ENHANCED YIELD OF MEDICINAL PRODUCTS FROM *TRIPTERYGIUM* CELL CULTURES

by

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(DEPARTMENT OF CHEMISTRY)

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

February 1992

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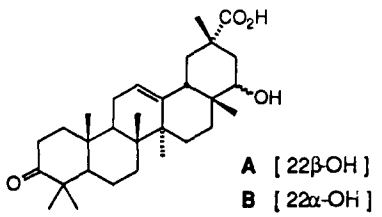
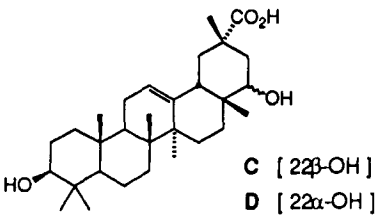
ABSTRACT

The growth of cell suspensions of the Asian medicinal plant *Tripterygium wilfordii* was manipulated in order to produce large amounts of pharmacologically active diterpene and triterpene natural products. A method was developed where elicitation with a strain of the fungus *Botrytis* stimulated the production of oleanane and friedelane triterpene acids. In rapidly growing twelve liter bioreactor cultures, triterpene yields were increased five to ten fold with this process, routinely providing more than 25 mg/L each of 22α-hydroxy-3-oxoolean-12-en-29-oic acid (B) and 3β,22α-dihydroxyolean-12-en-29-oic acid (D) and more than 5 mg/L each of 22β-hydroxy-3-oxoolean-12-en-29-oic acid (A) and 3β,22β-dihydroxyolean-12-en-29-oic acid (C).

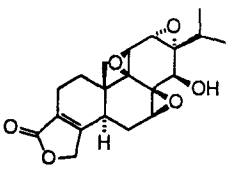
Yield improvement for the triptolide family of diterpenes (triptolide and tripdiolide) was approached through the synthesis of potential intermediates of the natural biosynthetic pathways, the first step in a technique where synthetic elaborations would be completed by cell cultures. These synthetic intermediates were also sought to establish the details of triptolide biosynthesis. Advances were made towards the synthesis of one potential intermediate, 18(4→3)-isodehydroabietenolide, starting from (L)-dehydroabietic acid.



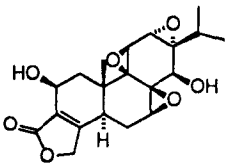
A [22β-OH]
B [22α-OH]



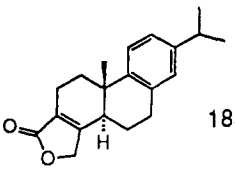
C [22β-OH]
D [22α-OH]



triptolide



tripdiolide



18(4→3)-isodehydro-abietenolide

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ACKNOWLEDGMENTS

I am very grateful for the opportunity and the careful direction provided by Professor J. P. Kutney in both synthetic and cell culture work. I extend my thanks also to Gary Hewitt for his fundamental input into the planning and execution of the elicitation project and to David Chen for providing such reliable day-to-day management of cell cultures. Elizabeth Buganté was an essential collaborator in the formative elicitation studies and Huifen Gu provided excellent work and most of all unwavering motivation throughout the cell culture triterpene analyses. I will miss these two, visiting scholars from The Philippines and China respectively. Thank-you to the others who contributed to our understanding of the elicitation of *Tripterygium*, including Stephen Cheung and especially Leo Law. I express highest regards for Malcolm Roberts who provided indispensable advice and encouragement throughout my synthetic studies. My worthy associates also included Kang Han, Francisco Kuri-Breña, Yong Huang Chen, Krystyna Piotrowska and several others who were never too busy for helpful suggestions and support. The staff and faculty of this Chemistry department provided a wonderful environment and excellent facilities that made my time here very enjoyable. Thank-you family and friends.

INTRODUCTION

THE PHARMACOLOGY AND COMPOSITION OF TRIPTERYGIUM

This thesis was written to describe methods for increasing production of pharmacologically useful compounds synthesized in the Asian medicinal plant *Tripterygium wilfordii.*

(I) MEDICINAL HISTORY

In China, the first written accounts of medicine (now 2000 years old) describe *Tripterygium* as a source of extracts used to treat fever, chills, joint pain, swellings and skin rashes[1].

In contrast, a recent article in the Chinese Medical Journal[2] names a commercial *Tripterygium* extract as the current most effective treatment of rheumatoid arthritis (RA). The oral ingestion of extract relieves the joint pain and inflammation that are symptoms of this autoimmune disorder. During the course of this treatment, two beneficial physiological responses are observed. The first is a rapid anti-inflammatory effect and the second a slow immunosuppressive effect [2,3]. These responses are similar to those obtained with corticosteroids, the most widely prescribed drugs for RA treatment* , and are presently the most valuable pharmaceutical activities displayed by *Tripterygium. Tripterygium* extracts are also prescribed in the treatment of other disorders related to the overactive or unregulated immune system: chronic nephritis (kidney) and hepatitis (liver), ankylosing spondilitis (joints) and systemic lupus erythematosis (skin and organs)[2,4].

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*corticosteroids are not among the compounds isolated from *Tripterygium* (table 1, p.17)

Tripterygium extracts exhibit a very effective male contraceptive activity that is reversed once ingestion ceases. This application is still at the stage of clinical trials[1,5] and work is underway to isolate the causative agent.

The pharmaceutical use of *Tripterygium* occurs mainly in China, where it is dispensed by two types of practitioner: the medical doctor and the expert in traditional herbal medicine. The first group has prepared a refined extract using university, industrial and clinical research facilities. The response of patients to this preparation is monitored closely to analyze the specific mode of action that the drug employs and to better understand the actual disease. The other group, the herbalists, use extracts that have been available for hundreds of years. The extraction procedures were developed by observing the overall health of patients following treatment, without any specific understanding of the therapeutic mechanisms involved. Controlled aqueous or ethanolic extraction of *Tripterygium* provides tonics free of the toxic components contained in the whole plant [1,6]. It is appropriate to note that the fevers, swellings and rashes for which herbal treatment is a long-standing remedy are often symptoms of the immunological disorders for which refined *Tripterygium* extracts are now prescribed.

(II) LABORATORY RESEARCH

The original studies on the chemical composition of *Tripterygium* began in the 1930's in China[7]. This research was carried into the U.S. by agricultural chemists [8 - 11] when it was learned that Chinese farmers spread powdered *Tripterygium* root over their crops to kill chewing insects and worms. The study produced a small catalog of compounds but was ended in the 1950's, possibly due to the availability of more potent synthetic pesticides.

In the 1970's, Prof. S.M. Kupchan and co-workers (Univ. Virginia) carried out a project to isolate the medicinal agents of *Tripterygium*. His analysis of the components present in a

utilized bio-assays to select only the active principles. The extracts were chromatographed into several pure compounds which demonstrated potent anti-leukemic activity[12,13]. This was a very important advance in *Tripterygium* research, providing a group of possible pharmaceuticals for further study and developing the methods for compound screening that would be used by subsequent groups.

The ability of *Tripterygium* extracts to cause a reversible suppression of male fertility was first noted in China during the course of a clinical trial on rheumatoid arthritis treatment. This led to separate studies of the extracts viability as a male contraceptive[1].

As well as searching for the antifertility agent, several groups are now interested in isolating compounds responsible for the anti-inflammatory and immunosuppressive properties that make *Tripterygium* useful against immune disorders[2,14,15]. Each group is screening the extracts for a single active principle with a narrow range of effects. For example, to prepare a useful male contraceptive it would be desirable to separate out the immunosuppressive agents so as not to weaken the disease resistance of the consumer. Prof. J.P. Kutney's laboratory is working on the isolation, identification and potential production of *Tripterygium*s medicinally useful anti-leukemic, anti-fertility, anti-inflammatory and immunosuppressive compounds. The methods used and the results of this research will be reviewed in this paper. The standard source of *Tripterygium* extracts is the dried root of the plant which is native to China. In contrast, Prof. Kutney has worked exclusively with tissue cultures of *Tripterygium* which demonstrate rapid growth and an increased production of metabolites.

(III) BOTANICAL INFORMATION

Tripterygium is a member of the family Celastraceae, which is a group of woody vines and shrubs. It grows wild in the forested hills of Southeast China and Taiwan. The species of *Tripterygium* include *T. wilfordii, T. hypoglaucum, T. regelii and T. forrestii*. Within this thesis, *Tripterygium* will refer to *Tripterygium wilfordii* Hook. f. (Hooker filius), on which most studies have been carried out. This plant is a perennial twining vine with pale green serrated leaves and a red pigment in the stems and roots. It bears small white flowers which develop into seeds having three longitudinal ridges, which is the source of the European name tri-pterygion (Greek; three small wings) and a Chinese name translating into "three wing nut". As a wild plant, the Chinese refer to *Tripterygium* as mang cao* (rank grass) while in the medicinal context it is referred to exclusively as léi gong teng (thunder god vine). The Celastraceae family contains other plants notable for the production of pharmacologically active compounds. *Maytenus*, for example, which produces the anti-cancer compound Maytansine[16,17]. The red quinone methide celastrol (68) is found, as the name suggests, in many Celastraceae and is a member of a group of cytostatic triterpene quinones.

Pharmaceutical extracts of *Tripterygium* are prepared from the root of mature plants. The roots two outer layers (referred to as the bark or the *cortex*) contain the toxic alkaloids that are used as an insecticide and the interior core (*xylem*) contains the medicinal agents[1]. The leaves of *Tripterygium* are exceedingly toxic to humans due to stored alkaloids [18 - 20].

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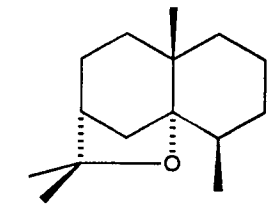
* these are Pinyin phonetic transcriptions of the Chinese pronunciation

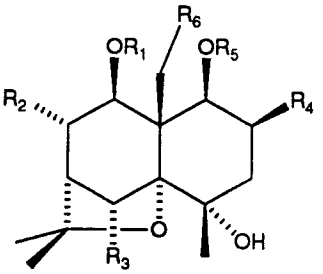
(IV) ANALYSIS OF PHARMACOLOGICALLY ACTIVE COMPONENTS

The screening of *Tripterygium* extracts for useful compounds began in China where Chou and Mei isolated sugar and pigment components (1936)[21]. In searching for an insecticide, the American chemist Haller identified the main pigment component, celastrol, and isolated insecticidal alkaloids (1941)[8 - 10]. This study was continued by Beroza, who identified several of the alkaloid structures (1953)[22,23]. In a search for medicinally useful compounds, Kupchan et al. discovered a group of anti-leukemic diterpenes (1972)[12]. These compounds were produced on a larger scale by plant tissue cultures in work carried out by Kutney's group [24,25] (1981), whose research is now focussed on improving the production method and identifying anti-inflammatory, immunosuppressive, and anti-fertility factors. The isolation of these compounds requires several repetitions of a sequence involving chromatography of biologically active extracts followed by an assay for active fractions. The following figures (figs. 1 - 4) provide a compendium of the natural products which have been isolated to date* . The source of these compounds was primarily *Tripterygium wilfordii*, with a few isolated from *T. regelii*, *T. hypoglaucum* and *T. forrestii*. Following the structures, *Table 1* correlates these compounds with the discovering groups and lists pharmacological activities that have been observed.

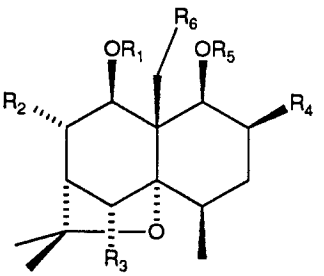
* A numbering scheme for terpene skeletons is provided in Appendix I.



dihydroagarofuran skeleton

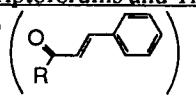


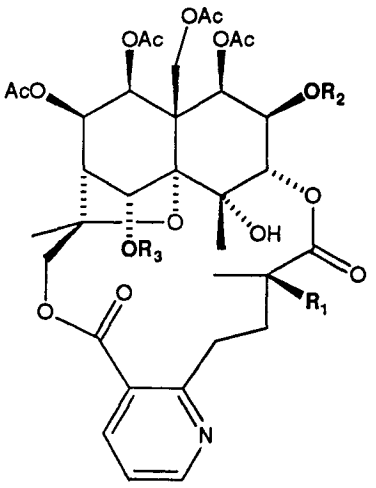
triptofordins



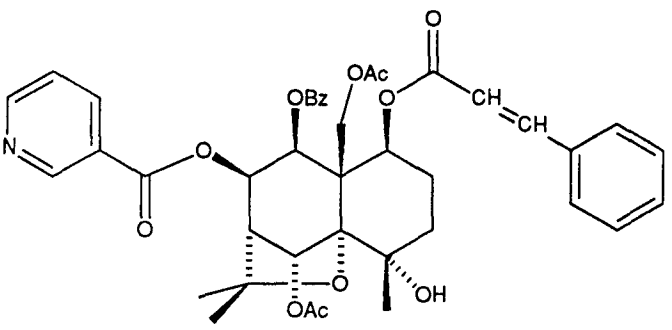
triptogelins

	R_1	R_2	R_3	R_4	R_5	R_6	Triptofordin
1	Cn	–	–	–	Bz	–	A
2	Bz	–	OH	–	Bz	–	B
3	Bz	–	OAc	=O	Ac	–	C-1
4	Bz	–	OAc	OH	Ac	–	C-2
5	Bz	=O	OAc	–	Cn	OAc	D-1
6	Bz	OH	OAc	–	Cn	OAc	D-2
7	Bz	OAc	OH	OAc	Cn	OAc	F-1
8	Bz	OBz	OH	OAc	Ac	OAc	F-2
9	Bz	OAc	OAc	OAc	Bz	OAc	F-3
10	Bz	OH	OH	OAc	Cn	OAc	F-4
11	Bz	=O	OAc	OAc	Bz	OAc	E
12	Bz	=O	OAc	OAc	Ac	OAc	- - -
13	Bz	=O	OAc	OAc	Cn	OAc	- - -
							Triptogelin
14	Bz	OBz	OAc	OBz	OBz	–	A-1
15	Bz	OBz	OAc	OH	OBz	–	A-2
16	Bz	OBz	OAc	OH	–	–	A-3
17	Bz	OBz	OAc	=O	–	–	A-4
18	Bz	OBz	OAc	–	–	–	B-1

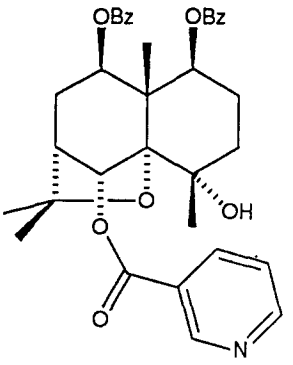
Figure 1 (a) SESQUITERPENES OF *TRIPTERYGIUM* (Triptofordins and Triptogelins)
Acetyl (Ac), benzoyl (Bz), (trans-)cinnamyl (Cn) 
unsubstituted (–).



	R_1	R_2	R_3	
19	–	Bz	–	wilforzine
20	–	Bz	Ac	wilforine
21	–	Ac	Ac	wilformine
22	–	Fr	Ac	wilforgine
23	–	Nc	Ac	wilfornine
24	OH	–	Ac	wilforidine
25	OH	Bz	Ac	wilfordine
26	OH	Fr	Ac	wilfortrine

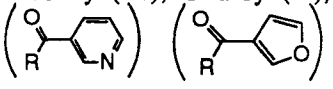


27 (*trans*- cinnamyl) triptofordinine A-1
28 (*cis*- cinnamyl) triptofordinine A-2



29 regilidine

Figure 1 (b) SESQUITERPENE ALKALOIDS OF *TRYPTERYGIUM*
Acetyl (Ac), benzoyl (Bz), nicotinyl (Nc), 3-furoyl (Fr), unsubstituted (–).



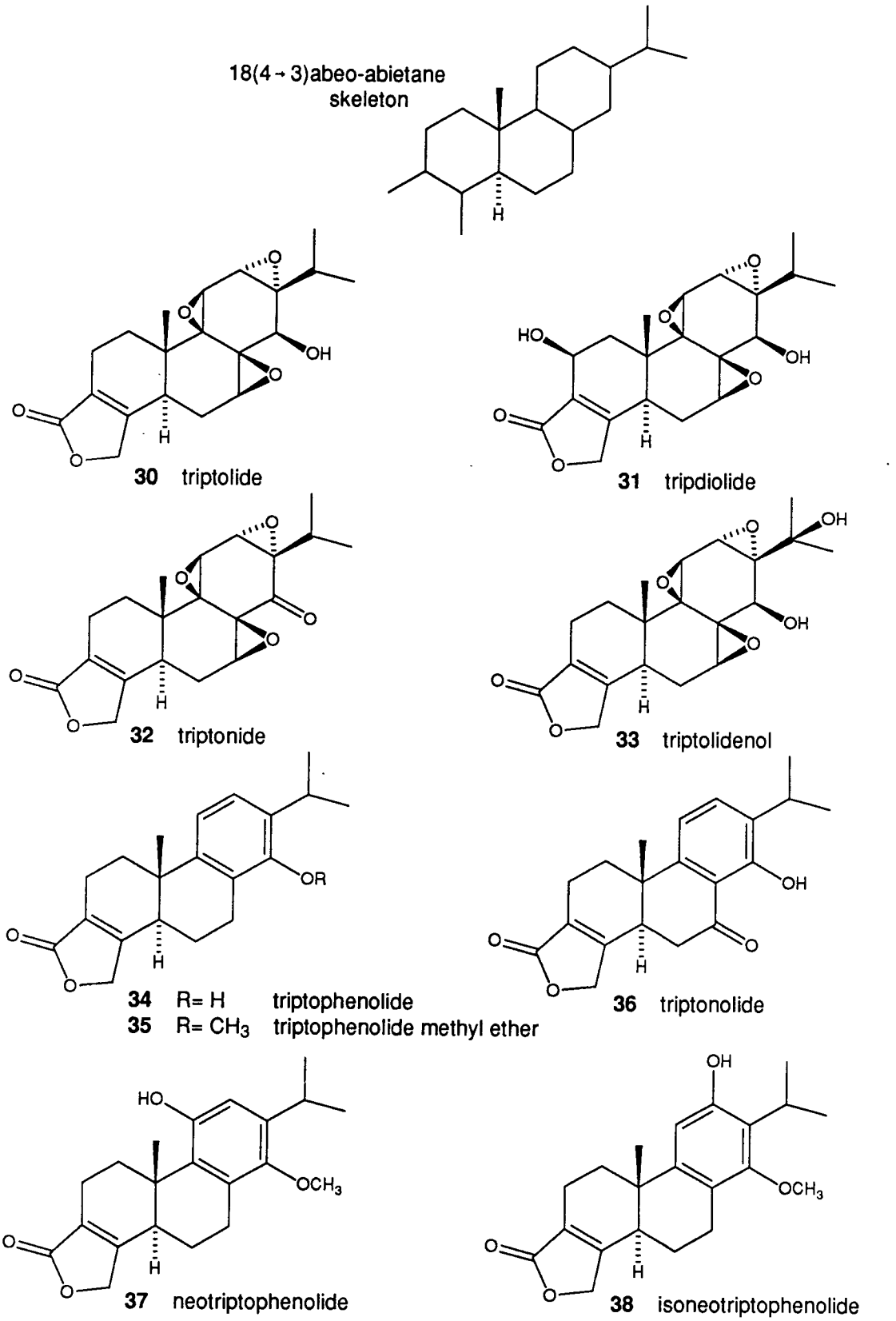
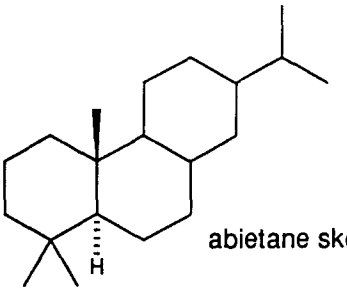
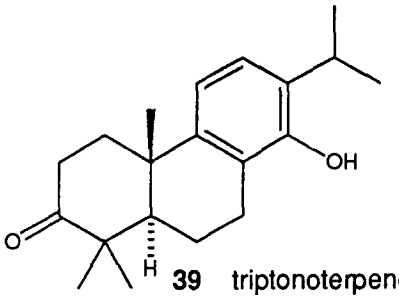


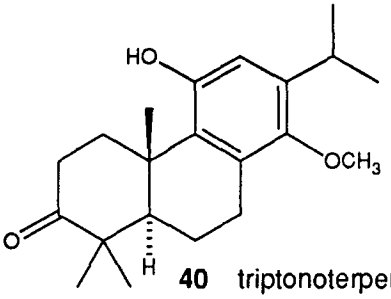
Figure 2 DITERPENES OF *TRIPTERYGIUM* -- ABEO-ABIETANE BUTENOLIDES AND ABIETANES



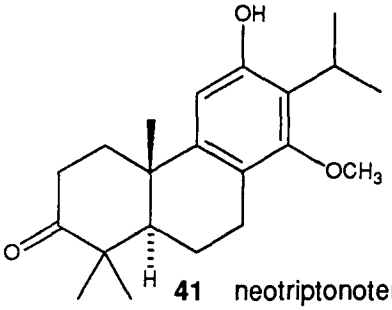
abietane skeleton



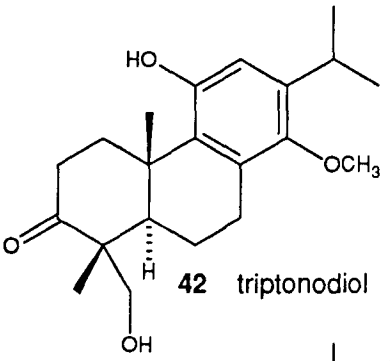
39 triptonoterpene (a)



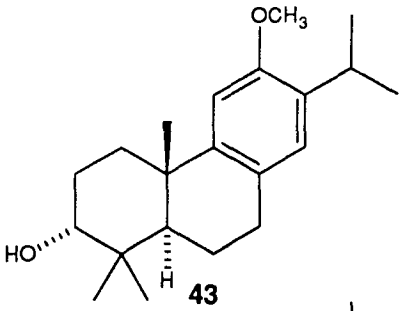
40 triptonoterpene (b)



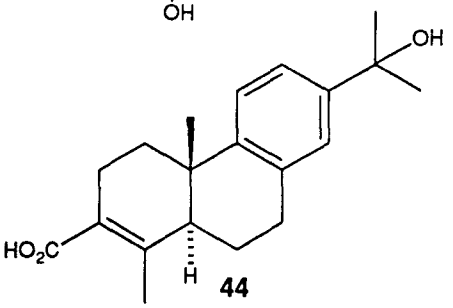
41 neotriptonoterpene



42 triptonodiol



43



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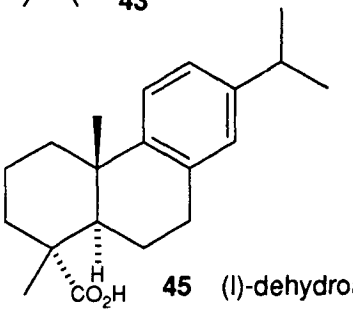
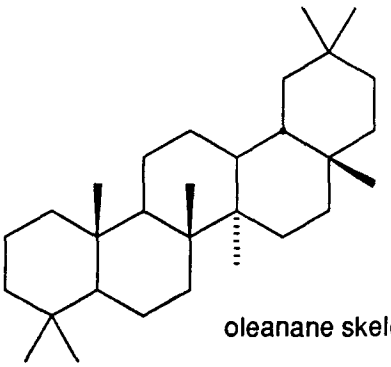
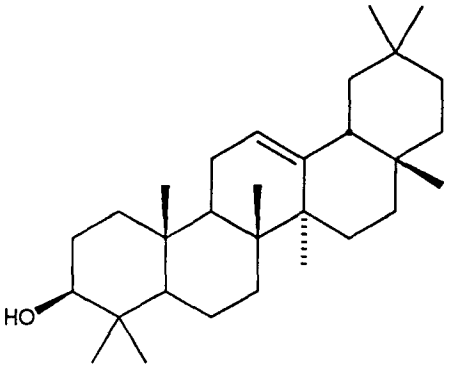


45 (l)-dehydroabietic acid

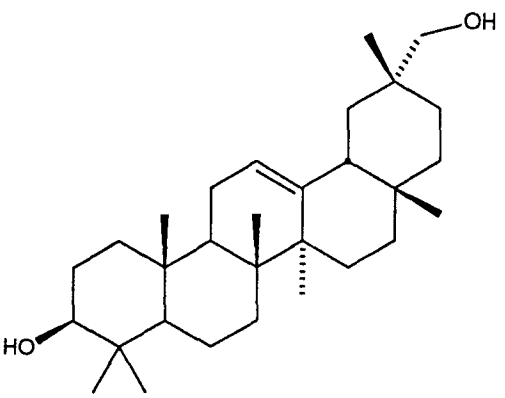
Figure 2 DITERPENES (cont.)



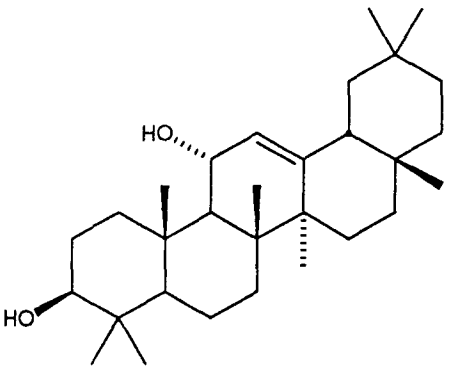
oleanane skeleton



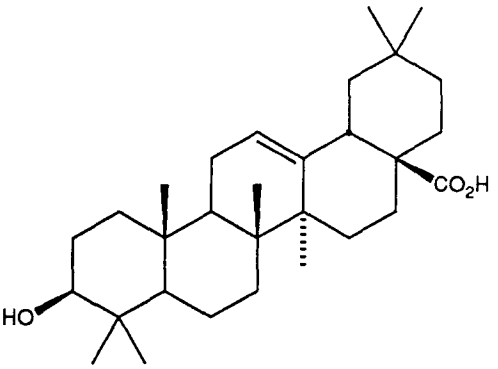
46 β - amyrin



47 3β,29-dihydroxyolean-12-ene

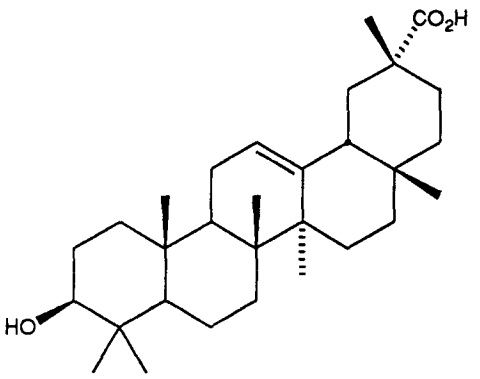


48 3β,11α-dihydroxyolean-12-ene

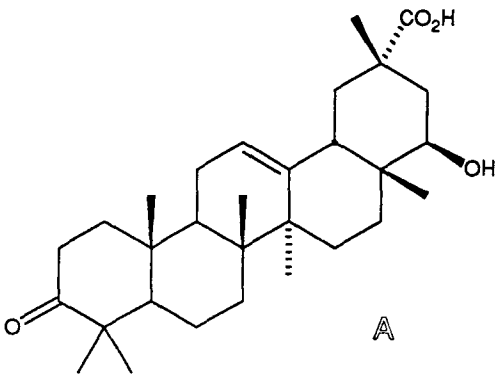


49 oleanolic acid

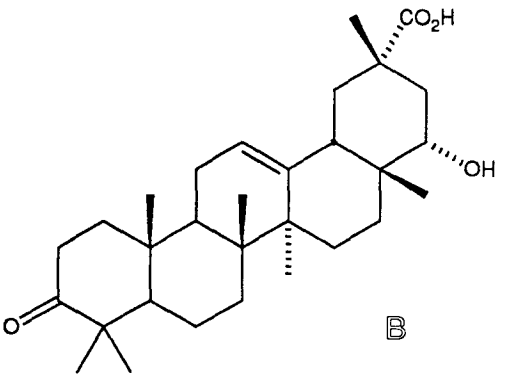
Figure 3 TRITERPENES OF *TRIPTERYGIUM* (a) OLEANANES (i)



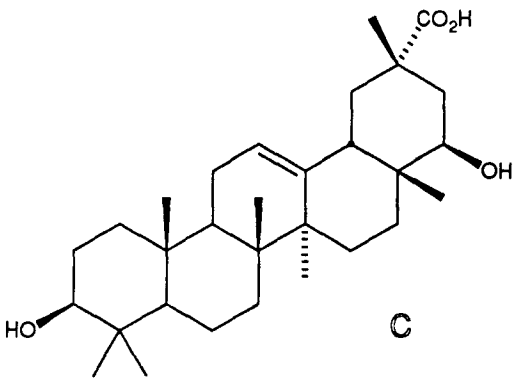
50 3β-epikatonic acid



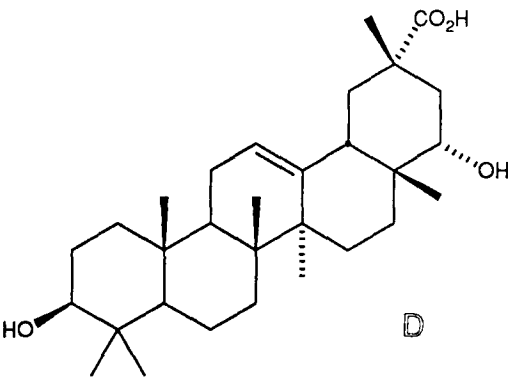
51 22β-hydroxy-3-oxoolean-12-en-29-oic acid



52 22α-hydroxy-3-oxoolean-12-en-29-oic acid

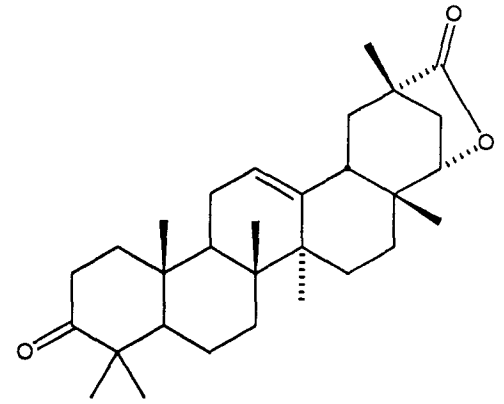


53 3β, 22β-dihydroxyolean-12-en-29-oic acid

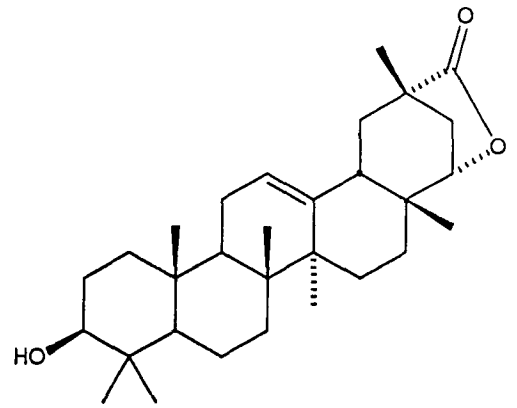


54 3β,22α-dihydroxyolean-12-en-29-oic acid

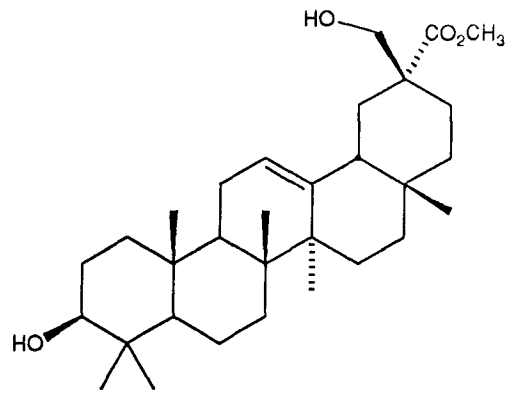
Figure 3 TRITERPENES OF *TRIPTERYGIUM* (a) OLEANANES (ii)



55 wilforlide B

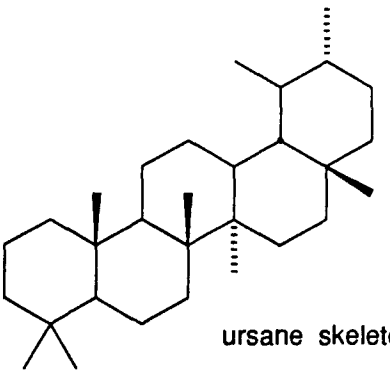


56 wilforlide A

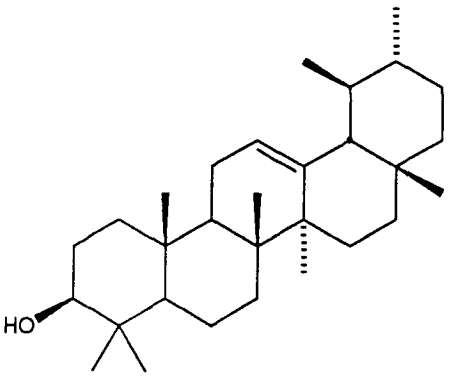


57 triptodihydroxy acid methyl ester

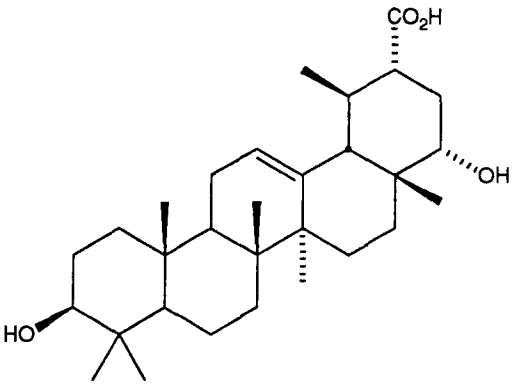
Figure 3 TRITERPENES OF *TRIPTERYGIUM* (a) OLEANANES (iii)



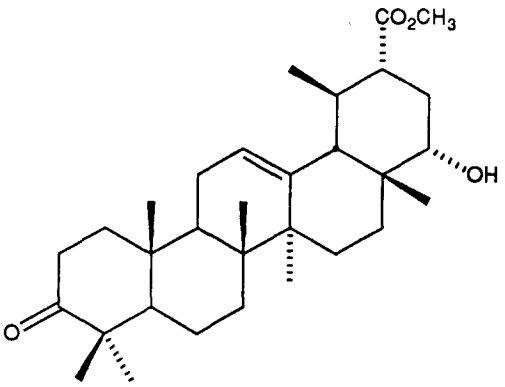
ursane skeleton



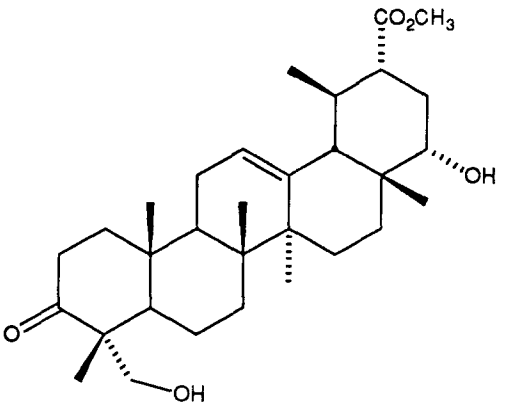
58 α- amyrin



59 triptotriterpenic acid C

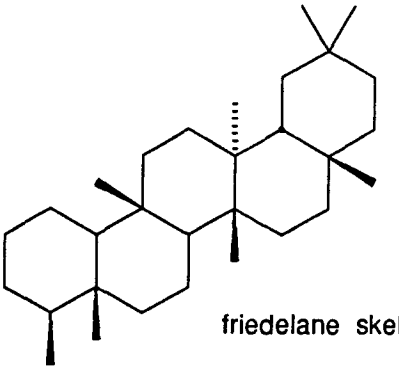


60 regelin

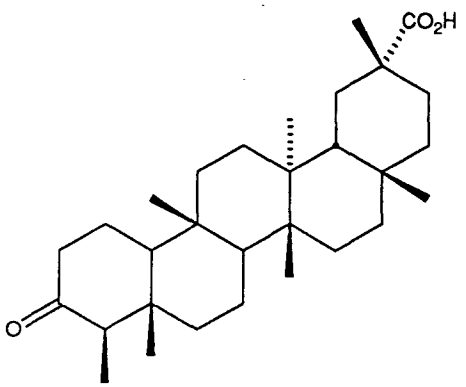


61 regelinol

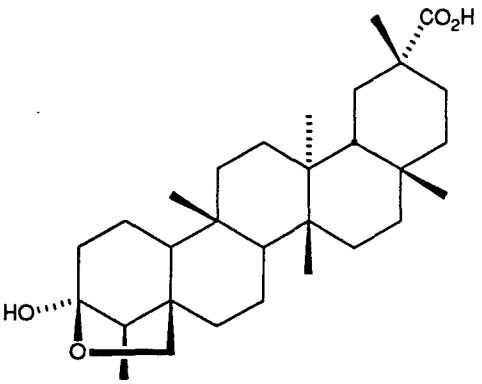
Figure 3 TRITERPENES OF *TRIPTERYGIUM* (b) URSANES



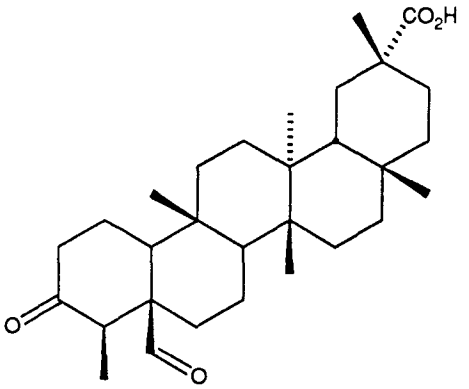
friedelane skeleton



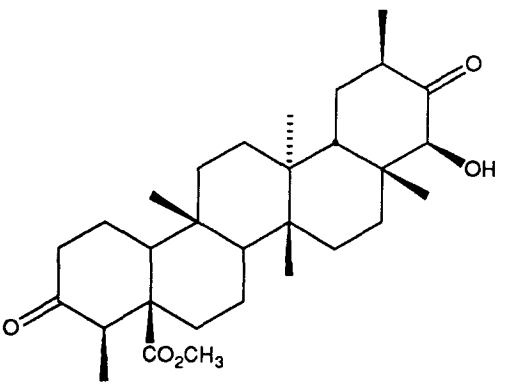
62 polpunonic acid



63 salaspermic acid



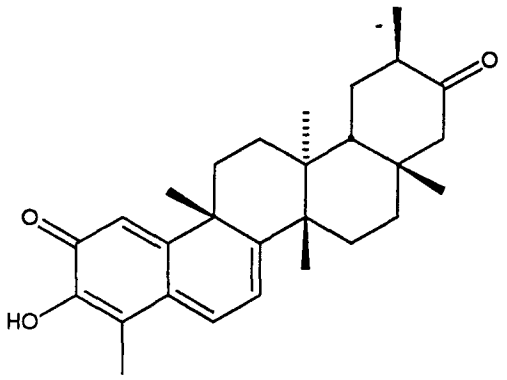
64 3,24 - dioxofriedelan-29-oic acid



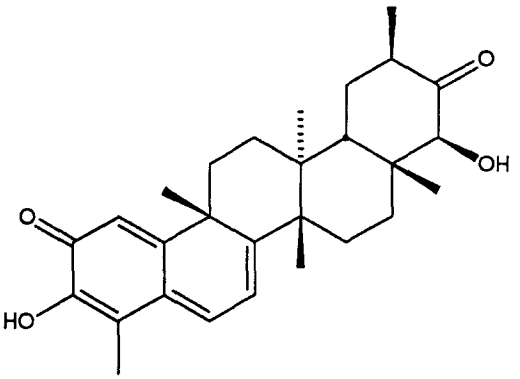
65

Figure 3 TRITERPENES OF *TRIPTERYGIUM* (c) FRIEDELANES

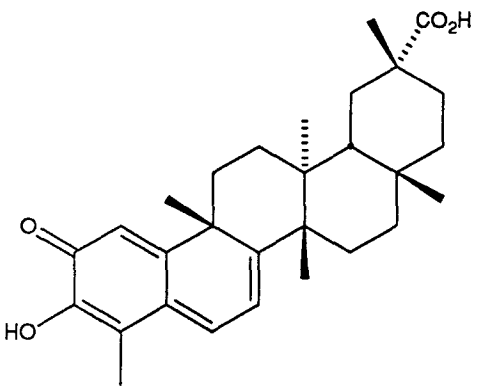
Quinone Methides



66 tingenone

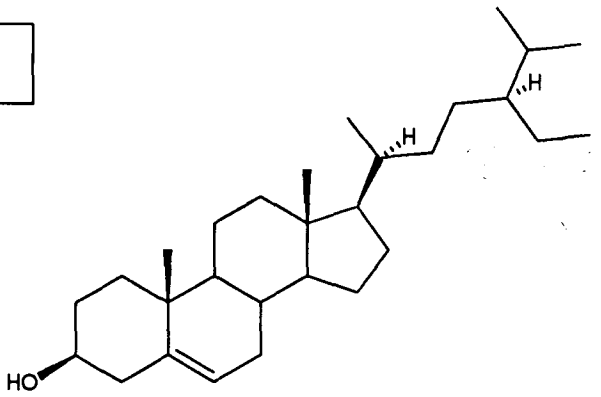


67 22β-hydroxytingenone



68 celastrol

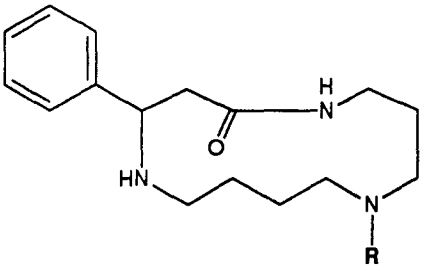
Sterols

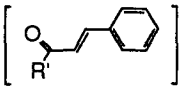
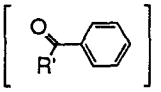
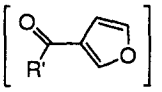


69 β-sitosterol

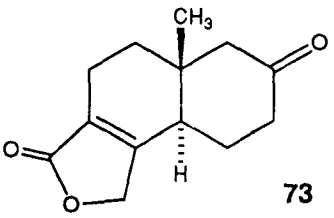
Figure 3 TRITERPENES OF *TRIPTERYGIUM* (d) OTHER

Spermidine Alkaloids

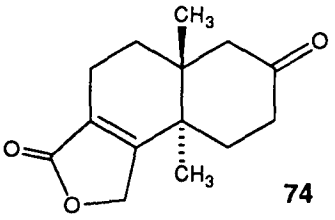


		R	
70	celacinnine	(*trans* - cinnamyl)	
71	celabenzine	(benzoyl)	
72	celafurine	(3-furoyl)	

Butenolides



73 wilforonide



74 neotriptonolide

Figure 4 OTHER NATURAL PRODUCTS OF *TRIPTERYGIUM*

Table 1 Isolation and Testing of Natural Products from *Tripterygium*

Compound	Reference	Source		Biological Activity * mice (m) human cell culture (c)
		whole plant	cell culture	
1 A	19	+	o	
2 B	19	+	o	
3 C-1	19	+	o	
4 C-2	19	+	o	
5 D-1	20, 26	+	o	
6 D-2	20, 26	+	o	
7 F-1	18	+	o	
8 F-2	18	+	o	
9 F-3	18	+	o	
10 F-4	18	+	o	
11 E	20, 26	+	o	
12 - - -	26	+	o	possibly insecticidal
13 - - -	26	+	o	possibly insecticidal

Table 1(a) Sesquiterpene Triptofordins

Compound	Reference	Source		Biological Activity mice (m) human cell culture (c)
		whole plant	cell culture	
14 A-1	27	+	o	
15 A-2	27	+	o	
16 A-3	27	+	o	
17 A-4	27	+	o	
18 B-1	27	+	o	

Table 1(b) Sesquiterpene Triptogelins

* few of the compounds have been subject to a wide range of tests; while human patients have been treated with plant extracts containing these components, purified compounds have yet to be used (except triptolide **30**)

Table 1 (cont.)

Compound	Reference	Source		Biological Activity mice (m) human cell culture (c)
		whole plant	cell culture	
19 wilforzine	22, 23, 28, 29	+	o	insecticidal
20 wilforine	22, 23, 30	+	o	insecticidal
21 wilformine (euonine)	28, 31, 32	+	o	insecticidal, immunosuppressive (m)
22 wilforgine	22, 23, 28	+	o	insecticidal
23 wilfornine	31	+	o	insecticidal, immunosuppressive (m)
24 wilforidine	33	+	o	insecticidal
25 wilfordine	22, 23, 33, 34, 35	+	o	insecticidal
26 wilfortrine	22, 23, 32, 33, 35, 36	+	o	insecticidal, antileukemic (m), immunosuppressive (m)
27 triptofordinine A-1	37	+	o	
28 triptofordinine A-2	37	+	o	
29 regilidine	38	+	o	

Table 1(c) Sesquiterpene Alkaloids

Table 1 (cont.)

Compound	Reference	Source		Biological Activity mice (m) human cell culture (c)
		whole plant	cell culture	
30 triptolide	12, 13, 24, 25, 39, 40, 41, 42, 43, 44, 45, 46	+	+	antileukemic (m), antitumor (c), cytostatic (c), anti-inflammatory (m), immunosuppressive (m)
31 tripdiolide	6, 12, 13, 24, 25, 42	+	+	antileukemic (m), antitumor (c)
32 triptonide	12	+	o	
33 triptolidenol	42, 47	+	o	
34 triptophenolide	48	+	o	
35 triptophenolide methyl ether	48	+	o	
36 triptonolide	49	+	o	
37 neotriptophenolide	48	+	o	
38 isoneotriptophenolide	50	+	o	
39 triptonoterpene (a)	47	+	o	
40 triptonoterpene (b)	51	+	o	
41 neotriptonoterpene	51	+	o	
42 triptonodiol	51	+	o	
43 - - -	25	o	+	
44 - - -	24, 25	o	+	
45 dehydroabietic acid	24	o	+	

Table 1(d) Diterpenes

Table 1 (cont.)

Compound	Reference	Source		Biological Activity mice (m) human cell culture (c)
		whole plant	cell culture	
(i) **46** β - amyrin	25, 52	o	+	
47 3β,29-dihydroxyolean-12-ene	25, 53	o	+	
48 3β,11α-dihydroxyolean-12-ene	25, 54	o	+	
49 oleanolic acid	24, 25	o	+	
50 3β-epikatonic acid	55	+	o	
51 A	25	o	+	
52 B	25, 26	o	+	
53 C	25	o	+	
54 D	16, 25, 55, 56, 57	+	+	antileukemic (m), anti-inflammatory (m)
55 wilforlide B	55, 58	+	+	partially converted to **52** in aqueous solution
56 wilforlide A	55, 58	+	+	partially converted to **54** in aqueous solution
57 triptodihydroxy acid methyl ester	36, 37	+	o	

Table 1(e) Triterpene Oleananes(i) Ursanes(ii) Friedelanes(iii) Quinone Methides(iv) Sterols(v)

Table 1 (cont.)

Compound	Reference	Source		Biological Activity mice (m) human cell culture (c)
		whole plant	cell culture	
(ii) **58** α - amyrin	25, 52	o	+	
59 triptotriterpenic acid C	56, 59, 60	+	o	
60 regelin	56, 61	+	o	
61 regelinol	56, 61	+	o	
(iii) **62** polpunonic acid	24, 25, 37	+	+	
63 salaspermic acid	55, 62	+	+	
64 3,24-dioxofriedelan-29-oic acid	55	+	o	
65 - - -	25	o	+	
(iv) **66** tingenone	24, 25, 63, 64, 65	+	+	cytostatic (c)
67 22β-hydroxytingenone	24, 25, 64, 65	o	+	cytostatic (c)
68 celastrol (tripterine)	1, 7, 9, 24, 55	+	+	immunosuppressive (c) cytostatic (c)
(v) **69** β-sitosterol	24, 25	+	+	

Table 1(e) (cont.) Triterpene Oleananes(i) Ursanes(ii) Friedelanes(iii) Quinone Methides(iv) Sterols(v)

Table 1 (cont.)

Compound	Reference	Source		Biological Activity mice (m) human cell culture (c)
		whole plant	cell culture	
(i) **70** celacinnine	66	+	o	
71 celabenzine	66	+	o	
72 celafurine	66	+	o	
(ii) **73** wiforonide	50	+	o	
74 neotriptonolide	51	+	o	

Table 1(f) Spermidine Alkaloids(i) Butenolides(ii)

(V) MEDICAL USES OF *TRIPTERYGIUM*

The pharmacological activities of these compounds and those of compounds yet undiscovered combine to produce the medicinal properties of whole plant extracts. In the preparation of a medicinal extract from the plant, a simple separation of compounds is available since the distribution of natural products is not uniform. The triptofordins and alkaloids are found mainly in leaves, stem and root bark, while the diterpenes and triterpenes occur in the root core[1, 3, 4, 6, 18]. Since the leaves and root bark demonstrate a high level of toxicity, pharmaceutical preparations primarily utilize the root core. The lower toxicity of root core preparations allow them to be administered in higher dosages than whole root extracts. The basic *Tripterygium* extract is prepared by boiling 15 to 25 grams of root core in water or by soaking 2 to 4 grams in ethanol[1, 3]. The resulting solutions provide one daily dose for rheumatoid arthritis treatment. A commercially refined preparation called *GTW* is produced by extracting the root core into hot water and then into chloroform. The extracted mixture is refined by column chromatography to produce GTW which has an unknown composition save that it contains glycosidic compounds*, diterpenes and triterpenes. The extract of 1.5 to 2.3 kilograms of root core provides the 60 to 90 milligrams GTW prescribed as a daily dosage[1].

The extracts of *Tripterygium* root core display anti-inflammatory / immunosuppressive properties due to the following physiological mechanisms: capillary permeability and the production of humoral inflammatory mediators are decreased and cell-based inflammation and immune responses are suppressed. These effects are due to an inhibition of the generation and activation of T-cells (the main regulatory agents of the immune system). Antibody production is also blocked, possibly another result of a lowered T-cell activity[4, 67].

---

* *GTW* from: "glycosides of *Tripterygium wilfordii*"

GTW was prescribed at 60-90 mg/day to treat rheumatoid arthritis (equal to approximately 1.0-1.5 mg/kg/day). The single dose toxicity (LD_{50})* in mice was 160 mg/kg, while in dogs 10 mg/kg/day caused chronic lethargy and weight loss[1]. This demonstrated an effective dosage approximately six times lower than the harmful dosage.

A smaller dosage of 20 mg of GTW per day was sufficient to produce infertility in human males. After two months at this level, the sperm number was reduced by 200 times and sperm motility was eliminated. Reversible damage to sperm generating cells accompanied the infertility observed in animals. Immunosuppressive side effects were not seen in human subjects at this level of GTW while higher dosages had produced an increase in secondary infections. Male fertility was totally recovered after two months without the drug[1, 4, 15, 68].

Triptolide (30) is the only pure compound that has been tested on human subjects and it was shown to be effective in treating rheumatoid arthritis (RA). This property was revealed when the majority of the anti-RA activity of *Tripterygium* was extracted into ethyl acetate and the triptolide isolated from this extract demonstrated a high level of activity. Treatment with pure triptolide impaired heart action in several patients, suggesting that some other components present in GTW enhance the activity of triptolide to keep the dosage below a harmful level[40].

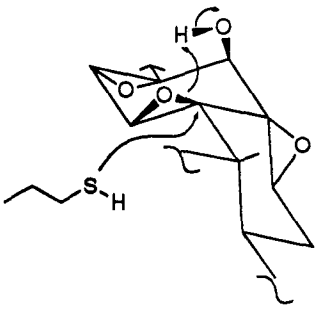
Triptolide has a strong anti-leukemic activity in mice. An injection with triptolide (at 0.1 mg/kg intravenous[12, 13] or at 0.25 mg/kg intraperitoneal[44]) greatly extended survival time after mice were injected with leukemic cells. The LD_{50} for triptolide (intravenous injection of mice) has been determined to be 0.8 mg/kg for a single dose and 0.16 mg/kg/day over seven days with concurrent degeneration of heart tissue and bone marrow[1]. This shows that the toxic dosage is very close to the therapeutic dosage and thus triptolide has not been applied as an anti-leukemic pharmaceutical for human patients.

---

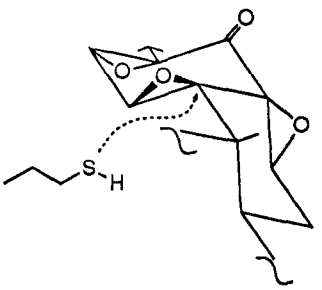
* LD_{50} ; the dosage lethal to 50% of test animals

The specific pharmacology of triptolide involves a strong inhibition of T-cell activity (cell-mediated immunity) that can be observed with mouse spleen cells *in vitro* at 50 ng/ml (0.14 μM), a cytostatic activity observed in human cell cultures at less than 3.0 μg/ml (8.3 μM) and a cytotoxic activity against human carcinoma cell cultures at 1.7 ng/ml (0.005 μM)[13, 41]. An inhibition of antibody production occurs in mice at 1.0 mg/kg (intraperitoneal)[39] which could result from the impairment of T-cell activity combined with cytostatic effects.

A mechanism for the action of triptolide has been demonstrated by Kupchan and Schubert[13]: triptolide may be able to modify the activity of cell growth regulating enzymes through the alkylation of thiol groups in cysteinyl residues. The alkylation process was modelled by opening the electrophilic 9,11-epoxide of triptolide and tripdiolide with propanethiol (pH 7.4) to produce the thio-ether at carbon 9. This opening can not occur without the donation of a hydrogen bond from the hydroxyl at carbon 14, as in the case of triptonide. The significance of epoxide opening is supported in that triptonide lacks anti-leukemic activity.



triptolide



triptonide

As well as the 9,11-epoxy-14β–hydroxy system, triptolide also contains an electrophilic α-methylene-γ-lactone. As a class these lactones have demonstrated anti-tumor activity[13]. The epoxide and lactone systems must both remain intact for triptolide analogs to exhibit anti-leukemic or anti-tumor activity[69, 70].

METHODS OF YIELD OPTIMIZATION IN PLANT CELL CULTURES

When a potential pharmacological application is found for a natural product, medical testing is begun and a large quantity of pure compound must be isolated. Several milligrams is sufficient for *in vitro* analysis and one hundred milligrams will supply a study on a population of mice, but gram quantities are often required for testing on human subjects as at least a dozen patients must be treated for a period of several months.

Tripterygium is harvested from the forests of southern China. Word of the plant appeared in North American publications after farmers, gathering the roots for insecticide, had caused major landslides[8]. Individual natural products are very dilute in the plant and so a large amount of material is required. In the example of tripdiolide, an isolation from dried root core of only 0.001% (10 mg/kg) is reported[12] along with 0.0005% from dried stem and leaves[42].

The level of natural products is affected by plant growth conditions such that the poorest environment can result in the highest yields. One experiences this effect with cultured food crops that display a milder flavour than the wild counterpart. Adverse conditions that are stressful to the plant increase the level of metabolites thought to act in defensive roles.

While the metabolite levels of cultivated crops can be manipulated through the growth conditions, the laboratory is a superior environment for this method. Kupchan's initial exposure of the anti-leukemic compounds of *Tripterygium* [12] initiated our group's interest in using plant cell cultures as a system for enhancing tripdiolide production and screening for further active compounds. *Tripterygium* cultures produced tripdiolide in 36-times the natural level once production was optimized (Kutney et al.[71]). Tripdiolide, assayed by TLC-fluorimetry, was found mainly in the nutrient medium after cells had been filtered out. This tripdiolide amounted to 0.036% of the whole culture dry weight, a level reached in 25-35 days compared to a crop time

of at least a year for whole plants. Seventy-eight liters of cell suspension (approximately 860 g dry weight) provided the isolation of 200 mg of pure tripdiolide (and 179 mg of pure triptolide) following several column chromatographies (a 0.023% yield of tripdiolide[25]). Two other groups have developed suspension cultures of *Tripterygium*[72,73] but the maximum yields of tripdiolide were relatively low (below 0.01 %).

While most of the commercially available natural products are currently isolated from plants, the drawbacks of this production method include a long growth time, difficulties in obtaining ton quantities of plant material and a general low yield which can be further decreased by climatic conditions.

Cell cultures offer the advantage of controlled growth conditions and allow media optimization for increased metabolite production as well as a ready selection of high yielding cell lines. The lack of extensive tissue formation in cell suspensions also provides a facilitated access to cells for enzyme isolation and the study of biosynthetic pathways.

Three basic methods for manipulating *Tripterygium* cultures to produce higher product yields are currently used by our laboratory. The primary method is alteration of nutrient and hormone levels to stimulate a metabolite production cycle. The second, a technique called elicitation, is a method used to stimulate accumulation of antimicrobial compounds by activation of the infection resistance process. The third method is the biotransformation of synthetic precursors. In a successful biotransformation one introduces a large quantity of a compound (similar or identical to a natural biosynthetic intermediate) into cell cultures and the plant enzyme systems convert it to a more elaborate product. The following text provides a description of these three methods.

(I) NUTRITIONAL STUDIES ON CELL CULTURES OF *TRIPTERYGIUM*

The natural products of *Tripterygium* listed in *figure 1* are classified as secondary metabolites (save β-sitosterol, 69). Plant biochemistry can be divided into two systems. The central one is primary metabolism which produces energy as well as the protein, carbohydrate and lipid compounds required in structural and homeostatic roles. Primary metabolic processes are mainly identical among various plant types. Of a lesser importance are the secondary metabolic processes since they produce compounds that are not essential for plant growth. A lack of demand has allowed the evolution of secondary pathways along various lines in different plant types. Assorted functions include the production of pigments, insect antifeedants, cytostatic or antimitotic compounds (which control infectious pathogens), toxins and insect pheromones. While these roles have been determined for some compounds, many others have no known biological activity. A diversity of secondary products allows one to identify genetic relationships between plant types according to the catalog of compounds produced. This is the technique of chemotaxonomy. *Tripterygium* is part of the family *Celastraceae* and many of its secondary products are observed in other family members such as *Euonymus, Celastrus* and *Maytenus*. Examination of *figure 1* reveals that most of the secondary metabolites from *Tripterygium* are terpenoid compounds. The process of terpene biosynthesis will be discussed later in this introduction.

In plant cell cultures as well as whole plants, primary and secondary metabolic activities are maximized at opposite stages of the growth cycle. Initially, a plant uses energy and chemical precursors for rapid growth. Then if a disease occurs, growth is inhibited while resources are diverted to secondary metabolism which provides compounds to resist further infection. When a plant has grown to reproductive readiness, growth slows and secondary metabolism is activated to produce compounds that may discourage foraging insects or animals. The inverse relationship between the growth cycle and secondary metabolism is carried over by plant cells grown in

culture, making metabolite production a two stage process. Cells are first grown up to a high mass and then stimulated to convert resources to the synthesis of secondary compounds.

As described in Kutney et al.[24], the first tripdiolide from cultures occurred in a level only slightly higher than that in the whole plant. A program to optimize tripdiolide production was subsequently carried out[71]. Tripdiolide was determined both directly by quantitative TLC-fluorimetry and by inference from the cytotoxicity against KB cells (human carcinoma).

To begin the plant cell cultures, small leaf cuttings from *Tripterygium* were incubated on a solid nutrient agar. Some of these explants developed a growth of callus tissue, a clump of rapidly dividing cells that lacks obvious structure and often occurs in plants at the site of injury. Callus lines with detectable tripdiolide were used to start suspension cultures by a transfer to stirred liquid medium. The suspensions were maintained by successive subculture at the end of each growth cycle with cells dividing rapidly until the point of nutrient depletion. This method provided a somewhat heterogeneous population of cells called a clonal variant which could reproduce for an indefinite period through mitotic division . One clonal variant of the many produced was selected for its stable growth and the ability to produce tripdiolide. This line was the TRP-4a variant and has been maintained in callus and suspension culture for the past eight years. Over this time the TRP-4a variant has altered from a stage where cells aggregated in large clumps with some root formation and has become a smooth suspension of small cell clumps with the appearance of a fine sand and a pale green to tan coloration.

The nutrient mixtures used to grow *Tripterygium* are based on either of two standard media, PRL-4 or MS(2% sucrose). These formulations supply all growth requirements for plant cells raised in darkness; sucrose is the carbon/energy source and simple salts supply nitrogen, phosphorous and sulphur for the synthesis of amino and nucleic acids. *Tripterygium* cultures are grown in the dark at 25°C in half-liter stock cultures swirled in erlenmeyers (shake flasks) or in ten liter production cultures grown in aerated bioreactors. Sterile conditions are maintained throughout the growth process.

The following additives, compounds that act as plant hormones to alter the cell state, were screened with respect to their effect on growth and the production of tripdiolide.

Additive *		Activity
I	indole-3-acetic acid	auxin (accelerates cell enlargement)
D	2,4-dichlorophenoxyacetic acid	auxin (synthetic) (accelerates cell enlargement)
NA	1-naphthaleneacetic acid	auxin (synthetic) (accelerates cell enlargement)
K	kinetin	cytokinin (induces cell division)
Co	fresh coconut milk	undefined mixture containing cytokinins, facilitates cell growth in culture medium

Leaf explants were found to initiate callus growth most readily on a solidified PRI_2Co agar. Callus and suspension cultures were then maintained with PRD_2Co. These media are the basic PRL-4 formula with the listed additions. I and D have similar biological activity; I is a natural product which is degraded rapidly in solution while D is a stable synthetic substitute. Coconut milk has many growth enhancing components which support cells in culture, though recent results (unpublished; Biological Services, UBC) have demonstrated good growth on a completely defined medium after a gradual reduction in coconut milk over many subcultures. PRD_2Co supported a rapid and stable growth (requiring 14-18 days for stock suspensions) but tripdiolide was not produced by these cultures. Other media were screened with respect to tripdiolide production and it was observed that maximal tripdiolide was produced following inoculation of $MSNA_{0.5}K_{0.5}$ medium (MS medium plus additions) with a stock culture that had

* The level added to media (mg/L) is denoted by a subscript (eg $D_{0.5}$). Co is used at 10% v/v .

been raised to the end of growth phase in PRD_2Co. From the time of this inoculation, tripdiolide reached a peak level after 25-35 days, which coincided with the end of the growth phase. To recover the tripdiolide, cells and medium were separated and extracted with ethyl acetate.

Growth was slower in $MSNA_{0.5}K_{0.5}$ and yielded a lower biomass, while tripdiolide in the filtered culture medium reached levels up to 4.0 mg/L (0.036% of culture dry weight), more than thirty times the yield from plants. The $MSNA_{0.5}K_{0.5}$ medium shares many basic constituents with the PRD_2Co formulation save the replacement of growth enhancing coconut milk with a pure cytokinin, kinetin. D and NA have equivalent activity as auxins. $MSNA_{0.5}K_{0.5}$ has a greater overall concentration of nutrients, including a doubling in the available nitrogen as well as increased calcium, decreased thiamine, the presence of glycine and small variations in the micronutrient levels (Mn^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}, etc).

Increases in the individual components of $MSNA_{0.5}K_{0.5}$ have demonstrated that no particular additive is responsible for the increased tripdiolide production[71]. It is a very good growth medium, yet the transition from PRD_2Co to $MSNA_{0.5}K_{0.5}$ results in a decreased biomass yield. The synthesis of other diterpene and triterpene secondary metabolites is activated along with that of tripdiolide[24, 25] with a visible darkening of cultures owing to the appearance of orange quinone methides and dark compounds which may be polyphenols.

The production of previously undetected secondary metabolites and a slower, low biomass growth are consistent with the hypothesis that a profound shift in metabolism occurs within the cultures on exposure to $MSNA_{0.5}K_{0.5}$: a transition from growth phase to a production phase, not caused by a specific additive so much as by a sudden change in the environment. In the past several years that $MSNA_{0.5}K_{0.5}$ has been used to produce tripdiolide, the biomass yield has been observed to vary from culture to culture. Following extraction, poor yields of tripdiolide were obtained from cultures exhibiting the low and high extremes of cell growth while the intermediate growth cultures produced maximal tripdiolide. This appears consistent

with a model in which stress on the culture is required to increase the population of cells in a production phase, to an upper limit above which the cells are not able to function.

$MSNA_{0.5}K_{0.5}$ medium stimulates the more mature "production" phase in *Tripterygium* cells in a fashion that cannot be directly linked to a particular culture component or physiological mechanism. Elicitation is an alternate method to increase the level of secondary metabolites. The technique appears to specifically activate disease resistance responses and in theory will produce only those metabolites involved in fighting infectious pathogens.What is known of the mechanism of elicitation is reviewed in the following section.

(II) ELICITATION

Some plant-derived secondary metabolites have antimicrobial properties which are displayed by the isolated compounds as well as from within the intact plants. With many plants, exposure to a pathogenic organism results in the rapid accumulation of these compounds and this lends resistance to spreading of the infection[74]. This type of metabolite induction is termed *elicitation.* Elicitation has been used to stimulate plant cell cultures such that the yield of antimicrobial metabolites was increased. The techniques used for this purpose will be reviewed following a brief summary of plant pathogen interactions.

(i) Disease Resistance in Plants

The laboratory applications of elicitation have been modelled mainly on the physiological response of plants following exposure to a pathogenic fungus. Similar responses are seen when the pathogen is bacterial, and to a lesser extent, when it is viral. During a successful infection process, a fungus penetrates the wall of a plant cell, digesting the wall and then the cellular contents. The plants defences are challenged in this order :

1. waxy cuticle or bark
2. cell wall
3. constitutive antimicrobial compounds
4. defences induced by the pathogen

Infection through the cuticle, a physical barrier of hydrocarbon wax, is usually fortuitous but the fungus has specific resources for penetrating the underlying plant cell wall. This cell wall is a laminar array of polysaccharides containing a small amount of protein and lipid. The outermost layer, cellulose, is composed of (1 → 4) linked β-D-glucose subunits. Beneath this, there is the pectin layer made up of galactose, rhamnose, arabinose and galacturonic acid

polymers and copolymers. The patterns of linkage and branching in these repeating sequences are common to many various plant types. It has been found that every type of plant cell wall polysaccharide can be digested into soluble oligomers by specific hydrolytic enzymes isolated from plant pathogens[75]. During an infection the plant cell wall acts to delay exposure of the vulnerable cell membrane, meanwhile the plant uses further resources to kill the fungus or inhibit its multiplication.

A plant's defenses can include constitutive antimicrobial compounds stored either in the cell wall or in intracellular compartments[74]. Maintaining this defence requires the continuous expenditure of energy and metabolites. The more economical defence is induced only on exposure to a pathogenic organism. Common components of a plants inducible defence are :

i)	Phytoalexins	-	low molecular weight secondary metabolites with antimicrobial activity produced in response to an imposed stress
ii)	cell wall reinforcement	-	polymer deposition (callose or lignans)
iii)	enzymes attacking the pathogen	-	cell wall hydrolases
iv)	necrogenesis	-	self destruction of plant cells following fungal exposure

When plant tissue is exposed to a fungal pathogen, rapid alterations may be observed in the plants respiration and hormonal balance[76]. A typical result is cell wall thickening. In pine seedlings, a layer of callose (β-(1 $\rightarrow$ 3)-glucan polymer) was produced precisely at the boundary of exposure to a fungus and this was detected only sixty minutes after the inoculation[77]. Such exposure may also stimulate a plant to rapidly accumulate fungal-specific cytotoxic or cytostatic secondary metabolites.

(ii) Phytoalexins

Examples of secondary metabolites produced by various plants following fungal exposure are presented by Eilert et al.[78] and Brodelius[79]. If compounds generated in this manner exhibit antifungal activity and are present in healthy plants in only trace amounts, they are classified as phytoalexins

The phytoalexin families include isoflavonoids, terpenes, polyacetylenes, stilbenoids and alkaloids. A good demonstration of the role of phytoalexins in plant defences is the response of soybean seedlings to fungal invasion[74]. The antifungal isoflavonoid glyceollin I is absent in control seedlings. Following a root-dip of the seedlings in fungal inoculum, this compound was produced rapidly and specifically at the site of infection. After 8 hours, an intracellular concentration of 0.6 mM was reached, and this was found to produce 90 % inhibition of fungal growth *in vitro*. The fungal infection was restricted to the exposed tissues; both the treated and control plants could be grown to maturity. When the seedlings where pre-treated with an inhibitor of isoflavonoid biosynthesis, inoculation resulted in widespread fungal invasion which stopped seedling growth. The mechanism of antifungal action for the glyceollin family involves direct disruption of fungal cell membranes [80].

(iii) Elicitors

Agents which cause the activation of plant defences (eg. phytoalexin production) are termed *elicitors*. While the term is normally used to describe activation by pathogenic organisms, other elicitors include physical stress, chemical agents and electromagnetic or ionizing radiation. A review by Darvill and Albersheim[81] is an extensive introduction to the elicitation of phytoalexin production. In one study, Eilert et al.[78] discovered a 300 fold stimulation of sanguinarine production from poppy cell cultures using a fungal elicitor (*Botrytis*). This antifungal alkaloid reached 2.9 % of the cells dry weight. Several mechanisms which are thought to be active in the elicitation of plant defences are outlined in the following scheme.

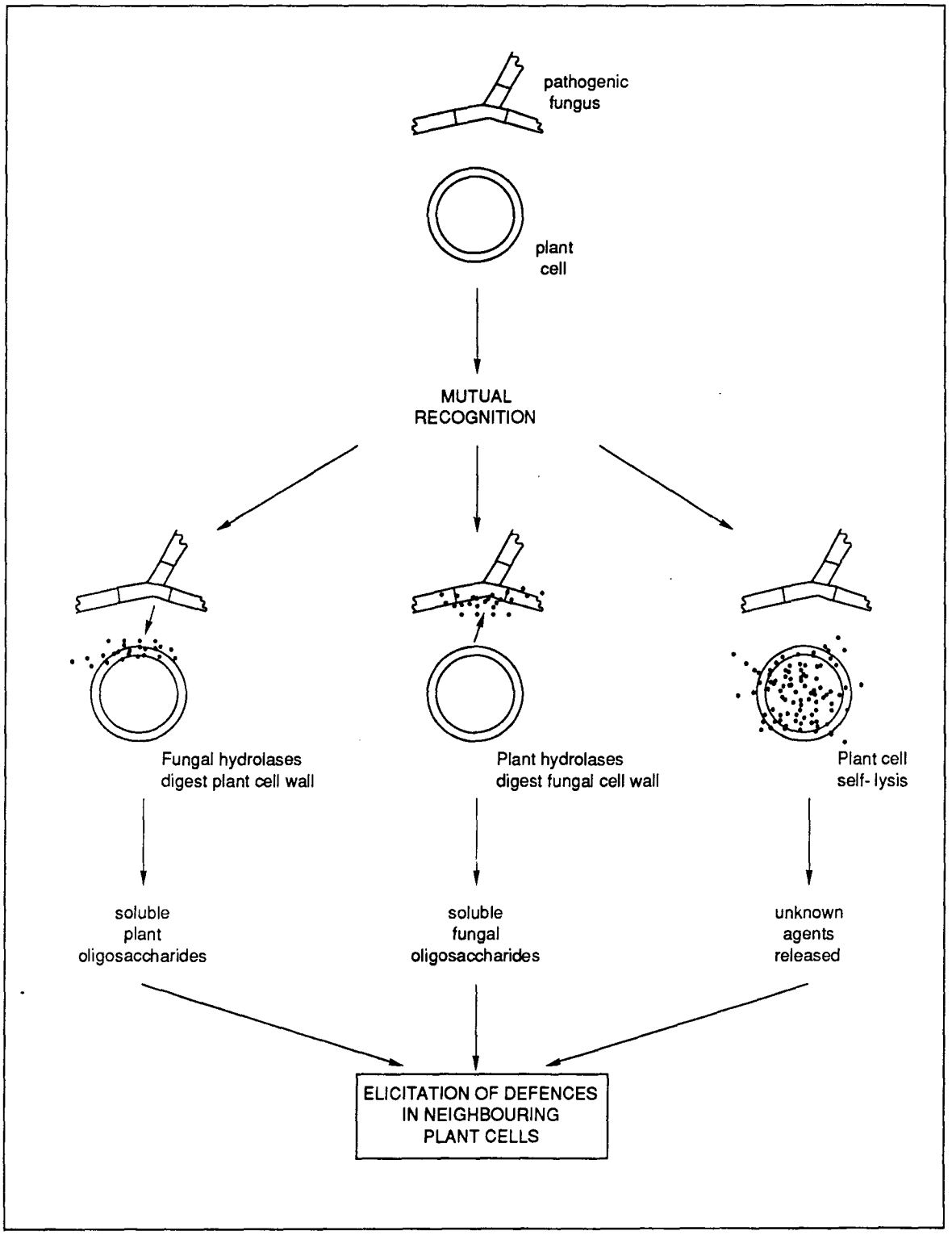
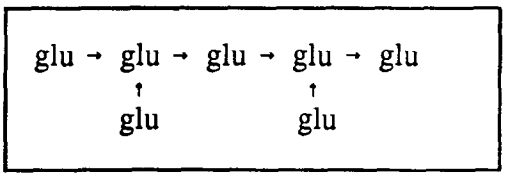


Figure 5 The Activation of Elicitation

This figure reveals that the effects of an eliciting fungus are brought on by the transmission of by chemical signals in the form of soluble oligosaccharides. This allows elicitation of neighbouring plant cells that have not been infected and therefore possess greater metabolic capabilities. It also means that a particular plant will respond to various types of fungi with the same set of defences (provided the fungi have similar cell walls). The two sources of soluble oligosaccharides have been demonstrated in the following experiments:

(a) pathogenic fungi synthesize a complement of hydrolytic enzymes able to degrade the plant cell wall[75] and the soluble oligosaccharide products have been shown to be elicitors. When pectin hydrolysates from cultured carrot cells[82] were re-introduced to parent cultures, the production of the phytoalexin 6-methoxymellein was initiated within 24 hours (also observed when only the hydrolysis enzyme, pectinase, was added to cultures). By this activation mechanism, plants are responding directly to damage caused by a fungus.

(b) alternately, some plants cells store hydrolases in their outer wall that are specific for fungal wall polysaccharides. Contact with a fungus brings the release of soluble fungal oligosaccharides which elicit the plants defences. To test this process, Darvill and Albersheim[81, 83] prepared an acid hydrolysate of polysaccharides from a fungal pathogen. The resulting oligosaccharide mixture stimulated phytoalexin production in soybean. Fractionation of this hydrolysate yielded the most active component, a seven unit glucan (polyglucose) with β-(1→6) connectivity and β-(1→3) branching:



Many fungi contain chitin (polyglucosamine) in their outer cell wall.. The hydrolytic enzyme chitinase, which degrades the polymer to oligomers, can be extracted from some plant species. The soluble digestion product of chitin (sold commercially as *chitosan)* often demonstrates elicitor activity with plant cell cultures[84].

Following fungal elicitation, some plants have demonstrated a rapid autolysis described as the necrogenic response[74]. This occurs in plant cells directly exposed to fungus and appears to cause the elicitation of more distant cells. Since mechanical cell disruption does not bring elicitation it is likely that necrogenesis is a specific physiological process for amplifying an elicitor signal.

(iv) Mechanism of Elicitor Action

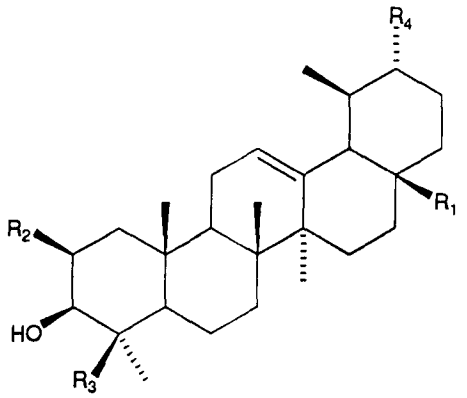
Protein receptors capable of binding oligosaccharides have been found on the surface of plant cells. Involvement of these receptors in the transduction of elicitor signals was demonstrated by Ebel and Grisebach with soybean seedlings[74]. A β-glucan elicitor was purified from a fungal hydrolysate and labelled with tritium. Cells from the seedlings had high affinity surface binding sites for this oligosacccharide and the radioactivity was exchangeable in the presence of the unlabelled glucan. Analogous glucans with different chain length or branching pattern had a decreased ability to compete for binding sites and this binding affinity *in vitro* was proportionate with elicitor response in whole plants. Surface receptors for the glucan elicitor isolated by Darvill and Albersheim ((b) on previous page) were also demonstrated[81,83]. In these studies, the extent of elicitation was dependant on the ratio of elicitor amount to cell biomass rather than on the elicitor concentration, thus plant cell receptors appear to bind elicitor tightly.

The mechanism for transduction of the elicitor signal within the interior of plants cells has not been identified. Calcium addition to the cell growth medium increased the sensitivity towards elicitation[74], thus oligosaccharide receptors may control calcium influx. The target for elicitor stimulation has been identified as gene activation by several groups[74,82,85] who

demonstrated that phytoalexin production is preceded by the synthesis of mRNA. As well, elicited increases of enzyme activity in the phytoalexin synthesis pathways was blocked by inhibitors of protein synthesis. In overview, elicitor stimulation leads to increased gene transcription producing a high enzyme population in the phytoalexin synthesis pathways.

(v) Elicitation of Triterpene Acid Biosynthesis

Experiments in our laboratory (to be detailed in this thesis) have demonstrated elicitation of triterpene acids from *Tripterygium* . A report in *Phytochemistry* (1989) by Threlfall and colleagues also describes the elicitation of cell cultures to produce triterpene phytoalexins[86]. The tropical shrub *Tabernaemontana divaricata* was cultured and elicited with a *Candida albicans* preparation (a yeast-like fungus parasitic on plants). Since Threlfall et al.'s findings were analogous in several ways to results from *Tripterygium*, I will give their work a brief review. The elicitation from *Candida* caused an inhibition of cell culture growth followed by the production of ursane (triterpene) phytoalexins to a maximum of 1.4 % of culture dry weight :



	R_1	R_2	R_3	R_4
i)	CO_2H	H	CH_3	CH_3
ii)	CO_2H	OH	CH_3	CH_3
iii)	CO_2H	H	CH_2OH	CH_3
iv)	CH_3	OH	CH_2OH	CO_2H

Figure 6 Elicited Ursane Triterpenes

Thirty-six hours after the elicitation, the capacity for synthesis of triterpenes had increased five-fold, assayed *in vitro* by the activity of squalene synthetase (an enzyme required for triterpene and phytosterol production).

Threlfall also used an elicited population of enzymes to carry out phytoalexin biosynthesis *in vitro* using disrupted cells[86]. A plant cell suspension culture was exposed to elicitor and after a short period a cell free enzyme extract was prepared. These enzymes, together with added cofactors, were able to convert radiolabelled isopentenyl pyrophosphate (IPP) into an ursane triterpene phytoalexin. In parallel controls using non-elicited cells, the label was only incorporated into phytosterols, which lack phytoalexin activity. Radiolabelled phytosterols were not detected in the elicited transformation, revealing that elicitation inhibits phytosterol production while activating phytoalexin production. Phytosterols are used for cell membrane formation. Inhibition of their synthesis could be responsible for the retarding effect of elicitation on cell growth. A summary of the effects of elicitation on metabolite production is presented on the following page.

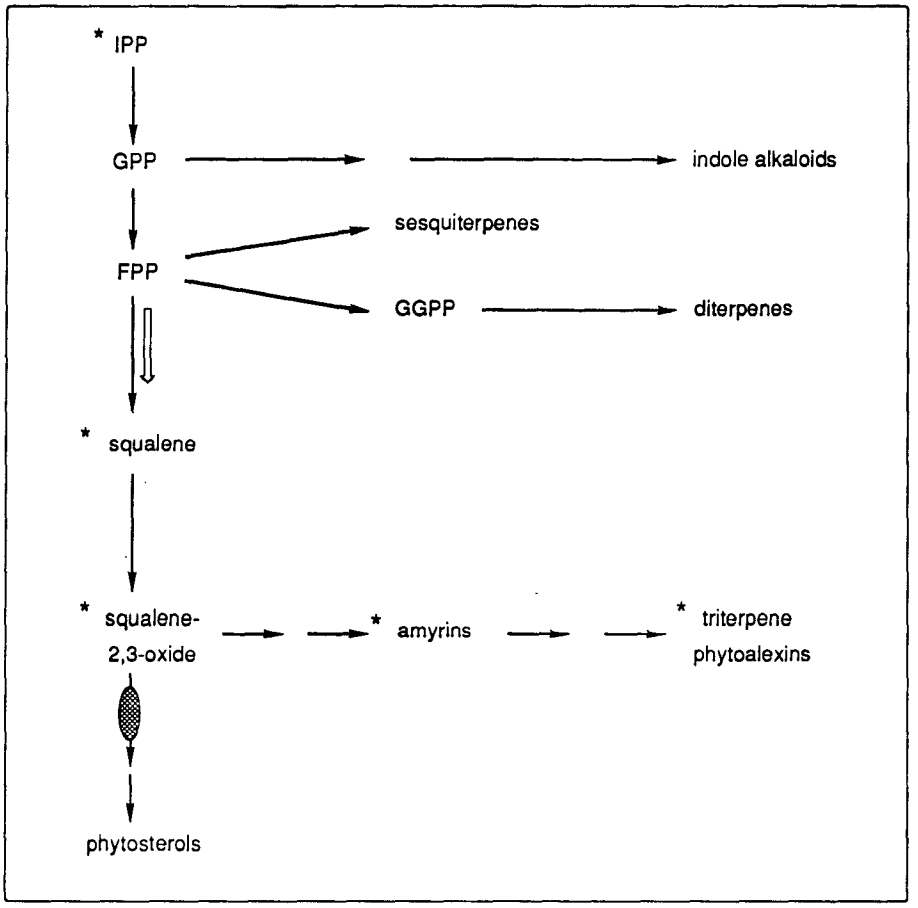


Figure 7 Partition of 1-^{14}C-IPP in Cell Free Biotransformations Using Elicited Cultures

Inhibited ( ), Enhanced ( ), Isolated with radiolabel (*)
IPP - isopentenyl pyrophosphate
GPP - geranyl pyrophosphate
GGPP - geranylgeranyl pyrophosphate
FPP - farnesyl pyrophosphate

In this model, elicitation creates a high activity of squalene synthetase, depleting the pool of FPP and thus decreasing the rate of sesquiterpene and diterpene synthesis. Indole alkaloids containing terpene components were not produced by the elicited cultures although they accumulate in non-elicited preparations. This may result from a depletion of GPP brought about by an increase in the metabolite flux towards squalene. At the branch-point following squalene oxidation, inhibition of phytosterol synthesis directs squalene-2,3-oxide exclusively towards pentacyclic triterpenes.

In our laboratory, the triterpene acids were selected as targets for elicitation due to their anti-inflammatory activity demonstrated in small animals (Table 1, page 20). This was part of our larger interest in isolating the anti-inflammatory, immunosuppressive, anti-fertility and anti-leukemic agents which give *Tripterygium* medicinal value. Threlfall et al. have provided a clear example of increases in non-essential triterpenes brought about through fungal elicitation. While it has not yet been proven that high triterpene content in whole plants is effective in inhibiting fungal infection, it seems very likely that the specific triterpenes synthesized after fungal exposure are part of the natural resistance process and thus function as true phytoalexins.

Anti-inflammatory activities from oleanane triterpene alcohols and hydroxy-acids have been revealed by Professor Shoji Shibata's group in a number of papers [87-91]. These compounds are structurally similar to the triterpene acids from *Tripterygium*, and were isolated in a study of traditional medicines parallel to the work on-going in our research group. The role of triterpenes as accessible plant phytoalexins and as compounds with medicinal potential is becoming well recognized. Further research will likely lead to their development into commercial pharmaceuticals.

(III) BIOTRANSFORMATION OF SYNTHETIC PRECURSORS

When plant cells are used to produce a compound, one limitation on yield is that secondary metabolite levels are regulated through enzymatic control. This is especially relevant for a toxic compound which must be compartmentalized within the cell. It is generally necessary to the cell that regulation of synthesis occurs early in a pathway to avoid the accumulation of intermediates. For example, in the mammalian synthesis of cholesterol regulation occurs at the six carbon stage where the transformation of hydroxy-methyl-glutaryl-CoA to mevalonic acid,the first dedicated terpene intermediate, is inhibited by the end product, a cholesterol ester. This type of regulation hinders the production level from bioreactor cultures, where it would be no drawback for cell death to occur from excess product accumulation. Regulation can be bypassed if one obtains quantities of a precursor that enters the synthesis after the regulation step, in which case high levels of biotransformation can be accomplished. Another attractive process is the use of soluble enzyme systems from cell-free plant extracts to carry out a biotransformation. These systems require only a few added cofactors, such as oxidizing or reducing agents to drive non-spontaneous processes. The major advantage here is simplified product recovery. The work carried out by Professor Kutney provides several examples of these methods[92]. In one case, cell cultures of *Catharanthus Roseus* provided enzyme extracts to couple indole precursors into a bis-indole product in an efficient synthesis of vinblastine. Also, a similar enzyme preparation effected a ring closure reaction in the production of the polycyclic phenol etoposide. Both products are widely used, valuable cancer therapy agents currently isolated from plants.

To utilize plant enzymes as synthetic tools, the natural biosynthetic intermediates of a production pathway must be determined. After this, an accessible intermediate can be targeted for laboratory synthesis. Terpenes share a biosynthetic pathway which forms isopentenyl pyrophoshphate (IPP) from acetic acid (as a thioester coenzyme adduct). All the various classes of terpenes are then generated from IPP[93] (figure 8).

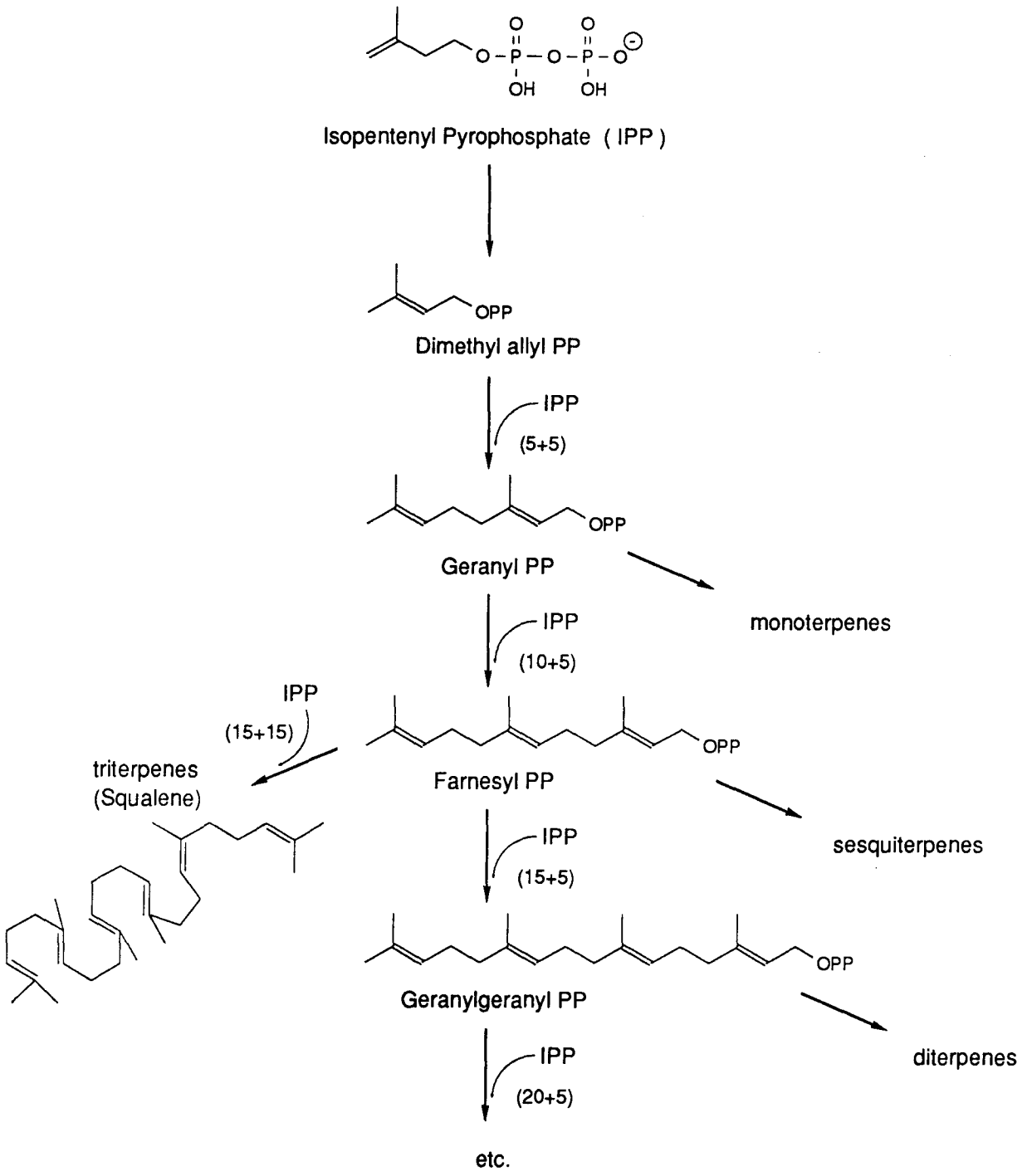


Figure 8 Biosynthesis of Terpene Classes

The phosphate esters of terpenols are either incorporated directly into other molecules or cyclized to yield the terpene classes of *figures 1-4* . The sesquiterpene agarofurans are produced through the cyclization of farnesyl pyrophosphate and the diterpene abietanes from

geranylgeranyl pyrophosphate. The oleanane, ursane, friedelane and quinone methide classes of triterpene are synthesized from squalene which is produced through a dimerization of farnesyl pyrophosphate. The biosynthesis proposed for the abietane diterpene dehydroabietic acid (which is produced by *Tripterygium*) follows this path:

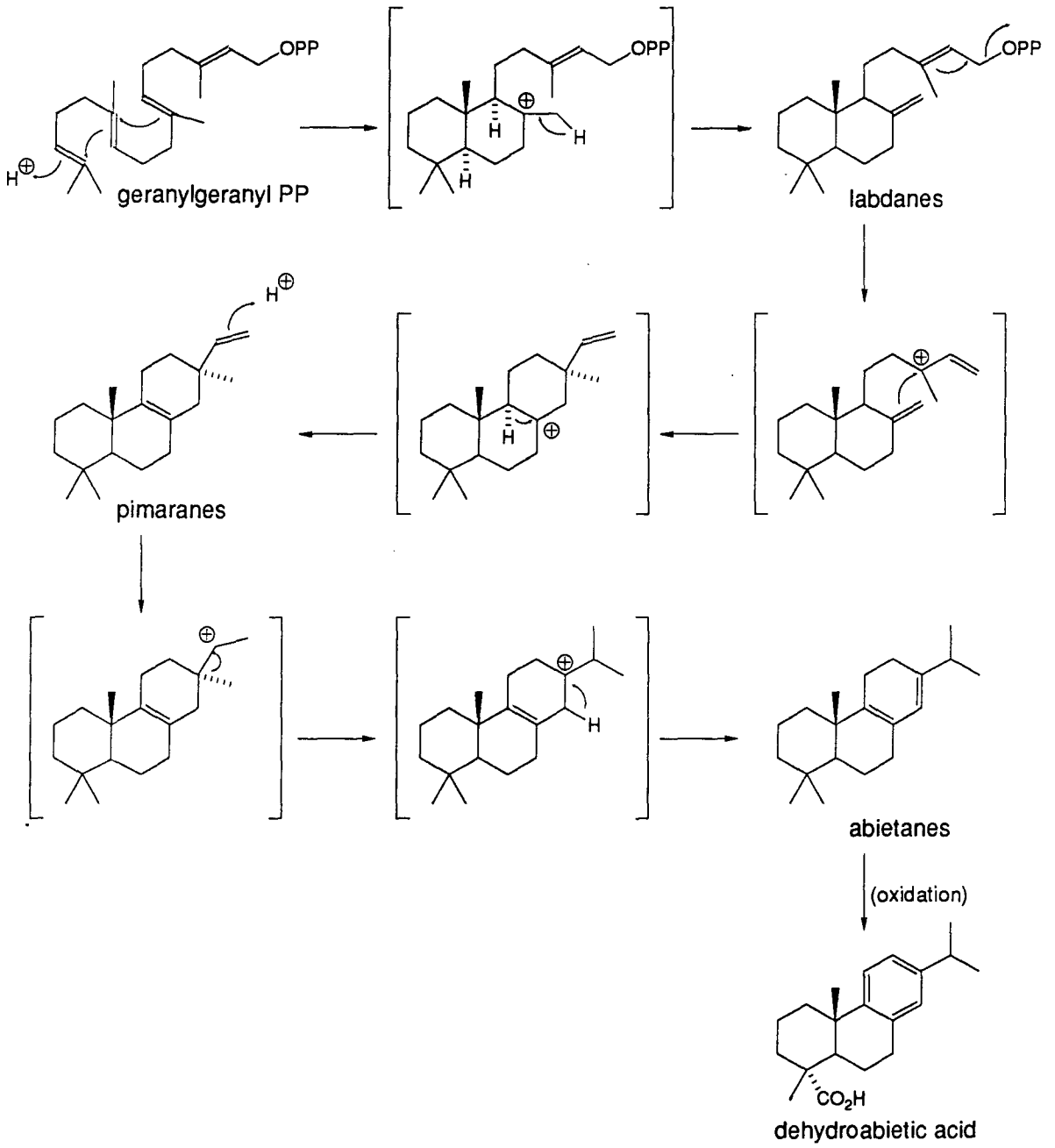


Figure 9 Biosynthesis of Abietane Diterpenes

In the plant cell, these processes occur on an enzyme surface where only one optical isomer is formed. The enzymes are thought to catalyze terpene synthesis by supplying electron donating systems which stabilize carbocations or act as bases in an elimination process.

To elucidate the synthesis of tripdiolide, one may work forward from an early intermediate such as geranylgeraniol or backward from the end products using late intermediate analogs. In the first method, one introduces radiolabelled geranylgeraniol for a short incubation with plant cells then extracts the products, most likely containing subsequent intermediates in labelled form. With this approach, Misawa et al.[72] determined that the addition of farnesol to cell culture medium caused a small increase in the level of tripdiolide produced.

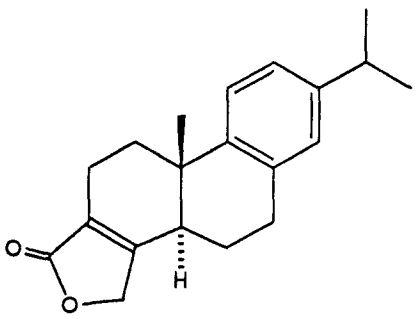
The other method of analysis, working backward from the product, requires a retrosynthetic analysis guided by any known intermediates. One synthesizes the labelled form of a possible precursor which closely resembles the end product and then tests for its incorporation into the end product. Attempts by our group demonstrated that no tritiated dehydroabietic acid is incorporated into tripdiolide, therefore it is likely that tripdiolide biosynthesis branches from an earlier abietane-type intermediate.

The late intermediate approach was chosen by our group since a very useful biotransformation, the epoxide incorporation, is likely to occur in a final stage of tripdiolide production. The triptolides have two main elaborations on the abietane skeleton, the butenolide at ring A and the hydroxy-triepoxy system of ring C. These functionalities are isolated on the carbon skeleton, making it likely that they are formed sequentially rather than concurrently. The butenolide is likely formed first due to its greater stability in solution. Support for this hypothesis is provided by the abeo-abietane compounds isolated from *Tripterygium* which display the completed butenolide at ring A but have only phenolic systems at ring C; conversely, epoxide compounds lacking the butenolide have not been isolated.

In the synthesis of potential precursors to tripdiolide, it was envisioned that a butenolide compound could be readily prepared from dehydroabietic acid which is in turn an available

component of tree resins. A program was begun to synthesize butenolides with suitable unsaturation at ring C for biological epoxide addition. In this respect, the presence of dehydroabietic acid in culture medium[3] suggests that the plant metabolism can recognize an aromatic ring.

Several other groups have developed synthetic strategies to the butenolide portion of the triptolides. These processes fall into two main classes, the synthesis of racemic butenolide from small achiral starting materials and the synthesis of optically pure butenolide from (l)-dehydroabietic acid. While the racemic product could not be used pharmacologically, it could be used in biotransformations to (l)-triptolides, though half of the compound would remain untransformed.



75 18(4→3)-isodehydroabietenolide

Synthetic planning to the butenolides must take into account a facile tautomerization to the A/B-*cis* isomer. The *trans* ring junction is stable in aqueous solution at neutral pH while in alkaline conditions a complete conversion to the *cis* junction takes place (Berchtold's group[70], using methoxide in methanol for 48 hours at room temperature). Alkaline or acidic equilibration though the extended enolate must therefore be avoided.

A synthesis of racemic butenolide by vanTamelen and Leiden[94] is one of the most facile pathways yet designed (figure 10). It results in 15% yield after 12 steps with only 4 purifications required.This is a biogenetic type synthesis inspired by the biosynthetic cyclization of geranylgeraniol. The key ring-forming step is the cyclization of a diterpene-like phenol derivative (A) which forms both A and B rings with the correct *trans* junction.

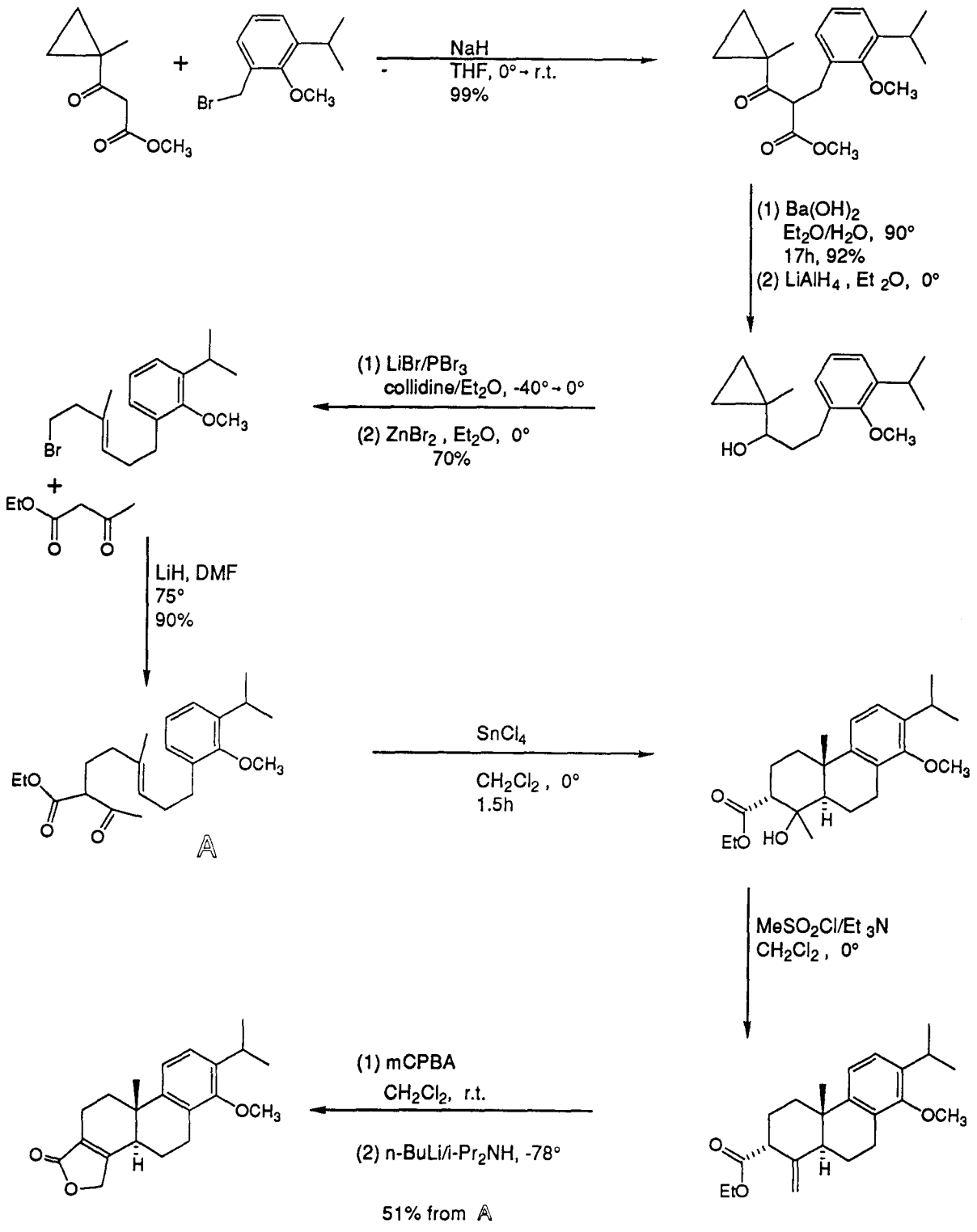


Figure 10 Synthesis of Racemic Butenolide by vanTamelen and Leiden (Biogenetic)

This and most other syntheses of butenolides lead up to a C_{14}-phenol product which facilitates the synthetic incorporation of the epoxide system. The absence of a phenolic hydroxyl (that is usually protected as an ether) would cause no apparent difficulties in these sequences.

Another racemic synthesis by Garver and vanTamelen[95] (figure 11) utilizes a *trans*-decalone for an A/B-ring nucleus. First ring C is added with a Diels-Alder reaction then ring A is modified to accept a one carbon unit at C_3 to prepare for the appropriate butenolide, a process outlined in figure 11. The C_3 addition occurs through a 2,3-sigmatropic rearrangement of a carbene intermediate generated by loss of methanol from a dimethylformamide mixed acetal.

A valuable tool for ring A transformations is introduced here at two stages of butenolide synthesis. This is the oxidation of an olefin to the rearranged allylic alcohol through epoxidation followed by ring-opening with base.

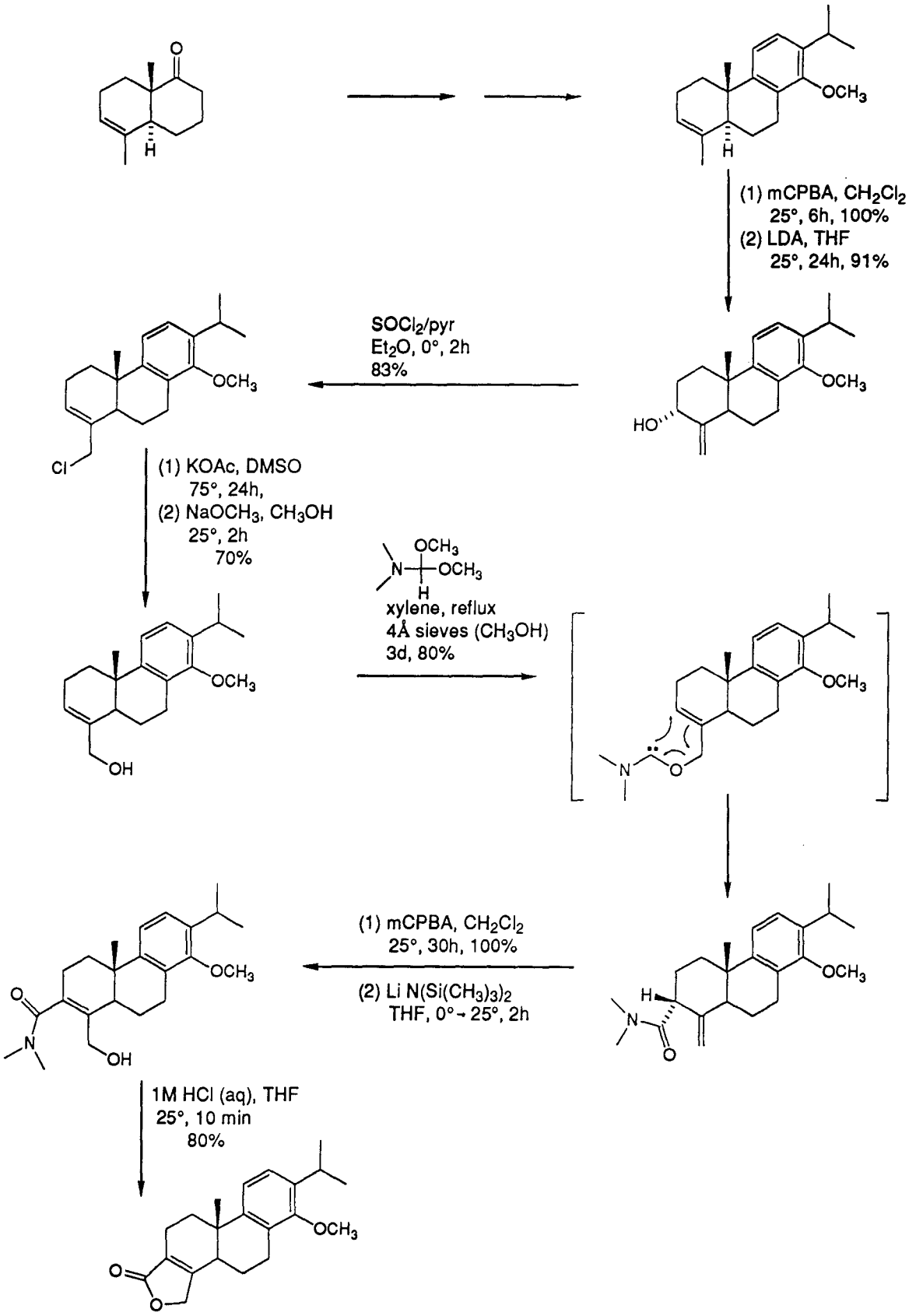


Figure 11 Synthesis of Racemic Butenolide by Garver and vanTamelen (Decalone)

The butenolide was also synthesized under the direction of Berchtold (Lai et al.[70]; figure 12) using a tetrahydronaphthalenone nucleus for rings B and C, then appending ring A through alkaline condensations. In this process, the formation of a large quantity of the more stable A/B-cis isomer was unavoidable during the conversion of a Δ4,5 olefin intermediate to the desired Δ3,4 olefin. The proper *trans* fusion was recovered by oxidation to a dienone and then catalytic hydrogenation (predominantly exo to the B-ring boat conformation).

vanTamelen et al.[96] carried out a chiral synthesis of the butenolide using (l)-dehydroabietic acid as a starting material (figure 13). After the aromatic C-ring was converted to the phenol ester at C_{14}, Curtius degradation was used to decarboxylate at C_4 followed by Cope elimination to the exocyclic olefin. Oxidative cleavage to the C_4 ketone allowed alkylation at C_3 using formaldehyde. The sequence used to complete the butenolide was a lengthy process demanding several protections (12% yield from the formaldehyde adduct). Benzyl methyllithium ether was added at C_4 and the product selectively oxidized to the correct hydroxy-acid. The release of a newer synthesis (figure 10) from the same group allows a more efficient butenolide completion if methyllithium is added to the C_4 ketone.

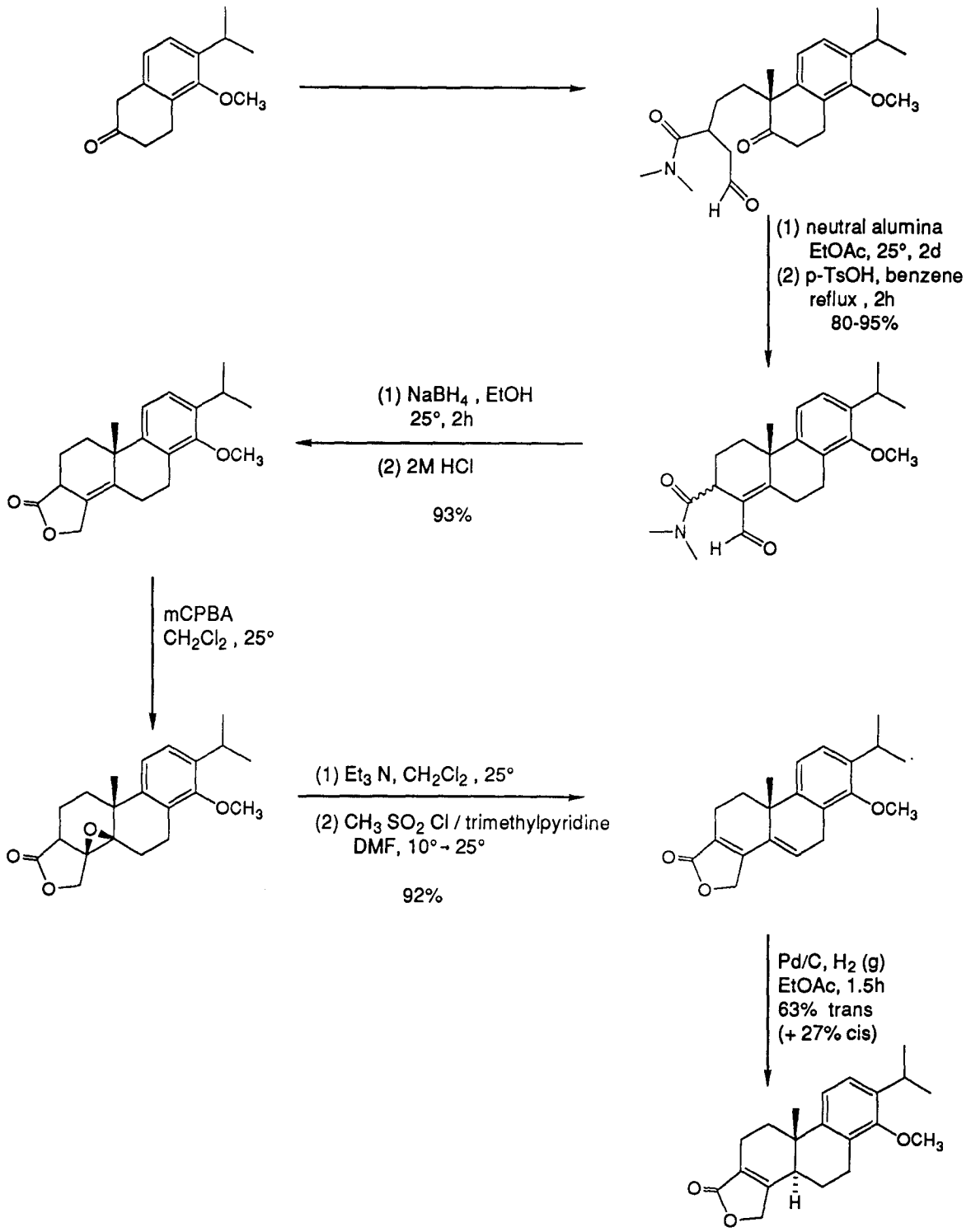


Figure 12 Synthesis of Racemic Butenolide by Berchtold et al. (Naphthalenone)

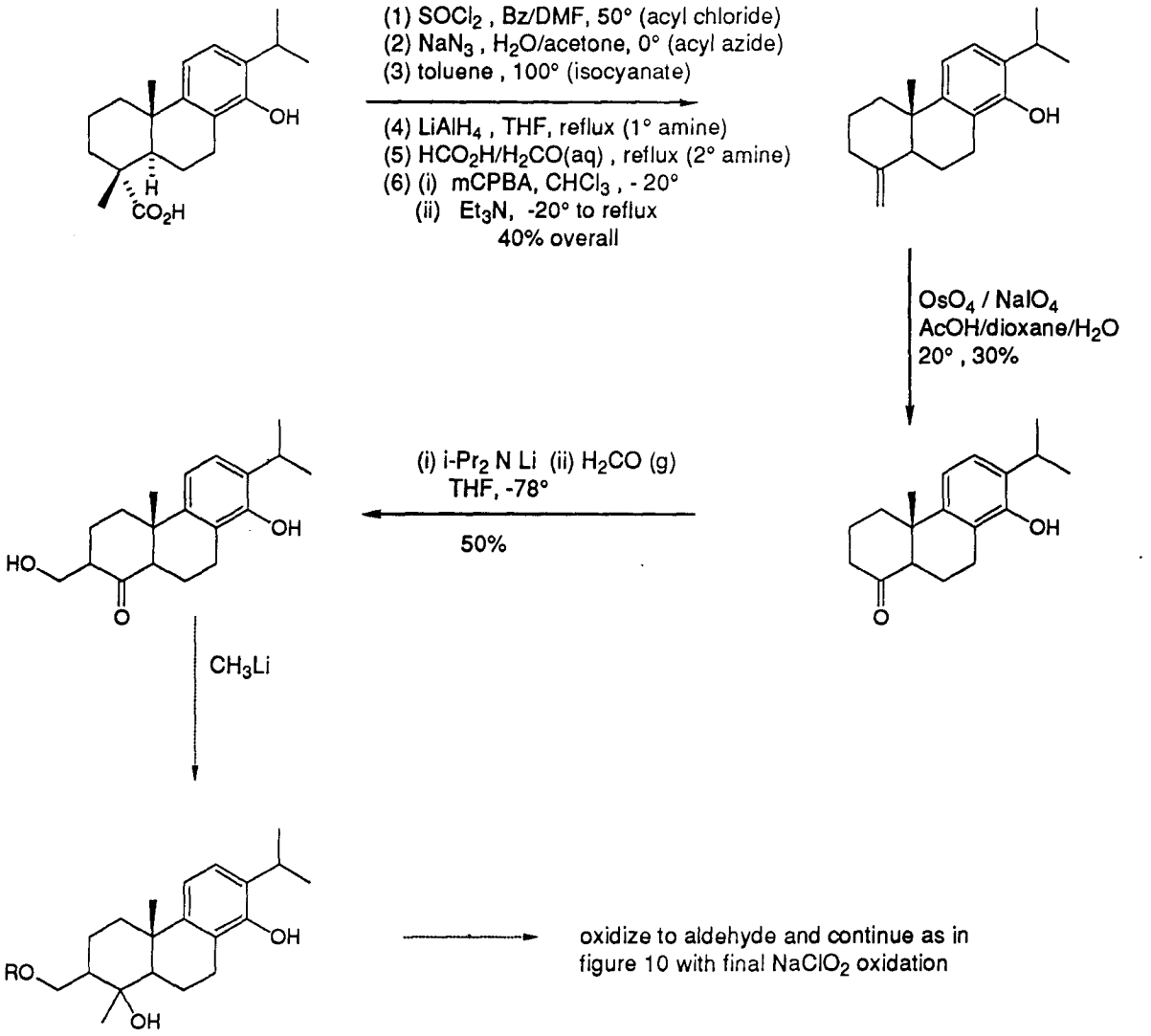


Figure 13 Synthesis of Chiral Butenolide by vanTamelen et al. (Dehydroabietic Acid)

Tokoroyama et al. developed an alternate synthesis of chiral butenolide[97, 98] (figure 14) from the (l)-dehydroabietic acid derived olefin of *figure 13*. Attempts at direct carbon addition by substitution of a C_3 alcohol had been unsuccessful, thus the butenolide was appended through Claisen rearrangement of a sulphonium ylid produced at the exocyclic methylene. With the carbon skeleton in place, two methods of oxidation were developed which produced either the butenolide precursor of triptolide or the C_2-hydroxylated butenolide precursor to tripdiolide.

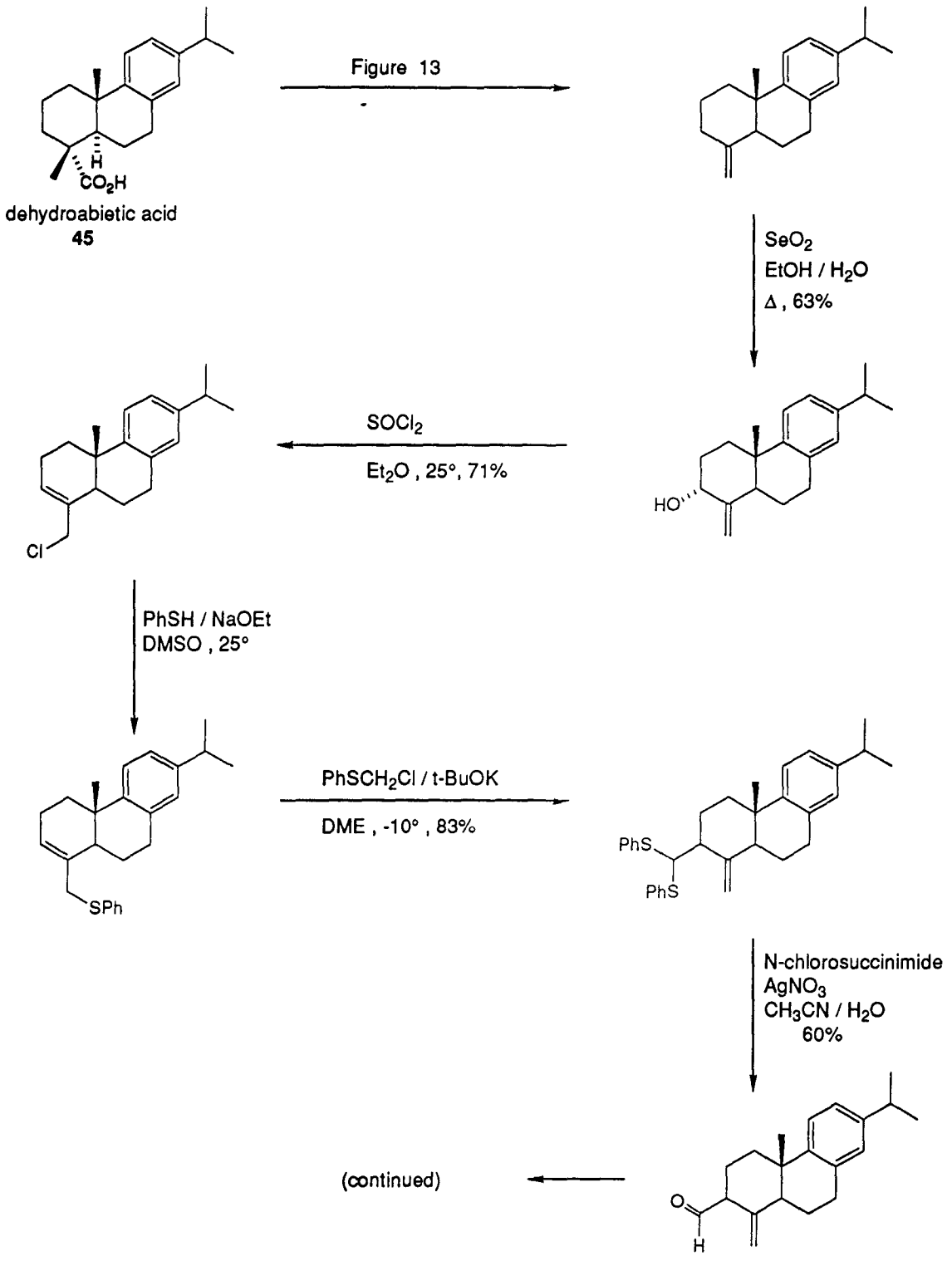


Figure 14 Synthesis of Chiral Butenolide by Tokoroyama et al. (Dehydroabietic Acid)

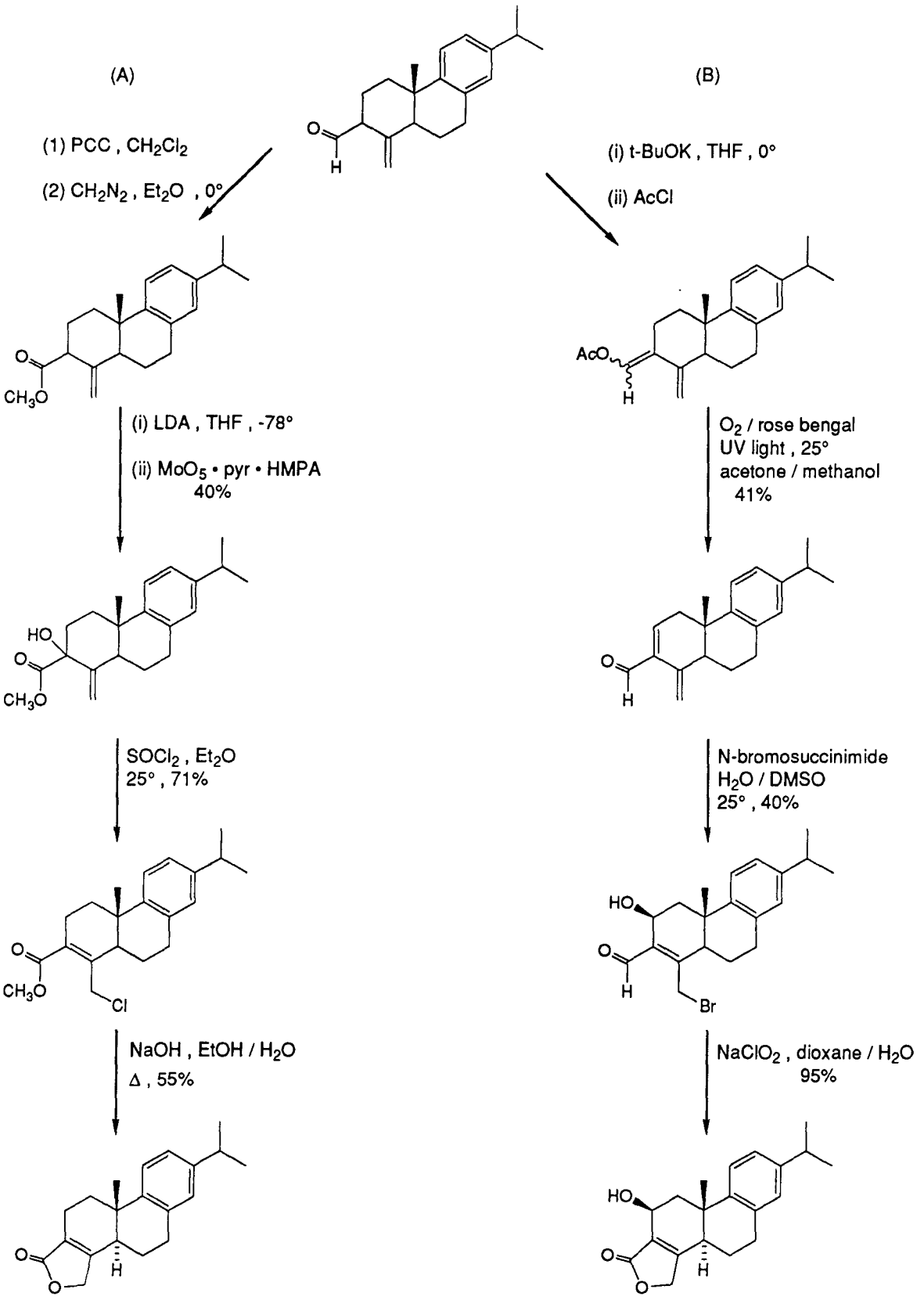


Figure 14 (continued)

In our group, interest in butenolide synthesis was directed towards biotransformation of readily prepared precursors rather than on a total chemical synthesis of the triptolides. A higher yielding pathway than those previously described would be necessary if the synthesis were to be adapted to commercial production. Also, in the initial studies on the biosynthetic pathway, it would be necessary to substitute a non-exchangeable atom of the precursor for its radioactive isotope. To this end, synthetic plans were envisioned to incorporate a single carbon atom into the butenolide skeleton at some late stage of the synthesis. The reagent used would have to be available in radioactive form, such as a ^{14}C isotope; its addition at a late stage would decrease losses of radioactivity in subsequent reactions.

Malcolm Roberts, working with Professor Kutney, developed one such route that incorporated $^{14}CH_3I$ into the butenolide ring. (A method that had been used previously by Garver and vanTamelen[95] to append ring C of the triptolides). In this synthesis[25] (figure 15), an exocyclic olefin was prepared by oxidative decarboxylation of (l)-dehydroabietic acid in one step using lead tetraacetate. Ozonolysis to the C_4 ketone was achieved in fair yield with by-products from benzylic oxidation. A masked carboxylic acid was added to C_3 through alkaline condensation with carbon disulphide followed by trapping in-situ as the ketene dithioacetal. The hindered, mild base used for the condensation did not cause isomerization to the A/B-cis form. The subsequent addition of a methylene unit to C_4 was a step that could be used to incorporate radioactive carbon. Methyl iodide (available enriched in ^{14}C) and dimethylsulphide were used to prepare a sulphur ylid that added to the C_4 carbonyl to form an exocyclic epoxide. At this stage, hydrolysis of the dithioacetal group led directly to the butenolide.

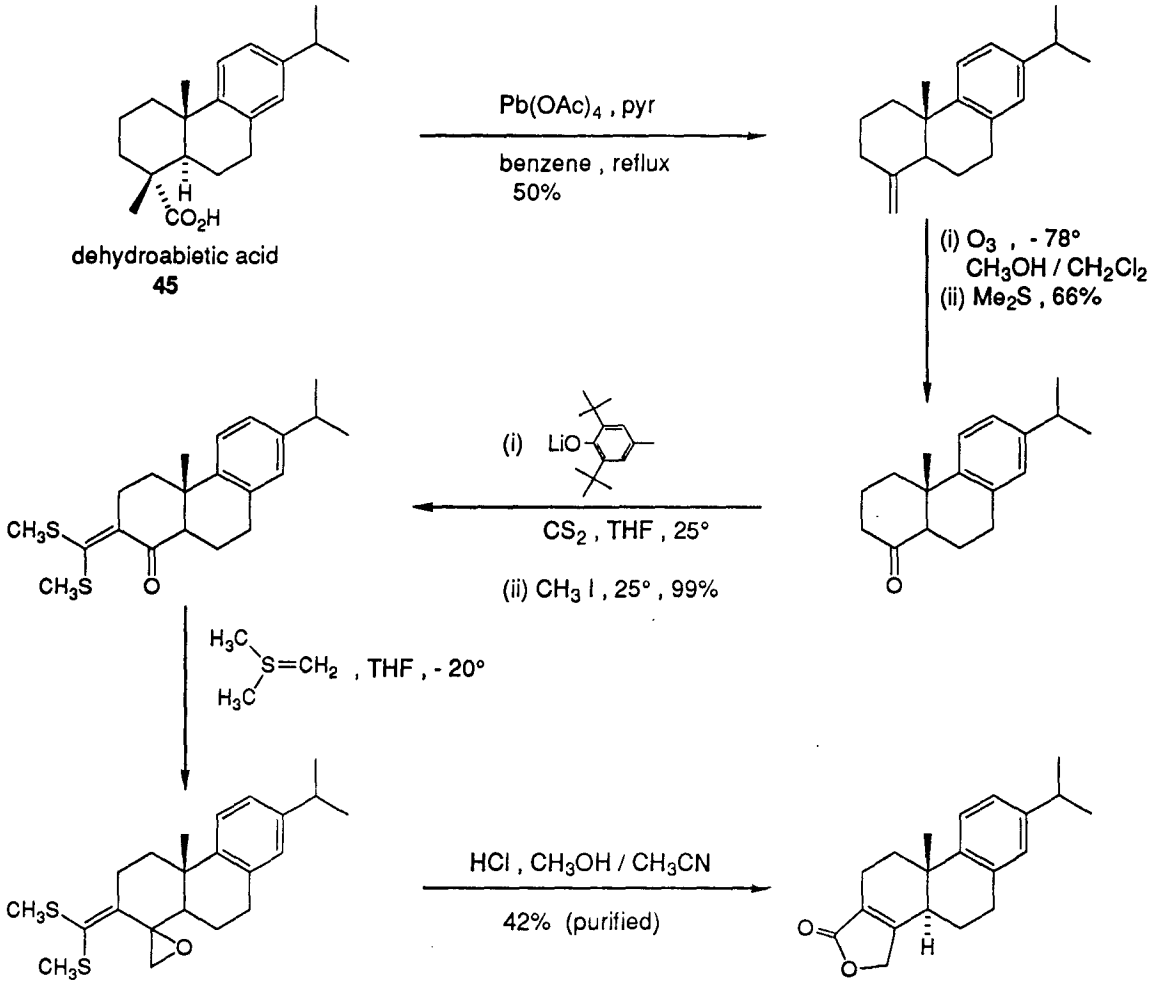


Figure 15 Synthesis of Chiral Butenolide by Roberts (Dehydroabietic Acid)

Prior to the development of the ketene dithioacetal approach, our group had studied the formation of a butenolide through addition of cyanide to C_3 of ring A. This addition, followed by elaboration of a C_4 hydroxymethyl group would allow hydrolysis of the nitrile product with spontaneous lactonization. This approach is attractive due to the addition of carbon in the appropriate oxidation level for hydrolysis to the butenolide and due to the commercial availability of ^{14}C-cyanide. The work that I have carried out in the synthesis of triptolide precursors utilizes this strategy.

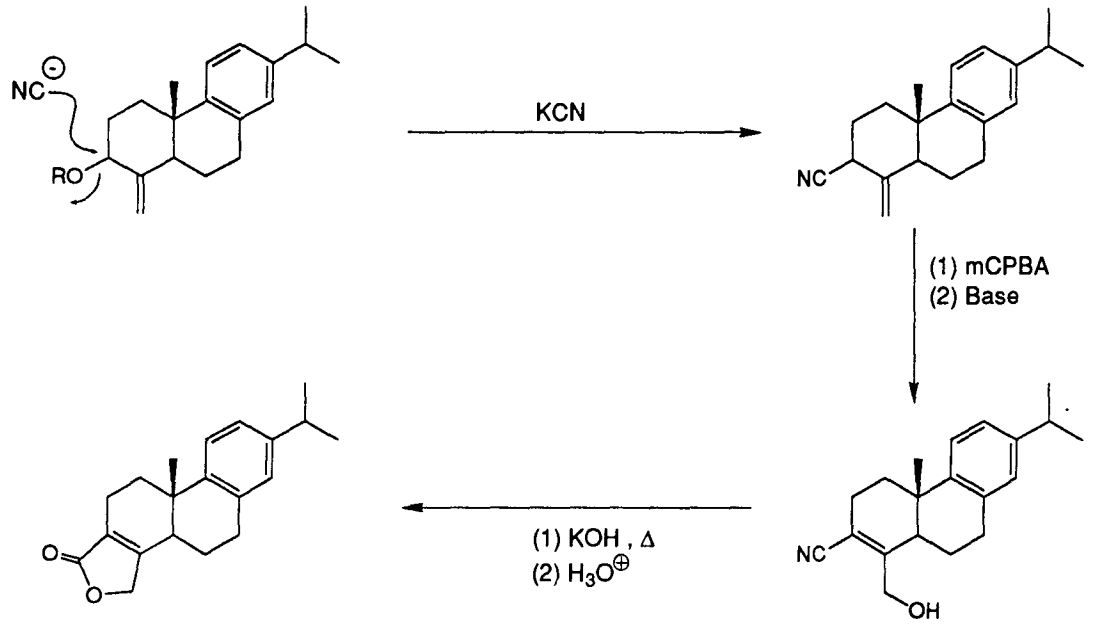


Figure 16 Planned Butenolide Synthesis (Dehydroabietic Acid)

The central objective of this research is to develop large scale production methods for the triptolides, which demonstrate antileukemic and antifertility properties, as well as for the oleanane triterpenes which have an anti-inflammatory activity. One approach taken has been the search for economical precursors that will allow production of the triptolides through biotransformation with plant cell cultures. Another approach has been to study elicitation conditions that will activate the biosynthetic pathways to the triptolides or the triterpenes. The results from these projects are described in the discussion which follows.

DISCUSSION

METABOLITE PRODUCTION FROM ELICITATION OF TRIPTERYGIUM

There is currently a demand for pure samples of the pharmacologically active compounds tripdiolide and triptolide which are used in clinical research studies. If testing supports the release of the triptolides as pharmaceuticals, the demand will be increased and a production industry will be established. At present, the richest source of tripdiolide is the TRP 4a cell culture of *Tripterygium wilfordii* developed in this laboratory. These cells produce 4 mg of tripdiolide per liter of culture medium in 30 days[71], 20-36 times more than can be isolated from plants. While the growth time is accelerated from years for the whole plant to weeks in culture, commercial cell culture is expensive and we have continued research on methods of improving tripdiolide production.

Promising results from the literature indicated that the technique of fungal elicitation would be well suited to these goals. The method was attempted using TRP 4a cultures and it proved successful in greatly enhancing the yield of some metabolites as well as reducing their production time. The initial studies of fungal elicitation described in this paper were aimed at several other products along with the triptolides. Foremost of these were a group of oleanane triterpenes, A,B,C and D (51,52,53 and 54), first isolated from TRP 4a by Malcolm Roberts[25]. Of these, triterpene D had been isolated from whole plants and demonstrated anti-inflammatory activity (see table 1). The linking of this activity to triterpene D was of great relevance as the primary medical use of *Tripterygium* has been the treatment of rheumatoid arthritis. The elicitation of TRP 4a cultures was therefore directed towards improving the production of oleanane triterpenes, tripdiolide and triptolide.

(I) GROWTH AND ELICITATION OF TRP 4A CULTURES

Stock cultures of TRP 4a were maintained in liquid PRD_2Co medium as shake flask cultures. The 500 ml volumes were grown in the dark and subcultured into fresh medium at 8 % v/v at the end of their growth phase (14-18 days). Small-scale elicitation experiments were also

carried out with cultures prepared in this manner. Large scale elicitations were run in 12 L bioreactors constructed from glass cylinders with stainless steel fittings. In the bioreactors, the medium, inoculum level and growth conditions were similar to those in shake flasks although air was bubbled up through the culture while in the shake flasks it entered solely by diffusion. To suspend the large cultures, a metal cone with open ends (immersed with large end downwards) was used to channel the rising air. This created a strong current in the medium. The "air-lift" circulation prevented settling of the cells with a minimum of shearing forces. Heated air filters on the inlet and outlet were used to maintain sterile conditions. Periodic sampling was carried out to assay growth (by refractive index) and culture purity (microscopically). This was carried out through a tube extending to the bottom of the bioreactor.

Since the first publication of cell culture methods for *Tripterygium* in 1981[24], stocks of TRP 4a have been continuously maintained in liquid culture by the Biological Services section of our chemistry department. The head of this section, Gary Hewitt, has noted that the growth of TRP 4a cultures in PRD_2Co medium (determined by the rate of dry weight accumulation) is inversely proportional to the refractive index (RI) of the medium (unpublished).The RI (25°C) of freshly inoculated PRD_2Co is 1.3371 compared to 1.3329 for water. The main contribution to increased RI is sucrose, the carbon and energy source for TRP 4a growth. At the end of growth, when sucrose is depleted, the RI reaches a minimum of 1.3333 ($\pm$ 0.0002). Cell debris is filtered out before measuring the RI and an increased amount of dead cells in older cultures does not noticeably affect the RI. As determination of the culture dry weight requires removal of a large sample (to insure that a representative ratio of cells to medium has been obtained), it was preferable to estimate growth through the RI which requires only a few drops of the medium.

The growth patterns of the bioreactor cultures used for the elicitation experiments to be described in this paper are presented in figure 17 (for data, see Appendix II). The open squares are RI data taken from all of the untreated PRD_2Co cultures while the crosses mark RI in cultures after the addition of elicitor.

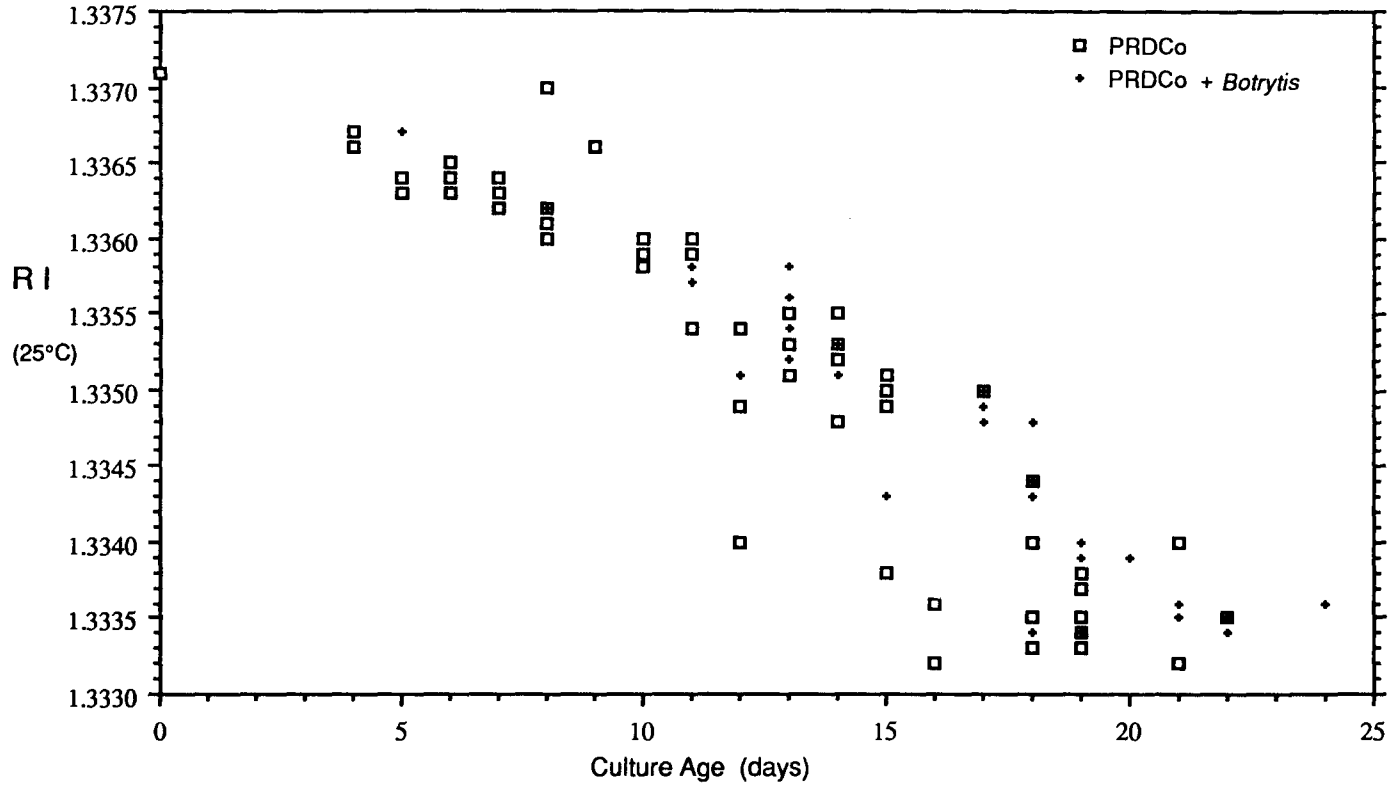


Figure 17 Growth Curves for PRD_2Co Cultures of TRP 4a in 12 Liter Aerated Bioreactors $RI_{25°C}$ of filtered broth from experiments 252, 254, 255, 256, 258, 259, 261, 262 and 266 (24 cultures). RI values recorded after the addition of Botrytis are marked with a (+).

This figure demonstrates that growth began at a slow rate and accelerated after the first ten days with the more rapid rate continuing until RI 1.3333. Cultures were harvested at this point to avoid a degradation of metabolites that has been observed after the depletion of sucrose [71]. The various contributors to sucrose consumption: cell division, cell growth and metabolite production, could not be distinguished using the RI curve. Three points on the growth curve were chosen as suitable times for the elicitation of cultures: early growth (RI 1.3360), mid growth (RI 1.3350) and late growth (RI 1.3333). It was thought that elicitation in the slow growth phase (before RI 1.3360) would be delay the onset of rapid growth.

The chosen method for elicitation was the addition of a fungal preparation directly into a growing TRP 4a culture. For this purpose, fungal cultures (cells and liquid medium) were disrupted using a tissue homogenizer and sterilized by heat treatment. Elicitors prepared in this fashion contained all of the heat-stable fungal products present before exposure to plant cells. Live fungi were not used as they would proliferate rapidly and kill the TRP 4a cells before secondary metabolites could accumulate. It was not known at the outset whether an elicitor would be a carbohydrate component of the fungal cell wall or a soluble factor.

(II) ANALYSIS OF THE METABOLITES FROM TRP 4A CULTURES

The quantification of metabolites from TRP 4a cultures involved three steps: extraction, purification and analysis. Ethyl acetate was used as the extraction solvent. While pharmaceutical extracts were prepared using hot water or ethanol[1,3], these solvents were not considered in the present study. Malcolm Roberts[25] had previously extracted TRP 4a cultures ($MSNA_{0.5}K_{0.5}$) into methanol and then partitioned the extract between ethyl acetate and water. While anti-inflammatory and anti-fertility activities were found in the ethyl acetate extract, no significant activity was observed in the water soluble material (also observed by Kupchan et al.[12]). The triptolides and triterpenes under investigation in the present study had been fully extracted into

ethyl acetate as they were absent in follow-up extractions of TRP 4a using methanol. A steady degradation of tripdiolide dissolved in methanol was be detected after one weeks storage at 4.0°C. This did not occur in ethyl acetate solutions.

To prepare for extraction, cultures were harvested and filtered to separate the cells from the liquid medium. If not extracted immediately, the cells were frozen. The medium was freeze-dried, dissolved in a small amount of water and extracted repeatedly with ethyl acetate. Freezing caused the cells to break open and they released 50 % of their mass as liquid after thawing and filtration. This liquid was extracted directly into ethyl acetate. The remaining cell solid was suspended in ethyl acetate, treated with the tissue homogenizer, then filtered. In each of these methods, extraction was repeated until the evaporation of solvent revealed no further mass. These crude ethyl acetate extracts were then purified or analyzed directly, depending on the composition of the extract and the metabolite being determined.

(i) Tripdiolide Determination

All of the crude ethyl acetate extracts were analyzed for tripdiolide without purification. This involved a fluorimetric method published by this group[99]. Samples were not purified as tripdiolide requires very careful chromatographic handling to prevent decomposition. The extracts from shake-flask cultures were often only 50 mg (with tripdiolide expected in quantities less than 5 mg) and thus the analysis would be disturbed by small losses on silica gel. For analysis, the extracts were dissolved in a fixed amount of ethyl acetate and spotted quantitatively to a silica gel plate using a microliter syringe. The extract spots were alternated with spots containing increasing amounts of pure tripdiolide. The silica plate was developed in methanol/chloroform and then sprayed with a cerium reagent which complexed with the tripdiolide to cause fluorescence under long wave UV light. A horizontal strip at the R_f of tripdiolide was bordered with black tape and the fluorescence was determined using a scanning fluorometer. The resulting peak areas were measured and the tripdiolide from extracts calculated with the standard curve generated from the pure samples present on each silica gel plate. It was

determined that this fluorescence assay was not specific for tripdiolide, thus a good degree of resolution had to be obtained with the plates prior to the determination to avoid detecting other compounds.

The fluorimetric determination was somewhat laborious as it required the development of many chromatography plates (each sample was determined twice and each plate incorporated a standard curve of tripdiolide). Gas chromatographic analysis (GC) was attempted, but no conditions were found in which tripdiolide did not decompose to numerous products. Tripdiolide has a melting point of 225°C, but is unstable at this temperature. Due to the greater stability of oleanane triterpenes, it was considered likely that they could be analyzed by GC.

(ii) Triterpene Determination

The oleanane triterpene acids A, B, C and D (51,52,53 and 54)have high molecular weights (A, B = 470 C, D = 472)and high melting points (260°-300°C), thus they are not suitably volatile for a direct GC analysis. HPLC (high pressure liquid chromatography) would not be immediately applicable either, as the acid functionality absorbs only weakly and in the short wavelength UV region. Previous attempts by this group had shown that the colored quinone methide compounds (66-68) created a strong interference with UV detection due to extensive peak tailing. For these reasons, either GC or HPLC would require derivitization of the triterpene acids: GC for increased volatility and HPLC to install a strong UV chromophore. GC analysis was chosen due to the high peak resolution obtainable with our capillary GC system.

The most logical target for derivitization of the triterpenes was the polar carboxylic acid functionality. Treatment of the triterpene acids with diazomethane in methanol provided complete conversion to methyl esters (mp 120°-230°C) under mild conditions. The triterpene esters would not elute from GC columns coated with polar stationary phases (cyanopropyl silicone or polyethylene glycol columns), even at their maximal operating temperatures ($\leq$ 250°C). The shift to non-polar stationary phases, dimethyl silicone or phenylmethyl silicone,

provided peaks for the triterpene esters. This was in part due to their higher operating temperatures of up to 300°C. The most rapid resolution of triterpene methyl esters was obtained using a 12 m dimethyl silicone capillary column at 280°C (isothermal). These conditions eluted the desired peaks in 28-35 minutes. This long analysis time might have been reduced by further derivitization of the triterpene esters, for example by a subsequent acetylation. This was not investigated as the chromatogram of methylated plant extracts was extremely crowded from 5-22 minutes. The late elution of triterpene methyl esters placed their peaks in the first region of the chromatogram that was relatively undisturbed by the peaks of other compounds.

The triterpene acids A, B, C and D (51, 52, 53 and 54) (a gift from my co-worker Malcolm Roberts) were treated with diazomethane and the methyl ester products were recrystallized from methanol/water. Standard mixed solutions of the triterpene esters in ethyl acetate were used to prepare a calibration curve. Calibrations for oleanolic and polpunonic acid (49, 62)were prepared in the same fashion. An internal standard (methyl cholate) was added to all solutions prior to injection.

During the analysis of plant extracts, methyl cholate was not a suitable internal standard. The peak from this compound eluted earlier than the triterpene esters and was often eclipsed by components of the extract. If samples were analyzed with a consistent technique, the internal standard was not required. Accurate repetition of the injection volume provided a repeatability of ± 5% in the triterpene ester integrations between duplicate analyses. To compensate for changes in the column conditions, re-calibration with the triterpene ester standards was repeated before each set of determinations.

The maximum resolution of triterpene ester peaks was achieved by injection of dilute samples. The triterpene esters under analysis constituted a very small proportion of the total extract mass (0-10 %), thus concentrated samples exceeded the column capacity and caused greatly decreased resolution. The extracts were diluted successively with accurate volumes of ethyl acetate until the triterpene peaks created less than 50 % of a full scale deflection in the

chromatogram (attenuation set at zero). With this method, the integrated area of triterpene peaks was kept well within the calibration range provided by the pure triterpene standards.

Injection of pure triterpene A or C, oleanolic or polpunonic acid methyl esters resulted in a single peak (comprising ≥ 97% of the run integration). The injection of pure triterpene B or D methyl esters resulted in the elution of two peaks. Through a process of co-injection, the additional peaks were identified as wilforlide B (55, the lactone isomer of triterpene B) and wilforlide A (56, the lactone isomer of triterpene D). Co-injection of the triterpene standards with their lactone analogs increased the integration of the early eluting peaks without altering the peak shape or retention time. Analysis of the triterpene B and D ester standards by TLC showed conclusively that lactones were not present prior to the GC analysis, demonstrating that lactonization must have occurred during the GC chromatography.

Lactonization of the ester and free acid forms of triterpenes B and D was observed to occur partially on silica gel and almost completely under acid catalysis (H_2SO_4 in ethanol) or in the mass spectrometer. This supports a conclusion that thermally induced lactonization had occurred during GC analysis. Lactonization was confined to the hot injector region (300°C) and did not occur along the length of the column (280°C) as the lactone and ester peaks were completely resolved. Prolonged conversion would have resulted in the elution of a single broad peak halfway between the normal retention times of the lactone and the methyl ester. The analyses of triterpene B and D esters in the plant extracts were reported using the combined areas of the lactone and ester peaks. The lactonization of the oleanane triterpenes (acid or ester forms) is outlined in figure 18 using the example of triterpene D.

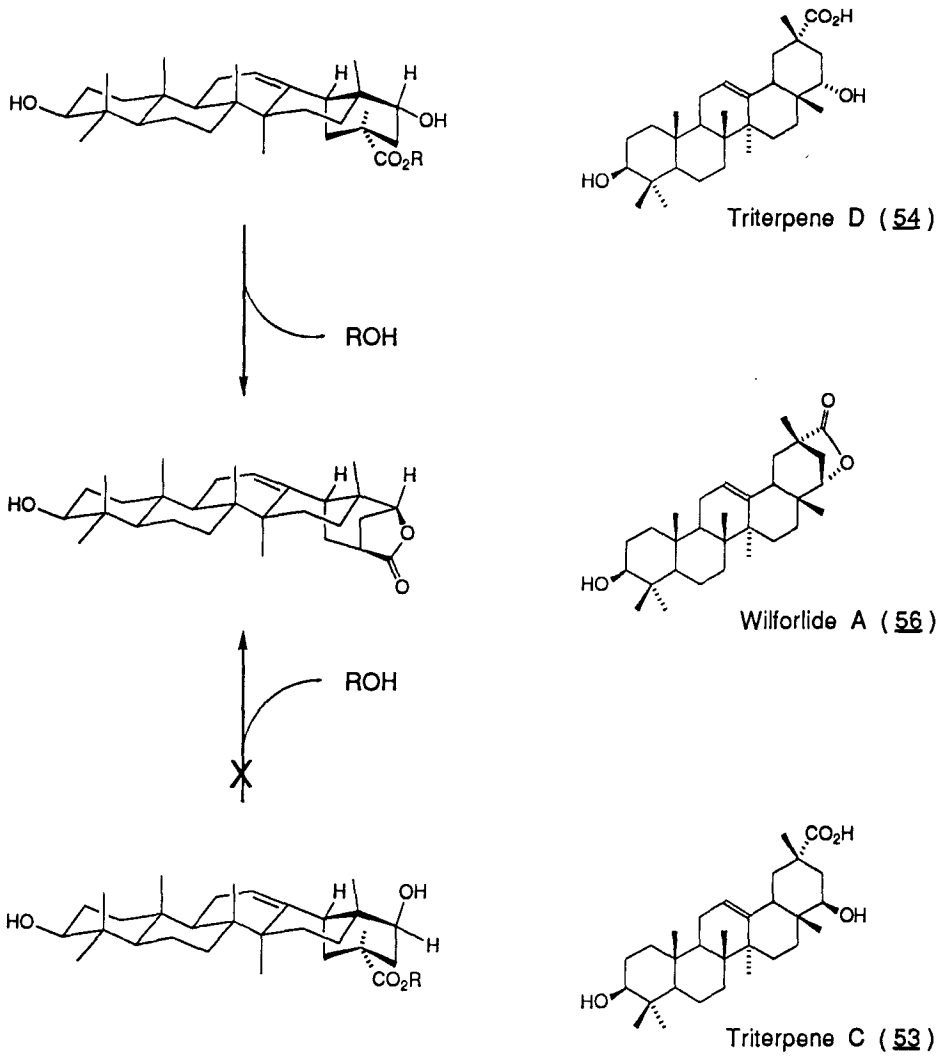


Figure 18 Lactonization of Oleanane Triterpenes
R=H or CH_3. The conformation of triterpene D acid was determined by X-ray crystallography[25].

(III) ELICITATION IN THE PRODUCTION OF TRIPDIOLIDE AND TRITERPENES

The PRD_2Co culture medium provided the most rapid growth of TRP 4a cultures. If fungal elicitation could be used to initiate tripdiolide synthesis in this medium, it would be superior to the slower $MSNA_{0.5}K_{0.5}$ culture medium. Several types of fungal elicitor were prepared by the Biological Services section using liquid cultures of fungi in PRL-4 medium

(PRD_2Co less D and Co). The fungi included a strain of *Botrytis*, *Sclerotinia sclerotiorum*, *Rhodotorula rubra*, *Trichoderma viride* and two unidentified types isolated by the biological services: POD 129 F-1 and POD 129 F-2. When the fungal cultures had grown to maturity (forming an aggregate of fungal mycelia rather than a loose suspension of cells) they were first treated with a tissue homogenizer until the mycelia had fragmented and were then autoclaved. These sterile fungal elicitor preparations were added to 500 ml TRP 4a cultures in amounts of 0.1% and 1.0% by volume. The TRP 4a cultures were elicited at the beginning of their rapid growth phase (RI 1.3355) to insure maximum cell vigor.

The first elicitation experiment was planned to screen the various fungi for the ability to initiate tripdiolide production. Elicited cultures and non-elicited controls were harvested 18, 24, 36, 48 and 72 hours after the time of fungus additions and extracted immediately with ethyl acetate. The TLC fluorimetric analysis of these extracts showed maximal tripdiolide accumulation in the 18-24 hour cell extracts of cultures elicited with the fungi *Botrytis*, *Trichoderma* and *Sclerotinia* at 0.1%. The levels of tripdiolide determined for these cultures were from 9 to 14 mg per liter of culture. The same fungi at 1.0% or 72 hours as well as the other fungi under all conditions produced tripdiolide levels in the cells close to those from control cultures, 2 to 3 mg per liter of culture. The tripdiolide levels from the medium of all elicited cultures were close to control levels of ≤ 1 mg per liter of culture. These results suggested that tripdiolide was produced rapidly after elicitation and was then degraded beyond 24 hours. Also, while tripdiolide produced in the $MSNA_{0.5}K_{0.5}$ cultures was found mainly in the medium (after 25-35 days), the more rapid production in elicited PRD_2Co cultures did not allow time for a release from the cells. The unusual presence of tripdiolide in control PRD_2Co cultures was attributed to interference from other compounds in the fluorimetric determination. The assay was not specific for tripdiolide and relied on a thin layer chromatography for resolution.

Elicitation had a notable effect on the appearance of cells. The cultures elicited with *Botrytis* and *Sclerotinia* (the fungi which induced a high tripdiolide assay) produced cells with a

dark gray/orange colour. The other elicited cultures were moderately dark green and the control cultures were a very pale green. One factor in this darkening was the accumulation of triterpene quinone methides. A member of this group of red/orange compounds, tingenone (66), was identified in the elicited extracts by TLC comparison with an authentic sample.

The 18 hour elicitation was scaled up from 500 ml shake flasks to a 12 liter bioreactor to allow isolation of the products. The large culture developed similarly to the small cultures and the elicited cells were dark in comparison with normal PRD_2Co cells. The elicited culture extract demonstrated a high tripdiolide level through fluorimetric analysis, 10 mg tripdiolide per liter of culture. Closer study of the cell extract by TLC in several solvent systems (chloroform/methanol, ethyl acetate/hexanes, chloroform ether) indicated that tripdiolide was not actually present, contrary to the positive fluorimetric assay result. After a purification of the extract using previously successful column chromatographies[24] produced no tripdiolide, it was concluded that the fluorimetric determination had not been accurate. Extracts from the small scale elicitor screening experiment were re-examined, and it was found that cultures with a high tripdiolide assay also contained higher levels of many other compounds. The TLC conditions used in the fluorimetric assay provided an R_f of 0.3 for pure tripdiolide while a large proportion of the extracted compounds travelled to a higher position. These compounds had left a trail of residual material back to the origin. This was especially true of the quinone methides (figure 1), where a tail of orange was visible behind pure standards after TLC. The high tripdiolide readings for elicited extracts had been caused by increased amounts of other compounds whose trailing residues were picked up by the sensitive fluorescence detector. This interference also accounted for the low tripdiolide levels determined for control PRD_2Co cultures that had not previously been observed to produce any tripdiolide.

The appearance of high levels of tripdiolide in the fluorimetric assay had demonstrated that the elicitation technique was successful in increasing the production of some other secondary metabolites besides the triptolides. Our initial efforts at identifying these compounds involved

screening the elicited sample extracts for oleanane triterpenes, the other family of pharmacologically active compounds under investigation in this group. The crude extracts were treated with diazomethane and directly analyzed by gas chromatography. The oleanane triterpenes A, B, C and D (51, 52, 53 and 54) were present in low levels in control cell extracts (< 5 mg/L) and were nearly undetectable in all extracts of the growth medium. On the other hand, some of the elicited cell extracts showed definite increases in triterpene yield. The 24 hour and 72 hour elicited cultures were tested by GC and the increases in the 72 hour levels were higher than those in the 24 hour levels. Also, the cultures treated with 1.0% elicitor had higher triterpene levels than those treated with 0.1% elicitor. Under the optimal conditions of 1.0% elicitor for 72 hours, the cultures treated with *Botrytis* and *Sclerotinia* produced the largest triterpene yields (20-40 mg of each triterpene per liter of culture). Treatment with *Rhodotorula* or the local isolates POD 129 F-1 and POD 129 F-2 resulted in moderate triterpene yields (10-20 mg per liter). Treatment with *Trichoderma* did not cause a significant increase in triterpene levels above those in the control cultures.

The discovery that fungal exposure produced increases in the related oleanane triterpenes A-D (figure 3, page 11) made elicitation a valuable addition to our metabolite production techniques since triterpene D has demonstrated anti-inflammatory activity (Table 1, page 20). The primary medicinal property of *Tripterygium* extracts is the reversal of inflammation caused by rheumatoid arthritis; a readily available triterpene compound capable of reproducing this effect would be a very useful pharmaceutical.

To confirm the elicited increase in triterpenes, large scale bioreactor experiments were designed which would provide enough extract to chromatographically purify the triterpenes. The fungus *Botrytis*, which had demonstrated positive results in both the GC and fluorimetric assays, was chosen as the elicitor for these experiments.

(IV) OPTIMIZATION OF TRITERPENE PRODUCTION IN 12 LITER BIOREACTORS

(i) The Growth Of Elicited Cultures

The growth of TRP 4a cells in 12 L bioreactors was described previously (see figure 17). The apparatus used allowed the operation of three bioreactors simultaneously , which provided for the growth of one control and two elicited cultures. In some cases, a bioreactor became contaminated with outside microorganisms. When this occurred, the affected culture was discarded and the experiment continued with the remaining cultures. TLC analysis did not show increased triterpene levels from these infections.

The growth curve for 12 L bioreactor cultures, figure 17, shows that elicitation slightly diminished the rate of RI decrease. In contrast, elicited cultures displayed a large drop in cell fresh weight in comparison with non-elicited cultures grown for an equal time (data to be presented in the following section). This indicates that the growth curve for elicited cultures included sucrose consumption that was not being directed towards the production of cell biomass. Increased secondary metabolite production could account for some of this alteration in the normal growth pattern.

Cell dry weights were sampled during the growth of the culture series 266. Figure 19 (data in appendix III) shows the growth curves determined by RI (open circles) and by cell dry weight (closed circles). The error bars of ± 10% included in the dry weight data reflect the difficulty in obtaining a homogeneous suspension of cells in a 25.0 ml culture sample. The nine culture samples analyzed revealed a cell water content of 92.9% (standard deviation ±1.5), indicating a close correlation of fresh weight to dry weight in both elicited and control cells. The cultures monitored in the upper two plots had been elicited with *Botrytis* before the sampling, while the third culture was elicited at the mid-point of sampling. The growth curves show that although elicited cultures accumulated cell mass more slowly than the control culture, a drop in refractive index still indicated increases in the cell dry weight. At the end of growth (RI 1.3333), the dry weight levelled off (lower plot) while the RI continued to fall at a slow rate.

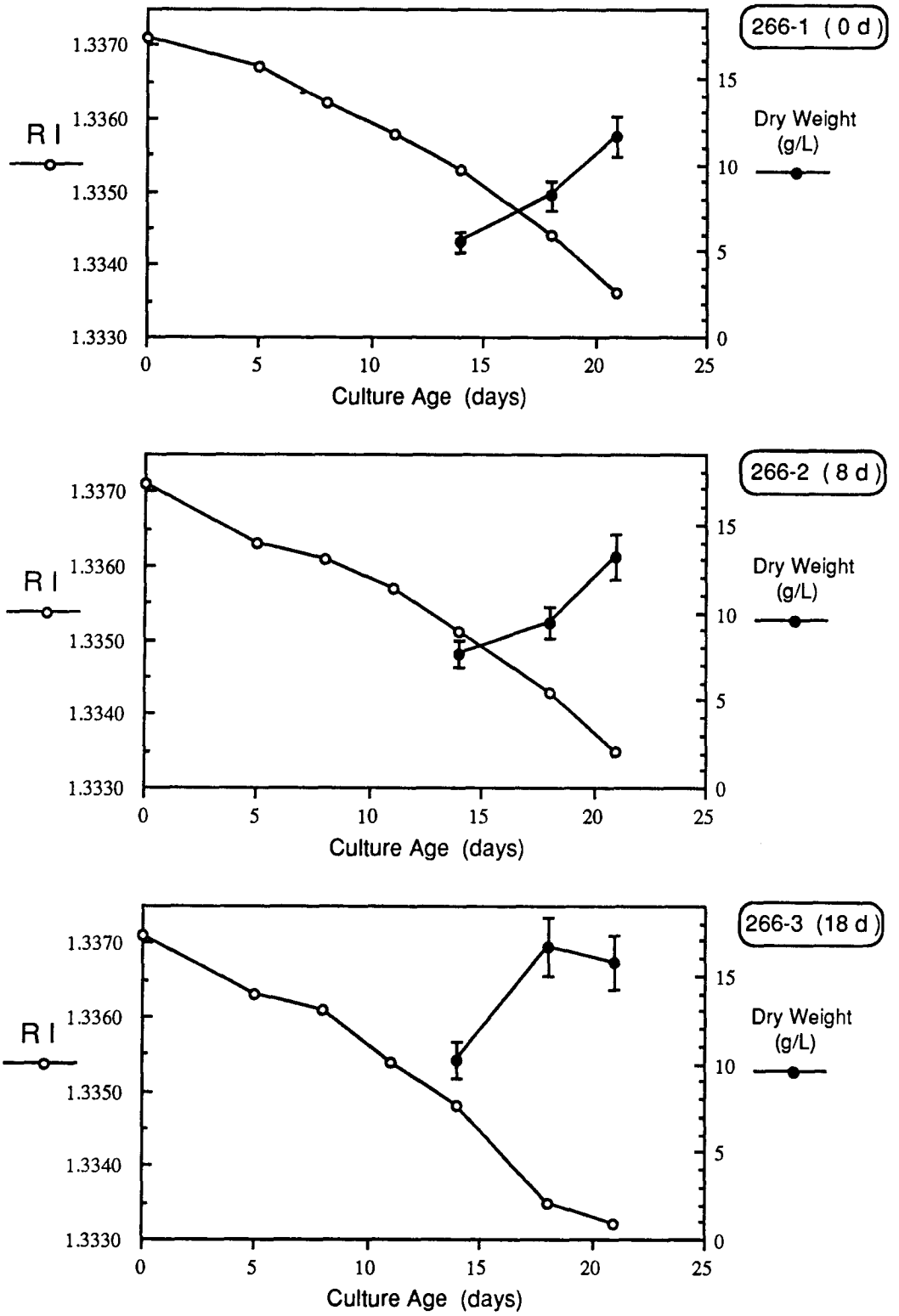


Figure 19 Growth Curves and Culture Dry Weight of TRP 4a Series 266
Refractive index at 25.0°C. Dry weights from freeze-dried cells in 25.0 ml culture samples. The culture number is followed by the age at which *Botrytis* was added (1.0 % v/v).

As well as decreasing the biomass yield of PRD_2Co cultures, elicitation decreased the proportion of viable cells. This was observed in a microscopic examination of samples from the culture series 266. Membrane integrity was assayed by dye exclusion (Biological Services, using Evans' blue stain).The three cultures were examined on the 18^{th} day of growth, culture 266-1 (elicitor from day 0) displayed 31% lysed cells, culture 266-2 (elicitor from day 8) had 34% lysed cells and culture 266-3 (no elicitor) had only 12% lysed cells (from counts of 200-300 cells in random fields). When live cultures were viewed without staining, the non-elicited cells were perfectly round while the elicited cells showed an irregular border and appeared shriveled. Also, when any of the culture series were harvested, the non-elicited cultures provided cells with a loose, granular texture while while cells from the elicited cultures packed into a firm, dense cake. The viability of the cells, the appearance and the texture could well be related to a single component of the elicitation response (such as increased cell permeability) though this could only be determined through a more detailed study of the elicitation mechanism.

(ii) Analysis of Culture Extracts

Ethyl acetate extraction of twelve liter cultures provided from two to five grams of material. For triterpene analysis, one half of this extract (in methanol) was treated with diazomethane. The esterification caused a lightening of the elicited extracts from orange to yellow, indicating an alteration in the chromophore (rings A and B) of the triterpene quinone methides. The extracts of the cells and the culture medium were kept separate. The medium extract mass was about 20% of the cell extract mass and the medium extract contained a much smaller number of compounds. This allowed an accurate GC analysis of the crude medium extract while the crude cell extract required purification to remove some compounds which eluted near the triterpene esters, disturbing the baseline of the chromatogram. Purification was accomplished by column chromatography on silica gel (with a gradient elution using increasing ethyl acetate in toluene). The triterpene region of the GC chromatograms from cell extracts of control cultures contained very few compounds, thus control extracts were also analyzed without

purification. As the elicited culture extracts were purified and not the control culture extracts, any loss of material on the silica gel would have diminished the reported magnitude of elicited triterpene yields. This increases the confidence in any elicited increases.

The reported triterpene yields are the total of the cell and medium results. Typically the medium produced much lower triterpene yields. These yields were reported in units of triterpene weight (mg) per unit volume of culture (L). The compounds oleanolic and polpunonic acid (compounds 49 and 62) were determined along with the triterpene acids A, B, C and D. While these two substances were of little interest in themselves, they provided a broader scope to our analysis of the elicitation effect, particularly with respect to determination of the friedelane triterpene family which includes polpunonic acid.

(iii) The Results of 72 Hour Elicitation

When the various fungal elicitors were screened, 500 ml cultures of TRP 4a were elicited in the early growth stage (RI 1.3355). A large increase in triterpenes was obtained using *Botrytis* at 1.0% v/v for 72 hours. The same conditions were incorporated into the first 12 L bioreactor experiment, culture series 252, save that this series was elicited in late growth (RI 1.3332). This was done in light of the results from tripdiolide production using TRP 4a in $MSNA_{0.5}K_{0.5}$, where maximum metabolite yield occurred at a late stage in growth[71]. Also, it was thought that the greater mass of cells in an older culture would provide a more rapid triterpene synthesis.

The conditions and the results of this experiment are presented in table 2. Culture 252-1 was the 1.0% *Botrytis*, 72 hour elicited culture and 252-2 the PRD_2Co control. The pattern of growth for the two cultures is presented in figure 20 and the triterpene yields are plotted in figure 21.

There was a significant increase in the production of triterpenes A, B, C and D due to elicitation with *Botrytis*. Moreover, the elicited yields compared favourably with those from the slower growing $MSNA_{0.5}K_{0.5}$ cultures (30-40 days growth time) from which the triterpenes were first isolated. The $MSNA_{0.5}K_{0.5}$ production levels were 1.2, 4.8, 2.7 and 9.3 mg/L for A, B, C and D acids[25]. This translates to methyl ester yields of 1.2, 4.9, 2.8 and 9.6 mg/L for comparison with the yields of the present discussion. (Triterpene acid yields are 0.97% of the ester yields.)

Culture	252-1	252-2
Initial Culture Volume (L)	11	11
Inoculum Level (v/v) [Age (d) / RI]	9.1 % [19 / 1.3332]	9.1 % [19 / 1.3332]
Age at Elicitation (d) [RI]	16 [1.3332]	-
Elicitor Level (v/v)	1.0%	not added
Elicitation Period / Age at Harvest (d)	3 / 19	0 / 19
Final RI of Culture Medium	1.3334	1.3333
Fresh Weight of Cells (g)	3303	2890
EtOAc Extract weight (mg/L)	448	321
Triterpene A (mg/L)	0.76	0.25
Triterpene B (mg/L)	4.49	0.97
Triterpene C (mg/L)	0.80	0.06
Triterpene D (mg/L)	3.83	0.22
Oleanolic Acid (mg/L)	-	-
Polpunonic Acid (mg/L)	-	-

Table 2 Elicitation at 1.0% in Late Growth (Culture Series 252)
Refined fractions for triterpene analysis were prepared by silica gel column chromatography of the esterified culture extracts (252-1 only). Oleanolic and polpunonic acids were not determined for this culture series.

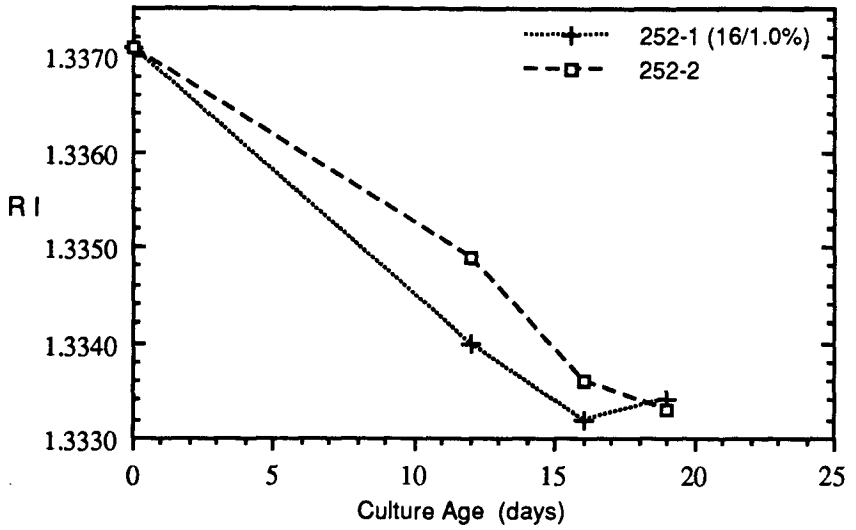


Figure 20 Growth Curves of TRP 4a Culture Series 252 Refractive index at 25.0°C. Legend symbols are followed by the age and level (v/v) at which *Botrytis* was added to cultures.

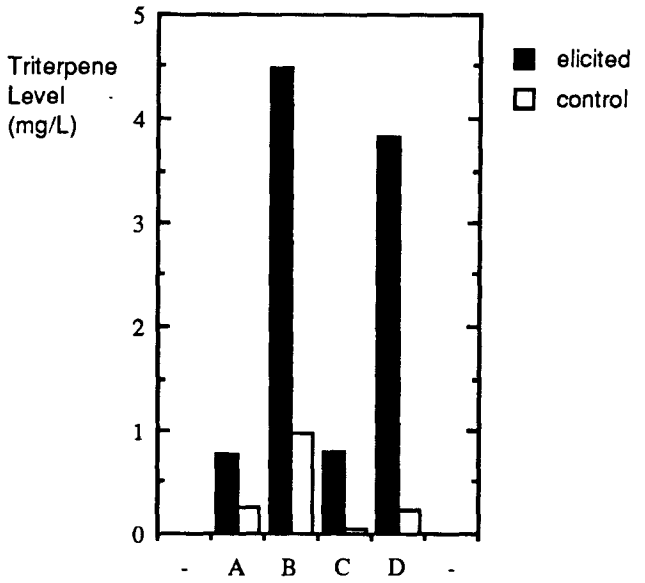


Figure 21 Triterpene Levels from Culture Series 252 Oleanane triterpenes A-D. Culture 252-1 is elicited with 1.0 % *Botrytis* at $RI_{25°}$ 1.3332 (late growth) for 72 hours. Culture 252-2 (control) has no additions.

The next experiment in 12 L bioreactors, series 254, was used to examine 72 hour elicitation in the middle of growth phase (RI 1.3354) rather than at the end. Culture 254-2 was similar to 252-1, with *Botrytis* added at 1.0%. Culture 254-1 was elicited with 0.1% *Botrytis* and culture 254-1 was the non-elicited control (table 3, figures 22 and 23).

Both elicitation conditions provided an increase in triterpenes A and B above the control levels, while triterpene C was only increased with 1.0% elicitation. Triterpene D appeared at control levels under both elicitor concentrations. Due to increased levels of the less available triterpenes A and C, the 1.0% elicitation was more useful than the 0.1% elicitation and the mid-growth elicitation of series 254 was more useful than the late growth elicitation of series 252. The low level of triterpene D in elicited 254 cultures was anomalous as it was more often the major triterpene product (as observed in the elicitor screening experiment and cultures 252, 259 and 261 through 266). The triterpenes B and D are subject to losses through lactonization on silica gel; this may have caused the appearance of lower levels.

Culture	254-1	254-2	254-3
Initial Culture Volume (L)	12	12	12
Inoculum Level (v/v) [Age (d) / RI]	8.3 % [18 / 1.3331]	8.3 % [18 / 1.3331]	8.3 % [18 / 1.3331]
Age at Elicitation (d) [RI]	14 [1.3355]	14 [1.33353]	-
Elicitor Level (v/v)	0.1 %	1.0 %	not added
Elicitation Period / Age at Harvest (d)	3 / 17	3 / 17	0 / 17
Final RI of Culture Medium	1.3350	1.3349	1.3350
Fresh Weight of Cells (g)	2400	2220	2590
EtOAc Extract weight (mg/L)	478	439	471
Triterpene A (mg/L)	1.10	1.73	0.55
Triterpene B (mg/L)	2.94	2.33	1.26
Triterpene C (mg/L)	0.59	1.83	0.88
Triterpene D (mg/L)	1.18	1.24	1.36
Oleanolic Acid (mg/L)	-	-	-
Polpunonic Acid (mg/L)	-	-	-

Table 3 Elicitation at 1.0% and 0.1% in Mid Growth (Culture Series 254)
Refined fractions for triterpene analysis were prepared by silica gel column chromatography of the esterified culture extracts (254-1 and 254-2 only). Oleanolic and polpunonic acids were not determined for this culture series.

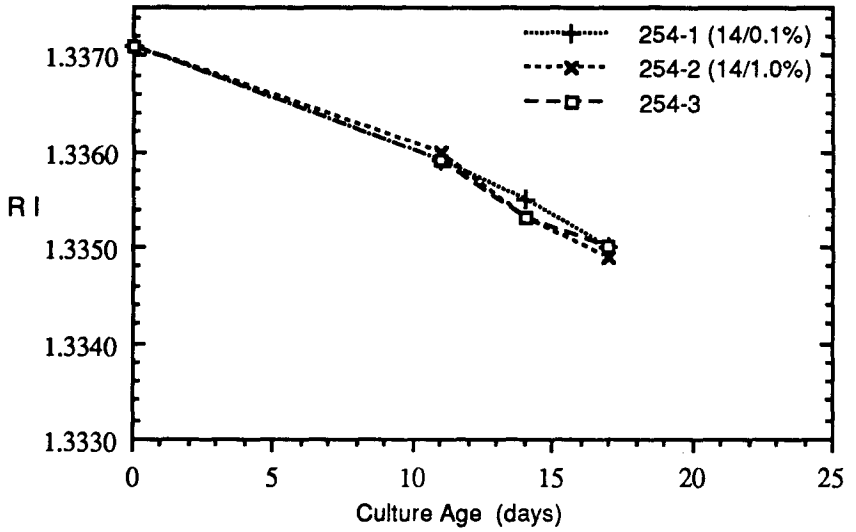


Figure 22 Growth Curves of TRP 4a Culture Series 254 Refractive index at 25.0°C. Legend symbols are followed by the age and level (v/v) at which *Botrytis* was added to cultures.

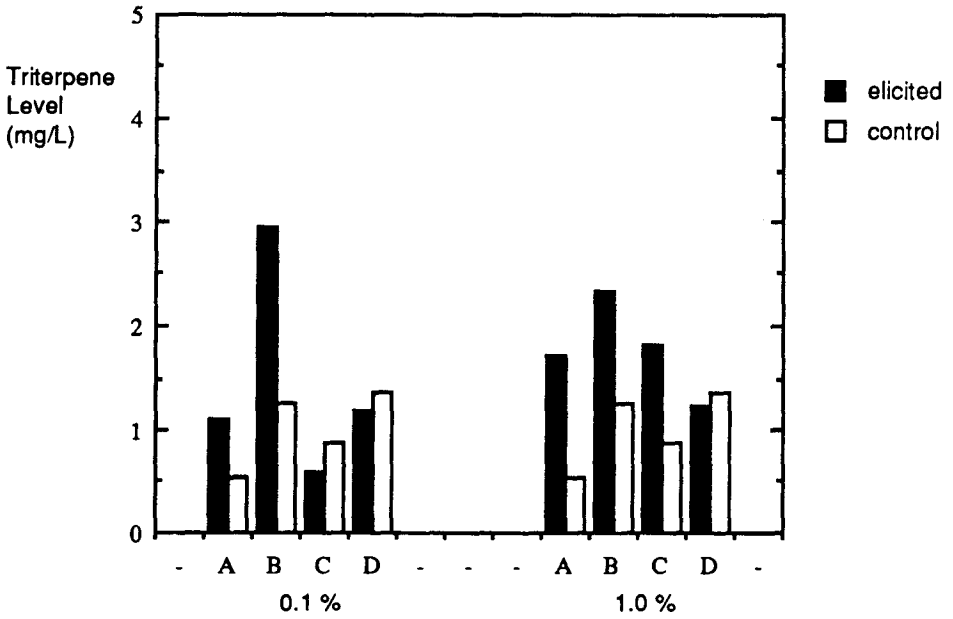


Figure 23 Triterpene Levels from Culture Series 254 Oleanane triterpenes A-D. Culture 254-1 is elicited with 0.1 % *Botrytis* at $RI_{25°}$ 1.3355 (mid growth) for 72 hours while culture 254-2 is elicited with 1.0 % *Botrytis* at $RI_{25°}$ 1.3353 (mid growth) for 72 hours. Culture 254-3 (control) has no additions.

Culture series 255 repeated the 1.0%, 72 hour, late growth elicitation (RI 1.3335) of culture series 252 and included a 0.1% elicited culture (table 4, figures 24 and 25). The triterpene yields from both elicitations were the highest yet obtained. Extracts from this series were analyzed for oleanolic and polpunonic acid methyl esters and these compounds were revealed to be major products of the elicitation response. The result of an increase from 0.1% to 1.0% elicitor was a large decrease in triterpenes D, O and P, a slight decrease in triterpene B and small increases in the levels of A and C. Cultures of series 254 and 255 indicated a possible advantage in triterpene A and C production from using 1.0% elicitation. As these were the minor triterpene products, 1.0% elicitation was accepted as the most useful course for further experiments .

The 1.0% elicited culture 255-3 was treated identically with the culture 252-1 and yet 255-3 provided a far superior triterpene yield. While there were no obvious causes for the difference, the slower growth of the 255 series prior to *Botrytis* addition (19 days to RI 1.3335 compared with 16 days to RI 1.3332) indicates that the TRP 4a inoculum had varying growth potential in the two culture series.

Culture	255-1	255-2	255-3
Initial Culture Volume (L)	12	12	12
Inoculum Level (v/v) [Age (d) / RI]	8.3 % [17 / 1.3333]	8.3 % [17 / 1.3333]	8.3 % [17 / 1.3333]
Age at Elicitation (d) [RI]	-	19 [1.3334]	19 [1.3335]
Elicitor Level (v/v)	not added	0.1 %	1.0 %
Elicitation Period / Age at Harvest (d)	0 / 22	3 / 22	3 / 22
Final RI of Culture Medium	1.3335	1.3334	1.3335
Fresh Weight of Cells (g)	4005	3212	3330
EtOAc Extract weight (mg/L)	530	564	569
Triterpene **A** (mg/L)	0.85	4.88	5.90
Triterpene **B** (mg/L)	4.69	18.2	17.8
Triterpene **C** (mg/L)	0.00	6.91	7.56
Triterpene **D** (mg/L)	0.61	9.83	5.25
Oleanolic Acid (mg/L)	0.88	12.8	6.34
Polpunonic Acid (mg/L)	0.41	10.8	6.53

Table 4 Elicitation at 1.0% and 0.1% in Late Growth (Culture Series 255) Refined fractions for triterpene analysis were prepared by silica gel column chromatography of the esterified culture extracts (255-2 and 255-3 only).

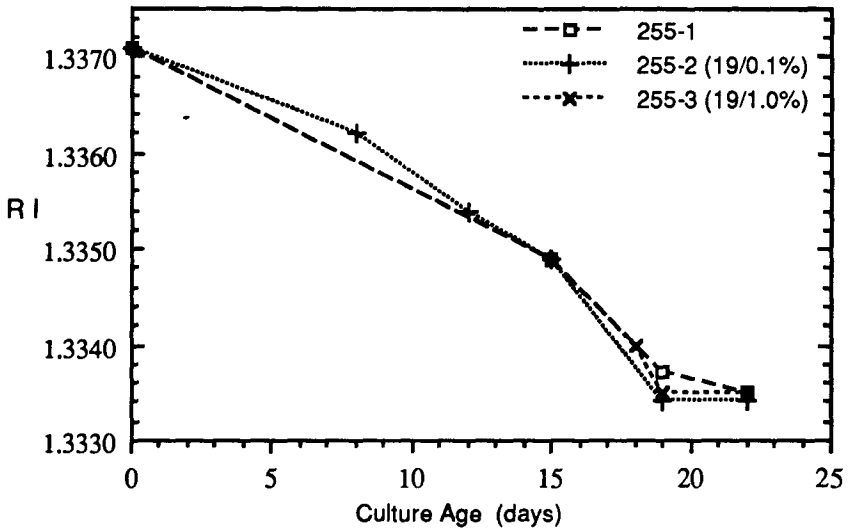


Figure 24 Growth Curves of TRP 4a Culture Series 255 Refractive index at 25.0°C. Legend symbols are followed by the age and level (v/v) at which *Botrytis* was added to cultures.

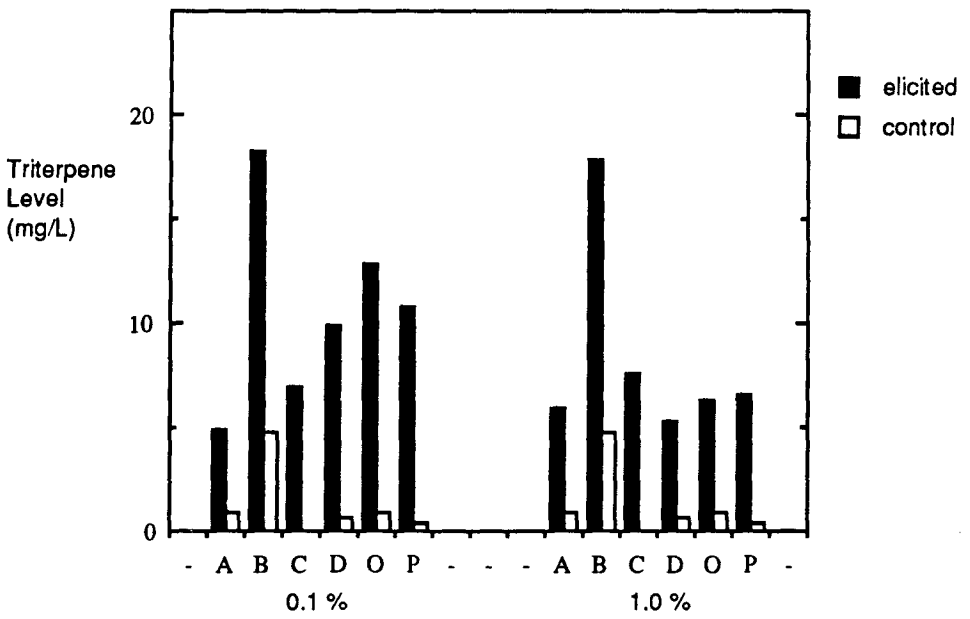


Figure 25 Triterpene Levels from Culture Series 255 Oleanane triterpenes A-D, oleanolic acid (O), polpunonic acid (P). Culture 255-2 is elicited with 0.1 % *Botrytis* at $RI_{25°}$ 1.3334 (late growth) for 72 hours while culture 255-3 is elicited with 1.0 % *Botrytis* at $RI_{25°}$ 1.3335 (late growth) for 72 hours. Culture 255-1 (control) has no additions.

Previous cultures had been elicited (for 72 hours at 1.0%) in late growth (252-1, 255-3) and mid growth (254-2) and so experiment 256 was designed to test the effects of elicitor addition in the early growth phase. At this stage, the cells had established a maximum rate of growth and the supply of nutrients was still high while the mass of cells was quite low. Culture 256-3 was elicited in early growth (RI 1.3360) with 1.0% *Botrytis* for 72 hours (table 5, figures 26 and 27).

The elicited triterpene levels from culture 256-3 were far higher than the control levels and were comparable with the highest results thus far, those from the late growth elicited culture 255-3. For these two cultures to have produced equal amounts of the triterpenes, the cells of the early elicited culture needed to synthesize triterpenes at a greater rate than the more numerous cells of the late elicited culture, revealing an increased elicitor response from the younger culture. The high triterpene yields from early elicitation was an important result in light of the overall growth time of 13 days for culture 256-3 compared to 22 days for the late elicited culture 255-3.

When the 1.0%, early growth elicitation was extended from 72 hours to 12 days, culture 256-1, the triterpene levels were doubled. This indicated that the TRP 4a cells could produce triterpenes continuously while in the presence of elicitor. This contrasted with the results obtained by Threlfall's group[86] where elicited plant cell cultures (*Tabernaemontana divaricata*) stopped accumulating triterpenes 48 hours after addition of the fungus. In the *Tabernaemontana* cultures, elicited pentacyclic triterpenes were not degraded after prolonged culture growth. The increase in triterpenes with *Tripterygium* elicited over 12 days suggested that either a degradation did not occur or that synthesis exceeded degradation.

Culture	256-1	256-2	256-3
Initial Culture Volume (L)	12	12	12
Inoculum Level (v/v) [Age (d) / RI]	8.3 % [17 / 1.3335]	8.3 % [17 / 1.3335]	8.3 % [17 / 1.3335]
Age at Elicitation (d) [RI]	10 [1.3360]	-	10 [1.3360]
Elicitor Level (v/v)	1.0 %	not added	1.0 %
Elicitation Period / Age at Harvest (d)	12 / 22	0 / 13	3 / 13
Final RI of Culture Medium	1.3334	1.3353	1.3354
Fresh Weight of Cells (g)	2480	1818	1490
EtOAc Extract weight (mg/L)	831	411	444
Triterpene A (mg/L)	21.4	1.09	6.18
Triterpene B (mg/L)	42.6	5.38	23.6
Triterpene C (mg/L)	32.4	1.48	6.74
Triterpene D (mg/L)	17.8	1.04	7.90
Oleanolic Acid (mg/L)	24.4	1.98	7.27
Polpunonic Acid (mg/L)	39.3	2.29	10.1

Table 5 Elicitation at 1.0% and Extended Elicitation in Early Growth (Culture Series 256) Refined fractions for triterpene analysis were prepared by silica gel column chromatography of the esterified culture extracts (256-1 and 256-3 only).

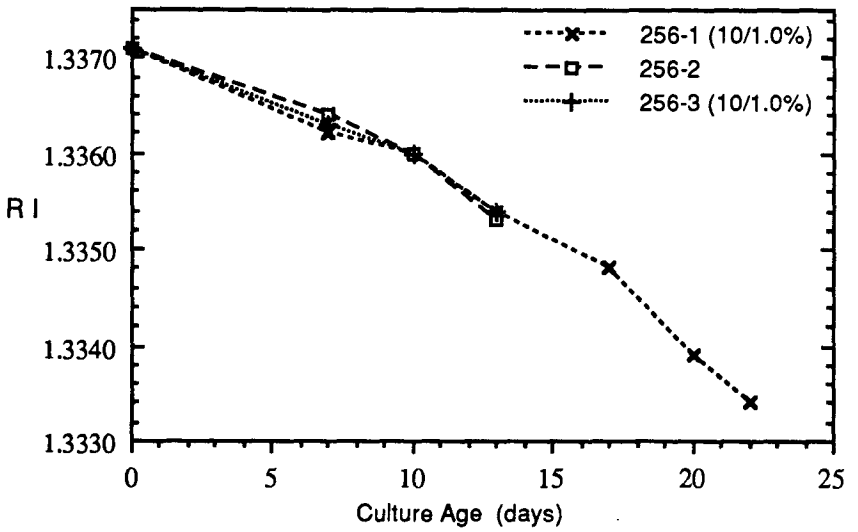


Figure 26 Growth Curves of TRP 4a Culture Series 256 Refractive index at 25.0°C. Legend symbols are followed by the age and level (v/v) at which *Botrytis* was added to cultures.

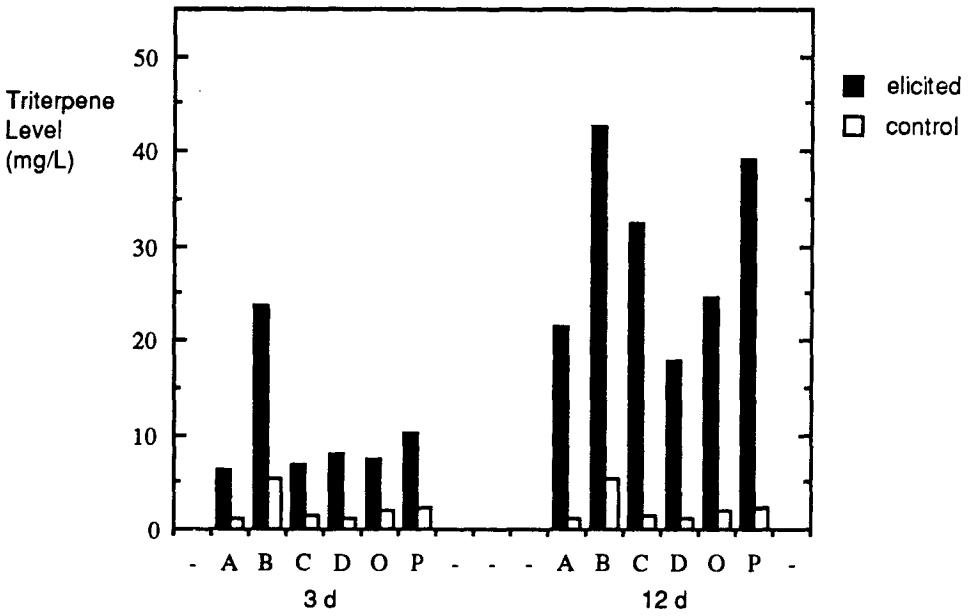


Figure 27 Triterpene Levels from Culture Series 256 Oleanane triterpenes A-D, oleanolic acid (O), polpunonic acid (P). Culture 256-3 is elicited with 1.0 % *Botrytis* at $RI_{25°}$ 1.3360 (early growth) for 72 hours while culture 256-1 is elicited with 1.0 % *Botrytis* at $RI_{25°}$ 1.3360 (early growth) for 12 days. Culture 256-2 (control) has no additions. Control triterpene levels were included beside the 12 day elicited trial for comparison, although the control was harvested 9 days earlier with the 3 day trial.

The results so far had shown an advantage to the use of 1.0% elicitor rather than 0.1% elicitor for the production of triterpenes A and C. The favourable triterpene increases from early growth elicitation in the 256 series could also be interpreted as an effect of higher elicitor levels. The degree of response to elicitation has previously been related to the elicitor/cell mass ratio, rather than simply to the elicitor concentration (introduction p. 38, ref's [81] and [83]). This suggested a tight binding of the elicitor to the cells. In light of this observation, 1.0% elicitation (v/v) would have provided a greater relative concentration of elicitor to early growth cultures than to late growth cultures due to the differences in cell mass. To further explore the effects of increased elicitor concentration, series 258 was carried out using 5.0% elicitor addition. Culture 258-1 was elicited with 5.0% *Botrytis* for 72 hours in the mid growth stage (RI 1.3351)and 258-2 was elicited equally in the mid-late growth stage (RI 1.3340). (An early elicited culture was lost to contamination.)The results are presented in table 6 and figures 28 and 29.

The growth of series 258 cultures was quite slow prior to elicitation. The 5.0% elicitation yielded triterpenes in the low range of previous 1.0% elicited results (levels similar to those of series 252 and 254) while the levels were well below those from the most successful 1.0% elicitations of series 255 and 256. With these comparisons, 5.0% elicitation did not appear advantageous to the lower elicitor levels. Due to the variances in triterpene production seen in the successive culture series, a set of fermentors with parallel 1.0% and 5.0% elicitation was deemed necessary to confirm the low triterpene yield from an increased elicitor level.

Of the two 5.0% elicitations, the earlier elicitation was more successful in generating triterpene production despite a much larger quantity of cells in the later elicited culture (fresh weight at harvest was 3102 g vs. 1918 g from the earlier elicited culture). This increased rate of elicited triterpene synthesis from younger cells was also noted in the comparison of 256-3 to 255-3, and so appears to be a dependable effect.

Culture	258-1	258-2
Initial Culture Volume (L)	12	12
Inoculum Level (v/v) [Age (d) / RI]	8.3 % [18 / 1.3333]	8.3 % [18 / 1.3333]
Age at Elicitation (d) [RI]	15 [1.3351]	21 [1.3340]
Elicitor Level (v/v)	5.0 %	5.0 %
Elicitation Period / Age at Harvest (d)	3 / 18	3 / 24
Final RI of Culture Medium	1.3348	1.3336
Fresh Weight of Cells (g)	1918	3102
EtOAc Extract weight (mg/L)	613	623
Triterpene **A** (mg/L)	1.05	0.48
Triterpene **B** (mg/L)	3.12	1.83
Triterpene **C** (mg/L)	1.78	0.77
Triterpene **D** (mg/L)	1.83	0.93
Oleanolic Acid (mg/L)	0.93	0.81
Polpunonic Acid (mg/L)	4.88	1.83

Table 6 Elicitation at 5.0% in Mid Growth (Culture Series 258)
Refined fractions for triterpene analysis were prepared by silica gel column chromatography of the esterified culture extracts (258-1 and 258-2).

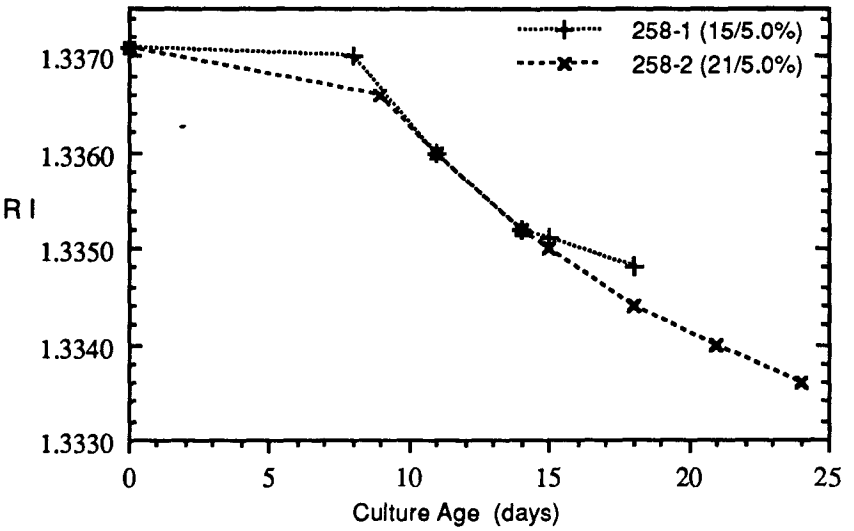


Figure 28 Growth Curves of TRP 4a Culture Series 258 Refractive index at 25.0°C . Legend symbols are followed by the age and level (v/v) at which *Botrytis* was added to cultures.

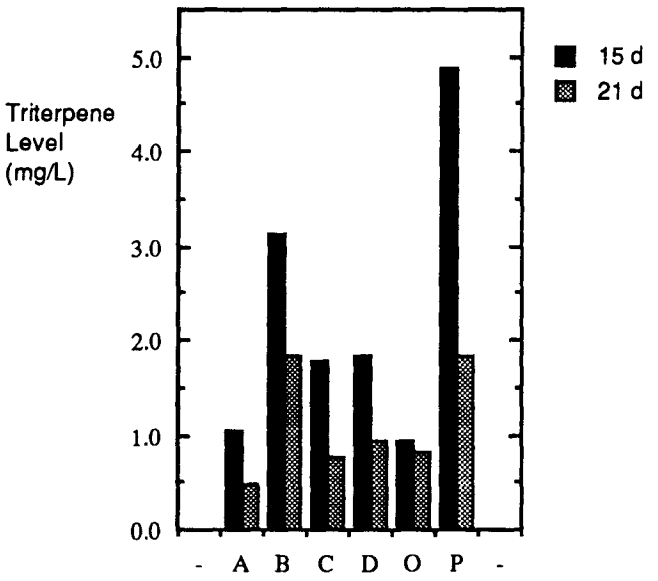


Figure 29 Triterpene Levels from Culture Series 258 Oleanane triterpenes A-D, oleanolic acid (O), polpunonic acid (P). Culture 258-1 is elicited with 5.0 % *Botrytis* at $RI_{25°}$ 1.3351 (mid growth) for 72 hours while culture 258-2 is elicited with 5.0 % *Botrytis* at $RI_{25°}$ 1.3340 (mid/late growth) for 72 hours.

Culture series 259 was planned to re-examine the effects of early growth elicitation using 1.0% and 5.0% elicitor additions. The 1.0% early growth elicitation, 259-2, was run under the same conditions as 256-3 (elicited 72 hours from RI 1.3360) and the 5.0% elicitation, 259-3, was treated the same way. Culture 259-1 was a non-elicited control. The results are presented in table 7 and figures 30 and 31.

The triterpene levels from the 1.0% elicited culture, 259-2, were moderately higher than control levels, save triterpenes C and O, which were not increased. The elicited triterpenes A, B, D and P were accumulated to only one third of the levels reached in culture 256-3 although the two cultures were grown and elicited under identical conditions. This indicated once more that additional conditions such as the state of the inoculum had a great effect on the responsiveness of cultures to elicitation.

The 5.0% elicited culture, 259-3, did not yield significantly more triterpenes than the control culture. Elicitation was not without any effect, as the harvest mass from 259-3 was 1600 g compared to 1991 g from the 1.0% elicited culture and 2169 g from the control culture. These observations suggested that an increase to 5.0% elicitation was detrimental to both cell growth and triterpene production.

Culture	259-1	259-2	259-3
Initial Culture Volume (L)	12	12	12
Inoculum Level (v/v) [Age (d) / RI]	8.3 % [16 / 1.3332]	8.3 % [16 / 1.3332]	8.3 % [16 / 1.3332]
Age at Elicitation (d) [RI]	-	10 [1.3360]	10 [1.3360]
Elicitor Level (v/v)	not added	1.0 %	5.0 %
Elicitation Period / Age at Harvest (d)	0 / 13	3 / 13	3 / 13
Final RI of Culture Medium	1.3355	1.3356	1.3358
Fresh Weight of Cells (g)	2169	1991	1600
EtOAc Extract weight (mg/L)	375	400	306
Triterpene A (mg/L)	0.70	1.79	0.83
Triterpene B (mg/L)	2.09	7.30	3.17
Triterpene C (mg/L)	0.88	0.69	0.55
Triterpene D (mg/L)	1.80	4.73	1.86
Oleanolic Acid (mg/L)	1.54	1.46	1.40
Polpunonic Acid (mg/L)	0.30	1.48	0.41

Table 7 Elicitation at 1.0% and 5.0% in Early Growth (Culture Series 259)
Refined fractions for triterpene analysis were prepared by silica gel column chromatography of the esterified culture extracts (259-2 and 259-3 only).

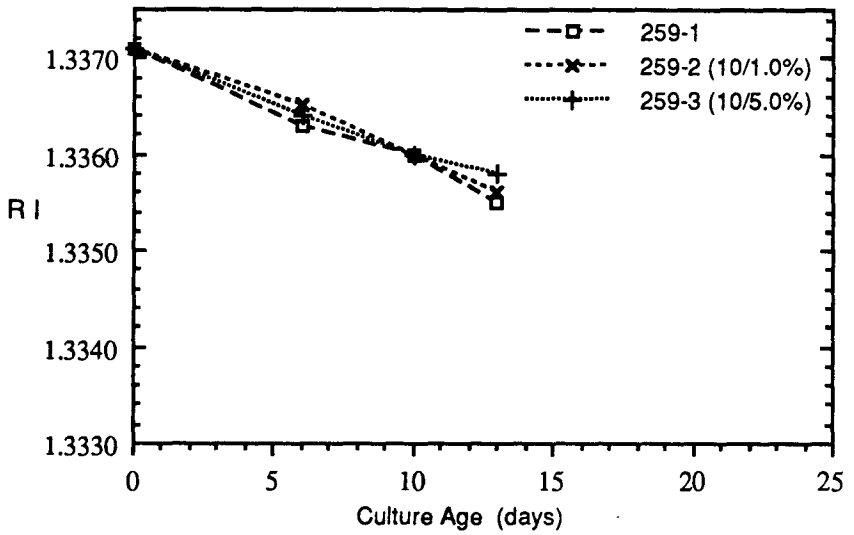


Figure 30 Growth Curves of TRP 4a Culture Series 259 Refractive index at 25.0°C. Legend symbols are followed by the age and level (v/v) at which *Botrytis* was added to cultures.

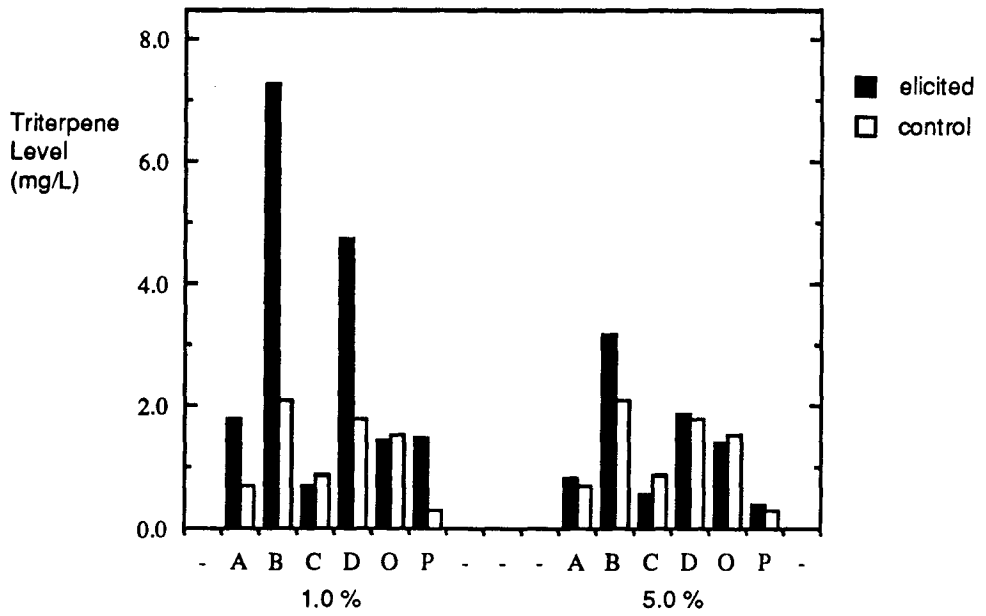


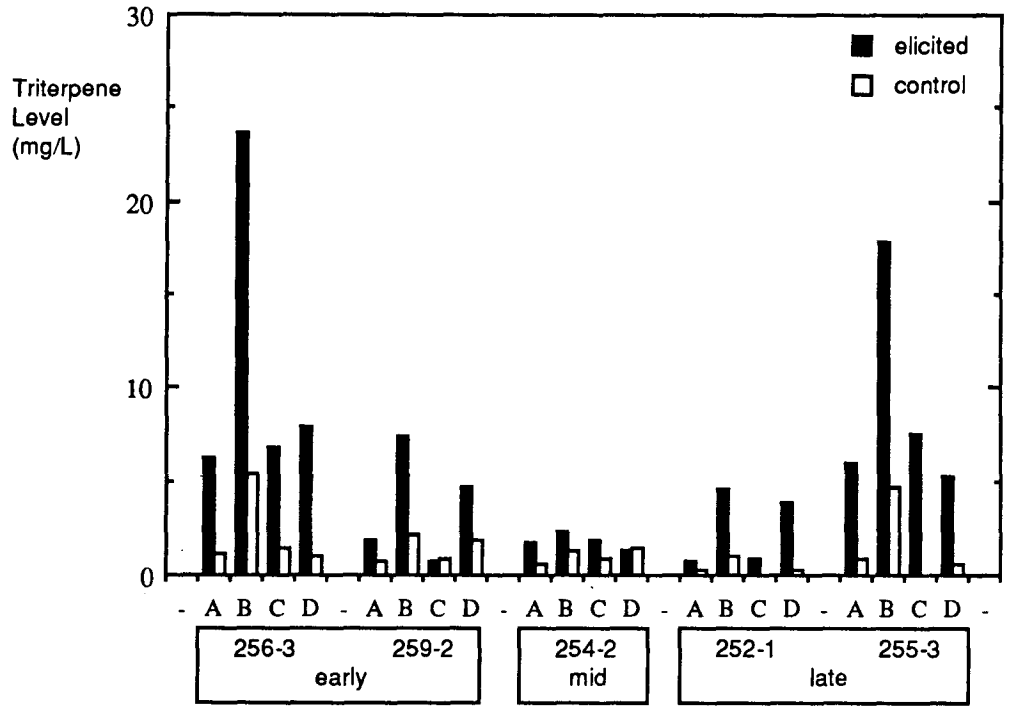
Figure 31 Triterpene Levels from Culture Series 259 Oleanane triterpenes A-D, oleanolic acid (O), polpunonic acid (P). Culture 259-2 is elicited with 1.0 % *Botrytis* at $RI_{25°}$ 1.3360 (early growth) for 72 hours while culture 259-3 is elicited with 5.0 % *Botrytis* at $RI_{25°}$ 1.3360 (early growth) for 72 hours. Culture 259-1 (control) has no additions.

(iv) A Summary of the 72 Hour Elicitations

(a) The effect on triterpene yield following elicitation with 1.0% *Botrytis* for 72 hours at various stages of culture growth is presented in figure 32. An excellent increase in triterpenes was possible from both early and late elicited cultures, although high yields were not entirely dependable. Early elicitation was clearly advantageous when one considered the time required for triterpene production and the mass of cell material that required extraction. When the triterpene levels presented in figure 32 were divided by the culture time before harvest and the harvested cell weight, an optimized production of triterpenes through early growth elicitation was revealed, figure 33.

When the elicitation was carried out using 0.1%, 1.0% and 5.0% *Botrytis*, the highest triterpene yields resulted from the 1.0% addition. The 0.1% elicitation resulted in slightly lower levels of the triterpenes A and C, while 5.0% elicitation did not raise the triterpene levels significantly above control levels and caused an inhibition in culture growth (with respect to the cell mass).

Elicitation with *Botrytis* produced other effects on cultures 252-259 besides increased triterpene production. The harvest weight of elicited cultures was less than that of parallel controls by a degree proportional to the level of *Botrytis* addition and to the duration of exposure. The most visible result of elicitation was an alteration of the colour of the growing cultures. Elicitation caused a darkening from the normal pale green/tan of control (PRD_2Co) cultures to a darker green and further to a dark green/gray/orange in proportion to the resulting triterpene yield rather than to the amount of elicitor added. The relation of a dark colour to production of the red-orange triterpene quinone methides was confirmed after the isolation of tingenone (66) and celastrol (68) from the elicited extracts (Huifen Gu, unpublished). Culture darkening provided a reliable estimation of triterpene synthesis prior to harvest and analysis.



elicited culture	$RI_{25°}$ at elicitation	control culture
256-3	1.3360	256-2
259-2	1.3360	259-1
254-2	1.3353	254-3
252-1	1.3332	252-2
255-3	1.3335	255-1

Figure 32 Triterpene Levels from Elicitation at Various Culture Ages
Oleanane triterpenes A-D. Elicitation with 1.0 % *Botrytis* for 72 hours. Controls were grown without *Botrytis*.

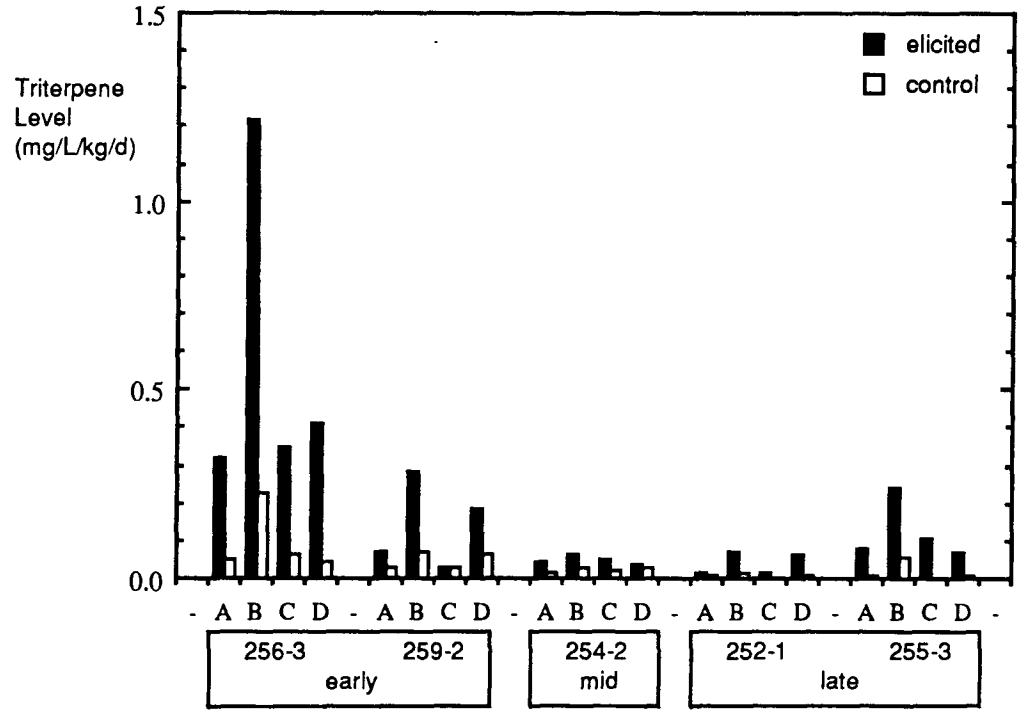


Figure 33 Optimization of Triterpene Production Oleanane triterpenes A-D. Elicitation with 1.0 % *Botrytis* for 72 hours as described in figure 32. Levels are quoted in triterpenes produced (mg/L) per fresh weight of cells (kg) per day of growth to harvest.

(b) Culture stress factors (such as temperature) are capable of stimulating secondary metabolite production[81]. The elicitation of TRP 4a with *Botrytis* caused stress on the cultures with regard to the decreased harvest mass and increased cell death. A study by Brodelius et al.[84] has demonstrated that a direct relationship can exist between culture stress and fungal elicitation . In cultured tobacco cells, the fungal elicitor chitosan increased secondary metabolite production in proportion to its concentration until an optimal level, above which the production was almost completely blocked. Studies measuring the electrical conductivity of the culture medium revealed that surpassing the optimal chitosan concentration produced a sudden increase in conductivity caused by cell membrane permeabilization (the release of ions to the medium). Chitosan is a cationic polyglucosamine that can be extracted from the outer cell walls of fungi. It may increase plant cell permeability by binding to anionic groups at the membrane surface to cause membrane disruption or by binding to specific elicitor receptors to open gated ion channels. If increased cell membrane permeability is linked to the elicitation of TRP 4a, it is reasonable to expect that high elicitor levels (5 %) would disturb the osmotic balance of the culture. Thus a single mechanism, osmotic stress, could cause increased metabolite production, decreased cell growth and increased cell lysis. The substance mediating the elicitation of TRP 4a by *Botrytis* has not been identified; chitosan or another type of polysaccharide may well be the active principle.

(c) One uncertainty in the analysis of triterpenes B and D originated in the column chromatography of extracts from the elicited cultures. Low triterpene D levels from elicited cultures 254-258 may have been caused by conversion of the methyl ester into the lactone wilforlide A (56). If this reaction occurred due to exposure to silica gel during chromatography, the lactone would have eluted well in advance of the triterpene ester fractions that were analyzed.

Triterpene levels from the crude esterified extract and from the purified triterpene fractions of culture 256-3 are presented below to demonstrate the results of purification. The decreased triterpene levels after purification resulted from the removal of overlapping peaks in the GC analysis and from any losses during column chromatography.

	Triterpene Level (mg/L)					
Triterpene	A	B	C	D	O	P
Crude extract	11.0	26.0	11.3	15.9	9.25	10.5
Purified extract	6.18	23.6	6.74	7.90	7.27	10.1

Table 8 The Effect of Column Chromatography on the Triterpene Assay
Triterpene determination from culture 256-3.

The increases in triterpene production through elicitation surpassed any effect of losses during chromatography as the control extracts were not purified. A greater uncertainty was provided by the lack of reproducible elicitation results from successive cultures treated under identical growth conditions. The growth rate of cultures prior to elicitation had often varied, thus our attention was directed towards the influence of the stock cultures used as inoculum. The TRP 4a stock cultures were grown in 500 ml volumes and required about 17 days to mature. The line was maintained in four groups, subcultured at intervals to provide mature inoculum at four day intervals. A correlation of elicited triterpene production to the inoculum cycle used for the successive cultures is presented in table 9. Culture series 255 and 256 provided an increased elicited triterpene production over other series, and this indicated that cultures from inoculum cycle B were the most responsive to elicitation. While the four inocula were derived from the same source, developmental differences must have occurred to cause varied levels of secondary metabolism.

Culture Series	Inoculum Cycle	Elicitation Results
252	A	+
254	A	+
255	B	+++
256	B	+++
258	C	+
259	A2	++

Table 9 The Effect of Inoculum Cycle on Elicited Triterpene Yields (+) indicates an increase in triterpene yields above control levels. (Culture age and elicitor levels are not considered in this comparison.)

(v) Extended Elicitation

An experiment was developed to monitor triterpene production at elicitation periods longer than 72 hours, since series 256 had demonstrated a doubling of triterpene levels from the 3 day to the 12 day elicitation. The time course of triterpene production was examined using cultures elicited in the early growth phase (RI 1.3360). Small samples were withdrawn from these cultures at intervals for triterpene assay. Purification of the triterpenes from these small samples was not practical, yet the previous large scale isolations had confirmed the high levels of triterpenes determined for the elicited cultures by GC analysis.

The time course experiments, 261, 262 and 266, were cultured with inoculum B to remove the effect of varying inoculum cycles on the triterpene production. The B sub-group of TRP 4a had previously demonstrated a maximum responsiveness to elicitation. The size of samples removed from the cultures was set at 200 ml to provide sufficient triterpene levels for analysis and to insure that the cell density was representative of the whole culture. Larger samples could not be taken as the air-lift bioreactors would not operate below a volume of 10 L. The culture samples (containing 30-70 g cells, fresh weight) were frozen and then extracted the

following day; ethyl acetate extraction of the solid and liquid material was rapid and efficient due to the small sample size.

As GC analysis was carried out on the crude esterified extracts, some inflation of the triterpene determinations was expected. While the assay was not highly accurate, the change in levels with time provided a good assessment of increases or decreases in the triterpene content.

A great advantage of determination from crude extracts was the removal of purification losses that occurred during column chromatography. Also, as the purification of triterpene ester fractions did not occur, determinations for triterpene B and D lactones (55 and 56) were included in the levels reported for B and D methyl esters. TLC analysis of the crude extracts had shown the lactones present in about 10% of the triterpene acid levels. Since treatment with KOH/ethanol could readily convert lactones to free hydroxy acids, the combined determination measured the total possible yield for the triterpene B and D acids.

(vi) The Results of Extended Elicitation

Culture series 261 incorporated 1.0% *Botrytis* addition in early growth (day 10, RI 1.3360); the same parameters were used for the most successful elicitation experiment 256-1. Cultures 261-1 and 261-2 were both elicited with these conditions while 261-3 was not elicited, providing a control culture. The results are presented in table 10 and in figures 34, 35. Throughout the growth, control triterpene levels remained below 3 mg per liter of culture. The elicited triterpene levels rose much higher. Triterpenes A, B and D increased over the first six days (261-1) and nine days (261-2) of elicitation and then began to decrease while triterpenes C, O and P increased over the entire elicitation period. A decrease in some triterpene levels after extended culture growth required a decrease in the rate of synthesis as well as a degradation process. This demonstrated that the cultures had become less sensitive to elicitation or the elicitor was metabolized by the culture or it was neutralized by irreversible binding. In the control culture, relatively stable triterpene levels indicated that above a low level of accumulation synthesis was balanced by degradation. An alternate interpretation was that triterpene synthesis was briefly elicited by the stress of transfer of the mature stock cultures (with depleted sucrose and depleted salts) into the concentrated PRD_2Co of the bioreactors at day 0 and the initially formed triterpenes remained throughout the culture period.

Culture	261-1	261-2	261-3
Initial Culture Volume (L)	12	12	12
Inoculum Level (v/v) [Age (d) / RI]	8.3% [18 / 1.3332]	8.3% [18 / 1.3332]	8.3% [18 / 1.3332]
Age at Elicitation (d) [RI]	10 [1.3358]	10 [1.3358]	-
Elicitor Level (v/v)	1.0 %	1.0 %	not added

	Culture 261-1			
Elicitation Period / Age (d)	3 / 13	6 / 16	9 / 19	12 / 22
RI of Culture Medium	1.3352	-	1.3340	1.3335
Volume Sampled (ml)	192	204	198	200
EtOAc Extract weight (mg/L)	578	760	1460	1220
Triterpene A (mg/L)	3.76	7.63	6.03	4.81
Triterpene B (mg/L)	15.7	25.6	24.4	21.8
Triterpene C (mg/L)	0.856	3.34	4.78	4.89
Triterpene D (mg/L)	7.08	18.6	14.6	13.3
Oleanolic Acid (mg/L)	5.24	6.75	9.58	11.2
Polpunonic Acid (mg/L)	2.36	6.71	12.2	15.1

Table 10 Extended Elicitation from Early Growth (Culture Series 261)
Triterpene analysis performed on crude esterified extracts of culture samples. Cultures originated with TRP 4a B inoculum. (cont. next two pages)

Culture 261-2				
Elicitation Period / Age (d)	3 / 13	6 / 16	9 / 19	12 / 22
RI of Culture Medium	1.3352	-	1.3339	1.3335
Volume Sampled (ml)	204	203	199	200
EtOAc Extract weight (mg/L)	691	956	1260	1080
Triterpene A (mg/L)	3.40	6.08	6.55	6.33
Triterpene B (mg/L)	14.7	20.3	28.4	26.6
Triterpene C (mg/L)	0.730	2.68	4.24	5.64
Triterpene D (mg/L)	6.61	12.2	20.4	20.6
Oleanolic Acid (mg/L)	5.11	6.13	8.09	10.4
Polpunonic Acid (mg/L)	2.07	5.13	10.1	14.8

Table 10 Extended Elicitation from Early Growth (Culture Series 261) (cont.)

Culture 261-3				
Elicitation Period / Age (d)	0 / 13	0 / 16	0 / 19	0 / 22
RI of Culture Medium	1.3351	-	1.3338	1.3335
Volume Sampled (ml)	202	208	186	200
EtOAc Extract weight (mg/L)	411	255	828	539
Triterpene **A** (mg/L)	0.39	0.35	0.780	0.23
Triterpene **B** (mg/L)	1.61	1.12	2.27	1.65
Triterpene **C** (mg/L)	0.00	0.00	0.00	0.28
Triterpene **D** (mg/L)	0.00	0.00	2.44	0.28
Oleanolic Acid (mg/L)	0.54	0.31	0.56	0.44
Polpunonic Acid (mg/L)	0.37	0.23	0.26	0.12

Table 10 Extended Elicitation from Early Growth (Culture Series 261) (cont.)

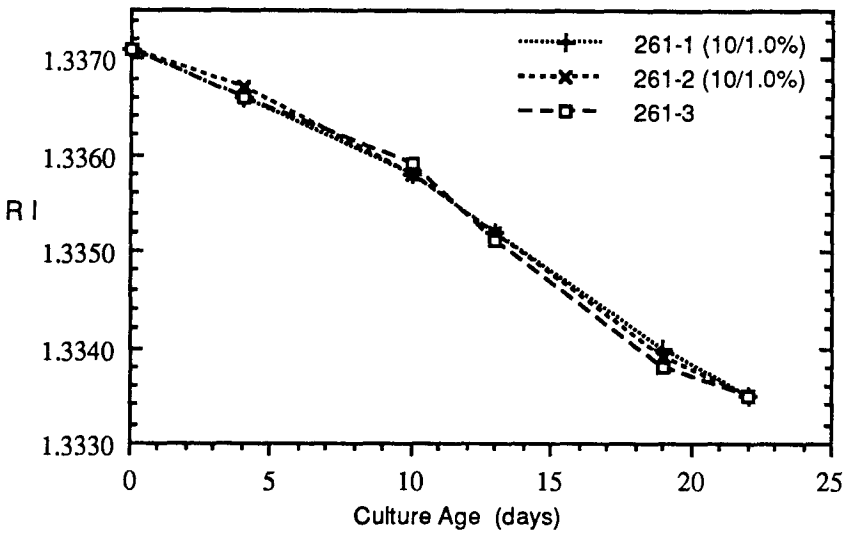


Figure 34 Growth Curves of TRP 4a Culture Series 261 Refractive index at 25.0°C. Legend symbols are followed by the age and level (v/v) at which *Botrytis* was added to cultures.

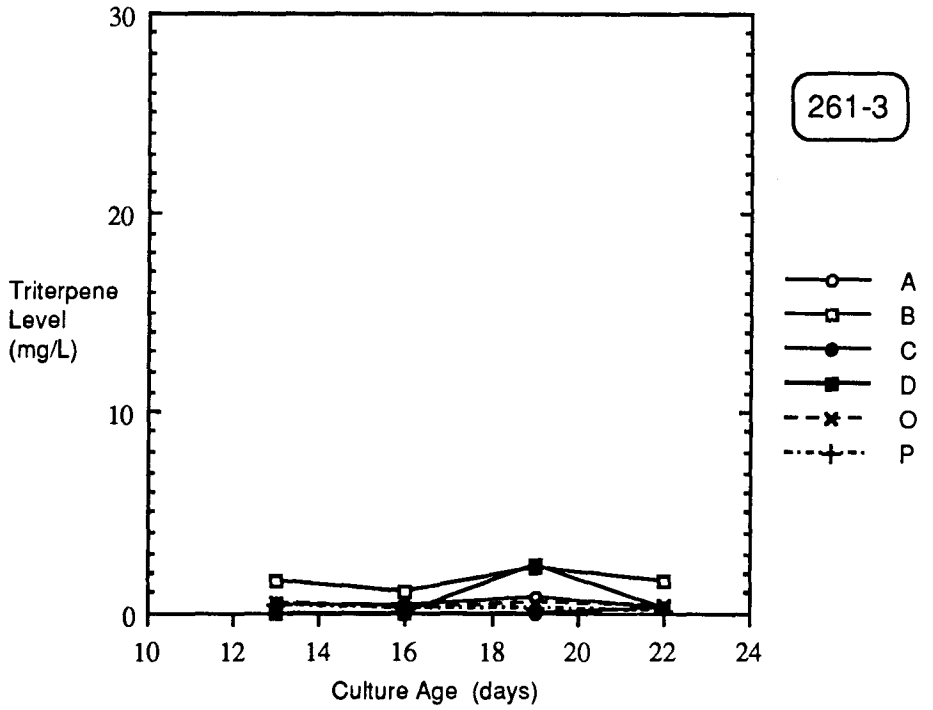


Figure 35 Triterpene Levels from Culture Series 261 Oleanane triterpenes A-D, oleanolic acid (O), polpunonic acid (P). Culture 261-3 is a control with no additions while 261-1 and 261-2 are elicited with 1.0% *Botrytis* (v/v) on day 10. (cont'd next page)

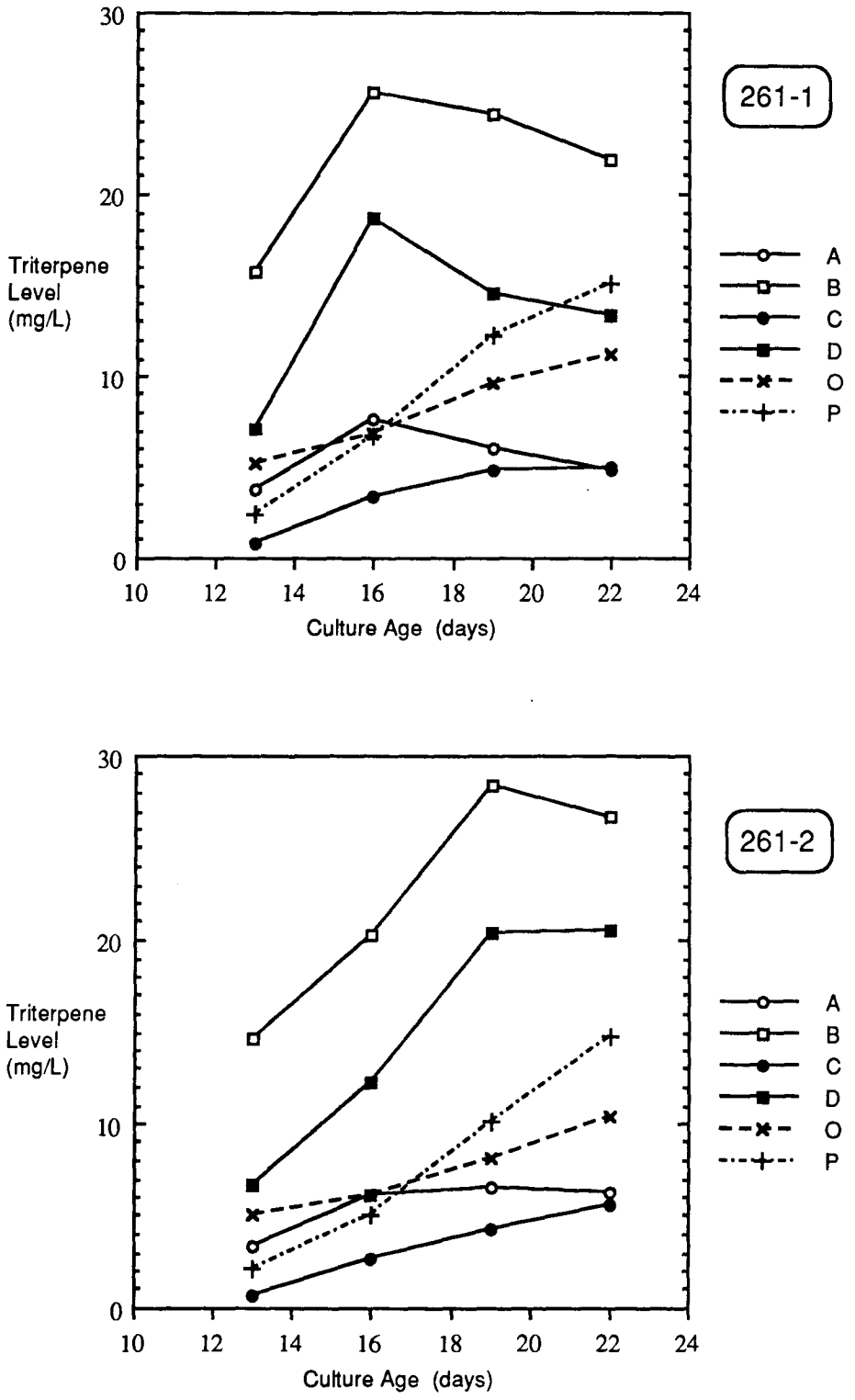


Figure 35 Triterpene Levels from Culture Series 261 Oleanane triterpenes A-D, oleanolic acid (O), polpunonic acid (P). Culture 261-3 is a control with no additions while 261-1 and 261-2 are elicited with 1.0% *Botrytis* (v/v) on day 10.

The elicitation conditions of culture series 262 repeated those used for series 261. Elicitor was added at 1.0% in early growth (day 8, RI 1.3360). The results are presented in table 11 and in figures 36, 37. Triterpene levels in the control culture (262-2) remained below 4 mg/L, falling from initially high levels which may have been stimulated by the osmotic stress of inoculation to fresh medium at day 0. The triterpene levels in the elicited cultures were first determined at the time of *Botrytis* addition. From initially low levels, the triterpenes increased greatly in the first 72 hours and continued to rise over the entire growth of the culture. The final triterpene levels were higher than the maximum levels of series 262. One apparent difference between 262 and 261 was the more rapid growth of 262 cultures (18 days to the end of growth compared to 22 days for 261 cultures). The more vigorous growth of series 262 may have allowed continued triterpene production instead of the diminishing synthesis seen in the late growth of series 261.

Culture	262-1	262-2
Initial Culture Volume (L)	12.5	12.5
Inoculum Level (v/v) [Age (d) / RI]	9.0% [16 / 1.3332]	9.0% [16 / 1.3332]
Age at Elicitation (d) [RI]	8 [1.3360]	-
Elicitor Level (v/v)	1.0 %	not added

Culture 262-1				
Elicitation Period / Age (d)	0 / 8	4 / 12	7 / 15	10 / 18
RI of Culture Medium	1.3360	1.3351	1.3343	1.3334
Volume Sampled (ml)	195	204	208	200
EtOAc Extract weight (mg/L)	349	559	586	975
Triterpene A (mg/L)	0.559	8.38	9.36	12.0
Triterpene B (mg/L)	2.54	34.7	42.2	58.3
Triterpene C (mg/L)	0.00	3.68	8.39	9.49
Triterpene D (mg/L)	0.48	20.2	28.9	42.0
Oleanolic Acid (mg/L)	1.13	9.59	11.1	17.5
Polpunonic Acid (mg/L)	1.09	6.64	18.2	21.7

Table 11 Extended Elicitation from Early Growth (Culture Series 262)
Triterpene analysis performed on crude esterified extracts of culture samples. Cultures originated with TRP 4a B inoculum. (cont. next page)

Culture 262-2				
Elicitation Period / Age (d)	0 / 8	0 / 12	0 / 15	0 / 18
RI of Culture Medium	1.3360	1.3349	1.3338	1.3333
Volume Sampled (ml)	200	208	199	203
EtOAc Extract weight (mg/L)	354	316	291	280
Triterpene A (mg/L)	0.830	0.26	0.23	0.17
Triterpene B (mg/L)	3.97	2.23	1.68	1.81
Triterpene C (mg/L)	0.00	0.00	0.00	0.00
Triterpene D (mg/L)	1.08	0.538	0.35	0.31
Oleanolic Acid (mg/L)	1.78	0.909	0.774	0.887
Polpunonic Acid (mg/L)	1.50	0.47	0.43	0.36

Table 11 Extended Elicitation from Early Growth (Culture Series 262) (cont.)

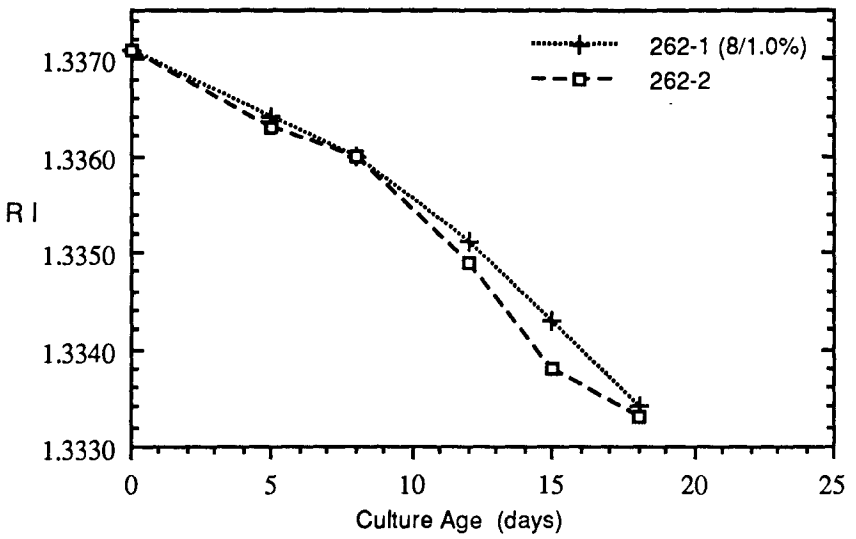


Figure 36 Growth Curves of TRP 4a Culture Series 262 Refractive index at 25.0°C. Legend symbols are followed by the age and level (v/v) at which *Botrytis* was added to cultures.

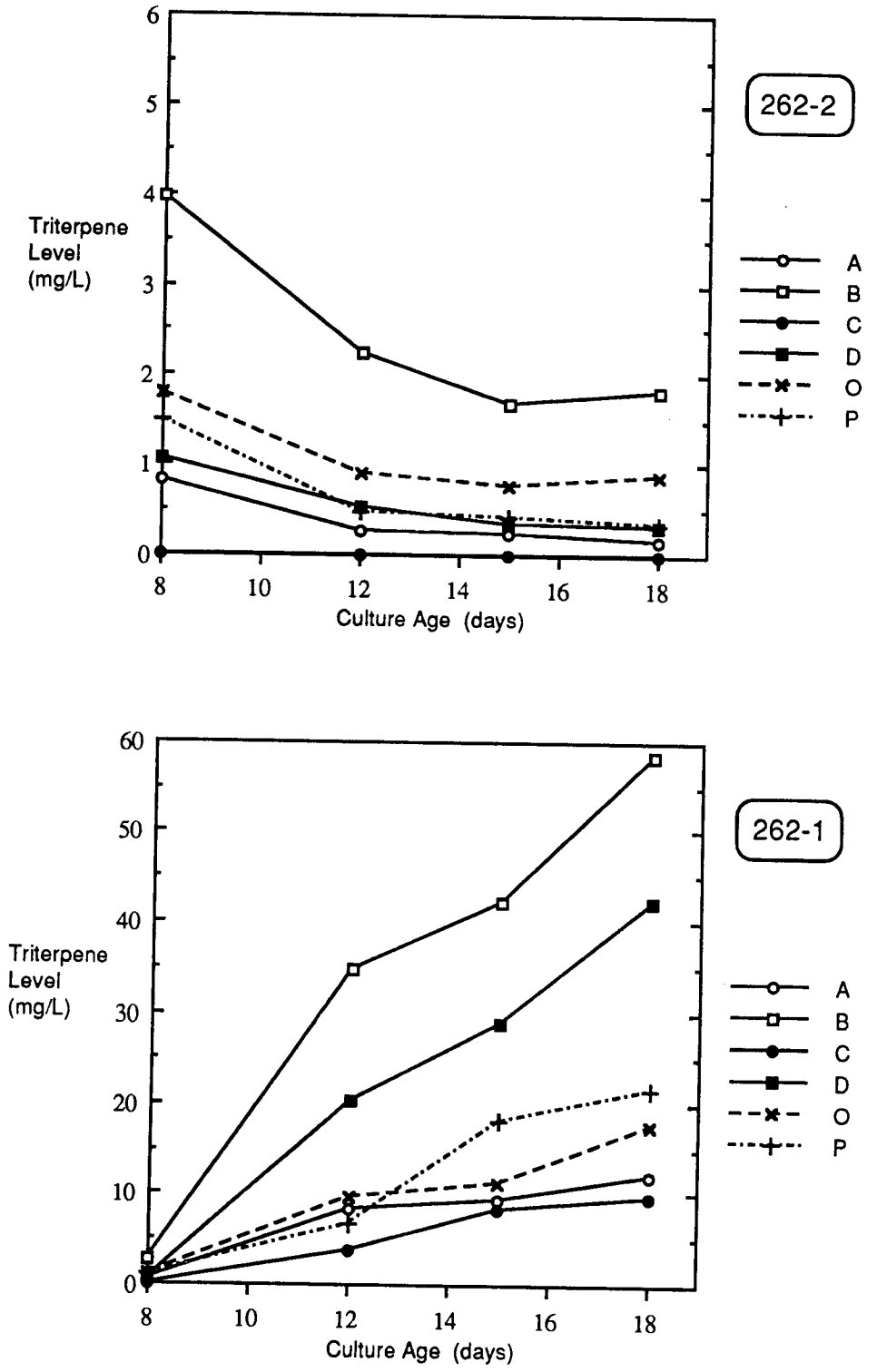


Figure 37 Triterpene Levels from Culture Series 262 Oleanane triterpenes A-D, oleanolic acid (O), polpunonic acid (P). Culture 262-2 is a control with no additions while 262-1 is elicited with 1.0% Botrytis (v/v) on day 8. Note a tenfold decrease in scale for the lower plot.

Culture 266-1 was designed to examine the effects of elicitation on very young cultures. For this purpose, 1.0% *Botrytis* was added at the time of inoculation (day 0, RI 1.3371). To match the elicited cultures of series 261 and 262, culture 266-2 was treated with 1.0% *Botrytis* in early growth (day 8, RI 1.3361). A control culture, 266-3, was maintained until close to the end of growth (day 18, RI 1.3335), but was then elicited with 1.0% *Botrytis* for 72 hours. This allowed a re-examination of late growth elicitation. The growth conditions and results are presented in table 12 and figures 38, 39.

All three elicitations resulted in large increases in triterpene production. Culture 266-2 produced higher triterpene levels than the similar cultures 261-1, 261-2 and 262-1. While it had been noted that increased triterpene production occurred in rapidly growing cultures, the growth rate of the 266 cultures was lower than that of the 262 cultures. This suggests that variations in the growth rate and in the elicitation response of successive cultures are not directly related. The control triterpene levels (266-3) remained low until a remarkably rapid synthesis of triterpenes was initiated immediately after elicitation. The 72 hour triterpene levels from this late elicitation were much higher than the 72 hour levels from the parallel early growth elicitation (266-2) and yet previous cultures, series 252-259, had demonstrated more rapid triterpene synthesis following early growth elicitation. The unusually large elicitation response of culture 266-3 may have resulted from elicitation at RI 1.3335 when the culture was not yet at the end of its growth phase (the RI decreased further to 1.3332 over 72 hours).

The day 0 elicited culture, 266-1, did not show increased triterpene levels until the day 11 analysis. At this time culture 266-2 displayed only slightly lower triterpene levels after just 72 hours of elicitation. At the end of growth (day 21) the day 0 elicited culture contained lower amounts of triterpenes than the parallel day 8 elicited culture. While both cultures had contained 1.0 % *Botrytis* from day 8 to the end of growth, a slower triterpene production from the day 0 culture may have been the result of desensitization to the elicitor during the first week of growth when triterpene synthesis had remained at control levels.

Culture	266-1	266-2	266-3
Initial Culture Volume (L)	13	13	13
Inoculum Level (v/v) [Age (d) / RI]	8.3 % [17 / 1.3333]	8.3 % [17 / 1.3333]	8.3 % [17 / 1.3333]
Age at Elicitation (d) [RI]	0 [1.3371]	8 [1.3361]	18 [1.3335]
Elicitor Level (v/v)	1.0 %	1.0 %	1.0 %

Culture 266-1						
Elicitation Period / Age (d)	5 / 5	8 / 8	11 / 11	14 / 14	18 / 18	21 / 21
RI of Culture Medium	1.3367	1.3362	1.3358	1.3353	1.3344	1.3336
Volume Sampled (ml)	202	204	202	206	197	200
EtOAc Extract weight (mg/L)	165	240	400	586	708	749
Triterpene A (mg/L)	0.703	1.01	4.81	8.51	12.2	14.9
Triterpene B (mg/L)	4.32	6.06	23.0	39.9	59.1	93.1
Triterpene C (mg/L)	0.00	0.966	2.94	4.02	8.40	8.36
Triterpene D (mg/L)	1.25	2.31	8.98	14.1	24.3	27.5
Oleanolic Acid (mg/L)	1.29	3.13	10.2	21.2	28.6	36.5
Polpunonic Acid (mg/L)	1.62	3.04	5.97	12.1	17.1	24.1

Table 12 Extended Elicitation from Early Growth and from Day 0 (Culture Series 266) Triterpene analysis performed on crude esterified extracts of culture samples. Cultures originated with TRP 4a B inoculum. (cont. next two pages)

Culture 266-2						
Elicitation Period / Age (d)	0 / 5	0 / 8	3 / 11	6 / 14	10 / 18	13 / 21
RI of Culture Medium	1.3363	1.3361	1.3357	1.3351	1.3343	1.3335
Volume Sampled (ml)	204	205	200	210	202	199
EtOAc Extract weight (mg/L)	238	299	452	688	983	1070
Triterpene A (mg/L)	0.45	0.634	3.57	16.1	16.6	29.4
Triterpene B (mg/L)	3.03	3.86	15.7	56.4	82.3	117
Triterpene C (mg/L)	0.00	0.47	0.930	7.77	15.5	20.8
Triterpene D (mg/L)	1.00	1.40	7.66	32.2	51.5	68.2
Oleanolic Acid (mg/L)	2.07	2.01	4.86	16.2	23.2	31.2
Polpunonic Acid (mg/L)	1.46	1.95	1.88	12.4	24.9	38.2

Table 12 Extended Elicitation from Early Growth and from Day 0 (Culture Series 266) (cont.)

. Culture 266-3						
Elicitation Period / Age (d)	0 / 5	0 / 8	0 / 11	0 / 14	0 / 18	3 / 21
RI of Culture Medium	1.3363	1.3361	1.3354	1.3348	1.3335	1.3332
Volume Sampled (ml)	202	196	200	196	199	200
EtOAc Extract weight (mg/L)	236	247	399	333	329	447
Triterpene A (mg/L)	0.540	0.34	-	0.724	0.618	9.66
Triterpene B (mg/L)	2.75	3.18	8.52	4.99	4.27	43.4
Triterpene C (mg/L)	0.00	0.11	0.41	0.21	0.40	3.11
Triterpene D (mg/L)	0.639	0.806	1.81	0.893	0.854	26.9
Oleanolic Acid (mg/L)	1.41	2.66	2.41	2.64	2.02	14.3
Polpunonic Acid (mg/L)	1.28	1.37	1.14	0.842	0.533	9.73

Table 12 Extended Elicitation from Early Growth and from Day 0 (Culture Series 266) (cont.)

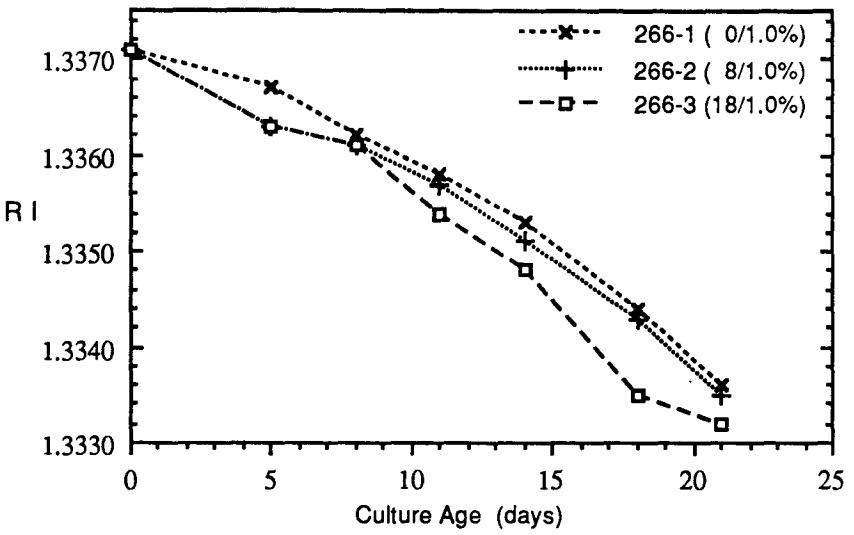


Figure 38 Growth Curves of TRP 4a Culture Series 266 Refractive index at 25.0°C. Legend symbols are followed by the age and level (v/v) at which *Botrytis* was added to cultures.

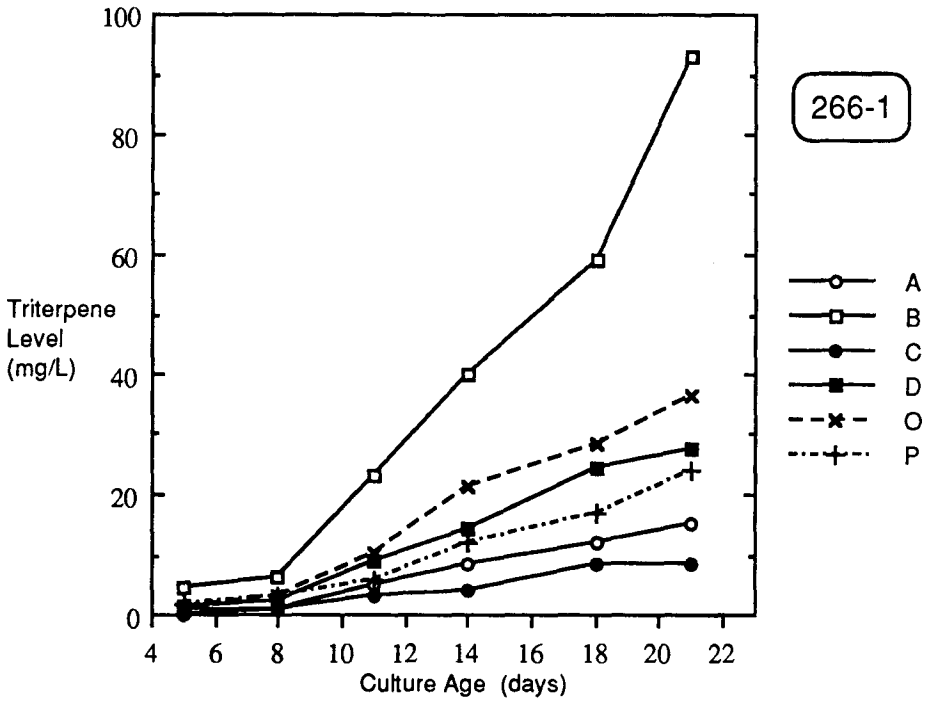


Figure 39 Triterpene Levels from Culture Series 266 Oleanane triterpenes A-D, oleanolic acid (O), polpunonic acid (P). Cultures are elicited with 1.0% *Botrytis* (v/v) on day 0 (266-1), 8 (266-2), or 18 (266-3). (cont'd next page)

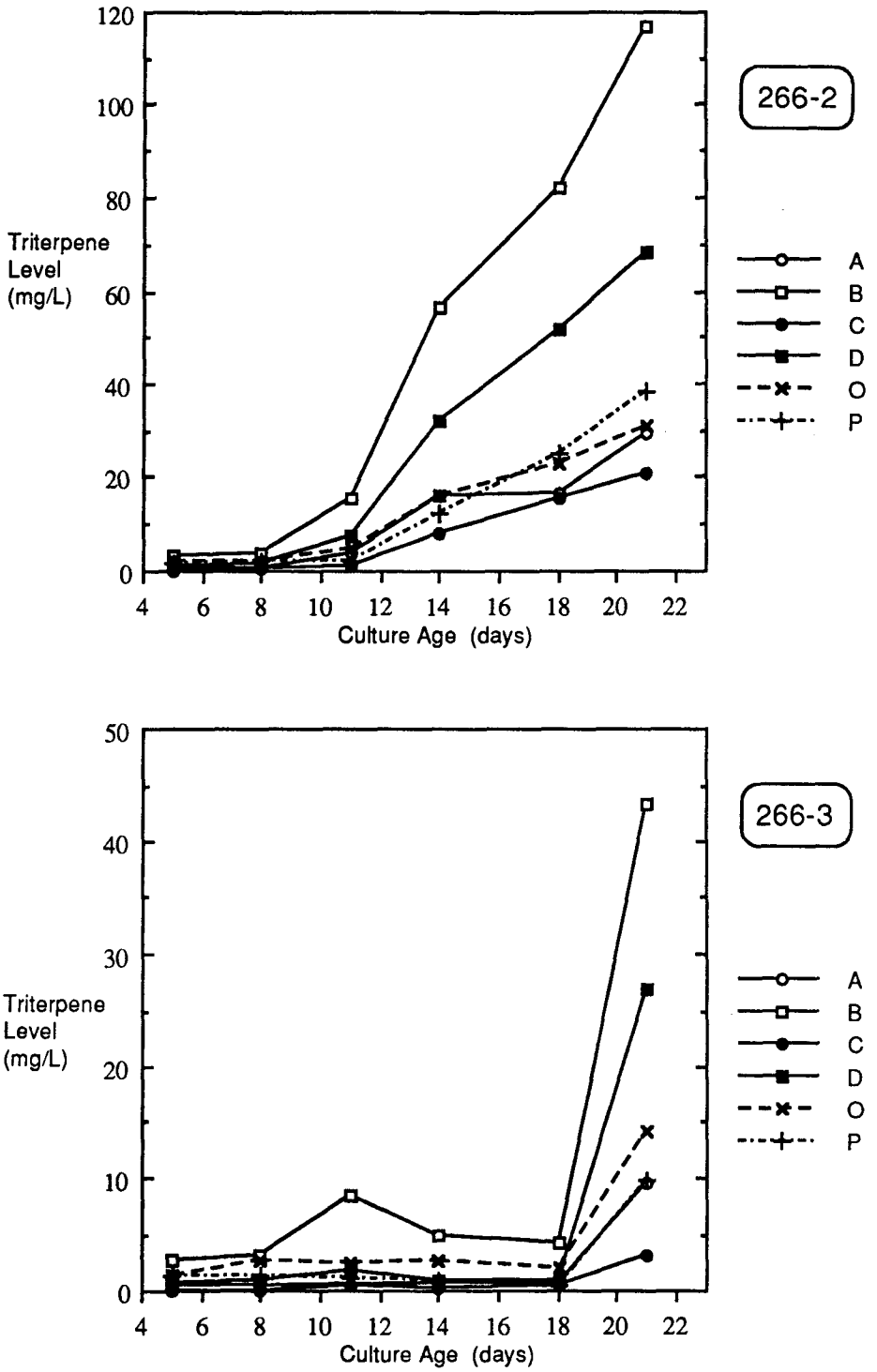


Figure 39 Triterpene Levels from Culture Series 266 Oleanane triterpenes A-D, oleanolic acid (O), polpunonic acid (P). Cultures are elicited with 1.0% *Botrytis* (v/v) on day 0 (266-1), 8 (266-2), or 18 (266-3).

(vii) Conclusions on Elicited Triterpene Production

The time course studies demonstrated that the maximum triterpene yield was obtained from cultures elicited at the beginning of the rapid growth phase. Elicitation with 1.0% *Botrytis* produced a steady increase in triterpene levels for at least six days, and usually until the end of culture growth. The growth time for the early elicited cultures ranged from 18 to 22 days. These culture parameters provide the highest yielding and most rapid biosynthetic production of the triterpene acids available.

The rate of triterpene production during the first 72 hours of elicitation varied between cultures. In some, a rapid and steady triterpene synthesis was initiated immediately after elicitation while in others an initially moderate synthesis rate accelerated after 72 hours. This variable time course of elicited triterpene production may have been a factor in the inconsistent elicitation results of culture series 252-259. An unpredictable delay before the induction of rapid triterpene synthesis made the time course analysis far more reliable than a single 72 hour harvest.

(viii) Further Studies on Triterpene Elicitation

Further studies of TRP 4a elicitation should first determine if freeze-dried preparations of *Botrytis* are effective as elicitors. Adding measured weights of dry fungus would allow a more precise control of elicitor levels. Experiments to date have used a *Botrytis* culture grown to the end of its growth phase, homogenized, sterilized and added in liquid form. As each new batch of *Botrytis* could have grown to a different final density, "1.0% *Botrytis*" was not an accurate statement of the elicitor content. To further refine the elicitations, the fungus could be fractionated to obtain the active principle.

The triterpene acids A, B, C and D have the same carbon skeleton and due to their similarity it is likely that they are formed from one precursor at a late stage in their biosynthesis.

Both epimers of the C_{22} hydroxyl group are present, the equatorial (α) hydroxyl of triterpenes B and D as well as the axial (β) hydroxyl of triterpenes A and C. This indicates that control of the C_{22} oxidation lacks the high degree of stereoselectivity present in most enzymatic conversions. Hydration of a planar carbocation would be a mechanism consistent with the observed ratio of products (B, D>>A, C) as the equatorial approach of water would be favoured over a hindered axial approach (inferred from the structures, figure 18).

In contrast, the C_3 triterpene alcohols produced by *Tripterygium* have only the equatorial (β) orientation. This is consistent with the biosynthesis of pentacyclic triterpenes from squalene-2,3-oxide[93, 86, which is in turn formed through the stereoselective oxidation of squalene. The cyclization of squalene-2,3-oxide to the C_3 equatorial alcohol is considered to be a final common step in the biosynthetic production of both the triterpene acids and the steroids.

In the laboratory, ketone acids A and B are converted completely to the dihydroxy acids C and D by treatment with sodium borohydride[25]. Conversely, the biosynthesis from squalene oxide would produce C and D initially followed by an oxidation to produce A and B. The time course of elicited triterpene production showed simultaneous formation of both C_3 oxidation levels. These studies could not determine whether the more rapid synthesis of C_3 ketones (A and B) over the C3 alcohols (C and D) was an indication of the actual order of biosynthetic production or a result of an equilibrium that favored the ketone form.

If the TRP 4a cells are capable of interconverting the C3 oxidation levels, it may be possible to influence the direction of the conversion. The enzymatic process would require a redox cofactor such as a nicotinamide or flavin nucleotide and the cellular pool of these cofactors can be driven (a) to the oxidized state through glycolysis inhibition in the presence of oxygen, or (b) to the reduced state by oxygen deprivation in the presence of sugars. The oxidized state would accumulate ketones A and B. This manipulation could be used to simplify the triterpene purification or to shift production towards a desired form of the triterpenes.

(ix) Error Analysis for the Determination of Elicited Triterpene Levels

Source of Error	Contribution to Reported Triterpene Levels	Magnitude
variance between successive cultures	increase or decrease	potentially large
peak overlap in GC chromatogram (series 261-266)	increase	30 %
chromatography losses (series 252-259)	decrease	10 - 20 %
GC analysis	increase or decrease	5 %

Table 13 Consistency in the Elicitation Results for the Triterpene Acids

The factors affecting the reproducibility of triterpene elicitations in successive cultures were ranked in the above order. Cultures elicited under identical conditions did not always produce similar triterpene levels. The main component of this variation was eliminated in experiments 261-266 by using a single line of TRP 4a inoculum. The remaining variation was attributed to spontaneous changes in the inoculum as well as in the *Botrytis* cultures.

While the triterpene ester peaks in the GC chromatogram of purified extracts were well resolved, the crude extracts analyzed for series 261-266 provided a less stable baseline with some peak overlap. A comparison of analysis results before and after purification (series 252-259) demonstrated that the crude extract triterpene levels were inflated by an average of 30 % (with a range of 0 - 80 %). For this reason, the crude extract analyses were only used in the time course studies where the relative levels from a single bioreactor could be compared.

Losses of 10 - 20 % during silica gel chromatography (culture series 252-259 only) were determined from the mass balance in column separations of the partially purified triterpene esters. The losses included irreversible binding to the silica gel as well as lactonization of the triterpenes B and D. These losses subtracted from the elicitation response as the elicited culture extracts were purified and not the control culture extracts.

The GC analysis techniques provided a high level of accuracy as the esterification proceeded cleanly and the extracts were assayed until an agreement of ± 5% was reached. Usually this was accomplished in two to three injections.

(V) THE NATURAL PRODUCTS ENHANCED BY ELICITATION

The majority of compounds observed in extracts of elicited cultures were triterpenes. These included (in the order of silica gel elution) tingenone (66), triterpene B lactone (wilforlide B, 55), a mixture of sitosterols, triterpene D lactone (wilforlide A, 56), polpunonic acid (62), oleanolic acid (49), celastrol (68), salaspermic acid (63) and the triterpene acids A, B, C and D (51, 52, 53 and 54). All of these compounds except salaspermic acid had been observed in $MSNA_{0.5}K_{0.5}$ cultures of TRP 4a by Malcolm Roberts[25], and were identified by TLC and GC comparisons with pure standards as well as by NMR, IR and mass spectroscopy. Salaspermic acid had been previously observed only in whole plant extracts[55]. The purification and identification of the above compounds, save the triterpenes A-D, was performed by Mrs. Huifen Gu in this laboratory (unpublished). The quinone methide triterpenes tingenone and celastrol were produced by elicited cultures but were completely absent from control culture extracts. The same was true of salaspermic acid, which was elicited to approximately the same levels as polpunonic acid. This was reasonable as the two compounds are closely related friedelane triterpenes. The GC analysis could not resolve salaspermic acid from the large group of early eluting compounds and thus it was not assayed in the elicitation experiments.

Oleanane triterpenes have often been isolated as water and methanol soluble glycoside derivatives (attachment at the C3 hydroxyl)[87-91]. Using the established conditions for hydrolysis, samples of methanol extracts of culture series 261 and 266 were treated with aqueous H_2SO_4 under reflux. This process caused considerable lactonization of triterpenes B and D but the triterpene assay was not increased, indicating that the triterpene diols, C and D, were not stored as glycosides by the TRP 4a cultures. Triterpene glycosides may be present in the parent plant, *Tripterygium*, since its aqueous extracts are active as medicinal tonics.

While the elicitation study has achieved good results with the production of triterpenes, conditions are still being sought for the elicitation of the diterpene compounds triptolide and tripdiolide. A screening for fungal elicitors and growth media that will support this process is planned for future studies.

(VI) PURIFICATION OF TRITERPENES A, B, C AND D

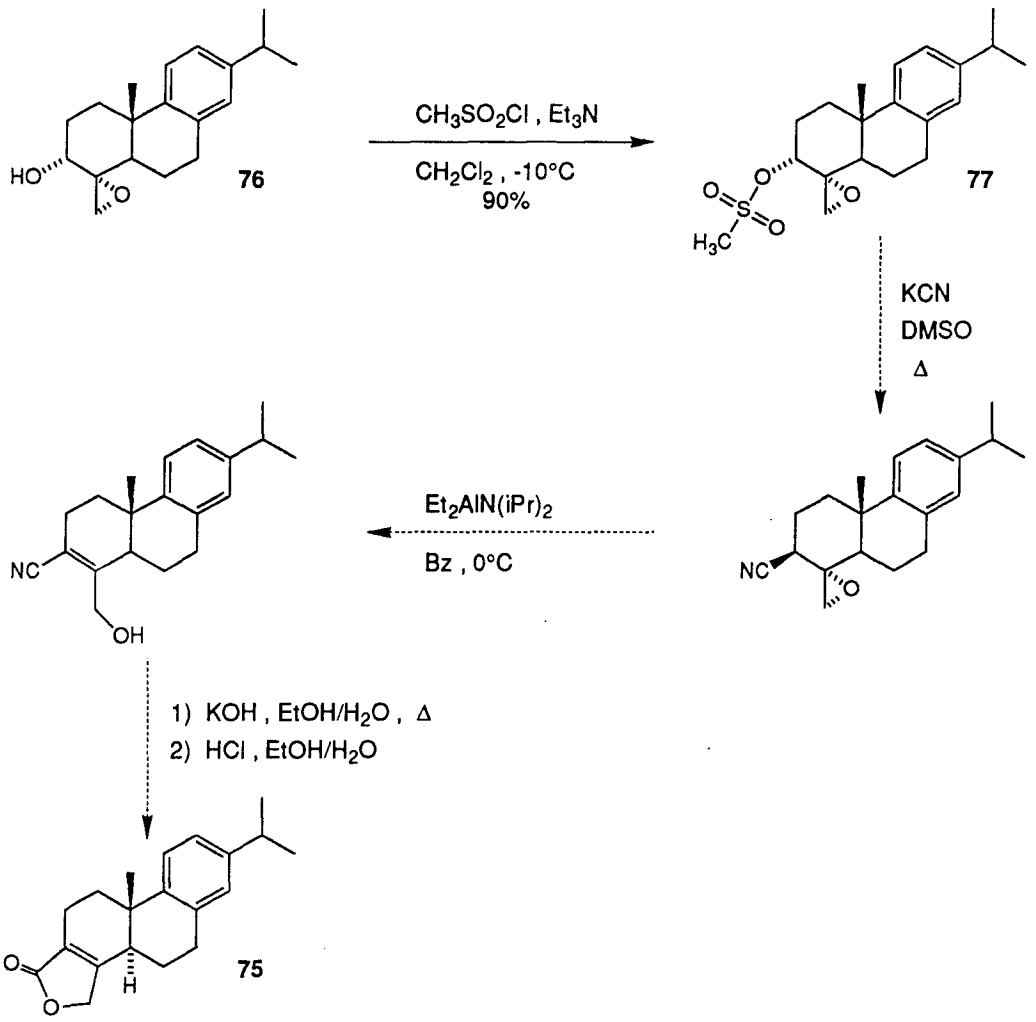
To accumulate the triterpene acids, ethyl acetate extracts of elicited cultures from series 261, 262 and 266 were combined with the non-esterified portions of series 252-259 extracts. The pooled extract was first subject to anion exchange chromatography in methanol / pyridine which separated an acid fraction. The acids were further purified using silica gel column chromatography, [methylene chloride / methanol / acetic acid (100:2:1)], and final separation of the acids required columns eluted with 3% methanol in chloroform and with hexanes / ethyl acetate / methanol / acetic acid (65:35:1:1).

Triterpene acids B and D were dissolved in methanol and recrystallized from methanol / water (4:1). Triterpene acids A and C were moistened with a few drops of methanol which allowed dissolution in methylene chloride. Recrystallization then proceeded following the partial evaporation of solvent.

Recrystallization provided 600 mg each of the triterpene acids B and D and 150 mg each of the triterpene acids A and C, pure by GC and TLC. The bulk of the triterpene acids remained in mixed column fractions and crystallization liquors (crystallization occurred in low yields and was successful only with fairly pure samples). The pure triterpene acids were set aside for pharmacological testing in other groups equipped for such determinations.

Elicitation had stimulated triterpene production but did not increase production of the triptolides. The most promising avenue to the triptolides remained the incorporation of synthetic precursors into the biosynthetic pathway used by *Tripterygium* cultures. To this end we undertook the synthesis of the butenolide 75 . With the ring A functionality of triptolide in place, there was potential that this compound was identical to one of the natural biosynthetic intermediates and that triptolide producing cultures (or enzyme preparations from these cultures) could be used to complete the synthesis through oxidations at rings B and C. For this study it was essential to insert an isotope into the skeleton of the precursor so that successful incorporation into the triptolide product could be verified. A reasonable site for isotope replacement was the carbonyl carbon of the butenolide which was the only additional carbon required in the synthesis from *l*-dehydroabietic acid (45). The addition of a ^{14}C-nitrile at carbon *3* introduces a label in the desired position and at the appropriate oxidation level for hydrolysis to the butenolide product.

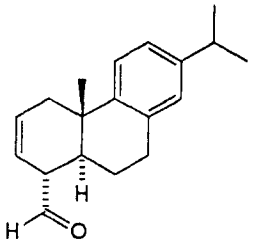
In my research project, C_3 nitrile synthesis was attempted using derivatives of the epoxy-alcohol 76 by a substitution with potassium cyanide (scheme I). Epoxidation was used to temper the reactivity at C_3 after all attempts at derivatization or displacement of the allylic hydroxyl had yielded the diene or products of allylic rearrangement[25,97]. The alcohol 76 was converted into the stable mesylate 77 in high yield. If this compound incorporated cyanide at C_3, the planned route to butenolide 75 (scheme I) continued with an epoxide opening to the allylic alcohol[100]. On treatment with hydroxide, a ready formation of imino-lactone was expected due to the proximity of the hydroxyl to the nitrile. Hydrolysis to the butenolide would be carried out in mild aqueous acid.



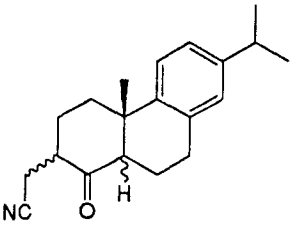
Scheme I

This sequence was checked at the cyanide substitution where no reaction occurred at room temperature and unwanted products were formed after heating (60°-80°C). The polar solvent DMSO had been selected for this reaction as as it supported a high concentration of potassium cyanide and could not supply protons to promote addition to the epoxide. The addition of HMPA to the reaction mixture did not alter the outcome nor did the addition of tetra-n-butyl ammonium iodide or replacement of DMSO with dimethyl formamide. Treatment of the mesylate in acetonitrile or benzene with a KCN-dicyclohexyl-18-crown-6 complex gave no reaction after 16 hours under reflux.

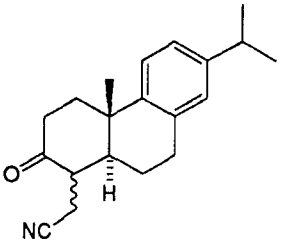
An examination of the KCN/DMSO reaction was carried out using 300 mg of the mesylate and 3 equivalents of KCN under mild reaction conditions (60°C for 24 hours). The elimination product 78 was recovered in 23% yield (it had formed a greater proportion of the product at higher temperatures) while 31% of the starting material remained unreacted. The remainder of the starting material had been converted into three nitrile products. One was isolated in a pure form (79 , 6%) while the other two could not be resolved (80 , 81; 35%). These compounds were isomers of each other and of the nitriles a and b produced in a mixture by a previous synthesis*.



78



79, 80, 81



a , b

The nitriles 79, 80 and 81 had identical mass spectra and produced the same parent mass (M^+) and ($M^+ - CH_3$) peaks formed by the nitriles a and b . All of the nitriles produced identical infrared spectra with the definitive stretches for nitrile (med, 2240 cm^{-1}) and ketone (str, 1720-1710 cm^{-1}). The isomers were readily distinguished using NMR spectroscopy with five unique sets of signals clearly present. The integration of axial methyl signals provided an estimate of the isomeric ratio in mixed nitrile fractions:

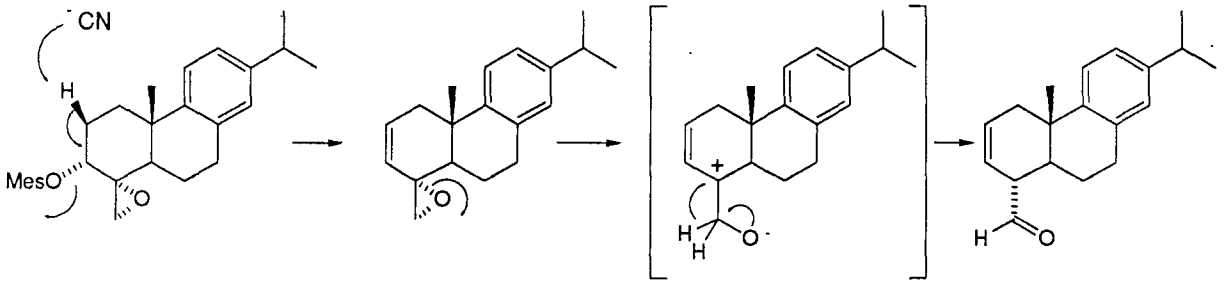
Compound	δ of axial methyl (ppm)	isomer ratio
79	1.39	---
80 , 81	1.31 / 1.03	1 : 2
a , b	1.45 / 1.23	2 : 1

Demers[101] had demonstrated that a transition from the A/B-trans to the A/B-cis ring junction moves the axial methyl shift downfield (probably due to phenyl ring re-orientation). This effect could not be used to assign the structures of the nitrile isomers as it was observed that

* prepared by Malcolm Roberts[25] through conjugate addition to the α-methylenecyclohexanone 85.

the a , b mixture contained one isomer with a downfield methyl shift in the presence of an A/B-trans ring junction. Structural assignment of the nitriles using NMR was therefore not straightforward; the shift of the axial methyl signals resulted from the combined anisotropic shielding influences of the carbonyl, nitrile and aromatic ring orientations.

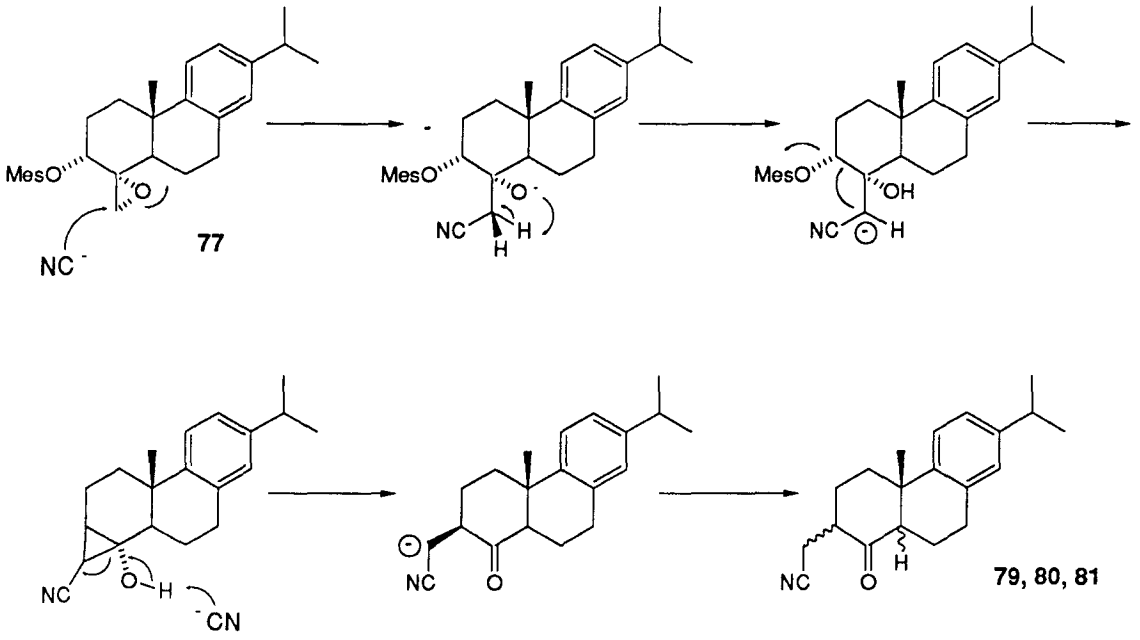
The formation of aldehyde 78 through elimination was expected as Malcolm Roberts[25] had observed this product in a similar reaction. The mechanism of its production is almost certainly an initial elimination followed by a pinacol-type rearrangement of the Δ-2,3-epoxide:



77 78

The formation mechanism for the nitrile products 79, 80 and 81 is less clear; I have proposed one possible route that allows a migration of acetonitrile from C_4 to C_3 (see below). While C_3 of the mesylate is likely to be the most electrophilic carbon, the exocyclic methylene is certainly more accessible to cyanide. Addition to the epoxide generates the powerfully basic alkoxide with the correct geometry to allow proton abstraction α to the nitrile. S_N2 substitution to form a cyclopropane would then be facile due to the antiperiplanar relationship of the carbanion to the mesylate leaving group*. After this, ring opening through base-catalyzed collapse of the tertiary alcohol forms the resonance stabilized α-nitrilate anion. Proton transfer to either of two enolate isomers followed by the aqueous work-up allows a maximum of four nitrile isomers; three of these were observed in the product . The A/B-trans-C_3 α compound is likely the missing isomer as it is the only isomer that cannot adopt a chair conformation with the C_3 substituent equatorial.

* A cyclization analogous to epoxide formation from an α-bromohydrin.

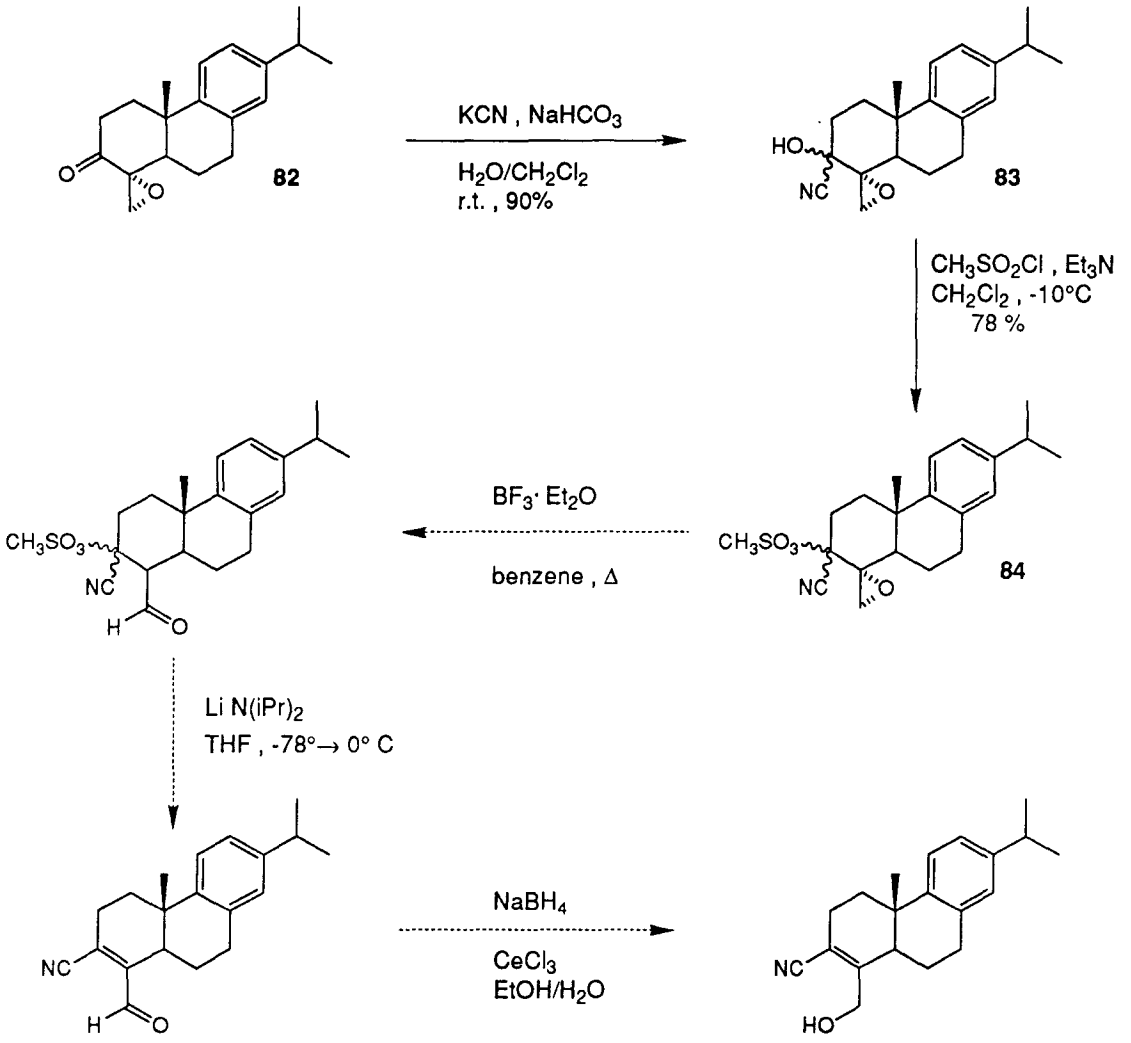


An alternate strategy was formation of the triflate derivative of epoxy-alcohol 76 to encourage cyanide attack at C_3. Although stable epoxy-triflates have been reported[102], all attempts at triflation of 76 (triflic anhydride, pyridine, dichloromethane, -78°→ 0°C) led to the formation of many products. This was not altered by the addition of KCN/crown ether to the reaction mixture at low temperature. Cyanide substitution of epoxy-alcohol derivatives was not continued beyond this point.

While cyanide could not be incorporated (at C_3) through substitution, it was found to be readily incorporated through addition to the carbonyl of epoxy-ketone 82 (scheme II). Cyanohydrin formation[103] was accomplished in a two-phase mixture of dichloromethane and water containing KCN and sodium bicarbonate*. The reaction produced a mixture of the cyanohydrin 83 with 5-10 % of the unreacted ketone. The cyanohydrin was stable in neutral and mildly acidic solutions but reverted to the ketone in the presence of bases and during silica gel chromatography.

* functioning here as a weak proton donor

A mesylate (84) was readily prepared in 70% yield from ketone. The strategy at this point was induction of the pinacol-type rearrangement of epoxide to aldehyde followed by elimination of the mesylate group and selective reduction to the allylic alcohol appearing in scheme I.



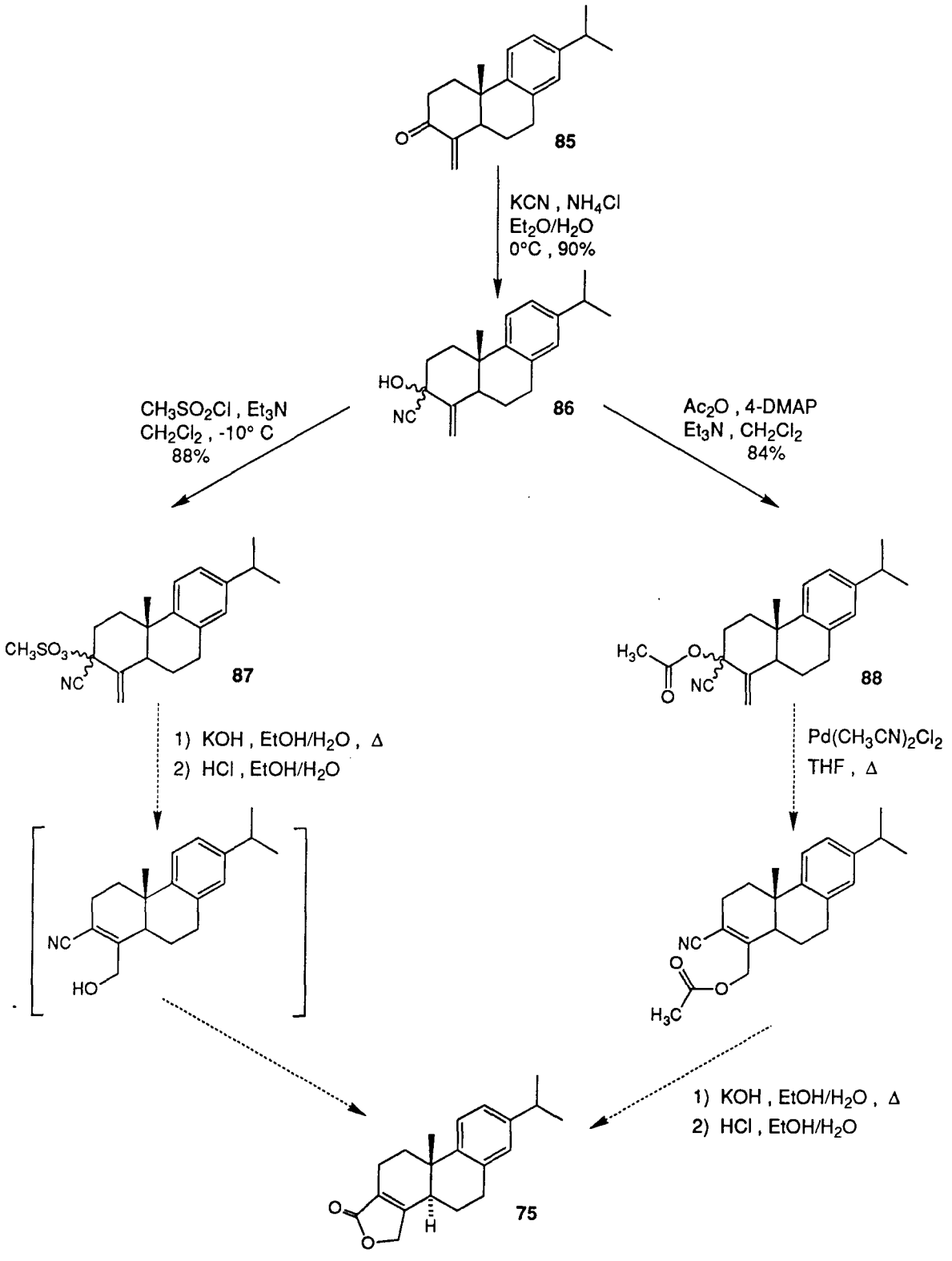
Scheme II

The rearrangement of 84 was attempted using excess boron trifluoride etherate in benzene[104]. No reaction was observed at room temperature or after 24 hours of at 40°C. Heating to reflux caused decomposition of the starting material to many products. The lack of epoxide opening was attributed to the presence of electron withdrawing groups adjacent to the epoxide which would suppress carbocation formation. The route was not continued beyond this point.

The formation of an epoxy-cyanohydrin was the first successful incorporation of cyanide at C_3. Using this addition strategy, another route to the butenolide was explored. The synthesis of allylic cyanohydrin 86 was attempted as this compound would allow several alternate approaches to the butenolide (scheme III).

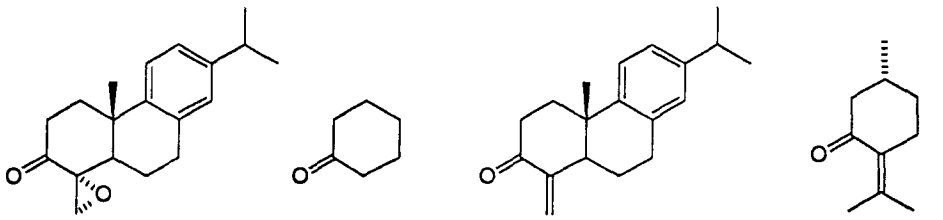
The first step of scheme III, formation of cyanohydrin 86 from the methylene-cyclohexanone 85 , was accomplished in high yield. While KCN· crown ether in DMF favoured the conjugate addition product[25], a repetition of the two-phase cyanide addition (KCN, $NaHCO_3$, H_2O, CH_2Cl_2) produced a small quantity of cyanohydrin (with a majority of the starting material unreacted). When sodium bicarbonate was replaced with the more acidic ammonium chloride and ether used instead of dichloromethane[105] a 90% conversion to cyanohydrin was achieved with no conjugate addition product. A high concentration of cyanide was essential for high yield and KCN was used at 3 equivalents with respect to ketone.

The presence of water may bring about 1,2-addition through several influences. As the hydrated cyanide ion is a much less powerful nucleophile than the "naked" cyanide produced by KCN· crown ether in aprotic solvents[106], it is likely that conjugate addition can occur much less readily in the aqueous system. Electron withdrawal by oxygen creates a higher density of positive charge at C_3 than at the exocyclic methylene thus if conjugate addition is not rapid, ionic attraction can draw the cyanide anion towards a 1,2-addition. This becomes very relevant in the presence of water as the accumulation of positive charge at the carbonyl carbon is expected to be increased due to a delocalization of oxy-anion charge resulting from hydrogen bonding.


85
KCN , NH4Cl
Et2O/H2O
0°C , 90%
HO
NC
86
CH3SO2Cl , Et3N
CH2Cl2 , -10° C
88%
Ac2O , 4-DMAP
Et3N , CH2Cl2
84%
CH3SO3
NC
87
H3C
O
NC
88
1) KOH , EtOH/H2O , Δ
2) HCl , EtOH/H2O
Pd(CH3CN)2Cl2
THF , Δ
NC
HO
NC
O
O
H3C
1) KOH , EtOH/H2O , Δ
2) HCl , EtOH/H2O
O
O
H
75

Scheme III

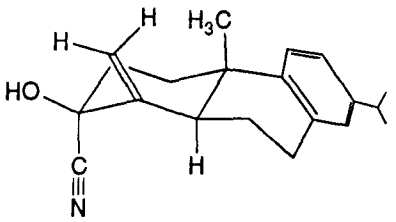
Another reason for the exclusive 1,2-addition of aqueous cyanide may be an incomplete conjugation in the enone system of 85, increasing the energy required for 1,4-addition. This was inferred from the infrared spectrum where the carbonyl stretching frequency was not low enough to have been produced by a fully conjugated enone. The following compounds illustrate the effect of π-overlap on the carbonyl infrared absorbance:



82	cyclohexanone	**85**	(+)-pulegone
1715 cm^{-1}	1710 cm^{-1}	1695 cm^{-1}	1675 cm^{-1}

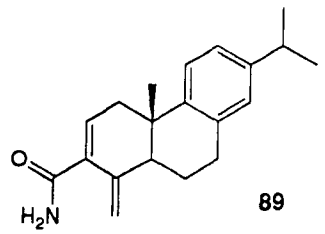
The enone 85 falls mid-way between the standard absorbance of ketone and enone. Decreased conjugation most likely indicates that the enone system is not planar (allowing less π-overlap). This is likely a result of torsional forces from the rigid A/B-trans ring junction which tend to drive the A-ring towards a chair conformation with the carbonyl directed downward. As well as suppressing congugate addition, this shields the upper face of the carbonyl with the methylene group so as to allow cyanide attack only from the lower face. (A boat conformation may also be available to the A-ring in which the upper face of the carbonyl is brought up close to an axial methyl, again allowing cyanide approach only from below (from the convex face)).

The proton NMR spectrum revealed that the cyanohydrin 86 was formed as a single isomer. An epimer would have been readily detected as the nitrile group exerts strong anisotropic shielding that would alter the A-ring shifts. Unfortunately, there was no readily available method to determine which isomer had been produced. Small nucleophiles are known to favour an axial approach to cyclohexanones which, as 85 , lack axial substitution in the *3* position[107]. At this point I will assume an axial attack by cyanide (with ring A in the chair conformation) to yield the α–nitrile. This assignment receives support from the reactivity of several cyanohydrin derivatives that will be discussed shortly.



The α-Nitrile Cyanohydrin

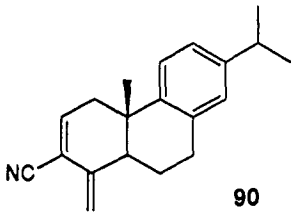
In the first strategy to butenolide (scheme III), the cyanohydrin mesylate 87 was prepared in 88% yield from the cyanohydrin. The allylic mesylate was considered an ideal candidate for S_N2' displacement[108] with an oxygen nucleophile to produce an allylic alcohol that would hydrolyze to the butenolide. Treatment with potassium hydroxide in tetrahydrofuran at room temperature for 60 minutes caused complete conversion to a more polar (TLC) compound. While the mesylate group was clearly missing (NMR and IR spectra) this was not the expected product. It was assigned this structure:



89

The NMR spectrum revealed 3 vinyl protons, two from the exocyclic methylene (δ 5.85 and 5.21 ppm, doublets, J = 1 Hz; coupling removed through irradiation of either signal) and the third attributed to the C_2 proton (δ 5.37 ppm, broad singlet). The amide (N-H) protons were revealed as a broad singlet (δ 4.38 ppm, 2H) which was removed by deuterium exchange. In the infrared spectrum the N-H stretching frequencies characteristic of amides were observed (3590, 3495 and 3400 cm^{-1}) along with a strong amide carbonyl stretch (1644 cm^{-1}).

The S_N2' displacement with mesylate 87 was then attempted using less basic oxygen nucleophiles. Reaction at room temperature with: (i) cesium acetate in DMF[109], (ii) cesium acetate in acetone, (i) and(ii) containing potassium iodide or (iii) sodium acetate in acetic acid led to a recovery of starting material. When these reaction mixtures were heated the major product



90

formed was the nitrile 90 which resulted from elimination. This compound was not converted to the amide 89 in KOH/THF, indicating that amide formation involved nitrile hydrolysis prior to the elimination of the mesylate group.

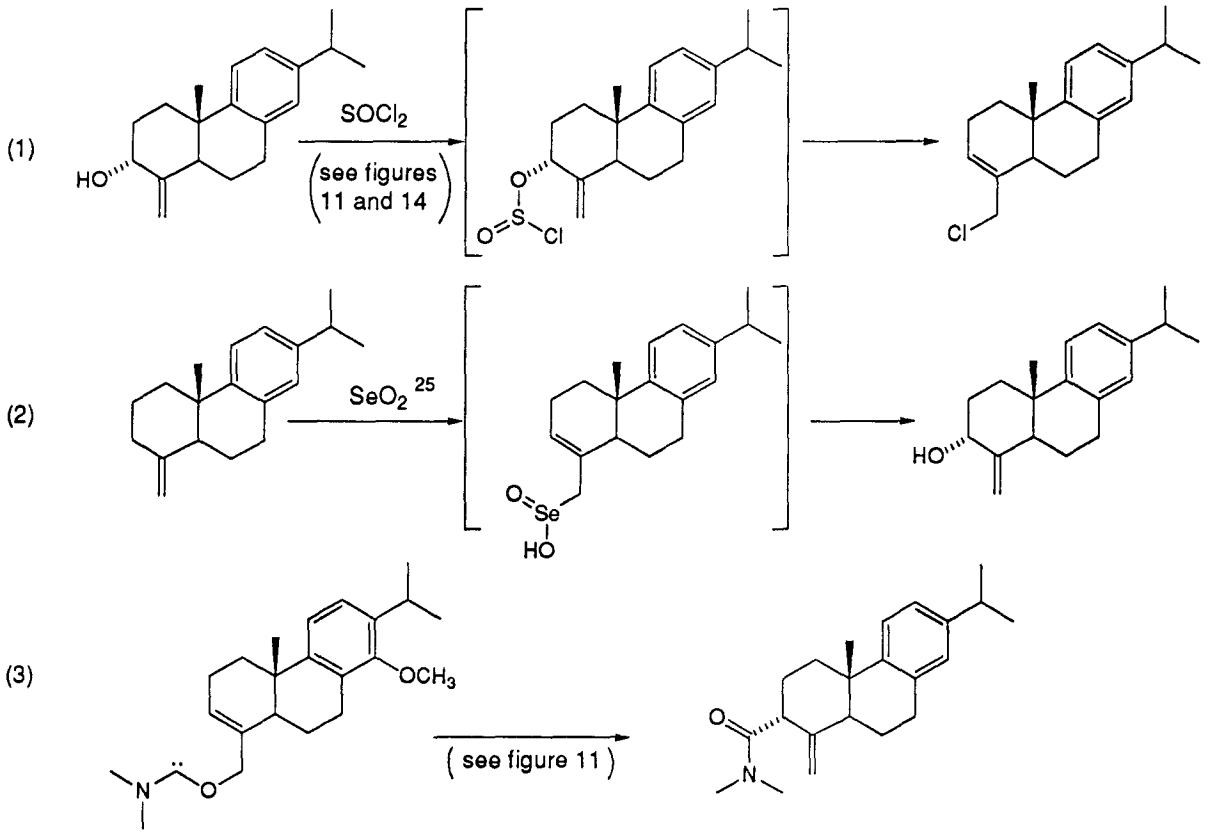
While the route was halted here, a conjugate addition to C_2 (hydride or hydroxyl or a Birch reduction) followed by epoxidation of the exocyclic olefin and then epoxide opening with base would produce an allylic alcohol similar to the originally desired intermediate of scheme III. Subsequently, nitrile hydrolysis would yield the butenolide.

As the S_N2' reaction with an external nucleophile was unsuccessful, a second pathway involving intramolecular 3,3-rearrangement was attempted. This route hinged on activation of the exocyclic olefin with a bis-acetonitrile palladium (II) chloride complex. Palladium catalysts have been used to initiate rearrangement of many allylic acetates[110] including α-cyanoallylic acetates[111]. The reactions had shown poor results with β-substituted allyl systems, but it was expected that conjugation with the nitrile would drive a conversion to the rearranged product. Cyanohydrin 86 was converted to a stable acetate 88 in high yield, but when the acetate was treated with the palladium complex no rearrangement was observed (reflux in dry THF under argon). A similar type of rearrangement was attempted through treating the free cyanohydrin with thionyl chloride in ether. No reaction took place at room temperature and heating caused a decomposition to many products (including the ketone precursor 85). This was somewhat surprising in light of the high yielding rearrangement of the corresponding hydroxy-acid methyl ester under the same conditions which was reported by Tokoroyama et al.[98] (fig. 14, route A, p. 55). The stereochemistry of this hydroxy-ester was not determined and the compound may have been inverted at C_3 with respect to cyanohydrin 86.

The lack of facile allylic rearrangement provided evidence towards the assignment of the cyanohydrin as the α-nitrile isomer. Studies of many rearrangements have indicated that allylic

displacements are favoured by a syn-diaxial relationship of nucleophile and leaving group[110,108]. In the cyanohydrin 86 , the A-ring is conformationally rigid with the C_3-β substituent equatorial and the C_3-α substituent axial. While the equatorial bond lies on the same plane as the exocyclic methylene, the axial bond is at an angle of just over 90°. Some rearrangement would therefore have been expected if the cyanohydrin contained an α (axial) hydroxyl.

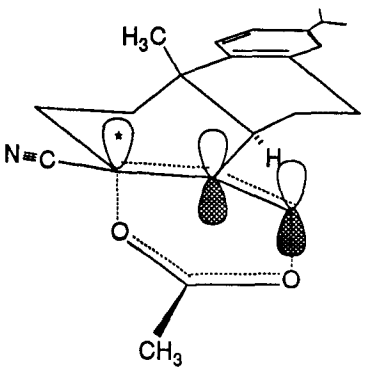
Allylic rearrangement between C_3 and the exocyclic methylene is well precedented for this ring system in cases involving an axial C_3 substituent. The following reactions illustrate this process:



In each of these examples an axial C_3 bond is formed or broken. Although reactions (2) and (3) are reversals of the attempted cyanohydrin rearrangement and are 2,3 rather than 3,3 processes, a cyclic transition state is still involved and a lack of β-addition to C_3 indicates that an equatorial bond is not reactive in the rearrangements. In these two examples, free rotation of the methylene substituent provided equal access to the upper and lower faces of the Δ 3,4-olefin

plane yet the migrating groups moved exclusively into the more hindered axial position. As steric factors were not responsible for the geometry of approach, the most probable explanation of the observed results is that the rearrangements were governed by electronic factors.

If the cyanohydrin had been formed with the β-nitrile geometry, an acetate derivative would be expected to rearrange through the cyclic intermediate pictured below.

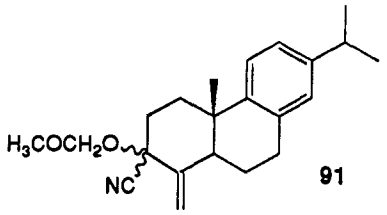


Of particular import is the alignment between the vacant σ* orbital of the C_3 - oxygen bond and the occupied π orbital of the exocyclic olefin. This should allow electron donation from the olefin to C_3 to form the 2,3-π bond of the rearranged product. This orbital alignment is minimal in the α-nitrile epimer, providing an explanation of why this compound would not readily undergo allylic rearrangement.

The same electronic factors would govern the $S_{N}2'$ reaction as the geometry of the leaving group rather than that of the approaching nucleophile is attributed with control over the reactivity. The observed lack of allylic rearrangement is the basis on which the nitrile was assigned to the α position in cyanohydrin 86 . The experimental results support these mechanistic considerations which indicate that the allylic rearrangement of cyanohydrin derivatives can not be used to access the butenolide product.

At this point, the most promising route remaining was through the dienes 89 or 90 that were formed from elimination of the allylic cyanohydrin mesylate 87 . This strategy was outlined earlier and remains to be explored. The synthesis of tripdiolide (31) had been carried out by Tokoroyama et al. using a similar diene intermediate (figure 14, route B, p. 55).

One attempt to gain more substantial proof of the stereochemistry of the allylic cyanohydrin 86 involved synthesis of the methoxymethyl derivative 91 . The reaction proceeded in 92% yield using methylal (formaldehyde dimethyl acetal) and the acid catalyst phosphorus pentoxide (P_2O_5)[112]. This compound was subject to NOE experiments with irradiation of the methylene signals of the protecting group. No enhancements were observed and thus no information was revealed with regards to the position of the oxygen substituent. Careful crystallization of one of the derivatives would allow an X-ray analysis for conclusive structural determination. The allylic cyanohydrin mesylate would be suitable as it recrystallized fairly readily.

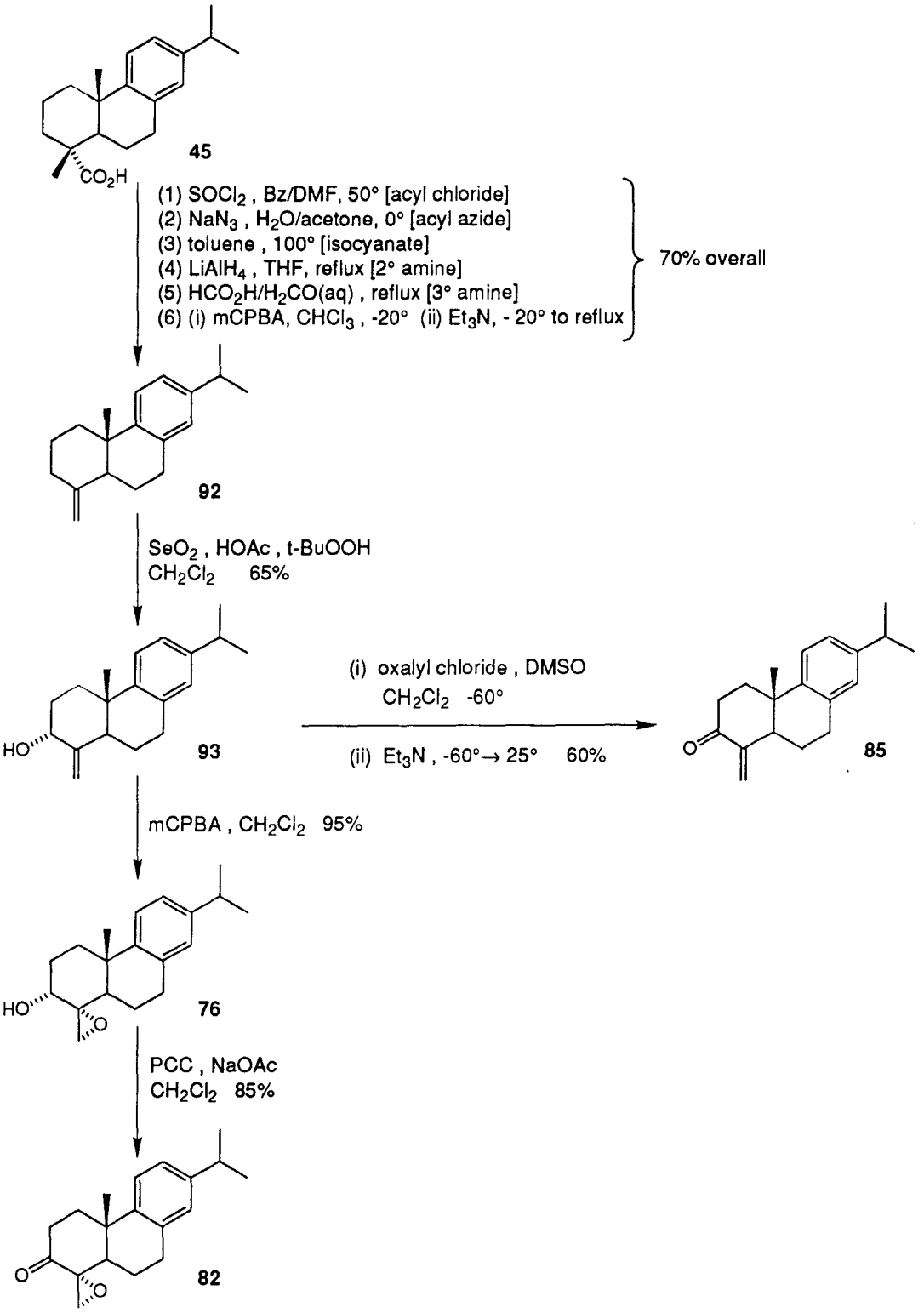


91

Origin of the Starting Materials

The starting materials for these syntheses were prepared as illustrated in scheme IV. The yields are reported for purified products. Using the methods of vanTamelen and Demers et al.[96,101], dehydroabietic acid (45) was purified from mixed resin acids through recrystallization of the ethanolamine salt; the exocyclic olefin 92 was obtained from the regenerated acid through Curtius rearrangement and Cope elimination. Further elaborations were carried out as reported by Malcolm Roberts[25]. Allylic oxidation (Sharpless) of 92 produced the α-alcohol 93*. Treatment of this alcohol with peroxy-acid produced a single epoxide 76 which had been demonstrated to be the α isomer through NOE enhancements between the axial methyl and the exocyclic methylene protons. A mild oxidation (Corey) then produced a fair yield of the epoxy ketone 82 . The allylic alcohol 93 was oxidized to the methylenecyclohexanone 85 using the Swern oxidation. The enone was stable for several weeks at -20°C but decomposed to many products after a few days at room temperature.

* In the NMR spectrum the C_3 proton produces a broad singlet, $w_{1/2}$ = 5 Hz, (δ 4.37 ppm) indicating its equatorial position which is β on C_3.


45
CO2H
(1) SOCl2 , Bz/DMF, 50° [acyl chloride]
(2) NaN3 , H2O/acetone, 0° [acyl azide]
(3) toluene , 100° [isocyanate]
(4) LiAlH4 , THF, reflux [2° amine]
(5) HCO2H/H2CO(aq) , reflux [3° amine]
(6) (i) mCPBA, CHCl3 , -20° (ii) Et3N, - 20° to reflux
70% overall
92
SeO2 , HOAc , t-BuOOH
CH2Cl2 65%
HO
93
(i) oxalyl chloride , DMSO
CH2Cl2 -60°
(ii) Et3N , -60°→ 25° 60%
O
85
mCPBA , CH2Cl2 95%
HO
O
76
PCC , NaOAc
CH2Cl2 85%
O
O
82

Scheme IV

EXPERIMENTAL

(I) IDENTIFICATION OF TRP 4A METABOLITES

^{1}H NMR spectra were recorded with a Bruker WH 400 spectrometer (400 MHz). Chemical shifts are reported in ppm relative to tetramethyl silane. Infrared spectra were recorded with a Perkin Elmer 710B infrared spectrometer from KBr pellets or from 5% solutions (sample minus reference). Ultraviolet spectra were recorded with a Unicam SP 800B spectrophotometer. Melting points were taken with a Reichert apparatus (heated stage) and are uncorrected. Electron impact low and high resolution mass spectra were recorded with a Kratos MS 50 spectrometer . (the Kratos MS 80 and 902 were also used for low resolution spectra). Elemental analyses were carried out in this department by Mr. P. Borda.

Silica gel column chromatographies were run with open or low pressure glass columns using Merck silica gel 60, 230-400 mesh. BDH Omnisolve™ grade solvents and BDH reagent grade hexanes were used for elution. Routine TLC analysis was carried out with Merck silica gel plates (0.2 mm, F-254, glass backed) sprayed with molybdic acid and heated at 110°C for visualization. A special colour reagent was used to visualize triterpenes: the developed plates were coated first with a spray of 30% conc. H_2SO_4 in glacial acetic acid and second with a spray of 5% anisaldehyde in isopropanol and were heated at 110°C for 5 minutes. This produced different colours for the various triterpenes (Appendix IV).

(i) Metabolite Production from Cell Cultures

Cell culture methods were developed by Gary Hewitt of the Biological Services section of our department. Mr. Hewitt supervised the growth of all cultures with the assistance of Mr. David Chen, Ms. Elizabeth Bugante and myself. TRP 4a suspension cultures[71] were grown in 500 ml stocks on a rotary shaker or in 12 L glass/stainless steel air-lift bioreactors. Cultures were raised in the dark at 26°C using a liquid PRL-4 medium with 2.0 mg/L 2,4-diclorophenoxyacetic acid and 10 % v/v coconut milk (from freshly picked young coconuts with husks). Cultures were

initiated with an 8% inoculum (v/v). Growth was monitored through the refractive index of samples removed at 72 hour intervals (Galileo refractometer, 25.0°C). The pH and microscopic purity were also determined from these samples.

For the fungal elicitor, a culture of *Botrytis* sp. (PRL# 2042) was grown on solid nutrient agar (OB5) in Roux bottles. The mycelial mass was suspended in Aerosol OT solution (450 µl/L, Fisher Scientific) and fragmented aseptically with an Ultra Turrax tissue homogenizer (28,000 rpm for 30 seconds, F25 head). The homogenate was used to inoculate fresh PRL-4 liquid medium and the culture was grown to maturity at 26°C on a rotary shaker(130-140 rpm, 7-10 days). At harvest, the culture was treated with the tissue homogenizer for 5 minutes and then autoclaved at 121°C for 20 minutes. The elicitor preparation was stored frozen at -20°C until needed and was added to TRP 4a cultures at 1.0 ml/L (0.1%), 10.0 ml/L (1.0%) or 50.0 ml/L (5.0%).

TRP 4a cultures were harvested rapidly by a mild suction filtration using miracloth (Calbiochem) followed by a rinse with distilled water (10% v/v). Fresh cells and medium were frozen immediately and stored at -20°C. The medium was freeze-dried in the bulk trays of an FTS Systems apparatus.

Prior to metabolite extraction, cells were thawed and vacuum filtered with Whatman #1 paper. The cell solid was extracted through suspension in ethyl acetate (at least an equal volume) and treatment with the tissue homogenizer for 2 minutes, followed by vacuum filtration. This was repeated three times. The cell liquid was extracted four times with ethyl acetate. The freeze-dried medium was also extracted four times after it was dissolved in a small volume of distilled water. The cells and medium were made slightly acidic (pH 4) by the addition of citric acid before extraction. This was done to insure complete protonation of the organic acids and was not done with samples assayed for tripdiolide, a compound degraded by acid. In the liquid-liquid extractions, emulsions were broken by Celite addition and vacuum filtration or by the addition of solid NaCl. All ethyl acetate extracts were washed with water then brine, followed by a drying

over anhydrous sodium sulphate. Solvent removal was accomplished by rotary evaporation and the extracts were stored for 24 hours under high vacuum.

(ii) Tripdiolide Analysis

The tripdiolide assay published by this group[99] was used for determinations. Seven samples, accurately diluted in ethyl acetate, were spotted onto Eastman/Kodak Chromagram sheets (plastic backed silica gel, 20 cm x 20 cm, stock #13181) at 2 cm intervals. Each plate held four extract samples which alternated with tripdiolide standards (2.0, 4.0 and 6.0 μg, recrystallized from ethanol/water 4:1). After development with 4% methanol in chloroform, the plates were air-dried then sprayed evenly with 5% v/v ceric sulphate in 10% aqueous H_2SO_4. Heating for exactly 5 minutes at 110°C produced faint brown spots at the location of tripdiolide ; most other compounds appeared as faint purple spots. A horizontal band, 1 cm x 20 cm, was centered on the tripdiolide spots and masked off with black vinyl tape. Fluorescence within the band was then recorded (30 minutes after the plates had been heated) using a Turner fluorometer fitted with a Camag plate scanner. The analysis was repeated with more dilute extract samples when peak integrations exceeded the range of the three point calibration generated by the tripdiolide standards on each plate.

(iii) Triterpene Analysis

Methyl esters were prepared from carboxylic acids by dissolving a culture extract or pure acid in methanol, cooling to 0°C with stirring and adding diazomethane in ether. Diazomethane was prepared from Diazald™ according to the Aldrich method supplied with the reagent. Diazomethane was added in ten-fold excess or until bubbling had ceased. The samples were covered and stirred for one hour and then a second addition was made to insure that excess diazomethane was present. The solvent and reagent were allowed to evaporate overnight in the fume hood and the samples were dried by rotary evaporation followed by 24 hours in high vacuum.

The treated samples were purified by silica gel column chromatography using a step-gradient of ethyl acetate in toluene (1:19 → 1:3). This provided the fractions for the gas chromatography analysis. Further purification of the esters could be carried out using columns eluted with toluene/ethyl acetate 4:1. The pure esters were obtained by recrystallization from methanol/water 4:1.

For the GC calibration, pure ester samples were accurately diluted with ethyl acetate containing 1.000 mg/ml methyl cholate. (Cholic acid was treated with diazomethane and recrystallized from ethanol/water.) Calibration factors were calculated from the linear response range of serial dilutions using the methyl cholate as an internal standard. The extract samples, accurately diluted with ethyl acetate, were injected in 5.00 µl volumes Dilutions were often necessary to bring sample concentrations within the calibrated range.

Analysis was carried out with the HP 5840A gas chromatograph fitted with a Hewlett Packard HP 1 column (12m x 0.2mm i.d. x 0.5µm film thickness; cross-linked dimethyl polysiloxane gum). The elution was isothermal at 280°C with a 290°C cleaning ramp after each analysis. Flame ionization detection was used (300°C). The injector was set at 300°C and the carrier gas was helium.

The retention times for all compounds analyzed are listed in Appendix V. Triterpene B and D were determined from the sum of the lactone and the ester peak integrations.

(iv) Isolation of Triterpene Acids A, B, C and D

In the initial stage of triterpene acid purification, acid fractions were obtained from crude culture extracts through anion exchange. This was carried out using BioRad AG-1-X4 resin (OH^- form) in a 3 cm x 30 cm glass column. Samples were added in methanol (5% pyridine), washed with methanol (5% toluene) and eluted with 0.5 M formic acid in methanol (5% toluene). The eluant fractions were concentrated by rotary evaporation, diluted with ethyl acetate, washed with water, dried with brine and Na_2SO_4, concentrated again and dried *in vacuo*. Pure triterpene acids were obtained from the acid fractions using silica gel column chromatography followed by recrystallization. The methods that were used are described in the discussion section (VI).

22β-Hydroxy-3-oxoolean-12-en-29-oic acid (51):

Triterpene A. Recrystallized from CH_2Cl_2 / CH_3OH. m.p. 267-270°C. UV(CH_3OH) λ_{max} 217 (ε 775). IR ($CHCl_3$; cm^{-1}): 3615, 2937, 2640, 1698, 1463, 1368, 1217, 724. ^1H NMR ($CDCl_3$) δ: 0.91 (3H, s), 1.04 (3H, s), 1.07 (3H, s), 1.09 (3H, s), 1.11 (3H, s), 1,41 (3H, s), 0.85-2.35 (m), 2.39 (1H, ddd, J=4,6,16 Hz), 2.55 (1H, ddd, J=4,11,16 Hz), 3.48 (1H, s, OH), 3.58 (1H, dd, J=3.6,7 Hz), 5.33 (1H, t, J=3.5 Hz). LRMS m/z (rel intensity): 470 (3.8, M^+), 452 (7.0), 437 (2.7), 426 (4.4), 424 (5.5), 408 (15.7), 391 (3.2), 340 (11.1), 325 (4.1), 264 (43.7), 246 (67.8), 217 (79.9), 205 (57.3), 201 (38.3), 189 (46.0), 171 (44.5), 159 (42.7), 147 (55.9), 135 (82.3), 119 (95.1), 107 (90.5), 81 (89.8), 69 (71.4), 55 (100), 43 (53.4). Elemental Analysis, calc. for $C_{30}H_{46}O_4$: C 76.55, H 9.85; found: C 76.02, H 9.97 .

22α-Hydroxy-3-oxoolean-12-en-29-oic acid (52):

Triterpene B. Recrystallized from CH_3OH / H_2O. m.p. 287-289°C. UV(CH_3OH) λ_{max} 212 (ε 1882). IR ($CHCl_3$; cm^{-1}): 3617, 2976, 2630, 1698, 1461, 1386, 1219, 1027, 910, 715. ^1H NMR ($CDCl_3$) δ: 1.02 (3H, s), 1.04 (3H, s), 1.07 (3H, s), 1.08 (3H, s), 1.10 (3H, s), 1.18 (3H, s), 1.29 (3H, s), 1.27-2.29 (m), 2.38 (1H, ddd, J=4,6,16 Hz), 2.55 (1H, ddd, J=4,11,16 Hz), 3.60

(1H, dd, J=4,12 Hz), 5.29 (1H, t, J=3.5 Hz). LRMS m/z (rel intensity): 470 (0.6, M^+), 452 (11.8), 437 (2.0), 408 (1.5), 391 (1.3), 353 (0.6), 340 (0.5), 326 (1.7), 299 (1.2), 285 (2.2), 264 (3.7), 246 (100), 228 (12.2), 218 (25.1), 205 (35.8), 201 (20.0), 185 (20.9), 173 (14.2), 159 (16.9), 145 (23.6), 131 (31.0), 119 (38.0), 107 (29.3), 95 (34.3), 81 (22.1), 69 (14.4), 55 (24.6), 43 (21.7). Elemental Analysis, calc. for $C_{30}H_{46}O_4$: C 76.55, H 9.85; found: C 76.40, H 9.90 .

3β,22β-Dihydroxyolean-12-en-29-oic acid (53):

Triterpene C. Recrystallized from CH_2Cl_2 / CH_3OH. m.p. 282-284°C. UV(CH_3OH) λ_{max} 216 (ε 840). IR (KBr pellet; cm^{-1}): 3470, 3421, 2976, 2640, 1698, 1471, 1393, 1298, 1240, 1048, 1032, 1005. ^1H NMR (C_5D_5N) δ: 0.76 (3H, s), 0.85 (6H, s), 1.02 (3H, s), 1.03 (3H, s), 1.08 (3H, s), 1.63 (3H, s), 0.65-2.63 (m), 3.22 (1H, dd, J=6,9.5 Hz), 3.80 (1H, d_{broad}, J=4.5 Hz), 5.21 (1H, t, J=3 Hz). LRMS m/z (rel intensity): 472 (6.1, M^+), 454 (5.0), 439 (2.6), 426 (2.0), 411 (2.1), 393 (1.5), 264 (99.7), 246 (53.0), 231 (15.4), 217 (100), 207 (59.9), 190 (45.2), 175 (39.3), 161 (22.7), 147 (37.9), 135 (74.1), 119 (49.9), 107 (48.2), 95 (52.4), 81 (49.9), 69 (48.6), 55 (48.7), 43 (30.7). Elemental Analysis, calc. for $C_{30}H_{48}O_4 \cdot H_2O$: C 73.43, H 10.27; found: C 73.53, H 10.40 .

3β,22α-Dihydroxyolean-12-en-29-oic acid (54):

Triterpene D. Recrystallized from CH_3OH / H_2O. m.p. 292-295°C. UV(CH_3OH) λ_{max} 212 (ε 1617). IR (KBr pellet; cm^{-1}): 3475, 3387, 2946, 2550, 1698, 1467, 1384, 1235, 1038. ^1H NMR (C_5D_5N) δ: 0.98 (3H, s), 1.03 (3H, s), 1.04 (3H, s), 1.23 (3H, s), 1.28 (3H, s), 1.37 (3H, s), 1.58 (3H, s), 0.82-2.73 (m), 3.42 (1H, dd, J=6,10 Hz), 4.01 (1H, dd, J=5.5,13 Hz), 5.38 (1H, t, J=3 Hz). LRMS m/z (rel intensity): 472 (0.2, M^+), 454 (2.6), 436 (1.7), 421 (1.3), 410 (0.6), 393 (1.2), 342 (1.1), 325 (0.6), 314 (0.9), 299 (2.0), 264 (2.7), 246 (100), 218 (21.6), 207 (18.1), 201 (15.5), 190 (35.6), 175 (20.3), 159 (14.8), 145 (20.1), 131 (22.5), 119 (32.2), 107 (23.2), 95 (25.9), 81 (17.7), 69 (12.2), 55 (14.7). Elemental Analysis, calc. for $C_{30}H_{48}O_4$: C 76.23, H 10.23; found: C 76.10, H 10.33 .

(II) SYNTHETIC REACTIONS TOWARD 18(4→3)-ISODEHYDROABIETENOLIDE

Chromatography and spectroscopy as described in section I. Dry solvents (BDH Omnisolve™ or freshly distilled) were stored over 4Å sieves while pyridine and triethylamine were stored over KOH pellets. Reagents were from Aldrich save potassium cyanide (BDH analaR™), methane sulphonyl chloride (BDH, 98%) and crude (l)-dehydroabietic acid (ICN). Solvent removal was carried out by rotary evaporation followed by a period of at least 16 hours in a high vacuum desiccator. Phosphate buffer (0.50 M, pH 7.0) was prepared by the addition of KOH to a solution of KH_2PO_4 in glass-distilled water.

3α-methane sulphonatyl-4(19)α-epoxy-18-norabieta-8,11,13-triene (77)

Triethyl amine (415 µl, 2.98 mmol) and methane sulphonyl chloride (130 µl, 1.68 mmol) were added to a stirred solution of epoxy-alcohol 76 (425 mg, 1.48 mmol) in dry dichloromethane (8 ml) with stirring under argon on an ice/salt bath. After 20 minutes the excess reagent was quenched with phosphate buffer (pH 7) The reaction mixture was diluted with ether (50 ml) and extracted with phosphate buffer, water and brine then dried over sodium sulphate. After solvent removal the product precipitated from a 1:1 mixture of hexanes and benzene to provide the mesylate 77 (485 mg, 1.33 mmol, 90%) as a white powder. Melting point 121°-123°C (decomp.). IR ($CHCl_3$; cm^{-1}): 2950 (str), 2860 (sh), 1502 (w), 1355 (str, O-S-O), 1180 (str, O-S-O), 1117 (med), 940 (str), 908 (med). ^1H NMR ($CDCl_3$) δ: 7.21 (1H, d, J= 8 Hz), 7.04 (1H, d, J= 8 Hz), 6.95 (1H, s), 4.42 (1H, t, J= 2.5 Hz), 3.10 (3H, s), 2.95 (1H, d, J= 4 Hz), 2.88 (3H, mult), 2.79 (1H, d/d, J= 4,1 Hz), 2.52 (1H, d/d, J= 13,2 Hz), 2.29 (1H, d/mult, J= 14 Hz), 2.21 (1H, d/mult, J= 14 Hz), 2.11 (1H, d/mult, J= 14 Hz), 1.97 (1H, t/d, J= 13,5 Hz), 1.68 (1H d/mult, J= 13 Hz), 1.43-1.30 (1H, mult), 1.24 (6H, d, J= 7 Hz), 1.17 (3, s). LRMS m/z (rel intensity): 364 (6, M^+), 349 (0.2), 268 (48), 253 (76), 237 (33), 222 (41), 211 (39), 193 (51), 181 (100), 164 (61), 141 (53), 129 (44), 115 (41), 91 (31), 77 (24), 55 (20), 43 (66). Elemental Analysis, calc. for $C_{20}H_{28}O_4S$ (364.50) : C 65.90, H 7.74; found: C 65.84, H 7.60 .

Cyanide Addition

Mesylate 77 (300 mg, 0.823 mmol) was dissolved in dry dimethyl sulphoxide (3 ml). Finely powdered potassium cyanide (163 mg, 2.50 mmol) was added and the mixture was heated on an oil bath (60°- 70°C) with stirring under nitrogen for 48 hours. The yellow solution was diluted with ether (200 ml) and extracted with water and brine then dried over sodium sulphate. Solvent removal gave 290 mg of a brown oil. Column chromatography using ethyl acetate (10) / chloroform (10) / hexanes (80) provided aldehyde 78 (52 mg, 0.19 mmol, 23%, a colourless oil which decomposed in air to a dark mixture beyond several days), a mixture of nitriles and unreacted 77 (92 mg, 0.25 mmol, 31 %). The nitrile mixture was partially resolved by column chromatography using ethyl acetate (7.5%) in a 1:1 mixture of toluene and hexanes to provide 79 (15 mg, 0.055 mmol, 6 %) and a mixture of 80 and 81 (85 mg, 0.288 mmol, 35%) which precipitated together from chloroform/hexanes.

19-norabieta-2,8,11,13-tetraen-18-al (78)

IR (CCl_4; cm^{-1}): 2960 (str), 2910 (str), 2850 (str),1722 (med),1500 (w),1460 (med),1380 (med),1353 (med),1282 (w),1120 (str),1080 (med). ^1H NMR ($CDCl_3$) δ: 9.57 (1H, d, J= 4 Hz), 7.21 (1H, d, J= 8 Hz), 7.05 (1H, d, J= 8 Hz), 6.93 (1H, s), 6.02 (1H, mult), 5.62 (1H, d/mult, J= 10 Hz), 2.85 (4H, mult), 2.61 (1H, d/d, J= 18, 6 Hz), 2.25 (1H, d/mult, J= 18 Hz), 2.05 (1H, t/d, J= 12, 3 Hz), 1.78 (1H, mult), 1.70 (1H, mult), 1.24 (6H, d, J= 7 Hz), 1.15 (3H, s). LRMS m/z (rel intensity): 268(55, M^+), 253(79), 239(16), 235(24), 225(30), 223(28), 211(34), 193(47), 186(72), 181(100), 177(51), 171(45), 164(60), 159(25), 152(22), 141(44), 129(44), 115(41), 105(32), 91(44), 83(85), 77(29), 65(14). 55(31), 43(75). HRMS, calc. for $C_{19}H_{24}O$: 268.1827 ; found: 268.1828 .

Nitrile 79

Yellow oil. IR (CCl_4; cm^{-1}): 2955(str), 2860(sh), 2240(med), 1740(sh), 1710(str), 1617(w), 1503(med), 1456(str), 1428(med), 1380(med), 1352(w), 1335(w), 1310(med), 1243(med), 1185(w), 1105(med), 1076(med), 918(med), 897(med), 832(med). ^1H NMR ($CDCl_3$) δ: 7.12

(1H, d, J= 8 Hz), 6.97 (1H, d, J= 8 Hz), 6.88 (1H, s), 3.10 (1H, quint/mult, J= 9 Hz), 2.81 (1H, sept, J= 7 Hz), 2.75-2.64 (4H, mult), 2.48 (1H, t, J= 3 Hz), 2.34 (1H, quar/mult, J= 7 Hz), 2.21-2.09 (2H, mult), 1.98 (2H, t/mult, J= 13 Hz), 1.39 (3H, s, H_3C-20), 1.33 (1H, d/d, J= 13, 2 Hz), 1.21 (6H, d, J= 7 Hz). Calc. for $C_{20}H_{25}ON$: 295.4236 .

Nitriles 80 , 81

White solid, melting point 139°- 142°C. IR ($CHCl_3$; cm^{-1}): 2995(sh), 2950(str), 2842(sh), 2240(med), 1710(str), 1605(w), 1500(med), 1465(med), 1419(med), 1384(med), 1105(w), 1068(w), 897(w), 830(med). ^1H NMR ($CDCl_3$) δ: 7.21 (d, J= 8 Hz), 7.19 (d, J= 8 Hz), 7.07 (d, J= 8 Hz), 7.05 (d, J= 8 Hz), 6.96 (s), 6.94 (s), 2.97-2.68 (mult), 2.65 (d/d, J= 12, 2 Hz), 2.58-2.42 (mult), 2.29-2.15 (mult), 2.10-1.96 (mult), 1.95-1.71 (mult), 1.31 (1H, s, H_3C-20), 1.25 (d, J= 7 Hz), 1.24 (d, J= 7 Hz), 1.03 (2H, s, H_3C-20). LRMS m/z (rel intensity): 295(36, M^+), 280(100), 262(14), 253(13), 238(38), 221(17), 213(29), 202(9), 193(23), 179(39), 169(22), 155(21), 141(45), 128(47), 115(40), 91(24), 77(24), 55(24), 51(16), 43(56). Calc. for $C_{20}H_{25}ON$: 295.4236 .

3-hydroxy-4(19)α-epoxy-18(4→3)-abeoabieta-8,11,13-triene-18-nitrile (83)

Aqueous potassium cyanide (3 ml, 373 mg KCN, 5.73 mmol) containing sodium bicarbonate (480 mg, 5.71 mmol) was added to a rapidly stirred solution of epoxy-ketone 82 (400 mg, 1.41 mmol) in dichloromethane (12 ml) at room temperature. A 95% conversion (TLC) was achieved after 3 hours and the mixture was taken up in ether (100 ml) and extracted with water and brine then dried over sodium sulphate. Solvent removal gave 410 mg of a white solid which was 90% cyanohydrin 83 and 10% starting material (NMR). The mixture was not purified as exposure to silica gel caused a reversion to ketone 82 . IR ($CHCl_3$; cm^{-1}): 3410(med, broad), 2940(str), 2850(sh), 1717(med), 1610(w), 1500(med), 1459(med), 1381(med), 1363(med), 1110(str, broad), 987(med), 925(med), 885(med), 827(med). ^1H NMR ($CDCl_3$) δ: 718 (1H, d, J= 8 Hz), 7.05 (1H, d, J= 8 Hz), 6.96 (1H, s), 3.17 (1H, d, J= 4 Hz), 3.10 (1H, s, D_2O exch.), 2.92-2.83 (3H, mult), 2.81 (1H, d, J= 4 Hz), 2.42 (3H, d/mult, J= 11 Hz), 2.07 (1H, t/d, J= 14, 4 Hz), 1.95 (1H, t/d, J=

14, 4 Hz), 1.78 (1H, d/mult, J= 12 Hz), 1.48-1.35 (1H, mult), 1.23 (6H, d, J= 7 Hz), 1.15 (3H, s). LRMS as starting material. Calc. for $C_{20}H_{25}O_2N$: 311.423 .

3-methane sulphonatyl-4(19)α-epoxy-18(4→3)-abeoabieta-8,11,13-triene-18-nitrile (84)

Triethyl amine (340 µl, 2.44 mmol) and methane sulphonyl chloride (130 µl, 1.67 mmol) were added to a stirred solution of cyanohydrin 83 (250 mg, 0.803 mmol, containing additional ketone 82) in dry dichloromethane (10 ml) with stirring under argon on an ice/salt bath. After 2 hours the excess reagent was quenched with phosphate buffer (pH 7) The reaction mixture was diluted with ether (100 ml) and extracted with phosphate buffer, water and brine then dried over sodium sulphate. Solvent removal gave 312 mg of a yellow oil and chromatography in ethyl acetate(2) / toluene(98) provided the mesylate 84 (244 mg, 0.626 mmol, 78%) as a white solid. IR (CCl_4; cm^{-1}): 2944(str), 2905(sh), 2850(sh), 1607(w), 1500(med), 1450(med), 1418(med), 1380(str), 1332(med), 1190(str), 1080(med), 1070(med), 1020(med), 962(str), 920(med), 888(med), 855(med). ^1H NMR ($CDCl_3$) δ: 7.17 (1H, d, J= 8 Hz), 7.06 (1H, d, J= 8 Hz), 6.97 (1H, s), 3.26 (1H, d, J= 4 Hz), 3.23 (3H, s), 2.98 (1H, d/mult, J= 13 Hz), 2.94-2.87 (3H, mult), 2.86 (1H, d, J= 4 Hz), 2.50-2.35 (3H, mult), 1.96 (1H, t/d, J= 13, 4 Hz), 1.80 (1H, d/mult, J= 13 Hz), 1.48-1.36 (1H, mult), 1.24 (6H, d, J= 7 Hz), 1.19 (3H, s). LRMS m/z (rel intensity): 389(24, M^+), 374(35), 344(4), 295(43), 293(22), 280(70), 278(100), 262(7), 248(28), 236(46), 220(21), 206(55), 193(28), 179(26), 165(27), 141(27), 128(32), 117(32), 97(52), 91(31), 76(23), 43(74). Calc. for $C_{21}H_{27}O_4NS$: 389.51 .

3-hydroxy-18(4→3)-abeoabieta-4(19),8,11,13-tetraene-18-nitrile (86)

Aqueous solutions of potassium cyanide (2.5 ml, 1020 mg KCN, 15.7 mmol) and ammonium chloride (2.5 ml, 892 mg NH_4Cl, 16.7 mmol) were added to a rapidly stirred solution of enone 85 (1.40 g, 5.22 mmol) in diethyl ether (5.0 ml) with cooling on an ice bath. After 90 minutes the reaction mixture was taken up in ether (200 ml) and extracted with water and brine then dried over sodium sulphate. Solvent removal gave 1.54 g of white solid which was 90% cyanohydrin 86 and 10% starting material (NMR). The mixture was not purified as exposure to silica gel

caused a reversion to ketone 85 . IR (CCl_4; cm^{-1}): 3580(sh), 3405(str, broad), 2945(str), 2850(sh), 2205(w), 1720(w), 1684(med), 1653(w), 1612(w), 1500(str), 1460(med), 1378(med), 1184(w), 1124(med), 1075(med), 1007(w), 920(str), 828(med). ^1H NMR ($CDCl_3$) δ: 7.22 (1H, d, J= 8 Hz), 7.07 (1H, d, J= 8 Hz), 7.01 (1H, s), 5.48 (1H, d, J= 1 Hz), 5.04 (1H, d, J= 1 Hz), 3.05-2.93 (3H, mult), 2.88 (1H, sept, J= 7 Hz), 2.59 (1H, d/d, J= 11, 2 Hz), 2.43-2.35 (2H, mult), 2.14-2.00 (1H, mult), 1.98 (1H, d, J= 10 Hz), 1.94-1.82 (2H, mult), 1.27 (6H, d, J= 7 Hz), 1.02 (3H, s), D_2O exchange is not observed. LRMS as starting material. Calc. for $C_{20}H_{25}ON$: 295.42 .

3-methane sulphonatyl-18(4→3)-abeoabieta-4(19),8,11,13-tetraene-18-nitrile (87)

Triethyl amine (1.12 ml, 8.46 mmol) and methane sulphonyl chloride (0.475 ml, 6.09 mmol) were added to a stirred solution of cyanohydrin 86 (1.00 g, 3.38 mmol, containing an additional quantity of ketone 85) in dry dichloromethane (20 ml) with stirring under argon on an ice/salt bath. After one hour the excess reagent was quenched with phosphate buffer (pH 7) The reaction mixture was diluted with ether (200 ml) and extracted with phosphate buffer, water and brine then dried over sodium sulphate. After solvent removal the product (1.41 g as an oil) was dissolved in chloroform (5ml). The addition of carbon tetrachloride (10ml) caused precipitation of the pure mesylate (0.561 g) as a white powder The remaining product was chromatographed with ethyl acetate (10%) in hexanes to provide a total of 1.11 g of mesylate 87 (2.97 mmol, 88%). Melting point 124°- 126°C (decomp.). IR ($CHCl_3$; cm^{-1}): 3000(sh), 2950(str), 2850(sh), 1645(w), 1604(w), 1497(med), 1457(med), 1417(med), 1375(str), 1332(sh), 1187(str), 1100(med), 1068(med), 1000(med), 960(str), 922(med), 828(med). ^1H NMR ($CDCl_3$) δ: 7.18 (1H, d, J= 8 Hz), 7.05 (1H, d, J= 8 Hz), 6.98 (1H, s), 5.56 (1H, s), 5.11 (1H, s), 3.29 (3H, s), 3.02-2.93 (2H, mult), 2.93-2.81 (2H, mult), 2.64 (1H, d/d, J= 11, 3 Hz), 2.41 (1H, d/t, J= 14, 3 Hz), 2.31 (1H, t/d, J= 14, 4 Hz), 2.00 (1H, t/d, J= 14, 4 Hz), 1.93-1.83 (2H, mult), 1.25 (6H, d, J= 7 Hz), 1.03 (3H, s). LRMS m/z (rel intensity): 373(13, M^+), 358(3), 279(20), 277(37), 262(78), 249(5), 234(32), 220(100), 199(20), 193(33), 186(12), 171(14), 143(15), 129(15), 117(14), 91(12), 79(7), 43(21). Elemental Analysis, calc. for $C_{21}H_{27}O_3NS$ (373.51): C 67.53, H 7.29, N 3.75; found: C 67.36, H 7.29, N 3.65 .

18(4→3)-abeoabieta-2,4,(19),8,11,13-pentaen-18-amide (89)

Potassium hydroxide (5% w/v in methanol, 0.5 ml, 0.45 mmol) was added to a solution of mesylate 87 (40 mg, 0.11 mmol) in THF (1.00 ml). The mixture was stirred at 22°C for one hour and was then acidified with 10% HCl_{aq} and extracted into ether (3 x 25 ml). The solvent was extracted with water and brine then dried over sodium sulphate. Solvent removal gave 35 mg of a more polar compound (TLC) as a brown oil. Preparative TLC using ethyl acetate (30) / toluene (70) provided 89 (22 mg, 0.074 mmol, 68%) as a colourless oil.

IR ($CHCl_3$; cm^{-1}): 3590(w), 3495(med, N-H stretch), 3400(med, N-H, stretch), 3350(sh), 2980(sh), 2900(str), 2845(sh), 1644(str, C=O stretch; amide), 1598(med), 1500(w), 1442(w), 1380(w), 1320(str), 1224(str), 1155(str), 1102(w), 1002(med), 967(w), 900(med), 832(med). ^1H NMR ($CDCl_3$) δ: 7.22 (1H, d, J= 8 Hz), 7.06 (1H, d, J= 8 Hz), 6.98 (1H, s), 5.85 (1H, d, J= 1 Hz), 5.37 (1H, s; broad; C_2-H), 5.21 (1H, d, J= 1 Hz), 4.38 (2H, s; broad; D_2O exchange; NH_2), 3.04-2.93 (2H, mult), 2.86 (1H, sept, J= 7 Hz), 2.54 (1H, d/t, J= 14,5 Hz), 2.48-2.39 (3H, mult), 2.04 (1H, d/mult, J= 13 Hz), 1.90-1.78 (3H, mult), 1.24 (6H, d, J= 7 Hz), 1.11 (3H, s). LRMS m/z (rel intensity): 220(0.3), 211(0.2), 205(1.5), 160(2), 149(0.6), 110(6), 95(11), 82(36), 67(52), 57(100), 43(57), 41(70). Calc. for $C_{20}H_{25}ON$: 295.42 .

18(4→3)-abeoabieta-2,4,(19),8,11,13-pentaene-18-nitrile (90)

Cesium acetate (412 mg, 2.14 mmol; from cesium carbonate and acetic acid[109])was added to a solution of the mesylate 87 (200 mg, 0.535 mmol) in dry DMF (9 ml). The mixture was stirred under nitrogen for 72 hours (room temperature) without reaction (TLC). When the mixture was heated to 60°C for 8 hours a new product was formed (less polar on TLC). The mixture was diluted with ether (150 ml) and extracted with water and brine then dried over sodium sulphate. Solvent removal gave a brown oil (190 mg). Preparative TLC using ethyl acetate (20) / hexanes (80) provided the unsaturated nitrile 90 (85 mg, 0.31 mmol, 57%) and unreacted starting material (75 mg, 37%). [Treatment of 90 with methanolic KOH in THF provided a mixture of two new compounds which were slightly less polar than the amide 89 (TLC).]

^{1}H NMR ($CDCl_3$) δ: 7.19 (1H, d, J= 8 Hz), 7.07 (1H, d, J= 8 Hz), 6.97 (1H, s), 6.71 (1H, d, J= 6 Hz), 5.58 (1H, s), 5.29 (1H, s), 2.98-2.82 (4H, mult), 2.56-2.47 (1H, mult), 2.43 (1H, d/d, J= 12,1 Hz), 2.15 (1H, d/t, J= 12,2 Hz), 1.78-1.66 (1H, mult), 1.25 (6H, d, J= 7 Hz), 1.02 (3H, s). LRMS m/z (rel intensity): 277(34, M^+), 262(40), 246(23), 234(24), 220(100), 204(12), 192(13), 186(18), 178(10), 171(25), 165(10), 153(8), 143(22), 129(22), 117(21), 115(20), 91(15), 77(11), 43(39). Calc. for $C_{20}H_{23}N$: 277.408 .

3-acetoxy-18(4→3)-abeoabieta-4(19),8,11,13-tetraene-18-nitrile (88)

Triethyl amine (0.283 ml, 2.03 mmol), acetic anhydride (0.287 ml, 3.05 mmol) and 4-dimethyl-aminopyridine (12 mg, 0.10 mmol) were added to cyanohydrin 86 (300 mg, 1.02 mmol; containing an additional quantity of ketone 85) in dry dichloromethane (5 ml) with stirring under argon on an ice bath. After 40 minutes the reaction was allowed to warm to room temperature and after a further 20 minutes the reaction mixture was quenched with aqueous phosphate buffer (pH 7). The mixture was extracted with ether (150 ml) and washed with phosphate buffer, water and brine then dried over sodium sulphate. Solvent removal gave 348 mg of a white solid which was chromatographed in ethyl acetate(15) / hexanes(85) to provide the acetate 88 (288 mg, 0.853 mmol, 84%). IR (CCl_4; cm^{-1}): 2950(str), 2860(sh), 1763(str), 1650(w), 1502(med), 1460(med), 1377(str), 1230(str), 1187(med), 1110(med), 1045(med), 1020(w), 930(med), 835(w). ^{1}H NMR ($CDCl_3$) δ: 7.19 (1H, d, J= 8 Hz), 7.05 (1H, d, J= 8 Hz), 6.98 (1H, s), 5.37 (1H, d, J= 1 Hz), 5.03 (1H, d, J= 1 Hz), 3.05-2.92 (2H, mult), 2.90-2.82 (2H, mult), 2.70 (1H, d/d, J= 12,1 Hz), 2.35 (1H, d/t, J= 13,3 Hz), 2.23 (3H, s, $COCH_3$), 2.04 (1H, t/d, J= 13,2 Hz), 1.98-1.82 (3H, mult), 1.24 (6H, d, J= 7 Hz), 1.02 (3H, s). Calc. for $C_{22}H_{27}O_2N$: 337.46 .

3-methoxymethoxy-18(4→3)-abeoabieta-4(19),8,11,13-tetraene-18-nitrile (91)

Phosphorus pentoxide (P_2O_5, 4.80 g, 33.8 mmol) was slowly added to a rapidly stirred solution of methylal (formaldehyde dimethyl acetal; 24 ml, 270 mmol) and cyanohydrin 86 (1.00 g, 3.38 mmol, containing additional ketone 85) in dry chloroform (20 ml) at room temperature under a blanket of argon. The mixture separated into an upper layer of pale yellow, transparent solution

and a lower layer of viscous red oil. The two phases were stirred together for five hours at which time the mixture was poured into an ice-cold solution of sodium carbonate (50 ml, 10% aqueous) with rapid stirring. This resulted in complete removal of the red material (P_2O_5/methylal). The chloroform was removed and the aqueous material extracted with ether (3x50 ml). The pooled solvent was extracted with water and brine then dried over sodium sulphate. Solvent removal gave 1.15 g of a yellow oil which was chromatographed with ethyl acetate(5) / hexanes(95) to provide the methoxymethyl ether 91 (1.057 g, 3.11 mmol, 92%) as a colourless oil.
IR (CCl_4; cm^{-1}): 2940(str), 1725(w), 1646(med), 1611(w), 1500(med), 1460(str), 1440(sh), 1378(med), 1302(w), 1218(med), 1182(str), 1120(str), 1057(sh), 1035(str), 982(med), 924(med), 897(med), 827(med). ^1H NMR ($CDCl_3$) δ: 7.20 (1H, d, J= 8 Hz), 7.05 (1H, d, J= 8 Hz), 6.98 (1H, s), 5.55 (1H, s), 5.14 (1H, d, J= 7 Hz, OCH_2O), 5.01 (1H, s), 5.00 (1H, d, J= 7 Hz, OCH_2O), 3.51 (3H, s), 3.02-2.95 (2H, mult), 2.86 (1H, sept, J= 7 Hz), 2.62 (1H, d/d, J= 10,3 Hz), 2.53 (1H, d/d, J= 9,3 Hz), 2.36 (1H, d/d, J= 9,3 Hz), 1.96 (2H, d, J= 10 Hz), 1.93-1.82 (2H, mult), 1.25 (6H, d, J= 7 Hz), 1.02 (3H, s). Calc. for $C_{22}H_{29}O_2N$: 339.45 .

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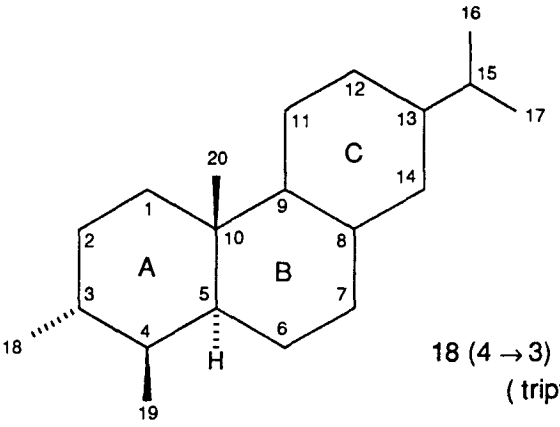
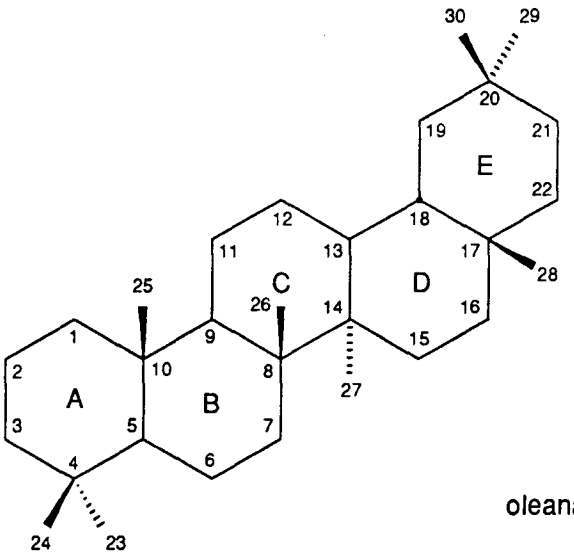
APPENDIX

APPENDIX I



18 (4 → 3) abeo-abietane
(triptolides)



oleanane

Terpene Skeleton Numbering

APPENDIX II

Age (d)	261-1	261-2	261-3	262-1	262-2	266-1	266-2	266-3
0	1.3371	1.3371	1.3371	1.3371	1.3371	1.3371	1.3371	1.3371
1								
2								
3								
4	1.3366	1.3367	1.3366					
5				1.3364	1.3363	1.3367	1.3363	1.3363
6								
7								
8				1.3360	1.3360	1.3362	1.3361	1.3361
9								
10	1.3358	1.3358	1.3359					
11						1.3358	1.3357	1.3354
12				1.3351	1.3349			
13	1.3352	1.3352	1.3351					
14						1.3353	1.3351	1.3348
15				1.3343	1.3338			
16								
17								
18				1.3334	1.3333	1.3344	1.3343	1.3335
19	1.3340	1.3339	1.3338					
20								
21						1.3336	1.3335	1.3332
22	1.3335	1.3335	1.3335					

Growth Curves for TRP 4-a in 12 L Aerated Bioreactors. $RI_{25°C}$ of filtered broth from experiments 266, 262, 261, 259, 258, 256, 255, 254, 252. Where not recorded, initial RI is estimated (1.3371). Double outlines mark the addition of *Botrytis*. [plotted in figure 17]

APPENDIX II (cont.)

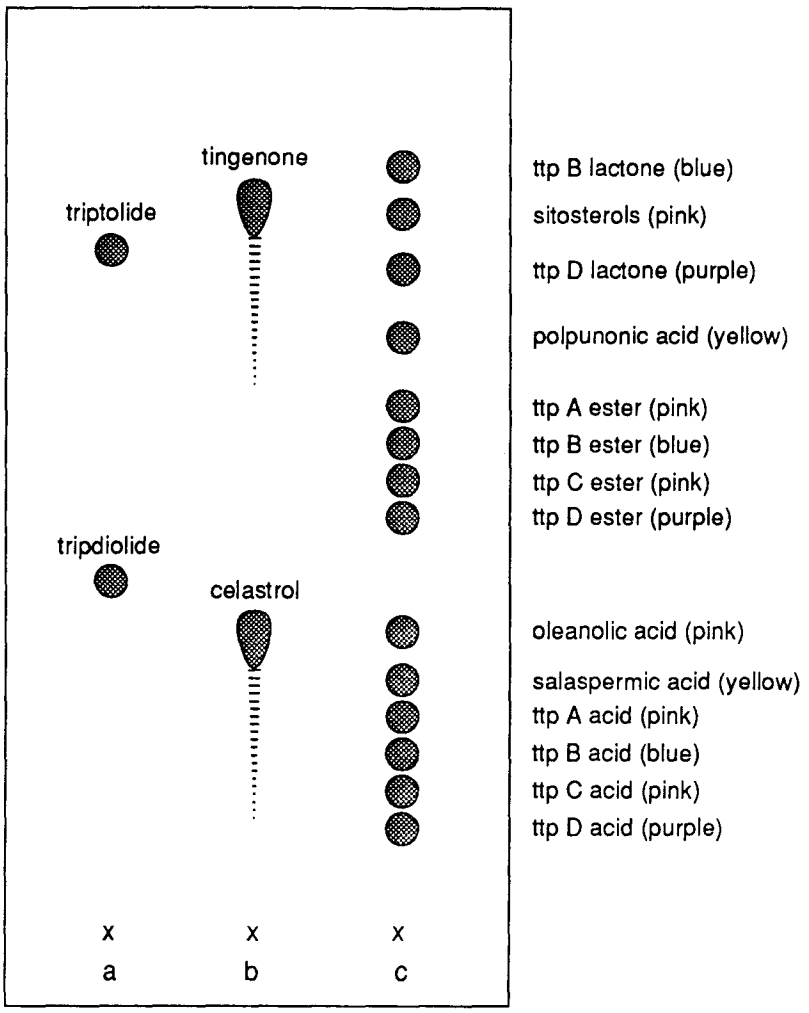
Age (d)	256-1	256-2	256-3	258-1	258-2	259-1	259-2	259-3
0	(1.3371)				1.3371	1.3371	1.3371	1.3371
1								
2								
3								
4								
5								
6						1.3363	1.3365	1.3364
7	1.3362	1.3364	1.3363					
8				1.3370				
9					1.3366			
10	1.3360	1.3360	1.3360			1.3360	1.3360	1.3360
11				1.3360	1.3360			
12								
13	1.3354	1.3353	1.3354			1.3355	1.3356	1.3358
14				1.3352	1.3352			
15				1.3351	1.3350			
16								
17	1.3348							
18				1.3348	1.3344			
19								
20	1.3339							
21					1.3340			
22	1.3334							
23								
24					1.3336			

APPENDIX II (cont.)

Age (d)	252-1	252-2	254-1	254-2	254-3	255-1	255-2	255-3
0	(1.3371)							
1								
2								
3								
4								
5								
6								
7								
8							1.3362	
9								
10								
11			1.3359	1.3360	1.3359			
12	1.3340	1.3349					1.3354	
13								
14			1.3355	1.3353	1.3353			
15						1.3349	1.3349	1.3349
16	1.3332	1.3336						
17			1.3350	1.3349	1.3350			
18								1.3340
19	1.3334	1.3333				1.3337	1.3334	1.3335
20								
21								
22						1.3335	1.3334	1.3335

Culture	Age (days)	Cell Fresh Weight (g/L)	Cell Dry Weight (g/L)	Water Content (%)
266-1	14	74.1	5.50	92.6
	18	107	8.25	92.3
	21	113	11.6	89.7
266-2	14	158	7.59	95.2
	18	141	9.42	93.3
	21	177	13.2	92.5
266-3	14	185	10.2	94.5
	18	228	16.7	92.7
	21	226	15.8	93.0

Culture Dry Weight in TRP 4a Series 266. Dry weights from the freeze-dried cells of 25.0 ml culture samples. The mean water content (± standard deviation) is 92.9 ±1.5 %.



Reference TLC Chromatogram

Samples:
(a) Diterpene standards
(b) Elicited Culture Extract Before Spray Reagent
(c) Elicited Culture Extract After Spray Reagent

A silica gel TLC plate developed in toluene (35): chloroform (16): ethyl acetate (15): formic acid (1) and sprayed with:

(i) 30% H_2SO_4 in glacial acetic acid
(ii) 5% anisaldehyde in isopropanol

followed by heating 5 minutes at 110°C. The triptolide spots are brown and the orange/brown quinone methides disappear after the spray is applied.

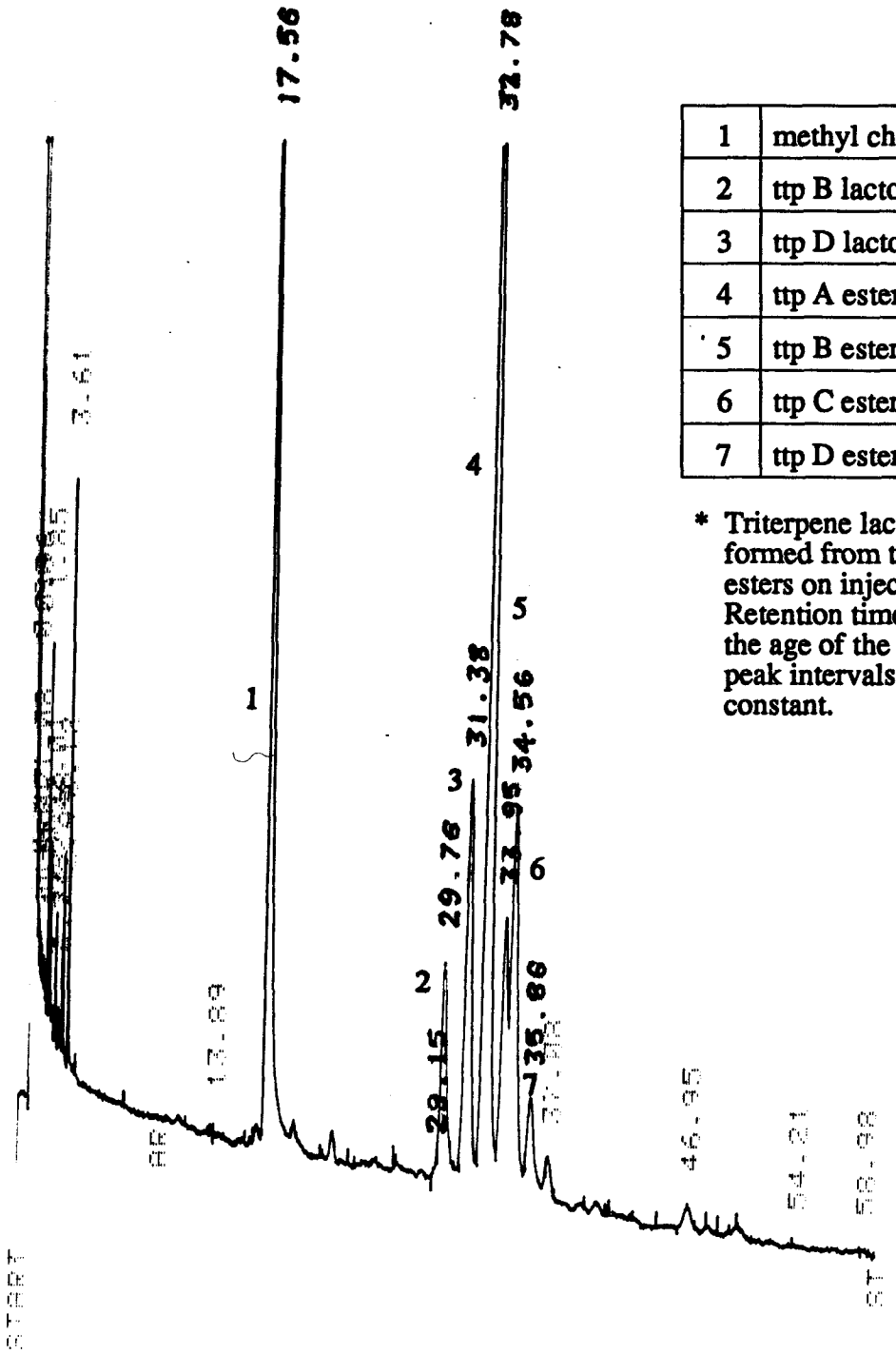
APPENDIX V

Compound	Parent Acid	Retention Time (min.)
methyl cholate	cholic acid	17.6
methyl oleanolate	49	22.0
methyl polpunonate	62	25.2
ttp B lactone	55	28.2
ttp D lactone	56	29.7
ttp A ester	51	30.9
ttp B ester	52	32.1
ttp C ester	53	32.8
ttp D ester	54	34.1

Retention Times in the Triterpene Analysis using Gas Chromatography

APPENDIX V

(continued)



1	methyl cholate
2	ttp B lactone *
3	ttp D lactone *
4	ttp A ester
5	ttp B ester
6	ttp C ester
7	ttp D ester

* Triterpene lactones are formed from the methyl esters on injection. Retention times vary with the age of the column but peak intervals are quite constant.

GC Chromatogram of a Mixture of Triterpene Esters (A,B,C,D) Co-injected with a Cell Culture Extract