Characterization of bioactive natural products from edible, medicinal and toxic plants

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Preface

This thesis is submitted for the degree of Philosophia Doctor (PhD) in the field of pharmacognosy and natural products chemistry at the University of Bergen. Work on this thesis has been performed during the period May 2011 to July 2015 at the Department of Chemistry, University of Bergen. Writing of papers and the thesis continued for another two years from august 2015 to September 2017. A collection of seven published papers preceded by an extended summary makes up the content of this thesis.

The work on European white-berry mistletoe (*Viscum album L.*) is part of an international collaboration where the fresh plant material was provided from the botanical garden of Department of Botany, Faculty of Pharmacy at University of Lille 2 in France. The host tree was a hybrid of *Populus nigra* L. with *P. deltoids* Bartrum ex Marshall = *P. x Canadensis* Moench (Salicaceae). A voucher specimen of *V. album* L has been deposited with accession number LIP-BOTA-12070201.

Fresh needles of the living fossil *Metasequoia glyptostroboides* were kindly provided by Professor Dag Olav Øvstedal from a tree growing in the botanical garden of the University of Bergen. At the Herbarium BG a voucher specimen of *M. glyptostroboides* has been deposited with accession number BG-S-159712.

Living plants of *Zamioculcas zamiifolia* were bought from the local IKEA store in Åsane, Bergen as this East African medicinal plant recently have seen widespread use as an indoor decorative plant because of its glossy, shiny and evergreen leaves.

Dried leaves of *Bromelia laciniosa*, *Neoglaziovia variegata* and *Encholirium spectabile*, family of Bromeliaceae were sent from Brazil as part of an international collaboration agreement between the Federal University of San Francisco Valley, Petrolina, State of Pernambuco, Brazil and University of Bergen, Bergen, Norway. Fresh plant material from the three species were collected from three separate locations within the municipality borders of the City of Petrolina, State of Pernambuco, Brazil in January 2013 and then dried in an oven for seven days prior to shipment to Bergen, Norway. Coordinates for

the locations are 08°59'16.90" S and 40°35'20.60" W for *B*. *laciniosa*, 09°07'54.30" S and 40°26'21.00" W for *E*. *spectabile* and 08°59'16.90" S and 40°35'20.60" W for *N*. *variegata*. Voucher specimens were deposited at the Herbarium Vale do São Francisco (HVASF) with accession numbers 6441 (*N*. *variegata*), 6442 (*B*. *laciniosa*) and 6443 (*E*. *spectabile*).

Narthecium ossifragum was harvested from a bog area 350 m above sea level on the south-western part of Ulriken, Bergen, Norway at 350 m above sea level (coordinates 60.36884 N 005.38237 E). Voucher specimen of *N*. *ossifragum* has been deposited at the ARBOHA at the University of Bergen (accession number BG/S-162115).

Acknowledgements

I wish to thank my main supervisor Professor Torgils Fossen for excellent supervision. Fossen has willingly shared of his knowledge and encouraged me in the daily struggle of making procedures work and figuring out the meaning of the results.

Assistant supervisor Professor Svein Haavik has provided valuable discussions and input at critical stages during the development of the thesis.

Professor Emeritus George W. Francis has most kindly used some of his time to discuss with me and share of his knowledge accumulated through more than four decades of natural products research. Francis has also contributed to improvement of language of both papers and thesis.

Special word of thanks to Professor Jackson Roberto Guedes da Silva Almeida (UNIVASF, Brazil), Professor Frederic Dupont (Universite Lille 2) and Professor Dag Olav Øvstedal (University of Bergen) for providing significant quantities of the plant material used in this thesis.

All co-authors on papers included in the thesis are appreciated for their collaboration and valuable contributions.

Dr. Bjarte Holmelid and Mr. Terje Lygre have my gratitude for the recording of high-resolution mass spectra. Now retired principal engineer Atle Aaberg have my gratitude for his technical assistance.

I wish to thank all the people at the Department of Chemistry including past and current members of the research group in Pharmacognosy. You have all contributed to a friendly, inspiring and good working environment.

Colleagues and my two closest superiors, apothecary Kine Risberg and manager Rikke Landsvik Berge at Apotek1 Oasen have my deepest gratitude. Their friendly attitude and flexibility has made it possible to continue writing papers and completing the thesis during the two years from August 2015 to September 2017 next to a full time job as pharmacist.

My family including my mother have my deepest gratitude for their support during all six years of work with this thesis. I would also like to express my gratitude towards my grandparents who have thought me the value of hard work and the meaning of "*don't put off until tomorrow what you can do today"* (Benjamin Franklin (1706-1790)). Lastly I would like to thank my wife for her patience during the PhD period by saying Oroka kely mamibe ho anao.

Funding for this work was provided by the University of Bergen by means of a position at the Centre of Pharmacy with workplace at the Department of Chemistry.

The research fund of Fanny Schnelle (1866-1953) for chemical research (created 11th of November 1949) has kindly provided some of the funding for the Biotek® EON™ Microplate Spectrophotometer (BioTek Instruments Incorporation, Winooski, Vermont, USA) used for absorbance readings in the bioassays.

A travel grant from the National PhD School of Pharmacy (Nasjonal forskerskole i farmasi) was used to participate at the international conference Nordic Natural Products Conference 2015 (NNPC 2015) 15-16th of June 2015 in Visby on Gotland, Sweden.

University of Bergen provided funding for Open Access publication of the review article "Growing with dinosaurs: natural products from the Cretaceous relict Metasequoia glyptostroboides Hu

Open Access publication of paper VI "Non-polar natural products from *Bromelia laciniosa*, *Neoglaziovia variegata* and *Encholirium spectabile* (Bromeliaceae)" were kindly funded by Centre for Pharmacy, University of Bergen.

"Blessed are those who find wisdom, those who gain understanding,"

Abstract

Research performed during work on this thesis has focused on isolation, structure elucidation and biological activity of natural products. Seven plant species have been examined, namely *Bromelia laciniosa* Mart. ex Schult f., *Encholirium spectabile* Martius ex Schult. Schult. f., *Metasequoia glyptostroboides* Hu & Cheng, *Narthecium ossifragum* L. Huds., *Neoglaziovia variegata* Arruda da Camara Mez, *Viscum album* L. and *Zamioculcas zamiifolia* Lodd. Engl. Fifty-five compounds are reported throughout the thesis. Among them are twelve novel natural products. *Artemia* toxicity test, 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity and 15-lipoxygenase inhibition test have been performed on pure compounds and in one case on crude extracts.

Four novel natural products and four known compounds are reported from European white-berry mistletoe (*V. album* L.). Two novel aminoalkaloids 4,5,4'-trihydroxy-3,3'-iminodibenzoic acid and 4,5,4',5'-tetrahydroxy-3,3'-iminodibenzoic acid were isolated and identified from the leaves of *V. album*. This is the first time alkaloids that appear to be specific to this medicinal plant used for millennia are reported in the scientific literature. The two novel natural products 3-(3'-carbomethoxypropyl) gallic acid and 3-(3'-carbomethoxypropyl)-7→3''-protocatechoyl galloate make up a novel group of natural products. They are derived of the methyl ester of γ-hydroxybutyric acid (GHB) coupled to aromatic hydroxybenzoic acids including gallic acid. Radical scavenging activity for 3-(3'-carbomethoxypropyl) gallic acid (IC_{50} value 222 μM) were twentyfold reduced by substitution of the 3-hydroxyl of gallic acid with the methyl ester of gamma-hydroxybutyric acid compared with that of gallic acid ((IC_{50} value 27.7 μM).

From dawn redwood (*M. glyptostroboides*) altogether eight compounds are reported including the novel natural product 6-carboxydihydroresveratrol 3-*O*-*β*-glucopyranoside. This dihydrostilbenoid exhibited both radical scavenging activity (IC_{50} value 164±39μM) and inhibition of 15-Lipoxygenase (IC_{50} value 246±30μM).

Apigenin-6-*C*-(6''-*O*-(3-hydroxy-3-methylglutaroyl)-*β*-glucopyranoside, a novel acylated C-glycosylflavone, and six other known natural products are reported

from *Z. zamiifolia*. This ancient African medicinal plant has recently seen widespread use as an indoor ornamental plant. *Z. zamiifolia* has gained a reputation for being toxic. Despite this no lethality was observed even at the highest concentrations (1 mg/ml) when crude extracts of both leaves and petioles were applied in initial toxicological tests with *Artemia salina*.

From the three species of the Bromeliaceae family, *B. laciniosa* (nine compounds), *N. variegata* (thirteen compounds) and *E. spectabile* (sixteen compounds), a total of twenty non-polar natural products are reported. Prior to this investigation very little information was available about the chemical composition of these three Bromeliaceae spp. Very long chain alkanes, fatty acids, tocopherols and triterpenoids are among the reported compounds.

Twelve aromatic compounds are reported from the fruits of bog asphodel (*N. ossifragum)*. Six novel natural products namely, (*E*)-4-(3-R-hydroxy-2,2-dimethylChroman-6-yl)but-3-en-3-one, (*E*)-4-(4-(((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)oxy)Phenyl)but-3-en-2-one, (2R,3S)-Naringenin(3→6'')luteolin, (2R,3S)-Naringenin(3→6'')chrysoeriol, liovil 4-*O*-*β*-glucopyranoside and (*E*)-2,6-dimethoxycinnamic acid were reported for the first time. Previous to this investigation the rare natural product 4-hydroxy-3-(3-methylbut-2-enyl) benzaldehyde was only known from the pathogenic fungus *Heterobasidion occidentale*. This fungal metabolite was shown to be cytotoxic towards both normal rat kidney (NRK) (IC_{50} value 430 μM) and MOLM13 acute myeloid leukemia (AML) cells (IC_{50} value 68 μM). Naringenin(3→6'')luteolin was the main aromatic compound, and was shown to be toxic to *A. salina* (LC_{50} value 130μM). This novel biflavonoid was cytotoxic for both NRK (IC_{50} value 230 μM) and MOLM13AML cells (IC_{50} value 115 μM).

Sammendrag

Forskningen utført i løpet av denne doktorgraden har fokusert på isolering, strukturoppklaring og biologisk aktivitet til naturprodukter. De syv planteartene *Bromelia laciniosa* Mart ex. Schult. f., *Encholirium spectabile* Martius ex Schult. Schult. f., *Metasequoia glyptostroboides* Hu & Cheng, *Narthecium ossifragum* (L.) Huds., *Neoglaziovia variegata* (Arruda da Camara) Mez, *Viscum album* L. and *Zamioculcas zamiifolia* (Lodd.) Engl har blitt undersøkt. Femtifem naturprodukter er rapportert i denne avhandlingen deriblant tolv hittil ukjente forbindelser. Rene forbindelser og i ett tilfelle et råekstrakt har blitt testet med Artemia toksistets test, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radikal sluk effekt og 15-lipoxygenase inhiberingstest.

Fire nye og fire kjente forbindelser er rapportert fra europeisk hvitbæret misteltein (*V. album L.*). De to aminoalkaloidene 4,5,4'-trihydroksy-3,3'-iminodibenzosyre og 4,5,4', 5'-tetraydroksy-3,3'-iminodibenzosyre er isolert og identifisert for første gang fra bladene til *V. album*. For første gang er det i den vitenskaplige litteraturen rapportert alkaloider som ser ut til å være spesifikke for denne medisinplanten som har vært brukt i årtusener. To nye naturstoffer basert på metyl esteren av gammahydroksysmørsyre (GHB) forbundet med aromatiske hydroksybenzosyrer inkludert gallussyre (3,4,5-trihydroksybenzosyre) er nå rapportert. De to nye forbindelsene 3-(3'-karbometoksypropyl) gallussyre og 3-(3'-karbometoksypropyl)-7→3''protocatechoyl galloat utgjør en ny gruppe av naturprodukter. Radikalslukeffekten av 3-(3'-carbometoksypropyl) gallussyre (IC_{50} verdi 222 μM) var redusert med en faktor på tjue i forhold til gallussyre uten substituering (IC_{50} verdi 27,7 μM).

Totalt åtte forbindelser er rapportert fra urtreet *M. glyptostroboides* inkludert det nye naturproduktet 6-karboksydihydroresveratrol 3-*O*-*β*-glukopyranosid. Det ble påvist både radikal sluk effekt (IC_{50} verdi 164±39 μM) og inhibering av 15-lipoksygenase (IC_{50} verdi 246±30 μM) for denne nye dihydrostilbenoid forbindelsen.

Apigenin-6-*C*-(6''-*O*-(3-hydroksy-3-metylglutaroyl)-*β*-glukopyranosid, en ny acylert C-glykosylflavon, og seks andre naturprodukter er rapportert fra *Z. zamiifolia*. I det siste har denne gamle afrikanske medisinplanten fått ustrakt

brukt som innendørs dekorasjonsplante. *Z. zamiifolia* har gjennom assosiasjon med andre planter i Araceae familien fått et rykte for å være giftig. Toksisitets test med *Artemia salina* viste imidlertid ingen dødelighet selv for de høyeste konsentrasjonene (1 mg/ml) for råekstrakt fra blader og stengel fra *Z. zamiifolia*.

Totalt tjue upolare naturstoffer er rapportert fra de tre Bromeliaceae artene *B. laciniosa* (ni forbindelser), *N. variegata* (tretten forbindelser) og *E. spectabile* (seksten forbindelser). Svært lite informasjon var tilgjengelig om den kjemiske sammensetningen til disse tre artene før denne studien ble gjennomført. Fettsyrer, tokoferoler, svært langkjedede alkaner og triterpenoider er blant de rapporterte forbindelsene.

Tolv aromatiske forbindelser er rapportert fra frukten til Rome (*N. ossifragum*) deriblant de seks nye naturproduktene (*E*)-4-(3-R-hydroksy-2,2-dimetylkroman-6-yl)but-3-en-3-on, (*E*)-4-(4-(((*E*)-4-hydroksy-3-metylbut-2-en-1-yl)oksy)fenyl)but-3-en-2-on, (2R,3S)-Naringenin(3→6'')luteolin, (2R,3S)-Naringenin(3→6'')chrysoeriol, liovil 4-*O*-*β*-glukopyranosid og (*E*)-2,6-dimetoksykanelsyre. Før denne studien var det sjeldne naturproduktet 4-hydroksy-3-(3-metylbut-2-enyl) benzaldehyd kun kjent fra den patogene soppen *Heterobasidion occidentale*. Denne soppmetabolitten ble vist å være cytotoksisk for både normal rat kidney (NRK) (IC_{50} verdi 430 μM) og MOLM13 acute myeloid leukemia (AML) celler (IC_{50} verdi 68 μM). Naringenin(3→6'')luteolin var den aromatiske hovedforbindelsen i fruktene av *N. ossifragum* og viste seg å være toksisk for *A. salina* (LC_{50} verdi 130 μM). Denne nye biflavonoiden var cytotoksisk for både NRK (IC_{50} verdi 230 μM) og MOLM13 AML (IC_{50} verdi 115 μM) celler.

Abbreviations and symbols

1D	1 Dimensional
2D	2 Dimensional
15 – LO	15 – Lipoxygenase
AML	Acute Myeolid Leukemia (cells)
C	Carbon
CAPT	Compensated Attached Proton Test
CD	Circular Dichroism
CI	Chemical Ionisation
COSY	COrrelation SpectroscopY
DART	Direct Analysis in Real Time
DMSO	DiMethyl SulfOxide
DPPH	1,1-DiPhenyl-2-PicrylHydrazyl
δ	Chemical shift in ppm
d	Doublet
dd	Double doublet
DI	Direct Inlet
EI	Electron Impact
ESI	Electro Spray Ionization
$[F^+]$	Positively charged Fragment ion
FID	Flame Ionization Detector
GC	Gas Chromatography
GHB	Gamma(γ)-hydroxybenzoic acid
H2BC	Heteronuclear-2-Bond Correlation
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High Performance Liquid Chromatography
HRMS	High Resolution Mass Spectrometry
HSQC	Heteronuclear Single Quantum Coherence
Hz	Herz
IC_{50}	Inhibitory concentration, 50%
L	Litre
LC_{50}	Lethal concentration, 50%
LC-MS	Liquid Chromatography Mass Spectrometry
m	Multiplet
min	Minute
ml	Millilitre
$[M+^{\cdot}]$	Positively charged Molecular ion
MS	Mass Spectrometry
Mp	Melting point
N	Nitrogen
NMR	Nuclear Magnetic Resonance Spectroscopy

NRK	Normal Rat Kidney (cells)
O	Oxygen
ppm	Parts per million
ROESY	Rotating frame Overhauser Effect SpectroscopY
s	Singlet
TFA	TriFluoroAcetic acid
TIC	Total Ion Chromatogram
TOCSY	Total Correlation SpectroscopY
UV-vis	UltraViolet to visible spectroscopy
V	Volt
VLCA	Very long chain alkanes

Papers

I. **Novel aminoalkaloids from European mistletoe (*Viscum album* L.).** Amer, Bashar; Juvik, Ole Johan; Dupont, Frédéric; Francis, George W.; Fossen, Torgils. (2012). Phytochemistry Letters, 5, 677-681

II. **Novel GHB-derived natural products form European mistletoe (*Viscum album*).** Amer, Bashar; Juvik, Ole Johan; Francis, George W.; Fossen, Torgils. (2013). Pharmaceutical Biology, ISSN 1388-0209 print / ISSN 1744-5116 online, DOI: 10.3109/13880209.2013.773520

III. **6-Carboxydihydroresveratrol 3-*O*-*β*-glucopyranoside – A novel natural product from the Cretaceous relict *Metasequoia glyptostroboides*.** Nguyen, Xuan Hong Thy; Juvik, Ole Johan; Øvstedal, Dag Olav; Fossen, Torgils. (2014). Fitoterapia, 95, 109 – 114

IV. **Growing with dinosaurs – Natural products from the Cretaceous relict *Metasequoia glyptostroboides* Hu & Cheng – a molecular reservoir from the ancient world with potential in modern medicine.** Juvik, Ole Johan; Nguyen, Xuan Hong Thy; Andersen, Heidi Lie & Fossen, Torgils. (2016). Phytochemistry Reviews, 15(2), 161-195

V. **First identification of natural products from the African medicinal plant *Zamiouculcas zamiifolia* – A drought resistant survivor through millions of years.** Le Moullec, Angharad; Juvik, Ole Johan; Fossen, Torgils. (2015). Fitoterapia, 106, 280 - 285

VI. **Non-polar natural products from *Bromelia laciniosa*, *Neoglaziovia variegata* and *Encholirium spectabile* (Bromeliaceae).** Juvik, Ole Johan; Holmelid, Bjarte; Francis, George W. ; Andersen, Heidi Lie; De Oliveira, Ana Paula; De Oliveira Junior, Raimundo Gonçalves; Da Silva Almeida, Jackson Roberto Guedes; Fossen, Torgils Molecules (2017) 22(9), 1478

VII. **Toxic aromatic compounds from fruits of *Narthecium ossifragum* L.** Vu, Marita; Herfindal, Lars; Juvik, Ole Johan; Vedeler, Anni; Haavik, Svein; Fossen, Torgils. (2016). Phytochemistry, 132, 76-85

Chapter 1
Introduction

Natural products from seven plant species (*Viscum album* L. *Metasequoia glyptostroboides* Hu & Cheng, *Zamioculcas zamiifolia* Lodd. Engl., *Bromelia laciniosa* Mart. ex Schult. f., *Neoglaziovia variegata* Arruda da Camara Mez, *Encholirium spectabile* Martius ex Schult. & Schult. f. and *Narthecium ossifragum* L. Huds. have been examined during the work with this thesis. This has resulted in the characterization of fifty-six natural products listed in Table 1. There, the compounds are listed alphabetically according to group. Below these chemical groups are presented. Where appropriate an explanation will be given for the choice of grouping compounds together.

1.1 Alkanes

Alkanes are saturated hydrocarbons with a linear or branched structure. It may be mentioned that saturated hydrocarbons containing rings are referred to as cycloalkanes. Very long chain alkanes (VLCA) are constituents of plant waxes. Intake of plant waxes has been associated with a cholesterol-lowering effect [1]. *In vivo* VLCA are hydroxylated at several positions to long chain alcohols by P450 – enzymes [2]. It is reported that very long chain fatty acids (VLFCA) inhibit cholesterol synthesis in cell culture [1]. Limited absorption and the need for *in vivo* conversion to VLFCA regulate the efficiency with which VLCA may contribute to the cholesterol-lowering effect of plant waxes. Six VLCA **(1-6)** shown in figure 38 are included in the current thesis (Paper VI).

1.2 Amino alkaloids

In amino alkaloids the nitrogen atom is located in an amino group, and not as part of a heterocyclic ring as in most other alkaloids [Paper I]. Examples of alkaloids in this group are capsaicin (from fruits of some *Capsicum* spp (Solanaceae)) [3], (-)-cathinone (from *Catha edulis* Forssk.) [4], colchicine (from *Colchicum autumnale* [5] and *Gloriosa superba* [6]), ephedrine (from *Ephedra sinica*) [7] and mescaline (from *Lophophora williamsii* (Cactaceae)) [8]. The two new amino alkaloids presented in this thesis (**7 and 8**) [Paper I]

define a new group of amino alkaloids where the amino group is situated between two benzoic acid moieties [9, 10].

1.3 Aromatic aldehydes and ketones

Four compounds belonging to this group are included in the current thesis, namely two aromatic aldehydes (Paper V and VII) and two aromatic ketones (Paper VII), which are grouped together due to their structural similarities.

1.4 Fatty acids and their derivatives

Fatty acids have a hydrocarbon chain of varying length with a carboxylate group at one end, and they may be saturated or unsaturated. From a nutritional perspective fatty acids may be a part of a healthy diet [11]. At the same time a diet including but not limited to a high intake of saturated fatty acids, often named as a westernized diet, is associated with obesity, development of diabetes and even with development an progression of the mucosal inflammatory diseases asthma and inflammatory bowel disease [12]. To provide an in-depth description of the role of intake of fatty acids in the human diet and its consequences for health and disease is beyond the scope of this introduction. The four fatty acids listed in Table 1 (**13-16**) [PaperVI] are common constituents in plants used for human nutrition including wheat [13], rice (*Oryza sativa* L.) [14]and sunflower (*Helianthus annuus* L.) oil[15].

1.5 Flavonoids

It is estimated that more than 12000 different flavonoids have been reported [16, 17]. Their structures consists of a fifteen carbon skeleton and with exceptions of chalcones, dihydrochalcones, chalcenes, chalcanes and chalcanols, three connected rings as shown in figure 1.

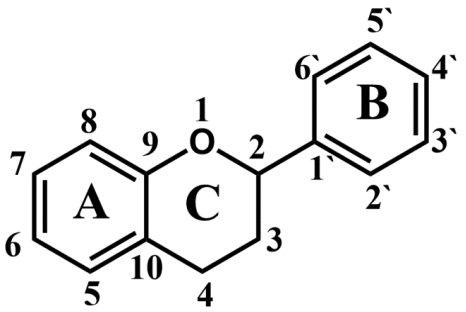


Figure 1. Basic ring structure of flavonoids.

The rings are named the A, B and C ring (Figure 1). The two aromatic rings are named the A and B ring while the heterocyclic ring is named the C ring. Based on substitution pattern and level of oxidation of the heterocyclic C ring the flavonoids are commonly divided into several subgroups (Figure 2). Brief descriptions of the flavonoid classes included in the thesis (Papers III, V and VII) are provided below.

Monomeric flavonoids

In the following text, a brief description of the majority of the flavonoid classes shown in Figure 2 (Paper III, V and VII and Table 1) is provided. The most common flavanols are also known as flavan-3-ols. Flavanols-3-ols have a hydroxy group (-ol) in 3 position of the C-ring, which has a saturated single bond between C-2 and C-3 of the aglycone. These compounds occur in nature as monomeric or polymeric flavonoids. Flavanones have a ketone group (-on) in 4-position of the C-ring and a saturated single bond between C-2 and C-3 of the aglycone. Several hundred flavanones have hitherto been identified [18]. Flavanonols are also known as dihydroflavonols. These compounds have a hydroxyl group (-ol) in 3-postion and a keto group (-on) in 4-position of a saturated C-ring. Flavones have a keto group (-one) in 4 position of the C ring as shown in Figure 2, in addition to an unsaturated bond between C-2 and C-3. Flavonols have both a hydroxyl group (-ol) in 3 position and a keto group (-on) in 4 position of the C ring as shown in Figure 2. The hydroxyl groups of the flavonoid aglycones are often substituted by glycosyl and methoxy groups The hydroxyls of the sugar units of flavonoids may be further substituted with glycosyl and acyl groups [19].

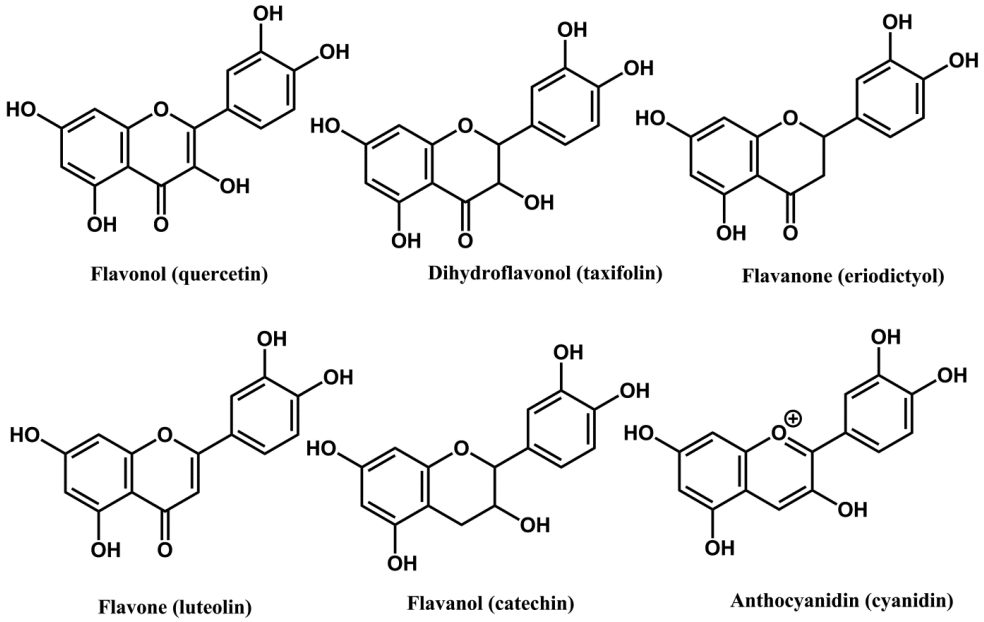


Figure 2. Overview of important flavonoid classes included in this thesis.

Biflavonoids

Biflavonoids consist of two flavonoids which are linked together. A large number of structural combinations are possible, both with respect to the nature of the monomeric flavonoid subunits, as well as the linkage positions between these subunits [20]. The monomeric sub-units can either belong to the same or a different class of flavonoids [20]. The latter is the case for compound 16 and 17 [Paper VII], which are flavanones linked with flavones (Figure 56). Most known bioflavonoids consist of flavonoid aglycone sub-units only, however, glycosylated biflavonoids have also been reported [21].

1.6 Dihydrostilbenoids

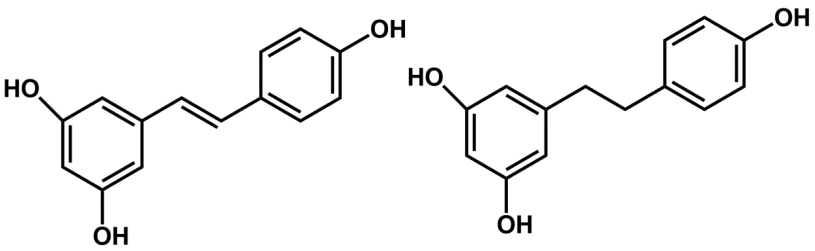


Figure 3. Resveratrol (left) and dihydroresveratrol (right).

Stilbenoids are bibenzyls e.g. resveratrol (Figure 3). Dihydrostilbenoids have two additional hydrogen atoms and thus a saturated linkage between the two aromatic rings e.g. dihydroresveratrol (Figure 3.). Only a single dihydrostilbenoid **(28)** is included in the current thesis. However this compound is a novel natural product from a unique species that is a relict from the Cretaceous period (Paper III).

1.7 γ-hydroxybutyric acid derivatives

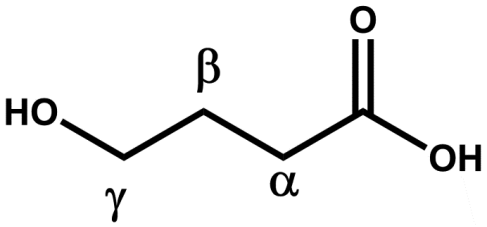


Figure 4. γ-hydroxybutyric acid

This class consists of compounds **(29 and 30)** where the methyl ester of γ-hydroxybutyric acid is merged with one or more phenolic acids through an ether bond [Paper II] [22].

1.8 Lignan glycosides

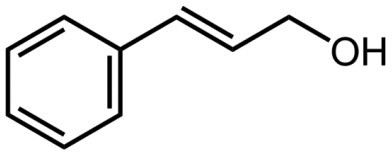


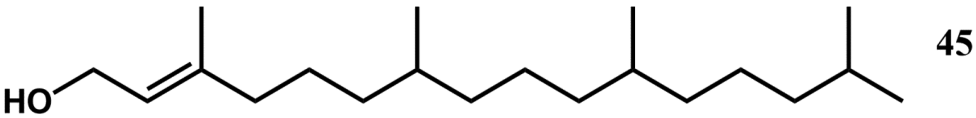
Figure 5. Cinnamoyl alcohol

Lignans are basically dimers of cinnamoyl alcohols (Fig 5.) Cyclization and further modifications ensures great structural variety within the natural products belonging to this group. Further modification is possible by linking the lignan aglycone to a sugar, as is the case for the single lignan glycoside included in this thesis **(31)** (Paper VII).

1.9 Phenolic acids and their derivatives

These natural products include benzoic acids (**42**, **44**) (Paper II), cinnamic acids (**32**, **36, 37**) (Paper III and VII) and their derivatives such as rosmarinic acid (**43**) (Paper V) and esters of cinnamic acids with quinic acid (**38-41**) (Paper III).

1.10 Phytol



45

Figure 6. Phytol

Phytol **(45)** (Figure 6.) is a relatively common natural product found in many edible plants (Paper VI). Spinach, beans, raw vegetables and asparagus are the main sources of phytol in the human diet [23]. Phytol can be converted to phytanic acid [24]. In individuals with Refsum's disease (named after neurologist Sigvald Bernhard Refsum (1907-1991)) phytanic acid is accumulated in plasma and tissue [25]. Coppack and co-authors have recommended that individuals suffering from Refsum's diseas should restrict the intake of food plants that contain free phytol [23]. However the importance of phytol as a source of phytanic acid in humans remains unclear [24].

1.11 Pyranosonic acids

The single pyranosonic acid (Paper V) presented in Table 1 **(46)** is a modified hexose and placed in a group of its own because of the structural uniqueness of the compound. Arabino-2-hexulopyranosonic acid is the systematic name for this heterocyclic compound.

1.12 Tocopherols

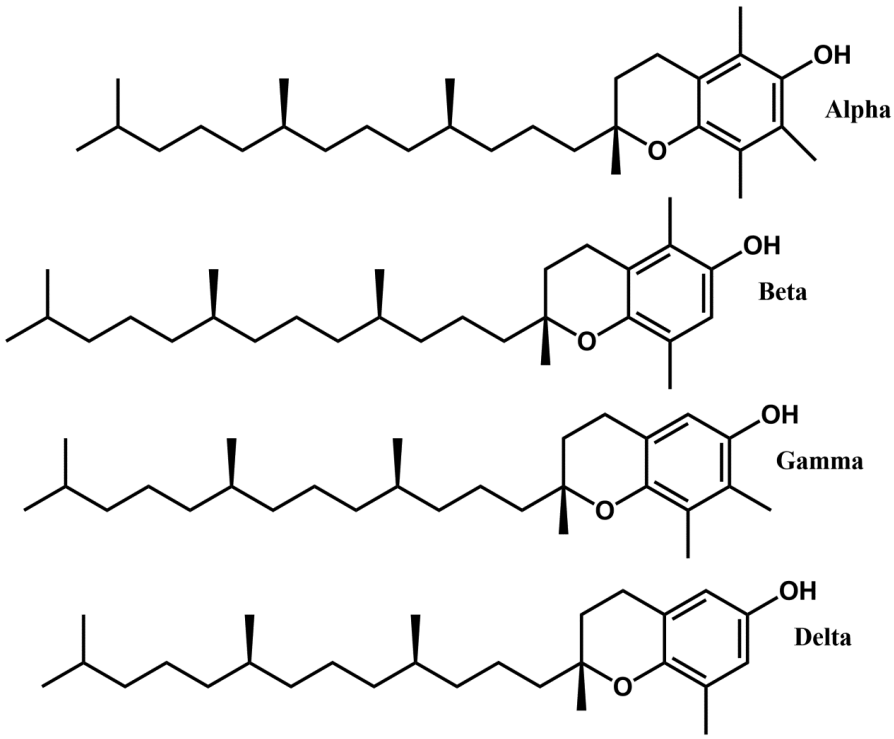


Figure 7. Alpha- (α), beta- (β), gamma- (γ) and delta- (δ) tocopherol.

Four naturally occurring tocopherols are shown in Figure 7. Position and number of methyl groups on the aromatic ring separates α-, β-, γ- and δ – tocopherol from each other. Tyrosine and phytol are the two main building blocks in the biosynthesis of tocopherols. Vitamin E is used as a name for mainly α-tocopherol, and also as a common name for the other tocopherols and their derivatives. Two tocopherols (**47** and **48**) are included in the current thesis (Paper VI).

1.13 Triterpenoids and derivatives

Triterpenes are hydrocarbons with 30 carbon atoms. Formally they can be regarded as consisting of six hemi-terpene (C_5) units or two fifteen carbon sesquiterpene units (C_{15}), according to their biosynthesis. Seven natural products of this type **(49-55)** are included in the current thesis (Paper VI). Phytosterols or plant sterols are derivatives of triterpenoids and are found in many plant products used for human alimentation. Intake of plant sterols are known to have cholesterol lowering effects [26-29].

Chapter 2
2. Aims of the study

The major objective of the study was to identify and perform initial testing of biological activity of the chemical constituents from selected plant species. The study had two main aims:

1. To extract, isolate and characterize novel natural products from selected plant sources.

2. To perform initial tests of biological activity.

The seven plant species which were examined and a brief statement about the reasons for their selection to the study are given below.

1. *Viscum album* L. has been used as a medicinal plant for millennia. However only a limited number of natural products have previously been reported from European white-berry mistletoe. The majority of the previously characterized natural products are not specific to European mistletoe.

2. *Metasequoia glyptostroboides* Hu & Cheng has remained unchanged for millions of years. Previous reports about compounds unique to dawn redwood, as well as the existing patent of an anticancer drug based on one of these compounds, are among the reasons for making this tree an interesting source in the search for further unique natural products.

3. *Zamioculcas zamiifolia* (Lodd.) Engl. is an ancient African medicinal plant. Recently it has seen widespread use as an indoor decorative plant in Europe, and it has gained a reputation for being toxic. However, prior to this study there were no reports about the chemical composition of this plant species.

4. *Bromelia laciniosa* Mart. ex Schult f. is widely used as fodder for livestock in Brazil and Argentina, medicinal uses are known and it is used as food for both humans and livestock. Very limited information about the

chemical composition of this Bromeliad species was available prior to this study.

5. *Neoglaziovia variegata* (Arruda da Camara) Mez produces an edible fleshy fruit at the begining of the rainy season. Current literature contain reports about antibacterial effect, antioxidant effect, antinociceptive effects in mice models, gastroprotective effects in a mice model of gastric ulcer as well as low toxicity in mice models for extracts from this Bromeliad. However, there were no reports about the chemical constituents of this plant species in current literature.

6. *Encholirium spectabile* Martius ex Schult. Schult. f. is used by rural communities in north-east Brazil during times of severe drought as a supplementary food supply. Additionally extracts of *E. spectabile* have been reported to exhibit favourable biological activities such as antioxidant and antibacterial activity. This study is the first to report on chemical constituents of this food source.

7. *Narthecium ossifragum* (L.) Huds. has been associated with poisoning and harmful effects on grazing livestock such as cattle, goats, and sheep since the 17th century. In current literature this perennial plant is reported to cause liver damage in lambs and kidney damage in goats. Ingestion of this plant is also a suspected cause of the photodynamic disease "Alveld" ("the elf's fire") in lambs. Reports in current literature on chemical constituents of *N. ossifragum* are limited to carotenoids, furanolactones, saponins and sterols and their derivatives. However, only limited information is available about the biological activity of the reported chemical constituents. Surprisingly, prior to this study, no aromatic compound had been reported from this suspected phototoxic plant.

Chapter 3
Experimental Methods

Details of the experimental methods are given in each paper (Paper I-VII) and references therein. The main experimental techniques and their function will be briefly summarized below.

3.1 Extraction

Methanol has been used to extract fresh plant material because of its ability to extract both polar and medium to low polar compounds. In one case isomers of hexane were used since the aim was to examine the non-polar constituents of three Bromeliaceae spp.

3.2 Purification

Liquid – Liquid partition of the concentrated aqueous solution against diethyl-ether or petroleum ether using a separation funnel was performed in order to remove hydrophobic compounds and less polar compounds such as chlorophylls.

3.3 Chromatographic separation

A combination of several chromatographic methods was used for separation and isolation of the natural products included in this thesis. In the following paragraphs the main methods used during this particular work will be briefly described.

3.3.1 Amberlite XAD-7 column chromatography

Absorption chromatography with Amberlite XAD-7 as stationary phase was used in several projects. When a gradient from distilled water to methanol is used as mobile phase free sugars and other polar aliphatic compounds are eluted first. Aromatic compounds are then eluted by increasing proportions of methanol in the mobile phase.

3.3.2 Sephadex LH-20 column chromatography

Sephadex LH-20 size-exclusion chromatography was used to purify fractions with aromatic compounds collected from Amberlite XAD-7 column. As mobile phase a mixture of methanol - distilled water with increasing amounts of methanol was used. With this aqueous methanolic gradient absorption chromatography will also play an important role for separation. Large molecules move between the particles of the stationary phase and are eluted first. Smaller molecules may enter the cavities of the particles. This gives a longer pathway through the column and consequently later elution. Aromatic polymers will be eluted last with this procedure because of strong absorption. In some cases it may become necessary to change the mobile phase from methanol to acetone in order to elute aromatic dimers and polymers such as polymeric flavonoids.

3.3.3 Preparative HPLC

A larger column and therefore larger volumes is the main difference between the utilized preparative HPLC and analytical HPLC. The latter is described below. Details about the C_{18} column and the applied gradients are described in papers I, II, III, V and VII.

3.4 Characterization and structure elucidation

3.4.1 Analytical HPLC with UV-vis multidiode array detector

Analytical HPLC was mainly used to determine the composition and purity of extracts and fractions. Figure 8 provides an example of this application of analytical HPLC. Distribution chromatography is the main principle for separation with the applied analytical HPLC systems. A gradient based on two solvents (distilled water and acetonitrile both with 0.5% trifluoroacetic acid) was used for elution. With a non-polar stationary phase and a polar mobile phase the polarity of the compounds as well as absorption plays a role for separation of the compounds. Analytical and preparative HPLC are techniques with a high resolving power. This makes it possible to separate even compounds with relatively similar structures.

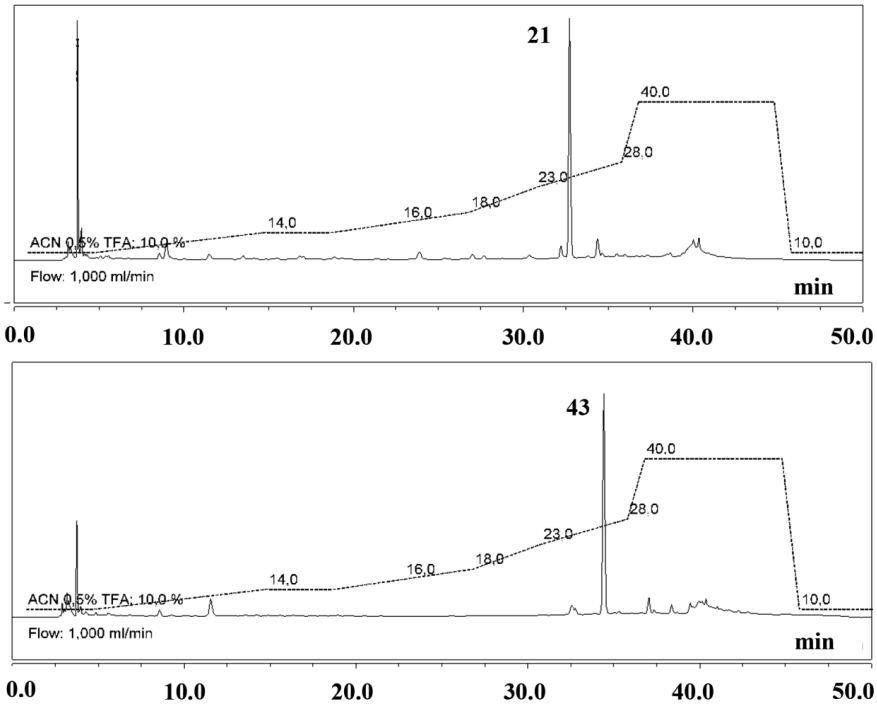


Figure 8. Comparison of the UV-vis chromatograms recorded during HPLC analysis of the crude extracts of the leaves (A) and the petioles (B) of *Z. zamiifolia*. The novel natural product apigenin-6-*C*-(6''-*O*-(3-hydroxy-3-methylglutaroyl)-*β*-glucopyranoside) **(21)** is the major aromatic component of the leaves while the known aromatic compound rosmarinic acid **(43)** is the main component of the petioles (Paper V).

3.4.2 Nuclear Magnetic Resonance

For several decades, Nuclear Magnetic Resonance (NMR) has been established as the single most important technique for structure elucidation of novel natural products and other organic compounds in solution, as well as determination of solution structures of macromolecules. In the current thesis NMR has been used to elucidate the structure of several novel natural products in addition to known compounds. A combination of several 1D and 2D NMR experiments has been applied (Paper I-III and V-VII). Below, the most important techniques used in this thesis are briefly explained, including selected examples of acquired high resolution spectra.

3.4.2.1 1D ^{1}H NMR

Individual peaks of a 1D ^{1}H NMR spectrum give information about the chemical shift values, and thus the chemical environment of each nucleus. Singlet peaks occur when all neighbouring atoms are quaternary carbons or NMR inactive nuclei like ^{16}O, or when the environment of the observed ^{1}H is equivalent for the ^{1}H attached to neighbouring carbons. Presence of one non-equivalent ^{1}H attached to neighbouring carbons will split the signal into two (a doublet). Splitting of the signal into three (a triplet) indicates the presence of two non-equivalent ^{1}H attached to neighbouring carbons, provided that the $^{3}J_{HH}$ coupling constants involved are similar or identical. This is known as the n+1 rule, since the peak number equals the number of non-equivalent ^{1}H attached to neighbouring carbons in addition to one (1). If the two $^{3}J_{HH}$ coupling constants involved are different, the signal occurs as a double doublet in the 1D ^{1}H NMR spectrum. Analogously, if a proton couples to three neighbouring protons with different $^{3}J_{HH}$ coupling constants, the signal appears as a double double doublet in the 1D ^{1}H NMR spectrum. The distance between peaks in one signal is measured in Herz (Hz) and is known as the coupling constant (J_{HH}). From a 1D ^{1}H NMR spectrum information about the chemical shift values and the chemical environment of the observed ^{1}H nuclei can be obtained. Additionally, integration of the area beneath the peaks can give information about the relative abundance of the observed signals. The ^{1}H isotope has a natural abundance of 99.985 % and a favourable magnetogyric ratio. Consequently 1D ^{1}H NMR is a sensitive and usually rapid technique.

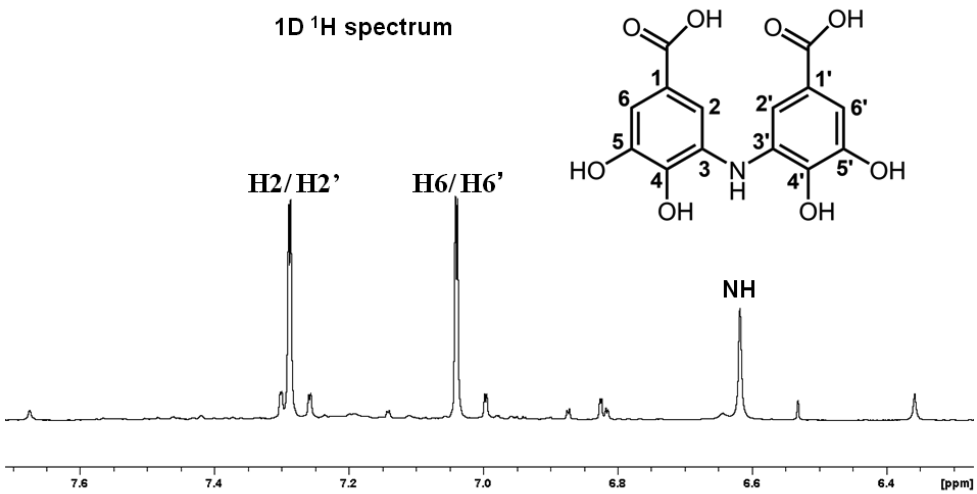


Figure 9. Assigned 1D ^{1}H spectrum of the novel aminoalkaloid 4,5,4',5'-tetrahydroxy-3-3'-iminodibenzoic acid **(8)** isolated from the leaves of *V. album*.

3.4.2.2 1D ^{13}C CAPT

Compensated Attached Proton Test (CAPT) is a 1D ^{13}C technique which has been used together with 2D experiments to acquire exact values for the chemical shifts of carbon [30, 31]. With this experiment Cq and CH_2 have opposite phase direction when compared to that of CH and CH_3. Conveniently, the 1D ^{13}C CAPT spectrum is phased so that signals of CH and CH_3 are negative while CH_2 and Cq are positive. Challenges with a low signal to noise ratio is a consequence of the low natural abundance of the ^{13}C isotope (1.1%) and relatively low magnetogyric ratio for ^{13}C.

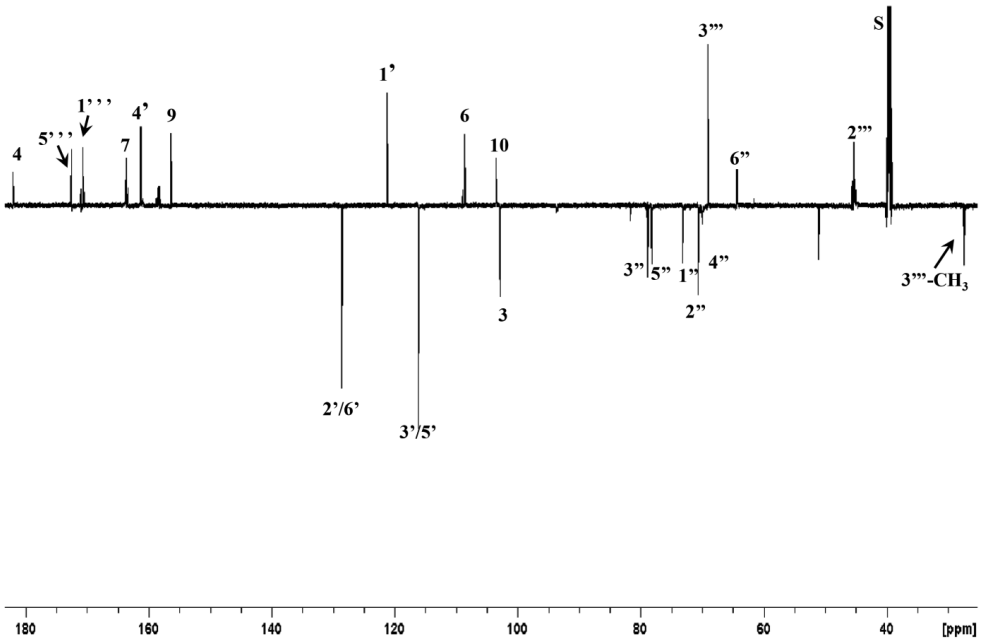


Figure 10. 1D ^{13}C CAPT spectrum of apigenin 6-*C*-(6″-(3-hydroxy-3-methyl-glutaroyl)-*β*-glucopyranoside) (**21**) isolated from leaves of *Z. zamiifolium*.

3.4.2.3 2D ^{1}H-^{1}H COSY

Correlation SpectroscopY (COSY) is a homonuclear 2D ^{1}H-^{1}H technique [32, 33]. Along both axes identical chemical shift values of ^{1}H are given. Plotting of these values gives diagonal peaks and cross peaks. The diagonal peaks distributed along the diagonal of the spectrum represent the chemical shift values of ^{1}H observed in the 1D ^{1}H NMR spectrum. When two protons are J-coupled to each other it is shown in the spectrum as cross peaks. They are symmetrically distributed along both sides of the diagonal. The cross peaks and the diagonal peaks form the corners of a square. Figure 11 gives an example of a square formed by diagonal and cross peaks. When a proton has couplings to more than one proton the diagonals will be the corner of more than one square.

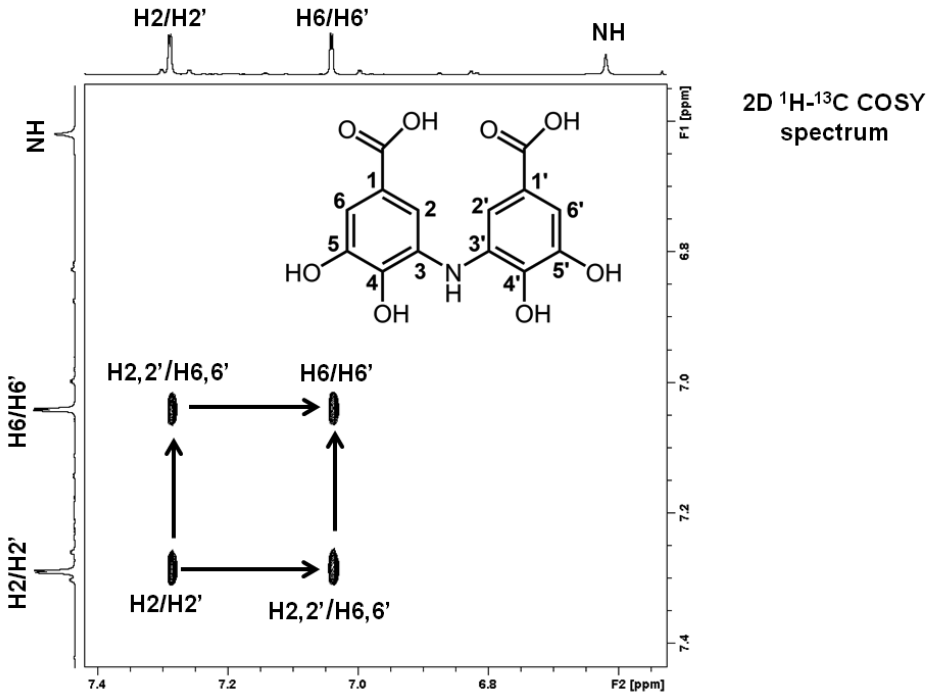


Figure 11. Assigned 2D ^{1}H-^{1}H COSY spectrum of the novel aminoalkaloid 4,5,4',5'-tetrahydroxy-3-3'-iminodibenzoic acid **(8)** isolated from the leaves of *V. album*.

3.4.2.4 2D ^{1}H-^{13}C HMBC and 2D ^{1}H-^{15}N HMBC

Heteronuclear Multiple Bond Correlation (HMBC) is a two dimensional and as the name indicates a heteronuclear technique [34, 35]. The experiment provides information about ^{1}H and ^{13}C chemical shifts which are correlated through multiple bonds. Cross peaks of geminal ($^{2}J_{CH}$) and vicinal ($^{3}J_{CH}$) correlations are usually the strongest couplings in the spectrum. One-bond couplings ($^{1}J_{CH}$) are not decoupled and may be observed in the spectrum. Because of the ability to observe long range couplings this method is especially important for the assignment of linkages between structural sub-units like aglycone, sugar units and acyl groups, and for assignment of quaternary carbons. In 2D ^{1}H-^{15}N HMBC correlations between proton and nitrogen are observed [36]. An example of this is shown in Figure 13.

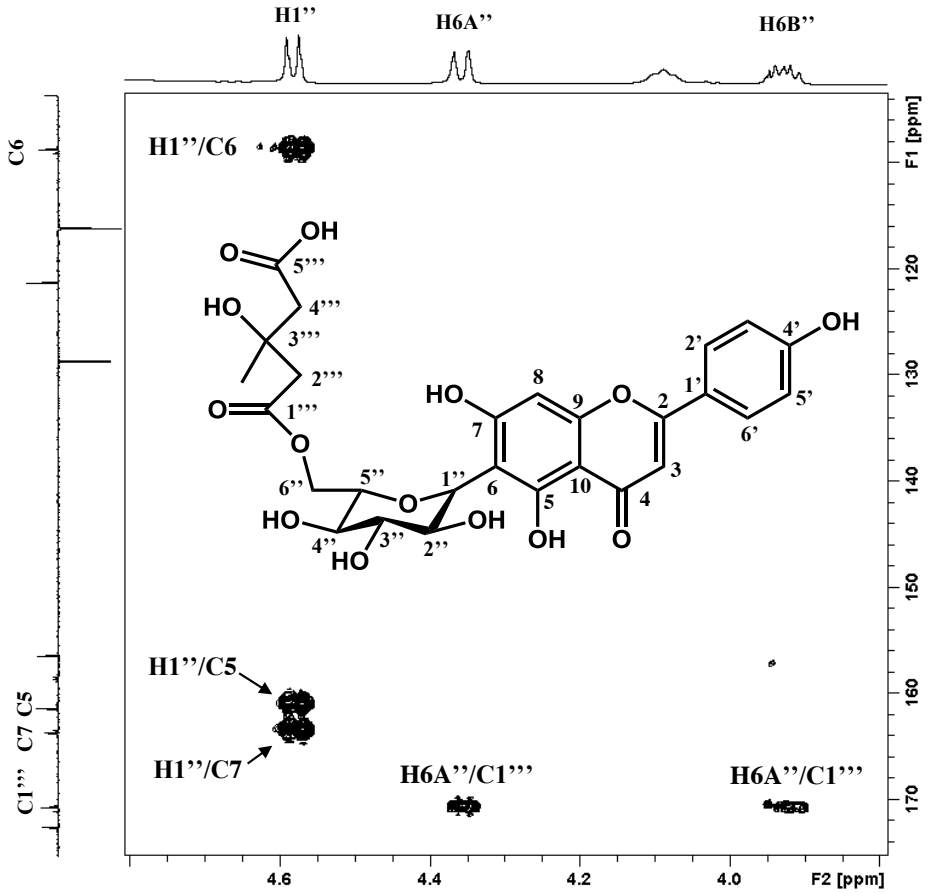


Figure 12. Expanded region of the ^{1}H-^{13}C HMBC spectrum of apigenin-6-*C*-(6′′-*O*-(3-hydroxy-3-methylglutaroyl)-*β*-glucopyranoside) **(21)** isolated from the leaves of *Z. zamiifolia*. The assigned cross peaks were decisive for determination of the linkages between the structural sub-units.

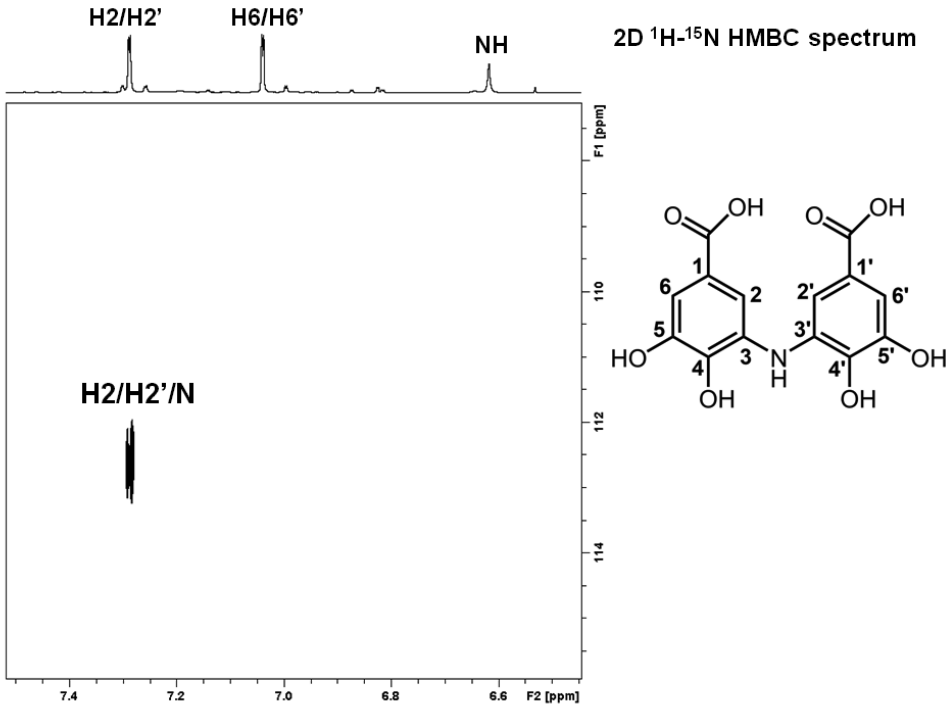


Figure 13. Expanded region of the ^{1}H-^{15}N HMBC spectrum of 4,5,4',5'-tetrahydroxy-3,3'-iminodibenzoic acid. The observed cross peak supported the determination of the linkages between the two benzoic acid moieties in this symmetrical compound.

3.4.2.5 2D ^{1}H-^{13}C HSQC and 2D ^{1}H-^{15}N HSQC

2D ^{1}H-^{13}C Heteronuclear Single Quantum Coherence (HSQC) is two dimensional and heteronuclear NMR technique [37]. The technique gives information about ^{1}H and ^{13}C coupled through a single bond ($^{1}J_{CH}$). Similarly, in ^{1}H-^{15}N HSQC spectra $^{1}J_{NH}$ correlations between proton and nitrogen (^{15}N) are observed.

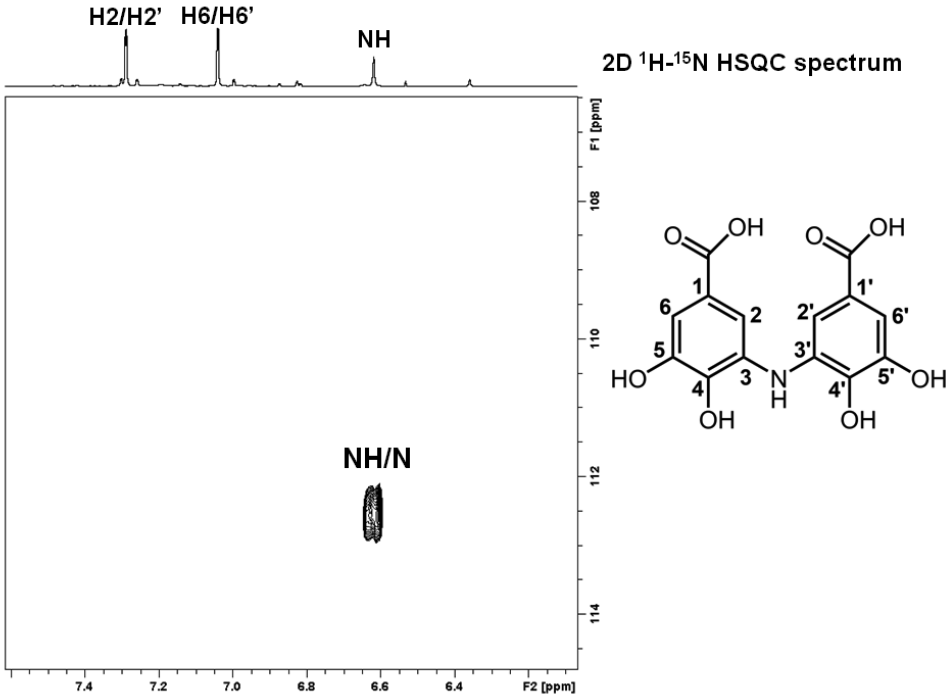


Figure 14. ^{1}H-^{15}N HSQC spectrum of 4,5,4',5'-tetrahydroxy-3-3'-iminodibenzoic acid **(8)** isolated from the leaves of *V. album*.

3.4.2.6 2D ^{1}H-^{13}C HSQC-TOCSY

This experiment name contains two abbreviations. The first part is explained in the paragraph above. With the HSQC pulse magnetisation is transferred from ^{13}C to ^{1}H through a single coupling ($^{1}J_{CH}$). Secondly during the Total Correlation SpectroscopY (TOCSY) sequence magnetisation is spread though the homonuclear spin network. Thus it is possible to observe cross peaks from an outstanding proton chemical shift value of a spin system to all carbons and protons belonging to the same spin system [38].

3.4.2.7 2D-Edited ^{1}H ^{13}C HSQC

2D- Edited ^{1}H ^{13}C HSQC is a two dimensional and heteronuclear NMR technique. It is distinguished from HSQC by different pulse angles in the sequence [39]. Its spectrum gives information about CH, CH_2 and CH_3 signals

and carbon-proton coupling through a single bond. The CH and CH_3 signals have opposite phase to that of CH_2 signals.

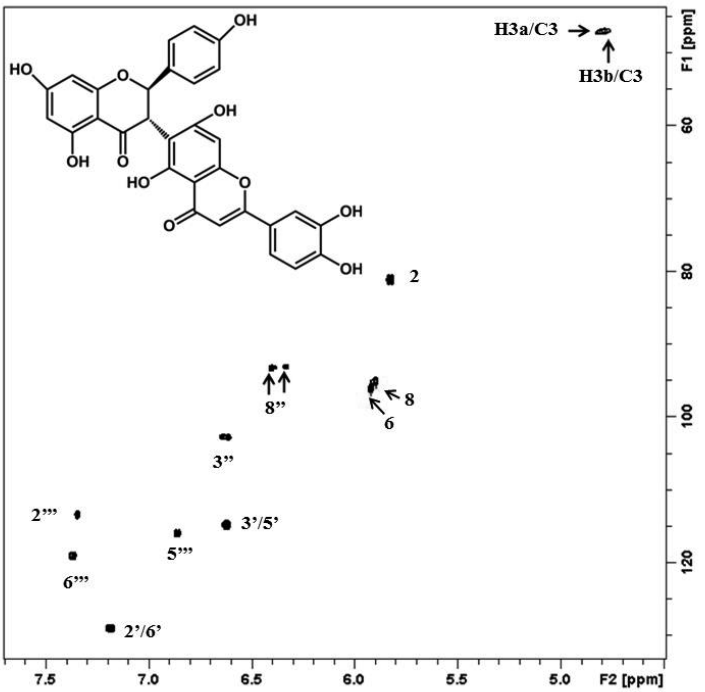


Figure 15. 2D-Edited ^{1}H- ^{13}C HSQC spectrum of naringenin(3→6'')luteolin (**17**) isolated from the fruits of *N. ossifragum*. Signals for 3'' and 8'' in this spectrum reveals the existence of two conformational isomers of this novel biflavonoid due to rotational hindrance around the bond between C3 and C6''. A numbered structure of compound **17** is shown in Figure 55.

3.4.2.8 2D ^{1}H ^{13}C H2BC

Heteronuclear-2-Bond Correlation (H2BC) is a HSQC-type two dimensional and heteronuclear NMR experiment [40]. It gives information about carbons with attached protons separated by two bonds ($^{2}J_{CH}$). Correlation to quarternary carbons is not shown. Since HMBC gives information about both geminal ($^{2}J_{CH}$) and vicinal ($^{3}J_{CH}$) correlations the information from H2BC is complementary and can be used to separate geminal ($^{2}J_{CH}$) from vicinal ($^{3}J_{CH}$) and long range correlations.

3.4.2.9 2D ^{1}H-^{1}H ROESY

Rotating frame Overhauser Enhancement Spectroscopy (ROESY) is a two dimensional and homo-nuclear technique [41]. This experiment is based upon the Nuclear Overhauser Enhancement effect (NOE). Magnetization is transferred through space because of dipole-dipole interactions. The observed crosspeaks provide information about proximity through space. Thereby the ROESY spectrum provides information about the stereochemistry of the compound. Diagonal peaks represent the signals of the 1D ^{1}H spectrum while the cross peaks represent proximity in space. Diagonal and cross peaks have opposite phases. Exchange peaks resulting from chemical exchange or conformational equilibria are often observed in the ROESY spectra. These crosspeaks can be distinguished from through-space crosspeaks because exchange peaks have the same phase as the diagonal peaks.

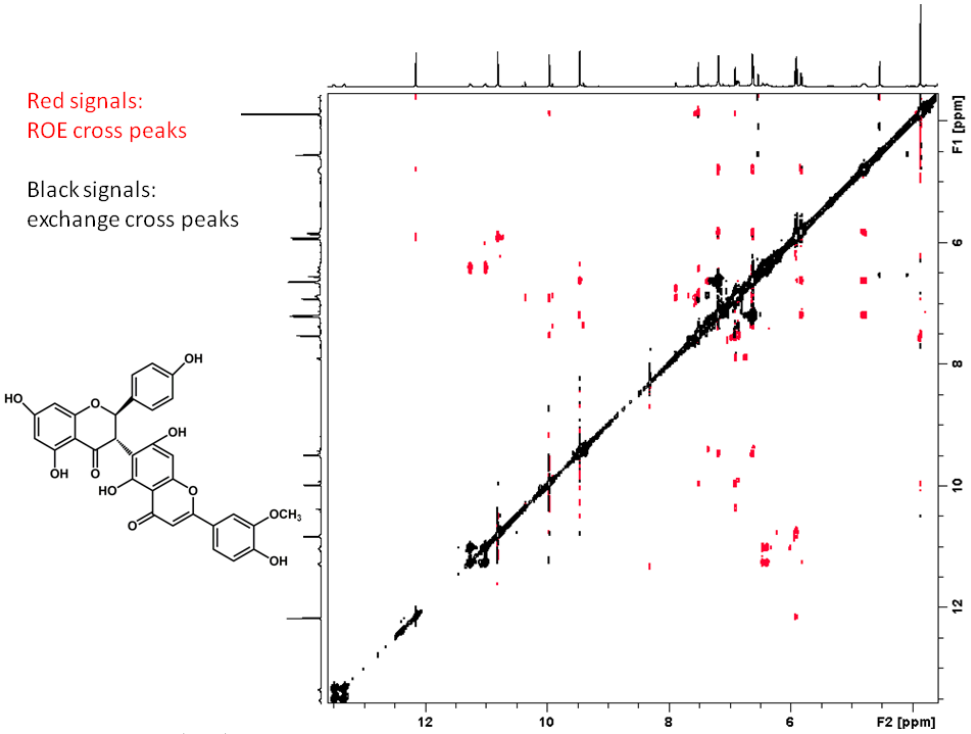


Figure 16. 2D ^{1}H-^{1}H ROESY spectrum of naringenin(3→6'')chrysoeriol isolated from the fruits of *N. ossifragum*.

3.4.3 Mass Spectrometry (MS)

High resolution Mass Spectrometry (HRMS) was mainly used to determine the molecular mass and elemental composition of the isolated natural products. Thereby the measured mass of the molecular ion confirmed the calculated mass based on the structure determined by NMR.

3.5 Biological activity

3.5.1 15-Lipoxygenase inhibition

Formation of a conjugated double bond system which absorbs light at 234 nm forms the basis of the test system. 15-Lipoxygenase catalyzes peroxidation of oxygen with polyunsaturated fatty acids such as linoleic and arachidonic acid [42]. The measured increase in absorption at 234 nm is proportional to the conversion of linoleic acid to the end product 13-hydroxyoctadecadienoic acid. Lipoxygenases takes part in the arachidonic acid pathway, which plays a central role in the inflammatory system [43]. In mammals there are three main types of lipoxygenase (5, 12 and 15- lipoxygenase) which catalyze peroxidation in positions C5, C12 and C15 respectively [44].+

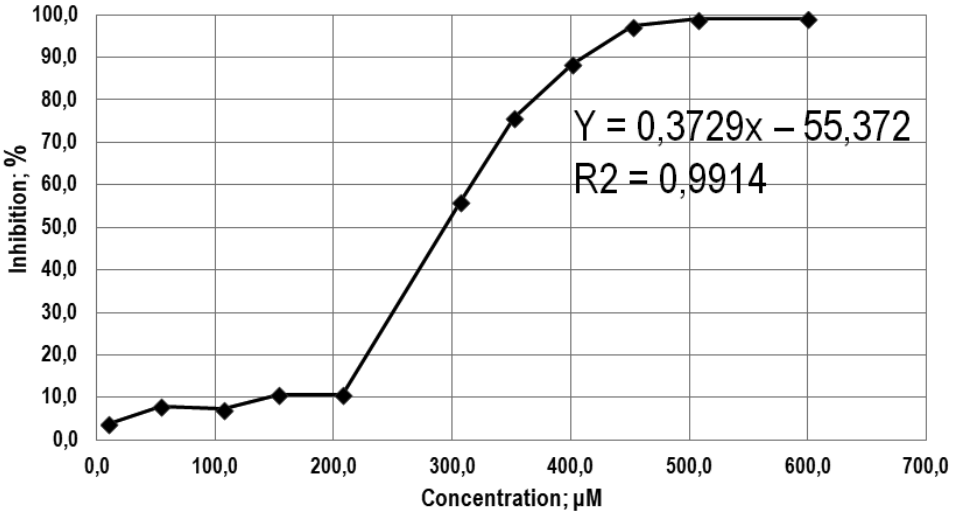


Figure 17. Plot of inhibiton of 15-Lipoxygenase by the novel natural product 6-carboxydihydroresveratrol 3-*O*-*β*-glucopyranoside.

3.5.2 DPPH scavenging activity

A solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) in ethanol has a deep violet colour and a strong absorption band at 517 nm. Decrease in absorption at 517 nm indicates pairing of the odd electron of this stable free radical. Low concentrations of DPPH may explain that a linear correlation between absorption and concentration according to the Lambert-Beer law is obeyed. Blois suggested the use of 1,1-diphenyl-2-picrylhydrazyl to test radical-scavenging activity in 1958 [45]. Glavind (1963) [46] and later Lyckander and Malterud (1996) [47] have described the procedure for the DPPH scavenging activity test. Adaption of the procedure to the BioTek® EON™ Microplate Spectrophotometer (BioTek Instruments Incorporations, Winooski, Vermont, USA) is described in Amer et al. (2012)[10].

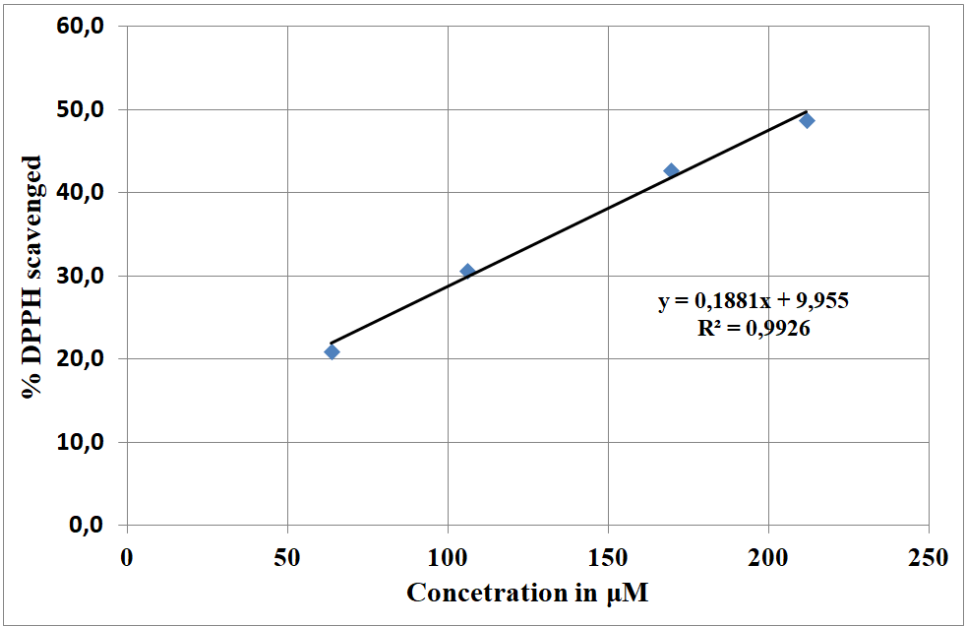


Figure 18. Plot of DPPH scavenging activity in series 1 for the novel compound 3-(3'-carbomethoxypropyl) gallic acid (Paper II).

3.5.3 *Artemia* toxicity test

Exposure of hatched *Artemia salina* nauplis to crude extracts or pure natural products in dilution series with a blank control is the basis of the *Artemia salina* toxicity test. After 24 hours living and dead larvae are counted in all wells and LC_{50} values are calculated [48]. Brine shrimp is the common name for *A. salina*. It is frequently used as a test organism since it provides a rapid, inexpensive and reliable method for initial toxicity tests [49, 50].

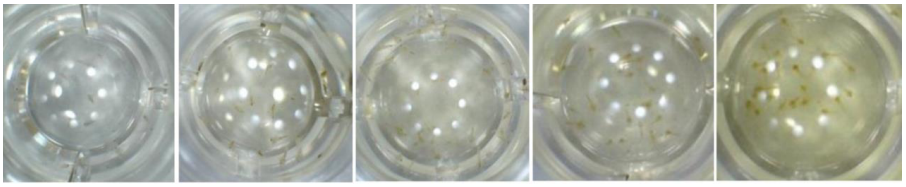


Figure 19. *A. salina* exposed to increasing concentrations of the yellow coloured (2R, 3S) naringenin(3→6 ´´)luteolin **(17)**. This novel natural product proved to be toxic for *A. salina* with LC_{50} value 130 µM. Notice the increasing yellow colouration of *A. salina* with increasing concentration of (2R, 3S) naringenin(3→6 ´´)luteolin **(17)**, suggesting that this compound is accumulated by *A. salina*.

Chapter 4
Results and discussion

4.1 Novel aminoalkaloids and GHB-derived natural products from European white-berry mistletoe (*Viscum album L.*)

For millennia European white-berry mistletoe (*V. album L.*) has been used in treatment of conditions such as hypertension, diabetes, arthrosis, epilepsy and cancer [51]. Cancer has been treated with preparations made from this old medicinal plant since the 1920's. Anticancer activity of extracts from mistletoe has been explained with the presence of lectins [52], viscotoxins [53] and alkaloids [54-61]. Extracts from *V. album* L. have a complex chemical composition [62] and limited information has been available about the content of natural products. This thesis reports on four novel natural products and an additional four previously known natural products characterized from *V. album*.

Figure 20. European mistletoe (*V. album* L) photographed in Amsterdam 22.10.2005. Photo: Professor Frédéric Dupont.

European white-berry mistletoe (*V. album L.*subs. album) is a dioecious small shrub [63] with linear lanceolate leathery evergreen leaves which lasts for several seasons and white translucent berries which develop in late fall and early winter [51] as shown in Figure 20.

V. album L. subspecies *album* is one of three recognised subspecies of *V. album L*. The other two subspecies are *V. album L*. subsp. *abietis* (Wiesb) Abrom and *V. album L*. subsp. *austriacum* (Wiesb) Vollm. They grow on conifer hosts while *V. album L*. subsp. *album* grows on deciduous trees. Fir (*Abies* Mill.) is as the systematic plant name suggests the host tree of *V. album L*. subsp. *abietis* (Wiesb) Abrom while Scots pine (*Pinus sylvestris L.*) is host for *V. album L*. subsp. *austriacum* (Wiesb) Vollm. Many tree species within the range may be host for *V. album L*. subsp. *album*. Mostly it grows on apple (Malus Mill.), poplar (*Populus* L.) and willow (*Salix* L.). Very rarely it is found on oak (*Quercus* L.) and never on beech (*Fagus* L).

V. album belongs to the family *Loranthaceae* within the order *Santalales*. Previously the genus belonged to the family *Viscaceae* [63], which has been included within the *Loranthaceae* [64].

V. album is native to Europe and western and southern Asia [63]. In Norway *V. album* is found on both sides of the Oslofjord. Along the western side mistletoe can be found within the municipalities of Tønsberg and Horten (both in Vestfold county) in the south and Hurum municipality (Buskerud) in the north. Along the eastern side of the fiord the shrub can be found growing on trees from Fredrikstad municipality (Østfold) in the south to Vestby and Ås municipalities (both in Akershus county) in the north [65].

Mistletoe is not used exclusively as a name for the genus Viscum, but for all genus of Loranthacae spp. which grows half-parasitically on branches of trees or shrubs [63]. This may lead to confusion, and makes it important to specify the genus and species.

Eight compounds were isolated and characterized from European white-berry mistletoe (*V. album*). Half of them were novel natural products (**7**, **8**, **29** and **30**).

Two novel aminoalkaloids have been isolated and characterized from *V. album* (**7** and **8**) [10]. The two compounds are shown in Figure 2. As may be readily observed, and as the systematic name indicates the two compounds are distinguished by the number of hydroxyl substituents. They define a new group of aminoalkaloids, and are the first alkaloids to be isolated from *V. album* [10]. NMR data are provided in Table 2. A selection of NMR spectra for both compounds is included in the supplementary material for Paper I. Several NMR spectra of the novel aminoalkaloid 4,5,4`,5`-tetrahydroxy-3-3`-iminodibenzoic acid (**8**) have been used as examples (Figure 9, 11, 13 and 14) in the section of Experimental Methods (Chapter 2).

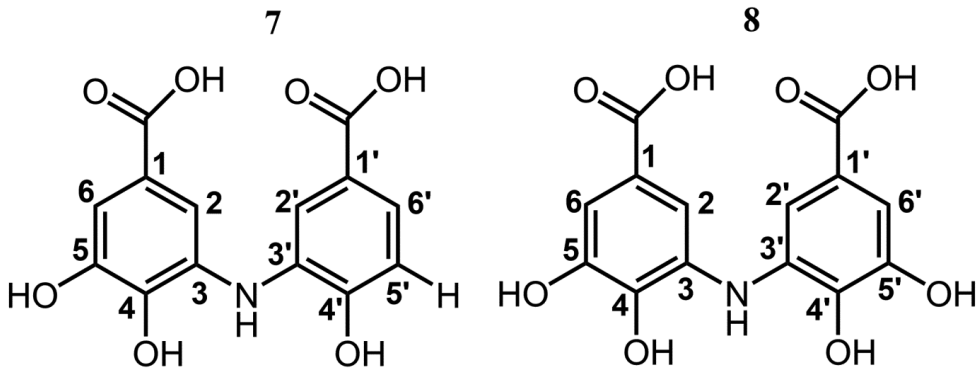


Figure 21. Structures of the two novel aminoalkaloids 4,5,4`-trihydroxy-3-3`-iminodibenzoic (**7**) and 4,5,4`,5`-tetrahydroxy-3-3`-iminodibenzoic acid (**8**).

Previous identifications of alkaloids from mistletoe have been from species other than *V. album* and always of natural products known to be present in the host tree at similar concentrations [66-70]. These findings support the assumption that mistletoe may also be able to incorporate natural products such as alkaloids from the host tree.

Caffeine has been found in Loranthaceae spp growing half parasitically on coffee trees. [65]. Antabasine, hyoscine, isopelletierine and nicotine have been reported from *Benthamina alyxifolia* F. Muell. ex Benth. Tiegh (Australian mistletoe) growing on *Duboisia myoporoides* R. Br. (Corkwood) [66, 67] belonging to the family Solanaceae. Five quinolizidine alkaloids, namely (-)-anagyrine, (-)-cytisine, (-)-lupanine, (-)-N-methylcytisine and (+)-retamine have been identified by ^{1}H NMR in *V. cruciatum* Sieber growing on Retama (syn. Lygos) sphaerocarpa belonging to the Fabaceae family [68]. Ammodendrine, an

bipiperidyl alkaloid, and the five quinolizidine alkaloids cytosine, *N*-methylcytisine, lupanine, 5,6-dehydrolupanine and retamine have been found in *V*. *cruciatum* parasitically attached to the roots of the host plant *Retama sphaerocarpa* Boissier [71]. One bipiperidyl alkaloid, ammodendrine, and two quinolizidine alkaloids, lupanine and retamine, have also been identified from the berries of *V*. *cruciatum* when parallel to the case above the mistletoe grew by root parasitism on *R*. *Sphaerocarpa* Boissier [70]. Presence of alkaloids has been indicated in the African mistletoe *Topinanthus dodoneifolius* (DC) Danser [72]. However these latter compounds still await structural characterisation.

Thirty years ago (1986) Khwaja and co-authors indicated presence of alkaloids in *V*. *album* [55]. The identification was solely based upon the applied extraction method and the substances reaction with the Dragendorf alkaloid reagent. Consequently, presence of alkaloids in European white-berry mistletoe remained controversial until recently [10]. Indeed as recently as at the beginning of the new millennium Pfüller wrote that *V*. *album* is devoid of alkaloids and only contains "alkaloid-like" compounds such as acetylcholine, choline phenylethylamine and tyramine [10, 73]. Hegnauer reviewed the presence of the latter compounds in *V*. *album* fifty years ago (1966) [74].

Fresh plant material for this study was collected in the botanical garden of Department of Botany, Faculty of Pharmacy, University of Lille 2. where *V*. *album* grew on *Populus x canadensis*. Neither the hybrid host tree nor its precursor trees *P*. *nigra* and *P*. *deltoides* are known to contain alkaloids. Sun and co-authors [Sun et al. 2009] have stated that *P*. *nigra* is devoid of alkaloids. Two nitrogenous compounds namely ortho-topolin-9-glucoside and *N*-6-(*O*-hydroxybenzylamino)purine have been reported from the hybrid host tree *P*. *x Canadensis* [75, 76]. However these nitrogenous compounds are not likely to be precursors for 4,5,4`-trihydroxy-3-3`-iminodibenzoic and 4,5,4`,5`-tetrahydroxy-3-3`-iminodibenzoic acid [10]. One structurally related alkaloid has previously been identified from *Drynaria fortune* [77]. There is structural resemblance between 4,4`-dihydroxy-3,3`-imino-di-benzoic acid and the two novel aminoalkaloids but there is no relation between the fern (*D*. *Fortunei*) from which it was isolated and *V*. *album*.

Gallic acid **(42)** and the two novel natural products 3-(3′-carbomethoxypropyl) -gallic acid **(29)** and 3-(3- carbomethoxypropyl)-7→3″-protocatechoyl galloate

(30) were isolated and charecterized from the leaves of *V*. *album (*Figure 22). These two compounds represent a novel group of natural products where γ-hydroxybutyric acid (GHB) is coupled to phenolic acids [22] [Paper II].

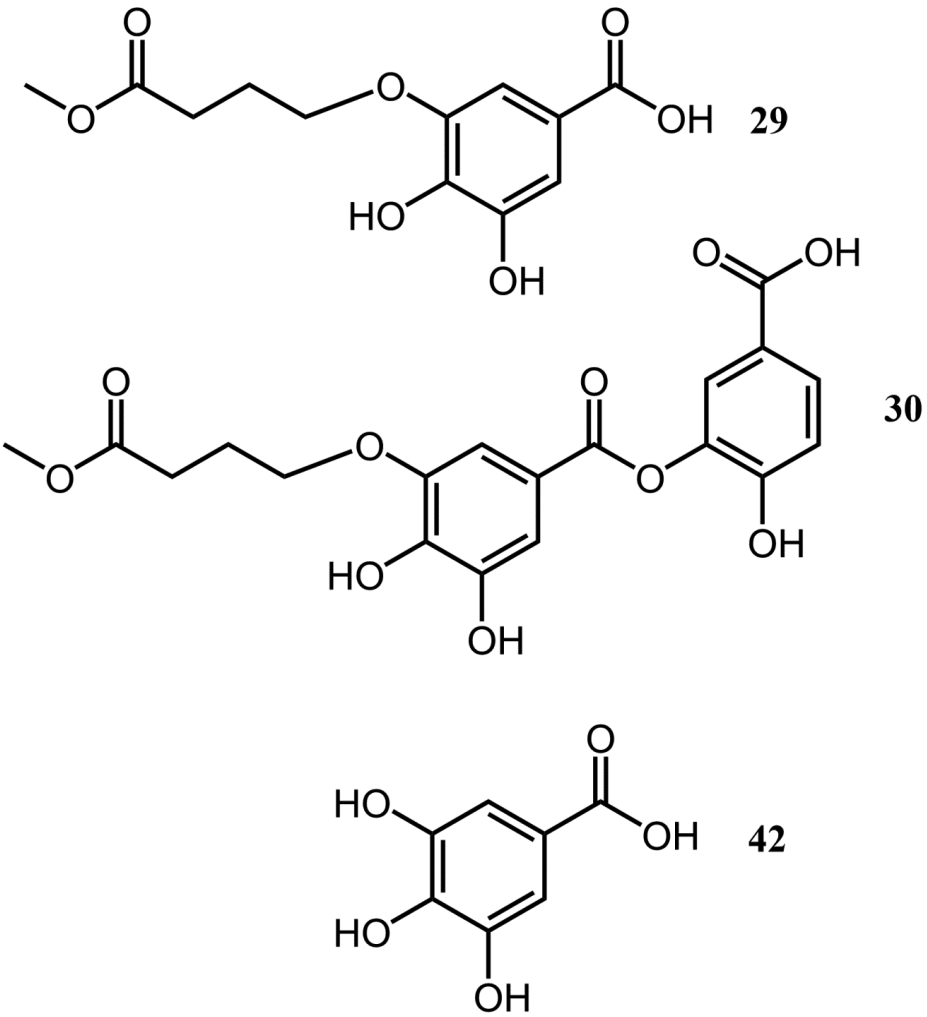


Figure 22. Structures of the two novel natural products 3-(3′-carbomethoxypropyl)gallic acid **(29)** and 3-(3′-carbomethoxypropyl)-7→3″-protocatechoyl galloate **(30)**, in addition to gallic acid **(42)**.

Table 12 gives ^{1}H and ^{13}C chemical shift values and coupling constants for 3-(3′-carbomethoxypropyl)gallic acid (**29**) and 3-(3′-carbomethoxypropyl)-7→3″-protocatechoyl galloate (**30**)**.** NMR data for gallic acid (**42**) and syringic acid (**44**) are found in Table 19.

In current literature gallic acid is described as a potent radical scavenger [78]. As a part of this study the radical scavenging activity for both 3-(3'-carbomethoxypropyl) gallic acid (**29**) and gallic acid (**42**) were determined with the DPPH assay as described in chapter 3.5.2. The calculated IC_{50} values were 27.7 μM for gallic acid and 222 μM for 3-(3'-carbomethoxypropyl) gallic acid. Thus it appears that substitution of the 3-hydroxyl of gallic acid with the methyl ester of γ-hydroyx butyric acid results in a significant reduction of radical scavenging properties when compared with gallic acid [Paper II].

Syringic acid (4-hydroxy-3, 5-dimethoxybenzoic acid) **(44)** was isolated from the leaves of *V*. *album* . A mixture of 1D and 2D ^{1}H and ^{13}C NMR techniques were used to identify the compound. Syringic acid has previously been identified from leaves of *V*. *album* growing on *Sorbus aucuparia L*., *Acer platanoides L*., *Malus domestica L*., *Pyrus communis L*. and in trace amounts when the host tree was *Populus nigra L*. However syringic acid was not detected when *Quercus robur L*. was the host tree. Qualitative and quantitative analysis were performed on a HPLC system equipped with an UV-detector [79]. Chemical shift values as described in Table 19 for syringic acid were in accordance with values in the current literature [80, 81].

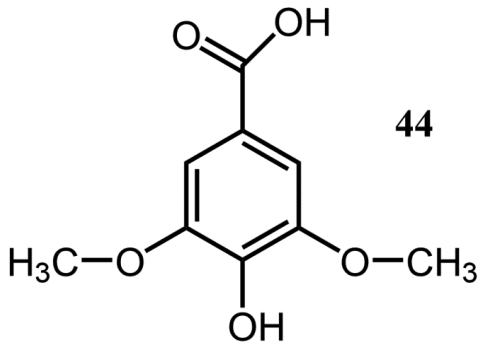
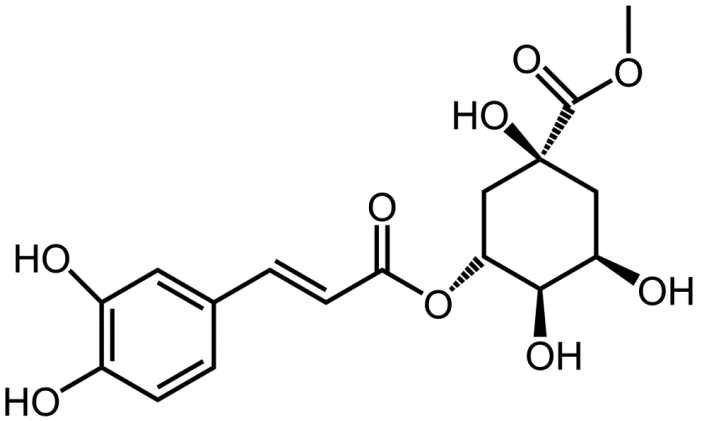


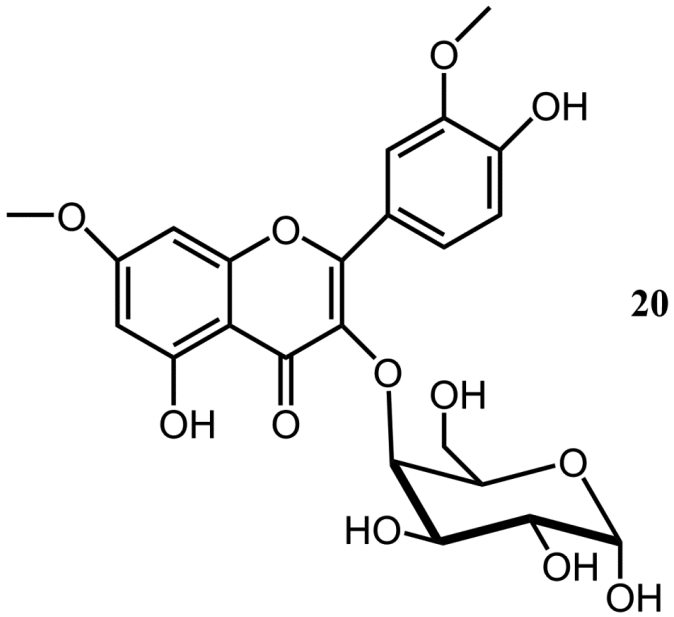
Figure 23. Syringic acid **(44)** isolated and identified from leaves of *V*. *album*.



35

Figure 24. Chlorogenic acid methyl ester **(35)** isolated and identified from the leaves of *V*. *album*.

Chlorogenic acid methyl ester is grouped with phenolic acids and their derivatives in Table 1. Chemical shift values and coupling constants for ^{1}H - and ^{13}C NMR for this compound isolated from the leaves of *V*. *album* are found in Table 15.



20

Figure 25. Rhamnazin-3-glucoside (**20**) isolated and identified from leaves of *V*. *album*.

Table 6 provide ^{1}H- and ^{13}C NMR chemical shifts and coupling constants for the known flavonol rhamnazin-3-glucoside. Nearly fifty years ago Ohta and

Yagishita reported the identification of rhamnazin-3-*O*-mono-D-glucoside from the leaves of a subspecies of mistletoe, namely *V*. *album* L var. *coloratum* Ohwi, also known as Japanese mistletoe [82].

4.2 A novel dihydrostilebenoid glucoside and seven other natural products isolated from dawn redwood (*Metasequoia glyptostroboides Hu & Cheng*)

M. *glyptostroboides* is a deciduous conifer and the only surviving species in the genus Metasequoia of the Cupressaceae family [83]. The tree is endemic to southeast China were native trees can be found in a large area covering an estimated 800 – 1000 km^2 within the western Hubei and Hunan provinces and the eastern Chongqing municipality [84-89]. The largest population of native trees is found in the Shui-Hsa river valley (also known as Xiaohe river vally) [84]. Since the discovery of *M*. *glyptostroboides* as a living species in 1948 it has been cultivated and grown in nearly 50 countries on four continents (Asia, America, Africa and Europe) [90].

Figure 26. *M*. *glyptostroboides* growing in the botanical garden of the University of Bergen 2013 Photo: Ole-Johan Juvik.

Natural products from this tree are of interest as potential new active principles of medicines because *M*. *glyptostroboides* as a species has resisted attacks from millions of generations of pathogens for approximately 100 million years since the tree first evolved during the Cretaceous period [91]. It should be mentioned that since shortly after the discovery of a living species of Metasequoia it has been questioned if *M*. *glyptostroboides* has remained unchanged at the molecular level. It has also been debated whether or not a present species and one existing millions of years ago should be given identical names [92].

Unsuccessful attempts have been made to recover DNA from millions of years old *M*. *glyptostroboides* needles encapsulated in amber [91]. However, recently a 53.3 ± 0.6 million years old piece of wood and resin from Metasequoia were discovered in the slave province of Canada [93]. Encapsuled in a kimberlite pipe the wood and resin had been preserved. Comparative IR spectral analysis with present *M*. *glyptostroboides* indicated preservation of the molecular composition [93].

Extracts from *M*. *glyptostroboides* have been shown to exhibit antioxidant activity [94, 95], antibacterial effects [96-98], antifungal activity [99], potential antidermatophytic activity [94, 99] and additionally a mixture of flavonoids referred to as total flavonoids from *M*. *glyptostroboides* has been reported to exhibit protective effects on cerebral-ischemia-reperfusion injury in rats [100]. Pure natural products identified from *M*. *glyptostroboides* have been reported to exhibit antioxidant activity [101, 102], inhibition of arachidonic acid metabolism [102], antibiotic activity towards bacteria [103-106], antifungal activity [107], antiviral activity [108] and anticancer activity [109]. Additionally Jung et al. have patented an anticancer drug based on 2, 3-dihydrohinokiflavone [110]. This flavonoid was first discovered from *M*. *glyptostroboides* [111, 112]. Eight natural products were isolated from *M*. *glyptostroboides* grown in Bergen, Norway [Paper III]. Seven of these were characterized from this tree for the first time [Paper III]. One of them was the novel natural product 6-carboxydihydroresveratrol 3-*O*-*β*-glucopyranoside **(28)** shown in Figure 5. This dihydrostilbenoid has been shown to exhibit DPPH-scavenging activity and inhibition of 15-Lipoxygenase [Paper III] [102]. Chemical shift values and coupling constants for this novel natural product are provided in Table 11.

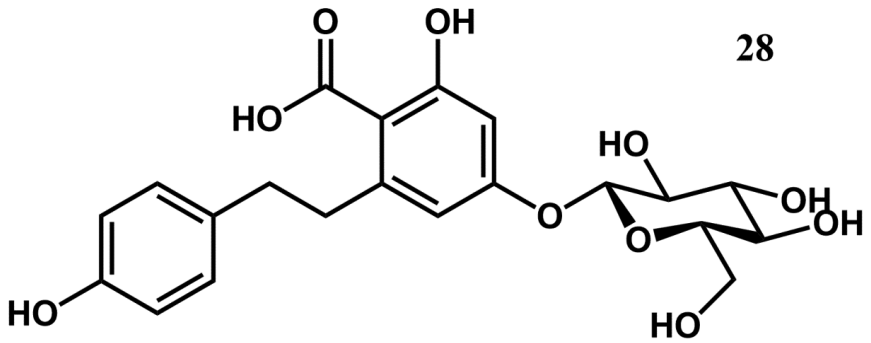


Figure 27. Structures of the novel dihydrostilbenoid 6-carboxydihydroresveratrol 3-*O*-*β*-glucopyranoside **(28)** isolated from *M. glyptostroboides*.

Metasequoia glyptostroboides Hu & Cheng and *Tragopogon porrifolius* subsp. Porrifolius (white salsify) are the only two known sources for the rare aglycone 6-carboxydihydroresveratol (Figure 3) [102, 113]. The glycosyl substituents are different and 6-carboxydihydroresveratrol has hitherto not been reported without a glycosyl subtituent [Paper III] [102].
Additionally two flavonoids namely the dihydroflavonol aromadendrin-7-*O*-*β*-glucopyranoside (**26**) and the flavonol quercetin-3-*O*-*α*-rhamnopyranoside-7-*O*-*β*-glucopyranoside (**27**) (Table 10), one flavanol (gallocatechin) (**19**) (Table 6) and four quinic acid derivatives (3-*O*-(*E*)-coumaroylquinic acid, 3-*O*-(*Z*)-coumaroylquinic acid (Table 17) and their methyl esters (**38-41**) (Table 18) were characterized from *M. glyptostroboides*. Gallocatechin **(19)** has previously been detected from the branches and stems [114] and the bark [95] of *M. glyptostroboides*. The other two flavonoids (**26** and **27)** and all four quinic acid derivatives **(38-41)** were detected in *M. glyptostroboides* for the first time [Paper III] [102].

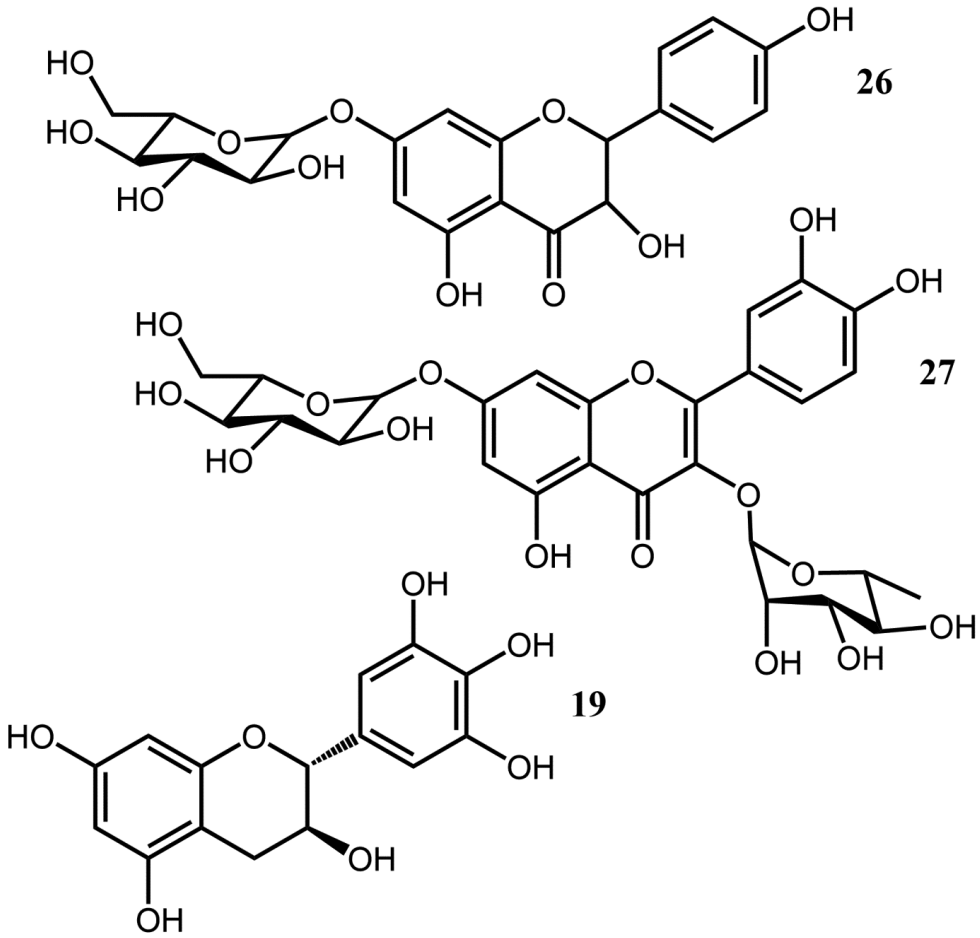


Figure 28. Structures of the three flavonoids characterized from *M. glyptostroboides*. Aromadendrin-7-*O*-*β*-glucopyranoisde **(26)**, quercetin-3-*O*-*α*-rhamnopyranoisde-7-*O*-*β*-glucopyranoside **(27)** and gallocatechin **(19)**.

Table 6 provides ^{1}H and ^{13}C chemical shift values and coupling constants for the flavanol gallocatechin (**19**).

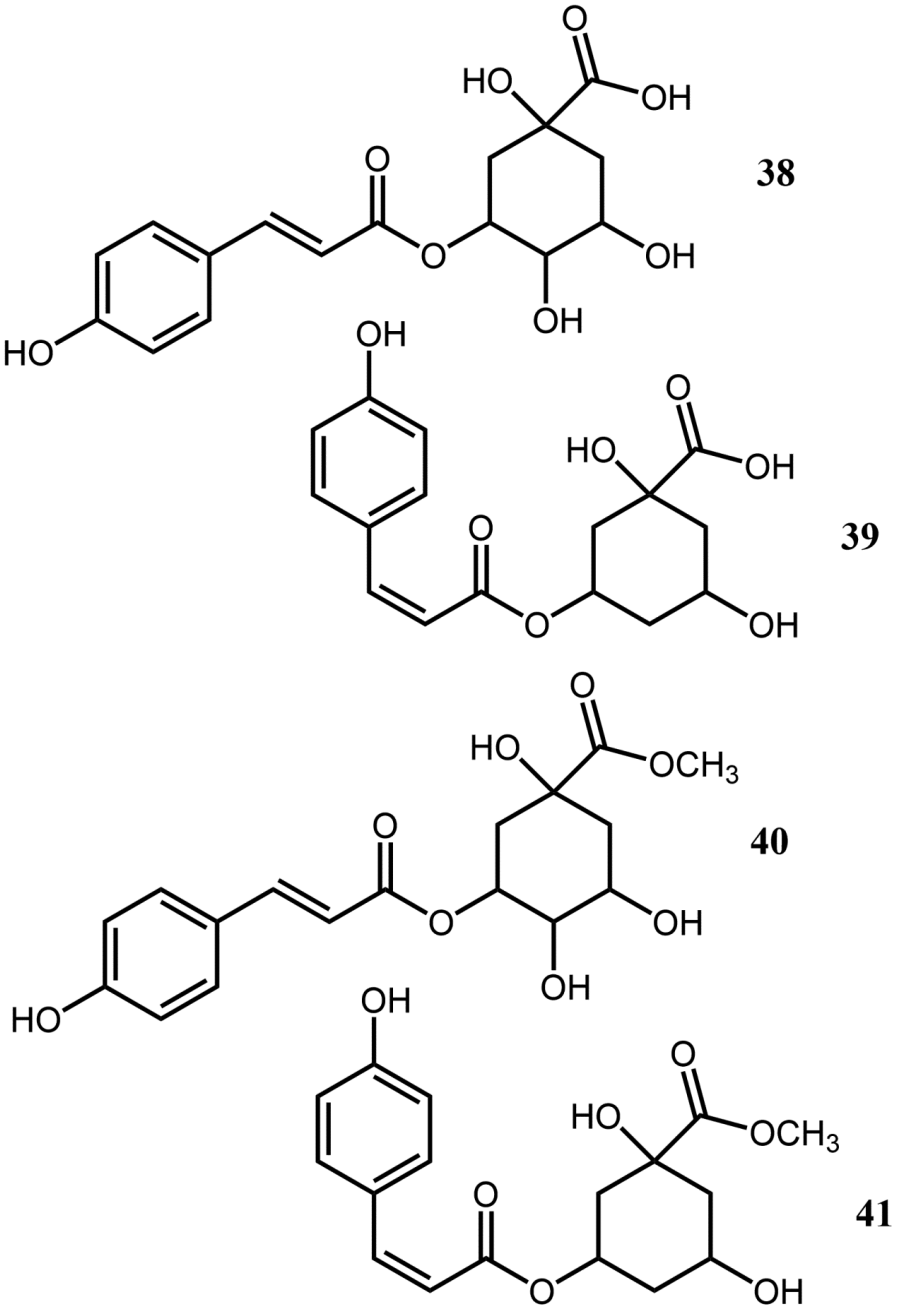


Figure 29. Structures of the quinic acid derivatives 3-O-(*E*)-coumaroylquinic acid **(38)**, 3-O-(*Z*)-coumaroylquinic acid **(39)**, 3-O-(*E*)-coumaroylquinic acid methyl ester **(40)** and 3-O-(*Z*)-coumaroylquinic acid methyl ester **(41)** characterized from the leaves of *M*. *glyptostroboides*.

Paper IV reviews all natural products reported from *M*. *glyptostroboides* during six decades (1954-2014) of phytochemical investigation of this relict of the cretaceous period [115].

Altogether 362 natural products have been reported from *M*. *glyptostroboides* [115]. This number is approximate since there were four cases including eight compounds where the terminology in the original research papers was not clear and structures of the compounds were not provided [96, 116-119]. If all these four cases are examples of double reporting this would reduce the number of reported natural products to 358. At the time of identification twenty-six natural products were unique to *M*. *glyptostroboides*. Some of these have later been identified from other sources. Numbers in brackets after these twenty-six unique natural products refer to their number in Table 1 of Paper IV [115]. Further details about the compounds are provided in Table 1 in Paper IV.

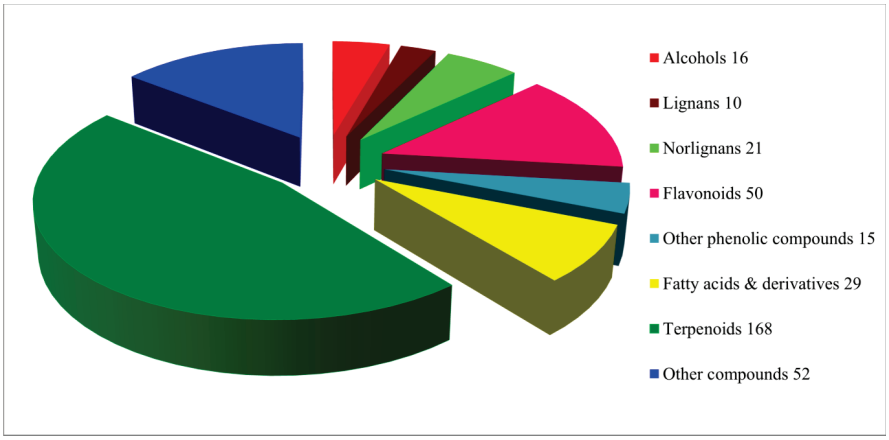


Figure 30. Classification of the 362 natural products identified from *M*. *glyptostroboides* into eight groups according to chemical properties.

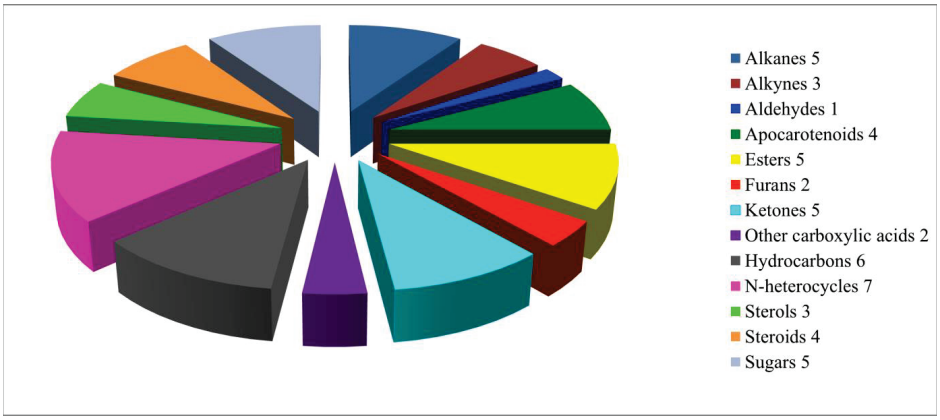


Figure 31. Overview of compounds characterized from *M. glyptostroboides* excluding the two large groups of terpenoids and flavonoids, as well as an amide (1), dihydrostilbenoid (1), aromatic hydrocarbon (1), quinic acid derivatives (4), phenolic compounds (5), phenylpropans (2) and phenylpropens (3). Numbers in brackets refers to the number of compounds in the groups.

According to compound class the 362 reported natural compounds were divided into 27 groups [Paper VI]. Some groups contained only one or a few compounds, which are therefore merged in the overview in Figure 30. The majority of these groups containing a relatively small number of compounds are shown in Figure 31, with some exceptions mentioned in the figure legend (Figure31).

Volatile compounds such as terpenoids constitute a majority (Figure 30) of the compounds reported from this plant source. This may reflect that several investigations of chemical constituents of *M. glyptostroboides* have been performed with GC-MS.

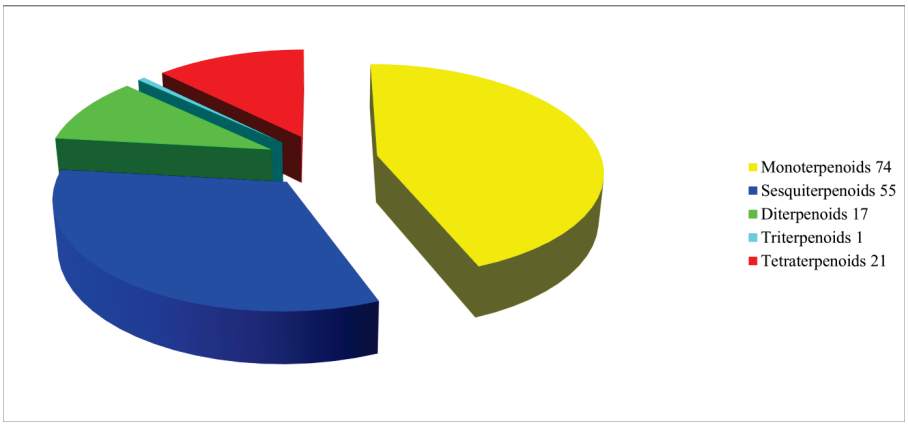


Figure 32. Groups of terpenoids identified from *M*. *glyptostroboides*.

Terpenoids are the largest group of reported natural products from *M*. *glyptostroboides* and constitute 47.7% (Figure 30 and 32) of the total number of reported compounds. All seventy-four monoterpenoids, the single triterpenoid and the twenty-one tetraterpenoids (carotenoids) are known from other plant sources. One of the fifty-five sesquiterpenoids namely (-)acora-2,4(14),8-trien-15-oic acid *(240)* is unique to *M*. *glyptostroboides* [120]. Additionally the six diterpenoids 3*β*-acetoxy-8 (17), 13-E-labdadien-15-oic acid *(297)* [121], 12*α*-hydroxy-8,15-isopimaradien-18-oic acid *(298)*[120], metasequoic acid A-C *(300-302)* [120, 122] and metaseglytorin A *(312)* [120] are unique to this plant source. Totally seven terpenoids are unique to *M*. *glyptostroboides*.

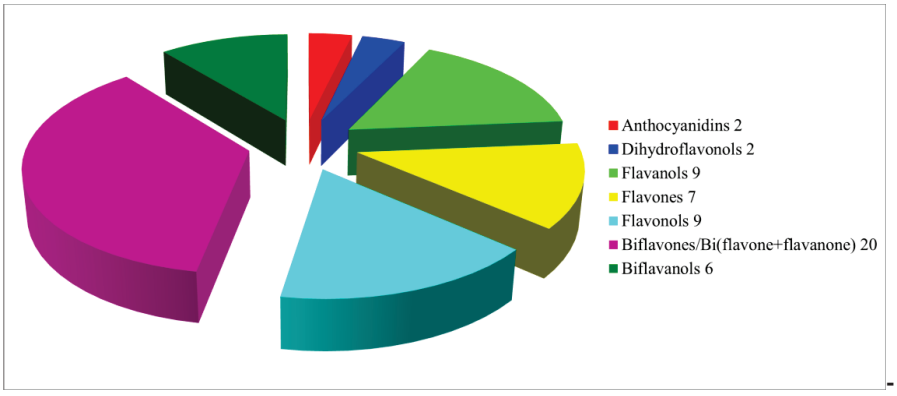


Figure 33. Flavonoid classes according to aglycone type characterized from *M*. *glyptostroboides*.

Figure 33 gives an overview of the number of compounds in each of the seven subgroups according to aglycone of flavonoids identified from *M*.

glyptostroboides. With fifty identified compounds flavonoids are the second largest (13.8%) group of natural products identified from this plant species. More than half of these are dimeric flavonoids of either the biflavone / bi(flavone + flavanone) or the biflavanol type. Three of the biflavone /bi(flavones+flavanone) type flavanoids, namely 2,3-dihydroamentoflavone-7'',4'''-dimethylether *(106)* [111], 2,3-dihydrohinokiflavone *(116)* [111, 112] and 2,3-dihydrosciadopitysin *(118)*[121] were unique to *M*. *glyptostroboides* at the time when they were first reported. Jung et al. have patented an anticancer drug based on 2,3-dihydrohinokiflavone [110].

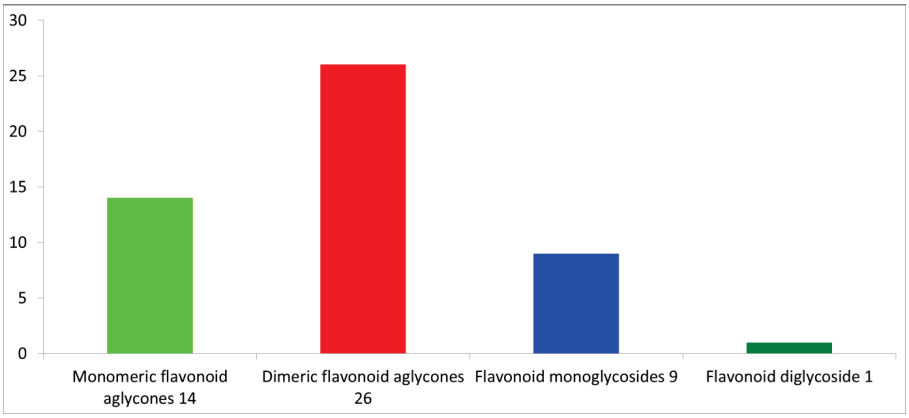


Figure 34. Flavonoid classes characterized from *M*. *glyptostroboides* grouped according to extent of glycosylation.

Eighty percent of the reported flavonoids from *M*. *glyptostroboides* are not glycosylated as can be seen from Figure 34. Only ten (20%) of the reported flavonoids are glycosylated with either glucose (6) or rhamnose (4). Of the flavonoids that are glycosylated the vast majority have only one glycosyl unit while one flavonoid is a diglycoside (Figure 34).

Twenty-one norlignans have been reported from *M*. *glyptostroboides* (Figure 30). More than half of them are unique to this plant species. This may indicate that the biosynthetic pathway for norlignans is relatively unique for *M*. *glyptostroboides*. The eleven norlignans that are unique to *M*. *glyptostroboides* are hydroxyathrotaxin *(143)*[123], metasequirin A *(146)* [124], hydroxymetasequirin A *(147)* [123] and metasequirin B-I *(148-155)* [109, 115, 120, 123, 125].

Metaseol (335) is one of five compounds in the group phenolic compounds which are merged into the wider group other phenolic compounds in Figure 30. Metaseol is both unique to *M*. *glyptostroboides* and the only symmetrical diphenylmethane reported as a natural product [103, 115].

Two phenylpropanoids namely 7-(3-ethoxy-5-methoxyphenyl)propane-7,8,9-triol (339) and 7-(3-hydroxy-5-methoxyphenyl)propane-7,8,9-triol (340) are reported from *M*. *glyptostroboides*. Cytotoxic activity towards the two cell lines A549 and Colo 205 have been reported for these natural products unique to *M*. *glyptostroboides* [109, 115].

Only one dihydrostilbenoid, namely the novel compound 6-carboxydihydroresveratrol-3-glucoside (Figure 27) (31) has been reported from *M*. *glyptostroboides* [102]. Further description is found in Figure 27 as well as in Paper III and IV.

M. *glyptostroboides* has since its sensational rediscovery as a living species often been described as a living fossil. Six decades of phytochemical investigation have resulted in the publication of a large number of natural products from this plant species (362), which have for the first time been reviewed in Paper IV. With twenty-six compounds that were unique to *M*. *glyptostroboides* at the time of identification this tree has proved to be a unique source of an unusually high number of novel natural products. Biological and pharmacological effects such as antibacterial effect, anticancer activity, antifungal and antidermatophytic effects, antioxidant activity, antiviral activity and inhibition of arachidonic acid metabolism have already been reported for a limited number of pure compound or extracts from *M*. *glyptostroboides*. However, the majority of the natural products unique to *M*. *glyptostroboides* have been tested to only a limited extent for their biological activity.

4.3 A novel acylated C-glycosylflavone and six other natural products from *Zamioculcas zamiifolia*

Zamioculcas zamiifolia (*Z. zamiifolia*) Lodd. Engl. is a traditional African medicinal plant [126-129]. Currently it is widely used as an indoor ornamental plant in Europe because it has shiny green leaves, and because it is extremely drought resistant and tolerates poor light conditions. It can therefore endure a lack of regular watering and thrive in households which neglect the care of plants [130].

Figure 35. *Z. zamiifolia* used as an indoor ornamental plant (Left: corridor decoration of nursing home in Kleppestø, Askøy, middle: plants for sale at IKEA in Åsane, Bergen, right: decoration plant in a living room). Photos: Ole-Johan Juvik

Z. zamiifolia is native to at least six countries in tropical east Africa and subtropical southeast Africa (Kenya, Malawi, Mozambique, South-Africa, Tanzania and Zimbabwe) [48, 126-129]. According to Mayo [126] *Z. zamiifolia* is frequently used for medicinal purposes in the Mulanje district of Malawi. Juice from the leaves are used to treat earache in Tanzania in the region of East Usambara mountains [126]. Among the Sukama people in northwestern Tanzania *Z. zamiifolia* is known in the Sukuma language as Ngulukesi [128, 129], and used as a local application to treat ulceration [127]. The inflammatory condition known as “mshipa” is treated with a poultice made from bruised *Z. zamiifolia* in Tanzania [127, 129].

As a member of the Araceae family known for toxic plant species [131, 132] *Z. zamiifolia* has a reputation for being poisonous [133]. In Norway all parents of newly born infants are given an information leaflet about poisoning from plants published by The Norwegian Directorate of Health [134]. Figure 37 shows a facsimile from the leaflet where *Z. zamiifolia* is described as a plant containing strongly irritating constituents, which can lead to severe irritation and wounds in the mouth. Furthermore it is pointed out that swallowing of parts of the plant may lead to stomach pain, nausea and vomiting [135]. However, there are not referred to any reports about such strongly irritating constituents. Nor are there listed any such references in the leaflet and such references are indeed non-existing in current scientific literature [Paper V].

Figure 36. Facsimile of The Norwegian Directorate of Health's information leaflet about plants and poisoning.

Prior to this investigation there were no reports in current literature dealing with the chemical composition of *Z. zamiifolia* [48].

Figure 37. *Z. zamiifolia*. Live plants photographed before harvesting. Photos: MSc Angharad Le Moullec.

Both leaves (91 %) and petioles (95 %) have very high water content [48]. This is in accordance with the fact that succulents are known to store water in petioles and rhizomes in order to survive in very dry habitats [132]. This was, to the best of our knowledge, the first time the water content of *Z. zamiifolia* has been determined and reported.

Figure 38. Petioles (left) and leaves (right) prepared for extraction with methanol. Photos: MSc Angharad Le Moullec.

Different retention times for the major peaks in the HPLC chromatograms of the crude extracts from the leaves and petioles indicated that the major aromatic compound of the leaves and the petioles were different. A HP 1050 multidiode array detector was used for recording at 280 nm. Gradient profile for the two solvents (water-TFA (99.5:0.5; v/v) and methanol-TFA (99.5:0.5; v/v)) used for elution is shown.

Five known aromatic compounds were isolated and characterized from the petioles (Figure 38). They were identified as rosmarinic acid (**43**) (Table 15), protocatechuic aldehyde (**12**) (Table 4), (*E*)-caffeic acid (**32**) and (*E*) - and (*Z*)-caffeic acid methyl ester (**33** and **34**) (Table 14). Rosmarinic acid was the main aromatic compound identified from the petioles of *Z. zamiifolia* (See frame B of Figure 8). Since methanol was used as extraction solvent and utilised during the isolation process it cannot be excluded that (*E*) – and (*Z*)-caffeic acid methyl ester are artefacts formed during the isolation process.

Two aromatic compounds were isolated and characterized from the leaves of *Z. zamiifolia*. Apigenin-6-C-(6´´-*O*-(3-hydroxy-3-methylglutaroyl)-*β*-glucopyranoside) (**21**) was identified as the main aromatic compound of the leaves (See frame A of Figure 8). Table 7 provide ^{1}H- and ^{13}C NMR date for this novel natural product. In addition, the rare natural product (3R,4S,5S)-2,3,4,5-tetrahydroxytetrahydro-2-H-pyran-2-carboxylic acid (Table 21) was isolated from the leaves of *Z. zamiifolia*. This heterocyclic compound is classified as a pyranosonic acid (See chapter 1.11), and has the systematic name arabino-2-hexulopyranosonic acid.

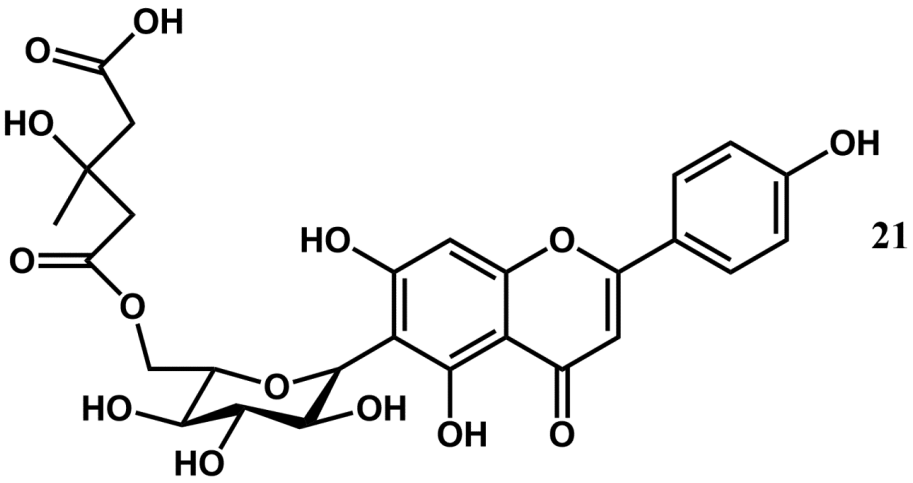


Figure 39. The novel acylated C-glycosylflavone apigenin-6-*C*-(6´´-*O*-(3-hydroxy-3-methylglutaroyl)-*β*-glucopyranoside) (**21**) the major flavonoid isolated from the leaves of *Z. zamiifolia*.

This is to the best of our knowledge the first time an acylated C-glycosylflavone has been isolated from species in the Araceae family. Flavone C-glycosides,

which are not acylated, are characteristic components of leaves in the Araceae family [136].

Williams et al. have reviewed the phenolic and flavonoid content of several species in the Araceae family. *C*-glycosylflavones, flavonol *O*-glycosides and structurally simpler phenolic compounds such as caffeic- and chlorogenic acid are some of the listed compounds [136, 137].

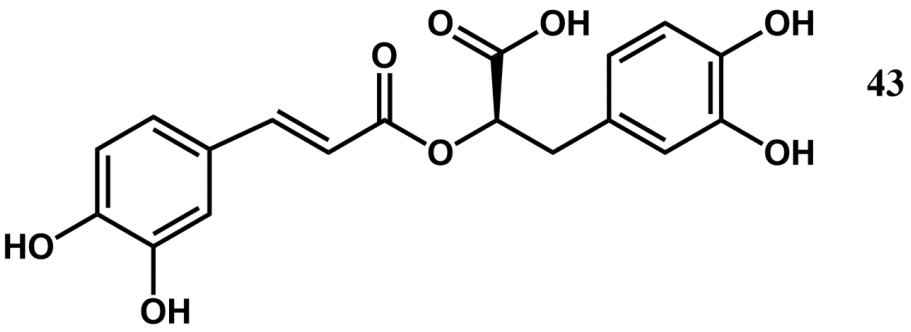


Figure 40. Rosmarinic acid **(43)**, the major aromatic compound isolated from the petioles of *Z. zamiifolia*.

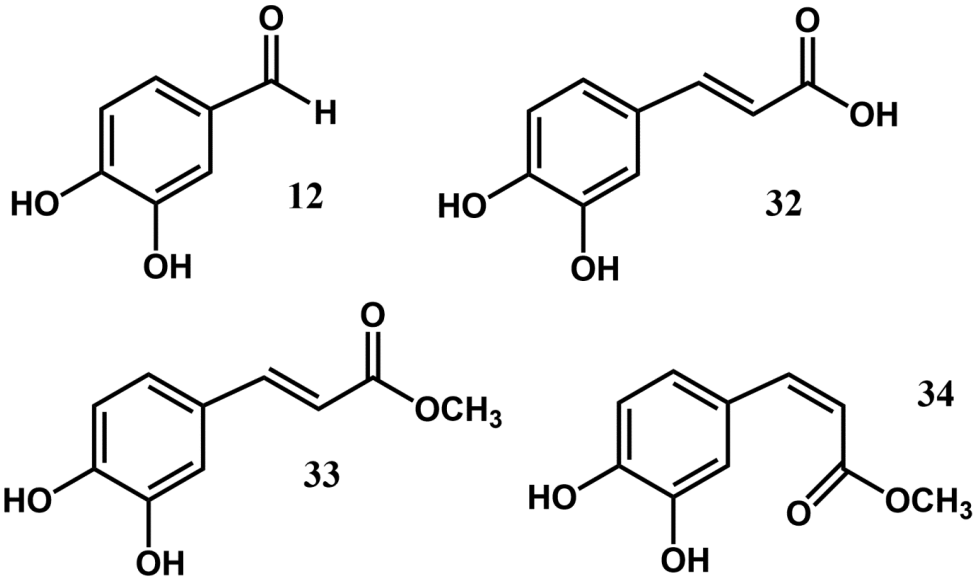


Figure 41. Protecatechuic aldehyde **(12)** and the three quinic acid derivatives (*E*)-caffeic acid **(32)** and (*E*) - and (*Z*)-caffeic acid methyl esters **(33** and **34** respectively) isolated from the petioles of *Z. zamiifolia*.

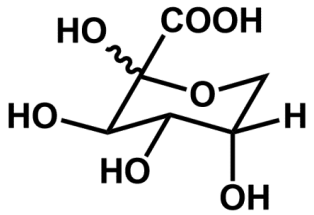


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Figure42. The rare natural product (3R,4S,5S)-2,3,4,5-tetrahydroxytetrahydro-2-H-pyran-2-carboxylic acid (**46**)isolated from the leaves of *Z. zamiifolia*.

It is well known that several species in the Araceae family contains toxic compounds. They are mainly cyanogenic glycosides, calcium oxalate and possibly alkaloids [131].

Brine shrimp lethality assay was used to evaluate the toxicity of crude extracts from leaves and petioles from *Z. zamiifolia*. Since the plant has a reputation for being toxic and the applied concentrations were ranging up to 1 mg/mL a low LD_{50} and a corresponding high mortality rate could be expected. However, the results showed no lethality for *A. salina* when exposed to any of the applied concentrations. Furthermore it appeared that exposure to the extracts improved the vitality of the larvae when compared to the unexposed control. This result indicates that *Z. zamiifolia* does not contain compounds of lethal toxicity towards *A. salina*. Improved vitality of *A. salina* after exposure to the extracts could be explained by presence of nutrients including free sugars in the extracts.

4.4 Twenty non-polar natural products from three Bromeliaceae spp.

Bromeliads are a large family of flowering plants. With the exception of one species which grows in western Africa all of the approximately 3200 species in the Bromeliaceae family are endemic to the tropical Americas. Currently the family is divided into 8 subfamilies [138].

Figure 43. Three Bromeliaceae spp. From left to right: *B*. *laciniosa*, *N*. *variegata* and *E*. *spectabile*. Photos: Professor Jackson Roberto Guedes da Silva Almeida.

Very limited authorative information about the chemical composition of these three Bromeliacee species was available prior to this investigation. For *E*. *spectabile* and *N*. *variegata* no natural products were previously reported in the current literature. From *B*. *lacinosa* one single flavonol namely quercetin 3,3',4'-trimethyl ether has been characterized from this plant species [139].

Three monosaccharides, namely galactose, arabinose and xylose have been detected in the gum from *B*. *lacinosa*. Additionally the gum also contained an acidic oligosaccharide composed of xylose and galacturonic acid [140].

Altogether twenty natural products were identified by a combination of HRMS and 2D NMR techniques from the three Bromeliaceae species *B*. *laciniosa* , *N*. *variegata* and *E*. *spectabile* [Paper VI]. This work has thereby significantly increased the knowledge about the chemical composition of these plant species compared to the prior level of knowledge.

Figure 44. *B*. *laciniosa* Photo Professor J. R. G. da Silva Almeida

B. *laciniosa* belongs to the Bromelioideae subfamily of Bromeliaceae and is found in both Brazil and Argentina. This Bromeliad species has been used for medicinal purposes [141, 142], as food for humans [142, 143] and as fodder for livestock [143-145]. In current literature it is reported that the leaves are rich in calcium (1.1%), proteins (4.9%) and starch (2.8%) [146]. Masses extracted from the base of the leaves can be used to make a kind of bread [143] or it is dried and powdered before mixing into food [142]. Dandruff, diarrhea, fever, infantile colic, intestinal diseases, jaundice and hepatitis are conditions for which *B*. *lacinosa* has been used as a medicinal plant [141, 142].

Figure 45. *N*. *variegata* Photo Professor J. R. G. S. Almeida

N. *variegata* also belongs to the Bromelioidea subfamily of Bromeliaceae. This species is endemic to northeastern Brazil where it traditionally has been used as a source for white, long and soft fibres used for handicrafts such as ropes and hammocks [147-150]. Edible fleshy fruits are produced by *N*. *variegata* at the beginning of the rainy season [151]. The literature reports antibacterial effect against gram negative [148, 152] and gram positive bacteria [148], antioxidant effect [148, 153], antinociceptive effect in experimental models in mice [153], gastroprotective effects in a mice model of gastric ulcer [154], photoprotective potential [155] and low toxicity in mice models [153]. Although named after the French landscape architect and botanist Auguste Francois Marie Glaziou (1828-1906) the species was first described by the Brazillian Manuel Arruda da Câmara (1752-1810) together with other fiber-producing plants of Brazil in 1810 [Paper VI].

Figure 46. *N*. *variegata* Photo Professor J. R. G. S. Almeida

E. *spectabile* belongs to the Pitcairnioidea subfamily of Bromeliaceae, and it is endemic to Brazil. Carbohydrates (28.7%), lipids (0.8%) and proteins (0,7%) are found in the leaf base of *E*. *spectabile* which is the edible part of the plant. Couscous can be made from the leaf base of the plant and is used as a food source during famine emergencies [156]. With a caloric value of 124.6 kcal/100g *E*. *spectabile* is comparable with rice (*Oryza sativa* L.) which is 130 kcal/100g [156]. Antibacterial activity towards Gram negative [152, 157] and Gram positive bacteria [157], antinociceptive activity in mice models [158], antioxidant [157, 159] and no signs of toxicity in mice models [158] is reported for extracts from *E*. *spectabile* in the current literature.

Figure 47. Extraction of *B*. *laciniosa* (left), *E*. *spectabile* (middle) and *N*. *variegata* (right) with isomers of hexane.

Dried leaves of the three Bromeliace spp. were extracted with hexane. The extracts were concentrated with a rotary evaporator prior to investigation on GC-HRMS.

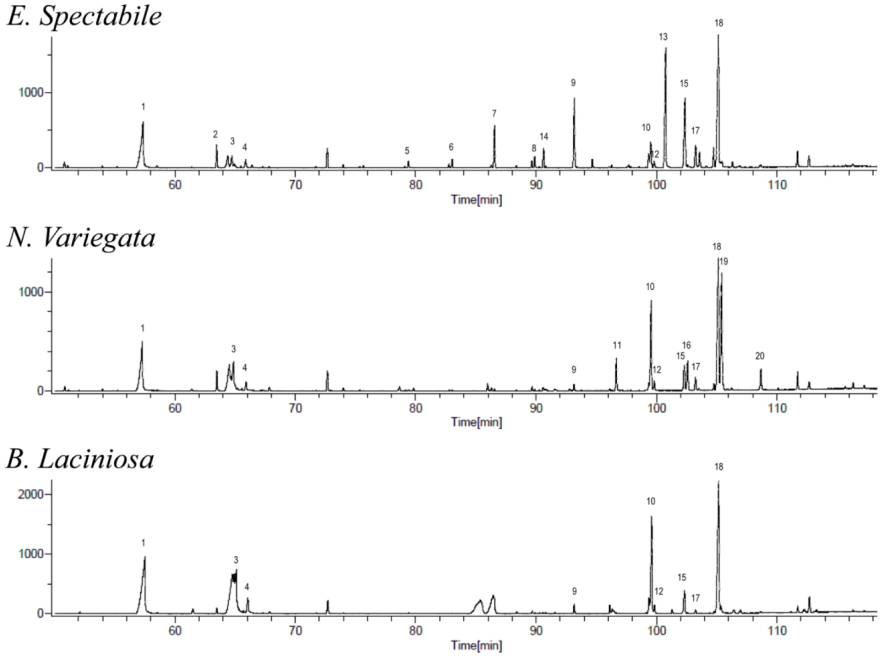

Figure 48. Comparison of Total Ion Chromatograms (TIC) comparison for the three extracts. Numbering in the figure refers to the number of these compounds given in Paper VI.

The ion chromatograms shown in figure 48 show the readily noticeable similarity of the three extracts. Nine compounds **(9, 10, 13, 15, 16, 47, 50,**

52 and 53), or nearly half of the identified compounds, were identified from all three species. This includes the plant sterol *β*-sitosterol, which is the main compound identified in all three extracts. However, differences are also observed. Eleven of the compounds **(1, 2, 34, 14, 45, 48, 49, 51, 54 and 55)**, or more than half, were only identified from one plant species. Below, all twenty compounds will be dealt with according to their structural group and differences between the three species will be described in further detail.

Figure 49. Six very long chain alkanes (n-pentacosane **(1)**, n-hexacosane **(2)**, n-heptacosane **(3)**, n-octacosane **(4)**, n-nonacosane **(5)** and n-triacontane **(6)**) identified from Bromeliaceae spp.

All the six very long chain alkanes **(1, 2, 3, 4, 5 and 6)** were identified from the leaves of *E. spectabile*. Only the two longest alkanes, namely n-nonacosane **(5)** and n-triacontane **(6)** were identified from *B. lacinosa* and *N. variegata*. Leaves of *B. lacinosa* and the leaf base of *E. spectabile* are used as a supplementary food source by rural communities in the Caatinga region of Brazil particularly at times of drought and threat of famine [143, 156, 160]. In vivo very long chain alkanes are converted to long chain alcohols by hydroxylation at several positions by cytochrome P450 enzymes [2]. Intake of plant waxes is associated with a cholesterol-lowering effect [1]. Through their long chain alcohol metabolites very long chain alkanes may contribute to the above mentioned cholesterol-lowering effect.

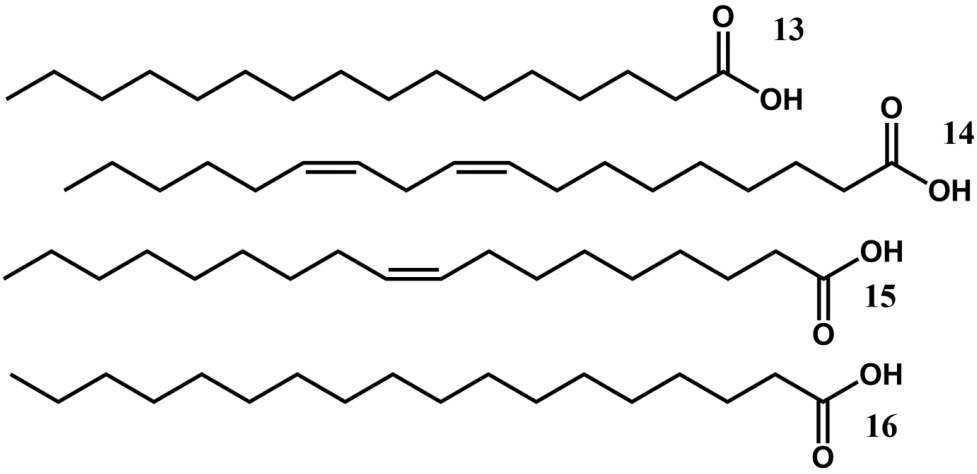

Figure 50. The four fatty acids n-hexadecanoic acid **(13)**, octadeca-(9, 12)dienonoic acid **(14)**, (9*Z*)-octadec-9-enoic acid **(15)** and octadecanoic acid **(16)** identified from Bromeliaceae spp.

All four fatty acids including octadeca-(9,12)-dienoic acid **(14)** were identified from E. spectabile which is at times used for human alimentation. From the other two species (*B*. *laciniosa* and *N*. *variegata*) three **(13, 15 and 16)** of the four fatty acids were identified. All four fatty acids are commonly found in plants of importance in the human diet such as wheat [13].

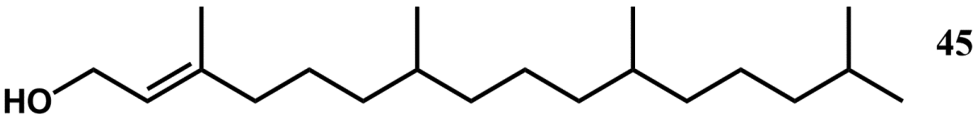

Figure 51. Phytol **(45)** identified from the leaves of *Encholirium spectabile*.

Phytol is also known as (2E,7R,11R)-3,7,11,15-tetramethyl-2-hexadecen-1-ol and was only identified with both HRMS and 2D NMR from *E*. *spectabile* (Table 1 and 20). In the human diet phytol is found in commonly used food plants such as beans, spinach, asparagus and raw vegetables [23, 161]. Phytanic acid is a metabolite of phytol [24] and accumulation of phytanic acid in plasma and tissue occurs in patients with Refsum's disease [25]. However, the role of phytol in the diet as a precursor of phytanic acid in humans is not clear [24].

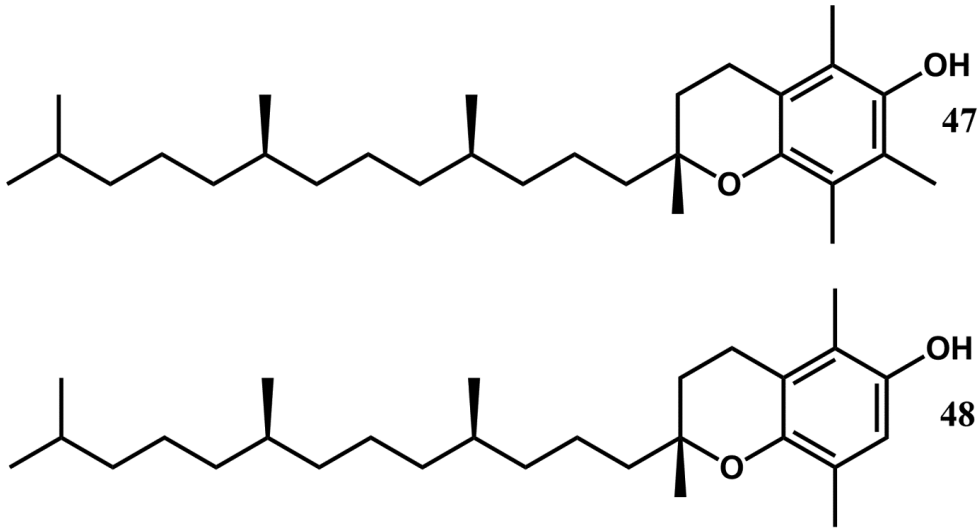

Figure 52. *α*- and *β*-tocopherol (**47** and **48** respectively) identified from leaves of Bromeliaceae spp.

Both *α*- and *β*-tocopherol (**47** and **48** respectively) were identified from *N*. *variegata*. *β*-tocopherol (**48**) was only found in *N*. *variegata*. The finding of an active form of Vitamin E in plants used both as human food (*B*. *lacinosa* and *E*. *spectabile*) and as fodder for livestock (*B*. *lacinosa*) underlines the nutritional value of these plants.

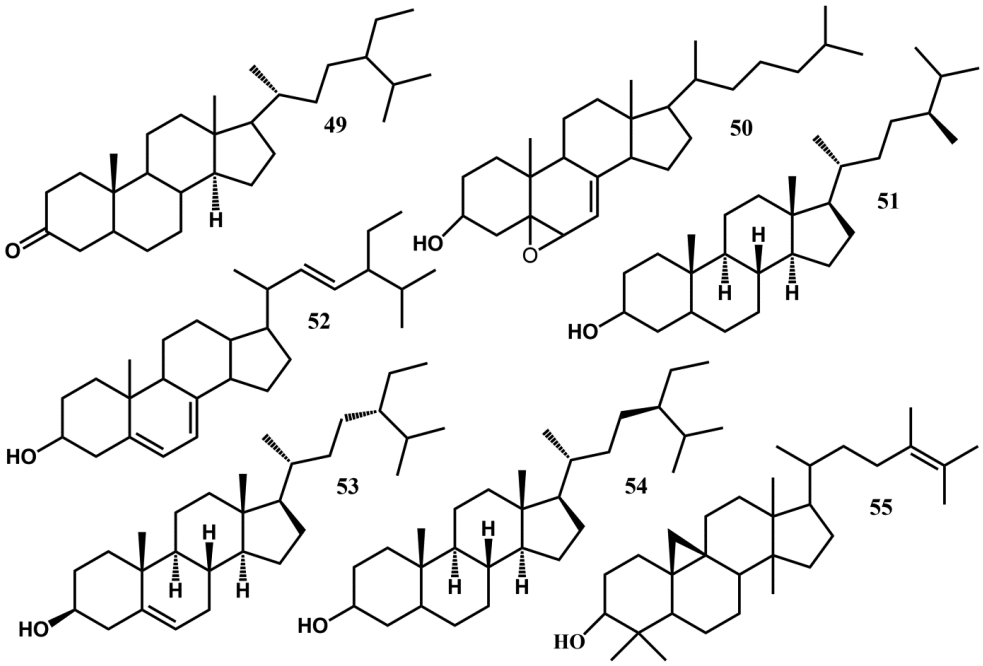

Figure 53 Seven triterpenoids and their derivatives (stigmastan-3-one (**49**), campesterol ((3*β*, 24 R)-ergost-5-en-3-ol) (**50**), ergostanol (**51**), stigmasta-4,22-dien-3-*β*-ol (**52**), *β*-sitosterol (**53**), stigmastanol (**54**) and 24-methyl-3-*β*-9,19-cyclolanost-24-en-3-ol (**55**)) identified from the leaves of Bromeliaceae spp.

Altogether seven triterpenoids and their derivatives were identified from the extracts. Three of these (**50, 52 and 53**) were identified from all the three extracts. Another three compounds (**51, 54 and 55**) were only identified from *N. variegata*. One compound (49) was only identified from *E. spectabile*.
The cholesterol-lowering effect of phytosterols such as *β*-sitosterol is well known [referanse 36-38]. Identification of phytosterols such as campesterol (**50**) and β-sitosterol (**53**) commonly found in edible plants strengthens the information about the nutritional value of these plant species. In fact *β*-sitosterol (**53**) was the main compound in all the three examined extracts (Table 1 and 20). The three peaks numbered 18 (numbering from Paper VI) in Figure 41 represents *β*-sitosterol (**53**)**.**

3.5 Novel aromatic compounds from *Narthecium ossifragum L.*

Figure 54. Flowers (left) and fruits (right) of *N. ossifragum* L.
Photos: Professor Svein Haavik (left) and Professor Torgils Fossen (right).

In summer from July to August *N. ossifragum* blooms [162] with bright yellow flowers [163]. The plant is perennial and grows to a height of 10-30 cm. Capsular red orange fruits are formed after flowering. At the same time the colour of the stems change from green to deep red orange. The leaves partially change colour from green to light orange [164].

N. ossifragum owes its name to a 350 year old reputation as a grass that causes the legs of domestic cattle to break first reported by Simon Paulli in 1667 [165]. In 1641 Jens Bjelke (1580-1659), at the time Chancellor of Norway (1614-1648), sent a letter with a description of the plant and a specimen to S. Paulli (1603-1680). Bjelke wrote that if cattle ate this plant they would become unable to walk and seem completely powerless. Bjelke therefore named the plant "Gramen ossifragum" which translates to the grass that breaks bones. Paulli used the information from Bjelke when he reported on the plant and its potential harmful effects in his work on medicinal plants published in 1667 [165]. Later Carl von Linnè systematically classified the species [166]. It is now known that *N. ossifragum* prevalently is found in soils deficient of calcium. Bog asphodel is

the common name for *N*. *ossifragum*. This offers a new explanation of the association between the plant and the breaking of bones of domestic livestock. Animals which graze in areas where *N*. *ossifragum* grows may suffer from calcium deficiency because of the low calcium levels in soil and fodder. A weakening of the skeleton and ultimately an inclination to break the bones may be the consequence [Paper VII].

Poisoning of livestock is both an animal welfare and an economically important problem, especially in the Nordic countries [167]. Intake of *N*. *ossifragum* is the suspected cause of a disease of photodynamic nature in lambs known as "Alveld" in Norwegian ("the elf's fire") [168]. Current literature does not provide an explanation at the molecular level for the observed phototoxic effects associated with intake of *N*. *ossifragum*. Hypotheses about the connection are that it is caused by metabolite(s) from the plant itself [169], or an associated fungus[170] or cyanobacteria [171].

Twelve aromatic compounds were isolated from the fruits of *N*. *ossifragum* for the first time. Six novel natural products (**10**, **11**, **17**, **18**, **31** and **36**) were characterized along with five known compounds (**22**, **23**, **24**, **25** and **37**) and one rare natural product previously known only as a fungal metabolite (**9**). Previously carotenoids [172], furanolactones [173-175], saponins [172-174] and sterols and their derivatives [168, 176-178] have been reported from *N*. *ossifragum*.

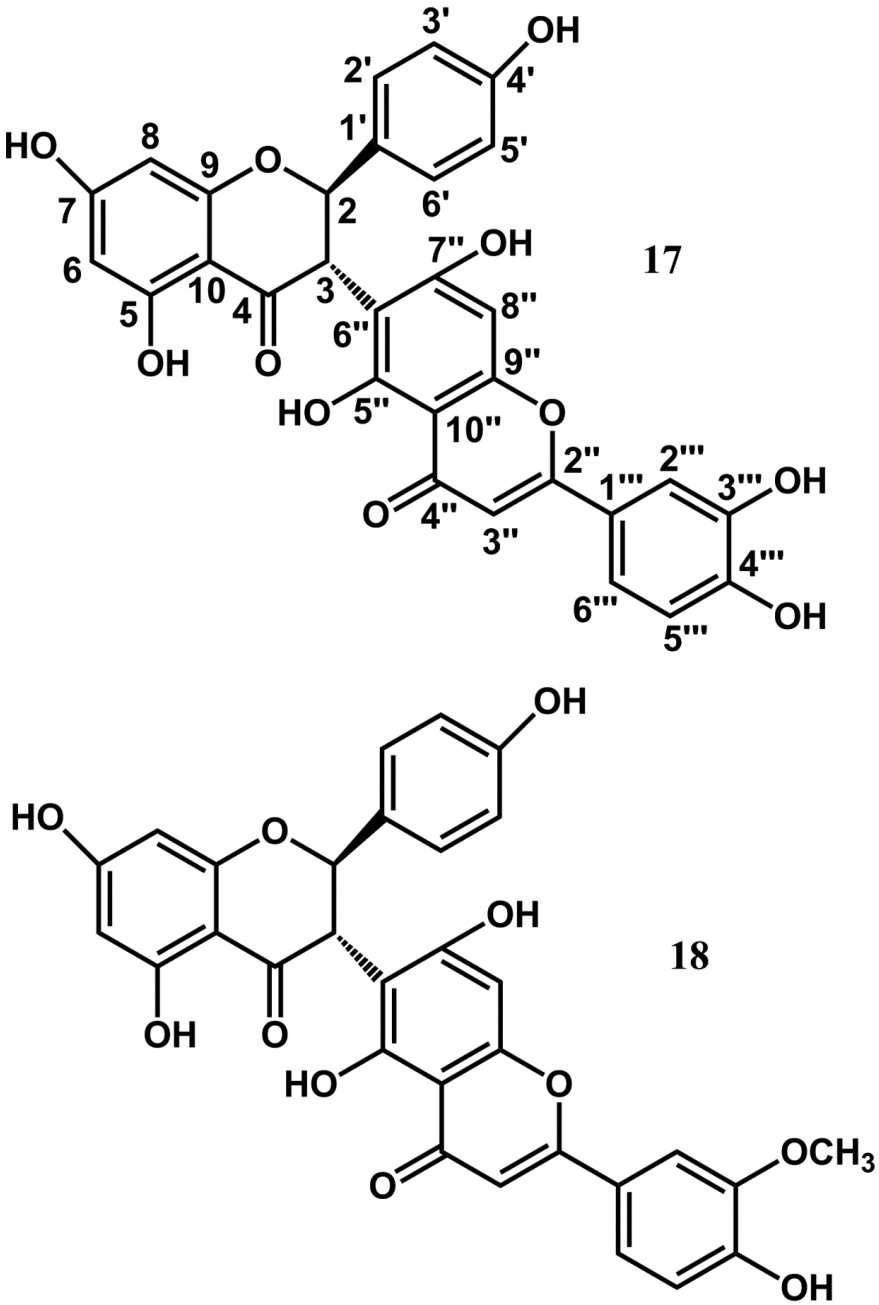

Figure 55. The two novel biflavonoids (2R, 3S)-naringenin(3→6``)luteolin **(17)** and (2R, 3S)-naringenin(3→6``)chrysoeriol **(18)** isolated from the fruits of *N. ossifragum*.

Figure 55 shows that the two novel biflavonoids identified from *N. ossifragum* consist of flavanone unit linked with a flavones unit (Table 5). In both compounds the flavanone unit is the same (Naringenin) while the flavone unit differs between the two compounds (Luteolin and Chrysoeriol respectively).

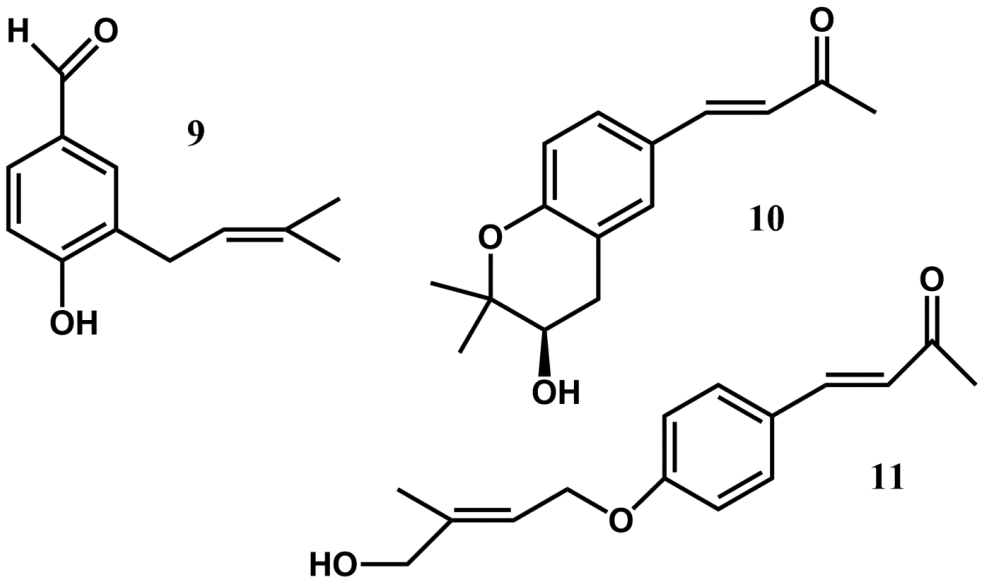

Figure 56. One aromatic aldehyde (4-hydroxy-3-(3-methylbut-2-enyl-)benzaldehyde **(9)**) and two aromatic ketones ((E) -4-(3R-hydroxy-2,2-dimethylchroman-6-yl)but-3-en-2-one) **(10)** and (E)-4-(4-(((E)-4-hydroxy-3-methylbut-2-en-1-yl)oxy)phenyl)but-3-en-2-one **(11)**) isolated from the fruits of *N. ossifragum*.

Previously, the rare natural product 4-hydroxy-3-(3-methylbut-2-enyl-)benzaldehyde (Table 3) has only been described as a fungal metabolite from *Heterobasidion occidentale*, a pathogenic fungus causing root rot in trees [179]. *H. occidentale* is found in western North-America and is one of five species in the fungal species complex *Heterobasidion annosum senso lato* (s.l.) [179]. These fungal species have wide distribution in the Northern Hemisphere, and two of them, namely *H. annosum* s.s. (H.an) and *H. parriporum* (H.pa) are known to be found in Norway [180].

Both ((E)-4-(3R-hydroxy-2,2-dimethylchroman-6-yl)but-3-en-2-one) **(10)** and (E)-4-(4-(((E)-4-hydroxy-3-methylbut-2-en-1-yl)oxy)phenyl)but-3-en-2-one **(11)**) were described for the first time as natural products in Paper VII. Their 1D

and 2D NMR spectra show many similarities (Table 3). Compound **10** has a six-membered heterocyclic ring fused to the aromatic ring while the latter compound (**12**) has an isoprenoid unit as substituent attached to the C4 position of the aromatic ring.

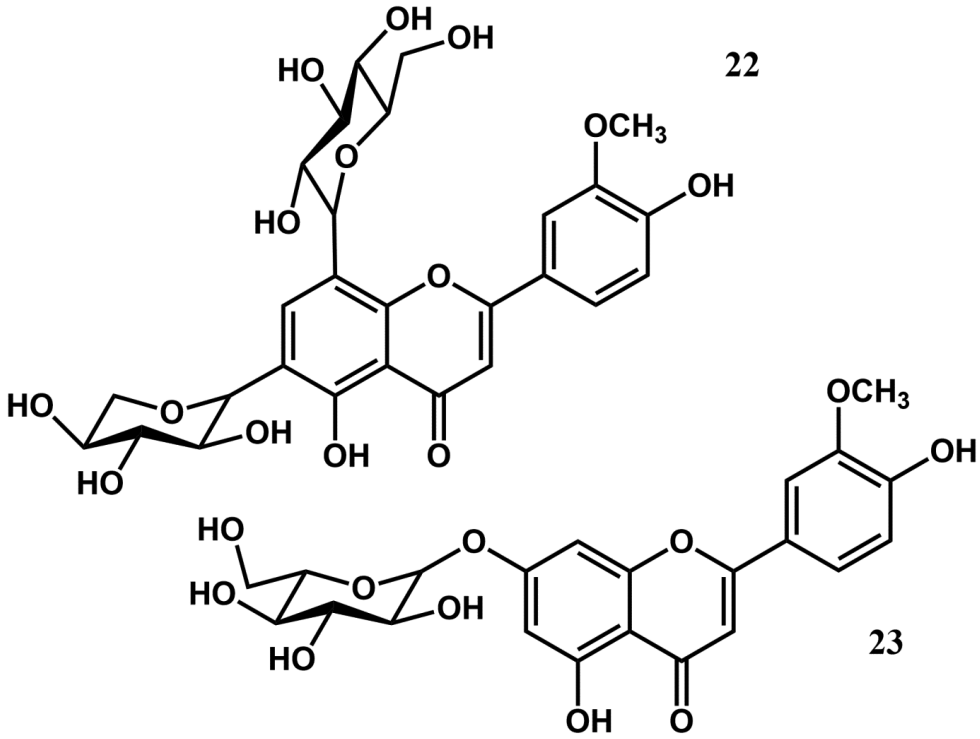

Figure 57. Two flavones isolated from the fruits of *N*. *ossifragum*. Isoschaftoside **(22)** and chrysoeriol-7-O-*β*-glucopyranoside **(23)** are *C*- and *O*-glycosyl flavones respectively.

This is the first time the known compounds isoschaftoside (Table 7) and chrysoeriol-7-*O*-*β*-glucopyranoside (Table 8) are reported from *N*. *ossifragum* [Paper VII].

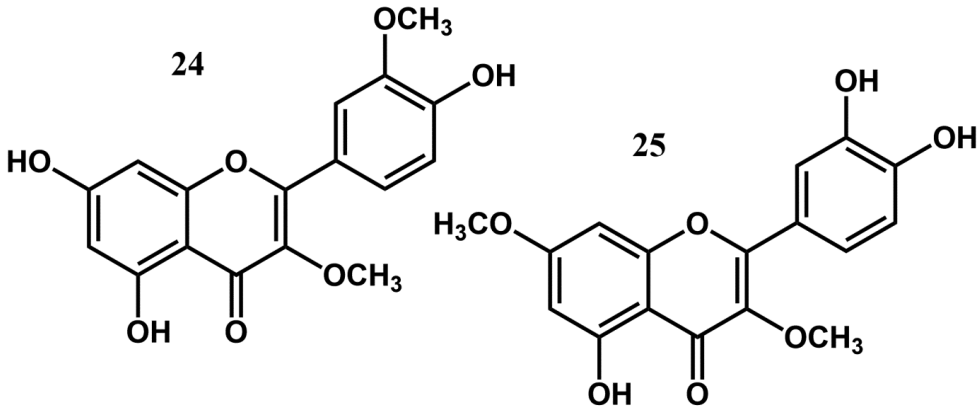

Figure 58. Two flavonols (quercetin-3,3`-dimethyl ether **(24)** and quercetin-3,7-dimethyl ether **(25)**) isolated from the fruits of *N*. *ossifragum*.

Position of one of the methyl substituents distinguishes quercetin-3,3`-dimethyl ether from quercetin-3,7-dimethyl ether (Figure 58 and Table 9). These known compounds were for the first time reported from *N*. *ossifragum* in Paper VII.

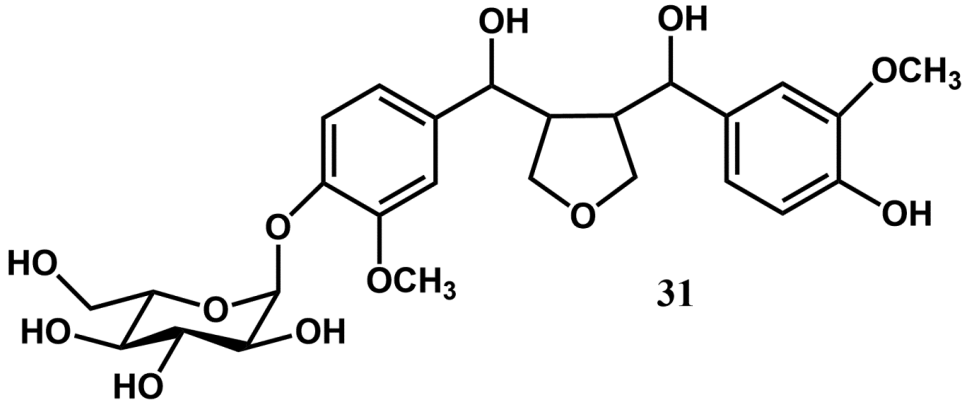

Figure 59. Structure of the novel lignan glycoside liovil-4-*O*-*β*-glucopyranoside **(31)** isolated from the fruits of *N*. *ossifragum*.

Table 13 provides ^{1}H and ^{13}C NMR data for the novel natural product Liovil-4-*O*-*β*-glucopyranoside. Structure elucidation including identification of the liovil and glucose subunits is described in the results and discussion section of Paper VII.

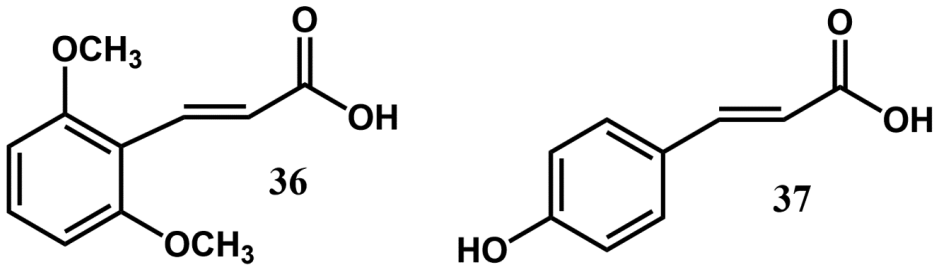

Figure 60. The two phenolic acids 2,6-dimethoxycinnamic acid **(36)** and (*E*)-*p*-coumaric acid **(37)** isolated from the fruits of *N*. *ossifragum*.

For the first time 2,6-dimethoxy cinnamic acid (**36**) (Table 16) is reported as a natural product [Paper VII]. Previously this compound has only been synthesized [181].

(*E*)-p-coumaric acid (Table 16) is well known as a natural product, and for the first time reported from *N*. *ossifragum* in Paper VII.

Brine shrimp lethality assay of naringenin(3→6'')luteolin (Figure 19.) showed that this novel natural product was toxic for *Artemia salina nauplii* with a LC_{50} value of 130 μM [Paper VII].

Naringenin(3→6'')luteolin and the rare natural product 4-hydroxy-3-(3-methylbut-2-enyl)benzaldehyde were shown to be cytotoxic for both MOLM13 acute myeoloid leukemia (AML) and Normal Rat Kidney (NRK) cells [Paper VII]. Plots of concentration versus metabolic conversion of the WST-1 tetrazolium reagent are shown in Figure 62.

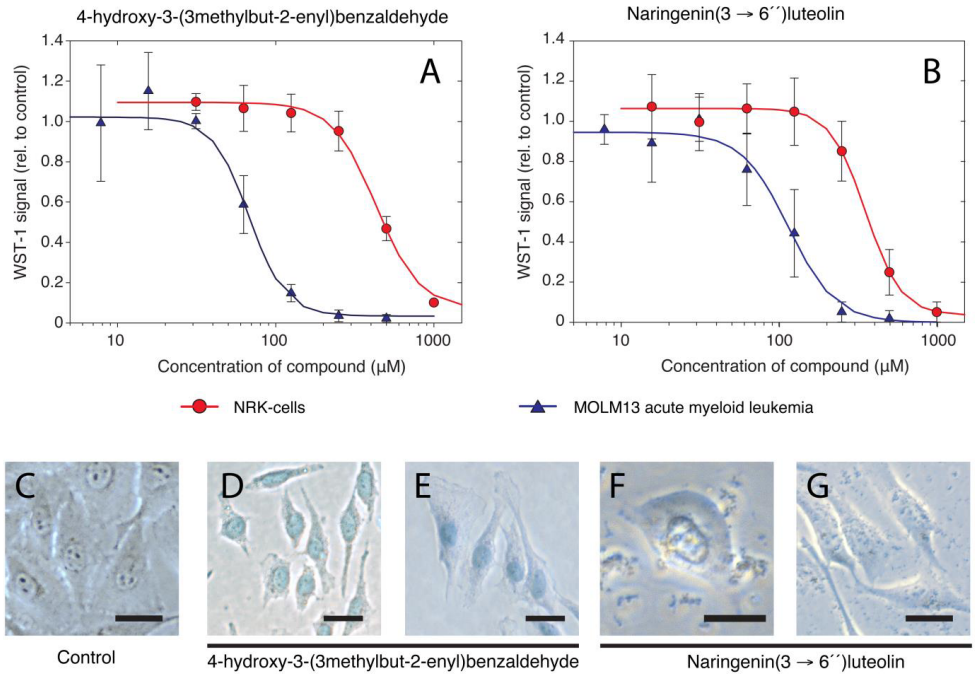

Figure 61. Cytotoxicity test of 4-hydroxy-3(3methylbut-2-enyl)benzaldehyde and naringenin(3→6'')luteolin. Top row: Plot of concentration in µM versus WST-1 signal relative to control. Left (A): 4-hydroxy-3(3methylbut-2-enyl)benzaldehyde. Right(B) naringenin(3→6'')luteolin. A four-parameter regression analysis was used for fitting the curve lines. Measurement was done after 24 h incubation. Bottom row: NRK Cells washed and coloured with Trypan blue after treatment with 0.5 % DMSO (C), 4-hydroxy-3(3methylbut-2-enyl)benzaldehyde (D and E) or naringenin(3→6'')luteolin (F and G) for 24h. Concentrations were 250 µM in E and G, and 500 µM in D and F. Black lines at the bottom of pictures C-G are scale bars which represent 10 µM.
Both compounds had a more potent cytotoxic effect towards MOLM12 AML cells than towerds NRK cells. The rare natural product 4-hydroxy-3-(3-methylbut-2-enyl)benzaldehyde had a IC_{50} value of 430 µM towards NRK cells and the much lower IC_{50} value of 68 µM towards MOLM13 AML cells. Thus this natural product showed selective toxicity towards AML cells. Naringenin(3→6'')luteolin had a IC_{50} value of 230 µM towards NRK cells and a lower value of 115 µm towards MOLM13 AML cells [Paper VII].

In the cells treated with 4-hydroxy-3(3methylbut-2-enyl)benzaldehyde (D and E) the nuclei became stained. This suggest that the membranes of these cells are permeabilized after treatment with 4-hydroxy-3(3methylbut-2-enyl)benzaldehyde. Conversion of WST-1 still took place in these cells, which were metabolically active even after treatment with 4-hydroxy-3(3methylbut-2-enyl)benzaldehyde . Cells treated with naringenin(3→6'')luteolin (F and G) still excluded Trypan blue from the nuclei, suggesting that the membranes are still ~~are~~ intact, and consequently a different cell death mechanism is involved from that seen with 4-hydroxy-3(3-methylbut-2-enyl)benzaldehyde [Paper VII].

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Appendix A

Natural products presented in the thesis

Table 1. Alphabetical presentation of compounds in the thesis

	Compounds alphabetically according to group	Plant	Methods of identification	NMR data in table	F	N	P
	Alkanes						
1	*n*-Pentacosane	*E. spectabile*	GC-MS	N/A	49	5	VI
2	*n*-Hexacosane	*E. spectabile*	GC-MS	N/A	49	6	VI
3	*n*-Heptacosane	*E. spectabile*	GC-MS	N/A	49	7	VI
4	*n*-Octacosane	*E. spectabile*	GC-MS	N/A	49	8	VI
5	*n*-Nonacosane	*B. laciniosa*	GC-MS	N/A			VI
		N. variegata	GC-MS	N/A	49	9	
		E. spectabile	GC-MS	N/A			
6	*n*-Triacontane	*B. laciniosa*	GC-MS	N/A			VI
		N. variegata	GC-MS	N/A	49	10	
		E. spectabile	GC-MS	N/A			
	Amino alkaloids						
7	4,5,4´-trihydroxy-3,3´-iminodibenzoic acid *	*V. album*	NMR, MS	2	21	1	I
8	4,5,4´, 5´-tetrahydroxy-3,3´-iminodibenzoic acid *	*V. album*	NMR, MS	2	21	2	I
	Aromatic aldehydes and ketones						
9	4-hydroyx-3-(3-methylbut-2-enyl)Benzaldehyde	*N. ossifragum*	NMR, MS	3	56	10	VII
10	(*E*)-4-(3-*R*-hydroxy-2,2-dimethylChroman-6-yl)but-3-en-2-one *	*N. ossifragum*	NMR, MS	3	56	11	VII

11	(*E*)-4-(4-(((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)oxy)Phenyl)but-3-en-2-one *	*N. ossifragum*	NMR, MS	3	56	12	VII
12	Protocatechuic aldehyde	*Z. zamiifolia*	NMR, MS	4	41	2	V
	Fatty acids and their derivatives						
13	*n*-Hexadecanoic acid (Palmitic acid)	*B. laciniosa*	GC-MS	N/A			VI
		N. variegata	GC-MS	N/A	50	1	
		E. spectabile	GC-MS	N/A			
14	Octadeca-(9,12)dienoic acid	*E. spectabile*	GC-MS	N/A	50	2	VI
15	(9*Z*)-Octadec-9-enoic acid (Oleic acid)	*B. laciniosa*	GC-MS	N/A			VI
		N. variegata	GC-MS	N/A	50	3	
		E. spectabile	GC-MS	N/A			
16	Octadecanoic acid (Stearic acid)	*B. laciniosa*	GC-MS	N/A			VI
		N. variegata	GC-MS	N/A	50	4	
		E. spectabile	GC-MS	N/A			
	Flavonoides						
	Biflavonoides						
17	(2*R*,3*S*)-Naringenin(3→6´´)luteolin *	*N. ossifragum*	NMR, MS	5	55	1	VII
18	(2*R*,3*S*)-Naringenin(3→6´´)chrysoeriol *	*N. ossifragum*	NMR, MS	5	55	2	VII
	Flavanols						
19	Gallocatechin	*M. glyptostroboides*	NMR, MS	6	28	4	III
20	Rhamnazin-3-glucoside	*V. album*	NMR	6	25	n/a	n/a
	Flavones						
	C-glycosyl flavones						

21	Apigenin 6-*C*-(6′′-*O*-(3-hydroxy-3-methylglutaroyl)-*β*-glucopyranoside) *	*Z. zamiifolia*	NMR, MS	7	39	7	V
22	Isoschaftoside	*N. ossifragum*	NMR, MS	7	57	7	VII
	O-glycosyl flavones						
23	Chrysoeriol 7-*O*- *β*-glucopyranoside	*N. ossifragum*	NMR, MS	8	57	6	VII
	Flavonols						
24	Quercetin 3,3′-dimethyl ether	*N. ossifragum*	NMR, MS	9	58	4	VII
25	Quercetin 3,7-dimethyl ether	*N. ossifragum*	NMR, MS	9	58	5	VII
	Dihydroflavonols						
26	Aromadendrin-7-*O*-*β*-glucopyranoside	*M. glyptostroboides*	NMR, MS	10	28	2	III
27	Quercetin-3-*O*-*α*-rhamnopyranoside-7-*O*-*β*-glucopyranoside	*M. glyptostroboides*	NMR, MS	10	28	3	III
	Dihydrostilbenoids						
28	6-carboxydihydroresveratrol 3-*O*-*β*-glucopyranoside *	*M. glyptostroboides*	NMR, MS	11	27	1	III
	γ-hydroxybutyric (GHB) acid derivatives						
29	3-(3′-carbomethoxypropyl) gallic acid *	*V. album*	NMR, MS	12	22	2	II
30	3-(3′-carbomethoxypropyl)-7→3′′-protocatechoyl galloate *	*V. album*	NMR, MS	12	22	3	II
	Lignan-glycosides						
31	Liovil 4-*O*-*β*-glucopyranoside *	*N. ossifragum*	NMR, MS	13	59	3	VII

	Phenolic acids and their derivatives						
32	(*E*)-Caffeic acid	*Z. zamiifolia*	NMR, MS	14	41	3	V
33	(*E*)-Caffeic acid methyl ester	*Z. zamiifolia*	NMR, MS	14	41	4	V
34	(*Z*)-Caffeic acid methyl ester	*Z. zamiifolia*	NMR, MS	14	41	5	V
35	Chlorogenic acid methyl ester	*V. album*	NMR	15	24	n/a	n/a
36	(*E*)-2,6-dimethoxycinnamic acid *	*N. ossifragum*	NMR, MS	16	60	8	VII
37	(*E*)-*p*-coumaric acid	*N. ossifragum*	NMR, MS	16	60	9	VII
38	3-*O*-(*E*)-Coumaroylquinic acid	*M. glyptostroboides*	NMR, MS	17	29	5	III
39	3-*O*-(*Z*)-Coumaroylquinic acid	*M. glyptostroboides*	NMR, MS	17	29	6	III
40	3-*O*-(*E*)-Coumaroylquinic acid methyl ester	*M. glyptostroboides*	NMR, MS	18	29	7	III
41	3-*O*-(*Z*)-Coumaroylquinic acid methyl ester	*M. glyptostroboides*	NMR, MS	18	29	8	III
42	Gallic acid (3,4,5-trihydroxybenzoic acid)	*V. album*	NMR, MS	19	22	1	II
43	Rosmarinic acid	*Z. zamiifolia*	NMR, MS	15	40	1	V
44	Syringic acid	*V. album*	NMR	19	23	n/a	n/a
	Phytol						
45	(2*E*,7*R*,11*R*)-3,7,11,15-Tetramethyl-2-hexadecen-1-ol (Phytol)	*E. spectabile*	GC-MS	20	51	13	VI
	Pyranosonic acids						
46	(3*R*,4*S*,5*S*)-2,3,4,5-tetrahydroxytetrahydro-2H-pyran-2-carboxylic acid	*Z. zamiifolia*	NMR, MS	21	42	6	V
	Tocopherols						
47	α-Tocopherol	*B. laciniosa* *N. variegata* *E. spectabile*	GC-MS GC-MS GC-MS	20	52	12	VI

48	*β*-Tocopherol	*N. variegata*	GC-MS	N/A	52	11	VI
	Triterpenoids and derivatives						
49	Stigmastan-3-one	*E. spectabile*	GC-MS	N/A	53	14	VI
50	Campesterol ((3*β*, 24*R*)-Ergost-5-en-3-ol)	*B. laciniosa*	GC-MS	N/A			VI
		N. variegata	GC-MS	N/A	53	15	
		E. spectabile	GC-MS	N/A			
51	Ergostanol	*N. variegata*	GC-MS	N/A	53	16	VI
52	Stigmasta-4,22-dien-3-*β*-ol	*B. laciniosa*	GC-MS	N/A			VI
		N. variegata	GC-MS	N/A	53	17	
		E. spectabile	GC-MS	N/A			
53	*β*-Sitosterol	*B. laciniosa*	GC-MS				VI
		N. variegata	GC-MS	20	53	18	
		E. spectabile	GC-MS				
54	Stigmastanol	*N. variegata*	GC-MS	N/A	53	19	VI
55	24-Methyl-3-*β*-9,19-cyclolanost-24-en-3-ol	*N. variegata*	GC-MS	N/A	53	20	VI

F = Figure number in thesis, N = Number of compound in paper, P = Paper

Appendix B

NMR data

Natural products were dissolved in deuterated dimethylsulfoxide and recorded at 298K on a Bruker 600 MHz instrument equipped with a ^{1}H, ^{13}C and ^{15}N triple resonance cryogenic probe at 600.13 MHz for ^{1}H and 150.90 MHz for ^{13}C.

Nomenclature:

s = singlet, d = doublet, ‘d’ = semi doublet, dd = double doublet, ddd = double double doublet, t = triplet, b = broad peak, m = multiplet

Amino alkaloids

Table 2. ^{1}H and ^{13}C NMR chemical shift values (ppm) and coupling constants (Hz) of the novel amino alkaloids 4,5,4´-trihydroxy-3,3´-iminodibenzoic acid **(7)** and 4,5,4´, 5´-tetrahydroxy-3,3´-iminodibenzoic acid **(8)** from European mistletoe (*Viscum album* L.).

Atom no.	**4,5,4´-trihydroxy-3,3´-iminodibenzoic acid**		**4,5,4´, 5´-trihydroxy-3,3´-iminodibenzoic acid**	
	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C
1		121.1		121.0
2	7.32 *d* 1.9 Hz	109.1	7.29 *d* 1.9 Hz	108.8
3		131.2		131.9
4		139.2		139.0
5		144.9		144.7
6	7.07 *d* 1.9 Hz	109.7	7.04 *d* 1.9 Hz	109.4
7		167.6		167.6
1’		121.7		121.0
2’	7.68 *d* 2.0 Hz	115.8	7.29 *d* 1.9 Hz	108.8
3’		131.2		131.9
4’		150.8		139.0
5’	6.89 *d* 8.3 Hz	114.2		144.7
6’	7.36 *dd* 2.0, 8.3 Hz	122.5	7.04 *d* 1.9 Hz	109.4
7’		167.5		167.6
NH	6.65 *s*		6.62 *s*	

Aromatic aldehydes and ketones

Table 3. ^{1}H- and ^{13}C- NMR chemical shifts (δ, ppm) and the coupling constants (J, Hz) for one rare aromatic aldehyde (4-hydroxy-3-(3-methylbut-2-enyl-)Benzaldehyde) **(9)** and two novel aromatic ketones ((E)-4-(3-R-hydroxy-2,2-dimethylChroman-6-yl)but-3-en-2-one **(10)** and (E)-4-(4-(((E)-4-hydroxy-3-methylbut-2-en-1-yl)oxy)Phenyl)but-3-en-2-one **(11)** isolated from fruits of *Narthecium ossifragum* L.

Atom no.	**4-hydroxy-3-(3methylbut-2-enyl) benzaldehyde**		**(E)-4-(3-hydroxy-2,2-dimethylChroman-6-yl)but-3-en-2-one**		**(E)-4-(4-(((E)-4-hydroxy-3-methylbut-2-en-1-yl)oxy)phenyl)but-3-en-2-one**	
	δ 1H	δ 13C	δ 1H	δ 13C	δ 1H	δ 13C
1		128.4				126.9
2	7.57 d 2.2 Hz	130.6		78.0	7.65 ‘d’ 8.8 Hz	130.2
3		128.5	3.65 dd 5.1, 7.5 Hz	67.6	6.98 ‘d’ 8.8 Hz	115.1
4a		161.1	2.93 dd 5.1, 16.7 Hz	30.8		160.4
4b			2.63 dd 7.5, 16.7 Hz			
4-OH	10.57 s					
5	6.94 d 8.2 Hz	115.1	7.45 d 2.1 Hz	130.6	6.98 ‘d’ 8.8 Hz	115.1
6	7.59 dd 2.8, 8.2 Hz	130.1		126.3	7.65 ‘d’ 8.8 Hz	130.2
7	9.74 s	191.5	7.41 dd 2.1, 8.5 Hz	128.0	7.56 d 16.3 Hz	143.2
8			6.74 d 8.5 Hz	117.0	6.66 d 16.3 Hz	125.0
9				155.2		197.9
10				121.0	2.29 s	27.2
1’	3.25 d (b) 7.3 Hz	27.8	2.27 s	27.2	4.64 dq 0.9, 6.6 Hz	64.4
2’	5.28 m	121.8		197.8	5.64 m	117.6
3’		132.2	6.62 d 16.3 Hz	124.5		141.1
4’	1.70 dd 1.3, 2.6 Hz	25.4	7.51 d 16.3 Hz	143.5	3.83 m	65.5
5’	1.67 d 1.3 Hz	17.6			1.65 dd 0.9, 1.5 Hz	13.8
2-Me			1.27 s	25.6		
2-Me			1.18 s	21.0		

Table 4. ^{1}H- and ^{13}C- NMR chemical shifts (δ, ppm) and the coupling constants (J, Hz) for Protocatechuic aldehyde **(12)** isolated from the petioles of *Zamioculcas zamiifolia*.

Atom no.	**Protocatechuic aldehyde**	
	δ ^{1}H	δ ^{13}C
1		129.2
2	7.23 d 2.0 Hz	114.6
3		146.3
4		152.8
5	6.91 d 8.1 Hz	n.a.
6	7.27 dd 2.0, 8.1 Hz	124.8
7	9.70	191.5

Table 5. ^{1}H- and ^{13}C- NMR chemical shifts (δ, ppm) and the coupling constants (J, Hz) for the novel biflavonoides (2R, 3S)-Naringenin(3→6``)luteolin **(17)** and (2R, 3S)-Naringenin(3→6``)chrysoeriol **(18)** isolated from the fruits of *Narthecium ossifragum* L. The duplicated signals represent rotational conformers.

Atom no.	**Naringenin(3→6´´)luteolin**		**Naringenin(3→6´´)chrysoeriol**	
	δ 1H	δ 13C	δ 1H	δ 13C
		Naringenin		
2	5.83 *d* 12.3 Hz	81.36	5.83 *d* 12.2 Hz	81.1
		80.91		
3	4.81 *d* 12.3 Hz	47.21	4.79 *m*	46.8
	4.78 *d* 12.3 Hz	46.74		
4		196.8		196.7
		196.7		
5		163.65		164.2
5-OH	12.15 s		12.15 s	
6	5.92 d 2.1 Hz	96.13	5.92 d 2.1 Hz	96.1
7		166.54		166.5
7-OH	10.80 s		10.80 s	
8	5.90 d 2.1 Hz	95.11	5.90 d 2.1 Hz	95.0
9		163.01		163.0
10		101.38		101.5
1´		127.97		127.8
2´/6´	7.19 ´d´ 8.7 Hz	128.98	7.18 ´d´ 8.5 Hz	128.9
3´/5´	6.62 ´d´ 8.7 Hz	114.82	6.62 ´d´ 8.5 Hz	114.7
4´		157.70		157.7
4´-OH	9.46 s		9.46 s	

Atom no.	Luteolin		Chrysoeriol	
	δ 1H	δ 13C	δ 1H	δ 13C
2′′	6.64 s (b)	164.01		163.8
3′′	6.61 s (b)	102.7	6.87 s (b)	103.1
		102.8	6.85 s (b)	103.2
4′′		181.8		N/A
		181.6		
5′′		159.8		N/A
	13.51 s (b)	159.2		
5′′-OH	13.38 s (b)		13.52 s (b)	
			13.34 s (b)	
6′′		N/A		N/A
7′′	11.26 s (b)	162.4		N/A
7′′-OH	11.01 s (b)		11.27 s (b)	
	6.40 s (b)		11.02 s (b)	
8′′	6.33 s (b)	93.37	6.48 s (b)	93.4
		93.02	6.40 s (b)	93.2
9′′		156.1		N/A
		155.9		
10′′		103.3		N/A
		103.2		

Atom no.	Luteolin		Chrysoeriol	
	δ 1H	δ 13C	δ 1H	δ 13C
1′′′	7.35 d 2.3 Hz	121.49		121.4
2′′′		113.42	7.51 m	110.2
3′′′	9.40 s	145.78		148.0
3′′′-OH				
4′′′′	9.91 s	149.81		150.0
4′′′-OH	6.86 d 8.3 Hz		9.96 s	
5′′′	7.37 dd 2.3, 8.3 Hz	116.07	6.91 d 8.9 Hz	115.8
6′′′		119.11	7.52 m	120.4
3′′′-OCH3			3.86 s	55.9

Flavanols

Table 6. ^{1}H- and ^{13}C- NMR chemical shifts (δ, ppm) and the coupling constants (J, Hz) for the flavanols Gallocatechin **(19)** and Rhamnazin-3-glucoside **(20)** isolated from respectively from *Metasequoia glyptostroboides* and leaves of *Viscum album* L.

Atom no.	**Gallocatechin**		**Rhamnazin-3-glucoside**	
	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C
1				
2	4.44 d 7.2 Hz	81.26		156.71
3	3.78 m	66.59		133.30
4	2.60 dd 5.4, 16.1 Hz	27.70		177.65
4 b	2.35 dd 7.7 , 16.1 Hz			
5		156.45		161.00
6	5.88 d 2.4 Hz	95.37	6.38 d 2.3 Hz	98.03
7		156.69		165.24
8	5.70 d 2.4 Hz	94.16	6.75 d 2.3 Hz	92.42
9		155.56		156.41
10		99.26		105.12
1’		130.14		121.04
2’	6.25 s	106.25	7.95 d 2.1 Hz	113.57
3’		145.90		149.64
4’		132.76		147.00
5’		145.90	6.92 d 8.4 Hz	115.28
6’	6.25 s	106.25	7.54 dd 2.1, 8.4 Hz	122.25

	Gallocatechin		Rhamnazin-3-glucoside	
	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C
1''			5.57 d 7.6 Hz	100.81
2''			3.22 s	74.42
3''			3.10 s	77.56
4''			3.10 s	69.88
5''			3.23 s	76.48
6a''			3.57 s	60.65
6b''			3.37 s	
5-OH	9.19 s			
7-OCH_3	8.97 s			
3'-OH	8.80 s			
5'-OH	8.80 s			

Table 7. ^{1}H- and ^{13}C- NMR chemical shifts (δ, ppm) and the coupling constants (J, Hz) for the novel natural product apigenin 6-C-(6''-(3-hydroxy-3-methyl-glutaroyl)- *β*-glucopyranoside **(21)** and Isoschaftoside **(22).** The novel acylated C-glycosylflavone **(21)** was isolated from the leaves of *Zamioculcas zamiifolia* while Isoschaftoside **(22)** was isolated from the fruits of *Narthecium ossifragum.*

Atom no.	**apigenin 6-*C*-(6''-(3-hydroxy-3-methyl-glutaroyl)-*β*-glucopyranoside**		**Isoschaftoside**	
	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C
	Apigenin		**Apigenin**	
2		163.65		164.0
3	6.77 s	102.92	6.80 s	102.2
4		182.08		182.4
5		161.31		158.4
5-OH			13.65 s	
6		108.65		108.2
7		163.39		160.9
7-OH			9.17 s	
8	6.50 s	93.70		105.2
9		156.40		155.0
10		103.55		103.8
1'		121.23		121.6
2'/6'	7.92 'd' 8.9 Hz	128.60	8.02 'd' 8.8 Hz	129.1
3'/5'	6.92 'd' 8.9 Hz	116.12	6.88 'd' 8.8 Hz	115.8
4'		161.30		161.4
4'-OH			10.35 s	

Atom no.	6-*C*-glucosyl		6-C-*β*-arabinoside	
	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C
1''	4.58 d 9.8 Hz	73.21	4.70 d 9.7 Hz	74.2
2''	4.09 dd 9.8, 8.7 Hz	70.00	3.81 m	68.5
3''	3.20 d 8.7 Hz	78.87	3.46 dd 9.2 , 2.9 Hz	73.8
4''	3.17 d 9.1 Hz	70.64	3.81 m	69.6
5''	3.37 ddd 2.9, 7.0, 9.1 Hz	78.25	3.82 m	70.2
6A''	4.36 dd 11.9, 2.0 Hz	64.38	3.66 dd 7.2 , 11.0 Hz	
6B''	3.93 dd 11.9, 7.9 Hz			
	6''-3-Methyl-Glutaroyl		**8-C-*β*-glucoside**	
1'''		170.70	4.74 d 10.0 Hz	73.3
2A'''	2.62, d 14.3 Hz	45.41	3.88 dd 10.0 , 8.8Hz	71.0
2B'''	2.52 d 14.3 Hz			
3'''		69.13	3.27 t 8.8 Hz	78.9
4A'''	2.52 m	45.40	3.39 t 9.2 Hz	70.6
4B'''	2.46 d 4.9 Hz			
5'''		172.53	3.23 m	82.0
6A'''			3.74 dd 2.0 , 12.3 Hz	61.3
6B'''			3.50 dd 6.2 , 12.3 Hz	
3'''-CH_3	1.23 s	27.46		

Table 8. ^{1}H- and ^{13}C- NMR chemical shifts (δ, ppm) and the coupling constants (J, Hz) for Chrysoeriol-*O*-*β*-glucopyranoside **(23)** isolated from the fruits of *N. ossifragum*.

Atom no.	**Chrysoeriol-*O*-*β*-glucopyranoside**				
	δ ^{1}H	δ ^{13}C		δ ^{1}H	δ ^{13}C
	Chrysoeriol			**7-*O*-*β*-glucopyranoside**	
2		164.1	1''	5.05 d 7.6 Hz	100.0
3	6.98 s	103.4	2''	3.25 dd 7.6 , 9.0 Hz	73.1
4		182.0	3''	3.29 t 9.0 Hz	76.5
5		161.3	4''	3.16 dd 9.0 , 9.4 Hz	69.6
5-OH	12.95 s		5''	3.44 m	77.3
6	6.44 d 2.2 Hz	99.5	6A''	3.71 dd 11.9 , 2.0 Hz	60.7
7		163.0	6B''	3.46 m	
8	6.86 d 2.2 Hz	95.1			
9		157.0			
10		105.4			
1'		121.2			
2'	7.58 m	110.4			
3'		148.1			
3'-OCH_3	3.88 s	56.0			
4'		150.9			
4'-OH	10.01 s				
5'	6.94 d 8.7 Hz	115.7			
6'	7.59 m	120.6			

Flavonols

Table 9. ^{1}H- and ^{13}C- NMR chemical shift values (δ, ppm) and coupling constants (Hz) for the two flavonols quercetin 3,3'-dimethyl ether **(24)** and quercetin 3,7-dimethyl ether **(25)** isolated from the fruits of *Narthecium ossifragum* L.

Atom no.	**Quercetin 3,3'-dimethyl ether**		**Quercetin 3,7-dimethyl ether**	
	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C
2		115.5		156.0
3		137.7		137.9
3-OCH3	3.79 s	59.8	3.79 s	59.7
4		178.0		178.1
5		161.4		161.0
5-OH	12.67 s		12.69 s	
6	6.20 d 2.1 Hz	98.6	6.36 d 2.3 Hz	97.8
7		164.1		165.1
7-OH	10.84 s			
7-OCH3			3.85 s	56.1
8	6.47 d 2.1 Hz	93.9	6.71 d 2.2 Hz	92.3
9		156.3		156.3
10		104.3		105.3

Atom no.	Quercetin 3,3'-dimethyl ether		Quercetin 3,7-dimethyl ether	
	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C
1'		120.8		120.7
2'	7.63 d 2.1 Hz	111.9	7.58 d 2.3 Hz	115.6
3'		147.6		145.4
3'-OCH3	3.85 s	55.7		
3'-OH			9.38 s	
4'		149.8		148.7
4'-OH	9.90 s		9.80 s	
5'	6.95 d 8.4 Hz	115.7	6.90 d 8.5 Hz	115.8
6'	7.57 dd 2.1, 8.4 Hz	122.0	7.47 dd 2.3, 8.5 Hz	120.7

Dihydroflavonols

Table 10. ^{1}H- and ^{13}C- NMR chemical shift values (**δ,** ppm) and coupling constants (Hz) for aromadendrin 7-*O*-*β*-glucopyranoside (**26**) and quercetin 3-*O*-*α*-rhamnopyranoside-7-*O*-*β*-glucopyranoside (**27**) isolated from the needles of *Metasequoia glyptostroboides*.

Atom no.	**Aromadendrin 7-*O*-*β*-glucopyranoside**		**Quercetin 3-*O*-*α*-rhamnopyranoside-7-*O*-*β*-glucopyranoside**	
	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C
2 a	5.11 d 11.5 Hz	83.12		n.a.
2 b				
3	4.64 d 11.5 Hz	71.65		n.a.
4 a		198.73		n.a
4b				
5		162.78		n.a
6 a	6.15 d 2.2 Hz	96.83	6.44 d 2.1 Hz	99.2
6 b				
7		165.48		n.a.
8	6.12 d 2.2 Hz	95.46	6.73 d 2.1 Hz	94.4
9		162.46		n.a.
10		102.10		n.a.

Atom no.	Aromadendrin 7-*O*-*β*-glucopyranoside		Quercetin 3-*O*-*α*-rhamnopyranoside-7-*O*-*β*-glucopyranoside	
	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C
1’		127.39		n.a.
2’	7.31 ‘d’ 8.7 Hz	129.58	7.31 d 2.2 Hz	115.5
3’	6.78 ‘d’ 8.7 Hz	147.0		114.99
4’		157.88		n.a.
5’	6.78 ‘d’ 8.7 Hz	114.99	6.86 d 8.3 Hz	115.2
6’	7.31 ‘d’ 8.7 Hz	129.58	7.26 dd 2.2,8.3 Hz	121.1
4’-OH	9.56 s(b)			
5-OH	11.79 s			
7-*O*-*β*-glucopyranoside			**3-*O*-*α*-rhamnopyranoside**	
1’’	4.94 d 7.6 Hz	99.70	5.27 d 1.7 Hz	99.7
2’’	3.19 dd 7.6, 9.0 Hz	73.07	3.97 m	69.9
3’’	3.24 d 8.8 Hz	76.37	3.49 m	69.7
4’’	3.12 dd 8.7, 9.7 Hz	69.56	3.14 m	71.0
5’’	3.36 m	77.14	3.20 m	70.6
6A’’	3.65 dd 2.2, 11.9 Hz	60.63	0.81 d 6.1 Hz	17.3
6B’’	3.42 dd 5.9, 11.9 Hz			
2’’-OH			4.96 d 4.4 Hz	
3’’-OH			4.62 m	
4’’-OH			4.73 d 4.8 Hz	

Atom no.			Quercetin 3-*O*-*α*-rhamnopyranoside-7-*O*-*β*-glucopyranoside	
	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C
			7-*O*-*β*-glucopyranoside	
1’’’			5.07 d 7.5 Hz	101.7
2’’’			3.24 m	73.0
3’’’			3.28 m	76.3
4’’’			3.15 m	69.4
5’’’			3.20 m	77.0
6A’’’			3.69 m	60.4
6B’’’			3.46 m	
2’’’-OH			5.39 d 5.0 Hz	
3’’’-OH			5.13 d 4.9 Hz	
4’’’-OH			5.06 m	
6’’’-OH			4.61 m	

Table 11. ^{1}H and ^{13}C NMR chemical shift values (**δ,** ppm) and coupling constants (Hz) for the novel bibenzyl glycoside 6-carboxydihydroresveratrol 3-*O*-*β*-glucopyranoside **(28)** isolated from the needles of *Metasequoia glyptostroboides*.

Atom no.	**6-carboxydihydroresveratrol 3-*O*-*β*-glucopyranoside**				
	δ ^{1}H	δ ^{13}C		δ ^{1}H	δ ^{13}C
1		145.47	1''	4.87 d 7.8 Hz	99.84
2	6.43 d 2.5 Hz	110.26	2''	3.20 dd 7.8 , 9.0 Hz	73.24
3		160.57	3''	3.27 t 9.0 Hz	76.67
4	6.41 d 2.5 Hz	101.55	4''	3.15 dd 9.0 , 9.6 Hz	69.74
5		162.13	5''	3.34 ddd 2.2 , 5.9 , 9.6 Hz	77.21
6		109.14	6''A	3.68 dd 2.2 , 12.0 Hz	60.72
7a	2.98 m	37.83	6''B	3.46 dd 5.9 , 12.0 Hz	
7b	2.94 m				
8	2.67 m	36.72			
1'		132.00			
2'/6'	6.99 'd' 8.5 Hz	129.15			
3'/5'	6.66 'd' 8.5 Hz	115.15			
4'		155.51			
1'''		172.14			
4'-OH	9.13 s(b)				
5-OH	11.57 s(b)				

Gamma-hydroxybutyric acid derivatives

Table 12. ^{1}H and ^{13}C NMR chemical shift values (**δ,** ppm) and coupling constants (Hz) for two novel compounds consisting of the methyl esther of γ-hydroxybutyric acid merged with gallic acid. They are 3-(3´-carbomethoxypropyl) gallic acid **(29)** and 3-(3´-carbomethoxypropyl)-7→3´´-protocatechoyl galloate **(30)** isolated from the leaves of European mistletoe (*V. album*).

Atom no.	**3-(3´-carbomethoxypropyl) gallic acid**		**3-(3´-carbomethoxypropyl)-7→3´´-protocatechoyl galloate**	
	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C
Galloyl				
1		120.7		120.65
2	7.06 d 2.0 Hz	116.6	7.07 d 2.0 Hz	116.80
3		136.5		136.09
3-OH				
4		144.5		144.56
4-OH	8.76 s		8.76 s	
5		145.4		145.30
5-OH	9.51 s		9.55 s	
6	7.14 d 2.0 Hz	112.1	7.14 d 2.0 Hz	112.06
7		167.3		167.40
7-COOH	12.31 s		12.35 s	

Atom no.	3-(3′-carbomethoxypropyl) gallic acid		3-(3′-carbomethoxypropyl)-7→3′′-protocatechoyl galloate	
	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C
3-GHB-OMe				
1′		173.3		173.32
2′	2.37 t 7.4 Hz	30.8	2.38 t 7.4 Hz	30.75
3′	1.69 p 7.4 Hz	23.1	1.70 p 7.4 Hz	22.96
4′	3.44 t 7.4 Hz	51.2	3.44 t 7.4	50.79
OMe	3.55 s	51.3	3.55 s	51.31
7→3″-PCA				
1″				121.70
2″			7.44 d 2.1 Hz	124.71
3″				136.44
4″				155.71
4″-OH			9.86 s	
5″			6.82 d 8.3 Hz	115.76
6″			7.48 dd 2.1, 8.3 Hz	125.82
7″				167.30

Lignan-glycosides

Table 13. ^{1}H and ^{13}C NMR chemical shift values (δ, ppm) and coupling constants (Hz) for the novel lignan-glycoside liovil 4-*O*-*β*-glucopyranoside **(31)** isolated from the fruits of *N*. *ossifragum*.

Atom no.	**Liovil 4-*O*-*β*-glucopyranoside**	
Liovil	δ ^{1}H	δ ^{13}C
1		135.2
2	6.94 d 2.0 Hz	110.6
3		149.0
3-OCH_3	3.76 s	55.8
4		145.9
5	7.03 d 8.5 Hz	115.3
6	6.84 dd 2.0, 8.5 Hz	118.2
7	4.66 d 4.2 Hz	84.9
7-OH	N/A	
8	3.03 dd 2.2, 4.4 Hz	53.7
9[a]	4.12 dd 2.1, 6.8 Hz	71.0
9B	3.74 m	
1’		132.2
2’	6.88 d 2.0 Hz	110.4
3’		147.5
3’-OCH_3	3.75 s	55.7
4’		146.0
4’-OH	8.88 s (b)	
5’	6.71 d 8. Hz	115.2
6’	6.74 dd 2.0, 8.1 Hz	118.7
7’	4.60 d 4.2 Hz	85.2
7’-OH	N/A	
8’	3.03 dd 2.2, 4.4 Hz	53.7
9A’	4.12 dd 2.1, 6.8 Hz	71.0
9B’	3.74 m	
	4-*O*-*β*-glucopyranoside	
1’’	4.86 d 7.5 Hz	100.2
2’’	3.23 m	73.3
3’’	3.23 m	76.9
4’’	3.14 m	69.8
5’’	3.27 m	77.1
6A’’	3.64 m	60.7
6B’’	3.43 m	

Table 14. ^{1}H and ^{13}C NMR chemical shifts (δ, ppm) and coupling constants (J, Hz) for (*E*)-Caffeic acid **(32)**, (*E*)-Caffeic acid methyl ester **(33)** and (*Z*)-Caffeic acid methyl ester **(34)** isolated from *Zamioculcas zamiifolia*.

Atom no.	**(*E*)-Caffeic acid**		**(*E*)-Caffeic acid methyl ester**	**(*Z*)-Caffeic acid methyl ester**
	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{1}H
1		n.a.		
2	7.01 d 2.1Hz	114.7	7.04 d 2.1 Hz	7.33 d 2.2 Hz
3				
3-OH	9.12	n.a	9.13 s	9.05 s
4				
4-OH	9.52	n.a	9.59 s	9.39 s
5	6.74 d 8.2 Hz	115.8	6.75 d 8.2 Hz	6.70 d 8.3 Hz
6	6.94 dd 2.1, 8.2 Hz	121.2	6.99 dd 2.1, 8.2 Hz	7.01 dd 2.2, 8.3 Hz
7	7.39 d 15.9 Hz	144.7	7.47 d 15.9 Hz	6.76 d 12.9 Hz
8	6.15 d 15.9 Hz	115.1	6.26 d 15.9 Hz	5.73 d 12.9 Hz
9				
9-OOH	12.09s (broad)			
9-OCH_3			3.67 s	3.64 s

Table 15. ^{1}H and ^{13}C NMR chemical shift values (ppm) and coupling constants (Hz) for Chlorogenic acid methyl ester **(35)** isolated from the leaves of *V. album* and Rosmarinic acid **(43)** isolated from the petioles of *Z. zamiifolia*.

Atom no.	**Chlorogenic acid methyl ester**		**Rosmarinic acid**	
	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C
1		73.60		125.3
1-OH	5.65 s			
2 a	1.97 dd 7.7, 13.6 Hz		7.04 d 2.1 Hz	114.9
2 b	1.85 ddd 1.0, 4.5 13.6 Hz	39.8		
3	3.95 m	64.50		145.6
3-OH	4.71 dd 3.1, 6.9 Hz		9.15 s	
4	4.71 dd 3.1, 6.9 Hz	75.77 b		148.5
4-OH			9.64 s	
5	4.07 m	65.47	6.76 d 8.3 Hz	115.8
5-OH	4.87 d 6.4 Hz			
6 a	2.07 dd 3.8, 13.4 Hz		7.00 dd 2.1, 8.3 Hz	121.6
6 b	1.79 ddd 7.3, 13.4, 1.0 Hz	38.09		
7		173.91	7.45 d 15.9 Hz	145.9
7-OCH_3	3.60 s	51.76		
8			6.23 d 15.9 Hz	113.2
9				165.9

Atom no.	Chlorogenic acid methyl ester		Rosmarinic acid	
	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C
1′		125.65		127.2
2′	7.04 d 1.9 Hz	114.82	6.66 d 2.1 Hz	116.7
3′		145.70		145.0
3′-OH	9.18 s		8.78 s	
4′		148.45		144.0
4′-OH	9.61 s		8.72 s	
5′	6.76 d 8.3 Hz	115.88	6.62 d 8.14 Hz	115.4
6′	6.99 dd 1.9, 8.3 Hz	121.32	6.51 dd 2.1, 8.1 Hz	120.0
7A′	7.48 d 15.9 Hz	144.98	2.97 dd 4.2, 14.5 Hz	36.1
7B′			2.89 dd 8.5, 14.5 Hz	
8′	6.25 d 15.9 Hz	114.60	5.02 dd 4.2, 4.5 Hz	72.8
9′		166.32		170.7
9-COOH′			13.03 s	

Table 16. ^{1}H and ^{13}C NMR chemical shift values (ppm) and coupling constants (Hz) for (E)-2,6-dimethoxycinnmic acid **(36)** which for the first time is described as a natural product and the known natural product (E)-p-coumaric acid **(37)** isolated from the fruits of *N. ossifragum*.

	(E)-2,6-dimethoxycinnmic acid		**(E)-p-coumaric acid**	
Atom no.	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C
1		121.0		125.3
2		159.5	7.52 'd' 8.7 Hz	130.0
3	6.71 d 8.4 Hz	104.2	6.79 'd' 8.7 Hz	115.8
4	7.34 t 8.4 Hz	131.7		159.5
4-OH			9.96 s	
5	6.71 d 8.4 Hz	104.2	6.79 'd' 8.7 Hz	115.8
6		159.5	7.52 'd' 8.7 Hz	130.0
7	7.92 d 16.3 Hz	134.6	7.50 d 15.9 Hz	144.2
8	6.70 d 16.3 Hz	121.0	6.29 d 15.9 Hz	115.4
9		168.8		168.0
9-OH			12.08 s (b)	
9-COOH	12.12 s (b)			
2/6-OC-H_3	3.85 s	56.0		

Table 17. ^{1}H and ^{13}C NMR chemical shift values (ppm) and coupling constants (Hz) for 3-*O*-(*E*)-coumaroylquinic acid **(38)** and 3-*O*-(*Z*)-coumaroylquinic acid **(39)** isolated from the needles of *Metasequoia glyptostroboides*.

Atom no.	**3-*O*-(*E*)-coumaroylquinic acid**		**3-*O*-(*Z*)-coumaroylquinic acid**	
	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C
1		73.23		73.12
2	1.87 d 5.3 Hz	39.4	1.87 m	39.20
3	3.88 dd 5.8, 12.1 Hz	67.55	3.82 m	67.69
4	3.56 m	71.29	3.56 s (b)	71.21
5	5.18 m	71.28	5.15 m	71.20
6[a]	2.03 dd 7.6, 13.4 Hz	35.33	2.01 m	35.11
6B	1.91 dd 7.6, 13.4 Hz		1.88 m	
7		176.34		176.28
1’		125.57		125.57
2’	7.51 'd' 8.8 Hz	130.41	7.69 d 8.7 Hz	132.93
3’	6.80 'd' 8.5 Hz	116.11	6.74 d 8.7 Hz	115.14
4’		159.89		158.91
4’-OH	10.00 s (b)		9.89 s	
5’	6.80 'd' 8.5 Hz	116.11	6.74 d 8.7 Hz	115.14
6’	7.51 'd' 8.8 Hz	130.41	7.69 d 8.7 Hz	132.93
7’	7.54 d 15.9 Hz	144.45	6.80 m	142.67
8’	6.32 d 15.9 Hz	115.52	5.76 d 12.9 Hz	116.77
9’		166.52		165.92

Table 18. ^{1}H and ^{13}C NMR chemical shift values (ppm) and coupling constants (Hz) for 3-*O*-(*E*)-coumaroylquinic acid methyl ester **(40)** and 3-*O*-(*Z*)-coumaroylquinic acid metyl ester **(41)** isolated from the needles of *Metasequoia glyptostroboides*.

Atom no.	**3-*O*-(*E*)-coumaroylquinic acid**		**3-*O*-(*Z*)-coumaroylquinic acid**	
	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C
2A	1.97 dd 5.9, 13.3 Hz	38.2	1.97 m	38.1
2B	1.83 dd 2.9, 13.3 Hz		1.81	
3	3.80 m	67.9	3.77 m	68.0
4	3.60 m	69.8	3.61 m	69.6
5	5.15 ddd 3.2, 4.2, 9.4 Hz	70.5	5.12 ddd 3.0, 4.3, 9.4 Hz	70.5
6[a]	2.05 ddd 12.5, 4.2, 1.3 Hz	34.9	2.03 m	34.9
6B	1.87 dd 12.5, 9.4 Hz		1.83 m	
7		174.2		N/A
1’		125.3		125.6
2’	7.52 'd' 8.7 Hz	130.3	7.68 'd' 8.8 Hz	132.7
3’	6.78 'd' 8.7 Hz	115.9	6.74 'd' 8.8 Hz	114.9
4’		159.7		158.7
4’-OH	10.00 s		9.89 s	
5’	6.78 'd' 8.7 Hz	115.9	6.74 'd' 8.8 Hz	114.9
6’	7.52 'd' 8.7 Hz	130.3	7.68 'd' 8.8 Hz	132.7
7’	7.54 d 15.9 Hz	144.2	6.80 d 12.9 Hz	144.2
7’-OCH_3	3.58 s	51.6	N/A	N/A
8’	6.33 d 15.9 Hz	115.1	5.75 d 12.9 Hz	116.4
9’		166.0		165.6

Table 19. ^{1}H and ^{13}C NMR chemical shift values (ppm) and coupling constants (Hz) for Gallic acid **(42)** and Syringic acid **(44)** isolated from the leaves of *V. album*.

	Gallic acid		**Syringic acid**	
Atom no.	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C
1		120.50		120.43
2	6.90 s	108.79	7.19 s	106.92
3		145.49		147.51
3-OH	9.16 s			
3-OCH_3			3.79 s	
4		138.06		140.27
4-OH	8.80 s			
5		145.49		147.51
5-OH	9.16 s			
5-OCH_3			3.79 s	
6	6.90 s	108.79	7.19 s	106.92
7		167.54		
7-COOH	12.26 s		12.54 s	167.31

Table 20. ^{1}H and ^{13}C NMR chemical shifts (δ, ppm) for pure samples of phytol **(45)**, *α*-tocopherol **(47)** and *β*-sitosterol **(53)** dissolved in $CDCl_3$ at 298K on a Bruker 600 MHz instrument equipped with a ^{1}H, ^{13}C and ^{15}N triple resonance cryogenic probe at 600.13 MHz for ^{1}H and 150.90 MHz for ^{13}C.

Atom no.	**Phytol**		***α*-tocopherol**		***β*-sitosterol**	
	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C
1A	4.08 s	59.0			1.83 s	37.3
1B					1.06 s	
2A	5.35 s	123.4		74.5	1.81 s	31.7
2B					1.48 s	
3A		139.7	1.82 s	31.5	3.50 s	71.8
3B			1.77 s			
4A	1.94 s	39.7	2.61 s	20.8	2.26 s	42.3
4B					2.21 s	
5	1.34 s	25.0		118.4		140.8
6A	1.22 s	36.5		144.5	5.33 s	121.7
6B	1.03 s					
7A	1.34 s	32.4		121.0	1.96 s	31.8
7B					1.50 s	
8A	1.21 s	37.1		122.6	1.43 s	32.0
8B	1.03 s					
9	1.22 s	24.4		145.5	0.91 s	50.1
10A	1.21 s	37.1		117.3		36.5
10B	1.03 s					
11	1.34 s	32.4			1.46 s	21.2
12a	1.21 s	37.1			1.99 s	39.9
12B	1.03 s				1.13 s	
13	1.21 s	24.6				42.2
14	1.09 s	39.1			0.97 s	56.7
15A	1.48 s	27.7			1.56 s	24.3
15B					1.05 s	
16A	0.82 s	22.4			1.82 s	28.3
16B					1.24 s	
17	0.82 s	22.4			1.08 s	56.0
18	0.81* s	19.6			0.66 s	11.9
19	0.80* s	19.6			0.99 s	19.3
20	1.61 s	15.9			1.33 s	36.1

Atom no.	Phytol		*α*-tocopherol		*β*-sitosterol	
	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C
21					0.90 s	18.7
22A					1.48 s	21.2
22B					1.44 s	
23					1.14 s	26.0
24					0.91 s	45.8
24^{1}a					1.25 s	23.2
24^{1}b					1.20 s	
24^{2}					0.83 s	12.0
25					1.64 s	29.2
26					0.79 s	18.0
27					0.81 s	19.7
1A’			1.58 s	39.8		
1B’			1.51 s			
2A’			1.46 s	21.0		
2B’			1.39 s			
3’			1.79 s	37.5		
4’			1.41 s	32.7		
5A’			1.26 s	37.4		
5B’			1.09 s			
6A’			1.26 s	37.4		
6B’			1.09 s			
7A’			1.26 s	37.4		
7B’			1.09 s			
8’			1.38 s	32.8		
9A’			1.26 s	37.4		
9B’			1.09 s			
10A’			1.32 s	24.7		
10B’			1.24 s			
11’			1.16 s	39.4		
12’			1.54 s	27.9		
2-Me			1.24 s	23.8		
5-Me			2.12 s	11.2		
6-OH			4.20 s			
7-Me			2.17 s	12.2		
8-Me			2.12 s	11.8		
4’-Me			0.87 s	19.7		
8’-Me			0.86 s	19.8		
12’-Me			0.88 s	22.6		
12’-Me			0.88 s	22.5		

Table 21. ^{1}H and ^{13}C NMR chemical shift values (ppm) and coupling constants (Hz) of the rare natural product (3R,4S,5S)-2,3,4,5-tetrahydroxytetrahydro-2H-pyran-2-carboxylic acid **(46)** isolated from the leaves *Zamioculcas zamiifolia*.

	(3*R*,4*S*,5*S*)-2,3,4,5-tetrahydroxytetrahydro-2H-pyran-2-carboxylic acid	
Atom no.	δ ^{1}H	δ ^{13}C
1A	4.02 m	67.01
1B	3.74 dd 12.9, 2.1 Hz	
2	4.01 m	72.04
3	3.83 dd 3.3, 10.2 Hz	72.70
4	4.06 d 10.2 Hz	71.98
5		99.92
6		177.3

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Paper I

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Novel aminoalkaloids from European mistletoe (*Viscum album* L.)

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^{1}H–^{15}N HMBC
2D NMR

ABSTRACT

The European white-berry mistletoe (*Viscum album* L.) has remained an important medicinal plant for millennia. Preparations of the plant have found application in the treatment of cancer and the anticancer activity of mistletoe extracts has been ascribed to the presence of lectins, viscotoxins and alkaloids. However, the alkaloids of this species have hitherto remained unidentified because of their claimed extreme lability. Here we report on the isolation and characterisation of the novel aminoalkaloids 4,5,4′-trihydroxy-3,3′-iminodibenzoic acid (**1**) and 4,5,4′,5′-tetrahydroxy-3,3′-iminodibenzoic acid (**2**) from *V. album* L. The compounds define a novel group of aminoalkaloids and are the first novel alkaloids ever identified in any mistletoe species. The structures were established using a combination of several 2D NMR spectroscopic techniques and high resolution mass spectrometry.

1. Introduction

The European white-berry mistletoe (*Viscum album* L.) (Fig. 1) belongs to the family *Loranthaceae* (APG III, 2009) within the order *Santalales*. Originally the plant belonged to *Viscaceae* (Mabberley, 2008) which has now been included in *Loranthaceae*. *V. album* L. is an evergreen dioecious small shrub growing half parasitically on a tree host in temperate Europe and western Asia. Three subspecies are recognised (Mabberley, 2008), one covering the whole range of host species. Two of them grow on conifer hosts: *V. album* L. subsp. *abietis* (Wiesb.) Abrom. on fir (*Abies* Mill.), *V. album* L. subsp. *austriacum* (Wiesb.) Vollm. on Scots pine (*Pinus sylvestris* L.). The best known European mistletoe is *V. album* L. *subsp. album*, growing on decidous trees, mostly on apple (*Malus* Mill.), poplar (*Populus* L.) and willow (*Salix* L.), and many other species growing in range but very rarely on oak (*Quercus* L.) and never on beech (*Fagus* L.). The species has linear lanceolate leathery leaves which last for several seasons, and whitish, translucent berries that develop in the late fall and early winter from the yellow to green flowers which grow in the sprout axil (Büssing, 2000). According to Mabberley (2008), the name mistletoe is applied not only to the genus *Viscum* but to any genus of *Loranthacae* spp. half parasitic on tree or shrub branches.

Hypertension, diabetes, arthrosis and cancer are some of the most important illnesses that have been treated by using the white-berry mistletoe as a traditional remedy in Europe. (Büssing, 2000 and references therein). The anticancer activity of mistletoe extracts has been ascribed to the presence of lectins (Franz, 1986), viscotoxins (Romagnoli et al., 2000) and alkaloids (Khwaja et al., 1980, 1986; Park et al., 1998, 1999; Chen et al., 2005 and references therein; Dong et al., 2009; Ge et al., 2009; Zhou et al., 2010). Mistletoe lectins are cytotoxic proteins whose cytotoxic activity results from inhibition of protein synthesis on the ribosomal level (Franz, 1986). Contrary to the viscotoxins and mistletoe alkaloids, mistletoe lectins are not thermally stable and lose their biological activity through heat treatment. Viscotoxins are toxic proteins with relatively low molecular weight (~5 kDa) which are produced in stems and leaves of the European mistletoe (*V. album* L.). The three-dimensional structure of the most important viscotoxin of this species, namely viscotoxin A3, has previously been characterised in detail (Romagnoli et al., 2000). Mistletoe alkaloids comprising the third group of anticancer natural products from this species have been scarcely characterised and only limited information about their structure and identities is available.

Mistletoe may be able to incorporate alkaloids from the host tree, in agreement with the observation that leaves of mistletoe contained the same alkaloids in similar concentrations to those found in the non-leafy parts of the host (Kanner, 1939; Trautner, 1952; Mortimer, 1957; Martin-Cordero et al., 1989, 1997). Kanner (1939) found caffeine in *Loranthaceae* spp. growing on coffee trees

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Fig. 1. European white-berry mistletoe (*Viscum album* L.) photographed in Amsterdam 22.10.2005.

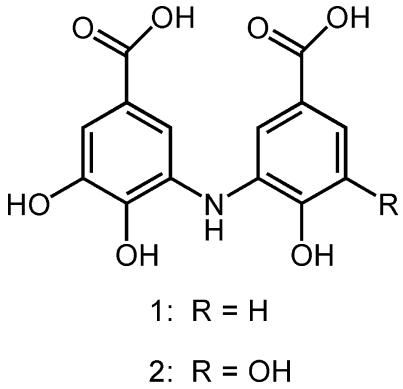

Fig. 2. Structures of the aminoalkaloids 4,5,4′-trihydroxy-3,3′-iminodibenzoic acid (**1**) and 4,5,4′,5′-tetrahydroxy-3,3′-iminodibenzoic acid (**2**) isolated from *Viscum album* L.

at levels equal to those found in the host three. The alkaloids hyoscine, anabasine, nicotine and isopelletierine have been reported from an Australian mistletoe (*Benthamina alyxifolia* (Benth.) Tieghem) growing on *Duboisia myoporoides* (Trautner, 1952; Mortimer, 1957), a small tree of the family *Solanaceae* that produces these alkaloids. Based on ^{1}H NMR Martin-Cordero et al. (1989) identified five quinolizidine alkaloids (+)-retamine, (−)-lupanine, (−)-anagyrine, (−)-cytisine, and (−)-*N*-methylcytisine, isolated *from Viscum cruciatum* Sieber growing on a shrub of the *Fabaceae* family *Retama* (*syn. Lygos*) *sphaerocarpa*. The same authors reported that plant material of *V. cruciatum* Sieber contained the bipiperidyl alkaloid ammodendrine and the quinolizidine alkaloids lupanine, 5,6-dehydrolupanine, retamine, cytosine and *N*-methylcytisine as a result of root parasitism on the host plant *Retama sphaerocarpa* Boissier (Martin-Cordero et al., 1993). Similarly, the berries of *V. cruciatum* Sieber also obtain the alkaloids ammodendrine, retamine and lupanine by root parasitism on the host plant *R. sphaerocarpa* Boissier (Martin-Cordero et al., 1997). Alkaloids have been indicated to occur in another African mistletoe (*Tapinanthus dodoneifolius* (DC) Danser) (Deeni and Sadiq, 2002) although these compounds still await structural characterisation. The existence of alkaloids in *V. album* L. has remained controversial until now. In fact, Pfüller (2000) stated that this species does not contain typical alkaloids, but only "alkaloid-like" compounds including tyramine, phenylethylamine, choline and acetylcholine (the latter compounds reviewed by Hegnauer, 1966). Khwaja et al. (1986) reported the presence of alkaloids in *V. album* L. However, the only identification criteria provided for these substances were the extraction procedure applied and their reaction with the Dragendorff alkaloid reagent. Due to their extreme lability none of these substances has hitherto been characterised.

The major objective of the present study was to characterise novel alkaloids of European mistletoe (*V. album* L.) on the basis that mistletoe alkaloids exhibit significant anticancer effect. They are thermally stable and remain active after heat treatment, a noteworthy property because mistletoe is often administered as tea (which is obviously prepared as a hot aqueous extract). Mistletoe alkaloids are more cytotoxic to tumour cells than to normal cells (Park et al., 1999). Mistletoe alkaloids have hitherto been poorly characterised. The previously reported alkaloids of mistletoe are all known substances of other plant species. No alkaloids have so far been identified in European mistletoe. In this paper we report on the isolation and characterisation of a novel group of aminoalkaloids isolated from *V. album* L. subsp. *album* (Fig. 2).

2. Results and discussion

Fresh plant material of *V. album* L. was extracted with methanol, concentrated under reduced pressure and purified by partition against diethyl ether. The aqueous phase was further purified with XAD-7 Amberlite absorption chromatography and Sephadex LH-20 chromatography. Pure compounds (**1** and **2**) were isolated by preparative HPLC.

The UV–vis spectra of **1** and **2** suggest that they are aromatic compounds based on substituted hydroxybenzoic acid core structures.

The 1D ^{1}H NMR spectrum of **1** showed a 2H AB system at δ 7.32 (d 1.9 Hz, H-2) and δ 7.07 (d 1.9 Hz, H-6), a 3H ABX system at δ 7.68 (d 2.0 Hz, H-2′), δ 7.36 (dd 2.0, 8.3 Hz, H-6′) and δ 6.89 (d 8.3 Hz, H-5′) and a 1H singlet at δ 6.65 (NH), which suggest a derivative of protocatechuic acid and *p*-hydroxybenzoic acid (Fossen et al., 1998; Lee et al., 2011) connected through a –NH– bridge. This was confirmed by the observation of 14 individual ^{13}C signals belonging to **1** which were assigned by the 2D ^{1}H–^{13}C HMBC and HSQC spectra of **1** (Table 1). The $^{4}J_{HH}$ correlations at δ 7.32/6.65 (H-2/NH) and δ 7.68/6.65 (H-2′/NH) confirmed the linkages between the NH bridge and the gallic and protocatechuic acids to be at the 3 and 3′ positions, respectively. This was further confirmed by the $^{3}J_{CH}$ multiple bond correlations at δ 6.65/109.1

Table 1
^{1}H and ^{13}C NMR chemical shift values (ppm) and coupling constants (Hz) for alkaloids **1**–**2** in DMSO-D_6 at 298 K.

Atom no.	Compound **1**		Compound **2**	
	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C
1		121.1		121.0
2	7.32 *d* 1.9	109.1	7.29 *d* 1.9	108.8
3		131.2		131.9
4		139.2		139.0
5		144.9		144.7
6	7.07 *d* 1.9	109.7	7.04 *d* 1.9	109.4
7		167.6		167.6
1′		121.7		121.0
2′	7.68 *d* 2.0	115.8	7.29 *d* 1.9	108.8
3′		131.2		131.9
4′		150.8		139.0
5′	6.89 *d* 8.3	114.2		144.7
6′	7.36 *dd* 2.0, 8.3	122.5	7.04 *d* 1.9	109.4
7′		167.5		167.6
NH	6.65 *s*		6.62 *s*	

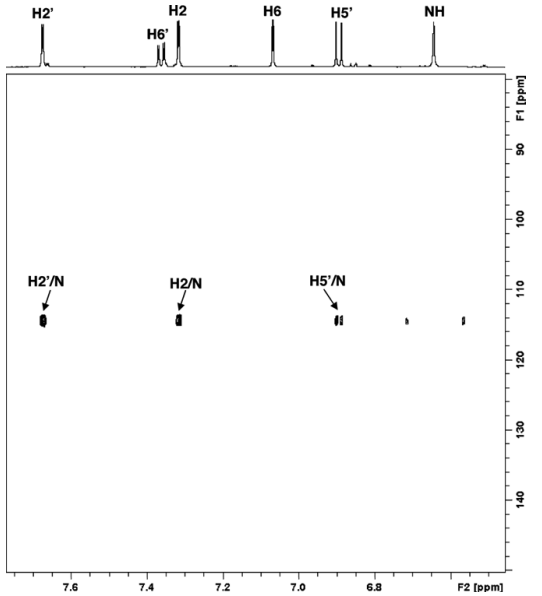

Fig. 3. 2D $^{1}H–^{15}N$ HMBC spectrum of 4,5,4′-trihydroxy-3,3′-iminodibenzoic acid (**1**).

(NH/C-2), δ 6.65/139.2 (NH/C-4), δ 6.65/115.8 (NH/C-2′) and δ 6.65/150.8 (NH/C-4′) observed in the 2D $^{1}H–^{13}C$ HMBC spectrum of **1** and the $^{3}J_{NH}$ multiple bond correlations at δ 7.68/114.3 (H-2′/N) and δ 7.32/114.3 (H-2/N) observed in the 2D $^{1}H–^{15}N$ HMBC spectrum of **1** (Fig. 3).

Thus, **1** was identified as the novel aminoalkaloid 4,5,4′-trihydroxy-3,3′-iminodibenzoic acid (Fig. 2). A quasi-molecular ion $[M+H]^+$ at *m*/*z* 306.075 corresponding to $C_{14}H_{12}O_7N$ (calculated 306.061) supported this identity. Further conformation was to be found in the observation of fragment ions corresponding to the loss of water (*m*/*z* 288.064) and carbon dioxide (*m*/*z* 262.083) from this ion.

The 1D and 2D NMR spectra of **2** shared many similarities with the corresponding spectra of **1**, although molecular symmetry resulted in a reduction of the number of signals observed in the NMR spectra of **2** (Table 1). The 1D ^{1}H NMR spectrum of **2** showed an AB system at δ 7.29 (d 1.9 Hz, H-2) and δ 7.04 (d 1.9 Hz, H-6) each integrating for 2H, in addition to a 1H singlet at δ 6.62 (NH), which suggest the presence of two units of protocatechuic acid connected through an –NH– bridge. The linkages between the NH bridge and the protocatechuoyl subunits were confirmed to be at C-3 and C-3′, respectively by the observation of the $^{4}J_{HH}$ correlations at 7.29/6.62 (H-2/NH and H-2′/NH) and the $^{3}J_{CH}$ multiple bond correlations at δ 6.62/108.8 (NH/C-2 and NH/C-2′) and δ 6.65/139.0 (NH/C-4 and NH/C-4′) observed in the 2D $^{1}H–^{13}C$ HMBC spectrum of **1**. A quasi-molecular ion $[M+H]^+$ at *m*/*z* 322.059 corresponding to $C_{14}H_{12}O_8N$ (calculated 322.056) confirmed the identity of 2 as the novel aminoalkaloid 4,5,4′,5′-tetrahydroxy-3,3′-iminodibenzoic acid (Fig. 2). Loss of carbon dioxide from this ion provided a prominent ion at *m*/*z* 278.067.

In previous literature, alkaloids identified in mistletoe have been ascribed to absorption from the host tree by root-parasitism. **1** and **2** define a new group of aminoalkaloids and are the first alkaloids to be identified from any mistletoe that are unique to the species. Although alkaloids previously detected in other *Viscum* spp. have been considered to originate from the host threes (Kanner, 1939; Trautner, 1952; Mortimer, 1957; Martin-Cordero et al., 1989, 1997), the aminoalkaloids identified from *V. album* appear to be specific for European mistletoe. Neither the host plant (*Populus* x *canadensis*) nor its precursors (*P. nigra* and *P. deltoids*) are known sources of alkaloids. In fact it has been stated that *P. nigra* is devoid of alkaloids (Sun et al., 2009). Ortho-topolin-9-glucoside and *N*-6-(*o*-hydroxybenzylamino)purine reported from *P.* x *canadensis* (Strnad et al., 1992, 1994), although nitrogenous compounds, appear unlikely to be precursors for the aminoalkaloids reported in this paper. Recently, Liang et al. (2010) characterised 4,4′-dihydroxy-3,3′-imino-di-benzoic acid which is the only structurally related alkaloid reported in current literature. This compound was isolated from the fern *Drynaria fortunei*, which is completely unrelated to the genus *Viscum*.

Extracts of European mistletoe (*V. album* L.) have been used in cancer treatment since the 1920s. Fractions of extracts from this species that have been indicated to contain alkaloids as the active substances (Khwaja et al., 1986) have been reported to exhibit significant anticancer activity. The finding of novel aminoalkaloids in this species reported in this paper suggests that European mistletoe has potential as a source of future anti-cancer drugs.

3. Experimental

3.1. Extraction of plant material

The plant material and the host three *V. album* L. (source: Lille, France, host tree: a hybrid of *Populus nigra* L. with *P. deltoides* Bartram ex Marshall = *P.* x *canadensis* Moench) was identified by Professor Frédéric Dupont, Department of Botany, Faculty of Pharmacy, University of Lille 2. The European mistletoe used in this work grows in the botanical garden of Department of Botany, Faculty of Pharmacy, University of Lille 2. A voucher specimen has been deposited (accession number LIP-BOTA-12070201). Fresh plant material of *V. album* L. (1.5 kg) was chopped using a blender. The chopped material was extracted three times with methanol (6 L) for 24 h. The mixture was continuously shaken during the extraction to ensure complete extraction. The crude extract was filtered through a glass wool filter. The plant residue was weighed to establish the dry weight (616.0 g). Water percentage in the plant was approximately 60%, since 10 g of the fresh plant material lost 6 g after drying in oven at 110 °C. The water-containing methanolic extract (water from the fresh plant material) was concentrated under reduced pressure (Büchi Rotavapor, R-205). The concentrated solution was purified by partition against diethyl ether to remove non-polar aliphatic compounds. A dark brown aqueous solution remained after removal of non-polar aliphatic constituents.

3.2. Amberlite XAD-7 column chromatography

Amberlite XAD-7 was chosen as stationary phase in order to purify aromatic constituents from the extract. The dark brown aqueous solution was concentrated by evaporating under reduced pressure until reaching a total volume of 400 ml prior to application on an Amberlite XAD-7 column (Amberlite XAD-7HP, Particle size: 20–60 mesh, wet, Sigma–Aldrich). The column was eluted with distilled water (5 L) to remove polar aliphatic constituents. The purified aromatic material was then eluted with methanol (5 L) and concentrated under reduced pressure to provide an aromatic concentrate.

3.3. Sephadex LH-20 column chromatography

The above aromatic concentrate was further purified on a Sephadex LH-20 column (Amersham Biosciences). The concentrate

was applied to a Sephadex LH-20 column (105 cm × 5 cm). The column was eluted with a solvent gradient starting with 3.6 L methanol–distilled water–TFA (60:40:0.2, v/v) as mobile phase followed by 2 L methanol–distilled water–TFA (70:30:0.2, v/v) and 1 L methanol–distilled water–TFA (75:25:0.2, v/v). The column was finally eluted with 1 L methanol–distilled water–TFA (80:20:0.2, v/v) as mobile phase. A total of 58 fractions were collected. Fractions 1–18 were eluted using 3.6 L methanol–distilled water–TFA (60:40:0.2, v/v) as mobile phase while fractions 19–43 were obtained using 2 L methanol–distilled water–TFA (70:30:0.2, v/v) as mobile phase. Fractions 44–48 were eluted using 1 L methanol–distilled water–TFA (75:25:0.2, v/v) as mobile phase. The last fractions (49–58) were eluted using 1 L methanol–distilled water–TFA (80:20:0.2, v/v) as mobile phase. The eluted fractions were concentrated under reduced pressure on a rotary evaporator and checked for purity by analytical HPLC. The aminoalkaloids were isolated from fraction 44 by preparative HPLC as described below.

3.4. Analytical HPLC

Analytical HPLC was performed with an Agilent 1100 HPLC equipped with a HP 1050 multidiode array detector, a 20 μl loop and a 250 mm × 4.6 mm, 5 μm Hypersil GOLD column. Two solvents: A, distilled water and 0.5% TFA (trifluoroacetic acid) and B, acetonitrile and 0.5% TFA (trifluoroacetic acid) were used for elution. Pre-injection conditions were established using a mixture of 90% A and 10% B: on injection an immediate steady gradient was employed to reach 14% B after 10 min; isocratic elution was carried out from 10–14 min; a new ramped gradient was then introduced to achieve the following conditions 16% B (at 18 min), 18% B (at 22 min), 23% B (at 26 min), 28% B (at 31 min), 40% (at 32 min); isocratic elution was employed from 32–40 min; gradient elution in the reverse sense was then utilised to achieve 10% B (40–43 min); finally isocratic elution at 10% B was employed from 43–46 min. The flow rate was 1.0 ml/min, and aliquots of 15 μl were injected with a Micro Auto-sampler (Agilent 1100 Series).

3.5. Preparative HPLC

Preparative HPLC was performed with a Gilson 321 HPLC with UV detection (UltiMate 3000 Variable Wavelength Detector). The system was equipped with a 250 mm × 22 mm, C18 Altech column. Two solvents: A, distilled water and 1.0% TFA (trifluoroacetic acid) and B, methanol and 1.0% TFA (trifluoroacetic acid) were used for elution. The elution profile consisted of 90% A and 10% B for 0–4 min, followed by gradient elution from 4 to 40 min (10% B–90% B), isocratic elution for 44–57 min and final linear elution from 57–58 min (90% B–10% B). The flow rate was 15.0 ml/min, and aliquots of 1000 μl were injected with a Microliter syringe. Following this multistep procedure approximately 4 mg of **1** and 2 mg of **2** were isolated. The purity of compound **1** was determined to be 95% based on integration of baseline-separated signals of the 1D ^{1}H NMR spectrum. The purity of compound **2** was determined to be 70% based on integration of baseline-separated signals of the 1D ^{1}H NMR spectrum.

3.6. Spectroscopy

High resolution mass spectra were recorded using a JEOL AccuTOF JMS T100LC instrument fitted with a DART inlet from Ion Sense. The isolated aminoalkaloids were dissolved in methanol. A glass needle was soaked into the analyte solution, and thereafter placed in the source at atmospheric pressure. The analysis was performed at helium flow rate 0.76 L/min, gas temperature 300 °C and a needle voltage of approximately 3000 V. Mass spectral data were recorded at orifice 1 voltage 19 V, orifice 2 voltage 7 V, ring lens voltage 8 V and detector voltage 2300 V. The spectrum was recorded over the mass range 50–1000 *m*/*z*.

UV–vis absorption spectra were recorded on-line during HPLC analysis over the wavelength range 240–600 nm in steps of 2 nm.

NMR sample was prepared by dissolving the isolated aminoalkaloids in deuterated dimethylsulphoxide (99.9 atom% D, Sigma–Aldrich).

The 1D ^{1}H and the 2D ^{1}H–^{15}N HMBC, ^{1}H–^{13}C HMBC, ^{1}H–^{13}C HSQC, ^{1}H–^{1}H COSY and ^{1}H–^{1}H ROESY NMR experiments were obtained at 600.13 MHz and 150.90 MHz for ^{1}H and ^{13}C, respectively, at 298 K on a Bruker 600 MHz instrument equipped with a cryogenic probe.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytol.2012.07.005.

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Supplementary data for

Novel aminoalkaloids from European mistletoe (*Viscum album* L.).

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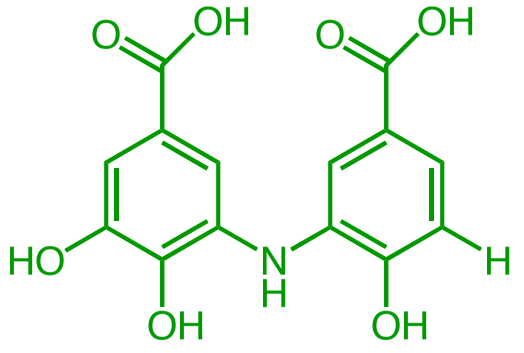
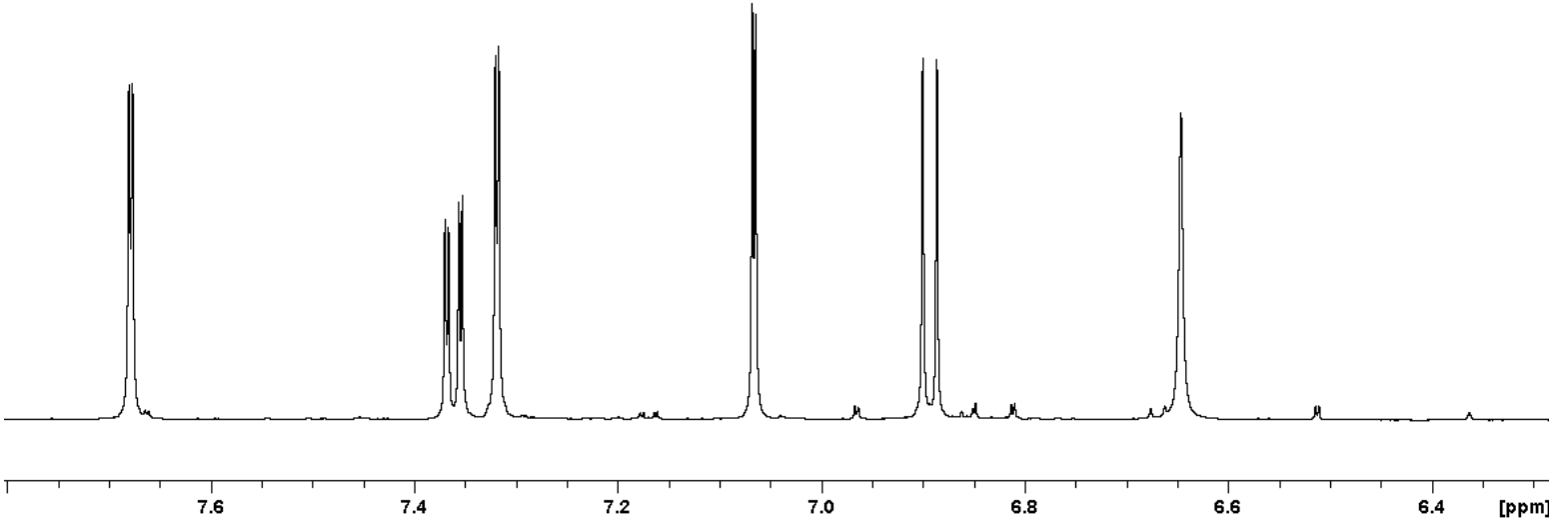


1D ^{1}H spectrum



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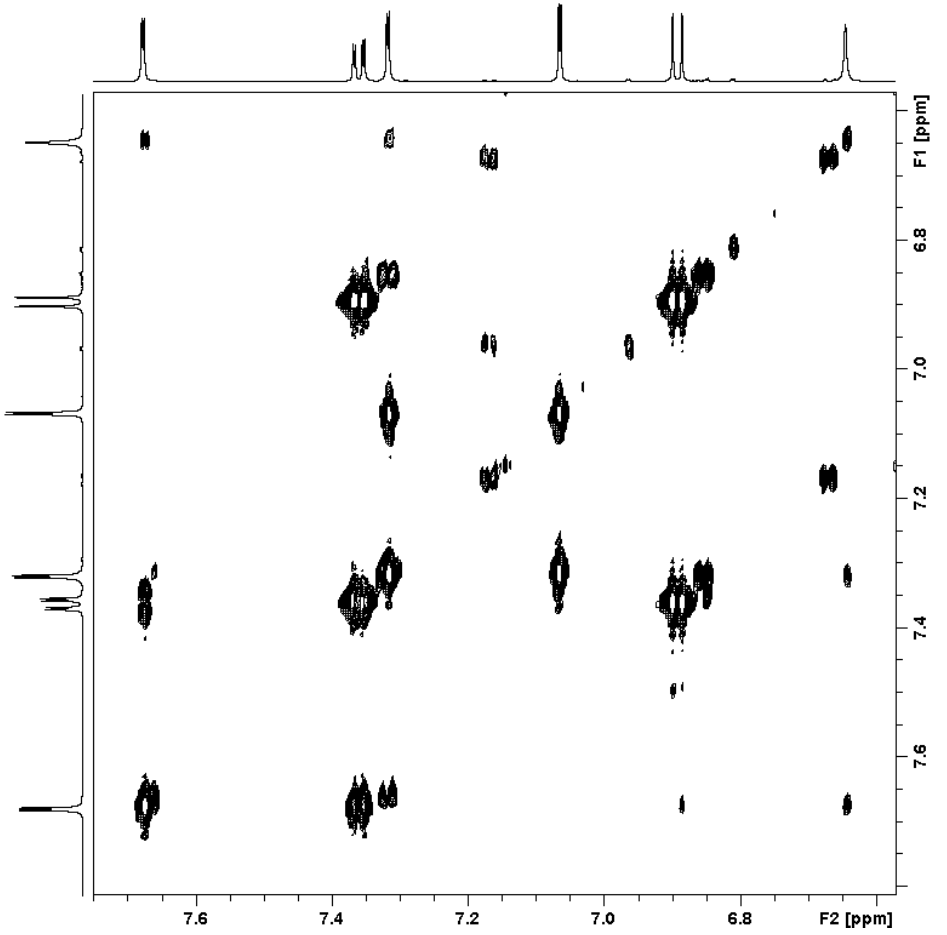

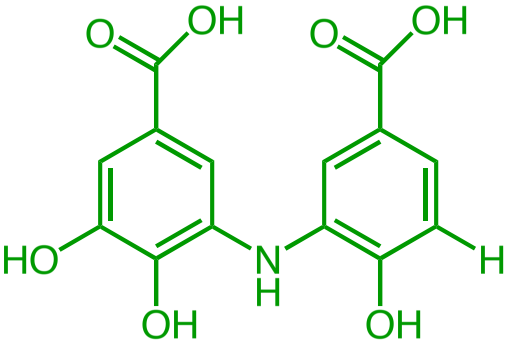

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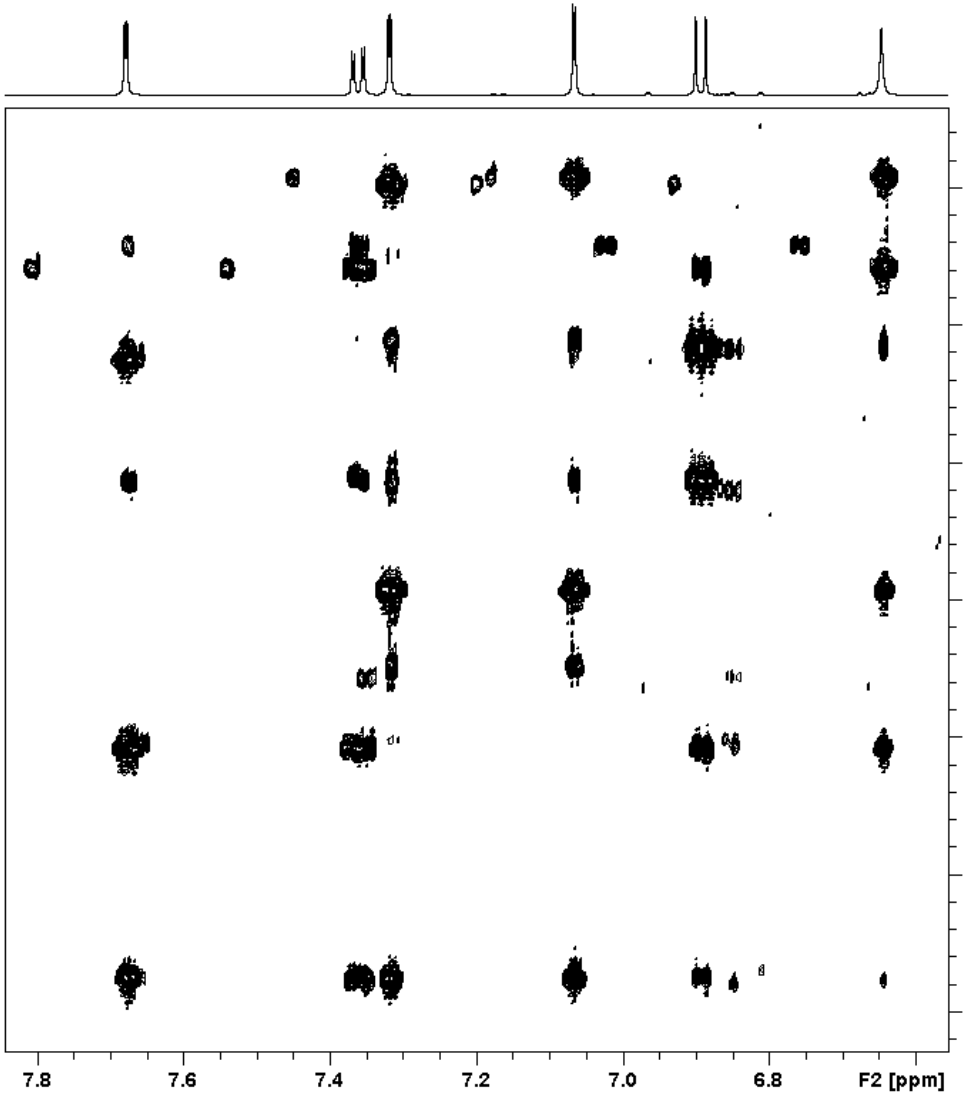

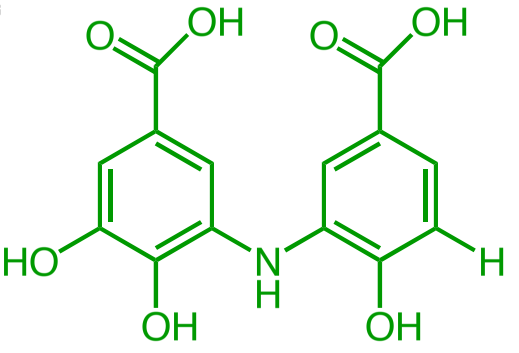

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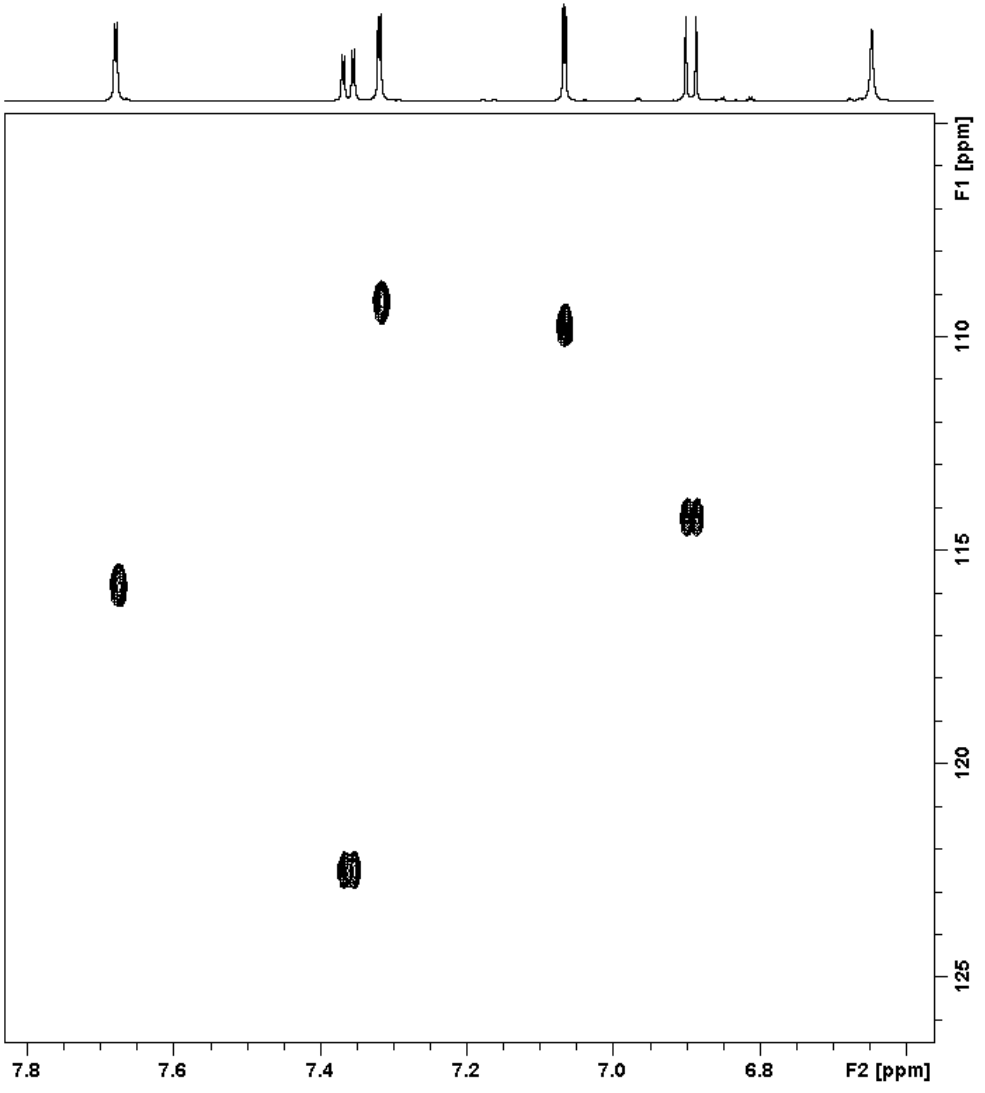

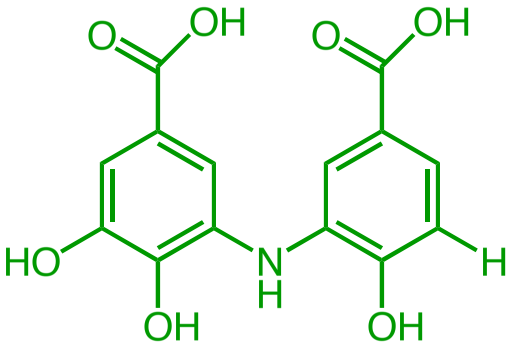

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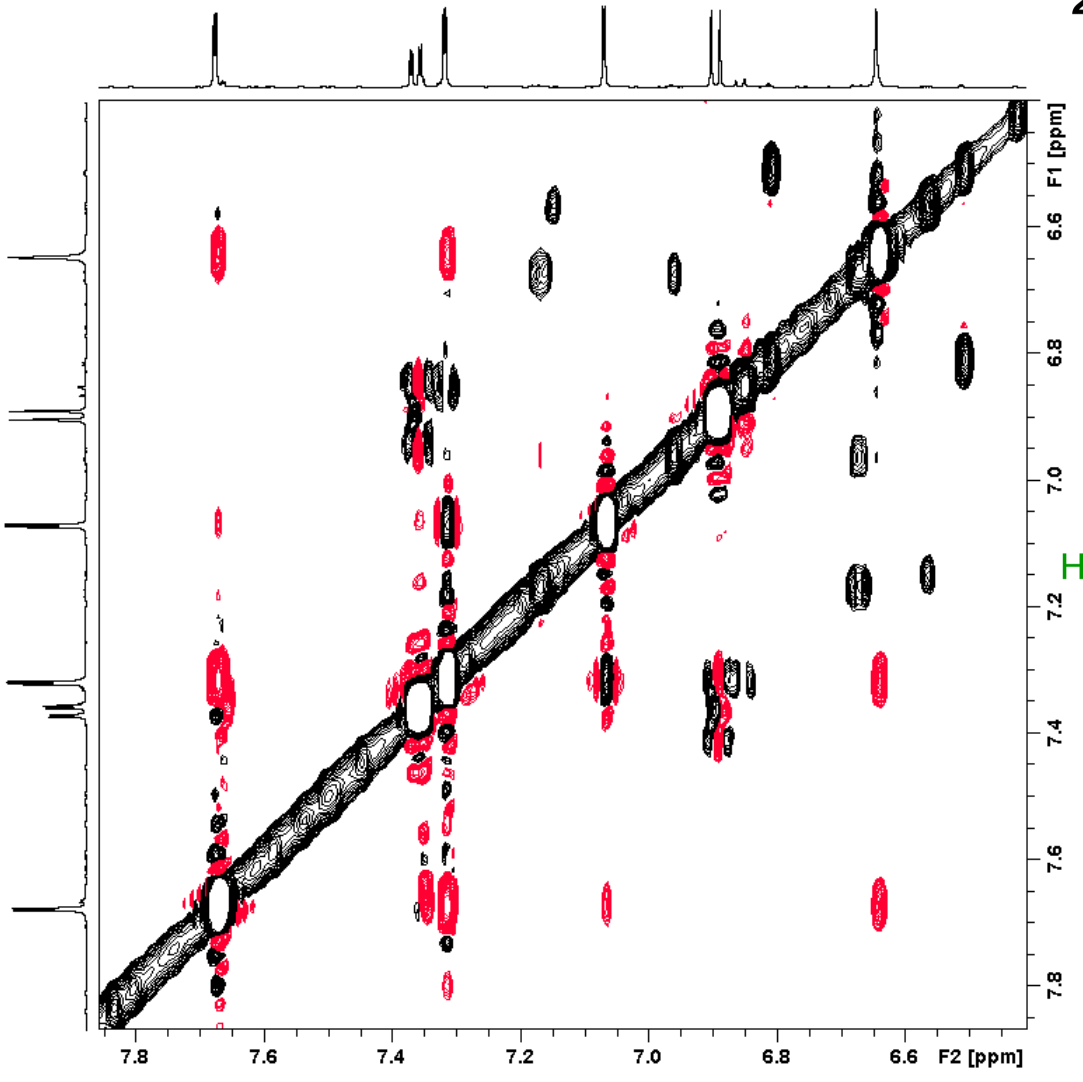

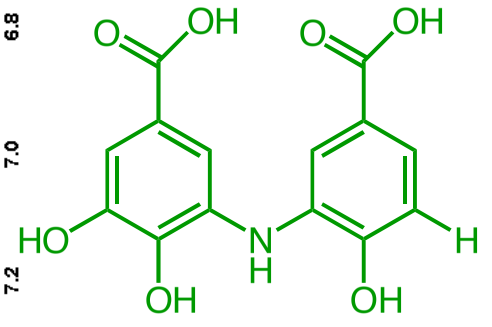

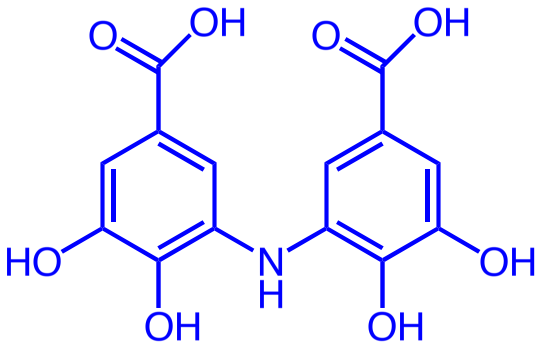

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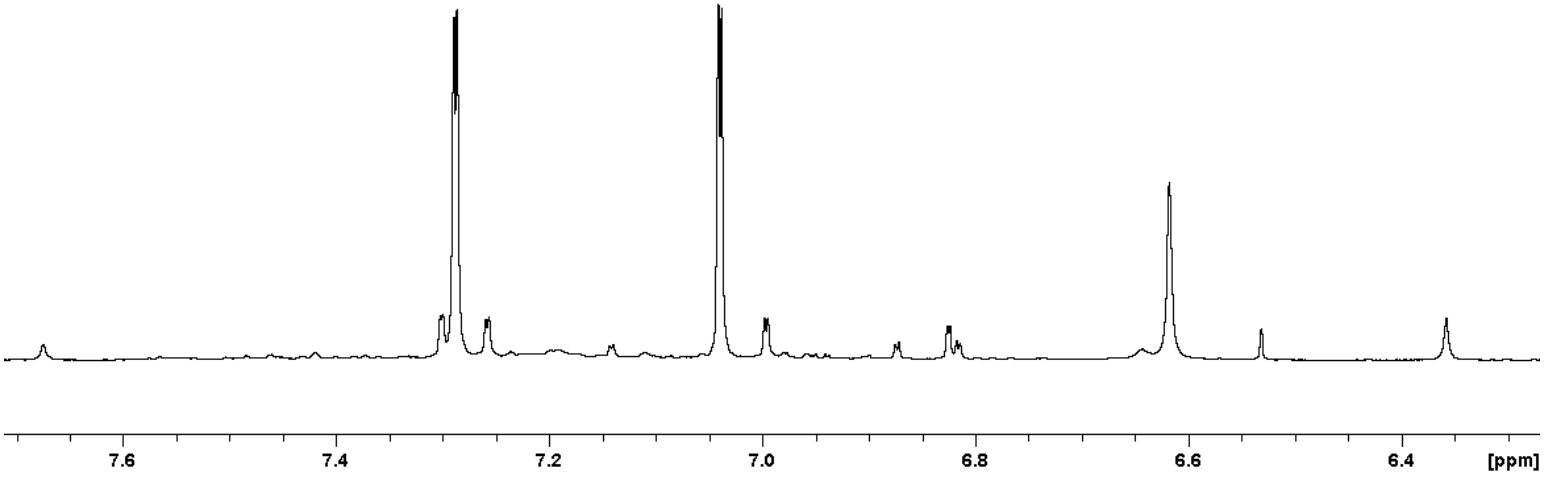

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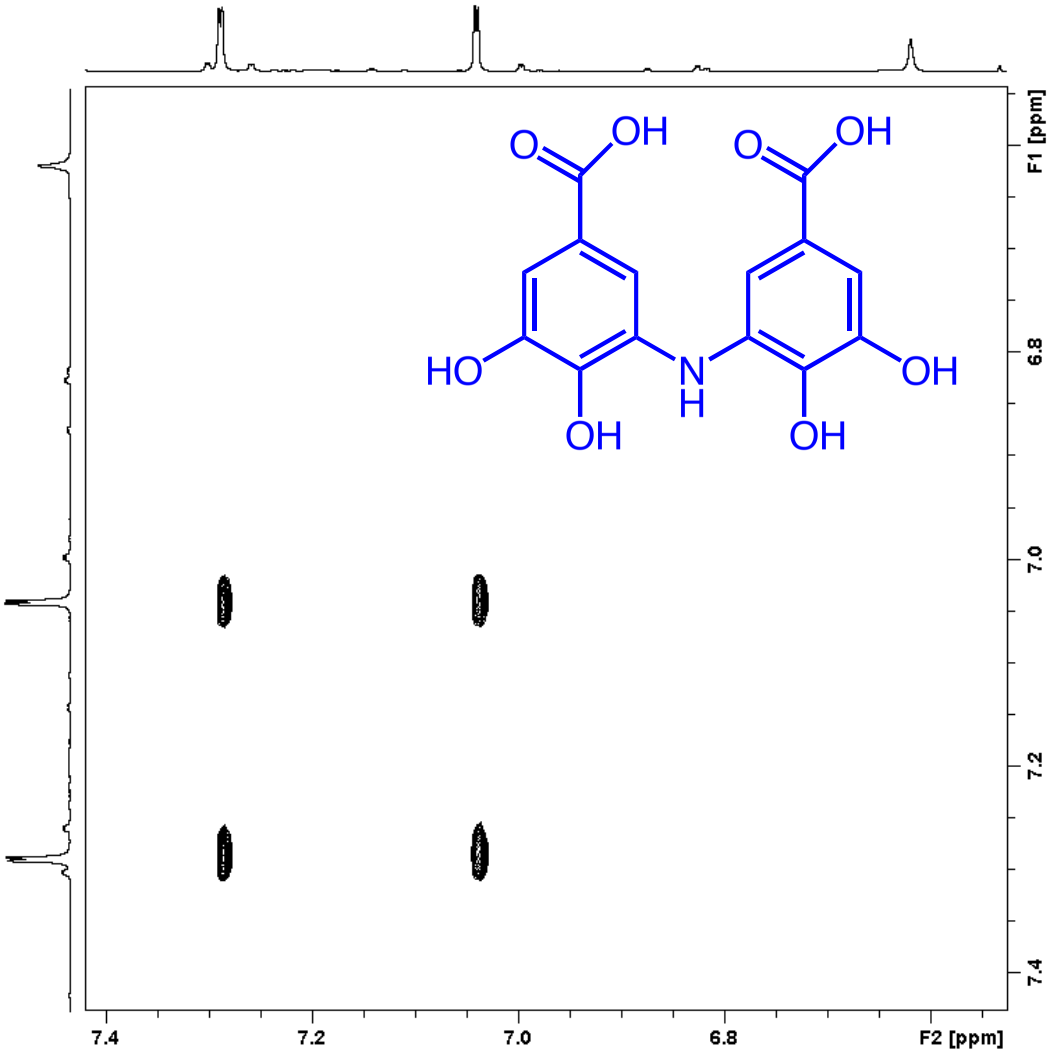
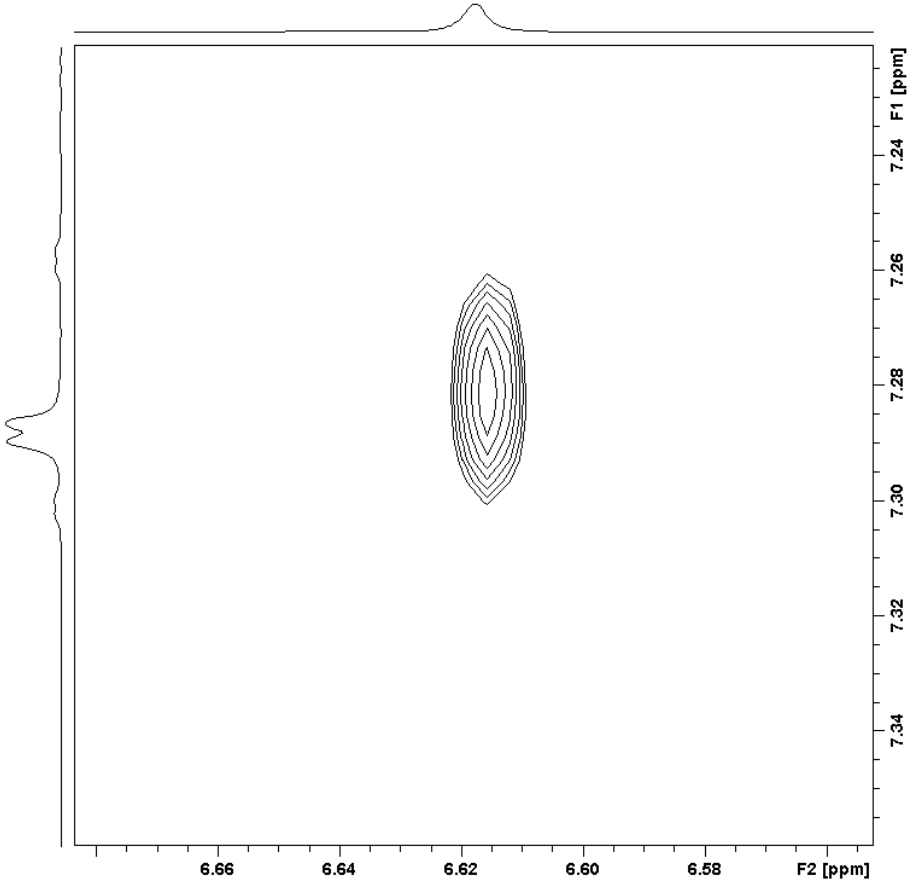
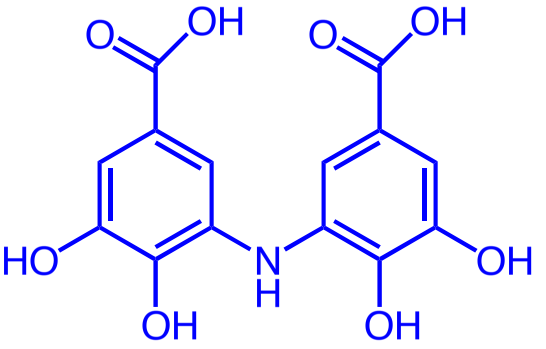
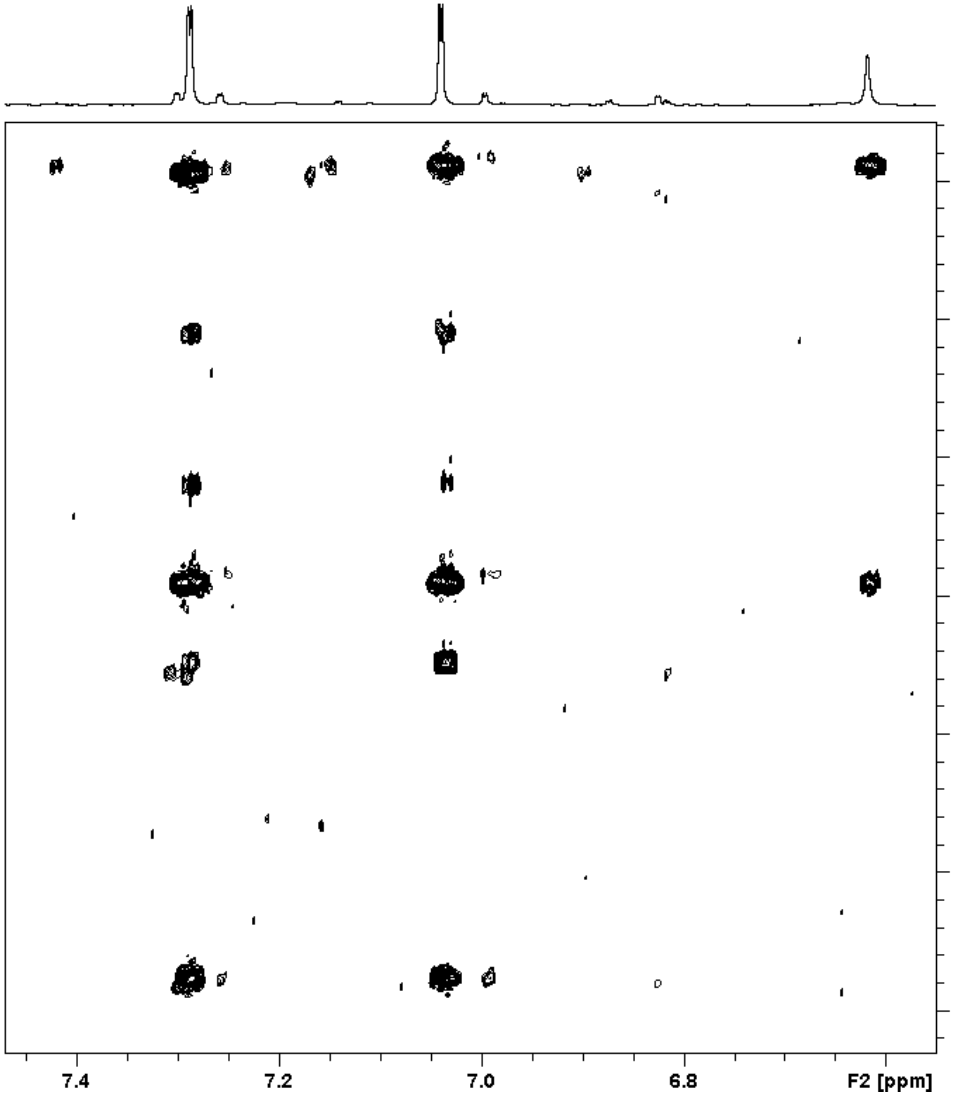


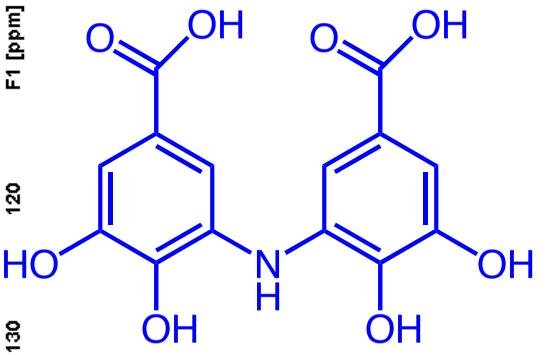




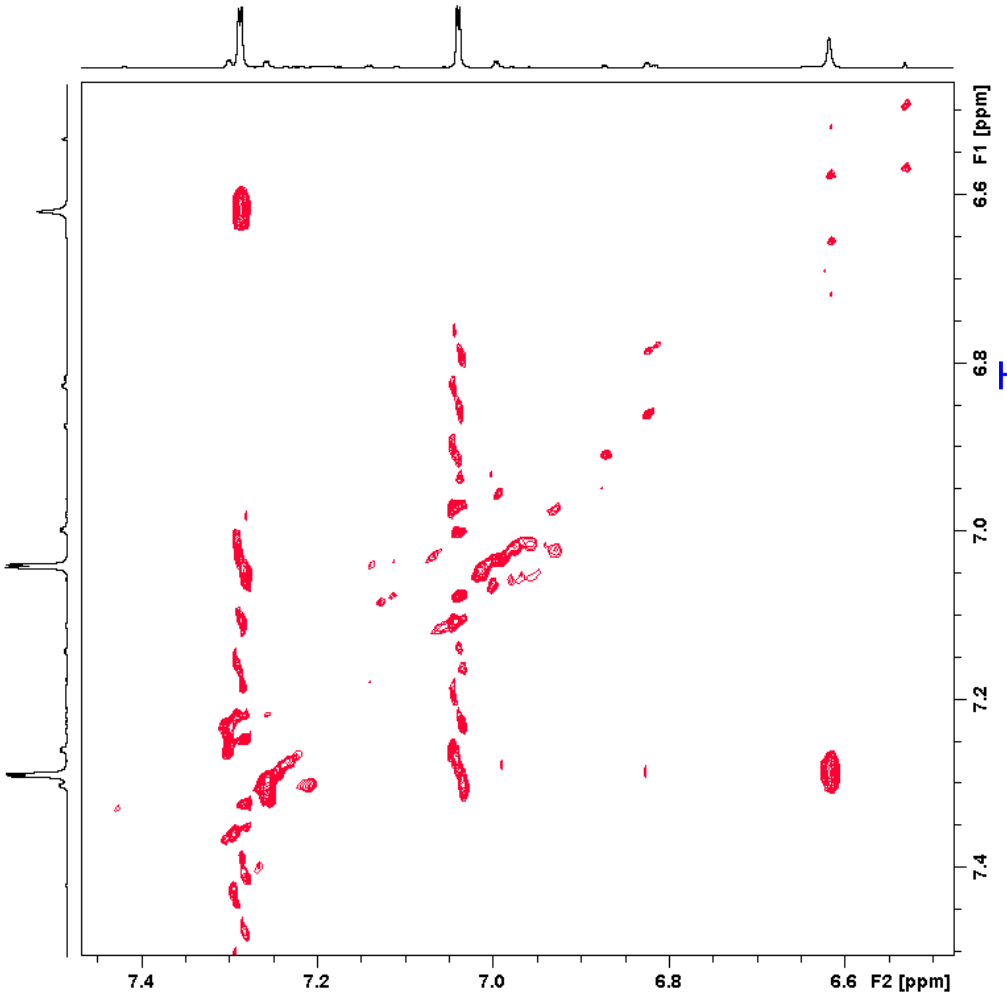
2D ^{1}H COSY spectrum at lower contour level showing the correlation between H-2/NH and H-2'/NH

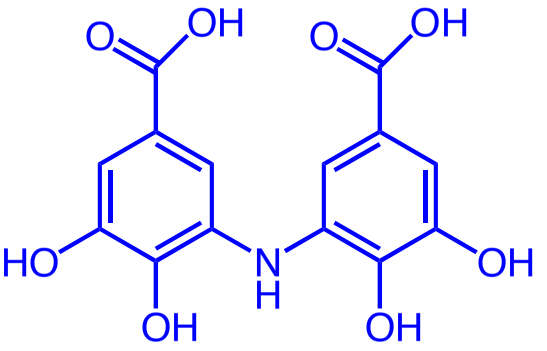
2D ^{1}H-^{13}C HMBC spectrum



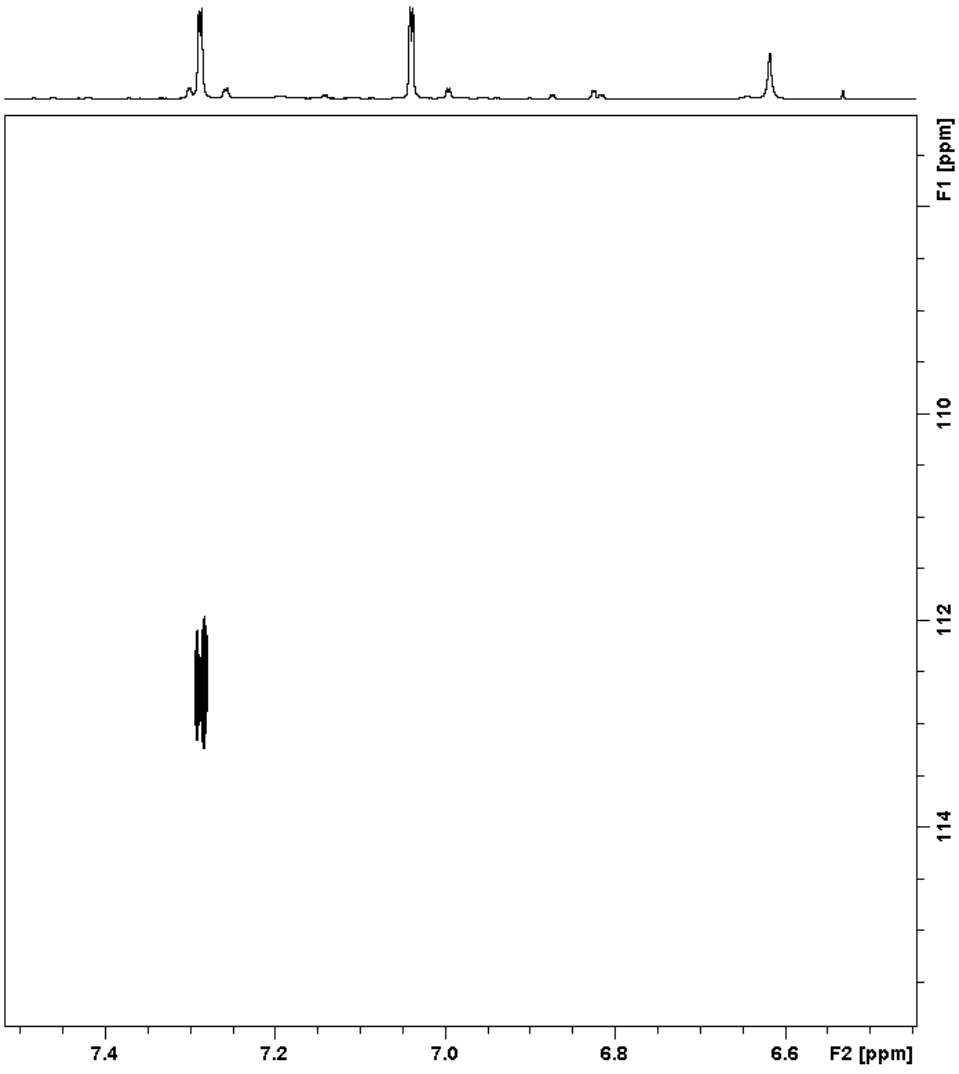


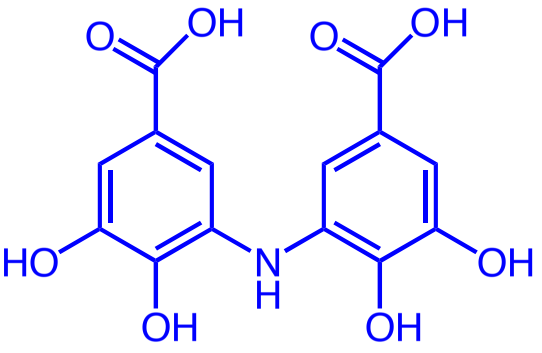
2D ^{1}H ROESY spectrum



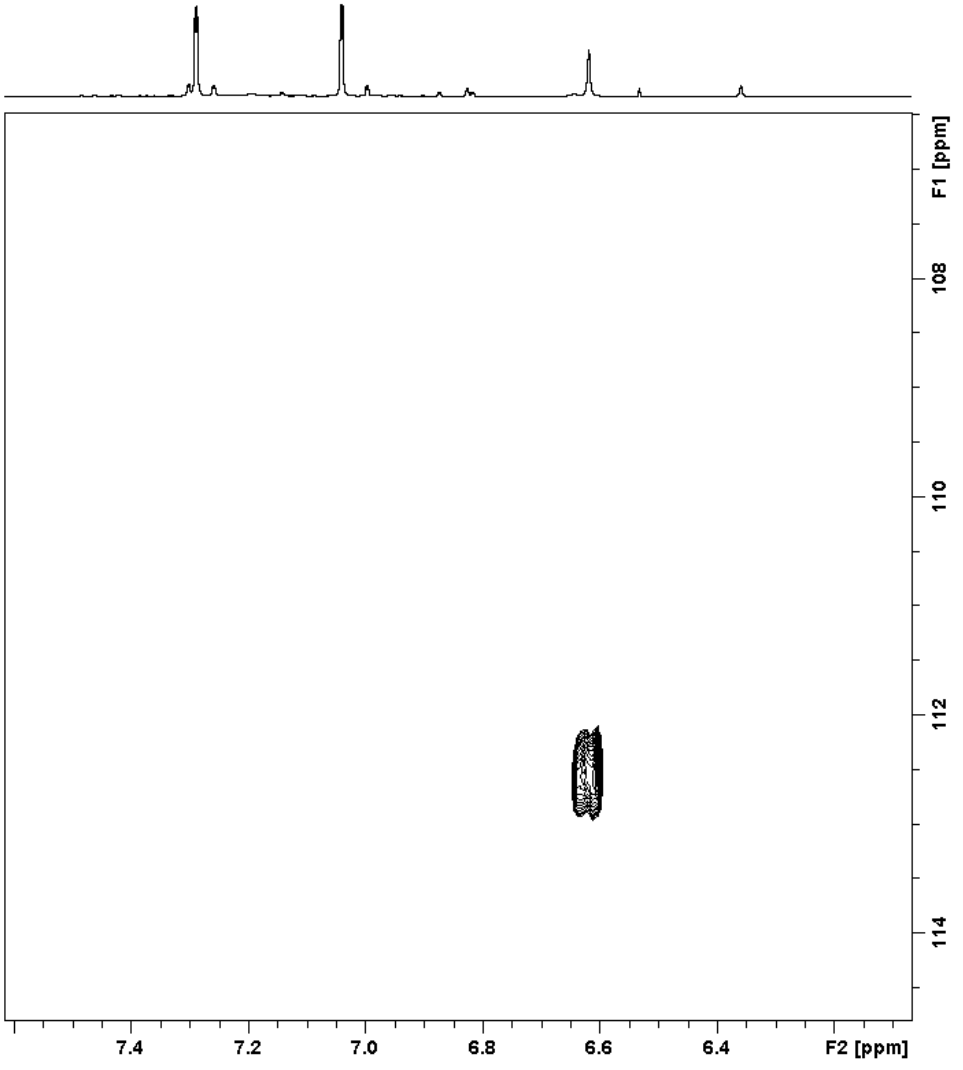


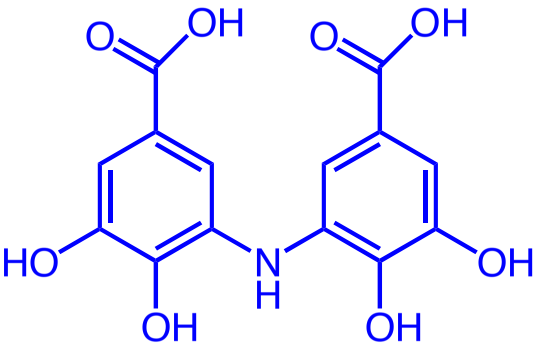
2D ^{1}H-^{15}N HMBC spectrum





2D ^{1}H-^{15}N HSQC spectrum





III

Paper III

6-Carboxydihydroresveratrol 3-*O*-*β*-glucopyranoside – A novel natural product from the Cretaceous relict *Metasequoia glyptostroboides*.

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6-Carboxydihydroresveratrol 3-*O*-β-glucopyranoside — A novel natural product from the Cretaceous relict *Metasequoia glyptostroboides*

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ABSTRACT

Metasequoia glyptostroboides, a tree native to China, is described as a living fossil and has existed for millions of years. The oldest fossils recorded have been dated to the late Cretaceous era. During the time of its existence, the molecular defence system of the tree has apparently resisted millions of generations of pathogens, which encouraged search for novel natural product from this source. Eight compounds have been characterised from needles of *M. glyptostroboides*, including the novel natural product 6-carboxydihydroresveratrol 3-*O*-β-glucopyranoside. The structure determinations were based on extensive use of 2D NMR spectroscopic techniques and high-resolution mass spectrometry.

1. Introduction

Metasequoia glyptostroboides Hu & W.C. Cheng (Taxodiaceae) is a tree native to the Chongqing municipality and the Hubei province in China. It is an ancient tree with a strong adaptability, and has survived since the late Cretaceous Era [1]. It was for the first time reported as a fossil in 1941 [2], and living specimens were sensationally discovered in Modaoxi, China ([3,4]; reviewed in [5]). In 1947 and 1948, seeds were sent on three occasions from Chinese and American botanists, to botanical institutions and nurseries around the world [6]. Some of these seeds reached the Dutch nursery Ruys. In 1950, five small trees were sold by Ruys to the botanical garden of Bergen. Today, two of these trees are still alive, as middle sized trees (Fig. 1). More than 50 countries are today cultivating trees of this species.

As medicinal plant *M. glyptostroboides* is constituent of a plant medicine used for treatment of diabetes [7] and has also applications in traditional Chinese medicine (TCM) [8]. Medicinal compositions for skin care have been prepared from *M. glyptostroboides* [9,10]. Moreover, an anticancer drug based on dihydrohinokiflavone isolated from *M. glyptostroboides* has been patented [11]. Since the late Cretaceous and until present, the tree has apparently been exposed to millions of generations of microorganisms and several significant environmental changes. *M. glyptostroboides* is therefore interesting as a source of natural products which could be potential new drug candidates. As a consequence, there has recently been an increased interest in the search for novel natural products from *M. glyptostroboides*. More than 250 compounds have been reported from this plant source in current literature. The studies previously performed are including, but not restricted to, characterization of flavonoids [12–15], norlignans [16,17], sesquiterpenoids [18,19], labdane-type diterpenoids [20,21], abietane-type diterpenoids [22], sterols [23,24] and carotenoids [25]. Zeng et al. [26] recently reported the isolation of no less than 52 compounds from branches and stems of *M. glyptostroboides* including thirteen norlignans, ten lignans, four phenylpropanoids, four apocarenoids, four flavan-3-ol derivatives, one fructoside, one sterol and fifteen other phenolic compounds. The majority of these compounds (28 compounds) were reported from the family Taxodiaceae for the first time.

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Fig. 1. *Metasequoia glyptostroboides* photographed in the Botanical garden of the University of Bergen 2013.
Photo: Torgils Fossen.

Moreover, a significant number of volatile compounds including monoterpenoids, low-molecular weight aromatic compounds, alcohols and other aliphatic compounds have been characterised from *M. glyptostroboides* using GC–MS [18,27–30].

Several compounds unique to *M. glyptostroboides* have hitherto been reported in current literature. These include norlignans [16,17,32], terpenoids [20,21,32] and a diphenylmethane derivative [31]. The fact that *M. glyptostroboides* has experienced an increasing importance as medicinal plant during the last decade [7–11] encouraged the search for novel natural products from this source. As part of our phytochemical work on medicinal plants, we investigated the needles of this species. In the present paper, we report the isolation and structure elucidation of several natural products reported for the first time from *M. glyptostroboides* including a novel bibenzyl glucoside.

2. Experimental

2.1. Extraction of plant material

Needles of *M. glyptostroboides* were collected from a tree located in the botanical garden of the University of Bergen. A voucher specimen has been deposited at the Herbarium BG (accession number BG-S-159712). Prior to extraction the plant material was stored in a freezer at −20 °C. Fresh needles were extracted. Water percentage in the plant was approximately 69%, since 12.58 g of the fresh plant material lost 8.63 g after drying in an oven (92 h) at 120 °C. The needles of *M. glyptostroboides* (1.9 kg) were extracted for three days (two times) with methanol at room temperature. The combined methanolic extracts were concentrated on a rotary evaporator to yield an aqueous concentrate. The aqueous concentrated extract was extracted (three times) with 1 L petroleum ether in a separation funnel. The aqueous phase was thereafter extracted (three times) with 1 L ethyl acetate. The ethyl acetate phase was further separated by column chromatography as described below.

2.2. Amberlite XAD-7 column chromatography

The concentrated ethyl acetate phase ~2.5 L from liquid–liquid partition was applied to the matrix surface of the column (column dimensions: 5 × 105 cm). Water residue was first used as mobile phase, and thereafter a gradient of mobile phase with increasing proportions of methanol (Tables S3–S4) was applied. Fractions around 500–1000 mL were collected in each conical flask. 1–1.5 mL of these were directly transferred to HPLC vials for later determination of their content and purity, as described below. All of the collected fractions were evaporated to dryness on a rotary evaporator. The dried fractions were redissolved in methanol and transferred to dram vials and dried under nitrogen gas stream. Fraction 7 (2.0 g) containing compounds **4**, **5** and **6** was analysed by NMR spectroscopy without further purification.

2.3. Sephadex LH-20 column chromatography

Fraction 8 from XAD-7 column chromatography of the ethyl acetate phase was further separated on a Sephadex LH-20 column (column dimensions: 5 × 100 cm) using varying proportions of methanol, super distilled water and trifluoroacetic acid (TFA). The gradient consisted of 0.5 L methanol–water–TFA 20:80:0.2; v/v (fractions 1–2), followed by 3 L methanol–water–TFA 50:50:0.2; v/v (fractions 3–28) and 10 L methanol–water–TFA 70:30:0.2; v/v (fractions 29–72). Prior to application the sample was dissolved in a total of 80 ml methanol-super distilled water (50:50). The dissolved sample was applied to the column and allowed to be absorbed by the stationary phase. A gradient of mobile phase was used (Table S4) to elute all the compounds. The purity of each fraction was determined by analytical HPLC. All the collected fractions were evaporated to dryness. The dried fractions were redissolved in methanol and directly transferred to dram vials and dried under nitrogen gas stream. Pure **1** (31 mg) was isolated in fraction 26. Fraction 14 was further purified by preparative HPLC.

2.4. Preparative HPLC

The HPLC instrument was equipped with a 250 × 22 mm, C_{18} Altech column. Two solvents were used for elution; A (water–TFA 99.5:1; v/v) and B (methanol–TFA 99.5:1; v/v). The elution profiles of the applied HPLC gradients are shown in Fig. S2. The sample was dissolved in a total of 1 mL of A–B (50:50 v/v). An HPLC gradient program was applied and started as described in Fig. S2. Portions of 100 μL of the sample were manually injected into the HPLC column. Each peak in the chromatogram was separately collected in vials. 1–1.5 mL of each of the collected fractions was transferred to HPLC vials for later identifications using analytical HPLC. Pure **2**

(3.5 mg) was isolated in fraction 5 whereas fraction 1 contained compounds **7** and **8** (relative proportions: 10:1; together 2 mg). Pure **3** (trace amounts) was isolated in fraction 8.

2.5. Analytical HPLC

The HPLC instrument was equipped with a HP 1050 multidiode array detector, a 20 μL loop and a 250 × 4.6 mm, 5 μm Thermo Scientific Hypersil GOLD column. Two solvents were used for elution; A (water–TFA 99.5:1; v/v) and B (acetonitrile–TFA 99.5:1; v/v). The elution profile of the applied HPLC gradient is shown in Fig. S1. The analytical HPLC pump system was purged with both solution A (super distilled water and 0.5% TFA) and solution B (acetonitrile and 0.5% TFA) for 15 min each with a flow of 5 mL/min. The column was thereafter equilibrated with a flow of 1 mL/min in 30 min with acetonitrile-super distilled water (10:90 v/v). 20 μL of each sample was injected with an autoinjector. An appropriate solvent gradient (Fig. S1) was applied for the separations. The flow rate was 1 mL/min.

2.6. Spectroscopy

High resolution mass spectra were recorded using a JEOL AccuTOF JMS T100LC instrument fitted with an electrospray ion source. The spectrum was recorded over the mass range 50–1000 m/z.

UV–Vis absorption spectra were recorded on-line during HPLC analysis over the wavelength range 240–600 nm in steps of 2 nm.

NMR samples were prepared by dissolving the isolated compounds in deuterated dimethylsulfoxide (99.9 atom % D, Sigma-Aldrich). The 1D ^{1}H and the 2D ^{1}H-^{13}C HMBC, ^{1}H-^{13}C HSQC, 2D ^{1}H-^{1}H COSY and ^{1}H-^{1}H ROESY NMR experiments were obtained at 600.13 MHz and 150.90 MHz for ^{1}H and ^{13}C, respectively, at 298 K on a Bruker 600 MHz instrument equipped with a cryogenic probe.

2.7. Bioassays

Measurement of DPPH scavenging activity and 15-Lipoxygenase inhibition were performed using a BioTek® EON™ Microplate Spectrophotometer (BioTek Instruments Inc., Winooski, Vermont, USA).

2.7.1. DPPH scavenging activity

Radical-scavenging activity was tested with DPPH as the radical as suggested by Blois (1958) [33] and in accordance with the procedure described by Glavind (1963) [34] and Lyckander and Malterud (1996) [35], with some modifications described in Amer et al. (2013) [36]. In brief, a methanolic solution of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) (180 μl, 291.64 μM) was added to each succeeding well on the 96 well UV-transparent microtiter plates (Greiner; cyclic olefin copolymer (COC) flat bottom) and absorbance at 517 nm were measured. The test substance 6-carboxydihydroresveratrol 3-*O*-β-glucopyranoside and reference compound gallic acid were dissolved in DMSO. Portions of these solutions were added to the wells and mixed with the DPPH solution. Concentrations of 10, 20, 30, 40, 60, 71, 81, 91, 101, 202, 404, 605, 807 and 1009 μM of 6-carboxydihydroresveratrol 3-*O*-β-glucopyranoside were tested. As positive control, concentrations of 10, 20, 30, 40, 50, 61, 71, 81, 91, 101, 202, 403, 605, 806 and 1008 μM of gallic acid were tested. Decrease in absorption at 517 nm was then measured initially each at 1 min. and then every 5 min. over a period of 30 min. All experiments were performed in triplicate at 25 °C.

2.7.2. 15-Lipoxygenase inhibition

Testing of 15-Lipoxygenase inhibition was performed as described by Lyckander and Malterud (1992) [37], with some modifications described below. A solution of linoleic acid (188 μL, concentration 0.004418%) in borate buffer (pH 8.95) were mixed with the test substances 6-carboxydihydroresveratrol 3-*O*-β-glucopyranoside and quercetin (2 μL) in each succeeding well of the 96 well UV-transparent microtiter plates. Concentrations of 10, 54, 107, 153, 207, 307, 352, 401, 453, 507, 600 and 688 μM of 6-carboxydihydroresveratrol 3-*O*-β-glucopyranoside were tested. As a positive control, concentrations of 11, 23, 32, 42, 51, 61, 74, 83, 93, 95, 112, 225, 305, 353, 401, 449, 497, 609, 706 and 802 μM of quercetin were tested. Absorbance at 234 nm were then measured before a solution of 15-Lipoxygenase (10 μL, 3333.218 U/mL) was added to the mixture. Increase in absorbance at 234 nm was then measured every 60 s over a period of 5 min. All experiments were performed in triplicate at 25 °C.

3. Results and discussion

The methanolic extract of needles of *M. glyptostroboides* was concentrated under reduced pressure and fractionated by extraction with petroleum ether and ethyl acetate. The ethyl acetate phase was further separated by gradient XAD-7 adsorption chromatography, Sephadex LH-20 gel filtration chromatography and preparative HPLC.

The seven known compounds aromadendrin 7-*O*-β-glucopyranoside (**2**), quercetin 3-*O*-α-rhamnopyranoside-7-*O*-β-glucopyranoside (**3**), gallocatechin (**4**), 3-*O*-(*E*)-coumaroylquinic acid (**5**), 3-*O*-(*Z*)-coumaroylquinic acid (**6**), 3-*O*-(*E*)-coumaroylquinic acid methyl ester (**7**) and 3-*O*-(*Z*)-coumaroylquinic acid methyl ester (**8**) were characterised from needles of *M. glyptostroboides* (Fig. 2). The structure determinations were based on NMR spectroscopy (supplementary tables S1–S2) and MS. Gallocatechin was isolated in a mixture together with 3-*O*-(*Z*)-coumaroylquinic acid and 3-*O*-(*E*)-coumaroylquinic acid (relative proportions: 1: 2: 1.4). Moreover, the isomers 3-*O*-(*Z*)-coumaroylquinic acid methyl ester and 3-*O*-(*E*)-coumaroylquinic acid methyl ester were isolated as a mixture (relative proportions: 1:10). Because these substances were known compounds, further separation was not attempted. With exception of gallocatechin [26], these compounds have, however, not previously been characterised from *M. glyptostroboides*. Methanol was used as extraction solvent and applied during the isolation process. Thus, we cannot exclude that the methyl esters of 3-*O*-coumaroylquinic acid may be formed as artefacts during the isolation process.

The aromatic region of the 1D ^{1}H NMR spectrum of **1** showed a 4H AA′XX′ system at δ 6.99 ('d' 8.5 Hz, H-2′/6′) and δ 6.66 ('d' 8.5 Hz, H-3′/5′) in accordance with a *para*-substituted

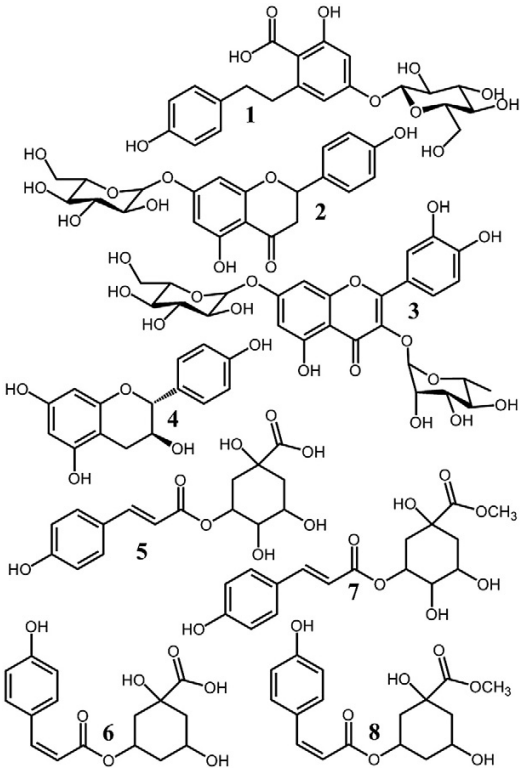

Fig. 2. Structures of compounds **1**–**8** isolated from needles of *Metasequoia glyptostroboides*.

aromatic moiety, in addition to a 2H AB system at δ 6.43 (d 2.5 Hz, H-2) and δ 6.41 (d 2.5 Hz, H-4). The 2D ^{1}H COSY and ROESY spectra and the 2D ^{1}H-^{13}C HMBC spectrum revealed that the aromatic rings were connected through an CH_2–CH_2 bridge at δ 2.98 (H-7a), δ 2.94 (H-7b) and δ 2.67 (H-8), which is in accordance with a substituted dihydroresveratrol moiety [38]. The ^{1}H and ^{13}C signals were completely assigned by the 1D ^{1}H and 1D ^{13}C CAPT spectra, the 2D ^{1}H COSY and ROESY spectra, in addition to the heteronuclear 2D ^{1}H-^{13}C HSQC, 2D-edited HSQC and HMBC spectra (Table 1). The aliphatic region of the 1D ^{1}H NMR spectrum and the 1D ^{13}C CAPT spectrum of **1** showed the presence of a sugar unit. The sugar unit was identified as glucose by the large axial–axial coupling constants observed in the 1D ^{1}H NMR spectrum, in addition to the 6 ^{13}C resonances belonging to the sugar unit observed in the 1D ^{13}C CAPT spectrum. All ^{1}H resonances belonging to the sugar unit were assigned by the 2D ^{1}H COSY spectrum. The corresponding ^{13}C resonances were then assigned from the 2D ^{1}H-^{13}C HSQC spectrum (Table 1). The anomeric coupling constant (7.7 Hz) confirmed the β-configuration of the glucosyl unit of **1**.

The crosspeak at δ 4.87/160.5 (H-1″/C3) confirmed that the glucosyl was attached to the 3-position of the aglycone through an oxygen bridge (Fig. 3). This connectivity was further supported by the crosspeaks at δ 6.43/4.87 (H-2/H-1″) and δ 6.41/4.87 (H-4/H-1″) observed in the 2D ROESY

Table 1
^{1}H and ^{13}C NMR chemical shift values (ppm) and coupling constants (Hz) for the novel natural product 6-carboxydihydroresveratrol 3-O-β-glucopyranoside (**1**) from *Metasequoia glyptostroboides*. Abbreviations: s = singlet, d = doublet, 'd' = semi doublet, dd = double doublet, ddd = double double doublet, t = triplet, b = broad signal.

Atom no.	6-carboxydihydroresveratrol 3-O-β-glucopyranoside	
	δ ^{1}H	δ ^{13}C
1		145.47
2	6.43 d 2.5	110.26
3		160.57
4	6.41 d 2.5	101.55
5		162.13
6		109.14
7a	2.98 m	37.83
7b	2.94 m	
8	2.67 m	36.72
1′		132.00
2′/6′	6.99 'd' 8.5	129.15
3′/5′	6.66 'd' 8.5	115.15
4′		155.51
1‴		172.14
4′-OH	9.13 s(b)	
5-OH	11.57 s(b)	
1″	4.87 d 7.8	99.84
2″	3.20 dd 7.8, 9.0	73.24
3″	3.27 t 9.0	76.67
4″	3.15 dd 9.0, 9.6	69.74
5″	3.34 ddd 2.2, 5.9, 9.6	77.21
6″A	3.68 dd 2.2, 12.0	60.72
6″B	3.46 dd 5.9, 12.0	

spectrum of **1**. The carboxyl substituent was determined to be attached to the 6-position of the dihydroresveratrol aglycone by the crosspeaks at δ 2.98/172.1 (H-7a/C=O), δ 2.94/172.1 (H-7b/C=O), δ 6.43/172.1 (H-2/C=O) and δ 6.41/172.1 (H-4/C=O) observed in the HMBC spectrum (Fig. 3). Thus, **1** was identified as the novel compound 6-carboxydihydroresveratrol 3-*O*-β-glucopyranoside (Fig. 2). A pseudomolecular ion $[M+H]^{+}$ at *m/z* 437.14489 observed in the high resolution mass spectrum of **1** corresponding to $C_{21}H_{24}O_{10}$ (calculated 437.14489), in addition to a fragment ion at *m/z* 275.0893 [M-glucose], corresponding to loss of the glucose unit, confirmed this identity.

Bibenzyls have not previously been identified from *M. glyptostroboides*. Bibenzyl glycosides based on the rare aglycone 6-carboxydihydroresveratrol seem to have a restricted occurrence in nature. 6-Carboxydihydroresveratrol without any glycosyl substituents has not been identified from any source. The only known structurally related compound with the same rare 6-carboxydihydroresveratrol aglycone but a different sugar unit, namely 6-carboxydihydroresveratrol 3-*O*-β-xylopyranoside, has been isolated from the sub-aerial parts of the completely unrelated species white salsify (*Tragopogon porrifolius* subsp. *porrifolius*) [38] belonging to the family Asteraceae (Compositae) which originated at a considerably later stage than *M. glyptostroboides*. The NMR data of the aglycone part of **1** are in good agreement with the published ones for 6-carboxydihydroresveratrol 3-*O*-β-xylopyranoside [38].

The radical scavenging activity of the novel compound (**1**) 6-carboxydihydroresveratrol 3-*O*-β-glucopyranoside exhibited IC_{50} value of 164 ± 39 μM. Since gallic acid is known to

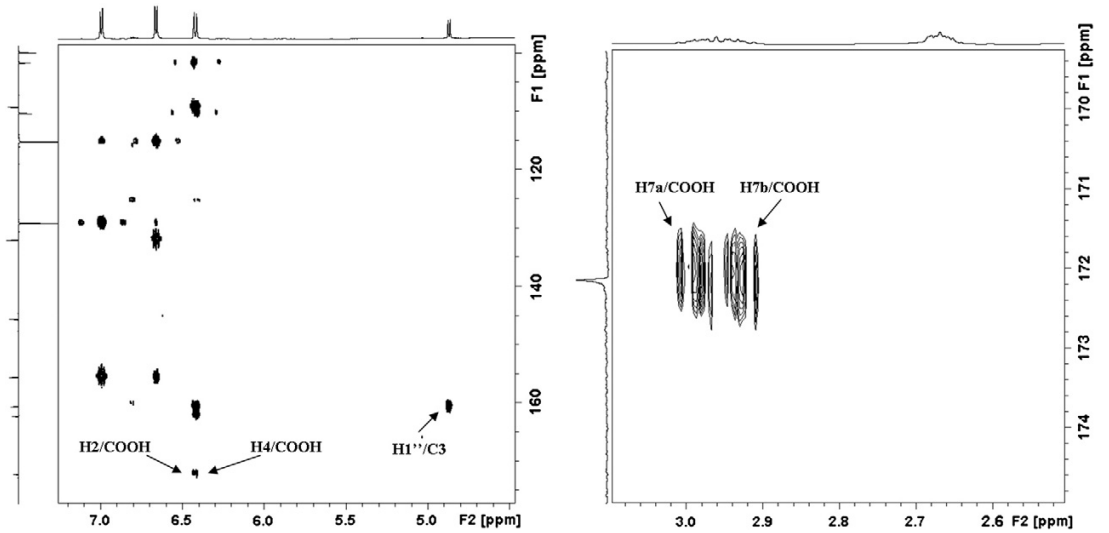

Fig. 3. Expanded regions of the HMBC spectrum of **1**. Selected cross peaks important for determination of the linkages between the structural sub-units are highlighted.

have potent radical scavenging activity [39] this compound was used as a positive control. In comparison, IC_{50} for gallic acid was determined to be 14 ± 2 μM, which is in accordance with previously reported values [Masaki et al., 1994]. Inhibition activities towards 15-Lipoxygenase from soybean were determined for the novel compound (**1**) and quercetin (positive control). The IC_{50} value for inhibition of 15-Lipoxygenase of 6-carboxydihydroresveratrol 3-*O*-β-glucopyranoside was determined to be 297 ± 15 μM, which is comparable to that of quercetin, which in our assay was determined to be 246 ± 30 μM. It may be noted that the IC_{50} value of quercetin was approximately 5 times higher than the values reported for this compound in current literature [35].

Stilbenoids and dihydrostilbenoids with carboxyl substituents have a restricted occurrence in nature. A limited number of identifications of such compounds from higher plants exist in current literature including white salsify (*T. porrifolius* subsp. *porrifolius*) [38] and *Hydrangea macrophylla* [40]. The fact that these compounds have mainly been found in species belonging to the oldest lineages of plant families like the fern *Hicriopteris glauca* [41], Liverworts [42–44] and algae [45] indicate that these compounds may be biogenetic precursor of modern plant stilbenoid, with the COOH group being a biogenetical archaicism.

A possible reason that the overwhelming majority of the isolated compounds have not previously been reported from *M. glyptostroboides* may be the fact that the plant material was harvested in Bergen, Norway. Because the original habitat of the tree is Hubei, it was grown under quite different climatic conditions in Norway and changes in the secondary metabolism might have been expected. Temperature and sunlight conditions are known to be important parameters for secondary metabolism of plants [46].

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.fitote.2014.03.001.

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Supplementary data for

6-Carboxydihydroresveratrol 3-*O*-*β*-glucopyranoside –

A novel natural product from the Cretaceous relict *Metasequoia glyptostroboides*

Xuan Hong Thy Nguyen, Ole Johan Juvik, Dag Olav Øvstedal, Torgils Fossen*

Table S1

^{1}H NMR chemical shift values (ppm) and coupling constants (Hz) for aromadendrin 7-*O*-*β*-glucopyranoside (**2**), quercetin 3-*O*-*α*-rhamnopyranoside-7-*O*-*β*-glucopyranoside (**3**), gallocatechin (**4**) , 3-O-(*E*)-coumaroylquinic acid (**5**) , 3-O-(*Z*)-coumaroylquinic acid (**6**) , 3-O-(*E*)-coumaroylquinic acid methyl ester (**7**) and 3-O-(*Z*)-coumaroylquinic acid methyl ester (**8**) and isolated from *Metasequoia glyptostroboides*. Nomenclature: s = singlet, d = doublet, 'd' = semi doublet, dd = double doublet, ddd = double double doublet, t = triplet, b = broad peak

Atom no.	**2**	**3**	**4**	**5**	**6**	**7**	**8**
	δ ^{1}H	δ ^{1}H	δ ^{1}H	δ ^{1}H	δ ^{1}H	δ ^{1}H	δ ^{1}H
2 a	5.11 d 11.5		4.44 d 7.2	1.87 d 5.3	1.87 m	1.97 dd 5.9 , 13.3	1.97 m
2 b						1.83 dd 2.9 , 13.3	1.81 m
3	4.64 d 11.5		3.78 m	3.88 dd 5.8 , 12.1	3.82 m	3.80 m	3.77 m
4 a			2.60 dd 5.4 , 16.1	3.56 m	3.56 s(b)	3.60 m	3.61 m
4b			2.35 dd 7.7 , 16.1				
5				5.18 m	5.15 m	5.15 ddd 3.2 , 4,2 , 9,4	5.12 ddd 3.0 , 4.3 , 9.4
6 a	6.15 d 2.2	6.44 d 2.1	5.88 d 2.4	2.03 dd 7.6 , 13.4	2.01 m	2.05 ddd 12.5, 4,2 , 1,3	2.03 m
6 b				1.91 dd 7.6 , 13.4	1.88 m	1.87 dd 12.5, 9.4	1.83 m
8	6.12 d 2.2	6.73 d 2.1	5.70 d 2.4				
2'	7.31 'd' 8.7	7.31 d 2.2	6,25 s	7.51 'd' 8.8	7.69 d 8.7	7.52 'd' 8.7	7.68 'd' 8.8
3'	6.78 'd' 8.7			6.80 'd' 8.5	6.74 d 8.7	6.78 'd' 8.7	6.74 'd' 8.8
5'	6.78 'd' 8.7	6.86 d 8.3		6.80 'd' 8.5	6.74 d 8.7	6.78 'd' 8.7	6.74 'd' 8.8
6'	7.31 'd' 8.7	7.26 dd 2.2,8.3	6,25 s	7.51 'd' 8.8	7.69 d 8.7	7.52 'd' 8.7	7.68 'd' 8.8
7'				7.54 d 15.9	6.80 m	7.54 d 15.9	6.80 d 12.9
8'				6.32 d 15.9	5.76 d 12.9	6.33 d 15.9	5.75 d 12.9
4'-OH	9.56 s(b)			10.00 s(b)	9.89 s	10.00 s	9.89 s

7’-OCH_3				3,58 s	N/A
5-OH	11.79 s		9,19 s		
7-OH			8,97 s		
3’-OH			8,80 s		
5’-OH			8,80 s		
			3-*O*-glycopyranoside		
1’’	4.94 d 7.6	5.27 d 1.7			
2’’	3.19 dd 7.6, 9.0	3.97 m			
3’’	3.24 d 8.8	3.49 m			
4’’	3.12 dd 8.7, 9.7	3.14 m			
5’’	3.36 m	3.20 m			
6A’’	3.65 dd 2.2,	0.81 d 6.1			
6B’’	11.9				
	3.42 dd 5.9,				
	11.9				
2’’-OH		4.96 d 4.4			
3’’-OH		4.62 m			
4’’-OH		4.73 d 4.8			
			7-*O*-β-glucopyranoside		
1’’’		5.07 d 7.5			
2’’’		3.24 m			
3’’’		3.28 m			
4’’’		3.15 m			
5’’’		3.20 m			
6A’’’		3.69 m			
6B’’’		3.46 m			
2’’’-OH		5.39 d 5.0			
3’’’-OH		5.13 d 4.9			
4’’’-OH		5.06 m			
6’’’-OH		4.61 m			

Table S2

^{13}C NMR chemical shift values (ppm) for aromadendrin 7-*O*-*β*-glucopyranoside (**2**), quercetin 3-*O*-*α*-rhamnopyranoside-7-*O*-*β*-glucopyranoside (**3**), gallocatechin (**4**) , 3-O-(*E*)-coumaroylquinic acid (**5**) , 3-O-(*Z*)-coumaroylquinic acid (**6**) , 3-O-(*E*)-coumaroylquinic methyl esther (**7**) and 3-O-(*Z*)-coumaroylquinic methyl esther (**8**) and isolated from *Metasequoia glyptostroboides*.

Atom no.	**2**	**3**	**4**	**5**	**6**	**7**	**8**
	δ ^{13}C	δ ^{13}C	δ ^{13}C	δ ^{13}C	δ ^{13}C	δ ^{13}C	δ ^{13}C
1				73.23	73.12		
2	83.12	n.a.	81.26	39.4	39.20	38.2	38.1
3	71.65	n.a.	66.59	67.55	67.69	67.9	68.0
4	198.73	n.a.	27.70	71.29	71.21	69.8	69.6
5	162.78	n.a.	156.45	71.28	71.20	70.5	70.5
6	96.83	99.2	95.37	35.33	35.11	34.9	34.9
7	165.48	n.a.	156.69	176.34	176.28	174.2	N/A
8	95.46	94.4	94.16				
9	162.46	n.a.	155.56				
10	102.10	n.a.	99.26				
1’	127.39	n.a.	130.14	125.57	125.57	125.3	125.6
2’	129.58	115.5	106.25	130.41	132.93	130.3	132.7
3’	114.99	147.0	145.90	116.11	115.14	115.9	114.9
4’	157.88	n.a.	132.76	159.89	158.91	159.7	158.7
5’	114.99	115.2	145.90	116.11	115.14	115.9	114.9
6’	129.58	121.1	106.25	130.41	132.93	130.3	132.7
7’				144.45	142.67	144.2	144.2
8’				115.52	116.77	115.1	116.4

9’			166.52	165.92	166.0	165.6
7-OCH_3					51.6	N/A
			3-*O*-glycopyranoside			
1’’	99.70	99.7				
2’’	73.07	69.9				
3’’	76.37	69.7				
4’’	69.56	71.0				
5’’	77.14	70.6				
6’’	60.63	17.3				
			7-*O*-β-glucopyranoside			
1’’’		101.7				
2’’’		73.0				
3’’’		76.3				
4’’’		69.4				
5’’’		77.0				
6’’’		60.4				

Table S3.

Mobile phase composition used for XAD-7 Amberlite column chromatography of the ethyl acetate phase of extract from needles of *Metasequoia glyptostroboides*

Mobile phase	100% Distilled water	Distilled water – Methanol (90:10 v/v)	Distilled water – Methanol (75:25 v/v)	Distilled water – Methanol (50:50 v/v)	Distilled water – Methanol (25:75 v/v)	100% Methanol
Volume (l)	2	1	1	2	1	5.4
Fractions	1-2	3	4	5-6	7	8-13

Table S4.

Mobile phase composition used for Sephadex LH-20 column chromatography of fraction 8 from XAD-7 column chromatography of the ethyl acetate phase of extract from needles of *Metasequoia glyptostroboides*

Mobile phase	Methanol- Super distilled water- TFA (20:80.0.2 v/v)	Methanol- Super distilled water- TFA (50:50:0.2 v/v)	Methanol Super distilled water TFA (70:30:0.2 v/v)
Volume (l)	0.5	3	10
Fractions	1-2	2-28	28-72

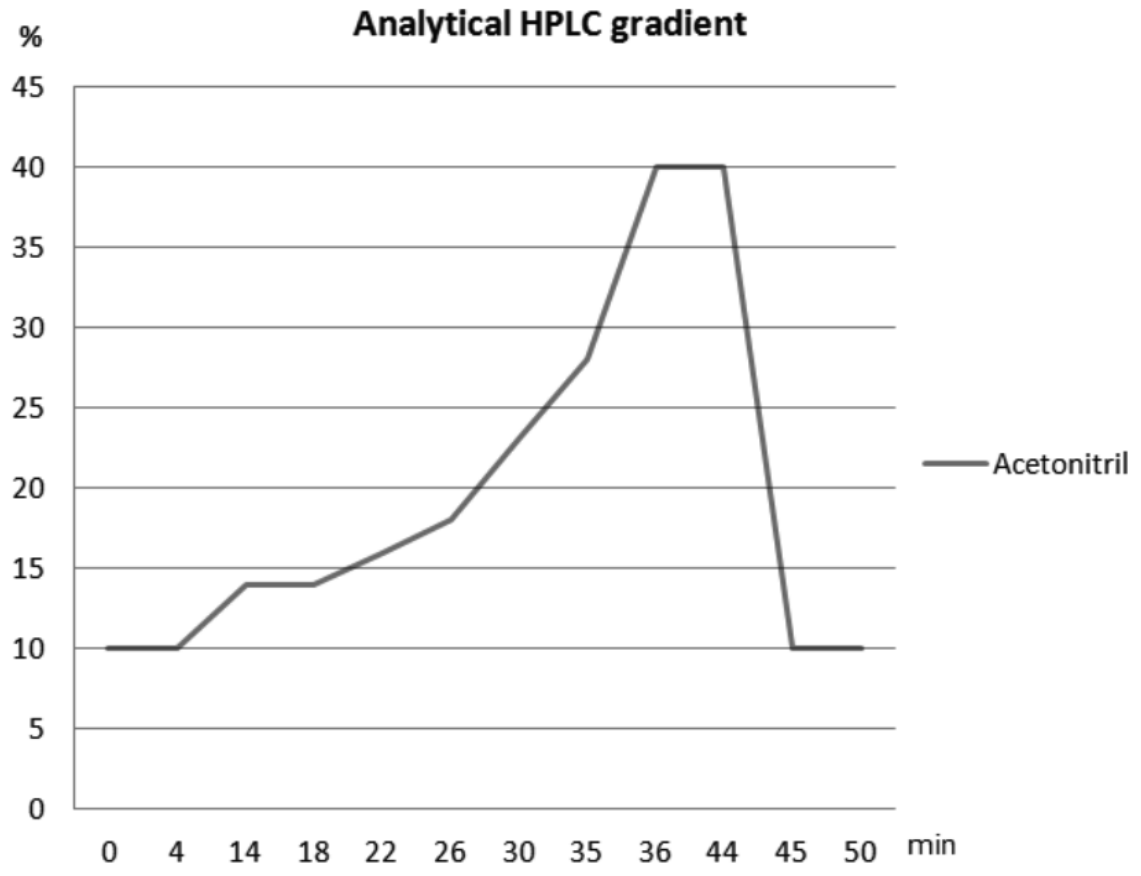

Fig. S1. Solvent gradient (%B in A versus time) for separation on analytical HPLC. Two solvents were used for elution; A (water-TFA 99.5:1; v/v) and B (acetonitrile-TFA 99.5:1; v/v).

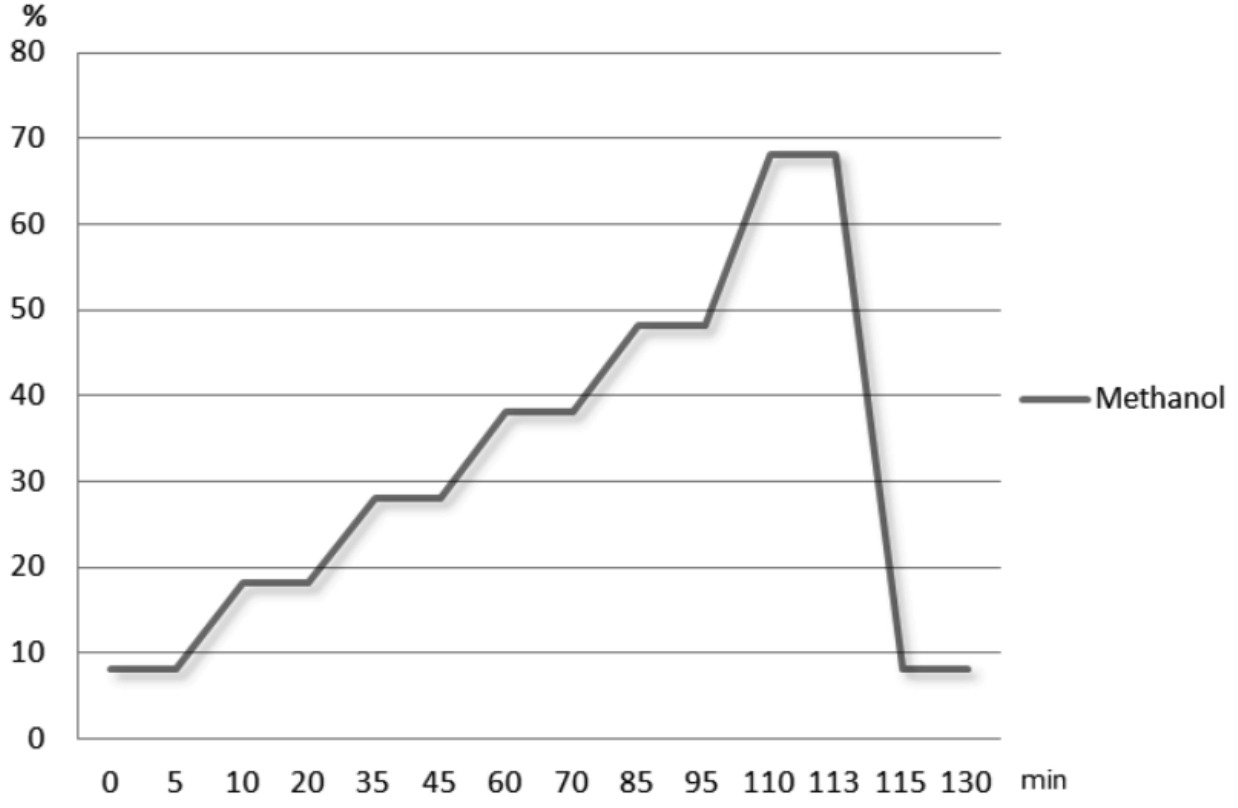

Fig. S2. Solvent gradient (%B in A versus time) applied for isolation of compounds on preparative HPLC. Two solvents were used for elution; A (water-TFA 99.5:1; v/v) and B (methanol-TFA 99.5:1; v/v).

Paper IV

Growing with dinosaurs – Natural products from the Cretaceous relict *Metasequoia glyptostroboides* Hu & Cheng – a molecular reservoir from the ancient world with potential in modern medicine..

IV

Growing with dinosaurs: natural products from the Cretaceous relict Metasequoia glyptostroboides *Hu & Cheng—a molecular reservoir from the ancient world with potential in modern medicine*

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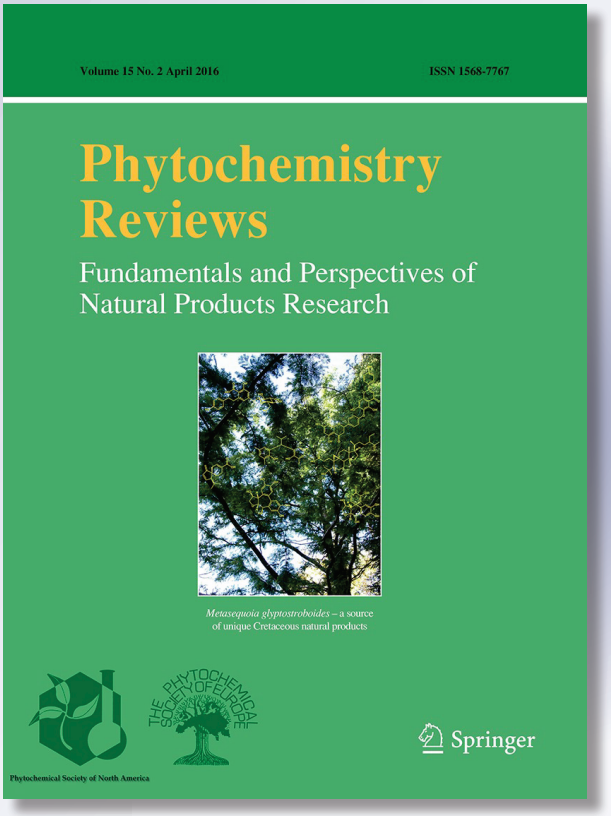

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Growing with dinosaurs: natural products from the Cretaceous relict *Metasequoia glyptostroboides* Hu & Cheng—a molecular reservoir from the ancient world with potential in modern medicine

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Abstract After the sensational rediscovery of living exemplars of the Cretaceous relict *Metasequoia glyptostroboides*—a tree previously known exclusively from fossils from various locations in the northern hemisphere, there has been an increasing interest in discovery of novel natural products from this unique plant source. This article includes the first complete compilation of natural products reported from *M. glyptostroboides* during the entire period in which the tree has been investigated (1954–2014) with main focus on the compounds specific to this plant source. Studies on the biological activity of pure compounds and extracts derived from *M. glyptostroboides* are reviewed for the first time. The unique potential of *M. glyptostroboides* as a source of bioactive constituents is founded on the fact that the tree seems to have survived unchanged since the Cretaceous era. Since then, its molecular defense system has resisted the attacks of millions of generations of pathogens. In line with this, some recent landmarks in *Metasequoia* paleobotany are covered. Initial spectral analysis of recently discovered intact 53 million year old wood and amber of *Metasequoia* strongly indicate that the tree has remained unchanged for millions of years at the molecular level.

Keywords *Metasequoia glyptostroboides* · Natural products · Biological activity · Paleobotany · Living fossil

Introduction

Metasequoia glyptostroboides Hu et Cheng (Cupressaceae) is a deciduous conifer native to southeast China (Hu 1948b). The tree is particularly interesting because it seems to have remained unchanged for millions of years since the Cretaceous period (145–66 million years ago). During this long timespan the tree has survived substantial ecological and climate changes and resisted attacks from countless generations of bacteria, viruses, fungi and other plant pathogens. Phytochemical investigations of natural products from *M. glyptostroboides* have been performed since the early 1950s (Bate-Smith 1954; Bate-Smith and Lerner 1954; Hattori et al. 1954). A significant number of natural products have hitherto been characterised from *M. glyptostroboides* although there is as yet no complete review of natural products thereof. In current literature a limited number of

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natural products from *M. glyptostroboides* have occasionally been included in reviews which focused on specific compound classes such as flavonoids (Beckmann et al. 1971; Gadek and Quinn 1989; Harborne and Mabry 1982; Hida 1958; Sawada 1958; Takahashi et al. 1960b), carotenoids (Ida 1981a, b) and sugars (Hida et al. 1962). A review reports on sources of shikimic acid including *M. glyptostroboides* (Hattori et al. 1954). Another review, which includes this tree, examines leaf waxes of several deciduous conifers without reporting any chemical constituents (Isoi 1958). The lack of complete, comprehensive literature of natural products from *M. glyptostroboides* has consequently led to cases of double reporting, where previous characterizations from this plant source have been overlooked.

The current review covers six decades of phytochemical investigation of *M. glyptostroboides* (1954–2014). A complete compilation of the considerable number of compounds characterized from *M. glyptostroboides* is presented for the first time (Table 1). Such a compilation may be invaluable for the increasing number of researchers working with natural products from this unique species. The exceptionality of *M. glyptostroboides* necessitates a particular focus on compounds unique to this species including available data regarding their biological activity. Consequently, the current paper also includes the first comprehensive review of studies on various biological activities of extracts and pure compounds from *M. glyptostroboides* as well as current medical applications. Moreover, the potential influence of geographical localization on secondary metabolite production of *M. glyptostroboides* is briefly discussed as this may be particularly relevant in view of the fact that since its rediscovery seven decades ago the tree has been extensively cultivated all over the world in regions where climatic conditions are suitable for this species, mainly covering its original prehistorical habitat.

The longevity of *M. glyptostroboides* may make this species a molecular window into the ancient world. Technological improvements allowing for characterization of modified and original natural products from fossil material, have consequently lead to characterization of such compounds from fossil leaves from *M. glyptostroboides*. These compounds which are included in the current review (Table 2) (Zhao et al. 2007) include two natural products reported both from fresh leaves and fossil leaves (Table 1 and 2) (Fujita 1990; Zhao et al. 2007). In line with this, correlations to recent identifications of natural products and modified derivatives thereof from well preserved fossil *M. glyptostroboides* originating from the Miocene era are discussed.

Brief History

Metasequoia was first described as a new extinct genus in 1941 by the Japanese paleobotanist Shigero Miki (1901–1974) (Miki 1941). He based his work on field samples of fossil remains from Japan, which he identified himself. Based on these observations Miki described two new species that were different from Sequoia, but with some common features, and renamed two published species previously ascribed to *Sequioa*. The first of these species was *Sequoia disticha Heer*, which was described in 1876 by Oswald Heer (1809–1883), a Swiss pioneer in paleobotany, based on field samples of fossil remains collected by a Swedish expedition to Svalbard in 1872–1873 (Heer and Nordenskiöld 1876). Five decades later the second species, Sequoia japonica Endô, was described in 1936 by the Japanese paleontologist Seidô Endô based on field samples from Korea and Japan (Endô 1936). The name of the new genus means "resemble a *Sequoia*", and acknowledges the fact that the two genera *Sequoia* and *Metasequoia* resemble each other.

During the early 1940′s a series of events in southeast China led to the sensational discovery of a living species of *Metasequoia*. At the centre of the events is a large deciduous tree, in the small village of Moudao in western Szechuan (Sichuan), locally known as "shui-sha" or water fir in English (Hsueh 1985; Hu 1948a). The story of the collection of specimens and identification of the tree covers seven years from 1941 to 1948, and a complete summary of events and the people involved is beyond the scope of this article. An account of the discovery of *Metasequoia* was written by Hu in 1948 (Hu 1948a). The great interest and rapid accumulation of botanical knowledge necessitated a botanical review as early as 1952 (Florin 1952). Fifty years after the first description of the tree a special thematic issue of *Arnoldia* (Madsen 1998–1999) celebrated the event and a

Table 1 Natural products identified from *Metasequoia glyptostroboides* Hu et Cheng

No.	Substances alphabetically according to group	Part of the tree	Methods of identification	References
	Alcohols			
1	Ethanol	Leaves	GC–MS	Fujita (1990)
2	Butylcarbinol (pentan-1-ol)	Leaves	GC–MS	Bajpai et al. (2009)
		Leaves	GC–MS	Bajpai and Kang (2011b)
3	*n*-Hexanol	Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
4	3-Hexen-1-ol	Shoots	N/A	Fujita et al. (1975)
5	Cis-3-Hexen-1-ol	Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
6	Trans-2-Hexen-1-ol	Leaves	GC–MS	Fujita (1990)
7	*n*-Octanol	Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
8	l-Octen-3-ol (Amyl vinyl carbinol)	Shoots, branchlet and trunk	N/A	Fujita et al. (1975)
		Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
		Leaves	GC–MS	Bajpai and Kang (2011b)
9	7-Octen-2-ol	Leaves	GC–MS	Bajpai et al. (2009)
		Leaves	GC–MS	Bajpai and Kang (2011b)
10	9,12-Tetradecadien-1-ol	Leaves	GC–MS	Bajpai et al. (2009)
		Leaves	GC–MS	Bajpai and Kang (2011b)
11	Ginnol [(+)-*n*-Nonacosanol-(10)]	Leaves	IR, MS, OR	Beckmann and Schuhle (1968)
12	2-Phenyl ethyl alcohol	Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
13	4-Methyl-1-(1-methylethyl)-3-cyclohexane-1-ol	Seeds	GC–MS	Mou et al. (2007)
14	3-Cyclohexene-1-ol	Leaves	GC–MS	Bajpai et al. (2009)
		Leaves	GC–MS	Bajpai and Kang (2011b)
15	Sequoyitol	Leaves	PC	Kariyone et al. (1958)
		Leaves	PPC	Takahashi et al. (1960a)
		Heartwood	IR, MP, EA	Sato et al. (1966)
16	Benzyl alcohol	Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
		Leaves	GC–MS	Bajpai et al. (2009)
		Leaves	GC–MS	Bajpai and Kang (2011b)
	Aldehydes			
17	Benzaldehyde	Leaves	GC–MS	Fujita (1990)
	Alkanes			
18	Tetracosane	Leaves	GC–MS	Fujita (1990)
		Fossil leaves	GC–MS	Zhao et al. (2007)
19	Pentacosane	Leaves	GC–MS	Fujita (1990)
		Fossil leaves	GC–MS	Zhao et al. (2007)
20	Cyclobutane	Leaves	GC–MS	Bajpai et al. (2009)
		Leaves	GC–MS	Bajpai and Kang (2011b)

Table 1 continued

No.	Substances alphabetically according to group	Part of the tree	Methods of identification	References
21	Cyclopentane	Leaves	GC–MS	Bajpai et al. (2009)
		Leaves	GC–MS	Bajpai and Kang (2011b)
22	2,3,3-Trimethyl tricycle heptane	Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
	Alkynes			
23	(Z)-3-Heptadecen-5-yne	Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
24	13-Heptadecyn-1-ol	Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
25	1-Dodecyn-4-ol	Leaves	GC–MS	Bajpai et al. (2009)
		Leaves	GC–MS	Bajpai and Kang (2011b)
	Amide			
26	Valeramide	Leaves	GC–MS	Bajpai et al. (2009)
		Leaves	GC–MS	Bajpai and Kang (2011b)
	Apocarotenoids			
27	Icariside B1	Branches and stems	N/A	Zeng et al. (2013)
28	Icariside B1 aglycon	Branches and stems	N/A	Zeng et al. (2013)
29	4′Dihydrophaseic acid	Branches and stems	N/A	Zeng et al. (2013)
30	4′-Dihydrophaseic acid 4′-O-β-D-glucopyranoside	Branches and stems	N/A	Zeng et al. (2013)
	Dihydrostilbenoids			
31	6-Carboxydihydroresveratrol 3-*O*-β-glucopyranoside	Leaves	NMR, MS	Nguyen et al. (2014)
	Esters			
32	Isopropyl acetate	Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
33	Methyl 4-methoxybutanoate	Seeds	GC–MS	Mou et al. (2007)
34	Cis-3-Hexenyl acetate	Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
35	l-Octen-3-yl acetate	Shoot, branchlet and trunk	N/A	Fujita et al. (1975)
36	Methyl-decanoate	Leaves	GC–MS	Eryin and Rongai (1997)
	Furans			
37	Furan	Leaves	GC–MS	Bajpai et al. (2009)
		Leaves	GC–MS	Bajpai and Kang (2011b)
38	5-Ethyl-2(5H)-furanone	Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
	Ketones			
39	2-Butanone	Leaves	GC–MS	Bajpai et al. (2009)
		Leaves	GC–MS	Bajpai and Kang (2011b)
40	3-Pinanone	Seeds	GC–MS	Mou et al. (2007)
41	6,10,14-Trimethyl pentadecan-2-one	Leaves	GC–MS	Fujita (1990)
42	β-Ionone	Leaves	GC–MS	Eryin and Rongai (1997)
43	Acetophenone	Leaves	GC–MS	(Bajpai et al. 2009)

Table 1 continued

No.	Substances alphabetically according to group	Part of the tree	Methods of identification	References
		Leaves	GC–MS	Bajpai and Kang (2011b)
	Fatty acids and their derivatives			
44	$C_5H_{11}COOH$ (Hexaenoic acid)	Cones	GC–MS	Bajpai et al. (2007a)
45	$C_7H_{15}COOH$ (Octanoic acid)	Leaves	GC–MS	Bajpai et al. (2009)
		Leaves	GC–MS	Bajpai and Kang (2011b)
46	$C_9H_{21}COOH$ (Capric acid)	Heartwood	GLC	Sato et al. (1966)
47	$C_{11}H_{23}COOH$ (Lauric acid/ Dodecanoic acid)	Heartwood	GLC	Sato et al. (1966)
		Twigs	IR, GC, S	Hayashi et al. (1969)
		Photosynthetic tissue	GLC	Mongrand et al. (2001)
48	$C_{12}H_{25}COOH$ (Tridecyclic acid/ Tridecanoic acid)	Twigs	IR, GC, S	Hayashi et al. (1969)
		Photosynthetic tissue	GLC	Mongrand et al. (2001)
49	$C_{13}H_{27}COOH$ (Myristic acid/ Tetradecanoic acid)	Heartwood	GLC	Sato et al. (1966)
		Twigs	IR, GC, S	Hayashi et al. (1969)
50	$C_{14}H_{29}COOH$ (Pentadecanoic acid)	Twigs	IR, GC, S	Hayashi et al. (1969)
51	$C_{14}H_{27}COOH$	Twigs	IR, GC, S	Hayashi et al. (1969)
52	$C_{15}H_{31}COOH$ (Palmitic acid/ Hexadecanoic acid)	Heartwood	GLC	Sato et al. (1966)
		Twigs	IR, GC, S	Hayashi et al. (1969)
		Leaves	GC–MS	Eryin and Rongai (1997)
		Photosynthetic tissue	GLC	Mongrand et al. (2001)
53	$C_{15}H_{29}COOH$	Twigs	IR, GC, S	Hayashi et al. (1969)
		Photosynthetic tissue	GLC	Mongrand et al. (2001)
54	$C_{15}H_{27}COOH$	Twigs	IR, GC, S	Hayashi et al. (1969)
55	16:2 Δ7,10	Photosynthetic tissue	GLC	Mongrand et al. (2001)
56	16:3 Δ7,10,13	Photosynthetic tissue	GLC	Mongrand et al. (2001)
57	$C_{16}H_{33}COOH$ (Margaric acid/ Heptadecanoic acid)	Twigs	IR, GC, S	Hayashi et al. (1969)
58	$C_{17}H_{35}COOH$ (Stearic acid/ Octadecanoic acid)	Twigs	IR, GC, S	Hayashi et al. (1969)
		Photosynthetic tissue	GLC	Mongrand et al. (2001)
59	$C_{17}H_{33}COOH$ (Oleic acid)	Twigs	IR, GC, S	Hayashi et al. (1969)
60	$C_{17}H_{31}COOH$ (Linoleic acid)	Twigs	IR, GC, S	Hayashi et al. (1969)
61	$C_{17}H_{29}COOH$	Twigs	IR, GC, S	Hayashi et al. (1969)
62	18:1 Δ9	Photosynthetic tissue	GLC	Mongrand et al. (2001)
63	18:2 Δ9,12	Photosynthetic tissue	GLC	Mongrand et al. (2001)
64	18:3 Δ9,12,15	Photosynthetic tissue	GLC	Mongrand et al. (2001)
65	$C_{19}H_{39}COOH$ (Eicosanoic acid/ Icosanoic acid)	Twigs	IR, GC, S	Hayashi et al. (1969)
		Photosynthetic tissue	GLC	Mongrand et al. (2001)
66	20:2 Δ5,11	Photosynthetic tissue	GLC	Mongrand et al. (2001)

Table 1 continued

No.	Substances alphabetically according to group	Part of the tree	Methods of identification	References
67	20:2 Δ11,14	Photosynthetic tissue	GLC	Mongrand et al. (2001)
68	20:3 Δ5,11,14	Photosynthetic tissue	GLC	Mongrand et al. (2001)
69	20:4 Δ5,11,14,17	Photosynthetic tissue	GLC	Mongrand et al. (2001)
70	22:0	Photosynthetic tissue	GLC	Mongrand et al. (2001)
71	6,9,12,15-Docosatetraenoic acid	Leaves	GC–MS	Bajpai et al. (2009)
		Leaves	GC–MS	Bajpai and Kang (2011b)
72	Methyl arachidonate	Seeds	GC–MS	Mou et al. (2007)
	Other carboxylic acids			
73	2-Hydroxypropanoic acid	Leaves	GC–MS	Bajpai et al. (2009)
		Leaves	GC–MS	Bajpai and Kang (2011b)
74	Shikimic acid	N/A	IR	Hattori et al. (1954)
	Flavonoids			
	I. Anthocyanidins			
75	Cyanidin	Leaves and other tissues	PC	Bate-Smith (91954)
		Leaves	PC,S	Hida (1958)
76	Delphinidin	Leaves	PC, S	Hida (1958)
	II. Flavones			
77	Apigenin	Leaves	TLC, UV, MS, NMR	Krauze-Baranowska (2004)
78	Apigenin-7-glucosid (Cosmosiin)	Leaves	TLC, PC	Beckmann and Geiger (1968)
79	Luteolin	Leaves	TLC, UV, MS, NMR	Krauze-Baranowska (2004)
80	Luteolin-7-glucosid	Leaves	TLC, PC	Beckmann and Geiger (1968)
81	Tricetin	Leaves	TLC, PC	Beckmann and Geiger (1968)
82	Tricetin-7-glucosid	Leaves	TLC, PC	Beckmann and Geiger (1968)
83	Tricetin 3′-O-glucoside	Leaves	TLC, UV, MS, NMR	Krauze-Baranowska (2004)
	III. Dihydroflavonols			
84	Aromadendrin-7-O-β-glucopyranoside	Leaves	NMR, MS	Nguyen et al. (2014)
85	Aromadendrin oxide	Leaves	GC–MS	Bajpai et al. (2009)
		Leaves	GC–MS	Bajpai and Kang (2011b)
	IV. Flavonols			
86	Kaempferol	N/A	PC	Takahashi et al. (1960b)
		Leaves	TLC, UV, MS, NMR	Krauze-Baranowska (2004)
87	Kaempferol-3-rhamnosid (Afzelin)	Leaves	TLC, PC	Beckmann and Geiger (1968)
88	Quercetin	N/A	PC	Takahashi et al. (1960b)
		Leaves	UV	Katou and Homma (1996)
		Leaves	TLC, UV, MS, NMR	Krauze-Baranowska (2004)
89	Quercetin-3-rhamnosid (Quercitrin)	Leaves	PPC	Takahashi et al. (1960a)
		Leaves	TLC, PC	Beckmann and Geiger (1968)

Table 1 continued

No.	Substances alphabetically according to group	Part of the tree	Methods of identification	References
		Leaves	UV	Katou and Homma (1996)
		Leaves	MP, UV, MS, NMR	Duan et al. (2009)
90	Quercetin 3-glucoside (isoquercetin, isoquercitrin)	Leaves	MP, MS, NMR, UV	Duan et al. (2009)
91	Quercetin-3-*O*-α-rhamnopyranoside-7-O-β-glucopyranoside	Leaves	NMR, MS	Nguyen et al. (2014)
92	Isorhamnetin	N/A	PC	Takahashi et al. (1960b)
93	Myricetin	N/A	PC	Takahashi et al. (1960b)
		Leaves	TLC, PC	Beckmann and Geiger (1968)
94	Myricetin-3-rhamnosid (Myricitrin)	Leaves	MP, MS, NMR, UV	Duan et al. (2009)
	V. Flavanols			
95	Catechin	Heartwood	IR, TLC, MP, EA	Sato et al. (1966)
		Branches and stems	N/A	Zeng et al. (2013)
		Bark	NMR, MS	Chen et al. (2014)
96	Epicatechin	Heartwood	IR, TLC, MP, EA	Sato et al. (1966)
		Branches and stems	N/A	Zeng et al. (2013)
		Bark	NMR, MS	Chen et al. (2014)
97	Gallocatechin	Branches and stems	N/A	Zeng et al. (2013)
		Leaves	NMR, MS	Nguyen et al. (2014)
		Bark	NMR, MS	Chen et al. (2014)
98	Epi-Gallocatechin	Branches and stems	N/A	Zeng et al. (2013)
		Bark	NMR, MS	Chen et al. (2014)
	Dimeric flavonoids			
	I. Biflavones and Bi(flavone + flavanone)			
99	Amentoflavone	Leaves	TLC, UV	Gadek and Quinn (1989)
		Leaves	MP, MS, NMR, UV	Duan et al. (2009)
100	7-Monomethyl Amentoflavone (Sequoiaflavone)	Leaves	TLC, UV	Gadek and Quinn (1989)
101	4′-Monomethyl Amentoflavone (Podocarpus flavone A)	Leaves	TLC, UV	Gadek and Quinn (1989)
102	7, 4‴-Dimethyl Amentoflavone (Podocarpus flavone B)	Leaves	TLC, UV	Gadek and Quinn (1989)
103	4′,4″-Dimethyl Amentoflavone (Isoginkgetin)	Leaves	TLC, UV	Gadek and Quinn (1989)
104	7, 4′,4″-Trimethyl Amentoflavone (Sciadopitysin)	Leaves	TLC, UV	Gadek and Quinn (1989)
105	2,3-Dihydro dimethyl Amentoflavone	Leaves	TLC, UV	Gadek and Quinn (1989)

Table 1 continued

No.	Substances alphabetically according to group	Part of the tree	Methods of identification	References
106	2,3-Dihydroamentoflavone-7″,4‴-dimethylether	Leaves	NMR, MP	Beckmann et al. (1971)
107	Amentoflavone-7″,4‴-dimethyl ether	Leaves	NMR, MP	Beckmann et al. (1971)
108	Bilobetin	Leaves	TLC, UV, MS, NMR	Krauze-Baranowska (2004)
109	Ginkgetin	Leaves	TLC, UV, MS, NMR	Krauze-Baranowska (2004)
110	Hinokiflavone	Leaves	N/A	Sawada (1958)
		Leaves	PC	Kariyone et al. (1958)
		Leaves	NMR, MP	Beckmann et al. (1971)
		Leaves	TLC, UV	Gadek and Quinn (1989)
111	Isocryptomerin	Leaves	NMR, MP	Beckmann et al. (1971)
112	Isoginkgetin	Leaves	MP, MS, NMR, UV	Duan et al. (2009)
113	Robustaflavone	Leaves	TLC, UV	Gadek and Quinn (1989)
114	Sciadopitysin	Leaves	MP, MS, NMR, UV	Duan et al. (2009)
115	Sotetsuflavone	Leaves	NMR, MP	Beckmann et al. (1971)
116	2,3-Dihydrohinokiflavone	Leaves	NMR, MP	Beckmann et al. (1971)
		Leaves	TLC, UV	Gadek and Quinn (1989)
117	2,3-Dihydroisoginkgetin	Leaves	TLC, UV, MS, NMR	Krauze-Baranowska (2004)
118	2,3-Dihydrosciadopitysin	Leaves	MP, MS, NMR, UV	Duan et al. (2009)
	II. Biflavanols			
119	Catechin (4 → 8) Catechin	Bark	NMR, MS	Chen et al. (2014)
120	Gallocatechin (4 → 8) Gallocatechin	Bark	NMR, MS	Chen et al. (2014)
121	Gallocatechin (4 → 8) Epigallocatechin	Bark	NMR, MS	Chen et al. (2014)
122	Gallocatechin (4 → 8) Catechin	Bark	NMR, MS	Chen et al. (2014)
123	Catechin (4 → 8) Gallocatechin	Bark	NMR, MS	Chen et al. (2014)
124	Gallocatechin (4 → 8) Epicatechin	Bark	NMR, MS	Chen et al. (2014)
	Hydrocarbons			
125	1-Methyl-4-(1-methylethyl)-benzene	Seeds	GC–MS	Mou et al. (2007)
126	1,2,3,4,4a,9,10,10α -Octahydro-1-phenanthrene	Seeds	GC–MS	Mou et al. (2007)
127	1,6,10-Dodecatriene	Leaves	GC–MS	Bajpai et al. (2009)
128	Ethylene (Ethene)	Stems	GC-FID	Du et al. (2004)
		Leaves	GC–MS	Bajpai and Kang (2011b)
129	Tricyclene	Leaves	GC–MS	Eryin and Rongai (1997)
		Cones	GC–MS	Bajpai et al. (2007a)

Table 1 continued

No.	Substances alphabetically according to group	Part of the tree	Methods of identification	References
	Aromatic hydrocarbons			
130	Perylene	Cones	GC–MS	Bajpai et al. (2007a)
	Lignans			
131	Arctigenin	Branches and stems	N/A	Zeng et al. (2013)
132	+(−)Lariciresinol	Branches and stems	N/A	Zeng et al. (2013)
133	Matairesinol	Branches and stems	N/A	Zeng et al. (2013)
134	(−)-Meridinol	Branches and stems	N/A	Zeng et al. (2013)
135	Pinopalustrin	Branches and stems	N/A	Zeng et al. (2013)
136	Pinoresinol	Branches and stems	N/A	Zeng et al. (2013)
137	Thujastandin	Branches and stems	N/A	Zeng et al. (2013)
138	1-(4-hydroxy-3-methoxyphenyl)-2-[4-(3-hydroxypropyl)-2-methoxyphenoxy]-propane-1,3-diol	Branches and stems	N/A	Zeng et al. (2013)
139	2-[2-hydroxy-4-(3-hydroxypropyl)phenoxy]-1-(4-hydroxy-3-methoxyphenyl)-1,3-propanediol	Branches and stems	N/A	Zeng et al. (2013)
140	(7S,8S)-3-methoxy-3′,7-epoxy-4′,8-oxyneoligna-4,9,9′-triol	Branches and stems	N/A	Zeng et al. (2013)
	Norlignans			
141	Agatharesinol	Heartwood	UV, IR, OR, NMR	Enoki et al. (1977a)
		Branches and stems	N/A	Zeng et al. (2013)
142	Athrotaxin	Heartwood	UV, IR, OR, NMR	Enoki et al. (1977a)
		Heartwood	N/A	Nagasaki et al. (2004)
143	Hydroxyathrotaxin	Heartwood	MP, OR, IR, UV, MS, NMR	Enoki et al. (1977b)
144	(−)-Evofolin	Branches and stems	N/A	Zeng et al. (2013)
145	Ficusal	Branches and stems	N/A	Zeng et al. (2013)
146	Metasequirin A	Heartwood	UV, IR, MS, NMR	Enoki et al. (1977a)
		Branches and stems	N/A	Zeng et al. (2013)
147	Hydroxymetasequirin A	Heartwood	MS, IR, UV, NMR	Enoki et al. (1977b)
		Branches and stems	N/A	Zeng et al. (2013)
148	Metasequirin B	Heartwood	MP, MS, IR, UV, NMR	Enoki et al. (1977b)
149	Metasequirin C	Heartwood	N/A	Nagasaki et al. (2004)
150	Metasequirin D	Stems and leaves	IR, MS, NMR, OR, UV	Dong et al. (2011)
151	Metasequirin E	Stems and leaves	IR, MS, NMR, UV	Dong et al. (2011)
152	Metasequirin F	Stems and leaves	IR, MS, NMR, UV	Dong et al. (2011)
153	Metasequirin G	Branches and stems	NMR, MS	Zeng et al. (2012)

Table 1 continued

No.	Substances alphabetically according to group	Part of the tree	Methods of identification	References
154	Metasequirin H	Branches and stems	NMR, MS	Zeng et al. (2012)
155	Metasequirin I	Branches and stems	NMR, MS	Zeng et al. (2012)
156	Sequirin C	Branches and stems	N/A	Zeng et al. (2013)
157	Sequosempervirin B	Branches and stems	N/A	Zeng et al. (2013)
158	Sequosempervirin F	Branches and stems	N/A	Zeng et al. (2013)
159	Threo-2,3-bis-(4-hydroxy-3-methoxyphenyl)-3-raethoxypropanol	Branches and stems	N/A	Zeng et al. (2013)
160	7′R,8′S-Threoguaiacylglycerol 8′-vanillic acid ether	Branches and stems	N/A	Zeng et al. (2013)
161	7′S,8′R-Threoguaiacylglycerol 8'-vanillic acid ether	Branches and stems	N/A	Zeng et al. (2013)
	Quinic acid derivatives			
162	3-*O*-(*E*)-Coumaroylquinic acid	Leaves	NMR, MS	Nguyen et al. (2014)
163	3-*O*-(*Z*)-Coumaroylquinic acid	Leaves	NMR, MS	Nguyen et al. (2014)
164	3-*O*-(*E*)-Coumaroylquinic acid methyl ester	Leaves	NMR, MS	Nguyen et al. (2014)
165	3-*O*-(*Z*)-Coumaroylquinic acid methyl ester	Leaves	NMR, MS	Nguyen et al. (2014)
	Terpenoids			
	I. Monoterpenoids			
166	Borneol	Leaves	GC–MS	Eryin and Rongai (1997)
		Leaves	GC–MS	Bajpai et al. (2009)
		Leaves	GC–MS	Bajpai and Kang (2011b)
167	Bornneol formate	Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
168	Bornylene	Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
169	Endo bornyl acetate	Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
170	Exo bornyl acetate	Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
171	Isobornyl acetate	Leaves	GC–MS	Bajpai et al. (2009)
		Leaves	GC–MS	Bajpai and Kang (2011b)
172	Camphene	Shoot, branchlet and trunk	N/A	Fujita et al. (1975)
		Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
		Leaves	GC–MS	Eryin and Rongai (1997)
		Seeds	GC–MS	Mou et al. (2007)
173	Camphene hydrate	Leaves	GC–MS	Fujita (1990)
		Leaves	GC–MS	Eryin and Rongai (1997)
174	α-Campholenone aldehyde	Leaves	GC–MS	Fujita (1990)
175	α-Campholene aldehyde	Leaves	GC–MS	Eryin and Rongai (1997)
176	Camphor	Shoot, branchlet and trunk	N/A	Fujita et al. (1975)
177	Cis-Carane	Cones	GC–MS	Bajpai et al. (2007a)

Table 1 continued

No.	Substances alphabetically according to group	Part of the tree	Methods of identification	References
		Cones	GC–MS	Bajpai et al. (2007b)
178	δ-3-Carene	Shoot, branchlet and trunk	N/A	Fujita et al. (1975)
		Leaves	GC–MS	Eryin and Rongai (1997)
		Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
		Seeds	GC–MS	Mou et al. (2007)
179	3-Caren-4-ol	Leaves	GC–MS	Bajpai et al. (2009)
		Leaves	GC–MS	Bajpai and Kang (2011b)
180	Carnosol	Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
181	Trans-carveol	Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
182	Carvone	Shoot, branchlet and trunk	N/A	Fujita et al. (1975)
183	*p*-Cymene	Shoot, branchlet and trunk	N/A	Fujita et al. (1975)
		Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
		Leaves	GC–MS	Eryin and Rongai (1997)
184	*p*-Cymene-8-ol	Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
185	Dihydrocarvyl acetate	Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
186	Cyclofenchene	Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
187	1,8-Cineole	Shoot, branchlet and trunk	N/A	Fujita et al. (1975)
188	Citronellyl acetate	Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
189	α-Fenchene	Seeds	GC–MS	Mou et al. (2007)
190	Fenchol	Leaves	GC–MS	Bajpai et al. (2009)
		Leaves	GC–MS	Bajpai and Kang (2011b)
191	Fenchone	Shoot, branchlet and trunk	N/A	Fujita et al. (1975)
192	α-Fenchyl alcohol	Leaves	GC–MS	Fujita (1990)
193	Geraniol	Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
194	Geranyl acetate	Leaves	N/A	Fujita and Kawai (1991)
		Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
195	Geranyl bromide	Leaves	GC–MS	Bajpai et al. (2009)
		Leaves	GC–MS	Bajpai and Kang (2011b)
196	Homomyrtenol	Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
197	Limonene	Shoot, branchlet and trunk	N/A	Fujita et al. (1975)
		Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
		Leaves	GC–MS	Eryin and Rongai (1997)

Table 1 continued

No.	Substances alphabetically according to group	Part of the tree	Methods of identification	References
		Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
		Seeds	GC–MS	Mou et al. (2007)
		Leaves	GC–MS	Bajpai et al. (2009)
		Leaves	GC–MS	Bajpai and Kang (2011b)
198	*cis*-Limonene oxide	Seeds	GC–MS	Mou et al. (2007)
199	Linalool	Shoot, branchlet and trunk	N/A	Fujita et al. (1975)
		Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
200	Linalool oxide	Leaves	GC–MS	Bajpai et al. (2009)
		Leaves	GC–MS	Bajpai and Kang (2011b)
201	Trans-Linalool oxide	Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
202	Cis-Linalool oxide	Leaves	GC–MS	(Fujita 1990)
		Leaves	N/A	(Fujita and Kawai 1991)
203	Linalyl acetate	Shoot, branchlet and trunk	N/A	Fujita et al. (1975)
		Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
204	Linaloyl propionate	Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
205	Methylol pinene (Nopol)	Cones	GC–MS	Bajpai et al. (2007a)
206	Myrcene	Leaves	GC–MS	Eryin and Rongai (1997)
207	β-Myrcene	Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
		Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
		Seeds	GC–MS	Mou et al. (2007)
		Leaves	GC–MS	Bajpai et al. (2009)
		Leaves	GC–MS	Bajpai and Kang (2011b)
208	Myrtenol	Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
		Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
		Leaves	GC–MS	Bajpai et al. (2009)
		Leaves	GC–MS	Bajpai and Kang (2011b)
209	Nerol	Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
210	Nopyl acetate	Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
211	Ocimene	Leaves	GC–MS	Bajpai et al. (2009)
		Leaves	GC–MS	Bajpai and Kang (2011b)
212	Perilla-aldehyde (tentative identification)	Shoot, branchlet and trunk	N/A	Fujita et al. (1975)
213	α-Phellandrene[a]	Leaves	GC–MS	Fujita (1990)

Table 1 continued

No.	Substances alphabetically according to group	Part of the tree	Methods of identification	References
		Leaves	N/A	Fujita and Kawai (1991)
214	1-Phellandrene[a]	Seeds	GC–MS	Mou et al. (2007)
215	β-Phellandrene	Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
		Leaves	GC–MS	Eryin and Rongai (1997)
		Seeds	GC–MS	Mou et al. (2007)
216	α-Pinene[b]	Heartwood	GLC	Sato et al. (1966)
		Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
		Leaves	GC–MS	Eryin and Rongai (1997)
		Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
		Seeds	GC–MS	Mou et al. (2007)
		Leaves	GC–MS	Bajpai et al. (2009)
		Leaves	GC–MS	Bajpai and Kang (2011b)
217	l-α-Pinene[b]	Shoot, branchlet and trunk	N/A	Fujita et al. (1975)
218	β-Pinene[c]	Shoot, branchlet and trunk	N/A	Fujita et al. (1975)
		Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
		Leaves	GC–MS	Eryin and Rongai (1997)
		Seeds	GC–MS	Mou et al. (2007)
219	1-β-Pinene[c]	Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
220	2-β-Pinene	Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
221	2-Pinen-4-ol	Leaves	GC–MS	Bajpai et al. (2009)
		Leaves	GC–MS	Bajpai and Kang (2011b)
222	Trans-Pinocarcved	Leaves	GC–MS	Eryin and Rongai (1997)
223	Sabinene	Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
		Leaves	GC–MS	Eryin and Rongai (1997)
		Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
		Seeds	GC–MS	Mou et al. (2007)
224	Cis-Sabinenehydrate	Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
225	α-Terpineol	Shoot, branchlet and trunk	N/A	Fujita et al. (1975)
		Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
		Leaves	GC–MS	Eryin and Rongai (1997)
		Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
		Leaves	GC–MS	Bajpai et al. (2009)
		Leaves	GC–MS	Bajpai and Kang (2011b)

Table 1 continued

No.	Substances alphabetically according to group	Part of the tree	Methods of identification	References
226	δ-Terpineol	Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
227	α-Terpinene	Leaves	GC–MS	Fujita (1990)
		Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
228	δ-Terpinene	Seeds	GC–MS	Mou et al. (2007)
229	γ-Terpinene	Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
		Cones	GC–MS	Bajpai et al. (2007b)
230	Terpinen-4-ol[d]	Shoot, branchlet and trunk	N/A	Fujita et al. (1975)
		Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
		Leaves	GC–MS	Eryin and Rongai (1997)
		Cones	GC–MS	Bajpai et al. (2007b)
231	Terpinolene	Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
232	α-Terpinolene	Leaves	GC–MS	Eryin and Rongai (1997)
		Seeds	GC–MS	Mou et al. (2007)
233	γ-Terpinolene	Leaves	GC–MS	Eryin and Rongai (1997)
234	Terpitineol-4[d]	Cones	GC–MS	Bajpai et al. (2007b)
235	α-Terpinyl acetate	Shoot, branchlet and trunk	N/A	Fujita et al. (1975)
236	α-Thujene	Leaves	GC–MS	Fujita (1990)
		Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
237	Thymol	Leaves	GC–MS	Bajpai et al. (2009)
		Leaves	GC–MS	Bajpai and Kang (2011b)
238	Tricyclene	Cones	GC–MS	Bajpai et al. (2007b)
		Seeds	GC–MS	Mou et al. (2007)
239	Verbenol	Leaves	GC–MS	Bajpai et al. (2009)
		Leaves	GC–MS	Bajpai and Kang (2011b)
	II. Sesquiterpenoids			
240	(−)-Acora-2,4(14),8-trien-15-oic acid	Stems, leaves	IR, MS, NMR, UV	Dong et al. (2011)
241	Bergamotene	Leaves	GC–MS	Bajpai and Kang (2011b)
242	α-Bisabolol	Leaves	GC–MS	Bajpai and Kang (2011b)
243	α-Bisabolene epoxide	Leaves	GC–MS	Bajpai and Kang (2011b)
244	β-Bisabolene	Leaves	GC–MS	Fujita (1990)
		Cones	GC–MS	Bajpai et al. (2007b)
245	β-Bourbonene	Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
		Leaves	GC–MS	Eryin and Rongai (1997)
246	α-Cadinol ($C_{15}H_{26}O_1$)	Twigs	IR,GC, standard	Hayashi et al. (1969)
		Shoot, branchlet and trunk	N/A	Fujita et al. (1975)

Table 1 continued

No.	Substances alphabetically according to group	Part of the tree	Methods of identification	References
		Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
247	δ-Cadinol	Leaves	GC–MS	Fujita (1990)
248	δ-Cadinene	Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
249	Calamenene	Shoot, branchlet and trunk	N/A	Fujita et al. (1975)
250	Calacorene	Leaves	GC–MS	Fujita (1990)
251	α-Calacorene	Shoot, branchlet and trunk	N/A	Fujita et al. (1975)
252	Caryophylla-1(12),8(15)-dien-9α-ol	Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
253	Caryophylla-1(12),8(15)-dien-9β-ol	Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
254	Caryophylla-1(12),7-dien-9α-ol	Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
255	Caryophylla-1(12),7-dien-9β-ol	Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
256	Caryophylla-1(12),7-dien-9-one	Leaves	N/A	Fujita and Kawai (1991)
257	Caryophylla-1(12),8(15)-dien-9-one	Leaves	N/A	Fujita and Kawai (1991)
258	Caryophyllene	Shoot, branchlet and trunk	N/A	Fujita et al. (1975)
259	β-Caryophyllene	Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
		Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
260	γ-Caryophyllene	Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
261	Caryophyllene oxide	Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
		Leaves	GC–MS	Eryin and Rongai (1997)
		Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
		Leaves	GC–MS	Bajpai et al. (2009)
		Leaves	GC–MS	Bajpai and Kang (2011b)
262	9,3 H-Caryophyllene	Leaves	N/A	Fujita and Kawai (1991)
263	Isocaryophyllene	Leaves	N/A	Fujita and Kawai (1991)
264	Trans-Caryophyllene	Leaves	GC–MS	(Eryin and Rongai 1997)
		Seeds	GC–MS	(Mou et al. 2007)
265	α-Chamigrene	Cones	GC–MS	Bajpai et al. (2007b)
266	β-Cubebene	Leaves	GC–MS	Eryin and Rongai (1997)
267	(R)-Cuparene	Cones	GC–MS	Bajpai et al. (2007b)
268	α-Elemene	Shoot, branchlet and trunk	N/A	Fujita et al. (1975)
269	β-Elemene	Shoot, branchlet and trunk	N/A	Fujita et al. (1975)

Table 1 continued

No.	Substances alphabetically according to group	Part of the tree	Methods of identification	References
		Leaves	GC–MS	Fujita (1990)
270	β-Famesene	Leaves	GC–MS	Bajpai et al. (2009)
271	α-Farnesene	Leaves	GC–MS	Bajpai and Kang (2011b)
272	Trans-β-Farnesene	Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
273	Cis-Farnesol	Cones	GC–MS	Bajpai et al. (2007a, b)
		Cones	GC–MS	
274	Hexahydrofarnesylacetone	Leaves	GC–MS	Eryin and Rongai (1997)
275	Humuladiene I: $C_{15}H_{24}O$	Leaves	GC–MS	Fujita (1990)
276	Humuladiene II: $C_{15}H_{24}O$	Leaves	GC–MS	Fujita (1990)
277	Humuladienone I	Leaves	N/A	Fujita and Kawai (1991)
278	Humuladienone II	Leaves	N/A	Fujita and Kawai (1991)
279	α-Humulene	Shoot, branchlet and trunk	N/A	Fujita et al. (1975)
		Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
		Cones	GC–MS	Bajpai et al. (2007b)
280	Humulene epoxide I	Leaves	N/A	Fujita and Kawai (1991)
281	Humulene epoxide II	Leaves	N/A	Fujita and Kawai (1991)
282	Humulenol II (tentative identification)	Leaves	N/A	Fujita and Kawai (1991)
283	Hurnulene	Leaves	GC–MS	Eryin and Rongai (1997)
284	Longipinenepoxide	Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
285	T-Muurolol	Leaves	N/A	Fujita and Kawai (1991)
286	Nerolidol	Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
287	β-Selinene	Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
288	Solanone	Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
289	Spathulenol	Leaves	GC–MS	Fujita (1990)
290	γ-Terpinine	Cones	GC–MS	Bajpai et al. (2007b)
291	Veridiflorol	Leaves	GC–MS	Bajpai and Kang (2011b)
292	α-Ylangene	Shoot, branchlet and trunk	N/A	Fujita et al. (1975)
293	$C_{15}H_{24}O$	Leaves	GC–MS	Fujita (1990)
294	$C_{15}H_{22}O$	Leaves	GC–MS	Fujita (1990)
	III. Diterpenoids and their derivatives			
295	Ferruginol	Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
296	3-Acetoxylabda-8(20),13-dien-15-oic acid	Brown autumn leaves	^{13}C NMR	Braun and Breitenbach (1977)
297	3β-Acetoxy-8 (17),13E-labdadien-15-oic acid	Leaves	MP, MS, NMR,	Duan et al. (2009)

Table 1 continued

No.	Substances alphabetically according to group	Part of the tree	Methods of identification	References
298	12α-Hydroxy-8,15-isopimaradien-18-oic acid	Stems, leaves	IR, MS, NMR, UV,	Dong et al. (2011)
299	Metaseglyptorin A	Stems and leaves	IR, MS, MP, NMR, UV	Dong et al. (2011)
300	Metasequoic acid A	Twig (branch)	NMR	Sakan et al. (1988)
301	Metasequoic acid B	Twig (branch)	NMR	Sakan et al. (1988)
302	Metasequoic acid C	Stems, leaves	IR, MS, NMR, UV	Dong et al. (2011)
303	Phytol	Leaves	GC–MS	(Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
304	Sugiol	Cones	GC–MS	Bajpai et al. (2007b)
		Cones	MP, NMR	Bajpai and Kang (2011a)
		Cones	MP, NMR	Bajpai et al. (2014a)
305	Taxaquinone	Cones	IR, MP, NMR, OR, TLC, UV	Bajpai and Kang (2014)
306	Taxodone	Cones	MP, NMR	Bajpai and Kang (2010a)
307	Totarol	Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
308	Totarol acetate	Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
309	2-Pentenoic acid, 5-(decahydro-6-hydroxy-5,5,8a-trimethyl-1-naphthalenyl)-3-methyl-, [1S-(1α,4aβ,6α,8aα)]- (9CI)	N/A	NMR, IR, MS	Asahi and Sakan (1984)
310	2-Pentenoic acid, 5-[6-(acetyloxy)decahydro-5,5,8a-trimethyl-1-naphthalenyl]-3-methyl-, [1S-(1α,4aβ,6α,8aα)]-(9CI)	N/A	NMR, IR, MS	Asahi and Sakan (1984)
311	2-Pentenoic acid, 5-(decahydro-5,5,8a-trimethyl-1-naphthalenyl)-3-methyl-, [1R-(1α,4aβ,8aα)]- (9CI)	N.A.	NMR, IR, MS	Asahi and Sakan (1984)
	IV. Triterpenoids			
312	Metaseglyptorin A	Leaves	NMR, MS, IR	Dong et al. (2011)
	V. Tetraterpenoids (Carotenoids)			
313	Adonirubin	Leaves	TLC	Czeczuga (1987)
314	Antheraxanthin	Leaves	TLC	Czeczuga (1987)
315	Apo-12′-violaxanthal	Leaves	TLC	Czeczuga (1987)
316	Astaxanthin	Leaves	TLC	Czeczuga (1987)
317	Auroxanthin	Leaves	TLC	Czeczuga (1987)
318	Canthaxanthin	Leaves	TLC	Czeczuga (1987)
319	α-Carotene	Leaves	UV	Hida and Ida (1961)
		Leaves	UV	Ida (1981b)
		Leaves	UV	Ida (1981a)
		Leaves	TLC	Czeczuga (1987)

Table 1 continued

No.	Substances alphabetically according to group	Part of the tree	Methods of identification	References
320	β-Carotene	Leaves	UV	Ida (1981b)
		Leaves	UV	Ida (1981a)
		Leaves	TLC	Czeczuga (1987)
321	γ-Carotene	Leaves	TLC	Czeczuga (1987)
322	α-Cryptoxanthin	Leaves	TLC	Czeczuga (1987)
323	β-Cryptoxanthin	Leaves	TLC	Czeczuga (1987)
324	Lycopene	Leaves	TLC	Czeczuga (1987)
325	Lutein	Leaves	UV	Hida and Ida (1961)
		Leaves	UV	Ida (1981b)
		Leaves	UV	Ida (1981a)
		Leaves	TLC	Czeczuga (1987)
326	Lutein epoxide	Leaves	TLC	Czeczuga (1987)
327	Luteoxanthin	Leaves	TLC	Czeczuga (1987)
328	Mutatochrome	Leaves	TLC	Czeczuga (1987)
329	Mutatoxanthin	Leaves	TLC	Czeczuga (1987)
330	Neoxanthin	Leaves	TLC	Czeczuga (1987)
331	Rhodoxanthin	Leaves	TLC	Czeczuga (1987)
332	Violaxanthin	Leaves	UV	Hida and Ida (1961)
		Leaves	UV	Ida (1981b)
		Leaves	UV	Ida (1981a)
		Leaves	TLC	Czeczuga (1987)
333	Zeaxanthin	Leaves	TLC	Czeczuga (1987)
	Phenolic compounds			
334	p-Cresol	Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
335	Metaseol	Root bark	IR, MP, MS, NMR, UV	Nakatani et al. (1991)
336	Phenol	Leaves	GC–MS	Fujita (1990)
		Leaves	N/A	Fujita and Kawai (1991)
		Leaves	GC–MS	Bajpai and Kang (2011b)
337	Protocatechuic acid	Heartwood	IR, TLC, MP, EA	Sato et al. (1966)
338	Protocatechuic aldehyde	Heartwood	IR, TLC, MP, EA	Sato et al. (1966)
	Phenylpropans			
339	7-(3-ethoxy-5-methoxyphenyl) propane-7,8,9-triol (1-(3-ethoxy-5-methoxyphenyl) propane-1,2,3-triol)	Branches and stems	NMR, MS	Zeng et al. (2012)
340	7-(3-hydroxy-5-methoxyphenyl) propane-7,8,9-triol (1-(3-hydroxy-5-methoxyphenyl) propane-1,2,3-triol)	Branches and stems	NMR, MS	Zeng et al. (2012)

Table 1 continued

No.	Substances alphabetically according to group	Part of the tree	Methods of identification	References
	Phenylpropens			
341	Chavicol	Leaves	GC–MS	Bajpai et al. (2009)
		Leaves	GC–MS	Bajpai and Kang (2011b)
342	Eugenol	Leaves	GC–MS	Bajpai et al. (2009)
		Leaves	GC–MS	Bajpai and Kang (2011b)
343	Guaiacol	Leaves	GC–MS	Bajpai et al. (2009)
		Leaves	GC–MS	Bajpai and Kang (2011b)
	N-heterocycles			
344	2,3-Benzopyrrole	Leaves	GC–MS	Bajpai et al. (2009)
		Leaves	GC–MS	Bajpai and Kang (2011b)
345	2-Cyanoaziridine	Cones	GC–MS	Bajpai et al. (2007a)
		Cones	GC–MS	Bajpai et al. (2007b)
346	2,3-Dimethyl 1,3 isopropylpyrazine	Leaves	GC–MS	Bajpai et al. (2009)
		Leaves	GC–MS	(Bajpai and Kang 2011b)
347	Imidazole	Leaves	GC–MS	Bajpai et al. (2009)
348	Indole-3-acetic acid	Stems	GC–MS	Du et al. (2004)
		Leaves	GC–MS	Bajpai and Kang (2011b)
349	Pyridine	Leaves	GC–MS	Bajpai et al. (2009)
		Leaves	GC–MS	Bajpai and Kang (2011b)
350	Pyrrolidine	Leaves	GC–MS	Bajpai et al. (2009)
		Leaves	GC–MS	Bajpai and Kang (2011b)
	Sterols			
351	β-Sitosterol	Leaves	MS, IR	Beckmann and Schuhle (1968)
		Twigs	LST, MP, IR	Hayashi et al. (1969)
		Leaves	MP, UV, MS, NMR	Duan et al. (2009)
		Branches and stems	N/A	Zeng et al. (2013)
352	Campesterol	Twigs	LST, MP, IR	Hayashi et al. (1969)
353	Stigmasterol	Twigs	LST, MP, IR	Hayashi et al. (1969)
	Steroids			
354	Campest-4-en-3-one	Twigs	IR, UV, NMR, MP	Hayashi et al. (1969)
355	Stigmast-4-en-3-one	Twigs	IR, UV, NMR, MP	Hayashi et al. (1969)
356	Stigmast-4-22-dien-3-one	Twigs	IR, UV, NMR, MP	Hayashi et al. (1969)
357	5α-Stigmastan-3,6-dione	Twigs	IR, ORD,MS, NMR	Hayashi et al. (1969)
	Sugars			
358	Fructose	Leaves	PC	Kariyone et al. (1958)
		Leaves	PC	Hida et al. (1962)
359	Galactose	Leaves	PC	Kariyone et al. (1958)
360	Glucose	Leaves	PC	Kariyone et al. (1958)

Table 1 continued

No.	Substances alphabetically according to group	Part of the tree	Methods of identification	References
		Leaves	PC	Hida et al. (1962)
361	Sucrose (Saccharose)	Leaves	PC	Kariyone et al. (1958)
		Leaves	PC	Hida et al. (1962)
362	α-*D*-Fructofuranoside	Branches and stems	N/A	Zeng et al. (2013)

EA elemental analysis, *FID* flame ionization detection, *GC* gas chromatography, *GLC* gas–liquid chromatography, *GC–MS* gas chromatography mass spectrometry, *IR* infrared spectroscopy, *LST* Liebermann and Salkowski color test, *MS* mass spectrometry, *MP* melting point, *NMR* nuclear magnetic resonance, *OR* optical rotation, *PC* paper chromatography, *PPC* paper partition chromatography, *S* standard [comparison of unknown with standard compound (s)], *TLC* thin layer chromatography, *UV* ultra violet to visible spectroscopy, *N/A* not available

[a,b,c,d] Compound names labelled with the same letter may refer to the same compound

detailed review of the chronology of the history of *M. glyptostroboides* was written by Ma in 2003 (Ma 2003).

Natural habitat and distribution of *M. glyptostroboides*

M. glyptostroboides is endemic to southeast China where the largest native population is found in the Shui-Hsa River valley, also called Xiaohe River Valley, in Zhonglu in Hubei Province (Wang et al. 2006). However, native trees have also been found in an estimated area of about 800–1000 km^2 within eastern Chongqing municipality, western Hubei, and western Hunan Provinces (Bartholomew et al. 1983; Chu and Cooper 1950; Gressit 1953; Leng et al. 2007; Tang et al. 2011; Wang et al. 2006). In this region the tree occurs as a constituent of the Mixed Mesophytic Forest and grows at an altitude ranging from 800 to 1500 m. Because of this limited distribution, the declining number of individuals, the decreasing available habitat, together with low genetic diversity (Li et al. 2005), *M. glyptostroboides* is classified as endangered on The IUCN Red List of Threatened Species (Farjon 2013). The natural habitat of the tree is in the humid and warm lower mountain slopes with river and stream valleys. In the nearby city of Lichuan, 1083 m above sea level and approximately 60 km from Zhonglu, the monthly mean temperature varies from around 1.9 °C in January to 22.6 °C in August with an annual mean temperature of 12.7 °C. Rainfall is seasonal with a mean annual precipitation of 1319 mm, most of which (85 %) falls during the seven months from April to October (Tang et al. 2011). After discovering *M. glyptostroboides* as a living species, there was an intense effort to cultivate the tree throughout the world (Chu and Cooper 1950). The tree is highly adaptable and since 1948, *M. glyptostroboides* has been successfully grown in nearly 50 countries in Asia, Africa, Europe and America (Ma 2007).

Botanical description

M. glyptostroboides is a large deciduous conifer that belongs to the family Cupressaceae (Fig. 1) and is the only living species in the genus. It is a fast growing tree that can reach a height of 45 m and 2.2 m in diameter (Ma 2007). *M. glyptostroboides* has a pyramidal shape when young, but can develop a more rounded shape with age. The bark is reddish brown in the early stage, and becomes darker and more greyish over time, with vertical furrows and armpits under the branches. The branchlets are up to about 7.5 cm long and usually arranged distichously with up to 50–60 leaves. The bright green opposite linear leaves provide foliage of feathery texture in mid-spring. During autumn the colour changes to orange, yellow and red-brown before the foliage falls off in wintertime. *M. glyptostroboides* is monoecious, with both male (pollen) and female cones growing on different branches of the same tree. The trees can in general produce cones when they are 9–15 m high, while pollen cones are produced when the tree attains a height of 18–27 m. Pollen cones are pendulous (5–6 mm long), and are produced mid-June, pollen

Table 2 Compounds identified from fossil leaves from *Metasequoia glyptostroboides* Hu et Cheng

No.	Substances alphabetically according to group	Part of the tree	Method of identification	References
	Alcohols			
1	2,3-Dimethyl-3-buten-2-ol	Fossil leaves	GC–MS	Zhao et al. (2007)
2	2-Methyl-Cyclopentanol	Fossil leaves	GC–MS	Zhao et al. (2007)
3	2-Hexanol	Fossil leaves	GC–MS	Zhao et al. (2007)
4	2-Heptanol	Fossil leaves	GC–MS	Zhao et al. (2007)
5	2-Hexyl-1-decanol	Fossil leaves	GC–MS	Zhao et al. (2007)
6	(*E*)-2-undecen-1-ol	Fossil leaves	GC–MS	Zhao et al. (2007)
7	2-methyl-3-(1-metylethenyl)-cyclohexanol	Fossil leaves	GC–MS	Zhao et al. (2007)
	Aldehydes			
8	2-(*E*)-hexenal	Fossil leaves	GC–MS	Zhao et al. (2007)
9	Decanal	Fossil leaves	GC–MS	Zhao et al. (2007)
	Alkanes			
10	Pentadecane	Fossil leaves	GC–MS	Zhao et al. (2007)
11	Hexadecane	Fossil leaves	GC–MS	Zhao et al. (2007)
12	2,6,10,14-tetramethyl-hexadecane	Fossil leaves	GC–MS	Zhao et al. (2007)
13	Heptadecane	Fossil leaves	GC–MS	Zhao et al. (2007)
14	Octadecane	Fossil leaves	GC–MS	Zhao et al. (2007)
15	Nonadecane	Fossil leaves	GC–MS	Zhao et al. (2007)
16	Eicosane (Icosane)	Fossil leaves	GC–MS	Zhao et al. (2007)
17	Heneicosane	Fossil leaves	GC–MS	Zhao et al. (2007)
18	Docosane	Fossil leaves	GC–MS	Zhao et al. (2007)
19	Tricosane	Fossil leaves	GC–MS	Zhao et al. (2007)
20	Tetracosane	Fresh leaves	GC–MS	Fujita (1990)
		Fossil leaves	GC–MS	Zhao et al. (2007)
21	Pentacosane	Fresh leaves	GC–MS	Fujita (1990)
		Fossil leaves	GC–MS	Zhao et al. (2007)
22	1,2-Dimethylcyclopentane	Fossil leaves	GC–MS	Zhao et al. (2007)
	Esters			
23	Dibutyl phthalate[a]	Fossil leaves	GC–MS	Zhao et al. (2007)
24	Diisobutyl phthalate[a]	Fossil leaves	GC–MS	Zhao et al. (2007)
25	Bis (2-ethylhexyl) phtalate[a]	Fossil leaves	GC–MS	Zhao et al. (2007)
	Furans			
26	Dibenzofuran	Fossil leaves	GC–MS	Zhao et al. (2007)
	Ketones			
27	1-(methylphenyl)-ethanone	Fossil leaves	GC–MS	Zhao et al. (2007)
28	3-(*E*)-Penten-2-one	Fossil leaves	GC–MS	Zhao et al. (2007)
29	4-Hydroxy-4-Methyl-2-pentanone	Fossil leaves	GC–MS	Zhao et al. (2007)
30	1-(-Naphthalenyl)-ethanone	Fossil leaves	GC–MS	Zhao et al. (2007)
31	1,7,7-trimethyl-bicyclo2.2.1hetpan-2-one	Fossil leaves	GC–MS	Zhao et al. (2007)
32	6,8-Dioxabicyclo [3.2.1] octane	Fossil leaves	GC–MS	Zhao et al. (2007)
33	Benzophenone	Fossil leaves	GC–MS	Zhao et al. (2007)
34	Tetrahydro-3,6-dimethyl-2H-pyran-2-one	Fossil leaves	GC–MS	Zhao et al. (2007)
	Fatty acids and their derivatives			
35	Dodecanoic acid, methyl ester	Fossil leaves	GC–MS	Zhao et al. (2007)

Table 2 continued

No.	Substances alphabetically according to group	Part of the tree	Method of identification	References
36	Formic acid octyl ester	Fossil leaves	GC–MS	Zhao et al. (2007)
37	Hexadecanoic acid methyl ester	Fossil leaves	GC–MS	Zhao et al. (2007)
38	Octadecanoic acid methyl ester	Fossil leaves	GC–MS	Zhao et al. (2007)
39	Tetradecanoic acid methyl ester	Fossil leaves	GC–MS	Zhao et al. (2007)
	Aromatic hydrocarbons			
40	Anthracene	Fossil leaves	GC–MS	(Zhao et al. 2007)
41	Naphthalene	Fossil leaves	GC–MS	Zhao et al. (2007)
42	1-Methyl-naphthalene	Fossil leaves	GC–MS	Zhao et al. (2007)
43	2-Methyl-naphthalene	Fossil leaves	GC–MS	Zhao et al. (2007)
44	Retene	Fossil leaves	GC–MS	Zhao et al. (2007)
	Isocyanate			
45	Isocyanato-cyclohexane	Fossil leaves	GC–MS	Zhao et al. (2007)
	Terpenoids			
	I. Monoterpenoids			
46	*L*-(−)-menthol	Fossil leaves	GC–MS	Zhao et al. (2007)
	II. Diterpenoids and their derivatives			
47	2,6,10-Trimethyl-hexadecane	Fossil leaves	GC–MS	Zhao et al. (2007)
	III. Triterpenoids			
48	Squalene	Fossil leaves	GC–MS	Zhao et al. (2007)
	N-heterocycles			
49	2,3-Dimethyl-*N*-phenylpyrrolidine	Fossil leaves	GC–MS	Zhao et al. (2007)
	Sulphur-containing compounds			
50	4-Hydroxybenzenesulfonic acid	Fossil leaves	GC–MS	Zhao et al. (2007)
51	1,2-Benzisothiazole	Fossil leaves	GC–MS	Zhao et al. (2007)

GC–MS gas chromatography mass spectrometry

[a] These compounds are known plasticizers and could as such be artefacts

forms in November, and is dispersed with wind in early spring, and is only produced in regions with relatively warm climates. The cones are globose to ovoid (1.5–2.5 cm long) with 16–28 scales in opposite pairs in four rows. The cone is produced early in July, but fertilization occurs in June the following year. The seeds mature 4–5 months after fertilisation (Li 1998/1999).

Natural products from *Metasequoia glyptostroboides*

To assist current and future researchers with interests in the vast number of natural products from *M. glyptostroboides*, all compounds hitherto reported from this species are systematized for the first time in Table 1, according to compound class. The information provided also includes from which part of the tree the compounds have been detected, as well as the methods used for identifications in each instance where such information is available. Approximately 362 natural products have been characterized from *M. glyptostroboides* (Table 1). The majority of these compounds have been characterized from the leaves, although seeds, branches, heartwood and bark have also been analyzed (Table 1). Twenty-six natural products were unique to *M. glyptostroboides* at the time they were characterized (Figs. 2, 3, 4, 5, 6). The structures of these novel compounds are shown in Figs. 2, 3, 4, 5, and 6. The compound classes, which include natural products specific to *M. glyptostroboides* are discussed in detail below. The various categories of natural products from this plant source are systematized in Figs. 7, 8, and 9.

Fig. 1 *Metasequoia glyptostroboides* grown in the Botanical Museum garden of University of Bergen, Bergen, Norway. Photo: Torgils Fossen

Characterization and structure elucidation

The majority of known compounds reported from *M. glyptostroboides* are relatively volatile, which may reflect the fact that the majority of samples from this plant source have been characterized by GC–MS. X-ray data have not been reported for any compound isolated from *M. glyptostroboides*. However, an increasing number of compounds have been characterized in detail at atomic resolution, mainly by using a combination of 2D NMR spectroscopy and MS (Table 1). Supporting structural information for a not insignificant minority of the characterized compounds has been achieved by using OR (for chiral compounds) and IR spectroscopy, as well as various forms of co-chromatography and MP determinations (Table 1).

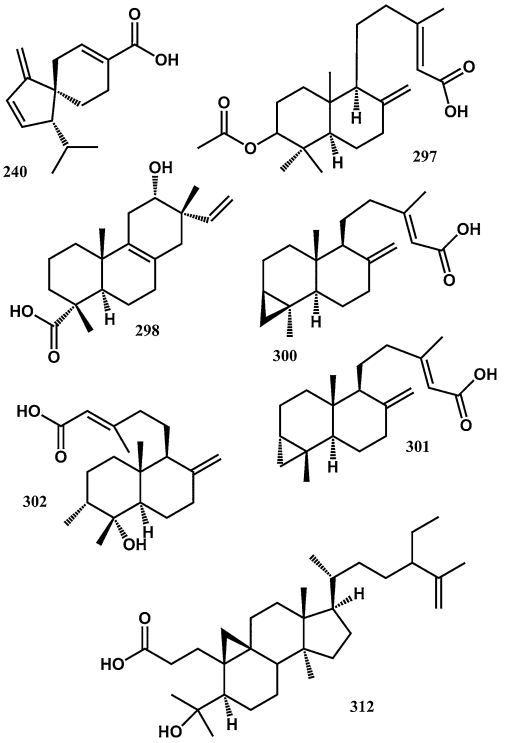

Fig. 2 Novel natural products isolated from *Metasequoia glyptostroboides*. I. Terpenoids

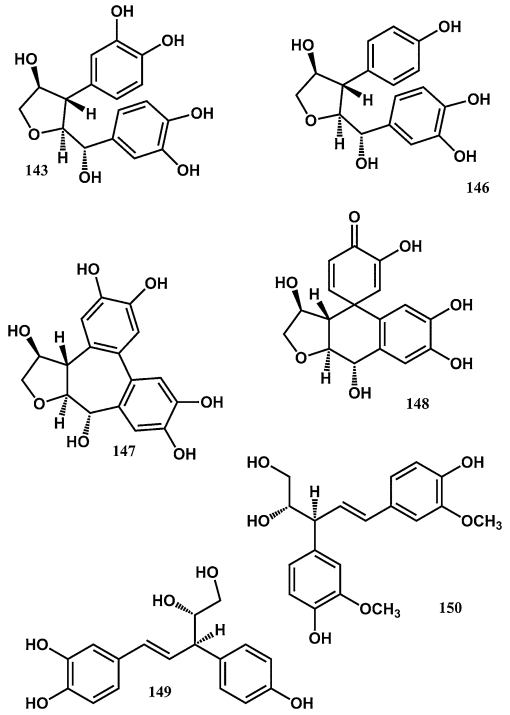

Fig. 3 Novel natural products isolated from *Metasequoia glyptostroboides*. II. Norlignans

Terpenoids

Terpenoids comprise the largest group of natural products characterized from *M. glyptostroboides*. Until now, 168 different terpenoids have been reported from this plant source (Table 1 and Fig. 7). The majority of these compounds are monoterpenoids, of which 74 have been identified (Fig. 8). Conifers are known to be rich sources of monoterpenoids (Cvrkal and Janak 1959). All of these monoterpenoids are known from other plant sources, as is also the case for the 21 tetraterpenoids (carotenoids) and the single triterpenoid identified. Sesquiterpenoids comprise the second largest group of terpenoids identified from *M. glyptostroboides* counting 55 different structures (Fig. 8). One of these, namely (−)-acora-2,4(14),8-trien-15-oic acid (**240**) is specific to *M. glyptostroboides* (Fig. 2). Among the 17 diterpenoids reported, the six compounds 3β-acetoxy-8 (17),13 E-labdadien-15-oic acid (**297**), 12α-hydroxy-8,15-isopimaradien-18-oic acid (**298**), metasequoic acid A-C (**300–302**), and metaseglyptorin A (**312**) are specific to *M. glyptostroboides* (Fig. 2).

Flavonoids

Flavonoids are the most important polyphenolic compounds synthesized by plants. According to Markham (1982) approximately 2 % of all carbon photosynthesized by higher plants are biosynthetically converted to flavonoids (Markham 1982). More than 10 000 different flavonoids have hitherto been reported (Tahara 2007). No less than 50 flavonoids have been reported from *M. glyptostroboides*, which means that they are one of the main groups of natural products characterized from this tree (Figs. 7, 9a). The majority of them are non-glycosylated monomeric (14) or dimeric (26) flavonoids (Fig. 9b). Nine flavonoid

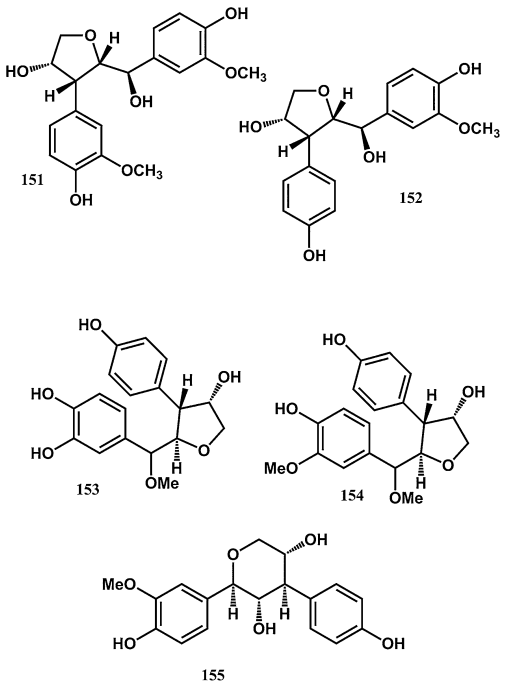

Fig. 4 Novel natural products isolated from *Metasequoia glyptostroboides*. III. Norlignans (continued)

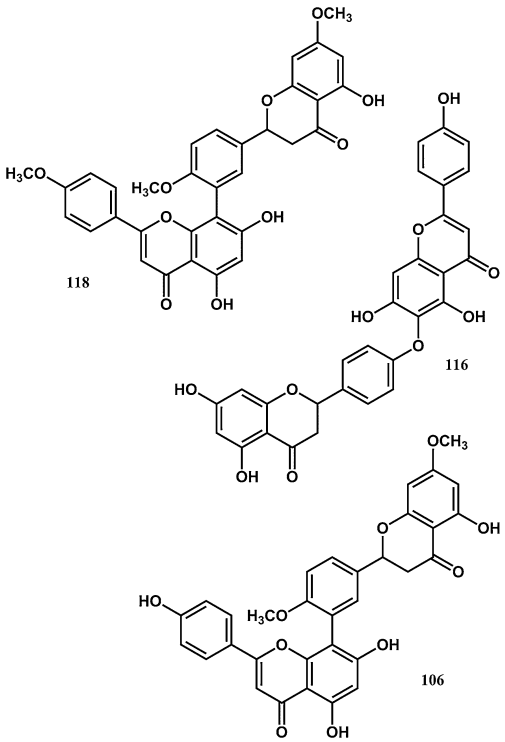

Fig. 5 Novel biflavonoids characterized from *Metasequoia glyptostroboides*

monoglycosides and one flavonoid diglycoside have been reported from *M. glyptostroboides*. The glycosylation positions in these compounds are restricted to the 3-, 7- and 3′-positions of the aglycones (Table 1). Glucose and rhamnose are the only sugar units found in the flavonoid glycosides reported from *M. glyptostroboides*, where glucose is the predominant glycosyl unit (Table 1). Acylated flavonoids have hitherto not been identified from this species. The flavonoids most characteristic for *M. glyptostroboides* are dimers of either two flavone units or a flavone and a flavanone unit (Table 1 and Fig. 5). Three such compounds, namely 2, 3-dihydroamentoflavone-7″,4‴-dimethylether (**106**), 2,3-dihydrohinokiflavone (**116**) and 2,3-dihydrosciadopitysin (**118**) were discovered in nature for the first time from this species (Fig. 5). Moreover, an anticancer drug based on one of these compounds (dihydrohinokiflavone) isolated from *M. glyptostroboides* has been patented (Jung et al. 2004).

Lignans and norlignans

The largest population of compounds specific to *M. glyptostroboides* belongs to the norlignans. Lignans and norlignans comprise classes of phenylpropanoid-derived natural products with abundant occurrence in nature (Suzuki and Umezawa 2007). Lignans are dimeric phenylpropanoids where the monomers are linked at the central carbon (C8) (Suzuki and Umezawa 2007). Norlignans are naturally occurring phenolic compounds based on a diphenylpentane carbon skeleton consisting of a phenyl–ethyl unit linked to a phenyl-propyl unit. Lignans are widely distributed within the plant kingdom (Suzuki and Umezawa 2007), while norlignans, on the other hand, are mainly found in conifers and monocotyledons (Suzuki and Umezawa 2007).

While some lignans are already established as active principles of anticancer drugs such as podophyllotoxines

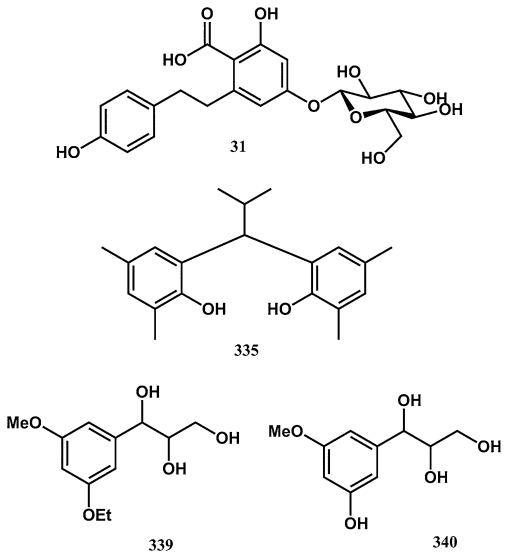

Fig. 6 Novel natural products isolated from *Metasequoia glyptostroboides*. V. Other phenolic compounds

(Stahelin and von Wartburg 1991), there is also an increased recent interest in research on norlignans with significant anticancer activity such as agatharesinol acetonide isolated from *Sequoia* (Zhang et al. 2005). Altogether 10 lignans have been reported from *M. glyptostroboides* (Table 1). All of these compounds are known from other plant sources. The biosynthetic pathways of the norlignans of *M. glyptostroboides* appear, however, to be more unique to this species. Among the 21 norlignans characterized from this plant source (Table 1), the majority of the compounds, namely hydroxyathrotaxin (**143**), hydroxymetasequirin A (**147**), and metasequirin A-I (**146, 148–155**) are unique to *M. glyptostroboides* (Figs. 3, 4).

Other aromatic compounds specific to *M. glyptostroboides*

Four further aromatic natural products unique to *M. glyptostroboides* deserve particular attention. The symmetric natural product metaseol (**335**), isolated from the root bark, belongs to the diphenylmethanes, a relatively rare class of natural product (Nakatani et al. 1991). Metaseol has only been detected in *M. glyptostroboides* and is the first and only symmetric diphenylmethane ever isolated from any natural source. The two new phenylpropanoids 7-(3-ethoxy-5-methoxyphenyl)propane-7,8,9-triol (**339**) and 7-(3-hydroxy-5-methoxyphenyl) propane-7,8,9-triol (**340**) (Fig. 6) isolated from branches and stems of *M. glyptostroboides* exhibited mild cytotoxic activity against A549 and Colo 205 cell lines (Zeng et al. 2012). 6-Carboxydihydroresveratrol-3-glucoside (**31**) is the only stilbenoid (bibenzyl) derivative hitherto reported from *M. glyptostroboides* (Nguyen et al. 2014). Bibenzyl aglycones with carboxylic substituents have a restricted occurrence in nature. The fact that these compounds have mainly been found in species belonging to the oldest lineages of plant families like the fern *Hicriopteris glauca* (Fang et al. 2012), Liverworts (Pryce 1971; Pryce 1972; Valio et al. 1969) and algae (Huneck and Pryce 1971) indicate that these compounds may be biogenetic precursors of modern plant stilbenoids, with the COOH group being a biogenetic archaicism (Nguyen et al. 2014).

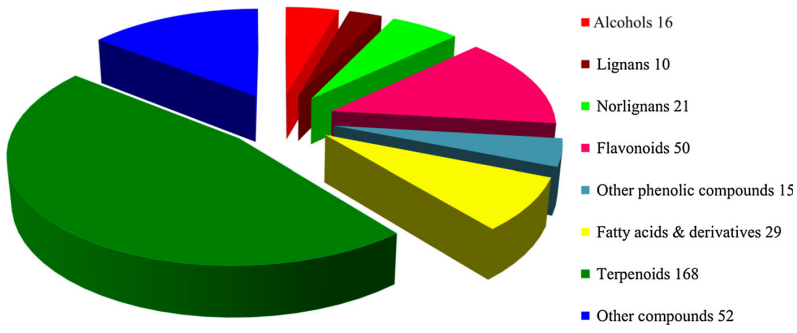

Fig. 7 Classes of natural product characterized from *Metasequoia glyptostroboides*

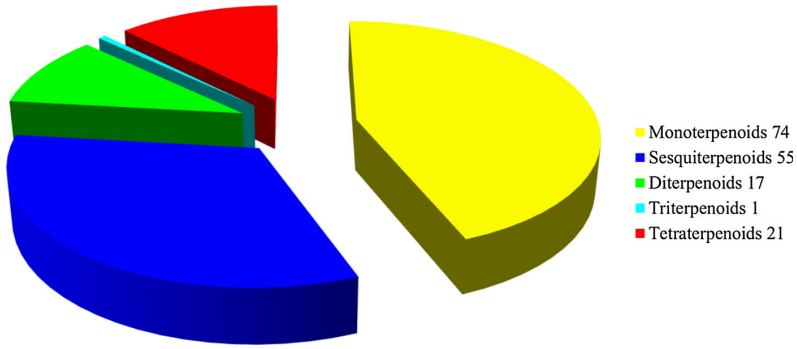

Fig. 8 Overview of groups of terpenoids characterized from *Metasequoia glyptostroboides*

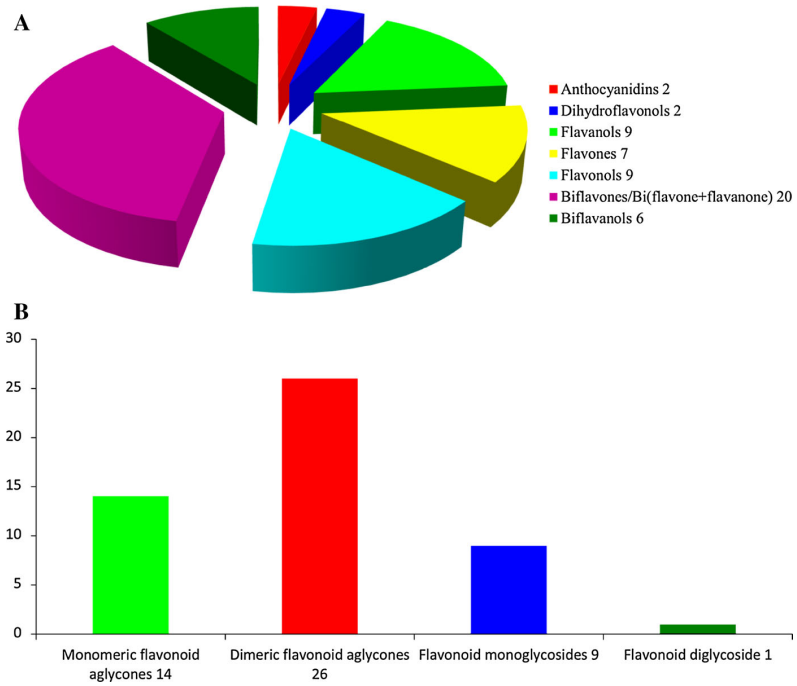

Fig. 9 Overview of flavonoid classes characterized from *Metasequoia glyptostroboides* based on type of aglycone (**a**) and extent of glycosylation (**b**)

Temperature and sunlight conditions –potential influential factors on secondary metabolite synthesis

Reports on natural products from *M. glyptostroboides* available in current literature have been conducted on trees growing at quite a few rather different localities, including several European and Asian countries, including China (Dong et al. 2011), South Korea (Bajpai and Kang 2010a, 2011a, b; Bajpai et al. 2007a, b, 2009, 2010, 2014a; Duan et al. 2009), Japan (Fujita 1990; Hayashi et al. 1969; Ida 1981a, b; Nakatani et al.

1991), Poland (Czeczuga 1987; Krauze-Baranowska 2004), Germany (Beckmann and Geiger 1968; Beckmann et al. 1971; Beckmann and Schuhle 1968; Braun and Breitenbach 1977), France (Mongrand et al. 2001) and Norway (Nguyen et al. 2014). The natural products isolated from *M. glyptostroboides* grown in Norway were mainly different from those reported from the tree grown at other localities (Nguyen et al. 2014). This may be accounted for by the fact that the sunlight conditions (particularly the day length) in the growth season in Norway are quite different from that of other locations from which plant material has been analyzed. Temperature and sunlight conditions are known to be important parameters for the secondary metabolism of plants (Radušienė et al. 2012). However, it should be mentioned that Northern Europe, as far north as Svalbard, was part of the natural habitat of *M. glyptostroboides* until the Miocene era (23–5 million years ago) from which fossils of this species were discovered for the first time in 1876 (but originally incompletely identified) (Heer and Nordenskiöld 1876). To date, however, no comprehensive systematic study has been performed to investigate the influences of any growth conditions or locality on secondary metabolite production of *M. glyptostroboides*.

Natural products of *Metasequoia glyptostroboides* – a molecular window into the Cretaceous era

Metasequoia is presumed to have evolved in eastern Russia during the early Late Cretaceous period, around 100 million years ago as the earliest dawn redwood fossils were reported from this region (Yang 1998/1999). The unique potential of the tree as a source of bioactive constituents is founded on the fact that it seems to have survived unchanged since the Cretaceous era. Since then, its unusually successful molecular defense system has resisted the attacks of millions of generations of pathogens. Unlike fossils, where the original molecules may be fragmentary at best, if present at all (Schweitzer et al. 2009), living fossils like *M. glyptostroboides* may provide a detailed, intact, high-resolution system from which ancient natural products can be uncovered and characterized. However, already at a very early stage after its discovery, doubt was cast about whether or not *M. glyptostroboides* had remained unchanged at the molecular level –or even if the present species could reasonably be named identically to a species existing in the Cretaceous era (Schopf 1948). The predominant view was that in fossils of plants, only the morphology was preserved, whereas the original molecules were lost (Calvin 1969). Until recently, detection or any identification of the original natural products of fossil material of any species appeared to be unlikely. However, recent development in analytical technology has made it possible to identify at least fragments of the original molecules, modified natural products or even unmodified natural products from well preserved fossils dating back as far as to the Cretaceous era (Bern et al. 2009; Schweitzer 2004; Schweitzer et al. 1997). As a consequence, Zhao et al. (2007) succeeded in identifying 51 different compounds from fossil *M. glyptostroboides* excavated at Svalbard, Norway, dating from the Miocene era (23–5 million years ago) (Table 2) (Zhao et al. 2007). Although the majority of these compounds were considered to be modified natural products, which may, however, in some instances had kept their original core structures (such as squalene and retene), two of these compounds, namely the hydrocarbons tetracosane and pentacosane, have also been identified from fresh plant material (Tables 1 and 2) (Fujita 1990; Zhao et al. 2007). Hydrocarbons are among the few natural products with sufficient expectable life time to be discovered intact in fossilized material which has been preserved over a time scale of millions of years (Calvin 1969). When keeping in mind that the growth conditions may influence the biosynthesis of natural products of *M. glyptostroboides* as indicated by Nguyen et al. (2014) (Nguyen et al. 2014), further compounds reported by Zhao may be either compounds with intact core structure or surviving original natural products from the relatively warm Miocene era, when the natural habitat of the tree included Svalbard in the far north. Very recently, an intact and significantly older piece of *Metasequoia* wood buried deeply in a kimberlite pipe that intruded northwestern Canada's Slave Province 53.3 ± 0.6 million years ago was discovered (Wolfe et al. 2012). Initial comparative IR spectral analysis of this intact 53 million year old wood and amber of *Metasequoia* with fresh wood from present *M. glyptostroboides* gave similar results, strongly indicating that the tree has remained unchanged for millions of years at the molecular level (Wolfe et al. 2012). Attempts to

recover DNA from well preserved fossilized *Metasequoia* needles encapsulated in amber have hitherto been unsuccessful (Yang 1998/1999). However, it may be possible that the 53 million year old intact *Metasequoia* wood recently discovered could contain intact DNA or sufficiently large fragments thereof required for a direct comparison with DNA of the present *M. glyptostroboides*.

Biological and pharmacological effects of substances and extracts of *M. glyptostroboides*

An increasing number of studies of various biological activities and medicinal applications of the title plant have been reported in current literature. These include studies performed on pure compounds, as well as extracts, and applications as plant medicines. Several recent patents exploiting substances or extracts of *M. glyptostroboides* visualize the increased commercial potential of medicinal applications based on the bioactive constituents from this species. (Ding 2003; Jung et al. 2004; Lee et al. 2009; Wu 2009). The different types of biological activities reported in current *Metasequoia* literature are treated in separate paragraphs below.

Antioxidant activity

Antioxidant activity, as well as radical scavenging activity has been determined for both extracts and pure compounds from *M. glyptostroboides*. Bajpai et al. (2009) tested the antioxidant activity of the essential oil and various organic extracts (n-hexane, chloroform, ethyl acetate and methanol) of *M. glyptostroboides*. DPPH was used to identify antioxidant activity. The study revealed that essential oil and ethyl acetate extracts showed higher or similar antioxidant activity compared to the standards, butylated hydroxyanisole and ascorbic acid. This might be accounted for by the high total phenolic content in the ethyl acetate extracts (Bajpai et al. 2009). Chen et al. (2014) reported significant DPPH radical, superoxide anion radical, and hydroxyl radical scavenging capacity, total antioxidative capacity, lipid peroxidation inhibitory activity, and metal ions chelating capacity of chromatographic fractions derived from bark extracts of *M. glyptostroboides*. The observed activities were correlated with the proanthocyanidin content of the active fractions isolated (Chen et al. 2014).

The DPPH scavenging activity of the pure compound 6-carboxydihydroresveratrol-3-*O*-β-glucopyranoside isolated from *M. glyptostroboides* was significant, though the IC_{50} value was approximately 11-fold higher than the reference compound gallic acid (Nguyen et al. 2014). Hinokiflavone, a biflavone which occurs in leaves of *M. glyptostroboides*, has been identified as a potent antioxidant using hyphenated HPLC-DPPH (Zhang et al. 2011). The compound used for these studies was, however, not isolated from *M. glyptostroboides*.

Arachidonic acid metabolism inhibition

Arachidonic acid metabolites play important roles in disease conditions such as inflammation and development of cancer (Hyde and Missailidis 2009). Therefore, there is an increasing interest in discovering inhibitors of key enzymes of the arachidonic acid cascade reaction, such as 15-lipoxygenase (Gillmor et al. 1997; Samuelsson et al. 1987). The dihydrostilbenoid glucoside 6-carboxydihydroresveratrol-3-*O*-β-glucopyranoside, a compound specific to *M. glyptostroboides*, proved to be a significant inhibitor of 15-lipoxygenase with IC_{50} at a comparable level to the standard inhibitor quercetin (Nguyen et al. 2014).

Antibacterial effect

There is a continuous need for the discovery of novel antibiotics, due to the observed development of bacterial resistance to the antibiotics presently known. Because *M. glyptostroboides* has resisted the attack of millions of generations of pathogens, apparently without changing, the tree may be a promising source of natural products with antibiotic activity. Indeed, significant antibiotic activity towards several types of bacteria has been reported for extracts, as well as for pure compounds derived from this species.

Bajpai et al. (2007a) identified 59 compounds from the floral cone of *M. glyptostroboides*, which mainly contained oxygentated mono- and sesquiterpenes and the corresponding hydrocarbons. These compounds

together with the complete methanol extract and methanol derived sub fractions were tested for antimicrobial effect against eleven different food spoilage and foodborne bacterial strains, four gram-positive bacteria and seven gram-negative bacteria. The essential oil, methanol extracts and various organic sub-fractions exhibited significant potential for antibacterial activity. The study indicated that mediated essential oils and extracts from *M. glyptostroboides* can be applied as natural preservatives or flavouring additives in the food industry to control spoilage and foodborne pathogenic bacteria which cause severe destruction of food (Bajpai et al. 2007a). Very recently, Bajpai et al. (2014a, b) reported anti-listeria activity of essential oils of *M. glyptostroboides*. The anti-listerial activity of essential oils of *M. glyptostroboides* acted synergistically with the peptide antibiotic nisin (Bajpai et al. 2014b).

The observed antibacterial activity of extracts derived from *M. glyptostroboides* may be rationalized by the fact that several pure compounds with significant antibacterial activity have been isolated from this plant source. Metaseol, a compound specific to *M. glyptostroboides*, exhibited potent antibacterial activity against *Bacillus subtilis* and *Escherichia coli* (Nakatani et al. 1991). Two abietane type diterpenoids, sugiol and taxodone, isolated from the ethyl acetate cone extract from *M. glyptostroboides*, proved to have antibacterial effect against several foodborne pathogenic bacteria, which may cause destruction and reduce the quality of food. Both studies showed that gram-positive bacteria were more sensitive to sugiol and taxodone than gram-negative bacteria. Sugiol exhibited higher antibacterial activity compared to the standard streptomycin in regard to gram-positive bacteria. Taxodone, on the other hand, exhibited lower antibacterial activity than the standard streptomycin. However both compounds inhibited gram-positive bacteria to some extent. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for sugiol against foodborne pathogens were lower than for taxodone. The MIC is determined by the lowest concentration of the compound that does not show any growth of the test organism. MBC is defined as the complete absence of growth of bacterial colonies on the agar surface in the lowest concentration of sample. MIC for sugiol and taxodone varied from 62.5 to 250 μg/ml and 250–1000 μg/ml against different foodborne pathogens while MBC varied from 125 to 250 μg/ml and 250–2000 μg/ml, respectively. Similar antibacterial effects have also been detected for taxoquinone (Bajpai et al. 2010). The findings indicate that sugiol, taxodone and taxoquinone could be possible candidates for application in the food industry for the control of foodborne pathogens. Such potential applications would, however, require further studies on the safety and toxicity of these compounds (Bajpai and Kang 2010a, 2011a; Bajpai et al. 2010).

Antifungal and antidermatophytic effects

The essential oil and various organic extracts (hexane, chloroform, ethyl acetate and methanol) of *M. glyptostroboides* have shown potential antidermatophytic effect against infectious fungal pathogens of the skin. They also inhibit some fungal spore germination at certain concentrations. Essential oils and extracts could therefore be used as a source of new antidermatophytic agents to control superficial human fungal infection (Bajpai et al. 2009). Bajpai and Kang have reported that the essential oil of *M. glyptostroboides* leaf has a moderate to high antifungal activity against seven different plant pathogenic fungal species namely *Botrytis cinerea* KACC 40573, *Rhizoctonia solani* KACC 4011, *Fusarium oxysporum* KACC 41083, *Sclerotinia sclerotiorum* KACC 41065, *Colletotrichum capsici* KACC 40978, *Fusarium solani* KACC 41092 and *Phytophthora capsici* KACC 40157. The results from the study also show that methanol, ethyl acetate and chloroform leaf extracts have strong antifungal activity against the tested plant pathogens. These findings indicate that the extracts and oil of *M. glyptostroboides* could be considered as potential antifungal agents to control several plant pathogenic fungi causing severe diseases in food, crops and vegetables (Bajpai and Kang 2010b).

Studies on antifungal activity of pure compounds isolated from *M. glyptostroboides* are hitherto limited to a few studies on diterpenoids. These include three antifungal diterpenoids reported by Asahi and Sakan (1984) (Table 1, compounds 305–307) and the diterpenoid taxoquinone (Bajpai and Kang 2014). The latter compound exhibited significant antifungal activity against pathogenic isolates of several *Candida* species.

Antiviral activity

In current literature, studies on antiviral activity of natural products isolated from *M. glyptostroboides* have hitherto only been performed on pure hinokiflavone. This dimeric flavonoid, isolated from *M. glyptostroboides*, exhibited antiviral activity against influenza viruses A and B (Miki et al. 2008). The mechanism at molecular level is based on the fact that hinokiflavone acts as an inhibitor of viral sialidase (also known as viral neuraminidase/exo-α-sialidase) (Miki et al. 2008), an enzyme which plays at least two important roles in the viral life cycle. These include the facilitation of virion progeny release and general mobility of the virus in the respiratory tract (von Itzstein 2007). The observed anti-influenza activity was amplified significantly when hinokiflavone was conjugated with sialic acid (Miki et al. 2008). Several identified antiviral natural products originate from the shikimic acid biosynthetic pathway (Andersen and Helland 1996; De Bruyne et al. 1999; Hayashi et al. 2003), which is also the case for hinokiflavone. The B-ring systems of this dimeric flavonoid, in addition to C-2, and C-2″ originate from this biosynthetic pathway. The observed antiviral activity of these compounds may be rationalized by the fact that the slightly modified shikimic acid derivative oseltamivir, which is the active constituent of the anti-influenza drug Tamiflu, possess its antiviral activity through inhibition of the influenza viral sialidase (von Itzstein 2007).

Anticancer activity

Recently, analyses of anticancer activity of extracts and pure compounds derived from *M. glyptostroboides* have been published. Zeng et al. (2012) reported that five pure compounds specific to *M. glyptostroboides*, namely the norlignans metasequirin G-I (**153–155**; Fig. 4) and the phenylpropans 7-(3-ethoxy-5-methoxyphenyl)propane-7,8,9-triol (**339**) and 7-(3-hydroxy-5-methoxyphenyl) propane-7,8,9-triol (**340**) (Fig. 6), exhibited cytotoxic activity against A549 and Colo 205 cell lines with IC_{50} values within the range 50–100 μM (Zeng et al. 2012). The fact that an anticancer drug based on dihydrohinokiflavone isolated from *M. glyptostroboides* has been patented (Jung et al. 2004) should encourage exploitation of the anticancer potential of the multitude of structurally relatively similar biflavonoids identified in leaves of this species (Table 1).

Protective effects on cerebral ischemia–reperfusion injury

Wang et al. (2004) reported that a mixture of flavonoids from *M. glyptostroboides* (referred to as total flavonoids) exhibited protective effects on cerebral ischemia–reperfusion injury in rats (Wang et al. 2004). This is in agreement with the previous findings that intake of flavonoid-rich food has been reported to significantly improve coronary circulation in healthy human adults (Shiina et al. 2009).

Other medicinal applications

As a medicinal plant *M. glyptostroboides* is a constituent of a plant medicine used for treatment of diabetes (Ding 2003) and has also applications in traditional Chinese medicine (TCM) (Wu 2009). Medicinal compositions for skin care have been prepared from *M. glyptostroboides* (Arashima et al. 2008; Lee et al. 2009).

Concluding remarks

The living fossil *M. glyptostroboides*, a tree which seems to have remained unchanged since the Cretaceous era, is a unique source of novel natural products. It is apparent that the chemical defense system of the tree, based on its bioactive secondary metabolites, has resisted the attack of millions of generations of pathogens during geological time. The potential of these compounds and extracts containing them has only very recently been exploited in modern medicine. As a consequence of the significant strides in the development of chromatographic methods and increasingly sensitive spectroscopic instruments, in particular the development of cryogenic probe technology for high-field NMR instruments, discovery of an increasing number of novel natural products from *M. glyptostroboides* is expected to continue in the near future. The fact that several medicinal applications based on compounds from this plant source as active

principles currently exist, would encourage such development, including extensive testing of biological activity of these new compounds. The latter point may be further reinforced by the fact that, at present, compounds specific for *M. glyptostroboides* have hitherto only been tested to a limited extent with respect to their biological activity. Indications that the growth and sunlight conditions may significantly influence the qualitative production of the selection of natural products of this species strongly encourage international research cooperation leading to a coordinated global exploitation of plant material from geographically exceptionally different localities.

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Paper V

First identification of natural products from the African medicinal plant *Zamiouculcas zamiifolia* – A drought resistant survivor through millions of years.

V

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First identification of natural products from the African medicinal plant *Zamioculcas zamiifolia* — A drought resistant survivor through millions of years

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(*E*)-caffeic acid methyl ester (PubChem CID: 689075)
(*Z*)-caffeic acid methyl ester
(3R,4S,5S)-2,3,4,5-tetrahydroxytetrahydro-2H-pyran-2-carboxylic acid and apigenin 6-C-(6″-(3-hydroxy-3-methyl-glutaroyl)-ß-glucopyranoside)

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ABSTRACT

Zamioculcas zamiifolia, an unusually drought resistant medicinal plant native to tropical east Africa and subtropical southeast Africa, including the countries Kenya, Malawi, Mozambique, South-Africa, Tanzania and Zimbabwe, is described as a living fossil which may have evolved as early as 42 million years ago. It belongs to the notoriously toxic family Araceae giving it, through association, a reputation for being toxic; despite little or no systematic evidence exists to support this claim. As an ancient plant it has sustained substantial climate changes and attacks from millions of generations of pathogenic microorganisms, which encouraged search for novel natural products from this source. Seven natural products have been characterized from leaves and petioles of *Z. zamiifolia*, including the novel main compound of the leaves, apigenin 6-C-(6″-(3-hydroxy-3-methyl-glutaroyl)-ß-glucopyranoside). The structure determinations were based on extensive use of 2D NMR spectroscopic techniques and high-resolution mass spectrometry. Initial toxicological experiment on extracts from *Z. zamiifolia* using brine shrimp lethality assay did not indicate lethality to the shrimps providing disproving evidence for the assumption of *Z. zamiifolia's* toxic character.

1. Introduction

Zamioculcas zamiifolia (Fig. 1) is an ancient plant species of the Araceae, a family which dates back to the early Cretaceous period, a geologic time period approximately 100–130 million years ago [1]. Fossils of plants of the Araceae family have been discovered [2,3]. An estimation of the evolution of the Araceae family based on fossils indicates that *Z. zamiifolia* may have evolved as early as 42 million years ago [1]. It seems remarkable that a species can exist for such a long period of time and survive significant factors such as substantial climate changes and attacks from millions of generations of pathogenic microorganisms, indicating the presence of an unusual successful chemical defense system at the molecular level [4].

Although little information is available, *Z. zamiifolia* is apparently frequently used medicinally in the Mulanje District of Malawi. In the region of the East Usambara mountains in Tanzania, the juice from the leaves is used medicinally to treat earache [5]. In Tanzania a poultice of bruised plant material from *Z. zamiifolia* is used as a treatment of the inflammatory condition known as "mshipa" [6,7]. Roots from *Z. zamiifolia* are used as a local application to treat ulceration by the Sukuma people in northwestern Tanzania [6,8]. Ngulukesi is the Sukuma name for *Z. zamiifolia* [7,8]. Investigations of biological activity of *Z. zamiifolia* are largely absent in current literature, with exception of early initial tests of antibacterial activity of aqueous extracts of the plant which, however, provided negative results [6,9].

As *Z. zamiifolia* is the only known species of the genus *Zamioculcas* it is not possible to compare it with close relatives. One wonders what distinguishes this species from others that have become extinct. The answer may very well lie in its chemical composition. In addition, coming from a family of notoriously toxic plants, *Z. zamiifolia* has gained

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Fig. 1. *Zamioculcas zamiifolia*. Top row: Living plants. Lowest row: Leaves and petioles prior to extraction with methanol. All photos: Angharad Le Moullec.

a reputation for being poisonous [10]. However, there is no systematic experimental evidence to support this belief.

The aim of this study was to isolate and identify compounds from the plant *Z. zamiifolia*. The extracts proceeded to be biologically tested to gain insight into their toxic potential. Very few studies have previously been carried out on this plant, and there is no previously existing record of compounds isolated from *Z. zamiifolia*. All compounds reported in this paper are therefore new compounds from *Z. zamiifolia*.

2. Experimental

2.1. Extraction of plant material

Z. zamiifolia were bought on a local market (IKEA, Bergen, Norway). Water percentages of petioles and leaves were determined to be 95% and 91%, respectively, since 13.4390 g petioles lost 12.7175 g after 96 h storage at 100 °C and 1.9644 g leaves lost 1.7932 g when stored for the same time at the same conditions. Leaves (860 g) and petioles (1318 g) of *Z. zamiifolia* were extracted for 48 h (two times) with methanol at room temperature. Prior to methanolic extraction, the leaves were extracted with petroleum ether to remove the wax layer at the surface of the leaves. The combined methanolic extracts were concentrated on rotary evaporator to yield an aqueous concentrate. The aqueous concentrated extract was extracted (three times) with 1 L petroleum ether in a separation funnel. The aqueous phase was thereafter extracted (three times) with 1 L ethyl acetate. The ethyl acetate phase was further separated by column chromatography as described below.

After the first extraction of the leaves using petroleum ether, two different phases emerged. A 100 mL glass pipette was utilized to separate the two different phases. Besides the pale green petroleum ether phase that made up the larger part of the extraction volume, a small amount of dark brown aqueous concentrate had accumulated at the bottom of the Erlenmeyer flask. This aqueous concentrate (20 mL) was further separated by XAD-7 amberlite column chromatography.

2.2. Amberlite XAD-7 column chromatography

The concentrated water phase from liquid–liquid partition of the leaf extract was applied to the surface of the Amberlite XAD-7 column (column dimensions: 5 × 105 cm). 5 L water was first used as mobile phase, and thereafter a gradient of mobile phase with increasing proportions of methanol was applied. Nine fractions of 800–950 mL were collected. 1–1.5 mL of these was directly transferred to HPLC vials for later determination of their content and purity, as described below. Fractions were evaporated to dryness on rotary evaporator. The dried fractions were redissolved in methanol and transferred to dram vials and dried under nitrogen gas stream.

Following a similar procedure the ethyl acetate phase of the extract of the petioles was separated on an identical Amberlite XAD-7 column. Thirty-three fractions were in this case collected from the column following the chromatography process varying in volumes from 150 to 250 mL. Fractions 2 and 10 were further separated on preparative HPLC as described below.

The volume of approximately 20 mL of aqueous concentrate from the initial extraction of the leaves, as described above, was gently applied onto a small Amberlite XAD-7 column. Seven fractions were collected from the column following the chromatography process varying in volumes from 500 to 650 mL. The purity of the fractions was determined by analytical HPLC. Compound **6** (isolated in fraction 1) was further analyzed by NMR spectroscopy.

2.3. Sephadex LH-20 column chromatography

Fraction 8 from XAD-7 column chromatography of the water phase was further separated on a Sephadex LH-20 column (column dimensions: 4 × 30 cm) using varying proportions of methanol, super distilled water and trifluoroacetic acid (TFA). The gradient consisted of 0.4 L methanol–water–TFA 20:80:0.2; v/v (fractions 1–4), followed by 0.2 L methanol–

water–TFA 40:60:0.2; v/v (fractions 5–6) and 1.8 L methanol–water–TFA 70:30:0.2; v/v (fractions 7–24). Fractions of 100 mL were collected. The purity of each fraction was determined by analytical HPLC. All the collected fractions were evaporated to dryness. The dried fractions were redissolved in methanol and directly transferred to dram vials and dried under nitrogen gas stream. Fraction 13 (29.7 mg) contained the major compound of the leaves (compound **7**).

2.4. Preparative HPLC

The compounds of fractions 2 and 10 from XAD-7 separation of the ethyl acetate phase of the extract of the petioles were isolated by preparative HPLC. The HPLC instrument was equipped with a 250 × 22 mm, C_{18} Altech column. Two solvents were used for elution; A (water–TFA 99.5:0.5; v/v) and B (methanol–TFA 99.5:0.5; v/v). The elution profile of the applied HPLC gradient is shown in Table S7. The sample was dissolved in a total of 1 mL of A-B (50:50 v/v). An HPLC gradient program was applied and started as described in Table S7. Portions of 200 μL of the sample were manually injected into the HPLC column. Each peak in the chromatogram was separately collected in vials. 1–1.5 mL of each of the collected fractions was transferred to HPLC vials for later identifications using analytical HPLC.

2.5. Analytical HPLC

The HPLC instrument was equipped with a HP 1050 multidiode array detector, a 20 μL loop and a 250 × 4.6 mm, 5 μm Thermo Scientific Hypersil GOLD column. Two solvents were used for elution; A (water–TFA 99.5:0.5; v/v) and B (acetonitrile–TFA 99.5:0.5; v/v). The elution profile of the applied HPLC gradient is shown in Fig. 2. The analytical HPLC pump system was purged with both solution A (super distilled water and 0.5% TFA) and solution B (acetonitrile and 0.5% TFA) for 15 min each with a flow of 5 mL/min. The column was thereafter equilibrated with a flow of 1 mL/min in 30 min with acetonitrile-super distilled water (10:90 v/v). 20 μL of each sample was injected with an autoinjector. An appropriate solvent gradient (Fig. 2) was applied for the separations. The flow rate was 1 mL/min.

2.6. Spectroscopy

High resolution mass spectra were recorded using a JEOL AccuTOF JMS T100LC instrument fitted with an electrospray ion source. The spectrum was recorded over the mass range 50–1000 m/z.

UV–Vis absorption spectra were recorded on-line during HPLC analysis over the wavelength range 240–600 nm in steps of 2 nm.

NMR samples were prepared by dissolving the isolated compounds in deuterated dimethylsulfoxide (DMSO-D_6; 99.9 atom% D, Sigma-Aldrich), with exception of compound **6**, which was only soluble in D_2O. The 1D ^1H and the 2D ^1H–^{13}C HMBC, the 2D ^1H–^{13}C HSQC, the 2D ^1H–^{13}C HSQCTOCSY, the 2D ^1H–^{13}C H2BC, the 2D ^1H–^1H COSY and 2D ^1H–^1H ROESY NMR experiments were obtained at 600.13 MHz and 150.90 MHz for ^1H and ^{13}C, respectively, at 298 K on a Bruker 600 MHz instrument equipped with a ^1H,^{13}C,^{15}N triple resonance cryogenic probe.

2.7. Brine shrimp lethality assay

Dried extracts of leaves (0.2181 g) and petioles (0.5484 g) were separately dissolved in 4.0 mL DMSO. Although the great majority of the extracts dissolved when applying DMSO, a small part did not, giving rise to a solution with some un-dissolved particles. From the stem solution of plant extract in DMSO a salt-water solution containing approximately 2.0 mg/mL of extract in salt water was prepared. 366.80 μL of leave extract was dissolved in 9.633 mL of salt water whereas 145.88 μL of petiole extract was dissolved in 9.854 mL salt water. The salt-water extract solutions proceeded to be transferred into micro wells of a 96 microwells plate filled with Artemia larvae using 100 μL micropipettes. After 24 h the Artemia larvae in each well were counted using a computer-connected Veho 400× USB microscope with 20 times magnification to determine the lethality rate caused by the extract in the varying concentrations.

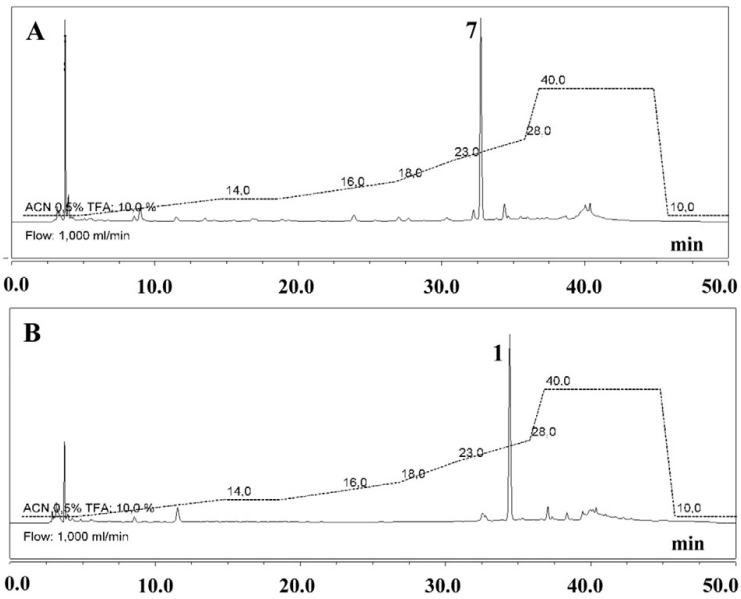

Fig. 2. HPLC chromatograms of the methanolic crude extracts of leaves (A) and petioles (B) of *Zamioculcas zamiifolia* recorded at 280 nm. Main aromatic components are annotated. The solvent gradient applied for separation is shown in the chromatograms.

3. Results and discussion

The HPLC chromatograms of leaves and petioles of *Z. zamiifolia* showed in each instance the presence of one major aromatic compound (compounds **7** and **1**, respectively) and several minor compounds (Fig. 2). The methanolic extracts of leaves and petioles of *Z. zamiifolia* were concentrated under reduced pressure and fractionated by extraction with petroleum ether and ethyl acetate. The ethyl acetate phase was further separated by gradient XAD-7 adsorption chromatography, Sephadex LH-20 gel filtration chromatography and preparative HPLC.

The five known compounds rosmarinic acid (**1**), protocatechuic aldehyde (**2**), (*E*)-caffeic acid (**3**) and (*E*)- and (*Z*)-caffeic acid methyl ester (**4** and **5**) were isolated from the petioles (Fig. 3). Rosmarinic acid is the major aromatic compound of the petioles (Fig. 2). These compounds are relatively common naturally occurring phenolic compounds reported from other plants. The rare natural product (3R,4S,5S)-2,3,4,5-tetrahydroxytetrahydro-2 H-pyran-2-carboxylic acid (**6**) was isolated from the leaves of *Z. zamiifolia* (Fig. 3). The ^{13}C NMR chemical shift values of the latter compound (Table S6) were in agreement with those presented in current literature [11]. The structure determinations were based on 2D NMR spectroscopy (supplementary Tables S1–S6). Methanol was used as extraction solvent and applied during the isolation process. Thus, we cannot exclude that the methyl esters of (*E*)- and (*Z*)-caffeic acid may be formed as artifacts during the isolation process.

The aromatic region of the 1D ^{1}H NMR spectrum of the main aromatic compound of the leaves (**7**) showed a 4 H AA′XX′ system at δ 7.92 ('d' 8.9 Hz, H-2′/6′) and δ 6.92 ('d' 8.9 Hz, H-3′/5′), and two 1 H singlets at δ 6.77 (H-3) and δ 6.50 (H-8), which is in accordance with a 6-*C*-substituted apigenin derivative. The *C*-glycosyl unit was identified as glucose by the seven ^{1}H signals in the region 4.58–3.17 ppm of the 1D ^{1}H NMR spectrum belonging to this unit, which correlated to six ^{13}C signals in the region 64–79 ppm of the heteronuclear 2D ^{1}H–^{13}C HSQC spectrum (Table 1). The large axial-axial coupling constants observed for each of the signals H-1″-H-5″ (Table 1) were of particular value for determination of the stereochemistry of the glucosyl unit. The anomeric coupling constant (9.8 Hz) was in accordance with a β-configuration of the anomeric carbon. The downfield shifts of H-6A″ (δ 4.36) and H-6B″ (δ 3.93) belonging to the glucose unit indicated the presence of acyl substitution. The acyl moiety was identified as 3-hydroxy-3-methylglutaroyl by the two 2 H doublets at δ 2.62 and δ 2.52 (J = 14.3 Hz; H-2A‴, H-2B‴) and δ 2.52 and δ 2.46 (J = 4.9 Hz; H-4A‴, H-4B‴), respectively, in the 1D ^{1}H NMR spectrum (assigned by the 2D COSY spectrum), a 3 H singlet (3-methyl) at δ 1.23 correlated to a ^{13}C signal at δ 27.46 (3‴-methyl) in the 2D HSQC spectrum, the five ^{13}C signals at δ 170.70 (C-1‴), δ 45.41 (C-2‴), δ 69.13 (C-3‴), δ 45.40 (C-4‴), and δ 172.53 (C-5‴) in the 1D ^{13}C CAPT spectrum, and the crosspeaks for this unit observed in the 2D HMBC spectrum. The ^{1}H and ^{13}C chemical shift values of the 3-hydroxy-3-methylglutaroyl unit were in agreement with reference data in current literature [12]. The linkages between the aglycone, sugar unit and 3-hydroxy-3-methylglutaroyl were determined by the long-range correlations in the 2D HMBC spectrum (Fig. 4). A pseudomolecular ion at m/z 577.1513 [MH^{+}] corresponding to $C_{27}H_{29}O_{14}$ (calculated 577.1513) in the high resolution ESI-MS spectrum, confirmed the identity of **7** to be the novel natural product apigenin 6-*C*-(6″-*O*-(3-hydroxy-3-methylglutaroyl)-β-glucopyranoside) (**7**) (Fig. 3).

During the extraction and isolation process, where methanol was extensively used, an artifact of compound **7** was formed by methyl esterification of the free carboxyl group of the 3-hydroxy-3-methylglutaroyl. The presence of this methylated artifact was confirmed by high resolution mass spectrometry. Methylation of free carboxylic acid groups of

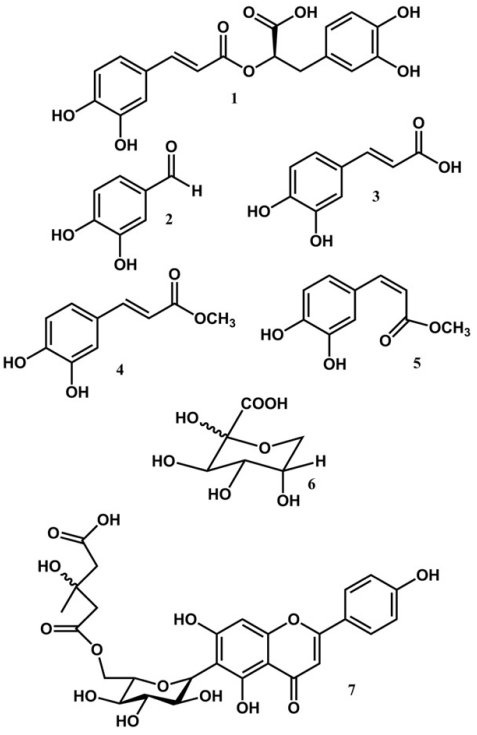

Fig. 3. Molecular structures of natural products (**1**–**7**) characterized from leaves and petioles of *Zamioculcas zamiifolia*.

Table 1
^{1}H and ^{13}C NMR chemical shifts (δ, ppm) and the coupling constants (J, Hz) for apigenin 6-*C*-(6″-(3-hydroxy-3-methyl-glutaroyl)-ß-glucopyranoside) (**7**). s = singlet; d = doublet; dd = double doublet; ddd = double double doublet; 'd' = semi-doublet and m = multiplet.

Position	δ ^{1}H	δ ^{13}C
Apigenin		
2		163.65
3	6.77 s	102.92
4		182.08
5		161.31
6		108.65
7		163.39
8	6.50 s	93.70
9		156.40
10		103.55
1′		121.23
2′/6′	7.92 'd' 8.9	128.60
3′/5′	6.92 'd' 8.9	116.12
4′		161.30
6-C-glucosyl		
1″	4.58 d 9.8	73.21
2″	4.09 dd 9.8, 8.7	70.00
3″	3.20 d 8.7	78.87
4″	3.17 d 9.1	70.64
5″	3.37 ddd 2.9, 7.0, 9.1	78.25
6A″	4.36 dd 11.9, 2.0	64.38
6B″	3.93 dd 11.9, 7.9	
6″-3-Methyl-glutaroyl		
1‴		170.70
2A‴	2.62, d 14.3	45.41
2B‴	2.52 d 14.3	
3‴		69.13
4A‴	2.52 m	45.40
4B‴	2.46 d 4.9	
5‴		172.53
3‴-CH_3	1.23 s	27.46

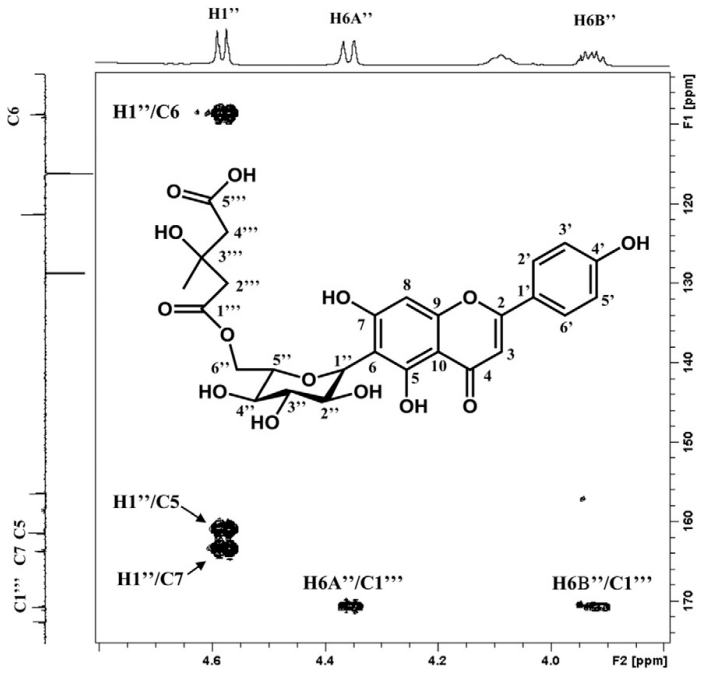

Fig. 4. Expanded regions of the HMBC spectrum of **7**. Selected cross peaks important for determination of the linkages between the structural sub-units are highlighted.

dicarboxylic acyl moieties of flavonoids have been documented in current literature [13].

Z. zamiifolia belongs to the Araceae family. Even though the chemical constituents of *Z. zamiifolia* has not been reported until now, extensive surveys focusing on the family members' flavonoid and phenolic contents have been performed [14]. Identified compounds include several *C*-glycosylflavones and flavonol *O*-glycosides, as well as structurally simpler phenolic compounds such as caffeic- and chlorogenic acids amongst others [15] (reviewed by Williams et al.) [14]. Flavone *C*-glycosides appear to be the most characteristic leaf components in the Araceae family (reviewed by Williams et al.) [14]. A comparison of data with that of other monocotyledon families suggests that the Araceae family may be more chemically primitive than most other groups [14]. This is due to the predominance of flavone *C*-glycosides and the presence of proanthocyanidins in over one third of the species surveyed. Flavone *C*-glycosides are considered to be primitively retained characters [14]. The diversity of flavone *C*-glycosylation along with the occurrence of methylated flavone *C*-glycosides in some species may however indicate a different form of chemical advancement in this family [14]. To our knowledge acylated *C*-glycosylflavones have not previously been reported from any plant source belonging to Araceae.

Several *Aracea* spp. are commonly known to possess toxic metabolites [16]. The toxicity in this family is thought to be largely attributed to the content of cyanogenic glycosides, calcium oxalates and perhaps alkaloids [16]. Moreover, saponins have also been identified from Araceae spp. (reviewed by Dring et al.) [16]. The cyanogenic glycosides triglochinin and isotriglochinin have been identified in *Alocasia macrorrhiza* [17]. The alkaloid coniine has been isolated in a number of plants in the family as well as anabasine and nicotine [16]. Lysicamine, nuciferine and liriodenine, a group of aporphine alkaloids were identified in *Lysichiton*. However, most of the family has not been examined for alkaloids [16].

Crude-extract from both leaves and petioles of *Z. zamiifolia*, a plant with assumed toxic character, was subjected to initial toxicological studies using brine shrimp lethality assay. Unexpectedly, no lethality was observed for *Artemia salina* when exposed to any of the applied concentrations of these extracts (up to 1 mg/mL). Thus, the outcome was not in accordance with the assumption that *Z. zamiifolia* is a toxic plant. On the contrary, it could appear as though the extract contributed to improvements in the vitality of the larvae. *Z. zamiifolia* like other succulents stores water in its petioles and rhizomes which provides a means of survival in very dry habitats [18]. For the first time the water content of *Z. zamiifolia* has been determined. These analyses revealed unusually high water contents of leaves (91%) and petioles (95%) of this drought resistant plant.

4. Conclusion

The previously completely uncharacterized ancient medicinal plant *Z. zamiifolia* proved to be a rich source of the novel acylated *C*-glycosylflavone apigenin 6-*C*-(6″-*O*-(3-hydroxy-3-methylglutaroyl)-β-glucopyranoside) which is the main aromatic component of its leaves. Initial toxicological studies using brine shrimp lethality assay provided disproving evidence for the assumption of *Z. zamiifolia's* toxic character.

Conflict of interest

The authors declare that there are no conflicts of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.fitote.2015.09.011.

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Supplementary data for:

First identification of natural products from the ancient African medicinal plant *Zamioculcas zamiifolia* –a drought resistant survivor for millions of years

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Table S1. ^{1}H and ^{13}C NMR chemical shifts (δ, ppm) and coupling constants (J, Hz) for rosmarinic acid (**1**). s=singlet, d=doublet and dd=double doublet.

Position	δ ^{1}H	δ ^{13}C
1		125.3
2	7.04 d 2.1	114.9
3		145.6
3-OH	9.15 s	
4		148.5
4-OH	9.64 s	
5	6.76 d 8.3	115.8
6	7.00 dd 2.1, 8.3	121.6
7	7.45 d 15.9	145.9
8	6.23 d 15.9	113.2
9		165.9
1’		127.2
2’	6.66 d 2.1	116.7
3’		145.0
3’-OH	8.78 s	
4’		144.0
4’-OH	8.72 s	
5’	6.62 d 8.14	115.4
6’	6.51 dd 2.1, 8.1	120.0
7A’	2.97 dd 4.2, 14.5	36.1
7B’	2.89 dd 8.5, 14.5	
8’	5.02 dd 4.2, 4.5	72.8
9’		170.7
9’-OOH	13.03 s	

Table S2. ^{1}H and ^{13}C NMR chemical shifts (δ, ppm) and coupling constants (J, Hz) for protocatechuic aldehyde (**2**). s=singlet; d=doublet and dd=double doublet; n.a. = not assigned

Position	**δ ^{1}H**	**δ ^{13}C**
1		129.2
2	7.23 d 2.0	114.6
3		146.3
4		152.8
5	6.91 d 8.1	n.a.
6	7.27 dd 2.0, 8.1	124.8
7	9.70 s	191.5

Table S3. ^{1}H and ^{13}C NMR chemical shifts (δ, ppm) and coupling constants (J, Hz) for *E*-caffeic acid (**3**). s=singlet, d=doublet and dd=double doublet, n.a. = not assigned

Position	**δ ^{1}H**	**δ ^{13}C**
1		n.a.
2	7.01 d 2.1	114.7
3-OH	9.12	n.a
4-OH	9.52	n.a
5	6.74 d 8.2	115.8
6	6.94 dd 2.1, 8.2	121.2
7	7.39 d 15.9	144.7
8	6.15 d 15.9	115.1
9-OOH	12.09s (broad)	

Table S4. ^{1}H NMR chemical shifts (δ, ppm) and coupling constants (J, Hz) for *E*-caffeic acid methyl ester (**4**). s=singlet, d=doublet and dd=double doublet.

Position	**δ ^{1}H**
1	
2	7.04 d 2.1
3	
4	
5	6.75 d 8.2
6	6.99 dd 2.1, 8.2
7	7.47 d 15.9
8	6.26 d 15.9
9	
3-OH	9.13 s
4-OH	9.59 s
9-OCH_3	3.67 s

Table S5. ^{1}H NMR chemical shifts (δ, ppm) and coupling constants (J, Hz) for *Z*-caffeic acid methyl ester (**5**). s=singlet, d=doublet and dd=double doublet.

Position	δ ^{1}H
1	
2	7.33 d 2.2
3	
4	
5	6.70 d 8.3
6	7.01 dd 2.2, 8.3
7	6.76 d 12.9
8	5.73 d 12.9
9	
3-OH	9.05 s
4-OH	9.39 s
9-OCH_3	3.64 s

Table S6. ^{1}H and ^{13}C NMR chemical shifts (δ, ppm) and the coupling constants (J, Hz) for (3R,4S,5S)-2,3,4,5-tetrahydroxytetrahydro-2H-pyran-2-carboxylic acid (**6**). d=doublet, dd=double doublet and m=multiplet.

Position	δ ^{1}H	δ ^{13}C
1		177.3
2		99.92
3	4.06 d 10.2	71.98
4	3.83 dd 3.3, 10.2	72.70
5	4.01 m	72.04
6A	4.02 m	67.01
6B	3.74 dd 12.9, 2.1	

Table S7. Gradient of solvents; (A) super distilled water + 0.1 % TFA and (B) methanol + 0.1 % TFA) applied during isolation of compounds using preparative HPLC.

No	Retention time (min)	Flow (ml/min)	A %	B%
1	0.000	14	90	10
2	0.000	14	90	10
3	4.000	14	90	10
4	44.000	14	60	40
5	60.000	14	60	40
6	70.000	14	30	70
7	88.000	14	30	70
8	89.000	14	90	10
9	104.000	14	90	10

Paper VI

Non-polar natural products from *Bromelia laciniosa*, *Neoglaziovia variegata* and *Encholirium spectabile* (Bromeliaceae).

VI

molecules

MDPI

Article

Non-Polar Natural Products from *Bromelia laciniosa*, *Neoglaziovia variegata* and *Encholirium spectabile* (Bromeliaceae)

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Abstract: Extensive regional droughts are already a major problem on all inhabited continents and severe regional droughts are expected to become an increasing and extended problem in the future. Consequently, extended use of available drought resistant food plants should be encouraged. *Bromelia laciniosa*, *Neoglaziovia variegata* and *Encholirium spectabile* are excellent candidates in that respect because they are established drought resistant edible plants from the semi-arid Caatinga region. From a food safety perspective, increased utilization of these plants would necessitate detailed knowledge about their chemical constituents. However, their chemical compositions have previously not been determined. For the first time, the non-polar constituents of *B. laciniosa*, *N. variegata* and *E. spectabile* have been identified. This is the first thorough report on natural products from *N. variegata*, *E. spectabile*, and *B. laciniosa*. Altogether, 20 non-polar natural products were characterized. The identifications were based on hyphenated gas chromatography-high resolution mass spectrometry (GC-HRMS) and supported by 1D and 2D Nuclear Magnetic Resonance (NMR) plant metabolomics.

Keywords: *Bromelia laciniosa*; *Neoglaziovia variegata*; *Encholirium spectabile*; nonpolar natural products; Hyphenated GC-HRMS; 2D NMR plant metabolomics

1. Introduction

1.1. Bromeliaceae

Several recent severe regional droughts have led to increased interest in exploiting drought resistant edible plants as human food sources and as forage for domesticated animals. Several drought-tolerant plants already utilized for such purposes belong to the Bromeliaceae and display features that make them especially capable of retaining water; the leaves are shaped adaxially concave to channel rainwater down to the overlapping, rosulate base for storage in a central cavity. The surface of the leaves bears absorptive, scale-like trichomes that take up water and nutrients. Bromeliaceae also have a Crassulacean acid *metabolism* (CAM) photosynthesis, where the stomata remain shut during daytime to avoid evaporation. The Bromeliaceae is a large family of flowering plants within the Monocots, which contains 58 genera and approximately 3200 species. Except for one western African

species, all Bromeliaceae are endemic to the American tropics. The family is currently divided into eight subfamilies [1], and the two species, *Bromelia laciniosa* Mart. ex Schult. & Schult. f. (Figure 1) and *Neoglaziovia variegata* (Arruda) Mez. (Figure 1), which belong to the subfamily Bromelioideae, and *Encholirium spectabile* Mart. ex Schult. & Schult. f (Figure 1),which belongs to the Pitcairnioideae subfamily. Bromeliads are used for alimentation (e.g., fruit production of *Ananas comosus* (L.) Merr., as fiber plants, and cultivated for ornamental and medicinal purposes.

Figure 1. *Bromelia laciniosa* (**left**); *Neoglaziovia variegata* (**middle**); and *Encholirium spectabile* (**right**) grown in Petrolina, Pernambuco, Brazil. Photos: JRGS Almeida.

1.2. B. laciniosa

B. laciniosa (Portuguese: macambira de porco) is native to Brazil and Argentina. Leaves from *B. laciniosa* are rich in proteins (4.9%), starch (2.8%) and calcium (1.1%) [2], and are therefore used in alimentation of both humans and domestic animals in northeastern Brazil [3,4]. Farmers in this region use the leaves as supplementary fodder for livestock [3,5,6]. The leaves are dried, powdered and mixed into the local cuisine [7]. A type of bread can be made from masses extracted from the base of the leaves [3]. Flowers, fruit and leaves from *B. laciniosa* are used in the treatment of infantile colic, diarrhea, fever, jaundice, hepatitis, and dandruff [8]. An aqueous root extract may be drunk as a treatment against intestinal diseases and hepatitis, and as a diuretic [7]. Only limited information is available which could rationalize these medicinal applications. Gum from *B. laciniosa* has been shown to contain galactose, arabinose and xylose, and an acidic oligosaccharide composed of xylose and galacturonic acid [9]. Quercetin 3,3′,4′-trimethyl ether is the only natural product isolated and characterized from *B. laciniosa* [10].

1.3. N. variegata

N. variegata (Portuguese: caroá) is one of only three species in the genus *Neoglaziovia*, all of which are endemic to northeastern Brazil. At the beginning of the rainy season, *N. variegata* produces edible fleshy fruits [11]. The plant was first described by the Brazilian Manuel Arruda da Câmara (1752–1810), while the genus is named after the French botanist Auguste Francois Marie Glaziou (1828–1906). *N. variegata* is used as a fiber plant by rural communities in the Caatinga region where a variety of products are made from the white, soft and flexible fibers [12–15]. Ethanol extracts of *N. variegata* have been reported to be of low toxicity [16] , and to exhibit antinociceptive effect in experimental models in mice [16], photoprotective potential, antioxidant effect [13,16], gastroprotective effects in a mice model of gastric ulcer [17] and antibacterial effect against both Gram-positive [13] and Gram-negative bacteria [13,18]. There is no report of natural products characterized from *N. variegata*.

1.4. E. spectabile

E. spectabile (Portuguese: macambira de flexa or macambira de pedra) is one of twenty five species in this genus, which is endemic to Brazil. This species is used as a supplementary food supply in famine emergencies by rural communities in the semi-arid Caatinga region [19]. The edible part of *E. spectabile* is the leaf base, which is rich in carbohydrates (28.7%), and contains some proteins (0.7%)

and lipids (0.8%) [19]. Flour made from the dried leaves is used to prepare a somewhat bitter tasting couscous. The nutritional value of *E. spectabile* (124.6 kcal/100 g) is in the same range as commercial grains such as rice (*Oryza sativa* L.) with 130 kcal/100 g [19]. Extracts have exhibited no signs of toxicity towards mice [20]; have been reported to exhibit antioxidant [21,22], photoprotective [22], and anti-nociceptive activity in mice models [20]; gastroprotective activity in a mice model of gastric ulcer [23]; and antibacterial activity towards Gram-negative [18,21] and Gram-positive bacteria [21]. No natural products have been characterized from this species.

Very little authoritative information is available about natural products from *Bromelia laciniosa*, and there is absolutely no previous information about *Encholirium spectabile* and *Neoglaziovia variegata*. In this paper, we report for the first time on the natural products characterized from hexane extracts of *Bromelia laciniosa*, *Encholirium spectabile* and *Neoglaziovia variegata*.

2. Results and Discussion

As part of our ongoing work on the characterization of natural products from food and medicinal plants aimed at rationalizing the molecular basis of their applications, the constituents of non-polar extracts of *B. laciniosa*, *N. variegata* and *E. spectabile* have been characterized. All identifications were based on hyphenated GC-HRMS. Altogether, 20 compounds were for the first time identified in the chromatograms of the hydrophobic crude extracts of *B. laciniosa*, *N. variegata* and *E. spectabile* (Table 1 and Figures 2–4). Compounds of each class are treated in separate paragraphs below.

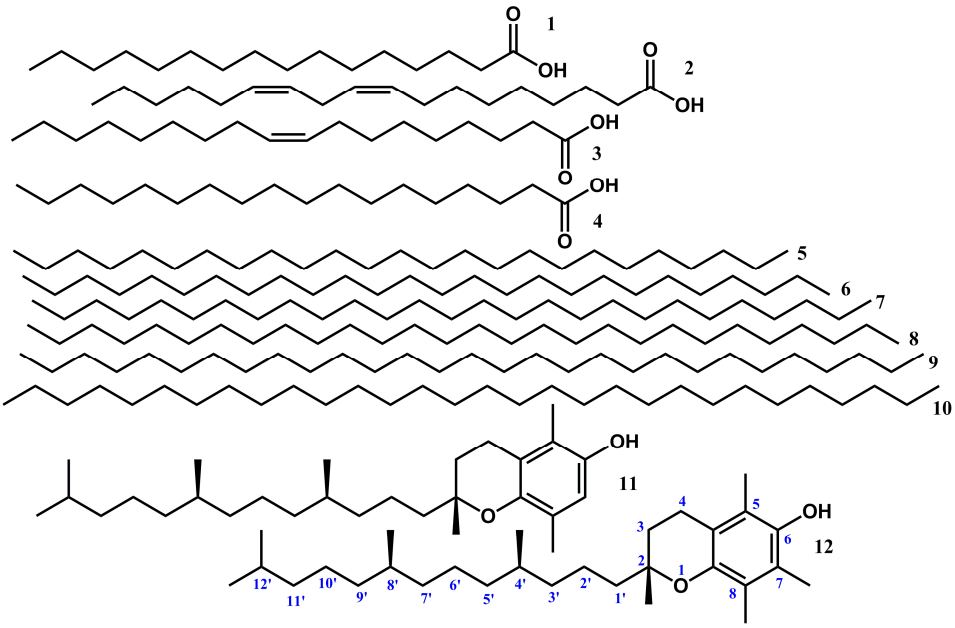

Figure 2. Structures of non-polar compounds identified from the leaves of *B. laciniosa*, *N. variegata* and *E. spectabile*. **1** *n*-Hexadecanoic acid (Palmitic acid); **2** 9*Z*,12*Z*-Octadecadienoic acid; **3** 9*Z*-Octadecenoic acid (Oleic acid); **4** Octadecanoic acid (Stearic acid); **5** *n*-Pentacosane; **6** *n*-Hexacosane; **7** *n*-Heptacosane; **8** *n*-Octacosane; **9** *n*-Nonacosane; **10** *n*-Triacontane; **11** β-Tocopherol; and **12** α-Tocopherol (Vitamin E).

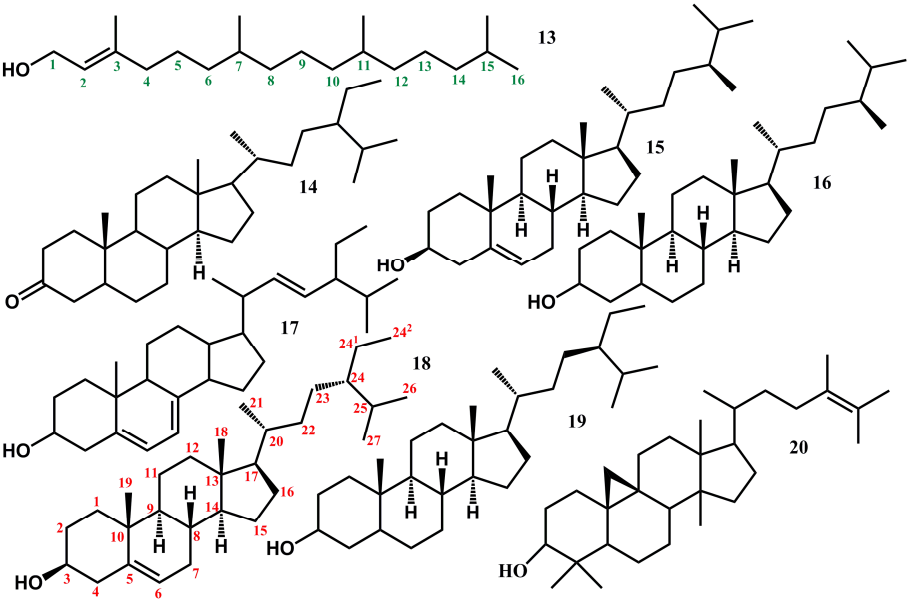

Figure 3. Structures of non-polar compounds identified from the leaves of *B. laciniosa*, *N. variegata* and *E. spectabile*. **13** Phytol; **14** Stigmastan-3-one; **15** Campesterol; **16** Ergostanol; **17** Stigmasta-4,22-dien-3-β-ol; **18** β-Sitosterol; **19** Stigmastanol; and **20** 24-Methyl-3-β -9,19-cyclolanost-24-en-3-ol.

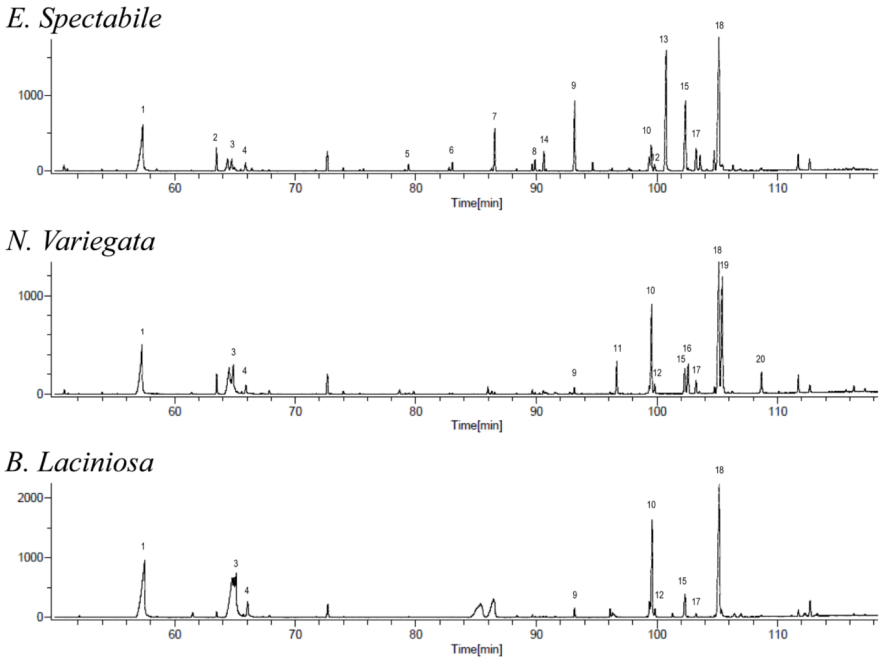

Figure 4. Comparison of TIC for *B. laciniosa*, *N. variegata* and *E. spectabile*.

Table 1. Compounds identified from hexane extracts of leaves of *B. laciniosa*, *N. variegata* and *E. spectabile*.

Nr.	Compounds According to Group	MF	Exact Mass *		Retention Time (min)			Content (%)		
			Observed	Calculated	*B. L.*	*N. V.*	*E.S.*	*B.L.*	*N.V.*	*E.S.*
	Fatty acids and their derivatives									
1	*n*-Hexadecanoic acid (Palmitic acid)	$C_{16}H_{32}O_2$	256.24110	256.24023	57.52	57.30	57.38	11.3	7.4	6.1
2	Octadeca-(9,12)dienoic acid	$C_{18}H_{32}O_2$	280.24398	280.24023			64.38			3.1
3	(9Z)-Octadec-9-enoic acid (Oleic acid)	$C_{18}H_{34}O_2$ $C_{18}H_{32}O$ [a]	n.a. 264.2434	264.24532	65.10	64.86	64.75	8.8	4.5	1.7
4	Octadecanoic acid (Stearic acid)	$C_{18}H_{36}O_2$	284.27187	284.27153	66.05	65.88	65.88	3.2	1.4	1.1
	Alkanes									
5	*n*-Pentacosane	$C_{25}H_{52}$					79.41			0.8
6	*n*-Hexacosane	$C_{26}H_{54}$					83.03			1.1
7	*n*-Heptacosane	$C_{27}H_{56}$					86.54			5.7
8	*n*-Octacosane	$C_{28}H_{58}$					89.90			1.4
9	*n*-Nonacosane	$C_{29}H_{60}$			93.16	93.14	93.18	2.0	1.0	9.3
10	*n*-Triacontane	$C_{30}H_{62}$			99.36	99.34	99.35	18.9	13.6	3.5
	Vitamins									
11	β-Tocopherol	$C_{28}H_{48}O_2$	416.36392	416.36543		96.67			4.9	
12	α-Tocopherol	$C_{29}H_{50}O_2$	430.38089	430.38108	99.60	99.56	99.52	1.8	1.5	0.9
	Phytol									
13	(2*E*,7*R*,11*R*)-3,7,11,15-Tetramethyl-2-hexadecen-1-ol (Phytol)	$C_{20}H_{40}O_1$	296.30982	296.30791			100.76			16.1
	Triterpenoids and derivatives									
14	Stigmastan-3-one	$C_{29}H_{50}O_1$	414.38688	414.38616			90.62			2.6
15	Campesterol ((3β,24*R*)-Ergost-5-en-3-ol)	$C_{27}H_{44}O_2$	400.33890	400.33413	102.33	102.33	102.37	4.5	3.9	9.4
16	Ergostanol	$C_{28}H_{50}O_1$	402.38723	402.38616		102.60			4.5	
17	Stigmasta-4,22-dien-3-β-ol	$C_{29}H_{48}O_1$	412.37123	412.37051	103.24	103.24	103.25	0.7	2.0	3.1
18	β-Sitosterol	$C_{29}H_{50}O_1$	414.38655	414.38616	105.18	105.14	105.16	25.8	19.8	17.8
19	Stigmastanol	$C_{29}H_{52}O_1$	416.40276	416.40181		105.42			17.6	
20	24-Methyl-3-β-9,19-cyclolanost-24-en-3-ol	$C_{31}H_{52}O_1$	440.40234	440.40181		108.67			3.2	
	Unidentified							23.0	14.7	16.3

Abbreviations: *B. L.* = *B. laciniosa*; *N. V.* = *N. variegata*; *E. S.* = *E. spectabile*; n.d. = not detected; MF = Molecular Formula. [a] Only the pseudomolecular ion $[M - H_2O]^+$ observed for this compound. * All exact masses are calculated for lowest monoisotopic mass.

2.1. Fatty Acids and Their Derivatives

Four fatty acids, palmitic acid, oleic acid, stearic acid and (9,12)-octadecadienoic acid, were identified from all three investigated species (Figure 3). These fatty acids are common phytochemical constituents from several plant species with significant nutritional value including wheat [24].

2.2. Very Long-Chain Alkanes (VLCA)

Very long-chain alkanes (VLCA) are considered to be of interest from a dietary point of view because their long-chain alcohol metabolites may contribute to the cholesterol-lowering effect associated with the intake of plant waxes [25]. The efficiency with which alkanes might contribute to a cholesterol-lowering effect of waxes is regulated by limited absorption and the need for hydroxylation of these compounds. Conversion to long-chain alcohols in vivo is achieved by the action of different cytochrome P450 enzymes, which hydroxylate alkanes at several positions [26]. Altogether, six very long-chain *n*-alkanes were identified (Figure 3). The identifications were initially based on the fragmentation pattern observed in the mass spectra. To further support these identifications, a standard solution containing alkanes with chain lengths varying from C_9–C_{40} was co-chromatographed with the samples. The results confirmed the presence and identities of several long-chain alkanes. The six *n*-alkanes identified in this work have a chain length ranging from C_{25} to C_{30}. *E. spectabile* differs from the two other species since the crude extract from this species contain all six *n*-alkanes from *n*-pentacosane (C_{25}) to *n*- triacontane (C_{30}). Meanwhile, only the two longest *n*-alkanes, *n*-nonacosane (C_{29}) and *n*-triacontane (C_{30}), were identified from the crude extracts from *B. laciniosa* and *N. variegata*.

The quantitative amount of the two *n*-alkanes identified from all of the species seems to be largest in the crude extract from *E. spectabile* while the amount in the crude extracts from *B. laciniosa* and *N. variegata* was much lower and at a comparable level. Seven alkanes with considerably shorter chain lengths, ranging C_{12}–C_{18}, have previously been identified from Bromeliaceae spp. [27]. Of particular importance may be that the chain lengths of the alkanes identified in *B. laciniosa*, *N. variegata* and *E. spectabile* were considerably longer than for those that have previously been reported to occur in Bromeliaceae species.

2.3. Vitamins

α-Tocopherol was present in all three investigated species (Figure 2). The discovery of the presence of an active form of vitamin E in the crude extracts from *B. laciniosa* and *E. spectabile* underlines the nutritional value of these plants as food for both humans and animals. Previously, α-tocopherol has not been identified from any plant source belonging to genus *Bromelia*. However, the vitamin has been found in the distant relative *A. erectifolius* belonging to genus *Ananas* in Bromeliaceae [28]. Moreover, another vitamin E, namely β-tocopherol (Figure 2), was identified in the crude extract of *N. variegata*.

2.4. Other Compounds

The relatively common natural product phytol (Figure 3) was identified in the crude extract from *E. spectabile*. According to Vetter et al. (2012), the presence of phytol in human food is mainly restricted to spinach, beans, raw vegetables, and asparagus [29,30]. It may be mentioned that intake of food plants containing free phytol should be restricted for individuals suffering from Refsum's disease [30].

2.5. Triterpenoids and Derivatives Therefrom

Altogether, seven triterpenoids were identified from the investigated Bromeliaceae species (Figure 4). While all seven were detected in *N. variegata*, only three (Compounds **15**, **17** and **18**) were identified in *B. laciniosa* and *E. spectabile*. With twice as many identified triterpenoids, *N. variegata* differs markedly from the two other species (Table 1). Five of the seven triterpenoids have previously been identified from other species of the family Bromeliaceae (Compounds **15**–**19**). The two triterpenoids stigmastan-3-one (Compound **14**) and 24-methyl-β-9,19-cyclolanost-24-en-3-ol

(Compound **20**) are identified from species of the family Bromeliaceae for the first time. 24-Methyl-β-9,19-cyclolanost-24-en-3-ol (Compound **20**) is also known as 24-methyl-cycloartenol. Campesterol (Compound **15**), ergostanol (Compound **16**), stigmasta-4,22-dien-3-β-ol (Compound **17**), β-sitosterol (Compound **18**) and stigmastanol (Compound **19**) have all been detected previously from species of the family Bromeliaceae. Although stigmastan-3-one (Compound **14**) has not been identified from Bromeliaceae previously the unsaturated form stigmast-4-en-3-one has previously been identified from *Ananas erectofolius* [28]. Campesterol (Compound **15**) is a common phytosterol found in many edible plants. It is therefore unsurprising that campesterol has been identified from six Bromeliaceae species. The species are *A. comosus* [31], *A. erectofolius* [28], *Tillandsia fasciculata* [32], *Tillandsia pohliana* (*T. pohliana* has been examined by Caiado and co-workers in an unpublished work according to Manetti et al. [33]), *Tillandsia streptocarpa* [34] and *T. usneoides* [35]. Ergostanol (Compound **16**) has previously been identified from the two species *A. comosus* [31] and *A. erectofolius* [28] from the family Bromeliaceae. Stigmasta-4,22-dien-3-β-ol (Compound **17**) is an unsaturated derivative of stigmastanol, which is known to inhibit absorption of cholesterol from the diet. Stigmasta-4,22-dien-3-β-ol has previously been identified from the three species *T. fasciculata* [32], *T. streptocarpa* [34] and *T. usneoides* [35]. All three species belongs to *Tillandsia,* a genus of the Bromeliaceae family. β-Sitosterol (Compound **18**) is the major compound from the non-polar (hydrophobic) extracts in all three investigated species. β-Sitosterol (Compound **18**) is a phytosterol with wide distribution throughout the plant kingdom including several plants used for human nutrition. Several dietary plant sterols including β-sitosterol exhibit significant cholesterol-lowering effects [36–38]. In the Bromeliaceae family β-sitosterol has been identified from the eight species: *A. comosus* [31,39] commonly known as pineapple, *A. erectofolius* [28], *Hechtia rosea* [40], *H. scariosa* [40], *T. fasciculata* [32], *T. pohliana* [33], *T. streptocarpa* [34], *T. usneoides* [35,41]. Stigmastanol (Compound **19**) is previously known from two species of the Bromeliaceae family, namely *A. comosus* [31] and *A. erectofolius* [28]. Stigmastanol is also known as sitostanol and is a phytosterol commonly found in many edible plants. 9,19-Cyclolanost-24-en-3-ol-3-β (Compound **20**) is not previously identified from species of the Bromeliaceae family. However, similar cycloartanol triterpenoids such as cyclolaudenol [32] and 24-methylenecycloartanol [35,42] are commonly found in Bromeliaceae species [33]. Identification of phytosterols with documented potential beneficial health effects, such as campesterol and β-sitosterol, from the leaves of *B. laciniosa* and *E. spectabile* strengthens the nutritional value of these plants.

2.6. NMR Plant Metabolomics

Even though GC-HRMS is an excellent method for characterizing mixtures of natural products, there are some limitations in connection with the application of this method. Some compounds may avoid detection because they are either not sufficiently volatile or insufficiently ionized. To further support the identifications of natural products achieved by GC-HRMS, we proceeded with a NMR metabolomics strategy by directly analyzing the dried extract of *N. variegata* (when dissolved in deuterated chloroform) on 600 MHz NMR equipped with a cryogenic probe, without any requirements for further workup of the sample. NMR plant metabolomics is, among others, a complementary strategy for identification of known plant metabolites of plant-derived extracts and allows for the detection of signals of all compounds present in the sample at sufficient quantities to be detected. Recent development in cryoprobe technology has made it possible to characterized complex natural products with concentrations as low as in the micromolar range. In current literature, NMR spectroscopy has been successfully applied in the evaluation of metabolites of plant extracts (NMR plant metabolomics) [43]. NMR metabolomics has gained importance because this strategy provides insight into complex systems of mixtures of natural products occurring at their natural relative abundance. NMR is able to provide a "holistic view" of the metabolites under certain conditions, and thus is advantageous for metabolomic studies [44]. Although most publications about NMR metabolomics, including plant metabolomics, only include application of 1D ^{1}H NMR (reviewed by Kim et al. 2011 [44]), several applications of 2D NMR exist in plant metabolomics [45]. Recent

development in cryoprobe technology has led to a four-fold increase of sensitivity and thus a 16-fold reduction of experiment time for 2D inverse experiments compared with those for similar NMR experiments recorded on analogous instruments equipped with conventional probes. This allows for applications of a broad selection of 2D NMR spectroscopic experiments, which are now accessible within an acceptable time scale of approximately 15–30 min per experiment.

To support the identifications achieved with GC-HRMS, 1D ^{1}H (Figure S1) and the 2D NMR experiments 2D ^{1}H-^{13}C Heteronuclear Single Quantum Coherence (HSQC), 2D ^{1}H-^{13}C Heteronuclear Multiple Bond Correlation (HMBC) (Figure S2), 2D ^{1}H-^{13}C Heteronuclear Single Quantum Coherence-Total Correlation Spectroscopy (HSQC-TOCSY), 2D ^{1}H-^{13}C Heteronuclear 2 Bond Correlation (H2BC), 2D ^{1}H-^{1}H Correlation Spectroscopy (COSY) and 2D ^{1}H-^{1}H Rotating frame Overhauser enhancement spectroscopy (ROESY) of the dried hexane extract of *B. laciniosa* dissolved in deuterated chloroform were recorded. The same NMR experiments were also recorded on samples of pure β-sitosterol, stigmasterol, α-tocopherol and phytol. The combined information from the 2D ^{1}H-^{13}C edited HSQC, HSQC-TOCSY, HMBC and H2BC were particularly helpful for assignment of ^{1}H and ^{13}C signals of both reference compounds (Tables S1–S3) and the analogous signals belonging to the mixture containing these compounds comprising the extract sample. The overlaid 2D NMR spectra of extract of *N. variegata* and pure β-sitosterol, α-tocopherol and phytol confirmed the presence of these compounds in the plant extract and allowed for identifications of individual signals (Figure 5). The multidimensional NMR data provided supportive evidence for the presence of the above-mentioned compounds, as well as significant amounts of long-chain alkanes and fatty acids identified by hyphenated GC-HRMS in *N. variegata* (Table 1).

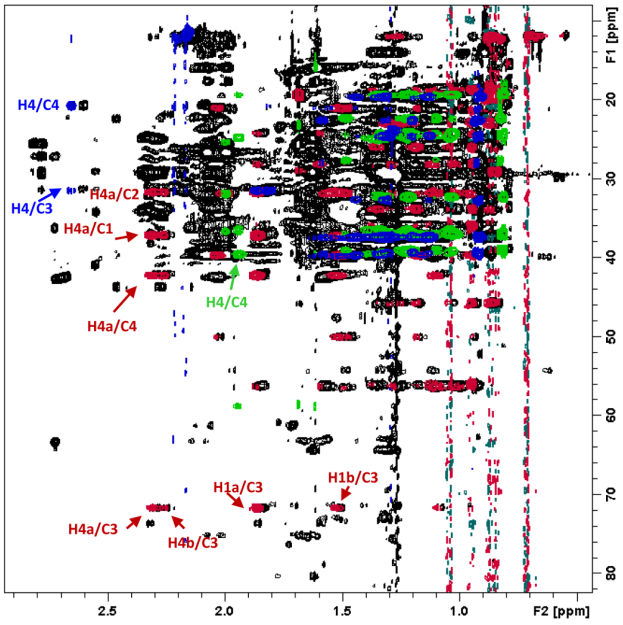

Figure 5. Expanded region of the superimposed 2D ^{1}H-^{13}C HSQC-TOCSY NMR spectra of *N. variegata* (black signals), β-sitosterol (red signals), phytol (green signals) and α-tocopherol (blue signals). Selected crosspeaks are assigned. Complete assignments are presented in Tables S1–S3. Notice that the characteristic signal patterns of the pure standard compounds matches the corresponding signals of the same compounds present as part of mixture comprising the extract of *N. variegata*.

3. Materials and Methods

3.1. Plant Material

Leaves of *B. laciniosa*, *E. spectabile* and *N. variegata* were collected within the municipality borders of Petrolina, Pernambuco, Brazil, in January 2013. Voucher specimens were deposited in the Herbarium Vale do São Francisco (HVASF) of the Federal University of Vale do São Francisco. The site for collecting the leaves of *B. laciniosa* was at 08°59′16.90″ S and 40°35′20.60″ W and the voucher specimen is No. 6442. The leaves of *E. spectabile* were collected at the coordinates 09°07′54.30″ S and 40°26′21.00″ W and the voucher specimen is No. 6443. *N. variegata* leaves were collected at the coordinates 08°59′16.90″ S and 40°35′20.60″ W and the voucher specimen is No. 6441. Identification of the collected plant species was done by the botanist André Paviotti Fontana from Centro de Recuperação de Áreas Degradadas da Caatinga (CRAD). Prior to shipment to Norway the leaves were dried in an oven with air circulation at a temperature of 50 °C for seven days. After drying, the plant materials were powdered in a mill.

3.2. Extraction and Concentration

Dried and pulverized leaves of *B. laciniosa* (100.86 g), *E. spectabile* (100.22 g) and *N. variegata* (100.49 g) were separately macerated in 800 mL of hexane for 89 h at room temperature. After extraction, solutions were filtered through glass wool before being concentrated under reduced pressure on a rotary evaporator. The volumes were reduced to 140 mL (*B. laciniosa*), 75 mL (*E. spectabile*) and 145 mL (*N. variegata*), respectively. Before examination by GC-FID and GC-HRMS the concentrated samples were filtered through a 0.45 μm Micropore Membrane Filter.

3.3. GC-FID

To optimize conditions for the GC-MS analysis, GC-FID was performed. These investigations of the composition and concentration of the extracts were performed on a Gas Chromatograph (GC) with a Flame Ionization Detector (FID). A Trace GC Ultra instrument (Thermo Electron Corporation S.p.A., Milan, Italy) fitted with an Ultra 1 column (crosslinked methyl siloxane, ID = 0.200 mm, L = 25 m, film thickness = 0.33 μm) (Santa Clara, CA, USA). Samples were dissolved in hexane and splitless mode was used for injection. The applied temperature gradient (initial temperature = 50 °C, holding for 2.5 min, then heating at 20 °C/min to 100 °C, holding for 10 min, finally heating at 2 °C/min to 300 °C applying a 15 min holding time provided both good separation of the compounds and the necessary information about the concentration of the extracts. Helium was used as carrier gas with a flow rate of 0.7 mL/min. An injector temperature of 260 °C was used.

3.4. GC-MS (TOF)

All samples were analyzed on an AccuTOF T100GC mass spectrometer from JEOL Ltd. (Tokyo, Japan) interfaced with an Agilent 6890 N gas chromatograph (Santa Clara, CA, USA). Samples dissolved in hexane were injected on a VF-50 MS GC column from Varian Inc. (Palo Alto, CA, USA), (silica column (5% phenyl)-metylpolysiloxane, ID = 0.200 mm, L = 25 m, film thickness = 0.33 μm) using splitless injection at 250 °C (injector temperature). Helium (5.0) was used as carrier gas at a constant gas flow rate of 0.7 mL/min (υ = 33.0 cm/s), and the following GC temperature program was applied; initial temperature = 40 °C, holding for 2.5 min, then heating at 20 °C/min to 100 °C, holding for 10 min, finally heating at 2 °C/min to 300 °C applying a 15 min holding time. The GC-MS interface was heated to 260 °C introducing the column flow into the electron ionization source four minutes after injection. The ion source operated at 260 °C generating positive ions at an ionization potential and ionization current of 70 eV and 300 μA, respectively. Settings for the time of flight mass analyser were optimized for ions in the mass range 40–800 amu, acquiring mass spectra after the following acquisition settings; spectral recording interval = 0.3 s, wait time = 0.005 s, spectra accumulation time = 0.295 s and data sampling interval = 1 ns. A total ion chromatogram (TIC) was acquired during the whole GC run and mass spectra were generated and transformed to centroided spectra using baseline correction

and smoothing using a weighted moving average. All mass spectra were calibrated against one of several polysiloxane background ions (m/z = 147.03290, 207.03290, 281.05169 or 355.07048) originating from the column and acquired during the same set of experiments. The relative quantities of each compound were calculated based on the peak heights in the total ion chromatogram. Mass spectral fragmentation patterns of individual compounds was compared with that of analogous standard compounds in NIST standard reference Mass Spectral library. Absolute configurations are based on mass spectral matches against known library mass spectra (NIST07), when applicable. Individual mass spectra are shown in Figures S3–S16.

3.5. NMR Spectroscopy

NMR spectroscopy was performed on samples of 19.1 mg dried heptane extract of *B. laciniosa* and on 164.9 mg dried hexane extract of *N. variegata*. In addition to pure samples of 20.0 mg of β-sitosterol, stigmasterol, 25 volume % of phytol and 76.2 mg of α-tocopherol, each individual sample was dissolved in, or mixed with, 0.75 mL chloroform-D. The 1D ^{1}H and the 2D ^{1}H-^{1}H COSY, 2D ^{1}H-^{1}H ROESY, the 2D ^{1}H-^{13}C HSQC, the 2D ^{1}H-^{13}C Edited HSQC, the 2D ^{1}H-^{13}C HSQCTOCSY, the 2D ^{1}H-^{13}C HMBC and the 2D ^{1}H-^{13}C H2BC NMR experiments were recorded on a Bruker Avance 600 MHz spectrometer (Bruker BioSpin AG, canton of Zürich, Switzerland) equipped with a ^{1}H-^{13}C-^{15}N triple resonance cryoprobe at 298 K. All 2D NMR experiments were recorded without spinning.

4. Conclusions

Using a combination of hyphenated GC-HRMS and NMR plant metabolomics, 20 natural products have been identified in the drought-resistant plants *B. laciniosa*, *N. variegata* and *E. spectabile* for the first time. A total of 13 natural products including six triterpenoids were identified from *N. variegata*. From the edible leaves of *B. laciniosa* and *E. spectabile*, 9 and 16 natural products were identified, respectively. The presence of significant amounts of vitamin E in leaves of *B. laciniosa* and *E. spectabile*, as well as nutrients such as fatty acids, and phytosterols with well documented potential beneficial health effects, as well as the absence of compounds with significant toxicity, underlines the nutritional values of the plants as food sources for humans and livestock.

Supplementary Materials: Supplementary materials are available online.

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Sample Availability: Samples of the compounds phytol, β-sitosterol and α-tocopherol are available from the authors.

Supplementary data

Non-polar natural products from *Bromelia laciniosa*, *Neoglaziovia variegata* and *Encholirium spectabile* (Bromeliaceae)

Ole Johan Juvik[a], Bjarte Holmelid[a], George W. Francis[a], Heidi Lie Andersen[b], Ana Paula de Oliveira[c], Raimundo Gonçalves de Oliveira Júnior[c], Jackson Roberto Guedes da Silva Almeida[c], Torgils Fossen[a]*

Table S1. ^{1}H and ^{13}C NMR data of β-sitosterol in $CDCl_3$ at 298K.

	^{1}H ppm	^{13}C ppm
1a	1.83	37.3
1b	1.06	
2a	1.81	31.7
2b	1.48	
3	3.50	71.8
4a	2.26	42.3
4b	2.21	
5		140.8
6	5.33	121.7
7a	1.96	31.8
7b	1.50	
8	1.43	32.0
9	0.91	50.1
10		36.5
11	1.46	21.2
12a	1.99	39.9
12b	1.13	
13		42.2
14	0.97	56.7
15a	1.56	24.3
15b	1.05	
16a	1.82	28.3
16b	1.24	
17	1.08	56.0
18	0.66	11.9
19	0.99	19.3
20	1.33	36.1
21	0.90	18.7
22a	1.48	21.2
22b	1.44	
23	1.14	26.0
24	0.91	45.8
24^{1}a	1.25	23.2
24^{1}b	1.20	
24^{2}	0.83	12.0
25	1.64	29.2
26	0.79	18.0
27	0.81	19.7

Table S2. ^{1}H and ^{13}C NMR data of α-tocopherol in $CDCl_3$ at 298K.

	^{1}H ppm	^{13}C ppm
2		74.5
3a	1.82	31.5
3b	1.77	
4	2.61	20.8
5		118.4
6		144.5
7		121.0
8		122.6
9		145.5
10		117.3
1a’	1.58	39.8
1b’	1.51	
2a’	1.46	21.0
2b’	1.39	
3’	1.79	37.5
4’	1.41	32.7
5a’	1.26	37.4
5b’	1.09	
6a’	1.26	37.4
6b’	1.09	
7a’	1.26	37.4
7b’	1.09	
8’	1.38	32.8
9a’	1.26	37.4
9b’	1.09	
10a’	1.32	24.7
10b’	1.24	
11’	1.16	39.4
12’	1.54	27.9
2-Me	1.24	23.8
5-Me	2.12	11.2
6-OH	4.20	
7-Me	2.17	12.2
8-Me	2.12	11.8
4’-Me	0.87	19.7
8’-Me	0.86	19.8
12’-Me	0.88	22.6
12’-Me	0.88	22.5

Table S3. ^{1}H and ^{13}C NMR data of phytol in $CDCl_3$ at 298K.

	^{1}H ppm	^{13}C ppm
1	4.08	59.0
2	5.35	123.4
3		139.7
4	1.94	39.7
5	1.34	25.0
6a	1.22	36.5
6b	1.03	
7	1.34	32.4
8a	1.21	37.1
8b	1.03	
9	1.22	24.4
10a	1.21	37.1
10b	1.03	
11	1.34	32.4
12a	1.21	37.1
12b	1.03	
13	1.21	24.6
14	1.09	39.1
15	1.48	27.7
16	0.82	22.4
17	0.82	22.4
18	0.81*	19.6
19	0.80*	19.6
20	1.61	15.9

*assignment may be reversed

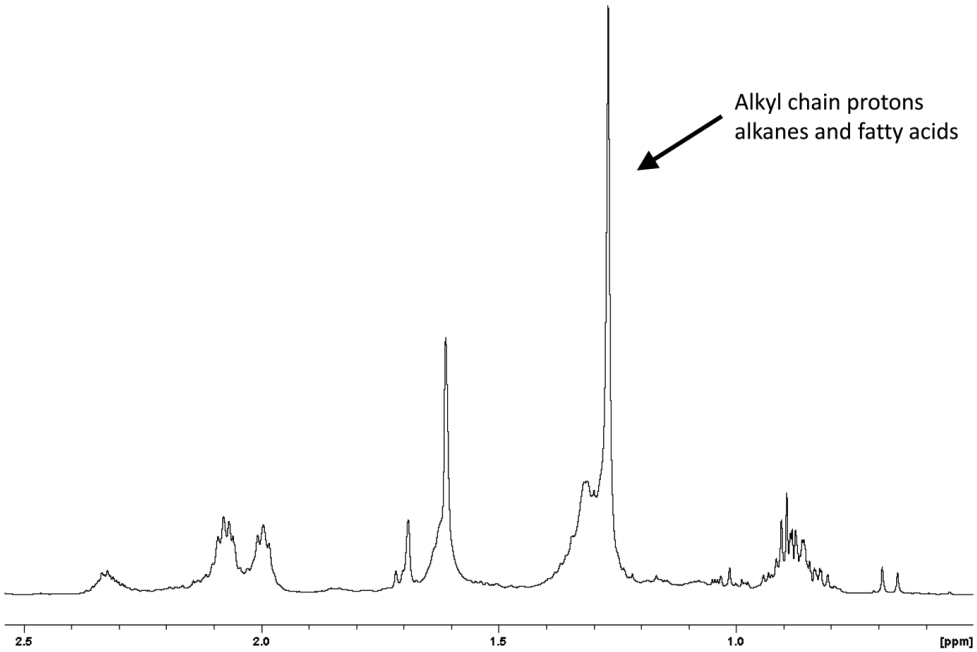


Figure S1A. Expanded region of the 1D ^{1}H NMR spectrum of hexane extract of *N. variegata*. The signal accounting for the majority of the alkyl protons of alkanes and fatty acids is highlighted.

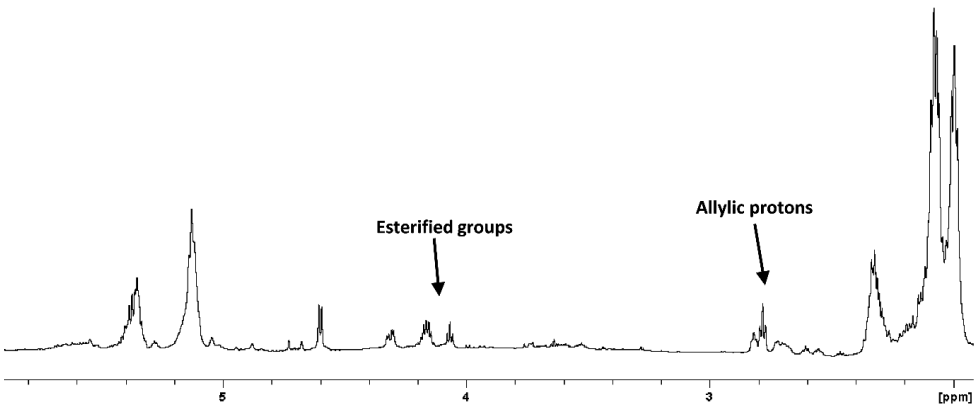


Figure S1B. Expanded region of the 1D ^{1}H NMR spectrum of hexane extract of *N. variegata* including the regions for signals belonging to esterified groups and allylic protons, respectively.

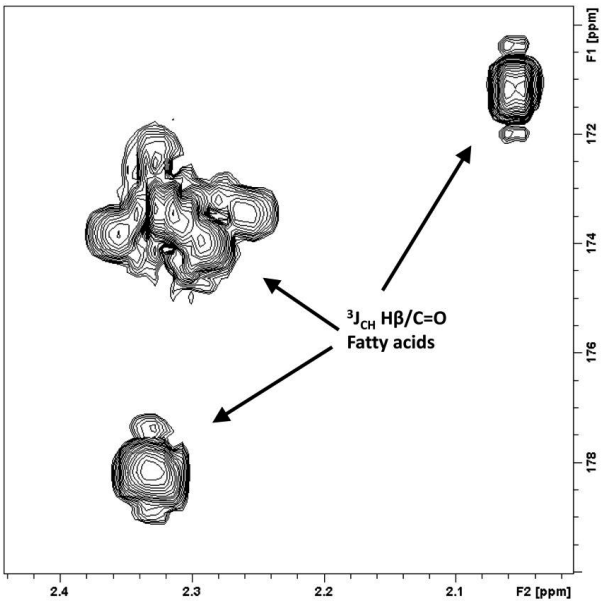
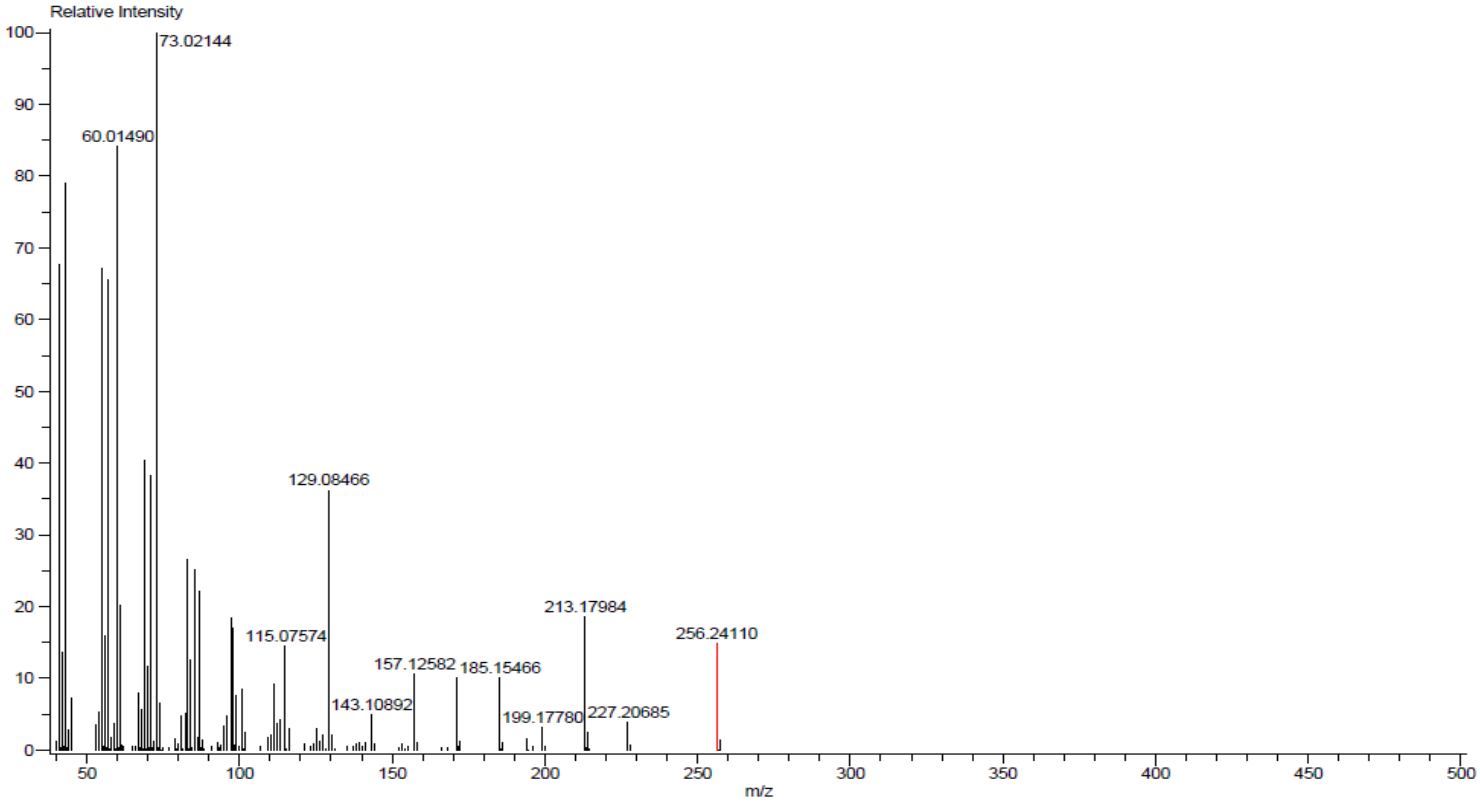
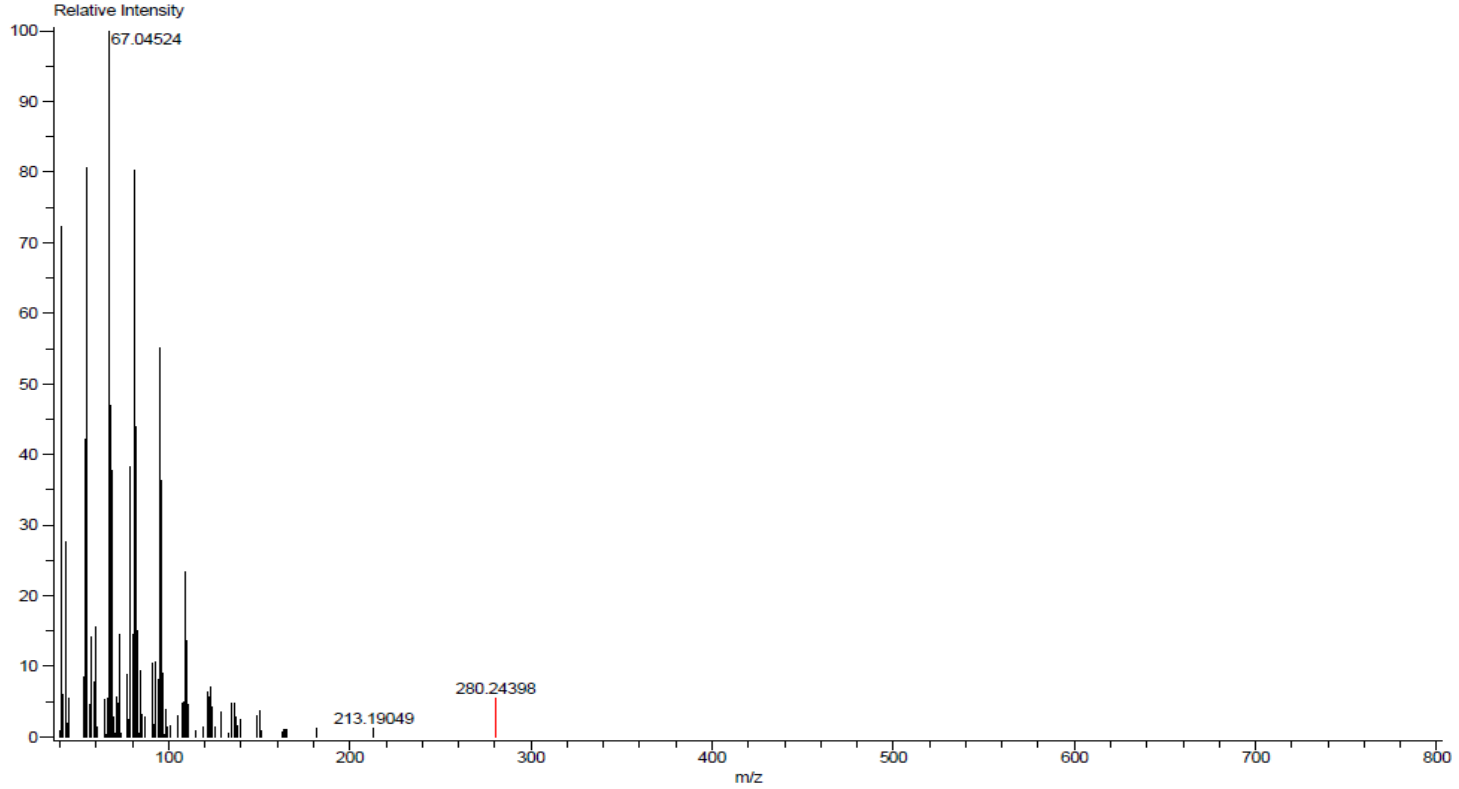


Figure S2. Expanded region of the 2D ^{1}H-^{13}C HMBC spectrum of hexane extract of *N. variegate* showing correlations between fatty acid carbonyls and their adjacent Hβ. The two groups of signals at ~171.6-174 ppm and ~178.2 ppm, respectively, may be accounted for by the presence of esterified and free fatty acids, respectively.



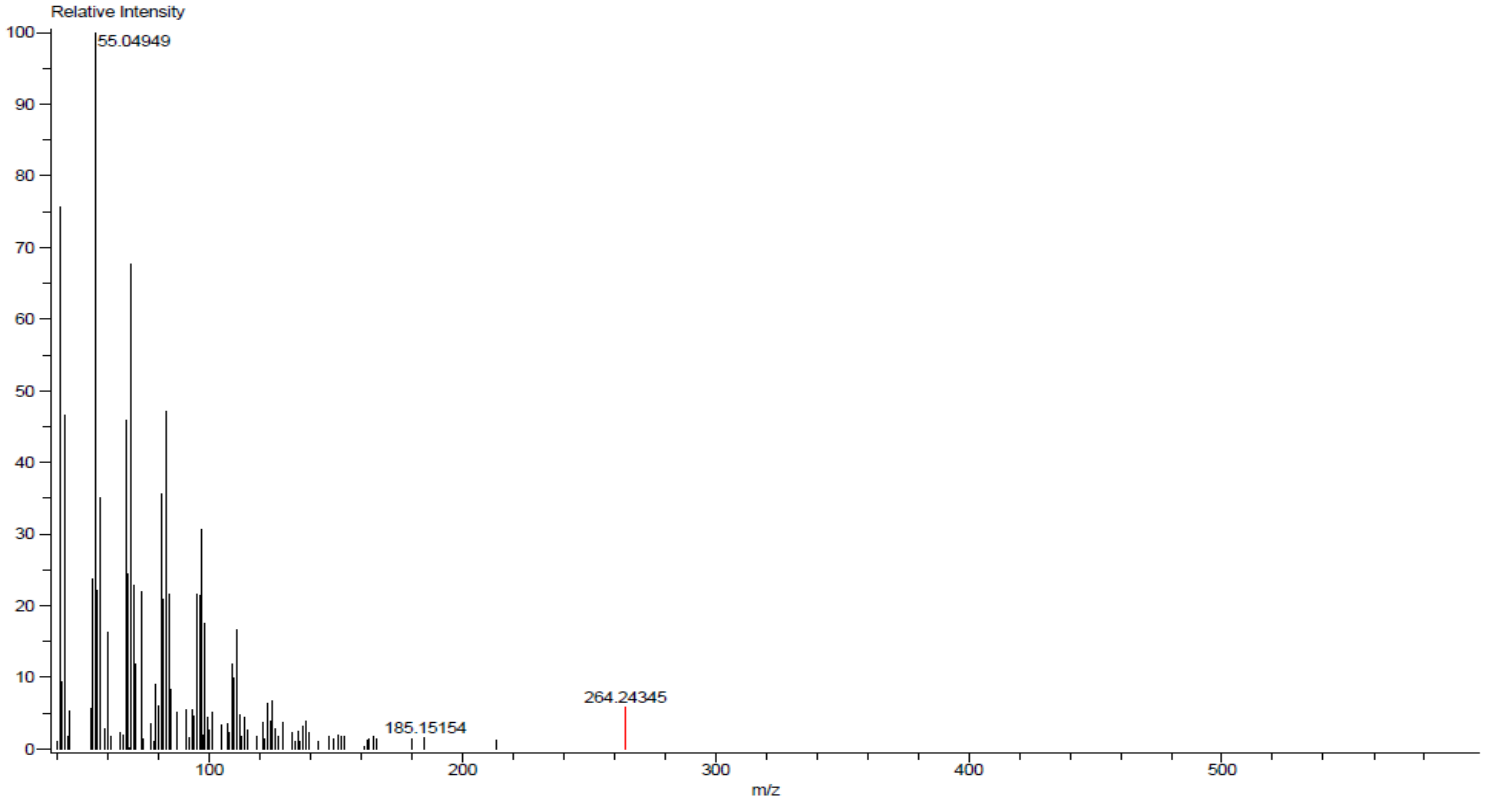
Mass	Intensity	Calc. Mass	Mass Difference (mmu)	Possible Formula	Unsaturation Number
256.24110	8781.95	256.24023	0.87	$^{12}C_{16}{}^{1}H_{32}{}^{16}O_2$	1.0

Figure S3. Mass spectrum of *n*-Hexadecanoic acid (Palmitic acid) (**1**)



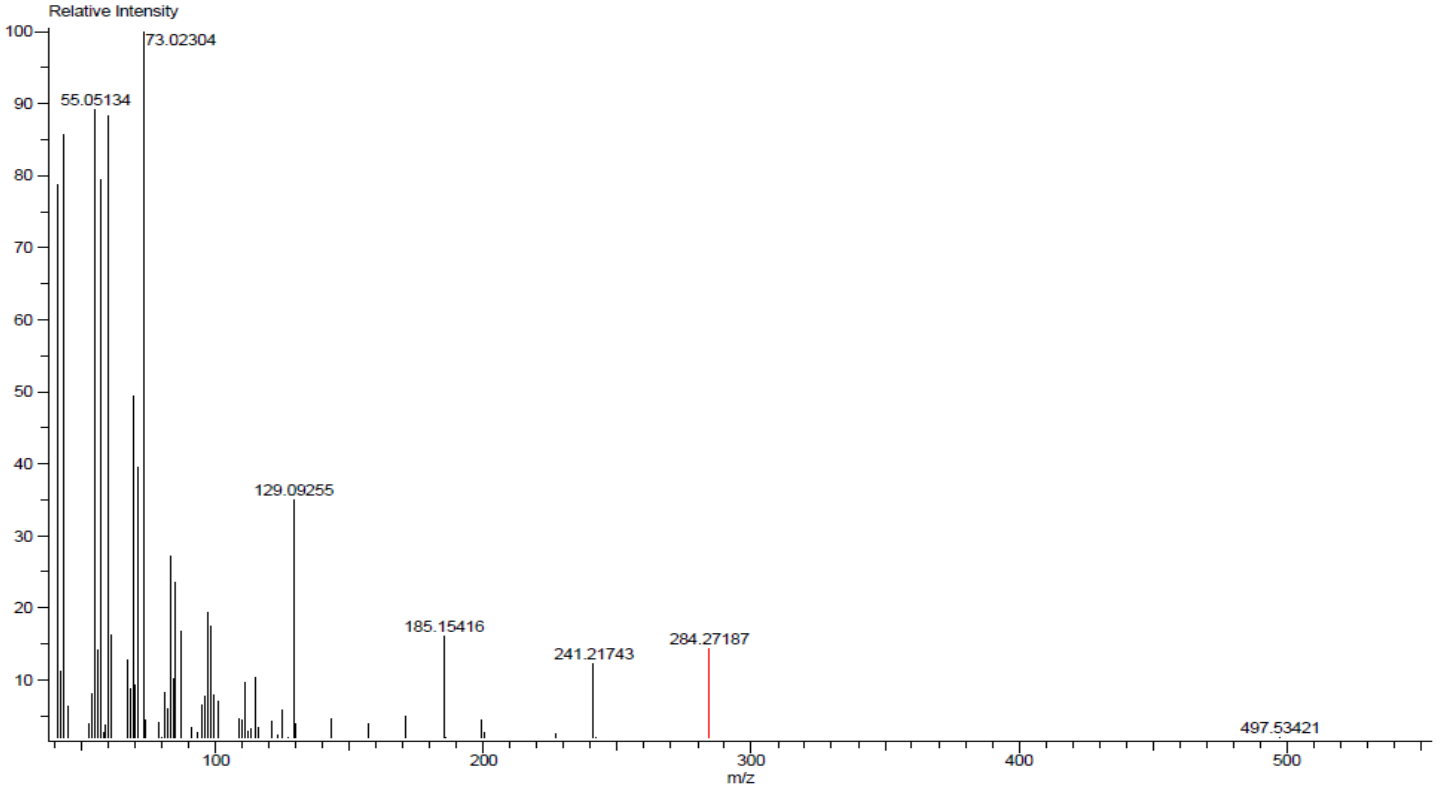
Mass	Intensity	Calc. Mass	Mass Difference (mmu)	Possible Formula	^{12}C	^{1}H	^{16}O	Unsaturation Number
280.24398	1027.48	280.24023	3.75	$^{12}C_{18}{}^{1}H_{32}{}^{16}O_2$	18	32	2	3.0

Figure S4. Mass spectrum of Octadecan-(9,12)-dienoic acid (**2**)



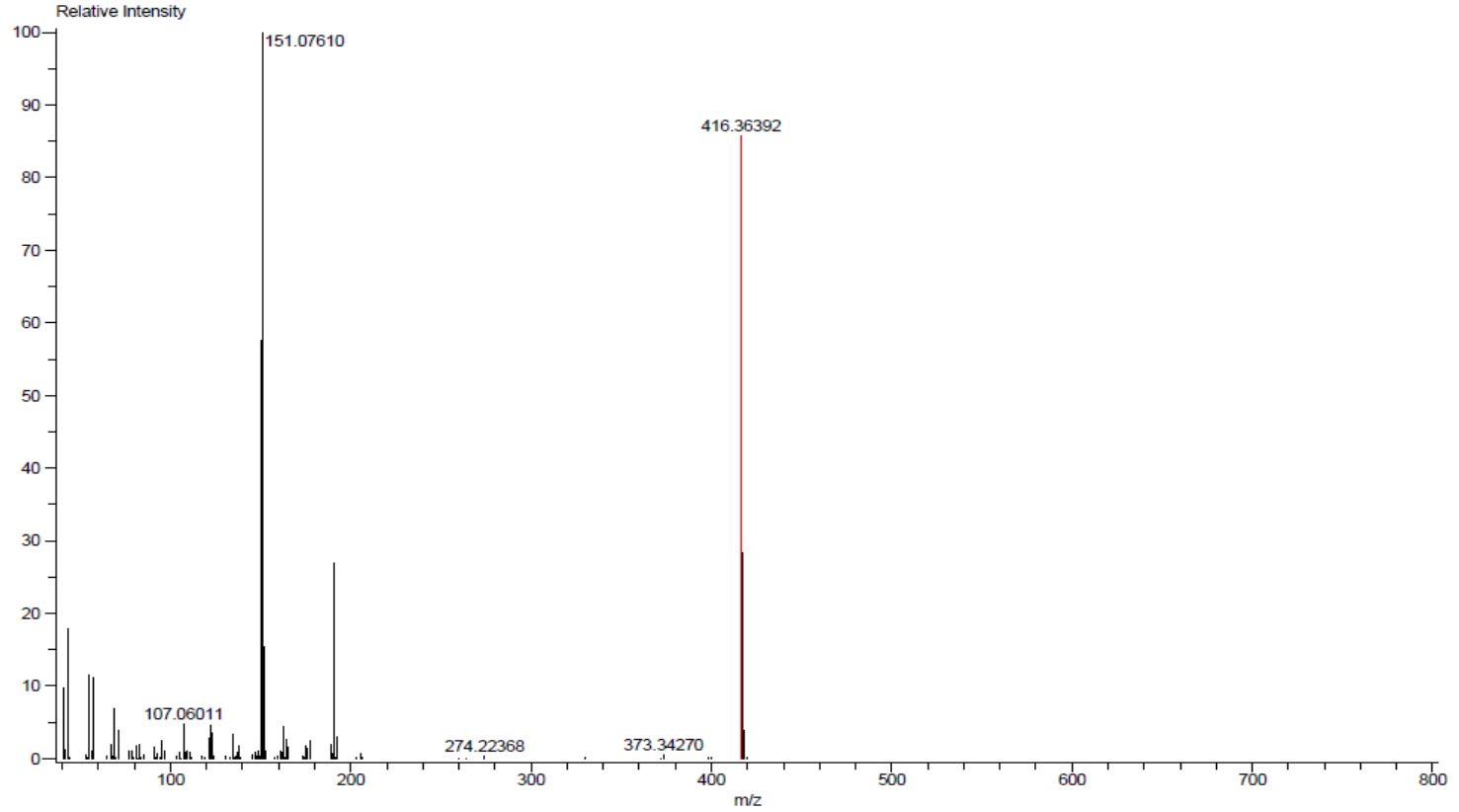
Mass	Intensity	Calc. Mass	Mass Difference (mmu)	Possible Formula	Unsaturation Number
264.24345	1240.14	264.24531	-1.86	$^{12}C_{18}{}^{1}H_{32}{}^{16}O_{1}$	3.0

Figure S5. Mass spectrum of (9*Z*)-Octadec-9-enoic acid (Oleic acid) (**3**)



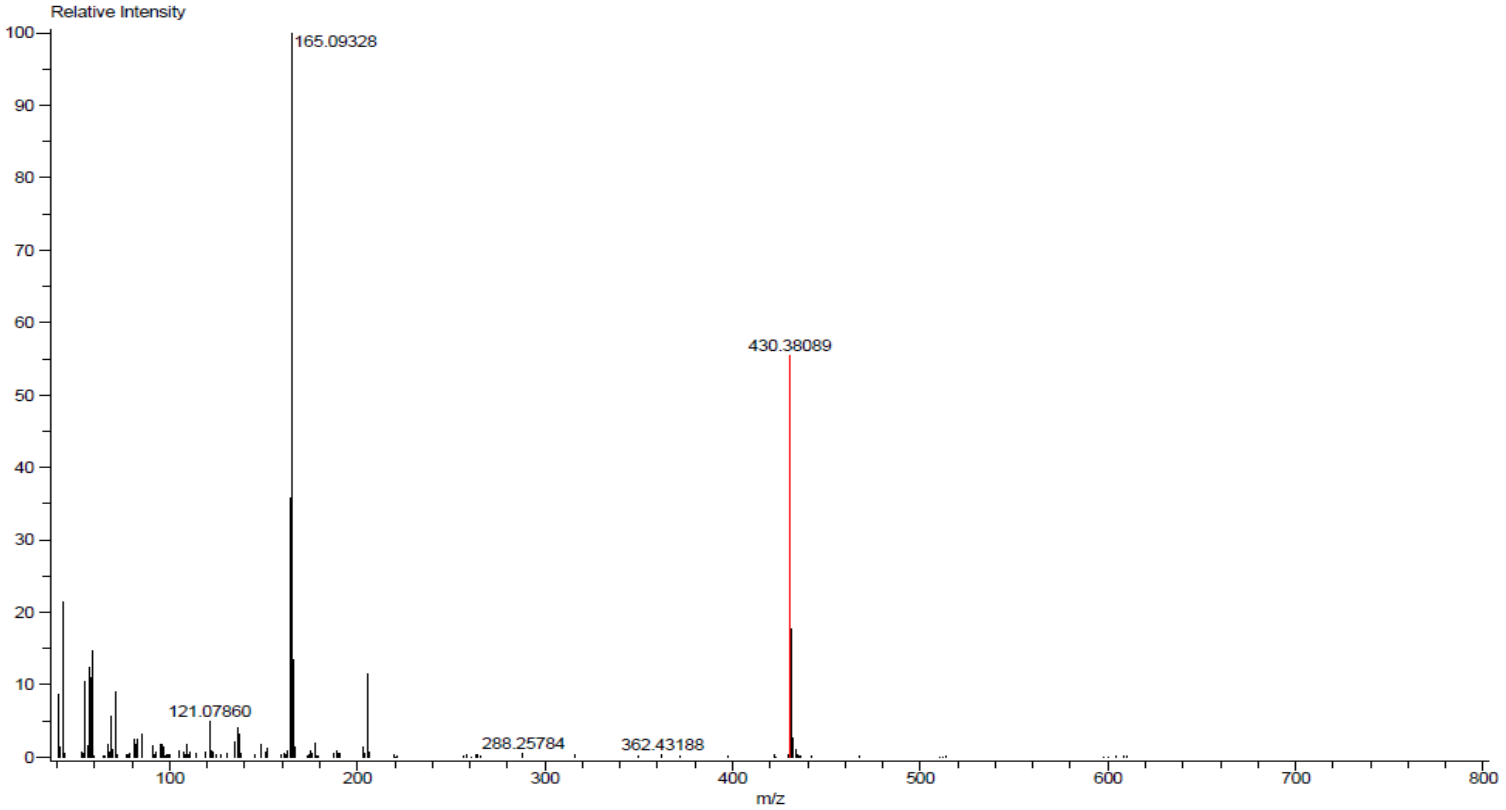
Mass	Intensity	Calc. Mass	Mass Difference (mmu)	Possible Formula	Unsaturation Number
284.27187	1780.55	284.27153	0.34	$^{12}C_{18}{}^{1}H_{36}{}^{16}O_2$	1.0

Figure S6. Mass spectrum of Octadecanoic acid (Stearic acid) (**4**)



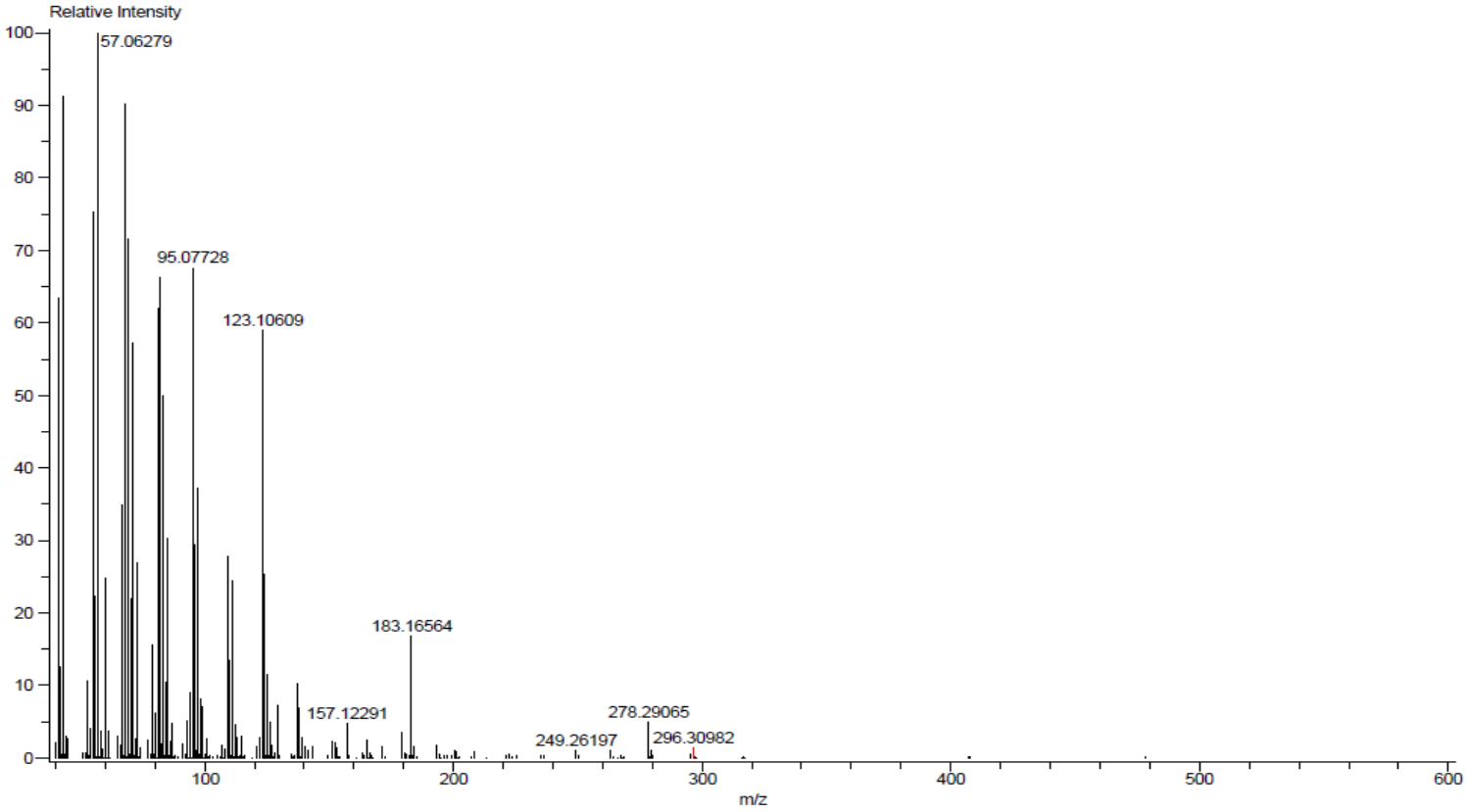
Mass	Intensity	Calc. Mass	Mass Difference (mmu)	Possible Formula	Unsaturation Number
416.36392	66051.91	416.36543	-1.51	$^{12}C_{28}{}^{1}H_{48}{}^{16}O_2$	5.0

Figure S7. Mass spectrum of β-Tocopherol (**11**)



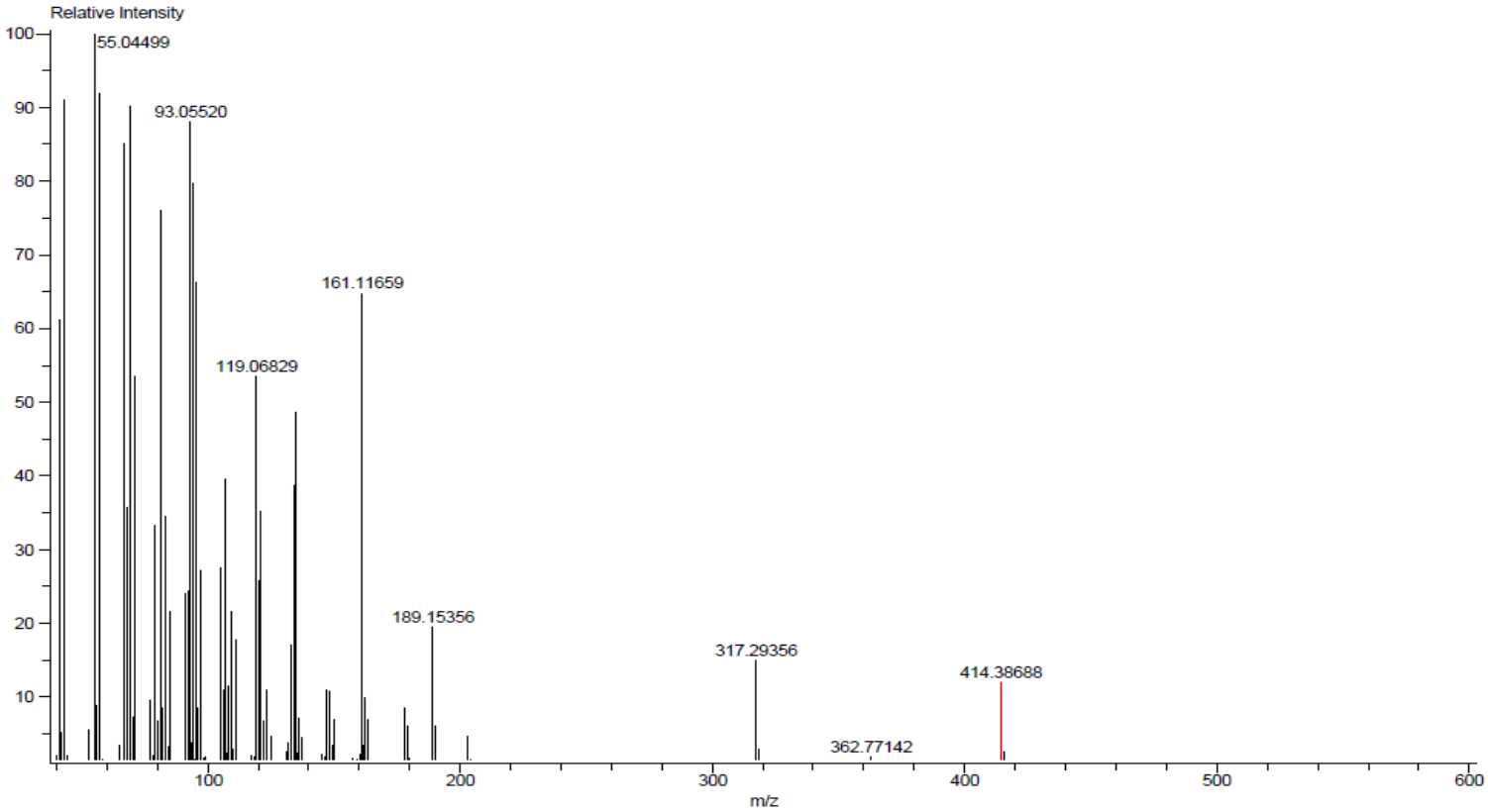
Mass	Intensity	Calc. Mass	Mass Difference (mmu)	Possible Formula	Unsaturation Number
430.38089	52136.45	430.38108	-0.19	$^{12}C_{29}{}^{1}H_{50}{}^{16}O_2$	5.0

Figure S8. Mass spectrum of α-Tocopherol (Vitamin E) (**12**)



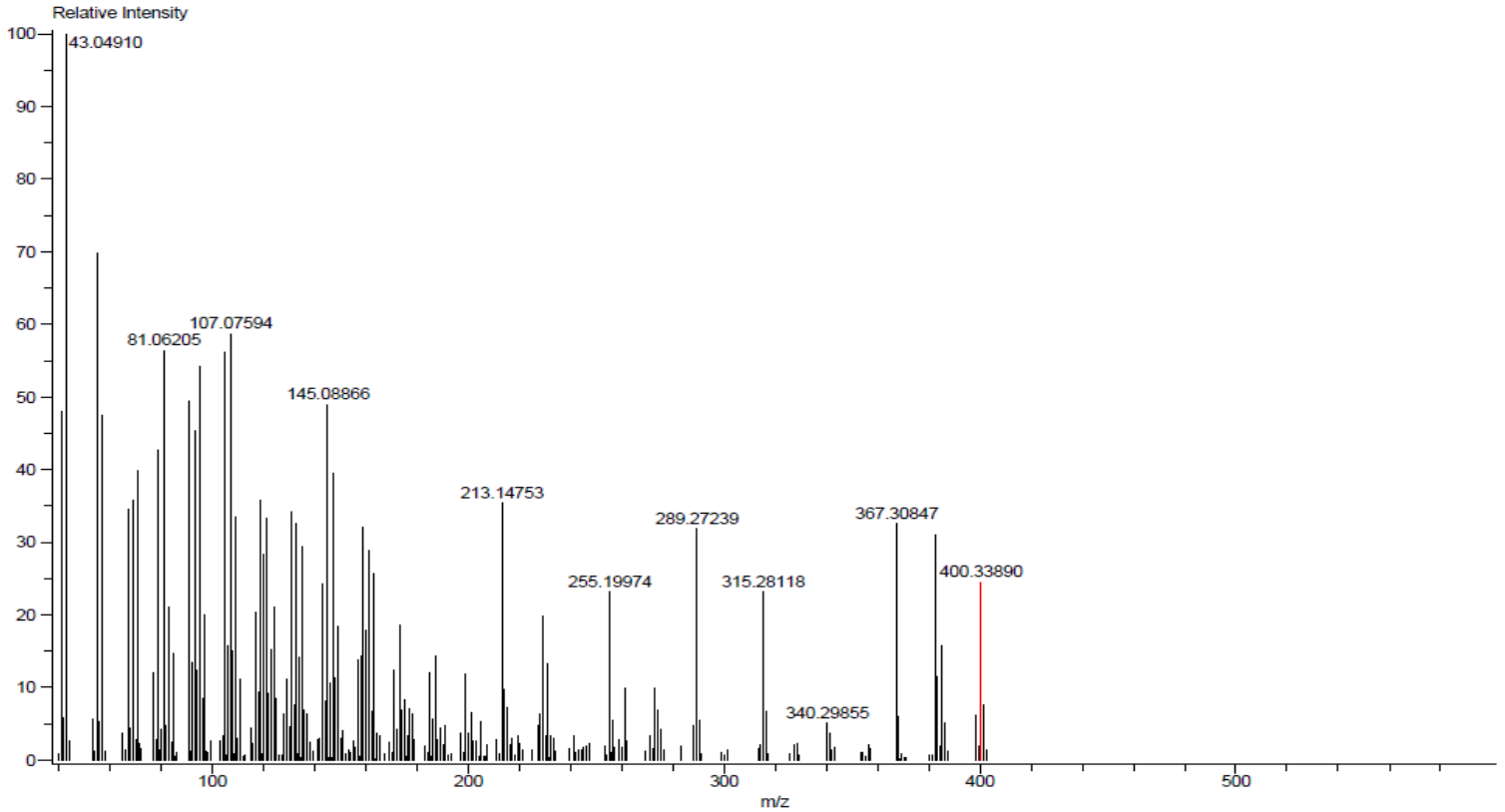
Mass	Intensity	Calc. Mass	Mass Difference (mmu)	Possible Formula	Unsaturation Number
296.30982	1582.69	296.30791	1.90	$^{12}C_{20}{}^{1}H_{40}{}^{16}O_{1}$	1.0

Figure S9. Mass spectrum of (2*E*,7*R*,11*R*)-3,7,11,15-Tetramethyl-2-hexadecen-1-ol (Phytol) (**13**)



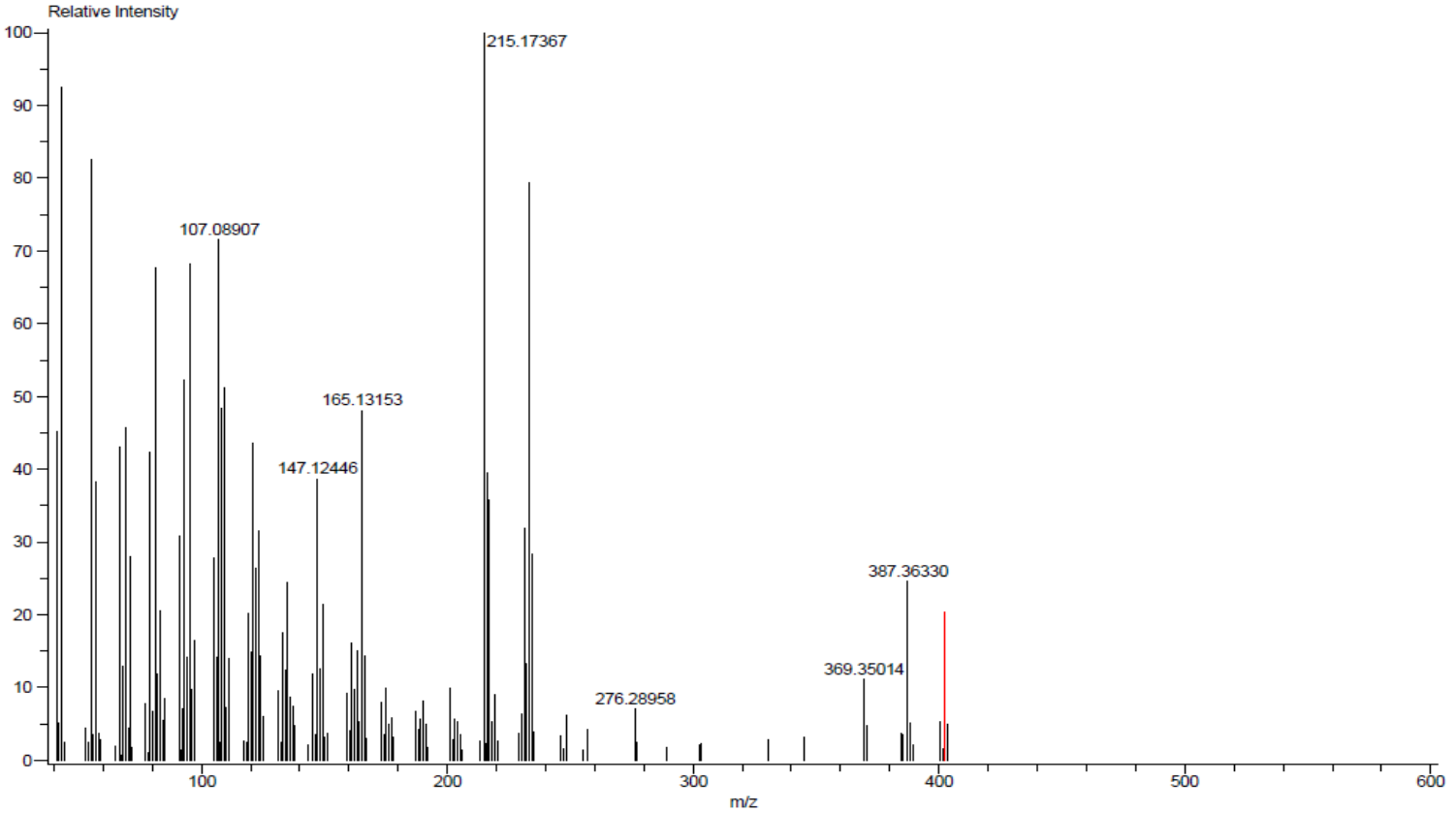
Mass	Intensity	Calc. Mass	Mass Difference (mmu)	Possible Formula	^{12}C	^{1}H	^{16}O	Unsaturation Number
414.38688	2122.34	414.38616	0.72	$^{12}C_{29}{}^{1}H_{50}{}^{16}O_{1}$	29	50	1	5.0

Figure S10. Mass spectrum of Stigmastan-3-one (**14**)



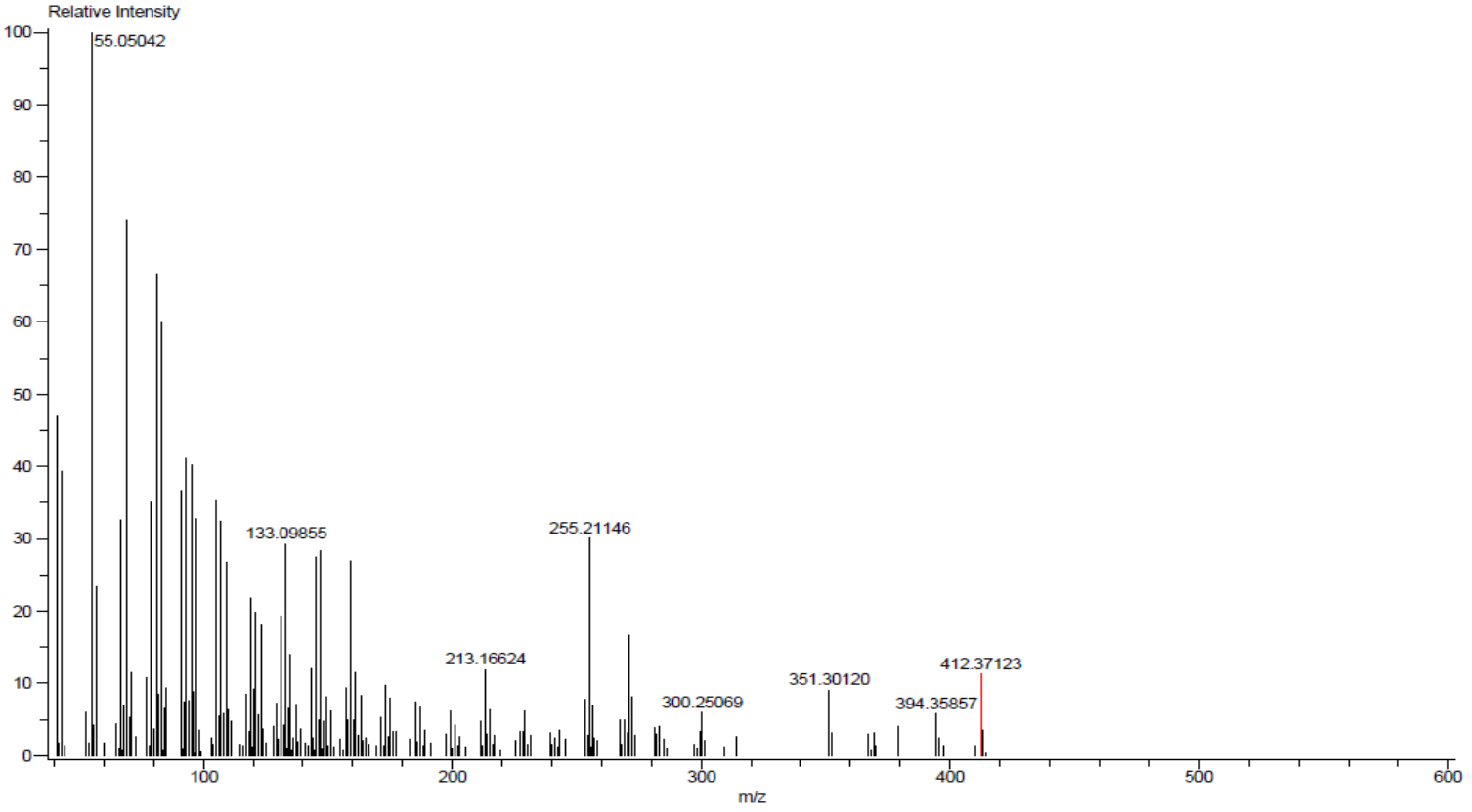
Mass	Intensity	Calc. Mass	Mass Difference (mmu)	Possible Formula	Unsaturation Number
400.33890	8900.78	400.33413	4.77	$^{12}C_{27}{}^{1}H_{44}{}^{16}O_2$	6.0

Figure S11. Mass spectrum of (3β, 24R) Ergost-5-en-3-ol (Campesterol) (**15**)



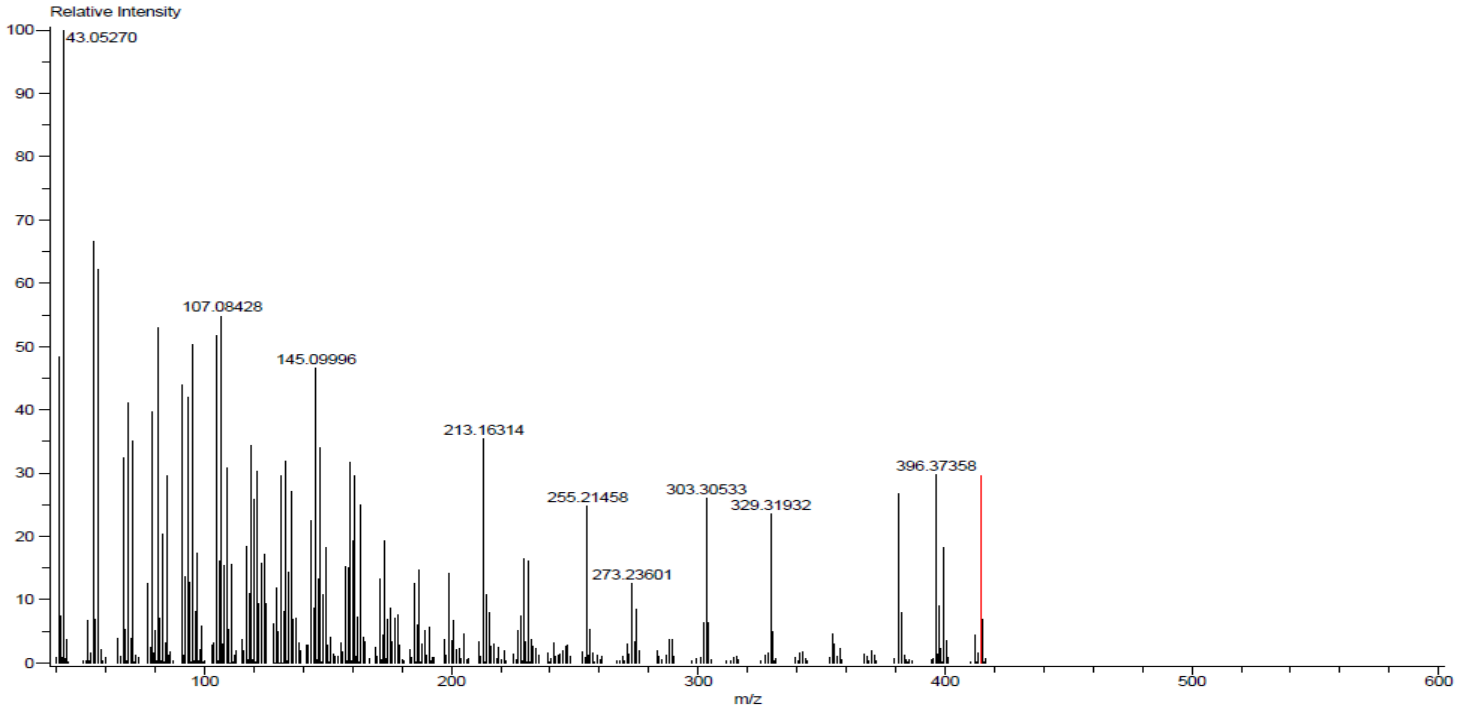
Mass	Intensity	Calc. Mass	Mass Difference (mmu)	Possible Formula	Unsaturation Number
402.38723	3503.88	402.38616	1.07	$^{12}C_{28}{}^{1}H_{50}{}^{16}O_{1}$	4.0

Figure S12. Mass spectrum of Ergostanol (**16**)



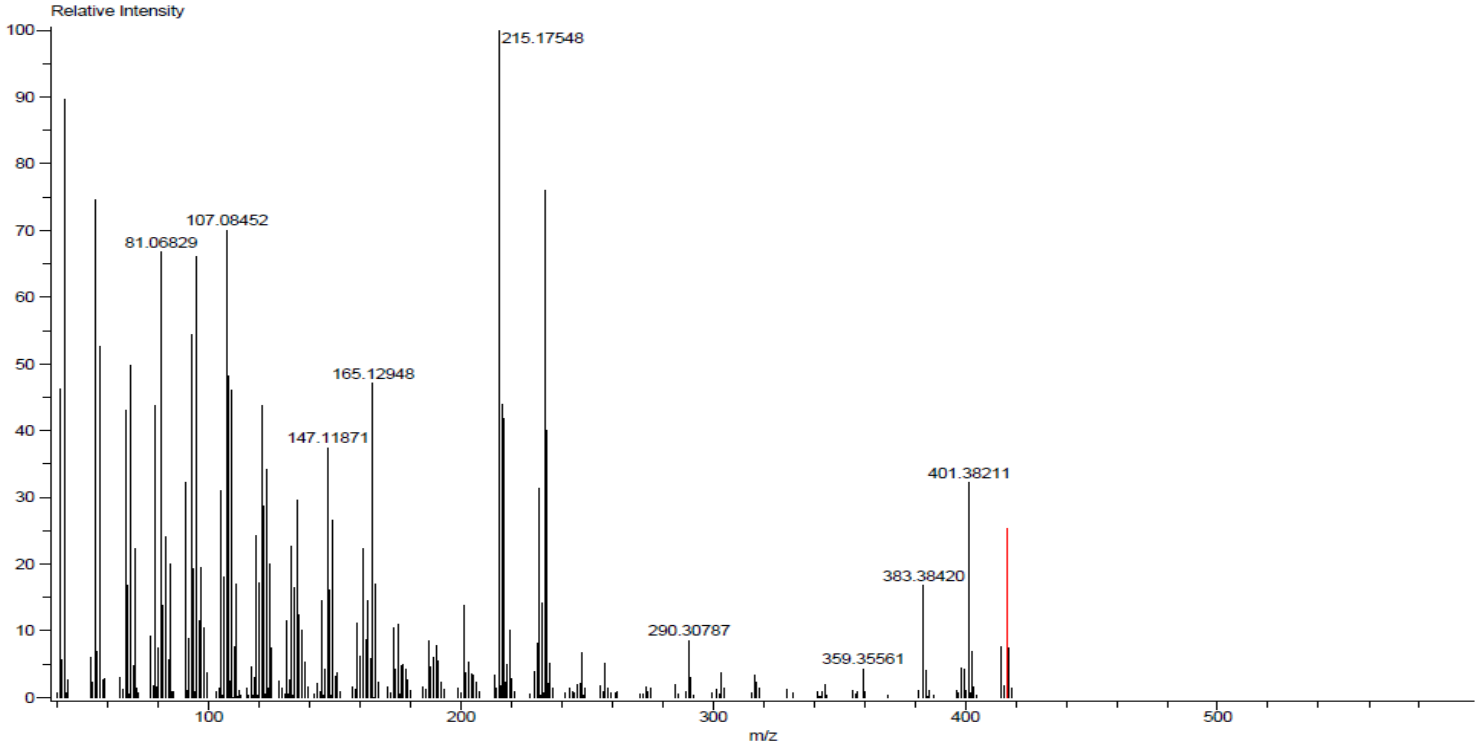
Mass	Intensity	Calc. Mass	Mass Difference (mmu)	Possible Formula	Unsaturation Number
412.37123	2734.74	412.37051	0.71	$^{12}C_{29}{}^{1}H_{48}{}^{16}O_{1}$	6.0

Figure S13. Mass spectrum of Stigmasta-4,22-dien-3-β-ol (**17**)



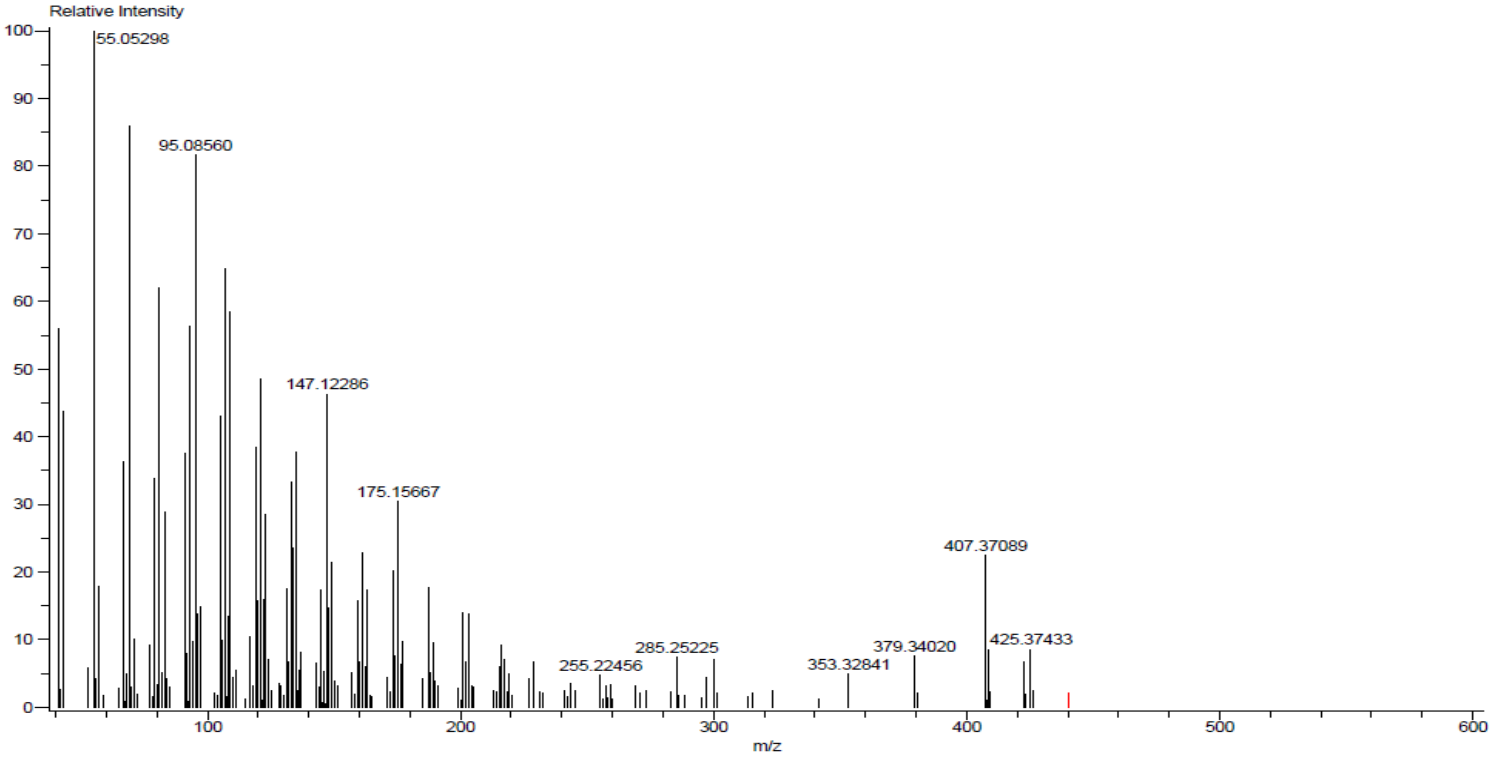
Mass	Intensity	Calc. Mass	Mass Difference (mmu)	Possible Formula	Unsaturation Number
414.38655	22998.02	414.38616	0.39	$^{12}C_{29}{}^{1}H_{50}{}^{16}O_{1}$	5.0

Figure S14. Mass spectrum of β-Sitosterol (**18**)



Mass	Intensity	Calc. Mass	Mass Difference (mmu)	Possible Formula	Unsaturation Number
416.40276	13472.06	416.40181	0.95	$^{12}C_{29}{}^{1}H_{52}{}^{16}O_{1}$	4.0

Figure S15. Mass spectrum of Stigmastanol (**19**)



Mass	Intensity	Calc. Mass	Mass Difference (mmu)	Possible Formula	Unsaturation Number
440.40234	333.96	440.40181	0.53	$^{12}C_{31}{}^{1}H_{52}{}^{16}O_1$	6.0

Figure S16. Mass spectrum of 24-Methyl-9,19-cyclolanost-24-en-3-ol-3-β (**20**)

Paper VII

Toxic aromatic compounds from fruits of *Narthecium ossifragum* L.

VII

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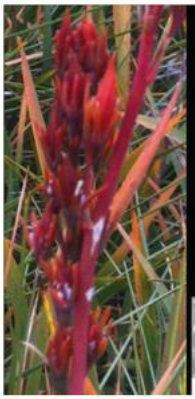
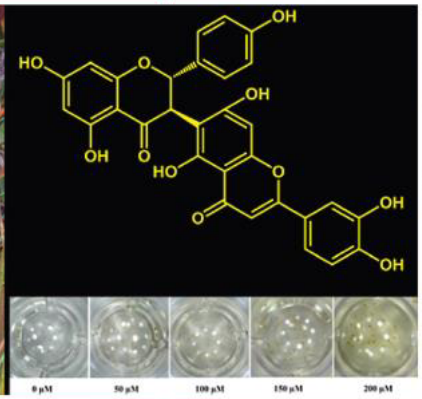
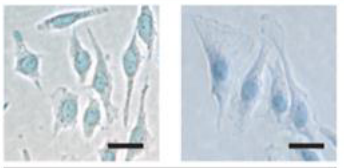
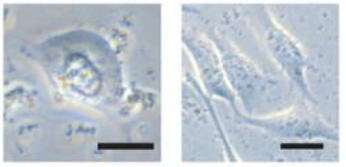






4-hydroxy-3-(3methylbut-2-enyl)benzaldehyde



Naringenin(3 → 6'')luteolin

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Toxic aromatic compounds from fruits of *Narthecium ossifragum* L.

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Naringenin(3 → 6″)luteolin
Naringenin(3 → 6″)chrysoeriol
Liovil 4-*O*-*β*-glucopyranoside
2,6-Dimethoxy cinnamic acid
(*E*)-4-(3-hydroxy-2,2-dimethylchroman-6-yl)but-3-en-2-one
(*E*)-4-(4-(((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)oxy)phenyl)but-3-en-2-one
Fungal metabolite

ABSTRACT

The intake of *Narthecium ossifragum*, commonly known as bog asphodel, has been associated with toxic effects observed in sheep for centuries. Although the plant has been studied for five centuries little is known about its chemical constituents. Six previously undescribed natural products, naringenin(3 → 6″) luteolin, naringenin(3 → 6″)chrysoeriol, liovil 4-*O*-*β*-glucopyranoside, 2,6-dimethoxy cinnamic acid, (*E*)-4-(3-hydroxy-2,2-dimethylchroman-6-yl)but-3-en-2-one and (*E*)-4-(4-(((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)oxy)phenyl)but-3-en-2-one, have been identified from fruits of *N. ossifragum* for the first time. In addition, the rare natural product 4-hydroxy-3-(3-methylbut-2-enyl)benzaldehyde and the five known compounds 4-hydroxycinnamic acid, quercetin 3,3′-dimethyl ether, quercetin 3,7-dimethyl ether, chrysoeriol 7-*O*-*β*-glucopyranoside and the di-*C*-glycosylflavone isoschaftoside were all characterized for the first time from the fruits of *N. ossifragum*. The discovery of sufficient amounts of 4-hydroxy-3-(3-methylbut-2-enyl)benzaldehyde in fresh plant material of *N. ossifragum* to allow complete structure elucidation by NMR and HRMS supports the possibility that fungi associated with *N. ossifragum* may be able to produce enough toxins to play a significant role in the pathogenicity of *N. ossifragum*. 4-Hydroxy-3-(3-methylbut-2-enyl)benzaldehyde showed mild toxicity towards normal rat kidney (NRK) and more profound activity towards MOLM13 acute myeloid leukemia cells (IC_{50} = 430 μM and 68 μM, respectively). Naringenin(3 → 6″)luteolin had IC_{50} of 230 μM towards NRK cells, and 115 μM towards MOLM13 cells. Microscopic evaluation suggests that these two compounds induce cell death by different mechanisms.

1. Introduction

Narthecium ossifragum L. Huds (Fig. 1). is a perennial 10–30 cm tall flowering plant, which blooms during summer from July to August (Mossberg et al., 1995). It has a creeping rhizome with long "cord" roots and short roots that are branched and fibrous (Summerfield, 1974). Its green leaves are 3–5 mm wide and 5–30 cm long (Stabursvik, 1959). The leaves are radical, often curved from base to apex, usually five-veined and are sheathed at the base in two opposite ranks. The stem on fertile shoots has also leaves but few and they are generally 2–3 cm long (Summerfield, 1974). The bright yellow flowers are 10–16 mm wide and are in a cluster with 6–20 flowers (Feilberg, 1999). The stamens have yellow woolly hairs on the filaments with bright red anthers. No nectar is produced but the plant has a fragrance like carnation. After flowering the plant forms capsular fruits. The flower stems change color from green to deep red orange and some parts of the leaves change color to light orange. The fruits have capsules up to 12 mm long, with six grooves containing red brown seeds with a small body and extended into a tail at each end (Summerfield, 1974). The species was systematically classified by Carl von Linné (von Linné., 1741).

Although the plant is mentioned among species which were harvested for hay production until the 1950s (Moen et al., 2012), *N. ossifragum* is mainly known for causing poisoning in cattle (Flåøyen et al., 1995), goats (Wisløff et al., 2003) and sheep (Stabursvik, 1953). Potential harmful effects of *N. ossifragum* was reported for the first time by Paulli (1667) based on a plant specimen and a letter he received from the Chancellor of Norway, Jens

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Fig. 1. Flowers (left) and fruits (right) of *N. ossifragum*. Photo: Svein Haavik and Marita Vu.

Bjelke in 1641, indicating that if the cattle ate this plant, they would be unable to walk and seem completely powerless. Bjelke named the plant "*Gramen ossifragum*", which means "the grass that breaks bones" (Paulli, 1667). More recent findings state that *N. ossifragum* is prevalently found in calcium-deficient soil (Mabey, 1996). Thus, livestock grazing in such areas may suffer calcium deficiency, which may lead to weakening of their skeletons because of relatively low levels of calcium in their forage and not because of any toxic effect of *N. ossifragum*.

In the current literature, *N. ossifragum* is reported to cause liver damage (Laksesvela and Dishington, 1983; Abdelkader et al., 1984; Flåøyen and Jensen, 1991) and in some instances damage to the kidneys (Flåøyen et al., 1997) indicating that the plant contains hepatotoxic compound(s). Moreover, ingestion of the plant is believed to be the cause of the disease alveld ("the elf's fire") in lambs (Stabursvik, 1953). Alveld is a disease of photodynamic nature (Stabursvik, 1953). This livestock poisoning is both economically important and an animal welfare problem, especially in the Nordic countries (Bernhoft, 2010). Alveld has been hypothesized to be caused by metabolite(s) produced by *N. ossifragum* (Abdelkader et al., 1984), or an associated fungus (di Menna et al., 1992) or cyanobacteria (Tønnesen et al., 2013). However, an explanation for the observed phototoxic effects associated with intake of *N. ossifragum* at the molecular level has not been provided in current literature. The existence of fungi associated with *N. ossifragum* has been known for more than a century (Rostrup, 1901). However, to date no fungal metabolites have been isolated either from plant material of *N. ossifragum* or from animals suffering from alveld.

Even though *N. ossifragum* has been extensively investigated, limited information is available with respect to its chemical constituents and their biological activity. The natural products hitherto identified from this plant source are limited to carotenoids (Stabursvik, 1959), furanolactones (Stabursvik, 1954a, b; Tschesche and Hoppe, 1971), saponins (Stabursvik, 1954a, b, 1959), sterols and sterol derivatives (Stabursvik, 1953; Tsuda and Hayatsu, 1959; Holen and Stabursvik, 1986; Stabursvik and Holen, 1988). To date, no aromatic compound has been reported from *N. ossifragum*.

Fruits of *N. ossifragum* (Fig. 1) are developed during the fall grazing season, when more limited amounts of other forage sources may be available, and it is thus likely that they may be eaten to a significant extent by domestic grazing livestock. The major objective of the current paper was to characterize aromatic constituents of fruits of *N. ossifragum* with a special focus on identification of substances unique to this plant source. In this paper we report on the isolation and characterization of twelve aromatic natural products from *N. ossifragum*, including six previously undescribed compounds (Figs. 2–4). Moreover, for the first time a fungal metabolite has been isolated from *N. ossifragum*.

2. Results and discussion

The methanolic extracts of fruits of *N. ossifragum* was concentrated under reduced pressure and fractionated by extraction with petroleum ether and ethyl acetate. The ethyl acetate phase was further separated by gradient XAD-7 adsorption chromatography, Sephadex LH-20 gel filtration chromatography and preparative HPLC. The five known compounds quercetin 3,3′-dimethyl ether (**4**), quercetin 3,7-dimethyl ether (**5**), chrysoeriol 7-*O*-*β*-glucopyranoside (**6**) the di-*C*-glycosylflavone isoschaftoside (**7**) and (*E*)-*p*-coumaric acid (**9**) (Figs. 3–4) were isolated from fruits of *N. ossifragum*. The identifications were based on a combination of several 2D NMR spectroscopic techniques and high resolution mass spectrometry (Tables S1–S4). All these compounds are reported for the first time in *N. ossifragum*. Chrysoeriol 7-*O*-*β*-glucopyranoside has previously been reported from the related species *N. asiaticum*. (Inoue et al., 1995).

Using a combination of 1D and several 2D NMR spectroscopic techniques and high-resolution MS, compound **10** was identified as the rare natural product 4-hydroxy-3-(3-methylbut-2-enyl)benzaldehyde (Fig. 4 and Table S4). The NMR chemical shift values of compound **10** were identical to that of commercial 4-hydroxy-3-(3-methylbut-2-enyl)benzaldehyde. In current literature, this compound has exclusively been reported as a fungal metabolite isolated from the root rotting pathogen *Heterobasidion occidentale* (Hansson et al., 2012). This is the first time a potential fungal metabolite has been identified from *N. ossifragum* although the existence of fungi

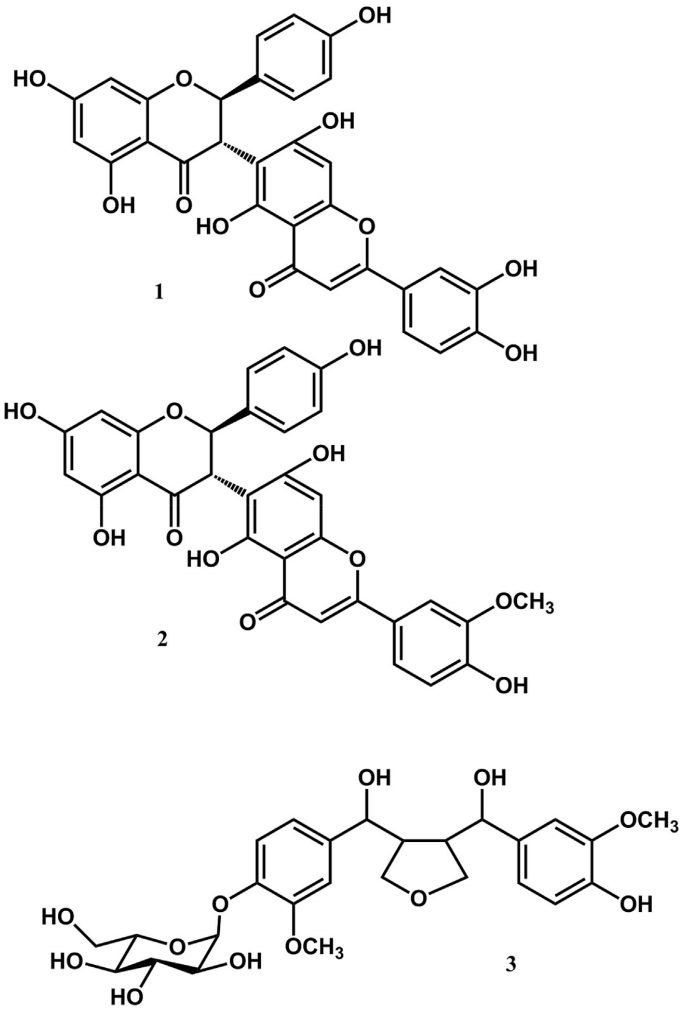

Fig. 2. Structures of naringenin(3 → 6″)luteolin (**1**), naringenin(3 → 6″)chrysoeriol (**2**) and liovil 4-*O*-*β*-glucopyranoside (**3**) isolated from fruits of *N. ossifragum*.

associated with this species has been known for more than a century (Rostrup, 1901; Aas and Løsvik, 1998). According to Aas and Løsvik (1998), fungi have been detected on approximately 10% of healthy plants of *N. ossifragum* (found on leaves, stem and inflorescence) (Aas and Løsvik, 1998). The fact that the amounts of **10** necessary for complete structural characterization could be isolated, strongly indicate that fungi associated with fresh plant material of *N. ossifragum* are present to an extent that their metabolites become quantitatively important and relevant from a toxicological point of view. Fungal metabolites are considered to be very diverse and at the same time very distinct (Moss, 1996). According to Aas and Løsvik a significant number of different fungi have been detected on *N. ossifragum* (Aas and Løsvik, 1998). It may be interesting to identify the potential fungal source of **10** among the multitude of different fungi hitherto associated with *N. ossifragum*, however, this is beyond the scope of the current manuscript.

The aromatic region of the ^{1}H NMR spectrum of the main aromatic compound (**1**) showed a 4H AA′XX′ system at δ 7.19 ('d' 8.7 Hz; H-2′/6′) and δ 6.62 ('d' 8.7 Hz; H-3′/5′), a 3H ABX system at δ 7.37 (dd 2.3 Hz, 8.3 Hz; H-6‴), δ 7.35 (d 2.3 Hz; H-2‴) and δ 6.86 (d 8.3 Hz; H-5‴), an AB system at δ 5.92 (d 2.1 Hz; H-6) and δ 5.90 (d 2.1 Hz; H-8), in addition to two duplicated 1H singlets at δ 6.64 and 6.61 (rotamers of H-3″) and δ 6.40 and 6.33 (rotamers of H-8″). This accords with a flavonoid dimer with monomeric units of different oxidation states of the C-ring consisting of naringenin and luteolin linked through a C–C bond between C-3 of naringenin and C-6″ of luteolin, respectively. The diagnostic chemical shift values of the rotamers of C-8″ of luteolin aglycone at δ 93.2 and δ 93.0, respectively in addition to the ROE crosspeak observed at δ 4.81/6.40 (H-3/H-8″) in the ROESY spectrum confirmed the inter-residual C–C linkage between C-3 of naringenin and C-6 of luteolin. Comparison with chemical shift values for the structural isomer morelloflavone obtained in the same solvent (Jamila et al., 2014), where the same aglycones are linked between C-3 of naringenin and C-8 of luteolin

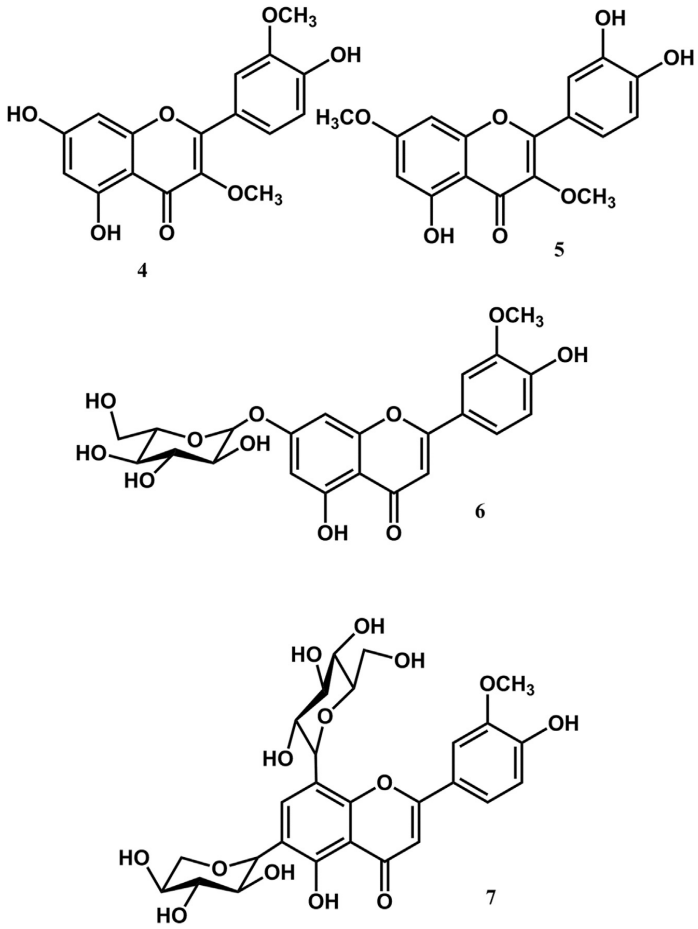

Fig. 3. Structures of the aromatic compounds quercetin 3,3′-dimethyl ether (**4**), quercetin 3,7-dimethyl ether (**5**), chrysoeriol 7-*O*-*β*-glucopyranoside (**6**) and the di-*C*-glycosyl-flavone isoschaftoside (**7**) isolated for the first time from fruits of *N. ossifragum*.

showed that the ^{13}C chemical shift values of the remaining CH group of the A-ring of luteolin (C-6; δ 98.5 and δ 97.9, respectively) were particularly different from the A-ring CH of **1** (C-8) observed at δ 93.2 and 93.0, respectively. Moreover, fewer signals of **1** are duplicated when compared with that of morelloflavone (Jamila et al., 2014), which is in line with similar observations of analogous C-6 versus C-8 substituted flavonoids in current literature (Rayyan et al., 2005). The absolute configuration of C-2 and C-3 of **1** was determined by a combination of circular dichroism (CD) spectroscopy and NMR. The high-amplitude positive Cotton effect near 290 nm observed in the CD spectrum of **1** (Supplementary data) indicates 2β-orientation of the B-ring (Li et al., 2002) and hence 2*R* absolute configuration of C-2. The large $^3J_{HH}$ coupling constant between H-2 and H-3 (12.3 Hz) confirmed the *trans* configuration of the bond between C-2 and C-3 and thus *S*-configuration of C-3. Thus, **1** was identified as the previously undescribed natural product (2*R*,3*S*)-naringenin(3 → 6″)luteolin (Fig. 2). A pseudomolecular ion $[M+H]^+$ at *m*/*z* 557.11012 corresponding to $C_{30}H_{21}O_{11}$ (calculated: 557.10845; 3.0 ppm) observed in the high resolution mass spectrum of **1** confirmed this identification.

The 1D and 2D NMR spectra of **2** shared many similarities with the spectra of **1** showing a flavonoid dimer consisting of naringenin with a 3 → 6″ linkage to a flavone aglycone with the same oxygenation pattern as in **1** (Table 1). The only structural difference between **2** and **1** was the presence of a methoxy group attached to the 3‴-position. The crosspeak at δ 3.86/148.0 (3‴−OCH_3/C-3‴) observed in the HMBC spectrum of **2** (supplementary data) and the crosspeak at δ 3.86/7.51 (3‴−OCH_3/H-2‴) observed in the ROESY spectrum of **2** (supplementary data) confirmed that the methoxy group is attached to the 3‴-position. The absolute configuration of C-2 and C-3 of **2** was determined by a combination of circular dichroism (CD) spectroscopy and NMR. The high-amplitude positive Cotton effect near 290 nm observed in the CD spectrum of **2** (Supplementary data) indicates 2β-orientation of the B-ring (Li et al., 2002) and hence 2*R* absolute configuration of C-2. The large $^3J_{HH}$ coupling constant between H-2 and H-3 (12.3 Hz) confirmed the *trans* configuration of the bond between C-2 and C-3 and thus

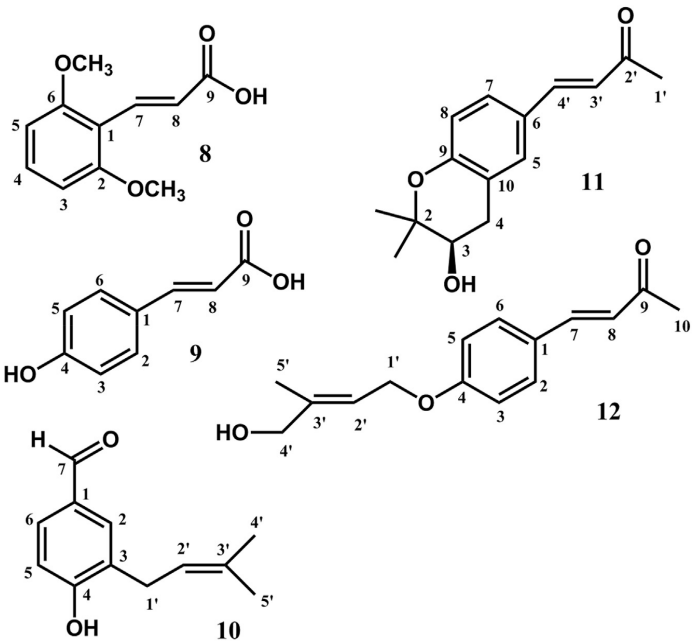

Fig. 4. Structures of (*E*)-2,6-dimethoxycinnamic acid (**8**), (*E*)-*p*-coumaric acid (**9**), 4-hydroxy-3-(3-methylbut-2-enyl)benzaldehyde (**10**), (*E*)-4-(3-*R*-hydroxy-2,2-dimethylchroman-6-yl)but-3-en-2-one (**11**) and (*E*)-4-(4-(((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)oxy)phenyl)but-3-en-2-one (**12**) isolated from fruits of *N. ossifragum*.

S-configuration of C-3. Thus, **2** was identified as the previously undescribed natural product (2*R*,3*S*)-naringenin(3 → 6″)chrysoeriol (Fig. 2). A pseudomolecular ion $[M+H]^+$ at *m*/*z* 571.12844 corresponding to $C_{31}H_{23}O_{11}$ (calculated 571.1241; 7.6 ppm) observed in the high resolution mass spectrum of **2** confirmed this identification.

The ^{1}H NMR spectrum of **3** showed the presence of liovil aglycone substituted with a glucose unit. Liovil aglycone was identified by the two 3H ABX systems at δ 6.94 (d 2.0 Hz; H-2), δ 7.03 (d 8.5 Hz; H-5), δ 6.84 (dd 2.0, 8.5 Hz; H-6) and δ 6.88 (d 2.0 Hz; H-2′), δ 6.71 (d 8.1 Hz; H-5′) and δ 6.74 (dd 2.0, 8.1 Hz; H-6′) with methoxy substituents at 3- and 3′-positions (Table 2), in addition to the signals belonging to the central oxygen-containing five-membered ring to which the aromatic rings were connected through its symmetrical CHOH substituents 7 and 7′, respectively (Table 2). The HMBC crosspeaks at δ 6.94/84.9 (H-2/C-7), δ 6.84/84.9 (H-6/C-7), δ 6.88/85.2 (H-2′/C-7′), δ 6.74/85.2 (H-6′/C-7′), δ 4.66/135.2 (H-7/C-1), δ 4.66/118.2 (H-7/C-6), δ 4.66/110.6 (H-7/C-2), δ 4.66/71.0 (H-7/C-9), δ 4.66/53.7 (H-7/C-8 and H-7/C-8′), δ 4.60/132.2 (H-7′/C-1′), δ 4.60/118.7 (H-7′/C-6′), δ 4.60/110.4 (H-7′/C-2′), δ 4.60/71.0 (H-7′/C-9′), δ 4.60/53.7 (H-7′/C-8 and H-7′/C-8′), δ 3.03/135.2 (H-8/C-1), δ 3.03/53.7 (H-8/C-8′ and H-8′/C-8), δ 4.12/84.9 (H-9A/C-7), δ 4.12/85.2 (H-9A′/C-7′), δ 4.12/53.7 (H-9A′/C-8, H-9A′/C-8′, H-9A/C-8 and H-9A/C-8′), δ 3.74/85.2 (H-9B′/C-7′) and δ 3.74/53.7 (H-9B′/C-8, H-9B′/C-8′, H-9B/C-8 and H-9B/C-8′) were important for identification of liovil aglycone. The positions of the methoxy groups at 3- and 3′-positions, respectively, were determined by the HMBC crosspeaks at δ 3.76/149.0 (3-OCH_3/C-3) and δ 3.75/147.5 (3′-OCH_3/C-3′), in addition to the ROESY crosspeaks at δ 3.76/6.94 (3-OCH_3/H-2) and δ 3.75/6.88 (3′-OCH_3/H-2′). The sugar unit was identified as glucose by the seven ^{1}H resonances in the spectral region δ 4.86–3.14, which correlated to six ^{13}C resonances at δ 100.2–60.7 in the ^{1}H-^{13}C HSQC spectrum and the edited ^{1}H-^{13}C HSQC, respectively (Table 2) (Fossen et al., 1998). The anomeric coupling constant (7.5 Hz) confirmed β-configuration of the anomeric carbon. The crosspeak at δ 4.86/145.9 (H-1″/C-4) observed in the ^{1}H-^{13}C HMBC spectrum and the crosspeak at δ 4.86/7.03 (H-1″/H-5) observed in the ^{1}H-^{1}H ROESY spectrum confirmed the linkage between the glucosyl and the aglycone at the 4-position. Thus, **3** was identified as the previously undescribed lignan liovil 4-*O*-β-glucopyranoside (Fig. 2). A molecular ion at *m*/*z* 538.20312 corresponding to $C_{26}H_{34}O_{12}$ observed in the HRMS spectrum confirmed this identification. A negative Cotton effect around 290 nm was observed in the CD spectrum of **3** (Supplementary data). However, due to the fact that there are no available CD reference data for liovil or any liovil derivatives in current literature, the absolute configuration of the chiral carbons remain undetermined for this compound.

The ^{1}H NMR spectrum of **8** showed a broad 1H singlet at δ 12.12 (9-OH), a 3H A_2X system at δ 6.71 (2H, d 8.4 Hz; H-3/5) and δ 7.34 (1H, t 8.4 Hz; H-4), a 2H AX system at δ 7.92 (d 16.3 Hz; H-7) and δ 6.70 (d 16.3 Hz; H-8), in accordance with a 2,6 symmetrically substituted cinnamic acid (Table 3). The substituents at the 2- and 6-positions of the aromatic ring were identified as methoxy groups by the 6H singlet at δ 3.85, which correlated to a ^{13}C shift at δ 56.0 in the ^{1}H-^{13}C HSQC spectrum. The substitution pattern of **8** was further confirmed by the fact that the signal at δ 3.85 belonging to the methoxy groups correlated strongly to the ^{13}C signal at δ 159.5 (C-2/6) and weakly to the ^{13}C signal at δ 104.2 (C-3). Thus, **8** was identified as the previously undescribed natural product (*E*)-2,6-dimethoxycinnamic acid (Fig. 4). A pseudomolecular ion at *m*/*z* = 209.08166 $[M+H]^+$ observed in the high resolution mass spectrum of **8** corresponding to $C_{11}H_{13}O_4$ (calculated 209.08138) confirmed this identity. **8** is reported as a natural product for the first time, although the compound has been synthesized previously

Table 1
^{1}H and ^{13}C chemical shifts of naringenin(3 → 6″)luteolin (**1**) and naringenin(3 → 6″) chrysoeriol (**2**) isolated from fruits of *N. ossifragum*. Duplicated signals represent rotational conformers.

Naringenin	1 δ ^{1}H ppm	1 δ ^{13}C ppm	2 δ ^{1}H ppm	2 δ ^{13}C ppm
2	5.83 *d* 12.3 Hz	81.36	5.83 *d* 12.2 Hz	81.1
		80.91		
3	4.81 *d* 12.3 Hz	47.21	4.82 *d* 12.3 Hz	47.0 46.8
	4.78 *d* 12.3 Hz	46.74	4.77 *d* 12.3 Hz	
4		196.8		196.7
		196.7		
5		163.65		164.2
5–OH	12.15 *s*		12.15 *s*	
6	5.92 *d* 2.1 Hz	96.13	5.92 *d* 2.1 Hz	96.1
7		166.54		166.5
7–OH	10.80 *s*		10.80 *s*	
8	5.90 *d* 2.1 Hz	95.11	5.90 *d* 2.1 Hz	95.0
9		163.01		163.0
10		101.38		101.5
1′		127.97		127.8
2′/6′	7.19 ‘*d*’ 8.7 Hz	128.98	7.18 ‘*d*’ 8.5 Hz	128.9
3′/5′	6.62 ‘*d*’ 8.7 Hz	114.82	6.62 ‘*d*’ 8.5 Hz	114.7
4′		157.70		157.7
4′–OH	9.46 *s*		9.46 *s*	
	Luteolin		***Chrysoeriol***	
2″		164.01		163.8
3″	6.64 *s* (b)	102.7	6.87 *s* (b)	103.1
	6.61 *s* (b)	102.8	6.85 *s* (b)	103.2
4″		181.8		N/A
		181.6		
5″		159.8		N/A
		159.2		
5″–OH	13.51 *s* (b)		13.52 *s* (b)	
	13.38 *s* (b)		13.34 *s* (b)	
6″		N/A		N/A
7″		162.4		N/A
		162.0		
7″–OH	11.26 *s* (b)		11.27 *s* (b)	
	11.01 *s* (b)		11.02 *s* (b)	
8″	6.40 *s* (b)	93.37	6.48 *s* (b)	93.4
	6.33 *s* (b)	93.02	6.40 *s* (b)	93.2
9″		156.1		N/A
		155.9		
10″		103.3		N/A
		103.2		
1‴		121.49		121.4
2‴	7.35 *d* 2.3 Hz	113.42	7.51 *m*	110.2
3‴		145.78		148.0
3‴–OH	9.40 *s*			
4‴		149.81		150.0
4‴–OH	9.91 *s*		9.96 *s*	
5‴	6.86 *d* 8.3 Hz	116.07	6.91 *d* 8.9 Hz	115.8
6‴	7.37 *dd* 2.3, 8.3 Hz	119.11	7.52 *m*	120.4
3‴–OCH_3			3.86 *s*	55.9

Table 2
^{1}H and ^{13}C chemical shifts of liovil 4-*O*-*β*-glucopyranoside (**3**) isolated from fruits of *N. ossifragum*.

Liovil	δ ^{1}H ppm	δ ^{13}C ppm
1		135.2
2	6.94 *d* 2.0 Hz	110.6
3		149.0
3–OCH_3	3.76 *s*	55.8
4		145.9
5	7.03 *d* 8.5 Hz	115.3
6	6.84 *dd* 2.0, 8.5 Hz	118.2
7	4.66 *d* 4.2 Hz	84.9
7–OH	N/A	
8	3.03 *dd* 2.2, 4.4 Hz	53.7
9A	4.12 *dd* 2.1, 6.8 Hz	71.0
9B	3.74 *m*	
1′		132.2
2′	6.88 *d* 2.0 Hz	110.4
3′		147.5
3′–OCH_3	3.75 *s*	55.7
4′		146.0
4′–OH	8.88 *s* (b)	
5′	6.71 *d* 8.1 Hz	115.2
6′	6.74 *dd* 2.0, 8.1 Hz	118.7
7′	4.60 *d* 4.2 Hz	85.2
7′–OH	N/A	
8′	3.03 *dd* 2.2, 4.4 Hz	53.7
9A′	4.12 *dd* 2.1, 6.8 Hz	71.0
9B′	3.74 *m*	
4-O-β-Glucopyranoside		
1″	4.86 *d* 7.5 Hz	100.2
2″	3.23 *m*	73.3
3″	3.23 *m*	76.9
4″	3.14 *m*	69.8
5″	3.27 *m*	77.1
6A″	3.64 *m*	60.7
6B″	3.43 *m*	

Table 3
^{1}H and ^{13}C NMR chemical shift values (ppm) and coupling constants (Hz) for (*E*)-2,6-dimethoxycinnamic acid (**8**) and (*E*)-4-(4-(((*E*)-4-hydroxy-3-methylbut-2-en-1-yl) oxy)phenyl)but-3-en-2-one (**12**) in DMSO-D_6 at 298 K.

	8 δ ^{1}H	8 δ ^{13}C	12 δ ^{1}H	12 δ ^{13}C
1		121.0		126.9
2		159.5	7.65 ‘d’ 8.8	130.2
3	6.71 d 8.4	104.2	6.98 ‘d’ 8.8	115.1
4	7.34 t 8.4	131.7		160.4
5	6.71 d 8.4	104.2	6.98 ‘d’ 8.8	115.1
6		159.5	7.65 ‘d’ 8.8	130.2
7	7.92 d 16.3	134.6	7.56 d 16.3	143.2
8	6.70 d 16.3	121.0	6.66 d 16.3	125.0
9		168.8		197.9
9–COOH	12.12 s (b)			
10			2.29 s	27.2
2/6–OC–H_3	3.85 s	56.0		
1A′			4.64 dq 0.9, 6.6	64.4
1B′				
2′			5.64 m	117.6
3′				141.1
4′			3.83 m	65.5
5′			1.65 dt 0.9, 1.5	13.8

(Limaye, 1934). A similarly substituted aldehyde has been reported as a natural product from Florida yew (*Taxus Floridana*) (Rao and Johnson, 1998).

The downfield region of the ^{1}H NMR spectrum of **11** showed a 3H ABX system at δ 7.45 (d, 2.1 Hz; H-5), δ 7.41 (dd, 2.1 Hz, 8.5 Hz; H-7) and δ 6.74 (d, 8.5 Hz; H-8) in addition to a 2H AX system at δ 7.51 (d, 16.3 Hz; H-4′) and δ 6.62 (d, 16.3 Hz; H-3′). The crosspeaks in HMBC at δ 7.51/197.8 (H-4′/C-2′), δ 6.62/197.8 (H-3′/C-2′) and δ 2.27/197.8 (H-1′/C-2′) confirmed that the methyl group at the 1′-position (3H, δ 2.27 s) together with H-3′ and H-4′ correlated with a ketone carbonyl at C-2′. The heterocyclic six-membered ring fused to the aromatic ring of **11** included a C_3 unit comprised by a methylene group at δ 2.93,2.63/30.8 (4) attached to C-10, a hydroxylated methine group at δ 3.65/67.6 (3) and a dimethyl substituted quaternary carbon at δ 78.0, which was attached to the oxygen substituent at 9-position of the aromatic moiety (Fig. 4 and Table 4). The HMBC crosspeaks at δ 7.45/30.8 (H-5/C-4), δ 6.74/30.8 (H-8/C-4), δ 2.93/155.2 (H-4A/C-9), δ 2.93/130.6 (H-4A/C-5), δ 2.93/126.3(w) (H-4A/C-6), δ 2.93/121.0 (H-4A/C-10), δ 2.93/78.0 (H-4A/C-2), δ 2.93/67.6 (H-4A/C-9), δ 2.63/155.2 (H-4B/C-9), δ 2.63/130.6 (H-4B/C-5), δ 2.63/126.3(w) (H-4B/C-6), δ 2.63/121.0 (H-4B/C-10), δ 2.63/78.0 (H-4B/C-2), δ 2.63/67.6 (H-4B/C-9), δ 3.65/121.0 (H-3/C-10), δ 3.65/78.0 (w) (H-3/C-2), δ 3.65/30.8 (w) (H-3/C-4), δ 3.65/25.6 (H-3/2-CH_3), δ 3.65/21.0 (H-3/2-CH_3), δ 1.27/155.2 (w) (2-CH_3/C-9), δ 1.27/78.0 (2-CH_3/C-2), δ 1.27/67.7 (2-CH_3/C-3), δ 1.27/30.8 (w) (2-CH_3/C-4), δ 1.27/21.0 (2-CH_3/2-CH_3), δ 1.18/78.0 (2-CH_3/C-2), δ 1.18/

Table 4
^{1}H and ^{13}C NMR chemical shift values (ppm) and coupling constants (Hz) for (*E*)-4-(3-hydroxy-2,2-dimethylchroman-6-yl)but-3-en-2-one (**11**) in DMSO-D_6 at 298 K.

	11 δ ^{1}H	11 δ ^{13}C
2		78.0
3	3.65 dd 5.1, 7.5	67.6
4a	2.93 dd 5.1, 16.7	30.8
4b	2.63 dd 7.5, 16.7	
5	7.45 d 2.1	130.6
6		126.3
7	7.41 dd 2.1, 8.5	128.0
8	6.74 d 8.5	117.0
9		155.2
10		121.0
1′	2.27 s	27.2
2′		197.8
3′	6.62 d 16.3	124.5
4′	7.51 d 16.3	143.5
2-Me	1.27 s	25.6
2-Me	1.18 s	21.0

67.7 (2-CH_3/C-3) and δ 1.18/25.7 (2-CH_3/2-CH_3), in addition to the ROESY crosspeaks at δ 7.45/2.93 (H-5/H-4A), δ 7.45/2.63 (H-5/H-4B), δ 6.74/1.27 (H-8/2-CH_3) and δ 6.74/1.18 (H-8/2-CH_3) were important for elucidation and assignment of the heterocyclic six-membered ring. The CD spectrum of **11** exhibited a negative Cotton effect at 245 nm (Supplementary data), which was indicative of *R* configuration of the chiral carbon of a structurally related chromane derivative (Batista et al., 2009). Thus, **11** was identified as the previously undescribed natural product (*E*)-4-(3-*R*-hydroxy-2,2-dimethylchroman-6-yl)but-3-en-2-one. A pseudomolecular ion observed at $m/z = 247.13377$ $[M+H]^+$ corresponding to $C_{15}H_{19}O_3$ (calculated 247.13342) in the high resolution mass spectrum of **11** confirmed this identification. A structurally analogous aldehyde exhibiting significant antitubercular activities against *Mycobacterium tuberculosis* H37Rv *in vitro*, with MIC values of 52.4 μg/mL, has previously been characterized from the root wood of *Zanthoxylum wutaiense* (Huang et al., 2011).

The 1D and 2D NMR spectra of **12** shared many similarities with that of **11** showing a benzalacetone moiety with oxygen substitution at the 4-position of the aromatic ring (Table 3). The oxygenated C_5 isoprenoid substituent attached to the oxygen at the 4-position of the aromatic ring was identified as (*E*)-3-methyl-2-en-4-ol by the signals at δ 4.64/64.4 (1′), δ 5.64/117.6 (2′), δ 141.1 (3′), δ 3.83/65.5 (4′) and δ 1.65/13.8 (5′) (Table 1). The strong crosspeak at δ 5.64/3.83 (H-2′/H-4′) observed in the ^{1}H-^{1}H ROESY spectrum of **12** confirmed the (*E*) configuration of the double bond of this substituent. Thus, **12** was identified as the previously undescribed natural product (*E*)-4-(4-(((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)oxy)phenyl)but-3-en-2-one. A pseudomolecular ion observed at $m/z = 247.13389$ $[M+H]^+$ in the high resolution mass spectrum of **12** corresponding to $C_{15}H_{19}O_3$ (calculated 247.13342) confirmed this identification. A possible biosynthesis of the latter compound based on the biosynthetic pathways of the individual building blocks of the molecule (Vanholme et al., 2012; Kakimoto, 2003) is shown in supplementary data.

Brine shrimp lethality assay is a simple *in vivo* test for cytotoxicity of biochemicals (Wu, 2014) where the test organisms are *Artemia salina* nauplii (Harwig and Scott, 1971; Meyer et al., 1982). Naringenin(3 → 6″)luteolin (**1**) was toxic to *Artemia salina* nauplii with LC_{50} value of 130 μM. Interestingly, a concentration-dependent significant yellow coloration of the *A. salina* nauplii, was observed (Fig. 5), indicating that they significantly enrich naringenin(3 → 6″)luteolin, suggesting that this compound is particularly bioavailable for *A. salina* nauplii.

We next evaluated the cytotoxic potential of 4-hydroxy-3-(3-methylbut-2-enyl)benzaldehyde (**10**) and the main aromatic compound naringenin(3 → 6″)luteolin (**1**) using Normal Rat Kidney (NRK) epithelial cells. 4-Hydroxy-3-(3-methylbut-2-enyl)benzaldehyde showed toxicity, with IC_{50} value of 430 μM measured by metabolic conversion of the WST-1 tetrazolium reagent (Fig. 6 A), whereas naringenin(3 → 6″)luteolin was more potent ($IC_{50} = 230$ μM). Both compounds were more potent towards the AML cell lines MOLM13 than the NRK-cells, with IC_{50} values of 68 for 4-hydroxy-3-(3-methylbut-2-enyl)benzaldehyde and 115 μM for naringenin(3 → 6″)luteolin. When cells treated with either of the two compounds were exposed to the vital stain Trypan blue, we noticed that cells treated with 4-hydroxy-3-(3-methylbut-2-enyl) benzaldehyde internalized the dye (Fig. 6 D, E), although the morphology resembled that of the control cells (Fig. 6C). The 4-hydroxy-3-(3-methylbut-2-enyl)benzaldehyde-treated cells were also metabolically active (Fig. 6A), suggesting that this compound permeabilizes the cellular membrane, without affecting the WST-1 conversion. Contrary to this, cells treated with naringenin(3 → 6″) luteolin were rounded and detached, but still excluded Trypan blue (Fig. 6 F, G), suggesting a cell death mechanism different from that found with 4-hydroxy-3-(3-methylbut-2-enyl)benzaldehyde. The general cytotoxicity of the former compound further confirms that naringenin(3 → 6″)luteolin is a previously undescribed toxic principle of *N. ossifragum*. We found no synergistic NRK cellular response when the two compounds were added in combination in a 1:1 M ratio (Data not shown).

2.1. Concluding remarks

For the first time, twelve aromatic natural products have been characterized from *N. ossifragum*. The six novel compounds, Naringenin(3 → 6)luteolin (**1**), naringenin(3 → 6″)chrysoeriol (**2**), liovil 4-*O*-β-glucopyranoside (**3**), 2,6-dimethoxy cinnamic acid (**8**), (*E*)-4-(3-hydroxy-2,2-dimethylchroman-6-yl)but-3-en-2-one (**11**) and (*E*)-4-(4-(((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)oxy)phenyl) but-3-en-2-one (**12**) are unique to this plant source. For the first time a fungal metabolite, namely 4-hydroxy-3-(3-methylbut-2-enyl)benzaldehyde has been characterized from *N. ossifragum*. The latter compound exhibited selective toxicity towards leukemia cells. The discovery of a bioactive fungal metabolite present in *N. ossifragum* at sufficient amounts to allow for isolation and complete structure elucidation by NMR and HRMS supports the suggestion that fungal metabolites may play a significant role in the observed pathogenicity of *N. ossifragum*.

3. Experimental

3.1. Plant material

Fresh plant material of *N. ossifragum* L. Huds. was collected on September 1st, 2014 at a bog area located at south western part of Ulriken, Bergen, Norway at 350 m above sea level (coordinates 60.36884 N 005.38237 E). Voucher specimen of *N. ossifragum* has been deposited at the ARBOHA at the University of Bergen (accession number BG/S-162115). The fruits, leaves and roots were separated from the stems and stored in a freezer at −20 °C prior to extraction.

3.2. Extraction of compounds and partitions with organic solvents

Fruits (3.0 kg) of *N. ossifragum* were extracted (twice) with 10 L methanol for 48–72 h at room temperature. The crude extract was filtered through glass wool and concentrated at reduced pressure by rotary evaporator to remove methanol. The resulting aqueous concentrated crude extract was partitioned (twice) with petroleum

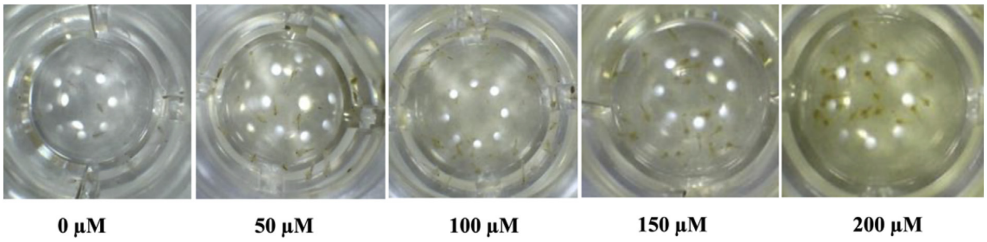

Fig. 5. Brine shrimp lethality assay performed by exposing *A. salina* nauplii to increasing concentrations of yellow naringenin(3 → 6″)luteolin (**1**). Notice that increased yellow coloration of *A. salina* occurs when increasing the concentration of **1** indicating that *A. salina* exhibit accumulation of naringenin(3 → 6″)luteolin in a concentration-dependent manner. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

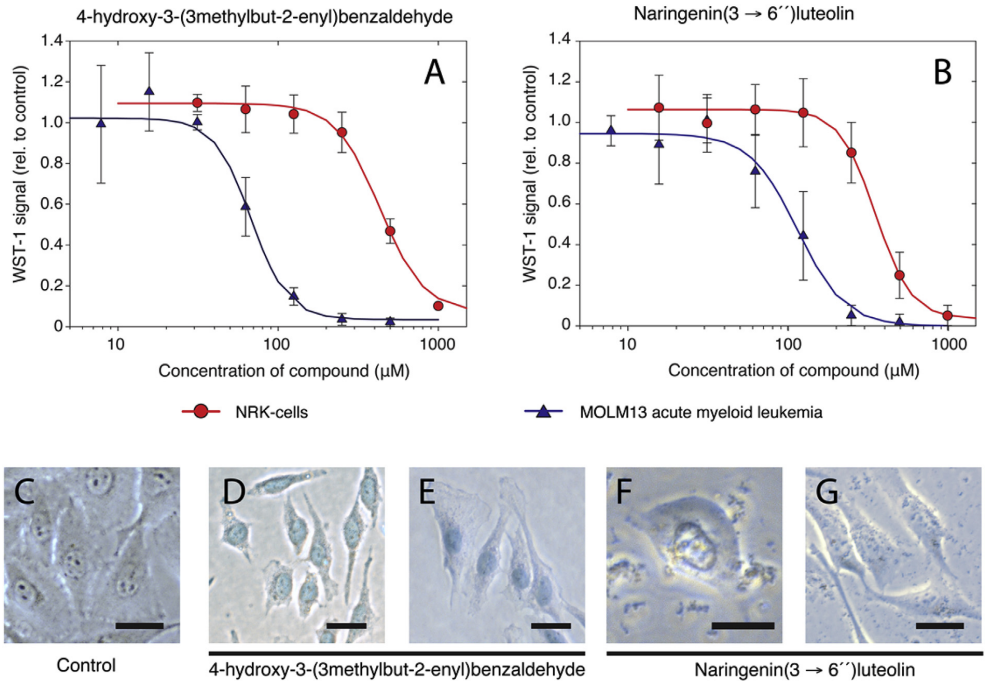

Fig. 6. Cytotoxicity of 4-hydroxy-3-(3-methylbut-2-enyl)benzaldehyde and naringenin(3 → 6″)luteolin towards mammalian cells. A and B: Viability of normal rat kidney (NRK) epithelial cells (red circles) or MOLM13 acute myeloid leukemia cells (blue triangles) after 24 h incubation with 4-hydroxy-3-(3-methylbut-2-enyl)benzaldehyde or naringenin(3 → 6″)luteolin. The lines are curve fittings from a four-parameter regression analysis. Note the higher activity towards MOLM13 cells in both compounds. C–G: NRK-cells were treated with 4-hydroxy-3-(3-methylbut-2-enyl)benzaldehyde (D and E) or naringenin(3 → 6″)luteolin (F and G) for 24 h, washed, and added Trypan blue. The images show phase-contrast micrographs of typical morphology of cells treated with the compounds at 500 µM in D and G, and 250 µM in E and G. C shows cell treated with 0.5% DMSO. Note the staining of the nuclei with Trypan blue in D and E, but not in C, F, and G, suggesting lack of intact membrane in D and E. The scale bars in C-G represent 10 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ether (4 L), followed by ethyl acetate (3.5 L). The ethyl acetate phase was concentrated to a volume of around 400 mL by rotary evaporator. A precipitate formed during concentration of the ethyl acetate phase was dissolved in acetone (20 mL).

3.3. Amberlite XAD-7 column chromatography

The concentrated ethyl acetate phase (400 mL) and the precipitated ethyl acetate extract (20 mL) were combined, and applied to an Amberlite XAD-7 column. The mobile phase gradient consisted of 4 L 100% water (fractions 1–5), followed by 1 L water: methanol 90:10; v/v (fractions 6–7), 1 L water: methanol 75:25; v/v (fractions 8–9), 1 L water: methanol 50:50; v/v (fractions 10–11), 1 L water: methanol 25:75; v/v (fractions 12–13) followed by 7 L 100% methanol (fractions 14–21). This gave in total 21 fractions with volumes of 200–1000 mL, which were analyzed individually on analytical HPLC as described below.

3.4. Sephadex LH-20 column chromatography

Fractions 12–16 from the XAD-7 column chromatography were combined and further separated on a Sephadex LH-20 column

using a gradient of water and methanol containing 0.2% TFA. The gradient consisted of 600 mL water: methanol: TFA 80:20:0.2; v/v/v (fractions 1–4), followed by 400 mL water: methanol: TFA 60:40:0.2; v/v/v (fractions 5–6), 600 mL water: methanol: TFA 30:70:0.2; v/v/v (fractions 7–12) and 1600 mL water: methanol: TFA 20:80:0.2; v/v/v (fractions 13–29). The above described gradient of water and methanol with constant relative proportions of TFA allowed collection of 29 fractions. The remaining substances were eluted from the column by using 700 mL 100% acetone as mobile phase (fractions 30–32). Fractions 10, 13, 31 and 32, in addition to the combined fractions 5 and 6 were further separated by preparative HPLC as described below. Fraction 32 contained pure compound **1**.

3.5. Preparative HPLC

Compounds of fractions 10, 13, 31 and 32, in addition to the combined fractions 5 and 6 from the Sephadex LH-20 separation were isolated by preparative HPLC. The HPLC instrument was equipped with a 250 × 22 mm, C_{18} Altech column. Two solvents were used for elution; A (water-TFA 99.5:0.5; v/v) and B (methanol-TFA 99.5:0.5; v/v). The elution profile of the applied HPLC gradient is shown in Fig. S1. The samples were dissolved in a total of 0.2–1.4 mL solvent. Portions of 200 μL of each of the samples were manually injected into the HPLC column. Each peak in the chromatogram was separately collected in vials. 1–1.5 mL of each of the collected fractions was transferred to HPLC vials for later identifications using analytical HPLC. Following this strategy 600 mg of the main compound (**1**) was isolated (including the amounts present in Sephadex fraction 32). The amounts of isolated compounds **2**–**12** were 2 mg (**2**), 1 mg (**3**), 3 mg (**4**), 1 mg (**5**), 8 mg (**6**), 0.5 mg (**7**), 1 mg (**8**), 3 mg (**9**), and 1 mg of each of compounds **10**–**12**, respectively.

3.6. Analytical HPLC

The HPLC instrument was equipped with a multi diode array detector, an autoinjector and a 250 × 4.6 mm, 5 μm Thermo Scientific Hypersil GOLD column. Two solvents were used for elution; A (water-TFA 99.5:0.5; v/v) and B (acetonitrile-TFA 99.5:0.5; v/v). The elution profile of the applied HPLC gradient is shown in Fig. S2. The analytical HPLC pump system was purged with both solution A (super distilled water and 0.5% TFA) and solution B (acetonitrile and 0.5% TFA) for 15 min each with a flow of 5 mL/min. The column was thereafter equilibrated with a flow of 1 mL/min in 30 min with acetonitrile-super distilled water (10:90 v/v). An aliquot (20 μL) of each sample was injected with an autoinjector. An appropriate solvent gradient (Fig. S2) was applied for the separations. The flow rate was 1 mL/min.

3.7. Spectroscopy

High resolution mass spectra were recorded using a JEOL AccuTOF JMS T100LC instrument fitted with an electrospray ion source. The spectrum was recorded over the mass range 50–1000 m/z.

UV-Vis absorption spectra were recorded on-line during HPLC analysis over the wavelength range 240–600 nm in steps of 2 nm.

CD spectra from 245 to 400 nm (light path 1 mm) of the compounds in methanol were recorded at 20 °C in a Jasco J-810 spectropolarimeter equipped with a Peltier temperature control unit. The spectra obtained were the average of four scans and methanol buffer scans were subtracted.

NMR samples were prepared by dissolving the isolated compounds in deuterated dimethylsulfoxide (DMSO-D_6; 99.9 atom % D, Sigma-Aldrich). The 1D ^{1}H and the 2D experiments ^{1}H-^{13}C HMBC, the ^{1}H-^{13}C HSQC, the ^{1}H-^{13}C HSQCTOCSY, the ^{1}H-^{13}C H2BC, the ^{1}H-^{1}H COSY and ^{1}H-^{1}H ROESY NMR experiments were obtained at 600.13 MHz and 150.90 MHz for ^{1}H and ^{13}C, respectively, at 298 K on a Bruker 600 MHz instrument equipped with a ^{1}H,^{13}C,^{15}N triple resonance cryogenic probe.

3.8. Brine shrimp lethality assay of the main aromatic compound

The stock solution was prepared by dissolving 5.6 mg pure compound **1** in 500 μL methanol. From the stem solution a salt-water solution was made of 200 μL stock solution, which was added to 10 mL seawater. The salt-water solution was then transferred to micro wells of a 96 micro wells plate filled with *A. salina* nauplii using 100 μL micropipettes. 100 μL of minimum 10 artemia shrimps were added to six different wells. The number of artemia shrimps in each well was controlled using an USB-microscope. 100, 75, 50, 50, 25 and 0 μL of seawater were added to the six wells. 0, 25, 50, 50, 75 and 100 μL of the solution of compound **1** described above were then added to the wells. The total volume in each well was 200 μL. Three parallels of each concentration and controls were made. After 24 h the *A. salina* nauplii in each well were counted using a computer-connected Veho 400× USB microscope with 20 times magnification to determine the toxicity of compound **1** at varying concentrations and the LC_{50} value was calculated. Rotenone was used as positive control for the artemia assay causing 100% lethality at all applied concentrations of this compound (2.5, 5, 10, 15 and 20 μM).

3.9. Cytotoxicity towards leukemia cells and normal rat kidney (NRK) epithelial cells

Pure compound **10** used in these experiments was purchased from Aldlab. The identity and purity of commercially available **10** was confirmed by NMR. Pure naringenin(3 → 6″)luteolin was isolated from fruits of *N. ossifragum* as described above. The stock solutions were prepared by dissolving 5.6 mg pure compound **10** and 18 mg naringenin(3 → 6″)luteolin in DMSO to a final concentration of 50 mM. Normal rat kidney epithelial cells (NRK, ATCC no.: CRL-6509) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA). When the cells reached 80% confluence, they were detached by mild trypsin treatment (0.33 mg mL^{-1} trypsin for 5 min at 37 °C), centrifuged (160 × g, 4 min) and reseeded in fresh medium to 25% confluence. The AML cell line MOLM13 (Matsuo et al., 1997; Quentmeier et al., 2003) were cultured in RPMI 1640 medium enriched with 10% FBS. The cells were kept in suspension cultures at a density between 150,000 and 600,000 cells/ml. Both NRK and MOLM13 cell lines were cultured in media supplemented with 100 IU/mL penicillin and 100 mg/mL streptomycin (both from Cambrex, Belgium) in a humidified atmosphere (37 °C, 5% CO_2).

For experiments with compounds **1** and **10**, the NRK cells were seeded in 96 wells tissue culture plates (5000 cells/well, 0.1 mL) and left overnight to attach whereas the MOLM13 cells were seeded in 96 wells tissue culture plates at 15,000 cells/well in 0.1 mL on the day of the experiment. Compounds dissolved in DMSO were added to the cells, and the plates were kept overnight before addition of the tetrazolium salt WST-1 according to the manufacturers instructions (Roche Diagnostics, Mannheim, Germany). The plates were further incubated for 2 h before the signal was recorded at 450 nm with reference at 620 nm. The cells were next fixed using 2% buffered formaldehyde (pH 7.4) with 0.01 mg mL^{-1} of the DNA-specific fluorescent dye, Hoechst 33342. The presence of apoptotic cells was determined by differential interference contrast and fluorescence microscopy (Axiovert 35M, Carl Zeiss, Oberkochen, Germany), as previously described (Oftedal et al., 2010; Myhren

et al., 2014). Cells with only DMSO added were used as control, and 1% DMSO gave less than 5% reduction in WST-1 signal, and less than 3% apoptotic nuclei in the cell culture. For blank subtraction, medium with only WST-1 and compound added were used. IC_{50} values were determined by four-parameter regression analysis. To assess membrane integrity, the vital stain trypan blue was added to viable cells, incubated for 5 min, and the cells washed with PBS. The cells were then studied by differential interference microscopy to visualize internalized trypan blue.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.phytochem.2016.09.010.

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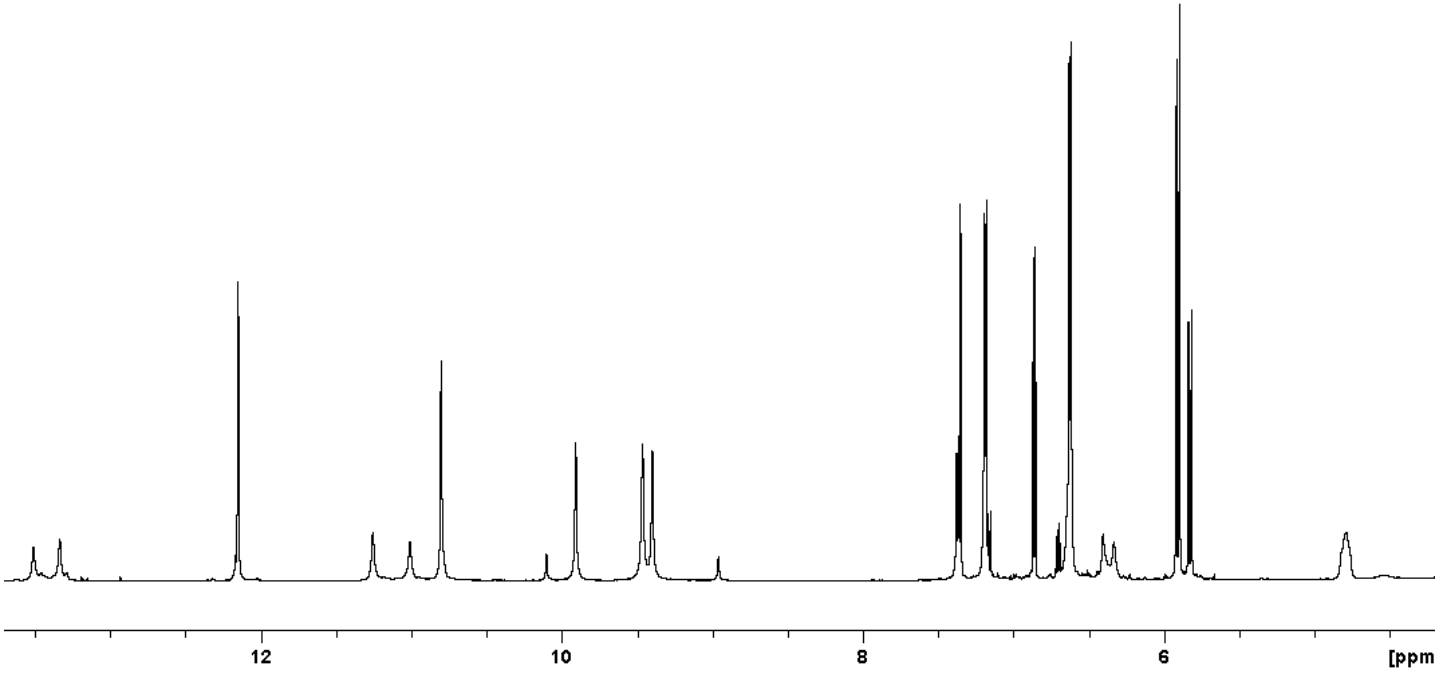
Toxic aromatic compounds from fruits of *Narthecium ossifragum* L.

Marita Vu, Lars Herfindal, Ole Johan Juvik, Anni Vedeler, Svein Haavik and Torgils Fossen

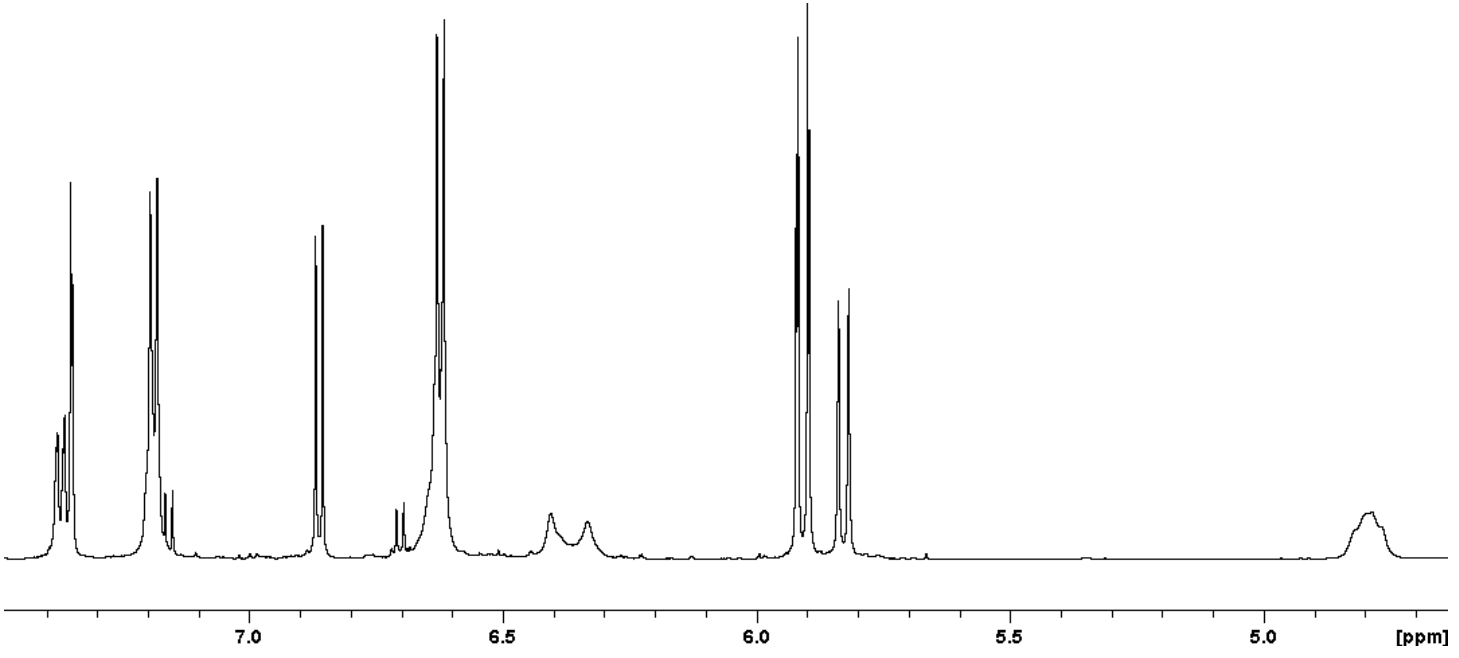
Supplementary data

NMR and CD spectra

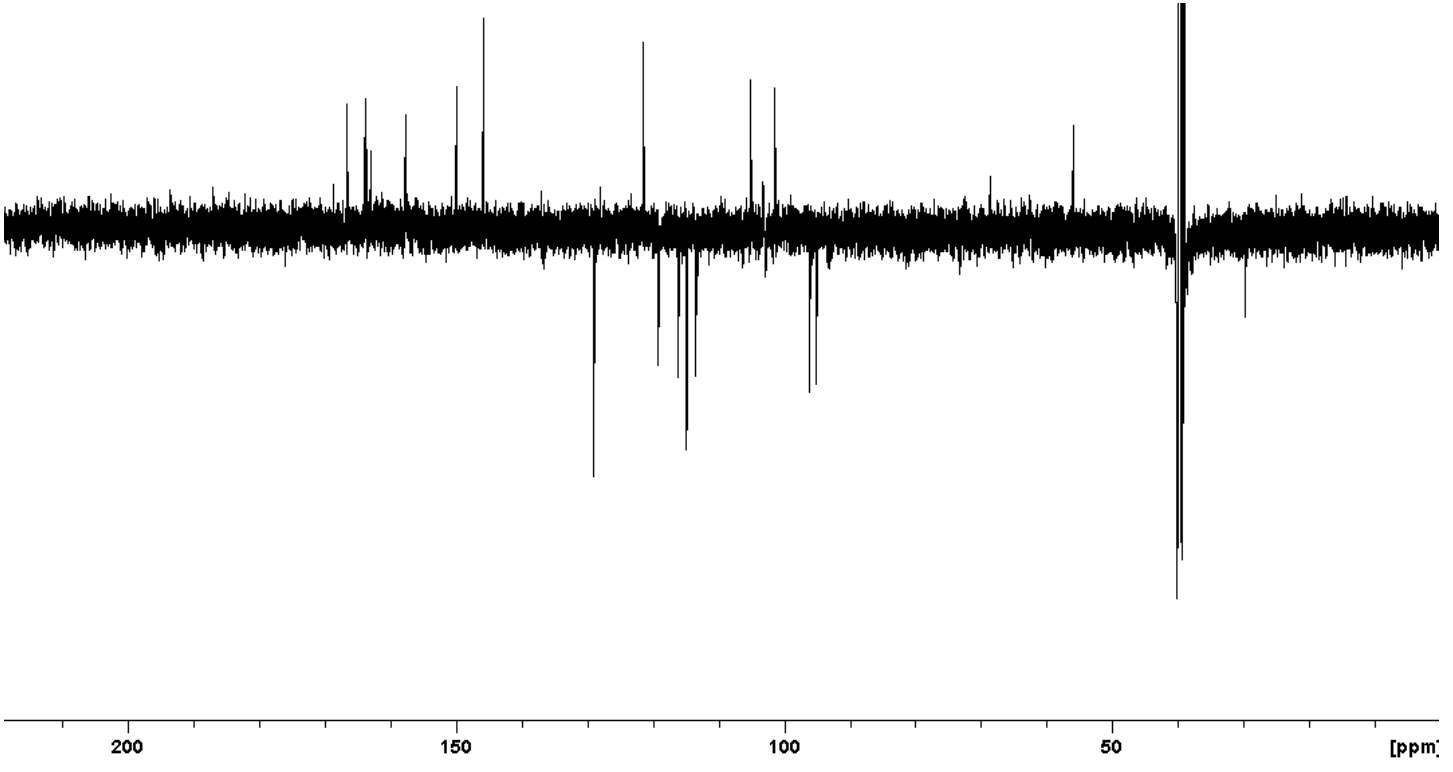
1D ^{1}H NMR spectrum of **1**



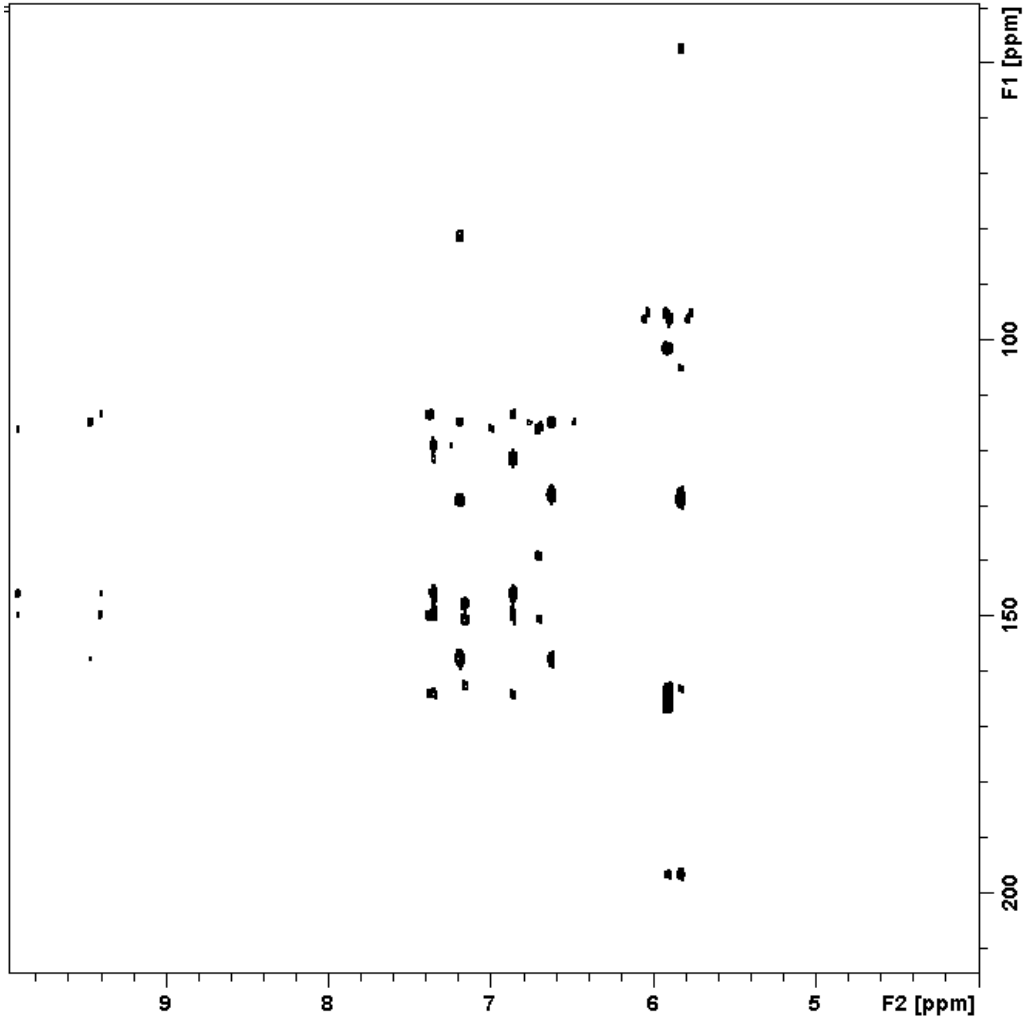
Expanded region of the 1D ^{1}H NMR spectrum of **1**



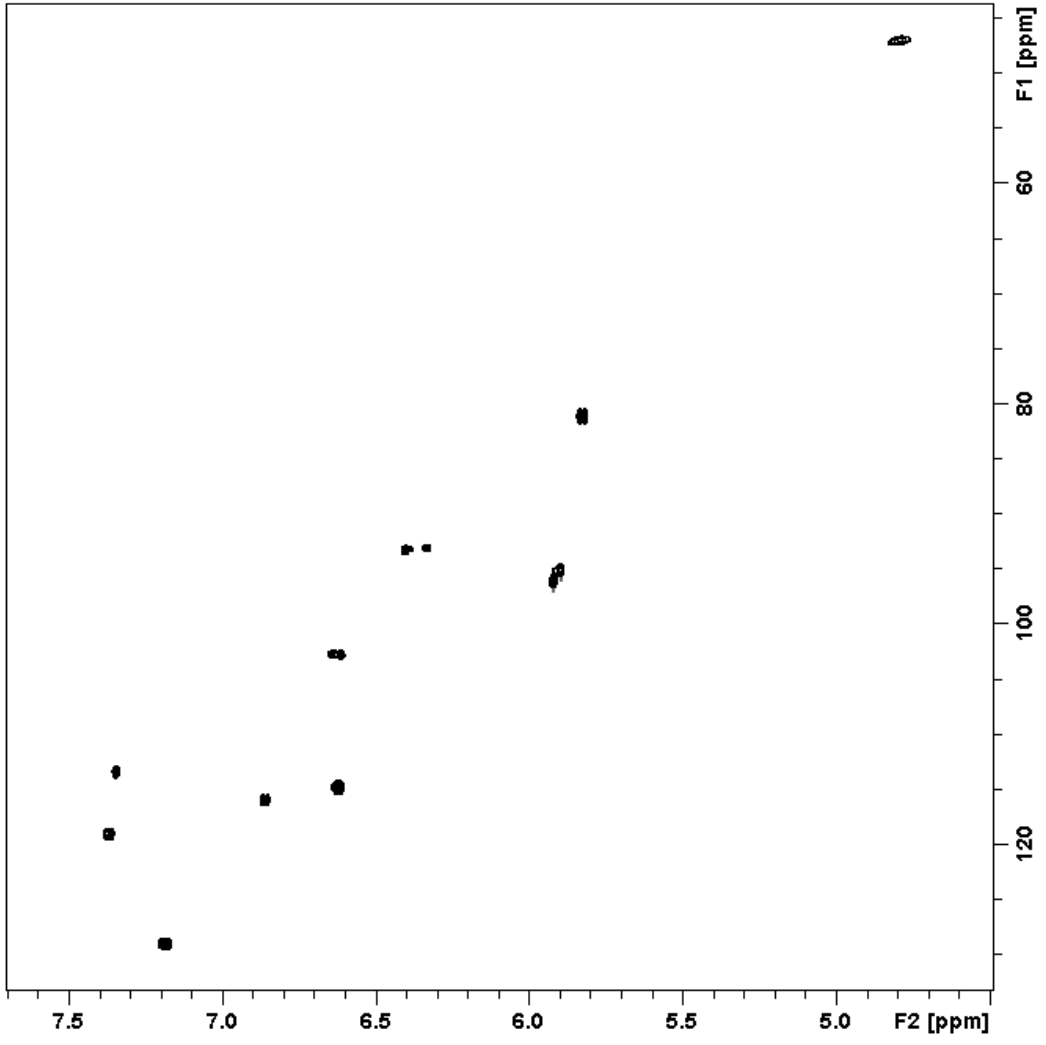
1D ^{13}C NMR spectrum of **1**



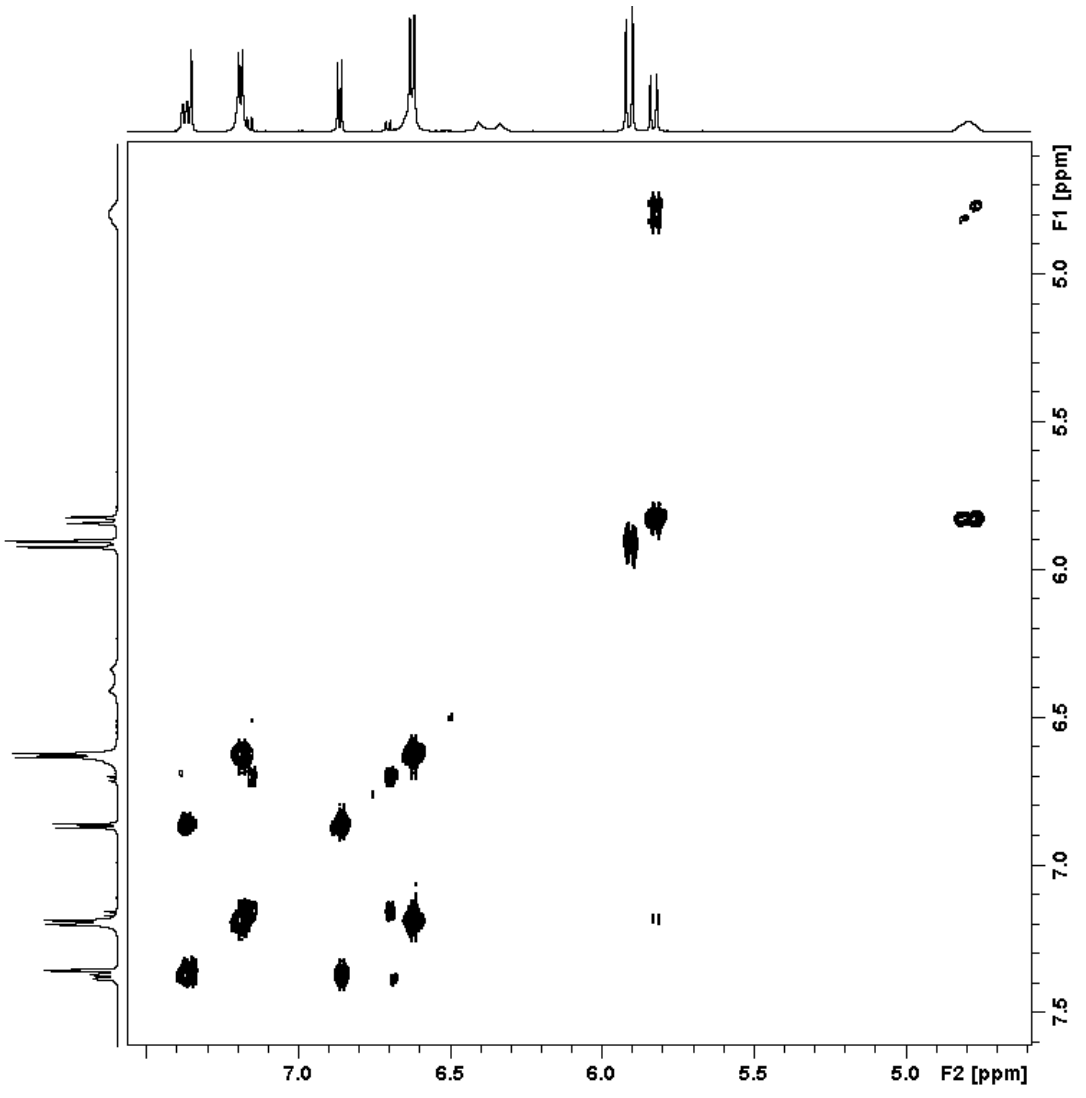
2D ^{1}H-^{13}C HMBC spectrum of **1**



2D edited ^{1}H-^{13}C HSQC spectrum of **1**



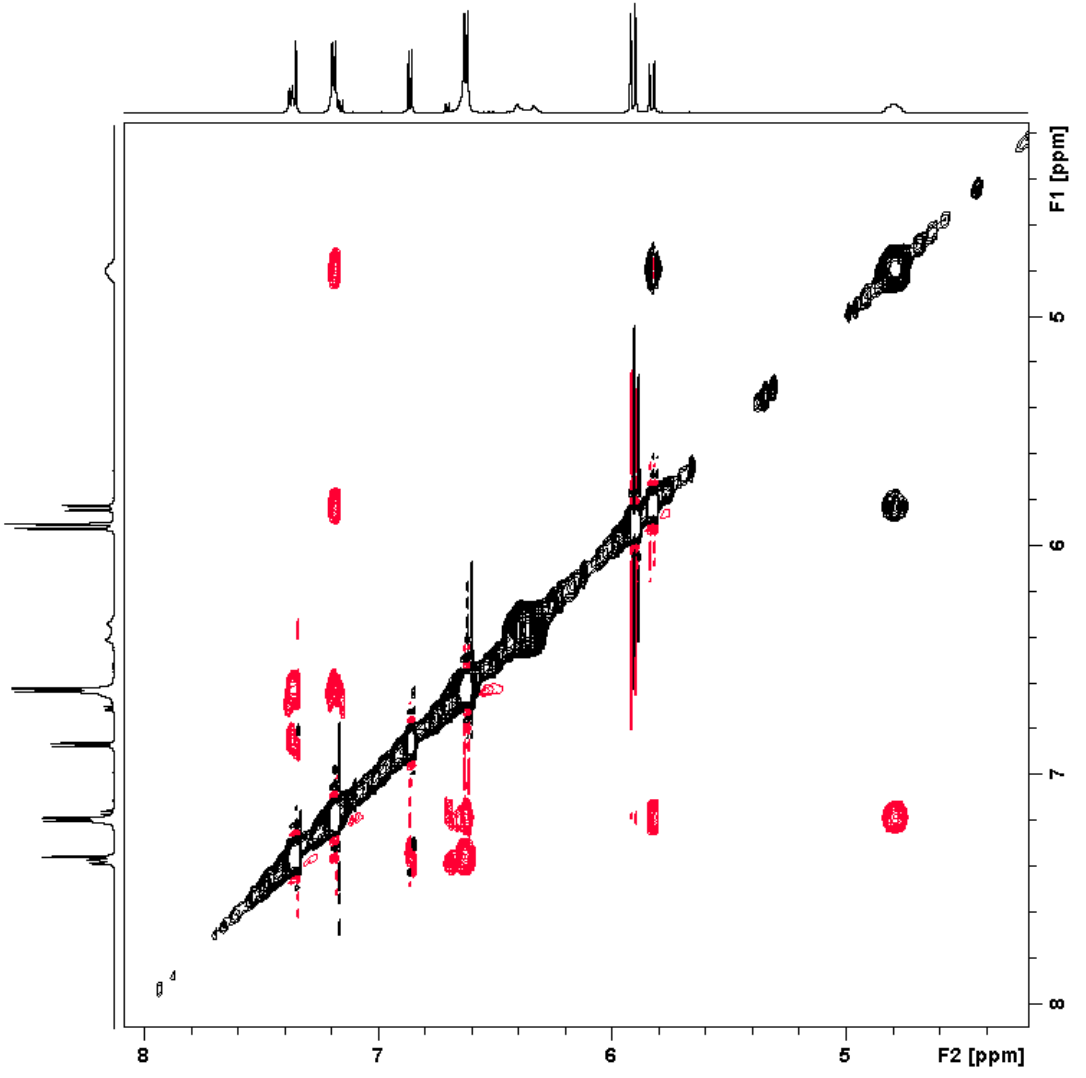
2D ^{1}H-^{1}H COSY spectrum of **1**



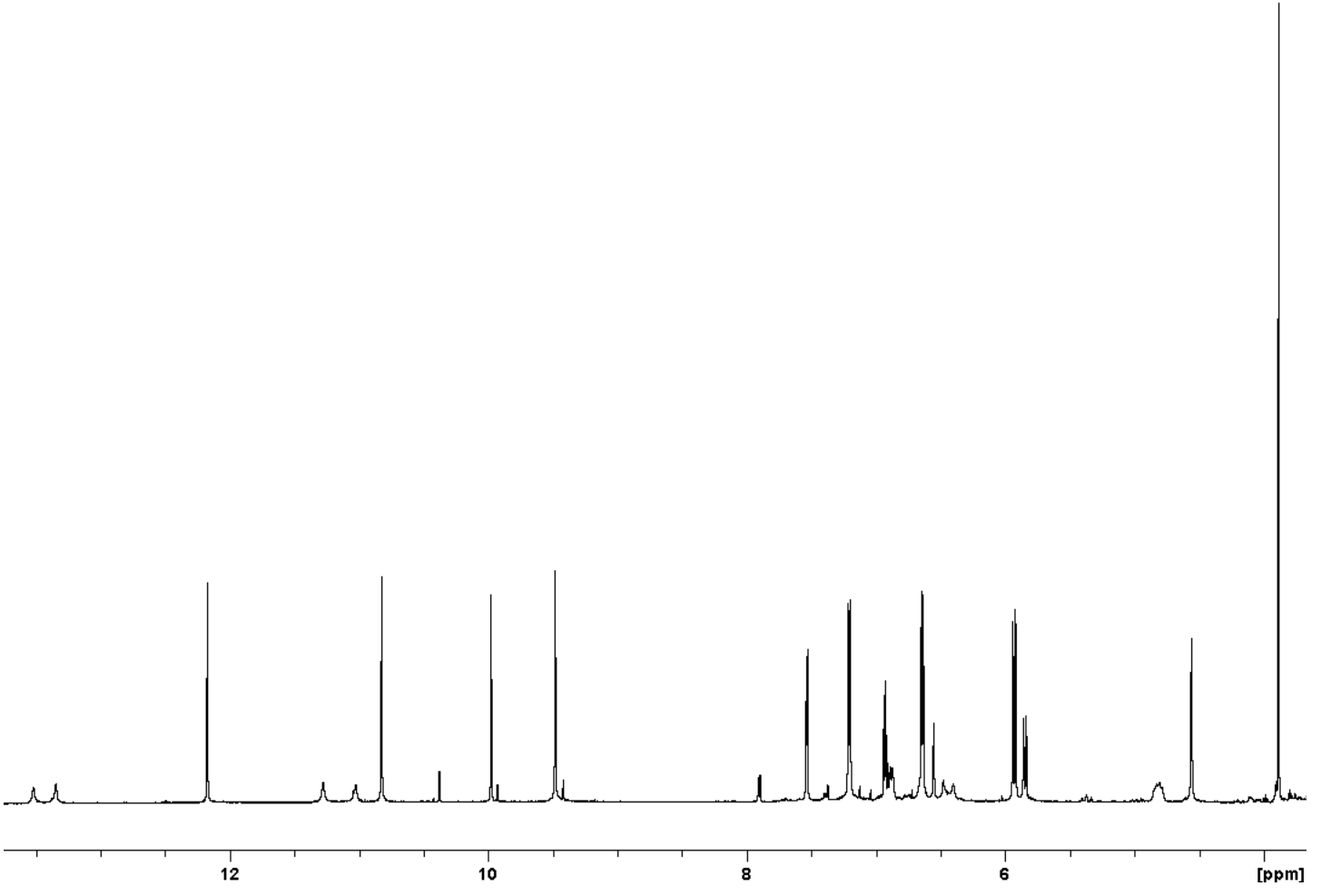
2D ^{1}H-^{1}H ROESY spectrum of **1**

Red signals:
ROE cross peaks

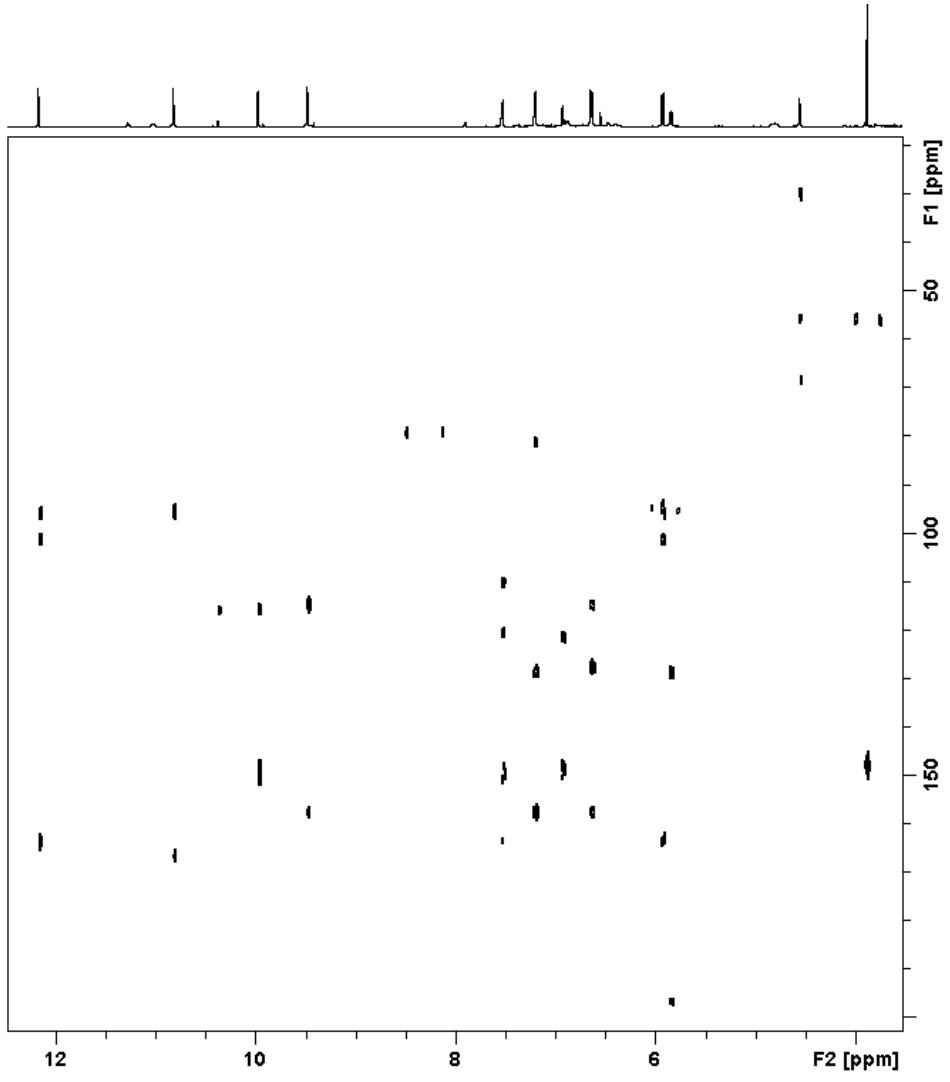
Black signals:
exchange cross peaks



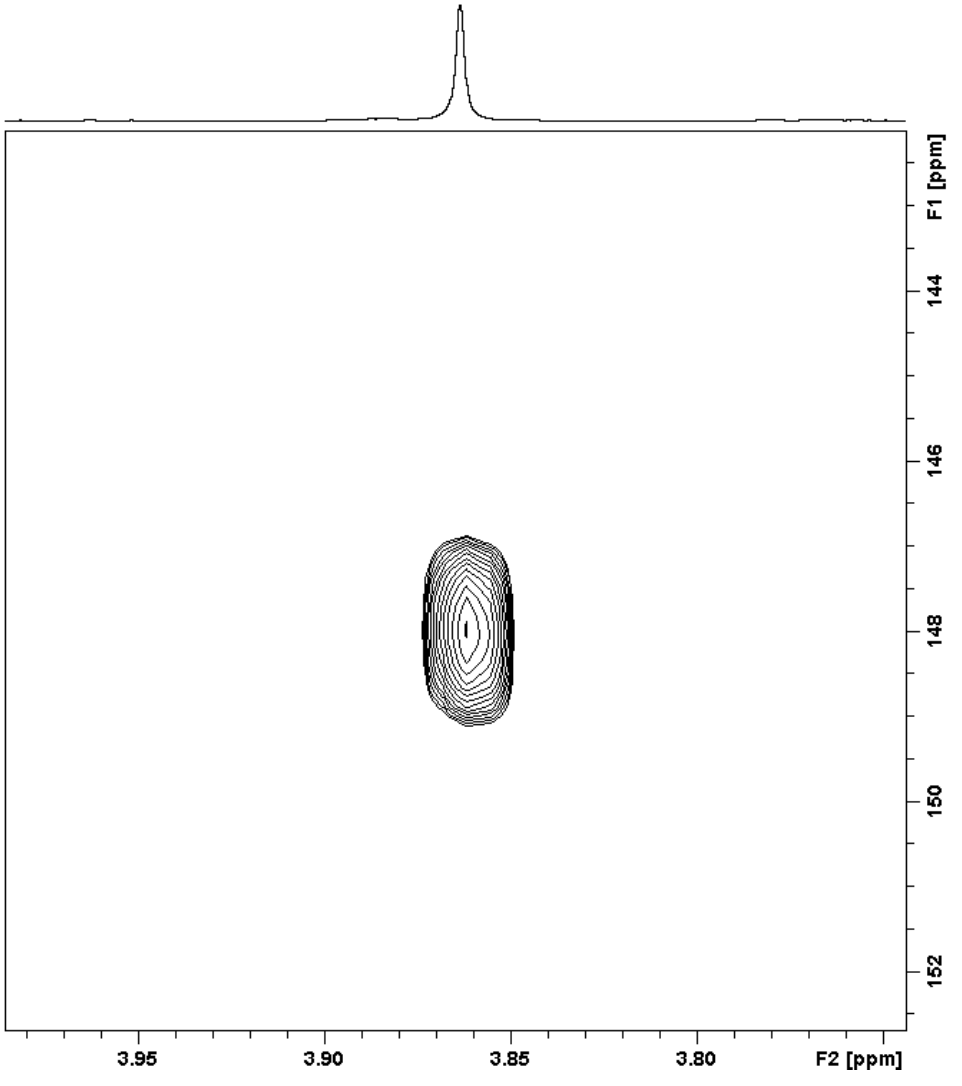
1D ^{1}H NMR spectrum of **2**



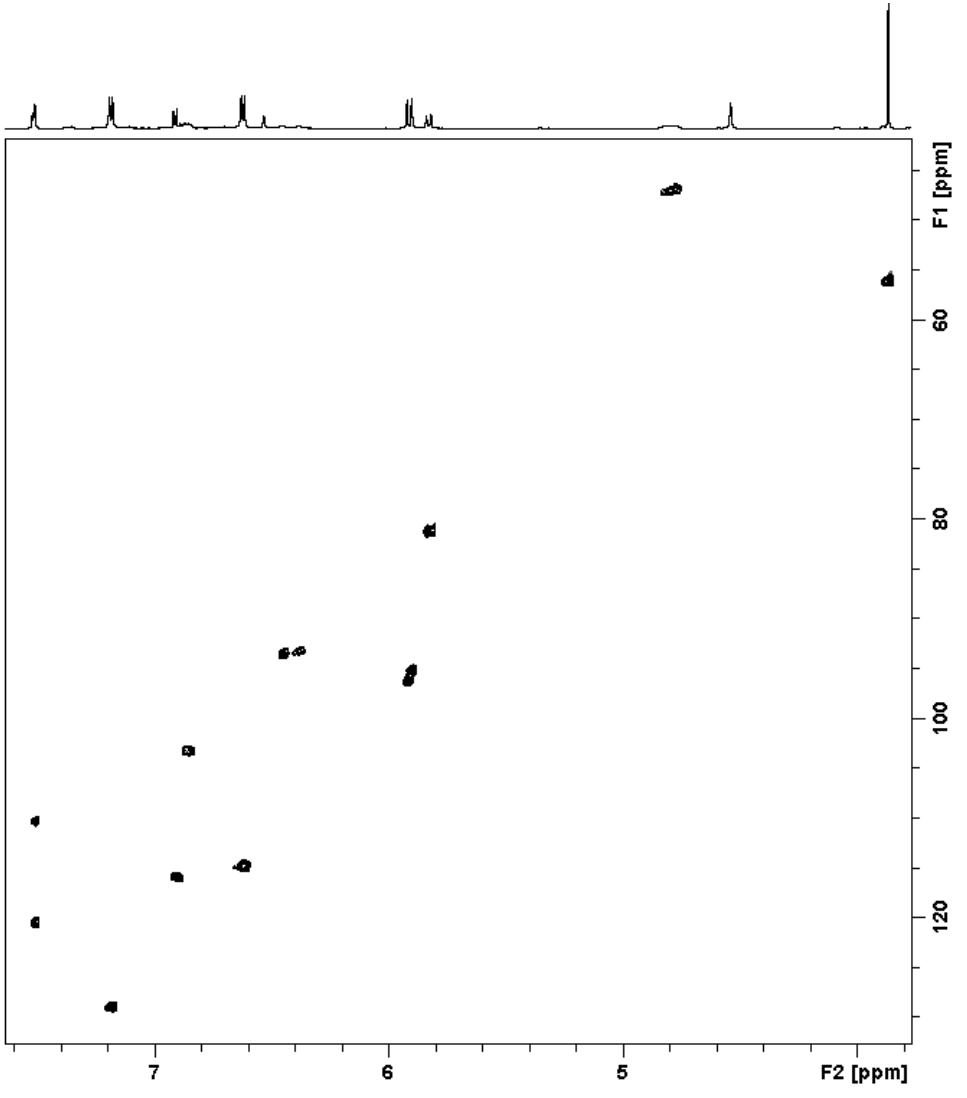
2D ^{1}H-^{13}C HMBC spectrum of **2**



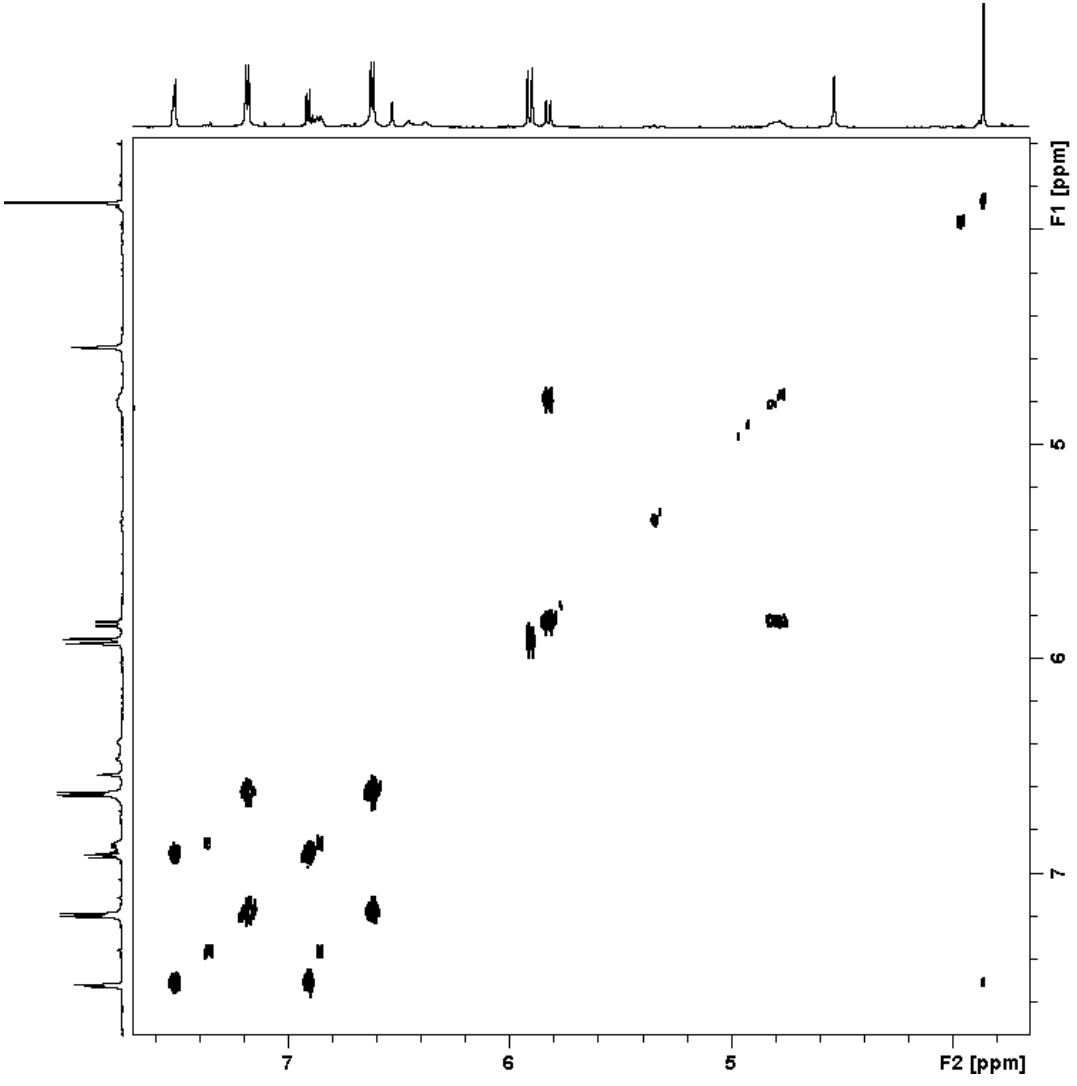
Expanded region of the 2D ^{1}H-^{13}C HMBC spectrum of **2**



2D ^{1}H-^{13}C HSQC spectrum of **2**



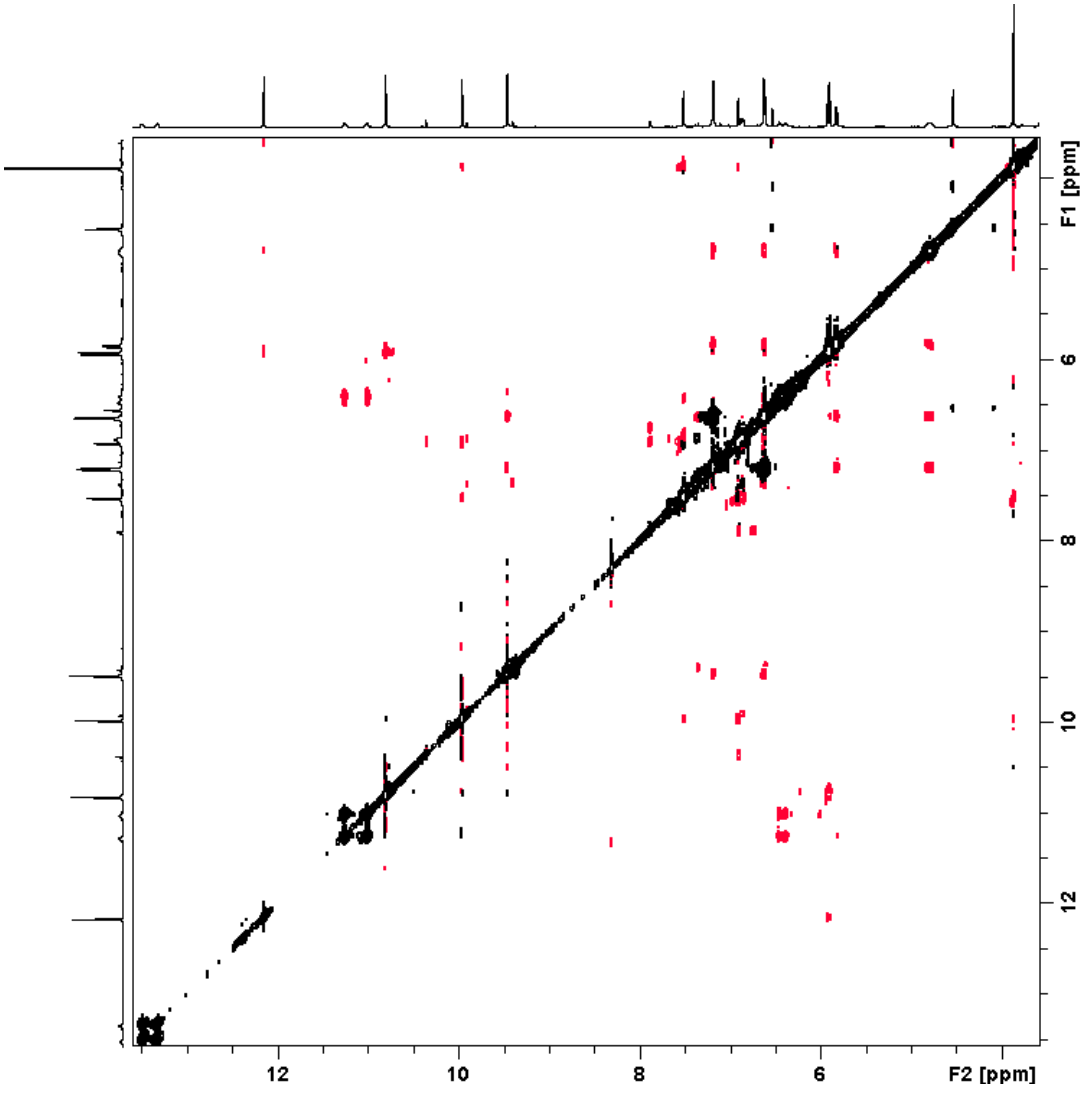
2D ^{1}H-^{1}H COSY spectrum of **2**



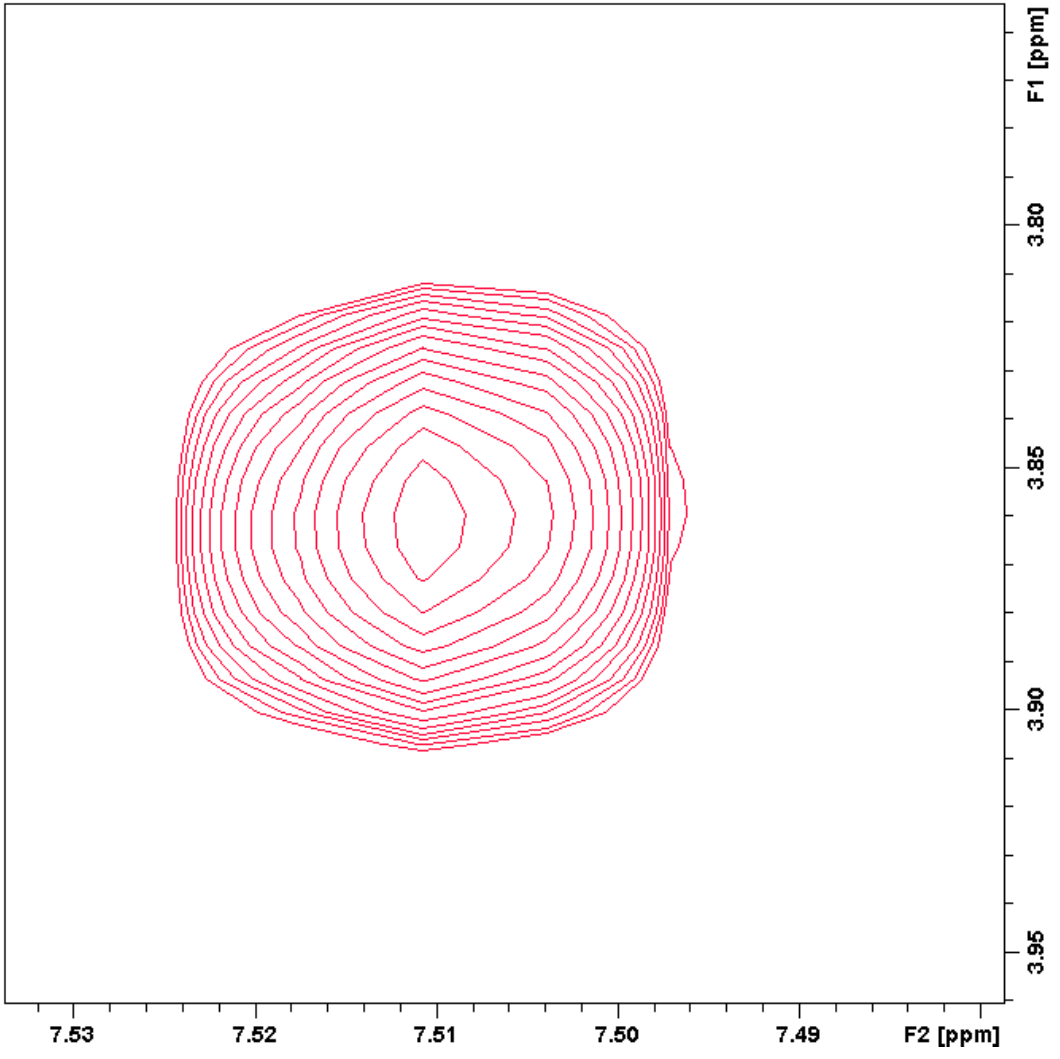
2D ^{1}H-^{1}H ROESY spectrum of **2**

Red signals:
ROE cross peaks

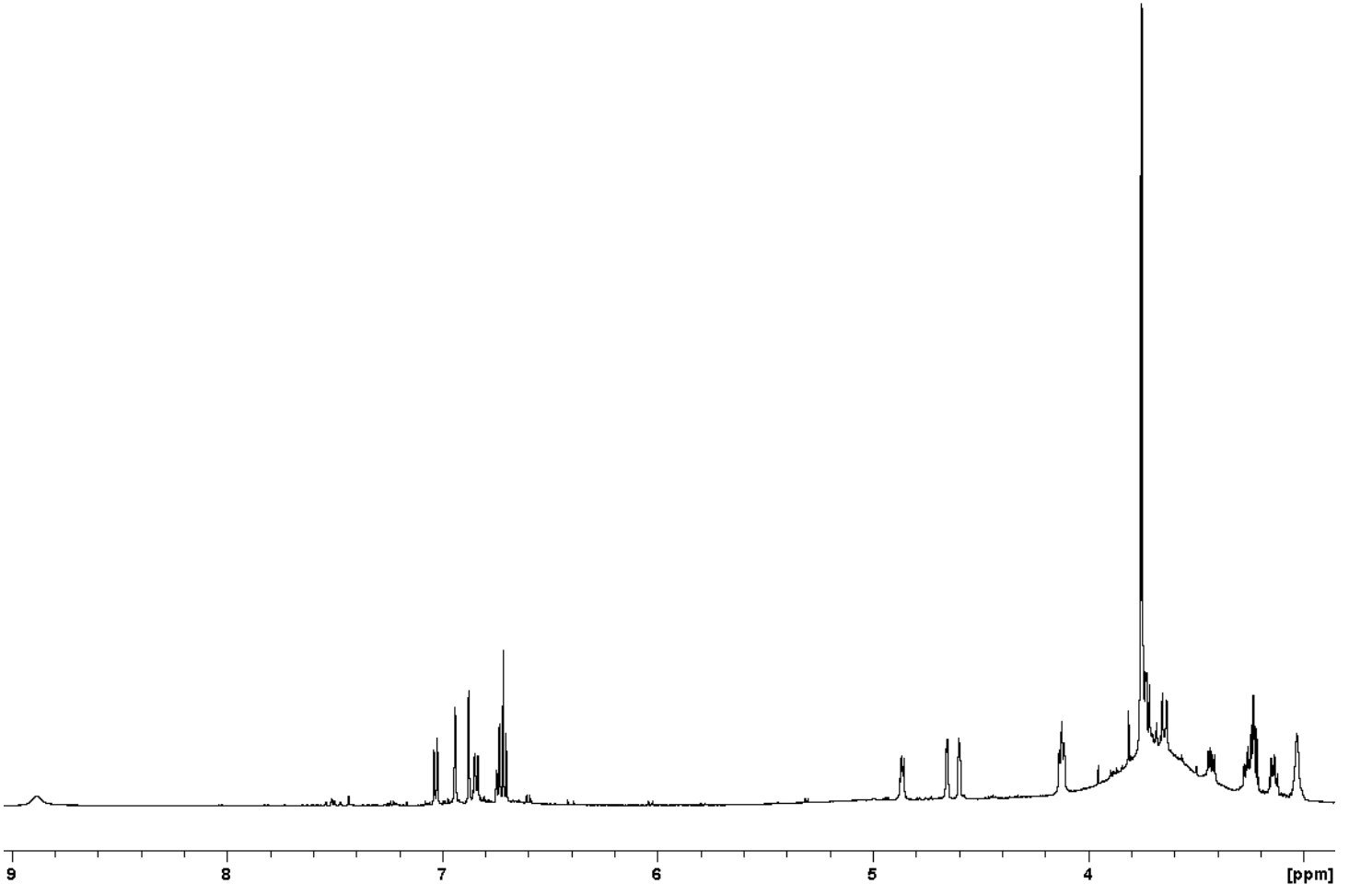
Black signals:
exchange cross peaks



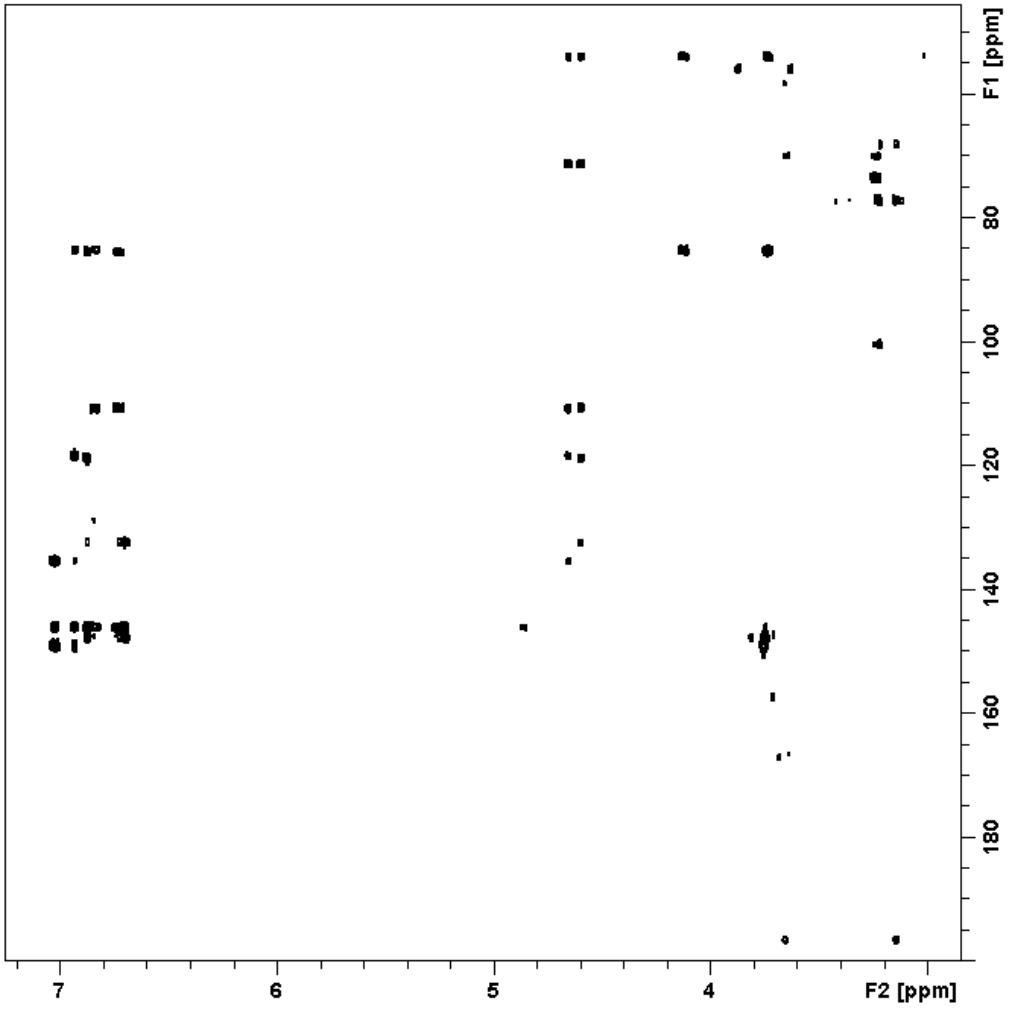
Expanded region of the 2D ^{1}H-^{1}H ROESY spectrum of **2**



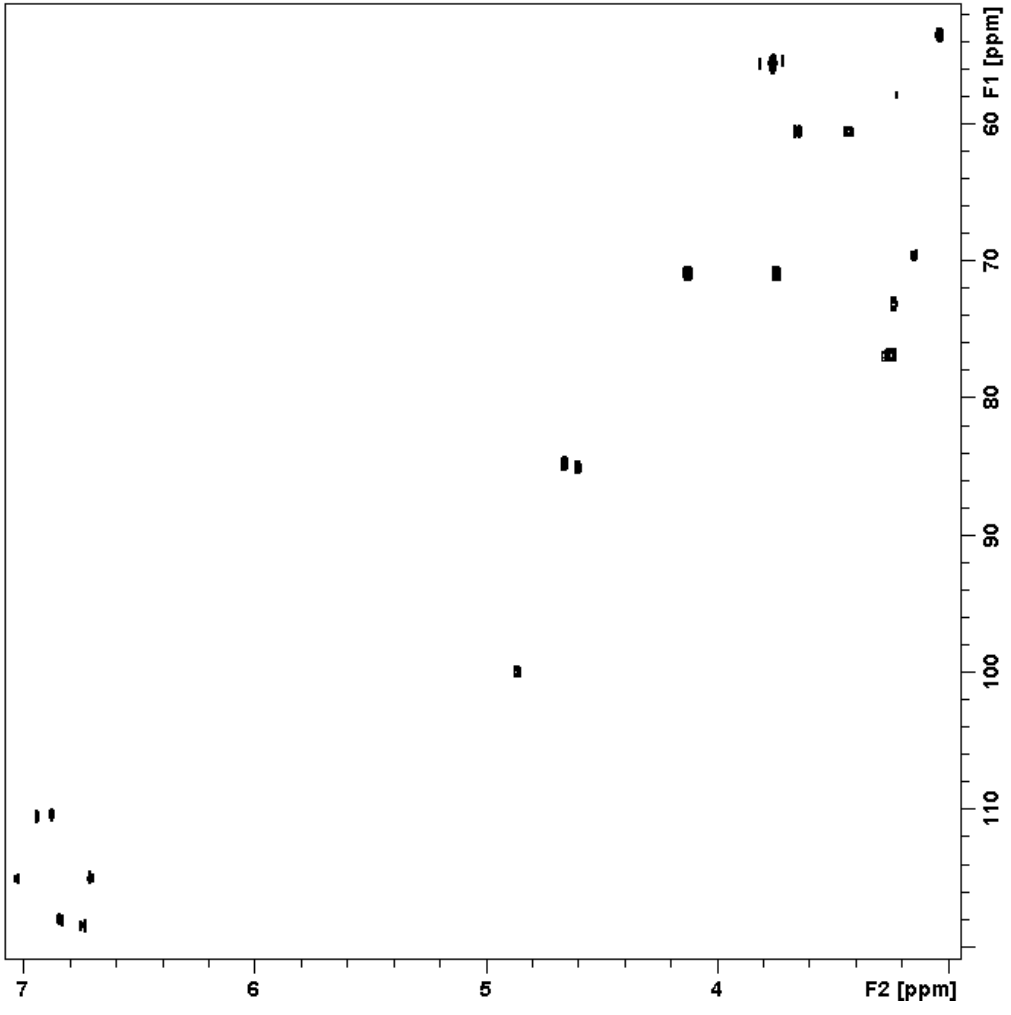
1D ^{1}H NMR spectrum of **3**



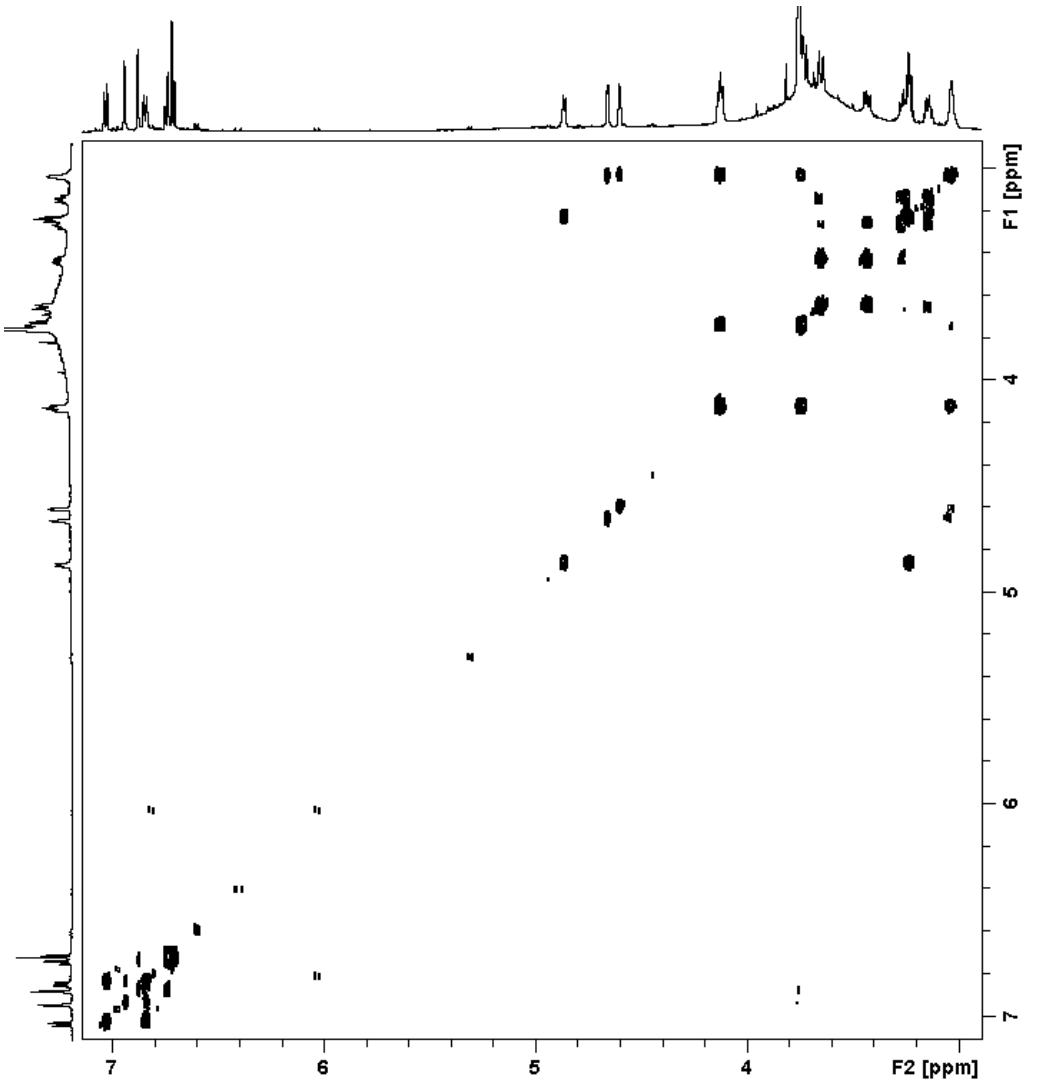
2D ^{1}H-^{13}C HMBC spectrum of **3**



2D ^{1}H-^{13}C HSQC spectrum of **3**



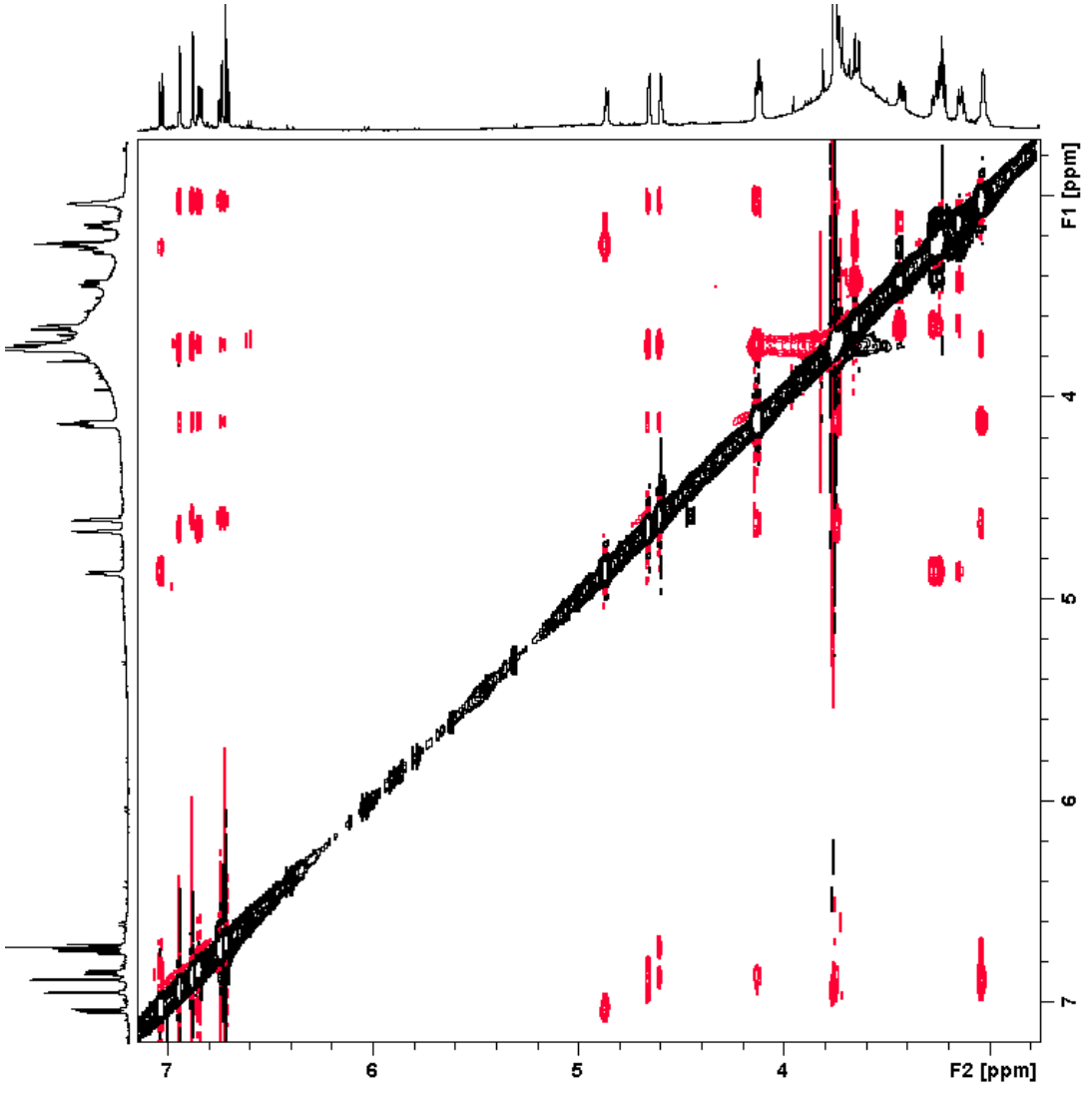
2D ^{1}H-^{1}H COSY spectrum of **3**



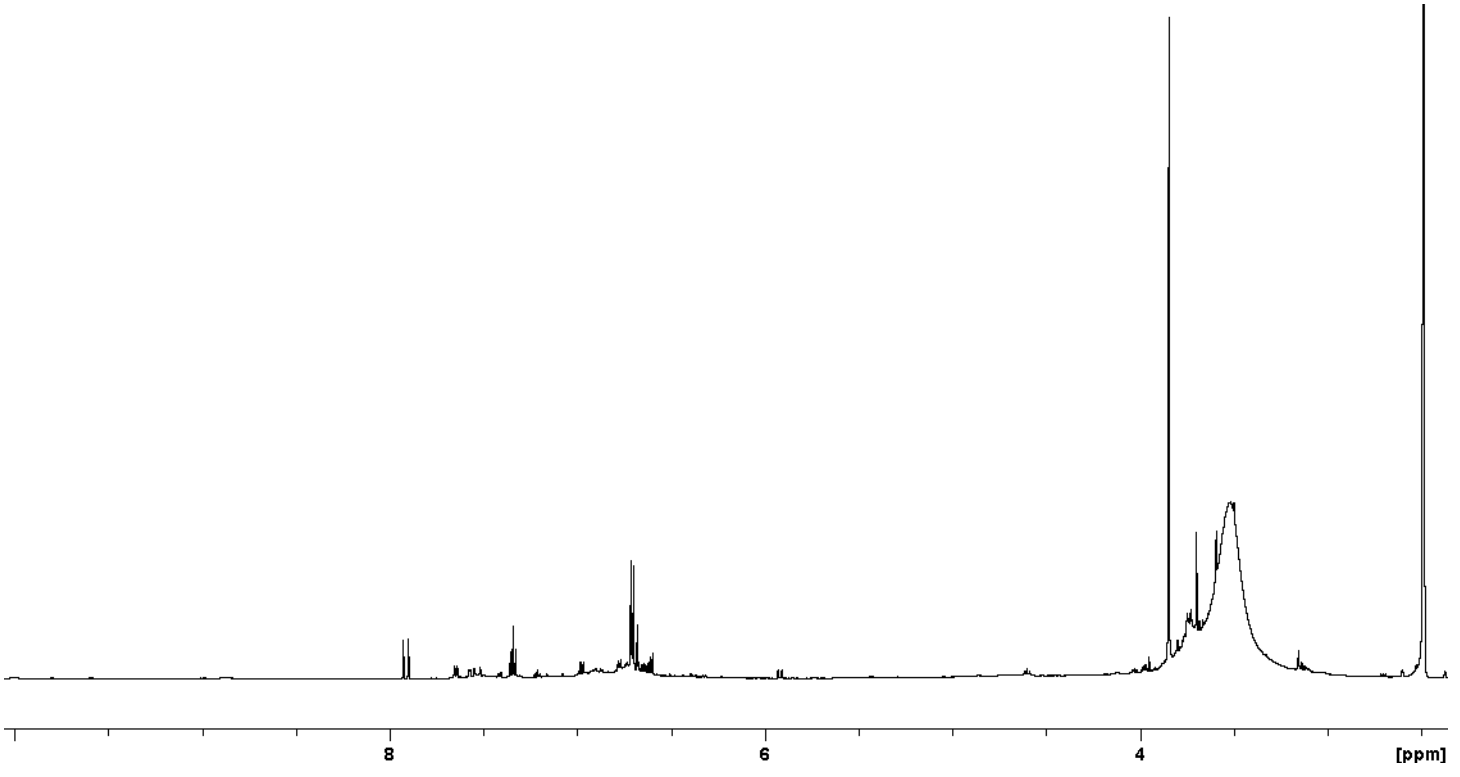
2D ^{1}H-^{1}H ROESY spectrum of **3**

Red signals:
ROE cross peaks

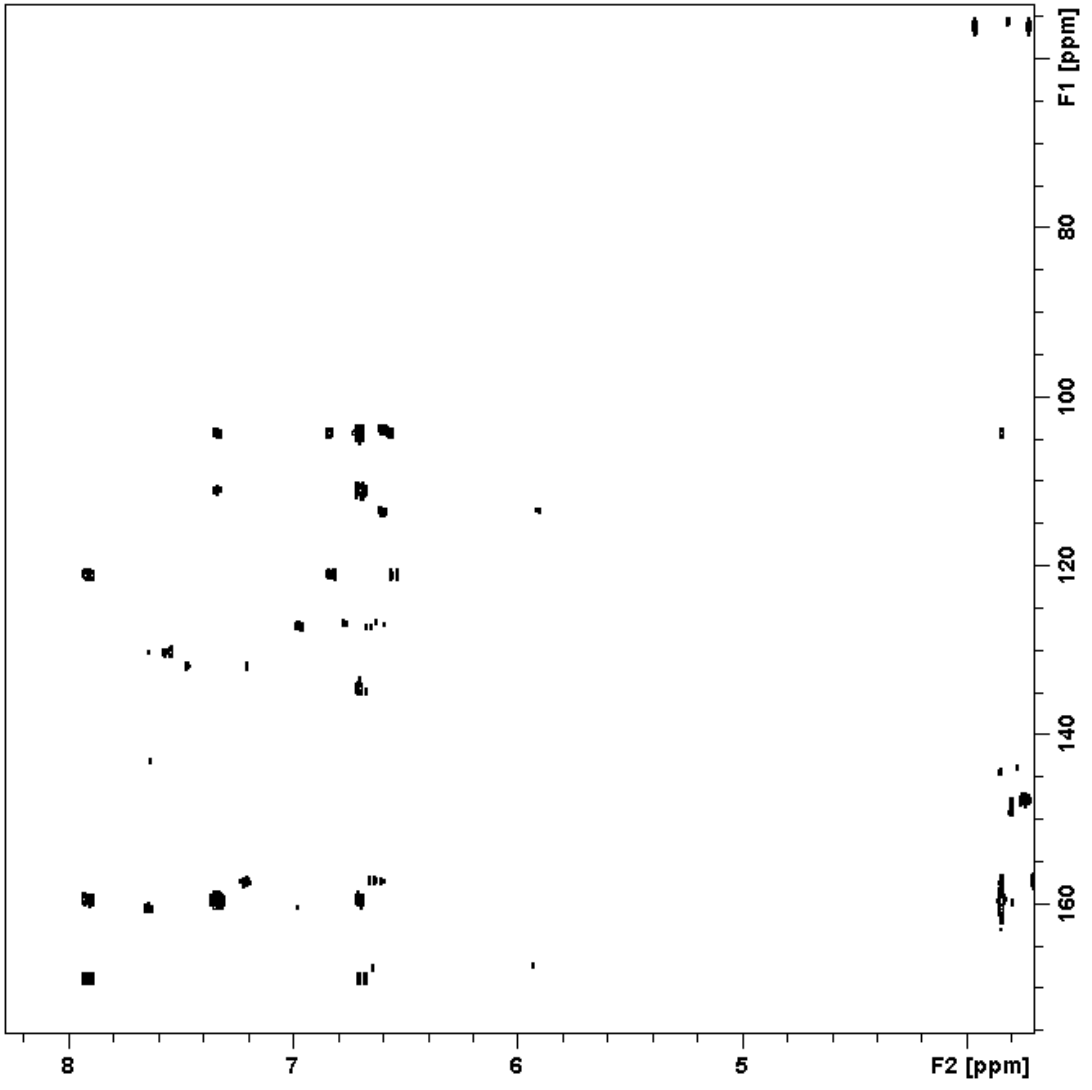
Black signals:
exchange cross peaks



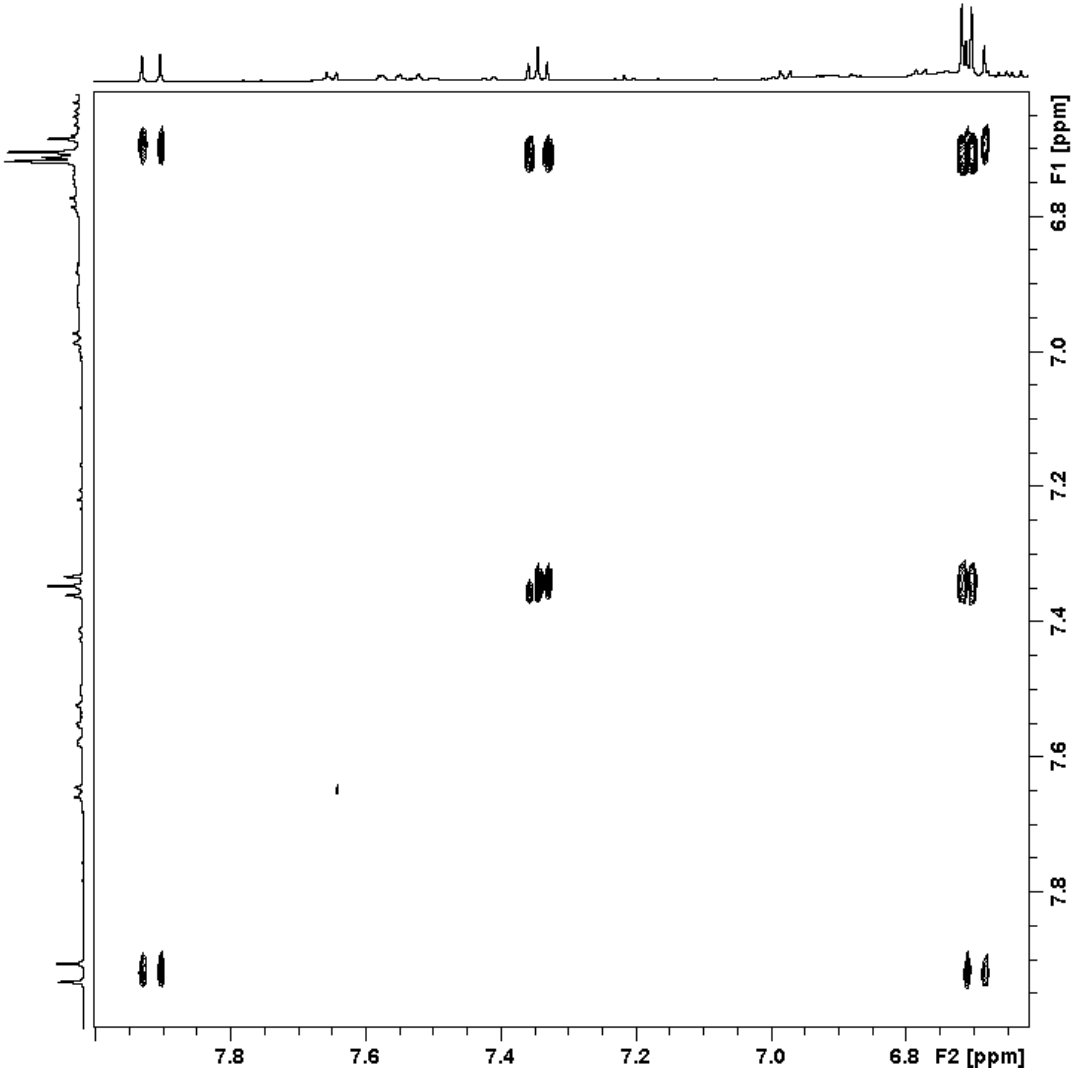
1D ^{1}H NMR spectrum of **8**



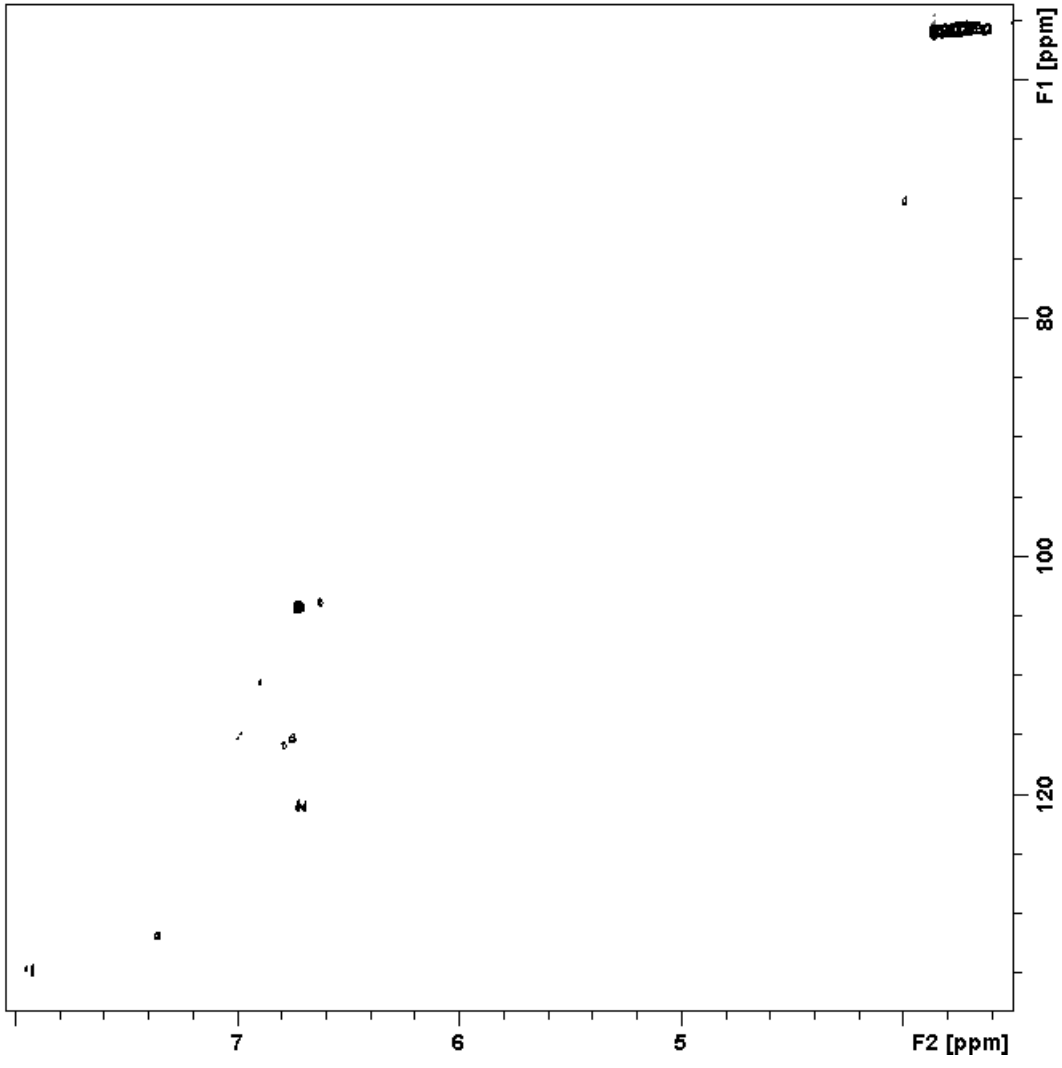
2D ^{1}H-^{13}C HMBC spectrum of **8**



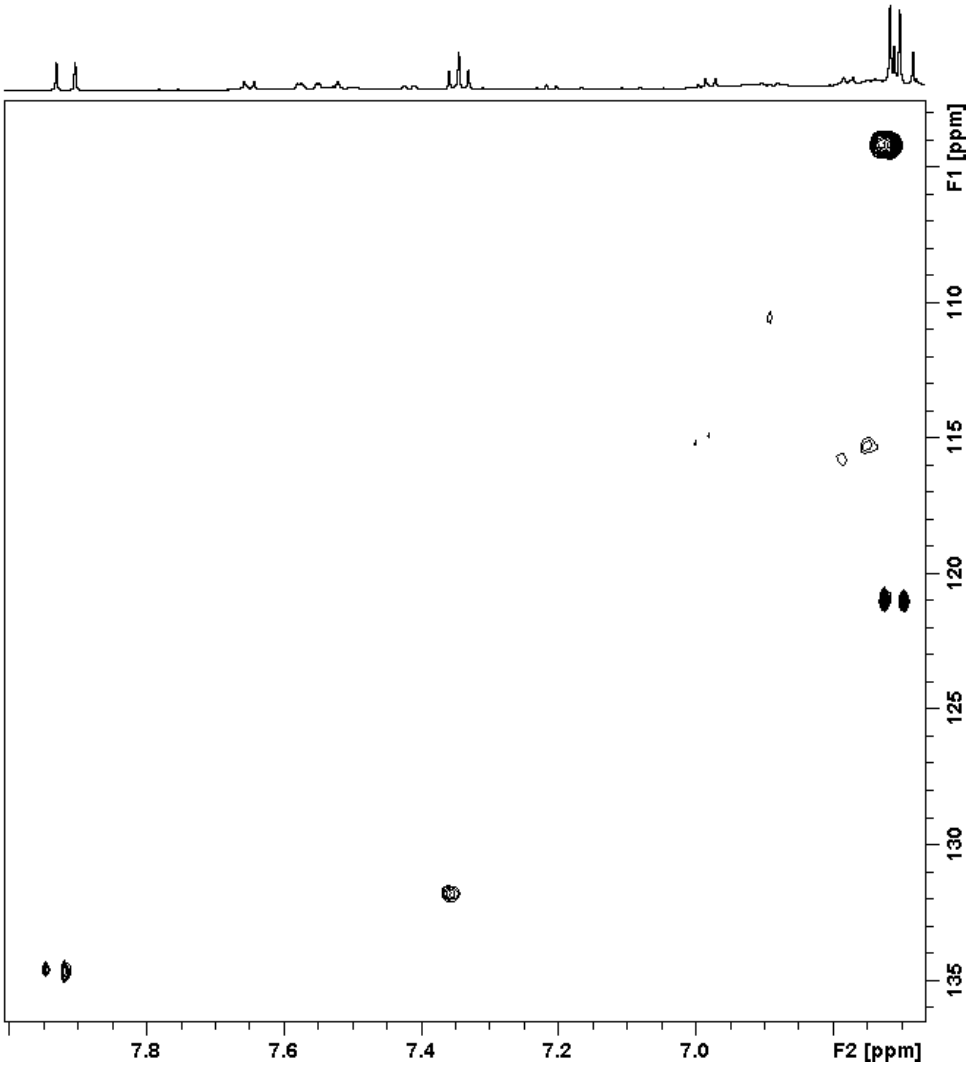
2D ^{1}H-^{1}H COSY spectrum of **8**



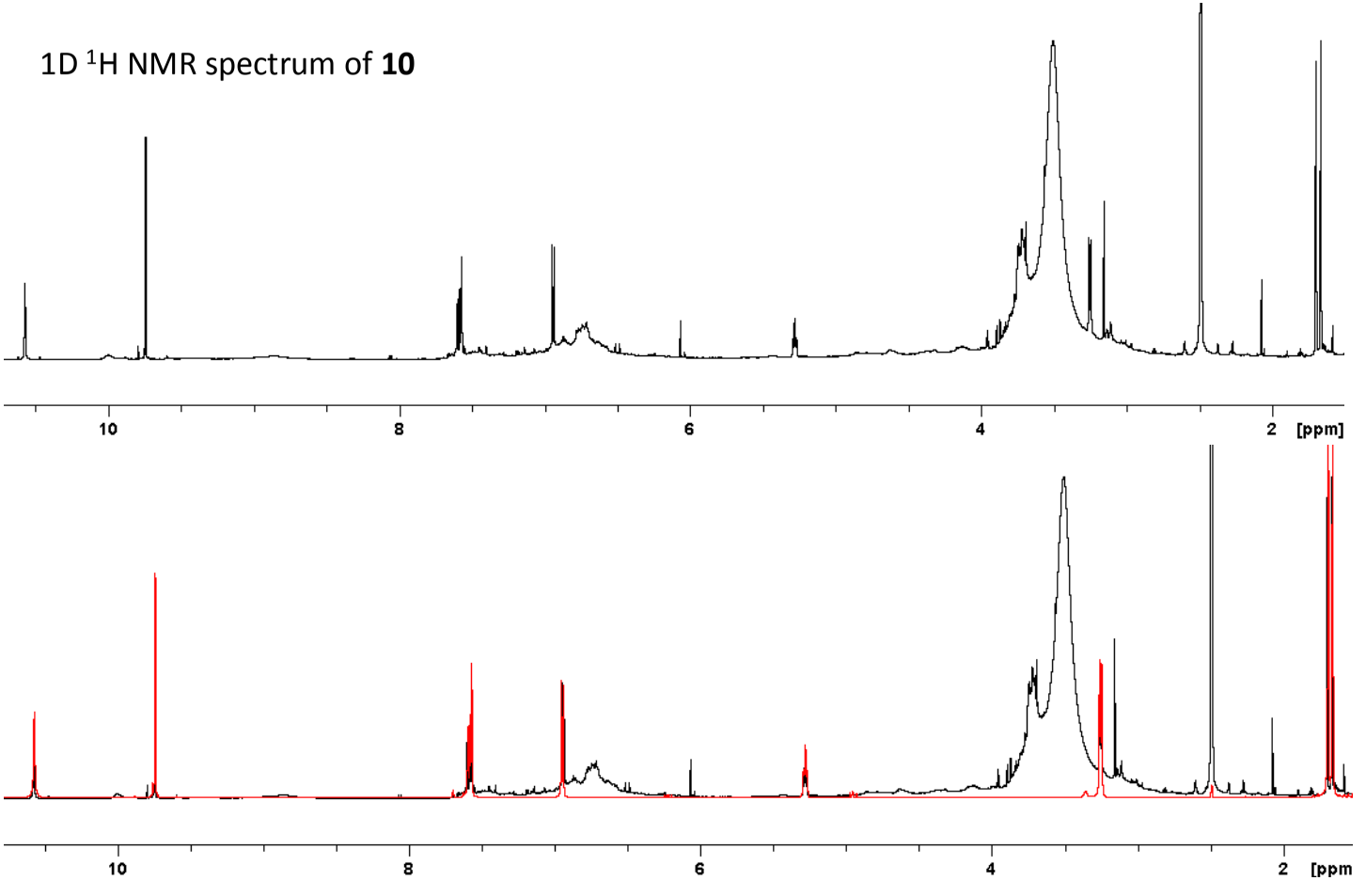
2D ^{1}H-^{13}C HSQC spectrum of **8**



Expanded region of the 2D ^{1}H-^{13}C HSQC spectrum of **8**



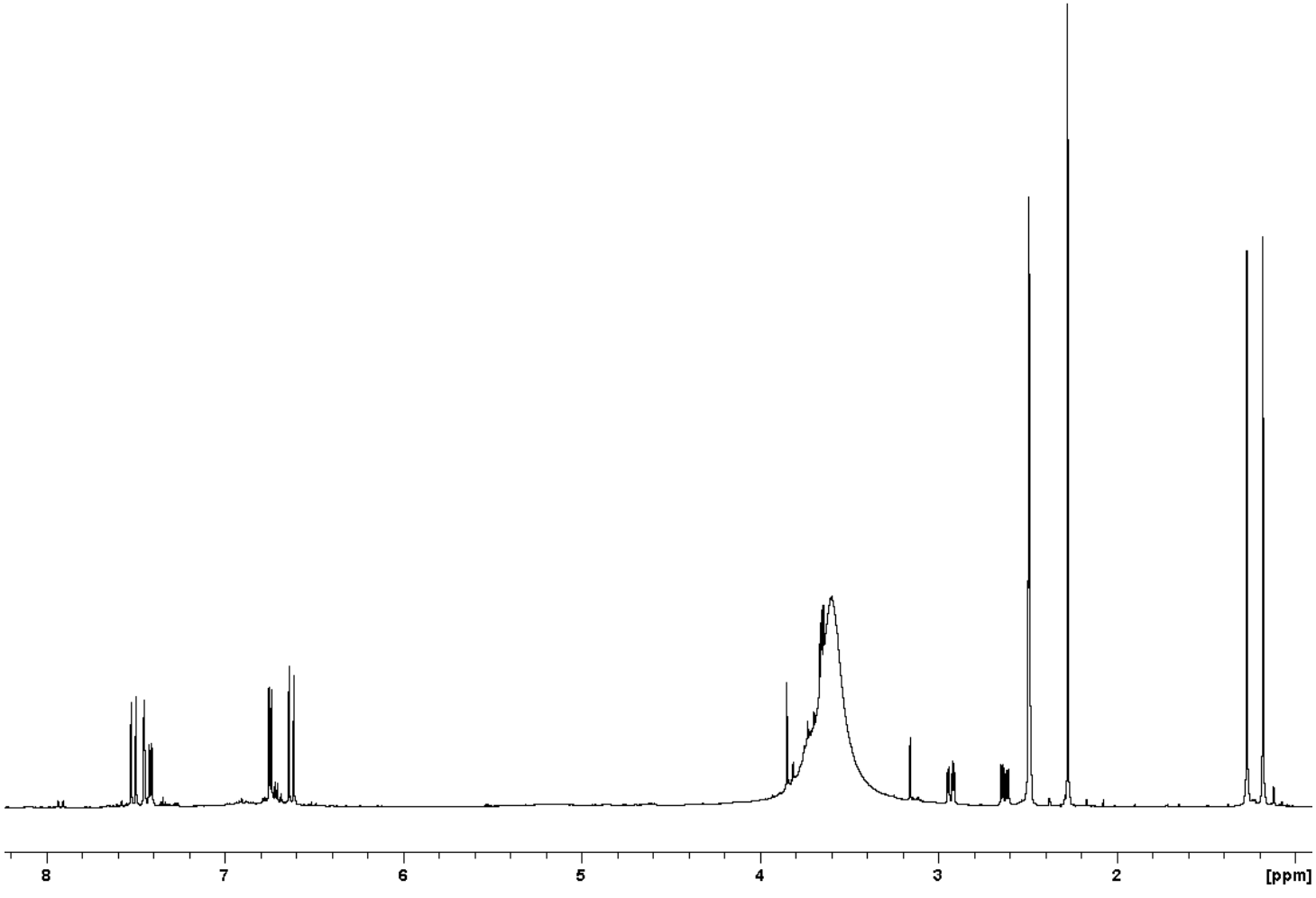
1D ^{1}H NMR spectrum of **10**



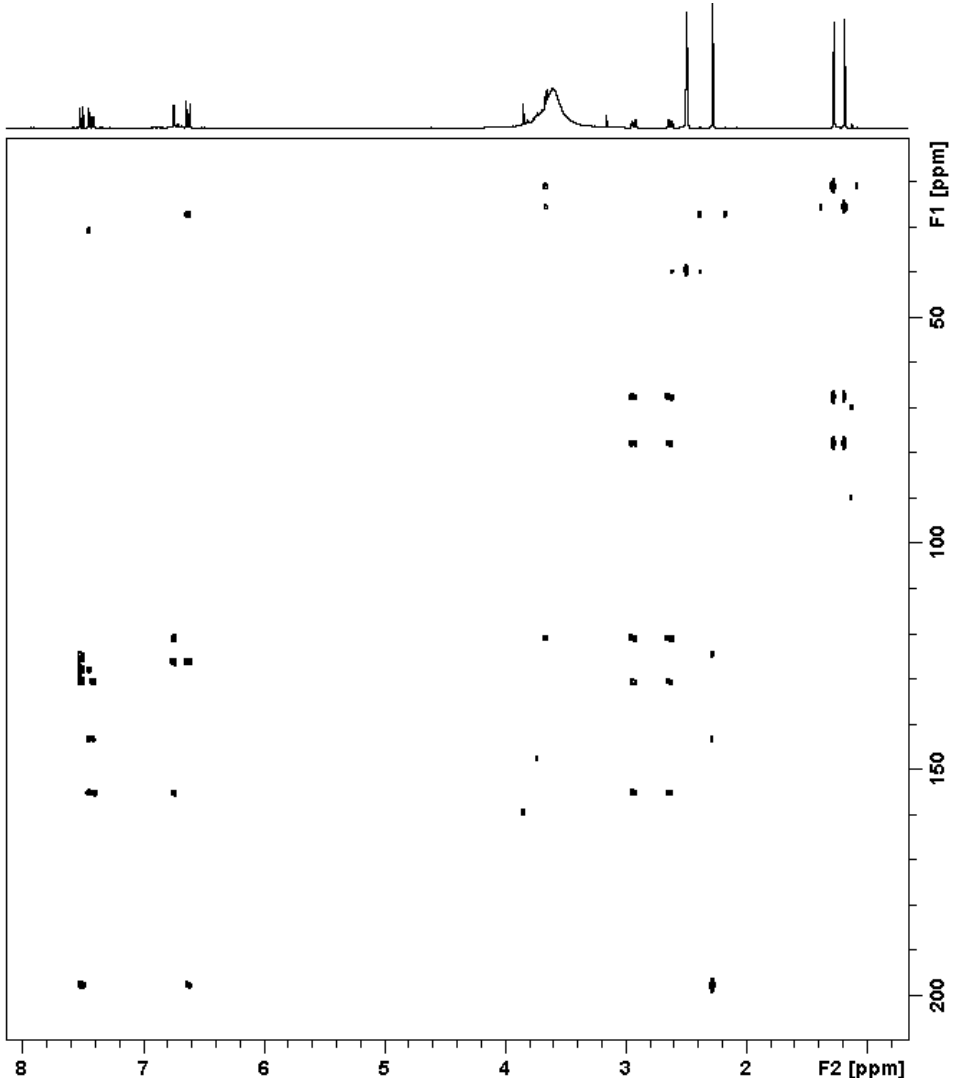
Red signals: 1D ^{1}H NMR spectrum of commercially available compound **10**

Black signals: 1D ^{1}H NMR spectrum of compound **10**

1D ^{1}H NMR spectrum of **11**



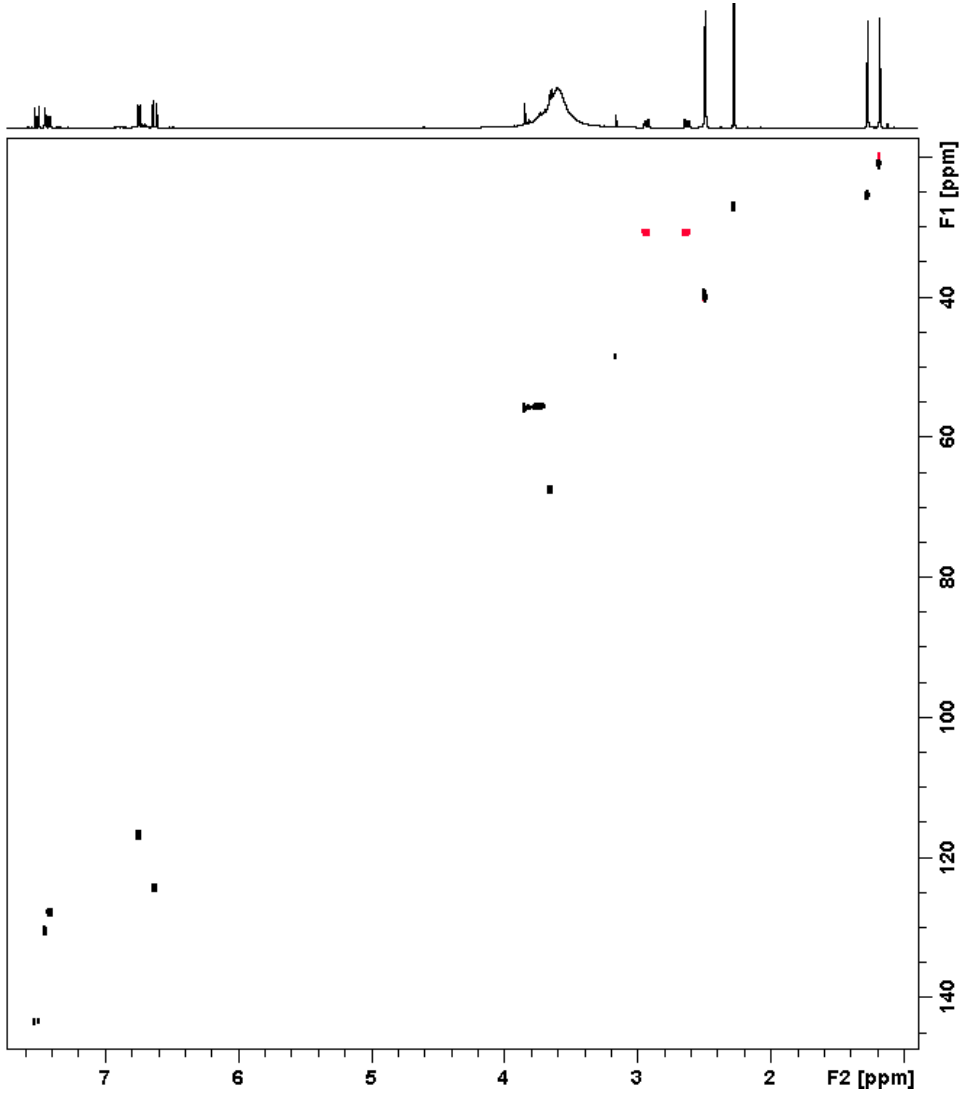
2D ^{1}H-^{13}C HMBC spectrum of **11**



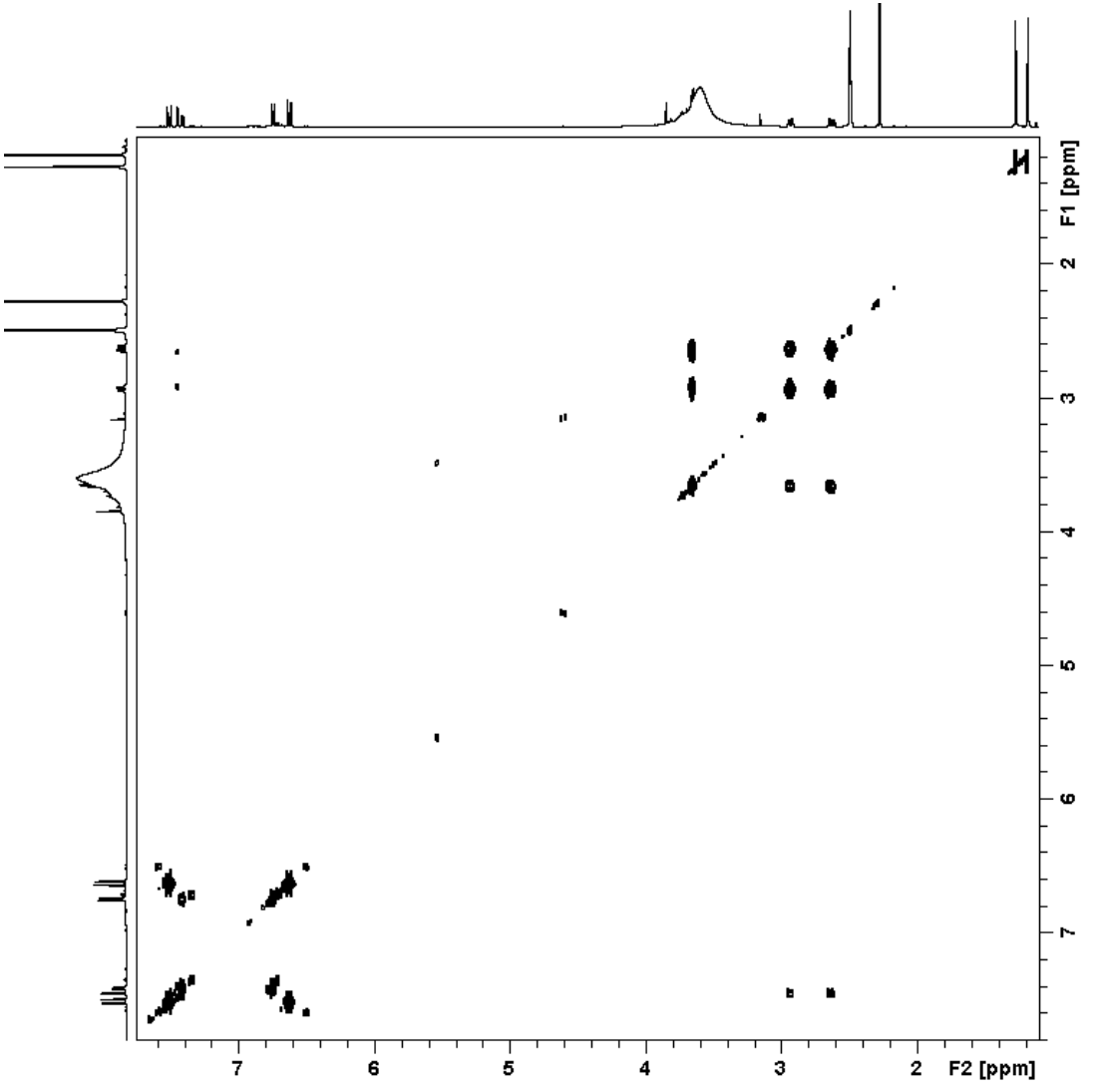
2D ^{1}H-^{13}C HSQC spectrum of **11**

Red signals: CH_2

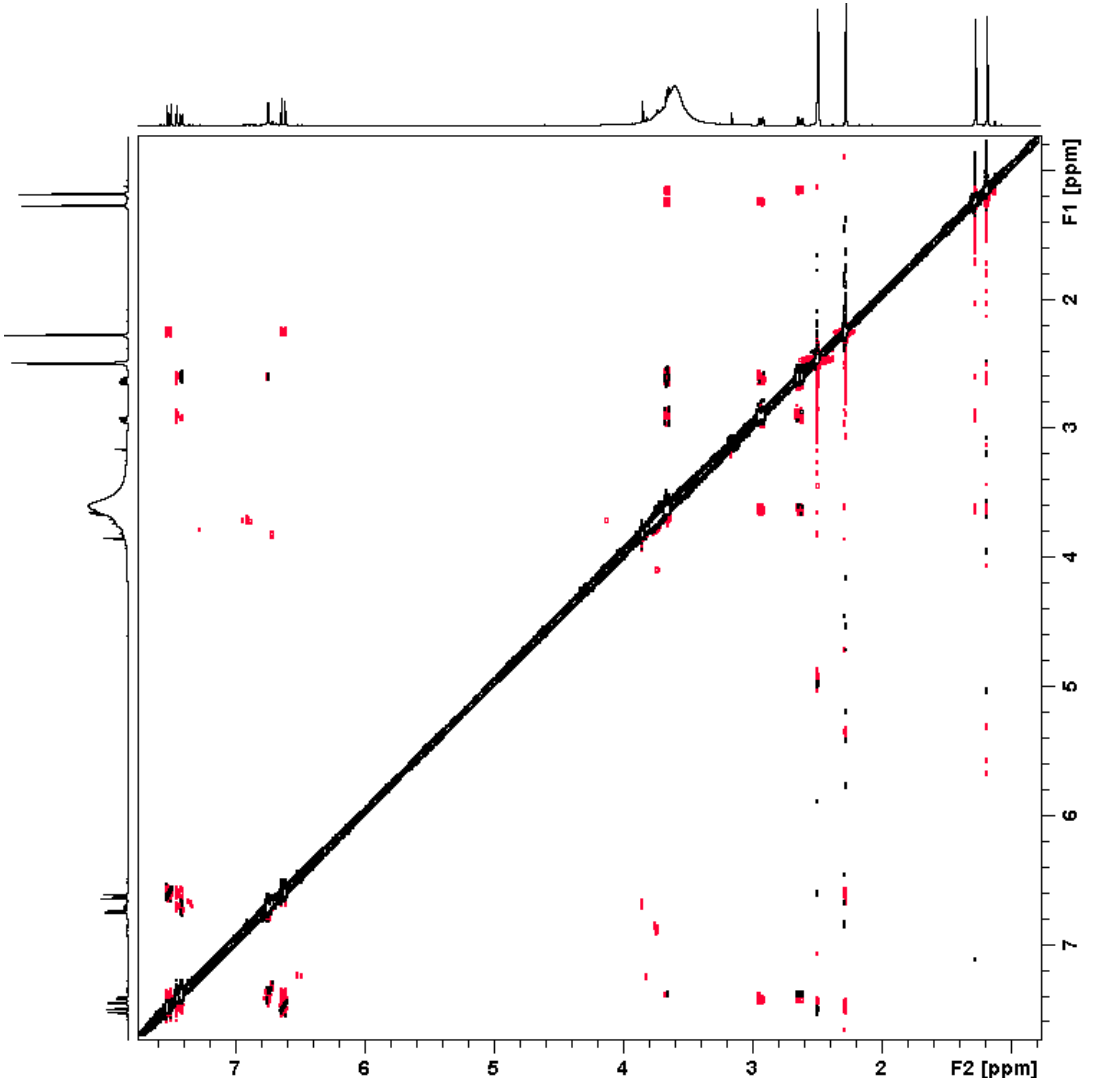
Black signals: CH_3 and CH



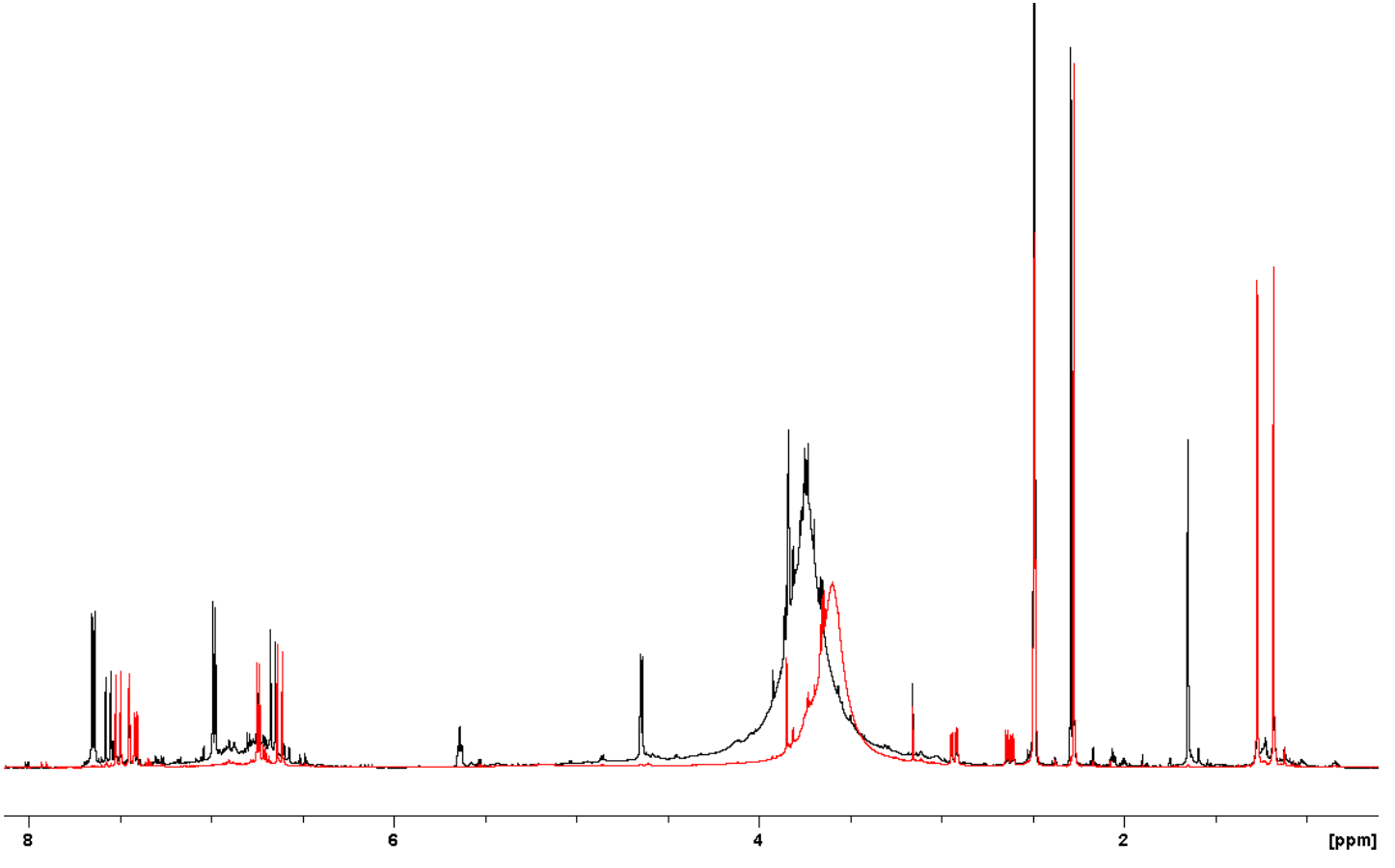
2D ^{1}H-^{1}H COSY spectrum of **11**



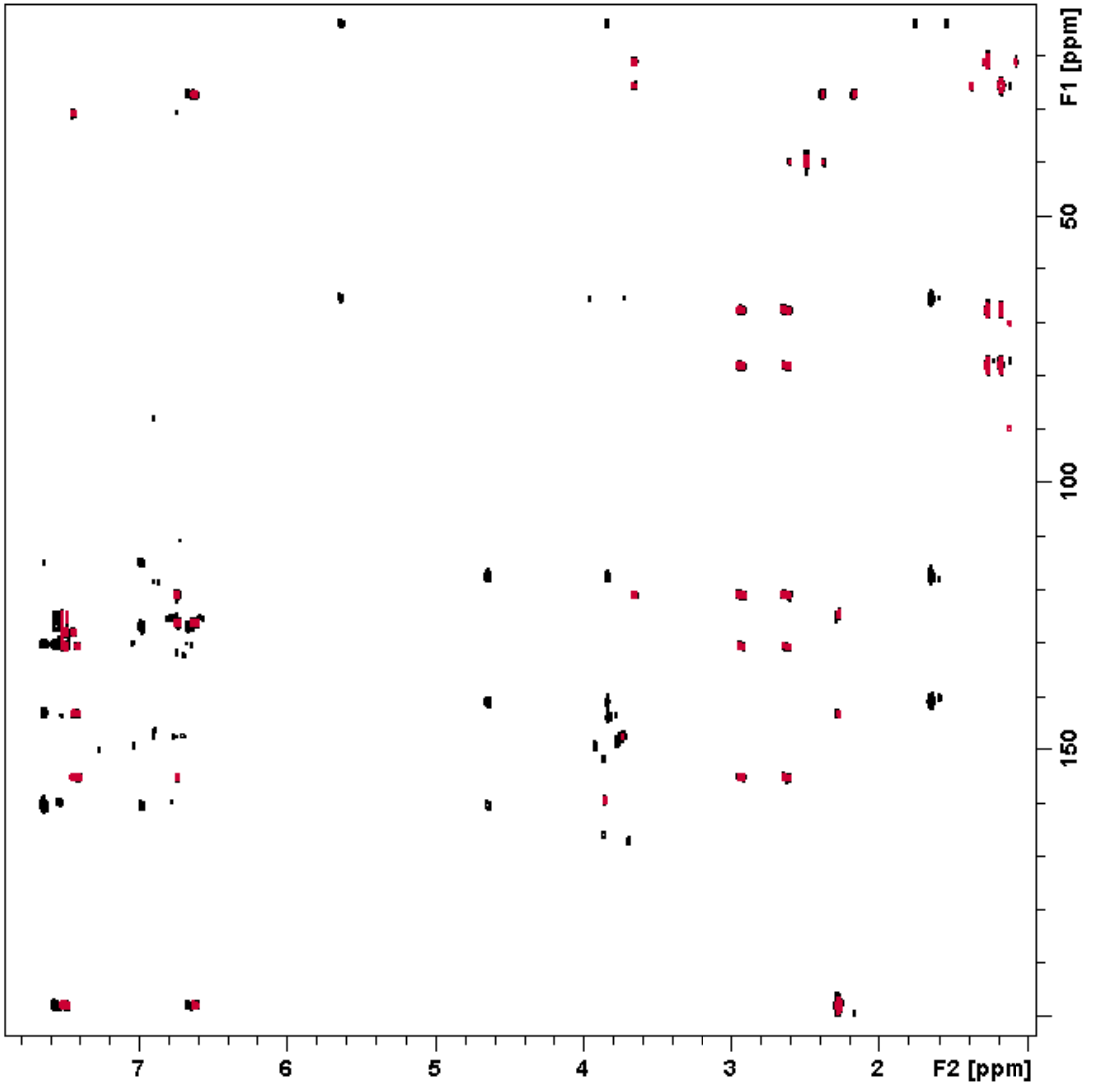
2D ^{1}H-^{1}H ROESY spectrum of **11**

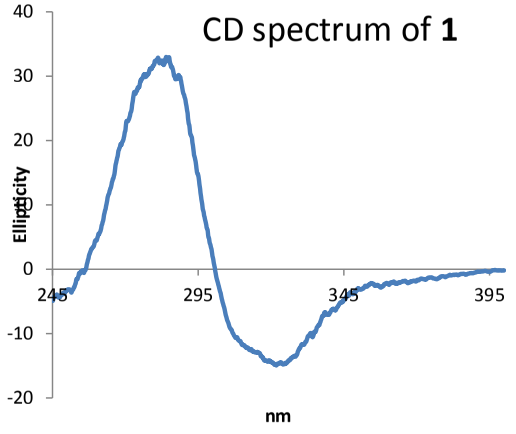


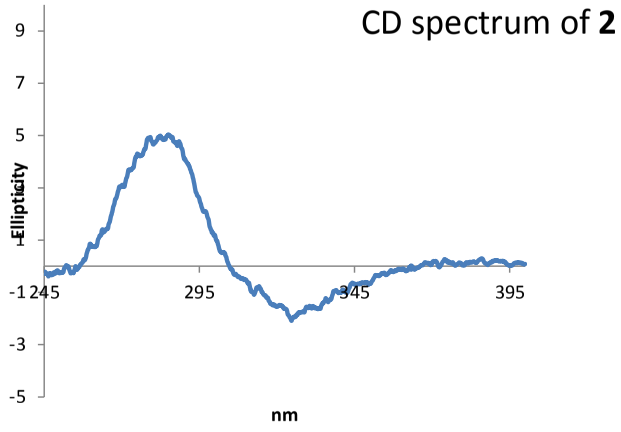
1D ^{1}H NMR spectrum of **12** (black signals)
and its structural isomer **11** (red signals)

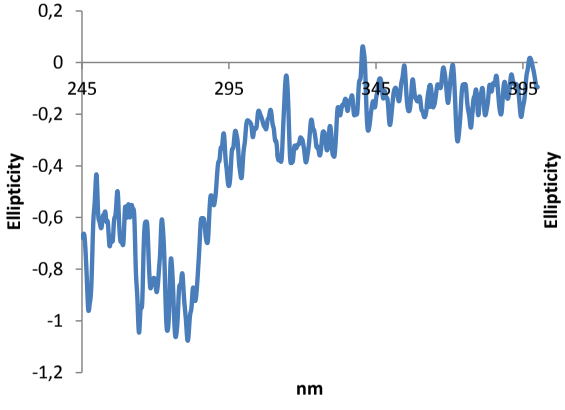


2D ^{1}H-^{13}C HMBC spectrum of **12** (black signals) and its structural isomer **11** (red signals)

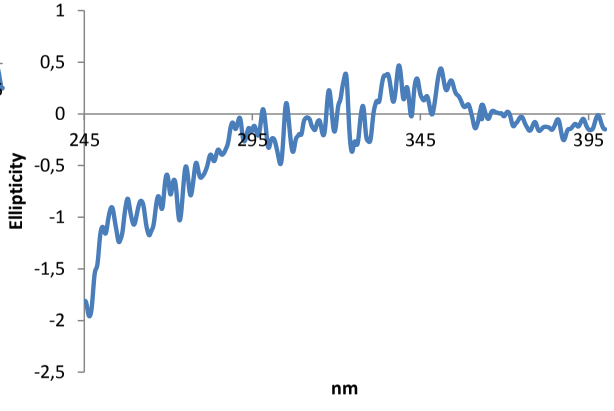




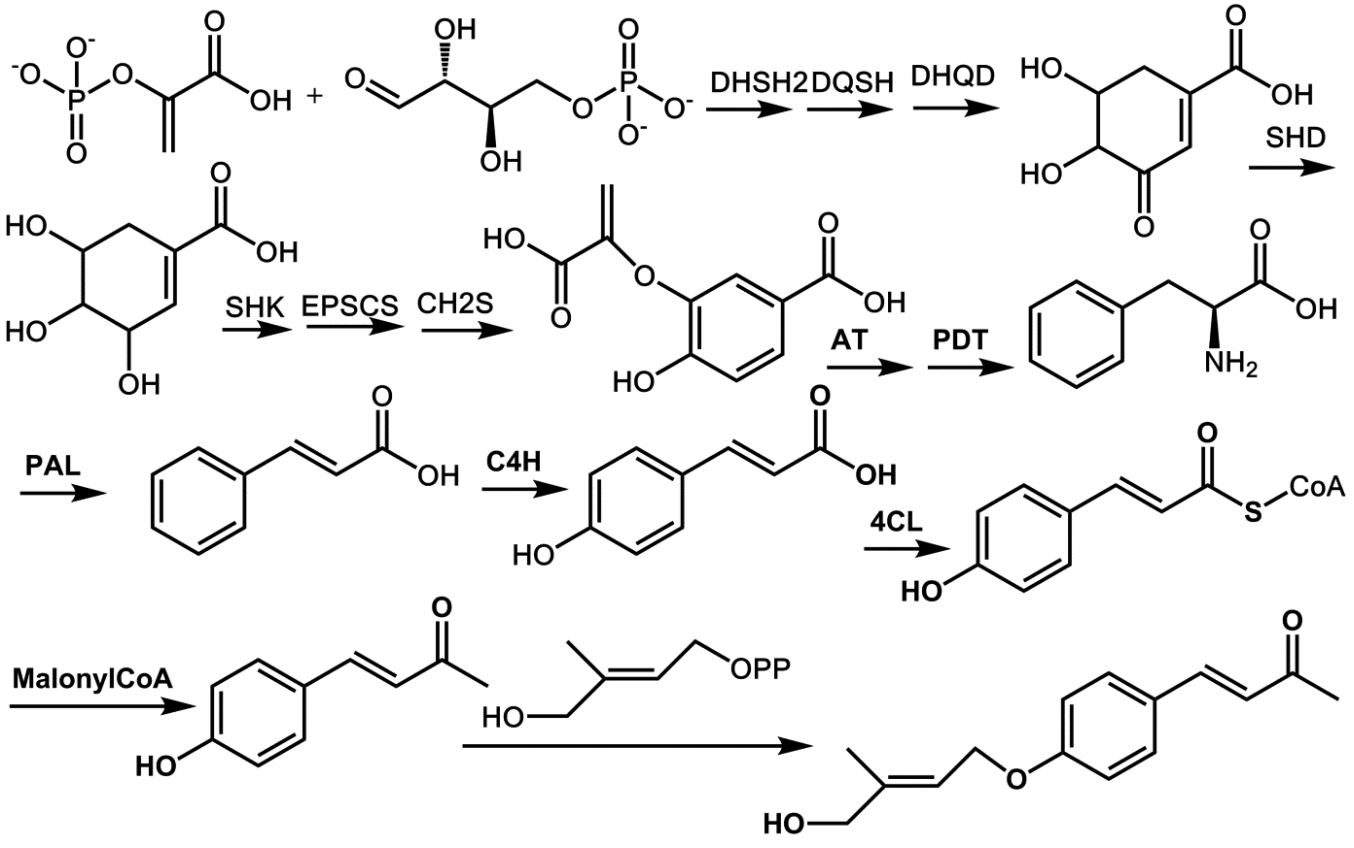




CD spectrum of **3**



CD spectrum of **11**



Proposed biosynthesis of compound **12** based on previously published biosynthetic pathways of the individual building blocks of the molecule (Kakimoto 2003; Vanholme et al. 2012). Compounds **1** and **4** are expected to be biosynthesized through analogous pathways sharing the majority of the biosynthetical precursors shown in the biosynthesis of **12**.