelberg: University of Munich, Germany, 1976; p 4.

(149) J.P. Kutney. *Synlett* **1991**, 11-19.

(150) J.D. Hamill and M.J.C. Rhodes. In *Biosynthesis and Manipulation of Plant Products*; D. Grierson Ed.; Blackie Academic & Professional (Chapman & Hall): Glasgow, 1993; Vol. 3; pp 178-209.

(151) J.P. Kutney. *Acc. Chem. Res.* **1993**, *26*, 559-566.

(152) R.J. Gautheret. In *Cell Culture and Somatic Cell Genetics of Plants: Cell Growth, Nutrition, Cytodifferentiation, and Cryopreservation*; 1 st ed.; I. K. Vasil Ed.; Academic Press: Orlando, Florida, 1985; Vol. 2; pp 1-59.

(153) O.L. Gamborg and D.E. Eveleigh. *Can. J. Biochem.* **1968**, *46*, 417-421.

(154) T. Murashige and F. Skoog. *Physiol. Plant.* **1962**, *15*, 473-497.

(155) M. Misawa, M. Hayashi, and S. Takayama. *Planta Medica.* **1983**, *49*, 115-119.

(156) L.W. Dujack, S.J. Pancake, and P.K. Chen. In *the 11th International Congress of Chemotherapy and the 19th Interscience Conference on Antimicrobial Agents and Chemotherapy*; The American Society for Microbiology: Washington, D.C., 1980; pp 1563-1565.

(157) J.P. Kutney, G.M. Hewitt, T. Kurihara, P.J. Salisbury, R.D. Sindelar, K.L. Stuart, P.M. Townsley, W.T. Chalmers, and G.G. Jacoli. *Can. J. Chem.* **1981**, *59*, 2677-2683.

(158) J.P. Kutney, G.M. Hewitt, G. Lee, K. Piotrowska, M. Roberts, and S.J. Rettig. *Can. J. Chem.* **1992**, *70*, 1455-1480.

(159) G.G. Jacoli, J.P. Kutney, P.M. Townsley, G.M. Hewitt, T. Kurihara, R.D. Sindelar, K.L. Stuart, and N. Kawamura. In *5th Intl. Cong. Plant Tissue & Cell Culture*; 1982; pp 373-374.

(160) M. Roberts. Ph. D. Thesis, University of British Columbia, 1989.

(161) F. Kuri-Brena. Ph. D. Thesis, University of British Columbia, 1992.

(162) J.P. Kutney, M.D. Samija, G.M. Hewitt, E.C. Bugante, and H.-F. Gu. *Plant Cell Reports* **1993**, *12*, 356-359.

(163) K. Li. M. Sc. Thesis, University of British Columbia, 1991.

(164) L. Zhang, Z.-X. Zhang, L.-S. Sheng, and D.-K. An. *Journal of China Pharmaceutical University* **1992**, *23*, 158-160.

(165) L. Zhang, Z.-X. Zhang, L.-S. Sheng, and D.-K. An. *Phytochemical analysis* **1993**, *4*, 105-108.

(166) M. Kitadani, A. Yoshikoshi, Y. Kitahara, J. de Paiva Campello, J.D. McChesney, D.J. Watts, and E. Wenkert. *Chem. Pharm. Bull.* **1970**, *18*, 402-405.

(167) J.P. Kutney. In *Cell Culture and Somatic Cell Genetics of Plants: Phytochemicals in Plant Cell Cultures*; I. K. Vasil Ed.; Academic Press: San Diego, 1988; Vol. 5; pp 159-177.

(168) E. Pretsch, T. Clerc, J. Seibl, and W. Simon. In *Tables of Spectral Data for Structure Determination of Organic Compounds*; English Translation of 2 nd German Edition (1981); F. L. Boschke, W. Fresenius, J. F. K. Huber, E. Pungor, G. A. Rechnitz, W. Simon and T. S. West Eds.; Spriger-Verlag Berlin Heideberg: Berlin, 1983.

(169) C.R. Enzell, R.A. Appleton, and I. Wahlberg. In *Biochemical Applications of Mass Spectrometry*; G. R. Waller Ed.; Wiley-Interscience: New York, 1972; pp 351-385.

(170) J.G. Urones, I.S. Marcos, J.F. Ferreras, and P.B. Barcala. *Phytochemistry* **1988**, *27*, 523-526.

(171) T. Matsumoto, S. Usui, H. Kawashima, and M. Mitsuki. *Bull. Chem. Soc. Jpn.* **1981**, *54*, 581-584.

(172) F.W. Wehrli, A.P. Marchand, and S. Wehrli. *Interpretation of Carbon-13 NMR Spectra*; 2 nd ed.; John Wiley & Sons: Chichester, 1988, pp 32-97.

(173) J.K. Whitesell and M.A. Minton. *Stereochemical Analysis of Alicyclic Compounds by C-13 NMR Spectroscopy*; 1 st ed.; Chapman and Hall: New York, 1987, p 5.

(174) J.D. Pascual, A.S. Feliciano, J.M.M.D. Corral, and A.F. Barrero. *Phytochemistry* **1983**, *22*, 300-301.

(175) G. Topcu and A. Ulubelen. *Phytochemistry* **1990**, *29*, 2346-2348.

(176) A.H. Conner, B.A. Nagasampagi, and J.W. Rowe. *Phytochemistry* **1980**, *19*, 1121-1131.

(177) T. Norin and B. Winell. *Phytochemistry* **1971**, *10*, 2818-2821.

(178) J.K. Sanders and B.K. Hunter. *Modern NMR Spectroscopy: A Guide for Chemists*; 2 nd ed.; Oxford University Press: New York, 1993, pp 205-234.

(179) E.S. Finne, J.R. Gunn, and T.S. Sorensen. *J. Am. Chem. Soc.* **1987**, *109*, 7816-7823.

(180) L.A. Paquette, J.W. Fischer, A.R. Browne, and C.W. Doecke. *J. Am. Chem. Soc.* **1985**, *107*, 686-691.

(181) Y. Fujimoto and T. Tatsuno. *Tetrahedron Lett.* **1976**, *37*, 3325-3326.

(182) H. Akita and T. Oishi. *Chem. Pharm. Bull.* **1981**, *29*, 1567-1579.

(183) R.V. Stevens and G.S. Bisacchi. *J. Org. Chem.* **1982**, *47*, 2396-2399.

(184) P. Manitto. *Biosynthesis of Natural Products*; Ellis Horwood: Chichester, West Sussex, 1981.

(185) T. Suga and T. Shishibori. *Phytochemistry* **1975**, *14*, 2411-2417.

(186) L.M. Jackman and S. Sternhell. *Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry*; 2 nd ed.; Pergamon Press: Oxford, 1969.

(187) W.-J. Zhang, R.-N. Zhang, D.-J. Pan, L.-X. Zhang, and G.-Y. Xu. *Acta Academeiae Medicinae Shanghai* **1986**, *13*, 267-272.

(188) J. March. *Advanced Organic Chemistry*; 3 rd ed.; John Wiley & Sons: New York, 1985, p 17.

(189) M.G. Peter. *Angew. Chem., Int. Ed. Engl.* **1989**, *28*, 555-570.

(190) A.J. Poss and R.K. Belter. *Tetrahedron Lett.* **1987**, *28*, 2555-2558.

(191) A.S. Kende, L.S. Liebeskind, J.E. Mills, P.S. Rutledge, and D.P. Curran. *J. Am. Chem. Soc.* **1977**, *99*, 7082-7083.

(192) J.P. Kutney. *Pure & Appl. Chem.* **1984**, *56*, 1011-1024.

(193) L.C. Garver. Ph. D. Thesis, Stanford University, 1981.

(194) M.E. Garst and T.A. Spencer. *J. Am. Chem. Soc.* **1972**, *95*, 250-252.

(195) B.J.M. Jansen, C.T. Bouwman, and A. de Groot. *Tetrahedron Lett.* **1994**, *35*, 2977-2980.

(196) M. Nakayama, S. Shinke, Y. Matsushita, S. Ohira, and S. Hayashi. *Bull. Chem. Soc. Japn.* **1979**, *52*, 184-185.

(197) W.G. Salmond, M.A. Barta, and J.L. Havens. *J. Org. Chem.* **1978**, *43*, 2057-2059.

(198) B.W. Finucane and J.B. Thomson. *J. Chem. Soc., Chem. Commun.* **1969**, 1220.

(199) E.J. Parish, S. Chitrakorn, and T.-Y. Wei. *Synth. Commun.* **1986**, *16*, 1371-1375.

(200) R. Rathore, N. Saxena, and S. Chandrasekaran. *Synth. Commun.* **1986**, *16*, 1493-1498.

(201) A.B. Turner. *Q. Rev.* **1964**, *18*, 347-360.

(202) H.-D. Becker. *J. Org. Chem.* **1969**, *34*, 1203-1210.

(203) H.-D. Becker. *J. Org. Chem.* **1967**, *32*, 2943-2947.

(204) L.K. Dyall and S. Winstein. *J. Am. Chem. Soc.* **1972**, *94*, 2196-2199.

(205) J.W.A. Findlay and A.B. Turner. *J. Chem. Soc. (C)* **1971**, 547-553.

(206) S.R. Angel and K.D. Turnbull. *J. Am. Chem. Soc.* **1990**, *112*, 3698-3700.

(207) O.L. Chapman, M.R. Engel, J.P. Springer, and J.C. Clardy. *J. Am. Chem. Soc.* **1971**, *93*, 6696-6698.

(208) S.R. Angle and J.D. Rainier. *J. Org. Chem.* **1992**, *57*, 6883-6890.

(209) J.P. Marino and S.L. Dax. *J. Org. Chem.* **1984**, *49*, 3672-3673.

(210) P. Capdevielle and M. Maumy. *Tetrahedron Lett.* **1983**, *24*, 5611-5614.

(211) C.D. Cook and N.D. Gilmour. *J. Org. Chem.* **1960**, *25*, 1429-1431.

(212) H.-D. Becker. *J. Org. Chem.* **1964**, *30*, 982-989.

(213) P.M. Brown, M. Moir, R.H. Thomson, T.J. King, V. Krishnamoorthy, and T.R. Seshadri. *J. Chem. Soc., Perkin. I* **1973**, 2721-2725.

(214) D.A. Bolon. *J. Org. Chem.* **1970**, *35*, 715-719.

(215) H.-D. Becker. *J. Org. Chem.* **1965**, *30*, 982-989.

(216) S.M. Kupchan, A. Karim, and C. Marcks. *J. Org. Chem.* **1969**, *34*, 3912-3918.

(217) K.M. Doxsee, M. Feigel, K.D. Stewart, J.W. Canary, C.B. Knobler, and D.J. Cram. *J. Am. Chem. Soc.* **1987**, *109*, 3098-3107.

(218) A.D. Fraser, S.J. Clark, and H.H. Wotiz. *J. Org. Chem.* **1976**, *41*, 170-171.

(219) E.H. Vickery, L.F. Pahler, and E.J. Eisenbraun. *J. Org. Chem.* **1979**, *44*, 4444-4446.

(220) F.W. Bachelor, A.A. Loman, and L.R. Snowdon. *Can. J. Chem.* **1970**, *48*, 1554-1557.

(221) G.A. Olah, R. Malhotra, and S.C. Narang. *Nitration: Methods and Mechanisms*; VCH Publishers: New York, Weinheim, Cambridge, 1989, p 11.

(222) N. Kornblum. In *Organic Reactions*; R. Adams *et al.* Eds.; John Wiley & Sons, Inc.: New York, 1944; Vol. 2; pp 262-340.

(223) J.I.G. Cadogan and G.A. Molina. *J. Chem. Soc., Perkin Trans. I* **1973**, 541-542.

(224) M.P. Doyle, J.F. Dellaria Jr., B. Siegfried, and S.W. Bishop. *J. Org. Chem.* **1977**, *42*, 3494-3498.

(225) T. Satoh, S. Suzuki, Y. Suzuki, Y. Miyaji, and Z. Imai. *Tetrahedron Lett.* **1969**, 4555-4558.

(226) A. Nose and T. Kudo. *Chem. Pharm. Bull.* **1981**, *29*, 1159-1161.

(227) T. Satoh, N. Mitsuo, M. Nishiki, Y. Inoue, and Y. Ooi. *Chem. Pharm. Bull.* **1981**, *29*, 1443-1445.

(228) A. Ono, M. Hiroi, and K. Shimazaki. *Chem. Ind. (London)* **1984**, 75.

(229) J.A. Cowan. *Tetrahedron Lett.* **1986**, *27*, 1205-1208.

(230) Y. He, H. Zhao, X.-F. Pan, and S.-F. Wang. *Synth. Commun.* **1989**, *19*, 3047-3050.

(231) M. Hudlicky. *Reductions in Organic Chemistry*; Ellis Horwood: Chichester, 1984, pp 62-69.

(232) M. Freifelder. *Practical Catalytic Hydrogenation*; Wiley-Interscience: New York, 1971, Chapter XX.

(233) *Org. Syn.*; E.C. Horning *et al.* Eds.; John Wiley & Sons: New York, 1955; Coll. Vol. 3, pp 69-70.

(234) H.H. Hodgson and E.R. Ward. *J. Chem. Soc.* **1947**, 327-331.

(235) T. Cohen, R.M. Moran Jr., and G. Sowinski. *J. Org. Chem.* **1961**, *26*, 1-5.

(236) A.H. Lewin, A.H. Dinwoodie, and T. Cohen. *Tetrahedron* **1966**, *22*, 1527-1537.

(237) T. Cohen, A.H. Dinwoodie, and L.D.M. Keever. *J. Org. Chem.* **1962**, *27*, 3385-3390.

(238) A. Tahara, M. Shimagaki, M. Itoh, Y. Harigaya, and M. Onda. *Chem. Pharm. Bull.* **1975**, *23*, 3189-3202.

(239) M.J. Harrison and R.O.C. Morman. *J. Chem. Soc. (C)* **1970**, 728-730.

(240) W.A. Bubb and S. Sternhell. *Tetrahedron Lett.* **1970**, 4499-4502.

(241) M.-L. Huang. *J. Am. Chem. Soc.* **1948**, *70*, 611-614.

(242) R.B. Woodward and T. Singh. *J. Am. Chem. Soc.* **1950**, *72*, 494-500.

(243) A.S. Dreiding, W.J. Pummer, and A.J. Tomasewski. *J. Am. Chem. Soc.* **1953**, *75*, 3159-3165.

(244) D.J. Bichan and P. Yates. *Can. J. Chem.* **1975**, *53*, 2054-2063.

(245) H. Achenbach, R. Waibel, M.H.H. Nkunya, and H. Weenen. *Phytochemistry* **1992**, *31*, 3781-3784.

(246) R. Csuk and B.I. Glanzer. *Chem. Rev.* **1991**, *91*, 49-97.

(247) H.G. Davies, R.H. Green, D.R. Kelly, and S.M. Roberts. *Biotransformations in Preparative Organic Chemistry*; Academic Press: London, 1989, p ix.

(248) H. Yamada and S. Shimizu. *Angew. Chem., Int. Ed. Engl.* **1988**, 27, 622-642.

(249) G.M. Whitesides. In *Ciba Foundation Symposium 111: Enzymes in Organic Synthesis*; Pitman Publishing: London, 1985; pp 76-90.

(250) H.G.W. Leuenberger. In *Biotransformations*; K. Kieslich Ed.; Verlag Chemie: Weinheim, 1984; Vol. 6a; pp 5-29.

(251) A.M. Klibanov. *Acc. Chem. Res.* **1990**, *23*, 114-120.

(252) B. Chance and A.C. Maehly. *Methods in Enzymology*; Academic Press: New York, 1955; Vol. 2, p 773.

(253) M.M. Bradford. *Anal. Biochem.* **1976**, *72*, 428-254.

(254) H.L. Holland. *Organic Synthesis with Oxidative Enzymes*; VCH Publishers: New York, 1992, pp 19-24.

(255) R.K. Scopes. *Protein Purification: Principles and Practice*; Springer-Verlag: New York, 1982, Chapter 1.

(256) P.A. Frey and E.D. Harris. In *Biochemistry*; G. Zubay Ed.; Addison-Wesley: Reading, Massachusetts, 1983.

(257) *Cytochrome P-450: Structure, Mechanism, and Biochemistry*; P.R.O. de Montellano, Ed.; Plenum Press: New York, 1986.

(258) N.C. Yang and R.A. Finnegan. *J. Am. Chem. Soc.* **1958**, *80*, 5845-5848.

(259) M.D. Samija. M. Sc. Thesis, University of British Columbia, 1992.

(260) C.M. Pearce and J.K.M. Sanders. *J. Chem. Soc., Perkin Trans. 1* **1994**, 1119-1124.

(261) A.H. Conner, B.A. Nagasampagi, and J.W. Rowe. *Phytochemistry* **1980**, *19*, 1121-1131.

Appendix

X-ray Structure Report on Epoxide 360

A. Crystal Data

Empirical Formula	$C_{20}H_{22}O_4$
Formula Weight	326.39
Crystal Color, Habit	Colorless, plate
Crystal Dimensions	0.03 x 0.20 x 0.35
Crystal System	monoclinic
Lattice Type	P
No. of Reflections Used for Unit	
Cell Determination (2 θ range)	25 (41.6 - 55.4°)
Omega Scan Peak Width at Half-height	0.48°
Lattice Parameters	a = 9.932(1)$\mathring{A}$
	b = 6.114(1)$\mathring{A}$
	c = 14.5035(6)$\mathring{A}$
	β = 109.105(5)°
	V = 832.3(1)$\mathring{A}^3$
Space Group	$P2_1$ (#4)
Z value	2
D_{calc}	1.302 g/cm^3
F_{000}	348
μ(MoKα)	7.29 cm^{-1}

B. Intensity Measurements

Diffractometer	Rigaku AFC6S
Radiation	CuKα (λ = 1.54178 $\mathring{A}$)
	graphite monochromated
Take-off Angel	6.0°
Detector Aperture	6.0 mm horizontal
	6.0 mm vertical
Crystal to Detector Distance	28.5 cm
Temperature	21.0°C
Scan Type	ω-2θ
Scan Rate	16.0°/min (in omega) (8 rescans)
Scan Width	(1.00 + 0.20 tan θ)°
$2\theta_{max}$	154.8°
No. of Reflections Measured	Total: 1982
	Unique: 1884 (R_{int} = 0.043)
Corrections	Lorentz-polarization
	Absorption
	(trans. factors: 0.63 - 1.00)
	Secondary Extinction
	(coefficient: 1.15(7) x 10^{-5})

C. Structure Solution and Refinement

Structure Solution   Direct Methods (SHELXS86)

Refinement   Full-matrix least-squares

Function Minimized $\sum \omega(|F_o| - |F_c|)^2$

Least Squares Weights $\frac{1}{\sigma^2(F_o)} = \frac{4F_o^2}{\sigma^2(F_o^2)}$

p-factor   0.00

Anomalous Dispersion   All non-hydrogen atoms

No. Observations (I>3σ(I))   1207

No. Variables   217

Reflection/Parameter Ratio   5.56

Residuals: R; Rw   0.038; 0.037

Goodness of Fit Indicator   2.02

Max Shift/Error in Final Cycle   0.0002

Maximum peak in Final Diff. Map $0.12\ e^-/Å^3$

Minimum peak in Final Diff. Map $-0.12\ e^-/Å^3$

Table I   Atomic Coordinates and B_{eq}

atom	x	y	z	B_{eq}
O(1)	0.4406(3)	0.3685	0.3780(2)	5.68(8)
O(2)	0.2821(3)	0.6126(8)	0.0075(2)	6.08(8)
O(3)	0.9422(3)	-0.3112(8)	0.4680(2)	6.92(9)
O(4)	1.0970(3)	-0.2359(9)	0.3900(2)	8.2(1)
C(1)	0.7429(4)	0.2586(9)	0.2445(3)	4.54(9)
C(2)	0.8691(4)	0.0990(10)	0.2684(3)	5.4(1)
C(3)	0.8695(4)	-0.0513(9)	0.3484(3)	4.8(1)
C(4)	0.7654(4)	-0.0678(9)	0.3871(2)	4.6(1)
C(5)	0.6360(3)	0.0743(8)	0.3568(3)	4.09(9)
C(6)	0.5039(4)	-0.0195(9)	0.3744(3)	5.0(1)
C(7)	0.3859(4)	0.1520(10)	0.3513(3)	5.0(1)
C(8)	0.3782(4)	0.3159(9)	0.2735(3)	4.31(9)
C(9)	0.4778(3)	0.3009(9)	0.2155(2)	3.77(9)
C(10)	0.6061(3)	0.1465(9)	0.2493(2)	3.60(8)
C(11)	0.4405(4)	0.4008(9)	0.1299(3)	4.28(9)
C(12)	0.3089(4)	0.5254(9)	0.0875(3)	4.27(10)
C(13)	0.2092(3)	0.5380(8)	0.1441(2)	3.82(9)
C(14)	0.2446(4)	0.4365(9)	0.2288(3)	4.58(10)
C(15)	0.0781(4)	0.6800(9)	0.1034(3)	4.58(10)
C(16)	0.1141(4)	0.9100(10)	0.1391(3)	5.6(1)
C(17)	-0.0511(4)	0.5926(10)	0.1271(3)	5.8(1)
C(18)	0.9823(5)	-0.200(1)	0.3993(3)	6.0(1)
C(19)	0.8049(4)	-0.229(1)	0.4670(3)	6.0(1)
C(20)	0.5674(4)	-0.0503(9)	0.1803(2)	4.68(10)

$$B_{eq} = \frac{8}{3}\pi^2(U_{11}(aa^*)^2 + U_{22}(bb^*)^2 + U_{33}(bb^*)^2 + 2U_{12}aa^*bb^* \cos\gamma + 2U_{13}aa^*cc^* \cos\beta + 2U_{23}bb^*cc^* \cos\alpha)$$

Table II   Bond Lengths (Å)

atom	atom	distance	atom	atom	distance
O(1)	C(7)	1.426(6)	O(1)	C(8)	1.473(4)
O(2)	C(12)	1.225(4)	O(3)	C(18)	1.369(5)
O(3)	C(19)	1.450(4)	O(4)	C(18)	1.211(4)
C(1)	C(2)	1.536(5)	C(1)	C(10)	1.543(4)
C(2)	C(3)	1.480(6)	C(3)	C(4)	1.332(4)
C(3)	C(18)	1.443(6)	C(4)	C(5)	1.494(5)
C(4)	C(19)	1.473(5)	C(5)	C(6)	1.527(4)
C(5)	C(10)	1.553(4)	C(6)	C(7)	1.502(6)
C(7)	C(8)	1.485(6)	C(8)	C(9)	1.496(4)
C(8)	C(14)	1.470(5)	C(9)	C(10)	1.532(5)
C(9)	C(11)	1.323(4)	C(10)	C(20)	1.531(5)
C(11)	C(12)	1.463(5)	C(12)	C(13)	1.480(4)
C(13)	C(14)	1.317(5)	C(13)	C(15)	1.515(5)
C(15)	C(16)	1.501(7)	C(15)	C(17)	1.529(5)

Table III   Bond Angles (°)

atom	atom	atom	angle	atom	atom	atom	angle
C(7)	O(1)	C(8)	61.6(3)	C(18)	O(3)	C(19)	108.5(3)
C(2)	C(1)	C(10)	111.9(3)	C(1)	C(2)	C(3)	111.3(3)
C(2)	C(3)	C(4)	125.1(4)	C(2)	C(3)	C(18)	126.5(4)
C(4)	C(3)	C(18)	108.4(4)	C(3)	C(4)	C(5)	123.0(4)
C(3)	C(4)	C(19)	109.8(4)	C(5)	C(4)	C(19)	127.0(3)
C(4)	C(5)	C(6)	115.8(3)	C(4)	C(5)	C(10)	108.8(3)
C(6)	C(5)	C(10)	112.5(3)	C(5)	C(6)	C(7)	109.2(3)
O(1)	C(7)	C(6)	114.3(3)	O(1)	C(7)	C(8)	60.8(3)
C(6)	C(7)	C(8)	120.3(3)	O(1)	C(8)	C(7)	57.6(2)
O(1)	C(8)	C(9)	117.3(3)	O(1)	C(8)	C(14)	111.1(3)
C(7)	C(8)	C(9)	119.1(3)	C(7)	C(8)	C(14)	120.4(3)
C(9)	C(8)	C(14)	116.8(3)	C(8)	C(9)	C(10)	119.4(3)
C(8)	C(9)	C(11)	117.8(3)	C(10)	C(9)	C(11)	122.2(3)
C(1)	C(10)	C(5)	106.9(3)	C(1)	C(10)	C(9)	111.3(3)
C(1)	C(10)	C(20)	110.9(3)	C(5)	C(10)	C(9)	111.2(3)
C(5)	C(10)	C(20)	111.0(3)	C(9)	C(10)	C(20)	105.7(3)
C(9)	C(11)	C(12)	124.8(3)	O(2)	C(12)	C(11)	120.8(3)
O(2)	C(12)	C(13)	121.7(4)	C(11)	C(12)	C(13)	117.5(3)
C(12)	C(13)	C(14)	118.0(3)	C(12)	C(13)	C(15)	117.3(3)
C(14)	C(13)	C(15)	124.5(3)	C(8)	C(14)	C(13)	125.1(3)
C(13)	C(5)	C(16)	109.0(3)	C(13)	C(15)	C(17)	112.9(4)
C(16)	C(15)	C(17)	111.9(4)	O(3)	C(18)	O(4)	120.0(4)
O(3)	C(18)	C(3)	108.9(4)	O(4)	C(18)	C(3)	131.2(5)
O(3)	C(19)	C(4)	104.3(3)				

Table IV   Hydrogen Atom Coordinates and B_{iso}

atom	x	y	z	B_{iso}
H(1)	0.7673	0.3791	0.2916	5.4
H(2)	0.7250	0.3165	0.1786	5.4
H(3)	0.8623	0.0129	0.2100	6.5
H(4)	0.9582	0.1827	0.2880	6.5
H(5)	0.6610	0.2074	0.3965	4.9
H(6)	0.4699	-0.1469	0.3322	5.9
H(7)	0.5280	-0.0636	0.4429	5.9
H(8)	0.3006	0.1121	0.3625	6.1
H(9)	0.5058	0.3905	0.0920	5.1
H(10)	0.1766	0.4418	0.2648	5.5
H(11)	0.0535	0.6816	0.0322	5.5
H(12)	0.1457	0.9118	0.2106	6.8
H(13)	0.1906	0.9656	0.1166	6.8
H(14)	0.0296	1.0028	0.1134	6.8
H(15)	-0.1341	0.6848	0.0956	6.9
H(16)	-0.0709	0.4422	0.1030	6.9
H(17)	-0.0310	0.5947	0.1979	6.9
H(18)	0.7346	-0.3473	0.4541	7.2
H(19)	0.8124	-0.1591	0.5293	7.2
H(20)	0.4810	-0.1197	0.1850	5.6
H(21)	0.5504	-0.0011	0.1131	5.6
H(22)	0.6459	-0.1559	0.1986	5.6

Table V   Bond Angles (°) Involving Hydrogen

atom	atom	atom	angle	atom	atom	atom	angle
C(1)	H(1)	C(2)	108.9	H(2)	C(1)	C(2)	108.9
C(1)	H(1)	C(10)	108.9	H(2)	C(1)	C(10)	108.9
C(1)	H(2)	C(1)	109.5	H(3)	C(2)	C(1)	109.0
C(2)	H(4)	C(3)	109.0	H(3)	C(2)	C(3)	109.0
C(2)	H(4)	C(2)	109.0	H(4)	C(2)	H(3)	109.5
C(5)	H(5)	H(3)	106.4	H(5)	C(5)	C(6)	106.4
C(5)	H(5)	C(6)	106.4	H(6)	C(6)	C(5)	109.5
C(6)	H(7)	C(5)	109.5	H(6)	C(6)	C(7)	109.5
C(6)	H(7)	C(7)	109.5	H(7)	C(7)	H(6)	109.5
C(7)	H(8)	H(6)	116.5	H(8)	C(7)	C(6)	116.5
C(7)	H(8)	C(6)	116.5	H(9)	C(11)	C(9)	117.6
C(11)	H(9)	C(9)	117.6	H(10)	C(14)	C(8)	117.5
C(14)	H(10)	C(8)	117.5	H(11)	C(15)	C(13)	107.6
C(15)	H(11)	C(13)	107.6	H(11)	C(15)	C(17)	107.6
C(16)	H(12)	C(17)	107.6	H(13)	C(16)	C(15)	109.5
C(16)	H(12)	C(15)	109.5	H(13)	C(16)	C(12)	109.5
C(16)	H(14)	C(12)	109.5	H(14)	C(16)	C(13)	109.5
C(16)	H(14)	C(13)	109.5	H(16)	C(17)	C(15)	109.5
C(17)	H(15)	C(15)	109.5	H(16)	C(17)	C(16)	109.5
C(17)	H(15)	C(15)	109.5	H(17)	C(17)	C(15)	109.5
C(17)	H(17)	C(16)	109.5	H(19)	C(19)	O(3)	109.5
C(19)	H(18)	C(15)	110.8	H(19)	C(19)	C(4)	110.8
C(19)	H(18)	C(16)	110.8	H(20)	C(20)	C(10)	110.8
C(19)	H(19)	C(10)	109.5				109.5

Table V   Bond Angles (°) Involving Hydrogen (continued)

atom	atom	atom	angle	atom	atom	atom	angle
C(10)	C(20)	H(21)	109.5	C(10)	C(20)	H(22)	109.5
H(20)	C(20)	H(21)	109.5	H(20)	C(20)	H(22)	109.5
H(21)	C(20)	H(22)	109.5				

Table VI   Torsion Angles(°)

atom	atom	atom	atom	angle	atom	atom	atom	atom	angle
O(1)	C(7)	C(6)	C(5)	-36.9(4)	O(1)	C(7)	C(8)	C(9)	105.6(4)
O(1)	C(7)	C(8)	C(14)	-97.0(4)	O(1)	C(8)	C(7)	C(6)	-102.6(4)
O(1)	C(8)	C(9)	C(10)	54.8(5)	O(1)	C(8)	C(9)	C(11)	-133.9(4)
O(1)	C(8)	C(14)	C(13)	135.4(4)	O(2)	C(12)	C(11)	C(9)	-179.8(4)
O(2)	C(12)	C(13)	C(14)	178.8(4)	O(2)	C(12)	C(13)	C(15)	-5.3(6)
O(3)	C(18)	C(3)	C(2)	-179.7(4)	O(3)	C(18)	C(3)	C(4)	1.7(5)
O(3)	C(19)	C(4)	C(3)	-2.2(5)	O(3)	C(19)	C(4)	C(5)	-177.0(4)
O(4)	C(18)	O(3)	C(19)	177.1(4)	O(4)	C(18)	C(3)	C(2)	0.1(8)
O(4)	C(18)	C(3)	C(4)	-178.5(5)	C(1)	C(2)	C(3)	C(4)	8.1(6)
C(1)	C(2)	C(3)	C(18)	-170.2(4)	C(1)	C(10)	C(5)	C(4)	-55.9(4)
C(1)	C(10)	C(5)	C(6)	174.3(3)	C(1)	C(10)	C(9)	C(8)	-134.9(3)
C(1)	C(10)	C(9)	C(11)	54.2(5)	C(2)	C(1)	C(10)	C(5)	64.1(4)
C(2)	C(1)	C(10)	C(9)	-174.3(3)	C(2)	C(1)	C(10)	C(20)	-56.9(4)
C(2)	C(3)	C(4)	C(5)	-3.1(7)	C(2)	C(3)	C(4)	C(19)	-178.2(4)
C(3)	C(2)	C(1)	C(10)	-39.1(4)	C(3)	C(4)	C(5)	C(6)	155.8(4)
C(3)	C(4)	C(5)	C(10)	28.0(5)	C(3)	C(18)	O(3)	C(19)	-3.1(5)
C(4)	C(5)	C(6)	C(7)	173.5(3)	C(4)	C(5)	C(10)	C(9)	-177.6(3)
C(4)	C(5)	C(10)	C(20)	65.1(4)	C(4)	C(19)	O(3)	C(18)	3.2(5)
C(5)	C(4)	C(3)	C(18)	175.5(4)	C(5)	C(6)	C(7)	C(8)	32.1(5)
C(5)	C(10)	C(9)	C(8)	-15.9(5)	C(5)	C(10)	C(9)	C(11)	173.3(3)
C(6)	C(5)	C(4)	C(19)	-29.9(5)	C(6)	C(5)	C(10)	C(9)	52.6(4)
C(6)	C(5)	C(10)	C(20)	-64.7(4)	C(6)	C(7)	O(1)	C(8)	112.4(4)
C(6)	C(7)	C(8)	C(9)	2.9(6)	C(6)	C(7)	C(8)	C(14)	160.4(4)
C(7)	O(1)	C(8)	C(9)	-108.7(4)	C(7)	O(1)	C(8)	C(14)	113.5(4)
C(7)	C(6)	C(5)	C(10)	-60.5(4)	C(7)	C(8)	C(9)	C(10)	-11.4(5)
C(7)	C(8)	C(9)	C(11)	159.8(4)	C(7)	C(8)	C(14)	C(13)	-160.6(4)
C(8)	C(9)	C(10)	C(20)	104.6(4)	C(8)	C(9)	C(11)	C(12)	0.1(6)
C(8)	C(14)	C(13)	C(12)	1.9(6)	C(8)	C(14)	C(13)	C(15)	-173.8(4)
C(9)	C(8)	C(14)	C(13)	-2.6(6)	C(9)	C(11)	C(12)	C(13)	-0.9(6)
C(10)	C(5)	C(4)	C(19)	-157.8(4)	C(10)	C(9)	C(8)	C(14)	-169.7(3)
C(10)	C(9)	C(11)	C(12)	171.1(4)	C(11)	C(9)	C(8)	C(14)	1.5(5)
C(11)	C(9)	C(10)	C(20)	-66.2(4)	C(11)	C(12)	C(13)	C(14)	-0.1(6)
C(11)	C(12)	C(13)	C(15)	175.9(3)	C(12)	C(13)	C(15)	C(16)	-87.5(4)
C(12)	C(13)	C(15)	C(17)	147.5(3)	C(14)	C(13)	C(15)	C(16)	88.1(5)
C(14)	C(13)	C(15)	C(17)	-36.8(5)	C(18)	C(3)	C(4)	C(19)	0.4(5)