Isolation and Structure Elucidation of Natural Products from Plants

DISSERTATION

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By

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List of Abbreviations

C	Carbon
CC	Column Chromatography
C_6D_6	hexadeuteriobenzene
$CDCl_3$	deuteriochloroform
CD	CycloDextrin
CI	Chemical Ionization
^{13}C-NMR	^{13}Carbon Nuclear Magnetic Resonance
COSY	COrrelation SpectroscopY
2D	two Dimensional
d	doublet
eV	electron Volt
EI	Electron Impact ionization
Fig.	figure
FPP	Farnesyl PyroPhosphate
GC	Gas Chromatography
H	proton
HPLC	High Performance Liquid Chromatography
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Coherence
^{1}H-NMR	Proton Nuclear Magnetic Resonance
Hz	Hertz
J	coupling constant
m/*z*	mass-to-charge ratio
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser Effect
NOESY	Nuclear Overhauser Enhancement SpectroscopY
MS	Mass Spectrometry
prep.	preparative
ppm	parts per million
PTLC	Preparative Thin Layer Chromatography
rel. int.	relative intensity
s	singlet
t	triplet
TLC	Thin Layer Chromatography

Arrangement of the contents of the dissertation

This dissertation encompasses two parts. The first part deals with general introduction to natural products, with emphasis on terpenes, an overview of analytical methods used in isolation and structural elucidation of natural products, and a synopsis of results and discussion. The second part presents compilation of papers arising from the the present work. These papers, which are listed below, comprise a published paper, submitted papers, papers under review and papers that are in press. The papers are referred to by the Roman numerals.

Paper I. Tesso, H. König, W. A. 2004. Terpenes from *Otostegia integrifolia*, Phytochemistry 65, 2054-2062.

Paper II. Tesso, H., König, W. A., Son, P. T., Giang, P. M. Composition of the Essential Oil from Flowers of *Chloranthus spicatus* (Thunb.) Makino, In Press.

Paper III. Tesso, H.,. Koenig, W. A., Asakawa, Y. Composition of the Essential Oil of a Liverwort *Radula perrottetii* of Japanese Origin, In Press.

Paper IV. Tesso, H.,. Koenig, W. A., Kubeczka, K.-H., Bartnik, M. Glowniak, K. 2005. Secondary Metabolites from *Peucedanum tauricum* Fruits. Phytochemistry 66, 707-713.

Paper V. Tesso, H., Koenig, W. A., Kubeczka, K.-H. Isoligustilide: Isoligustilide: A New Phthalide from the Essential oil of *Meum athamanticum*, In Press

Paper VI. Tesso, H.,. Koenig, W. A. Kubeczka, K.-H Melanene-a new sesquiterpene hydrocarbon with a novel skeleton and other terpenes from the essential oil of the leaves of *Melanoselinum decipiens*, Submitted.

In paper I, I was responsible for collection of the plant material, isolation as well as structural elucidation of the compounds. The preparation of the article was the joint effort of Prof. Koenig and myself.

In Paper II, my responsibility was in the isolation as well as structural elucidation of the compounds. The preparation of the article was the joint effort of Prof. Koenig and myself.

In Paper III, my responsibility was in the isolation and structural elucidation of the compounds. The preparation of the article was the joint effort of Prof. Koenig and myself.

In Paper IV, my responsibility was in the isolation as well as structural elucidation of the compounds. The preparation of the article was the joint effort of Prof. Koenig and myself.

In Paper V, my responsibility was in the isolation as well as structural elucidation of the compounds. The preparation of the article was the joint effort of all the authors.

In Paper VI, my responsibility was in the isolation as well as structural elucidation of the compounds. The preparation of the article was the joint effort of Prof. Koenig and myself.

ABSTRACT

Plant materials obtained from different geographical locations including Africa, Asia and Europe were investigated for their secondary metabolites using *the-state-of-the-art* separation and structural elucidation techniques comprising analytical and preparative Gas Chromatography (GC), GC-Mass Spectrometry (MS), one-dimensional (1D) and two-dimensional (2D) Nuclear Magnetic Resonance (NMR) techniques. Many volatile compounds, mainly mono- and sesquiterpenes and their derivatives could be identified. In addition, a number of hitherto unknown compounds could be isolated and their structures elucidated by extensive spectral analysis. These include, a prenyl bisabolane type diterpene (+)-axinyssene (**11**), and two furanolabdanediterpenes, preotostegindiol (**12**) and otostegindiol (**13**) from *Otostegia integrifolia* collected in Ethiopia (Paper I), two guaiane sesquiterpenes, guaia-1(10),11-diene (**14**), and guaia-9,11-diene (**15**) from *Peucedanum tauricum* collected in Poland (Paper IV), four sesquiterpenoids: chloranthalactone A (**49**), isogermafurenolide (**50**), eudesma-4(15),7(11),9-trien-12-olide (**51**), and 7α-hydroxyeudesm-4-en-6-one (**52**) from *Chloranthus spicatus* flower oil from Vietnam (Paper II), a phthalide named isoligustilide (**55**) from *Meum athamanticum* from Germany (Paper V), two viscidane diterpenes and four bisabolane sesquiterpenes (**38-43**) from *Radula perrottetii* of Japanese origin (Paper III) and a sesquiterpene hydrocarbon with novel skeleton, melanene (**56**), from *Melanoselinum decipiens* grown in Hamburg, Germany (Paper VI).

1. Introduction

Secondary metabolites are chemical compounds derived from living organisms. The study of natural products involves isolation in a pure form of these compounds and investigation of their structure, formation, use, and purpose in the organism. Secondary metabolites appear to function primarily in defense against predators and pathogens and in providing reproductive advantage as intraspecific and interspecific attractants. They may also act to create competitive advantage as poisons of rival species. Most natural products can be classified into a few groups only: acetogenins as well as propanogenins, terpenoids, derivatives of aminoacids, and aromatic compounds. Many plant terpenoids are toxins and feeding deterrents to herbivores or are attractants, and many possess pharmacological activity. Phenolic compounds play important roles in plants. Tannins, lignans, flavonoids, and some simple phenolic compounds serve as defenses against herbivores and pathogens. Lignins strengthen cell walls, and many flavonoid pigments are important attractants for pollinators and seed dispersers. Some phenolic compounds have allelopathic activity and may adversely influence the growth of neighboring plants. Throughout evolution, plants have developed defenses against herbivory and microbial attack and produced other natural products to foster competitiveness. The better defended, more competitive plants have generated more progeny, and so the capacity to produce and safely store such ecologically useful metabolites has become widely established in the plant kingdom.[1].

The study of natural products has had a number of rewards. It has led to the discovery of a variety of useful drugs for the treatment of diverse ailments and contributed to the development of separation science and technology, spectroscopic methods of structure elucidation and synthetic methodologies that now make up the basics of analytical organic chemistry.

One of the most important areas of application of natural products is in the treatment of human and veterinary ailments. Currently, at least 119 chemical substances derived from 90 plant species can be considered important drugs that are in use in one or more countries [2]. Although the use of natural products as medicinal agents presumably predates the first recorded history as the earliest humans used various, but specific plants to treat illness, the treatment of diseases with pure pharmaceutical agents is a relatively modern phenomenon. Nevertheless, the role of traditional medicine in the discovery of potent chemicals is quite crucial. Among some of the earliest successes in developing drugs from natural products, one can mention the isolation of the antimalarial agents such as the Cinchona tree alkaloids, pain relievers such as the morphine alkaloids as well as the development of aspirin. Quinine (**1**)

(Fig. 1) originally isolated from the bark of Cinchona trees, *Cinchona succirubra*, was one of the principal antimalarial agents. Morphine (**2**) the major alkaoid of *Papaver somniferum* was first isolated between 1803/06. It was widely used for pain relief beginning in the 1830's, but was also recognized as addictive. The "Ebers papyrus", the Egyptian pharmaceutical record, indicates the use of willow leaves as an antipyretic agent [3]. Following on this knowledge, chemists began to isolate the compounds responsible for the remedy, and salicin (**3**) was isolated from the bark of the white willow, *Salix alba*, in 1825-26 [3]. It was subsequently converted to salicylic acid (**4**) via hydrolysis and oxidation, and proved potent as an antipyretic that was manufactured and used worldwide [3]. To overcome the severe gastrointestinal toxicity of salicylic acid, it was converted into acetylsalicylic acid (ASA) (**5**) via acetylation and started to be marketed under the trade name aspirin in 1899 [3]. Aspirin is still the most widely used analgesic and antipyretic drug in the world.

More recently, the vinca alkaloids, vinblastine (**6**) and vincristine (**7**) were isolated as antineoplastic agents from the Madagascan periwinkle, *Catharanthus roseus*, and subsequently derivatized to vinorelbine and vindesine, the drugs that are currently in use for cancer treatment [2]. Similarly, a potent antimalarial drug, a sesquiterpenoid endoperoxide, named artemisinin (**8**) was isolated from *Artemisia annua* as a remedy against the multidrug resistant strains of *Plasmodium*, following on the long use of this plant material as an antimalarial drug in the traditional Chinese medicine. Using the basic structure of artemisinin, semisynthetic compounds were synthesized with the aim of optimizing the pharmacology of the principal molecule leading to the identification of artemether (**9**) and dihydroartemisinin (**10**) as potent antimalarial agents that are now in a widespread use around the world [2].

These few accounts underscore not only the potential of natural products as a source of drugs as well as the solid link between the folk medicine and drug development but also the necessity of natural products research. Today, drugs derived from natural products must be pure and completely characterized compounds. Structures are elucidated primarily by spectroscopic techniques, and the elaboration of the stereochemistry is an important feature of the characterizations.

2. Terpenes

Terpenes is the generic name of a group of natural products, structurally based on isoprene (isopentenyl) units. The term may also refer to oxygen derivatives of these compounds that are known as the terpenoids. The theory that provided the first conceptual framework for a common structural relationship among the terpenes was first formulated by Wallach in 1887 [4] after carrying out structural investigations of several terpenes. His theory stated that

terpenes can be viewed as made up of one or more isoprene (2-methyl-1,3-diene) units joined together in a head to tail manner. Wallach's idea was further refined in the 1950 by Ruzicka's formulations of the biogenetic isoprene rule [5], emphasizing mechanistic considerations of terpene synthesis in terms of electrophilic elongations, cyclizations and rearrangements.

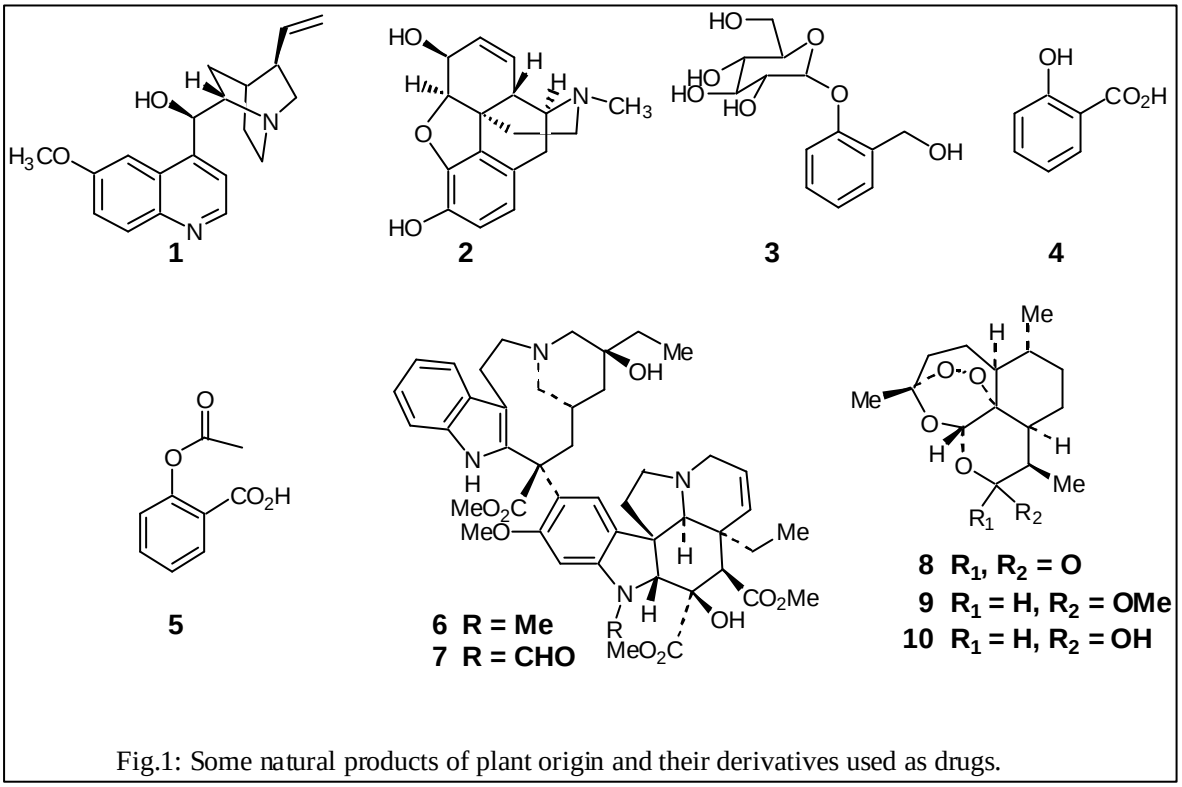


Fig.1: Some natural products of plant origin and their derivatives used as drugs.

2.1. Classifications of Terpenes

Terpenes are normally classified into groups based on the number of isoprene units from which they are biogenetically derived. The higher terpenes are further subdivided in to several subclasses based on the particular type of skeletons they possess.

2.1.1. Hemiterpenes

These are terpenes made up of merely one isoprene unit. The best known hemiterpene is isoprene itself. [6]

2.1.2. Monoterpenes

This class of terpenes contains two isoprene units. These are widely distributed in nature, particularly in essential oils. They are important in perfumery and flavor industries. They are also found in marine organisms. The biosynthetic pathways of the main classes of monoterpenes have been well studied. [7, 8]

2.1.3. Sesquiterpenes

This class of terpenes contains three isoprene units. They are found in many living systems but particularly in higher plants.[9,10] There is a vast number of sesquiterpenoid carbon skeletons, which all, however, arise from the common precursor, farnesyl pyrophosphate (Fig. 5), by various modes of cyclizations, followed in many cases by skeletal rearrangements.[11, 12, 13, 14]

2.1.4. Diterpenes

These are terpenes that contain twenty carbon atoms in their basic skeletons, made from four isoprene units. They are derived from geranylgeranyl pyrophosphate (Fig. 5). [11, 15, 16, 17] They occur in almost all plant families and belong to more than 20 major structural types.

2.1.5. Triterpenes

These are compounds containing thirty carbon atoms made from six isoprene units. They are believed to be derived from squalene which in turn is formed upon head to head coupling of two sesquiterpenoid units. [18, 19] They may be tetracyclic or pentacyclic and best classified biogenetically. [20]

2.1.6. Tetraterpenes

Tetraterpenes are compounds based on eight isoprene units. They are formed by head to head coupling of two geranylgeranyl pyrophosphate molecules. [21] Important among these are the C_{40} carotenoids.

3. Terpenoid Biosynthesis

Terpenes are mainly biosynthesised from two precursors: isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). [22] At present, in addition to the mevalonate pathway, a second pathway known as the non-mevalonate or the deoxyxylulose pathway has been discovered. [23] It is now apparent that the mevalonate pathway formerly regarded as the universal route to terpenoids and steroids is much less prominent in secondary metabolism than the deoxyxylulose pathway. [24].

3.1. The Mevalonate Pathway

Initial observations by Folkers, Tavormina and co-workers indicated that the isoprenoid monomers are biosynthetically derived from mevalonate. [25, 26] Subsequent work by several scientists elucidated in detail the steps of the mevalonate pathway reviewed in Dewick; [23, 24]

As summarized in Fig. 2, the reaction consists of the following steps.

- A formal Claisen condensation type reaction of two acetyl-CoA molecules yields acetoacetyl-CoA catalyzed by acetoacetyl-CoA thiolase.
- An aldol condensation type addition of another acetyl-CoA molecule forms 3-hydroxy-3-methylglutaryl-CoA(HMG-CoA) that is catalyzed by HMG-CoA synthase.
- Subsequent reduction of the 3-hydroxy-3-methylglutaryl-CoA to (R)-mevalonic acid by HMG-CoA reductase under the employment of NADPH molecules
- Phosphorylation of the formed mevalonic acid to mevalonic acid-5-diphosphate catalyzed by mevalonate and phosphomevalonate kinases.
- Phosphorylation assisted decarboxylation yielding isopentenyl diphosphate (IPP).
- Isomerization of IPP to dimethylallyl diphosphate (DMAPP).

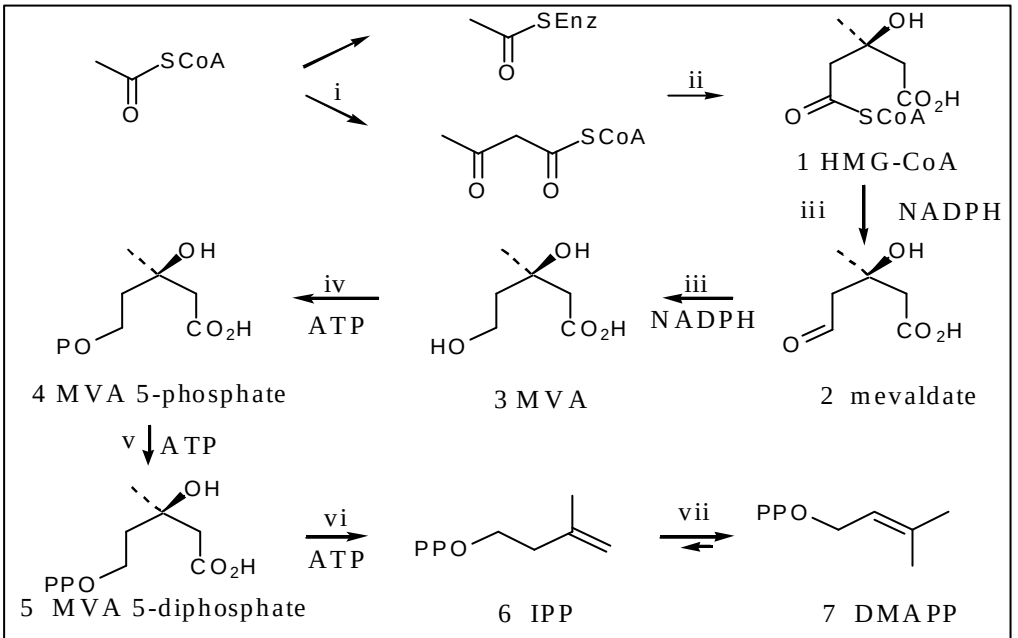


Fig.2:Key steps in mevalonate pathway. *Enzymes*: i, acetoacetyl CoA thiolase (AACT); ii, HMG-CoA synthase; iii, HMG-CoA reductase (HMGR); iv, mevalonate kinase; v, phosphomevalonate kinase; vi, mevalonate 5-diphosphate decarboxylase; vii, IPP isomerase

3.2. The Deoxyxylulose Phosphate Pathway (Fig. 3)

- A transketolase-like condensation between pyruvate and D-glyceraldehyde 3-phosphate to form 1-deoxy-D-xylulose 5-phophate (DXP).
- Transformation of DXP into 2-C-methyl-D-erythritol 4-phosphate (MEP)
- Conversion of MEP into 4-diphosphocytidyl-2-C-methyl-D-erythritol (CDP-ME) in the presence of CTP.
- Conversion of CDP-ME in to CDP-ME2P through phosphorylation.

- Transformation of 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate into 2-C-methyl-D-erythritol 2,4-cyclodiphosphate.
- Intramolecular eliminations followed by reductions and dehydrations to form IPP.

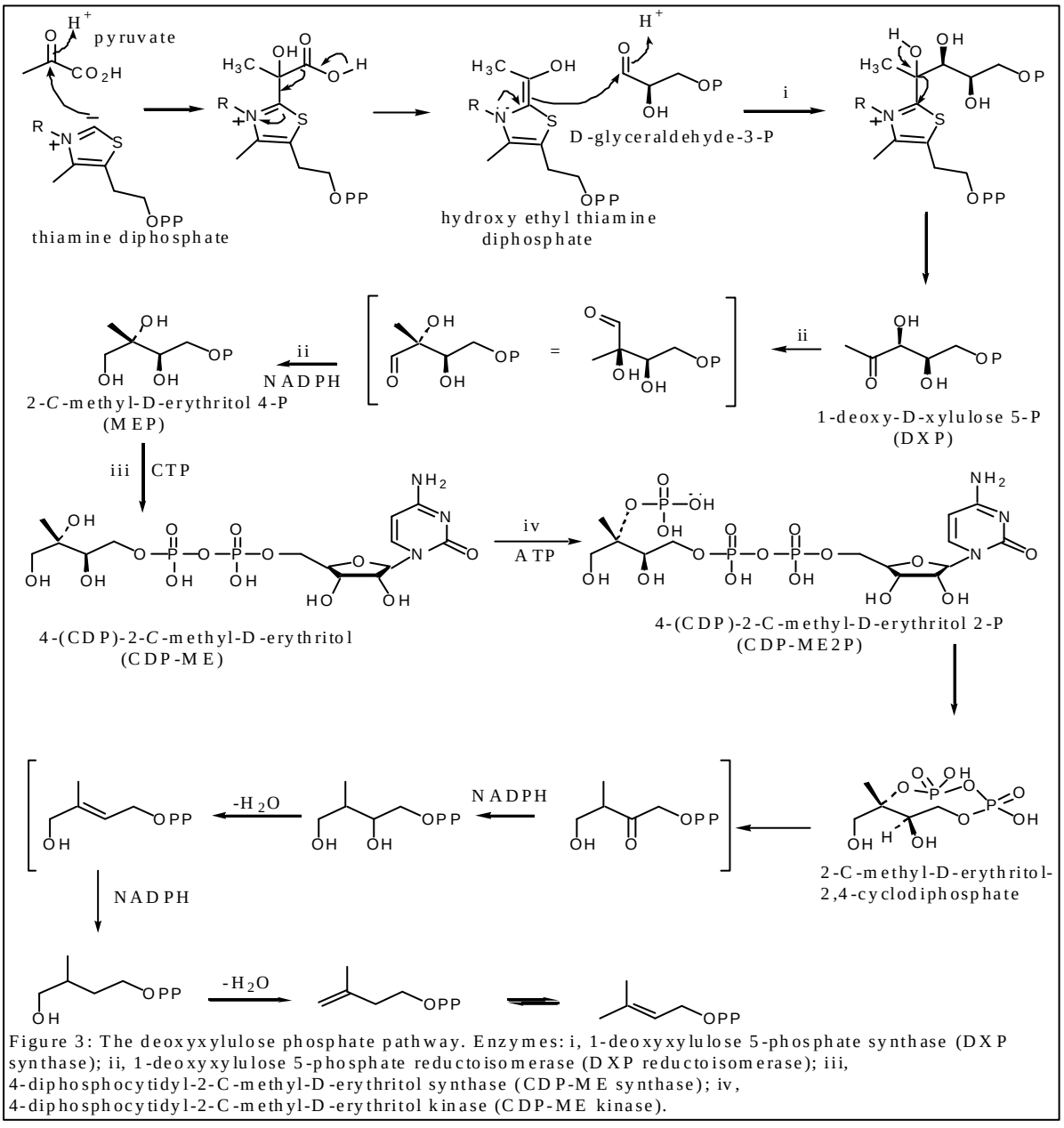


Figure 3: The deoxyxylulose phosphate pathway. Enzymes: i, 1-deoxyxylulose 5-phosphate synthase (DXP synthase); ii, 1-deoxyxylulose 5-phosphate reductoisomerase (DXP reductoisomerase); iii, 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase (CDP-ME synthase); iv, 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (CDP-ME kinase).

3.3 Isomerization of IPP to DMAPP

Dimethylallyl diphosphate that serves as the immediate precursor for isoprenoid biosynthesis is formed from isopentenyl diphosphate through an enzyme catalyzed 1,3-allylic rearrangement reaction (Fig. 4).

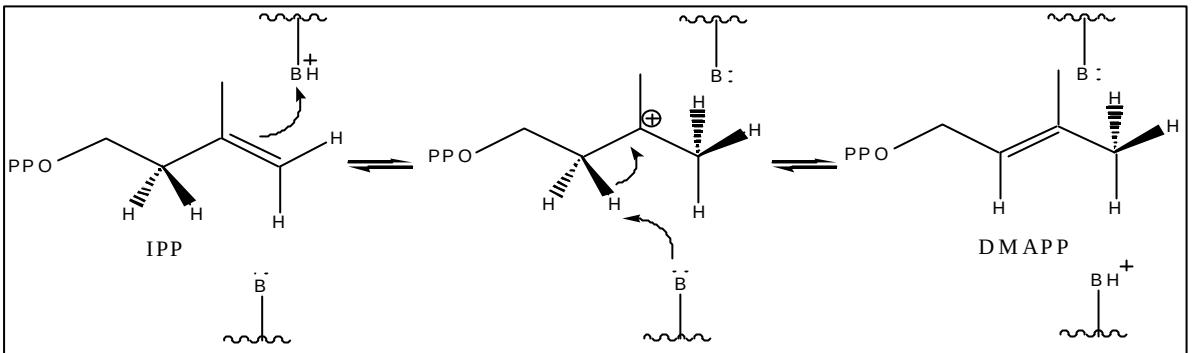


Fig. 4. Enzyme catalysed isomerisation of IPP to DMAPP

3.4. Prenyl Transferases

Dimethylallyl diphosphate serves as the immediate precursor of the different families of terpenoids. As shown in figure 5, it undergoes elongation by the sequential addition of one, two or three IPP molecules to form geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP), respectively. GPP is the 10 carbon precursor for monoterpenes, FPP is the 15 carbon precursor for sesquiterpenes, and GGPP is the 20 carbon precursor for diterpenes. A family of enzymes, known collectively as prenyltransferases, catalyze this elongation sequence. Specific prenyltransferases exist for the formation of GPP, FPP, and GGPP. The reaction catalyzed by prenyltransferases involves the initial ionization of the allylic diphosphate, DMAPP, to generate a delocalized allylic carbocation. This enzyme bound cation attacks the double bond of IPP followed by deprotonation to generate the next allylic diphosphate homologue. As shown in figure 5, in the case of GPP synthase, the first condensation product is released from the enzyme. In the case of FPP and GGPP synthases, the resulting GPP undergoes further reaction with the addition of another IPP to generate FPP, which is either released in the case of FPP synthase or which undergoes reaction with a third IPP to generate GGPP. [27]

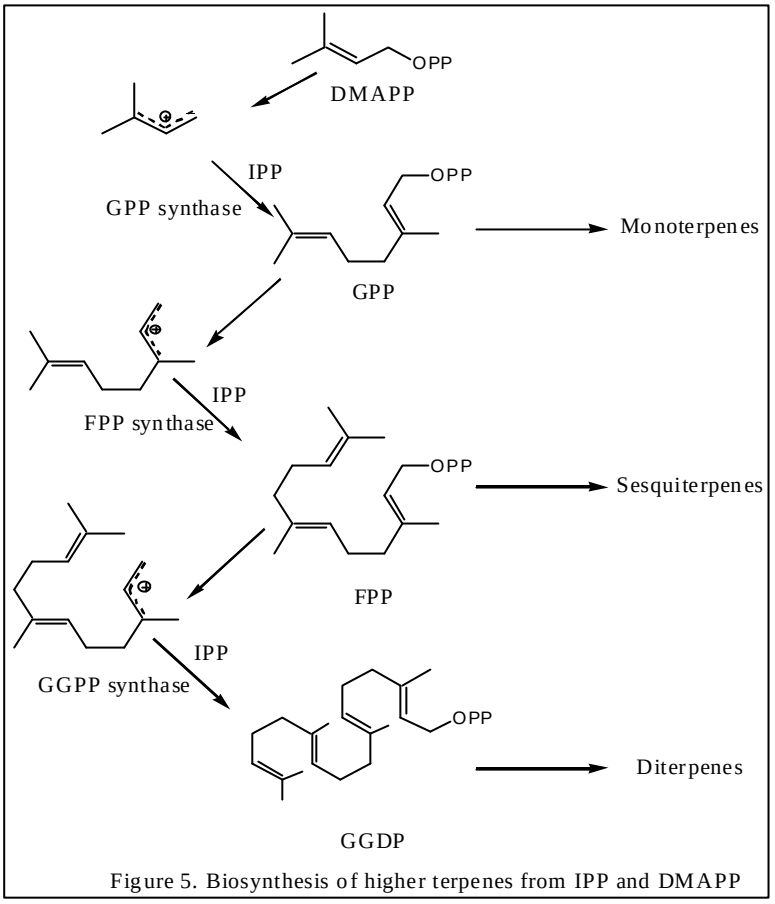


Figure 5. Biosynthesis of higher terpenes from IPP and DMAPP

4. An Overview of the Analytical Methods

4.1. Chromatographic Methods

Chromatography is the method of choice in handling the problem of isolation of a compound of interest from a complex natural mixture. Therefore, the chromatographic methods used during the present work are briefly described.

4.1.1 Thin Layer Chromatography (TLC)

TLC involves the use of a particulate sorbant spread on an inert sheet of glass, plastic, or metal as a stationary phase. The mobile phase is allowed to travel up the plate carrying the sample that was initially spotted on the sorbant just above the solvent. Depending on the nature of the stationary phase, the separation can be either partition or adsorption chromatography. The advantage of TLC is that the samples do not have to undergo the extensive cleanup steps, and the ability to detect a wide range of compounds, using reactive spray reagents. Non destructive detection (fluorescent indicators in the plates, examination

under a UV lamp) also makes it possible for purified samples to be scraped off the plate and be analyzed by other techniques.[28, 29, 30]

4.1.2 Column Chromatography (CC)

CC consists of a column of particulate material such as silica or alumina that has a solvent passed through it at atmospheric, medium or low pressure. The separation can be liquid/solid (adsorption) or liquid/liquid (partition). The columns are usually glass or plastic with sinter frits to hold the packing. Most systems rely on gravity to push the solvent through, but medium pressure pumps are commonly used in flash CC. The sample is dissolved in solvent and applied to the front of the column (wet packing), or alternatively adsorbed on a coarse silica gel (dry packing). The solvent elutes the sample through the column, allowing the components to separate. Normally, the solvent is non polar and the surface polar, although there are a wide range of packings including chemically bound phase systems. Bonded phase systems usually utilize partition mechanisms. The solvent is usually changed stepwise, and fractions are collected according to the separation required, with the eluting products usually monitored by TLC. The technique is not efficient, with relatively large volumes of solvent being used, and particle size is constrained by the need to have a flow of several mls/min. The advantage is that no expensive equipment is required, and the technique can be scaled up to handle sample sizes approaching gram amounts.[28, 29, 30]

4.1.3 High Pressure Liquid Chromatography (HPLC)

HPLC is a development of column chromatography. To improve resolution, HPLC columns are packed with small sized particles (3, 5, 10μm) with a narrow size distribution. Flow rate and column dimensions can be adjusted to minimize band broadening. The required pressures are supplied by pumps that could withstand the involved chemicals. In addition to the normal phase columns, (non polar solvent and polar surface such as silica), there are reverse phase (RP) columns as well. The latter, normally, involves the use of a polar solvent (water, methanol, acetonitrile etc.) and a non polar surface. RP HPLC is the method of choice for larger non volatile molecules. The commonly used detector (UV detector) in HPLC systems not only places constraints on the solvents that can be used but also is limited to absorbing compounds. Refractive index detectors although considered "universal" can not easily be used with solvent gradients. However, recently, the evaporative light-scattering detector has emerged as a universal detector.[28, 29, 30]

4.1.4 Gel Permeation Chromatography

Gel permeation chromatography (Size Exclusion Chromatography) is based on the ability of molecules to move through a column of gel that has pores of clearly defined sizes. The larger molecules can not enter the pores, and therefore, they move faster through the column and elute first. Slightly smaller molecules can enter some pores, and so take longer to elute, while small molecules can be delayed further. The advantage of the technique is simplicity, is isocratic, and large molecules rapidly elute. However, the columns are expensive and sensitive to contamination; consequently they are mainly used in applications where alternative separation techniques are not available, and samples are fairly clean. The commonly used gel in natural products lab is sephadex LH-20 to separate chlorophyll from compounds of interest, where usually chlorophyll elutes first. [28, 29, 30]

4.1.5 Gas Chromatography (GC)

GC is the use of a carrier gas to convey the sample in a vapor state through a narrow column made from usually fused silica tubes (0.1 to 0.3mm ID) that have refined stationary phase films (0.1 to 5µm) bound to the surface and cross linked to increase thermal stability. The column is installed in an oven that has temperature control, and the column can be slowly heated up to 350-450 °C starting from ambient temperature to provide separation of a wide range of compounds. The carrier gas is usually hydrogen or helium under pressure, and the eluting compounds can be detected several ways, including flames (flame ionization detector), by changes in properties of the carrier (thermal conductivity detector), or by mass spectrometry. The availability of "universal" detectors such as the FID and MS, makes GC the appropriate tool in the investigation of essential oils. The availability of modified cyclodextrins as stationary phases made it possible to separate enatiomers, the determination of enatiomeric ratios and absolute configurations. However, GC is restricted to molecules (or derivatives) that are sufficiently stable and volatile to pass through the GC system intact at the operating temperatures. [31, 32, 33].

4.1.6. Chromatographic solvent "polarity"

There are four major intermolecular interactions between sample and solvent molecules in liquid chromatography, dispersion, dipole, hydrogen-bonding, and dielectric. Dispersion interactions are the attraction between each pair of adjacent molecules, and are stronger for sample and solvent molecules with large refractive indices. Strong dipole interactions occur when both sample and solvent have permanent dipole moments that are aligned. Strong hydrogen-bonding interactions occur between proton donors and proton acceptors. Dielectric

interactions favor the dissolution of ionic molecules in polar solvents. The total interaction of the solvent and sample is the sum of the four interactions. The total interaction for a sample or solvent molecule in all four ways is known as the "polarity" of the molecule. Polar solvents dissolve polar molecules and, for normal phase partition chromatography, solvent strength increases with solvent polarity, whereas solvent strength decreases with increasing polarity in reverse-phase systems. The subject is discussed in detail in Snyder and Kirkland. [30]

4.2. Extraction Techniques

4.2.1 Solvent Extraction

Solvent extraction is usually used to recover a component from either a solid or liquid. The sample is contacted with a solvent that will dissolve the solutes of interest. Some extraction techniques involve partition between two immiscible liquids, others involve either continuous extractions or batch extractions. During the present work, dried and pulverized plant materials are soaked in an organic solvent to extract the secondary metabolites.

4.2.2 Hydrodistillation

This was the main method used for extraction of the analyzed essential oils. The process is carried out in a Clevenger type apparatus, where the material to be extracted is chopped, and immersed in water, which is then boiled. During hydrodistillation the essential oil components form an azeotropic mixture with water. The vapors of the volatile components are carried by the steam to a condenser. Upon condensation, the droplets are continuously extracted by a ca. 1 mL HPLC grade hexane layer at the front of the receiver and separated by decantation. The distillation period can take from 2 to 2.5 hr. The extraction period influences not only the yield but also the extract composition. The sample is exposed to temperatures close to 100ºC, which can lead to changes in 'thermolabile' components. Prolonged heating in contact with water can lead to hydrolysis of esters, polymerization of aldehydes, or decomposition (e.g. dehydration) of other components.[34]

4.3. Spectroscopic Techniques

4.3.1. Nuclear Magnetic Resonance Spectroscopy (NMR)

Spectroscopy is the study of the interaction of electromagnetic radiation (EMR) with matter. NMR spectroscopy is the study of interaction of radio frequency (RF) of the EMR with unpaired nuclear spins in an external magnetic field to extract structural information about a given sample. NMR spectroscopy is routinely used by chemists to study chemical structure of

simple molecules using simple one dimensional techniques (1D-NMR). Two-dimensional techniques (2D-NMR) are used to determine the structure of more complicated molecules. [35]. The organic chemist is principally concerned with the study of carbon compounds. As a consequence, he/she is interested in 1D and 2D NMR involving protons (^{1}H) and carbons (^{13}C).

4.3.1.1. One Dimensional NMR

4.3.1.1.1. 1D-Proton NMR (^{1}H-NMR)

Proton NMR is a plot of signals arising from absorption of RF during an NMR experiment by the different protons in a compound under study as a function of frequency (chemical shift). The area under the plots provides information about the number of protons present in the molecule, the position of the signals (the chemical shift) reveals information regarding the chemical and electronic environment of the protons, and the splitting pattern provides information about the number of neighboring (vicinal or geminal) protons. [36, 37, 38]. For instance in Figure 6, proton NMR of a diterpene hydrocarbon, viscida-4,11(18),14-triene, isolated from the liverwort *Radula perrotteetii* (Paper III) is presented. The abscissa shows the chemical shift (δ) values of the different type of protons and the ordinate shows the intensities of the signals. The signals of protons attached to saturated carbon atoms such as methyl, methylene as well as methine groups appear between δ 0.8 and 2.4 ppm in the spectrum. The most intense peaks arise from the methyl groups. The less intense peaks arise from both the methylene as well as the methine groups. Further, the signals between δ 4.8 and 5.1 ppm correspond to the olefinic methylene groups and the signals between δ 5.2 and 5.4 are due to the olefinic methine groups. The detailed assignment of the chemical shift values are give in Paper III.

4.3.1.1.2. 1D-Carbon NMR (^{13}C-NMR)

Similar to proton NMR, carbon NMR is a plot of signals arising from the different carbons as a function of chemical shift. The signals in ^{13}C-NMR experiments normally appear as singlets because of the decoupling of the attached protons. Different techniques of recording of the 1D carbon NMR has been developed so that it is possible to differentiate between the various types of carbons such as the primary, secondary, tertiary and quaternary from the 1D ^{13}C-NMR plot. The range of the chemical shift values differs between the ^{1}H (normally 0-10) and ^{13}C NMR (normally 0-230) that arises from the two nuclei having different numbers of electrons around their corresponding nuclei as well as different electronic configurations. [36,

37, 38]. Figure 7 shows a proton decoupled ^{13}C-NMR of a furanolabdane diterpene isolated from *Otostegia integrifolia* (Paper I). Between δ 15 and 45 ppm ^{13}C signals arising from saturated methyl, methylene, methine and quaternary carbons are seen. Between δ 70 –80 ppm, besides the solvent ($CDCl_3$) peaks, signals arising from saturated oxygenated groups are seen. From around δ 105- 145 ppm, signals arising from olefinic as well as oxygenated carbons such as olefinic methines, olefinic quaternaries and olefinic oxygenated carbons are seen. The detailed assignments are given in Paper I.

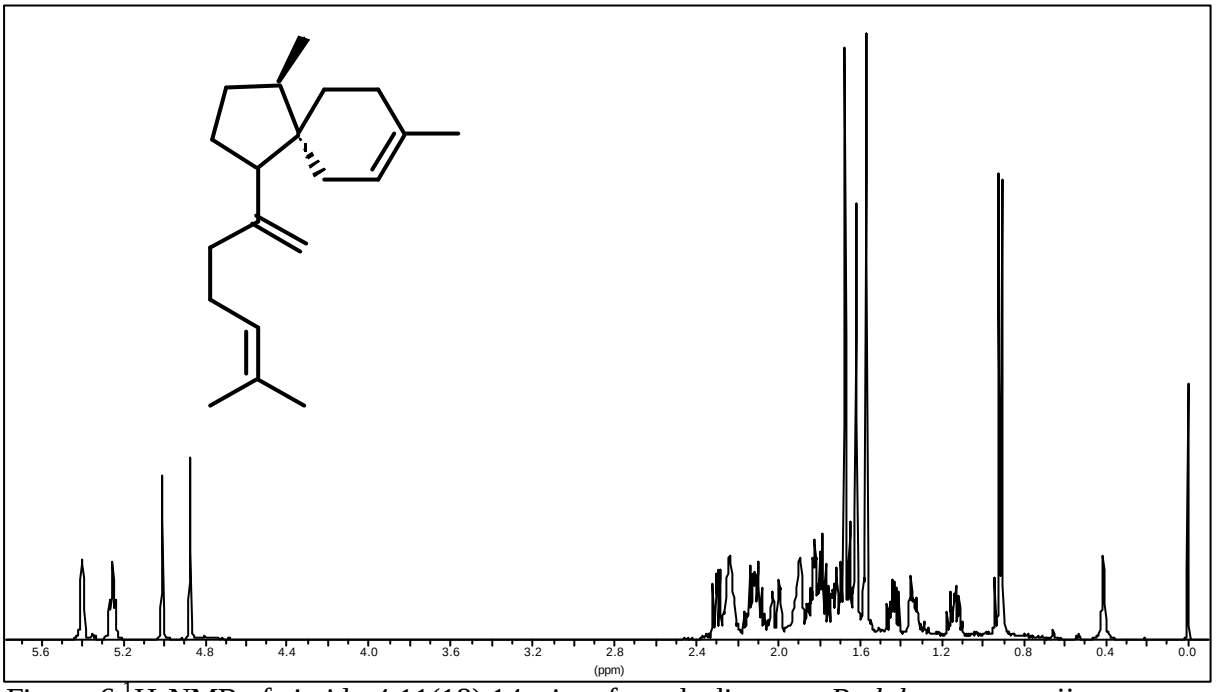


Figure. 6 ^{1}H-NMR of viscida-4,11(18),14-triene from the liverwort *Radula perrotteetii*

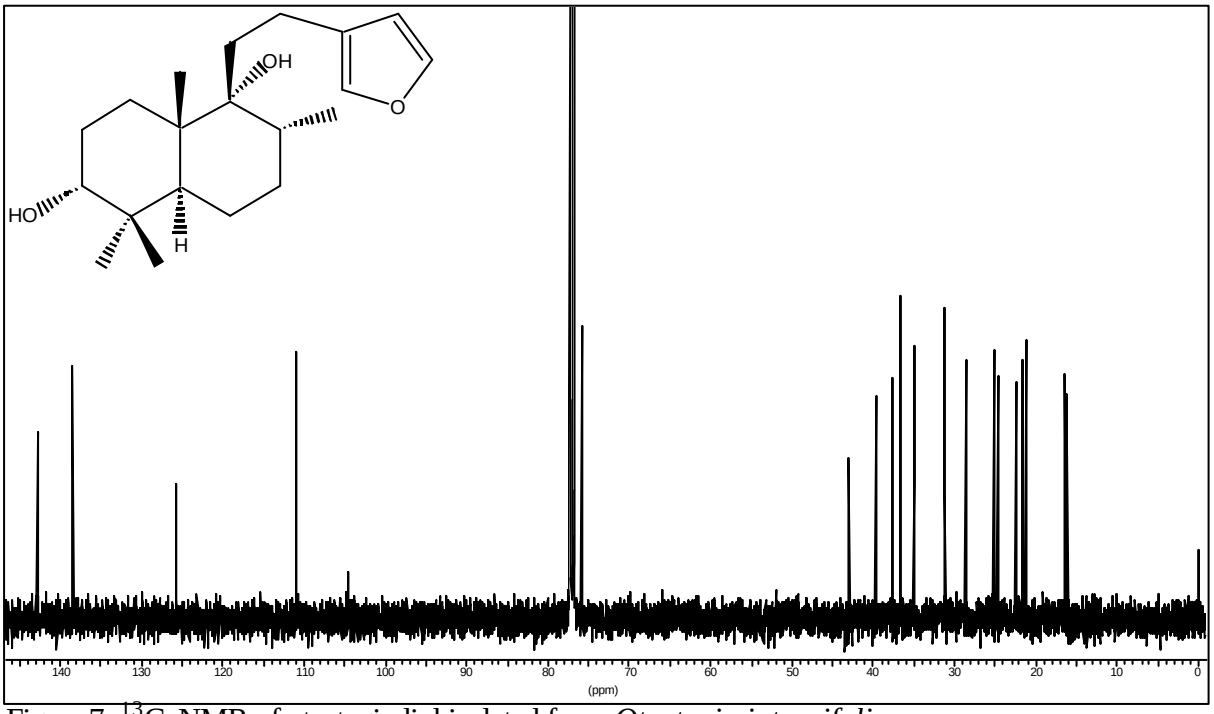


Figure 7: ^{13}C-NMR of otostegindiol isolated from *Otostegia integrifolia*

4.3.1.2. Two dimensional NMR

Currently, the common 2D-NMR experiments that appear in papers concerned with structural elucidation of natural products include the homonuclear ^{1}H, ^{1}H-COSY as well as NOESY and the hetronuclear ^{1}H, ^{13}C-HMQC as well as HMBC.

4.3.1.2.1 2D ^{1}H,^{1}H-COSY (COrrelated SpectroscopY)

^{1}H,^{1}H-COSY is one of the most useful experiments. It is a plot that shows coupling among neighboring protons involving ^{2}J, ^{3}J as well as ^{4}J. It provides information on the connectivity of the different groups within the molecule. [39, 40]. Figure 8 displays ^{1}H,^{1}H-COSY of a sesquiterpene hydrocarbon, guaia-1(10),11-diene isolated from *Peucedanum tauricum* fruits (Paper IV). On both axes are shown the ^{1}H-NMR of the compound. By drawing a straight line from any of the dark spots to each axis, one can see which protons couple with one another, and which are therefore attached to neighboring carbons.

4.3.1.2.2. 2D Nuclear Overhauser Enhancement SpectroscopY (NOESY)

2D NOESY is a homonuclear correlation via dipolar coupling; dipolar coupling may be due to NOE or chemical exchange. It is one of the most useful techniques as it allows to correlate nuclei through space (distance smaller than 5Å) and enables the assignment of relative configuration of substituents at chiral centers. [39, 40]. Figure 9 shows the NOESY spectrum of otostegindiol (Paper I). Similar to the COSY, it is possible to see which protons are nearer to each other in space by drawing a straight line from any of the dark spots to each axis of the plot.

4.3.1.2.3. HMQC (Heteronuclear Multiple Quantum Correlation)

The HMQC experiment provides correlation between protons and their attached heteronuclei through the heteronuclear scalar coupling. This sequence is very sensitive as it is based on proton detection instead of the detection of the least sensitive low gamma heteronuclei. The basic idea behind this experiment is related to the echo difference technique which is used to eliminate proton signals not coupled to the heteronuclei. From this experiment important information regarding the number and chemical shifts of methyl, methylene and methine groups can be extracted. [39, 40]. For instance, Figure 10 shows part of the HMQC spectrum of viscida-4,9,14-triene isolated from the liverwort *R. perrotteetii*. The abscissa of the spectrum shows the proton signals, and the ordinate shows the carbon signals. The spots in the spectrum indicate which protons signal is attached to which carbon and the nature of the

signals, i.e. whether the signal is due to a methyl, methylene or methine carbon. Since quaternary carbons have no attached protons, they don't appear in HMQC plots.

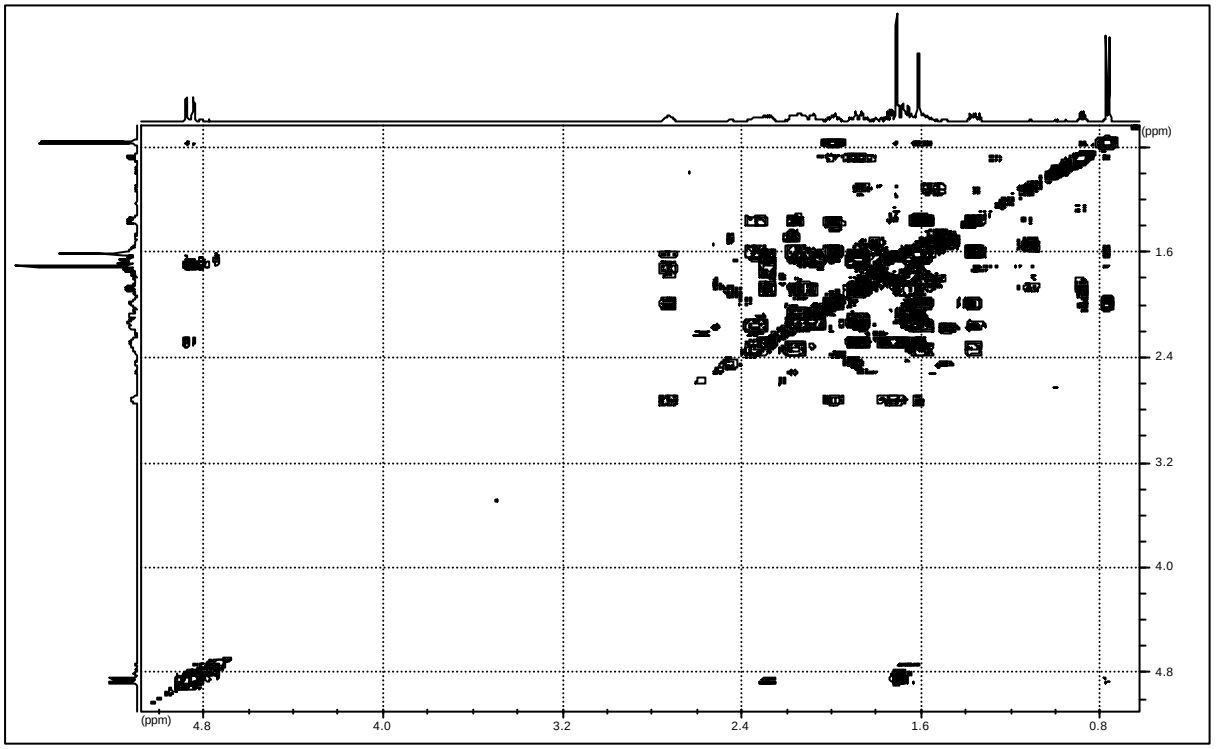


Figure 8. $^{1}H,^{1}H$-COSY of guaia-1(10),11-diene from *Peucedanum tauricum* fruits

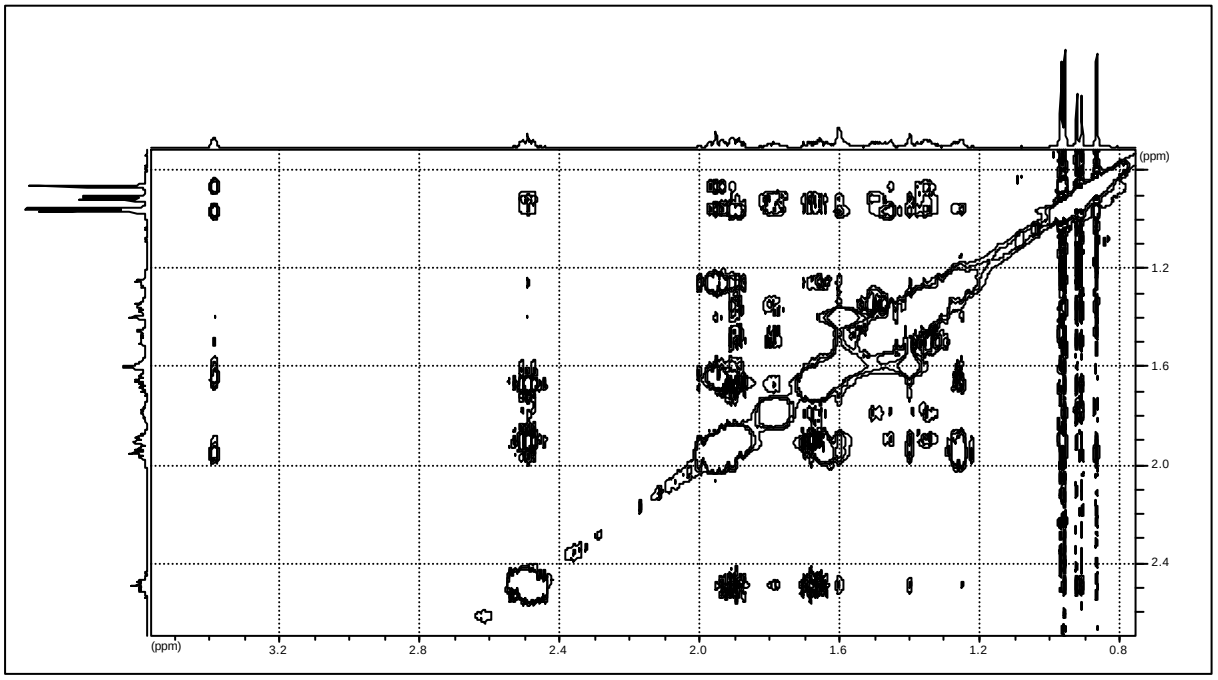


Figure 9. NOESY spectrum of otostegindiol isolated from *Otostegia integrifolia* leaves.

4.3.1.2.4. HMBC (Heteronuclear Multiple Bond Correlation)

The HMBC experiment detects long range coupling between proton and carbon (two or three bonds away) with great sensitivity. The experiment can be adjusted to detect relatively large coupling constants (4-10 Hz) or smaller. This experiment in conjugation with ^{1}H, ^{1}H-COSY enables the elucidation of the skeleton of the compound under study. [39, 40]. Figure 11 shows the HMBC spectrum of viscida-4,11(18),14-triene. The detailed correlations are described in Paper III.

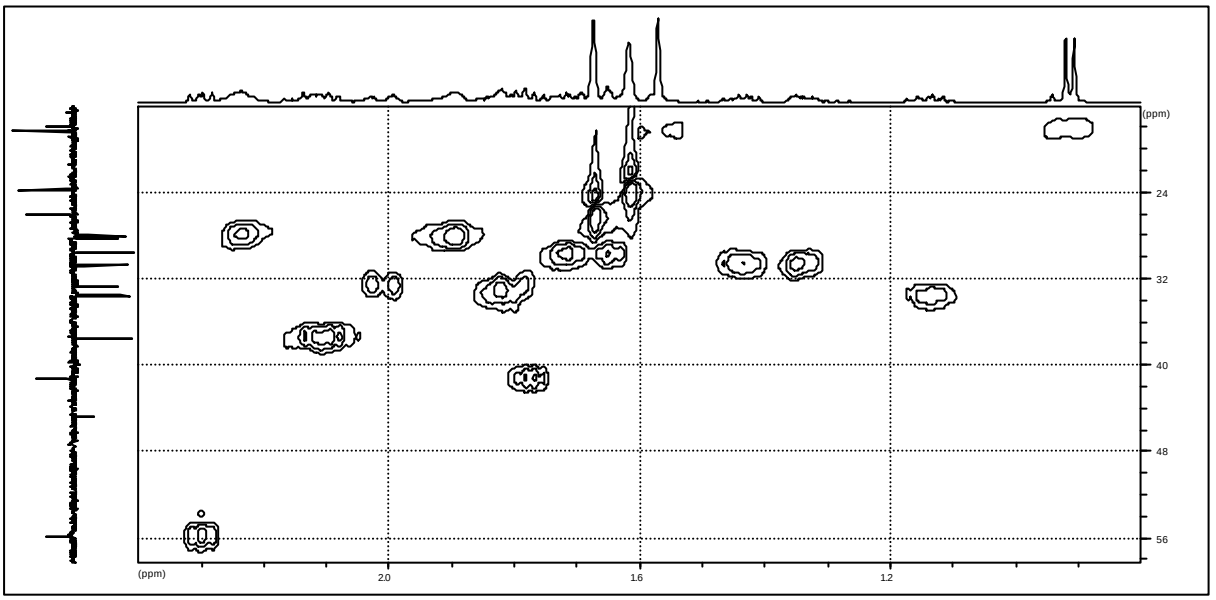


Figure 10. HMQC spectrum of viscida-4,9,14-triene from the liverwort *Radula perrotteetii*

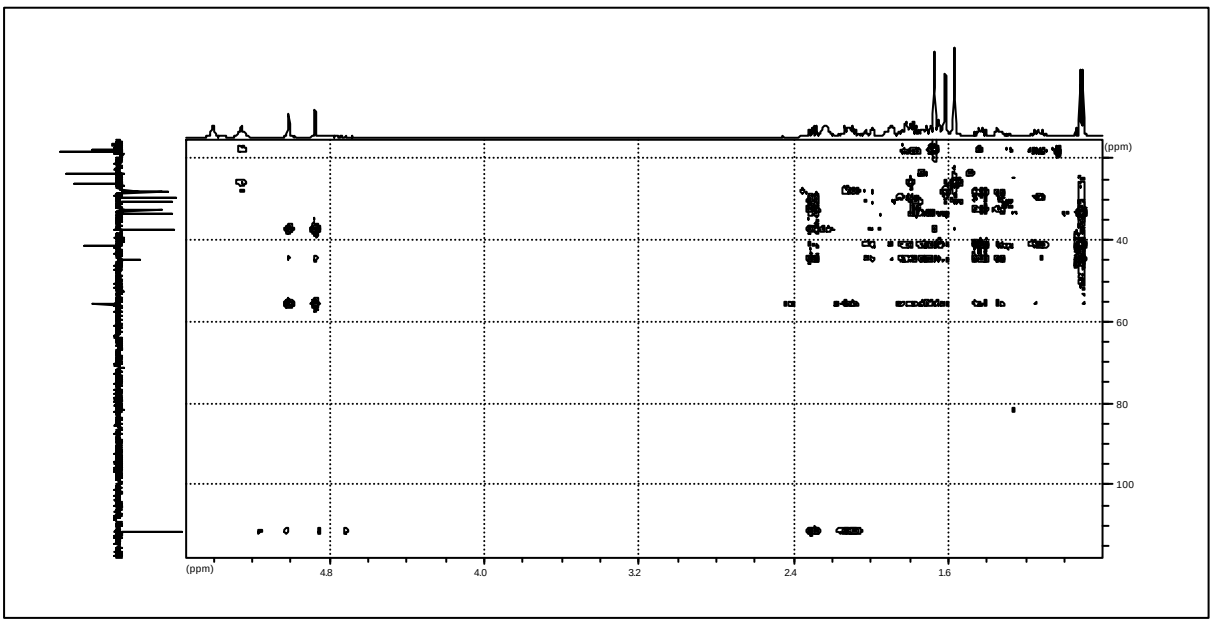


Figure 11. HMBC spectrum of viscida-4,11(18),14-triene from the liverwort *R. perrotteetii*

4.3.1.3. Other Spectoscopic methods

These include the infrared (IR) spectroscopy which offers information relating to the functional groups, and the ultraviolet (UV) spectroscopy which reveals information relating to the presence of sites of unsaturations in the structure. These two methods are becoming less important in structure elucidation of natural products due to the superiority of information obtained from the NMR experiments with much less sample amounts.

4.3.1.4. Gas Chromatography/Mass Spectrometry (GC/MS)

In GC/MS a mixture of compounds to be analyzed is initially injected into the GC where the mixture is vaporized in a heated chamber (injector). The gas mixture travels through a GC column carried by a carrier gas, where the compounds become separated as they interact with the stationary phase of the column. The separated compounds then immediately enter the mass spectrometer that generates the mass spectrum of the individual compounds.

4.3.1.4.1. Mass Spectrometery (MS)

MS is an analytical technique that involves generating charged particles (ions) from molecules of the analyte. The generated ions are analyzed to provide information about the molecular weight of the compound and its chemical structure. There are many types of mass spectrometers and different sample introduction techniques which allow a wide range of samples to be analyzed. The widely utilized practice of coupling Gas Chromatography (GC) with Mass Spectrometry (MS) was routinely employed for the analysis of the compositions of various essential oils. All mass spectrometers consist of three distinct regions that can be described as ionizer, ion analyzer, and detector.

Ionizer

In GC/MS, normally the charged particles (ions) required for mass analysis are formed by Electron Impact (EI) ionization technique. The gas molecules eluting from the GC are bombarded by a high energy electron beam (70 eV). An electron which strikes a molecule may impart enough energy to remove another electron from that molecule thereby creating a positively charged ion. EI ionization usually produces singly charged ions containing one unpaired electron. An array of ionization methods different from EI is available to meet the needs of many types of chemical analysis. A few are listed here with a highlight of their usefulness, (Table 1).

Table 1: The different sample ionization methods currently in use.

Ionization method	Typical Analytes	Sample Introduction	Method Highlights
Electron Impact (EI)	Relatively small volatile	GC or liquid/solid probe	Hard method versatile provides structure info
Chemical Ionization (CI)	Relatively small volatile	GC or liquid/solid probe	Soft method molecular ion peak $[M+H]^+$
Electrospray (ESI)	Non-volatile	Liquid Chromatography or syringe	Soft method ions often multiply charged
Fast Atom Bombardment (FAB)	Nonvolatile	Sample mixed in viscous matrix	Soft method but harder than ESI or MALDI
Matrix Assisted Laser Desorption (MALDI)	Peptides Proteins Nucleotides	Sample mixed in solid matrix	Soft method very high mass

Ion Analyzer

Molecular ions and fragment ions are accelerated by manipulation of the charged particles through the mass spectrometer. Uncharged molecules and fragments are pumped away. A summary of some of the different types of mass analyzers is displayed in Table 2.

Table 2: Some of the various mass analyzers and their system highlights.

Analyzer	System Highlights
Quadrupole	Unit mass resolution, fast scan
Sector (Magnetic and/or Electrostatic); double focusing	High resolution, exact mass
Time-of-Flight (TOF)	Theoretically, no limitation for m/z maximum, high throughput
Ion Cyclotron Resonance (ICR)	Very high resolution, exact mass

The GC/MS employed during this work was a one with a sector analyzer having magnetic and electric sector. The magnetic sector analyzes the momentum of the ions ($mv = zeBr_m$ *or* $r_m = mv/zeB$, where m denotes the mass, v velocity, z charge, e unit charge, B magnitude of the magnetic field and r_m radius). That is only ions that travel at the right velocity (momentum) can successfully follow the path (r_m) through the sector. The electric sector selects ions possessing a given energy value. That is for a given electrostatic sector, $mv^2/r_e = zeV_e/d$ where

V_e is the applied potential, d the distance between the plates and r_e the radius the ions follow in the sector. For a given geometry, r_e and d are fixed, therefore, the setting of V_e selects the kinetic energy $mv^2/2$.

As the ions move with the same velocity through both the magnetic and electrostatic sectors, we can equate the velocities used for electric and magnetic sectors which yield:

$$\frac{zeBr_m}{m} = \sqrt{\frac{zeV_e r_e}{md}}$$

From this equation we can measure a mass spectrum by scanning the magnetic field, B, and/or the electrostatic field, V_e.

$$\frac{m}{z} = \frac{ed}{V_e r_e} B^2 r_m^2$$

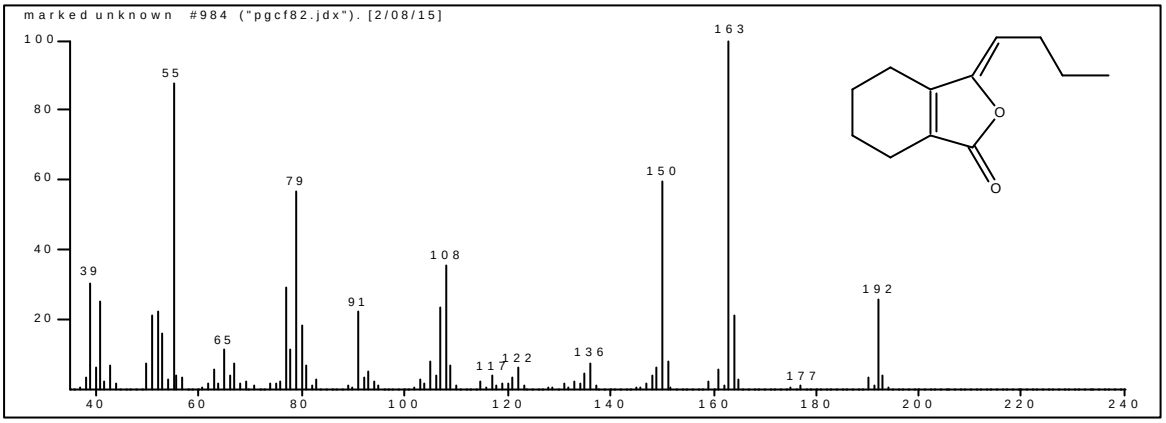
Detector

There are many types of detectors, but most function by producing an electronic signal when struck by an ion. Timing mechanisms which integrate those signals with the scanning voltages allow the instrument to report which *m/z* strikes the detector. The mass analyzer sorts the ions according to *m/z* and the detector records the abundance of each *m/z*. Regular calibration of the *m/z* scale is performed to maintain accuracy in the instrument.

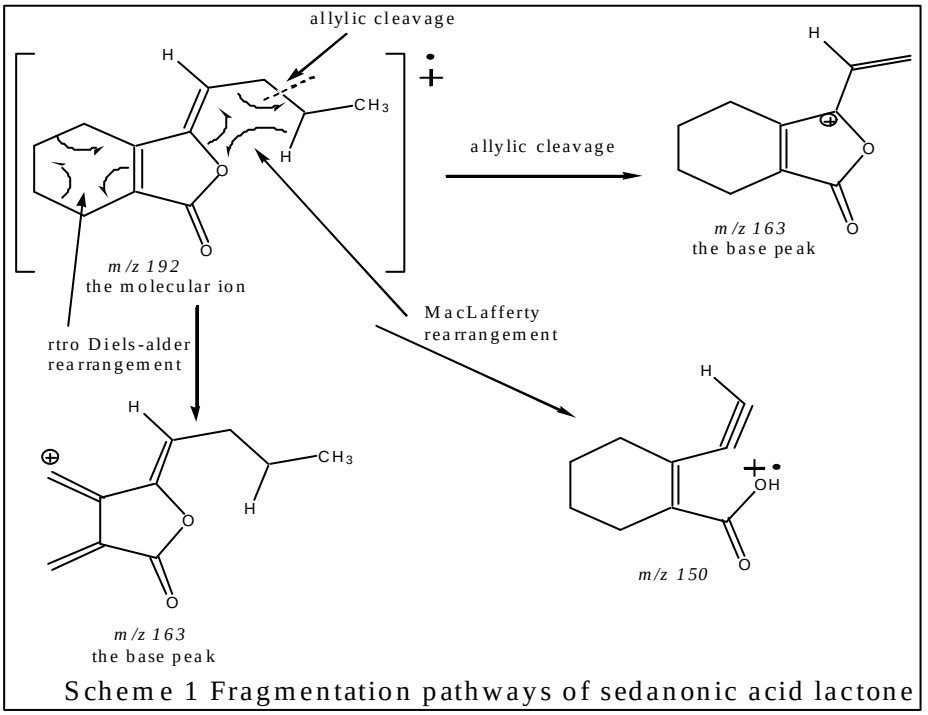
4.3.1.4.2. Interpreting mass spectra

a. Molecular ion ($M^{\cdot +}$): The mass spectrum of sedanonic acid lactone isolated from the essential oil of *Meum athamanticum* Jacq. is shown in Fig.12. $C_{12}H_{16}O_2^{+\bullet}$ (the molecular ion) and several fragment ions appear in this spectrum. The ordinate represents the relative abundance of each ion. Assignment of the relative abundance begins by assigning the most abundant ion a relative abundance of 100% ($C_{10}H_{11}O_2^+$ in this spectrum, also known as the base peak). All other ions are shown as a percentage of that most abundant ion. If the molecular ion appears (as it does in this case), it will be the highest mass in an EI spectrum (except for isotope peaks). This peak will represent the molecular weight of the compound and correspond to the elemental composition which is a key information in structural elucidation problems. Its appearance depends on the stability of the compound. Double bonds, cyclic structures and aromatic rings stabilize the molecular ion and increase the probability of its appearance. [41].

Figure 12. 70 eV mass spectrum of sedanonic acid lactone from *Meum athamanticum* Jacq.



b. Fragmentation: Energy imparted by the electron impact can cause the molecular ion to split into fragment ions. In general, bond cleavages may take place at σ-bonds, or σ-bonds next to a hetro atom such as oxygen, or σ-bonds allylic to sites of unsaturations. In addition, cleavage may take place through rearrangement reactions such as the retro Diels-Alder and the MacLafferty rearrangements. Weaker bonds tend to break easily, particularly next to structural features that support the stability of the formed fragment ion through resonance (aromatic compounds) and inductive effects as well as donation of lone pairs of electrons. Functional groups and overall structure determine how some portions of molecules will resist fragmenting, while other portions will fragment easily. Some of these are shown for sedanonic acid lactone in scheme 1 and in Fig. 14 for (+)-axinyssene.



Scheme 1 Fragmentation pathways of sedanonic acid lactone

c. Reference spectra: Mass spectral patterns are reproducible. The mass spectra of many compounds have been published and can be used to identify unknowns. Computers generally contain spectral libraries which can be searched for matches. During this work, a spectral library generated under identical experimental conditions was routinely used to identify known components.

d. Isotopes: Since isotopes occur in compounds analyzed by mass spectrometry in the same abundances as they occur in nature, they can aid in peak identification.

5. Plant Materials

Six different plant species obtained from various geographical locations including Africa, Asia and Europe were investigated. These were leaves of a herbaceous plant *Otostegia integrifolia* Benth. (Lamiaceae (Labiatae)) [42] collected in Ethiopia (Paper I), flowers of the herbaceous plant *Chlorantus spicatus* (Chloranthaceae) [43] from Vietnam (Paper II), fruits of an umbelliferous plant known as *Peucedanum tauricum* [44] collected in Poland (Paper III), aerial parts of the herbaceous plant *Meum athamanticum* (Apiaceae) from Germany (Paper IV), the liverwort *Radula perrottetii* (Hepaticae) of Japanese origin (Paper V) and leaves of the rare umbellifer *Melanoselinum decipiens* [45] grown in Hamburg, Germany (Paper VI).

6. Experimental Aspects

Prior to the practical analysis, a proper study of the botanical information, chemical background, and ethnobotany (if available) of each of the investigated plants were carried out. The isolation of the compounds started with extracting of the mixture of secondary metabolites present in the plant material. Air-dried or fresh plant materials were subjected to either hydrodistillation or solvent extraction. The former yielded a complex mixture of volatile compounds known as essential oil while the latter gave a complex mixture of volatile or non-volatile compounds depending on the polarity of the solvent employed for the extraction process.

6.1. Analysis of the Essential Oils

The essential oils were diluted to appropriate concentrations (ca. 1 µg/ml) and were preliminarily analyzed by capillary GC as well as GC/MS. Mass spectrum and retention index of each component was compared with a library spectra of authentic samples. The known components were identified. The components that couldn't be identified by simple comparisons were marked as unknowns and subsequently isolated. The isolation of the

unknowns from the complex mixtures was carried out by using a number of techniques. The essential oils were first fractionated into a hydrocarbon fraction and an oxygenated fraction by using a simple flash silica gel column. The former was obtained by eluting the column with hexane and the latter by ethyl acetate, consecutively. Each fraction was analyzed on several capillary GC equipped with columns coated with a range of stationary phases (from the non-polar CPSil-5 to various modified cyclodextrins) until optimum resolution of the components was obtained. The hydrocarbon and the oxygenated fractions were further fractionated by preparative GC equipped with a prep. column packed with the stationary phase that gave the optimal resolution. This process was repeated as many times as required until pure compounds were obtained.

6.2. Analysis of the Solvent Extracts

In the cases of solvent extraction, chloroform or dichloromethane was used. This gave mainly non volatile mixtures of compounds. The composition of the crude extracts was inspected by using TLC. The visualizations were aided by either observing the TLC under an UV lamp or by spraying with anisaldehyde reagent followed by heating. The TLC was repeatedly improved by changing the solvent systems until a system that gave the best separation was obtained. The crude extracts were repeatedly chromatographed on columns packed with either silica-gel, sephadex-LH 20 or on HPLC until pure compounds were obtained (Papers I &IV).

6.3. Identification and characterization of Isolated Unknowns

The isolated unknowns were identified by using a combination of MS as well as 1D and 2D-NMR techniques. Relative configurations were determined from 2D-NOESY spectra and, where possible, absolute configurations were determined through chemical transformations, correlations with references of known absolute configurations, and enantioselective capillary GC analysis (Paper IV).

7. Results and Discussion

7.1. Otostegia integrifolia Benth.

7.1.1 Description of the plant and Literature Survey

O. integrifolia is a herbaceous plant belonging to plants of the Lamiaceae (Labiatae), [42]. The plant grows in the wild but is also cultivated in gardens. It is one of the plants used in traditional medicine in Ethiopia. The plant has insecticidal properties and is often used as fumigant for pots and houses. The roots are used for treating lung diseases, [42]. No previous

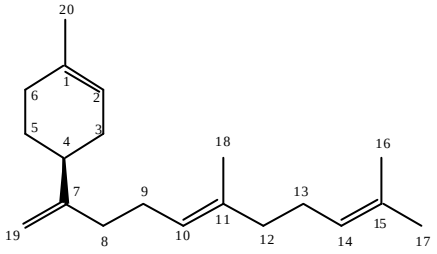
phytochemical investigation of the plant has been reported. However, a literature review on the genus otostegia has revealed that thymol, γ-terpinene and p-cymene were identified as major constituents in the essential oil of *O. fruticosa* analyzed by gas chromatography-mass spectrometry (GC-MS) [46]. Furthermore, from aerial parts of the same plant, isolation of three new and five known prefuranic and furanic labdane diterpenes together with iridoid glucoside was reported. These were otostegin A, otostegin B, 15-*epi*-otostegin B [47], preleoheterin, leoheterin [48], and related compounds leopersin C, 15-*epi*-leopersin C [49], ballonigrin [50], vulgarol [51], and 8-O-acetylharpagide [52].

7.1.2 Results and Discussion on O. integrifolia

Air-dried and pulverized leaves of *O. integrifolia* were subjected to both hydrodistillation that gave the essential oil and solvent extraction using chloroform.

7.1.2.1. Essential oil of the leaves

GC and GC/MS analysis of essential oil of air-dried leaves of *O. integrifolia* made possible the identification of a total of 40 constituents comprised of monoterpenes, sesquiterpenes, diterpenes and their derivatives (Paper I). The major component was identified as a prenylbisabolane diterpene known as (+)-axinyssene (**11**). Axinyssene is 1-methyl-4-(5,9-dimethyl-1-methylenedeca-4,8-dienyl)cyclohexene. The structure of the compound was established from MS, 1D- and 2D-NMR data after the compound was isolated by preparative GC.



11

7.1.2.1.1 (+)-Axinyssene (1-methyl-4-(5,9-dimethyl-1-methylenedeca-4,8-dienyl)cyclohexene)

The mass spectrum of the compound (Fig. 13) showed a molecular ion peak at *m/z* 272. The fragmentation pattern is indicative of a presence of a polyunsaturated branched hydrocarbon chain. The spectrum exhibits close similarity to that of geranyl linalool, but in its NMR data no oxygenated group could be observed excluding the possibility of geranyl linalool. Instead, its ^{1}H- and HMQC-NMR revealed the presence of a total of 32 protons. These were four

allylic methyl, one exocyclic and seven saturated methylene as well as three olefinic and one saturated methine protons. From the ^{13}C-NMR of the compound, presence of twenty carbon atoms comprised of four methyl, eight methylene, four methine and four olefinic quaternary carbons was established. The ^{1}H- and ^{13}C-NMR data in combination with the mass spectrum confirmed an elemental composition of $C_{20}H_{32}$, a diterpenoid hydrocarbon with five degrees of unsaturation. The fact that eight of the twenty carbon atoms were olefinic was indicative of four double bonds in the molecule. Therefore, the compound had to be a tetraunsaturated monocyclic diterpene. Extensive analysis of the 2D-^{1}H,^{1}H COSY as well as HMBC spectra of the compound led to the actual structure of 1-methyl-4-(5,9-dimethyl-1-methylenedeca-4,8-dienyl)cyclohexene. This structure was also supported by its mass spectrum. The base peak at *m/z* 69 arises from an allylic cleavage at the C-13/C-14 bond, typical for alkenes. It owes its stability to the resonance effect of the double bond as shown in the scheme. The other fragments can also be accounted for by a similar pattern of allylic cleavages with rapid rearrangements of the double bonds. This diterpene hydrocarbon is the first of its kind to be isolated from the genus *Otostegia* or any other plant. The optical antipode of this compound, named (-)-axinyssene, was recently reported as a constituent of a Japanese marine sponge and exhibited anti-tumor activity [53]. In addition, it was observed that the composition of the essential oil of *O. integrifolia* is different from that of *O. fruticosa*.

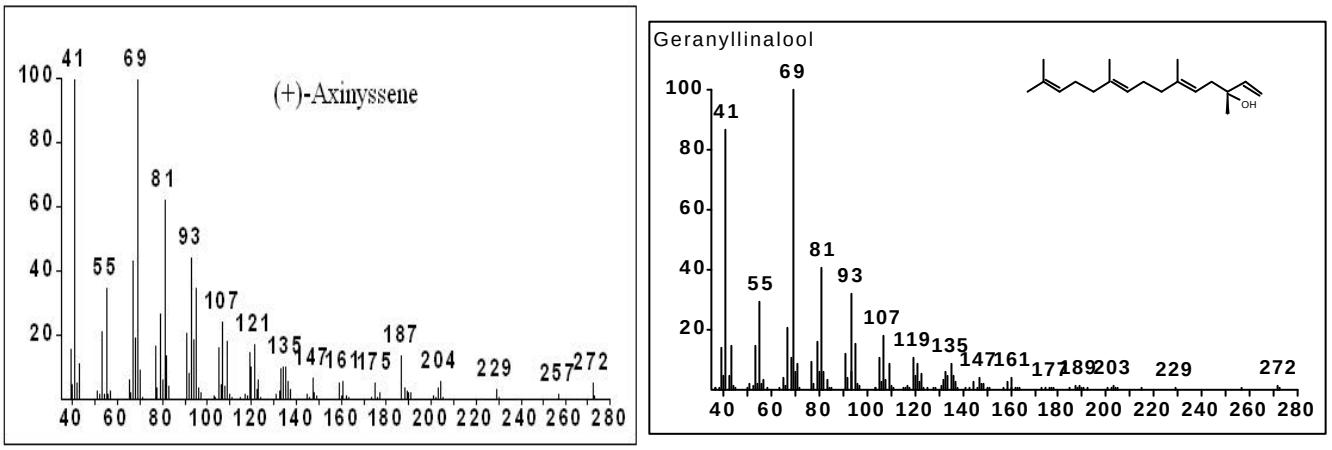


Figure. 13. 70 eV mass spectrum of axinyssene from the essential oil of *O. integrifolia* and a reference geranyl linalool showing a similar mass spectrum.

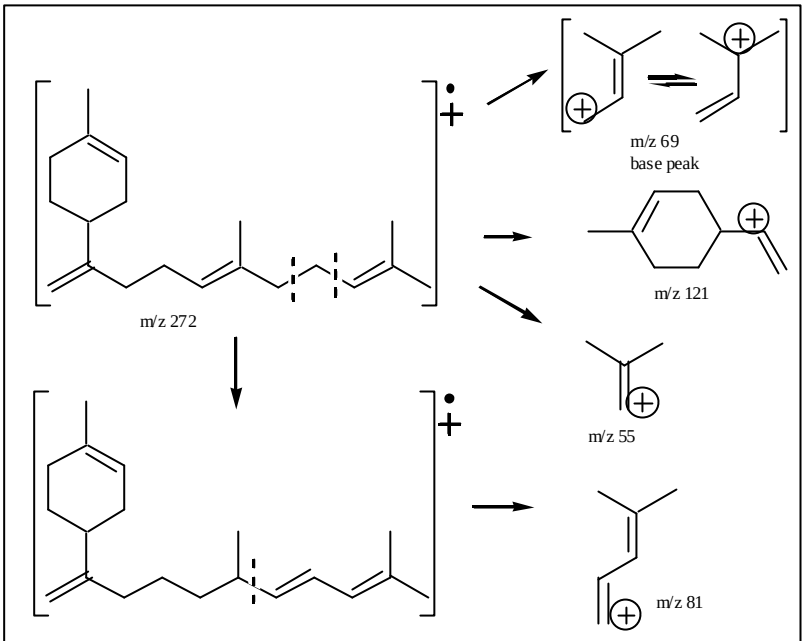


Figure 14. Possible fragmentation modes in compound **11**

7.1.2.2. Chloroform extract of the leaves

Repeated column chromatography on silica gel and Sephadex LH-20 columns of the chloroform extract resulted in the isolation of two new natural products, a prefuranic and furanic labdanediterpenoids named otostegindiol (**12**) and preotostegindiol (**13**), pentatriacontane, and stigmasterol.

7.1.2.2.1 Otostegindiol (12)

Otostegindiol (**12**) was obtained as a white solid. Its mass spectrum exhibited a molecular ion peak at m/z 320, which in combination with its ^{1}H- (Fig.16) and ^{13}C-NMR (Fig. 7) data led to an elemental composition of $C_{20}H_{32}O_3$, an oxygenated diterpenoid with five degrees of unsaturation. In its ^{1}H NMR spectrum, typical signals of a β-monosubstituted furan ring at δ 6.27 (1H, *bs*, H-14), 7.34 (1H, *bs*, H-15) and 7.22 (1H, *bs*, H-16) were observed. This indicated that three of the unsaturations were due to the furan ring. The remaining two should be due to two rings since no more signals arising from multiple bonds were present in the spectra. Moreover, the appearance of resonances of several methylene protons connected to the same carbon at different chemical shifts substantiated the presence of rings in the molecule. Extensive analysis of the 2D-^{1}H,^{1}H COSY as well as HMBC spectra of the compound led to the depicted structure.

7.1.2.2.2.Preotostegindiol (13)

Preotostegindiol (**13**) was obtained as a white solid. Its ^{1}H NMR (Fig 17) was similar to that of otostegindiol**,** except that the former instead of the furan ring signals exhibited signals typical of a β,β-disubstituted dihydrofuran ring. These signals appeared at δ 6.43 (1 H, *d, J*=2.84, H-15), 5.14 (1 H, *d, J*=2.84, H-14) and a two proton AB system at δ 4.53 (1 H, *d, J*=10.1) and 4.05 (1 H, *d, J*=10.1) corresponding to the methylene group of the dihydrofuran ring (H_2-16). This was further confirmed by the signals in the ^{13}C NMR spectrum at δ 93.3 (C-9) and 93.0 (C-10), which were joined by the ether linkage of the 9(13)-epoxy group and the appearance of an oxygenated methylene signal at δ 81.5 (C-16) instead of the olefinic methine signal at δ 138.9 in otostegindiol. The observed conversion of preotostegindiol to otostegindiol under mild acidic conditions was a further proof of its structure (Figure 15). Such observation was earlier reported. [48].

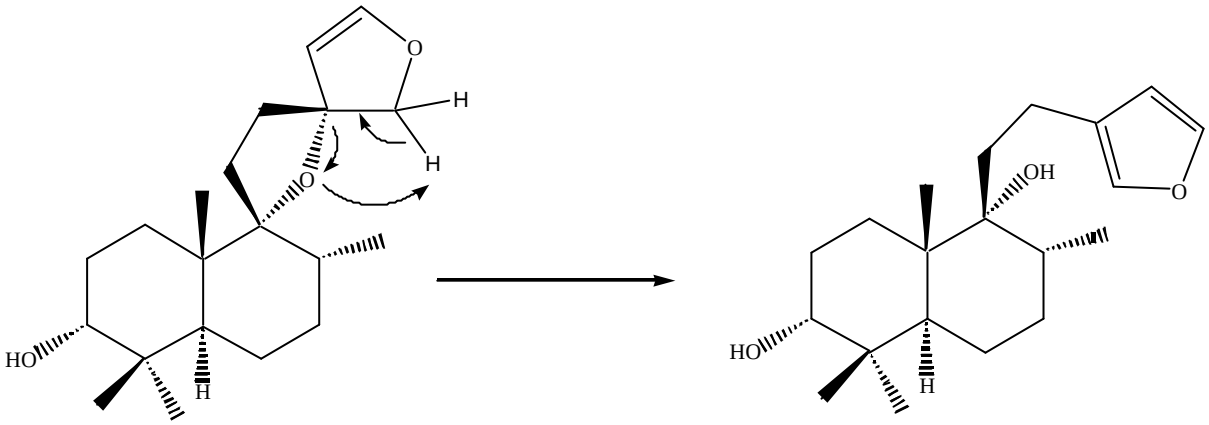


Figure 15. Conversion of preotostegindiol to otostegindiol

Prefuranic and furanic labdane diterpenoids are commonly encountered in many species of the Lamiaceae family, such as *Leonurus heterophyllus,* [44], and *L. persicus*, [46]. It was mentioned above that prefuranic and furanic labdanediterpenoids were identified from *O. fruticosa*. [45]. This new identification of prefuranic and furanic labdanediterpenoids from yet another *Otostegia* species may have a chemotaxonomical significance. *O. integrifolia* is the only species, so far, in which C-3 hydroxylated prefuranic and furanic labdanes are found. In addition, unlike the labdanes from other species, these two labdanes from *O. integrifolia* are missing C-6-, C-7- or C-8-oxygenations, (Paper I).

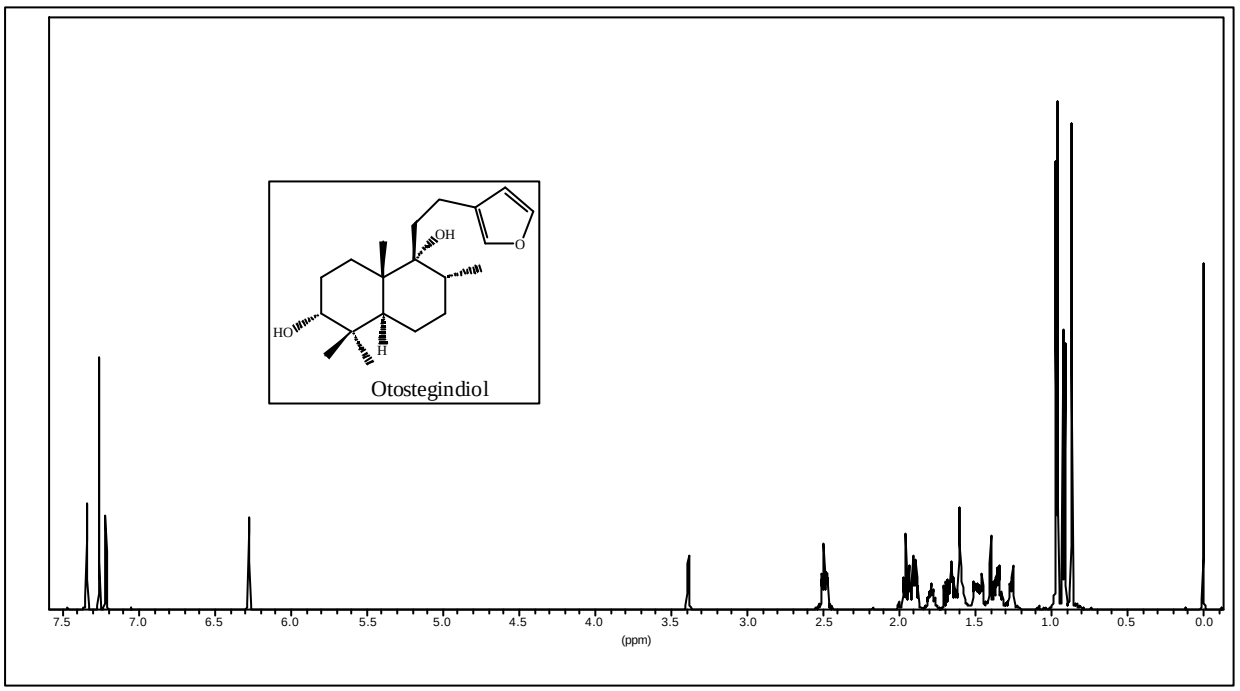


Figure 16. Proton NMR of otostegindiol (500 MHz)

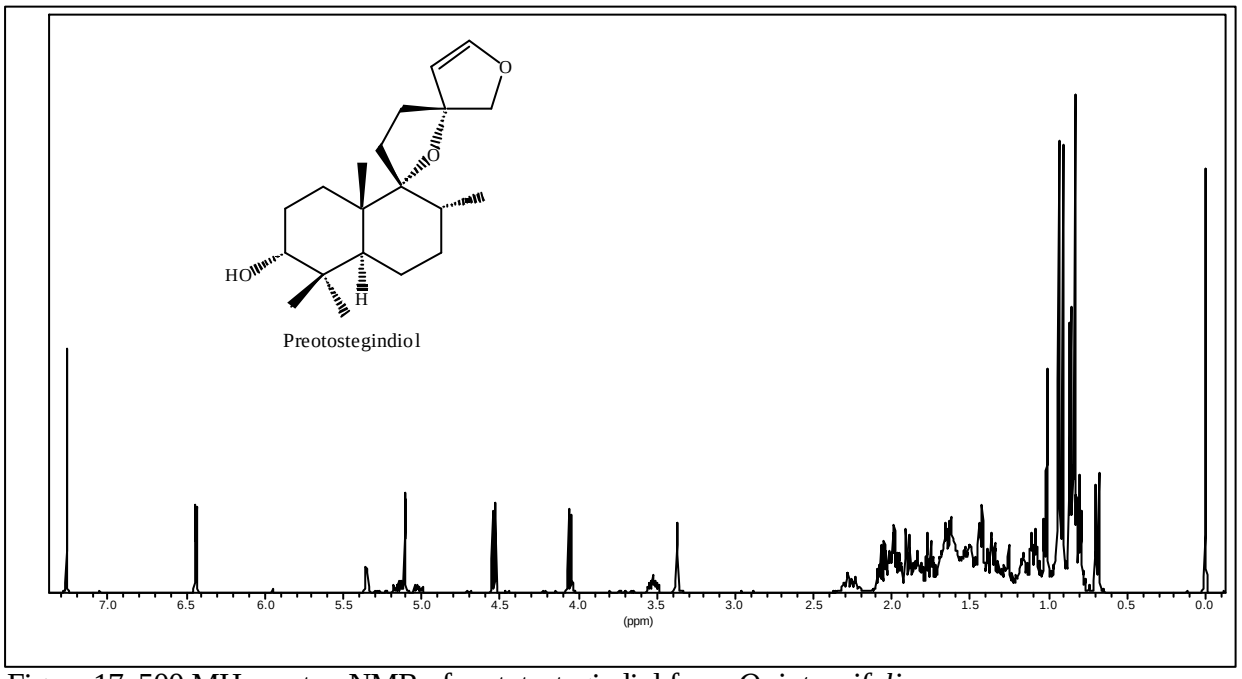


Figure 17. 500 MHz proton NMR of pretotostegindiol from *O. integrifolia*

7.1.2.2.3.Pentatriacontane

Pentatriacontane was eluted from a silica gel column in the first two fractions by a gradient of pure n-hexane to 5% n-hexane in ethyl acetate during the initial fractionation of the crude chloroform extract. Upon removal of the solvent under vacuum a product was obtained which upon recrystallization from methanol gave a white crystalline solid. The mass spectrum of the compound showed a molecular ion peak at *m/z* 492 which suggested an elemental

composition of $C_{35}H_{72}$. Moreover, a fragmentation pattern with a difference of 28 mass units corresponding to a loss of ethylene typical of long chain hydrocarbons, was observed.

The ^{1}H NMR of the compound showed two peaks, a broad singlet at δ 0.95 corresponding to six protons of the two terminal methyl groups and a broad singlet at δ 1.45 corresponding to 66 protons for the remaining 33 methylene groups. The ^{13}C NMR displayed signals at δ 14.6 ($2xCH_3$), 23.4 (CH_2), 30.1 (CH_2), 30.4 ($30xCH_2$) and 32.6 (CH_2).

7.1.2.2.4. Stigmasterol

Stigmasterol was obtained as a white solid upon repeated chromatography on silica-gel and Sephadex LH 20 columns. This compound was quickly recognized as a steroid from its mass spectrum, which exhibited a molecular ion peak at m/z 412 corresponding to an elemental composition of $C_{29}H_{48}O$. Its proton NMR spectrum was found to be identical with that of reported values.

7.2. Peucedanum tauricum

7.2.1 Description of the plant and Literature Survey

P. tauricum Bieb. is an endemic perennial plant of the Apiaceae family, growing in nature at dry hillsides and pinewoods in Crimea, Caucasus, and in Romania (Paper IV). Previous chemical studies of the plant concerned the identification of phenolic acids in the foliage and fruits, GC/MS analysis of the essential oil of the fruits in which a number of sesquiterpene hydrocarbons were identified, isolation of coumarins from the fruits, isolation of an analogue of chlorogenic acid and a chromone from the roots, determination of saponins in roots and fruits as well as isolation of peucedanin from a combined extract of *P. tauricum* and *P. calcareum*. (Paper IV)

7.2.2 Results and Discussion on P. tauricum

Powdered and pulverized fruits of *P. tauricum* were subjected to both hydrodistillation and solvent extraction using dichloromethane.

7.2.2.1. Essential oil of the fruits

Analysis of the essential oil from the fruits of *P. tauricum* by GC and GC/MS was carried out. Mass spectra and retention indices of the oil constituents were compared with a library of mass spectra of authentic compounds established under identical experimental conditions. A total of 22 components comprised of mainly mono and sesquiterpene hydrocarbons could be identified. (Fig. 19) In addition, two hitherto unknown sesquiterpene hydrocarbons were

isolated by preparative GC. Their structures were elucidated by 1D- and 2D-NMR techniques to be guaia-1(10),11-diene (**14**) and guaia-9,11-diene (**15**). The relative configurations of the new compounds were established through 2D NOESY experiments. Their absolute configurations were assigned according to chemical correlations and capillary GC analysis using modified cyclodextrins as stationary phases. [54, 55].

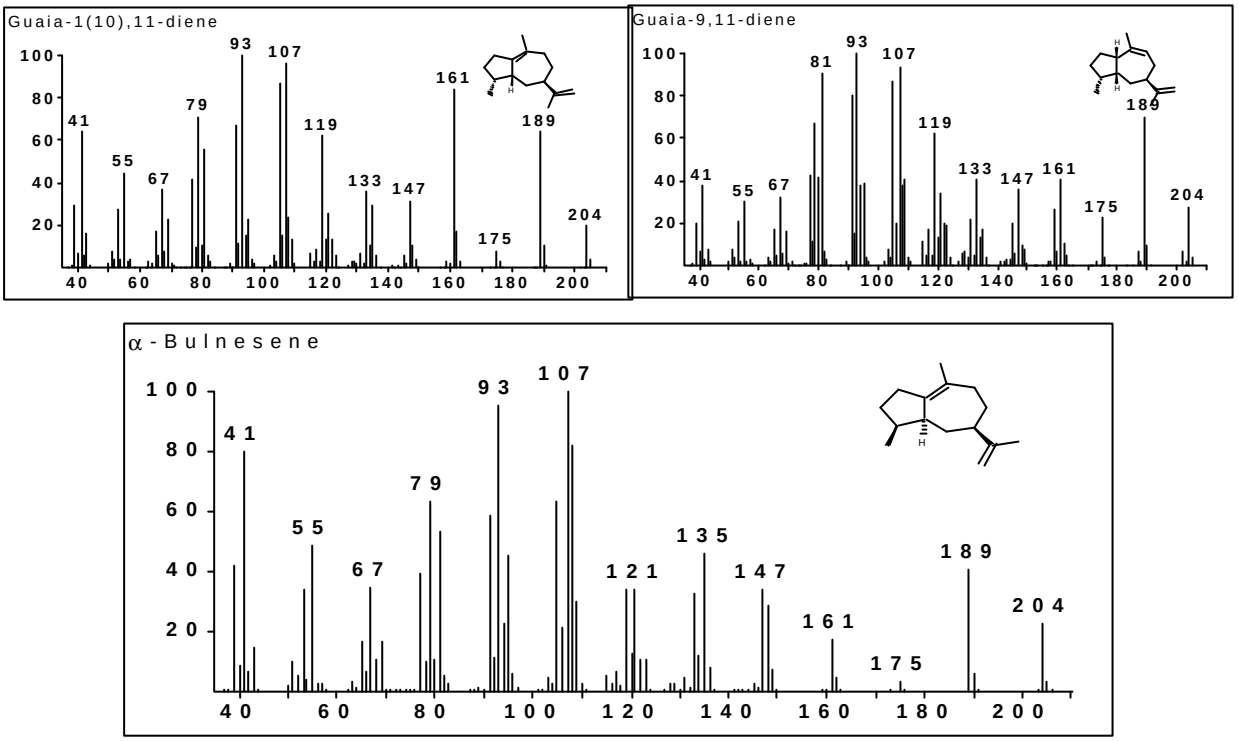


Figure 18. Mass spectra of guaia-1(10),11-diene, guaia-9,11-diene and α-bulnesene

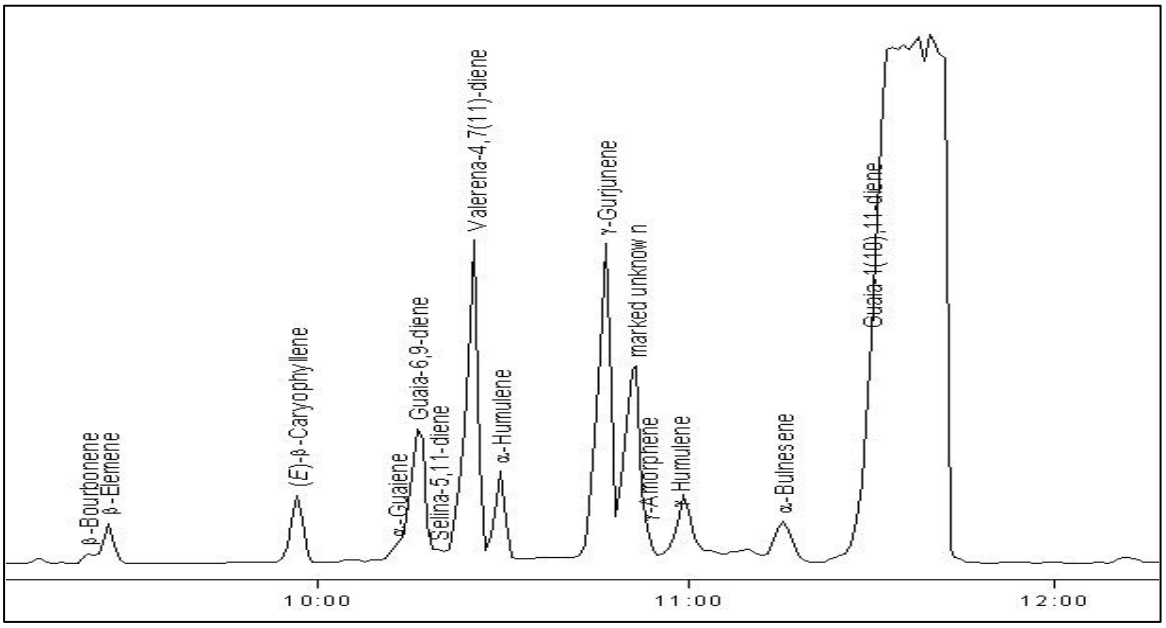


Figure 19. Total Ion Current Chromatogram (TIC) of the essential oil of *P. tauricum* fruit from Poland

*7.2.2.1.1 Guaia-1(10),11-diene (**14**)*

The compound exhibited a mass spectrum (Fig. 18) typical of a sesquiterpene hydrocarbon with a molecular ion signal at m/z 204 (RI = 1516). This indicated an elemental composition of $C_{15}H_{24}$, a sesquiterpene hydrocarbon with four degrees of unsaturations. The mass spectrum was very similar to that of α-bulnesene (RI = 1503) (Fig. 18), but the retention index was quite different. The NMR data confirmed a $C_{15}H_{24}$ molecular formula and the presence of two double bonds. Therefore, the compound was a doubly unsaturated bicyclic sesquiterpene hydrocarbon. Extensive analysis of the 2D-^{1}H,^{1}H COSY as well as HMBC spectra of the compound led to the actual structure of guaia-1(10),11-diene.

*7.2.2.1.2 Guaia-9,11-diene (**15**)*

Compound **15** (RI = 1522) exhibited spectral properties (MS, ^{1}H-, ^{13}C-NMR) similar to those of **14** except that in the ^{13}C-NMR spectrum instead of the olefinic quaternary resonance a methine resonance was displayed and instead of one of the ring methylene resonances an olefinic methine resonance appeared. Examination of the 2D-^{1}H,^{1}H COSY and HMBC NMR data revealed compound **15** to show a ring double bond between C-9 and C-10 instead of C-1 and C-10.

*7.2.2.1.3.Relative and Absolute Configuration of **14** and **15***

The relative configuration of **14** was determined by a 2D NOESY experiment (Fig. 20). Correlations between H-4/H-5 indicated that the two methine protons were oriented in the same direction. Moreover, the absence of NOESY correlations between the methine protons H-5 and H-7 suggested a trans configuration. Considering the bulky isopropenyl group to keep a *pseudo* equatorial position and being β-oriented, H-7 had to be α (axial orientation). Consequently, H-5 and H-4 had to be β-oriented as depicted.

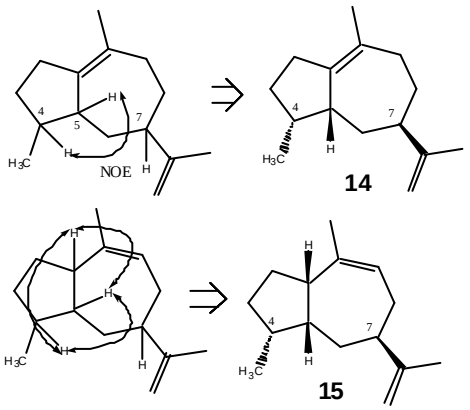


Figure 20. Key NOESY correlations in **14** and **15**.

The depicted relative stereochemistry in **15** was established through a 2D NOESY experiment. Key NOESY correlations were seen between H-5/H-1, H-5/H-4 and H-1/H-4 which led to the conclusion that these methine protons were on the same side of the ring. In addition, the fact that a NOESY correlation was exhibited between H-5/H-1 indicated that the ring was *cis*-fused.

In order to determine the absolute configurations of the new guaiane sesquiterpenes, authentic reference substances, (+)-γ-gurjunene (**16**), (+)-α-bulnesene (**17**) and (+)-α-guaiene (**18**) (Sigma-Aldrich)**,** all showing guaiane skeletons of known absolute configurations, were hydrogenated (Fig. 21). Each of the two new compounds was also hydrogenated separately. The hydrogenation products of the reference compounds and the hydrogenation products of **14** and **15** were analyzed by capillary GC under identical conditions using modified cyclodextrins as stationary phases which separated the diastereomeric products formed upon hydrogenation [54, 55]. The GC traces of the hydrogenation products of the compounds were compared with those of the references. At least one of the hydrogenation products of **14**, **15** and the reference compounds (compound **19**, in Fig. 21) should exhibit identical retention times, provided that the corresponding chiral centers of compounds **14** and **15** show the same absolute configuration as the reference compounds. This was found to be the case only with (+)-γ-gurjunene on both 2,6-di-OMe-3-O-pentyl-γ-cyclodextrin and 6-O-TBDMS-2,3-di-OMe-β-cyclodextrin. As a result, the absolute configuration of **14** is (4R,5R,7R)-guaia-1(10),11-diene while **15** is (1S,4R,5R,7R)-guaia-9,11-diene.

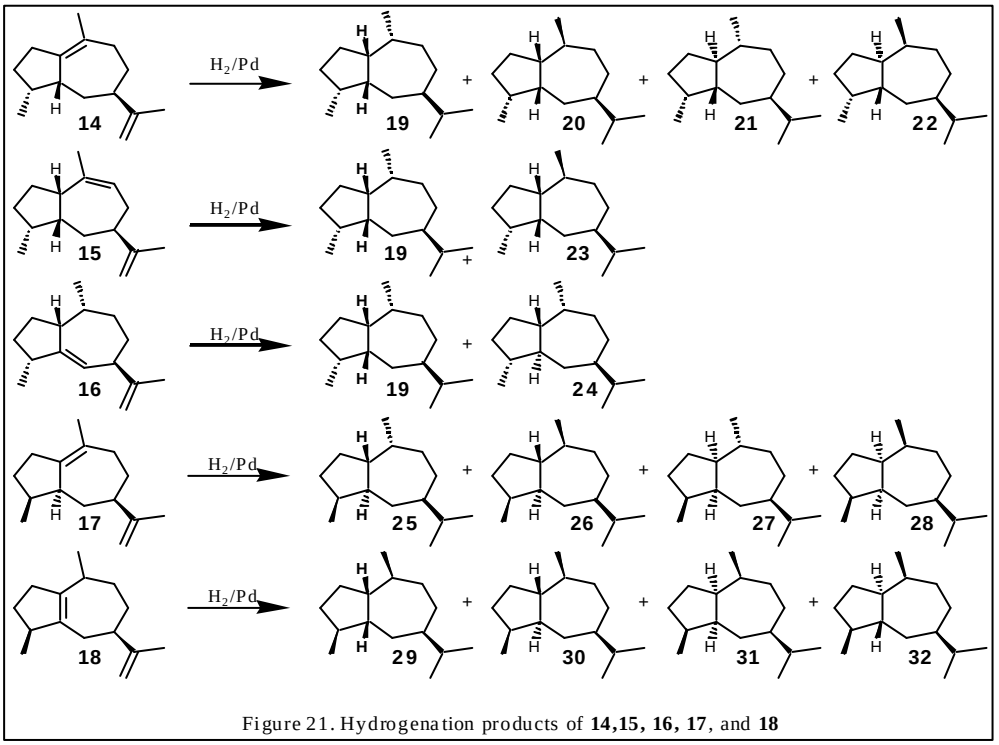
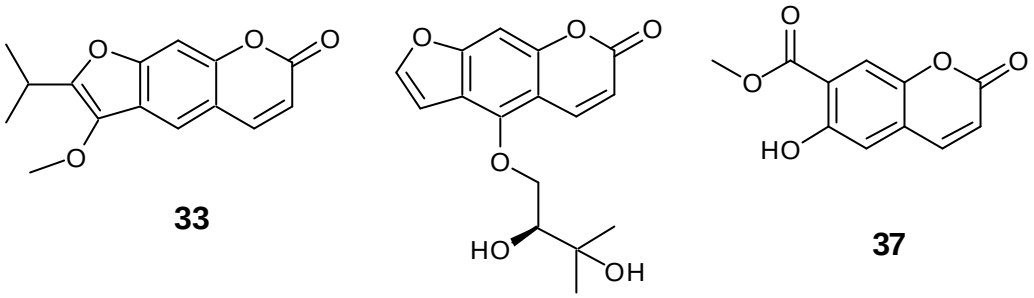


Figure 21. Hydrogenation products of **14,15, 16, 17**, and **18**

7.2.2.2. Dichloromethane extract of the fruits

Repeated chromatography of the dichloromethane extract of the fruits enabled the isolation of known coumarins: peucedanin (**33**), oxypeucedanin hydrate (**34**), and officinalin isobutyrate (**35**) (Scheme 2), (Paper IV). Their structures were established by MS, 1D and 2D NMR data as well as comparison with literature data (Paper IV).



Scheme 2. Selected compounds from *P. tauricum*.

7.2.2.2.1 Structure of officinalin isobutyrate

Officinalin isobutyrate, earlier reported from *P. officinale* [56], can be considered an ester formed from 6-carbomethoxy-7-hydroxycoumarin (**36**) and isobutyric acid as shown in Fig. 22.

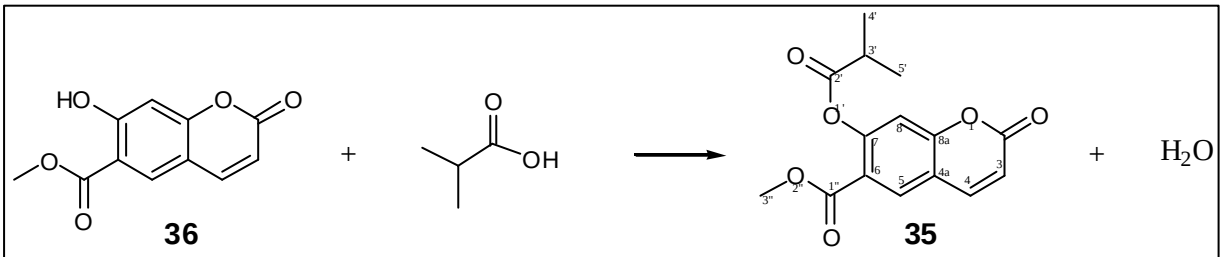


Figure 22. Proposed formation of officinalin isobutyrate

However, in the literature there are two different compounds represented by the same structure of 6-carbomethoxy-7-hydroxycoumarin. These are officinalin isolated from *P. officinale* [40], and peuruthenicin (**37**) isolated from *P. ruthenicum* [54]. The two coumarins exhibited the same molecular formula, very similar patterns of ^{1}H NMR but different melting points indicating they are actually isomeric. This raised the questions as to whether the reported structure of officinalin isobutyrate is correct, and whether the compound the *iso*-butyrate of officinalin or peuruthenicin. The confusion could be clarified by comparing the ^{1}H NMR of the isolated compound and the reported data which were found to be in good agreement. In addition, the melting point of the compound was found to be in agreement with

the reported value (Paper IV). Furthermore, the isolated compound was subjected to alkaline hydrolysis and the product showed good agreement in its ^{1}H-NMR data with that of officinalin rather than that of peuruthenicin. Furthermore, in the HMBC spectrum of the compound (Fig.22), the methoxy protons as well as the aromatic methine proton attached to C-5 correlated strongly to the carbomethoxycarbonyl carbon. In addition, one of the AB system aromatic methine doublets at C-4 was correlated to C-5. This observation clearly showed the carbomethoxy substituent to be linked to C-6 leading to 6-carbomethoxy-7-isobutyroxycoumarin as the structure of the compound, exactly the same as that reported for officinalin isobutyrate [56].

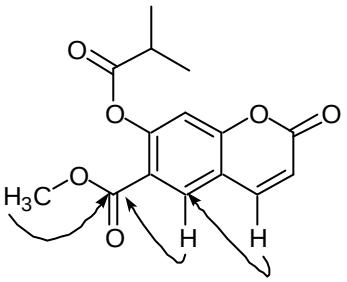


Figure 23. Key HMBC correlations showing the position of the carbomethoxy group in compound **35**

This demonstrated that the compound is indeed the isobutyrate of officinalin. Therefore the earlier reported structure of peuruthenicin [57], an isomer of officinalin, is amended to be 6-hydroxy-7-carbomethoxycoumarin (Paper IV). This is the first report on the isolation of officinalin isobutyrate and oxypeucedanin hydrate from *P. tauricum*.

7.3. Radula perrottetii

7.3.1 Description of the plant and Literature Survey

The liverwort *R. perrottetii* Gott. belongs to the Jungermanniales (Hepaticae). Earlier, several prenylated bibenzyls and derivatives thereof have been reported from *R. perrottetii* (Paper III)

7.3.2 Results and Discussion on R. perrottetii

The essential oil obtained by hydrodistillation from the powdered and pulverized *R. perrottetii* sample was analyzed by GC and coupled GC/MS. Mass spectra and retention indices of the oil constituents were compared with a library of mass spectra of authentic compounds established under identical experimental conditions. A number of mono- and sesquiterpenes and their derivatives could be identified. Eleven components that could not be identified by this method were isolated by preparative GC, and, according to their MS, 1D- and 2D-NMR data, their structures were established as viscida-4,9,14-triene (**38**), viscida-4,11(18),14-triene

(**39**), bisabola-2,6,11-triene (**40**), bisabola-1,3,5,7(14),11-pentaene (**41**), bisabola-1,3,5,7,11-pentaene (**42**), 6,7-epoxybisabola-2,11-diene (**43**), 2-methyl-1-(4-methoxy phenyl)propene (**44**), bisabola-1,3,5,7(14),10-pentaene (**45**), *ar*-tenuifolene (**46**), α-helmiscapene (**47**) and β-helmiscapene (**48**) from their respective.

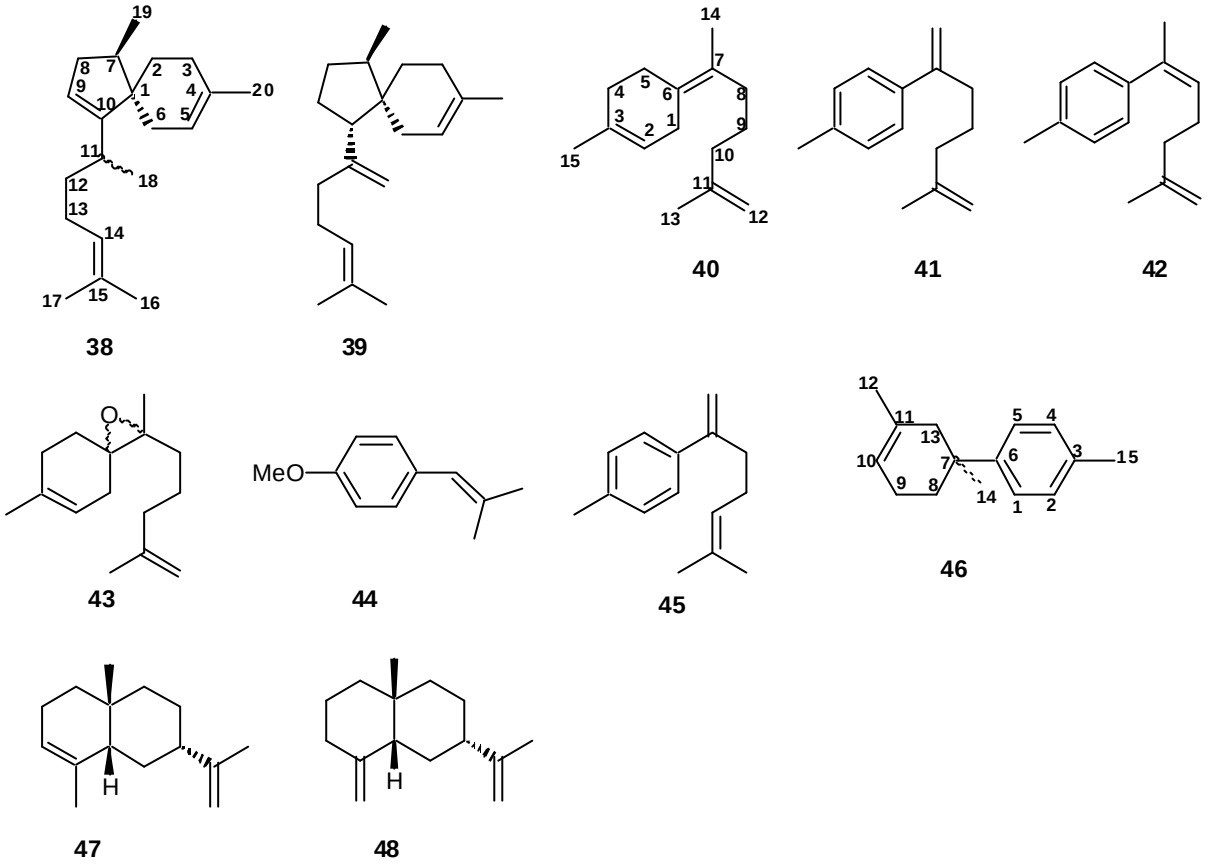


Figure 24. Selected compounds from R. perrottetii essential oil

The two viscidane diterpenes, viscida-4,9,14-triene as well as viscida-4,11(18),14-triene, and the four bisabolane sesquiterpenes, bisabola-2,6,11-triene, bisabola-1,3,5,7(14),11-pentaene, bisabola-1,3,5,7,11-pentaene, 6,7-epoxybisabola-2,11-diene, and 1-(4-methoxyphenyl)-2-methyl propene are novel natural products. This is the first finding of viscidane diterpenes in liverworts. *ar*-Tenuifolene, hitherto described only from the East African Sandalwood plant *Osyris tenuifolia* as the (-)-enantiomer was found to exist in the unusual racemic form in the oil of *R. perrottetii*. It was earlier reported that biosyntheses of racemic mixtures of sesquiterpene hydrocarbons occur in liverworts only occasionally. (Paper III)

7.4. Chloranthus spicatus

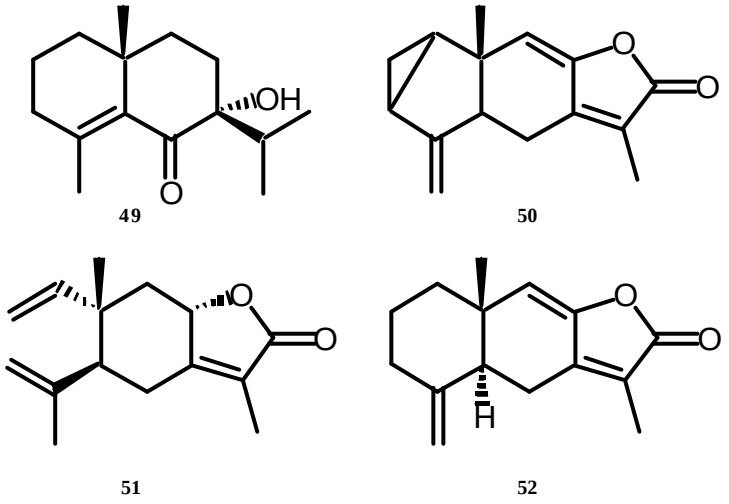
7.4.1 Description of the plant and Literature Survey

C. spicatus (Vietnamese name: *Soi gie*) is a herb belonging to the family Chloranthaceae reaching the height of 1.5 m with pleasant-smelling yellow flowers in summer and autumn.

The plant is grown in Vietnam to produce flowers for scenting tea. Earlier investigation dealt with the sesquiterpene constituents of *C. serratus*, *C. glaber*, *C. japonicus* and the constituents of the volatiles of flowers of *C. spicatus* growing in China.

7.4.2 Results and Discussion on C. spicatus

The essential oil composition of *C. spicatus* flowers was investigated using capillary gas chromatography (GC), GC-mass spectrometry (MS), preparative GC and NMR techniques. A total of 47 compounds were identified either by comparing the retention indices and mass spectra with a library of authentic data established under identical experimental conditions' or, where deemed necessary, by isolating the compounds using preparative GC and establishing their structure using NMR techniques. Thus, four minor components *viz*. 7α-hydroxyeudesm-4-en-6-one (**49**), chloranthalactone A (**50**), isogermafurenolide (**51**) and eudesma-4 (15), 7(11), 9-trien-12-olide (**52**) were isolated for the first time as constituents of the oil of *C. spicatus*. Their structures were established according to their MS, 1D- and 2D-NMR data. (Paper II).



49 **50**

51 **52**

Figure 25. 7a-Hydroxyeudesm-4-en-6-one (49), chloranthalactone A (50), isogermafurenolide (51) and eudesma-4 (15), 7(11), 9-trien-12-olide (52) from Chloranthus spicatus flower essential oil

7.5. Meum athamanticum

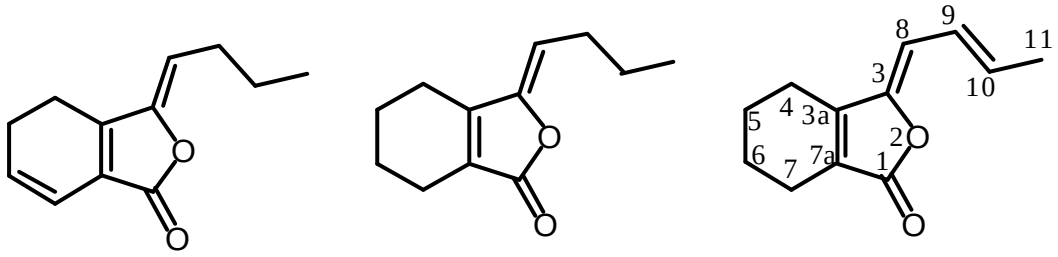
7.5.1 Description of the plant and Literature Survey

Meum athamanticum (L.) Jacq. is a strongly aromatic smelling herb which is used for the preparation of a special liquor. Its essential oil has previously been investigated several times. An earlier analysis of the oil from the fruits of a sample from Germany has revealed (*Z*)-3-butylidenephthalide, butylphthalide and (*Z*)-ligustilide as the chief constituents while from the

root oil the identification of several monoterpenes and the phthalides, in addition to sedanonic acid lactone was reported. In a further analysis aimed at the investigation of sesquiterpene hydrocarbons of the oil from the roots, the identification of sesquiterpenoids such as β-bazzanene, α- and β-barbatene was reported for the first time as constituents of higher plants. From the aerial parts of a sample from Italy, the monoterpene hydrocarbons, (*E*)-β-ocimene (34.9%), p-cymene (12.1%), (*Z*)-β-ocimene (10.2%), Δ-3-carene (6.2%) were reported as major constituents while from the oil of the roots, (*Z*)-ligustilide (36.2%,), (E)-β-ocimene (14.4%), and (Z)-3-butylidene phthalide (6.3%) were reported as major constituents. Recently, from the oil of the leaves and stems of *M. athamanticum* of Spanish origin, (*E*)-β-ocimene (29.6%), γ-terpinene (17.9%), terpinolene (17.0%) and p-cymene (9.7%) were reported as major components.

7.5.2 Results and Discussion on M.athamanticum

The essential oil of the aerial parts of *M. athamanticum* was analyzed by capillary and preparative GC, GC/MS and NMR techniques. Mass spectra and retention indices on a non-polar stationary phase (CpSil-5) of the components of the oil were compared with library spectra of authentic compounds generated under identical experimental conditions. Several mono- and sesquiterpenes and their derivatives could be identified. Three unknown phthalides were isolated and their structures established according to their MS, 1D and 2D NMR data. These were 3-but-2-enylidene-4,5,6,7-tetrahydro-3H-isobenzofuran-1-one (**53**), the previously reported *Z*-butylidene-4,5,6,7-tetrahydrophthalide (sedanonic acid lactone) (**54**), and Z-ligustilide (**55**). Isoligustilide is a new natural product. (Paper V)



53 **54** **55**

Figure 26. Selected compounds from the essential oil of *M.athamanticum*

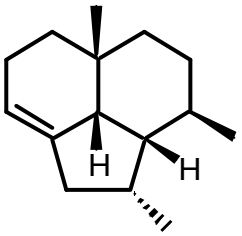
7.6. Melanoselenium decipiens

7.6.1 Description of the plant and Literature Survey

Melanoselinum decipiens is a rare umbelliferous shrub which inhabits rocky slopes in the interior of Madeira. But the leaves of *M. decipiens* used in this study were collected in Hamburg (Pinneberg), Germany, from a private garden. Earlier, the essential leaf oil of *M. decipiens* from Madeira was shown to contain β-pinene (53.1%) as the major component among 31 other compounds. According to a second investigation, the essential oils of the aerial parts of the plants, collected during the vegetative phase on Madeira and on the Azores, were composed mainly of monoterpene hydrocarbons (ca. 90 %), β-pinene (72%) and limonene (82%) being the main components of the Madeiran and Azorean oils, respectively. Other investigations on *M. decipiens* resulted in the isolation of a series of sesquiterpene lactones, called decipienins A–H, and a number of eudesmanolides.

7.6.2 Results and Discussion on M. decipiens

GC and GC/MS analysis of the essential oil of leaves of *M. decipiens* resulted in the isolation of a new sesquiterpene hydrocarbon possessing a novel carbon skeleton. The compound, named melanene, was isolated by using preparative GC, and its structure was established by extensive NMR analysis as (1*R*,4*E*,7*R*,8*R*,9*R*,12*S*)-1,7,9-trimethyltricyclo[6.3.1.$0^{5,12}$]dodec-4-ene (**56**) or its antipode The relative configuration was determined by a 2D NOESY experiment. This is the first detection of a hydroacenaphthylene structure as a sesquiterpene skeleton in nature. The presence of a second similar sesquiterpene hydrocarbon was detected by its mass spectrum (Fig.2). The amount was not sufficient for structure elucidation, however, in its ^{1}H-NMR the presence of 3 methyl signals at δ 0.89, 0.97, and 1.04 as well as an olefinic methine proton at δ 5.35 indicated that the unknown is not a stereoisomer of melanene.



56

In addition, several mono- and sesquiterpenes were identified as components of the oil by comparing their retention indices and mass spectra with a library of authentic samples established under identical experimental conditions. Similar to that of the Madeiran plants, the

oil from the plant grown in Hamburg contains β-pinene (55.1 %) as the main component. By enantioselective GC, this was shown to be represented by enantiomerically almost pure (1R)-(+)-β-pinene. The oil of the plant grown in Hamburg contains higher amounts of sesquiterpenes (ca. 17 %) than that of the Madeiran plants. (Paper VI).

7.7. Hazard symbols, risk and safety phrases for chemicals used

Chemical	Hazard symbol	Risk phrase	Safety phrase
Hexadeutratedbenzene	T, F	42.2-11-23/24/25-48	53-16-29-44
Deuteratedchloroform	X_n	20-40	2-24/25
Ethylacetate	Xi, F	11-36-66-67	(2)16-26-33
n-Hexane	X_n, F	11-20/21-40	9-16-29-33
Methanol	T, F	11-23/24/25-39/11-23/24/25	(1/2-)7-16-36/37-45
Petrolether	F	11	9-16-29-33
Sodium hydroxide	X	35	26-27/39-45
p-Anisaldehyde	X	22-36/37/38	26-36
Sulphuric acid	X, C	35	26-30-45
Hydrochloric acid	X, F, C	11-20/21/22-35-37	7/9-16-26-36-/37/39-44
Acetone	X, F	11-36-66-67	9-16-26
10 % Palladium on activated charcoal	X, F^+	-	-
Silica gel	X	20-37	9-36
Aluminum oxide	-	37	22-36-38
Dichloromethane	X	40	23b-24/25-36/37
Acetonitrile	X, F	11-20/21/22-36	16-36/37-60
Tetramethylsilane	F	12	9-16-29-43h
Pentane	F	11	9-16-29-33
Diethylether	F^+	12-19	9-16-29-33
Palladium	A	-	-
Lithium aluminum hydride	F	15	7/8-24/25-43
Hydrogen	F	12	9-16-29-33
Amberlite	Xi	36/37/38	26/36

Summary and conclusions

Leaves of a herbaceous plant known as *Otostegia integrifolia* collected in Ethiopia (Paper I), flowers of a plant called *Chlorantus spicatus* from Vietnam (Paper II), fruits of an umbelliferous plant known as *Peucedanum tauricum* collected in Poland (Paper IV), aerial parts of a herbaceous plant known as *Meum athamanticum* from Germany (Paper V), a liverwort called *Radula perrottetii* of Japanese origin (Paper III) and leaves of a rare umbellifer known as *Melanoselinum decipiens* grown in Hamburg, Germany (Paper VI) were investigated for their secondary metabolites. The isolation of the compounds started with extracting of the mixture of secondary metabolites present in the plant material. Air-dried or fresh plant material was subjected to either hydrodistillation or solvent extraction. The former yielded a complex mixture of volatile compounds known as essential oil while the latter gave a complex mixture of volatile or non-volatile compounds depending on the polarity of the solvent used for the extraction process. The essential oils were analyzed by capillary GC as well as GC/MS. Mass spectra and retention index of each component was compared with a library spectra of authentic samples. The known components were identified. The components that couldn't be identified by simple comparisons were marked as unknowns and subsequently isolated. The isolation of the unknowns from the complex mixtures were carried out by chromatographic techniques. By using flash silica gel column chromatography, the essential oils were first fractionated in to a fraction containing hydrocarbons and a fraction containing oxygenated compounds. The former was obtained by eluting the column with hexane and the latter with ethyl acetate, consecutively. Each fraction were analyzed by using several capillary columns coated with a range of stationary phases varying from the non-polar CPSil-5 (Chrompack) to various modified cyclodextrins until optimum resolution of the components was obtained. The hydrocarbons and the oxygenated compounds were further fractionated by preparative GC equipped with a preparative column packed with the stationary phase that gave the optimal resolution. This process was repeated as many times as necessary until pure compounds were obtained. In the cases of solvent extraction, chloroform or dichloromethane were employed. This gave mainly mixtures of non volatile compounds. The composition of the crude extracts was examined by using TLC. Visualizations were supported by either observing the TLC under a UV lamp or by spraying with an anisaldehyde reagent followed by heating. TLC was repeatedly developed by changing the solvent systems until a system that gave the best separation was obtained. The crude extracts were repeatedly chromatographed on columns packed with either silica-gel, sephadex-LH 20 or on HPLC until the pure compounds were obtained (Papers I & IV).

From the essential oil of air-dried leaves of *O. integrifolia* Benth., a total of 40 constituents including monoterpenes, sesquiterpenes, diterpenes and their derivatives were identified. A prenylbisabolane type diterpene, (+)-1-methyl-4-(5,9-dimethyl-1-methylene-deca-4,8-dienyl)cyclohexene (**11**), also called (+)-axinyssene, was identified as a major component. The chloroform extract of the leaves yielded two new furanolabdane diterpenoids, 15,16-epoxy-3α,9α-dihydroxy-labda-13(16),14-diene (**12**) and 9(13),15(16)-diepoxy-3α-hydroxy-16-dihydrolabda-14-ene (**13**), a saturated hydrocarbon, pentatriacontane, and stigmasterol.

From the essential oil of flowers of *C. spicatus***,** 47 compounds could be identified among which chloranthalactone A (**49**) (0.5 %), isogermafurenolide (**50**) (0.7 %), eudesma-4(15),7(11),9-trien-12-olide (**51**) (0.5 %), and 7α-hydroxyeudesm-4-en-6-one (**52**) (3.3 %), were isolated for the first time as constituents of the essential oil of the flowers.

From the essential oil of the liverwort *R. perrottetii* two novel viscidane diterpenes, viscida-4,9,14-triene (**38**), viscida-4,11(18),14-triene (**39**), four bisabolane sesquiterpenes, bisabola-2,6,11-triene (**40**), bisabola-1,3,5,7(14),11-pentaene (**41**), bisabola-1,3,5,7,11-pentaene (**42**), 6,7-epoxybisabola-2,11-diene (**43**), and 1-(4-methoxyphenyl)-2-methyl propene (**44**) were identified as new natural products. This is the first finding of viscidane diterpenes in liverworts. In addition, bisabola-1,3,5,7(14),10-pentaene (**45**), *ar*-tenuifolene (**46**), α-helmiscapene (**47**), and β-helmiscapene (**48**) were also isolated for the first time.

From the essential oil of fruits of *P. tauricum*, two new guaiane type sesquiterpene hydrocarbons guaia-1(10),11-diene (**14**) and guaia-9,11-diene (**15**) were isolated. The relative configurations of the new compounds were established by 2D-NOESY experiments while the absolute configurations were deduced through chemical correlations with (+)-γ-gurjunene (**16**) and capillary GC analysis using modified cyclodextrins as the stationary phases. From the less volatile dichloromethane extract of the fruits, coumarins, *viz*. peucedanin (**33**), oxypeucedanin hydrate (**34**) and officinalin isobutyrate (**35**) were isolated. This is the first report on the isolation of oxypeucedanin hydrate and officinalin isobutyrate from *P. tauricum*. Officinalin isobutyrate was confirmed to be 6-carbomethoxy-7-isobutyroxycoumarin. Peuruthenicin, a positional isomer of officinalin, is assigned a correct structure (**37**). Bergapten was identified by its mass spectrum.

From the essential oil of *M. athamanticum* a new phthalide, named isoligustilide (**55**) (3,5 %) was isolated together with Z-ligustilide (**53**) (0.1 %) and sedanonic acid lactone (**54**) (0.5%). In addition, a total of 23 components accounting for 93.2 % of the oil could be

identified. The major components of the oil were shown to be monoterpene hydrocarbons, limonene (33.5 %), α-phellandrene (15.3 %), myrcene (13.4 %) and (E)-β-ocimene (11.6 %).

From the essential oil of the leaves of *M. decipiens*, melanene, a tricyclic sesquiterpene hydrocarbon showing a novel carbon skeleton represented by a trimethyldecahydroacenaphthylene (**56**) was isolated. The structure of melanene was established to be (1*R*,4*E*,7*R*,8*R*,9*R*,12*S*)-1,7,9-trimethyltricyclo[6.3.1.$0^{5,12}$]dodec-4-ene or its antipode. In addition, 45 compounds (mostly monoterpenes and sesquiterpenes) were identified, among which enantiomerically almost pure (1R)-(+)-β-pinene forms the major component (55.1 %).

It can be concluded that currently GC/MS in combination with computerized mass libraries is the best method for the study of the chemical composition of essential oils, particularly, if supplemented with 1D- and 2D-NMR techniques as well as enatioselective GC using modified cyclodextrin phases as chiral selectors. The availability of automated preparative gas chromatographs GC equipped with autosampler and preparative fraction collector such as the Gerstel PFC turned the isolation of new components from volatile plant constituents into a much more easier task, the limiting factor being the selection of a stationary phase capable of providing the required resolution of the components to be isolated.

Zusammenfassung und Schlussfolgerungen

Die Blätter der krautigen Pflanze *Otostegia integrifolia* aus Ethiopia (Publikation I), der Blüten von *Chlorantus spicatus* aus Vietnam (Publikation II), der Früchte des Doldengewächses *Peucedanum tauricum* aus Polen (Publikation IV), der oberirdischen Teile der krautigen Pflanze *Meum athamanticum* aus Deutschland (Publikation V), des Lebermooses *Radula perrottetii* japanischer Herkunft (Publikation III) und der Blätter der seltenen Umbelliferae *Melanoselium decipien*, gezüchtet in Hamburg, Deutschland (Publikation VI) wurden untersucht. Die Isolierung der Inhaltsstoffe begann mit der Extraktion der sekundären Metaboliten aus dem Pflanzenmaterial. Luftgetrocknetes oder frisches Pflanzenmaterial wurde einer Wasserdampfdestillation oder einer Lösungsmittelextraktion unterzogen. Während erstere ein komplexes Gemisch flüchtiger Verbindungen, bekannt als ätherisches Öl, lieferte, ergab die Extraktion ein komplexes Gemisch aus flüchtigen und nicht-flüchtigen Substanzen, in Abhängigkeit der Polarität des für die Extraktion verwendeten Lösungsmittels. Die ätherischen Öle wurden mittels Kapillar GC und GC/MS untersucht. Die Massenspektren und Retentionsindices jeder Verbindung wurden mit den Datenbankspektren authentischer Proben verglichen. Bekannte Verbindungen wurden

identifiziert. Die Komponenten, welche durch einfachen Vergleich nicht identifiziert werden konnten, wurden als unbekannte Verbindungen markiert und anschließend isoliert. Die Isolierung der unbekannten Substanzen aus den komplexen Gemischen wurden mittels chromatographischer Verfahren durchgeführt. Die ätherischen Öle wurden zuerst mittels Flash Säulen Chromatographie an Silicagel in eine Kohlenwasserstoff- und eine oxygenierte Fraktion aufgetrennt. Erstere wurden mittels Hexan aus der Säule eluiert, während letztere anschließend mit Ethylacetat eluiert wurden. Jede Fraktion wurde mit mehreren Kapillar GC's, ausgestattet mit einer Auswahl beschichteten Säulen, deren stationäre Phasen von der unpolaren CPSil-5 (Chrompack) bis zu unterschiedlich modifizierten Cyclodextrinen variieren, untersucht, bis eine optimale Trennung der Verbindungen erhalten wurde. Die Kohlenwasserstoff und die oxygenierten Fraktionen wurden unter Verwendung eines preparativen GC mit einer gepackten präparativen Säule mit der stationären Phase, welche die optimale Auflösung ergab, weiter aufgetrennt. Dieser Prozess wurde so lange wie notwendig wiederholt, bis reine Stoffe erhalten wurden. Im Falle der Lösungsmittelextraktion wurde Chloroform oder Dichlormethan angewendet, welche hauptsächlich Gemische nicht flüchtiger Verbindungen ergaben. Die Zusammensetzung der Rohextrakte wurde mittels TLC Methoden untersucht. Die Detektion der Substanzen wurde entweder mit Hilfe der Betrachtung unter UV-Licht oder durch Besprühen mit Anisaldehyd-Schwefelsäure Reagenz und anschließendes erhitzen, durchgeführt. Die Dünnschichtchromatogramme wurden unter Variation des Lösungsmittelsystems wiederholt entwickelt, bis ein optimales Laufmittelgemisch erhalten wurde. Die Rohextrakte wurden wiederholt an gepackten Säulen mit Silicagel oder Sephadex-LH 20, oder mittels HPLC chromatographiert, bis reine Stoffe erhalten wurden (Publikation I & IV).

Aus dem ätherischen Öl luftgetrockneter Blätter von *O. intergrifolia* Benth. wurden insgesamt 40 Inhaltstoffe, darunter Monoterpene, Sesquiterpene, Diterpene und ihre Derivate identifiziert. Ein Prenylbisabolan-artiges Diterpen, (+)-1-Methyl-4-(5,9-dimethyl-1-methylen-deca-4,8-dienyl)cyclohexen (**11**), welches auch (+)-Axinyssen genannt wird, wurde als eine Hauptkomponente identifiziert. Der Chloroform Extrakt der Blätter ergab zwei neue Furanolabdan-Diterpenoide, 15,16-Epoxy-3α,9α-dihydroxy-labda-13(16),14-dien (**12**) und 9(13),15(16)-Diepoxy-3α-hydroxy-16-dihydrolabda-14-en (**13**), einen gesättigten Kohlenwasserstoff, Pentatriacontan und Stigmasterol.

Aus dem ätherischen Öl der Blüten von *C. spicatus* wurden 47 Verbindungen identifiziert. Chloranthalacton A (**49**) (0.5 %), Isogermafurenolid (**50**) (0.7 %), Eudesma-

4(15),7(11),9-trien-12-olid (**51**) (0.5 %), und 7α-Hydroxyeudesm-4-en-6-on (**52**) (3.3 %) wurden erstmalig als Inhaltsstoffe des ätherischen Öls der Blüten isoliert.

Aus dem ätherischen Öl des Lebermooses *R. perrottetii* wurden zwei neue Viscidane Diterpene, Viscida-4,9,14-trien (**38**), Viscida-4,11(18),14-trien (**39**), vier Bisabolan-Sesquiterpene, Bisabola-2,6,11-trien (**40**), Bisabola,1,3,5,7(14),11-pentaen (**41**), Bisabola-1,3,5,7,11-pentaen (**42**), 6,7-Epoxybisabola-2,11-dien (**43**), und 1-(4-Methoxyphenyl)-2-methylpropen (**44**) als neue Naturstoffe isoliert. Dies ist die erste Entdeckung von Viscidane Diterpenoiden in Lebermoosen. Darüberhinaus wurden auch Bisabola-1,3,5,7(14),10-pentaen (**45**), *ar*-Tenuifolenen (**46**), α-Helmiscapen (**47**), und β-Helmiscapen (**48**) erstmalig isoliert.

Aus dem ätherischen Öl der Früchte von *P. tauricum* wurden zwei neue Guaian-artige Sesquiterpene, Guaia-1(10),11-dien (**14**) und Guaia-9,11-dien (**15**), isoliert. Die relative Konfiguration dieser neuen Verbindungen wurde mittels 2D-NOESY Experimenten bestimmt, während die absoluten Konfigurationen durch chemische Korrelation mit (+)-γ-Gurjunene (**16**) und Kapillar-GC Analyse mittels modifizierter Cyclodextrine als stationäre Phase, abgeleitet wurde. Aus dem weniger flüchtigen Dichlormethan Extrakt der Früchte wurden die Coumarine Peucedanin (**33**), Oxypeucedaninhydrat (**34**) und Officinalinisobutyrat (**35**) isoliert. Dies ist der erste Bericht über die Isolierung von Oxypeucedaninhydrat und Officinalinisobutyrat aus *P. tauricum*. Officinalinisobutyrat wurde als 6-Carbomethoxy-7-isobutyroxycoumarin bestätigt. Dem Peuruthenicin, einem Stellungsisomer von Officinalin, wurde eine korrekte Struktur (**37**) zugeordnet. Bergapten wurde anhand des Massenspektrums identifiziert.

Aus dem ätherischen Öl von *M. athamanticum* wurde ein neues Phtalid, bezeichnet als Isoligustid (**55**) (3.5 %), zusammen mit Z-Ligustid (**53**) (0.1 %) und Sedanonsäurelacton (**54**) (0.5 %), isoliert. Darüberhinaus wurden insgesamt 23 Komponenten, welche 93.2 % des Öles ausmachen, identifiziert. Es wurde gezeigt, dass die Hauptkomponenten aus Monoterpenkohlenwasserstoffen bestehen: Limonen (33.5 %), α-Phellandren (15.3 %), Myrcen (13.4 %), und (*E*)-β-Ocimen (11.6 %).

Aus dem ätherischen Öl der Blätter von *M. decipiens*, wurde Melanen, ein tricyclischer Sesquiterpenkohlenwasserstoff mit dem neuen Kohlenstoffgerüst eines Trimethyldecahydroacenaphthylens (**56**), isoliert. Die Struktur des Melanens wurde als (*1R,4E,7R,8R,9R,12S*)-1,7,9-Trimethyltricyclo[6.3.1.$0^{5,12}$]dodec-4-en oder dessen Spiegelbild aufgeklärt. Darüberhinaus wurden 45 Verbindungen (hauptsächlich Monterpene und Sesquiterpene) identifiziert, von denen beinahe enantiomerenreines (*1R*)-(+)-β-Pinen eine Hauptkomponente ausmacht (55.1 %).

Es kann geschlussfolgert werden, das die GC/MS in Kombinantion mit computerunterstützten Spektren-Datenbanken zur Zeit als eine der besten Methoden für die Untersuchung der chemischen Zusammensetzung ätherischer Öle angesehen werden kann. Dies gilt insbesondere wenn diese durch 1D- und 2D-NMR Techniken, sowie die enantioselektive Gaschromatographie unter Verwendung modifizierter Cyclodextrinphasen als chirale Selektoren, ergänzt wird. Die Verfügbarkeit automatisierter präparativer Gaschromatographen, welche mit einem automatischen Probengeber und einem präparativen Fraktionssammler, wie dem Gerstel PFC ausgerüstet sind, macht die Isolierung neuer Verbindungen der flüchtigen Pflanzeninhaltsstoffe zu einer weitaus einfacheren Aufgabe. Der begrenzende Falktor stellt dann das Auffinden einer stationären Phase dar, welche die benötigte Auflösungen der zu isolierenden Komponenten gestattet.

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Part II

Papers I-VI

Paper-I

Terpenes from *Otostegia integrifolia*[1]

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Abstract

The essential oil and chloroform extract of air-dried leaves of *Otostegia integrifolia* Benth. were investigated for the first time using analytical and preparative gas chromatography (GC), GC-mass spectrometry (MS) and NMR techniques. A total of 40 constituents including monoterpenes, sesquiterpenes, diterpenes and their derivatives were identified. A prenylbisabolane type diterpene, 1-methyl-4-(5,9-dimethyl-1-methylene-deca-4,8-dienyl)cyclohexene was identified as a major component. The chloroform extract of the leaves yielded two new labdane type diterpenoids, 15,16-epoxy-3α,9α-dihydroxy-labda-13(16),14-diene and 9(13),15(16)-diepoxy-3α-hydroxy-16-dihydrolabda-14-ene, a saturated hydrocarbon, pentatriacontane, and stigmasterol. The structures of the isolated compounds were established by spectroscopic methods.

Keywords: *Otostegia integrifolia* Benth.; Lamiaceae; Essential oil, Furanolabdane diterpenes, Otostegindiol, Preotostegindiol, (+)-Axinyssene, Pentatriacontane, Stigmasterol

1. Introduction

Otostegia integrifolia Benth. belongs to the Lamiaceae (Labiatae) family (Fichtl et al., 1994). It is one of the plants used in traditional medicine in Ethiopia. The plant has

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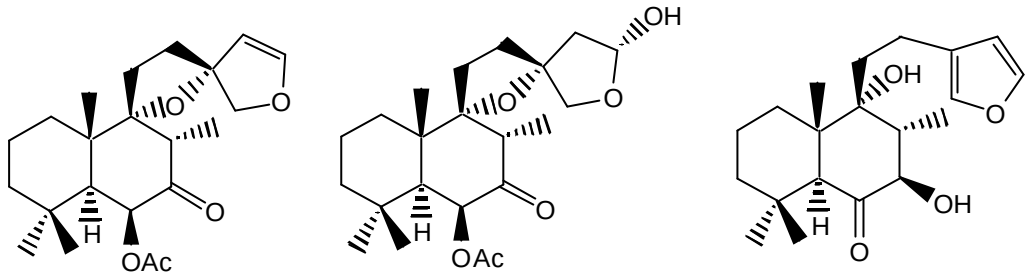
insecticidal properties and is often used as fumigant for pots and houses. The roots are used for treating lung diseases (Fichtl et al., 1994). No previous phytochemical investigation on the plant has been reported. However, there are reports on the chemical investigation of other members of the genus *Otostegia*. Thymol, γ-terpinene and p-cymene were reported as major constituents in the essential oil of *O. fruticosa* analysed by GC-MS (Aboutabl et al., 1995). Furthermore, from aerial parts of the same plant, isolation of three new and five known labdane diterpenes together with iridoid glucoside was reported. These were otostegin A (**1**), otostegin B (**2**), 15-*epi*-otostegin B (Al-Musayeib et al., 2000), preleoheterin, leoheterin (**3**) (Hon et al., 1993), and related compounds leopersin C, 15-*epi*-leopersin C (Tasdemir et al., 1996), ballonigrin (Brian and James, 1977), vulgarol (Popa and Pasechnik, 1975) and 8-O-acetylharpagide (Scarpati et al., 1965) (Figure 1).



1 **2** **3**

Figure 1. Selected labdane diterpenes from Otostegia fruticosa

Our investigation of air-dried leaves from *O. integrifolia* resulted in the isolation of a prenylbisabolane type diterpene, axinyssene, from the essential oil and two new furanolabdane type diterpenes, otostegindiol and preotostegindiol, a saturated hydrocarbon, pentatriacontane, and stigmasterol from the chloroform extract.

2. Results and discussion

The essential oil of air-dried leaves of *O. integrifolia* was analysed by GC and GC-MS. Mass spectra and retention indices on an non-polar stationary phase (CPSil-5) of the components were compared with a library of mass spectra of authentic compounds established under identical experimental conditions (Joulain and König, 1998; Hochmuth et al., 2002): *trans*-2-hexenal, *trans*-hex-3-ene-1-ol, 1-hexanol, α-thujene, α-pinene, thuja-2,4(10)-diene, 1-octene-3-ol, β-pinene, 3-octanol, phenylacetaldehyde, limonene, (*Z*)-β-ocimene, linalool, *trans*-sabinol (**4**), mentha-1,5-diene-8-ol, terpinene-4-ol, α-terpineol, β-cyclocitral (**5**), nerol, geraniol, vinylguajacol, dihydroedulan (**6**), theaspirane (**7**), eugenol, α-ylangene, β-bourbonene, *E*-β-caryophyllene, geranylacetone, α-humulene, β-ionone, γ-

muurolene, germacrene D, 4,5-di-epi-aristolochene, α-muurolene, δ-amorphene, *E*-nerolidol, spathulenol, caryophyllenoxide, and β-eudesmol were identified. The major component which could not be identified by this method was isolated by preparative GC and its structure was established as (+)-1-methyl-4-(5,9-dimethyl-1-methylene-deca-4,8-dienyl)-cyclohexene (**8**) from its MS, 1D- and 2D-NMR data. The dried and pulverised leaves of the plant were also soaked in chloroform at room temperature for 48 hours. Unlike the essential oil, the chloroform extract contained mainly non-volatile substances. Part of the extract was subjected to repeated column chromatography on silica gel and Sephadex LH-20 columns and two new furanolabdane type diterpenes, **9** and **10** (Figure 2), a saturated hydrocarbon pentatriacontane, and stigmasterol were isolated.

*2.1. (+)-1-Methyl-4-(5,9-dimethyl-1-methylene deca-4,8-dienyl)cyclohexene (**8**)*

8 was isolated as an oil by preparative GC from the essential oil of the dried leaves of the plant. It exhibited a positive optical rotation. Its mass spectrum showed a molecular ion peak at m/z 272. Its ^{1}H- (Table 1) and HMQC-NMR data indicated the presence of four allylic methyl singlets, three olefinic and one aliphatic methine signals, one exocyclic methylene singlet and seven aliphatic methylene multiplets. The ^{13}C-NMR (Table 1) contained signals due to four olefinic quaternary carbons in addition to the signals of the groups observed in the ^{1}H-NMR. The ^{1}H- and ^{13}C-NMR data in combination with the mass spectrum confirmed an elemental composition of $C_{20}H_{32}$, a diterpenoid hydrocarbon with five degrees of unsaturation. Analysis of connectivities in the ^{1}H-^{1}H COSY- and HMBC spectra confirmed that four of the five unsaturations were due to double bonds and the fifth was due to a ring.

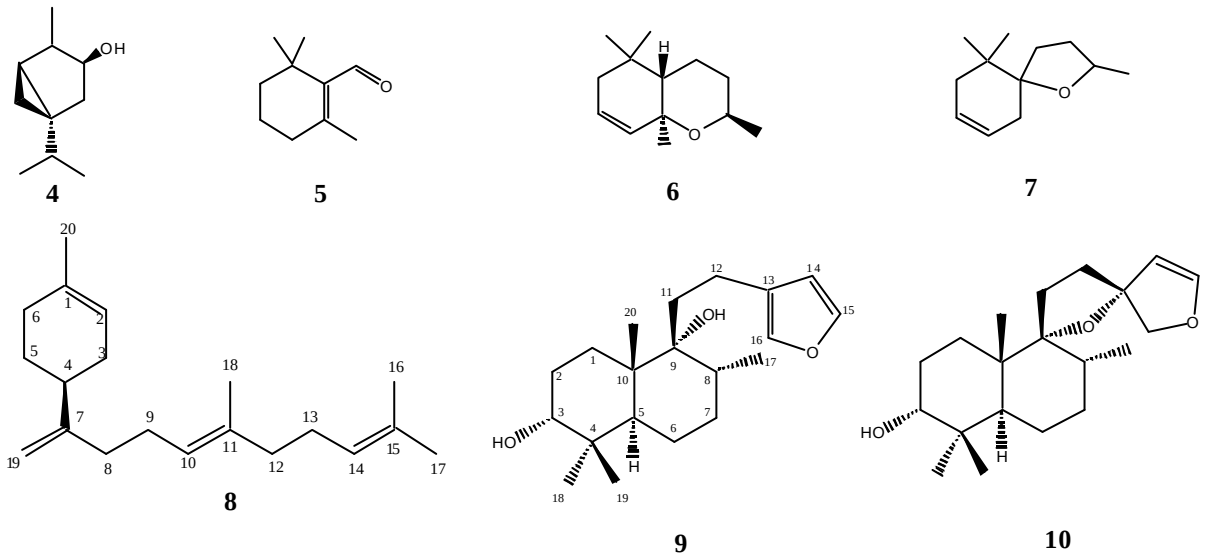


Figure 2. Selected constituents of *O. integrifolia* leaves

The connectivities within the molecule were established by interpretation of the 2D ^{1}H-^{1}H COSY- and HMBC spectra. In the HMBC spectrum it was observed that two of the allylic methyl groups at δ 1.57 (H_3-16) and 1.68 (H_3-17) exhibited long range couplings with each other and also with the olefinic methine triplet at δ 5.24 (H-14). In addition, in the HMBC spectrum, each of these methyls as well as the methine signal were coupled to the olefinic quaternary carbon at δ 129.9 (C-15). This clearly indicated that these two methyl groups must be geminal and connected to the olefinic quaternary carbon that itself was connected to the olefinic methine carbon. The olefinic quaternary carbon at δ 129.9 (C-15) also exhibited long range coupling in the HMBC to the methylene multiplet centred at δ 2.17 (H_2-13), which in the ^{1}H-^{1}H COSY spectrum exhibited coupling to the olefinic methine signal (δ 5.24, H-14) as mentioned above and another methylene multiplet centred at δ 2.09 (H_2-12). The latter was coupled to the third allylic methyl singlet at δ 1.59 (H_3-18) whose carbon (δ 26.4, C-18) showed long range coupling to the same methylene multiplet (H_2-12) in the HMBC spectrum. Further this methylene multiplet (H_2-12) in the ^{1}H-^{1}H COSY spectrum also exhibited allylic coupling to the second olefinic methine triplet at δ 5.29 (H-10) whose carbon (δ 125.1, C-10) showed long range coupling to the same methylene multiplet (H_2-12) in the HMBC. In the ^{1}H-^{1}H COSY spectrum this olefinic methine triplet (δ5.29, H-10) was coupled to another methylene multiplet centred at δ 2.22 (H_2-9). The latter coupled to another methylene group at δ 2.11 (H_2-8), whose protons were coupled to the olefinic methine carbon at δ 125.1 (C-10) in the HMBC spectrum, as well as to the protons of the exocyclic methylene singlet (allylic coupling) at δ 4.90 (H_2-19) in the ^{1}H-^{1}H COSY. This constituted the 5,9-dimethyl-1-methylene-4,8-decadienyl side chain of the compound. In the ^{1}H-^{1}H COSY the aliphatic methine multiplet centred at δ 2.14 (H-4) coupled to each of the methylene multiplets centred at δ 1.48 (H_a-5) and 1.79 (H_b-5). The latter further coupled to each of the two methylene protons at δ 1.86 (H_a-6) and at δ 1.95 (H_b-6). On the other side, the aliphatic methine (δ 2.14, H-3) also coupled to each of the methylene protons at δ 1.97 (H_a-3) and at δ 2.15 (H_b-3). The latter coupled to the third olefinic methine signal (*bs*) at δ 5.43 (H-2) which itself exhibited allylic coupling to the fourth allylic methyl singlet at δ 1.62 (H_3-20). This constituted the 1,4-disubstituted cyclohexene ring moiety of the compound. This diterpene hydrocarbon is the first of its kind to be isolated from the genus *Otostegia* or any other plant. But the optical antipode of this compound, named axinyssene, was recently reported as a constituent of a Japanese marine sponge and exhibited anti-tumour activity (Kodama et al., 2003).

Table 1: ^{1}H and ^{13}C NMR data of compounds **8, 9** and **10**.

	8			**9**			**10**		
	^{1}H	m (*J*)	^{13}C	^{1}H	m (*J*)	^{13}C	^{1}H	m (*J*)	^{13}C
1		-	133.8	1.25	*m*	25.1	1.24	*m*	25.1
				1.93	*m*		1.63	*m*	
2	5.41	*bs*	121.6	1.64	*m*	25.6	1.65	*m*	25.2
				1.96	*m*		1.93	*m*	
3	1.97	*m*	32.1	3.38	*bs*	76.3	3.37	*bs*	76.1
	2.15	*m*							
4	2.14	*m*	40.6	-	-	43.2	1.87	-	42.4
5	1.48	*m*	28.9	1.90	*t*	39.8		*m*	39.8
	1.79	*m*							
6	1.86	*m*	31.2	1.34	*m*	21.6	1.35	*m*	21.2
	1.95	*m*		1.50	*m*		1.47	*m*	
7	-	-	154.4	1.35	*m*	31.4	1.32	*m*	31.6
				1.46	*m*		1.46	*m*	
8	2.11	*m*	35.6	1.79	*m*	37.1	1.79	*m*	37.3
9	2.22	*m*	27.5	-		77.5		-	93.3
10	5.29	t (7.0)	125.1	-		37.8		-	37.7
11	-	-	135.4	1.67	*m*	35.5	1.66	*m*	32.0
				1.91	*m*		1.91	*m*	
12	2.09	*m*	40.1	2.46	*m*	21.9	1.81	*m*	35.6
13	2.17	*m*	27.5	-		126.1	-	-	93.0
14	5.22	*t* (7.0)	125.2	6.27	*bs*	111.3	5.14	*d* (*2.84*)	107.6
15	-	-	131.5	7.34	*bs*	143.2	6.43	*d* (*2.84*)	147.8
16	1.57	*s*	17.9	7.22	*bs*	138.9	4.53	*d* (*10.1*)	81.5
							4.05	*d* (*10.1*)	
17	1.65	*s*	26.1	0.92	*d* (*6.62*)	16.5	0.86	*d* (*6.62*)	17.6
18	1.59	*s*	16.4	0.97	*s*	22.6	0.93	*s*	22.4
19	4.39	*s*	108.1	0.87	*s*	29.0	0.83	*s*	29.5
20	1.61	*s*	23.9	0.96	*s*	16.8	0.91	*s*	21.1

*2.2. Otostegindiol (**9**)*

9 was obtained as a white solid upon repeated column chromatography on silica gel and Sephadex LH 20 columns. Its mass spectrum exhibited a molecular ion peak at m/z 320, which in combination with its ^{1}H- and ^{13}C-NMR data (Table 1) led to an elemental composition of $C_{20}H_{32}O_3$, an oxygenated diterpenoid with five degrees of unsaturation. Fragment peaks at m/z 81 and 95 in the mass spectrum indicated the presence of a β-monosubstituted furan ring. This was confirmed by its ^{1}H NMR spectrum that showed typical signals of a β-monosubstituted furan ring at δ 6.27 (1H, *bs*, H-14), 7.34 (1H, *bs*, H-15) and 7.22 (1H, *bs*, H-16). This indicated that three of the unsaturations were due to the furan ring. The remaining two should be due to two rings since no signal arising from multiple bonds was present in any of the spectra. Moreover, the appearance of resonances of several methylene protons connected to the same carbon at different chemical shifts substantiated the presence of rings in the molecule.

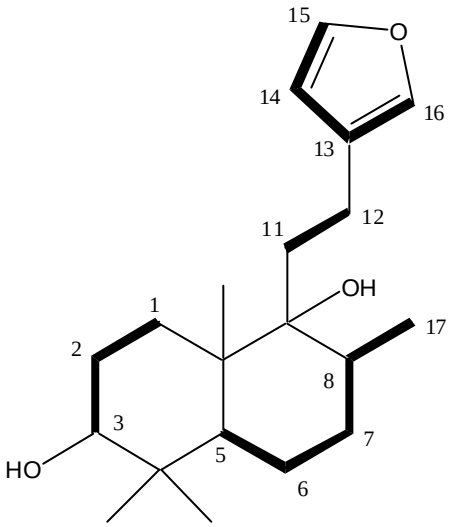


Figure 3. Key 1H-1H COSY couplings (bold face bonds) observed for compound 9

The ^{1}H NMR data (Table 1) also contained three methyl singlets at δ 0.87 (H_3–19), 0.96 (H_3–20), 0.97 (H_3–18) and a methyl doublet at δ 0.92, (*J*=6.62, H_3-17). The proton NMR also contained a methine multiplet at δ 1.79 (H-8) which was coupled to the secondary methyl doublet (δ 0.92, H_3-17) as observed in the ^{1}H-^{1}H COSY spectrum. Presence of a methine carbinol group (H-3) was indicated by one proton signal at δ 3.35 (1H, *bs*). A multiplet at δ 2.46 due to the H_2-12 methylene group was also observed. The remaining proton signals were overlapping in the range of δ 1.24 to 2.00. These include five methylene protons due to H_2-1 (δ 1.25, 1.93), H_2-2 (δ 1.64, 1.96), H_2-6 (δ 1.34, 1.50), H_2-7 (δ 1.35, 1.46) H_2-11 (δ 1.67, 1.91) and a methine multiplet due to H-5 (δ 1.90).

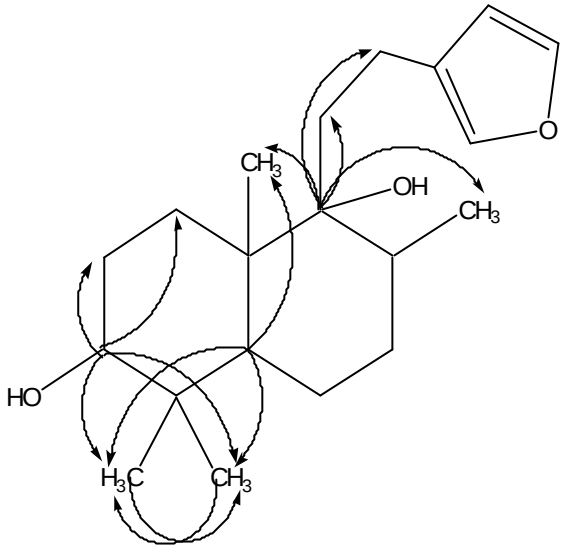


Figure 4. Key HMBC couplings observed for compound 9

The ^{13}C NMR of **9** contained signals due to a total of twenty carbon atoms. These were four primary, six secondary, six tertiary and four quaternary carbons. This was also confirmed by a PENDANT experiment (Homer et al., 1994). Among these, two carbons were oxygenated. One was a methine carbinol at δ 76.3 and the second was a quaternary carbinol at δ 77.5. In the ^{1}H-^{1}H COSY spectrum of the compound (Figure 3), key couplings that indicated the connectivity in the molecule were observed between the methylene protons at δ 1.25 (H_a-1), 1.93 (H_b-1) and at δ 1.64 (H_a-2) and 1.96 (H_b-2). The latter were coupled to the carbinol methine proton at δ 3.35 (H-3). Furthermore, couplings were observed between the methine multiplet centred at δ 1.90 (H-5) and each of the two methylene protons at δ 1.34 (H_a-6) and 1.50 (H_b-6) that were themselves coupled to another two methylene protons at δ 1.35 (H_a-7) and 1.46 (H_b-7). The latter were further correlated to a methine multiplet centred at δ 1.79 (H-8) that was itself coupled to the methyl doublet at δ 0.92 (H_3-17). Additional ^{1}H-^{1}H couplings were observed between each of the two methylene protons at δ 1.67 (H_a-11) and 1.91 (H_b-11) and the methylene multiplet centred at δ 2.46 (H_2-12). Similarly in the HMBC spectrum (Figure 4) of compound **9** several key couplings were observed that enabled us to elucidate the complete connectivity of atoms in the compound. Two of the primary methyl groups at δ 0.87 (H_3–18) and 0.97 (H_3–19) must be geminal because they exhibited couplings with each other in the HMBC spectrum, i.e. the carbon at δ 29.0 (C-18) was coupled to the methyl proton singlet at δ 0.97 (H_3–19) and the carbon at δ 22.6 (C-19) was coupled to the methyl protons singlet at δ 0.87 (H_3–18). Furthermore, in the HMBC spectrum the methine carbinol at δ 76.3 (C-3) exhibited couplings to each of the geminal methyl proton singlets at δ

0.87 (H_3–18) and 0.97 (H_3–19) and to each of the two methylene protons at 1.25 (H_a-1) and 1.93 (H_b-1) and the two methylene protons at δ 1.64 (H_a-2), 1.96 (H_b-2).(see also ^1H-^1H COSY). This indicated that the secondary hydroxyl group has to be at C-3 on the A-ring of the molecule. This was further substantiated by the observation of couplings in the HMBC spectrum between the aliphatic methine carbon at δ 39.8 (C-5) and the two geminal primary methyl proton singlets. The aliphatic methine carbon at δ 39.8 (C-5) was also coupled to another primary methyl singlet at δ 0.96 (H_3-20).

Another key structural information was obtained from the couplings observed between the quaternary carbinol at δ 77.5 (C-9) and the methyl proton doublet at δ 0.92 (H_3-17), the methyl proton singlet at 0.96 (H_3-20), the methylene proton multiplets at 1.67 (H_a-11), 1.91 (H_b-11) and 2.46 (H_2-12) which clearly indicated that the tertiary alcohol should be at C-9. At this stage it became clear that **9** had a furanolabdane diterpene skeleton.

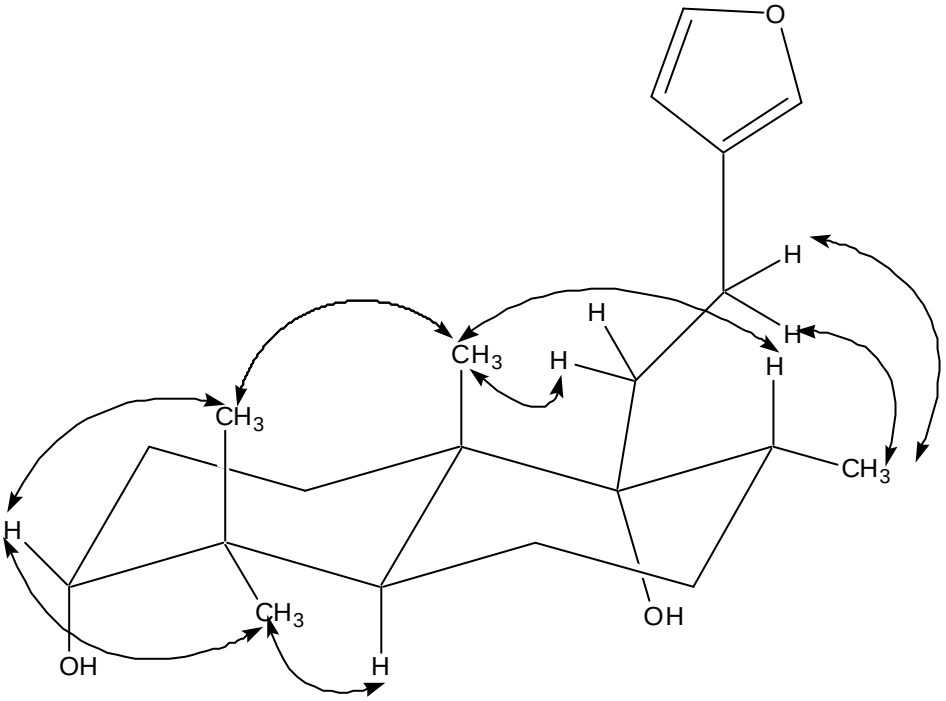


Figure 5. Key NOESY correlations observed for 9

The relative stereochemistry of the chiral centres at C-3, C-5, C-8, C-9 and C-10 was deduced from the NOESY spectrum of the compound and *J*-values. In the NOESY of **9** (Figure 5) couplings were seen between H-3/H_3-18, H-3/H_3-19 and H-3/H_a-2 and H-3/H_b-2. This indicated that H-3 must be equatorial and on the β–side of the molecule. This was also supported by the small *J*-values for the equatorial-axial and equatorial-equatorial couplings between H-3/H_a-2 and H-3/H_b-2.

The presence of NOESY couplings between H-5/H_3-18 indicated that H-5 had to be α−oriented like the C-18 methyl. NOESY correlations between H-8/H_3-20, H-8/H-11, H-11/H_3-20 and H_3-19/H_3-20 indicated the close proximity of these groups and their β−disposition. Therefore the C-17 methyl group had to be equatorial and α-oriented. Thus, all the NMR data of the compound are in agreement with the proposed structure of 15,16-epoxy-3α,9α-dihydroxy-labda-13(16),14-diene (**9**). In addition, the stereochemistry observed for the compound is similar to that of other labdane diterpenes reported from the Lamiaceae family.

*2.2. Preotostegindiol (**10**)*

10 was obtained as a white solid. Its ^{1}H NMR (Table 1) was similar to that of **9,** except that **10** instead of the furan ring signals exhibited signals typical of a β,β-disubstituted dihydrofuran ring. These signals appeared at δ 6.43 (1 H, *d*, *J*=2.84, H-15), 5.14 (1 H, *d*, *J*=2.84, H-14) and a two proton AB system at δ 4.53 (1 H, *d*, *J*=10.1) and 4.05 (1 H, *d*, *J*=10.1) corresponding to the methylene group of the dihydrofuran ring (H_2-16). This was further confirmed by the signals in the ^{13}C NMR spectrum at δ 93.3 (C-9) and 93.0 (C-10), which were joined by the ether linkage of the 9(13)-epoxy group and the appearance of an oxygenated methylene signal at δ 81.5 (C-16) instead of the olefinic methine signal at δ 138.9 in **9**. The remaining NMR data of **10** resembled those of **9**. The conversion of **10** to **9** under mildly acidic conditions was further proof of structure of **10**.

Prefuranic and furanic labdane diterpenoids are commonly encountered in many species of the Lamiaceae family, such as *O. fruticosa* (Al-Musayeib et al., 1995), *Leonurus heterophyllus* (Hon et al., 1993), *L. persicus* (Tasdemir et al., 1998) *Marrubium vulgare* (Bergeron et al., 1995), and *Ballota aucheri* (Rustaiyan et al., 1992). *O. integrifolia* is the only species, so far, in which C-3 hydroxylated prefuranic and furanic labdanes are found. In addition, unlike the labdanes from other species, these two labdanes from *O. integrifolia*, are missing C-6-, C-7- or C-8-oxygenations.

2.3. Pentatriacontane

This compound was eluted in the first two fractions by a gradient of pure n-hexane to 5% n-hexane in ethyl acetate from a silica gel column during the initial fractionation of the crude chloroform extract. Upon removal of the solvent under vacuum an impure solid was obtained which on recrystallization from methanol gave a white crystalline solid. The mass spectrum of the compound showed a molecular ion peak at m/z 492 which led to the elemental composition of $C_{35}H_{72}$. Moreover, a fragmentation pattern with a difference of 28

mass units corresponding to a loss of ethylene typical of long chain hydrocarbons, was observed. The ^{1}H NMR of the compound showed only two signals, a broad singlet at δ 0.95 corresponding to six protons of the two terminal methyl groups and a broad singlet at δ 1.45 corresponding to 66 protons for the remaining 33 methylene groups. The ^{13}C NMR displayed signals at δ 14.6 ($2xCH_3$), 23.4 (CH_2), 30.1 (CH_2), 30.4 ($30xCH_2$) and 32.6 (CH_2).

2.4 Stigmasterol

This was obtained as a white solid upon repeated chromatography on silica-gel and Sephadex LH 20 columns. This compound was quickly recognized as a steroid from its mass spectrum, which exhibited a molecular ion peak at m/z 412 corresponding to an elemental composition of $C_{29}H_{48}O$. Its proton NMR spectrum was found to be identical with that of an authentic sample.

3. Experimental

3.1. General

The plant material used in this study was collected near the town of Ambo, Ethiopia. Melting points were measured with an Electrothermal Melting Point apparatus. The NMR spectra were recorded on a Bruker WM 400 or 500 MHz spectrometer in either deuterated benzene or deuterated chloroform. The chemical shift values are reported with reference to TMS and the coupling constants are given in Hz. Optical rotations were measured as solutions in methanol or benzene on a Perkin Elmer 341 polarimeter at 589 nm and 20 °C .

3.2. Hydrodistillation, extraction and isolation procedure

Ca. 500 g of cleaned, air dried and pulverized leaves of *O. integrifolia* were divided into two equal portions. The first part was homogenized and hydrodistilled in a Clevenger type apparatus for 2.5 hours and a slightly greenish oil was collected in HPLC grade hexane. The oil was analysed on an Orion capillary GC with FID detector, containing 2 columns, a 25 m/0.25 mm i.d. non-polar CPSil-5-CB and a slightly polar CPSil-19-CB column of identical dimensions. The oven temperature was programmed from 50°C to 230°C at a rate of 3°C/min. The injector and detector temperatures were kept at 200°C and 250°C, respectively. The oil was then further analysed using GC-MS on a HP 5890 GC coupled to a VG Analytical 70-250S mass spectrometer with electron impact (70 eV) ionization. Retention indices and mass spectra of the components were compared with library spectra generated under identical experimental conditions Joulain and König, 1998; Hochmuth et al., 2002). Some high boiling

components, including **8**, were apparently unknown. Then the oil was fractionated into 12 fractions on a modified Varian 1400 preparative gas chromatograph, equipped with a stainless steel column (1.85m x 4.3mm) packed with 10% polydimethylsiloxane SE 30 on Chromosorb W-HP. Then **8** was isolated from fraction 9 using the same preparative GC equipped with a column (2m x 4.3mm) packed with the slightly polar SE 52 stationary phase.

The second part of the dried pulverized leaves of *O. integrifolia* was soaked in chloroform at room temperature for 48 hours. The extract was filtered and the chloroform was removed under vacuum to give a dark oily residue. Part of the extract was subjected to column chromatography on a silica gel column with hexane as eluent containing increasing amounts of ethyl acetate. Fourteen fractions were collected. Fractions 1 and 2 eluted with pure and 5 % hexane in ethyl acetate, respectively, and yielded pentatriacontane. Fractions 7, 8 and 10 gave impure solids upon evaporation of the solvents under *vacuo*. Each of these was re-chromatographed separately on silica gel columns using n-hexane with increasing amounts of ethyl acetate (20-30%, v/v) as eluent, followed by purification from chlorophyll on a Sephadex LH-20 column using chloroform/methanol (2:1, v/v) as eluent. By this method, fraction 7 gave stigmasterol, fraction 8 afforded compound **10** and fraction 10 yielded compound **9**.

*3.3. (+)-Axinyssene (**8**)*

Colourless oil, RI_{CPSil5}=1142, sense of optical rotation (benzene): (+); MS (EI, 70 eV), m/z (rel. inten.): 272 (5), 257 (2), 229 (3), 187 (12), 175 (4), 159 (4), 147 (4), 133 (5), 119 (13), 107 (20), 93 (36), 81 (50), 69 (100), 55 (25), 41 (75). ^{1}H- and ^{13}C-NMR data, see Table 1.

*3.4. Otostegindiol (**9**)*

White crystals from hexane; mp 124–125°C; $[\alpha]_{20}^{589}$ = (+) 25 (c=0.01, methanol); ^{1}H- and ^{13}C-NMR (see Table 1); MS (EI, 70 eV), *m/z* (rel. inten.): 320 [M^{+}] (33), 302 (20), 284 (3), 259 (6), 241 (8), 207 (10), 189 (10), 179 (14), 165 (53), 150 (88), 135 (34), 123 (58), 109 (26), 95 (56), 81 (100), 69 (34), 55 (22), 43 (35).

*3.5 Preotostegindiol (**10**)*

White crystals. NMR data, see Table 1. **10** rearranged completely to **9** when submitted to GC or GC-MS.

3.6 Pentatriacontane

MS (EI, 70 eV), *m/z* (rel. inten.): 492 (1), 464 (8), 436 (7), 365 (6) 351 (6), 337 (6), 323 (6), 309 (6), 295 (6), 281 (7), 267 (7), 253 (7), 239 (7), 225 (8), 211 (9), 197 (9), 183 (10),

169 (11), 155 (12), 141 (14), 127 (17), 113 (20), 99 (28), 97 (18), 85 (69), 83 (19), 71 (85), 69 (17), 57 (100), 55 (16), 43 (48).

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Paper-II

Composition of the Essential Oil from Flowers of *Chloranthus spicatus* (Thunb.) Makino[2]

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ABSTRACT: The composition of the essential oil from the flowers of *Chloranthus spicatus* (Thunb.) Makino (Chloranthaceae)**,** was investigated using capillary GC, GC/MS, preparative GC and NMR techniques. Forty-seven compounds were identified either by comparing their retention indices and mass spectra with a library of authentic samples established under identical experimental conditions or, by isolating the compounds and establishing their structures by 1- and 2-dimensional NMR investigations. Thus, four minor components, *viz.* chloranthalactone A (0.5 %), isogermafurenolide (0.7 %), eudesma-4(15),7(11),9-trien-12-olide (0.5 %), and 7α-hydroxyeudesm-4-en-6-one (3.3 %), were isolated for the first time as constituents of the essential oil of the flowers of *C. spicatus* and their structures established. The major components of the oil include (Z)-β-ocimene (6.3 %), *allo*-aromadendrene (6.2 %), sarisane (2-allyl-4,5-methylenedioxyanisol, 4.2 %) and selina-4 (15), 7 (11)-diene (6.4 %).

KEY WORDS: *Chloranthus spicatus*; essential oil; 7α-hydroxyeudesm-4-en-6-one; chloranthalactone A; isogermafurenolide; eudesma-4(15),7(11),9-trien-12-olide; (Z)-β-ocimene; *allo*-aromadendrene; sarisane; selina-4 (15), 7 (11)-diene.

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[2] In press.

Introduction

Three *Chloranthus* species of the family Chloranthaceae are listed in the Flora of Vietnam. They consist of *C. erectus* (Benth & Hook. f.) Verdc., *C. japonicus* Sieb. and *C. spicatus* (Thunb.) Makino. The *C. spicatus* species (Vietnamese name: *Soi gie*) is a herb reaching the height of 1.5 m with pleasant-smelling yellow flowers in summer and autumn[1,2]. The plant is grown in Vietnam to produce flowers for scenting tea[1,2]. Earlier investigation concerned the sesquiterpene constituents of *C. serratus*[3-5], *C. glaber*[6,7], *C. japonicus*[8-14] and the constituents of the volatiles of flowers of *C. spicatus* growing in China[15,16]. We now report on the constituents of the flower essential oil of *C. spicatus* of Vietnamese origin.

Results and Discussion

The essential oil composition of *C. spicatus* was investigated using capillary gas chromatography (GC), GC-mass spectrometry (MS), preparative GC and NMR techniques. Forty-seven compounds (Table 1) were identified either by comparing the retention indices and mass spectra with a library of authentic data established under identical experimental conditions[17, 18] or, where deemed necessary, by isolating the compounds using preparative GC and establishing their structure using NMR techniques. Thus, four minor components (Fig. 1) *viz*. 7α-Hydroxyeudesm-4-en-6-one (**1**), chloranthalactone A (**2**), isogermafurenolide (**3**) and eudesma-4 (15), 7(11), 9-trien-12-olide (**4**) were isolated for the first time as constituents of the oil of *C. spicatus* and their structures established from their MS, 1D- and 2D-NMR data. (Z)-β-ocimene (6.3 %), allo aromadendrene (6.2 %), sarisane (2-allyl-4,5-methylenedioxyanisol, 4.2 %) and selina-4 (15), 7 (11)-diene (6.4 %) were found to be the major components. The major components in the flower essential oil of *C. spicatus* of Chinese origin were methyl jasmonate[15, 16], (Z)-β-ocimene[15], β-pinene[15] and 4-hydroxy-**b** -ionone[16].

*1. 7**a**-Hydroxyeudesm-4-en-6-one (**1**)*

The ^{1}H- and HMQC-NMR spectra of compound **1** exhibited the presence of two secondary methyl groups at δ 0.96 (d, J =7.0) and δ 0.97 (d, J =7.0) and two tertiary methyl groups at δ 0.83 and δ 1.82. The chemical shift of δ 1.82 is typical for an allylic proton. The presence of a methine septet centred at δ 2.37 (J =7.0) and five methylene multiplets at δ (1.22, 1.30), (1.31, 1.38), (1.77), (1.59, 1.74) and (1.23, 1.82) was also observed (Table 2). The ^{13}C-NMR of the compound contained signals of a total of 15 carbon atoms (Table 2) including four methyl, five methylene,

an aliphatic methine and five quaternary carbons (one aliphatic, one carbinol, two olefinic and one keto carbonyl group). In the EI-MS of **1**, the molecular ion signal appeared at m/z 236. This, in combination with the ^{1}H-NMR and ^{13}C-NMR data suggested an elemental composition of $C_{15}H_{24}O_2$, corresponding to an oxygenated sesquiterpene with four degrees of unsaturation. Two of the unsaturations were due to two double bonds and therefore the remaining two must be due to two rings.

Table 1 Constituents of the Flower Essential oil of *Chloranthus spicatus*.

Name	Ret.index[3]	% Composition
Benzaldehyde	941	trace
α-Pinene	943	0.2
1-Octene-3-ol	969	1.1
β-Pinene	982	0.8
Myrcene	991	0.1
p-Cymene	1021	0.1
Limonene	1031	trace
(Z)-β-Ocimene	1033	6.3
(E)-β-Ocimene	1044	2.8
o-Guiacol	1083	trace
Rose furan	1087	0.2
Linalool	1090	0.2
1-Oct-3-enylacetate	1099	2.2
trans-Pinocarvylformate	1249	0.1
Safrol	1267	1.6
Bicycloelemene	1341	trace
(E)-Isosafrol	1354	0.2

[3] Retention Index on a 25 m x 0.25 mm CPSil-5 polydimethylsiloxane.

α-Copaene	1381	0.8
β-Elemene	1392	1.3
Cascarilladiene	1420	0.7
(E)-β-Caryophyllene	1423	0.6
γ-Elemene	1431	0.2
trans-α-Bergamotene	1436	0.3
(E)-β-Farnesene	1448	0.2
Selina-4(15),7-diene	1454	0.2
α-Humulene	1456	0.4
Allo aromadendrene	1462	6.2
Sarisane	1466	4.2
5-epi-Aristolochene	1474	0.4
γ-Muurolene	1480	0.8
Furanoelemene (Furanodiene)[4]	1485	2.8
β-Selinene	1488	0.5
(Z)-α-Bisabolene	1492	2.5
Bicyclogermacrene	1495	0.8
β-Bisabolene	1501	2.2
δ-Cadinene	1517	0.8
Selina-4(15),7(11)-diene	1533	6.4
Germacrene B	1555	1.4
Spathulenol	1567	3.0
Methyl jasmonate	1607	2.4

[4] Under the used GC conditions inter-conversion is possible.

Germacrone	1675	2.2
7α-Hydroxyeudesm-4-en-6-one	1702	3.3
Eudesma-4(15),7(11)-dien-8-one	1707	0.2
(Z)-Lanceol	1739	0.1
Isogermafurenolide	1869	0.7
Chloranthalactone A	1943	0.8
Eudesma-4(15),7(11),9-trien-12-olide	1968	0.5

In the ^{1}H-^{1}H COSY of compound **1** (Table 3), couplings were observed between the methylene protons at δ 1.22 (H_a-1), 1.30 (H_b-1) and 1.31 (H_a-2), 1.38 (H_b-2). The latter were further coupled to another methylene group at δ 1.77 (H_2-3). In addition, two methylene groups at δ 1.59 (H_a-8), 1.74 (H_b-8) and δ 1.23 (H_a-9), 1.82 (H_b-9) showed coupling correlations with each other. Again, both of the secondary methyl doublets at δ 0.96 (H_3-12) and δ 0.97 (H_3-13) were not only coupled to the methine septet at δ 2.37 (H-11) but also coupled to each other indicating the presence of an isopropyl group. In the HMBC spectrum of the compound (Table 3), the carbinol carbon at δ 78.68 (C-7) was coupled to the methine septet at δ 2.37 (H-11), the two secondary methyl protons at δ 0.96 (H_3-12) and 0.97 (H_3-13) and the two methylene groups at δ 1.59 (H_a-8), 1.74 (H_b-8) and δ 1.23 (H_a-9), 1.82 (H_b-9). This indicated that the isopropyl group must be connected to the carbinol carbon. The keto carbon at δ 202.44 (C-6) was coupled to the methine septet at δ 2.37 (H-11), the methylene protons at δ 1.59 (H_a-8), 1.74 (H_b-8) and the olefinic methyl singlet at δ 1.82 (H_3-15). One of the olefinic quaternary carbons at δ 138.17 (C-5) was coupled to the tertiary methyl singlet at δ 0.83 (H_3-14) and the olefinic methyl singlet at δ 1.82 (H_3-15) while the other olefinic quaternary at δ 141.55 (C-4) was coupled solely to the olefinic methyl singlet at δ 1.82 (H_3-15). From this data it was concluded that the compound had an eudesmane skeleton with a double bond between C-4 and C-5, the keto group at C-6 and the carbinol group at C-7. In addition, the MS and NMR data were found to be similar to the only report of the compound from a different *chlorantus* species, *C. serratus*[3].

Table 2. ^{1}H and ^{13}C –NMR data of Compounds **1**, **2**, **3** and **4**.

C No.	**1**		**2**		**3**		**4**	
	^{1}H, ppm	^{13} C, ppm	^{1}H, ppm	^{13}C, ppm	^{1}H, ppm	^{13} C, ppm	^{1}H, ppm	^{13} C, ppm
1	1.22, 1.30	38.57	1.17	27.05	5.38, dd, J = 2.4, 14. 5	146.98	1.03 m	39.32
2	1.31, 1.38	18.69	0.56, 0.72	17.36	4.70, 4.78	111.40	1.19 m	30.47
3	1.77	33.43	1.71	22.91	4.48, 4.84	113.64	1.59,1.95	36.67
4	-	141.55	-	150.79	-	144.89	-	147.82
5	-	138.17	2.59	62.45	1.56	52.55	1.66 m	47.90
6	-	202.44	1.67, 2.04	21.26	1.82, 2.11	28.00	1.19,1.83	23.51
7	-	78.68	-	150.24	-	160.63	-	147.31
8	1.59,1.74	26.58	-	147.68	4.20	77.08	-	148.58
9	1.23,1.82	35.51	5.81	118.66	0.94, 1.81	45.68	5.04 s	117.96
10	-	37.27	-	40.08	-	40.68	-	37.63
11	2.37 sep. J =7.0	32.60	-	123.52	-	120.30	-	120.93
12	0.96 d, J =7.0	16.22	-	170.73	-	174.86	-	170.36
13	0.97 d, J =7.0	18.42	1.52	8.75	1.61	8.23	1.40 s	8.66
14	0.83	25.27	0.48	22.23	0.67	16.62	0.49 s	18.74
15	1.82	22.11	4.59, 5.00	106.61	1.50	24.71	4.19 s, 4.59 s	107.50

*2. Chloranthalactone A (**2**)*

The ^{1}H- and HMQC-NMR spectrum of **2** exhibited the presence of two tertiary methyl groups at δ 0.48 and 1.52, the latter being obviously allylic. The presence of three aliphatic (δ 1.17, 1.70, 2.59) and one olefinic (δ 5.81) methine groups were also observed. Furthermore, the presence of two aliphatic methylene groups at δ (0.56, 0.72) and δ (1.67, 2.04), respectively, and one exocyclic olefinic methylene group δ (4.59, 5.00) was observed.

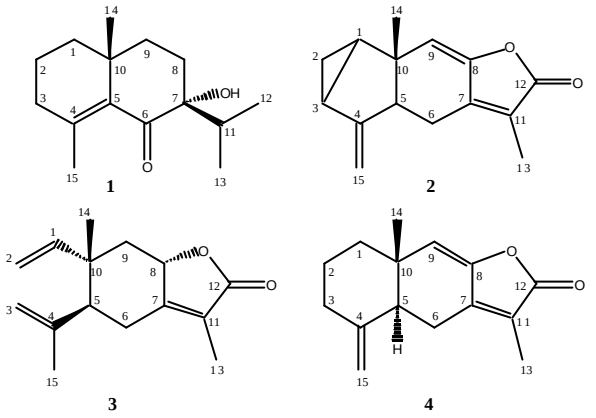

Figure 1. 7a-Hydroxyeudesm-4-en-6-one (1), chloranthalactone A (2), isogermafurenolide (3) and eudesma-4(15), 7(11), 9-trien-12-olide (4) from Chloranthus spicatus flower essential oil

The ^{13}C-NMR of the compound contained signals of a total of 15 carbon atoms. These were assigned to two methyl, two aliphatic and one exocyclic methylene, three aliphatic and one olefinic methine, one aliphatic and five olefinic quaternary carbons (Table 2). The presence of a lactone function in the compound was readily recognized from the ^{13}C-NMR shift at δ 170.73 (C-12) of the lactone carbonyl group. In the EI-MS of **2** the molecular ion signal appeared at m/z 228. This, in combination with the ^{1}H- and ^{13}C NMR data indicated an elemental composition of $C_{15}H_{16}O_2$, an oxygenated sesquiterpene with eight degrees of unsaturations. Four of the unsaturations were due to four double bonds and the remaining four must be due to four rings.

In the ^{1}H-^{1}H COSY spectrum (Table 4) of compound **2** correlations were observed between the methine multiplet at δ 1.17 (H-1) and each of the two methylene proton multiplets at δ 0.56 (H_a-2) and 0.72 (H_b-2). The latter was coupled to another methine group at δ 1.71 (H-3). Furthermore, the two methine groups were coupled to each other. These high field methylene signals indicated the presence of a cyclopropane ring in the compound. On the other hand, allylic couplings were observed between the methine group at δ 1.71 (H-3) and the exocyclic methylene protons at δ 4.59 (H_a-15) and 5.00 (H_b-15). Also, the latter showed an allylic coupling to the

methine group at δ 2.59 (H-5) which indicated the position of the exocyclic methylene group between the C-3 and C-5 methines connected to C-4. The C-5 methine proton was further coupled to methylene protons at δ 1.67 (H_a-6) and δ 2.04 (H_b-6). The latter exihibited J^4 coupling to the allylic methyl singlet at δ 1.52.

In the HMBC spectrum of **2** (Table 4)**,** several important correlations were observed that substantiated the structural evidences observed in the ^1H-^1H COSY. Thus the aliphatic quaternary carbon at δ 40.08 (C-10) was correlated with the cyclopropane methylene protons at δ 0.56 (H_a-2) and 0.72 (H_b-2), the aliphatic tertiary methyl singlet at δ 0.48 (H_3-14), the olefinic methine singlet at δ 5.81 (H-9) and the aliphatic methine multiplet at δ 2.59 (H-5). Furthermore, the aliphatic methine carbon at δ 62.45 (C-5) was correlated to the exocyclic olefinic methylene protons at δ 4.59 (H_a-15), 5.00 (H_b-15), the aliphatic tertiary methyl singlet at δ 0.48 (H_3-14) and the methylene protons at δ 1.67 (H_a-6) and δ 2.04 (H_b-6). The latter were also correlated with the olefinic quaternary carbons at δ 150.24 (C-7) and δ 123.52 (C-11). Additional coupling correlations were observed between the allylic methyl singlet at δ 1.52 (H_3-13) and the olefinic quaternary carbon at δ 123.52 (C-11) and the lactone carbonyl carbon at δ 170.73 (C-12). One of the methine carbons of the cyclopropane ring at δ 22.91 (C-3) was coupled to the exocyclic methylene protons at δ 4.59 (H_a-15) and 5.00 (H_b-15). All the NMR data of the compound are in agreement with the proposed structure. This compound was first reported from *C. glaber*[19] where structural elucidation was done partly by spectroscopic and partly by chemical methods. Its presence in *Sarcandra glabra*[20] was also reported.

*3. Isogermafurenolide (**3**)*

The ^1H- and HMQC-NMR spectra of compound **3** exhibited the presence of one aliphatic and two allylic tertiary methyl groups at δ 0.67, δ 1.50 and δ 1.61, respectively. Also the presence of two aliphatic and one olefinic methine signal centred at δ 1.56, δ 4.20 and δ 5.38 (dd, J = 2.4, 14.5 Hz) were observed. Two aliphatic methylene multiplets at δ (0.94, 1.81), δ (1.82, 2.11), and two exocyclic olefinic methylene signals at δ (4.48, 4.84) and δ (4.70, 4.78) were also present (Table 2). The ^{13}C-NMR of the compound contained signals for a total of 15 carbon atoms (Table 2). These were three methyl, four methylene (two aliphatic and two exocyclic olefinic), three methine (one aliphatic, one oxygenated and one olefinic) and five quaternary (one aliphatic, three

olefinic and a lactone carbonyl) carbon signals. In the EI-MS of **3** the molecular ion signal appeared at m/z 232. This, in combination with the ^{1}H- and ^{13}C-NMR data, indicated an elemental composition of $C_{15}H_{20}O_2$, a sesquiterpene lactone with six degrees of unsaturation. Four of the unsaturations were attributed to four double bonds and therefore the remaining two must be due to the two rings. Inspection of the NMR and MS data of the compound led to the proposed structure. The compound was previously reported from *Lindera strychnofolia*[21], *Neolitsea hiiranensis*[23] and has also been synthesized[22]. The NMR data of **3** are in good agreement with the reported data.

Table 3. Important ^{1}H-^{1}H COSY and HMBC correlations observed in **1**.

^{1}H-^{1}H COSY		HMBC	
Hydrogen	Correlated with	Carbon	Correlated with
H_2-1	H_2-2	C-1	H_3-14
H_2-2	H_2-1, H_2-3	C-2	H_2-3
H_2-3	H_2-2	C-3	H_3-15
H_2-8	H_2-9	C-4	H_3-15
H_2-9	H_2-8, H_3-14 (J^4)	C-5	H_3-14, H_3-15
H-11	H_3-12, H_3-13	C-6	H-11, H_2-8, H_3-15
H_3-12	H-11	C-7	H_a-8,H_a-9,H-11,H_3-12, H_3-13
H_3-13	H-11	C-8	H-11, H_b-9
H_3-14	H_2-9 (J^4)	C-9	H_b-8, H_3-14
		C-10	H_3-14
		C-11	H_3-12, H_3-13
		C-12	H-11, H_3-13
		C-13	H-11, H_3-12
		C-14	H_2-1, H_2-9

Table 4. Important ^{1}H-^{1}H COSY and HMBC correlations observed in **2**

^{1}H-^{1}H COSY		HMBC	
Hydrogen	Correlated with	Carbon	Correlated with
H-1	H_2-2, H-3	C-1	H_3-14
H_2-2	H-1, H-3	C-2	H_3-14
H-3	H_2-2, H-1, H_2-15 (J^4)	C-3	H_2-15
H-5	H_2-6, H_2-15 (J^4)	C-4	H_2-2
H_2-6	H-5	C-5	H_b-6, H-9, H_3-14, H_2-15,
H_2-15	H-3 (J^4), H-5 (J^4)	C-7	H_b-6, H-9, H_3-13
		C-8	H_b-6, H-9, H_3-13
		C-9	H_3-14
		C-10	H_2-3, H_b-6, H-9, H_3-14
		C-11	H_b-6, H_3-13
		C-12	H_3-13

*4. Eudesma-4(15),7(11),9-trien-12-olide (**4**)*

The ^{1}H- and HMQC-NMR spectra of compound **4** exhibited the presence of one aliphatic and one allylic tertiary methyl groups at δ 0.49 and δ 1.40, respectively. In addition, the presence of an aliphatic methine multiplet centred at δ 1.66, an olefinic methine singlet at δ 5.04, four aliphatic methylene multiplets at δ (1.03), δ (1.19), δ (1.59, 1.95) and δ (1.19, 1.83) and one exocyclic olefinic methylene group at δ (4.19, 4.59) was observed (Table 2). The ^{13}C-NMR spectrum of the compound contained signals due to a total of 15 carbon atoms (Table 2). These were two methyl, five methylene (four aliphatic and one exocyclic olefinic), two methine (one aliphatic and one olefinic) and five quaternary carbon signals (one aliphatic, four olefinic and a lactone carbonyl). In the EI-MS of **4** the molecular ion peak appeared at m/z 230. In combination with the ^{1}H- and ^{13}C-NMR data this indicated an elemental composition of $C_{15}H_{18}O_2$, a

sesquiterpene lactone with seven degrees of unsaturations. Four of the unsaturations were due to four double bonds and therefore the remaining three must be due to the three rings. Inspection of these NMR and the MS data of the compound led to the proposed eudesmanolide. This compound has previously been reported from Asteraceae *Aster umbellatus*[24], *Mikania banisteriae*[25] and *Atractylodes chinensis*[26].

Experimental

Plant material and Isolation of the Essential Oil

The flowers of *C. spicatus* were collected in Phu Tho Province, Vietnam, in July 2001. The plant was identified by Dr. Tran Ngoc Ninh, a botanist at the Institute of Ecology and Biological Resources, Vietnam National Centre for Natural Science and Technology, Hanoi, Vietnam. A voucher specimen (No. CS.IEB 601) was deposited at the Herbarium of the same Institute. Hydrodistillation of the dry flowers of *C. spicatus* yielded 0.7% (w/w) of the essential oil.

Table 5. Important $^{1}H^{1}H$ COSY and HMBC couplings observed in **4**.

^{1}H-^{1}H COSY		HMBC	
Hydrogen	Correlated with	Carbon	Correlated with
H_2-1	H_2-2, H_3-14	C-1	H_2-2, H_2-3, H_3-14
H_2-2	H_2-1, H_2-3	C-2	H_2-1, H_2-3
H_2-3	H_2-2	C-3	H_2-15
H-5	H_2-6	C-4	H_2-2
H_2-6	H-5	C-5	H-9, H_3-14, H_2-15,
H-9	H_3-14	C-7	H-9, H_3-13
H_3-14	H_2-1, H-9	C-9	H_3-14
		C-10	H_3-14
		C-11	H_3-13
		C-12	H_3-13

Capillary GC Analysis

The oil was preliminary analysed on an Orion Micromat 412 GC equipped with double columns, 25 m x 0.25 mm polydimethylsiloxane CP-Sil-5-CB and CP-Sil-19-CB (chrompack) capillaries and flame ionisation detectors. The oven temperature was programmed linearly from 50°C to 230°C at a rate of 3 °C/min. The injector and detector temperatures were 200 °C and 250 °C respectively and with a split injection. The carrier gas used was hydrogen at a flow rate of 0.5 ml/ min..

GC-MS Analysis

GC-MS measurements were carried out on a Hewlett-Packard HP 5890 gas chromatograph equipped with a 25 m x 0.25 mm polydimethylsiloxane CP-Sil-5-CB (chrompack) capillary column and coupled to a VG Analytical VG 70-250S mass spectrometer with electron impact (70 eV) ionisation. The oven was operating under a linear temperature program from 80 °C to 270 °C at the rate of 10°C/min. Helium was used as a carrier gas at a flow rate of 0.5 ml/ sec.. The injector, transfer line and ion source temperatures were 220 °C, 230°C and 220°C respectively.

Preparative GC

Preparative GC was carried out on a modified Varian 1400 preparative gas chromatograph, equipped with stainless steel columns (1.85 m x 4.3 mm) packed with either 10 % polydimethylsiloxane SE 30 on Chromosorb W-HP or a modified β-cyclodextrin (6-O-TBDMS-2,3-di-OMe-β-cyclodextrin) stationary phases. This analysis was under taken in order to isolate the minor components of the oil that could not be identified by the usual method of comparison of mass spectra and retention indices of the unknowns with a library of mass spectra and retention indices. There fore, in order to obtain enough material for recording of NMR data, the unknowns were enriched by repeated fractionation of the oil on the preparative GC equipped with a column packed with the SE 30 stationary phase. During the fractionation, the oven temperature was programmed from 80°C to 180°C at the rate of 2°C/min. Then, each of the fractions were analysed by GC/MS to verify that no transformation has taken place during the fractionation process. Then, the individual compounds were isolated using the preparative GC equipped with a stainless steel column packed with the modified β-cyclodextrin (6-O-TBDMS-2,3-di-OMe-β-cyclodextrin) stationary phase. By this method it was possible to achieve ca. 90 % or more purity.

NMR spectroscopy

NMR measurements were carried out with a Bruker WM 400 or 500 MHz instrument using TMS as internal standard in deuterated benzene, C_6D_6.

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Paper-III

Composition of the Essential Oil of a Liverwort *Radula perrottetii* of Japanese Origin[5]

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Abstract

Analysis of the essential oil of the liverwort *Radula perrottetii* afforded two novel viscidane diterpenes, viscida-4,9,14-triene (**1**), viscida-4,11(18),14-triene (**2**), four bisabolane sesquiterpenes, bisabola-2,6,11-triene (**3**), bisabola-1,3,5,7(14),11-pentaene (**4**), bisabola-1,3,5,7,11-pentaene (**5**), 6,7-epoxybisabola-2,11-diene (**6**), and 1-(4-methoxyphenyl)-2-methyl propene (**7**) as new natural products. In addition, the known compounds bisabola-1,3,5,7(14),10-pentaene (**8**), *ar*-tenuifolene (**9**), α-helmiscapene (**10**), and β-helmiscapene (**11**) were also isolated. Isolation was carried out by preparative gas chromatography, and the structures were established by extensive NMR analysis. This is the first finding of viscidane diterpenes in liverworts. Compounds **8**, **9** and the rarely encountered eudesmane sesquiterpene hydrocarbons **10** and **11** are reported for the first time from *R. perrottetii*.

Keywords: *Radula perrottetii*; Liverwort; Essential oil; Viscidane diterpenes; Bisabolane and Eudesmane sesquiterpenes; *ar*-Tenuifolene.

Submitted.

1. Introduction

The liverwort *Radula perrottetii* Gott. belongs to the Jungermanniales (Hepaticae). Earlier, several prenylated bibezyls and derivatives thereof have been reported from *R. perrottetii* (Asakawa, et al., 1982, 1991; Toyota et al., 1985, 1994). Further investigation of the chemical constituents of *R. perrottetii* resulted in the isolation of two diterpene hydrocarbons with viscidane structure (**1, 2**), (Fig. 1) four bisabolane sesquiterpenes (**3-6)** as well as 1-(4-methoxy phenyl)-2-methyl propene (**7**) as new compounds. In addition, bisabola-1,3,5,7(14),10-pentaene (**8**), *ar*-tenuifolene (**9**) as well as two eudesmane sesquiterpenes (**10, 11**) were isolated.

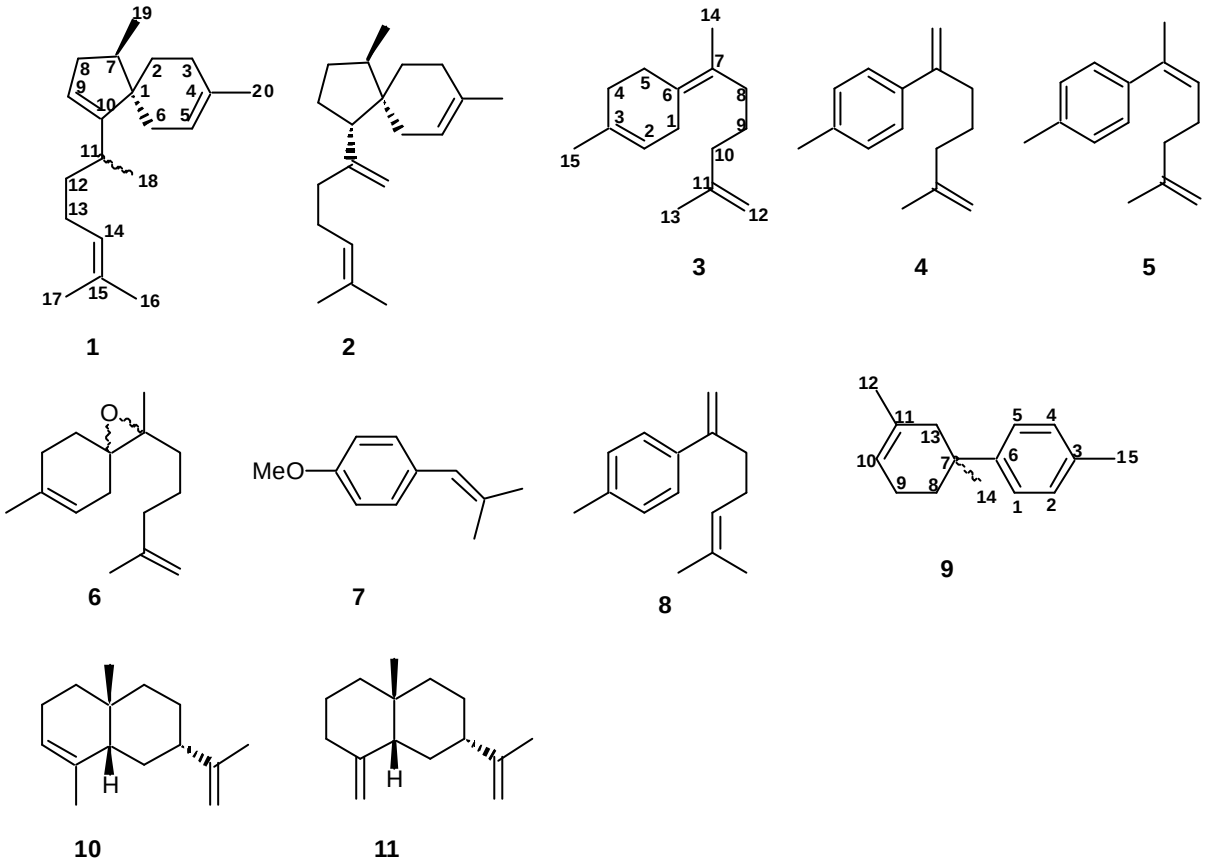

Figure 1. Structures of compounds isolated from the essential oil of *R. perrottetii*

2. Results and discussion

The essential oil of *R. perrottetii* was analysed by gas chromatography (GC) and coupled gas chromatography-mass spectrometry (GC/MS). Mass spectra and retention indices of the components of the essential oil on a non-polar stationary phase (CPSil-5) were compared with a library of mass spectra of authentic compounds established under identical experimental conditions (Joulain and König, 1998; Hochmuth et al., 2003): Δ-3-Carene, α-terpinene, p-

cymene, (Z)-β-ocimene, γ-terpinene, terpinolene, β-elemene, α-gurjunene, 7-*epi*-α-cedrene, α-gurjunene, α-cedrene, aristolene, γ-maaliene, eremophila-1(10),6-diene, calarene, valerena-4,7(11)-diene, selina-3,7-diene, β-acoradiene, *allo*-aromadendrene, 4,5-*di-epi*-aristolochene, selina-4,7-diene, β-chamigrene, eremophila-1(10),7-diene, eremophilene, hinesene, cuparene, α-chamigrene, (E)-γ-bisabolene, γ-cuprenene and bicyclohumulenone were identified. Eleven components that could not be identified by this method were isolated by preparative GC and their structures were established as viscida-4,9,14-triene (**1**), viscida-4,11(18),14-triene (**2**), bisabola-2,6,11-triene (**3**), bisabola-1,3,5,7(14),11-pentaene (**4**), bisabola-1,3,5,7,11-pentaene (**5**), 6,7-epoxybisabola-2,11-diene (**6**), 2-methyl-1-(4-methoxy phenyl)propene (**7**), bisabola-1,3,5,7(14),10-pentaene (**8**), *ar*-tenuifolene (**9**), α-helmiscapene (**10**) and β-helmiscapene (**11**) (Fig. 1) from their respective MS, 1D- and 2D-NMR data and comparison with reported data for the known compounds. Compound **8** was previously reported from *Biota orientalis* wood (Tomita, et al, 1969). Compound **9**, hitherto described only from the East African Sandalwood plant *Osyris tenuifolia* as the (-)-enantiomer (Kreipl et al., 2004) was found to exist in the unusual racemic form in the oil of the liverwort *R. perrottetii*. It was earlier reported (Asakawa, 1995; König et al., 1998; Toyota et al., 1999) that biosyntheses of racemic mixtrures of sesquiterpene hydrocarbons occur in liverworts only occasionally. Both compounds **10** and **11** were previously described as constituents of liverworts (Huneck et al, 1978).

*2.1. Viscida-4,9,14-triene (**1**)*

Compound **1** was isolated as oil by preparative GC from the essential oil of the plant. Its mass spectrum displayed a molecular ion signal at m/z 272. Its ^{1}H (Table 1) and HMQC NMR data exhibited the presence of three allylic methyl singlets, two methyl doublets, five methine proton signals (three of them olefinic) as well as six methylene multiplets. The ^{13}C NMR (Table 1) showed signals of twenty carbon atoms including four quaternary carbons, three of them olefinic, besides the signals of the groups observed in the ^{1}H and HMQC NMR. The ^{1}H and ^{13}C NMR data in combination with the mass spectrum confirmed an elemental composition of $C_{20}H_{32}$, a diterpene hydrocarbon with five degrees of unsaturation. Detailed analysis of the two dimensional NMR spectra of the compound confirmed that three of the five unsaturations were due to double bonds revealing a bicyclic diterpene. The exact connectivity of atoms within the molecule that led to the viscidane type skeleton was established by interpretation of the 2D $^{1}H,^{1}H$ COSY and HMBC spectra.

Table 1: ^{1}H and ^{13}C NMR data of compounds **1, 2**, **3** and **6**

C-	**1**			**2**			**3**			**6**		
No.	δ ^{1}H	m (*J*)	δ ^{13}C	δ ^{1}H	m (*J*)	δ ^{13}C	δ ^{1}H	m (*J*)	δ ^{13}C	δ ^{1}H	m (*J*)	δ ^{13}C
1	-	-	50.3	-	-	44.8	1.97	*m*	32.3	1.86	*m*	29.3
										2.24	*m*	
2	1.86	*m*	28.8	1.80	*m*	32.8	2.35	*m*	27.2	1.67	*m*	27.3
	1.97	*m*		2.00	*m*							
3	5.42	*bs*	121.5	5.40	*bs*	122.1	-	-	134.2	-	-	134.5
4	-	*m*	133.2	-	-	133.8	5.39	*bs*	121.5	5.31	*s*	120.2
5	1.36	*m*	32.9	1.34	*m*	30.8	2.75	*bs*	30.4	2.00	*d(15)*	31.9
	1.75	*m*		1.43	*m*					2.24	*d(15)*	
6	1.87	*m*	28.4	1.89	*m*	28.4	-	-	128.9	-	-	63.3
7	2.08	*m*	40.8	1.77	*m*	41.3	-	-	126.3	-	-	64.0
8	2.45	*m*	39.1	1.13	*m*	33.6	2.08	*t(7.5)*	34.2	1.40-	*m*	34.7
	1.85	*m*		1.82	*m*					1.58		
9	5.35	*t(2.2)*	120.6	1.68	*m*	29.6	1.54	*m*	27.3		*m*	24.18
10	-	-	156.8	2.29	*m*	55.9	1.98	*m*	38.3	1.92	*t(7)*	38.5
11	1.96	*m*	31.3	-	-	150.6	-	-	146.1	-	-	146
12	1.43	*m*	38.9	2.11	*m*	37.6	4.82	*bs*	110.5	4.78	*s*	110.8
	1.61	*m*								4.80	*s*	
13	2.05	*m*	26.9	2.24	*m*	28.1	1.66	*s*	22.8	1.62	*s*	22.6
14	5.25	*t 5.2*	125.5	5.25	*t(7)*	125.3	1.62	*s*	18.7	1.18	*s*	18.8
15	-	-	130.9	-	*bs*	131.5	1.64	*s*	23.9	1.61	*s*	23.7
16	1.59	*s*	17.8	1.57	*s*	18.4						
17	1.69	*s*	25.9	1.67	*s*	26.2						
18	1.06	*d(7)*	23.0	4.87	*s*	111.6						
				5.00	*s*							
19	0.98	*d(7)*	18.6	0.91	*d(7)*	18.0						
20	1.62	*bs*	23.7	1.62	*bs*	23.8						

*2.2. Viscida-4,11(18),14-triene (**2**)*

Compound **2** that eluted next to **1** on the CPSil-5 capillary column was isolated as an oil. Its mass spectrum showed a molecular ion peak at m/z 272 similar to **1** but the base peak for the latter was at m/z 122 while that of the former was at m/z 119. Its ^{1}H (Table 1) and HMQC NMR data indicated the presence of three allylic methyl singlets, a methyl doublet, four methine protons signals (two of them olefinic) and eight methylene multiplets (including one exocyclic methylene). The ^{13}C NMR (Table 1) displayed signals due to four quaternary carbons three of them olefinic besides the signals of the groups observed in the ^{1}H and HMQC NMR spectra. Thus similar to compound **1**, the ^{1}H and ^{13}C NMR data in combination with the mass spectrum revealed an elemental composition of $C_{20}H_{32}$, a diterpenoid hydrocarbon with five degrees of unsaturation. Examination of connectivity of atoms in the ^{1}H,^{1}H COSY and HMBC spectra verified that three of the unsaturations were due to double bonds and, consequently, the carbon skeleton was bicyclic. Further interpretation of the observed correlations in the 2D ^{1}H,^{1}H COSY, and HMBC spectra of the compound led to the depicted structure for compound **2** that differed from compound **1** in the position of one of the double bonds.

The relative configurations of compounds **1** and **2** was determined by 2D-NOESY spectrum of compound **2**, where a key correlation was observed between the methyl doublet at δ 0.91 (H_3-19) and the methine proton multiplet at δ 2.29 (H-10). This indicated these substituents to be situated at the same side of the molecule furnishing the depicted relative configuration. Assuming close biogenetic relationships between **2** and **1** the configuration at C_7 in the latter was suggested to be the same as in the former. This is the first isolation of viscidane diterpene hydrocarbons from the genus *Radula*. Diterpenes with viscidane skeleton were hitherto reported from plants of the genus *Eremophila* only (Ghisalberti, et al., 1984; Forster et al., 1986 and 1993; Syah et al., 1997).

The absolute configuration of the viscidane diterpenes from *Eremophila* species was determined to be opposite to that of the sesquiterpene hydrocarbon, (+)-α-acoradiene (Ghisalberti, et al., 1984). It is long recognized that liverworts produce terpenes that are predominantly optical antipodes to those found in higher plants (Hayashi, et al., 1975, König, et al., 1998). Therefore, it is expected that the viscidane diterpenes from the liverwort *R. perrottetii* could show the opposite absolute configuration to those reported from the *Eremophila* species although this remains to be clarified.

*2.3. Bisabola-2,6,11-triene (**3**)*

The ^{1}H NMR (Table 1) of compound **3** indicated the presence of three allylic methyl groups, seven methylene groups including one exocyclic methylene as well as one olefinic methine proton. The ^{13}C NMR (Table 1) indicated the presence of fifteen carbon atoms. The MS of **3** exhibited a molecular ion signal at m/z 204 confirming an elemental composition of $C_{15}H_{24}$. Analysis of the 2D $^{1}H,^{1}H$ COSY and HMBC spectra of the compound led to a monocyclic bisabolane skeleton having three double bonds as indicated. While this is the first report of the compound from a natural source, it has been synthesized earlier as an intermediate in the preparation of iso-β-bisabolol reported from East Indian and western Australian sandalwood oil. (Braun, N.A. et al., 2003).

*2.4. Bisabola-1,3,5,7(14),11-pentaene (**4**)*

The ^{1}H NMR (Table 2) of **4** indicated the presence of an allylic and an aromatic methyl group, five methylene groups (including two exocyclic methylene groups) as well as four aromatic methine protons. The aromatic methine signals appeared as a pair of doublets, each representing two protons, a pattern, typical for a para-disubstituted benzene system. The ^{13}C NMR (Table 2) indicated the presence of fifteen carbon atoms. The MS of **4** exhibited a molecular ion signal at m/z 200 confirming an elemental composition of $C_{15}H_{20}$. Analysis of the 2D $^{1}H,^{1}H$ COSY and HMBC spectra of the compound revealed a monocyclic aromatic bisabolane skeleton having two exocyclic double bonds as depicted. This is the first report of the compound from a natural source. The synthesis of the compound as a new sesquiterpene from 4-methylacetophenone via successive enolization, Michael reaction and Wittig reaction was described (Zhang, et al., 1995).

*2.5. Bisabola-1,3,5,7,11-pentaene (**5**)*

Its ^{1}H NMR (Table 2) indicated the presence of two allylic and one aromatic methyl singlet, three methylene groups (including one exocyclic methylene groups) four aromatic methine protons as well as one olefinic methine proton. Again, the aromatic methine signals appeared in a pattern typical for a para-disubstituted benzene system. The ^{13}C NMR (Table 2) indicated the presence of fifteen carbon atoms. The MS of **5** exhibited a molecular ion signal at m/z 200 confirming an elemental composition of $C_{15}H_{20}$. Interpretation of the 2D $^{1}H,^{1}H$ COSY and HMBC spectra of the compound led to the aromatic bisabolane skeleton having one exocyclic double bond as indicated. This is the first report of the compound from a natural source. The synthesis of the compound and related derivatives was described earlier. (Kuznetsov, et al., 1971; Wu, et. al., 1992).

Table 2: ^{1}H and ^{13}C NMR data of compounds **4, 5** and **7**

	4			**5**			**7**		
Atom-no.	δ ^{1}H	m (*J*)	δ ^{13}C	δ ^{1}H	m (*J*)	δ ^{13}C	δ ^{1}H	m (*J*)	δ ^{13}C
1	7.00	*d(8)*	129.3	7.03	*d(8)*	129.2	-	-	158.5
2	7.31	*d(8)*	126.5	7.32	*d(8)*	126.0	6.81	*d(9)*	114.0
3	-	-	137.1	-	-	136.3	7.16	*d(9)*	130.3
4	7.31	*d(8)*	126.5	7.32	*d(8)*	126.0	-	-	131.7
5	7.00	*d(8)*	129.3	7.03	*d(8)*	129.2	7.16	*d(9)*	130.3
6	-	-	138.9	-	-	141.6	6.81	*d(9)*	114.0
7	-	-	148.7	-	-	135.3	6.3	*s*	125.5
8	2.45	*t(7)*	35.3	5.82	*t(8)*	127.2	-	-	133.5
9	1.58	*m*	26.5	2.26	*q(7)*	27.3	1.74	*s*	26.8
10	1.96	*t(7)*	37.7	2.06	*t(8)*	37.8	1.76	*s*	26.8
11	-	-	145.6	-	-	145.4	3.33	*s*	54.8
12	4.76	*s*	110.6	4.81	*s*	110.7			
	4.77	*s*		4.82	*s*				
13	1.58	*s*	22.4	1.65	*s*	22.6			
14	5.03	*s*	111.7	1.93	*s*	15.9			
	5.32	*s*							
15	2.12	*s*	21.1	2.16	*s*	21.0			

*2.6. 6,7-Epoxybisabola-2,11-diene (**6**)*

The ^{1}H NMR spectrum (Table 1) of compound **6** indicated the presence of a tertiary methyl singlet connected to an oxygenated carbon atom and two allylic methyl groups, seven methylene groups (including one exocyclic methylene group) as well as one olefinic methine proton. The ^{13}C NMR (Table 1) indicated the presence of fifteen carbon atoms. As the EI-MS of **6** failed to show a molecular ion signal, CI-MS measurement was carried out, and the corresponding spectrum exhibited a strong $[M+1]^{+}$ signal at m/z 221, confirming an elemental composition of $C_{15}H_{24}O$. Analysis of the 2D $^{1}H,^{1}H$ COSY and HMBC spectra of the compound led to the depicted bisabolane skeleton having two C-C double bonds and an epoxide ring as indicated. The presence of the epoxide ring was evident by the two up-field shifted signals at δ 63.3 and 63.4 indicating oxygenated quaternary carbon atoms in the ^{13}C-NMR spectrum (Table 1). The fact that the tertiary methyl singlet protons at δ 1.18 (H_3-14)

were strongly correlated with these two carbons in the HMBC spectrum, further substantiated the position of the epoxy function. This is the first report of the compound from a natural source. This compound has been synthesised as a precursor of the previously mentioned alcohol, iso-β-bisabolol (Braun, et al., 2003).

*2.7. 1-(4-methoxyphenyl)-2-methyl propene (**7**)*

The ^{1}H NMR (Table 2) of **7** indicated the presence of two allylic methyl groups, one methoxy singlet, an olefinic methine, and four aromatic methine protons that appeared in a pattern characteristic of a para-disubstituted benzene system. The ^{13}C NMR (Table 2) indicated the presence of a total of eleven carbon atoms. The MS of **7** exhibited a molecular ion peak at m/z 162 confirming an elemental composition of $C_{11}H_{14}O$. Interpretation of the spectra of the compound led to the indicated structure. This is the first isolation of the compound from a natural source.

3. Experimental

3.1. General

The Liverwort used in this study was collected in Aioicho, Nakagun, Tokushima, Japan, in October 2003. The NMR spectra were recorded on a Bruker WM 400 or 500 MHz spectrometer in deuterated benzene (C_6D_6). The chemical shift values are reported with reference to TMS and the coupling constants are given in Hz. Optical rotations were measured as solutions in benzene on a Perkin Elmer 341 polarimeter at 589 nm and 20 °C.

3.2. Hydrodistillation and isolation procedure

Cleaned and pulverized plant material was homogenized and hydrodistilled in a Clevenger type apparatus for 2.5 hours, and a slightly greenish oil was collected in HPLC grade hexane. The oil was analysed on a HRGC 5300 MEGA series GC with FID detectors, equipped with double columns: a 25 m/0.25 mm i.d. non-polar CPSil-5-CB and a more polar CPSil-19-CB column of identical dimensions. The oven temperature was programmed from 50°C to 230 °C at a rate of 3 °C/min. The injector and detector temperatures were kept at 200°C and 250°C, respectively. The oil was then further analysed using GC/MS with a HP 5890 GC coupled to a VG Analytical 70-250S mass spectrometer with electron impact (70 eV) ionisation. Retention indices and mass spectra of the components were compared with library spectra generated under identical experimental conditions (Joulain and König, 1998; Hochmuth et al., 2003). Unknown components were marked and their mass spectra and retention properties noted. Subsequently, the oil was fractionated on a modified Varian 1400 preparative gas

chromatograph, equipped with a stainless steel column (1.85 m x 4.3 mm) packed with 10% polydimethylsiloxane SE 30 on Chromosorb W-HP (Chrompack). Compounds **1**, **2** and **7** were isolated from their respective fractions using the same preparative GC equipped with a column (1.95 m x 5.3 mm) packed with 6.5% 6-O-TBDMS-2,3-di-OMe-β-cyclodextrin in SE-52 (1:1, w/w) on chromosorb W-HP (Chrompack). All the other compounds were isolated on an Agilent 6890N Network preparative GC System equipped with a DB-1 column (30 m, 0.53 mm I.D and 5 µm film thickness) coupled to a preparative fraction collector (Gerstel, Germany).

*3.3. Viscida-4,9,14-triene (**1**)*

(1-(1,5-Dimethyl hex-4-enyl)-4,8-dimethyl spiro[4.5]deca-1,7-diene)

Colourless oil, $RI_{CPSil-5}$ = 1862, sense of optical rotation (benzene): (-); MS (EI, 70 eV), m/z (rel. inten. %): 272 [M^+] (12), 229 (3), 215 (2), 204 (21), 190 (40), 175 (4), 161 (66), 147 (56), 134 (15), 122 (100), 107 (72), 91 (35), 79 (24), 67 (26), 55 (32), 41 (65). ^{1}H- and ^{13}C-NMR data, see Table 1.

*3.4. Viscida-4,11(18),14-triene (**2**)*

(1,8-Dimethyl-4-(5-methyl-1-methylene hex-4-enyl) spiro[4.5]dec-7-ene)

Colourless oil, $RI_{CPSil-5}$ = 1911, sense of optical rotation (benzene): (-) MS (EI, 70 eV), *m/z* (rel. inten. %): 272 [M^+] (21), 257 (4), 243 (1), 229 (5), 215 (5), 203 (14), 187 (16), 175 (4), 161 (31), 147 (18), 132 (34), 119 (100), 105 (44), 93 (81), 81 (40), 69 (92), 55 (27), 41 (40). ^{1}H- and ^{13}C-NMR (see Table 1).

*3.5. Bisabola-2,6,11-triene (**3**)*

(4-(1,5-Dimethyl hex-5-enylidene)-1-methyl cyclohexene)

Colourless oil, $RI_{CPSil-5}$ = 1525: MS (EI, 70 eV), *m/z* (% rel. inten.): 204 [M^+] (26), 189 (5), 171 (4), 161 (9), 148 (20), 133 (35), 119 (89), 105 (50), 93 (100), 79 (45), 69 (20), 55 (42), 41 (74). ^{1}H- and ^{13}C-NMR (see Table 1).

*3.6. Bisabola-1,3,5,7(14),11-pentaene (**4**)*

(2-Methyl-6-(4-methylphenyl)-1,6-heptadiene)

Colourless oil, $RI_{CPSil-5}$ = 1509: MS (EI, 70 eV), m/z (% rel. inten.): 200 [M^+] (11), 185 (3), 172 (8), 157 (6), 145 (6), 132 (100), 117 (22), 105 (8), 91 (13), 65 (5), 41 (11). ^{1}H- and ^{13}C-NMR (see Table 2).

*3.7. Bisabola-1,3,5,7(14),10-pentaene (**5**)*

(2-Methyl-6-(4-methylphenyl)-1,5-heptadiene)

Colourless oil, $RI_{CPSil-5}$ = 1576, MS (EI, 70 eV), m/z (% rel. inten.): 200 [M^+] (8), 185 (5), 171 (3), 161 (5), 145 (100), 130 (12), 115 (9), 105 (13), 91 (7). ^{1}H- and ^{13}C-NMR (Table 2).

*3.8. 6,7-Epoxybisabola -2,11-diene (**6**)*

(2,6-Dimethyl-2-(4-methylpent-4-enyl)-1-oxaspiro[2.5]oct-5-ene)

Colourless oil, $RI_{CPSil-5}$ = 1142, sense of optical rotation (benzene): (-); MS (EI, 70 eV), m/z (% rel. inten.): 205 (1), 152 (4), 137 (3), 110 (33), 95 (100), 79 (59), 68 (17), 55 (31), 41 (35). ^{1}H- and ^{13}C-NMR (see Table 1).

*3.9. 1-Methoxy-4-(2-methylpropenyl)-benzene (**7**)*

Colourless oil, $RI_{CPSil-5}$ = 1325, MS (EI, 70 eV), m/z (% rel. inten.): 162 [M^+] (100), 147 (73), 131 (13), 121 (18), 115 (16), 103 (14), 91 (39), 77 (21), 65 (10), 51 (10), 41 (14). ^{1}H- and ^{13}C-NMR (see Table 2).

*3.10. Bisabola-1,3,5,7(14),10-pentaene (**8**)*

(2-Methyl-6-(4-methylphenyl)-2,6-heptadiene)

Colourless oil, $RI_{CPSil-5}$ = 1517, MS (EI, 70 eV), m/z (% rel. inten.): 200 [M^+] (2), 185 (2), 157 (100), 142 (4), 132 (10), 115 (12), 91 (11), 69 (67), 41 (52). ^{1}H-NMR: (500 MHz, C_6D_6): 1.46 (3H, *s*, H_3-12), 1.65 (3H, *s*, H_3-13), 2.12 (3H, *s*, H_3-15), 2.19 – 2.24 (2H, *q*, *J* = 8, H_2-9), 2.52 – 2.54 (2H, *t*, *J* = 7, H_2-8), 5.06 (1H, *s*, H_a-14), 5.20 – 5.23 (1H, *t*, *J* = 7, H-10), 5.34 (1H, *s*, H_b-14), 7.00 (2H, *d*, *J* = 8, H-1, H-5), 7.32 (2H, *d*, *J* = 8, H-2, H-4).

*3.11. ar-Tenuifolene (**9**)*

Colourless oil, $RI_{CPSil-5}$ = 1531, MS (EI, 70 eV), m/z (% rel. inten.): 200 [M^+] (13), 185 (1), 171 (1), 157 (2), 143 (2), 132 (100), 117 (11), 105 (6), 91 (8), 39 (5). ^{1}H-NMR: (500 MHz, C_6D_6): 1.22 (3H, *s*, H_3-14), 1.56 – 1.60 (1H, *m*), 1.67 (3H, *s*, H_3-12), 1.77-1.85 (2H, *m*), 1.88 – 1.98 (2H, *m*), 2.17 (3H, *s*, H_3-15), 2.25 – 2.33 (1H, *m*), 5.34 (1H, *bs*, H-10), 7.05 (2H, *d*, *J* = 8, H-1, H-5), 7.19 (2H, *d*, *J* = 8, H-2, H-4).

*3.12. α-Helmiscapene (**10**)*

Colourless oil, $RI_{CPSil-5}$ = 1451, sense of optical rotation (benzene): (-); MS (EI, 70 eV), m/z (rel. inten. %):204 [M^+] (54), 189 (100), 175 (13), 161 (46), 147 (27), 133 (38), 121 (40),

107 (96), 93 (84), 81 (49), 67 (32), 55 (41), 41 (61). ^{1}H-NMR: (500 MHz, C_6D_6): 0.77–0.81 (1H, *m*, H_a-1), 0.93 (3H, *s*, H_3-14), 1.10–1.18 (1H, *m*, H_a-6), 1.27–1.53 (5H, *m*, H_2-8, H_2-9, H-5), 1.62–1.69 (1H, *m*, H_a-2), 1.65 (3H, *s*, H_3-15), 1.66 (3H, *s*, H_3-13), 1.78–1.86 (2H, *m*, H_a-1, H-7), 1.91–1.98 (2H, *m*, H_b-2, H_b-6), 4.79 (1H, *s*, H_a-12), 4.82 (1H, s, H_b-12), 5.27 (1H, *s*, H-3). ^{13}C-NMR: (400 MHz, C_6D_6): 21.2 (C-13), 23.1 (C-15), 23.7 (C-2), 26.7 (C-1), 27.4 (C-14), 27.8 (C-9), 29.3 (C-10), 35.3 (C-6), 41.4 (C-8), 46.3 (C-7), 48.8 (C-5), 109.0 (C-12), 119.8 (C-3), 137.1 (C-4), 150.7 (C-11).

*3.13. **b**-Helmiscapene (**11**)*

Colourless oil, $RI_{CPSil-5}$ = 1446, sense of optical rotation (benzene): (-); MS (EI, 70 eV), m/z (% rel. inten.): 204 [M^+] (12), 189 (73), 176 (62), 161 (33), 147 (100), 133 (46), 121 (40), 107 (57), 93 (71), 79 (61), 67 (44), 55 (38), 41 (52). ^{1}H-NMR: (500 MHz, C_6D_6): 0.77–0.78 (1H, *m*, H_a-3), 0.92 (3H, *s*, H_3-14), 1.13–1.23 (1H, *m*, H_a-1), 1.37–1.57 (6H, *m*, H_b-1, H_2-2, H_a-6, H_2-8), 1.62–1.64 (1H, *m*, H_b-1, H_2-2, H_a-6, H_2-8), 1.67 (3H, *s*, H_3-14), 1.72–1.94 (3H, *m*, H-5, H-7, H_a-9), 1.97–2.12 (2H, *m*, H_b-3, H_b-9), 4.74 (2H, *bs*, H_2-15), 4.81 (1H, *s*, H_a-12), 4.83 (1H, *s*, H_b-12). ^{13}C-NMR: (400 MHz, C_6D_6): 21.3 (C-13), 23.5 (C-2), 27.4 (C-8), 28.2 (C-14), 29.9 (C-9), 30.5 (C-3), 33.9 (C-10), 34.2 (C-6), 41.0 (C-1), 45.8 (C-7), 52.6 (C-5), 108.6 (C-15), 108.7 (C-14), 150.3 (C-4, C-11).

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Paper-IV

Secondary Metabolites from *Peucedanum tauricum* Fruits[6]

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Abstract

From the essential oil of fruits of *Peucedanum tauricum* Bieb., two new guaiane type sesquiterpene hydrocarbons guaia-1(10),11-diene (**1**) and guaia-9,11-diene (**2**) were identified. The structures of **1** and **2** were assigned by 1D- and 2D-NMR analysis. The relative configurations of the new compounds were established by 2D-NOESY experiments while the absolute configurations were deduced through chemical correlations with (+)-γ-gurjunene (**9**) and capillary GC analysis using modified cyclodextrins as the stationary phases. From the less volatile dichloromethane extract of the fruits, coumarins, *viz*. peucedanin (**3**), oxypeucedanin hydrate (**4**) and officinalin isobutyrate (**5**) were isolated. Compound **5** was confirmed to be 6-carbomethoxy-7-isobutyroxycoumarin by its 1D- and 2D-NMR data as well as by conversion into officinalin (**7**) by alkaline hydrolysis. Peuruthenicin, a positional isomer of officinalin, is assigned structure **8**. Bergapten (**6**) was identified by its mass spectrum. This is the first report on the isolation of compounds **4** and **5** from *P. tauricum*.

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[6]Submitted and currently under review.

Key words: *Peucedanum tauricum* Bieb.; Apiaceae; Essential oil; Guaia-1(10),11-diene; Guaia-9,11-diene; Coumarins; Peucedanin; Officinalin isobutyrate; Oxypeucedanin; Peuruthenicin; Bergapten.

1. Introduction

Peucedanum tauricum Bieb. is an endemic perennial plant of the *Apiaceae* family, growing in nature at dry hillsides and pinewoods in Crimea, Caucasus, and in Romania (Tutin et al., 1968, Grosgiejm, 1967, Sãvulescu, 1958, Sziszkin, 1955). Previous chemical studies of the plant concerned the identification of phenolic acids in the foliage and fruits (Bartnik et al., 2003), GC/MS analysis of the essential oil of the fruits in which a number of sesquiterpene hydrocarbons were identified (Bartnik et al., 2002), isolation of coumarins from the fruits (Glowniak et al., 2002), isolation of an analog of chlorogenic acid and a chromone from the roots (Baranauskaite, 1970), determination of saponins in roots and fruits (Baranauskaite, 1968) as well as isolation of peucedanin from a combined extract of *P. tauricum* and *P. calcareum* (Baranauskaite et al., 1965). Here we report the isolation and structural elucidation of two new guaiane type sesquiterpene hydrocarbons from the essential oil of the fruits of the title plant and isolation of coumarins from the high boiling dichloromethane fraction. In addition, revision of the structures of officinalin isobutyrate, officinalin as well as peuruthenicin is reported.

2. Results and Discussion

The essential oil obtained from the fruits of *P. tauricum* was analysed by coupled gas chromatography and mass spectrometry (GC/MS). Mass spectra and retention indices of the oil constituents were compared with a library of mass spectra of authentic compounds established under identical experimental conditions (Joulain and König, 1998; Hochmuth et al., 2003). Monoterpene hydrocarbons consisting of tricyclene, myrcene, limonene, (Z)-β-ocimene, 2,4(8)-p-menthadiene, the oxygenated monoterpene linalool and sesquiterpene hydrocarbons comprising α-ylangene, α-copaen, β-bourbonene, guaia-6,9-diene, selina-5,11-diene, valerena-4,7(11)-diene, γ-amorphene, γ-humulene, α-bulnesene, β-elemene, (E)-β-caryophyllene, α-guaiene, α-humulene, and γ-gurjunene were identified. The latter 5 sesquiterpenes have been earlier identified along with α- and β-selinene and γ-cadinene (Bartnik et al., 2002). Two unknown components were isolated by preparative gas chromatography. Their structures were elucidated by 1D and 2D NMR techniques to be guaia-1(10),11-diene (**1**) and guaia-9,11-diene (**2**), (Fig. 1). The relative configurations of the

new compounds were established through 2D NOESY experiments. Their absolute configurations were assigned according to chemical correlations and capillary GC analysis using modified cyclodextrins as stationary phases (König, 1992, König et al., 1999).

In addition, from the less volatile dichloromethane fraction, known coumarins, peucedanin (**3**), oxypeucedanin hydrate (**4**), and officinalin isobutyrate (**5**) were isolated. (Fig. 1). Their structures were established by MS, 1D and 2D NMR data as well as comparison with literature data (Perel'son et al., 1971, Gonzales et al., 1976, 1978, Patra et al, 1981). In addition, the structure of compound **5** was confirmed according to its 1D and 2D NMR data as well as by converting it into officinalin (**7**) by alkaline hydrolysis, thereby clearing the confusion concerning its structure. The earlier reported structure of peuruthenicin (Soine et al., 1973), an isomer of officinalin, is amended to be **8**. Bergapten (**6**) previously reported from the fruits (Glowniak et al., 2002) was identified by its mass spectrum. This is the first report on the isolation of officinalin isobutyrate and oxypeucedanin hydrate from *P. tauricum*.

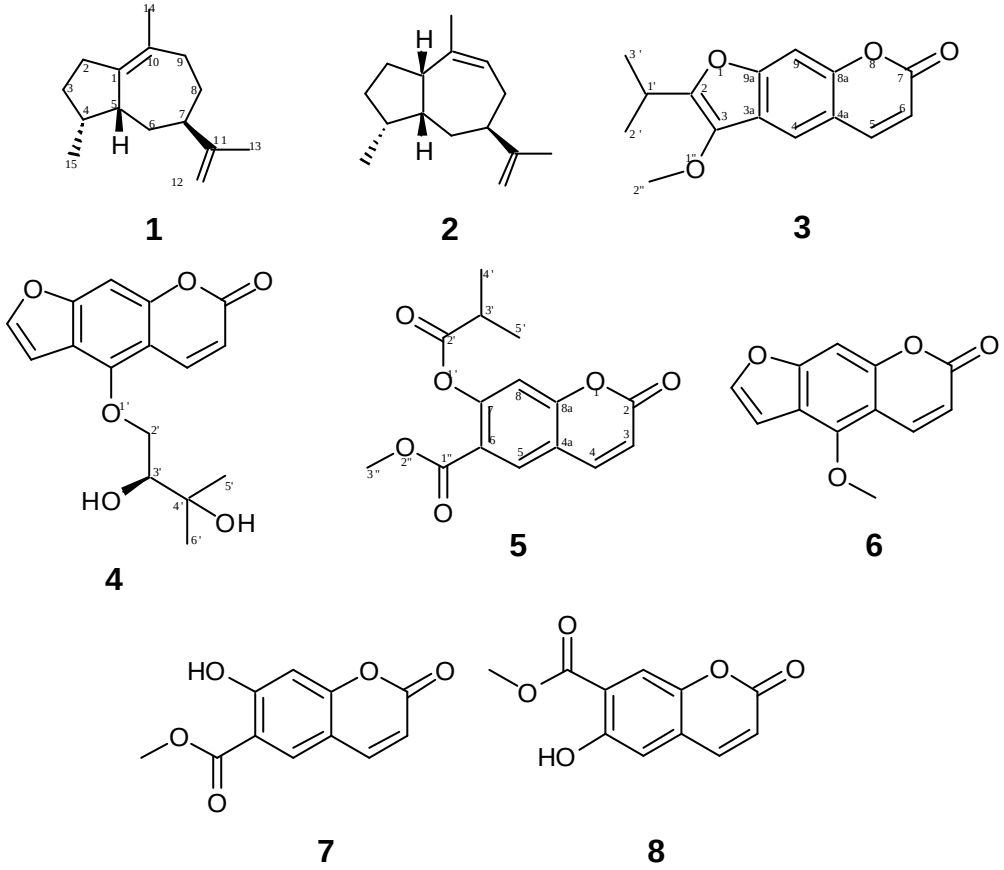

Figure 1. Selected compounds from *P. tauricum.*

*2.1. Guaia-1(10),11-diene(**1**)*

The mass spectrum of **1** exhibited a molecular ion signal at m/z 204 indicating an elemental composition of $C_{15}H_{24}$, typical for a sesquiterpene hydrocarbon with four degrees of unsaturations. The ^1H NMR spectrum and 2D HMQC experiment indicated six methylene groups, three methine and three methyl groups confirming the presence of twenty four protons directly attached to carbon atoms. Signals at δ 1.61 (H_3-14) and 1.71 (H_3-13) of two of the methyl groups were indicative of an allylic position while the signal at δ 4.84 (H_2-12) was indicative of an exocyclic methylene group. The ^{13}C NMR spectrum showed signals of a total of 15 carbon atoms comprising three primary, six secondary (including one exocyclic olefinic), three tertiary and three olefinic quaternary carbon atoms. Therefore, two of the four unsaturations were due to double bonds, suggesting a structure of a doubly unsaturated bicyclic sesquiterpene hydrocarbon for **1**. In the $^1H,^1H$ COSY spectrum of **1** (Table 1), the coupling between the protons of the allylic methyl singlet at δ1.71 (H_3-13) and the exocyclic olefinic methylene protons at δ 4.84 (H_2-12) indicated the presence of an isopropenyl group. This was further substantiated by an HMBC experiment (Table 1), which exhibited couplings between protons of the allylic methyl singlet at δ 1.71 (H_3-13) with both the exocyclic olefinic methylene carbon at δ 108.92 (C-12) and an olefinic quaternary carbon at δ 150.32 (C-11). The presence of a methyl substituted five membered ring substructure was concluded from couplings observed in the $^1H,^1H$ COSY spectrum between each of the two methylene protons centred at δ 2.15 (H_a-2) and 2.32 (H_b-2) and methylene protons centred at δ 1.36 (H_a-3) and 1.58 (H_b-3). The latter was also coupled to a methine multiplet at δ1.99 (H-4) that in turn coupled to the secondary methyl doublet at δ 0.77 (H_3-15) and to a methine proton at δ 2.73 (H-5). These observations and examination of the remaining signals in the 2D $^1H,^1H$ COSY and HMBC spectra indicated a guaiane skeleton with double bonds between C_1 and C_{10} as well as between C_{11} and C_{12} as depicted in Fig.1.

The relative configuration of **1** was determined by a 2D NOESY experiment. Correlations between H-4/H-5 indicated that the two methine protons were oriented in the same direction. Moreover, the absence of NOESY correlations between the methine protons H-5 and H-7 suggested a trans configuration. Considering the bulky isopropenyl group to keep a *pseudo* equatorial position and being β-oriented, H-7 has to be α (axial orientation). Consequently, H-5 and H-4 have to be β-oriented.

Table 1. Observed ^{1}H,^{1}H COSY and HMBC Correlations for compound **1**

^{1}H-^{1}H COSY		HMBC	
Hydrogen	Correlated with	Carbon	Correlated with
H_2-2	H_2-3	C-1	H_2-3, H-5, H-4, H_3-14
H_2-3	H_2-2, H-4	C-2	H_2-3, H-4
H-4	H_3-15, H_2-3, H-5	C-3	H_3-15, H-4
H-5	H_2-6, H-4	C-4	H_3-15, H_2-3, H_2-6
H_2-6	H-5, H-7	C-5	H_3-15, H_2-3, H-4, H-7
H-7	H_2-6, H_2-8, H_2-12 (^3J)	C-6	H_2-8, H-7
H_2-8	H_2-9	C-7	H_2-12, H_2-8, H_3-13
H_3-13	H_2-12 (^3J)	C-8	H_2-6, H-7
		C-9	H_3-14, H_2-8
		C-10	H_3-14, H_2-8, H_2-9
		C-11	H_2-12, H-7, H_3-13
		C-12	H_3-13, H-7
		C-15	H_2-3, H-4

*2.2. Guaia-9,11-diene (**2**)*

Compound **2** exhibited similar spectral properties to **1** except that in the ^{13}C-NMR spectrum instead of the olefinic quaternary resonance at δ127.6 a methine resonance was displayed at δ49.8 and instead of one of the ring methylene resonances at δ33.8 an olefinic methine resonance at δ122.0 appeared. Analysis of ^{1}H,^{1}H COSY and HMBC NMR data (Table 2) revealed compound **2** to show the position of the ring double bond between C-9 and C-10. The depicted relative stereochemistry in **2** was established through a 2D NOESY experiment. Key NOESY correlations were seen between H-5/H-1, H-5/H-4 which led to the conclusion that these methine protons were on the same side of the ring. In addition, the fact that a NOESY correlation was exhibited between H-5/H-1 indicated that the ring was *cis*-fused.

Table 2. Observed $^1H,^1H$ COSY and HMBC Correlations in compound **2**

$^1H,^1H$ COSY		HMBC	
Hydrogen	Correlated with	Carbon	Correlated with
H-1	H_2-2	C-1	H_2-3, H_2-2, H_3-14, H-5
H_2-2	H_2-3	C-2	H_2-3, H-5
H_2-3	H_2-2, H-4	C-3	H_3-15, H_2-2, H-5
H-4	H_3-15, H_2-3, H-5	C-4	H_2-3, H_2-2, H-5
H-5	H_2-6, H-4, H-1	C-5	H_3-15, H_2-3, H-7
H_2-6	H-5, H-7	C-6	H_2-8, H-5,
H-7	H_2-6, H_2-8, H_2-12 (^3J)	C-7	H_2-6, H_3-13, H_2-8, H-5
H_2-8	H-9	C-8	H_2-6, H-7
H_3-13	H_2-12 (^3J)	C-9	H_3-14
		C-10	H_3-14
		C-11	H_2-6, H_3-13, H-7
		C-12	H_3-13, H-7
		C-15	H_2-3, H-5

*2.3. Absolute Configurations of **1** and **2***

In order to determine the absolute configurations of the new guaiane sesquiterpenes, an authentic reference substance, (+)-γ-gurjunene (**9**) (Sigma-Aldrich)**,** showing a guaiane skeleton of known absolute configuration, was hydrogenated. Each of the two new compounds was also hydrogenated separately. The hydrogenation products of **9** and the hydrogenation products of **1** and **2** were analysed by capillary GC using modified cyclodextrins as stationary phases which separated the diastereomeric products formed upon hydrogenation (König, 1992, König et al., 1999). At least one of the hydrogenation products of **1**, **2** and **9** (compound **10**, in Fig. 2) should exhibit identical retention times, provided that the corresponding chiral centres of compounds **1** and **2** show the same absolute configuration as in compound **9**. This was found to be the case on both 2,6-di-OMe-3-O-pentyl-γ-cyclodextrin and 6-O-TBDMS-2,3-di-OMe-β-cyclodextrin. As a result, the absolute

configuration of **1** is (4R,5R,7R)-guaia-1(10),11-diene while **2** is (1S,4R,5R,7R)-guaia-9,11-diene.

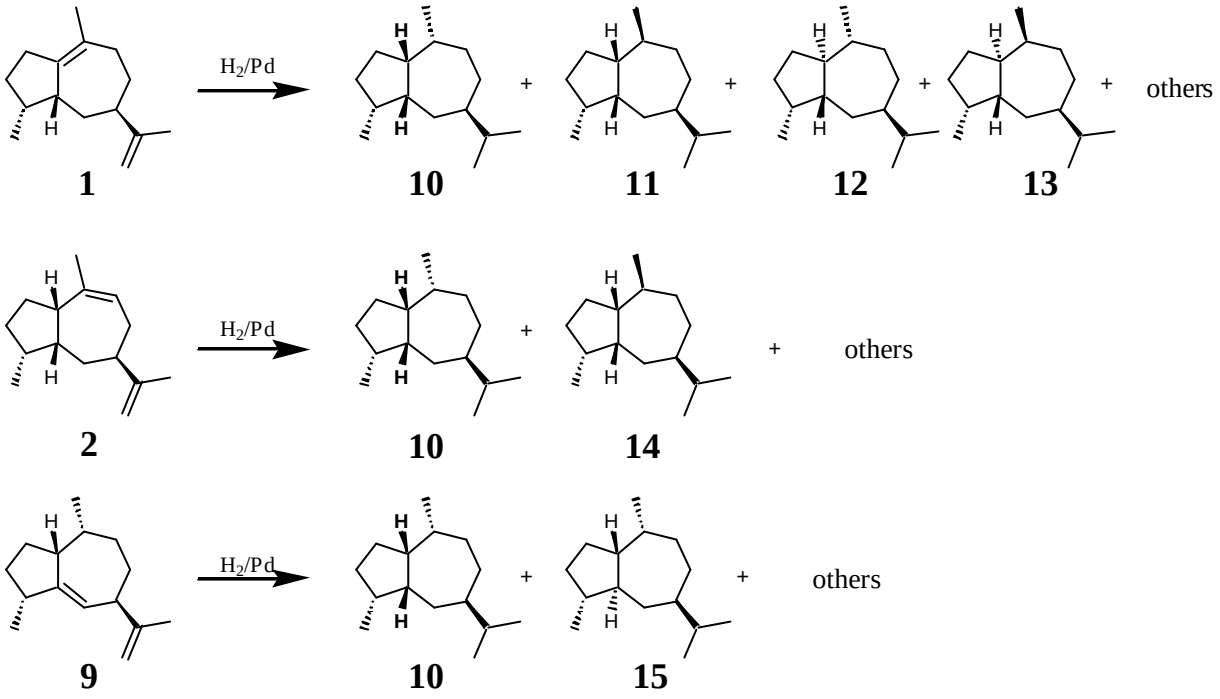

Figure 2 : Hydrogenation products of compounds 1, 2 and 9.

*2.4. Officinalin isobutyrate (**5**)*

There is some confusion in the literature concerning the structures of the coumarins peuruthenicin (Soine et al., 1973) and officinalin (Gonzalez et al. 1976) as well as their corresponding acetates and isobutyrates. Officinalin isolated from *P. officinale* was reported to be 6-carbomethoxy-7-hydroxycoumarin (**7**) on the basis of its spectral data (Gonzalez et al. 1976). Peuruthenicin isolated from *P. ruthenicum* was independently assigned the same structure as officinalin on the basis of its spectral data as well as on chemical evidence (Soine et al., 1973). Soon, it was noted (Ahluwalia et al., 1976) that the two natural products that show the same molecular formula and very similar patterns of ^{1}H-NMR spectra, exhibit different m.ps., 197–198 °C for the former and 162–164 °C for the latter. The observed discrepancy led to the re-evaluation of the structures of peuruthenicin as well as its acetate and isobutyrate by comparison of mainly m.ps. with synthetic samples. As a result, the proposed structure of peuruthenicin was concluded to be correct while that of officinalin, its acetate, and isobutyrate were reported to be untenable and had to be revised (Ahluwalia et al., 1976). The confusion essentially concerns the positions of the carbomethoxy group as well as the hydroxyl group: the question is which of the two substituents is at position 6 and which

one is at position 7 of the coumarin nucleus. This could be clarified by the 2D-HMBC experiments described below.

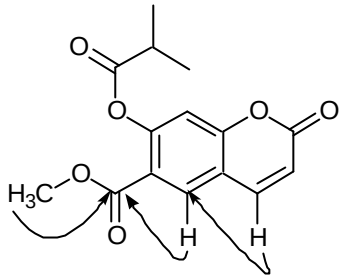

Figure 3. Key HMBC correlations showing the position of the carbomethoxy group in compound **5**

Sample of compound **5** obtained as white crystals exhibited a molecular ion peak at *m/z* 290. NMR spectral data revealed the presence of 15 carbon atoms indicating an elemental composition of $C_{15}H_{14}O_6$. In addition, it was observed that its ^{1}H-NMR data as well as the m.p. (see experimental) were in good agreement with data reported for officinalin isobutyrate (Gonzalez et al., 1976). In the HMBC spectrum of **5**, both the methoxy proton signal at δ 3.9 (3H, *s*) as well as the aromatic methine signal at δ 8.18 (1H, *s*, H-5) correlated strongly to the carbomethoxycarbonyl carbon at δ 164.3 (Fig. 3). In addition, one of the AB system methine doublets at δ 7.71 (H-4, 1H, *d*, *J* = 10) was correlated to C-5 (δ 131.9). This observation clearly showed the carbomethoxy substituent to be linked to C_6 leading to 6-carbomethoxy-7-isobutyroxycoumarin as the structure of **5**, exactly the same as that reported for officinalin isobutyrate (Gonzalez et al., 1976). Furthermore **5** could be hydrolysed to yield **7,** that still kept the carbomethoxy moiety. The ^{1}H-NMR of **7** was recorded (see experimental) and was found to be in agreement with that of officinalin (Gonzalez et al., 1976) rather than that of peuruthenicin (Soine et al., 1973). While this evidence confirms the structures of officinalin isobutyrate as well as officinalin, it also shows that the structure of peuruthenicin (Soine et al., 1973) has to be 7-carbomethoxy-6-hydroxycoumarin (**8**).

3. Experimental

3.1. General

The plant material used in this study was collected in September 2002 and 2003 in the Botanical Garden of Maria Curie-Skodowska University (UMCS) in Lublin, Poland. The identity of plant material was established by K. Dbrowska, a botanical specialist from UMCS. Voucher specimen are deposited in the herbarium of the Medicinal Plant Laboratory,

Department of Pharmacognosy (Skubiszewski Medical University in Lublin). The NMR spectra were recorded on Bruker WM 400 or 500 MHz spectrometers in either deuterated benzene (C_6D_6) or deuterated chloroform ($CDCl_3$). The chemical shift values are reported with reference to TMS, and the coupling constants are given in Hz. Optical rotations were measured as solutions in benzene on a Perkin Elmer 341 polarimeter at 589 nm and 20 °C.

3.2. Hydrodistillation, extraction and isolation procedure

Cleaned, air dried, and pulverized fruits of *P. tauricum* were powdered under liquid nitrogen, homogenized and hydrodistilled in a Clevenger type apparatus for 2.5 hours (Peyron, 1992), and a slightly greenish oil was collected in HPLC grade hexane. The oil was preliminarily analysed on an Orion capillary GC with FID detector, containing 2 columns, a 25 m x 0.25 mm i.d. non-polar CPSil-5 and a more polar CPSil-19 (chrompack) column of identical dimensions. The oven temperature was programmed from 50°C to 230°C at a rate of 3°C/min. The injector and detector temperatures were kept at 200°C and 250°C respectively. The oil was then further analysed using GC/MS on a HP 5890 GC coupled to a VG Analytical 70-250S mass spectrometer with electron impact (70 eV) ionization. The crude oil was fractionated on a modified Varian 1400 preparative gas chromatograph, equipped with a stainless steel column (1.85m x 4.3mm) packed with Chromosorb W-HP coated with 10% polydimethylsiloxane SE 30. Subsequently, **1** and **2** were isolated from the fifth fraction using the same preparative GC equipped with a column (2 m x 5.3 mm) packed with chromosorb G-HD coated with 2.5% 2,6-OMe-3-O-pentyl-γ-cyclodextrin in OV 1701 (1:1, w/w).

The aqueous portion of the residue from the hydrodistillation was separated from the solid through filtration and was extracted with dichloromethane in order to obtain the high boiling fractions. The dichloromethane was removed under vacuum to give a dark oily residue. Part of the extract was subjected to column chromatography on a silica gel column using dichloromethane and a methanol gradient. Fractionation was monitored by thin layer chromatography and UV detection. Fractions with similar compositions were combined. Further separations were carried out by HPLC (MERK-HITACHI) using a RP-18 column (Prodigy 5u ODS(3), 250 x 4.6 mm, Phenomenex) and acetonitrile as the mobile phase at a flow of 1 ml/min.

3.3. Hydrogenation

Hydrogenation of sesquiterpene hydrocarbons was performed by bubbling hydrogen gas through stirred solutions of ca. 0.5 mg samples in 1 ml hexane and 0.25 mg Pd/C at room temperature for 1 h. The reaction mixture was filtered, and the products were analysed by GC and GC/MS.

3.4. Hydrolysis of officinalin isobutyrate

Officinalin isobutyrate (ca. 1 mg) was stirred overnight with 20 % aqueous sodium hydroxide solution (5 ml). The solution was diluted to 20 ml with water, acidified, and extracted with chloroform. The chloroform was removed under *vacuo*, and the conversion was confirmed by MS and NMR.

*3.5. Guaia-1(10),11-diene (**1**)*

*(5**b**-Isopropenyl-3**a**,8-dimethyl-1,2,3,3a**b**,4,5,6,7-octahydroazulene)*

$C_{15}H_{24}$, Colourless oil, RI_{CPSil5} = 1517, sense of optical rotation (benzene): (+) ^{1}H NMR (500 MHz, C_6D_6): δ 0.77 (3H, *d*, *J* = 7.0, H_3-15), 1.36 (1H, *m*, H_a-3), 1.58 (1H, *m*, H_b-3), 1.61 (3H, *s*, H_3-14), 1.68 (1H, *m*, H_a-8), 1.71 (3H, *s*, H_3-13), 1.74 (2H, *m*, H_2-6), 1.88 (1H, *m*, H_b-8), 1.99 (1H, *m*, H-4), 2.14 (2H, *m*, H_2-9), 2.15 (1H, *m*, H_a-2), 2.28 (1H, *m*, H-7), 2.32 (1H, *m*, H_b-2), 2.73 (1H, *m*, H-5), 4.84 (2H, *2s*, H_2-12); ^{13}C NMR (400 MHz, C_6D_6): δ 14.5 (C-15), 22.1 (C-13), 22.2 (C-14), 29.8 (C-8), 30.1 (C-2), 32.1 (C-6), 32.3 (C-3), 33.8 (C-9), 39.1 (C-4), 41.7 (C-5), 43.5 (C-7), 108.9 (C-12), 127.6 (C-10), 139.1 (C-1), 150.3 (C-11); MS (EI, 70 eV), *m/z* (rel. inten.): 204 [M^+] (30), 189 (70), 175 (25), 161 (82), 147 (38), 133 (41), 119 (62), 107 (87), 93 (100), 79 (87), 67 (40), 55 (58), 41 (39).

*3.6. Guaia-9,11-diene (**2**)*

*(5**b**-Isopropenyl-3**a**,8-dimethyl-1,2,3,3a**b**,4,5,6,8a**b**-octahydroazulene)*

$C_{15}H_{24}$, Colourless oil, RI_{CPSil5} = 1521, sense of optical rotation (benzene): (+) ^{1}H NMR (500 MHz, C_6D_6): δ 0.88 (3H, *d*, *J* = 7.0, H_3-15), 1.10 (1H, *m*, H_a-3), 1.50 (2H, *m*, H_2-6), 1.52 (1H, *m*, H_a-2), 1.55 (1H, *m*, H_b-3), 1.65 (3H, *s*, H_3-14), 1.68 (3H, *s*, H_3-13), 1.80 (1H, *m*, H_b-2), 1.86 (1H, *m*, H-4), 1.90 (1H, *m*, H_a-8), 2.18 (1H, *m*, H-5), 2.44 (1H, *m*, H-7), 2.45 (1H, *m*, H_b-8), 2.51 (1H, *m*, H-1), 4.83 (2H, *2s*, H_2-12), 5.47 (1H, *m*, H-9); ^{13}C NMR (400 MHz, C_6D_6): δ 16.1 (C-15), 21.5 (C-14), 24.2 (C-13), 25.9 (C-6), 28.9 (C-2), 29.8 (C-8), 30.1 (C-3), 39.2 (C-4), 40.2 (C-5), 42.7 (C-7), 49.8 (C-1), 109.2 (C-12), 122.0 (C-9), 139.0 (C-10), 150.3 (C-11); MS (EI, 70 eV), *m/z* (rel. inten.): 204 [M^+] (30), 189 (70), 175 (25), 161 (42), 147 (38), 133 (41), 119 (62), 107 (87), 93 (100), 81 (87), 67 (40), 55 (58), 41 (39).

*3.7. Peucedanin (**3**)*

$C_{15}H_{14}O_4$, White solid, mp. 108-109 °C; MS (EI, 70 eV), *m/z* (rel. inten.): 258 [M^+] (25), 243 (100), 228 (10), 200 (10), 189 (5), 171 (5), 160 (5), 144 (2), 115 (5), 108 (4), 88 (2), 76

(2), 69 (2), 51 (2), 39 (2). ^{1}H NMR, ($CDCl_3$, 500 MHz): 1.36 (6H, *d*, *J* = 7.0, H_3-2', H_3-3'); 3.25 (1H, *sep*. *J* = 7.0, H-1'); 3.95 (3H, *s*, H3-2"); 6.37 (1H, *d*, *J* =10.0, H-5); 7.33 (1H, *s*, H-9); 7.57 (1H, *s*, H-4); 7.79 (1H, *d*, *J* = 10.0, H-6). ^{13}C NMR, ($CDCl_3$, 400 MHz): 21.6 (C-2', C-3'); 26.8 (C-1'); 62.4 (C-2''); 100.1 (C-9); 114.6 (C-6); 115.0 (C-8a); 117.0 (C-4); 122.2 (C-9a); 136.7, (C-3); 144.2 (C-5);151.9 (C-4a); 152.9 (C-2); 154.3 (C-3a); 161.6 (C-7).

*3.8. Oxypeucedanin hydrate (**4**)*

$C_{16}H_{16}O_6$, ^{1}H NMR, ($CDCl_3$, 500 MHz): δ1.31 (3H, *s*); 1.36 (3H, *s*); 3.91 (1H, *dd*, *J* = 10.0, 3.0, H-3'); 4.45 (1H, *dd*, *J* = 10.0, 3.0, H_a-2'); 4. 54 (1H, *dd*, *J* = 10.0, 3.0, H_b-2'); 6.31 (1H, *d*, *J* = 10.0, H-5); 6.99 (1H, *d*, *J* = 2.5, H-3); 7.20 (1H, *s*, H-9); 7.61 (1H, *d*, *J* = 2.5, H-2); 8.18 (1H, *d*, *J* = 10.0, H-6). ^{13}C NMR, ($CDCl_3$, 400 MHz): **d** 25.6 (C-5'); 27.2(C-6'); 72.1 (C-3'); 74.9 (C-2'); 76.9 (C-4'); 95.4 (C-9); 105.1 (C-3); 107.8 (C-4a); 113.5 (C-3a); 114.7 (C-6) ; 138.6 (C-2); 146.4 (C-5);149.0 (C-8a); 153.0 (C-9a); 161.4 (C-7); 188.5 (C-4).

*3.9. Officinalin isobutyrate (**5**)*

White crystals; mp. 118–120 °C; $C_{15}H_{14}O_6$; MS (EI, 70 eV), *m/z* (rel. inten.): 290 [M^+] (10), 274 (10), 259 (15), 243 (25), 220 (25), 188 (50), 160 (20), 71 (80), 43 (100). ^{1}H NMR, ($CDCl_3$, 500 MHz): 1.37 (6H, *d*, *J* = 7, H_3-4', H_3-5'), 2.90, (1H, *septet J = 7*, H-3'); 3.9 (3H, *s*, H_3-3"); 6.44 (1H, *d*, *J* = 10, H-3); 7.04 (1H, *s*, H-8); 7.71, (1H, *d*, *J* = 10, H-4); 8.18 (1H, *s*, H-5). ^{13}C NMR, ($CDCl_3$, 400 MHz): **d**18.8 (C-4', C-5'); 34.3 (C-3'); 52.5 (C-3''); 112.5 (C-8); 117.3 (C-3); 119.8 (C-4a); 120.4 (C-6); 131.9 (C-5); 142.5 (C-4); 153.7 (C-7); 157.3 (C-8a); 159.9, (C-2); 164.3, (C-1''); 175.3, (C-2').

*3.10. Bergapten (**6**)*

$C_{12}H_8O_4$, MS (EI, 70 eV), *m/z* (rel. inten.): 216 [M^+] (100), 201 (25), 188 (10), 157 (2), 145 (35), 89 (20), 74 (10), 63 (15), 51 (23), 38 (10).

*3.11. Officinalin (**7**)*

$C_{11}H_8O_5$, MS (EI, 70 eV), *m/z* (rel. inten.): 220 [M^+] (60), 188 (100), 160 (48), 132 (18), 104 (12), 76 (15). ^{1}H NMR, ($CDCl_3$, 500 MHz): 3.99 (3H, *s*, -OMe); 6.29 (1H, *d*, *J* = 10, H-3); 6.88 (1H, *s*, H-8); 7.61, (1H, *d*, *J* = 10, H-4); 8.02 (1H, *s*, H-5); 11.20 (1H, *s*, -OH)

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Paper-V

Isoligustilide: A New Phthalide from the Essential oil of *Meum athamanticum*[7]

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ABSTRACT: A new phthalide isoligustilide (3,5 %) was isolated from the essential oil of the aerial parts of *Meum athamanticum* (L.) Jacq together with Z-ligustilide (0.1 %) and sedanonic acid lactone (0.5%) and their structures established by MS and NMR techniques. In addition, a total of 23 components accounting for 93.2 % of the oil could be identified by GC/MS. The major components of the oil were found to be monoterpene hydrocarbons, limonene (33.5 %), α-phellandrene (15.3 %), myrcene (13.4 %) and (E)-β-ocimene (11.6 %).

KEY WORDS: *Meum athamanticum*; Apiaceae; essential oil; Phthalides; 3-but-2-enylidene-4,5,6,7-tetrahydro-3H-isobenzofuran-1-one; *Z*-ligustilide; *Z*-butylidene-4,5,6,7-tetrahydrophthalide.

Introduction

The European *Meum athamanticum* (L.) Jacq., is a strongly aromatic plant which is used for the preparation of a special liquor. Its essential oil has previously been investigated several times. An earlier analysis of the oil from the fruits of a sample from Germany has revealed (*Z*)-3-butylidenephthalide (**1**), butylphthalide (**2**) and (*Z*)-ligustilide (**3**) as the chief constituents[1] while from the root oil the identification of several monoterpenes and the phthalides, in addition to sedanonic acid lactone (**4**) was reported.[2] In a further analysis aimed

[7] Submitted and currently under review.

at the investigation of sesquiterpene hydrocarbons of the oil from the roots, the identification of sesquiterpenoids such as β-bazzanene (**5**), α- and β-barbatene (**6**, **7**) for the first time as constituents of higher plants was reported.[3] From the aerial parts of a sample from Italy, the monoterpene hydrocarbons, (*E*)-β-ocimene (34.9%), p-cymene (12.1%), (*Z*)-β-ocimene (10.2%), Δ-3-carene (6.2%) were reported as major constituents while from the oil of the roots, (*Z*)-ligustilide (36.2%, **3**), (E)-β-ocimene (14.4%), and (Z)-3-butylidene phthalide (6.3%, **1**) were reported as major constituents.[4] Recently, from the oil of the leaves and stems of *M. athamanticum* of Spanish origin, (*E*)-β-ocimene (29.6%), γ-terpinene (17.9%), terpinolene (17.0%) and p-cymene (9.7%) were reported as major components.[5] In this communication, we report on the isolation of a new phthalide, *(3Z),(9E)*-isoligustilide (**8**), from the essential oil of the above ground parts of *M. athamanticum* from Germany.

Results and discussion

The essential oil of *M. athamanticum* was analysed by capillary and preparative GC, GC/MS and NMR techniques. Mass spectra and retention indices on a non-polar stationary phase (CpSil-5) of the components of the oil were compared with library spectra of authentic compounds generated under identical experimental conditions.[6,7] Several mono-, sesquiterpenes and their derivatives could be identified (Table 1). Three unknown phthalides were isolated and their structures established from interpretation of their MS, 1D and 2D NMR data (Fig. 1). These were 3-but-2-enylidene-4,5,6,7-tetrahydro-3H-isobenzofuran-1-one (**8**), the previously reported *Z*-butylidene-4,5,6,7-tetrahydrophthalide (sedanonic acid lactone, **4**) and Z-ligustilide (**6**). **8** is a new natural product.

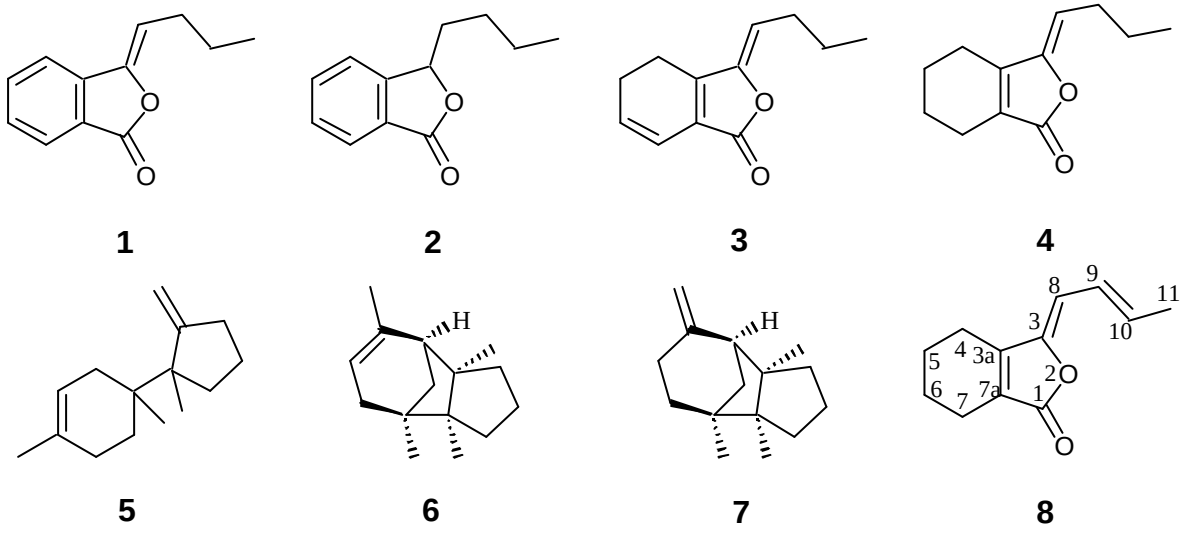

Figure 1. Selected compounds reported from the essential oil of Meum athamanticum (1-7) and the new compound (8).

Table 1. Identified components in the aerial parts of *Meum athamanticum* essential oil

Name	Ret.index[8]	% Composition
α-Thujene	928	0.2
α-Pinene	935	1.3
Camphene	950	0.1
Sabinene	971	0.2
β-Pinene	976	2.5
Myrcene	985	13.4
α-Phellandrene	1003	15.3
Δ-3-Carene	1011	0.1
p-Cymene	1020	1.2
Limonene	1029	33.5
(*Z*)-β-Ocimene	1034	2.3
E-β-Ocimene	1043	11.6
γ-Terpinene	1057	0.9
Terpinolene	1089	0.7
Citronellylformate	1252	0.5
(*E*)-β-Caryophyllene	1422	1.1
α-Humulene	1456	0.1
allo-Aromadendrene	1463	0.4
Germacrene D	1482	2.5
Bicyclogermacrene	1495	0.9
δ-Cadinene	1518	0.1
10-*epi*-Junenol	1579	0.1
α-Cadinol	1644	0.1
(3*Z*)-Butylidene phthalide	1711	0.5
(*Z*)-Ligustilide	1717	0.1
(3*Z*),(9*E*)-Isoligustilide	1804	3.5

[8] Retention Indices on CPSil-5 capillary column

*(3Z),(9E)-Isoligustilide (**8**)*

8 that was present as 3.5 % of the oil was obtained as a waxy substance. The mass spectrum of **8** exhibited a molecular ion at m/z 190 suggesting an elemental composition of $C_{12}H_{14}O_2$. Its ^{1}H- and HSQC-NMR exhibited presence of signals due to four methylenes, three olefinic methines and one allylic methyl, confirming the presence of fourteen protons directly connected to carbon atoms in **8.** The ^{13}C NMR contained signals of a total of twelve carbon atoms which include four aliphatic methylene, three olefinic methine, three olefinic quaternary one of which was oxygenated, a lactone carbonyl and one methyl carbons. These NMR data in combination with the mass spectral data, confirmed the molecular formula of $C_{12}H_{14}O_2$ for **8**, that requires six-degrees of unsaturations. Four of the unsaturations were due to double bonds suggesting a bicyclic structure for the compound.

Table 2. Important HMBC correlations observed in compound **8**.

HMBC	
Carbon	Correlated with
C-3	H-8, H-9
C-3a	H-8, H_2-5
C-4	H_2-7, H_2-6, H_2-5
C-6	H_2-4
C-7	H_2-6, H_2-5, H_2-4
C-7a	H_2-7, H_2-6
C-8	H-10
C-9	H-11
C-10	H-11
C-11	H-10, H-9

In the ^{1}H-^{1}H COSY of **8,** two separate sets of protons were observed. The first set comprised signals due to four methylenes that were coupling among themselves. These couplings were between a methylene multiplet at δ 1.67 (H_2-4) and a two methylene multiplet centred at δ 1.10 (H_2-5, H_2-6). The latter were further coupled to a methylene multiplet centred at δ 1.89 (H_2-7). These four methylenes must be a part of the six-membered ring of the compound. The second set contained signals due to three olefinic methines and a methyl group. Couplings were observed between the olefinic methine doublet at δ 5.28 (1H, *d*, *J = 11*, H-8) and another

olefinic methine signal at δ 6.69 (1H, *ddd, J = 15.0, 11.0, 2.0,* H-9). The latter was further coupled to the third olefinic methine doublet of doublet at δ 5.64 (1H, *dd, J = 15.0, 7.0,* H-10) and this was further coupled to the allylic methyl doublet of doublet at δ 1.56 (1H, *dd, J = 7.0, 1.3,* H_3-11). These second set of protons signals that were due to the side chain of the compound exhibited no correlations in the ^1H-^1H COSY with the first set of protons indicating their separated nature within the structure. Analysis of these NMR data and comparison of the data with phthalides already reported from the plant led to the depicted structure for compound **8**. The HMBC data (Table 2) of **8** further substantiated the proposed structure. The stereochemistry at the double bonds between C-3/C-8 and C-9/C-10 were determined from 2D NOESY spectrum. In the NOESY spectrum of **8**, correlations were observed between H-8/H-4 and H-9/H_3-11 indicating a ***Z*** configuration at C-3/C-8 and an ***E*** configuration at the C-9/C-10 double bonds.

Experimental

Plant material and Isolation of the Essential oil

M. athamanticum sample was collected in July 2002 from Altenau, Harz Mountains, in Germany. The fresh plant material was cleaned, homogenized and hydrodistilled in a Clevenger type apparatus for 2.5 hours and the oil was collected in HPLC grade n-hexane.

GC and GC/MS Analysis

GC analysis was carried out on an Orion Micromat 412 GC equipped with two capillary columns, a 25 m x 0.25 mm polydimethylsiloxane CP-Sil-5-CB and a CP-Sil-19-CB (chrompack) and flame ionisation detectors. The oven temperature was programmed linearly from 50°C to 230°C at a rate of 3 °C/min. The injector and detector temperatures were 200 °C and 230 °C respectively. The carrier gas used was hydrogen at a flow rate of 0.5 ml/ min.. GC/MS measurements were carried out on a Hewlett-Packard HP 5890 gas chromatograph equipped with a 25 m x 0.25 mm polydimethylsiloxane CP-Sil-5-CB (chrompack) capillary column and coupled to a VG Analytical VG 70-250S mass spectrometer with electron impact (70 eV) ionisation. The oven was operating under a linear temperature program from 80 °C to 270 °C at a rate of 10 °C/min. Helium was used as a carrier gas at a flow rate of 0.5 ml/ sec.. The injector, transfer line and ion source temperatures were 220 °C, 230°C and 220°C respectively.

Preparative GC

Preparative GC analysis was carried out on a modified Varian 1400 preparative gas chromatograph, equipped with stainless steel columns, a (1.85 m x 4.3 mm) packed with 10 % polydimethylsiloxane SE 52 on Chromosorb W-HP and a (1.95 m x 5.3 mm) packed with 6.5% 6-O-TBDMS-2,3-di-OMe-β-cyclodextrin in SE-52 (1:1, w/w) on chromosorb W-HP.

NMR spectroscopy

NMR measurements were carried out with a Bruker WM 400 or 500 MHz instrument using TMS as internal standard in deuterated benzene, C_6D_6.

*1. (Z)-Ligustilide (**3**)*

Colour less oil; MS (EI, 70 eV), m/z (%) = 190 ([M^+], 50), 161 (90), 148 (82), 133 (31), 120 (18), 105 (76), 91 (34), 77 (55), 65 (10), 55 (100), 40 (57). ^{1}H-NMR: (500 MHz, C_6D_6): 0.82 (3H, *t, J = 7.6,* H_3-11), 1.27-1.30 (1H, *sep., J = 7.6,* H-10), 1.78-1.82 (4H, *m*, H_2-4, H_2-5), 2.20 (1H, *q, J = 7.6,* H-9), 4.63 (1H, *t, J = 8.0,* H-8), 5.44 (1H, *dt, J = 9.8, 4.1*, H-6), 6.25 (1H, *d, J = 10.0,* H-7). ^{13}C-NMR: (400 MHz, C_6D_6): 14.1 (C-11), 18.5 (C-10), 22.7 (C-4), 22.9 (C-5), 28.6 (C-9), 111.6 (C-8), 117.8 (C-7), 127.9 (C-7a), 129.9 (C-6), 146.7 (C-3), 149.9 (C-3a), 167.1 (C-1).

*2. Sedanonic acid lactone (**4**)*

MS (EI, 70 eV), m/z (%) = 192 ([M^+], 32), 177 (1), 163 (100), 150 (67), 135 (11), 122 (8), 107 (43), 91 (24), 79 (61), 65 (9), 55 (62), 40 (24). ^{1}H-NMR: (500 MHz, C_6D_6): 0.83 (3H, *t, J = 7.6,* H_3-11), 1.10-1.13 (4H, *m,* H_2-5, H_2-6), 1.31-1.33 (1H, *sep., J = 7.6,* H-10), 1.67 (2H, *bs,* H_2-4), 1.97 (2H, *bs,* H_2-7), 2.23 (1H, *q, J = 7.6,* H-9), 4.62 (1H, *t, J = 8.0,* H-8). ^{13}C-NMR: (400 MHz, C_6D_6): 13.9 (C-11), 20.1 (C-4), 23.7 (C-2), 20.4 (C-7), 20.7 (C-5, C-6), 21.4 (C-10), 28.0 (C-9), 108.6 (C-8), 127.4 (C-3a), 149.9 (C-3), 150.1 (C-7a), 169.0 (C-1).

*3.. (3Z),(9E)-Isoligustilide (**8**)*

(3-But-2-enylidene-4,5,6,7-tetrahydro-3H-isobenzofuran-1-one)

MS (EI, 70 eV), m/z (%) = 190 ([M^+], 100), 175 (18), 162 (25), 147 (47), 134 (25), 119 (41), 105 (16), 91 (50), 79 (28), 65 (9), 53 (21), 39 (19). ^{1}H-NMR: (500 MHz, C_6D_6): 1.10 (4H, *m,* H_2-5, H_2-6), 1.56 (3H, *dd, J = 7.0, 1.3,* H_3-11), 1.67 (2H, *m,* H_2-4), 1.89 (2H, *m,* H_2-7), 5.28 (1H, *d, J = 11.0,* H-8), 5.64 (1H, *dd, J = 15.0, 7.0,* H-10), 6.69 (1H, *ddd, J = 15.0, 11.0, 2.0,* H-9). ^{13}C-NMR: (400 MHz, C_6D_6): 17.5 (C-11), 19.2 (C-5), 19.4 (C-6), 20.2 (C-4), 20.4 (C-7), 106.5 (C-8), 124.4 (C-9), 126.4 (C-3a), 132.4 (C-10), 146.4 (C-3), 148.9 (C-7a), 167.4 (C-1).

Acknowledgements

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Paper-VI

Melanene-a new sesquiterpene hydrocarbon with a novel skeleton and other terpenes from the essential oil of the leaves of *Melanoselinum decipiens*[9]

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Abstract-Melanene, a tricyclic sesquiterpene hydrocarbon showing a novel carbon skeleton represented by a trimethyldecahydroacenaphthylene was isolated from the essential oil of the leaves of the umbelliferous shrub *Melanoselinum decipiens* (Schrad. Et Wendl.) Hoffm. (Apiaceae). The structure of melanene was established to be (1*R*,4*E*,7*R*,8*R*,9*R*,12*S*)-1,7,9-trimethyltricyclo[6.3.1.$0^{5,12}$]dodec-4-ene or its antipode. In addition, 45 compounds (mostly monoterpenes and sesquiterpenes) were identified, among which enantiomerically almost pure (1R)-(+)-β-pinene forms the major component (55.1 %).

Keywords: *Melanoselinum decipiens* (Schrad. Et Wendl.) Hoffm.; Apiaceae (Umbelliferae); Essential oil; Melanene; Sesquiterpene; β-Pinene.

1. Introduction

Melanoselinum decipiens is a rare umbelliferous shrub which inhabits rocky slopes in the interior of Madeira.[1,2,3] The essential leaf oil of plants from Madeira was shown to contain β-pinene (53.1%) as the major component among 31 other compounds.[4] According to a second investigation,[5] the essential oils of the aerial parts of the plants, collected during the vegetative phase on Madeira and on the Azores, were composed mainly of monoterpene hydrocarbons (ca. 90 %), β-pinene (72%) and limonene (82%) being the main components of

[9] Submitted.

the Madeiran and Azorean oils, respectively. Other investigations on *M. decipiens* resulted in the isolation of a series of sesquiterpene lactones, called decipienins A-H,[6,7,8] and a number of eudesmanolides.[9,10] In our investigation, we analysed the essential oil obtained from a plant grown in Hamburg, Germany.

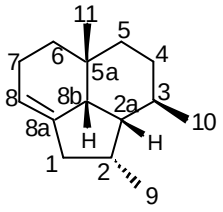
2. Results and Discussion

GC and GC/MS analysis of the essential oil of leaves of *M. decipiens* grown in Hamburg, Germany, resulted in the isolation of the new sesquiterpene hydrocarbon, **1**, possessing a novel carbon skeleton. Compound **1** was isolated by using preparative GC, and its structure was established by extensive NMR analysis. The mass spectrum of **1** (Fig. 1) exhibited a molecular ion peak at m/z 204 consistent with an elemental composition of $C_{15}H_{24}$, typical for a sesquiterpene hydrocarbon with four degrees of unsaturations. This was supported by ^{1}H-NMR and 2D-HMQC experiments which indicated five methylene groups, one olefinic proton, four saturated methine protons, and three methyl groups confirming the presence of 24 protons in **1**. Two of the methyl signals appeared as doublets while the third was a singlet. The ^{13}C-NMR spectrum showed signals of 15 carbon atoms. These were 3 primary, 5 secondary, 5 tertiary (including an olefinic tertiary), and 2 quaternary carbons (including an olefinic quaternary). ^{1}H- and ^{13}C-NMR δ- values are given below. One of the 4 unsaturations was due to a double bond (2 olefinic carbons) indicating a tricyclic structure of **1**. Extensive analysis of 2D-NMR experiments ^{1}H,^{1}H COSY as well as HMBC (Table 1) spectra of **1** revealed the depicted structure. The relative configuration of **1** was determined by a 2D NOESY experiment that exhibited correlations between H_3-15 and H-5, H-5 and H-6, H-6 and H_3-14. The new compound that we like to term melanene, therefore, is (1*R*,4*E*,7*R*,8*R*,9*R*,12*S*)-1,7,9-trimethyltricyclo[6.3.1.0^{5,12}]dodec-4-ene or its antipode. This is the first detection of a hydroacenaphthylene structure as a sesquiterpene skeleton in nature. The presence of a second similar sesquiterpene hydrocarbon was detected by its mass spectrum (Fig.2). The amount was not sufficient for structure elucidation, however, in its ^{1}H-NMR the presence of 3 methyl signals at δ 0.89, 0.97, and 1.04 as well as an olefinic methine proton at δ 5.35 indicated the unknown is no stereoisomer of **1**.

In addition to melanene, several mono- and sesquiterpenes were identified as components of the oil[13] by comparing their retention indices and mass spectra with a library of authentic samples established under identical experimental conditions.[11,12] Similar to that of the Madeiran plants,[4] the oil from the plant grown in Hamburg contains β-pinene (55.1 %) as the main component. By enantioselective GC, this was shown to be represented by

enantiomerically almost pure (1R)-(+)-β-pinene. The oil of the plant grown in Hamburg contains higher amounts of sesquiterpenes (ca. 17 %) than that of the Madeiran plants.[5]

Leaves of *M. decipiens* were collected in Hamburg (Pinneberg), Germany, from a private garden. Extraction of the oil, GC and GC/MS analysis as well as preparative GC analysis was performed the same way as reported earlier.[14] Enantioselective GC was carried out on two Carlo Erba GC series 2150 instruments equipped with two 25 m fused silica capillaries coated with modified cycodextrins: (3'-*O*-acetyl-6-*O*-TBDMS-2,3-di-*O*-methyl-β-cyclodextrin, and 6-*O*-methyl-2,3-*O*-pentyl-γ-cyclodextrin both in 50 % OV 1701) as stationary phases. The injector, oven, and detector temperatures were 200 °C, 50 °C and 250 °C, respectively. NMR measurements were carried out with a Bruker WM 400 and 500 MHz instruments using TMS as internal standard in deuterated benzene, C_6D_6. Optical rotation was determined on a Jasco DIP 370 polarimeter at 589 nm and 20 °C.



1

Table 1. Key ^{1}H,^{1}H COSY and HMBC correlations observed for **1**.

^{1}H,^{1}H COSY		HMBC	
H-atom	Correlated with	H-atom	Correlated with
H_2-1	H-2	H_2-1	C-2, C-9
H-2	H-2a, H_2-1, H_3-9	H_2-1, H_3-9, H_3-10	C-2a
H-2a	H-5, H-7, H-11	H_2-5, H_3-10	C-3
H-3	H-2a, H_2-4, H_3-10	H_2-5	C-4
H_2-4	H-3, H_2-5	H_2-6, H_2-5, H_2-4, H_3-11	C-5a
H_2-6	H_2-7	H_2-6, H_2-1	C-8
H_2-7	H_2-6, H-8	H_2-1	C-8a
H-8	H_2-7	H_2-6, H_2-1	C-8b
H-8b	H-2a		
H_3-9	H-2		
H_3-10	H-3		

Figure 1: 70 eV EI mass spectrum of compound **1** (RI^{10} = 1455).

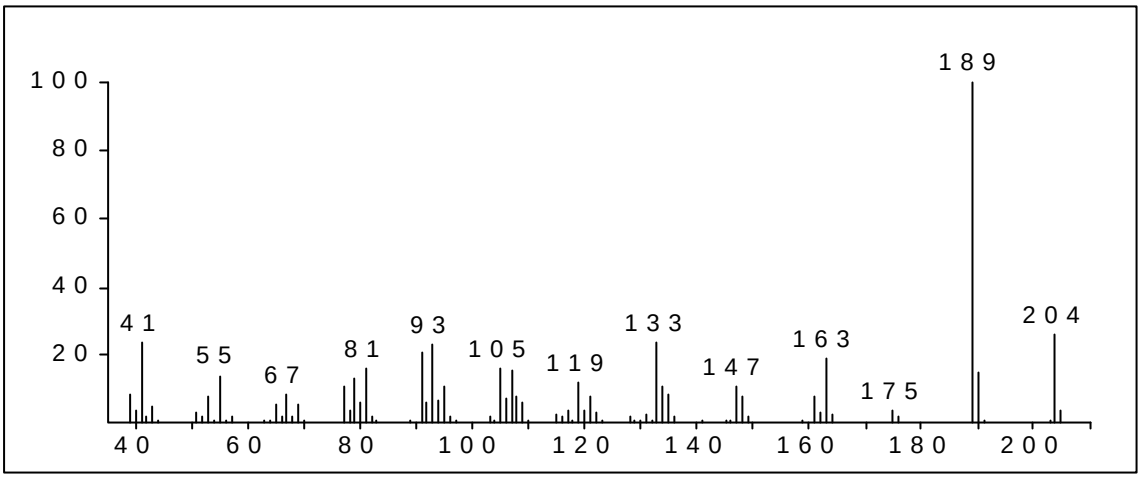
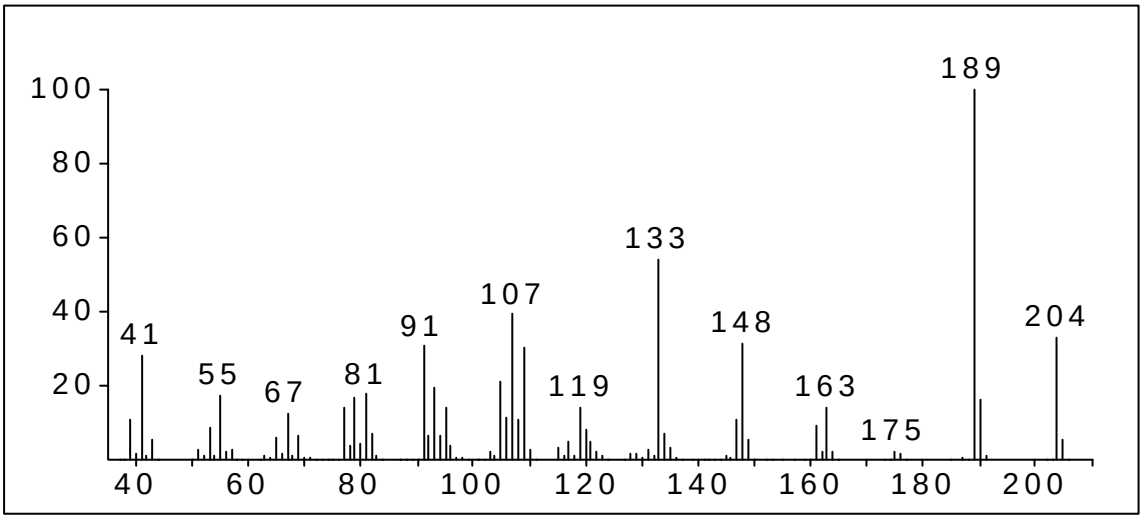


Figure 2: 70 eV EI mass spectrum of the unidentified trace compound (RI = 1397).



2.1 Melanene (2,3,5a-Trimethyl-1,2,2a,3,4,5,5a,6,7,8b-decahydroacenaphthylene = (1R,4E,7R,8R,9R,12S)-1,7,9-trimethyltricyclo[6.3.1.$0^{5,12}$]dodec-4-ene or its antipode)

Colourless oil, sense of optical rotation: (+); ^{1}H-NMR: (500 MHz, C_6D_6): 0.89 (3H, *d*, *J = 7.0*, H_3-10), 0.92 (3H, *d*, *J = 7.0*, H_3-9), 1.05 (3H, *s*, H_3-11), 1.09-1.19 (3H, *m*, H-2, H-2a, H-3), 1.14 (1H, *m*, H_a-4), 1.25 (1H, *d*, *J = 11.0*, H-8b), 1.38 (3H, *m*, H_2-1, H_a-5), 1.62 (1H, *m*, H_b-5), 1.75 (2H, *m*, H_b-4, H_a-1), 1.85 (1H, *m*, H_a-7), 2.07 (1H, *m*, H_b-7), 2.21 (1H, *m*, H_b-1), 5.37 (1H, *bs*, H-8); ^{13}C-NMR: (400 MHz, C_6D_6): 17.4 (C-10), 19.8 (C-9), 23.3 (C-7), 27.2 (C-11), 30.5 (C-4), 35.6 (C-6), 37.2 (C-5a), 41.4 (C-5), 42.2 (C-2), 44.5 (C-1), 44.8 (C-2a), 54.6 (C-3), 56.6 (C-8b), 115.1 (C-8), 140.1 (C-8a).

[10] Retention index on CPSil-5 capillary

Acknowledgements

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13. α-Pinene (1.1%), sabinene (0.1%), **(1R)-(+)-b-pinene (55.1%)**, myrcene (5.0%), limonene (0.8%), linalool (0.3%), *trans*-pinocarveol (0.9%), pinocarvone (tr[11]), myrtenal (0.9%), myrtenol (0.6%), citronellol (0.1%), methyl citronellate (0.7%), *trans*-pinocarvyl acetate (0.3%), dihydroedulan (0.2%), methyl geranate (tr), myrtenyl acetate (tr), citronellylacetate (tr), cyclosativene (0.7%), α-copaene (1.0%), β-cubebene (0.4%), selina-4(15),6-diene (0.3%), (E)-β-caryophyllene (0.9%), dauca-8,11-diene (0.1%), *trans*-α-bergamotene (0.5%), (E)-β-farnesene (0.6%), **Melanene (2.3%)**, β-ionone (tr), *ar*-curcumene (3.6%), germacrene D (tr), zingiberene (2.7%), bicyclogermacrene (0.1%), β-bisabolene (1.5%), γ-cadinene (tr), β-sesquiphellandrene (2.1), elemol (0.4%), E-nerolidol (0.4%), dihydro-(ar)-turmerone (0.1%), spathulenol (tr), 10-(*epi*)-junenol (2.9%), α-guaiol (3.3%), rosifoliol (tr), hinesol (tr), 5-guaiene-11-ol (0.5%), β-eudesmol (0.2%), α-eudesmol (0.2%), and neophytadiene (0.9%).

14. Tesso, H., König, W.A. Terpenes from *Otostegia integrifolia*, *Phytochemistry* 2004, 65, 2057-2062.

[11] trace < 0.1%

Curriculum Vitae

1. Personal Description

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2.3.5. Dates attended: October 1994-February 1997

2.3.6. Credential obtained: Masters of Science in Chemistry

3. Work Experience

3.1. Research assistant: Nov. 1993-Sep. 1994; April 1997-Sep. 1998 at Chemistry Department, Addis Ababa University

3.2. Lecturer: Oct.1998-Sep. 2001; at Chemistry Department, Alemaya University

3.3. April 2002- : Working on a Ph.D. degree project.