SECONDARY METABOLITES FROM *TAXUS × MEDIA* PLANT CELL CULTURE

by

ALEKSANDRA MARKOVIC

B. Sc. (Chemistry), University of Nis, 1993

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES
(Department of Chemistry)

We accept this thesis as conforming
to the required standard.

THE UNIVERSITY OF BRITISH COLUMBIA
June, 1999

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Chemistry

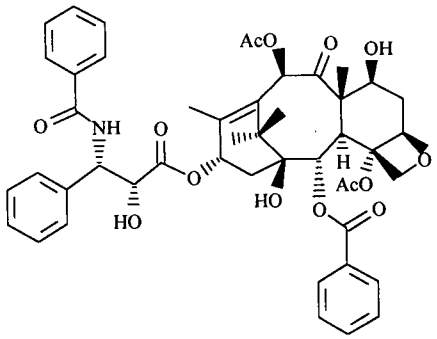
The University of British Columbia
Vancouver, Canada

Date Sep 17, 1999

DE-6 (2/88)

Abstract

Taxol (**1**) is a novel anti-cancer agent isolated from the Pacific Yew *Taxus* species. Effective activity against breast and ovarian cancer has been clinically proven, and led to Taxol being approved for marketing (Bristol-Myers Squibb Company) in 1992. However, Taxol's utility was limited not by safety or efficacy but by the lack of sufficient supplies of the therapeutic agent. Pacific Yew trees are scarce; to obtain only one gram of Taxol approximately 3-6 trees need to be harvested. Hence the intensive search for alternative methods to mass-produce this precious anti-cancer agent.

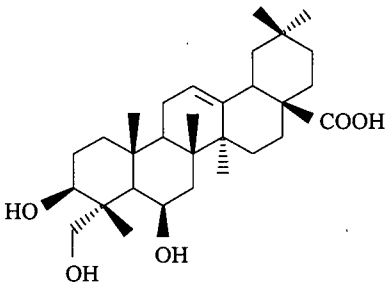


1

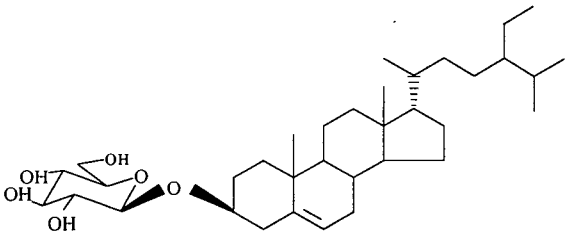
The main goal of my research was to examine the plant cell culture of *Taxus × media* for the production of secondary metabolites, especially for the production of Taxol and its simpler analogs that can, by semisynthetic routes be converted to Taxol. This thesis is based on two different types of experiments with the same culture:

1. Microferm experiments, where the culture was grown in bioreactors with mechanical stirring, and
2. Shake flask experiments, where the culture was grown in flasks placed on a rotary shaker.

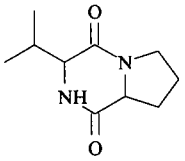
After extensive separation procedure, the secondary metabolites were isolated from the culture, and on the basis of spectroscopic data, the following structures could be assigned.



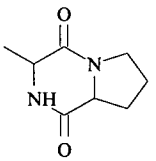
36



38



39



40

Compounds **36** and **38** were isolated from the culture grown in the Microferm bioreactor. The culture grown in shake flasks produced compounds **36**, **38**, **39** and **40**.

It was found that the *Taxus × media* plant cell culture was not producing taxanes at levels measurable by our techniques. However, the culture was found to be stable and to give reproducible results in terms of metabolite production. We believe that further experiments with a combination of strategies, including nutrient and osmotic manipulations, gas composition effects, and elicitor treatments, to mention a few, would eventually lead to a Taxol and taxane producing cell line.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

Ac	acetyl
ACD	Advanced Chemistry Development Inc., Toronto
atm	atmosphere
B-5	standard cell culture medium developed by Gamborg and Eveleigh
BMS	Bristol-Myers Squibb
br	broad
Bu	butyl
c	concentration (g/100mL)
°C	degree Celsius
c.c.	column chromatography
cm^{-1}	wave number
COSY	^1H-^1H 2-dimensional COrrelated NMR SpectroscopY
CRADA	Cooperative Research and Development Agreement
δ	chemical shift
d	doublet
2,4-D	2,4-Dichlorophenoxyacetic acid
3D	three dimensional
10-DAB	10-deacetylbaccatin III
DCI MS	Desorption Chemical Ionization Mass Spectrometry
dd	doublet of doublets
ddd	doublet of doublet of doublets
dec	decomposition
EI MS	Electron Impact Mass Spectrometry
ELISA	Enzyme-Linked Immuno Sorbent Assay
Et	ethyl
FDA	Food and Drug Administration
Fr.	fraction
g	gram

GGPP	geranylgeranylpyrophosphate
h	hour
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Coherence
HPLC	high pressure (performance) liquid chromatography
HRMS	high resolution mass spectrum
Hz	hertz
IR	infrared
IUPAC	International Union of Pure and Applied Chemistry
J	coupling constant
KB	a tissue culture cell line derived from human carcinoma
kg	kilogram
λ	wavelength
L	liter
LD_{50}	median lethal dose
m	multiplet
M	molar
M^+	molecular ion
Me	methyl
MF	Microferm
Mg	miligram
MHz	megahertz
min	minute
mL	mililiter
mp	melting point
μ	micro (10^{-6})
m/z	mass to charge ratio
ν	frequency
NCI	National Cancer Institute
nm	nanometre

NMR	nuclear magnetic resonance
P-388	a tissue culture cell line derived from mouse leukemia
Ph	phenyl
ppm	parts per milion
PVP	polyvinylpyrolidine
rpm	revolutions per minute
s	singlet
SF	shake flask
t	triplet
THF	tetrahydrofuran
TLC	thin layer chromatography
UV	ultraviolet

Acknowledgements

I would like to thank Dr. James Kutney for giving me the opportunity to work in his group, and for his guidance throughout my research.

I would also like to thank all the members of his research group for great collaboration, support and help during the experimental work and preparation of this thesis.

My family back home, has been constantly supporting me, giving me love and strength when I needed it most, and I am very thankful for that. I am especially grateful to my grandmother who read the first books to me, and gave me that great desire for knowledge that inspired me through my work and studies.

Last but not least, I would like to express my gratitude to my husband for his patience, encouragement and love that made me persist through difficult moments.

Thanks go to the UBC faculty and staff for accepting me as a member of this creative and positive environment.

INTRODUCTION AND SCOPE:

In all groups of living organisms there are similar primary metabolic reactions such as the formation and degradation of nucleic acids, proteins and their precursors, carbohydrates, and carboxylic acids. However, a wide variety of biochemical pathways are characteristic of only a few species of organisms, of single "chemical races" or even of a certain stage of differentiation of specialized cells. These pathways are referred to as secondary metabolism and they lead to the formation of special chemical compounds, the so-called secondary products or secondary metabolites. One of the main characteristics of these compounds is the lack of importance for the synthesizing cell itself, but importance for the organism as a whole.

The diversity of chemical structures of secondary metabolites is enormous, and there is also a great diversity of biological activity, too. The plant world has always been a rich reservoir of secondary metabolites that are used as pharmaceuticals for medicine. The majority of secondary metabolites used medicinally are terpenoids (mono-, sesqui-, di-, tri-, steroids), quinones, lignans, flavonoids and alkaloids.

Monroe Wall and his collaborators discovered the novel diterpenoid anticancer agent Taxol (**1, Figure 1**) in 1971[1], and this discovery ranks in retrospect as one of the most significant discoveries ever made in the field of naturally occurring anticancer agents. Plant extracts have been used as anticancer agents for centuries, but only a handful of plant derived natural products have been found to show clinically useful activity. Taxol is one such natural product.

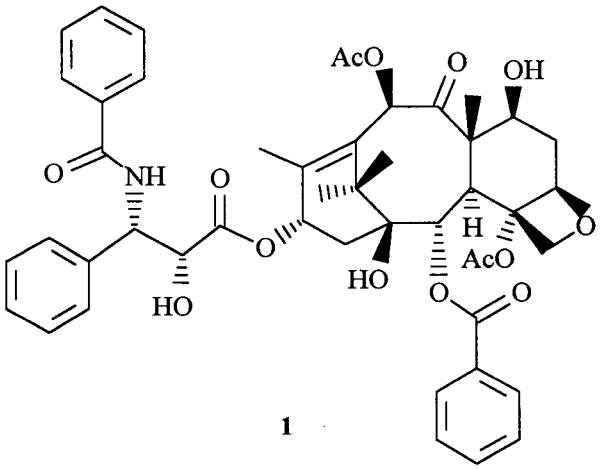
Taxol attracted and still has a lot of attention as "the best anticancer agent developed in last 15 years"[2] with its:

– Unusual nature as a novel, complex, natural product with fascinating chemistry

– Unique mechanism of action

– As a difficult to produce compound, found in low yield in a slow growing tree, and above all

– As an important new therapeutic agent in the treatment of breast and ovarian cancer.



1

Figure 1. Taxol

Taxol is obtained by extraction from the bark of *Taxus* species, but the amount extracted is low, and the tree is slow growing, which brings up the problem of supply.[3] From the environmental point of view, a great number of living trees need to be cut to get a very small amount of Taxol. On the other hand, Taxol has a complex structure, making it a very difficult target for total synthesis. The need for Taxol increases, but also the need to protect nature so we turned to plant cell cultures of *Taxus*, to try to find new ways for obtaining this precious anticancer agent without destroying living trees.

The main goal of our research was to examine the plant cell culture of *Taxus × media* for the production of secondary metabolites, especially for the production of Taxol and its simpler analogs that can be, by semisynthetic routes, converted to Taxol. In this thesis I will try to introduce the reader to the history and the importance of this compound, as well as to the sources of supply with the main focus on plant cell culture, which may be a major contributor to the future supply of Taxol.

1. HISTORY OF TAXOL

1.1. DISCOVERY AND ISOLATION OF TAXOL

Back in the nineteenth century it was already known that *Taxus* species contained toxic compounds. In 1856, a mixture of toxic alkaloids was isolated from the English yew, *Taxus baccata*, and given the name taxine[4] but solving structural problems was a difficult task for the techniques of that time. However, it was very early recognized that the taxines had an unusual skeletal structure, known as the taxane skeleton.

But the real story of Taxol started not with *Taxus*, but with the state of cancer treatment research in the 1950s. After the discovery of penicillin, the so-called "antibiotic era" started which led to change in mortality patterns. That is, pneumonia and many infectious diseases were not the major killers any more, and this turned attention to cancer and heart disease. This led to a strong interest in cancer research, and in the United States in 1955, Congress directed the National Cancer Institute (NCI) to organize a cancer drug-screening program of plant extracts.[5-7] This program used tumor system models *in vivo* and tumor cell lines *in vitro*. These studies led to a discovery that the stem bark extract of the Pacific Yew tree, *Taxus brevifolia* Nutt., showed cytotoxicity in the KB assay and also activity against carcinosarcoma in rats and leukemia in mice.

At that time there were less methods available for separation then today, and of course, they were not so sophisticated. More steps had to be done in separation with the multiplication in time for the return of assay results. The methodology for Taxol isolation began with ethanol extraction of 12 kg of Pacific yew bark followed by partitioning of the extract between

chloroform and water (the activity went into the chloroform phase). It took about two years to isolate 0.5 g of pure Taxol (**1, Figure 1**), from *T. brevifolia* bark, and the first pure sample was isolated in 1966. The molecular formula $C_{47}H_{51}NO_{14}$ obviously indicated a complex structure. Some conclusions were made that the compound belonged to the taxane family, but at that time not many members had been elucidated.

The isolation of Taxol was first presented at the American Chemical Society meeting in Miami Beach, Florida in 1967.[8] In the main paper[1] published in 1971 the reported yield of Taxol was 0.02% from dried bark of *T. brevifolia* and it was also reported that Taxol was present in other *Taxus* species including *T. baccata* and *T. cuspidata.*

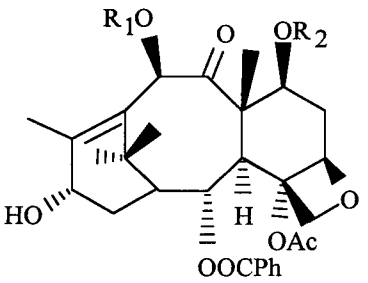
1.2. STRUCTURE ELUCIDATION

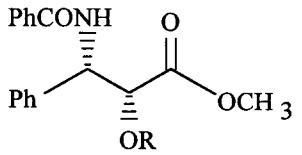
On the basis of the ^{1}H-NMR spectrum, the structure of Taxol was elucidated, and this paper was published in 1971.[1] However, the full solution of the structural problem required the use of X-ray crystallography. Although Taxol was poorly soluble in various solvent systems and readily crystallized, the crystals were fine needles, unsuitable for X- ray crystallography. Today, almost 30 years later there are still no reports of X-ray crystal structure of Taxol. Mild methanolysis of Taxol resulted in a methyl ester (**5**) and a tetraol (**3**). The ester was characterized using X-ray crystallography as its p-bromobenzoate derivative (**6**), and the tetraol was characterized as its bisiodoacetate (**4**) (**Figure 2**). X-ray crystallography data, together with ^{1}H NMR data gave the complete structure of Taxol.

IUPAC recommended the numbering system[9] (IUPAC, 1978) shown in **Figure 3**.

Taxol is differentiated structurally from most other taxane diterpenoids by its ester side chain at C-13 and by its oxetane ring D. It can be viewed as the N-benzoyl-β-phenylisoserine

ester of baccatin III (**2**). The conventional planar representation of Taxol can be misleading, since it's structure is best described as an inverted cup shape[10] as shown in **Figure 4**.





2	Baccatin III	R_1= Ac, R_2= H	**5**	Methyl ester	R=H
3	Tetraol	R_1= R_2= H	**6**	p-Bromobenzoate	R=COC_6H_4Br
4	Bisiodoacetate	R_1= R_2= $COCH_2I$			

Figure 2. Baccatin III and its derivatives, and derivatives of the Taxol ester side chain

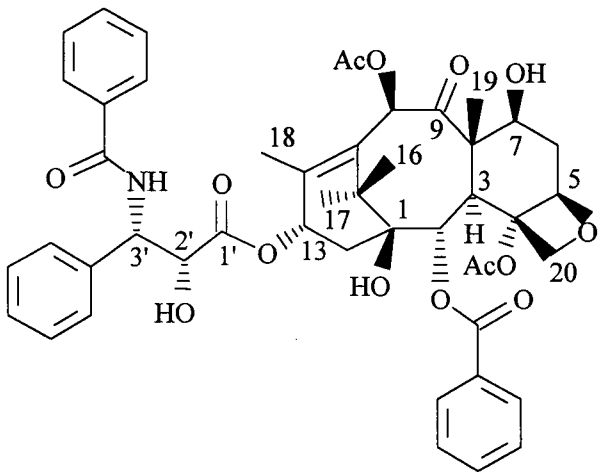


Figure 3. IUPAC numbering system of Taxol

By using the ACD-3D program (Advanced Chemistry Development Inc., Toronto) and incorporating the data from NMR and X-ray studies the stereoview of Taxol was obtained.

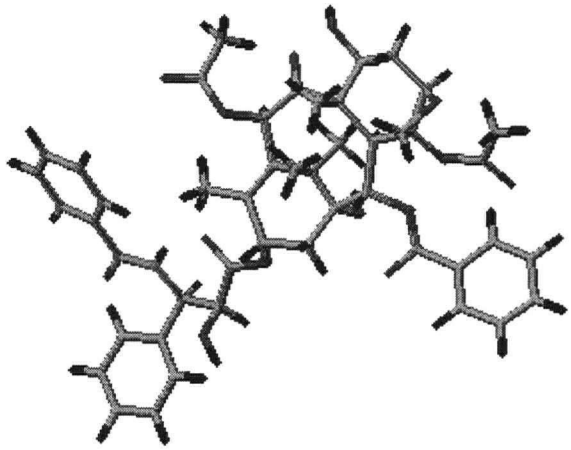


Figure 4. 3D structure of Taxol

1.3. MECHANISM OF ACTION

It was obvious that Taxol did not have a structural relation to any other known antitumor agents. In 1979 Susan B. Horwitz and her coworkers in the Department of Molecular Pharmacology and Cell Biology at Albert Einstein College of Medicine, Bronx, proved[11] that it also had a unique mechanism for antitumor activity involving cell microtubules, which play a key role in mitosis, maintenance of cell shape, cell motility, and intracellular transport. Microtubules are self-assembling, and self-disassembling structures that are in dynamic equilibrium with tubulin dimers, the protein sub-units of which they are composed. A substance that interferes with microtubules can disrupt cell growth and function. The 1979 study by Horwitz and coworkers reported that binding of Taxol to tubulin acts to stabilize cell microtubules and to prevent their depolymerization. Other drugs were known to bind tubulin, but they all enhanced disassembly (depolymerization) of microtubules, and because tumor cells usually divide much more rapidly than normal cells, Taxol inhibits tumor activity. Discovery of

the novel mechanism, by which Taxol disrupts cancer cells, intensified research interest in the drug, and the NCI began a concerted effort to obtain it for clinical trials.

1.4. CLINICAL TRIALS

Phase I trials of Taxol began in 1983, and were aimed at determining the maximum tolerated dose in humans and any dose-limiting toxicities. At first the drug produced some serious hypersensitivity reactions.[12] Researchers soon found that slowing the rate of infusion or premedicating patients with antihistamines and steroids avoided such reactions. Studies done in this phase showed the important activity of Taxol against ovarian cancer.

In phase II trials, the drug's effectiveness was tested on a larger number of patients, and these trials are still being conducted. Some of the completed trials caused great excitement, and were quite encouraging. A phase II trial of Taxol in 1988 by the medical researchers E. K. Rowinsky, and associates at Johns Hopkins Oncology Center, Baltimore, showed a 30% improvement[13] (combined partial and complete responses) among patients with advanced ovarian cancer. These were all refractory cases that had not responded to standard treatment.

Another phase II Taxol study by G. H. Hortobagyi[14] and coworkers at the M. D. Anderson Cancer Center of the University of Texas, Houston, showed tumor shrinkage in 48% of patients with metastatic (advanced) breast cancer whose prior chemotherapy had failed. Taxol has also shown preliminary indications of activity against lung cancer and malignant melanoma.

When Taxol entered phase III studies the major problem was that these required large amounts of the drug. This provided the necessity for larger scale production by the NCI, which put out a request for applications for a Cooperative Research and Development Agreement (CRADA) to expand supply and clinical trials leading to the marketing of Taxol. The CRADA

competition was won by the Bristol-Myers Company (soon to become Bristol-Myers Squibb) and they did an outstanding job of making supplies of Taxol available and conducting the studies in cooperation with the NCI that led to the successful marketing approval in refractory ovarian cancer in 1992. The next major clinical development after the report of activity in ovarian cancer was the discovery that Taxol as a single agent had remarkable activity in primary metastatic breast cancer with a response rate of 56%.[15] This exciting publication by Holmes and collaborators in 1991 stimulated further clinical research. The Food and Drug Administration (FDA) in April 1994 gave marketing approval for the treatment of metastatic breast cancer insensitive to anthracyclines. There are insufficient data for most tumor types at this time to predict other clinical uses for Taxol, although antitumor activity seen thus far implies that breast and ovarian cancer are not the only Taxol-treatable cancers.

2. TAXOL AND OTHER TAXANES-

BIOSYNTHESIS

Taxol is the most popular and the most studied member of the taxane diterpenoids or the taxoids, a large family of over 100 identified compounds. Almost all of them have the basic pentadecene ring system. There are a few others that have modified structures from this one, but these also belong to the taxane family. As previously mentioned for Taxol (**Figure 4**), the 3D shape for other taxanes is also similar to an inverted cup. This representation is very important for a better understanding of the chemistry of Taxol and taxanes, because some of the functional groups that seem to be far apart in the planar representation are actually very close.

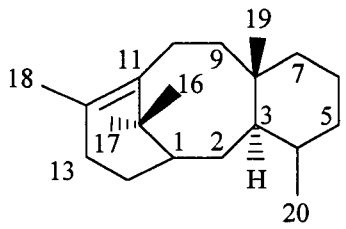
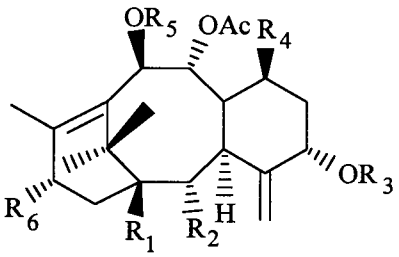


Figure 5. Basic taxane ring system

2.1. CLASSIFICATION OF NATURAL TAXANES

A. Taxanes with a C-4(20) double bond, and an oxygen function at C-5. These and other compounds with the same structural characteristic (over 50 members in this class[16]) form the most abundant group of natural taxanes. This class can be divided into the following subclasses, depending on the nature of the oxygen functionality:

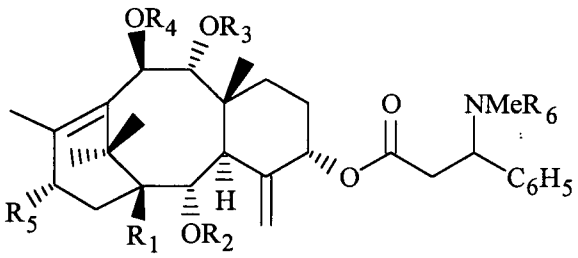
1. Taxanes with a hydroxyl group or acetate at C-5 (**Figure 6**). Taxusin (7) is given as an example from this group.



	R_1	R_2	R_3	R_4	R_5	R_6
7	H	H	Ac	H	Ac	OAc

Figure 6. Taxanes with a hydroxyl group or acetate at C-5

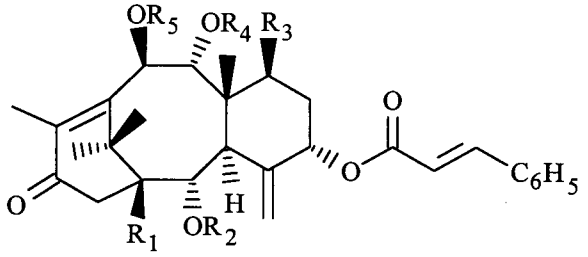
2. Taxanes with a basic side chain at C-5 (**Figure 7**). One example from this subclass is one of the toxic constituents of yew, taxine B (**8**), which will be mentioned later.



	R_1	R_2	R_3	R_4	R_5	R_6
8	OH	H	H	Ac	=O	Me

Figure 7. Taxanes with a basic C-5 side chain

3. Taxanes with a C-5 cinnamoyl group (**Figure 8**). Taxinine B (**9**) is an interesting example because it was one of the first taxanes whose relative stereochemistry was assigned by nuclear Overhauser effect.

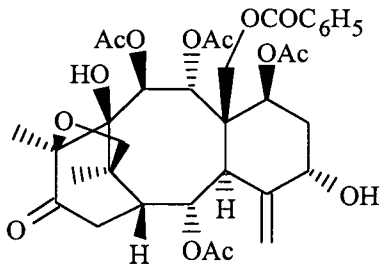


	R_1	R_2	R_3	R_4	R_5
9	H	Ac	OAc	Ac	Ac

Figure 8. Taxanes with a C-5 cinnamoyl group

B. Taxanes with a transannular bond.

1. Taxanes with a C-12(16)-oxido bridge (**Figure 9**).



10

Figure 9. Taxinine M, a taxane with a C-12(16)-oxido bridge

2. Taxanes with a C-3(11) bridge (**Figure 10**).

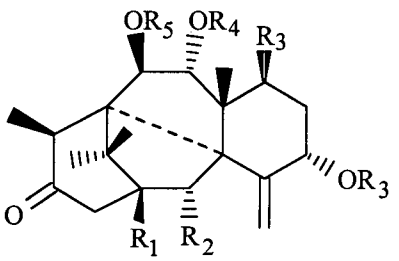


Figure 10. Taxanes with a C-3, C-11 bridge

C. Taxanes with a C-4(20) epoxide.

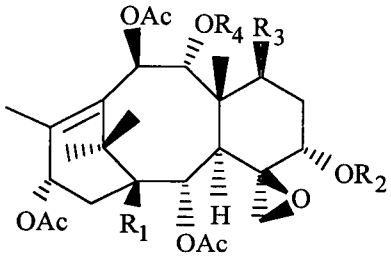
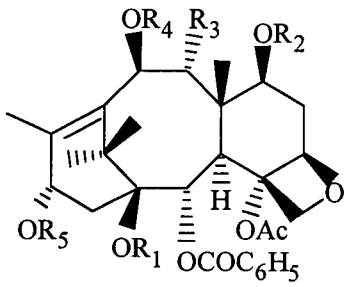


Figure 11. Taxanes with a C-4(20) epoxide

D. Taxanes with an oxetane ring. This is the second largest, and the most important class, from the chemotherapeutic point of view. It includes:

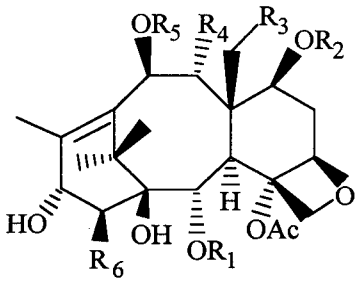
1. Taxanes with an oxetane ring (**Figure 12**). This class includes baccatin III[17], (**2**) as the diterpenoid portion of Taxol, and its important analogue 10-deacetylbaccatin III (**11**). The latter can be isolated in reasonably good yield from many *Taxus* species, and converted to Taxol by a semisynthetic route.[18]



	R_1	R_2	R_3	R_4	R_5
2	H	H	=O	Ac	H
11	H	H	=O	H	H

Figure 12. Taxanes with an oxetane ring

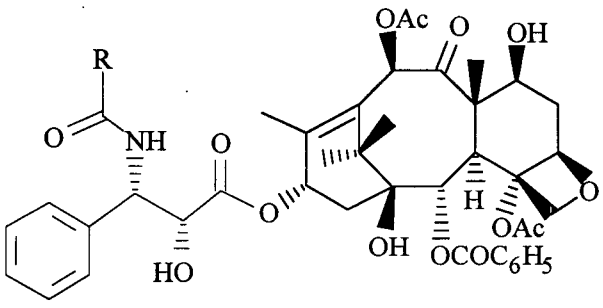
The other compound of greater interest for the synthesis of Taxol is 14β-hydroxy-10-deacetylbaccatin III shown in **Figure 13, (12)**.



	R_1	R_2	R_3	R_4	R_5	R_6
12	Bz	H	H	=O	H	OH

Figure 13. Other taxanes with an oxetane ring

2. Taxanes with an oxetane ring and a complex C-13 side chain. This class includes the most important taxane Taxol (**1**) and related cephalomannine[19] (**13**) shown in **Figure 14**. The latter was isolated from a plant that was at first thought to be *Cephalotaxus mannii.* Later, it was shown that the plant was *Taxus wallichiana.*[20] However, the original name of the compound has been retained.



1 R=C_6H_5

14 R=

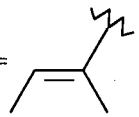


Figure 14. Taxanes with an oxetane ring and a complex C-13 side chain

E. A-nor taxanes. This is a small group of taxanes, with a five-membered A-ring in place of the normal six-membered ring (**Figure 15**).

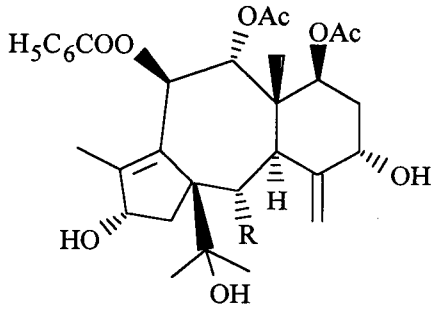
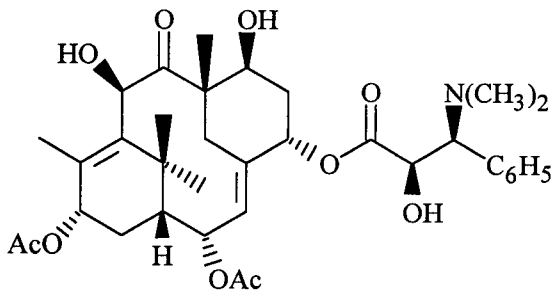


Figure 15. A-nor taxanes

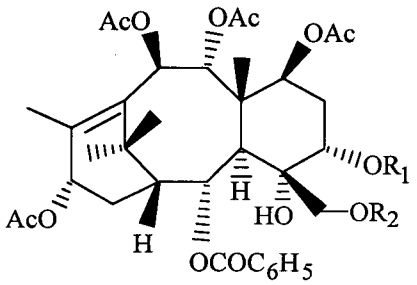
F. Miscellaneous taxanes. As mentioned earlier, a mixture of toxic compounds was isolated in 1856 from *Taxus baccata,* and named taxine. From this mixture two basic components were isolated. One of these compounds is taxine A (**14, Figure 16**), the other is named taxine B, and it was mentioned earlier (**8**).



14

Figure 16. Taxine A

The other two compounds from this group were isolated recently from *Taxus mairei* (**Figure 17**). These compounds (**15** and **16**) are hydroxylated analogues of the taxanes with a C-4(20) exocyclic double bond.[21]



15 $R_1 = H$, $R_2 = Ac$
16 $R_1 = Ac$, $R_2 = H$

Figure 17. Hydroxylated taxanes from *Taxus mairei*

2.2. BIOSYNTHESIS OF TAXOL

There is little information in the literature on the biosynthesis of Taxol and other taxanes, and much of what has been published are only biosynthetic proposals and model studies. Until recently, virtually nothing was known about the Taxol biosynthetic process, especially about the enzymes that catalyze the process. The few experimental studies on the biosynthesis of taxanes, that were reported were all done *in vivo* by feeding radioactive or stable isotope labeled precursors to intact plants or plant parts. Now, after an incredible amount of work has been done, scientists are beginning to understand the detailed biosynthetic pathway by which yew plants produce the anticancer agent Taxol. Those findings are very important, because they could lead to the development of a more efficient cell culture process for commercial production of Taxol as an alternative to the semisynthetic process currently used to make the drug. As a matter of fact, in the long run it would be highly desirable to be able to produce Taxol and other

taxanes by a process that doesn't have to rely on the extraction of always deficient plant material, but instead is able to be carried out under entirely controllable conditions. Such a process would be based on a detailed knowledge of the biosynthetic pathway by which the plant assembles Taxol and by characterizing the enzymes that catalyze those reactions, or eventually the genes coding for them.

Although taxanes had only been observed as constituents of plants, Stierle et al.[22] reported the production of Taxol and related taxanes by an endophytic fungus isolated from a specific yew tree in Montana. This organism, named *Taxomyces andreanae* produces 24 to 50 ng/L of Taxol. A number of control experiments have been carried out to demonstrate that the compound is formed by the fungus and is not a contaminant carried over from the plant. The authors think that the fungus may have inadvertently picked up the genes for Taxol biosynthesis from the yew tree. However those findings still don't have any scientific confirmation or any verification on the reproducibility of the results.

Taxol biosynthesis consists of three distinct aspects:

–Formation and functionalization of the diterpene moiety

–Formation of phenylisoserine side chain, and

–Assembly of Taxol from these components

2.2.1. The Formation and Functionalization of the Diterpene Moiety

2.2.1.1. The Formation of the Diterpene Moiety

Terpenes share a biosynthetic pathway that forms isopentenyl pyrophosphate (IPP) from acetic acid (as a thioester coenzyme adduct). All the various classes of terpenes are then generated from IPP (**Figure 18**).

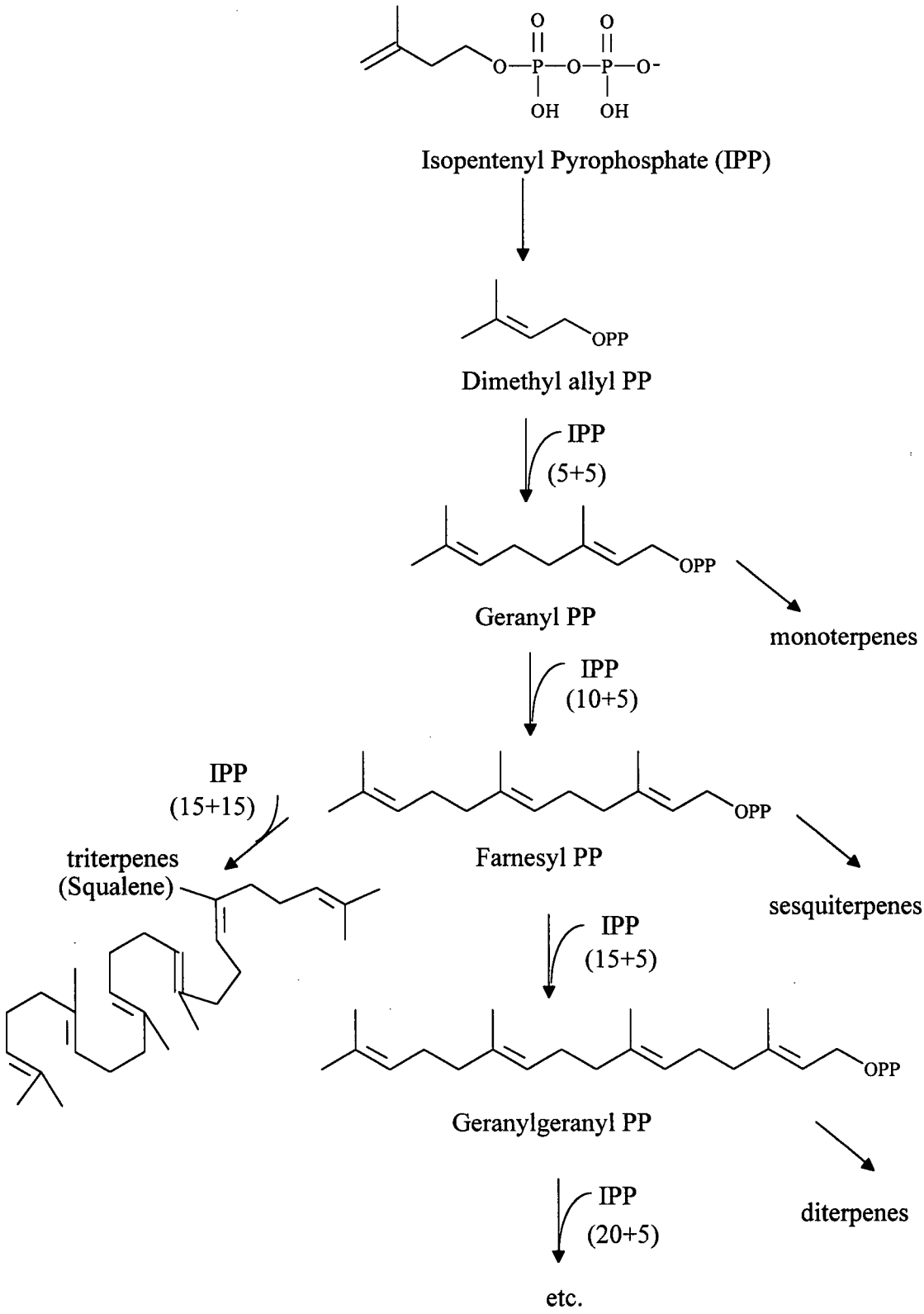


Figure 18. Biosynthesis of terpene classes

As shown in **Figure 18** diterpenes are produced through the cyclization of geranylgeranyl pyrophosphate. Using the general biochemical knowledge on biosyntheses pathways, the taxane ring system formation can be proposed to go by an electrophylic cyclization of geranylgeranyl pyrophosphate (GGPP), possibly via cembrene or verticillene intermediates[23,24,25] (**Figure 19**) to give, ultimately, a hydrocarbon precursor, 4 (20),11-taxadiene (**17**).

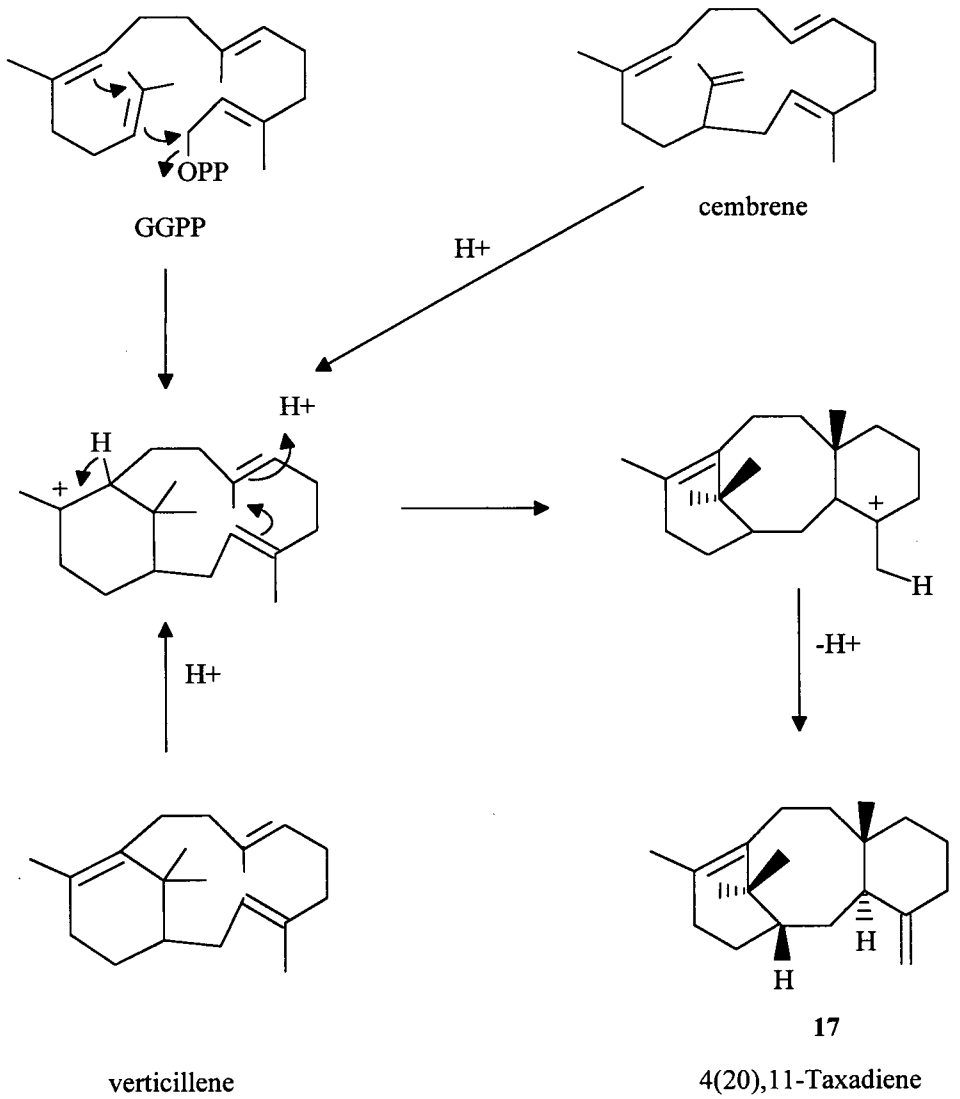


Figure 19. Possible biosynthetic scheme for the taxane skeleton

This hypothetical precursor is not a known compound. Both cembrene and verticillol (gives as the dehydration product verticillene) are constituents of a conifer wood. However, model studies on transannular cyclization of cembrenes[26] and verticillene (**Figure 19**) and epoxyverticillenes[27] failed to yield products with a taxane skeleton. These experiments do not invalidate the hypothesis that the taxane skeleton is formed from verticillene-type intermediates, since cyclizations of this type can be quite sensitive to the actual conditions. However, they suggest that the biogenetic sequence is much more delicate than supposed, maybe involving isomers of geranylgeranyl pyrophosphate, or involving alternative reactive intermediates such as radicals.

Early attempts to verify experimentally the isoprenoid origin of the taxane ring system by feeding radiolabeled mevalonic acid to *T. baccata* were unsuccessful.[28] However, two groups have recently reported positive results. Zamir et al.[29] fed [5-^{3}H]mevalonate to ground new grown leaves of *T. canadensis* for 3 weeks and obtained 0.12% incorporation of radioactivity into Taxol. Although this was without any supporting evidence, the authors stated that the product had been purified to constant specific radioactivity by HPLC. Sodium [^{3}H]acetate gave an obviously lower incorporation of 0.02%.

Strobel et al.[30] fed precursors to prepared pieces of the inner bark of *T. brevifolia* and observed 0.1% specific incorporation* of [2-^{14}C]mevalonate and 0.38% incorporation of [1-^{14}C]acetate.

In 1995, Rodney B Croteau, the biochemistry professor of the Institute of Biological Chemistry at Washington State University, and his coworkers, identified the first committed step of Taxol biosynthesis.[31] Three carbon-carbon bonds are formed in this reaction, which creates

---

* Specific incorporation= $\frac{\text{Specific mol.radioactivity of product}}{\text{Specific mol.radioactivity of precursor}}$ x 100 [%]

the tricyclic taxane core structure. In this reaction geranylgeranyl diphosphate cyclizes to form taxa-4(5),11(12)-diene (**18, Figure 20**), and it is only this process that funnels the starting material toward Taxol, others are all leading to other diterpenoid compounds. Croteau et al. discovered that the structure proposed in the 1960s for this taxadiene was incorrect, and that a double bond that had been predicted to lie outside one of the taxane rings (**17**, **Figure 19**) is according to their studies inside the ring (**18**, **Figure 20**). They characterized taxadiene synthase, the enzyme that catalyzes the cyclization, and then cloned and expressed the gene for the enzyme.[32] They believe that the cyclization is relatively slow and therefore a rate-determining step in Taxol biosynthesis. The detailed mechanism of the taxadiene synthase-catalyzed cyclization[33] has also been determined by Croteau, Floss, and their coworkers. By using deuterium labeling they found that an intramolecular proton transfer from C-11 to C-7 is a key aspect of the reaction. The resulting cation at C-8 would then initiate the transannular cyclization.

This kind of approach is interesting for our group's work with plant cell cultures. Williams believes that the potential applications of this is to take "genes and put them on high-expression vectors in cell culture in hopes that it might significantly boost cell culture's production to commercially viable quantities".[33] Work with this type of approach by Croteau, Wiliams, Floss and their coworkers continues with identification of other metabolites and enzymes in the Taxol biosynthetic pathway. As Williams said "harnessing and manipulating the awesome power of complex secondary metabolism in plants, fungi and microbes for commercial syntheses of complex molecules promises to be an area that will continue to open up numerous collaborative opportunities for synthetic chemistry and molecular biology".[33]

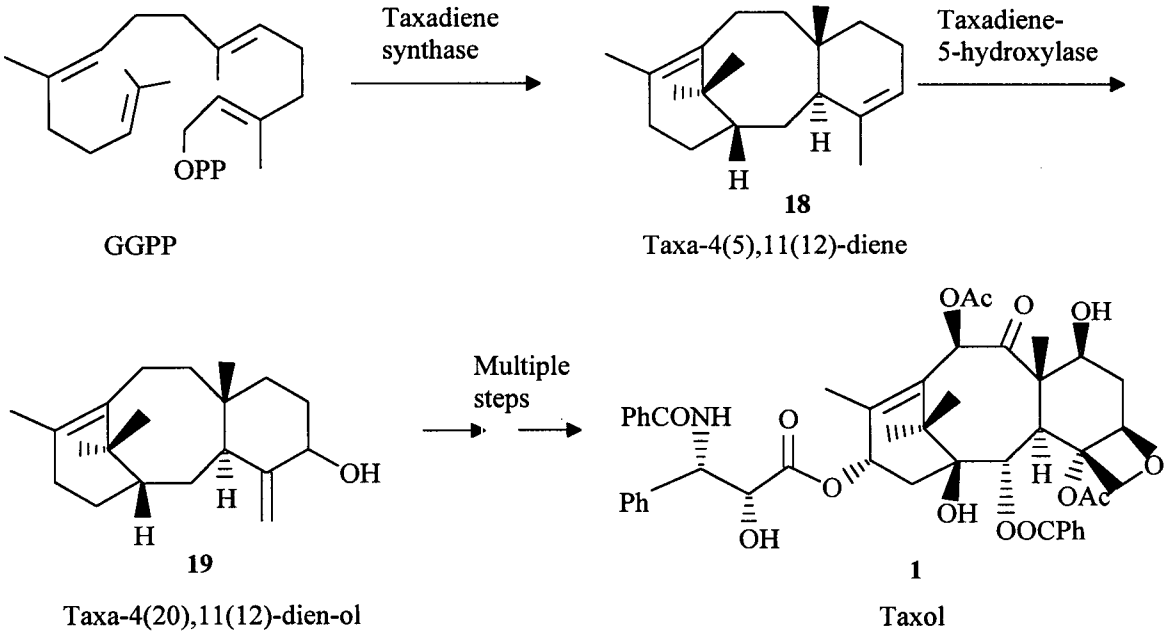
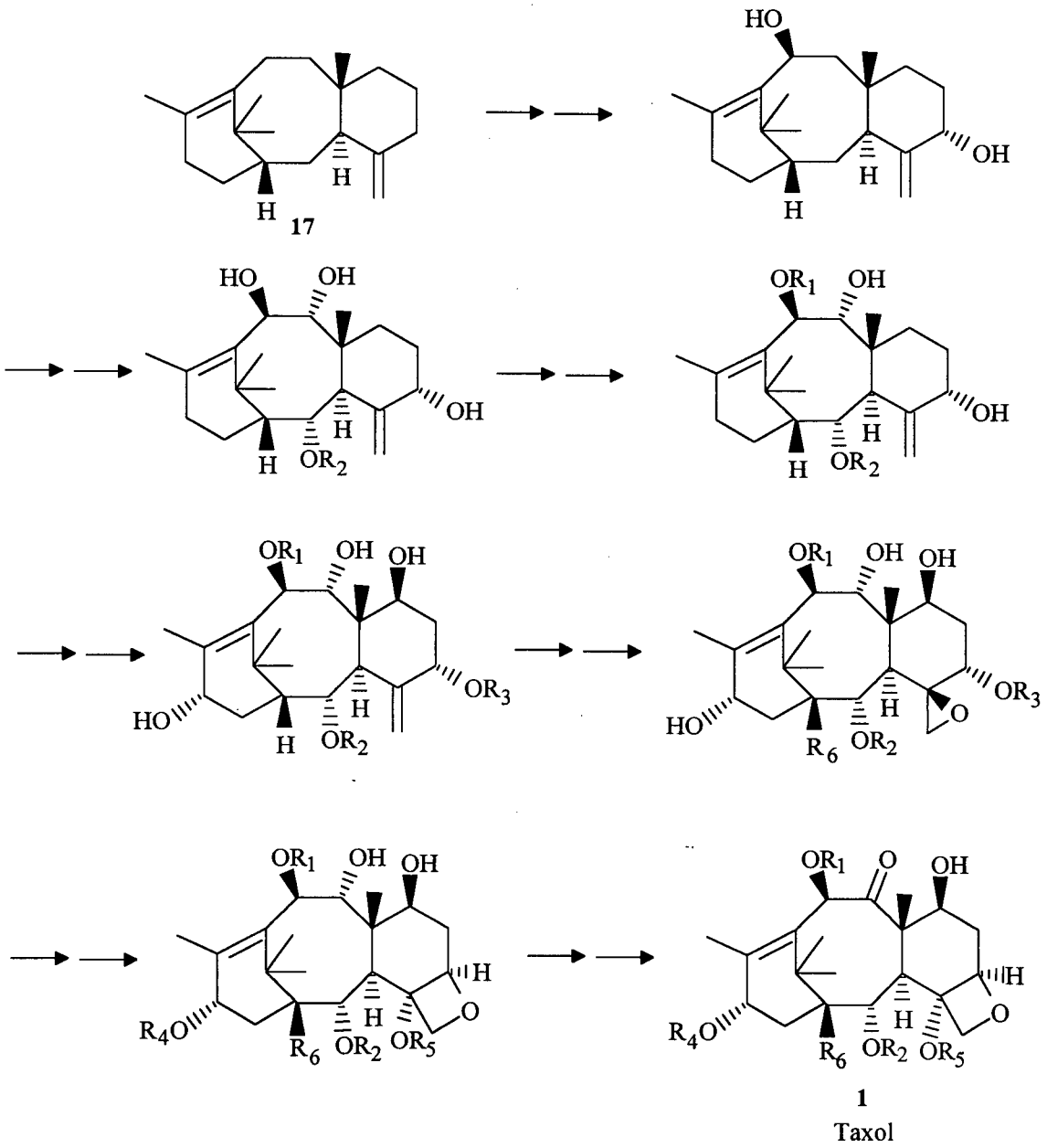


Figure 20. First steps in Taxol biosynthesis

2.2.1.2. Functionalization of the Ring System

The large number of functionalities present in Taxol must be introduced successively into the diterpenoid ring system following the generation of the taxane ring system and functionalization include hydroxylations, acylations of hydroxy groups, oxidation to a ketone, and generation of the oxetane ring system.

Comparison of the structures of all naturally occurring taxanes provides some suggestions about the order in which these reactions take place (**Figure 21**). The isolated compounds that are least functionalized have at least two oxygens, namely on carbons 5 and 10. Therefore, these two oxygens must be introduced first in the reaction sequence. Next in abundance are oxygens at C-2 and C-9 followed by C-13.



R_1, R_3 = H or acetyl; R_2 = H or benzoyl; R_4 = H,N-benzoyl-phenylisoserinyl or precursor thereof; R_5 = acetyl; R_6 = H or OH

Figure 21. Hypothetical pathway for the functionalization of taxadiene to Taxol

Acylation of some of the existing oxygens may however precede the introduction of the new ones. The last introduced are probably oxygens at C-7 and C-1, the latter possibly even

after oxetane ring formation. Definitely, epoxidation and oxetane ring formation are late steps, preceding the acylation of C-13 with a side chain precursor, and oxidation of C-9 to the ketone.

There are three different mechanisms for the elaboration of the unique oxetane moiety attached to ring C of Taxol and related compounds that have been suggested by various groups. They are all based on the occurrence of three major structural classes of taxanes, namely, **A**, **B**, and **C** given in **Figure 22**. The proposed mechanisms are given in **Figure 23**.

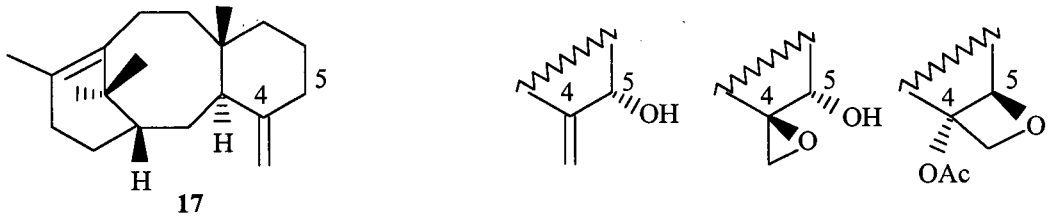


Figure 22. Structural arrangement around C4/C5 found in natural taxanes

Della Casa de Marcano et al.[34] considered opening of the epoxide to a triol which then cyclizes (**Figure 23a**).

Swindell and Britcher[35] have suggested a direct rearrangement of the α-hydroxyepoxide (**Figure 23b**). However, they failed to observe this transformation in their model system.

The most plausible proposal by Potier and colleagues[36] suggested a rearrangement of an α-acetoxyepoxide in which the acetoxy group migrates from C-5 to C-4 (**Figure 23c**). This proposal still remains untested.

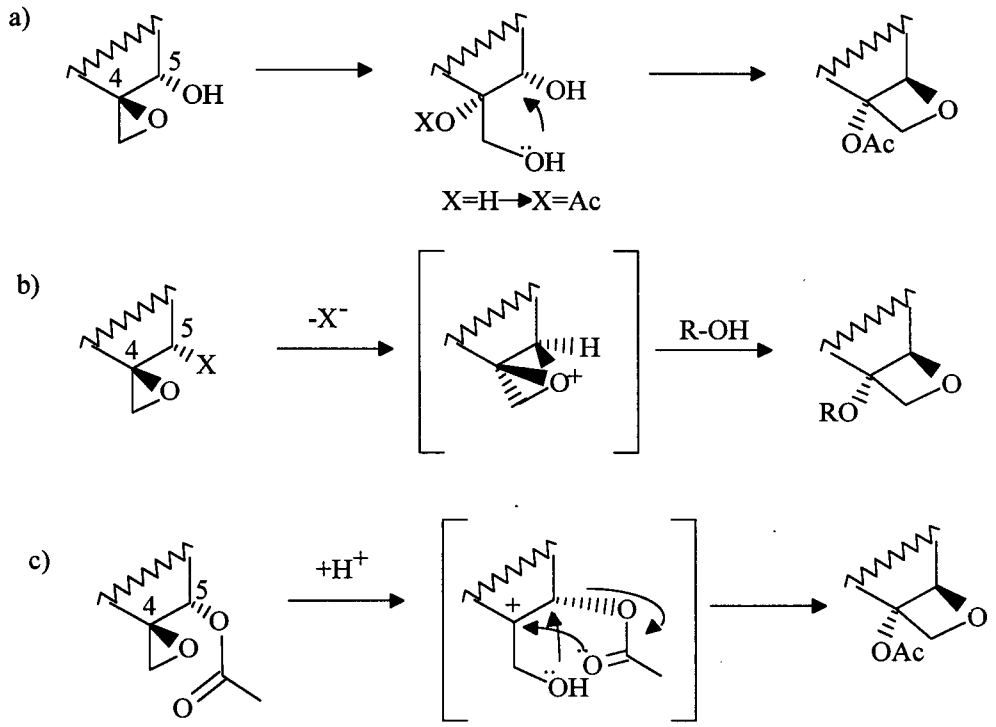


Figure 23. Possible mechanisms for the elaboration of the oxetane ring

However, the general idea about the oxetane ring elaborated from an α-hydroxymethylene compound via the epoxide remains plausible, but still unsupported by any biochemical experiments.

2.2.2. The Formation of the Phenylisoserine Side Chain

This is the only part of the molecule on which actual biosynthetic studies have been carried out. Phenylpropanoid side chains can be found in taxanes in three versions (**Figure 24**): as N-acylated (2R,3R)-phenylisoserine (**20**), as (3R)-N,N-dimethyl-β-phenylalanine called Winterstein's acid (**21**), and as cinnamic acid (**22**).

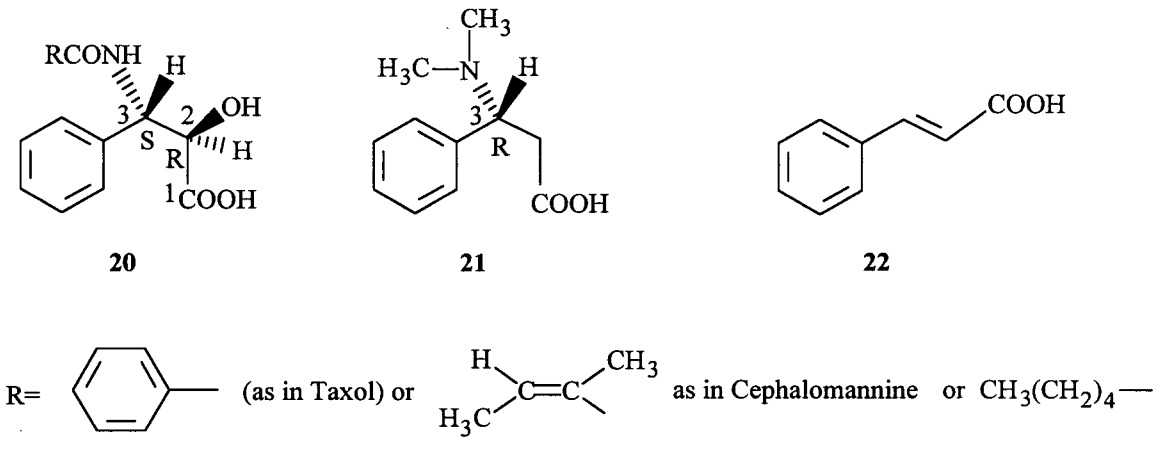


Figure 24. Structures of phenylpropanoid side chains found in taxanes

Biosynthetic studies on Winterstein's acid, which is related to the Taxol side chain, have been carried out by Leete and Bodem[37] and Haslam.[38] In *T. baccata* phenylalanine serves as the best precursor of Winterstein's acid and the bioconversion occurs stereospecifically, with loss of the 3-*pro*-R proton and retention of the 3-*pro*-S proton (**Figure 25**).

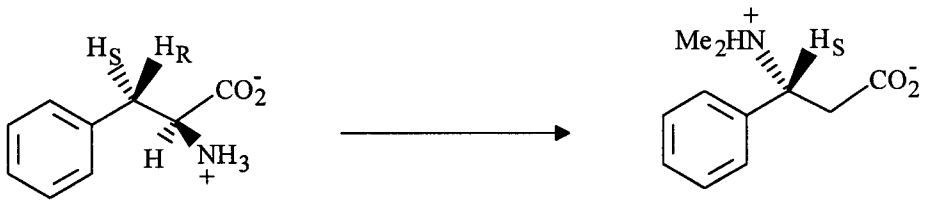


Figure 25. Bioconversion of phenylalanine to Winterstein's acid

Zamir and coworkers[29] and Strobel et al.[30] reported the incorporation of radiolabeled phenylalanine into the phenylisoserine side chain of Taxol. Zamir et al. fed a mixture of L-[ring-2,6-^{3}H]phenylalanine and (3R)-[2-^{14}C]mevalonate (^{3}H/^{14}C = 3.6) to ground new growth leaves of *T. canadensis* and obtained Taxol of a ^{3}H/^{14}C ratio of 4.4, which upon hydrolysis gave

baccatin III of $^{3}H/^{14}C$=1.75. This experimental result is consistent with the earlier work on Winterstein's acid.

In higher plants, the benzoic acid system studied so far is synthesized from phenylalanine via cinnamic acid. The observation in the feeding experiments with the deuterated precursors in the innerbark system that cinnamic acid was not at all incorporated into the benzoic acid moiety of the Taxol side chain, was therefore surprising. It was found that both β-phenylalanine and phenylisoserine gave not only pentadeuterated Taxol molecules but also ones containing ten atoms of deuterium, i.e., two aromatic rings, derived from the added precursor. All of the deuterium atoms were found in the side chain, showing that both the phenylisoserine moiety and the benzoate moiety of the side chain must have been formed from β-phenylalanine and phenylisoserine. This result gave a novel pathway for the formation of benzoate units in a plant metabolite.

2.2.3. The Formation of Taxol from its Components

Finally, there remains the question of the stage at which the phenylpropanoid side chain and the diterpene moiety of Taxol are united together. Based on other studies of the existing natural structures, the assumption would be that the terpene moiety is fully elaborated to the level of baccatin III and that the C-13 side chain is attached last.

However, Potier et al.[36] suggested an intriguing alternate mechanism for the introduction of the Taxol C-13 side chain. They noted the existence of three major groups of taxanes shown in **Figure 22**: group **A** with an exocyclic methylene at C-4, group **B** with a β epoxide (oxirane) at C-4, and group **C** with an oxetane ring. Many of the group **A** compounds contain ester groups at C-5 that are structurally related to the Taxol C-13 ester side chain, and Potier suggested that the

C-13 ester side chain of Taxol is the result of the intramolecular transfer from C-5. Those parts of the molecule seem to be far apart in the conventional representation of the taxane skeleton, but they are fairly close in a 3D structure (**Figure 4**), supporting this suggestion. The resulting allylic alcohol is proposed to lead to the oxetane ring through an epoxide, which undergoes ring opening to a tertiary cation and cyclization to an oxetane. However, experiments with radioactive labeled compounds couldn't confirm this proposal.

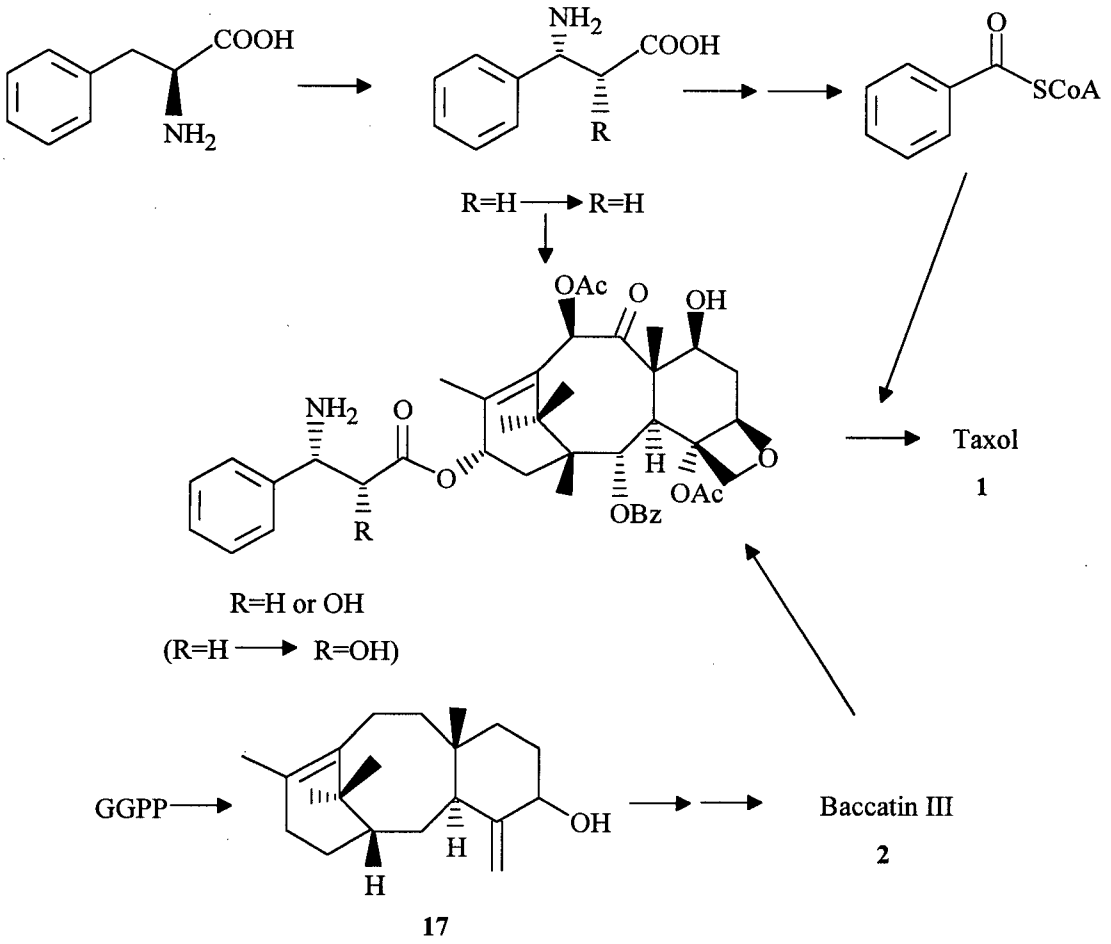


Figure 26. The late steps in the biosynthesis of Taxol

These experiments also demonstrate the presence of an enzyme in *T. brevifolia* tissue, which is capable of linking a phenylpropanoid side chain to C-13 of baccatin III, suggesting that side chain attachment is a late step in the biosynthesis of Taxol.

Figure 26 shows the summarized late stages of Taxol assembly. The diterpene moiety is fully functionalized and built up to the level of baccatin III (**2**). Phenylalanine is separately transformed into β-phenylalanine, and/or phenylisoserine, which is attached to C-13 of baccatin III. β-phenylalanine, or phenylisoserine is also transformed into benzoate, which is attached to the β-phenylalanyl- or phenylisoserinyl- baccatin III, most likely as the final step in the sequence.

3. SOURCES AND SUPPLY OF TAXOL

The only original official source of Taxol® as a formulated pharmaceutical was the bark of the Pacific yew *T. brevifolia* Nutt. However, Taxol's utility was limited by the lack of sufficient supplies. Factors such as relatively large therapeutic doses of Taxol, low concentrations in the bark and limited availability of Pacific yew bark led to the insufficiency of Taxol for the treatment of cancer patients.

A number of alternative taxane supplies sources range from the use of genetic engineering and tissue culture, to partial and total syntheses. According to BMS the most promising alternative source is Taxol® made from 10-deacetylbaccatin III (**11**) by a semisynthetic process licensed from Robert Holton of Florida State University.[39] In 1994 the FDA approved the use of pharmaceutical Taxol, produced by semisyntheses. Currently, *T. wallichiana* is a major commercial source of 10-deacetylbaccatin III and also appears to be a large potential source of 14β-hydroxy-10-deacetylbaccatin III (**12**).

Other alternative methods for Taxol production, as for other complex natural products molecules, have proven to be excellent scientific tools, but they are rarely economical sources of plant derived drugs. Until tissue culture or other newer technologies prove otherwise, the most economical and abundant Taxol supplies will be based on the use of wild and cultivated *Taxus* plant material. In addition to the original process of the direct isolation of Taxol from plant material, the partial synthesis approach for new sources of Taxol and Taxol analogues begins with taxanes, such as 10-deacetylbaccatin III, also isolated from *Taxus* species.

To understand the potential plant resource for Taxol supplies, I will begin with a brief overview of the diversity, distribution and abundance of *Taxus* species. After that a review of

alternative sources follows, with the main accent on plant cell culture which was the center of our study as a future potential method for Taxol and taxanes production.

3.1. *TAXUS* SPECIES AS A SOURCE FOR TAXOL AND TAXANES

Taxol has been isolated and detected only from the tissue of *Taxus* species. There are no reports that it has been found in any other genus. Furthermore, in every species of *Taxus* that has been examined, Taxol has been detected and is accompanied by numerous analogs, many of which show cytotoxic activity.

3.1.1. About the Yew Tree

"...the yew, from it's perpetual verdure, the durability of its wood, and its power of rejuvenescence has from the earliest times been used in funeral rites, and has been held to symbolize the resurrection, and immortality of the soul."

John Lowe, 1897[40]

The yew has been there for our predecessors when they needed a wood to make a fire, or just a tree for shelter, or as a source of needles for poisons and potions. The wood is red and heavy, hard and durable. A yew wood spear-point found near Clacton-on Sea, England, is thought to be more than 50,000 years old.[41] All the cultures that grew up around the yew – Egyptians, Greeks, Romans, Celts, Japanese, Indians, Native Americans, and others, used its wood for tools, weapons, personal implements, and sacred objects and its foliage and bark for poisons and medicines.

"The yew is a dioecious evergreen; the males bear golden anthers, the females scarlet arils. Its gnarly, fluted red-barked boles and twisted limbs give thee tree a sinuous, almost human appearance. It grows slowly, lives a long time and can adapt to a wide range of habitats, although it grows in dense groves.[42]"

3.1.2. Geographical Distribution and Diversity of *Taxus* and Related Genera

Taxus is today widespread, but rarely abundant in North America and Eurasia. *Taxus* grows in moist, temperate forests in the Northern Hemisphere but also occurs in subtropical and tropical areas of Southeast Asia and Central America. In the Southern Hemisphere *Taxus* occurs in Sumatra and Celebes.[42-46] The oldest fossils identified as Taxus have been recovered from the Jurassic period.[47] The Jurrasic began approximately 200 million years ago, and has been characterized as a time of a uniform, mild climate when conifers achieved worldwide distribution and dinosaurs were abundant.[48] The seeds of *Taxus* and related genera such as *Torreya* are partly or completely enclosed by a fleshy envelope termed the aril. Similarly as in pines (*Pinus*) or firs (*Abies*), the lack of true seed cones in *Taxus* has resulted in fact that some taxonomists place it together with one or more closely related genera into a separate order of plants, the Taxales, instead of the Coniferales.[49]

Four native species of *Taxus* are generally accepted by their wide geographical separation in North and Central America.[46] *T. brevifolia* Nutt., called the Pacific or western yew, is widely distributed in western parts of Canada and the U.S.[50] It grows from the southern tip of Alaska to the Sierra Nevada mountains in California. *T. globosa* Schlechtendal, called the Mexican yew, is native to Mexico, Honduras, Belize, El Salvador and Guatemala.[46-49] The rare Florida yew, *T. floridana* Nutt. ex Chapman, is endemic to two counties in Northern Florida. The Canadian yew,

T. canadensis Marshall, has a range from Newfoundland to Manitoba and south from Iowa to Tennessee and North Carolina.[46] *T. baccata* Linnaeus, called the English or Common yew is native to Europe, Asia Minor, and north Africa.[46] The Japanese yew, *T. cuspidata* Siebold & Zuccarini, is native to eastern Asia including China, Korea, Japan, and Manchuria in Russia.[47,48] *T. wallichiana* Zuccarini, the Himalayan yew, can be found from eastern Afghanistan to Tibet and China.[48] In Asia, the southern range of *Taxus* includes Burma, Vietnam, Taiwan, Sumatra, the Philippines, and the Celebes.[44]

The division of *Taxus* into distinct species can be problematic except on the basis of populations that are widely separated geographically. Today, more morphological differences have been described between cultivars within the species of *T. baccata* than between all the described species. Nowadays, in the U.S. and in Europe there are hundreds of cultivars that have been introduced into cultivation.[49] Almost all the ornamental yews cultivated in the U.S. are reported to be selections of *T.baccata* L., *T. cuspidata* Sieb. & Zucc., and the hybrid of these two species *T. × media* Rehd.[49]

The future *Taxus* sources of Taxol and Taxol analogues for clinical supplies will include both wild and cultivated sources that have a high content of taxanes such as Taxol (**1**) and 10-deacetylbaccatin III (**11**) as well as species that are high in more than one taxane that can be readily reduced to a simpler taxane such as 10-deacetylbaccatin III. Cultivated taxane-rich species should increase the concentration of taxanes a minimum of five to ten times the current yields from different wild sources. The advantage of higher taxane production by superior plant selections grown under optimum field conditions, and, also, the better recovery of these taxanes by on site harvesting and drying should in the future offer important opportunities to establish nurseries dedicated to producing taxanes for the pharmaceutical industry.[51]

3.2. ALTERNATIVE SOURCES OF TAXOL

The new alternative methods for Taxol supply should prove that the new source can provide significant quantities of pure compound in a reliable, economical, and environmentally acceptable manner. Significant resources are exploring the potential alternatives, which include:

(1) **Plant tissue culture**, an emerging technology that would require significant development before it could be scaled up to industrial production levels,

(2) **Semisynthesis**, i.e., synthesis of Taxol from a simpler and more readily available analog, and

(3) **Total synthesis,** although improving still far away from industrial application.

3.2.1. Plant Cell Culture

Undoubtedly, plant cell culture has a number of important advantages over field-grown material since it's not subject to various factors of disease, weather, and season. Furthermore, material from exotic plants can be grown via cell culture independent of its original, sometimes very remote location. Finally, cell culture can offer reliable, year-round production using a renewable resource.

3.2.1.1. History of Plant Cell Culture

There is a long practice of cloning of many herbaceous and woody plants. For example, *Cryptomeria japonica* has been vegetativly propagated for more than 1000 years, and this practice has its origin in Neolithic Britain. However, for many plants the simple, traditional cloning techniques are ineffective, and as a result, there is a long history of attempts to obtain controlled regeneration of plants *in vitro*.[52,53]

Back in the eighteenth century Duhamel du Monceau observed that wounded trees produced callus. Later, in the early nineteenth century Schwann had noted that in lower plants any cell could be separated from the plant and could then be induced to reconstitute the plant. This observation eventually lead to the totipotency concept that postulates that cells are autonomic, and, in principle, capable of giving rise to a new plant (Schleiden 1838, Schwann 1839). It also encouraged attempts to regenerate plants from smaller tissue masses, and, eventually, from single cells (Haberlandt 1902). In the early part of the twentieth century cell culture was generally a failure. When organ cultures were first established the future started to look brighter. The roots were the first organs that grew in culture. Attempts to grow root cultures of maize (Robins 1922), and tomato (White 1939), and buds of asparagus (Loo 1945) were successful. Another milestone was the aseptic germination of orchids on a simple nutrient medium (Knudson 1922). Went (1926) made a significant breakthrough with the discovery of auxins, the growth regulating substances, and their characterization was done by Koegl et al. (1934). Gautheret (1938), Nobecourt (1938) and White (1939) used auxin subsequently to initiate formation of callus and to maintain its continuous growth, which without auxin had been impossible. Organogenesis became possible after another group of growth regulating substances, the cytokinins, became readily available. This study started in the 1950s after

Blakeslee and van Overbeek had found that coconut milk contained unknown growth regulating substances that stimulated the growth of some plant cell cultures. Tissue culture techniques improved very rapidly after the chain of events that culminated in the discovery of a number of compounds with cytokinin-like activity by Miller et al. (1955). The increasing interest in 'biotechnology' in the 1970's carried plant cell culture with it. In 1971 regeneration of plants from protoplasts was realized by Takebe et al. After that rapid developments followed including the success in establishment of often "disobedient" species in culture, micro-screening techniques for natural products such as ELISA system, process technology in the form of large scale culture, continuous culture systems and immobilized cell technology. However, lack of information on the biosynthetic pathways to secondary metabolites, on the properties of key enzymes and their control systems, and on cellular transport phenomena[53] are still limiting further developments.

3.2.1.2. *Taxus* Plant Cell Cultures

Production of Taxol and taxanes by plant cell and tissue culture is a new and developing technology that could provide sufficient quantities of Taxol for cancer treatment.[54] It is expected that plant cell culture will be a major contributor to Taxol supply in the next decade, eliminating the need to use the limited natural resource of *Taxus* species worldwide. Although the biosynthetic pathway for Taxol and taxanes has not been elucidated yet, Lewis and Croteau have made significant progress[55] in identifying some new taxanes and enzymes which may be rate limiting in the pathway. Definitely, more efficient manipulations of the pathway will be possible to increase production of desired products once the basic information on biosynthesis is known.

Plant cell and tissue cultures are usually established by choosing and isolating living plant tissue called an explant, away from the intact plant. The explant is grown in strictly controlled and defined conditions regarding both medium composition, and physical conditions such as temperature, light, humidity, etc. Because of the fact that conditions that support the growth of plant culture also support the growth of algal, bacterial, and fungal contaminants the explant has to be maintained in an aseptic environment.[56]

Taxus plant cell cultures are grown as:

A. Differentiated Cultures

This kind of culture contains tissues that maintain distinct cellular, tissue, and organ interactions. Genetically, differentiated tissues can be more stable since they usually show slower growth in an organized state and maintain cellular, tissue and organ interactions similar to the intact plant. A differentiated culture is sometimes necessary, and has many advantages for secondary product formation since it may keep the temporal and spatial expression of the genes needed for the biosynthesis of the desired compound. *Taxus* differentiated cultures can be grown as:

1. Root Cultures
2. Hairy Root Cultures
3. Embryo Cultures
4. Shoot Cultures
5. Nodule Cultures

B. Undifferentiated Cultures

This kind of culture was of greater interest for our group. It can be roughly divided into two kinds of cultures:

1. Callus Cultures

"Callus is an unorganized, proliferating mass of undifferentiated cells."[56] The source tissue from the intact plant in which the secondary metabolites of interest are synthesized can be the main guideline for the choice of the explant tissue for use in initiating callus although other sources, such as embryos, can be used. For the induction of callus an environment in which some of the cells can divide and proliferate is necessary. The nutrient medium is the strongest factor in allowing induction, and it is supplemented with plant growth regulators. Two kinds of media, Murashige and Skoog (MS)[57] and Gamborg B5[58] have been widely used with a number of plant tissues although many other plant media have been tried and described.

1. Suspension Culture

The cells in the callus, when shaken continuously, can disperse to produce a suspension culture. This kind of plant cell culture is induced usually by transferring callus from established callus cultures into one of a number of liquid media favoring suspension culture growth.[59] The work of our group was done on suspension cultures. Aggregates of cells are typical for plant cell cultures because of the failure of dividing cells to separate completely. Mechanical or enzymatic disruption can decrease aggregate size or this can be accomplished by selectively subculturing only the fine suspension cells. The culture regime itself can tend to select for a cell type that produces a fine suspension culture by rapid subculturing. This kind of system has many advantages especially regarding scale-up production. Bioreactors up to 75,000 L have been

successfully used to culture this kind of plant cells. When designing model bioreactor systems for production of desirable plant-derived products suspension cultures remain the method of choice, because they can be grown in a relatively controlled environment and are relatively homogenous. They can grow to much higher cell densities than animal cell systems and their ease of handling regarding manipulation and maintenance are more similar to microbial systems.

3.2.1.3. Recent Developments of *Taxus* Plant Cell Culture

After considering the problem of Taxol supply, great attention has been paid to development of plant cell culture that would produce Taxol. In 1989, Christen et al. first reported the production of Taxol in *Taxus* callus culture. Further work of this group resulted in the first patent for production of taxanes by cell culture in the United States Department of Agriculture in 1991.[60] Many groups have also been working on the development of a high producing cell line, and this work caused a great interest. Only in the period between 1991 and 1996 around 75 papers including 18 patents were published on *Taxus* cell and tissue culture.

Some companies (Mitsui Petrochemical Industries, Phyton Catalytic Inc. and ESCA Genetics) have patented processes and are almost ready to start commercial production of Taxol by plant cell culture.[61,62] Mitsui Petrochemicals and Phyton Inc. have managed to manipulate *Taxus* cell cultures to produce almost 100 times more Taxol and some other taxanes than the amounts reported in starting experiment with *Taxus* plant cell cultures. They also reported being on the brink of going into commercial production.

To make Taxol via cell culture commercially viable needed productivity has to be 1-2/mg/L/day.[63] These amounts have been exceeded just recently but only in 1-liter shake flasks, and not industrial size bioreactors.

Monitoring progress that has been made in this field it is to be expected that the production of Taxol and taxanes from plant cell culture will be marketed in just a matter of few years.

3.2.2. Semisynthesis of Taxol

There is a logical separation of Taxol's structure (**1**) into a tetracyclic core (Baccatin III, **2**) and a carboxylic acid side chain (**23**, **Figure 27**).

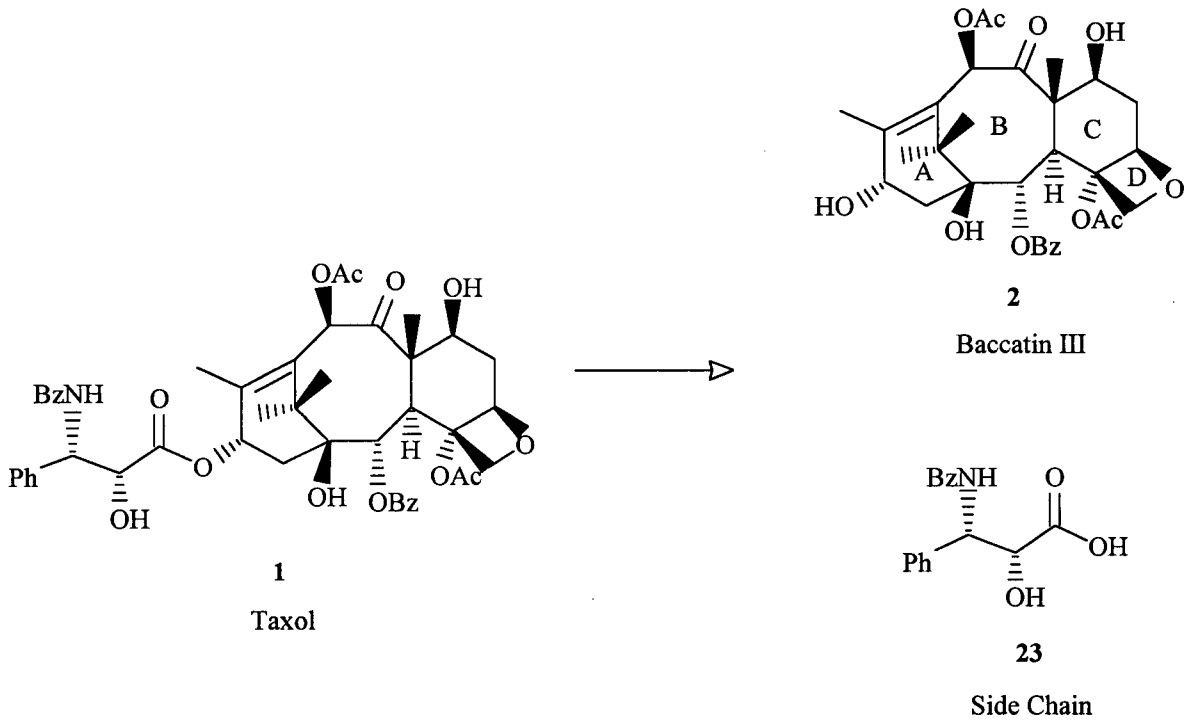


Figure 27. Separation of Taxol's structure

Potier first recognized the potential of semisynthesis after founding that 10-deacetyl baccatin III (10 DAB) (**11**, **Figure 13**) is a relatively abundant taxane.[18] Potier et al. reported in 1981 that the least active, but most abundant compound in the dried leaves was 10 DAB with an isolated yield of 0.02%.[64] This compound can be extracted from a renewable resource, the

needles of *Taxus baccata,* the English yew, which can be harvested from the wild or from the cultivated plantations. The isolation of 10-DAB was significantly simpler since the separation of Taxol and the structurally similar cephalomannine, was a problem. Additionally, while Taxol must be isolated in ultra-pure state for direct incorporation into formulated drug, 10-DAB must only be pure enough for use as a starting material for semisynthesis. The isolation of 10-DAB was also more economical than the isolation of Taxol, because the yield of 10-DAB isolated in this fashion was approximately six- to tenfold greater than the yield of Taxol from *T. brevifolia* bark.

The synthesis of Taxol from baccatin III (**2**) appeared at first sight deceivingly simple, since the key step is the esterification of the C-13 hydroxyl group using a suitably protected derivative of the side chain acid. Acetylation at this position is however very difficult, because the C-13 hydroxyl group of baccatin III is very hindered, and is also hydrogen bonded to the C-4 acetoxy carbonyl group. The relative reactivity of the four hydroxyl groups of 10-DAB toward acetylation was determined by Potier et al.[65] They found that the most reactive was C-7 hydroxyl, followed by the C-10 hydroxyl, the C-13 hydroxyl, and the C-1 hydroxyl, which couldn't be acetylated under various conditions. These acetylations were not completely selective, but this study determined the relative reactivity of the four hydroxyl groups and has served as a foundation for a number of selective protection schemes.

In 1988 Potier et al. published the first semisynthesis of Taxol with the direct attachment of side chain for the conversion of 10-deacetyl baccatin III to Taxol in 38% yield.[65]

A number of attempts to achieve semisynthesis by direct esterification of baccatin III derivatives with an intact suitably protected N-benzoyl-3-phenylisoserine ended up with obvious limitations such as harsh conditions, low conversion, loses of the expensive baccatin III derivative, formation of substantial amounts of C-2' epimerized products, and instability of the protected carboxylic acid. After that significant effort has been made in a search for alternative

acylating agents and processes that could potentially address some of the issues mentioned above.

Palomo and coworkers[66] came up with the most efficient proposal and their semisynthetic work has been scaled up to a highly efficient industrial process. FDA approval for the process was obtained in late 1994, and now multi-kilogram quantities of Taxol have been prepared in this way. Because of the successful development of this process, Bristol-Myers Squibb Company in January 1993. announced that no more yew bark would be harvested from public lands to provide a supply of Taxol, and there is currently a plentiful supply of Taxol for marketing as well as continued clinical trials.

3.2.3. Total Synthesis of Taxol

For years the Taxol's complex structure (**1**) has eluded the successful total synthesis. From the synthetic work of the past few decades it is clear that molecules even more complex than Taxol can be prepared through total synthesis. However, only very few molecules of Taxol's complexity have been synthesized in a practical fashion. Thus, while total syntheses of Taxol and its analogues have been achieved, and there are important advances in the field, the main challenge in this area is the development of a *practical* synthesis of this compound. The following section will review and summarize only the general approach to Taxol and taxane synthesis. Reviewing only the work of some of the authors would be unjust to the effort and work of others equally important. Reviewing separately all of them is beyond the scope of this thesis since a different doctoral dissertation could be written on each of these syntheses.

The practicality of a total synthesis of Taxol or its analogues will ultimately be determined by a cost/benefit comparison with alternative means of production. The number of steps, as well as their efficiency and ease of execution, the cost of materials, safety and

environmental factors, and the time and labor required would all be of consequence in such a comparison. Industrial syntheses of 20 to 30 steps have been achieved on the scale needed to produce Taxol (approximately 300 kg/year now, but greater if ongoing clinical trials establish that Taxol or its analogues can be used against other cancer types). In the end, however, the synthesis would have to do (much) better than current supply options in order to provide incentives to change sources. It is not unreasonable to expect that these goals could be met through synthesis provided that development in this area is driven by an emphasis on practicality.

3.2.3.1. The Design of a Total Synthesis of Taxol and its Analogs: Strategic Considerations

As previously mentioned, there is a logical separation of Taxol's structure into a tetracyclic core and a carboxylic acid side chain (**Figure 27**).

This tetracyclic core is actually baccatin III (**2**), a natural product, and there are many elegant methods developed for attaching it or one of its protected derivatives to the side chain. Therefore, the problem of synthesizing Taxol is somewhat simplified to the synthesis of baccatin III or its derivatives.

Usually development of a synthetic plan goes through a combination of retrosynthetic analysis, intuition, experience, creativity, and, not rarely serendipity. There are certain guidelines associated with the chemistry of the target and the objectives of the synthesis that influence the selection of a plan. This plan should include acceptable length, reagent types, overall yield, cost, time requirements, novelty, safety, and environmental and regulatory concerns.[67] In the most ideal case, a developed plan would be short, efficient, fast, novel, cheap and safe. Taxol possesses several functional groups exhibiting a range of reactivities, and in

molecules such as this it is strategically better to introduce the more reactive functionality near the end of the sequence in order to minimize the number of reactions that it needs to be exposed to.

Another important issue in the design of a Taxol synthesis is how absolute stereochemistry is regulated. The methods for the absolute stereochemistry control are limited to a racemic mixture resolution, enantiocontrolled elaboration of a prochiral intermediate, or the use of an enantiomerically pure starting material. There are several commercially available, enantiomerically pure or enriched starting materials, including carbohydrates and terpenes such as camphor[68] and pinene[69], that have been used in efforts to make taxanes.

Also of strategic significance in synthetic design is synthetic convergency. Convergency does not necessarily reduce the overall number of steps in a synthesis plan. It however rather reduces the number of steps in part of a plan, usually the longest in the sequence. There are also other advantages of convergency since several converging sequences can be developed simultaneously. Furthermore, apart from these purely synthetic considerations, convergency often offers more flexible and efficient access to analogs.

The process of selecting a plan is a final point of strategic significance. One of the options is a retrosynthetic analysis is in which one considers how the various bonds of the target can be formed from simpler precursors based on known reactions. Thus, in Taxol, the β-hydroxy ketone for example, brings the idea of a retrosynthetic disconnection of the C7-C8 bond since in the synthetic direction a condensation of a ketoaldehyde would be expected to form this bond. The problem of preparing Taxol could thus be simplified to that of preparing its less complex precursor. Another kind of approach to retrosynthetic planing involves a complete analysis of options for disconnection of the target structure into less complex precursor structures conducted before chemical analysis. In this approach the target molecule is treated as a graph, without any chemical bias, i.e., as points-which are corresponding to atoms connected with lines that are

corresponding to bonds. Disconnections of these points-atoms produce simpler graphs-precursor molecules. The length of a synthesis is directly determined by the average complexity increase per step. It therefore always advantageous to choose graphical disconnections that would provide the most simplified structure since when the graphical concept is translated into a chemical reaction these will provide the greatest increase in complexity in the synthesis direction.

# Author	Index	# Author	Index
1. Shea	L-9	22. Hudlicky	J-8
2. Jenkins	L-9	23. Cha	D-2
3. Lansbury	C-2	24. Ghosh	J-8
4. Kende	F-4	25. Arseniyadis	F-4
5. Kuwajima	F-4	26. Pattenden	B-7
6. Fetizon	D-2	27. Pattenden	C-6
7. Nicolaou	C-2	28. Winkler	C-8
8. Kishi	D-2	29. Oishi	B-2, A-1
9. Wang	F-10, C-2	30. Funk	D-2
10. Fetizon	D-2	31. Yadav	L-9, C-2
11. Blechert	E-4	32. Paquette	E-4
12. Wender	A-5	33. Martin	B-7
13. Inouye	E-4	34. Snider	E-4
14. Berkowitz	E-4	35. Zucker	E-4
15. Swindell	J-8, E-4	36. Gadwood	B-1
16. Winkler	G-7	37. Kahn	B-1
17. Kraus	L-4	38. Levine	B-1
18. Kanematsu	L-7	39. Wender	C-8
19. Trost	D-2	40. Sieburth	C-8
20. Holton	D-10	41 Sakan	E-8
21. Yamada	A-6	42. Fallis	E-6
		43. Fallis	A-7

Table 1. Taxane Two-Bond Disconnections[67]

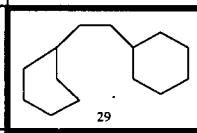
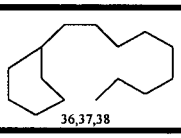
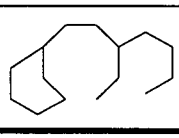
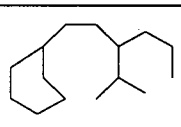
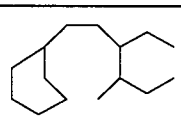
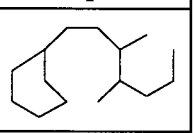
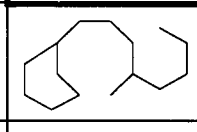
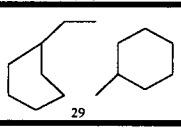
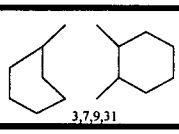
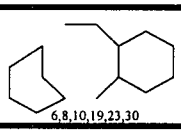
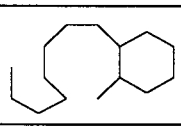
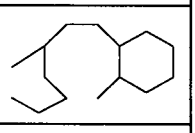
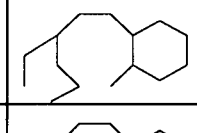
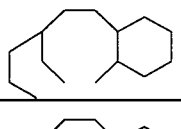
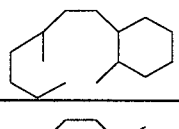
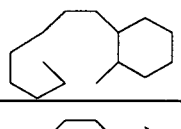
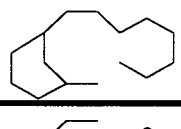
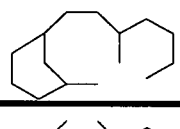
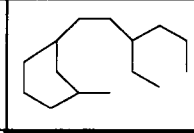
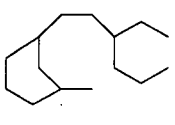
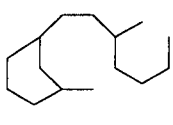
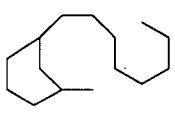
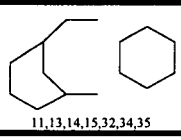
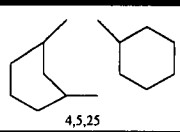
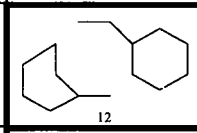
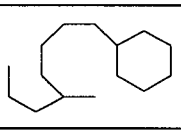
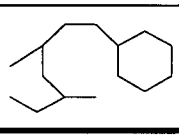
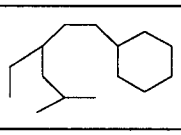
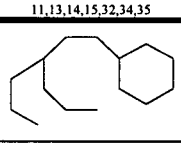
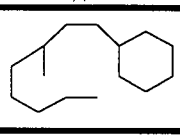
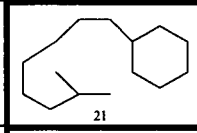
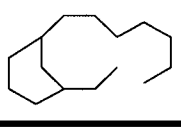
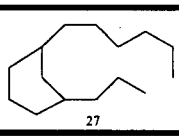
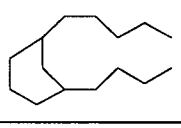
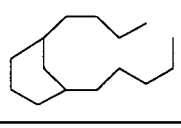
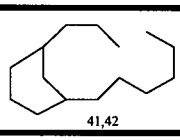
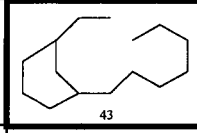
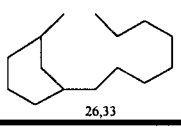
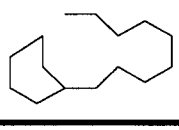
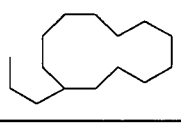
	A	B	C	D	E	F
1	 29	 36,37,38				
2		 29	 3,7,9,31	 6,8,10,19,23,30		
3						
4					 11,13,14,15,32,34,35	 4,5,25
5	 12					
6	 21		 27			 41,42
7	 43	 26,33				
8			28,39,40			
9						
10				20		9
11						
12						
13						

Table 1. (*Continued*) Taxane Two-Bond Disconnections

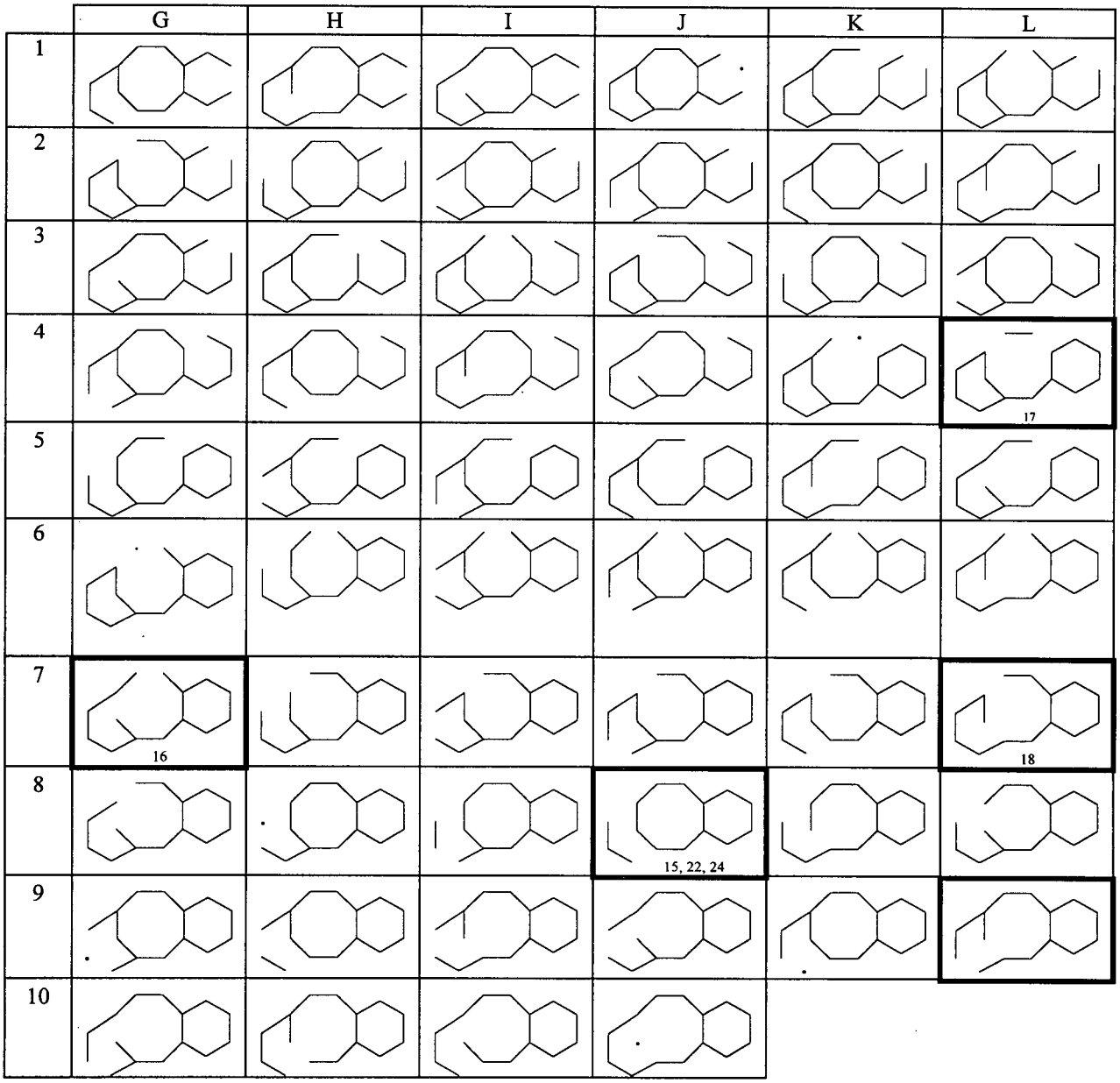


Table 1. (*Continued*) Taxane Two-Bond Disconnections

There are many advantages of the graph-based approach such as that it allows one to rapidly identify without chemical bias all one- and two- bond disconnections. This identification is needed because most organic reactions allow for the formation of only one or two bonds.

Then one can make selection of these on the basis of complexity change, convergency, and other factors that influence design.[67]

In this way, by using one- or two-line bond disconnections, it can be shown that the carbocyclic core of Taxol can be simplified in (only!) 153 ways, 17 one-line (bond) disconnections and 136 two-line (bond) disconnections. As given in **Table 1**, examination of the latter can provide further points of interest. The graphical representation from **Table 1** corresponding to each strategy is noted parenthetically after the main author's name.[67]

The solution of the problem of making the tricyclic taxane structure can in this way be greatly simplified to that of making two much less complex monocyclic precursors. When the graphical, chemically unbiased analysis of a target is completed, some of the best graphical options can be translated into chemical transformations. In **Figure 28** an example is shown of one translation of a graphical concept into a chemical one. This is illustrated for the L-9 disconnection taken from **Table 1**.

The L-9 graph is derived from the taxane graph after the disconnection of lines corresponding to the C1-C15 and C13-C14 bonds. The reconnection of these lines, representing the addition of a 4-atom segment across a 2-atom segment could be achieved through the Diels-Alder reaction, a [4+2] cycloaddition. With further analysis of this possibility directed to improve its viability or to introduce target-related functionality, leads are made to numerous chemical precursors such as substrates **25** to **27** which have in fact figured in published approaches to taxanes.[67]

Of special interest are disconnections that produce equivalent fragments because they offer the benefit of symmetry in a plan. The complex target is reduced to two fragments in this case, and the fact that they are identical simplifies the problem to that of making only one fragment.

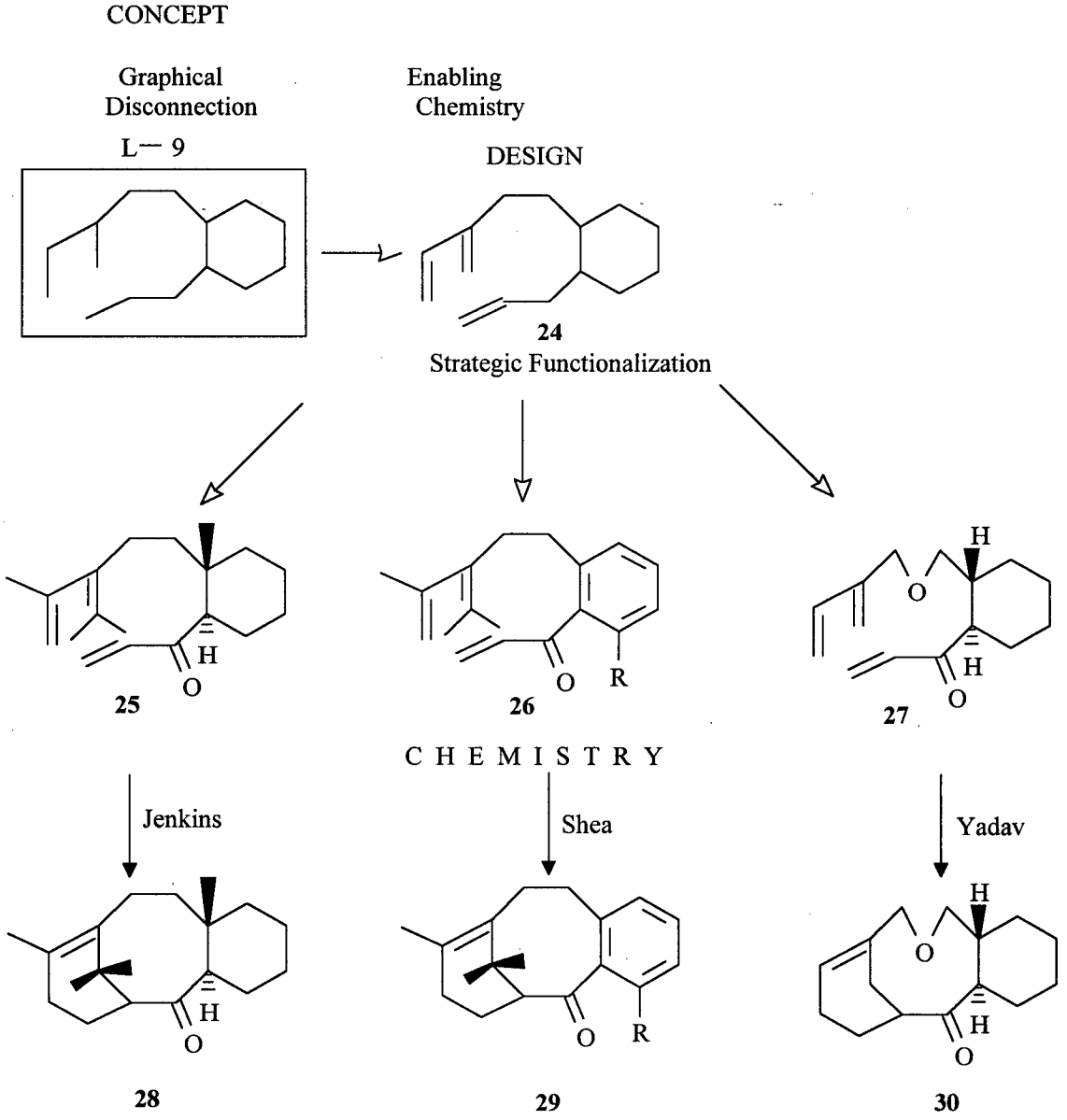


Figure 28. From Concept to Chemistry

One example is the disconnection C-2, in which the taxane tricycle is simplified to two monocycles. Addition of an appendage to one (disconnections can be modified in this fashion if it serves the purpose of design simplification) would thus produce two identical fragments **31** (**Figure 29**). When chemistry is added to this graphical concept this can lead to a powerful new

process for taxane synthesis based on a dimerization. This basic research was still not developed for the purpose of making taxanes, but it describes one of the shortest methods for producing the carbotricyclic core of taxanes.[70]

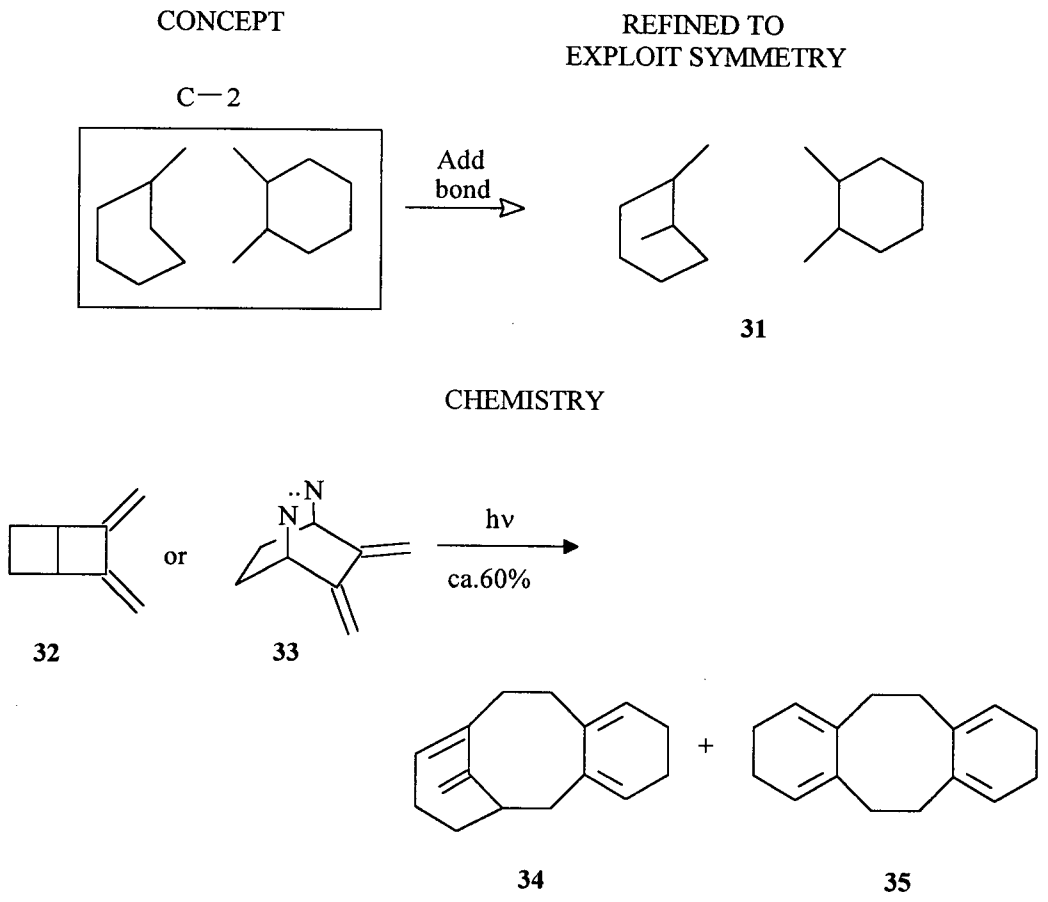


Figure 29. Conceptual Refinements

This concept of complex molecule synthesis design has great value in synthetic planning. It can be also used to classify approaches to the problem, which are grouped according to the ring (A, B, or C) that is addressed by the strategy level reaction.

4. *TAXUS × MEDIA* PLANT CELL CULTURE: INITIATION, GROWTH, AND PRODUCTION EVALUATION

The cell line derived from *Taxus × media* was studied as a potential source for the industrial production of taxanes. The callus culture was initiated from stems and established in 1994 by Dr. Shan Lin Gao in the Biological Services Laboratories of the UBC Chemistry Department. Dr. Elena Polishchuk and David Chen carried out propagation and the culture maintenance in the same laboratory. The cell suspension was developed and grown in shake flasks, airlift bioreactors and mechanically stirred bioreactors. My involvement in this part consisted of inoculating and harvesting microferm bioreactors and shake flasks for my further chemical experiments. The process of stabilization of this culture took about two years and included 60 subcultures. During this time more than 100 bioreactor incubations were performed.

4.1. INITIATION OF *TAXUS × MEDIA* CALLUS

The following steps were performed to initiate callus:

1. Stems of *Taxus x media* were cut into 1 cm length segments and sterilized with 30 % commercial bleach solution for ten minutes.
2. Sterilized inoculate material was washed with sterilized water five times.
3. Explants were placed in petri dishes on solidified nutrient media (Gambourg B5 media[58] supplemented with 2.5 - 3 % sucrose, double vitamins, 0.2% casein hydrolysate and 1 mg/mL of 2,4-D) and kept in darkness at 26 °C.

4.2. MAITENANCE OF CALLUS

Induced callus may turn brown after subculturing. Tannin compounds present in cut stems cause this. Also, oxidation products of phenolic compounds found in the cells lead to darkening or reddening of the culture and inhibit growth. Polyvinylpolypyrolidine (PVP) was added at 1-1.5% of media in order to suppress production of tannins or phenolic compounds. It has been found that PVP is the most effective in uptake of these phenolic compounds in callus culture without affecting growth.[71]

Callus was transferred to new solid media every two weeks and was maintained using conditions identical to those of initiation with the exception of the addition of PVP.

4.3. ESTABLISHMENT OF SUSPENSION CULTURES

Callus is subcultured for about 10 times. If it grows quickly and does not turn brown, then it can be used for the establishment of suspension culture.

Suspension cultures were established by transferring callus tissue into Erlenmeyer flasks containing Gambourg B5 liquid media. The cultures were kept in the dark at 26 °C on a rotary shaker set at 135 rpm. Every 14 days the cell lines were propagated by transferring the culture into new media at a 1: 10 inoculum ratio.

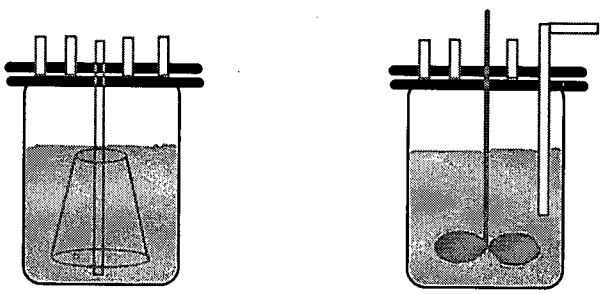
4.4. SHAKE FLASKS AND SCALE-UP IN BIOREACTORS

After achieving successful flask suspension cultures, the cells can be further grown in shake flasks and scaled up in bioreactors. Media composition is the same as in the suspension culture.

In shake flasks experiments the inoculation rate was 1:10. Each flask contained 300 mL of media, at temp. =26°C. The shaker speed was adjusted to 120-135 rpm.

In bioreactor experiments, 14-L bioreactors were used for scaling-up. The inoculation rate was 1:10. In case foam formed, an anti-foam solution (1-2 mL) was added.

There are two kinds of bioreactors used for our experiments (**Figure 30**). First is the Microferm, with mechanical stirring. The second is the Labroferm, with airlift.



Labroferm Microferm

Figure 30. Schematic representation of the two different bioreactors in use

4.5 PREPARATION AND COMPOSITION OF MEDIA

The modified Gambourg B5 medium[58] was prepared from stock solutions described in **Table 2**.

Stock solution / Component	Volume of stock solution (mL/L)	Amount used (g/L)	Components	Concentration (g/L)
B5 Macronutrients	50.00		KNO_3	50.000
			$(NH_4)_2SO_4$	2.680
			$NaH_2PO_4 \cdot H_2O$	3.000
			$MgSO_4 \cdot 7\ H_2O$ *	5.000
Calcium chloride	3.75		$CaCl_2 \cdot 2\ H_2O$	40.000
Iron chelate	9.30		$Na_2EDTA \cdot 2\ H_2O$	4.010
			$FeSO_4 \cdot 7\ H_2O$	2.990
B5 Micronutrients	5.00		H_3BO_3	0.600
			$MnSO_4 \cdot H_2O$	2.000
			$ZnSO_4 \cdot 7\ H_2O$	0.600
B5 Modified trace elements	1.00		KI	0.750
			$Na_2MoO_4 \cdot 2\ H_2O$	0.250
			$CoCl_2 \cdot 6\ H_2O$	0.250
			$CuSO_4 \cdot 5\ H_2O$	0.250
B5 Vitamins	20.00		Inositol	10.000
			Nicotinic acid	0.100
			Pyridoxine · HCl	0.100
			Thiamine · HCl	1.000
2,4-D	2.00		2,4-Dichlorophenoxyacetic acid**	0.500
Sucrose		25.000		
Casein hydrolysate		2.000		

* Dissolve $MgSO_4 \cdot 7\ H_2O$ separately and then add to solution of the other components.

** Dissolve in 200 ml EtOH and make up the volume with water to 1000 mL.

Table 2. Media Composition

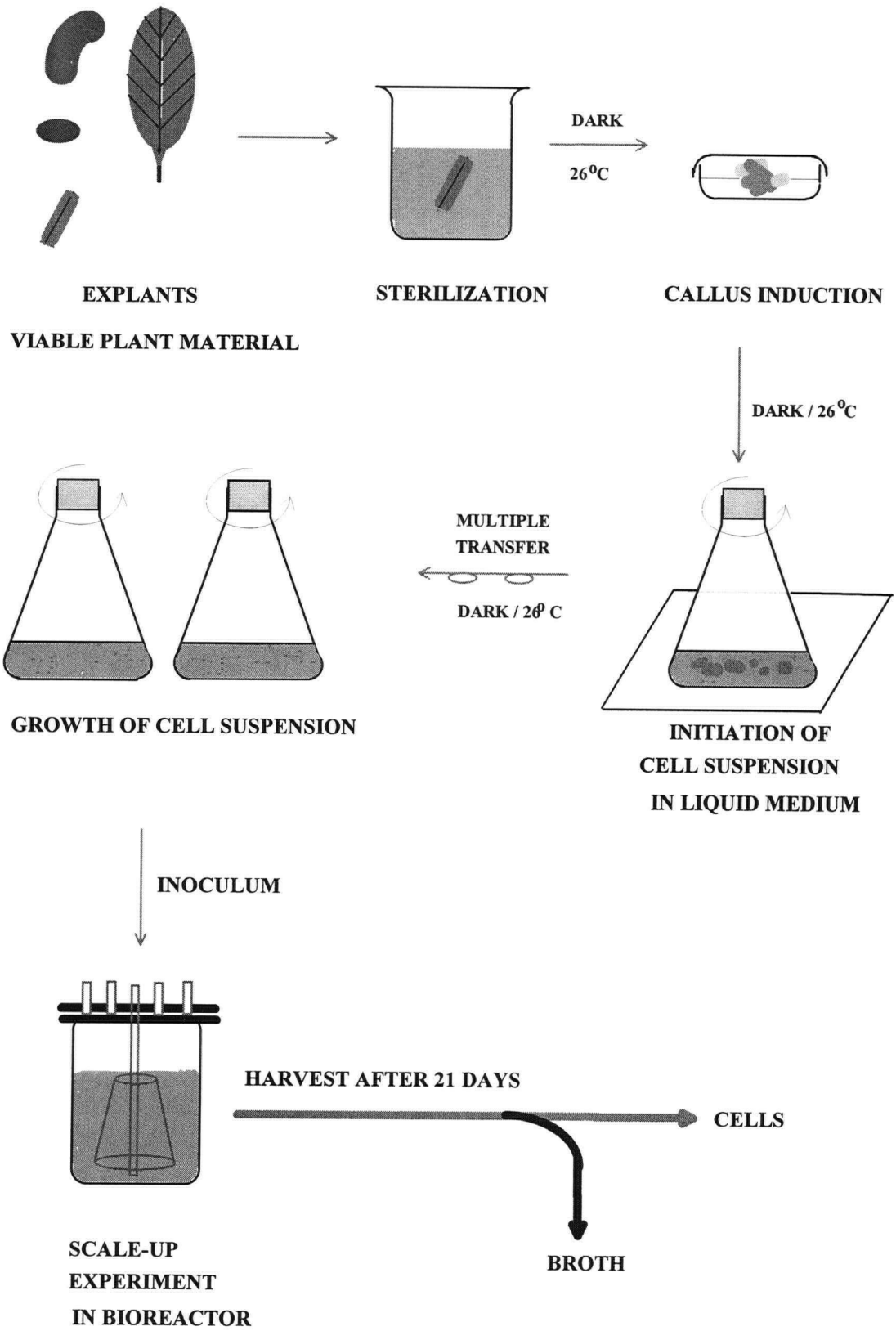


Figure 31. Initiation, propagation and scale-up of cell suspension culture

Medium prepared in this way was used as a standard media for subculture and growth experiments. Solid media is created by addition of 8 g/L of agar. pH is adjusted to 5.5 prior to autoclavation.

The whole procedure of initiation, propagation and scale-up of cell suspension culture in bioreactors is summarized in **Figure 31.**

4.6. EVALUATION OF TAXANE PRODUCTION IN *TAXUS × MEDIA* PLANT CELL CULTURE

In order to examine plant cell culture for Taxol production, rapid screening methods for taxanes had to be applied. Although several methods have been developed for this purpose, HPLC analysis remains the method of choice for screening of cell culture for taxane production.

In our group several HPLC conditions have been developed for the analysis of taxanes in extracts of *Taxus × media* culture. The analysis, which we have carried out, was reverse phase separation with a gradient solvent system containing water/acetonotrile/tetrahydrofuran (**Table 3**). This separation system allows the complete separation of Taxol, baccatin III, and their analogues (which were used as standards). The UV absorbance at 254 nm was monitored in four separate solvent systems (B4, B6, D1 and D4) at a flow rate of 1 mL/min.

Solvent systems	CH_3CN	THF	H_2O
B4	20	20	60
B6	15	20	65
D1	20	-	80
D4	40	-	60

Table 3. Composition of solvent systems used for HPLC analysis (%)

4.6.1 HPLC Calibration for Taxanes

Calibration was done for eight standard compounds given in **Table 4** with their retention times. The standard compound (0.2-1.0 mg) was weighed on a precision balance (±0.005 mg) and dissolved in 1.00 mL of methanol. The concentration of each solution was calculated. From each standard solution 1.0, 2.0, 5.0, 10.0, 20.0, 50.0 and 100.0 μL were injected. The area of the peaks (absorbance units × minutes) was plotted against the injected amount. The best-fit line gave equations, which can be used for quantitative determination of taxanes by HPLC.

Calibration curves were prepared for each of the eight standards by correlating peak area with known amounts of the pure compounds in methanol. These curves were used to obtain the retention times and the amount of the various taxanes in mg/L as well as minimum detection limits. Sensitivity of the system was found to be 0.5 μg/mL for all the compounds except baccatin III, which was 0.03 μg/mL and cephalomannine which was 0.9 μg/mL.

Standards	**Solvent System**			
	B4	**B6**	**D1**	**D4**
Baccatin III	-	8.36	32.63	5.80
14-hydroxybaccatin	4.46	5.43	12.63	4.13
14-hydroxy-10-desacethylbaccatin	4.50	5.43	12.66	4.16
10-desacethylbaccatin	4.56	5.46	12.56	4.23
Taxol	14.63	30.80	-	14.46
Cephalomannine	12.36	23.73	-	12.03
7-epitaxol	20.30		18.03	22.06
7-methyltaxol	24.46	-	-	25.16

Table 4. Standards and their retention times (in minutes)

4.6.2. HPLC Analysis of *Taxus × media* extracts

HPLC analysis was performed for our extracts in order to check the stability of the cell line and to obtain information about possible taxane production. A Supelcosil LC-F column 150 x 4 mm was used in a Waters HPLC system (HPLC pump model 6000A, Auto sampler Model 712, Model 440 UV Absorbance Detector, and 730 Data module). The UV absorbance at 254 nm was monitored in four separate solvent systems (B4, B6, D1 and D4) at a flow rate of 1 mL/min.

Before injection, all samples were filtered through HV filter paper (pore size 0.45 nm, Millipore) and prepared in methanol solution at a concentration of 1 mg/mL. Samples were run with different injection volumes and one or more times when necessary. Optimum sample injections volumes were determined to be 20.0μL and 40.0μL (+ 0.3%).

Although standard retention times for each solvent have been determined, usually standards are run again each time with a sample to compare results. Some fluctuation of the retention times from the tabulated ones were observed and they are due to variations in temperature, in pressure that was applied or in solvent composition.

Also comparison of HPLC and TLC profiles of the extracts after each subculture is important to determine if the cell culture is stable (produce roughly the same compounds consistently).

Additionally, this comparison was useful for decisions made on combining of certain extracts, which was very useful for accumulation of extracted material for further successful chromatographic separation, and for obtaining sufficient quantities of compounds for complete spectral analysis.

5. MICROFERM EXPERIMENTS

RESULTS AND DISCUSSION

Bioreactors with mechanical stirring were inoculated with suspension cell cultures from Erlenmeyer flasks harvested at 14 days of growth. The inoculum ratio was 1:10. In this way a "stabilized" cell line was scaled-up and grown in 14-L bioreactors. Each Microferm bioreactor culture was harvested after 28 days of growth. Prior to harvesting the refractive index was checked. This was done to ensure that sugars had been consumed and therefore the cells had commenced secondary metabolism. At the time of harvest the RI was ~1.3333 for all processed bioreactors. The pH value ranged between 5-6 at the time of harvest for "healthy" bioreactors. High pH (9-10) was an indication of a possible contamination. Higher pH was also related to a darker, brownish culture color.

After harvesting, the content of each bioreactor was filtered through three layers of Miracloth® in order to separate cells from broth. Broth was immediately extracted with ethyl acetate (EtOAc) three times and methanol once (MeOH). Cells were freeze-dried and after that processed in a similar manner as described later.

5.1. EXTRACTION OF BROTH

After filtration around 8 to 9 L of broth was obtained from each bioreactor experiment. Broth was extracted immediately after filtration from cells using the following procedure. In a 4-L separatory funnel portions of broth (two liters in one portion) were extracted three times each with 700 mL of EtOAc. If smaller amounts of EtOAc were used, there was usually an emulsion

formed after shaking in the separatory funnel. The EtOAc extract was dried with Na_2SO_4 for at least two hours. After drying it was filtered using #1 Whatman filter paper. On the same day extracts were concentrated on a rotoevaporator and dried using a high vacuum.

5.2. EXTRACTION OF CELLS

After filtration, cells were freeze-dried. Dried cells were weighed, and after that, frozen using liquid nitrogen and then pulverized using a mortar and pestle. Processed in this way, cells were extracted three times with EtOAc. The last EtOAc extract was then filtered from cells (#1 Whatman filter paper), and added to other portions of EtOAc extract. After that the same cells were extracted once with MeOH, and this extract was filtered again. Both extracts were separately concentrated on rotovaps and dried using a high vacuum.

The whole procedure with bioreactor culture is summarized in **Figure 32**.

As previously mentioned, the process of stabilization of this culture took about two years. There were numerous difficulties to avoid contamination. Contaminated bioreactor cultures were discarded. During this time of optimization and stabilization more than 100 bioreactor incubations were performed including both microferm and labroferm experiments.

Based on our experience with early attempts to chromatograph bioreactor extracts, it was necessary to accumulate sufficient amounts of extract to perform successful chromatography. On the other hand, if one has in mind the length of each experiment's growth period, and problems with contamination, it is obvious that the accumulation of extract required a lot of time. Our goal was to increase the production of biomass. Starting with Microferm number 79 it was decided that double inoculum (ratio 1:5) should be used to increase the amount of biomass and therefore the amount of extract. After comparison of the amount of extract produced from broth and cells

before and after double inoculation it was obvious that the amount of cell extract from each bioreactor increased and was greater than the corresponding amount of broth extract. This data is summarized in **Table 5**.

Although TLC comparison showed similarities it was decided not to combine them, and to go on with further purification with the cell extract. Studies on related broth extracts were done by Catia Seri.[74]

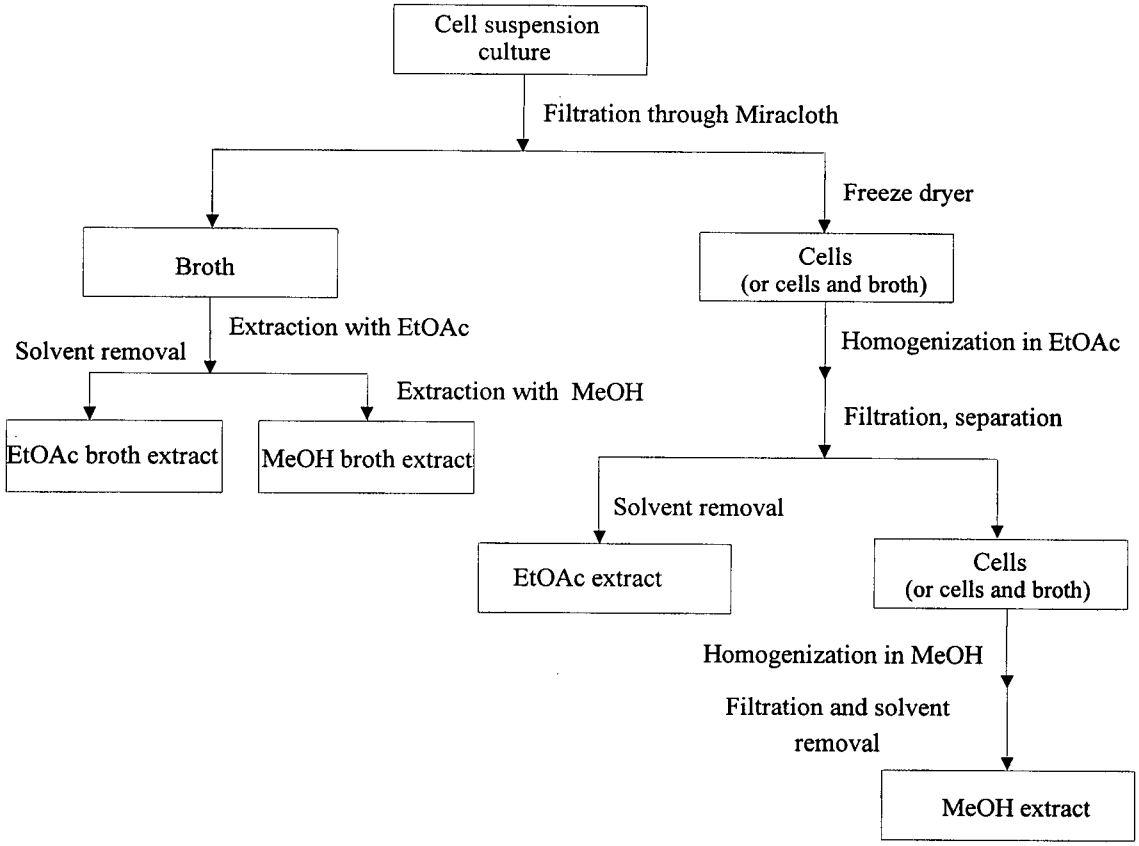


Figure 32. Bioreactor procedure after harvesting

Once we decided that a sufficient amount of extract was accumulated, extracts from different bioreactors were compared by TLC (mobile phase used was CH_2Cl_2: MeOH = 94: 6). A 10% sulfuric acid in methanol (immersion solution) was used, and then the plates were heated at 110°C for 2-3 minutes.

Figure 33 shows a typical TLC comparison of different bioreactor extracts.

For reasons of simplicity in further text F will be used instead of Fermentor and MF will be used instead of Microferm. The number that stands next to it is the experimantal number of the incubated bioreactor.

Microferm experiments in which isolation and purification of secondary metabolites was attempted included F58, F64, F68, F70, F71, F76, F78, F79, F82, and F90. Important data regarding those bioreactors is summarized in **Table 5.**

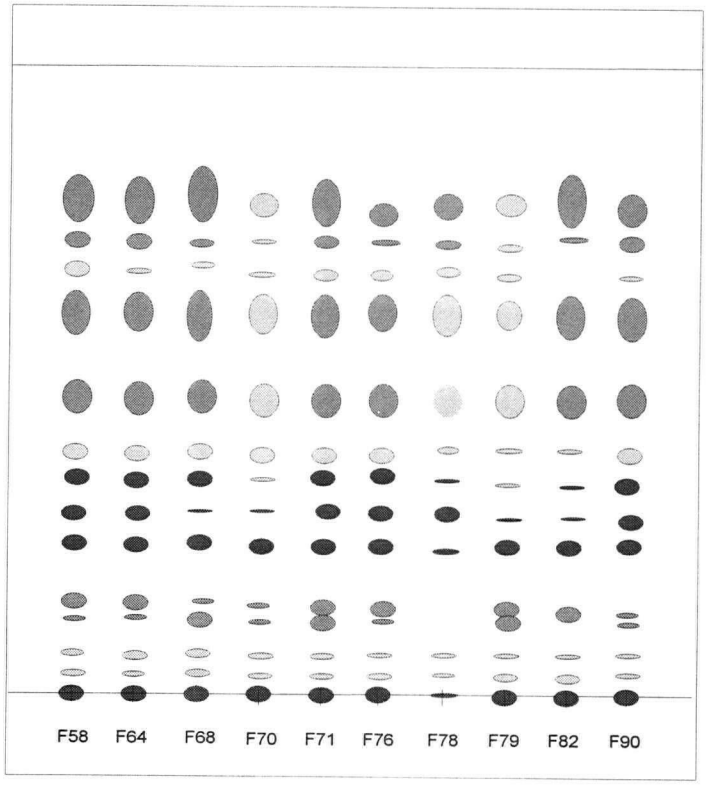


Figure 33. TLC comparison of F58, F64, F68, F70, F71, F76, F78, F79, F82, and F90 EtOAc extract

As previously mentioned, my experiments included cell EtOAc extracts. These extracts originating from the above bioreactors were compared by TLC and combined. Total weight of the combined extracts was 6.6717 g.

#	stirring	antifoam	initial pH	final pH	initial RI	final RI	cells dry weight (g)	broth EtOAc extr. (mg)	cells EtOAc extr. (mg)
F58	mechanical	200/10	5.70	8.41	1.3342	1.3340	36.58	775	317
F63	airlift	250/14	5.53	8.26	1.3339	1.3334	30.98	521	203
F64	mechanical	250/13	5.00	7.52	1.3339	1.3338	38.46	731	279
F66	airlift	1ml/15	5.74	7.34	1.3339	1.3336	45.24	403	218
F67	airlift	1ml/15	5.39	6.62	1.3340	1.3334	35.74	175	163
F68	mechanical	450/7	5.88	7.04	1.3339	1.3338	59.01	271	542
F70	mechanical	-	5.72	7.99	1.3340	1.3338	38.95	396	504
F73	airlift	-	5.96	6.62	1.3339	1.3330	47.43	160	410
F76	mechanical	-	5.36	8.44	1.3340	1.3339	33.73	593	699
F78	mechanical	-	5.20	9.10	1.3348	1.3340	49.91	542	460
F79	mechanical	-	5.40	5.90	1.3342	1.3338	54.55	358	1436
F82	mechanical	-	5.60	5.35	1.3340	1.3342	51.37	1183	1354
F90	mechanical	-	5.00	6.63	1.3349	1.3339	48.67	685	1081

Table 5. Bioreactor Experiments

5.3. PARTITION WITH SOLVENTS

Our group decided to start processing material by partition with various solvents. This procedure was based on a modified method published by Cardellina[75]. We chose this procedure after numerous unsuccessful chromatographies of crude extracts (previous work in our group) stemming from a complexity of extracts. An ideal extraction system for natural products is one, which would remove only the product(s) of interest, would do so completely, and would leave behind all the other compounds (components). In our case, processed in this way, the complex extract would be divided based on solubility in different solvents (polarity) into smaller sub-extracts suitable for further chromatographic procedures.

Combined cell EtOAc extract from F58, F64, F68, F70, F71, F76, F78, F79, F82, and F90 (total weight 6.6717 g) was dissolved in 250 mL MeOH: distilled water = 9: 1, and kept overnight at ~4°C. A precipitate was obtained and it was filtered off. Precipitated material was checked by TLC and it contained mainly compounds with similar or higher R_f than β-sitosterol.

The filtrated solution was extracted with hexane – 3 × 250 mL. The lower phase (MeOH: water) was left again at ~4°C overnight. No precipitate was obtained. This solution was adjusted to 25% water and extracted three times with 200 mL hexane: methylene chloride=2:1. The aqueous phase was then adjusted to 35% water and extracted with methylene chloride – 4 × 300 mL. Finally, the aqueous phase was freeze-dried, with previous elimination of organic solvent traces at reduced pressure. Organic phases were dried with Na_2SO_4, filtered and solvent removed under vacuum.

The whole procedure is summarized in the following scheme (MF= Microferm):

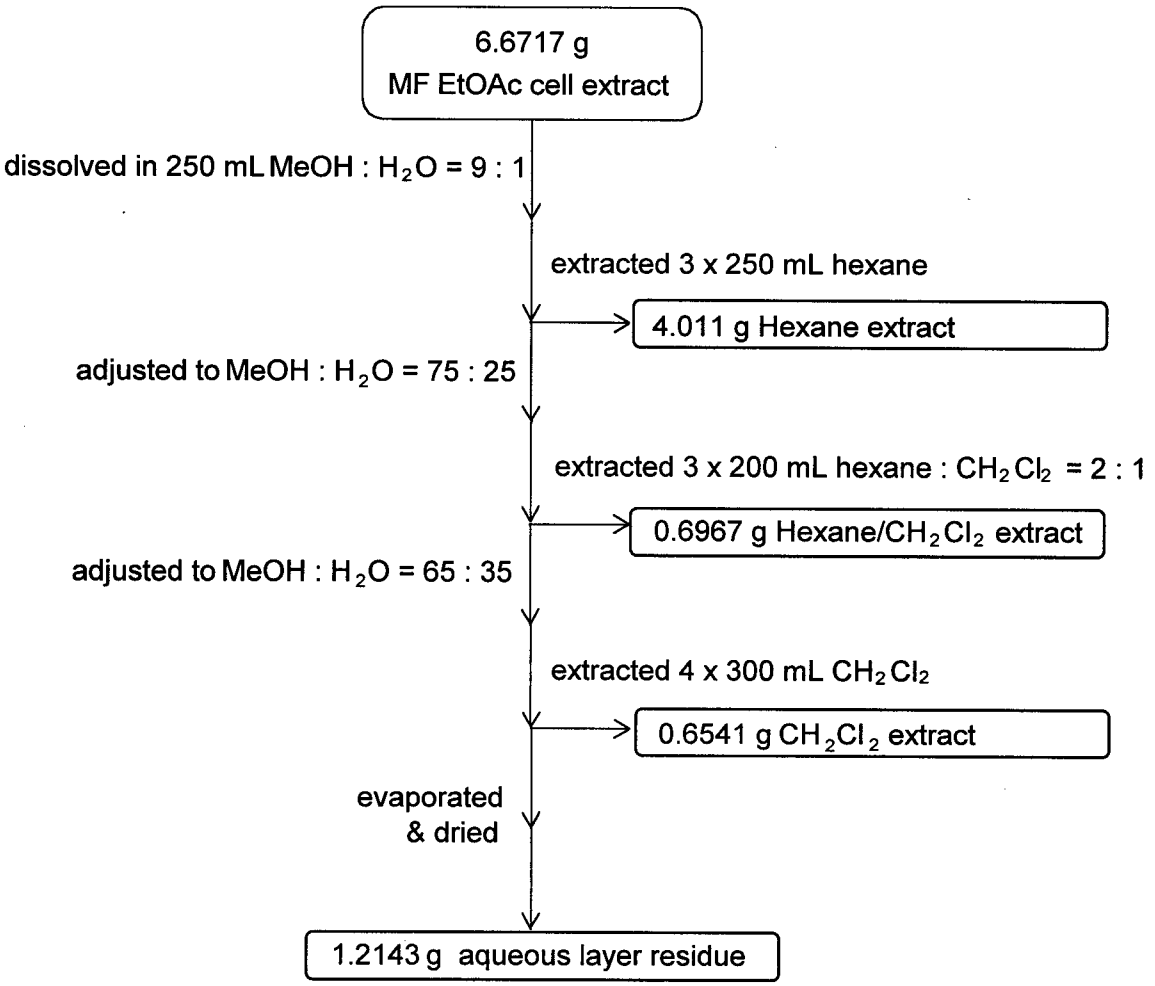


Figure 34. Partition of MF cell EtOAc extract

5.3.1. TLC Analysis of Sub-extracts

The mobile phase used depended on the processed sub-extract. For a non-polar, Hexane sub-extract, usually hexane: ethyl acetate = 8: 2. For those more polar, Hexanes: CH_2Cl_2 and CH_2Cl_2 sub-extracts, the solvent system CH_2Cl_2: MeOH = 94: 6 was used. A 10% sulfuric acid in methanol (immersion solution) was used, and then the plates were heated at 110°C for 2-3 minutes.

The Hexane sub-extract mostly showed the presence of compounds with R_f in the same region or higher than sterol standards.

The Hexane: Methylene Chloride sub-extract showed the presence of some non-polar compounds in the sterol region, but also showed spots in the taxane region.

The Methylene Chloride sub-extract showed presence of compounds with R_f in the taxane region and more polar compounds.

5.4. SEPARATION OF SUB-EXTRACTS

Based on the TLC comparison with our taxane standards it was decided that if taxanes were present they would be found in the Hexane: CH_2Cl_2 or CH_2Cl_2 sub-extracts. Therefore, a procedure was developed for further separation of these extracts.

5.4.1. Separation of the Hexane: CH_2Cl_2 Sub-extract

Flash chromatography, on a sintered glass funnel, (SiO_2 40–60 μ, Merck, 40g), was performed with the following solvent systems:

I	Hexane	200 mL
II	Hexane: CH_2Cl_2 = 1: 1	200 mL
III	CH_2Cl_2	200 mL
IV	CH_2Cl_2: EtOAc = 8: 2	200 mL
V	CH_2Cl_2: EtOAc = 1: 1	200 mL
VI	EtOAc	200 mL

VII	MeOH (twice)	200 mL

For each solvent system one fraction was collected, and the results are summarized in **Figure 35.**

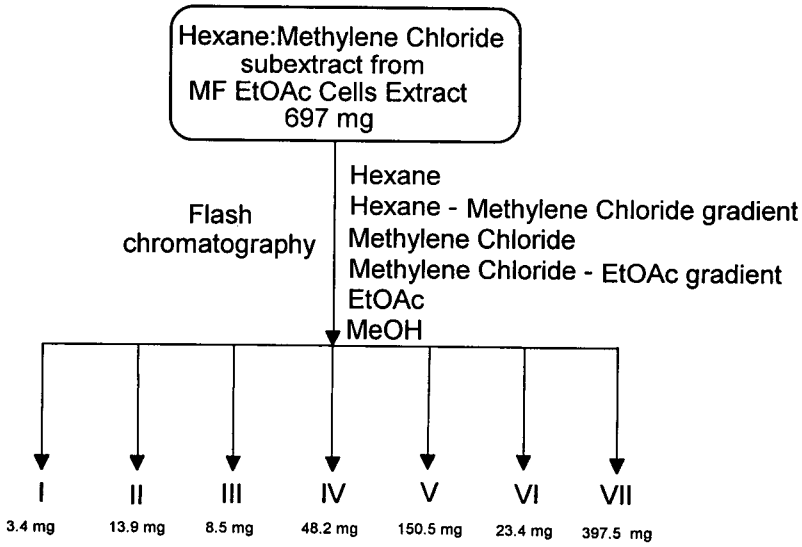


Figure 35. Separation of the Hexane: CH_2Cl_2 sub-extract

5.4.2. Separation of the CH_2Cl_2 Sub-extract

Flash chromatography, on a sintered glass funnel, (SiO_2 40–60 μ, Merck, 40g), was performed with the following solvent systems:

I	Hexane	200 mL
II	Hexane: EtOAc = 8: 2	200 mL
III	Hexane: EtOAc = 1: 1	200 mL
IV	Hexane: EtOAc = 3: 7	200 mL

V	EtOAc	200mL
VI	EtOAc: MeOH = 8: 2	200 mL
VII	MeOH (twice)	200 mL

For each solvent system one fraction was collected, and the results are summarized in **Figure 36.**

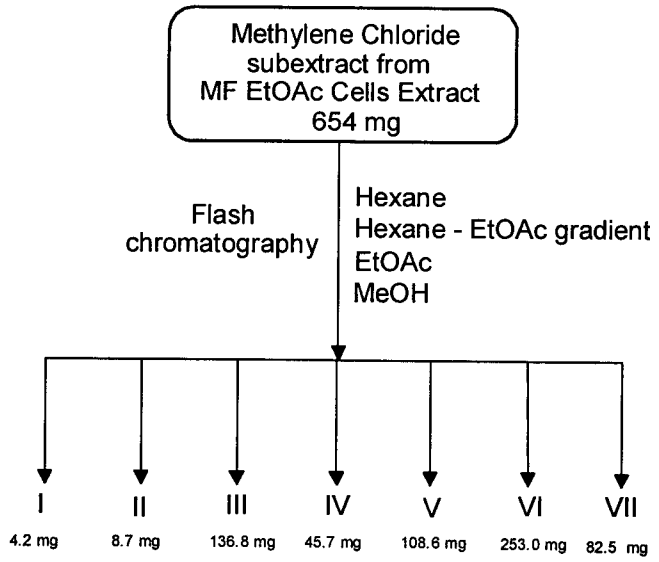


Figure 36. Separation of the CH_2Cl_2 sub-extract

5.4.3. TLC Analysis of Fractions

The TLC analysis of the fractions (CH_2Cl_2: MeOH = 94: 6) showed that fractions V and VI from Hexane: CH_2Cl_2 sub-extract and the fractions III to VI CH_2Cl_2 sub-extract were in the region of our taxane standards. HPLC analysis was performed on those fractions.

5.4.3. HPLC Analysis of Fractions

Sample	Inj. vol.	B4	B6	D1	D4
Fraction V Hex: CH_2Cl_2 sub-ext. (150 mg)	50 µL	2.03, 2.66, 3.06, 3.50, 3.93, **4.33**, 5.10, 11.66, 13.00, 18.26, 22.50, **24.50**, 26.00, 28.50, 31.00, 35.00	2.06, 3.03, 3.83, 4.93, 6.16, 7.50, **8.75**, 10.00, 13.50, 16.00, 17.00, 18.75, 20.75, **23.75**	1.33, 2.13, 2.56, 3.06, 3.73, 4.06, 4.86, 6.23, 8.16, 10.40	1.36, 1.96, 2.93, 3.73, **4.30**, 5.00, 8.26, 10.06, 11.75 17.33, 24.23, 30.43, 35.00
Fraction VI Hex: CH_2Cl_2 sub-ext. (23.4 mg)	40 µL	2.03, 3.06, 3.46, 6.93, 10.00, **12.50**, 15.50, 18.26	3.03, 3.43, 3.80, 10.66, 10.90, 16.25, 20.00, **23.25**, 26.50, 33.25	1.33, 2.10, 3.03, 3.80, 4.10, 4.86, 5.60	1.96, 2.93, 3.60, 4.63, **5.40**, 6.50, 7.86, 9.40, **12.00**, 17.23
Fraction III CH_2Cl_2 sub-ext. (136.8 mg)	40 µL	3.06, 3.53, 7.23, 10.50, 29.25	3.03, 3.50, 3.73, 4.83, 10.00, 12.50, 20.00	1.30, 2.33, 3.03, 3.80, 4.80, 5.80	2.93, 3.60, **4.26**, 5.03, 5.50, 13.96, 27.25
Fraction IV CH_2Cl_2 sub-ext. (45.7 mg)	50 µL	2.03, 3.06, **4.46**, 5.20, 38.26, 39.26	2.10, 3.03, **5.10**, 6.33	1.33, 2.06, 3.03, 3.73, 6.73, 7.46, 8.96, 10.50, 14.66	1.36, 1.96, 2.93, 3.70, **4.26**, 4.96, **5.63**, 8.96, 18.25, 20.50, 27.26, 33.20
Fraction V CH_2Cl_2 sub-ext. (108.6 mg)	50 µL	3.06, 3.50, 3.70, **4.58**, **4.75**, 6.66, 10.10, 13.00, 19.03, **21.90**	2.06, 3.03, 3.53, 3.86, 4.86, **5.68**, **5.80**, 12.23, 17.30, 18.36, 22.50, 26.30, **47.90**	1.36, 2.10, 3.00, 3.80, 4.63, 5.53, 6.60, 9.03, **13.50**, 15.50, 21.00, **34.53**, 55.00	1.36, 2.03, 2.96, 3.66, 3.96, **4.28**, **5.80**, 7.56, 9.43, 10.96, **12.53**, **14.70**, 17.06
Fraction VI CH_2Cl_2 sub-ext. (253 mg)	40 µL	2.13, 3.06, 3.40, **4.67**, **4.90**, 7.43, 10.36, 13.00, **15.75**, 30.75	3.03, 3.43, 3.86, **5.83**, 6.20, **8.86**, 9.90, 10.26, 11.03, 12.20, 22.00, **23.98**, 26.13, (31.16)*	1.33, 2.16, 3.03, 4.06, 4.73, 5.70, 10.50, **13.56**, 15.93, 19.00, 21.30, 25.30, **34.10**	2.16, 2.93, 3.63, **4.53**, 5.06, **5.56**, 6.43, 9.33, 11.03, **14.25**, **24.54**, 27.00

Retention times that match standard's retention time in a narrow range are reported in bold.

Table 6. HPLC data collected for different fractions from the Hexane: Methylene Chloride and the Methylene Chloride sub-extracts

HPLC samples of each of the sub-extracts were injected under the same conditions as described in section 4.6.2. to screen for the presence of our taxane standards. Injection volumes were optimized for each sample. **Table 6** summarizes the HPLC data.

As can be seen from the HPLC data, the more polar fractions V and VI from the CH_2Cl_2 sub-extract were the most interesting for us from the taxane point of view. HPLC analysis showed retention times that match the standard's retention time in a narrow range. It was decided that further separation of those fractions should be done.

5.5. SEPARATION OF FRACTION V FROM THE CH_2Cl_2 SUB-EXTRACT

Column chromatography was performed with a CH_2Cl_2-MeOH gradient and MeOH as a mobile phase (**Figure 37**). There was one major compound in fraction V and this compound was pure in fraction V-9. Other fractions were composed of three or more compounds making the further separation difficult because of an insufficient amount of each fraction. Other fractions were submitted for mass spectroscopic analysis to see if there was a fragmentation pattern characteristic for taxanes.

There was no fragmentation pattern observed that was characteristic for taxanes.

5.5.1. Mass Spectrometry Analysis

Studies have been done using Electron Impact Mass Spectrometry (EI MS) and Desorption Chemical Ionization Mass Spectrometry (DCI MS) in the positive and negative

mode. Our studies showed that the positive mode was the most sensitive with the equipment available.

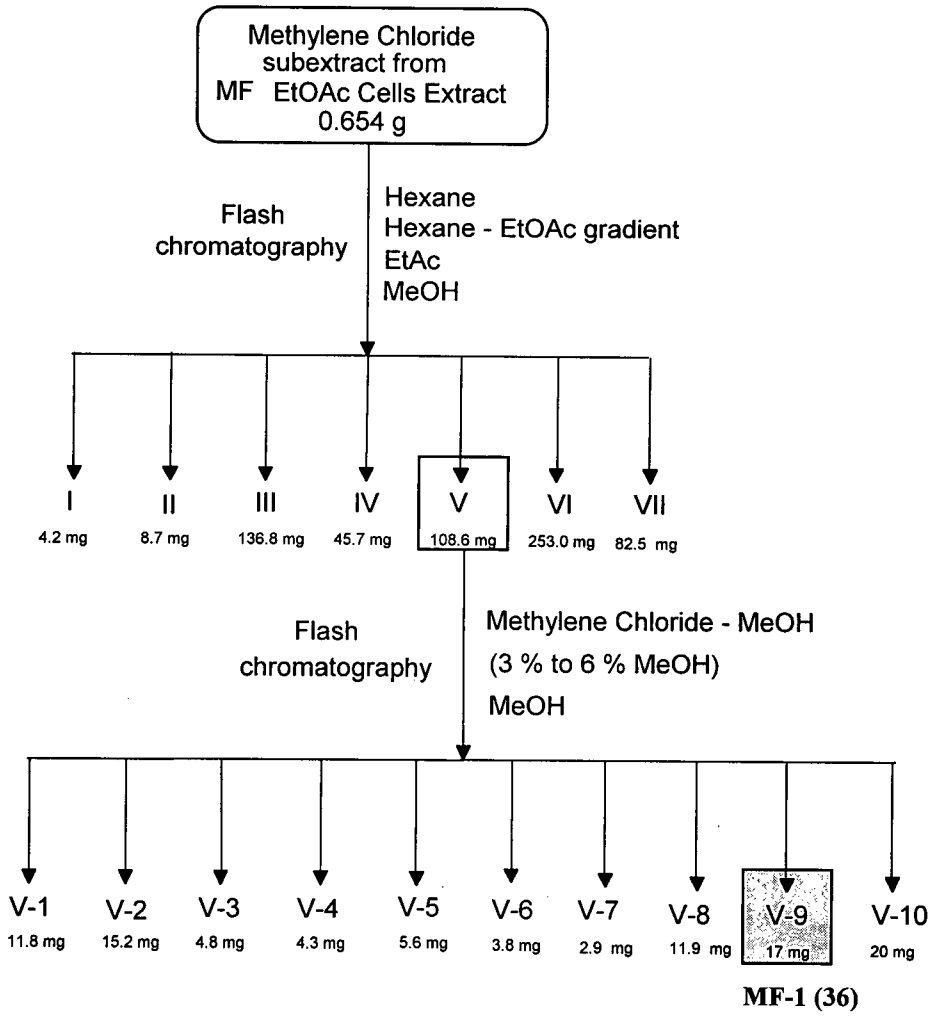


Figure 37. Separation of fraction V from the CH_2Cl_2 sub-extract

Table 7 summarizes fragmentation patterns obtained for some standards. Mass spectra of some sterols are also reported since those compounds are usually present in plant extracts and in plant cell extracts.

We found this kind of analysis very useful especially for the analysis of some fractions that contained from one to three compounds. In this way it was possible to get a general idea about the presence of any taxanes.

Standard	Method	Fragmentation
Taxol	DCI (+), NH_3	871, 838, 587, 528, 510, 466, 406, 389, 303, 286, 240, 212, 156, 139, 122, 98, 77
Baccatin III	DCI (+), NH_3	562, 544, 440, 424, 139, 112, 77
10-Deacetyl baccatin	DCI (+), NH_3	562, 544, 527, 440, 139, 126, 112, 98, 77
β-Sitosterol	DCI (+), NH_3	432, 414, 397, 381, 303, 273, 233, 156, 126, 112, 98, 86, 77
	EI (+)	414, 396, 381, 354, 329, 303, 273, 255, 231, 213, 199, 173, 159, 145, 133, 107, 105, 95, 91, 81, 69, 55,
Campesterol	EI (+)	400, 382, 315, 289, 273, 255, 231, 213, 199, 173, 159, 145, 119, 107, 95, 71, 43
Stigmastanol	EI (+)	416, 401, 383, 290, 233, 215, 165, 147, 121, 107, 95, 69, 55,

Table 7. Mass spectrometry data of some standards

5.5.2. Analysis of Compound **MF-1** (**36**)

Compound **MF-1** was identified using spectroscopic methods.

The high resolution EI MS gave a molecular mass, which corresponds to a molecular formula of $C_{30}H_{48}O_5$, which suggested a triterpene structure. The two signals in low resolution EI MS (**Table 8**), 203 and 248 were characteristic of retro- Diels Alder fragmentation of oleananolic acid type pentacyclic triterpenes[74] (**Figure 38**).

MF-1	EI MS (%)	488(0.5), 470(2), 452(1.5), 440(1), 393(1.5), 288(6), 248(100), 222(20), 203(90), 189(17)

Table 8. Low resolution EI MS data of **MF-1** (**36**)

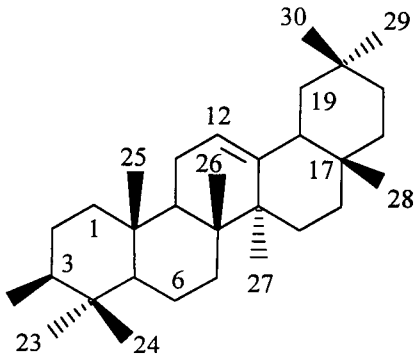


Figure 38. Oleanane skeleton

Signals at 222 and 204 gave an idea about the positions of the hydroxyl and carboxyl groups (**Figure 39**).

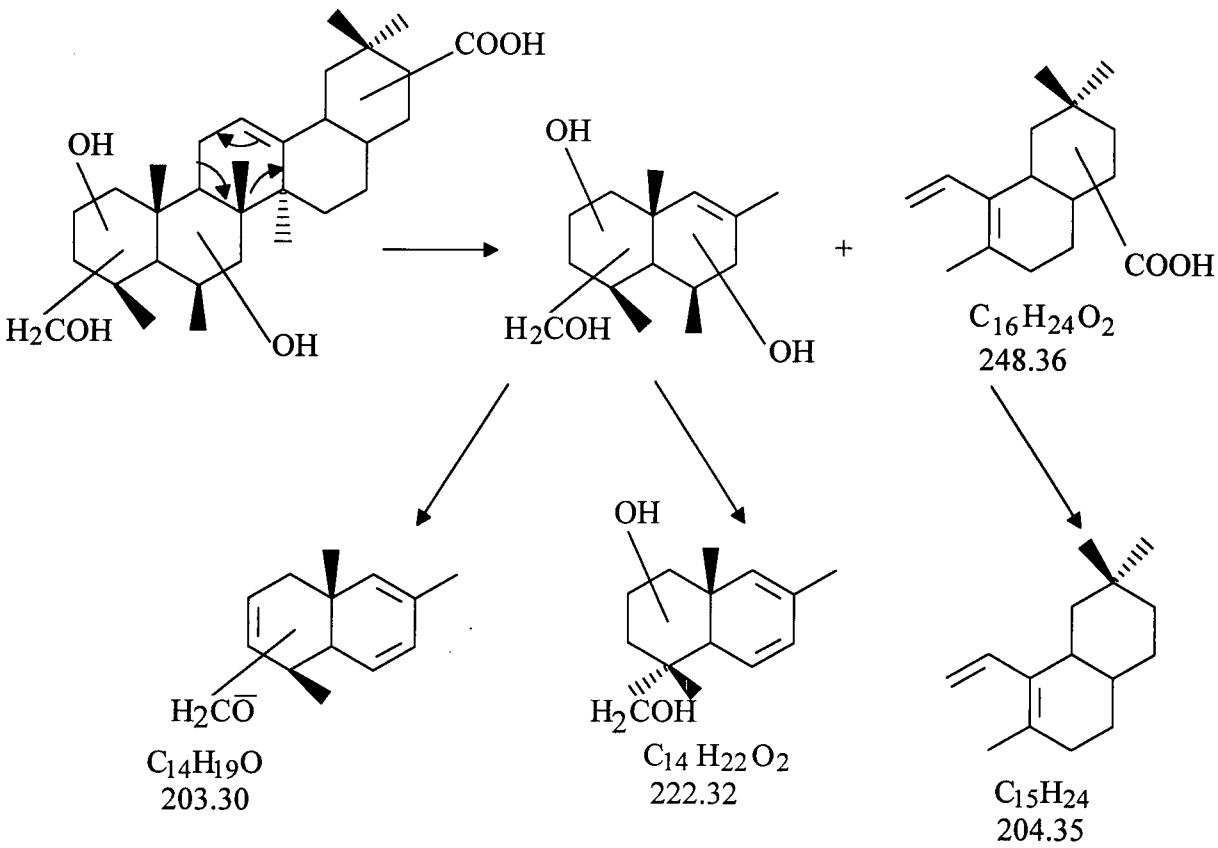
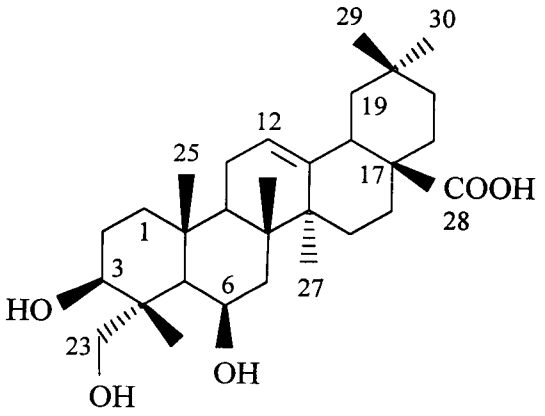


Figure 39. Suspected mass fragmentation of **MF-1 (36)**

Position	^{13}C δ	1H δ (J in Hz)
1	40.9	
2	27.8	
3	73.1	4.24 dd (4.0, 9.5)
4	44.0	
5	49.2	
6	67.4	5.02, br s
7	40.9	
8	39.0	
9	48.5	
10	36.8	
11	23.6	
12	122.6	5.56, t (3.5)
13	144.1	
14	42.5	
15	28.1	
16	23.6	
17	46.3	
18	41.9	3.33, dd (4.0, 9.2)
19	46.5	
20	30.8	
21	34.1	
22	33.1	
23	67.0	4.00, d (10.0) 4.37, d (10.0)
24	14.5	1.70, s
25	17.3	1.64, s
26	18.4	1.60, s
27	26.1	1.23, s
28	180.0	
29	23.7	0.97, s
30	33.1	0.89, s

Table 9. ^{1}H NMR (400 MHz) and ^{13}C NMR (75 MHz) data of compound **MF-1 (36)** in pyridine

A further literature search led us to the work of Mahato et al.[75] and 3β,6β,23-trihydroxy olean-28-oic acid as a correct structure of our compound (**Figure 40**).



36

Figure 40. Structure of **MF-1 (36)**

A comparison to published spectra[75] helped us to assign the resonances (**Table 9**).

Identification of compound **MF-1 (36)** was confirmed with additional analysis of ^{1}H COSY and ^{13}C APT.

5.6. SEPARATION OF FRACTION VI FROM THE CH_2Cl_2 SUB-EXTRACT

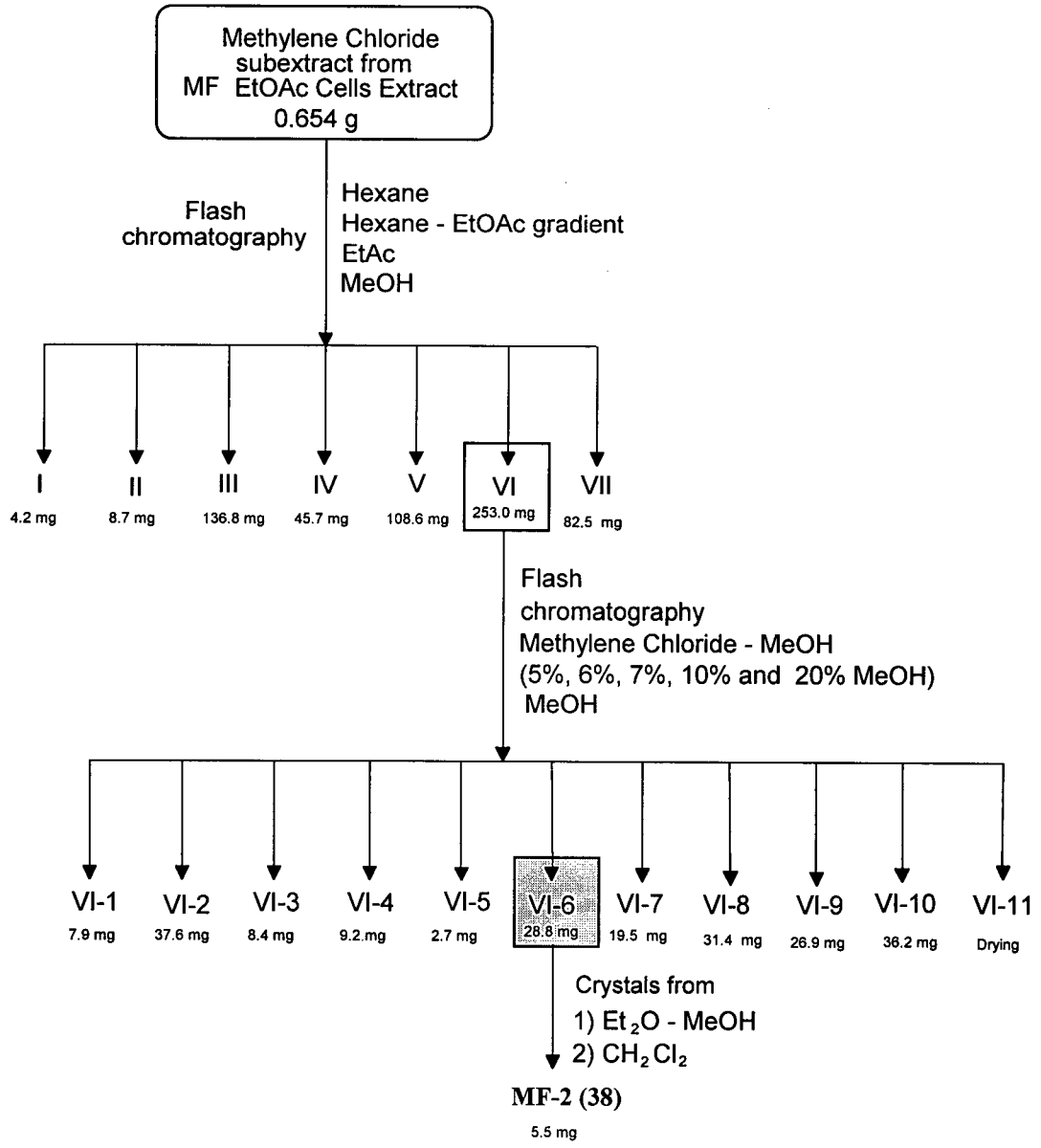


Figure 41. Separation of fraction VI from the CH_2Cl_2 sub-extract

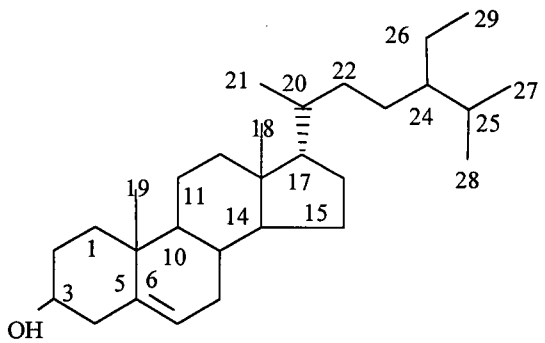
5.6.1. Analysis of Compound **MF-2 (38)**

The low resolution EI MS of **MF-2 (38)** was obtained and compared to β-Sitosterol **(Table 10)**.

MF-2	EI MS (%)	576(1.39), 415(9.68), 396(100), 382(23.8), 275 (11.3), 255(22.7), 229(8.0), 213(20.7), 121(26.5)
β-Sitosterol	EI MS (%)	414 (77), 396 (18), 382 (13), 275(3.5), 255(15), 213(17), 173(13), 121(14), 43(100)

Table 10. Low resolution EI MS data of **MF-2 (38)** in comparison to β-Sitosterol

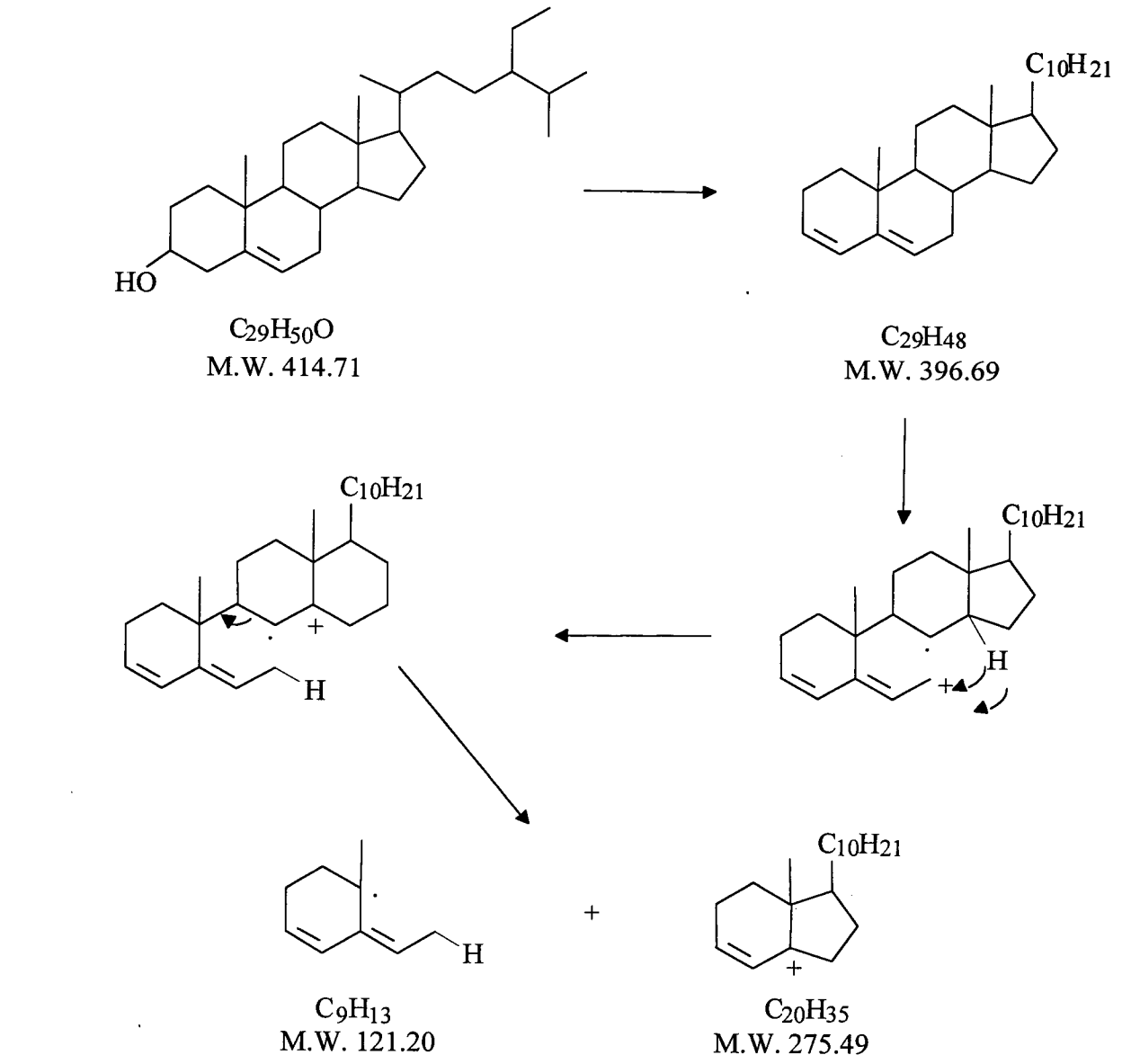
The mass spectrum of **MF-2** was obviously similar to that of β-sitosterol **(37,Figure 42)**. On the basis of this it was correctly assumed that this compound was a glycoside of β-sitosterol.



37

Figure 42. β-sitosterol

The high resolution EI MS gave a molecular mass corresponding to a molecular formula $C_{35}H_{60}O_6$. MS showed that after the loss of glucose the compound followed the fragmentation pattern of β-sitosterol shown in **Figure 43.**



also:

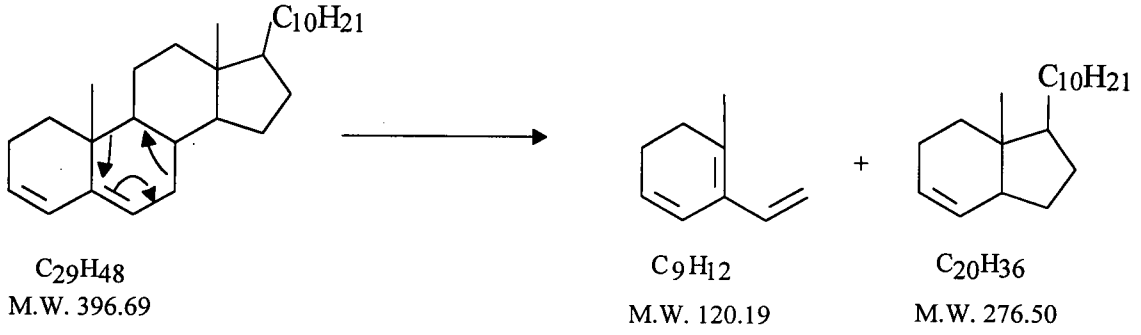
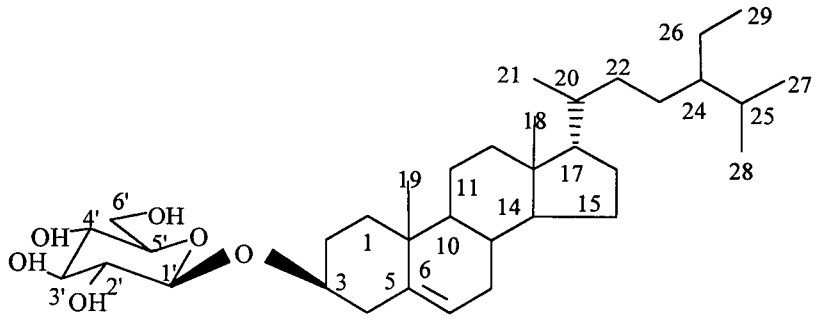


Figure 43. Suspected mass fragmentation of **MF-2 (38)**

Position	^{13}C δ	1H δ (J in Hz)
1	37.5	
2	30.2	
3	78.1	3.92, m
4	39.3	
5	140.9	
6	121.9	5.31, m
7	32.2	
8	32.0	
9	50.3	
10	36.9	
11	21.3	
12	39.9	
13	42.5	
14	56.8	
15	24.5	
16	28.5	
17	56.5	
18	12	0.62
19	19.4	0.90, d (6.5)
20	36.4	
21	19.0	0.95
22	34.2	
23	26.3	
24	46.0	
25	29.4	
26	19.2	0.82, d (6.5)
27	20.0	0.85, d (6.5)
28	23.4	
29	12.2	0.86, t (6.5)
1'	102.6	5.03, d (7.9)
2'	75.3	4.03, dd (7.9, 9.0)
3'	78.6	4.25, t (9.0)
4'	71.7	4.27, t (9.0)
5'	78.5	3.95, ddd (2.5, 5.0, 9.0)
6'	62.8	4.38, dd (5.0, 12.5) 4.53, dd (2.5, 12.5)

Table 11. ^{1}H NMR (400 MHz) and ^{13}C NMR (75 MHz) data of compound **MF-2 (38)** in pyridine

An inspection of the literature data led us to the work of Koizumi et al.[76] A thorough analysis of the ^{1}H and ^{13}C NMR spectra and comparison to the NMR shifts published by the same group allowed us to assign the resonances. Compound **MF-2** was identified as β-sitosterol-3-O-β-D-glucopyranoside (**38, Figure 44**).



38

Figure 44. Structure of **MF-2 (38)**

Table 11 summarizes the ^{1}H and ^{13}C NMR data and the assignments for the compound **MF-2 (38).** Those assignments were in the accordance with ^{1}H COSY and ^{13}C APT data.

6. SHAKE FLASK EXPERIMENTS

RESULTS AND DISCUSSION

Moving on to the shake flask experiments had a multiple importance. First of all we wanted to see if taxanes were produced under different conditions of growing from Microferm experiments. Secondly, we wanted to compare the production of secondary metabolites with Microferm experiments and in this way to see if the culture was producing the same compounds although it was grown under different conditions. This would be of great importance in further proposals on how to increase or initiate the taxane production once the cell culture is stable.

6.1. GROWTH OF SHAKE FLASK SUSPENSION CULTURE

After determining that the suspension cell culture was stable, it was grown further in shake flasks. Media composition was the same as in suspension culture. In shake flask experiments the inoculation rate was 1: 10. Each flask contained 300 mL of media, at temp. =26°C. The shaker speed was adjusted to 120-135 rpm. Shake flask culture was harvested after 28 days of growth. A check of pH and RI was important to give information about the behavior of the cell line. A high pH (9-10) for example is an indication of a possible contamination, while a high RI (1.3344) might be related to a low consumption of nutrients. Just like in the Microferm experiments, prior to harvesting, the refractive index was checked to ensure that sugars had been consumed and therefore the cells had commenced secondary metabolism (**Table 12**). The pH value ranged between 5-6.5 at the time of harvest for "healthy" shake flasks (**Table 12**).

6.2. EXTRACTION OF BROTH AND CELLS

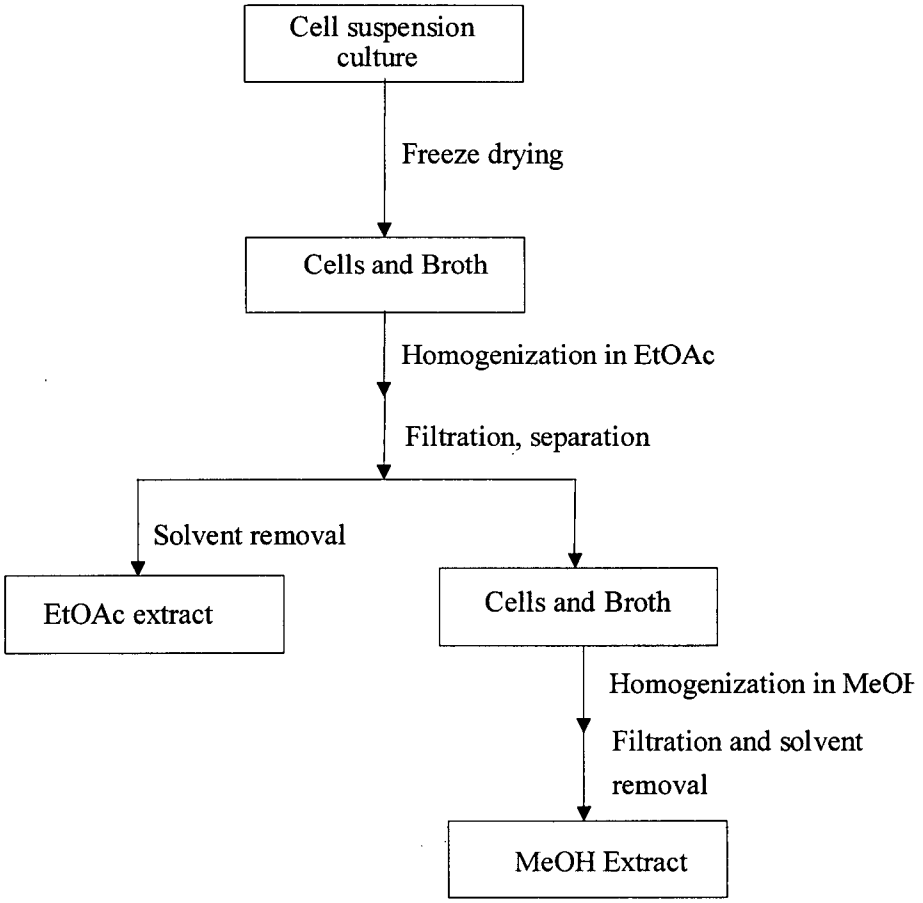


Figure 45. Shake flask procedure after harvesting

After harvesting, the next procedure with the culture was somewhat different from the one with bioreactors. Due to good growth of cells and thickness of the harvested biomass, cells were not filtered from the broth. Cells and broth together were freeze-dried. After that the weight of the biomass was recorded, after what it was frozen using liquid nitrogen and then pulverized using a mortar and a pestle. Processed in this way the biomass was extracted three times with EtOAc. The last EtOAc extract was then filtered from the biomass (#1 Whatman filter paper), and added to other portions of EtOAc extract. After that the biomass was extracted

once with MeOH, and this extract was filtered again from the biomass (#1 Whatman filter paper). Both extracts were separately concentrated on rotovaps and dried using a high vacuum. The whole procedure with shake flask culture is summarized in **Figure 45**.

# of Transfer	# of Flasks (approx. 440ml/ flask)	initial pH	final pH	initial RI	final RI	cells dry weight (g)	broth and cell EtOAc extr. (g)
56T	6	5.35	6.62	1.3341	1.3337	31.80	1.4546
57T	19	5.53	6.83	1.3340	1.3334	101.65	2.6470
58T	15	5.00	6.05	1.3339	1.3334	81.45	1.1845
59T	28	5.28	6.68	1.3339	1.3333	170.28	2.6792
63T	10	5.39	6.66	1.3343	1.3334	56.90	1.5472
64T	26	5.48	6.78	1.3341	1.3333	142.48	0.6788
66T	27	5.30	6.59	1.3340	1.3333	165.87	2.9786
67T	36	5.38	6.62	1.3339	1.3334	192.78	3.6664
68T	27	5.18	5.98	1.3343	1.3335	143.65	3.405

Table 12. Shake flask experiments

After the experience with Microferm experiments it was shown that it was a good idea to accumulate larger amounts of EtOAc extract and then start with separations. This time it was decided to start with an even larger amount of extract – at least 20 grams. The accumulated material was differentiated in the following way. Each transfer of the suspension culture to shake flasks for further growth had a number and T standing next to it. For example, 63T tells about the 63rd experiment or transfer of suspension culture (inoculation) into shake flasks. One

experiment included from 20 to 40 shake flasks. The volume of culture grown in one shake flask was 440 mL.

Important data regarding shake flask experiments is summarized in **Table 12**.

Once a sufficient amount of extract was accumulated, extracts from different shake flask transfers were compared by TLC (CH_2Cl_2: MeOH = 94: 6) and combined. My shake flask experiments included 56T, 57T, 58T, 59T, 63T, 64T, 66T, 67T and 68T. The total amount of accumulated extract was 20.2863 g.

6.3. PARTITION WITH SOLVENTS

The partition with various solvents was based on a modified method published by Cardellina[73] was carried out. The procedure is similar to that done in Microferm experiments (section 5.3). Results are summarized in **Figure 46** (SF = shake flask).

6.3.1. TLC Analysis of the Sub-extracts

For a non-polar sub-extract (hexane), usually hexane: ethyl acetate = 8: 2 was used as a mobile phase. The solvent system CH_2Cl_2: MeOH = 94: 6 was used as a mobile phase for more polar, Hexane: CH_2Cl_2 and CH_2Cl_2 sub-extracts.

A 10% sulfuric acid in methanol (immersion solution) was used, and then the plates were heated at 110°C for 2-3 minutes.

The Hexane sub-extract mostly showed the presence of compounds with an R_f in the same region as sterol standards.

The Hexane: Methylene Chloride sub-extract besides the presence of some non-polar compounds in the sterol region also showed spots in the taxane region.

The Methylene Chloride sub-extract showed the presence of compounds with an R_f less polar than for taxanes, in the taxane region and more polar compounds.

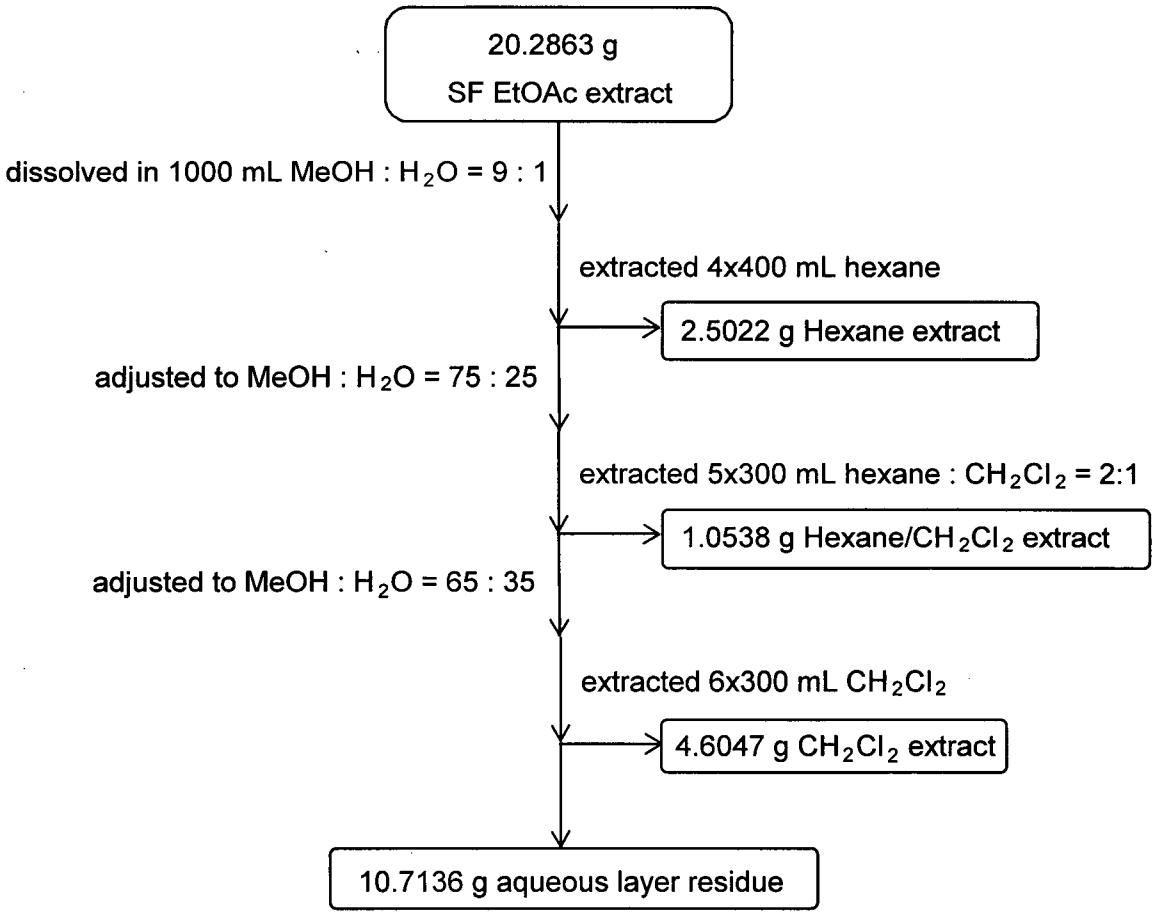


Figure 46. Solvent partition of the shake flask EtOAc extract

6.4. SEPARATION OF SUB-EXTRACTS

Similar to the conclusions in the Microferm experiments, and based on this TLC comparison with our taxane standards, it was again decided that if taxanes were present they would be found in the Hexane: CH_2Cl_2 or CH_2Cl_2 sub-extracts. A further separation of these extracts followed.

6.4.1. Separation of the Hexane: CH_2Cl_2 Sub-extract

Flash chromatography on a sintered glass funnel (SiO_2 10–40 μm, Sigma, 80 g) was performed, similar to that on p. 67. The following solvent systems were used as a mobile phase:

I	Hexane	500 mL
II	Hexane: CH_2Cl_2 = 1: 1	500 mL
III	CH_2Cl_2	500 mL
IV	CH_2Cl_2: EtOAc = 8: 2	500 mL
V	CH_2Cl_2: EtOAc = 1: 1	750 mL
VI	EtOAc	750 mL
VII	EtOAc: MeOH = 8: 2	750 mL
VIII	MeOH	1000 mL

One fraction for each solvent was collected and the results are summarized in **Figure 47.**

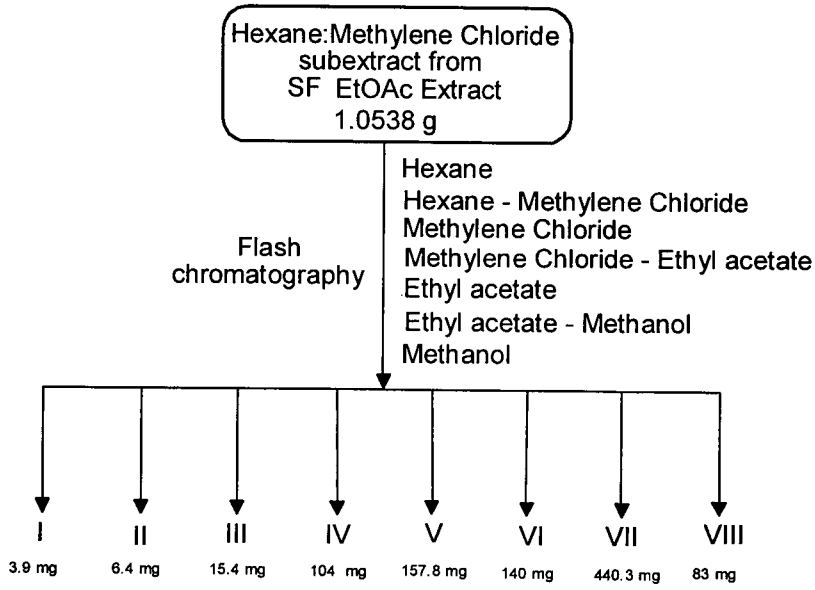


Figure 47. Separation of the Hexane: CH_2Cl_2 sub-extract

6.4.2. Separation of the CH_2Cl_2 Sub-extract

Flash chromatography, on a sintered glass funnel (SiO_2, 10–40 μm, Sigma, 300 g) was performed. The procedure is identical to that on p. 68, section 5.4.2., with the exception that different volumes of solvent systems were used.

I	Hexane	1200 mL
II	Hexane: EtOAc = 8: 2	1200 mL
III	Hexane: EtOAc = 1: 1	1200 mL
IV	Hexane: EtOAc = 3: 7	2000 mL
V	EtOAc	2000 mL
VI	EtOAc: MeOH = 8: 2	4000 mL

VII	MeOH	4000 mL

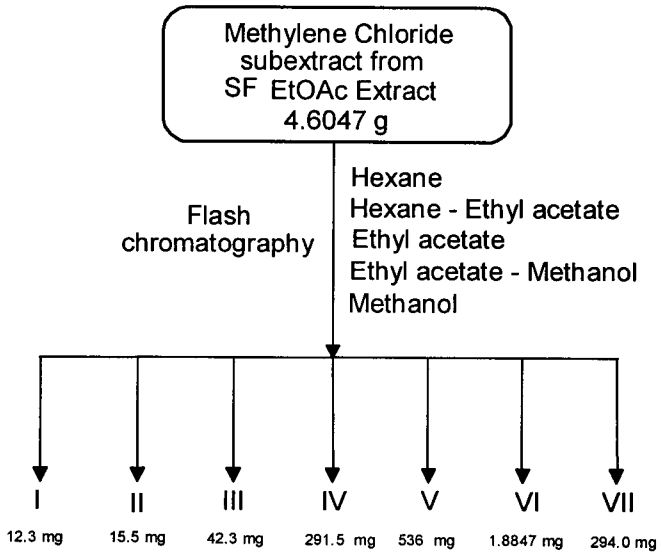


Figure 48. Separation of the CH_2Cl_2 sub-extract

For each solvent system one fraction was collected and the results are summarized in the **Figure 48**.

6.4.3. TLC Analysis of Fractions

For better separation and TLC results a new mobile phase was examined. This was isopropylether: acetone: acetic acid = 90: 5: 5. It showed excellent results, in fact the best results from all the examined solvent systems for the separation of this type of compounds, and it was decided that it should be employed in further separation and TLC analysis. A 10% sulfuric acid in methanol (immersion solution) was used, and then the plates were heated at 110°C for 2-3 minutes.

TLC analysis of the fractions showed that fractions IV, V and VI from the Hexane: CH_2Cl_2 sub-extract and fractions V and VI from the CH_2Cl_2 sub-extract were in the region of our taxane standards. It was decided that HPLC analysis was to be performed on those fractions.

Fraction VI from the CH_2Cl_2 sub-extract was especially interesting from the taxane point of view. It showed two gray spots (the same color taxanes develop under the above conditions), of which one had an R_f value very close to Taxol (**1**) and baccatin III (**2**) and the other showed an R_f value similar to that of 10-deacetylbaccatin (**11**).

6.4.4. HPLC Analysis of Fractions

HPLC samples of each of the sub-extracts were injected under the same conditions as described in section 5.4.3 to screen for the presence of our taxane standards. Injection volumes were optimized for each sample. **Table 14** summarizes the HPLC data.

As can be seen from the HPLC data, the more polar fractions V and VI from the CH_2Cl_2 sub-extract were the most interesting for us from the taxane point of view. HPLC analysis showed retention times that match the standard's retention time in a narrow range. It was decided that further separation of those fractions was to be done. I started with the fraction VI from the CH_2Cl_2 sub-extract because it was more interesting in regards to TLC analysis. The weight of this fraction was 1885 mg, which was relatively large in comparison to the other fractions. For that reason and especially because we considered it relatively important from the taxane point of view, it was decided that this fraction should be divided into two fractions, so that one could serve as a back up, in case different separation conditions were needed.

Sample	Inj. vol.	B4	B6	D1	D4
Fraction IV Hex: CH_2CL_2 sub-ext. (104 mg)	50μL	2.33, 2.86, 3.06, 3.50, 3.96, **4.33**, 7.88, 11.46, 22.50, **24.52**, 26.90, 28.88, 36.00	2.30, 2.90, 3.06, 3.50, 3.96, 4.83, **5.63**, 8.26, 12.25, **23.48**, 28.75, 39.25	2.36, 3.10, 3.90, 4.70, 5.53, 5.83, 6.76, 8.43, 10.36, 12.10, **12.66,** 24.11, 28.34	1.40, 2.90, 3.66, 5.00, 10.63, **14.75,** 15.31, 16.50, 20.03, 28.50
	20μL	2.30, 3.10, 3.63, **4.36**, **4.83**, 6.10, 6.63, 7.46, 18.30, 19.36	2.06, 3.06, 3.43, 3.80, 4.26, **5.70**, 6.13, 7.56, 9.03, 9.63, 11.60, 37.53	3.13, 3.60, 3.90, 4.20, 4.63, 6.83, 10.13, 11.13, **12.43**, 16.80	2.96, 3.63, **4.26**, 5.26, 5.63, 7.50, 12.20, 16.50, 20.00, **25.10**
Fraction V Hex: CH_2Cl_2 sub-ext. (157.8)	50μl	2.13, 2.76, 3.66, **4.33**, **4.86**, 6.10, 6.73, 7.66, 14.23, 18.90, 27.25, 36.50	2.76, 3.03, 3.83, 4.40, 8.50, 10.25, **27.75**, 41.50, 47.00	1.36, 2.23, 3.06, 3.96, 4.66, 5.75, 8.75, 10.50	0.83, 1.43, 2.20, 3.63, 4.96, 6.90, 7.90, 12.06, **16.73**, 42.75
Fraction VI Hex: CH_2Cl_2 sub-ext. (140 mg)	50μL	2.33, 2.86, 3.43, 5.96, 9.46, 13.72, 15.75, 19.00, **20.26**, 27.50, 33.00	2.06, 2.33, 2.90, 3.10, 3.33, 3.83, 4.40, 4.90	1.03, **1.33,** 2.30, 2.56, 3.16, 3.73, 4.46, 5.53, 5.80, 10.40	1.00, 1.40, 2.40, 2.90, 3.40, **4.86**
Fraction IV CH_2Cl_2 sub-ext. (291.5 mg)	50μL	3.73, 6.96, 7.33, 8.25, 9.86, **11.75**, 13.13, 30.25	3.93, 4.26, 4,73, **5.56**, 6.13, 9.63, 12.80, 13.46, 15.25, 16.63, 17.63, 28.50, **29.00**, 37.75, 42.75	4.33, 5.13, 5.86, 6.80, 8.83, 10.00, 10.76, **12.23**, 14.00, 16.50, 44.13, 67.50	3.76, **4.46**, 5.00, **5.90**, 7.25, 7.93, 29.7, 38.00
	20 μL	3.66, **4.43**, 7.03, 8.00	3.30, 3.86, 4.33, 5.03, **5.60**, 13.13, 13.88, 18.75	3.70, 4.10, 4.83, 6.13, 7.00, 8.70, 10.33, **12.83**	3.60, **4.45**, 5.00, **5.86**, 7.96

Retention times that match standard's retention time in a narrow range are reported in bold.

Table 14. HPLC data collected for different fractions from the Hexane: Methylene Chloride and the Methylene Chloride sub-extracts.

Fraction V CH_2Cl_2 sub-ext. (536 mg)	100μL	3.56, **4.58**, 5.30, 5.83, 8.13, 9.75, 11.50, **12.53**, 17.16, **19.89**	3.50, 3.90, **5.23**, **8.80**, 9.50, 12.63	4.16, 4.50, 5.20, 5.63, 6.23, 8.00, 9.25, **12.62**, 14.05 **18.00**	3.90, **4.40**, **6.03**, 7.26, 9.75, **12.30**, 17.00, **22.25**, **23.13**, 29.00, 29.25, 30.00
	20μL	4.03, **4.46**, **4.86**, 6.33, 7.03	3.36, 3.83, **5.66**, **8.73**, 9.50, 11.38	3.86, 4.13, 4.50, 5.36, 6.20, 7.63, 8.75, **18.13**	3.60, **4.43**, **5.40**, **6.25**, 7.38, 9.63, **12.50**, 17.13, 20.00
Fraction VI CH_2Cl_2 sub-ext.. (1884 mg)	20μL	**4.16**, **4.58** 7.00, 8.60, 10.00, 13.50, **14.80**,**15.75**, 18.30, 32.38, 40.00, 42.75, 51.25, 55.00	3.60, 4.30, 4.86, 6.20. 6.90, **8.80**, 9.00, 10.50, 14.00, 24.60	3.76, 4.06, 5.53, 7.00, 9.00, **11.75**, **18.13**, 25.00	3.83, **4.36**, 4.80, 7.50, 8.40, 9.75, **11.86**, **14.40**, 17.50, **23.50**, **25.00**, 35.00
	7μL	3.76, 4.00, **4.33**, **4.68**, 6.50, 8.50, 10.25, **14.25**, 15.88, 34.5, 35.25, 37.25	3.36, 3.73, 4.26, 4.83, **5.23**, 6.93, **8.88**, 14.00, 25.13, 25.75	3.86, 4.46, 5.26, 7.38, 10.13, **12.13**, 14.54, 15.66, **17.93**	3.60, 4.06, **4.06**, **6.02**, 7.50, 8.30, 9.75, **12.00**, **14.38**, 17.5, 25.00

Retention times that match standard's retention time in a narrow range are reported in bold.

Table 14. (continued) HPLC data collected for different fractions from the Hexane: Methylene Chloride and the Methylene Chloride sub-extracts.

6.5. SEPARATION OF FRACTION VI FROM SF CH_2Cl_2 SUB-EXTRACT

Figure 49 gives a graphical presentation of the separation work done on fraction VI from the CH_2Cl_2 shake flask sub-extract.

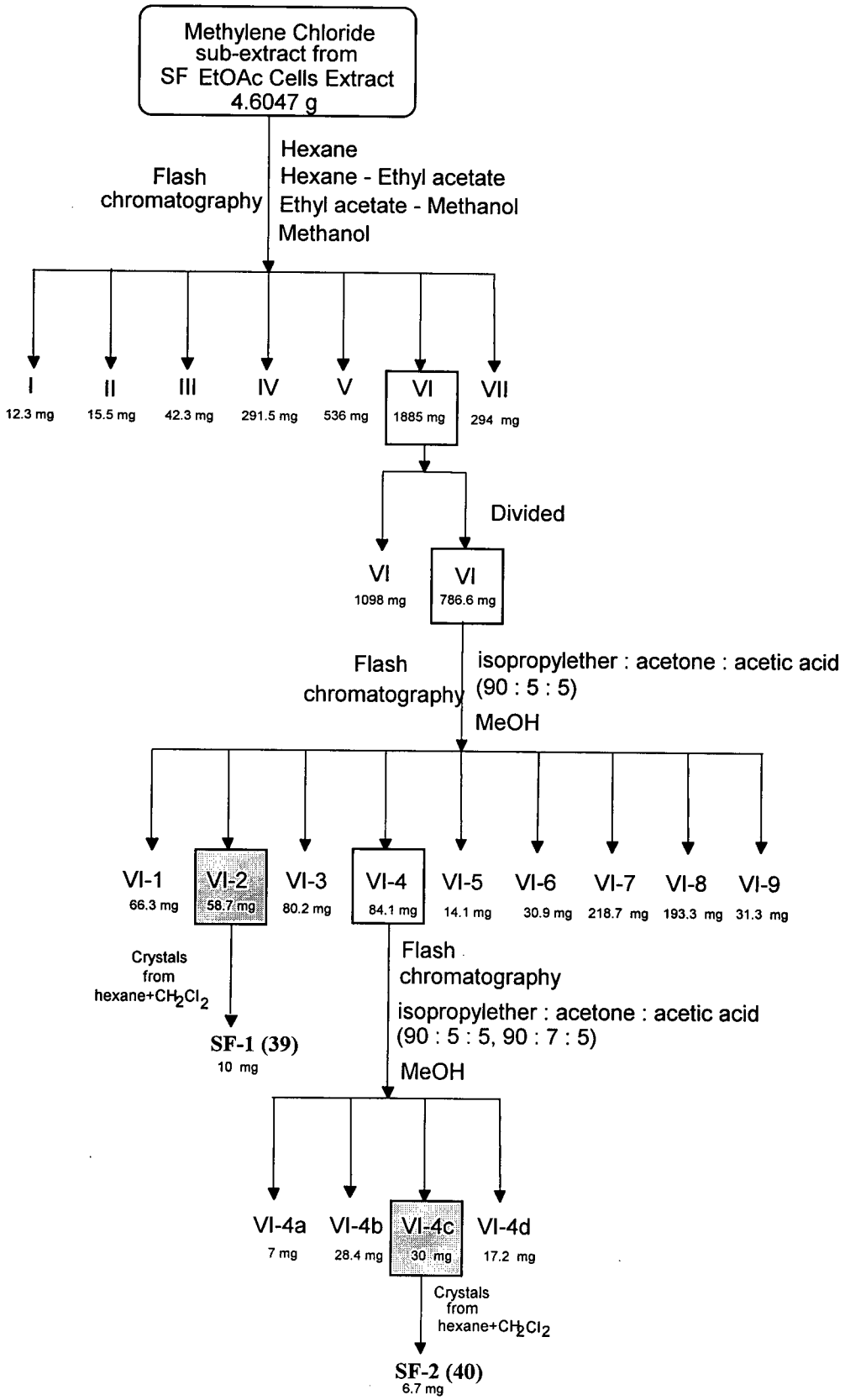


Figure 49. Separation of fraction VI from the SF CH_2Cl_2 sub-extract

6.5.1. Analysis of Compound **SF-1 (39)**

We paid special attention to the isolation and purification of this compound for two reasons. First, it developed a gray spot on a TLC plate (the same color taxanes develop under the same conditions) after being treated with 10% sulfuric acid in methanol (immersion solution) and secondly it had an R_f value very close to Taxol (**1**) and baccatin III (**2**).

Table 15 gives the low resolution EI MS of **SF-1 (39)**.

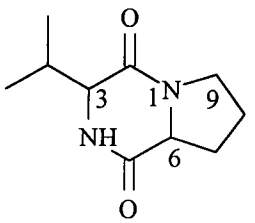
SF-1	EI MS (%)	196 (M^+, 3.4), 169 (1.4), 167 (1.3), 154 (100), 140 (1.4), 138 (4.4), 125 (40), 98 (7.7), 97 (5.7), 72 (29), 71 (8.0), 70 (88)

Table 15. Low resolution EI MS data for **SF-1 (39)**

It was obvious that this was not a taxane. Needless to say we were very disappointed.

The high resolution EI mass spectra gave a molecular formula of $C_{10}H_{16}O_2N_2$.

After an extensive literature search and comparison of the mass spectral and ^1H NMR characteristics with those published by various groups[77,78] we finally gathered enough clues to identify the compound. This data was identical to that previously published. The compound was identified as a diketopiperazine: cyclo-(Pro-Leu) (**39, Figure 50**).



39

Figure 50. Structure of **SF-1 (39)**

Position	1H δ (J)
methyls	0.93, d (7.05)
methyls	1.1, d (7.05)
	~1.8-2.6, m
H-9	3.6, m
H-3,6	3.98-4.10, m
H-4	5.87, b s

Table 16. ^{1}H NMR (400 MHz) of compound **SF-1** (**39**) in CD_2Cl_2

This compound had been previously isolated from fungi and plants.[77,78] We examined the possibility that it also could have been an artifact from the media, but we did not find any evidence for this.

6.5.2. Analysis of Compound **SF-2** (**40**)

This compound also developed a gray spot on a TLC plate (like taxanes) after being treated with 10% sulfuric acid in methanol (immersion solution), and it had an R_f value very close to 10-deacetylbaccatin III (**11**).

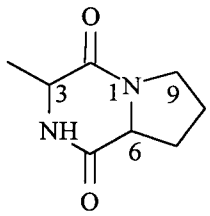
The low resolution EI mass data is given in **Table 17**:

SF-2	EI MS (%)	168 (M^+, 78), 140 (7.2), 125 (27.8), 97 (39.4), 70(100)

Table 17. Low resolution EI MS data for **SF-2** (**40**)

High resolution EI mass spectra suggested a molecular formula $C_8H_{12}O_2N_2$.

Comparison of the mass spectral and ^1H NMR signals with those published by various groups[77,78] helped us to identify this compound. Our data was identical to that published. The compound **SF-2** was identified as a diketopiperazine: cyclo-(Pro-Ala) (**40**, **Figure 54**).



40

Figure 51. Structure of **SF-2** (**40**)

Isolation of this metabolite from fungi and plants had also been previously reported.[77,78]

Position	1H δ (J)
methyl	1.43, d (6.80)
	~1.8-2.4, m
H-9	3.6, m
H-3,6	4.0-4.3, m
H-4	6.5, s

Table 18. ^1H NMR (400 MHz) data of compound **SF-2** (**40**) in CD_2Cl_2

6.6. SEPARATION OF FRACTION V FROM THE SF CH_2Cl_2 SUB-EXTRACT

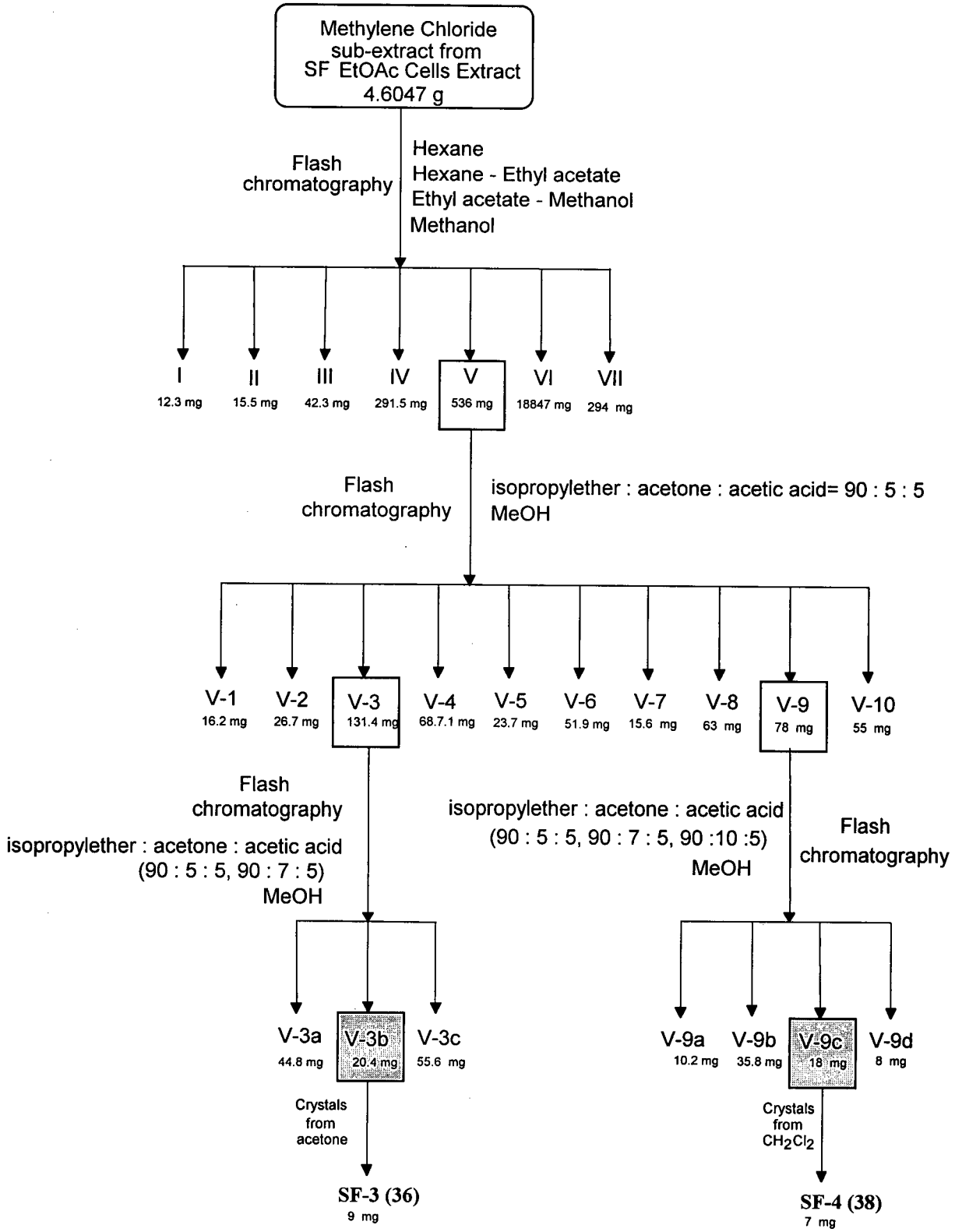


Figure 52. Separation of fraction V from the SF CH_2Cl_2 sub-extract

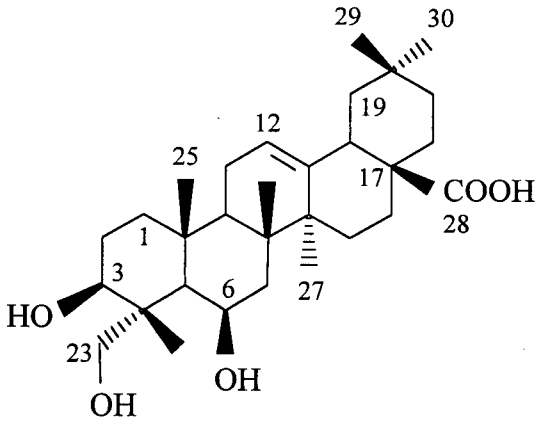
6.6.1. Analysis of Compound **SF-3 (36)**

Low resolution EI MS of **SF-3** gave the following signals (**Table 19**):

SF-3	EI MS (%)	488(0.5), 470(2), 452(1.5), 440(1), 393(1.5), 288(6), 248(100), 222(20), 203(90), 189(17)

Table 19. Low resolution EI MS data for **SF-3 (36)**

High resolution EI MS gave a molecular mass, which corresponds to a molecular formula of $C_{30}H_{48}O_5$. Other data obtained was also identical to that obtained for **MF-1** (refer to **Table 9**). The compound **SF-3** was therefore identified as 3β,6β,23-trihydroxy olean-28-oic acid (**36**).



36

Figure 53. Compound **SF-3 (36)**

6.6.2. Analysis of Compound **SF-4 (38)**

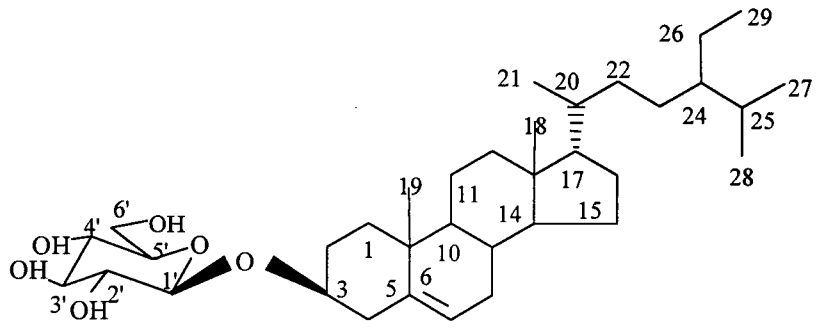
Low resolution EI MS of **SF-4** gave the following signals (**Table 19**):

SF-4	EI MS (%)	576(1.39), 415(9.68), 396(100), 382(23.8), 275 (11.3), 255(22.7), 229(8.0), 213(20.7), 121(26.5)

Table 19. Low resolution EI MS data for **SF-4 (38)**

High resolution EI MS gave a molecular mass corresponding to a molecular formula of $C_{35}H_{60}O_6$.

The mass spectrum of **SF-4** was obviously identical to that of **MF-2 (38, Figure 47)** as was the R_f value. ^1H and ^{13}C NMR data was also identical to that obtained for **MF-2** (refer to **Table 11**). On the basis of this, the compound **SF-4** was identified as β-sitosterol-3-O-β-D-glucopyranoside (**38**, **Figure 54**).



38

Figure 54. Compound **SF-4 (38)**

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

From our results it was obvious that the *Taxus × media* plant cell culture grown at UBC under different conditions is not producing Taxol or any taxanes. However, our group showed significant success in the work, because it came up with a strategy that allowed easier separation and purification of compounds. It showed that accumulation of a larger amount of extract (now that the culture is stable) is necessary in order to get sufficient amounts of pure compound(s) and to perform structure elucidation. A new solvent partitioning of the extract was applied and it was very effective in the elimination of less polar and very polar compounds that were not of interest.

The cell line now appears to be stable and to give reproducible results in applied purification steps. On the basis of this one can conclude that the culture is stable, problems with contamination have been solved, and that the culture, although grown under different conditions, is mostly producing the same compounds.

Considering these results it is our belief that a further combination of strategies should be examined in order to develop a taxane producing cell line.

These strategies should include the following:

- **Nutrient Manipulations** have been shown to influence the production and accumulation of secondary metabolites. For example, precursor-feeding approaches may be used for elevating taxane production.[79]

- **Osmotic Manipulations** should also be examined. Elevated carbohydrate levels, particularly sucrose, have been used to increase the accumulation of secondary products.[80] In addition to sucrose's role as a carbon source for cell growth, elevated levels of it can increase osmotic pressure, while inhibiting growth significantly. Other compounds[79] have also been used to induce osmotic stress in culture, which leads to the production of secondary metabolites.

- **Gas Composition Effects** are important because cells require an aerobic environment in addition to the nonvolatile dissolved nutrients.[80] Some gaseous components in the medium, primarily oxygen, carbon dioxide and ethylene play critical roles in growth and product formation, and their relative concentrations are also important in plant growth regulation.

- **Elicitor Treatments** are thought to mimic stress, similarly to those induced by pathogens. In this way they are thought to stimulate the production of secondary metabolites, which may act as defensive compounds inside the plant. It has been shown that Taxol could be induced as early as 26 h after addition of fungal elicitor preparations from *Cytospora abietis* and *Penicillium miniolutem* in *Taxus brevifolia* suspension cultures[60]. Methyl jasmonate as an elicitor has been shown to be very successful in increasing Taxol and baccatin III production in cell lines by twenty folds.[81, 82]

There are also other strategies that are important and that need to be examined. Also, much basic information remains to be discovered in order to manipulate the taxane biosynthetic pathway. Definitely, a further integration of strategies to optimize a Taxol producing plant cell culture would be a great challenge.

7. EXPERIMENTAL

7.1 GENERAL

Anhydrous Na_2SO_4 was used as a drying agent for organic extracts. Prior to use, tetrahydrofuran, diethyl ether and isopropyl ether were freshly distilled.

Analytical TLC was performed on aluminum-backed, silica gel plates (Merck silica gel 60 F_{254}). Developed TLC plates were initially visualized with UV illumination, λ= 254 nm and λ= 365 nm. A 10% sulfuric acid in methanol (immersion solution) was used, and then the plates were heated at 110°C for 2-3 minutes.

Column chromatography was carried out using silica gel 60, 230-400 mesh (Merck), while short column chromatography was performed with 10-40 μm silica gel type H (S-6628, Sigma), or with 40-60 μm (Merck). Small-scale preparative chromatography was performed on a Chromatotron (Model 7924T, Harrison Research, Palo Alto, CA, USA) and glass disks coated with silica gel 60 PF_{254} (7794-2, EM Science/Merck). All solvents for column chromatography were reagent grade and were used without any additional purification or drying. Flash column chromatography on silica gel (Merck silica gel 60, 230-400 mesh) was run under moderate air pressure to maintain a proper eluent flow rate.

^1H NMR spectra were recorded on Bruker WH-400, AE-200 or AMX-500 spectrometers. Chemical shifts (δ) are reported in ppm relative to tetramethylsilane (TMS). COSY experiments were run on Bruker AE-200 or WH-400 spectrometers.

^{13}C NMR spectra were obtained on Varian XL-300 at 75 MHz. Chemical shifts (δ) are cited in ppm relative to TMS. HMBC and HMQC were performed on the AMX-500 spectrometer.

Low-resolution EI mass spectra (LR EI MS) were determined on Kratos MS 50 or MS 80 mass spectrometers. High-resolution EI mass spectra (HR EI MS) were recorded on the Kratos MS 50 spectrometer. Desorption Chemical Ionization mass spectra (DCI MS) were recorded on a Delsi-Nermag R10-IOC mass spectrometer using ammonia as the carrier gas (for low resolution spectra) and an ammonia - methane mixture (for high resolution).

IR spectra were recorded in a chloroform film or KBr pellet on a Perkin Elmer 1710 infrared Fourier transform spectrophotometer.

UV spectra were recorded on a Perkin Elmer Lambda 4B UV/VIS spectrophotometer, using quartz cells of 1cm path length.

All melting points were recorded on a Gallenkamp capillary melting point apparatus and are uncorrected.

HPLC analysis: A Supelcosil LC-F column 150 x 4 mm was used in a Waters HPLC system (HPLC pump model 6000A, Auto sampler Model 712, Model 440 UV Absorbance Detector, and 730 Data module). The flow rate was adjusted to 1 mL/min and the absorbance monitored at 254 nm. Before injection, all samples were filtered through HV filter paper (pore size 0.45 nm, Milipore) and prepared in 1mg/mL methanol solution. A mobile phase consisting of acetonitrile, tetrahydrofuran and water was developed in previous studies and indicated as B4, B6, D1, and D4 solvent systems (**Table 4**).

Initiation, maintenance and growth of *Taxus × media* plant cell culture are given in detail on p. 55-60. Composition of the media is given in **Table 2**. Medium prepared in this way was used as a standard medium for subculture and growth experiments. Solid medium was created by the addition of 8g/L of agar. The pH was adjusted to 5.5 prior to autoclavation.

7.2 MICROFERM EXPERIMENTS

Microferms (bioreactors with mechanical stirring) were inoculated with suspension cell cultures from Erlenmeyer flasks harvested at 14 days of growth. The inoculum ratio was 1:10. Each Microferm bioreactor culture was harvested after 28 days of growth. Prior to harvesting the RI was checked and it was ~1.3333 for all processed bioreactors. The pH value ranged between 5-6 at the time of harvest for healthy bioreactors. Cells were filtered out from the broth through three layers of Miracloth®. Cells were freeze dried, weighed and extracted three times with EtOAc and once with MeOH. Cell EtOAc extracts from different bioreactors were compared by TLC and combined. Microferm experiments in which isolation and purification of secondary metabolites was attempted included F58, F64, F68, F70, F71, F76, F78, F79, F82 and F90 (**Table 5**). Total weight of the combined extract was 6.6717 g. Next partition of the extract was performed with various solvents (p. 72-73 and **Figure 34**). After TLC analysis it was decided that separation of the Hexane: Methylene Chloride extract and Methylene Chloride extract should be performed next.

Purification of the Hexane: Methylene Chloride extract: 697 mg of Hexane: Methylene Chloride extract from the standard partition procedure (**Figure 34**) was chromatographed (see **Figure 35** for a graphical presentation of this isolation work) on a sintered glass funnel (ϕ=10 cm, height 12 cm) and 40 g of silica gel, 40-60 μm (Merck), by consecutive elution with (200 mL each): hexane, hexane: methylene chloride = 1: 1, methylene chloride, methylene chloride: ethyl acetate = 8: 2, methylene chloride: ethyl acetate = 1: 1, ethyl acetate, ethyl acetate: methanol = 8: 2, methanol (twice).

Purification of the Methylene Chloride extract: 654 mg of the Methylene Chloride extract from the standard partition procedure (**Figure 34**) was chromatographed (see **Figure 36** for a graphical presentation of this isolation work) on a sintered glass funnel (ϕ=10 cm, height 12 cm) and 45 g of silica gel, 40-60 μm (Merck), by consecutive elution with (200 mL each): hexane, hexane: ethyl acetate = 8: 2, hexane: ethyl acetate = 1: 1, hexane: ethyl acetate = 3: 7, ethyl acetate, ethyl acetate: methanol = 8: 2, methanol (twice). On the basis of TLC and HPLC analyses, it was decided that fractions V and VI from CH_2Cl_2 should be purified next.

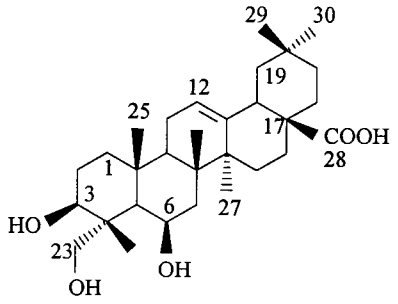
Separation of fraction V from the CH_2Cl_2 sub-extract (see **Figure 37** for a graphical presentation of this isolation work): Fraction V was separated into 10 fractions (V-1 to V-10) by flash column chromatography using methylene chloride: methanol (3% to 6% methanol) and methanol.

Isolation of **MF-1**: Fraction V-9 yielded 9 mg and when checked by TLC showed the presence of only one compound.

Separation of fraction VI from the CH_2Cl_2 sub-extract (see **Figure 41** for a graphical presentation of this isolation work): Fraction VI was separated into 11 fractions (VI-1 to VI-11) by flash column chromatography using methylene chloride: methanol (5%, 6%, 7%, 10% and 20% methanol) and methanol.

Isolation of **MF-2**: Fraction VI-6 crystallized. Upon recrystalization from MeOH with addition of Et_2O it yielded~5.5 mg (refer to **Figure 41**).

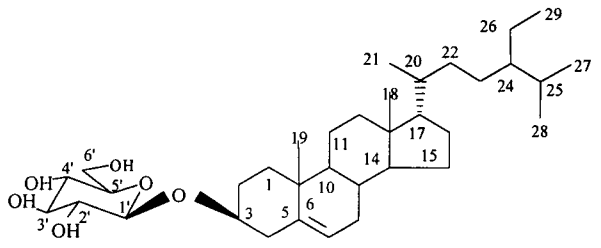
MF-1 (36)



36

White powder. mp=272-275°C; IR ($CHCl_3$) cm^{-1}: 3238 (O–H), 2970 (O–H, acid), 1664 (C=C), 1435, 1347, 1294, 1235 (C–O), 1152 (C–O), 918, 753; EI MS, m/e, (% abundance) 488 [M^+] (5), 470 (2), 452 (1.5), 440 (1), 288 (6), 248 (100), 222 (20), 203 (90), 189 (17); 400 MHz ^1H NMR (pyridine) 0.89 (s, 3H), 0.97 (s, 3H), 1.23 (s, 3H), 1.60 (s, 3H), 1.64 (s, 3H), 1.70 (s, 3H), 3.33 (d of d, J = 3.9, 6.8, 1 H), 4.00 (d, J = 8.3, 1H), 4.24 (d of d, J= 4.3, 11.6 Hz, 1H), 4.37 (d, J = 10.3, 1H), 5.02 (br s, 1H), 5.38 (d, J = 3.6, 1H), 5.56 (distorted t, J =3.3, 1H); 75 MHz ^{13}C NMR (pyridine) 14.5, 17.3, 18.4, 23.6, 23.7, 26.1, 27.8, 28.1, 30.8, 33.1, 34.1, 36.8, 39.0, 40.9, 41.9, 42.73, 44.0, 46.3, 46.5, 48.5, 49.2, 67.00, 67.61, 73.1, 122.6, 144.1, 180.0.

MF-2 (38)



38

White powder. mp=240-242°C (dec); IR ($CHCl_3$) cm^{-1}: 3300 (O–H), 1620 (C=C), 1216 (C–O), 775, 670; EI MS, m/e, (% abundance) 576 [M^+] (1.39), 415(9.68), 396 (100), 382 (23.8), 275 (11.3), 255 (22.7), 229 (8.0), 213 (20.7), 121 (26.5); 400 MHz ^1H NMR δ (pyridine) 0.62 (s, 3H), 0.82 (d, J = 6.5, 3H), 0.85 (d, J = 6.5, 3H), 0.95 (s, 3H) 0.99 (d, J = 6.5, 3H) 1.1 - 2.0 (overlapping m), 2.12 (overlapping m), 2.47 (t of d, J = 13.6, ~2), 2.73 (d of q, J = 12, ~2), 3.92 (m, 2H), 4.03 (t, J = 8.4, 1H), 4.24-4.29 (m, 2H), 4.56 (d of d, 5.6, 2.3, 2H), 4.8- 5.0 (br s), 5.05(d, J = 7.8, 1H), 5.34 (d, J = 5.0, 1H); 75 MHz ^{13}C NMR (pyridine) 11.98, 12.16, 19.0, 19.2, 19.43, 20.00, 21.28, 23.38, 24.52, 26.36, 28.55, 29.44, 30.26, 32.05, 32.18, 34.19, 36.40, 36.92, 37.48, 39.33, 39.94, 42.47, 46.03, 50.33, 56.23, 56.82, 62.81, 71.66, 75.34, 78.06, 78.51, 78.61, 102.6, 121.9, 140.9.

7.3 SHAKE FLASK EXPERIMENTS

The media composition was the same as in the suspension culture (**Table 2**). In shake flask experiments the inoculation rate was 1: 10. Each flask contained 300 mL of media, at temp. =26°C. Shaker speed was adjusted to 120-135 rpm. Each shake flask culture was harvested after 28 days of growth. A check of pH (~ 6 to 6.8) and RI (~ 1.3334) was important to give information about the behavior of the cell line. Cells and broth together were freeze-dried. Biomass was frozen using liquid nitrogen and then pulverized using a mortar and a pestle. Processed in this way the biomass was extracted three times with EtOAc (**Figure 45**). The last EtOAc extract was then filtered from the biomass (#1 Whatman filter paper), and added to other portions of the EtOAc extract. After that the biomass was extracted once with MeOH, and this extract was filtered again from the biomass (#1 Whatman filter paper). Both extracts were separately concentrated on rotovaps and dried using a high vacuum. Once a sufficient amount of

extract was accumulated, extracts from different shake flask transfers were compared by TLC (CH_2Cl_2: MeOH = 94: 6) and combined. Shake flask experiments included 56T, 57T, 58T, 59T, 63T, 64T, 66T, 67T and 68T. The total amount of accumulated extract was 20.2863 g. Next partition of the extract was performed with various solvents (p. 72-73 and **Figure 46**). After TLC analysis it was decided that separation of the Hexane: Methylene Chloride extract and the Methylene Chloride extract should be performed next.

Purification of the Hexane: Methylene Chloride extract: 1.0538 g of the Hexane: Methylene Chloride extract from the standard partition procedure (**Figure 46**) was chromatographed (see **Figure 47** for a graphical presentation of this isolation work) on a sintered glass funnel (ϕ=10 cm, height 12 cm) and 80 g of silica gel, 10-40 μm (Sigma), by consecutive elution with: hexane (500 mL), hexane: methylene chloride = 1: 1 (500 mL), methylene chloride (500 mL), methylene chloride: ethyl acetate = 8: 2 (500 mL), methylene chloride: ethyl acetate = 1: 1 (750 mL), ethyl acetate (750 mL), ethyl acetate: methanol = 8: 2 (750 mL), methanol (1000mL).

Purification of the Methylene Chloride extract: 4.6047 g of the Methylene Chloride extract from the standard partition procedure (**Figure 46**) was chromatographed (see **Figure 48** for a graphical presentation of this isolation work) on a sintered glass funnel (ϕ=10 cm, height 21.5 cm) and 300 g of silica gel, 10-40 μm, by consecutive elution with: hexane (1200 mL), hexane: ethyl acetate = 8: 2 (1200 mL), hexane: ethyl acetate = 1: 1 (1200 mL), hexane: ethyl acetate = 3: 7 (2000 mL), ethyl acetate (2000 mL), ethyl acetate: methanol = 8: 2 (4000 mL), methanol (4000 mL). On the basis of the TLC and HPLC analyses, it was decided that fractions VI and V from CH_2Cl_2 should be purified next.

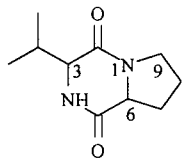
Separation of fraction VI from the SF CH_2Cl_2 sub-extract (see **Figure 49** for a graphical presentation of this isolation work): Fraction VI was divided into two portions. One portion of

786.6 mg was separated into 9 fractions (VI-1 to VI-9) by flash column chromatography using isopropyl ether: acetone: acetic acid (90: 5: 5) and methanol.

Isolation of **SF-1**: Fraction VI-2 yielded 58.7 mg. After dissolving it in methylene chloride and upon gradual addition of a small amount of hexane, 10 mg of crystals of **SF-1** formed.

Isolation of **SF-2**: Fraction VI-4, 84.1 mg (refer to **Figure 49**) was further chromatographed on a short column with isopropyl ether: acetone: acetic acid (90: 5: 5, 90: 7: 5) and methanol. Fraction VI-4c yielded 30 mg. After dissolving it in methylene chloride and upon gradual addition of a small amount of hexane, 6.7 mg of crystals of **SF-2** formed.

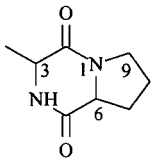
SF-1 (39)



39

Colorless needles. Recrystallized from CH_2Cl_2/hexane. mp=172-174°C; IR ($CHCl_3$) cm^{-1}: 3411 (N–H), 2459, 2266, 1651 (C=O), 1537, 1300 (C–N), 963, 887, 690; ^1H NMR (400 MHz, CD_2Cl_2) 0.92 and 1.10 (each d, methyls), ~1.8–2.6 (m), 3.6 (m, 2H, H-9), 3,98-4.10 (m, 2H, H-3,6); EI MS (70 eV), m/e (% abundance) 196 (M^+, 3.4) 169 (1.4), 167 (1.3), 154 (100), 140 (1.4), 138 (4.4), 125 (40), 98 (7.), 97 (5.7), 72 (29), 71 (8.0), 70 (88).

SF-2 (40)



40

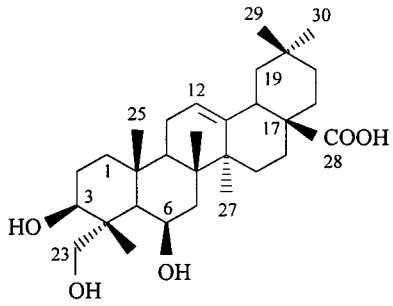
White powder. Recrystallized from CH_2Cl_2/hexane. mp=162-167°C; IR ($CHCl_3$) cm^{-1}: 3460 (N–H), 2537, 2386, 1701 (C=O), 1537, 1228 (C–N), 950, 890, 824, 720; ^1H NMR (400 MHz, CD_2Cl_2) 1.43, (d, Me), 1.8-2.4 (m), 3.6 (m, 2H, H-9), 4.0-4.3 (m, 2H, H-3 and H-6); EI MS, m/e (% abundance), 168 (M^+, 78), 140 (7.2), 125 (27.8), 97 (39.4), 70 (100).

Separation of fraction V from the SF CH_2Cl_2 sub-extract (see **Figure 52** for a graphical presentation of this isolation work): Fraction V was separated into 10 fractions (V-1 to V-10) by flash column chromatography with isopropyl ether: acetone: acetic acid (90: 5: 5) and methanol.

Isolation of **SF-3**: Fraction V-3 weighed 131.4 mg (refer to **Figure 52**) and was further chromatographed on a short column with isopropyl ether: acetone: acetic acid (90: 5: 5, 90: 7: 5) and methanol. Fraction V-3b yielded 20.4 mg. After dissolving it in acetone and leaving it overnight in the fridge ~4°C, 9 mg of crystals of **SF-3** formed.

Isolation of **SF-4**: Fraction V-9, 78 mg (refer to **Figure 52**) was further chromatographed on a short column with isopropyl ether: acetone: acetic acid (90: 5: 5, 90: 7: 5) and methanol. Fraction V-9c yielded 18 mg. Fraction V-9c crystallized, and upon recrystallization from methylene chloride gave ~7 mg of **SF-4**.

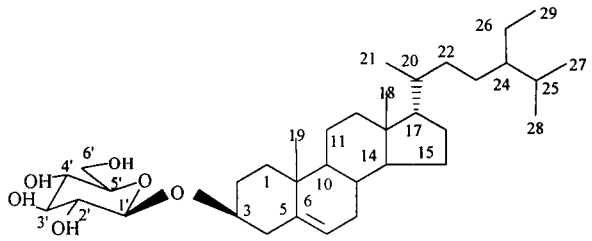
SF-3 (36)



36

White powder. mp=272-275°C; IR ($CHCl_3$) cm^{-1}: 3238 (O–H), 2970 (O–H; acid), 1664 (C=C), 1435, 1347, 1294, 1235 (C–O), 1152 (C–O), 918, 753; EI MS, m/e, (% abundance) 488 [M^+] (5%), 470 (2%), 452(1.5%), 440 (1%), 288 (6%), 248 (100%), 222 (20%), 203(90%), 189(17%); 400 MHz ^1H NMR (pyridine) 0.94 (s, 3H), 1.08 (s, 3H), 1.28 (s, 3H), 1.66 (s, 3H), 1.67 (s, 3H), 1.72 (s, 3H), 3.34 (d of d, J = 3.9, 6.8, 1 H), 4.04 (d, J = 8.3, 1H), 4.26 (d of d, J= 4.3, 11.6 Hz, 1H), 4.39 (d, J = 10.3, 1H), 5.04 (br s, 1H), 5.38 (d, J = 3.6, 1H) 5.52 (distorted t, J =3.3, 1H); 75 MHz ^{13}C NMR (pyridine) 14.82, 17.62, 18.66, 23.79, 23.95, 26.29, 28.08, 28.35, 31.00, 33.28, 34.25, 37.02, 39.29, 41.14, 42.11, 42.73, 44.08, 46.51, 46.71, 48.75, 49.37, 67.15, 67.61, 73.29, 123.0, 144.4, 180.7.

SF-4 (38)



38

White powder. mp=240-242°C (dec); IR ($CHCl_3$) cm^{-1}: 3300 (O–H), 1620 (C=C), 1216 (C–O), 775, 670; EI mass spectum, m/e, (% abundance) 576 [M^+] (1.39), 415(9.68), 396 (100), 382 (23.8), 275 (11.3), 255 (22.7), 229 (8.0), 213 (20.7), 121 (26.5); 400 MHz ^1H NMR δ (pyridine) 0.68 (s, 3H), 0.88 (d, J = 6.5, 3H), 0.90 (d, J = 6.5, 3H), 0.98 (s, 3H), 1.1 (d, J = 6.5, 3H), 1.1 - 2.0 (overlapping m), 2.12 (overlapping m), 2.47 (t of d, J = 13.6, ~2), 2.73 (d of q, J = 12, ~2), 3.92 (m, 2H), 4.03 (t, J = 8.4, 1H), 4.24-4.29 (m, 2H), 4.56 (d of d, 5.6, 2.3, 2H), 4.8- 5.0 (br s), 5.05(d, J = 7.8, 1H), 5.34 (d, J = 5.0, 1H); 75 MHz ^{13}C NMR (pyridine) 11.96, 12.10, 19.0, 19.2, 19.43, 20.00, 21.28, 23.38, 24.52, 26.36, 28.55, 29.44, 30.26, 32.05, 32.18, 34.19, 36.40, 36.92, 37.48, 39.33, 39.94, 42.47, 46.03, 50.33, 56.23, 56.82, 62.81, 71.66, 75.34, 78.06, 78.51, 78.61, 102.6, 121.7, 140.7.

References

1. Wani, M.C., Taylor, H.L.,Wall, M.E.,Coggon,P., and McPhail, A.T., *J. Am.Chem.Soc.*, 93, 2325, 1971.

2. Gibson, D. M., Ketchum, R. E. B., Hirasuna, T. J., Shuler, M. L., (Suffness, M., Ed.), TAXOL® Science and Applications, Chapter 4, Potential of plant cell culture for Taxane production, 72, 1995.

3. Borman, S., *Chem. Eng. News,* 69, 11, 1991.

4. Adams, J. D., Flora, K. F., Goldspiel, B.R., Wilson, J.W., Arbuck, S. G., and Finley, R., *Monogr. Natl. Cancer Institute,* 15, 141, 1993.

5. Zubrod, C, G., Schepartz, S., Leiter, J.,Endicott, K. M., Carrese, L. M., and Baker, C. G., *Cancer Chemother. Rep.*, 50, 349, 1966.

6. Schepartz, S. A., *Cancer Treat. Rep.,* 60, 975, 1976.

7. Suffness, M., *Gann Monogr.Cancer Res.*, 36, 21, 1989.

8. Wall, M. E. and Wani, M. C., Recent progress in plant antitumor agents, Paper M-006, 153rd National Meeting, American Chemical Society, Miami Beach, FL, 1967.

9. IUPAC Commision on the Nomenclature of Organic Chemistry (1978). Nomenclature of Organic Chemistry. Section F: Natural Products and Related Compounds, *Eur. J. Biochem.*, 86, 1-8.

10. Kingston, D. G. I., Molinero, A.A., and Rimoldi, J. M., Progress in the Chemistry of Organic Natural Products, The Taxane Diterpenoids, (Herz, W., Kirby, G. W., Moore, R. E., Steglich, W., and Tamm, Ch., Eds.), Springer-Verlag, New York, 1993.

11. Schiff, P.B., Fant., and Horwitz, S. B., *Nature*, 22, 665, 1979.

12. Weiss, R., Donehower, R. C., Wiernik, P. H. Ohnuma, T., Gralla, R. J., Trump, D. L. Baker, J. R., Jr., Van Echo, D. A., Von Hoff, D. D., and Leyland Jones, B., *J. Clin. Oncol.*, 8, 1263, 1990.

13. Rowinsky, E. K., Donehower, R. C., Jones, R. J. and Tucker, R. W., *Cancer Res.,* 48, 4093, 1988.

14. Hortobagyi, G. N., Holmes, F. A., Valero, V., Walters, R. S., Theriault, R. L., Booser, D. J., Fraschini, G., Buzdar, A. U., Frye, D. K., Gibbs, H. R., *Monogr. Natl. Cancer Inst.,* 15, 161, 1993.

15. Holmes, F. A., Walters, R. S., Thierault, R. L., Forman, A. D., Newton, L. K., Raber, M. N., Buzdar, A. U., Frye, D. K., and Hortobagyi, G. N., *J. Natl. Cancer Inst.,* 83, 1797, 1991.

16. Gueritte-Voegelein, F., Guenard, D., and Potier, P., *J. Nat. Prod.*, 50, 9, 1987.

17. Della Casa de Marcano, D. P. and Halsall, T. G., *J. Chem. Soc. Chem.Commun.*,365, 1975.

18. Holton, R. A., Biediger, R. J., Boatman, D, P., TAXOL® Science and Applications, (Suffness, M. ed.), Chapter 5, Semisynthesis of Taxol and Taxotere, 97, 1995.

19. Powell, R. G., Miller, R. W., and Smith, C.R., Jr., *J. Chem. Soc. Chem. Commun.*, 102, 1979.

20. Miller, R. W., Powell, R. G., Smith, C. R., Jr., Arnold, R., and Clardy, J., Antileukemic alkaloids from *Taxus wallichiana* Zuc., J. Org. Chem., 40, 1469, 1981.

21. Liang, J. and Kingston, D.G.I., *J. Nat. Prod.*, 56, 594, 1993.

22. Stierle, A., Strobel, G., and Stierle, D., *Science,* 260, 154, 1993.

23. Harrison, J. W. and Lythogoe, B., *J. Chem. Soc. C.,* 1932, 1966.

24. Harrison, J. W., Scrowston, R. M. and Lythgoe, B. *J. Chem. Soc. C.,* 1933-1945, 1966.

25. Erdtman E., et al, *Tetrahedron Lett.,* 3879-3886, 1964.

26. Dauben W. G., et al, *J. Org. Chem.,* 44, 669-673, 1979.

27. Begley, M. J., et al, *Tetrahedron,* 46, 4907-4924, 1990.

28. Leete, E. and Scott, I. A., personal communication, 1992.

29. Zamir L. O., Nedea, M. E., et al, *Tetrahedron lett.,* 33, 5235, 1992.

30. Strobel, G. A., Stierle, A., et al, *Plant Sci.,* 84, 65, 1992.

31. Croteau R. B., Koepp, A. E., Hezari, M., et al, *J. Biol. Chem.,* 270, 8686, 1995.

32. Croteau R. B., Hezari, M., et al, *J. Biol. Chem.*, 271, 9201, 1996.

33. Croteau R. B., Hezari, M., et al, *Biochemistry,* 35, 2968, 1996.

34. Della Casa de Marcano, D. P., et al, *J. Chem. Soc., Chem. Commun.,* 1382, 1970.

35. . Swindell, C. S. and Britcher, S. F., *J. Org. Chem.,* 51, 793, 1986.

36. Potier P., et al, *J. Nat. Prod.*, 50, 9, 1987.

37. Leete, E. and Bodem, G. B., *Tetrahedron Lett.*, 3925, 1966.

38. Platt, R. V., Opie, C. T. and Haslam, E., *Phytochem.,* 23, 2211, 1984.

39. Swindell, C. S., Krauss, N. E., *J. Med. Chem.,* 34, 1176, 1991.

40. Lowe, J., The Yew-Trees of Great Britain and Ireland, Macmillan, London, 1897.

41. Wilkinson, G., Trees in the Wild: And other Trees and Shrubs, Stephen Hope Books, London, 1973, 71.

42. Hartzell, R. H. Jr., TAXOL® Science and Applications (Suffness, M. ed.), Chapter 2, Yew and Us: A Brief History of Yew Tree, 27, 1995.

43. Li, H, L., *Evolution,* 7, 245, 1953.

44. Florin, R., *Acta Horti Berg.,* 20(4), 121, 1963.

45. Price, R, A., *J. Arnold arb.,* 71, 69, 1990.

46. Florin, R., *Acta Hor. Berg.,* 17, 257, 1958.

47. Gifford, E. M. and Foster, A. S., Morphology and Evolution of Vascular Plants, 3rd ed., W. H. Freeman, New York, 626, 1989.

48. Rushfort, K., Conifers, Facts on File, New York, 1987.

49. den Ouden, P. and Boom, B. K., Manual of the Cultivated Conifers, Martinus Nijhoff, The Hague, 1965.

50. van Gelderen, D. M. and van Hoey Smith, J. R. P., Conifers, 2nd ed., Timber Press, Portland, OR, 1989.

51. U.S. House of Representatives, Pricing of drugs codeveloped by federal laboratories and private companies, Hearing before Subcommittee on Regulation, Business Opportunities, and Technology, Committee on Small Business, Washington, DC, Serial No. 103-2, January 25, 1993.

52. Morris P., Scragg A. H., Stafford A., Fowler M. W., Secondary metabolism in plant cell culture, Section 1, Secondary products from plant cell culture, 1, 1986.

53. Charlwood, B. V., Rhodes, M. J. C., Secondary Products from Plant Tissue Culture, Chapter 1, Plants as sources of valuable products, 1, 1990.

54. Christen, A. A., Gibson, D. M., and Bland, J., Production of taxol or taxol like compounds in cell culture, U. S. Patent 5, 918, 504, May 28, 1989.

55. Lewis, N. and Croteau, R., Taxol biosynthesis, in Proc.2nd Natl. Cancer Institute Workshop in Taxol and *Taxus,* 1992.

56. Bonga, J. M., von Aderkas, P., In Vitro Cultures of Trees, Chapter 4, Collection, Sterilization, Excision and Culture, 55, 1992.

57. Murashige, T. and Skoog, F., *Physiol. Plant,* 15, 473, 1962.

58. Gamborg, O. L., Miller, R. A., and Ojima, K., *Exp. Cell Res.,* 50, 151, 1968.

59. Grothaus, P. G., Raybould, T. J. G., Bignami, G. S., Lazo, C. B., and Byrnes, J. B., *J. Immunol. Methods,* 158, 5, 1993.

60. Christen, A.A., Gibsom, D.M. and Bland, J. Production of Taxol or Taxol -like Compounds in Cell Culture. US Patent 5,019,504. 1991.

61. Wickremesinhe, E. R. M. and Arteca, R. N., Vol. 41, Biotechnology in Agriculture and Forestry. (Bajaj, Y.P.S ed) Medicinal and Aromiatic Plants, Springer-Verlag. Berlin. 1998.

62. Yukimune, Y., Hara, Y., et al, Method of Producing a Taxane-Type Diterpene and a Method of Obtaining Cultured Cells which Produce the Taxane -Type DiTerpene at a High Rate. US Patent 5, 637, 484. 1997.

63. Jaziri, M., Zhiri, A., et al, Plant Cell Tissue and Organ Culture. *46*. 59-75. 1996.

64. Chauviere, G., Guenard, D., Picot, F., Senilh, V., and Potier, P., *C. R. Acad. Sci. Paris, II*, 293, 501, 1981.

65. Denis, J. N., Greene, A. E., Guenard, D., Gueritte-Voegelein, F., Mangatal, L., and Potier, P., *J. Am. Chem. Soc.*, 110, 5917, 1988.

66. Palomo, C., Arrieta, A., Cossio, F. P., Aizpurua, J. M., Mielgo, A., and Aurrekoetxea, N., *Tetrahedron Lett.*, 31, 6429, 1990.

67. Wender, P. A., Natchus, M. G., and Shuker, A. J., TAXOL® Science and Applications (Suffness, M. ed.), Chapter 6, Toward the total synthesis of Taxol and its analogs, 123, 1995.

68. Paquette, L. A., Elmore, S. W., Combrink, K. D., Hickey, E. R., and Rogers, R. D., *Helv. Chim. Acta*, 75, 1755, 1992.

69. Wender, P. A. and Mucciaro, T. P., *J. Am. Chem. Soc.*, 114, 5878, 1992.

70. Roth, W. R. and Erker, G., *Angew. Chem. Int. Ed. Engl.*, 12, 503, 1973.

71. DiCosmo, F., Fett-Neto, A. G., et al, *Bio/Technology* , 10, 1572, 1992.

72. Serri, C, Unpublished Final Report, 1997.

73. Cardellina, J. H., *J. Liq. Chromatogr.*, 14, 659, 1991.

74. Yamaguchi, K., Spectral Data of Natural Products, Vol. 1. Elsevier Publishing Co. New York. 1970.

75. Mahato, S., and Kundu, A., *Phytochemistry*, 37, 1517, 1994.

76. Koizumi, N., Fujimoto, Y., et.al, *Chem. Pharm. Bull.*, 27, 38, 1979.

77. Schmitz, F. J. et al, *Journal of Organic Chemistry*, 48, 3941, 1983.

78. Grove, F. J., Pople, M., *Phytochemistry,* 20, 815, 1981.

79. Stroebel, G.A., Stierle, A., and Van Kuijk, F. J. G. M., *Plant Sci.,* 86, 65, 1992.

80. Sakuta, M. and Komamaine, A., Vol. 4, Cell growth and accumulation of secondary metabolites, in Cell culture and Somatic Cell Genetics of Plants, (Constabel, F. and Vasil, I. K., Eds.), 97, 1987.

81. Linden, J. C. and Mirjalili, N., *Biotech.Bioeng.* 48, 123, 1995.

82. Yukimune, Y. et al, *Nature Biotechnology*, 14, 1129, 1996.