AN ABSTRACT OF THE DISSERTATION OF

Mohammad A. Khasawneh for the degree of Doctor of Philosophy in Chemistry presented on December 5, 2003.
Title: Natural and Semi-Synthetic Compounds With Biocidal Activity Against Arthropods Of Public Health Importance.

Abstract approved: Redacted for privacy

Joseph J. Karchesy

This study identified new compounds with pest control activities. The two sources of candidates that were followed here were the main heartwood extract of Alaska Yellow Cedar (AYC) constituents and several semi-synthetic counterparts.

Five compounds were isolated and identified for the first time in AYC heartwood in this research: two monoterpenes, two sesquiterpenes, and one lignan. The two monoterpenes were (1S)-2-oxo-3-p-menthenol (**41**) and (4R)-4-hydroxy-4-isopropyl-cyclohex-1-enecarboxylic acid (**63**). The two sesquiterpenes were (5S,7R,10R,11R)-eudesm-4(14)-ene-11,12-diol (**46**) and (4R,5S,7R)-1(10)-eremohpilen-11,12-diol (**59**). The lignan was (1R,2S,5R,6S)-2,6-bis-(3,5-dimethoxy-4-hydroxyphenyl)-3,7-dioxabicyclo-[3.3.0]octane, (**67**). Structures for

these compounds were confirmed on the basis of spectroscopic techniques such as 1- and 2-D NMR, high resolution MS and IR.

The pest control activity studies of 15 compounds isolated or semi-synthesized from AYC heartwood were conducted at the Centers for Disease Control and Prevention (CDC). Two types of studies were conducted – short-term (24h) and residual (over 1-4 weeks) activity for application against three types of pests related to human health – nymphal *I. scapularis* ticks, adult *X. cheopis* fleas and adult *Ae. eagypti* mosquitoes.

The 24 h studies revealed that nootkatone, valencene-13-aldehyde and valencene-13-ol were the most active among the studied compounds against the three pests. They exhibited highly improved pest control activities compared to valencene. This suggests that oxidation on both positions C-2 and C-13 of the eremophilane ring structure has an important effect on the activity. For compounds where the conformation of the eremophilane bicyclic ring has been altered, the activity seemed to diminish greatly. The above mentioned three compounds can be good candidates as pest control lead compounds.

The residual studies revealed that the most active compounds exhibited activity profiles that generally decreased with time. Although the long-term safety of these compounds has yet to be evaluated, the natural origin and the long history of use of these compounds suggest that they can be promising candidates. This study revealed that the three most promising compounds in the 24 h study

exhibited reasonably promising behavior, which makes them even stronger as pest control candidates.

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Natural and Semi-Synthetic Compounds With Biocidal Activity Against Arthropods Of Public Health Importance

by

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Dean of the Graduate School

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Mohammad A. Khasawneh, Author

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LIST OF ABBREVIATIONS

AYC	Alaska Yellow Cedar
br	broad
CI	chemical ionization
COSY	correlated spectroscopy
d	doublet
EI	electronic ionization
EtOAc	ethyl acetate
GC	gas chromatography
Hex	hexane
HMBC	heteronuclear multiple bond correlation
HPLC	high performance liquid chromatography
HR	high resolution
HRCIMS	high resolution chemical ionization mass spectormetry
HSQC	heteronuclear single quantum correlation
hr	hour
IR	infrared spectroscopy
LC_{50}	concentration that kills 50% of test population
LD_{50}	dose that kills 50% of test population
m	multiplet
Me	methyl
MeOH	methanol
MS	mass spectrometry
min	minute
NMR	nuclear magnetic reonance
NOE	nuclear Overhauser effect
q	quartet
RH	relative humidity
s	singlet
sec	second
t	triplet
UV	ultraviolet spectroscopy
wk	week

Natural and Semi-Synthetic Compounds With Biocidal Activity Against Arthropods Of Public Health Importance

1. Introduction

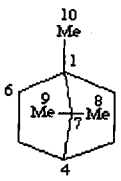
Living organisms (and especially plants) have been used since antiquity and continue to be used for their important bioactivity (medicinal, pest control etc.) by different cultures throughout the world. Currently, there are more than 120 plant derived chemical substances used as drugs throughout the world. More specifically, research in natural products has contributed about 65% of today's anti-infective and anti-cancer drugs and overall 25% of the drugs in use currently [1]. Yet less than 20% of more than 300,000 known plant species have been studied, often incompletely, for their therapeutic value. Other kinds of bioactivities (e.g., pest control activity) have been studied even less extensively. Therefore, plants represent an immense, mostly untapped source of novel, complex chemicals with potential bioactivity.

1.1 Terpenes and terpenoids

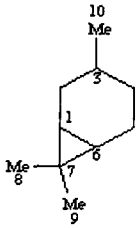
The name terpene was originally given to primarily describe hydrocarbons found in the resinous turpentine oil, which is one of the major products of the "pine chemicals industry". Terpenes are generally one major group of secondary metabolitic hydrocarbons present in many living organisms. Terpenoids are the oxygenated (hydroxylated, carboxylated, and carbonylated) terpene derivatives.

For simplicity, the term "terpenoids" is used to include both categories collectively. Terpenoid structures may generally be dissected into several five-carbon isoprene (2-methylbutadiene) units joined together in a head-to-tail fashion, which is known as the isoprene rule. Terpenoids are divided into groups depending on the number of "isoprene units" present. Hemiterpenoids contain a basic skeleton of one isoprene unit (C5). Other terpenoids include mono-(C10), sesqui-(C15), di-(C20), sester-(C25), tri-(C30) and tetraterpenoids (C40). Terpenoids containing more than eight isoprene units are called polyterpenoids.

Scheme 1 shows different skeletons found in plants for the two most important classes of secondary metabolites encountered in this study—namely, monoterpenes and sesquiterpenes. In the monoterpene class, there are five main structure types found in plants—Bornane, Carane, Fenchane, p-Menthane and Pinane (**1-5**). It is worthwhile noting that all these skeletons follow the isoprene rule. In the sesquiterpenes family, 15 unique skeletons could be identified (scheme 1) representing more than a thousand known sesquiterpene derivatives known mostly in the higher plants. Reflected by the higher degree of diversity among these skeletons, there are five skeletons that do not follow the isoprene rule—Aristolane (**7**), Drimane (**12**), Eremophilane (**13**), Gibbane (**16**), and Trichothecane (**20**). The nomenclature and numbering systems shown on each of the structures are according to the latest recommendations of the IUPAC found in reference [2].



Bornane
1



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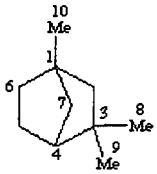
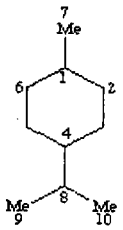
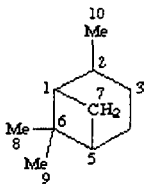


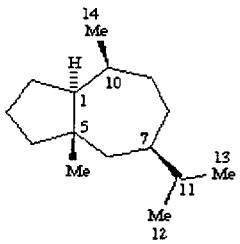
Fenchane
3



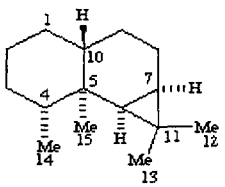
p-Menthane
4



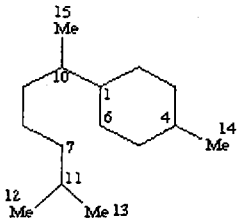
Pinane
5



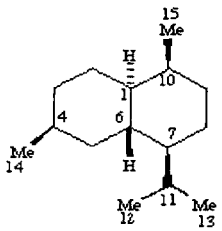
Ambrosane
6



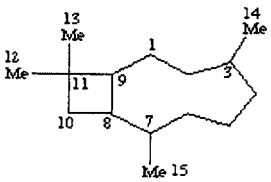
Aristolane
7



Bisabolane
8



Cadinane
9



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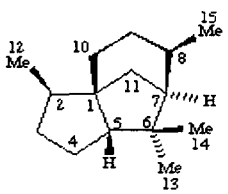

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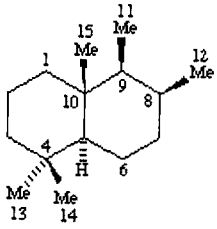

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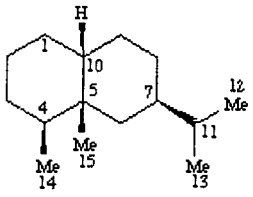

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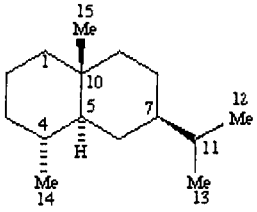

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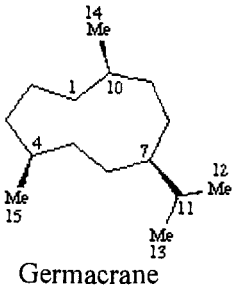

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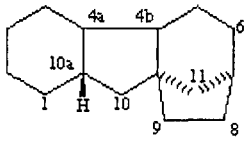

Gibbane
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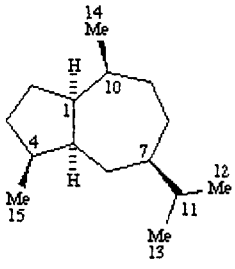

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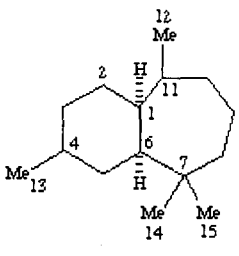

Himachalane
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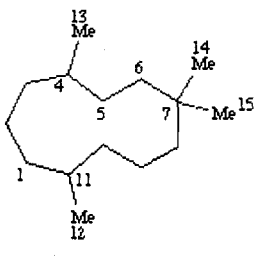

Humulane
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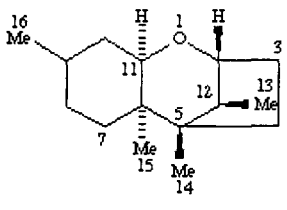


Trichothecane
20

Scheme 1: Different skeletons of monoterpenes and sesquiterpenes found in natural sources (Continued).

1.2 The Alaska Yellow Cedar (AYC) Tree

Alaska yellow cedar [*Chamaecyparis nootkatensis* (D. Don) spach], also known as yellow cedar, Alaska cedar, yellow cypress, is a medium-sized tree of up to 24 meters tall and 90 cm in diameter. It is one of the eleven genera that make the *Cupressaceae* (Cypress) family. The genus *Chamaecyparis* includes, in addition to *Chamaecyparis nootkatensis*, other common species such as *Chamaecyparis obtusa* (Japanese cypress), *Chamaecyparis lawsoniana* (Port-Orford cedar), *Chamaecyparis formosensis*, *Chamaecyparis thyoides* (Atlantic white cedar), *Chamaecyparis taiwanensis*, etc. This taxonomic hierarchy of AYC is currently being questioned by some prominent botanists [3].

The AYC tree is common to the pacific coast of Alaska, Canada and higher elevations of the US western states (figure1). It grows well on deep, slightly acidic, moist soils [4] usually as single trees or in small clumps. The AYC tree was described in 1793 by Archibald Menzies in the Nootka Sound area of Vancouver Island. The tree was then described by Davis Don and introduced into Europe through the Imperial Botanical Garden at Leningrad, Russia [5]. The heartwood of the tree has a distinctive and uniform yellow color. It is very valuable commercially due to many superlative qualities such as: 1. Its exceptionally fine texture and unusual straight grain. 2. Its being one of the

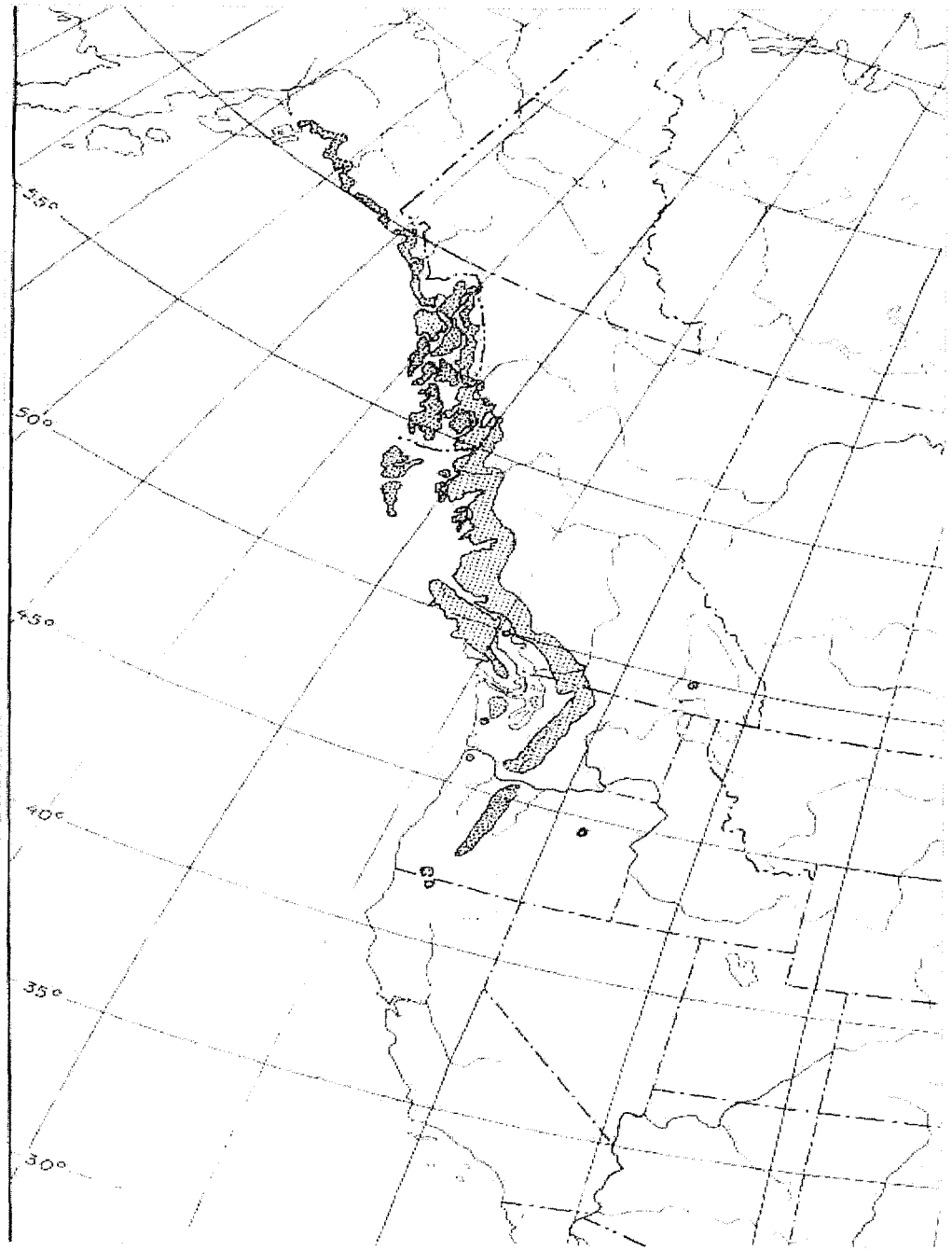


Figure 1: Distribution map for AYC.

world's most durable timbers with excellent resistance to decay properties and 3. Its relatively hard wood, with excellent strength and wear properties as well as good impact resistance. These high qualities made the AYC wood a highly prized timber throughout the world. Aboriginal people along the pacific coast have extensively utilized the wood in both their indoor and outdoor activities. They used the wood for shipbuilding, dishes, paddles, masks and bows and wove the bark to make clothing and blankets. Presently, AYC wood is being used for making outdoor furniture, garden products and boat building.

The most notable property of the AYC wood, in which lies the concentration of this work, is its high durability and resistance to decay [6-8]. The exact mechanism responsible for this property has not been fully understood yet. However, Shultz and Nicholas, reported that some limited number of wood samples (AYC was not among them) showed an enhanced short-term laboratory decay properties when treated with a mixture of commercial antioxidant (2,6-dimethyl-di-*tert*-butyl-4-methylphenol, BHT) and different commercial organic biocides compared to those samples treated with either antioxidant or biocide only [9]. Based on this limited result, these authors proposed that a dual defensive mechanism could be behind the outstanding durability of the wood. In the proposed mechanism, two properties of the extractives have to be available in order to prevent decay of the wood—fungicidal activity and free radical scavenging (antioxidant) activity. This hypothesis is based on the fact that most fungi are believed to use some type of free radical species in order to initiate the disruption

of cell walls to increase the pore size so that the extracellular fungal enzymes can penetrate into cell walls [10].

If this hypothesis holds for the case of AYC heartwood, it can be presumed that the unique set of secondary metabolites found in this heartwood can account for the AYC heartwood durability. For example, nootkatin, a sesqueterpenoid tropolone isolated from the heartwood of AYC in 1955, was shown to have high antifungal activity by Rennerfelt and Nacht [11]. Other constituents such as chamic acid , chaminic acid and carvacrol were all shown to have some antifungal activities [11-13], respectively. These compounds are discussed in more detail in the literature review and their structures are shown therein.

AYC trees have been facing a mysteriously high rate of mortality since the 1880s in southeastern Alaska. There are over 500,000 acres of dead Alaska Yellow Cedar trees in the forest [14a,b]. It should be mentioned here that because of durability properties of the heartwood of AYC, dead snags of essentially heartwood stay standing in the forest for up to a century as the foliage, twigs and sapwood are weathered away. Hennon and Shaw studied the mortality phenomenon of AYC and suggested that climatic warming observed coincided with the onset of extensive AYC mortality could be responsible for triggering some stress factor that has led to this phenomenon [14b]. However, more research should be done before a definitive conclusion can be drawn.

1.3 Arthropods and Human Health

Vector-borne diseases are transmitted to humans and animals by blood-feeding arthropods, such as mosquitoes, ticks and fleas. In certain countries, vector-borne diseases are still causing epidemics that have large health and economic impact on those countries. For example, it was estimated that at least one million children die each year in Africa as a result of malaria and its symptoms' complications [15]. On the other hand, mosquito vectors are still widely spread in all over the world, which makes them of concern to all countries. Also of special interest to the scientific community is the issue of global warming, deforestation and other resulting problems, which help provide more suitable conditions for those vectors' spread especially in this era of rapid and efficient worldwide transportation. Table 1 lists some of the vector-borne diseases and insects transmitting them along with some statistics of the number of cases reported in recent years in the United States.

Lyme disease, for example, is the most common vector borne disease in the United States resulting in an all time high of 17,730 cases in 2001. Lyme disease is transmitted by the deer tick *Ixodes scapularis* and is endemic in the northeast and upper mid-west of the United States. Methods to control vector populations of Lyme disease include habitat modification, host targeted control products, host reduction/exclusion, the use of desiccants and soaps and area-wide pesticide application. Clearly, the latter method has proven to be the most effective means

Table 1: Some common vector-borne diseases transmitted to humans and animals.

Disease	Pathogen	Vector	Annual number of cases reported in the United States
Viral encephalitis	West Nile Virus	*Aedes aegypti* mosquitoes	7718 total cases; 166 deaths as of October 29, 2003 *
Malaria	*Plasmodium falciparum* and *plasmodium vivax*	*Anopheles* mosquitoes	1,200- nearly all of which are imported
Lyme disease	*Borrelia burgdorferi*	*Ixodes scapuaris* and *Ixodes pacificus* (hard ticks)	15,934 in 1998
Rocky Mountain	*Rickettsia rickettssii*	Several species of *Ixodid* (hard ticks)	In recent years, 800 cases annually; 3-5% of which are fatal. Case fatality increases to 13-25% if the disease is left untreated.
Human ehrlichiosis	Several species of bacteria of the subfamily *Rickettsiaceal*	*Ixodid* tick	Since 1986,more than 500 cases have been reported (occasionally fatal).
Babesiosis	Babesia micorti	*Ixodid* tick	Co-infection occurs with Lyme disease and ehrlichiosis

* Source: CDC website (http://www.cdc.gov/od/oc/media/wntrend.htm).

of tick control, but also the least desirable for a majority of homeowners in Lyme endemic areas.

In 1999, another vector borne disease emerged in the United States that had previously been unreported. This infectious disease is known as West Nile virus (WNV) disease and is responsible for 7718 reported human cases in 2003 across the United States (current case count as of October, 29, 2003). Although not nearly as numerous as the number of cases of Lyme disease, 166 of them were fatal, an outcome not associated with Lyme borreliosis. This virus is transmitted by *Culex* species of mosquitoes, which is found throughout the U.S. making it of high potential danger to human health in this country. All but a few states have confirmed cases of WNV disease, the vast majority of the affected states have human cases. Other vector-borne diseases are also known and affecting human life. Some examples are given in table 1 include encephalitis, malaria, rocky mountain, human ehrlichiosis and babesiosis.

Aside from these vector-borne diseases that directly affect human life, there are many other indirect effects pests have on human life through their effect on livestock animals and agricultural crops of economical importance. These effects can be through inter-transmitting of diseases to animals and plants or through damaging or downgrading crops affecting the efficiency and quality of food production.

One approach to these problems is to control pests by using synthetic insecticides. During and following World War II, many chlorinated insecticides, including DDT, were discovered, which were used in large quantities worldwide to combat vector-borne diseases such as malaria, typhoid and cholera. Many years later, beginning in 1960s, after this widespread use of synthetic pesticides, many significant disadvantages of most of these compounds were discovered. Most notably, contamination of ground water and soil (bioaccumulation), adverse effects on non-target species (acute toxicity), and the development of pesticide resistant strains of insects became evident. For example, the principal control agents employed by pest management professionals and homeowners to combat Lyme disease include both liquid and granular formulations of carbaryl, cyfluthrin, diazinon and chlorpyrifos. Both diazinon and chlorpyrifos will be removed from the market by 2004 because of health risks to children and birds. Additionally, both are extremely toxic to fresh water fish and aquatic invertebrates. Although other kinds of insecticides have been made available, their toxicity and unknown bioaccumulation have resulted in growing public concerns and strict regulations about their use.

Since there is no medication or vaccine available for the WNV disease to date, the only approach is to control its vector, mosquitoes. During the initial outbreaks in New York City in 1999, authorities of the mosquito control districts used Anvil and Scourge to control vector mosquitoes. Both of these are pyrethroid derivatives, which pose a lower health risk to humans overall, but have recently

come under scrutiny for being linked to breast cancer, endocrine disruptions, neurological damage and childhood cancers.

The problems associated with the use of synthetic pesticides triggered an increasing trend against their use in the control of disease-transmitting vectors as well as agricultural pests. These concerns triggered, in turn, a movement towards the use of other less toxic alternatives for pest control. This trend is highly noticeable in the recent demand for organic food. In organic agriculture, no synthetic pesticides or fertilizers can be used. Natural biocides with improved properties compared to those available nowadays are highly desirable. Therefore the need for research with the aim of identifying new lead compounds having the desired activity but with much lower health hazards to humans and the environment is obvious.

1.4 Historical use of plants as pest control agents

The use of plants and plant extracts as pest control materials have been known to human civilizations since early stages of recorded human history. For example, the ancient Romans used false hellebore (*Veratrum album*) as rodentcides and the Chinese discovered the insecticidal properties of *Derris* species [16]. Also in China, the *Classical Pharmacopoiea of the Heavenly Husbandsman* as early as 25-220 A.D. mentioned the anthelmintic effect of *Melia azadarach*, a tree related to the neem tree (see later) [17]. The Arab scholar Abu

Mansur in 970 A.D. listed 584 natural materials, most of which are plants, as possessing pharmacological and poisonous properties [18]. The Pyrethrum plant was known and used in Persia and Dalmatia as an insecticide [19]. More recently, tobacco preparations have been used as insecticidal materials for over two centuries [20]. In 1983, Secoy and Smith have listed about 700 plant species, which have been reported to have been used in pest control by different civilizations of the world [21]. More recently, Yang and Tang have listed 267 pesticidal plants that have been studied in China in the late 1950s in an effort of using indigenous plants based on the rich Chinese knowledge of herbs [22].
In the above discussion, the mentioned literature tends to include the use of the plant (or the most efficacious part of it) in its entirety mainly in the protection of livestock and crops. However, it was mentioned in some of these references that in some cases the plant was soaked or treated in boiling water for a specified period of time and the "extract" was then used rather than the entire plant.

1.5 Examples of plant-derived commercial pest control agents and lead compounds

After the brief historic review of the use of plants in pest control given above, it becomes useful to give a few examples of some plant derived commercial pest control agents and promising lead compounds available these days, which were inspired from the knowledge acquired over the long human history. All these

examples demonstrate the strong potential of plants as an immense source of novel pest control agents highly desirable. It should also be emphasized that the trend for agricultural products with no or very low "pesticide residue" is increasing with time through the increasing demand for "organic food" observed these days. The following examples are given in an order of commercial importance with some promising lead compounds that have not been commercialized to date yet. These examples and others resemble the basis for the modern pest control agents industry. Structures of these compounds are shown in scheme 2.

1.5.1 Pyrethrins

Pyrethrins are one of the first and probably most important plant-derived insecticides. This group of compounds is found in the leaves and flowers of *Chrysanthemum cineriaefolium* [23]. It was estimated that the annual world market for Pyrethroids to be as high as $ 400 million in 1992. The structures of the active compounds pyrethrin I (**21**) and II (**22**) are given in scheme 2. Pyrethroids effectively control a wide range of pests with much lower overall health hazards, which makes them preferable to synthetic pesticides. However, they are very toxic to fish, such as bluegill and lake trout. Additionally, some insects have already developed some kind of resistance to Pyrethroids after continued use.

1.5.2 Azadirachtins

Azadirachtin (**24**) is another commercially important pest control compound derived from plants. It is a triteterpenoid isolated from the well-known neem tree (*Azadirachta indica*), which also contains other triterpenoids such as meliantriol (**25**) and salannin (**26**). The collection of the three compounds reportedly control more than 100 species of insects, mites and nematodes [24]. Azadirachtin itself is mainly used to effectively control *Lepidopetra* and *Dipetra* species by both contact and ingestion. It has the advantages of being less toxic to nontarget species, easily degradable and nonmutagenic. The mechanism of Azadirachtin disappearance in soil was found to be related to its photodegradation [25]. Its short half-life, while good for environmental reasons, is substantially limiting its efficacy.

1.5.3 Quassin

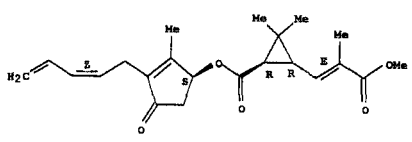
The original active ingredient, quassin (**23**), which is a wood extract found in the genus *Quassina*, has been approved as a biocide in Organic Agriculture [26]. Quassin has been found to be selectively effective against sawfly larvae, aphids and locusts among more than 100 species of insects tested [27].

1.5.4 Examples of natural-based synthetic pest control agents

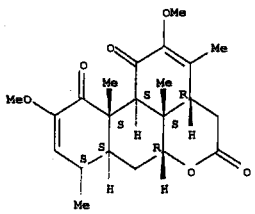
The group of synthetic pesticides called the carbamates falls in this category, which was developed following a natural lead provided by the alkaloid physostigmine (**27**) found in the plant *Phystigma venenosum*. Another example in this category is a group of commercial pesticides such as Padan and Cartap, both of which were developed following a natural lead compound called nereistoxin (**28**), a toxin isolated from the marine organism *Lumbriconeries heteropoda* [28].

1.5.5 Non-Commercial promising lead compounds

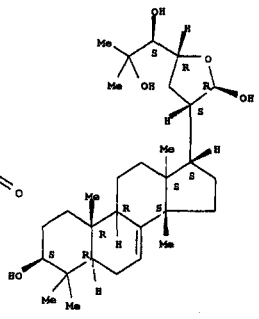
As for the promising natural lead compounds still in the research stage, the following examples can be ideally representing (scheme 3). In the first one, some monoterpenes, which are found in high concentrations in the higher plants have been found to have good pest control properties. The insecticidal, repellent and antimicrobial activity and phytotoxicity of monoterpenes have been recently reviewed by Ibrahim et al. [29]. In this review, a special reference to (+)-limonene (**29**) and its suitability for control of insect pest has been thoroughly given. In the second example, the class of polyester sesquiterpenoids isolated from the *Celastraceae* family (or the bittersweet family) is interesting for its insecticidal



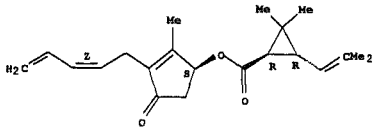
Pyrethrin I
21



Quassin
23



Meliantril
25



Pyrethrin II
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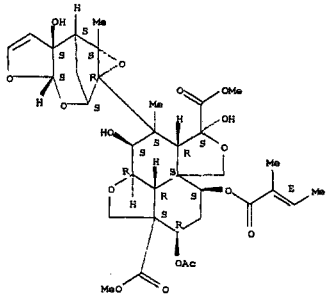
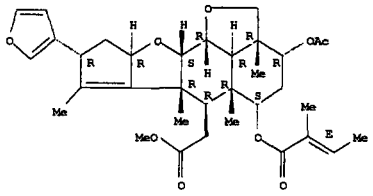
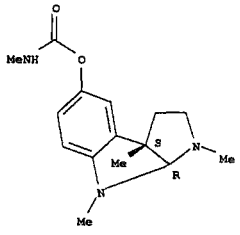


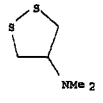
Azadirachtin
24



Salannin
26



Physostigmine
27

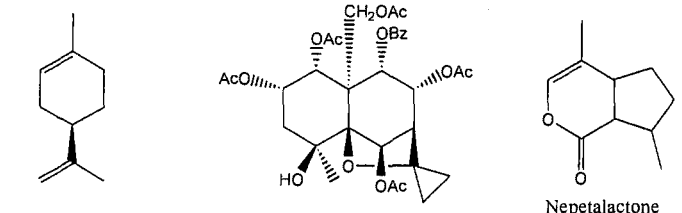


Nereistoxin
28

Scheme 2: Structures of some natural-based commercial pesticides and natural lead compounds.

and insect antifeedant properties (for example, Celangulin **30** (scheme 3)). The bittersweet family, which is consisting of 55 genera and 850 species distributed pantropically is an inspiring family for its nature of secondary metabolites [30].

The third example is nepetalactone (**31**) found in the catnip tree (*Nepeta cataria*). Nepetalactone has been found to have excellent insect repellency properties against mosquitoes, houseflies and cockroaches [31].



(+)-Limonene
29

Celangulin
30

Nepetalactone
31

Scheme 3: Structures of promising pesticide lead compounds.

All previous examples of commercially important pest control agents or promising lead compounds demonstrate the importance of natural products derived from plant sources in the search for novel pest control compounds with improved properties compared to synthetic counterparts.

The need for research with the aim of finding new and improved pest control agents never stops for more than one reason. One of these reasons is that most of the currently available insecticides suffer from problems such as acute

toxicity and/or long-term environmental effects, which can be known only long after their use. Another common problem is that most pest strains develop resistance to compounds after continued use. It is interesting to note that even with the demonstrated need for new pest control agents to replace the existing synthetic ones, there are only a limited number of lead structures. Therefore, concerted efforts with the aim of identifying new candidates for lead structures should be made.

1.6 Preliminary research

Preliminary tests in collaboration with the Centers for Disease Control and Prevention (CDC), Division of Infectious Borne Diseases, Fort Collins, CO have indicated that the essential oil and diethyl ether extract of the AYC heartwood are effective as acaricides and insecticides against adult fleas, ticks and mosquitoes [32-34]. Initial isolations have also indicated that the compounds carvacrol and a family of sesquiterpenes called eremophilanes are responsible for this activity [35].

1.7 Goals of this research

In this research, the primary long-range goal is to identify new pesticides and pesticide lead compounds based on encouraging results of the preliminary research and the well-known durability and antifungal (wood rotting fungi)

properties of AYC heartwood. In order to achieve this ultimate goal, the immediate objectives were to:

1. Complete identification of bioactive compounds in AYC heartwood both by bioassay guided fractionation and isolating and testing of previously known compounds in this wood.
2. Make semi-synthetic derivatives of eremophilane structure to test structure/bioactivity relationships in these systems.
3. Establish the stereochemistry of active eremophilane structures previously isolated from AYC heartwood where the stereochemistry was not proven.

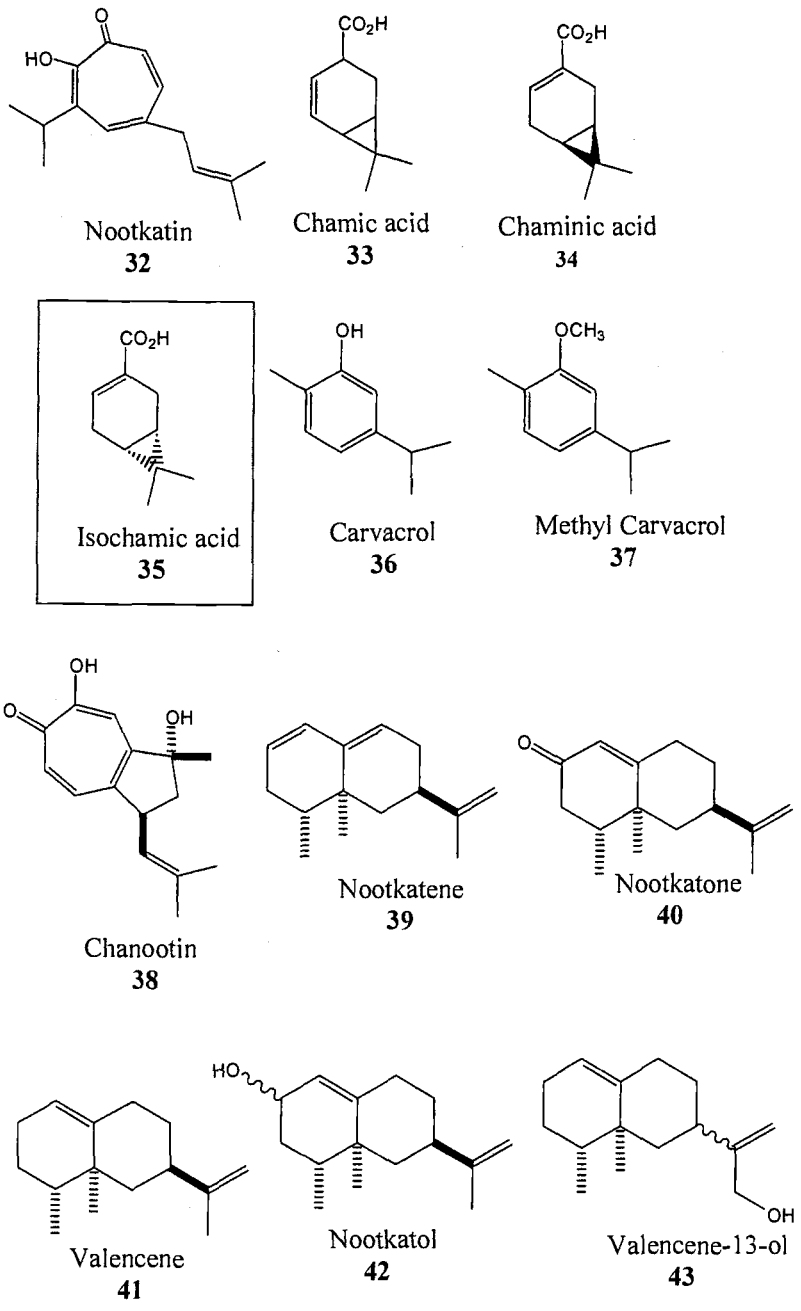
A secondary long-range goal is to identify major compounds in AYC extract and to compare to the literature for other *Chamaecyparis* species. Reasons to doing this were to:

1. Shed light on the issue of taxonomic hierarchy of AYC as a member of the *Chamaecyparis* family and doubts raised by some prominent botanists.
2. Discover other compounds, which may be of value in chemical utilization of this species.

2. Literature Review

AYC has been the topic of hundreds of scientific investigations since the early 1900s. The topics of these investigations include the tree's distribution, its ecological aspects, properties of its timber, phytochemistry of its different parts and challenges facing this valuable species. Hennon and Harris prepared a comprehensive bibliography of *Chamaecyparis nootkatensis* species [36], which contains over six hundred annotated references that appeared in the literature up to the end of 1996. However, only a few papers are concerned with the chemistry of this species.

The first documented phytochemical investigation of AYC foliage dates back to 1926 when Clark and Lucas studied both the heartwood and leaf oils of AYC [37]. In the leaf oil, they identified α-pinene and limonene as the main constituents and obtained evidence of β-pinene, sabinene and β–cymene. As for the heartwood oil, no constituents were described for reasons that were probably related to the primitive isolation and identification techniques available at that time. Following, we will give a detailed survey of the literature dealing with the phytochemical and bioactivity investigations of AYC dealing with one compound at a time as much as possible. Scheme 4 lists all structures of the secondary metabolites isolated from the heartwood of AYC.



Scheme 4: Secondary metabolites isolated from AYC heartwood. Isochamic acid (**35**) is included for reference.

The first compound to be isolated from AYC heartwood oil was nootkatin (**32**), which was isolated by Erdtman et al [38]. In this reference, no structure was established for nootkatin except that it was concluded to have a seven-membered ring tropolone based on a comparative UV study with other known tropolones and a cryoscopic molecular weight determination. In another paper by Carlsson and Erdtman et al, the steam-distilled AYC heartwood oil was studied [39]. During the distillation process, two distinct fractions were obtained. The first was a pale yellow, which distills fairly easily and the second was a colorless slower-distilling fraction, in which a colorless crystalline compound material formed upon cooling. The crystalline compound was purified and identified as nootkatin. Structure of nootkatin was unambiguously characterized by Duff and Erdtman using both chemical methods and X-ray analysis of its copper complex in 1954 [40]. Along with nootkatin, a monoterpenoid acid was isolated from the AYC oil for which the name chamic acid (**33**) was proposed [39]. It remained until 1956 when Erdtman et al correctly established the structure of chamic acid by using degredative chemical experiments [41]. A *trans* relationship between the carboxyl group and the cyclopropyl group was deduced based on catalytic hydrogenation study of chamic acid and the known isochamic acid by studying the properties of these reduction products [41]. In the same work performed by Carlsson and Erdtman [39], yet another monoterpenoid acid was isolated for which the name chaminic acid (**34**) was proposed. The structure of chaminic acid was later deduced to be the enantiomeric isomer of isochamic acid (**35**) based on IR and optical rotation as

well as other experiments [41]. The absolute configurations of chamic, chaminic and isochamic acids were ultimately reported by Norin in 1964 [42].

The well-established natural product carvacrol (**36**) was also isolated in significant amounts from the heartwood of AYC. It is responsible for the pungent thymol odor of the wood of AYC. Carvacrol appears to be generally found in the heartwoods of *juniperus* and *Cupressus* genera [43]. Carvacrol is found in much larger quantities in herbal species such as thyme and oregano. A numerous number of reports is still being published each year about the identification of the volatile constituents of herbal species in which carvacrol is found (for example, from 2000-2002 there were about 60 references reported in the literature). Another derivative of carvacrol, namely methyl carvacrol (**37**), was also isolated from the heartwood of AYC in 1953 by Duff and Erdtman [44].

Nootkatin (**32**), chamic acid (**33**) and even the neutral fraction of AYC heartwood oil were found to possess fungicidal activity [45]. Nootkatin was found to be the most active fungicidal among the studied compounds or fractions. It was found to inhibit fungal growth at concentrations as low as 0.001-0.002% (w/v); while chamic acid was found to inhibit fungal growth at concentrations of 0.01-0.02% (w/v). In the same year, Erdtman confirmed the activity of nootkatin and chamic acid and found chaminic acid to have fungicidal activity as well [46]. These results were used to explain the durability of this wood to decay.

Chanootin (**38**), another C-15 tropolone, was isolated from the heartwood of AYC in 1964 by Norin [47]. In 1973, Karlsson et al reported on the crystal

structure of chanootin [48]. To date, there have been no direct bioactivity studies on chanootin in the literature.

AYC is known to have several eremophilane-type sesquiterpenoids in its heartwood. Nootkatene (**39**), nootkatone (**40**), valencene (**41**), nootkatol (**42**) and valencene-13-ol (**43**) have been isolated thus far from the heartwood oil. This fact makes AYC a unique species in the *Cupressaceae* family since the eremohpilane-type sesquiterpenes are extremely rare in this family. This observation has been cited as one of the indications that the systematic position of this species should be reconsidered. This issue will be addressed in the discussion in chapter 4.

Nootkatene (**39**) was the first eremophilane-type sesquiterpene to be isolated from the heartwood of AYC. It was discovered by Erdtman et al in 1957 when these authors further examined the neutral fraction of the extract [49]. The structure of nootkatene was later established by MacLeod in 1965 [50]. Nootkatene was later isolated from valencia orange peel (*citrus sinesis*) by Lund et al in 1970 [51]. To date, no bioactivity was correlated to nootkatene in the literature.

Nootkatone (**40**) is probably one of the most important and exhaustively studied constituents of the heartwood of AYC. It has first been isolated from AYC in 1962 by Erdtman et al [52]. Later, it was also found in grapefruit [53], orange [54], lemon [55] and Virginia cedarwood (*Juniperus Virginiana L.*) [56]. Nootkatone has a mildly pungent flavor, which is the major contributor to the distinctive aroma of grapefruit [57] with a very low odor threshold [58]. Several

structures were initially debated for nootkatone, until it was undoubtedly established by MacLeod in 1965 [59]. Compelling evidence of the structure was based on NMR, IR, UV, and chemical transformations of nootkatone and some corresponding authentic samples.

The importance of nootkatone comes from the fact that it is highly valued for its commercial use as a flavoring and a fragrant. Resources of nootkatone could be natural or semisynthetic from the more available natural product valencene (**41**). There are numerous reports on the conversion of valencene into nootkatone including many patents (for example, see references [59-61]).

Nootkatone has also been totally synthesized stereoselectively starting from (-)-(β)-pinene [62]. Both enantiomers of nootkatone were also synthesized from the naturally occurring (+)- and (-)- Sabinene [63]. In this unique study, it was found that the naturally occurring enantiomer of nootkatone (the (+)-form) to have about 1000-fold concentration threshold lower than that of the non-natural (the (-)-form) in water and 2000-fold in the vapor phase [64].

Nootkatone is a well-studied natural product for its remarkable bioactivity properties. It was found to exhibit insecticidal activity [65], fungicidal activity [66], antiulcer activity [67] and inhibitory activity against cytochrome P_{450} enzymes responsible for drug inactivation [68, 69].

Another alternative to the above mentioned processes for obtaining this highly prized product is by the use of plant cultures, by bioconversion of likely precursors. Drawert et al used this technique utilizing valencene as a precursor in

the production of nootkatone in cell suspension cultures of some citrus species [70]. Del Rio et al. reported that *Callus* cultures of various citrus species are capable of synthesizing nootkatone and valencene [71] though in much smaller quantities compared to those found in mature fruits. Furthermore, these authors reported the decrease of valencene levels and the increase of nootkatone levels with the aging of the *Callus* cultures. These results suggested that valencene might be considered as a precursor in the biosynthetic pathway of nootkatone in this particular system. These results agreed with those obtained when Del Rio et al studied the variations of nootkatone and valencene levels during the development of grapefruit [72].

Valencene (**41**) was first isolated from orange juice and peel oils [73, 74]. Then it was isolated from AYC [50], grapefruit [75, 76], lemon [76], mango fruit [77] and American cedarwood [78]. The structure of valencene was established by MacLeod [50]. Moshonas et al [54] reported that the quantity of valencene in whole essence oil of overripe valencia oranges increased to more than double its quantity in ripe fruit (about 1.5% by weight of the oil). These authors suggested that overripe valencia oranges could be a viable source for valencene, which can be converted to the more useful product nootkatone. To date, there have been a few reports describing the biological activity of valencene [79, 80].

Nootkatol (**42**) was first isolated from the essential oil of blond and blood orange juices [81]. Recently, Xiong isolated nootkatol from AYC [32, 33, 34]. This was the first report for the presence of nootkatol in this species. More

recently, it was isolated from *Alpinia oxyphylla* Miq. By Luo et al [82]. Nootkatol was described in many reports dealing with oxidations of valencene (for example, see [83]) or reduction of nootkatone [84].

Although nootkatol has been known for quite some time now, there is some confusion about the stereochemistry of the hydroxyl group and about naming it as nootkatol or epi-nootkatol. This issue will be addressed in detail in this work. Nootkatol was found to have efficacy as a Ca antagonist, inhibiting the constriction of rabbit isolated artery [85].

The last sesquiterpene to be isolated from the heartwood of AYC is valencene-13-ol (**43**). This natural product was recently isolated by Karchesy et al [32, 34] and its structure was based on compelling spectroscopic data. However, the stereochemistry around C-7 has never been proven, which is an issue that will be addressed in this research. This compound has never been described as a natural product in any species before AYC. However, quite recently, it has been briefly described as a hydroxylation product of valencene by enzymes from chicory (*Chicorium intybus L.*) roots [83].

All the above discussion was devoted to the literature related to the heartwood of AYC. As for the leaf oil of this species, the first comprehensive study must be accredited to Cheng and von Rudloff, who in 1970 identified (-)-α-pinene, (+)- carene and (+)-limonene as the main constituents [86]. In addition many other compounds were isolated along with these compounds (table 2) using the technique of gas-liquid chromatography, which was newly developed at that

time. Another important work on the AYC leaf oil is that of Anderson and Syrdal also in 1970. In their work, these authors reported on the stereochemistry aspects of the sesquiterpenes found in the leaf oil and discussed the biogentic importance of these stereo assignments [87, 88]. It is worth mentioning that none of the sesquiterpenoids isolated from the heartwood of AYC were detected in the leaf oil.

The complex nature of constituents found in the leaf oil of AYC was used by Erdtman and Norin as a basis for the discussion of the issue of species change through chemotaxonomy towards the reclassification of AYC. This argument was supported by the fact that AYC contains in its heartwood a unique set of eremophilane-type sesquiterpenoids. However, there was insufficient chemical evidence to justify the reclassification at that time [89]. This issue will be discussed in more detail later in chapter 4.

Table 2: Constituents of the leaf oil of AYC

COMPOUND	% IN TOTAL OIL	COMPOUND	% IN TOTAL OIL
(-)-α-Pinene	38.8	Isopimara-8(9), 15-diene	0.3
(+)-3-Carene	30.8	Isohibaene	0.3
(+)-Limonene	9.2	Sandaracopimaradiene	0.1
Fenchene	1.1	Isophyllocladene	0.1
Camphene	0.5	Isopimaradiene	0.1
(β)-Pinene	2	Manoyl oxide	0.3
Sabinene	0.2	13-epimanoyl oxide	0.3
Myrcene	2.3	Phyllocladene	0.3
(α)-Phellandrene	0.5	Abieta-7,13-diene	Trace
(β)-Phellandrene	2.9	Dehydroavietadiene	Trace
(α)-Terpinene	0.2	8-13-diepimanoyl (IV)	Trace
p-Cymene	0.3	8-Epimanoyl (III)	1.1
Terpinolene	2	Phyllocladan-16-ol	0.3
1-Methyl-4-isopropenyl benzene	0.3	3-Methyl-3-butenylisovalerate	0.4
Bornyl acetate	0.2	Pelargonaldehyde	0.2
Terpinen-4-ol	0.2	3-Methyl-2-butenyl isovalerate	0.2
(α)-t Terpineol	0.5	3-Methyl-3-butenyl-(3-methyl)-3-butenoate	Trace
(α)-Terpinyl acetate	1.2	n-Pentadecane	Trace
Piperitone	0.1	3-Methyl-3-butenyl-(3-methyl)-2-butenoate	0.2
Citronellol	0.1	3-Methyl-2-butenyl-(3-methyl)-3-butenoate	Trace
1-Methyl-4-(α-hydroxy isopropyl) benzene	Trace	n-Heptadecane	Trace
(α)-Cubebene	Trace	Benzyl isovalerate	0.1
(α)-Copaene	Trace	n-Nonadecane	Trace
Thujopsene	0.1	n-Teradecanal	Trace
(γ+δ)-Cadinene	0.1	(β)-Phenylethyl isovalerate	0.1
ar-Curcumene	Trace	Benzyl-(3-methyl)-2-butenoate	0.1
Calamene	0.1	n-Heneicosane	Trace
Nerolidol	0.4	Pelargonic acid	0.1
Cedrol	Trace	(β)-Phenylethyl-(3-methyl)-2-butenoate	0.2
(+)-Bisabolol	0.7	n-Tricosane	Trace
(α)-Cadinol	0.3	n-Pentacosane	Trace
(Diterpene I)	0.5	n-Docosanal	Trace

3. Experimental Section

3.1 Materials and General Methods

3.1.1 Materials

Hexane (EM Science, Gibbstown, NJ, glass distilled 99.4%) was used as received. Acetone (99.6%), Ethyl Acetate (99.9%), Dichloromethane (99.9%) and Methanol (99.9%) were all purchased from Fisher Scientific, Fair Loan, NJ and distilled prior to use. Diethyl ether (EM Science, Gibbstown, NJ 98%) was used as received. Chloroform (Mallinckrodt Baker Inc., Paris, KY, 99.9%) was used as received. Deuterated chloroform (Aldrich Chemical company, Milwaukee, WI, 99.9 atom %D) was used as received for NMR experiments.

SeO_2 (Matheson Company, Inc., East Rutherford, NJ), $NaBH_4$ (Mallinckrodt, Paris, KY 96%, H_2O_2 (Mallinckrodt, 30%solution in water), t-BuOOH (Aldrich Chemical Company, 5.0-6.0M solution in nonane), Triethylamine (EM Science, 98% min), Pyridine (Fisher, 99.9%), Na_2CO_3 (Mallinckrodt, 99.7%), H_2SO_4 (EM, 95.0-98.0%), HCl (J.T. Baker, Inc., PhillipsBurgh, NJ, 37.4%), KMO_4 (Aldrich, 97%), $NaHCO_3$ (Mallinckrodt, 99.7%), m-CPBA (Aldrich Chemical Company, Inc. 80-90%, remainder m-chlorobenzoic acid), KOH,(Mallinckrodt, minimum 86%), Acetic Anhydride (Mallinckrodt, 99.8%), (R)-(-)-α-Methoxy-α-trifluoromethyl acetic acid chloride (Fluka, ≥99%), (S)-(+)-α-Methoxy-α-trifluoromethyl acetic acid chloride (Fluka,

≥99%), Dimethylaminopyridine (DMAP, Aldrich, 99%) were all used as received from vendors. (+)-valencene (Fluka Chemical Company, Switzerland) and (+)-Nootkatone (Lancaster, Pelham, NH) were used in the synthesis of derivatives and were purified by a silica gel column prior to use and NMR spectroscopy was used to check purity. Commercial samples of nootkatone used in the bioassay screening for comparison with the nootkatone sample isolated from AYC were obtained from Bedoukian Research Inc., Danbury, CT (semisynthetic crystalline, 98%) and Frutarom, Inc. N.J. (from grapefuit oil, >70%). These samples were also purified by column chromatography prior to use. Anhydrous Na_2SO_4 (Fisher Scientific, Fair Lawn, NJ, 100%), Silica gel (EM Science, particle size 0.063-0.200mm, 70-230 mesh ASTM), TLC plates (EM Science, Kieselgel 60 F_{254} coated on Aluminum support) were all used as received.

3.1.2 General Methods

3.1.2.1 NMR experiments

In a typical NMR experiment, the sample to be analyzed (typically 2-25mg) was purified, and concentrated in vacuum to remove any traces of solvent. Sometimes the sample was left under vacuum for 10-60 min depending on amount of impurities and volatility of analyte and impurities. The pure sample was then

dissolved in a suitable NMR solvent (typically 0.5mL $CDCl_3$ unless indicated otherwise). The solution was transferred to an NMR tube (Aldrich, 5mm id, 528-PP series) and the tube was stoppered with a plastic cover. The measurements were recorded on a Bruker Avance NMR (400MHz in the case of ^1H and 100 MHz in the case of ^{13}C) spectroscopy instrument. The workup of the spectra was performed using XWIN-NMR software. Multiplicities were determined by DEPT techniques.

3.1.2.2 Optical rotation measurements

Typically, standard solutions of the compounds were prepared by dissolving 2-5 mg of the compounds to be analyzed in chloroform (2.0mL) and the resulting solution was transferred to a micro cell (3.5mm id) for measurement. Blank zeroing of the instrument reading was done on the same micro cell prior to each reading. Solutions that are not clear were filtered prior to taking readings. Measurements were conducted on a JASCO (model DIP-370, Tokyo, Japan) digital polarimeter. The values of specific rotation listed in the text ($[\alpha]_D^{20}$) are calculated according to the formula:

$$[\alpha]_D^{20} = \frac{\alpha,^\circ}{l(cm)XC(g/mL)}$$

, where α is the observed rotation angle, *l* is the length of sample in cm and C is the concentration of the sample in g/mL. The subscript D indicates that the light used to measure the rotation was the sodium D line (589 nm wavelength).

3.1.2.3 IR measurements

A thin film of a solution in CH_2Cl_2 of each of the samples to be analyzed was deposited on an NaCl plate and the plate was left to air dry for 3 min and then placed in the sample holder. Measurements were conducted on a Nicolet SDXB FT-IR instrument equipped with OMNIC E.S.P. software.

3.1.2.4 Thin Layer Chromatography (TLC)

The TLC was used as a guiding technique for the monitoring of fractionation of AYC extract samples and following the progress of reactions. A TLC plate was carefully cut to warrant sharp and even edges to a length of 7-10cm and width depending on the number of spots needed. A line was made with pencil to mark the point of application of spots. The solution to be analyzed was applied on this line using a TLC spotting capillary. The spotted TLC plate was placed

(with the spotted edge down) in an eluting jar containing the eluent in the bottom and securely covered. A filter paper was placed inside the developing jar to help saturate the atmosphere and maximize evaporation. R_f values were calculated by dividing the distance traveled by each component by that traveled by the solvent (solvent front). The R_f value was used as a quick way of identity of compounds until the NMR spectroscopy was run. TLC solvent system used to report R_f values was in most cases a mixture of Hex:EtOAc (1:1 v/v), unless otherwise stated. Spots on the TLC plates were visualized under UV light and then dipped in either a saturated aqueous solution of KMnO4 solution or a freshly prepared acidic vanillin solution made by dissolving vanillin (1g) in absolute ethanol (50 mL) and concentrated HCl (10mL) followed by heating.

3.1.2.5 Column Chromatography

Column chromatography was extensively utilized throughout this study for the fractionation of the components of AYC heartwood. In a typical column chromatography experiment, silica gel in Hexane was slurry packed in glass columns equipped at their exits with glass frits to hold solids in place. The packing was continued with agitation using a cork to make packing beds of different sizes (10-80 cm in length and 0.5-4.5cm in diameter) depending on the sample to be fractionated. The exit frit of the column is connected through Teflon tubing to a

time programmable fraction collector (Gilson, France, model 203) equipped with a rack containing 105 culture tubes (13X100mm). The sample to be fractionated was applied on the top of the packing bed and the eluent was slowly supplied by a separatory funnel. The collection of fractions was then started and continued until all constituents of interest were eluted. In most cases a gradient elution of Hex (100%) to Hex:EtOAc (50:50 v/v) was necessary to be applied for optimum separation efficiency. The similar fractions were then combined and concentrated in vacuum and further investigated by NMR, MS and IR. Fractionation was typically monitored by TLC and/or GC. Known compounds for bioassays were isolated over silica gel columns as previously described by Xiong [35]. New compounds are isolated as described in section 3.3.3 below.

3.1.2.6 Gas Chromatography

Gas chromatography was used to monitor separation of known compounds throughout this study by comparing to standards. A GC-17A model (Shimadzu, Japan) gas chromatograph equipped with a flame ionization detector (FID) was used. Conditions used were according to those reported in [35] in which an open-tube column (30m X0.25mm DB-5, 0.25μm, J&W Scientific) was used with temperature programming from initial temperature 100°C for 1 min to 150 °C at a

rate of 5°C /min, then to 220°C at 3°C/min, and finally to 240°C at 5°C/min and held at this temperature for 2 min.

3.2 Plant Materials

AYC tree was collected from the Hungry Mountain area on the Sol Duc River Drainage on the western slopes of the Olympic Mountains in the Olympic National Forest, WA. A collection permit was obtained from the Sol Duc ranger station in Forks, WA. A botanical voucher specimen (#188046) is deposited in the Oregon State University Herbarium. Heartwood was separated from the rest of the components and chipped in a grinder to a size of approximately 2X1 cm chips and kept at room temperature in the dark until used.

3.3 Extractions and Isolations

3.3.1 Steam Distillation

The chipped wood sample (1000g) was steam distilled as previously described by Xiong [35].

3.3.2 Solvent Extraction

The chipped AYC heartwood sample (1000g) was soaked in methanol (2.5L) in a 5.0L Erlinmeyer flask with continuous stirring for 24h. The mixture was gravity filtered through a 15cm filter paper. Anhydrous Na_2SO_4 was added to the filtrate and the mixture was re-filtered. The filtrate was concentrated in vacuum to give thick dark brown oil (43±10 g, 4.3±1.0% based on the wet wood mass).

3.3.3 Fractionation of the extract constituents

Carvacrol, methylcarvacrol, valencene, nootkatin, nootkatene, Nootkatol, nootkatone, and valencene-13-ol were isolated as previously described by Xiong [35]. Briefly, this was achieved by open column chromatography over silica gel using solvent gradients of Hex (100%) to Hex:EtOAc (80:20 v/v) and monitoring of fractionation by TLC and GC.

Compounds reported here for the first time were isolated from the methanol extract of AYC described in section 3.3.2 according to the following procedure. The methanol extract residue (0.5g sample) was chromatographed on a silica gel column using a gradient elution system of increasing polarity from 100:0 to 50:50 Hex-EtOAc (v/v) resulting in nine main fractions— I-IX: fraction I (mainly Valencene and Nootkatene as checked by TLC and GC against standards),

fraction II (mainly carvacrol by TLC and GC), fraction III (mainly nootkatol and nootkatone). Fractions I - III were not investigated any further here since their constituents were isolated from the steam-distilled oil as described above. Fraction IV was carefully chromatographed over a silical gel column with Hex-EtOAc (80:20) to provide unknown 1 (12mg, R_f=0.56, Hex-EtOAc (50:50)) as a colorless oily residue and unknown 2 (11mg, R_f=0.46, Hex-EtOAc (50:50)) as a colorless amorphous solid. Fraction V was mainly composed of unknown 3 (43mg, R_f=0.36 50:50 Hex:EA) as yellow oil with fair purity (>90%) by NMR. Fraction VI contains mainly chamic acid (13mg) and chaminic acid (8mg) both with good purity (checked by TLC and GC). This fraction was not investigated any further since these two compounds have been described before. Fraction VII contains mainly unknown 4 (7mg, R_f=0.24 in Hex-EtOAc (50:50)) with purity of more than 95% shown by NMR. Fraction VIII mainly contains unknown 5 along with other complex mixture of highly polar components. After a repeated silica column chromatography with a gradient elution of 60:40 to 30:70 Hex:EtOAc, unknown 4 (8mg, R_f=0.14 in Hex:EtOAc (50:50)) was recovered in good purity. Fraction IX contains mainly unknown 7 (10mg, R_f=0.12 in 50:50 Hex:EA), which was purified by a small scale silica column chromatography (elution with Hex-EtOAc (70:30)). Structure of this unknown follows the eremophilane sesquiterpene skeleton, but is still not clear.

3.4 List of Isolated Compounds with data

(1S)-2-Oxo-3-p-menthenol, 44 (Unknown 1): yellow oil; $[\alpha]_D^{20}$ - 85° (c 0.455, $CHCl_3$); IR (thin film) ν_{max} 3450, 1715, 1620 cm^{-1}; EIMS *m/z* $[M]^+$ 168, 153, 151, 125; HRCIMS m/z $[M]^+$ 168.1143, calculated for $C_{10}H_{16}O_2$, requires 168.1150. ^1H NMR spectral data ($CDCl_3$, 400 MHz) δ 1.16 (3H, d, *J*=6.8 Hz, H-9 or H-10), 1.14 (3H, d, J=6.8Hz, H-10 or H-9), 1.33 (3H, s, H-7), 1.98-2.05 (1H, m, H-6-equatorial), 2.16 (1H, dt, *J*=12.9, 3.02Hz, H-6-axial), 2.46 (2H, m, H-5), 2.48 (1H, m, H-8), 5.93 (1H, *J*=1.7Hz, H-3) and 3.77 (1H, bs, O-H). ^{13}C NMR spectral data ($CDCl_3$, 100MHz) δ (multiplicity) 21.1, 21.4 (CH_3, C-9 and C-10), 24.6 (CH_3, C-7), 27.3 (CH_2, C-5), 36.0 (CH, C-8), 36.1 (CH_2, C-6), 73.1 (C, C-1), 120.5 (CH, C-3), 172.8 (C, C-4) 203.3 (C, C-2). 2-D NMR data are shown in figures 2-4 and summarized in table 2.

(5S, 7R, 10R, 11R)-Eudesm-4(14)-ene-11,12-diol, 49 (Unknown 2): white amorphous solid; $[\alpha]_D^{20}$ +66.8° (c 0.25, $CHCl_3$); IR (thin film) ν_{max} 3450 cm^{-1}; EIMS *m/z* $[M]^+$ 238 (M^+), 220 (M^+-H_2O), 207 (M^+-CH_2OH), 202 (M^+-$2H_2O$) and 189 (M^+-CH_2OH-OH); HRCIMS m/z $[M]^+$ 238.19377, calculated for $C_{15}H_{26}O_2$, requires 238.1933. ^1H NMR spectral data ($CDCl_3$, 400 MHz) δ 1.88 (1H, m, H-1), 1.31 (1H, m, H-1), 1.70 (2H, ddd, *J*=5.7, 5.0, 5.0 Hz, H-2), 2.33 (1H, md, J=12.2, H-3), 2.04 (1H, mq, J=12.2, H-3), 2.16 (1H, dd, J=12.7, 3.5, H-5), 1.63 (1H, m, H-6); 1.70 (1H, m, H-6), 1.52 (1H, m, H-7), 1.78 (1H, dq, *J*=14.3, 3.1 Hz, H-8), 1.57

(1H, m, H-8), 1.32 (1H, m, H-9); 1.46 (1H, m, H-9), 3.67 (1H, bd, *J*=10.8, 3.5 Hz, H-12), 3.46 (1H, bd, *J*=10.8, 3.5 Hz, H-12), 1.28 (3H, s, H-13), 4.76 (1H, dd, *J*=1.6,1.6 Hz, H-14), 4.49 (1H, dd, *J*=1.6, 1.6 Hz, H-14), 0.78 (3H, s, H-15). ^{13}C NMR data ($CDCl_3$, 100MHz) δ (multiplicity) 38.9 (CH_2, C-1), 21.6 (CH_2, C-2), 37.6 (CH_2, C-3), 151.6 (C, C-4), 45.2 (CH, C-5), 23.7 (CH_2, C-6), 38.7 (CH, C-7), 24.0 (CH_2, C-8), 42.8 (CH_2, C-9), 35.7 (C, C-10), 76.6 (C, C-11), 69.5 (CH_2, C-12), 24.0 (CH_3, C-13), 105.8 (CH_2, C-14), 17.3 (CH_3). 2-D NMR data are shown in figures 5-7 and summarized in table 6.

(4R, 5S, 7R, 11)-1(10)-Eremohpilen-11,12-diol (Unknown 3), 62: yellow oil; $[\alpha]_D^{20}$ -17.3 (c 0.66, $CHCl_3$); IR (thin film) ν_{max} 3450, 1620 cm^{-1}; EIMS *m/z* 238 (M^+), 220 (M^+-H_2O), 207 (M^+-CH_2OH), 202 (M^+-$2H_2O$), 189(M^+-(CH_2OH+OH), 161 (189-$CHCH_3$, the decaline system residue); HRCIMS m/z $[M]^+$ 238.1938, calculated for $C_{15}H_{26}O_2$, requires 238.1933). ^1H NMR spectral data ($CDCl_3$, 400 MHz) δ 5.36 (1H, m, H-1), 1.98 (2H, m, H-2), 1.46 (2H, m, H-3), 1.46 (1H, m, H-4), 2.02 (1H, ddd, *J*=12.6, 3.4, 2.5 Hz, H-6), 0.92 (1H, m, H-6), 1.87 (1H, apparent tt, *J*=12.6, 3.02 Hz, H-7), 1.74 (1H, dddd, *J*=12.2, 4.7, 2.6, 1.8 Hz, H-8); 1.03 (1H, dd, *J*=12.6, 4.01 Hz, H-8), 2.31 (1H, mt, J=13.5 Hz, H-9); 2.10 (1H, ddd, J=14.3, 5.5, 2.8 Hz, H-9), 3.62 (1H, bd, *J*=10.9 Hz, H-12), 3.46 (1H, bd, *J*=10.9 Hz, H-12), 1.11 (3H, s, H-13), 0.94 (3H, s, H-14), 0.90 (3H, d, *J*=6.1 Hz, H-15). ^{13}C NMR data ($CDCl_3$, 100MHz) δ (multiplicity) 120.6 (CH, C-1), 26.3 (CH_2, C-2), 27.6 (CH_2, C-3), 41.5 (CH, C-4), 38.0 (C, C-5), 39.9 (CH_2, C-6), 40.4 (CH, C-7), 29.6

(CH_2, C-8), 32.9 (CH_2, C-9), 143.4 (C, C-10), 75.1 (C, C-11), 69.0 (CH_2, C-12), 20.9 (CH_3, C-13), 18.8 (CH_3, C-14), 16.1 (CH_3, C-15). 2-D NMR data are shown in figures 9-11 and summarized in table 9.

(4R)-4-Hydroxy-4-isopropyl-cyclohex-1-enecarboxylic acid. 64 (Unknown 4): white amorphous solid; $[\alpha]_D^{20}$ + 25.5 (c 0.275, $CHCl_3$); IR (thin film) ν_{max} 2500-3500, 1620 cm^{-1}; EIMS *m/z* 184 [M^+], 167 [184-OH], 166 [M^+-H_2O], 151 [166-Me], 141 [[M^+-CH_3CHCH_3], 138 [166-CO], 123 [138-Me]; HRCIMS $[M]^+$ 184.1088 calculated for $C_{10}H_{16}O_3$, requires 184.10995. ^1H NMR spectral data ($CDCl_3$, 400 MHz) δ 7.07 (1H, m, H-2), 2.37 (1H, dd, *J*=7.0, 3.1 Hz, H-3 equatorial), 2.25 (1H, m, H-3 axial), 1.81 (1H, dddd, *J*=13.6, 5.7, 3.3, 2.1 Hz, H-5 ax); 1.59 (1H, ddd, *J*=8.4, 4.3, 3.8 H-5 eq), 2.45 (1H, m, H-6); 2.42 (1H, m, H-6), 1.72 (1H, q, *J*=6.86 Hz, H-7), 1.01 (3H, d, *J*=6.86 Hz, H-8 or H-9), 0.99 (3H, d, *J*=6.86 Hz, H-9 or H-8). ^{13}C NMR data ($CDCl_3$, 100MHz) δ (multiplicity) 129.6 (C, C-1), 140.2 (CH, C-2), 35.5 (CH_2, C-3), 72.1 (C, C-4), 30.8 (CH_2, C-5), 21.3 (CH_2, C-6), 37.8 (CH, C-7), 17.1 (CH_3, C-8 or C-9), 17.2 (CH_3, C-9 or C-8), 172.1 (C, C-10). 2-D NMR data are shown in figures 15-17.

(1R, 2S, 5R, 6S)-2, 6-bis(3, 5-dimethoxy-4-hydroxyphenyl)-3,7-dioxabicyclo-[3.3.0]octane, (+)-Syringaresinol (68), (Unknown 5): white amorphous solid; $[\alpha]_D^{20}$ + 8.4 (c 0.175, $CHCl_3$); IR (thin film) ν_{max} 1610,1510, 3450-3550 cm^{-1}; HRCIMS *m/z* $[M]^+$ 418.16193 calculated for $C_{22}H_{26}O_8$, requires 418.16277. ^1H

NMR spectral data ($CDCl_3$, 400 MHz) δ 3.13 (2H, m, H-1, H-5), 4.77 (2H, d, *J* = 4.5 Hz, H-2, H-6), 3.92 (2H, m, H-4, H-8(α)), 4.32 (2H, ddd, *J*= 9.1, 6.9, 2.1Hz, H-4, H-8(β)), 6.62 (4H, bs, H-2', H-6'), 5.54 (2H, bs, OH), 3.94 (12H, s, OCH_3). ^{13}C NMR data ($CDCl_3$, 100MHz) δ (multiplicity) 54.8 (CH, C-1, C-5), 85.3(CH, C-2, C-6), 71.0 (CH_2, C-4, C-8), 132.5 (C, C-1'), 103.1 (CH, C-2', C-6'), 147.6 (CH, C-3', C-5'), 134.7 (CH, C-4'), 56.8 (CH_3). 2-D NMR data are shown in figure 18-20 and summarized in table 12.

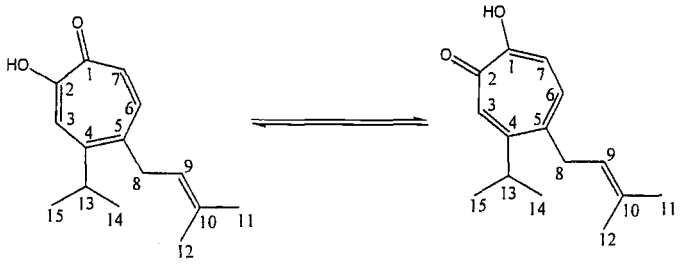
Nootkatol (42): colorless oil; $[\alpha]_D^{20}$ + 41.0 (c 1.00, $CHCl_3$); R_f 0.60 (Hex:EtOAc 50:50 v/v); IR (thin film) ν_{max} 3400, 1610 cm^{-1}; EIMS *m/z* 220 [M^+], 203 [M^+-OH], 187 [M^+-H_2O-CH_3], 177, 162,138,123, 121, 107; HRCIMS [M]$^+$ 220.18165 calculated for $C_{15}H_{24}O$, requires 220.18272. ^1H NMR spectral data ($CDCl_3$, 400 MHz) δ 5.32 (1H, d, *J*=1.6Hz, H-1), 4.25 (1H, m, H-2), 1.76 (1H, td, *J*=6.5, 2.0 Hz, H-3 *eq*), 1.37 (1H, td, *J*=12.4, 10.0 Hz, H-3 *ax*), 1.51 (1H, brd, J=2.1Hz, H-4), 1.85 (1H, dd, *J*=12.6, 2.7 Hz, H-6 *ax*), 0.95, m, H-6 *eq*), 2.25 (1H, tt, *J*=12.4, 3.0 Hz, H-7 *ax*), 2.33 (1H, m, H-8 *eq*), 2.1, 1H, ddd, *J*=14.1, 4.2, 2.6 Hz, H-8 *ax*), 1.79 (1H, dd, *J*=2.0, 4.5 Hz, H-9), 1.20 (1H, dm, J=4.3 Hz, H-9), 4.68 (2H, brs, H-12), 1.71 (3H, s, H-13), 0.99 (3H, s, H-14), 0.89 (3H, d, *J*=6.86 Hz, H-15). ^{13}C NMR data ($CDCl_3$, 100MHz) δ (multiplicity) 124.7 (CH, C-1), 68.4 (CH, C-2), 37.6 (CH_2, C-3), 39.7 (CH, C-4), 38.6 (C, C-5), 45.0 (CH_2, C-6), 41.2 (CH, C-7), 32.8

(CH_2, C-8), 33.3 (CH_2, C-9), 146.5 (C, C-10), 150.6 (C, C-11), 108.9 (CH_2, C-12), 21.1 (CH_3, C-13), 18.6 (CH_3, C-14), 15.8 (CH_3, C-15).

Eremophil-1(10), 11-dien-13-ol (Valencene-13-ol), 43: pale yellow oil. $[\alpha]_D^{20}$ + 62 (c 1.25, $CHCl_3$); R_f 0.65 (Hex:EtOAc 50:50 v/v); IR (thin film) ν_{max} 3400, 1610 cm^{-1}; EIMS *m/z* 220 (100), 202 (40), 189 (81), 161 (77), 105 (67), 91 (65), 79 (54). ^1H NMR spectral data ($CDCl_3$, 400 MHz) δ 5.33 (1H, t, *J*=2.4 Hz, H-1), 2.01 (2H, m, H-2), 1.41 (3H, m, H-3, H-4), 1.01 (1H, d, *J*=12.6 Hz, H-6), 1.93 (1H, m, H-6), 2.31 (1H, m, H-7), 2.09 (1H, td, J=13.4, 3.4 Hz, H-8), 2.31 (1H, m, H-8), 1.82 (1H, m, H-9), 1.21 (1H, dd, 13.3, 4.1 Hz, H-9), 5.02 (1H, s, H-12), 4.88 (1H, s, H-12), 4.12 (2H, s, H-13), 0.95 (3H, s, H-14), 0.87 (3H, d, *J*=6.0 Hz, H-15). ^{13}C NMR data ($CDCl_3$, 100MHz) δ (multiplicity) 120.8 (CH, C-1), 26.3 (CH_2, C-2), 27.5 (CH_2, C-3), 41.3 (CH, C-4), 38.3 (C, C-5), 45.8 (CH_2, C-6), 37.1 (CH, C-7), 33.1 (CH_2, C-8), 34.0 (CH_2, C-9), 143.2 (C, C-10), 154.5 (C, C-11), 108.3 (CH_2, C-12), 65.7 (CH_2, C-13), 18.8 (CH_3, C-14), 16.0 (CH_3, C-15).

Nootkatin (32): white crystals m.p. 91-92°C. This compound has been described before, but with no NMR data reported in the literature. Therefore, a complete ^1H and ^{13}C-NMR data will be listed here. ^1H-NMR data ($CDCl_3$, 400 MHz) δ 7.43 (1H, H-3, s) 7.36(1H, H-6, d, *J*=12 Hz), 7.24 (1H, H-7, d, *J*=12 Hz), 5.14 (1H, mt, J=6.5 Hz, H-9), 3.41 (2H, d, J=6.8 Hz, H-8), 3.29(1H, septet, *J*=6.7 Hz, H-13), 1.77 (6H, two s, H-11 and H-12), 1.28 (6H, d, *J*=6.7 Hz, H-14 and H-15). ^{13}C-

NMR data ($CDCl_3$, 100MHz) δ (data for one regioisomer only is given here) 172.6 (C-1), 167.9 (C-2), 157.4 (C-4), 141.4 (CH, C-6), 140.7 (C-5), 133.9 (C, C-10), 125.5 (CH, C-7), 122.6 (CH, C-9), 121.2(CH, C-3), 36.4 (CH_2, C-8), 32.7 (CH, C-13), 26.1 and 18.4 (CH_3, C-11 and C-12), 23.6 (CH_3, C-14 and C-15).



3.5 Stereochemistry of nootkatol (**42**) and 13-hydroxy-valencene (**43**)

Nootkatol and 13-hydroxy-valencene were isolated as previously outlined with their data are listed in the previous section. 13-hydroxy-valencene was also synthesized as outlined in the next section and spectral data (NMR and optical rotation) were compared with those of the natural product. The absolute stereochemistry of nootkatol was assigned by the modified Mosher ester method. Both the (R)- and (S)-esters of nootkatol were synthesized as outlined in sections 3.6.1. and 3.6.2 below and ^{1}H-NMR spectra of these two esters were used to assign the absolute stereochemistry around C-2 of nootkatol.

3.6 Syntheses

3.6.1 Synthesis of Nootkatol-(R)-α-Methoxy-α-(trifluoromethyl)phenyl acetate

To a stirred solution of nootkatol (6mg, 0.028mmol) in CH_3Cl (0.5mL) at room temparature was added triethyl amine (5.5mg, 0.054mmol) followed by (S)-α-Methoxy-α-(trifluoro-methyl)phenyl acetyl chloride (6.8 mg, 0.027mmol) as a solution in CH_3Cl (1.0mL) and the resultant mixture was stirred for 24 hr. The reaction mixture was washed with ice cold water (3.0mL), 2.0M HCl (1.0mL), saturated $NaHCO_3$ solution (3.0mL) and the organic layer was dried (anhydrous Na_2SO_4), and concentrated in vacuum to yield the title product (12.2 mg, 0.028mmol, 100% yield). The product was purified by using a small silica column with Hex-EtOAc (90:10) as eluent.

3.6.2 Synthesis of Nootkatol-(S)-α-Methoxy-α-(trifluoro-methyl)phenyl acetate

The same procedure as in section 3.6.1 was followed here with the exception of the use of the (R)-enantiomer of the Mosher reagent instead of the (S)-enantiomer.

3.6.3 Synthesis of unknown 2 monoacetate

Unknown 2 (10mg, 0.042 mmol) was dissolved in excess acetic anhydride (1mL) and pyridine (9.9mg, 0.126mmol) was added to the reaction mixture with continuous stirring at room temperature. After 12 hr, the reaction was stopped and water (10mL) was added followed by CH_2Cl_2 (10mL) to break up the excess acetic anhydride. The mixture was transferred to a separatory funnel and the organic layer separated. The aqueous layer was washed with CH_2Cl_2 (2X5mL) and the combined organic layer was washed with 10% acetic acid solution (10mL), saturated $NaHCO_3$ (20mL), brine (10mL), dried (anhydrous Na_2SO_4), filtered and concentrated in vacuum to give the title compound (10.6 mg, 0.038mmol, 90%). The purity was found to be $\geq$ 95% by NMR spectroscopy.

3.6.4 Synthesis of unknown-3-monoacetate

The same procedure as in section 3.6.3 above was followed except with the use of unknown 3 as the starting material in this case.

3.6.5 Synthesis of Valencene-13-aldehyde

To a stirred solution of Valencene (1.00g, 4.89mmol, 1.00equivalent) dissolved in freshly distilled pyridine (10mL) at room temperature was added SeO_2 (1.00g, 9.01mmol, 1.84eq).

The resultant yellow mixture was refluxed for 5hr until it turned black. The mixture was then filtered to remove the selenium dust and passed through a funnel packed with a mixture of 1:1 silica gel:Na_2CO_3 (w/w) washing with diethyl ether. The filtrate was concentrated in vacuum to remove the diethyl ether and pyridine was removed by vacuum distillation. The resulting oil was chromatographed (silica gel-Na_2CO_3 1:1 mixture, elution with hexane to give the title compound (0.125g, 0.572mmol, 12% yield) as a yellow oil. R_f = 0.2 in Hexane (100%).

3.6.6 Synthesis of valencene-13-ol

To a solution of valencene-13-aldehyde (0.100g, 0.458mmol) dissolved in ethanol (5mL) and stirred at room temperature was added with stirring a solution of $NaBH_4$ (0.04g, 1mmol) dissolved in ethanol (4mL). Stirring was continued for 2hrs and then the reaction mixture was diluted with water (5mL). The mixture was made slightly acidic to destroy excess hydride by addition of a few drops of dilute HCl solution. The ethanol was evaporated and the product was extracted with

ether (3X5mL). The combined organic layer was dried (Na_2SO_4), filtered and concentrated in vacuum to give a residue that was purified by silica column eluting with Hex:EtOAc 8:2 v/v to the title product as pale yellow oil (0.081g, 0.37mmol, 81%yield).

3.6.7 Synthesis of nootkatone-11, 12-epoxide

To an ice bath cooled solution of Nootkatone (4.00g, 18.32, 1.00eq) in diethyl ether (30mL) was added with stirring m-chloroperoxybenzoic acid (m-CPBA, 3.79g, 80% weight, 18.32mmol, 1.00eg). After 2 hours, an excess of 1.00eq of m-CPBA was added to the reaction mixture in portions. The mixture was stirred at room temperature for two additional hrs and then cold deionized water (30mL) was added followed by saturated aqueous solution of $NaHCO_3$ (30mL) to quench the reaction. The resultant mixture was transferred into a separatory funnel and the organic layer was separated. The aqueous layer was extracted with diethyl ether (2x30mL). The combined organic layer was dried (anhydrous Na_2SO_4), filtered and concentrated in vacuum to yield 2.50g of a pale yellow residue that eventually crystallized (R_f=0.24 in 50:50 Hex:ether (v/v)). NMR analysis of the crude product showed a purity of at least 90%. Efforts to purify the crude product yielded a mixture of ring-opened products, probably the diols. A few crystals of the pure product were obtained by passing a small quantity of the crude epoxide

through a small column packed with Na_2CO_3-silica gel (70:30w/w) yielding white crystals that melted at 35.2-35.7 °C (uncorrected).

3.6.8 Synthesis of Nootkatone-1, 10-epoxide

To a cooled (10°C) solution of nootkatone (5.00g, 22.29mmol, 1.00eq) in methanol (30mL) while stirring was added H_2O_2 (4.67g, 30% by weight, 133.74mmol, 15.57mL, 6.00eq) followed by 6.0N KOH (10mL) drop wise over a period of 20 min. The resultant mixture was then stirred at room temperature for 3hr. The methanol was then removed by vacuum and then the product was extracted from the aqueous layer by diethyl ether (3x20mL). The combined organic layer was dried (Na_2SO_4), filtered, and concentrated in vacuum to yield the title compound (2.97g, 12.3mmol, 55% yield based on the purity of 97%) as colorless oil (R_f=0.44 in Hex-Acetone (90:10)). NMR analysis showed high purity (>97%) of the title compound.

3.6.9 Synthesis of nootkatone-1,10-11,12-diepoxide

To a cold solution of nootkatone-11, 12-epoxide (1.70g, 7.26mmol, 1.00eq) in anhydrous methanol (50mL) was added H_2O_2 (1.48g, 30% by weight, 43.56mmol, 4.94mL, 6.00eq) followed by 6.0N KOH (2.5mL) over a period of 10 min and the resultant mixture was stirred for 3hr. The reaction mixture was then quenched with cold deionized water (30mL), and methanol was eliminated in vacuum. The product was extracted from the aqueous solution by diethyl ether (3x20mL). The combined ethereal layer was dried (Na_2SO_4), filtered, and concentrated in vacuum to yield the title compound (0.735g, 40.5% yield) as a semisolid white residue (R_f=0.50 in Hex-Acetone (50:50)). NMR showed a mixture of diastereomers with high purity.

3.6.10 Synthesis of nootkatin acetate

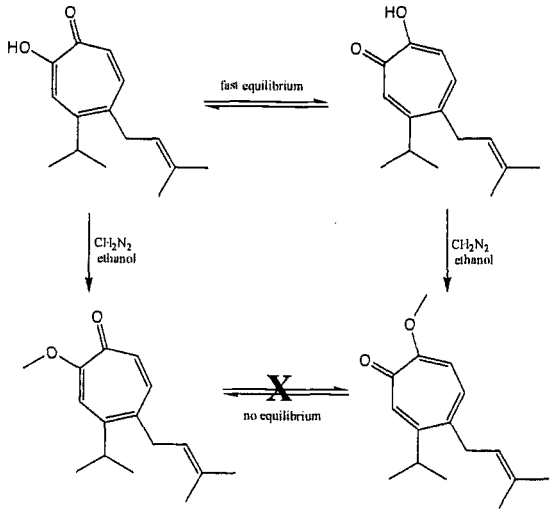
Nootkatin (0.500g, 0.0021 mol) was dissolved in excess acetic anhydride (10mL) and pyridine (0.4977g, 0.0063 mol) was added to the reaction mixture with continuous stirring at room temperature. After 12 hr, the reaction was stopped and water (30mL) was added followed by CH_2Cl_2 (30mL) to break up the excess acetic anhydride. The mixture was transferred to a separatory funnel and the organic layer separated. The aqueous layer was washed with CH_2Cl_2 (2X25mL) and the combined organic layer was washed with 10% acetic acid solution (25mL),

saturated $NaHCO_3$ (50mL), brine (50mL), dried (anhydrous Na_2SO_4), filtered and concentrated in vacuum to give the title compound (0.489g, 0.00179mol, 85%).

3.6.11 Synthesis of nootkatin methyl ether

Methylation of nootkatin was achieved by the reaction of nootkatin and freshly generated diazomethane according to the following procedure. The reaction set-up for diazomethane generation is similar to a simple distillation apparatus equipped with an addition funnel attached to a Claisen adapter. Glassware should be of special type with no rough surfaces and joints as these are known to initiate diazomethane explosion. In the generation vessel, a mixture of KOH (5g) dissolved in water (8mL) and 95% ethanol (10 mL) was warmed to 65°C. Diazlad® (N-methyl-N-nitroso-*p*-toluenesulfonamid) (5g, 0.0233mol) solution dissolved in ether (45mL) and placed in the separatory funnel was added slowly to the generation mixture over a period of 20 min. The side arm of the of the vacuum adapter is equipped with ether (2mL) trap and cooled with an NaCl-ice bath (a mixture of 33g NaCl and 100g ice gives a temperature of –21.3°C). A solution of nootkatin (0.5g, 0.0022 mol) dissolved in ether (5mL), cooled with dry ice and stirred was placed in the receiver flask where reaction took place with the generated diazomethane. The separatory funnel was rinsed with ether (10mL) and the warming underneath the generation flask was continued until the distillate was colorless. Stirring in the reaction flask was continued for additional 45 min after

the end of the distillation. The reaction mixture was concentrated in vacuum to give colorless oil as the methylated nootkatin product (0.49g, 0.00199mol, 92% yield).



3.7 List of synthesized derivatives with data

It should be mentioned that because the synthesized compounds' structures were expected based on starting materials and the known reaction mechanisms, only the most necessary data were collected for these compounds.

Valencene-13-aldehyde: yellow oil; (R_f=0.2, Hexane 100%); HRCIMS $[M]^+$ 218.16711 calculated for $C_{15}H_{22}O$, requires 218.16706. Selected ^1H NMR spectral data ($CDCl_3$, 400 MHz) δ 9.45 (1H, s, H-13), 6.20 (1H, s, H-12 cis to CHO), 5.85

(1H, s, H-12 trans to CHO), 5.25 (1H, brs, H-1). ^{13}C-NMR data ($CDCl_3$, 100MHz) δ 120.9 (C-1), 26.3 (C-2), 27.5 (C-3), 41.3 (C-4), 38.4 (C-5), 45.3 (C-6), 31.8 (C-7), 32.9 (C-8), 33.5 (C-9), 142.9 (C-10), 155.6 (C-11), 133.2 (C-12), 195.0 (C-13), 16.0 (C-14), 18.6 (C-15).

Valencene-13-ol: pale yellow oil. $[\alpha]_D^{20}$ + 59 (c 0.85, $CHCl_3$); Other data are exactly the same as those listed for the natural product (section 3.4).

Nootkatone-1,10-epoxide: colorless oil; (R_f=0.44, Hex:Acetone 90:10 v/v); HRCIMS $[M]^+$ 234.16209 calculated for $C_{15}H_{22}O_2$, requires 234.16198. Selected ^{1}H-NMR data ($CDCl_3$, 400 MHz) δ 3.01 (1H, s, H-1), 2.36 (1H, dd, *J*=19.7, 6.6 Hz, H-3), 1.89 (dd, *J*=19.7, 11.5 Hz, H-3), 4.71 (2H, m, H-12). ^{13}C-NMR data ($CDCl_3$, 100MHz) δ 63.1 (C-1), 207.0 (C-2), 42.4 (C-3), 40.5 (C-4), 37.0 (C-5), 41.1 (C-6), 32.8 (C-7), 29.5 (C-8), 30.7 (C-9), 69.2 (C-10), 149.6 (C-11), 109.7 (C-12), 21.1 (C-13), 15.5 (C-14), 14.9 (C-15).

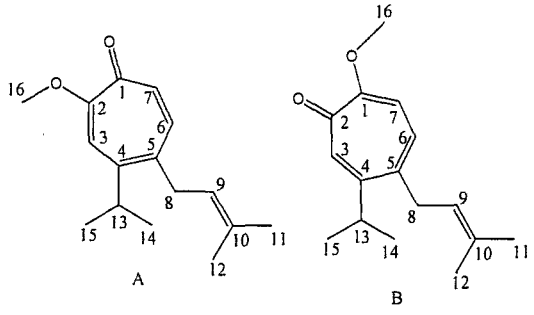
Nootkatone-11,12-epoxide: white crystals; m.p. 35.2-35.7 °C (uncorrected); (R_f=0.24, Hex:ether 50:50 v/v); HRCIMS $[M]^+$ 234.16180 calculated for $C_{15}H_{22}O_2$, requires 234.16198. ^{1}H-NMR data ($CDCl_3$, 400 MHz) δ 5.75 (1H, brs, H-1), 2.60 (2H, dd, *J*=5.0, 5.3 Hz, H-12), 1.22 (3H, s, H-13). ^{13}C-NMR data

($CDCl_3$, 100MHz) showed an approximately 2:1 mixture of two inseparable diastereomers δ 125.3 (C-1), 199.8 (C-2), 41.1 (C-3), 42.4 (C-4), 39.4 (C-5), 40.9 (C-6), 39.8 (C-7), 32.8 (C-8), 28.8 (C-9), 170.3 (C-10), 54.0 (C-11), 59.5 (C-12), 18.5 (C-13), 17.2 (C-14), 15.3 (C-15).

Nootkatone-1,10-11,12-diepoxide: semisolid residue; (R_f=0.50, Hex:Acetone 50:50 v/v); HRCIMS $[M]^+$ 250.15670 calculated for $C_{15}H_{22}O_3$, requires 250.15689. Distinguishable from the starting material, ^{1}H-NMR data ($CDCl_3$, 400 MHz) δ 3.08 (1H, s, H-1), 1.28 (3H, s, H-13), 0.93 (3H, s, H-14), 0.78 (3H, d, J=6.8 Hz, H-15). ^{13}C-NMR data ($CDCl_3$, 100MHz) δ 63.0 (C-1), 207.3 (C-2), 42.3 (C-3), 39.9 (C-4), 36.8 (C-5), 38.0 (C-6), 32.8 (C-7), 30.1 (C-8), 26.7 (C-9), 69.2 (C-10), 54.3 (C-11), 60.0 (C-12), 18.3 (C-13), 15.3 (C-14), 14.8 (C-15).

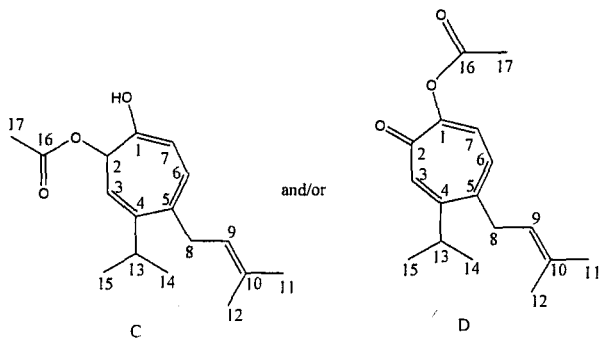
Methyl nootkatin: colorless liquid; (R_f=0.75, Hex:EtOAc 50:50 v/v); HRCIMS $[M]^+$ 246.16210 calculated for $C_{16}H_{22}O_2$, requires 246.16198. ^{1}H and ^{13}C-NMR data showed a 1:1 mixture of regioisomers. ^{1}H-NMR data ($CDCl_3$, 400 MHz) δ 6.65 (0.5H, H-3, s, (in A)) and 7.13 (0.5H, H-3, s, (in B)), 6.88 (0.5H, H-6, d, *J*=11.8 Hz (in A)) and 6.83 (0.5H, H-6 (in B), d, *J*=11.8 Hz), 6.51 (0.5H, H-7, d, *J*=11.8 Hz (in A)), 7.02 (0.5H, H-7, d, *J*=11.8 Hz (in B)), 4.98 (1H, m, H-9 in both structures A and B), 3.97 and 3.74 (3H total, H-16, both s), 3.18 (2H, d, *J*=6.3 Hz, H-8 in both structures A and B), 3.16 and 2.98 (1H total, septet, *J*=6.8 Hz, H-13 in both structures), 1.55 and 1.53 (3H total, two s, H-11 and H-12 in one

regioisomer), 1.56 (3H, s, H-11 and H-12 for the other isomer), 1.09 and 1.05 (3H each, d, J=6.8 Hz, H-14 and H-15 in both structures). ^{13}C-NMR data ($CDCl_3$, 100MHz) δ (data for one regioisomer only is given here) 180.1 (C=O), 163.2 (C-OH), 158.6 (C, C-4), 150.4 (C, C-5), 142.1 (CH, C-6), 135.1 (C, C-10), 132.2 (CH, C-7), 122.5 (CH, C-9), 113.7 (CH, C-3), 56.3 (OCH_3, C-16), 36.0 (CH_2, C-8), 32.7 (CH_2, C-13), 26.0 and 18.3 ($2XCH_3$, C11-, C-12, 24.0 (CH_3), 23.4 ($2XCH_3$, C-14, C-15).



Nootkatin acetate: white amorphous solid; (R_f=0.55, Hex:EtOAc 50:50 v/v); HRCIMS $[M]^+$ 274.15701 calculated for $C_{17}H_{22}O_3$, requires 274.15689. ^{1}H-NMR data ($CDCl_3$, 400 MHz) δ 7.23 (1H, s, H-3), 7.09 (2H, two d, *J*=4.2 Hz H-6 and H-7), 5.08 (1H, tq, *J*=6.7, 1.4 Hz, H-9), 3.34 (2H, d, *J*=6.7 Hz, H-8), 3.22 (1H, Septet, *J*=6.8 Hz, H-13), 2.36 (3H, s, H-17), 1.75 and 1.74 (3H each, two s, H-11 and H-12), 1.22 (6H, d, *J*=6.8 Hz, H-14 and H-15). ^{13}C-NMR data ($CDCl_3$, 100MHz) δ 172.5 (C, C=O, inside the ring), 168.7 (C=O, acetate), 167.9 (C, C-

OH), 157.4 (C, C-4), 141.5 (CH, C-6), 140.7 (C, C-5), 134.5 (C, C-10), 125.5 (CH, C-7), 122.6 (CH, C-9), 121.6 (CH, C-3), 36.4(CH_2, C-8), 32.7 (CH, C-13), 26.0 and 18.4 (2XCH_3, C-11, C-12), 23.6 and 23.2 (CH_3, C-14 and C-15), 21.1 (CH_3, C-17).



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3.8 Bioassays

Bioassays were done by Marc Dolan, Senior Research Biologist, Division of Vector-Borne Infectious Diseases (DVBID), Lyme Disease Vector Section and Nick Panella, Research Biologist, DVBID, Epidemiology and Ecology Section, CDC, Ft. Collins, CO.

3.8.1 Tick Colonies

Nymphal *I. Scapularis* ticks (8-12 weeks old) were used in all trials and were obtained from the F1 offspring of adult *I. Scapularis* ticks collected in Bridgeport, CT. There was no known pesticide used in this location. Ticks were maintained at 21°C, 90% RH, and recived a 16:8 h (light:dark) cycle as described in reference [90].

3.8.2 Flea Colonies

Adult *X. cheopis* fleas (1-3 wk old) were obtained from a colony founded more than a decade ago using adults received from Tom Schwan, Rocky Mountain Laboratories, Hamilton, MT. The area from which these original adult fleas were obtained, and the resulting colony, has no known history of pesticide exposure. Colonies were maintained in glass jars containing a 4:1:1:1 ratio of sawdust, dried beef blood, powdered milk, and powdered mouse chow and were held at 23°C, 85% RH and received a 24 h dark cycle.

3.8.3 Mosquito Colonies

Aedes aegypti adult mosquitoes were obtained from an existing colony at the Centers for Disease Control and Prevention (CDC), Division of Vector-Borne infectious Diseases (DVBID), Fort Collins, CO. This colony has been maintained for over fifteen years with no known history of exposure to pesticides. Mosquitoes were reared at 28°C, 85% RH, and a 14:10 h (light:dark) cycle. Larvae were grown in de-ionized water and fed ground liver powder solution *ad libitum*. Four instars were removed and placed in emergence cages, and adults were fed a 2% sucrose solution until assayed. Adults were exposed to test products at 4-7 d after emergence.

3.9 Bioassay methods

3.9.1 Tick and Flea Bioassays

Concentrations of the compounds to be assayed were prepared by 2-fold serial dilutions of a 0.5% (w/v) stock solution of the corresponding compounds in acetone. The approximate toxicity of individual compounds was determined with a total of 8 doses ranging from 0.002% to 0.25%. All tested compounds were run in duplicates with more active compounds replicated once. A control treated with

blank (acetone only) was run with each series. Tick and flea susceptibility was evaluated using a modified disposable pipette method as outlined in reference [91].

Groups of 10 nymphal *I. Scapularis* and *X. cheopis* fleas were used in all tests, resulting in a total of 5,240 nymphs and 4, 860 fleas exposed to the compounds tested in this study. The inner surfaces of 2-dram friction cap vials were treated with an acetone solution of known concentration of the compound to be tested (or acetone for the blank solution) and left to air-dry overnight. Three to four holes were made in the plastic cap to allow for air exchange. Nymphal ticks and adult fleas were then placed directly into the vials using forceps. Vials containing ticks or fleas were placed in desiccators for 24 h at 21°C and 90% RH. Morbidity and mortality was recorded at 1, 2, 4, 8, and 24 h after initial exposure. After 24 h, ticks were considered alive if they exhibited normal behavior when breathed upon or physically stimulated with wooden dowels. For each time point, if ticks were incapable of movement, maintaining normal posture, leg coordination, ability to right themselves, or any other sign of life, they were considered moribund or dead. Results from tests where more than 10% of the control population died were discarded and repeated.

Efficacies of individual compounds were determined by calculating lethal concentration 50% (LC_{50}) and/or 90% (LC_{90}) w:v by probit analysis using the LdP line software (copyright 2000 by Ehab Mostafa Bakr), available via the Internet.

3.9.2 Mosquito Bioassay

Adult mosquitoes were tested using the bottle bioassay method as outlined in reference [92] with minor modifications. Compounds to be tested were two-fold serially diluted for a total of 8 concentrations ranging from 0.002% to 0.25% in 1.5mL of acetone. Individual dilutions were added to 250 mL Wheaton glass bottles and capped. Bottles were manipulated to evenly coat all inner surfaces. The caps were removed and bottles were allowed to air-dry overnight. Once bottles were completely dry, 10-50 adult mosquitoes were aspirated into the bottles. Mosquitoes were held in the bottles for 24h at 23°C and 85% RH. Morbidity and mortality were recorded at 15, 30, 45 and 60 min and 24 h intervals. One bottle for each replicate was treated with acetone only and served as a control. The dose-mortality data was evaluated with probit analysis, using the LdP line software, to determine LC_{50} and LC_{90} values.

3.9.3 Residual Activity Experiments

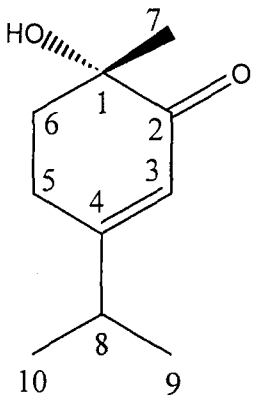
To determine the persistence of pesticidal activity of the compounds, 2-dram friction cap vials and 250 mL Wheaton glass bottles were treated using the same series of dilutions as described above. Treated vials and bottles, minus vector test species, were held at 21°C with the ability of air exchange to take place (3-4

holes in the lids of 2-dram vials and lids on Wheaton bottles loosely applied). On day 7 after treatment, 2-dram vials were loaded with 10 ticks or 10 fleas and Wheaton bottles with 10-50 mosquitoes and held as previously described. Morbidity and mortality data for each test subject were recorded using the same time points. Any compounds that displayed acaricidal /insecticidal activity were retested at 2 and 4 wk after treatment. Dose mortality data were evaluated using probit analysis via the LdP line software.

4. Results and Discussion

4.1 Structures of newly isolated compounds from AYC heartwood

4.1.1 Unknown 1: (1S)-2-Oxo-3-p-menthenol- (**44**)



44

This unknown was isolated from the AYC extract as a colorless liquid. The molecular formula was determined on the basis of HRCIMS ion peak at 168.1143 to be $C_{10}H_{16}O_2$ (required for this formula is 168.1150). The EIMS showed a molecular ion peak at m/z 168 [M^+] in addition to peaks at 153 [M^+-Me], 151 [M^+-H_2O], 125 [[M^+-CH_3CHCH_3] as the main fragmentation pattern. The IR spectrum showed absorption bands ascribable to hydroxyl (3450 cm^{-1}), carbonyl (1715cm^{-1}) and alkene (1620 cm^{-1}) groups.

The ^{1}H-NMR spectrum ($CDCl_3$, 400MHz) indicated the presence of three methyl groups: δ_H 1.16 (3H, d, J=6.8 Hz, H-9 or H-10), 1.14 (3H, d, J=6.8Hz, H-10 or H-9), 1.33 (3H, s, H-7), two methylene groups: 1.98-2.05 (1H, m, H-6-equatorial) and 2.16 (1H, dt, J=12.9, 3.02Hz, H-6-axial), 2.46 (2H, m, H-5), one sp^3 methine: 2.48 (1H, m, H-8), one sp^2 methine 5.93 (1H, J=1.7Hz, H-3) and 3.77 (1H, bs, O- H). The ^{13}C-NMR ($CDCl_3$, 100MHz) spectrum showed ten signals as follows: δ_C (multiplicity from DEPT spectra) 21.1, 21.4 (CH_3, C-9 and C-10), 24.6 (CH_3, C-7), 27.3 (CH_2, C-5), 36.0 (CH, C-8), 36.1 (CH_2, C-6), 73.1 (C, C-1), 120.5 (CH, C-3), 172.8 (C, C-4), and 203.3 (C, C-2).

From the molecular formula of $C_{10}H_{16}O_2$ it follows that there are three degrees of unsaturation in this compound and from the NMR data (both ^{1}H and ^{13}C), there is one C-C double bond. The IR spectrum showed a strong absorption band at 1717cm^{-1} and 3450cm^{-1} characteristic of carbonyl and hydroxyl groups, respectively. To make up for the third degree of unsaturation, there must be a ring in the structure of this molecule. The presence of two closely similar methyl groups that exhibit doublet signals suggests that there is an isopropyl group. The third methyl group being a singlet and shifted downfield suggests that it is attached to the ring where a hydroxyl group is also attached. Therefore, it can be deduced that the ring is six membered based on simple atom count.

The carbon atom signal at 172.8 ppm is generally of higher value than expected for alkenes, but a closer look at the literature reveals that high values like what we have here are observed for α,β-unsaturated carbonyl compounds. Based

Table 3: NMR data for unknown 1 (**44**) in $CDCl_3$ (400MHz for ^1H and 100MHz for ^{13}C)

Position	^1H (Integration, multiplicity, *J* in Hz)	^{13}C	HSQC[a]	HMBC[b]
1	_	73.1	C-1	_
2	_	203.3	C-2	_
3	5.93 (1H, q, *J*=1.3)	120.5	C-3	C-5, C-8, C-1
4	_	172.8	C-4	_
5	2.46 (2H, m)	27.3	C-5	C-8, C-6, C-1, C-3, C-4
6	2.16 (1H, dt, *J*=12.9, 3.02); 1.98-2.05 (1H, ddd, *J*=12.0, 8.0, 8.0)	36.1	C-6	C-2, C-7, C-1, C-4
7	1.33 (3H, s)	24.6	C-7	C-2, C-6, C-1
8	2.48 (1H, m)	36.0	C-8	C-9, C-10, C-3, C-4
9[c]	1.16 (3H, d, *J*=6.8)	21.1	C-9	C-10, C-8, C-4
10[c]	1.14 (3H, d, *J*=6.8)	21.4	C-10	C-9, C-8, C-4

[a] Proton showing one-bond correlation to the indicated carbon with the exception of carbons C-1, C-2 and C-4, which as expected show no correlation to any H atoms. [b] Proton showing long-range (mainly 2- and 3-bond) correlation to indicated carbon(s). [c] Protons and carbons assignments are interchangeable.

on that and the presence of a ketone functionality (from IR and the ^{13}C-NMR signal at 203ppm), it can be concluded that there is an α,β-unsaturated ketone arrangement in this molecule. Finally, the isopropyl group is deduced to be connected at the β-position of the double bond based on the position of the ^{1}H NMR signal of 5.93ppm, because one would expect that this value should be around 7.1ppm if the connection was through the α–position with the H on the β–position. Confirmation of the isopropyl groups attached to C-4 is seen in the HMBC, which shows connectivity of H-8 to C-3 and C-5.

Further confirmation of structure **41** was verified by 2-D NMR spectra including HSQC (figure 2), HMBC (figure 3) and COSY (figure 4). These results reveal that the structure follows the menthane-type skeleton pattern frequently observed in plant species. The downfield-shifted methyl group (at δ_H1.33) is located at C-1 as evidenced by the HMBC correlations of its protons with C-2, C-6 and C-1 (203.3, 36.1 and 73.1ppm, respectively). The absence of correlations between the vinylic proton and the carbonyl carbon from one side and that between the proton at δ_H 2.48 with the same carbon support the earlier conclusion that the isopropyl group is located at the β–carbon. Table 3 summarizes HMBC correlations observed for this molecule the most important of which are shown graphically in scheme 5. Key COSY correlation of H-5 with H-6 and H-8 with H-9 and H-10 add further support for structure **44**.

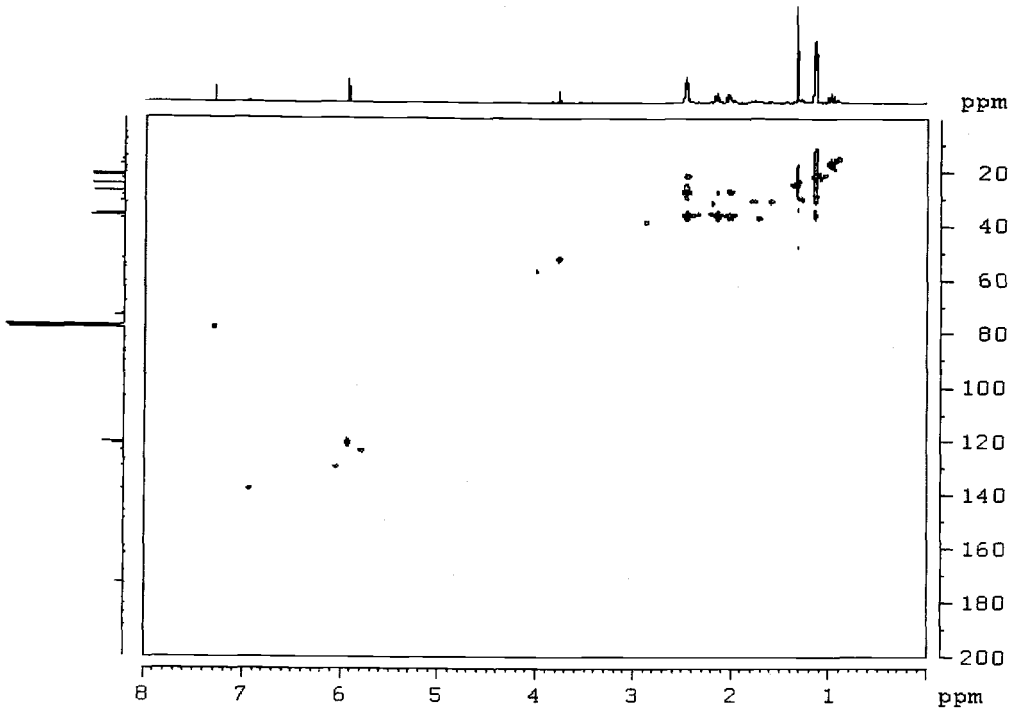


Figure 2: HSQC spectrum for unknown 1 (**44**).

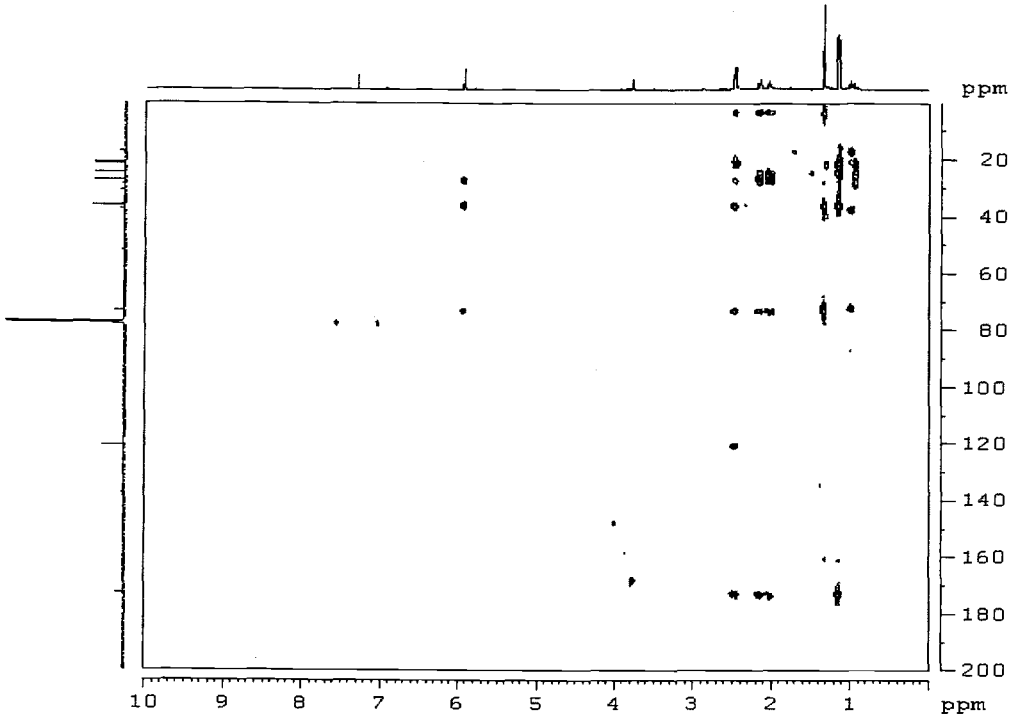


Figure 3: HMBC spectrum for unknown 1 (**44**).

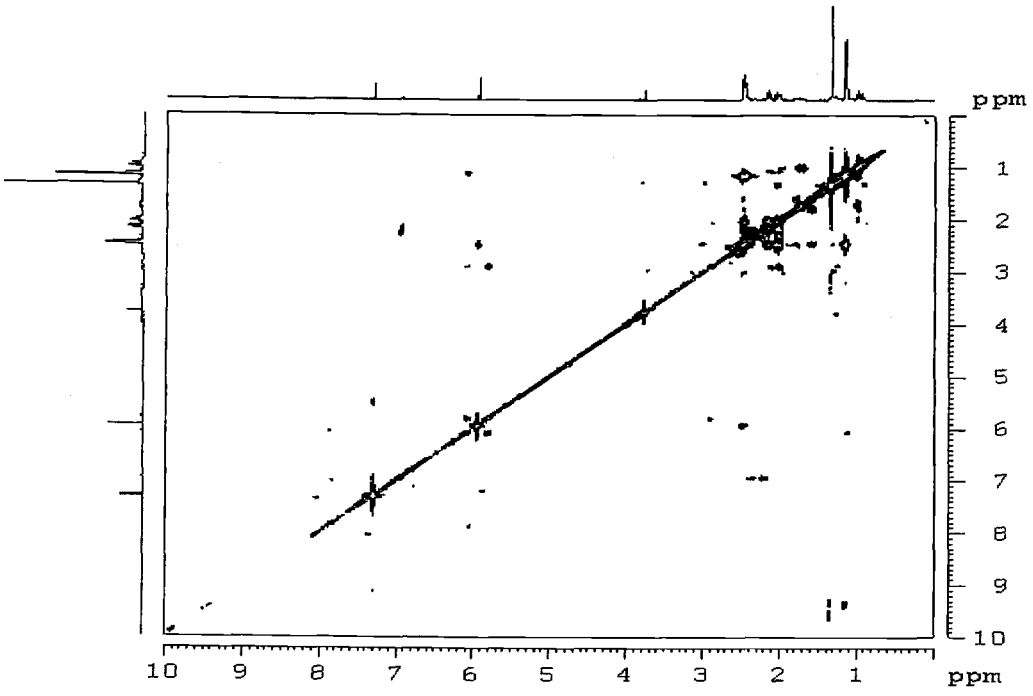
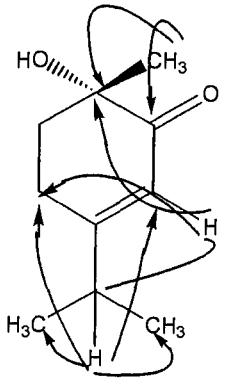


Figure 4: COSY spectrum for unknown 1 (**44**).

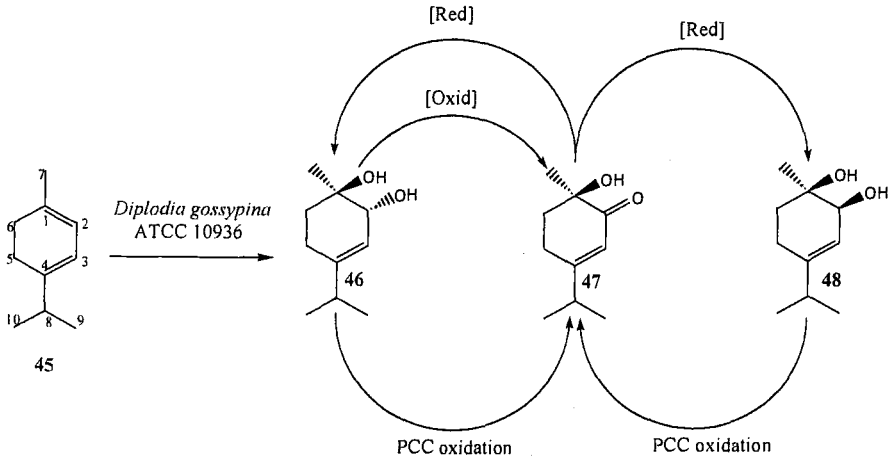


Scheme 5: The most important HMBC correlations observed for compound **44**.

The absolute stereochemistry around C-1 of this unknown is extremely difficult to establish because it is a tertiary alcohol. This is opposed to the many methods available for the identification of absolute configuration of secondary alcohols. The Mosher method and its modifications are extremely successful in the assignment of absolute configuration of secondary alcohols (for example, see [93-95]. One of the reasons for the difficulty encountered in the application of the Mosher method for tertiary alcohols is that it is difficult to predict the preferred conformer of the α–methoxy-α-(trifluoromethyl)-phenylacetyl (MTPA) moiety in the MTPA ester of tertiary alcohols [96a, b, c].

However, **44** is related to a compound previously described in the literature in which the microbial transformation of α–terpinene (**45**) by the fungus *Diplodia gossypina* ATCC 10936 was found to give three products as shown in scheme 5 [97]. The absolute configuration of the C-2 alcohol of product **46** in scheme 6, being a secondary alcohol, was determined by a partial resolution method [98]. It was observed by Abraham et al.[97] and others (for example, see [99]) that the dihydroxylations by *Diplodia gossypina* ATCC 10936 usually proceed via an epoxide intermediate (which was isolated in some cases) and cleave enzymatically in a *trans*-1,2 fashion to form the corresponding *trans*-1,2-diols. Based on this notion, the absolute configuration around C-1 was indirectly deduced to be "R". Compound **47** was isolated from the fermentation medium as a presumably intermediate for the formation of the "unusual" *cis*-1, 2-diol (compound **48**). Both compounds **45** and **48** gave compound **47** on PCC oxidation. Optical rotation data

obtained for **44** suggest that it is the enantiomer of compound **47** (**44** gave $[\alpha]_D$ - 85° (c 0.455, $CHCl_3$), while compound **47** gave $[\alpha]_D$ + 92.9 (c 1.00, $CHCl_3$, [97]). These two compounds have essentially the same ^1H and ^{13}C NMR spectra (see table 4 and compare to table 3).

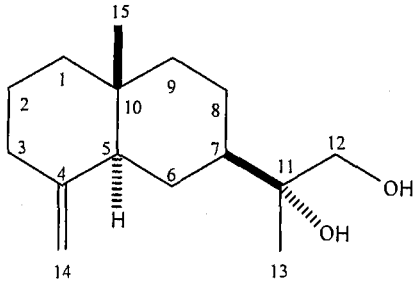


Scheme 6: Products of the microbial fermentation of **45** and chemical transformations on them [97].

Table 4: NMR data for **47** in $CDCl_3$. Data obtained from reference [97].

Position	^{1}H (Integration, multiplicity [a], J in Hz)	^{13}C
1	_	72.6
2	_	202.8
3	5.89 (1H, ddd, *J*=1, 1, 1)	120.1
4	_	172.3
5	2.44 (2H, m)	26.8
6	2.13 (1H, ddd, *J*=12, 4, 4); 2.00 (1H, ddd *J*=12.0, 8.0, 8.0)	35.7
7	1.305 (3H, s)	24.1
8	2.44 (1H, m)	35.5
9	1.12 (3H, d, *J*=7.0)	20.9
10	1.12 (3H, d, *J*=7.0)	20.6

4.1.2 Unknown 2: (5S, 7R, 10R, 11R)-Eudesm-4(14)-ene-11,12-diol, **49**



49

Unknown 2 (**49**) was isolated from the AYC heartwood extract after repeated column chromatographic separations (see the experimental) as an amorphous solid. The molecular formula was determined as $C_{15}H_{26}O_2$ on the basis of HRMS ion peak at m/z 238.19377 (required for $C_{15}H_{26}O_2$ is 238.1933), and therefore possessed three degrees of unsaturation. LREIMS showed peaks at 238 (M^+), 220 (M^+-H_2O), 207 (M^+-CH_2OH), 202 (M^+-$2H_2O$) and 189 (M^+-CH_2OH-OH) as the main fragment peaks. IR spectroscopy indicated the presence of an alcoholic functional group absorption band at 3450 cm^{-1}.

The ^{1}H-NMR spectrum indicated the presence of an exo-methylene group [δ_H 4.76, 1H (dd, *J*=1.6, 1.6Hz), 4.49 (1H, dd, *J*=1.6, 1.6Hz)], one methylene attached to an oxygen [3.67 (1H, bd, *J*=10.8Hz), 3.46 (1H, bd, *J*=10.8Hz)], two methyl groups [0.78 (3H, s), 1.28 (3H, s)] along with other signals as summarized in table 5. Both ^{1}H and ^{13}C spectra (table 5) indicated the presence of one carbon-

carbon double bond. Thus, two rings were required to account for the remaining two degrees of unsaturation.

The ^{13}C-NMR spectrum indicated the presence of 15 carbon atoms supporting the earlier conclusion from HRMS. ^{13}C polarization techniques (DEPT 90 and 135) revealed the presence of two methyl (δ_C 24.0, 17.3), seven sp^3 methylene (δ_C 38.6, 21.6, 37.6, 23.7, 24.0, 42.8, 69.5), two sp^3 methine (δ_C 45.2, 38.7), two sp^3 quaternary (δ_C 35.7, 76.6), one sp^2 methylene (δ_C 105.8) and one sp^2 quaternary (δ_C 151.6) carbon atoms in this molecule. In addition, these data suggested the presence of two oxygenated sp^3 carbons (δ_C 76.6 (C) and 69.5 (CH_2)) indicating the presence of a quaternary and primary oxygenated moieties on these carbon atoms. These results along with results from EIMS and IR suggested that this unknown is in fact a diol.

The structural details of **49** were mainly deduced based on results of 2-D NMR experiments, including HSQC (figure 5), HMBC (figure 6) and COSY (figutre 7). A summary of the results of these experiments is listed in table 6.

Table 5: NMR data for unknown 2 (**49**) in $CDCl_3$ (400MHz for ^1H and 100MHz for ^{13}C).

Position	^1H (Integration, multiplicity [a], J in Hz)	^{13}C (type)
1	1.88 (1H, m); 1.31 (1H, m)	38.9 (CH_2)
2	1.70 (2H, ddd, J=5.7, 5.0, 5.0)	21.6 (CH_2)
3	2.33 (1H, md, J=12.2); 2.04 (1H, mq, J=12.2)	37.6 (CH_2)
4		151.6 (C)
5	2.16 (1H, dd, J=12.7, 3.5)	45.2 (CH)
6	1.63 (1H, m); 1.70 (1H, m with H-2 signal)	23.7 (CH_2)
7	1.52 (1H, m)	38.7 (CH)
8	1.78 (1H, dq, J=14.3, 3.1); 1.57 (1H, m)	24.0 (CH_2)
9	1.32 (1H, m); 1.46 (1H, m)	42.8 (CH_2)
10		35.7 (C)
11		76.6 (C)
12	3.67 (1H, brd, J=10.8, 3.5); 3.46 (1H, brd, J=10.8, 3.5)	69.5 (CH_2)
13	1.28 (3H, s)	24.0 (CH_3)
14	4.76 (1H, dd, J=1.6,1.6); 4.49 (1H, dd, J=1.6, 1.6)	105.8 (CH_2)
15	0.78 (3H, s)	17.3 (CH_3)

[a] m= multiplit, d=doublet, q= quartet, s= singlet, br= broad.

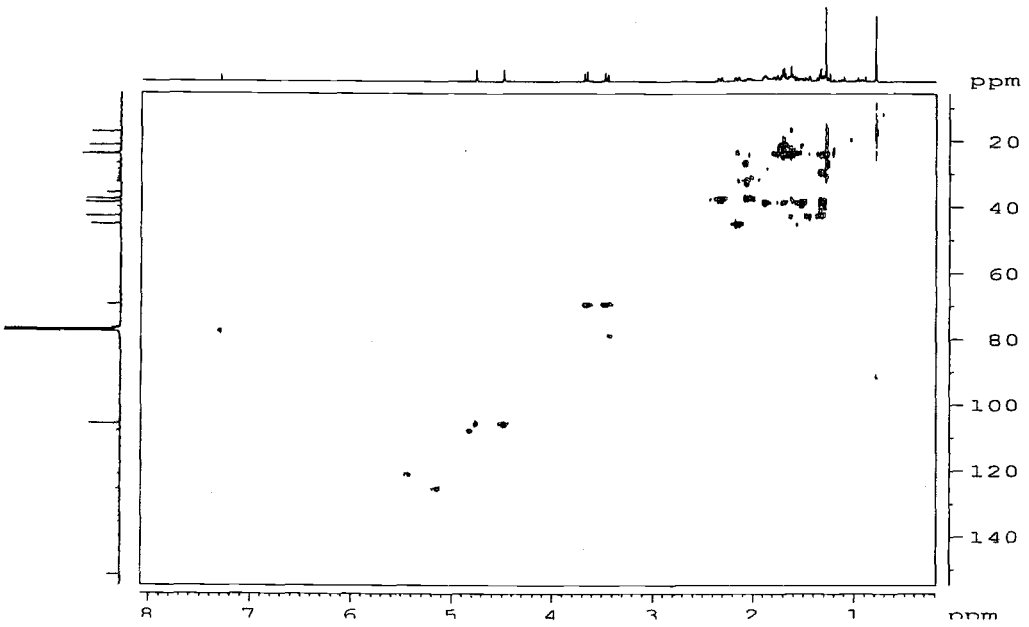


Figure 5: HSQC spectrum for unknown 2 (**49**).

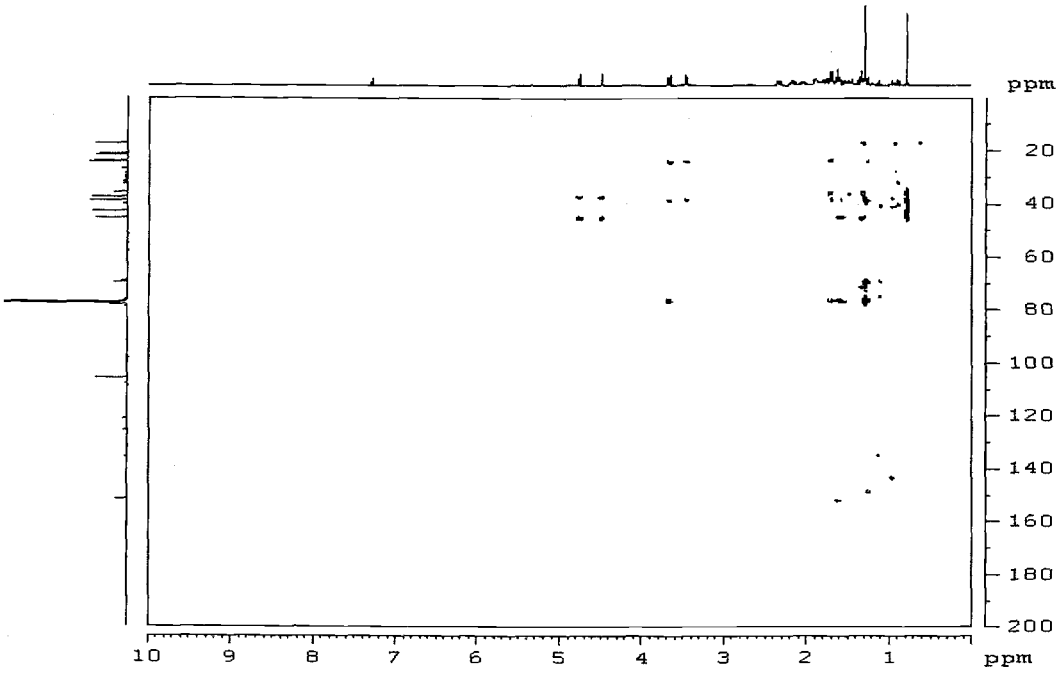


Figure 6: HMBC spectrum for unknown 2 (**49**).

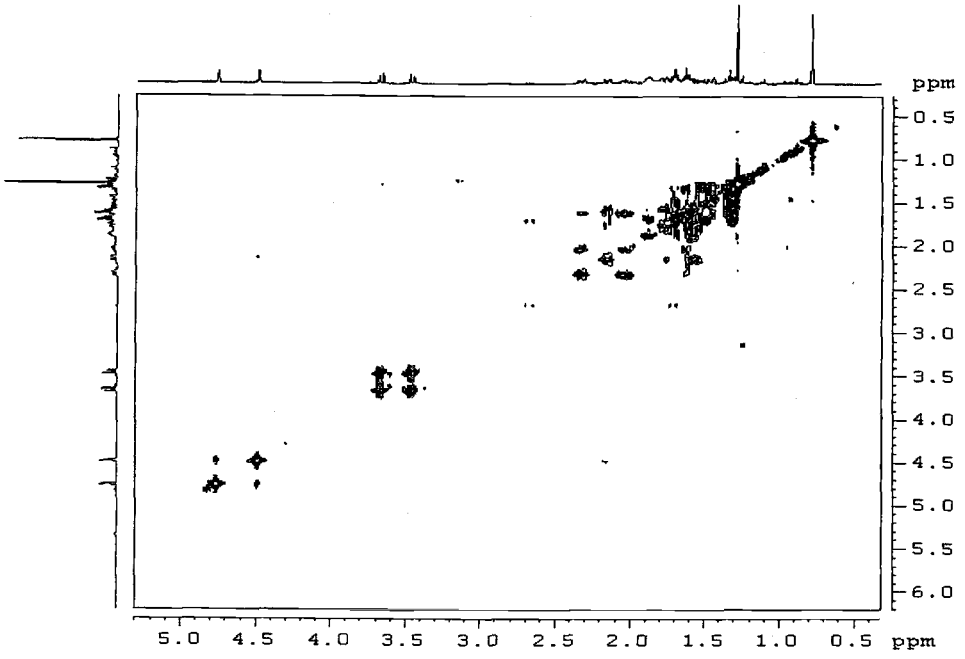


Figure 7: COSY spectrum for unknown 2 (**46**)

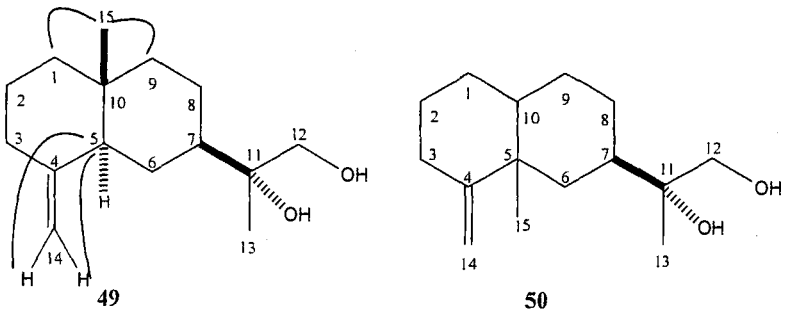
At first, examination of both the ^{1}H and ^{13}C-NMR data suggested that the structure could be related to those secondary metabolites previously reported for AYC, namely the eremophilane-type structure. However, a tremendous effort to come up with an eremophilane-type structure for this unknown that correctly accounts for all observed NMR data has failed. For example, structure **50** below (scheme 7) cannot explain the observed HMBC correlations between H-14 and the methane carbon at 45.2 ppm, which would be C-10 in **50** and C-5 in **49**. Similarly, H-15 observed correlations with C-1 and C-9 along with the absence of C-15 and H-6 correlation would be impossible to explain for structure **50**. On the hand, structure **49** successfully accounted for all HMBC results obtained for this compound. The diol subunit structure was evident from HMBC data of correlation

Table 6: 2-D NMR results for unknown 2 (**49**) in $CDCl_3$.

Position	HSQC[a]	HMBC[b]	COSY[c]
1	38.9	17.3, 35.7, 45.2	2
2	21.6	35.7, 38.9, 37.6	1, 2
3	37.6	151.6	2, 3
4	151.6[a]		
5	45.2	151.6	6
6	23.7	24.0, 35.7, 38.7, 76.6, 151.6, 45.2	5
7	38.7	76.6, 45.2	6
8	24.0	NR[d]	8, 7
9	42.8	17.3, 35.7, 45.2	9
10	35.7[a]		
11	76.6[a]		
12	69.5	76.6, 38.7, 24.0	12
13	24.0	76.6, 38.7, 69.5	
14	105.8	45.2, 37.6	14, 5, 3
15	17.3	35.7, 38.9, 45.2, 42.8	

[a] Proton showing one-bond correlation to the indicated carbon with the exception of carbons C4, C10 and C11, which as expected show no correlation to any H atoms. [b] Proton showing long-range (mainly 2 and 3 bond) correlation to indicated carbon(s). [c] Protons showing (vicinal) correlations to the indicated proton(s). [d] NR = not resolved under the experiment conditions.

between H-12 and H-13 with C-11. The connectivity of the diol subunit and the bicyclic system through C-7 was also evident from the H-7 correlation with C-11 and H-12, H-13 correlation with C-7.



Scheme 7: Two possible structures for unknown 2. The main HMBC correlations are shown on structure **49**.

Unknown 2 (**49**) belongs to a family of sesquiterpenes called the eudesmanes, which is a very large group of sesquiterpenes widely occurring in the plant kingdom. **49** has been previously isolated from the aerial parts of *Jasonia glutinosa* and named as kudtdiol [100] and it has not been reported for any other species to date. Therefore, the isolation of kudtdiol (**49**) from AYC is a new and important finding. This is in fact the first report to indicate the presence of a eudesmane-type skeleton in AYC. *Chamaecyparis lawsoniana* (Port Orford Cedar) on the other hand, has been shown to have eudesmane-type skeleton as the dominant sesquiterpenes and no eremophilane ring system has been reported

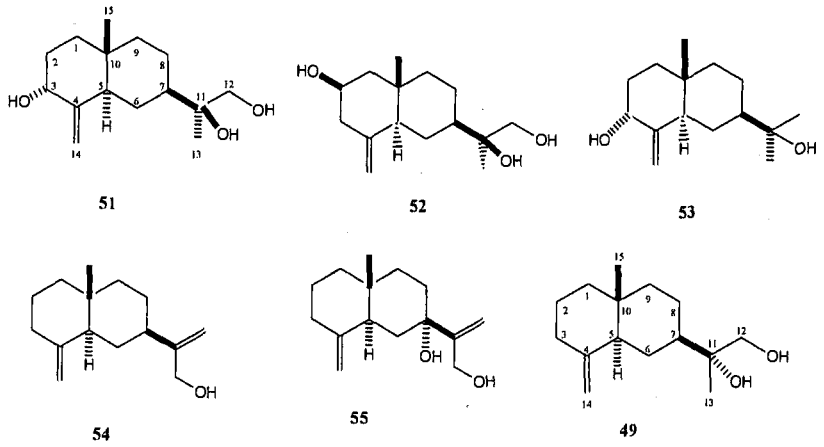
[101]. For this reason and other considerations, some botanists are now questioning whether AYC belongs in the genus *Chamaecyparis* [3].

It is worth mentioning that although this compound has been described in the literature before, there was no ^{13}C or two-dimensional NMR data reported anywhere in the literature for this compound. Furthermore, only the most obvious part of the ^{1}H NMR spectrum of kudtdiol has been documented with the structure based only on this ^{1}H NMR data as well as chemical transformations. Results of the present work do confirm this assignment of the structure of kudtdiol and also produce more comprehensive and dependable NMR data to be reported in the literature.

With the absence of ^{13}C NMR data for this compound in the literature, one approach is to compare data collected for kudtdiol isolated in this study from AYC to a compilation of ^{13}C data of a series of closely related compounds in the literature. For this purpose, five compounds (**51-55**) were chosen for direct comparison with ^{13}C data for kudtdiol isolated here (see table 7 and scheme 8). From data in this table, it follows that among the five model compounds, there is good agreement between the values reported for kudtdiol and those of the corresponding reference compound(s) when structural similarities between the two compounds (**49** and each of the reference compounds) do exist. For example, kudtdiol carbon atoms C1-C5, C9, C10, C14 and C15 chemical shifts are in good agreement with the corresponding carbons in compounds **54** and **55**, while C6-C9,

C11-C13 are generally in good agreement with the corresponding carbons of compounds **51** and **52** (and **53** except for C-12).

Acetylation of kudtdiol afforded the monoacetylation product evident from both ^{1}H and ^{13}C-NMR experiments of the product (see the Appendix). It should be noted that the 2-D NMR data of this monoacetylated product played a central role in the clarification of the ambiguity of some parts of the 2-D NMR data of kudtdiol itself. For example, in the ^{13}C-NMR spectrum of **46** there are two sets of carbon atoms occurring at very close chemical shifts (CH_2 at 24.02, CH_3 at 23.98 and CH_2 at 23.74 in one set and CH at 38.7 and CH_2 at 38.6 in another) and therefore it was much of a challenge to resolve all the correlations in the 2-D NMR spectra. These two sets are well resolved in the ^{13}C spectrum of the acetylated product, which made it much easier to draw more decisive conclusions about the structure. Figure 8 shows parts of the ^{13}C-NMR spectra of **49** and its acetylated product.



Scheme 8: Structures of five model compounds structurally related to **49**.

Table 7: ^{13}C NMR data for **49** and other related compounds (**51-55**).

Position	**51**[a]	**52**[a]	**53**[b]	**54**[b]	**55**[c]	**49**[d]
1	35.2	48.9	35.8	41.9	41.5	38.6
2	29.3	67.9	29.8	23.5	23.4	21.6
3	73.0	42.2	73.6	36.9	36.9	37.6
4	151.9	151.9	152.1	154.2	154.8	151.6
5	49.2	50.8	43.6	50.0	43.9	45.2
6	23.1	23.5	24.6	30.0	35.6	24.0
7	44.3	44.8	49.4	41.6	74.6	38.7
8	22.3	22.1	22.5	27.3	32.5	23.7
9	40.4	40.6	NR	41.2	35.6	42.8
10	35.4	35.6	35.8	36.1	36.1	35.7
11	74.4	74.7	73.0	NR	150.0	76.6
12	67.7	67.9	27.3[e]	107.0	110.9	69.5
13	19.1	20.0	27.0[e]	65.0	64.8	24.0
14	108.2	108.1	109.0	104.9	105.9	105.8
15	15.0	15.5	15.6	16.3	15.4	17.3

[a] Ref [102]. [b] Ref [103], [c] Ref [104]. [e] Assignment may be exchangeable. [d] This work. NR= not reported.

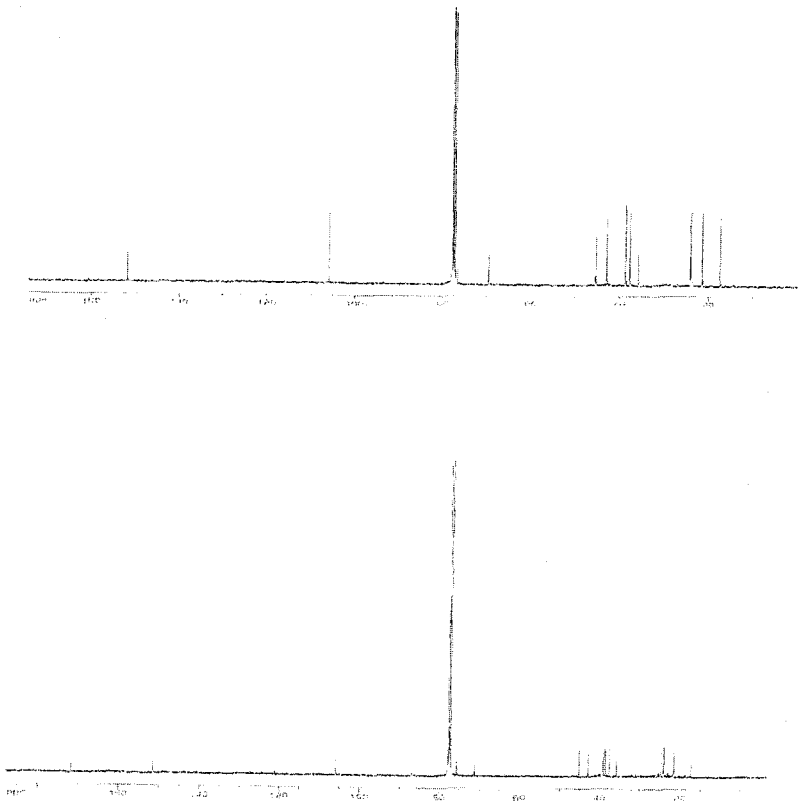
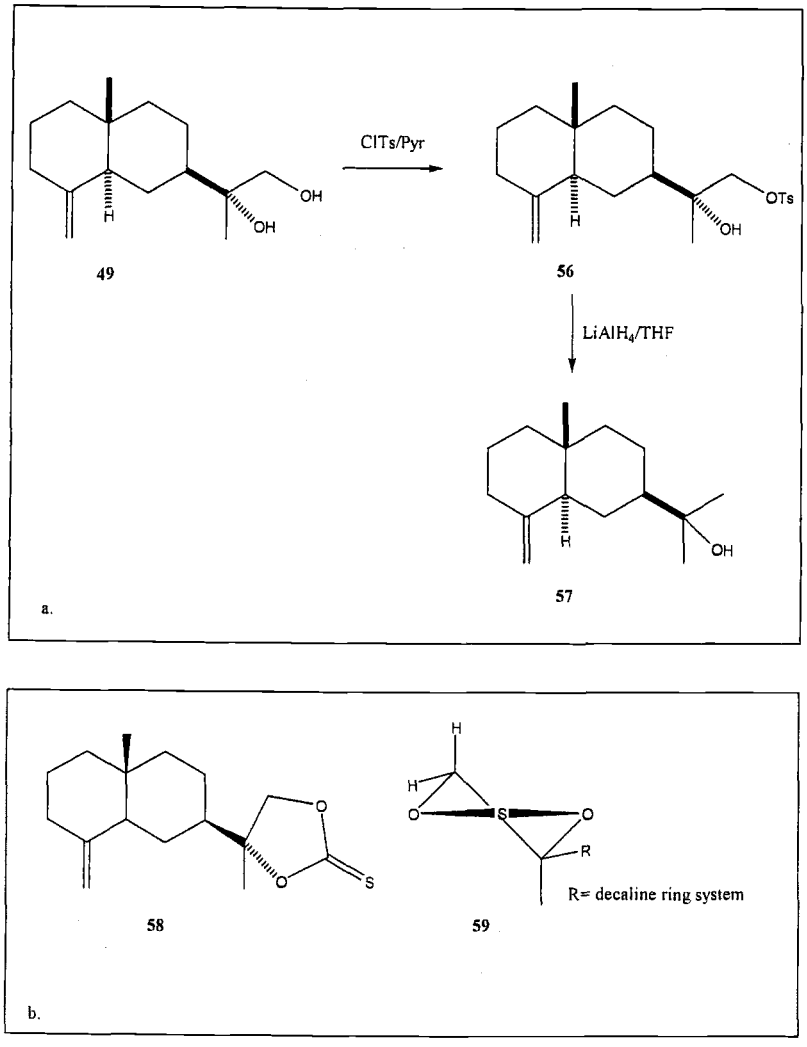


Figure 8: Part of the ^{13}C spectra of **46** (upper spectrum) and its monoacetylated product.

The value of the optical rotation for kudtdiol isolated in this work from AYC ($[\alpha]_D$ +66.8° (c 0.25, $CHCl_3$)) is in good agreement with the reported value for the same compound in the literature($[\alpha]_D$ +72.9° ($CHCl_3$)). The absolute stereochemistry around the bicyclic ring chirality centers (C-5, C-7, and C-10) of **49** was established by Teresa et al [100] by converting **49** into the previously known (+)-β-eudesmol (**57**) via **56** as outlined in scheme 8.a (Reference for the literature optical rotation value for **57** is [105]).

The absolute configuration at the C-11 was also established by the same authors by studying the Cotton effect (CE) of the thiocarbonate (**58**) derivative of kudtdiol [100]. It was found that the CE for the thiocarbonate derivative is negative (ΔE_{308}= -0.21, MeOH) suggesting that the thiocarbonate ring adopts the conformation shown below (**59**) and since the decaline system must be equatorial (sterically favored), the absolute configuration at C-11 was concluded to be "R" (scheme 9.b).

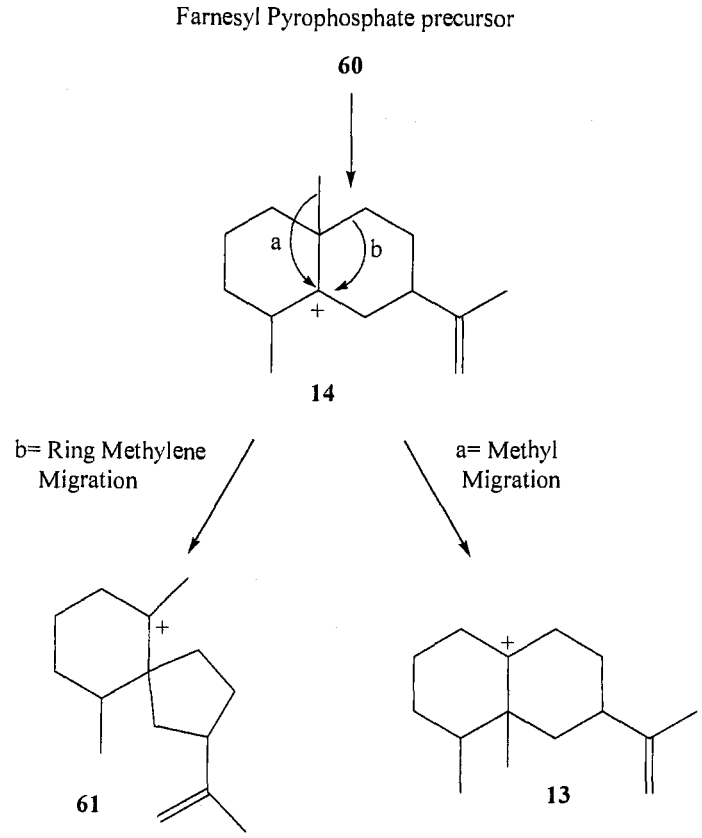
It should be mentioned that structure **49** was proposed for sesquibenhiddiol, a substance isolated from *Chamaecyparis formosensis* [106], but Teresa et al. expressed that physical constants are different for **49** and sesquibenhiddiol. Obviously, our data support that the correct structure for the compound isolated from AYC, which is identical to that isolated by Teresa et al. is **49**.



Scheme 9: Absolute stereochemistry determination around C-5, C-7 and C-10 of **49** [100].

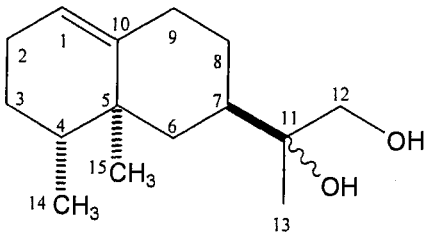
The new and important isolation of kudtdiol (**49**) from AYC should be discussed in terms of the biosynthetic origin of the eudesmane-type skeleton and its relationship with the eremophilane-type skeleton common in AYC. It is worth

noting that the eudesmane skeleton follows the "isoprene rule", while the eremophilane skeleton does not. It was suggested by Robinson in 1939 that the eremophilane skeleton **13** could be derived from a eudesmane-type precursor **14** [107], which is directly derived from the farnesyl pyrophosphate precursor **60**. Later on, this proposal had been extended to include a similar rearrangement from eudesmanes to form the vetispiranes **61** through a similar ring methylene migration [108]. Scheme 10 resembles the now acceptable proposal for the biosynthetic origin of eremophilanes and vetispiranes.



Scheme 10: The acceptable proposal for the biosynthesis of eremophilanes and vetispirans from the eudesmane-type precursor.

4.1.3 Unknown 3: (4R, 5S, 7R, 11?)-1(10)-Eremohpilen-11,12-diol, **62**



62

This unknown was isolated from the AYC heartwood extract by a series of column chromatography procedures as an amorphous solid. It has similar TLC properties as those of kudtdiol **49** (R_f values are 0.36 and 0.45, respectively in 1:1 Hexane: EtOAc(v/v)). The molecular formula was determined on the basis of HRCIMS ion peak at 238.1938 to be $C_{15}H_{26}O_2$ (required for $C_{15}H_{26}O_2$ is 238.1933). EIMS showed peaks at 238 (M^+), 220 (M^+-H_2O), 207 (M^+-CH_2OH), 202 (M^+-$2H_2O$), 189(M^+-(CH_2OH+OH), 161 (189-$CHCH_3$, the decaline system residue) as the main fragment peaks. IR spectroscopy indicated the presence of an alcoholic functional group in the molecule.

The ^{1}H-NMR spectrum indicated the presence of a vinylic (δ_H 5.36 (multiplet), two methylene protons (δ_H 3.62 (d, J=10.9Hz), 3.46 (d, J=10.9Hz) three sets of methyl (δ_H 1.11 (3H, s), 0.95 (3H, s), 0.90 (3H, d, J=6.1Hz) protons

along with other signals (see table 8 for a complete listing and analysis of the ^{1}H-NMR signals).

The ^{13}C-NMR spectrum and polarization techniques (DEPT 90 and 135) confirmed the presence of 15 carbon atoms as follows: three methyl (δ_C 20.9, 18.8, 16.1), six sp^3 methylene (δ_C 26.3, 27.6, 39.9, 29.6, 32.9, 69.0), two sp^3 methine (δ_C 41.5, 40.4), two sp^3 quaternary (δ_C 38.0, 75.1), one sp^2 methine (δ_C 120.6) and one sp^2 quaternary (δ_C 143.4) carbon atoms in this molecule (table 8).

It is noteworthy to mention that these assignments were made based on the common knowledge of various types of ^{1}H and ^{13}C NMR data, comparison with closely related structures from the literature and were confirmed by 2-D NMR data as will be discussed later. All the above results suggest that the structure of this unknown is related to that of **49** described above. However, investigating the results of a combination of 2-D NMR (HSQC, HMBC and COSY, figures 9-11, respectively) reveals that the structure follows the eremophilane skeleton, which is widely found in the heartwood of AYC. Table 9 summarizes the results of HMBC, HSQC and COSY experiments. Among the key correlations observed in the HMBC spectrum, which played a major role in the assignment of the structure of this unknown, shown in scheme 10, are the H-15 correlations with C-10, C-4 and C-6. These correlations show that this methyl is attached to C-5. Other important correlations are those of H-14 with C-14, C-3, C-4 and C-5, which suggests that the C-14 methyl is attached to C-4 and that C-14 and C-15 methyls are vicinal to each other. Correlations of H-12 and H-13 with C-7 on one hand and

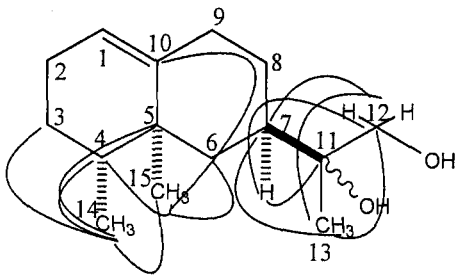
those of H-7 with C-11, C-12 and C-13 on the other hand demonstrate that the attachment between the bicyclic ring and the side chain occurs through C-7.

Table 8: NMR data for **62** in $CDCl_3$ (400MHz for ^1H and 100MHz for ^{13}C).

Position	^1H (Integration, multiplicity [a], *J* in Hz)	^{13}C (type[b])
1	5.36 (1H, m)	120.6 (CH)
2	1.98 (2H, m)	26.3 (CH_2)
3	1.46 (2H, m)	27.6 (CH_2)
4	1.46 (1H, m)	41.5 (CH)
5	_	38.0 (C)
6	2.02 (1H, ddd, *J*=12.6, 3.4, 2.5); 0.92 (1H, m)	39.9 (CH_2)
7	1.87 (1H, apparent tt, *J*=12.6, 3.02)	40.4 (CH)
8	1.74 (1H, dddd, *J*=12.2, 4.7, 2.6, 1.8); 1.03 (1H, dd, *J*=12.6, 4.01)	29.6 (CH_2)
9	2.31 (1H, mt, J=13.5); 2.10 (1H, ddd, J=14.3, 5.52, 2.83)	32.9 (CH_2)
10	_	143.4 (C)
11	_	75.1 (C)
12	3.62 (1H, bd, *J*=10.9); 3.46 (1H, bd, *J*=10.9)	69.0 (CH_2)
13	1.11 (3H, s)	20.9 (CH_3)
14	0.90 (3H, d, *J*=6.1)	16.1 (CH_3)
15	0.94 (3H, s)	18.8 (CH_3)

[a] s= singlet, d=doublet, t= triplet, q= quartet, m= multiplit, b= broad. [b] results obtained from DEPT 90 and 135 spectra.

These results strongly suggest that the skeleton of this compound follows the eremophilane structure. The correlations of H-12 with C-11 and C-13 and correlations of H-13 with C-11 and C-12 suggest that the side chain diol has the arrangement shown in the structure. COSY spectrum correlations provide a support for these conclusions (scheme 11).



Scheme 11: HMBC correlations of unknown 3 (**62**).

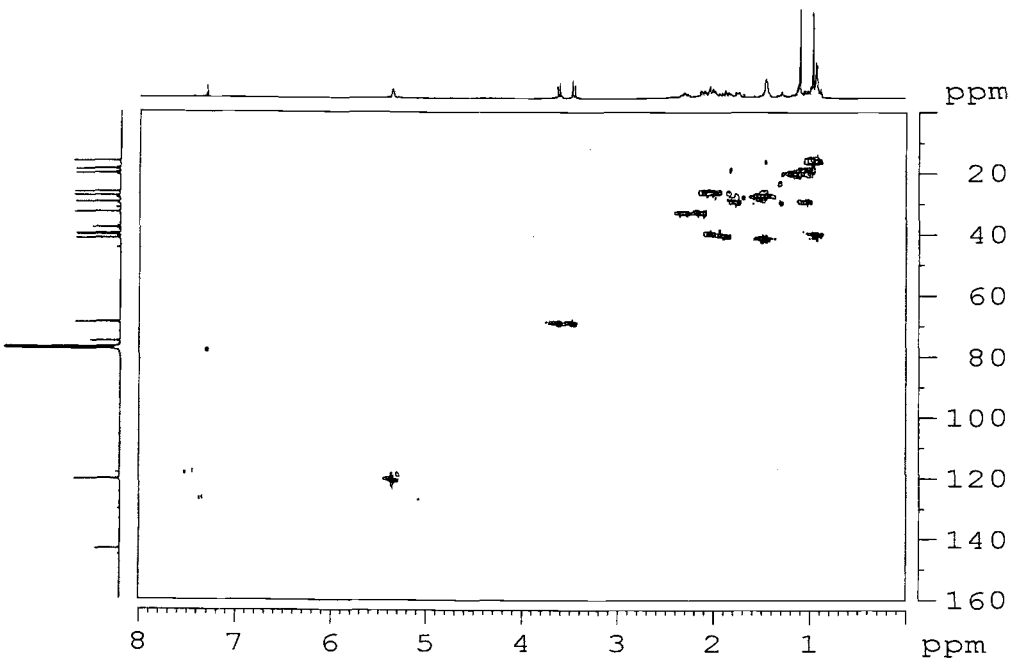


Figure 9: HSQC spectrum of unknown 3 (**62**).

Table 9: 2-D NMR data for unknown 3 (**62**).

Position	Proton	HSQC[a]	HMBC[b]	COSY[c]
1	5.36	120.6	38.0, 32.9, 27.6, 26.3	2
2	1.98	26.3	NR	3, 4, 2′
3	1.46	27.6	120.6, 41.5, 16.1, 38.0, 143.4	4, 3′
4	1.46	41.5	27.6, 16.1, 38.0	15, 3
5	–	38.0[a]	–	–
6	2.02, 0.92	39.9	NR	(7, 8) NR
7	1.87	40.4	69.0, 75.1	8, (6) NR
8	1.74, 1.03	29.6	32.9	9, 8′
9	2.31, 2.01	32.9	120.6, 143.4	8, 9′
10	–	143.4[a]	–	–
11	–	75.1[a]	–	–
12	3.62, 3.46	69.0	20.9, 75.1, 40.4	12′
13	1.11	20.9	75.1, 69.0, 40.4	–
14	0.90	16.1	27.6, 18.8, 38.0, 41.5	4
15	0.94	18.8	143.4, 38.0, 39.9, 41.5	–

[a] Proton showing one-bond correlation to the indicated carbon with the exception of carbons C5, C10 and C11, which as expected show no correlation to any H atoms. [b] Proton showing long-range (mainly 2- and 3-bond) correlation to indicated carbon(s). [c] Protons showing (vicinal) correlations to the indicated proton(s). NR = not observable because the proton signals are not resolved from larger signals.

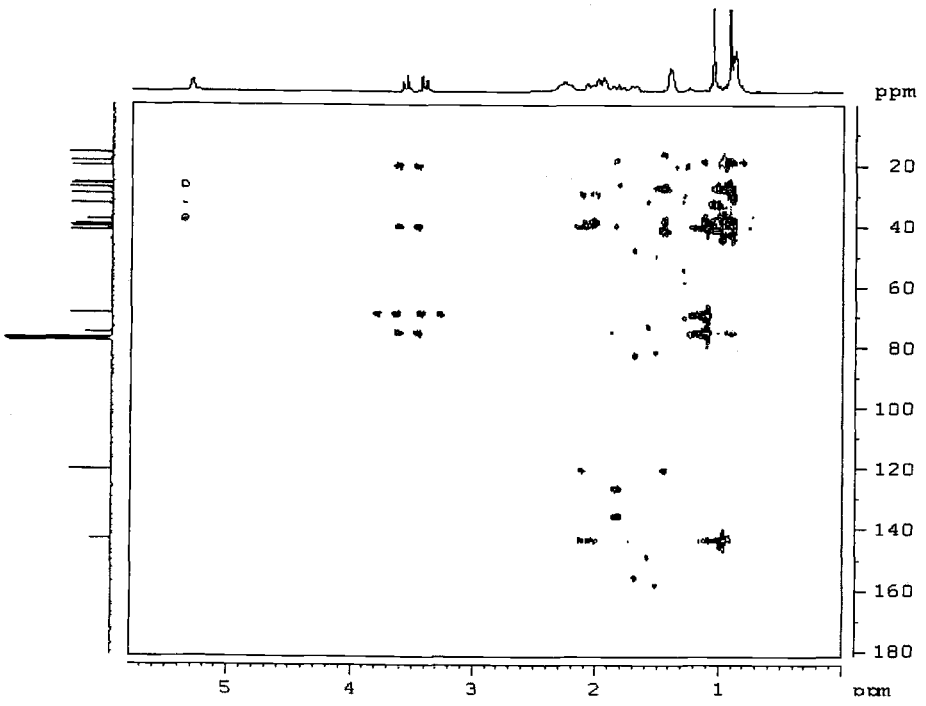


Figure 10: HMBC spectrum of unknown 3 (**62**).

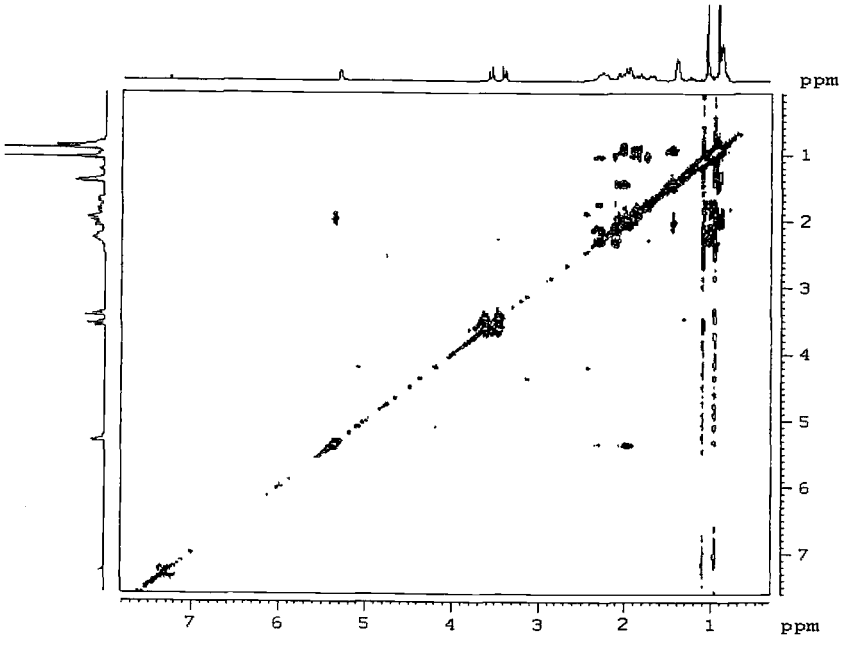
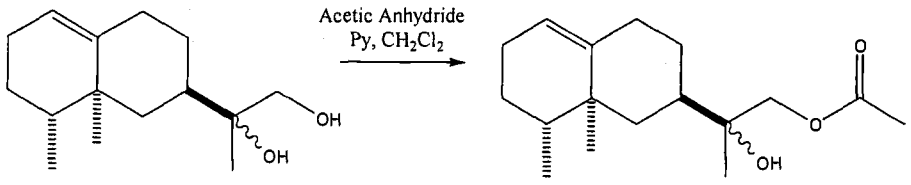


Figure 11: COSY spectrum of unknown 4 (**62**).

This unknown (**62**) was described twice in the literature [109, 110]. In the first reference, **62** was isolated as a natural product from the plant *Tessaria dodonelfolia* and named as todonodiol. The structure was assigned mainly based on chemical transformations as well as ^{1}H NMR data, of which only the most important part was described while no ^{13}C-NMR data were reported. A comparison of the data reported in the present work and those in the reference reveals that there is good agreement in the ^{1}H NMR data except that there is a systematic shift of about positive 0.07-0.10 δ_H units in the data reported here for most of the signals. The second reference in which this compound has appeared was an article dealing with the methodological development for conversion of vicinal diols into carbonyl compounds [110]. In this article, todonodiol was synthesized by treatment of (+)-valencene with $Pb(OAc)_4$ and subsequent hydrolysis of the diacetate. No spectroscopic, physical or chemical properties were reported for this compound at all. Having said that, it is necessary to report all the data that were collected for todonodiol **62** in this research.

To gain more insight on this compound, the monoacetylated product (**63**) of todonodiol was synthesized (scheme 12). ^{1}H and ^{13}C-NMR data for **63** provided additional support to the previous conclusions obtained for those of unknown 3. This compound (**63**) showed a similar ^{1}H NMR spectrum with the exception of the added acetyl protons at 2.10 ppm (3H, s) and the downfield shift of the H-12 protons to occur at 4.02 ppm (2H, q).



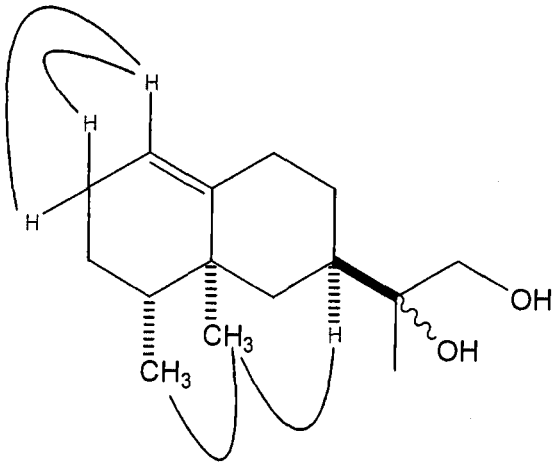
62 63

Scheme 12: Synthesis of the monoacetylated product of unknown 3.

As discussed earlier, **62** belongs to the family of sesquiterpenoids called eremophilanes, which is known to be found in the heartwood of AYC. Therefore, the isolation of this compound from the heartwood of AYC is not a surprising finding. As was discussed in the literature review, many eremophilane-type sesquiterpenoids were isolated from this plant. It was also discussed in section 4.1.2 the biosynthetic origin of the eremophilane skeleton in relation to the eudesmane skeleton.

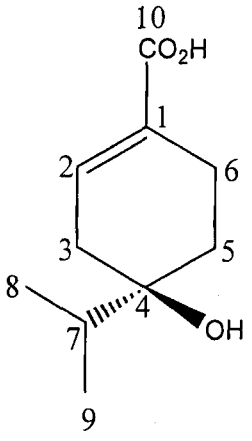
The absolute stereochemistry of **62** was not discussed in both the references mentioned above. The value of the optical rotation was measured to be $[\alpha]_{589} = -17.3$ (c 0.66, $CHCl_3$) [109]. To shed some light on the conformational arrangement of the bicyclic system of **62**, the nuclear overhauser effect (NOE) should be useful. In the NOESY spectrum measured in this study, the correlation between H-7 and H-14 suggests that these two types of protons occur in spatial proximity (scheme 13). The correlation of H-14 with H-15, which is more

difficult but can be observed suggests that **62** has the usual conformation of eremophilanes isolated previously from AYC.



Scheme 13: NOE correlations observed for **62**.

4.1.4 Unknown 4: (4R)-4-Hydroxy-4-isopropyl-cyclohex-1-enecarboxylic acid, **64**



64

This unknown was isolated from the AYC extract as a white amorphous solid. The molecular formula was determined on the basis of HRCIMS ion peak at 184.1088 to be $C_{10}H_{16}O_3$ (required for this formula is 184.10995). The EIMS showed a molecular ion peak at m/z 184 [M^+] in addition to peaks at 167 [184-OH], 166 [M^+-H_2O], 151 [166-Me], 141 [[M^+-CH_3CHCH_3], 138 [166-CO], 123 [138-Me] as the main fragmentation pattern. The IR spectrum showed absorption bands ascribable to an acid (2500-3500 cm^{-1}) and olefin (1620 cm^{-1}) groups.

The ^{1}H-NMR spectrum ($CDCl_3$, 400MHz) indicated the presence of two methyl groups: δ_H 1.01 and 0.99 (2X3H, two d, J=6.8 Hz, H8 and H9), three methylene groups: δ_H 2.37 and 2.25 (1H each, H-3), 1.81 and 1.59 (1H each, H-5), 2.45 and 2.42 (1H each, H-6), one sp^3 methine: 1.72 (1H, H-7), one sp^2 methine 7.07 (1H, H-3). The ^{13}C-NMR ($CDCl_3$, 100MHz) spectrum showed the presence of ten signals as follows: δ_C (multiplicity from DEPT 90 and 135 spectra) 129.6 (C, C-1), 140.2 (CH, C-2), 35.8 (CH_2, C-3), 72.1 (C, C-4), 30.8 (CH_2, C-5), 21.3 (CH_2, C-6), 37.8 (CH, C-7), 17.1 and 17.2 (CH_3 each, C-8 and C-9), 172.1 (C, C10). These assignments were based on the results of 2-D NMR experiments including HSQC (figure 12), HMBC (figure 13) and COSY (figure 14). All ^{1}H and ^{13}C-NMR data are summarized in table 10.

From the molecular formula of $C_{10}H_{16}O_3$ it follows that there are three degrees of unsaturation (DUS) in this compound and from NMR data (both ^{1}H and ^{13}C), there is only one C-C double bond. The IR spectrum showed a strong absorption band at 2500-3500 cm^{-1} characteristic of a carboxylic acid, which accounts for another DUS. Therefore, there must be a ring in the structure of this molecule. The presence of two closely similar methyl groups in the ^{1}H NMR spectrum that exhibit doublet signals suggests that there is an isopropyl group. This conclusion is supported by the presence of a single proton that has a quartet signal correlated with two methyl groups in the COSY (figure 14). Therefore, it can be deduced that the ring is six membered based on simple atom count.

Table 10: NMR data for **64** in $CDCl_3$ (400MHz for ^1H and 100MHz for ^{13}C).

Position	^1H (Integration, multiplicity [a], J in Hz)	^{13}C
1	_	129.6 (C)
2	7.07 (1H, m)	140.2 (CH)
3	2.37 (1H, dd, 7.0, 3.1, equatorial)	35.5 (CH_2)
	2.25 (1H, m, axial)	
4	_	72.1 (C)
5	1.81 (1H, dddd, *J*=13.6, 5.7, 3.3, 2.1);	30.8 (CH_2)
	1.59 (1H, ddd, *J*=8.4, 4.3, 3.8)	
6	2.45 (1H, m); 2.42 (1H, m)	21.3 (CH_2)
7	1.72 (1H, q, *J*=6.86)	37.8 (CH)
8*	1.01 (3H, d, J=6.86)*	17.1* (CH_3)
9*	0.99 (3H, d, *J*=6.86)*	17.2* (CH_3)
10	_	172.1 (C)

* Assignment may be interchangeable.

The proton signal at 7.07ppm is generally of higher value than expected for vinylic protons, but a closer look at literature reveals that high values like what we have here are observed for β- protons when there is an electron withdrawing group such as the carboxyl group attached at the α-position of the alkene.

Finally, the isopropyl group is deduced to be connected to the ring through C-4 based on HMBC correlations of this carbon with H-8, H-9 and H-7 protons. Other key HMBC correlations include those of H-2 with C-10 and C-4, H-3 with C-2, H-5 with C-4 and C-6 (scheme 14).

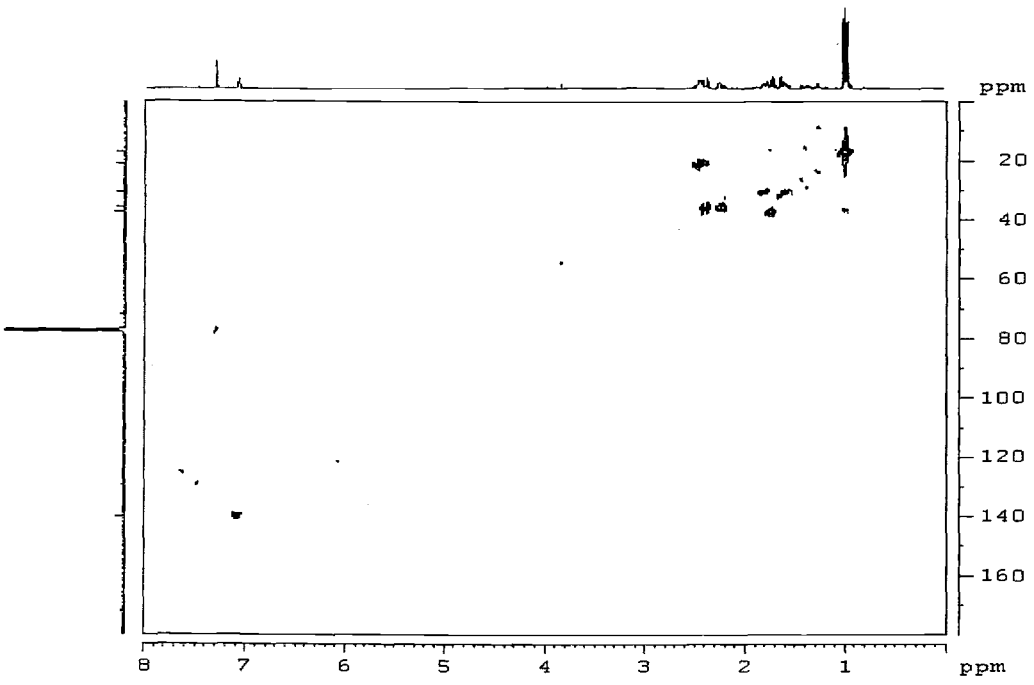


Figure 12: HSQC spectrum of **64**.

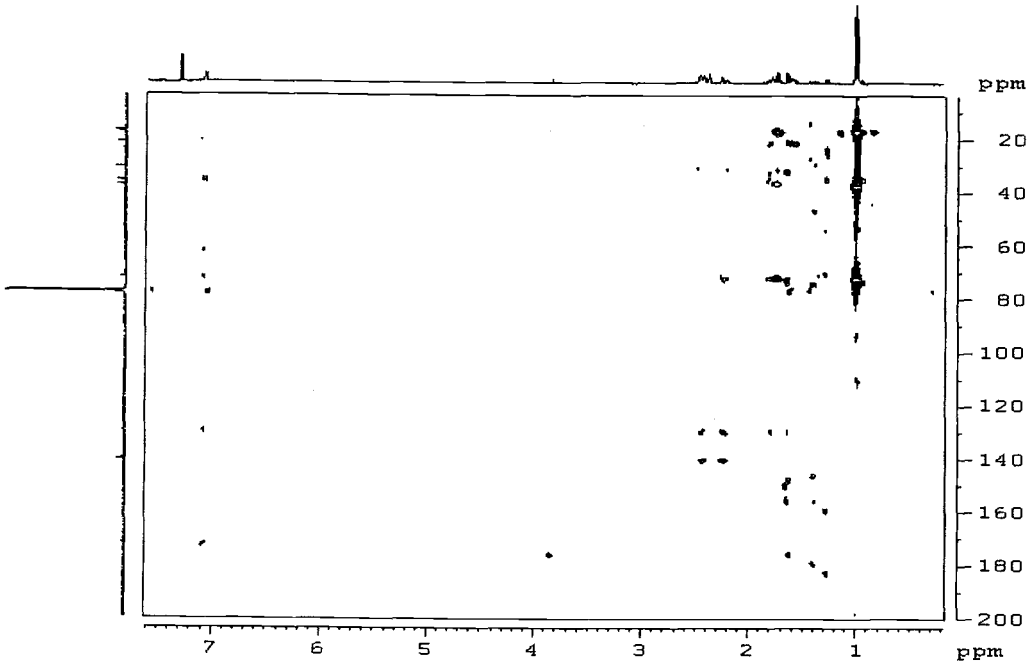


Figure 13: HMBC spectrum of **64**.

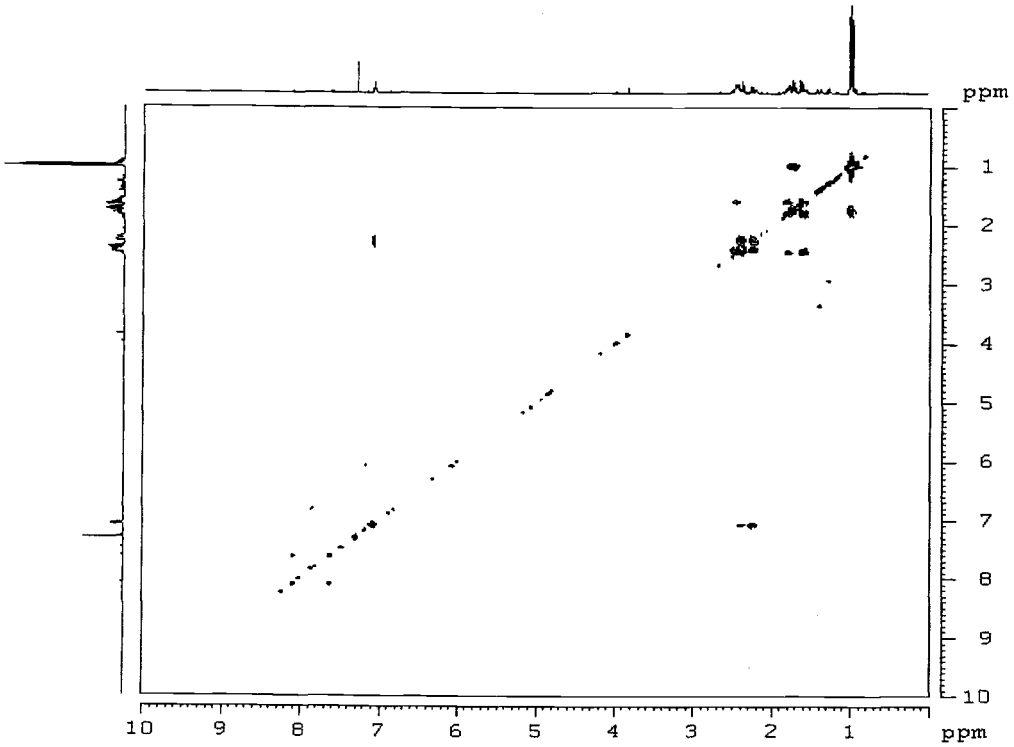
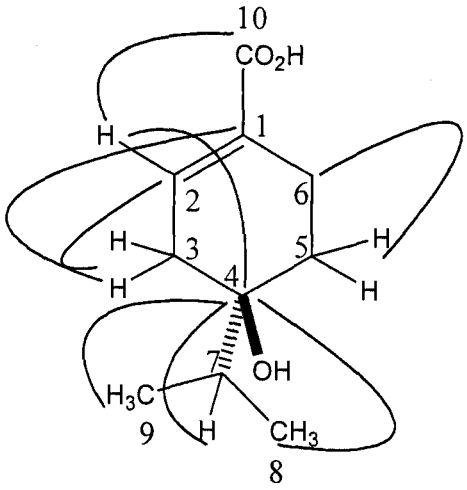


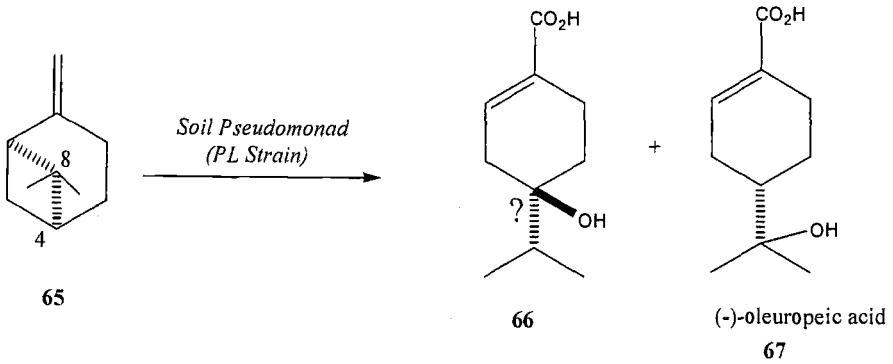
Figure 14: COSY spectrum of **64**.



Scheme 14: Key HMBC correlations for **64**.

As for the stereochemistry of this compound, what has been said for the case of unknown 1 (**44**) is true here since we have tertiary alcohols in both cases. However, it was reported in the literature that the microbial fermentation of (-)-β-pinene (**65**) by *Soil Pseudomonad (PL-Strain)* produced a mixture of hydroxy acids **66** and **67** [111] (scheme 15). Compound **66** had a similar ^{1}H NMR spectrum to unknown 5 (**64**). The structure of compound **66** was deduced based on ^{1}H-NMR, IR and UV data. The specific rotation for this product was reported to be of negative sign with no value given [111]. The specific rotation ($[\alpha]_D^{20}$) value for unknown 4 was measured to be + 25.5 (c 0.275, $CHCl_3$). Since the value observed in this research is the opposite in sign to that reported in reference [111], it can be

assumed that the stereochemistry of unknown 4 is opposite to that of **66**. Further studies are necessary to verify this conclusion. One approach to this problem is by derivatization of these acids to form the corresponding salts and the subsequent x-ray diffraction of single crystals. Unfortunately, this approach is hampered by the paucity of material obtained for **64**. It can be hypothesized that the cyclobutane ring does not cleave at the C4-C8 bond in the process and it can be assumed that the isopropyl group is α-directed in compound **66** and therefore it is β-directed in **64**, because **66** and **63** have opposite signs for $[\alpha]_D$.

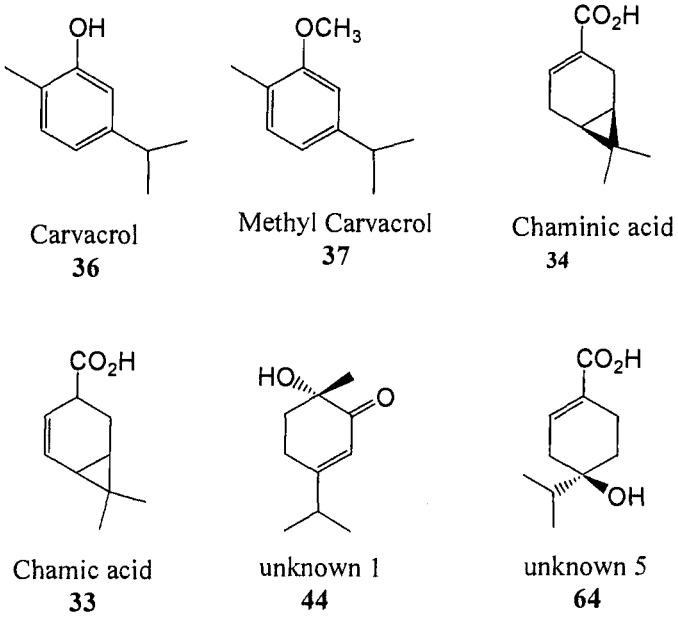


65 **66** (-)-oleuropeic acid **67**

Scheme 15: Microbial fermentation of (-)-β-pinene **65** [111].

It should be pointed out that structures of unknown 1 (**44**) and unknown 4 (**64**) to follow the menthane monoterpene skeleton common in the plant kingdom. These two structures have the same carbon skeleton of other monoterpenes isolated previously from the heartwood of AYC—carvacrol (**36**), methyl carvacrol

(**37**), chamic acid (**33**) and chaminic acid (**34**). It is likely that all these monoterpenes share the same biosynthetic origin. This observation needs to be explored in order for a more definitive conclusion to be made.

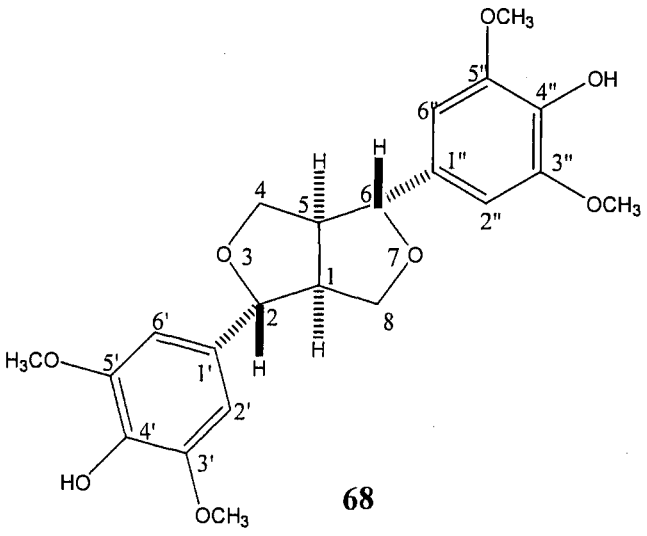


Carvacrol **36** | Methyl Carvacrol **37** | Chaminic acid **34**

Chamic acid **33** | unknown 1 **44** | unknown 5 **64**

Scheme 16: Monoterpenes isolated from AYC heartwood share the same carbon skeleton.

4.1.5 Unknown 5: (1R, 2S, 5R, 6S)-2, 6-bis(3, 5-dimethoxy-4-hydroxyphenyl)-3,7-dioxabicyclo-[3.3.0]octane, (+)-Syringaresinol (**68**)



68

This compound was isolated from the AYC heartwood extract as an amorphous solid after a series of column chromatographic separations (see the experimental). The molecular formula was determined on the basis of HRCIMS to be $C_{22}H_{26}O_8$ (required 418.16277, found 418.16193) and therefore it contained ten degrees of unsaturation. The IR spectrum showed the presence of an aromatic moiety (1610 and 1510 cm^{-1}) and a phenolic functionality (3450-3550 cm^{-1}). The ^{1}H-NMR spectrum showed the presence of aromatic protons (δ_H 6.62, 4H, bs), methoxyl protons (δ_H 3.94, 12H, s), phenolic protons (δ_H 5.54, 2H, bs) as well as other four types of aliphatic protons (see table 11).

The ^{13}C-NMR spectrum showed the presence of eight types of signals at δ_C147.6 (C), 134.7 (C), 132.5 (C), 103.1 (CH), 86.5 (O-CH), 72.2 (OCH_2), 56.8 ($O-CH_3$) and 54.8 (CH). Since there are only four signals of sp^2 hybridized carbons, and we know from IR that there is an aromatic character, then there should be an even number of aromatic rings with some kind of symmetry in this molecule. Since there are only 22 carbon atoms with 10 degrees of unsaturation, it can be concluded that there are two aromatic rings. To make up for the remaining 10 carbon atoms, which belong only to four types according to ^{13}C-NMR spectrum, and given the symmetry in the molecule, these 10 carbons should be distributed into a 4:2:2:2 manner. The four carbons must make up for the methoxy groups observed in ^{1}H and ^{13}C-NMR spectra and the three sets of two carbons each must form a bicyclic ring structure since there are two more degrees of unsaturation unaccounted for (the other 8 are for the two aromatic rings). The last conclusions are supported by ^{1}H-NMR integration of the signals.

To account for oxygen atoms, we know from the formula that there are eight of them in this molecule, four of which make up the methoxy groups. Investigating the HSQC spectrum (figure 15) reveals that the proton signal at δ_H5.54, which integrates to two protons, is not correlated with any carbon atoms. Therefore, these two protons must be attached to oxygen atoms and it can be concluded that there are two OH groups and they should be attached to the two aromatic rings as shown by IR (one for each to make symmetry). The remaining

two oxygen atoms must therefore be contained in the bicyclic ring structure containing the 6 carbon atoms concluded earlier. All the above evidences suggest that this compound has the structure shown above (**68**), which belongs to a family called furofuran lignans.

HMBC (figure 16) and COSY (figure 17) spectral data are summarized in table 12 and also shown graphically in scheme 16. From the HMBC results, H-2 is correlated to C-1′, C-2′ and C-6′ (and similarly H-6 is correlated with C-1″, C-2″ and C-6″). This suggests that the place of attachment between the bicyclic moiety and the phenyl ring occurs through C-2 to C-1′ bond (and C-6 to C-1″). The other key correlation, which supports the same conclusion, is that of H-1 with C-1′ (and H-5 with C-1″). COSY correlations of H-1 with H-2 and H-8 (and H-5 with H-4 and H-6) add further support for the shown structure.

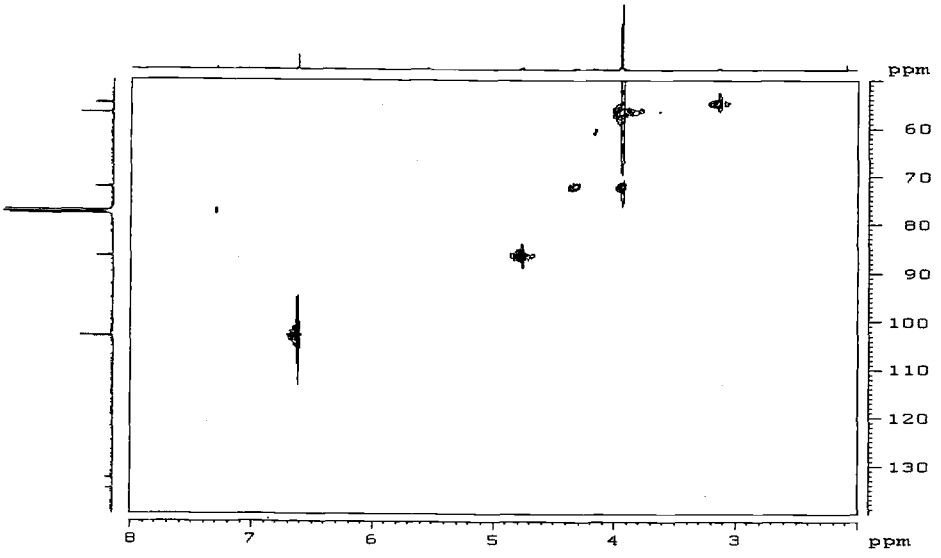


Figure 16: HSQC spectrum for **68**.

Table 11: NMR data for **68** in $CDCl_3$ (400MHz for ^1H and 100MHz for ^{13}C).

Position	^1H (Integration, multiplicity [a], *J* in Hz)	^{13}C (type[b])
1, 5	3.13 (2H, m)	54.8 (CH)
2, 6	4.77 (2H, d, $J = 4.5$)	85.3(CH)
3, 7	_	_
4, 8-α	3.92 (2H, m)	
4, 8-β	4.32, 2H, ddd, J= 9.1, 6.9, 2.1	71.0 (CH_2)
1′	_	132.5 (C)
2′, 6′	6.62 (4H, bs)	103.1 (CH)
3′, 5′	_	147.6 (CH)
4′	_	134.7 (CH)
OH	5.54 (2H, bs)	_
OCH_3	3.94 (12H, s)	56.8 (CH_3)

[a] s= singlet, d=doublet, t= triplet, q= quartet, m= multiplit, b= broad. [b] results obtained from DEPT 90 and 135 spectra.

Table 12: 2-D NMR results for **68**.

Position	HSQC [a]	HMBC [b]	COSY [c]
1, 5	54.8	1′ (4, 8 not observed!)	2, 4, 6, 8
2, 6	85.3	(1, 5), (4, 8), (2′, 6′), (1′)	1, 5
3, 7	–	–	–
4, 8-α!	71.0	(1, 5), (2, 6)	1, 4, 5, 8
4, 8-β!			
1′	132.5	–	–
2′, 6′	103.1	(2, 6,) (4′), (2′, 6′), (1′), (3′), (5′)	2, 6
3′, 5′	147.6		–
4′	134.7		–
OH	–		–
OCH_3	56.8	5′, 3′	–

[a] Proton showing one-bond correlation to the indicated carbon with the exception of carbons C1′, 3′, 4′, 5′, which as expected show no correlation to any H atoms. [b] Proton showing long-range (mainly 2- and 3-bond) correlation to indicated carbon(s). [c] Protons showing (vicinal) correlations to the indicated proton(s).

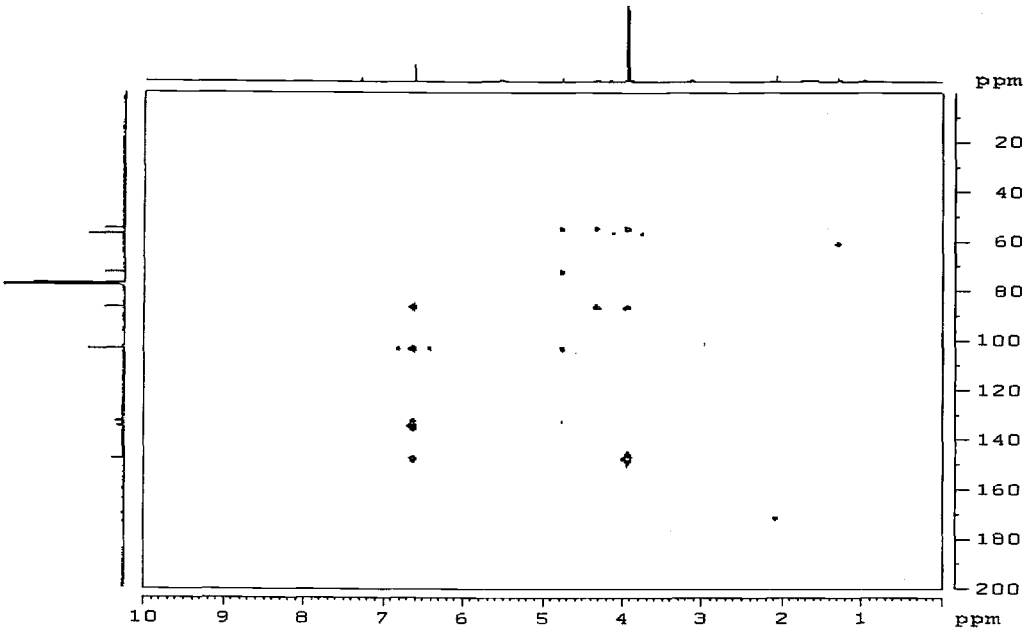


Figure 16: HMBC spectrum for **68**.

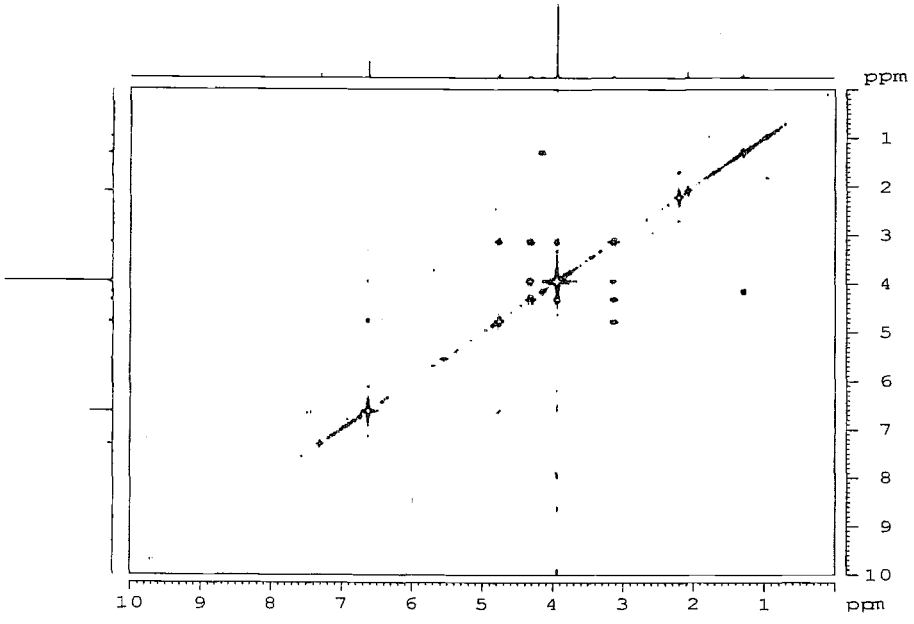
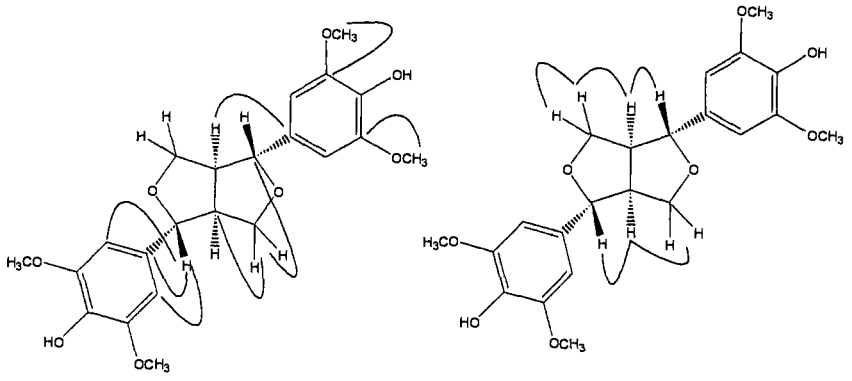


Figure 17: COSY spectrum for **68**.

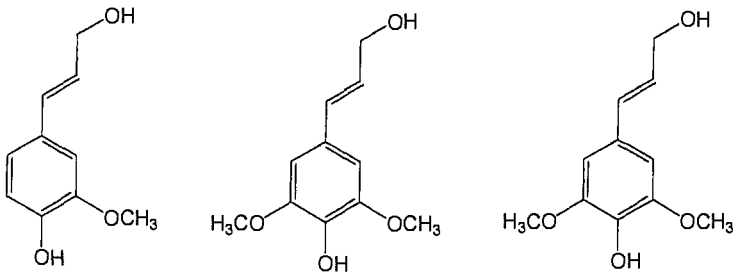


Scheme 17: HMBC and COSY correlations of **68**.

This compound has been previuosly isolated from several plant species such as *Liriodendron tulipifera* [112], *Berberis ermpetrifolia* [113], *Berberis darwinii* [114], *Passerina vulgaris* [115], *Gyrinops walla* [116], *Cocculus hirsutus* [117], *Desfontainia spinosa* [118], just to name a few. However, this is the first report for this compound in AYC. It belongs to an important family of natural products called the lignans. A lignan assay is given in the following few paragraphs to shed some light on this family of secondary metabolites.

Lignans are phenylpropane dimers that commonly occur in woody plants. The phenylpropane units are linked by a central carbon (the β-position) of the aliphatic side chain. This coupling is produced in the shikimic acid pathway from

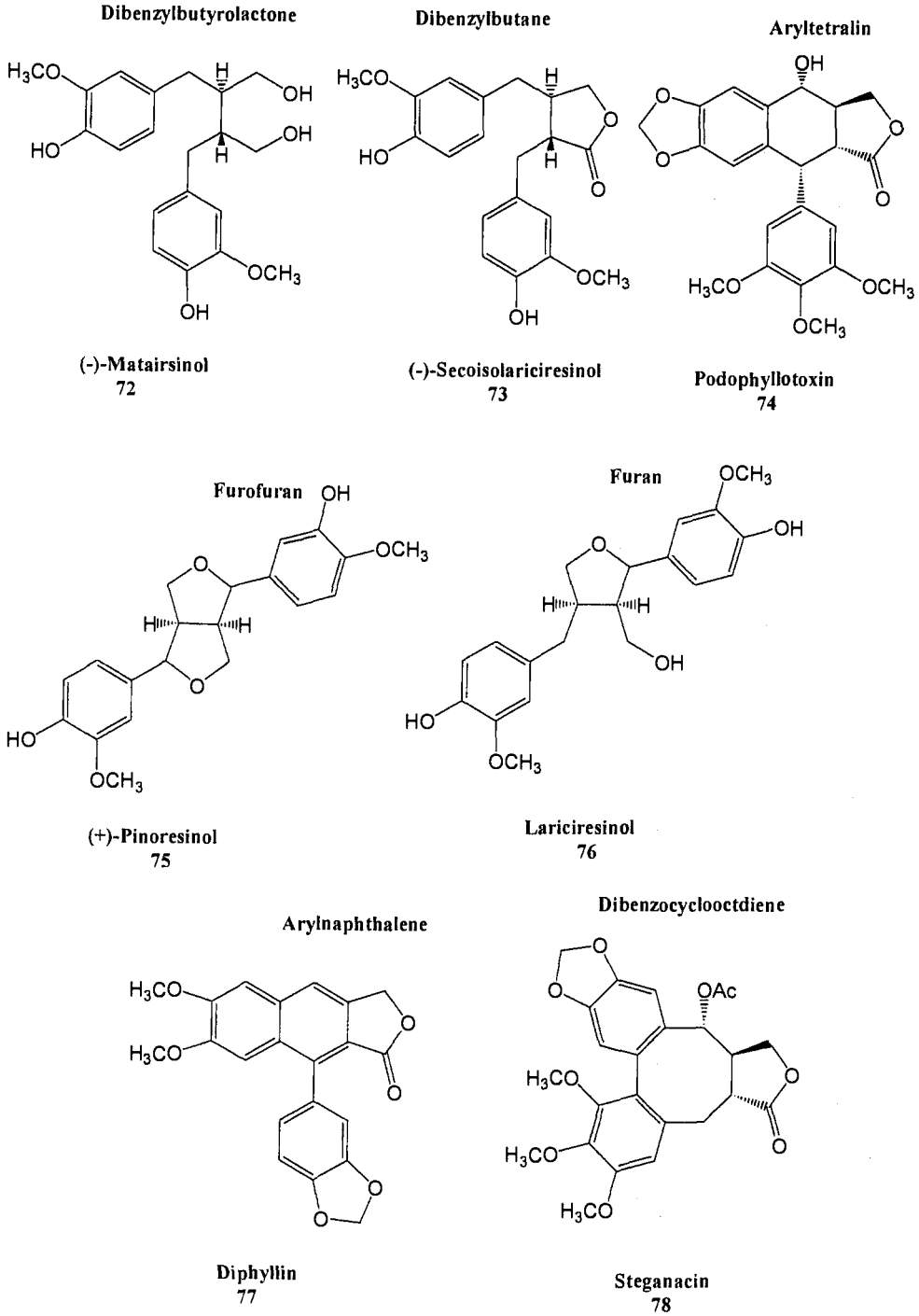
cinnamyl alcohol (mono-lignol) precursors. In trees these are most commonly coniferyl alcohol (**69**) and sinapyl alcohol (**70**) and to a lesser degree coumaryl alcohol (**71**) (scheme 18). Coniferyl alcohol is most often associated with softwoods and sinapryl alcohol with hardwood [119, 120].



69 **70** **71**

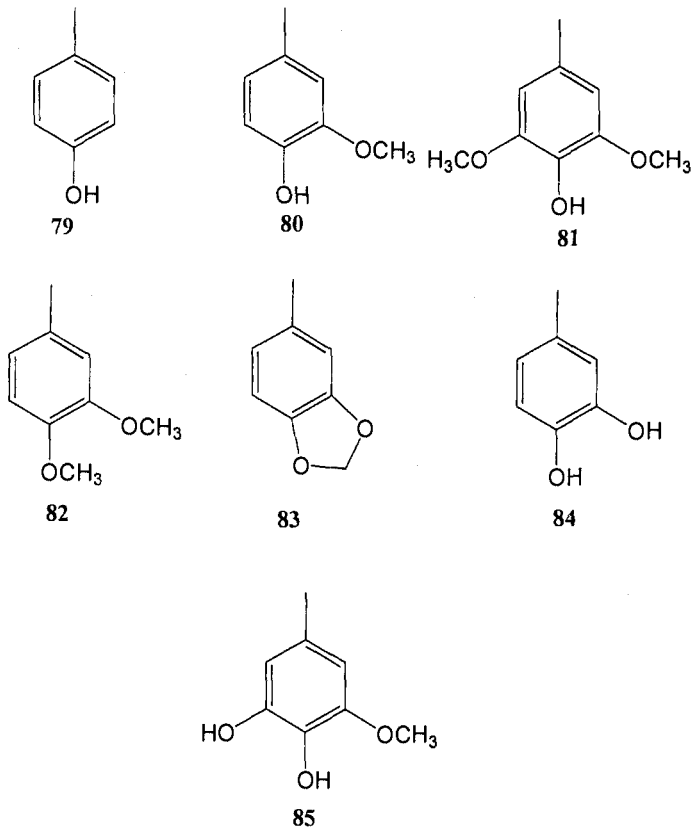
Scheme 18: Structures of the three monolignols that form lignans in trees.

Lignans are classified into several structural groups based on a parent ring structure. These are the dibenzylbutyrolactone (**72**), dibenzylbutane (**73**), aryltetralin (**74**), furofuran (**75**), furan (**76**), arylnaphthalene (**77**), and dibenzocyclooctadiene (**78**) parent structures (scheme 19).



Scheme 19: Examples of lignans.

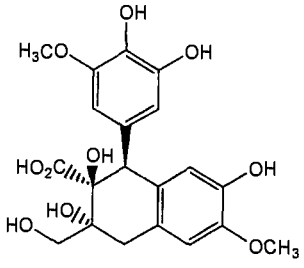
The phenyl groups found in lignans all have some form of hydroxylation. No lignans have been isolated with an unsubstantiated phenolic ring. Seven hydroxylation patterns have been observed, depending on species. These are the p-hydroxy (**79**), guaiacyl (**80**), syringyl (**81**), veratryl (**82**), methylene dioxy (**83**), catechol (**84**), and 3,4-di-hydroxy-5-methoxy phenyl (**85**) groups (scheme 20).



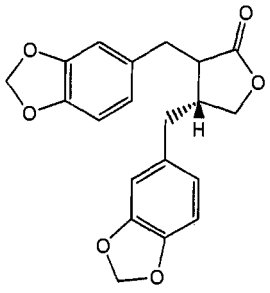
Scheme 20: Phenyl groups in lignans.

The enantiomeric purity, abundance and biological activities of many lignans have made them of commercial importance [120]. Some plant resins and tree heartwoods are particularly rich in these compounds with yields of 20% or more. Lignan rich plants have had a long history of folk medicine and lignans have been lead compounds for drug development as well as semi-synthetic starting materials. Most notably podophyllotoxin (**74**) is an anticancer compound that has lead to the development and use of etoposide and teniposide, two very important anticancer drugs. Other lignans have also shown antimitotic, antioxidant, and antiviral activities. Some lignans have also been associated with human health problems. Plicatic acid (**86**) from western redcedar (*Thuja plicata*) is the cause of occupational asthma to workers exposed to cedar sawdust in saw mills [121, 122].

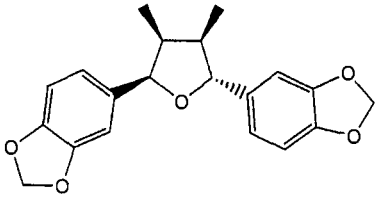
The various lignan groups and oxidation patterns of their aromatic rings is commonly reflected chemotaxonomically [123,124]. For example, pinoresinol (**75**), a furofuran lignan with the phenyl 3-methoxy-4-hydroxy (**80**) group is commonly associated with pines, spruce, and fir trees. In the case of the genus *Chamaecyparis*, *C. obtusa* has been most investigated for its heartwood lignans and it is known or the unusual abundance of hinokinin (**87**) a dibenzylbutyrolactone. Reports of as much as 40% of the heartwood have been noted [120]. Other reports have shown other dibenzylbutyrolactones related to hinokinen and furan types (e.g., zuonin A, **88**, scheme 20) are also present [125,126]. Recently, three novel anthone derivatives (e.g., **89**) were found in the



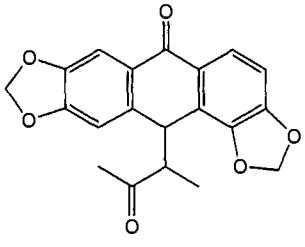
Plicatic acid
86



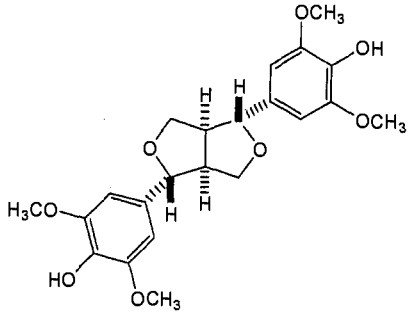
Hinokinin
87



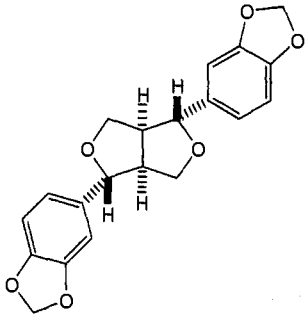
Zuonein A.
88



Chamaecypanone A.
89



(+)-Syringaresinol
68



(+)-Sesamin
90

Scheme 21: Some more examples of lignans.

heartwood of *C. obtusa* var. Formosa [127]. These structures were derived from arylnaphthalene-type lignans.

Recently, it has been shown that some furofuran type lignans (e.g. (+)-sesamin (**90**) have been found in the leaves and young shoots of *C. obtusa* in addition to the dibenzylbutane and dibenzylbutyrolactone types [125]. However, there have not been any reports of the furofuran types in the heartwood. Sesamin and the other furofuran lignan types found in the leaves and shoots also have a common 3,4-oxo-substitution in their aromatic rings.

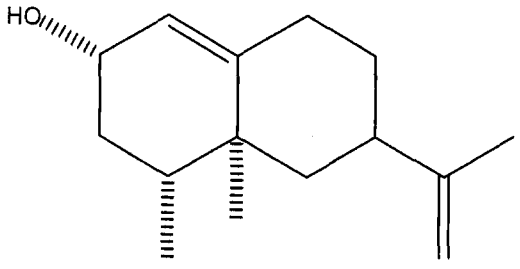
In this research, (+)-syringaresinol (**68**) was the only lignan found in Alaska cedar heartwood. This is an unusual finding.

Biosynthetically, lignan formation is believed to be under enzymatic control in the shikimic acid pathway [123,124]. Oxidation of the aromatic rings is generally believed to be complete on the monolignol precursors before they undergo oxidative coupling to form lignans. Coniferyl alcohol (**69**) and to a lesser extent p-coumaryl alcohol (**71**) are the associated precursors in softwoods or gymnosperms. The hardwoods or angiosperms are usually associated with coniferyl alcohol (**69**) and sinapyl alcohol (**70**). Sinapyl alcohol is not normally associated with softwoods such as Alaska cedar. Biosynthetic studies on the hardwood *Lirodendron talipifera* have confirmed that the (+)-enantiomer of the lignan syringaresinol was enantoselectively formed from two molecules of synapyl alcohol [128,129].

4.2 Settlement of the stereochemical questions about nootkatol and valencene-13-ol

As mentioned in the introduction, some stereochemical issues need to be clarified for two previously isolated sesquiterpenes from AYC. In the following two sections the stereochemistry issues for nootkatol and valencene-13-ol will be addressed in detail.

4.2.1 Nootkatol ((2S, 4R, 5S, 7R)- Eremophil-1 (10), 11-diene-2-ol), **42**



Nootkatol
42

Nootkatol was isolated from the neutral fraction of AYC as a colorless oil after a series of column chromatographic separations as outlined in the experimental following a protocol developed previously in our laboratory [35]. All spectroscopic data were in agreement with those observed for nootkatol (**42**).

Nootkatol was isolated from several plant sources such as citrus temple [54, 130], the Haitian vetiver oil [131], Alpinia Oxyphylla [132]. Both C-2 epimers of nootkatol were obtained from the reduction of nootkatone with lithium aluminum hydride [133]. Another report on the reduction of nootkatone to produce both epimers of nootkatol was made by Ohizumi et al [134]. Most recently, nootkatol was produced along with valencene-12-ol and nootkatone by the enzymatic hydroxylation of valencene using chicory (*Cicorium intybus* L.) roots [135].

By reviewing the above references, one can conclude that there is some confusion in the literature about the absolute configuration of the alcohol moiety at C-2 of the natural product, which was sometimes called nootkatol ([130] and [135]) and sometimes epinootkatol [59, 132, 134]). To clarify this point in the literature, a clear-cut method with the ability to unambiguously determine the absolute configuration of C-2 on nootkatol needs to be applied.

The absolute stereochemistry of secondary alcohols can generally be assigned by several methods described in the literature. Of these methods, the Mosher method and its modifications have been the most widely used. The modified Mosher method described by Ohtani et al is currently the method of choice [89]. In this method, both the (S)- and (R)-2-methoxy-2-(trifluromethyl)-2-phenyl-acetic acid (MTPA) esters of the secondary alcohol under question are synthesized. Originally, Mosher proposed a model for the assignment of the arrangement of the groups around the secondary alcohol in which the methine proton, ester carbonyl and trifluromethyl groups of the MTPA moiety lie in the

same plane in solution [136, 137] and for convenience this plane is called the MTPA plane. Later, x-ray studies on the (R)-MTPA esters of 4-trans-tert-butylcyclohexanol [138] and 1-(R)-hydroxy-(2R)- bromo-1,2,3,4-tetrahydronaphthalene [139] revealed that the MTPA ester moiety was in almost identical conformation to the one proposed by Mosher. Other methods based on theoretical calculations [140] revealed that the coformation proposed by Mosher was one of the two most energetically preferred conformations. Therefore, since it is possible to correctly predict the MTPA plane of the ester and due to the anisotropic effect of the phenyl ring, the upfield shift of protons on both sides of the MTPA plane will depend on the configurations of the alcohol as well as the MTPA moiety. The MTPA plane is shown in the box in figure 18A and a schematic representation of the used model is shown in figure 18 C. As shown in this figure, the MTPA plane divides the MTPA ester into two parts: one lies to the right and the other to the left. The parameter $\Delta\delta$, which is defined arbitrarily as δ_S-δ_R, can be used as a guide to determine the absolute configuration of the secondary alcohol as follows. Protons with positive $\Delta\delta$ values should be placed on the right side of the MTPA plane and those with negative $\Delta\delta$ values to the left using model A (figure 18 C.). A molecular model can then be constructed and configuration can be assigned.

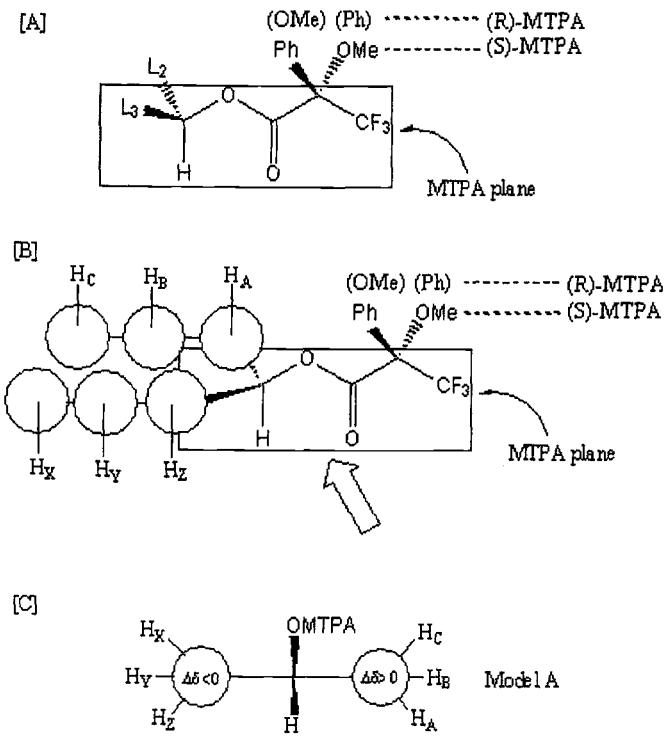
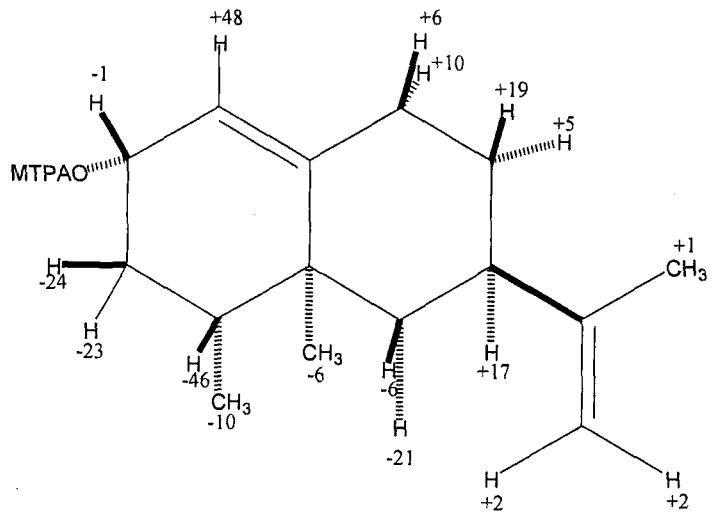


Figure 18: Model for predicting stereochemistry of secondary alcohols as proposed by Mosher et al. [93].

The above method was applied for the assignment of the absolute stereochemistry of nootkatol isolated from AYC at the controversial C-2 position. Towards that end, both the (S)- and (R)-2-methoxy-2-(trifluromethyl)-2-phenyl-acetic acid (MTPA) esters were prepared for nootkatol according to a standard procedure (see the experimental) and their ^{1}H-NMR spectra were recorded (see figure 19). The values for $\Delta\delta=\delta_S-\delta_R$ (in units of Hz) were calculated for each of the protons in nootkatol (see scheme 22). If nootkatol shown in scheme 22 is

portrayed according to model A shown as **62** then protons H-3, H-6, H-14 and H-15 lie to the left of the MTPA plane ($\Delta\delta$ values are negative) and H-1, H-7, H-8, H-9 (and to a much lesser extent H-12 and H-13) lie to the right of the MTPA plane ($\Delta\delta$ values are positive). From these results, it can be concluded that the alcohol in nootkatol has an α-orientation and therefore the absolute configuration at C-2 is (S).



42

Scheme 22: $\Delta\delta$ values (in Hz) for nootkatol (**42**) isolated from AYC.

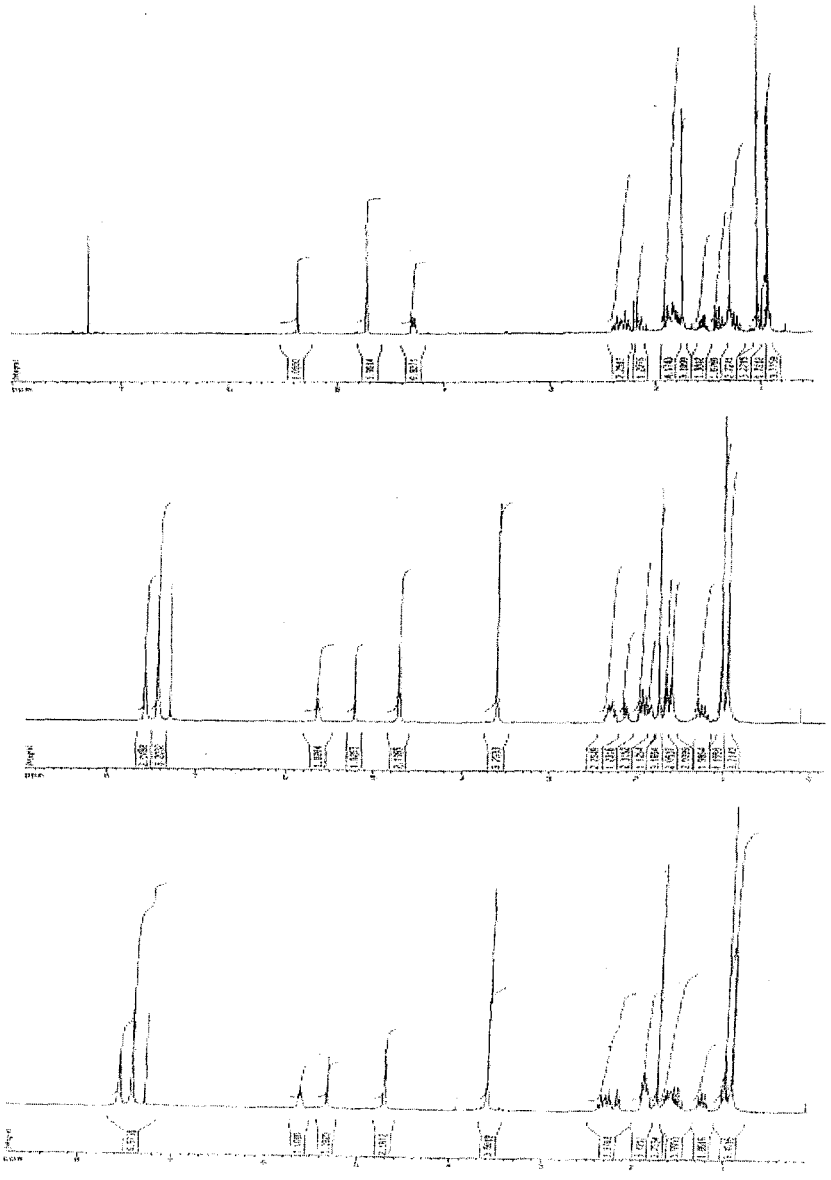
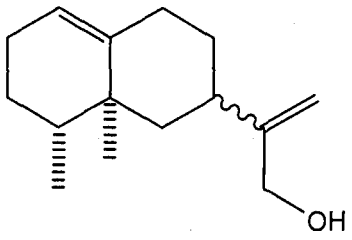


Figure 19: ^{1}H NMR spectra for Nootkatol (top), (R)- MTPA ester (middle) and (S)-MTPA ester (bottom) of Nootkatol.

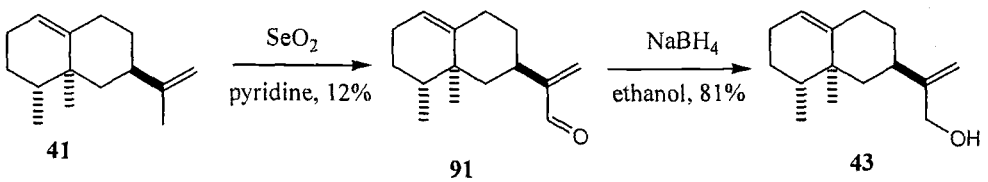
4.2.2 Valencene-13-ol, (Eremophil-1(10)-,11-dien-13-ol), **43**



Valencene-13-ol
43

This compound was isolated as previously described by Xiong [35] and outlined in section 3.3.3. Structure of this compound was established on the basis of MS and NMR data [35]. However, the stereochemistry around C-7, which was based solely on other eremophilanes isolated from AYC, has not been proven. Although this "assignment" seems reasonable, there is still a need for a clear-cut method to establish the stereochemistry. One possible route to tackle this issue is to synthesize the same compound starting from valencene, which has a well-established stereochemistry around all chirality centers includind C-7 and then compare the two compounds' spectral data. The fact that valencene-13-ol can be semi-synthesized from valencene in two steps without the manipulation of any chirality center simplifies this approach. Therefore, this compound was semi-synthesized as outlined in scheme 21 starting from valencene. In this route, valencene (**41**) was refluxed with selenium dioxide in the presence of pyridine to

give valencene-13-aldehyde (**91**) as the main product, which was isolated and reduced with $NaBH_4$ in ethanol yielding valencene-13-ol (**43**). The purified product exhibited MS, NMR and optical rotation data as those of the natural product, which proves that the original assignment of C-7 was correct. Therefore, the assignment of stereochemistry around C-7 is (R) and the name of this compound is (4R)-(5S)-(7R)-valencene-13-ol or (4R)-(5S)-(7R)- eremophil-1(10)-,11-dien-13-ol.



41 91 43

Scheme 23: Semi-synthesis of valencene-13-ol **43** from valencene **41**.

4.3 Semi-synthetic compounds

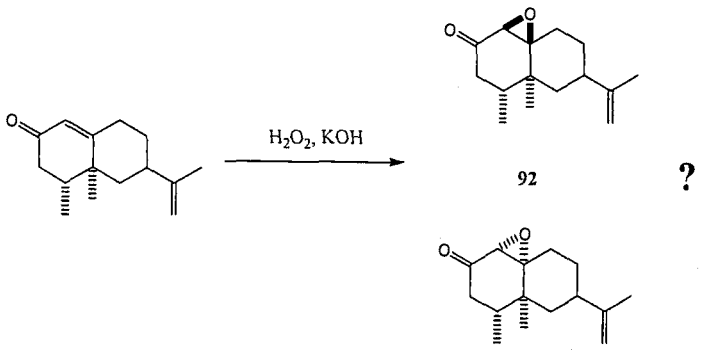
Most of the following structures are in fact new compounds not described in the literature before. Therefore, their structures need to be discussed in appropriate detail. In the following few sections, synthetic routes and spectral properties will be given for compounds synthesized in this research. It should stressed however that since these compounds are synthesized from known starting

materials and given the reagents' known mechanisms of action, the concentration in structure determination will be made on the difference between starting materials and products.

4.3.1 Nootkatone-1, 10-epoxide (**92**)

This compound was synthesized by reacting nootkatone with alkaline H_2O_2 solution (scheme 22). HRCIMS revealed a molecular ion of 234.16209 calculated for $C_{15}H_{22}O_2$, requires 234.16198. ^{1}H-NMR showed some important signals and in remarkable difference from those of the previous compound. For example, the methyl group at δ 1.71 (3H, s, H-13) for the allylic methyl group C-13 and the broad singlet at δ 4.71 (2H, brs, H-12) demonstrate that the side chain remains unaltered. The emergence of a new signal at δ 3.01 (1H, s, H-1) and the disappearance of the vinylic protons at δ 5.75 demonstrate that the C-1, C-10 double bond has been epoxidated. ^{13}C-NMR spectrum revealed the presence of one C=C bond between δ 149.6 (C-11) and 109.7 (C-12), which are the typical values for side chain carbons. The shift of the ketonic carbon signal from 199. 8 in nootkatone to 207.0 confirms the previous conclusion. The rest of the data for both ^{1}H and ^{13}C-NMR are listed in the experimental section and the spectra in the appendix. One observation worth of mentioning is that unlike nootkatone-11,12-

epoxide, the 1,10-isomer showed only one set of signals in both ^{1}H and ^{13}C-NMR spectra indicating the presence of only one diastereomer as the sole product in very high purity. This may be explained by the argument that the C-1, C-10 double bond has one side that is more favorable for epoxidation. This side may be the upper side, since the bottom side is partially blocked by the methyl groups at C-14, C-15 and α protons at C-7 and C-9. However, more clarification is needed for this point.



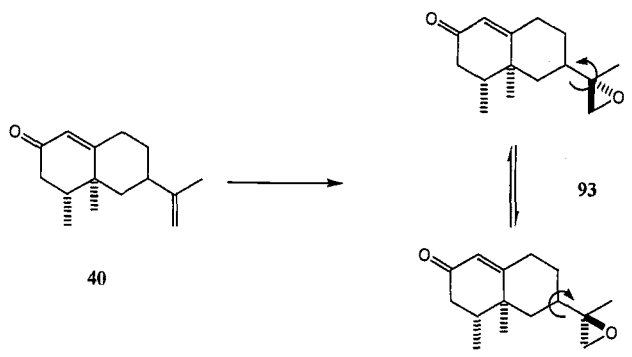
Scheme 24: Synthesis of nootkatone-1,10-epoxide from nootkatone.

4.3.2 Nootkatone-11, 12-epoxide (**93**)

This compound was synthesized by reacting nootkatone with m-chloroperoxybenzoic acid (m-CPBA) in ether (scheme 23). HRCIMS data revealed a molecular weight of 234.16180 calculated for $C_{15}H_{22}O_2$, requires 234.16198. ^{1}H-NMR data ($CDCl_3$, 400 MHz) showed only one alkene proton at δ

5.75 (1H, brs, H-1), which is in good agreement with calculated chemical shift of H-1 (δ 5.88). The two alkenic protons for nootkatone starting material should be at about δ 4.62 have disappeared. Also there are two protons at 2.60 (2H, two d, *J*=5.0, 5.3 Hz, H-12), which are typical for epoxides (both the chemical shifts and coupling constants). One more piece of evidence is the disappearance of the allylic methyl group usually observed at δ 1.7-1.8, which confirms that the epoxidation has occurred at the side chain. ^{13}C-NMR spectrum showed only two olefinic carbons at δ125.3 (C-1) and 170.3 (C-10), which are prctically the same values of chemical shifts for C-1 and C-10, respectively [141]. The above data confirm that using the electrophilic reagent m-CPBA, the epoxidation takes place at the electron rich side-chain double bond as expected.

The rest of the ^{1}H and ^{13}C-NMR data are reasonably as expected and are listed in the experimental section with the original spectra listed in Appendix A. The ^{13}C-NMR data revealed that there are two inseparable diastereomers produced in a ratio of approximately 2:1 the reaction owing to the fact that epoxidation produces a new chirality center at C-11. The reason for the observation of the two diastereomers in the ^{13}C but not in the ^{1}H spectrum is probably because ^{1}H-NMR signals are broad and more complex compared to the much simpler ^{13}C-NMR signals. Another closely related factor is that the difference between the two diastereomers is so small because of the facile free rotation about C7-C11 single bond.

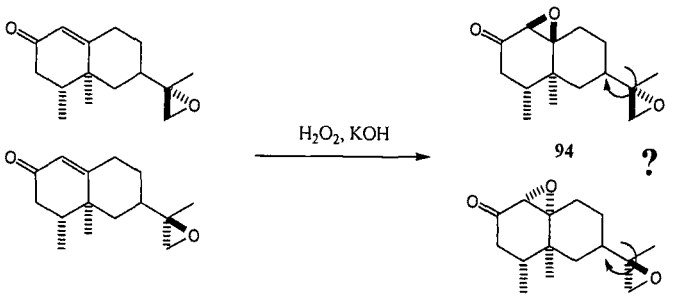


Scheme 25: Synthesis of nootkatone-11,12-epoxide from nootkatone.

4.3.3 Nootkatone-1,10-11,12-diepoxide (**94**)

This compound was synthesized by reacting nootkatone-11, 12-epoxide with alkaline H_2O_2 solution (scheme 24). HRCIMS revealed a molecular ion peak at 250.15670 calculated for $C_{15}H_{22}O_3$, requires 250.15689. ^{1}H-NMR spectrum revealed the absence of any vinylic protons in this molecule. The three methyl groups are present at their expected position for this compound δ 1.28 (3H, s, H-13), 0.93 (3H, s, H-14), 0.78 (3H, d, J=6.8 Hz, H-15). Similar to nootkatone 1,10-epoxide, H-1 appeared at δ 3.08 (1H, s) in the diepoxide. ^{13}C-NMR spectrum confirmed the disappearance of all alkenic carbons and the emergence of four sp^3 hybridized carbons attached to oxygen at δ 63.0 (C-1) 69.2 (C-10), 54.3 (C-11), 60.0 (C-12). The ketonic carbon at δ 207.3 (C-2) is in complete agreement with that of nootkatone-1,10-epoxide. The ^{13}C-NMR spectrum of nootkatone diepoxide in fact showed two sets of signals for many of the carbon atoms. This can be

explained by the presence of two diastereomers in the same way nootkatone-11,12-epoxide behaved. However, these suggestions need more experiments to be proven.

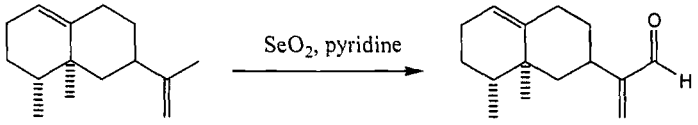


Scheme 26: Synthesis of nootkatone-1,10-11,12-diepoxide from nootkatone.

4.3.4 Valencene-13-aldehyde (**91**)

This compound was synthesized by refluxing of valencene with SeO_2 in the presence of pyridine (scheme 25). HRCIMS revealed a molecular ion peak at 218.16711 calculated for $C_{15}H_{22}O$, requires 218.16706. ^{1}H NMR spectrum indicated the emergence of aldehyde proton at δ 9.45 (1H, s, H-13), three vinylic protons at 6.20 (1H, s, H-12 cis to CHO), 5.85 (1H, s, H-12 trans to CHO), 5.25 (1H, brs, H-1). The last three signals appeared at reasonably close chemical shifts as those calculated using NMR tables, respectively at δ 6.09, 5.87 and 5.12. ^{13}C-NMR spectrum showed the emergence of an aldehyde carbon at δ 195.0 (C-13) in

addition to the four alkenic carbons at δ 120.9 (C-1), 142.9 (C-10), 155.6 (C-11), 133.2 (C-12). Compared to valencene starting material C-1 and C-10 occurred at virtually the same chemical shift (120.9 and 142.9 in valencene), but C-11 and C-12 have been shifted dramatically from δ 150.2 and 108.5 for C-11 and C-12 in valencene, respectively. These considerations provide a compelling evidence for the structure of this compound as valencene-13-aldehyde.



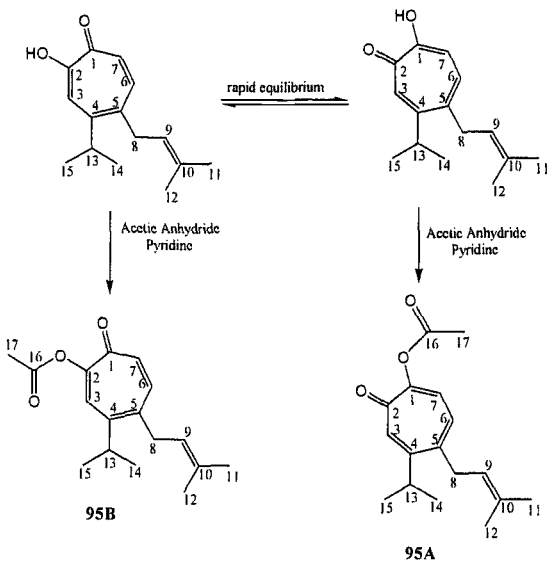
91

Scheme 27: Synthesis of valencene-13-aldehyde from valencene.

4.3.5 Nootkatin acetate (**95**)

This compound was synthesized by treatment of nootkatin isolated from AYC with acetic anhydride in pyridine (scheme 26). HRCIMS revealed a molecular ion peak as expected at 274.15701 calculated for $C_{17}H_{22}O_3$, requires 274.15689. ^{1}H-NMR spectrum showed signals at δ 7.23 (1H, s, H-3), 7.09 (2H, two d, *J*=4.2 Hz H-6 and H-7), 5.08 (1H, tq, *J*=6.7, 1.4 Hz, H-9), 3.34 (2H, d,

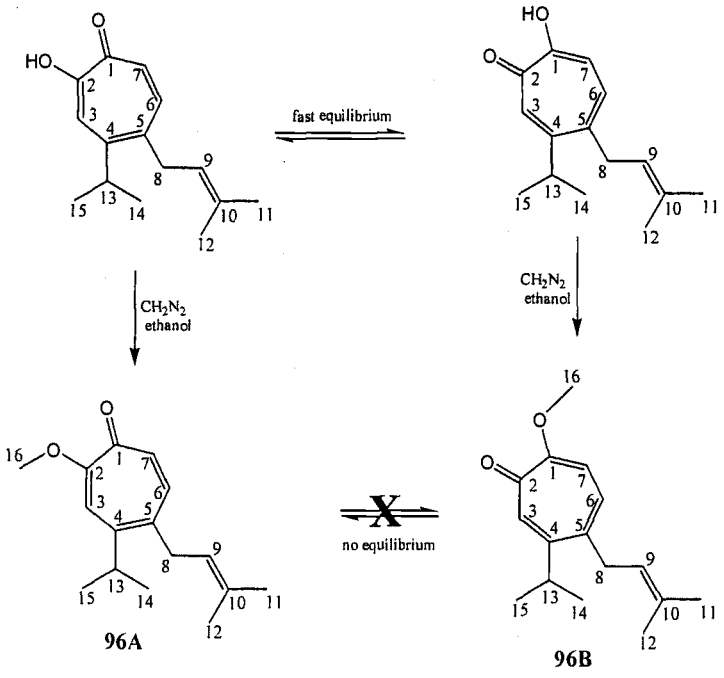
J=6.7 Hz, H-8), 3.22 (1H, Septet, *J*=6.8 Hz, H-13), 2.36 (3H, s, H-17), 1.75 and 1.74 (3H each, two s, H-11 and H-12), 1.22 (6H, d, *J*=6.8 Hz, H-14 and H-15). ^{13}C-NMR data showed 17 carbon signals at δ 172.5 (C), 168.7 (C), 157.4 (C), 141.5 (CH), 140.7 (C), 134.5 (C), 133.8 (C), 125.5 (CH), 122.6 (CH), 121.6 (CH), 36.4(CH), 32.7 (CH_2), 26.0 (CH_3), 23.6 (CH_3), 23.2 (CH_3), 21.1 (CH_3), 18.4 (CH_3).



Scheme 29: Synthesis of nootkatin acetate from nootkatin.

4.3.6 Nootkatin methyl ether (**96**)

This compound was synthesized by reacting nootkatin with diazomethane in ether, which is known to give a clean product with relatively high yield (scheme 27). HRCIMS revealed a molecular ion peak at 246.16210 calculated for $C_{16}H_{22}O_2$, requires 246.16198. ^{1}H and ^{13}C-NMR data showed a 1:1 mixture of regioisomers. ^{1}H-NMR data ($CDCl_3$, 400 MHz) δ 6.65 (0.5H, H-3, s, (in A)) and 7.13 (0.5H, H-3, s, (in B)), 6.88 (0.5H, H-6, d, *J*=11.8 Hz (in A)) and 6.83 (0.5H, H-6 (in B), d, *J*=11.8 Hz), 6.51 (0.5H, H-7, d, *J*=11.8 Hz (in A)), 7.02 (0.5H, H-7, d, *J*=11.8 Hz (in B)), 4.98 (1H, m, H-9 in both structures A and B), 3.97 and 3.74 (3H total, H-16, both s (A and B)), 3.18 (2H, d, *J*=6.3 Hz, H-8 (A and B)), 3.16 and 2.98 (1H total, septet, *J*=6.8 Hz, H-13 (A and B)), 1.55 and 1.53 (3H total, two s, H-11 and H-12 in one regioisomer), 1.56 (3H, s, H-11 and H-12 for the other isomer), 1.09 and 1.05 (3H each, d, J=6.8 Hz, H-14 and H-15 (A and B)). ^{13}C-NMR data ($CDCl_3$, 100MHz) δ (data for one regioisomer only is given here) 180.1 (C), 163.2 (C), 158.6 (C), 150.4 (C), 142.1 (CH), 135.1 (C), 132.2 (CH), 122.5 (CH), 113.7 (CH), 56.3(OCH_3), 36.0 (CH), 32.7 (CH_2), 26.0 (CH_3), 24.0 (CH_3), 23.4 (CH_3), 18.3 (CH_3).



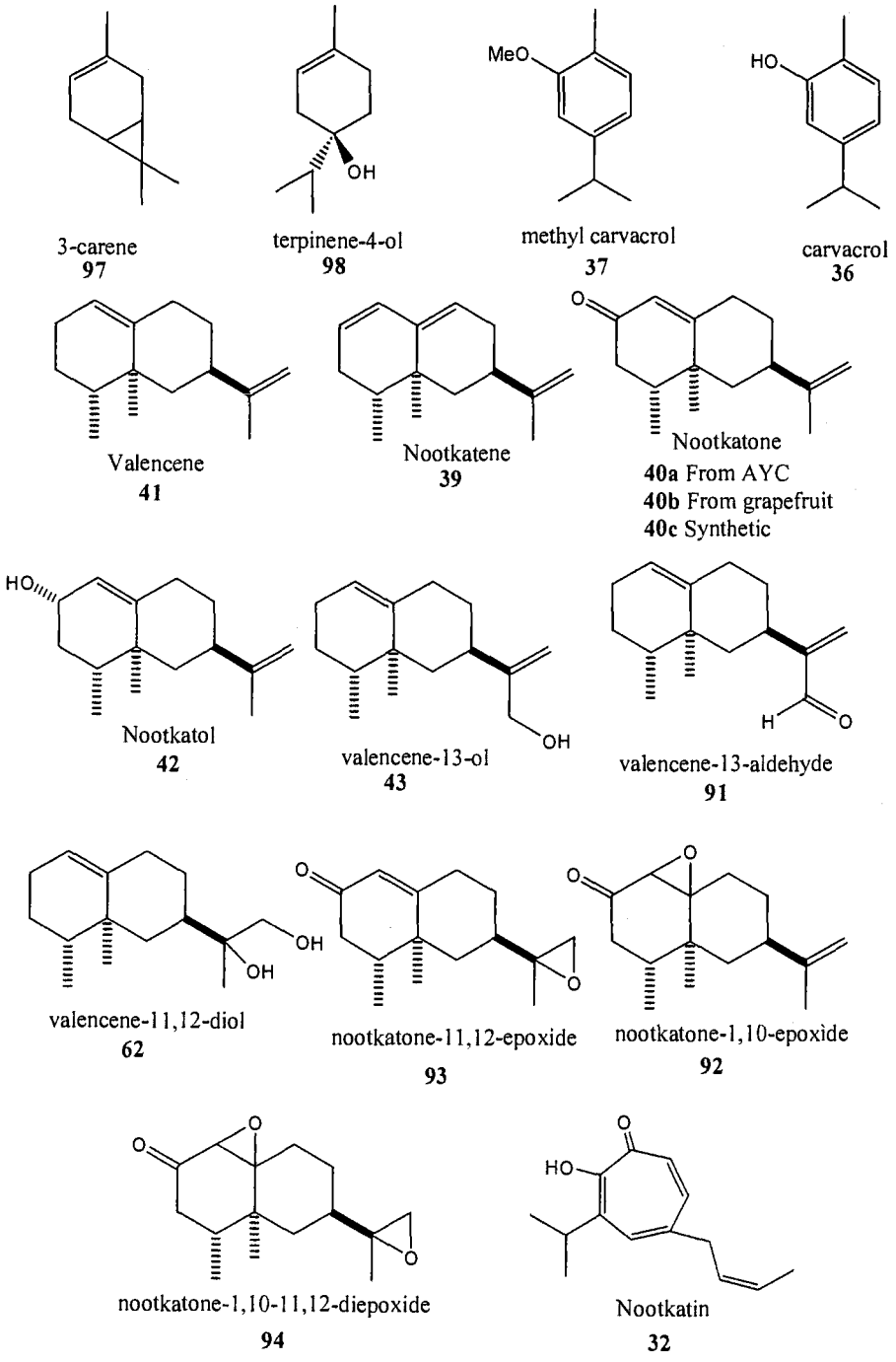
Scheme 30: Synthesis of methyl nootkatin from nootkatin.

4.4 Bioactivity studies

After the isolation and synthesis of several new and previously known compounds derived from the heartwood of AYC along with detailed structural elucidations have been given in the previous sections, the ultimate goal of studying bioactivities of these compounds becomes the natural following step. As was mentioned in the introduction, AYC heartwood is known to be highly resistant to decay processes, making its wood highly prized. Therefore, compounds isolated

and semi-synthesized in this research were screened for their pest control activity in an effort to evaluate each individual compound's activity. Discussions of the trends of the activity as a function of structure of these compounds will be given where possible.

Scheme 30 lists structures and names of the 15 compounds used in this study of screening their biocidal activity against insects and acarines, including members of the taxonomic orders *Acarina*, *Dipetra*, *Homopetra*, or *Siphonopetra*. Specifically, the biocidal activities against nymphal *I. scapularis* ticks, adult, *X. cheopis* fleas and adult *Ae. eagypti* mosquitoes were investigated. This study was initially designed so that the main constituents found in AYC heartwood are screened for biocidal activity in an attempt to identify the most active compounds in this species. However, after reviewing the initial screening results, the focus of the study was brought on the eremophilane sesquiterpenes found in AYC — valencene, nootkatol, nootkatone, valencene-11,12-diol and nootkatene as well as other semi-synthetic derivatives of some of these compounds. 3-Carene (**97**) and terpinene-4-ol (**98**) were detected in the AYC heartwood extract using GC-MS, and commercially available samples were used in the screening experiments.



Scheme 30: Compounds tested in this study for their pest control activity.

Methods of controlling the arthropod are described in which the arthropod is brought into contact with a "pesticidally effective amount" of a compound described herein, which is sufficient to cause an adverse effect on at least some number of the test pests such as causing mortality or repellence from a locus (see the experimental). The term "pest control agent" is used throughout this discussion and taken to describe the control of the behavior of a pest both as a repellent or a pesticide.

The 24 h and residual (up to 4 weeks) susceptibility studies of three pests including *I. scapularis* nymphs, *X.cheopis* adults, and *Ae. Aegypti* adults were conducted. The short-term studies resemble the single-dose mortality of test pests observed after 24 h of exposure. Residual susceptibility studies of the test pests for the test compounds resemble the study of the activity of test compounds 1-4 weeks after the first application to the test vessels. These studies test the persistence of these compounds and the persistence of their activity several weeks after the application.

LC_{50} and LC_{90} values given in the results are expressed in terms of absolute values and 95% confidence intervals (CI, given in parentheses) of the results obtained for concentration ranges for each tested compound. The slope values given in these results resemble the slope of the "dose-response curve", which is a measure of the sensitivity of the response (here % pest mortality) to the change in the used dose (here the concentration) of the test compound. In general terms, a large slope of the dose-response curve (steep curve) indicates that a small change

in the dose (concentration) of the test compound will result in a significant difference in the activity of that compound. Therefore, depending on the threshold concentration, compounds with large slope values of the dose response curves are generally more preferable over those with smaller slope values since with the former ones, smaller concentrations can achieve high rates of pest mortality. Conversely, a somewhat less steep dose-response curve (moderate slope) indicates that a relatively large increase in the concentration has relatively less effect on the pest mortality rate. For simplicity, the term "slope" will be used here, which indicates the slope of the does-response curve.

4.4.1 Short-term (24 h) activity studies

4.4.1.1 Activity against *I. scapularis* nymphs

The complete results of these studies are summarized in table 13. These results reflect the variety of structure types and functional groups exhibited by the compounds investigated. Therefore, it is useful to divide these compounds into categories to facilitate the understanding of the trends of the activity exhibited. Three categories can be readily recognized—category I including the monoterpenic compounds **97**, **98**, **35** and **36** (scheme 30), category II including

sesquiterpenic compounds of the eremophilane skeleton (compounds **39-43, 62, 91-94**), and category III including the tropolone sesquiterpenoid nootkatin (**32**).

Figure 20 depicts the activity of each of the studied compounds in terms of an arbitrary parameter named as relative activity (R.A.) and defined as:

$$R.A. = \frac{(LC_{50})_{valencene}}{(LC_{50})_{compound}}$$

, where valencene is chosen to be the reference compound.

For category I compounds, 3-carene, terpinen-4-ol and methyl carvacrol (compounds **97**, **98** and **36**) exhibited no activity towards *I scapularis* nymphs under the experimental conditions. Carvacrol (**35**), however, exhibited a significant biological activity under the same conditions (LC_{50} = 0.0068% w/v) with a slope of 3.9. This compound is in the same order of activity as compounds **43** and **93** and comes second only to the most active compound (nootkatone **40a, b, c**). The relatively high content of carvacrol in the AYC heartwood and the relative ease of its isolation with an acceptable purity make it of high importance as a candidate lead compound. The failure of its methyl derivative, compound **36**, to exhibit any detectable biological activity suggests that the polar functionality (OH) may have an important role in the activity, which can be through polar interactions (H-bonding or dipole-dipole) with the biological receptor of the test pest. This hypothesis needs further investigation in order for a more definitive

Table 13: Response of *I scapularis* nymphs after 24h exposure.

Compound	LC_{50}	LC_{90}	Slope
35	0.0068 (0.0054-0.0084)	0.014 (0.011-0.022)	3.906
41	0.598	44.837	0.684
39	0.011 (0.0086-0.014)	0.06 (0.043-0.098)	1.763
40a	0.0029 (0.0025-0.0034)	0.0055 (0.0046-0.0073)	4.708
40b	0.0061 (0.005-0.0072)	0.015 (0.012-0.021)	3.211
40c	0.0033 (0.0027-0.004)	0.0087 (0.0069-0.012)	3.061
42	0.023 (0.017-0.03)	0.059 (0.042-0.116)	3.069
43	0.0051 (0.0041-0.0062)	0.016 (0.013-0.023)	2.562
91	0.0059 (0.0044-0.0076)	0.017 (0.012-0.029)	2.755
62	21.219	3713.114	0.571
93	NE	NE	NE
92	0.02 (0.015-0.026)	0.055 (0.04-0.092)	2.883
94	0.061 (0.045-0.094)	0.245 (0.142-0.756)	2.131
32	NE	NE	NE

NE = not effective under the concentration range of the experiment.

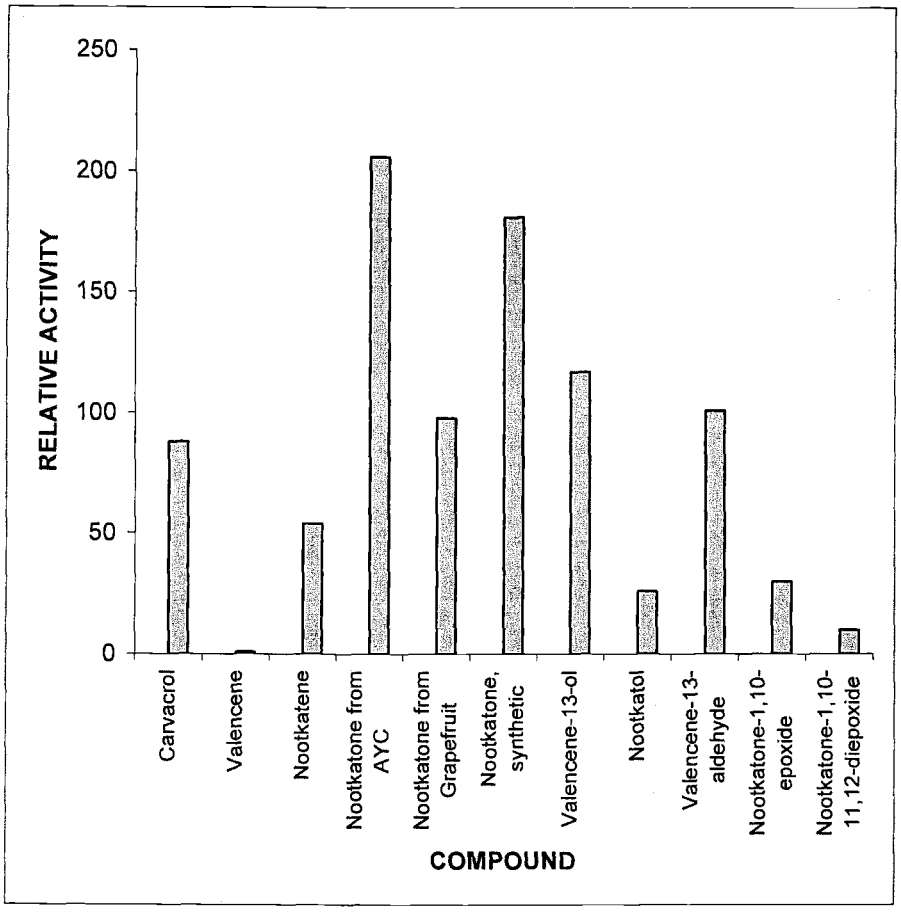


Figure 20: Relative activities of studied compounds against *I. scapularis* nymphs. Compounds not exhibiting activity (i.e. RA≈0) are not shown.

conclusion to be made. Other compounds of this category (compounds **97, 98**) are either lacking one or more of the structural, configurational or functional group requirements for the activity process.

In category III, nootkatin (compound **32**) was surprisingly found to be ineffective against *I. scapularis* even at relatively high concentrations. It is interesting to stress this finding as opposed to the previously known high

antifungal activity of nootkatin [142]. The reason for this difference in activity behavior could be due to the fact that the working mechanism of activity in the case of pest control activity is different from that in the case of antifungal activity.

Category II compounds resemble the most important category of this study. Valencene (compound **41**) exhibited a very weak activity against *I. scapularis* nymphs (LC_{50} = 0.598% w:v) with a small slope, which is reflected by the very high LC_{90} of 44.8% w:v. Nootkatene (compound **39**) is about 60 times more active than valencene (in terms of LC_{50}) with a larger slope. This increase in activity on going from valencene to nootkatene suggests that the increased unsaturation and that the subsequent change in the configuration of the bicyclic moiety seems to play a role in the activity.

As was mentioned before, three samples of nootkatone from different sources (from AYC (compound **40a**), from grapefruit oil (compound **40b**) and semisynthetic (compound **40c**)) were tested for comparison. The activities of these three samples were essentially in the same order within experimental variables and taking into account different levels of impurities found in these samples.

Nootkatol (compound **42**) exhibited a relatively moderate activity against *I. scapularis*. Compared to nootkatone, its activity was reduced by a factor of 8 in terms of LC_{50} values, while in comparison to valencene, it was found to be about 26 times more active with a much larger slope. Valencene-13-aldehyde (compound **91**) exhibited activity that was comparable to its reduced counterpart

(compound **43**) both in terms of LC_{50} and slope. This compound is over 100 times more active than valencene, but about 2 times less active than nootkatone (compound **40a**). Valencene-11,12-diol (compound **62**), on the other hand, exhibited some activity against *I .scapularis* nymphs only at extremely high concentrations, which makes it essentially ineffective (LC_{50} = 21.2%). In fact, this compound is one of the weakest compounds in activity in category II.

The last three compounds (compounds **92-94**) are the epoxidated derivatives of nootkatone prepared semisynthetically. Nootkatone-11,12-epoxide (compound **93**) was found to be ineffective at the test conditions. However, the 1,10-epoxidated isomer (compound **92**) and the diepoxidated derivative (compound **94**) showed moderate activities under the same conditions (LC_{50}=0.02, 0.06 % w/v, respectively).

To simplfy the study and discussion of category II ten compounds, five distinct subcategories can be identified as follows:

- Valencene series I: including valencene (**41**), Nootkatol (**42**) and nootkatone (**40a-c**).
- Valencene series II: including valencene (**41**), valencene-13-ol (**43**), valencene-13-aldehyde (**91**).
- Valencene series III: including valencene (**41**) and valencene-11, 12-diol (**62**).
- Nootkatone series I: including nootkatone (**40a-c**), nootkatone-1,10-epoxide (**92**), nootkatone 1,10-11,12-diepoxide (**94**).
- Nootkatone series II: including nootkatone (**40a-c**) and nootkatone-11,12-epoxide (**93**) and nootkatone-1,10-11,12-diepoxide ((**94**).

In each subcategory, the compounds are arranged in an order of increasing "oxidation level". This division of compounds will allow some basis for

comparison of activities among compounds of the same subcategory and therefore more conclusions can be drawn. These subcategories will be discussed separately or collectively whenever possible. The concept "oxidation level" is used here as a general guide to express the extent of introduction of different functional groups and the subsequent change in the structure and properties of the compound. On oxidation, several factors may be changing simultaneously, which leads to the change in activity. These inter-related factors include polarity, conformation, volatility, and H-bonding donor or acceptor ability. One or a combination of these factors may be the deciding factor(s) for activity in each of the above subcategories. Since we have no idea about the receptor shape of the studied test pests, we will use general statements in describing these effects.

4.4.1.1.1 Valencene series I, II and III

The following diagram (figure 21) represents a sketch drawing of the relative activity as defined above versus oxidation level of these three subcategories.

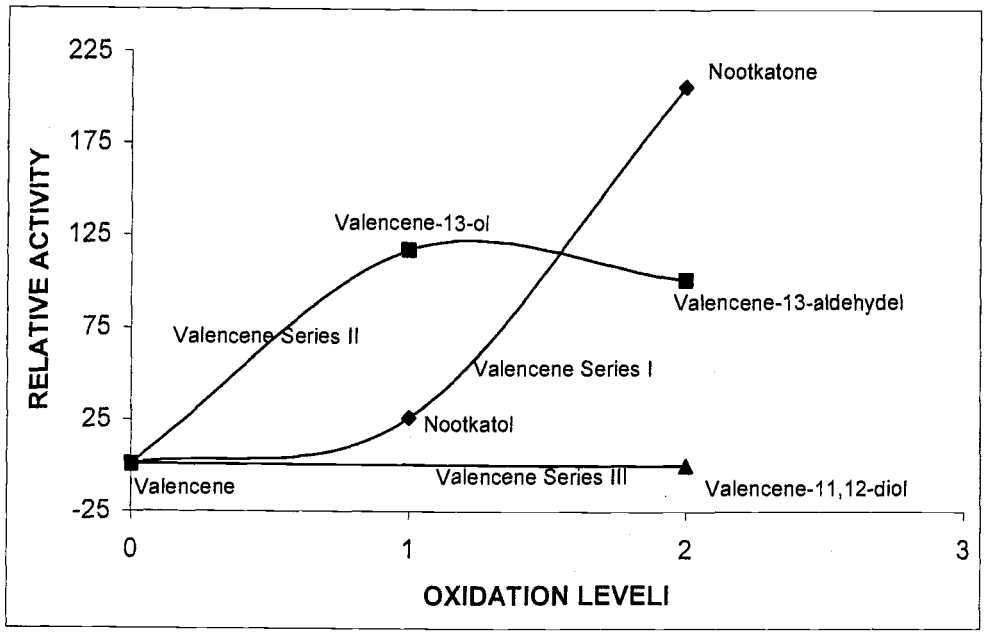


Figure 21: Relative activity (R.A.) versus oxidation level of valencene series I, II, and III.

From this figure, it can be seen that in the valencene series I, activity increases with increasing the oxidation level of the test compound from 1 for valencene to about 220 for nootkatone. For the case of valencene series II, a similar trend can be observed on going from valencene to valencene-13-ol (compound **43**, with R.A. of about 117). However, further oxidation of **43** to form compound **91** has only a marginal effect on R.A. value. This is contrasted to the continuous trend observed in the valencene series I. In the valencene series I and II, activity increases with the introduction of polar functional groups with the

ability for H-bonding and polarity as in nootkatone (**40a-c**) and nootkatol (**42**) and with the decrease in the volatility of the test compounds. In the valencene series III, the trend is opposite to that observed in both valencene I and II series. The activity decreases dramatically with the introduction of the two hydroxyl functional groups at C-11 and C-12 positions as in compound **62**. This finding suggests the importance of the olefinic side-chain in the activity process and stresses that there are several factors playing role in the activity process.

4.4.1.1.2 Nootkatone series I and II

The relative activity values for compounds of these subcategories are graphed against the oxidation level as shown in figure 22. As can be seen in this figure, the activity of the test compound decreases as the oxidation level increases. This is the opposite of the trend observed for valencene series I and II and the same trend for valencene series III. This shows that, for valencene and nootkatone series compounds, nootkatone (**40a-c**) and valencene-13-aldehyde (**91**) resemble the "optimum oxidation level" that is reflected by the highest activity observed against *X. cheopis*.

In the nootkatone series I and II, the activity decreases with the increase in polarity and decrease in volatility. Conformation may be more important in this series, because of the substantial change in hybridization of the olefinic carbon

atoms. There is another interesting observation to note in the comparison of nootkatone series I and II. In the series I, the activity decreases on the epoxidation of the 1,10-double bond (compound **92**) and continue to decrease on the second epoxidation (compound **94**). This is contrasted to the complete loss of activity on first epoxidation of the 11,12-double bond (compound **93**) and then the subsequent restoration of the activity upon epoxidation of the 1,10-double bond (compound **94**) in the series II. In addition to the previously mentioned factors, this comparison suggests that the side chain double bond may be important. This last suggestion is supported by the previous finding that valencene-11,12-diol (compound **62**) is about 35 times less active than valencene itself while compounds **43** and **91** (where the side chain double bonds are reserved) are both about 100 times more active than valencene.

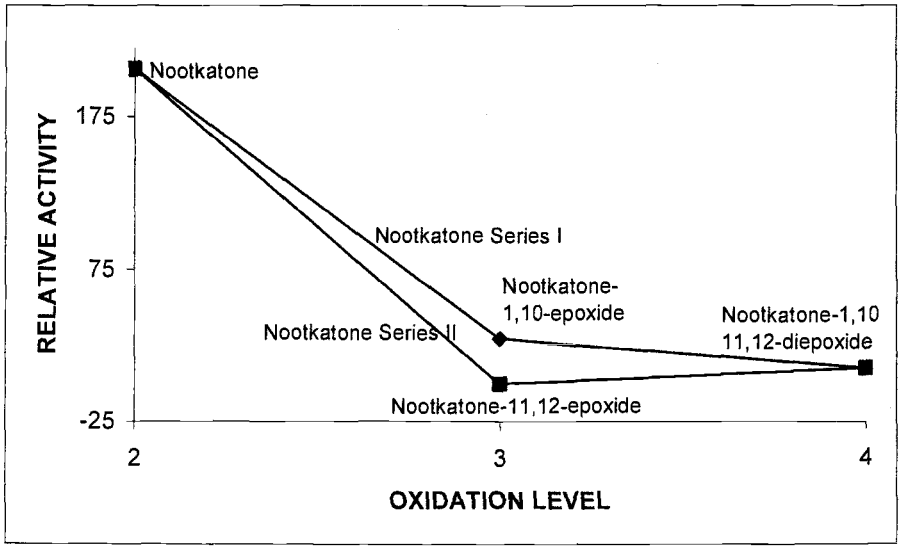


Figure 22: R.A. versus oxidation level of nootkatone series I and II.

4.4.1.2 Activity against *X. cheopis* adult fleas

The short-term (24h) single-dose mortality susceptibility results of *X. cheopis* adult fleas to test compounds are presented in table 14. LC_{50}, LC_{90}, along with their 95% confidence intervals and slope values of the dose-response curves are listed for test compounds. In this discussion, the same classification of test compounds that was used in the discussion of the results of *I. scapularis* in section 4.4.1.1 will be used here. The data are shown graphically in figure 23 in terms of relative activity (which is defined as the ratio of LC_{50} reference / LC_{50} compound, with valencene chosen as the reference) against compound name. This figure serves as a general display of the activities of the tested compounds regardless of their structure or category.

Table 14: Response of *X. Cheopis* adult after 24h exposure.

Compound	LC_{50}	LC_{90}	Slope
93	0.0059 (0.0047-0.0075)	0.014 (0.011-0.023)	3.413
94	0.041 (0.034-0.049)	0.063 (0.052-0.093)	6.925
95	0.017 (0.012-0.024)	0.075 (0.049-0.151)	2.022
96	0.0083 (0.0064-0.01)	0.019 (0.015-0.031)	3.527
97	0.0029 (0.002-0.0038)	0.008 (0.0059-0.014)	2.949
98	0.0066 (0.0046-0.0086)	0.018 (0.013-0.032)	2.9
99	0.024 (0.018-0.034)	0.1 (0.065-0.196)	2.101
100	0.0083 (0.0064-0.011)	0.021 (0.016-0.035)	3.143
101	0.0049 (0.0039-0.0058)	0.0085 (0.0071-0.011)	5.366
102	NE	NE	NE
103	NE	NE	NE
104	0.017 (0.012-0.022)	0.059 (0.041-0.108)	2.326
105	0.064 (0.044-0.101)	0.484 (0.284-1.609)	1.455
106	NE	NE	NE

NE = not effective under the conditions of experiment.

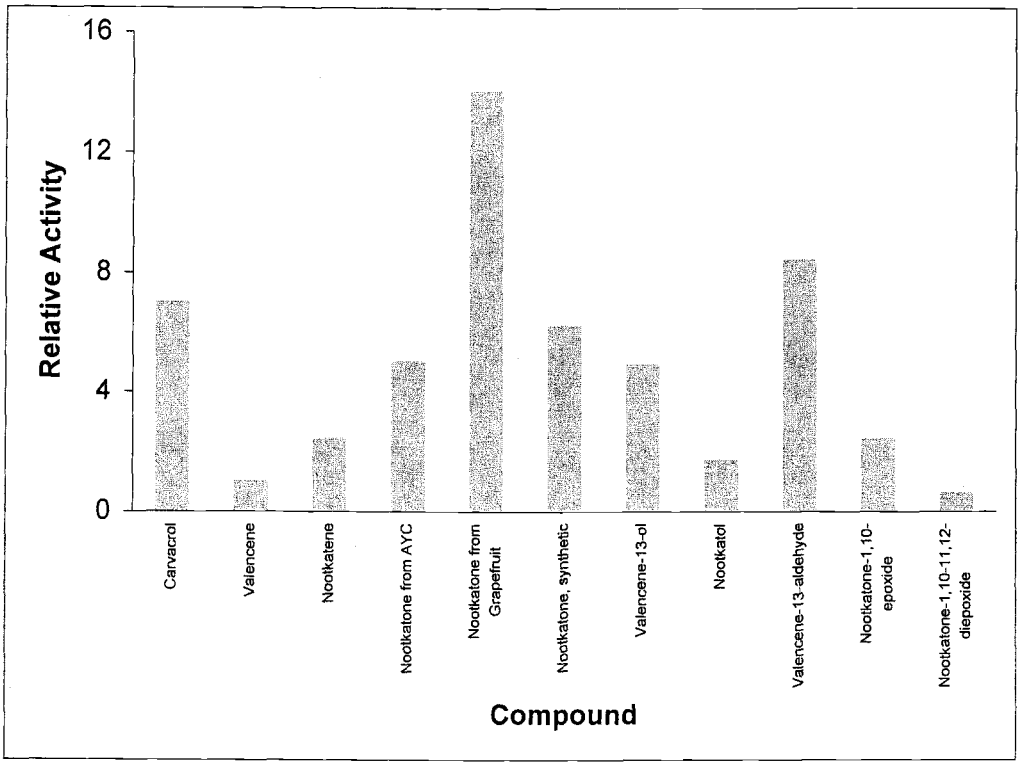


Figure 23: R.A versus oxidation level for compounds against adult *X. cheopis*.

In category I, the first three compounds—3-carene (**97**), terpinene-4-ol (**98**) and methyl carvacrol (**36**) were found to be ineffective against *X. cheopis* under all concentration studied in this work. This observation is in agreement with that made for the same compounds against *I. scapularis*. Carvacrol (**35**), however, was found to have good activity (LC_{50} = 0.0059% w:v), which is in the same order as the activity of carvacrol against *I. scapularis*. Nootkatin (**32**) was found to be

ineffective against *X. cheopis* at all concentrations tested, which also is in good agreement with the results found for nootkatin against *I. scapularis*.

For category II compounds, valencene (compound **41**) exhibited a relatively good activity against *X.cheopis* ticks (LC_{50} = 0.041% w:v) with a large slope, which is reflected by the relatively low LC_{90} of 0.063% w:v. This activity is about 15 times greater than the activity of the same compound against *I scapularis* in terms of LC_{50} and more than 700 times in terms of LC_{90}. The reason for the improved activity in terms of LC_{90} is the very larger value of the slope of the dose-response curve against *X. cheopis* compared to that against *I. scapularis*. Nootkatene (compound **39**) is only about 2 times more active than valencene in terms of LC_{50} with a smaller slope, which makes valencene slightly more active in terms of LC_{90} values.

The three samples of nootkatone (compounds **40a-c**) exhibited comparable activities against *X. cheopis*, with compound **40b** (nootkatone from grapefruit) having twice the activity as the other two compounds.

Nootkatol (compound **42**) exhibited a relatively moderate activity against *X. cheopis*. Compared to nootkatone, its activity was reduced by a factor of 3 in terms of LC_{50} values. In comparison to valencene, it was found to be only about 2 times more active in terms of LC_{50} and about 2 times less active in terms of LC_{90} values.

Valencene-11,12-diol (compound **43**) was found completely inactive against *X. cheopis* ticks. This result is in general agreement with the result of the activity of compound **62** against *I. scapularis*.

Valencene-13-aldehyde (compound **91**) exhibited activity that was comparable to compound 97 in terms of LC_{50} and LC_{90}. These two compounds (**91** and **40b**) are therefore the most active against *X. cheopis* among all the studied compounds. Compound **91** is about two times more active than valencene-3-ol (compound **43**) both in terms of LC_{50} and LC_{90}.

Compound **92** exhibited reduced activity compared to its parent compound (nootkatone) with LC_{50} value of 0.017% w:v and a slope of 2.3. This is about half of the activity of nootkatone against *X. cheopis*. Compound **94** exhibited an even lower activity than compound **92** with an LC_{50} value of 0.064% and a slope of 1.4. Finally, compound **93** was found inactive against *X. cheopis* at all the concentration range.

For discussion of the trends obtained for the activity values of the studied compounds against *X. cheopis*, the same subcategories discussed in section 4.4.1.1. will be used here.

4.4.1.2.1 Valencene series I, II and III

In valencene series I, from figure 24, it can be seen that the activity slightly increases from valencene to nootkatol and from valencene to nootkatone. A similar trend was observed for the same compounds against *I. scapularis*, but to a much smaller degree mainly because of the enhanced activity observed for valencene against *X. cheopis*. This increase in activity is parallel to the introduction of polar groups on position 2 of valencene. Therefore, in the valencene series I, activity increases with increasing the oxidation level of the compound at position 2. A similar observation can be detected in the valencene series II, in which the oxidation was performed on the side-chain. In fact, valencene-13-aldehyde (compound **91**) was found to have the best activity against *X. cheopis* among all the studied compounds. Therefore, compounds **91** and nootkatone can be good candidates for lead structures. On the other hand, in valencene series III, the dihydroxylated product of valencene (compound **62**) was found to be completely inactive against *X. cheopis*, a finding that goes in line with the results of activity against *I scapularis* nymphs.

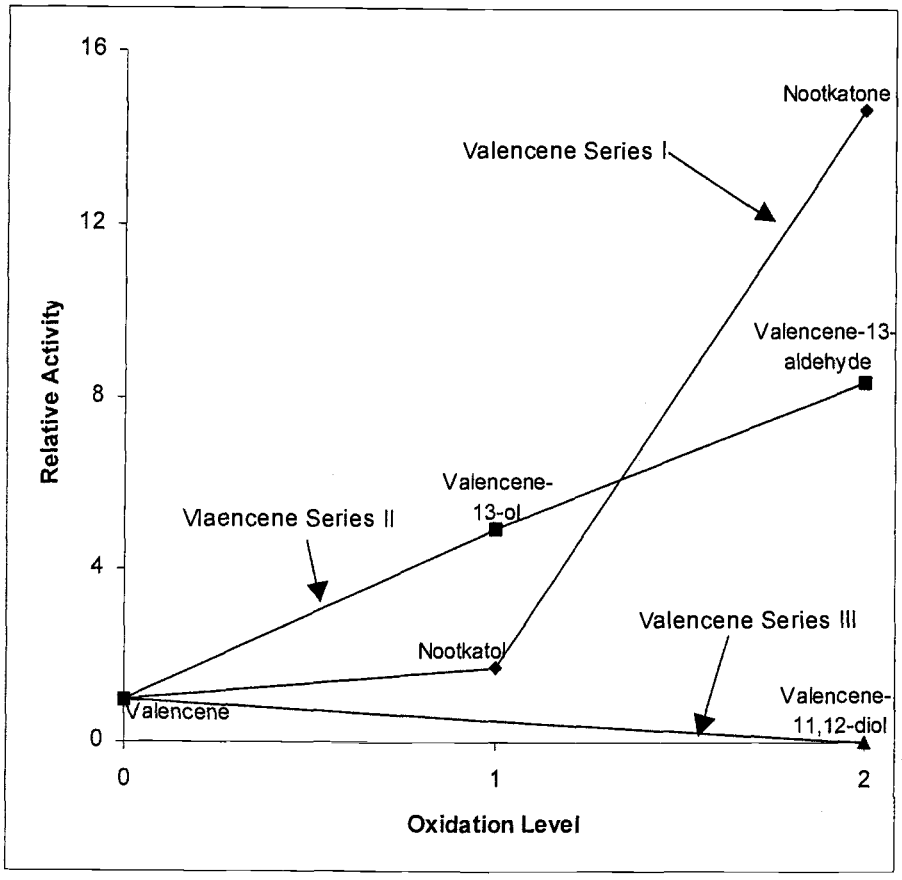


Figure 24: R.A. versus oxidation level of valencene series I, II and III against *X. cheopis* adult fleas.

These results demonstrate that in the valencene series I and II, activity increases with the introduction of polar functional groups with the ability for H-bonding and other polar interactions in both C-2 and C-13 as in compounds **40a-c** and **42** and with the associated decrease in the volatility of these compounds. It should be pointed out here that the same trends observed here were observed in the case of activity against *I. scapularis*.

4.4.1.2.2 Nootkatone series I and II

The relative activity values for compounds of these subcategories are graphed against the oxidation level as shown in figure 25. As can be seen in this figure, the activity of the test compound decreases as the oxidation level increases. This is the opposite of the trend observed for valencene series I and II and the same trend for valencene series III.

In the nootkatone series I and II, the activity decreases with the increase in polarity and decrease in volatility. Conformation may be more important than polarity in these series, because of the substantial change in hybridization of the olefinic carbon atoms. There is another interesting observation to note in the comparison of nootkatone series I and II. In the series I, the activity decreases on the epoxidation of the 1,10-double bond (compound **92**) and continue to decrease on the second epoxidation (compound **94**). This is contrasted to the complete loss of activity on first epoxidation of the 11,12-double bond (compound **93**) and then the subsequent restoration of the activity upon epoxidation of the 1,10-double bond (compound **94**) in the series II. In addition to the previously mentioned factors, this comparison suggests that the side chain double bond may be important. This last suggestion is supported by several findings mentioned earlier.

This shows that, for valencene and nootkatone series compounds, nootkatone,valencene-13-ol (**43**), and valencene-13-aldehyde (**91**) resemble the

"optimum oxidation level" that is reflected by the highest activity observed against *X. cheopis*.

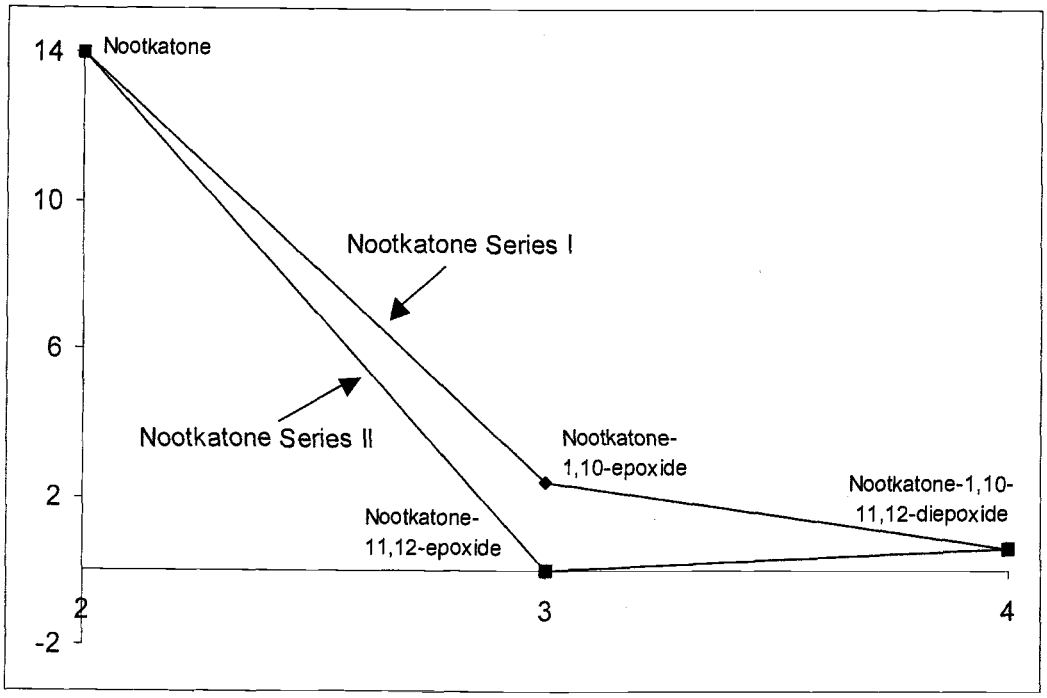


Figure 25: R.A. versus oxidation level of nootkatone series I and II against *X. cheopis* adult fleas.

4.4.1.3 Activity against *Ae.aegypti* adult mosquitoes

The complete results of this study is summarized in table 15 and graphed in figure 26. For category I compounds, 3-carene, terpinen-4-ol and methyl carvacrol (compounds **97, 98 and 36**) showed no activity towards *Ae. aegypti* mosquitoes

under the studied conditions. Carvacrol (**35**), however, exhibited moderate biological activity under the same conditions (LC_{50} = 0.0051% w/v) with a slope of 2.9. These results are comparable with those obtained for the same compounds against *I. scapularis* and *X. cheopis* discussed in the previous two sections.

Nootkatin (compound **32**) was found to have very week activity against *ae. aegypti* under all concentrations studied (LC = 0.85% w:v). Although nootkatin exhibited this low activity against *ae. aegypti*, it is still considered practically inactive, a finding observed for the same compound against other pests studied here. It is interesting to stress this finding as opposed to the previously known high antifungal activity of nootkatin [142].

Valencene (**41**) exhibited a reasonably moderate activity against *ae. aegypti* mosquitoes (LC_{50} = 0.015% w:v) with a relatively high slope. In terms of LC_{50} and LC_{90} values, this activity is the highest observed for valencene against the three tested pests. Nootkatene (**39**) is now about 2 times less active than valencene, a unique observation against *ae. aegypti* among the studied pests. It should be pointed out that because of this relatively high activity of valencene against *ae. aegypti*, the relative activity values (R.A.) for the study compounds are smaller in this case compared to other pests.

Table 15: Response of *A. aegypti* after 24 h exposure.

Compound	LC_{50}	LC_{90}	Slope
35	0.0051	0.014	2.908
41	0.015 (0.008-0.029)	0.037 (0.031-0.162)	3.199
39	0.027	0.059	3.696
40a	0.0057	0.0092	6.22
40b	0.0046 (0.004-0.0053)	0.0087 (0.0072-0.011)	4.635
40c	0.0075	0.021	2.845
42	0.004 (0.0032-0.0048)	0.01 (0.008-0.014)	3.201
43	0.0034 (0.0024-0.0045)	0.014 (0.01-0.023)	2.094
91	0.0024	0.0034	8.714
62	0.295 (0.21-0.522)	1.911 (0.922-7.257)	1.581
93	NE	NE	NE
92	0.223 (0.158-0.402)	2.001 (0.873-10.537)	1.346
94	0.059	0.114	4.472
32	0.852	7.869	1.327

NE = not effective under conditions of the experiment.

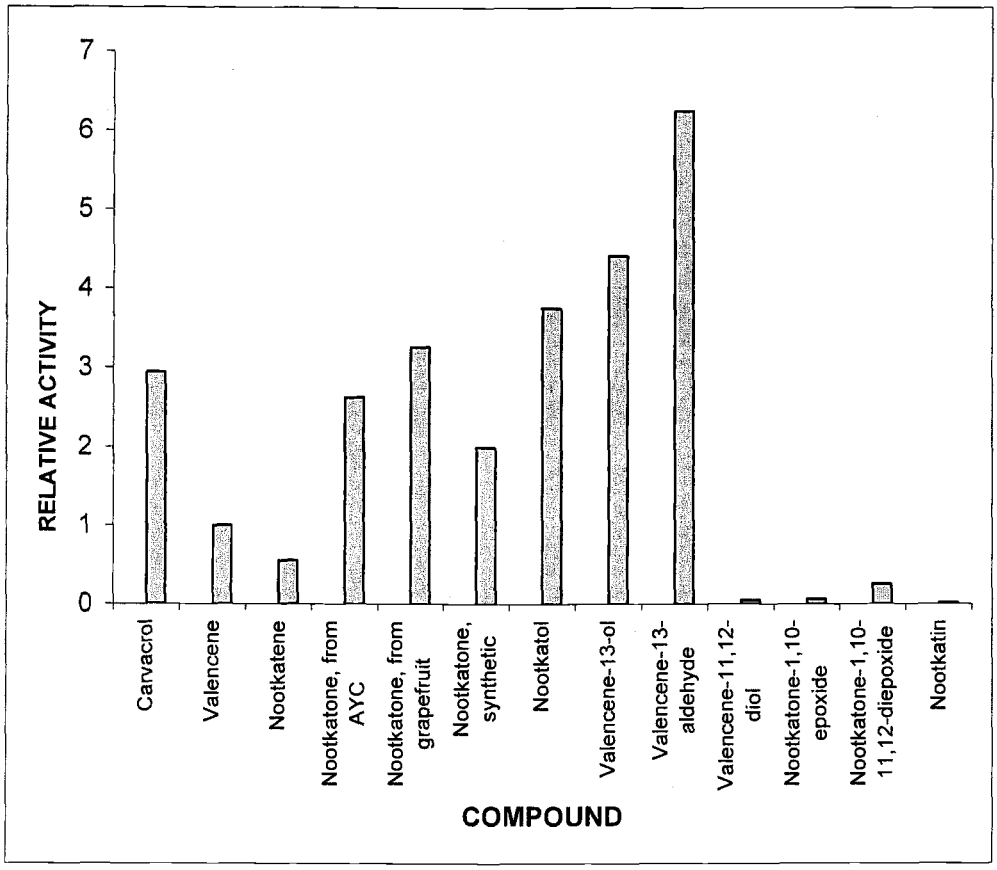


Figure 26: R.A. versus name of the test compound against *A. aegypti* adult mosquitoes.

The three samples of nootkatone (**40a-c**) exhibited activity values that are comparable to each other with the first two compounds having a slightly higher activity (LC_{50} values = 0.0057, 0.0046, 0.0075% w:v, respectively). These activity values are in general agreement with those obtained for three samples of nootkatone against *I. scapularis* and *X. cheopis*.

Nootkatol (**42**) exhibited a relatively good activity against *ae.aegypti.* Compared to nootkatone, its activity was in the same order in terms of LC_{50} and

LC_{90} values, and in comparison to valencene, it was found to be about 4 times more active with the same value of slope. Valencene-11,12-diol (**62**), exhibited low activity against *ae. aegypti* (LC_{50} = 0.295% w:v). This activity is much higher than those observed for the same compound against *I. scapularis* and *X. cheopis*.

Valencene-13-aldehyde (**91**) exhibited the best activity among all compounds tested in terms of LC_{50} and LC_{90} (values were 0.0024 and 0.0034% w:v, respectively) with a large slope value. Compared to the results against other pests, this compound exhibits the best results against *ae. aegypti*. This compound is about 6 and 11 times more active than valencene in terms of LC_{50} and LC_{90}, respectively.

Valencene-13-ol (**43**) is also one of the best compounds studied here in activity against *ae.aegypti*. Its activity is slightly less than that of **91** and in the same order as nootkatone. When compared to valencene, it is about 4 times more active in terms of LC_{50} values. Compared to its activity against other pests, compound **43** has a relatively improved activity (LC_{50} = 0.0034 against *ae. aegypti* versus 0.0051 and 0.0083% w:v against *I. scapularis* and *X. cheopis*, respectively).

The last three compounds (**92-94**) have reduced activity values compared to their parent compound (nootkatone). In general terms, these compounds are among the poorest in activity among compounds of category II (except compound **62**) against *ae. aegypti* as well as other pests tested in this study (LC_{50} values for compounds **92** and **94** are 0.223 and 0.059% w:v, respectively, while compound **93** was found inactive).

4.4.1.3.1 Valencene series I, II and III

The following diagram (figure 27) represents a sketch drawing of the relative activity as defined above versus oxidation level of compounds belonging to these subcategories.

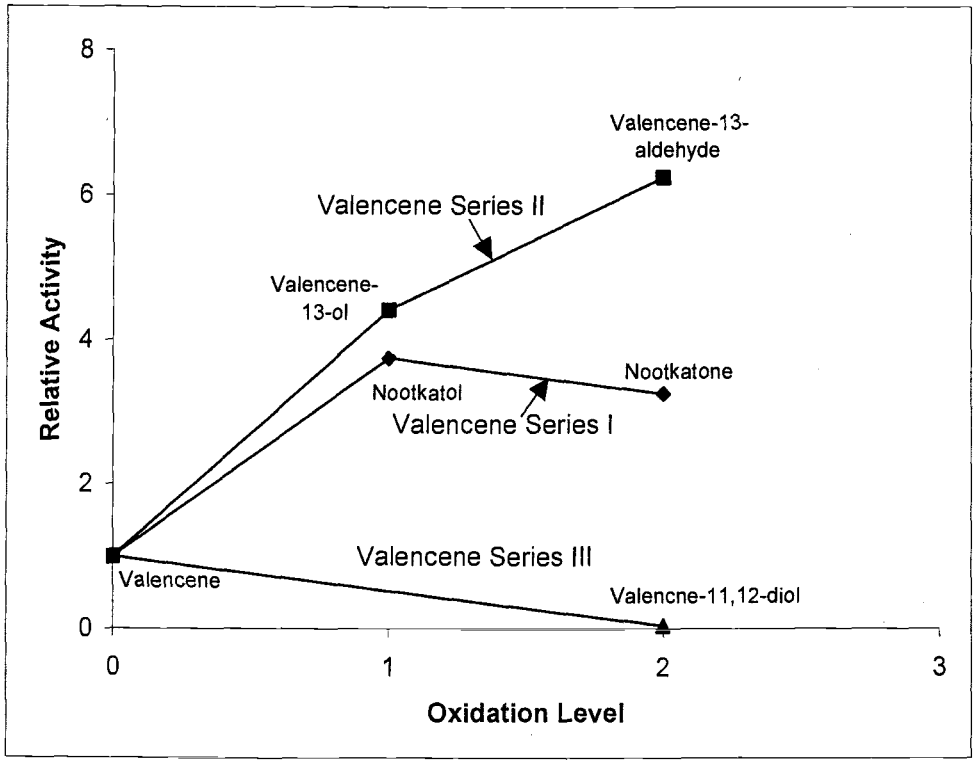


Figure 27: R.A. versus oxidation level for valencene series I, II and III compounds against *A. aegypti* adult mosquitoes.

From this figure, it can be seen that in the valencene series I, activity increases with increasing the oxidation level of the test compound from 1 for the case of valencene to about 4 for nootkatone. It is noteworthy to mention that this increase in the activity is less steep compared to that in the case of *I. scapularis*, but in the same order of that in the case of *X. cheopis*. The reason for this is the higher activity of the reference compound (valencene) observed against *X. cheopis* and *Ae. aegypti*. For the case of valencene series II, a similar trend can be observed on going from valencene to valencene-13-ol (compound **43**, with R.A. of 4 in terms of LC_{50}) and to valencene-13-aldehyde (compound **91**, with R.A. of 6 in terms of LC_{50}). This trend is similar to that observed for valencene II series in the case of *X. cheopis*, but slightly different from that observed for the same compounds in the case of *I. scapularis*.

As was observed in the case of activity against *I.scapularis* and *X. cheopis*, activity of valencene series I and II compounds generally increases with the introduction of polar functional groups with the ability for H-bonding/polarity as in nootkatone (**40a-c**) and nootkatol (**42**) compared to valencene itself and with the decrease in the volatility of the test compounds.

In the valencene series III, the trend is opposite to that observed in both valencene I and II series. The activity decreases dramatically with the introduction of the two hydroxyl functional groups at C-11 and C-12 positions as in compound **62**. This finding is in full agreement with those observed in the case of activity against *I.scapularis* and *X. cheopis*.

4.4.1.3.2 Nootkatone series I and II

The relative activity values for compounds of compounds of these subcategories are graphed against the oxidation level as shown in figure 28. As can be seen in this figure, the activity of the test compound decreases as the oxidation level increases. This is in general similar to the trend observed for activity of nootkatone series I and II against *I. scapularis* and *X. cheopis*.

In the nootkatone series I and II, the activity decreases with the increase in polarity and decrease in volatility. Conformation may be more important in these compounds, because of the substantial change in hybridization of the olefinic carbon atoms. There is however an interesting observation to note in the comparison of nootkatone series I and II. In the series I, the activity decreases on the epoxidation of the 1,10-double bond (compound **92**) and continue to decrease on the second epoxidation (compound **94**). This is contrasted to the complete loss of activity on first epoxidation of the 11,12-double bond (compound **93**) and then the subsequent restoration of the activity upon epoxidation of the 1,10-double bond (compound **94**) in the series II. In addition to the previously mentioned factors, this comparison suggests that the side chain double bond may be important.

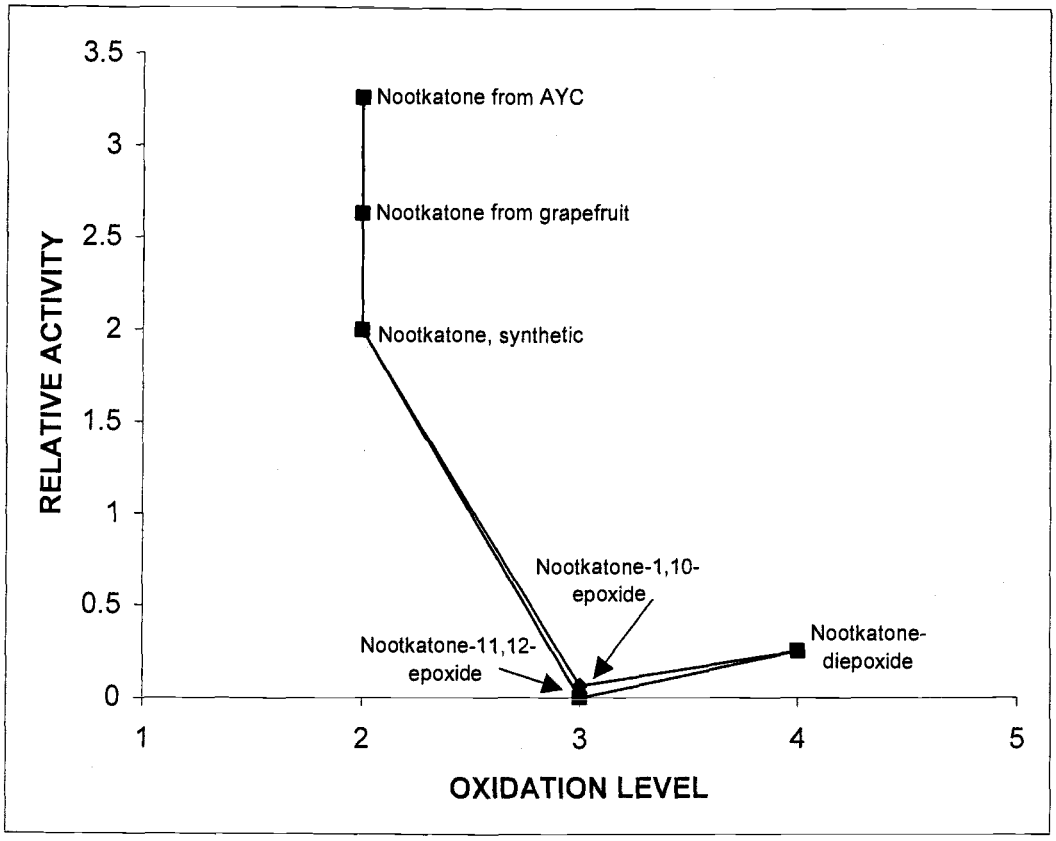


Figure 28: R.A. versus oxidation level of nootkatone series I and II against *A.aegypti* adult mosquitoes.

4.4.1.4 Conclusions of the 24 h study

In summary, 15 natural and semisynthetic compounds derived from the heartwood extract of AYC were studied for their short-term (24 h) pesticidal activities against three common pests of great health importance. These compounds were divided into three main categories: monoterpenes (category I),

sesquiterpenes with eremophilane ring structure type (category II) and sesquiterpenes with the tropolone ring type (category III).

Of category I, carvacrol showed good activity against all the three pests used in this study while other monoterpenes were found completely inactive. The wide availability of carvacrol in many natural sources and the ease of its isolation in a pure form make it a good pesticide lead candidate.

Of category III, the single tropolone sesquiterpene nootkatin was found to exhibit no detectable activity against *I. scapularis* nymphs and *X. cheopis* adults and only a very weak activity against *Ae. aegypti* adults.

For category II compounds, the studied compounds were divided into subcategories and results were discussed in terms of structural similarities among compounds of one subcategory. For the three studied pests, the following generalizations of activity patterns can be made:

- Valencene series I: Valencene < Nootkatol < Nootkatone
- Valencene series II: Valencene < Valencene-13-ol≤ Valencene-13-aldehyde
- Valencene series III: Valencene > Valencene-11,12-diol
- Nootkatone series I: Nootkatone > Nootkatone-1,10-epoxide > Nootkatone-1,10-11,12-diepoxide
- Nootkatone series II: Nootkatone >> Nootkatone-1,10-11,12-diepoxide >> Nootkaton-11,12-epoxide

In the valencene series I and II, the trend was that activity increases with oxidation level, while in the series III, the opposite trend was observed. In the nootkatone series I and II, the activity decreases with the change in the conformation and the decrease in volatility of the test compound.

In general, among the 15 compounds tested in this study, nootkatone, valencene-13-aldehyde, and valencene-13-ol resemble the best performing candidates for pesticide lead compounds. These compounds therefore resemble the optimum structures for this biological activity.

4.4.2 Residual Activity Studies

The purpose of residual activity experiments was to determine the persistence of compounds that exhibited the most promising activity patterns in the short-term exposure studies. Towards that end, the promising compounds were studied for their activities against the same three pests studied in the short-term experiments 1, 2 and 4 weeks after their first application. In all experiments, the exposure period was limited to the final 24 h of the experiment as outlined in the experimental. It is worthwhile mentioning that one of the limitations of these experiments was the scarcity of materials available for testing, which was mainly because of the difficulty encountered in obtaining those compounds in enough quantities from the natural source. Since valencene was not included in these studies, nootkatene was chosen as a reference to calculate relative activities against *I. scapularis* and *A. Aegypti* according to the equation:

$$R.A. = \frac{(LC_{50})_{(Nootkatene)_{week1}}}{(LC_{50})_{compound}}$$

For the residual activity against *X. cheopis*, valencene-13-aldehyde was used as a reference as can be seen in the following equation:

$$R.A. = \frac{(LC_{50})_{(valencene-13-aldehyde)_{week1}}}{(LC_{50})_{compound}}$$

4.4.2.1 Residual activity against *Ixodes scapularis* nymphs

The residual activity patterns of compounds **39**, **40a**,**b**,**c**, **43**, and **91** against *I. scapularis* are given in table 16. These results are shown graphically in figure 29.

From these data, we can see that nootkatene (**39**) has a rapidly decaying activity pattern. Its LC_{50} value increases from 0.011% (w:v) in the short-term 24 h exposure experiment to 2.06% (w:v) after week 1 of the residual activity experiment. It showed no detectable activity beyond week 1 in the residual activity experiment.

For nootkatone sample isolated from AYC heartwood (compound **40a**), the activity decreased substantially from the short-term experiment value (LC_{50} = 0.0029% w/v) but stays essentially the same beyond week 1 through week 4 of

study. On the other hand, nootkatone isolated from grapefruit (compound **40b**) showed a fast activity decay pattern (the short-term LC_{50} = 0.0061%, 0.025 in week 1 and 0.25% w:v in week 4). Nootkatone sample synthesized from valencene (compound **40c**) exhibited a similar activity (to compound **40b**) pattern in the sense that the activity decreased in the first week (LC_{50} = 0.0033 in the short-term to 0.0084% w:v) and stayed essentially the same through week 2. Unfortunately, the activity was not monitored in week 4 after application of compound 98.

13-Hydroxy valencene (compound **43**) exhibited a steadily decreasing activity pattern from the short-term experiment through week 4 ((LC_{50} = 0.0051, 0.0071, .0083, 0.031% for the short-term experiment and week 1, 2 and 4, respectively). Finally, valencene-13-aldehyde (compound **91**) exhibited a relatively faster decreasing activity pattern after week 1 (LC_{50} = 0.026 increasing from 0.0059 % in the short-term activity experiment). This compound was not monitored beyond week 1 of the residual activity experiment.

Nootkatene (**39**) completely lost its activity two weeks after application. Since nootkatene was found to have only weak activity in the 24h study, it is generally not a good candidate for a lead compound according to these results. Nootkatone isolated from AYC (**40a**) suffered approximately a factor of 10 decrease in the activity after one week of the application, but showed essentially the same activity beyond that. Nootkatone from grapefruit oil (**40b**) exhibited a somewhat unusual

Table 16: Residual activity against *I. scapularis* nymphs at 1, 2 and 4 weeks.

Compound	Weeks	LC_{50}	LC_{90}	Slope
39	1	2.062	524.6	0.533
39	2	NE	NE	NE
39	4	NE	NE	NE
40a	1	0.023	0.13	1.682
40a	2	0.026 (0.018-0.039)	0.168 (0.094-0.448)	1.586
40a	4	0.026 (0.018-0.039)	0.164 (0.093-0.429)	1.609
40b	1	0.025	0.071	2.871
40b	2	0.019 (0.014-0.024)	0.056 (0.04-0.094)	2.727
40b	4	0.25	0.71	2.871
40c	1	0.0084 (0.005-0.013)	0.08 (0.045-0.22)	1.304
40c	2	0.0089 (0.0068-0.012)	0.027 (0.02-0.045)	2.649
43	1	0.0071 (0.0055-0.0091)	0.019 (0.014-0.031)	3.005
43	2	0.0083 (0.0057-0.011)	0.044 (0.029-0.081)	1.781
43	4	0.031	0.303	1.285
91	1	0.026 (0.02-0.033)	0.072 (0.052-0.121)	2.859

NE = not effective at concentrations tested.

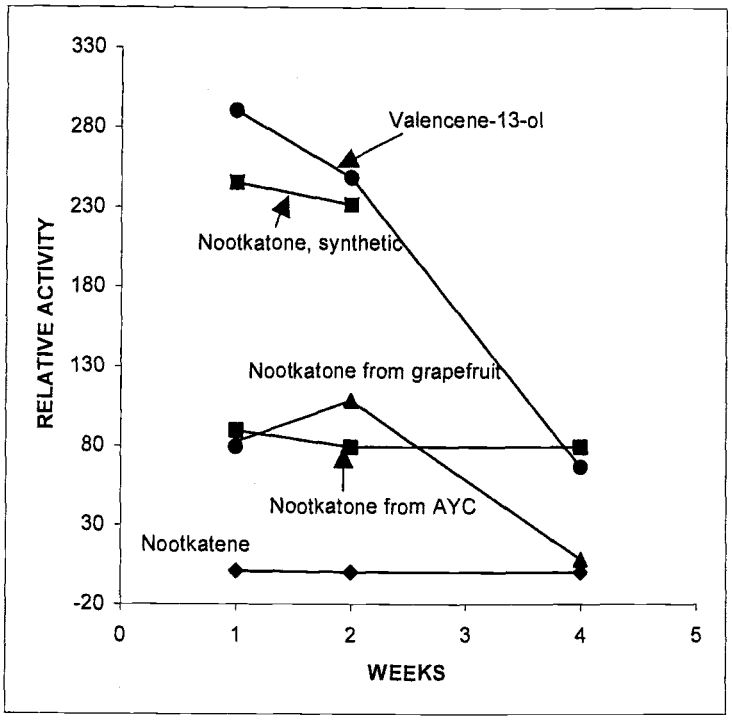


Figure 29: Residual activity of studied compounds against *I. scapularis* nymphs.

behavior. Its activity decreased by a factor of 4 in week 1, marginally increased in week 2 and eventually deteriorated in week 4 of the residual study.

Regardless of the difference in behavior among the three samples of nootkatone, the general finding that nootkatone exhibited a strong activity in the 24 h studies and the fact that nootkatone is generally safe both positively testify for its potential as a candidate for a promising lead compound. The difference in behavior between the three samples of nootkatone could be referred to the difference in the impurity nature and concentration among the three samples. One

observation needs to be stressed here is that compound **40c** (semisynthetic nootkatone) maintained a higher residual activity over the test period.

Valencene-13-ol (**43**) exhibited only a slightly decreased residual activity through week 3, which largely decreased after week 4 of application. This behavior can be ideal in some applications that require moderately persistent pest control agents and therefore this compound can be a good lead compound. Compound **91** has an activity behavior that is similar to compound **40a** up to one week after application. Although this compound was not monitored beyond week 1 of the application, it is generally still a good candidate given its good activity in the 24h study.

In summary, the residual activity experiments against *I. scapularis* produced that nootkatone was the most persistent component among the eremophilane sesquiterpenoids derived from AYC or synthesized from its constituents showing promising insecticidal properties against *I. scapularis* nymphs. This finding emphasizes the importance of this compound as an insecticidal lead compound, from which improved candidates could be derived. Since nootkatone has long been used as food flavoring it is generally safe for repeated usage, which increases its value in this context. The surprising finding that the nootkatone isolated from grapefruit has a much faster rate of decay is rather intriguing and needs more investigation. Vlaencene-13-ol (**43**) and valencene-13-aldehyde (**91**) showed promising potential for lead compounds similar to nootkatone.

4.4.2.2 Residual Activity against *Xenopsylla cheopis* adults

The activity results of compounds **39**, **40a**,**b**,**c**, **43** and **91** are given in table 17 and the results are graphed in figure 30.

Nootkatene (compound **39**) showed no detectable activity for week 1 experiment and beyond even though it exhibited a medium activity pattern in the short-term experiment. This finding is in good agreement with the previous finding for nootkatene residual activity against *I. scapularis*.

Nootkatone isolated from AYC (compound **40a**) suffered a slight decrease in activity pattern (LC_{50} = 0.0083, 0.016, 0.031% for the short-term, week 1, and week 2). This sample was not monitored beyond week 2 for residual activity. This observation is in clear disagreement with those observations made for the same sample against *I. scapularis*. Nootkatone isolated from grapefruit (compound **40b**) showed a substantial decrease in the residual activity after week 1 compared to the 24 h (LC_{50} values were 0.02 and 0.0029% w/v, respectively). This trend continued in the same manner in weeks 2 and 4 after first application of compound **40b**. For synthetic nootkatone (compound **40c**), there was a substantial decrease in the activity of week 1 compared to the short-term activity (LC_{50} = 0.043 compared to 0.0075%, respectively). This decrease in activity was followed by a sudden *increase* in the activity on going to week 2 activity (LC_{50} = 0.027%). The activity for week 4 returned to the normal trend of decreasing value (LC_{50} = 0.043%). This is opposed to the activity pattern observed for the same compound

Table 17: Residual activity against *Xenopsylla cheopis* nymphs at 1, 2 and 4 weeks.

Compound	Weeks	LC_{50}	LC_{90}	Slope
39	1	NE	NE	NE
40a	1	0.016 (0.012-0.02)	0.05 (0.036-0.081)	2.546
40a	2	0.031	0.134	2.033
40b	1	0.02 (0.016-0.026)	0.053 (0.04-0.082)	3.107
40b	2	0.018 (0.013-0.024)	0.081 (0.054-0.156)	1.931
40b	4	0.035 (0.026-0.051)	0.161 (0.098-0.387)	1.950
40c	1	0.043	0.259	1.633
40c	2	0.027	0.085	2.571
40c	4	0.043 (0.034-0.056)	0.111 (0.08-0.193)	3.135
43	1	0.03 (0.022-0.041)	0.113 (0.074-0.226)	2.218
43	2	0.039 (0.029-0.053)	0.131 (0.087-0.268)	2.442
91	1	0.059	0.148	3.211

NE = not effective at concentrations tested.

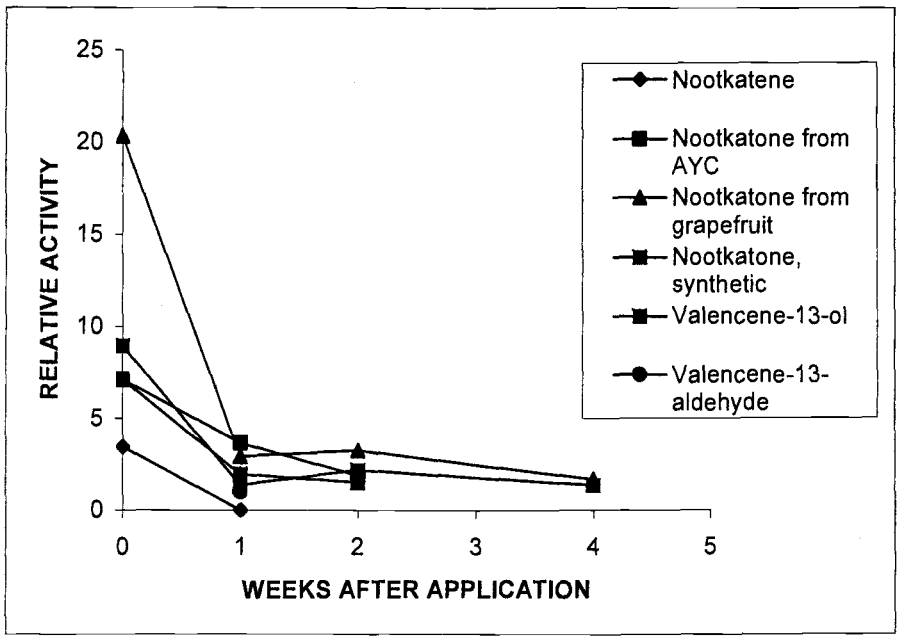


Figure 30: Residual activity results of studied compounds against *X. cheopis*.

against *I. scapularis* in which the activity decreased slightly after one week of application and stayed essentially the same beyond that.

Activity pattern for compound **43** showed a continuously decreasing pattern with time (LC_{50} = 0.0083, 0.03 and 0.039% for the short-term, week 1 and week 2 activity results, respectively). This trend is similar to that observed for the same compound against *I. scapularis* nymphs.

Finally, valencene-13-aldehyde (compound **91**) exhibited a decreased activity after 1 week of application compared to the short-term experiment results

(LC_{50} = 0.059 compared to 0.0049%). This observation is in line with activity pattern of the same compound against *I. scapularis*.

4.4.2.3 Residual Activity Against *Aedes Aegypti*

The 1, 2, and 4 week residual activity results of compounds **39**, **40a**,**b**,**c**, **43** and **91** against *Aedes Aegypti* are given in table 18 and are graphed in figure 31.

Nootkatene (compound **39**) exhibited a reduced residual activity for week 1 experiment (LC_{50} = 0.132%) and lost any detectable activity against *ae. aegypti* beyond week 1. This finding is in good agreement with the previous finding for nootkatene residual activity against *I. scapularis* and to some degree for activity against *x. cheopis* in the sense that the activity decreases with time and is completely lost after one or more weeks of the first application of nootkatene.

Nootkatone isolated from AYC (compound **40a**) suffered a slight decrease in activity pattern with time (LC_{50} = 0.0057, 0.02, 0.019 and 0.013% for the short-term, week 1, week 2, and week 4, respectively). This observation is in agreement with the activity pattern observed for the same compound against *x. cheopis*, but in clear disagreement with those observations made for the same sample against *I. scapularis*, in which the activity stayed essentially the same beyond week 1 of application.

Table 18: Residual activity against *Aedes Aegypti* adults at 1, 2 and 4 weeks.

Compound	Weeks	LC_{50}	LC_{90}	Slope
39	1	0.132 (0.096-0.236)	0.659 (0.329-0.855)	1.833
39	2	NE	NE	NE
39	4	NE	NE	NE
40a	1	0.02 (0.014-0.029)	0.054 (0.041-0.105)	3.043
40a	2	0.019 (0.016-0.022)	0.046 (0.037-0.061)	3.342
40a	4	0.013 (0.011-0.0105)	0.038 (0.031-0.051)	2.698
40b	1	0.0065	0.014	3.858
40b	2	0.0067 (0.0058-0.0076)	0.012 (0.01-0.015)	4.953
40b	4	0.02	0.029	7.772
40c	1	0.0089 (0.0077-0.01)	0.018 (0.015-0.023)	4.336
40c	2	0.0042 (0.0036-0.0049)	0.0088 (0.0074-0.011)	4.014
40c	4	0.018	0.036	4.169
43	1	0.024 (0.02-0.028)	0.058 (0.046-0.081)	3.286
43	2	0.014 (0.012-0.016)	0.021 (0.019-0.027)	6.932
43	4	0.013 (0.0089-0.017)	0.04 (0.029-0.074)	2.645
91	1	0.011 (0.0095-0.013)	0.027 (0.022-0.037)	3.392
91	2	0.014 (0.0074-0.025)	0.037 (0.029-0.127)	3.136

NE = not effective at concentrations tested.

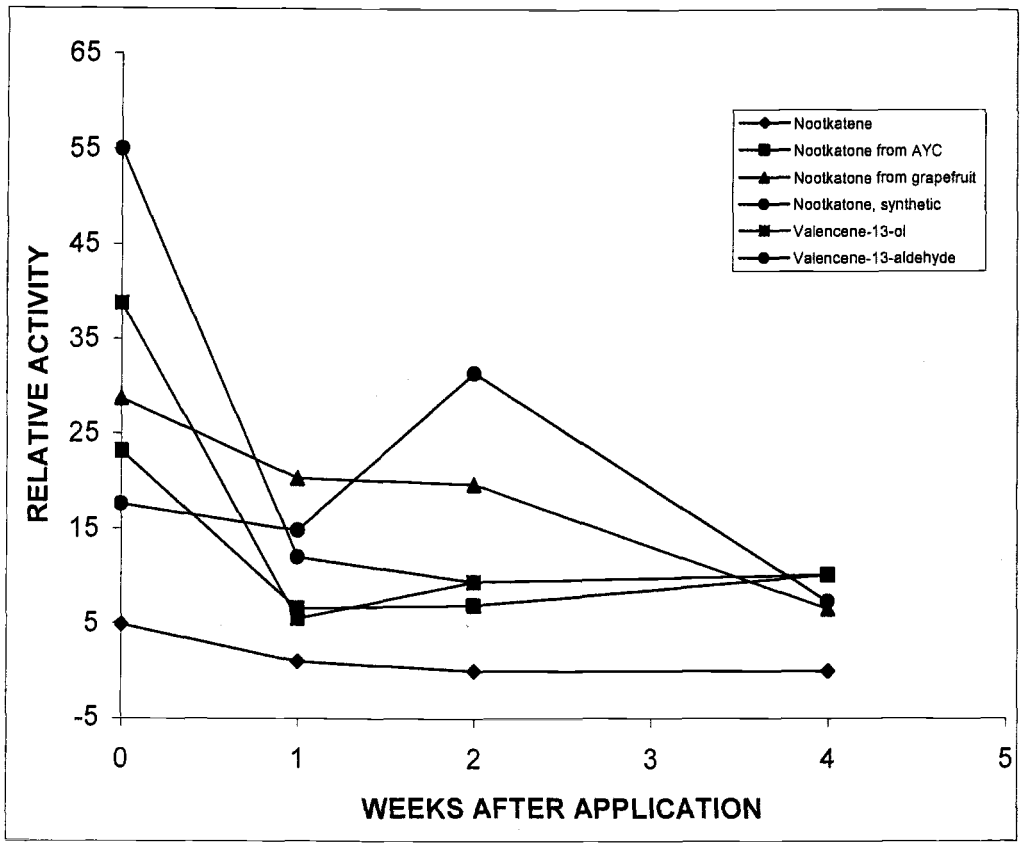


Figure 31: Residual activity results of studied compounds against *A .aegypti.*

Nootkatone isolated from grapefruit (compound **40b**) has an activity pattern that stayed essentially unchanged until beyond week 2 when activity started to degrade. This behavior is in good agreement with those observed for the same compound against *I. scapularis* and *X.cheopis*.

For the synthetic nootkatone (compound **40c**), there was a slight decrease in the activity of week 1 compared to the short-term activity (LC_{50} = 0.0089 compared to 0.0075%, respectively). This decrease in activity was followed by a

substantial *increase* in the activity on going to week 2 activity (LC_{50} = 0.0042%). The activity for week 4 returned to the normal trend of decreasing value (LC_{50} = 0.018%). This is good agreement with the activity pattern observed for the same compound against *X. cheopis* in which the activity decreased slightly after one week of application, but increased in the second week and then decreased again beyond that.

Activity pattern for compound **43** showed a substantial decrease in the activity after week 1 and a reversed trend beyond that period (LC_{50} = 0.0034, 0.024, 0.014 and 0.013% for the short-term, week 1, week 2 and week 4 activity results, respectively). This trend is different from both trends observed for this compound against *I. scapularis* and *X. cheopis*.

Finally, valencene-13-aldehyde (compound **91**) exhibited a somewhat steep decrease in activity after 1 week of application compared to the short-term experiment results and then a slower decrease beyond that period (LC_{50} = 0.011, 0.014 for week 1 and 2 compared to 0.0024% for the short-term experiment). While there is no good basis for comparison the behavior of activity beyond week 1 since the residual activity patterns were not recorded for *X. cheopis* and *I. scapularis*, the data till week 1 indicates that there is a general agreemnet.

4.4.2.4 Conclusions of the residual activity study

In summary, six compounds of the eremophilane sesquiterpenoids family that are isolated from the heartwood extract of AYC or semisynthesized from its constituents have been studied to evaluate their long-term performance as pest control agents. The three human health-related pests that were used for the 24 h study were also used in these experiments and include *I. scapularis* nymphs, *X. cheopis* adults, and *Ae. aegypti adults*.

In general terms, all studied compounds have suffered some degree of deterioration in their activities as a function of time against test pests. Some of the tested compounds were relatively more persistent throughout the experiment period than others. Reasons for these activity patterns could be related to stability or volatility of test compounds or a combination of these two variables.

The criterion of evaluation of these compounds depends on the specific application required. For example, when a long effect persistent pest control agent is required, then compounds such as **40c** and relatively **43** are recommended to be used against *I. scapularis*, and compounds **40b** and **40c** against *Ae. aegypti*. Other applications may require other compounds with optimized properties based on these compounds.

4.5 Implications of phytochemical compounds found in AYC

The unique and complex nature of constituents found in the heartwood oil of AYC was used by Erdtman and Norin as a basis for the discussion of the issue of species change through chemotaxonomy towards the reclassification of AYC [89]. The basis for this issue is that the major constituents present in AYC heartwood being carvacrol, chamic acid, chaminic acid, nootkatin, chanootin, nootkatone, nootkatene and valencene, are unique to this species within the genus *Chamaecyparis*. The closest species to AYC, *Chamaecyparis lawsoniana* (Port Orford Cedar) contains none of these compounds, with the major constituents being β–thujaplicin, β-bisabolene, and α-cadinol [101]—compounds common to most other *Chamaecyparis* species. Carvacrol is an extremely rare compound in the genus *Chamaecyparis*, with the only documented isolation from *Chamaecyparis thoides* [89]. Nootkatin, chamic acid and chaminic acid have been found in no other *Chamaecyparis* species whatsoever. Nootkatin and carvacrol, however are extremely common constituents in the *Juniperus* and *Cupressus* genera [89]. Furthermore, other previously isolated constituents of AYC including nootkatone, nootkatene, valencene, nootkatol, valencene-13-ol have not been found in any other *Chamaecyparis* species. Additionally, the new compounds isolated in this research from the AYC—valencene-11,12-diol, eudesmane-11,12-diol, (+)-syringaresinol, (1S)-2-oxo-3-p-menthenol, (4R)-4-hydroxy-4-isopropyl-cyclohex-1-enecarboxylic acid, which are not found in any *Chamaecyparis* species studied so far add support to the previous argument.

The other piece of evidence that supports the above argument is the absence of any cedrenes or cadinenes common to the genus *Chamaecyparis* from the heartwood of AYC. All the above evidences should be taken into account in the reconsideration of the taxonomic classification of this important species.

5. Conclusions and Future Aspects

5.1 Conclusions

The heartwood of the well-known AYC tree is very valuable for its remarkable properties of durability and resistance to decay. Theses outstanding properties inspired this research in an effort to identify more constituents of this wood (which is focus 1 of this research) and to evaluate the biological activities of these constituents especially in the identification of new pest control lead compounds (focus 2 of this research).

In the first focus of this research, the AYC heartwood was found to contain many constituents that have never been described before in AYC. Other constituents have never been described in any natural source before. In the monoterpene group, two compounds were isolated and identified (unknowns 1 and 4) in this research. These two compounds add to the previously known series of monoterpenes in this tree. The compounds share together a general skeleton, which is the menthane- type skeleton widely encountered in plants. In the sesquiterpene family, two compounds were isolated. One of the compounds has an eremophilane skeleton type common to AYC heartwood. The other compound follows the eudesmane skeleton type, which is the first eudesmane-type compound to be isolated from AYC. This finding hints to the common biosynthetic origin for both eremohpilanes and eudesmanes in natural sources. Finally in this focus, a furfuran-type lignan (unknown 5) was isolated from the heartwood of AYC. This

lignan follows a structure type not commonly encountered in heard woods such as AYC, but rather in softwoods.

In the second focus of this research, fifteen of AYC constituents or derivatives of them were screened for their pest control activity in both 24 h and residual activity over a period of 4 weeks against three pests of public health importance. The 24 h studies revealed that nootkatone, valencene-13-aldehyde and valencene-13-ol were the most active among the studied compounds against the three pests. This suggests that oxidation on both positions C-2 and C-13 of the eremophilane ring structure has an important effect on the activity. These three compounds can be good candidates as pest control lead compounds. However, more compounds need to be synthesized to verify this theory.

The residual studies revealed that the most active compounds exhibited activity profiles that decreased with time. Although the long-term safety of these compounds has yet to be evaluated, the natural origin and the long history of use of these compounds suggest that they can be promising candidates. These dose-mortality results indicate that these compounds—such as nootkatone, carvacrol, 13-hydroxy-valencene, and valencene-13-aldehyde function as effective pest control agents and pesticides. These compounds have the ability to kill insects quickly and maintain a comparable level of activity for several weeks.

5.2 Outlook and Future Aspects

The outcome of this research resembles a very important step of the effort aimed at producing environmentally friendly pest control agents desperately needed these days to replace the much less desired synthetic pesticides currently available. This research suggests that several constituents of AYC heartwood extract and some of their synthetic derivatives are important lead compounds. The natural continuation of this research should include the following goals:

1. More constituents seem to be present in the polar fraction of the heartwood extract of AYC, although in minute quantities. These compounds can be isolated, identified and studied for their bioactivity.
2. From the results of chapter 4, the activity of the eremophilane sesquiterpenes was found to be greatly affected by the oxidation of positions C-2 and C-13 while keeping the general shape of the eremophilane untouched. This hypothesis awaits more studies to provide more insights. In this context, more compounds with different oxidation levels need to be synthesized and tested.
3. Other pests can be tested to provide information about whether the results obtained in this research are general to more species or limited to the studied ones only.

4. The compounds studied in this research can be tested for other bioactivities. In this regard, pest repellent, antifeedant and other related activities are among those to be studied. Anticancer activity can also be studied based on a promising preliminary screening study performed on the heartwood extract of AYC.

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APPENDIX

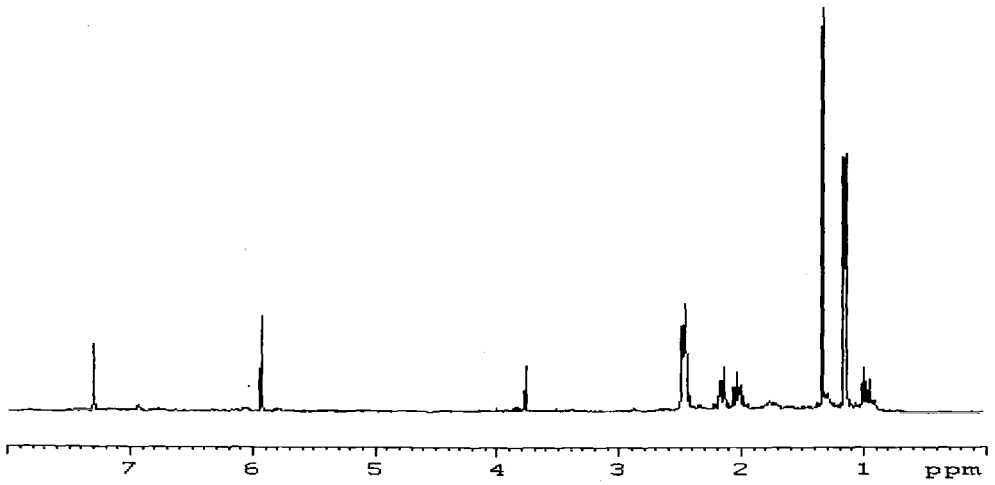

Figure 33: ^{1}H NMR spectrum of unknown 1.

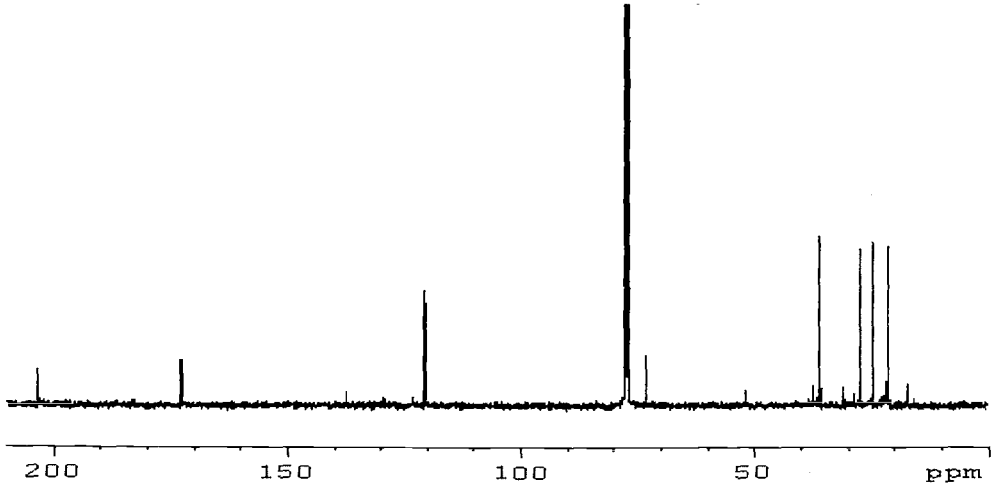

Figure 34: ^{13}C NMR spectrum of unknown 1.

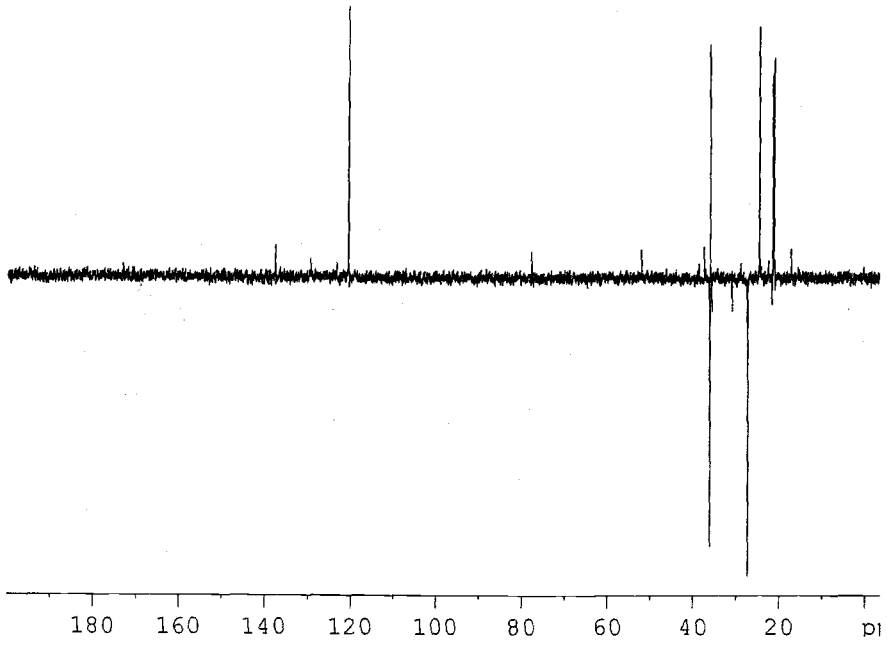

Figure 35: DEPT 135 spectrum of unknown 1.

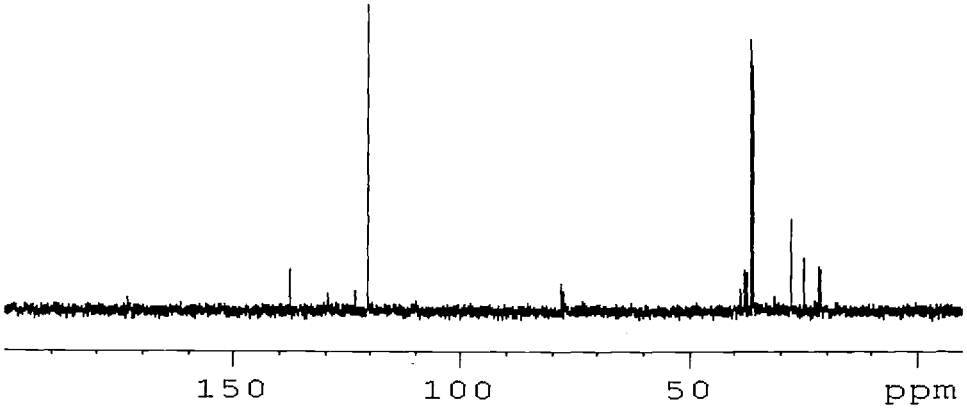

Figure 36: DEPT 90 spectrum of unknown 1.

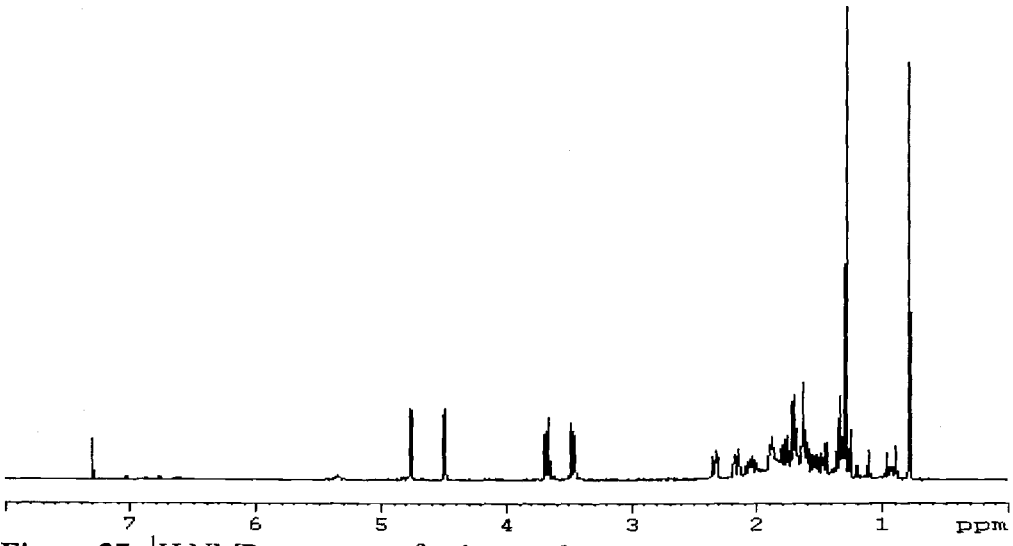

Figure 37: ^{1}H NMR spectrum of unknown 2.

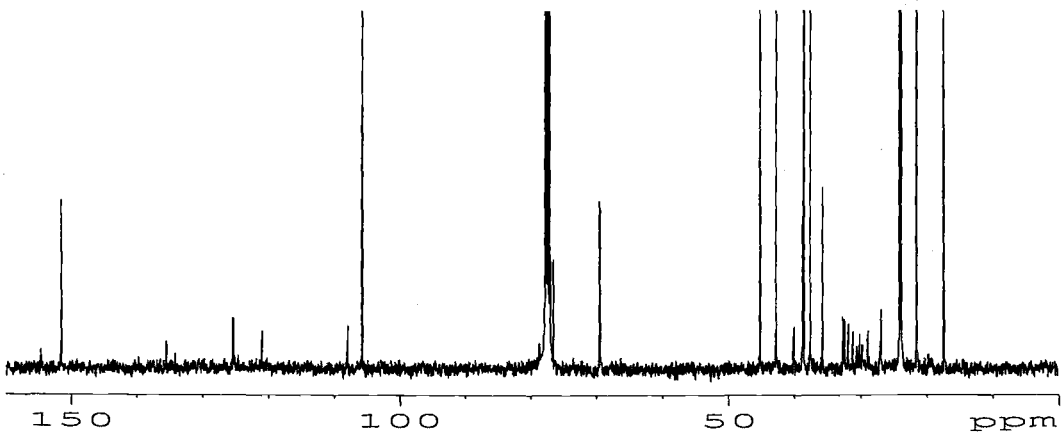

Figure 38: ^{13}C NMR spectrum of unknown 2.

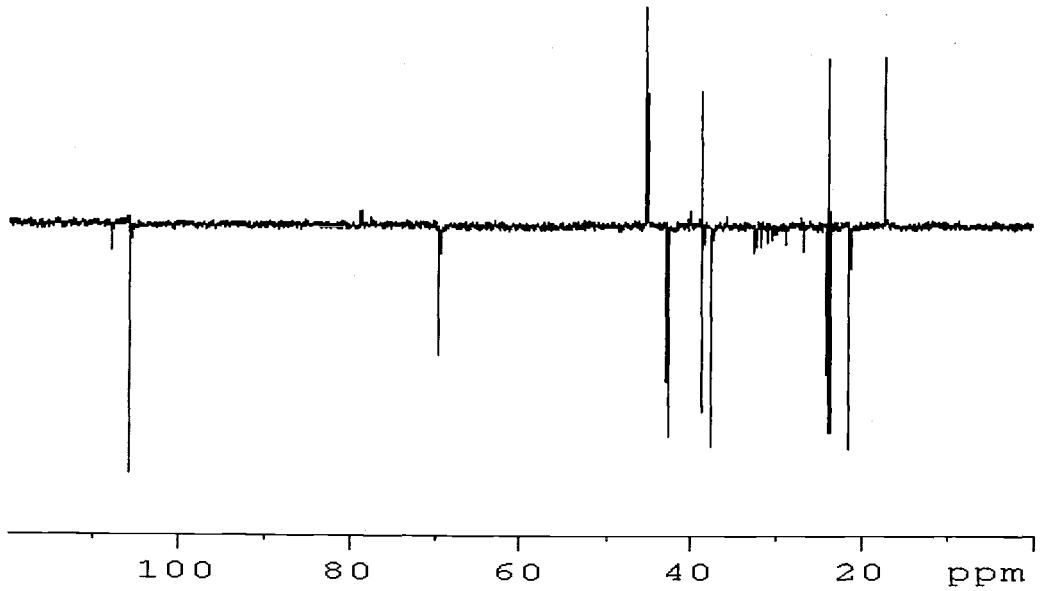

Figure 39: DEPT 135 spectrum of unknown 2.

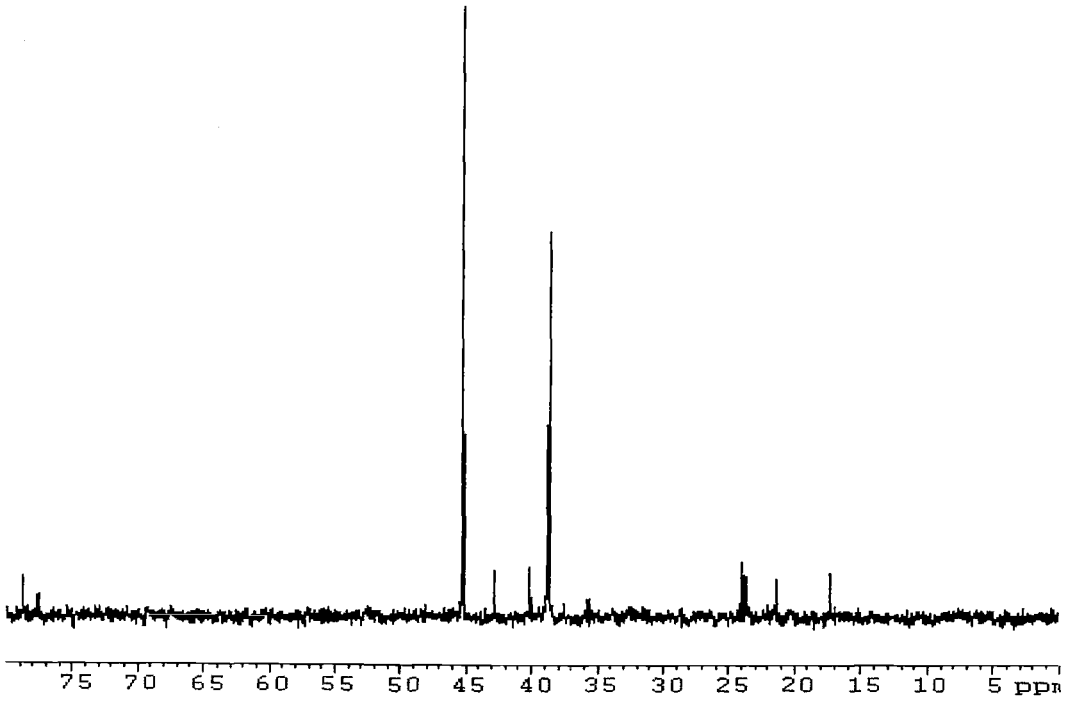

Figure 40: DEPT 90 spectrum of unknown 2.

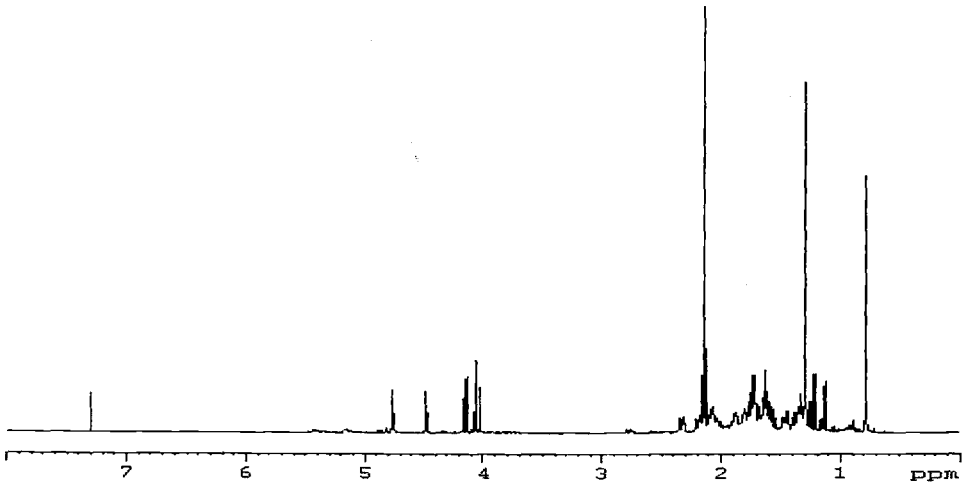

Figure 41: ^{1}H NMR spectrum of the acetylated unknown 2 derivative.

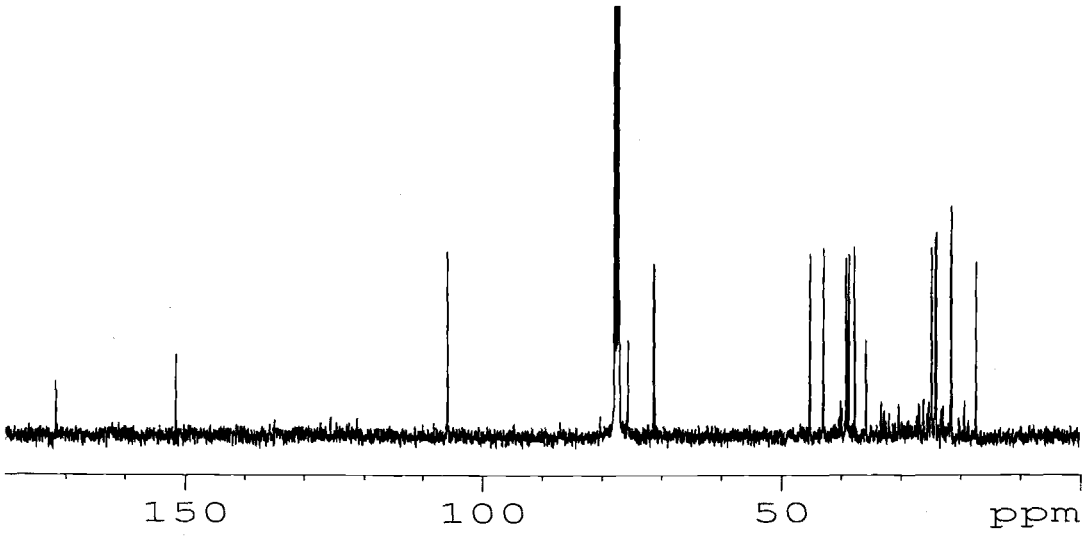

Figure 42: ^{13}C NMR spectrum of the acetylated unknown 2 derivative.

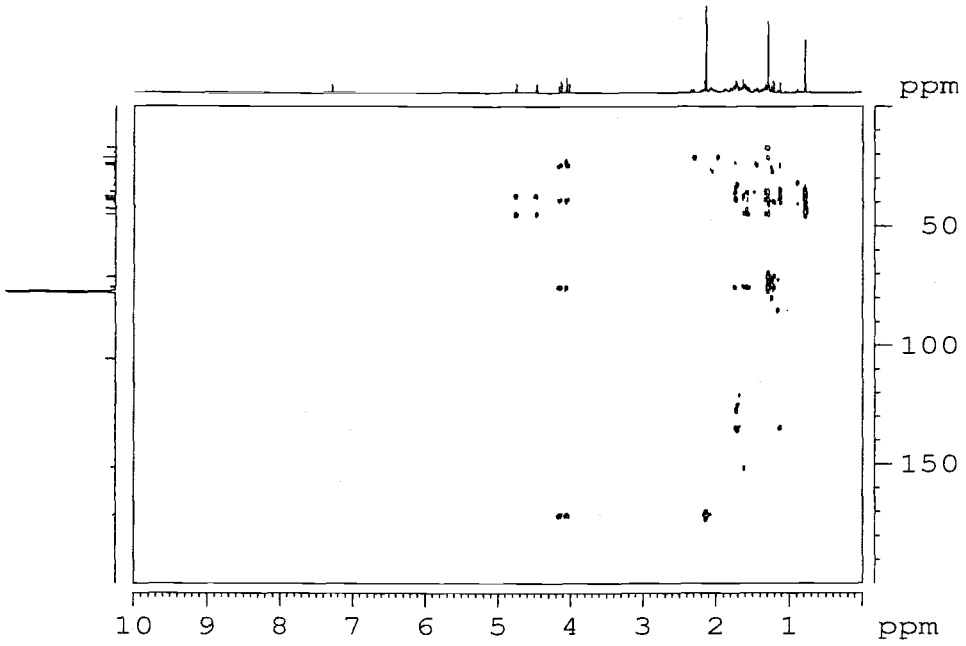

Figure 43: HMBC spectrum of the acetylated unknown 2 derivative.

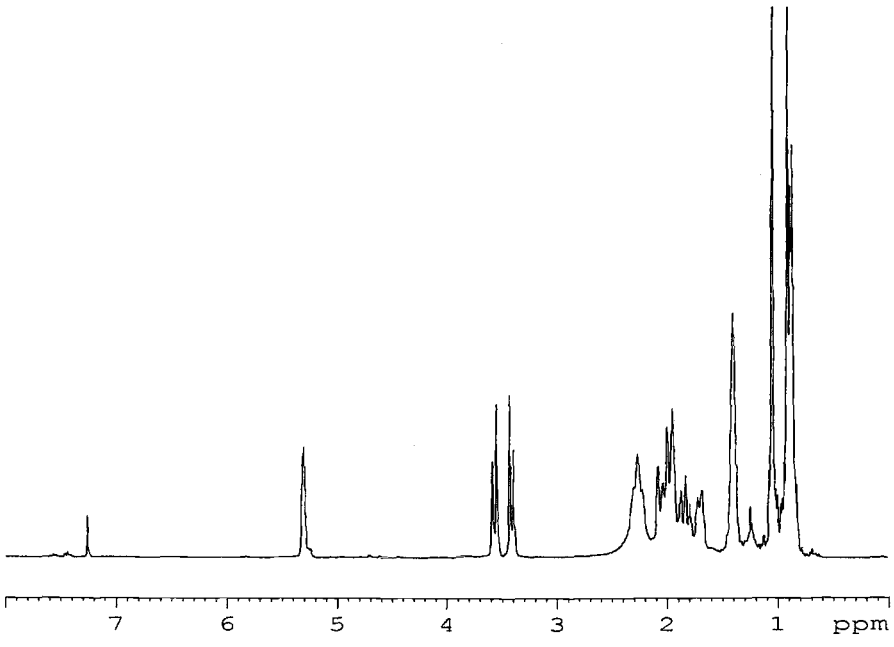

Figure 44: ^{1}H NMR spectrum of unknown 3.

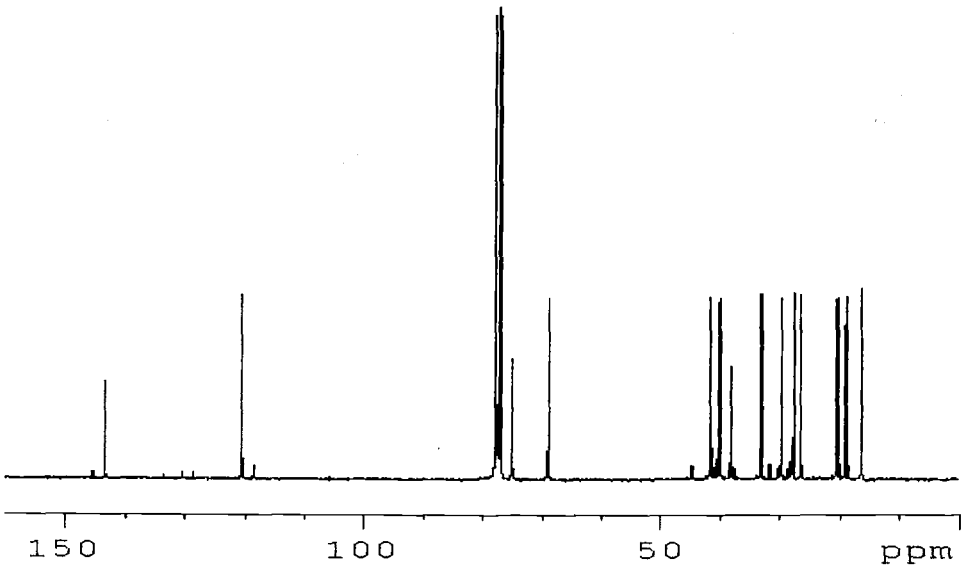

Figure 45: ^{13}C NMR spectrum of unknown 3.

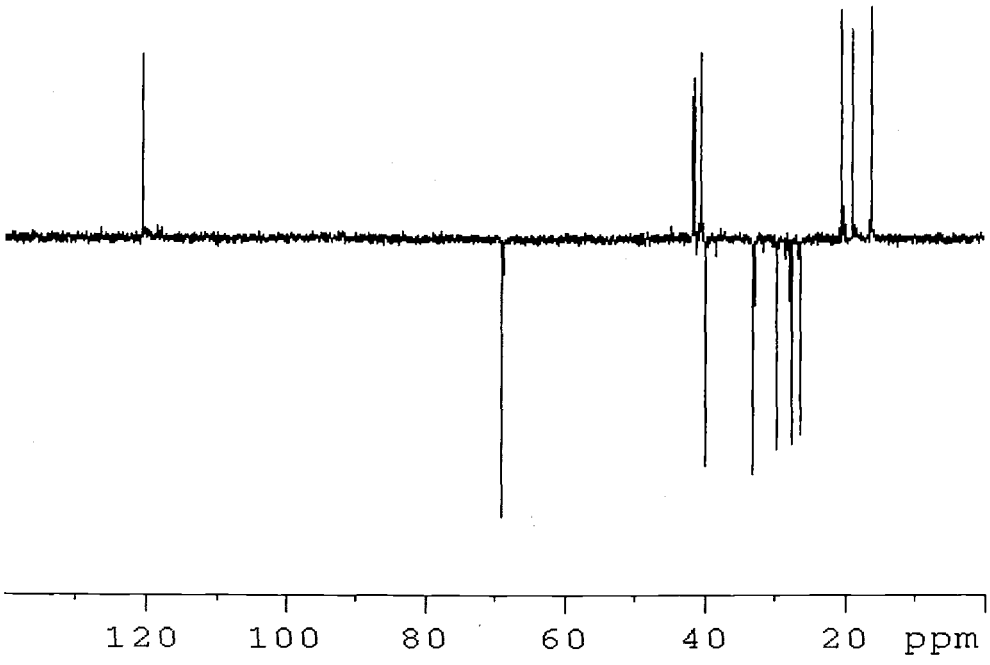

Figure 46: DEPT 135 spectrum of unknown 3.

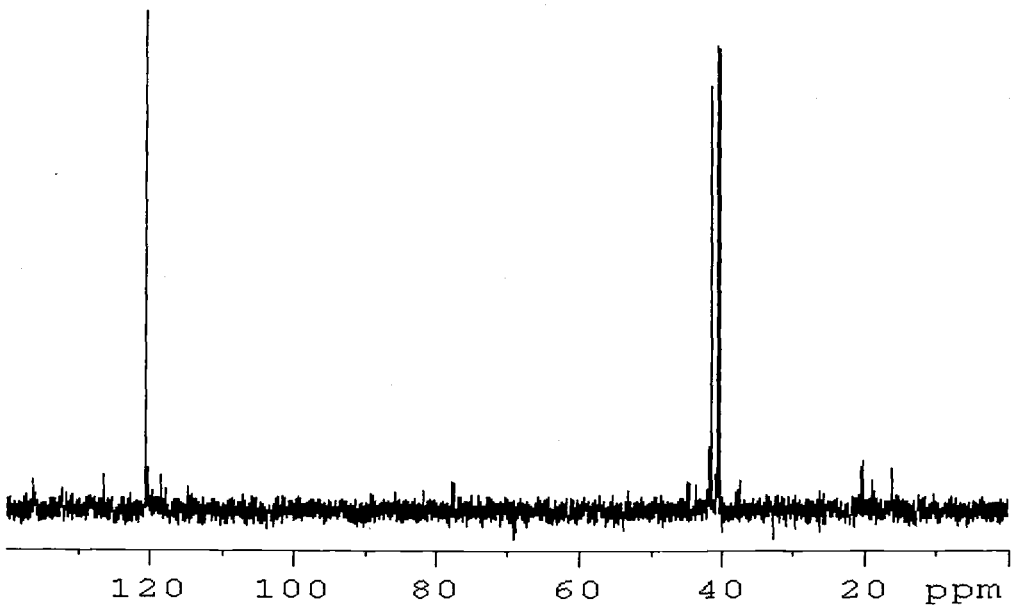

Figure 47: DEPT 90 spectrum of unknown 3.

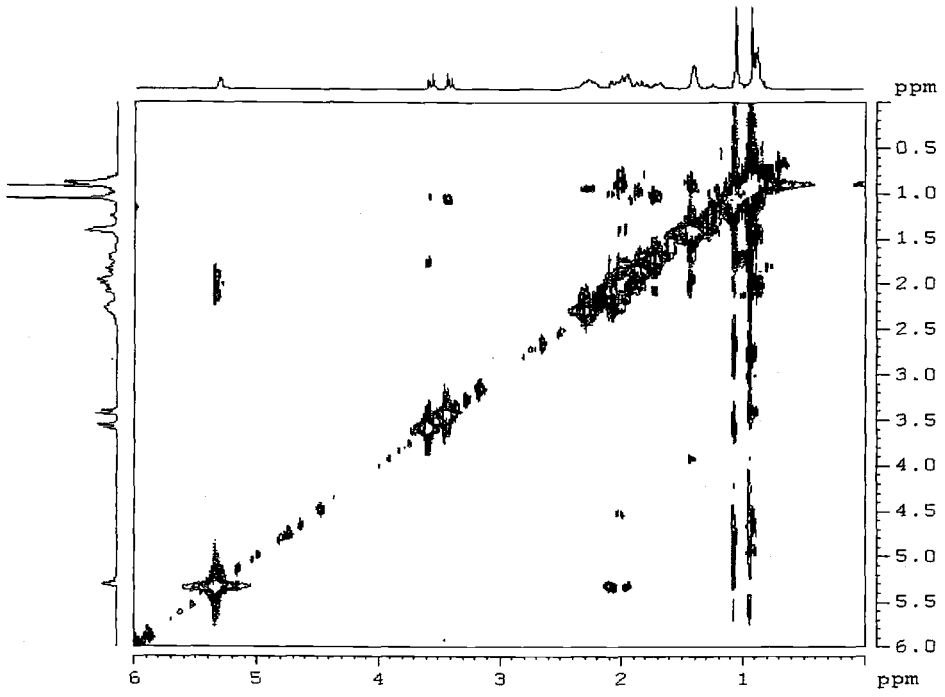

Figure 48: NOESY spectrum of unknown 3.

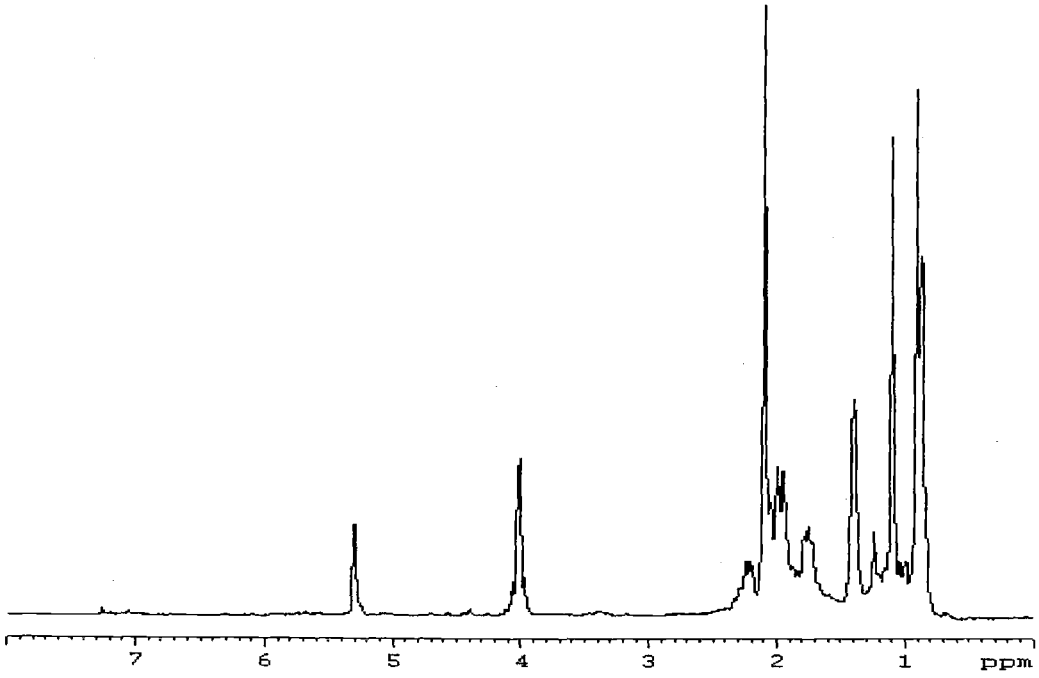

Figure 49: ^{1}H NMR spectrum of the acetylated unknown 3 derivative.

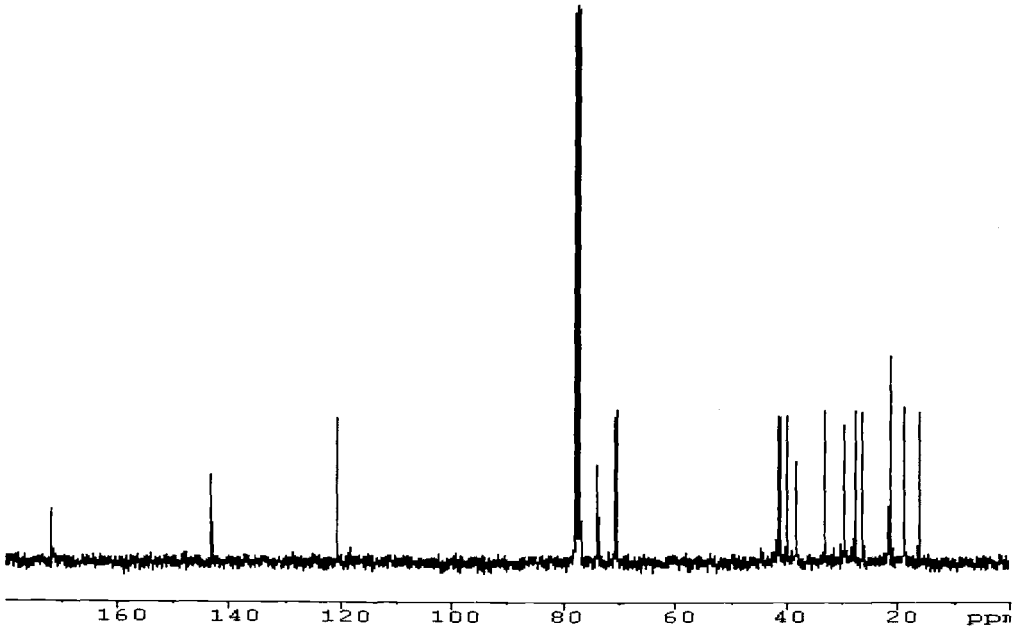

Figure 50: ^{13}C NMR spectrum of the acetylated unknown 3 derivative.

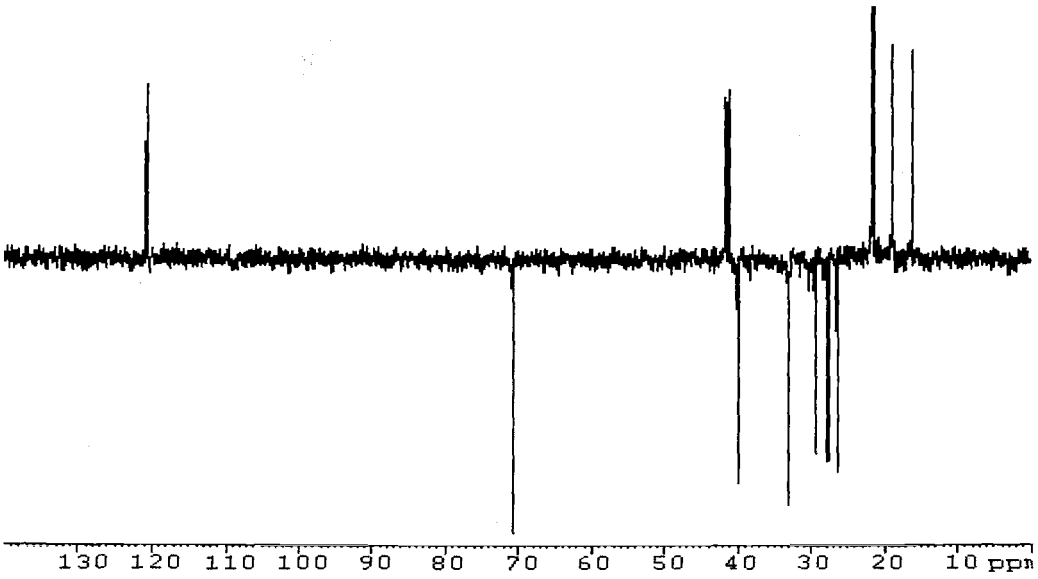

Figure 51: DEPT 135 spectrum of the acetylated unknown 3 derivative.

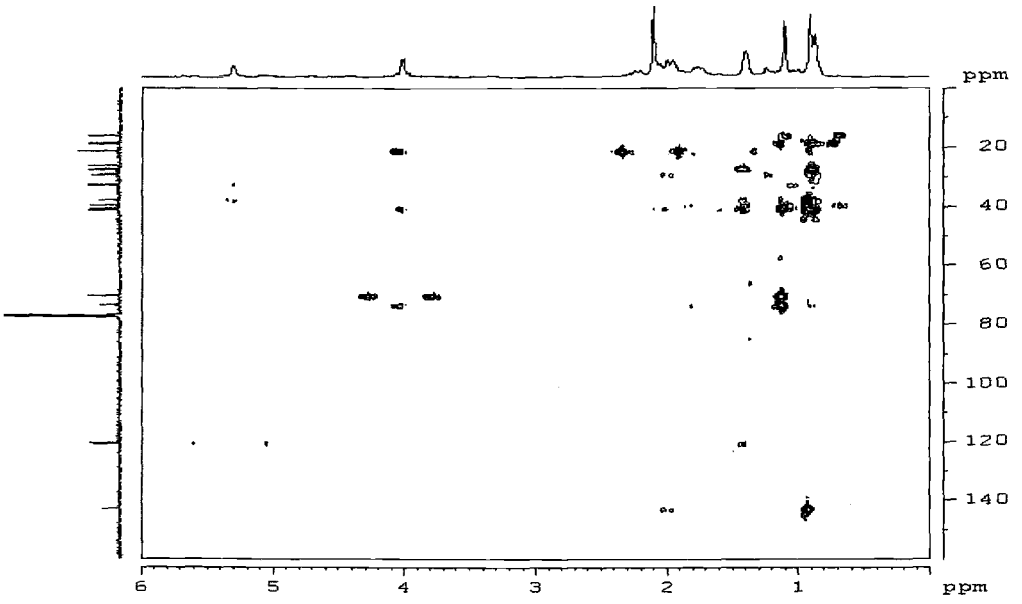

Figure 52: HMBC spectrum of the acetylated unknown 3 derivative.

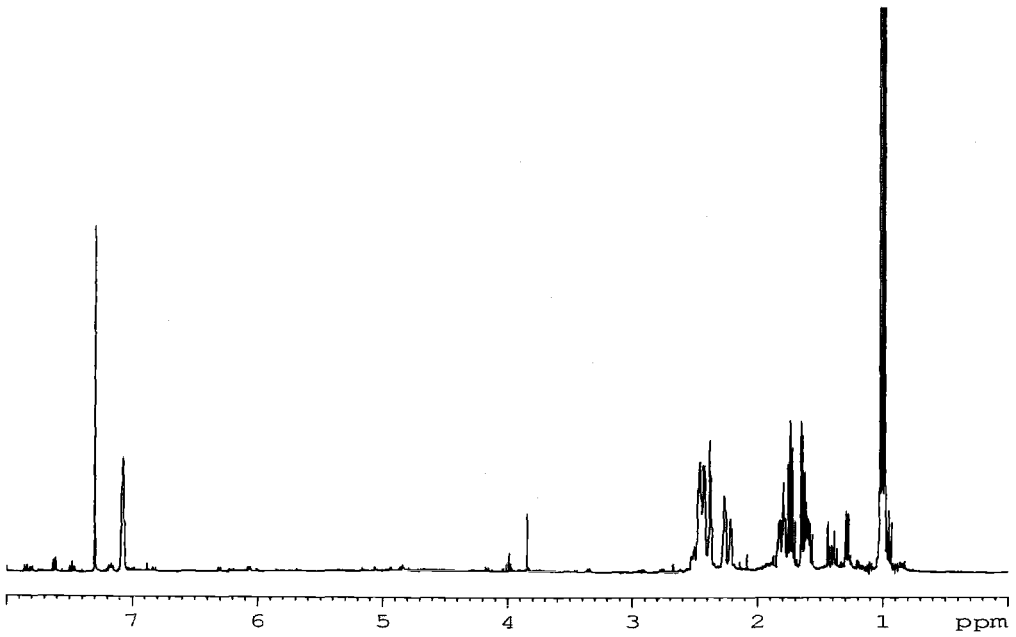

Figure 53: ^{1}H spectrum of unknown 4.

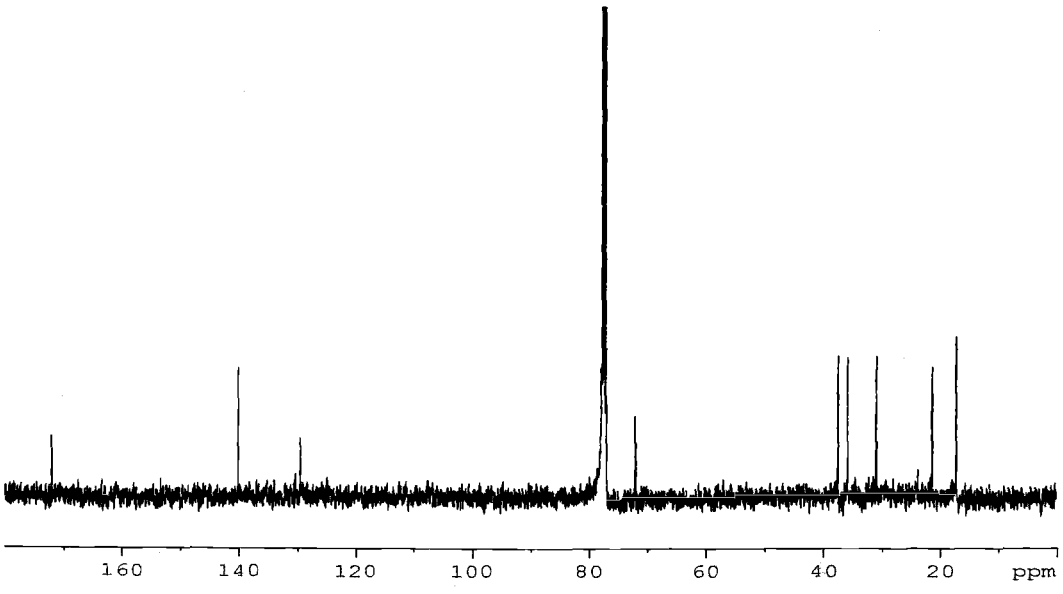

Figure 54: ^{13}C spectrum of unknown 4.

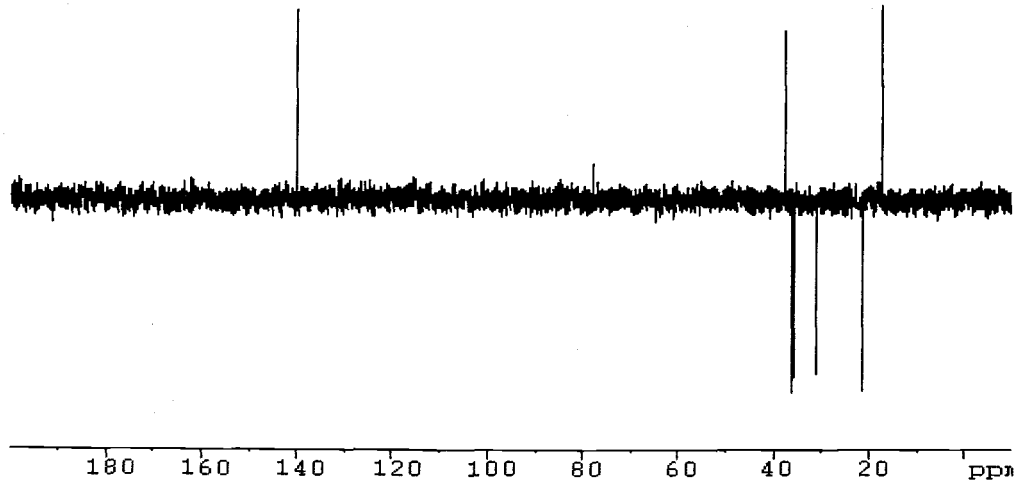

Figure 55: DEPT 135 spectrum of unknown 4.

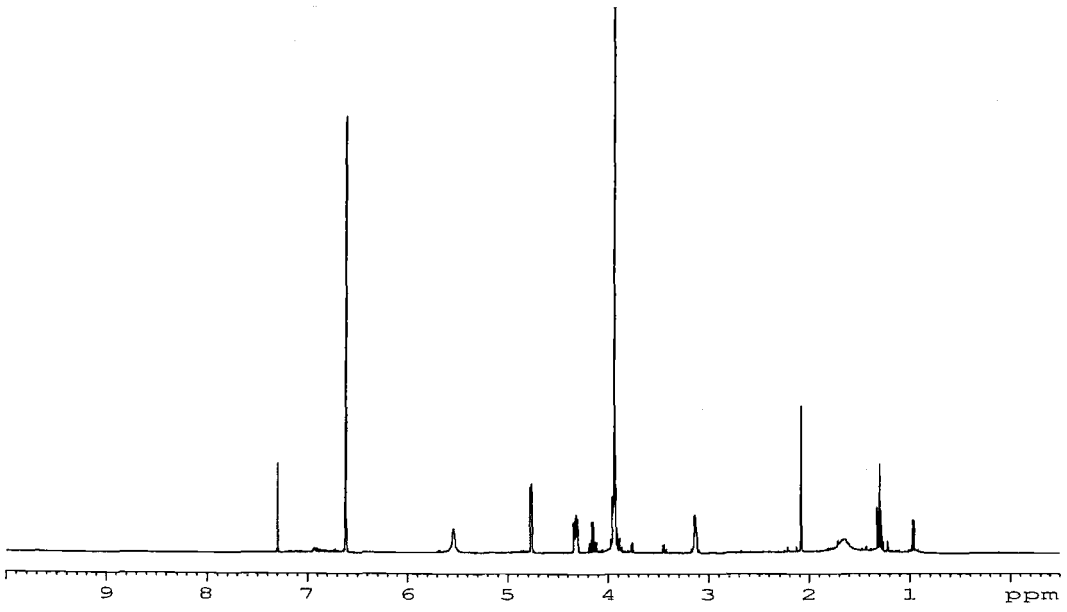

Figure 56: ^{1}H NMR spectrum of unknown 5.

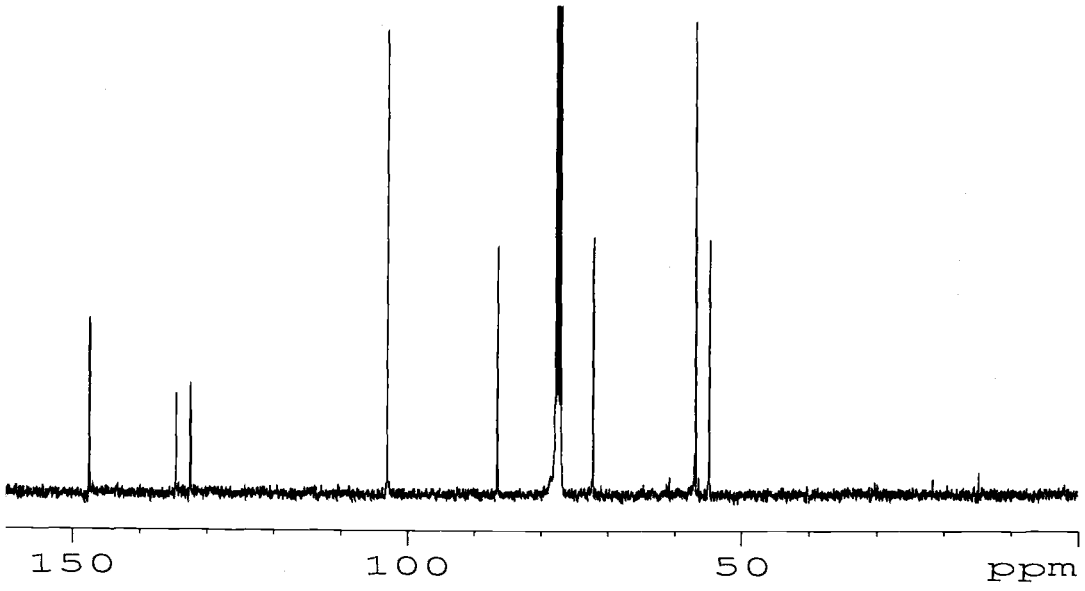

Figure 57: ^{13}C NMR spectrum of unknown 5.

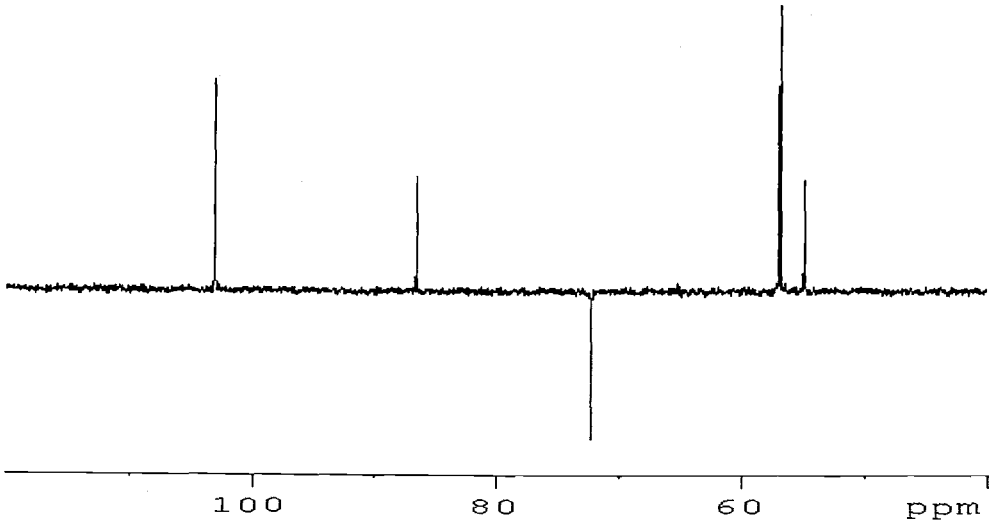

Figure 58: DEPT 135 spectrum of unknown 5.

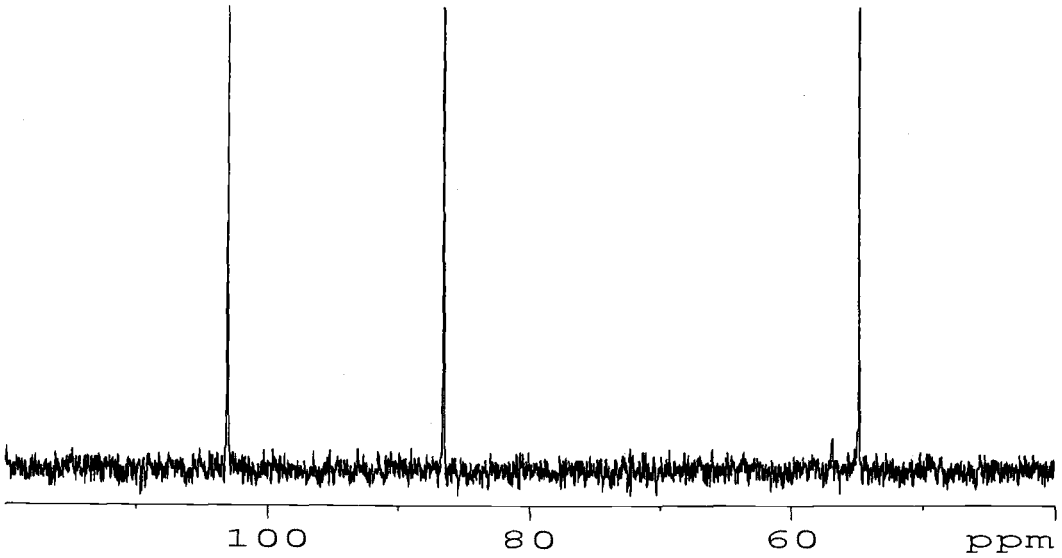

Figure 59: DEPT 90 spectrum of unknown 5.

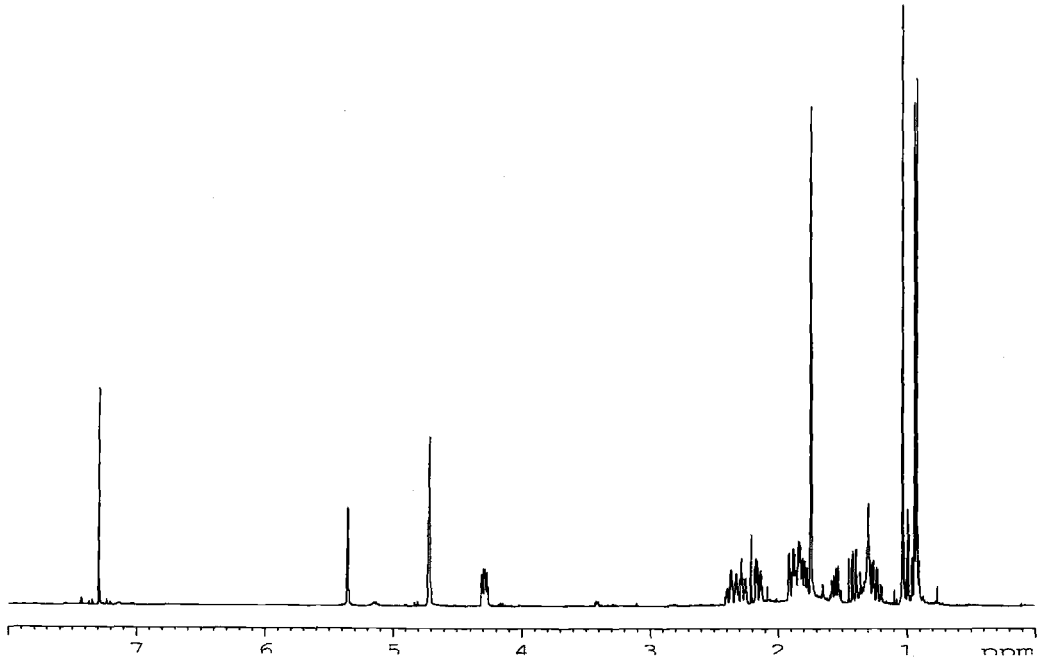

Figure 60: ^{1}H spectrum of nootkatol.

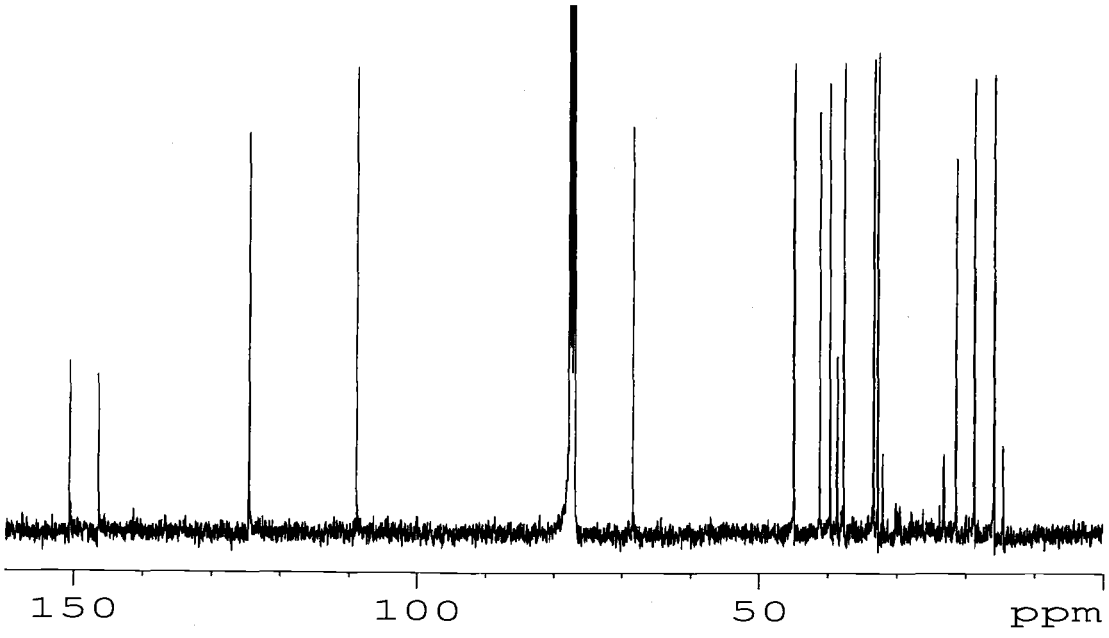

Figure 61: ^{13}C spectrum of nootkatol.

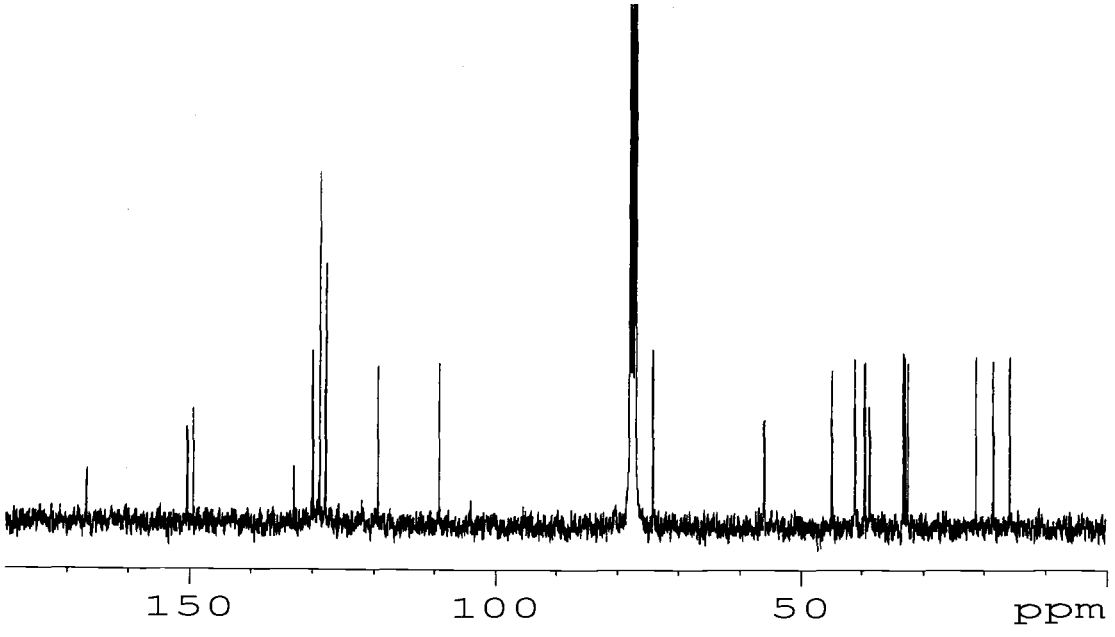

Figure 62: ^{13}C spectrum of Nootkatol-(S)-MTPA Mosher ester.

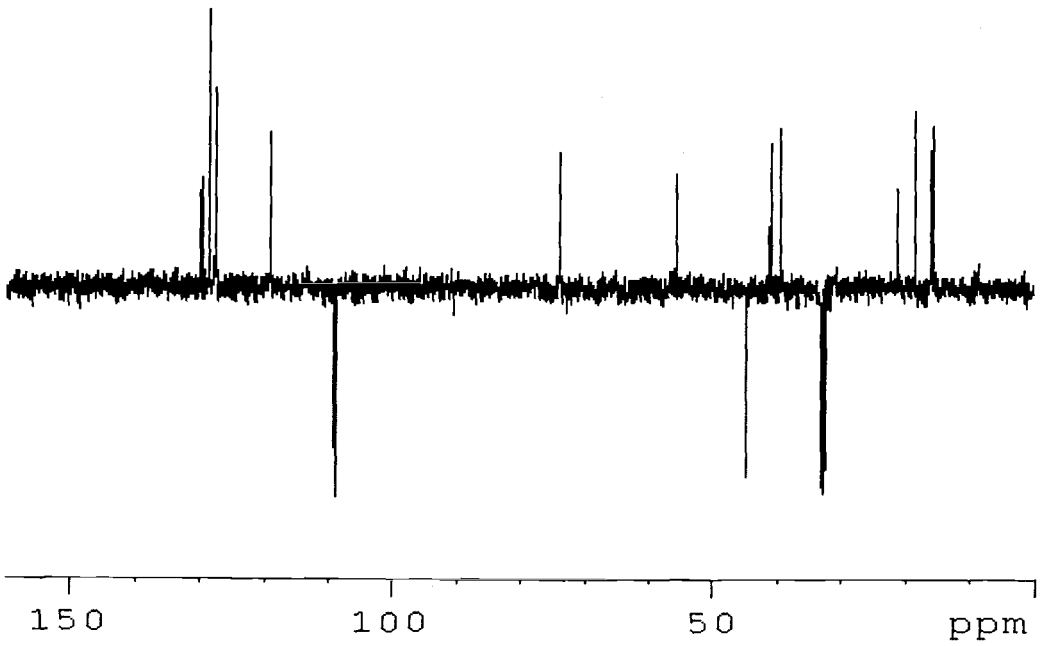

Figure 63: DEPT 135 spectrum of Nootkatol-(S)-MTPA Mosher ester.

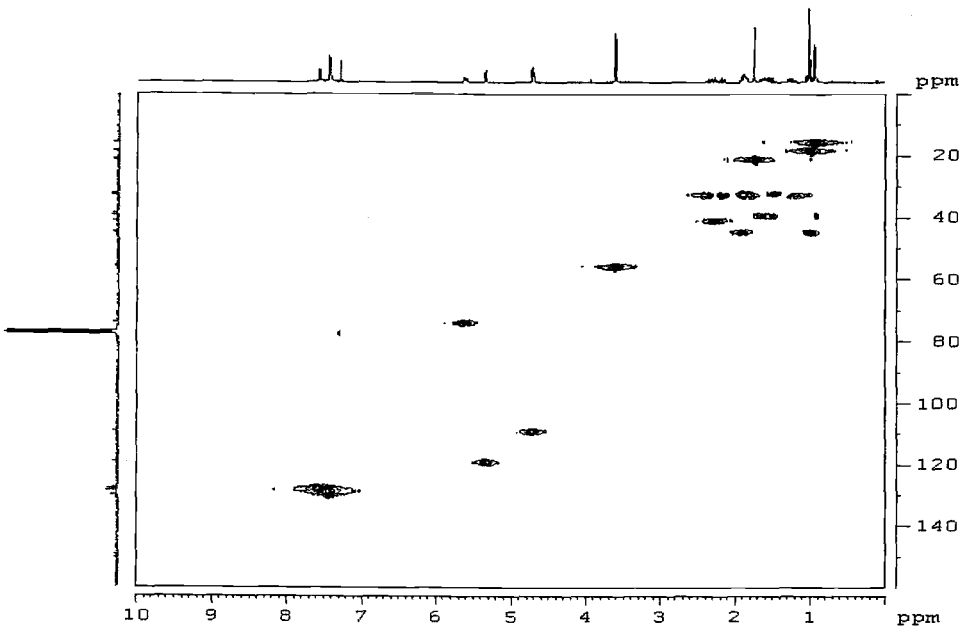

Figure 64: HSQC spectrum of Nootkatol-(S)-MTPA Mosher ester.

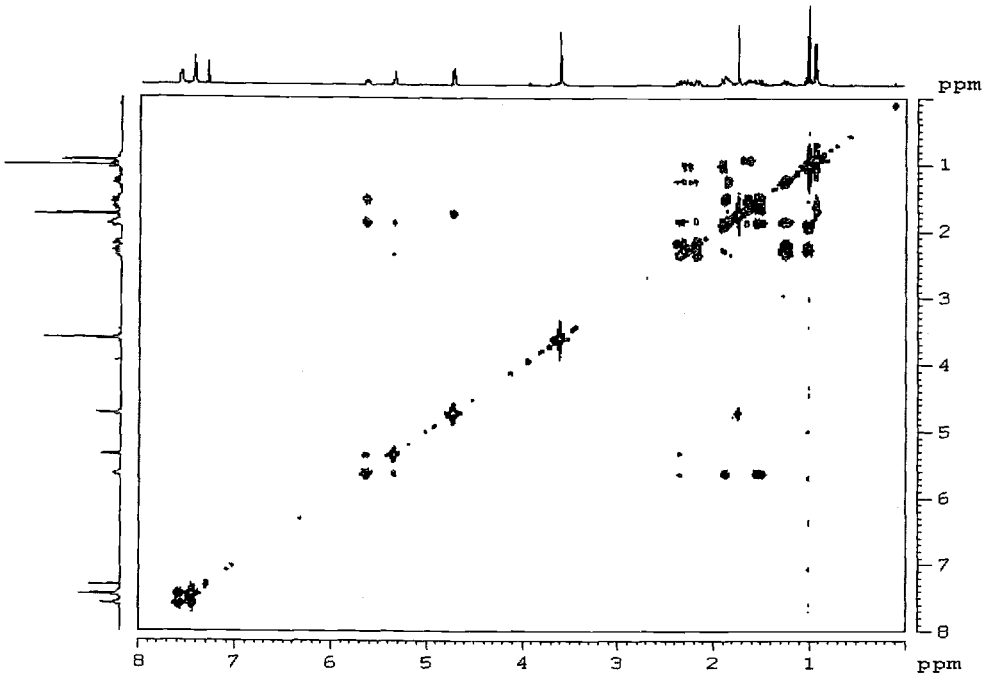

Figure 65: COSY spectrum of Nootkatol-(S)-MTPA Mosher ester.

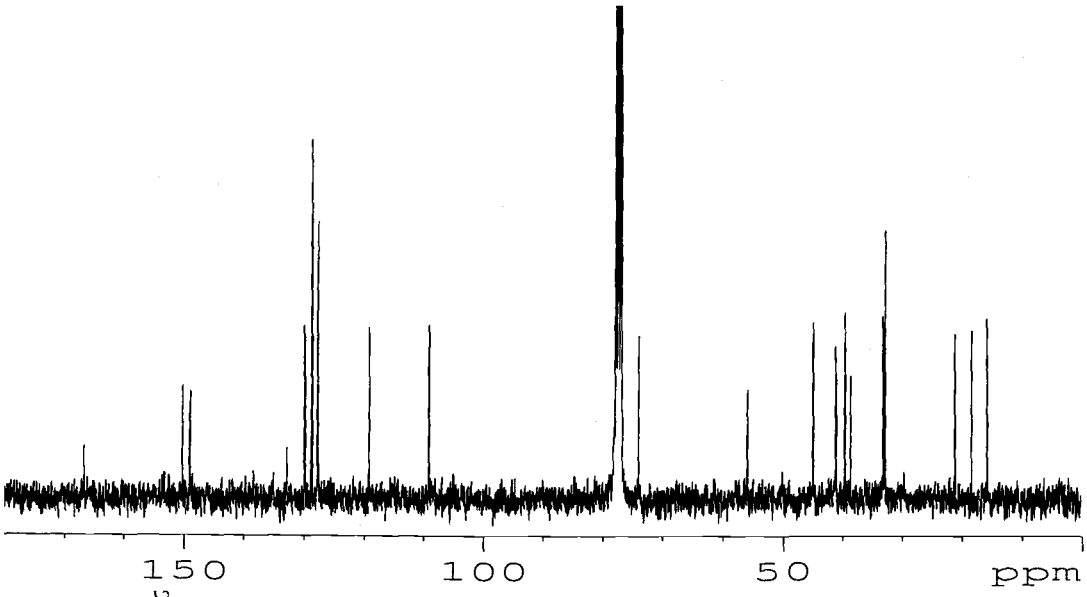

Figure 66: ^{13}C spectrum of Nootkatol-(R)-MTPA Mosher ester.

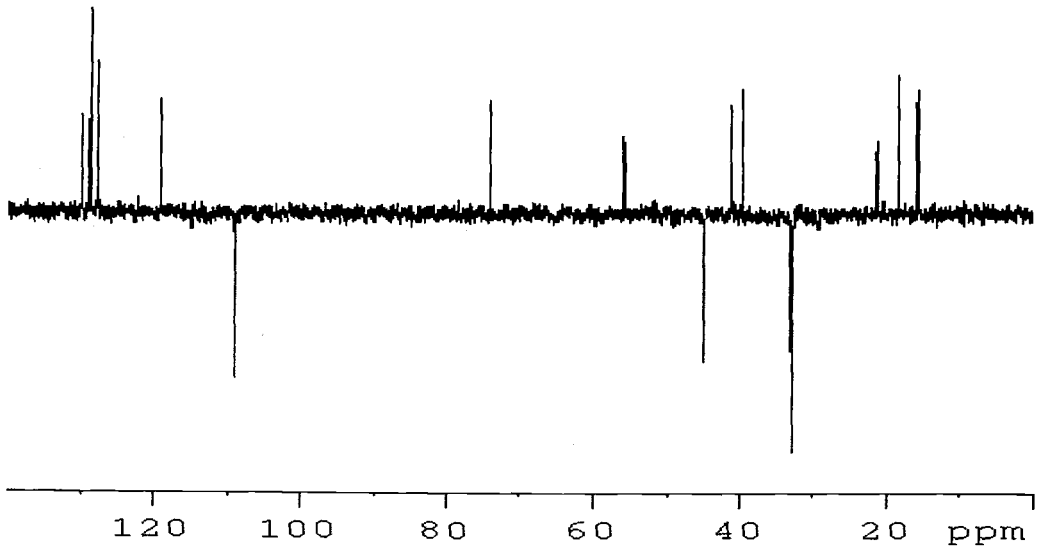

Figure 67: DEPT 135 spectrum of Nootkatol-(R)-MTPA Mosher ester.

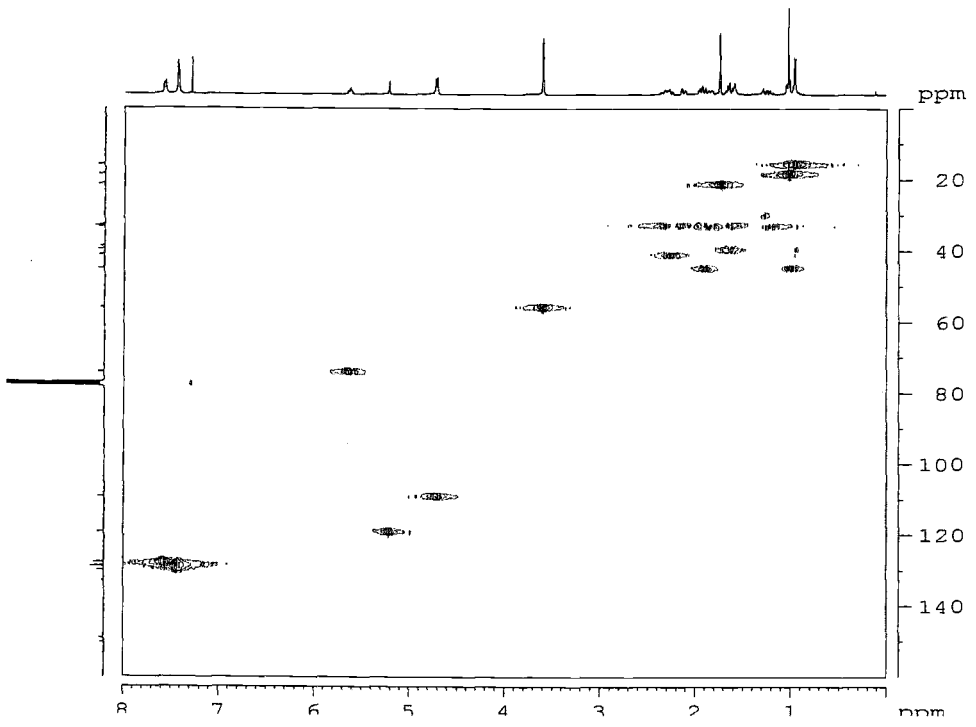

Figure 68: HSQC spectrum of Nootkatol-(R)-MTPA Mosher ester.

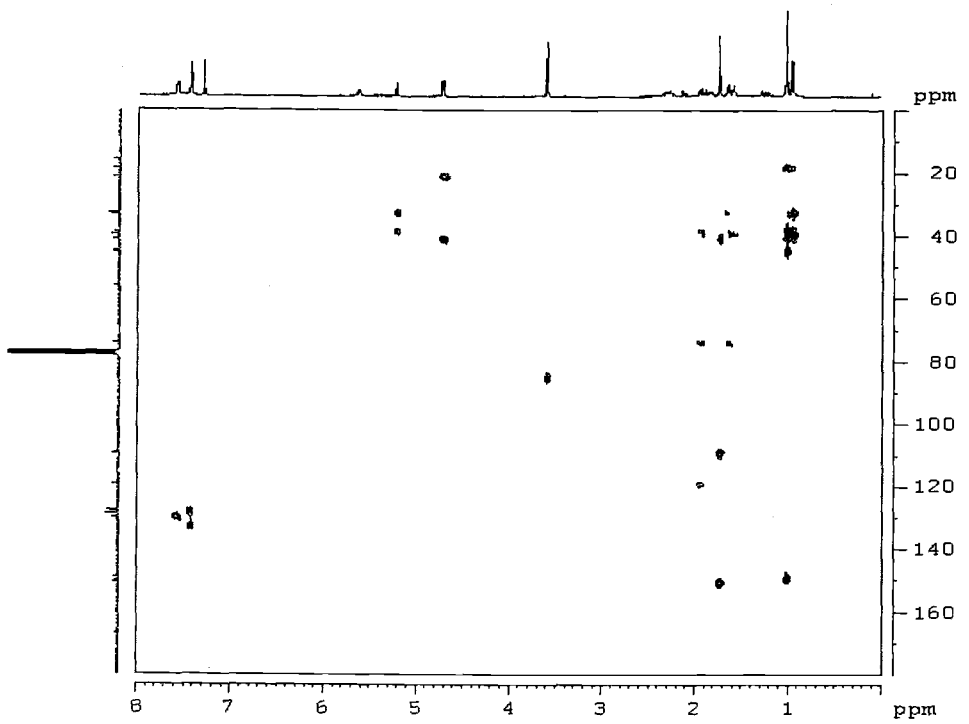

Figure 69: HMBC spectrum of Nootkatol-(R)-MTPA Mosher ester.

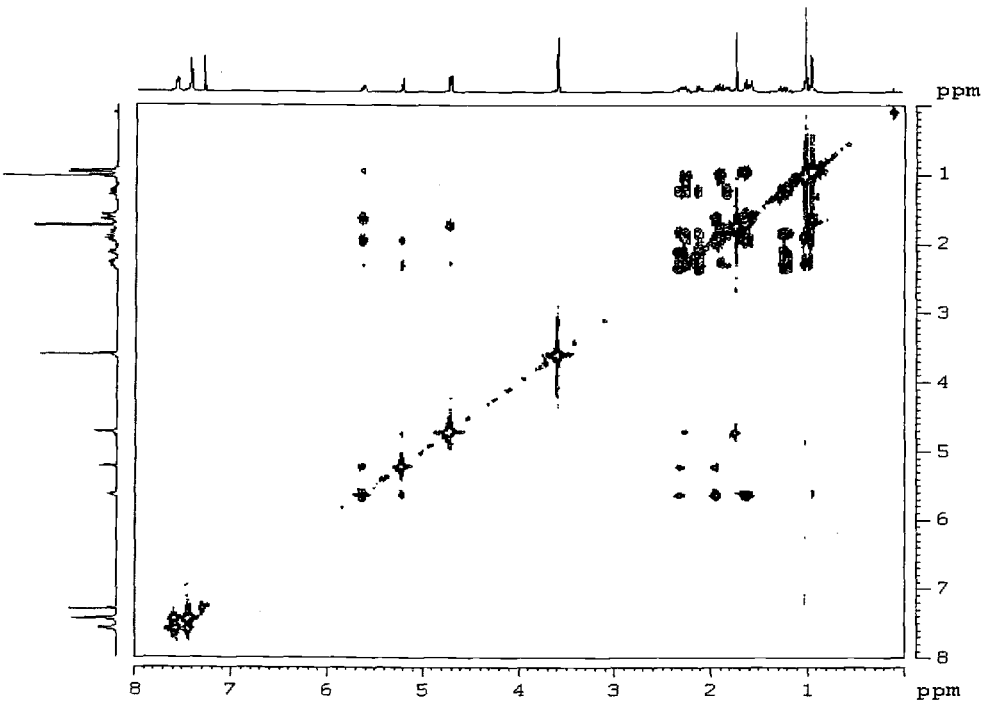

Figure 70: COSY spectrum of Nootkatol-(R)-MTPA Mosher ester.

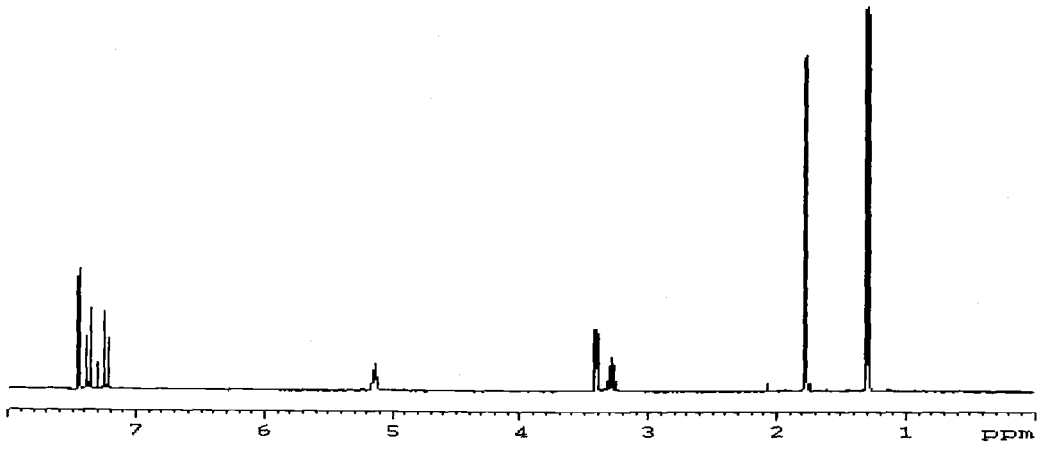

Figure 71: ^{1}H spectrum of Nootkatin.

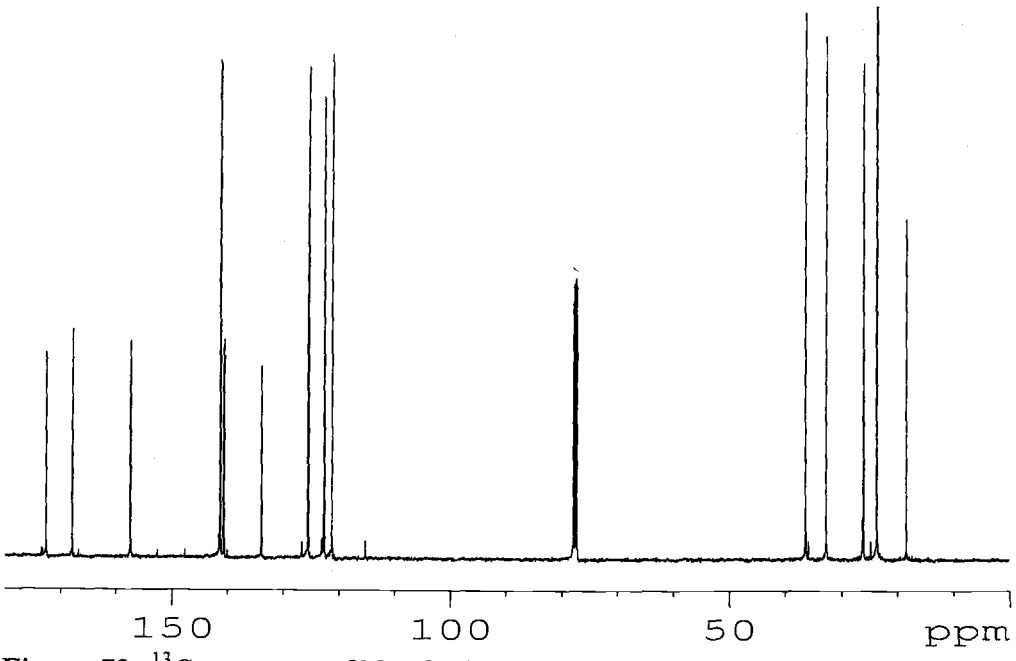

Figure 72: ^{13}C spectrum of Nootkatin.

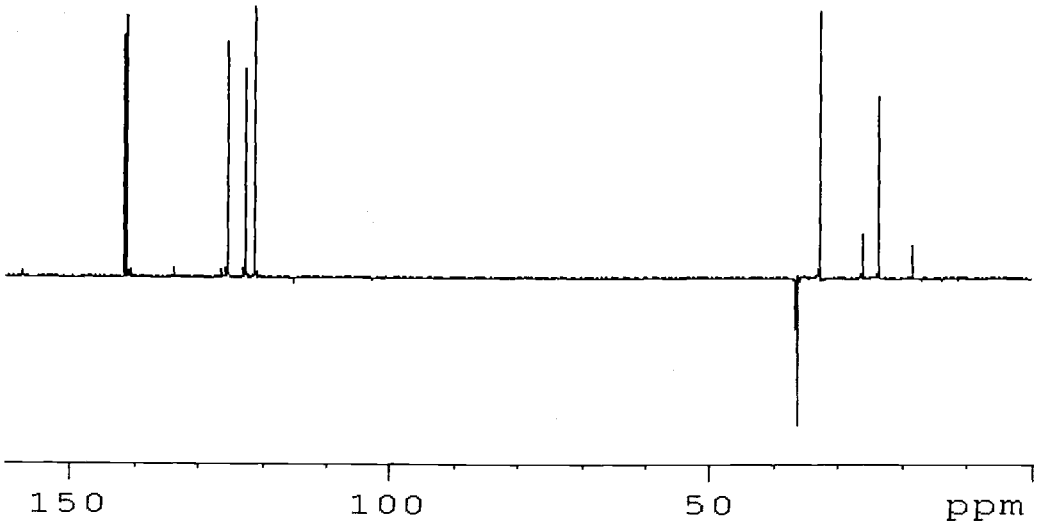

Figure 73: DEPT 135 spectrum of Nootkatin.

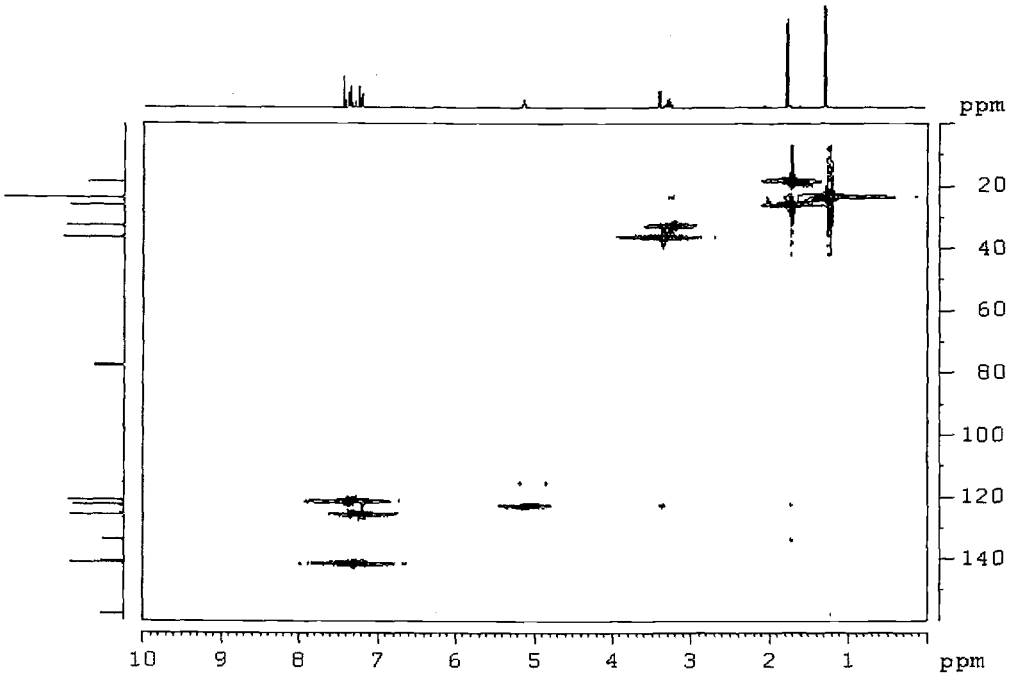

Figure 74: HSQC spectrum of Nootkatin.

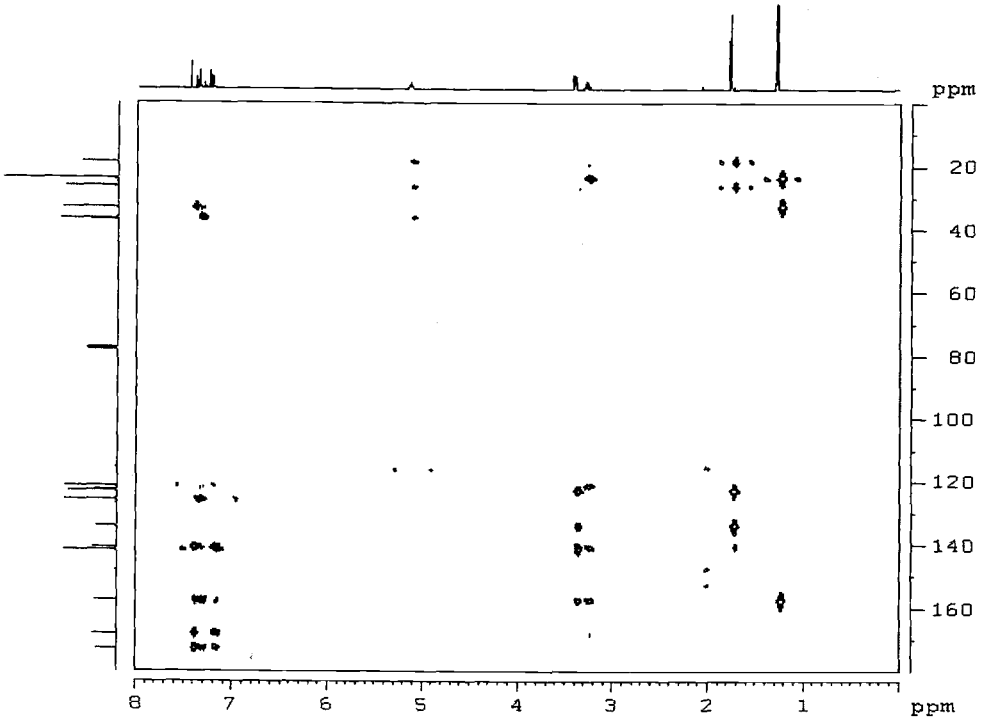

Figure 75: HMBC spectrum of Nootkatin.

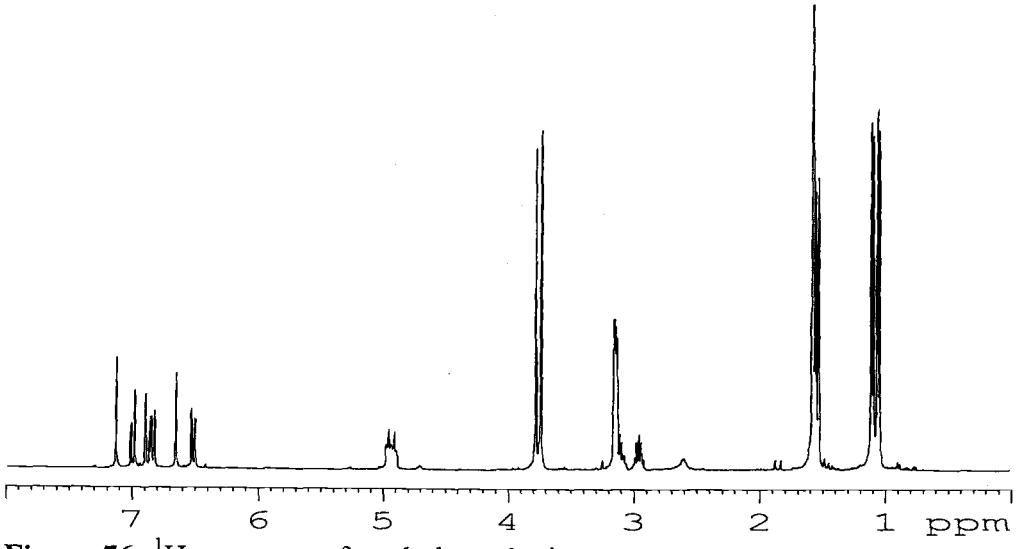

Figure 76: ^{1}H spectrum of methyl nootkatin.

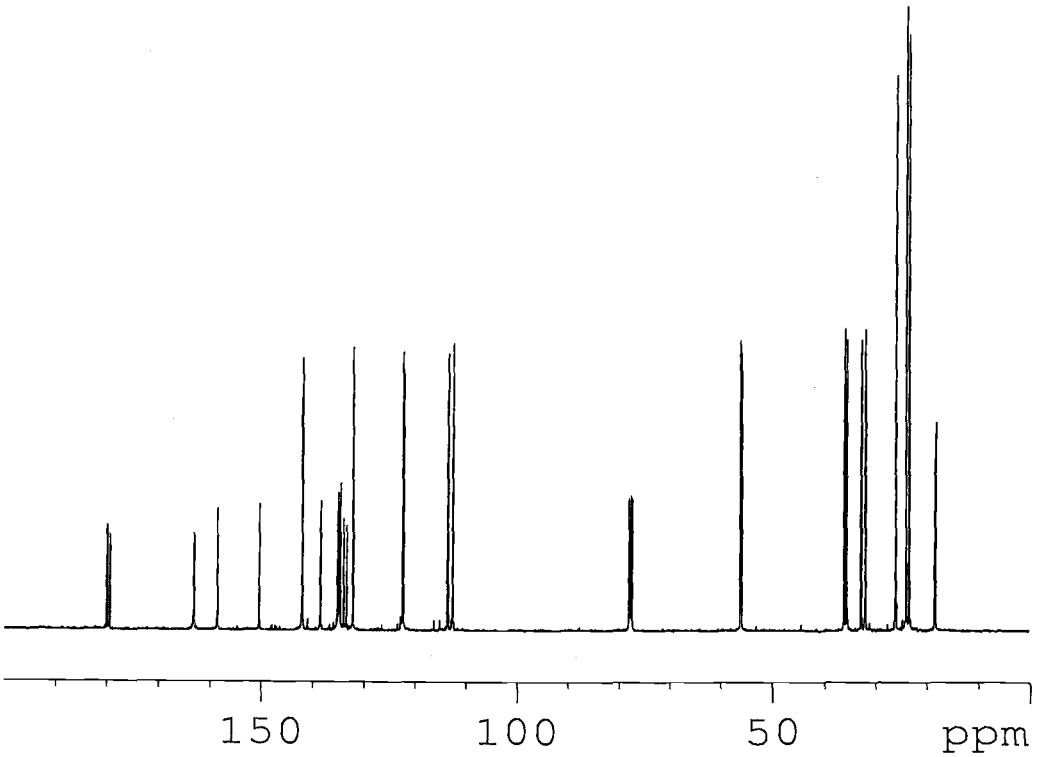

Figure 77: ^{13}C spectrum of methyl nootkatin.

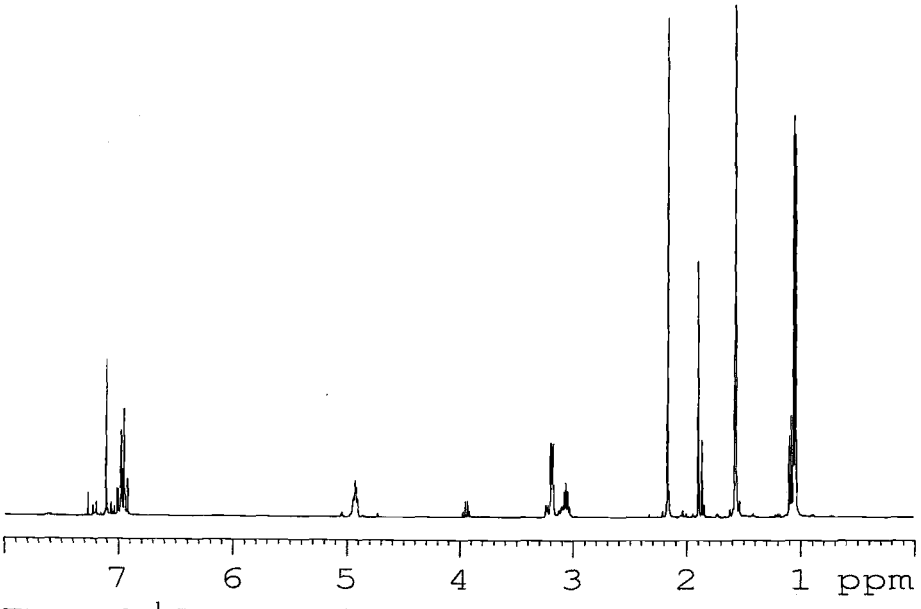

Figure 78: ^{1}H spectrum of nootkatin acetate.

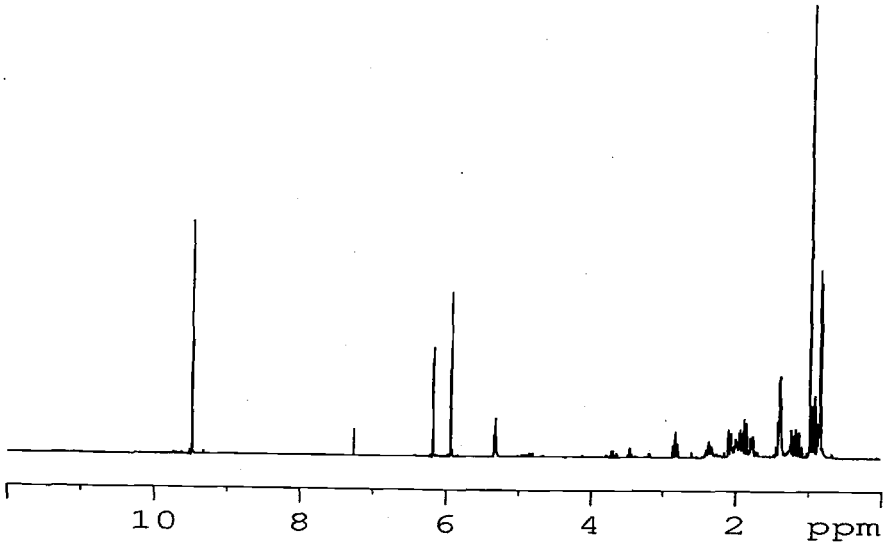

Figure 79: ^{1}H spectrum of valencene-13-aldehyde.

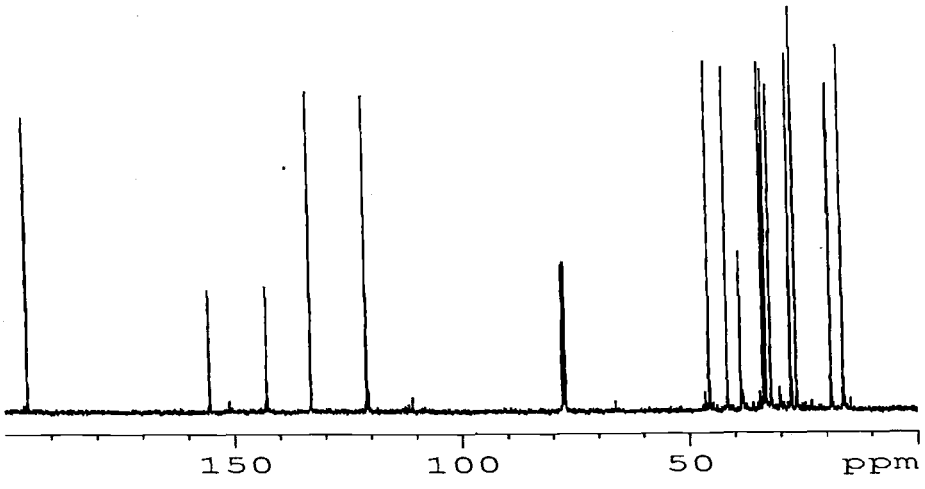

Figure 80: ^{13}C spectrum of valencene-13-aldehyde.

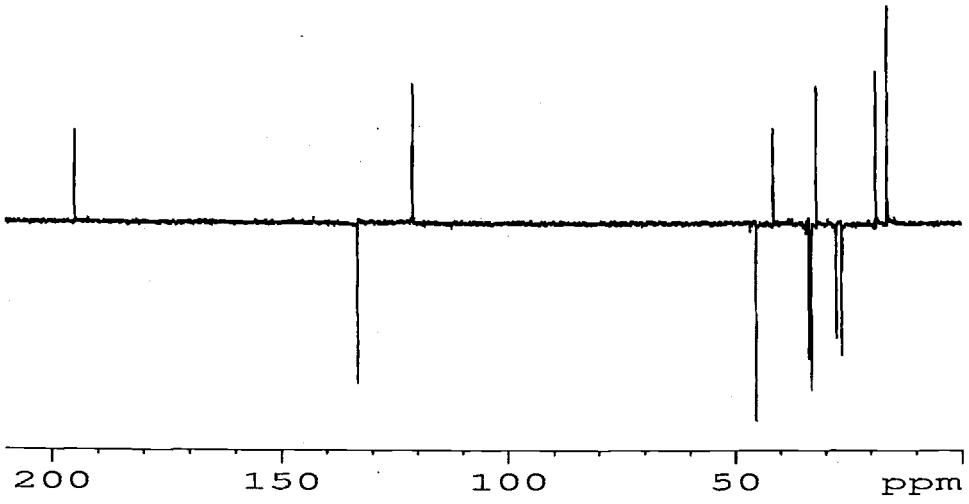

Figure 81: DEPT spectrum of valencene-13-aldehyde.

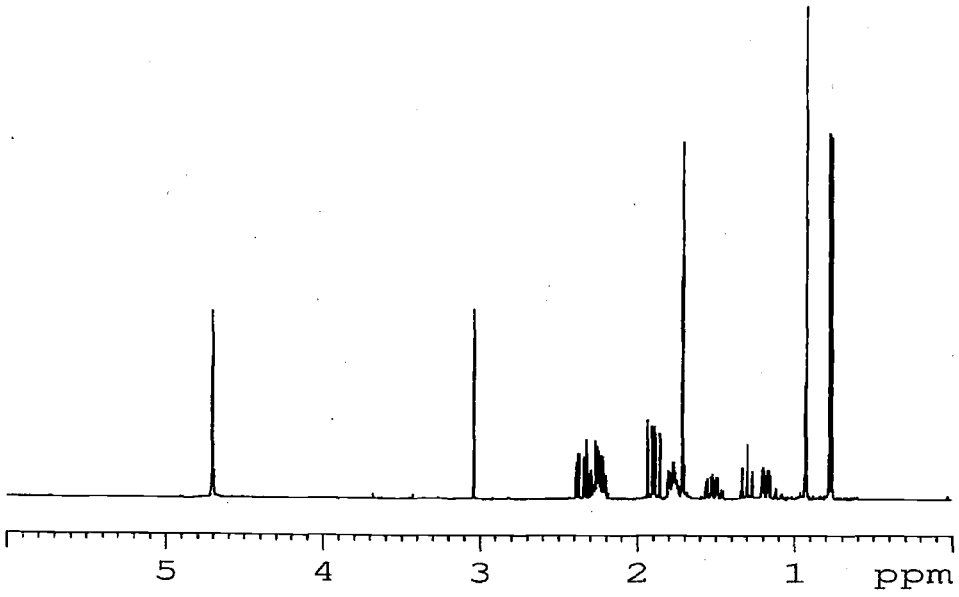

Figure 82: ^{1}H spectrum of nootkatone-1,10-epoxide.

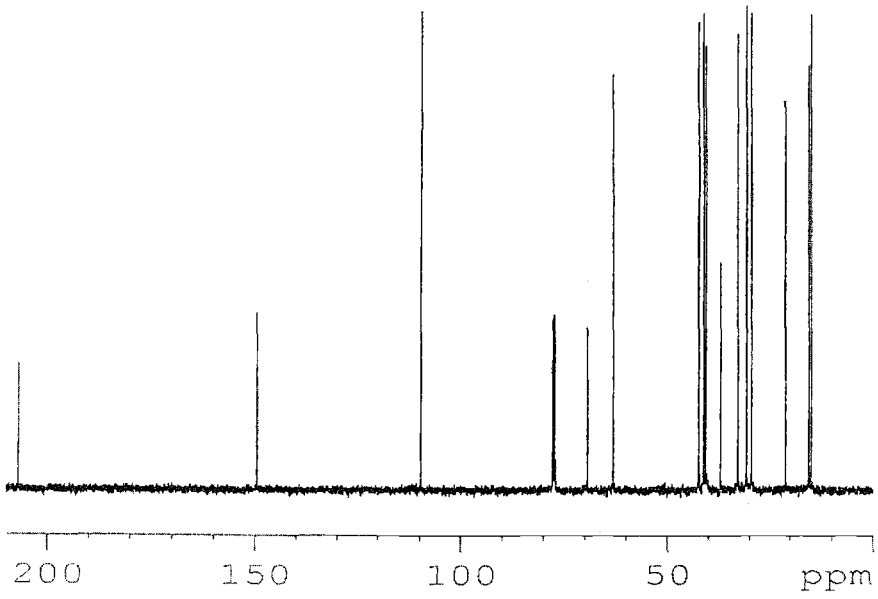

Figure 83: ^{13}C spectrum of nootkatone-1,10-epoxide.

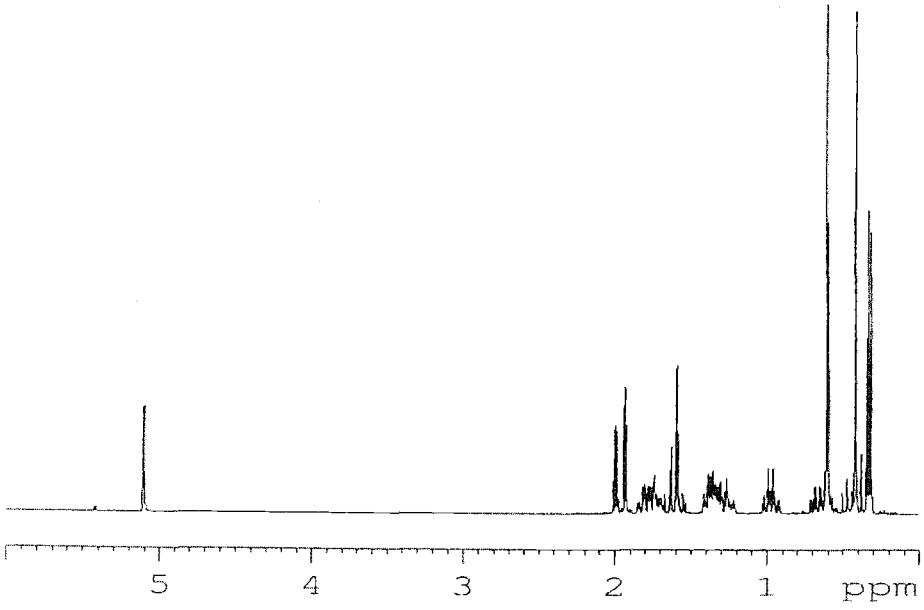

Figure 84: ^{1}H spectrum of nootkatone-11,12-epoxide.

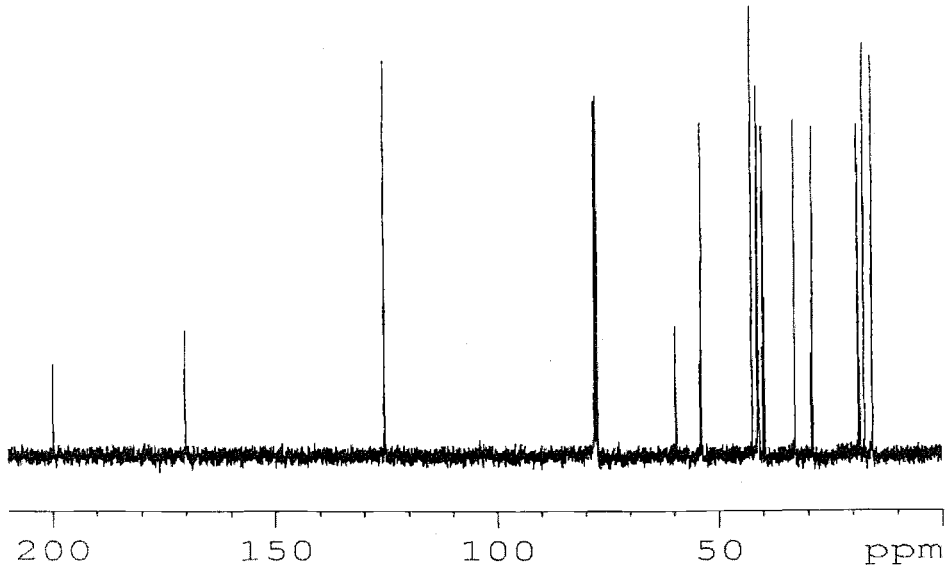

Figure 85: ^{13}C spectrum of nootkatone-11,12-epoxide.

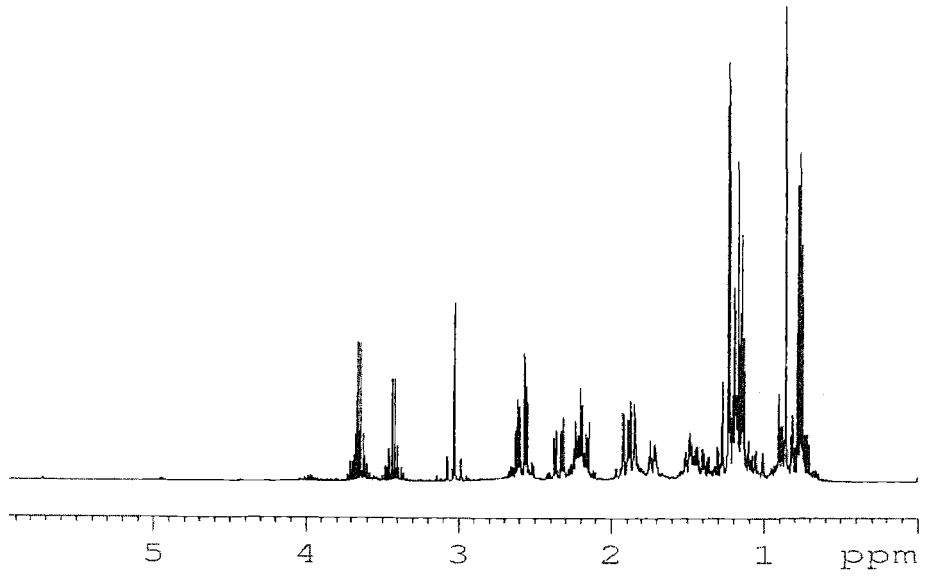

Figure 86: ^{1}H spectrum of nootkatone-1,10-11,12-diepoxide.

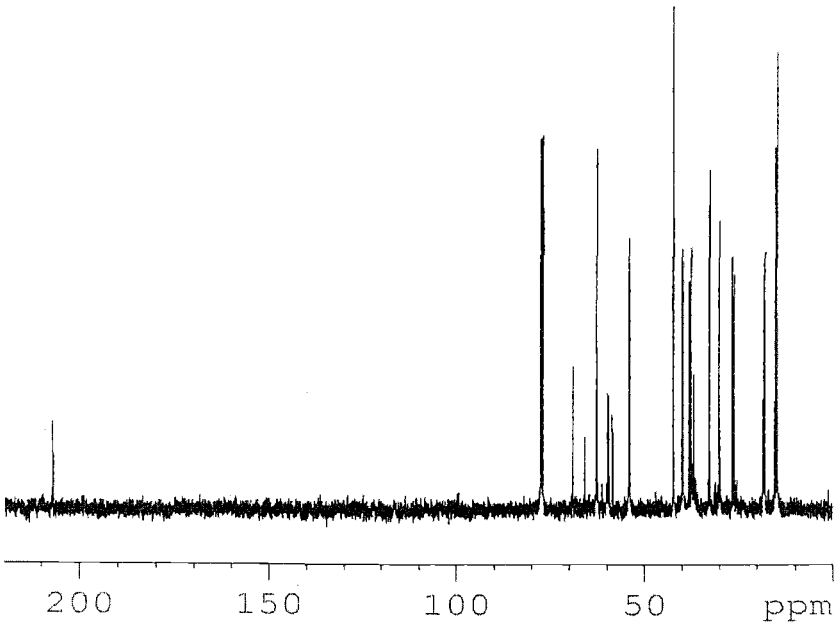

Figure 87: ^{13}C spectrum of nootkatone-1,10-11,12-diepoxide.

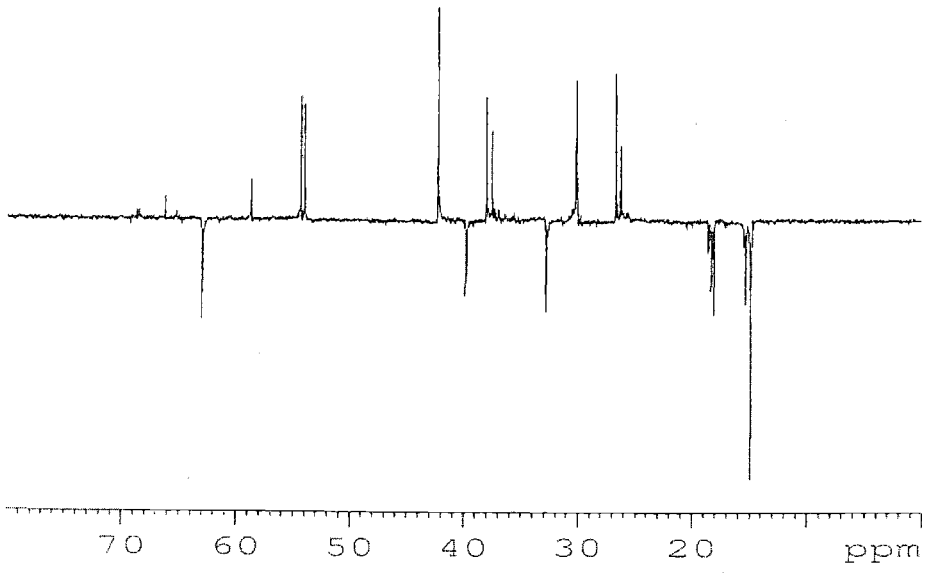

Figure 88: DEPT spectrum of nootkatone-1,10-11,12-diepoxide.