SYNTHESIS OF [1-^{3}H]-GERANYLGERANYL PYROPHOSPHATE

THE SYNTHESIS OF [1-^{3}H]-GERANYLGERANYL PYROPHOSPHATE AND ITS INCUBATION WITH *TAXUS X MEDIA* DENSIFORMIS

by

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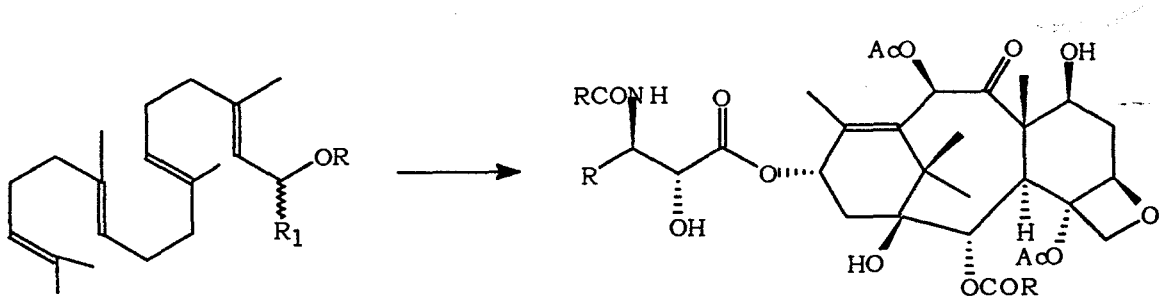
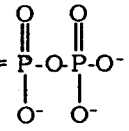
1. Abstract

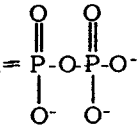
Recently, the discovery that taxol (**2**), a highly modified diterpenoid available from *Taxus* species, possesses anticancer activity has attracted considerable interest. The synthesis of [1-^{3}H]-geranylgeranyl pyrophosphate(**1b**), a labelled form of the precursor to the diterpenoids(**1a**), and its incubation with a cell-free enzyme preparation from *Taxus x media* densiformis, a species of yew tree, were investigated.

The investigation into the biosynthesis of taxol(**2**) was to be accomplished by monitoring the enzymatic transformations of (**1b**). The synthesis of **1b** was accomplished via a convergent method using geraniol, a ten carbon compound, as the starting material. Essentially two molecules of geraniol were chemically manipulated and condensed to give geranyl geraniol which was oxidized to the corresponding aldehyde and then reduced using sodium borotritide. The radioactive alcohol was subsequently converted to the pyrophosphate (**1b**) using standard methodology. Cell-free extracts of *Taxus x media* densiformis were prepared and incubated with [1-^{3}H]-GGPP (**1b**), the intermediates were extracted, and then analyzed by scintillation counting and radioactive gas chromatography. Identification of these labelled intermediates was attempted in order to provide information about the biosynthesis of **2**.



1a , R_1= H, R= 

2 , R= C_6H_5

1b , R_1= T, R= 

Acknowledgements

I would like to express my gratitude to my supervisor, Dr. Paul Harrison, for his guidance and enthusiasm throughout this research. I would also like to thank the members of our research group for their helpful advice and friendship. I wish to thank the staff of the Royal Botanical Gardens for their generous supply of yew. I would like to thank the faculty, technical staff, and fellow graduate students of the chemistry department, many of who have become good friends. Finally, I want extend my appreciation to my family, who have always supported me.

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2. Introduction

2.A. General Introduction:

Naturally occurring substances have captured peoples attention since the beginning of time. It was not until 1950 that this field of chemistry began to progress with leaps and bounds due to the development of effective and efficient isolation and separation techniques, and powerful spectroscopic methods. Add to this the recent advances in theoretical and practical organic chemistry, and the understanding of the chemistry and biochemistry associated with natural products has accumulated at a rapid rate.

Natural products encompass a wide variety of compounds such as alkaloids, terpenoids, carbohydrates, aliphatic and aromatic compounds, and steroids. Natural product chemistry has advanced our understanding of enzyme catalyzed processes occurring in living systems. Through time, the separation, purification, and analysis of the compounds produced in living cells has had a profound effect on organic chemistry. The imitation of bio-organic processes in the laboratory has led to important advances in synthetic methods.

The terpenes are amongst the most widespread and chemically interesting groups of natural products[1]. Over the past number of years the terpenoids have revealed an

abundance of fascinating structural, synthetic, biosynthetic, and mechanistic problems with compounds that have the added interest of a wide range of biological activities. The terpenes possess a common unifying feature despite their diversity in structure and biological activity, namely, they are a family of natural products which biosynthetically derive their carbon atoms from isopentenyl pyrophosphate (3) (IPP), a 5-carbon compound.

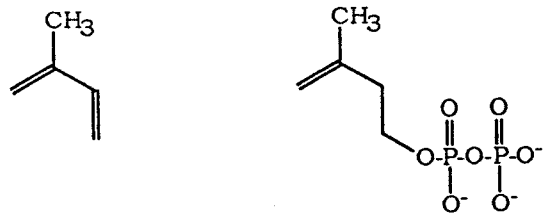

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Figure 1.

The classical isoprene rule[2] states that terpenes are composed of repeating C_5H_8 units linked together in a regular (head-to-tail) manner so that their structure may be divided into isoprene units. It was during this period of structural determination, that the isoprene rule was found not to be applicable to the explanation of structures in all cases. The biogenetic isoprene rule was then established to identify those compounds which could be derived through cyclization/rearrangement reactions from an aliphatic precursor

which did obey the classical isoprene rule. This family of compounds can then be classified according to the number of isoprene units contained in their structure and the way in which these isoprene units are joined.

Two major classes of terpenoids are the mono- and di-terpenes. Monoterpenes denote 10-carbon compounds while diterpenes designate 20-carbon compounds. The classes may be further subdivided into acyclic, monocyclic, bicyclic, tricyclic, tetracyclic, and pentacyclic. Generally in the mono-, sesqui-, di-, and sester-terpenes the isoprene units are connected in a head-to-tail fashion (Figure 2); however, in the tri-terpenes (C_{30}) and carotenoids (C_{40}), the two C_{15} and C_{20} fragments respectively are joined together tail-to-tail.

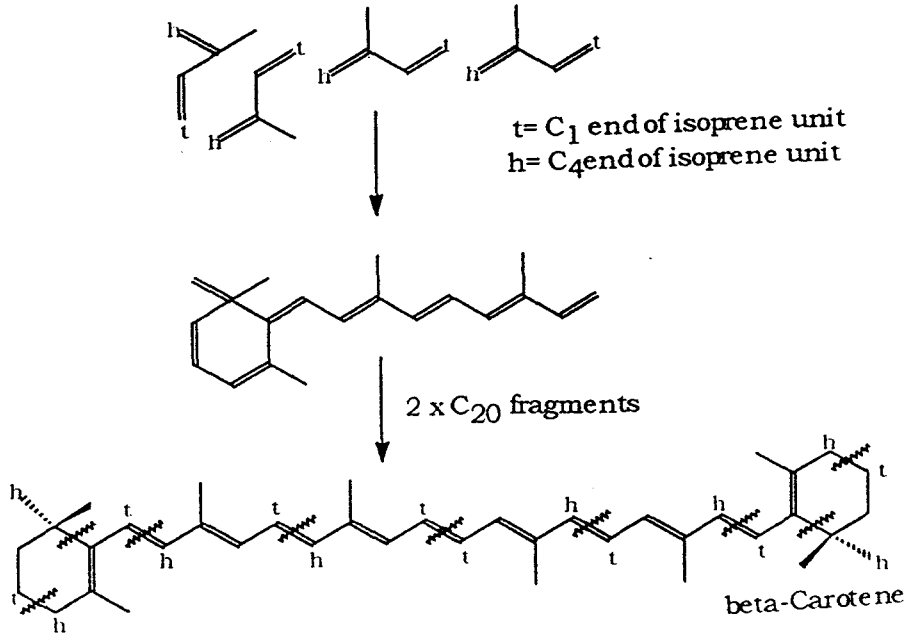
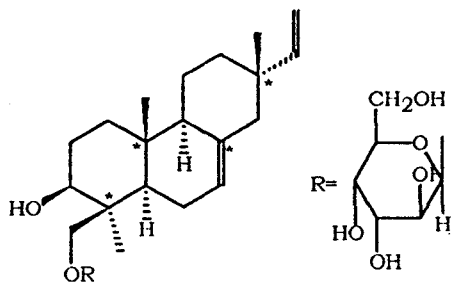
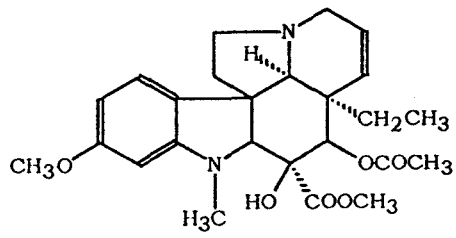


Figure 2

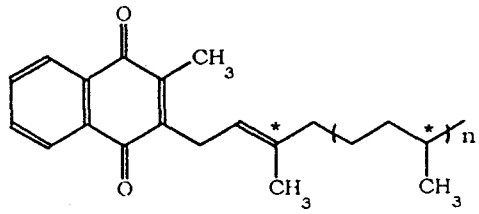
Compounds possessing a C_{20} moiety covalently bonded to other moieties of differing biogenetic origin are also classed among the diterpenoids, e.g. (-)-virescenoside-B (**4**), a metabolite of *Oospora virescens*. Isoprene building blocks are not only the foundation of the terpenoids, but are less obviously found within the framework of other natural products such as vindoline (**5**), an alkaloid from the leaves of *Vinca rosea* which is the pentacyclic moiety of vinblastin, an anticancer agent, and vitamin K (**6**), a group of polyisoprenoid substituted naphthoquinones.



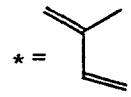
(-)-Virescenoside-B
4



Vindoline
5



Vitamin K derivative
n= 1-12
6

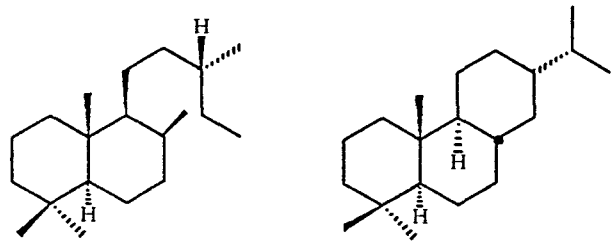


isoprene unit

Figure 3

2.B. The Diterpenoids

The diterpenes which have been identified to date are a family of compounds numbering over 2000 and categorized into 176 skeletal types[3]: this shows the skeletal and functional group diversity. The diterpenes are varied in structure: some acyclic diterpenes, e.g. (**7**) are known, but the majority are cyclic containing from one to as many as five rings. At present the diterpenes shown in Figure 4 account for approximately 50% of the known diterpenes. Monocyclic diterpenes are rare while bicyclic diterpenes are much more common: there are 320 known compounds which possess the labdane skeleton. Tricyclic diterpenoids such as abietane (**8**) are also common. Over 150 tetra- and pentacarbocyclic diterpenes have been isolated and characterized; for example, the gibberellanes (**9**), plant growth promoting hormones, are important biologically active tetracyclic diterpenoids of which over 70 are known at present.



Labdane

Abietane

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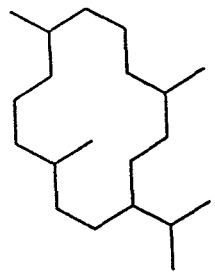
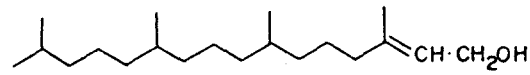


Cembrane



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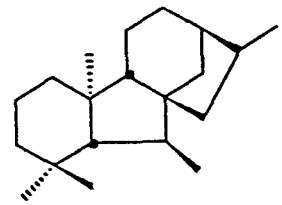


Gibberellane

9

Figure 4.

Most of the known diterpenes have been isolated from both higher and lower terrestrial plant species, and are believed to be produced there. Diterpenes are also available from other sources in nature including: fungi, algae, bacteria, and marine organisms. It has been determined that most, if not all, of the animals from which diterpenes have been isolated live symbiotically with algae. This seems to suggest that these algae are responsible for the *de novo* synthesis of the diterpenes in these cases[4].

The most abundant diterpenes are not the hydrocarbons, which are generally found in small quantities, but rather are the more oxygenated polycyclic derivatives[5]. It is assumed that these have been generated by oxidative transformations from a small

group of parent hydrocarbons. The introduction of oxygen-containing, and in some cases nitrogen-containing, functional groups, may be accompanied by modification of skeletons resulting from rearrangement, ring-opening, further cyclization, elimination of appended groups, or some combination of the aforementioned transformations.

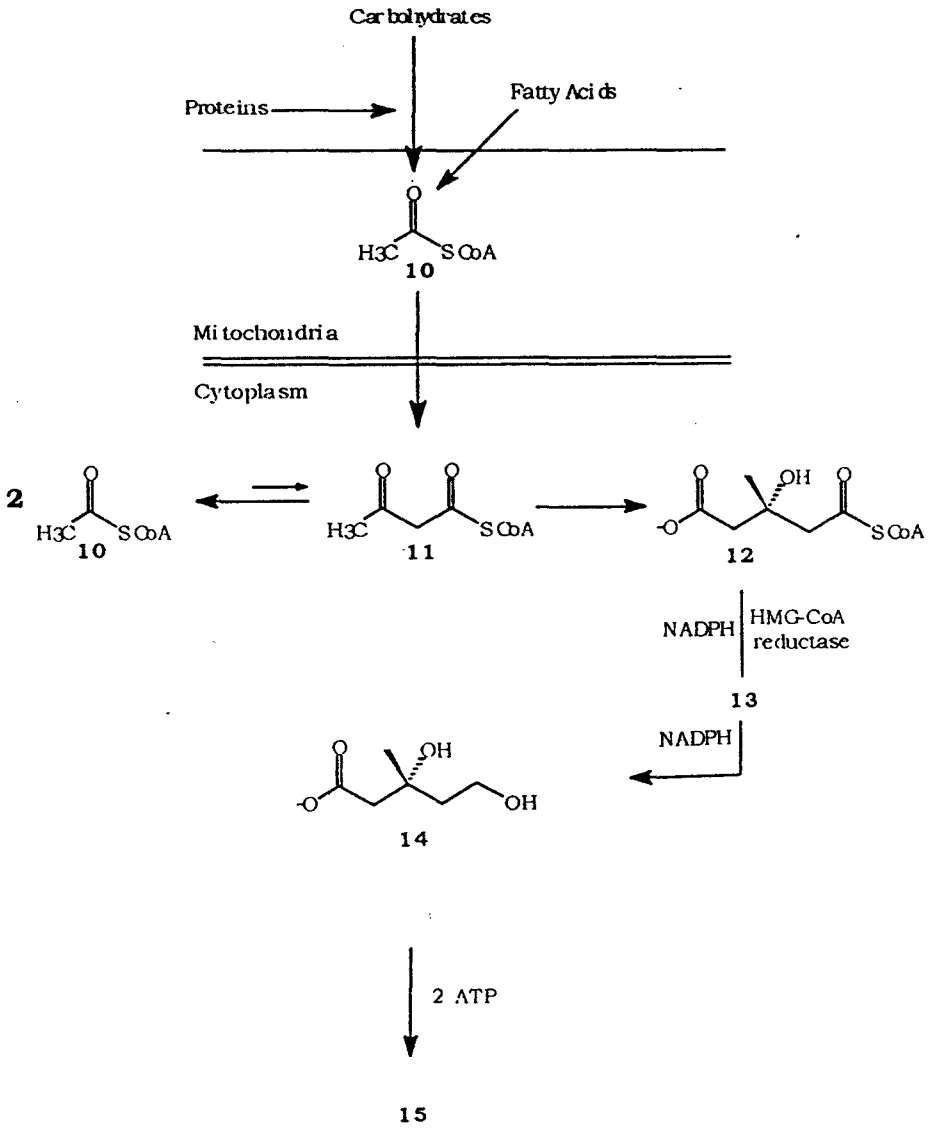
2.C. Biosynthetic Origin of Diterpenes: Mevalonic Acid Pathway:

It has become well-established[6,7] that diterpenes, and terpenes in general, are derived biosynthetically from acetyl-coenzyme A (**10**), obtained from the catabolism of carbohydrates, fats, and possibly proteins. Two molecules of acetyl-coenzyme A (**10**) undergo a Claisen-like condensation catalyzed by acetyl-CoA acetyl transferase to produce acetoacetyl-coenzyme A (**11**) (Figure 5). Further condensation with another molecule of acetyl-coenzyme A, in an aldol-type reaction, results in β-hydroxy-β-methylglutaryl-coenzyme A (**12**). Two consecutive, irreversible reductions requiring NADPH (reduced nicotinamide-adenine dinucleotide phosphate, each the donor of a hydride), via intermediate **13**, occur to produce *R*-mevalonic acid (**14**)[8], a six carbon compound with a chiral center at C-3 and the building block of almost all isoprenoids. Phosphorylation of mevalonic acid (**14**) by ATP (adenosine triphosphate), in two steps (two enzymes) leads to mevalonic acid 5-pyrophosphate (**15**). It is believed that the primary hydroxyl at C-5 of (**14**) is initially converted to the 5-phosphate and then the 5-pyrophosphate by kinases, followed by phosphorylation of the tertiary hydroxyl at C-3 of (**15**) by another

molecule of ATP. This provides an example of the use of ATP for the conversion of a poor leaving group into a good one. Decarboxylation and dehydration of (**15**) gives isopentenyl pyrophosphate (**16**), the long-sought biological isoprene unit. This elimination reaction, which had no previous analogy in biochemistry, proceeds by a concerted *trans*-elimination. Next, isopentenyl pyrophosphate (**16**) (IPP) is equilibrated enzymatically with dimethylallyl pyrophosphate (**17**) (DMAPP): the latter component predominates in the equilibrium. This isomerization proceeds in a stereospecific manner: the incoming proton, from the surrounding environment, attacks the *re,re* face of the ethylenic double bond. Finally, loss of the pro-(*R*)-hydrogen from C-2 (which was the 4-pro-(*S*)-hydrogen of mevalonic acid (**14**)) generates **17**. Rose *et al*[9] have shown in experiments with tritium labelled **17** that there is no intramolecular hydrogen transfer: this is consistent with the hypothesis of a carbonium-ion intermediate in the allylic isomerization.

The two intermediates react in a stereospecific condensation, with the aid of a "prenyl transferase", generating geranyl pyrophosphate(**18**), along with a molecule of liberated inorganic pyrophosphate (PPi). The stereochemistry of this step as well as that of the earlier steps was clarified using suitably double-labelled (^{14}C and ^{3}H) precursors[10]. Further condensations with IPP (**16**) will lead to farnesyl pyrophosphate(**18**), geranylgeranyl pyrophosphate(**1a**), and so on. In each condensation, the PPi released originates from the allylic molecule, therefore the new carbon-carbon bond forms between the C-4 (head) of the isopentenyl molecule and the C-1 (tail) of the allylic molecule representing a head-to-tail chain elongation. By this sequence of reactions geranylgeranyl

pyrophosphate (**1a**) is generated and represents Ruzicka's acyclic precursor to the diterpenoids[11].



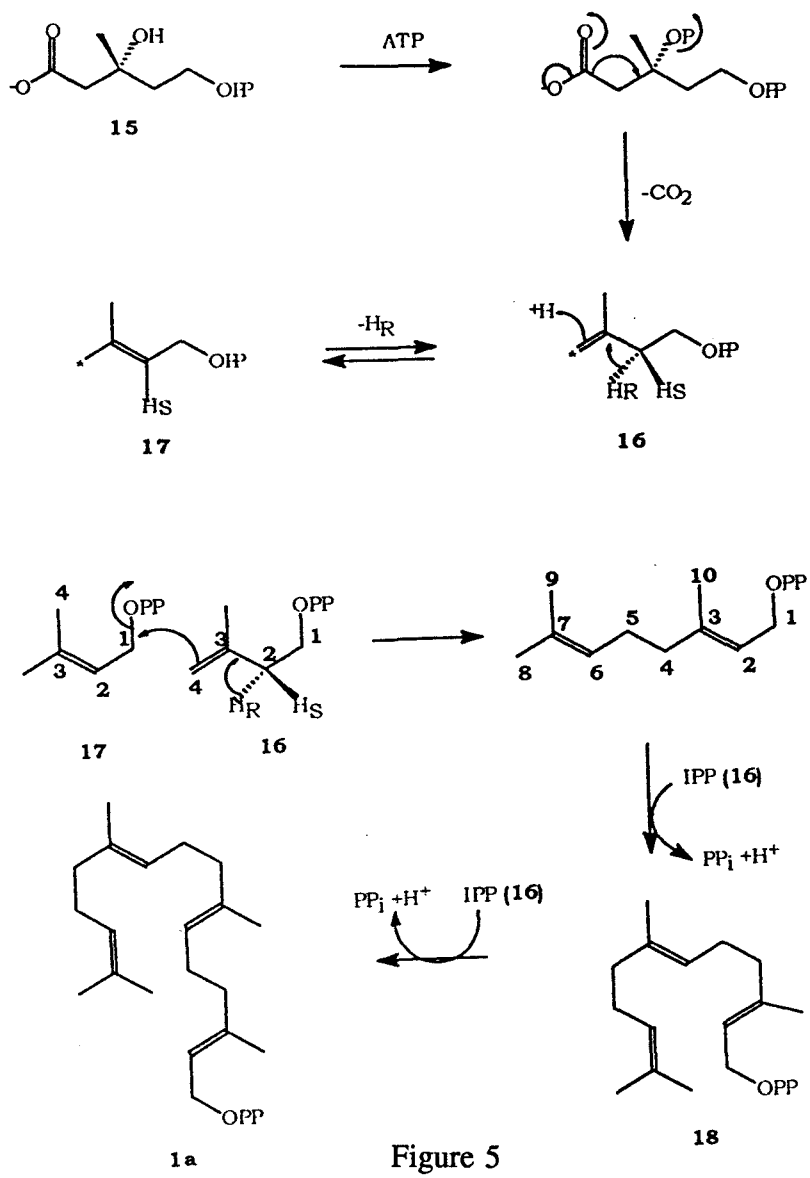


Figure 5

Most of the diterpenes, and isoprenoids in general, found in nature have all-*trans* stereochemistry due to the stereochemistry of the IPP-DMAPP isomerization process. The 2-pro-(*R*)-hydrogen of IPP is lost to give a *trans* (E) double bond, and when the 2-pro-(*S*)-hydrogen is lost, a new *cis* (Z) double bond is produced. Although some

plant species have the ability to synthesize *cis*-isoprenoids, the *trans*-polyisoprenoids predominate.

The above knowledge has been gathered essentially from studies on the biosynthesis of steroids in animals and microorganisms[12,13]. It was soon established that mevalonic acid(**14**) is indeed the precursor of almost all classes of isoprenoids.

2.D. Role of Geranylgeranyl Pyrophosphate (1a):

Geranylgeranyl pyrophosphate (**1a**) [(E,E,E)-3,7,11,15-tetramethyl-2,6,10,14-hexadecatetraen-1-ol dihydrogen pyrophosphate] is believed to be the precursor of all the diterpenes[14]. The pyrophosphate esters of the acyclic prenyl alcohols serve as the natural activated precursors of the terpenic compounds rather than the free alcohols themselves. A majority of the bicyclic and higher diterpenes are formed via cyclization of (**1a**) in its chair-chair conformation (Figure 6). An A:B ring junction with the 5α,10β configuration is the one most commonly encountered, but kaurene, a metabolite in the gibberellane (**9**) family biogenesis, has the 5β,10α A:B ring junction.

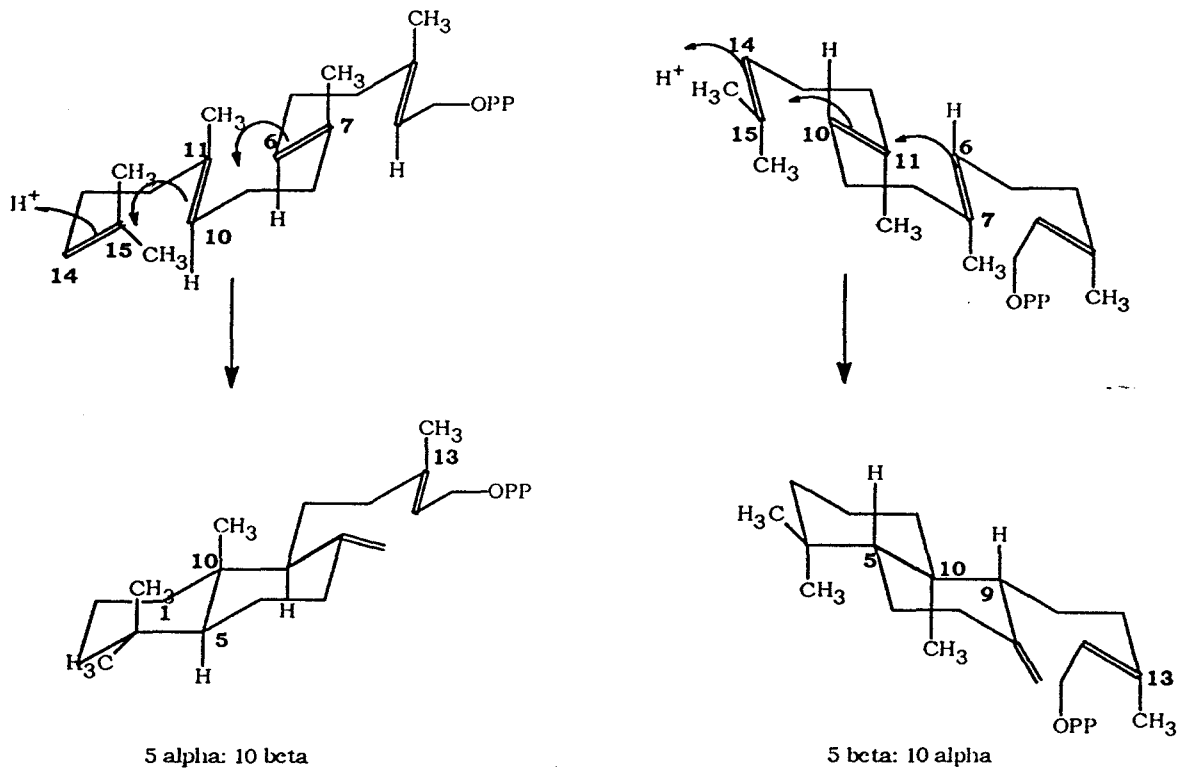
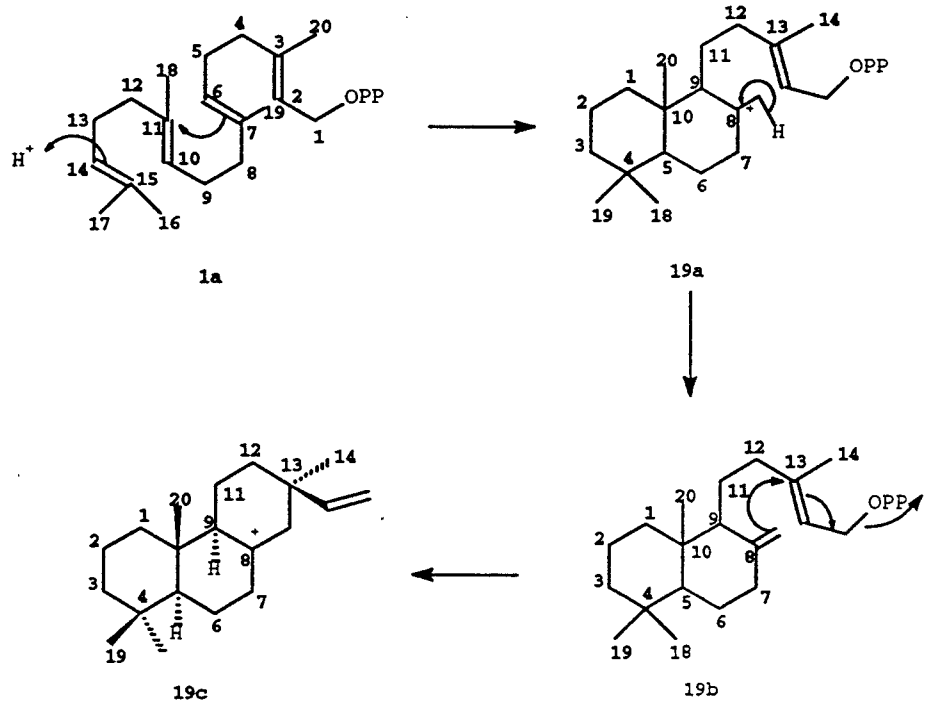


Figure 6.

Evidence supporting the role of (**1a**) in the synthesis of polycyclic and macrocyclic diterpenes was obtained from studies of cell-free enzyme preparations from immature seeds[15] and germinating seedlings[16] and from feeding studies with fungi[17].

For example, it has been shown by tracer studies[18] that the biosynthesis of the diterpenoid fungal metabolite, rosenonolactone(**19**) proceeds by way of geranyl (**16**), farnesyl (**17**), and geranylgeranyl pyrophosphates (**1a**). The formation of (**19**) (Figure 7)

from (**1a**) can be rationalized by nucleophilic attack by the C-6-C-7 double bond on C-11, nucleophilic attack by the C-10-C-11 double bond on C-15, proton addition to C-14, and proton elimination from the methyl group at C-7 to generate labdadienyl pyrophosphate (**19b**). The cyclization reactions lead to the formation of the **A** and **B** rings with an 5α,10β A:B ring junction. This bicyclic diterpene then undergoes further cyclization by electrophilic attack at C-13 by the exocyclic double bond at C-8 to produce the primarenyl cation **19c**. A hydride shift from C-9 to C-8 and a methyl shift from C-10 to C-9 in **19c**, yields a tertiary carbocation at C-10 which can then be neutralized by water to give **19e**. Oxidation of the C-19 methyl in **19e** to the carboxylic acid generates the lactone **19f** which is further oxidized at C-7 to produce rosenonolactone (**19**).



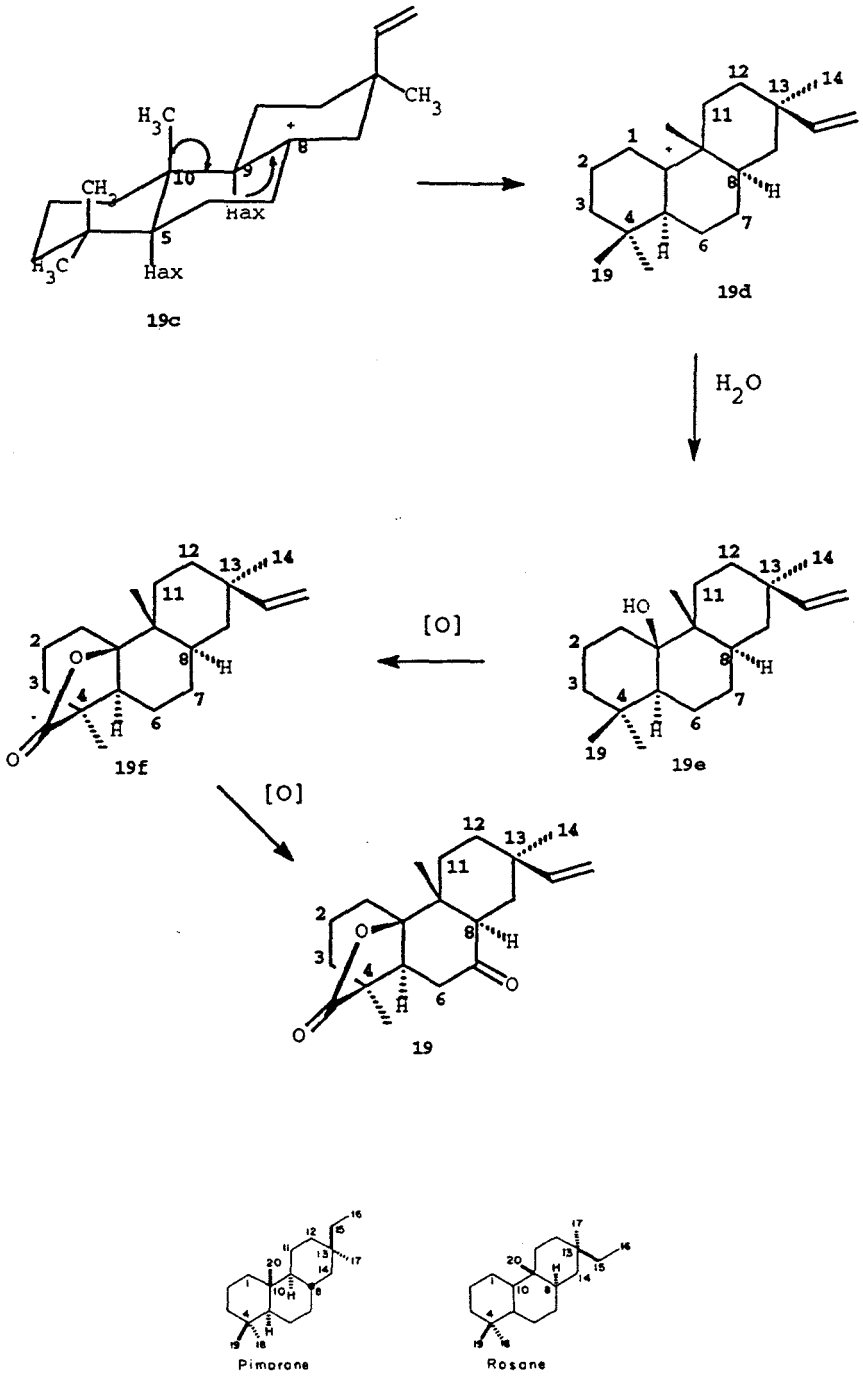


Figure 7.

Various incorporation experiments utilizing labelled substrates have been performed to provide evidence to support the role of geranylgeranyl pyrophosphate (**1a**) as the biological precursor to **19**. In accordance with the mevalonic acid pathway discussed previously, the use of [4*R*-4-^{3}H]mevalonic acid would produce GGPP (**1a**) with tritium atoms at any of the following sites: C-14, C-10, C-6, and C-2 (IUPAC numbering system). If cyclization of **1a** to **19c** proceeds as shown in figure 7, the tritium atom at C-9 (was C-6 in **1a**) will become attached to C-8 (was C-7 in **1a**) as a result of the 1,2-shift. The labelling experiments using [4*R*-4-^{3}H]mevalonic acid as the substrate[19] showed that ^{3}H was indeed recovered at C-8 in **19** confirming the C-9-C-8 1,2-H shift in **19c**. In another set of experiments[20], when tritium labelled copalyl pyrophosphate(**19b**) was used to biosynthesize labdadienyl pyrophosphate(**19a**), followed by incubation of **19a** in the intact fungus, **19a** was incorporated into **19** without degradation. This provides further evidence for the pathway proposed in Figure 7, since **19b** is a proposed intermediate. Further support for the role of geranylgeranyl pyrophosphate (**1a**) as a precursor to rosenonolactone (**19**) comes from other labelling studies. These studies[21] have shown that the C-9 methyl of **19** and the C-10 methyl of primarane (**20**), a diterpenoid similar in structure to rosane (**21**) which has a methyl substituent at C-10 rather than C-9, both originate from mevalonate and as such should follow the mevalonic acid pathway and produce **1a**. Experiments using 5(*S*)- and 5(*R*)-monodeutero-mevalonic acid (MVA)[22] proved that the 5-pro-(*R*)-hydrogen of MVA becomes the (Z)-hydrogen of the C-15-C-16 double bond in rosenonolactone. Alternatively, the 5-pro-(*S*)-hydrogen of MVA becomes

the (E)-hydrogen. Although some intermediates proposed in this scheme are hypothetical, the pattern of incorporation of isotopically labelled precursors, combined with the knowledge of the stereochemistry of the cyclization/ rearrangement reactions supports geranylgeranyl pyrophosphate (**1a**) as the precursor to rosenonolactone (**19**). Incorporation of geranylgeranyl pyrophosphate (**1a**) into many other diterpenoids, for example gibberellic acid[15], has also been demonstrated.

On the basis of present understanding of biosynthetic relationships, geranylgeranyl pyrophosphate (**1a**) (GGPP) occupies a position as a key branch point metabolite of polyisoprenoid metabolism since it serves as the precursor of the carotenes and xanthophylls as well as the various subclasses of diterpenes (Figure 8).

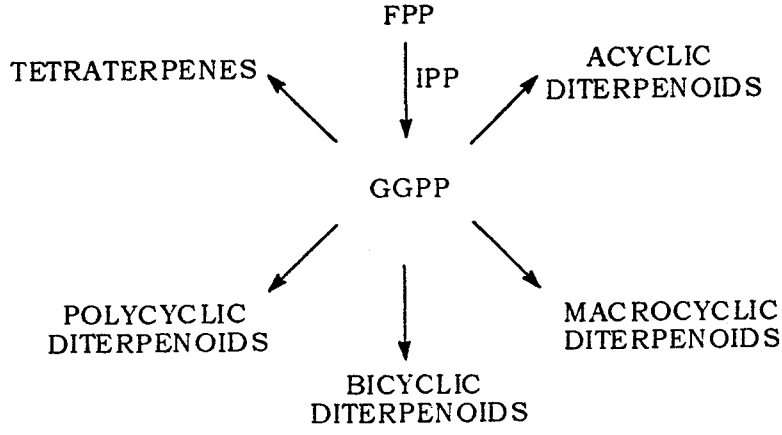
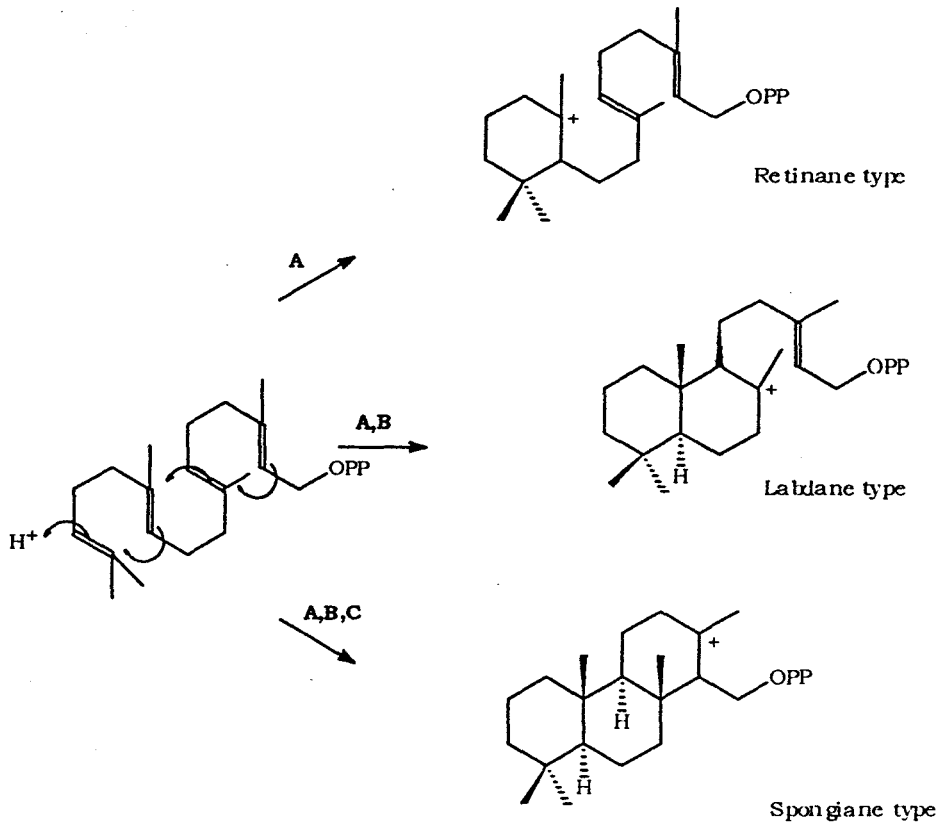
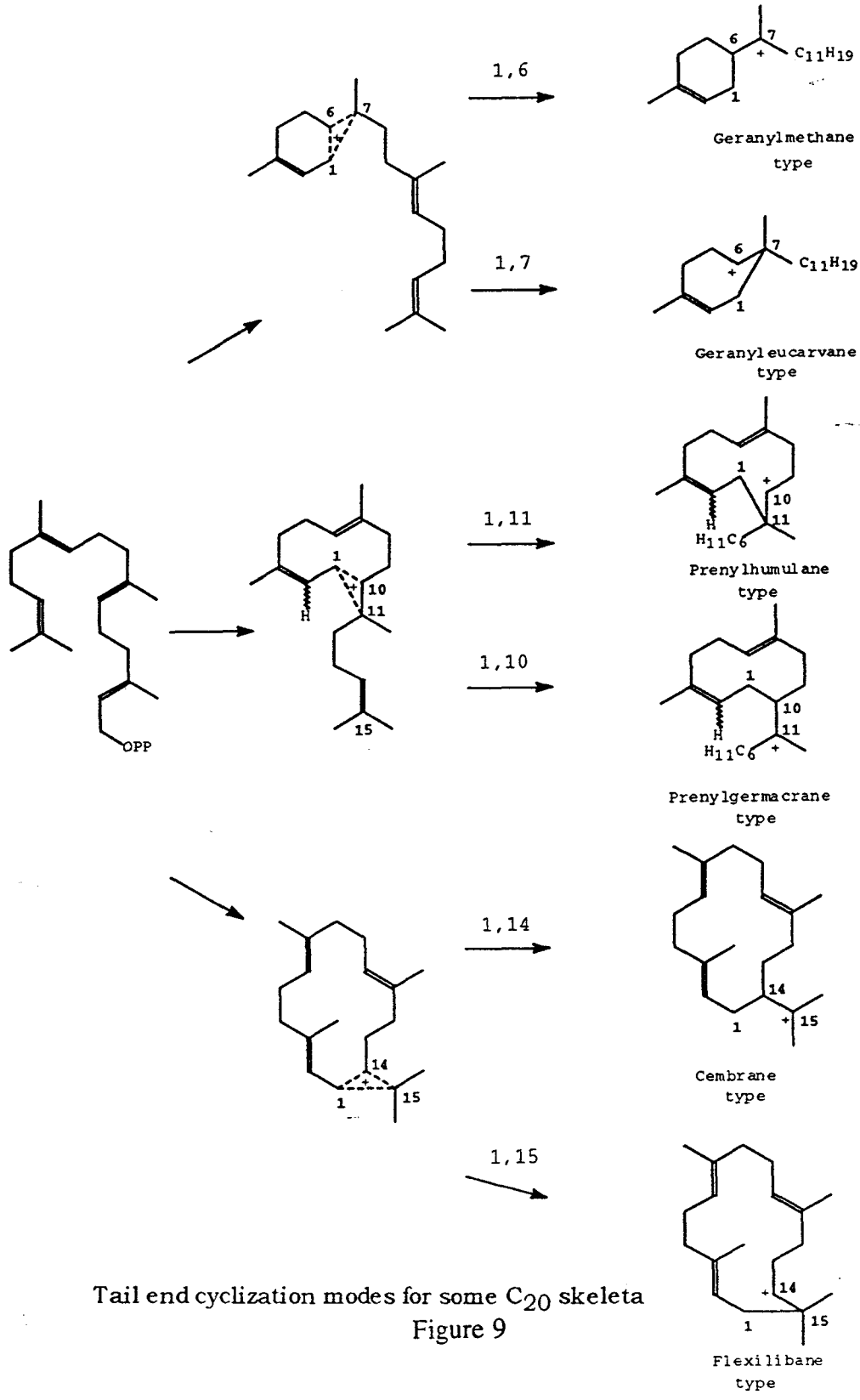


Figure 8.

Specifically, in the area of diterpenoids, Ruzicka proposed rationalization of structures in terms of initial cyclization of geranyl geraniol, the allylic alcohol analogue of **1a**, or geranyl-linalool, employing ionic or free-radical pathways[23]. At present, almost all known diterpene structures can be rationalized this way. Initiation of cyclization of the acyclic precursors as pyrophosphates can occur from either side of the molecule: tail (alcohol end) or head (isopropylidene end). Though free-radical cyclizations have been considered, cyclizations involving carbocations have generally been implicated (Figure 9). The resulting carbocations can then cyclize by π-bond participation generating different cyclic systems depending on the conformation of the substrate on the enzyme(s) involved.



Head end cyclization modes for some C_{20} skeleta



Tail end cyclization modes for some C_{20} skeleta
Figure 9

Cell-free extracts have been used to probe the mechanisms of action of the cyclases that convert acyclic precursors into the various classes of terpenes with differing ring systems[24]. By using such systems as cell-free extracts, micro-organisms, and tissue-cultures, information leading to enzyme purification, control of metabolic pathways, and effect of environmental factors on the production of terpenes can be obtained. Cell-free systems have been established from a number of fungal and higher plant species to enable the elucidation of detailed, particularly stereochemical, features of terpenoid biosynthetic cyclizations to be made.

2.E. Taxol and its Mechanism of Action:

Although taxol(**2**) was first identified in 1971 by Wall and co-workers[25] as the active constituent of *Taxus brevifolia* and showed promising antitumour activity, difficulty associated with the isolation[26,27], extraction[28,29], and its poor aqueous solubility retarded the drug's development. Recently, however, rekindled interest by institutes such as NCI (National Cancer Institute) has stimulated research into the mechanism of action of taxol.

Because cancer cells divide rapidly they are susceptible to drugs that interfere with the mechanisms responsible for cell division; for example, the formation of the mitotic spindle.[30,31] The mitotic spindle is composed of microtubules and is responsible for separating chromosomes during mitosis. Microtubules are the largest cytoskeletal elements and, along with various cell functions such as being the primary constituent of

the mitotic spindle apparatus, are responsible for definition and maintenance of overall cell shape.[32,33] Microtubules form by the reversible polymerization of α and β tubulin, two similar but non-identical protein subunits (Figure 10).

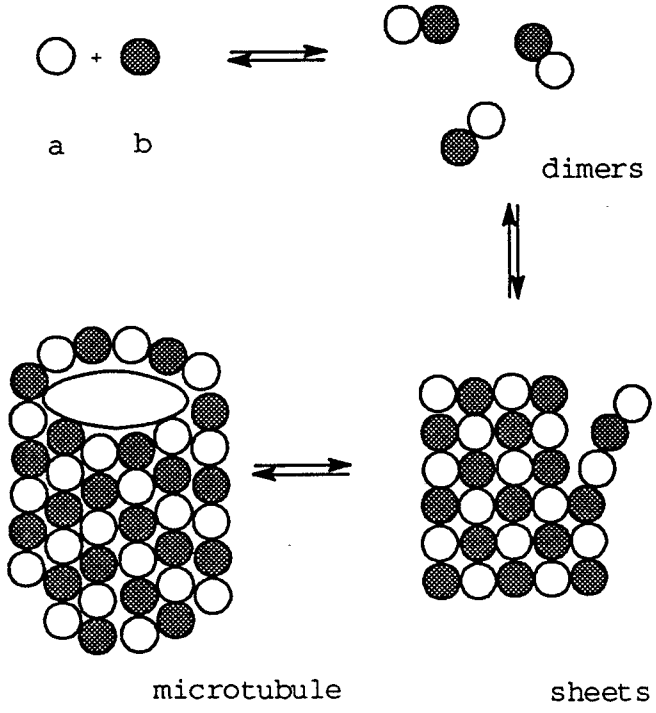
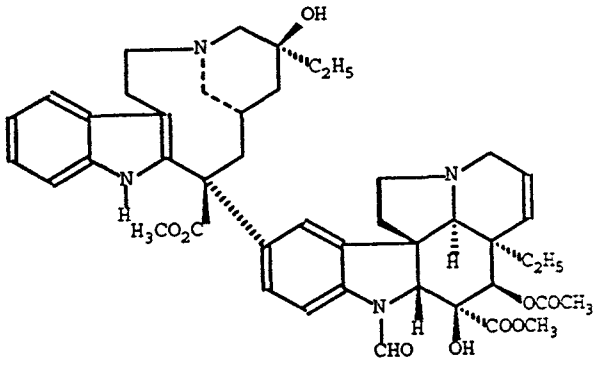


Figure 10.

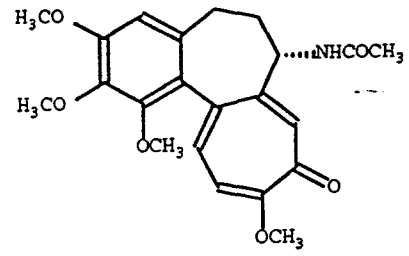
There is typically a lag time required for dimers to come together in the correct configuration to initiate microtubule formation. Studies have shown that taxol, introduced into the cell in concentrations as low as 0.05μmol/L, has the effect of decreasing this lag time, promoting microtubule formation.[34] In contrast to classical antimicrotubule agents such as colchicine(**20**) and the vinca alkaloids, e.g. (**21**), which

promote disassembly, taxol(2) binds tightly to microtubules, stabilizing them and promoting microtubule assembly[35-38].



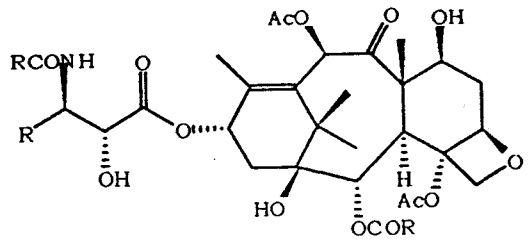
Vincristine

21



Colchicine

20



2 , R= C_6H_5

Microtubules treated with taxol are stable in the presence of Ca^{+2} and low temperatures[39], conditions that usually promote disassembly. This unusual stability results in the inhibition of the normal dynamic reorganization of the microtubule network, that is it "poisons" the microtubule network responsible for cell division. It is this unique mechanism of cytotoxic action that has renewed interest in taxol(2).

The other factor which has given taxol(2) research new life is its spectrum of antitumour activity; namely, its cytotoxicity against various types of cancer such as ovarian cancer, breast cancer, lung cancer, and leukaemia[40]. Encouraging results in phase I and II trials[41] of taxol(2), phase I trials were primarily designed to assess the maximum dosage to human patients and any related side effects, while phase II trials are being performed on a larger number of patients to investigate, in greater detail, the biological activity of taxol, have stimulated investigation into the supply problem for further studies on taxol's antitumour activity against other cancers. Future supplies of **2** are threatened since 25,000 pounds of bark (2,000-3,000 trees) are required to produce 1 Kg of taxol[42,43]. The average dosage of **2** per treatment is 300 mg and at least four treatments are given per patient[44]. As positive responses to treatment increase, the demand for taxol (**2**) will increase. Due to the extremely low quantities of taxol present in the species of yew and the limited supply of the tree itself, it has been necessary for more extensive studies on the drug to be performed. Research is also being performed on the genetic and environmental factors which affect the supply of taxol(**2**)[45]. It is with this data and information concerning the structural modifications[46], partial[47-50] and complete synthesis[51], and the structure-reactivity relationships[52] of taxol that a long term solution to the current problem of demand exceeding supply will be achieved.

At the time of writing, a study involving 300 women in Canada and Europe is attempting to determine how much taxol(**2**) is required to effectively reduce tumours[53]. Previous studies have shown that taxol(**2**) has produced tumour remissions in 1/3 of

advanced ovarian cancers. This is important since it is estimated that at least 1250 Canadian women/year die of ovarian cancer. Recently[54], the Oncological Drug Advisory Committee of the FDA (Food and Drug Administration) has unanimously approved taxol (**2**) for treatment of ovarian cancer patients who failed to respond to at least one round of chemotherapy. This approval will most definitely result in an increased demand for the drug, a demand that as yet can only be supplied by the Pacific yew. Investigation of the biosynthesis of taxol (**2**) will provide valuable information concerning its synthesis.

2.F. Biogenetic hypothesis of taxol(2):

No studies have yet been published on the biosynthesis of taxol(**2**); however, some biosynthetic work has been reported on related compounds, and various speculations and model reactions for the biosynthesis of the taxane ring system also have been published. The most commonly accepted hypothesis for the formation of the taxanes is that they are formed from geranylgeranyl pyrophosphate (**1a**) by electrophilic cyclization, possibly via cembrene (**22**)[55a-d] or verticillene (**23**)[56a-c] (Figure 11) since a similar biogenesis has been suggested for each of these compounds. Preliminary cyclization (Pathway 1->5) is believed to begin with attack by the nucleophilic C-14-C-15 double bond on the electrophilic C-1 bearing the pyrophosphate moiety. This attack results in a tertiary carbocation at C-15, a cembrane type structure, which through H^+ loss (Pathway 3,4,5) generates cembrene (**22**). This neutral intermediate can then undergo ring formation

between C-3 and C-8 followed by H^+ addition (Pathway 3,4) to generate a verticillene type structure (**22a**). The bicyclic diterpene, with a tertiary carbocation at C-12, can be derived directly from the cembrane type structure by attack of the nucleophilic olefin at C-11-C-12 on the tertiary carbcation at C-15 (Pathway 1,2). Loss of H^+ at C-11 of **22a** (Pathway 2,3) produces verticillene (**23**) which may ring-close between C-3 and C-8 to form the taxane type structure (**24**). **24** could also be produced from **22a** (Pathway 1,4) or from cembrene (**22**) through the mechanisms outlined in Figure 11. Another route to the formation of the taxane type structure (Pathway 5) could be through cembrene (**22**) via the bicyclic intermediate having a tertiary carbocation at C-12. In this pathway the tricyclic taxane type structure could result from the formation of a six-membered ring between C-3 and C-8.

It is because of these relationships that the biogenetic hypothesis of the taxane skeleton is expected to involve cembrene and/or verticillene. There are many pathways by which the taxane type structure can be produced and only a few have been discussed here. The biosynthetic route to taxol is therefore a complex one and much work must be done in order to understand what the precursor is, what the intermediates are, and the mechanisms by which the intermediates and the compound of interest are produced.

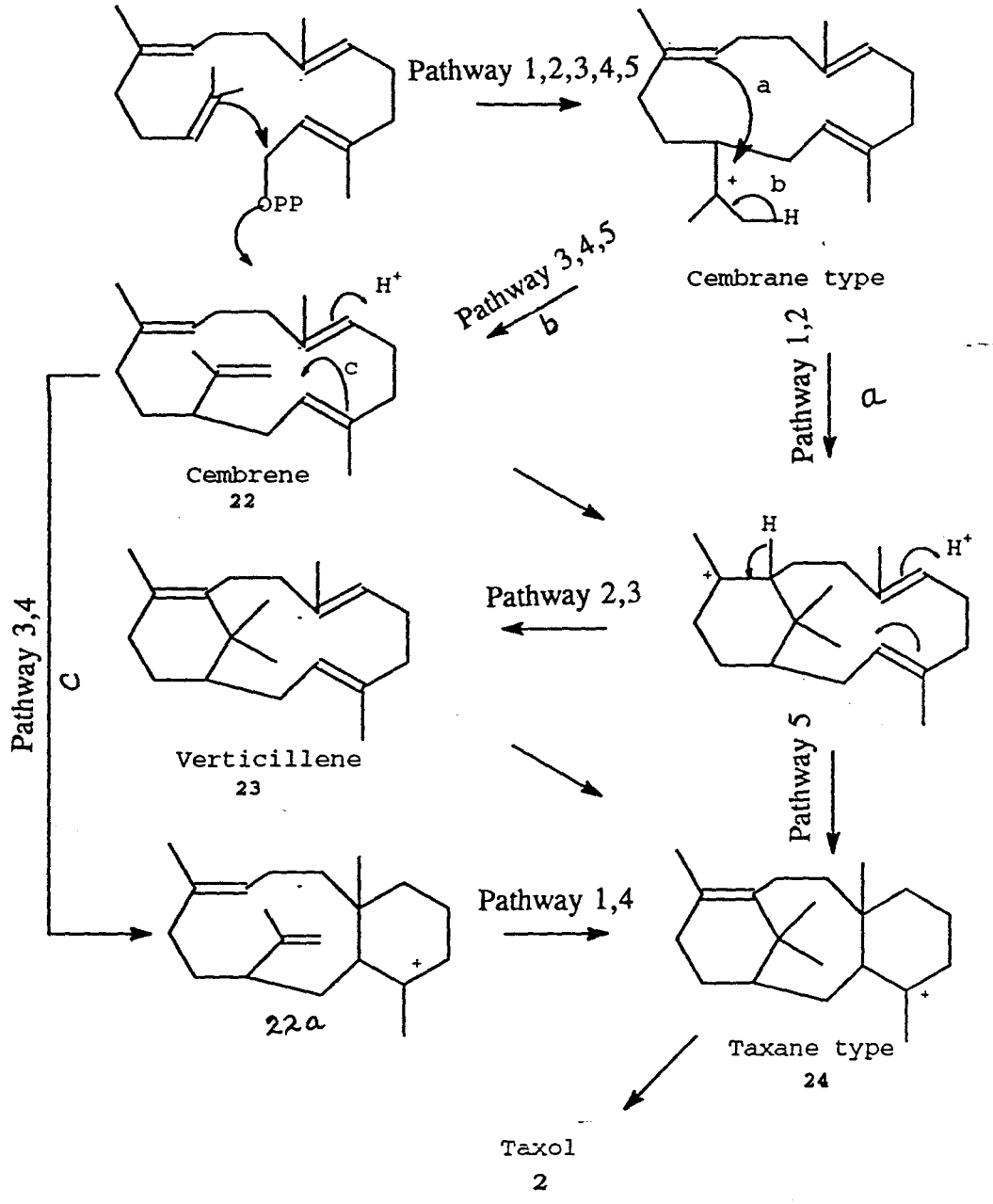
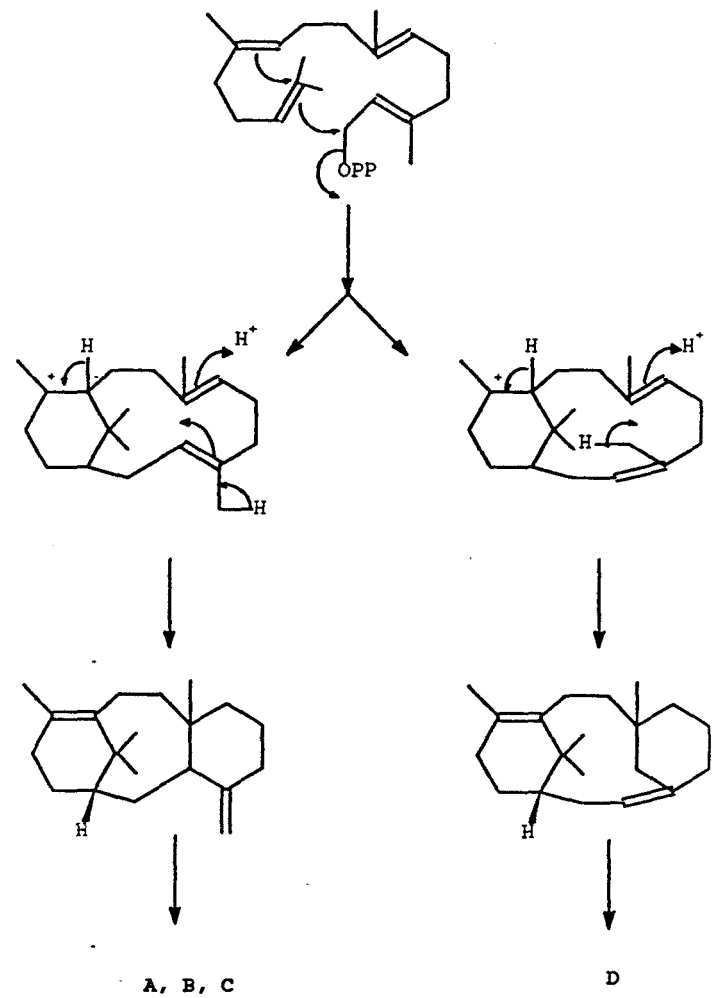

Pathway 1,2,3,4,5
OPP
a
b
H
Cembrane type
Pathway 3,4,5
b
H+
c
Cembrene
22
Pathway 1,2
a
Pathway 3,4
c
H
H+
Pathway 2,3
Verticillene
23
Pathway 5
Pathway 1,4
22a
Taxane type
24
Taxol
2

Figure 11.

Studies[57] involving the treatment of verticillene, and some verticillene epoxides with Lewis acids, failed to effect the electrophilic cyclization to the tricyclic taxane skeleton. Although verticillene (**23**) has yet to be shown to undergo *in vitro* cyclization to the tricyclic system, it cannot be ruled out as a possible biosynthetic intermediate. This result suggests that the biosynthetic sequence is more subtle, perhaps involving alternative geometric/positional isomers of GGPP. The fact that the taxane skeleton is a rare one also suggests that the biosynthetic pathway to it is unusual.

To date, more than 40 derivatives of taxane have been isolated from different species of *Taxus*[58]. The biosynthetic relationship between each of these taxanes can be based on their structural similarities. For example, it is postulated that those taxanes possessing an exocyclic CH_2 moiety at C_4 originate via **A**; taxanes with an oxirane at C_4 are formed from **B**; taxanes having an oxetane ring **C**; and those taxanes with an endocyclic C_4-C_{20} double bond belong to group **D**. Compounds resembling **B,C** can be related to **A** via epoxidation at C_4 of **A** to give **B**, and **C** can be produced by ring opening of the epoxide, **B**, and subsequent cyclization to form an oxetane ring. Taxine A, the only known taxane to date with an endocyclic double bond, is believed to undergo a cyclization route separate from the other known taxanes. A proposed biogenetic scheme from geranylgeranyl pyrophosphate through to each of these groups is shown in Figure 12.



Biogenetic approach to taxane ring system

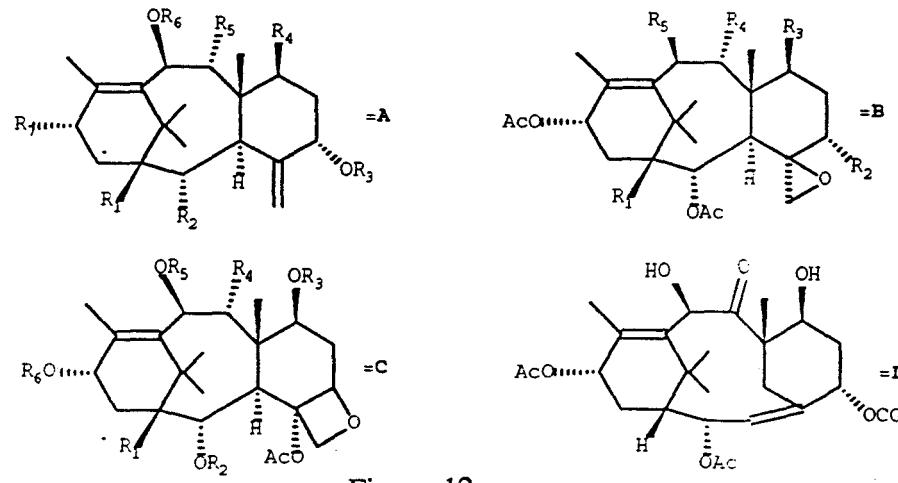


Figure 12.

It is the goal of this research to probe the biosynthesis of taxol (**2**) through the synthesis of a radioactively labelled precursor, [1-^{3}H]-geranylgeranyl pyrophosphate (**1b**), and its subsequent incubation with a cell-free extract from a species of yew. It is hoped that these extracts will contain those enzymes responsible for the biosynthetic conversion of **1b** to **2**, and that some hydrocarbon intermediates (formed through the proposed cyclization pathways discussed herein, Figures 11 and 12) related to taxol (**2**) will contain the tritium label. Detection of these labelled intermediates, and their isolation and identification could provide a vigorously sought route to the synthesis of taxol (**2**) since these intermediates would have the basic taxane skeleton and the correct stereochemistry necessary for the drug's antitumour activity. This may provide an increased supply of **2** which would result in a more extensive investigation into its role as an anticancer agent.

3.Results and Discussion

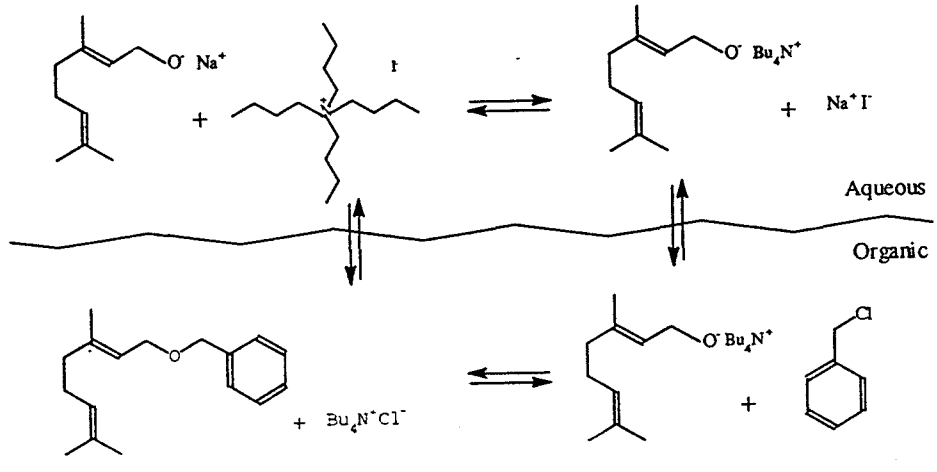
It has yet to be determined if geranylgeranyl pyrophosphate (**1a**) has a role in the biosynthesis of taxol and what that role is. It is the goal of this research to establish whether or not **1a** is a precursor to **2** as is generally accepted. In order to verify that **1a** is a precursor to taxol (**2**), geranylgeranyl pyrophosphate labelled with tritium (**1b**) was prepared from commercially available geraniol (Schemes 1-3). Tritium was chosen as the radioactive label because it is a relatively inexpensive radioisotope, it has a relatively long half-life, and it has high specific activity[59]. The radiolabelled pyrophosphate (**1b**) was used in incubation experiments with cell-free enzyme preparations of *Taxus x media* densiformis. The extracts from the incubation experiments were analyzed by scintillation counting for radioactivity content and then analyzed by radioactivity gas chromatography for compound content.

3.A. Synthesis of Geranyl Geraniol

In the synthesis of the benzyl protected geraniol compound (**26**), the phase-transfer reagent, tetra-*N*-butylammonium iodide(**25**) was prepared. An acid-base reaction is utilized to give **25** as a salt which precipitates out of solution and is dried over P_2O_5. The ^{1}H-NMR, ^{13}C-NMR, and MS confirm the formation of **25**.

The Williamson reaction was used in the preparation of geranyl benzyl ether (**26**). The alkoxide was generated by treatment with NaH in THF at 0°C. Tetrabutylammonium iodide (**25**), Bu_4NI, was added in order to make the alkoxide soluble in the benzyl chloride so that nucleophilic substitution to produce **26** could occur.

The quaternary ammonium salt, despite its ionic nature, is soluble in nonpolar media due to the presence of the alkyl substituents. These alkyl substituents shield the positively charged nitrogen atom imparting nonpolar character upon the ion. This property is exploited in phase-transfer catalysis, because the sodium alkoxide salt is not soluble in the benzyl chloride: these two reactants would only encounter each other at the surface of the sodium salt resulting in very little reaction product.The Bu_4NI forces the alkoxide into the organic phase where it undergoes a nucleophilic substitution reaction with benzyl chloride to produce **26** (Figure 13).



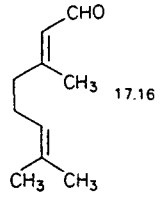
26

Figure 13.

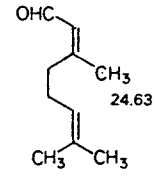
The structure of (**26**) was confirmed by ^{1}H-NMR, ^{13}C-NMR, IR, and MS analysis. The ^{1}H-NMR (Figure 14) contains a broad singlet at 7.35 ppm representing the phenyl protons. The triplets at 5.40 and 5.11 ppm each integrate for one proton and fall within the C=C<u>H</u> region of the ^{1}H-NMR spectrum. The presence of the electronegative oxygen atom causes deshielding of the nuclei attached to or near it[60], therefore the triplet at 5.40 ppm should be assigned to the olefinic proton at C-2 while the triplet at 5.11 ppm is assigned to the olefinic proton at C-6. The doublet corresponding to the methylene protons at C-1 in **26** has a chemical shift of 4.03 ppm which is 0.12 ppm upfield compared to the C-1 methylene chemical shift for geraniol[61]. The multiplet at 2.07 ppm represents the methylene protons at C-4 and C-5. Unlike the geraniol ^{1}H-NMR spectrum, which shows only two singlets at 1.68 and 1.61 ppm for the nine methyl protons, the spectrum of **26** has three singlets at 1.68, 1.64, and 1.60 ppm each integrating for three protons. The ^{13}C-NMR spectrum (Figure 15) contains 15 carbon signals, there are two aromatic carbon signals, 127.38 and 127.80 ppm, which actually represent the four aromatic carbons C-13, C-17 and C-14, C-16. The ipso carbon, C-12, resonates at 139.93 ppm, while the signal at 127.07 ppm is assigned to the para aromatic carbon, C-15[62]. The methylene carbon at C-11 resonates at 71.41 ppm and the methylene carbon at C-1 resonates at 66.06 ppm. The ^{13}C-NMR spectral data of geraniol and farnesol[63] support this last assignment as their C-1 carbons resonate at 58.6 ppm. The signals at 25.93 and 39.20 ppm have been assigned to the internal methylenes at C-4 and C-5 respectively. The C-9 and C-10 carbons are substitutents of the olefinic carbons at C-3 and C-7, both having *cis*

geometry, resonate at 17.18 and 16.00 ppm: the signal at 25.27 ppm has been assigned as the methyl carbon at C-8, this carbon atom being *trans*. The effect on the resonance of an sp^3 carbon attached to an sp^2 carbon in either a *cis* of *trans* geometry is quite different. The *trans* substituted methyl carbon will be shielded to a greater extent than the corresponding *cis* isomer. This can be seen in such examples as *cis*- and *trans*-citral[64].

Citral a (Geranial) $C_{10}H_{16}O$



Citral b (Neral) $C_{10}H_{16}O$



The infrared spectrum contains an absorbance at 1383cm^{-1} which is characteristic of the C-H stretch of a geminal dimethyl group, the sp^2 C-C aryl absorbance at 1450cm^{-1}, and the C-O-Ar absorption peak at 1250cm^{-1}. The mass spectrum (Figure 16) shows the $[M+NH_3]^+$ peak, m/z 262 and a relative intensity of 48%, the m/z 137 peak due to the $C_{10}H_{17}^+$ fragment, 100% relative intensity, and the m/z 81 peak corresponds to the $C_6H_9^+$ fragment, 12% relative intensity.

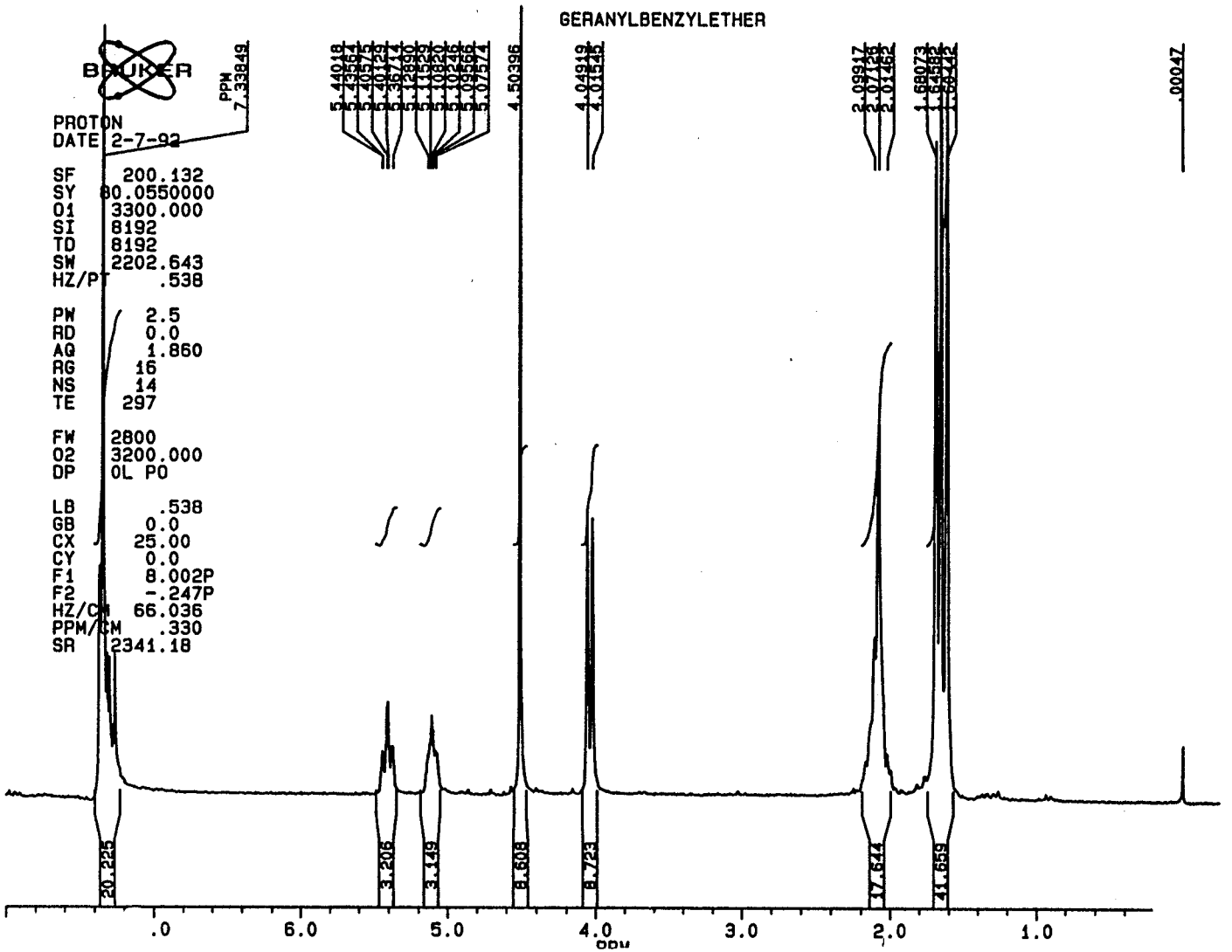
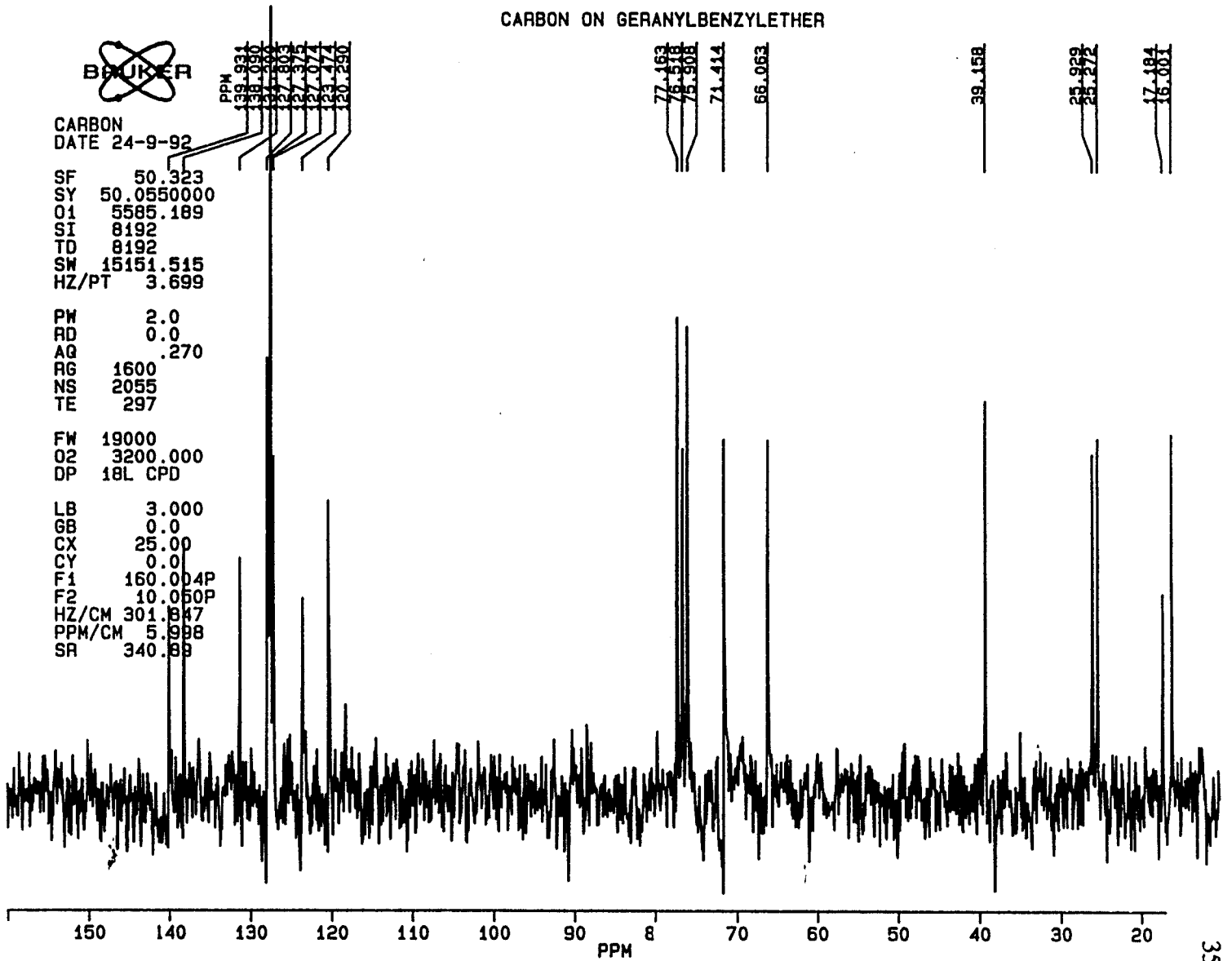


Figure 14.

^{1}H-NMR (200 MHz, $CDCl_3$) spectrum of Geranyl Benzyl Ether

Figure 15.



^{13}C-NMR (200 MHz, $CDCl_3$) spectrum of Geranyl Benzyl Ether

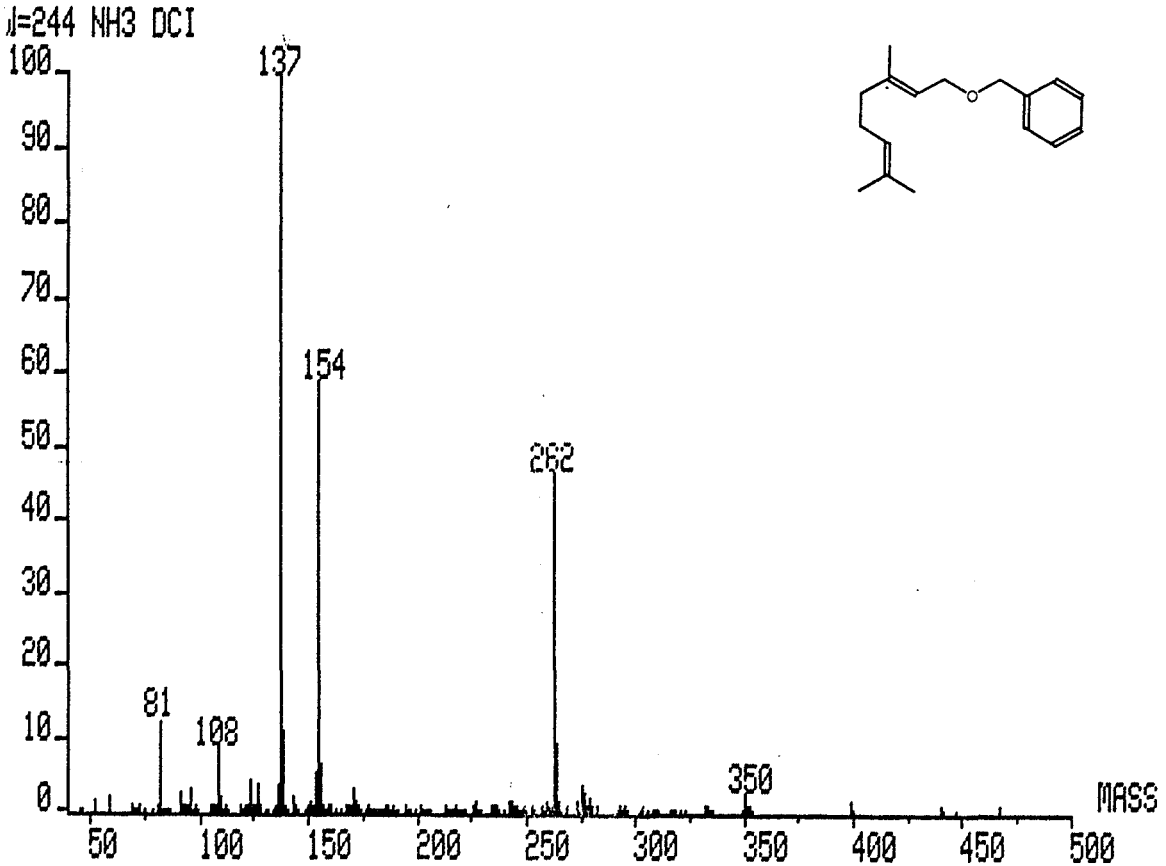


Figure 16.

Mass Spectrum (NH_3-CI) of Geranyl Benzyl Ether

Oxidation of unsaturated compounds by selenium dioxide introduces an OH into the allylic position[66,67]. Chemical manipulation of the primary hydroxyl is a very useful way to introduce other functional groups into the allylic position. Treatment of **26** with selenium dioxide and 95% ethanol at reflux allows the formation of the α,β-unsaturated primary alcohol **28**, but further oxidation can also lead to formation of the aldehyde **27**[68]. In fact, both **27** and **28** were produced, separated, purified, and characterized by ^{1}H-NMR, ^{13}C-NMR, IR, and MS analysis.

The ^{1}H-NMR spectrum for alcohol **28** contained a triplet at 5.40 ppm integrating for both the C-2 and C-6 vinylic protons. The presence of the allylic OH group withdraws electron density from the unsaturated C<u>H</u> carbon, C-6, thereby affecting the proton and deshielding it by approximately 0.3 ppm resulting in overlap with the resonance of the C-2 proton. The C<u>H</u>$_2$OH protons appear as a singlet at 3.96 ppm. There is also a singlet at 1.65 ppm which integrates for approximately six protons: these would be the protons of the methyl groups at C-10 and C-7. The rest of the spectrum resembles that of **26**.

The ^{1}H-NMR of **27** (Figure 17) has an aldehyde resonance at 9.39 ppm, and two olefinic triplets at 6.47 and 5.45 ppm. These signals have been assigned to the vinylic protons at C-6 and C-2 respectively.

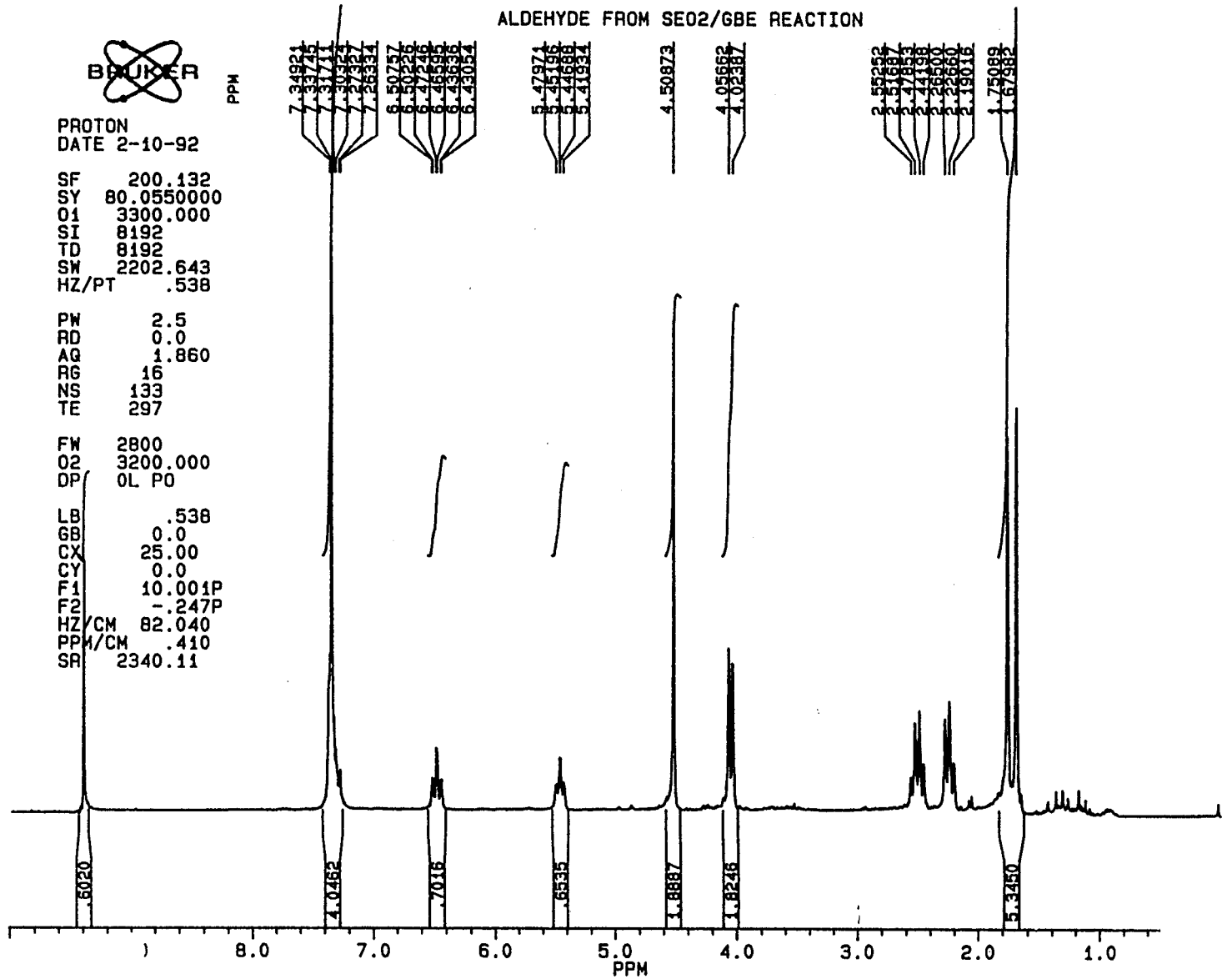


Figure 17.

^{1}H-NMR (200 MHz, $CDCl_3$) spectrum of 8-O-Benzyl-2,6-dimethyl- 2,6-octadienal

The proton resonance at C-6 has moved downfield by 1.37 ppm (relative to that in starting material) as a result of the deshielding effect of the aldehyde C=O. The multiplet at 2.03 ppm has now been replaced by a quartet at 2.50 ppm representing the $C\underline{H}_2$ at C-5 and a triplet at 2.22 ppm which has been assigned to the $C\underline{H}_2$ at C-4. Again, the remainder of the resonances have the same chemical shift and multiplicity as in **26**.

The ^{13}C-NMR spectrum of **28** is similar to that for geranyl benzyl ether(**26**) except for the replacement of the $\underline{C}H_3$ signal at 25.93 ppm by a $\underline{C}H_2$ signal at 68.28 ppm which is the carbon chemical shift of the primary alcohol. The ^{13}C-NMR spectrum of **27** contains the aldehyde carbon signal at 194.54 ppm and the other resonances indicative of the benzyl ether (see Figure 18).

The aldehyde **27** was reduced in the presence of the mild reducing agent, $NaBH_4$, so as not to affect the other functionalities in the molecule. Spectral characteristics of the reduction product were identical to **28**.

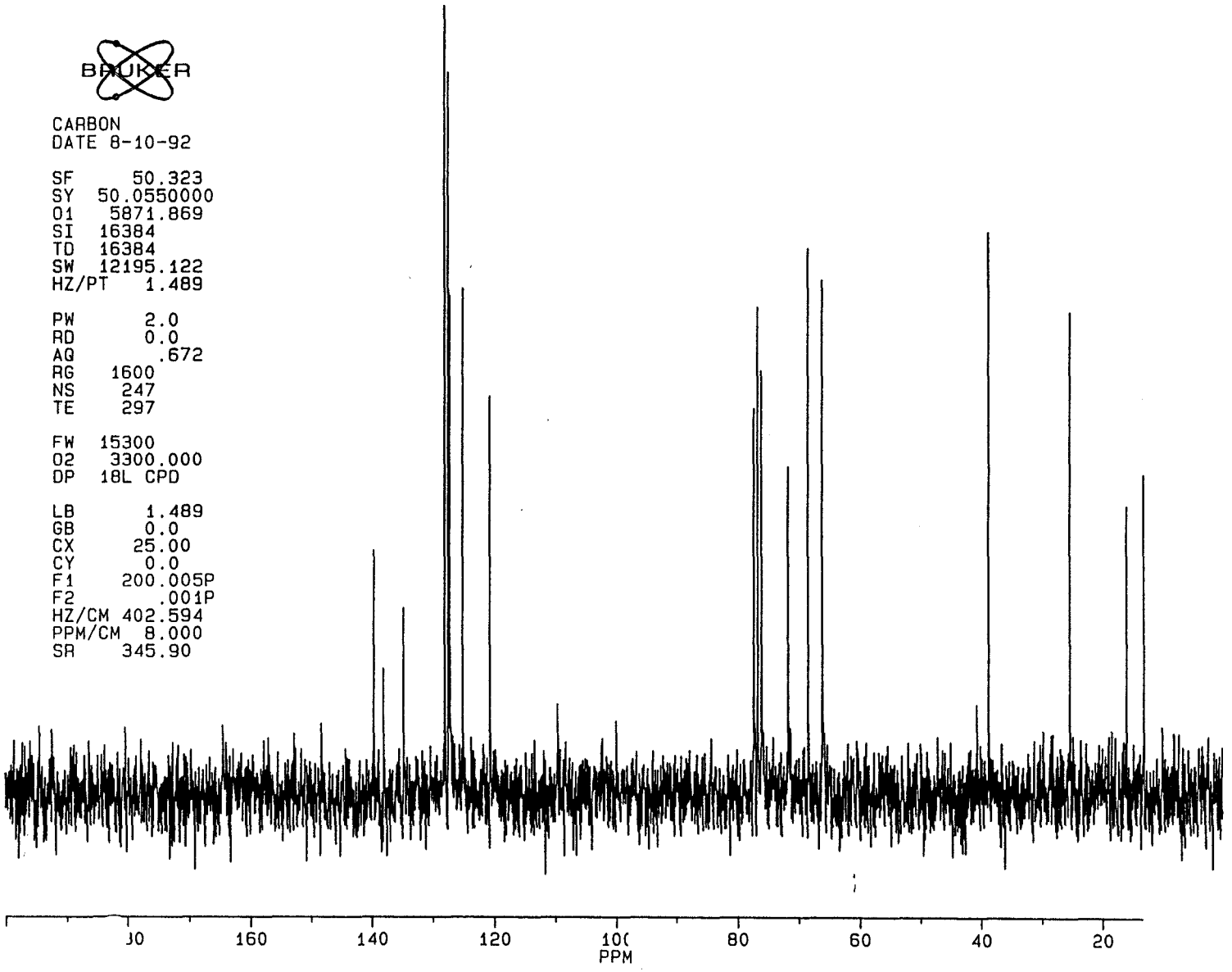
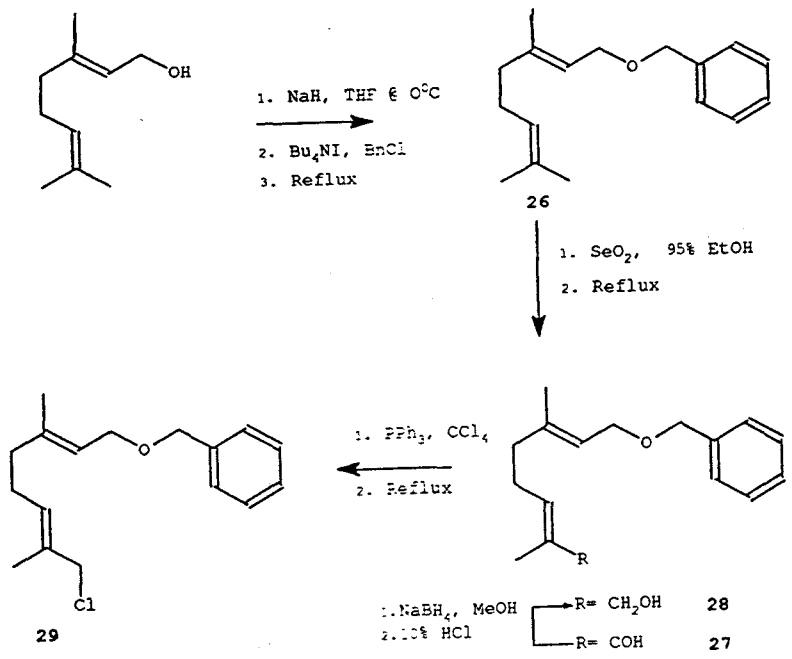


Figure 18.

^{13}C-NMR (200 MHz, $CDCl_3$) spectrum of 8-Hydroxy-3,7-dimethyl- 2,6-octadienyl Benzyl Ether

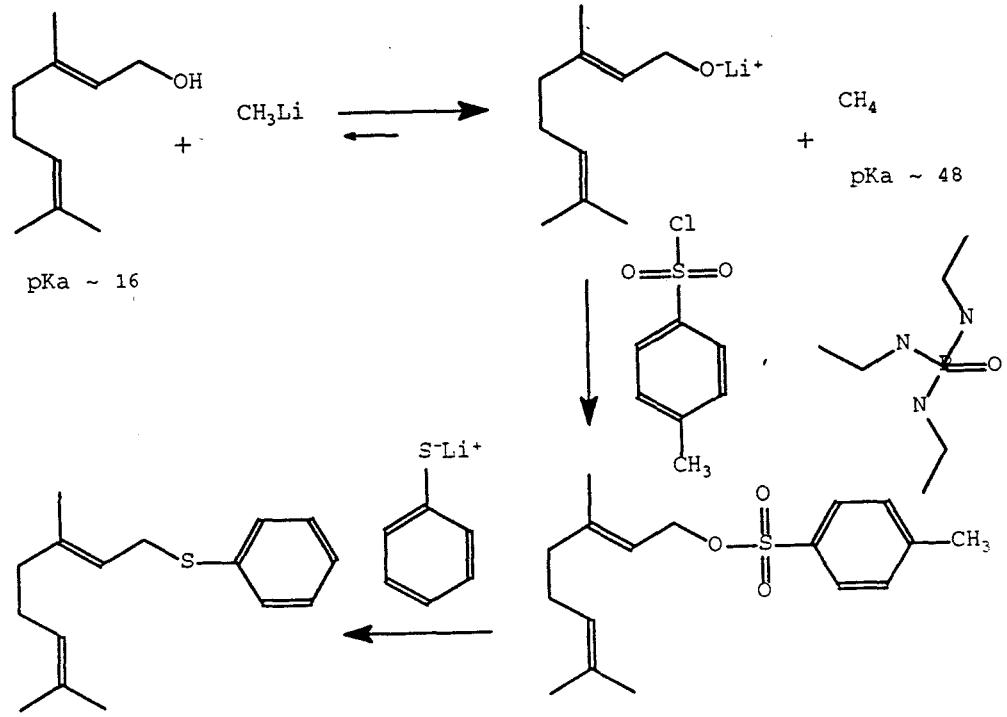
The alcohol **28** was converted to the chloride **29**[69] in the presence of triphenylphosphine and carbon tetrachloride under reflux conditions (see Scheme 1). The chloride was produced in 78% yield and its structure confirmed by NMR, IR, and MS. The ^{1}H-NMR spectrum is similar to that of **28** except that the $\underline{C}H_2Cl$ doublet, at 3.91 ppm, is shifted upfield by 0.05 ppm due to the replacement of the oxygen atom by the less electronegative chlorine atom. The ^{13}C-NMR has all the appropriate carbon resonances as in **28**, but the $\underline{C}H_2OH$ carbon resonance has been replaced by a signal at 52.15 ppm which can be assigned to the $\underline{C}H_2Cl$ carbon. The IR and MS also confirm the formation of 8-chloro-geranyl benzyl ether (**29**).



Scheme 1.

Now a C_{10} compound (**29**) has been synthesized with the alcohol end, C-1, protected as a benzyl ether and the isopentenyl end converted to a primary halide. This primary alkyl halide can undergo nucleophilic substitution since the Cl^- is a good leaving group.

The next step in the synthesis of geranyl geraniol (**33**) is to prepare *trans*-geranyl phenyl thioether (**31**)[70]. Geraniol is subjected to basic conditions, methyl lithium, in order to generate the alkoxide. Geraniol has a pK_a of approximately 16, a typical value for RCH_2OH compounds[71], making it slightly acidic. Methyllithium is a relatively strong base: the pK_a of its conjugate acid, CH_4, is 48 making it a very weak acid. Therefore the acid-base equilibrium will lie to the right as is shown in Figure 19.



31

Figure 19.

Once the alkoxide is generated, *p*-toluenesulphonyl chloride is added to the reaction mixture and nucleophilic substitution takes place with Cl^- as the leaving group. This produces the allylic alcohol activated as the tosylate, a very good leaving group by virtue of the fact that the tosylate anion can be stabilized through resonance. Upon addition of lithium thiophenoxide, the primary carbon of the protected alcohol, C-1, undergoes nucleophilic substitution to yield the phenyl thioether (**31**). It is important to note that the primary solvent in this series of reactions is HMPA, hexamethyl phosphorus(V) triamide, a polar aprotic solvent, which was chosen for its ability to solvate the large allylic alkoxide so that the nucleophilic substitution reaction with tosyl chloride could proceed. Formation of **31** was confirmed by ^{1}H-NMR, ^{13}C-NMR, MS, and IR.

The ^{1}H-NMR signals in the spectrum of **31** were assigned as follows: the olefinic proton at C-2 was in the expected region (4-6 ppm) but shifted downfield (5.31 ppm) of the other olefinic proton (5.06 ppm) due to the effect of the electronegative sulphur atom of the S-phenyl substituent at C-1. The doublet at 3.55 ppm was assigned to the $C\underline{H}_2$ protons at C-1: this is in agreement with the olefinic proton assignment at C-2. The multiplet at 2.02 ppm represents the methylene protons at C-4 and C-5. There are two singlets in the 1.5-1.7 ppm region which have been assigned to the protons of the geminal methyls at C-7 and the methyl protons at C-3. The methyl protons at C-3 should have a chemical shift downfield relative to the methyl protons at C-7 due to the inductive effect of the S-phenyl substituent at C-1. The spectrum also contains the resonances for the

protons of the monosubstituted aromatic moiety at 7.23 ppm which integrates for 5 protons.

In the ^{13}C spectrum of **31** the $\underline{C}H_3$ signals at 15.84, 17.51, and 25.48 ppm have been assigned to C-9, C-10, and C-8 respectively. The resonance for the $\underline{C}H_2SPh$ carbon is present at a chemical shift of 32.14 ppm which is characteristic of an alkyl group with a thiophenyl substituent[72]. The mass spectrum in Figure 20 shows the fragmentation pattern for **31**. The EI spectrum of **31** contains the molecular ion peak at m/z 246, 8% relative intensity, m/z 81 from the monoterpene moiety of the molecule as discussed previously, and the peak at m/z 69 is the $C_5H_9^+$ fragment due to cleavage of the C-4-C-5 bond[65]. There is also a peak at m/z 110 representing the $[M-C_{10}H_{16}]^+$ fragment resulting from cleavage of the carbon-sulphur bond at C-1.

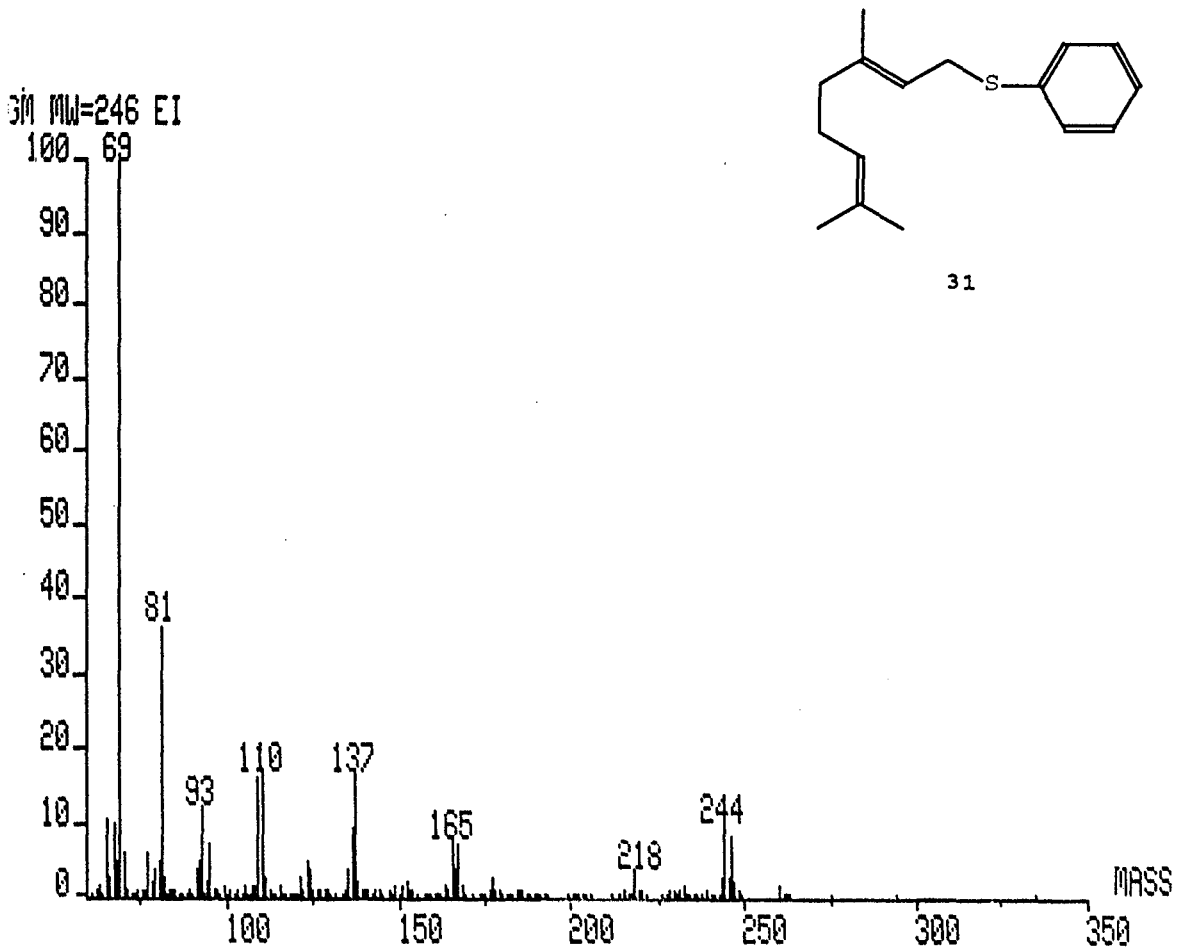
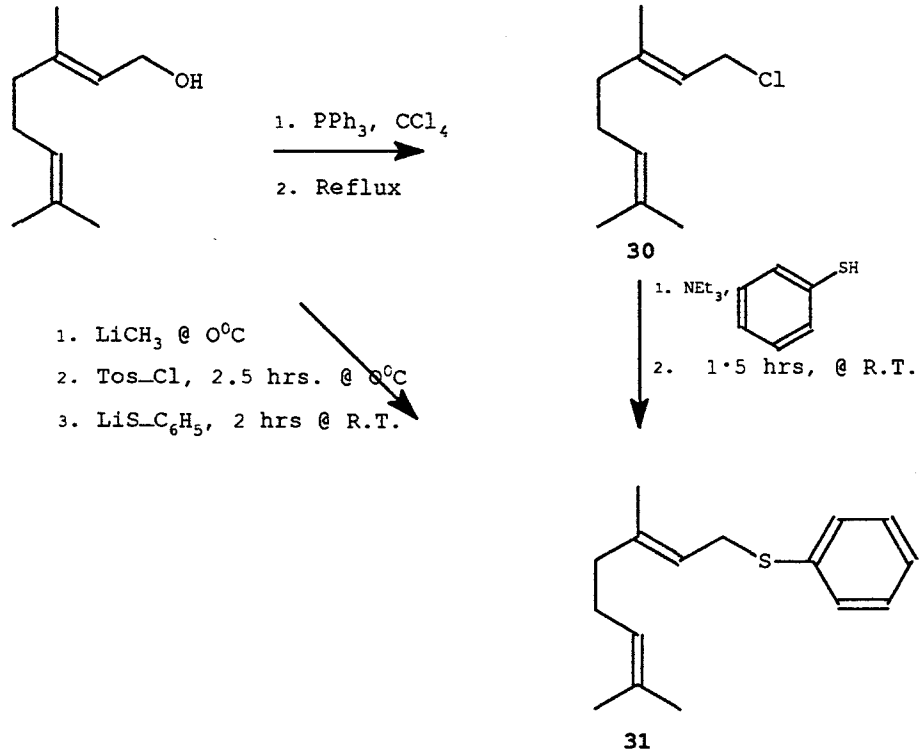


Figure 20.

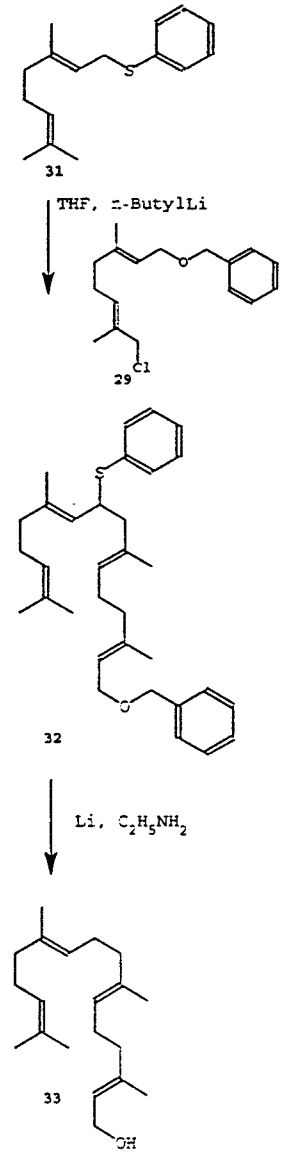
Mass Spectrum (EI) of 3,7-Dimethyl-2,6-octadienyl Thiophenyl Ether

An alternative approach to the synthesis of **31** was investigated so as to avoid use of HMPA, a cancer suspect agent. The method[69] involved synthesis of a primary halide derivative of geraniol, **30**, by halogenation of geraniol using triphenylphosphine (PPh_3) and carbon tetrachloride (CCl_4) under reflux conditions. The chloride was produced in 79% yield and its structure confirmed by ^{1}H-NMR, ^{13}C-NMR, IR, and MS analysis. Geranyl chloride (**30**) was combined with triethylamine, NEt_3 and thiophenol at room temperature (Scheme 2)[73] to give **31** in 76% yield. Spectral characteristics are identical to those described above.



Scheme 2.

With the synthesis of **31** complete, a Biellmann[74,75] coupling reaction on the two C_{10} compounds would produce the desired C_{20} skeleton (Scheme 3). This coupling method has an advantage over other such methods in that the original positions and configurations of the allylic double bonds are preserved[76].



Scheme 3.

Geranylphenyl thioether (**31**) in THF was treated with *n*-butyl lithium at -78°C to generate the anion. The allylic chloride (**29**) in THF was added to the anion and nucleophilic attack by the anion on the primary alkyl chloride resulted in the formation of 3,7,11,15-tetramethyl-9-phenylthio-2,6,10,14-hexadecatetraenyl benzyl ether (**32**) in 76% yield. The ^{1}H-NMR spectrum contains a broad multiplet at 7.20 ppm which integrates for the 10 aromatic protons. The vinylic protons at C-2, C-6, C-10, and C-14 overlap to produce the broad multiplet 5.50-4.80 ppm. The methine proton at C-9 is a singlet which overlaps with the doublet of the C$\underline{H}_2$Bn protons at C-1. All of the methylene resonances occur as a broad multiplet at 2.47-1.80 ppm. There are two signals for the fifteen C$\underline{H}_3$ protons, one singlet at 1.55 ppm integrates for twelve protons while the other sharp singlet at 1.33 ppm represents the other methyl protons.

Reduction of the thiophenyl[77] and benzyl groups in **32** is achieved by using lithium in ethylamine. The reduction is facilitated by the resonance stabilizing ability of the aromatic moieties and also the electronegative sulphur and oxygen atoms. This results in relatively easy cleavage of the hetero-atom/carbon bonds. Not only are the allylic double bonds at C-10-C-11 and C-2-C-3 important in stabilizing the negative charges at C-9 and C-1, but the allylic substituents are cleaved without reducing the olefinic double bonds[76].

The reduction product, geranyl geraniol (**33**) was homogeneous by TLC and produced in 68% yield. The compound was characterized by ^{1}H-NMR, ^{13}C-NMR, IR, and MS. The ^{1}H-NMR spectrum contains the signals corresponding to the vinylic protons at C-2, C-6, C-10, and C-14. The triplet at 5.42 ppm integrates for one proton assigned as

the vinylic proton at C-2 since the electronegative oxygen atom should deshield the C-2 hydrogens. The triplet at 5.10 ppm integrates for three protons and represents the protons at C-6, C-10, and C-14. The doublet at 4.15 ppm has been assigned as the $C\underline{H}_2$ at C-1, and the multiplet at 2.03 ppm represents the six $C\underline{H}_2$ groups at C-4, C-5, C-8, C-9, C-12, and C-13. There are two singlets remaining in the spectrum: the singlet at 1.68 ppm integrates for six protons while the other singlet at 1.60 ppm integrates for nine protons. These singlets represent the methyl protons at C-16, C-17, C-18, C-19, and C-20.

The ^{1}H-NMR, ^{13}C-NMR, MS, and IR spectra of **33** were compared to those of authentic geranyl geraniol and are in agreement. Due to the small quantity of **33** produced, the commercial supply of geranyl geraniol was used in the conversion to the radiolabelled pyrophosphate (**39**). Although the radioactive label was attached to C-1 of the diterpene precursor, the synthetic approach outlined in this research thesis was chosen because of the potential for radioactive labelling at many other sites in the C_{20} molecule. The other advantage to this synthetic route is that different radioactive nuclides could be contained in the same molecule.

3.B. Preparation of [1-^{3}H]-Geranylgeranyl Pyrophosphate:

In order to introduce a tritium atom into the C_{20} skeleton, geranyl geraniol (**33**) is oxidized to the aldehyde in the presence of MnO_2 in hexanes at room temperature in 85% yield[78]. There are the absorption peaks at 1240 cm^{-1} and 1570 cm^{-1} in the IR spectrum

which correspond to the geminal dimethyl C-H stretch and the conjugated (C=O)-H aldehydic stretch. The ^{1}H-NMR has the appropriate signals for the protons in the isoprenoid skeleton as well as the doublets at 10.01 and 9.91 ppm, the region of the proton spectrum characteristic of an aldehyde proton. Each doublet has a coupling constant of 8.2 Hz and the ratio of peak heights was different each time the aldehyde (**34**) was produced by this method. This may be a result of *E-Z* isomerization about the double bond, but this proposal was not confirmed. However, there is only one CHO carbon signal at 191.24 ppm in the ^{13}C-NMR spectrum. The remainder of the ^{13}C-NMR spectrum is similar to that of geranyl geraniol.

The aldehyde (**34**) was diluted with methanol and the solution was injected into a vial containing 5 mCi of $NaBH_3T$ at 0°C and stirred for 3 hours[77]. The reduction was completed by the addition of excess $NaBH_4$ followed by dilute acid. The mixture was extracted several times with hexanes to recover [1-^{3}H]-geranyl geraniol (**35**). The alcohol (**35**) was purified by flash chromatography and the product found to be homogeneous by TLC. A ^{1}H-NMR spectrum was obtained and in comparison to the ^{1}H-NMR spectrum of geranyl geraniol (**33**), the spectra are almost identical (see Table 1).

Table 1.

PROTON TYPE AND LOCATION	^{1}H-NMR δ(ppm); MULTIPLICITY	
	[1-^{3}H]-GGOH	GGOH
vinylic @ C-2	5.42;triplet	5.42;triplet
vinylic @ C-6,C-10,C-14	5.10;triplet	5.10;triplet
* C<u>H</u>T @ C-1; C<u>H$_2$</u> @ C-1	4.14;doublet	4.15;doublet
C<u>H$_2$</u> @ C-4,-5,-8,-9,-12,-13	2.10;multiplet	2.09;multiplet
C<u>H$_3$</u> @ C-20	1.68;singlet	1.68;singlet
C<u>H$_3$</u> @ C-16,-17,-18,-19	1.60;singlet	1.60;singlet

A small portion of the alcohol (**35**) was prepared for scintillation counting to determine the specific activity (1.95 x 10^8 DPM/mg(+/-5.6%))[79].

[1-^{3}H]-Geranyl geraniol (**35**) was converted to the corresponding radioactive chloride (**36**) according to the PPh_3/CCl_4 method previously mentioned[69]. This procedure was also carried out on a sample of authentic geranyl geraniol to give geranylgeranyl chloride so that TLC and ^{1}H-NMR data could confirm the structure of **36** (see Table 2). The ^{1}H-NMR spectrum of geranylgeranyl chloride differs from the ^{1}H-NMR spectrum of **36** in that there are three singlets at 1.68, 1.64, and 1.60 ppm with an integration ratio of 3:3:9 instead of two singlets at 1.68 and 1.60 ppm. The ^{1}H-NMR spectrum of geranylgeranyl chloride is shown in Figure 21.

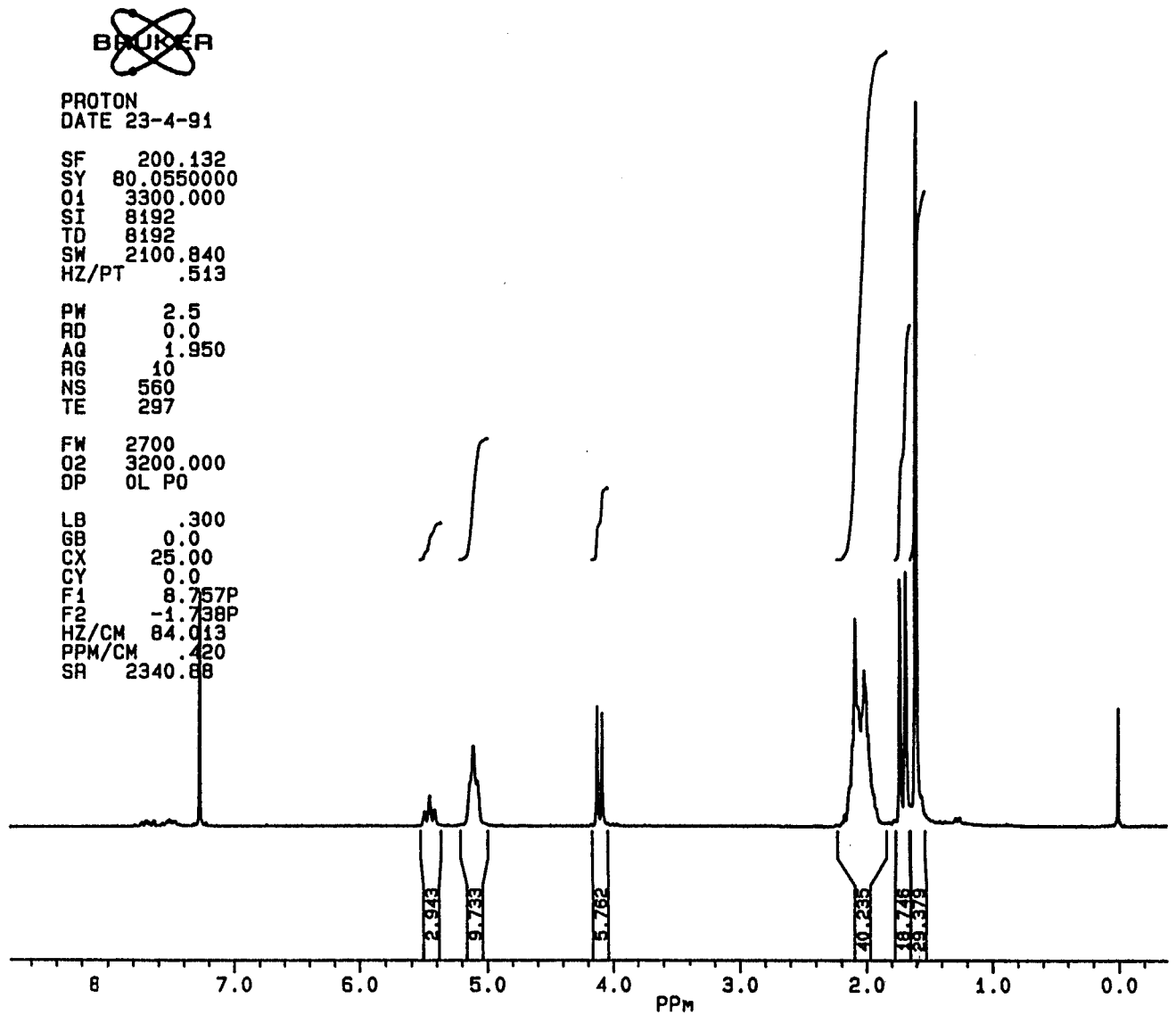


Figure 21.

^{1}H-NMR (200 MHz, $CDCl_3$) spectrum of Geranylgeranyl Chloride

Table 2.

PROTON TYPE AND LOCATION	^{1}H-NMR δ(ppm); MULTIPLICITY	
	[1-^{3}H]-GGCl	GGCl
vinylic @ C-2	5.44;triplet	5.43;triplet
vinylic @ C-6,C-10,C-14	5.10;triplet	5.09;triplet
*CHT @ C-1; CH_2 @ C-1	4.10;doublet	4.10;doublet
CH_2 @ C-4,-5,-8,-9,-12,-13	2.04;multiplet	2.04;multiplet
CH_3 @ C-20	1.68;singlet	1.68;singlet
CH_3 @ C-16,-17,-18,-19	1.60;singlet	1.64,1.60;s

The final reaction in the synthesis of [1-^{3}H]-geranylgeranyl pyrophosphate (**39**) involves the pyrophosphorylation of **36** using tris(tetra-*N*-butylammonium) hydrogen pyrophosphate (**37**) in acetonitrile at room temperature[80] to afford the corresponding salt. Tris(tetra-*N*-butylammonium) hydrogen pyrophosphate (**37**) is prepared from disodium dihydrogen pyrophosphate and a solution of tetra-*N*-butylammonium hydroxide[81]. This solution is loaded onto a cation exchange resin and eluted with deionized water. The resulting solution is titrated with Bu_4NOH to pH 7.3. Lyophilization gives the white solid **37**. The ^{1}H-NMR contains a triplet at 3.02 ppm assigned to the CH_2 closest to the nitrogen atom, a quintet at 1.47 ppm represents the next CH_2 in the alkyl chain, a sextet at 1.20 ppm is assigned to the next CH_2 in the butyl group, and a triplet at 0.77 ppm belongs to the CH_3 at the end of each butyl substituent on the nitrogen atom. There is also the proton from the pyrophosphate counter ion as a singlet resonating at 2.05 ppm. The ^{1}H-NMR spectrum is shown in Figure 22.

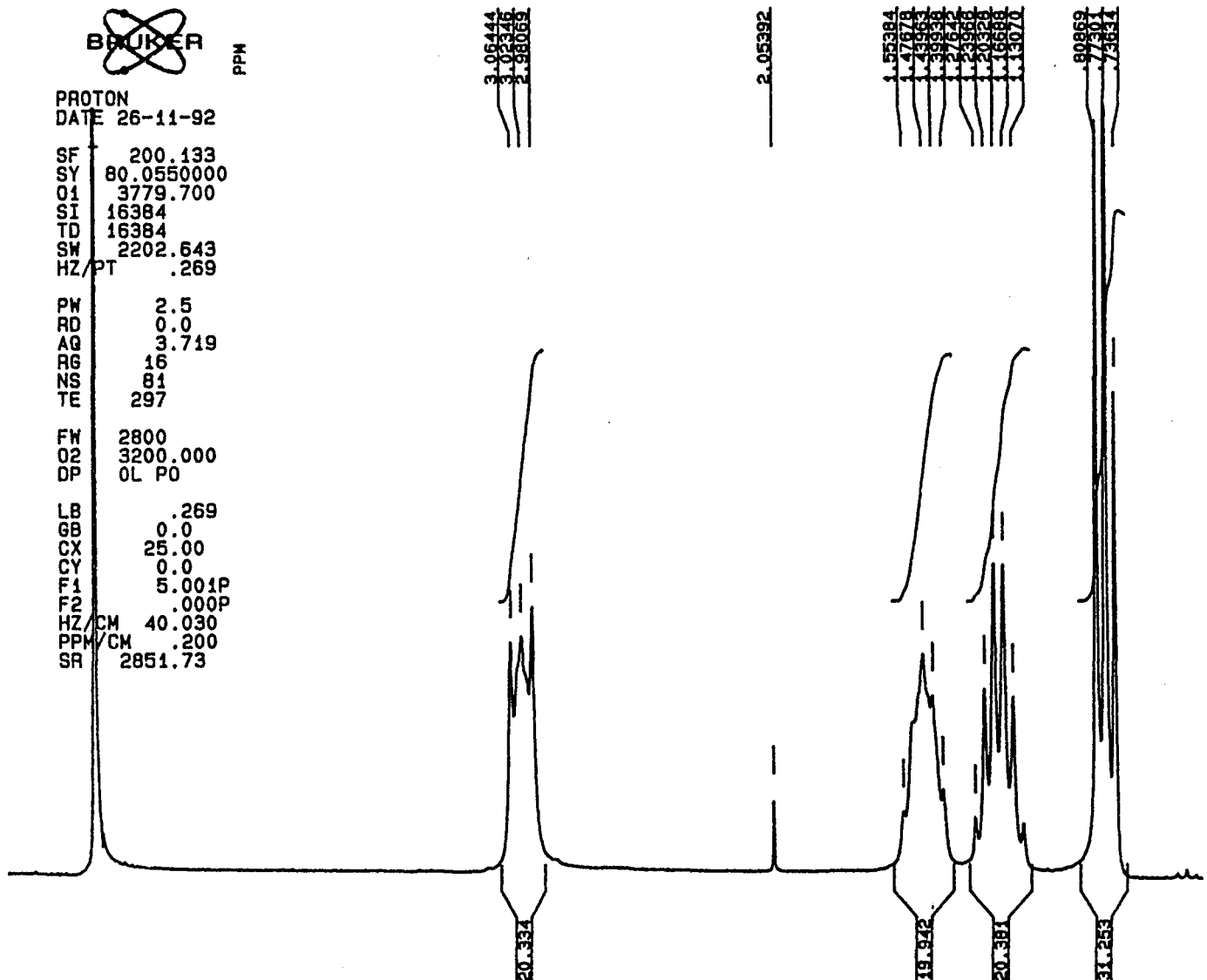
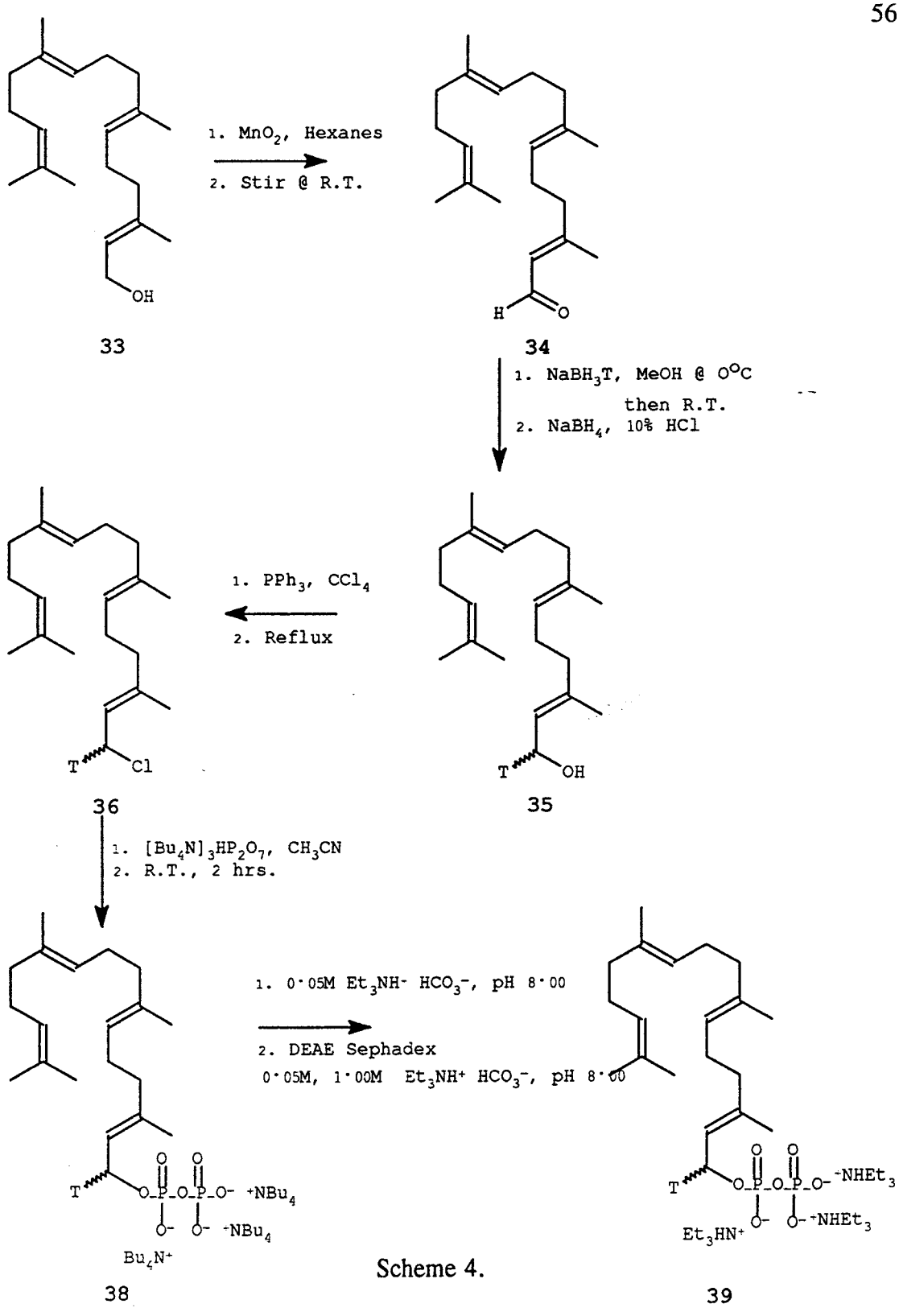


Figure 22.

^{1}H-NMR (200 MHz, D_2O) spectrum of Tris(tetra-*N*-butylammonium) Hydrogen Pyrophosphate

An aqueous solution of the salt, from the reaction with geranylgeranyl chloride, was then loaded onto an ion-exchange resin and eluted with a linear gradient of 0.05 to 1.00 M $HNEt_3^+$ HCO_3^- buffers to give radioactive fractions containing **39**[82]. The fractions were combined and lyophilized to give [1-^{3}H]-geranylgeranyl pyrophosphate (**39**). A sample of **39** was prepared as a solution in D_2O for ^{1}H-NMR and ^{31}P-NMR analysis. The ^{1}H-NMR spectrum contained resonances for the triethylamine and ammonium protons, but the signals corresponding to the geranylgeranyl portion of the molecule could not be found. Although the ^{31}P-NMR spectrum only contained the resonance for the inorganic pyrophosphate phosphorus, at -6.83 ppm, the lack of signals for the α and β phosphorous atoms of the pyrophosphate group did not disprove the formation of **39**. Rather the formation of **39** was rationalized from the radioactive data obtained by scintillation counting of the fractions. The presence of inorganic pyrophosphate supports the formation of **39** since they should elute at similar molarity from the Sephadex column. The elution of the corresponding non-radiolabelled geranylgeranyl pyrophosphate from the DEAE Sephadex column at the same stage as **39**, and the assumption that the concentration of **39** must have been too low for detection by ^{31}P-NMR also tend to support the successful production of **39**. The pyrophosphate (**39**) was used in the incubation experiments as a solution in D_2O, NH_4OH pH>10, 8.28 μmol, 4.39 X 10^6 DPM ^{3}H, 15.6(+/-0.5%) chemical yield.

These reactions are summarized in Scheme 4.



33 34 35 36 38 39

Scheme 4.

3.C. Specific Activity Determination:

The decay process of an unstable nucleus is governed by the nuclear forces present in the element. The disintegration or decay of the nucleus is a random process, the result of which is known as radioactivity.

Over a short period of time, Δt, a vast number of decays will occur. These disintegrations are proportional to Δt and to the total number of radioactive nuclei, N. The rate of decay is a first order process.

That is,

$$-\frac{d[N]}{dt} = k[N] \qquad \text{eq. 3.C.1.}$$

Following integration of this rate equation, a very useful equation may be obtained.

$$\ln[N]_0^t = -kt \qquad \text{eq. 3.C.2.}$$

$$N_t = N_0 e^{-\epsilon t} \qquad \text{eq. 3.C.3.}$$

In this particular case the rate constant, k, is equivalent to the decay constant, ϵ, of the isotope under study. The half life of the reaction, $t_{1/2}$, is a constant and does not depend on the initial concentration of the isotope. Therefore, when half of the initial concentration of N has decayed, and $t= t_{1/2}$, equation 3.C.3. can be manipulated to show that ϵ and $t_{1/2}$ are inversely related[83]. This information can reduce the previous equation into a more simple form into which experimental data may be substituted directly.

That is,

$$N = N_0 e^{(-0.693t/t_{1/2})} \qquad \text{eq. 3.C.4.}$$

Therefore, to determine the activity of the [^{3}H]-toluene standard, in terms of

disintegrations per minute(DPM), at the time of measurement several factors must be known or be determined. The initial activity of the standard is known to be $2.06x10^6$ DPM/mL as calibrated on January 2,1990[84]. The time elapsed since the calibration date and the date on which a portion as counted was calculated to be 2.392 years. The half life of tritium, $t_{1/2}$, is known to be 12.35(±0.01) years[84]. Substitution of these values into equation 3.C.4. gives the activity of the standard.

That is,

$$N = (2.06x10^6 \text{ DPM/mL})e^{[-0.693x2.392]/12.35(\pm 0.01)} \qquad \text{eq. 3.C.4.}$$

$$N = 1.80x10^5 \text{ DPM/mL}(\pm 0.08\%)$$

Since a series of 12.5 μL samples from the (^{3}H)-toluene standard were prepared for and counted by liquid scintillation, the average radioactivity, R, will be:

$$R = [1.80x10^5 \text{ DPM/mL}(\pm 0.08\%)] \text{ x } [12.5x10^{-3} \text{ mL}(\pm 1\%)]$$

$$R = 2.25x10^4 \text{ DPM}(\pm 1.0\%)$$

This value may be used to determine the counting efficiency, CE, of the sample, or to compare the activities of samples which are counted with different efficiencies.

Counting Efficiency determination:

The counting efficiency, CE, is the ratio of the number of observed counts per minute, CPM, to the number of disintegrations per minute, DPM, occurring in the sample

and is usually less than 100%[59]. The counting efficiency must be calculated to determine the specific activity of the sample. One of the basic methods used to determine the CE is internal standardization. A known amount of non-quenching radioactive standard, such as [^{3}H]-toluene, is counted while the same quantity is added to a known amount of sample. The counts of the sample are subtracted from the counts of the sample plus standard and this number is divided by the disintegration rate of the standard. That is,

$$CE = \frac{[(CPM_{St+S}) - (CPM_S)]}{[DPM_{St}]} \times 100 \qquad \text{eq. 3.C.5.}$$

This equation can be manipulated to calculate the counting efficiency if the DPM of the standard is known.

$$CE = \frac{[(CPM_{St}]}{[DPM_{St}]} \times 100$$

$$CE = \frac{[11300\ CPM(\pm 3.1_8\%)]}{[2.25 \times 10^4\ DPM(\pm 1.0\%)]} \times 100$$

$$CE = 50.2(\pm 3.33\%)$$

$$CE = (50.2 \pm 1.87)\ \%$$

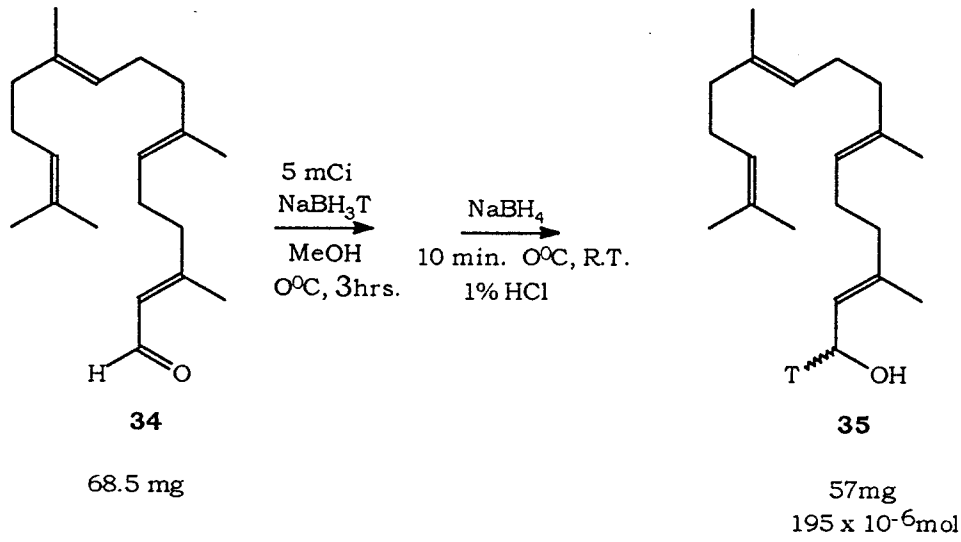
The value of CE can now be used to determine the specific activity of [1-^{3}H]-geranyl geraniol.

Determination of Specific Activity of [1-^{3}H]-Geranyl Geraniol (35):

[1-^{3}H]-Geranyl geraniol (**35**) was prepared from geranyl geranial (**34**) using 5 mCi $NaBH_3T$ in methanol. The tritium labelled reducing agent had a total activity of 1.11 x

10^{10} DPM[85]. During the course of the reaction excess $NaBH_4$ was added to ensure total reduction of the aldehyde (Scheme 5).

The product was found to be homogeneous by TLC and had the same R_f value as geranyl geraniol (0.41 in 50% Et_2O/hexanes). The chemical yield of the reaction was 82%. The specific activity of **35** was determined by counting 17.5 μL(±1%) samples of a 22.6 μM(±2.0%) solution of **35** in hexanes. The DPM values for these aliquots can be found in Table 3. The DPM values were calculated from the measured, corrected CPM values for the samples and the CE determined as described above. Since the radioactivity associated with the 17.5 μL samples was known and the concentration of the sample known, the specific activity was calculated to be 1.95 x 10^8 DPM/mg(± 5.6%).



34

68.5 mg

35

57mg
195 x 10^{-6}mol

Scheme 5.

Table 3.

[1-^{3}H]-GGOH[!],A	CPM	2SIG%	DPM[*]
17.5µL	10960	1.96	19500
17.5µL	10890	1.97	19380
17.5µL	12030	1.98	21400
^{3}H-Toluene[$],B	CPM	2SIG%	DPM
12.5µL	10900	1.96	19400
12.5µL	11780	0.85	20960
12.5µL	11230	1.99	19980
A & B	CPM	2SIG%	DPM
30.0µL	23690	1.94	42150
30.0µL	23210	1.96	41300
30.0µL	24930	1.89	44360

! Corrected CPM values were obtained by subtracting the background CPM from the measured CPM value of the sample. This background CPM correction value was calculated from a series of 5 mL scintilllation cocktail samples, the average CPM value being 32.01 CPM.
* These values were calculated using the CE value determined from the tritiated toluene standard.
$ Each sample was removed from the 10 mL standard, du Pont Lot # S004008, and delivered into a scintillation vial containing 5 ml cocktail.

3.D. Incubation Experiments:

The method of Munck and Croteau[87], for the purification and characterization of a sesquiterpene cyclase, was modified in order to prepare a cell-free enzyme preparation

from a sample of *Taxus x media* densiformis, obtained from the Royal Botanical Gardens, Hamilton. The 200 mM Tris, HCl pH 8.4 buffer used throughout the experiments contained 10 mmol $MgCl_2$, 5 mmol β-mercaptoethanol, 200 mmol Tris, made to 1000 mL with deionized water, and was titrated to pH 8.4 with concentrated HCl. The enzyme preparation was made from freshly ground yew needles: the needles had been frozen in liquid nitrogen and the cell walls broken using a mortar and pestle. Immediately after the needles were ground to a powder, the buffer was added in order to maintain the enzymatic activity. While half of the extract was maintained at 0°C, the other portion was boiled for several minutes to denature the enzymes present in the cell-free extract: this sample serves as a control for the experiment. The conditions of the incubation experiments were varied according to volume of [1-^{3}H]-geranylgeranyl pyrophosphate used in the incubation (TGGPP), the incubation time (IT), the quantity of polyvinylpolypyrrolidone used in the cell-free extract preparation (PVPP), and the volume of cell-free extract (2 mL -TGGPP) used. After the samples had incubated for the specified time at ambient temperature, they were extracted with hexanes and loaded onto a pasteur pipette flash silica column and eluted with equal portions of hexanes and then with ether. Each of the samples were collected into separate scintillation vials and the radioactivity content measured. The abbreviations "exp" and "contr" refer to the nondenatured and denatured cell-free enzyme extract preparation used in the particular experiment and the values found in these columns are in units of DPM.

From the results of the experiment (Table 4) the conditions under which the

hexanes and ether extracts yielded the highest DPM values were from the incubation sample treated with 10 g PVPP. A second series of incubation samples were prepared in a similar manner, with an additional elution of the extract, after hexanes, using a 3% ether/hexanes solution[88].

Table 4.

Expt #	IT (min)	PVPP (g)	TGGPP exp.	TGGPP contr.	Hex. exp.	Hex. contr	Eth exp.	Eth contr
1	30	5	400µL	400µL	47	33	3380	700
2	30	5	600µL	600µL	1000	87	7060	1390
3	60	5	400µL	400µL	29	22	3800	340
4	60	5	600µL	600µL	31	46	4620	580
5	30	10	400µL	400µL	4050	73	2460	610
6	30	10	600µL	600µL	120	83	4620	980
7	60	10	400µL	400µL	8360	N/A	3100	N/A
8	60	10	600µL	600µL	1420	110	5480	680

The results show an increased DPM value for the ether extract from each of the

non-denatured incubation samples relative to those from the denatured sample. The highest DPM measured are for those ether extracts in which 10 g PVPP was used in the cell-free enzyme preparation and those samples incubated at ambient temperature for 60 minutes. These results agree with those obtained in the previous experiment. However, the DPM measured in the 3% ether/hexanes extract did not have as high a radioactivity count as was hoped.

The final experiment concerning the incubation of [1-^{3}H]-geranylgeranyl pyrophosphate (**39**) with the cell-free extract of *Taxus x media* densiformis involved the isolation of the ether extract from the denatured and non-denatured incubated samples (results can be found in Table 5), concentrating the extract, and combining the extract with a fresh preparation of carrier yew material.

The carrier yew material was prepared through a modification of the procedure of Muschik *et al*[26], for the analysis and isolation of taxol content in needles of various *Taxus* species, using dichloromethane as the extraction solvent. The CH_2Cl_2 soluble residue obtained from extraction of the needles (frozen in N_2(l) and ground to a powder with a mortar and pestle) was vortexed with a portion of ether to isolate the polar compounds. The ether extract was transferred to a 5 mL pear shaped flask and concentrated to give a thick green solution, the carrier material.

The ether extract from the radioactive incubation experiment was added to the concentrated yew extract and the sample injected into the Hewlett Packard GC which was connected to a GCR Radioactivity detector. The conditions established for the run were

as follows:

Oven initial temp(°C)	: 100 , hold time 2.00 min.
Oven final temp(°C)	: 300 , hold time 5.00 min.
Injector temp(°C)	: 325
Detector temp(°C)	: 325
Ramping rate	: 20°C/min.
Total run time	: 17 min.

Gas flow rates were set within the guidelines according to the Hewlett Packard Operating Instructions Reference manuals.

Table 5.

Expt #	IT (min)	TGGPP (μL)	Hex. expt	Hex. contr	3%E/H expt	3%E/H contr	Eth. expt	Eth. contr
A	60	150	280	140	180	60	2900	130
B	60	150	130	N/A	330	14	2430	110
C	60	150	77	N/A	300	18	2350	100

The cell-free extract was prepared in the same manner as in previous experiments. Six samples were prepared, three containing 850 μL denatured CFE and the others containing 850 μL non-denatured CFE. To these samples, 150 μL of a 7.39 μM solution

of [1-^{3}H]-geranylgeranyl pyrophosphate (**39**) was added. The mixtures were incubated for 60 minutes at 23.2°C in a water bath. The samples were extracted as in earlier experiments.

No peaks were detected in the chromatograms for the isotope channel (Radioactivity detector for ^{3}H) nor the mass channel (TCD-GC). This result does seem strange since earlier experiments involving yew extraction material, the ether extract from the CH_2Cl_2 soluble material obtained from the extraction of crushed *Taxus x media* densiformis, and the TCD (the GC runs were performed under the same conditions as those for this experiment) showed numerous peaks for a small sample volume. There should have been peaks observable in the mass channel representing the polar compounds present in the carrier material regardless of the presence of the radioactive material.

4. Conclusion

Geranyl geraniol (**33**) was synthesized in six steps from geraniol using a modified version of the convergent method of Altman[70]. The radioactively labelled pyrophosphate (**39**) was prepared in five steps, 4.39 x 10^6 DPM ^{3}H, 8.28 μmol, 15.6(±0.5%)% yield, from [1-^{3}H]-geranyl geraniol (**35**), 1.95 x 10^8 DPM(±5.6%) ^{3}H.

Incubation experiments involving **39** and a cell-free enzyme extract of *Taxus x media* densiformis were performed. The results of these experiments showed that the ether extract from the incubated samples contained the highest level of radioactivity. In one case 3.34% of the radioactivity introduced into the incubation was maintained in the ether extract after the incubation was complete. A general method for the preparation of a cell-free extract has been completed and a method for the preparation of the carrier yew material has also been accomplished.

5. Future Work

Future work on this project could involve the introduction of ^{3}H and ^{14}C labels into other sites in the geranyl geraniol molecule, since the synthetic approach to **33** involved the condensation of two C_{10} molecules. The total protein content from the cell-free extract should also be determined so as to establish whether or not the cell walls are being broken under the conditions used in the cell-free enzyme preparation. Studies into the preparation of radiolabelled pyrophosphates mention the importance of maintaining the pH of the pyrophosphate between 9.5 and 6.5. This is necessary since it has been observed that above pH 9.5 the pyrophosphate may degrade to the monophosphate; below pH 6.5 the pyrophosphate may hydrolyze to give the corresponding alcohol and inorganic pyrophosphate. Also, further investigation into the incubation experiments, manipulating the many variables such as: the buffer system, the cell-free extract preparation, and the optimum GC conditions under which to analyze the ether extract may lead to the detection of a radioactive intermediate. Once this intermediate is isolated and identified, valuable information concerning the biosynthetic conversion of **1b** to taxol (**2**) could be obtained.

6. Experimental

General:

All reactions were performed in acetone rinsed, oven- or flame-dried glassware under a positive pressure of nitrogen. All solvents were distilled unless otherwise stated. Reagents were reagent grade or better. All organic layers from extractions were dried over anhydrous magnesium or sodium sulphate. The term *in vacuo* refers to the removal of solvent by rotary evaporation followed by evacuation to constant sample weight. Commercially available radio-labelled reagents were purchased from E.I. duPont de Nemours & Co.. Deionized water was obtained from a Millipore purification system. All organic reactions were followed by thin layer chromatography (TLC) utilizing either UV fluorescence, iodine staining, or *p*-anisaldehyde staining for visualization. Commercial glass-backed TLC plates were Merck Kieselgel 60F-254. Silica gel for column and flash chromatography was EM Science 60, 230-400 mesh.

All masses were measured on a mechanical Gram-Atic balance from Fisher Scientific Co. which determined weights to the fourth decimal place. Plastic tipped automatic pipettes from Nichiryo, Model 5000, were used to deliver microliter volumes. The accuracy and precision are 1-2% and 0.5% respectively. Glass syringes were also used to deliver volumes ranging from 5 μL-20 mL to reaction systems, with

accuracy and precision values of approximately 1%[89].

Liquid scintillation counting was performed on a Beckman LS 5801 scintillation counter using Fisherbrand 20 mL plastic screw-cap vials and Beckman Ready Safe liquid scintillation cocktail for aqueous and nonaqueous samples.

Melting points (mp) were determined on a Gallenkamp melting point apparatus using open capillary tubes and are uncorrected. All buffers were made using an Orion Research, model SA250, portable pH meter. Proton nuclear magnetic resonance spectra were recorded on an AC200 Bruker NMR instrument in the specified deuterated solvent, br=broad, d=doublet, m=multiplet, s=singlet, and t=triplet. Tetramethylsilane (TMS) was used as the internal standard for ^{1}H spectra determined in $CDCl_3$, and solvent as internal standard for ^{13}C spectra. Carbon spectra were recorded with a spectrometer frequency of 50 MHz. Phosphorus-31 spectra were recorded at 32 MHz using 85% H_3PO_4 as external standard at a chemical shift of 0 ppm. Infrared (IR) samples were prepared as 5%(v/v) solutions in CCl_4 and spectra were determined either on a Bio-Rad FTS-40 FT-IR spectrometer or a Perkin Elmer IR spectrometer. Chemical Ionization (CI) and Electron Impact (EI) mass spectra were recorded on a VG Analytical ZAB-E mass spectrometer.

Gas chromatography (GC) was performed on a Hewlett Packard (HP) 5890A gas chromatograph fitted with a 61+ 3% OV-101 whp 100/120 19001a D32 packed column and a HP-1 methyl silicone gum 5m x 0.53mm x 2.65μm capillary column with helium as the carrier gas. Compounds were detected using a flame ionization detector

(FID) for the capillary column and a thermal conductivity detector (TCD) for the packed column. Flow Radiochromatography for GC was performed on a Radiomatic Model GCR connected to the HP GC. The GCR was established for ^{3}H analysis with a computer controlled reaction chamber temperature of 700°C and a plateau analysis to determine flow through the gas proportional counter using ^{133}Ba, 7.8 μCi.

Tetra-*N*-butylammonium Iodide (25)[60]:

To tetra-*N*-butylammonium hydroxide (4 mL, 15.3 mmol, 40 wt.% solution in water) was added hydriodic acid (2.34 mL, 15.2 mmol, 47-51 wt.% solution in water). A flocculant white precipitate was produced, collected by filtration, washed with distilled water, and the crystals dried over P_2O_5 under vacuum. The melting point of the product was 145-148°C, which is identical to the literature mp[93], yield was 5.54 g, 99% yield.

^{1}H NMR (200 MHz, $CDCl_3$) δ 3.38 (t, 2H, $C\underline{H}_2$ at C-1), 1.70 (quintet, 2H, $C\underline{H}_2$ at C-2), 1.48 (sextet, 2H, $C\underline{H}_2$ at C-3), 1.02 (t, 3H, $C\underline{H}_3$ at C-4).

^{13}C NMR (50 MHz, $CDCl_3$) δ C-1 59.1, C-2 24.2, C-3 19.7, C-4 13.6.

MS (NH_3-DCI); m/z 242 ($[M-I]^+$, 6%), m/z 186 ($[M-C_4H_9]^+$, 100%).

3,7-Dimethyl-2,6-octadienyl Benzyl Ether (26)[90]:

A solution of geraniol (1.0133 g, 6.57 mmol, 98%) in 7 mL tetrahydrofuran (THF) was added dropwise at 0°C to sodium hydride (0.2179 g, 7.26 mmol, 80% dispersion in oil washed with 3x4 mL distilled hexanes prior to use) under a positive

pressure of nitrogen. The reaction mixture was stirred at 0°C for 10 min. and then at ambient temperature for 2.5 hours, after which Bu_4NI **25** (0.2629 g, 0.712 mmol) and benzyl chloride (280 μL, 2.43 mmol) were added. The reaction mixture was brought to reflux and left overnight under a positive pressure of nitrogen. After cooling the mixture to room temperature the reaction was quenched by slow addition of distilled water. The reaction product was extracted with diethyl ether (3x10 mL). The combined ethereal extracts were washed with saturated NaCl solution, dried over anhydrous $MgSO_4$, filtered through celite, and concentrated *in vacuo* to give a yellow oil. The reaction product (Rf=0.66, 20% v/v ethyl acetate/hexanes) was purified by flash column chromatography with mixed eluting solvents, 100% hexanes then 20% ethyl acetate/hexanes, to yield 0.5939 g, 2.43 mmol (100%) of pure product.

^{1}H NMR (200 MHz, $CDCl_3$) δ 7.35 (br.s., 5H, phenyl), 5.40 (t, 1H, $CH_2\underline{CH}{=}CCH_3$ at C-2, $J_{2,1}$ = 6.8Hz), 5.11 (t, 1H, $CH_2\underline{CH}{=}CCH_3$ at C-6, $J_{6,5}$ = 4.1 Hz), 4.50 (s, 2H, $C\underline{H_2}Ph$), 4.03 (d, 2H, $C\underline{H_2}$ at C-1, $J_{1,2}$ = 6.8 Hz), 2.07 (m, 4H, $C\underline{H_2}$ at C-4 and C-5), 1.68 (s, 3H, $C\underline{H_3}$), 1.64 (s, 3H, $C\underline{H_3}$), 1.60 (s, 3H, $C\underline{H_3}$).

^{13}C NMR (50 MHz, $CDCl_3$) δ C-12 139.90, C-13,C-17 127.32, C-14,C-16 127.82, C-14 126.99, C-1 66.06, C-11 71.42, C-2 123.48, C-3 138.05, C-4 39.09, C-5 25.85, C-6 120.28, C-7 131.13, C-8 25.18, C-9 17.17, C-10 15.98.

IR (CCl_4); 3017 cm^{-1} (sp^2 C-H), 2967, 2917, 2833 cm^{-1} (sp^3 C-H), 1650 cm^{-1} (sp^2 C=C), 1450 cm^{-1} (sp^2 C-C aryl), 1383 cm^{-1} (gem-dimethyl), 1250 cm^{-1} (C-O-Ar, C-O-C), 1100 cm^{-1} (C-O).

MS (NH_3-CI); m/z 262 ($[M+NH_3]^+$, 48%), m/z 137 ($[M-BnO]^+$,100%)

8-O-Benzyl-2,6-dimethyl-2,6-octadienal (27), and 8-Hydroxy-3,7-dimethyl-2,6-octadienyl Benzyl Ether (28)[67]:

Selenium dioxide (1.15 g, 10.4 mmol, 99.8%) was added to **26** (4.96 g, 20.3 mmol) in 95% ethanol (40mL) and the reaction mixture heated to reflux for 2 hours. An initial fine red precipitate became black and coarse during the course of the reaction. The mixture was cooled to room temperature, filtered through celite, and the ethanol removed *in vacuo* to leave an orange-yellow oil which was dissolved in 90 mL ether. The solution was filtered, washed with water then saturated sodium chloride solution, dried over $MgSO_4$, filtered, and the solvent removed *in vacuo* to afford a yellow oil. The reaction products (R_f= 0.09 20% EtOAc/hexanes for the alcohol (**28**) and R_f= 0.23 20% EtOAc/hexanes for the aldehyde (**27**)) were separated by column chromatography using mixed eluting solvents, ethyl acetate/ hexanes (2%, 5%, 10%, and then 20%). The yields were 2.52 g, 9.74 mmol, 48% for **27** and 0.74 g, 2.84 mmol, 14% for **28**.

^{1}H NMR (28) (200 MHz, $CDCl_3$) δ 7.32 (br.s., 5H, phenyl), 5.40 (t, 2H, $CH_2\underline{CH}=CCH_3$ at C-2 and C6, $J_{2,1}$= 6.7 Hz and $J_{6,5}$= 6.7 Hz), 4.50 (s, 2H, $C\underline{H_2}Ph$), 4.02 (d, 2H, $C\underline{H_2}$ at C-1, $J_{1,2}$= 6.7 Hz), 3.96 (s, 2H, $C\underline{H_2}$ at C-8), 2.10 (m, 4H, $C\underline{H_2}$ at C-4 and C5), 1.65 (s, 6H, $C\underline{H_3}$ at C-3 and C-7).

^{13}C NMR (28) (50 MHz, $CDCl_3$) δ C-1 68.32, C-2 124.03, C-3 137.02, C-4 38.58, C-5 25.23, C-6 120.53, C-7 134.49, C-8 71.53, C-9 15.91, C-10 13.10, C-11 66.05, C-12

139.48, C-13,17 127.28, C-14,16 127.81, C-15 127.05.

IR (28) (CCl_4); 3440 cm^{-1} (O-H), 3017 cm^{-1} (sp^2 C-H), 3000, 2880, 2820 cm^{-1} (sp^3 C-H), 1465 cm^{-1} (C-C aryl), 1180 cm^{-1} (C-O-Ar), 1100 cm^{-1} (C-0),

MS (28) (NH_3-CI); m/z 278 ($[M+NH_3]^+$, 11%); m/z 243 ($[M-OH_2]^+$, 21%); m/z 135 ($[M-C_7H_7O]^+$, base peak); m/z 108 ($[M-C_{10}H_{17}O]^+$, 20%)

^{1}H NMR (27) (200 MHz, $CDCl_3$) δ 9.39 (s, 1H, C<u>H</u>O), 7.32 (br.s., 5H, phenyl), 6.47 (t, 1H, $CH_2C\underline{H}=CCH_3$ at C-6, $J_{6,5}$= 7.2 Hz), 5.45 (t, 1H, $CH_2C\underline{H}=CCH_3$ at C-2, $J_{2,1}$= 6.6 Hz), 4.51 (s, 2H, C<u>H</u>$_2$Ph), 4.03 (d, 2H, C<u>H</u>$_2$ at C-1, $J_{1,2}$= 6.6 Hz), 2.50 (q, 2H, C<u>H</u>$_2$ at C-5, $J_{5,6}$= 7.2 Hz, $J_{5,4}$= 7.7 Hz), 2.22 (t, 2H, C<u>H</u>$_2$ at C-4, $J_{4,5}$= 7.7 Hz), 1.75 (s, 3H, C<u>H</u>$_3$), 1.65 (s, 3H, C<u>H</u>$_3$).

^{13}C NMR (27) (50 MHz, $CDCl_3$) δ C-8 194.54, C-7/C-3 137.97, C-6 153.12, C-5 37.31, C-4 26.57, C-2 121.64, C-1 65.97, C-9 31.03, C-10 15.92, C-11 71.74, C-12 139.06, C-13,-17 127.23, C-14,16 127.85, C-15 127.06.

IR (27) (CCl_4); 3010 cm^{-1} (sp^2 C-H), 2880, 2820 cm^{-1} (sp^3 C-H), 2670 cm^{-1} (aldehydic C-H), 1670 cm^{-1} (C=O conjugated), 1490, 1440 cm^{-1} (sp^2 C-C), 1350 cm^{-1} ((C=O)-H aldehydic bend), 1220 cm^{-1} (C-O-Ar).

MS (27) (EI); m/z 259 ($[M+H]^+$, 3%), m/z 150 ($[M-C_7H_8O]^+$, 70%).

Reduction of (27) to (28):

A mixture of **27** (0.5485 g, 2.12 mmol), sodium borohydride (0.1043 g, 2.76 mmol), and methanol (4.23 mL) was allowed to stir at ambient temperature for 2.5 hours. The reaction was quenched by addition of a few drops of 10% HCl, the solution was extracted with three portions of hexanes, passed through a silica column using 15% EtOAc/hexanes as eluent, and the solvent removed to yield a clear oil (R_f= 0.09 in 20% EtOAc/hexanes). Spectral characteristics identical to **28**.

8-Chloro-3,7-dimethyl-2,6-octadienyl Benzyl Ether (29)[91]:

A mixture of **28** (200 mg, 768 μmol), PPh_3 (413 mg, 1575 μmol), and carbon tetrachloride (2.28 mL, 23.6 mmol) was brought to reflux under N_2 for 9 hours. The mixture was cooled to room temperature, diluted with pentane (10 mL), and the precipitated triphenylphosphine oxide was removed by filtration through celite. The filtrate was concentrated under reduced pressure at 0°C, diluted with pentane (10 mL), and filtered again. The solvent was removed *in vacuo* at 0°C to give a yellow oil which still contained traces of triphenylphosphine oxide (white needles). This dilution, filtering, and removal of solvent was repeated until no PPh_3O was present (R_f= 0.72 in 20% EtOAc/ hexanes). Yield 167 mg, 599 μmol, 78%.

^{1}H NMR (200 MHz, $CDCl_3$) δ 7.23 (br.s., 5H, phenyl), 5.42 (t, 1H, $CH_2C\underline{H}=CCH_3$ at C-2, J= 6.9 Hz), 5.33 (t, 1H, $CH_2C\underline{H}=CCH_3$ at C-6, J= 6.1 Hz), 4.42 (s, 2H, $C\underline{H}_2C_6H_5$), 3.94 (d, 2H, $C\underline{H}_2$ at C-1, J= 6.9 Hz), 3.91 (s, 1H, $C\underline{H}_2Cl$), 2.03 (m, 4H, $C\underline{H}_2$ at C-4 and

C-5), 1.68 (s, 3H, $C\underline{H}_3$), 1.57 (s, 3H, $C\underline{H}_3$).

^{13}C NMR (50 MHz, $CDCl_3$) δ C-1 66.30, C-2/C-6 121.14, C-3/C-7 131.94 or 137.05, C-4 38.49, C-5 26.03, C-8 52.12, C-9 16.24, C-10 13.90, C-11 71.82, C-12 138.26, C-13,17 128.29, C-14,16 128.44, C-15 127.53.

3,7-Dimethyl-2,6-octadienyl-1-chloride (30):

The procedure for the preparation of **30** was repeated for the synthesis of **31**. A solution of geraniol (1.0026 g, 6.50 mmol), triphenylphosphine (3.2444 g, 12.4 mmol), and CCl_4 (19.3 mL, 200 mmol) was brought to reflux for 6 hours. The product was obtained as a yellow/green oil **31** (R_f= 0.78 in 20% EtOAc/hexanes), 0.8913 g, 5.16 mmol, 79% yield.

^{1}H NMR (200 MHz, $CDCl_3$) δ 5.45 (t, 1H, $CH_2C\underline{H}=CCH_3$ at C-2, J= 8.0 Hz), 5.09 (t, 1H, $CH_2C\underline{H}=CCH_3$ at C-6, J= 2.7 Hz), 4.10 (d, 2H, $C\underline{H}_2$ at C-1, J= 8.0 Hz), 2.12 (m, 4H, $C\underline{H}_2$ at C-4, C-5), 1.73 (s, 3H, $C\underline{H}_3$), 1.69 (s, 3H, $C\underline{H}_3$), 1.61 (s, 3H, $C\underline{H}_3$).

^{13}C NMR (50 MHz, $CDCl_3$) δ C-1 41.09, C-2/C-6 120.32 and 123.59, C-3/C-7 131.94, C-4 39.43, C-5 26.40, C-8 25.62, C-9 17.66, C-10 16.09.

IR (CCl_4); 2940,2880,2820 cm^{-1} (sp_3 C-H), 1640cm^{-1} (C=C), 1450 cm^{-1} (sp^2 C-C), 1350 cm^{-1} (gem. dimethyl), 700cm^{-1} (C-Cl).

MS (EI); m/z 157 ($[M-CH_3]^+$, 32%), m/z 137 ($[M-Cl]^+$, 20%), m/z 69 ($[M-C_5H_8Cl]^+$, base peak).

3,7-Dimethyl-2,6-octadienyl Thiophenyl Ether (31)[70]:

Method A:

A solution of geraniol (0.1998 g, 1.30 mmol), in 3:1 anhydrous ether/ hexamethylphosphorous(V) triamide (8.25 mL) was titrated with methyl lithium (1.4 M solution in Et_2O) to a triphenylmethane (yellow) endpoint at 0°C under a positive pressure of nitrogen. With stirring, p-toluenesulphonyl chloride (0.2925 g, 1.53 mmol) in HMPA (0.99 mL) was added. After the mixture was stirred for 2.5 hours at 0°C, lithium thiophenoxide (1.53 mmol) in HMPA (5.35 mL) was added dropwise and the solution was allowed to stir at ambient temperature for 2 hours. Distilled water was added followed by extraction with three portions of hexane. The combined hexane layers were washed with saturated $NaHCO_3$ solution, saturated NaCl solution, dried over $MgSO_4$, and the solvent removed *in vacuo* to give a yellow oil. Chromatographic purification using silica gel (100 g/g) and elution with CH_2Cl_2 yielded a yellow oil **31** (R_f= 0.27 in 100% hexanes); 0.2550 g, 80% yield.

Method B:

Geranyl chloride **30** (0.1041 g, 603 μmol), triethylamine (92.7 μL, 666 μmol), and thiophenol (79.8 μL, 777 μmol) were stirred together under a nitrogen atmosphere at room temperature for 3 hours[77]. The reaction mixture was diluted with 10 mL of ethyl acetate, washed with 5 mL 1N HCl, and washed with two portions of distilled water. The organic phase was dried over $MgSO_4$ and the solvent removed *in vacuo* to give a crude yellow oil. Chromatographic purification on silica gel using 1:1 CH_2Cl_2/hexanes as solvent

afforded product **31** pure by TLC (R_f= 0.27 in hexanes). Yield 0.113 g, 76%.

^{1}H NMR (200 MHz, $CDCl_3$) δ 7.23 (br.s., 5H, phenyl), 5.31 (t, 1H, $CH_2C\underline{H}=CCH_3$ at C-2, J= 7.7 Hz), 5.06 (t, 1H, $CH_2C\underline{H}=CCH_3$ at C-6), 3.55 (d, 2H, $C\underline{H}_2$ at C-1, J= 7.7 Hz), 2.02 (m, 4H, $C\underline{H}_2$ at C-4, C-5), 1.67, 1.58 (s, 9H, $C\underline{H}_3$ at C-8, C-9, C-10).

^{13}C NMR (50 MHz, $CDCl_3$) δ C-1 32.14, C-2/C-6 123.88, C-3/C-7 131.60, 136.68, C-4 39.56, C-5 26.40, C-8 25.62, C-9 15.98, C-10 17.66, C-11 139.83, C-12/16 126.24, C-13/15 128.43, C-14 125.90.

MS (EI); m/z 246 ($[M]^+$, 10%), m/z 137 ($[M-C_{10}H_{17}]^+$, 17%), m/z 81 ($C_6H_9^+$, 37%), m/z 69 ($C_5H_9^+$, base peak).

3,7,11,15-Tetramethyl-9-phenylthio-2,6,10,14-hexadecatetraenyl Benzyl Ether (32)[74]:

A solution of geranyl thiophenyl ether **31** (100 mg, 406 μmol) in THF (3 mL) at -78°C was treated with butyllithium (367 μmol) resulting in a yellow solution. After the mixture was stirred for 2.5 hours under a positive pressure of nitrogen, the allylic chloride (**29**) (901 mg, 323 μmol) in THF (440 μL) was added dropwise with stirring and the solution allowed to stir for an additional 1.5 hours. The reaction was quenched at -78°C with a 1:1 methanol/ether mixture (265 μL) and the yellow color dissipated. The solution was allowed to come to room temperature and then equal portions of distilled water and ether were added. Separation of the phases followed by additional extraction of the aqueous layer afforded a combined organic mixture which was washed with distilled water, saturated NaCl solution, dried over $MgSO_4$, filtered through celite, and the

solvent removed *in vacuo* to yield a colorless oil. Chromatographic purification on silica gel (100g/g) using CH_2Cl_2 as eluent gave the product homogeneous by TLC (R_f= 0.85 in CH_2Cl_2), yield 117 mg, 240 μmol, 76%.

^{1}H NMR (200 MHz, $CDCl_3$) δ 7.20 (br.m., 10H, phenyl), 5.50-4.80 (br.m., 4H, $CH_2C\underline{H}=CCH_3$), 4.40 (s, 2H, $C\underline{H}_2Ph$, J= 7.7 Hz), 3.94 (m, 3H, C<u>H</u> at C-9 and $C\underline{H}_2$ at C-1, overlapping singlet and doublet, J= 7.7 Hz), 2.47-1.80 (br.m., 10H, $C\underline{H}_2$), 1.55 (br.s., 12H, $C\underline{H}_3$), 1.33 (s, 3H, $C\underline{H}_3$).

3,7,11,15-Tetramethyl-2,6,10,14-hexadecatetraen-1-ol (Geranyl geraniol) (33):

Reduction of both the thiophenyl and benzyl groups was performed based on the method of van Tamelen[75]. To dry ethylamine (4.00 mL) at -78°C was added lithium wire (23 mg) as small pieces[92]. The temperature of the blue solution was brought to 0°C for 1.25 hours to ensure dissolution and then decreased to -78°C and **32** (117 mg, 240 μmol) in THF (2.40 mL) was added dropwise and the resulting solution allowed to stir an additional 15 minutes. Maintaining the temperature at -78°C, 3-hexyne was added until the blue color was totally dissipated and the yellow solution was then quenched with methanol until colorless. After attaining ambient temperature, distilled water was added to the mixture until all of the solids dissolved and the volatiles were carefully evaporated under reduced pressure. The resulting cloudy solution was extracted with four portions of ether and the combined organic layers washed with water, saturated NaCl, dried over $MgSO_4$, filtered, and the solvent removed *in vacuo* to give a clear oil. Chromatographic

purification on silica gel (100 g/g) with 40% ether/hexanes as eluent yielded a clear oil pure by TLC (R_f= 0.41 in 50% Et_2O/hexanes). Yield 47 mg, 68%.

^{1}H NMR (200 MHz, $CDCl_3$) δ 5.42 (t, 1H, $CH_2C\underline{H}=CCH_3$ at C-2, $J_{2,1}$= 6.9 Hz), 5.10 (t, 3H, $CH_2C\underline{H}=CCH_3$ at C-6, C-10, C-14), 4.15 (d, 2H, $C\underline{H}_2$ at C-1, $J_{1,2}$= 6.9 Hz), 2.09 (br.m., 12H, C_2 at C-4, C-5, C-8, C-9, C-12, C-13), 1.68 (s, 1H, CH_3), 1.60 (s, 9H, CH_3).

IR (CCl_4); 3500 cm^{-1} (O-H), 3033 cm^{-1} (sp^2 C-H), 2983, 2933, 2866 cm^{-1} (sp^3 C-H), 1667 cm^{-1} (sp^2 C=C), 1225 cm^{-1} (gem. dimethyl), 1100 cm^{-1} (C-O).

MS (NH_3-DCI); m/z 308 ($[M + NH_3]^+$, 10%), m/z 290 ($[M]^+$, 70%, spectrum #9a), m/z 273 ($[M - H_2O]^+$, base peak), m/z 205 ($[M - C_5H_9O]^+$, 50%), m/z 137 ($[M - C_{10}H_{17}O]^+$, 60%), m/z 81 ($C_6H_9^+$, 64%), m/z 69 ($C_5H_9^+$, 6%).

3,7,11,15-Tetramethyl-2,6,10,14-hexadecatetraenal (34)[78]:

A mixture of geranyl geraniol (91.2 mg, 314 μmol), manganese(IV) oxide (1060 mg, 12.19 mmol), and hexanes (5 mL) was stirred under a positive pressure of nitrogen at room temperature for 2 hours. The mixture was filtered through celite and the solvent removed to afford 76.8 mg (266 μmol, 85%) of product as a pale yellow oil (R_f= 0.39 in 15% EtOAc/hexanes). The aldehyde was used in the next step without further purification.

^{1}H NMR (200 MHz, $CDCl_3$) δ 1.68 (s, 3H, C_3 at C-20), 1.60 (s, 12H, C_3 at C-16, C-17, C-18, C-19), 2.00 (m, 12H, C_2 at C-4, C-5, C-8, C-9, C-12, C-13), 5.89 (d, 1H, $C_2C\underline{H}=CCH_3$ at C-2, J= 8.2 Hz), 5.09 (t, 3H, $C_2C\underline{H}=CCH_3$ at C-6, C-10, C-14), 10.0 (d,

1H, C-1, J = 8.2Hz), 9.91 (d, 1H, C-1, J = 8.2Hz).

^{13}C NMR (50 MHz, $CDCl_3$) δ C-1 191.24, C-2/C-6/C-10/C-14 127.38, 124.35, 123.97, 122.44, C-3 unassigned, C-4 40.61, C-5/C-9/C-13 25.66, 26.75, C-7/C-11/C-15 136.10, 135.10, C-8/C-12 39.69, C-9 26.75, C-16 25.06, C-17 17.64, C-18 and C-19 15.98, C-20 18.72.

IR (CCl_4); 2880, 2800 cm^{-1} (sp^3 C-H), 1600 cm^{-1} (C-C), 1570 cm^{-1} ((C=O)-H, conjugated), 1390 cm^{-1} ((C=O)-H aldehydic), 1240 cm^{-1} (gem. dimethyl).

MS (NH_3-CI); m/z 306 ($[M+NH_3]^+$, 14%), m/z 289 ($[M+H]^+$, 76%), m/z 271 ($[M-H_2O]^+$, base peak), m/z 137 ($[M-C_{10}H_{16}O]^+$, 48%).

[1-^{3}H]-3,7,11,15-Tetramethyl-2,6,10,14-hexadecatetraen-1-ol (35)[78]:

A mixture of geranyl geranial **34** (68.5 mg, 238 μmol) and methanol (0.57 mL) was injected into a vial of $NaBH_3T$ (5 mCi, 490 mCi/mmol, 102 μmol) at 0°C. After stirring for 3 hours at 0°C excess sodium borohydride (6.9 mg, 182 μmol) was added and the reaction mixture allowed to stir for another 5 minutes followed by addition of 2 drops of 10% HCl. The solution was extracted with three portions of hexanes. The combined extracts were passed through a flash silica column, to remove any residual water, utilizing 10% EtOAc/hexanes as the eluent. The solvent was removed *in vacuo* to give a clear oil 57.0 mg (1.11×10^{10} DPM ^{3}H, 195 μmol, 82%) homogeneous by TLC (R_f= 0.41 in 50% EtOAc/hexanes which is identical to authentic unlabelled material). The radioactive sample was stored in benzene in the freezer until further use.

^{1}H NMR (200 MHz, $CDCl_3$) δ 5.42 (t, 1H, $C_2C\underline{H}=CCH_3$ at C-2), 5.10 (t, 3H, $C_2C\underline{H}=CCH_3$ at C-6, C-10, C-14), 4.14 (d, 1H, C at C-1, J= 6.97 Hz), 2.10 (m, 12H, C_2 at C-4, C-5, C-8, C-9, C-12, C-13), 1.68, 1.60 (s, 15H, C_3 at C-16, C-17, C-18, C-19, C-20).

[1-^{3}H]-1-chloro-3,7,11,15-Tetramethyl-2,6,10,14-hexadecatetraen (36):

A mixture of **35** (15.5 mg, 53 μmol), triphenylphosphine (303 mg, 116 μmol), and carbon tetrachloride (178 μL, 1845 μmol) was brought to reflux in a 1 mL reacti-vial at 86°C under a nitrogen atmosphere for 6 hours. The mixture was cooled and worked up as in the preparation of **29**. The solvent was removed *in vacuo* at 0°C to afford 23.2 mg, 74.6 μmol, as a yellow oil which still contained traces of triphenylphosphine oxide. **^{1}H NMR (200 MHz, $CDCl_3$)** δ 5.44 (t, 1H, vinyl at C-2), 5.10 (t, 3H, vinyls at C-6, C-10, C-14), 4.10 (d, 1H, methine at C-1, J = 8.0 Hz), 2.04 (m, 12H, methylenes at C-4, C-5, C-8, C-9, C-12, C-13), 1.68, 1.60 (s, 15H, methyls at C-16, C-17, C-18, C-19, C-20).

Tris(tetra-*N*-butylammonium) Hydrogen Pyrophosphate (37)[81,82]:

A mixture of $Na_2H_2P_2O_7$ (3.1311 g, 14.1 mmol) in distilled water/ conc. ammonium hydroxide (24 mL:1 mL) was stirred at room temperature until all the solid dissolved. The resulting clear solution was loaded onto a column of cation exchange resin (DOWEX AG 50W-X8; 20-50 mesh, H^+), which had been pre-washed with deionized water, and the column was eluted with deionized water. The first 150 mL of eluent was

collected and the solution was titrated to pH 7.3 by slow addition of Bu_4NOH (40 wt.% solution in water). The flask was spun slowly in a dry ice/isopropanol bath in order to uniformly freeze the contents onto the inside wall of the flask. The sample was lyophilized for 3 days to afford a flocculant white solid (9.85 g, 77% yield). The compound was hygroscopic and was stored in a desiccator over P_2O_5.

^{1}H NMR (200 MHz, D_2O) δ 3.07 (t, 2H, $C\underline{H}_2$ at C-1, $J_{1,2}$= 8.4 Hz), 1.51 (quintet, 2H, $C\underline{H}_2$ at C-2, $J_{2,1}$= 8.4 Hz), 1.23 (sextet, 2H, $C\underline{H}_2$ at C-3, $J_{3,4}$= 7.3 Hz), 0.82 (t, 3H, $C\underline{H}_3$ at C-4, $J_{4,3}$= 7.3 Hz).

^{13}C NMR (50 MHz, $CDCl_3$) δ C-1 58.5, C-2 24.0, C-3 19.5, C-4 13.6.

Preparation of Et_3NH^+ HCO_3^-, pH 8.00 Buffers (38):

Carbon dioxide was bubbled through a solution of freshly distilled triethylamine (6.97 mL, 0.05 mol) and deionized water (993 mL) until the pH was 8.00 to give 0.05 M Et_3NH^+ HCO_3^{-3}. The 1.00 M buffer was prepared in the same manner.

3,7,11,15-Tetramethyl-2,6,10,14-hexadecatetraen-1-[^{3}H]-pyrophosphate (39)[89]:

A mixture of **36** (16.2 mg, 52.1 μmol), tris-(tetra-*N*-butylammonium) hydrogen pyrophosphate **37** (108 mg, 120 μmol), and acetonitrile (600 μL) was stirred at room temperature under a nitrogen atmosphere for 4.5 hours. The solvent was removed *in vacuo* to yield a dark yellow oil which was dissolved in 0.05 M Et_3NH^+ HCO_3^-, pH 8.00 (**38**) (2 mL), and extracted with two portions of diethyl ether. The clear aqueous phase was

loaded onto a column (1 cm dia. x 25 cm) of DEAE Sephadex A-25, 40-120 μ at 4°C. The Sephadex had been equilibrated to the bicarbonate form by swelling and washing (5 times) with 1.00 M triethylammonium bicarbonate, pH 8.00, and then with 0.05 M triethylammonium bicarbonate, pH 8.00. Finally, the packed column was washed with several column volumes of 0.05 M Et_3NH^+ HCO_3^-, pH 8.00. The column was developed with a linear gradient (300 mL, 0.5 mL/min.) from 0.05 M to 1.00 M triethylammonium bicarbonate, pH 8.00. Fractions eluting at ca. 0.65 M were analyzed by scintillation counting and found to contain [1-^{3}H]-geranyl geranyl pyrophosphate. The fractions were combined and lyophilized to the point at which the white solid just disappears to give **39** (8.28 μmol, 15.6%, 4.39 x 10^6 DPM ^{3}H) which was stored frozen in 2.8 mL D_2O, 2 drops conc.NH_4OH, pH > 10 until further use.

Preparation of TRIS-HCl, pH 8.4 Buffer (40):

A solution of magnesium chloride hexahydrate, (2.0331 g, 10 mmol), β-mercaptoethanol (351 μL, 5.00 mmol), tris(hydroxymethyl)aminomethane (24.228 g, 200 mmol), and 1000 mL distilled water were stirred at room temperature until all solids were dissolved. The solution was titrated with conc.HCl to pH 8.4.

Content of Yew (*Taxus x media* densiformis):

Yew samples from the Royal Botanical Gardens (RBG), Hamilton, Ontario, were analyzed for polar and nonpolar compound content. Bark, pulp, needle, and seed

samples were washed, dried, frozen in liquid nitrogen and ground to a fine powder. Pre-weighed portions of each powder were added to various solvents (25 mL each of dichloromethane, methanol, absolute ethanol, and ethyl acetate) and stirred for 5.5 hours at room temperature under nitrogen. The resulting mixtures were filtered, the solvent removed, and the resulting residues weighed. Each solid was vortexed with a portion of absolute ethanol and then the solvent removed under reduced pressure to remove any remaining water. The residues were combined with 1.5 mL hexanes to isolate the nonpolar compounds, vortexed, and analyzed by GC. The residue which was not soluble in hexanes was dissolved in a portion of ether, vortexed, and analyzed by GC for polar compound content.

Agitation Procedure for Preparation of Yew (*Taxus x media* densiformis) Carrier Material :

A modification to the method of Muschik *et al*[26], for the analysis and isolation of taxol content in the needles of various *Taxus* species, was used to prepare carrier yew material. Fresh-cut yew branches from the RBG were washed with water, air-dried, and the needles (23.47 g) were frozen in liquid nitrogen and ground to a fine powder. The powder was combined with 150 mL CH_2Cl_2 and stirred for 22 hours at room temperature under a nitrogen atmosphere. The mixture was filtered and the filtrate removed *in vacuo* leaving the CH_2Cl_2-soluble residue. The solid residue was scraped off the inside wall of the flask, 5 mL of absolute ethanol was added to remove any residual water, and the

sample mixed at maximum speed for 2 minutes and the solvent was removed *in vacuo*. A portion of hexanes was added to the residue to dissolve the nonpolar compounds. The solution was vortexed at maximum speed for 1 minute, the organic layer was transferred to a sample vial, concentrated, and stored at 4°C under nitrogen until further use. In the same manner, the ether layer was prepared, transferred to a vial, concentrated, and stored until further use.

General Procedure for Preparation of Yew (*Taxus x media* densiformis) Cell-Free Extract :

The procedure of Munck and Croteau[87], for the purification and characterization of a sesquiterpene cyclase, was followed for the preparation of the cell-free extract. *Taxus x media* densiformis needles (20 g) were washed, dried, frozen in liquid nitrogen, and ground to a fine powder with a mortar and pestle. The powder was combined at 0°C with polyvinylpolypyrrolidone (PVPP, 5 g) and 150 mL 200 mM Tris buffer, titrated to pH 8.4 with conc. HCl, and allowed to stir for 30 minutes. The stirred mixture was filtered through 4 layers of cheesecloth and the filtrate treated with a second portion of PVPP (5 g). Again the solution was stirred for 30 minutes in the ice bath, filtered through cheesecloth, and the resulting filtrate was centrifuged at 6000 rpm for 20 minutes at 0-4°C to provide a supernatant which was used as the cell-free enzyme preparation.

General Procedure for [1-^{3}H]-Geranylgeranyl Pyrophosphate (39) Incubation with Yew Cell Free Extract :

The cell-free extract was made according to the above procedure. One aliquot of the enzyme extract was denatured, by exposure to high temperature, to serve as a control while the other aliquot was kept at 0°C. The four factors in the experiment, incubation time (IT), volume of [1-^{3}H]-geranylgeranyl pyrophosphate (T-GGPP), volume of cell-free extract (CFE), and amount of total PVPP used in preparation of the CFE (PVPP), were varied to determine the best conditions under which to perform the incubation. The appropriate volume of CFE was delivered via automatic pipette into borosilicate test tubes. The T-GGPP was added to the CFE at time zero and the test tubes were placed in a water bath at 20°C and remained there for the specified IT. Once the pre-determined time period had elapsed, the incubation was stopped by addition of hexanes (2 mL), swirling of the mixture on the vortex mixer, and removal of the hydrocarbon layer. This layer was loaded onto a flash silica pasteur pipette column and delivered directly into a scintillation vial containing 5mL scintillation cocktail. The incubated sample was extracted once more with a portion of hexanes; this layer was loaded onto the same pipette column, delivered into the same scintillation vial, and was followed by three portions of hexanes. The material remaining in the column was then washed with three portions of 3% v/v ether/hexanes to elute any slightly polar compounds: these eluents were delivered directly into a second scintillation vial. Finally, the column was subjected to elution with three portions of ether and the eluents were

collected into a third scintillation vial. This process was repeated for each of the denatured and non-denatured incubation samples. The samples were mixed and counted on the Beckman scintillation counter to determine radioactivity content.

Procedure for the Determination of the Specific Activity of [1-^{3}H]-Geranyl Geraniol (39) :

A sample of [1-^{3}H]-3,7,11,15-tetramethyl-2,6,10,14-hexadecatetraen-1-ol (2 μL, 1.65 mg, 564 nmol) was diluted with 5 mL hexanes. A small aliquot (200 μL) of this solution was removed and diluted with 10 mL hexanes to give a 22.6 μM solution of **39**. A series of samples were removed from this stock solution and prepared for scintillation counting. In order to determine the specific activity of the tritiated alcohol (**39**), 17.5 μL of the 22.6 μM solution was mixed with 12.5 μL of a [^{3}H]-toluene standard of known activity. The counted samples activity values were corrected for background noise and the specific activity of **39** was determined to be 1.95 x 10^8 DPM/mg.

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