STUDIES ON PLANT CELL CULTURES OF PODOPHYLLUM PELTATUM AND TRIPTERYGIUM WILFORDII FOR BIOSYNTHESIS OF BIOLOGICALLY ACTIVE COMPOUNDS.

by

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We accept this thesis as conforming

to the required standard.

THE UNIVERSITY OF BRITISH COLUMBIA

26 August 1992

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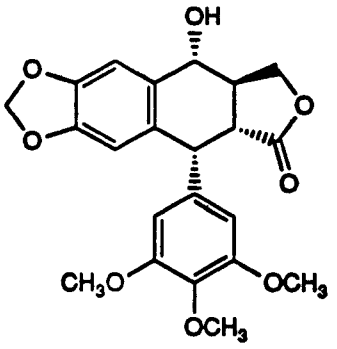
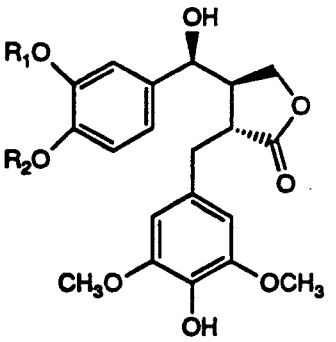
ABSTRACT

This thesis investigates the use of plant cell cultures in combination with synthetic chemistry to provide new routes to biologically active compounds. The first chapter consists of approaches to the lignan podophyllotoxin (**4**), by means of enzyme catalyzed ring closure reactions of dibenzylbutyrolactone intermediates **56** and **57** mediated by *Podophyllum peltatum* cells. The substrates **56** and **57** studied were synthesized from readily available aromatic aldehydes employing a "one pot", 1,4-addition-enolate alkylation sequence, as the key step. Semi-continuous biotransformation experiments performed with **56** illustrate the potential of the technique yielding the ring-closed products **75** and **76**. A possible mechanism for the ring closure *in vivo* is provided, as well as suggestions to alter the stereochemical outcome of the process.

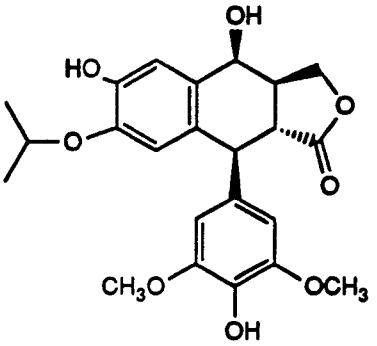
The second chapter of this thesis concerns the attempts to oxidize dehydroisoabietanolide (**132**) with crude enzyme preparations (CFEs) from *Tripterygium wilfordii*, as part of an ongoing study regarding the biogenesis of the diterpene triepoxides tripdiolide (**5**) and triptolide (**91**). The 12-hydroxy analogue of **132**, isotriptophenolide (**190**), is also studied. Both compounds were prepared in gram quantities from dehydroabietic acid (**122**). The results from the biotransformation experiments, suggest that triptophenolide (**163**), an isomer of **190** and bearing a phenolic group at C-14, is a likely candidate for future biotransformation experiments. The present studies also suggest that an "activated" diterpene, that is, a substrate bearing a hydroxyl group in the aromatic ring (**190** or an isomer), is a biogenetic precursor for the triepoxide system present in **5** and **91**.



Podophyllotoxin (**4**)



56: R_1= H, R_2= i-Pr
57: R_1,R_2= methylene



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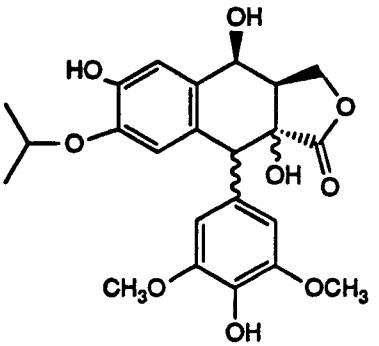

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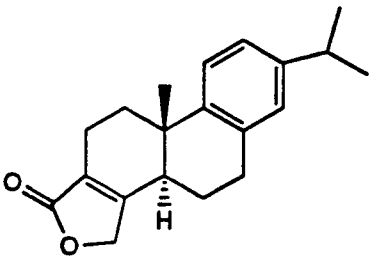
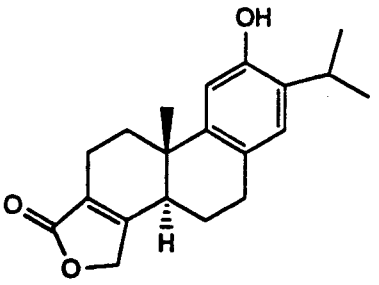
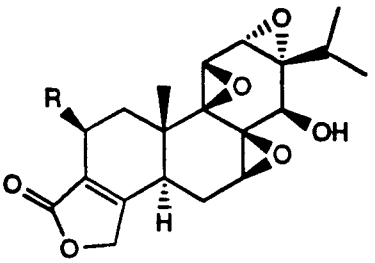


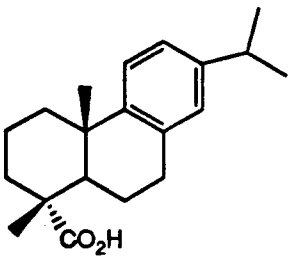
Dehydroisoabietanolide (**132**)



Isotriptophenolide (**190**)



R= H; Triptolide (**91**)
R= OH; Tripdiolide (**5**)



Dehydroabietic acid (**122**)

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Ac	acetyl
AC-3	a cell line of tissue culture developed from *Catharanthus roseus*
aq.	aqueous
B-5	standard tissue culture medium developed by Gamborg and Eveleigh
br	broad
Bn	benzyl
CFE	cell free extract
d	doublet
dd	doublet of doublets
DMF	dimethylformamide
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DCC	dicyclohexyl carbodiimide
DME	dimethoxy ethane
DYE	dextro yeast extract
Et	ethyl
GC	gas chromatography
[H]	reduction
HPLC	high pressure liquid chromatography
HRMS	high resolution mass spectrum
hexanes	generally a mixture of several isomers of hexane (C_6H_{14}) predominantly *n*-hexane, and methylcyclopentane (C_6H_{12})
HMPA	hexamethylphosphoramide
hv	light radiation
i-Pr	*iso*-propyl
IR	infra-red
J	coupling constant
KB	a tissue culture cell line derived from human carcinoma of the nasopharynx
L-1210	a tissue culture cell line derived from mouse leukemia
LDA	lithium diisopropyl amide
m	multiplet
MCPBA	*meta*-chloroperoxybenzoic acid
Me	methyl
MES	2-(4-morpholino)-ethane sulfonic acid
$MSNA_{0.5}K_{0.5}$	MS medium of Murashige and Skoog supplemented with naphthalene acetic acid (NA, 0.5 mg/L) and kinetin (K, 0.5 mg/L)
Na_2EDTA	ethylene diamine tetraacetic acid disodium salt
NBS	*n*-bromosuccinimide
NOE	Nuclear Overhauser Effect
n-Bu	*normal*-butyl
nmr	nuclear magnetic resonance
NiRa	Nickel-Raney
[O]	oxidation
P-388	a tissue culture cell line derived from mouse leukemia
PCC	pyridinium chlorochromate
PDA	potato dextrose agar
Ph	phenyl
pp	pyrophosphate

ppm	parts per million
PRI_2Co_{100}	PRL-4 medium of Gamborg and Eveleigh supplemented with indole-3-acetic acid (I, 2 mg/L) and coconut milk (100 ml/L)
PRD_2Co_{100}	PRL-4 medium of Gamborg and Eveleigh supplemented with 2,4-dichlorophenoxy acetic acid (D, 2 mg/L) and coconut milk (Co, 100 mL/L)
PRL-4	standard tissue culture medium developed by Gamborg and Eveleigh.
pyr	pyridine
R	alkyl residue
RI	refractive index
R.T.	room temperature
s	singlet
sp.	species
t	triplet
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
TMS	tetramethylsilane
TRP-4a	a cell line of tissue culture developed from *Tripterygium wilfordii*
THP	tetrahydropyrane
UV	ultraviolet
Δ	heat
ν	wave number (cm^{-1})

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Professor J. P. Kutney, for his guidance and support throughout this work. I would like to thank also the following people for their contribution: Dr. Malcolm Roberts, Mijo Samija, Carles Cirera, and Kang Han for their help and invaluable suggestions during the preparation of this thesis; Gary Hewitt, David Chen, Fay Hutton, and Elizabeth Bugante for their hard work in producing the cultures and for having the patience to teach me how to work with them; Radka Milanova and Nikolay Stoynov who were never too busy to lend a hand; Johanne Renaud, Jacques Roberge and Krystyna Piotrowska for their advice throughout my synthetic studies; Andres Gonzalez, Martha Kline and Antonio Kuri-Brena who helped keep my sanity; the Staff and Faculty of this Chemistry Department. Financial support from Syntex, S.A. is gratefully acknowledged. Thank you, family and friends.

FOREWORD

Biotechnology has been widely used since the early days of mankind; microbial and yeast-mediated transformations in particular, were applied to the production of bread, dairy products, and alcoholic beverages. All of these early applications used mixed cultures of microorganisms, and pertained to agriculture and human nutrition. In 1862 Pasteur[1] laid the scientific foundation of one of these early applications, namely, the oxidation of alcohol to acetic acid by using a pure culture of *Bacterium xylinum*[2]. Investigations of the oxidation of glucose to gluconic acid[3] by *Acetobacter aceti* and of sorbitol to sorbose by *Acetobacter sp.* followed these earlier studies[4]. The reducing action of fermenting yeast, *Saccharomyces cerevisiae*, was first observed by Dumas in 1874[5]. He reported that, on addition of finely powdered sulfur to a suspension of fresh yeast in a sugar solution, hydrogen sulfide was liberated. The reduction of furfural to furfuryl alcohol under the anaerobic conditions of fermentation by means of living yeast[6,7] was the first "phytochemical reduction"[8] of an organic molecule described in the literature. Numerous further enzymatic or microbial biotransformations, biodegradations and fermentations followed, and in the initial excess enthusiasm that invariably accompanies the birth of a new field, biotransformations were hailed as a panacea that would ultimately displace traditional organic chemistry.

However, the role of such biotransformations is one of support rather than supplantation; biotransformations should be employed when a given reaction step is not easily accomplished by "ordinary" chemical methods[9].

Contrary to the very early applications, biotransformations are carried out today by pure cultures of microorganisms or animal and plant cells or with purified enzymes, and they should always be considered as a way of performing selective modifications of defined pure compounds into defined final products[10]. The main

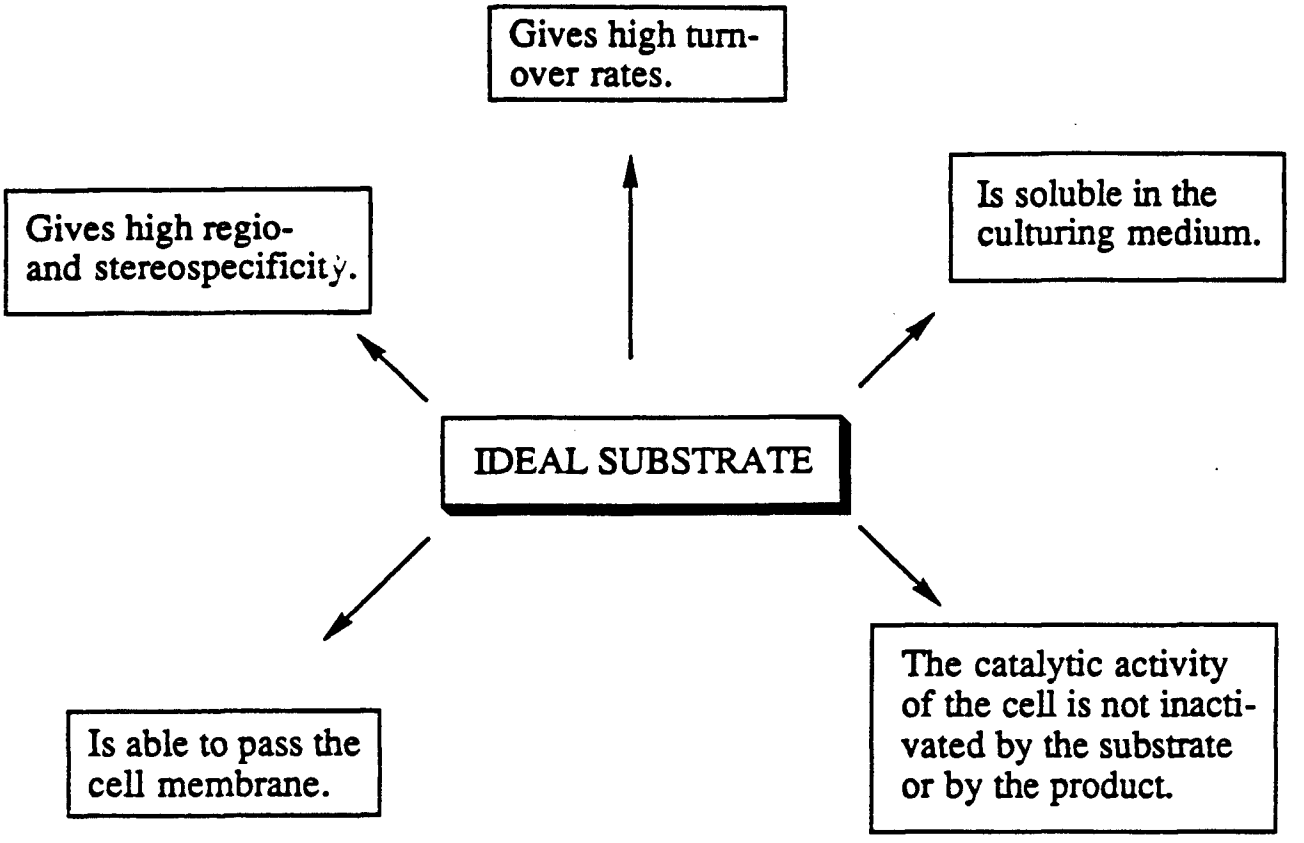
differences between biotransformation and fermentation have been clearly listed by Yamada[11].

The general goal of biotransformations may be considered to be as follows: resolution of racemates, selective conversion of functional groups among several groups of similar reactivities, introduction of a chiral center, and functionalization of a certain nonactivated carbon[12].

There are two different biotransformation systems: cells or isolated enzymes. Availability of a certain organism can be a deciding factor for an organic chemist turning to biotransformations in synthesis. For example, Baker's yeast (*Saccharomyces cerevisiae*) is readily available and easily used, but access to other microorganisms may require help by a microbiologist and access to fermentation facilities. Disadvantages of the use of whole cells for laboratory or industrial-scale operations are that aseptic growth of the cells is usually required, and the work-up is time consuming and messy due to separation of the product from the huge amount of biomass.

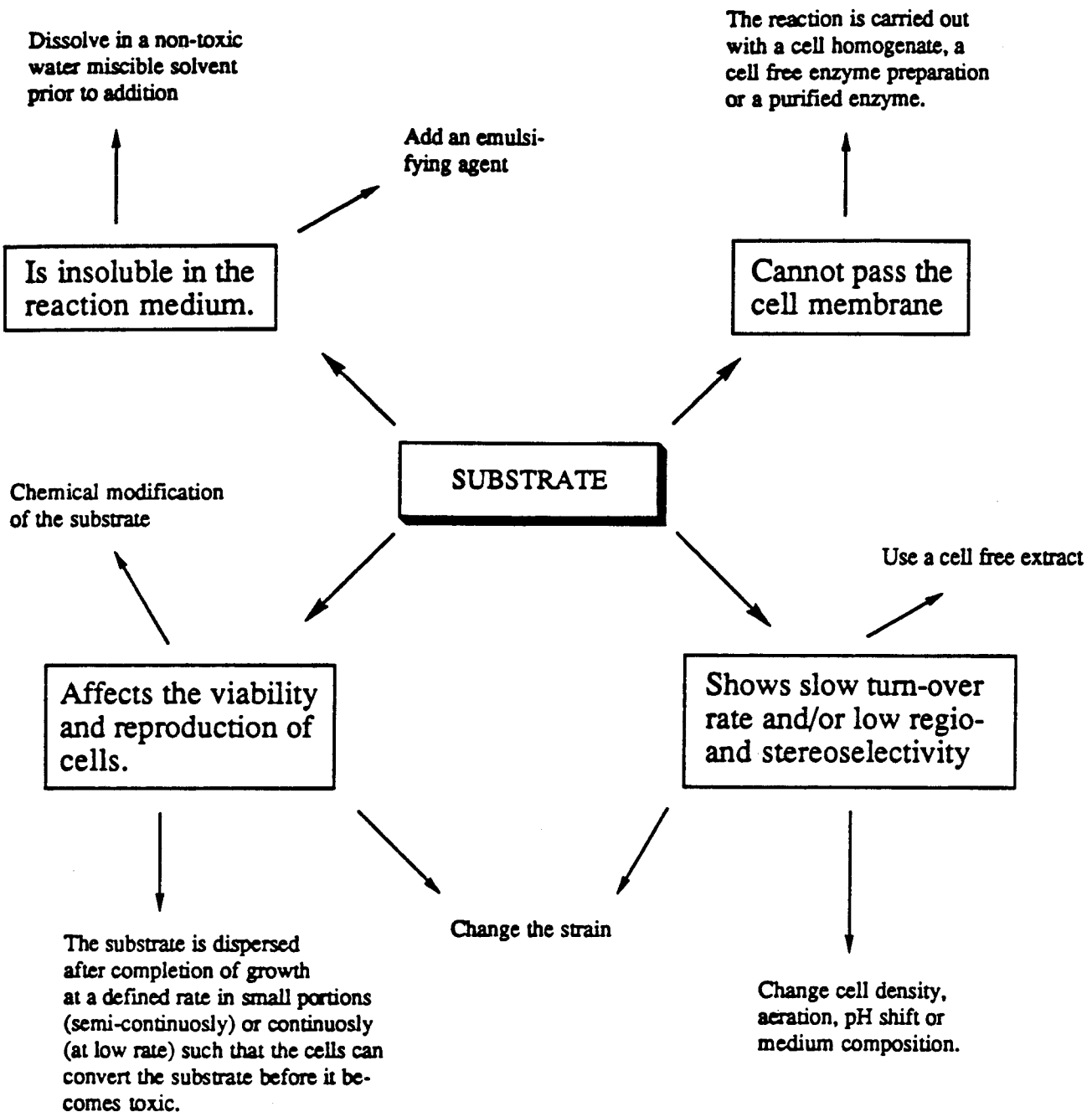
The advantages of isolated enzymes are specificity for selected reactions and that their use may require small equipment and simple work-up[13]. But enzymes are more expensive, and addition of enzyme cofactors or enzyme cofactor recycling might be necessary[14,15]. The present work consists of general biotransformation methodology applied to plant cell cultures and derived crude enzyme preparations (cell free extracts). Ideal interaction between substrate and cell (Scheme 1)[16] is rarely found in practice. Some advice on how to deal with basic problems often encountered with such biotransformations is provided in Scheme 2[16].

Plants are the most important source of land-based foods, oils, and fibers, and represent an immense repository of biochemicals, including flavors, essences, pigments, fine chemicals, pharmaceuticals, and novel biologically active substances[17-20]. In most instances, plant-derived biochemicals are secondary metabolites with wide structural variety, although specific taxonomic plant groups yield specific homologues.



Scheme 1. Ideal interactions between substrate and cell.

The plants sought for their secondary products are sometimes native to remote or politically unstable countries. Biotechnology provides a potential means of securing stable supplies of these compounds[21]. Secondary metabolites are derived biosynthetically from the products of primary metabolism. The main categories include compounds derived from 1) acetate, 2) mevalonate, 3) shikimic acid, 4) non-aromatic amino acids, or 5) are of mixed biosynthetic origin. The structural diversity and complexity of plant secondary products account for the unique properties and biological activities of these compounds.



Scheme 2. Basic problems and solutions for substrate-cell interactions.

Chemical synthesis is the obvious approach for obtaining supplies of these compounds; however, even in cases where synthetic routes can be established, the total synthesis of the desired product cannot always be achieved economically.

In vitro plant culture systems provide an alternate route to plant biochemicals. Plants cultured *in vitro* represent an excellent experimental system for biochemical investigations and potentially a vast commercial source of valuable chemicals. For example, plantlets, specific plant organs or cells growing on solid medium may produce the substances of interest. However, these cultures grow relatively slowly and are labour intensive because they require extensive subculture to generate the large quantities of biomass needed for commercial product isolation.

However, differentiated plant tissue may not be required. If one puts an excised plant tissue on semi-solid nutrient media, an undifferentiated callus tissue may form. This can be dissociated into a fine cell suspension by putting the callus into liquid media and allowing further growth in rotary shakers. The advantages of cell suspensions as potential sources of secondary metabolites include a defined culture environment, the ease of manipulation of culture conditions, and the rapid generation of large volumes of relatively uniform tissue. But, despite the tremendous versatility of the system, the technique has as yet resulted in few commercial successes. One of the major reasons is that in contrast to microbial fermentations, where many years of research have been available, the technique of producing secondary metabolites in plant cell cultures is a relatively new area of research, so much further development must occur.

Another way to exploit a specific enzyme of a cultured plant cell for the purpose of biotransformation of a relatively inexpensive substrate to a more valuable product is found in a cell free system. This involves the isolation and use of enzyme(s), thus maintenance of the biomass is not required. Chemical production based on isolated enzymes, either in solution or as an immobilized system, is an

established technology; the production of plant derived chemicals by using this technique, however, is not yet common, again for the reasons noted above. The potential of this approach to commercial phytochemical production has been demonstrated in our laboratories. For example, the discovery of a catharanthine-vindoline coupling enzyme which yields 3′,4′-anhydrovinblastine (3)[22,23], an analogue of the anticancer drugs vinblastine and vincristine, has resulted in an important process (Figure 1).

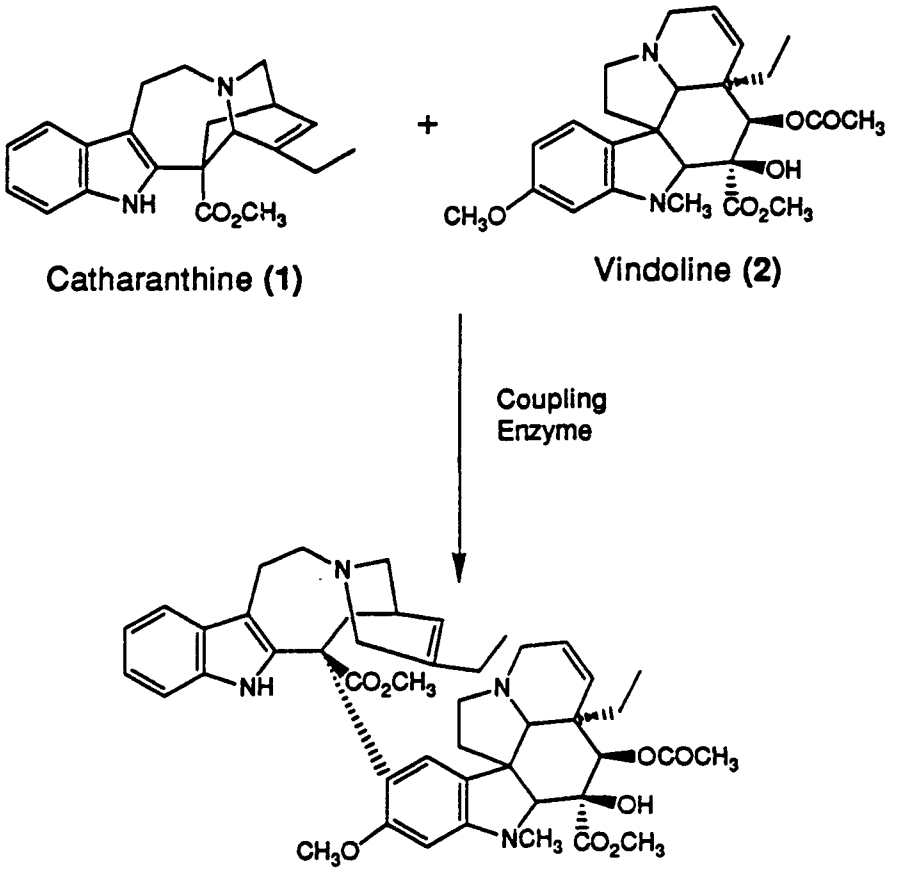
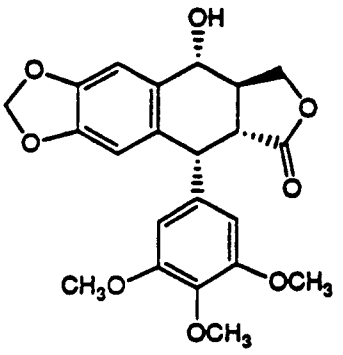


Figure 1. Coupling of catharanthine (1) and vindoline (2).

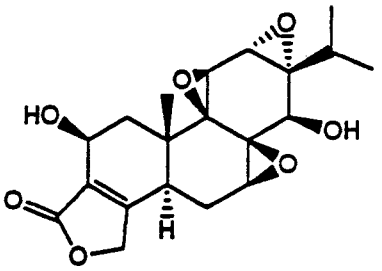
This example couples the use of "precursors" believed to be the biosynthetic building units with an elegant biotransformation step. As chemists, the clue to the use

of cultured cells for biochemical synthesis is the understanding of secondary metabolic processes, so that we may logically and consistently control and manipulate the relevant parameters.

In the present study, two different pharmacologically interesting and synthetically challenging products were chosen for studies of plant-cell mediated biotransformation: (i) the lignan podophyllotoxin (4), the commercial starting material in the synthesis of the anti-cancer drugs etoposide and teniposide, and (ii) tripdiolide (5), a diterpene with significant male anti-fertility activity. One chapter of this thesis is devoted to each of these studies.



Podophyllotoxin (4)



Tripdiolide (5)

CHAPTER 1. THE PODOPHYLLOTOXIN FAMILY

INTRODUCTION

Podophyllotoxin (4)[24,25] is an interesting member of the naturally-occurring family of compounds known as lignans which are derived biosynthetically from the shikimate pathway[26-28]. Podophyllotoxin has been the subject of much study (Figure 2) as it presents a challenging synthetic target and, more importantly, has proven anti-cancer properties. Natural sources of podophyllotoxin are relatively few[29,30], but species of *Podophyllum* produce quite large amounts of this lignan in their roots[31-33].

Podophyllotoxin is isolated commercially from the American Mandrake (*Podophyllum peltatum* Linnaeus) and the related Indian species *P. emodi* Wallich (*P. hexandrum* Royle). The dried roots and rhizomes, known as podophyllum, are extracted with alcohol to produce a resin (podophyllin) containing podophyllotoxin. For example, *P. peltatum* yields 4-6% podophyllin[34] which affords 9-10% podophyllotoxin upon chromatographic separation[35]. Although the medicinal properties of *P. peltatum* had long been known by North American natives, it was not until 1942 that Kaplan[36] demonstrated the efficacy of podophyllin in the treatment of venereal warts. This finding sparked much interest in the biological and chemical properties of podophyllin and its constituents, and this continues today as can be seen from the present publications on this topic.

It has been found that certain glycosides of 4′-demethylepipodophyllotoxin (6) (Figure 3) are also effective anti-cancer drugs but lack the unacceptable side effects of podophyllotoxin[37]. The most important compound of this class is etoposide (7)[38], which is used clinically in the treatment of myelocytic leukemia, neuroblastoma, bladder cancer, testicular cancer, and small-cell lung cancer[39].

Unlike podophyllotoxin, these compounds do not inhibit microtubule assembly; they are believed to derive their anticancer properties from interference with DNA topoisomerase II, an enzyme which makes reversible double-stranded breaks in DNA

1700's:	Folklore medicine. Uses of podophyllin, a resin obtained from alcohol extraction of *Podophyllum peltatum.* North American Indians used preparations as a purgative and emetic and also as a suicide agent. It was also used for diseases of the liver, kidneys, syphilis gonorrhea, urinary obstruction, and as a cathartic.
1820:	Included in the first US pharmacopoeia as a cathartic.
1850:	Commercial production of podophyllin begins.
1880:	Isolation of components from podophyllin starts. Active compounds believed to be podophyllotoxin and peltatin.
1942:	Pharmacological studies are continued and reports about possible use of podophyllotoxin in cancer treatment emerge.
1950:	Clinical studies initiated at Sandoz Laboratories, Basel, Switzerland with a variety of synthetic analogues of podophyllotoxin are discontinued due to toxicity.
1970:	Clinical studies with synthetic analogues of podophyllotoxin are reassumed.
1978:	Etoposide is selected as clinical drug, licensed by Sandoz to Bristol-Myers, USA.
1983:	FDA approves drug etoposide (VP-16-213), commercial name: vepesid.

Figure 2. Brief history of etoposide development - from folk medicine to clinical drug.

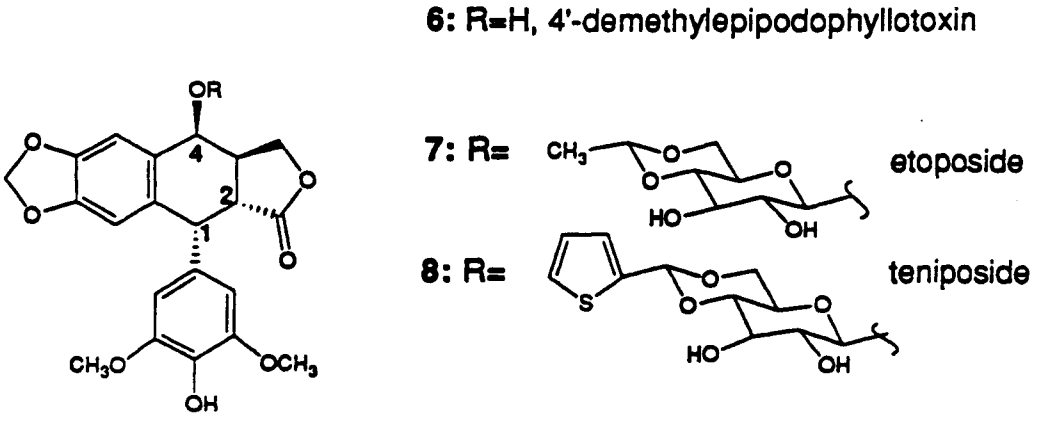


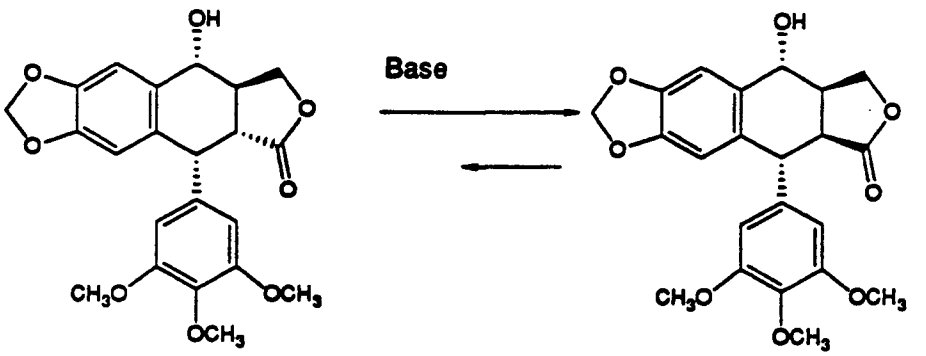
Figure 3. The structures of 4′-demethylepipodophyllotoxin (**6**), etoposide (**7**), and teniposide (**8**).

in order to prevent the tangling resulting from DNA replication[40]. Recent work has suggested that the role of etoposide is to promote both DNA cleavage and inhibit its subsequent ligation[41,37], the presence of a hydroxyl group at C(4′)[42] and a β-orientation of the C(4) substituent have been shown to be critical for such anti-tumor activity. The actual nature of the C(4) substituent is much less important[43].

The drugs are produced from podophyllotoxin by a demethylation and epimerization sequence[25]. Podophyllotoxin, rather than its demethylated analogue, 4′-demethylpodophyllotoxin, which is also present in *Podophyllum* species, was chosen as starting material because of its greater natural abundance.

The structure of podophyllotoxin is only deceptively simple as the 1,2-*cis*-2,3-*trans* stereorelationship, which is of crucial importance for the biological activity[24], constitutes a synthetic difficulty, due to facile epimerization to the less strained, but inactive *cis*-lactone picropodophyllotoxin (**9**)[44] (Figure 4).

It should be noted that the stereochemistry of C(4) at this point is of less concern as podophyllotoxin and epipodophyllotoxin are easily interconvertible[45,46].



Podophyllotoxin (**4**) Picropodophyllotoxin (**9**)

Figure 4. The equilibrium between podophyllotoxin (**4**) and picropodophyllotoxin (**9**).

Furthermore, for the synthesis of etoposide and teniposide the C-4 hydroxyl group can either be α- or β-oriented because in the glycosidation reaction only the C-4 β-glycoside is obtained[47].

TOTAL SYNTHESES

The first total synthesis of podophyllotoxin was achieved in 1966 by Gensler[48]. Now, a quarter of a century later, synthetic chemistry continues to be enchanted with the tactical complexity of establishing the four contiguous stereocenters and *trans*-fused γ-lactone ring of this natural product[49-61].

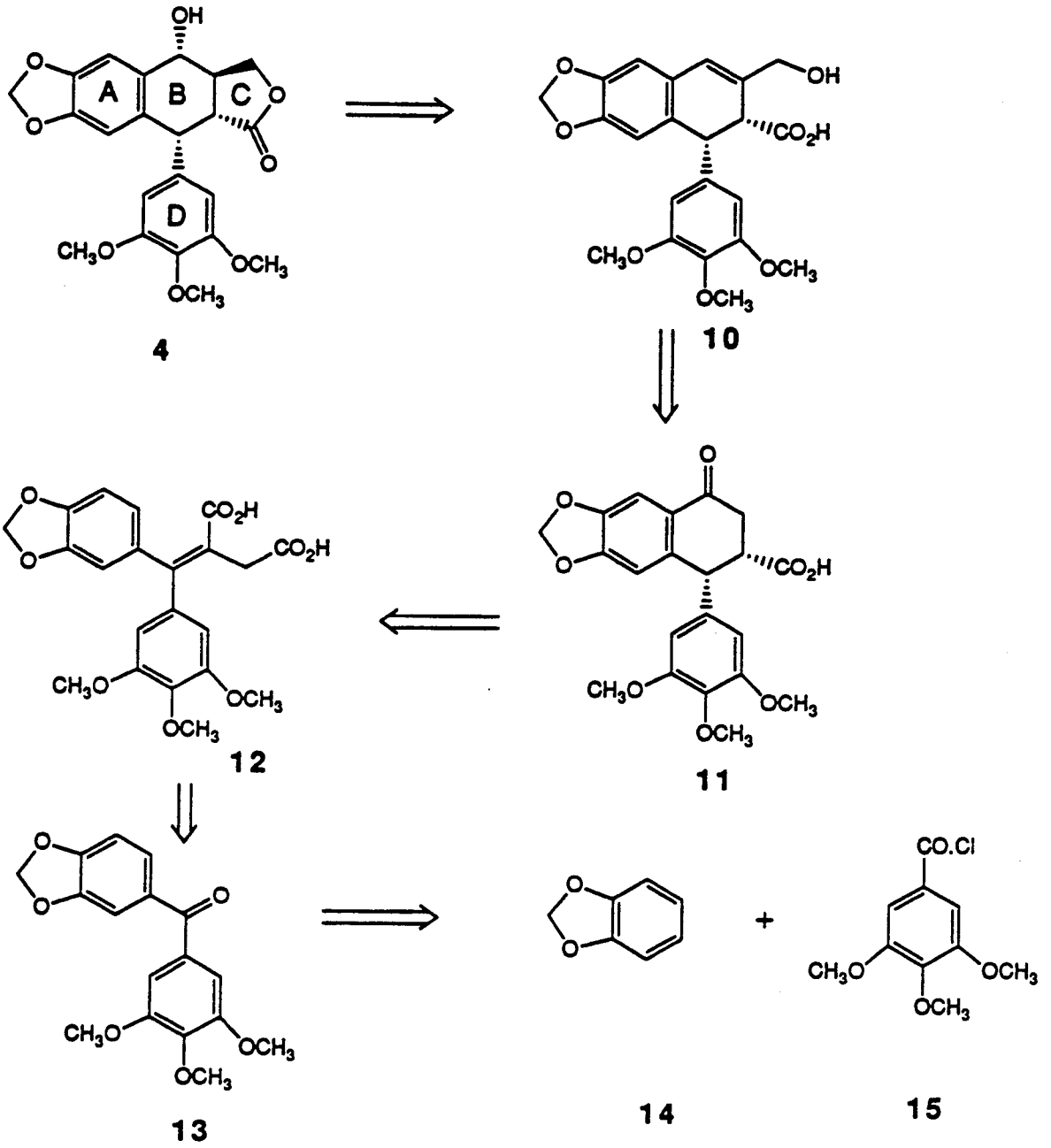
Three different strategies have been used; and I have arbitrarily selected three syntheses from the literature to illustrate these approaches.

1. Kinetic protonation of picropodophyllotoxin.

Several approaches[54,56,49], including the first enantioselective synthesis reported by Meyers et al[56], are directed towards picropodophyllotoxin and rely on Gensler's observation of kinetic protonation of the enolate anion of picropodophyllotoxin[48] yielding podo- and picropodophyllotoxin in a 45:55 ratio.

More recently, equilibration at an earlier stage of the sequence was found to be more efficient[7].

Gensler's retrosynthetic analysis is shown in Scheme 3.



Scheme 3. Retrosynthetic analysis of podophyllotoxin (4) by Gensler.

Disconnection on ring C and dehydration leads to DL-α-apopodophyllinic acid (10) which can be thought of as being derived from intermediate 11 via formylation followed by reduction and dehydration. The ketone functionality in 11 is derivable from the intermediate product 12, the latter arising from condensation between a succinic ester and the ketone 13. This diaryl ketone could, in turn, be prepared via Friedel-Crafts acylation of an appropriately substituted benzene with 3,4,5-trimethoxy benzoyl chloride (15).

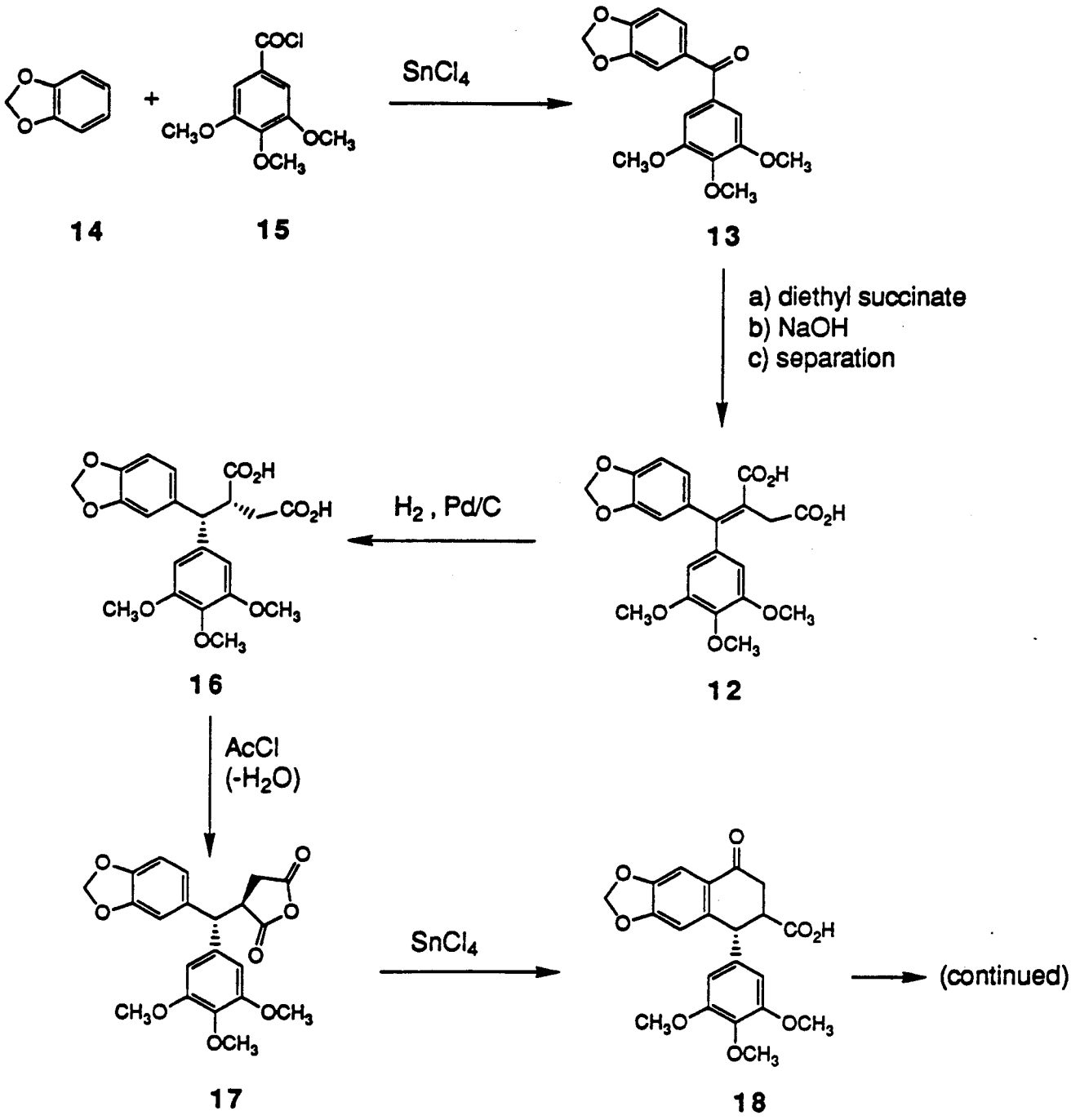
Ring B of podophyllotoxin was constructed by a condensation reaction between the ketone 13 and diethyl succinate (Scheme 4) to produce the unsaturated acid 12. This acid was further elaborated to produce the anhydride 17, which was cyclized using tin (IV) chloride to afford intermediate 18.

Ring C was constructed by the sequence, 19 → 20 → 21 → 22 (Scheme 4). Reaction of intermediate 19 with sodium hydride and ethyl formate provided enol 20, the latter was then further elaborated to the diol 21 which was then cyclized under acidic conditions to produce the lactone 22. The key intermediate 22 was then hydrolyzed to the corresponding acid 23, resolved with quinine and relactonized to give the (-)-enantiomer of 22.

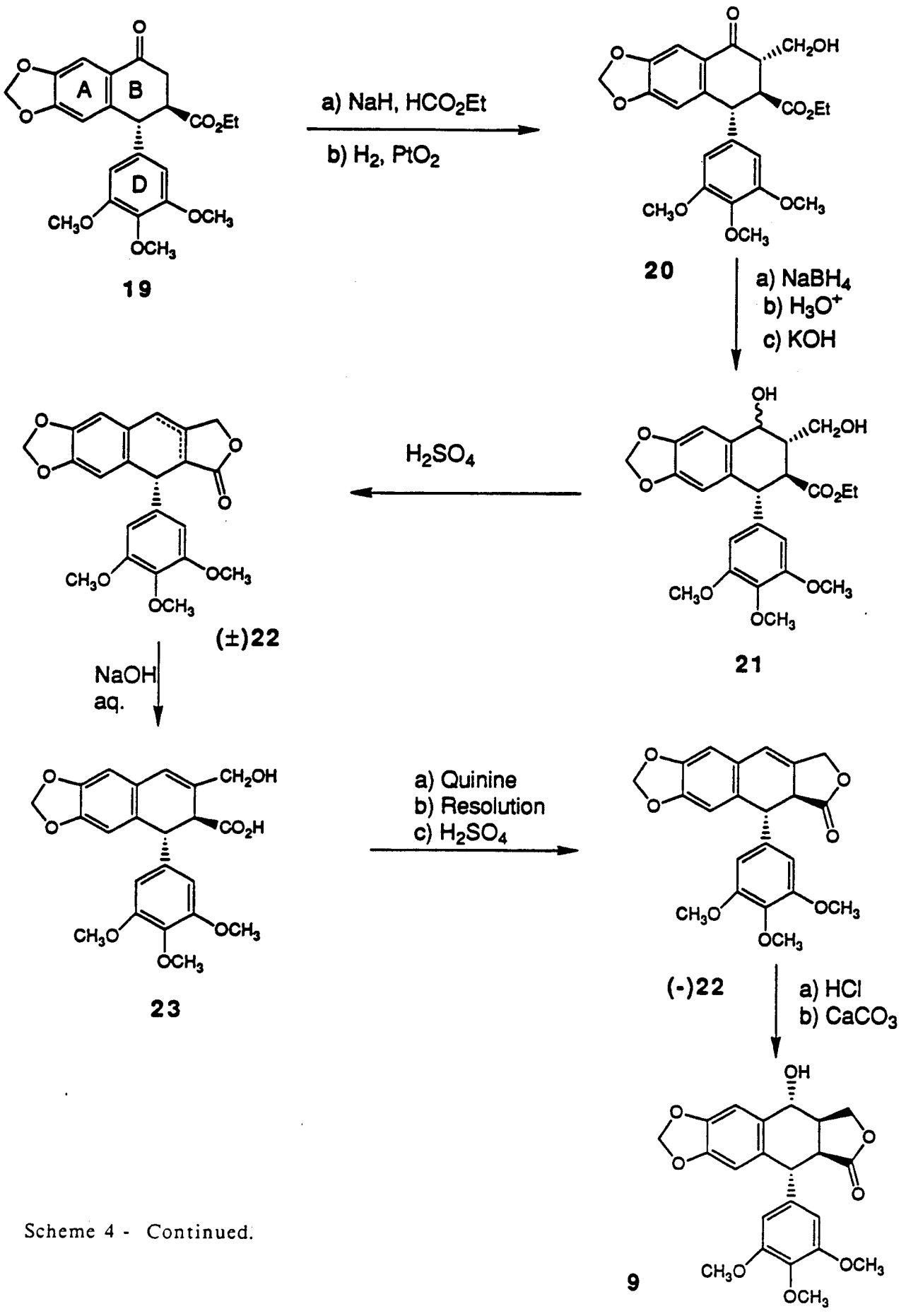
The synthesis was completed when the tetrahydropyranyl ether of picropodophyllotoxin (24) (Scheme 5) was treated with triphenylmethyl sodium to produce the enolate. Irreversible protonation of the enolate with glacial acetic acid followed by removal of the protective group with dilute aqueous acid gave a 45:55 mixture of podophyllotoxin (4) and picropodophyllotoxin (9) which could be chromatographically separated.

2. Diels-Alder approach.

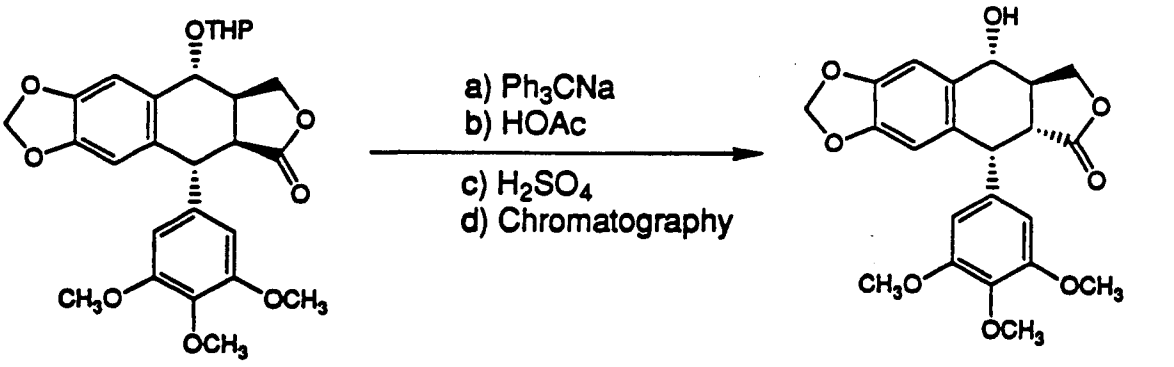
The intermediacy of picropodophyllotoxin was avoided first by Rodrigo et al[63] who constructed ring B via a Diels-Alder reaction involving an isobenzofuran.



Scheme 4. Total synthesis of picropodophyllotoxin (**9**) by Gensler.



Scheme 4 - Continued.



24 Podophyllotoxin (**4**)

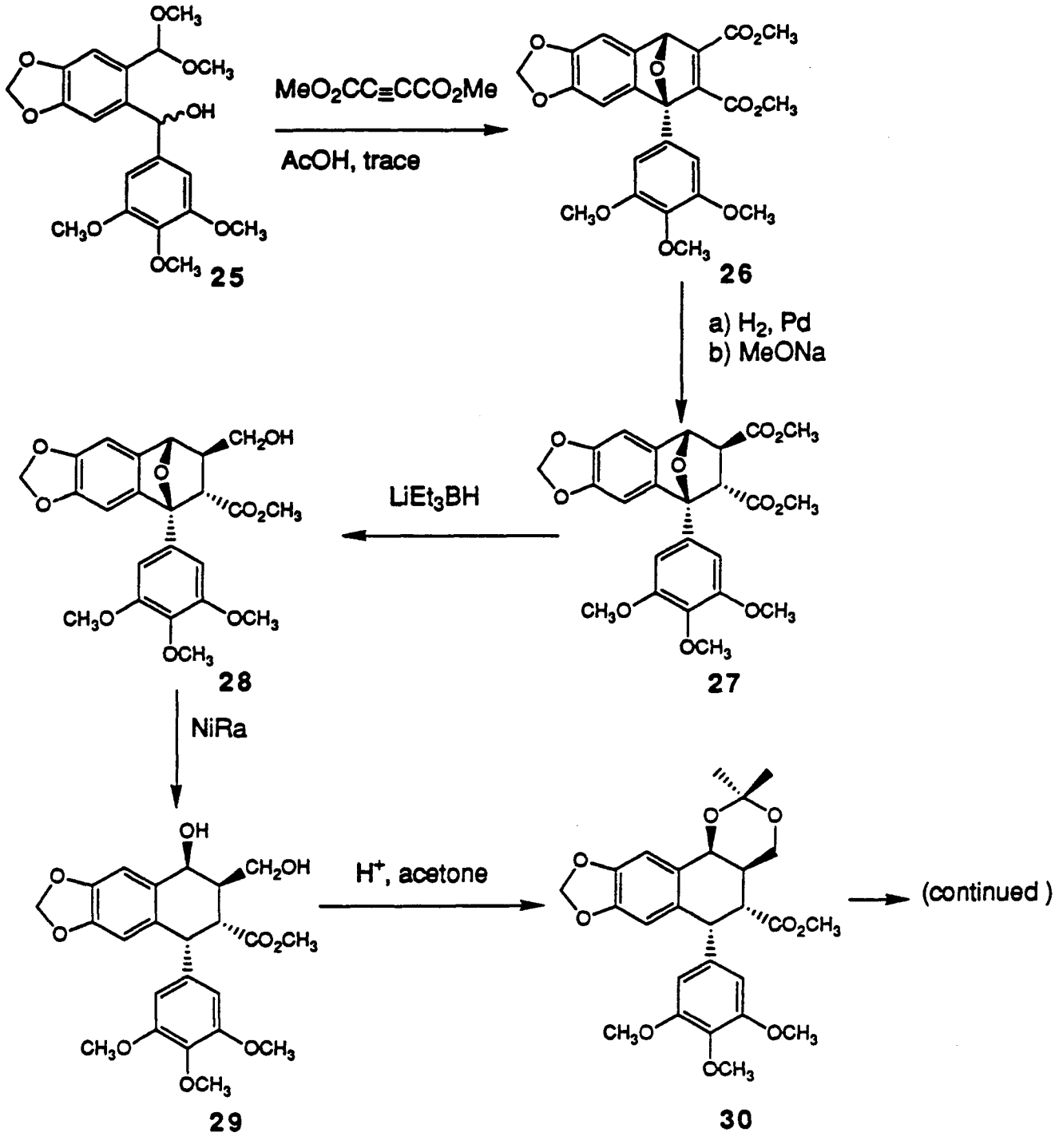
Scheme 5. Synthesis of podophyllotoxin (**4**) from the tetrahydropyranyl ether of picropodophyllotoxin (**24**) by Gensler.

The initial step in this synthesis (Scheme 6) involves a cycloaddition of compound **25** (prepared by lithiation of the dimethylacetal of 6-bromo piperonal and reaction with 3,4,5-trimethoxybenzaldehyde) with dimethylacetylene dicarboxylate to give the isobenzofuran **26**. This is further elaborated to produce the diol **29**. The acid **31** produced from the diol **29** by protection as an acetonide and saponification, proved to be the key intermediate in the synthesis. When this compound was treated with dilute acid for 24 h, the acetonide was cleaved and subsequent treatment with dicyclohexylcarbodiimide (DCC) yielded epipodophyllotoxin (**33**). However, if compound **31** was treated with aqueous acid for 48 h, then neopodophyllotoxin (**32**) was produced. On treatment of **32** with sodium hydroxide followed by treatment with DCC, podophyllotoxin was produced.

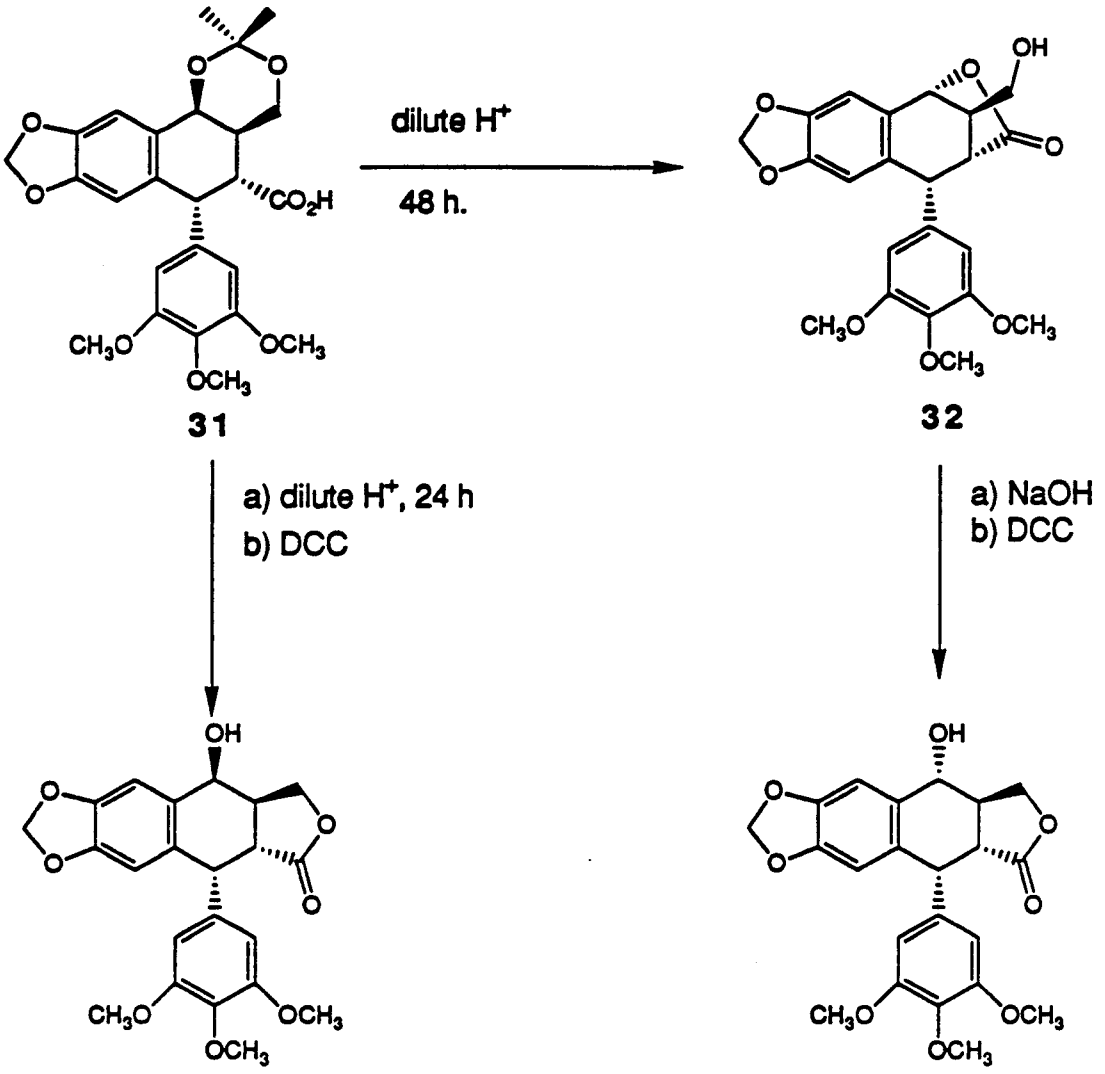
Other groups have also drawn on this strategy[51,52,64,65].

3. **Tandem Michael addition-aldol condensation approach.**

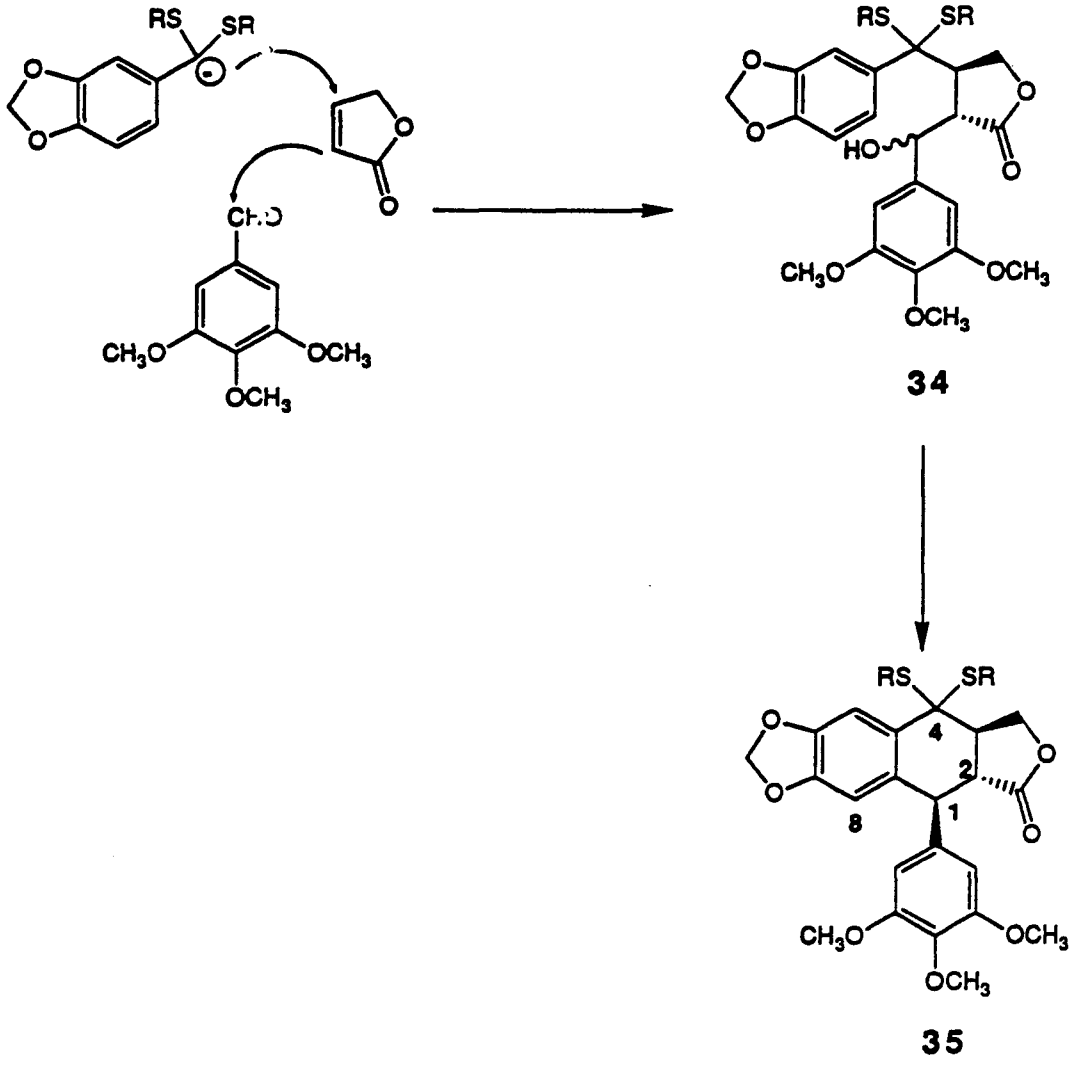
An interesting approach has been explored by Ziegler et al[66]. In this approach (Scheme 7) the carbon framework is assembled during a one-pot tandem Michael



Scheme 6. Synthesis of podophyllotoxin (4) from 25 by Rodrigo et al.

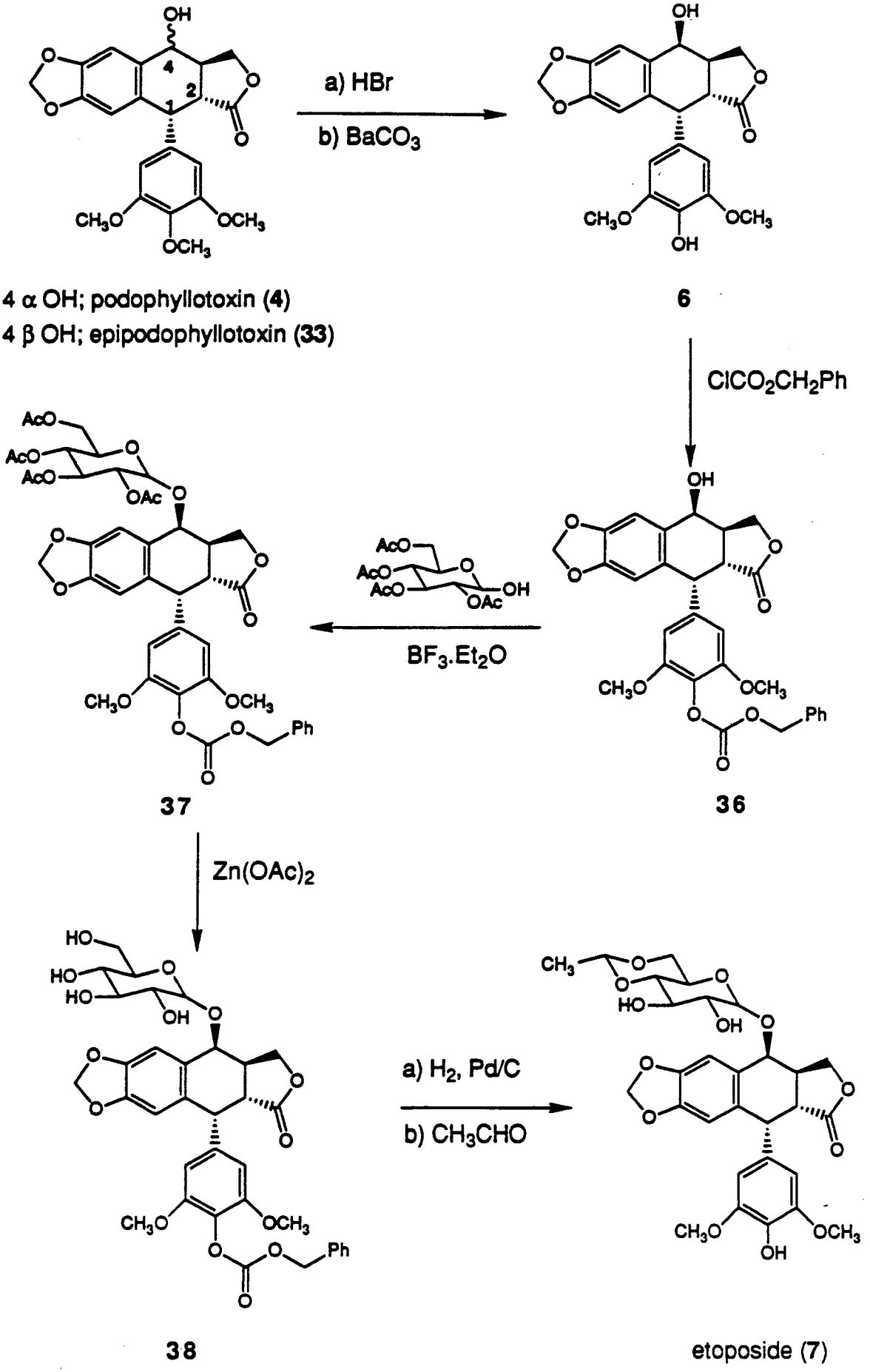


Scheme 6 - Continued.



Scheme 7. Ziegler's approach to aryl tetralin synthesis.

addition-aldol condensation resulting in the desired 2,3-*trans* relationship. However, electrophilic ring closure (i.e. formation of the 1-8a bond) of 34 leads to the all *trans* product 35, belonging to the pharmacologically uninteresting isopodophyllotoxin series. Also a "biomimetic" ring closure with thallium (III) trifluoroacetate has provided only the 1,2-*trans*-substitution pattern[49].



Scheme 8. The synthesis of etoposide (7) from podophyllotoxin (4).

Deoxypodophyllotoxin[57], and epipodophyllotoxin[53] have also been synthesized. Other routes into the lignan system have also been investigated and are discussed in a review by Whiting[67].

The etoposide precursor, 4′-demethylepipodophyllotoxin (6) was synthesized by Kende[49] in 1981 with an overall yield of 2.4% in a 13 step sequence. The stereochemical problem of the lactone (ring C) was solved by Gensler's methodology. The conversion of podophyllotoxin (4), epipodophyllotoxin (33) or 4′-demethylepipodophyllotoxin (6) to etoposide is outlined in Scheme 8[68,47], in which 4′-demethylepipodophyllotoxin (6) is treated with benzyl chloroformate to produce the protected derivative 36. Treatment of 36 with 2,3,4,6-tetra-0-acetyl-β-D-glucopyranose and boron trifluoride etherate followed by zinc acetate afforded the glycosylated intermediate 38. Deprotection via catalytic hydrogenolysis of the carbonate and condensation with acetaldehyde produced the drug etoposide (7).

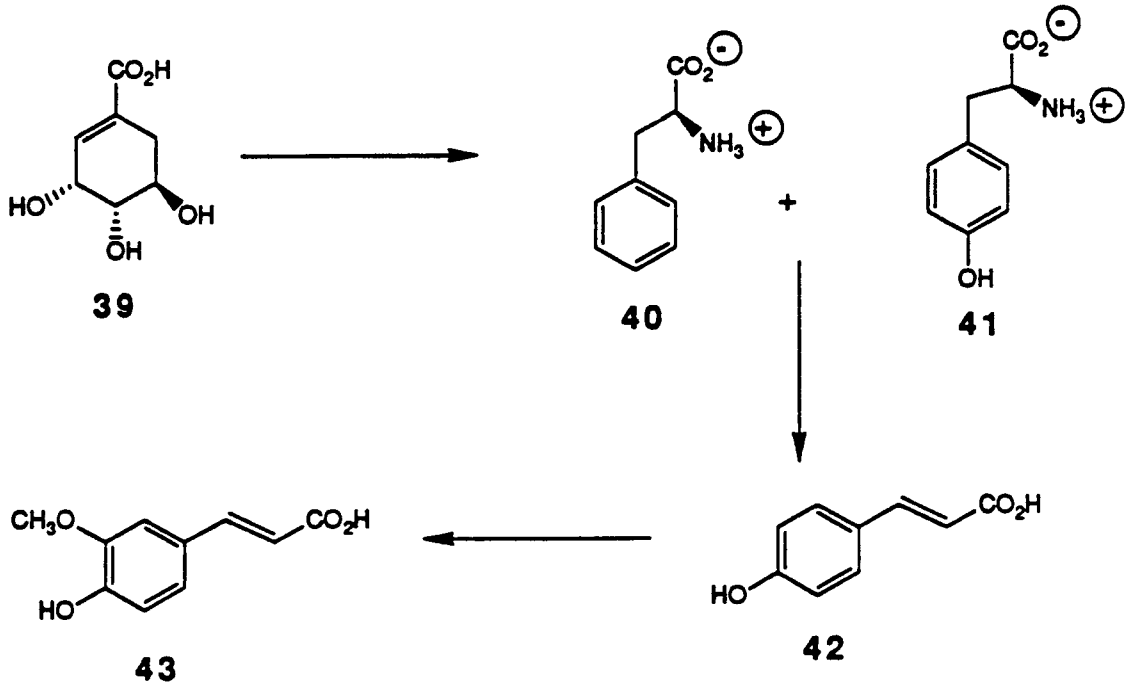
Because Ziegler's strategy is inherently straightforward and easily adaptable to different ring substitution patterns, the reinvestigation of a stereoselective 1-8a bond formation would be highly rewarding. An approach which may solve this problem could involve biotransformations by plant cell cultures in which suitable precursors are subjected to such enzymatic processes. Such processes may be more amenable to scale up than the corresponding synthetic counterparts.

Any investigation of the use of biotransformations in the production of the podophyllotoxins must first consider their biosynthesis. It is this information which will direct research towards the most potentially rewarding precursors.

BIOSYNTHESIS OF THE PODOPHYLLOTOXINS

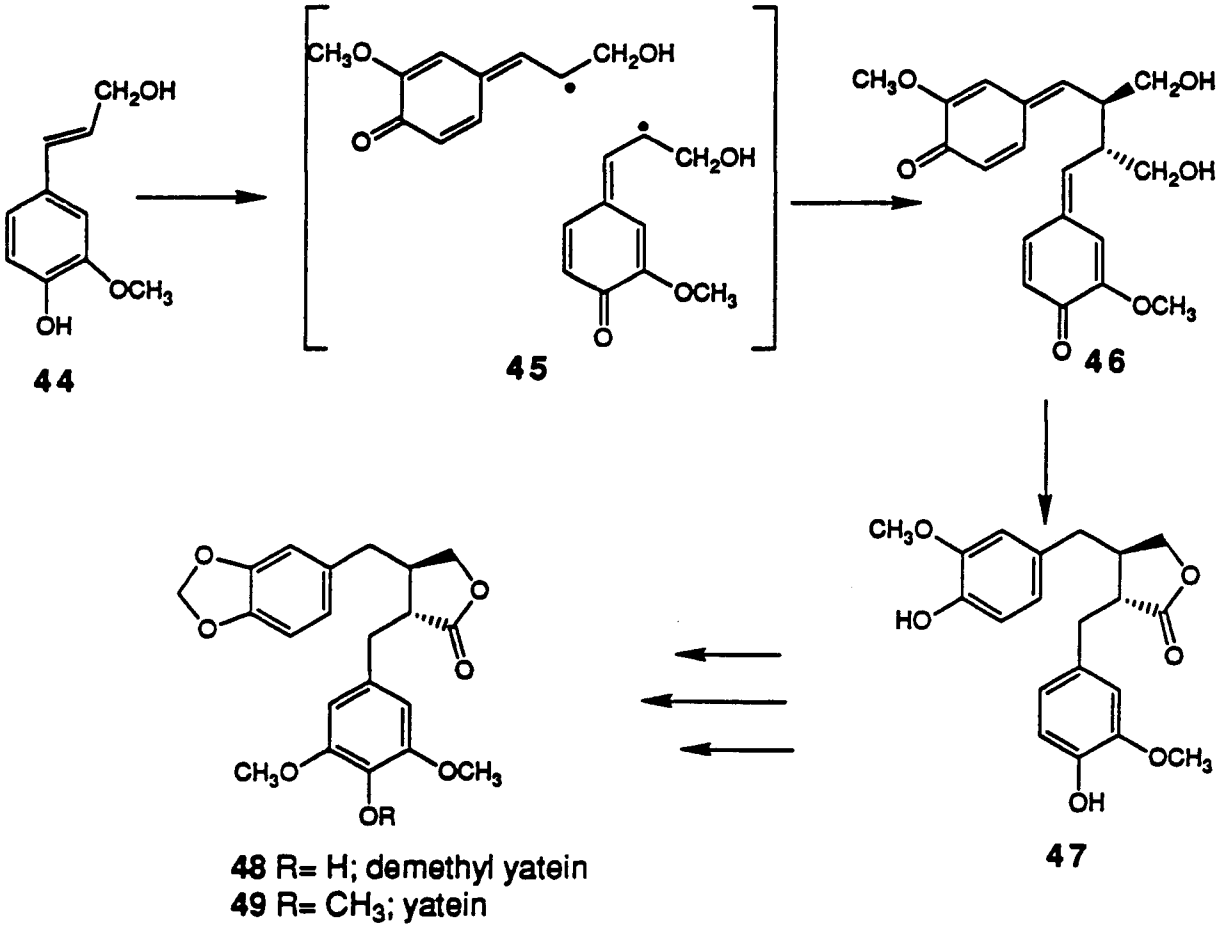
The biosynthesis of lignans is a rather neglected experimental area. Studies that have been reported to date have concentrated on the demonstration of the incorporation of C6-C3 units into lignans and on the nature of the preferred units. Little is known about the nature or the transformations of the immediate products formed from the coupling of such C6-C3 units. The skeleton of the *Podophyllum* lignans is known to be derived from two C6-C3 molecules, and a phenolic oxidative coupling process is generally believed to operate[69].

The biosynthesis of C6-C3 compounds is clearly germane to the origin of lignans. Some of the intermediates believed to be involved in this pathway are shown in Scheme 9[70].



Scheme 9. Biosynthesis of ferulic acid (43) from shikimic acid (39).

The aromatic rings are formed from shikimic acid (39), which is enzymatically transformed into (L)-phenyl alanine (40) and (L)-tyrosine (41). These intermediates are then converted by further enzymatic processes into 4-hydroxycinnamic acid (42) and then into ferulic acid (43). Two of these nine carbon units are then envisaged to couple together in a head-to-head fashion to produce the lignan intermediate (46) (Scheme 10).

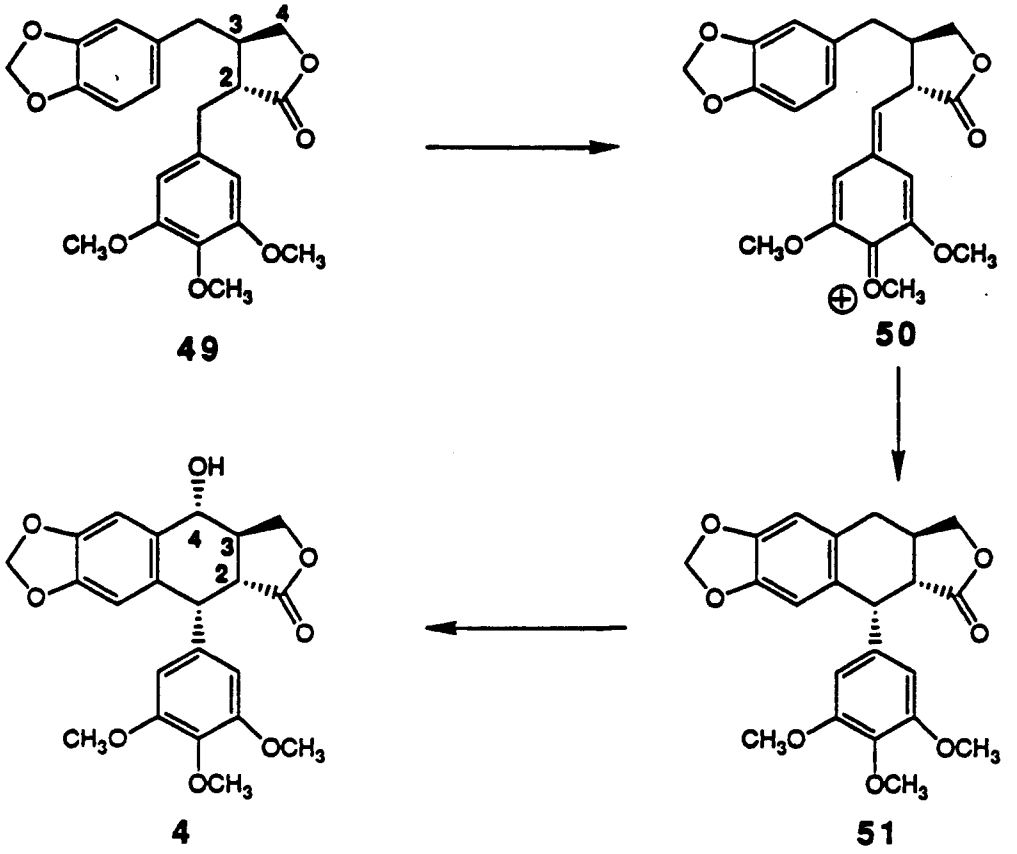


Scheme 10. Proposed biosynthesis of yatein (49) from coniferyl alcohol (44).

From the results of the feeding experiments with *Podophyllum hexandrum* living plants, this coupling has been shown to involve two phenylpropane "precursors" with the same 4-hydroxy-3-methoxy substitution pattern[71], and coniferyl alcohol (44) is almost certainly the intermediate concerned[72]. Stereospecific enzyme-catalyzed coupling of free radical mesomers 45 derived from coniferyl alcohol would lead to a diquinomethide (46). The dibenzobutyrolactones are thought to be produced from 46 via reduction, lactone ring formation and appropriate modification of the aromatic substitution pattern. Matairesinol (47) has been confirmed to be a common intermediate in the pathway of *Podophyllum* lignans[73], such as yatein (49). Other experiments involving labelled compounds have shown that matairesinol and yatein are incorporated into podophyllotoxin. The incorporation of yatein (49) into podophyllotoxin (4) involves a stereoselective cyclization in which the newly created chiral centers (C1 and C2) place the protons attached to these centers in a *cis* relationship. These results are summarized in Scheme 11.

In considering which substrates are suitable for a potential biotransformation, a number of factors must be taken into account. Firstly, the substrate must be easily synthesized on a large scale; secondly, the substrate must possess structural features similar to presumed late stage biosynthetic intermediates, for example, 49, in order that its transformation to the desired product may "mimic" the reactions thought to occur in the biosynthetic process. Only in cases where the substrate is very inexpensive can low incorporation into the product be tolerated. For these reasons, compounds structurally related to yatein were chosen, and it was hoped that a stereoselective ring closure to produce compounds with the podophyllotoxin structure could be achieved.

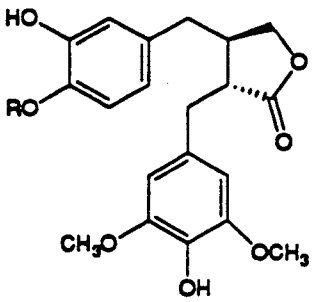
Terry Jarvis[74] and Jan Palaty[75] in our group have addressed some of these problems in investigating the biotransformation of compounds 52 to 55 (Figure 5) with *Catharanthus roseus* cell free extracts (crude enzymes).



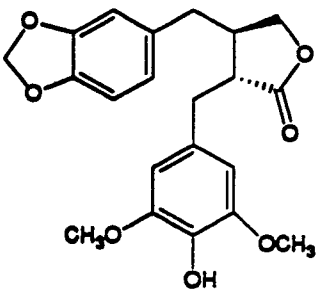
Scheme 11. The incorporation of yatein (**49**) into podophyllotoxin (**4**).

Preliminary evaluation of compound **56** by these workers showed some encouraging results. However, even in the cases where they achieved ring closure, the stereochemistry at C_1 was incorrect. It was therefore concluded that enzymes from the *C. roseus* cell line, the latter producing indole alkaloids as secondary metabolites, are not suitable for the stereoselective ring closure, for example, **49** → **51**. In these studies, high yields (approximately 90%) of ring closure did occur to achieve the "wrong" cyclic isomer in which the aromatic unit at C_1 in **51**, for example, was attached to the β-orientation.

While the above studies with *C. roseus* were underway, a cell line of *P. peltatum* (see next section), the plant from which the podophyllotoxin family of compounds was isolated, was developed. It was shown that this cell line produces the "podophyllotoxins" and it was presumed that enzymes for the correct ring closure (49 → 51, for example) were now available. It was therefore of interest to turn our attention to studies of biotransformations of appropriate precursors with this cell line.



52; R=H
53; R=CH_3
54; R= *i*-Pr



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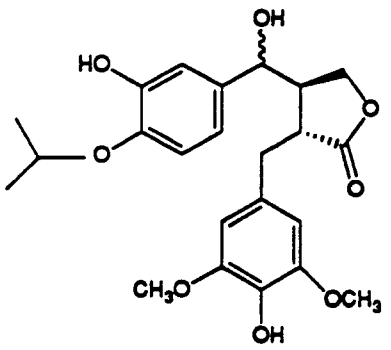


56

Figure 5. The synthetic precursors used in previous biotransformation experiments in Prof. Kutney's group, and involving cell free extracts of *Catharanthus roseus*.

TECHNIQUES OF CELL SUSPENSION CULTURES. PREMISE OF THIS WORK.

An appreciation of the basic techniques of cell suspension culture is essential to any assessment of their potential application.

Suspension cultures are normally initiated by transfer of undifferentiated callus pieces to a liquid medium which is agitated during growth. Successful establishment of a fine suspension culture depends on the initial callus being friable, a condition that may depend upon appropriate phytohormone supplements. During the initial growth phase in the liquid medium, some cells are released from the callus and multiply. For subculture, a wide-mouth pipette is usually used to exclude any persistent large cell aggregates derived by only partial break-up of the initiate callus fragment. The suspension transferred on subculture should consist of free cells and some cell aggregates. The culture medium used for suspension culture is usually based upon that which maintains good growth of callus; it may, however, be necessary to modify this medium (particularly in its phytohormone content) to achieve a high growth rate and good cell separation in liquid medium.

Batch cultures (or cultures in a fixed volume of medium) increase in biomass by cell division and cell growth until a factor in the culture environment (nutrient or oxygen availability) becomes limiting. The cells then enter a stationary phase and finally a declining phase in which cell dry weight decreases: the stability of the cells in stationary phase depends upon the species and on the nature of the growth-limiting factor (cells brought to stationary phase by nitrogen limitation retain viability longer than when carbohydrate-starved)[82]. When a stationary phase cell suspension is subcultured, the cells in succession pass through a lag phase, a short lived period of exponential growth, a period of declining relative growth rate and then again enter stationary phase (Figure 6).

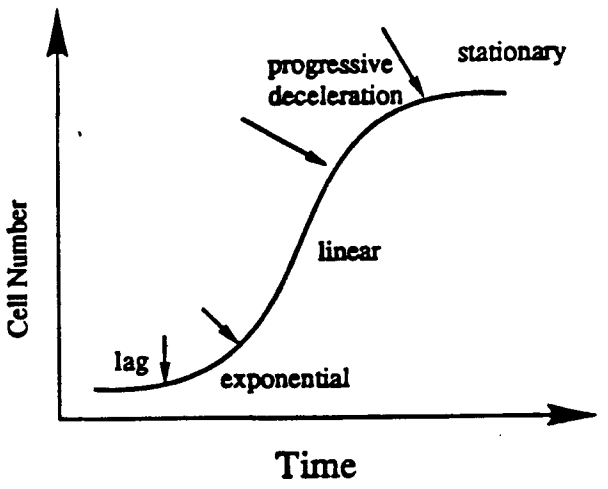


Figure 6. Model curve relating cell number per unit volume of culture to time in a batch-grown plant cell suspension culture. Phases of the growth cycle are labelled.

Traditionally at each subculture, cultures are initiated by a relatively high cell density inoculum and the cells therefore accomplish only a very limited number of divisions (cell number doublings) before again entering stationary phase.

The basic pattern of increase in cell number per unit volume in batch culture is illustrated in Figure 6[76]. During lag phase the stationary phase cells of the inoculum embark upon a massive synthesis of new cytoplasm and associated organelles, replicate their DNA and then begin to divide. Then for a strictly limited period of time, cell division rate is constant and maximal. During this period the mean cell volume declines sharply. Following this the growth begins to decline (slowly at first and later at an ever-increasing rate), mean cell volume increases, and the cells move into a stationary phase. There is evidence that subsequent differentiation may be determined by conditions operating during the period of cell multiplication (meristematic phase)[77], and that the production of many secondary plant products normally occurs in non-dividing cells, often in non-dividing cells which have embarked upon a special pathway of differentiation. Thus, during the progress of

batch culture, cultured cells pass through a series of contrasted physiological states which encompass cells in transition to a meristematic state; cells expressing high meristematic activity; cells undergoing expansion and becoming either metabolically quiescent or geared to express certain restricted metabolic pathways.

The production of secondary products by cell cultures has been shown to be most active during a restricted phase of the growth cycle.

Secondary plant product biosynthesis within whole plants range from products widely distributed in the plant kingdom and whose synthesis is not confined to specialized cells or cell groups, to those characteristic of particular families, genera or species, and whose synthesis takes place in localized and highly specialized cells or cell groups. As might be expected, compounds of the first group (e.g. coumarins, particular flavonoids, phytosterols) either identical with or chemically related to those of the whole plant, have been frequently detected in plant tissue and cell cultures[78]. In contrast, compounds at the other end of the range have, with few if any exceptions, only been detected in tissue cultures in which a high level of organization (cytodifferentiation or organ initiation) has developed[79]. This suggests that progress toward inducing particular specialized patterns of cytodifferentiation in cell cultures will need to be achieved before they can be utilized for the study of the synthesis of volatile oils, resins, latex constituents, and so on. Certainly the most promising immediate application of cell cultures in the synthesis of secondary plant products would be expected to come from work with appropriately highly dispersed cell suspensions in which the desired class of compounds can be demonstrated even if currently at very low yield.

There are now a number of instances where not only primary metabolites (e.g. amino acids, nucleotides) but also secondary products produced by cell suspension cultures, have been shown to be released in significant amounts into their culture medium. As examples may be mentioned lignans[80] and diterpene derivatives[81]. In

such cases a maintenance culture in a closed continuous system should enable the chemical product to be continuously harvested from a fixed culture biomass. This would discount the objection sometimes raised with respect to plant cell cultures as potential commercial biosynthetic systems in that they are very slow growing compared with microorganisms.

The premise on which the present work is based is that if a large biomass could function to release, over a long period, a secondary plant product or a desired derivative from a supplied substrate, then the time required to grow up the biomass would be of small economic consequence. Further, the ability in the closed continuous system of constantly removing the released metabolite could mimic a biological "sink", preventing any feedback inhibition of synthesis. It would also stop the product from exerting any "stalling" effect (toxicity due to the action of the product at the exposed surfaces of the cells) by preventing its accumulation.

PRODUCTION OF PODOPHYLLOTOXINS BY PLANT TISSUE CULTURES

Podophyllotoxins as mentioned previously, have been isolated from several plant species. However, isolation of the target compounds by extraction from the plant can be associated with various problems, seasonal variation being one of them. Detailed analysis of *P. peltatum* plants raised in the United Kingdom and also in the United States of America, has confirmed variation in lignan pattern between individual plants, but in general, dormant plants contain podophyllotoxin (**4**), α-peltatin (**58**), and β-peltatin (**59**), with β-peltatin usually predominating[31] (Figure 7).

Subsequent analysis of root specimens excised from plants during the growth season (March-August) show the peltatin content rapidly disappearing, and by May only traces can be detected. Podophyllotoxin is now the main constituent. Towards the end of July, some peltatin can again be observed in many plants, coinciding with

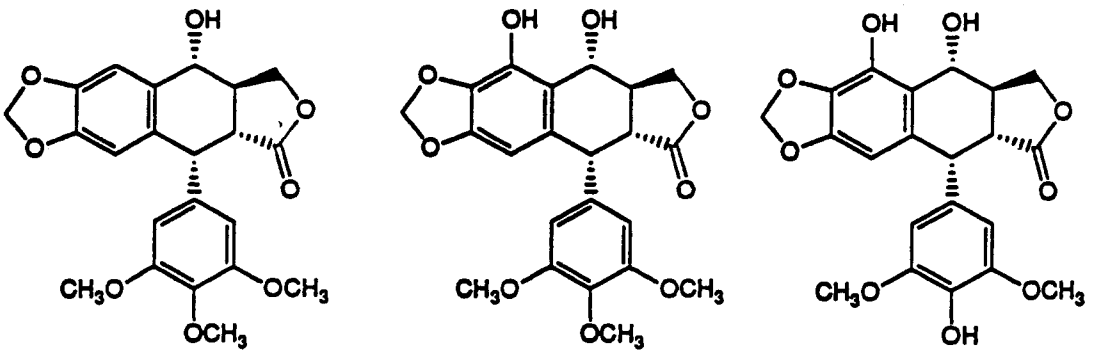


Figure 7. Some of the lignans of *P. peltatum.*

the onset of senescence. Dormant plants are then shown to have regained the normal lignan pattern.

The advantages afforded by direct production of podophyllotoxin by plant cell culture, together with the possible isolation of new metabolites, led to an interest in the culturing of *Podophyllum* species.

An article by van Uden et al[83] in 1989 described the first isolation of podophyllotoxin from cell suspension cultures. The cell suspension culture was initiated from a callus culture of *Podophyllum hexandrum.* It was reported that the callus was very difficult to initiate. The yield of podophyllotoxin was typically between 0 and 0.1% based on dry weight.

Earlier work in our laboratory led to the development of a cell suspension culture of *Podophyllum peltatum.* Leaf, stem, and root explants of *P. peltatum* were initiated on one of Kadkade's[84] variations of Murashige-Skoog[85] (MS) medium that contained 2,4-D (0.1 mg/L), kinetin (0.2 mg/L), casamino acids (Difco: 500 mg/L) and agar (Difco Bacto: 8 g/L). As in the case of the Dutch workers, it was extremely difficult to propagate the calli due to severe fungal contamination, especially in root material, and to lethal tissue browning. Although many stem and 3 root-derived calli

survived, extreme sensitivity to the effects of tissue browning persisted and required scrupulous removal of any dark tissue at each transfer.

Currently calli are maintained on one-half MS medium supplemented with naphthalene acetic acid (1.0 mg/L), kinetin (0.2 mg/L), and casamino acids (100 mg/L).

Shake flask studies for growth improvement of suspension cultures led to media with low auxin content in which root-derived cell line R3 showed signs of organogenesis. Repeated manual selections of root structures from filtered culture provided inocula that eventually gave healthy, differentiated suspensions.

Subsequent removal of hormones, optimization of sucrose content (15 g/L) and rebalancing of the medium to one half standard salts concentration gave a healthy habituated cell line that has been perpetuated in hormone free one-half MS broth (initial pH = 5.8) since September of 1988. Subculture requires filtration to obtain the cell aggregates free of spent medium and inoculation of fresh broth at a rate of 15 ml aggregates per 300 ml medium. Biomass doubles once (from 4- 8.5 g/L) and stationary phase is reached in 14-16 days.

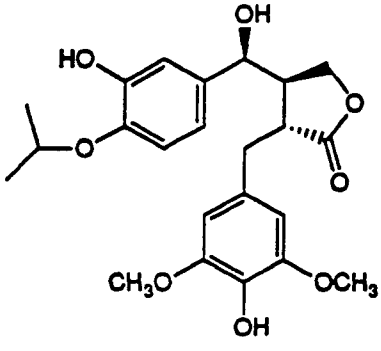
Inoculum from shake flasks transferred into Microferm (New Brunswick Scientific) bioreactors grows successfully (5.5 - 15 L) and provides sufficient material for characterization of the metabolites produced. In a 5.5 L bioreactor culture, the following yields were obtained: podophyllotoxin (49 mg), 4′-demethylpodophyllotoxin (36 mg), and 17 mg of an inseparable mixture of deoxypodophyllotoxin and podophyllotoxone. The dry biomass of mature suspension cultures has been determined to be 7.5-8.5 g/L and on this basis the yield of podophyllotoxin in this bioreactor study was estimated at 0.32-0.36%[80].

In conclusion, it is clear that the developed cell culture of *Podophyllum peltatum* is not only an excellent potential source of the desired podophyllotoxins, but can also serve as a means for the biotransformation of appropriate synthetic substrate to the desired end products.

OBJECTIVES OF THIS INVESTIGATION

Based on the previous information, we set the objectives of this work as follows:

1. Synthesis of substrate dibenzylbutyrolactones **56** and **57** and use of the developed differentiated *Podophyllum peltatum* cell cultures in biotransformation experiments to produce the aryl tetralin products **60** and **61** respectively.
2. Explore the use of a semi-continuous biotransformation process to produce the above mentioned aryl tetralin products, and,
3. Investigate the relationship between cell suspension age and biotransformation abilities to determine the best biotransformation conditions.



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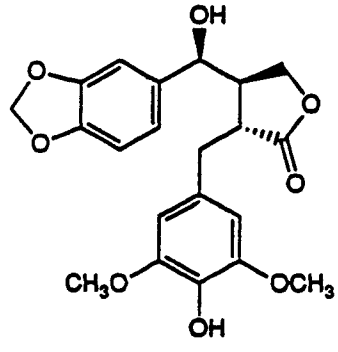

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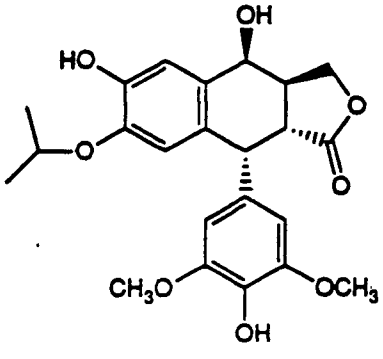

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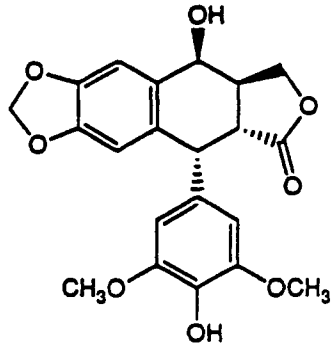


61

RESULTS AND DISCUSSION

Synthesis of Trans 2-(4-hydroxy-3,5-dimethoxybenzyl)-3-(3-hydroxy-4-isopropoxy-α-hydroxybenzyl)butanolide (56).

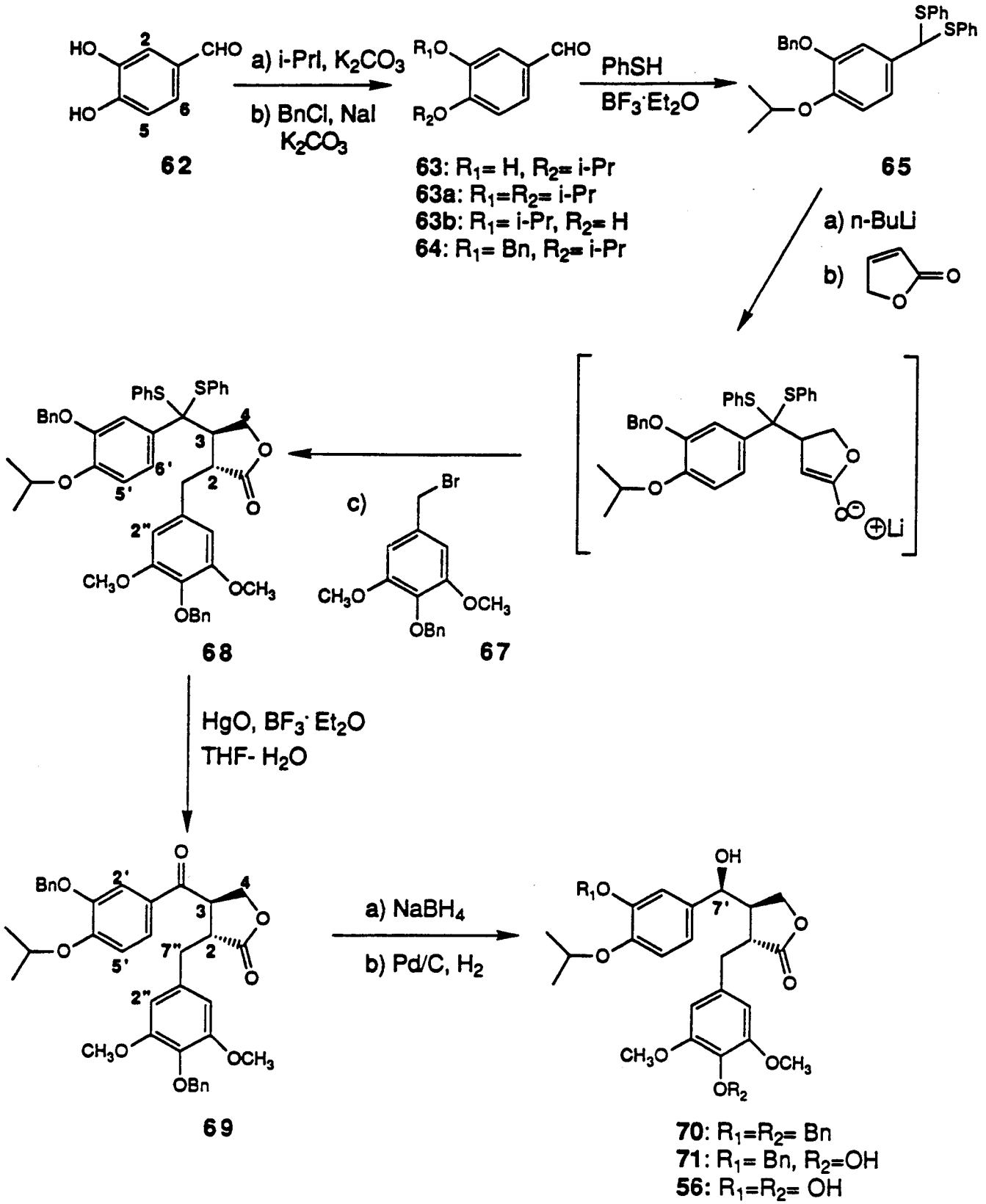
As mentioned, previous work on the synthesis of compounds such as **54** has already been done in our group. The sequences described in this thesis are modified versions of these previous synthetic routes, both in terms of substrate substitution and reaction conditions, particularly in the tandem Michael addition-aldol condensation step and subsequent functional group deprotections. We anticipated no surprises during the synthesis of precursor **56** and, in fact, were able to perform all of the following reactions on gram scale. The synthetic route is shown in Scheme 12.

The sequence starts with the selective protection of 3,4-dihydroxybenzaldehyde (62). Reaction of this cathecol with 1.3 equivalents of isopropyl iodide in DMSO using potassium carbonate as a base produced a mixture of isopropyl ethers with the 3-hydroxy-4-isopropoxy derivative **63** being the predominant isomer (70% isolated yield).

The position of the isopropyl side chain can be confirmed by NMR spectroscopy, since a positive Nuclear Overhauser Effect (NOE) is found for the signal at δ 6.95 (H5) when the signal attributed to the methine heptet (δ 4.75) is irradiated. Benzylation of this phenol with benzyl chloride in ethanol using potassium carbonate as a base and a catalytic amount of sodium iodide proceeded in 70% yield.

Reaction of this protected benzaldehyde with thiophenol catalyzed by boron trifluoride etherate afforded the corresponding thioketal in 56% isolated yield. The reaction has to be carried out under reduced temperature (-40^{o} C) to avoid ether hydrolysis. It was found that if the reaction time exceeded 30 minutes the yield was reduced, presumably due to benzyl ether and/or isopropyl ether cleavage.

The other fragment (67) needed for the construction of dibenzyl butyrolactone **56** was prepared from commercially available syringaldehyde by benzylation (benzyl



62

63: R_1= H, R_2= i-Pr
63a: R_1=R_2= i-Pr
63b: R_1= i-Pr, R_2= H
64: R_1= Bn, R_2= i-Pr

65

68

67

69

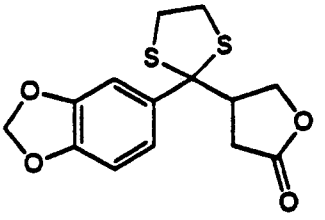
70: R_1=R_2= Bn
71: R_1= Bn, R_2=OH
56: R_1=R_2= OH

Scheme 12. The synthesis of substrate 56 from 3,4-dihydroxybenzaldehyde (62).

chloride, K_2CO_3, catalytic amount of NaI in ethanol, 85% yield), aldehyde reduction ($NaBH_4$ in ethanol, 97% yield) and reaction with phosphorus tribromide in ether (76% yield).

The key step of the synthetic plan, i.e. the Michael addition of the anion of thioketal **65** to γ-crotonolactone and subsequent alkylation of the intermediate anion with aryl bromide **67**, deserves some attention. This reaction is of special importance because it allows one to synthesize a great variety of lignan precursors depending on the aromatic substitution pattern.

Metallated thioketals show, in general, a high preference for non-conjugate 1,2-addition to α,β-unsaturated carbonyl compounds. It was found additionally that anion-stabilizing groups enhance the tendency of metallated dithio-acetals towards conjugate addition. In fact, the first published example of a facile 1,4-addition of a lithio-dithiane to an unsaturated carbonyl compound (γ-crotonolactone) is that leading to **72**[86]. On the other hand, Ishibashi et al[62] reported the addition of a



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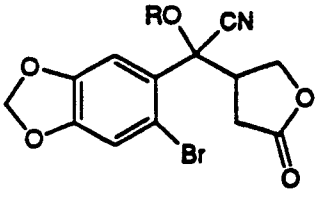


73

protected cyanohydrin to γ-crotonolactone leading to **73** while the dithio-diphenyl thioketal failed to give the desired conjugate addition product. In our hands, the addition of metallated dithio-diphenyl thioketals proved to be a sensitive reaction with dramatic yield variations if the conditions were not carefully controlled. However, we succeeded eventually in achieving yields consistently in the order of 75%.

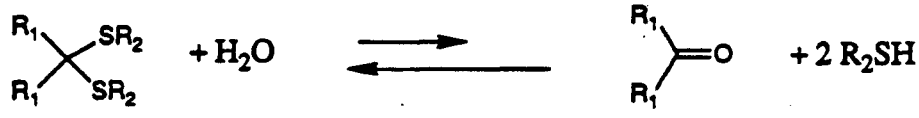
Dithianes are in most cases converted to the lithio derivatives by treatment with *n*-butyl lithium[87] at temperatures between -30° and -10° C. Lower temperatures can be used if additional activation (for instance, aryl groups) is present. In these

cases, other metallating reagents and metals other than lithium are applicable. The origin of the acidity of hydrogen atoms adjacent to divalent sulfur has been the subject of some research[88].

The best results were obtained when thioketal **65** was reacted with 1.1 equivalents of *n*-butyl lithium at -70° C for 25 min. Stringent temperature control was required as well as a very slow rate of γ-crotonolactone addition to the metallated thioketal solution. After stirring this mixture for 40 min, the benzyl bromide was introduced (again at a slow rate) and the temperature allowed to rise slowly to room temperature. Quenching with water and the usual work-up afforded the desired dibenzylbutyrolactone **68**. The IR spectrum of this compound showed the carbonyl absorption at 1770 cm^{-1} corresponding to a γ-lactone, both electron impact and high resolution mass spectroscopy showed the fragment corresponding to M^+-2SPh but elemental analysis agreed favorably for the empirical formula $C_{49}H_{49}O_7S_2$. The ^1H nmr spectrum of the product showed a doublet for the methyl groups of the isopropyl side chain at δ 1.38 ppm and the corresponding methine heptet situated at δ 4.59 ppm. The methylene protons of C7″ appear as an ABX system at δ 2.7 (dd, J = 5, 14 Hz) and δ 3.10 (dd, J = 4, 14 Hz) due to their coupling with the proton located at C2. This proton appears as a multiplet at δ 2.83-2.90, while the corresponding multiplet for the proton at position 3 is located at δ 3.2-3.25 ppm. The ABX system for the methylene protons of C4 shows clearly at δ 3.35 (dd, J = 8, 11 Hz) and δ 4.21 (dd, J = 3, 11 Hz). The fact that the latter component of this ABX system shows a coupling constant of 3 Hz, suggests that this signal belongs to the proton *cis* to the proton at position 3. A singlet integrating for 6 protons located at δ 3.68 was assigned to the methoxy groups present on ring D, while two additional singlets integrating for two protons each at δ 4.97 amd 5.03 ppm were attributed to the methylene protons of the benzyl protecting groups. The aromatic proton signals are visible as a singlet for two protons at δ 6.19 corresponding to the protons on ring D, a doublet at δ 6.80 (J = 8 Hz) for the proton at

position 5′ on ring A and a doublet of doublets at δ 7.03 (J = 2, 8 Hz) for the proton located at C6′ of the same ring. A multiplet at δ 7.15-7.50 integrated for the remaining 21 protons present in the molecule. The *trans* relationship between H2 and H3 was assumed on a thermodynamic basis and could be confirmed at a later stage of the synthesis.

The next step was the hydrolysis of the dithio-ketal derivative to the corresponding ketone. This step was a crucial one since it is known that this class of compounds does not hydrolyze easily. The problem is that the equilibrium shown below lies far to the left. Therefore, only irreversible removal of the thiol or of



the ketone can push it to the right. The irreversible removal of the thiol can be done by one of the following methods: distilling low molecular weight thiols, transacetallization, formation of a transition metal thiolate (Ag, Cd, Hg), oxidation to a higher oxidation state of sulfur and alkylation to a sulfide. Most of these procedures go back to Fischer[89] who used 5% HCl, $HgCl_2$, $AgNO_3$, Br_2, and HNO_2 on his sugar ethyl dithio-acetals. Jan Palaty[75] in our group found all too well these difficulties, but fortunately a relatively recent procedure[90] which uses mercury (II) oxide and boron trifluoride etherate proved to be suitable for our purposes.

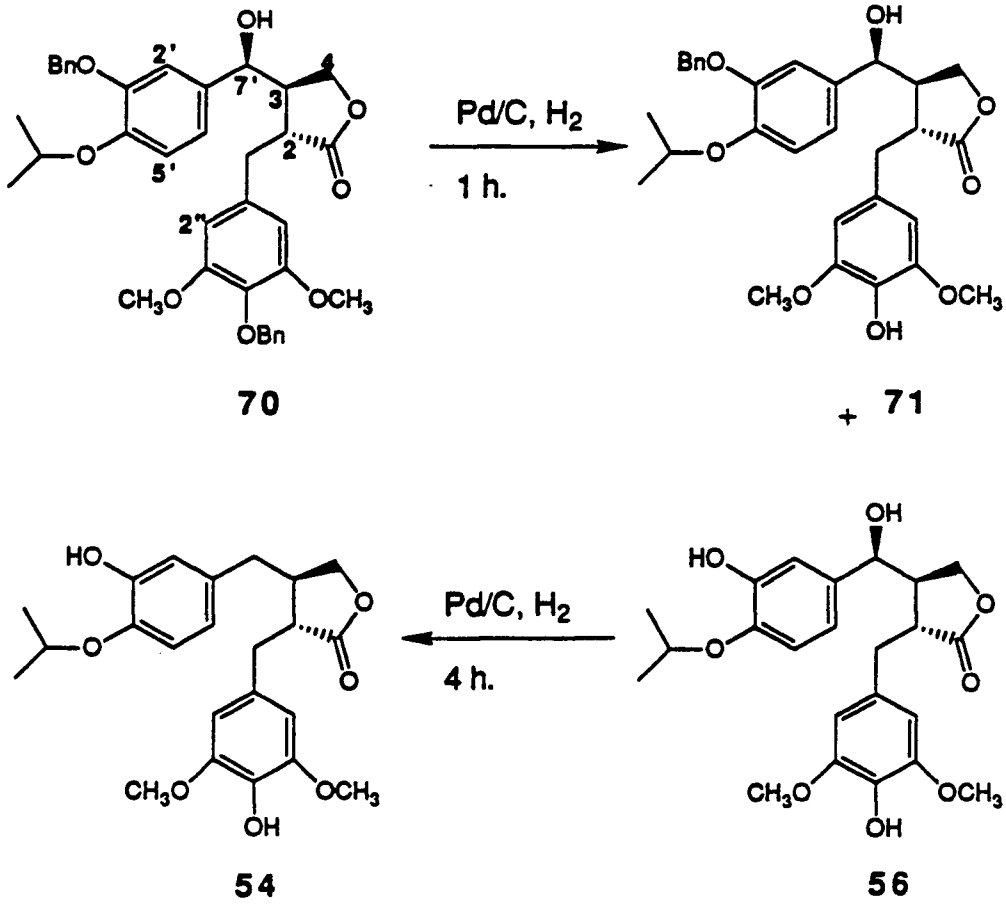
Care had to be taken in controlling the temperature at 0^0 C to avoid benzyl ether hydrolysis by the boron trifluoride during the initial stage of the reaction. A rapid filtration through silica gel removed easily the biphenyl disulphide byproduct of the reaction affording the pure ketone **69** in excellent yield. This compound could be crystallized from ethanol and showed in the IR spectrum absorptions at 1775 (lactone) and 1663 (ketone) cm^{-1} respectively. The ^1H nmr spectrum of **69** when compared to that of dithioketal **68**, showed a reduction in the number of phenyl protons to 10, and

a considerable downfield shift of the H3 multiplet to δ 3.57 ppm. Mass spectroscopy showed the molecular ion at m/z 610, and the only significant fragments at m/z 519 (loss of benzyl) and m/z 91 (tropilium ion), the latter being the base peak.

Reduction of the ketone 69 with sodium borohydride gave the corresponding alcohol as a mixture of epimers in 83% yield. The IR spectrum showed a band at 3492 cm^{-1} characteristic of alcohols, the band of the lactone carbonyl at 1767 cm^{-1}, and the disappearance of the ketone band at 1663 cm^{-1}. In the ^1H nmr spectrum, the signals for the proton at C7′ of both epimers can be seen at δ 4.2 and 4.5 ppm. The presence of the mixture of epimers at C7′ complicates the assignment of the signals for the other protons that now present closer chemical shifts. The doublet for the methyl groups of the isopropyl side chain is located at δ 1.45 ppm and the protons at positions 2, 3 and 7″ give rise to a multiplet at δ 2.50-3.2 ppm. The multiplet corresponding to the methylene protons of C4, is centered at δ 3.70 ppm, and the singlet for the methoxy groups on ring D is at δ 3.78 ppm. The isopropyl methine heptet is located at δ 4.50 and the signals corresponding to the methylene protons from the benzyl protecting groups appear at δ 4.98 and 5.10 ppm. Two singlets attributed to the aromatic protons of ring D, are located at δ 6.26 and 6.40 ppm, and their integration reflects the same epimeric ratio than the signals for the C7′ proton. The aromatic protons of ring A (positions 2′, 5′ and 6′) appear as a multiplet at δ 6.7-6.9 and the phenyl protons from the benzyl protecting group produce a multiplet at δ 7.2-7.5 ppm. The major isomer was assigned as the β-alcohol based on the relative accessibility of the carbonyl faces in the most favorable configuration of the starting material as shown by Dreiding models. Other closely related dibenzylbutanolides also have been shown to produce mainly the β-hydroxy epimer upon reduction of the ketone[91]. This assignment was not entirely crucial since both isomers can be used to produce the glycoside without the need of isolation. In the reaction conditions of glycosidation, only the C-4 β-glycoside is obtained[47]. Since sodium borohydride is an

inexpensive reagent, and the reactions proceeded in good yield, we did not investigate the improvement of the epimer ratio by, for example, using sterically hindered hydride donors.

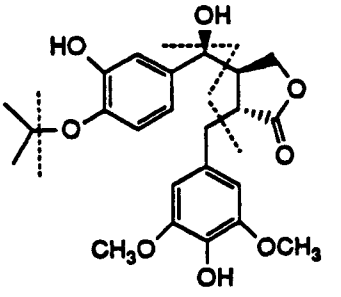
The alcohol (70) was then subjected to hydrogenolysis using Pd/C (10%) as catalyst. The reaction proceeded at a fast rate using 1 atm. of hydrogen and producing 56 in 74% isolated yield (Scheme 13). If the reaction was left for a longer period, however, over-hydrogenolysis occurred to give product 54 (84%) in which the C-7 alcohol has been lost. The spectroscopic data of this compound proved to be



Scheme 13. The hydrogenolysis of compound 70.

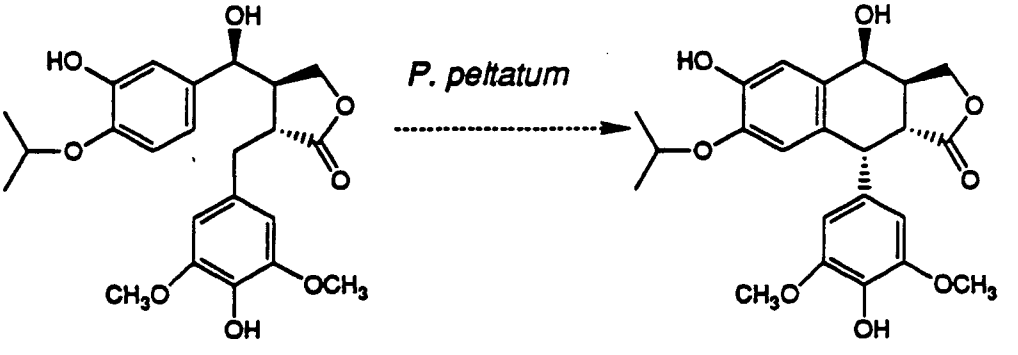
identical with a sample prepared by Terry Jarvis[74] via the desulfurization-hydrogenolysis of dithioketal **68** with Raney-Nickel. On the other hand, too short reaction times afforded mixtures of mono- and didebenzylated products **71** and **56**. The ^1H nmr spectrum of the monobenzylated product **71** showed a reduction in the number of phenyl protons to 5, and the disappearance of one of the benzylic methylene signals due to a benzyl group and present in the starting material (δ 4.98 ppm), while the other (δ 5.10 ppm) remained. In general, the position and multiplets of the rest of the signals were consistent with the remaining benzyl ether being at position 3′ . The mass spectrum of compound **71** showed the molecular ion at m/z = 522, being the fragment at m/z = 91, the base peak.

We found that upon running the hydrogenolysis reaction for 50 min, yields in the order of 75% for compound **56** could be achieved. The product presents in the IR spectrum absorptions at 3459 (alcohol) and 1757 (lactone) cm^{-1} respectively. In the ^1H nmr spectrum, the doublet for the isopropyl methyl is located at δ 1.4 ppm. The protons at positions 2 and 3 produce multiplets centered at δ 2.98 and 2.64 ppm respectively. The ABX system characteristic of the C7″ methylene protons is situated at δ 2.85 (dd,d J = 5.4, 13.6 Hz) and 3.10 (dd, J = 4.9, 13.6 Hz) while the signal due to the protons at C4 appears together with the ring D methoxy signals as a multiplet at δ 3.8-3.95 ppm. The isopropyl methine, together with the proton at C7′ produce a multiplet at δ 4.52 - 4.62. The aromatic protons are evident as a singlet at δ 6.4 for ring D and a multiplet at δ 6.65-6.86 ppm for ring A. Three one-proton signals located at δ 1.7, 5.4 and 5.75 ppm disappeared upon addition of deuterated water. Mass spectrometry showed the molecular ion at m/z 432 and a fragmention pattern that is characteristic of dibenzyl butyrolactones of this kind, giving rise to a base peak with m/z 167 corresponding to the fragmentation of the C7″-C2 bond.



56

Having thus developed a short and efficient synthesis of precursor **56**, we turned our attention to the biotransformation experiments, with the intention of performing the oxidative coupling to the aryl tetralin **60** (Scheme 14).

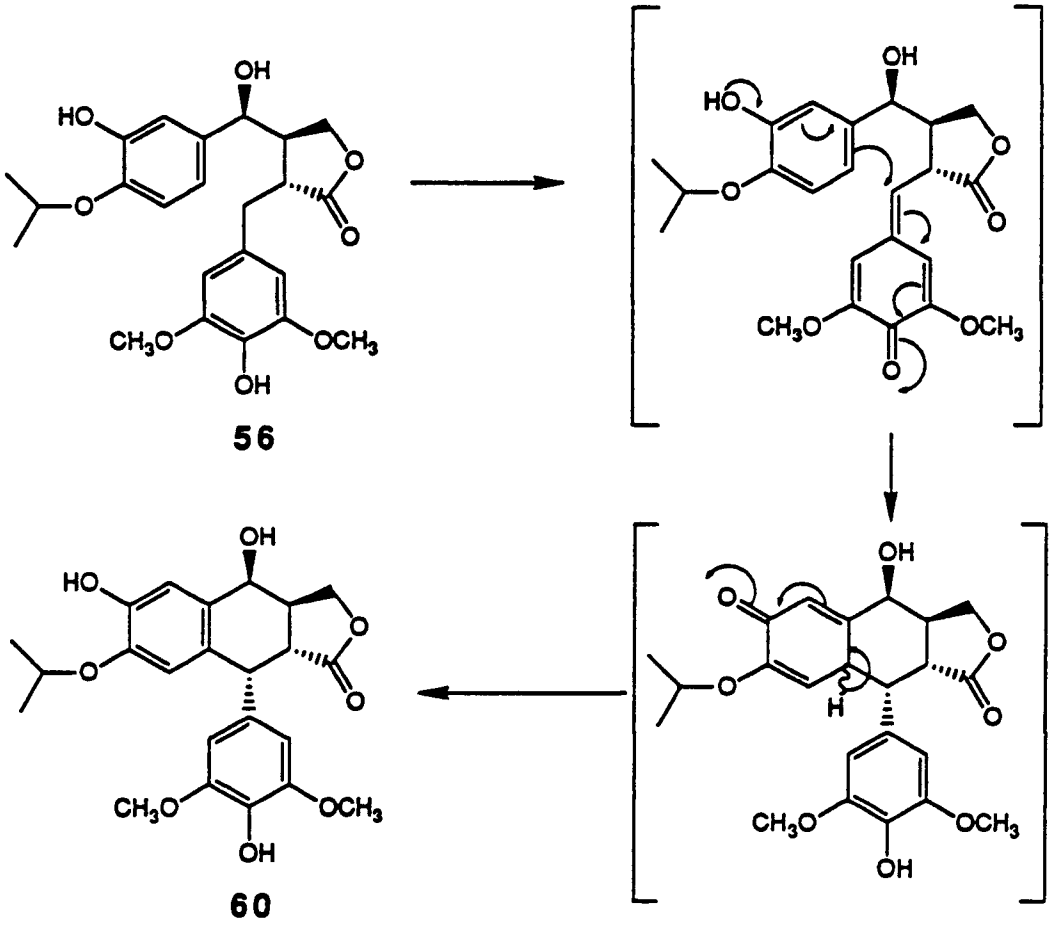


56 **60**

Scheme 14. The proposed biotransformation of substrate 56.

The Biotransformation of Trans-2-(4-hydroxy-3,5-dimethoxybenzyl)-3-(3-hydroxy-4-isopropoxy-α-hydroxybenzyl)butanolide (56) with *Podophyllum peltatum* cell suspension cultures.

We felt that precursor **56** incorporated many of the characteristics previously shown to be important for successful biotransformation. It has a hydroxy group at position 4″ and a sufficiently nucleophilic ring A to make the cyclization of the hypothetical quinone methide intermediate possible (Scheme 15).



Scheme 15. Hypothetical mechanism for the ring closure of substrate **56** to aryl tetralin 60.

It also has a protective group (isopropyl) which can be cleaved[92] in the presence of the methoxy groups of ring D to give an entry to the methylenedioxy system[93] and the hydroxyl group already in position 7'. If the enzyme's active site is reached, this would clearly provide an efficient entry to the podophyllotoxins.

Isolation and Structure Elucidation of the Major Biotransformation Products of 56 with *Podophyllum peltatum* Cell Suspension Culture.

In order to isolate sufficient quantities of extracts (and later, metabolites) from the biotransformation of substrate **56** with *P. peltatum* cells, a large scale biotransformation was set up, using cells grown in a Microferm reactor for 2 days (450 mL drained cells, 18 days old, in 3 L 1/2 MS - 1.5% sucrose medium) and 3.0 g of substrate dissolved in ethanol (70 mL). Biotransformation was allowed to proceed while the consumption of the precursor was monitored by chromatographic analysis of broth samples. We were pleased to find that both starting material and products were present in the culture medium. The analysis of the broth samples also indicated a fairly rapid biotransformation; substrate consumption was >50% after 48 h, and only a trace remained after 4 days of incubation. At this point, the Microferm was harvested, and cells and broth were extracted separately.

Extraction of the cells was carried out using ethyl acetate and methanol (Figure 8). The cells were homogenized in ethyl acetate, followed by filtration through Celite 545, and the cell residue was washed with ethyl acetate. The aqueous filtrate was separated, extracted with ethyl acetate, and the organic layers were combined. Solvent removal yielded the ethyl acetate extract. Sonication of the cell residue in methanol, followed by filtration through Celite 545, washing with methanol and solvent removal constituted the methanol extract.

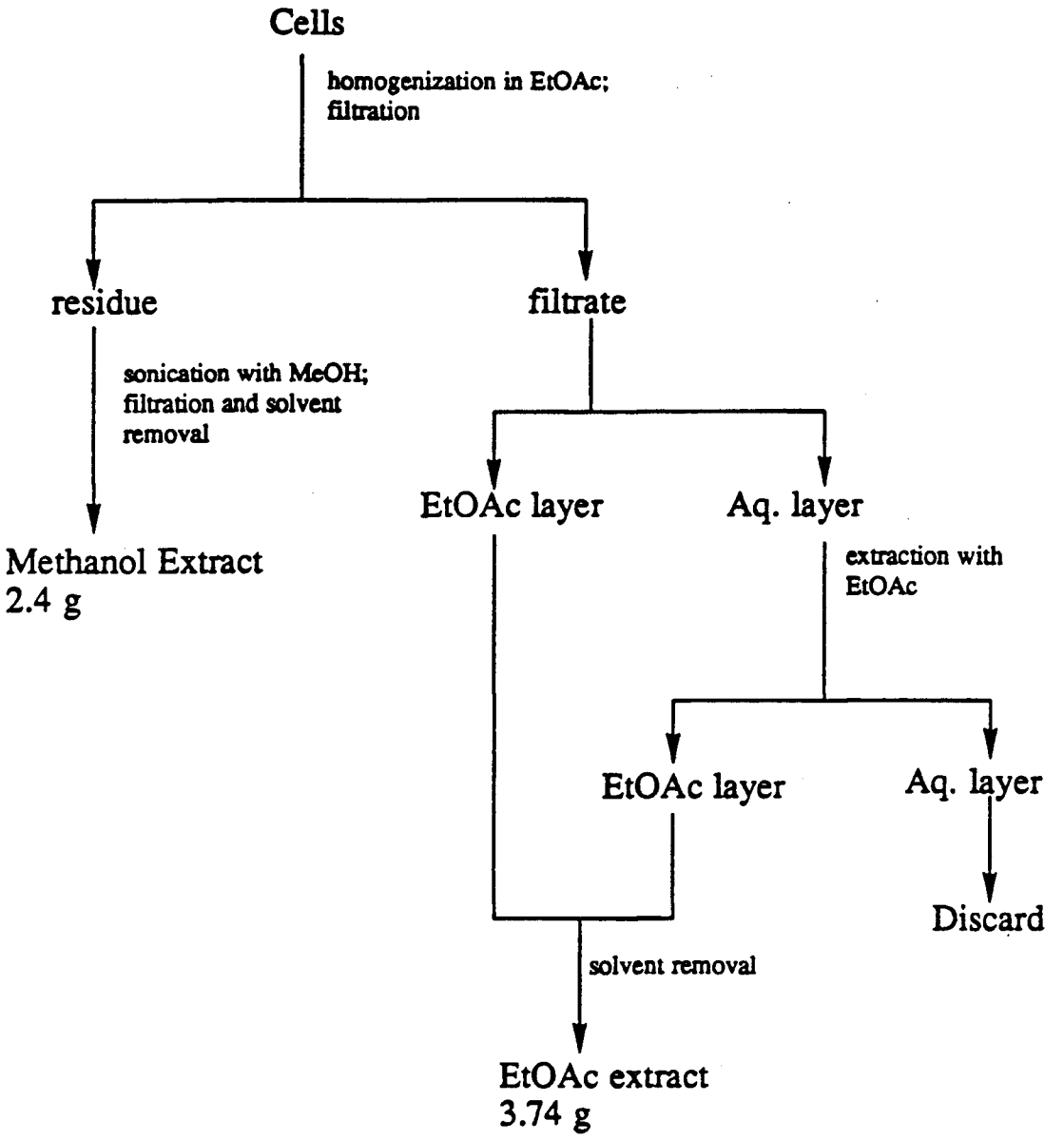


Figure 8. Extraction procedure for the *P. peltatum* cells.

The broth was saturated with sodium chloride and extracted with ethyl acetate (Figure 9). A methylene chloride extract was also obtained. Chromatographic analysis of these extracts showed lignan-type compounds only in the ethyl acetate extracts of broth and cells. The methanol and methylene chloride extracts were not investigated further. The ethyl acetate extracts were subjected to chromatographic separation (Figure 10).

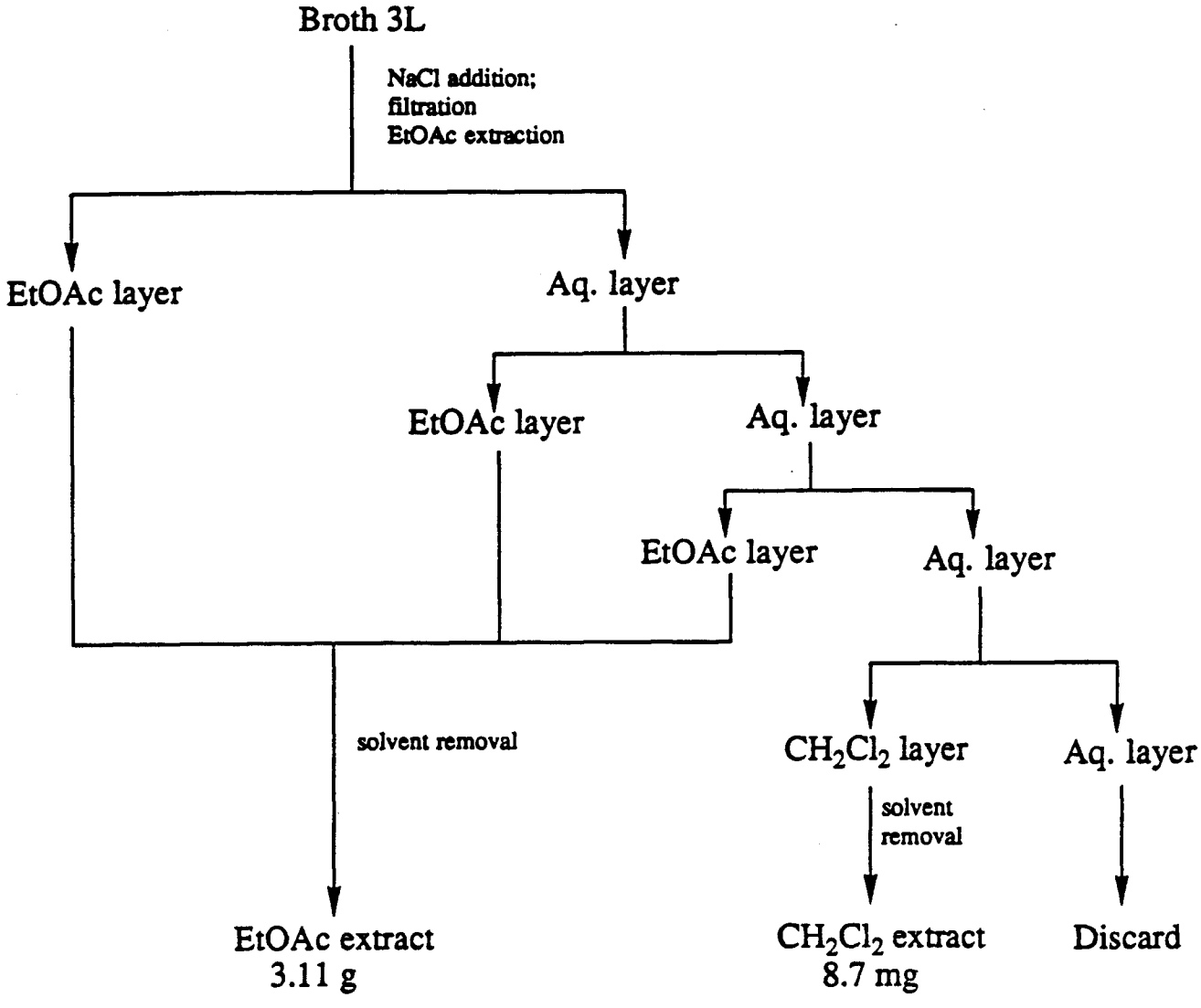


Figure 9. Extraction procedure for *P. peltatum* spent medium.

Partial separation of the broth extract was carried out by a quick filtration through silica gel, eluting with chloroform:methanol 25:1. The column was also

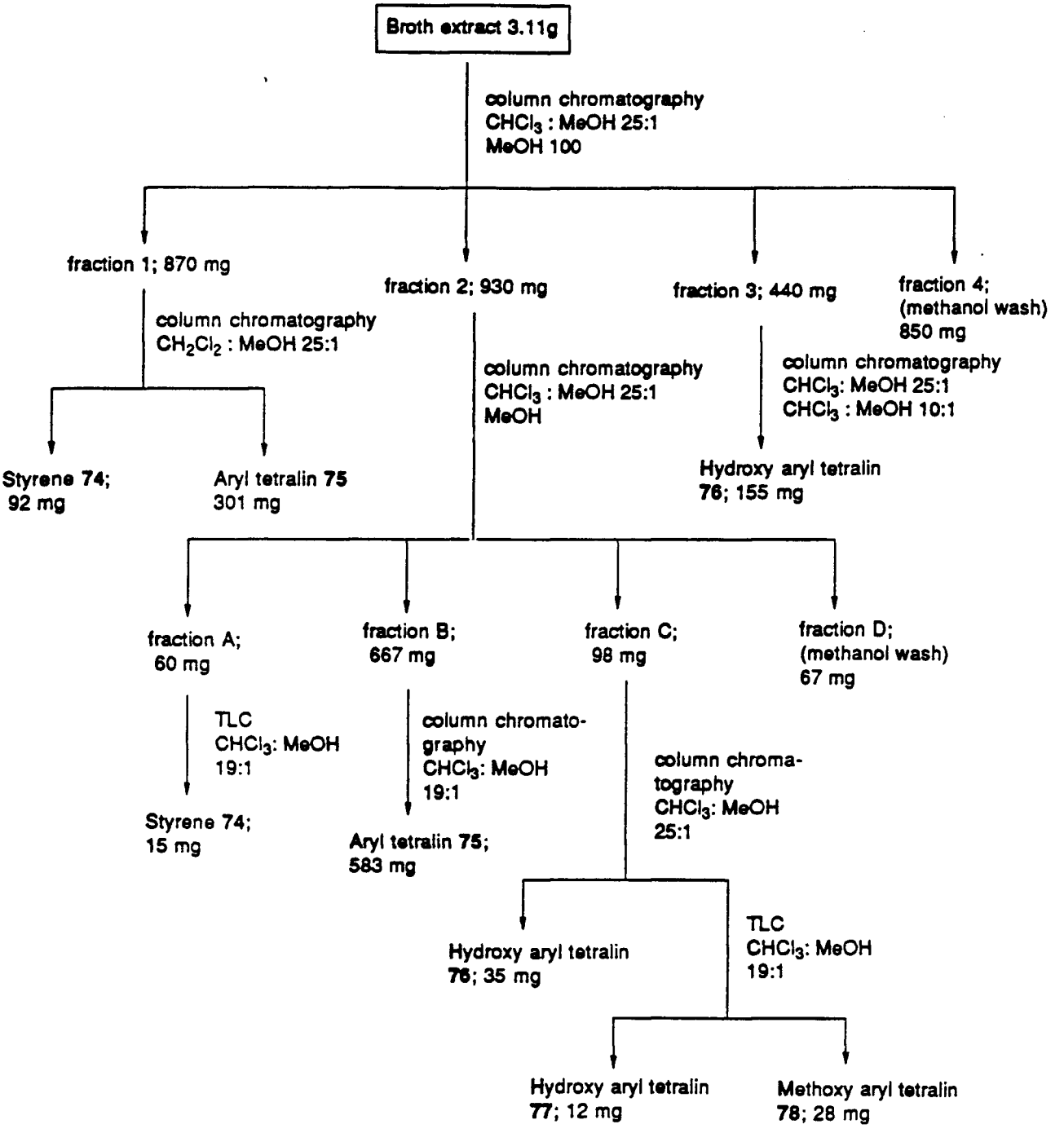


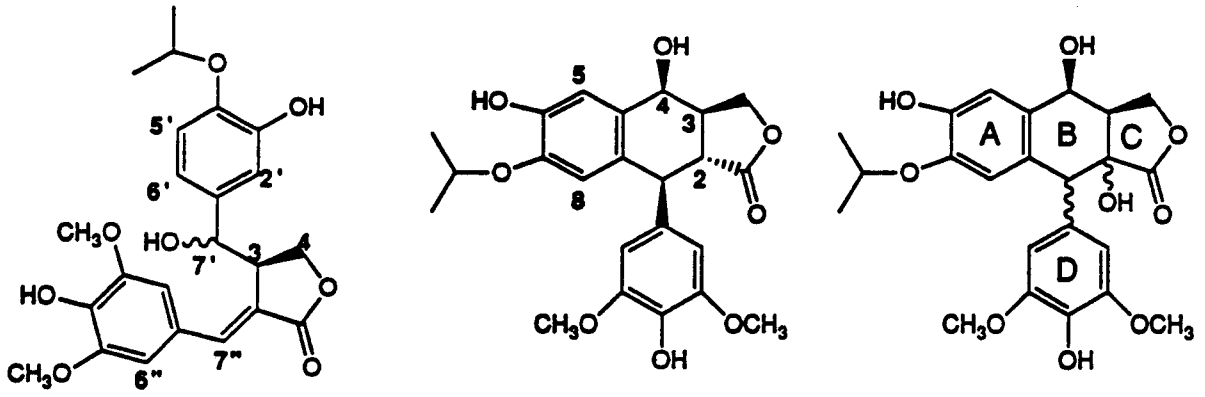
Figure 10. Chromatographic separation of the broth extract.

flushed with methanol to remove the polar material. Four crude fractions containing complex mixtures of metabolites were collected. Fraction 1 contained mostly non-polar cell produced material (870 mg). Fractions 2 (930 mg) and 3 (440 mg) contained lignans and were orange-brown solids. Fraction 4 (850 mg) was a dark brown solid containing the polar material.

Fraction 1 yielded upon chromatographic separation (chloroform:methanol 25:1) 92.0 mg of **74** and 301 mg of **75**.

Fraction 2 was still very complex and required a partial purification by chromatography eluting with chloroform:methanol 25:1, and a final wash with methanol to remove the polar material. Again 4 fractions were obtained. Fraction A (60.2 mg) contained mostly cell material but when applied to preparative thin-layer chromatography afforded 15.0 mg of compound **74**. Fraction B (667.8 mg) contained the main metabolic product, and was further purified by flash column chromatography (chloroform:methanol 19:1) to yield 583.8 mg of compound **75**. Fraction C (98.2 mg) was a mixture of three compounds possessing very similar retention times on TLC, and after a column chromatography (chloroform:methanol 25:1) and preparative TLC (chloroform:methanol 19:1), afforded 35.5 mg of **76**, 12.7 mg of **77**, and 28.0 mg of **78**. Fraction D (67.6 mg) was a dark solid containing polar material. Fraction 3 consisted mostly of compound **76** and indigenous metabolites, and was purified by flash column chromatography (chloroform:methanol 25:1) to obtain an additional 155.5 mg of compound **76** as a white solid.

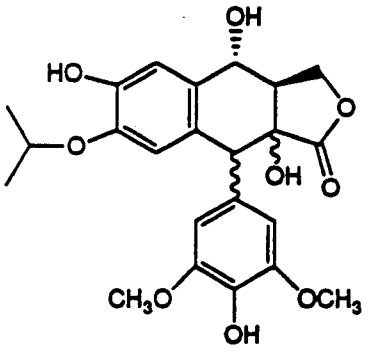
Evidence that led to the structure elucidation of these compounds follows (Figure 11).



74

75

76



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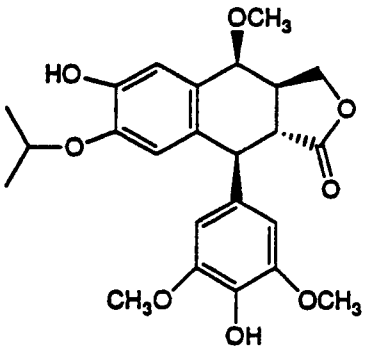


78

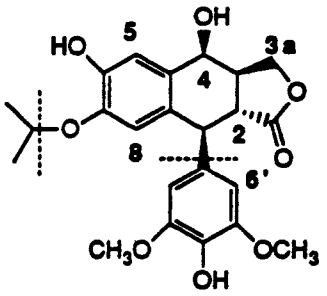
Figure 11. Biotransformation products from substrate 56 with *P. peltatum* cells.

Compound **74** was isolated as a light yellow solid with a molecular formula of $C_{23}H_{26}O_8$ (high resolution mass spectrometry). Its IR spectrum shows the carbonyl band at 1720 cm^{-1}, well below the value of 1770 cm^{-1} observed for substrate **56**. The UV spectrum showed a bathochromic shift with a maximum at 324 nm relative to 281 nm for compound **56**, consistent with a cinnamic ester. The product presented in the ^1H nmr spectrum the signals characteristic for a ring opened compound, particularly the aromatic region in which *ortho* and *meta* coupling gives rise to multiplicity. The absence for the ABX signal for the protons of C7″ and of the multiplet for the proton at C2 indicated the presence of unsaturation at these positions. Accordingly, a new singlet was located at δ 7.6 ppm and assigned to the vinylic proton at C7″. The rest of the signals are down-field with respect to those of compound **56**. The proton at C3 presents a multiplet centered at δ 4.08 ppm and the signals for the C4 methylene protons are located at δ 4.28 and 4.57 ppm. The methoxyl signal is at δ 3.93 and the proton at C7′ presents a doublet centered at δ 5.02 ppm. The aromatic protons of ring D produce a singlet at δ 7.10 while those of ring A give rise to a multiplet at δ 6.64-6.82 ppm. The stereochemistry of the double bond is believed to be E based on the chemical shift of the proton at position 7″. It has been shown in similar lignans[94] that when the geometry of the olefin is Z, the vinylic proton signal appears at chemical shifts around δ 6.6 ppm, while in the E configuration, the deshielding effect of the neighboring carbonyl group shifts this signal down-field to about δ 7.5 ppm. The chemical shift of δ 7.6 ppm for the proton at C7″ in our case suggests an E configuration for the double bond.

Compounds **75** to **78** showed in the ^1H nmr spectrum an aromatic proton signal pattern consisting exclusively of singlets, implying substitution at C6′ of compound **56**, and therefore, that ring closure was achieved. The appearance of only one signal for both H2′ and H6′ indicated that ring D had essentially complete freedom of rotation.

The main metabolite was identified as aryl tetralin 75.

This product showed an IR absorption at 1774 cm^{-1} which is consistent with the presence of the lactone carbonyl group. The mass spectrum gave a molecular ion at m/z 430 which is 2 mass units less than the molecular ion peak of the substrate **56** and, in contrast to the fragmentation pattern presented by **56**, the product on hand showed at base peak at m/z 154, i.e. loss of ring D, and a fragment with m/z 388 (loss of iso-propyl). These fragments are characteristic of ring closed compounds.



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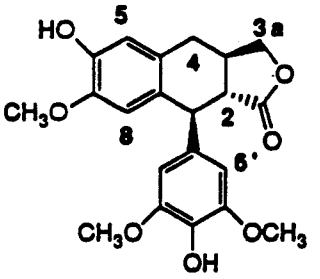


75a

As mentioned earlier, the ^{1}H nmr spectrum of compound 75 shows no *ortho* or *meta* coupling between the aromatic protons. The protons at position 2′ and 6′ gave a singlet at δ 6.46, the protons at positions 5 and 8 occurred as one proton singlets at δ 6.38 and 6.88 ppm respectively. The *trans* stereochemistry of the lactone was confirmed by analysis of the signals produced by the aliphatic protons. H2 appears as a doublet of doublets at δ 3.19 ppm with coupling constants of 11.4 and 14 Hz, suggesting also a *trans* relationship with H1 whose signal shows as a doublet at δ 3.96 ppm with coupling constant of 11.4 Hz. These values are in good agreement with the reported coupling constants of H2 in compound **75a** of 11 and 14 Hz. The product **75a** was prepared by Jan Palaty[75] via the peroxidase catalyzed cyclization of the corresponding dibenzylbutyrolactone using *Catharanthus roseus* cell free extracts, and the stereochemistry was unambiguously determined by X-ray analysis.

Also in the ^1H nmr spectrum of 75, the isopropyl methyl signals appear now as two doublets at δ 1.14 and 1.28 ppm. The signal corresponding to the isopropyl methine, appears together with the multiplet for the C3a methylene protons at δ 4.25-4.47 ppm. The multiplet for H3 is located at δ 2.68, while the doublet for H4 is at δ 4.85 ppm (J = 2.7 Hz). The methoxy groups singlet is located at δ 3.86 and three signals integrating for one proton each at δ 1.55, 5.5 and 5.7 ppm exchanged with heavy water. The UV spectrum showed maxima at 283 and 215 nm.

Compound 76 was isolated as a white powder with molecular formula $C_{23}H_{26}O_9$ (high resolution mass spectrometry). The presence of a strong M^+-18 peak in the electron impact mass spectrum suggested that the extra oxygen formed part of a hydroxyl group. The product showed an IR band at 3484 cm^{-1} characteristic of hydroxy groups and a band at 1746 cm^{-1} consistent with the lactone carbonyl. The UV spectrum showed maxima at 290 and 206 nm consistent with the aromatic rings of the aryl tetralin. In the ^1H nmr spectrum, the aromatic singlets can be seen at δ 7.08, 6.61 and 6.21 ppm for H8, H5 and H2′, H6′ respectively. An additional one proton singlet is found at δ 5.22 ppm assigned to H1, while the signal for H2 is missing. The system for the aliphatic protons is clearly visible. H4 shows as a doublet with J = 7.8 Hz at δ 4.03 ppm, H3 occurs together with the methoxy signal at δ 3.62 ppm, and the ABX system for the methylene protons is located at δ 4.21 (dd, J = 8.3, 9.6 Hz) and 4.37 (dd, J = 9.6, 9.6 Hz) ppm. A positive NOE between H3 and H4 indicates that they have a *cis* relationship and, therefore, the hydroxyl group at C4 must be beta-oriented. The stereochemistry of the lactone ring appears to be *cis* according to the similarity of the coupling constants of H3 with both protons at C3a, since a rigid *trans* fusion would be likely to produce very different dihedral angles between these two positions (3 and 3a). Such a situation would translate into much different J values for this system. NOE irradiation experiments failed to provide conclusive evidence to assign

the orientation of the aromatic ring attached to C1, and attempts to crystallize the material for X-ray analysis were unsuccessful.

Compound 77 shared many of the characteristics of 76. The high resolution mass spectrum indicated the same molecular formula and similar fragmentation pattern; it had IR absorptions at 3424 (OH) and 1709 (lactone) cm^{-1}, and UV maxima at 289 and 210 nm. The ^1H nmr spectrum showed again only singlets for the aromatic protons and an additional one proton singlet at δ 5.10 ppm assigned to H1. The system for the aliphatic protons is again clearly visible. The proton at H3 is seen as a multiplet at δ 3.40 ppm, the ABX system for the C3a methylene protons is located at δ 4.04 (dd, J = 8, 8 Hz) and 4.22 (dd, J = 8, 9.6 Hz) ppm, and the doublet for H4 at δ 4.62 ppm with J = 6.0 Hz. These assignments were confirmed by multiple irradiation experiments. For example, upon irradiation at δ 3.4 ppm, the signals due to the protons at C3a (δ 4.04, 4.22 ppm) collapse to doublets with J = 8 Hz, while the doublet for the proton at C4 (δ 4.62 ppm) turns into a singlet. Again the stereochemistry of the lactone ring fusion is most probably *cis* as suggested by the similarity of the coupling constants of H3 with both protons at C3a, but this evidence is not conclusive.

Finally, compound 78 presented a very similar ^1H nmr spectrum when compared to aryl tetralin 75, except that an additional methyl group singlet resonance at δ 3.84 ppm was noted, and now only two protons could be exchanged with heavy water (δ 5.47, 5.68 ppm). High resolution mass spectrometry indicated the molecular formula $C_{24}H_{28}O_8$ (14 mass units more than compound 75), and the electron impact spectrum showed the characteristic fragmentation pattern with a strong molecular ion peak and the fragment at m/z = 154 (ring E) as in the case of compound 75 confirming a ring-closed structure.

Chromatographic purification of the ethyl acetate extract obtained from the cells was done in a similar way. The majority of the material, by weight, was cell produced material, but an additional 124.4 mg of ring closed product **75** were obtained.

In summary, this experiment afforded 5 biotransformation products in the following yields: styrene **74**: 107 mg (3.5%) ; aryl tetralin **75**: 1.00 g (33.5%); hydroxy aryl tetralin **76**: 191 mg (6.18%); hydroxy aryl tetralin **77**: 12.7 mg (0.4%) and O-methyl-aryl tetralin **78**: 28.0 mg (0.9%). The total recovery was 1.34 g (44.6%). We concluded that the long biotransformation time of this experiment had been detrimental in the final recovery, the biotransformation proceeded possibly to give water soluble compounds (e.g. glycosides), or the starting precursor was catabolized or otherwise irreversibly bound to cellular components. However, we felt encouraged to investigate the possibility of a semi-continuous process to exploit the same biomass to effect several biotransformation experiments. Since only a small fraction of product was obtained from the cell extract, harvesting of the spent medium under aseptic conditions should be a practical way of adding and removing substrate and biotransformation products.

The Semi-continuous Biotransformation of Substrate 56 with *Podophyllum peltatum* Cell Suspension Culture.

The ideal state for a cell suspension culture is that of morphological, biochemical and genetic homogeneity, grown in a fully controllable environment.

The majority of investigations involving plant cell suspension cultures have been performed under batch culture conditions. Under these conditions, the cells grow in a limited amount of medium and multiply to form a population in which succeeding generations progressively modify their environment. This situation produces a sequence of changes in the culture that is referred to as the culture cycle.

Under these conditions, the metabolism of the cells is also altered and it is extremely difficult, if not impossible, to determine any factor responsible for these fluctuations.

In continuous culture, medium is added into a constant volume of growing culture producing a steady-state condition for growth[95]. The environment imposes a constant growth rate on the cells so that the doubling time and overall metabolism of the cells remain constant and thus characteristic of the steady-state.

It must be noted, however, that the cells divide randomly. The ideal conditions would include synchronicity of the cells so that instead of an average condition as is obtained in steady-state conditions from the randomly dividing population of cells, a synchronous pattern of change, which coincides with the cell cycle and repeats itself with each successive doubling of the cell population, would be produced. This would have the advantage that enzymes or metabolites occurring only at certain stages of the cell cycle, could be obtained at maximal yields.

In our experiments, we envisaged a semi-continuous process in which the cells would be grown for a fixed period of time corresponding to the lag, growth or stationary phase of the culture cycle, and resuspend them in diluted medium (with just enough nutrients to maintain the live cells) containing the substrate (Figure 12). In this way, we would minimize the time required to prepare biomass. This "draw and fill" process would result in a series of partial batch cultures that would have abbreviated lag phases and would probably not reach stationary phase provided resuspension is done quickly. Once this draw and fill process is initiated, the physiology of the culture would be that which is created by this process, somewhat between batch and continuous culture. The age of the initiating culture may still play a role in the biotransformation outcome, in that it may be related to the production of the relevant enzymes.

We first attempted this type of study with 7-day old cells (growth phase). The Microferm reactor was fitted with a peristaltic pump and a filter that would enable

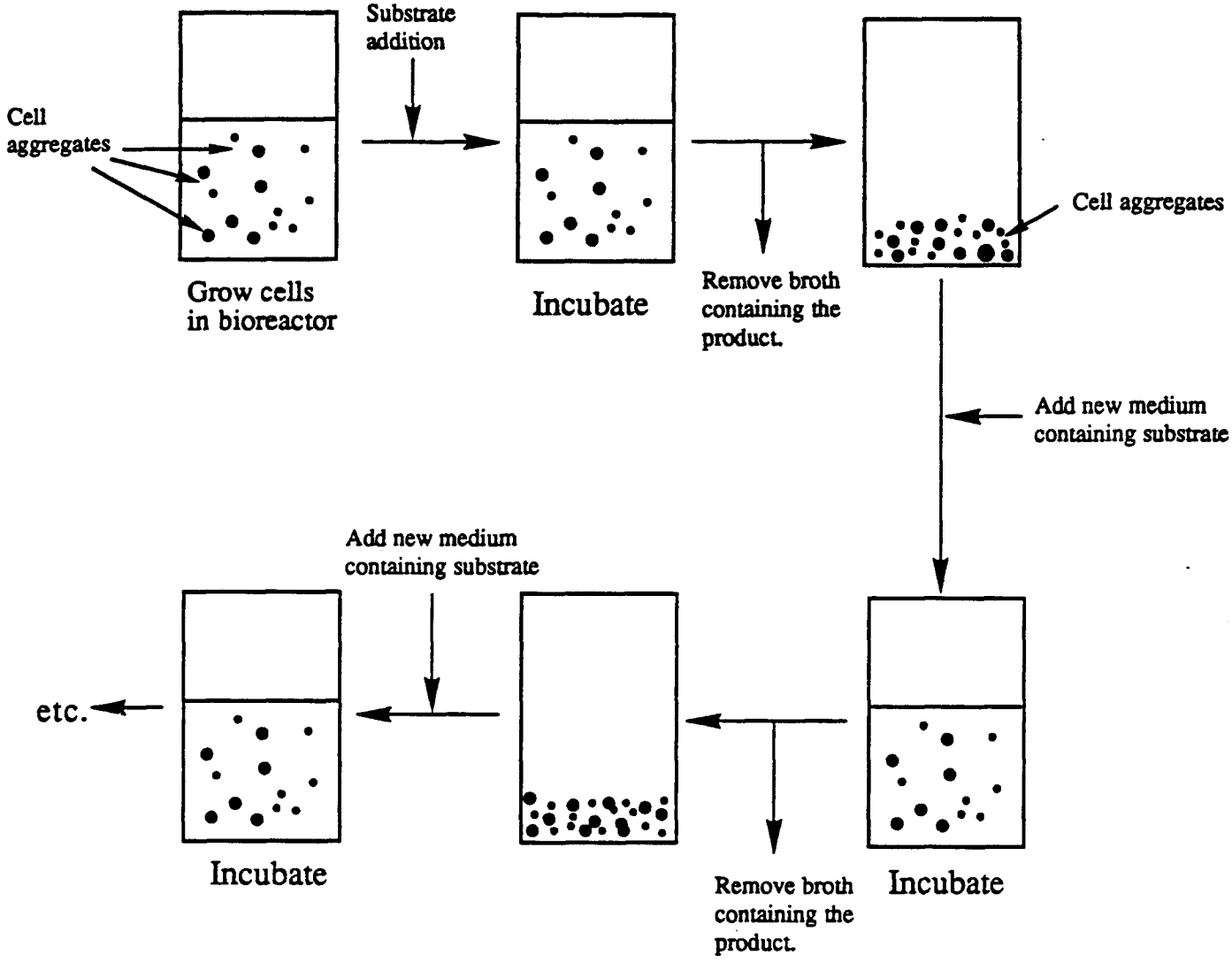


Figure 12. Schematic representation of the semi-continuous biotransformation process.

the removal of the broth and resuspension of the cells *in situ*. The reactor was inoculated with 180 mL drained cells (RI = 1.3330, pH = 4.5, inoculum, 15 days old) and grown for a full cycle (18 days), after which the spent medium was removed and the cells were resuspended in fresh medium. Seven days after resuspension, the cells were healthy and uncontaminated (pH = 4.43, RI = 1.3342) indicating that the peristaltic pump worked satisfactorily. The cells, now in growth phase, were resuspended in 1/10 MS - 0.3% sucrose medium supplemented with 2.5 mM MES as buffer and the pH was adjusted to 6.5 which was considered the optimum for the ring closure to occur according to data available from Jan Palaty[75]. Substrate **56** (168.3 mg) was added, dissolved in ethanol (5 mL), and biotransformation was allowed to proceed for 22 h. After this time, the broth was removed and replaced with 1/10 MS - 0.3% sucrose medium containing 200 mg of precursor **56** in 7.5 mL ethanol and 2.5 mM MES as buffer. Again pH was automatically controlled to 6.5 and the Microferm was harvested (cells and broth) after 22 h of incubation. The results are shown in Table 1.

Substrate added (mg)	Extract obtained (mg)	Recovered substrate %	Yield of 74 %	Yield of 75 %	Yield of 76 %	Recovery %
168.3	281.7	0.3	1.1	29.9	11.0	42.2
200.0	513.0	11.5	1.8	51.3	12.8	69.9
cells	2190.0	12.5	-	-	-	12.5

Table 1. Semi-continuous biotransformation of substrate **56** with 7-day old *P. peltatum* cells. Incubation time was 22 h, pH = 6.5.

As can be seen, similar yields to the ones obtained in the previous biotransformation were obtained in the first experiment. However, in entry #2, the overall recovery and ring closure yields are higher. This could be explained in several ways. Firstly, the cells may be accumulating products or starting material from the

first biotransformation, and are being released in the second experiment; but the starting material recovered from the cell extract (24.9 mg) indicates the contrary. Cells do not retain either products or starting material. Another explanation would be that the cells are able to respond better to biotransformation after they have been exposed to the substrate. We wanted to know if this trend of increasing biotransformation ability would be maintained throughout a larger number of biotransformations. We also wanted to check if the cells retained the biotransformation ability after repeated resuspension, and to what extent the stress to which they were subjected affected the biotransformation outcome.

To answer these questions, another set of experiments was done. Stationary phase cells would be assessed under the same experimental conditions, but a large number of biotransformations was planned. To this effect, a Microferm was started (180 mL drained cells, RI = 1.3335, pH = 4.63, inoculum 19 days old) and the cells were grown for 17 days (RI = 1.3334, pH = 4.8). The spent medium was removed and the cells were resuspended in 1/10 MS - 0.3% sucrose medium containing substrate **56** (200 mg in 5 mL ethanol) and 2.5 mM MES buffer. The pH was automatically controlled to 6.5, and biotransformation was allowed to proceed for 24 h. After this time the broth was removed and extracted, and the cells were resuspended in broth containing substrate **56** under the same conditions. The operation was repeated three times (entries 1-4, Table 2) after which time it was found that some cells were dying, presumably due to lack of nutrients. It was decided to do the following biotransformations at a higher sucrose concentration, and 1/5 MS - 0.6% sucrose medium was arbitrarily chosen (entries 5 and on). Also during these biotransformations (entries 5 and 6), the pH control system failed leading us to believe that this was not a relevant parameter for our biotransformations since conversion occurred apparently at the same rate when the pH was left to drift. Problems during the extraction of these experiments in particular, forced us to resuspend the cells in

full strength (1/2 MS - 1.5% sucrose) medium for one day to give them a chance to recover (entry 7).

Three more experiments were done after this time using 1/5 MS - 0.6% sucrose medium (entries 8-10), before resuspension in 1/2 MS - 1.5% sucrose medium and normal growth conditions were established (entries 11-14). After one month of normal culture conditions, the cell suspension was found to be able to effect biotransformation (entries 15 and 16) even though many cells had died. The results are shown in Table 2.

The same trend as with the 7-day old experiments was observed, namely, that the second biotransformation of a given series afforded best results (Entries #1 vs. 2, and #15 vs. 16). In these cases, the recoveries are highest as are the ring closure yields, but in any subsequent biotransformation the percentage of ring-closed compounds decreases and tends toward stabilization at about 23% for aryl tetralin 75.

In entry #8, the recovery is good, but there is a poor biotransformation since a significant amount of substrate was not transformed indicating that the cells had not recovered from the previous experiments.

In general, we thought that enzymatic modification of the substrate and/or products gave water soluble compounds which would remain in the aqueous phase since the cell extract did not reveal the presence of these metabolites. In fact, glycosidation processes (amongst others) might be in effect in the cell suspension culture.

It is also worth mentioning the high yield of compound 76 in entry #16 (40%), possibly indicating that longer incubation times favored the production of this compound, but results are difficult to interpret because of the low recoveries. However, this was an exciting experiment on the one hand, in that enzymatic activity was retained throughout a large series of experiments.

Entry	Substrate added(mg)	medium strength	time (h)	Extract obtained (mg)	Substrate recovery %	Yield of 74 %	Yield of 75 %	Yield of 76 %	Recovery %
1	200	1/10	24.5	331.8	26.2	1.1	32.2	5.9	55.2
2	200	1/10	26.8	251.4	37.2	9.2	47.0	9.5	78.5
3	200	1/10	45.0	243.7	17.5	-	26.7	1.4	40.8
4	200	1/5	43.2	230	10.9	-	21.2	9.9	38.7
5	200	1/5	55.8	286	-	-	-	-	-
6	200	1/5	46.1	242.9	-	-	-	-	-
7	-	1/2	23.7	97.8	-	-	-	-	-
8	200	1/5	23.7	152	52.3	1.9	26.9	12.8	72.2
9	200	1/5	25.8	246	5.0	-	18.3	11.0	32.9
10	200	1/5	23.5	244	13.3	-	25.7	7.0	41.7
11	-	1/2	167.0	271	-	-	-	-	-
12	-	1/2	192.2	299	-	-	-	-	-
13	-	1/2	240.6	667	-	-	-	-	-
14	-	1/2	96.1	350	-	-	-	-	-
15	500	1/5	50.7	434	3.1	-	12.9	12.6	27.8
16	814	1/2	182.8	1632	-	-	22.3	40.1	62.3
cells	-	-	-	6241	-	-	-	-	-

Table 2. Semi-continuous biotransformation of substrate **56** with 17-day old *P. peltatum* cells. Entries 1-5 with pH controlled to 6.5.

We thought next of assessing the influence of pH drift in the biotransformations since it could be important for better yields and recoveries.

With the experience gained from this bioreactor, we returned to younger cells still in their growth phase (7 days) and performed seven consecutive biotransformation experiments. In these studies, 1/5 MS - 0.6% sucrose medium was used and the pH was allowed to drift. A much better reproducibility was obtained, and normal cell growth could be continued after the biotransformation experiments were finished, the cells being healthy and normal in all respects. The results are shown in Table 3.

Entry	Substrate added (mg)	Extract obtained (mg)	Substrate recovery %	Yield of 74 %	Yield of 75 %	Yield of 76 %	Recovery %
1	200.0	375	5.4	2.1	32.6	7.7	45.6
2	200.0	229	7.0	3.9	25.7	6.9	41.0
3	200.0	237	7.2	1.8	27.1	14.7	47.8
4	200.0	220	9.3	3.8	28.8	10.3	48.2
5	200.0	223	5.8	2.5	27.2	9.9	43.2
6	200.0	192	2.7	0.3	20.1	11.6	33.9
7	200.0	208	4.0	0.9	22.6	10.3	36.5

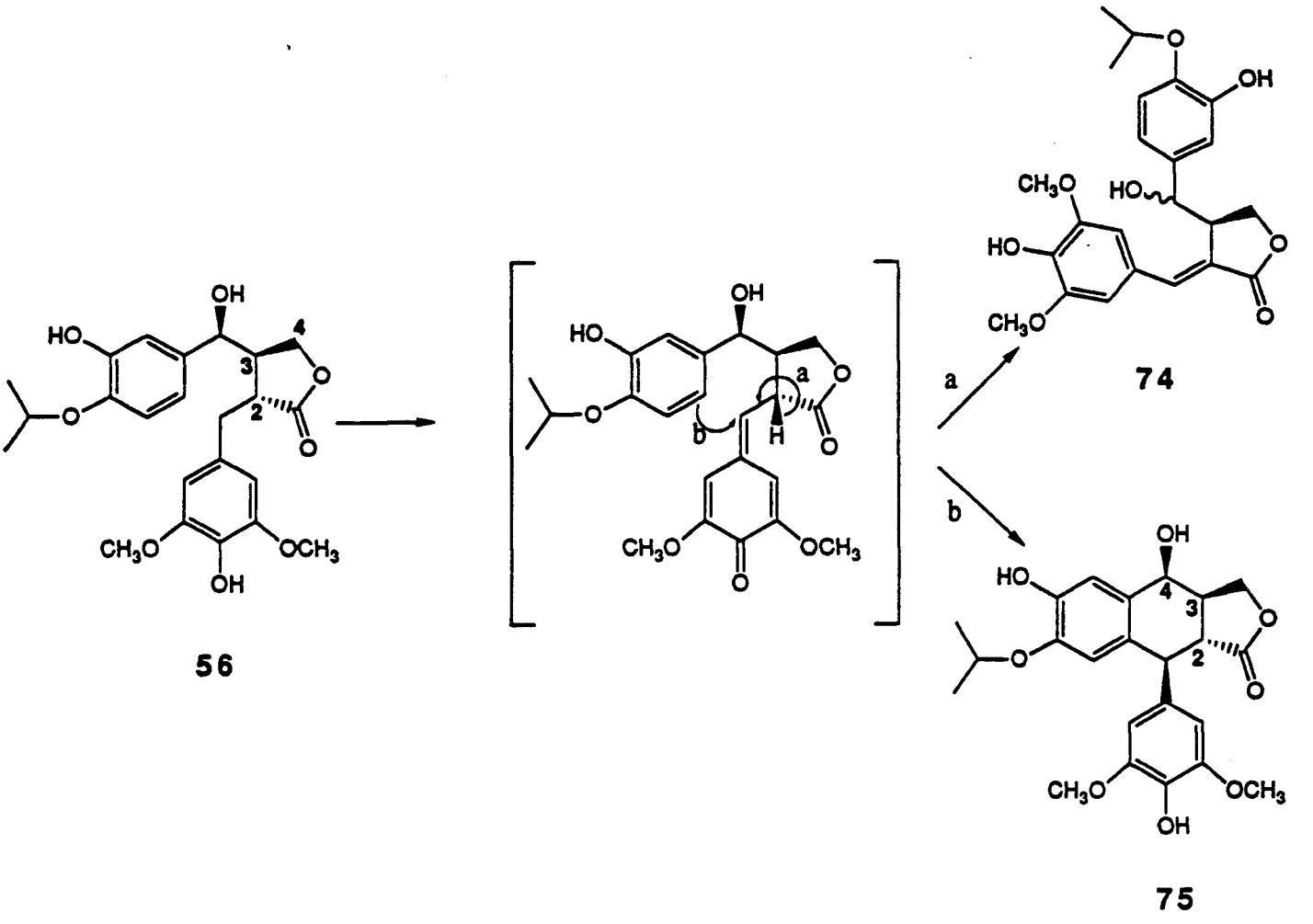
Table 3. Semi-continuous biotransformation of substrate **56** with 7-day old *P. peltatum* cells. PH was not controlled.

We found that there were no more abrupt variations in yields, but only half of the material was accounted for, again glycosidation or degradation of the precursor and/or products being the suspected cause.

With the data gathered from all of these experiments, we concluded that the enzymes were more dependent on pH (6.5 gave better yields and recoveries) than on cell age.

The biotransformation products can be explained using a common intermediate, namely, the hypothetical quinone methide which could tautomerize to give styrene **74**

or undergo nucleophilic attack by ring A to produce the ring-closed compound 75 (Scheme 16).



Scheme 16. The intermediacy of a hypothetical quinone methide in the formation of products 74 and 75.

The aryl tetralin 75 could then undergo O-methylation to 78 or enzymatic hydroxylation to 76. This latter process might involve multiple steps in nature, for example, conversion of 75 to a styrene, that is, dehydrogenation at C1-C2, epoxidation followed by epoxide opening or perhaps direct hydroxylation at the activated C2 carbon atom. No evidence concerning these mechanistic speculations is available from our studies.

It should also be emphasized that an alternative mechanism involving diradical coupling of **56**, and involving peroxidase enzymes, cannot be excluded from the present studies (see later).

Biotransformation Reproducibility. Summary of the Semi-continuous Biotransformation Experiments.

The average yields for the semi-continuous biotransformation of substrate **56** with *P. peltatum* cells, in the experiments discussed above, are presented in Table 4.

Cell Age	Recovered substrate %	Yield of 74 %	Yield of 75 %	Yield of 76 %	Recovery %
7-day (table 1)	8.1	1.4	40.6	11.9	41.5
17-day (table 2)	18.3	1.3	25.9	12.2	50.0
7-day (table3)	5.9	2.1	26.3	10.2	42.3

Table 4. Average yields for the semi-continuous biotransformation of substrate **56** with *P. peltatum* cells.

As can be seen from this table, the average biotransformation results fall within experimental error. It is worth mentioning the yield of 40.6% for the ring closed product **75** when the biotransformation is done under controlled pH (6.5, entry 1) versus not controlled conditions (entry 3).

These experiments illustrate also the reproducibility of the biotransformation outcome between different batches of cell culture. For example, entries 2 and 3 compare the average yields obtained over the space of several months. The experiment summarized in Table 2 required 69 days to completion while that of Table 3, 14 days.

Clearly, the ability of the cells to biotransform the supplied substrate is maintained for relatively long periods of time without significant yield variations.

The Biotransformation of 1-(3,5-Dimethoxy-4-hydroxy-phenyl)-6,4-dihydroxy-3-hydroxymethyl-7-isopropoxy-1,2,3,4-tetrahydro-2-naphthoic acid γ-lactone (75) with *Podophyllum peltatum* Cell Suspension Cultures.

To check the stability of aryl tetralin 75 under the biotransformation conditions, a series of experiments were done and 3, 11, 17, and 21-day old shake flask cultures were assessed.

Aryl tetralin 75 in 3 mL ethanol was added to each flask containing 300 mL of cell suspension culture. A series of control flasks were set up adding only 3 mL ethanol and reaction progress was followed by withdrawing 2.5 mL aliquots. HPLC (Rad-Pak C_{18} Waters, H_2O-MeOH 55:45 with 0.1% AcOH, UV detector) and TLC analysis ($CHCl_3$-MeOH 9:1) of the ethyl acetate extracts were done after 0, 3, 6, 9 and 24 h, after which the flasks were harvested by homogenizing together cells and spent medium prior to ethyl acetate extraction. The analytical conditions used are suitable for the detection of compounds 75-78.

The data (Figure 13) indicate a fair stability for the aryl tetralin 75 under the biotransformation conditions. In one case (21-day old cells experiment) the concentration of aryl tetralin 75 dropped by more than 50% after 3 h of incubation, but a repeat of this experiment revealed a similar behavior to the rest of the experiments suggesting that this particular case was exceptional and likely due to the inevitable variation between different shake flasks; particularly when a differentiated culture like ours is used. The fact that the hydroxylated compound 76 was not detected in every case and that the rate of consumption of product 75 was relatively slow (89-91% of the product was recovered unchanged after 24 h) suggests

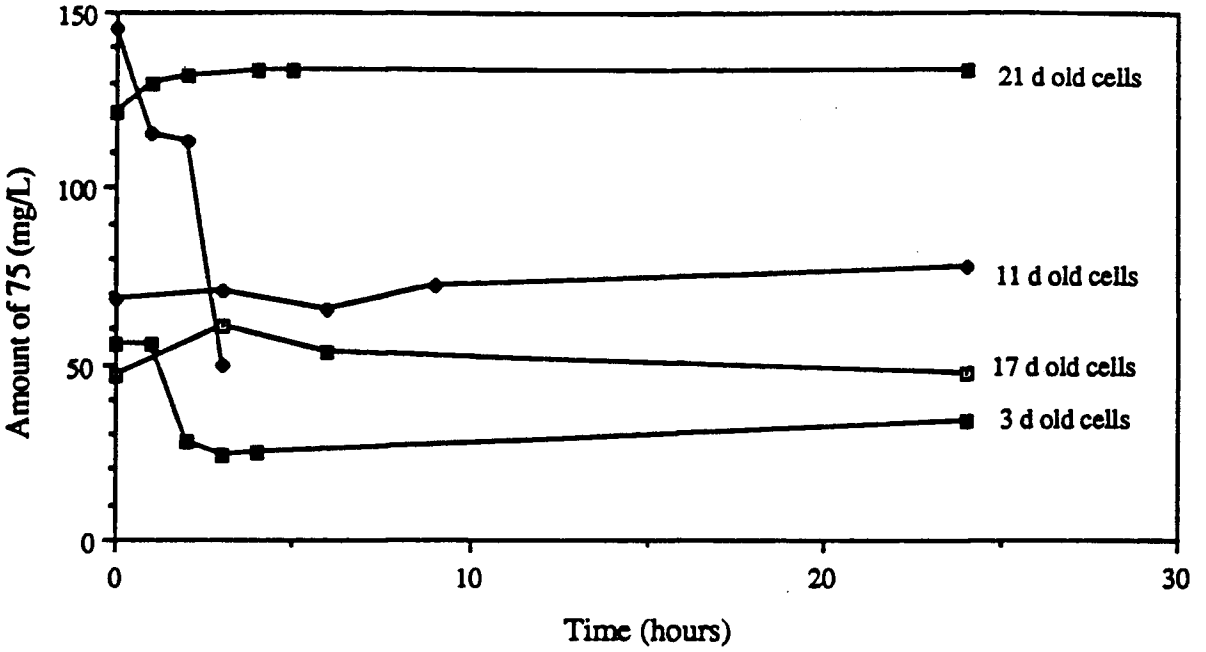


Figure 13. Time-course study to check stability of aryl tetralin 75 under the biotransformation conditions.

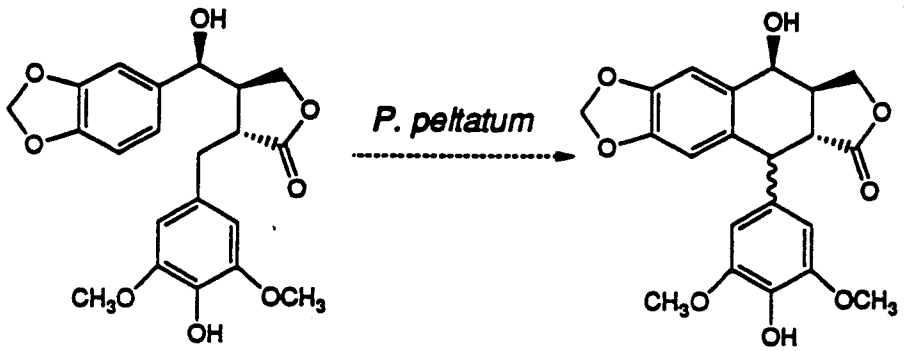
that the process going from 75 to 76 is likely to require long biotransformation times increasing the possibility of 75 being biotransformed further along the way, but not exclusively to product 76, thereby providing for a more complex situation. Perhaps CFE technology would be a better option for these studies since variations between cell batches are eliminated and experiments are run on a much shorter time scale. Such studies will be undertaken by other coworkers in our laboratory.

We were prompted next to investigate the biotransformation of a more closely related analogue of podophyllotoxin, and compound 57 seemed appropriate (Scheme 17).

The fact that 57 incorporates the methylenedioxy group, would make it presumably more stable towards oxidations or degradations during biotransformation, and if ring closure could be achieved, a more direct way into the podophyllotoxins would be on hand.

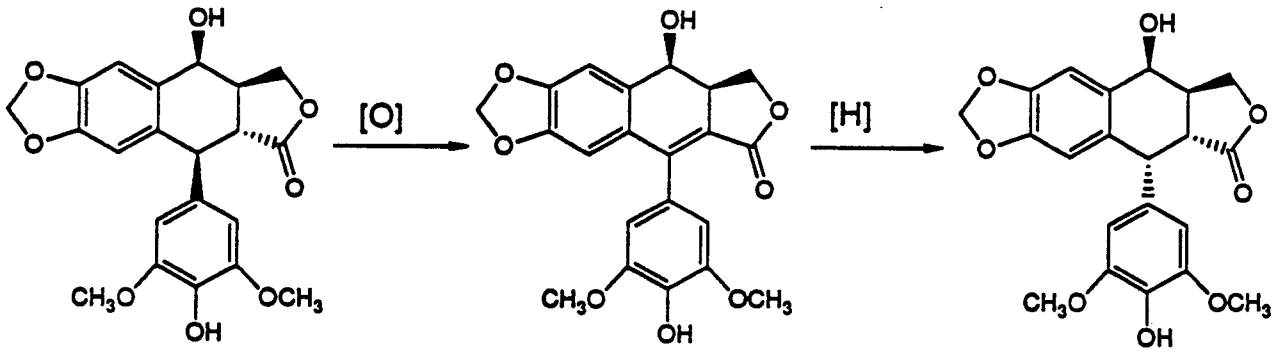
Should the ring closure produce the wrong stereochemistry at C(1), we intended to employ a synthetic pathway involving the formation of a double bond (see 80),

followed by directed catalytic hydrogenation to give the desired *trans*-1,2-aryl tetralin system (Scheme 18). Very similar hydrogenations have been reported in the literature[96].



57 61

Scheme 17. The proposed biotransformation of substrate 57.



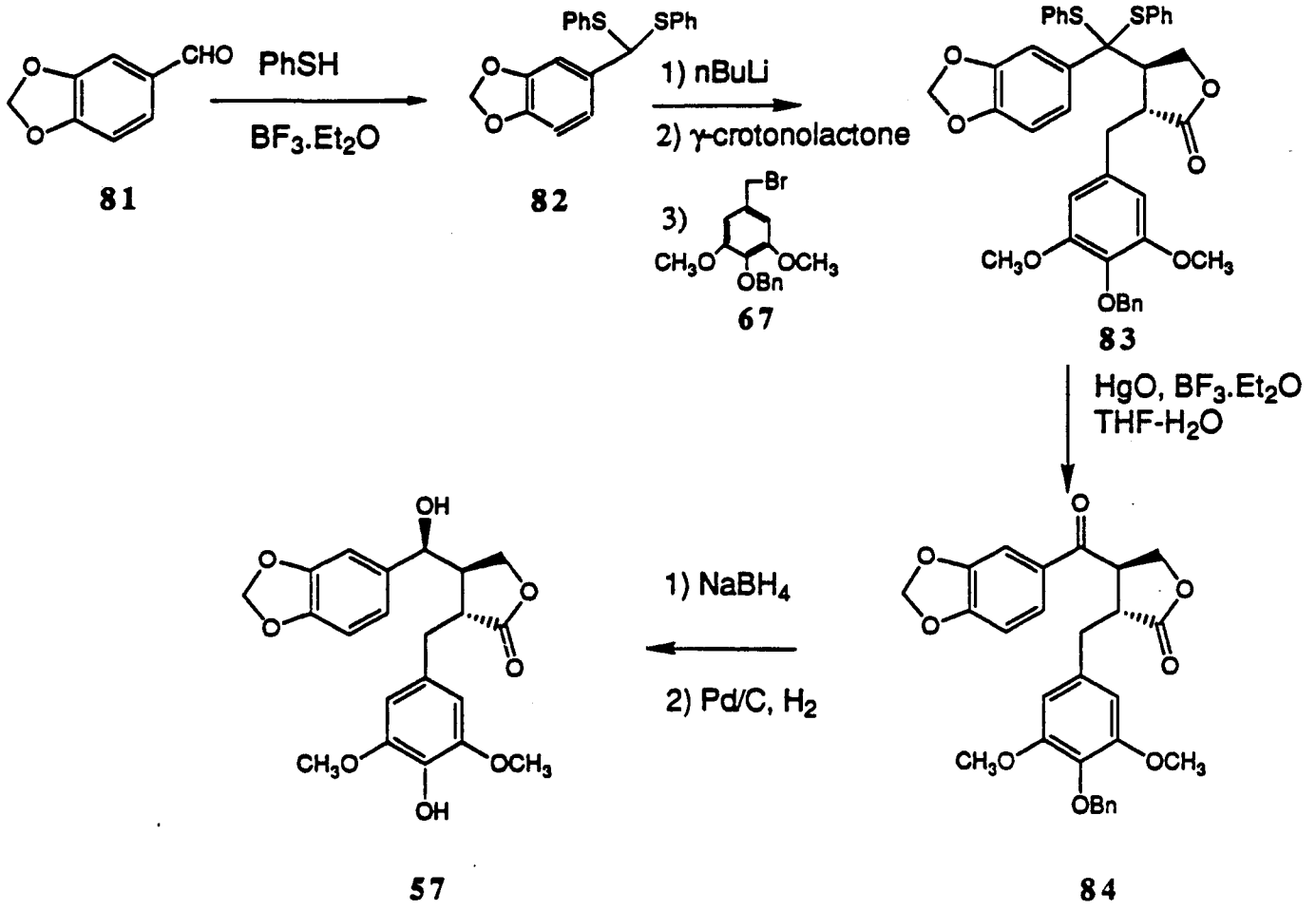
79 80 61

Scheme 18. Proposed C(1) epimerization sequence from compound 79 to epipodophyllotoxin (6).

Synthesis of Trans-2-(4-hydroxy-3,5-dimethoxybenzyl)-3-(3,4-methylenedioxy)-7-hydroxybenzyl)butanolide (57).

The synthesis of substrate **57** was done along the same lines as that of substrate **56**, and is outlined in Scheme 19.

The sequence starts with readily available piperonal (**81**) which upon treatment with thiophenol and boron trifluoride etherate at -40° C for 15 min afforded thioketal **82** (98% yield) as a yellow oil. Michael addition of the anion of **82** generated by the addition of *n*-butyl lithium, to γ-crotonolactone followed by *in situ* alkylation with the aryl bromide 67, produced the desired dibenzylbutyrolactone **83** in 55% yield. The



Scheme 19. The synthesis of substrate 57 from piperonal (81).

product could be crystallized from acetone and showed a fragment corresponding to M^+-PhS on electron impact mass spectroscopy. However, both HRMS and elemental analysis were in agreement with the molecular formula $C_{40}H_{36}O_7S_2$. The IR spectrum of this compound showed the carbonyl band at 1770 cm^{-1} corresponding to a γ-lactone. In the ^1H nmr spectrum, the signal for the methylenedioxy group was present at δ 6.05 ppm, H2 produced a multiplet at δ 2.85-2.92 ppm while H3 produced another multiplet at δ 3.29-3.34 ppm. Again, the ABX system for the lactone methylene was clearly visible at δ 3.50 and 4.45 ppm. The coupling constants of the lower field component of this system were 3 and 10 Hz, indicating that the proton responsible for this component is most likely *cis* to H3. Thioketal hydrolysis was carried out in 85.5% yield by treatment with red mercuric oxide and boron trifluoride etherate to produce ketone **84** as white crystals when crystallized from ethyl ether. The IR spectrum showed two bands for carbonyl groups at 1770 (lactone) and 1670 cm^{-1} (ketone) while the ^1H nmr spectrum showed a reduction in the aromatic protons to 5 and a downfield shift for H3 compared to that of compound **83** which appeared now as a doublet of doublets of doublets at δ 4.00 ppm. The signal for H2 is seen as a multiplet at δ 3.58 ppm. The protons at position 7″ are located at δ 3.0 (dd, J = 6, 14 Hz) and 3.08 (dd, J = 5, 14 Hz), the singlet for the methoxy groups on ring D is situated at δ 3.7. The ABX system for the methylene protons of C4 is located at δ 4.10 (dd, J = 8, 9 Hz) and 4.39 (dd, J = 8, 9 Hz) ppm, and the methylene singlet from the benzyl protecting group is seen at δ 4.92 ppm. The aromatic protons appear as a two proton singlet at δ 6.29 (H2″, 6″), a one proton doublet at δ 6.80 (H5′, J = 8 Hz) and a seven proton multiplet at δ 7.22-7.48 ppm. Electron impact mass spectroscopy showed the molecular ion at m/z = 488 with a base peak at m/z 91 arising from the loss of the benzyl protecting group.

Treatment of this compound with sodium borohydride (1.4 equiv.) in methanol at 0^o C produced alcohol **85** in 95% yield as a white foam. The product was present

as a mixture of epimers at C7′ and the major isomer was assumed to be the β-alcohol on the same grounds as those presented earlier for compound 70. The ^{1}H nmr spectrum showed the new proton at C7′ as a doublet with J = 6.7 Hz at δ 4.60 ppm, a new broad signal that could be exchanged with heavy water at δ 1.70, the singlet for the methoxy groups at δ 3.80 and the multiplet for the methylene protons of C4 centered at δ 3.9 ppm. The protons at positions 2, 3 and 7″ create a multiplet at δ 2.50-3.10 ppm, while the protons of ring A (5′, 6′, and 2′) appear as a multplet at δ 6.6-6.8 ppm. The rest of the aromatic protons are under a singlet at δ 6.4 (H2″, 6″) and a multiplet at δ 7.24-7.50 (5H from benzyl ether) ppm. The IR spectrum shows absorptions at 3600 (alcohol) and 1770 (lactone) cm^{-1} and mass spectrometry showed the molecular ion at m/z 490.

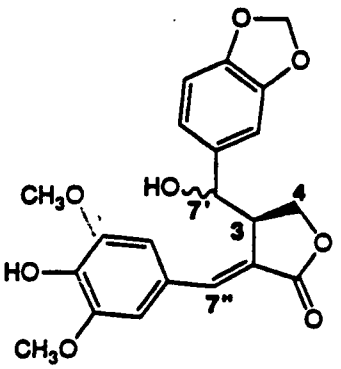
Finally, catalytic hydrogenation of compound **85** under 1 atm of hydrogen for 6 h with Pd/C 10% as catalyst afforded substrate **57** in 86.6% yield. The loss of the benzyl protecting group was evidenced by the disappearance in the ^{1}H nmr spectrum of the signals at δ 5.0 and 7.24-7.50 ppm, and the molecular ion at m/z 402 shown in the mass spectrum of **57**. We had now a 5-step synthetic route for substrate **57** starting with piperonal and could turn once again to biotransformation experiments.

The Biotransformation of Trans-2-(4-hydroxy-3,5-dimethoxybenzyl)-3-(3,4-methylenedioxy)-7-hydroxybenzyl)butanolide (57) with *Podophyllum peltatum* Cell Suspension Cultures.

Biotransformation experiments with substrate 57 were carried out using *P. peltatum* cells at various ages and different incubation periods, while maintaining the pH at 6.5. In all cases, biotransformation was observed to produce a major, more polar metabolite, which after extensive chromatographic purification of the ethyl acetate extract from the harvested bioreactors, was isolated as a pale yellow solid with mp = 222-225^{o} C (EtOAc).

High resolution mass spectrometry indicated a molecular formula of $C_{21}H_{20}O_8$ (two hydrogen atoms had been lost) but the high degree of fragmentation observed in the electron impact spectrum suggested a ring-opened type compound. The product showed an IR band at 1715 cm^{-1} for a cinnamic ester carbonyl band and the UV spectrum presented maxima at 335, 240 and 208 nm supporting the presence of a strong chromophore.

The ^{1}H nmr spectrum demonstrated clearly that the compound in hand was the styrene 86.



86

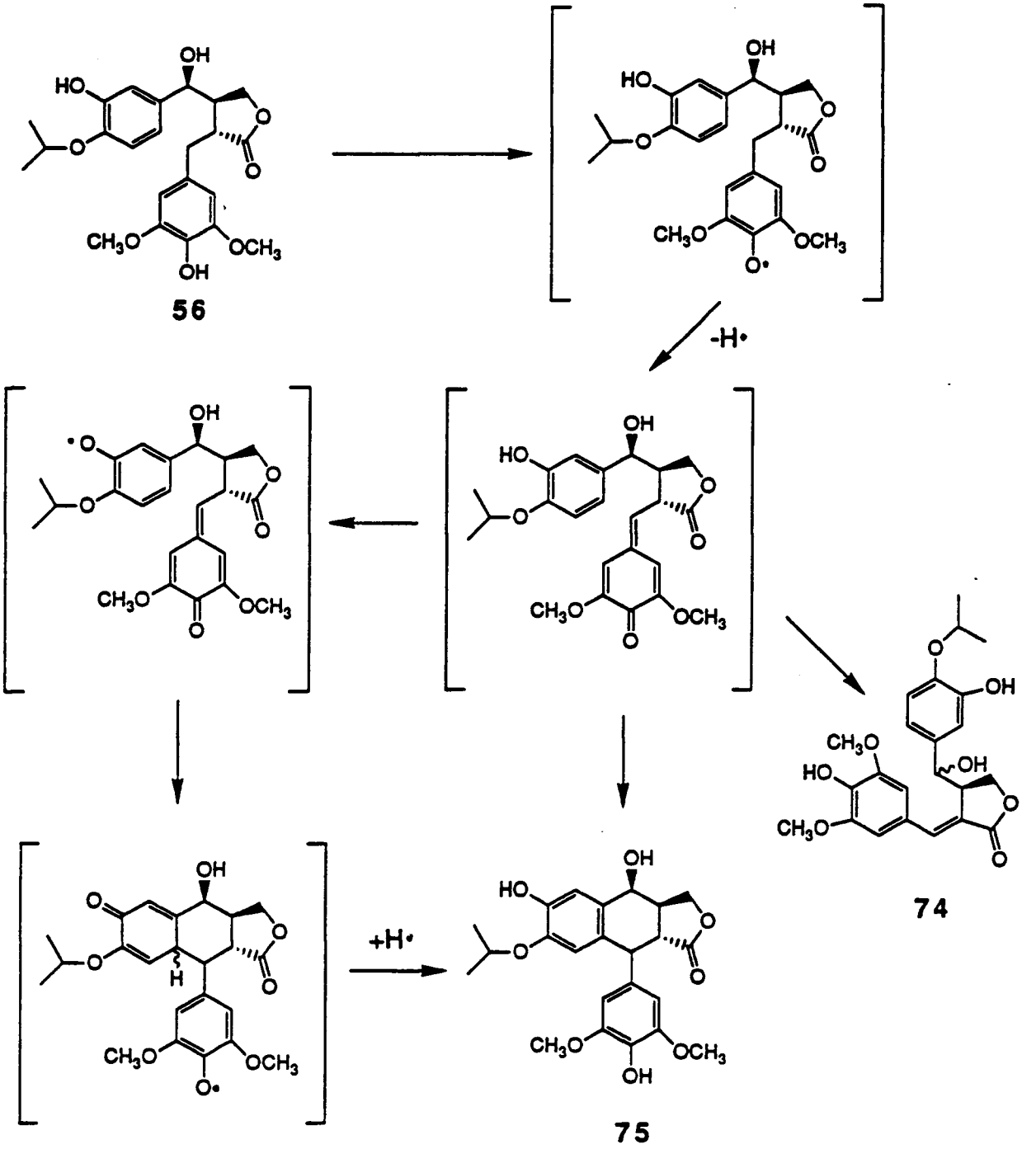
The aromatic region presented multiplets similar to the precursor compound 57; however, a new singlet whose integration corresponded to one proton was present at δ 7.40 ppm, and was assigned to the olefinic proton H7". The system of the aliphatic protons was again clearly visible as in the case of compound 74 and the assignments

were made making use of multiple irradiation experiments. The methoxy groups' singlet appears at δ 3.85 ppm while H3 presents a broad signal centered at δ 4.10 ppm. The ABX system for the methylene protons of C4 is seen as a broad triplet centered at δ 4.24 and a broad doublet at 4.40 ppm. The proton at H7′ is located at δ 4.95 ppm, and the aromatic protons are present at δ 6.6 (H6′), 6.75 (H5′ and 2′) and 7.10 ppm (H2″ and 6″). As mentioned earlier, H7″ is located at δ 7.4 ppm which permits the assignment of an E configuration for the double bond on the same grounds as with compound **74**.

The yield of **86** varied according to incubation times and oscillated around 60% for 20 h. It was observed that shorter biotransformation times lead to higher yields based on recovered starting material. However, extensive research on this area by us and other members of the group[97] failed to produce any ring-closed compound. We concluded, therefore, that the presence of a free hydroxyl group in ring B was essential for this process to occur.

Considering this fact and the excellent parallel of the present studies, involving the cyclization of **56**, with those of earlier experiments concerning a similar biotransformation of the closely related dibenzylbutanolide to **75a** by J. Palaty[75], it seems likely that a free radical process in the conversion, **56** to **75**, is involved. It is pertinent to note that Palaty's experiments were pursued with crude enzyme preparations (CFE) derived from the cell culture of *C. roseus*, an indole alkaloid producing culture containing significant levels of peroxidase enzyme. In those studies, Palaty was able to relate the yield of **75a** versus the number of peroxidase units involved. Since the present studies were performed with cells of *P. peltatum*, the levels of "peroxidases" within the growing cells versus yield of **75** were not evaluated. However, such studies with crude enzyme preparations from *P. peltatum* and biotransformation yields of **56** to **75** are presently underway in our laboratory.

In conclusion, the mechanistic pathway shown in Scheme 20 is presented to provide a rationale for the results obtained.



Scheme 20. Proposed mechanistic pathway for the formation of products 74 and 75 in the biotransformation of substrate 56 by *P. peltatum* cell suspension cultures.

PHARMACOLOGICAL SCREENING OF THE CELL PRODUCED METABOLITES, AND SYNTHETIC SUBSTRATES.

A number of samples from both synthetic and plant cell derived metabolites was forwarded for pharmacological screening at CIBA-GEIGY, Basel, Switzerland. In order to best characterize the biological activity of these new derivatives in comparison with etoposide (VP-16), an assessment of their activity in some cell lines and particularly on VP-16 resistant cells was proposed. In view of the known toxicity of etoposide and podophyllotoxin, it was thought important to ascertain the toxicological profiles of the preparations at this early stage of research, if possible, in cell systems.

The compounds were tested for inhibition of cell proliferation *in vitro*. Two cell lines were used, the human KB31 cell and a derivative thereof, KB8511 which was selected for resistance toward colchicine and expresses the mdr-1 (multi-drug resistance) phenotype.

The results obtained indicated that some of the compounds possessing the "wrong" stereochemistry at C1, do lack cross-resistance and are apparently more potent than etoposide. However, since these products are likely to be subjected to patent protection, the details of these studies will not be published until later.

In conclusion, the biotransformation of the synthetic substrate by the cell cultures of *P. peltatum* to the "wrong" stereochemistry, for example, **56** to **75**, may turn out to be highly desirable in producing a novel family of pharmacologically active compounds.

SUMMARY

The present studies have provided an efficient and versatile route to a series of dibenzylbutanolides to be utilized in biotransformation experiments involving whole cells of *P. peltatum* and/or crude enzyme preparations derived therefrom.

The use of the semi-continuous fermentation process applied in these studies is novel and has an exciting potential in the future even for large scale commercial processes. The shortcomings in the use of plant cell cultures as expressed by various practitioners in the field, such as long term periods for metabolite production, are clearly eliminated by such methodology.

We have shown that "enzymatic activity" in converting, for example, the dibenzylbutanolide **56** to **75**, can be maintained for several months, within a given batch of *P. peltatum* cells. Although, additional studies are required to optimize conditions, this "biological factory" is certainly of interest for future experiments.

Finally, it should be noted that the conversion of **56** to **75**, although providing, at present, the "wrong" stereochemistry in terms of intermediates for etoposide synthesis, does afford the opportunity to obtain novel podophyllotoxin analogues which may exhibit interesting pharmacological properties.

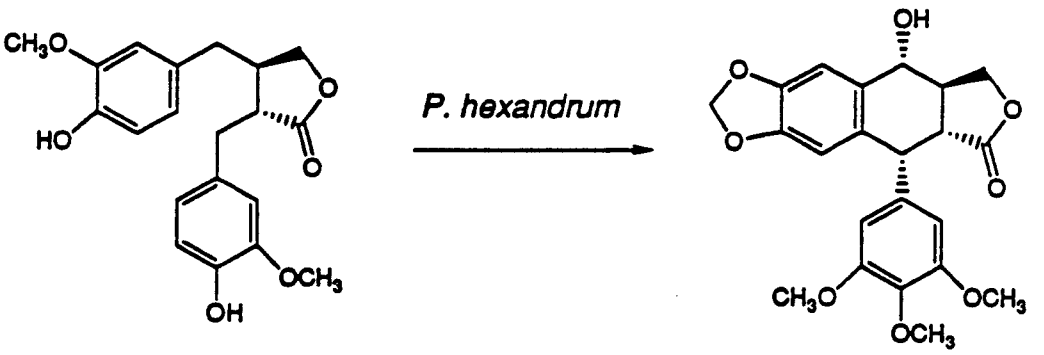
FUTURE WORK

These studies provide an excellent basis for the direction of future research. The cell line has been shown to produce the relevant enzymes for ring closure but the fact that the configuration at C(1) of the biotransformation product is epimeric with that of the natural product gives room for speculation.

There are many parameters influencing the outcome of the reaction under study, but in the light of our results, it is unlikely that factors such as cell age, medium composition, and biotransformation time or pH are responsible for the observed stereochemistry.

One can speculate that if the precursor is reaching the enzyme's active site, something is preventing it from adopting the appropriate conformation for ring closure, resulting in the obtained product. Since both phenolic groups on rings A and D appear to be critical for enzymatic ring closure to occur, one can put forward the hypothesis that the enzyme uses these particular groups as "handles" for the transformation, in a manner such that the substrate "sits" in the enzymatic active site in a way that determines the stereochemical outcome of the process.

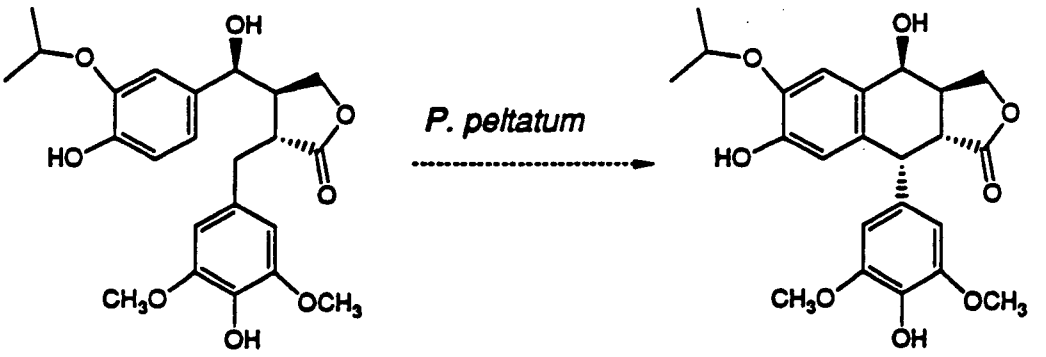
The fact that matairesinol (47) which possesses the phenolic hydroxyl groups at other centers, has been shown to be incorporated into podophyllotoxin (4) when fed to *P. hexandrum* plants[73] provides a possible support for this hypothesis (Scheme 21) although clearly the relationship between the enzymes within our cell culture and those within the plant has not been established.



Matairesinol (47) podophyllotoxin (4)

Scheme 21. The incorporation of matairesinol (47) into podophyllotoxin (4) by *P. hexandrum* plants.

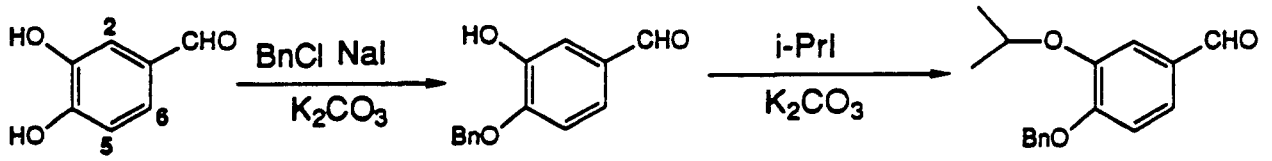
A possible experiment to test this hypothesis could involve the biotransformation of a precursor such as 87 (Scheme 22).



87 88

Scheme 22. Proposed biotransformation for substrate 87.

The synthesis of this precursor could be achieved in a similar way to that of substrate 56, with the only essential change in the overall sequence being an inversion of the initial step involving introduction of the isopropyl and benzyl groups in the starting aldehyde. The protection of 3,4-dihydroxy benzaldehyde (62), as outlined in Scheme 23, would produce a regio isomer of 64 that would eventually be elaborated into precursor 87. Studies along this line have been initiated.



62 89 90

Scheme 23. Proposed synthesis of 3-isopropoxy-4-benzyloxy-benzaldehyde (90) from 3,4-dihydroxybenzaldehyde (62).

Alternatively, the glycosidation of the biotransformation substrate at position 7′ could have some effect in the biotransformation outcome, since a more soluble product would be more readily available for enzymatic transformation in the cell suspension culture. Also, the incorporation of a bulky substituent in this position may change the conformation adopted for ring closure and therefore, the stereochemical outcome of the reaction. We were thus prompted to explore biotransformations of podophyllotoxin (**4**) and related compounds aiming to identify a whole cell or enzymatic system capable of glucosylating regioselectively these compounds. This would have the added advantage of obviating the need for the protecting groups employed in the chemical transformation.

Work along this line has been started in collaboration with the group of Professor Bruno Botta in Italy to whom we have forwarded some of our synthetic precursors. Work is being considered in our group to attempt this transformation using microbial transglucosylation technology[98].

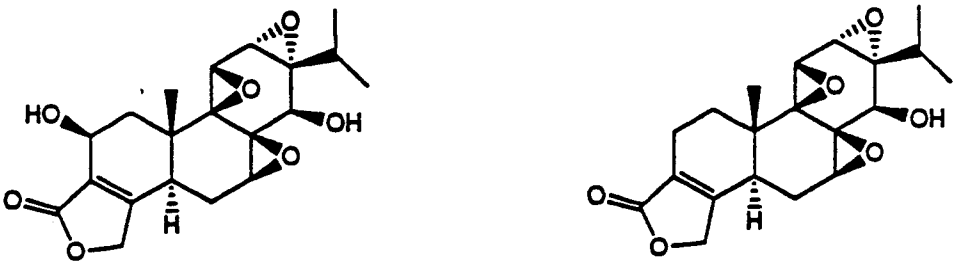
The synthetic sequences described in this work provide access to the racemic modification of the dibenzylbutenolides. However, the synthesis of homochiral **56** is being considered.

The chiral synthesis of **56** would make use of the chiral synthon 4-menthyloxybutenolide as described in a recent paper by Ward et al[91] (1992) which follows also the tandem Michael addition-alkylation approach used in our synthesis. Advances in this direction will certainly be part of the future work in our group.

CHAPTER 2. THE TRIPDIOLIDE FAMILY.

INTRODUCTION.

Tripdiolide (5) and triptolide (91) are the first recognized naturally occurring diterpene triepoxides, and represent the first reported natural products containing the 18(4 → 3)*abeo*-abietane skeleton. These compounds were first isolated from extracts of *Tripterygium wilfordii* Hook by Kupchan and co-workers in 1972[99] (Figure 14).



tripdiolide (5) triptolide (91)

Figure 14. The structures of tripdiolide (5) and triptolide (91).

The plant *Tripterygium* is a vine indigenous to China belonging to the family *Celastraceae.* There are three species. *Tripterygium wilfordii* grows in the mountainous areas of south-east and southern China, *Tripterygium hypoglaucum* Level occurs in south-western China, and *Tripterygium regelii* Sprague et Takeda is found in the north-east of China. The most common species is *T. wilfordii*, and it has been the most studied.

The plant has a long history of being used as a herbal medicine in the treatment of rheumatoid arthritis, chronic hepatitis, and various skin disorders with more or less promising results.

The chemistry of the plant has been studied for half a century and several alkaloids, diterpenoids, triterpenoids and sesquiterpenes, have been isolated and characterized.

Interest in the plant was renewed when the contraceptive properties of a "refined extract" from the root xylem were discovered[100].

Preliminary results from tests on the effects of this extract in male rats and in men showed a strong male anti-fertility effect as well as some anti-inflammatory and immunosuppressive activities. An alcoholic extract of the plant showed significant activity *in vivo* against mice L-1210 and P-388 leukemias and *in vitro* against KB cells from human carcinoma of the nasopharynx[100].

The male anti-fertility effect attracted our attention since the crude extract induced reversible infertility in men without significant side effects, and with the levels of testosterone in serum, potency and libido apparently unaffected.

There is a great deal of research aimed at developing a safe, fully reversible method for family planning for men. At present, the male partner has only a narrow choice if he wishes to participate in family planning, namely, the choice between the condom, a vasectomy, or withdrawal. No acceptable anti-fertility drug for men has yet been produced despite major research efforts by several agencies. A method based on chemical anti-fertility agents would be highly desirable, and in view of the interesting activity of the plant extracts, efforts have been directed towards the isolation, characterization, and mode of action of the chemical responsible for the anti-fertility effect of *Tripterygium wilfordii*.

It is believed that *T. wilfordii* extracts damage the epidydimal spermatozoa, and to a lesser extent, the spermatogenic cells. At present, it is difficult to comment on the events underlying the reduction of sperm density without apparent testicular damage. The inhibition of spermatogenesis by one of the components of the extract may serve as a possible explanation.

The data gathered so far indicated that spermatogenic cell types most sensitive to the action of the extract are the spermatids and the spermatocytes.

To understand this, it is necessary to consider some basic facts about spermatogenesis and its chemical impairment.

CHEMICAL IMPAIRMENT OF SPERMATOGENESIS.

A normal fertile adult male produces in excess of 100 million new sperm every day[103]. However, sperm are not produced within a day. Each takes ten weeks to generate[103] and during this process, there are many complex changes in shape and function that are needed to transform the stem germ cells (spermatogonia) into the characteristic sperm[103]. These include the formation of a long tail for swimming, massive condensation of the cell's DNA so that it can be packaged inside the sperm's nucleus, and the formation of an acrosome or cap over the head of the sperm to store important enzymes used by the sperm to penetrate the protective layer which surrounds the female egg. All these changes occur in an orderly and precisely timed sequence, and while it is the germ cells which are undergoing these changes, it is the sertoli cells (sometimes called "nurse" cells) which orchestrate and control these changes[103]. The sertoli cells do so by secreting many different proteins, each of which has a different function and may target a particular germ cell type. The details on this control network are still very poorly understood, but research is gradually putting together the story by identifying the "messenger" proteins and their relevant functions.

Chemicals that impair normal sperm production probably do so by disrupting the normal protein messenger system in a highly specific way, which will differ between chemicals.

Of all the components of the extract of *T. wilfordii*, the diterpenoid epoxides, triptolide and tripdiolide, are the most likely to be responsible for this effect.

Detailed toxicity studies of tripdiolide in mice and dogs have been reported[101]. Structural data from X-ray crystallographic studies and from spectroscopic studies

clearly indicate, as can be seen with molecular models, that the C14 β-hydroxyl group of this compound is hydrogen bonded to the C9,11 epoxide oxygen atom[99]. Both compounds suffer selective nucleophilic attack by propanethiol at C9, while C14 epitriptolide is recovered unchanged under the same reaction conditions[102] (Figure 15). A proposal relating to the hydrogen bonding observed in **5** and **91** to their chemical reactivity and biological activity has been presented[102].

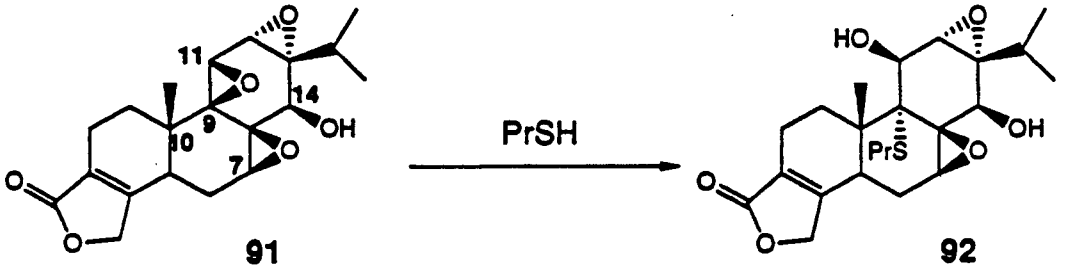


Figure 15. Alkylation of triptolide (**91**) with propane thiol.

OTHER CLINICAL AND PHARMACEUTICAL USES OF THE PLANT.

The past two decades have seen increasing clinical use, by Chinese physicians, of extracts from the plant. The preparations which have been used are extracts from the dried root xylem. Various disorders have been treated ranging from rheumatoid arthritis and ankylosing spondilitis[100a] through a variety of skin disorders. The extract used in these treatments, is found to be more potent than the conventional non-steroidal antirheumatic agents such as salicylates, indomethacin, and phenylbutazone, and can be substituted for corticosteroids in some skin diseases and in some patients who are steroid-dependent or who have contraindications to steroids. Its therapeutic effectiveness is believed to be related to its anti-inflammatory and immunosuppressive effects.

A comparison of the pharmacological effects of different preparations of the plant available in Chinese markets, showed that a refined extract, prepared by extraction of the root xylem with water and chloroform followed by column chromatography[100], contained the main anti-inflammatory constituent of *Tripterygium wilfordii*[100b]. Pentacyclic triterpenes and diterpenes are present in this preparation, as well as other constituents (possibly glycosides)[100].

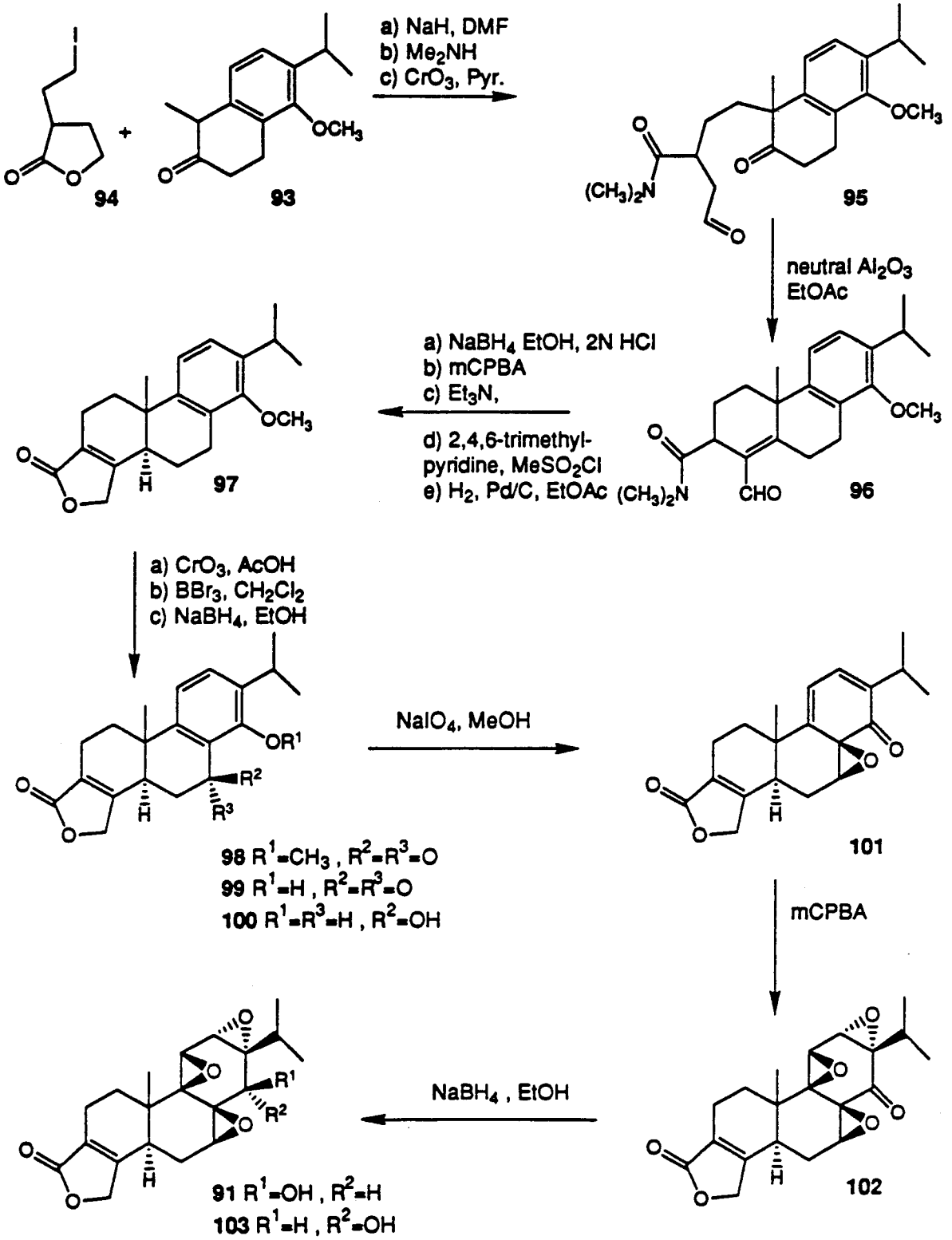
In view of this, research in the area of the diterpene biosynthesis in *Tripterygium* sp. could provide novel analogues with interesting activities in the area of auto-immune disorders such as rheumatoid arthritis.

TOTAL SYNTHESES.

A number of approaches have been adopted in the total synthesis of tripdiolide and triptolide. The major obstacles that have to be overcome are the construction of the triepoxide system in ring C and the butenolide in ring A. The *trans* A/B ring junction is stable in aqueous solution at neutral pH while in alkaline conditions, a complete conversion to the *cis* junction takes place[104]. Alkaline or acidic equilibration through the extended enolate must therefore be avoided.

The approach adopted by Berchtold et al[104] was to synthesize the dihydronaphthalenone **93**, as a starting material, providing the B/C ring fragment of the abietane skeleton (Scheme 24).

Construction of ring A via annulation of the napthhalenone provided a suitable functionalized tricyclic intermediate, **96**, for the construction of the ring C triepoxide system and the butenolide in ring A. Annulation was achieved via alkylation of **93** with the iodobutyrolactone **94**. Opening of the lactone gave **95** which yielded **96** after aldol condensation. Reduction of the aldehyde, acidic hydroysis and rearrangement of the double bond completed the synthesis of the lactone ring.



Scheme 24. Synthesis of triptolide (91) by Berchtold et al.

Hydroxylation at C7 and periodate oxidation produced the epoxy dienone **101**. Further epoxidation gave racemic triptonide (**102**), while reduction gave a 3:1 mixture of racemic 14_{β}-epitriptolide (**103**) and triptolide (**91**) respectivley. This and most other syntheses lead to a C14-phenol product which facilitates the synthetic incorporation of the epoxide system.

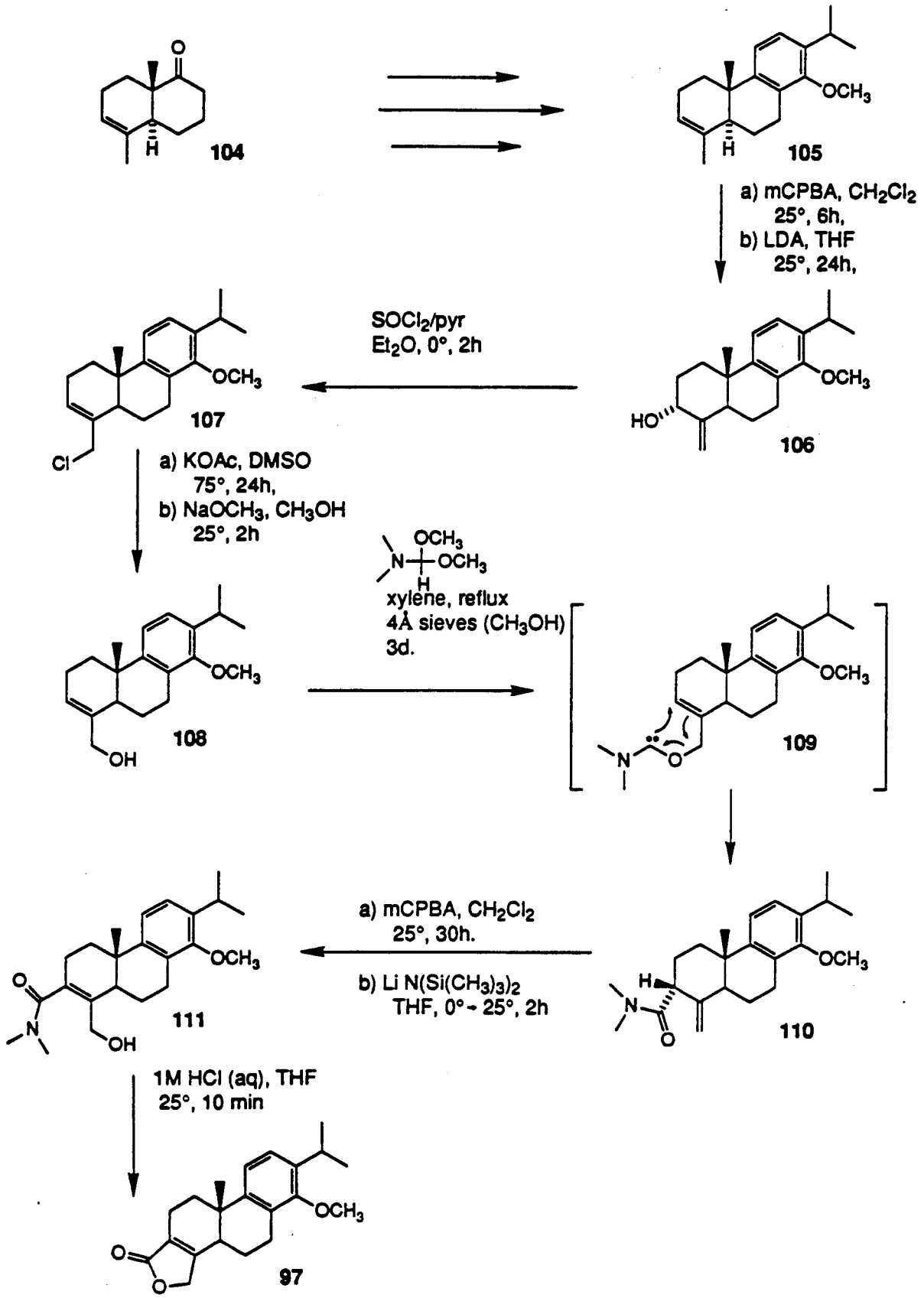
A synthesis of (±)-triptolide, involving fewer steps, comprised construction of ring C onto an appropriate AB fragment (Scheme 25), derived from decalone **104**.

In this synthesis Garver and van Tamelen[105] make use of the alkene intermediate **105**, to construct the butenolide **97**, via introduction of a hydroxyl group at C3, rearrangement with thionyl chloride to afford the allylic halide **107** and finally conversion of the latter to the allylic alcohol **108**. Addition of dimethylformamide dimethylacetal to this alcohol, was followed by a carbene [2,3]-sigmatropic rearrangement to **110**. Further elaboration yielded the key intermediate **97**.

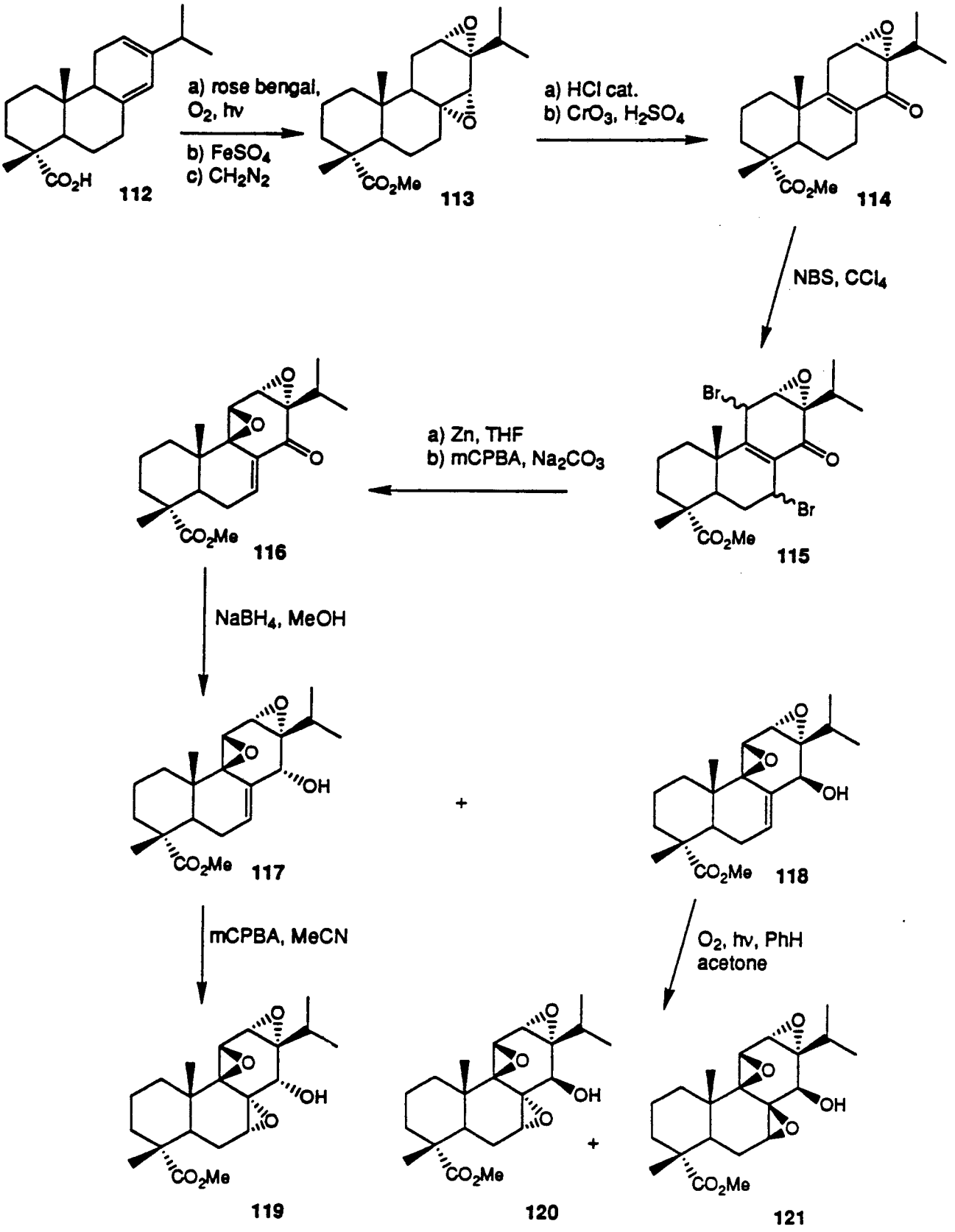
Tokoroyama et al devised alternative routes to the C ring system (Scheme 26) from laevopimaric acid[106] (**112**) and to the butenolide ring of triptolide (Scheme 27) and tripdiolide from dehydroabietic acid[107] (**122**).

The ring C construction shown in Scheme 26 involves the endoperoxide formation and rearrangement to the diepoxide **113**. Double bond manipulations and further epoxidations and reductions yielded the desired triepoxide system **121**, and the epimeric triepoxides **119** and **120**.

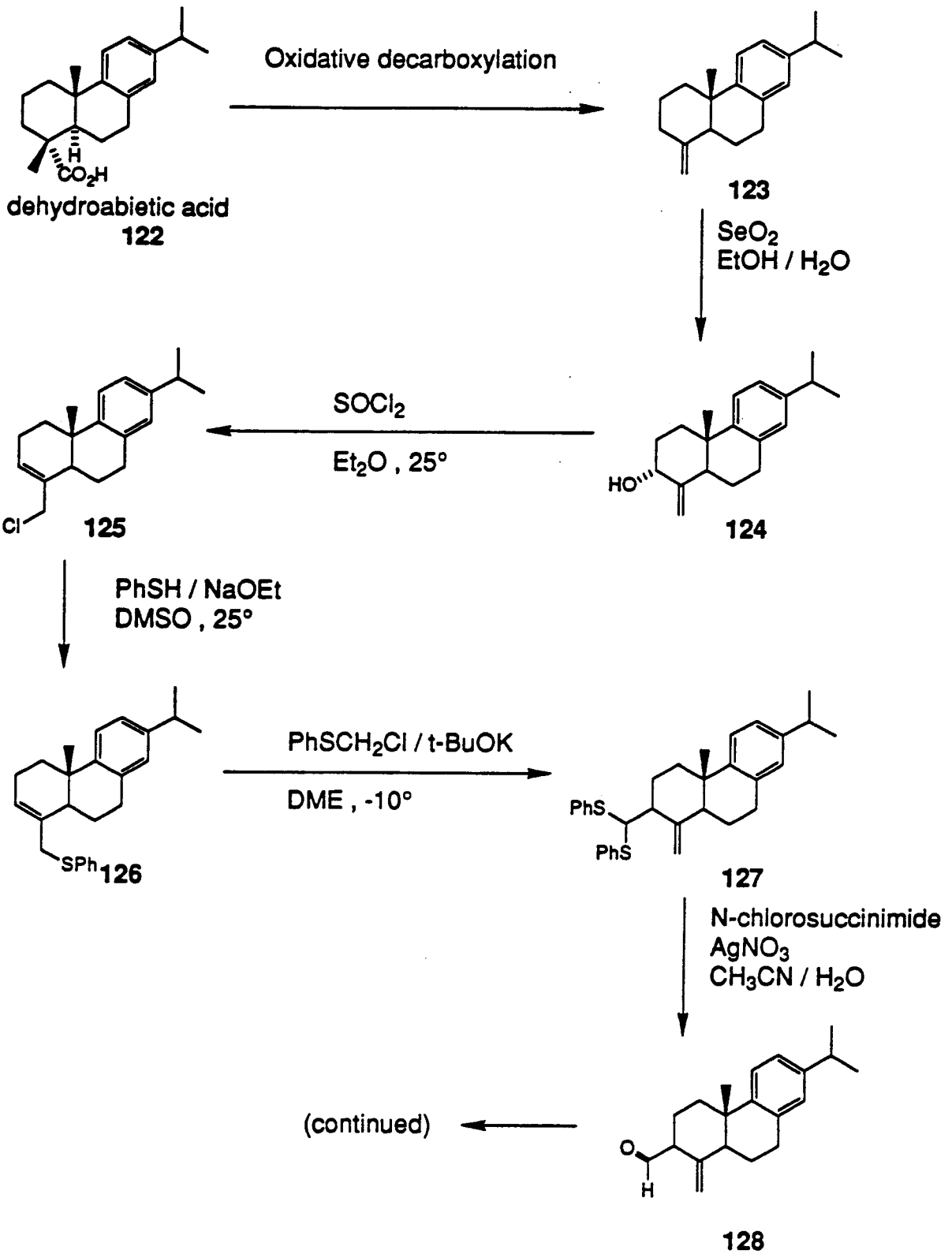
Scheme 27 shows Tokoroyama's construction of the butenolide ring from dehydroabietic acid (**122**). This is a multistep sequence through the exocyclic olefin **123**, Claisen rearrangement to give **127**, and finally SN_1' attack on **130** by chloride and closure to the butenolide ring **132**. Synthesis of the tripdiolide analogue **136** consisted of an elimination process via hydroperoxide to give the diene **134** and ring closure of the C2 hydroxylated intermediate **135** yielding the desired tripdiolide analogue **136**.



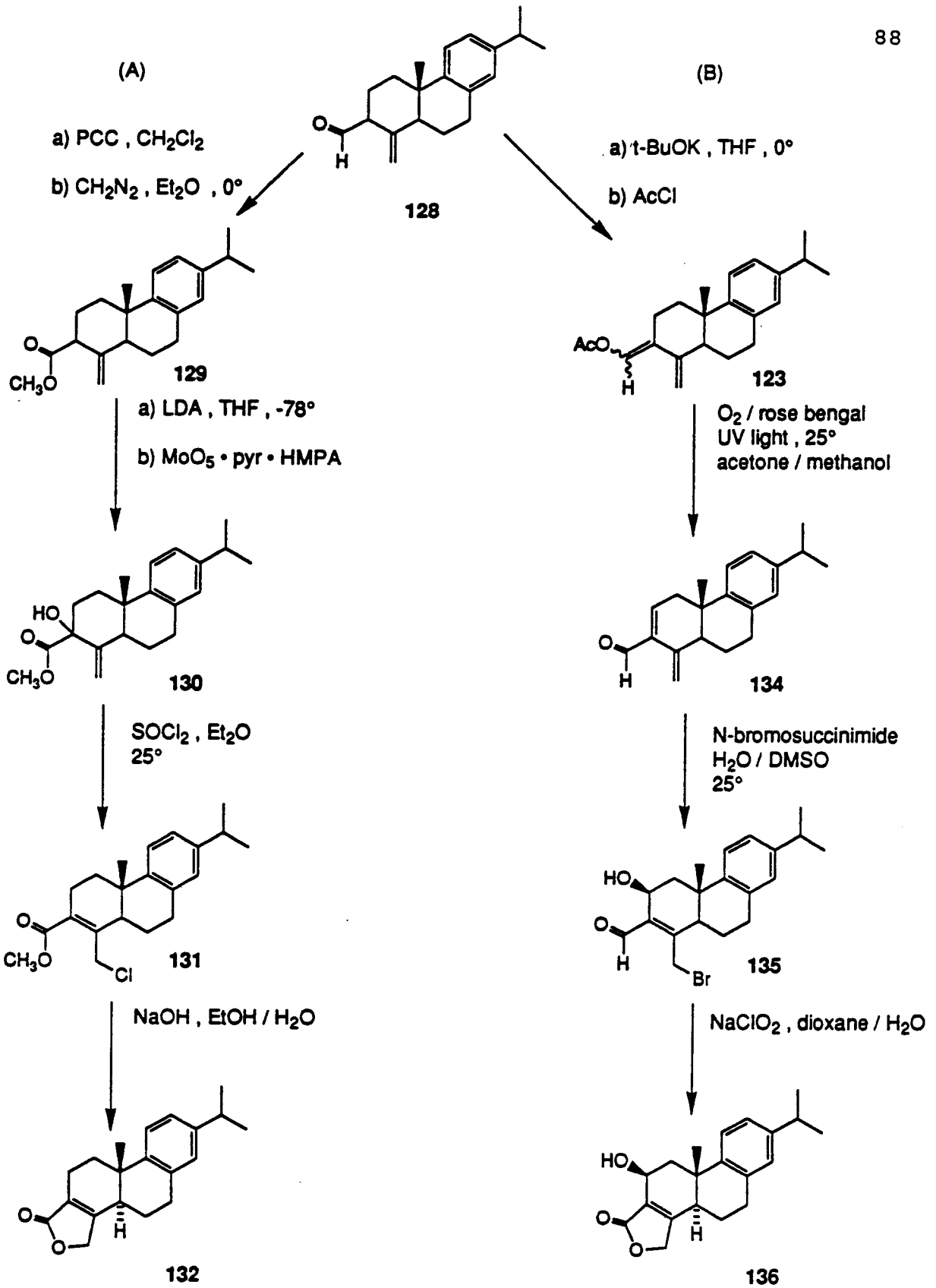
Scheme 25. Synthesis of 97 by Garver and van Tamelen.



Scheme 26. Triepoxide construction from laevopimaric acid (112) by Tokoroyama et al.



Scheme 27. Synthesis of the butenolide ring of tripdiolide and triptolide from dehydroabietic acid (122) by Tokoroyama et al.

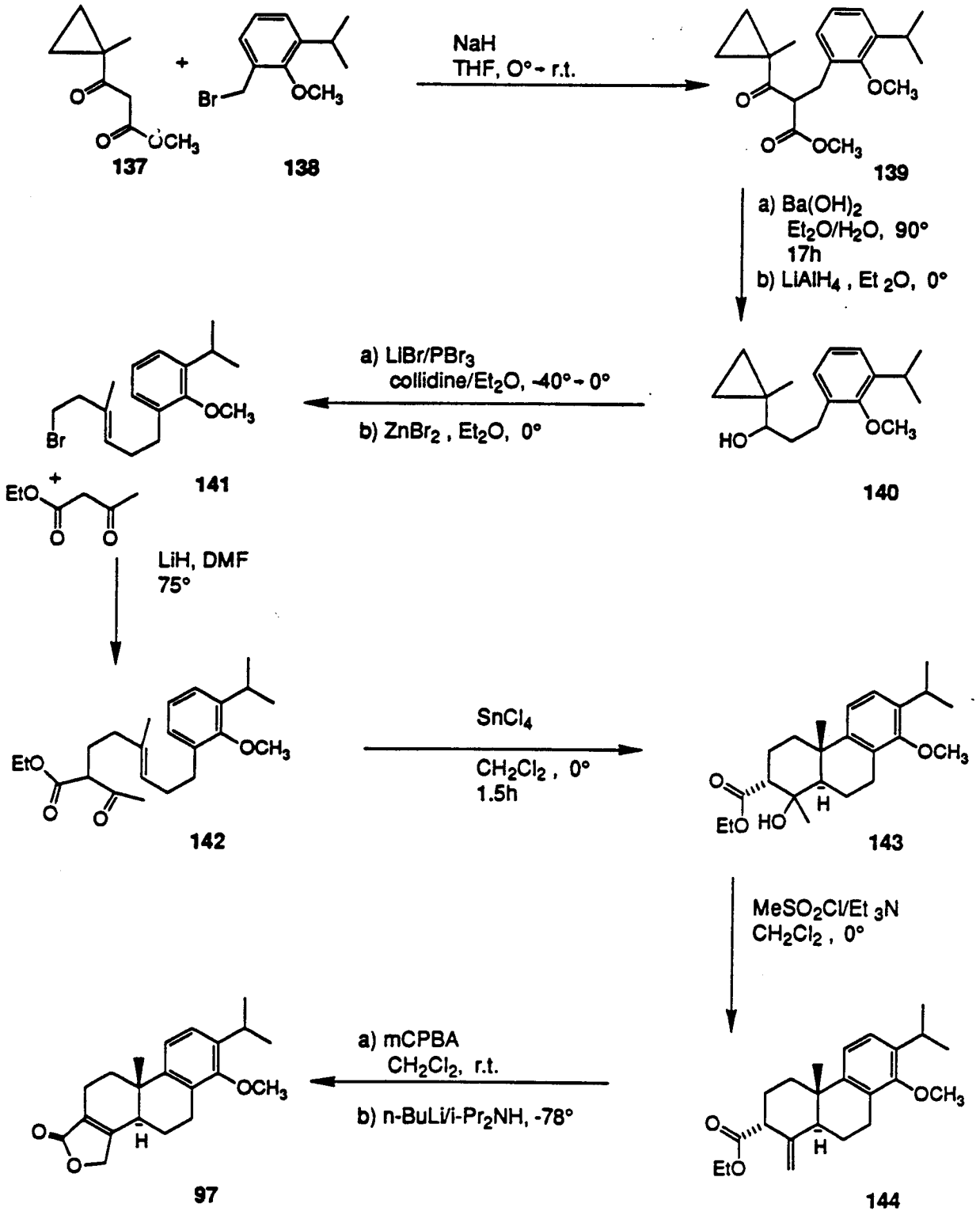


Scheme 27 - Continued.

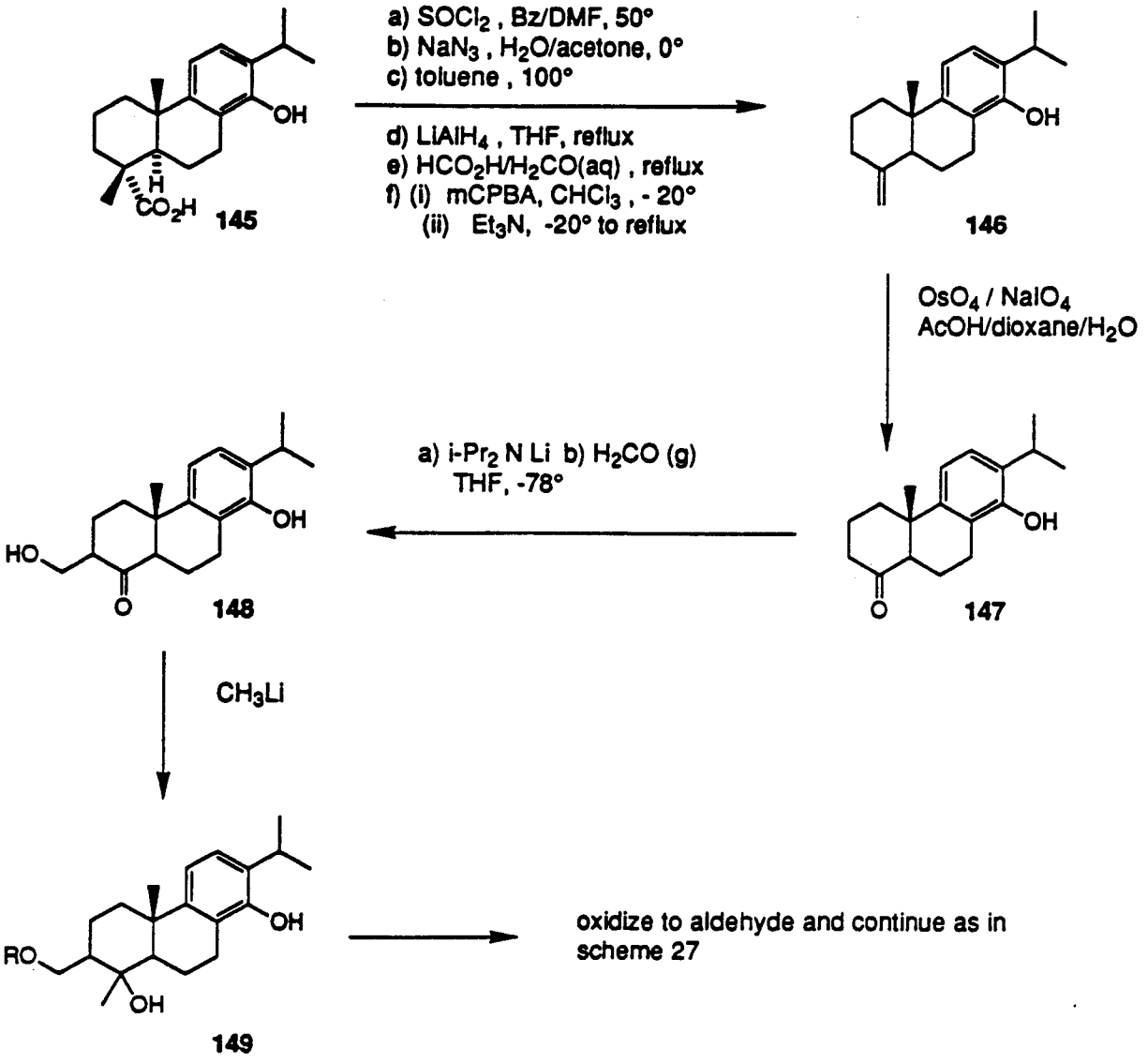
A synthesis of racemic butenolide **97** by van Tamelen and Leiden[108] is one of the most efficient yet designed (Scheme 28). It results in 15% yield after 12 steps with only four purifications required. It is also a biogenetic type synthesis modeled on the biosynthetic cyclization of geranylgeraniol. The key step is the cyclization of intermediate **142** which forms both A and B rings with the correct *trans* junction.

A chiral synthesis passing through the same intermediate **143** was carried out by van Tamelen et al[109], using dehydroabietic acid (**122**) as starting material. The starting material **145** (Scheme 28) is available from **122** via the sequence shown in Scheme 37 (see later). Hydrolysis of **189** in the latter scheme affords **145**.

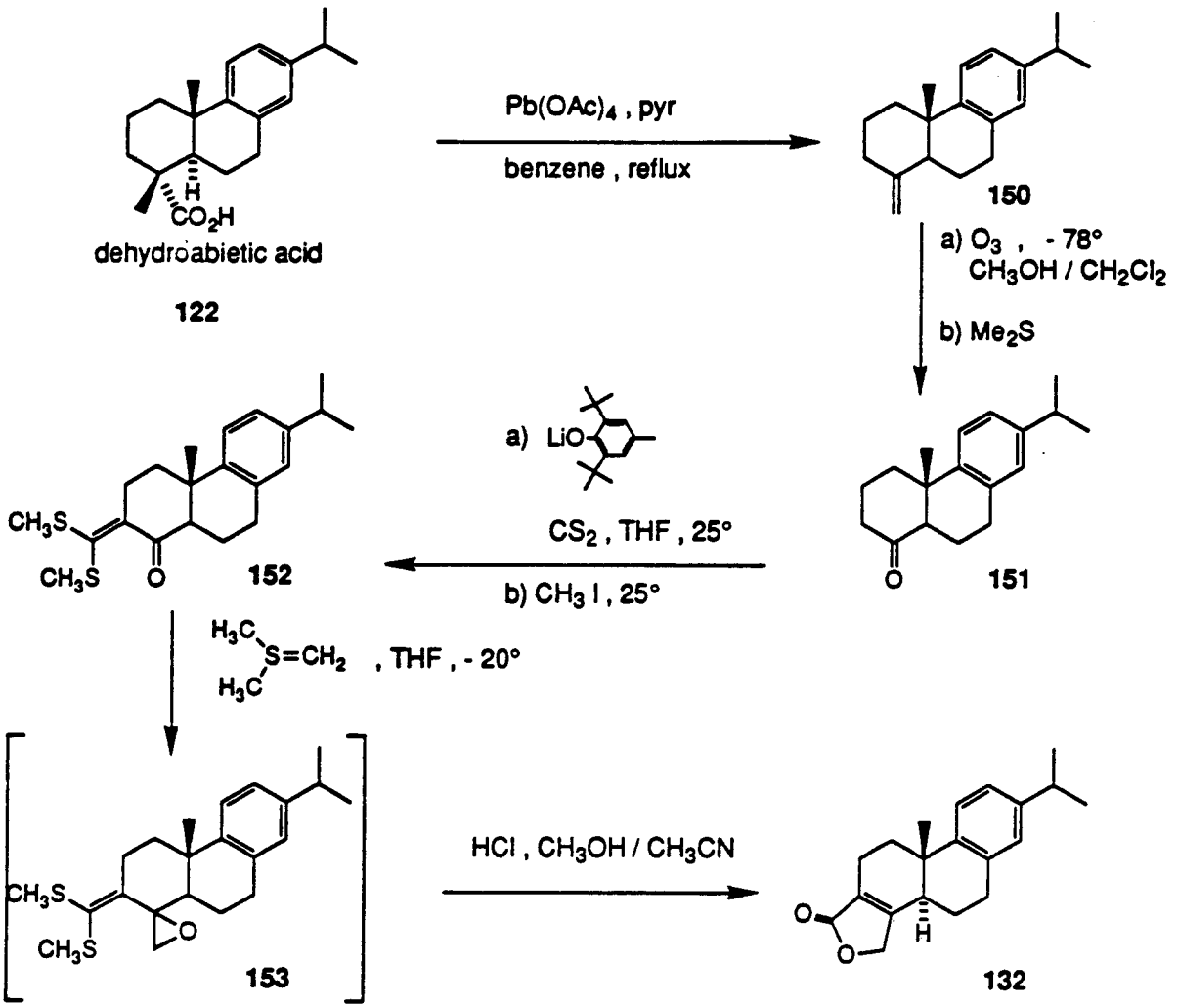
Malcolm Roberts[110] in our group, developed a synthetic route to dehydroisoabietanolide (**132**) from dehydroabietic acid (**122**). In his synthesis (Scheme 30), oxidative decarboxylation of dehydroabietic acid led in one step to the exocyclic olefin (**150**). Ozonolysis and condensation with carbon disulphide followed by methyl iodide quenching afforded ketene thioketal **152** in good yield. Dimethyl sulphonium methylide addition followed by acidic hydrolysis produced dehydroisoabietanolide (**132**) in a one pot reaction. This latter reaction involves intermediate epoxide **153** and provides for a short and efficient synthesis.



Scheme 28. Biogenetic-type synthesis of 97 by van Tamelen and Leiden.



Scheme 29. Chiral synthesis of 97 by van Tamelen et al.

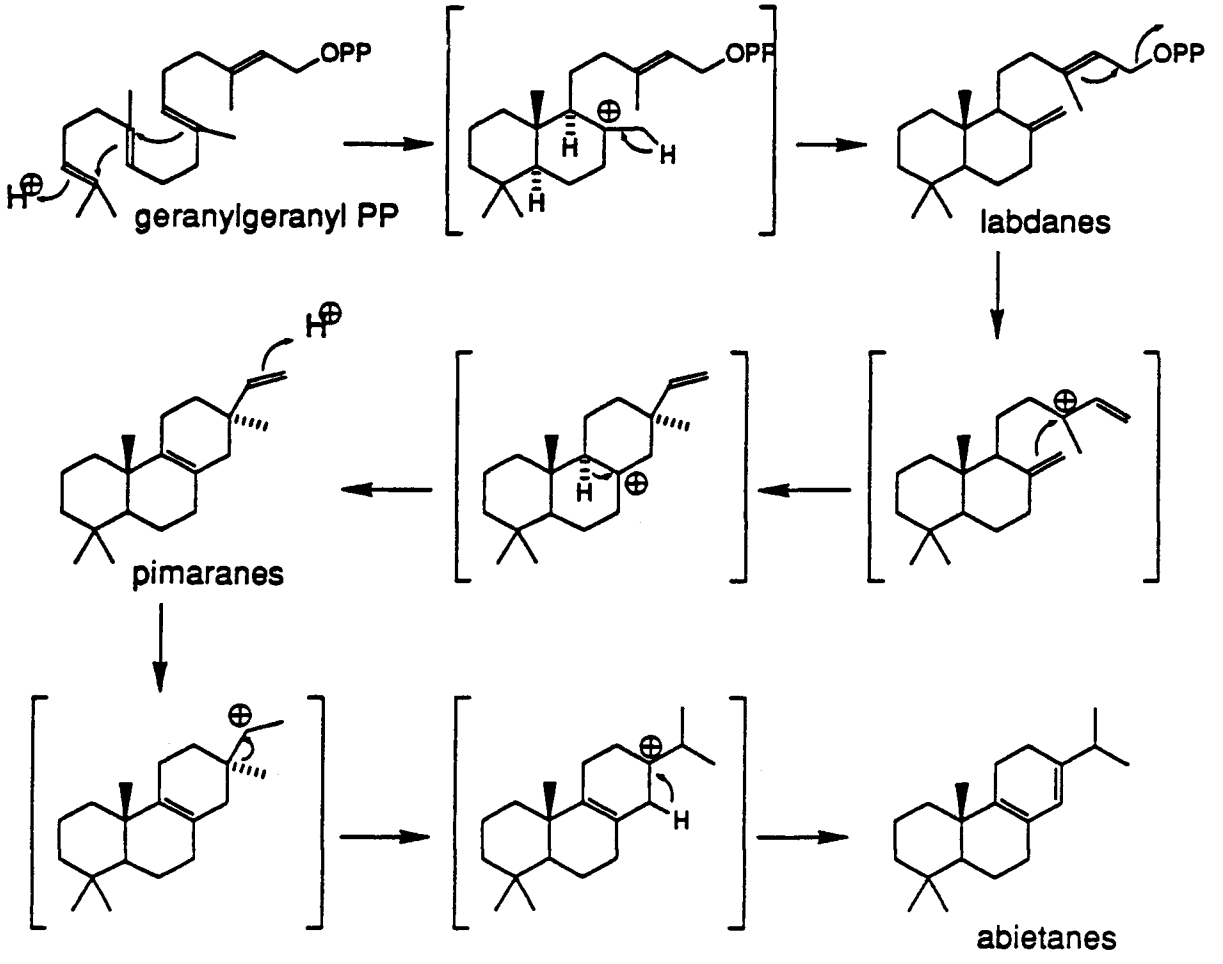


Scheme 30. Chiral synthesis of dehydroisoabietanolide (132) by Roberts.

ABIETANE AND *abeo*-ABIETANE BIOSYNTHESIS.

The biosynthesis of this class of diterpenes is believed to occur via geranylgeraniol (154) cyclization to the abietane type skeleton (Scheme 31). While most research has been directed to the understanding of the early stages of diterpene biosynthesis, it is the last transformations (butenolide and triepoxide formation) that are most relevant to our study.

The structures of the *abeo*-abietane butenolides and abietanes isolated from living plants of *Tripterygium sp.* are shown in Figure 16. Figure 16a shows the diterpenes isolated from *T. wilfordii* cell cultures. These compounds vary only in the



Scheme 31. Biosynthetic generation of the abietane skeleton.

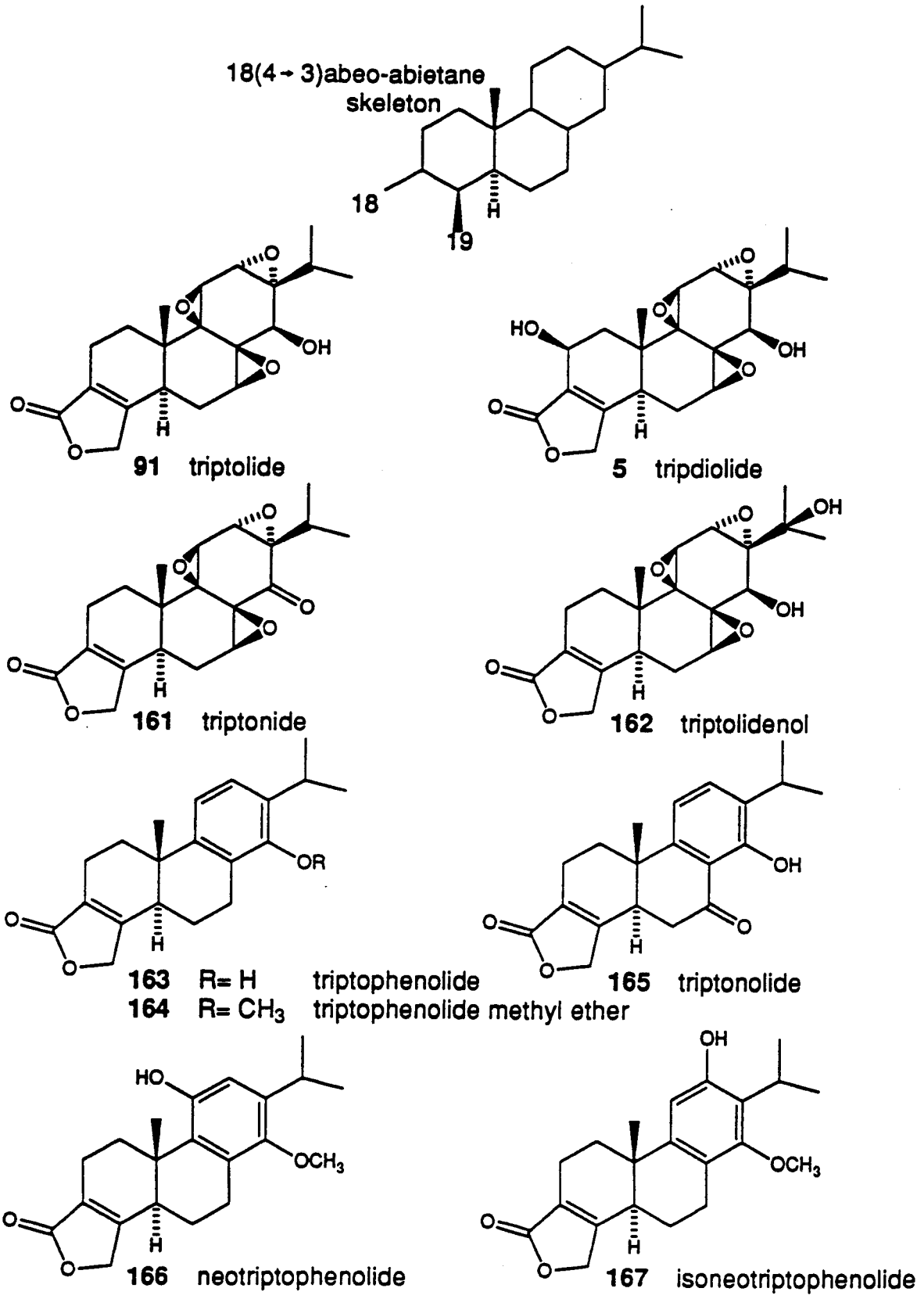
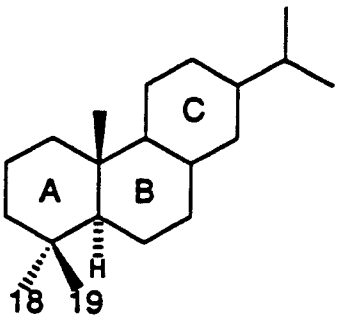
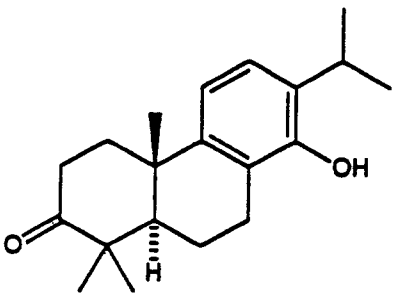


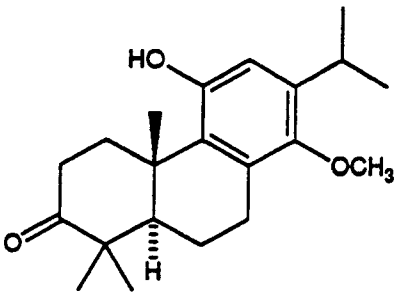
Figure 16. Diterpenes isolated from *Tripterygium* plants.



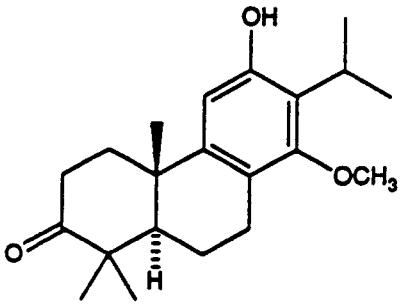
abietane skeleton



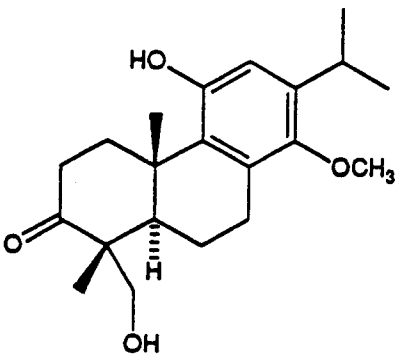
155 triptonoterpene (a)



156 triptonoterpene (b)



157 neotriptonoterpene



158 triptonodiol

Figure 16 - Continued.

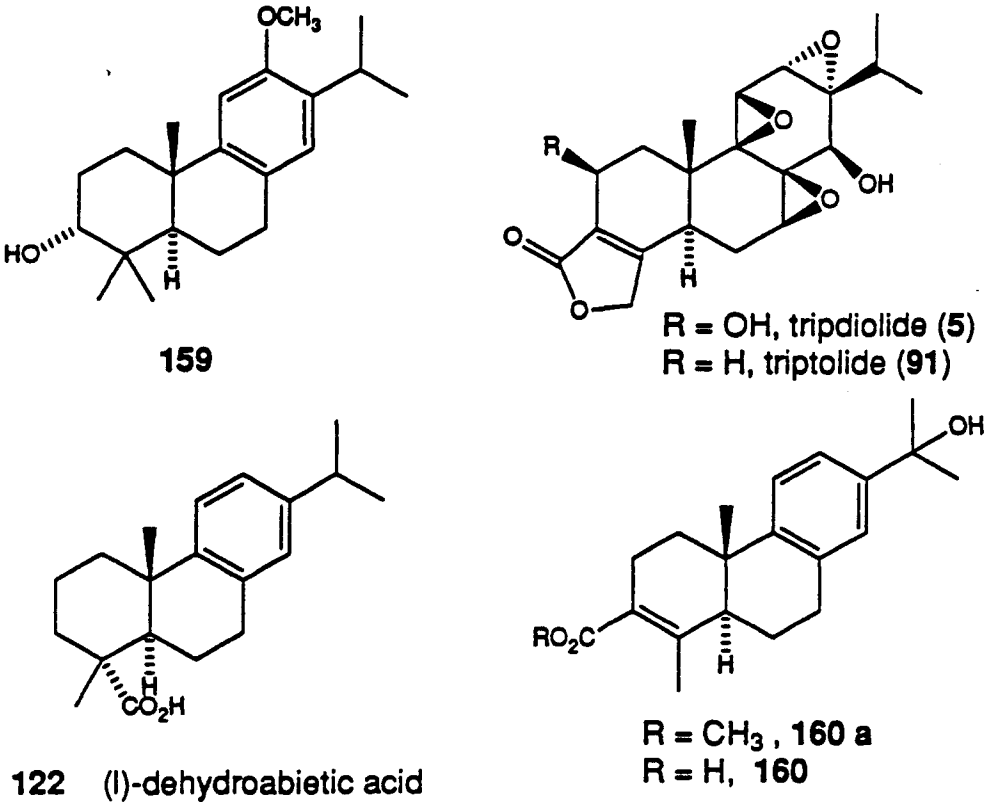
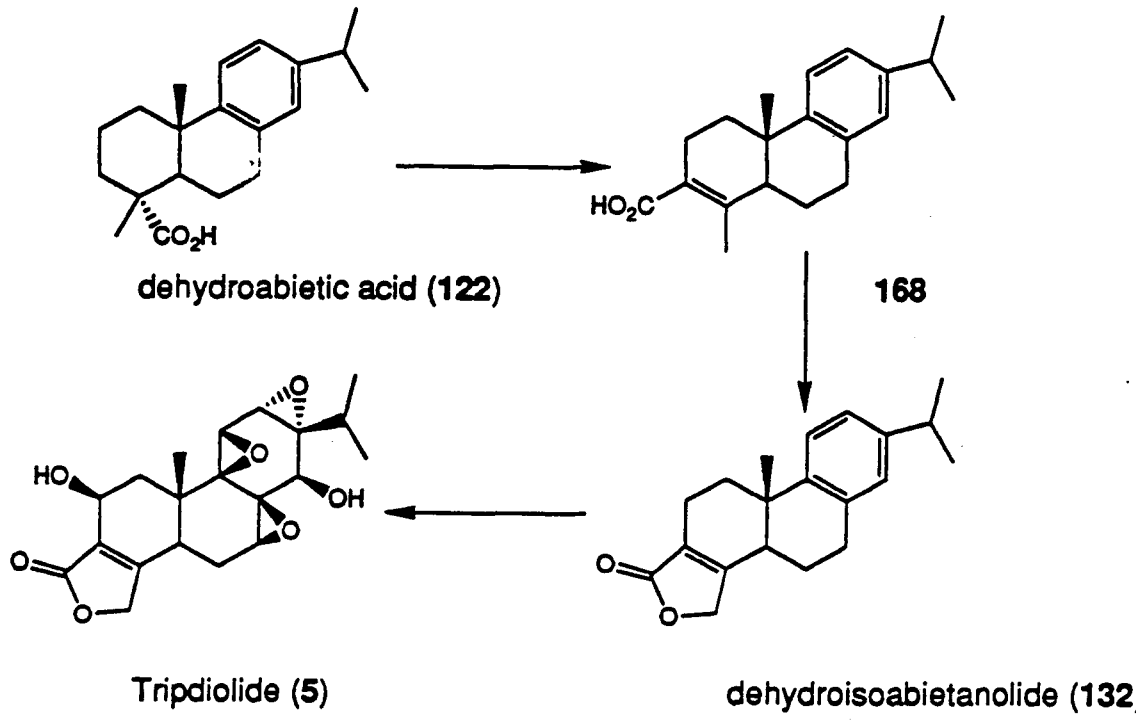


Figure 16a. Diterpenes isolated from *Tripterygium wilfordii* tissue cultures.

degree of oxidation and can provide some clues as to the order of events leading to tripdiolide (5). The isolation of dehydroabietic acid (122) and the hydroxy acid 160 from the cell cultures of *T. wilfordii* led to proposals of the biosynthetic pathway to tripdiolide (5)[111], in which dehydroabietic acid 122 and the acid 168 were implicated (Scheme 32).



Scheme 32. Proposed biosynthetic pathway to tripdiolide (5) via dehydroabietic acid (122).

Previously, chemically related precursors possessing an abietane-type structure have been synthesized in our laboratory in an effort to investigate the biosynthetic pathway subsequent to dehydroabietic acid (122). Biotransformation studies of dehydroabietic acid, as well as of the synthetic precursors 132 and 168-174, were carried out using *Tripterygium wilfordii* cell cultures[112] (Figure 17).

The most promising results were obtained by Malcolm Roberts using precursor 132[110], which showed considerable utilization by the cells to yield C2 and C7 oxidation products (Scheme 33).

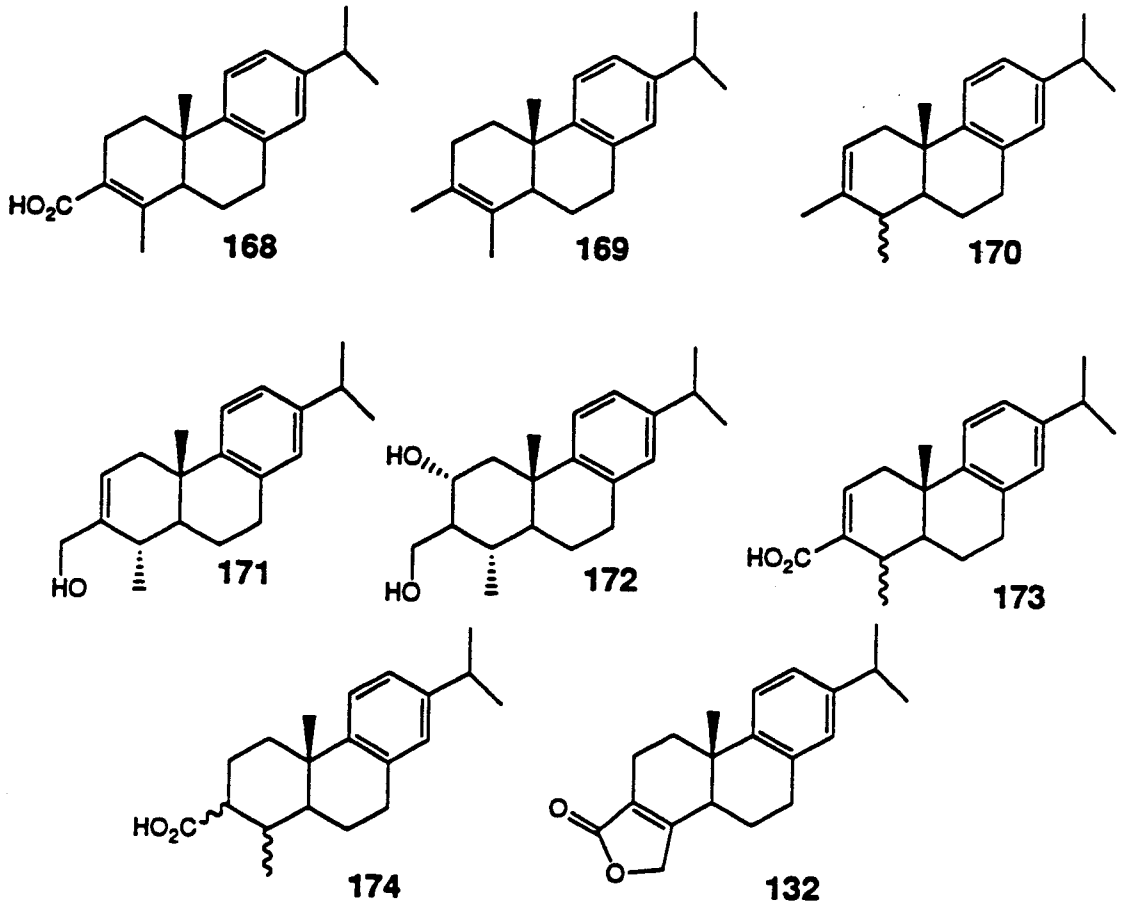
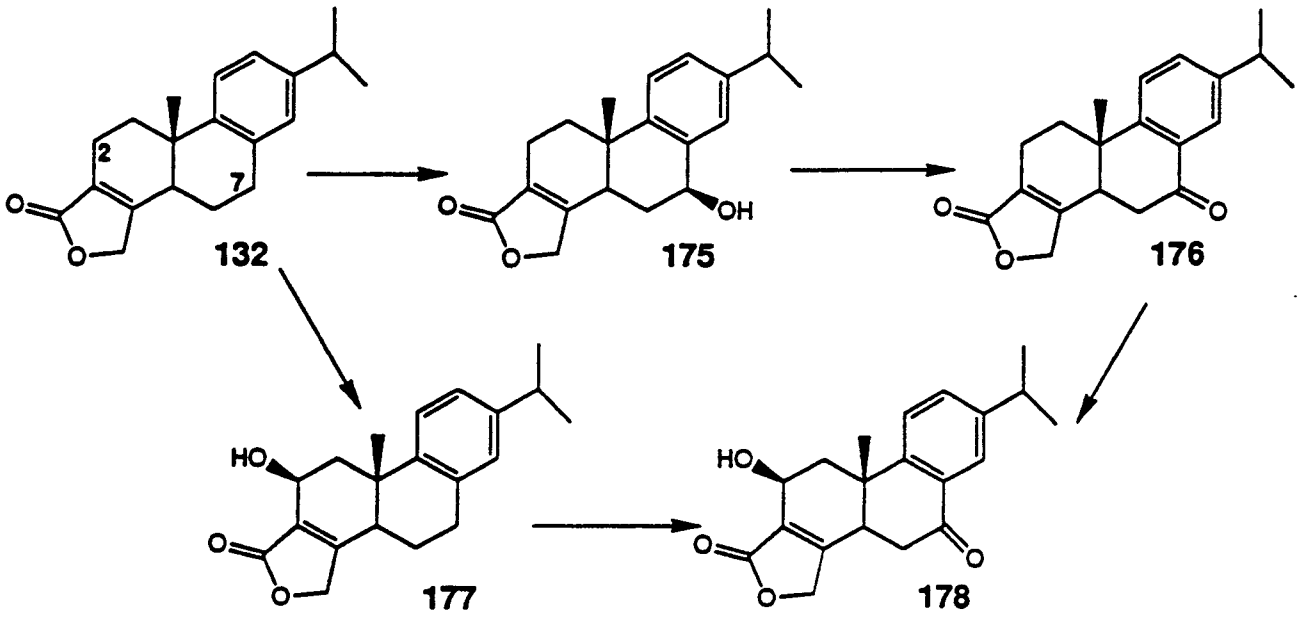
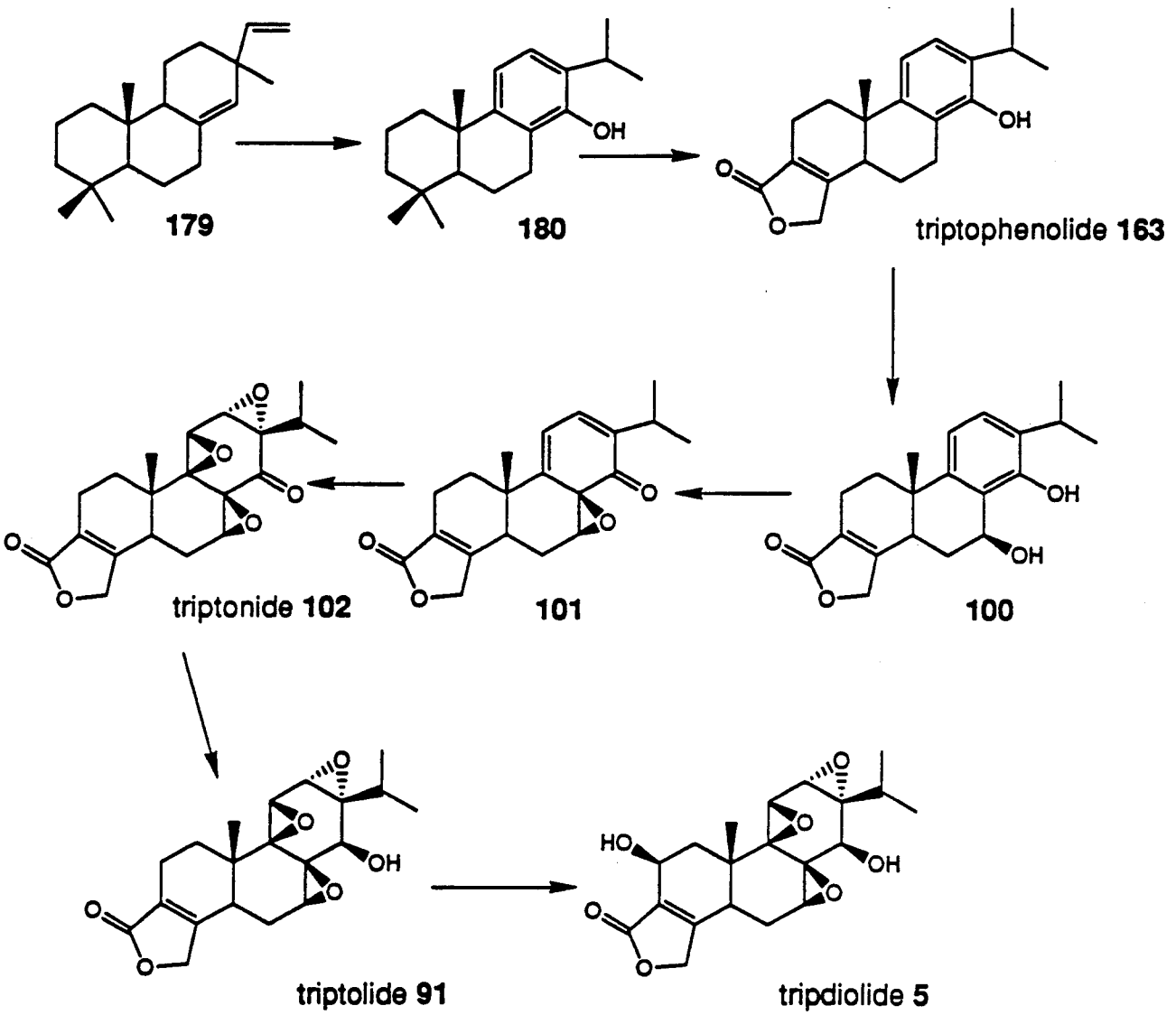


Figure 17. Synthetic precursors utilized in previous biotransformation studies in Prof. Kutney's group.



Scheme 33. Biotransformation of dehydroisoabietanolide (132) with *Tripterygium* cells.

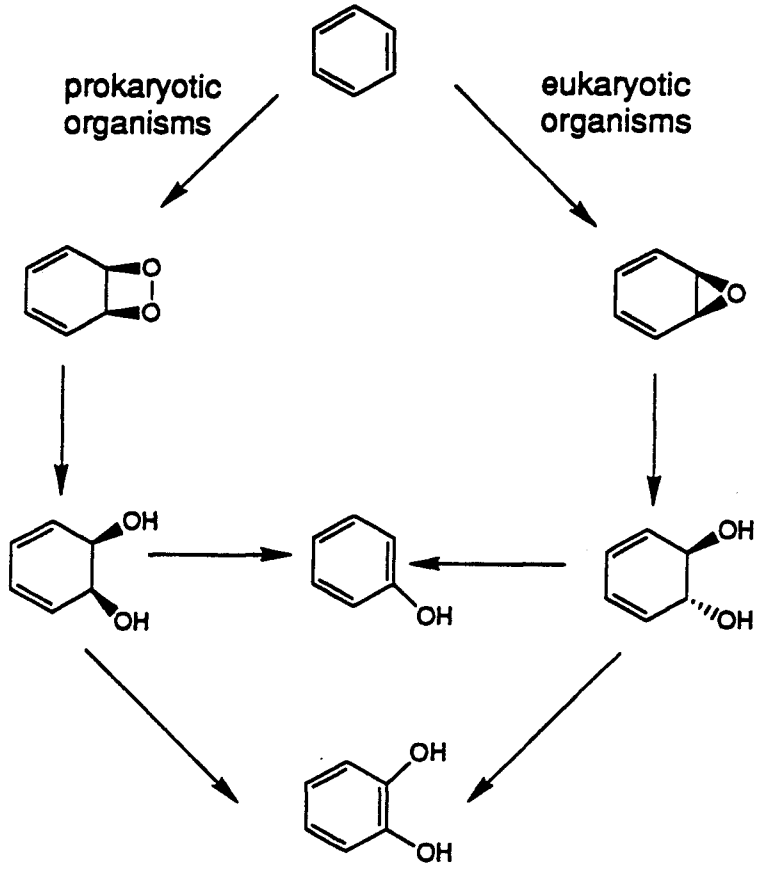
In light of these results, a new biosynthetic pathway was proposed from pimaradiene (179)[110] (Scheme 34). In this scheme, hydroxylation of ring C is



Scheme 34. Biosynthetic pathway to the diterpene triepoxides proposed by Roberts.

presumed as well as lactone ring formation before oxidation at C2 or C7 can occur. The hydroxylation of the aromatic ring seemed a requisite for the formation of the epoxide system. However, direct epoxidation of aromatic rings have been observed in some microorganisms (e.g. *Pseudomonas putida*)[113] to give the corresponding arene

oxides (Scheme 35). Eukaryotic organisms (fungi, yeasts, and higher organisms) utilize mono-oxygenases such as cytochrome P-450 to give these arene epoxides which can be hydrolyzed to a *trans* diol. Oxidation of this diol yields a cathecol, while loss of water results in the effective hydroxylation of the original aromatic ring. In contrast,



Scheme 35. Oxidation of benzene by prokaryotic and eukaryotic organisms.

prokaryotic organisms (bacteria) hydroxylate aromatic compounds by dioxygenase enzymes which catalyze a cycloaddition reaction with molecular oxygen to yield a dioxetane. This can be reduced to a *cis*-diol and/or cathecol.

Hydroxylation is often the initial step in the degradation of aromatic compounds by microorganisms in the environment, and by the liver. If a similar enzymatic system is present in the *T. wilfordii* cell culture, dehydroisoabietanolide

(132) would still be considered as a strong candidate for biotransformation studies. Although in Malcolm Roberts' work no incorporation into the diterpene triepoxide was obtained, changes in experimental parameters such as time of addition of the precursor, length of incubation, culture medium used, etc., might also yield the desired biotransformations.

Production of Tripdiolide (5) and Triptolide (91) by Plant Cell Tissue Cultures.

As part of the tissue culture program of our group, a cell suspension culture of *Tripterygium wilfordii* has been established[114].

Stem and leaf explants were obtained from *T. wilfordii* plants maintained under normal greenhouse conditions. Explants were placed on B5 and PRL-4 media (standard tissue culture medium developed by Gamborg and Eveleigh) solidified with Bacto-agar (8 g/L) and supplemented with numerous combinations of 2,4-dichlorophenoxyacetic acid (D), kinetin (K), 1-naphthaleneacetic acid (NA), indole-3-acetic acid (I), 6-benzylaminopurine (B), 4-aminobenzoic acid (P), and coconut milk (Co). The explants and resulting calli were incubated at room temperature in darkness. Many calli grew and were transferred to fresh media of the same or different composition. Preliminary selection of promising cell lines was based on growth vigor as well a qualitative thin-layer chromatography and cytotoxic activity analyses[114].

The cell line designated TRP4a was selected for further investigation after these screenings.

This cell line was initiated as a leaf explant on PRI_2Co_{100} (PRL-4 medium supplemented with indole-3-acetic acid (2 mg/L) and coconut milk (100 mL/L)), transferred to PRD_2Co_{100} agar (PRL-4 medium supplemented with 2,4-dichlorophenoxyacetic acid (2 mg/L) and coconut milk (100 mL/L)), and maintained in this medium.

Suspension cultures of TRP4a were generated in PRD_2Co_{100} medium and were maintained as stock cultures by regular subculture at 3-week intervals.

A rapid TLC assay of tripdiolide using fluorimetric detection that was accurate for tripdiolide concentrations of 0.2 to 3.6 μg was developed[115] and a detailed investigation in terms of tripdiolide production versus variations in growth conditions was done[116]. It was found advantageous to maintain TRP4a stock cultures in the rich PRD_2Co_{100} medium and encourage tripdiolide production by transferring into $MSNA_{0.5}K_{0.5}$ medium (MS medium supplemented with naphthaleneacetic acid (0.5 mg/L) and kinetin (0.5 mg/L)). Tripdiolide production was found to be at a level of 4.0 mg/L, or 36 times greater than that reported for the plant by Kupchan et al[99]. More recent investigations on the isolation and characterization of natural products from *T. wilfordii* plants led to the discovery of nine novel diterpenoids[117]. A review that summarizes the earlier studies with the TRP4a cell line of *T. wilfordii* is available[81] while more recent and detailed studies with TRP4a derived metabolites is described in the Ph.D. thesis of M. Roberts and in a recent publication[110].

In order to produce large amounts of pharmacologically active diterpene and triterpene natural products, a method was developed by Mijo Samija[118] in our group, where elicitation with a strain of the fungus *Botrytis* stimulated the production of oleanane and friedelane triterpene acids. An excellent review of the chemistry of the plant is also available in his thesis[118].

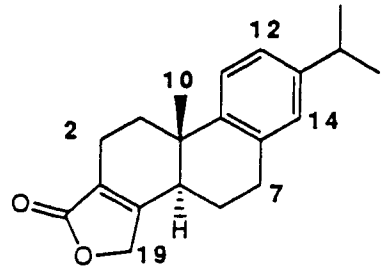
In order to derive additional information about the biosynthesis and production of tripdiolide and triptolide, a number of biotransformation experiments were envisaged. Such experiments would involve substrates possessing the abietane skeleton, at a lower oxidation level, and their incubation with the TRP4a cell line to evaluate their role, if any, in the production of the target compounds.

It is in this area that the present investigation was directed.

OBJECTIVES OF THIS INVESTIGATION.

1. Biotransformation of dehydroisoabietanolide (**132**) with cell free extracts (CFE, crude enzyme preparations) from the TRP4a cell line to provide a more direct entry into the C7 and/or C2 oxidation products, and if possible, an entry to the triepoxide system.

2. In the event that the crude enzyme preparations were incapable of significant biotransformation, synthesize a ring C activated precursor and evaluate its biotransformation by the TRP4a cell line. A likely candidate for this study would be the C12 or C14 hydroxy derivatives of **132**.



RESULTS AND DISCUSSION

Synthesis of Dehydroisoabietanolide (132).

At the time the present work was being done, studies in our laboratories were directed towards a short synthesis of dehydroisoabietanolide (132) to be used on our biotransformation studies. Shortly after my arrival at the University of British Columbia, Malcolm Roberts completed his synthesis of 132 and it was decided to use this sequence for our own investigations.

Minor changes to the original synthetic plan were done in order to optimize the yield of the sequence or to facilitate its repetition.

The starting material, dehydroabietic acid (122) is available in unlimited quantity at insignificant cost, is optically active, and incorporates most of the carbon skeleton of tripdiolide (5). The only disadvantage to starting 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. This became more apparent when ring C functionalization was attempted (*vide infra*). Consequently, the strategy for the synthesis was one of opportunism: to find out what would work, then attempt to exploit the results.

In the original synthesis of Roberts (Scheme 30), the exo-olefin 150 was produced by oxidative decarboxylation of 122 with lead tetraacetate yielding a mixture of the endo-, and exo-olefins, which requires careful chromatography on silver nitrate treated silica gel for the final purification (Figure 18). In our studies we followed a reported approach[119], involving the concerted elimination of an amine N-oxide in order to avoid the production of the isomeric olefins (Scheme 36). Thus, treatment of dehydroabietic acid with excess thionyl chloride and a catalytic amount of DMF in benzene, afforded the acid chloride. This crude product was treated with sodium azide in acetone and subsequent heating of the resulting acyl azide in toluene

effected the Curtius rearrangement to **183**. All of these reactions were conveniently monitored by IR spectroscopy. Reduction of the isocyanate with lithium

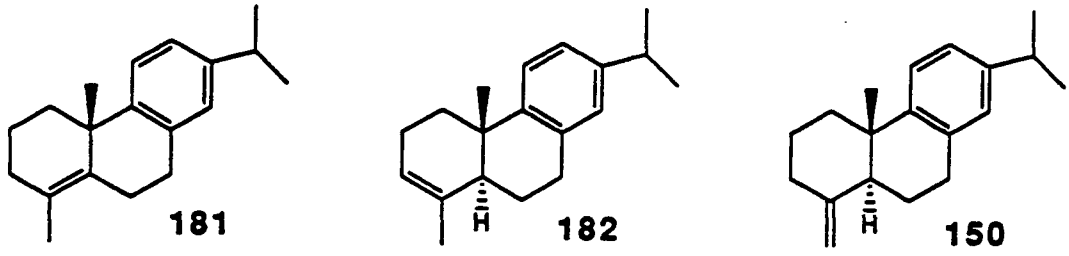
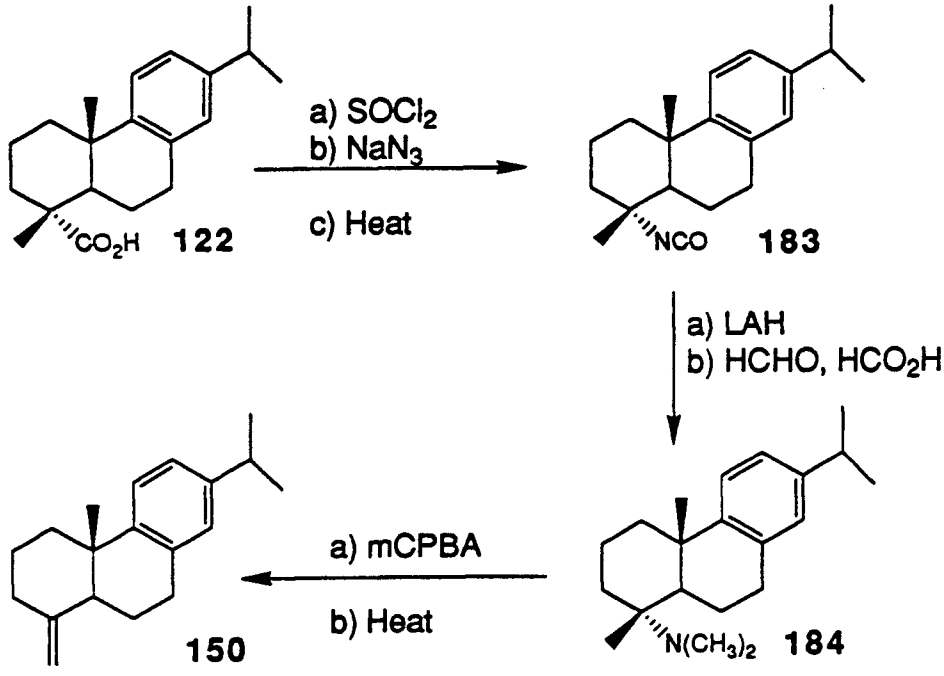


Figure 18. The isomeric olefins from the oxidative decarboxylation of dehydroabietic acid.



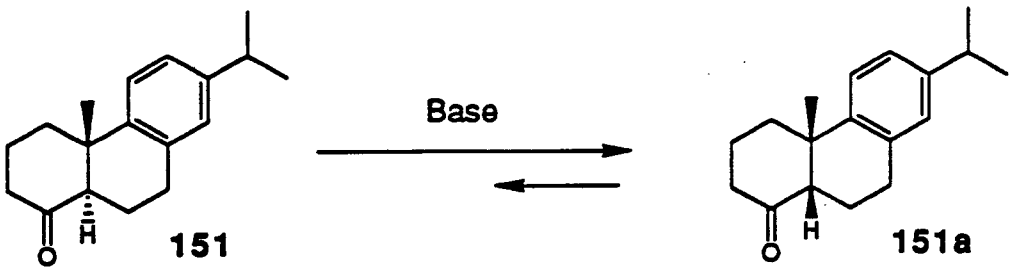
Scheme 36. Production of the exo-olefin **150** from dehydroabietic acid (**122**).

aluminum hydride proceeded in good yield to the secondary methyl amine, and the latter was then subjected to Eschweiler-Clarke methylation with aqueous formaldehyde in refluxing formic acid. The resulting tertiary amine was converted to the

corresponding amine oxide by treatment with *meta*-chloroperoxybenzoic acid at -20° C. After quenching the excess peracid with triethyl amine the reaction mixture was brought to reflux, at which point the elimination of dimethyl hydroxyamine took place. The isomerically pure product was readily isolated by column chromatography upon elution with hexanes. The overall yield was typically in the order of 70%.

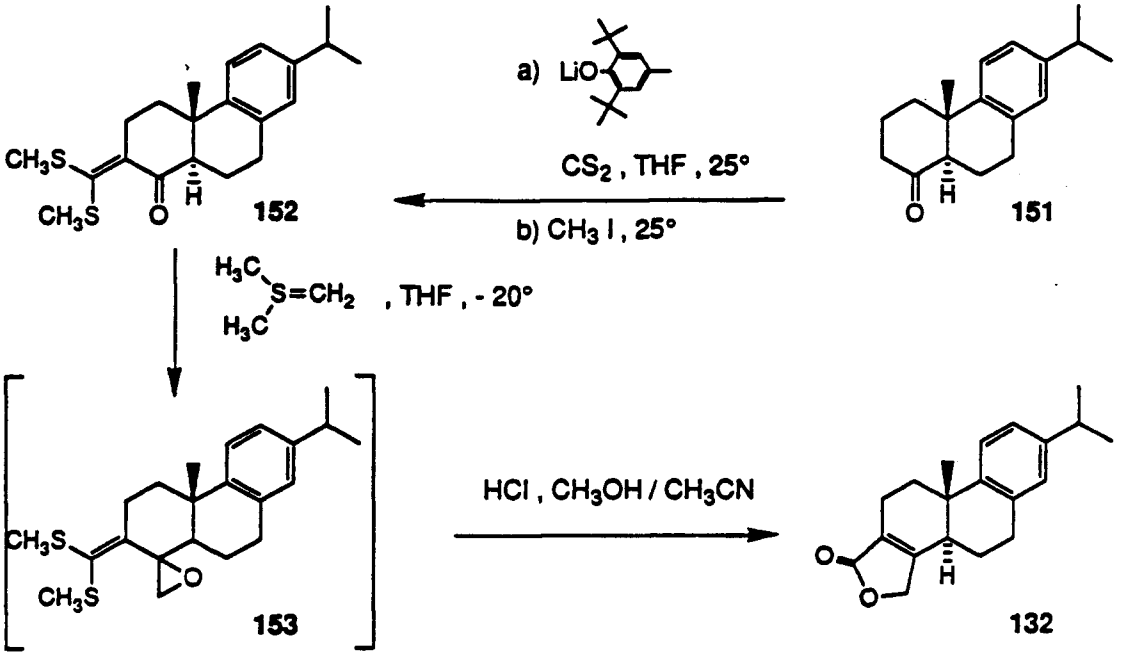
The direct production of the isocyanate **183** from dehydroabietic acid was also attempted. Treatment of **122** with diphenylphosphoryl azide[120] afforded poor yields of the isocyanate, and in view of the excellent results obtained with the Curtius sequence, this route was not investigated further.

The next step in the sequence was the ozonolysis of the exo-olefin to afford ketone **151**. This step was carried out using the same conditions reported by Roberts[110], that is, treatment of **150** with ozone in a 5:1 mixture of methanol:methylene chloride to afford an average yield of 90% of **151**. The ketone **151** is stable to mild acid, but is rapidly epimerized by base to a 3:1 mixture of the A/B-*cis* and A/B-*trans* ketones. The two ketones are inseparable by chromatography but can be readily distinguished by ^{1}H nmr spectroscopy. In general, it has been found[121] that there is a downfield shift of the C10 angular methyl signal of all A/B-*cis* compounds of this class, relative to the corresponding A/B-*trans* compounds. Typically, epimerization at C5 resulted in a 0.2 to 0.3 ppm downfield shift for the angular methyl signal. The sensitivity of the C5 stereochemistry in **151** required certain precautions in handling the ketone (Scheme 37). The product, however, was sufficiently stable to be stored as a solid in the refrigerator for several weeks.



Scheme 37. The epimerization of C5 in ketone 151.

The attachment of a C3 substituent (later to become C18) was thoroughly explored by van Tamelen[109]; the ketone failed to add carbon dioxide, ethyl-, methyl-, or phenyl formates. Malcolm Roberts[110] found that the alkylation with carbon disulphide followed by methyl iodide quenching resulted in a near quantitative yield of the α-oxo ketone dithioketal **152**. The synthetic possibilities of this class of compounds were first foreseen by Corey[122] who also used the lithium salt of 4-methyl-2,6-di-*t*-butylphenol as a sterically shielded base which would not attack an electrophile, but would deprotonate the substrate. In our case, the base did not epimerize C5 as shown from the angular methyl position of δ 1.1 ppm in the ^{1}H nmr spectrum of the ketene-dithioketal **152** when compared to δ 1.06 ppm for the corresponding signal in the *trans*-fused ketone **151**. Product **152** could be crystallized from isopropyl alcohol. The final transformation (Scheme 38) involved a one pot conversion of the dithioketal **152** to the lactone **132** via the epoxide **153**. Treatment of **152** with dimethylsulphonium methylide in THF presumably gave a mixture of epoxides, which was hydrolysed without purification to give directly dehydroisoabietanolide (**132**). The yield of this reaction tended to vary and after an investigation of different hydrolytic conditions an optimized yield of 73% could be achieved. The lactone was an oil which slowly solidified to a white mass and could be crystallized from ethanol (Mp = 97-99^0 C). The product presented in the



Scheme 38. The synthesis of dehydroisoabietanolide (132) from ketone 151.

IR spectrum, absorptions at 1757 (lactone) and 1678 (unsaturation) cm^{-1}. In the ^{1}H nmr spectrum, the signal for the angular methyl is located at δ 1.02 ppm, while the lactone methylene (C19) protons are positioned at δ 4.78 ppm. The aromatic protons appear at δ 6.99 (brs, H14), 7.06 (brd, H12), and 7.27 ppm (d, H11). The lactone presents an absorption maximum at 221 nm in the UV spectrum and a molecular ion at m/z 296 in electron impact mass spectroscopy. Having this precursor in hand, we turned our attention to biotransformation experiments.

Dehydroisoabietanolide (132) Biotransformation with Cell Free Extracts (CFE) derived from the TRP4a Cell Line.

From the work of Malcolm Roberts, we knew that the TRP4a cells would oxidize the lactone 132 at positions 7 and 2, when incubated for a long period of time.

We now wished to establish a CFE capable of effecting the same transformation, and if possible, evaluate the oxidation of ring C.

A cell free extract is prepared after cell disintegration, by removing, via centrifugation, insoluble material. Before centrifuging, the mixture is described as an homogenate; after centrifugation as much as possible of the enzyme should be present in the supernatant.

There are many methods for cellular disintegration, and there are many types of cells. Most cells have particular characteristics which need special attention during disintegration. Plant cells are generally more difficult to disrupt than animal cells because of the cellulosic cell wall. Plant cells are highly compartmentalized; in most cases, there is a large vacuolar space which can be filled with proteases or other compounds, and the chloroplasts, starch granules and other organelles occupy much of the cytoplasmic space. On occasion, the desired enzyme is present in an organelle, and in such instances one can either isolate the organelle or do a complete tissue disruption. If the cells are not broken properly, an otherwise excellent source might be overlooked because much of the activity was not released but removed with the residue while preparing the extract.

The particular problem of marked changes in enzyme composition during different growth phases must be carefully studied. Preliminary studies on the cell culture should be carried out to determine what physiological state contains the highest concentration of the enzyme required.

In this work, *Tripterygium wilfordii* cell suspension cultures were grown in shake flasks in the appropriate medium for the required period of time. A flask was harvested after every two days of culture, and pH and RI were measured, and the microscopic purity was evaluated. The contents of the flasks were filtered through Mira-cloth in a Buchner funnel attached to a water aspirator to allow thorough draining and the resultant cell mass was washed with distilled water. The cells were

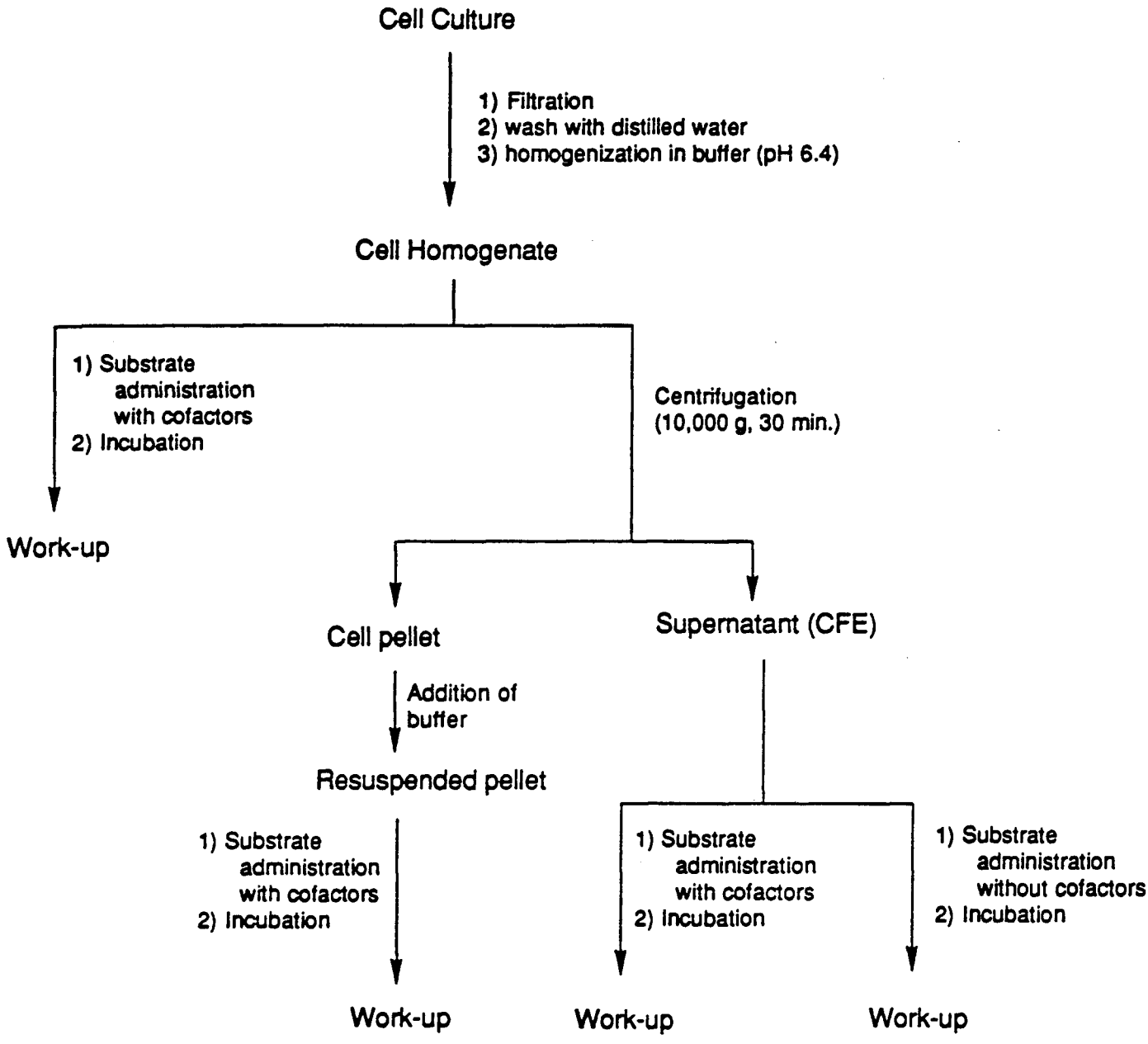
taken into the cold room (4° C), mixed in a plastic container with 180 mL of 0.02 M phosphate buffer (pH = 6.4), homogenized with an Ultra Turrax T-25 disperser for 30 sec, allowed to stand for an additional 30 sec., and homogenized again for 30 sec.

The homogenization was repeated three times, the resulting homogenate was transferred to plastic centrifuge flasks and centrifuged at 10,000 g for 30 min (T = 4° C). The supernatant (CFE) thus obtained was decanted, and peroxidase activity and protein content were measured.

For these determinations, the Bio-Rad protein assay[128] was used for the measurement of protein concentration and peroxidase activity was evaluated by the pyrogallol-purpurogallin assay[129] method. (For details, see Experimental.) Biomass (dry) was also determined for every harvested flask.

A number of biotransformation experiments using cells at different ages were performed (Scheme 39). For each experiment, a number of flasks were set adding 22 peroxidase units of the CFE to a mixture of the lactone 132 (10 mg) dissolved in ethanol (2 mL), 15 mL of distilled water, 35 mL of phosphate buffer (pH = 6.4), and 2.6 equivalents of a 0.5% solution of hydrogen peroxide.

Experiments were also carried out to assess membrane bound enzymes and cofactors (hydrogen peroxide, flavine mononucleotide, manganese dichloride). Biotransformations using the cell homogenate, the resuspended pellet, and the cell free extract were carried out with and without the cofactors. A control flask was set with everything except the CFE. The flasks were stirred at room temperature for different periods of time (typically 30 min, 2 h, and overnight), and harvested adding 25 mL of ethyl acetate. The resulting emulsion was quickly filtered through a short pad of Celite 545 in a fritted glass filter funnel and the Celite was washed with more ethyl acetate (25 mL). The filtrate was extracted twice with ethyl acetate (25 mL each time) and the Celite was sonicated for 20 min with enough ethyl acetate to cover it



Scheme 39. Preparation of the cell homogenate, resuspended pellet and CFE, and biotransformation.

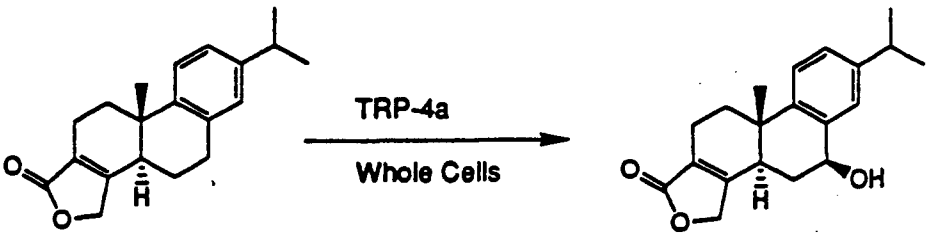
completely, then filtered and rinsed. The combined organic extracts were dried, filtered and rotary evaporated to dryness.

TLC (toluene:ethyl acetate 4:1 and chloroform:methanol:acetic acid 95:5:1) and HPLC (reverse phase, water:methanol 25:75) analyses were carried out on each sample. The results indicated no oxidation of the lactone **132**, and the substrate was recovered in essentially quantitative yield (97-98% recovery). The lack of oxidation products suggested either that aromatic ring activation is necessary prior to biotransformation, or that the relevant enzymes are extracellular or are destroyed during the CFE preparation.

Several experiments following the podophyllotoxin line of research made by Jan Palaty and using the AC3 cell line of *Catharanthus roseus* for biotransformation of 132 were done. In other studies, this cell line had demonstrated high peroxidase activity and the capacity to oxidize structurally diverse substrates, but biotransformation was not detected in our case.

Dehydroisoabietanolide (132) Biotransformation with TRP4a Cells.

During preparation of CFE, some enzymatic activity may have been lost so experiments were also conducted using whole cells of the TRP4a cell line to verify the ability, if any, to biotransform **132**. Experiments with cells of different ages (0, 7, and 10 days old) and different incubation periods (24, 48, 72 and 92 h) were qualitatively analyzed by TLC. The results indicated that, although the enzymes responsible for C7 oxidation, that is, conversion of **132** to **175** (Scheme 40), are present in the early stage of the culture, older cells and longer incubation times give better yields (i.e. a higher biomass:precursor ratio favours the conversion). Experiments with CFE prepared from the same batch of cells resulted in the recovery of only the substrate, therefore, indicating that indeed, "hydroxylase" activity is being lost during CFE preparation.



132 175

Scheme 40. The biotransformation of dehydroisoabietanolide 132 into 175 by *T. wilfordii* cell suspension culture.

It was clear at this point that CFE technology would not be useful for our purposes and, since whole cells produced relatively uninteresting results in the above studies, our attention was directed into functionalization of other sites of the substrate molecule (see later).

Dehydroisoabietanolide (132) Oxidation with *Mortierella isabellina*.

While the above studies were underway, consideration was given to the possibility of obtaining C2 hydroxylated analogues of 132 by means of microbial transformation. If success could be achieved, such substrates may prove of interest in future biotransformation studies with TRP4a, particularly since tripdiolide possesses a C2-hydroxyl function. The fungus, *Mortierella isabellina*, was selected after screening various microorganisms, since it consistently and selectively hydroxylates resin acids at C2 under appropriate conditions[123].

An experiment with this fungus was carried out using five Erlenmeyer flasks, each containing 50 mL of DYE (Dextro yeast extract) medium and 5 mg of the lactone 132 in 0.5 mL of ethanol. Addition was made at time zero (i.e. at inoculation time) and the cultures grown in a rotary shaker at 30^{o} C and 200 rpm for 4, 8, 24, 48, and 56 h. The cultures were harvested to obtain samples for analysis. The results

indicated the production of a new metabolite, more polar than the precursor, as well as almost complete consumption of the starting material after 48 h of incubation.

The new spot on TLC was not present in a culture grown under the same conditions. Microscopic inspection of the culture treated with the substrate, showed significant growth inhibition, where practically all cells remained in the lag phase.

The product was isolated in 37% yield and characterized as the C7-β-hydroxyl derivative **175**. All spectroscopic data were identical to those reported previously by M. Roberts[110]. No attempt to improve this yield was pursued.

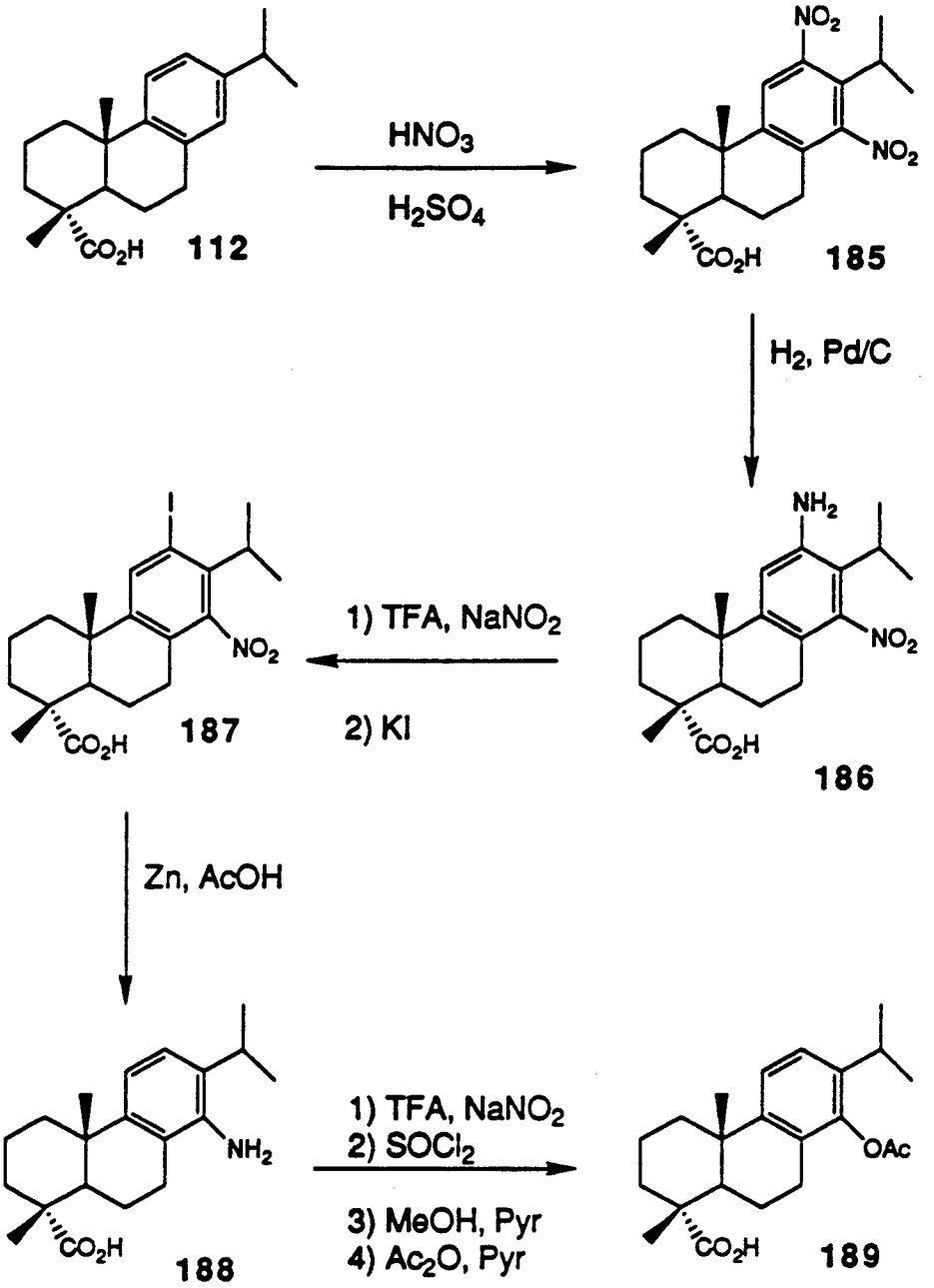
All these results made clear to us that further "activation" of the aromatic ring in **132** was required. Perhaps, a ring C hydroxylated compound would be an interesting candidate based on the previously proposed biosynthetic pathway (see, for example, **163** in Scheme 34).

The Synthesis of a Ring C-activated Substrate.

A search of the literature revealed only two reactions which proceeded efficiently on the C ring of dehydroabietic acid: acetylation at C12[124] and dinitration at C12 and C14[125]. We did not enjoy the luxury of flexibility in the substitution pattern of the aromatic nucleus, and this loss of synthetic flexibility caused considerable difficulty in this portion of the project.

It was felt that the ideal precursor would be the C14 hydroxy derivative triptophenolide (**163**) since the plant cell probably effects the biological equivalent to Alder's oxidation of *ortho*-hydroxymethyl phenols with periodate as utilized by Berchtold[104] in his synthesis of triptolide (Scheme 24, *vide supra*).

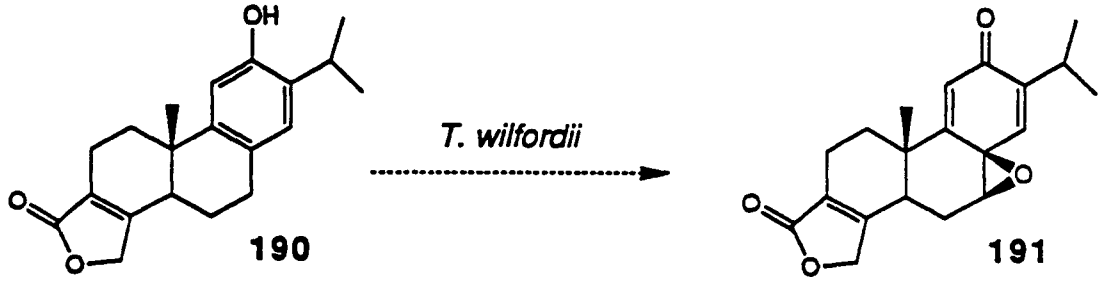
Published routes to 14-hydroxy-dehydroabietic acid are lengthy and based on dinitration, reduction and diazotizations reactions (Scheme 41)[121]. We speculated that the butenolide functionality may not endure the reaction conditions involved in this process. Problems were also anticipated for the synthesis of the lactone from 14-



Scheme 41. Synthesis of 14-acetoxydehydroabietic acid (189) by Demers.

hydroxy-dehydroabietic acid and, therefore, it was decided to proceed initially with a synthesis of the C12 hydroxy derivative, isotriptophenolide (190).

We hoped that this precursor would be transformed by the cell culture derived enzyme system into the corresponding epoxy-dienone (191) via benzylic hydroxylation and subsequent 1,4-addition (Scheme 42), or even further along the biosynthetic pathway of triptolide to generate novel analogues of the triepoxide system. The latter compounds could be very useful in providing a "family" of diterpene epoxides for pharmacological screening within the areas of contraception, immunosuppression, etc. For this reason an efficient synthesis of the hydroxy lactone 190 was pursued.

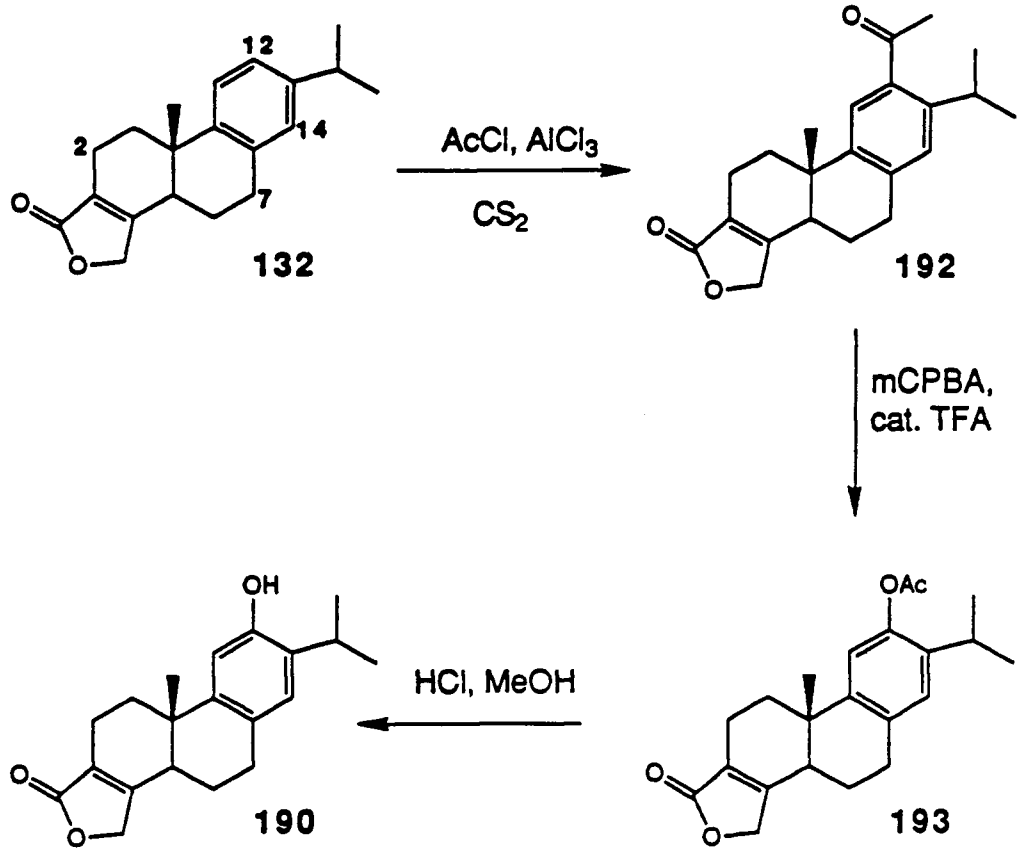


Scheme 42. The proposed biotransformation of substate 190.

The synthesis of substrate 190 involved Friedel-Crafts acylation of dehydroisoabietanolide (132), followed by Baeyer-Villiger oxidation and hydrolysis of the resulting ester to the desired phenol (Scheme 43).

Dehydroisoabietanolide (132) and acetyl chloride in carbon disulphide were added to aluminum chloride in anhydrous carbon disulphide as solvent. After overnight reflux, the solvent was removed and the residue treated with a cold (ice) aqueous solution of hydrochloric acid. Workup of the hydrolyzed mixture afforded the 12-acetyl derivative 192 and none of the isomeric 14-acetyl derivative. This compound could be purified by crystallization from methanol (68% yield) to give the pure product as white crystals. The product showed two carbonyl bands in the IR

spectrum at 1751 and 1669 cm^{-1}, and in the ^1H nmr spectrum, a new methyl singlet appeared at δ 2.58 ppm. The C14 and C11 aromatic protons were present as singlets at δ 7.15 and 7.47 ppm respectively. The Baeyer-Villiger oxidation was performed under the conditions reported by Chamberlin and Canan Koch in 1989[126]. They reported the use of *meta*-chloroperoxybenzoic acid catalyzed by trifluoroacetic acid as a simple and effective means of effecting the reaction. In our case, the reaction proceeded in almost quantitative yield. The reaction took usually 20 h to completion, and was best monitored by TLC utilizing isopropyl ether as eluting solvent. Also this was the only solvent found capable of resolving the starting material from the product. In the IR spectrum of **193**, the carbonyl band for the acetate appears at 1680 cm^{-1}, and in the ^1H nmr spectrum, the methyl singlet is centered at δ 2.33 ppm



Scheme 43. Synthesis of substrate **190** from dehydroisoabietanolide (**132**).

while the singlets for the aromatic protons at positions C14 and C11 have moved upfield to δ 6.96 and 7.06 ppm respectively.

The final hydrolysis proved to be sensitive to basic conditions since coloured impurities were noted, and was thus best performed under acidic conditions. A reported selective hydrolysis of aryl acetates with p-toluenesulfonic acid adsorbed on silica gel[127] proved ineffective but when the acetate was treated with concentrated hydrochloric acid in methanol, the phenol **190** could be isolated in 95% yield after crystallization from acetone-water. The product showed IR bands at 3320 (hydroxyl) and 1748 (lactone carbonyl) cm^{-1}, and in ^1H nmr spectrum, the aromatic protons were present as singlets at δ 6.72 (C14) and 6.91 (C11) ppm. We were now in a position to test the biotransformation of this "activated" substrate with *T. wilfordii* cell cultures.

Isotriptophenolide (190) Biotransformation with TRP4a Cells.

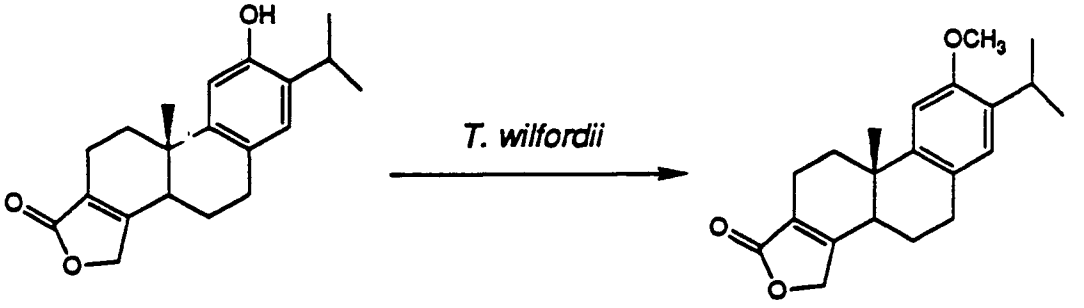
Preliminary studies on the biotransformation of isotriptophenolide (**190**) were done using whole cells since the likelihood of success is greater in this type of experiment. Several experiments were carried out using the TRP4a cell line.

An initial experiment of incubating **190** with TRP4a cells grown in $MSNA_{0.5}K_{0.5}$ medium for 7 days was performed. A solution of the phenol **190** (50 mg) in ethanol was added to the cultures (1 L) in shake flasks and the culture was incubated for 7 days. Even though analytical TLC of the reaction mixture still showed a considerable quantity of starting material remaining, further incubation was not considered in order to avoid low recovery of products - a situation encountered in the podophyllotoxin experiments, where overoxidation of the substrate was consistently observed. The flasks were harvested by homogenizing cells and broth together, and extracting the mixture with ethyl acetate in accord with the general procedure developed in the first part of this thesis. TLC analysis of the crude extract

did not show a significant difference between the control flask and the biotransformation experiment except for a very faint spot slightly less polar than the starting material.

The crude extract was purified by column chromatography with gradient elution (hexanes:ethyl acetate 8:2 - to hexanes: ethyl acetate 2:8) and the polar material was finally removed with ethyl acetate:methanol 1:1. The fractions containing the new spot and the recovered substrate were further purified by preparative thin layer chromatography eluting with hexanes:ethyl acetate 7:3 to afford 38.0 mg (76%) of recovered substrate and 4.3 mg (8%) of a new metabolite, the latter obtained as a yellowish solid.

The new product was characterized as the C12 methyl ether (194). The ^{1}H nmr spectrum was very similar to the phenol 190 but the signal for the additional methyl appears at δ 3.84 ppm. In the mass spectrometry, the molecular ion peak was noted at m/z 326, with a base peak at m/z 311 (M^{+}-15). No C7 or C2 oxidation products were detected.



isotriptophenolide (190) methyl isotriptophenolide (194)

Scheme 44. The biotransformation of isotriptophenolide (190) by *T. wilfordii* whole cells.

In an attempt to force the cells to metabolize the supplied substrate, two experiments with "starved" cells (0% sucrose and 0.2% sucrose $MSNA_{0.5}K_{0.5}$

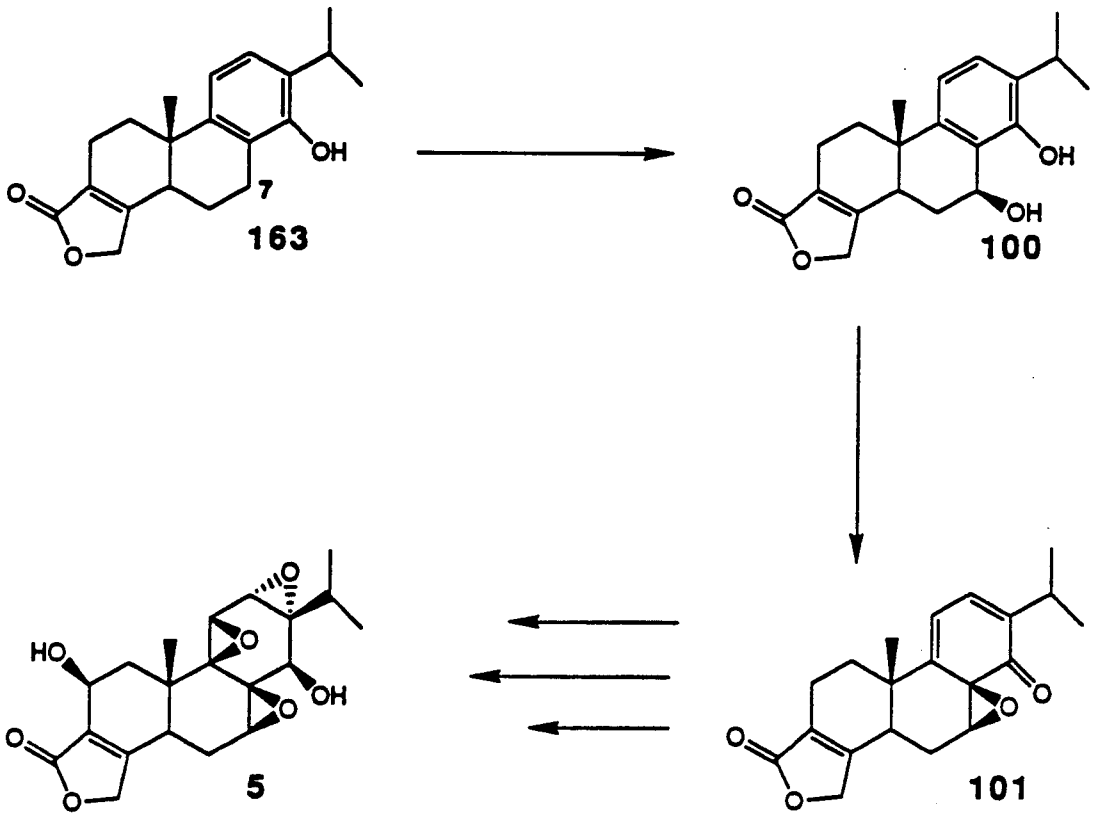
respectively) and adding the substrate at inoculation time, were carried out. In the first experiment (0% sucrose) the cells died and the substrate was recovered unchanged, whereas with the other culture only a trace of the methyl ether could be detected along with significant amounts of recovered substrate.

CONCLUSIONS.

While a more thorough investigation on the biotransformation of isotriptophenolide (190) was not possible within the constraints of this research effort, Mr. Kang Han in our group has been continuously studying this problem. Interestingly enough, he has found that *T. wilfordii* cell suspension cultures are indeed capable of oxidizing this substrate to the epoxy dienone **191** under appropriate conditions. This appears to indicate that the biosynthesis of the diterpene triepoxides may well proceed through an intermediate such as triptophenolide (163) via the biological analogue of Alder's oxidation of *ortho*-hydroxymethyl phenols and subsequent epoxidations (Scheme 45).

It is also most probable that the introduction of the hydroxyl group at C14 occurs prior to ring C aromatization since no aromatic hydroxylation could be demonstrated.

Future research in this area should involve the biotransformation of triptophenolide (163) to clarify this situation. Such studies are currently underway in our laboratory.



Scheme 45. The proposed biogenesis of the triepoxide system.

EXPERIMENTAL SECTION

EXPERIMENTAL

All experiments involving moisture-sensitive reagents were carried out under a positive pressure of nitrogen or argon gas, as indicated. Solvents were commercial reagent grade except that technical grade ether, methylene chloride, and ethyl acetate were used for extractions in most instances. Unless otherwise noted, anhydrous magnesium sulphate was used to dry organic solutions prior to solvent removal.

Tetrahydrofuran, ether, benzene, and toluene were freshly distilled from sodium and benzophenone ketyl under argon prior to use if strictly anhydrous solvents were required; otherwise, reagent grade anhydrous ethers, benzene, and toluene were used.

Column chromatography was carried out using Merck silica gel 60, 230-400 Å mesh, while analytical and preparative TLC was performed using Merck pre-coated silica gel 60 F_{254} TLC plates. All solvents for column chromatography were reagent grade and received no additional purification or drying prior to use.

Synthetic samples were visualized on analytical TLC plates by UV and by spraying with a 5% solution of ammonium molybdate in 10% sulphuric acid, followed by heating at 125° C until blue spots developed. Tissue culture extracts were visualized on TLC by spraying first with a 30% solution of concentrated sulphuric acid in glacial acetic acid, then with a 5% solution of anisaldehyde in isopropanol, followed by heating at 125° C for approximately 10 minutes. Lignan-type compounds were developed by spraying with a 10:3 mixture of glacial acetic acid and concentrated nitric acid followed by heating at 125° C for 5 min, giving typically red or brown spots.

^1H nmr spectra were run in $CDCl_3$ at 400 MHz using a Bruker WH 400 spectrometer, unless otherwise quoted. Tetramethylsilane was used as the internal standard and all peaks were recorded in ppm (δ) relative to TMS (δ 0.00 ppm).

Low resolution mass spectra were recorded using Kratos MS 50 and MS 80 mass spectrometers. High resolution (HRMS) mass spectra were run on a Kratos MS 50 mass spectrometer. Chemical ionization mass spectra were recorded on a Delsi-Nermag R10-10C mass spectrometer using isobutane as the carrier gas.

IR spectra were recorded on a Perkin Elmer 710B infrared spectrophotometer, and Fourier transform IR spectra were recorded on a Perkin Elmer 1710 infrared Fourier transform spectrophotometer.

UV spectra were recorded on a Perkin Elmer Lambda 4B UV/VIS spectrophotometer, using quartz cells of 1 cm path length. All melting points were recorded on a Reichert melting point apparatus and are uncorrected.

Elemental analyses were carried out by Mr. P. Borda, of the Microanalytical Laboratory, University of British Columbia, Vancouver.

Plant cell tissue culture production was carried out by Gary Hewitt, David Chen, Fay Hutton, Radka Milanova, Nikolay Stoynov, and myself at the Biological Services Laboratories, Chemistry Department, University of British Columbia. The cultures were grown in shake flasks and in glass airlift or mechanically stirred fermentors (Labroferm, New Brunswick Scientific or Microferm, New Brunswick Scientific, respectively). Cell culture methods were developed by Gary Hewitt of the Biological Services section of our Department. Cultures were raised in the dark at 26^{o} C using an appropriate liquid medium (see individual culturing methods). Growth was monitored through the refractive index (Galileo refractometer, 25^{o} C) and pH and microscopic purity were also determined at the end of the growth cycle.

For the fungal experiments, a culture of *Mortierella isabellina* was grown on 10 ml of PDA in test tubes at 25^{o} C or in a Roux bottle with 15 ml of PDA at 25^{o} C for 10 days by Ms. Elizabeth Bugante who also provided technical assistance for these experiments.

Cell free extracts were prepared according to the methods described in this Experimental Part by Ms. Radka Milanova.

Hydrogen peroxide was prepared from a stock solution (30% w/v). The solutions were standardized by iodimetric titration with sodium thiosulphate.

N-Butylithium solutions were standardized by tritration against diphenyl acetic acid in anhydrous THF.

High pressure liquid chromatography was performed using a Waters C_{18} "Radial Pack" liquid chromatography cartridge, a Waters 440 Absorbance Detector set at 280 and 254 nm, and a methanol/water eluent, containing 0.1% of acetic acid.

Gas chromatography analyses were carried out with an HP 5840A gas chromatograph fitted with a Hewlett Packard HP-1 column (100% dimethylpolysiloxane, non-polar fused silica capillary column) 25 m x 0.2 mm x 0.5 μm film thickness.

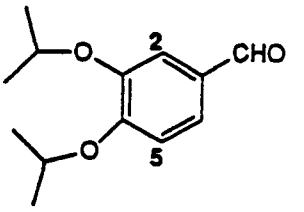
Ozone was generated in a Welsbach model T-23 laboratory ozonator.

3-HYDROXY-4-ISOPROPOXYBENZALDEHYDE (63):

To a solution of 3,4-dihydroxybenzaldehyde (62) (5.0 g, 36.2 mmol) in DMSO (18 mL) was added anhydrous potassium carbonate (5.6 g, 3.12 equiv.) and 4.8 mL (8.2 g, 1.33 equiv.) of distilled isopropyl iodide, and the solution was stirred overnight in the dark. The reaction was quenched by pouring into brine (50 mL), followed by slow acidification with 1 N HCl (114 mL). The mixture was extracted with dichloromethane (3 x 50 mL), the combined organic extracts were washed with acidic brine (50 mL), dried, filtered and evaporated *in vacuo* to yield 6.39 g of the crude product. Column chromatography was carried out using the flash technique (4 cm diameter column, 6" silica gel), collecting 10-mL fractions, and eluting with benzene:

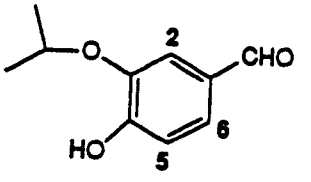
ethyl acetate:acetic acid 9:0.5:0.5 (500 mL). The following compounds were isolated and characterized in order of elution:

3,4-DIISOPROPOXYBENZALDEHYDE (63a):[74]



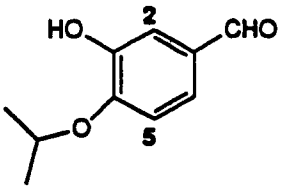
0.40 g, 5%. IR (NaCl neat): ν_{max} 1705 cm^{-1} (aldehyde). ^1H nmr δ: 1.41 (12H, m, $CH(CH_3)_2$), 4.53 (1H, heptet, $CH(CH_3)_2$, J = 6 Hz), 4.64 (1H, heptet, $CH(CH_3)_2$, J = 6 Hz), 6.98 (1H, d, H5, J = 8 Hz), 7.44 (2H, m, H2, H6), 9.83 (1H, s, CHO). UV (MeOH) λ_{max} (log ϵ): 303 (4.00), 278 (4.11), 232 (4.13), 213 (4.09). MS m/z: 222 (M^+), 180, 138. HRMS calc. for $C_{13}H_{18}O_3$: 222.1256; found: 222.1258. Anal. calcd. for $C_{13}H_{18}O_3$: C 70.25, H 8.15; found: C 70.15, H 8.23. Previously unpublished UV and elemental analysis data.

4-HYDROXY-3-ISOPROPOXYBENZALDEHYDE (63b):[74]



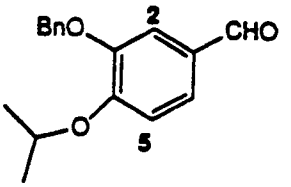
0.26 g, 4%. IR (NaCl neat) ν_{max}: 1705 cm^{-1} (aldehyde); ^1H nmr δ: 1.38 (6H, d, $CH(CH_3)_2$, J = 6 Hz), 4.75 (1H, heptet, $CH(CH_3)_2$, J = 6 Hz), 6.35 (1H, brs, OH), 7.05 (1H, d, H5, J = 8 Hz), 7.42 (2H, m, H2 H6), 9.83 (1H, S, CHO). MS m/z: 180 (M^+), 137, 109. HRMS calc. for $C_{10}H_{12}O_3$: 180.0786, found: 180.0783. Anal. calcd. for $C_{10}H_{12}O_3$: C 66.66, H 6.70; found: C 66.40, H 6.84. Previously unpublished UV and elemental analysis data.

3-HYDROXY-4-ISOPROPOXYBENZALDEHYDE (63):[74]



Initially 5.6 g (70%) as an oil that crystallized to white needles. Mp = 63-65° C (EtOAc-hexanes). IR (KBr pellet): ν_{max} 1705 cm^{-1} (aldehyde); ^1H nmr δ: 1.40 (6H, d, $CH(CH_3)_2$, J = 6 Hz), 4.75 (1H, heptet, $CH(CH_3)_2$), J = 6 Hz), 5.80 (1H, brs, OH), 6.95 (1H, d, H5, J = 8 Hz), 7.43 (2H, m, H2, H6), 9.84 (1H, s, CHO). UV (methanol) λ_{max} (log ϵ) : 311 (3.12), 276 (4.00), 229 (4.00). MS m/z: 180 (M^+), 137, 109. HRMS calc. for $C_{10}H_{12}O_3$: 180.0786, found: 180.0783. Anal. calcd. for $C_{10}H_{12}O_3$: C 66.66, H 6.70; found: C 66.74, H 6.75. Previously unpublished UV and elemental analysis data.

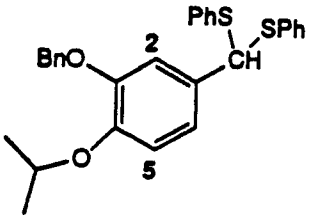
3-BENZYLOXY-4-ISOPROPOXYBENZALDEHYDE (64):[74]



4-isopropoxy-3-hydroxybenzaldehyde (3.47 g, 0.0192 mol) was dissolved in ethanol (25 mL). Potassium carabonate (3.0 g), sodium iodide (0.1 g) and benzyl chloride (3.0 g, 2.72 mL, 0.0237 mol, 1.23 equiv.) were added. The mixture was refluxed protected from moisture for 4 h, when the reaction was complete. The reaction was cooled and water (10 mL) was added. After evaporation of the ethanol *in vacuo*, the residue was diluted with water (25 mL) and extracted with dichloromethane (3 x 10 mL). The combined organic extracts were washed with 1 N NaOH (10 mL) and with water (10 mL) before drying, filtration, and rotary evaporation. The crude product (5.0 g) was purified by flash column chromatography (4.0 cm diameter, 5.5" silica gel) eluting with 1 L of petroleum ether:ethyl acetate 9:1, and collecting 20-mL

fractions, to give 3.68 g (70.78%) of the product as a colorless oil. IR (neat) ν_{max}: 1686 cm^{-1} (aldehyde), ^1H nmr δ: 1.40 (6H, d, $CH(CH_3)_2$, J = 6 Hz), 4.65 (1 H, heptet, $CH(CH_3)_2$, J = 6 Hz), 5.22 (2H, s, Ph-CH_2-O), 7.0 (1H, d, H5, J = 8 Hz), 7.30-7.50 (7H, m, aromatic), 9.82 (1H, s, CHO). UV (methanol) λ_{max} (log ϵ): 305 (4.11), 275 (4.28), 230 (4.38), 207 (4.48). MS m/z: 270 (M^+), 228 (1.2), 179 (6.5), 91 (100). HRMS calcd. for $C_{17}H_{18}O_3$: 270.1256, found: 270.1249. Previously unpublished UV and elemental analysis data.

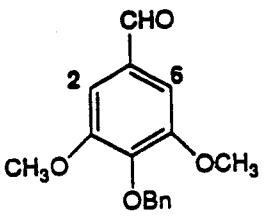
3-BENZYLOXY-1-BIS(PHENYLTHIO)METHYL-4-ISOPROPOXYBENZENE (65):[74]



3-benzyloxy-4-isopropoxybenzaldehyde (3.68 g, 13.6 mmol) was dissolved under nitrogen in dry chloroform (30 mL). The solution was cooled to -40° C with an acetonitrile-dry ice bath and 2.94 mol (2.1 equiv.) of thiophenol were slowly added maintaining the inner temperature of -40° C, followed by the addition of 4 mL (2.3 equiv.) of boron trifluoride etherate maintaining good stirring and temperature of -40° C. The resulting brown mixture was stirred at -40° C for 20 min, and then quenched by pouring it over ice cold water (40 mL). The resulting mixture was extracted with dichloromethane (3 x 30 mL) and the combined organic extracts were washed successively with KOH (50 mL), water (50 mL), and brine (50 mL). Drying, filtration and rotary evaporation afforded 4.82 g of the crude product as a yellow-brownish oil, which was purified by flash column chromatography (4.5 cm diameter, 5.5" silica gel) eluting with 5% ethyl acetate in petroleum ether. 20-mL fractions were collected, to afford 3.58 g (55.63%) of the product as a clear oil which slowly solidifies to a white solid. IR (neat) ν_{max}: 2895 (aromatic), 1480 cm^{-1}. ^1H nmr δ: 1.30 (6H, d,

$CH(CH_3)_2$, J = 6 Hz), 4.45 (1H, heptet, $CH(CH_3)_2$, J = 6 Hz), 5.0 (2H, s, CH_2-Ph), 5.33 (1H, s, $CH(SPh)_2$), 6.78 (2H, m, H2′, H6′), 6.98 (1H, d, H5′, J= 2 Hz), 7.20-7.50 (15H, m, aromatic). UV (methanol) λ_{max} (log ϵ): 216 (4.56). MS m/z: 363 (M^+- SPH) (100), 321 (33.1), 91 (38.5). HRMS calcd. for $C_{29}H_{28}S_2O_2$: 472.1531, found: 472.1525. Anal. calcd. for $C_{29}H_{28}S_2O_2$: C 73.70, H 5.96, S 13.56; found: C 73.68, H 6.02, S 13.45. Previously unpublished UV data.

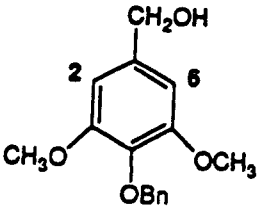
4-BENZYLOXY-3,5-DIMETHOXYBENZALDEHYDE (67a):



Syringaldehyde (5.0 g, 0.027 mol) was dissolved in ethanol (50 mL) and anhydrous potassium carbonate (5.0 g), sodium iodide (0.5 g), and benzyl chloride (4.4 g, 4 mL, 1.28 equiv.) were added. The mixture was refluxed protected from moisture for 3 h, when the reaction was complete. After cooling the mixture, water (50 mL) was added and the ethanol was removed *in vacuo*. The residue was diluted with water (50 mL) and extracted with dichloromethane (3 x 25 mL). The combined organic extracts were successively washed with 1 N NaOH (25 mL), and water (25 mL). Drying, filtration and rotary evaporation afforded 7.88 g of crude product which was purified by flash column chromatography (4.5 cm diameter, 5.5" silica gel) eluting with 1.5 L of a 20% solution of ethyl acetate in petroleum ether and collecting 20-mL fractions. Rotary evaporation yielded 6.357 g (85.05%) of the pure product as an oil which slowly crystallized to off-white crystals. Mp = 52-54^o C (EtOAc-hexanes). IR (KBr) ν_{max}: 1685 (aldehyde) cm^{-1}. ^1H nmr δ: 3.90 (6H, s, $-OCH_3$), 5.23 (2H, s, CH_2-Ph), 7.12 (2H, s, H2, H6), 7.24-7.5 (5H, m, aromatic), 9.86 (1H, s, CHO). UV (methanol) λ_{max} (log ϵ): 287 (4.05), 223 (4.13), 220 (4.13). MS m/z: 272 (M^+, 26.9), 181 (10.8),

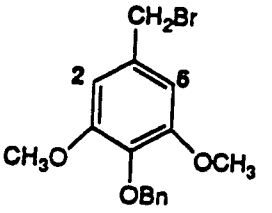
125 (22.0), 110 (22.4), 91 (100). HRMS calcd. for $C_{16}H_{16}O_4$: 272.1049; found: 272.1048. Anal. calcd. for $C_{16}H_{16}O_4$: C 70.58, H 5.91; found: C 70.58, H 5.92.

4-BENZYLOXY-3,5-DIMETHOXYBENZYL ALCOHOL (67b):



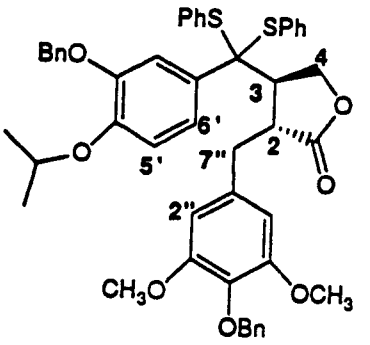
4-benzyl-syringaldehyde (6.35 g, 0.023 mol) was dissolved in ethanol (100 mL), at room temperature. Sodium borohydride (635 mg, 2.9 equiv.) was added in one portion and the mixture was stirred at room temperature for 1 h. Analytical TLC on 30% ethyl acetate in petroleum ether showed reaction completion. The reaction was quenched by the addition of 1 N HCl (20 mL) (the mixture turns clear at pH = 2.0) and the ethanol was removed *in vacuo*. The residue was diluted with water (25 mL) and extracted with dichloromethane (3 x 25 mL). The combined organic extracts were dried, filtered, and rotary evaporated to yield 6.47 g of crude product as a light yellow oil. The crude was purified by flash column chromatography (4.0 cm diameter column, 5.5" silica gel) eluting with 2 L of 30% solution of ethyl acetate in petroleum ether and collecting 20-mL fractions. Rotary evaporation yielded 6.18 g (96.7%) of the pure product as a colorless oil. IR (neat) ν_{max}: 3436 (alcohol), 1127 (ether) cm^{-1}. ^1H nmr δ: 1.75 (1H, brs, D_2O+, OH), 3.83 (6H, s, -OCH_3), 4.62 (2H, d, CH_2-OH, J = 4 Hz), 5.0 (2H, s, CH_2-Ph), 6.60 (2H, s, H2, H6), 7.24-7.5 (5H, m, aromatic). UV (methanol) λ_{max} (log ϵ): 210 (4.91). MS m/z: 274 (M^+, 16.9), 183 (46.9), 155 (24.2), 127 (36.3), 123 (12.9), 95 (14.4), 91 (100). HRMS calcd. for $C_{16}H_{18}O_4$: 274.1205, found: 274.1205. Anal. calcd. for $C_{16}H_{18}O_4$: C 70.07, H 6.60; found: C 70.23, H 6.76.

4-BENZYLOXY-3,5-DIMETHOXYBENZYL BROMIDE (67):



6.18 g (22.5 mmol) of 4-benzyloxy-3,5-dimethoxybenzyl alcohol were dissolved in anhydrous ether (40 mL) under argon. To this solution were slowly added (over 20 min) 2.47 g (1.2 equiv.) of phosphorus tribromide dissolved anhydrous ether (10 mL) maintaining the reaction temperature at 25-30^{0} C (water bath). At the end of the addition, the reaction mixture was stirred at room temperature until control TLC (s.s. hexanes-EtOAc 1:1) showed reaction completion. The mixture was then poured into a separatory funnel and washed with brine (3 x 25 mL), then with saturated sodium carbonate solution (25 mL) and brine (25 mL). The organic phase was dried, filtered and concentrated *in vacuo* to yield 6.44 g of the crude product as a light yellow oil. The aryl bromide was purified by flash column chromatography (4.5 cm diameter, 6" silica gel) eluting with 2 L of petroleum ether:ethyl acetate 9:1 and collecting 20-mL fractions. Rotary evaporation afforded 5.76 g (75.9%) of a clear oil that solidified upon cooling to a white mass. IR (neat) ν_{max}: 3003, 2938, 2838, 1593 cm^{-1}. ^{1}H nmr δ: 3.85 (6H, s, -OCH_3), 4.49 (2H, s, CH_2-Br), 5.0 (2H, s, CH_2-Ph), 6.62 (2H, s, H2, H6), 7.30-7.53 (5H, m, aromatic). UV (methanol) λ_{max} (log ϵ): 260 (3.45). MS m/z: 336 (M^+, 3.4), 338 (M^+, 3.4), 257 (10.6), 91 (100). HRMS calcd. for $C_{16}H_{17}O_3Br$: 338.0342, 336.0362; found: 338.0357, 336.0358. Anal. calcd. for $C_{16}H_{17}O_3Br$: C 56.99, H 5.07, Br 23.69; found: C 57.13, H 5.17, Br 23.53.

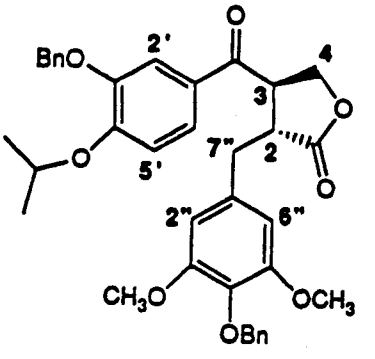
TRANS-2-(4-BENZYLOXY-3,5-DIMETHOXYBENZYL)-3-(3-BENZYLOXY-4-ISOPROPOXY-α,α-BIS(PHENYLTHIO)BENZYL)BUTANOLIDE (68):[74]



6.05 g (0.0128 mol) of the phenyl thioketal **65** were dissolved in dry THF (60 mL) and the solution was cooled under nitrogen to -70° C (acetone-dry ice bath). 8.83 mL (1.1 equiv.) of a 1.6 M solution of *n*-butyl lithium in hexanes were slowly added via the addition funnel, keeping the reaction temperature ≤ -68° C. After the addition was finished, the funnel was rinsed with dry THF (3 mL) and the resulting deep-yellow solution was stirred at -70° C for 25 min. After this time, a solution of butenolide (1.21 g, 1.1 equiv.) in dry THF (10 mL) was added very slowly (dropwise, over a 1 h period) via the addition funnel while keeping the reaction temperature ≤ -68° C. After the addition was completed, the mixture was stirred at -70° C for 40 min and then, a solution of the aryl bromide **67** (6.5 g, 1.5 equiv.) in THF (13 mL) was slowly added via the addition funnel keeping the temperature ≤ -68° C at all times. After the addition was finished, the reaction mixture was stirred at -70° C for 1 h, then slowly allowed to reach room temperature, and stirred for a further hour at this temperature. The reaction was quenched adding water (90 mL) and removing most of the THF by rotary evaporation. The resulting suspension was extracted with ethyl acetate (3 x 100 mL) and the combined organic extracts were dried, filtered and concentrated to dryness to yield 15.09 g of crude product as an orange oil. The product was purified by flash chromatography using a 4.5 cm diameter column (6"

silica gel) and eluting with 1.6 L of hexanes:ethyl acetate 3:1 collecting 20-ml fractions. Solvent removal afforded 7.91 g (76%) of the pure product as a white foam. IR (KBr) ν_{max}: 1770 (lactone) cm^{-1}. ^1H nmr δ: 1.38 (6H, d, $CH(CH_3)_2$, J = 6 Hz), 2.7 (1H, dd, H7", J = 5, 14 Hz), 2.83-2.90 (1H, m, H2), 3.10 (1H, dd, H7", J = 4, 14 Hz), 3.2-3.25 (1H, m, H3), 3.35 (1H, dd, H4, J = 8, 11 Hz), 3.68 (6H, s, OCH_3), 4.21 (1H, dd, H4, J = 3, 11 Hz), 4.55 (1H, septet, $CH(CH_3)_2$, J = 6 Hz), 4.97 (2H, s, CH_2-Ph), 5.03 (2H, s, CH_2-Ph), 6.19 (2H, s, H2", H6"), 6.80 (1H, d, H5', J = 8 Hz), 7.03 (1H, dd, H6', J = 2, 8 Hz), 7.15-7.50 (21H, m, aromatic). UV (methanol) λ_{max} (log ϵ): 219 (4.70). MS m/z: 595 (M^+-2SPh). HRMS calcd. for $C_{37}H_{39}O_7$ (M^+-2SPh): 595.2696, found: 595.2699. Anal. calcd. for $C_{49}H_{48}O_7S_2$: C 71.97, H 6.04, S 8.0; found: C 72.18, H 5.95, S 7.83. Previously unpublished UV data.

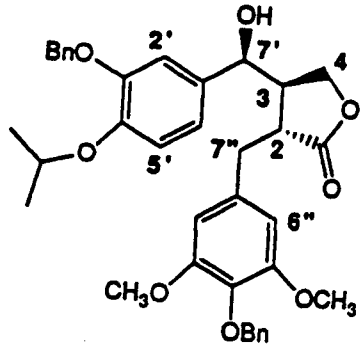
TRANS-2-(4-BENZYLOXY-3,5-DIMETHOXYBENZYL)-3-(3-BENZYLOXY-4-ISOPROPOXY-α-OXO-BENZYL)BUTANOLIDE (69):



2.31 g (1.8 equiv.) of red mercuric oxide were suspended in 15% aqueous THF (36 mL) under nitrogen, and the suspension was stirred at -5° C (methanol-ice bath). Boron trifluoride etherate (12.5 mL, 17 equiv., recently distilled) was added dropwise via addition funnel keeping the temperature ≤ 0° C. After the addition was complete, the reaction mixture was stirred for 10 min at -5° C and the thioketal **68** (4.9 g, 0.0059 mol) in dry THF (833.5 mL) was added dropwise maintaining the

temperature at 0^{o} C. The addition funnel was washed with THF (20 mL) and the washing added to the mixture. The orange suspension was allowed to reach room temperature and stirred for 2 h at this temperature. During this time the mixture was clear. After this time, the reaction was heated to 35^{o} C for 1 h after which the control TLC (s.s. hexanes:ethyl acetate 4:2) showed reaction completion. The reaction was quenched by adding saturated sodium bicarbonate solution (230 mL), shaking well and separating the organic layer. This was then diluted with dichloromethane (250 mL) and washed with sodium bicarbonate solution (3 x 120 mL). The combined aqueous washings were extracted with dichloromethane (120 mL) and the mixed organic extracts dried, filtered and rotary evaporated to yield 7.25 g of crude product. This was purified by flash column chromatography (4.5 cm diameter, 6" silica gel) packed initially with dichloromethane. The crude product was dissolved in dichloromethane (146 mL) (with warming) and applied to the column top. The column was run with 1.5 L of dichloromethane collecting 75-ml fractions and then the solvent was changed to hexanes:ethyl acetate 4:2 (1.2 L) and continued the elution collecting 20-mL fractions. Rotary evaporation of the solvent yielded 3.22 g (88.2%) of pure product as white crystals. Mp = 111-113^{o} C (EtOH). IR (KBr) ν_{max}: 1775 (lactone), 1663 (ketone) cm^{-1}. ^{1}H nmr δ: 1.40 (6H, d, $CH(CH_3)_2$, J = 6 Hz), 3.0 (2H, d, H7", J = 6.1 Hz), 3.62 (6H, s, $-OCH_3$), 3.57 (1H, m, H3), 3.99 (1H, dd, H4, J = 8, 9 Hz), 4.01 (1H, dd, H4, J = 9, 9 Hz), 4.3 (1H, m, H2), 4.65 (1H, septet, $CH(CH_3)_2$, J = 6 Hz), 4.90 (2H, s, CH_2-Ph), 5.12 (2H, 5, CH_2-Ph), 6.26 (2H, s, H2″, H6″), 6.82 (1H, d, H5′, J = 8 Hz), 7.2-7.50 (12H, m, aromatic). UV (methanol) λ_{max} (log ϵ): 310 (4.37), 280 (3.44), 231 (4.76), 211 (5.03). MS m/z: 610 (M^{+}, 0.4), 91 (100). HRMS calcd. for $C_{37}H_{38}O_8$: 610.2567; found: 610.2568. Anal. calcd. for $C_{37}H_{38}O_8$: C 72.78, H 6.26; found: C 73.00, H 6.29.

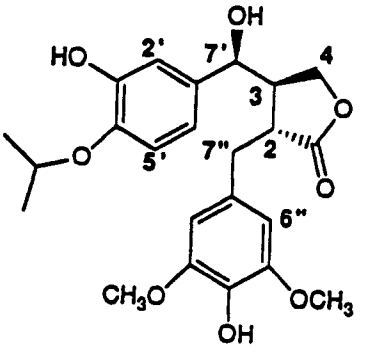
TRANS-2-(4-BENZYLOXY-3,5-DIMETHOXYBENZYL)-3-(3-BENZYLOXY-4-ISOPROPOXY-7′ β-HYDROXYBENZYL)BUTANOLIDE (70):



The ketone 69 (4.48 g, 7.3 mmol) was suspended in methanol (530 mL), and the suspension cooled to 0-5° C (ice-water bath). 417 mg (6.0 equiv.) of sodium borohydride were added in one portion, and the mixture was stirred under nitrogen at 0-5° C for 4 h when the control TLC (s.s. hexanes:ethyl acetate 1:1) showed reaction completion. The reduction was quenched adding enough 1 N HCl solution to obtain acid pH (50 mL) and most of the methanol was removed *in vacuo*. The resulting suspension was diluted with brine (55 mL) and extracted with dichloromethane (3 x 200 mL). The organic extracts were washed with water (140 mL) dried, filtered and rotary evaporated to afford 4.19 g of crude product as a white foam. Flash column chromatography (4.5 cm diameter, 5.5" silica gel) eluting with 1.5 L of hexanes:ethyl acetate 6:4, and collecting 20-mL fractions afforded 3.76 g (83.65%) of the pure alcohol, which appeared as one spot on TLC. IR (KBr) ν_{max}: 3492 (OH), 1767 (lactone) cm^{-1}. ^1H nmr δ: the product consists of approximately 20% α-OH and 80% 7′ β-OH epimers. 1.45 (m, $CH(CH_3)_2$), 1.58 (brs, D_2O+, OH), 2.50-3.2 (m, H2, H3, H4, H7″), 3.70 (m, H4β), 3.78 (s, OCH_3), 4.2 (m, H7′α), 4.3 (m, H4α), 4.50 (m, H7′β, $CH(CH_3)_2$), 4.98 (s, CH_2-Ph), 5.10 (s, CH_2-Ph), 6.26 (s, H2″α), 6.40 (s, H2″β), 6.7-6.9 (m, H2′, H5′, H6'), 7.2-7.5 (m, aromatic). UV (methanol) λ_{max} (log ε): 278 (3.74), 214 (4.72). MS m/z: 612 (M^+, 2.8), 594 (M^+-H_2O, 1.6), 91 (100). HRMS calcd. for

$C_{37}H_{40}O_8$: 612.2723; found: 612.2716. Anal. calcd. for $C_{37}H_{40}O_8$: C 72.54, H 6.57; found: C 72.70, H 6.66.

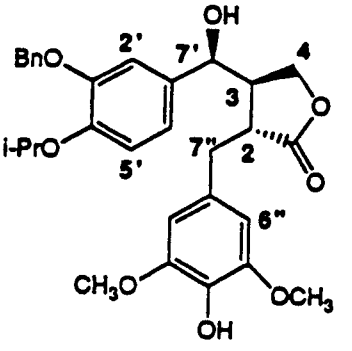
TRANS-2-(4-HYDROXY-3,5-DIMETHOXYBENZYL)-3-(3-HYDROXY-4-ISOPROPOXY-7′ β-HYDROXYBENZYL)BUTANOLIDE (56):



In a 500 mL RB flask, 2.54 g of 10% Palladium on charcoal were suspended in ethanol (75 mL). The vessel was purged with vacuum - hydrogen three times, pressurized to 1 atm of hydrogen, and stirred for 1 h at room temperature. A solution of 3.76 g (6.1 mmol) of the benzyl ether **70** in reagent grade ethyl acetate (75 mL) was added to the suspension via canule with hydrogen pressure. The reaction was stirred under 1 atm. of hydrogen until analytical TLC of a sample (s.s. hexanes-ethyl acetate 2:3) showed reaction completion (approx. 50 min. Over-hydrogenation must be avoided). The mixture was then filtered in a Schott packed with Celite 545 and the Celite and flask were washed with ethanol (75 mL) and ethyl acetate (75 mL). The filtrate was rotary evaporated to dryness to afford 2.71 g of crude poduct which was purified by flash column chromatography (4.5 cm diameter column, 5.5" silica gel) eluting with 1.5 L of hexanes:ethyl acetate 1:1, collecting 20-mL fractions. Removal of the solvent *in vacuo* yielded 1.97 g (74.3%) of the product (which consisted of approximately 8% α-OH and 92% 7′β-OH epimers), as a white foam. IR (KBr) ν_{max}: 3459 (OH), 1757 (lactone). ^{1}H nmr δ: 1.4 (6H, d, $CH(CH_3)_2$, J = 6 Hz), 2.64 (1H, m, H3), 2.85 (1H, dd, H7″, J = 5.4, 13.6 Hz), 2.98 (1H, m, H2), 3.10 (1H, dd, H7″, J = 4.9,

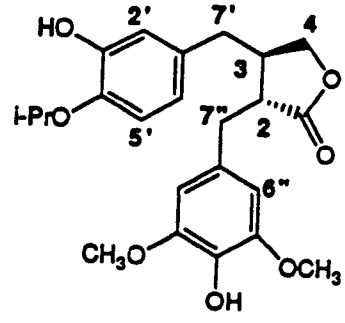
13.6 Hz), 3.8-3.95 (8H, m, H4, OCH_3), 4.41 (d, H7′α, J = 7 Hz), 4.52-4.62 (2H, m, H7′β, $CH(CH_3)_2$), 1.7 (1H, brs, D_2O+, -OH), 5.4 (1H, brs, D_2O+, -OH), 5.75 (1H, brs, D_2O+, OH), 6.30 (s, ,H2″, 6″α), 6.40 (2H, s, H2″, 6″β), 6.65-6.86 (3H, m, aromatic). UV (methanol) λ_{max} (log ϵ): 281 (3.75), 213 (4.47). MS m/z: 432 (M^+, 0.3), 414 (M^+-H_2O, 0.3), 373 (44.3), 262 (11.7), 224 (11.1), 205 (14.6), 189 (14.2), 181 (14.2), 178 (28.3), 175 (12.4), 167 (100). HRMS calcd. for $C_{23}H_{28}O_8$: 432.1784; found: 432.1778. Anal. calcd. for $C_{23}H_{28}O_8$: C 63.89, H 6.52; found: C 63.06, H 6.77.

TRANS-2-(4-HYDROXY-3,5-DIMETHOXYBENZYL)-3-(3-BENZYLOXY-4-ISOPROPOXY-7′β-HYDROXYBENZYL)BUTANOLIDE (71):



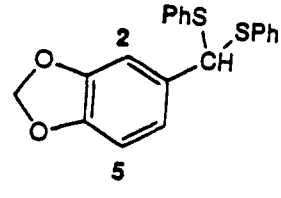
This product is found when the hydrogenolysis reaction is not complete and can easily be hydrogenolysed to the trihydroxy compound. White foam: IR (KBr) ν_{max}: 3471 (OH), 1762 (lactone). ^1H nmr δ: 1.35 (6H, d, $CH(CH_3)_2$, J = 6 Hz), 1.51 (1H, brs, D_2O+, OH), 2.5-3.15 (4H, m, H2, H3, H7″), 3.70 (2H, m, H4), 3.85 (6H, s, OCH_3), 4.54 (2H, m, $CH(CH_3)_2$, H7′), 5.10 (2H, m, CH_2-Ph), 5.40 (1H, brs, D_2O+, OH), 6.41 (2H, s, H2″, H6″), 6.7-6.9 (3H, m, H2′, H5′, H6′), 7.25-7.42 (5H, m, aromatic). UV (methanol) λ_{max} (log ϵ): 280 (0.47), 209 (4.65). MS m/z: 522 (M^+, 9.2), 504 (M^+-H_2O, 2.5), 432 (12.2), 207 (12.5), 167 (49.2), 149 (10.6), 139 (13.5), 91 (100). HRMS calcd. for $C_{30}H_{34}O_8$: 522.2253; found: 522.2256. Anal. calcd. for $C_{30}H_{34}O_8$: C 68.96, H 6.55; found: C 68.11, H 6.61.

TRANS-2-(4-HYDROXY-3,5-DIMETHOXYBENZYL)-3-(3-HYDROXY-4-ISOPROPOXYBENZYL)BUTANOLIDE (54):[74]



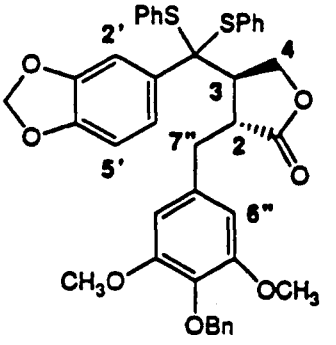
Subproduct of hydrogenolysis. If the reaction is carried out for 5 h, an isolated yield of 84% can be achieved. White foam: IR (KBr): 3540 (OH), 1775 (lactone). ^{1}H nmr δ: 1.35 (6H, d, $CH(CH_3)_2$, J = 6 Hz), 2.48-2.65 (4H, m, H2, H3, H7″), 2.87 (1H, dd, H7′, J = 5, 14 Hz), 2.95 (1H, dd, H7′, J = 6, 14 Hz), 3.82-3.95 (7H, m, H4, OCH_3), 4.11 (1H, dd, H4, J = 6, 8 Hz), 4.52 (1H, heptet, $CH(CH_3)_2$, J = 6 Hz), 5.40 (1H, brs, D_2O+, OH), 5.68 (1H, brs, D_2O+, OH), 6.40 (2H, s, H2″, H6″), 6.47 (1H, dd, H6′, J = 2, 8.5 Hz), 6.62 (1H, d, H2′, J = 2 Hz), 6.74 (1H, d, H5', J = 8.5 Hz). UV (methanol) λ_{max} (log ϵ): 281 (3.62), 216 (4.20). MS m/z: 416 (M^+, 21.9), 414 (11.2), 374 (24.5), 372 (18.6), 209 (40.5), 167 (100). HRMS calcd. for $C_{23}H_{28}O_7$: 416.1835, found: 416.1829. Anal. calcd. for $C_{23}H_{28}O_7$: C 63.58, H 6.96; found: C 63.58, H 6.49.

1-BIS(PHENYLTHIO)METHYL-3,4-METHYLENEDIOXYBENZENE (82):[75]



Piperonal (1.0 g, 6.7 mmol) was dissolved in dry chloroform (10 mL) and the solution cooled under nitrogen to -40° C. 1.4 mL (2.0 equiv.) of thiophenol and 2.5 equiv. of boron trifluoride etherate were added via syringe and the resulting mixture was stirred for 15 min at -40° C. The reaction was quenched by pouring into ice cold water (10 mL) and extracting with chloroform (3 x 10 mL). The combined organic extracts were washed with 10% KOH solution (2 x 10 mL), water (10 mL), and brine (10 mL). Drying and rotary evaporation afforded the product (2.3 g, 98%) which was not purified further, as a yellow oil homogeneous by TLC. IR (neat) ν_{max}: 2890 (C stretch). ^{1}H nmr δ: 5.37 (1H, s, $CH(SPh)_2$), 5.96 (2H, s, -O-CH_2-O-), 6.66 (1H, d, H5, J = 4 Hz), 6.77 (1H, dd, H6, J = 2, 4 Hz), 6.98 (1H, d, H2, J = 2 Hz), 7.22-7.44 (10H, m, aromatic). UV (methanol) λ_{max} (log ϵ): 248 (4.14), 282 (3.88). MS m/z , 352 (M^+). HRMS calcd. for $C_{20}H_{16}O_2S_2$: 352.0592; found: 352.0598. Anal. calcd. for $C_{20}H_{16}O_2S_2$: C 68.15, H 4.58; found: C 68.11, H 4.63. Sulfur not determined.

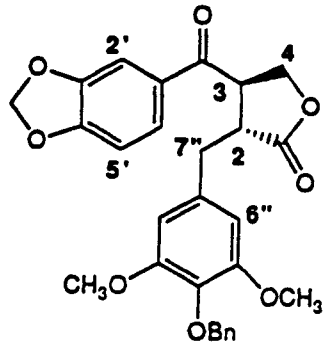
TRANS-2-(4-BENZYLOXY-3,5-DIMETHOXYBENZYL)-3-[3,4-METHYLENEDIOXY-α,α-BIS(PHENYLTHIO)BENZYL]BUTANOLIDE (83):[75]



Piperonal diphenyl dithioketal **82** (2.0 g, 5.7 mmol) was dissolved in anhydrous THF (10 mL) under nitrogen and cooled to -78° C. To this were added 3.6 mL (1.0 equiv.) of a 1.6 M solution of *n*-butyllithium in hexanes controlling the reaction temperature at -70° C. The resulting solution was stirred for 2.5 h at -70° C, and a

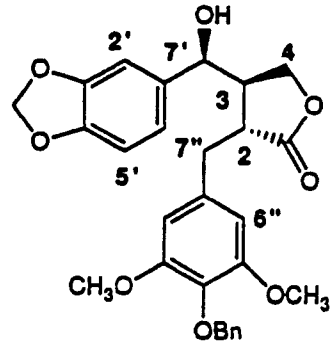
solution of 480 mg (1 equiv.) of the butenolide in anhydrous THF (8 mL) was slowly added via syringe. After this, the mixture was stirred at -70° C for another 2 h, and a solution of 2.06 g (1.07 equiv.) of the benzyl bromide 67 in THF (10 mL) was introduced. The reaction mixture was allowed to warm to room temperature, and quenched with water (25 mL) and extracted with ethyl acetate (3 x 50 mL). The combined organic extracts were washed with water (25 mL), dried, and evaporated *in vacuo* to give 4.48 g of crude mixture which from flash column chromatography (4.5 cm diameter, 5.5" silica gel) eluting with dichloromethane, yielded the pure produce as a yellow solid (2.16 g, 55%). Mp = 55-57° C (acetone). IR (KBr) ν_{max}: 1770 (lactone). ^{1}H nmr δ: 2.79 (1H, dd, H7″, J = 5, 14 Hz), 2.85-2,92 (1H, m, H2), 3.12 (1H, dd, H7″, J = 4, 14 Hz), 3.29-3.34 (1H, m, H3), 3.50 (1H, dd, H4, J = 8, 10 Hz), 3.70 (6H, s, $-OCH_3$), 4.45 (1H, dd, H4, J = 3, 10 Hz), 5.02 (2H, s, CH_2-Ph), 6.05 (2H, d, O-CH_2-O), 6.18 (2H, s, H2″, H6″), 6.73 (1H, d, H5′, J = 8 Hz), 7.12 (1H, dd, H6′, J = 2, 8 Hz), 7.20-7.47 (14H, m, aromatic). MS m/z: 583 (M^+, -PhS). UV (methanol) λ_{max} (log ϵ): 279 (3.82), 286 (3.80). HRMS calcd. for $C_{34}H_{31}O_7S$: 583.1790; found: 583.1792. Anal. calcd. for $C_{40}H_{36}O_7S_2$: C 69.34, H 5.24, S 9.25; found: C 69.18, H 5.42, S 9.23.

TRANS-2-(4-BENZYLOXY-3,5-DIMETHOXYBENZYL)-3-(3,4-METHYLENEDIOXY-α-OXO-BENZYL)BUTANOLIDE (84):[75]



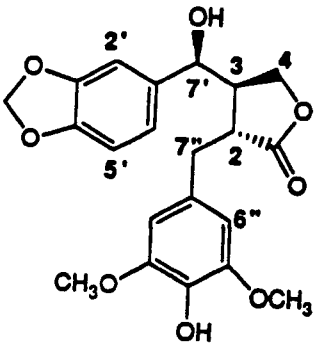
Red mercuric oxide (2.48 g 11.4 mmol) was suspended in 15% aqueous THF (40 mL) and cooled under nitrogen to 0-5^{o} C (ice-water bath). 12.92 mL of recently distilled boron trifluoride etherate were added keeping the temperature at about 0^{o} C. After stirring the suspension for 10 min at -5-0^{o} C, a solution of the thioketal **83** (4.0 g, 6.3 mmol) in anhydrous THF (80 mL) was slowly added maintaining the inner temperature at 0^{o} C. After the addition was complete, the mixture was allowed to reach room temperature, stirred for 2 h at this temperature, and then heated to 30-35^{o} C (warm water bath) for a further hour. Once the reaction was judged complete by analytical TLC (hexanes:ethyl acetate 4:2), the mixture was cooled to room temperature, and diluted with dichloromethane (400 mL) and saturated aqueous sodium carbonate (400 mL). The organic layer was separated and washed with water (200 mL), dried, filtered, and concentrated *in vacuo* to afford 4.8 g of a creamy solid. Purification of the crude mixture by flash column chromatography (4.0 cm diameter, 6.0" silica gel) packed initially with dichloromethane (dissolving the sample in warm dichloromethane (100 mL) and forerunning with 1 L ofthe same solvent followed by 700 mL of hexanes:ethyl acetate 4:2 collecting 20-mL fractions yielded 2.67 g (85.57%) of the pure product as a white solid. Mp = 43-44^{o} C (ethyl ether). IR (KBr) ν_{max}: 1770 (lactone), 1670 (ketone). ^{1}H nmr δ: 3.00 (1H, dd, H7″, J = 6, 14 Hz), 3.08 (1H, dd, H7″, J = 5, 14 Hz), 3.58 (1H, m, H2), 3.70 (6H, s, OCH_3), 4.00 (1H, ddd, H3, J = 8, 8, 8 Hz), 4.10 (1H, dd, H4, J = 8, 9 Hz), 4.39 (1H, dd, H4, J = 8, 9 Hz), 4.92 (2H, s, CH_2-Ph), 6.01 (1H, d, -O-CH_2-O-, J = 1 Hz), 6.04 (1H, d, O-CH_2-O, J = 1 Hz), 6.29 (2H, s, H2″, H6″), 6.80 (1H, d, H5′, J = 8 Hz), 7.22-7.48 (7H, m, aromatic). UV (methanol) λ_{max} (log ϵ): 275 (3.82), 314 (3.90). MS m/z: 488 (M^{+}), 91 (100).

TRANS-2-(4-BENZYLOXY-3,5-DIMETHOXYBENZYL)-3-(3,4-METHYLENEDIOXY-7′ β-HYDROXYBENZYL)BUTANOLIDE (85):



The ketone **84** (3.0 g, 6.1 mmol) was suspended in methanol (430 mL) and the suspension cooled in an ice water bath. Sodium borohydride (335 mg, 1.45 equiv.) was added and the mixture was stirred at 0° C for 5 h. When the reaction was judged complete by TLC analysis (s.s. hexanes:ethyl acetate 1:1) 53 mL of 1 N HCl were added to quench it. The mixture was concentrated *in vacuo* to remove most of the methanol, and the resulting suspension was diluted with brine (40 mL), and extracted with dichloromethane (3 x 460 mL). The combined organic extracts were washed with water (110 ml), dried, filtered and rotary evaporated to yield 2.88 g (95.68%) of the product as a white foam homogeneous by TLC. IR (KBr) ν_{max}: 3600 (OH), 1770 (lactone). ^{1}H nmr δ: 1.70 (1H, brs, D_2O+, OH), 2.50-3.10 (4H, m, H2, H3, H7"), 3.80 (6H, s, OCH_3), 3.9 (2H, m, H4), 4.60 (1H, d, H7′, J = 6.7 Hz), 5.0 (2H, s, CH_2-Ph), 5.98 (2H, d, O-CH_2-O, J = 1 Hz), 6.40 (2H, s, H2″, H6″), 6.6-6.8 (3H, m, H5′, H6′, H2′), 7.24-7.50 (5H, m, aromatic). Also the 7α-OH isomer is present in 18%. UV (methanol) λ_{max} (log ϵ): 282 (3.60). MS m/z: 490 (M^+). HRMS calcd. for $C_{24}H_{26}O_8$: 490.1627; found: 490.1627. Anal. calcd. for $C_{24}H_{26}O_8$: C 68.56, H 5.34; found: C 68.78, H 5.56.

TRANS-2-(4-HYDROXY-3,5-DIMETHOXYBENZYL)-3-(3,4-METHYLENEDIOXY-7′ β-HYDROXYBENZYL)BUTANOLIDE (57):[75]



Palladium on charcoal (10%, 550 mg) was suspended in ethanol (26 mL) in a hydrogenation flask. The vessel was purged evaporating with vacuum and breaking it with hydrogen (3 times). The flask was pressured to 1 atm of hydrogen and the suspension stirred for 1 h at room temperature. A solution of the benzylether (1.298 g, 2.6 mmol) in ethyl acetate (26 mL) was added via canule with hydrogen pressure, and the reaction mixture was stirred under 1 atm of hydrogen for a further 6 h when control TLC (s.s. hexanes:ethyl acetate 2:3) showed reaction completion. The suspension was filtered through a small pad of Celite 545, washing the bed with ethanol (50 mL). Evaporation of the filtrate produced 1.0496 g of a light green foam which upon flash chromatography purification (4 cm diameter, 6.0" silica gel), eluting with hexanes:ethyl acetate 2:3 (1.5 L) and collecting 15-mL fractions afforded 918.5 mg (86.6%) of the pure product as a white foam. IR (KBr) ν_{max}: 3.449 (OH), 1762 (lactone). ^{1}H nmr δ: 1.52 (1H, brs, D_2O+, OH), 2.52-3.16 (4H, m, H2, H3, H7"), 3.87 (6H, s, OCH_3), 3.94 (2H, m, H4), 4.65 (1H, d, H7′, J = 6.8 Hz), 5.98 (2H, m, O-CH_2-O), 6.40 (2H, s, H2″, H6″), 6.52-6.78 (3H, m, aromatic). Approximately 14% of the C7 epimer could be observed. UV (methanol) λ_{max} (log ϵ): 214 (4.39), 283 (3.79). MS m/z: 402 (M^+). HRMS calcd. for $C_{21}H_{20}O_8$: 402.1314; found: 402.1302. Anal. calcd. for $C_{21}H_{20}O_8$: C 62.69, H 5.50; found: C 62.39, H 5.70.

GENERAL PROCEDURE FOR SUBCULTURE OF PLANT SUSPENSION CULTURES.

Stringent precautions are needed for the protection of cell line purity. Information on medium, inoculum age and volume, and usual subculture interval for each stock culture is provided under the individual plant species headings.

The suspensions to be subcultured were assessed visually (usual colour?, usual degree of aggregation?, usual cell density?, gross contamination?) and microscopically (at 100 and 400X for obvious contaminants such as filamentous fungi or motile bacteria). A small sample (10-25 mL) was filtered and pH and R.I. measured. For transfer to new media, only cultures that appeared normal in all aspects were used.

Transfer routine: Using aseptic technique, in the clean air station, the stipulated aliquots of inocula were transferred into fresh media. Absolute accuracy of inocula volumes is not needed, but a measuring device should be used. More than one flask of inoculum was used for each new set of flasks to ensure that all new stock cultures came from more than a single source and a note was made as to which flasks were derived from which flasks.

Whenever possible, not all flasks of a set of stock cultures were used or discarded. One or two flasks remained on the shaker in case the new culture behaved abnormally, became contaminated, or it was discovered that there was a mistake in the preparation of the new media.

From each flask of inoculum, some culture was plated onto nutrient agar (1 mL streaked on the agar surface), incubated at 25^{o} C for 72 h, and examined for bacterial or fungal growth. Any contamination positives were not used for experiments or for future stock culture transfers.

Some culture was also plated on agar-solidified stock culture broth as an additional safeguard to perpetuate each stock culture in callus form. These cultures were kept at room temperature, in the dark, for at least one month after subculture.

At approximately monthly intervals, calli maintained as stocks for each cell line were transferred to fresh agar media. Old flasks of stock cultures were discarded when it was obvious that their respective progeny was healthy.

Podophyllum peltatum cell line 128-B.

Medium: MS (1/2) - 1.5% sucrose, vessel size: 1 L, medium volume: 300 mL, inoculum volume: 25 mL of drained cells, normal period between transfers: 17 days.

Tripterygium wilfordii cell line TRP4a-B.

Medium: PRD_2Co_{100}, vessel size: 1 L, medium volume: 500 mL, inoculum volume: 50 mL, normal period between transfers: 14 days.

Media Preparations.

(a) MS medium for *P. peltatum* and *T. wilfordii* (production)

	Stock Solution		Use	Final Concentration
	NH_4NO_3	66.0 g/L		1650 mg/L
MACRO	KNO_3	76.0 g/L	25 mL/L	1900 mg/L
	$KH_2PO_4 \cdot H_2O$	6.8 g/L		170 mg/L
	$MgSO_4 \cdot 7H_2O$	14.8 g/L		370 mg/L
	H_3BO_3	1.24 g/L		6.2 mg/L
MICRO	$MnSO_4 \cdot H_2O$	4.46 g/L	5 mL/L	22.3 mg/L
	$ZnSO_4 \cdot 7H_2O$	1.72 g/L		8.6 mg/L

	Stock Solution		Use	Final Concentration
	KI	0.166 g/L		0.83 mg/L
TRACE	$Na_2MoO_4 \cdot 2H_2O$	0.05 g/L	5 mL/L	0.25 mg/L
	$CoCl_2 \cdot 6H_2O$	0.005 g/L		0.025 mg/L
	$CuSO_4 \cdot 5H_2O$	0.005 g/L		0.025 mg/L
	$CaCl_2 \cdot 2H_2O$	40.0 g/L	11 mL	440.0 mg/L
	Na_2EDTA	4.0 g/L	9.3 mL	37.35 mg/L
	$FeSO_4 \cdot 7H_2O$	3.0 g/L		27.85 mg/L
	Thiamine-HCl	0.01 g/L		0.1 mg/L
VITAMINS	Nicotinic acid	0.05 g/L		0.5 mg/L
	Pyridoxine HCl	0.05 g/L	10 mL	0.5 mg/L
	Glycine	0.2 g/L		2.0 mg/L
	Myo inositol	10.0 g/L		100.0 mg/L
	Sucrose	20 g/L	20 g/L	2%
	NAA	0.05 mg/mL	1 mL/L	0.5 mg/L
	Kinetin	0.5 mg/mL	1 mL/L	0.5 mg/L

For $MSN_{(0.5\ mg/L)}K_{(0.5\ mg/L)}$, use as indicated, final pH=5.8.

For MS (1/2) - 1.5%, use half of everything but leave out the hormones (NAA and kinetin), and use 15 g/L of sucrose so that the final concentration is 1.5%. Adjust the pH to 5.8 once the final volume has been reached, using 1 M NaOH or 1 M HCl as required.

To prepare agar, add 7-8 g of Difco agar per liter of medium.

(b) PRDCo medium for *T. wilfordii* (maintenance)

Stock Solution		Use	Final Concentration
$(NH_4)_2SO_4$	40.0 g/L		200 mg/L
$NaH_2PO_4 \cdot H_2O$	18.0 g/L		90 mg/L
Na_2HPO_4	6.0 g/L		30 mg/L
KI	0.15 g/L	5 mL/L	75 mg/L
H_3BO_3	0.6 g/L		0.30 mg/L
$Na_2MoO_4 \cdot 2H_2O$	0.05 g/L		0.25 mg/L
$CoCl_2 \cdot 6H_2O$	0.05 g/L		0.25 mg/L
KNO_3	100.0 g/L	10 mL/L	1000 mg/L
KCl	30.0 g/L		300 mg/L
$MgSO_4 \cdot 7H_2O$	50.0 g/L		250 mg/L
$MnSO_4 \cdot 4H_2O$	2.64 g/L	5 ml/L	13.2 mg/L
$ZnSO_4 \cdot 7H_2O$	0.6 g/L		30 mg/L
$CuSO_4 \cdot 5H_2O$	0.05 g/L		0.25 mg/L
$CaCl_2 \cdot 2H_2O$	40.0 g/L	3.75 mL/L	150 mg/L
Na_2EDTA	4.0 g/L	9.3 mL/L	37.2 mg/L
$FeSO_4 \cdot 7H_2O$	3.0 g/L		27.9 mg/L
Casein hydrolysate		2.0 g/L	0.2%
Sucrose		20.0 g/L	2%
Coconut milk		100 mL/L	10%
2,4-D		4 mL/L	2 mg/L

Stock Solution		Use	Final Concentration
nicotinic acid	0.1 g/L		1 mg/L
thiamine·HCl	1.0 g/L	10 mL/L	10 mg/L
pyridoxine·tHCl	0.1 g/L		1 mg/L
myo inositol	10.0 g/L		100 mg/L

Final pH adjusted to 6.2 with 1 M NaOH or 1 M HCl as required.

BIOTRANSFORMATION OF SUBSTRATE 56 WITH *P. PELTATUM* CELL SUSPENSION CULTURE.

P. peltatum cells were grown in a Microferm for 2 days (450 mL drained cells, R.I. = 1.3333, pH = 4.8, inoculum 18 days old) in 3 L of 1/2 MS - 1.5% sucrose medium at T = 26^{o} C, 300-400 mL/min L air and stirring at 440 rpm with two impellers. After this period, no apparent contamination was found (microscopic inspection and NA plate), the pH was 4.9 and R.I. = 1.3349. Substrate **56** (3.0 g) dissolved in ethanol (70 mL) was injected into the Microferm while filtering with a sterile HV (0.2 μ) membrane to keep aseptic conditions. The broth turned milky and biotransformation was allowed to proceed withdrawing samples to check reaction progress after 2 days (48 h, 40 min), 4 days (89 h, 30 min) and 5 days (111 h, 40 min). Analytical data indicated that the disappearance of substrate **56** was >50% after two days and only a trace of it remained after 4 days.

The Microferm was harvested (pH = 4.35, R.I. = 1.3352, no apparent contamination shown by microscopic inspection) and the broth and cells were extracted separately.

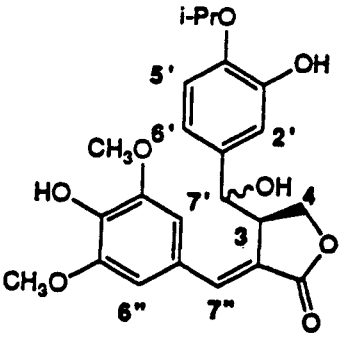
The cells were soaked overnight in 700 mL EtOAc, drained and homogenized in EtOAc (500 mL) using an Ultra Turrax T25 blender. The mass was filtered through a short pad of Celite 545 and the filtrate was mixed with the first EtOAc extract, dried, filtered and evaporated to dryness to yield 3.74 g of extract. The cell debris and Celite were sonicated with methanol (700 mL) for 30 min, filtered, dried and concentrated to yield 2.4 g of methanolic cell extract.

The broth was extracted as follows: ethyl acetate (800 mL) were added and the mixture shaken vigorously for 15 min. Then salt was added to saturate the aqueous layer and the mixture was stirred vigorously for another 15 min. Some protein separated forming a gelatinous layer that was filtered off with the aid of Celite (30 g). The cake was washed with ethyl acetate (100 mL) and the filtrate transferred to a separatory funnel. The aqueous layer was drained and extracted with ethyl acetate (2 x 800 mL). The organic extracts were combined, dried, filtered, and rotary evaporated to give 3.1 g of extract as a brown foam. A second extraction of the broth was made using dichloromethane (1.5 L) to give 8.7 mg of an orangey extract.

Analysis of the extracts indicated the presence of lignan-type compounds in the ethyl acetate extracts, and these were purified by repeated column chromatography using chloroform-methanol mixtures as described in the discussion. Final purifications were made by preparative TLC using the same eluant.

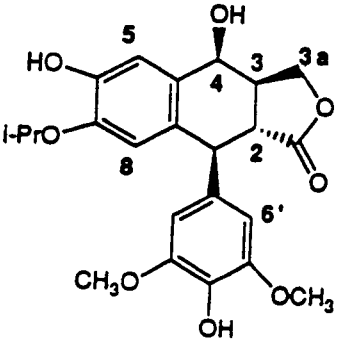
The following metabolites were identified in order of elution:

E-2-(3,5-DIMETHOXY-4-HYDROXYBENZYLIDENE)-3-(3,7-DIHYDROXY-4-ISOPROPOXYBENZYL)BUTANOLIDE (74):



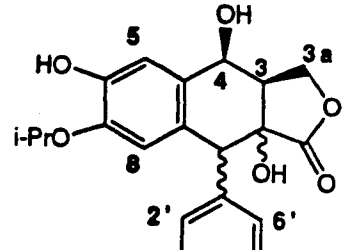
107 mg (3.5%). IR (KBr) ν_{max}: 1720 cm^{-1} (carbonyl). ^1H nmr (methanol-d 4) δ: 1.3 (6H, d, $CH(CH_3)_2$), 4.08 (1H, m, H3), 4.28 (1H, m, H4), 4.49 (1H, septet, $CH(CH_3)_2$), 4.57 (1H, m, H4), 5.02 (d, 1H, H7′), 3.93 (6H, s, OCH_3), 6.64-6.82 (3H, m, aromatic), 7.10 (2H, s, H2", H6"), 7.50 (1H, s, H7"). UV (methanol) λ_{max}: 324 (3.77), 203 (4.19). MS m/z: 430 (M^+, 18.0), 412 (M^+-H_2O, 7.8), 372 (11.4), 248 (28.1), 220 (15.8), 211 (14.9), 191 (52.7), 181 (43.6), 154 (85.8), 137 (100). HRMS calcd. for $C_{23}H_{26}O_8$: 430.1628; found: 430.1621. The product failed to give satisfactory elemental analysis.

1-(3,5-DIMETHOXY-4-HYDROXYPHENYL)-6,4β-DIHYDROXY-3-HYDROXYMETHYL 7-ISOPROPOXY-1,2,3,4-TETRAHYDRO-2-NAPHTHOIC ACID γ LACTONE (75):



The data presented are for an inseparable mixture of **75** and the 4α-isomer (ratio of **75** to 4α-isomer 10:1). Nmr data as cited below allowed the analysis of the isomer ratio. 1.00 g (33.5%). IR (KBr) ν_{max}: 3495 (OH), 1774 (lactone) cm^{-1}. ^1H nmr δ: 1.14 (3H, d, $CH(CH_3)_2$), 1.28 (3H, d, $CH(CH_3)_2$), 1.55 (1H, brs, D_2O+, OH), 2.58 (m, H3α), 2.68 (1H, m, H3β), 3.19 (1H, dd, H2, J = 11.4, 14 Hz), 3.86 (6H, s, OCH_3), 3.96 (1H, d, H1, J = 11.4 Hz), 4.25-4.47 (3H, m, H3a, $CH(CH_3)_2$), 4.85 (1H, d, H4β, J = 2.7 Hz), 4.9 (d, H4α, J = 12.3 Hz), 5.5 (1H, brs, D_2O+, OH), 5.7 (1H, brs, D_2O+. OH), 6.30 (s, H5α), 6.38 (1H, s, H5β), 6.42 (s, H2″, 6″α), 6.46 (2H, s, H2″, Hβ6″), 6.88 (1H, s, H8). UV (methanol) λ_{max} (log ε): 283 (3.68), 215 (4.53). MS m/z: 430 (M^+, 35.3), 388 (44.0), 154 (100). HRMS calcd. for $C_{23}H_{26}O_8$: 430.1628; found: 430.1622. The product failed to give satisfactory elemental analysis. Attempts to crystallize the material afforded only a sticky solid.

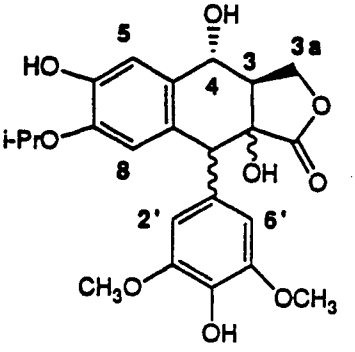
1-(3,5-DIMETHOXY-4-HYDROXYPHENYL)-2,4β,6-TRIHYDROXY-3-HYDROXYMETHYL-7-ISOPROPOXY-1,2,3,4-TETRAHYDRO-2-NAPHTHOIC ACID LACTONE (76):



191 mg (6.18%). IR (KBr) ν_{max}: 3484 (CH), 1746 (lactone). ^1H nmr δ: 1.33 (3H, d, $CH(CH_3)_2$), 1.38 (3H, d, $CH(CH_3)_2$), 3.51-3.62 (7H, m, H3, OCH_3), 4.03 (d, 1H, H4, J = 7.2 Hz), 4.21 (dd, 1H, one of lactone, J = 8.3, 9.6 Hz), 4.37 (dd, 1H, one of lactone, J = 9.6, 9.6 Hz), 4.66 (1H, septet, $CH(CH_3)_2$), 5.22 (1H, s, H1), 6.21 (2H, s, H2′,

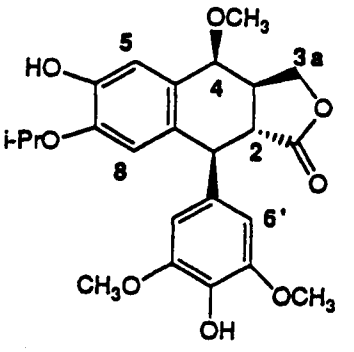
H6′), 6.61 (1H, s, H5), 7.08 (1H, s, H8). UV (methanol) λ_{max} (log ϵ): 290 (3.56), 206 (4.40). MS m/z: 446 (M^+, 0.9), 428 (M^+-H_2O, 2.1), 386 (2.1), 286 (81.6), 264 (100). HRMS calcd. for $C_{23}H_{26}O_9$: 446.1577; found: 446.1583. No satisfactory elemental analysis could be obtained. Attempts to crystallize the material produced only a white mass.

1-(3,5-DIMETHOXY-4-HYDROXYPHENYL)-2,4α,6-TRIHYDROXY-3-HYDROXYMETHYL-7-ISOPROPOXY-1,2,3,4-TETRAHYDRO-2-NAPHTHOIC ACID LACTONE (77):



12.7 mg (0.4%). IR (neat): 3424 (OH), 1709 (lactone) cm^{-1}. ^1H nmr δ: 1.32 (two doublets, 6H, $CH(CH_3)_2$), 3.39 (1H, m, H3), 3.72 (6H, s, OCH_3), 4.04 (dd, 1H, one of lactone, J = 8, 8 Hz), 4.24 (dd, 1H, one of lactone, J = 8, 9.6 Hz), 4.56 (1H, septet, $CH(CH_3)_2$), 4.61 (1H, d, H4, J = 6 Hz), 5.08 (1H, s, H1), 6.49 (2H, s, H2′, H6′), 6.78 (1H, s, H5), 7.0 (1H, s, H8). UV (methanol) λ_{max} (log ϵ): 289 (3.68), 210 (4.53). MS m/z: 446 (M^+, 0.7), 428 (M^+-H_2O, 10.2), 386 (10.0), 264 (100). HRMS calcd. for $C_{23}H_{26}O_9$: 446.1577; found: 446.1585. No satisfactory elemental analysis could be obtained. Attempts to crystallize the product produced a sticky solid.

1-(3,5-DIMETHOXY-4-HYDROXYPHENYL)-6-HYDROXY-3-HYDROXYMETHYL-7-ISOPROPOXY-4β-METHOXY-1,2,3,4-TETRAHYDRO-2-NAPHTHOIC ACID LACTONE (78):



28.0 mg (0.9%). IR ($CHCl_3$) ν_{max}: ^1H nmr δ: 1.4 (3H, d, $CH(CH_3)_2$), 1.26 (3H, d, $CH(CH_3)_2$), 1.61 (1H, brs, D_2O+, OH), 2.68 (1H, m, H3), 3.18 (1H, dd, H2, J = 11.5, 14 Hz), 3.84, 3.86 (two singlets, 9H, OCH_3), 3.96 (1H, d, H1, J = 11.5 Hz), 4.25-4.47 (3H, m, H3a, $CH(CH_3)_2$), 4.85 (1H, d, H4, J = 2.6 Hz), 5.48 (1H, brs, D_2O+, OH), 5.68 (1H, brs, D_2O+, OH), 6.38 (1H, s, H5), 6.47 (2H, s, H2′, H6′'), 6.88 (1H, s, H8). MS m/z: 444 (M^+, 18.8), 402 (15.5), 154 (42), 40.0 (100). HRMS calcd. for $C_{24}H_{28}O_8$: 444.1784; found: 444.1791. This thick oil could not be crystallized. No satisfactory elemental analysis could be obtained.

ARYL TETRALIN 75. STABILITY STUDIES.

P. peltatum cells were grown in shake flasks for 3, 11, 17, and 21 days and eight experiments with one flask each (containing 300 mL of cell suspension) were started. Aryl tetralin 75, dissolved in 3 mL of ethanol was added under aseptic conditions to four flasks (one of each age), and a control was set adding 3 mL ethanol to the remaining flasks.

All flasks were incubated in the rotary shaker and reaction progress was followed by withdrawal of 2.5 mL aliquots and reverse phase HPLC analysis every hour. After 5 h of incubation, the flasks were incubated overnight and harvested the next morning.

For harvesting, the cells and spent medium were homogenized together using an Ultra Turrax T-25 disperser and filtered through Celite 545. The filtrate was saturated with NaCl and extracted with EtOAc (3 x 150 mL). The cell debris and Celite were sonicated for 30 min in EtOAc (150 mL), filtered and washed with more EtOAc (50 mL). The combined organic extracts (from spent medium and cell debris) were dried, filtered, and rotary evaporated to dryness. The ethyl acetate extracts (usually c.a. 250 mg) were purified separately by flash column chromatography using a 3 cm diameter column, packed with 7.0" silica gel using a 19:1 mixture of chloroform:methanol (600 mL) collecting 10-mL fractions. The pure recovered product, typically fractions 12-24, was pooled together and rotary evaporated, affording yields in the range of 72-94.1%.

BIOTRANSFORMATION OF SUBSTRATE 57 WITH *P. PELTATUM* CELL SUSPENSION CULTURE.

P. peltatum cells were grown in a bioreactor for 7 days. After this period, no contamination was apparent upon microscopic inspection (pH = 4.5, R.I. = 1.3340). The pH was adjusted to 6.5 with 0.2 M KOH solution, and 1 mL of polypropylen glycol was added as anti-foam agent. A solution of substrate 57 (491 mg) in ethanol (10 mL) was added to the cell culture filtering through a sterile HV (0.2 μ) membrane, and biotransformation was allowed to proceed for 5 h. TLC analysis of a sample indicated partial conversion to a more polar compound with strong UV absorption. The broth was filtered out under aspectic conditions, and the cells were resuspended in 1/10 MS medium (3.0 L) containing a sterile solution of substrate 57 (501.7 mg) in ethanol (10 mL). Biotransformation was allowed to proceed for 20 h. After this time, the broth was again removed and the cells resuspended in 1/10 MS medium (3.0 L) containing a solution of the substrate 57 (488.2 mg) in ethanol (10 ml). Biotransformation

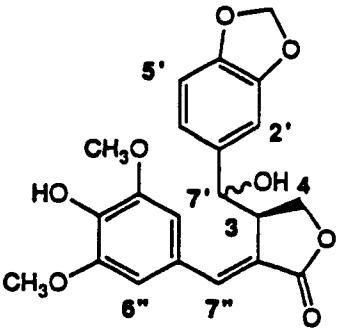
proceeded for 20 h before the broth was removed and the cells resuspended in fresh 1/2 MS - 1.5% sucrose medium (3.0 L).

The three broths from this experiment were extracted immediately following the same procedure as with the previous biotransformations (*vide supra*) affording 1.66 g, 469 mg, and 324 mg, respectively.

A typical purification procedure is exemplified with the first broth extract:

The extract was dissolved in ethyl acetate and applied to a 4 cm diameter column packed with the flash technique (6" silica gel) using hexane:EtOAc 2:3 (2 L). The column was run collecting 72, 10-mL fractions and then 25-mL fractions until the solvent was finished. The column was then eluted successively with EtOAc (500 mL) collecting 25-mL fractions, 10% methanol in EtOAc (500 mL) collecting 25-mL fractions and methanol (500 mL) collecting 50-mL fractions. The fractions were spotted on TLC and those which had similar composition were grouped together and rotary evaporated to dryness. Fraction 1 (from 1 to 31) contained non polar material (40.4 mg), fraction 2 (from 32-72) contained recovered starting material (136.4 mg), fraction 3 (from 73-112) contained a mixture of recovered starting material and the styrene **86** (102.5 mg). Fraction 4 (from 113 to 124) contained the product **86** (243.9 mg), fraction 5 (from 125 + 132) contained indigenous metabolites (186.0 mg), fraction 6 (from 132-158) consisted of recovered polypropylen glycol (822.6 mg) and fraction 7 (methanol wash) contained the polar material as a brown solid (120 mg). The total recovery was 1.65 g (98.9%). Yield of styrene **86** based on recovered starting material = 273.9 mg (90.3%) (50 mg recovered starting material + 30 mg styrene from fraction 3).

2-(3,5-DIMETHOXY-4-HYDROXYBENZILIDENE -3-(3,4-METHYLENEDIOXY-7-HYDROXYBENZYL)BUTANOLIDE (86):



IR (KBr) ν_{max}: 3357 (OH), 1715 (lactone). ^{1}H nmr (DMSO-d 6) δ: 3.85 (6H, s, OCH_3), 4.10 (1H, brs, H3), 4.24 (1H, brt, H4), 4.40 (1H, brd, H4), 4.95 (1H, brs, H7′). 5.80 (1H, brs, D_2O+, OH), 5.94 (2H, brd, O-CH_2-O), 6.6 (1H, d, H6), 6.75 (2H, m, H5′, H2′), 7.10 (2H, s, H2″, H6″), 7.40 (1H, s, H7″), 9.07 (1H, brs, D_2O+, OH). UV (methanol) λ_{max} (log ϵ): 208 (4.34), 240 (4.24), 335 (4.28). MS m/z: 400 (M^+, 100), 353 (36), 312 (11.6), 311 (52), 287 (31.6), 286 (96.8), 285 (28.9), 258 (11.8), 251 (16.2), 250 (87.6). HRMS calcd. for $C_{21}H_{20}O_8$: 400.1158; found: 400.1161. Despite several attempts aimed to crystallize the product, no satisfactory elemental analysis could be obtained.

DEHYDROABIETIC ACID (122) PURIFICATION.

Technical grade dehydroabietic acid (ICN, 200.0 g) was dissolved in warm ethanol (488 mL) in a 1.5 L capacity liquid-liquid extractor. Ethanolamine (40.0 mL) and water (488 mL) were added while stirring, and the resulting mixture extracted continuously with petroleum ether (60-80^{o} C) for 20 h, while being kept at 50-55^{o} C. The resulting aqueous solution, thus freed of neutral material, was boiled briefly to drive out any dissolved petroleum ether. Cooling this solution to 0-5^{o} C overnight, resulted in a semi-solid mass, which was broken up manually. Filtration provided the ethanolamine salt of dehydroabietic acid, which was stirred with 50% aqueous ethanol (132 mL) at 4^{o} C and vacuum filtered. The moist salt was dissolved in hot ethanol (334 mL), acetic acid (33.2 mL) was added, and water (160 mL) was gradually added to the boiling solution until the cloud point was reached. The hot solution was filtered, and cooled to room temperature. The acid that crystallized was isolated by filtration, rinsed with 50% aqueous ethanol (40 mL), and air-dried to provide 59.8 g of dehydroabietic acid, 88.7% pure by G.C. analysis. MP = 160-161^{o} C (Lit. = 171-172^{o} C).

DEHYDROABIETIC ACID ANALYSIS BY GAS CHROMATOGRAPHY.

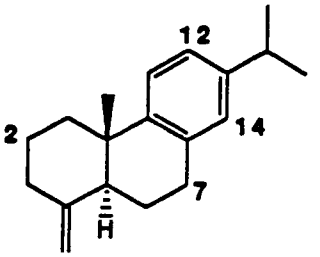
This method analyzes acids as methyl esters and is comparable to diazomethane treatment.

Sample preparation: dissolve approximately 5.0 mg of sample in 1 mL of methanol. Add one drop of 1% w/v phenolphtalein in methanol solution, and then dropwise 10% w/v tetramethyl ammonium hydroxide in methanol solution mixing well until the pink colour remains for 5 min without change.

Instrument conditions: Initial temperature: 220^{o} C, final temperature: 245^{o} C, stop time: 20 min; rate: 0.7^{o} C/min, Attn: 1; Detector temp. : 300^{o} C, Injector/temp: 300 ^{o} C.

With these parameters the retention time for methyldehydro abietate is 15 min.

18-NORABIETA-4(19),8,11,13-TETRAENE (150):[119]



Dehydroabietic acid (44.2 g, 0.147 mol) was dissolved in benzene (250 ml). Thionyl chloride (13 mL, 0.18 mol) and DMF (0.5 mL) were added, and the solution stirred for 1 h at room temperature. After this time, the mixture was warmed to 68-73^{o} C and this range of temperature was maintained for 30 min. The solution was then refluxed for 15 min, then cooled and rotary evaporated. The crude acid chloride (IR (neat) ν_{max} 1786 cm^{-1}) was taken up in reagent grade acetone (300 mL), and the solution cooled to -5-0^{o} C in a methanol-ice bath. A solution of sodium azide (12 g, 0.18 mol) in water (40 mL) was added dropwise, and with vigorous stirring. Toluene (100 mL) was then added, and the organic layer decanted, dried, filtered, and the acetone was removed by rotary evaporation. The solution of acyl azide (IR (toluene) ν_{max}: 2120 (azide), 1692 (carbonyl) cm^{-1}) was brought to 300 mL with additional toluene and heated slowly to reflux. After refluxing for 30 min, an IR of a sample of the solution showed complete reaction. The solvent was then removed *in vacuo.* The crude isocyanate (183) (IR (neat) ν_{max}: 2250 (NCO) cm^{-1}) was added to a suspension of LAH (7 g, 0.18 mmol) in anhydrous THF (600 mL), and the mixture refluxed with stirring under nitrogen overnight.

Successive dropwise addition of acetone (10 mL), water (7 mL), 15% aqueous sodium hydroxide (7 mL), and more water (20 mL) produced a thick white suspension which was filtered and triturated with hot THF (200 mL). The combined organic

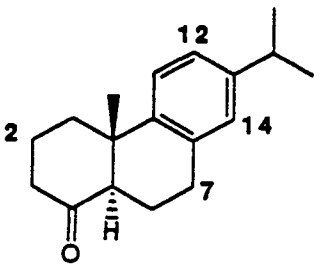
solutions were rotary evaporated to give the crude monomethyl amine. This was refluxed with formic acid (100 mL) and 35% aqueous formaldehyde (50 mL) for 3 h, and the solvents removed *in vacuo*. The resulting tarry mass was shaken with ether (300 mL) and 4 N NaOH (200 mL) until dissolved. The organic layer was drained, dried, filtered, and evaporated. The crude dimethylamine (**184**) was obtained as a golden syrup. To a solution of 40.9 g (0.13 mol) of this product in chloroform (680 mL), cooled to -40° C, was added 85% MCPBA (34.8 g, 0.17 mol) in small portions over a 20 min period. Once the addition was complete, the reaction was stirred 10 min at -40° C. Triethylamine (8.2 mL, 82 mmol) was added, and the solution brought to reflux. After 30 min, the reaction mixture was cooled and the solvent removed *in vacuo*. The residue was taken up in ether (550 mL) and washed with 10% sulfuric acid (500 mL), 10% potassium carbonate (2 x 500 mL), dried, filtered, and rotary evaporated.

The crude exo-olefin (31.8 g) was purified by gravity column chromatography (200 g of silica gel) eluting with hexanes to afford the pure exo-olefin as a colourless oil (20.8 g, 55.8% overall). IR (neat) ν_{max}: 3050 ($C=CH_2$), 2925 (CH), 1650 (C=C). ^1H nmr δ: 1.02 (3H, s, C10, CH_3), 1.25 (6H, d, $CH(CH_3)_2$, J = 6 Hz), 1.51-2.92 (m, aliphatic H), 4.61 (1H, d, H4, J = 2 Hz), 4.86 (1H, d, H4, J = 2 Hz), 6.94 (1H, brs, H14), 7.01 (1H, brd, H12), 7.22 (1H, d, H11). MS m/z: 254 (M^+, 42.9), 239 (92.9), 211 (4.5), 197 (100). HRMS calcd. for $C_{19}H_{26}$: 254.2034; found: 254.2030. UV (methanol) λ_{max} (log ϵ): 207 (4.19). Anal. calcd. for $C_{19}H_{26}$: C 89.70, H 10.30; found: C 89.90, H 10.22.

18,19-DINORABIETA-8,11,13-TRIEN-4-ONE (151):

A stock solution of the olefin **150** (20.8 g, 0.081 mol) in methanol-methylene chloride 5:1 (945 mL) was divided in 4 portions of 240 mL each. Ozone was passed into the stirred solutions at -78° C until they turned a pale blue colour (approximately 1 h per flask at 2.2 psi of oxygen, 90 volts, and a flow of 0.015 L/min). The reaction mixtures were stirred for a further 30 min at -70° C without the ozone stream. Analytical TLC on hexanes of the reaction media showed reaction completion. Dimethyl sulphide (2 mL, 26.5 mmol) was added to each flask and the reaction mixtures were stirred at room temperature for 20 h. Analytical TLC (s.s. hexanes:ethyl acetate 9:1) showed that all the reactions were alike, and they were mixed at this point, and the solvent removed *in vacuo*. The residue was dissolved in hexanes:ethyl ether 2:1 (690 mL) and washed with water (3 x 86 mL) and brine (86 mL). The aqueous layer was extracted back with ether (130 mL) and the combined organic layers were dried, filtered, and rotary evaporated. The yellow residue was separated by flash column chromatography (4.5 cm diameter, 6" silica gel) using hexanes:ethyl acetate 9:1 to yield 18.0 g (90.6%) of the ketone **151** as a white solid and 2.0 g (9.04%) of diketone **151b** as a white solid.

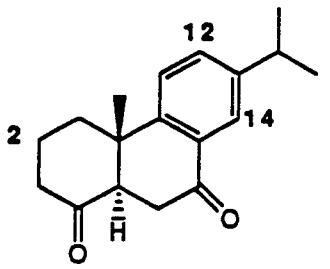
18,19-DINORABIETA-8,11,13-TRIEN-4-ONE (**151**):[110]



Mp = 40-42° C. IR ($CHCl_3$) ν_{max}: 2950 (CH), 1710 (carbonyl). ^1H nmr δ: 1.06 (3H, s, C10 CH_3), 1.24 (6H, d, $CH(CH_3)_2$), 1.76-2.94 (m, aliphatic), 6.95 (1H, brs, H14), 7.04 (1H, brd, H12), 7.22 (1H, d, H11). UV (methanol) λ_{max} (log ϵ): 214 (3.98).

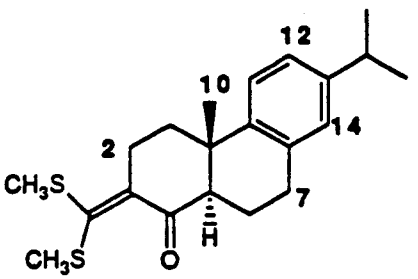
MS m/z: 256 (33.3, M^+), 241 (100). HRMS calcd. for $C_{18}H_{24}O$: 256.1827; found: 256.1826. Anal. calcd. for $C_{18}H_{24}O$: C 84.33, H 9.43; found: C 84.30, H 9.21.

18,19-DINORABIETA-8,11,13-TRIEN-4,7-DIONE (151b):[110]



Mp = 107-108° C. IR ($CHCl_3$) ν_{max}: 2950 (CH), 1715 (carbonyl), 1680 (carbonyl). ^1H nmr δ: 1.17 (3H, s, C10, CH_3, J = 6 Hz), 1.27 (6H, d, $CH(CH_3)_2$), 1.98-3.30 (m, aliphatic), 7.38 (1H, d, H11, J = 8 Hz), 7.45 (1H, dd, H12, J = 3, 8 Hz), 7.95 (1H, d, H14, J = 3 Hz). UV (methanol) λ_{max}: (log ϵ): 254 (3.56), 204 (4.09). MS m/z: 270 (M^+, 35.6), 255 (100). HRMS calcd. for $C_{18}H_{22}O_2$: 270.1620; found: 270.1613. Anal. calcd. for $C_{18}H_{22}O_2$: C 79.96, H 8.20; found: C 80.18, H 8.23.

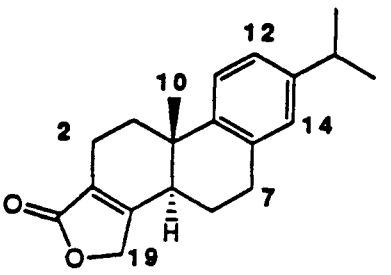
3-DIMETHYLTHIOMETHYLENE-18,19-DINORABIETA-8,11,13-TRIEN-4-ONE (152):[110]



N-Butyllithium (1.6 M in hexanes, 41.5 mL, 66.4 mmol) was added to a stirred solution of 4-methyl-2,6-di-*t*-butylphenol (14.7 g, 66.6 mmol) in anhydrous THF (348 mL) at 0-5° C under argon. Carbon disulphide (13.4 mL, 0.22 mmol) was added, and the resulting light yellow solution was allowed to warm to room temperature. A

solution of the ketone **151** (6.6 g, 25.7 mmol) in dry THF (52 mL) was added, and the flask that contained the ketone solution was washed with 18 mL dry THF and this washing was added to the reaction mixture. Stirring was continued at room temperature for 48 h. Analytical TLC of a sample (s.s. hexanes:ethyl acetate 9:1) showed no remaining starting material. Methyl iodide (8.94 mL, 142.01 mmol) was then added and the reaction mixture was stirred (wrapped in aluminum foil) for a further 20 h. The solvent was evaporated and the residue dissolved in ether (910 mL), washed with water (3 x 280 mL) and brine (280 mL), dried, filtered, and concentrated *in vacuo*. The orange residue (26.3 g) was purified by gravity column chromatography (263 g of silica gel) using 1.3 L of hexanes as eluent, to recover the phenol and followed by hexanes:ethyl acetate 9:1 (2.1 L) to yield 8.44 g (91.37%) of the ketone thioketal as an orange oil which solidifies upon cooling. Mp = 68-70^{o} C (i-PrOH). IR (KBr) ν_{max}: 2956 (CH), 1714, 1680 (C=O). ^{1}H nmr δ: 1.1 (3H, s, C10 CH_3), 1.23 (6H, d, $CH(CH_3)_2$), 1.76-3.42 (m, aliphatic, H), 2.37 (3H, s, CH_3-S), 2.39 (3H, s, CH_3-S), 6.95 (1H, brs, H14), 7.04 (1H, brd, H12), 7.22 (1H, d, H11). UV (methanol) λ_{max} (log ϵ): 211 (4.23), 314 (3.81). MS m/z: 360 (M^+, 15.7), 345 (27.1), 313 (10.3), 256 (24.7), 253 (71.1), 241 (62.4), 220 (20.8), 213 (19.0), 205 (81.8), 141 (22.5), 1129 (29.3), 115 (22.1), 91 (100). HRMS calc. for $C_{21}H_{28}OS_2$: 360.1583, found: 360.1584. Anal. calcd. for $C_{21}H_{28}OS_2$: C 69.99, H 7.77, S 17.79; found: C 70.19, H 7.87, S 17.94.

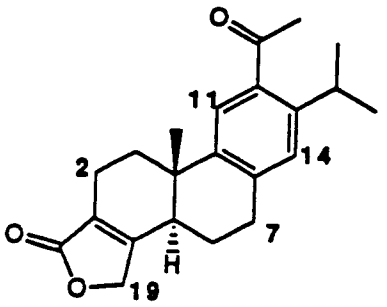
19-HYDROXY-18(4 → 3)*ABEOABIETA*-3,8,11,12-TETRAEN-18-OIC ACID LACTONE (132):[110]



N-butyllithium (1.6 M in hexanes, 5.3 mL, 8.48 mmol) was added to a stirred suspension of trimethyl sulphonium iodide (1.92 g, 9.39 mmol) in dry THF (40 mL) at -70° C under argon, and the reaction mixture was allowed to warm to -10° C over 30 min. The reaction mixture was cooled again to -70° C and a solution of the ketene thioketal **152** (2.26 g, 6.26 mmol) in THF (12 mL) was added slowly via canule. Some precipitate appeared. The flask that contained the thioketal was washed with 1.0 mL dry THF and the washing was added to the reaction flask. The mixture was stirred at -70° C for 30 min, and then allowed to slowly reach room temperature (in approximately 45 min). After stirring at room temperature for 2 h, control TLC (s.s. hexanes:ethyl acetate 9:1) showed no remaining starting material. The solvent was evaporated at room temperture, and the residue was dissolved in ethyl ether (160 mL) and washed with water (2 x 20 mL). Removal of the solvent left a residue which was dissolved in acetonitrile (13.5 mL) and the methanol (40 mL), and the solution was cooled in an ice bath. Concentrated HCl (4.8 mL) was added while stirring and the cooling bath was removed immediately after the addition. The reaction mixture was stirred at room temperature for 40 h, and the methanol and acetonitrile were evaporated. The residual suspension was extracted into ether (160 mL) and the organic solution was washed with saturated sodium bicarbonate (3 x 20 mL) and water (20 mL), dried, filtered, and rotary evaporated to afford an orangey-brown residue which was purified by gravity column chromatograph (100.0 g of silica) using 1 L of hexanes:ethyl acetate 8:2 as eluent to give 1.374 g (73.9%) of the pure lactone **132** as an oil which slowly solidifies to give a white solid. Mp = 97-99° C (EtOH). IR (KBr) ν_{max}: 2959 (CH), 1757 (C=O), 1678 (C=C). ^{1}H nmr δ: 1.02 (3H, s, C10 CH_3), 1.24 (6H, d, $CH(CH_3)_2$), 1.71-2.60 (m, aliphatic), 2.73 (1H, m, H5), 2.86 (1H, septet, H15), 3.02 (2H, m, H7), 4.78 (2H, m, H19), 6.99 (1H, brs, H14), 7.06 (1H, brd, H12), 7.27 (1H, d, H11). UV (methanol) λ_{max} (log ϵ): 221 (4.00). MS m/z: 296 (M^+, 28.8), 281 (100).

HRMS calcd. for $C_{20}H_{24}O_2$: 296.1776; found: 296.1776. Anal. calcd. for $C_{20}H_{24}O_2$: C 81.05, H 8.15; found: C 81.06, H 8.20.

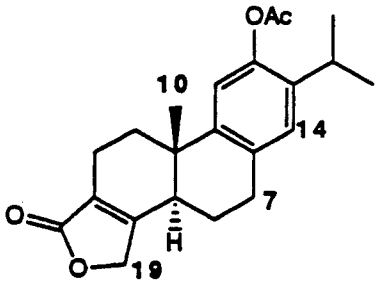
12-ACETYL-19-HYDROXY-18(4→3)*ABEOABIETA*-3,8,11,13-TETRAEN-18-OIC ACID LACTONE (192):



Anhydrous aluminum trichloride (2.93 g, 22 mmol) was suspended in carbon disulphide (150 mL), and a solution of the lactone (2.07 g, 6.9 mmol) and acetyl chloride (2.34 mL, 33 mmol) in carbon disulphide (40 mL) was added under efficient stirring. A brown precipitate appeared and the mixture was refluxed under argon with good stirring overnight. The carbon disulphide was evaporated at room temperature and iced HCl (112 mL) (3:10) was added to the residue. The mixture was stirred for 15 min in the ice-water bath and then 30 min at room temperature. After all the complex had been destroyed, the product was extracted into ethyl ether (225 mL), washed with water (2 x 75 mL), saturated sodium bicarbonate solution (3 x 75 mL), more water (75 mL), and finally with brine (75 mL). The organic solution was dried, filtered, and rotary evaporated to yield the crude product which was crystallized from methanol (30 mL) and vacuum dried at 50° C for 1 h to yield 1.61 g (68.36%) of the pure product as white crystals. Mp = 161-163° C (MeOH). IR (KBr) ν_{max}: 2952 (CH), 1751 (C=O), 1669 (C=O). ^{1}H nmr δ: 1.05 (3H, s, C10 CH_3), 1.23 (6H, dd, $CH(CH_3)_2$), 1.70-2.63 (m, aliphatic), 2.58 (3H, s, CH_3C=O), 2.73 (1H, m, H5), 3.07 (2H, brt, H7), 3.48 (1H, septet, H15), 4.80 (2H, m, H19), 7.15 (1H, s, H14), 7.47 (1H, s, H11). UV (methanol) λ_{max} (log ϵ): 253 (3.89), 217 (4.39). MS m/z: 338 (M^+, 35.4),

323 (100). HRMS calcd. for $C_{22}H_{26}O_3$: 338.1882; found: 338.1882. Anal. calcd. for $C_{22}H_{26}O_3$: C 78.08, H 7.73; found: C 78.14, H 7.60.

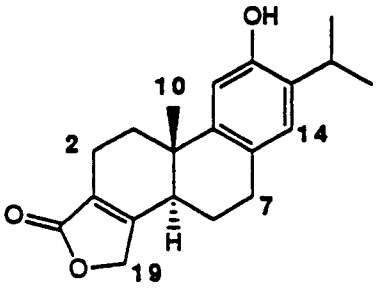
12-ACETOXY-19-HYDROXY-18(4 → 3)*ABEOABIETA*-3,8,11,13-TETRAEN-18-OIC ACID LACTONE (193):



To a solution of the acetyl lactone **192** (250 mg, 0.735 mmol) in dichloromethane (1.5 mL) was added m-chloroperoxybenzoic acid (330 mg, 2.6 equiv.) and the resulting suspension was cooled to -5-0° C (ice-methanol bath) under argon. Trifluoroacetic acid (60 μL) was added as catalyst, and the mixture was allowed to reach room temperature protected from light, and stirred overnight, until analytical TLC showed reaction completion (s.s. i-propyl ether, developed twice). The reaction took usually 20 h to completion, then it was diluted with ethyl acetate (10 mL) and washed once each with 10% Na_2SO_3 (1.5 mL), saturated $KHCO_3$ (1.5 mL) and brine (1.5 mL), the organic layer was dried, filtered and concentrated *in vacuo* to yield the essentially pure compound as a white foam (261.3 mg, 99.8%). The ester can be used as is or purified by flash column chromatography (1.5 cm diameter, 5.5" silica gel) eluting with 200 mL of diisopropyl ether and collecting 5-mL fractions to yield 188.2 mg (71.8%) of the pure acetoxy lactone. IR (neat): 2950 (CH), 1750 (C=O), 1680 (C=O). ^{1}H nmr δ: 1.04 (3H, s, C10 CH_3), 1.21 (6H, dd, $CH(CH_3)_2$), 1.70-2.57 (m, aliphatic), 2.33 (3H, s, OAc), 2.73 (1H, brm, H5), 2.91-3.07 (3H, m, H7, H15), 4.80 (2H, brd, H19), 6.96 (1H, s, H14), 7.06 (1H, s, H11). UV (methanol) λ_{max} (log ϵ): 218 (4.21). MS m/z: 354 (M^+, 2.8), 312

(36), 297 (25.6), 149 (31.9), 110 (100). HRMS calcd. for $C_{22}H_{26}O_4$: 354.1831; found: 354.1830.

12,19-DIHYDROXY-18(4 → 3)*ABEOABIETA*-3,8,11,13-TETRAEN-18-OIC ACID LACTONE (190):



To a solution of the acetoxy lactone 193 (159.6 mg, 0.45 mmol) in methanol (10 mL), was added 1 mL of concentrated HCl at room temperature. The mixture was stirred at room temperature until TLC showed reaction completion (i-propyl ether developed twice, approximately 5 h), the reaction mixture was then diluted with water (10 mL) and the bulk of methanol was removed *in vacuo*. The resulting suspension was extracted with ethyl acetate (3 x 10 mL) and the combined organic extracts were washed once with water (10 mL), 5% sodium bicarbonate (10 mL), water (10 mL), and brine (10 mL), dried, filtered, and rotary evaporated. The resulting solid (156.4 mg) was crystallized from acetone:water (1.5:1 mixture, charcoal treatment included) to yield 133.7 mg (95%) of the pure product as off-white crystals. Mp = 194-197° C (decomposition). IR (KBr) ν_{max}: 3320 (OH), 2965 (CH), 1748 (C=O). ^{1}H nmr δ: 1.00 (3H, s, C10 CH_3), 1.23 (6H, dd, $CH(CH_3)_2$), 1.70 (1H, m, H1), 1.90 (2H, m, H6), 2.32-2.59 (3H, m, H1, H2), 2.72 (1H, brm, H5), 2.97 (2H, m, H7), 3.16 (1H, septet, H15), 4.67 (1H, brs, D_2O+, OH), 4.78 (2H, brd, H19), 6.72 (1H, s, H14), 6.91 (1H, s, H11). UV (methanol) λ_{max} (log ϵ): 283 (3.57). MS m/z: 312 (M^+, 100), 297 (53.3), 149 (42.8), 115 (14.7), 91 (15). HRMS calcd. for $C_{20}H_{24}O_3$: 312.1726; found: 312.1724. Anal. calcd. for $C_{20}H_{24}O_3$: C 76.9, H 7.73; found: C 77.0, H 7.83.

DEHYDROISOABIETANOLIDE BIOTRANSFORMATION: TRP4a CELL FREE EXTRACT EXPERIMENTS, AND CULTURE CHARACTERIZATION.

T. wilfordii cell suspension cultures were grown in PRDCo or MSNAK media for the required period. The cells were harvested filtering through Mira-cloth. The R.I. and pH of the filtrate were measured and a sample of the cells was taken for dry weight determination after weighing the wet cell mass. The cells were rinsed with distilled water (500 mL) and taken into the cold room where the remainder of the preparation was done (T = 0-4^{o} C).

Phosphate buffer (0.02 M, pH = 6.6, 180 mL) was added to the cells in a plastic container and the mixture was homogenized with an Ultra Turrax T-25 disperser for 30 sec, then allowed to cool for another 30 sec. The homogenization was repeated three times and the homogenate was transferred to plastic centrifuge flasks and centrifuged at 10,000 g for 30 min (T = 4^{o} C). The supernatant was decanted and peroxidase activity (pyrogallol) and protein content (Bio Rad) were determined.

For each cell age, a minimum of 3 Erlenmeyer flasks were set mixing phosphate buffer (pH = 6.6, 35 mL), lactone **132** (10 mg dissolved in 2 mL ethanol), distilled water (15 mL), 0.5% hydrogen peroxide (1 mL, 2.6 equiv.) and the equivalent of 22 peroxidase units of CFE. The flasks were stirred at room temperature for the required period of time (usually 30 min, 2 h, and overnight), and quenched adding 25 mL of ethyl acetate. The mixture was quickly filtered through a short pad of Celite 545 in a fritted glass filter funnel under vacuum and the Celite was washed with ethyl acetate (25 mL). The filtrate was extracted with ethyl acetate (2 x 25 mL) while the Celite was sonicated in ethyl acetate (20 mL) for 20 min, filtered, and the filtrate was mixed with the organic extracts, dried, filtered, and concentrated *in vacuo*.

HPLC analysis was done on each sample (reverse phase, C_{18} cartridge, methanol:water 75:25 with 0.1% acetic acid) and TLC in toluene:ethyl acetate 4:1 and chloroform:methanol:acetic acid 95:5:1.

The lactone was recovered by flash column chromatography of the crude extracts eluting with an 8:2 mixture of hexanes:ethyl acetate, yielding typically 93-97% recovered product.

MEASUREMENT OF PROTEIN CONCENTRATION: BIO-RAD PROTEIN ASSAY

One part of the dye reagent (Bio-Rad Protein Assay Dye Reagent Concentrate) was diluted with four parts of distilled water. The diluted dye reagent solution (5 mL) was then added to a test tube containing CFE (0.1 mL) and the solution was mixed thoroughly. After using a reference sample (prepared by mixing phosphate buffer (0.1 mL, 0.1 M, pH 6.6) and the diluted dye solution (5 mL) to adjust the reading of the UV spectrometer at 595 nm to zero, the absorbance of the CFE was then measured at the same wavelength. The protein concentration can be calculated from the standard curve which was produced by dissolving known amounts of bovine serum albumin (BSA) powder in the same buffer to produce a set of standard solutions (0.1 mg/mL to 0.01 mg/mL), adding aliquots (0.1 mL) of these solutions to the diluted dye (5 mL) and measuring absorbances at 595 nm.

MEASUREMENT OF PEROXIDASE ACTIVITY: PYROGALLOL-PURPUROGALLIN ASSAY.

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 solution (1 mL) and distilled water (14 mL) at 20^{o} C. This mixture was allowed to stand for 20 seconds at 20^{o} C, then 2M sulphuric acid (1 mL) was added to quench the reaction and the solution was then extracted with ether (2 x 25 mL). After the reading of the UV spectrometer was adjusted to zero to 420 nm by a reference sample which was an ether extract (2 x 25 mL) from a mixture of 5% pyrogallol solution (2 mL), 0.1 M phosphate buffer (3 mL, pH 6.6), freshly prepared

0.5% hydrogen peroxide solution (1 mL) and distilled water (14 mL), the absorbance of the organic extract from the CFE reaction was then recorded at the same wavelenth. The standard curve can be obtained by measuring absorbance at 420 nm of a set of standard solutions prepared by dissolving purpurogallin (0.5 to 3.5 mg) in ether (50 ml).

DEHYDROISOABIETANOLIDE BIOTRANSFORMATION: ASSESSMENT OF MEMBRANE-BOUND ENZYMES AND COFACTORS.

T. wilfordii cells were grown in PRDCo medium for 14 days and the cells were harvested and homogenized in phosphate buffer (pH = 6.6). One half of the cell homogenate was set aside for biotransformation experiments and the other half was centrifuged at 10,000 g. The supernatant (CFE) was decanted and the pellet was resuspended in pH 6.6 buffer. The following experiments were done:

a) Cell homogenate. (3.86 units of peroxidase/mL, 0.804 mg of protein/mL). In two 500 mL Erlenmeyer flasks were mixed distilled water (75 mL), phosphate buffer (pH 6.6, 175 mL), hydrogen peroxide solution (0.5%, 5 mL), lactone **132** (50 mg in 10 mL ethanol) and the equivalent amount of 125 units of peroxidase of cell homogenate. The flasks were incubated for 3 and 24 h respectively, and quenched by the addition of ethyl acetate (125 mL). Sodium chloride was added to saturation and the mixture was filtered using some Celite 545 as a filter aid. The filtrate was extracted with ethyl acetate (2 x 100 mL) and the Celite and cell debris were extracted by sonication in ethyl acetate (100 mL) for 30 min. The mixed organic extracts were dried, filtered, and concentrated *in vacuo.*

b) Resuspended pellet. (2.5 units of peroxidase/mL, 0.80 mg protein/mL). In two 500-mL Erlenmeyer flasks were mixed distilled water (75 mL), phosphate buffer (pH 6.6, 175 mL), hydrogen peroxide solution (0.5%, 5 mL, 2.5 equiv.), lactone **132** (50 mg in 10 mL of ethanol), and the equivalent amount of **275** peroxidase units of resuspended

pellet. The flasks were incubated for 24 and 48 h, and worked up as with the cell homogenate.

c) Cell free extract plus cofactors. (4.72 peroxidase units/mL, 0.636 mg protein/mL). In two 500-mL Erlenmeyer flasks were mixed distilled water (75 mL), phosphate buffer (pH 6.6, 175 mL), hydrogen peroxide solution (0.5%, 2.5 equiv., 5 mL), lactone 132 (50 mg in 10 ml ethanol), manganese chloride tetrahydrate (16.6 mg, 0.5 equiv.). Flavine mononucleotide monohydrate (50.8 mg, 0.5 equiv.) and the equivalent of 125 peroxidase units of cell free extract. The flasks were incubated for 3 and 24 h, and worked up as before.

The crude extracts were analyzed by HPLC and the lactone recovered by flash column chromatography in hexanes:ethyl acetate 8:2.

DEHYDROISOABIETANOLIDE BIOTRANSFORMATION: TRP4a WHOLE CELL EXPERIMENTS.

T. wilfordii cells were grown in MSNAK shake flasks for 0, 7 and 10 days. Lactone 132 (8.8 mg in 1 mL ethanol) was added to the cultures and the flasks were incubated for 48, 72 and 92 h. A control flask without precursor was also run for every experiment. One flask containing the lactone 132 (12.0 mg in 1 mL ethanol) in $MSNA_{0.5}K_{0.5}$ medium (125 mL) was also incubated without cells for 48 h.

The samples were harvested homogenizing the cells and broth with an Ultra Turrax T-25 disperser and filtering through Celite. The filtrate was saturated with sodium chloride and extracted with ethyl acetate (2 x 100 mL). The cell debris was sonicated with ethyl acetate (50 mL) for 30 min, filtered through Celite, and the Celite was washed with ethyl acetate. The organic layers (from the spent medium and the cell debris) were combined, dried, filtered, and the solvent was evaporated.

TLC analysis of the extracts (hexane:ethyl acetate 6:4) showed that oxidation had occurred, with the C7 alcohol 175 and C7 ketone 176 being the main products.

Their concentration increased in the longer time/older cell experiments. The blank experiment had only unchanged lactone **132** after incubation on MSNAK medium.

DEHYDROISOABIETANOLIDE BIOTRANSFORMATION: *MORTIERELLA ISABELLINA* EXPERIMENTS.

A culture of *M. isabellina* was grown in a Roux bottle with 15 mL of PDA at room temperature for 10 days. Inoculum was prepared by washing the surface of this culture with sterile DYE medium (10 mL). The spore suspension was diluted to 25 mL with DYE medium. The resulting suspension contained approximately 9.84×10^8 spores per mL (as determined using a Howard Mold counting chamber) and was inoculated into 550 mL of DYE medium (i.e. a volumetric ratio of 0.45:10) so as to give 1.96×10^6 spores per mL.

50 mL of this mixture were transferred into each of 11 500-mL Erlenmeyer flasks. A blank flask contained only medium and lactone but no fungus. All cultures used for the biotransformation contained 5.0 mg of lactone **132** dissolved in 0.5 mL ethanol. The cultures were incubated at 30° C and 200 rpm in a rotary shaker for 48 h. Microscopic inspection of the cultures showed that the fungus was still on the lag phase (only some spores had germinated). The flasks were harvested combining the filtrates (after Whatman No. 1 paper) and rinsing well both flasks and spores with ethyl acetate in 25 mL portions (275 mL total). The broth was extracted with ethyl acetate (2 x 275 mL), the organic fractions were combined, washed with brine (250 mL), dried, filtered, and concentrated *in vacuo*, to yield 94.0 mg of a yellow thick oil.

TLC analysis of the extract showed consumption of the starting material and the appearance of the C7 alcohol **175**.

The crude was purified by flash column chromatography (1.5 cm diameter column, 5.5" silica gel) eluting with hexanes:ethyl acetate 6:4 (250 mL) and collecting 5-mL fractions. After this, the column was flushed with ethyl acetate (100 mL). The

product was a sticky oil (23.4 mg, 37%). Spectroscopic data was identical with the reported values of M. Roberts[110].

ISOTRIPTOPHENOLIDE BIOTRANSFORMATION WITH *T. WILFORDII* CELLS.

T. wilfordii cells were grown in $MSNA_{0.5}K_{0.5}$ medium in shake flasks for 7 days. A solution of isotriptophenolide (**190**) (50 mg) in ethanol (2 mL) was added to 1 L of the suspension culture in 500 mL batches.

A control experiment was conducted in which only ethanol was incubated with 500 mL of culture. The flasks were incubated until TLC analysis showed partial consumption of the precursor (7 days) to avoid overoxidation and were harvested (pH = 4.7, R.I. = 1.3352). The cells and spent medium were homogenized and extracted with ethyl acetate as in the other experiments (*vide supra*) to yield a brown solid extract (448.6 mg for the experiment and 279.2 mg for the control). The crude extract was purified by column chromatography (4.5 cm diameter, 6" silica gel) with the following eluants: hexanes:ethyl acetate 8:2 (1 L), hexanes:ethyl acetate 6:4 (1 L), hexanes:ethyl acetate 2:8 (500 mL) and ethyl acetate:methanol 1:1 (500 mL). The fractions that contained the recovered precursor and the new metabolite were finally purified by preparative thin layer chromatography eluting twice with hexanes:ethyl acetate 7:3 to yield 38.0 mg (76%) of recovered isotriptophenolide and 4.3 mg (8.23%) of the methyl ether **194**.

IR ($CHCl_3$) ν_{max} (cm^{-1}): 2944 (CH), 1748 (C=O). ^1H nmr δ: 1.60 (3H, s, C10 CH_3), 1.21 (6H, dd, $CH(CH_3)_2$), 1.76 (1H, m, H1), 1.92 (2H, m, H6), 2.42 (1H, m, H1), 2.52 (2H, m, H2), 2.35 (1H, brm, H5), 2.97 (2H, m, H7), 3.26 (1H, septet, H15), 3.84 (3H, s, OCH_3), 4.78 (2H, brd, C19), 6.81 (1H, s, H14), 6.93 (1H, s, H11). MS m/z: 326 (M^+, 80.6), 311 (100), 163 (73.4). HRMS calcd. for $C_{21}H_{26}O_3$: 326.1882, found: 326.1882.

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