UNIVERSITY OF KWAZULU-NATAL

PHYTOCHEMICAL INVESTIGATION AND TISSUE CULTURE STUDIES ON THE SOUTH AFRICAN KNOB TREES, *ZANTHOXYLUM CAPENSE* AND *SENEGALIA NIGRESCENS*

BY

BODEDE OLUSOLA SUNDAY

2017

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A thesis submitted to the School of Chemistry and Physics, College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville, for the degree of Doctor of Philosophy.

This Thesis has been prepared according to **Format 4** as outlined in the guidelines from the College of Agriculture, Engineering and Science which states:

This is a thesis in which chapters are written as a set of discrete research papers, with an overall introduction and final discussion where one (or all) of the chapters has already been published. Typically, these chapters will have been published in internationally-recognised, peer-reviewed journals.

As the candidate's supervisor(s), I have approved this thesis for submission.

Supervisor:

Signed: ----------Name: ---------- Date: ----------

Co-Supervisor:

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ABSTRACT

Zanthoxylum capense and *Senegalia nigrescens*, of the Rutaceae and Fabaceae families, respectively, are both South African plant species used in traditional medicine and have a common knob-bearing feature. However, there are limited reports on the phytochemistry of these plants that could provide a scientific basis for their ethnomedicinal use. Due to the depletion of these medicinal plants in the wild and the global search for new pharmacologically-active compounds of plant origin, alternative routes to conventional systems of propagation and the harvesting of their bioactive phytocompounds for pharmaceutical applications is deemed necessary. The aim of this research was therefore twofold; firstly, to isolate and characterise the secondary metabolites in the plants and to test them for biological activity and secondly, to promote their conservation using tissue culture techniques.

The phytochemical analysis of *Z. capense* yielded ten compounds including alkaloids, coumarates, lignans, flavonoids, triterpenoids and pigments. Amongst the compounds isolated, chelerythrine (IC_{50} = 95.4 and 153.9 μM) and dodecyl-*trans*-*p*-coumarate (IC_{50} = 15.1 and 182.4 μM) had the highest cytotoxicity in MCF-7 and in Caco-2 tumor cell lines, respectively. The seeds of *Z. capense* were subjected to pre-sowing treatments prior to germination, and soaking in hot water or GA_3 was found to overcome dormancy in the species. The potential of the leaves, knobs and roots of *Z. capense* was further evaluated for the green synthesis of silver nanoparticles (AgNPs) and the results showed the leaves to be the most effective bioreductants. The biosynthesised AgNPs were more effective than sodium hypochlorite (NaOCl) and sodium dichloroisocyanurate (NaDCC) in controlling *in vitro* fungal contamination. Still, the former did not eliminate the fungi but delayed their emergence, thereby promoting the impetus for the

identification and characterisation of *Z. capense* fungal endophytic strains using standard DNA extraction and sequencing methods.

In *S. nigrescens*, a series of lupane-type triterpenoids, kaurene diterpenoids, flavonoids and a long-chain alcohol were isolated. A new *ent*-kaurene diterpenoid (*ent*-kaur-15-en-18,20-diol) and *ent*-kaur-15-en-18-ol, found for the first time in the plant, were amongst the compounds isolated. Compounds and crude extracts from *S. nigrescens* were tested for their antimicrobial potential against nine bacterial strains including a well-known quorum sensing inhibitor indicator strain, *Chromobacterium violaceum*. The novel *ent*-kaurene diterpene (*ent*-kaur-15-en-18,20-diol) and the flavonoids (quercetin-3-*O*-methyl ether and melanoxetin) showed promising anti-quorum sensing activity, following a qualitative agar-overlay assay. A micropropagation protocol was developed for *S. nigrescens* using explants derived from its seedlings obtained from mechanically-scarified seeds sown on full strength Murashige and Skoog basal medium (MS). There were no significant differences in the number of shoots produced across all the treatments of kinetin (KIN) and benzylaminopurine (BAP) that were tested, although the treatment containing 1.0 mg L^{-1} KIN produced a significantly higher shoot length (14.17 mm) than 0.5, 1.0 and 2.0 mg L^{-1} BAP (7.67, 6.75 and 8.70 mm, respectively). Rooting of *S. nigrescens* was best achieved using one quarter strength MS supplemented with either indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA). The auxin, 2,4-dichlorophenoxyactic acid, was found to be effective for inducing callus from *S. nigrescens* explants. Spectrochemical analysis of the root-derived calli revealed their potential to produce the phytocompounds, quercetin, quercetin-3-*O*-methyl ether, *ent*-kaur-15-en-18-ol and *ent*-kaur-15-en-18,20-diol.

Chemomorphological distribution in *Z. capense* showed knobs to possess the most biologically-active compounds as chelerythrine and dodecyl-*trans*-*p*-coumarate were isolated from knobs only. On the other hand, there was no difference in the phytochemical profile of

the stem bark and knobs of *S. nigrescens*. The findings of this research provide a scientific rationale for the use of both species in traditional medicine. The therapeutic potential of the new compounds found in *S. nigrescens* may be synthetically enhanced to produce new drugs. The identification of the fungal endophytes that caused extensive fungal contamination in *Z. capense in vitro* cultures can provide much needed information for decontamination protocols such as the selection of fungicides. The micropropagation and callus induction studies on *S. nigrescens* provide preliminary information towards the large-scale propagation of the plant.

SUMMARY OF ISOLATED COMPOUNDS

Compounds (A1 – A10) isolated from *Z. capense* (chapter three)

A1 chelerythrine

A2 6-hydroxydihydrochelerythrine

A3 rutaecarpine

A4 sesamin

A5 dodecyl-*trans*-*p*-coumarate

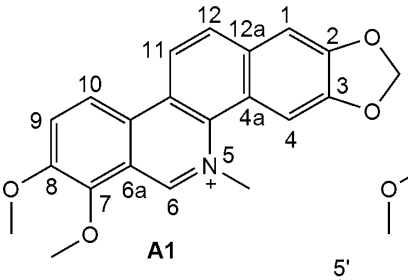
A6 catechin

A7 lupeol

A8 *β*-sitosterol

A9 pheophytin a

A10 lutein



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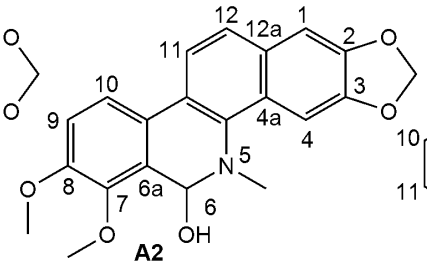

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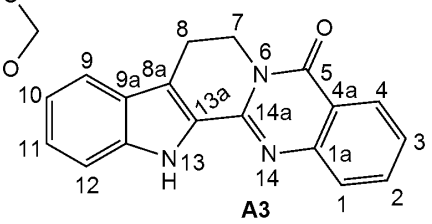

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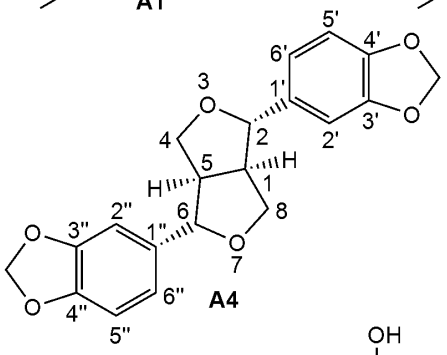

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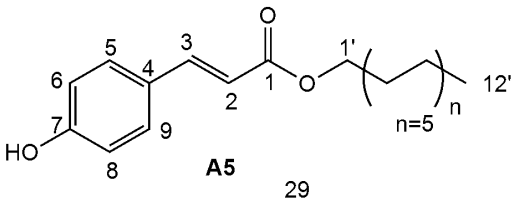

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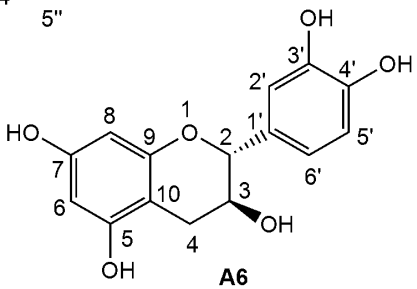

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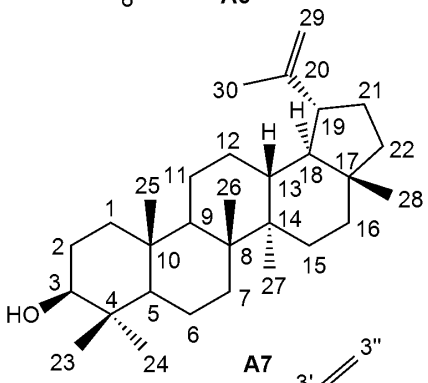

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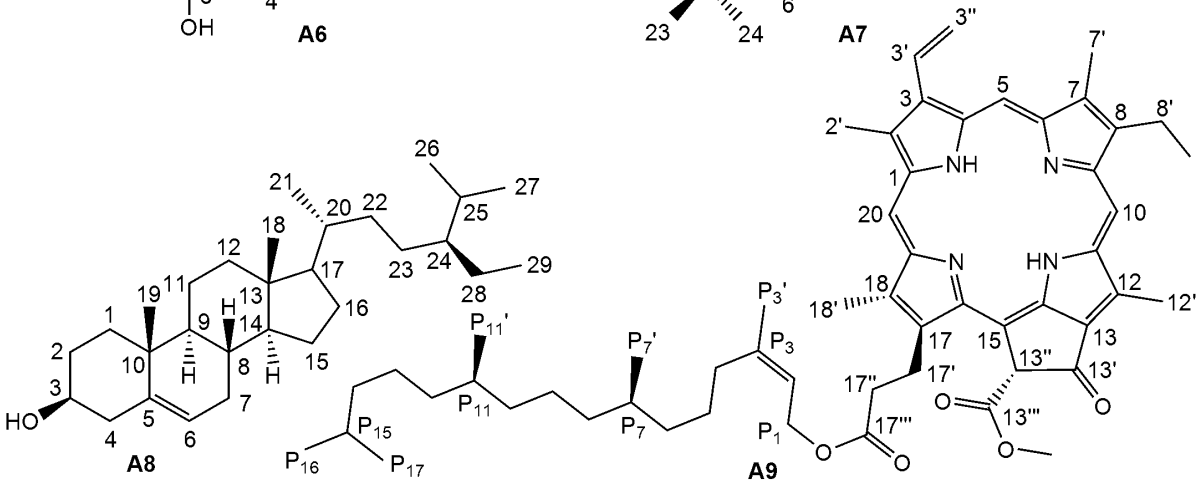
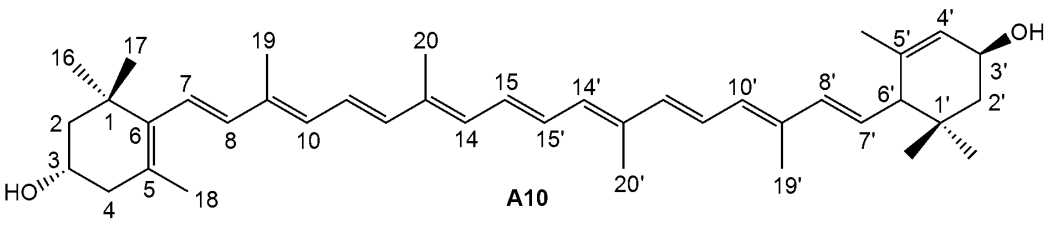


A8 A9



A10

Compounds (B1 – B11) isolated from *S. nigrescens* (chapter six)

B1 30-hydroxylup-20(29)-en-3*β*-ol

B2 3*β*-hydroxy-20(29)-en-lupan-30-al

B3 lupeol (same as **A7**)

B4 stigmasterol

B5 *ent*-kaur-15-en-18-ol

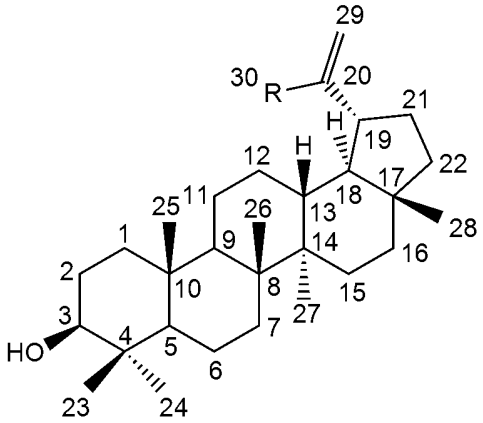
B6 *ent*-kaur-15-en-18,20-diol

B7 tetracosan-1-ol

B8 melanoxetin

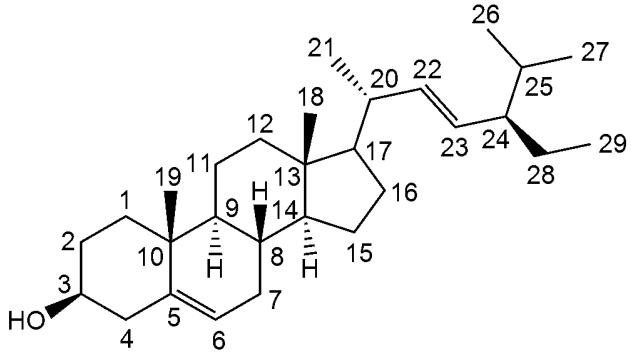
B9 quercetin

B10 quercetin-3-*O*-methyl ether



B1 R = CH_2OH **B3** R = CH_3

B2 R = CHO



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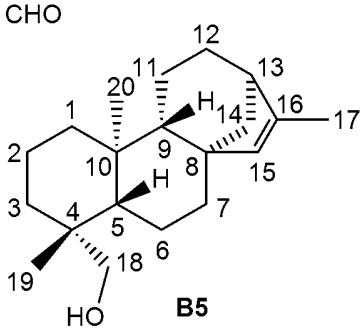

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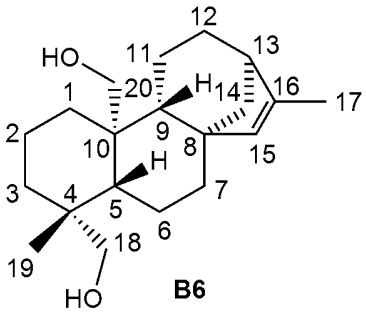

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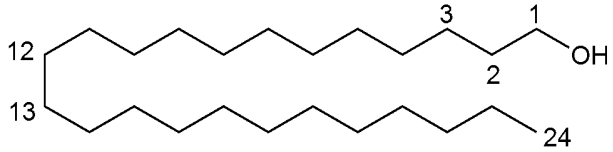

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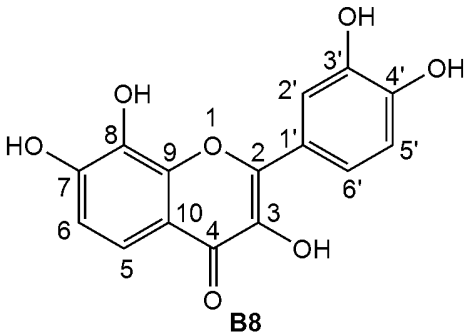

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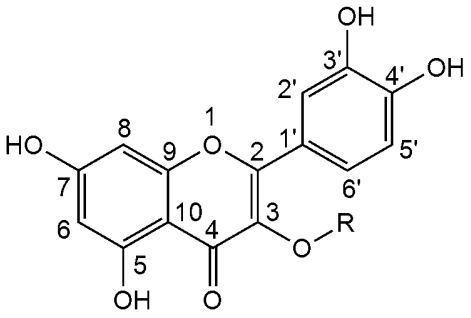

B9 R = H **B10** R = CH_3

ABBREVIATIONS

^{13}C-NMR	C-13 nuclear magnetic resonance spectroscopy
^{1}H-NMR	proton nuclear magnetic resonance spectroscopy
2,4-D	2,4-dichlorophenoxyacetic acid
Ac	acetate
ANOVA	analysis of variance
BAP	benzylaminopurine
br	broad resonance
c	concentration
CC	column chromatography
COSY	correlated spectroscopy
d	doublet
dd	double doublet
DEPT	distortionless enhancement by polarization transfer
DPPH	2,2-diphenyl-1-picrylhydrazyl
EIMS	electron impact mass spectroscopy
FRAP	ferric reducing antioxidant potential
FTIR	fourier transform infrared
GC-MS	gas chromatography-mass spectrometry
HMBC	heteronuclear multiple bond coherence

HRMS	high resolution mass spectroscopy
HSQC	heteronuclear single quantum coherence
Hz	hertz
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
IR	infrared
KIN	kinetin
m	multiplet
Me	methyl
mp	melting point
MS	Murashige and Skoog basal medium
NOESY	nuclear overhauser effect spectroscopy
PGR	plant growth regulator
RSA	radical scavenging activity
s	singlet
t	triplet
TDZ	thidiazuron
TLC	thin layer chromatography
UV	ultraviolet

DECLARATIONS

DECLARATION 1 - PLAGIARISM

I, Bodede Olusola Sunday, declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

   a. Their words have been re-written but the general information attributed to them has been referenced.

   b. Where their exact words have been used, then their writing has been placed in italics and inside quotation marks, and referenced.

5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

Signed: …….

DECLARATION 2 - PUBLICATIONS

Publication 1

Bodede, O., Moodley, R., Shaik, S., Singh, M. **2017**. Phytochemical analysis with antioxidant and cytotoxicity studies of the bioactive principles from *Zanthoxylum capense* (small knobwood), *Anticancer Agents in Medicinal Chemistry*, 17 (4), 627 – 634.

Publication 2

Bodede, O., Shaik, S., Moodley, R. **2015**. Germination response of *Zanthoxylum capense* (small knobwood) seed to different pre-treatment protocols, *African Journal of Traditional, Complementary and Alternative Medicine,* 12 (5), 70-73.

Publication 3

Bodede, O., Shaik, S., Govinden, R., Moodley, R. **2017**. Evaluating the bioreducing potential of the leaves, knobs and roots of *Zanthoxylum capense* (small knobwood) for the synthesis of silver nanoparticles, applicable to *in vitro* fungal contamination control, *Advances in Natural Sciences: Nanoscience and Nanotechnology*, 8 (2017) 045007. https://doi.org/10.1088/2043-6254/aa84ed

Publication 4

Bodede, O., Shaik, S., Cheniah, H., Singh, P., Moodley, R. Quorum sensing inhibitory potential and *in silico* molecular docking of novel diterpenoids and flavonoids from *Senegalia nigrescens*. *Journal of Ethnopharmacology*.

Publication 5

Bodede, O., Shaik, S., Moodley, R. Establishment of *in vitro* seed germination and micropopagation protocols for *Senegalia nigrescens*, a potential anti-virulent species. Manauscript prepared for submission.

Publication 6

Bodede, O., Shaik, S., Moodley, R. Evaluation of the bioactive flavonols and *ent*-kaurenes in the 2,4-dichlorophenoxyacetic acid-induced calli of *Senegalia nigrescens* using FTIR and GC-MS. Manauscript prepared for submission.

Publication 7

Bodede, O., Shaik, S., Moodley, R. **2016**. Knob-derived phytocompounds: A case study on two South African knob trees, *South African Journal of Botany,* 103, 309. (Published conference paper)

In all the above publications, I carried out all the experimental work and wrote the manuscripts. The co-authors' respective expertise was employed to verify the scientific content and accuracy of my findings. They were also involved in the editing of the manuscripts.

Signed: …….

DECLARATION 3 - CONFERENCES

- 2nd International Symposium on Natural Products, 23 – 25 September 2014, Cape Town, South Africa.
    - **Poster**: Phytochemical and biotechnological studies of *Zanthoxylum capense*: An endangered South African medicinal species.
- Joint South African Association of Botanists (SAAB)-Southern African Society for Systematic Biology (SASSB) Conference, 10 – 13 January 2016, Bloemfontein, South Africa.
    - **Oral**: Knob-derived Phytocompounds: A case study on two South African knob trees (*Zanthoxylum capense* and *Acacia nigrescens*).
- The 35th National Medicinal Chemistry Symposium, 26 – 29 June 2016, Chicago, Illinois, USA.
    - **Poster**: Antibacterial activity and anti-quorum sensing potential of triterpenes and flavonoids from *Senegalia nigrescens*.

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Lastly, I would like to specially thank my family; my parents Mr and Mrs Bodede, for wholesome support, encouragement and sincere love that they have shown me from my cradle to date, my brother Oluwatobiloba Bodede for his understanding, love and support throughout my journey to PhD.

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CHAPTER ONE

INTRODUCTION

Plants are an inexhaustible source of naturally occurring biologically-active compounds that form the bedrock of modern drug discovery. South Africa is a country of robust plant biodiversity with about 22 000 indigenous taxa, of which, about 3 500 are used medicinally by approximately 200 000 traditional healers who base their healthcare practice on folklore (Wynberg, 2002; Willis, 2006; Locher et al., 1995; Rabe and Van Staden, 1997). Since about 80% of the world's population still depend on herbal medicine to meet their primary healthcare needs, studies on the ethnomedicinal use of plants and their role in modern medicine will continue to be of global interest (Ekor, 2014). Phytochemical research is one of the most reliable means of providing a scientific rationale for the use of plant materials in the management of diseases and infections in ethnomedicine (Efferth et al., 2007; Folashade et al., 2012). Plants biosynthesise secondary metabolites as part of their defence mechanism against herbivores and frugivores (Bennett and Wallsgrove, 1994). These metabolites are not only deterrents, but also represent a myriad of phytocompounds from which pharmacologically-active compounds are formulated or synthetically modified to produce drugs with enhanced biological activities in conventional medicine (Efferth et al., 2007).

Plant-based therapeutic agents have recorded great success in the past, amongst these being the analgesic compound morphine (from *Papaver somniferum*), and the 'wonder' cancer drug taxol (from *Taxus brevifolia*) (Atanasov et al., 2015). However, commercial supply of most plant-derived compounds is unsustainable due to shortage in the wild because of environmental mutilation, overexploitation by medicinal herb collectors, dormancy and increasing market demand (Staniek et al., 2014; Arora et al., 2010; Aghilian et al., 2014). As a result of the threatened or endangered status of many potent medicinal plant species, conservation

initiatives and rescue approaches such as cell and tissue culture have been explored. The latter are biotechnological methods that manipulate the totipotent nature of plant cells for the rapid reproduction of whole plants or desired plant parts (Yoshimatsu, 2008). In addition, plant biotechnology applications such as elicitation of biosynthetic pathways can result in increased production of the chemical constituents (Chandra and Chandra, 2011).

Rationale for the research

There is an increasing global demand for new chemotherapeutic drugs for the management of cancer and infectious diseases. Plant-derived compounds have proven to be promising candidates in the development of new drugs to combat these diseases (Hosseini and Ghorbani, 2015; Savoia, 2012). Biologically-active compounds of plant origin are mostly derived from medicinal plants, which do not only provide pharmaceutical industries with raw materials, but are also readily available for traditional medicine. Traditional healers sometimes use different parts of the same plant to treat different diseases. This may be due to variations in the distribution of phytocompounds across different parts of the same plant (del Valle et al., 2015; Singh et al., 2015). Certain plant taxa develop knobs and thorns as defence mechanisms and these modified plant parts have received minimal attention by phytochemists even though their use in traditional medicine is documented (Batool et al., 2010; Lalitharani et al., 2010). This study, therefore, focuses on two South African, knob-bearing, medicinal plant species, *Zanthoxylum capense* and *Senegalia nigrescens*. Previous phytochemical studies on *Z. capense* focused on the twigs and roots of the species and not leaves, bark and knobs which are also used in traditional medicine (Steyn et al., 1998; Luo et al., 2010). A complete phytochemical analysis would provide a scientific rationale for the wide array of traditional uses of this species (Watt and Breyer-Brandwijk, 1962; Steyn et al., 1998; Steenkamp, 2003). For *S. nigrescens*, the hardwood is the only part of the plant that has been phytochemically studied (Malan, 1993, Howell et al., 2002). The phytochemical documentation of the leaves, stem bark, knobs and

roots are therefore necessary to justify the plants use in traditional medicine (Dharani, 2006; Chauke et al., 2015). Furthermore, since both these species are currently in the Red List of threatened South African plants (Raimondo et al., 2009), micropropagation studies could provide, not only a means for survival of these species, but also the potential for mass propagating materials of interest to harness their rich natural products.

Aims and objectives of the study

The aim of this study was to investigate two indigenous South African medicinal plant species that contain knobs (*Zanthoxylum capense* and *Senegalia nigrescens*) for their biologically active secondary metabolites, to validate their uses in traditional medicine, and to identify novel phytocompounds for cancer and microbial chemotherapeutics. This work also aimed to enhance seed germination and to investigate micropropagation protocols directed towards the conservation of these species and possible harvesting of phytocompounds from *in vitro* cultures.

The research objectives were:

- To extract, isolate and identify the secondary metabolites from various morphological parts of *Z. capense* using spectroscopic techniques such as infra-red (IR), ultraviolet-visible (UV-Vis), nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry.
- To test the compounds isolated from *Z. capense* for their biological activity using suitable bioassays (antioxidant and cytotoxicity) thus validating the ethnomedicinal use of the species.
- To assess the impact of various pre-sowing treatments on *in vitro* germination in *Z. capense* due to reports on problematic natural propagation.

- To biosynthesise silver nanoparticles using *Z. capense* aqueous extracts, to characterise them using UV-Vis, Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) and to test them for *in vitro* fungal contamination control of fungal endophytes in *Z. capense*.

- To extract, isolate and identify the secondary metabolites from various morphological parts of *S. nigrescens* using spectroscopic techniques such as IR, UV-Vis, NMR and mass spectrometry.

- To test the compounds isolated from *S. nigrescens* for their biological activity using suitable bioassays (antibacterial and anti-quorum sensing) thus validating the ethnomedicinal use of the species.

- To develop seed germination and micropropagation protocols for *S. nigrescens*.

- To establish callus cultures using materials from *in vitro* germinated plantlets of *S. nigrescens* and to evaluate them for the bioaccumulation of phytocompounds.

Outline of the research presented in this thesis

The findings of the various investigations reported in this thesis are as follows: In chapter 3, the extraction, isolation and identification of the secondary metabolites from *Z. capense* with subsequent evaluation for their antioxidant activity and cytotoxicity is described. Chapter 4 describes the germination response of *Z. capense* seeds to different pre-sowing treatment protocols. Chapter 5 evaluates the bioreducing potential of *Z. capense* extracts in the synthesis of silver nanoparticles for possible application in the control of *in vitro* fungal contamination. Also, the isolation and identification of *Z. capense* fungal endophytes is described. Chapter 6 describes the extraction, isolation and identification of novel bioactive compounds from *S. nigrescens* which were subsequently evaluated for their antibacterial and anti-quorum sensing

activities. Chapter 7 covers the establishment of a micropropagation protocol for *S. nigrescens* using explants from *in vitro* seedlings. Chapter 8 describes callus induction in *S. nigrescens* using 2,4-dichlorophenoxyacetic acid and the evaluation of the calli for the presence of bioactive flavonoids and diterpenoids. A summary of the findings from previous chapters is presented in chapter 9. In chapter 10, the conclusion and recommendations for further work are presented.

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CHAPTER TWO

LITERATURE REVIEW

2.1. The genus *Zanthoxylum*

2.1.1. Background of the genus *Zanthoxylum*

The genus *Zanthoxylum* belongs to the family Rutaceae. The *Zanthoxylum* genus was created in 1757 by Linnaeus. The name *Zanthoxylum* originates from the Greek term "xanthon xylon" meaning "yellow wood" which gave rise to the use of *Xanthoxylum* or *Zanthoxylum* in literature (Patiño et al., 2008). The genus, also referred to as *Fagara* by some authors, has over 250 species distributed mainly across the temperate and tropical regions of the world. *Zanthoxylum* comprises of shrubs and trees, most of which are dioecious. The trees, whose branches are often canopy-like, may be up to 20 m in height or taller in some species. Species of *Zanthoxylum* are characterised by sharp thorns on their trunks and branches.

2.1.2. Ethnobotanical overview of the genus *Zanthoxylum*

Several species of *Zanthoxylum* have been used in ethnomedicine. A summary of *Zanthoxylum* species describing their use in traditional medicine is given in Table 2.1. *Zanthoxylum* species occurring within Africa include *Zanthoxylum capense*, *Z. chalybeum*, *Z. davyi*, *Z. gillettii*, *Z. leprieurii*, *Z. macrophylla*, *Z. tessmannii*, *Z. usambarense* and *Z. xanthoxyloides*. Differences in the plant part used depends on the variation in ethnobotanical practices across different geographical locations. Various morphological parts (including leaves, fruits, stem bark and roots) of *Zanthoxylum* species are reported to be used in ethnomedicine. However, there are very limited reports on the use of thorns. The thorns of *Z. alatum* are reported to be used as a stomachic (assists digestion) and carminative (reduces flatulence) (Batool et al., 2010) whilst

the knobs (modified thorns) of *Z. rhetsa* are reported to be used by an Indian tribe for pain relief and to increase lactation in nursing mothers (Lalitharani et al., 2013).

2.2. The genus *Senegalia*

2.2.1. Background of the genus *Senegalia* (formerly known as *Acacia*)

The genus *Senegalia* is one of the five genera that was borne out of the split of the genus *Acacia*. *Acacia* is a genus made of shrubs and trees, which belongs to the family Fabaceae and sub-family, Mimosoideae. The *Acacia* genus has about 1500 species, most of which are native to Australia, while others are spread across Africa, America and Asia (Dyer, 2014). The name "Acacia" is known to be derived from the Greek word "akakia" meaning thorny tree (Morris, 1969). Pre-Linnean history of the genus *Acacia* claimed that some of its species thrived along the banks of the river Nile in Egypt. Also, the biblical word "Shittim" which was commonly used by King James Version referred to a species of *Acacia* (Ross, 1980). Species of *Acacia* are usually characterised by thorns (although may be absent in a few), seed-bearing pods which represent the fruit and compound pinnated leaves. Previous phylogenetic studies on *Acacia* reveal that certain subgenera were polyphyletic while others monophyletic, thus the need for reclassification (Murphy, 2008). Upon proposal of the reclassification of *Acacia* by a group of Australian botanists in 2003, intense debate ensued but a revision of *Acacia* was approved at the 17th International Botanical Congress held in Melbourne in 2011 (Dyer, 2014). In the new nomenclature, species that were native to Australia retained the name *Acacia*, being the largest with about 900 species, while African species of *Acacia* were grouped into two genera (*Senegalia* and *Vachellia*) (Kyalangalilwa et al., 2013).

Table 2.1. *Zanthoxylum* species used in traditional medicine.

Plant species	Plant part	Traditional use	References
Z. acanthopodium	Fruits and bark	Cholera, dysentery, stomach ache and toothache	Suryanto et al., 2004; Gupta and Mandi, 2013
Z. ailanthoides	Fruits and bark	Epigastric pain, diarrhoea, abdominal pain, colds and snake bites	Wine-Show et al., 1994
Z. alatum	Fruits, bark, stem and thorns	Dyspepsia (indigestion), headache, toothache, stomach ache and carminative	Batool et al., 2010
Z. americanum	Whole plant	Rheumatism, sore throat, toothache, burns and malaria	Bafi-Yeboa et al., 2005; Arun and Paridhavi, 2012
Z. armatum	Whole plant	Fever, dyspepsia, hepatosis (any functional disorder of the liver), skin diseases, round worm expulsion, cholera, diarrhoea, toothache, cardioprotective and analgesic	Ramanujam and Ratha, 2008; Ranawat et al., 2010; Singh and Singh, 2011
Z. avicennae	Stem	Snake bites and stomach tonic	Thuy et al., 1999

Z. beecheyanum	Leaves	Stomach ache and skin diseases	Cheng et al., 2004
Z. budrunga	Leaves and bark	Dyspepsia, diarrhoea, dysentery, coughs and headache	Islam et al., 2001
Z. bungeanum	Pericarp, seeds, leaves and root	Dyspepsia, gastralgia, anti-inflammatory, diuretic, carminative, epigastric pains, eczema and snake bites	Zhang et al., 2014
Z. capense	**Leaves and root**	**Mouth ulcers, toothache, flatulent colic, infertility, snake bites and convulsion**	**Steyn et al., 1998; Steenkamp, 2003**
Z. caribeum	Leaves and bark	Asthma, spasm, fever, herpes and skin diseases	Martínez, 1969; Schnee, 1984
Z. chalybeum	Leaves, fruits, bark and root	Colds, pneumonia, malaria, coughs, toothache, headache and wounds	Kamikawa et al., 1996; Matu and Van Staden, 2003
Z. chiloperone	Root bark	Malaria, emmenagogue (increases menstrual flow) and antirheumatic	Ferreira et al., 2007

Z. davyi	Leaves, spines, stem bark and root	Snake bites, coughs, colds, infected wounds, boils, toothache, pleurisy, sore throat and aphrodisiac	Tarus et al., 2006
Z. dipetalum	Leaves and pericarp	Insecticide	Marr and Tang, 1992
Z. dugandii	Bark	Diuretic and sudorific (causes sweating)	Schnee, 1984
Z. ekmanii	Leaves and roots	Malaria and toothache	Facundo et al., 2005
Z. fagara	Leaves, fruits and seeds	Sedative and sudorific	Amaro-Luis et al., 1988
Z. flavum	Bark	Toothache	DeFilipps et al., 2004
Z. gillettii	Leaves and wood	Hypertension, analgesic and gonorrhoea	Addae-Mensah et al., 1989; Jirovetz et al., 1999; Adesina, 2005
Z. hawaiiense	Leaves and pericarp	Insecticide-ovicide	Marr and Tang, 1992
Z. hyemale	Leaves	Pain killer, sudorific and emetic (causes vomiting)	Guy et al., 2001
Z. integrifoliolum	Bark	Snake bites	Cheng et al., 2007

Z. leprieurii	Leaves, fruits, stem bark, root and wood	Stomatitis, gingivitis, bilharzia, ulcer, antiseptic, diarrhoea, cancer and anti-odontologic (prevent dental diseases)	Ngane et al., 2000; Adesina, 2005; Ngoumfo et al., 2010
Z. liebmanianum	Bark	Amebiasis, intestinal parasites and anaesthetic	Navarrete and Hong, 1996; Arrieta et al., 2001
Z. limonella	Bark	Febrifugal, sudorific and diuretic	Somanabandhu et al., 1992
Z. macrophylla	Bark and seeds	Cold, fever, malaria, stomach ach	Kuete et al., 2011
Z. monophyllum	Bark	Runny nose, jaundice, ophthalmic and anaesthetic	Patiño and Cuca, 2011
Z. naranjillo	Leaves	Pain killer, sudorific and emetic	Guy et al., 2001
Z. nitidum	Fruits, branches, stem bark and root	Cough, stomach ache, vomiting and diarrhoea, colic, piscicide, toothache, fever, rheumatism, paresis and cholera	Bhattacharya et al., 2009; Chen et al., 2011
Z. piperitum	Pericarp and whole plant	Vomiting, diarrhoea and abdominal pain	Yamazaki et al., 2007; Lee and Lim, 2008

Z. rhetsa	Fruits, seeds, spine and bark	Digestion problems, urinary complaint, dyspepsia, diarrhoea, breast pain, to increase lactation in nursing mothers and snake bites	Lalitharani et al., 2010; Arun and Paridhavi, 2012
Z. riedelianum	Not stated	Inflammations, rheumatism and skin stains	Fernandes et al., 2009
Z. rigidum	Leaves and wood	Toothache	Schnee, 1984; Moccelini et al., 2009
Z. rhoifolium	Bark and root bark	Toothache, ear ache, antivenom, antitumor, haemorrhoids, inflammations, febrifuge and malaria	Da Silva et al., 2007; Pereira et al., 2010
Z. rubescens	Stem bark	Malaria, toothache, prevent abortion in pregnant women	Penali et al., 2007
Z. schinifolium	Leaves and pericarp	Used as culinary applications and for epigastric pain	Chang et al., 1997; Cao et al., 2009; Cui et al., 2009
Z. simulans	Root	Snake bites and gastrointestinal disorders	Chen et al., 1994; Ih-Sheng et al., 1994

Z. tessmannii	Stem bark and wood	Tumours, inflammation and gonorrhoea	Mbaze et al., 2007
Z. tetraspermum	Stem bark	Dyspepsia, rheumatism and diarrhoea	Nissanka et al., 2001
Z. tingoassuiba	Stem bark	Antispasmodic, analgesic, sudorific, antifungal, diuretic, antiplatelet, antiparasitic and antihypertensive	Silva et al., 2008
Z. usambarense	Seeds, bark and twigs	Respiratory tract infections, malaria, catarrhal fevers and rheumatism	Matu and Van Staden, 2003; Nanyingi et al., 2008
Z. xanthoxyloides	Leaves, seeds, stem bark and root	Cough, fever, colds, toothache, snake bites, antiseptic, anti-sickler, digestive aid, parasiticide, antirheumatic, anti-odontalgic and diuretic	Ngane et al., 2000; Ngassoum et al., 2003; Kassim et al., 2009

2.2.2. Ethnobotanical overview of the genus *Senegalia*

Although there are several identified species in the genus *Senegalia*, only a limited percentage have a long record of usage in ethnomedicine. The properties of most *Senegalia* plants, specifically, hardness and termite resistance, have enabled them to find applications in construction works like building, mine props and furniture. Some of the *Senegalia* species with documented traditional usage are presented in Table 2.2. The leaves, fruits, stem bark and roots of various *Senegalia* species have been used in traditional management of several ailments (Table 2.2).

2.3. Plants used in this study

Figure 2.1 shows the trunk and branches of the two selected plant species, *Z. capense* and *S. nigrescens*.

Figure 2.1. Pictures of plant species used in this research

A: *Z. capense* stem, B: *Z. capense* branches, displaying fruits (Kondlo, 2012), C: *S. nigrescens* stem, D: *S. nigrescens* branches displaying flowers (Masupa, 2011).

Table 2.2. *Senegalia* (formerly *Acacia*) species used in traditional medicine.

Plant species*	Plant part	Traditional use	References
A. albida	Bark and root	Chest pain, colds, diarrhoea, haemorrhage, leprosy and ophthalmic	Umar et al., 2014
A. arabica	Leaves, flowers, bark, gum and root	Skin diseases, diarrhoea, dysentery, cough, diabetes, eczema, wound healing, burning sensation, mucoprotective, asthma and astringent	Rajvaidhya et al., 2012
A. aroma	Leaves, fruits and bark	Wound healing, antiseptic, gastrointestinal disorder, diuretic and anti-inflammatory	Arias et al., 2004
A. auriculiformis	Seeds	Spermicidal, anti-filariasis (deworming) and anthelmintic (antiparasite)	Mandal et al., 2005; Pal et al., 2009
A. catechu	Stem	Anti-inflammatory, anti-virus, anti-bacterial, anti-cancer and cardiovascular application	Srivastava et al., 2011
A. confusa		Wound healing, anti-blood stasis	Kan, 1978; Chang et al., 2001
A. farnesiana	Bark and root	Mucous membrane inflammation and diarrhoea	Williams, 2011

A. karoo	Leaves, stem bark and gum	Dysentery, diarrhoea and haemorrhage	Olajuyigbe and Afolayan, 2012
A. ligulata	Bark	Cough and medicinal wash	Williams, 2011
A. melanoxylon	Bark	Rheumatism	Williams, 2011
A. mellifera	Stem bark	Syphilis and pneumonia	Mutai et al., 2009
A. nigrescens	**Stem bark and root**	**Toothache, snake bite and dysentery**	**Dharani, 2006; Chauke et al., 2015**
A. nilotica	Leaves, flowers, pods and bark	Cancer, cold, congestion, cough, diarrhoea, dysentery, fever, haemorrhoids, ophthalmia, sclerosis, small pox, tuberculosis, leprosy, leukoderma and menstrual problems	Singh et al., 2009
A. senegal	Gum	Cough and diarrhoea	Williams, 2011
A. sinuata	Not stated	Skin diseases, burning sensation, constipation, renal (stony) calculi, haemorrhoids, vitiligo and eczema	Mehta et al., 2014
A. tortilis	Stem bark and root	Asthma and cough	Arbonnier, 2004

* *Senegalia* species, herein referred to as *Acacia* as referenced.

2.3.1. *Z. capense*: description and phytochemical review

Z. capense (Figure 2.1A and B) is a multi-branched tree that grows to about 4 – 10 m high. Its leaves are alternate, unevenly compound, having 4 – 8 pairs of leaflets. The leaves are characterised by a citrus smell while the trunk and branches have thorns which develop into cone-shaped knobs on mature stems (Schmidt et al., 2002). *Z. capense* is geographically distributed through South Africa, Mozambique and Zimbabwe (Luo et al., 2010) where it has been employed for a variety of traditional medicinal uses (Table 2.1).

Previous phytochemical studies on the twigs of *Z. capense* indicated the presence of pellitorine (**1**), *β*-sitosterol (**2**), sitosterol-*β*-D-glucoside (**3**) and xanthoxylol-*γ*,*γ*-dimethylallyl ether (**4**) (Steyn et al., 1998). Compounds found in the roots were decarine (**5**), norchelerythrine (**6**), dihydrochelerythrine (**7**), 6-acetonyldihydrochelerythrine (**8**), tridecanonchelerythrine (**9**), 6-acetonyldihydronitidine (**10**), zanthocapensine (**11**), rutaecarpine (**12**), skimmianine (**13**), (-)-sesamin (**14**), (-)-episesamin (**15**), (-)-savinin (**16**), zanthocapensol (**17**), zanthocapensate (**18**), *N*-isobutyl-(*2E,4E*)-2,4-tetradecadienamide (**19**) and lupeol (**20**) (Luo et al., 2013). The structures of compounds **1** to **20** (listed above) are presented in Figure 2.2.

2.3.2. *S. nigrescens*: description and phytochemical review

S. nigrescens (Figure 2.1C and D) is a multi-branched tree which may grow from 6 to 30 m in height. A leaf of *S. nigrescens* consists of 2 or 3 pairs of pinnae with 1 or 2 pairs of leaflets per pinna. Its fruit are dark brown, thinly textured pods borne in pendant clusters (Dharani, 2006). Its trunk and branches have hooked-shape thorns which develop into knobs on the mature stem but may be absent in some specimens. *S. nigrescens* is found in South Africa, Namibia, Botswana, Tanzania and Zimbabwe (Dharani, 2006).

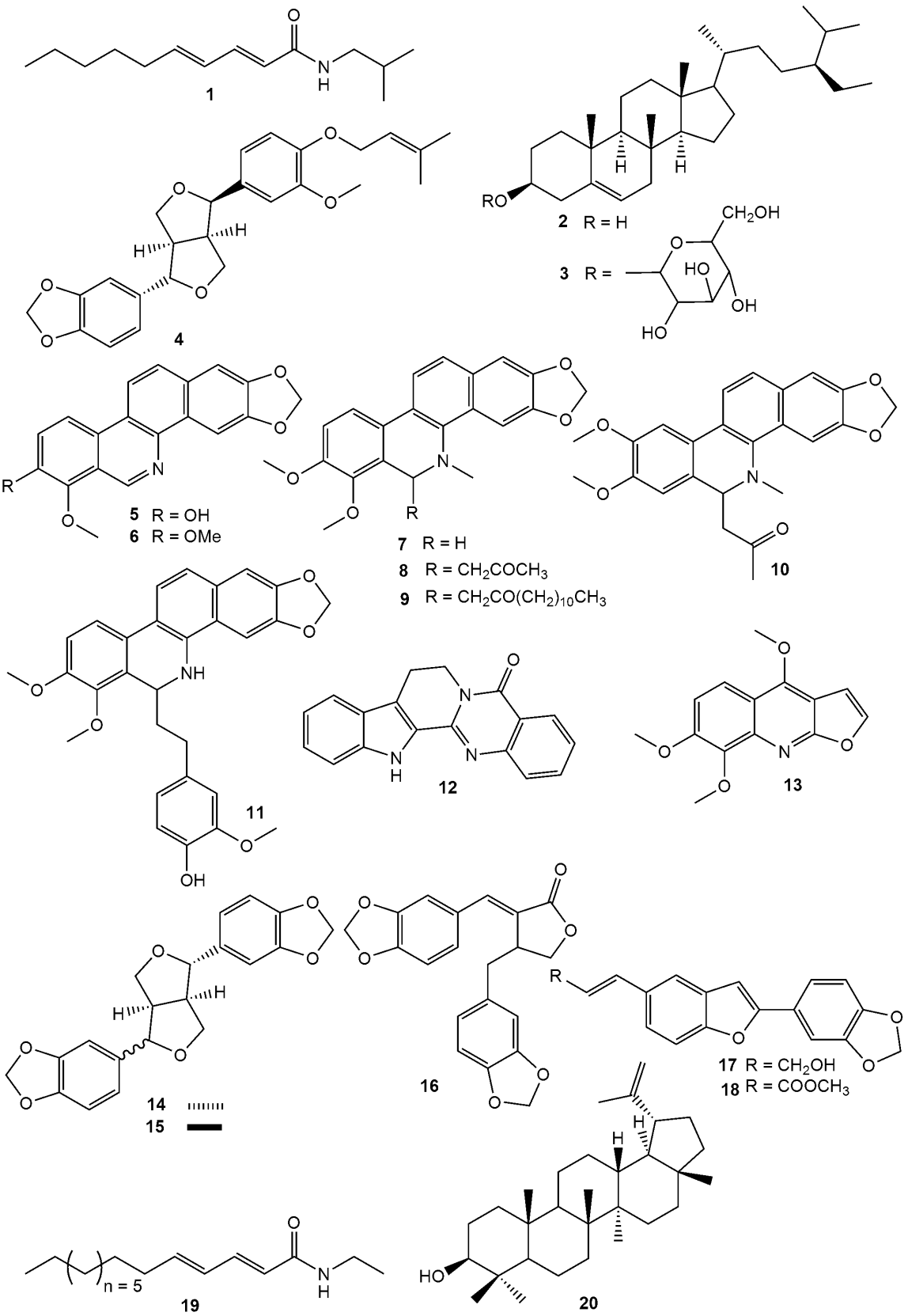

Figure 2.2. Compounds previously isolated from *Z. capense*

Compounds **1** - **4** isolated from the twigs of *Z. capense* (Steyn et al., 1998); Compounds **5** - **20** isolated from the roots of *Z. capense* (Luo et al., 2013)

Previous phytochemical investigations of the heartwood of *S. nigrescens* afforded the following compounds: 7,8,3',4'-tetrahydroxy-3-methoxyflavone (**1**), 7,8,4'-trihydroxy-3,3'-dimethoxyflavone (**2**), 7,8,4'-trihydroxyflavone (**3**) (Malan, 1993), mesquitol-(4α→5)-3,3',4',7,8-pentahydroxyflavonone (**4**), and epimesquitol-(4β→5)-3,3',4',7,8-pentahydroxyflavonone (**5**) (Howell et al., 2002). The structures of compounds **1** to **5**, listed above are presented in Figure 2.3.

From the phytochemical review of *Z. capense* and *S. nigrescens*, it is evident that studies on the knobs, a modified part of the plant are lacking.

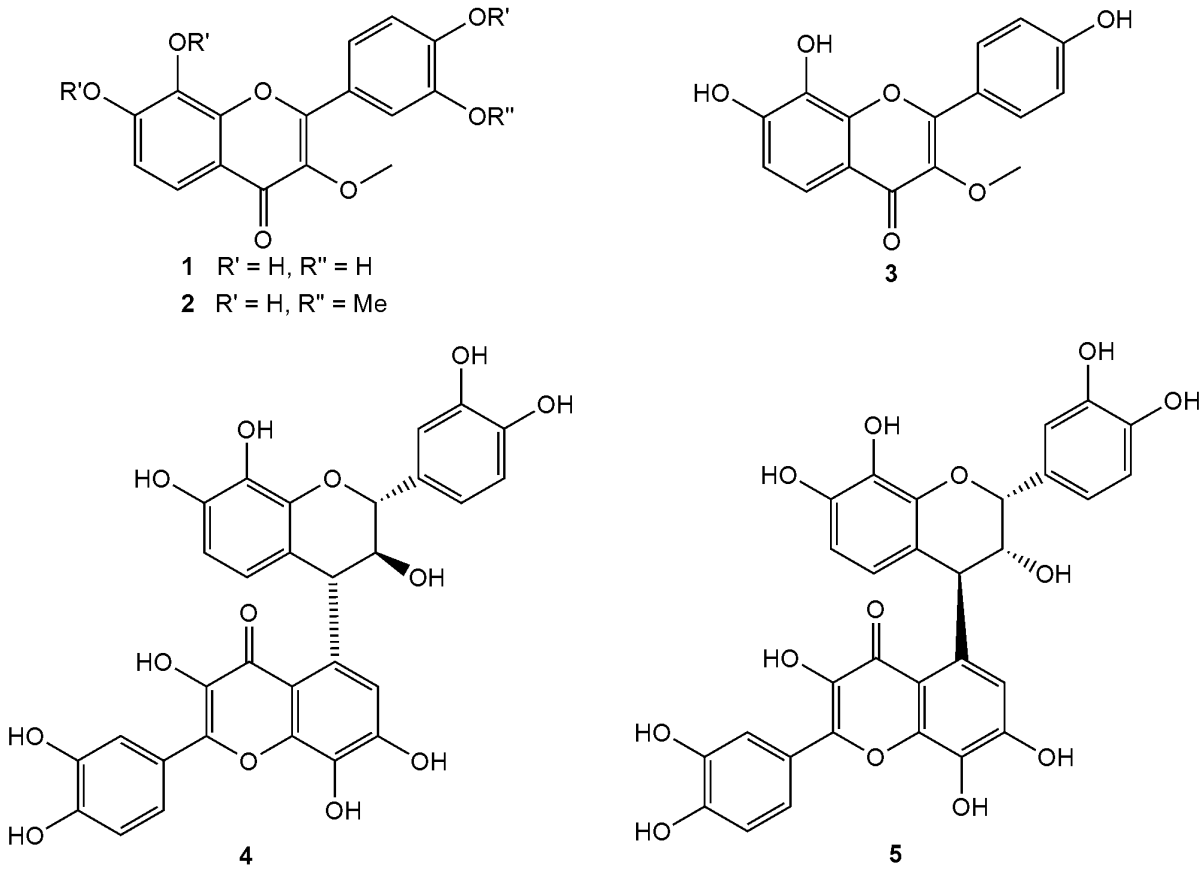

Figure 2.3. Compounds previously isolated from the heartwood of *S. nigrescens*
(Malan, 1993; Howell et al., 2002)

2.4. A brief overview of the *ent*-kaurene diterpenoids

The *ent*-kaurene diterpenoids are a group of cyclic diterpenoids with extensive biological activities resulting from their unique structural diversity (Sun et al., 2006; Zhu et al., 2015). Species from the genus *Isodon* are known to produce a variety of *ent*-kaurenes with over 1300 *ent*-kaurenes being isolated from this genus (Sun et al., 2006). The biosynthesis of *ent*-kaurenes occurs via cyclisation of geranylgeranyl pyrophosphate (GGPP) by the enzymes *ent*-copalyl diphosphate synthase (CPS) and entkaurene synthase (KS) (Dewick, 2009; Keeling et al., 2010), resulting in the bicyclo[3.2.1]octane framework of *ent*-kauranoids (Figure 2.4). GGPP, the common precursor for twenty-carbon diterpenes is first converted to a bicyclic intermediate copalyl diphosphate (CPP) through protonation-initiated cyclisation. This is followed by loss of the diphosphate group from *ent*-CPP which allows a carbocation-mediated cyclisation to afford the pimarenyl carbocation A from which a secondary carbocation, beyeranyl cation B is generated by cation-alkaene cyclisation. A [1,2]-alkyl migration from C13 to C16 then occur via Wagner-Meerwein rearrangement, changing the secondary carbocation into a tertiary one (Dewick, 2009), the *ent*-kauranyl cation C. Upon deprotonation of either the C17 methy or C15 methylene group, scaffolds of *ent*-kaurene or ent-isokaurene are obtained, respectively (Hedden and Phinney, 1979).

2.5. Extraction, isolation and identification of phytocompounds

2.5.1. Extraction and isolation of bioactive compounds from plants

Bioactive compounds of plant origin represent a selected fraction of the products of plant secondary metabolism and are often bound with other molecules and spread throughout the plant (Cannell, 1998). The therapeutic potential of extracts from medicinal plants depend on the pharmacological effect produced by the active compounds individually or synergistically

with other compounds within the extracts. However, the identity of each bioactive compound is essential for their incorporation in modern drug development, hence the need for their isolation and identification.

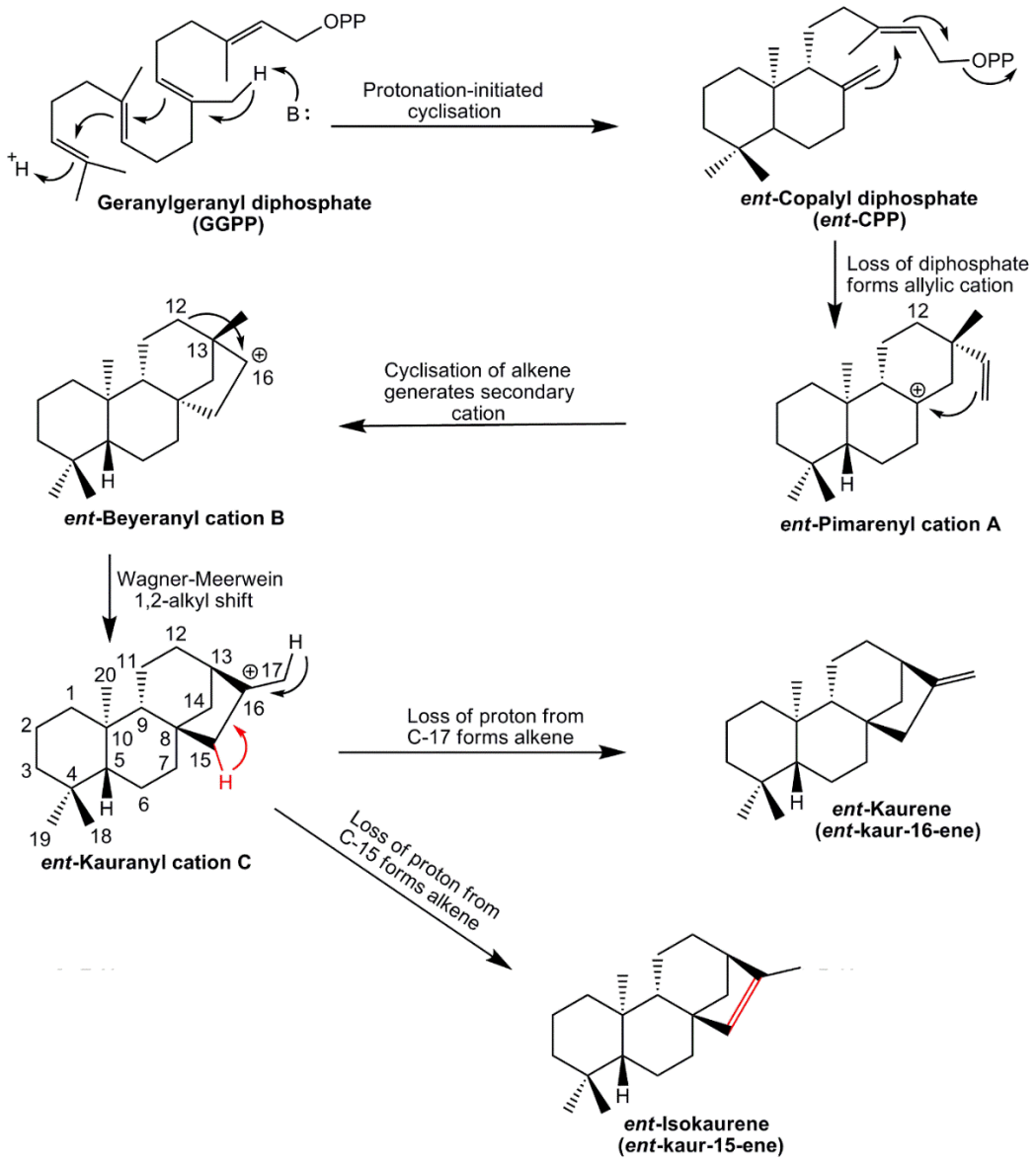

Figure 2.4. Biogenetic pathway to the *ent*-kaurene diterpenes

(Hedden and Phinney, 1979; Ding et al., 2017)

Extraction is a process that involves obtaining compounds from the tissues of plant. The plant material is first subjected to pre-extraction treatments such as pre-washing, air-drying or freeze drying and grinding. The grinding is necessary to achieve sample homogeneity and increase sample surface area for increased contact with the extracting solvent (Sasidharan et al., 2011). Careful selection of solvents or solvent systems is important to extract the compounds of interest, since different compounds have an affinity for different solvents. For a bioassay guided extraction, hydrophilic compounds are extracted with polar solvents (water, ethanol, methanol or ethyl acetate) and lipophilic compounds are extracted with less-polar solvents (dichloromethane or dichloromethane/methanol mixture) (Cos et al., 2006). A sequential method of extraction may also be applied when isolation is more general. In this method, extraction is initiated with non-polar solvents (hexane), then solvents of intermediate polarity (dichloromethane) and finally with polar solvents (methanol). Resulting crude extracts may then be concentrated using a rotary evaporator before proceeding to the isolation step.

The techniques that have been employed for the isolation and purification of plant-derived compounds are mostly based on chromatography. In a chromatographic separation process, components of a mixture are distributed between two phases, usually a stationary and a mobile phase. Thus, separation is possible due to the components differential partitioning between the stationary and mobile phase (Scott, 2003). Examples of stationary phase include silica, alumina and sephadex while mobile phases are usually organic solvents (hexane, dichloromethane, acetonitrile, ethyl acetate and methanol). The commonly used chromatographic techniques are thin layer chromatography (TLC), column chromatography (CC), gas chromatography (GC), flash chromatography (FC) and high performance liquid chromatography (HPLC). In this research, qualitative and quantitative analysis were achieved using TLC, CC and GC for the isolation, purification and identification of the bioactive compounds from *Z. capense* and *S. nigrescens*.

2.5.2. Identification of isolated bioactive compounds

Having isolated the phytochemicals and a satisfactory degree of purity has been established using various chromatographic techniques, the exact identity of the compounds is known by characterisation using different spectroscopic techniques, the masses determined by mass spectrometry and molecular structures proposed. The spectroscopic techniques employed for the elucidation of the compounds' molecular structures in this research are infra-red (IR), ultraviolet-visible (UV-vis) and nuclear magnetic resonance (NMR) spectroscopy.

IR spectroscopy is a technique that provides phytochemists and other scientists with information regarding the functional groups present in a compound or sample. This technique involves the analysis (through the measurement of absorption, emission and reflection) of infrared light upon interaction with a molecule. Vibrational frequencies which are characteristics of the atom size, bond length and strength within a molecule are thus obtained. The Fourier-transform infrared (FTIR) spectrophotometer is a common laboratory instrument used for analysis of a sample using IR. The FTIR performs mathematical data processing and produces a result in the form of an IR spectrum (a plot of % transmittance versus wavenumber). The IR spectrum, commonly measured in units of wavenumbers (cm^{-1}) gives the vibrational frequency range of each bond type, thus, individual functional groups are identified (Stuart, 2004). Unlike IR, UV-Vis spectroscopy occurs in the ultraviolet and visible regions of the electromagnetic spectrum. It may also be referred to as electronic spectroscopy since it involves the transfer of electrons from a low-energy to a high-energy atomic or molecular orbital upon irradiation of a compound with light. UV-Vis spectroscopy is used to identify organic chromophores and compounds bearing unsaturated heteroatoms (Kalsi, 2007).

NMR spectroscopy is to date, the most reliable technique used for the structural identification of organic compounds. The magnetic properties of atomic nuclei form the basis for NMR

studies. Atomic nuclei with non-zero nuclear magnetic moment interact with radio frequency (RF) in an external field (B_o). This leads to a nuclear energy level diagram (by quantum mechanical rules) because nuclear magnetic energy is restricted to certain discrete values *E*i, called *eigenvalues*. These *eigenvalues* are associated with *eigenstates*, also called *stationary states*. Transitions between the states are stimulated by RF transmitter followed by the absorption of energy which is then detected by a receiver and then recorded as a spectral line, which is referred to as the resonance signal. A spectrum, which is a plot of frequency versus absorption is thus obtained (Günther, 2013). The characteristic signal of magnetic nuclei in a molecule is distributed along the x-axis of the spectrum and the chemical shift (frequency of signal) of the signal peaks provide important information regarding the local environment of the atom. Detailed information regarding the structure of compounds can be obtained using one dimension (1D)-NMR, of which proton (^{1}H) and carbon-13 (^{13}C) NMR are the most commonly used. ^{1}H and ^{13}C-NMR have chemical shifts which range from 0-13 and 0-230 ppm, respectively. Other 1D-NMR techniques are Distortionless Enhancement by Polarisation Transfer (DEPT 90 and 135). Additional information can be provided by two-dimensional (2D)-NMR in cases where structure elucidation is not possible with 1D-NMR only because of unresolved or overlapping signals resulting from compounds with complex structures. Examples of 2D-NMR are Correlation Spectroscopy (COSY), Nuclear Overhauser Effect Spectroscopy (NOESY), Heteronuclear Single Quantum Correlation (HSQC) and Heteronuclear Multiple Bond Correlation (HMBC).

In modern drug development, compounds isolated from medicinal plants are subjected to preliminary testing, using *in vitro* assays. These assays are often selected based on the ethnomedicinal use of the plants and the class of compounds isolated. Crude extracts as well as the pure compounds isolated are tested to assess the effects of synergy or antogonism on the active compounds.

2.6. Antioxidant activity

Free radicals, derived from normal metabolic processes in the human body or exposure to ozone, X-rays, air pollutants, cigarette smoking and industrial chemicals (Bagchi and Puri, 1998), are responsible for several diseases including cancer (Alam et al., 2013). The oxidation reactions that produce free radicals can be prevented by an antioxidant. Various methods exist for determining the antioxidant property of compounds and other samples like plant extracts, food and commercial antioxidants (Alam et al., 2013). Amongst these methods are the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay and ferric reducing antioxidant power (FRAP) assay which were used in this research.

The DPPH method is a simple and rapid antioxidant test that is based on free radical scavenging. The DPPH molecule possesses a stable free radical due to delocalisation of the spare electron over the entire molecule thereby preventing dimerisation (common with other free radicals). The molecule is characterised by a deep violet colour because of the delocalisation of electrons and has an absorption band around 517 nm. When a substrate (AH) is mixed with DPPH solution, the substrate (hydrogen donating) reduces the DPPH and the latter loses the violet colour (Figure 2.5) (Alam et al., 2013). The antioxidant potential of a test sample may then be evaluated as a measure of the change in the optical density of DPPH radical relative to the various sample concentrations.

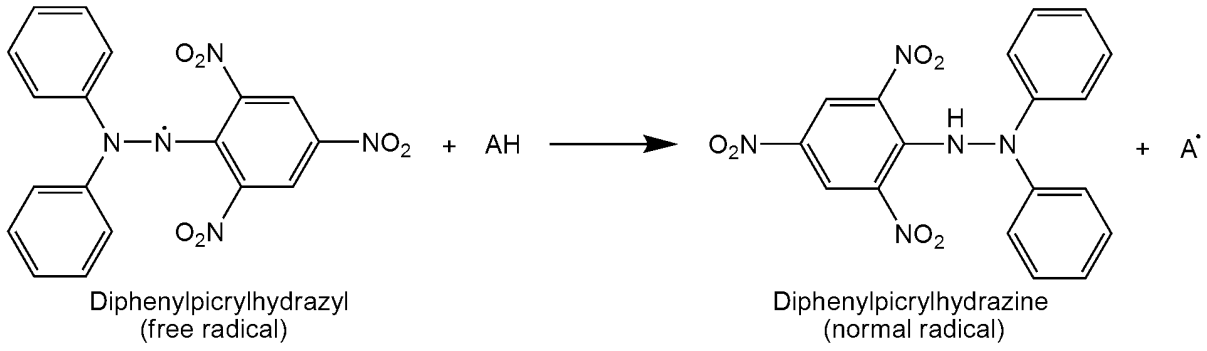

Figure 2.5. Reaction of a hydrogen donating substrate with DPPH

The FRAP assay employs the ability of antioxidants to reduce a ferric iron complex (ferric tripyridyltriazine) to its ferrous form at low pH. At a wavelength of 593 nm, changes in absorption are used to monitor reduction of the Fe^{3+} to Fe^{2+} ion which is measured with the use of a diode-array spectrophotometer (Alam et al., 2013). A standard protocol describing the preparation of the FRAP reagent and test method was reported by Benzie and Strain (1999). The change in absorbance of a test sample may then be compared with those obtained increasing concentrations of Fe^{3+} and expressed as mM of Fe^{2+} equivalents per mass or volume of the sample.

2.7. Cytotoxicity

Most of the earliest cancer chemotherapeutic agents were plant-derived or semi-synthetic derivatives of the compounds of plant origin (Pezzuto, 1997). The following anticancer agents, paclitaxel, vincristine, podophyllotoxin and camptothecin isolated from *Taxus brevifolia* L., *Catharanthus roseus* G. Don, *Podophyllum peltuturn* L. and *Camptotheca acuminata* Decne, respectively, came into the lime light in the late 1940s. Subsequently, antitumor potentials of most of these compounds were improved through structural modifications which in turn modified their mechanisms of action and water solubility (Pezzuto, 1997). Cancer drugs may be classified (based on their curative approach) into, cytotoxic drugs, endocrine (hormonal) therapy, targeted therapies (which includes small molecules and antibodies) and vaccines (Hawthorn and Redmond, 2006). To date, most cancer chemotherapeutic agents work based on their cytotoxicity to tumour cells. Cytotoxicity assays measure cell viability in primary and immortalised mammalian cell lines, with high specificity and reproducibility over a wide range of concentrations. Most of these assays are colorimetric, where cell death is measured based on the change in colour of a compound. The 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyl tetrazolium bromide (MTT) assay is the most commonly used colorimetric assay employed for the measurement of cell viability. Here, the yellow water-soluble MTT salt is converted to

insoluble purple-blue formazan in metabolically active cells (Mosmann, 1983), whereas dead cells resulting from toxic damage cannot convert MTT. Formazan production is thus, proportionate to the number of viable cells and inversely proportional to the extent of cytotoxicity (Senthilraja and Kathiresan, 2015). Cytotoxicity may then be followed by *in vivo* studies using animal models before being subjected to clinical trials. These drug development processes have since suffered serious limitations due to selectivity (ability of the compound to significantly induce mortality in tumour cells while normal human cells are spared) and drug resistance issues (Narang and Desai, 2009) associated with existing chemotherapeutic agents. Thus, new candidates of natural origin or synthetic derivatives with extensive pharmacophores capable of selective cytotoxicity are being sought after through research involving *in vitro* cytotoxicity, apoptosis and animal model studies.

2.8. New targets in the development of antimicrobials

The discovery of antimicrobial drugs recorded great success in the mid-1900s, with the discovery of penicillin in 1928 (Saga and Yamaguchi, 2009) which came into clinical application in the 1940s (Saga and Yamaguchi, 2009). In the 1950s, chloramphenicol, tetracycline, macrolide and aminoglycoside were discovered (Saga and Yamaguchi, 2009). However, the prospect of these antimicrobial agents was short-lived due to rapid development of drug resistance by the pathogens (Gold and Moellering Jr, 1996). The basic mechanisms by which bacteria develop resistance to antimicrobial drugs are enzymatic degradation of the drugs, mutation in the genetic make-up of the target bacteria and changes in the permeability of antibiotics to the microbial cell membrane (Dever and Dermody, 1991). Whenever an antimicrobial agent attacks a bacterial colony, a few bacteria survive due to the law of natural selection and through evolutionary process. Such species develop adaptive features that leads to drug resistance (Davies and Davies, 2010). It has been observed that the space from which

a community of bacteria has been eliminated is soon colonised by other microorganisms which later become pathogenic to the host (Bjarnsholt and Givskov, 2008).

Recent research is being directed towards the development of drugs that could shut down the communication among bacteria within their colony, a process known as anti-quorum sensing. Quorum sensing (QS) is a form of corporative cell-cell communication that are usually mediated through the production of auto-inducers which are small signal molecules. For example, the most widely studied auto-inducer compound in Gram-negative bacteria is N-acyl homoserine lactone (AHL) (Fuqua et al., 2001). Among the Gram-negative bacteria is *Chromobacterium violaceum* which responds to QS regulated gene expression by producing the pigment violacein (Vasavi et al., 2013). The anti-quorum sensing potential of a compound or extract can therefore be determined as a function of its capacity to inhibit violacein production relative to increasing concentration of the compound or extract. Extracts of several plant species have been tested for QS inhibitory potential (Koh et al., 2013) among which is the *Acacia* species *A. nilotica* (Singh et al., 2009). The knowledge of the bioactive compounds in the plant extracts is central to the future production of commercially-available antimicrobial drugs from these species.

In vitro pharmacological studies utilise the isolated purified compounds and crude extracts from selected medicinal plants. The results obtained often give clues to whether the compounds produce pharmacological effects singly or synergistically with other compounds in the extracts. Thus, traditional uses of medicinal plants are scientifically justified (Taylor et al., 2001). Nevertheless, progressive studies on these plant species and their active constituents in the process of developing novel drugs are limited by availability of medicinal plants in the wild (Cordell, 2009). One of the rapidly-growing techniques employed for overcoming this challenge is plant biotechnology.

2.9. A brief background to plant tissue culture technology

Plant biotechnology is a general term used to define the use of the various *in vitro* culture techniques for plant improvement, applicable to disease control, food production and pharmaceuticals. Biotechnology was first named by a Hungarian engineer, Karl Ereky, in 1919 (Chawla, 2002). Plant tissue culture involves the aseptic culture of plant cells, tissues, organs or any other morphological part under controlled physical and chemical conditions *in vitro* (Thorpe, 2007). The theory behind tissue culture technology is the ability of individual cells to regenerate into whole plants when provided with the right environment. This concept, totipotency, was established by Gottlieb Haberlandt in his address to the German Academy of Science in 1902 on his experiments on the culture of single cells (Thorpe, 2007). Gottlieb's submission finds its root from the earlier proposition of Schleiden and Schwann, in 1839, that the cell is the basic unit of life in all organisms (Bechtel, 2006).

Plant growth and regeneration is known to be aided by plant hormones also referred to as plant growth regulators (PGRs). These are chemical substances usually produced in small amounts in specialised parts of the plant and are transported to other areas of the plant to produce specific responses (Weyers and Paterson, 2001). Naturally-occuring PGRs are classified into auxins, cytokinins, gibberellins, abscisic acid and ethylene, and auxins and cytokinins are considered as the most important PGRs required for growth regulating and organised development in plant tissue culture (Gasper et al., 1996).

As plants undergo morphological differentiation during growth and development, they exhibit stress responses to environmental factors, including PGRs. One of the stress responses is the production of secondary metabolites (Selmar and Kleinwächter, 2013). The chemical structure, concentration of the PGRs and their control of the signalling pathways (Westfall et al., 2013) influence the biosysnthesis of secondary metabolites. Further, enzymes regulate the amount

and exposure time of the hormones at their receptor sites through storage, degradation or structural modification (Westfall et al., 2013). Thus, the PGRs often represent intermediate metabolites or precursors for biologically-active secondary metabolites. For instance, the gibberellins are tetracyclic diterpenoids which are derived biosynthetically from gibberellin A_{12}-aldehyde, with over 100 members (Macmillan and Beale, 1999).

2.10. Plant tissue culture for the propagation of medicinal plants

Over the years, medicinal plants have been playing a crucial role in curative and preventative medicine (Rahmatullah et al., 2010), being a reservoir of nutraceuticals and pharmacologically-active compounds. These compounds are usually products of the plants' secondary metabolism, a process that plants develop as part of their means of adaptation to the environment (Bourgaud et al., 2001). Phytocompounds are therefore bioaccumulated by plants for chemical defence and signalling. Although plants are widely available all over the globe, only a selected fraction is applicable to disease management, following a series of trial-and-error usage and extensive experimentation (Halberstein, 2005). These medicinal species do not only feed the drug development industry with active components (Oksman-Caldentey and Inzé, 2004) but they are also directly utilised by about 80% of the world's population for primary health care (Ekor, 2014). Today, increasing demand for plant-derived bioactive compounds by the pharmaceutical industry, over-exploitation by medicinal herb collectors and unfavourable biotic and abiotic factors contribute to the rapid depletion of medicinal plants.

Even though vegetative (asexual) propagation provides an easy and reproducible means of propagating plants from stem, root, leaf or bud, there are no reports on this method of propagation for *Z. capense* or *S. nigrescens*. Vegetative propagation has been attempted using *Z. leprieurii* stem cuttings but this was unsuccessful (Nyamukuru et al., 2014). In the case of *Senegalia*, some species (*S. senegal* and *Acacia tortillis*) could not successfully regenerate

vegetatively as poor rooting rate was obtained from stem cuttings (Dick and East, 1992; Elbasheer and Elkalifa, 2010). Tissue culture is a collection of techniques which can be used for the sustainable commercial propagation of medicinal plants (Rout et al., 2000). One such technique is *in vitro* seed germination which can be adapted for plants with limited germination and growth using conventional propagation. Low seed germination mostly results from dormancy, a condition whereby a seed is unable to germinate in a specified period of time under a combination of environmental factors that are normally suitable for the germination of the non-dormant seed (Baskin and Baskin, 2004). Seeds of *Zanthoxylum* and *Senegalia* species are characterised with seed coat-imposed dormancy (Bonner, 1974; Sanon et al., 2005; Rasebeka et al., 2014) and in these cases germination can be promoted by physical or chemical pre-sowing treatments.

Other ways to propagate plants *in vitro* are by using the direct and indirect organogenesis routes of morphogenesis. Direct organogenesis is the approach using axillary buds or shoot tips (the explants) to generate whole plants directly from the explant surface usually with the help of plant growth hormones (Rout et al., 2000). It also involves the induction of adventitious shoots or roots directly from leaf, stem and root segments. But when the adventitious shoot or root is derived from an intermediate callus (a mass of undifferentiated cells) phase, it is called indirect organogenesis (George et al., 2008). Shoots derived via direct or indirect organogenesis can be rooted on nutrient media containing a controlled amount of auxin, with or without cytokinins in low concentration, and sometimes on hormone-free media (Gantait et al., 2016). Each step of the micropropagation process may then be optimally standardised, thus providing effective regeneration and mass production of medicinal plants. When using tissue culture techniques to conserve plant germplasm, direct organogenesis methods are preferred over indirect methods since the risk of somaclonal variation is lower when there is no callus phase (Singh, 2002).

However, callus production is useful to generate cell suspension cultures from which important phytochemicals can be harvested.

Direct and indirect approaches to the regeneration of plants in the genus *Acacia* have been extensively reviewed (Beck and Dunlop, 2001; Gantait et al., 2016). A summary of the past successes on direct and indirect organogenesis of *Acacia* are presented in Tables 2.3 and 2.4, respectively. In the case of the *Zanthoxylum* species, there are a few reports of *in vitro* propagation including those on *Z. armatum* (Purohit et al., 2014), *Z. piperitum,* (Son et al., 1995) *Z. simulans* (Ducci and Malentacchi, 1993) and *Z. xanthoxyloides* (Etsè et al., 2011).

2.11. Application of tissue culture to the production of bioactive phytochemicals

Tissue culture technology provides effective means for sustainable commercial production of plant-derived compounds (Pant, 2014). These compounds are biosynthesised by plants in response to several physiological factors imposed by the natural environment (Borges et al., 2016). To a large extent, tissue culture allows for control of physiological factors and can thereby significantly reduce production time and increase yield of compounds within the plants. Large-scale production of phytochemicals involves mainly cell suspension or hairy root cultures (Hussain et al., 2012).

In cell suspension culture, callus is first initiated from the plant, friable portions of the callus are then transferred onto liquid nutrient media and maintained on a shaker under controlled light, temperature, aeration and other physical parameters (Chattopadhyay et al., 2002). It is often desirable to initiate callus from the part of the plant known for high production of the compound of interest (Chattopadhyay et al., 2002). In this way, the callus can be bulked up while suitable solvents are used to isolate the compound.

Table 2.3. Direct organogenesis in the genus *Acacia*.

Species	Explant used	Medium used	PGR (mg L^{-1})	Result obtained	References
A. albida	Cotyledon	MS	0.5 NAA + 3.0 BAP	Multiple shoots	Duhoux and Davies, 1985
			0.1 NAA	Rt	
A. auriculiformis	Node	MS	2.0 BAP + 0.1 NAA	Multiple shoots	Girijashankar, 2011
		½ MS	PGR-free	Rt	
A. catechu	Shoot tip	MS	1.5 BAP + 1.5 KIN	Multiple shoots	Kaur and Kant, 2000
		¼ MS	3.0 IAA	Rt	
A. farnesiana	Node	MS	1 BAP	Multiple shoots	Khalisi and Al-Joboury, 2012
		½ MS	0.5 IBA + 0.05 NAA	Rt	
A. melanoxylon	Node	Q-LP	1.0 BAP + 0.5 NAA	Multiple shoots	Jones and Smith, 1988
A. nilotica	Node	MS	0.6 NAA	Multiple shoots	Dhabhai et al., 2010
		½ MS	0.5 IBA	Rt	
A. senegal	Node	MS	1.0 BAP	Multiple shoots	Khalafalla and Daffalla, 2008
			1.0 IBA	Rt	

A. sinuata	Node	MS	2.0 BAP + 0.5 TDZ + 50.0 AdS	Multiple shoot	Vengadesan et al., 2003
		½ MS	1.5 IBA	Rt	
A. tortilis	Node	MS	2.5 BAP	Multiple shoot	Aziz et al., 2002

AdS – Adenine sulfate, BAP – 6-Benzylaminopurine, IAA – Indole-3-acetic acid, IBA – Indole-3-butyric acid, KIN – Kinetin, MS – Murashige & Skook medium (Murashige and Skoog, 1962), NAA – α-naphthalene acetic acid, PGR – Plant growth regulator, Q-LP – Quoirin Lepoivre medium (Quoirin and Lepoivre, 1977), Rt – Root, TDZ – Thidiazuron.

Table 2.4. Indirect organogenesis in the genus *Acacia*.

Species	Explant used	Medium used	PGR (mg L^{-1})	Result obtained	References
A. arabica	Immature zygotic	MS	2.0 BAP + 1.5 2,4-D	Ca	Nanda and Rout, 2003
	embryo		1.5 BAP + 1.5 2,4-D	SR	
		½ MS	0.01 BAP + 0.2 IBA	Rt	
A. auriculiformis	Cotyledon	MS	0.2 2iP + 4.0 NAA	Ca	Kaur and Kant, 2000
			2.0 2iP + 0.2 NAA	SR	
			1.0 NAA	Rt	
A. catechu	Immature cotyledon	WPM	3.0 KIN + 0.5 NAA	Ca	Rout et al., 1995
			3.0 KIN + 0.5 NAA + L-proline	SE	
A. confusa	Leaf	MS	3.0 2,4-D + 0.01 NAA + 0.05	Ca	Arumugam et al., 2009
			KIN		
		WPM	3.0 BAP + 0.05 NAA + 0.1	SR	
			zeatin + 5.0 AdS		
		MS	4.0 IBA + 0.05 KIN	Rt	

A. melanoxylon	Shoot	MS	0.2 BAP + 0.2 IAA	Ca	Meyer and Van Staden,
				SR	1987
A. nilotica	Cotyledon	MS	0.4 2,4-D + 0.2 BAP	Ca	Dhabhai and Batra, 2010
			0.4 2,4-D + 0.2 BAP + 200 AC	SR	
		½ MS	0.5 IBA	Rt	
A. senegal	Cotyledon	MS	0.1 2,4-D + 0.5 KIN	SE	Rathore et al., 2012
			0.05 BAP	SR	
A. sinuata	Leaf	MS	1.0 2,4-D + 0.5 BAP	Ca	Vengadesan et al., 2002
		MS	1.0 2,4-D + 10% CW	SE	
		(Liquid)	PGR-free	SR	

2,4-D – 2,4-Dichlorophenoxyacetic acid, 2iP – 2-Isopentyl adenine, AC – Activated charcoal, AdS – Adenine sulfate, BAP – 6-Benzylaminopurine, Ca – callus, CW – Coconut water, IAA – Indole-3-acetic acid, IBA – Indole-3-butyric acid, KIN – Kinetin, MS – Murashige & Skook medium (Murashige and Skoog, 1962), NAA – α-naphthalene acetic acid, PGR – Plant growth regulator, Rt – Root, SE – Somatic embryogenesis, SR – Adventitious shoot regeneration, WPM – Woody Plant Medium (Lloyd and McCown, 1980).

Whenever the callus is suspended in liquid media, cell aggregates can disperse into smaller clumps and rapidly-divide into single cells which become evenly distributed throughout the media (Loyola-Vargas and Vázquez-Flota, 2006). Each cell is capable of the biochemical transformation required to produce secondary metabolites (Yue et al., 2016). This method has been optimised for industrial applications using bioreactors. Bioreactors are stirred-tank or airlift systems that can carefully control culture parameters to allow for large cell suspension cultures for massive production of compounds of interest. For example, some *Taxus* natural products; taxane and paclitaxel (Chattopadhyay et al., 2002), Ginseng saponin (Zhong et al., 1999), *Catharanthus roseus*-derived ajmalicine, catharanthine, serpentine and tryptamine (Zhao et al., 2001) have been successfully mass-produced using bioreactors.

2.12. *In vitro* contamination

In commercial tissue culture laboratories, one of the greatest challenges to culture success is the *in vitro* fungal and bacterial contamination (Leifert et al., 1994). Control of these contaminants is more difficult with woody plant species especially when explants are obtained from field-grown plants, compared to their greenhouse counterparts (Niedz and Bausher, 2002). Chemicals employed for control of *in vitro* contaminants include alcohols, mercuric chloride, hydrogen peroxide, halogenated compounds (sodium hypochlorite (NaOCl)), antibiotics and fungicides (Niedz and Bausher, 2002). In addition, silver nanoparticles are recently gaining popularity for their microbial contamination control.

2.13. Plant-mediated synthesis of silver nanoparticles

Nanotechnology is a rapidly-growing field that involves the manufacture, measurement and manipulation of the size and morphology of nanometre (usually between 1 and 100 nm) scale matter (Donaldson and Stone, 2004). Nanotechnology explores the physicochemical properties of metallic and non-metallic nanoparticles for applications in biomedical and clinical sciences,

environmental sciences, agricultural engineering, pharmaceuticals and drug delivery (Liong et al., 2008; Brar et al., 2010; Prasad, 2014). Silver nanoparticles (AgNPs), one of the most applied nanomaterials, can be synthesised through physical methods, chemical reduction (Iravani et al., 2014) and the recently-emerging green synthesis (Rauwel et al., 2015). Physical and chemical methods of synthesis are decreasing in popularity due to their hazardous impact on the environment and the cost factor (Prasad, 2014; Rauwel et al., 2015). Sizes and shapes of the AgNPs depend largely on the synthetic route adopted, although, green-synthesised AgNPs are often spherical (Srikar et al., 2016). The morphology and other physical properties of AgNPs have made them applicable to water treatment, food packaging (Tran and Le, 2013), textile coatings, as catalysts (El-Nour et al., 2010) and in *in vitro* contamination control (Table 2.5).

In the last few decades, plants have proven to be reliable, reproducible and inexhaustible bio-factories for the green synthesis of AgNPs (Prasad, 2014). Plant extracts are known to contain phytochemicals which are responsible for the reduction of the silver ion (Ag^{+}) to nano-sized metallic silver (Ag^{0}). The AgNPs are obtained bio-capped with phytocompounds thus imparting biostability to the AgNPs (Rauwel et al., 2015). In a study involving the correlation of the cytotoxicity of green-synthesised AgNPs on tumour cells to biocapping agents (phytochemicals), differences in bioreducing extracts significantly influenced the mechanism of DNA damage and duration to achieve apoptosis in hepatocellular liver carcinoma (HepG2) cells and human cervical cancer (HeLa) cells (Chunyan and Valiyaveettil, 2013). The antimicrobial property of AgNPs has made them find applications in medicine, water treatment and consumer products (Ahmed et al., 2016).

Moreover, AgNPs have been employed for the control of *in vitro* bacterial and fungal contamination. Table 2.5 summarises previous reports on the use of AgNPs for the control of *in vitro* contaminants in some plant species. This review reveals the use of different explant

types across the species with the concentration of AgNPs ranging between 4 and 2000 mg L^{-1} while the soaking time of explants ranged between 5 and 180 mins. Total elimination of *in vitro* contaminants with AgNPs was recorded in the leaf and stem explants culture of *Olea europaea* (Rostami and Shahsavar, 2009). *Arabidopsis thaliana*, *Lycopersicon esculentum* and *Solanum tuberosum* all had 100% healthy plantlets from seed, cotyledon and leaf explants, respectively, upon soaking in AgNPs (Mahna et al., 2013).

Table 2.5. Achievements on the control of *in vitro* fungal and bacterial contaminations using AgNPs.

Species	Explant used	Concentration of AgNPs (mg L^{-1})	Soaking time (mins)	% of non-contaminated explant	Reference
Valeriana officinalis	Stem	100	180	89	Abdi et al., 2008
Olea europaea	Leaf and stem	4	Ag-in-medium	100	Rostami and Shahsavar, 2009
Araucaria excelsa	Stem	200	180	89	Sarmast et al., 2011
		400	Ag-in-medium	81	
Gerbera jamesonii	Immature capitulum	200	15	90	Fakhrfeshani et al., 2012
Vitis vinifera	Leaf	1000	20	89	Gouran et al., 2013
Arabidopsis thaliana	Seed	100 – 2000	5 – 20	100	Mahna et al., 2013
Lycopersicon	Cotyledon	25 – 100	5	100	Mahna et al., 2013

esculentum					
Solanum tuberosum	Leaf	100 – 500	5 – 20	100	Mahna et al., 2013
Nicotiana tabacum	Leaf	10	20	89	Bansod et al., 2015
Hevea brasiliensis	Leaf	10	20	94	Moradpour et al., 2016

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CHAPTER THREE

Phytochemical analysis with antioxidant and cytotoxicity studies of the bioactive principles from *Zanthoxylum capense* (small knobwood)

ABSTRACT

Zanthoxylum capense (small knobwood) is a South African species known for a wide range of anecdotal uses. However, there is a dearth of information on its phytoconstitutional make-up, specifically its knobs, with only a few reports on the bioactive compounds that could justify its ethnomedicinal use. This work aimed to identify the active principles in *Z. capense* and evaluate their cytotoxicity against breast and colon cancer tumour cells. Extracts from the stem bark, knobs and leaves were purified using chromatographic methods and characterised using spectroscopic techniques. Cytotoxicity of isolated compounds was evaluated on mammalian MCF-7, Caco-2 tumour cell lines and HEK295, a normal kidney cell line. The following known compounds were isolated from the plant: a quaternary benzophenanthridine-type alkaloid (chelerythrine) along with its alkanoamine derivative (6-hydroxydihydrochelerythrine), an indolopyridoquinazoline alkaloid (rutaecarpine), an alkyl *p*-coumaric acid ester (dodecyl-*trans*-*p*-coumarate), a lignan (sesamin), a flavanol (catechin), two triterpenoids (lupeol and sitosterol) and two pigments (pheophytin a and lutein). In the cytotoxicity study, all tested samples decreased the viability of the MCF-7 tumour cells by at least 23% at a concentration of 1 μg mL^{-1} and Caco-2 tumour cells by at least 15% at 5 μg mL^{-1} but a mild toxic effect on HEK295 across the tested samples. The cytotoxicity assay revealed that chelerythrine and dodecyl-*trans*-*p*-coumarate exhibited good-to-moderate activity with IC_{50} values of 95.4 and 15.1 μM, respectively for MCF-7 and 153.9 and 182.4 μM, respectively for Caco-2 cells. Dodecyl-*trans*-*p*-coumarate was isolated for the first time in the genus *Zanthoxylum*, while chelerythrine, 6-hydroxydihydrochelerythrine, catechin, pheophytin a and lutein are reported

for the first time in *Z. capense*. Bioactive constituents of *Z. capense* display interesting chemo-morphological distribution.

Keywords: benzophenanthridine alkaloid; ethnomedicine; tumour cells; breast cancer; antioxidant; chromatography.

INTRODUCTION

The genus *Zanthoxylum* (Rutaceae) comprises approximately 250 species, among which is *Zanthoxylum capense*, a native South African species (Steyn et al. 1998). It is distributed widely across northern and southern South Africa (van Wyk et al., 1997) but also extends to Mozambique and Zimbabwe (Luo et al., 2010). *Z. capense* is a small to medium tree that grows to a height of approximately 10 m. It has cone-shaped knobs on the bark and the leaves have a characteristic citrus smell when crushed. *Z. capense* is used in traditional medicine to treat mouth ulcers, tooth ache, flatulent colic, bronchitis, fever and infertility (Steyn et al. 1998; Steenkamp, 2003). It has also found application in the management of epilepsy (Watt and Breyer-Brandwijk, 1962) and the Human Immunodeficiency Virus (HIV) (Semenya et al., 2013). The *Zanthoxylum* genus is well known for its medicinal uses due to the presence of alkaloids, amides, lignans, flavonoids, coumarins and phytosterols (Krane et al., 1984; Jang et al., 2008; Chen et al., 1999; Saqib et al., 1990; Adesina, 2005). Essential oils of *Zanthoxylum* species have been widely used in perfumery and food industries (Patino et al., 2008) with the most widely used essential oils being obtained from *Z. xanthoxyloides* (Ngassoum et al., 2003), *Z. gillettii* (Jirovetz et al., 1999) and *Z. simulans* (Chyau et al., 1996). Benzophenanthridine alkaloids, amides and lignans have also been said to have chemotaxonomic significance to the genus (Patino et al., 2008).

The benzophenanthridines are well known for their pharmacological activities including antibacterial, antifungal, antimalarial and antitumour activity (Tavares et al., 2014). Structure-activity relationships reveal that the activities of the benzophenanthridine alkaloids are mostly influenced by various substituents on the isoquinoline unit (Tavares et al., 2014). The quaternary form of the position 5 nitrogen has been found to significantly enhance activity which has led to the development of synthetically modified analogs, among which is NK314, a benzophenanthridine fused at the N5-C6 position with a pyrrolidine ring and which is a unique anticancer agent possessing specific and potent inhibitory activity against topoisomerase IIα (Top2α) (Hisatomi et al., 2011). It is also reported to be a promising candidate for the treatment of Adult T-cell leukemia-lymphoma (ATL) (Hisatomi et al., 2011).

The search for new leads in cancer chemotherapy is receiving global attention as cancer is one of the world's leading causes of mortality (Stewart and Wild, 2014) due to chemo-resistance and emergence of new types of cancer. Previously, 6-acetonyldihydrochelerythrine and zanthocapensate were isolated from the root of the Mozambican *Z. capense* and identified as potential colon cancer treatment compounds (Mansoor et al., 2013). In the present study, our quest for further exploration of the anticancer potential of *Z. capense* led to the isolation of ten compounds from the stem bark, knobs and leaves, three of which were evaluated for their cytotoxicity against the mammalian MCF-7, Caco-2 tumour cell lines alongside a normal kidney cell line (HEK295) after an initial antioxidant activity test was conducted on the compounds. The crude extracts were also evaluated for their synergistic effects.

MATERIALS AND METHODS

General experimental procedures

Infrared (IR) spectra were recorded on a Perkin Elmer Spectrum 100 Fourier transform infrared spectrophotometer (FT-IR) with universal attenuated total reflectance (ATR) sampling accessory. The ultraviolet (UV) spectra were obtained using a Varian Cary ultraviolet – visible (UV-Vis) spectrophotometer. ^{1}H, ^{13}C and 2D nuclear magnetic resonance (NMR) spectra were recorded using deuterated chloroform ($CDCl_3$) or methanol (CD_3OD) at room temperature on a Bruker Avance^{III} 400 or 600 MHz spectrophotometer. The high resolution mass spectra (HRMS) were obtained on a Waters Micromass LCT Premier TOF-MS instrument. All column chromatography was done using Merck silica gel 60 (0.040-0.063 mm) and preparatory thin-layer chromatography (TLC) was carried out using Merck pre-coated kieselgel 60 (20 cm × 20 cm, 0.5 mm thick) F-254 PLC plates while Merck 20 cm × 20 cm silica gel 60 F254 aluminum sheets were used for TLC. The TLC plates were analysed under UV (254 and 366 nm) before further visualization by spraying with a 10% H_2SO_4 in methanol (MeOH) solution followed by heating. Solvents (analytical grade) and other chemicals used were supplied by either Merck (Darmstadt, Germany) or Sigma (St. Louis, USA) chemical companies.

Plant material

The leaves and stem bark of *Z. capense* were collected in January 2014 from the Westville Campus of the University of KwaZulu-Natal (UKZN). The plant was identified and authenticated by curator Syd Ramdhani (School of Life Sciences, UKZN, Westville, Durban). A voucher specimen (No. Bodede 01) was deposited at the Ward Herbarium of the University.

Extraction and isolation

The stem bark of *Z. capense* was separated into knobs and bark. Air-dried powdered bark (1.5 kg), knobs (670 g) and leaves (700 g) were sequentially extracted with hexane,

dichloromethane (DCM), ethyl acetate (EtOAc) and (MeOH) and concentrated using a rotary evaporator. Crude MeOH extracts were further partitioned with DCM and EtOAc. Extracts with similar TLC profiles (retention factor (Rf) were similar) were combined before subjecting them to gravity column chromatography (CC). Hexane extracts from the knobs and bark were combined (28.5 g), loaded onto a silica gel column and eluted with a hexane:EtOAc (100:0-60:40, v/v) gradient to give 81 fractions of 100 mL each. Fractions with similar TLC profiles were combined. Fractions 27-33 were combined and crystallised in MeOH to give compound **A7**, a white solid (910 mg) while fractions 46-51 were further purified to obtain white crystals of compound **A4** (536 mg).

The EtOAc extract of the knobs (11 g) was subjected to CC using a hexane:EtOAc (100:0-0:100, v/v) gradient. The fractions were combined based on TLC profiles to yield six sub-fractions B1-B6. B1 was further purified on a silica gel column and compound **A5**, a yellow solid (8 mg) was obtained while B5 was thoroughly washed with MeOH to give compound **A6**, a white solid (1100 mg). The DCM fraction partitioned from the MeOH extract of the knobs was loaded onto a silica gel column and eluted with a hexane:EtOAc (100:0-0:100, v/v) gradient to give sub-fractions C1-C6. C5 was further purified using preparatory TLC with a solvent system of DCM:MeOH (85:15, v/v). Two zones (A and B) were observed on the plate and zone A was scraped, re-dissolved in MeOH, filtered and subsequently concentrated to produce a yellow solid (153 mg), a mixture of compounds **1** and **2**. The DCM fraction partitioned from the MeOH extract of the bark afforded dull yellow needles of compound **A3** (23 mg), following a similar procedure to that above.

The hexane and DCM extracts of the leaves (20 g) were combined due to similar TLC profiles and was subjected to silica gel CC using a hexane:DCM (100:0-0:100, v/v) gradient. Thereafter, 50 × 10 mL fractions were collected and those with similar TLC profiles were

combined. From these combined fractions, compound **A8** (40 mg), compound **A9** (405 mg), a dark green amorphous solid and compound **A10** (59 mg), a yellow solid, were obtained

Antioxidant activity

Estimation of free radical scavenging activity using the DPPH assay

The antioxidant activity of the isolated phytochemicals and crude extracts from the leaves, bark and knobs of *Z. capense* were evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay by an established procedure (Shirwaikar et al., 2006) with slight modifications. One mL of 0.1 mM DPPH solution was added to 1 mL each of the various concentrations (7.5, 15, 25, 50, 125 and 250 µg mL^{-1} of MeOH) of the compounds and extracts. An equal amount of MeOH and DPPH was used as a control. The solutions were left to incubate in the dark for 20 min after which, absorbance was recorded at 517 nm. The experiment was carried out in triplicate.

Evaluation of the reducing potential using the Ferric Reducing Antioxidant Power (FRAP) assay

The total reducing power of the isolated compounds and extracts from *Z. capense* was determined according to the Ferric Reducing Antioxidant Power (FRAP) method (Ferreira et al., 2007). Different concentrations (7.5, 15, 25, 50, 125 and 250 µg mL^{-1}) of the compounds were prepared and used for the assay.

Cytotoxicity

Cell culture

MCF-7, Caco-2 tumour cells and HEK295 normal kidney cells were procured from the American Tissue Culture Collection (ATCC) (Virginia, USA) and propagated in the laboratory. The cells were grown to 100% confluency in 25 mL tissue culture flasks in Eagles's Minimum Essential Medium (EMEM) (Lonza Biowhittaker, Walkersville, USA)

supplemented with fetal bovine serum (10%) and antibiotics (100 U mL^{-1} penicillin & 100 µg mL^{-1} streptomycin). Cells at a seedling cell density of 1.8×105 cells per well were plated onto a 96 well plate containing 100 µL of medium. The cells were then incubated at 37 °C overnight in a humid atmosphere of 5% CO_2. The medium was subsequently removed and 100 µL fresh medium added.

Sample preparation and exposure

Compounds **A1**, **A5** and **A6** along with crude extracts were tested for cytotoxicity. 5-fluorouracil (5-FU), an anticancer agent used for the treatment of breast cancer, was used as a positive control. Stock solutions of compounds, extracts and positive control were prepared in dimethylsulfoxide (DMSO). These were added at various concentrations (1 µg mL^{-1}, 5 µg mL^{-1}, 25 µg mL^{-1} and 50 µg mL^{-1}) in triplicate to the cells, along with fresh medium, following removal of the medium from incubated cells.

Cell viability and cytotoxicity evaluation

The viability of the cells was evaluated after 48 h of cell incubation in the presence of the test compounds, extracts and control by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This assay measures cell metabolic activity and their MTT to formazan reducing ability using the succinate-tetrazolium reductase system (Mosmann, 1983). Approximately, 100 µL of 5 mg mL^{-1} solution of MTT in phosphate buffer saline (PBS) was added to each well. The cells were incubated with the MTT solution at 37 °C for 4 h after which the MTT with the medium was removed from the wells and 100 µL of DMSO was added to each well to dissolve the formazan salt. Cell viability is indicated by the presence of the formazan salt that produces a purple colour after DMSO addition. The absorbance was then measured using a Mindray MR-96A microplate reader at 570 nm. The absorbance read for each well is proportional to the number of viable cells in it. The tests were conducted in triplicate

and the concentrations at 50% cell death (IC_{50}) were calculated from the graphs generated using Microsoft Excel™ 2010 (Microsoft Corporation, Redmond, USA).

Statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used to determine if there were significant ($P < 0.05$) differences among the means of the data sets. All statistical analyses were done using the Statistical Package for Social Sciences (PASW Statistics 22, IBM Corporation, Cornell, New York).

RESULTS AND DISCUSSION

Phytochemistry

Ten compounds were isolated from the stem bark and leaves of *Z. capense* (Fig. 3.1). The compounds, belonging to the benzophenanthridine alkaloids, indolopyridoquinazoline alkaloids, coumarates, lignans, flavonoids, triterpenes and natural pigments were identified based on their ^{1}H and ^{13}C-NMR spectra and 2D-NMR spectra which were compared to values in literature. Cytotoxicity of selected compounds and extracts are also presented.

Compound **A1**, chelerythrine, was obtained as a yellow solid. Its molecular formula, $C_{21}H_{18}NO_4^+$ was established by NMR spectroscopy and HRMS which gave an exact mass of 348.1231 (calc. for $C_{21}H_{18}NO_4^+$, 348.1236), corresponding to 13.5 Double Bond Equivalent (DBE). The IR spectrum revealed strong aromatic C=C and C=N absorption bands at 1600 cm^{-1}. The ^{1}H-NMR spectrum acquired in CD_3OD showed 3 singlets for aromatic protons at δ_H 9.95 (H-6), 8.17 (H-4) and 7.57 (H-1) which were supported by 4 overlapping *ortho*-coupled (*J* ranging between 7.9 and 9.2 Hz) doublets at δ_H 8.67 (H-10), 8.64 (H-11), 8.22 (H-12) and 8.20 (H-9). Four other singlets were also observed at δ_H 6.28 (methylenedioxy), 4.99 (N-CH_3), 4.30 (7-OMe) and 4.16 (8-OMe). The downfield chemical shifts were consistent with the

benzophenanthridine skeleton and all other resonances were similar to those reported in literature (Miao et al., 2012).

Compound **A2** was identified as 6-hydroxydihydrochelerythrine with molecular formula $C_{21}H_{19}NO_5$. The ^{1}H-NMR spectrum revealed a benzophenanthridine-type system with two aromatic singlets at δ_H 7.66 (H-4) and 7.14 (H-1), four *ortho*-coupled doublets (all having *J*=8.6 Hz) at δ_H 7.77 (H-11), 7.67 (H-10), 7.48 (H-12) and 7.17 (H-9), one singlet for the methylenedioxy group (δ_H 6.07), a N-CH-O methine (δ_H 5.56), two methoxy resonances (δ_H 3.94, 3.94) and one nitrogenated methyl resonance (δ_H 2.74). The ^{13}C-NMR spectrum showed signals for four oxygenated-aromatic quaternary carbons (δ_C 152.0 (C-8), 148.1 (C-3), 147.5 (C-2), 146.5 (C-7)), six aromatic quaternary carbons (δ_C 137.9 (C-4b), 131.1 (C-12a), 126.5 (C-4a), 126.0 (C-6a), 124.8 (C-10a), 122.4 (C-10b)), six aromatic methine resonances (δ_C 123.3 (C-12), 119.4 (C-11), 118.6 (C-10), 113.2 (C-9), 103.7 (C-1), 99.8 (C-4)), one methylenedioxy resonance (δ_C 101.1), one N-CH-O methine resonance (δ_C 86.1 (C-6)), two methoxy resonances (δ_C 60.5 (7-OCH_3), 55.1 (8-OCH_3)) and a nitrogenated methyl resonance (δ_C 39.5 (-NCH_3)), totaling twenty one carbons which were resolved using DEPT experiments. In the IR spectrum, absorption bands were observed at 3360 cm^{-1} due to the hydroxyl group and 1640 cm^{-1} which is characteristic of double bond. These spectral data agree with those previously reported (Cho et al., 2006).

Compound **A3**, rutaecarpine, which was isolated as yellow needles was found to have the molecular formula of $C_{18}H_{13}N_3O$ (DBE = 14). In the ^{1}H-NMR spectrum, eight protons corresponding to a pair of di-substituted aromatic ring systems were observed. The quinazolinic part showed characteristic downfield signals at δ_H 8.29, 7.67, 7.60 and 7.39 while similar de-shielded signals were displayed by the indolyl unit at δ_H 7.58, 7.31, 7.27 and 7.14. The signals have common *ortho*-couplings around *J*=7.7 Hz. The spectrum also exhibited two methylene signals at δ_H 4.56 and 3.20. A broad singlet was observed at δ_H 9.59 ascribable to the indolyl

NH. This was further confirmed by N15-HSQC which showed a single correlation between the nitrogen and the δ_H 9.59 singlet. The ^{13}C-NMR spectrum showed 18 carbon resonances of the indolopyridoquinazoline skeleton consisting of a carbonyl at δ_C 161.6, two non-aromatic methylenes at δ_C 41.1 and 19.6 and fifteen aromatic carbon resonances between δ_C 147.4 and 112.1. The spectra data for rutaecarpine in the present study compared well with those published in literature (Lee et al., 2008; Wattanapiromsakul et al., 2003).

Compound **A4** was identified as sesamin, which has a molecular formula $C_{20}H_{18}O_6$ that was established by its NMR and HRMS (377.0991 [M+Na]) data. The ^{1}H-NMR spectrum exhibited 6 aromatic proton resonances of the two phenyl rings. The signal at δ_H 5.92 integrated to 4 protons and corresponded to the pair of methylenedioxy groups on both ends of the molecule. Ten distinct carbon resonances were observed in the ^{13}C-NMR spectrum which is expected for the C20 symmetrical molecule, sesamin (Laggoune et al., 2011).

Compound **A5**, dodecyl-*trans*-*p*-coumarate, was obtained as a yellow crystalline solid. Its molecular formula was determined to be $C_{21}H_{32}O_3$ based on NMR and HRMS (355.0833 [M+Na]) data. The ^{13}C-NMR spectrum showed characteristic resonances of a carbonyl (δ_C 161), eight unsaturated carbons (δ_C 157.5-115.8), one oxygenated carbon (δ_C 64.6) and other saturated carbons (δ_C 31.9-14.1). In the ^{1}H-NMR spectrum, the cinnamate protons were observed at δ_H 7.58 (*J*=16 Hz, H-3) and 6.26 (*J*=16 Hz, H-2). Two signals at δ_H 7.39 (2H, d, *J*=8.6 Hz, H-5/9), and δ_H 6.81 (2H, d, *J*=8.6 Hz, H-6/8) which are indicative of a *p*-substituted benzene ring were noticed in the aromatic region. The presence of hydroxyl group was indicated by the broad absorption band of 3377 in the IR spectrum and other functionalities, C=C (2846, 1674), C=O (1709) and C-H (2917) were also observed. These spectral data agree with those previously reported (Saleem et al., 2011; Singh and Singh, 2014).

Catechin (**A6**), lupeol (**A7**), sitosterol (**A8**), pheophytin a (**A9**) and lutein (**A10**) were also isolated. The identification of these compounds and their structural determinations was achieved by comparing their ^{1}H-NMR, ^{13}C-NMR and HRMS spectroscopic data with those in literature (Galotta et al., 2008; Kamboj and Saluja, 2011; Wang et al., 2009; Aman et al., 2005). Several benzophenanthridines have been reported in the genus *Zanthoxylum* (Patino et al., 2008) and in the roots of *Z. capense* (Luo et al., 2010). However, no quaternary type has previously been reported. Chelerythrine and 6-hydroxydihydrochelerythrine has not previously been reported in *Z. capense* but has been isolated from other species such as *Z. davyi* (Tarus et al., 2006) and *Z. nitidum* (Hu et al., 2006). It has also been found in other Rutaceae species (Rajkumar et al., 2008). Rutaecarpine has previously been isolated from *Z. budrunga* (Mukhlesur et al., 2005), *Z. integrifolium* (Sheen et al., 1996), *Z. pistaciiflorum* (Chen et al., 2004) and the roots of *Z. capense* (Luo et al., 2010), but is now isolated from the stem bark. Earlier studies on *Z. capense* reported sesamin and sitosterol as contributing to its bioactivity (Steyn et al., 1998). Sesamin and several other members of lignans are widely distributed among the the Rutaceae family but common among the *Zanthoxylum* species are the diarylbutirolactones and the 2,6-diaryl-3,7-dioxabicyclo[3.3.0]octanes (Patino et al., 2008) while sitosterol and lupeol are ubiquitous phytosterols that have been isolated from several *Zanthoxylum* species (Adesina, 2005).

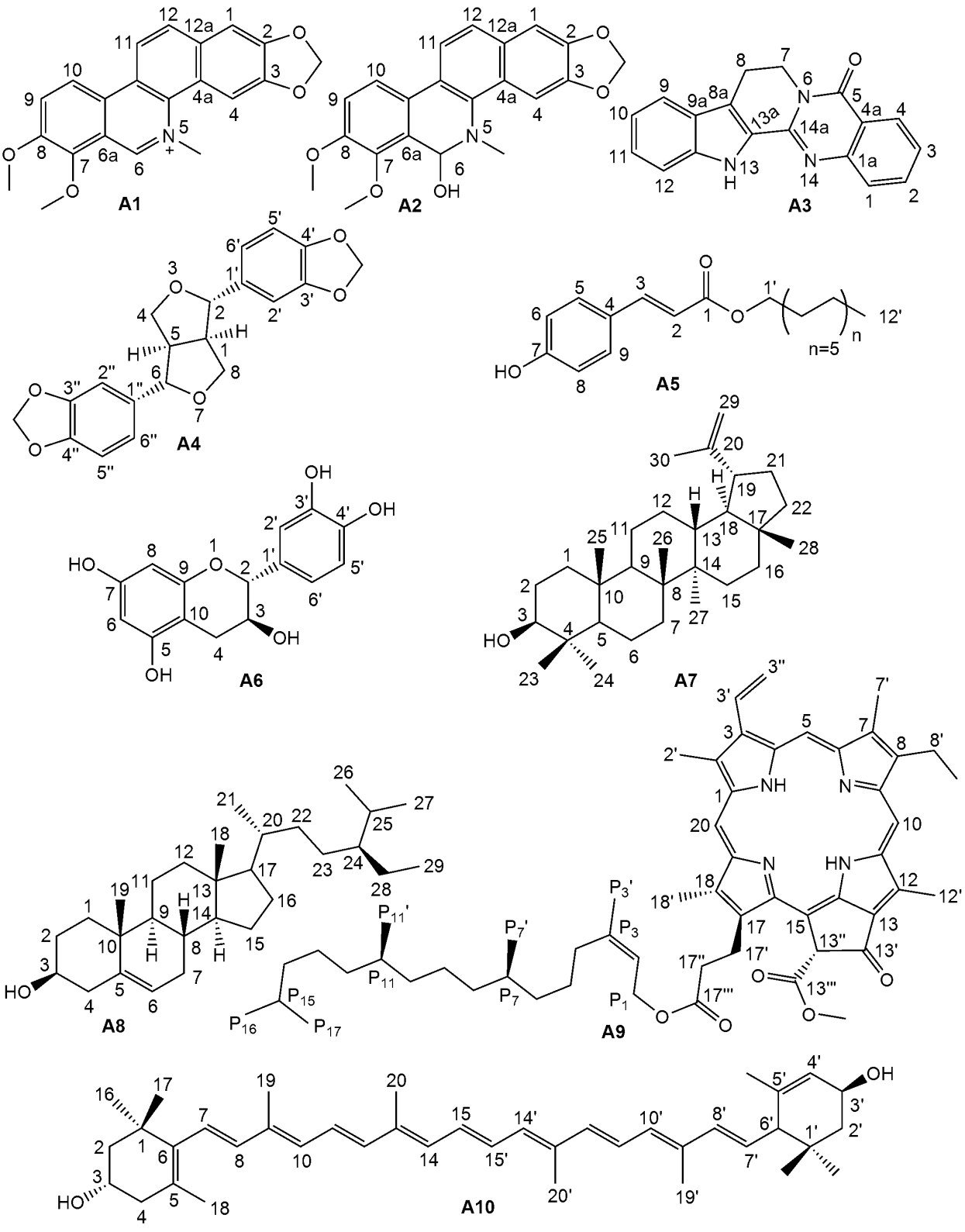

Figure 3.1. Chemical structures of compounds **A1 – A10** isolated from different morphological parts of *Z. capense*

Antioxidant activity

Radical-scavenging activity

The results from the DPPH radical scavenging assay are presented in Fig. 3.2. The graph revealed that the scavenging activity of ascorbic acid was higher than that of the test samples at all concentrations. At 50 µg mL^{-1}, the order of decreasing activity was ascorbic acid > MeOH extract of knobs (KM) > EtOAc extract of knobs (KE) > DCM extract of bark (BD) > MeOH extract of bark (BM) for the crude extracts and ascorbic acid > catechin > rutaecarpine > chelerythrine > dodecyl-*trans*-*p*-coumarate for the selected compounds.

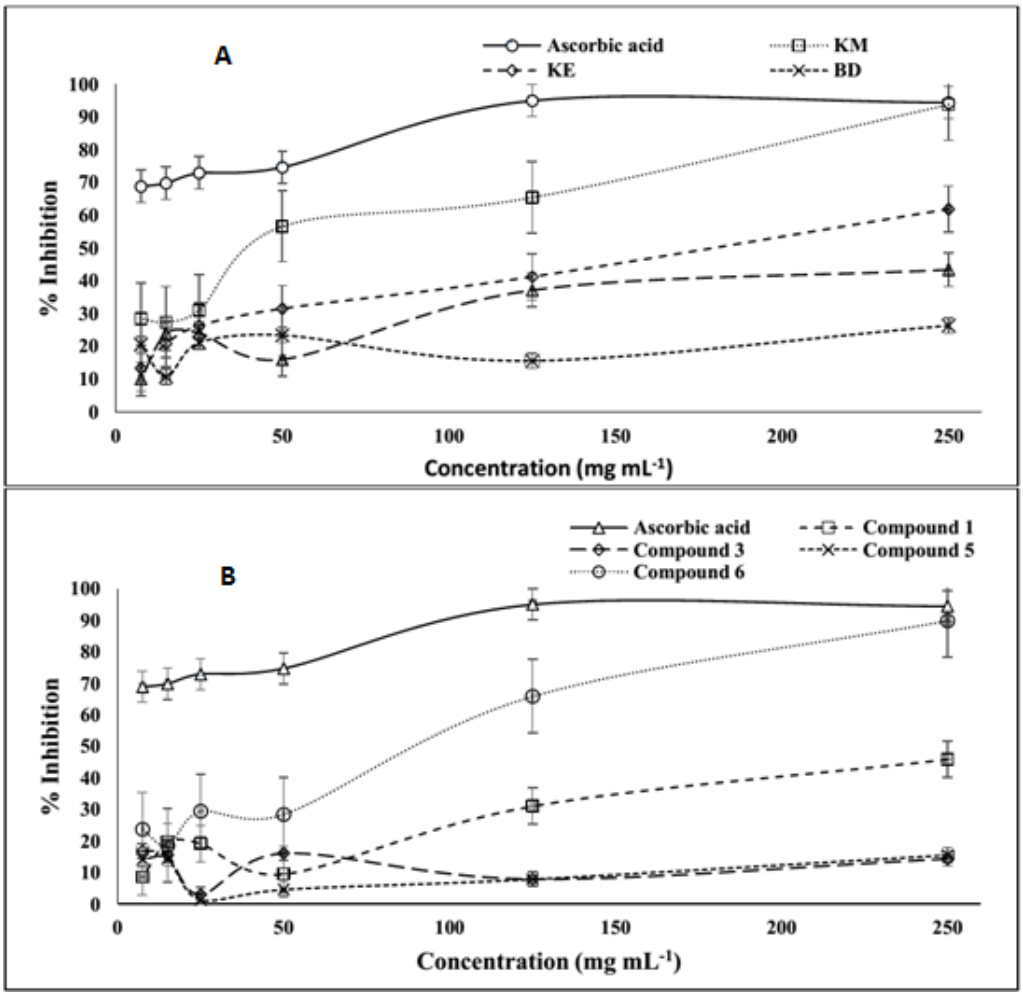

Figure 3.2. Free Radical Scavenging Activity of selected crude extracts (A) and compounds (B) measured by the DPPH method

Values represent mean ± SD (n = 3). KM: methanol extract of knobs, KE: ethyl acetate extract of knobs, BD: dichloromethane extract of bark, BM: methanol extract of bark.

Ferric reducing antioxidant power (FRAP) assay

The reducing capacity of the tested samples, indicated by the change of the yellow test solution to various shades of blue and recorded at 700 nm is presented in Fig. 3.3. At 50 µg mL^{-1}, the order of decreasing activity was ascorbic acid > BD > KM > KE > BM, for the crude extracts and catechin > ascorbic acid > chelerythrine > rutaecarpine > dodecyl-*trans*-*p*-coumarate, for the selected compounds. The extracts (KE and BM) and pure phytocompounds chelerythrine, dodecyl-*trans*-*p*-coumarate and catechin were selected for cytotoxicity studies.

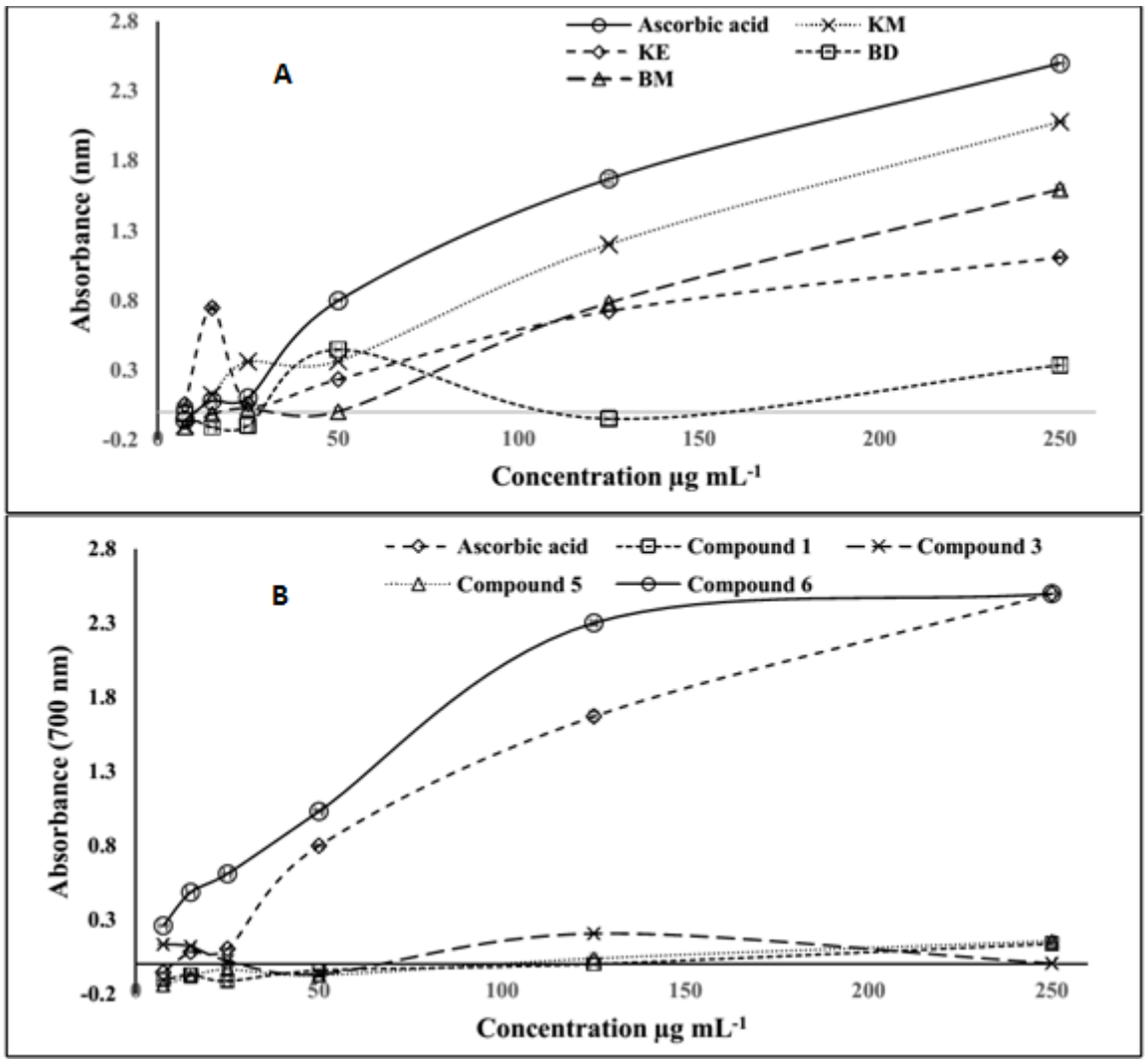

Figure 3.3. Ferric Reducing Antioxidant Power (FRAP) of selected crude extracts (A) and compounds (B)

Values represent mean ± SD (n = 3). KM: methanol extract of knobs, KE: ethyl acetate extract of knobs, BD: dichloromethane extract of bark, BM: methanol extract of bark.

Cytotoxicity

In this study, selected crude extracts and phytocompounds from *Z. capense* were exposed to MCF-7, Caco-2 tumour cell lines and HEK295 normal kidney cell line for 48 h *in vitro* for their cytotoxicity/cell viability evaluation via the MTT assay. Their cell death inducing capacities are presented relative to the anticancer drug 5-FU and DMSO vehicle control in Fig. 3.4. Results showed that all tested samples decreased the viability of the tumour cell by at least 23% at concentration 1 µg mL^{-1}. Chelerythrine and dodecyl-*trans*-*p*-coumarate significantly induced cell death up to at least 66% across the following concentrations 1, 5 and 25 µg mL^{-1}. Chelerythrine, was the most active cytotoxic agent against both MCF-7 and Caco-2 cells. Its activity can be related to the presence of the cationic iminium unit which possesses the ability to inter-convert into the alkanoamine form (a pseudo-base) through nucleophilic attack thereby enhancing its permeating ability through the cell membrane. Afterwards, this lipophilic hydroxide adduct forms the reactive quaternary cation in its attempt to set a new equilibrium inside the cell (Fig. 3.5) (Jash et al., 2015). This behavior is consistent with that of sanguinarine and other quaternary benzophenanthridine alkaloids (Slaninova et al., 2001; Schmeller et al., 1997; Wu et al., 2013). Dodecyl-*trans*-*p*-coumarate displayed good cytotoxic activity in this study, with the lowest IC_{50} value (5.0 µg mL^{-1}) among the compounds tested. The mechanism of action of this compound still remains unclear. Its antioxidant and DNA polymerase α and β inhibitory activity were reported as low (Singh and Singh, 2014; Nishimura et al., 2009). However, the unesterified form (*p*-coumaric acid) has been reported to have good antioxidant and anticancer properties (Kong et al., 2013). This may provide an explanation to the cytotoxicity of the coumarate, in that, the lipophilicity of the long chain alkyl ester enhanced permeability of the compound while the cells probably provided an environment conducive for the hydrolysis of the ester into the weak acid thereby enhancing the activity.

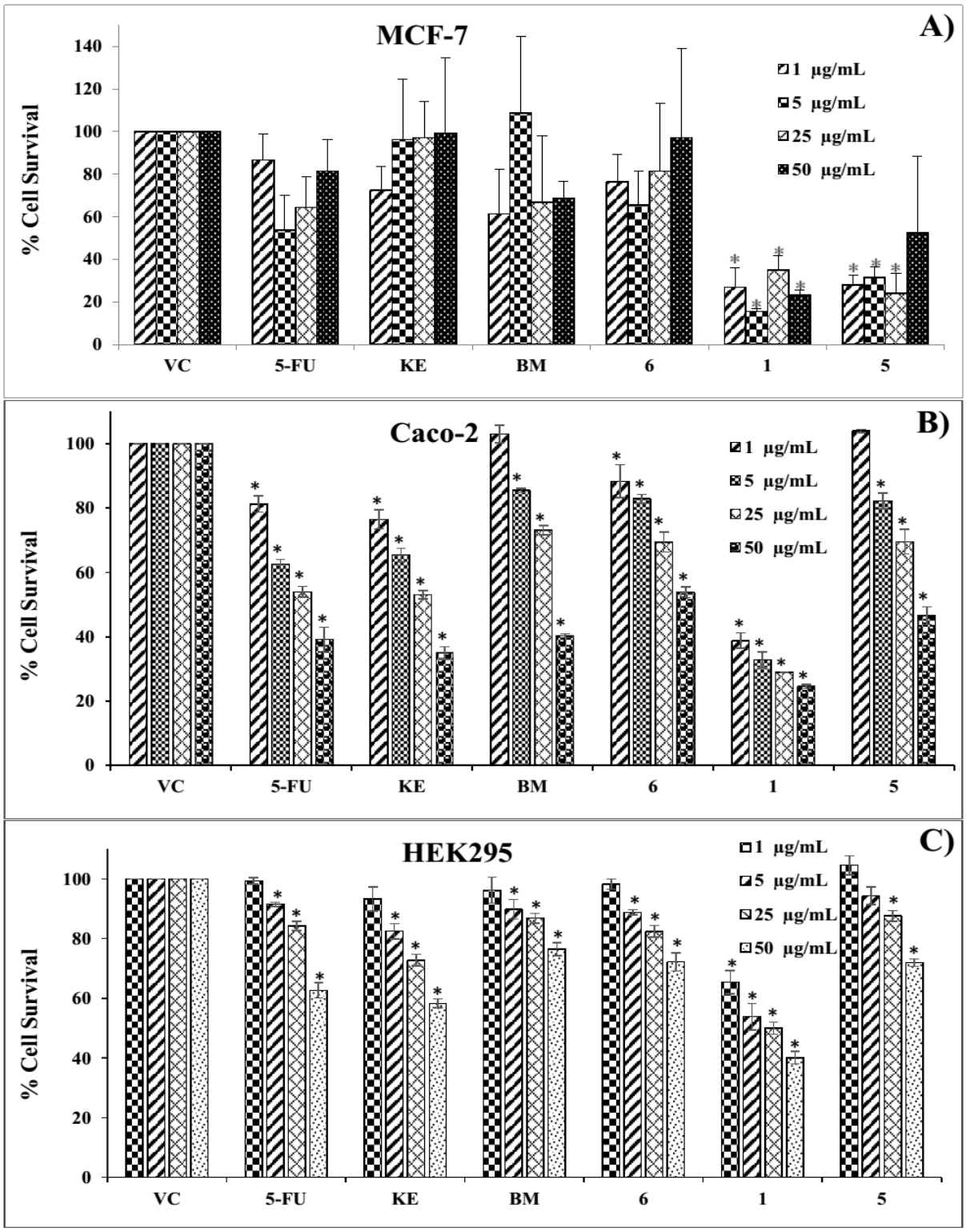

Figure 3.4. Cytotoxicity of selected *Z. capense* extracts and isolated compounds towards MCF-7 (5A), Caco-2 (5B) tumour cell lines and HEK295 (5C) normal kidney cell line as determined by the MTT assay

Values represent mean ± SD (n = 3), *p < 0.05 from vehicle control (VC, DMSO). KE: ethyl acetate extract of knobs, BM: methanol extract of bark.

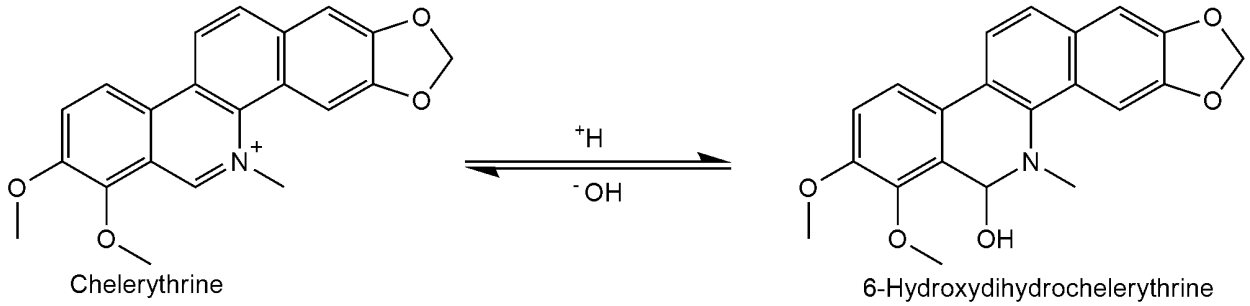

Figure 3.5. Equilibrium between chelerythrine iminium ion and its alkanolamine form (Jash et al., 2015)

The activity profiles of the crude extracts and individual compounds evaluated in this study showed a synergistic effect for free radical scavenging (Fig. 3.2), while an antagonistic effect (Fig. 3.4A) was observed in the cytotoxicity study as the isolated compounds significantly reduced the viability of the MCF-7 tumour cells which was not the case when in the presence of other compounds (crude extracts, KE and BM). However, only chelerythrine has results far better than the crude extracts while the other compounds could be said to have synergistic cytotoxicity effect on the Caco-2 cells as the compounds have comparable activity with the crude extracts. An overall average of 78.3% cell survival was obtained across all the tested isolated compounds and extract from *Z. capense* on the normal kidney cell (HEK295) (Fig. 3.4C). This is indicative of the selectivity of the plant towards cancer cell. Even though chelerythrine had better activity across the two cancer cell lines, dodecyl-*trans*-*p*-coumarate had better selectivity. Both compounds represent potential candidates for cancer chemotherapeutics in that they provide a biologically-active skeletal base for which cytotoxicity, apoptosis and required selectivity can be improved on through synthetic modification of their basic functionalities and structure-activity relationships.

Among the other pure isolates in this study, sitosterol was reported to effectively promote apoptosis in the human breast cancer cell line, MCF-7 (Awad et al., 2007). A related finding was also observed for lupeol which selectively induced apoptosis in MCF-7 cells (Pitchai et

al., 2014). Rutaecarpine was also reported to be cytotoxic on selected cancer cell lines although better activity was observed in some of its derivatives (Yang et al., 1995). Lutein is classified as a cancer chemopreventive carotenoid (Tanaka et al., 2012) while pheophytin a is known for its moderate antioxidant power (Liu et al., 2014). Sesamin was reported to inhibit MCF-7 cell proliferation through the down-regulation of cyclin D1 protein expression (Yokota et al., 2007)

Chemo - morphological findings

Phytochemicals are often distributed across all plant parts. However, some classes of compounds have been proven to be confined to specific locations in the plant. Literature revealed that there is still a dearth of information on the phytoconstitutional make-up of *Z. capense* knobs. Thus, the knobs were carefully removed from the bark and investigated in this study. The two benzophenanthridines (chelerythrine and 6-hydroxydihydrochelerythrine) together with dodecyl-*trans*-*p*-coumarate and catechin were all obtained from the knobs. It is therefore worthy to note that the only flavonoid isolated from *Z. capense* was from the knobs. A similar study was conducted previously on *Z. rhetsa* whose knobs were reportedly used by the Kanikkar tribes of Tamil Nadu to reduce pain and increase lactation in nursing mothers (Lalitharani et al., 2013; Lalitharani et al., 2010). The preliminary phytochemical study showed the presence of several classes of compounds among which were alkaloids, coumarins, catechins and terpenoids (Lalitharani et al., 2013). Triterpenes are ubiquitous to many plant species (Babalola and Shode, 2013). Sitosterol and lupeol were obtained in the leaves and bark of *Z. capense*, respectively even though trace amounts were found in other plant parts. The two pigment molecules (pheophytin a and lutein) were only isolated from the leaves. Previously, studies on *Z. capense* roots from Mozambique revealed the presence of bezophenanthridine, quinazoline and quinoline alkaloids, lignans and neolignans, an amide and a triterpene (Luo et al., 2013).

CONCLUSION

The bioactive principles in *Z. capense* stem bark, knobs and leaves have been identified as alkaloids, coumarates, lignans, flavonoids, triterpenes and pigment molecules. The medicinal uses of the plant are due to the individual or synergistic biological effects of the identified secondary metabolites present. The bioassay for the cytotoxic activities of the compounds revealed the alkaloid (chelerythrine) and alkyl *p*-coumaric acid ester (dodecyl-*trans*-*p*-coumarate) which produced lower MCF cell viability compared to 5-FU, to be the most bioactive constituents. This study reveals the therapeutic potential of tree thorns (knobs) and validates their ethnomedicinal use.

CONFLICT OF INTEREST

The authors report no conflict of interest.

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CHAPTER FOUR

Germination response of *Zanthoxylum capense* (small knobwood) seeds to different pre-treatment protocols

ABSTRACT

Zanthoxylum capense has been widely used in ethnomedicine for the treatment of fever, bronchitis, infertility, stomach ache and epilepsy. Nevertheless, it has been reported threatened due to problematic propagation and over-exploitation. We therefore aim to study the germination of *Zanthoxylum capense* seeds in response to various scarification and stratification treatments. Seeds were procured and preserved at 18 ^{o}C before the experiment commenced. Seeds were either chilled, mechanically scarified, soaked in water or treated with different acids at varying durations. This was followed by surface sterilization before sowing onto plain agar in either light or dark. The highest final germination percentage (FGP) (71.1%) was obtained from seeds soaked in hot water for 15 minutes and grown in dark conditions. The 30 days chilling treatment also gave a good response (57.8%) when grown under light or dark conditions. Other FGPs of note included seeds soaked with 500 ppm gibberellic acid (GA_3) (60%, light; 44.4%, dark) and 1000 ppm GA_3 (46.7%, light; 48.9%, dark) and soaking in H_2SO_4 for 5 minutes (42.2%, dark). Overall, the seeds sown under dark conditions produced better FGPs than those sown in light. These results reveal that *Z. capense* seeds display combinational dormancy, that imposed physically by the seed coat and that imposed physiologically by the embryo. These dormancy traits can be easily overcome by either chilling or soaking in hot water or GA_3.

Keywords: Dormancy, scarification, germination, chilling, hot water treatment.

INTRODUCTION

The genus *Zanthoxylum* which belongs to the Rutaceae family, comprises about 200 species distributed globally. It includes the medicinally important *Zanthoxylum capense*, native to South Africa (Steyn et al., 1998). It is widely distributed across eastern and northern South Africa (van Wyk et al., 1997) and is also found in the flora of Mozambique and Zimbabwe (Luo et al., 2010). *Z. capense* is a small multi-branched tree about 4-10 m high with the bark armed with thorny prickles on younger branches which develop into cone-shaped knobs often scattered on mature trunks (Schmidt et al., 2002). The leaves are alternate and unevenly compound with 4-8 pairs of leaflets which have a strong smell of citrus when crushed (Schmidt et al., 2002). Ethnobotanically, the root or stem bark has been used to treat mouth ulcers and tooth ache (Steyn et al., 1998). Decoction of the leaves or root has found application in the treatment of fever, influenza, bronchitis, colds and infertility (Steyn et al., 1998; Steenkamp, 2003). Other uses are the treatment of syphilis, gastro-intestinal disorders, flatulent colic and epilepsy (van Wyk et al., 1997; Amabeoku and Kinyua, 2010). In addition to the wide array of anecdotal uses, recent scientific submissions have revealed the anticonvulsant (Amabeoku and Kinyua, 2010), antitubercular (Luo et al., 2013) and anticancer (Mansoor et al., 2013) potential of *Z. capense*.

Z. capense is propagated by seeds which are primarily dispersed by monkeys, birds and insects but rarely germinate even when provided with favourable germination conditions (Netshiluvhi, 1996). This could be the result of dormancy, a phenomenon which describes the absence of germination of a viable and intact seed (Hilhorst, 1995). Many species use seed dormancy as a survival strategy (Li and Foley, 1997) but this could contribute to the declining numbers of many important plants. *Z. capense* is currently listed by the South African National Biodiversity Institute among the threatened species in the Red List of South African Plants

(Raimondo et al., 2009). The survival of this species is at risk probably as a result of hardseededness and over-exploitation by medicinal herb collectors.

Seed dormancy could be imposed either by the seed coat (physical/exogenous) or the embryo (physiological/endogenous) (Bewley and Black, 1994). Over the years, extensive studies on dormancy breaking for the enhancement of seed propagation have brought about the application of diverse seed pre-treatments. Mechanical scarification, used to overcome impermeability of seed coats, can be carried out by rubbing the seed between two pieces of sandpaper (Faria et al., 2012). Cold stratification and chemical scarification have been reported to also stimulate germination (Baskin and Baskin, 2004). Sometimes, seeds with hydrophilic germination-inhibiting chemicals in their seed coats are subjected to leaching with water (Baskin and Baskin, 2004). Seed germination in several *Zanthoxylum* species has been reportedly poor and is ascribed majorly to the dormancy imposed by the hard seed coat (Bonner, 1974, Sanon et al., 2005). *Z. capense* produces hard seeds but an extensive search of the literature did not produce any reports on whether the seeds are exogenously dormant. Therefore, the aim of this work was to establish the best pre-treatment protocol to overcome dormancy and enhance seed germination in *Z. capense*.

MATERIALS AND METHODS

Seeds of *Z. capense* were procured from Silver Hill Seeds, Kenilworth, Cape Town in October 2013. The seeds were preserved in air-tight containers under ambient laboratory temperature (18^{o}C) prior to the commencement of the experiment. The seed moisture content (determined as an average of four replicates, n=25) was 11.20%. This was obtained by the difference in the fresh and dry weight as a percentage of the fresh weight. Various pre-sowing treatments were used including scarification and stratification. Seeds were stratified using chilling by placing intact seeds on moist filter paper in sterile Petri plates at 4^{o}C for 7 and 30 days. Mechanical scarification was achieved by rubbing intact seeds over sandpaper for 8 to 10 seconds or long

enough to slightly expose the embryo but not damage it. For water scarification, intact seeds were soaked separately in room temperature distilled water for 6 and 24 hours. Also, seeds were soaked separately in hot distilled water (80ºC) for 5 and 15 minutes. In the acid scarification treatments, intact seeds were separately soaked in 32% hydrochloric acid (HCl) for 10 and 30 minutes and in 98% sulphuric acid (H_2SO_4) for 1 and 5 minutes. In addition, mechanically scarified seeds were soaked separately for 24 hours in gibberellic acid (GA_3) (500 and 1000 ppm) and for 24 hours in potassium nitrate (KNO_3) (1% and 4%). Intact seeds that were not pre-treated were used as a control.

After pretreatments, all seeds were surface sterilised by washing in sterile distilled water with a few drops of Tween 20 for 3 minutes and then rinsed in sterile distilled water for 20 minutes. This was followed by a 60 second rinse in 70% ethanol and then two sterile distilled water rinses. The seeds were then transferred into a 3.5% sodium hypochlorite solution for 10 minutes and finally rinsed three times in sterile distilled water before sowing onto 1% agar plates. There were 16 treatments with 6 replicates in each. Each replicate had 15 seeds. Three plates per treatment were incubated in a 16-hour photoperiod supplied by a fluorescent white light at 40 $\mu mol\ m^{-2}\ s^{-1}$, while the other three were kept in the dark (the plates were wrapped with foil). All the plates were incubated in a growth room at 23ºC for 30 days. Germinated seeds were counted every 3 days (seeds in the dark were counted under a green light). A seed was considered germinated following a visible emergence of the radicle. The final germination percentage was recorded after day 30 in culture. Final germination percentages were subjected to one-way analysis of variance (ANOVA) followed by Tukey's test using Statistical Package for the Social Sciences (SPSS version 21) to determine if there were significant ($P< 0.05$) differences among the treatment means.

RESULTS AND DISCUSSION

The final germination percentage (FGP) across all treatments was in the range of 6.7 to 71.1% (Table 4.1). The FGP of the control in the light was higher (26.7%) than the dark (17.8%). However, out of the 16 treatments, 11 treatments had higher FGPs in the dark compared to the light. Fourteen treatments had higher FGPs compared to the control in the dark, while 10 treatments had higher FGPs than the control in the light. In addition, 12 treatments in the dark had higher FGPs than either the dark or light control. The highest FGP was achieved in the 15 minutes-hot water soak in the dark (71.1%) which was significantly different from the control (17.8%). In the light, the highest FGP was achieved in 500 ppm GA_3 (60%), although this was not significantly different from the FGP for 30 days chilling (57.8%). The thirty-day chilling was the only treatment that had equal FGP in both light and dark conditions (57.8%). The acid-treated seeds resulted in low germination both in HCl 10 minutes (6.7%, light; 20%, dark) and 30 minutes (13.3%, light; 15.6%, dark) while those of H_2SO_4 were higher, particularly H_2SO_4 5 minutes (28.9%, light; 42.2%, dark). There was no significant difference between the KNO_3 treated seeds and the control in both light and dark conditions. Both 1% and 4% KNO_3 had equal FGPs in the dark (33.3%). Seeds soaked for 6 hours in cold water had higher FGPs than the control in both light and dark, while seeds soaked in cold water for 24 hours had a higher FGP than the control in the dark only. Mechanically scarified seeds had higher FGPs (42.2, light; 55.6%, dark) than the control but this difference was only significant under dark conditions.

The conditions that determine and regulate germination and survival of seedlings cannot be over emphasised as key factors that influence the expansion or extinction of populations (Rasmussen and Whigham, 1998). Seed dormancy, usually a survival strategy employed by plants, is one of the important phenomena that can affect successful expansion of a species. Thus, pre-treatment of seeds prior to sowing has gained much attention in seed germination

studies. It is evident from the findings of this work that the best pre-sowing treatment for seeds of *Z. capense* is soaking them in hot water to promote germination. Hot water pre-treatment promotes germination by influencing factors such as permeability of the seed coat to water and gases, and the release of germination inhibitors (Sharma et al., 2008). It is likely that the hot water softens the hard seed coat of *Z. capense* making it more conducive to water and gas uptake. Germination-inhibiting chemicals (like phenolics) may have been leached with water. It is unknown whether *Z. capense* seeds are covered by an oil film as is the case with *Zanthoxylum gilletii* and some other species of Rutaceae (Okeyo et al., 2011). If so, washing in NaOCl and pre-treating in hot water may have removed the oil film thereby promoting germination. Germination has been reported to be appreciably increased in other species by hot water treatment (Fariman et al., 2011; Gupta and Bandopadhyay, 2013; Irvani et al., 2012; Missanjo et al., 2013).

Cold stratification is known to promote seed germination by causing an increase and a decrease in the endogenous GA_3 and the abscisic acid (ABA) concentrations, respectively (Diaz and Martin, 1972). The latter has been reported to be involved in the control of dormancy whilst GA_3 promotes germination (Nicolás et al., 1996). It could be assumed that GA_3 production was enhanced by low temperature during cold stratification of *Z. capense* seeds in this study hence promoting its germination. Furthermore, the FGP of the chilling treatments were not significantly different to that of the GA_3 treatments.

Table 4.1. Effect of different pre-treatments on the final germination percentage (FGP) of *Zanthoxylum capense* seeds after a 30-day culture period in the light and dark.

Treatment	Average Final Germination Percentage	
	Light	Dark
Control	26.7^{abcd}	17.8^{b}
Chilling 7 days	46.7^{acd}	42.2^{abcd}
Chilling 30 days	57.8^{d}	57.8^{cd}
Cold Water 6 h	35.6^{abcd}	28.9^{abc}
Cold water 24 h	20.0^{abc}	31.1^{abc}
GA_3 500 ppm	60.0^{d}	44.4^{abcd}
GA_3 1000 ppm	46.7^{acd}	48.9^{abcd}
HCl 10 min	6.7^{b}	20.0^{be}
HCl 30 min	13.3^{ab}	15.5^{b}
H_2SO_4 1 min	13.3^{ab}	22.2^{abc}
H_2SO_4 5 min	28.9^{abcd}	42.2^{abcd}
Hot Water 5 min	42.2^{acd}	53.3^{cde}
Hot Water 15 min	53.3^{cd}	71.1^{d}
KNO_3 1%	26.7^{abcd}	33.3^{abc}
KNO_3 4%	28.9^{abcd}	33.3^{abc}
Mechanical Scarification	42.2^{acd}	55.6^{acd}

Numbers with different superscript letters (a–e) are significantly different by Tukey's Post Hoc Test at $p < 0.05$.

This finding agrees with an earlier study on seed germination in *Pistacia khinjuk* seeds in which it was suggested that chilling treatments could be replaced by GA_3 treatments to overcome dormancy (Baninasab and Rahemi, 2008). It is also likely that chilling promotes germination

of *Z. capense* seed by activating the mobilization system of the embryo's reserve food (Davies and Slack, 1981). These results suggest non-deep physiological dormancy in *Z. capense* seeds. The positive response of *Z. capense* seeds to cold stratification in this study is consistent with a few *Zanthoxylum* species and some other genera (Bonner, 1974, Fariman et al., 2011, Gupta and Bandopadhyay, 2013, Irvani et al., 2012, Soltani, 2003).

In this study, mechanical scarification also promoted germination. The hard seed coat of *Z. capense* contributes largely to its germination barrier which mechanical scarification has appreciably overcome. The seed coat crack is assumed to ease the permeability of the pericarp to water and oxygen (Dewir et al., 2011) and also facilitate emergence of the radicle (Bewley, 1997). These results are similar to those obtained from some other *Zanthoxylum* species (Etse et al., 2011, Perez, 2001). Mechanically scarified seeds of *Sabal palmetto* and *Thrinax morrisii* palms were also reported to have higher FGPs than their controls (Dewir et al., 2011).

Acids and other chemicals can break dormancy partially or completely, although their effects depend on the duration of the treatments and their respective concentrations. These include acids like H_2SO_4 and HCl; peroxides like H_2O_2 and $KMnO_4$; nitrogen compounds like KNO_3 and $NaNO_3$; and plant growth regulators like BA, GA_3, GA_7 and kinetin (Dewir et al., 2011, Yang et al., 2007). Nevertheless, seeds of some plant species either do not respond or demonstrate a poor response to them. Acid treatments did not significantly promote germination of *Z. capense* seeds in the current study. The higher FGP observed in H_2SO_4 5 minutes may be due to longer time exposure compared with H_2SO_4 1 minute and control which probably helped release chemical inhibitors. This shows that exposure time and concentration influences the effectiveness of acid scarification in breaking seed dormancy. Seed germination in many plant species is well known to be increased by pre-treating with GA_3 (Bewley and Black, 1994; Phillips et al., 2003, Pipinis et al., 2012). In this study, GA_3 also promoted

germination possibly by increasing the embryonic physiological activities (Miransari and Smith, 2014).

There are only a few reports on the conditions required for effective seed propagation in the genus. The present study presents a simple, economical approach to promote germination in *Z. capense* using either hot water or cold stratification treatments. It is concluded that *Z. capense* seeds display both exogenous and non-deep physiological dormancy which require pre-treatment to promote germination.

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CHAPTER FIVE

Evaluating the bioreducing potential of the leaves, knobs and roots of *Zanthoxylum capense* (small knobwood) for the synthesis of silver nanoparticles, applicable to *in vitro* fungal contamination control

ABSTRACT

In this study, we report on the green synthesis of silver nanoparticles using extracts from selected morphological parts of *Zanthoxylum capense*. UV-Vis spectra of the biosynthesised silver nanoparticles (AgNPs) revealed absorption peaks at around 450 nm, indicative of the nanoparticles' surface plasmon resonance. The nature, shape and morphology of the biosynthesised AgNPs were examined using Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray (EDX) analysis. *Zanthoxylum capense* AgNPs were mostly spherical in shape with particle sizes in the range of 4-28 nm, 7-20 nm and 4-32 nm for leaves, knobs and roots, respectively. Leaf extracts were the most efficient in the synthesis of AgNPs with an average yield of 0.027 g AgNPs per g of plant (dry mass). The AgNPs were more effective than sodium hypochlorite (NaOCl) and sodium dichloroisocyanurate (NaDCC) in the control of *in vitro* fungal contamination in nodal explants of Z. *capense* up to two weeks. Shoots induced from the surface sterilised explants were further used for shoot multiplication on benzyl aminopurine (BAP) and kinetin (KIN). BAP at 0.5 mg L^{-1} gave the highest percentage (88.6%) of explants bearing shoots with an average of 4.78 shoots per explant. A total of 15 fungal endophyte strains associated with *Z. capense* were identified using molecular methods.

Keywords: Biosynthesis, microscopy, nanoparticles, pathogenicity, spectroscopy.

INTRODUCTION

Research geared towards the synthesis and application of nanoscale silver particles have gained serious attention in recent years due to their unique physicochemical and biological properties. The biointeractions of silver nanoparticles (AgNPs) are significantly enhanced by their large surface area-to-volume ratio as compared to their bulk counterparts (El Nour et al., 2010). This, along with their chemical, conductive and optical properties allow them to find applications in modern medicine (Wong and Liu, 2010; Pantic, 2014), biosensing (Ren et al., 2005; Doria et al., 2012), drug delivery (Brown et al., 2013; Benyettou et al., 2015), renewable energy (Prasad et al., 2013) and catalysis (Crooks et al., 2001; Dong et al., 2015; Kumar et al., 2016). Their broadened spectrum of antifungicidal and antibactericidal properties have made them popular in consumer products like cosmetics and textiles. These properties have also enabled them to find application in food processing (El Nour et al., 2010) and water purification (Zhang, 2013).

Biocatalysed synthesis of AgNPs have been reported from various bio-resources and is a Ag^1 to Ag^0 reduction route that is preferred over chemical and physical approaches due to cost-effectiveness, it being less hazardous to living organisms and being environmentally friendly (Prasad, 2014). AgNPs have been successfully synthesised from medicinal plant species (Banerjee et al., 2014), leafy vegetables (Sigamoney et al., 2016) and seaweeds (Kannan et al, 2013; Selvi et al., 2016). Other biofactories include animal tissues, animal waste (Jha and Prasad, 2013), endophytic fungi (Chandrappa et al., 2016; Govindappa et al., 2016) and other microorganisms (Mahgoub and Samaras, 2014; Ebrahiminezhad et al., 2016). Optimisation of these bio-inspired synthetic methods is on-going with the introduction of the microwave-assisted technique (Abdel-Fattah et al., 2015) and the use of the statistical experimental design tool, central composite design (CCD) which incorporates biosynthesis parameters like incubation time, concentration and volume of reaction mixture components (Biswas and Mulaba-Bafubiandi, 2016). Medicinal plants are known to be rich sources of biologically active

compounds (Zulkipli et al., 2015). The presence of diverse secondary metabolites in addition to primary metabolic products in these plants contribute largely to their efficacy in the green synthesis of AgNPs. Since there are variations in the distribution of secondary metabolites across different morphological parts of plants, different plant parts including leaves, fruits, bark and roots are used for the synthesis of AgNPs (Mittal et al., 2014; Gavade et al., 2015; Singh et al., 2015).

Zanthoxylum capense is a woody South African species of the Rutaceae family and is found in KwaZulu-Natal and Eastern Cape and distributed across Mozambique and Zimbabwe. It grows to a height of about 10 m with its trunk covered in knobs (Amabeoku and Kinyua, 2010). The fruits possess a strong smell of citrus. The roots and stem bark of *Z. capense* are used traditionally for the management of snake bite, epilepsy, toothache (Steyn et al., 1998) and HIV/AIDS-related diseases (Semenya et al., 2013). Amabeoku and Kinyua (2010) recently reported on the anticonvulsant potential of *Z. capense* leaf extracts. The incessant collection by traditional healers and problematic propagation resulting from its hard seededness (Netshiluvhi, 1996) has resulted in *Z. capense* being classified as a threatened species (Raimondo et al., 2009) with a high risk of extinction. *In vitro* micropropagation can provide an avenue for the mass production of desired plant species which can be used to produce secondary metabolites of interest (Murashige, 1974; Allan, 1981; Khosroushahi et al., 2006).

However, asepsis plays a vital role in the establishment of *in vitro* protocols for either research or commercial tissue culture purposes (Leifert et al., 1994). Since fungi and bacteria rapidly proliferate in *in vitro* cultures (Enjalric et al., 1987) and compete with plant cultures for media nutrients (Pierik, 1997), explants for *in vitro* propagation must first undergo surface sterilisation using conventional sterilants such as sodium hypochlorite, calcium hypochlorite, ethanol or mercuric chloride at varying concentrations and soaking durations (Meghwal et al., 2000; Etsè et al., 2011) while antibiotics and fungicides are sometimes incorporated into the

culture media (Jena and Samal, 2011). But commonly practiced surface sterilisation techniques are not very effective in woody plant species (Reed et al., 1998). Moreover, it has been reported that antibiotics can be phytotoxic, inhibiting *in vitro* shoot multiplication and plantlet regeneration (Leifert et al., 1992; da Silva et al., 2003). Silver nanoparticles have recently emerged as more efficient and less toxic (to humans during handling) for the control of *in vitro* microbial contaminants owing to their multidimensional modes of microbial inhibitory action (Ge et al., 2014). Nanosilver-mediated elimination of contamination has been reported in some plant species (Abdi et al., 2008; Rostami Shahsavar, 2009; Safavi et al., 2011; Mahna et al., 2013).

To the best of our knowledge, there are only two reports on micropropagation in the genus *Zanthoxylum* (Hwang, 2005; Etsè et al., 2011). This may be associated with the high level of fungal or bacterial contamination in culture. In addition, no *Zanthoxylum* species have been studied for their potential in the green synthesis of AgNPs or the use of synthesised nanoparticles in controlling *in vitro* microbial contamination. The present study, therefore, aimed to explore the rich phytoconstituents of *Z. capense* as bioreductants, capping and stabilising agents in the synthesis of AgNPs, which in turn were tested for the management of microbial contamination during *in vitro* culture. In addition, endophytic fungi associated with *Z. capense* during *in vitro* culture were isolated and identified.

MATERIALS AND METHODS

Collection of plant materials

The leaves, knobs and roots of *Z. capense* were collected in June 2015 from the University of KwaZulu-Natal (UKZN), Durban, South Africa. The plant was identified by Dr Syd Ramdhani and a voucher specimen was deposited in the Ward Herbarium (No. Bodede 01) at UKZN, School of Life Sciences.

Preparation of plant extracts

The leaves, knobs and roots of *Z. capense* were air dried for four weeks and ground to near-powder using a metallic mortar and pestle. Each plant part (5 g) was boiled with 100 mL of Millipore™ water for 15 min and the resulting aqueous solution was filtered through Whatman No. 1 filter paper. The crude aqueous extracts were stored at 4 ºC and used within 48 h.

Synthesis of silver nanoparticles (AgNPs)

The reduction of Ag^{+} was achieved by adding 5 mL of each *Z. capense* crude aqueous extract to 20 mL $AgNO_3$ (1 mM) (Sigma Aldrich, South Africa). The solution was incubated for 24 h in the light at room temperature. The change in colour from light yellow to dark brown was indicative of the presence of AgNPs (Kannan et al., 2013; Sharma et al., 2013). The procedure was repeated with the incubation for 30 min at 80 ºC. All syntheses were performed in triplicate.

Quantification of AgNPs

Each AgNP solution was subjected to centrifugation using an Eppendorf Centrifuge (Model:5804/5804 R, USA). The six treatment solutions (leaves, knobs and roots at room temperature and at 80 ºC) were separately transferred into pre-weighed Eppendorf tubes and purified for 2 h at 5000 rpm and at 4 ºC. The supernatant from each solution was decanted, while the pellet was reconstituted in 20 mL sterile distilled water and the centrifugation step repeated three times for effective removal of unreacted materials. The samples were then oven-dried at 50 ºC for 24 h after which the tubes were re-weighed to obtain the yield of the synthesised AgNPs.

Morphological analysis of AgNPs

The bioreducibility of Ag^1 to Ag^0 was evaluated using a SHIMADZU UV-2600 UV-Vis Spectrophotometer (Japan) at a range of 200-700 nm with a resolution of 1 nm. The size and morphology of the AgNPs were examined using transmission and scanning electron microscopy (TEM and SEM, respectively). For TEM measurements, solutions of synthesised AgNPs were sonicated using a sonication bath (SONICLEAN, England) and evenly dispersed AgNPs were coated onto carbon-coated TEM grids and placed under a lamp for the evaporation of the solvent before viewing. The SEM images were obtained on an ultra plus field emission gun scanning electron microscope (FEGSEM) (Carl Zeiss, Germany) operated at 5 kV accelerating voltage and equipped with energy-dispersive X-ray analysis (EDX) (Aztec Analysis Software, United Kingdom) to allow for the determination of the elemental composition. Dried samples were placed on aluminium stubs using carbon tape.

Surface sterilisation of *Z. capense* explants

Nodal explants of *Z. capense* from the nursery purchased and greenhouse maintained plants were cut to a length of about 12 ± 2 mm, washed 5 times under running tap water before being subjected to various surface sterilisation protocols. Explants were first rinsed in 70% ethanol for 1 min followed by a 5-min rinse in 3.5% sodium hypochlorite (NaOCl) and finally rinsed for 5 min in sterile distilled water three times. This procedure was repeated by replacing NaOCl with sodium dichloroisocyanurate (NaDCC). For NaDCC, explants were left in the dark (exposure times were 0.17, 0.5, 1.0, 6.0, 12.0 and 24.0 h) to reduce contamination. This was followed by washing once with sterile distilled water followed by a 1 min 70% ethanol rinse, then 3 times sterile distilled water rinse for 3 min each. In another sterilisation treatment, explants were treated with NaDCC as above for 6 h. They were further treated with the synthesised AgNP solution from the knobs at different concentrations (0, 25 and 50 mg L^{-1}) for 30 min before culture. AgNPs synthesised from the knobs were selected as it showed

minimal agglomeration and was the preferred particle-size range compared to the leaves and roots.

In vitro culture

Following surface sterilisation, all explants were edge trimmed and cultured on MS (Murashige and Skoog, 1962) basal salt medium supplemented with 3% (w/v) sucrose, 2.0 mg L^{-1} benzylaminopurine (BAP), 1.0 mg L^{-1} indole-3-butyric acid (IBA) and AgNP solutions at concentrations of 0, 25, 50 or 100 mg L^{-1} in 20 × 100 mm culture tubes. All media were solidified with 1% (w/v) agar. The pH of the media was adjusted to 5.8 ± 0.2 before autoclaving for 20 min at 121 ºC and 1.2 kg cm^{-2}. All two-week-old sterile cultures, regardless of surface sterilisation treatment used, were removed and placed onto media containing BAP at 0, 0.5 and 1.0 mg L^{-1} singly or in combination with kinetin (KIN) at 0, 0.5, 1.0 and 2.0 mg L^{-1} following the procedure as described above.

Isolation and identification of fungal endophytes associated with *Z. capense*

Sample preparation and isolation of endophytic fungi

Healthy leaf and stem cuttings of *Z. capense* were obtained from juvenile plants growing in the greenhouse or from a young tree at the School of Life Sciences, University of KwaZulu-Natal, Westville Campus. All samples were surface sterilised using an established method (Kaewkla and Franco, 2013). Potato dextrose agar (PDA) or Rose Bengal agar (RBA) solutions (39 g in 1 L distilled water) were autoclaved at 121ºC for 15 min then cooled to 40-50 ºC for 20 min. The agar media were prepared with or without antibiotic supplements i.e. chloramphenicol (10 µg mL^{-1}), ampicillin (10 µg mL^{-1}) or streptomycin (50 µg mL^{-1}) and then poured into 9 cm Petri dishes. Thereafter, plates were inoculated with aseptically dried plant tissue segments (length = 7.5 mm, 4 explants per plate), in triplicate for each sample type, incubated at 25ºC and observed daily for fungi growth.

Morphological and molecular identification of isolated fungi endophytes

After 3 days in culture, plates with sufficient fungal growth were examined and fungal material transferred to fresh PDA plates to obtain pure cultures. Selection was based on differences in colony colour, size, growth pattern and consistency. For the extraction of DNA, mycelia were transferred from the PDA plates into potato-dextrose broth in 250 mL Erlenmeyer flasks, incubated for 3 days at 28ºC, harvested and subjected to genomic DNA extraction using standard procedures (Godwin and Lee, 1993) with the ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research, Irvine, California) following the manufacturer's instructions. The isolated DNA was diluted with tris-ethylenediaminetetraacetic acid (TE) buffer and stored at 4ºC for further use.

Polymerase chain reaction (PCR) was performed using the primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') (White et al., 1990). The reaction was done in a 50 µL final volume containing 10 µL of genomic DNA, 2.5 µL of each primer (10 pM), 5 µL of Taq buffer, 0.25 µL Taq, 2 µL of $MgCl_2$ (25 mM), 1 µL of dNTPs (10 mM) and made up with nuclease-free distilled water. The parameters employed for the PCR thermal cycle were: 95ºC for initial denaturation (2 min) and denaturation (30 s), annealing at 53ºC (45 s), extension at 72ºC (1 min) and final extension at 72ºC. The amplified PCR products were examined using gel electrophoresis (1.5% agarose gels in tris-acetate and TE buffer) and further purified using a GenElute PCR Clean-Up Kit® (Sigma, South Africa). Purified PCR products were sequenced by the Central Analytical Facilities DNA Sequencing service of the University of Stellenbosch (South Africa). Edited sequences were subjected to the Basic Local Alignment Search Tool Nucleotide (BLASTN) program and comparisons were made with data available from the GenBank® databases (National Centre for Biotechnology Information, U.S. National Library of Medicine, Bethesda, Maryland).

Statistical analyses

One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used to determine if there were significant ($P < 0.05$) differences among the means of the data sets for nanoparticle yield and size and percentage contamination during *in vitro* culture. All statistical analyses were done using the Statistical Package for Social Sciences (SPSS, Version 23, IBM Corporation, Cornell, New York).

RESULTS AND DISCUSSION

Synthesis and quantification of AgNPs

The aqueous solution of the biosynthesised AgNPs from leaves, knobs and roots had similar shades of dark brown indicative of AgNPs (Mulvaney, 1996; Kannan et al., 2013) due to the reduction of Ag^1 to Ag^0 as reflected from the changes in electronic energy level (Dare et al., 2012). The temperature-enhanced synthesis (80 ºC) produced a more intensely coloured solution than the synthesis at room temperature. At elevated temperature, the molecules' kinetic energy increases which speeds up the consumption of silver ions thus reducing the chances for particle size growth (Verma and Mehata, 2016). The yields of the leaves at either room temperature (RT) or 80 ºC were significantly higher than the knobs (at RT and 80 ºC) and the roots at 80 ºC (Fig. 5.1). Plant pigments in addition to other secondary metabolites present in the leaves of *Z. capense* may have contributed to the increase in the yield of AgNPs. Recently, the pigments, pheophytin a and lutein were isolated from the leaves of *Z. capense* (Bodede et al., 2017). Lutein is a naturally occurring carotenoid, having a structure analogous to fucoxanthin which was previously reported to be responsible for the bioreduction of silver ions using *Amphora* sp. (Jena et al., 2015). Leaves and roots produced higher yield of AgNPs compared to knobs (Fig. 5.1). This may be due to higher concentration of the phytochemicals in the leaves and roots.

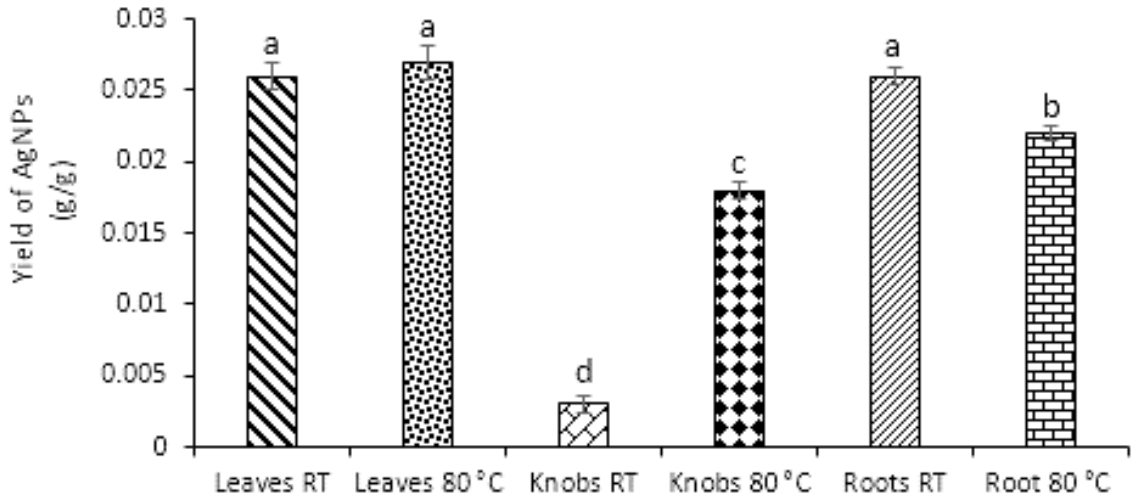

Figure 5.1. Yield of AgNPs produced from leaves, knobs and roots of *Z. capense* at room temperature (RT) and at 80 ºC (g AgNPs per g of plant material (dry mass))

Values represent mean ± SD (n = 3). Columns labelled with different letters (a - d) are significantly different at $p < 0.05$.

UV-visible spectral analysis

The formation of AgNPs was confirmed by the absorption peaks observed along the absorption band of the surface plasmon resonance of the AgNPs free electrons (Mulvaney, 1996). For both room temperature and temperature-enhanced sysnthesis, absorption peaks were observed at 440, 435 and 450 nm for the leaves, knobs and roots, respectively (Fig 5.2). The occurrence of the SPR band around 440 nm and the broadness of the peaks are indicative of the spherical shape (Lokina et al., 2014) and dispersion (Jain et al., 2009) of nanoparticles, respectively. The nanoparticles synthesised from knobs were the best dispersed as confirmed in both TEM (Fig. 5.5B) and SEM (Fig. 5.3B & E) images. Higher absorption peak intensities were observed for AgNPs synthesised from leaf extracts and knob extracts at 80 ºC while AgNPs synthesised from root extracts at room temperature had a higher intensity. A progressive increase in the intensity of absorption peaks was also observed in previously reported temperature-enhanced AgNP biosynthesis (Emeka et al., 2014; Ibrahim, 2015).

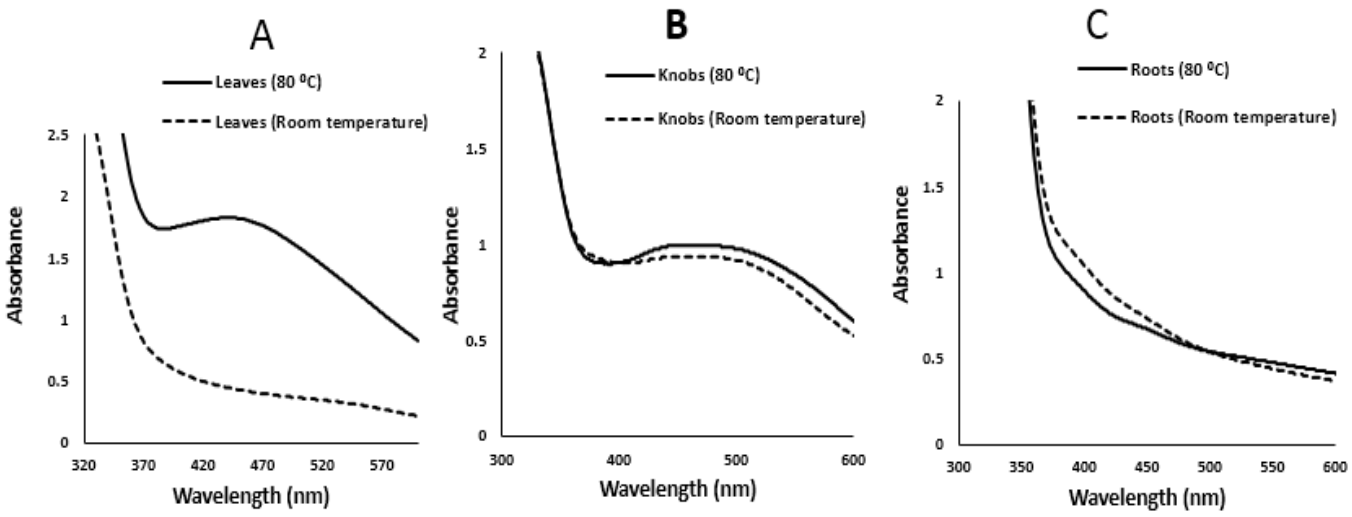

Figure 5.2. UV – visible spectra of AgNPs synthesised from *Z. capense* leaves (A), knobs (B) and roots (C) at room temperature and at 80 ºC

Scanning electron microscopy and EDX analysis

Figure 5.3 shows the SEM images of the synthesised AgNPs. Particles of similar size were well distributed and far less agglomerated in the knobs (Fig. 5.3B & E) compared to leaves and roots. The difference in degree of agglomeration may be attributed to slight differences in the structures of bioreducing compounds (Sahu et al., 2016) or some physicochemical parameters like pH and concentration (Prathna et al., 2011). Flavonoids are known to be good stabilisers of AgNPs and, thus far, the only flavonoid isolated from *Z. capense* was obtained from the knobs during the phytochemical investigation earlier conducted on the plant (Bodede et al., 2017). This may have also contributed to the low agglomeration of the AgNPs biosynthesised using knobs. This was further substantiated with the EDX profiles (Fig. 5.4) with strong Ag peaks and weaker peaks for carbon, oxygen and other elements arising from the biomolecules that are surface-bound to the AgNPs. Silicon was observed only in the roots at RT. At RT, percentage elemental silver in the knobs (48.4%) was significantly lower than leaves (69.3%) and roots (70.0%). Percentage elemental silver increased with temperature in all cases

(difference was only significant for the knobs), leaves (RT = 69.3%; 80 ºC = 74.4%), knobs (RT = 45.4%; 80 ºC = 66.9%) and roots (RT = 70.0%; 80 ºC = 77.5%).

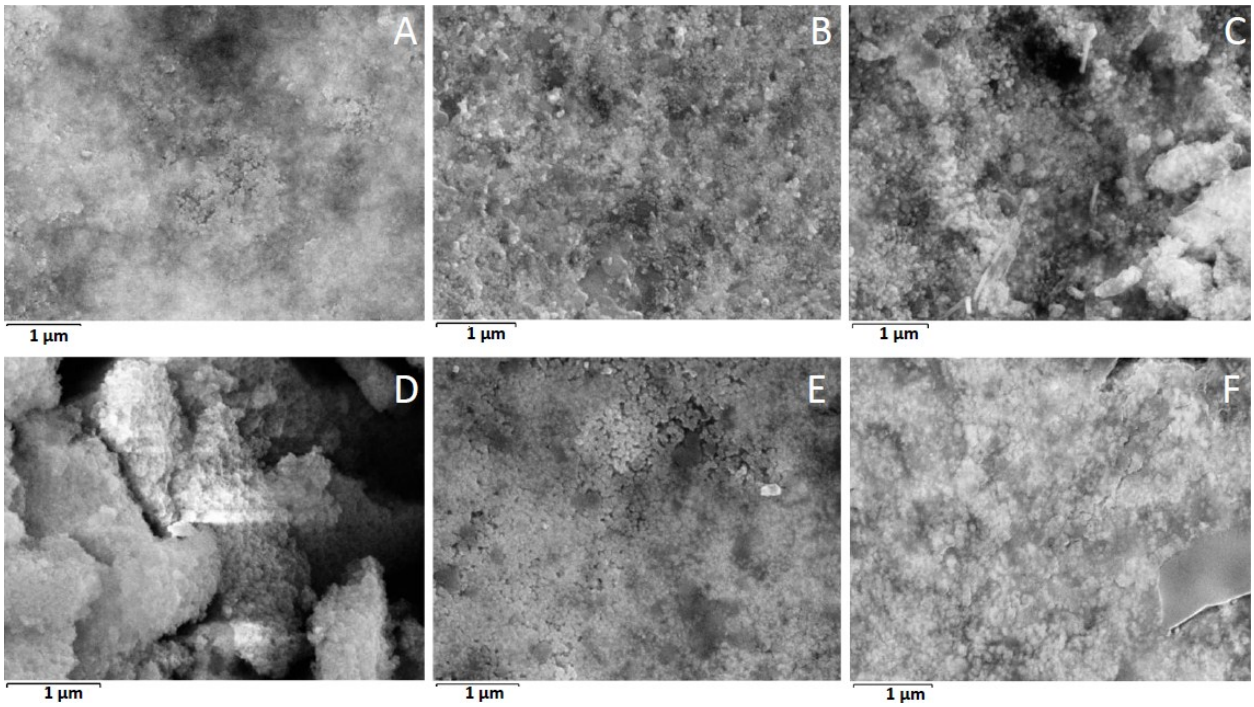

Figure 5.3. SEM images of AgNPs biosynthesised from the leaves, knobs and roots of *Z. capense* at room temperature (A, B and C, respectively) and at 80 ºC (D, E and F), respectively.

Biosynthesis at elevated temperature was also observed to have increased the yield for the knobs but a decrease was noticed with the roots while there was no significant difference between the yield of leaves at room temperature and at an elevated temperature. These differences can best be explained by the nature of phytocompounds involved in the bioreduction and subsequent stabilisation of the AgNPs. In previous studies on green synthesis, temperature has been proven to have a strong influence on the yield and morphological characteristics of AgNPs (Jiang et al., 2011; Kalpana et al., 2014; Lee et al., 2014).

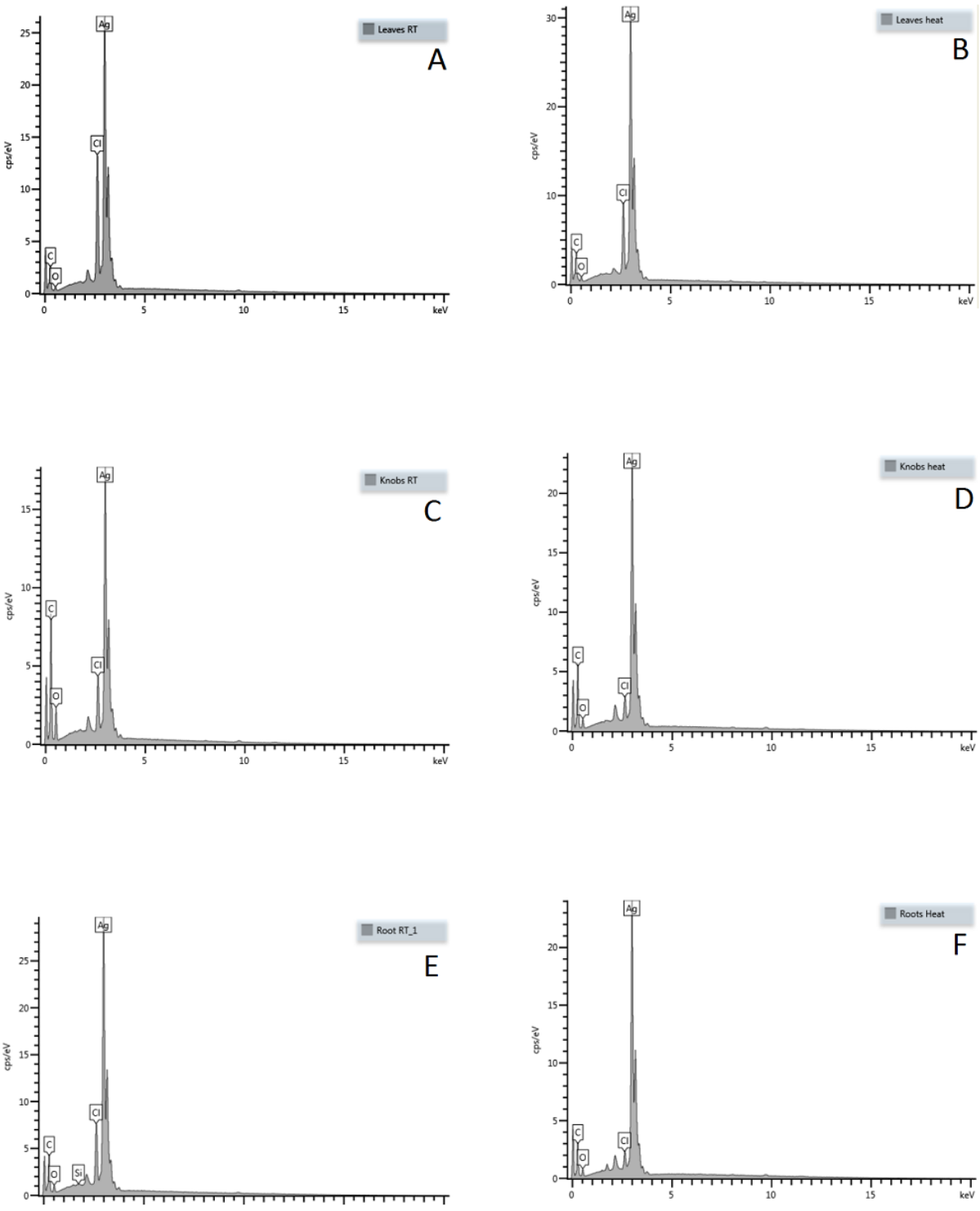

Figure 5.4. EDX spectra of AgNPs synthesised from Z. capense leaves (A&B), knobs (C&D) and roots (E&F) at room temperature and at 80 ºC, respectively

Transmission electron microscopy

The shapes of the AgNPs as revealed by the TEM images were found to be predominantly spherical (Fig. 5.5) for the three extracts with a few nanorods seen from the leaf extract (Fig. 5.5A). The knobs gave the best nano-sized particle distribution ranging from 7 to 20 nm in the entire sample population while a wider range was observed in both leaves (4-28 nm) and roots (4-32 nm) (Fig. 5.6). Films of the biocapping molecules were observed around the AgNPs. This is important in that the former is responsible for the stabilisation of the AgNPs. Extracts from plants are known to contain phytocompounds serving dual purposes of both reducing Ag^{1} and stabilising Ag^{0} in the green-synthesis of size and shape-controlled AgNPs (Rauwel et al., 2015).

Morphological characterisation of AgNPs was better achieved using TEM than SEM. TEM clearly reveals the individual nanospheres and nanorods of the particles with their sizes as compared to SEM which shows mainly the conglomerated environment of the AgNPs. The class, concentration and combining ratios of the numerous secondary metabolites in plants are known to vary across different plant organs (Kumar et al., 2010). Thus, explaining the observed differences in the characteristics of the AgNPs synthesised by each plant organ as observed in this study.

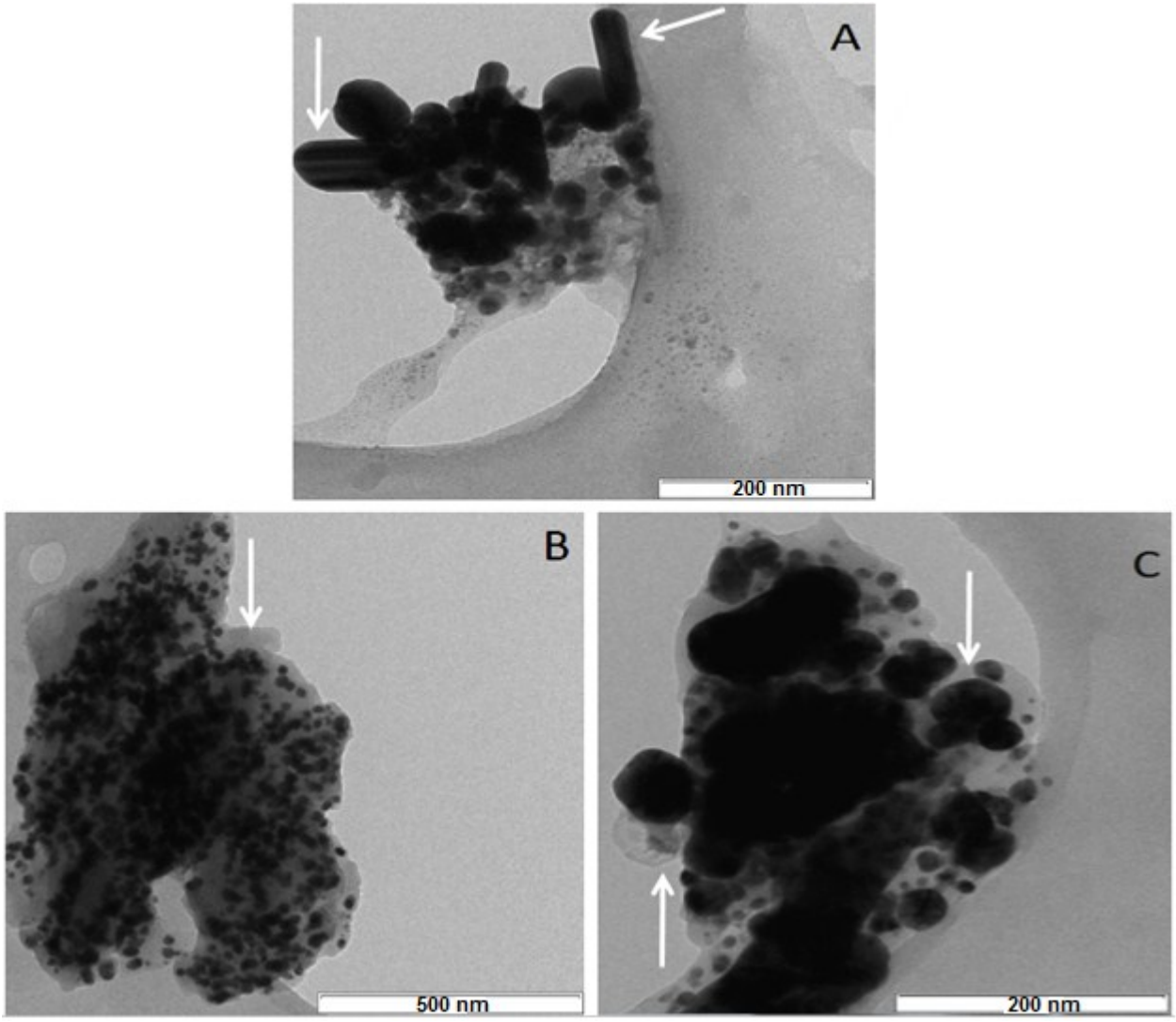

Figure 5.5. TEM images of AgNPs biosynthesised at 80 ºC from the leaves (A), knobs (B) and roots (C) of *Z. capense*

Arrow heads point to nanorods (A) and films of biocapping molecules (B and C).

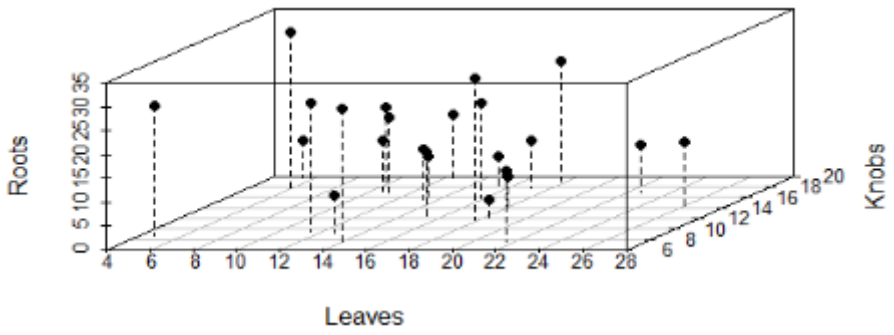

Figure 5.6. Particle size (nm) distribution of AgNPs synthesised (at 80 ºC) from the leaves, knobs and roots of *Z. capense*

Surface sterilisation of *Z. capense* explants using NaOCl and NaDCC

The treatment using 70% ethanol with NaOCl did not show any potential for the sterilisation of *Z. capense* explants as all explants were contaminated with fungi after 3 days in culture (data not shown). Replacing NaOCl with NaDCC gave better results (Table 5.1) probably due to the source of available chlorine. NaDCC is an organic form of chlorine having a solution pH of around 7, at which point hypochlorous acid (HOCl) makes up 80% of the solution's ionic constitution (Bausher and Niedz, 1998).

Table 5.1. Percentage *Z. capense* explants contaminated after various treatments with NaDCC after a one-week culture period.

Treatment	**Treatments**		**% contaminated**
	NaDCC (ppm)	Time (h)	
N1	1000	0.17	100.0 ± 0.00^{a}
N2	1000	0.5	98.7 ± 1.15^{a}
N3	1000	1.0	96.7 ± 2.89^{a}
N4	2000	0.17	80.0 ± 0.00^{b}
N5	2000	0.5	78.3 ± 2.89^{b}
N6	2000	1.0	60.7 ± 1.15^{c}
N7	5000	6.0	40.0 ± 0.00^{d}
N8	5000	12.0	15.0 ± 0.00^{e} dns
N9	5000	24.0	1.7 ± 2.89^{f} dns

All explants that did not survive one week of culture are denoted (dns). NADCC = sodiumdichloroisocyanurate. N = NADCC treatment number. Percentages are given as mean ± SD. Means followed by the same superscript letter are not significantly different using Tukey HSD test at 5% probability level (n = 3).

Explants from treatments N8 and N9 had the highest percentage of sterilisation. However, necrosis set in within one week of culture. Exposing *Z. capense* explants to NaDDC beyond 12

h is therefore not suitable since this resulted in very high necrosis even though explants displayed little to no contamination. As concentration of NaDCC increases with the exposure time, the level of fungal contamination decreases with subsequent increase in necrosis.

AgNP-mediated decontamination of *Z. capense* explants

Table 5.2 shows the potential of synthesised AgNPs in the control of *in vitro* contamination in *Z. capense* explants. Treatments T3, T5 and T10 had the best results with only 25% contamination. About half of the explants from T4, T6, T8 and T11, 27% from T7 and 25% of explants in treatments T9 were free of contamination. Nearly all explants were contaminated in T12 while all explants in the control (T1) (which had no AgNP treatment during both surface sterilisation or in the culture medium) were contaminated. Increasing concentration of AgNPs from 50 to 100 mg L^{-1} in the media (T3 and T4) showed a decline in the percentage of explants without contamination (75 to 50%, respectively). In the Ag rinse-only treatment, a good result (75% of explant not contaminated) was obtained at 25 mg L^{-1} concentration (T5).

Higher concentrations of AgNPs in both the surface sterilisation stage and in the culture media were not effective in the control of contamination as observed in treatment T12 which had no uncontaminated explants after the culture period. It is possible that the high Ag concentration was lethal for the cell wall (Tripathy et al., 2014), thereby allowing for the release of endogenous fungi. More so, there was emergence of fungi after the two-week culture period suggesting that AgNPs did not completely inhibit fungi contamination in *Z. capense* but rather delayed its emergence.

All sterile cultures, regardless of sterilisation treatment, were used for shoot multiplication and the results are presented in Table 5.3. The highest percentage of explants with shoots (88%) was recorded in explants cultured on 0.5 mg L^{-1} BAP while 1 mg L^{-1} KIN resulted in 74% explant producing shoots. Combining plant growth regulators (PGRs) for shoot multiplication

led to a decrease in the percentage of explants with shoots as observed in treatments 5 to 8 (Table 5.3).

Table 5.2. Percentage *Z. capense* explants contaminated after various treatments with AgNPs after a two-week culture period.

Treatment	Treatments		% contaminated
	AgNPs rinse (mg L^{-1})	AgNPs in medium (mg L^{-1})	
T1	0	0	100.0 ± 0.00^{a}
T2	0	25	97.7 ± 2.52^{a}
T3	0	50	25.0 ± 5.00^{d}
T4	0	100	50.3 ± 2.08^{c}
T5	25	0	25.0 ± 10.44^{d}
T6	25	25	50.0 ± 0.00^{c}
T7	25	50	73.3 ± 16.07^{b}
T8	25	100	51.0 ± 3.61^{c}
T9	50	0	75.0 ± 5.00^{b}
T10	50	25	25 ± 3.00^{d}
T11	50	50	51.0 ± 1.00^{c}
T12	50	100	99.4 ± 0.81^{a}

T = rinse treatment with AgNP solution with or without AgNP added to the culture medium. Percentages are given as mean ± SD. Means followed by the same superscript letter are not significantly different using Tukey HSD test at 5% probability level (n = 3).

The inhibitory effect of combined PGRs in this study shows that *Z. capense* may possess an appreciable concentration of some endogenous PGRs, thus limiting its requirement from external source. The result obtained from the use of BAP in the present study agrees with those reported in the propagation of *Zanthoxylym piperitum* (Hwang, 2005) and *Zanthoxylym zanthoxyloides* (Etsè et al., 2011).

Table 5.3. Effect of benzylaminopurine (BAP) and kinetin (KIN) on the development of shoots from the nodal explants of *Z. capense* for a culture period of 21 days.

Treatment	Medium		% of explants with shoots	Average no of shoots/explant (mean ± sd)	% fungal contamination
	BAP ($mg\ L^{-1}$)	KIN ($mg\ L^{-1}$)			
Control	0	0	33.3 ± 8.30^{e}	2.42 ± 3.1^{ab}	58.1 ± 0.35^{b}
1	0.5	0	88.6 ± 7.60^{a}	4.78 ± 3.3^{a}	22.2 ± 2.31^{f}
2	1.0	0	52.0 ± 5.29^{c}	1.50 ± 2.0^{b}	66.6 ± 0.51^{a}
3	0	1.0	74.3 ± 4.04^{ab}	2.83 ± 2.8^{ab}	41.6 ± 1.50^{d}
4	0	2.0	50.9 ± 1.62^{c}	1.58 ± 2.3^{ab}	66.7 ± 0.06^{a}
5	0.5	0.5	66.9 ± 2.16^{bd}	1.67 ± 2.0^{ab}	41.8 ± 0.95^{d}
6	0.5	1.0	NS	NS	16.8 ± 1.11^{g}
7	1.0	0.5	33.5 ± 4.85^{e}	0.67 ± 1.2^{b}	50.0 ± 0.50^{c}
8	1.0	1.0	52.7 ± 6.42^{cd}	1.17 ± 1.9^{b}	26.3 ± 1.53^{e}

NS = No shoot. Percentages are given as mean ± SD. Means followed by the same superscript letter are not significantly different using Tukey HSD test at 5% probability level (n = 3).

The present study shows that AgNPs controlled, to a large extent, the contaminants associated with *Z. capense* explants in *in vitro* culture, having a sterilisation potential greater than those of conventionally utilised sterilants, NaOCl and NaDCC. However, the synthesised nanoparticles could not achieve a complete removal of the fungal pathogens but rather delayed their emergence. This suggests that *Z. capense* may be a natural habitat for endophytic fungi which are mostly beneficial to their host plants in a symbiotic relationship (Herman, 1990). A few members of the genus *Zanthoxylum* were reported to be hosts for certain endophytic fungi (Ho et al., 2012). It is not fully understood whether the tissue culture of such plants allows the mutual benefit to continue or the relationship becomes lethal, but it has been reported that some fungi may not be pathogenic *in vitro* despite their prolificity in cultures (Herman, 1990). This

may explain why some shoots appear healthy even in fungal contaminated culture tubes. The role played by PGRs cannot be overemphasised in micropopagation studies as they control several physiological processes in plants either as biological stimulants or inhibitors (Harms and Oplinger, 1988). The cytokinin, BAP has been used for induction or proliferation of shoots in several plants including two species of *Zanthoxylum*, *Z. piperitum* and *Z. zanthoxyloides*. PGRs are generally used in low concentrations either singly or combined with others. BAP was found to be more effective when used singly in this study. A similar result was obtained for the shoot proliferation experiment in *Z. piperitum* (Hwang, 2005). Nevertheless, a contrasting result was obtained in the micropropagation studies of *Z. zanthoxyloides* where a combination of BAP and IBA gave better results (Etsè et al., 2011). These also show that the PGR requirements vary from species to species even within the same genus.

***Z. capense*'s associated endophytic fungi**

Morphological screening and the sequencing results of the ITS-18S rDNA led to the identification of 15 isolates of fungal endophytes from *Z. capense*. The fungal strains (Accession number) included *Alternaria sp*. YLN10 (KC139496.1), *Colletotrichum boninense* (KX197406.1), *Colletotrichum gloeosporioides* (KP145439.1), *Colletotrichum sp*. X4 (KJ958362.1), *Diaporthe actinidiae* (KT163360.1), *Diaporthe kongii* (KR024740.1), *Fusarium equiseti* (HM008677.1), *Guignardia mangiferae* (EU677817.1), *Neofusicoccum sp.* GT4 (KC507279.1), *Penicillium glabrum* (KJ475813.1), *Phomopsis sp*. F89 (KM979832.1), *Stagonosporopsis cucurbitacearum* (KR085970.1), *Xylaria* sp. D14b2 (JQ341084.1), an uncultured Ascomycota clone ITS-11 AR (KJ461402.1) and an uncultured fungal clone ITS11 AR (EU718657.1). A total of 12 fungal species were identified in the field-derived stem explant while nine species were found in stem explants obtained from the green house. Overall, stems had a higher diversity of fungi compared to leaves where only six of the 15 isolates were found. Only 2 fungal strains (*C. boninense* and *C. gloeosporioides*) were present in both greenhouse

and field samples indicating a preference of fungal strains to different environments. Environmental factors, differing across geographical locations, are known to have a significant influence on the abundance of fungal species in a specific plant (Chowdhary and Kaushik, 2015).

CONCLUSION

In this study, a bio-inspired synthesis of AgNPs using the leaves, knobs and roots of *Z. capense* is reported. The results show that biosynthesis at elevated temperature is needed to increase the yield of nanosilver from leaves and knobs only. The size of the particles and their corresponding elemental Ag content were different across the three plant parts. Knobs, a rather ignored plant organ, can be employed for the biosynthesis of AgNPs with widely applicable morphological properties. A self-rescue approach to microbial contamination control was carried out as the leaves, knobs and roots of *Z. capense* were used for nanosilver biosynthesis which was in turn employed for decontamination of its explants used in preliminary tissue culture studies of this threatened species. Biosynthesised AgNPs could not prevent fungal contamination but rather delayed its emergence. However, identification of the endophytic fungi in *Z. capense* may lead to effective strategies for the control of *in vitro* fungal contamination in this species. BAP is a good candidate for shoot proliferation of *Z. capense*. This can be employed in further plant regeneration studies of this species by combination with varying ratios of low concentration auxins and/or other cytokinins.

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CHAPTER SIX

Novel terpenoids from *Senegalia nigrescens*: isolation, characterisation, antibacterial and anti-quorum sensing activities

ABSTRACT

A phytochemical investigation of *Senegalia nigrescens* resulted in the isolation of a new *ent*-kaurene diterpenoid (*ent*-kaur-15-en-18,20-diol) alongside *ent*-kaur-15-en-18-ol, which has been isolated for the first time in a plant species. Other compounds isolated included, 30-hydroxylup-20(29)-en-3*β*-ol, 3*β*-hydroxy-20(29)-en-lupan-30-al, lupeol, stigmasterol, and three flavonoids (melanoxetin, quercetin and quercetin-3-*O*-methyl ether). The structures of isolated compounds were elucidated using different spectroscopic techniques including 1D and 2D nuclear magnetic resonance. All compounds, along with the crude extracts, were tested for their antimicrobial potential against nine bacterial strains including *Chromobacterium violaceum*, a commonly used quorum sensing inhibitor indicator strain. Crude extracts from *S. nigrescens* together with the novel *ent*-kaurene diterpene (*ent*-kaur-15-en-18,20-diol) and the flavonoids (quercetin, quercetin-3-*O*-methyl ether and melanoxetin) demonstrated promising anti-quorum sensing activity using the qualitative agar-overlay assay. *Senegalia nigrescens* may represent a new phyto-therapeutic candidate for the control of existing and emerging infectious diseases.

Keywords: *ent*-kaurene; quorum sensing inhibition; chromatography; spectroscopy

INTRODUCTION

For centuries, pharmacologically-active molecules of plant origin have shown great potential for the control of infectious diseases (Cowan, 1999; Silva et al., 2012). Compounds of diverse classes have been identified as plant-derived antimicrobial agents (Cowan, 1999; Upadhyay et al., 2014). Having established the chemical signalling of microbial communities as the key disease-causing characteristic feature of most bacteria and viruses, compounds that interfere or prevent quorum sensing among these microorganisms are becoming sought-after due to a more reduced possibility of having drug resistance issues (Finch et al., 1998; Koh et al., 2013).

The genus *Senegalia*, among the newly reclassified *Acacia* genera, has 61 species being native to Africa (Kyalangalilwa et al., 2013). These species were, in the past, included among the approximately 1500 species of the *Acacia* genus world-wide (Dyer, 2014), with over 60% occurring in Australia and the rest spread across the world's subtropical and tropical regions (Saini et al., 2008). *Senegalia nigrescens* (knobthorn) is a South African species of the family Fabaceae. It grows to a height of about 18 m, with most specimens bearing hook-shaped thorns on the trunk and branches (Van Wyk, 1984). The leaves and pods of *S. nigrescens* are good fodder while the wood is termite resistant, making the plant economically important (Howell et al., 2002). *Senegalia nigrescens* has been used traditionally for the management of convulsions (Gelfand, 1985) and dysentery (Chauke et al., 2015). Several species of the genus have displayed antibacterial activity (Saini et al., 2008; Mutai et al., 2009) however, there is limited information on their anti-quorum sensing potential (Singh et al., 2009). Previous studies on *S. nigrescens* reveal the presence of oligomeric flavonoids in the heartwood (Howell et al., 2002). The root, stem bark and leaves of *S. nigrescens* have not been studied phytochemically. The aim of this study was, therefore, to isolate the secondary metabolites from *S. nigrescens* and to evaluate them relative to the crude extracts for their antibacterial activity and anti-quorum sensing potential.

MATERIALS AND METHODS

General experimental procedures

The Infrared (IR) spectra were obtained on a Perkin Elmer Spectrum 100 Fourier transform infrared spectrophotometer (FT-IR) with universal attenuated total reflectance (ATR) sampling accessory. The ultraviolet (UV) spectra were obtained using a Varian Cary ultraviolet – visible (UV-Vis) spectrophotometer. ^{1}H, ^{13}C and 2D nuclear magnetic resonance (NMR) spectra were recorded using deuterated chloroform ($CDCl_3$) or methanol (CD_3OD) at room temperature on a Bruker Avance^{III} 400 or 600 MHz spectrometer. High resolution mass spectrometry (HRMS) was done on a Waters Micromass LCT Premier TOF-MS instrument. All column chromatography (CC) was carried out using Merck silica gel 60 (0.040-0.063 mm) and preparatory thin-layer chromatography (TLC) was carried out using Merck pre-coated kieselgel 60 (20 cm × 20 cm, 0.5 mm thick) F-254 PLC plates while Merck 20 cm × 20 cm silica gel 60 F_{254} aluminum sheets were used for TLC. The TLC plates were analysed under UV (254 and 366 nm) before further visualisation by spraying with 10% sulfuric acid in methanol (MeOH) solution followed by heating. Solvents (analytical grade) and other chemicals used were supplied by either Merck (Darmstadt, Germany) or Sigma (St. Louis, USA) chemical companies.

Plant material

The root, stem bark (separated into bark and knobs) and leaves of *S. nigrescens* were collected in January 2015 from the Westville Campus of the University of KwaZulu-Natal (UKZN). The plant was identified and authenticated by the curator in the School of Life Sciences, UKZN, Westville, Durban. A voucher specimen (No Bodede 02) was deposited at the ward Herbarium of the University.

Extraction, isolation and purification

Approximately 500 g each of air-dried crushed leaves, knobs, stem bark and roots of *S. nigrescens* were extracted sequentially using n-hexane, dichloromethane (DCM), ethyl acetate (EtOAc) and MeOH. Their corresponding crude extracts were qualitatively profiled using TLC and those with similar TLC profiles were combined based on the retention factor (R_f). Hexane and DCM extracts from the knobs and stem bark were combined to give 18 g which was then fractionated using CC with a hexane:EtOAc gradient as eluent, beginning with 100% hexane for the first 1 L after which the polarity of the solvent system was gradually increased by 10% EtOAc per 400 mL of eluent until 100% EtOAc was reached. This gave 100 aliquots of 100 mL each which was then combined into 6 fractions; aliquots 1-21 (A_1), 22-25 (A_2), 36-43 (A_3), 44-60 (A_4), 61-71 (A_5) and 72-100 (A_6). A_4 yielded compound **B1** (white solid, 60 mg) while A_3 was further subjected to CC using a similar approach as described above to yield compound **B2** (white solid, 40 mg) and **B7** (white solid, 36 mg).

The MeOH extracts from the knobs and stem bark were combined (based on similar TLC profiles) to give the extract (50 g) which was further partitioned between DCM and EtOAc. The resulting DCM extract from the partitioning was loaded onto a silica gel column and eluted using hexane:EtOAc and EtOAc:MeOH solvent systems. Initially, 100% hexane was used, after which, the polarity was gradually increased by 10% EtOAc to 100% of EtOAc and then completed by adding 10% MeOH to yield 60 aliquots of 40 mL each. Fractions 17 and 18 (combined) yielded compound **B3** (white solid, 35 mg) while compound **B4** (white solid, 20 mg) was obtained from fraction 22. The EtOAc extracts of the knobs and stem bark were also combined (6 g) which gave 55 aliquots of 25 mL each following a similar CC procedure as described above for MeOH extracts of knobs and stem bark. Aliquots 35-43 were combined and further purified using PTLC (DCM:MeOH; 8:2 mobile phase) which afforded compound **B8** (yellow solid, 80 mg).

The combined hexane and DCM extracts of the root (14 g) was subjected to CC (procedure is the same as for the combined hexane and DCM of the knobs and stem bark). Fifty aliquots were collected, the sixteenth of which yielded compound **B5** (white solid, 200 mg). Aliquot 41 was further purified using CC to give compound **B6** (white solid, 18 mg). The EtOAc extract of the roots was chromatographed in a similar procedure as for the EtOAc extracts of the knobs and stem bark. Thirty aliquots were obtained and aliquot 20 gave compound **B10** (yellow needles, 65 mg). The MeOH extract of the leaves was partitioned between DCM and EtOAc. The resulting EtOAc extract (1 g) was then subjected to CC using the procedure adopted for the MeOH extract of the knobs and stem bark (total volumes of the solvent systems are scaled down). Thirty aliquots were obtained, the fifteenth of which yielded compound **B9** (yellow solid, 32 mg).

Spectroscopic data of compounds

*Ent-kaur-15-en-18-ol (**B5**)*: White solid; m.p. 120-124 ºC. IR (KBr) ν_{max} cm^{-1}: 3291 (-OH), 2921, 1436, 1028, 811. HR-ESI-MS at *m/z* 311.2358 $[M+Na]^+$ (calcd, 311.2351). The ^1H, ^{13}C and DEPT ($CDCl_3$, 400 MHz) NMR spectral data are given in Table 6.1.

*Ent-kaur-15-en-18,20-diol (**B6**)*: White solid; m.p. 135-139 ºC. IR (KBr) ν_{max} cm^{-1}: 3234 (-OH), 2917, 1476, 1029, 813. HR-ESI-MS at *m/z* 327.2309 $[M+Na]^+$ (calcd, 327.2300). ^1H-NMR ($CDCl_3$, 400 MHz). The ^1H, ^{13}C and DEPT NMR spectral data are given in Table 6.1.

*Melanoxetin (**B8**)*: Yellow solid; m.p. 215-219 ºC. IR (KBr) ν_{max} cm^{-1}: 3454, 2923, 2568, 1561, 1363,1284, 1111, 969, 768. API-TOF-MS at *m/z* 303 $[M+H]^+$ (calcd. 302.0427). ^1H-NMR (CD_3OD, 400 MHz) δ_H: 6.90 (1H, d, J = 8.4 Hz, H-5'), 6.96 (1H, d, J = 8.8 Hz, H-6), 7.54 (1H, d, J = 8.8 Hz, H-5), 7.79 (1H, dd, J = 8.4 & 1.8 Hz, H-6'), 7.89 (1H, d, J = 1.8 Hz, H-2'). ^{13}C-NMR (CD_3OD, 400 MHz) δ_C: 115.0 (C-6), 116.2 (C-2'), 116.3 (C-5, C-10), 116.6 (C-5'), 122.0

(C-6'), 124.6 (C-1'), 134.1 (C-8), 138.3 (C-3), 146.1 (C-9, C-3'), 147.6 (C-2), 148.7 (C-4'), 151.3 (C-7), 174.7 (C-4).

Evaluation of antibacterial activity

Bacterial strains

The microorganisms employed in this study were five Gram-negative indicator bacteria (*Chromobacterium violaceum* ATCC 12472, *Escherichia coli* ATCC 25922, *E. coli* ATCC 35218, *Klebsiella pneumoniae* ATCC 700603 and *Pseudomonas aeruginosa* ATCC 27853) and four Gram-positive indicator bacteria (*Enterococcus faecalis* ATCC 29212, *Ent. faecalis* ATCC 51299, *Staphylococcus aureus* ATCC 29213 and *S. aureus* ATCC 43300).

Bacterial susceptibility assay

Pure compounds (**B1- B10**; 5 mg) and six crude extracts of *S. nigrescens* were subjected to antibacterial screening using the agar-well diffusion method (CLSI, 2012). Test samples were dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 5 mg mL^{-1}. Wells (6 mm) were loaded with 100 µg, 200 µg and 500 µg of the pure compounds or crude extracts, respectively. Two standard antimicrobial agents (Oxoid, UK), i.e., ampicillin (AMP10, 10 µg per disc) and tetracycline (TE30, 30 µg per disc) as well as a negative control (DMSO-impregnated discs) were also assessed. The following zone diameter criteria were used to assign susceptibility or resistance to compounds tested: Susceptible (S) ≥ 15 mm, Intermediate (I) = 11-14 mm, and Resistant (R) ≤ 10 mm (Chenia, 2013). The criteria for assigning susceptibility or resistance to AMP10 was as follows: (S) ≥ 17 mm, (I) = 14–16 mm, (R) ≤ 13 mm, while those for TE30 were: (S) ≥ 19 mm, (I) = 15–18 mm, (R) ≤ 14 mm (CLSI, 2012).

Quorum sensing (QS) inhibition assay

All isolated compounds and *S. nigrescens* crude extracts were evaluated for their anti-QS activity using the qualitative agar-overlay diffusion assay (Chenia, 2013). This assay employs

the biomonitor strain, *C. violaceum* ATCC 12472. Molten Luria-Bertani (LB) agar (0.5% w/v, 5 mL) was inoculated with 50 µL of the violacein (purple pigment)-producing strain, *C. violaceum* 12472 grown overnight in LB broth at 30 °C with agitation. The agar-culture solution was then poured over the surface of pre-warmed LB agar plates. Varying volumes (50 and 100 µL; 250 and 500 µg) were pipetted from 5 mg L^{-1} stock solutions of pure compounds and *S. nigrescens* crude extracts and onto sterile paper discs, placed on solidified agar plates and then incubated overnight at 30 ºC and then examined for violacein production. A known anti-quorum sensing agent, (*Z*-)-4-bromo-5-(bromomethylene)-2(5*H*)-furanone (20 µg) was used as a QSI-positive control. The inhibition of QS, indicated by a colourless, opaque but viable halo around the disc, was estimated by measuring the diameter of the transparent and opaque zones for growth inhibition (GI) and quorum sensing inhibition (QSI), respectively.

Statistical analysis

Data are expressed as mean ± SD from three independent experiments. One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used to determine if there were significant differences ($P < 0.05$) among the means of the data sets. Statistical analysis was done using the Statistical Package for Social Sciences (PASW Statistics 23, IBM Corporation, Cornell, New York).

RESULTS AND DISCUSSION

Identification of isolated phytocompounds from *S. nigrescens*

The combined hexane and DCM extracts of the stem bark yielded 30-hydroxylup-20(29)-en-3*β*-ol (**B1**) (Mutai et al., 2004), 3*β*-hydroxy-20(29)-en-lupan-30-al (**B2**) (Elya et al., 2010), lupeol (**B3**) (Soumia et al., 2012) and stigmasterol (**B4**) (Jam and Ban, 2010). Hexane and DCM extracts of the root yielded the new *ent*-kaurenoids (*ent*-kaur-15-en-18,20-diol (**B6**) and *ent*-kaur-15-en-18-ol (**B5**), being isolated for the first time in plant) with a long chain alcohol,

tetracosan-1-ol (**B7**) (Oliveira et al., 2016). The flavonol, melanoxetin (**B8**) (Sousa et al., 2016) with quercetin (**B9**) (Sikorska and Matlawska, 2000) and quercetin-3-*O*-methyl ether (**B10**) (Krenn et al., 2003) were obtained from MeOH extracts of leaves and roots, respectively (Fig. 6.3).

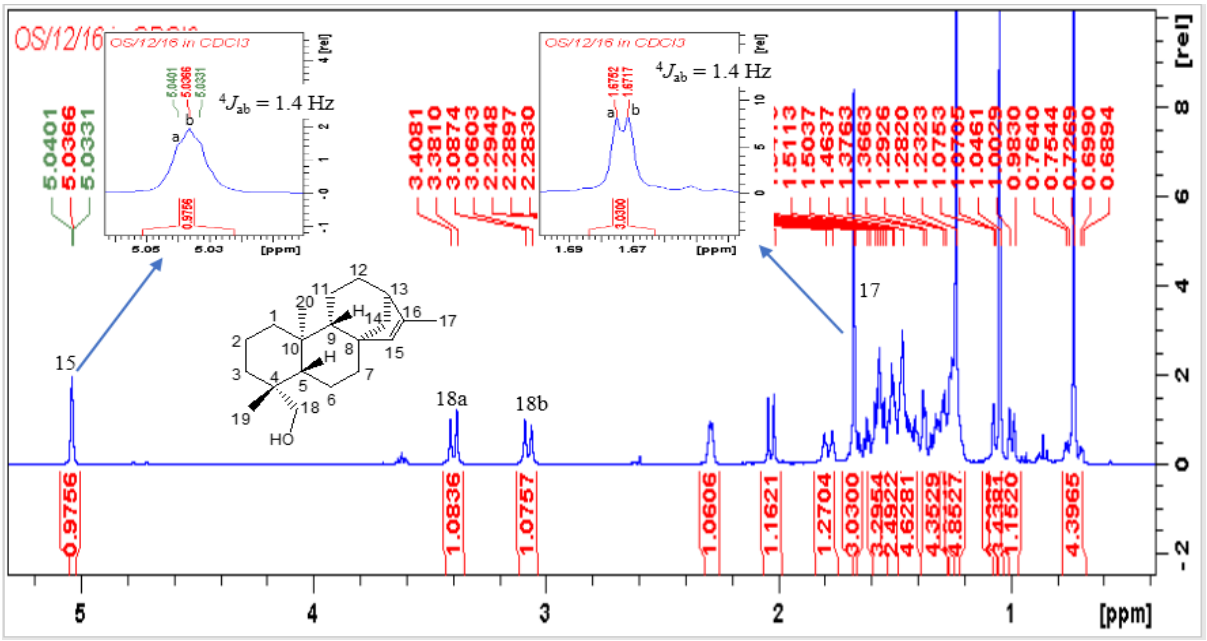

Figure 6.1. Expanded ^{1}H-NMR spectrum of compound **B5** showing the long-range "W"-type allylic coupling between protons at H-15 and H-17

Compound **B5** was obtained as a white amorphous solid. The HR-ESI-MS spectrum showed a molecular ion peak at *m/z* 311.2358 $[M+Na]^+$ (calcd for $C_{20}H_{32}ONa$, 311.2351). The ^{1}H-NMR and ^{13}C-NMR spectral data (Table 6.1) are consistent with a typical *ent*-kaurene diterpene. The doublet at δ_H 1.67 (J = 1.4 Hz) was assigned to the CH_3 at position 17. Although a singlet is expected for this quaternary methyl, a doublet was observed which is due to long-range, allylic "W"- type, (^4J) coupling. An expansion of the H-15 resonance at δ_H 5.0 revealed the presence of shoulder peaks which support the "W"- type coupling (Barfield and Chakrabarti, 1969) of methyl protons (H-17) to the methine proton (H-15) (Fig. 6.1). Similar observations for *ent*-kaurenes have been reported in previous studies (Diaz et al., 1987; Tanaka et al., 2004). The

assignments were further substantiated by the DEPT experiments which resolved 3 methyl, 9 methylene, 4 methine and 4 quaternary carbons. The resonances at δ_H 3.09 (1H, d, *J*=10.9 Hz) and δ_H 3.41 (1H, d, *J*=10.9 Hz) were assigned to the germinal protons at position 18. The HMBC correlations (Fig. 6.2) of the hydroxylated methylene protons (H-18) with C-3 and C-5 confirm the hydroxyl group to be at position 18. In addition, correlations of protons (H-17) with C-13, C-15 and C-16 justify the proximity of the terminal methyl group to the ethylenic carbons. Thus, compound **B5** was identified as *ent*-kaur-15-en-18-ol. To the best of our knowledge, this is the first report on the isolation of *ent*-kaur-15-en-18-ol from a plant species. Piozzi et al. (1973) reported the partial synthesis of *ent*-kaur-15-en-18-ol from *ent*-kaur-15-en-18-oic acid upon dissolution in ether and further treatment with lithium aluminium hydride ($LiAlH_4$). In a similar semi-synthetic approach, *ent*-kaur-15-en-18-ol was obtained by reducing methyl *ent*-kaur-16-en-19-oate with $LiAlH_4$ in anhydrous tetrahydrofuran (de Andrade et al., 2011).

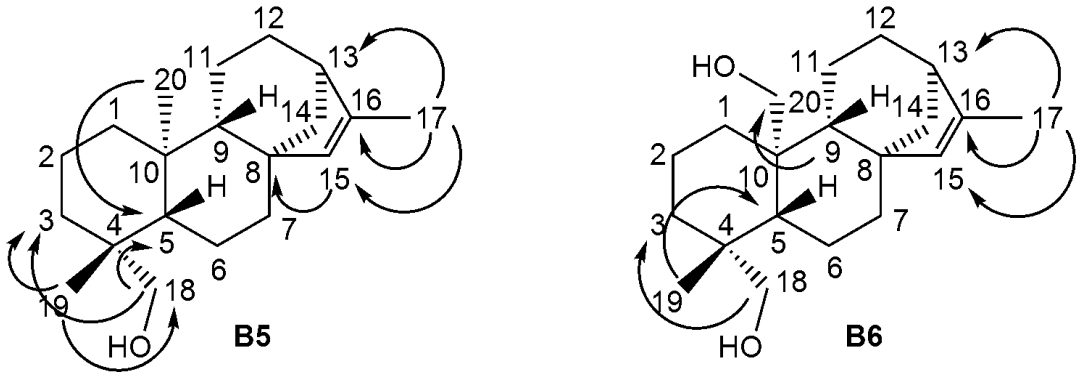

Figure 6.2. Major HMBC correlations observed in compounds **B5** and **B6**

Compound **B6** was obtained as a white amorphous solid. The HR-ESI-MS spectrum showed a molecular ion peak at *m/z* 327.2309 $[M+Na]^+$ (calcd for $C_{20}H_{32}O_2Na$, 327.2300). The ^{1}H-NMR and ^{13}C-NMR spectral data were similar to compound **B5**, with a typical *ent*-kaurene skeleton (Table 6.2). The presence of the hydroxy group at position 20 reduced the methyl groups to

two instead of three, as observed in compound **B5**. This however, led to slight differences in the chemical shifts especially around the first and second kaurene rings. The two hydroxylated methylene carbons observed in the ^{13}C-NMR spectrum at δ_C 73.9 and δ_C 64.9 were assigned to positions 18 and 20, respectively over positions 19 and 17 due to differences in the chemical shift values and HMBC correlations. Computational calculations showed that both hydroxylated methylene carbons at both positions 18 and 19 would have had similar resonances due to similar chemical environments, which was not the case. There were also no HMBC correlations (Fig. 6.1) between the hydroxymethylene protons (H-20) and either of the ethylenic carbons. However, there was a correlation between the resonance at δ_H 0.96 (H-9) and δ_C 64.9 (C-20) indicating the proximity of the hydroxylated methylene to C-9. Thus, compound **B6** was identified as *ent*-kaur-15-en-18,20-diol.

Compound **B8** was obtained as a yellow amorphous solid. The LC-ESI-MS spectrum gave a molecular ion peak at *m/z* 303 $[M+H]^+$ (calc. 302.0427) which agrees with the molecular formula, $C_{15}H_{10}O_7$. The ^{1}H-NMR and ^{13}C-NMR spectrum was typical of flavonols. More so, the observed coupling constants within the A and B ring agree with the 7,8,3,4' hydroxylation pattern. In the A-ring, resonances at δ_H 7.54 (d, *J*=8.8 Hz) and at δ_H 6.96 (d, *J*=8.8 Hz) were assigned to H-5 and H-6, respectively due to *ortho* coupling. In the B-ring, the resonance at δ_H 7.79 (1H, dd, *J*=8.4 and 1.8 Hz) was assigned to H-6' which was *ortho* coupled to δ_H 6.9 (H-5') and meta coupled to δ_H 7.89 (H-2'). The spectral data agree with literature values for the flavonol melanoxetin (Sousa et al., 2016). Melanoxetin has been previously reported from some species of the genus, *Acacia*, *A. confusa* (Tung et al., 2010), *A. melanoxylon* (Foo, 1987), *A. burkittii* (Grace et al., 2009). The present study, having found this flavonol as a major active compound suggests that melanoxetin may represent a chemotaxonomic marker for the *Acacia* genus.

Table 6.1. ^{1}H-NMR, ^{13}C-NMR and DEPT (90, 135) spectral data for compounds **5** and **6** (400 MHz, δ in ppm, $CDCl_3$).

Position	Compound B5			Compound B6		
	δ_H	δ_C	DEPT	δ_H	δ_C	DEPT
1	0.75, m; 1.79, dd	40.0	CH_2	0.76, m; 1.85, dd	40.1	CH_2
2	[a]1.41-1.49, m	18.7	CH_2	[a]1.47-1.62, m	18.7	CH_2
3	1.22-1.27; 1.35-1.43	35.3	CH_2	1.98-2.00, m; [b]0.95-0.96, m	30.1	CH_2
4		37.6	C		39.2	C
5	[b]1.01-1.09, dd	48.9	CH	[b]0.93-0.98, dd	53.1	CH
6	1.30	19.0	CH_2	[a]1.55-1.66, m	19.6	CH_2
7	[a]1.50-1.61, m	39.1	CH_2	[a]1.49-1.52, m	39.6	CH_2
8		49.1	C		48.9	C
9	[b]1.01-1.09, dd	49.0	CH	[b]0.96-1.00, dd	49.0	CH
10		39.3	C		41.9	C
11	[a]1.53-1.55, m	17.9	CH_2	[a]1.53-1.63, m	17.6	CH_2
12	[a]1.44-1.49, m	25.0	CH_2	[a]1.48, m	24.9	CH_2
13	2.29, m	44.9	CH	2.28, m	44.8	CH
14	1.30 (1H, m); 2.04 (1H, d)	43.9	CH_2	1.26-1.28, m; 1.95-1.96, m	43.7	CH_2
15	5.0 (1H, s)	135.4	CH	5.05 (1H, s)	135.2	CH
16		142.4	C		142.6	C
17	1.67 (3H, d; *J*=1.4 Hz)	15.3	CH_3	1.66 (3H, d, *J*=1.47 Hz)	15.3	CH_3
18	3.09 (1H, d); 3.41 (1H, d); *J*=10.9 Hz	72.2	CH_2	3.96 & 3.33 (1H each, d, *J*=10.6 Hz)	73.9	CH_2
19	0.72 (3H, s)	17.4	CH_3	[b]0.98 (3H, s)	17.9	CH_3
20	1.04 (3H, s)	18.0	CH_3	3.87 & 3.70 (1H each, d, *J*=10.6 Hz)	64.9	CH_2

[a,b] - Chemical shifts having the same letter are within similar overlapping signals.

The phyto-constitutional similarity between the knobs and stem bark of *S. nigrescens,* as revealed by the pre-fractionation TLC analysis of the crude extracts is consistent with the fact

that the knobs are not always well differentiated from the stem bark and may sometimes be lacking in some specimens of *S. nigrescens*. The chemo-morphological distribution of the phytochemicals of *S. nigrescens* showed that the triterpenoids and diterpenoids are confined to the stem bark and root, respectively. While the flavonoids are likely distributed across the leaves, stem bark and roots, the *ent*-kaurenes appear to be confined to the roots. In a related study recently conducted on another knob tree, *Z. capense*, the knobs were found to bioaccumulate phytocompounds different from the stem bark. More so, these knob-confined phytocompounds were found to possess higher cytotoxic activity against MCF-7 and Caco-2 tumour cell-lines compared to compounds isolated from the stem bark and the leaves (Bodede et al., 2017).

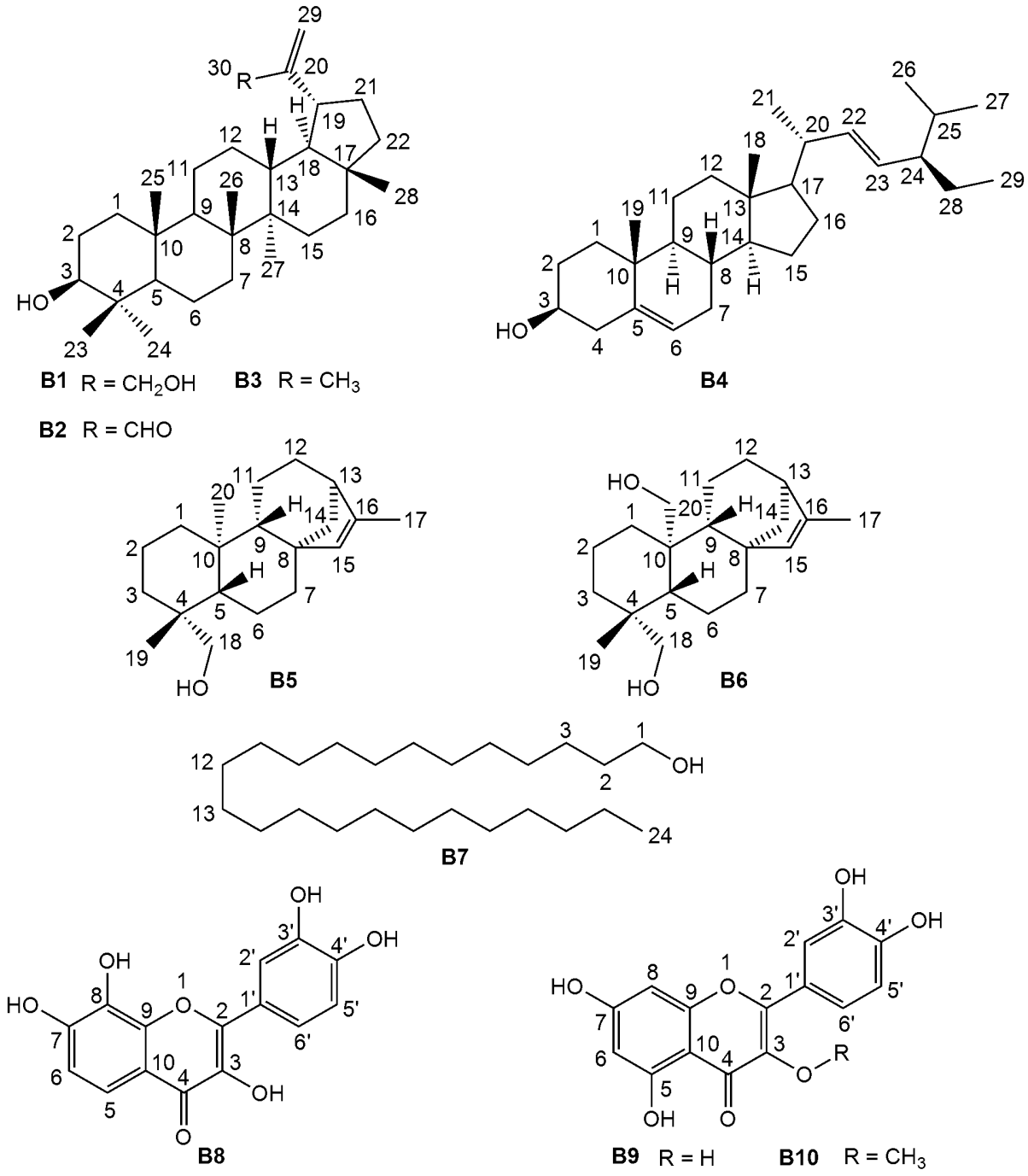

Figure 6.3. Chemical structures of compounds **B1** – **B10** isolated from *S. nigrescens*

Antibacterial activity

The results of the susceptibility test for the 10 compounds and six crude extracts of *S. nigrescens* are presented in Table 6.2. The SNB-EA and SNR-DCM extracts appeared promising due to their antimicrobial effect against the extended-spectrum *β*-lactam resistant *K.*

pneumoniae ATCC 700603, since *Klebsiella* species are important nosocomial pathogens (Podschun and Ullmann, 1998). The SHV-containing *K. pneumoniae* ATCC 700603 strain (extended-spectrum *β*-lactamase-expressing) demonstrated intermediate susceptibility to compounds **B1 - B4** and **B7**. Promising bacterial inhibition was observed with the flavonoids, melanoxetin (**B8**) and quercetin-3-*O*-methy ether (**B10**), which were active against the susceptible Gram-positive *S. aureus* ATCC 29213 and vancomycin-resistant *Ent. faecalis* ATCC 51299 at 0.5 mg, as well as susceptible Gram-negative *E. coli* ATCC 25922. Methicillin-resistant *S. aureus* ATCC 43300 and multi-drug-resistant *P. aeruginosa* ATCC 27853 demonstrated intermediate susceptibility to these flavonoids. Of particular interest, was that both sensitive *S. aureus* ATCC 29213 and methicillin-resistant *S. aureus* ATCC 43300 were susceptible to quercetin (**B9**). The results obtained for flavonoids in this study, is consistent with the findings of other researchers who have identified the class as having potent antimicrobial activity (Cushnie and Lamb, 2005). Additionally, the methicillin-resistant *S. aureus* ATCC 43300 as well as the sensitive *E. coli* ATCC 25922 demonstrated intermediate susceptibility towards *ent*-kaur-15-en-18-ol (**B5**) and *ent*-kaur-15-en-18,20-diol (**B6**) (Table 6.2). Generally, *ent*-kaurene compounds are reported to be inactive against Gram-negative but active against Gram-positive bacteria (Pereira et al., 2012).

Anti-quorum sensing activity

The quorum sensing (QS) inhibitory potential of the compounds and crude extracts of *S. nigrescens* was assessed at 250 and 500 µg (Figs. 6.4 – 6.5). Inhibition of violacein (opaque halos) by extracts or isolated compounds was examined in comparison to the QSI-positive control (*Z*-)-4-bromo-5-(bromomethylene)-2(5*H*)-furanone). All tested crude extracts demonstrated QSI at both 250 and 500 µg, however, increased growth inhibition zone diameters were observed at 500 µg (Fig. 6.4). The data for growth inhibition zone diameters *vs* quorum sensing inhibition zone diameters (opaque halos) at 500 µg are presented in Fig.

6.5. The opaque halos were greatest with the SNR-DCM extract at both tested concentrations, followed by SNR-EA. The root extracts appeared to be more potent inhibitors of QS than the stem bark extracts.

All tested compounds (except compound **B5**) showed varying levels of quorum sensing inhibition, with zone diameters (ZD) ranging from 11.5 - 30 mm compared to the furanone control (17 mm). Except for *ent*-kaur-15-ene-18-ol (**B5**), compounds **B1 - B7** demonstrated quorum sensing inhibition at 500 µg. *Ent*-kaur-15-en-18,20-diol (**B6**) demonstrated the best quorum sensing activity among the diterpenoids at 500 µg. Increased functionalities on the kaurene pharmacophore may be responsible for higher QSI activity of the *ent*-kaur-15-en-18,20-diol (**B6**) since the mono-hydroxylated derivative (**B5**) did not display QSI at both 250 and 500 µg. The flavonoids, melanoxetin (**B8**), quercetin (**B9**) and quercetin-3-*O*-methyl ether (**B10**) demonstrated the highest QSI at both 250 and 500 µg (Figs. 6.4 - 6.5). Flavonoids have been identified as QS inhibitors (Nazzaro et al., 2013). The unusual 7,8,3',4'-tetrahydroxy substitution pattern may be responsible for the activity of melanoxetin as observed in this study.

Vasavi et al. (2014) observed that quercetin and quercetin-3-*O*-arabinoside from the flavonoid fraction of *Psidium guajava* leaves inhibited violacein production in *C. violaceum* ATCC 12472, at 50 and 100 µg, respectively. As for quercetin-3-*O*-arabinoside (Vasavi et al., 2014), quercetin (**B9**) quercetin-3-*O*-methyl ether (**B10**) and melanoxetin (**B8**) demonstrated both antibacterial (Table 6.2) and anti-quorum sensing activity (Figs 6.4 - 6.5), suggesting multiple modes of action against Gram-negative and Gram-positive bacteria. The search for new antimicrobials has currently been focusing on the QS inhibitory activity of potential plant-based candidates, with some plant extracts and isolated compounds proving to be promising (Koh et al., 2013; Singh et al., 2015; Borges et al., 2016). This trend has been observed among the genus *Senegalia* (formerly *Acacia*), however, it appears to be species-specific. Acid-treated

MeOH extract of *Acacia nilotica* pods showed a concentration-dependent inhibitory effect on violacein production by the biomonitor strain, *C. violaceum* 12472 (Singh et al., 2009), while methanolic extract of *Acacia arabica* (Shukla and Bhathena, 2016) had low potential for the same strain.

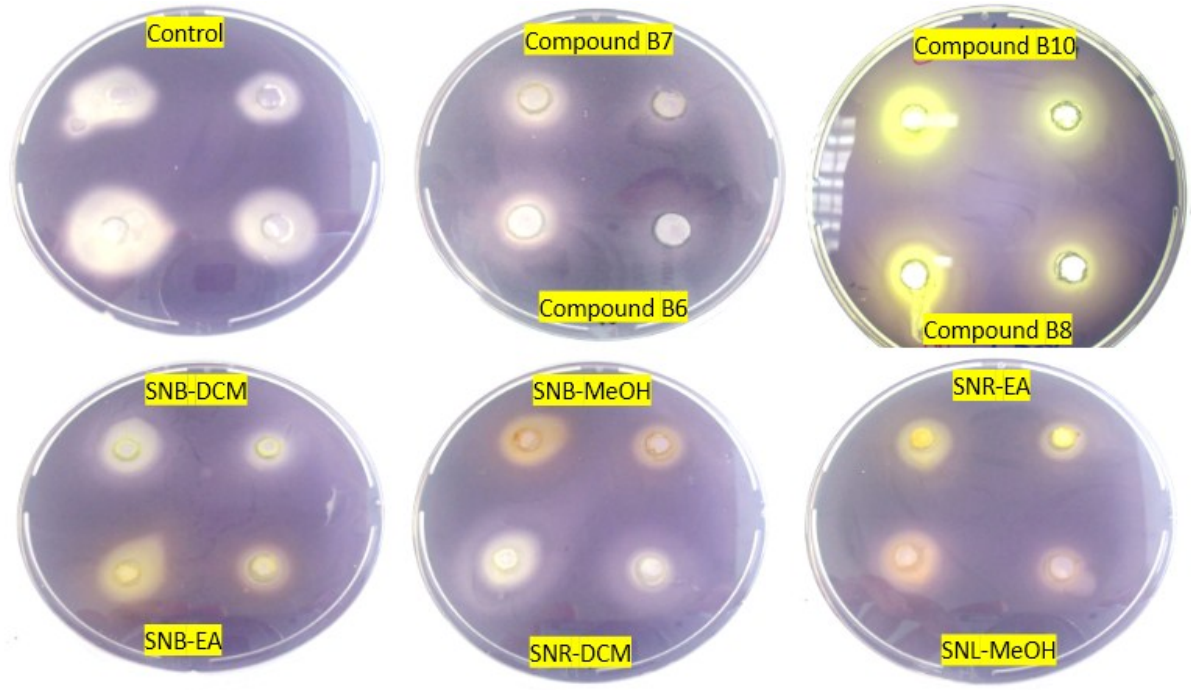

Figure 6.4. Inhibitory effect of compounds **B6**, **B7**, **B8** and **B10** and *S. nigrescens* crude extracts, in comparison with the quorum sensing inhibition-positive control (*Z*-)-4-bromo-5-(bromomethylene)-2(5*H*)-furanone) on violacein production by *Chromobacterium violaceum* ATCC 12472 strain

SNB-DCM = *S. nigrescens* bark DCM extract, SNB-EA = *S. nigrescens* bark ethyl acetate extract, SNB-MeOH = *S. nigrescens* bark methanol extract, SNR-DCM = *S. nigrescens* root DCM extract, SNR-EA = *S. nigrescens* root ethyl acetate extract, SNL-MeOH = *S. nigrescens* leaf methanol extract.

Table 6.2. Antibacterial susceptibility profile of selected gram positive and gram-negative bacteria upon exposure to *S. nigrescens* phytocompounds and crude extracts.

Test compounds	Zone diameter (mm)																	
	Gram-negative bacteria												Gram-positive bacteria					
	Ec ATCC 25922		*Ec* ATCC 35218		*Cv* ATCC 12472		*Kp* ATCC 700603		*Pa* ATCC 27853		*Sa* ATCC 29213		*Sa* ATCC 43300		*Ef* ATCC 29212		*Ef* ATCC 51299	
	Concentration (µg)																	
	200	500	200	500	200	500	200	500	200	500	200	500	200	500	200	500	200	500
Compound (**B1**)	0	10.5^{d}	0	0	0	13^{cde}	0	12^{b}	0	0	0	0	0	0	0	0	0	0
Compound (**B2**)	6^{d}	11.5^{cd}	0	0	0	13.5^{cd}	0	13^{b}	0	0	0	0	0	0	0	0	0	0
Compound (**B3**)	0	0	0	0	0	12^{e}	0	13.5^{ab}	0	0	0	0	0	0	0	0	0	0
Compound (**B4**)	0	0	0	0	0	13.5^{cd}	0	14.5^{a}	9^{b}	9	0	0	0	0	0	0	0	0
Compound (**B5**)	8.5^{c}	12^{c}	0	0	0	12.5^{de}	0	0	0	0	0	0	0	12^{d}	9.5^{cd}	9.5	0	0
Compound (**B6**)	10^{bc}	12.5^{bc}	0	0	0	13.5^{cd}	7.5^{c}	7.5	7.5^{c}	7.5	10^{fg}	11.5^{e}	8.5^{f}	14^{c}	9^{cd}	9	10^{cd}	10
Compound (**B7**)	0	0	0	0	0	0	0	14^{ab}	0	0	0	0	0	0	0	0	0	0
Compound (**B8**)	9^{c}	15^{a}	9^{c}	9	0	14^{c}	0	0	10.5^{ab}	14^{a}	9.5^{g}	20.5^{c}	8.5^{f}	14.5^{c}	10.5^{bc}	14^{c}	9^{de}	15.5^{c}
Compound (**B9**)	0	0	0	0	15^{a}	19.5^{a}	0	7^{d}	0	12^{bc}	15^{d}	17.5^{d}	14^{b}	18^{b}	0	0	0	0

Compound (**B10**)	8^{c}	15^{a}	0	0	0	12.5^{de}	7.5^{c}	7.5	10^{b}	11^{c}	10^{fg}	25^{b}	9^{ef}	12^{d}	9.5^{c}	15^{b}	8^{e}	18^{b}
SNB-DCM	0	0	8^{d}	8	9de	13.5^{cd}	0	14^{ab}	11.5^{a}	12^{bc}	17^{c}	17	9^{ef}	9	11^{b}	11	10.5^{c}	10.5
SNB-EA	0	0	9^{c}	9	9.5^{cd}	13.5^{cd}	10^{b}	15^{a}	10^{b}	12.5^{b}	11^{ef}	11	10de	10	10.5^{bc}	12^{d}	11^{c}	11
SNB-MeOH	0	0	0	0	8^{e}	12.5^{de}	0	13.5^{ab}	0	12^{bc}	9^{g}	9	11.5^{c}	11.5	8.5^{d}	8.5	10^{cd}	10
SNR-DCM	0	0	0	0	10.5^{bc}	13.0^{c}	10.5^{b}	15^{a}	0	12^{bc}	11.5^{e}	12^{e}	10.5^{cd}	10.5	11^{b}	11.5^{d}	13.5^{b}	14.5^{cd}
SNR-EA	10^{bc}	10	10^{b}	10	11^{b}	16^{b}	11.5^{a}	12^{b}	11^{ab}	11	11^{ef}	11	10de	10	11^{b}	11	10.5^{c}	14.5^{d}
SNL-MeOH	0	0	0	14.5^{b}	0	13^{c}	0	10^{c}	0	0	0	0	0	0	0	0	0	0
Ampicillin (AMP10)	22		0		0		0		0		20^{b}		0		18.5^{a}		20^{a}	
Tetracycline (TE30)	27		23^{a}		30		10.5		15		26^{a}		19^{a}		23		23	

Means (of ZD) followed by the same superscript letter(s) are not significantly different along each column using Tukey HSD test at 5% probability level (n = 3)
Ec - *Escherichia coli*, *Cv* - *Chromobacterium violaceum, Kp* - *Klebsiella pneumoniae, Pa* - *Pseudomonas aeruginosa*, *Sa* - *Staphylococcus aureus*, *Ef* - *Enterococcus faecalis.*
Compounds: 30-hydroxylup-20(29)-en-3*β*-ol (**B1**), 3*β*-hydroxy-20(29)-en-lupan-30-al (**B2**), lupeol (**B3**), stigmasterol (**B4**), *ent*-kaur-15-en-18-ol (**B5**), *ent*-kaur-15-en-18,20-diol (**B6**), tetracosan-1-ol (**B7**), melanoxetin (**B8**), quercetin (**B9**), quercetin-3-*O*-methyl ether (**B10**).
Extracts: SNB-DCM (*S. nigrescens* bark DCM extract), SNB-EA (*S. nigrescens* bark ethyl acetate extract), SNB-MeOH (*S. nigrescens* bark methanol extract), SNR-DCM (*S. nigrescens* root DCM extract), SNR-EA (*S. nigrescens* root ethyl acetate extract), SNL-MeOH (*S. nigrescens* leaf methanol extract).

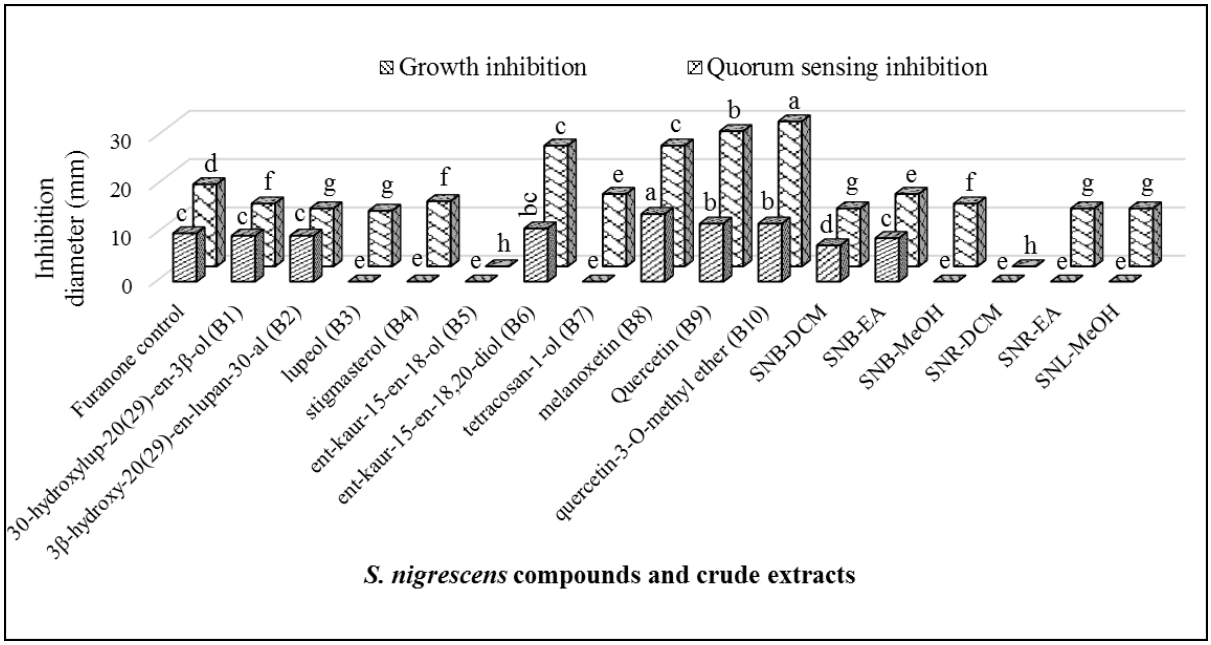

Figure 6.5. Quorum sensing inhibitory potentials of 500 µg of *S. nigrescens* isolated compounds and crude extracts relative to (*Z*-)-4-bromo-5-(bromomethylene)-2(5*H*)-furanone)

Bars with the same letter(s) are not significantly different within each group (Growth inhibition or Quorum sensing inhibition) using Tukey HSD test at 5% probability level (n = 3).
SNB-DCM = *S. nigrescens* bark DCM extract, SNB-EA = *S. nigrescens* bark ethyl acetate extract, SNB-MeOH = *S. nigrescens* bark methanol extract, SNR-DCM = *S. nigrescens* root DCM extract, SNR-EA = *S. nigrescens* root ethyl acetate extract, SNL-MeOH = *S. nigrescens* leaf methanol extract.

CONCLUSION

Isolation of ten phyto-constituents from *S. nigrescens* was reported, among which was a new diterpene (*ent*-kaur-15-en-18,20-diol) with *ent*-kaur-15-en-18-ol, found for the first time in plant. *Ent*-kaur-15-en-18,20-diol, melanoxetin and quercetin-3-*O*-methyl ether, having shown promising anti-quorum sensing and antimicrobial bioactivities, represent a set of active phytochemicals which could be developed as future antimicrobials through synthetic derivatisation and structure-activity relationships. The occurrence of melanoxetin suggests that it may be a chemotaxonomic marker for the genus *Acacia*.

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DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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CHAPTER SEVEN

Establishment of seed germination and micropopagation protocols for *Senegalia nigrescens*, a potential anti-virulent species.

ABSTRACT

This study describes seed germination and micropropagation of *Senegalia nigrescens*, an economic, medicinal and nitrogen-fixing species of South Africa. Seeds of *S. nigrescens* were subjected to pre-sowing treatments including soaking in cold water for 24 hours, sulphuric acid (H_2SO_4) or hydrochloric acid (HCl) for 4, 8 and 12 minutes and mechanically scarifying seeds before sowing on Petri plates with wet filter paper. Mechanically-scarified seeds were also sown aseptically on a filter paper bridge, plain agar, ½ strength Murashige and Skoog (MS) basal medium or full strength MS. Single nodal explants from MS-derived seedlings were grown on MS media supplemented with different concentrations and combinations (0.0, 0.5, 1.0 and 2.0 mg L^{-1}) of benzylaminopurine (BAP) or kinetin (KIN) to investigate shoot multiplication. No significant differences were observed in the number of shoots produced across all treatments. However, the treatment containing 1.0 mg L^{-1} KIN produced a significantly higher shoot length (14.17 ± 5.20 mm) than 0.5, 1.0 and 2.0 mg L^{-1} BAP (7.67 ± 3.87, 6.75 ± 2.93 and 8.70 ± 3.56 mm), respectively. The highest rooting rate (16.7 %) was obtained on ¼ strength MS supplemented with either of the auxins, indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) at concentrations 1.0 mg L^{-1} and 0.5 mg L^{-1}, respectively. Rooted plantlets were successfully acclimatised with a 66.7% survival rate. The findings from this study would be of great benefit for the commercial propagation of *S. nigrescens*.

Keywords: *Acacia*, dormancy, pre-sowing, *in vitro* regeneration.

INTRODUCTION

The continuous increase in the global human population accompanied by rapid industrialisation has contributed to climate change over the years. Afforestation, a major climate change control system (Zomer et al., 2008) is being developed as part of South Africa's effort towards ensuring environmental sustainability. With over one million hectares of land currently serving commercial forestry, South Africa's agro-forestry industry now contributes about 2.3% to the Gross Domestic Product (GDP) of the nation (Greyling et al., 2015) via the cultivation and harvesting of exotic timber species like *Pinus*, *Eucalyptus* and *Acacia* (now called *Senegalia*) (Van Der Zel, 1997). Although the genus *Acacia*, which belongs to the family Fabaceae and sub-family Mimosoideae, has about 1500 species across the globe (Dyer, 2014), only 61 of these are African which were recently re-classified under the genus *Senegalia* among which is *Senegalia nigrescens* (previously *Acacia nigrescens*) (Kyalangalilwa et al., 2013). *S. nigrescens* represents a nodulating member of the genus, known for its nitrogen fixing activity, a property that significantly contributes to improved agriculture and land rehabilitation (Brockwell et al., 2005).

S. nigrescens is a deciduous, pod-bearing, white flowered species. The tree is characterised with a thick stem bark having knobs equipped with small black hooked thorns (Fornara, 2005). *S. nigrescens* is widely distributed across South Africa through Zimbabwe up to Tanzania. It is a medium-to-tall (6 to 30 meters in height), slow growing tree (Dharani, 2006). Its hardness, drought- and termite-resistance properties make it suitable for use as mine props and fence posts (Howell et al., 2002). Its roots and stem bark are traditionally used for the treatment of snake bite, toothache (Dharani, 2006) and dysentery (Chauke et al., 2015), respectively. *S. nigrescens* wood is also used as fuel as it burns with severe heat yielding long-lasting coal (Liengme, 1983).

A series of flavonoids have been previously reported from the heartwood of *S. nigrescens* (Fourie et al., 1972; Malan, 1993) and our recent phytochemical study on the leaves, stem bark and roots of this species revealed its potential as an antibacterial and anti-quorum sensing agent (manuscript in preparation). The economic importance, nitrogen-fixing properties and its reservoir of biologically active constituents with anti-virulent potential suggest a higher demand for *S. nigrescens* in the near future. Moreover, species of *Senegalia* are known for delayed, uneven germination due to seed coat-imposed dormancy (Rasebeka et al., 2014). It thus becomes imperative to investigate a sustainable multiplication method for this multi-purpose species. Micropropagation studies within the genus *Acacia* have been widely documented (Beck et al., 1998; Beck and Dunlop, 2001; Dhabhai et al., 2010; Shahinozzaman et al., 2012). Nevertheless, there is no report to date on the micropropagation of *S. nigrescens*. Therefore, the aims of this work were to find the most effective pre-sowing treatment to promote *S. nigrescens* seed germination and to utilise the resulting *in vitro* seedlings for micropropagation studies of the plant.

MATERIALS AND METHODS

Pre-sowing treatments and seed germination

S. nigrescens seeds were purchased from Silver Hill Seeds, (Cape Town, South Africa) in February 2015. The seeds were preserved in air-tight containers under ambient temperature and stored in the dark before the start of the experiment. The pre-sowing treatments included soaking seeds in cold water for 24 hours, H_2SO_4 or HCl for 4, 8 and 12 minutes each and mechanically scarifying seeds using commercial sandpaper (60 Grit). After each treatment, the seeds were thoroughly rinsed under tap water before sowing onto 9 cm Petri plates with wet filter paper. Twelve seeds were used for each treatment unless otherwise stated. Seeds were

considered germinated when radicle emergence was visible (Tobe et al., 2000). The final germination percentage was recorded after 2 weeks.

In vitro germination

Mechanically-scarified seeds were subjected to surface sterilisation by first rinsing in a solution of sterile distilled water (SDW) and Tween 20® for 3 min. These were immediately transferred into 70% ethanol (EtOH) for 1 min and then rinsed with two lots of SDW. The seeds were then placed into a solution of 3.5% (v/v) sodium hypochlorite (NaOCl) for 10 min and finally rinsed 3 times with SDW. Surface sterilised seeds (10 seeds per treatment) were sown on different substrates and nutrient media viz. filter paper bridge, plain agar, ½ strength Murashige and Skoog (MS) (Murashige and Skoog, 1962) basal medium or full strength MS.

Shoot multiplication

Two-week-old, single nodal explants (1.5 cm long) were aseptically excised from full strength MS-derived seedlings, rinsed in 3.5% (v/v) NaOCl for 30 secs, followed by SDW (3 rinses, 30 secs per rinse) after which the bleached edges were trimmed before transferring onto fresh MS media supplemented with benzylaminopurine (BAP) (0.0, 0.5, 1.0 or 2.0 mg L^{-1}) singly or in combination with kinetin (KIN) (0.0, 0.5, 1.0 or 2.0 mg L^{-1}). The carbon source and hardening agents were 3% (w/v) sucrose and 1.0% (w/v) agar, respectively, while the culture vessels were 10 × 4 cm glass bottles with 3 explants in each. Each treatment had four replicates. The pH of media was adjusted to 5.8 ± 0.1 before autoclaving at 121 ºC and 1.2 kg cm^{-2} pressure for 20 min. All cultures were maintained at 25 °C under diffuse white light of 55 µmol m^{-2} s^{-1} with an 18 h photoperiod for 6 weeks after which the number of shoots, shoot length, height of plantlet, percentage of explants forming callus and percentage of explants with contamination were recorded. The multiple shoots were then aseptically excised into 1.5 cm nodal segments and elongated for 1 week on plain MS under the same growth conditions here-mentioned.

Rooting and acclimatisation

Shoot tips (1 cm long) from elongated plantlets were aseptically excised and cultured on different rooting media which comprised ½ or full strength MS supplemented with indole-3-acetic acid (IAA) (0.5, 1.0, and 2.0 mg L^{-1}, with or without 0.1 mg L^{-1} BAP) or indole-3-butyric acid (IBA) (0.5 or 1.0 mg L^{-1}, with or without 0.1 mg L^{-1} BAP). Six-week-old rooted plantlets were then rinsed with SDW to remove agar from the roots before being carefully transferred into flower pots (height = 85 mm, top diameter = 100 mm) containing potting soil, watered once-off with ¼ strength liquid MS and then maintained at 25 °C under diffuse white light of 55 µmol m^{-2} s^{-1} with an 18 h photoperiod in a growth room for 14 days. The pots were covered with plastic bags for the first 7 days and thereafter, the bags were perforated to decrease humidity for another 7 days. The plantlets were watered when the soil appeared dry. Thereafter, they were transferred to a greenhouse, where they were watered daily (for 2 minutes), morning and evening, by an automatic watering system. The % survival was recorded after 4 weeks.

Statistical analyses

All statistical analyses were carried out using the Statistical Package for the Social Sciences (SPSS, Version 23, IBM Corporation, Cornell, New York). The means of various data sets were subjected to one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test to determine if there were significant ($P < 0.05$) differences among them.

RESULTS AND DISCUSSION

Germination response of *S. nigrescens* to pre-sowing and substrate treatments

The final germination percentages (FGPs) after each pre-sowing and substrate/nutrient media treatment are presented in Table 7.1. The germinated *S. nigrescens* seeds sown on wet filter paper (in either Petri plates or as a bridge in a culture tube) and on plain agar are pictorially represented in Figure 7.1 (A to C). Overall, the success rate for the germination of *S. nigrescens*

across all pre-sowing treatments was high (83.33±28.97 - 100.00±0.00%). The highest FGP (100%) was recorded for mechanically-scarified seeds, sown on filter paper and on full MS. This result is in agreement with previous findings in another *Acacia* species i.e. mechanical scarification gave the best result (100% germination) in *A. polyacantha* (Missanjo et al., 2014). However, when soaking *S. nigrescens* seeds in H_2SO_4 for 4, 8 and 12 min in the present study, FGPs of 83.33±28.97, 91.67±14.43 and 98.33±2.89% (not significantly different), respectively, were obtained, which contrast to a previous report on the same species where the maximum germination percentage achieved was only 30% from seeds pre-soaked in H_2SO_4 for 3 min (Rasebeka et al., 2014). Therefore, longer H_2SO_4 soaking times enhance *S. nigrescens* seed germination. Alternatively, the increase in FGP in the present study could be an effect of geographical location on seed varieties as was observed with the ecotypes of *Arabidopsis thaliana* (Vaistij et al., 2013).

Having established mechanical scarification as the best pre-sowing treatment for *S. nigrescens*, mechanically-scarified seeds were surface-sterilised as before and sown on different substrates and nutrient media (Table 7.1). Seeds cultured on full strength MS produced 100% FGP as was obtained from the original mechanical scarification treatment although this was not significantly different from plain agar (80.57±14.16%) or ½ strength MS (80.57±17.33%). Only seeds sown on the filter paper bridge had a significantly lower FGP (61.13±9.64%) than the seeds sown on full strength MS. A similar study was carried out on a leguminous species, *Vigna subterranea* (Fabaceae), where the effect of MS strength on germination and seedling development was examined (Koné et al., 2015). It was reported that the best growth parameters were obtained from media containing full strength and ½ strength MS (with no significant difference between them) in comparison to those with ¼ strength MS. This study has shown that hard seededness in *Acacia* can be overcome by mechanical and chemical pre-sowing

treatments, thereby allowing for water imbibition by the embryo (Rasebeka et al., 2014) promoting successful germination.

Shoot multiplication

Bud break was observed within a week and shoots were well developed after 4 weeks (Figure 7.1D). The response of *S. nigrescens* nodal explants to the cytokinins BAP and KIN are presented in Table 7.2. The number of shoots produced across the seven treatments was in the range of 2.58±1.31 - 4.50±1.62, with no significant differences among the values. It has been widely reported that BAP is more effective in *in vitro* shoot organogenesis than KIN for *Acacia* species (Mittal et al., 1989; Galiana et al., 1991; Dewan et al., 1992; Beck et al., 1998; Khalafalla and Daffalla, 2008; Rout et al., 2008; Shahinozzaman et al., 2012) but in the present study, there were no significant differences between KIN and BAP in shoot multiplication, or when used in combination. Since the number of shoots obtained from the plant growth regulator (PGR)-free medium was not significantly different from the PGR treatments, it is likely that *S. nigrescens* contains endogenous PGRs whose concentrations meet the plant's need to induce shoots. This is in agreement with other reports which show that hormone-free media produced multiple shoots in some *Acacia* species (Jones et al., 1990; Zhang et al., 1995; Beck et al., 2000).

Table 7.1. Effect of pre-sowing treatments and substrate/nutrient media type on the final germination percentage (FGP) of *S. nigrescens* after 2 weeks of growth.

Treatment category	Treatment type	Final germination %
*Pre-sowing	Cold Water 24 h	91.67±14.43[a]
	H_2SO_4 4 min	83.33±28.87[a]
	H_2SO_4 8 min	91.67±14.43[a]
	H_2SO_4 12 min	98.33±2.89[a]
	HCl 4 min	83.33±14.43[a]
	HCl 8 min	91.67±10.41[a]
	HCl 12 min	91.67±5.77[a]
	Mechanical Scarification	100.00±0.00[a]
**Substrate/nutrient media	Filter paper bridge	61.13±9.64[b]
	Plain agar	80.57±14.16[ab]
	½ MS	80.57±17.33[ab]
	Full MS	100.00±0.00[a]

*Seeds were sown on filter paper in Petri plate. **Mechanically scarified seeds were used. The FGPs with the same alphabet are not significantly different by Tukey's Post Hoc Test at $p < 0.05$ within the column.

PGRs significantly influenced the shoot length and height of plantlets for some treatments (Table 7.2). KIN at 1.0 mg L^{-1} produced a significantly higher shoot length (14.17±5.20 mm) than BAP at 0.5 mg L^{-1} (7.67±3.87 mm), 1.0 mg L^{-1} (6.75±2.93 mm) and 2.0 mg L^{-1} (8.70±3.56 mm). Likewise, KIN at 1.0 mg L^{-1} produced significantly higher plantlet height (27.42±9.76 mm) than BAP at 0.5 mg L^{-1} (16.75±5.31 mm) and 1.0 mg L^{-1} (14.75±3.77 mm). Although KIN has been successfully used for shoot development in some plant species when used singly (Hesar et al., 2011; Jerzy et al., 2013; Abu-Romman et al., 2015) or combined with other PGRs (Shaheenuzzaman et al., 2011; Tolera et al., 2014), this was not the same for most *Acacia*

species where BAP, used singly or in combination with other PGRs were found to be superior to KIN for shoot development (Galiana et al., 1991; Girijashankar, 2011; Rathore et al., 2014). Plants within the same or different genera often exhibit variations in their morphogenic response to the same PGRs. These are caused by differences in genotype, explant type and culture conditions which in turn modifies the mechanisms of action of the PGRs (Gaspar et al., 1996).

In vitro shoot regeneration from nodal explants is sometimes characterised by the formation of callus around the basal end of the explant due to the effect of the auxin accumulation in this plant part (Marks and Simpson, 1994). Such masses of undifferentiated cells sometimes inhibit desirable morphological differentiation of the plantlet (Mittal et al., 1989; Nanda et al., 2004; Dhabhai et al., 2010) although they also represent platforms on which indirect organogesis of plant organs can be obtained (Vengadesan et al., 2003). Even though some treatments resulted in up to 66.67±0.49% explants forming callus (Table 7.2), the callus did not inhibit the induction of multiple shoots from the axillary bud and no evidence of indirect organogenesis was observed. The combination of BAP and KIN in this study resulted in the highest percentage of explants forming callus, which is in agreement with other reports on *Acacia* (Ortiz et al., 2000; Xie and Hong, 2001; Rathore et al., 2012).

There was no record of fungal contamination in *S. nigrescens* explants cultured on plain MS, 2.0 mg L^{-1} BAP and 2.0 mg L^{-1} KIN (Table 7.2). Furthermore, there were no significant differences in the percentage contaminated explants between BAP or KIN treatments of the same concentration (16.70±0.39% in BAP and KIN at 0.5 mg L^{-1} and 25.00±0.45% in BAP and KIN at 1.0 mg L^{-1}). The factors contributing to the occurrence of fungal contaminants in *S. nigrescens*, like other woody species, is yet to be fully understood. Different surface sterilisation methods have been employed for *Acacia* species (Gantait et al., 2016). Sterilisation involving dipping explants in 70% ethanol for 30 – 90 secs followed by 0.1% (w/v) mercuric

chloride ($HgCl_2$) for 5 – 10 mins and repeated washing in sterile water, are the most common. However, $HgCl_2$ is known to be environmentally hazardous (Saha, 1972) thus, more environmentally-friendly alternatives are being sought for tissue culture application. In the present study, the sequential and time-controlled use of Tween 20®, 70% EtOH, 3.5% NaOCl and SDW effectively removed surface contaminants, as there was no record of fungal contamination in the seeds of *S. nigrescens* germinated *in vitro*. However, as seen in Table 7.2, fungi were observed in some treatments during *in vitro* shoot multiplication. This may be attributed to endogenous fungal pathogens, which to date, are still a subject of concern in *Acacia* micropropagation (Gantait et al., 2016).

Table 7.2. Effect of BAP and KIN on shoot multiplication, plantlet height, callus formation and fungal contamination in *S. nigrescens* nodal explants after 6 weeks of *in vitro* culture.

Treatment (mg L^{-1})	No. of shoots	Shoot length (mm)	Height of plantlet (mm)	% explants forming callus	% contaminated explants
Plain MS (control)	3.92 ± 1.78^{a}	11.75 ± 3.14^{ab}	20.42 ± 5.05^{abc}	16.70 ± 0.39^{d}	0.00 ± 0.00^{c}
BAP 0.5	3.25 ± 1.76^{a}	7.67 ± 3.87^{bc}	16.75 ± 5.31^{c}	33.30 ± 0.49^{c}	16.70 ± 0.39^{b}
BAP 1.0	2.58 ± 1.31^{a}	6.75 ± 2.93^{c}	14.75 ± 3.77^{c}	0.00 ± 0.00^{e}	25.00 ± 0.45^{a}
BAP 2.0	2.90 ± 1.45^{a}	8.70 ± 3.56^{bc}	20.30 ± 7.13^{abc}	50.00 ± 0.53^{b}	0.00 ± 0.00^{c}
KIN 0.5	3.17 ± 1.75^{a}	11.50 ± 5.71^{abc}	20.50 ± 10.26^{abc}	25.00 ± 0.45^{c}	16.67 ± 0.39^{b}
KIN 1.0	3.67 ± 1.87^{a}	14.17 ± 5.20^{a}	27.42 ± 9.76^{a}	50.00 ± 0.52^{b}	25.00 ± 0.45^{a}
KIN 2.0	3.08 ± 1.31^{a}	11.25 ± 3.08^{abc}	21.67 ± 6.61^{abc}	16.67 ± 0.39^{d}	0.00 ± 0.00^{c}
BAP 0.5 + KIN 0.5	4.50 ± 1.62^{a}	12.08 ± 2.81^{ab}	29.58 ± 9.33^{a}	66.67 ± 0.49^{a}	33.30 ± 0.49^{a}

BAP - benzylaminopurine, KIN - kinetin. Values are represented as mean ± standard deviation (where n =12). Values with similar letters are not significantly different to one another by Tukey's Post Hoc Test at $p < 0.05$. (Shoot length: length of longest shoot; Height of plantlet: distance between the longest root and shoot tip. Both measurements were taken after 6 weeks of culture).

Rooting and acclimatisation

In vitro shoot tips of *S. nigrescens* were cultured on media which contained different MS salt strengths and varying concentrations of IAA, IBA and BAP, singly or combined (Figure 7.2). The highest percentage of rooted explants (16.7%) was obtained on ¼ strength MS supplemented with either 1.0 mg L^{-1} IAA or 0.5 mg L^{-1} IBA. This contrasts with reviews on micropropagation in the genus *Acacia* which showed that rooting was more successful using ½ strength MS supplemented with various concentrations of PGRs (Beck and Dunlop, 2001). In other rooting studies among the *Acacia* species, ½ strength MS was used with very low auxin (IAA and IBA) concentration (Galiana et al., 1991) or hormone free (Girijashankar, 2011). When using ½ strength MS in the present study, rooting was only observed when combined with 0.5 mg L^{-1} IBA (8.3%). The results imply that reduced MS strength is required for rooting in *S. nigrescens* similar to *Acacia catechu* which showed the best rooting (60 – 80%) on ¼ strength MS but supplemented with higher auxin concentration (3 mg L^{-1} IAA) (Kaur et al., 1998). The choice of the concentration range (0 – 2 mg L^{-1}) for rooting PGRs in this study was based on literature which mostly use PGRs of concentrations between 0 and 1 mg L^{-1} in *Acacia* species (Gantait et al., 2016). Few reports exist on *Acacia* species using PGR concentration above 2 mg L^{-1} for rooting (Gantait et al., 2016). More studies are therefore needed in order to fully understand the requirements for rooting in *S. nigrescens*. Acclimatisation was achieved on potting soil. The controlled exposure of plantlets to hardening conditions (Figure 7.1, F to H) resulted in a 66.7% survival rate of *S. nigrescens*.

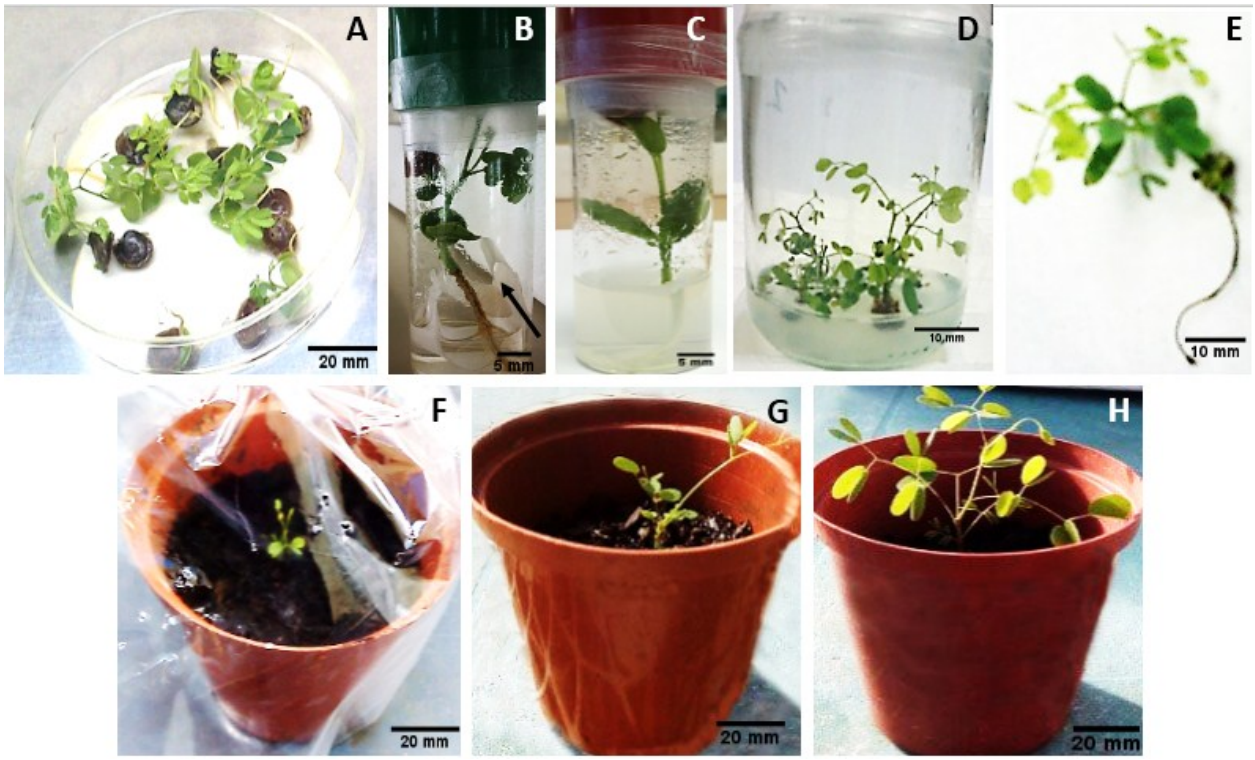

Figure 7.1. Developmental stages observed in the propagation of *S. nigrescens*

Seed germination on filter paper in Petri plate (A), filter paper bridge, indicated by an arrow (B), plain agar (C) after 2 weeks of sowing. Multiple shoot formation (D) after 4 weeks in culture, *in vitro* rooting (E) after 6 weeks in culture. Acclimatisation after 7 days in a growth room (F) and then after 4 weeks (G) and 10 weeks (H) in the greenhouse.

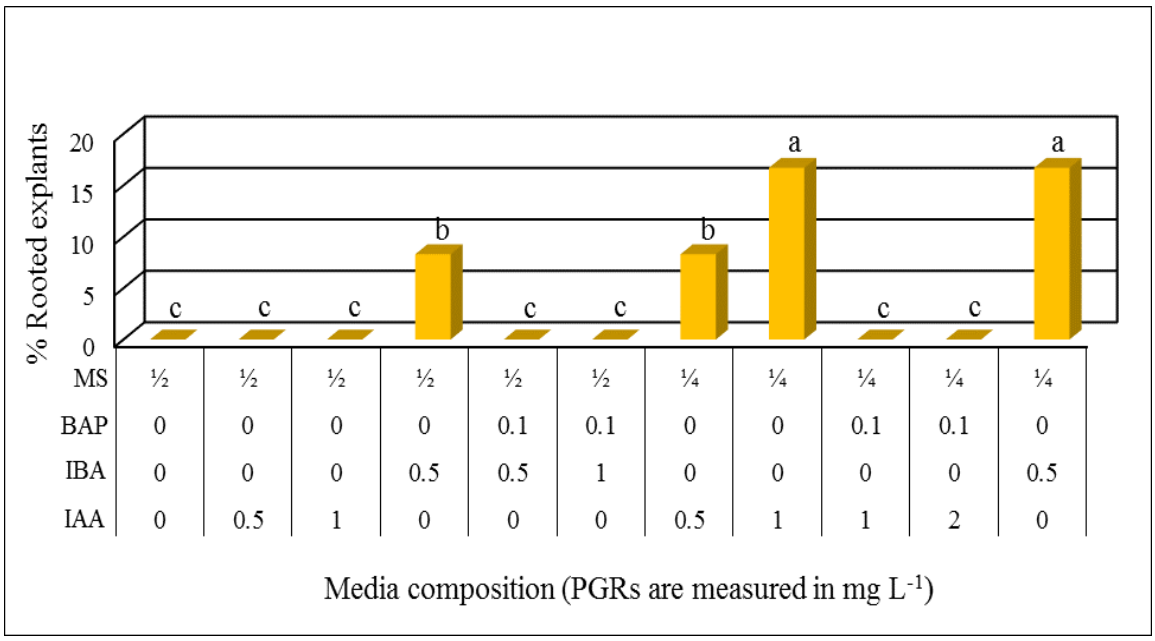

Figure 7.2. Effect of MS strength and PGRs on the *in vitro* rooting of *S. nigrescens* after 6 weeks of culture

Bars with similar letters are not significantly different from one another by Tukey's Post Hoc Test at $p < 0.05$.

CONCLUSION

To the best of our knowledge, this work has described the first report on the micropropagation of *S. nigrescens*. Mechanical and chemical pre-sowing treatments were effective for overcoming the barrier of hard seededness in the germination of *S. nigrescens*. Different PGR types and concentrations used in this work had no significant effect on shoot multiplication but significantly influenced the shoot length and plantlet height while the best rooting was achieved using ¼ strength MS in the presence of either 1.0 mg L^{-1} IAA or 0.5 mg L^{-1} IBA. The survival rate for the acclimatisation stage was 66.7%. Further studies are therefore necessary for the optimisation of the rooting stage of this species and more insight is needed to eliminate endogenous fungal contamination *in vitro*. However, the germination, shoot multiplication and acclimatisation protocols reported in this study provide valuable information for subsequent works in the regeneration of *S. nigrescens*.

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CHAPTER EIGHT

Evaluation of bioactive flavonols and *ent*-kaurenes in the 2,4-dichlorophenoxyacetic acid-induced calli of *Senegalia nigrescens* using FTIR and GC-MS

ABSTRACT

The demand for plant-derived pharmacologically-active compounds is increasing as industries search into sources of alternative, more natural and environmentally friendly therapeutic agents. In this study, an alternate source of biologically active compounds was investigated via the development of a callogenesis protocol for *Senegalia nigrescens*. The plant growth regulators (2,4-dichlorophenoxyacetic acid (2,4-D) and thidiazuron (TDZ)) at concentrations 0.5 and 1.0 mg L^{-1}, were tested for callus induction in the leaf, stem, cotyledon and root explants of *S. nigrescens*. 2,4-D was more productive across the explant sources with that of stem, root and cotyledon having between 85 to 100% explants forming callus. Calli from explants of stem were hard and compact with nodular structures, of root were soft and moist and of cotyledon were glassy and friable. Methanol extracts of calli obtained from the stem and root explants were characterised using fourier transform infrared spectroscopy (FTIR) and gas chromatography-mass spectrometry (GC-MS). The presence of four compounds (quercetin, quercetin-3-*O*-methyl ether, *ent*-kaur-15-en-18-ol and *ent*-kaur-15-en-18,20-diol) in both 4 and 12-week-old, root-derived calli was observed. This study, therefore, provides preliminary information for callus induction and phytochemical harvesting in *S. nigrescens*.

Keywords: *Senegalia nigrescens*, auxin, callus induction, chromatogram.

INTRODUCTION

The role played by plant-derived molecules cannot be overemphasised in modern drug discovery as they feed synthetic medicinal chemistry with diverse skeletal frameworks of pharmacological significance (Ganesan, 2008). However, sustainability of plant-based drugs is increasingly being threatened because of the depletion of medicinal plants in the wild. This is due to habitat destruction, climate change and over-exploitation by medicinal herb collectors who harvest these plants for local management of diseases and for the commercialisation of their herbal products (Roberson, 2008). Thus, alternative sources of plant-derived bioactive compounds and effective means of conserving medicinal plants with potential as therapeutic agents is being sought (Rasool Hassan, 2012).

In vitro bioprocessing technology is a tool that has attracted global attention in recent years for plant propagation and improvement, plant disease control and the production of secondary metabolites (Hussain et al., 2012a). Techniques in micropropagation and genetic engineering are currently being employed to meet conservational demands of medicinal plants and for rapid multiplication of species with desirable traits (Sidhu, 2011; Teng and Shen, 2015). In contrast to field plants that require several years to bioaccumulate the compounds at low concentrations, the use of *in vitro* cultures for producing phytocompounds allows for the manipulation of phytohormones and culture conditions to increase the yield of the biologically-active secondary metabolites within a short period (Hagendoorn et al., 1997). Specifically, *in vitro* callus cultures can be used to successfully produce plant-derived compounds in a short time (Dörnenburg and Knorr, 1995). Callus can be bulked up after the establishment of an effective callogenesis protocol and the compounds of interest can thereafter be extracted and purified from the bulk callus. Some acridone and furoquinoline alkaloids and coumarins were isolated from hypocotyl-derived calli of *Ruta bracteosa*, *R. chalepensis* and *R. macrophylla* (Baumert et al., 1992). The popularly-known cytotoxic drug, camptothecin and its 9-methoxy derivative were

found to be hyper-produced in callus cultures derived from immature cotyledons of *Nothapodytes foetida* (Thengane et al., 2003). Also, Jedinák et al. (2004) reviewed on flavonoid production from callus cultures of different plant species. It was found that isoflavones were produced more in callus cultures than parent plants of some *Genista* species, of which *G. tinctorial* had the highest isoflavone content. In addition, quercetin along with several other secondary metabolites, were identified in calli derived from both leaf and internode explants of *Citrullus colocynthis* (Tanveer et al., 2012). Meanwhile, some biologically-active compounds derived from plants have been found to accumulate more in callus cultures than intact plants (Janarthanam et al., 2010). Calli can be further utilised to generate cell suspension cultures for the commercial production of selected phytocompounds (Hussain et al., 2012b), like the well-known taxol (Filová and Rovna, 2011; Malik et al., 2011), shikonin (Gupta et al., 2014) and rosmarinic acid (De-Eknamkul and Ellis, 1988; Hippolyte et al., 1992).

Senegalia nigrescens is a leguminous South African species that was recently found to contain flavonoids and diterpenoids which inhibit quorum sensing in a *Chromobacterium violaceum* bacterial community (see Chapter six). Compounds causing quorum sensing inhibition are gaining popularity in the development of new antimicrobial drugs (Hong et al., 2012). Although micropropagation studies in the genus *Acacia,* (now *Senegalia*, for African species) (Kyalangalilwa et al., 2013) are well documented (Beck and Dunlop, 2001; Xie et al., 2007; Gantait et al., 2016), there are no reports on the potential of *S. nigrescens* for callus production intended for either the micropropagation of this species or its phytocompound production. In this study, we therefore report on the establishment of callus cultures from the leaf, stem, root and cotyledon explants of *S. nigrescens* for the purpose of characterising flavonoids and *ent*-kaurenoids from the methanol (MeOH) extracts using fourier transform infrared spectroscopy (FTIR) and gas chromatography-mass spectrometry (GC-MS).

MATERIALS AND METHODS

Callus induction

Seeds of *S. nigrescens* were mechanically scarified using a 60-Grit sandpaper and subjected to surface sterilisation by rinsing in a solution of sterile distilled water (SDW) and Tween 20® for 3 min. The seeds were immediately transferred into 70% ethanol (EtOH) for 1 min and then rinsed twice with SDW. The seeds were then placed into a solution of 3.5% sodium hypochlorite (NaOCl) for 10 min and finally rinsed 3 times with SDW. The sterilised seeds were sown on full strength Murashige and Skoog basal medium (MS) (Murashige and Skoog, 1962) in culture vessels (10 × 4 cm glass bottles) with 3 seeds in each. The cultures were maintained under diffuse white light of 55 µmol m^{-2} s^{-1} with 18 h photoperiod at 25 °C. Explants (leaf, stem, root and cotyledon segments) of *S. nigrescens* were excised from aseptic 2-week-old *in vitro* seedlings. The leaf and cotyledon explants were cut into 0.4 × 0.7 cm segments while the stem and root explants were cut into 0.7 cm segments. All explants were cultured on MS supplemented with sucrose (20 or 30 g L^{-1}) and 2,4-dichlorophenoxyacetic acid (2,4-D) (0.5 and 1.0 mg L^{-1}) or with thidiazuron (TDZ) (0.5 and 1.0 mg L^{-1}). Explants were cultured in 9 cm sterile Petri plates containing 20 mL medium. Each treatment consisted of 4 plates with 5 explants per plate. The cultures were maintained in the dark (the Petri plates were wrapped with foil) in a growth room at 25 °C. The pH of all media was adjusted to 5.8 and then solidified with 8 g L^{-1} agar before autoclaving at 121°C and 1.2 kg cm^{-2} pressure for 15 min.

The surface morphology of the calli was examined after 4 weeks in culture, using a LEICA MZ16 stereo microscope (USA). The percentage of explants forming callus (% EFC) was recorded after 4 weeks of culture. The means of the various percentages were subjected to one-way analysis of variance (ANOVA) after which Tukey's post-hoc test was used to determine if there were significant ($P < 0.05$) differences among them. These statistical analyses were

carried out using the Statistical Package for the Social Sciences (SPSS, Version 23, IBM Corporation, Cornell, New York).

Extraction

After 4 and 12 weeks, stem and root-derived calli (using 2,4-D) were subjected to spectroscopic characterisation. The calli were subjected to freeze-drying for 72 hours at -60°C and 10^{-3} mbar using an Edwards EF4 Modulyo bench-top freeze dryer (Sussex, England). Thereafter, 1 g each of the various calli were powderised using a Fisherbrand™ Porcelain Mortar and Pestle and then extracted overnight with MeOH, on an orbital shaker. The MeOH extract was thereafter concentrated using a rotary evaporator then air-dried before analysis.

Fourier Transform Infrared Spectroscopy

The MeOH extracts of the calli (4-week and 12-week-old stem-derived and root-derived), were subjected to FTIR analysis using a Perkin Elmer Spectrum 100 Fourier transform infrared spectrophotometer (USA) with universal attenuated total reflectance (ATR) sampling accessory. This utilises the PerkinElmer's revolutionary Spectrum™ FTIR software for the acquisition of quality spectrum and subsequent interactive spectra processing.

Gas chromatography-mass spectrometry

GC-MS analysis was carried out on a Shimadzu GCMS-QP 2010SE instrument. The instrument was fitted with Shimadzu HP-5MS capillary column (0.25 µm film thickness) having a dimension of 30 m (length) × 0.25 µ (I.D.). Reference samples of quercetin, quercetin-3-*O*-methyl ether, *ent*-kaur-15-en-18-ol and *ent*-kaur-15-en-18,20-diol previously isolated from field plants of *S. nigrescens* and MeOH extracts of the calli (4-week and 12-week-old stem-derived and root-derived), were subjected to GC-MS analysis. Helium was used as the carrier gas at 7.5 kPa pressure with oven temperature programmed at 60 ºC (for 2 min) to 300 ºC (for 30 min) at a ramping rate of 4 ºC per min. A 2 µL sample was manually injected at an

injection temperature of 300 ºC with a split ratio of 1:10. For the GC-MS detection, a sample ionisation energy of 70 eV was used. The system software was driven by Shimadzu GCMS solution workstation software (Japan). The compounds of interest were identified from the calli chromatograms by comparing the retention time of each peak and their respective fragmentation patterns with those of the reference samples. The relative amount of each compound in the extracts as a percentage was then computed by comparing the area under a compound's peak to the total area.

RESULTS AND DISCUSSION

Leaf, stem, root and cotyledon explants of *S. nigrescens* were cultured on MS media containing two concentrations each of sucrose and plant growth regulators (PGRs) (2,4-D or TDZ) for callus formation (Table 8.1). Only stem explants produced significantly higher % EFC than the leaf explants in treatment 1 (without PGRs). Within all the other treatments (treatments 2 - 9), the stem, root and cotyledon explants produced significantly higher % EFC than that obtained from the leaf explants. All the treatments containing 2,4-D (treatments 2 - 5) had significantly higher % EFC than the treatments containing TDZ (treatments 6 - 9) in all explant types. At the same concentration of either 2,4-D or TDZ (0.5 mg L^{-1} or 1.0 mg L^{-1}), there were no significant differences in % EFC between the treatments containing 20 g L^{-1} and 30 g L^{-1} of sucrose. Moreover, at 20 g L^{-1} sucrose, 1.0 mg L^{-1} 2,4-D produced significantly higher % EFC (100%) than 0.5 mg L^{-1} 2,4-D (85%) within stem explants. A similar trend was observed for root explants which had higher % EFC (100%) in 1.0 mg L^{-1} 2,4-D than 0.5 mg L^{-1} 2,4-D (90%) when cultured with 20 g L^{-1} sucrose. When using 30 g L^{-1} sucrose, there were no significant differences in the % EFC amongst the explants at the two concentrations (0.5 and 1.0 mg L^{-1}) of 2,4-D. For treatments containing TDZ, stem, root or cotyledon explants resulted in significantly higher % EFC in 1.0 mg L^{-1} TDZ (65%) than in 0.5 mg L^{-1} TDZ (50%, 55% and 45%, respectively), when cultured using 20 g L^{-1} sucrose. Similarly, the treatments containing

TDZ and 30 g L^{-1} sucrose resulted in higher % EFC with 1.0 mg L^{-1} TDZ (70%, 65% and 65%) for the stem, root and cotyledon explants, respectively than 0.5 mg L^{-1} TDZ (45%, 55% and 50%, respectively).

Callus may be defined as a mass of rapidly-dividing, unorganised cells (Jensen and Malter, 1995). Callus is commonly induced in plants by exogenous application of auxins and cytokinins, singly or in combination (Ikeuchi et al., 2013). It can be deduced from the results of the present work that exogenous growth hormones are important for callus formation in *S. nigrescens*, as the hormone-free medium had % EFC that were significantly lower than nearly all other treatments, for each explant type (Table 8.1). The synthetic auxin, 2,4-D was more effective for inducing callus than TDZ. All treatments with 2,4-D had significantly higher % EFC than TDZ across all the explants. An increase in PGR concentration from 0.5 to 1.0 mg L^{-1} did not have a significant effect on callus formation in most of the treatments containing 2,4-D except in the stem and root explants at 20 g L^{-1} sucrose. For TDZ, an increase in concentration gave higher % EFC in both 20 and 30 g L^{-1} sucrose across all explants except for the leaf in which the % EFC were not significantly different when cultured on similar concentrations of sucrose, irrespective of the concentration of the PGR supplement. It has been reported that 2,4-D is one of the most effective auxins for callus production (Osman et al., 2016), although many species respond better when it is used in combination with appropriate concentrations of a cytokinin or different auxin. The single or combinatorial use of 2,4-D in *Acacia* species has been reported to successfully produce callus. From seedling shoot tips of *Acacia koa* cultured on liquid medium of Schenk and Hildebrandt medium (Schenk and Hildebrandt, 1972) supplemented with 0.2 mg L^{-1} 2,4-D, healthy white callus was produced (Skolmen and Mapes, 1976). The embryonic axes and cotyledon explants of *A. farnesiana* produced embryogenic callus after 3 weeks of culture on MS medium supplemented with a combination of 2.0 mg L^{-1} 2,4-D and 1.0 mg L^{-1} KIN (Ortiz et al., 2000), cotyledonary node

explants from *A. nilotica* seedlings produced green callus after 25 days of culture with 2,4-D used singly (2 mg L^{-1}) or in combination (0.4 mg L^{-1}) with benzylaminopurine (0.2 mg L^{-1}) (Dhabhai and Batra, 2010) and the immature leaflets from *A. confusa* produced friable and compact nodular calli after 35 days of culture on MS supplemented with a combination of 2,4-D (3.0 mg L^{-1}), α-naphthaleneacetic acid (0.01 mg L^{-1}) and KIN (0.05 mg L^{-1}) (Arumugam et al., 2009).

The effectiveness, relative abundance and low cost of sucrose have made it a carbon source of choice in tissue culture, although some other carbohydrates may also be used (Muslihatin and Ratnadewi, 2012). In the present study, media containing 20 or 30 g L^{-1} (2 or 3%) sucrose were used. There were no significant differences in the % EFC in all explant types due to an increase in the concentration of sucrose from 2 to 3% when supplemented with the same concentration of PGRs. Even though early tissue culture research revealed that 2-4% sucrose is usually preferred (Gamborg et al., 1976), the use of 2% may be preferred over 3% sucrose due to reduction in the cost.

The source of explant contributes largely to the success of any tissue culture experiment. This is because cell division rates are higher in some plant tissues than others, while some may not respond to growth at all (Gantait et al., 2016). Tissue from seedling roots and stems or from non-dormant buds and shoot tips are suitable materials for producing friable callus (Gamborg et al., 1976). In *Acacia* species, leaf, stem, cotyledon, immature zygotic embryo and hypocotyl explants have been used for callus induction and proliferation (Gantait et al., 2016).

Table 8.1. Effect of explant type, sucrose, 2,4-D and TDZ concentrations on the callogenesis of *S. nigrescens* after 4 weeks in culture.

Treatments				% Explant forming callus (EFC)			
	Sucrose (g L^{-1})	**2,4-D (mg L^{-1})**	**TDZ (mg L^{-1})**	**Leaf**	**Stem**	**Root**	**Cotyledon**
1	20	0.0	0.0	10^{Db}	30^{Ea}	20^{Eab}	15^{Db}
2	20	0.5	0.0	50^{Ab}	85^{Ba}	90^{Ba}	95^{Aa}
3	20	1.0	0.0	60^{Ac}	100^{Aa}	100^{Aa}	95^{Ab}
4	30	0.5	0.0	55^{Ac}	90^{ABb}	95^{ABab}	100^{Aa}
5	30	1.0	0.0	55^{Ab}	100^{Aa}	100^{Aa}	100^{Aa}
6	20	0.0	0.5	20^{CDc}	50^{Dab}	55^{Da}	45^{Cb}
7	20	0.0	1.0	30^{BCb}	65^{Ca}	65^{Ca}	65^{Ba}
8	30	0.0	0.5	30^{BCb}	45^{Da}	55^{Da}	50^{Ca}
9	30	0.0	1.0	35^{Bb}	70^{Ca}	65^{Ca}	65^{Ba}

2,4-D - 2,4-dichlorophenoxyacetic acid, TDZ – thidiazuron. Percentages with similar uppercase letters are not significantly different from one another within the same column. Likewise, percentages with similar lowercase letters are not significantly different from one another within the same row as determined by Tukey's Post Hoc Test at $p < 0.05$.

In the present study, stem, root and cotyledon explants were more effective for callus induction than leaf explants. There are only a few reports where different explant types were compared for callus induction in *Acacia* species. In *Acacia mangium*, explants from embryonic axes, cotyledons, seedling leaves, stems and petioles, all had 100% callus formation when cultured on MS media supplemented with a combination of 2.0 mg L^{-1} 2,4-D and 3.0 mg L^{-1} kinetin (Xie and Hong, 2001) while the phyllode, which serves the function of a true leaf, had the least score for callus proliferation, compared to the node explants of *A. holosericea*, when cultured on MS media supplemented with 0.88 mg L^{-1} indole-3-acetic acid combined with 0.23 mg L^{-1}

benzylaminopurine (Jones et al., 1990). These show that the PGR requirement of similar explant type may vary across species of a genus due to the differential reactivity of their micro-environments to media components. To fully understand the requirements for callus proliferation in *S. nigrescens*, it will be necessary to subject the explants to other PGRs and test singly and in combination.

Table 8.2 shows the morphological characteristics of the calli obtained from various *S. nigrescens* explants using 2,4-D. Stem-derived calli were mostly hard and compact with nodular structures and varied in colour from cream to green. White, fluffy, wool-like calli were also observed in the stem explants. Root-derived calli were mostly soft, moist, creamish brown in colour while cotyledon-derived calli were glassy, friable and white in colour. Representative images of calli obtained from stem and cotyledon explants, observed with the aid of a stereo microscope, are presented in Figure 8.1. Generally, callus may be classified into two broad categories, compact and friable (Bhatia et al., 2015). Compact calli are commonly hard, consisting of differentiated structures that may be separated into individual components and sometimes representing different morphological stages of somatic embryos. This is often cultured on plant regeneration media for the induction of shoots, roots or whole plantlets. On the other hand, friable calli are usually soft and require less effort to break apart compared to compact calli (Bhatia et al., 2015). This type of callus is preferred in cell suspension media for the purpose of producing bioactive compounds *in vitro* (Hussain et al., 2012a). In the present study, hard and compact calli were obtained from the stem explants while soft, moist callus was obtained from root explants. With the aid of comparative morpho-histological studies of *S. nigrescens* calli, the embryogenic competence of the stem-derived calli may be substantiated, thus, revealing its potential for indirect organogenesis while the root-derived calli may be cultured to produce bioactive compounds via cell suspension cultures.

Table 8.2. Morphological variations in 4-week-old calli derived from *S. nigrescens* stem, root and cotyledon explants using 2,4-D.

Explant type	Colour	Texture
Stem	White	Fluffy
	Cream	Hard, compact
	Green	Hard, compact
Root	Brown	Soft, moist
Cotyledon	White	Glassy, friable

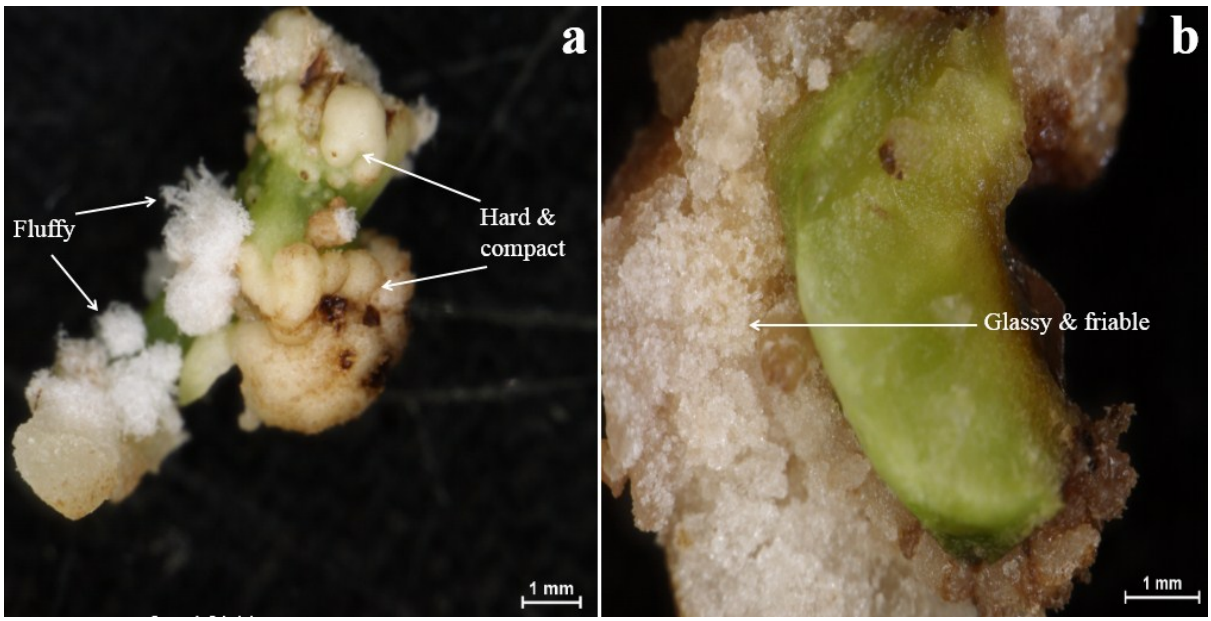

Figure 8.1. Stereo microscope images of 2,4-D - induced calli of *S. nigrescens* after 4 weeks in culture.

Calli obtained from stem and cotyledon are labelled a and b, respectively

The factors responsible for the observed morphological variations in the calli are yet to be fully understood. Nevertheless, the hardness and compactness of the stem-derived calli may be desirable for indirect organogenesis while the softness of the root-derived calli is characteristic of calli commonly used for cell suspension cultures to produce phytochemicals *in vitro* (Filova, 2014).

Fourier transform infrared spectroscopy (FTIR) analysis

The FTIR spectra of the MeOH extracts of calli (Figure 8.2) revealed the presence of the characteristic vibrational frequencies of the target phytocompounds (quercetin, quercetin-3-*O*-methyl ether, *ent*-kaur-15-en-18-ol and *ent*-kaur-15-en-18,20-diol) whose structures are presented in Figure 8.3. The vibrational frequencies between 3292 and 3274 cm^{-1} are characteristic of the H-bonded OH stretch which can be assigned to the hydroxy functional groups on the flavonols and *ent*-kaurenes. The peak intensities, as observed by the height and width of each peak, provide important information regarding the concentrations of the compounds giving rise to the respective peaks in the samples analysed (Stuart, 2004). It is likely that the presence of the two highly hydroxylated flavonols alongside the mono and dihydroxylated kaurenes in RCE1 and RCE2 was responsible for the higher intensities of the OH peaks compared to those of SCE1 and SCE2 from which only quercetin and the monosubstituted kaurene were detected. The sp^3 C-H stretch observed between 2935 and 2921 was present in all spectra. This supports the presence of the sp^3-rich *ent*-kaurenes in all extracts. The other vibrational frequencies observed were around 1628 – 1594 cm^{-1} (C=C stretch of alkenes and aromatics), 1399 – 1348 cm^{-1} (C-O-H in-plane bending and CH_2, CH_3 bending) and 1053 – 1050 cm^{-1} (C-O stretch of alcohols).

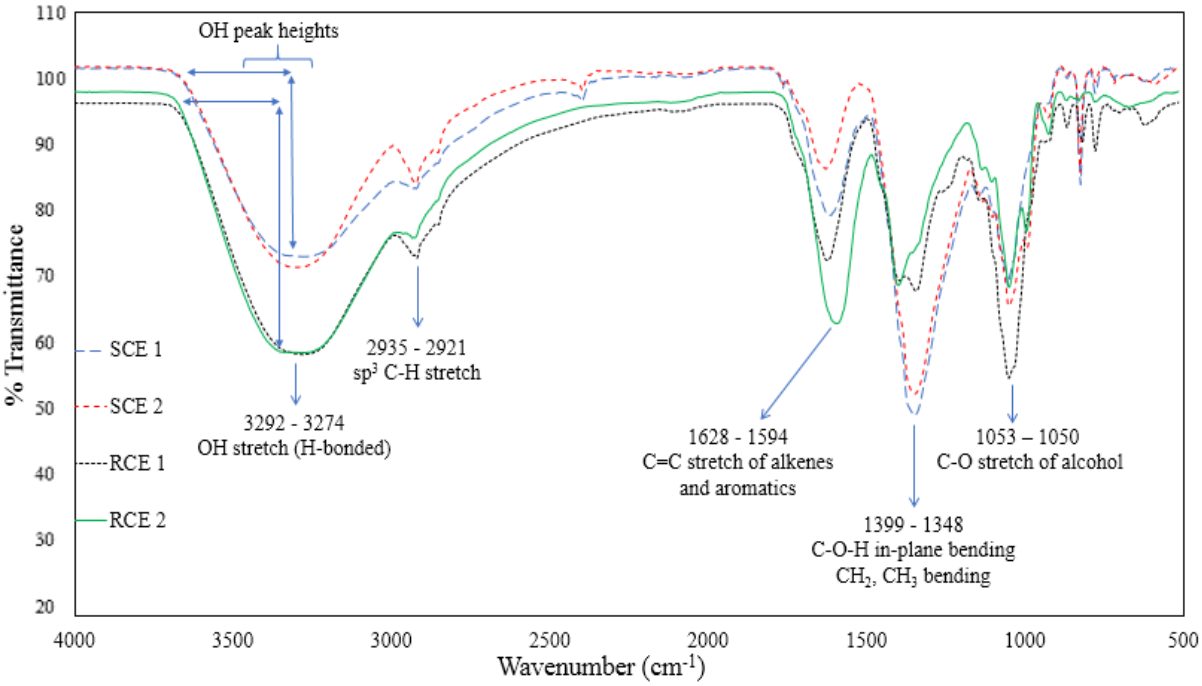

Figure 8.2. FTIR spectra of *S. nigrescens* calli MeOH extracts

SCE1 - 4 wk-old stem-derived callus extract, SCE2 - 12 wk-old stem-derived callus extract, RCE1 - 4 wk-old root-derived callus extract, RCE2 - 12 wk-old root-derived callus extract.

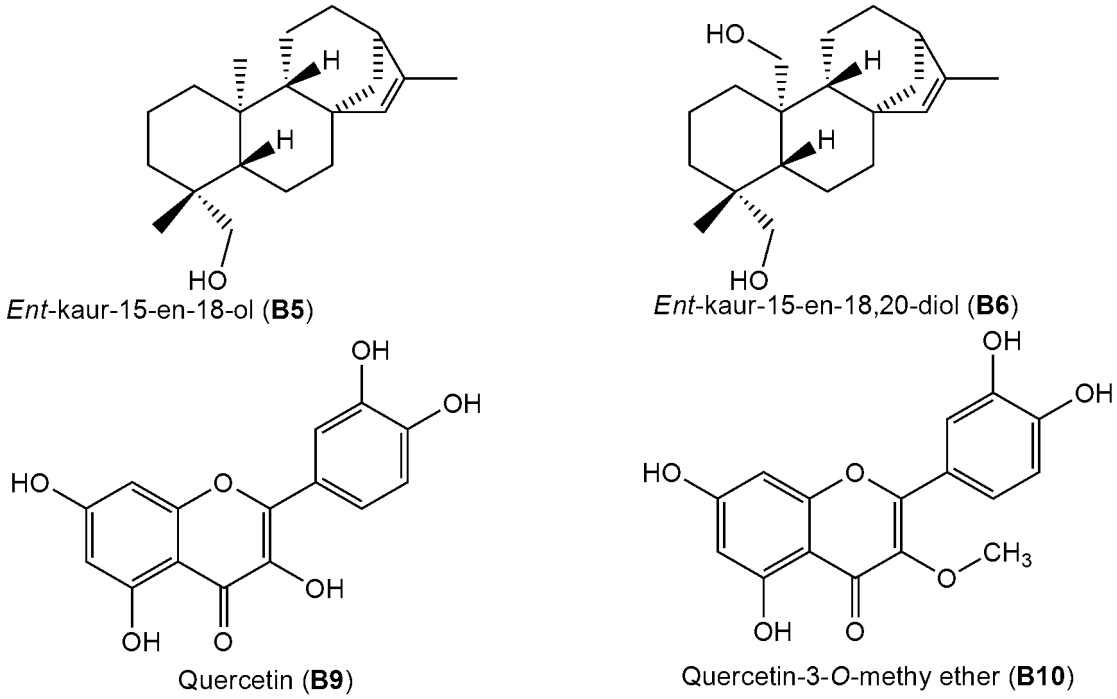

Figure 8.3. Chemical structures of the *ent*-kaurenes and flavonols identified from the methanolic extracts of *S. nigrescens* calli

Gas chromatography-mass spectrometry (GC-MS) analysis

The GC-MS chromatograms of the target compounds and those of the MeOH extracts of calli are shown in Figures 8.4 and 8.5, respectively while Table 8.3 shows the reports of the GC-MS analysis. Only quercetin and *ent*-kaur-15-en-18-ol were found in the extracts of the stem-derived callus extracts (SCE1 and SCE2) while all four compounds (quercetin, quercetin-3-*O*-methyl ether, *ent*-kaur-15-en-18-ol and *ent*-kaur-15-en-18,20-diol) were detected in the root-derived callus extracts (RCE1 and RCE2). Callus cultures had higher percentages of quercetin after 12 weeks (SCE2: 2.3%, RCE2: 2.9%) compared to 4 weeks (SCE1: 1.8%, RCE1: 1.8%). Adversely, for roots, percentage of quercetin-3-*O*-methyl ether was 2.6% after 4 weeks and 0.5% after 12 weeks.

Ent-kaurene has been identified as a biosynthetic precursor in the synthesis of either the biologically-active hydroxylated *ent*-kaurene derivatives or the growth-regulating gibberellins (Barrero et al., 1999). On the other hand, flavonoids are polyphenolic compounds, most of which are derived from the phenylpropanoid biosynthetic pathway (Ferreyra et al., 2012). The plastid is believed to be the site for the initiation step in *ent*-kaurene biosynthesis (Kasahara et al., 2002) while the enzymes involved in flavonoid biosynthesis can bind to membranes belonging to different organelles like plastids, vacuoles and nucleus (Petrussa et al., 2013). Plastids are known to possess a highly dynamic, tube-like feature called the stromule, which contains important phenylpropanoid biosynthetic enzymes and is also involved in intracellular transport of synthesised compounds (Hanson and Sattarzadeh, 2008; Bross et al., 2017). Stromules of striking biosynthetic significance are said to be found in callus and suspension cultures (Hanson and Sattarzadeh, 2008). This is likely responsible for the accumulation of flavonols in the callus cultures of *S. nigrescens* in this study.

The translocation and resulting bioaccumulation of secondary metabolites play key roles in their overall chemo-morphological distribution in the plant. In the development of callus

cultures to produce bioactive compounds, environmental factors that affect the response of plant organelles to the biosynthesis and translocation of phytocompounds are removed or systematically controlled to produce compounds of interest with desirable yield. The yield of bioactive compounds from plants has a direct relationship with the rate of biosynthesis, a process that depends on the biotic and abiotic stress imposed on the plant (Akula and Ravishankar, 2011; Naik and Al-Khayri, 2016). The well-controlled artificial environment (temperature, light, water, mineral salts provided by the MS basal medium, carbon source and plant growth regulators) provided for *S. nigrescens*' cell proliferation (callus formation) in this study is believed to have influenced the biosynthesis of the target secondary metabolites. This is further substantiated by the observed differences in the percentage compositions of the compounds obtained after 4 or 12 weeks of culture, as implied by exhaustion of media nutrients.

Structural similarity between gibberellins and the *ent*-kaurenes suggests a direct relationship between the two compound classes with regards to their bioaccumulation in plants. That is, high kaurene may be indicative of high gibberellin content. Although gibberellins are common plant growth hormones, differences in the intracellular concentration of the specific biosynthetic enzymes in different species, with variations in their genetic make-up or expression (Pichersky and Gang, 2000) are likely to contribute to either the presence of bioactive *ent*-kaurene derivatives (as found in *S. nigrescens*) or their absence in some other species. The auxin (2,4-D) may have triggered the synthesis of the flavonoids in addition to its high callusing potential. Although the mechanism of action of 2,4-D on the flavonoid biosynthesis is beyond the scope of the present research, previous reports have it that 2,4-D regulates certain biosynthetic enzymes and were found to have inhibitory effects on the phenylpropanoid metabolism (Ozeki and Komamine, 1985) thereby leading to low flavonoid production in media fortified with 2,4-D (Ozeki and Komamine, 1981; Ban, et al., 2003).

However, species-specific and tissue specific differences in the response of the cells to biosynthesis exist when culture media are treated with auxins at various concentrations (Smolenskaya et al., 2007). Nikolaeva et al. (2009) reported the efficiency of 2,4-D in the growth of *Camellia sinensis* callus but no significant increase in production of phenolic compounds and flavans. A deeper insight into the relationship between 2,4-D and selected biosynthetic enzymes in *S. nigrescens* is inevitable for future work.

Table 8.3. Flavonols and diterpenes identified in *S. nigrescens* calli extracts using GC-MS.

	Rt	**% Peak area**			
	(min)	SCE1	SCE2	RCE1	RCE2
Quercetin (B9)	28.0	1.8	2.3	1.8	2.9
Quercetin-3-*O*-methyl ether (B10)	27.3	-	-	2.6	0.5
***Ent*-kaur-15-en-18-ol (B5)**	25.3	1.7	1.5	2.4	0.6
***Ent*-kaur-15-en-18,20-diol (B6)**	29.7	-	-	2.7	1.31

SCE1 - 4 wk-old stem-derived callus extract, SCE2 - 12 wk-old stem-derived callus extract, RCE1 - 4 wk-old root-derived callus extract, RCE2 - 12 wk-old root-derived callus extract, Rt - retention time.

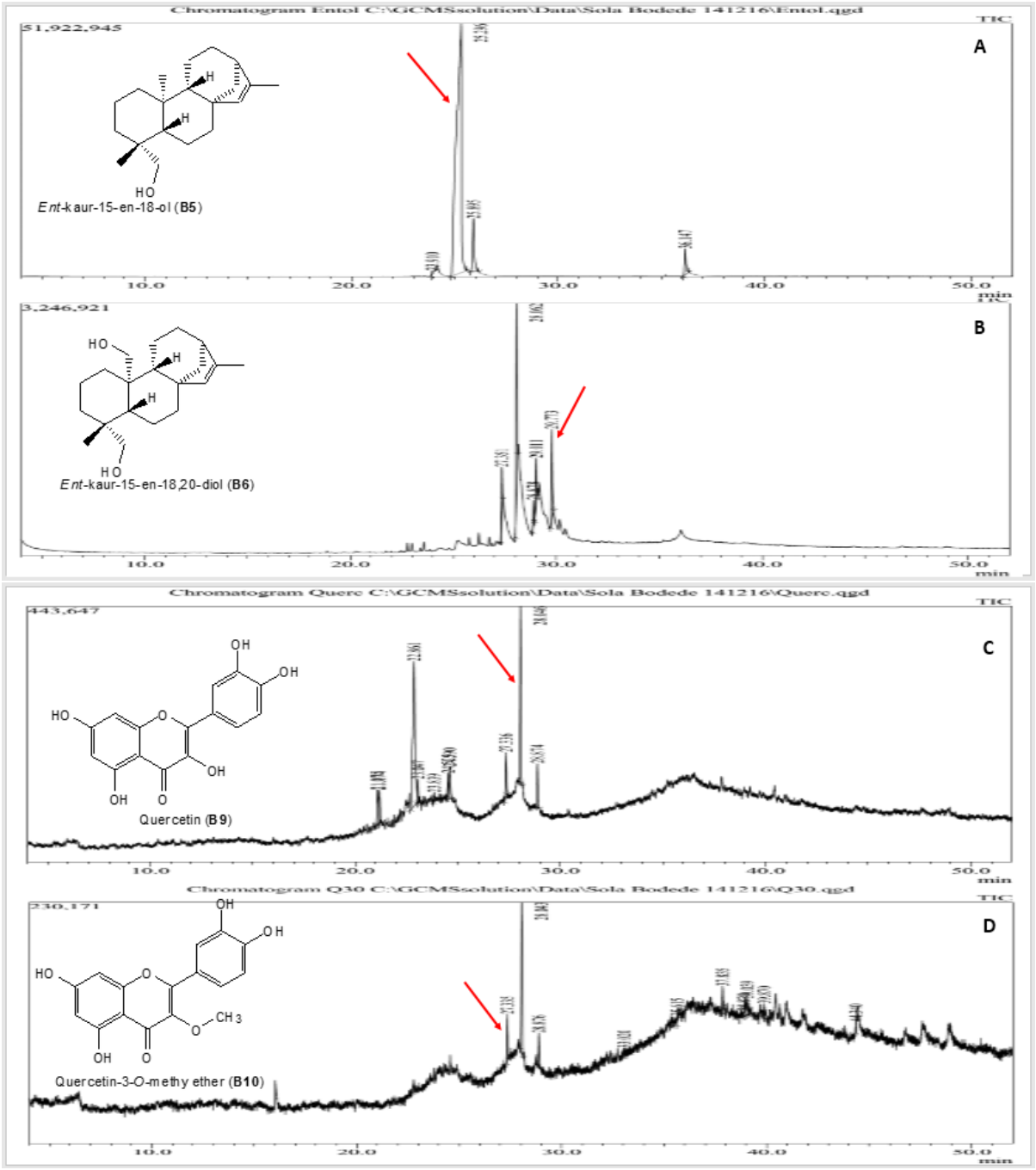

Figure 8.4. GC-MS chromatograms of compounds identified in *S. nigrescens* calli

A - *Ent*-kaur-15-en-18-ol, B - *ent*-kaur-15-en-18,20-diol, C - quercetin, D - quercetin-3-*O*-methyl ether. Arrows indicate peaks of compounds.

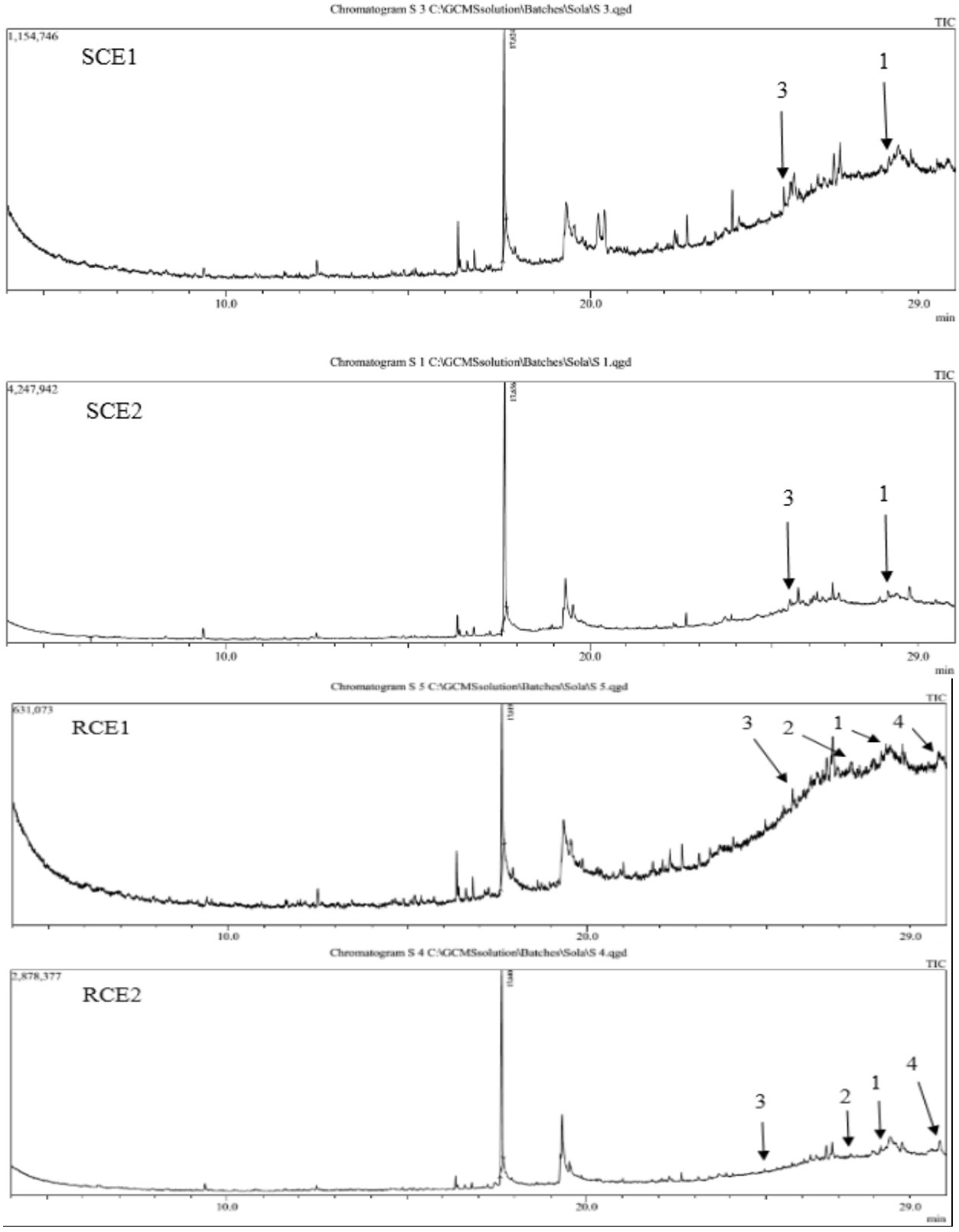

Figure 8.5. GC-MS chromatograms of the *S. nigrescens* callus extracts

SCE1 - 4-wk-old stem derived callus extract, SCE2 - 12-wk-old stem derived callus extract, RCE1 - 4-wk-old root derived callus extract, RCE2 - 12-wk-old root-derived callus extract, Peaks of the compounds (quercetin, quercetin-3-*O*-methyl ether, *ent*-kaur-15-en-18-ol and *ent*-kaur-15-en-18,20-diol) are represented by 1, 2, 3 and 4, respectively. Arrows indicate peaks of compounds.

CONCLUSION

A simple callogenesis protocol for *S. nigrescens* using different explant types was reported in this study. When comparing the PGRs, 2,4-D and TDZ at either 0.5 or 1.0 mg L^{-1} concentrations, 2,4-D proved to be better than TDZ for callus induction in *S. nigrescens*. Explants derived from the stems, roots and cotyledons of *S. nigrescens* seedlings were more effective than leaf explants for the induction of callus. The morphological characteristics of stem-derived calli revealed their potential for the micropropagation of *S. nigrescens* via indirect organogenesis. FTIR and GC-MS analysis showed that calli derived from the stem and root explants of *S. nigrescens* produced the phytocompounds of interest. Thus, more work is required in elicitation, cell suspension culture and bioreactor studies for mass production of the bioactive principles from *S. nigrescens*.

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CHAPTER NINE

OVERALL SUMMARY

Planet earth is endowed with inexhaustible natural resources, amongst which are plants that provide humans with food and to meet their healthcare needs. Knowledge on the chemistry and biology of plants that are used in healthcare is essential to heighten their effective, profitable and sustainable usage as therapeutic agents. *Zanthoxylum capense* and *Senegalia nigrescens* are two South African species with dissimilar phylogeny but share one unique morphological feature, "the knobs". A phytochemical probe and tissue culture studies of these knob trees were presented in this work.

Bioactive compounds were isolated from the leaves, stem bark and knobs of *Z. capense*, some of which showed cytotoxicity against the breast cancer (MCF-7) and human colorectal tumour (Caco-2) cell lines. Chemo-morphological findings showed that there were compounds localised in the knobs that were not found in the stem bark. The effect of pre-sowing treatments on seed germination of *Z. capense* showed that dormancy in its seeds could be overcome by soaking in hot water or GA_3. However, micropropagation studies of *Z. capense* was not successful due to extensive fungal contamination. This led to the evaluation of the crude extracts of *Z. capense* for the biosynthesis of AgNPs which were tested for their *in vitro* fungal contamination control. AgNPs delayed the emergence of fungi but did not eliminate them which led to the use of molecular methods to identify the prevailing endogenous fungal strains in the plant.

Phytocompounds with anti-quorum sensing potential were isolated from the leaves, stem bark and roots of *S. nigrescens*. The knobs and stem bark of *S. nigrescens* were reported as "stem bark" due to similarity in their TLC profiles. Chemo-morphological findings revealed that *ent*-

kaurenoids were localised in the root while flavonoids were spread across various parts of the plant. The micropropagation studies of *S. nigrescens* suggest that the species has a high content of endogenous growth hormones that promote shoot multiplication but not necessarily rooting, while callogenesis was successfully induced by 2,4-D using seedling-derived explants of *S. nigrescens*. 2,4-D has strong influence on the calli biomass but its regulatory effect on the enzymes involved in certain biosynthetic pathways in *S. nigrescens* needs further study. FTIR and GC-MS analyses showed that calli from *S. nigrescens* (4 and 12-week-old) had the potential for producing bioactive flavonoids and *ent*-kaurenes.

CONCLUSION

This work described the phytochemistry and *in vitro* culture of *Z. capense* and *S. nigrescens*, both known for their use in traditional medicine. Scientific justification for the ethnomedicinal use of these plants has been provided in this research, having isolated a range of biologically active secondary metabolites that could be responsible for the plants therapeutic potency. Chelerythrine and dodecyl-*trans*-*p*-coumarate isolated from *Z. capense* showed potential as cytotoxic phytocompounds against MCF-7 and Caco-2 cancer cell lines. Similarly, *ent*-kaur-15-en-18,20-diol, quercetin, quercetin-3-*O*-methyl ether and melanoxetin, isolated from *S. nigrescens* were found to be promising quorum sensing inhibitors. The phytocompound-rich extracts from *Z. capense* was used to synthesise AgNPs which delayed the emergence of fungal contaminants in *Z. capense* cultures and the fungal endophytic strains were isolated and identified. Micropropagation and callogenesis protocols, which are foundational steps for flora conservation, were established for *S. nigrescens*.

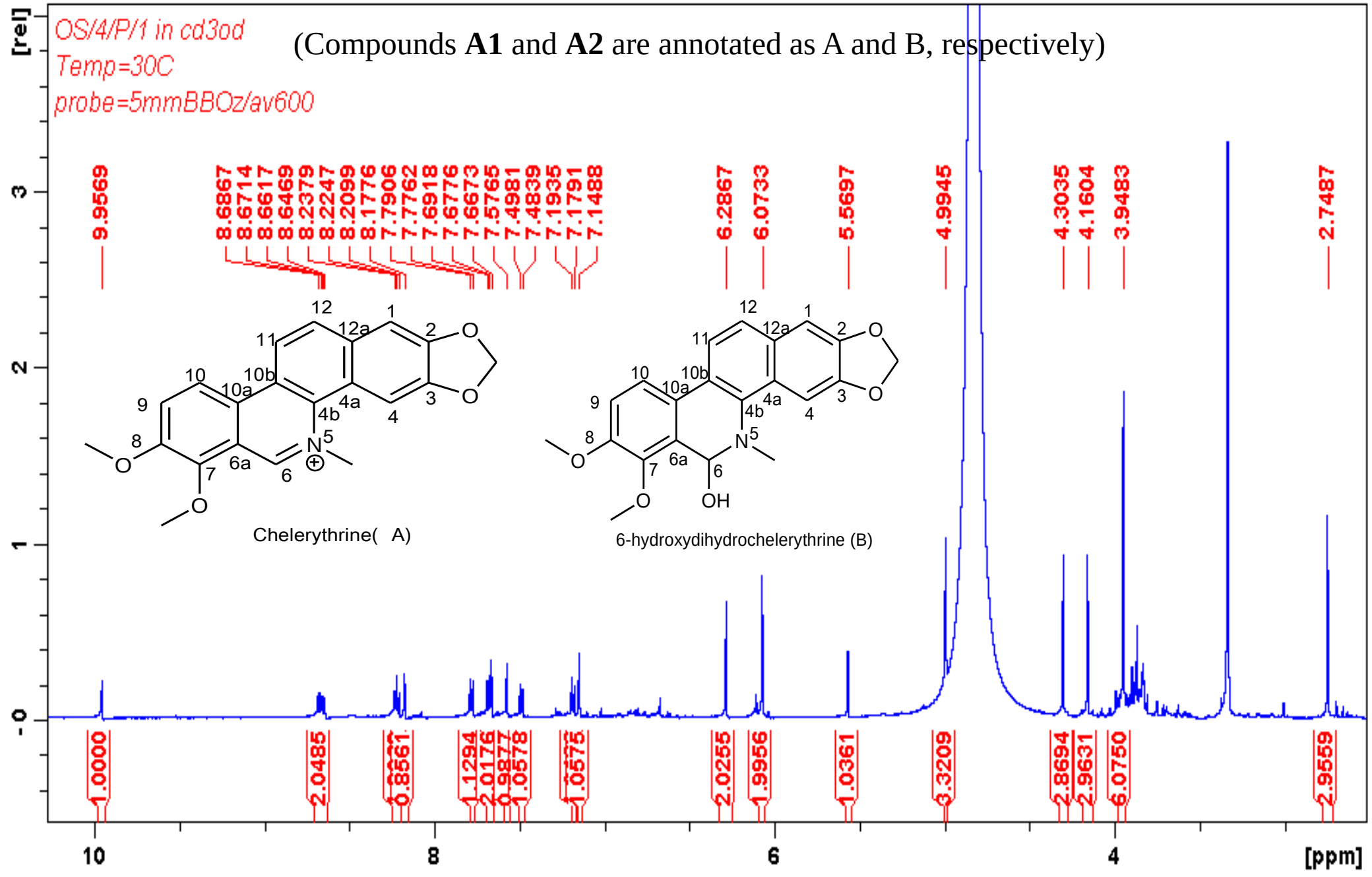
RECOMMENDATIONS FOR FUTURE RESEARCH

Having identified the fungal endophytes associated with *Z. capense*, further purification of the fungal strains and subsequent bulk culture is recommended, since fungal endophytes have potential for the biosynthesis of bioactive compounds. This is expected to open grounds for the *in vitro* commercial production of the bioactive compounds of *Z. capense*, thus, reducing the strain on field plants.

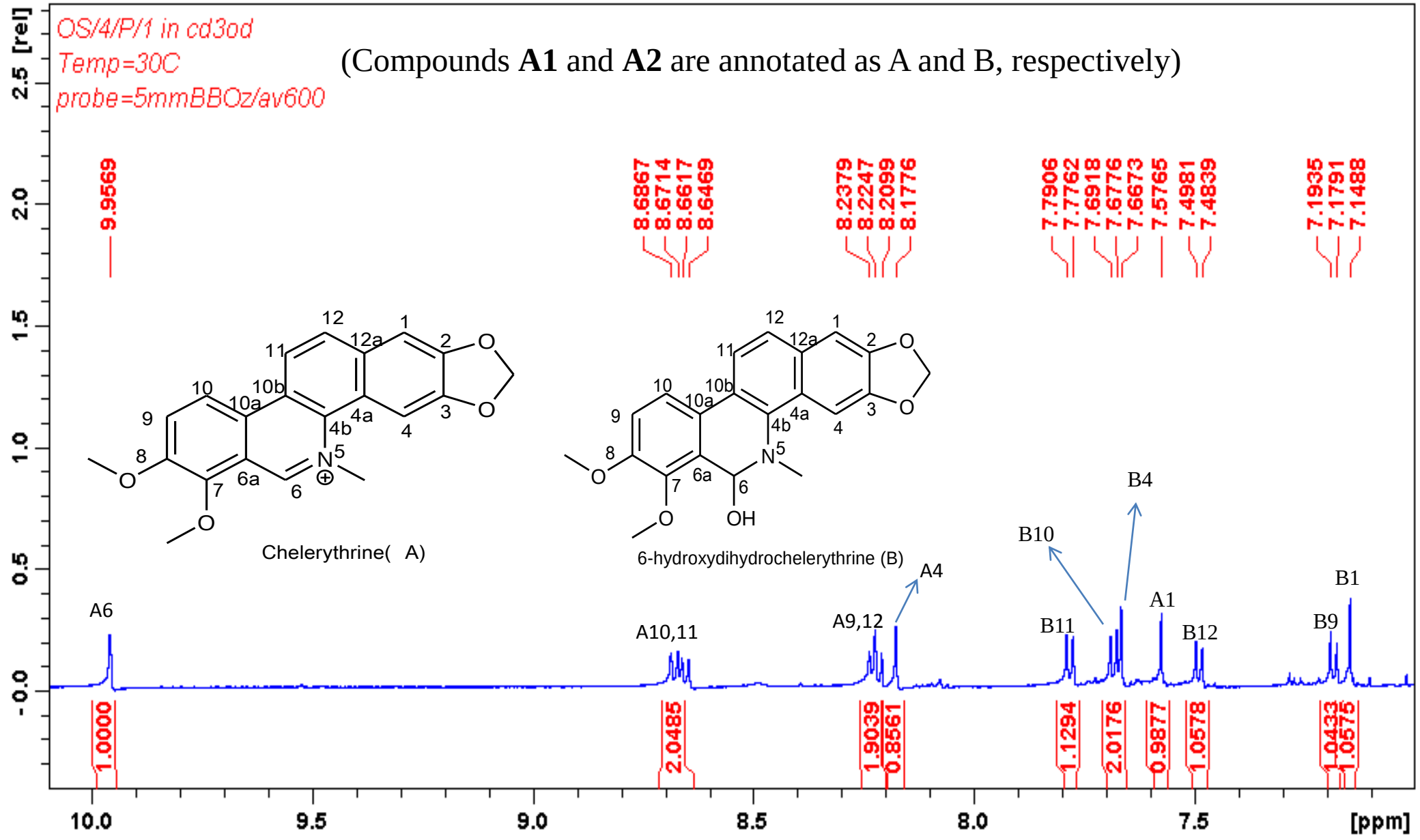
Further variation of media constituents and physical parameters can be employed to optimise *in vitro* proliferation of *S. nigrescens*. Cell suspension and bioreactor studies can be developed for the commercialisation of the bioactive compounds from this plant. The pods of *S. nigrescens* can be phytochemically investigated. This will help to fully describe the chemo-morphological distribution of phytocompounds in the plant.

APPENDICES

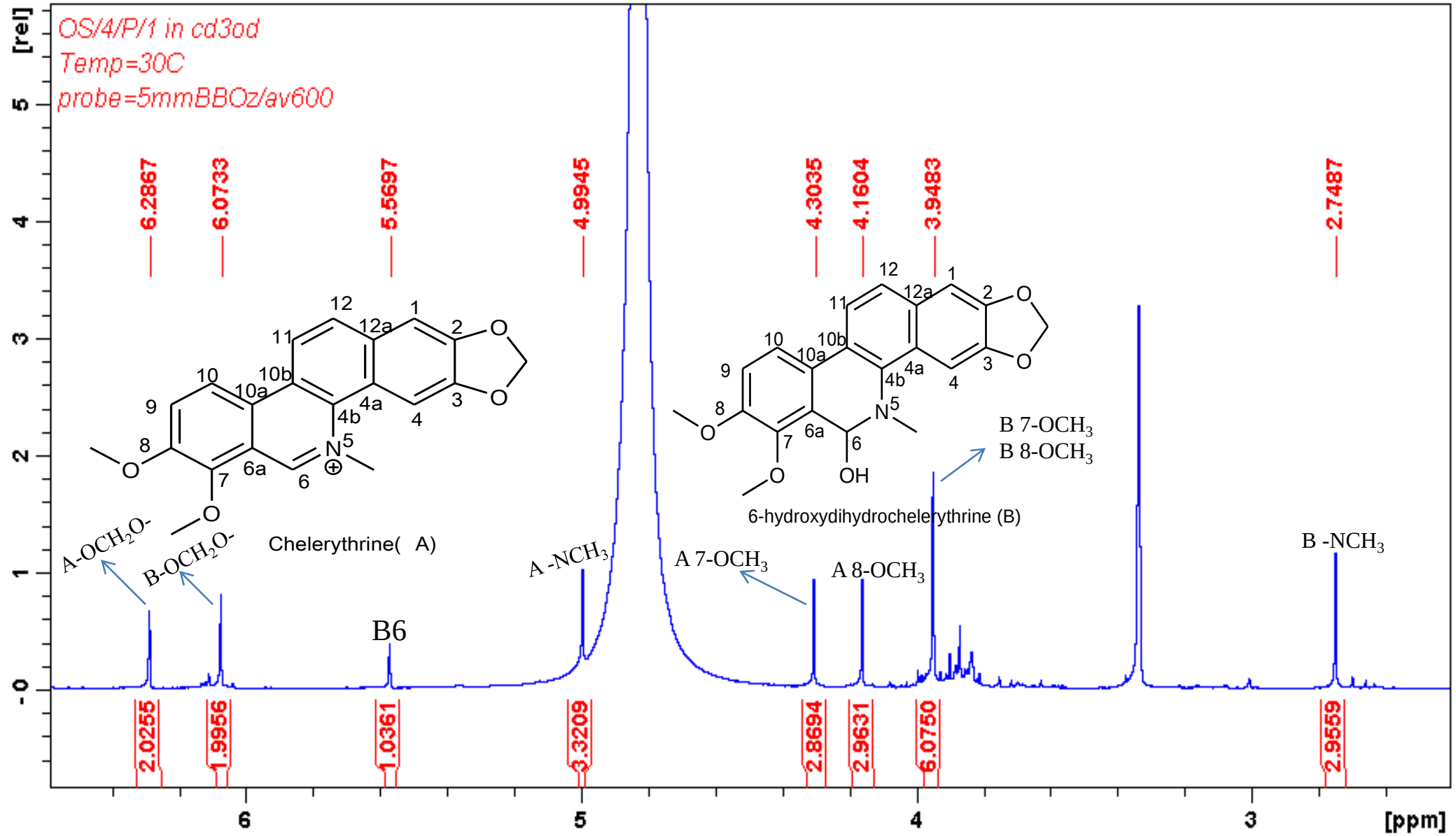
Supporting information consisting the NMR, IR, UV and MS data



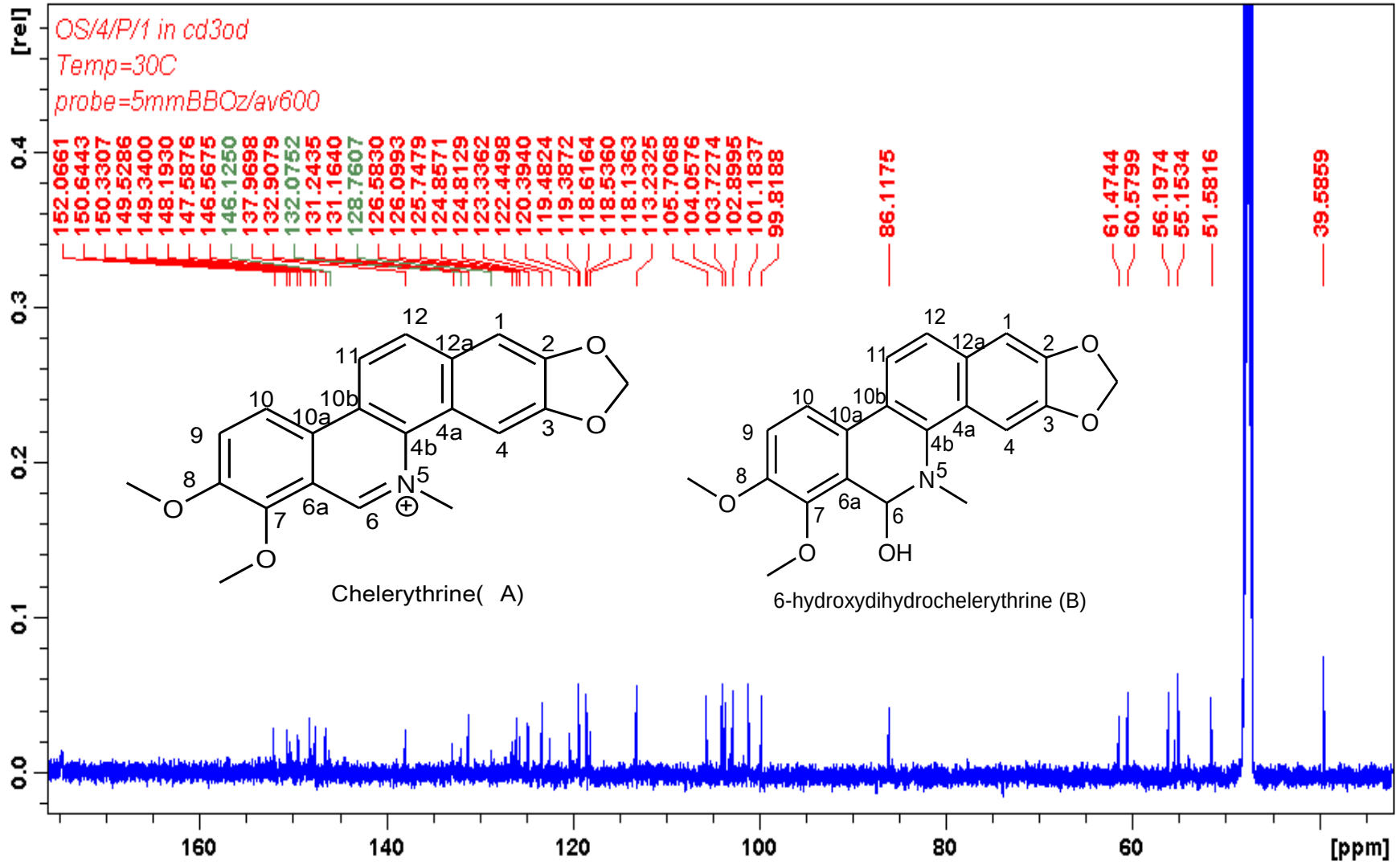
^{1}H NMR spectrum of chelerythrine (**A1**) and 6-hydroxydihydrochelerythrine (**A2**)



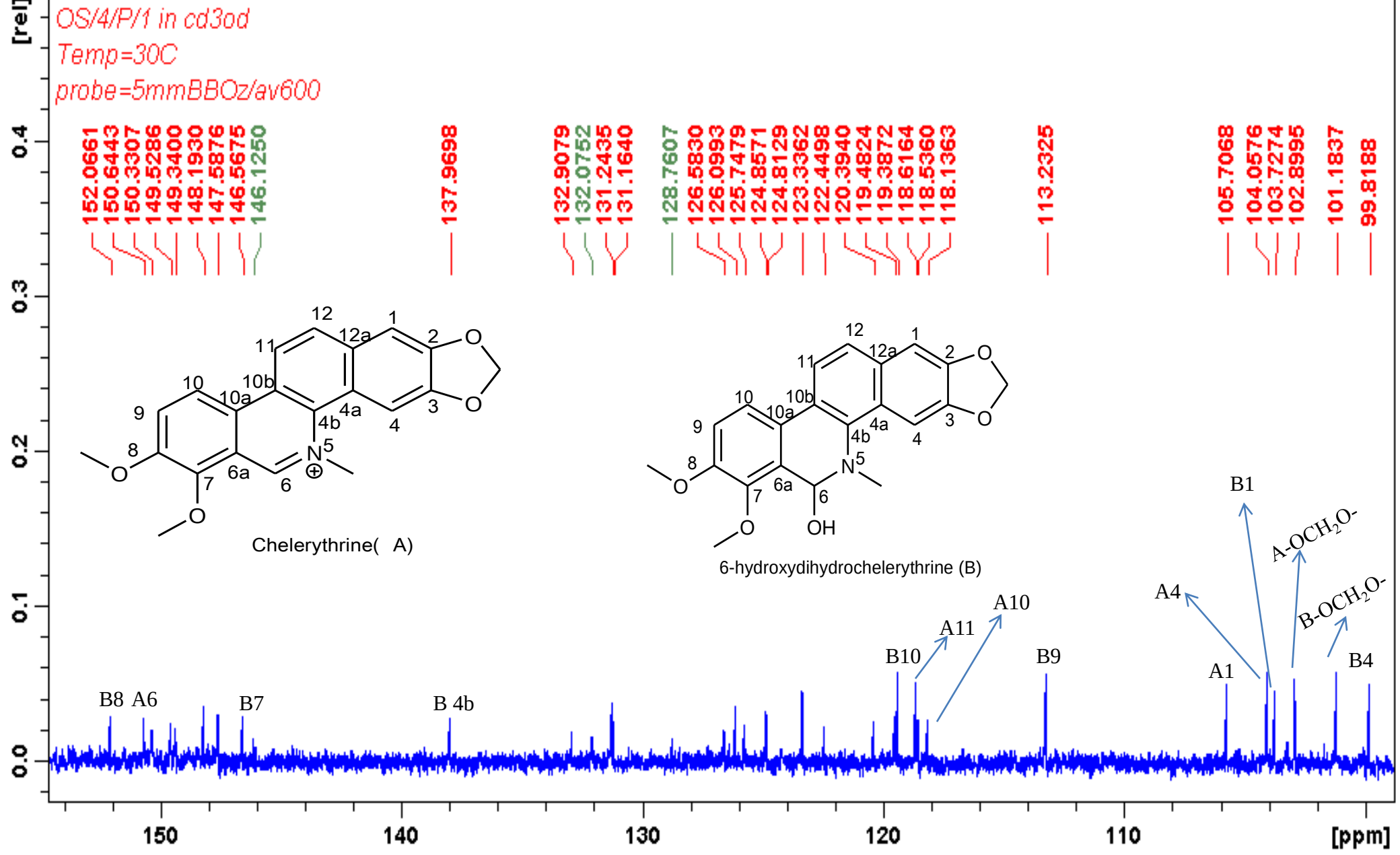
Expanded ^{1}H NMR spectrum of chelerythrine (**A1**) and 6-hydroxydihydrochelerythrine (**A2**)



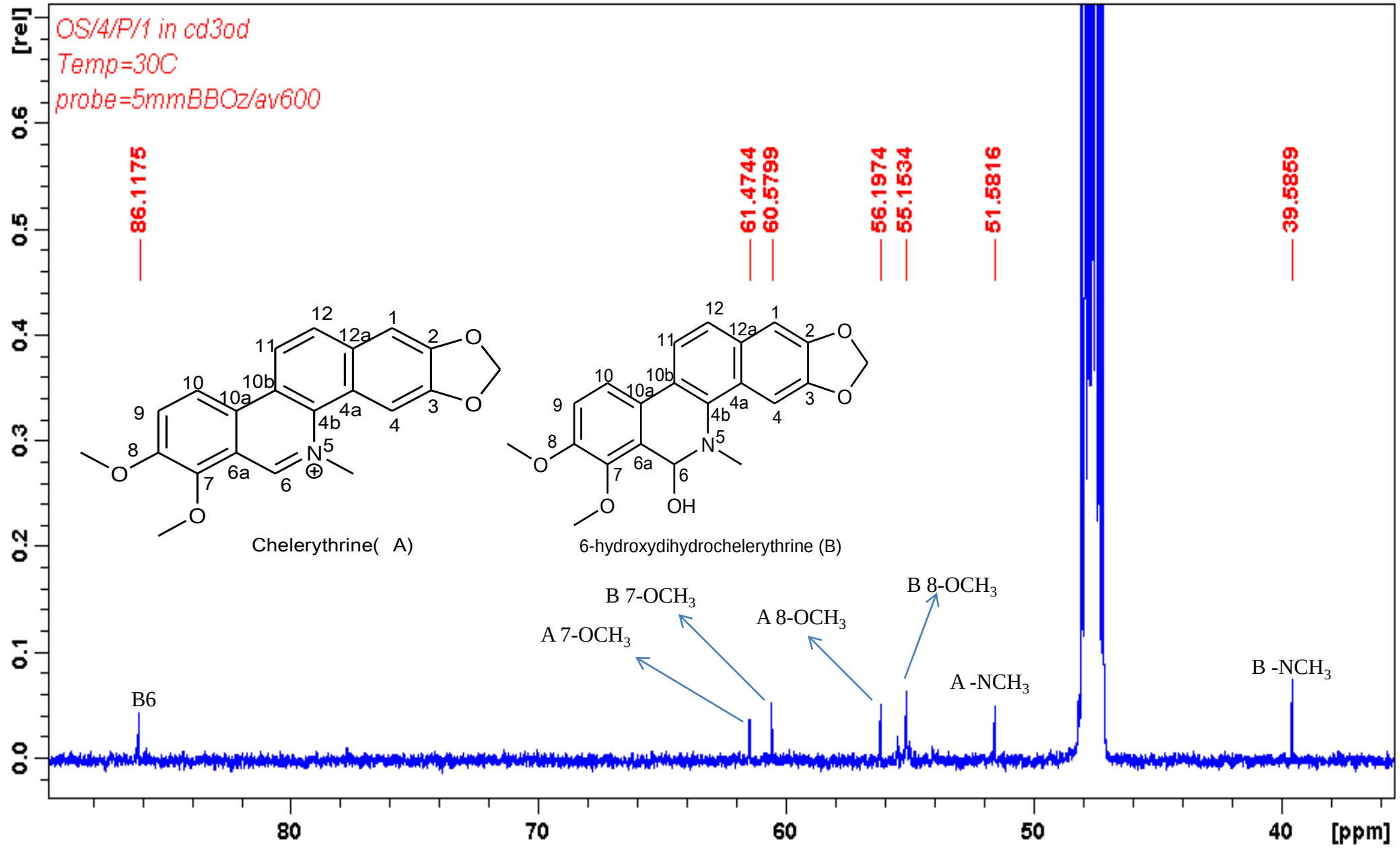
Expanded ^{1}H NMR spectrum of chelerythrine (**A1**) and 6-hydroxydihydrochelerythrine (**A2**)



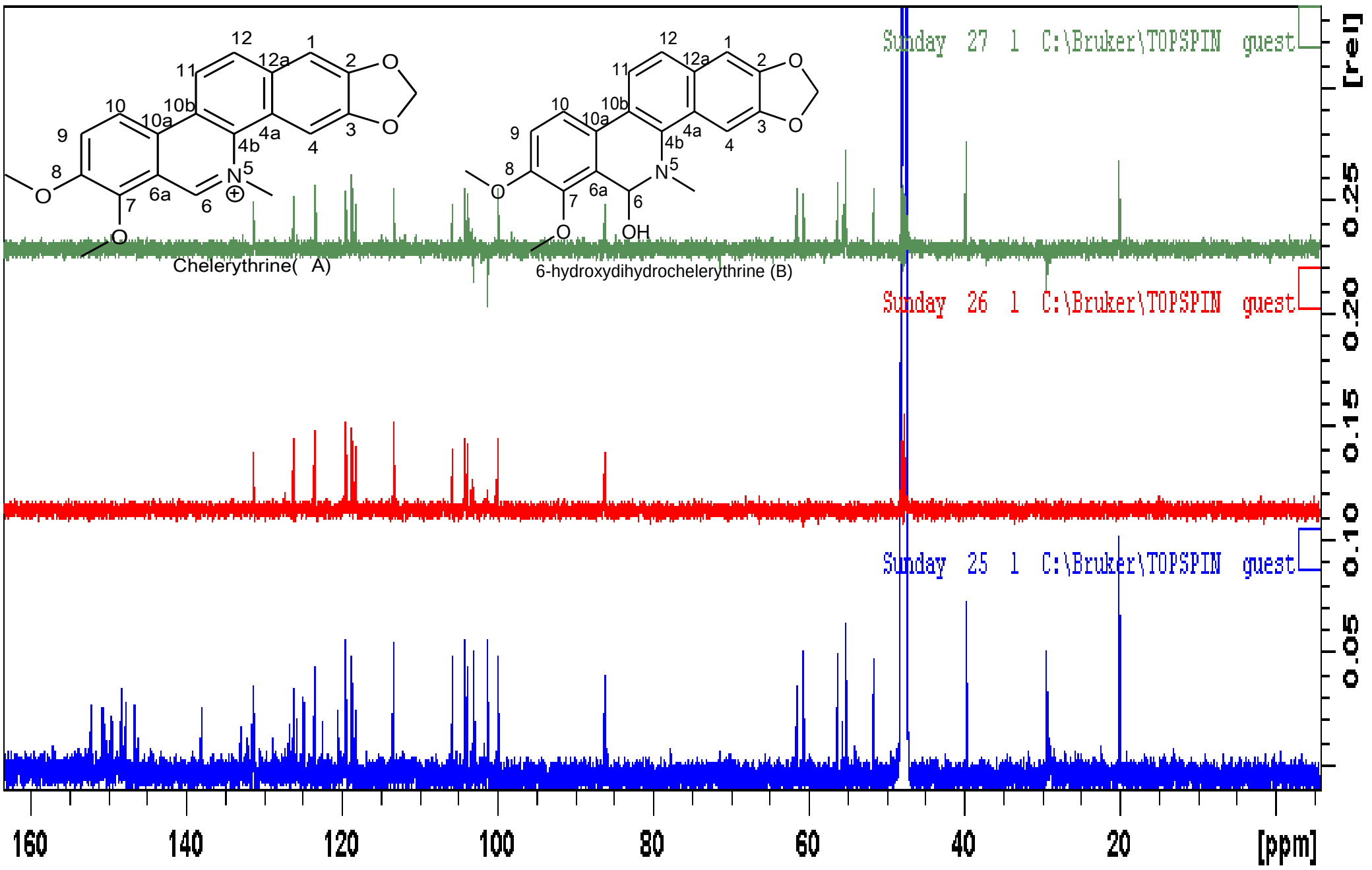
^{13}C NMR spectrum of chelerythrine (**A1**) and 6-hydroxydihydrochelerythrine (**A2**)



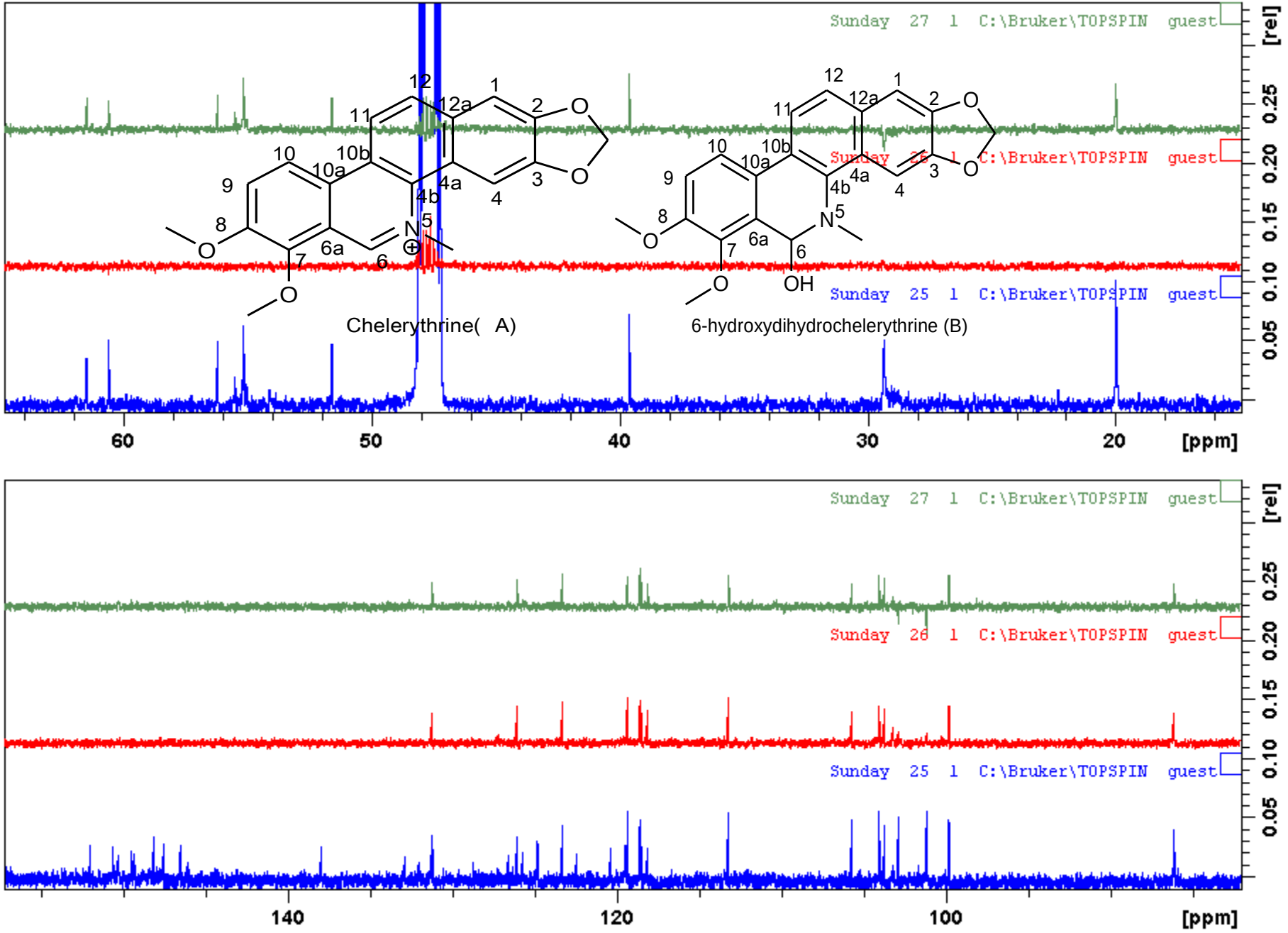
Expanded ^{13}C NMR spectrum of chelerythrine (**A1**) and 6-hydroxydihydrochelerythrine (**A2**)



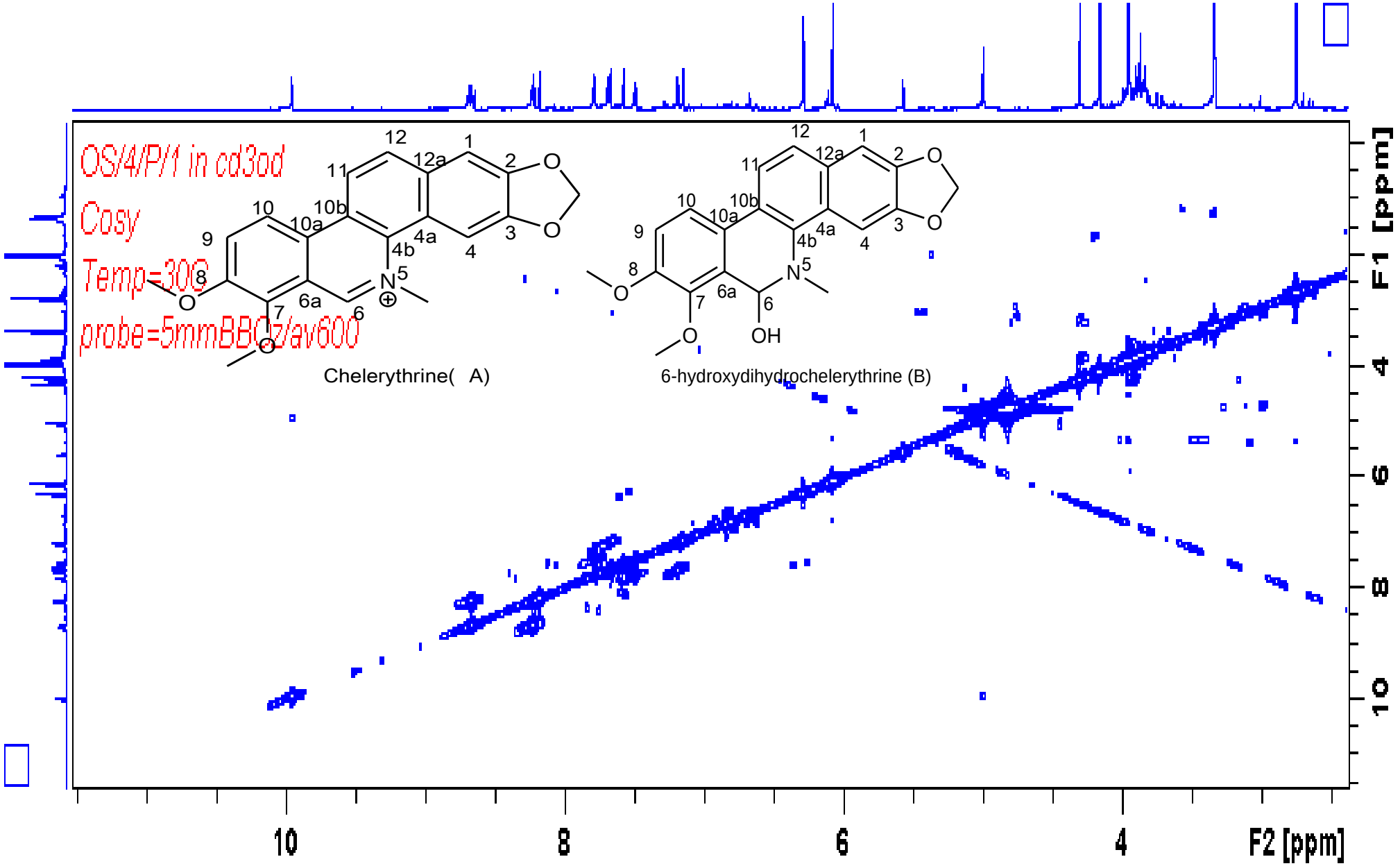
Expanded ^{13}C NMR spectrum of chelerythrine (**A1**) and 6-hydroxydihydrochelerythrine (**A2**)



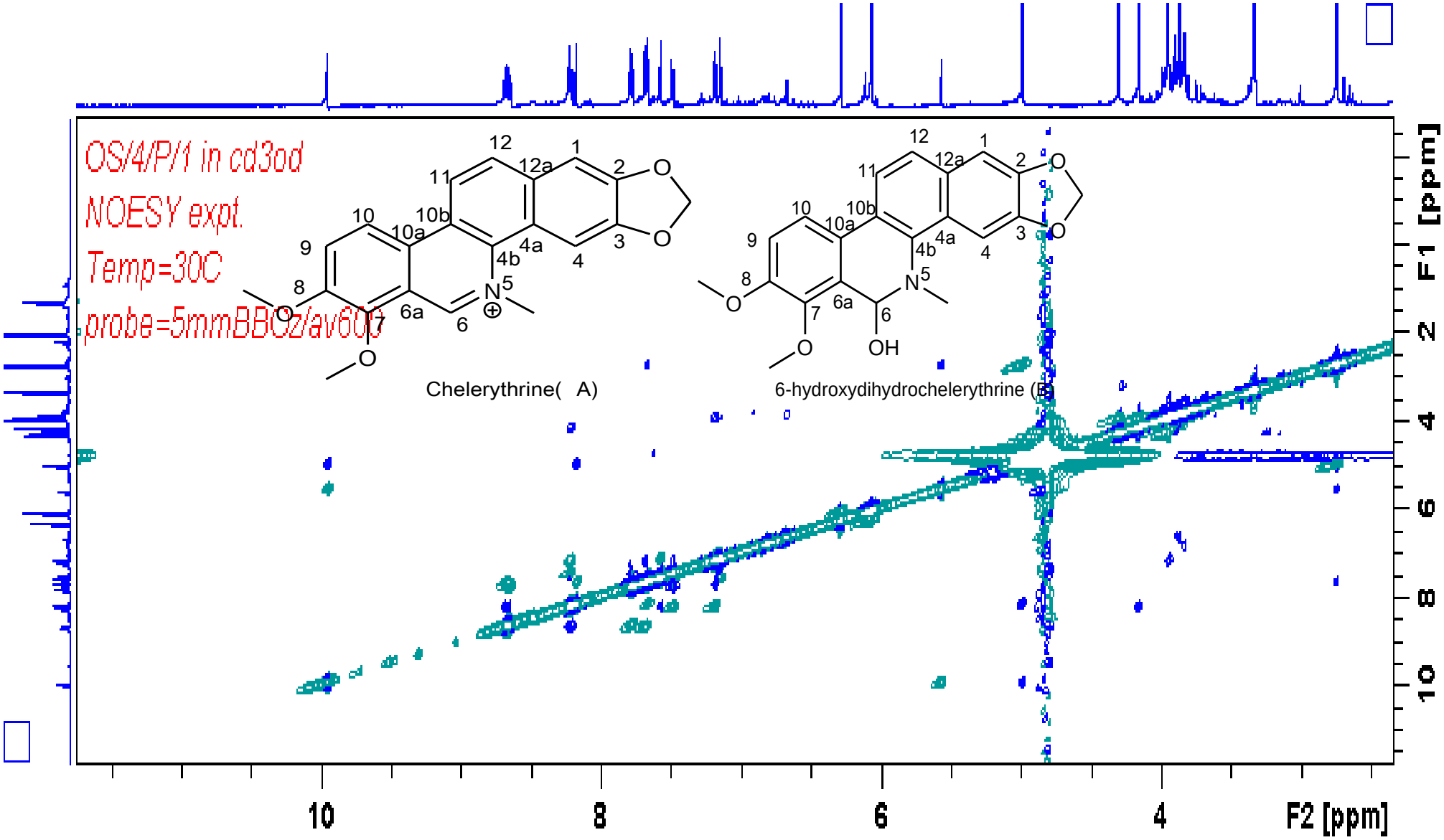
DEPT spectrum of chelerythrine (**A1**) and 6-hydroxydihydrochelerythrine (**A2**)



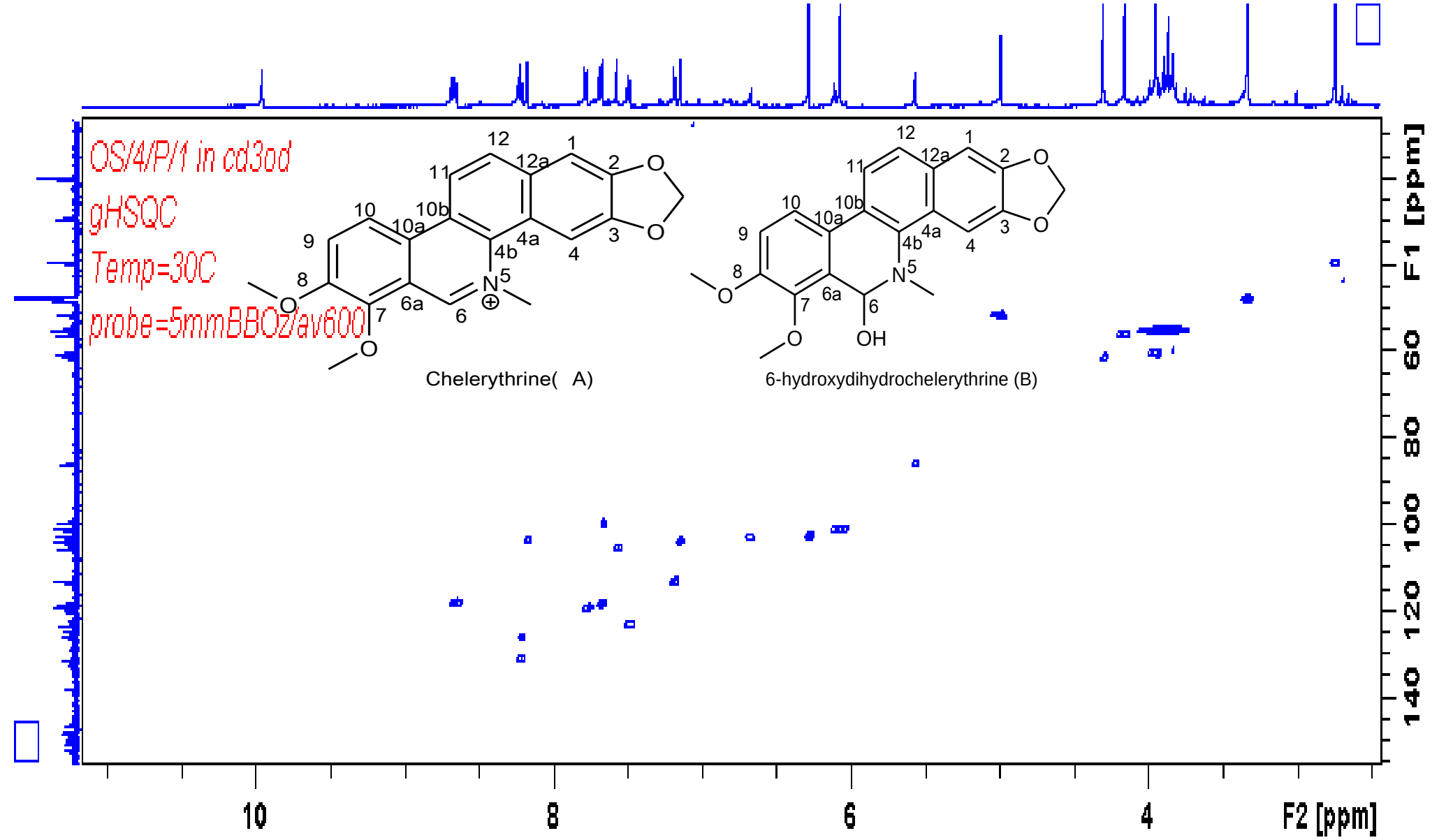
Expanded DEPT spectrum of chelerythrine (**A1**) and 6-hydroxydihydrochelerythrine (**A2**)



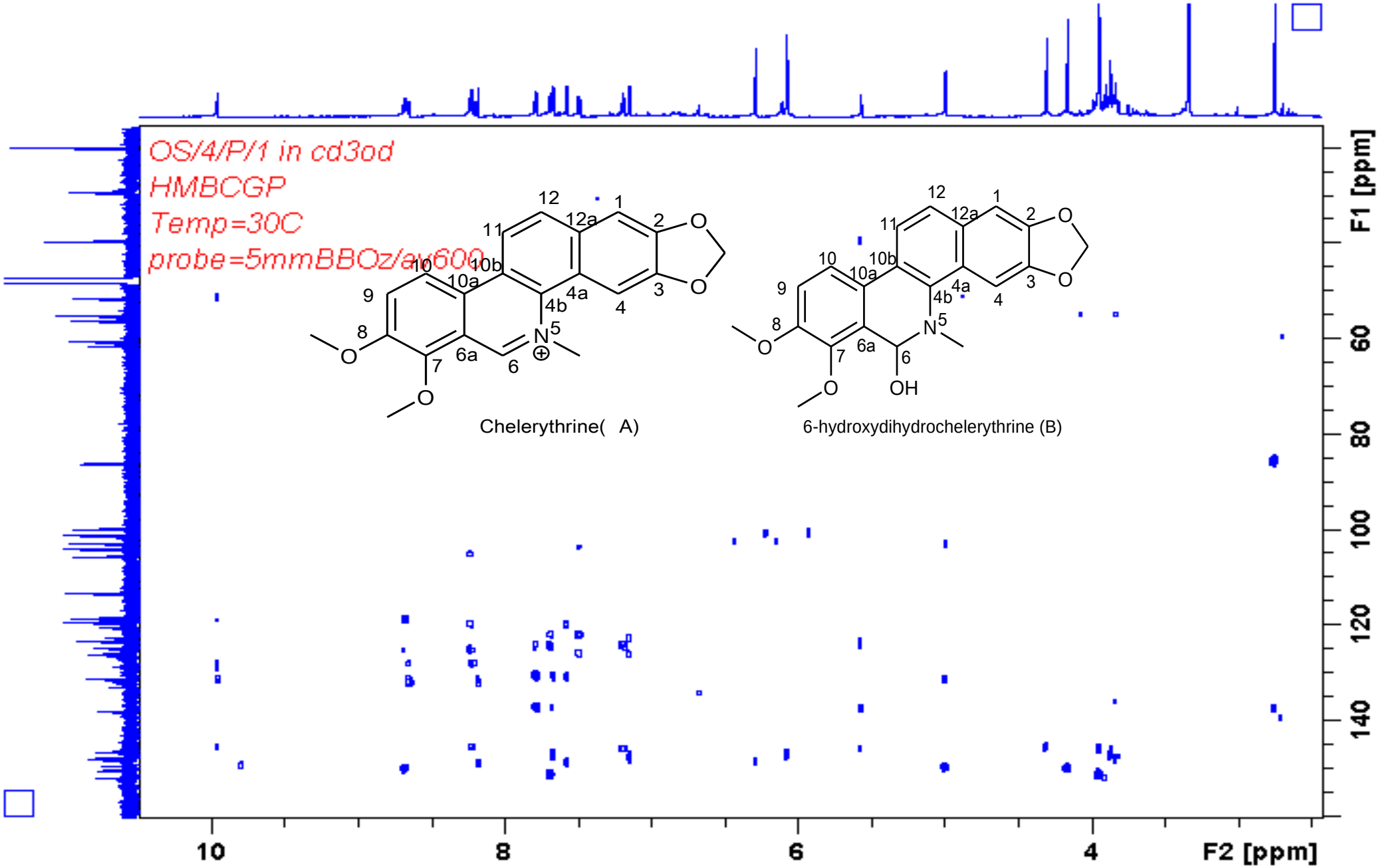
COSY spectrum of chelerythrine (**A1**) and 6-hydroxydihydrochelerythrine (**A2**)



NOESY spectrum of chelerythrine (**A1**) and 6-hydroxydihydrochelerythrine (**A2**)



HSQC spectrum of chelerythrine (**A1**) and 6-hydroxydihydrochelerythrine (**A2**)

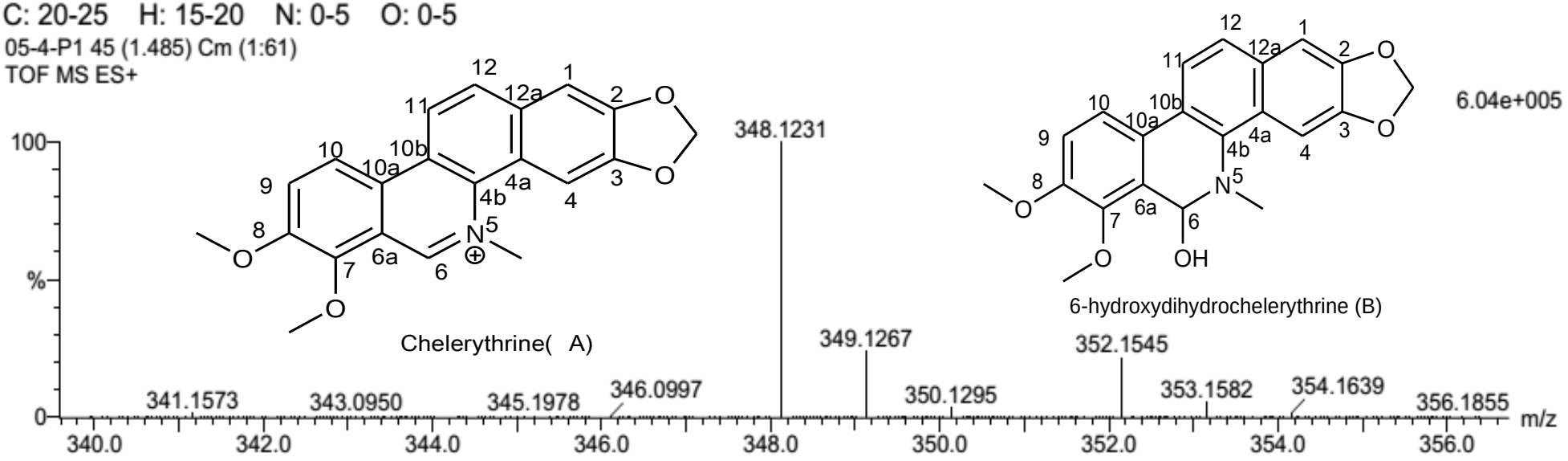


HMBC spectrum of chelerythrine (**A1**) and 6-hydroxydihydrochelerythrine (**A2**)

Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

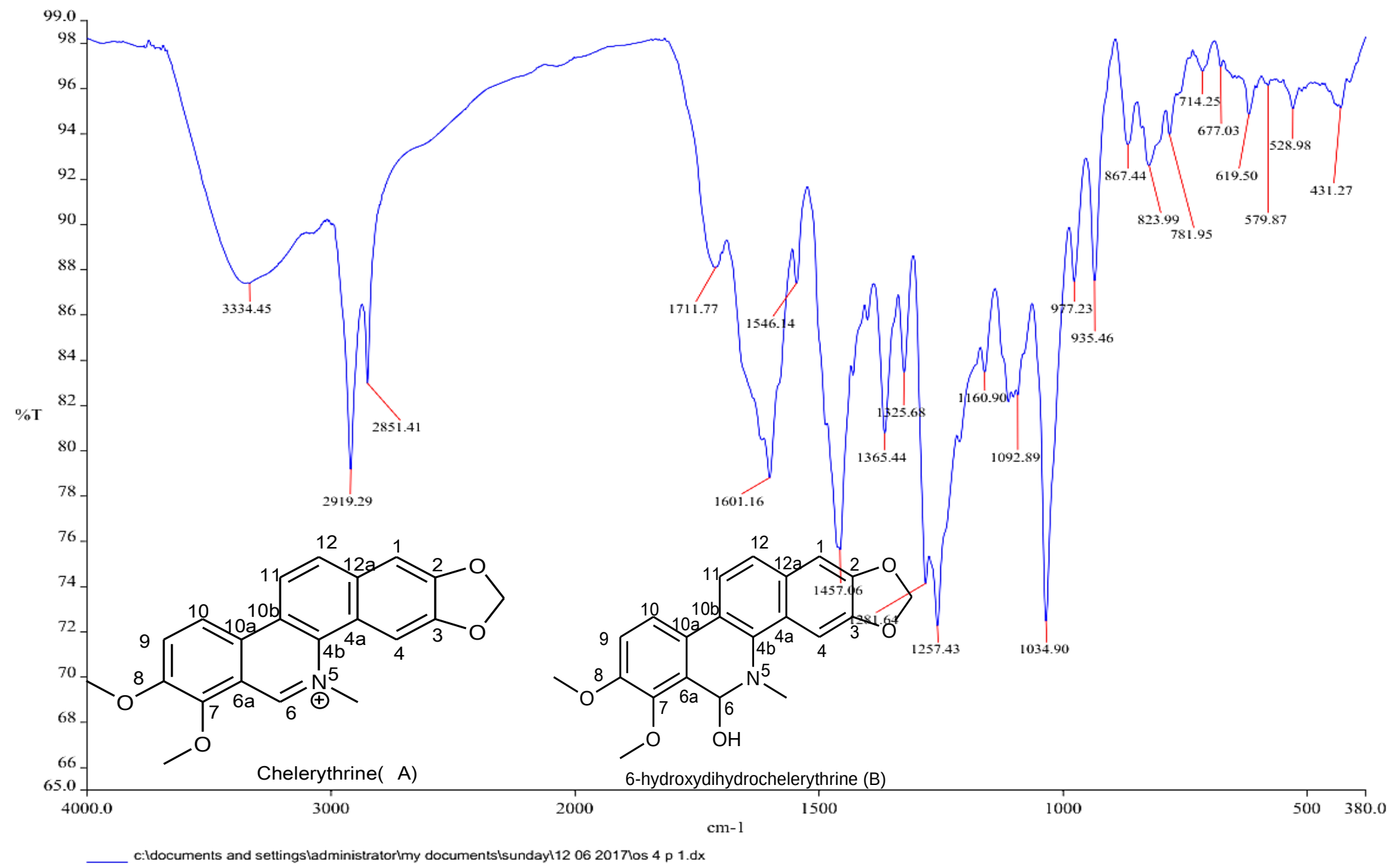
Monoisotopic Mass, Even Electron Ions
19 formula(e) evaluated with 1 results within limits (up to 20 closest results for each mass)
Elements Used:
C: 20-25 H: 15-20 N: 0-5 O: 0-5

05-4-P1 45 (1.485) Cm (1:61)
TOF MS ES+

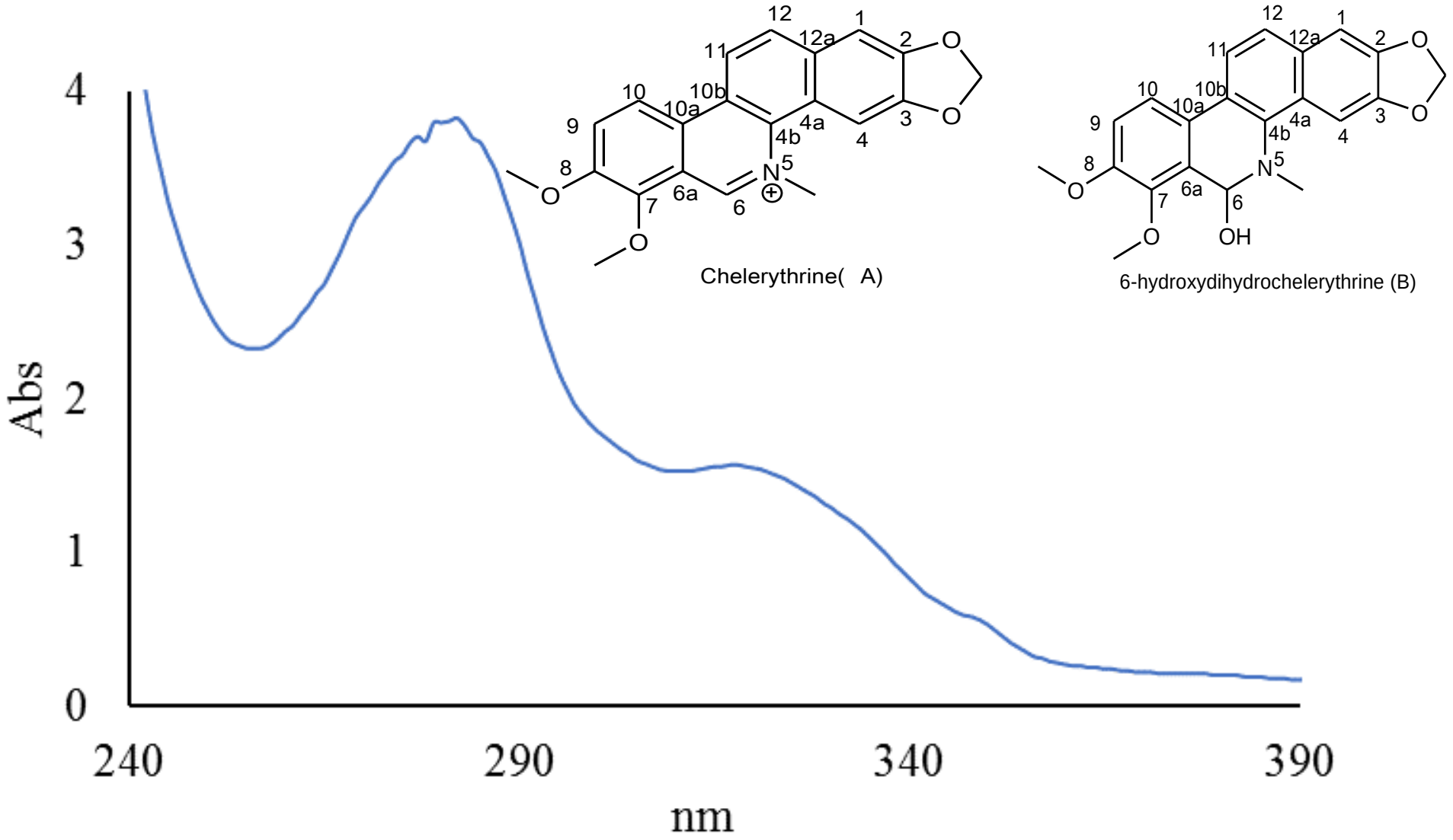


Minimum:				-1.5			
Maximum:		5.0	5.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
348.1231	348.1236	-0.5	-1.4	13.5	649.3	0.0	C21 H18 N O4

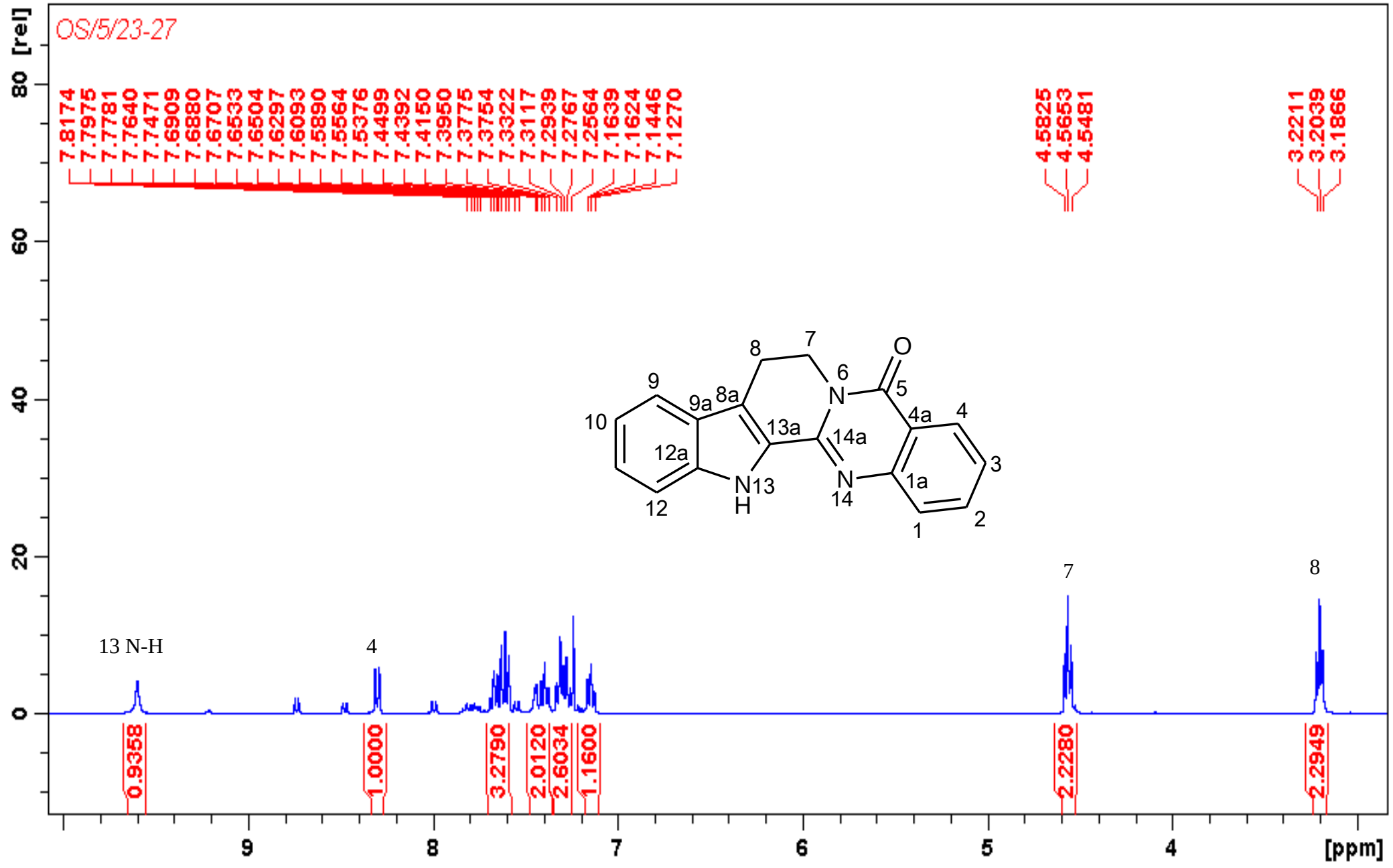
Mass spectrum of chelerythrine (**A1**) and 6-hydroxydihydrochelerythrine (**A2**)



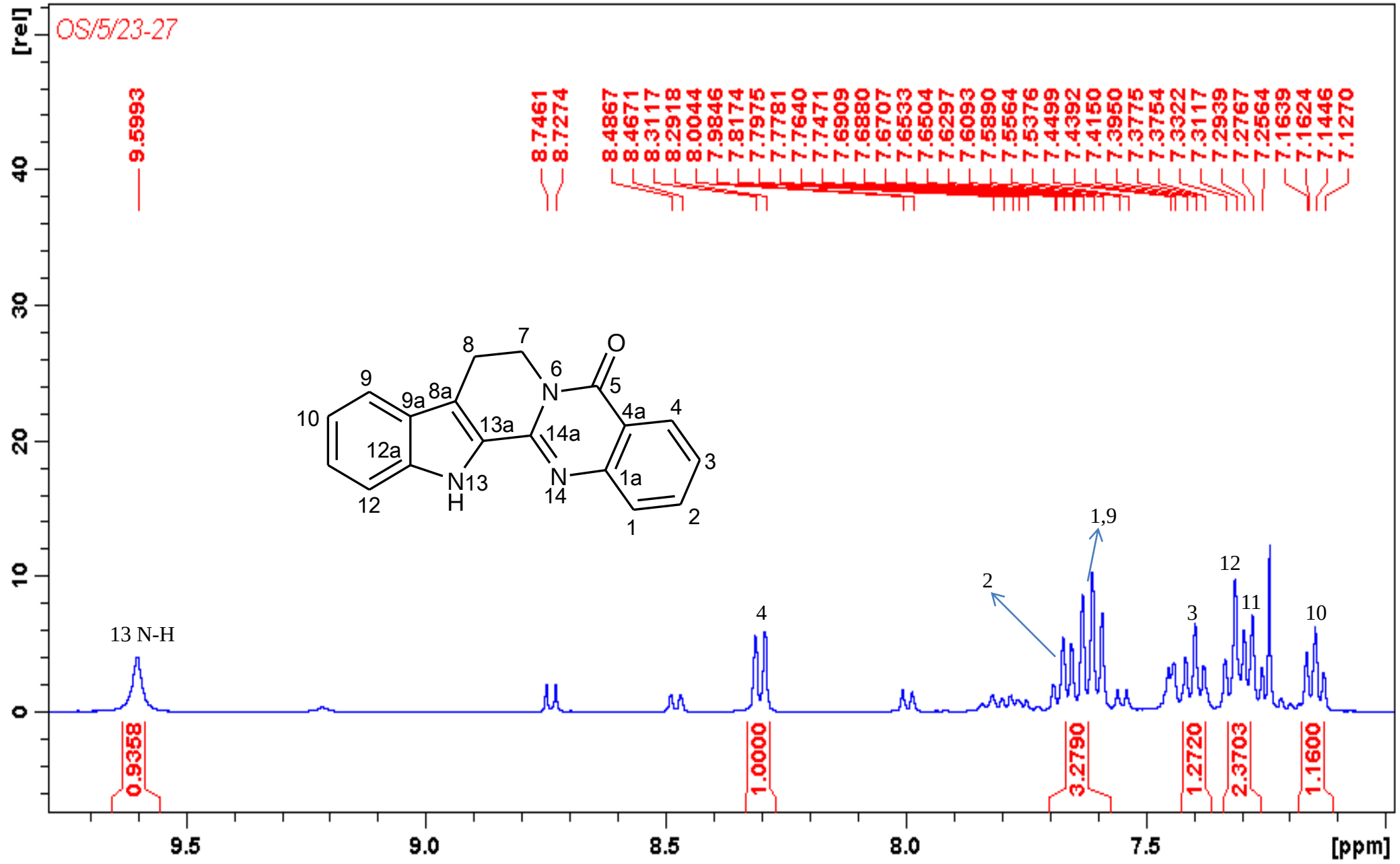
IR spectrum of chelerythrine (**A1**) and 6-hydroxydihydrochelerythrine (**A2**)



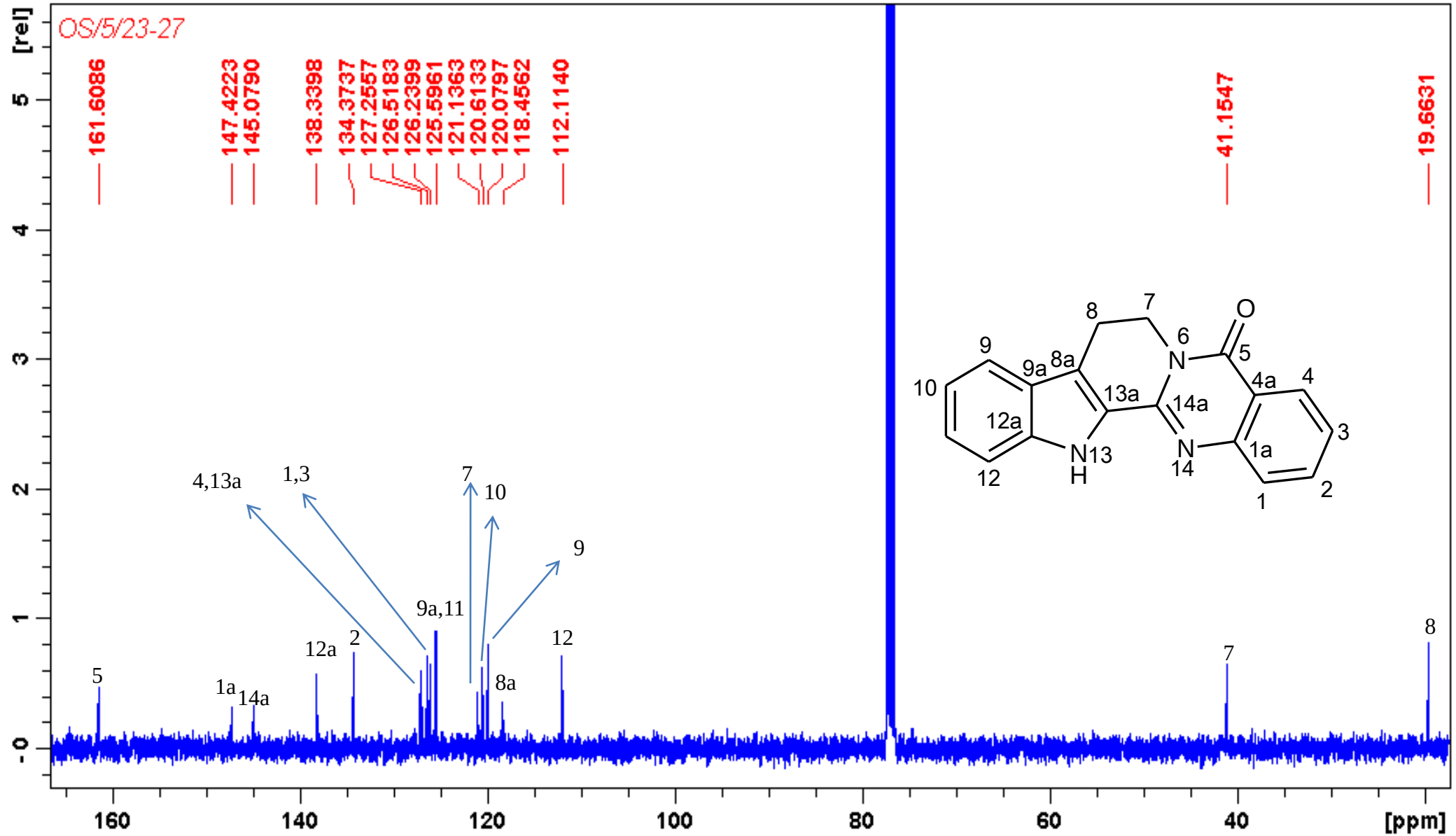
UV spectrum of chelerythrine (**A1**) and 6-hydroxydihydrochelerythrine (**A2**)



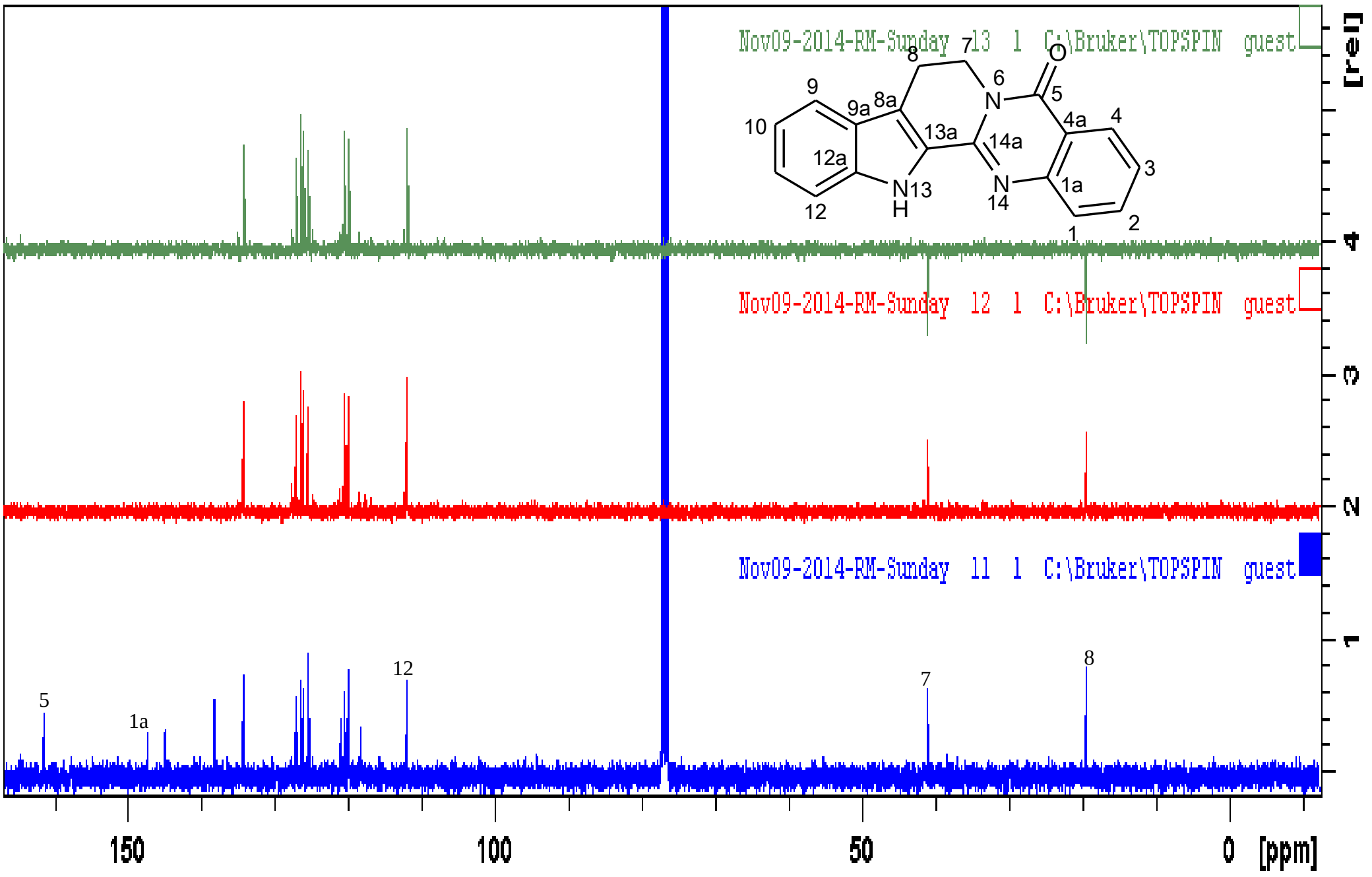
^{1}H NMR spectrum of rutaecarpine (**A3**)



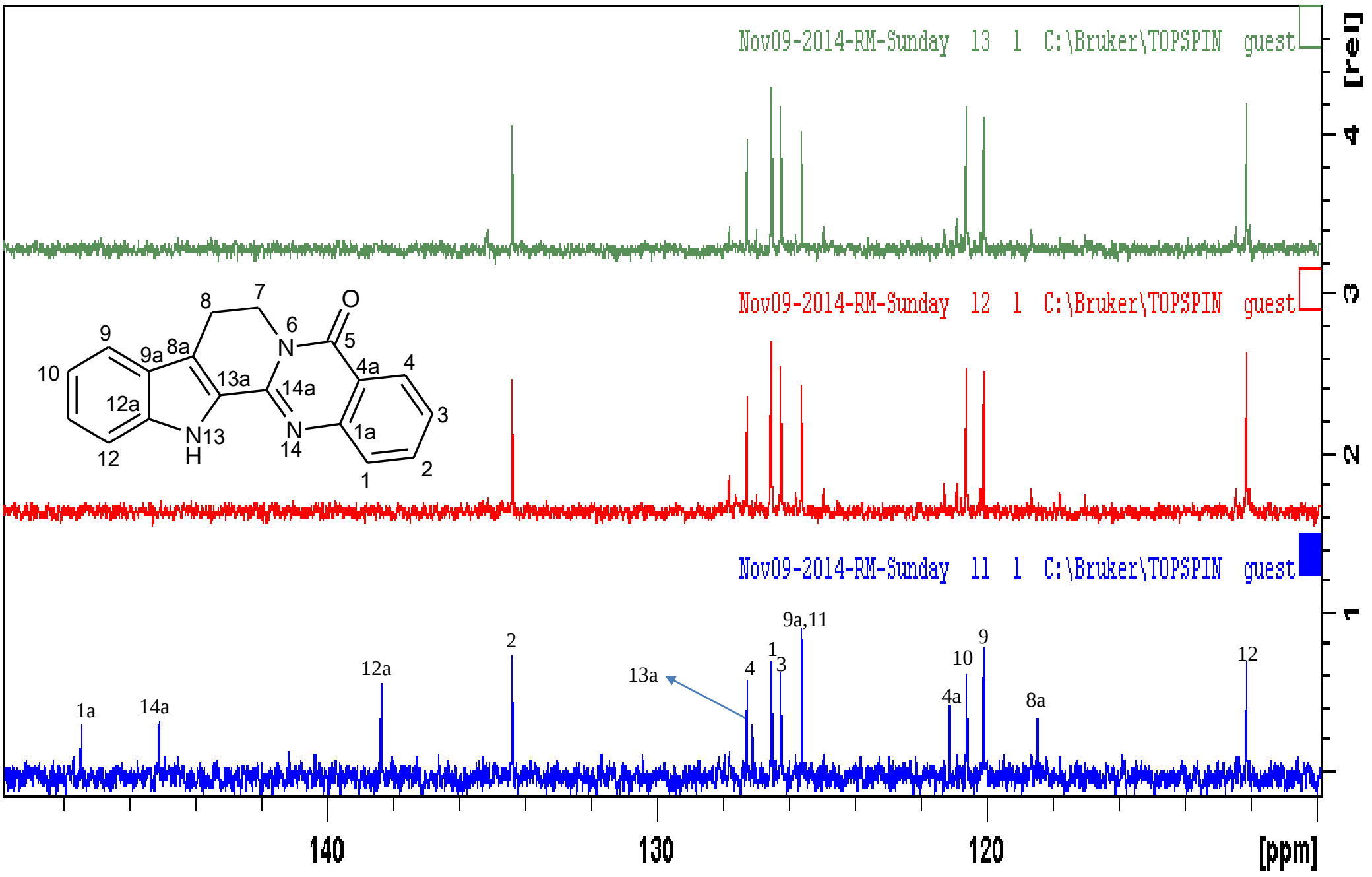
Expanded ^{1}H NMR spectrum of rutaecarpine (**A3**)



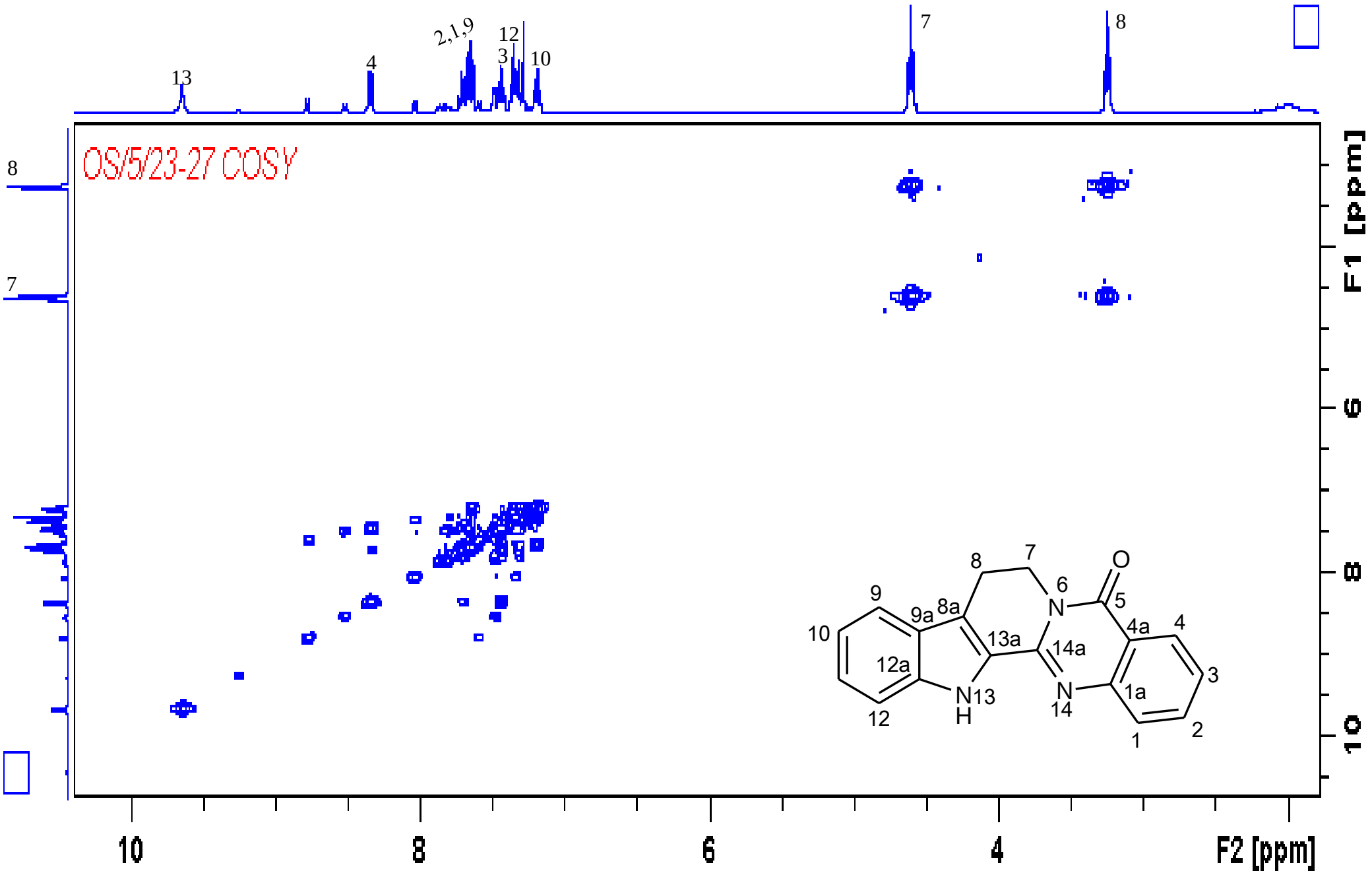
^{13}C NMR spectrum of rutaecarpine (**A3**)



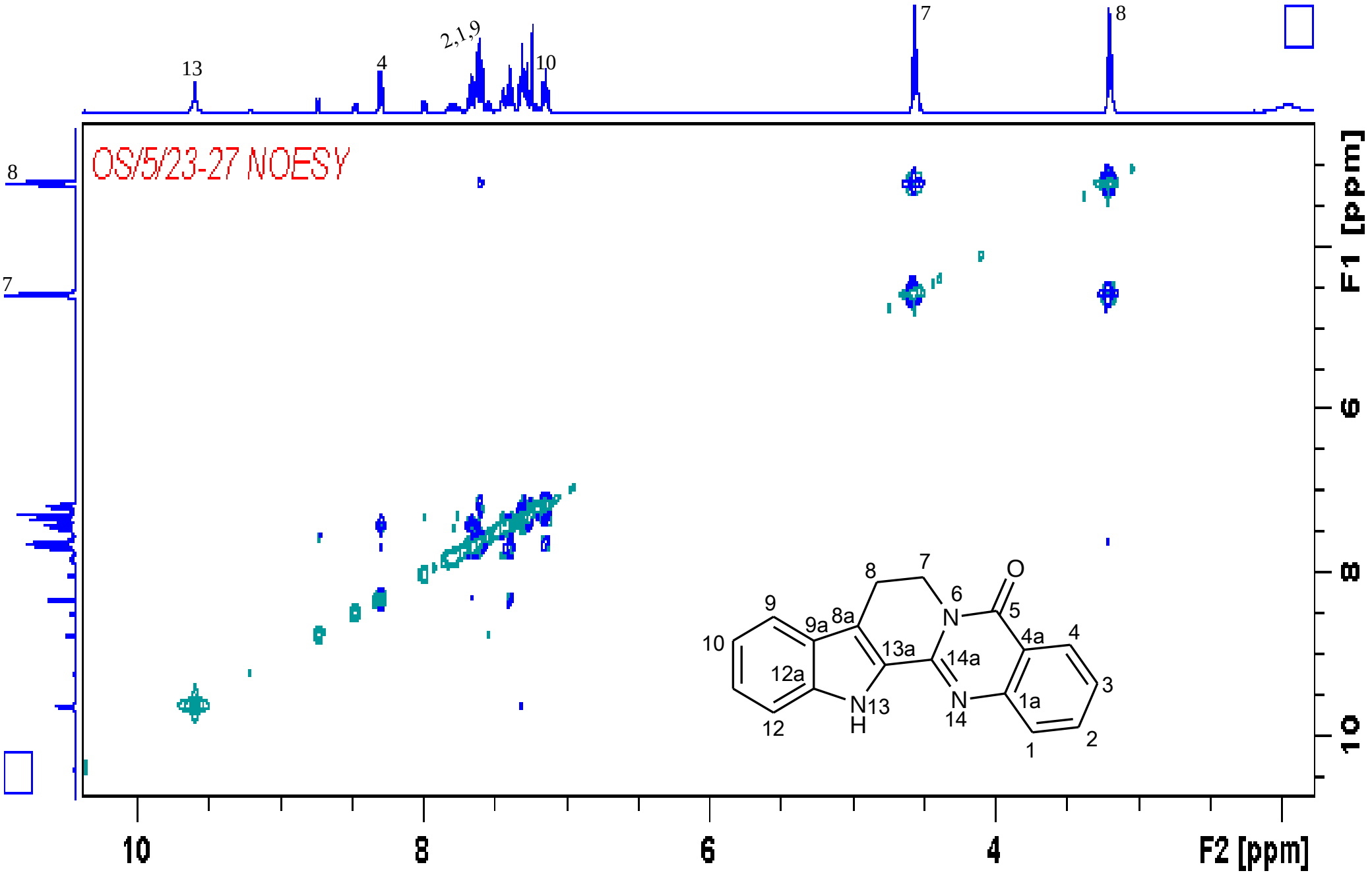
DEPT spectrum of rutaecarpine (**A3**)



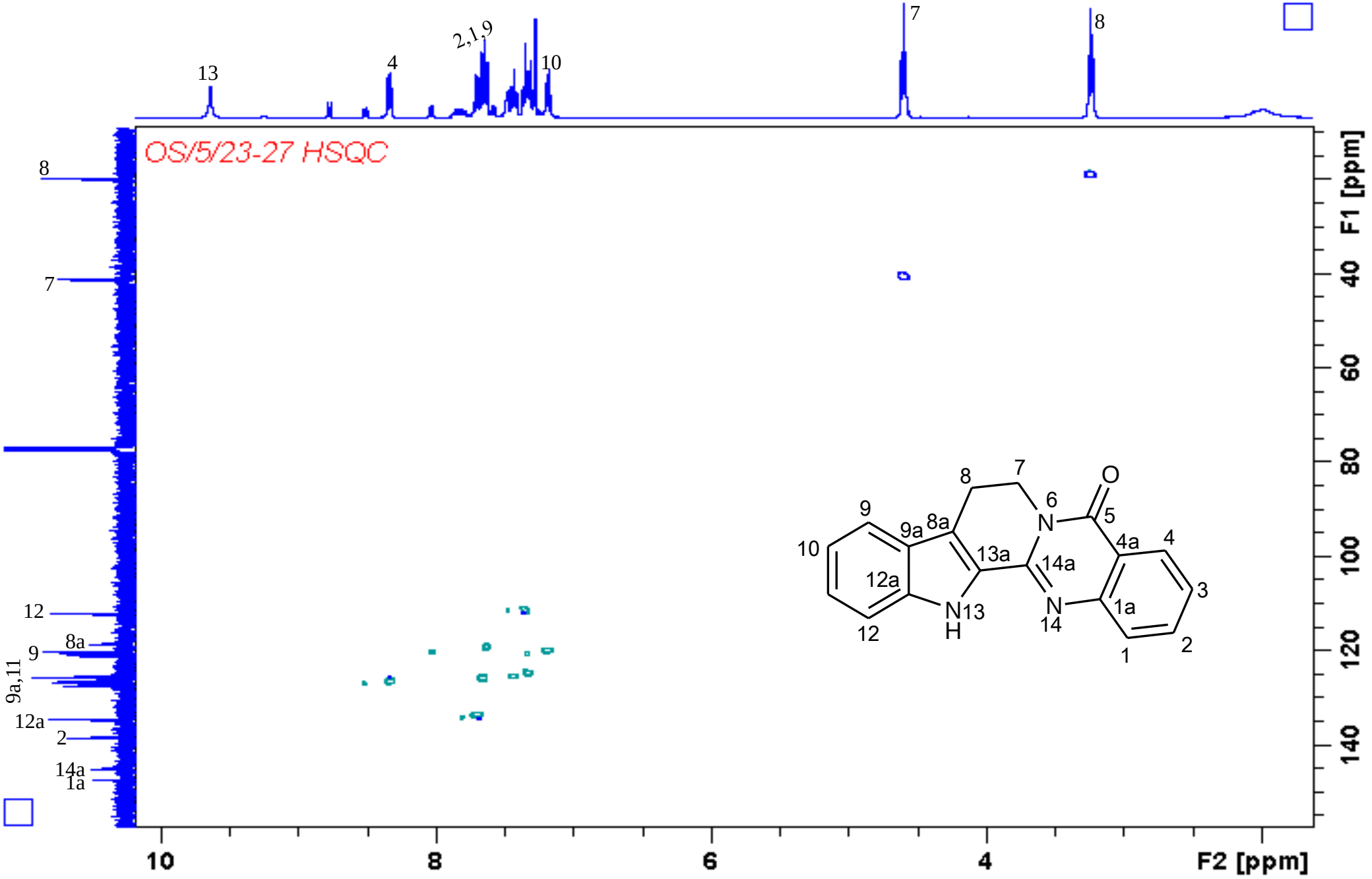
Expanded DEPT spectrum of rutaecarpine (**A3**)



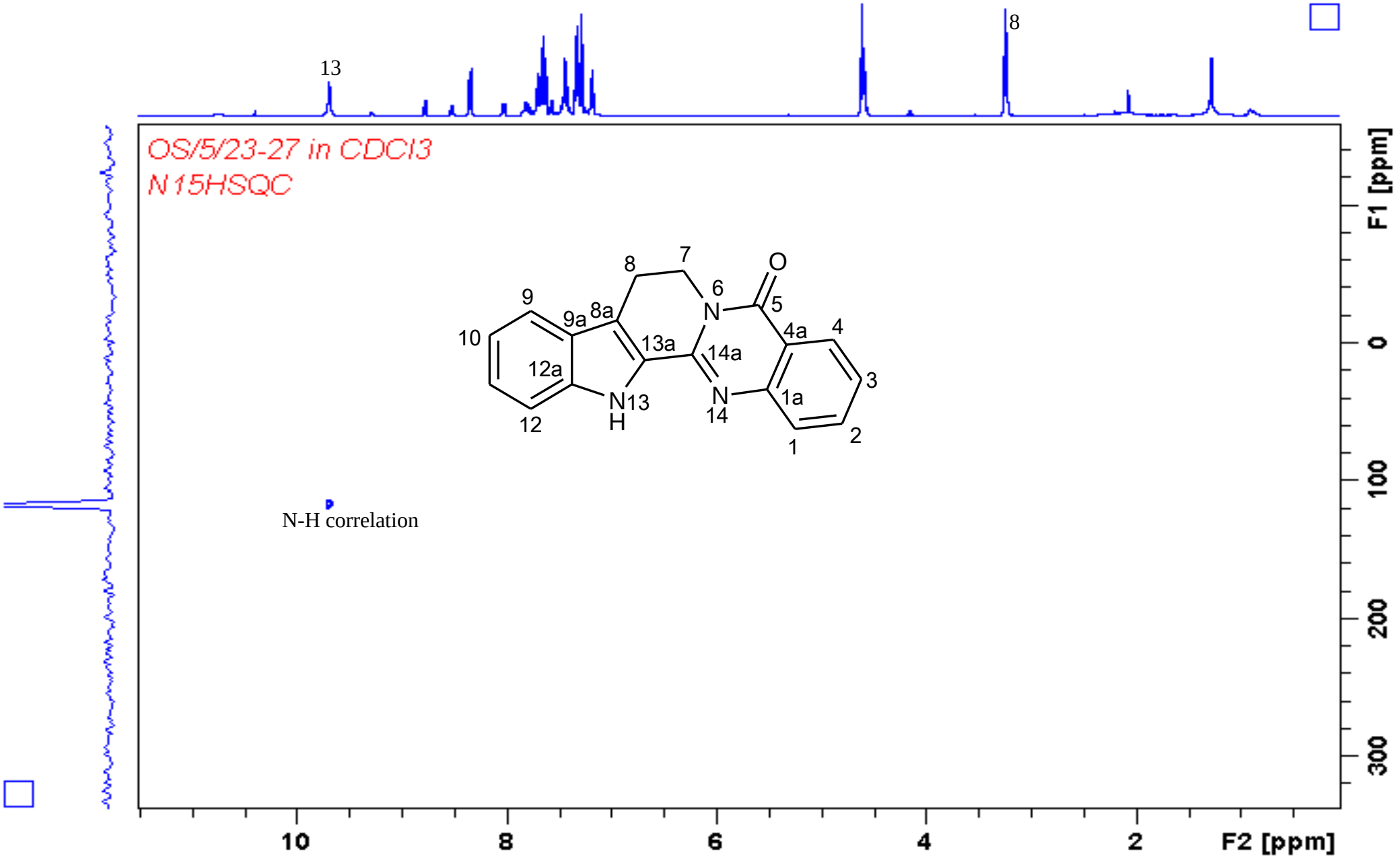
COSY spectrum of rutaecarpine (**A3**)



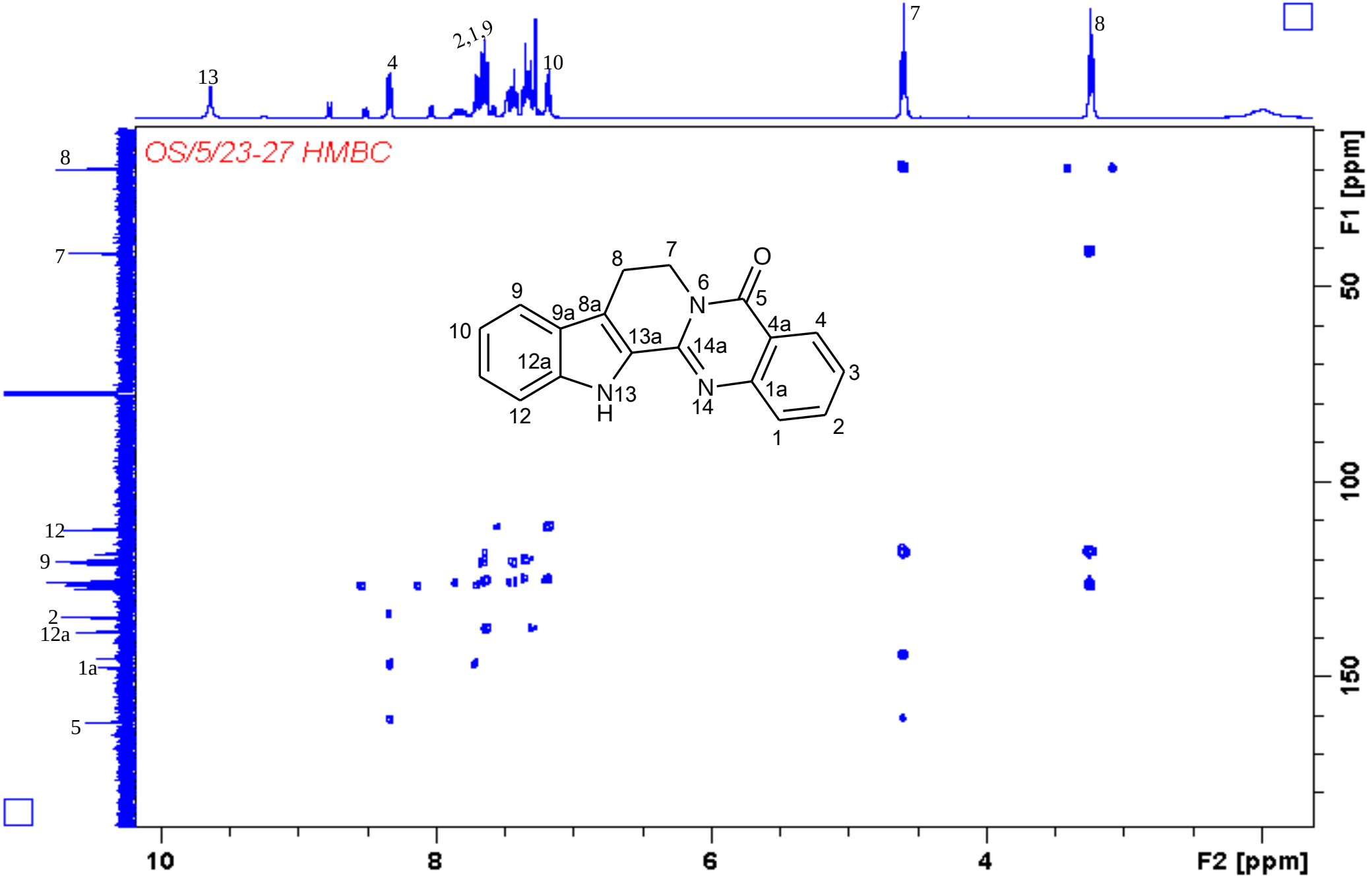
NOESY spectrum of rutaecarpine (**A3**)



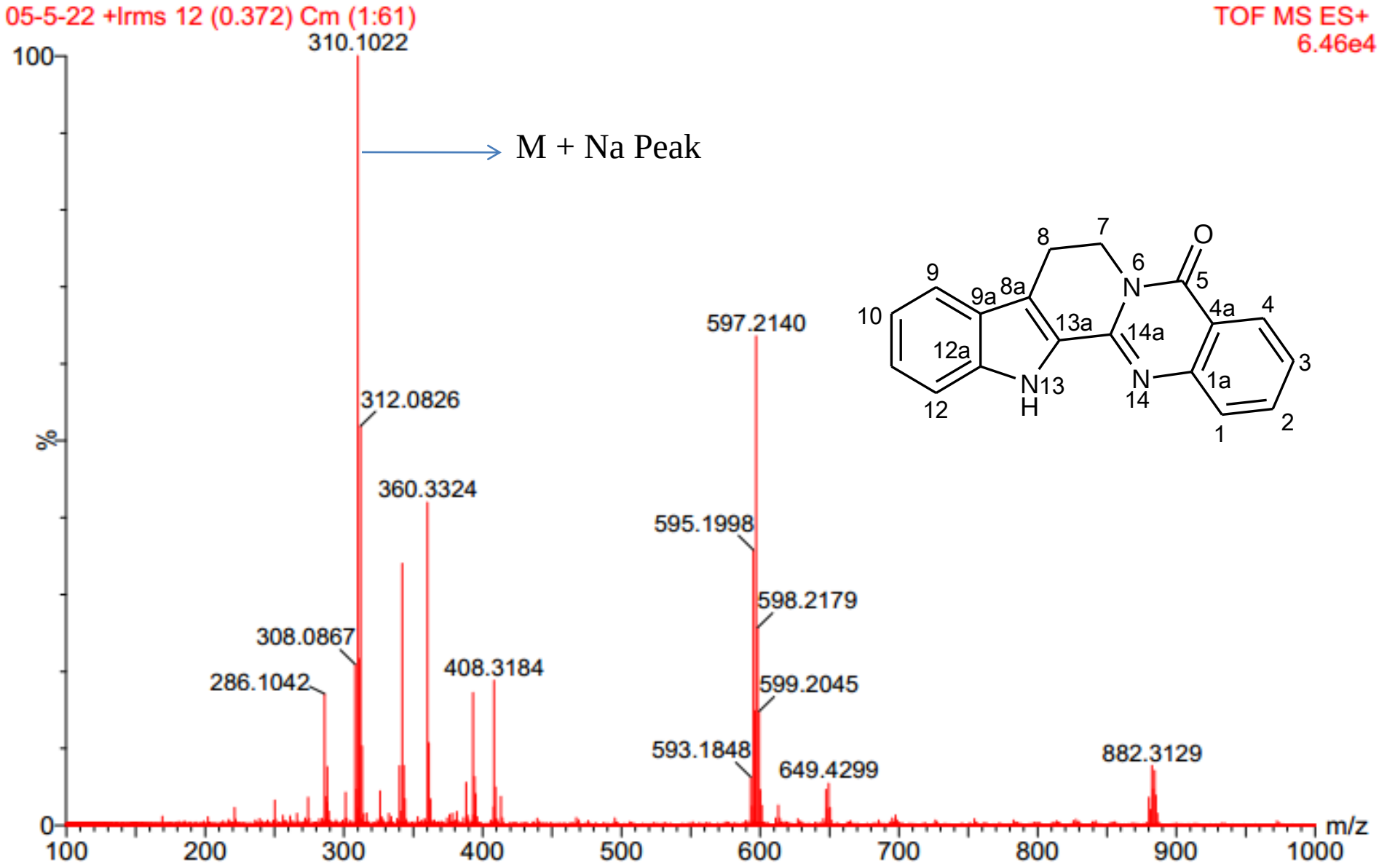
HSQC spectrum of rutaecarpine (**A3**)



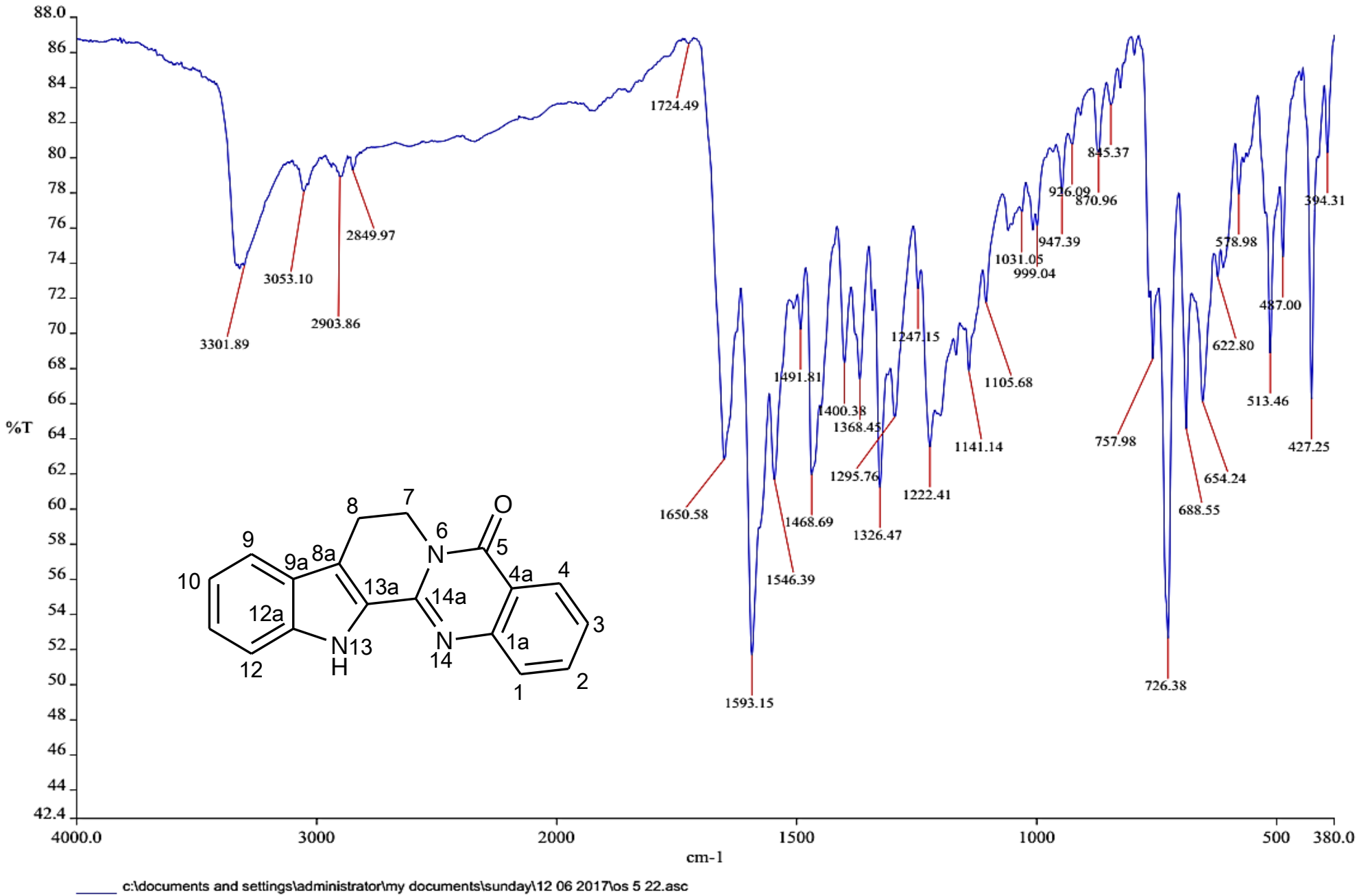
N15 HSQC spectrum of rutaecarpine (**A3**)



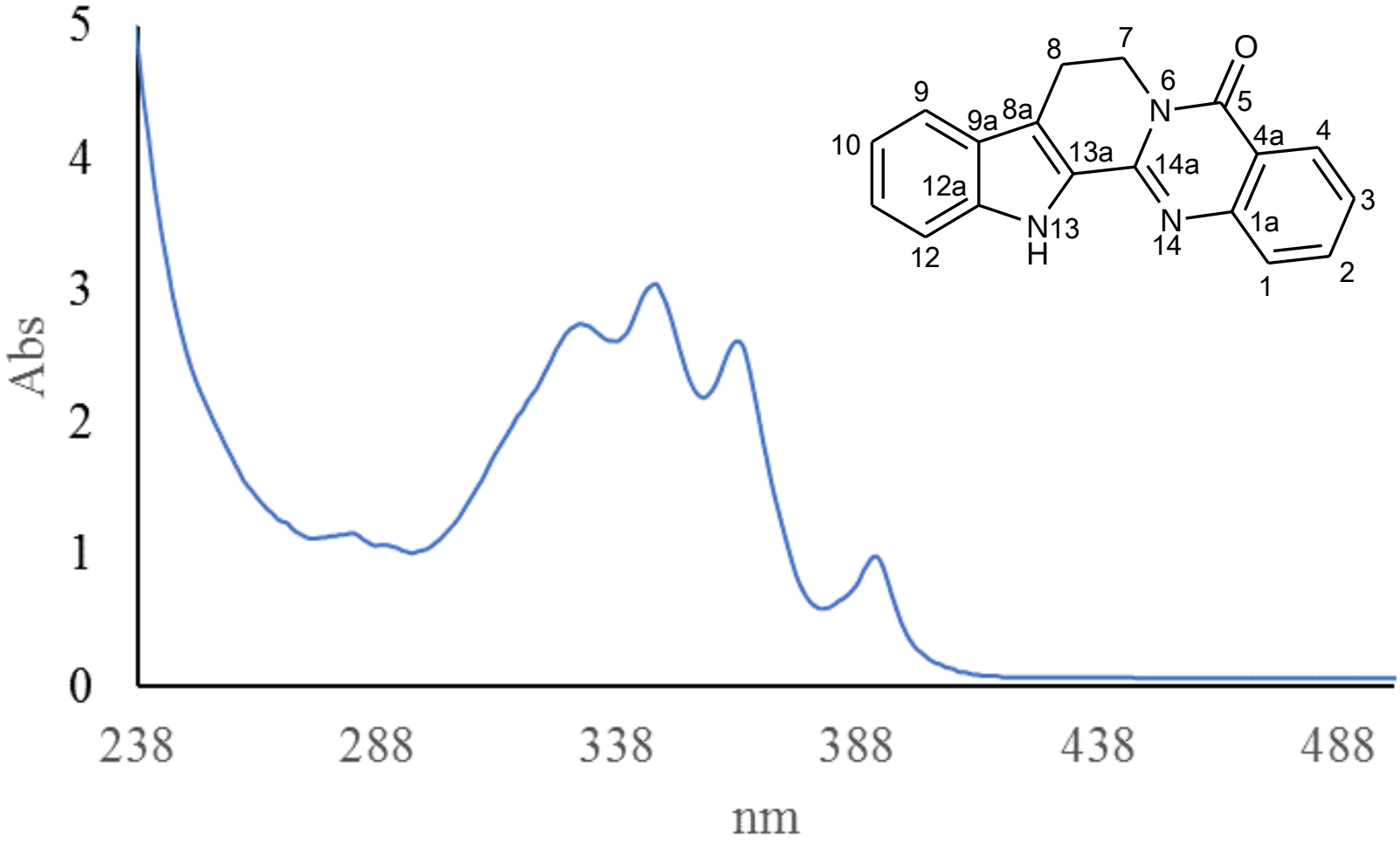
HMBC spectrum of rutaecarpine (**A3**)



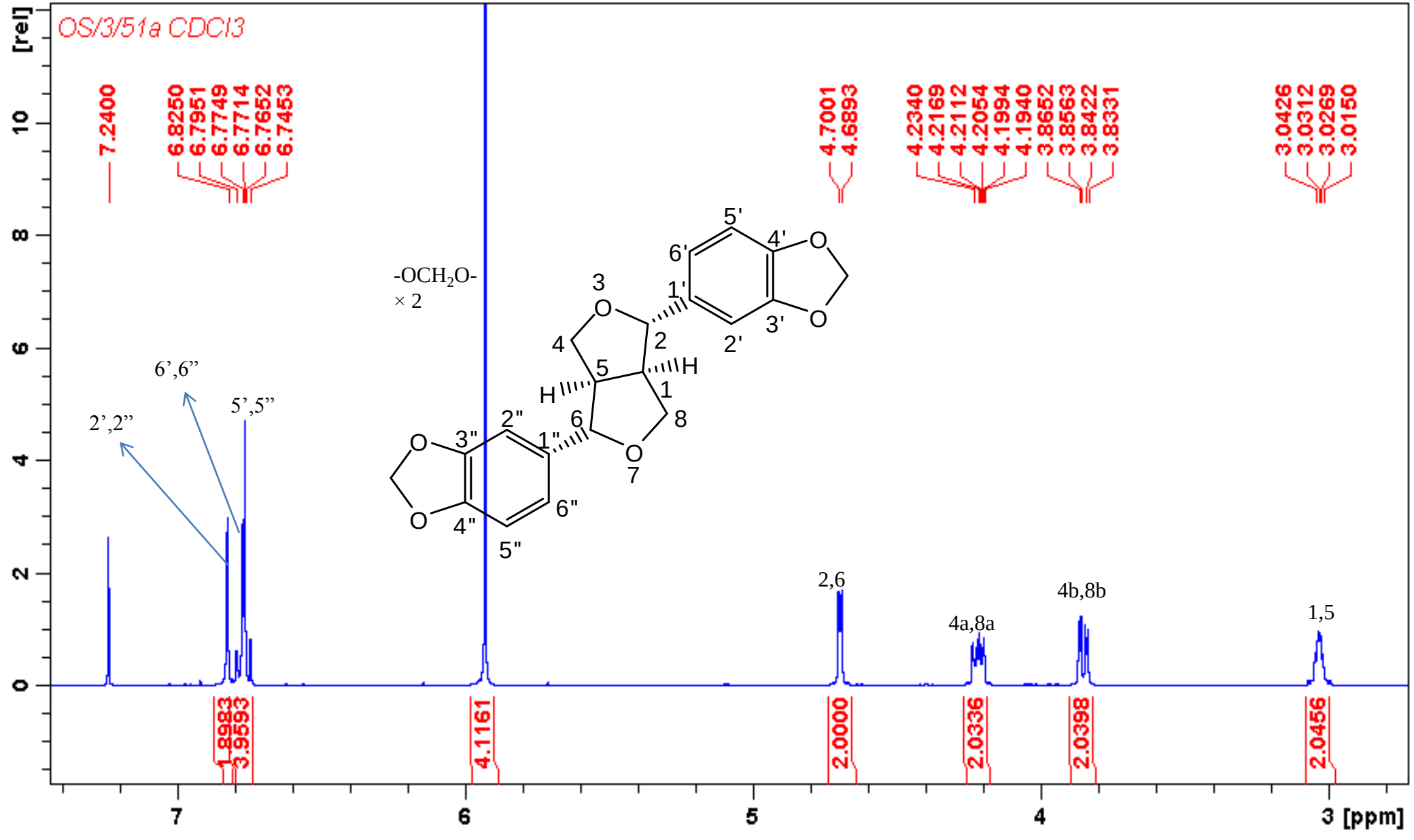
Mass spectrum of rutaecarpine (**A3**)



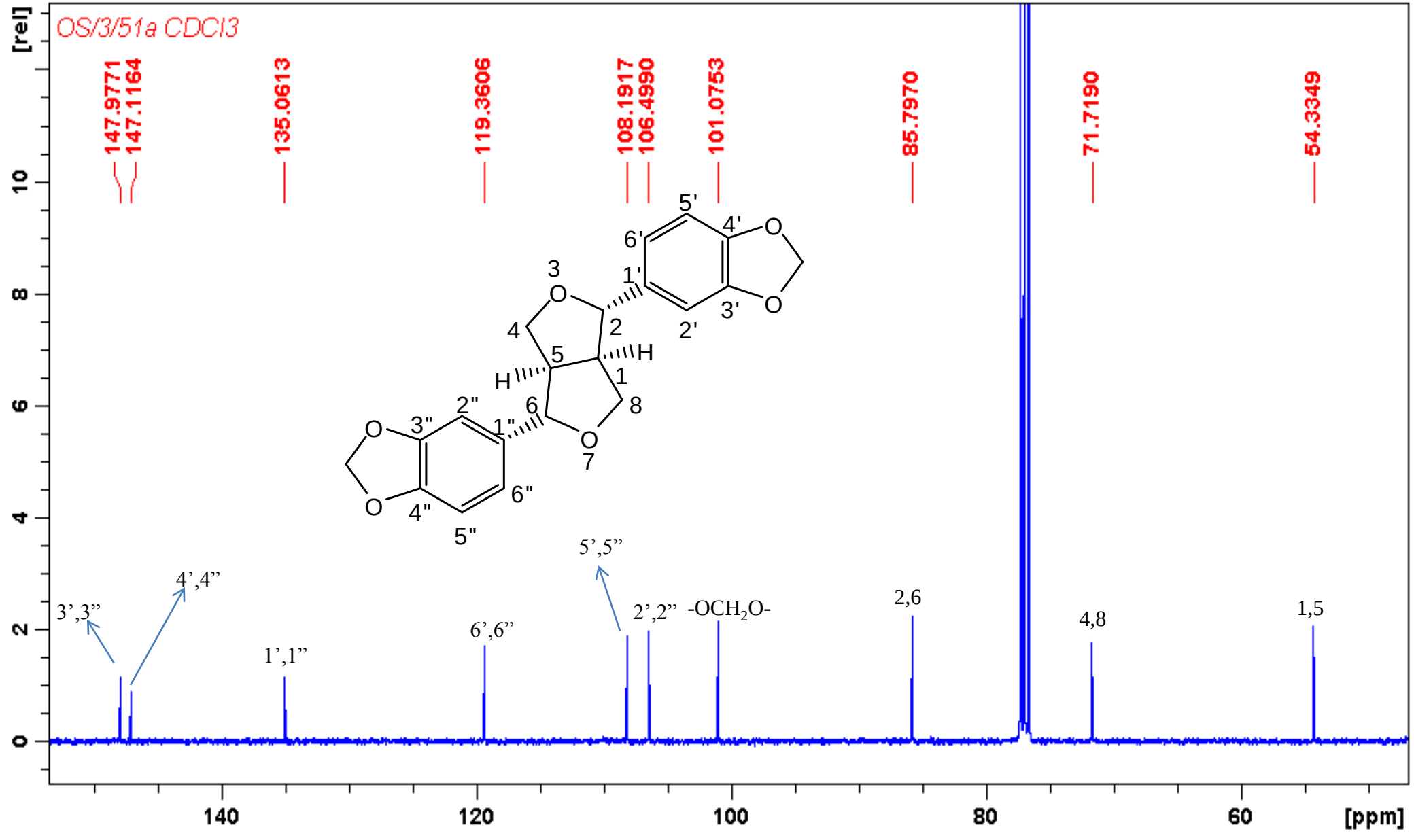
IR spectrum of rutaecarpine (**A3**)



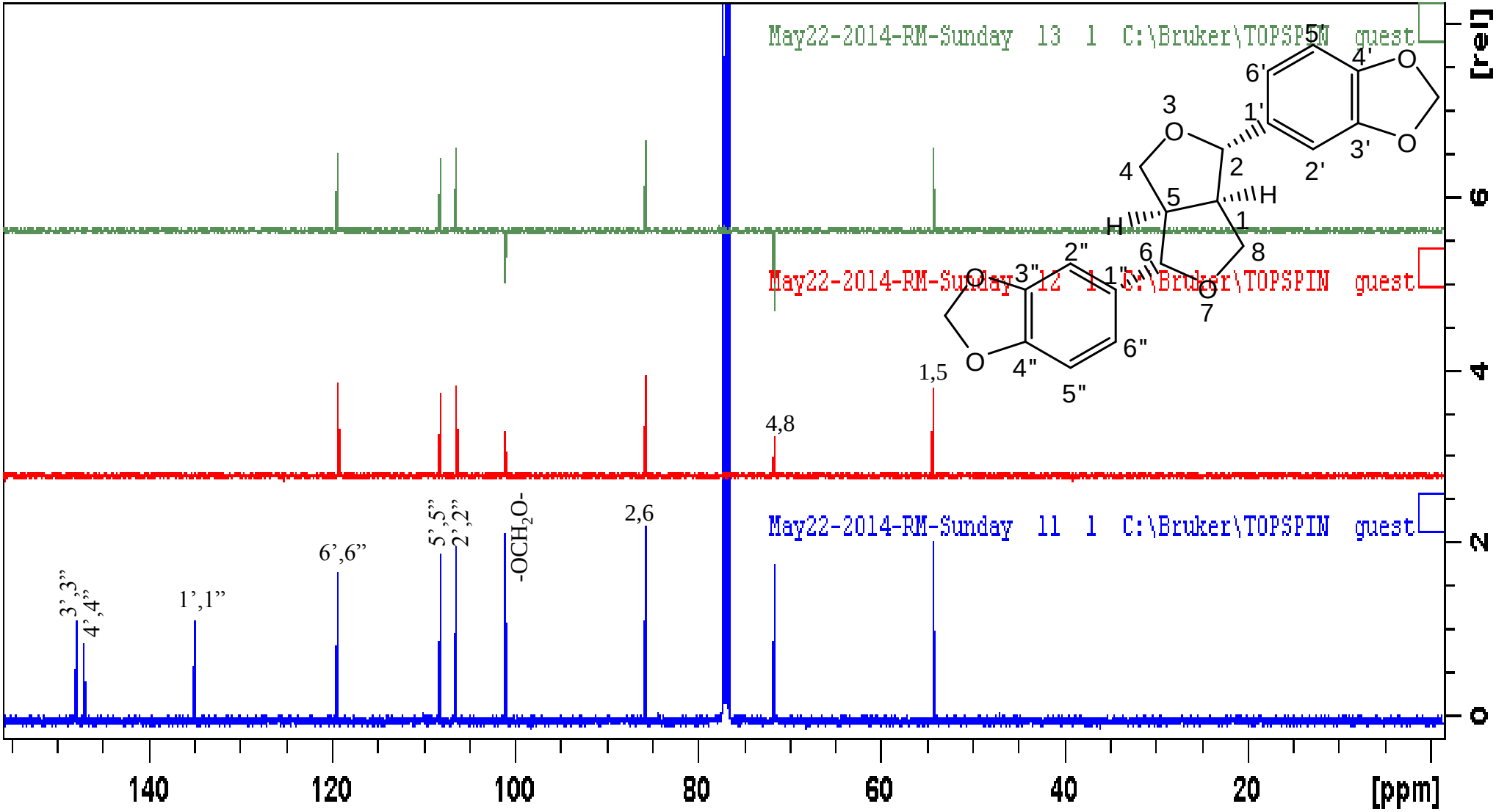
UV spectrum of rutaecarpine (**A3**)



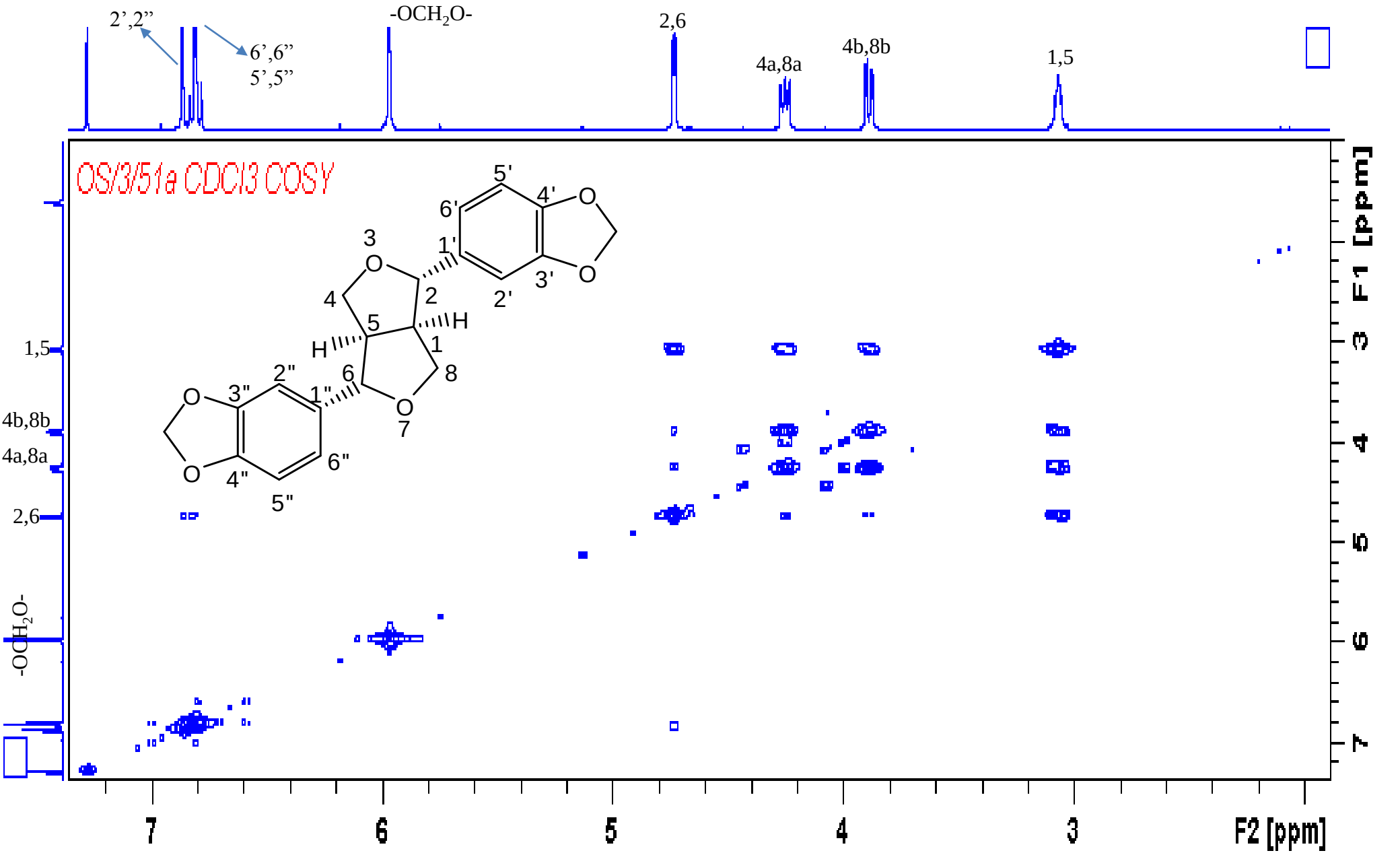
^{1}H NMR spectrum of sesamin (**A4**)



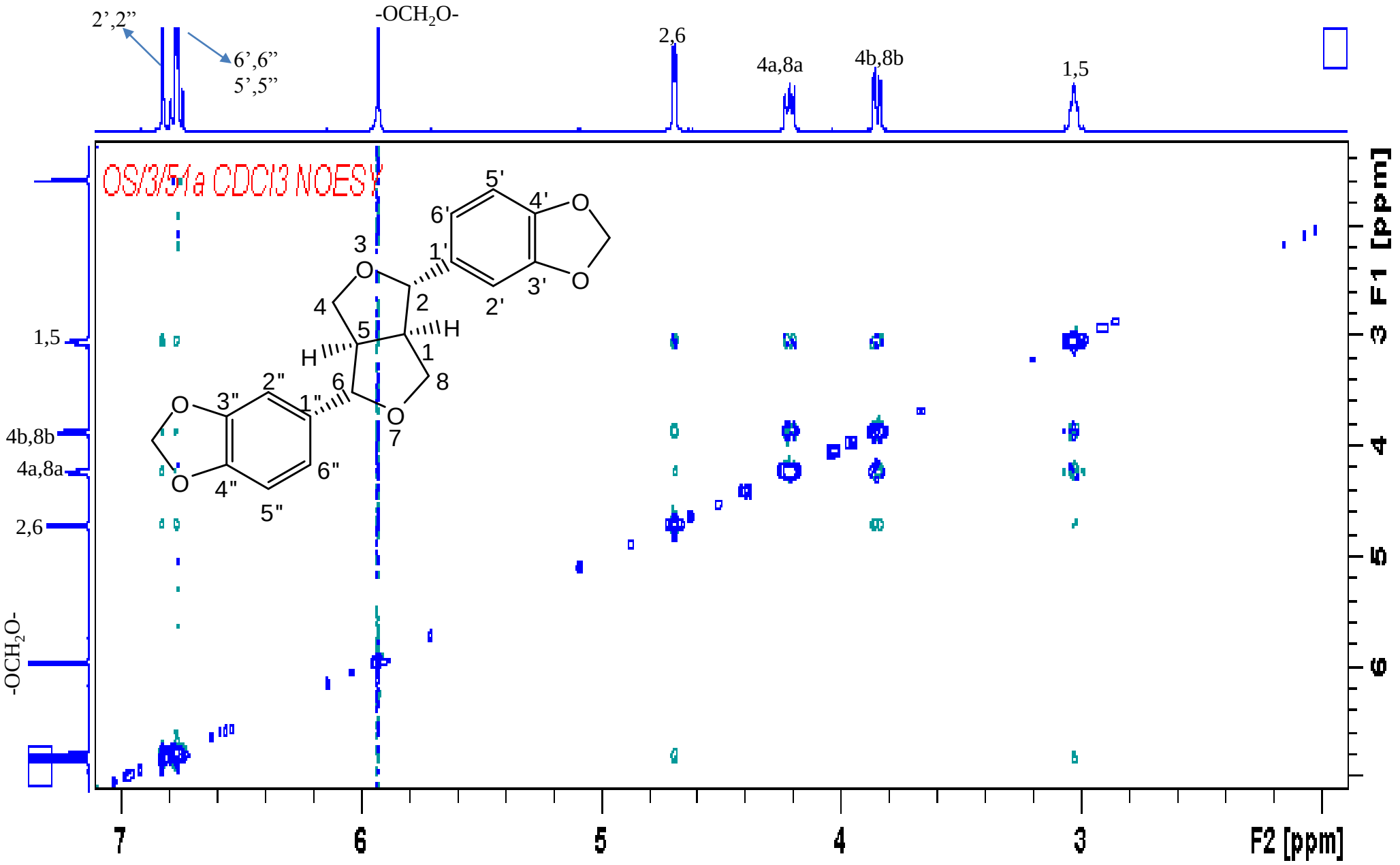
^{13}C NMR spectrum of sesamin (**A4**)



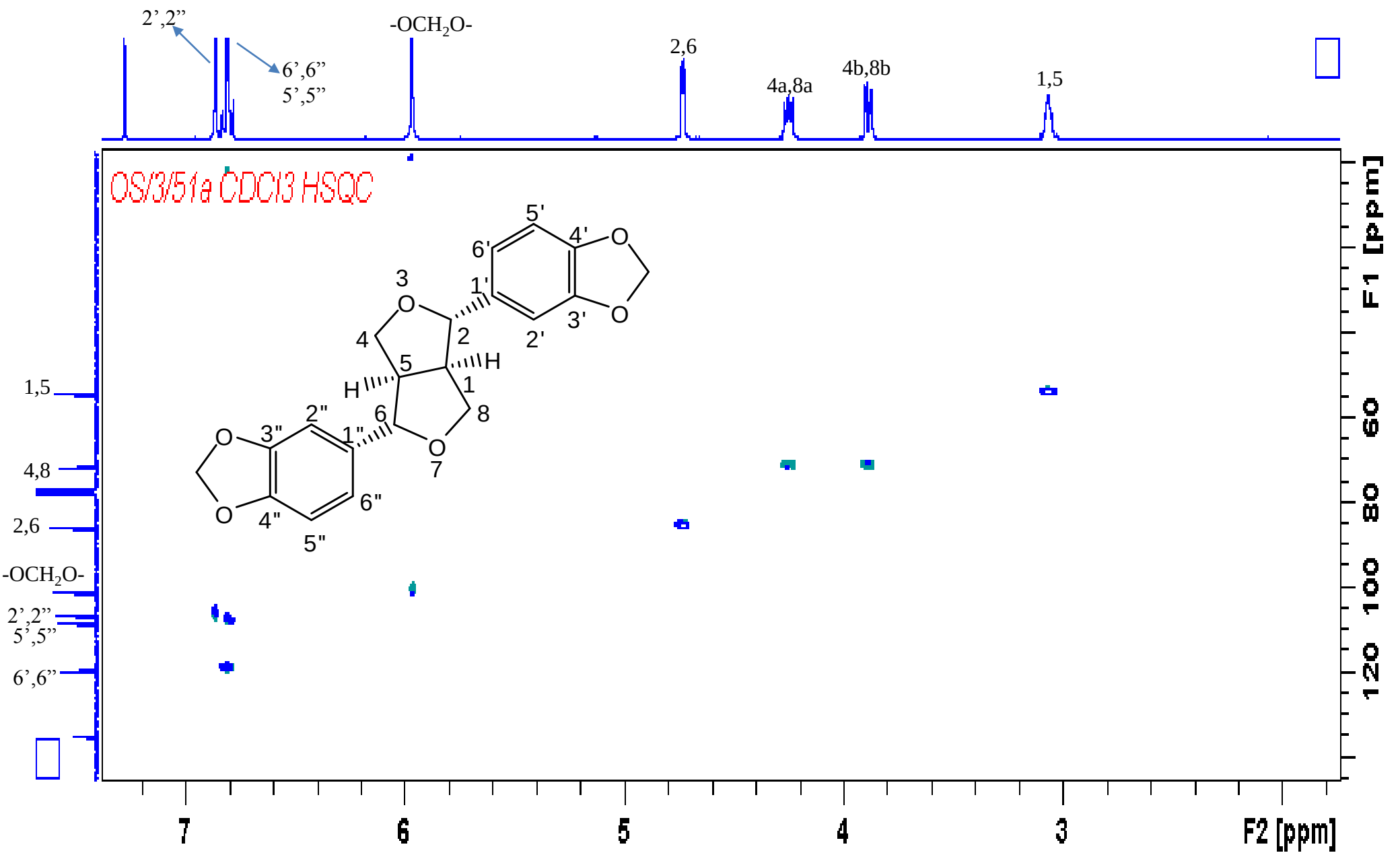
DEPT spectrum of sesamin (**A4**)



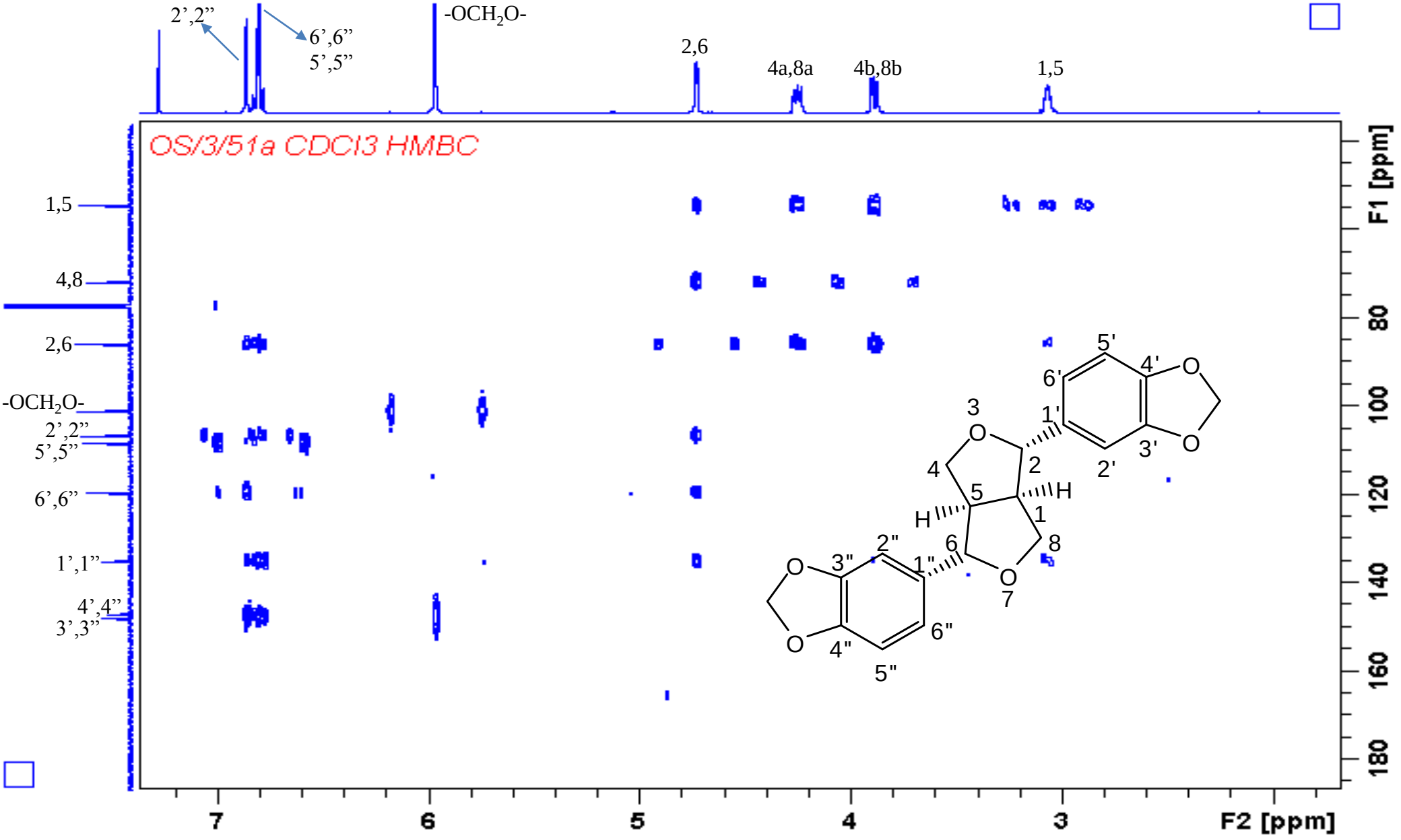
COSY spectrum of sesamin (**A4**)



NOESY spectrum of sesamin (**A4**)



HSQC spectrum of sesamin (**A4**)



HMBC spectrum of sesamin (**A4**)

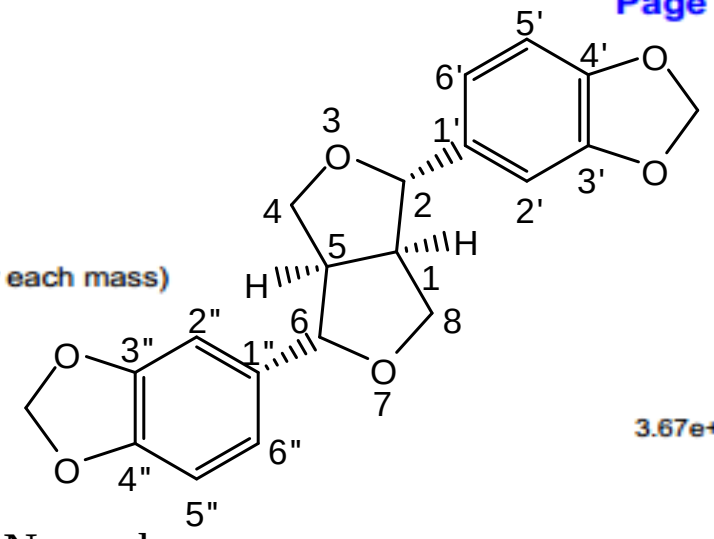
Elemental Composition Report

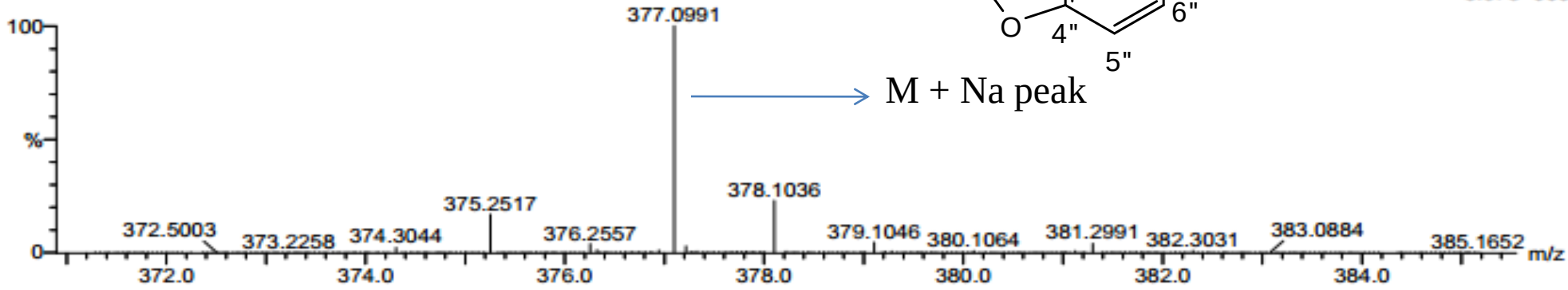
Single Mass Analysis

Tolerance = 5.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
795 formula(e) evaluated with 5 results within limits (up to 20 best isotopic matches for each mass)
Elements Used:
C: 5-50 H: 5-50 N: 0-10 O: 0-10 Na: 0-1

05-3-51a 32 (1.047) Cm (1:61)
TOF MS ES+

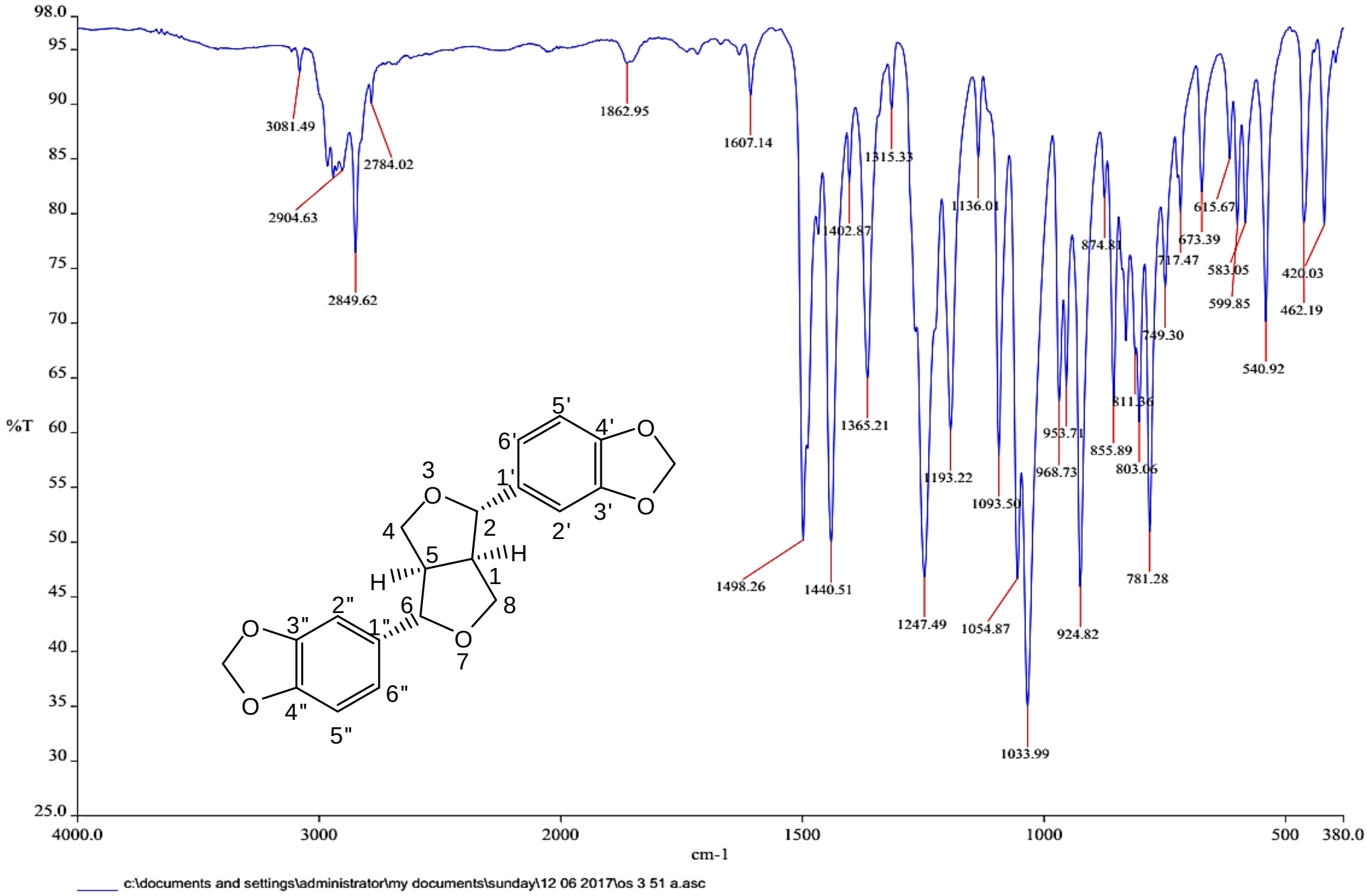




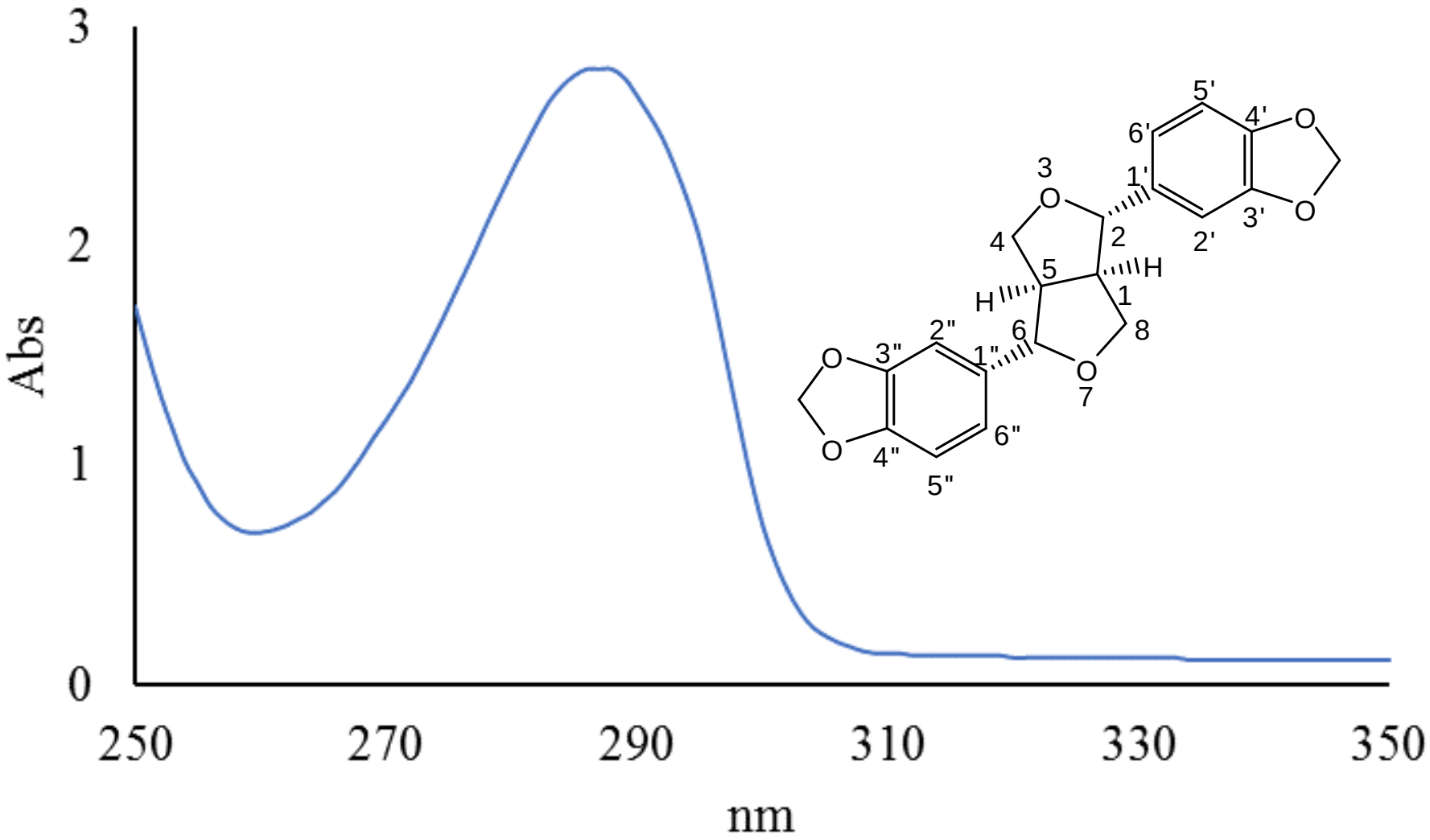
M + Na peak

Minimum:				-1.5			
Maximum:		5.0	5.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
377.0991	377.0988	0.3	0.8	17.5	658.3	5.5	C17 H10 N10 Na
	377.0985	0.6	1.6	10.5	655.4	2.6	C17 H17 N2 O8
	377.0998	-0.7	-1.9	15.5	655.2	2.5	C18 H13 N6 O4
	377.1001	-1.0	-2.7	11.5	653.0	0.2	C20 H18 O6 Na
	377.0974	1.7	4.5	12.5	658.4	5.6	C16 H14 N6 O4 Na

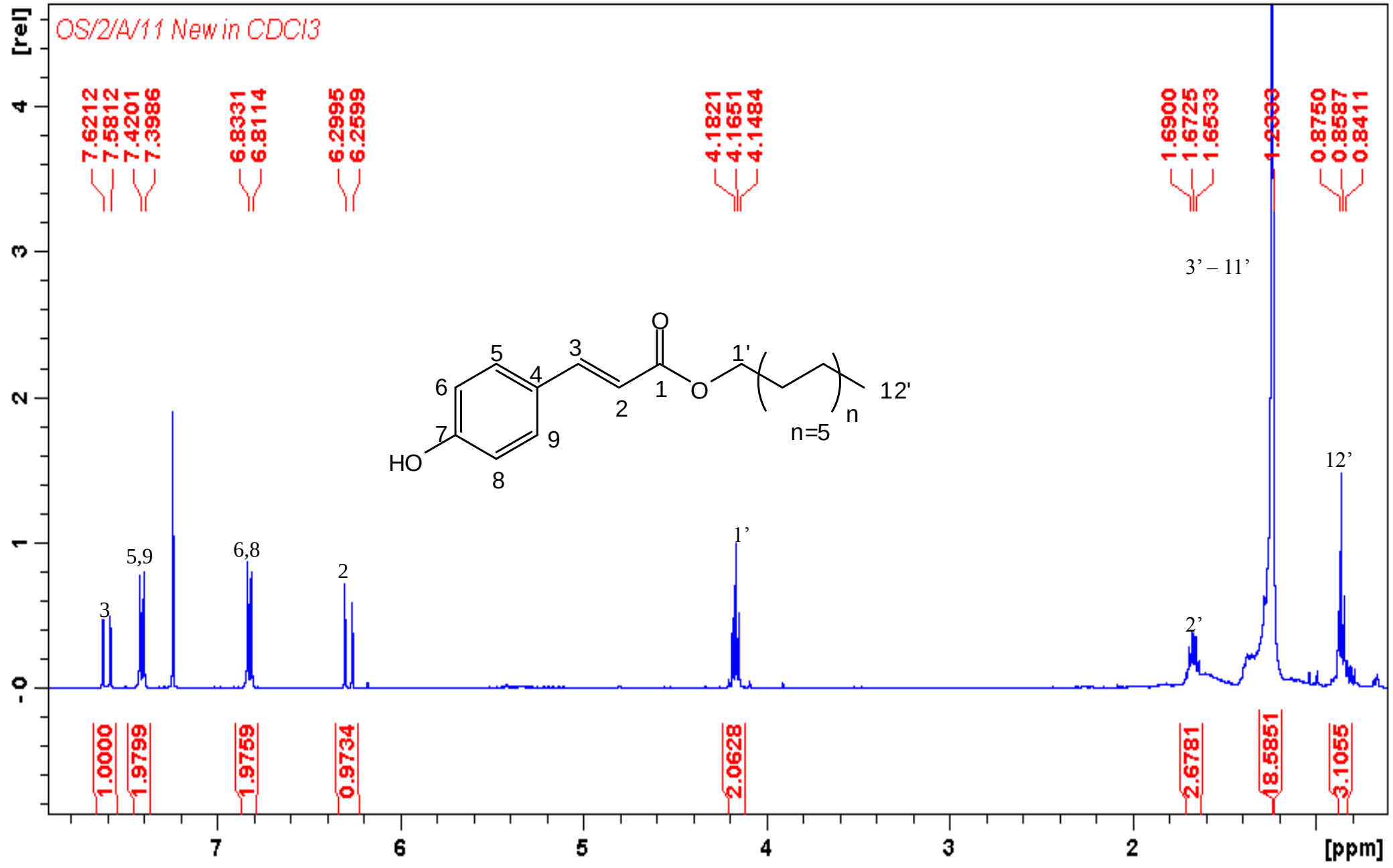
Mass spectrum of sesamin (**A4**)



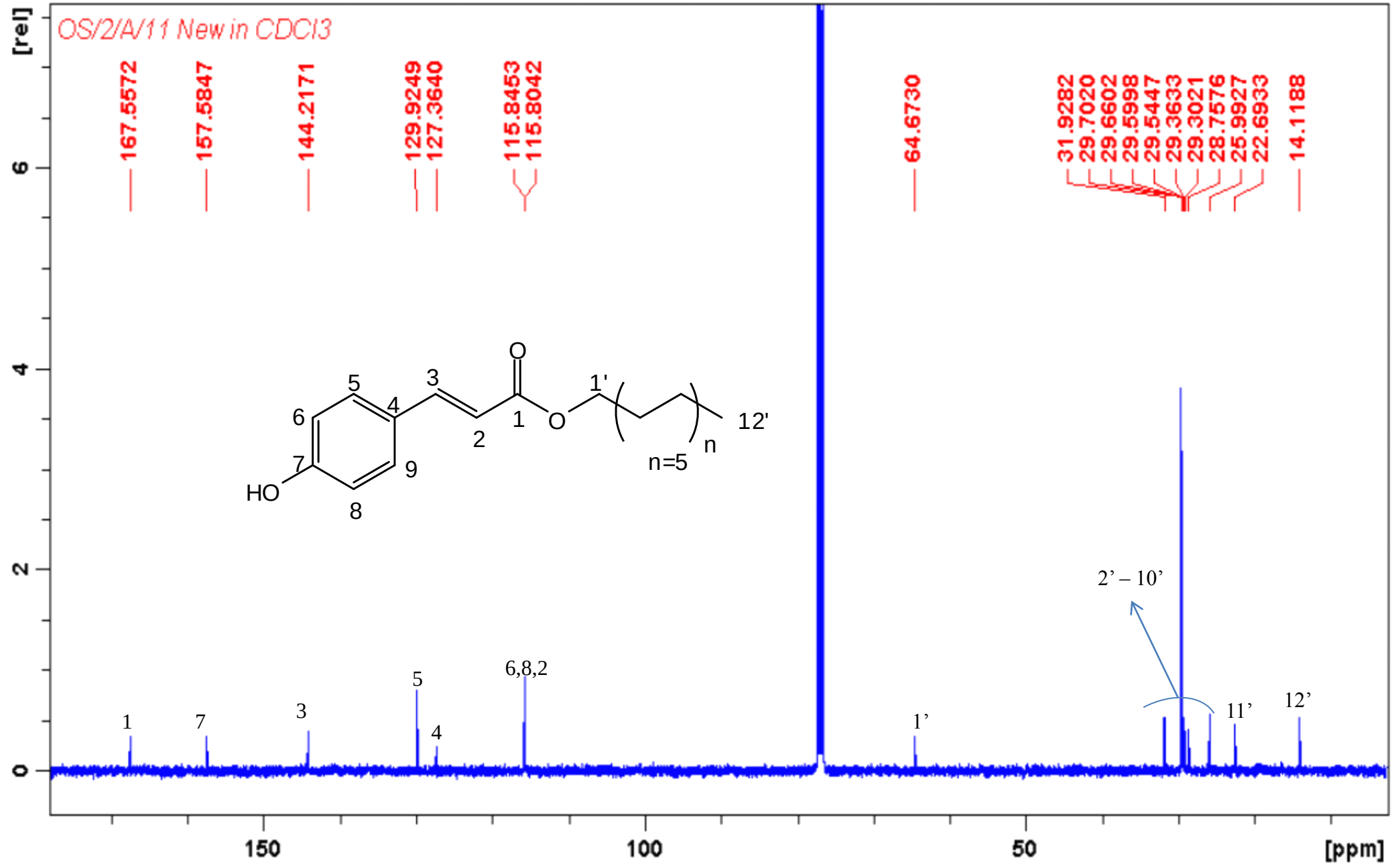
IR spectrum of sesamin (**A4**)



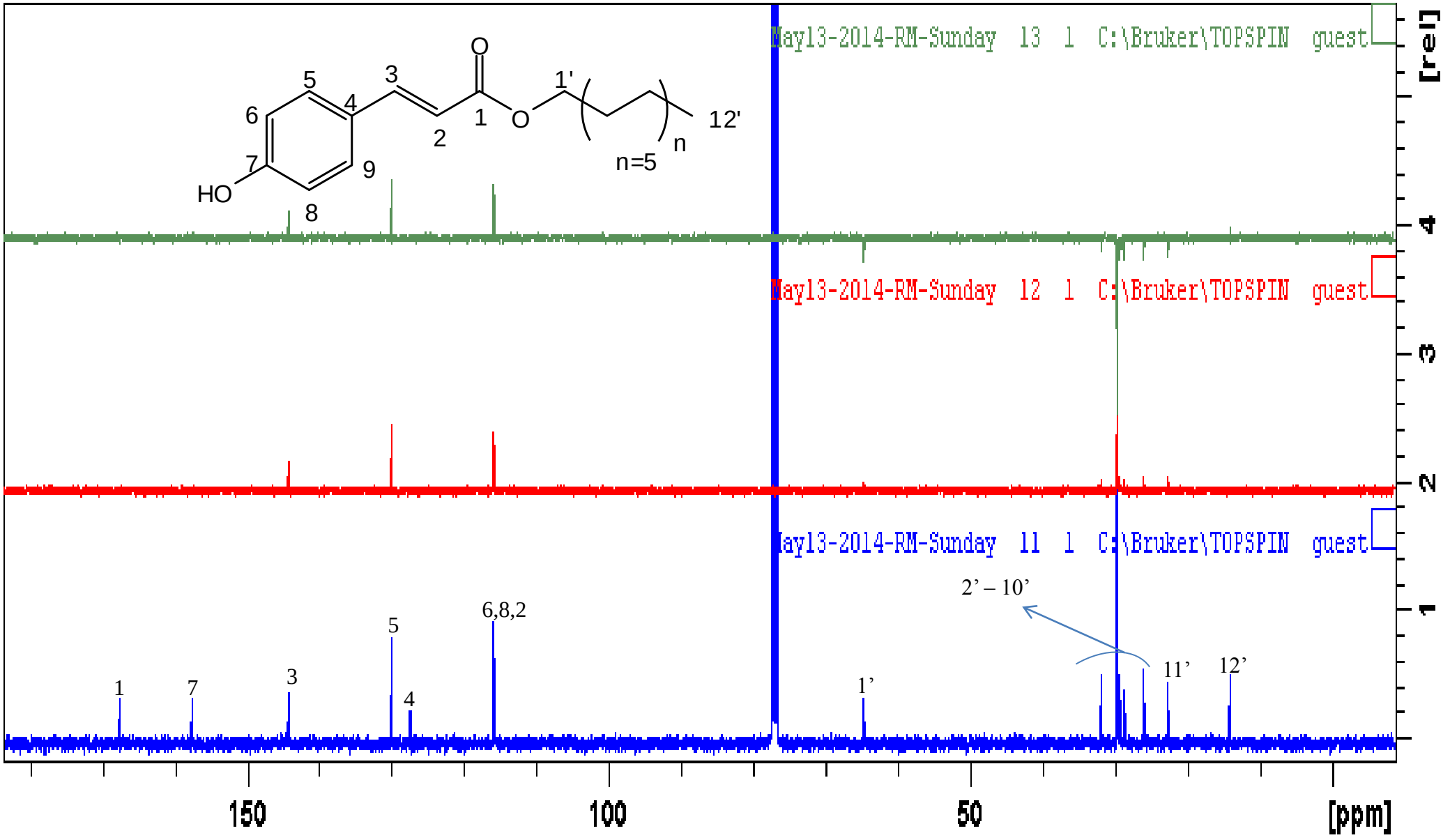
UV spectrum of sesamin (**A4**)



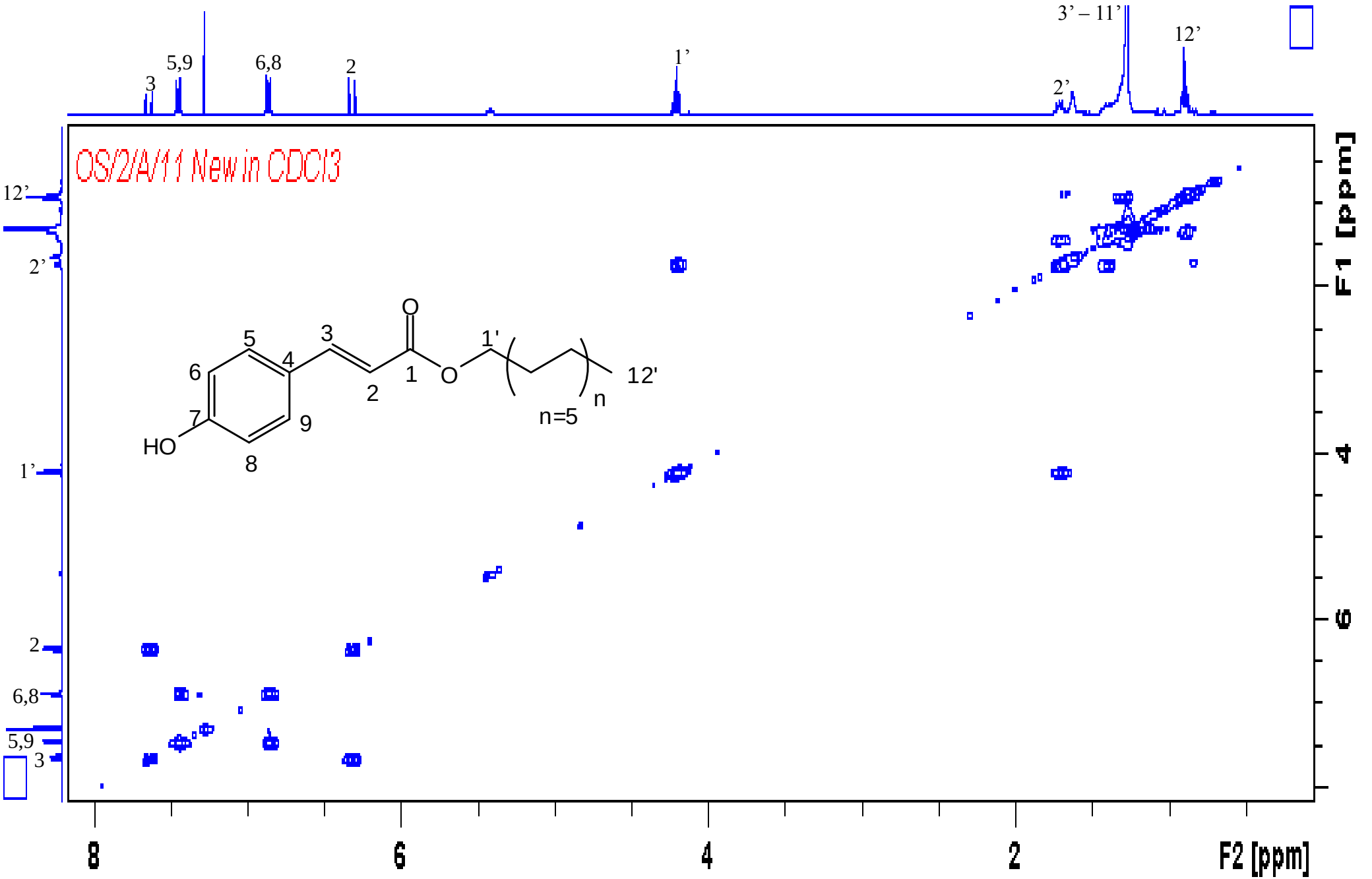
^{1}H NMR spectrum of dodecyl-*trans*-*p*-coumarate (**A5**)



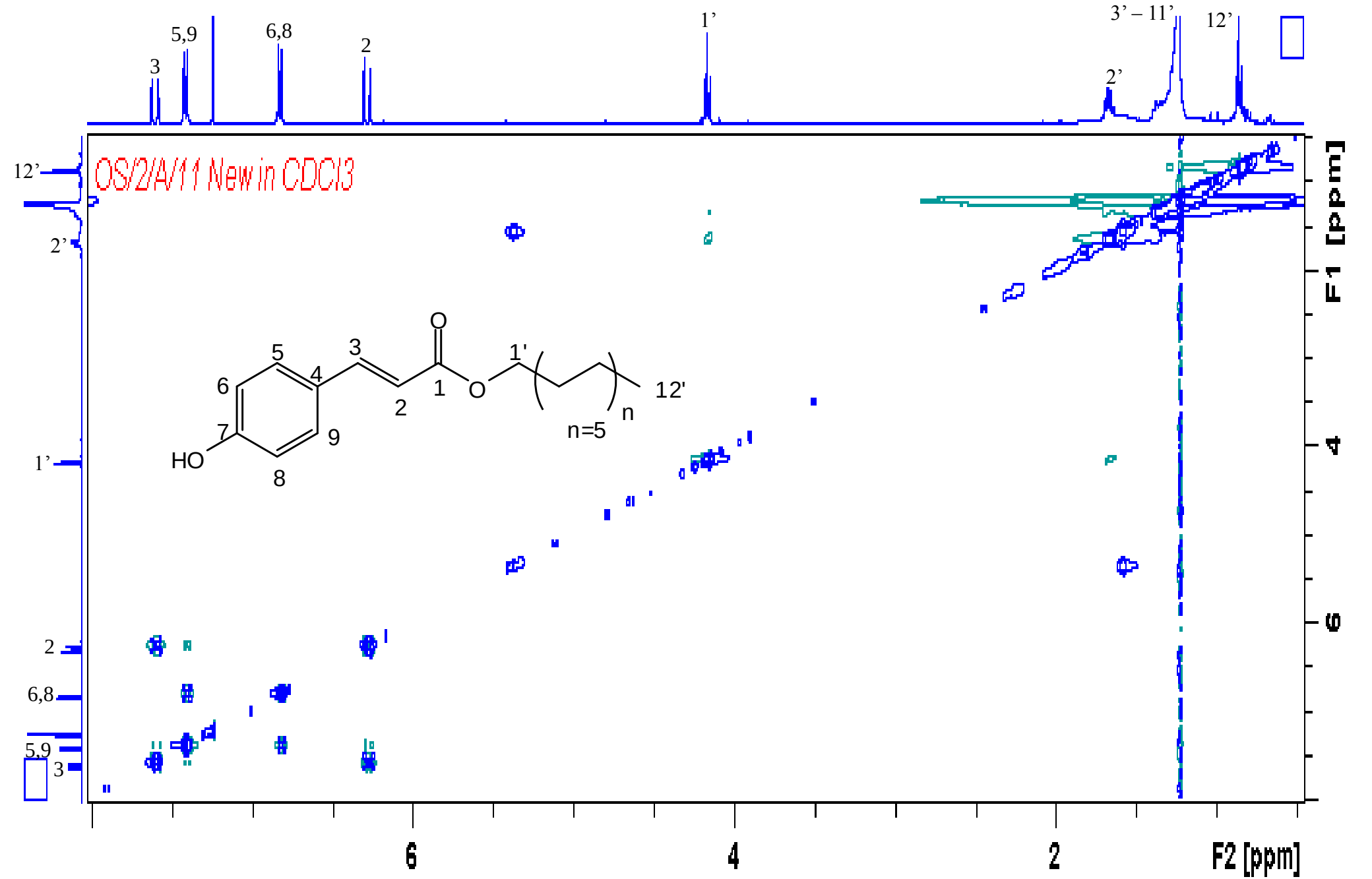
^{13}C NMR spectrum of dodecyl-*trans*-*p*-coumarate (**A5**)



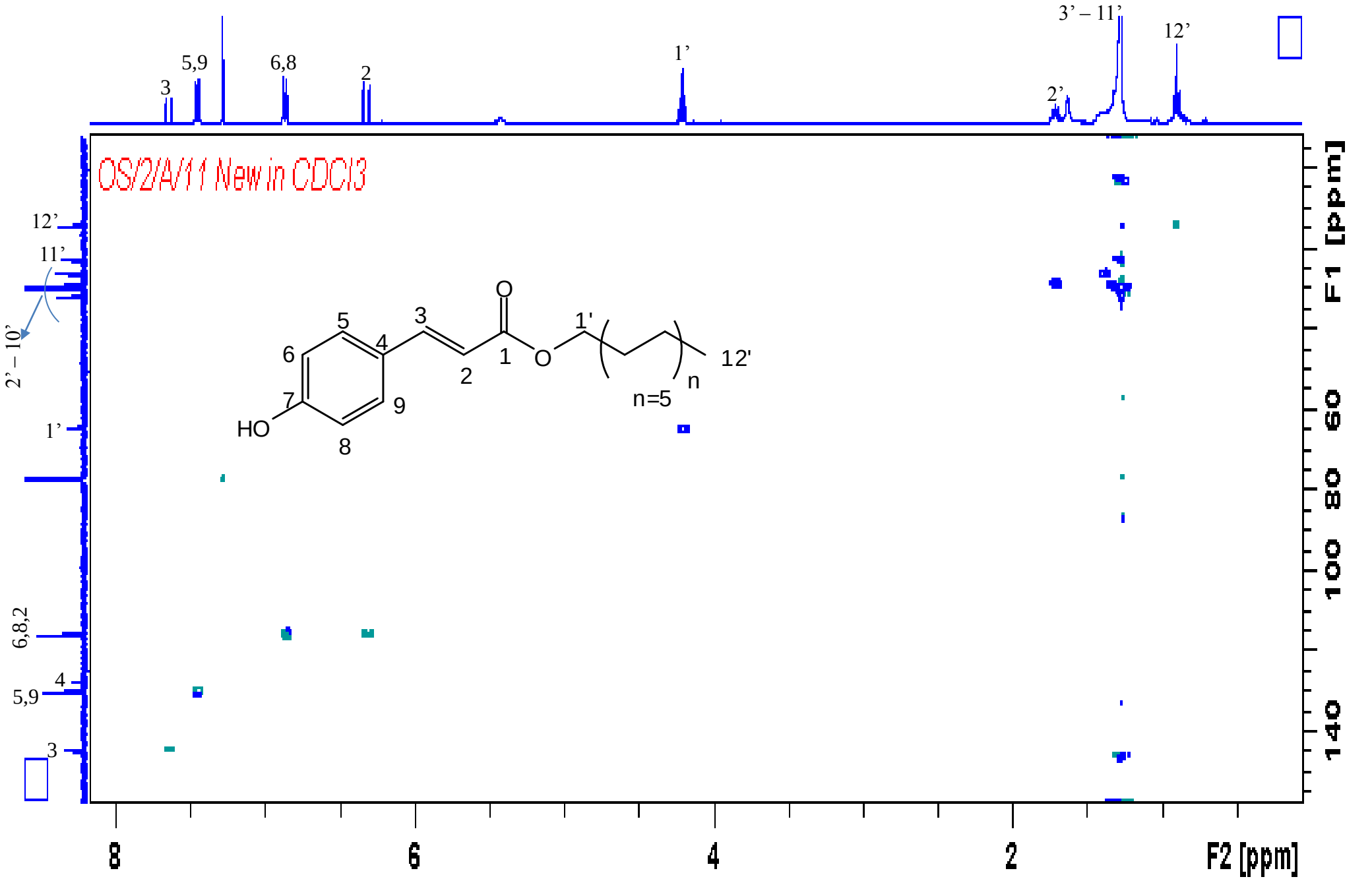
DEPT spectrum of dodecyl-*trans*-*p*-coumarate (**A5**)



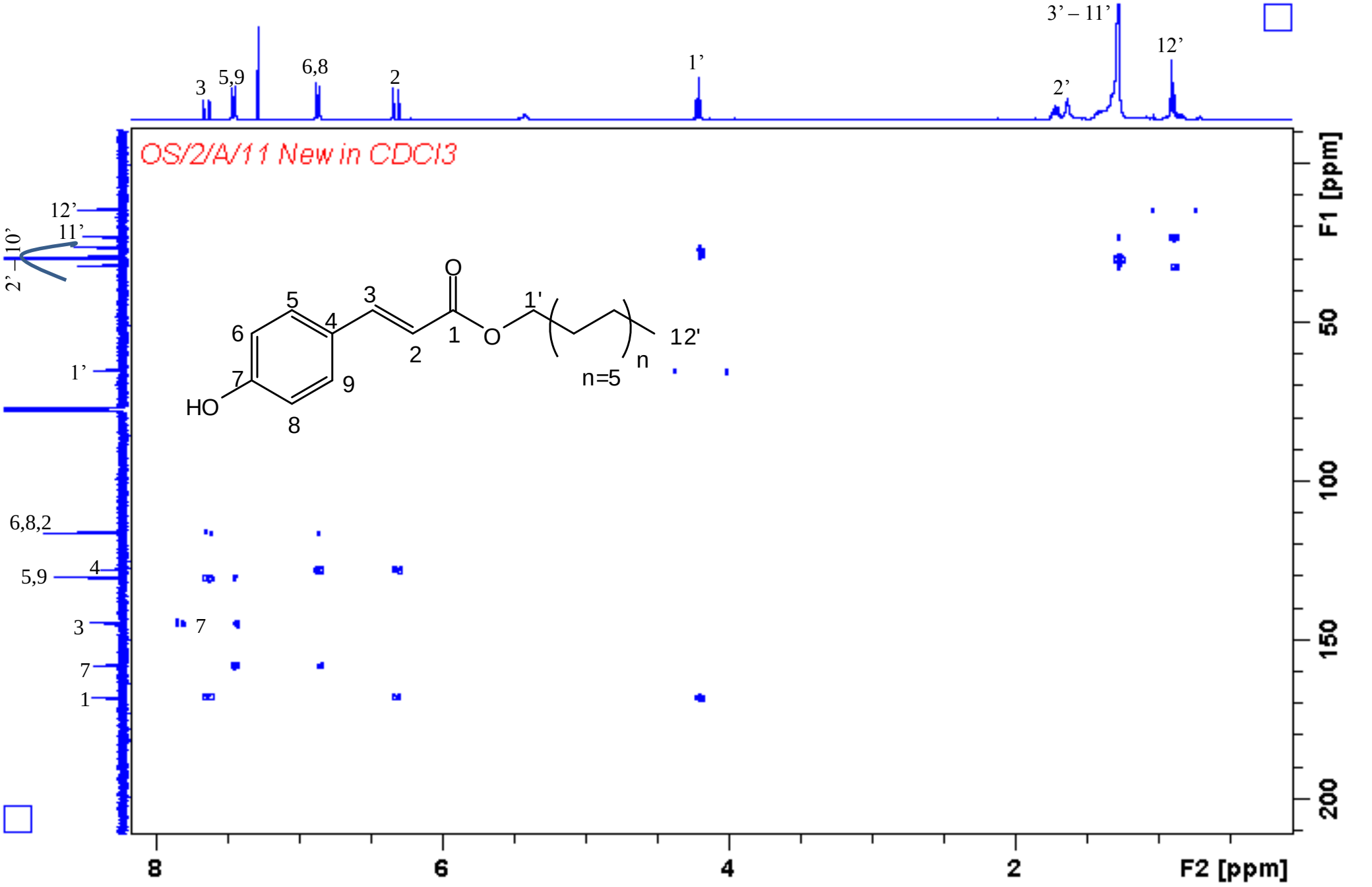
COSY spectrum of dodecyl-*trans*-*p*-coumarate (**A5**)



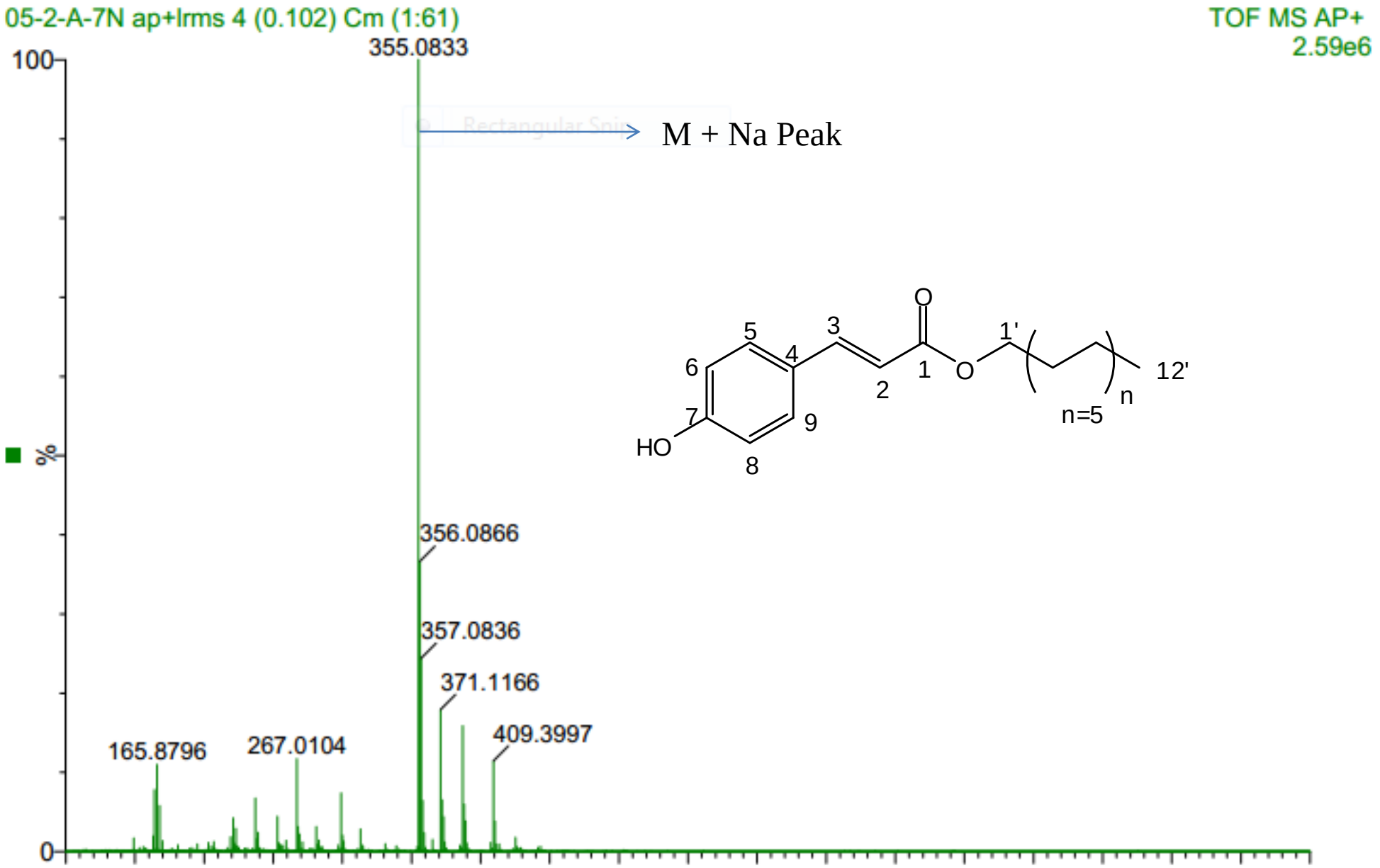
NOESY spectrum of dodecyl-*trans*-*p*-coumarate (**A5**)



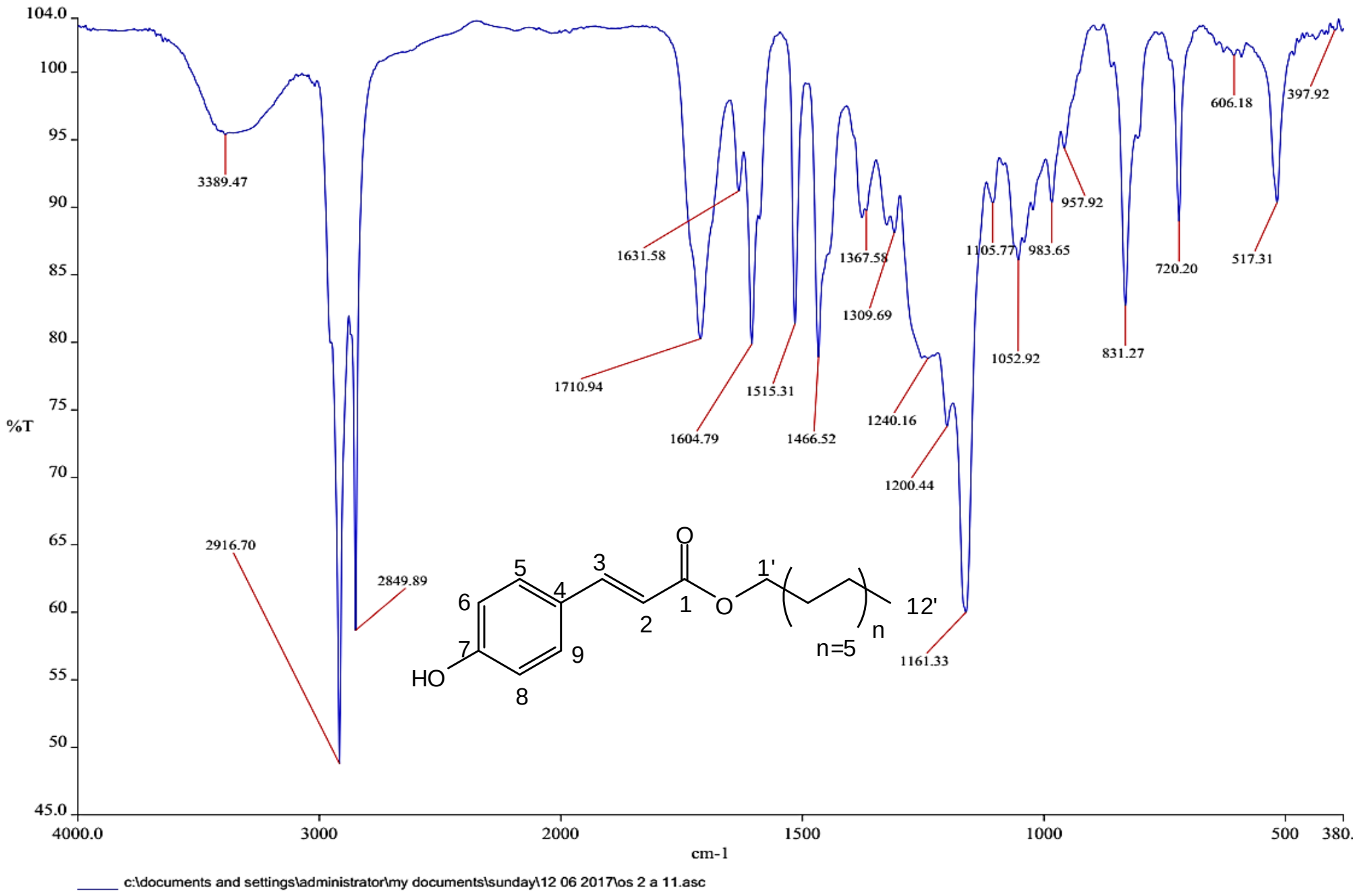
HSQC spectrum of dodecyl-*trans*-*p*-coumarate (**A5**)



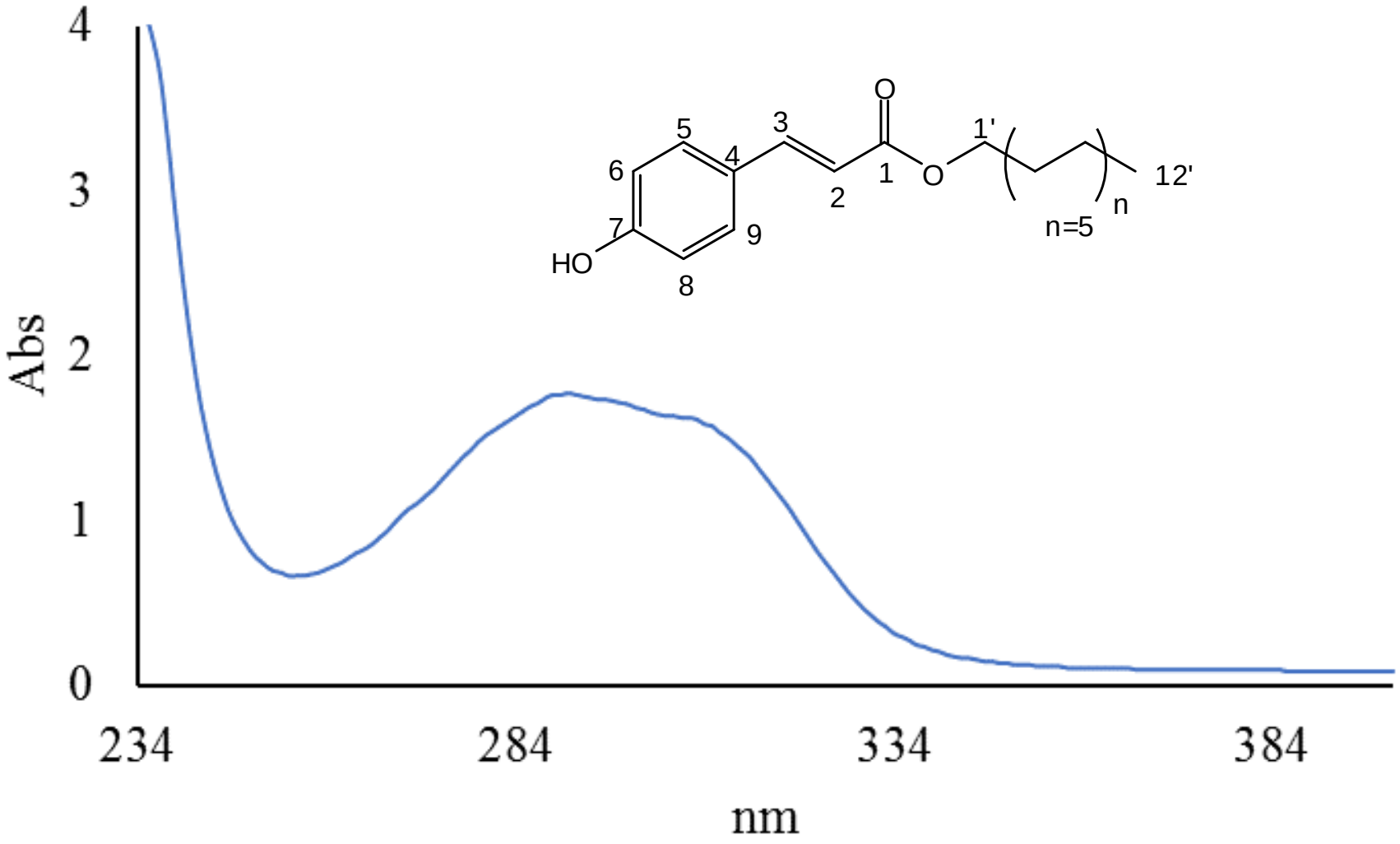
HMBC spectrum of dodecyl-*trans*-*p*-coumarate (**A5**)



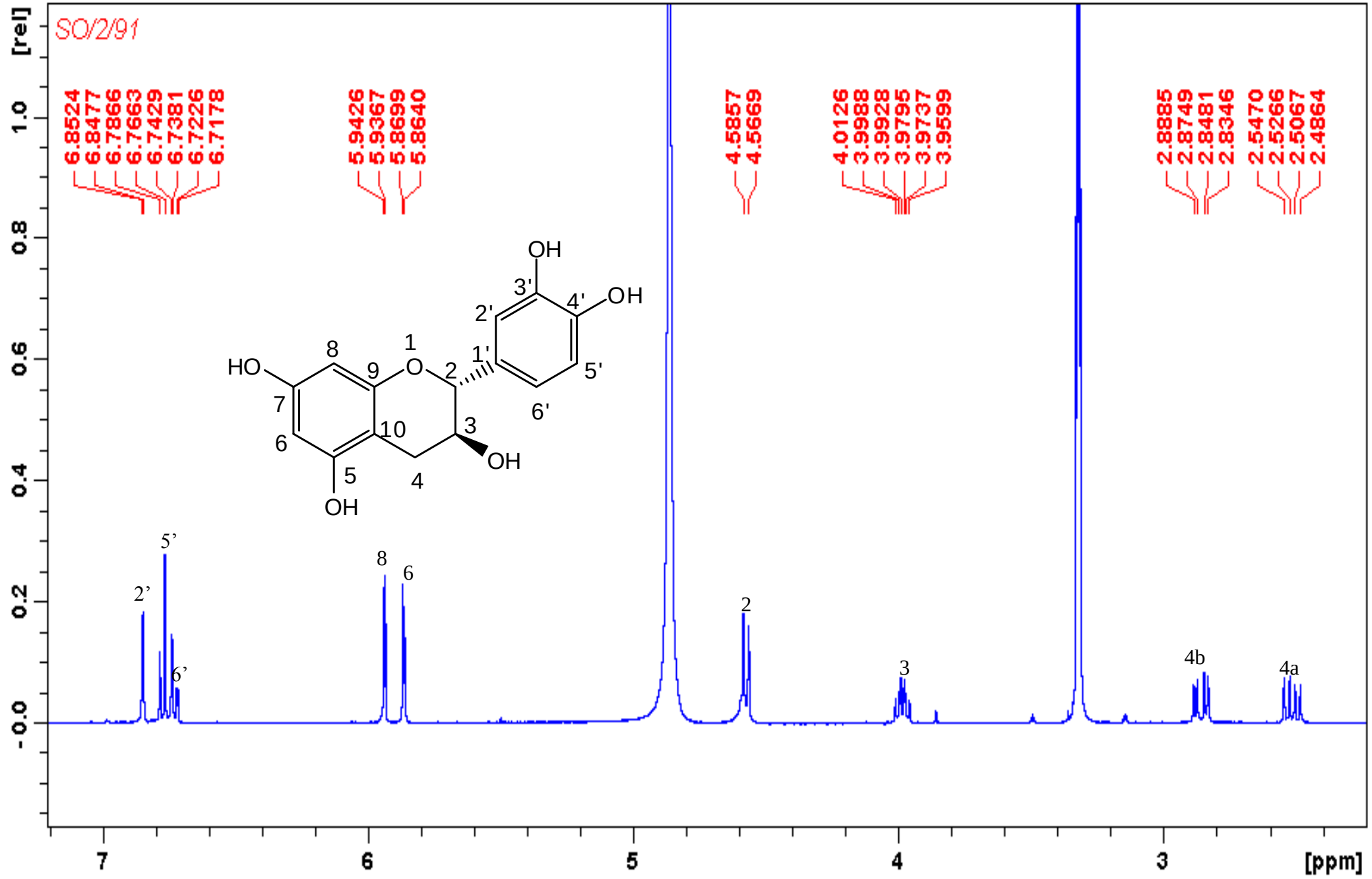
Mass spectrum of dodecyl-*trans*-*p*-coumarate (**A5**)



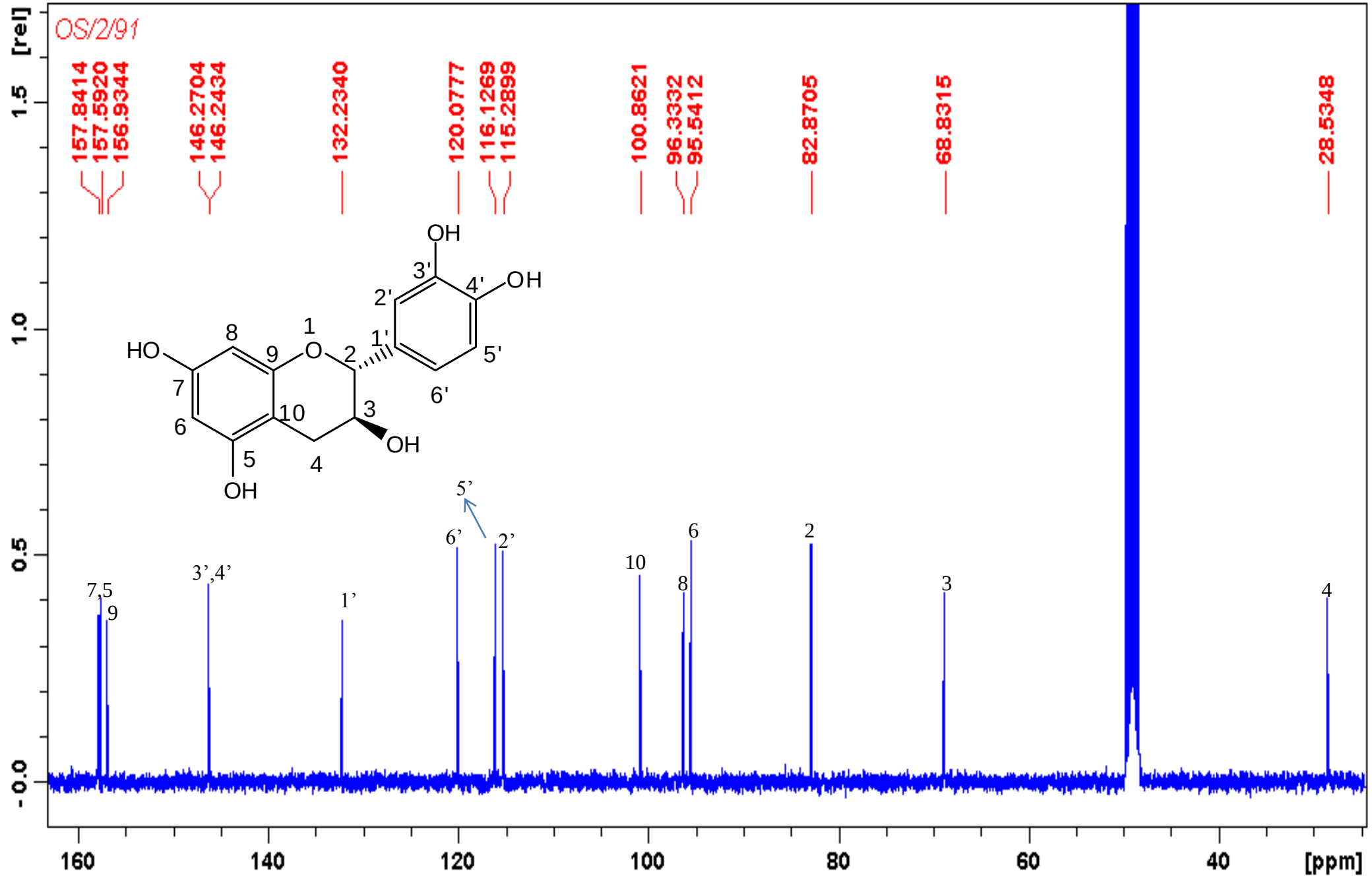
IR spectrum of dodecyl-*trans*-*p*-coumarate (**A5**)



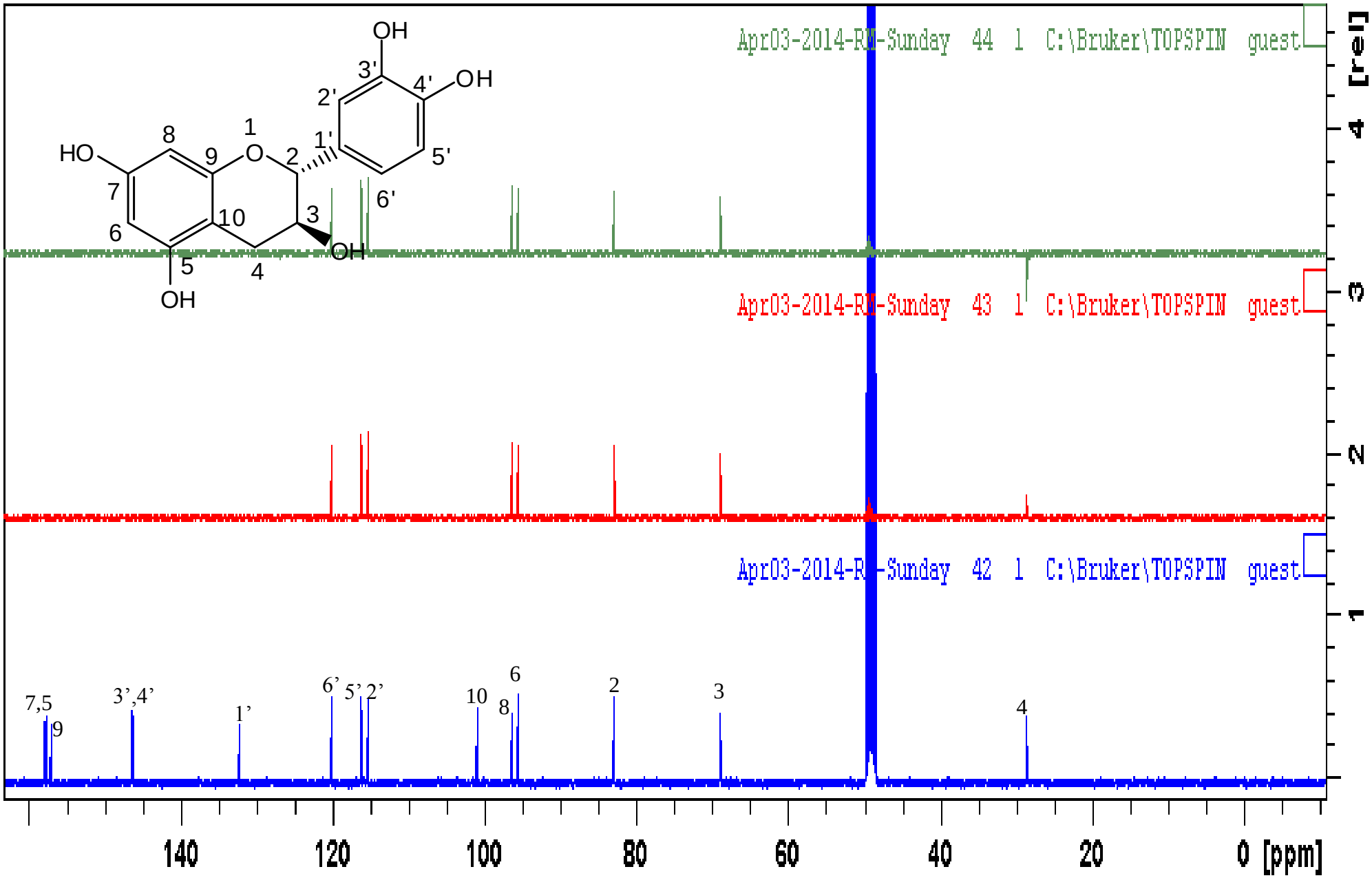
UV spectrum of dodecyl-*trans*-*p*-coumarate (**A5**)



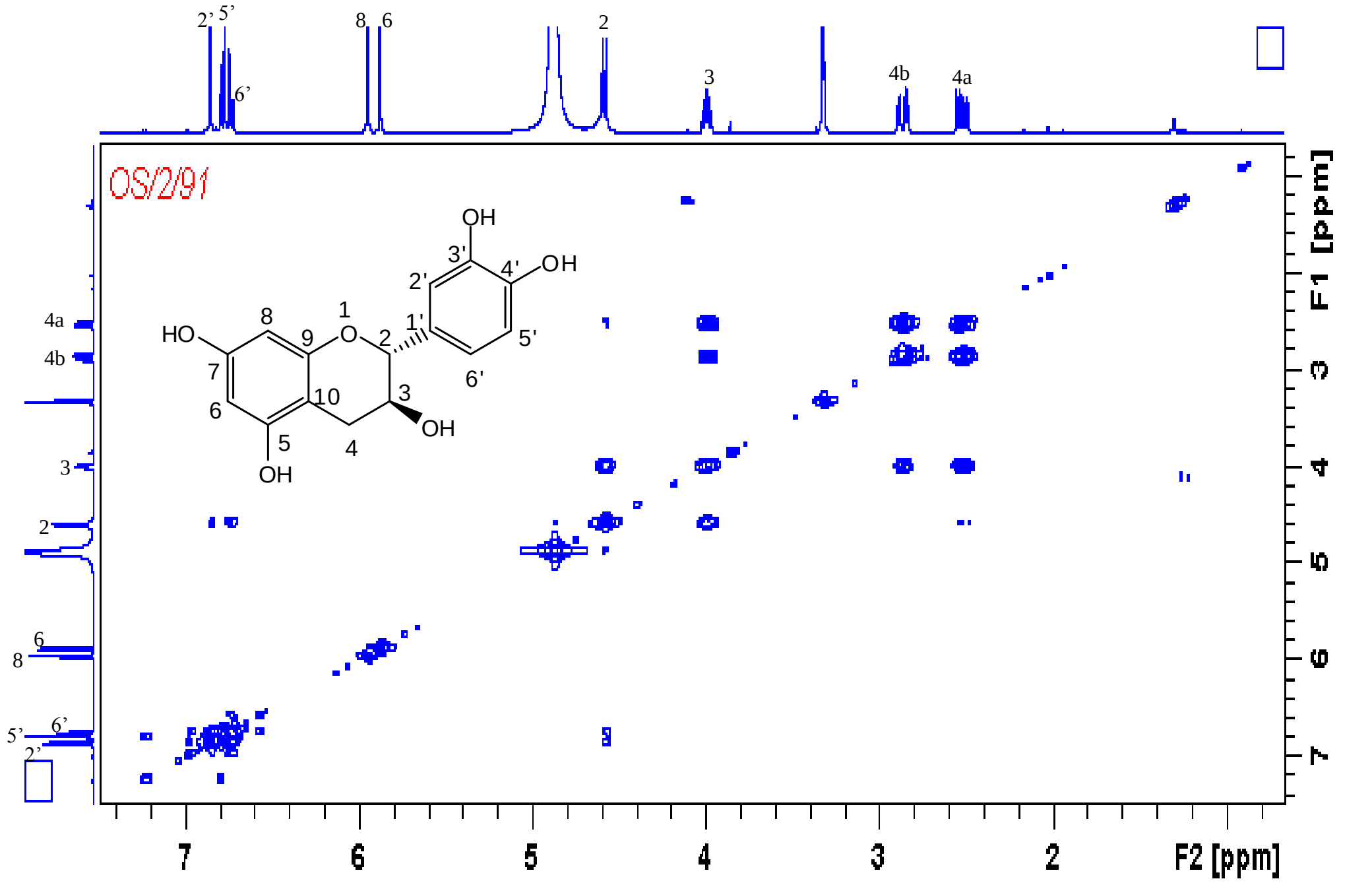
^{1}H NMR spectrum of catechin (**A6**)



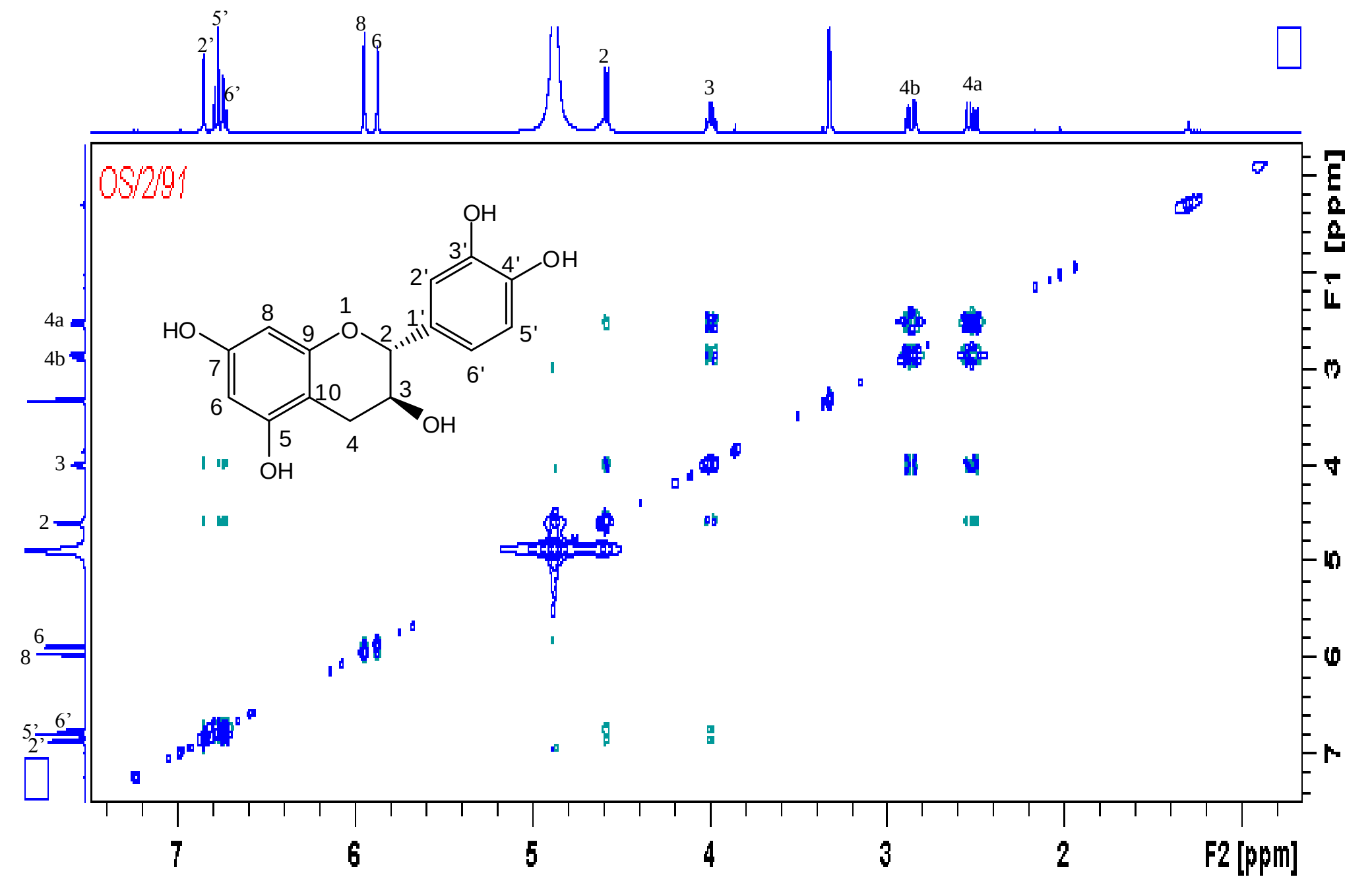
^{13}C NMR spectrum of catechin (**A6**)



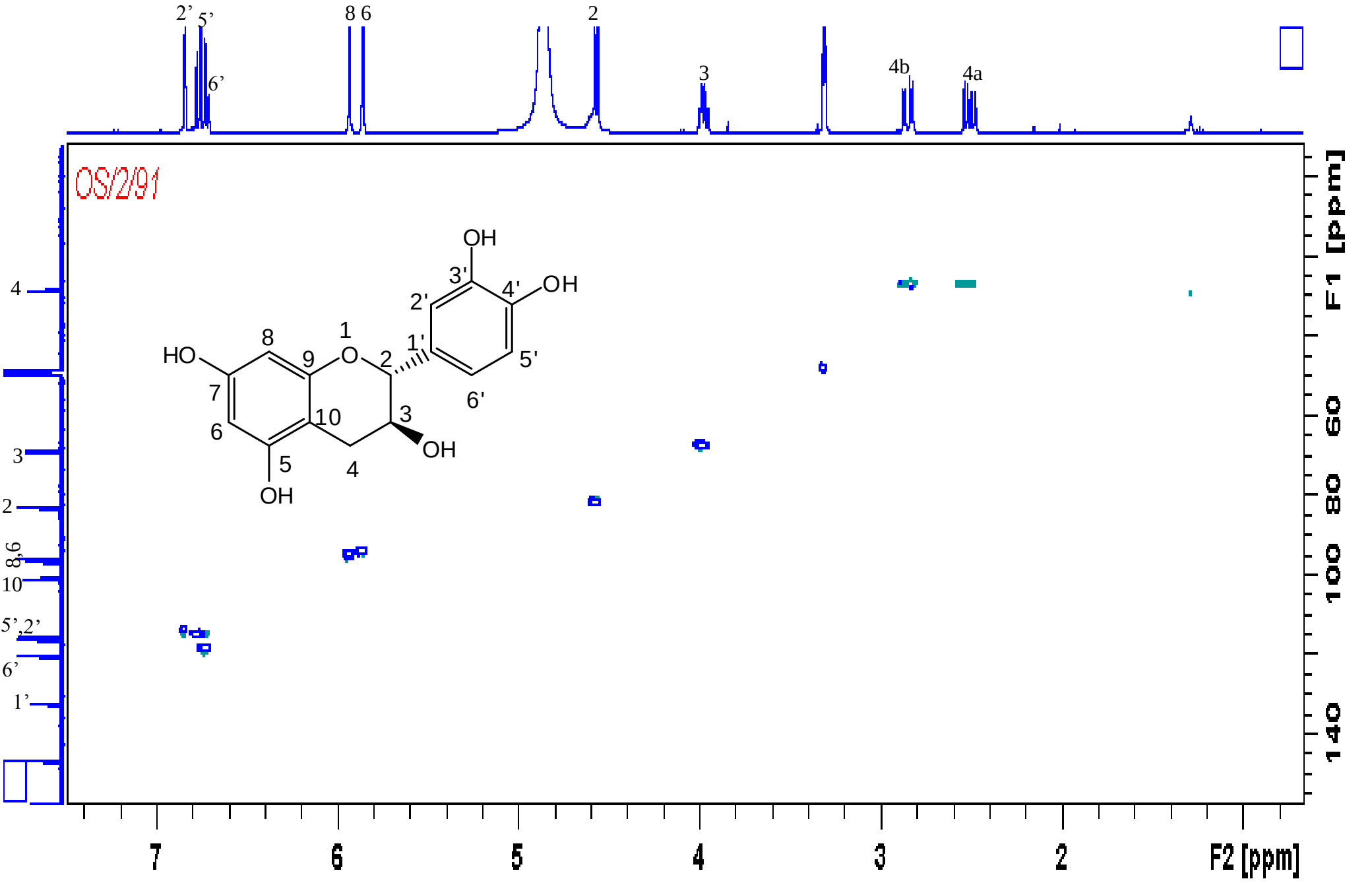
DEPT spectrum of catechin (**A6**)



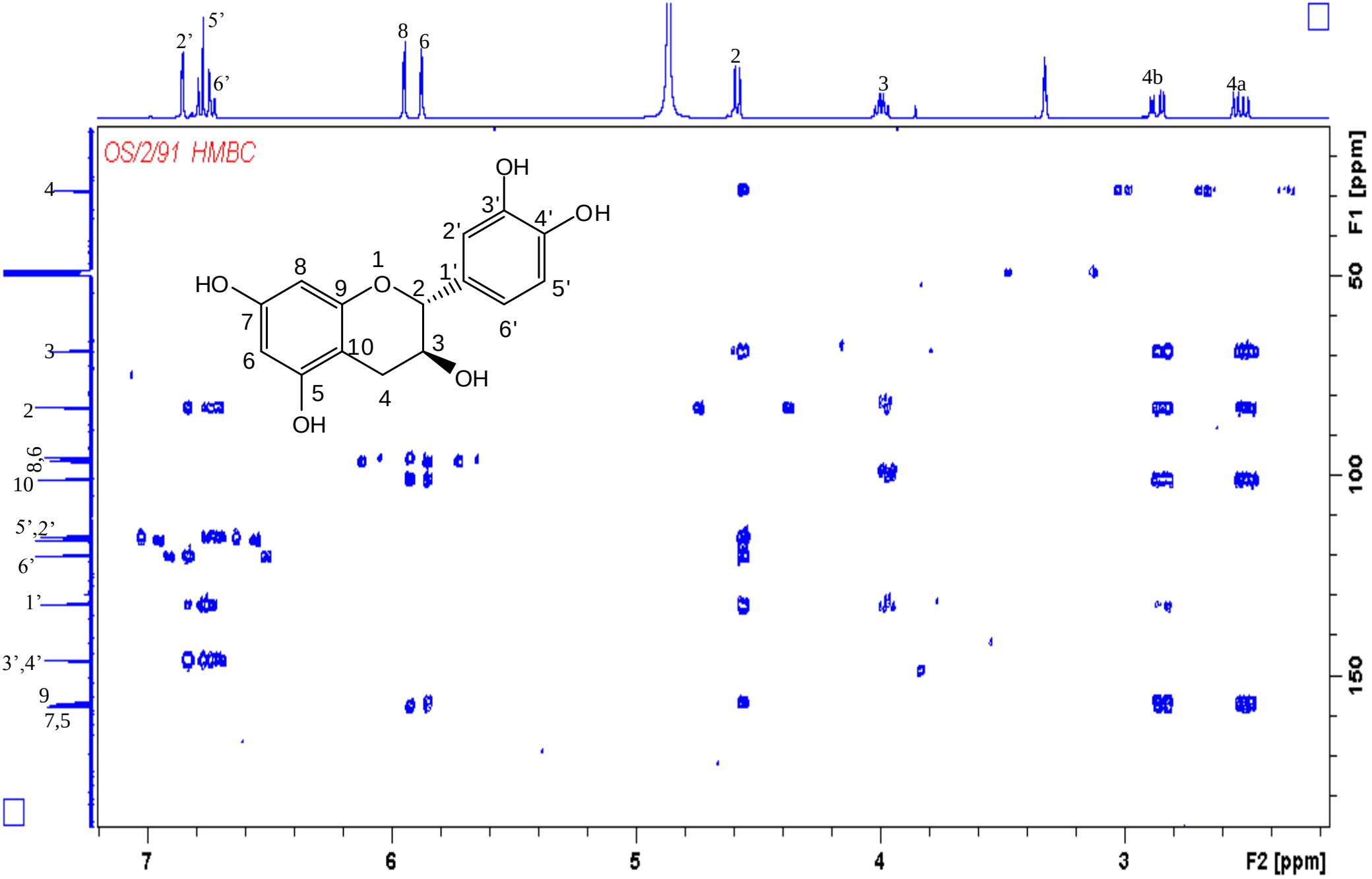
COSY spectrum of catechin (**A6**)



NOESY spectrum of catechin (**A6**)



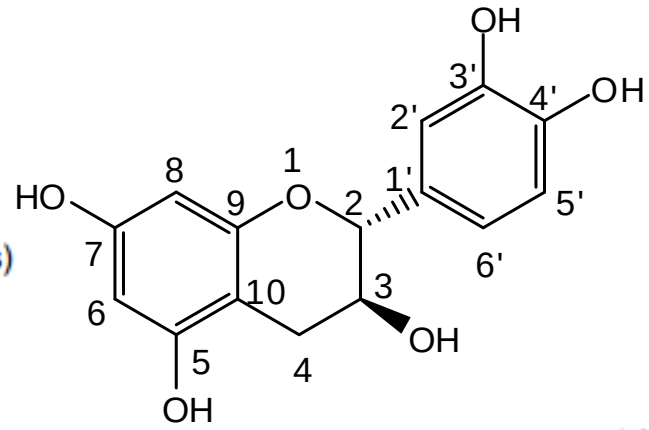
HSQC spectrum of catechin (**A6**)



HMBC spectrum of catechin (**A6**)

Single Mass Analysis

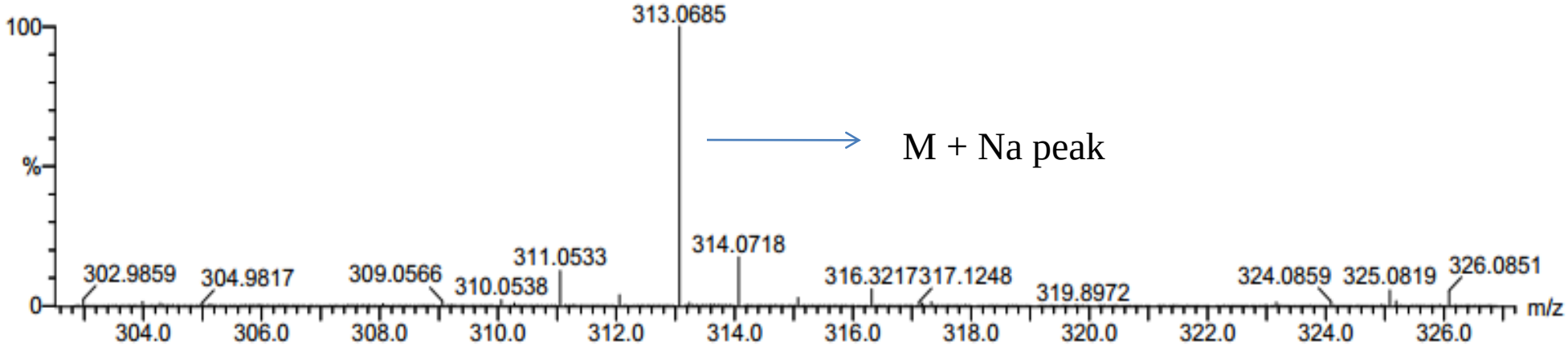
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3



Monoisotopic Mass, Even Electron Ions
6 formula(e) evaluated with 1 results within limits (up to 20 closest results for each mass)
Elements Used:
C: 10-15 H: 10-15 O: 5-10 Na: 0-1

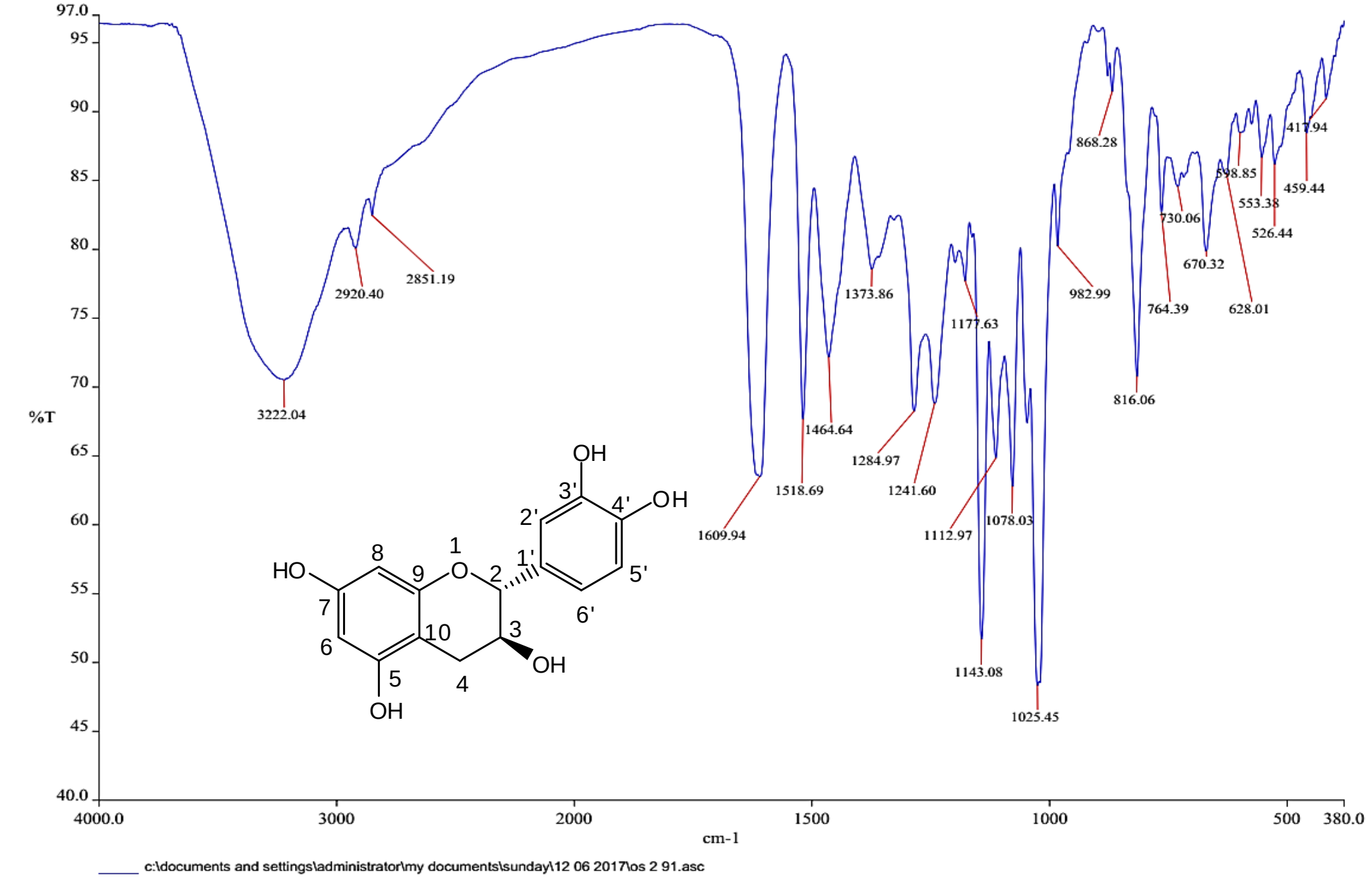
02-2-91 28 (0.911) Cm (1:61)
TOF MS ES+

1.61e+005

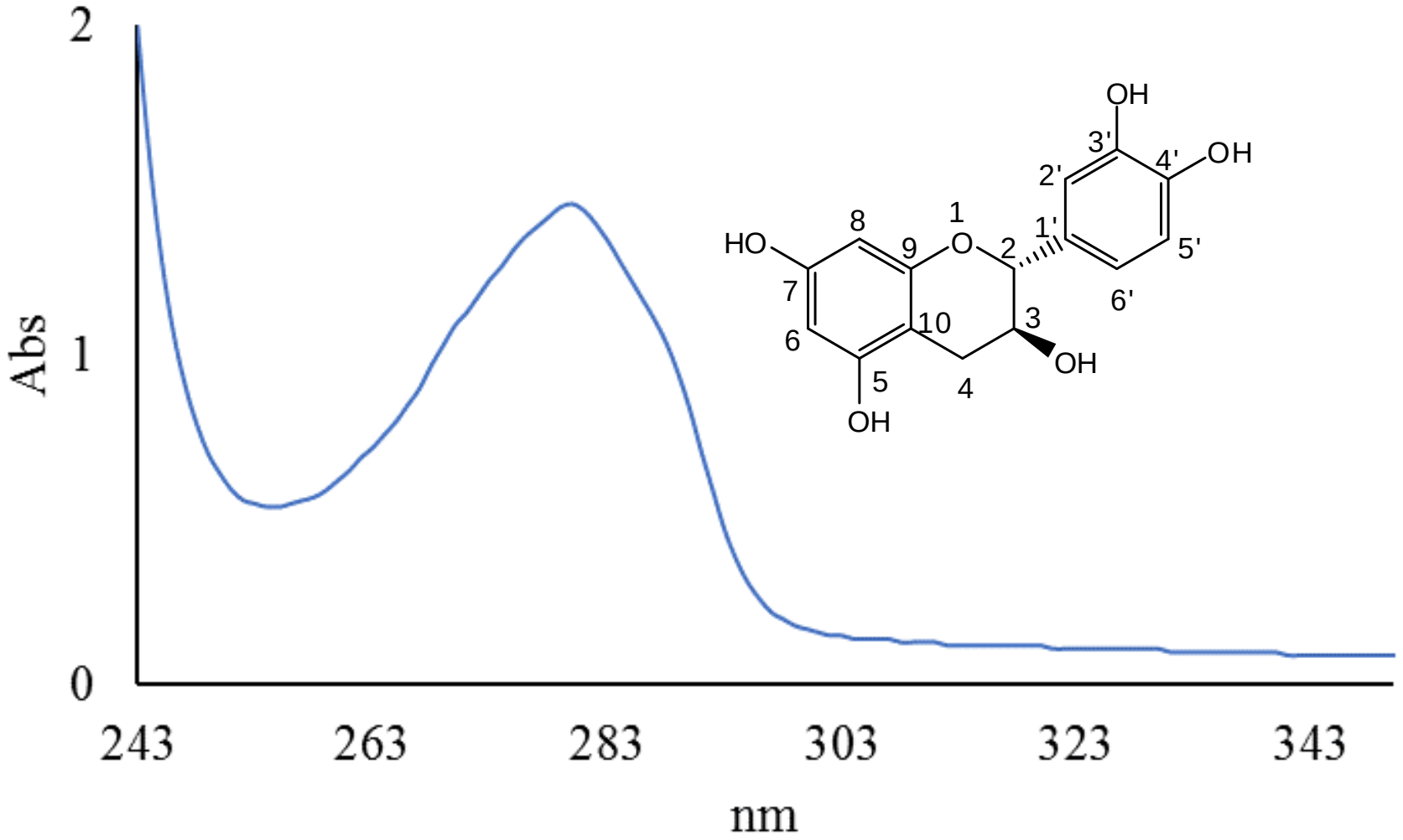


Minimum:				-1.5			
Maximum:		5.0	5.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
313.0685	313.0688	-0.3	-1.0	8.5	617.3	0.0	C15 H14 O6 Na

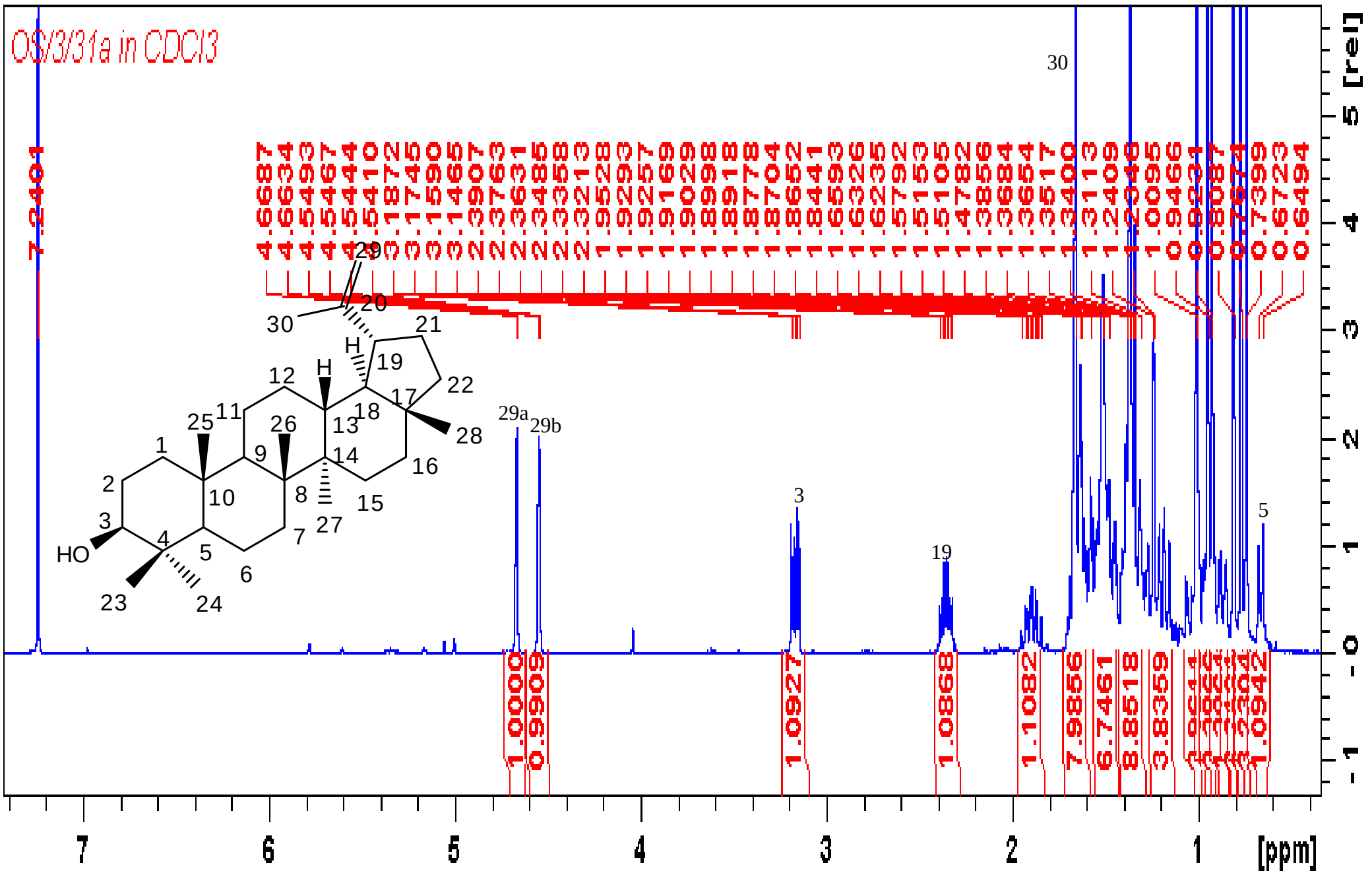
Mass spectrum of catechin (**A6**)



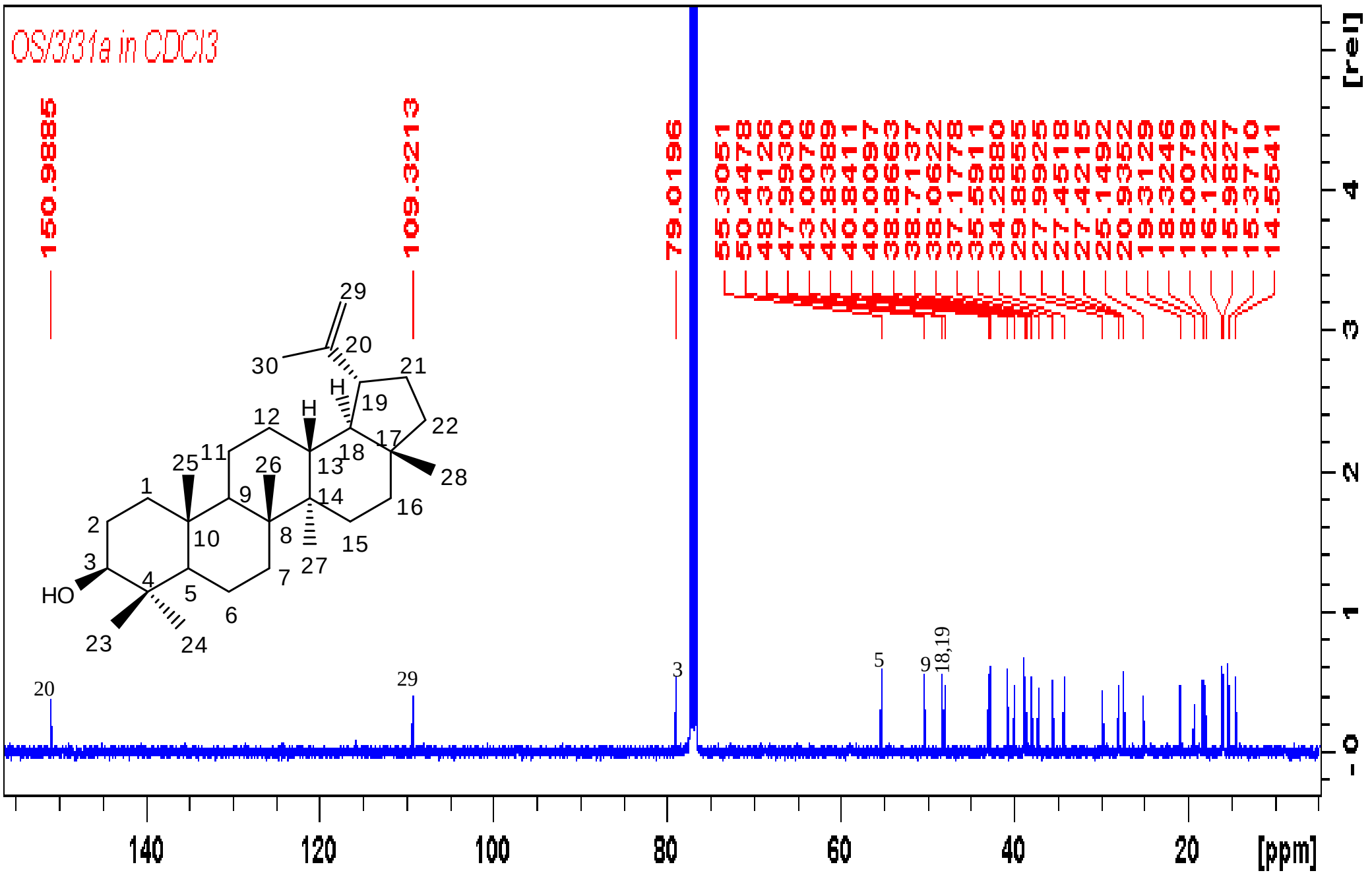
IR spectrum of catechin (**A6**)



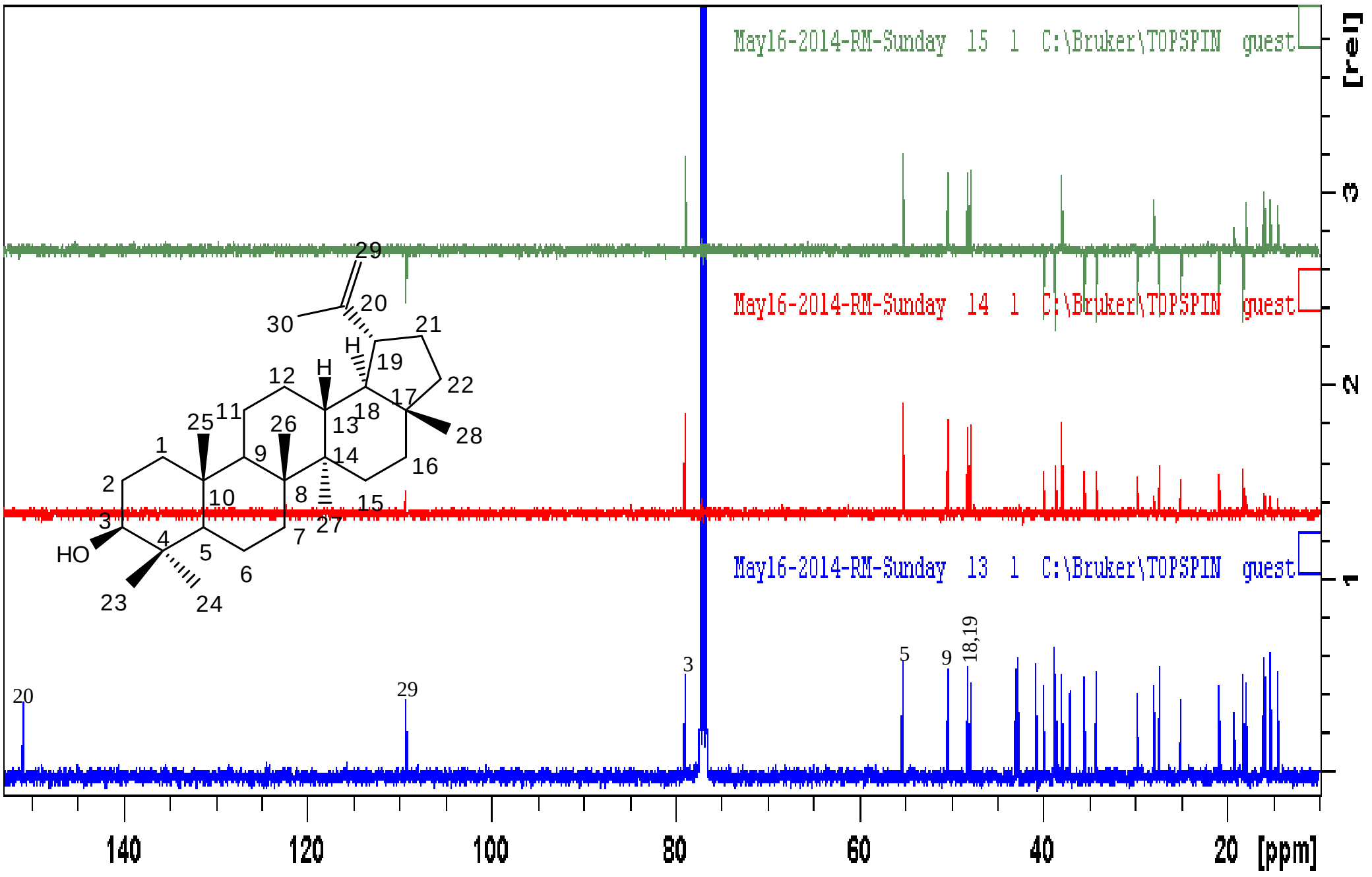
UV spectrum of catechin (**A6**)



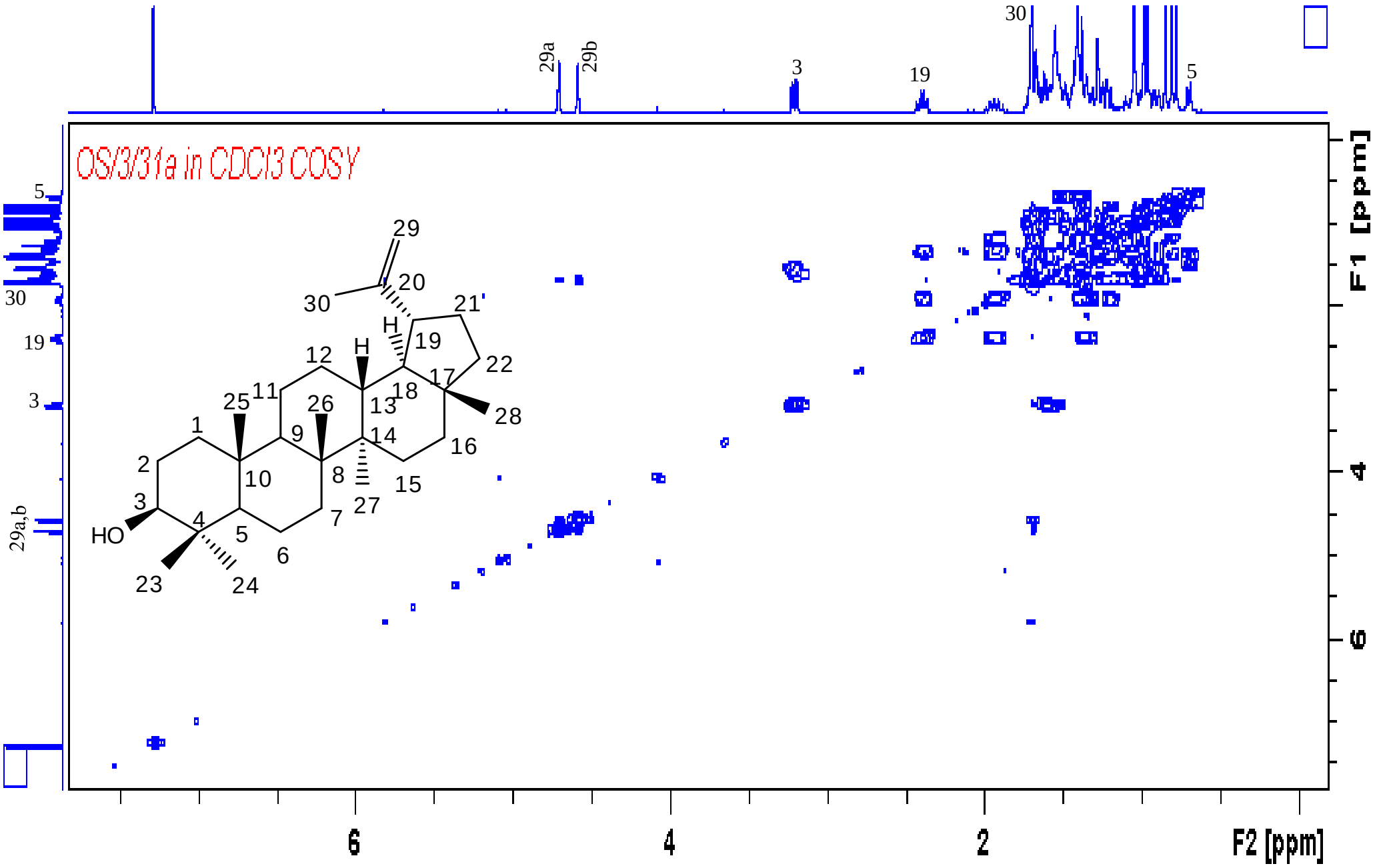
^{1}H NMR spectrum of lupeol (**A7**)



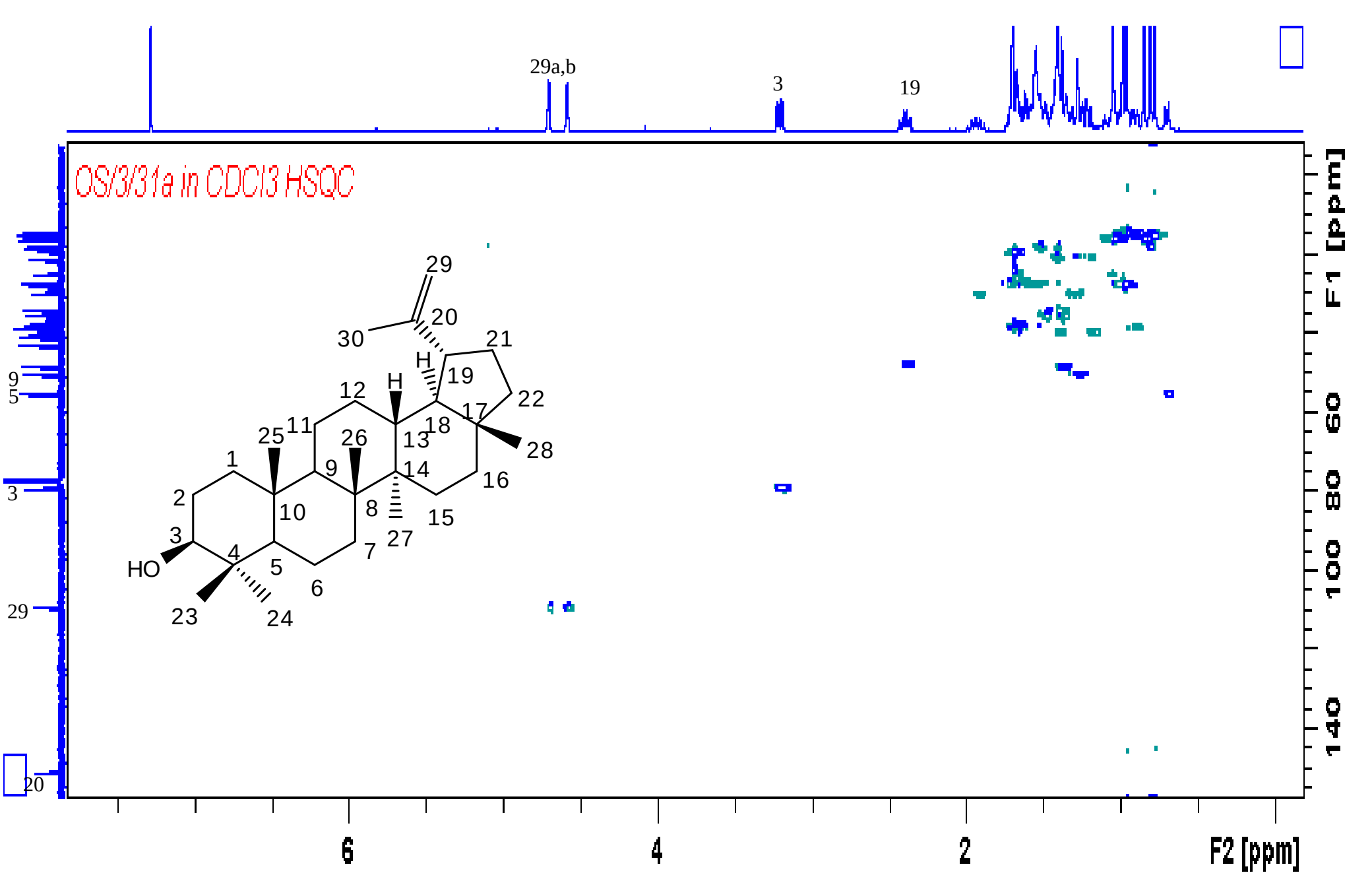
^{13}C NMR spectrum of lupeol (**A7**)



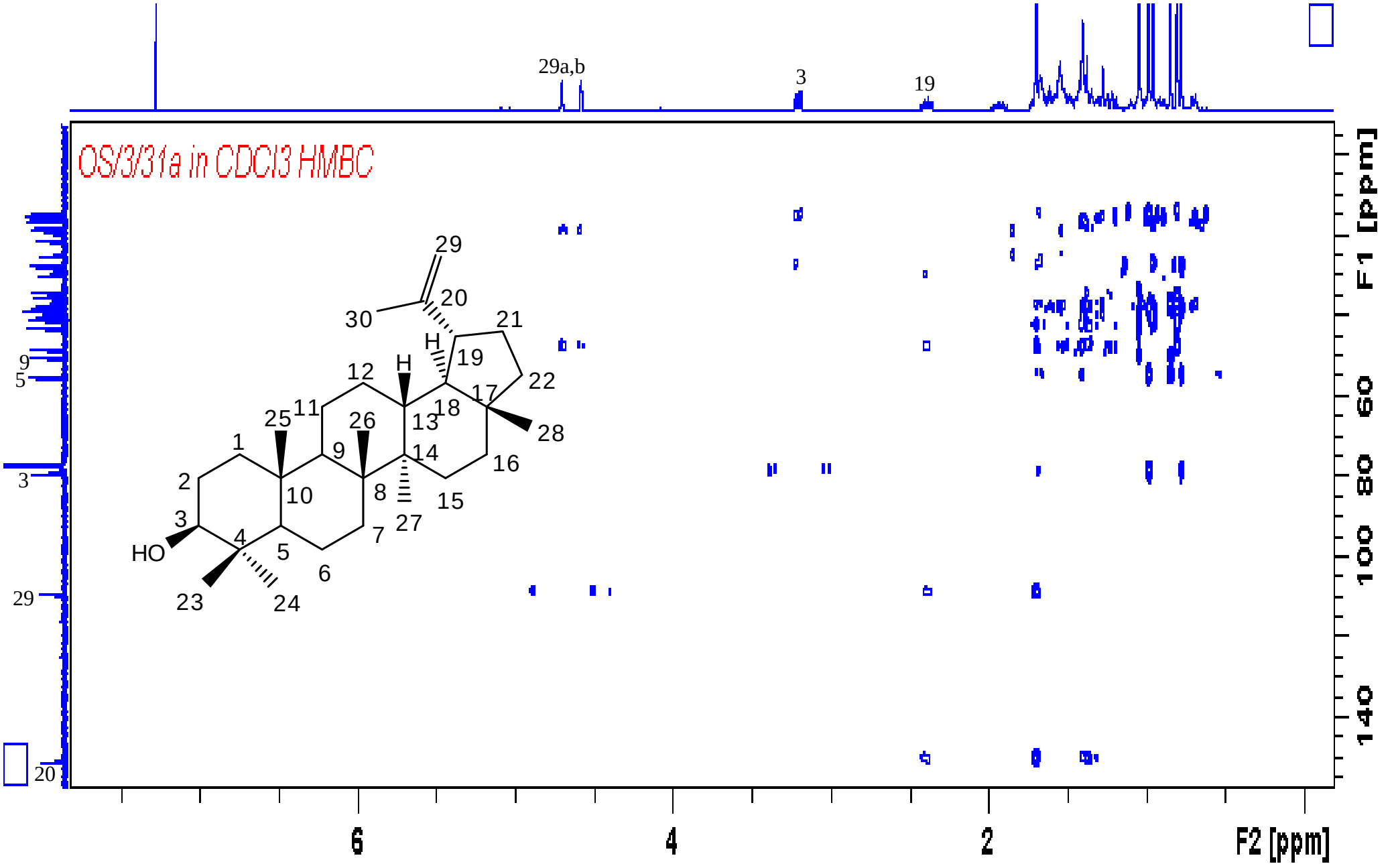
DEPT spectrum of lupeol (**A7**)



COSY spectrum of lupeol (**A7**)



HSQC spectrum of lupeol (**A7**)



HMBC spectrum of lupeol (**A7**)

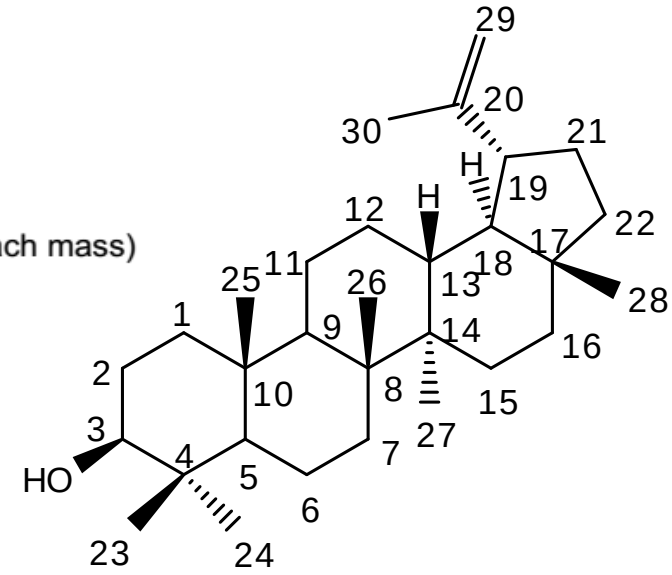
Elemental Composition Report

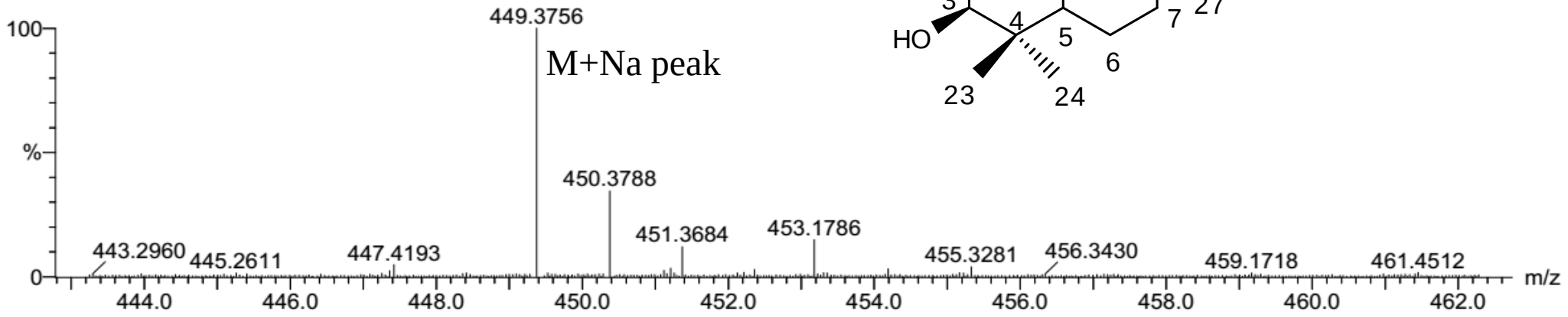
Single Mass Analysis

Tolerance = 5.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
8 formula(e) evaluated with 1 results within limits (up to 20 closest results for each mass)
Elements Used:
C: 20-35 H: 40-55 O: 0-5 Na: 1-1

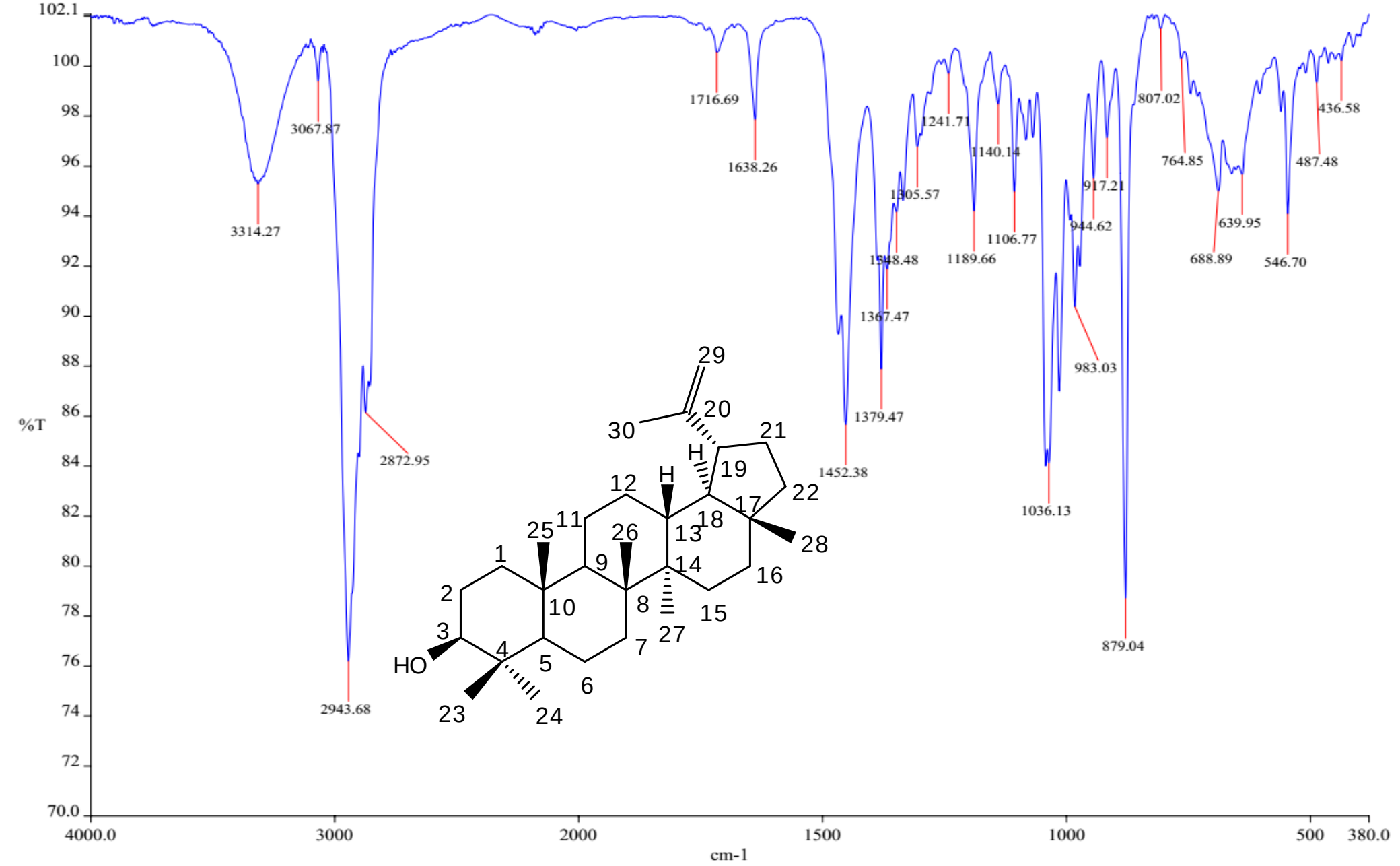
05-8-17-18 31 (0.512) Cm (1:60)
TOF MS ES+



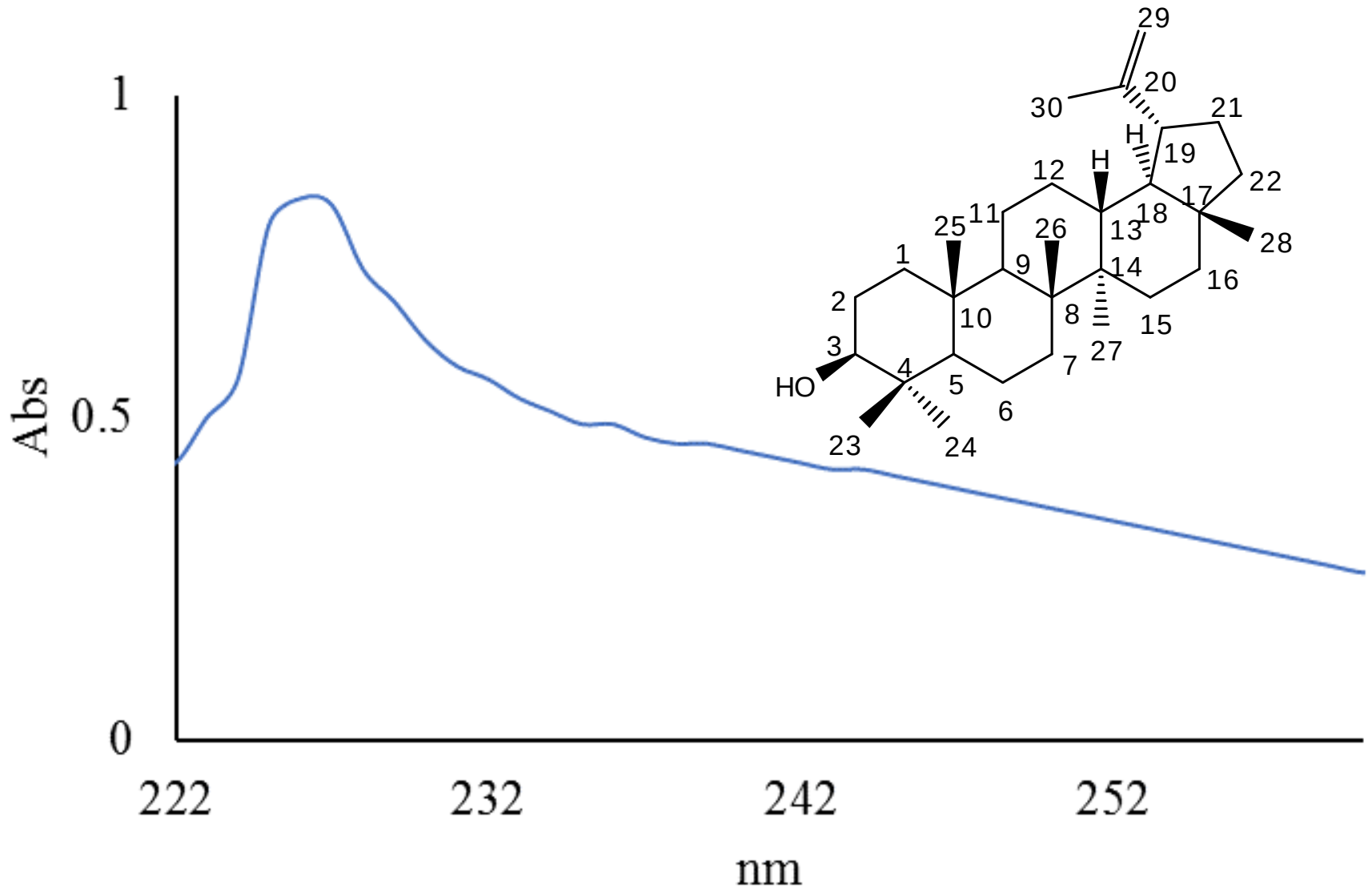


Minimum:				-1.5			
Maximum:		5.0	5.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
449.3756	449.3759	-0.3	-0.7	5.5	462.3	0.0	C30 H50 O Na

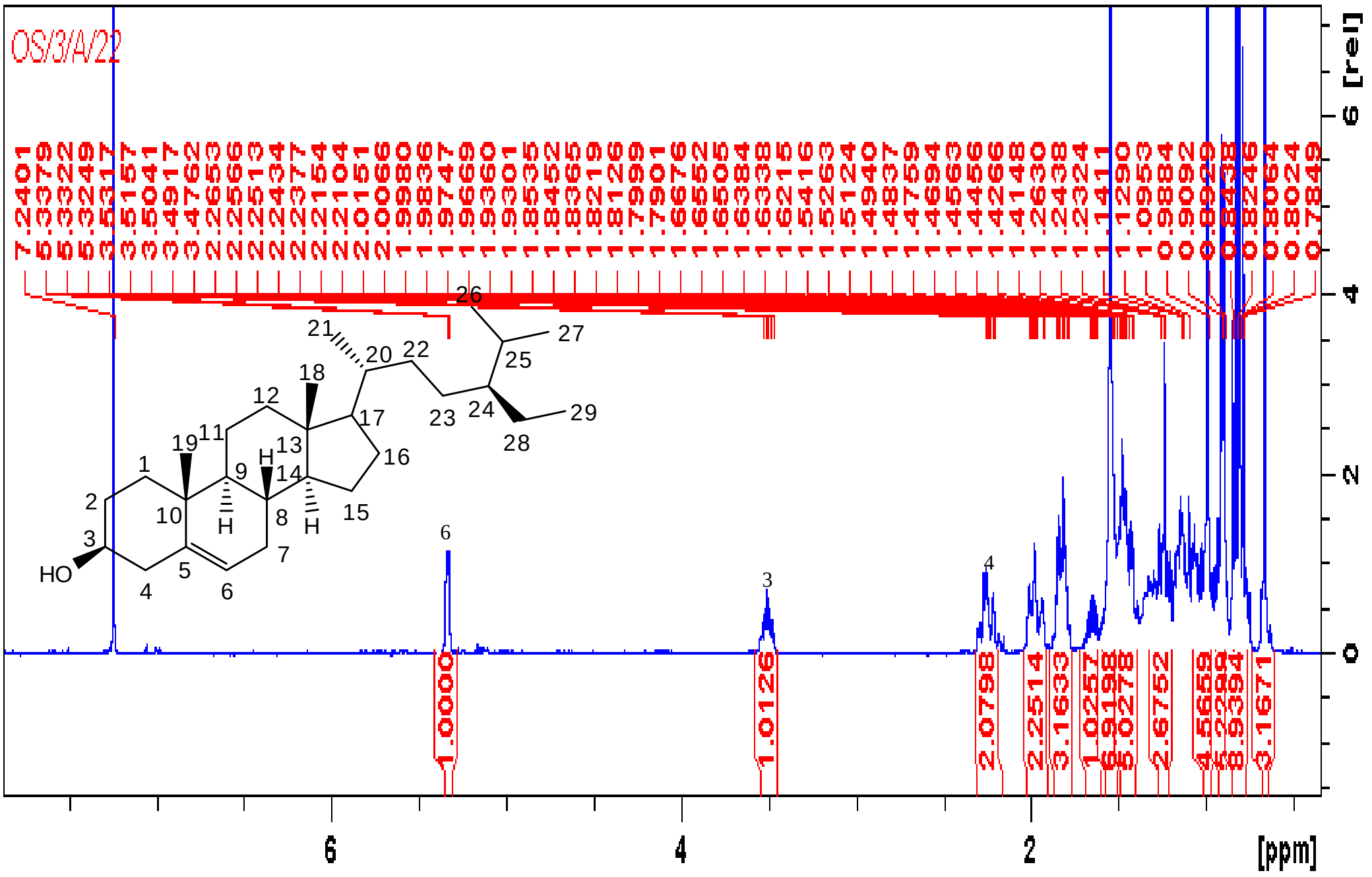
Mass spectrum of lupeol (**A7**)



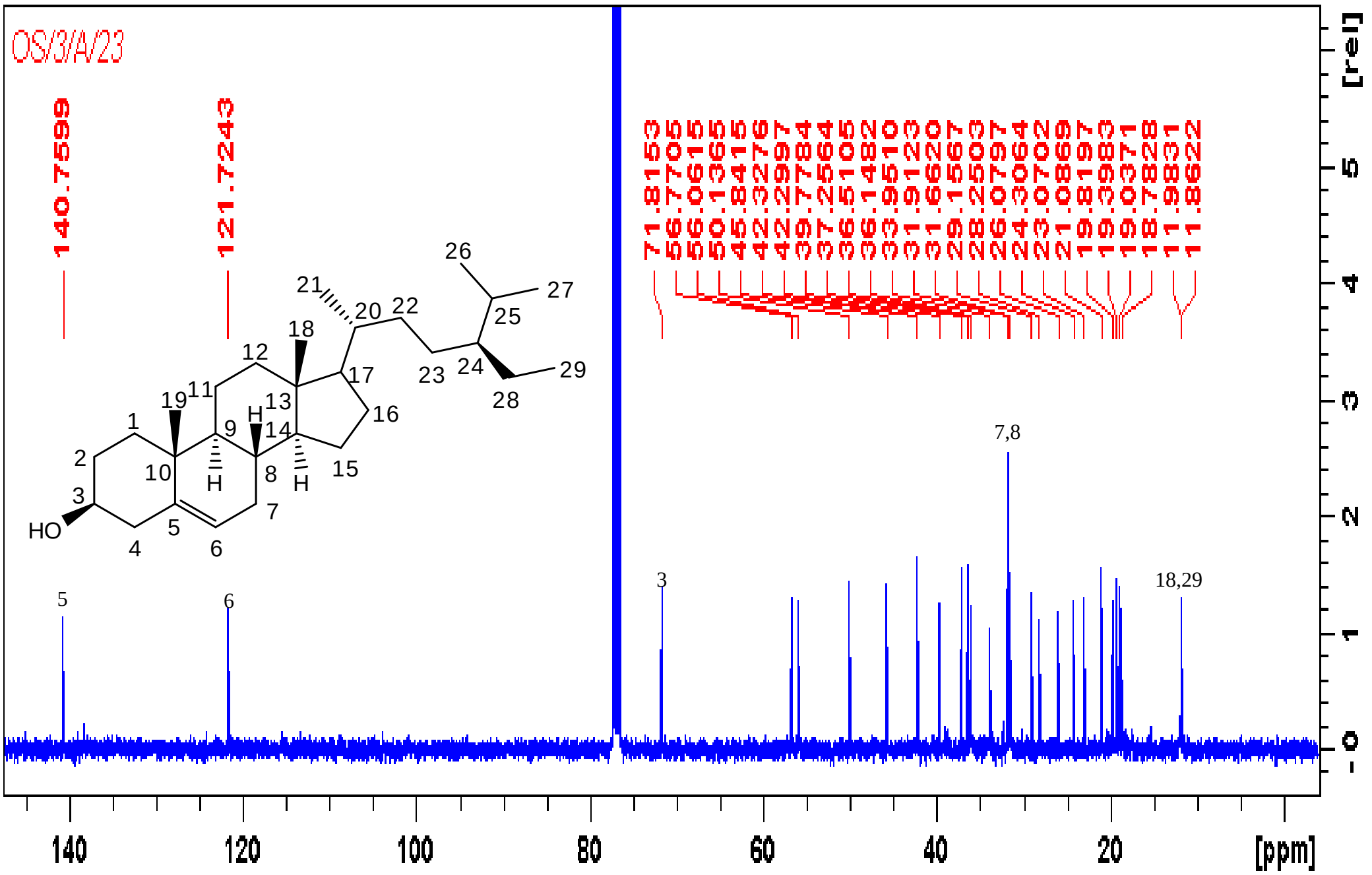
IR spectrum of lupeol (**A7**)



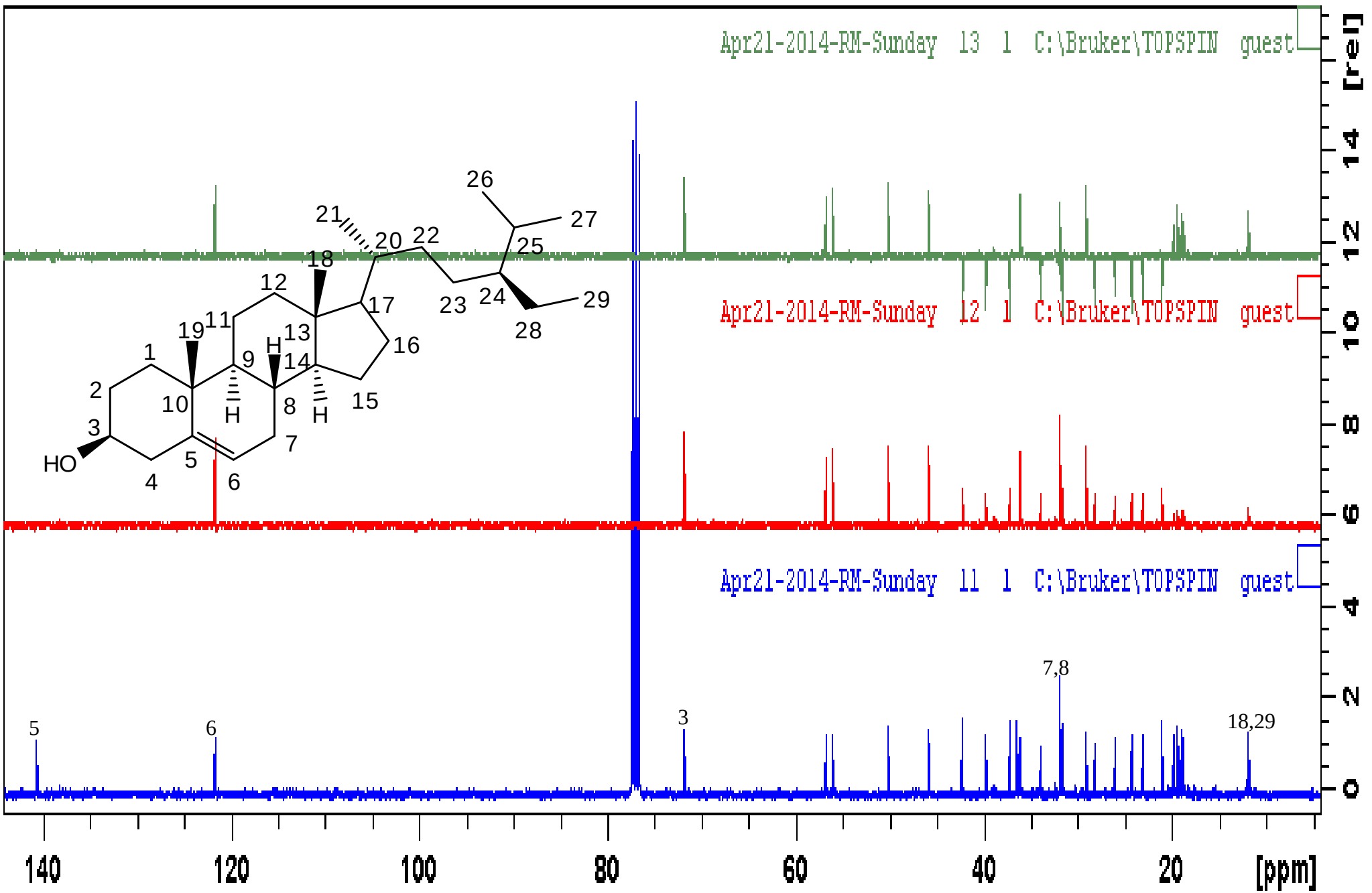
UV spectrum of lupeol (**A7**)



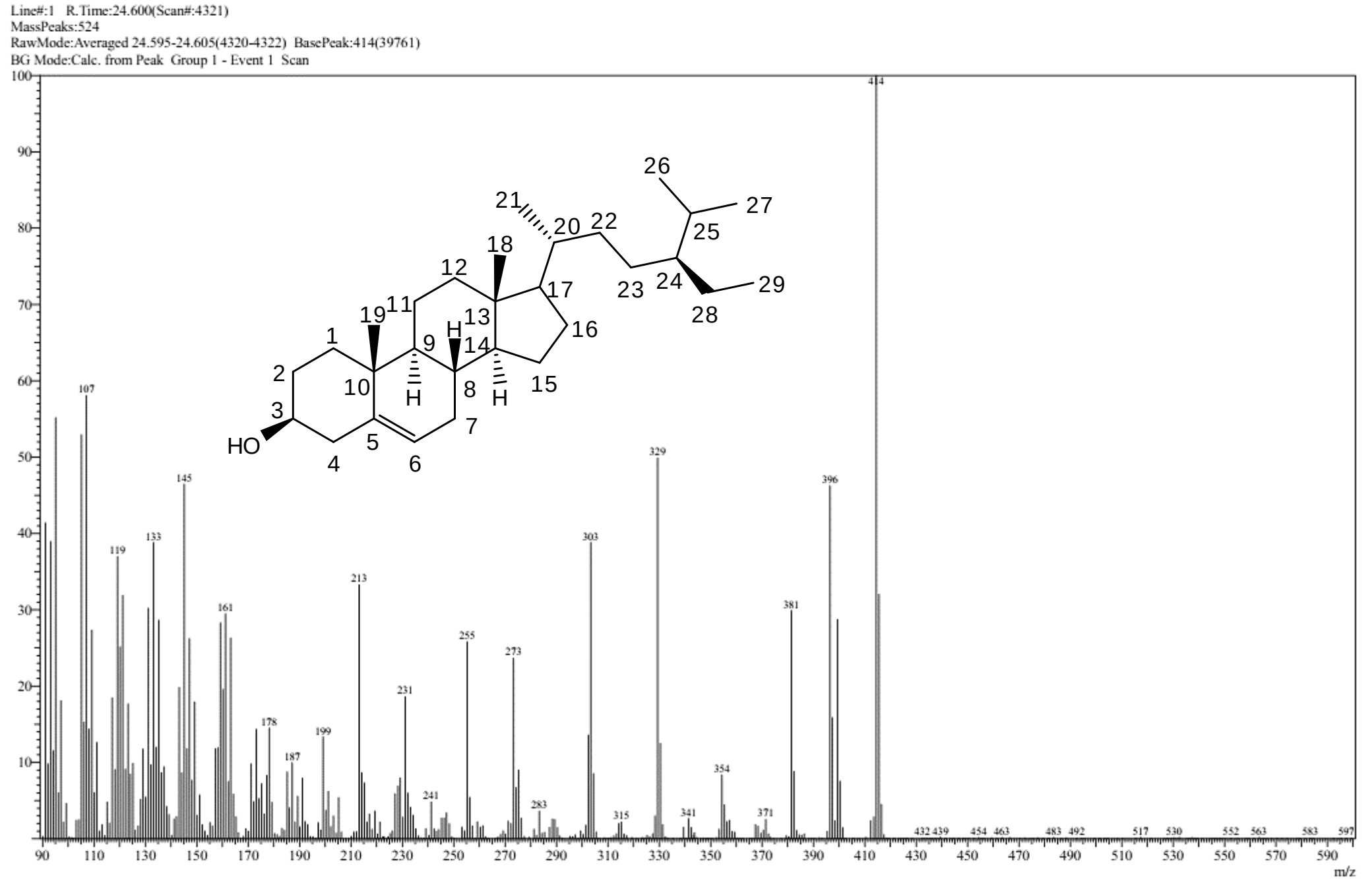
^{1}H NMR spectrum of β-sitosterol (**A8**)



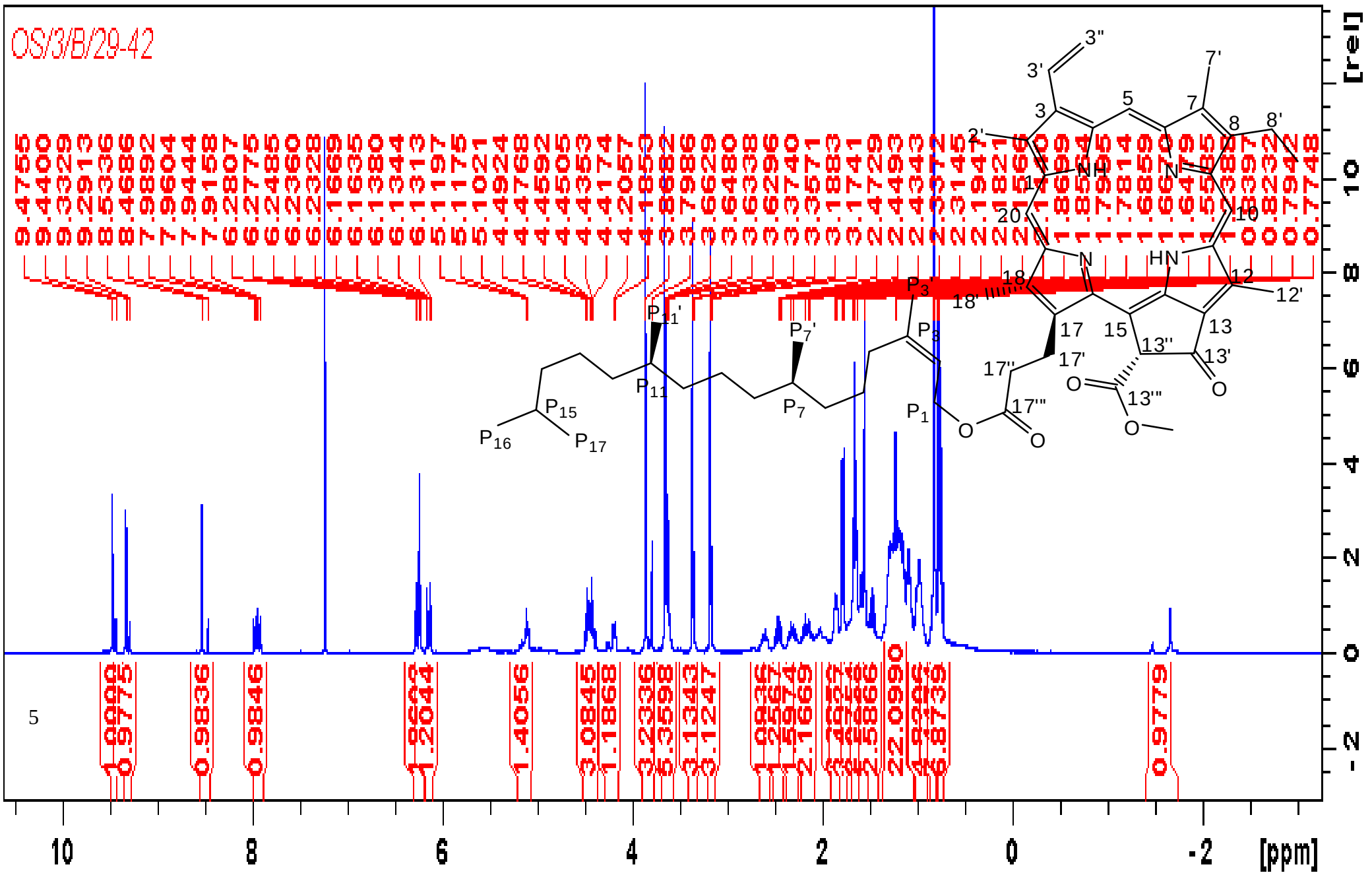
^{13}C NMR spectrum of *β*-sitosterol (**A8**)



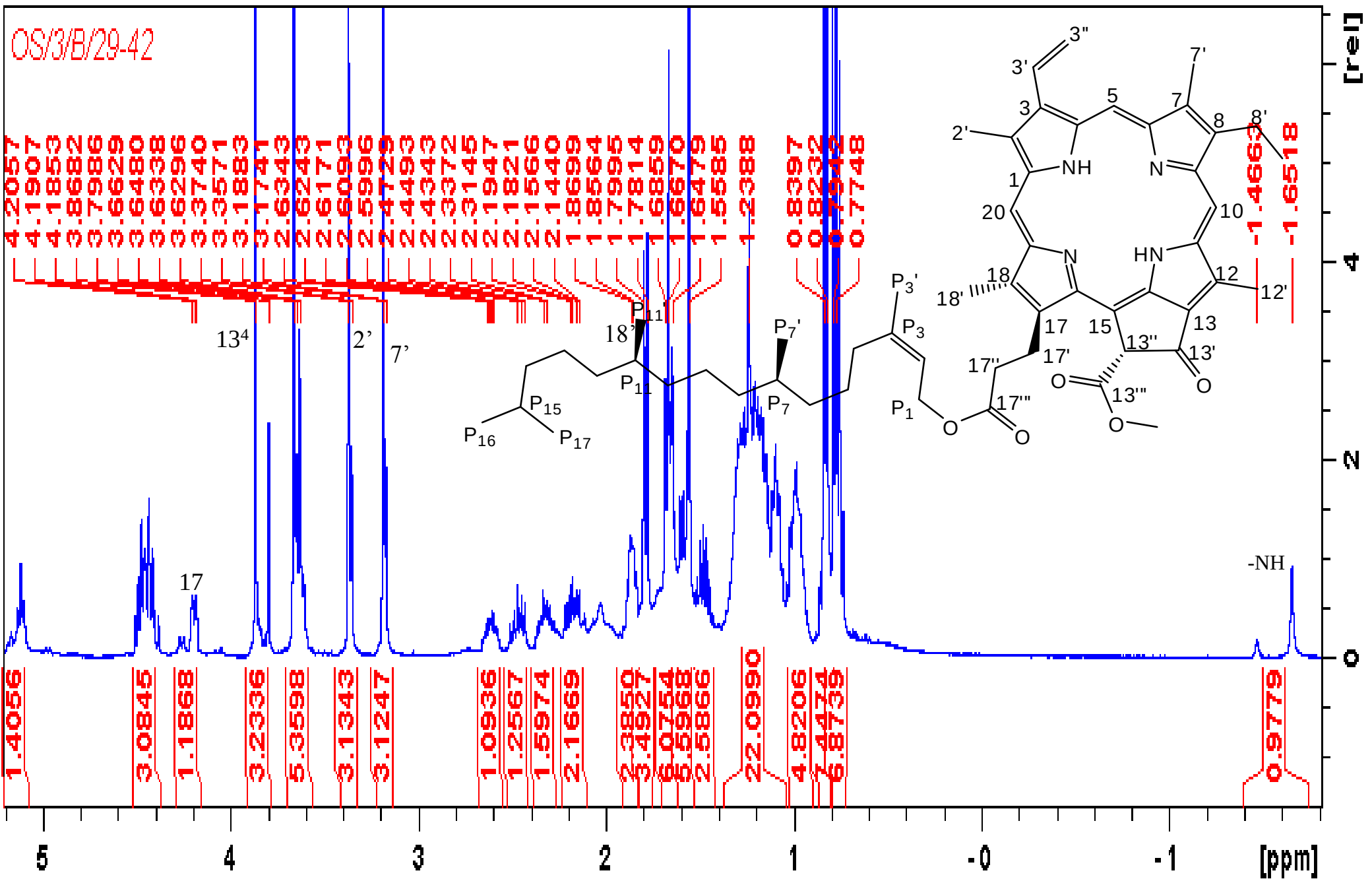
DEPT spectrum of *β*-sitosterol (**A8**)



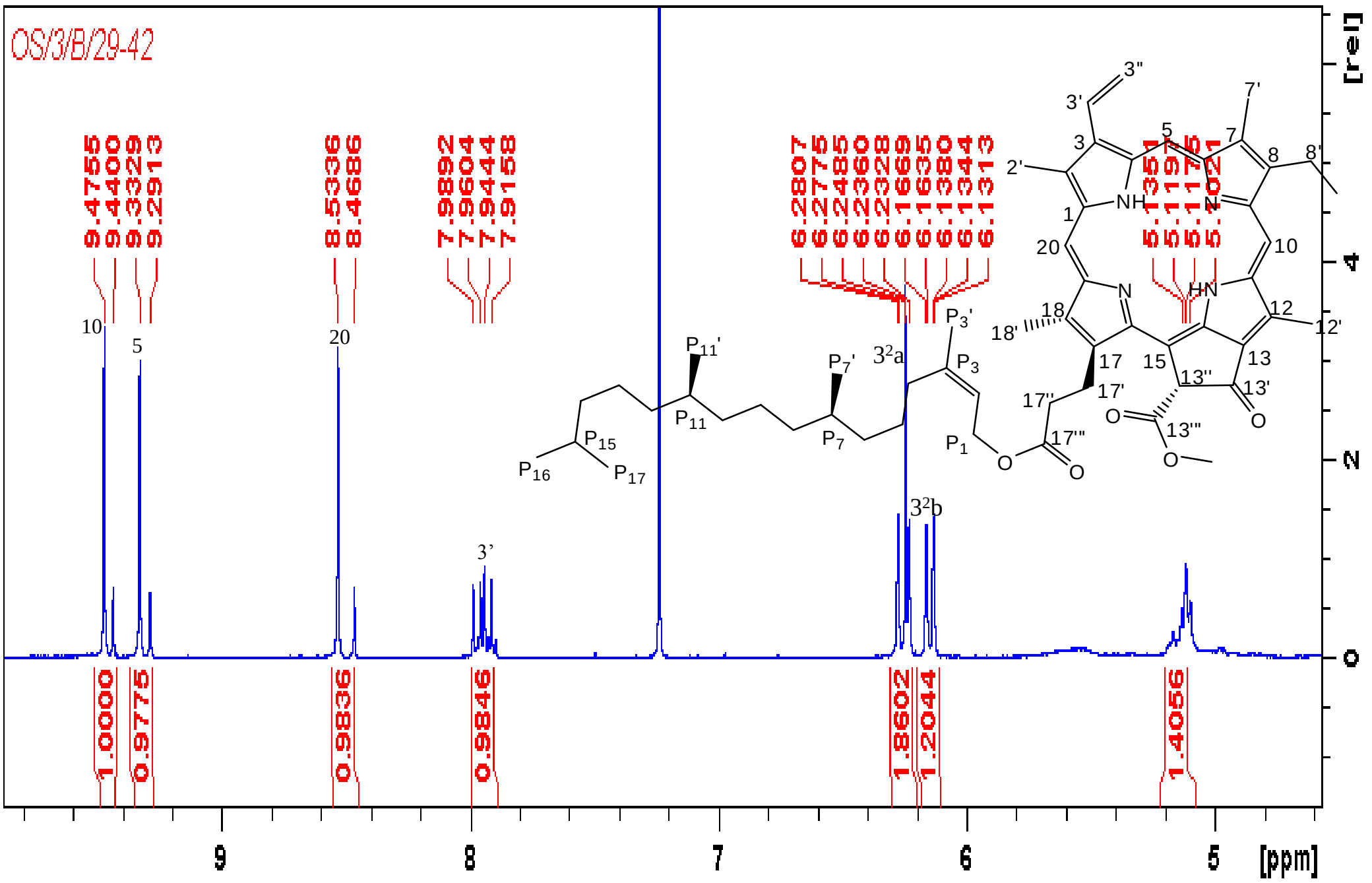
Mass spectrum of β-sitosterol (**A8**)



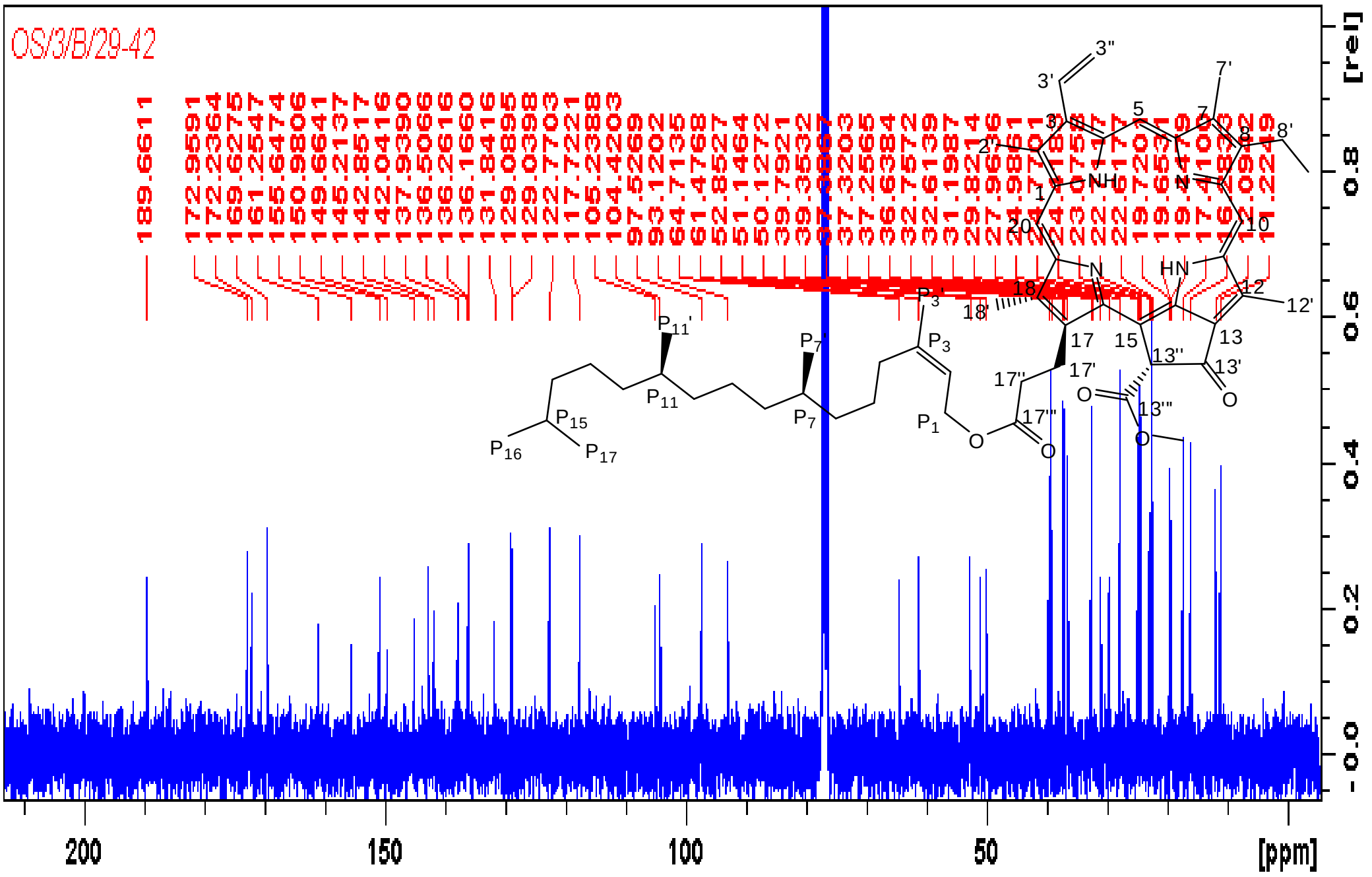
^{1}H NMR spectrum of pheophytin a (**A9**)



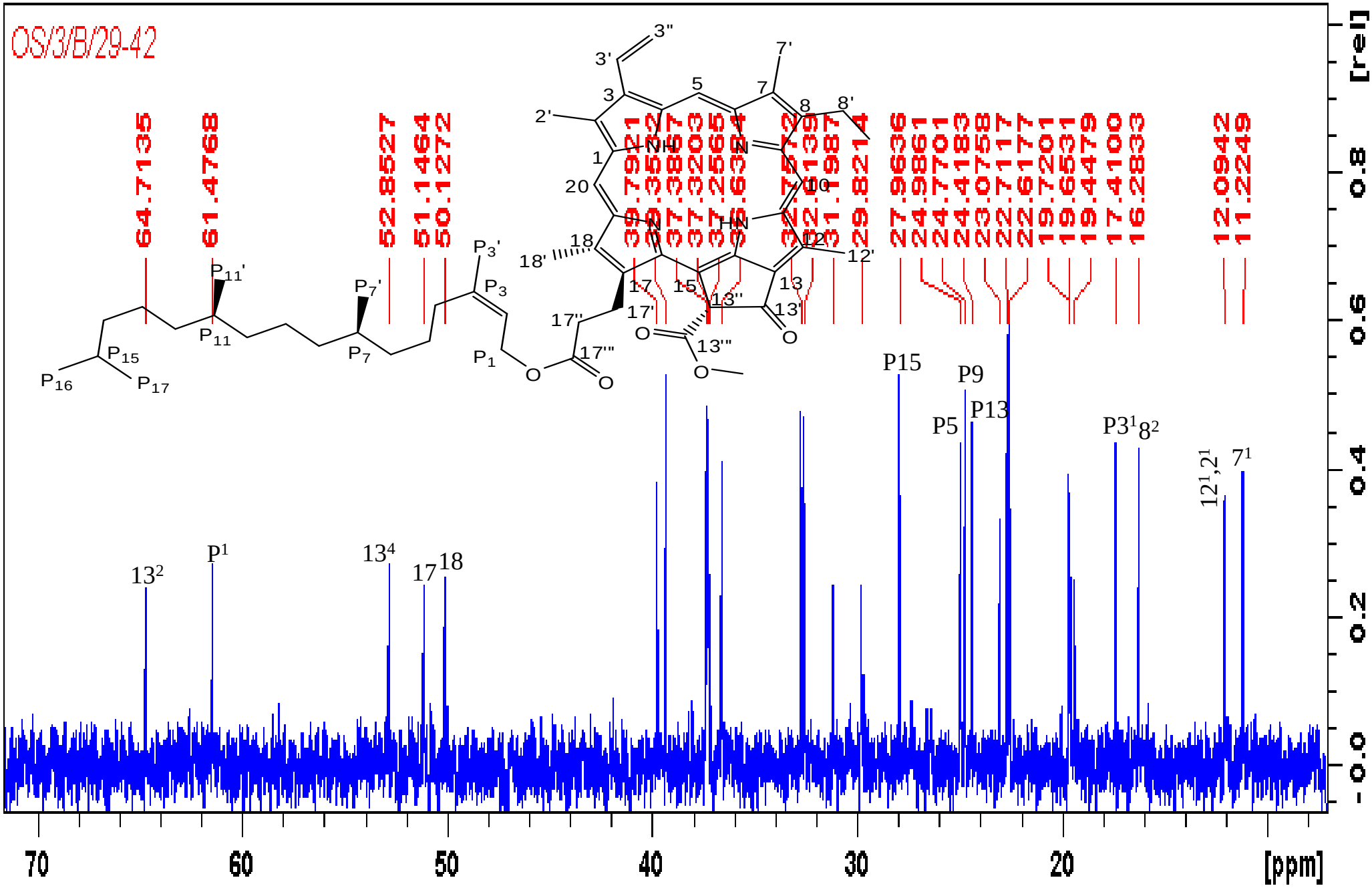
Expanded ^{1}H NMR spectrum of pheophytin a (**A9**)



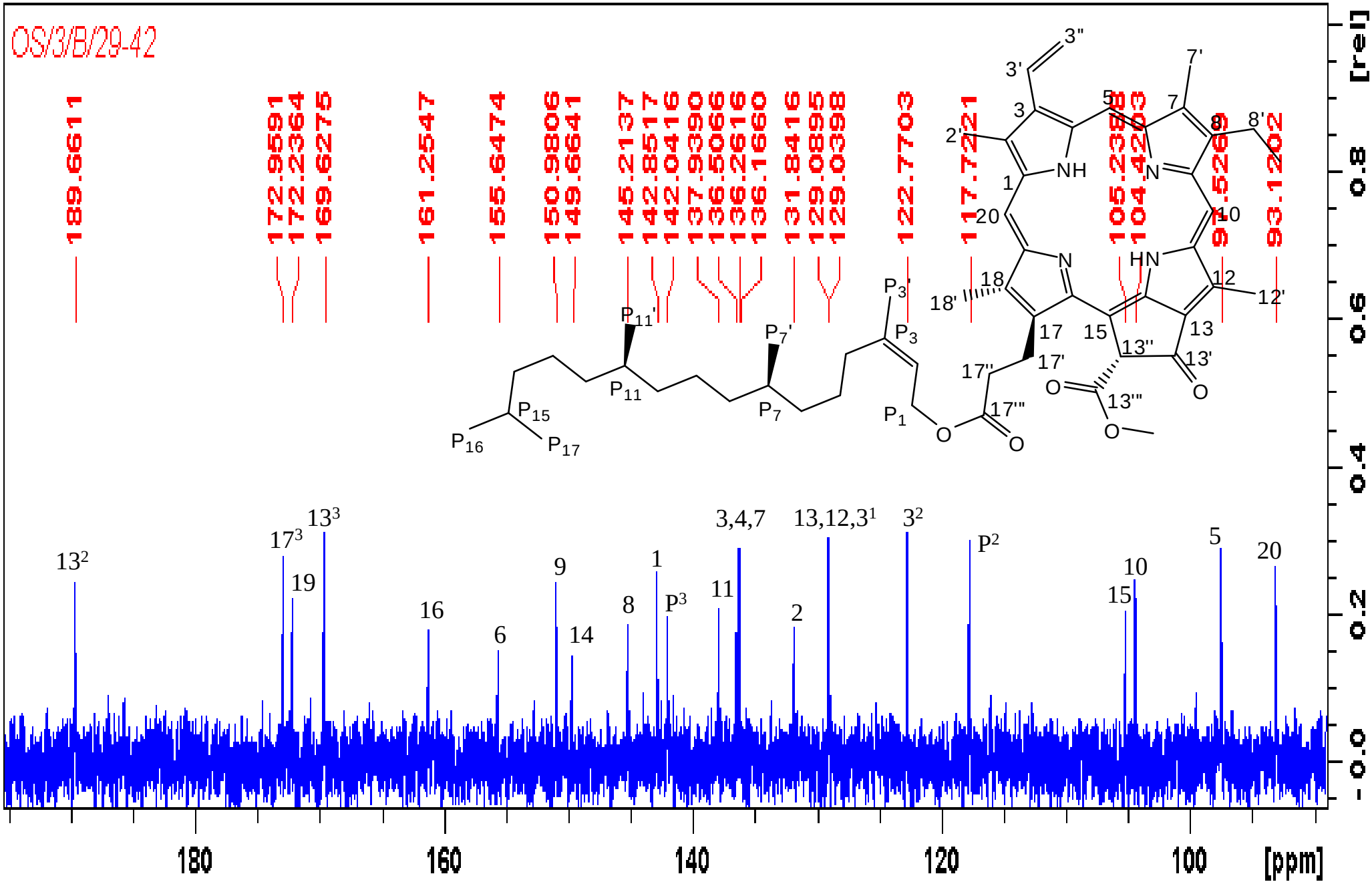
Expanded ^{1}H NMR spectrum of pheophytin a (**A9**)



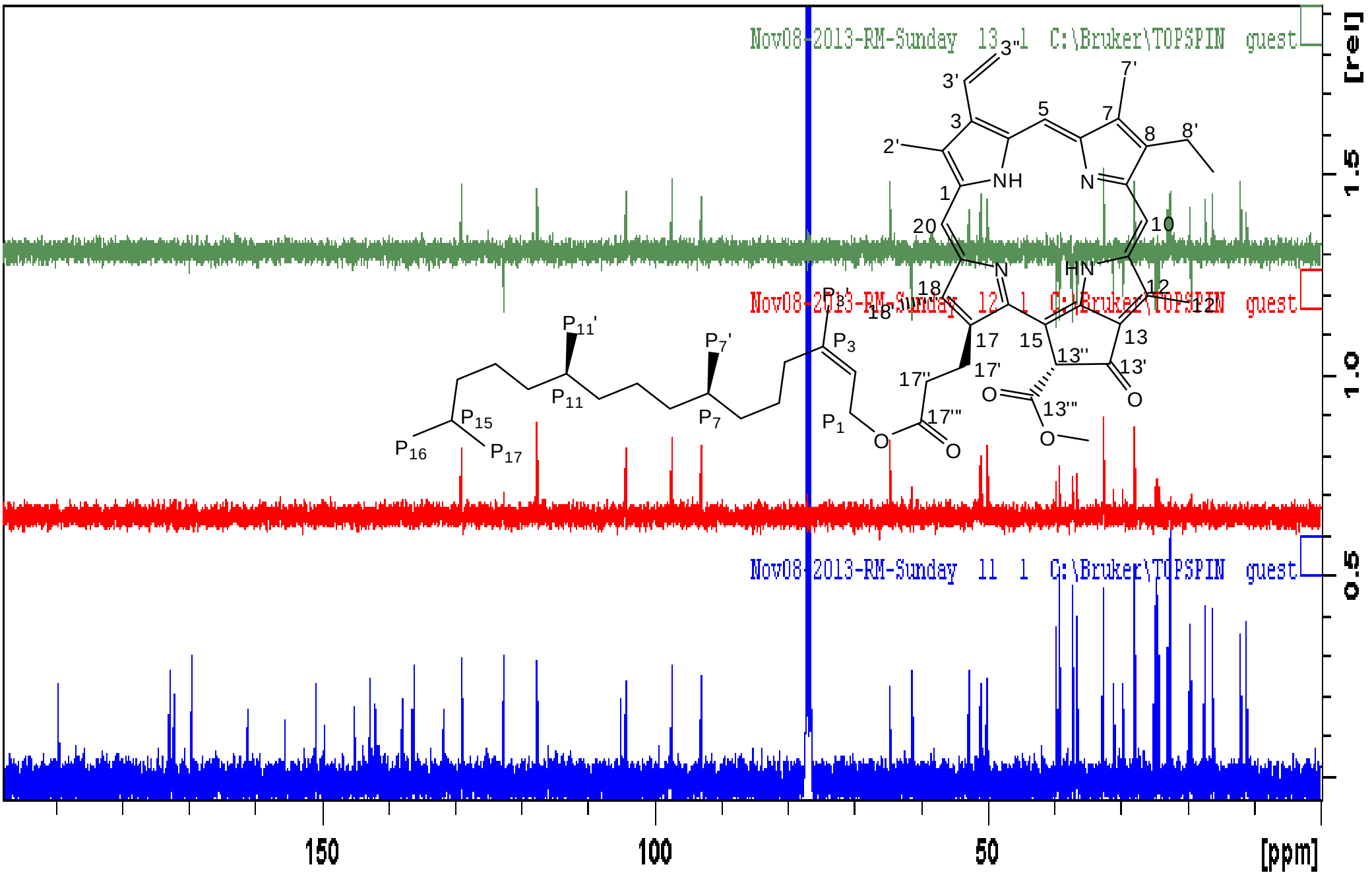
^{13}C NMR spectrum of pheophytin a (**A9**)



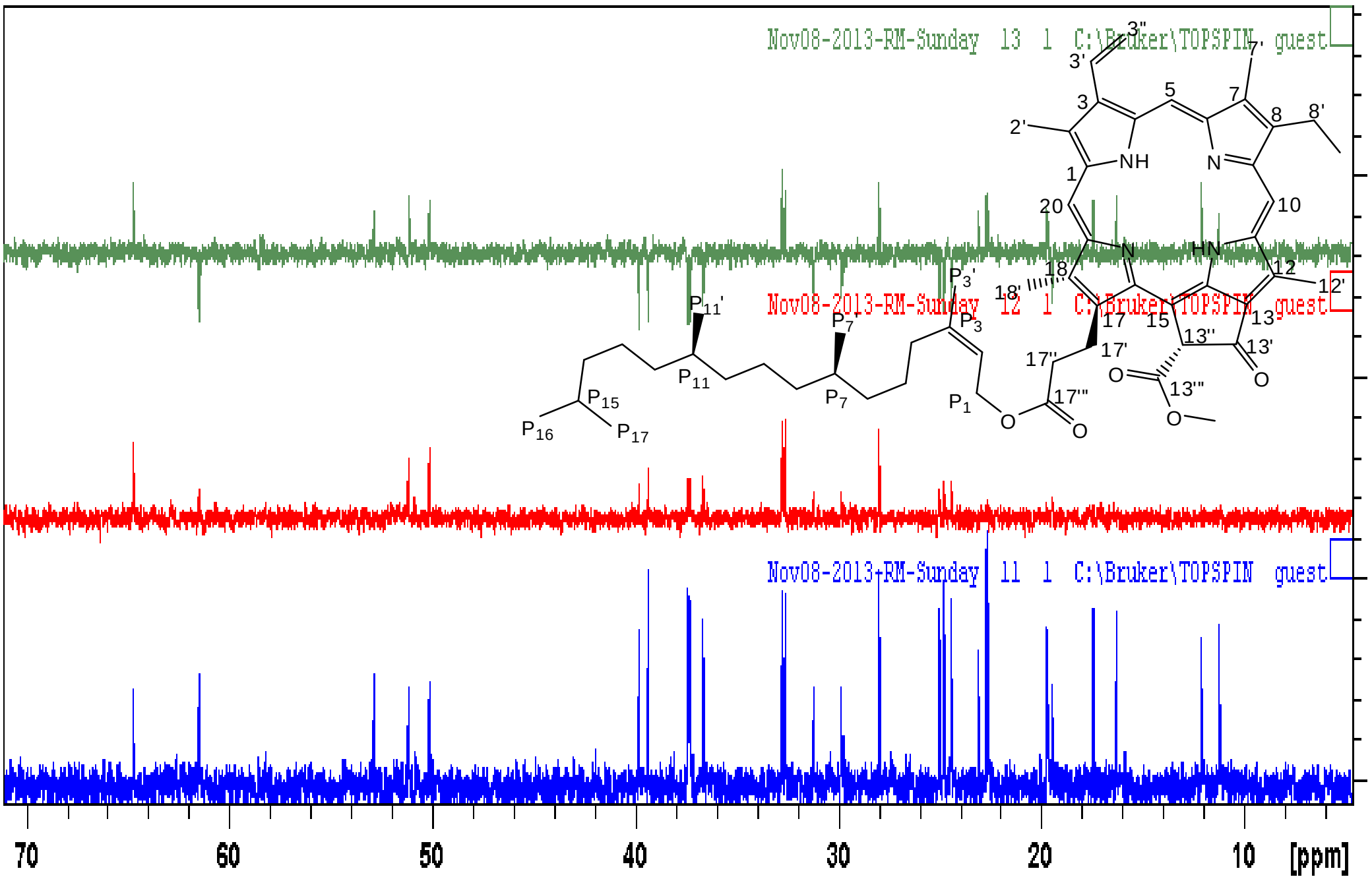
Expanded ^{13}C NMR spectrum of pheophytin a (**A9**)



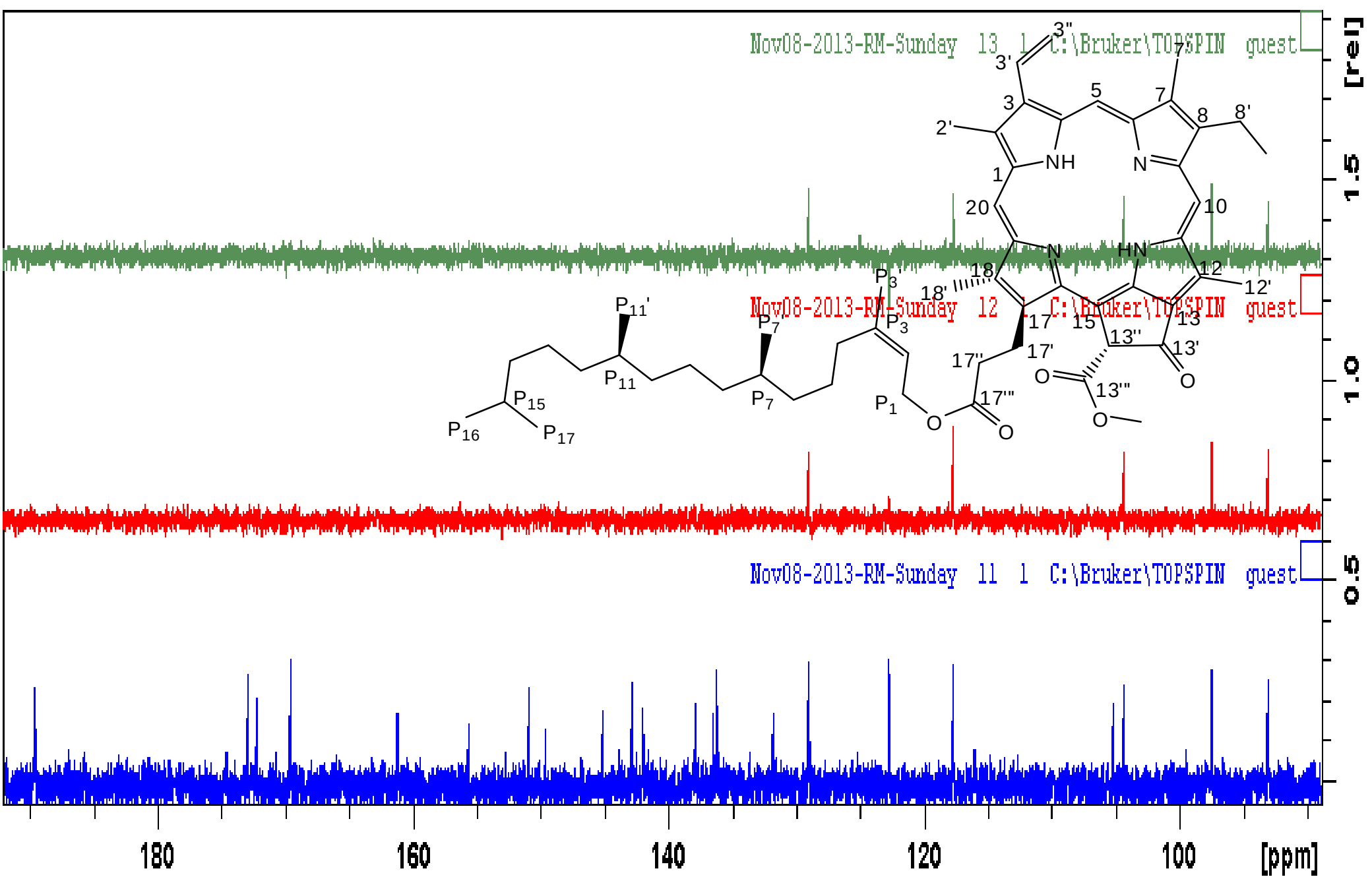
Expanded ^{13}C NMR spectrum of pheophytin a (**A9**)



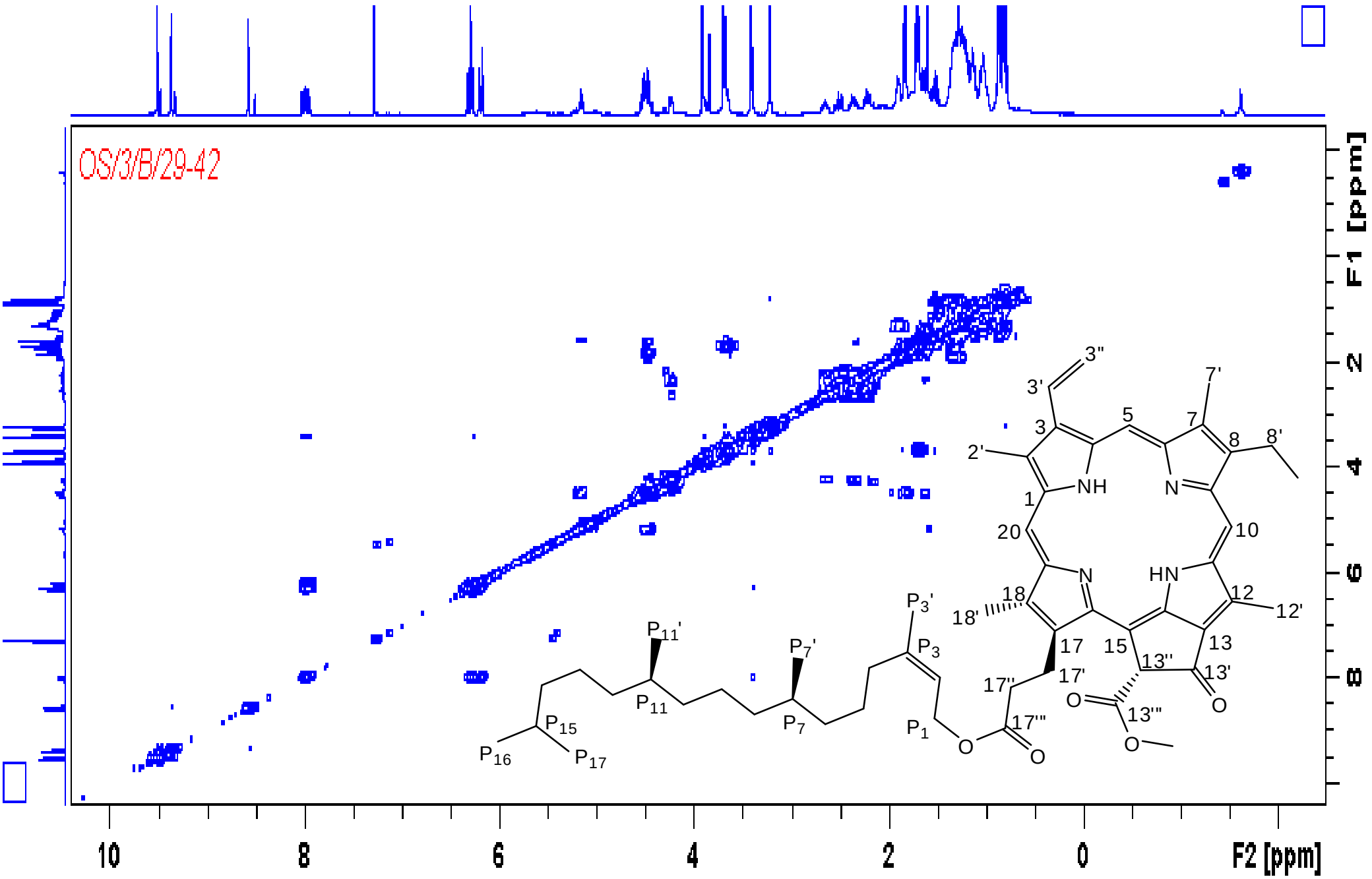
DEPT spectrum of pheophytin a (**A9**)



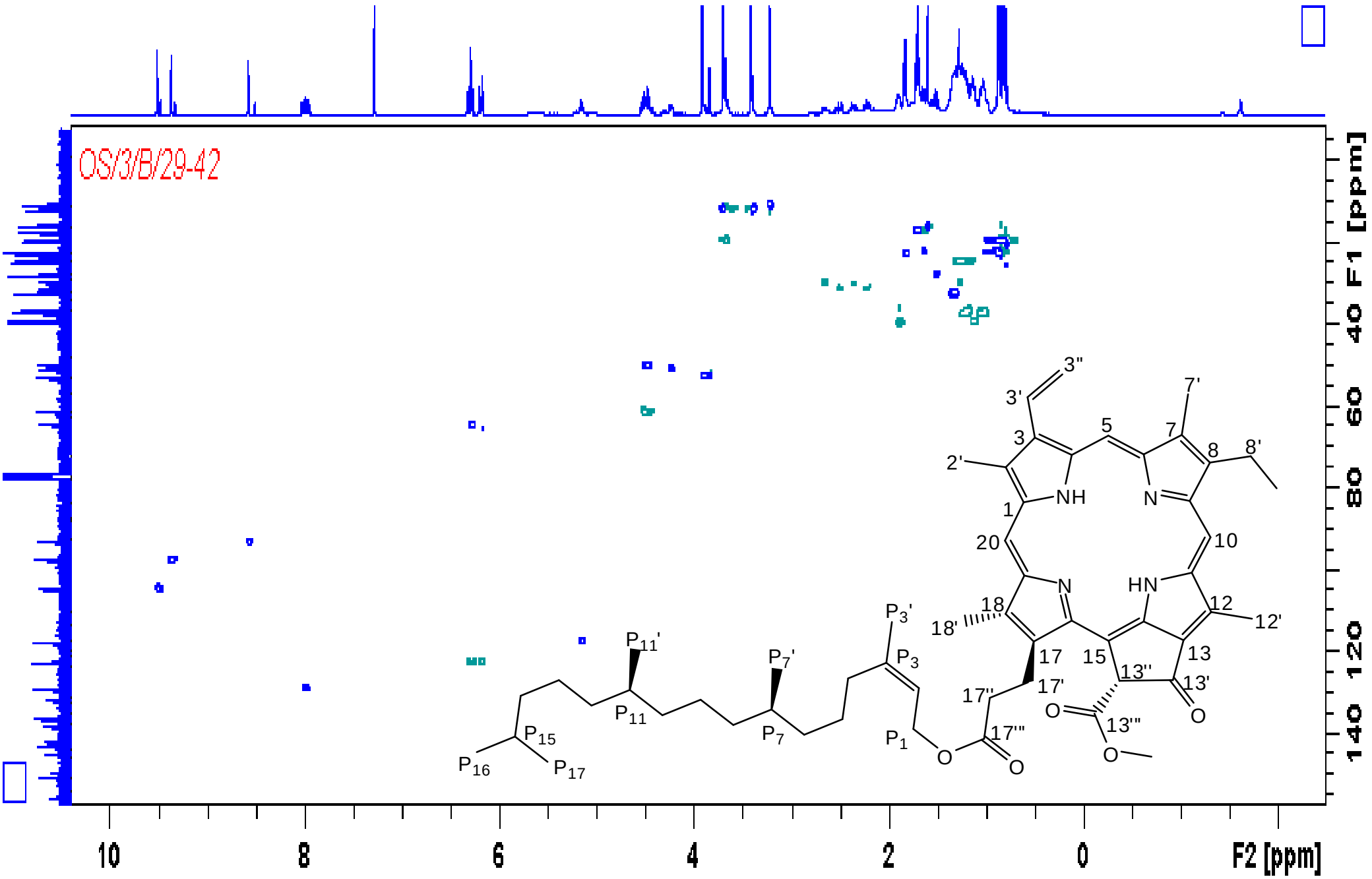
Expanded DEPT spectrum of pheophytin a (**A9**)



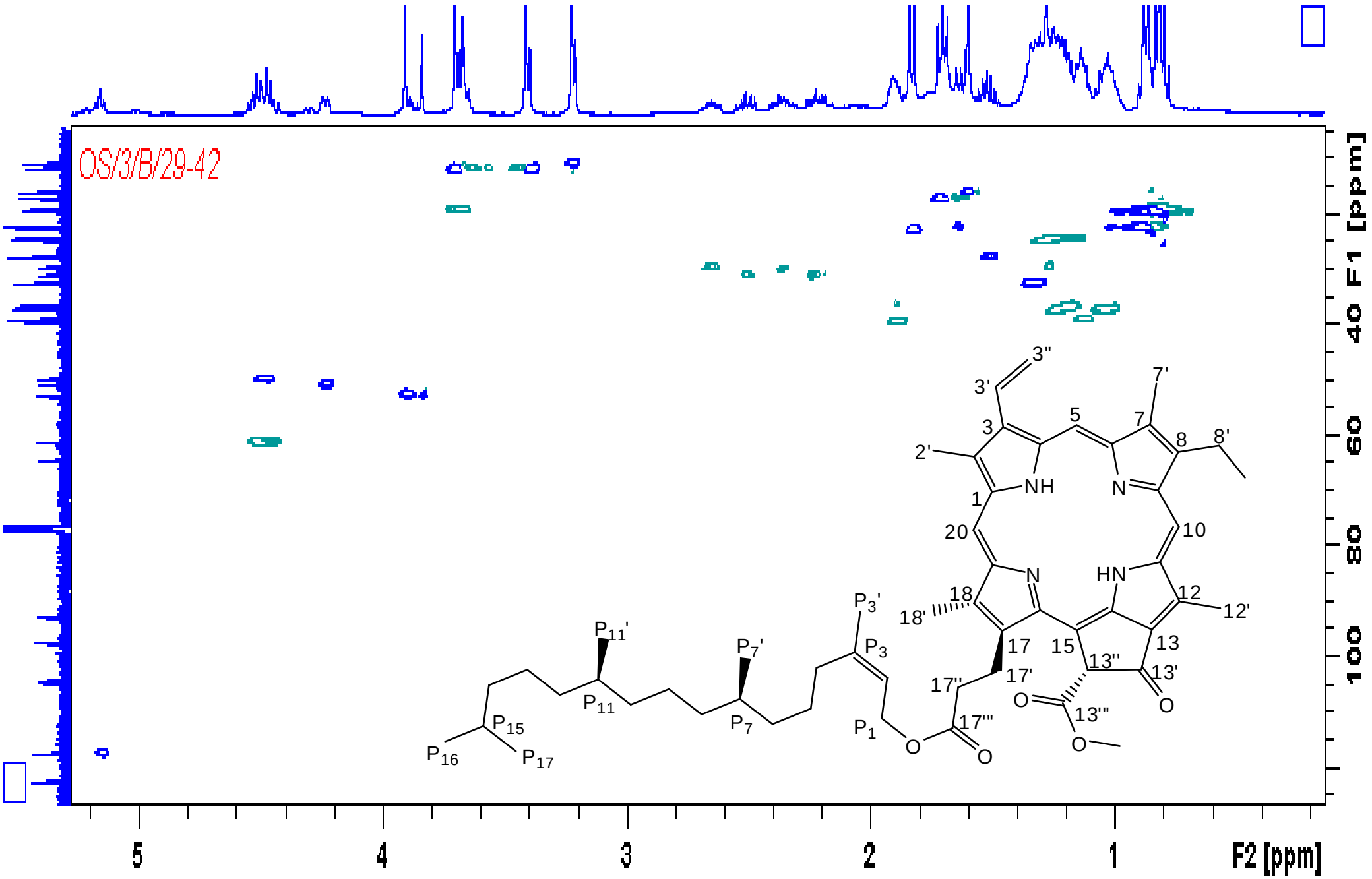
Expanded DEPT spectrum of pheophytin a (**A9**)



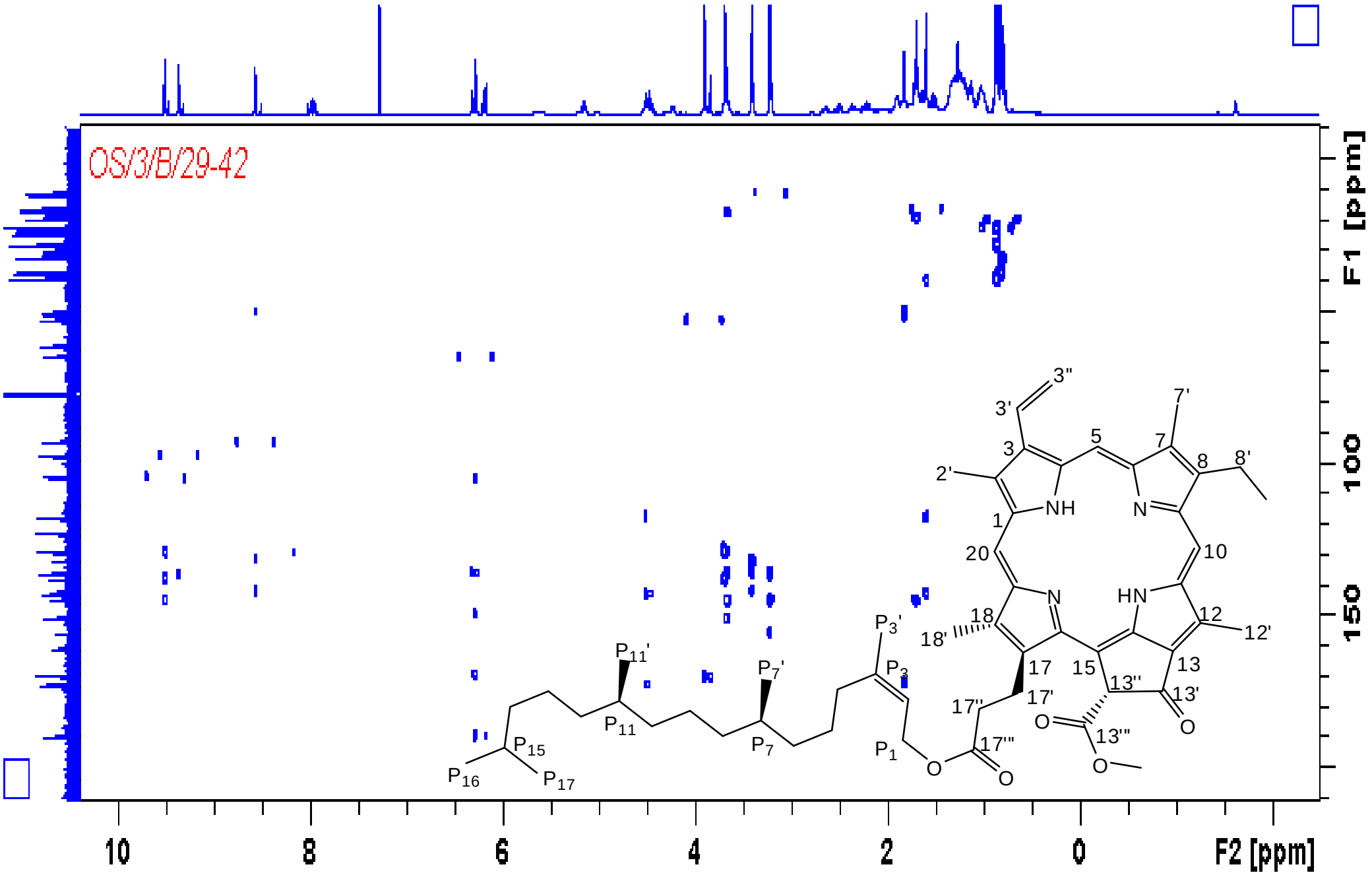
COSY spectrum of pheophytin a (**A9**)



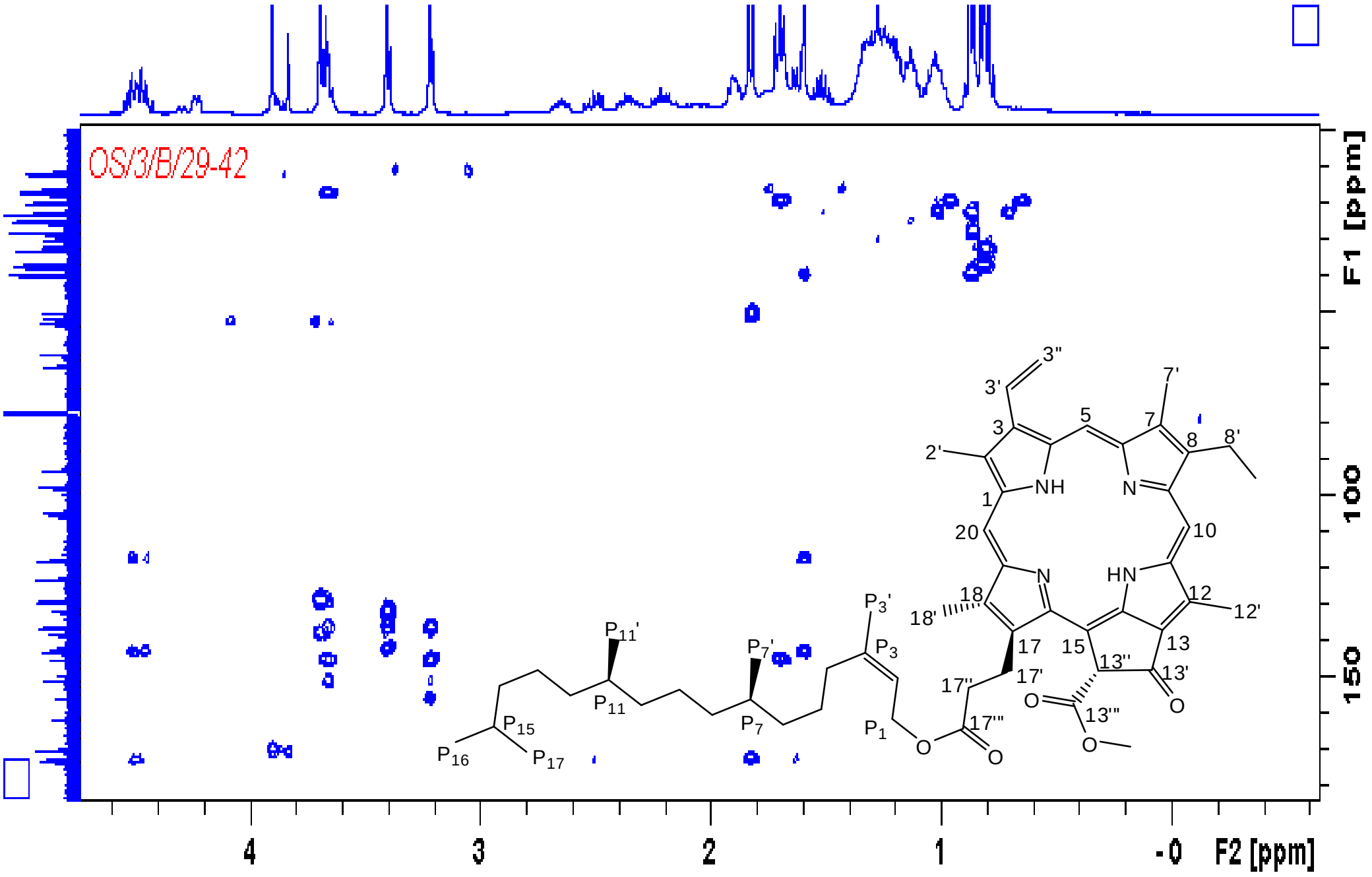
HSQC spectrum of pheophytin a (**A9**)



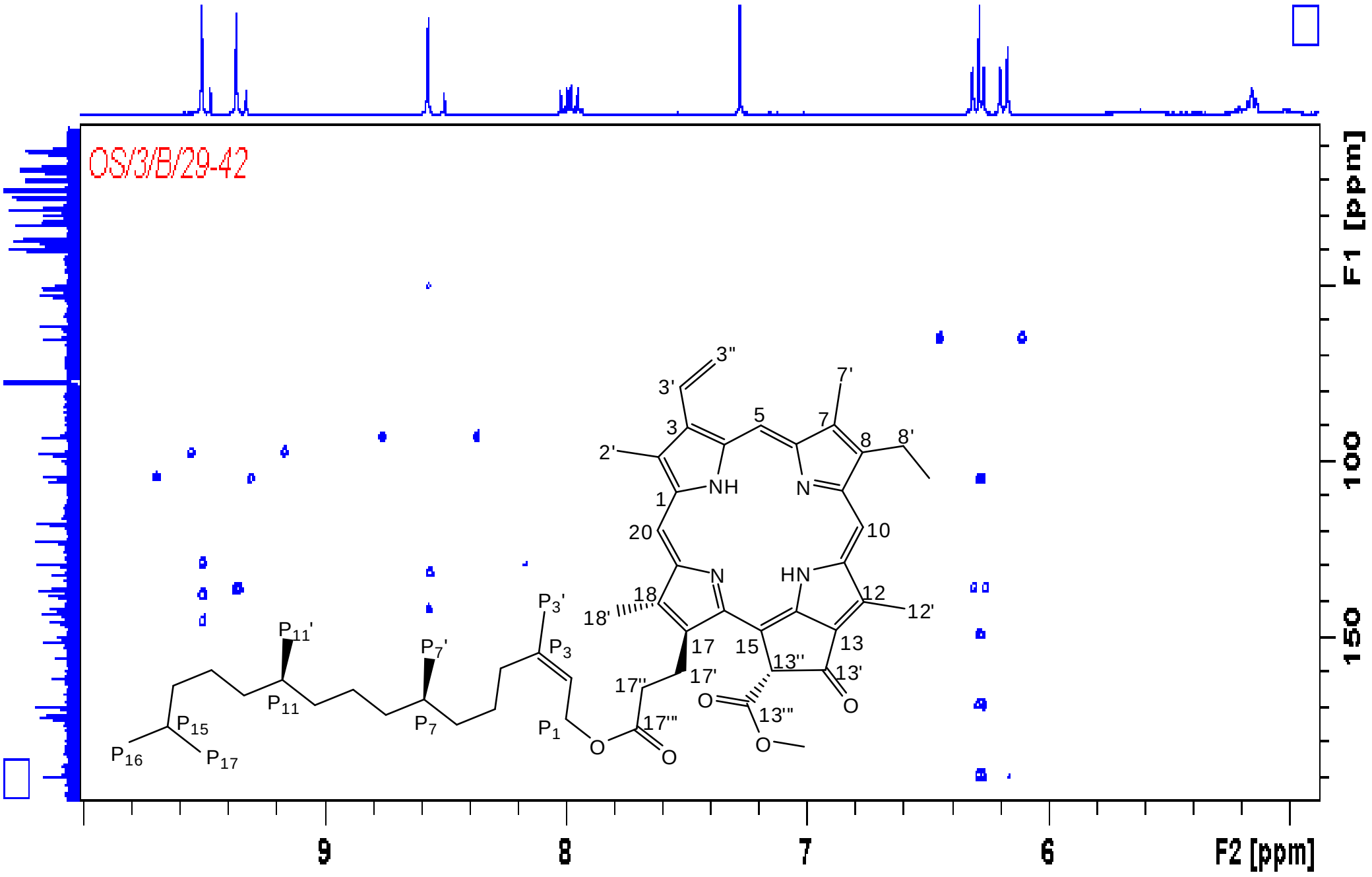
Expanded HSQC spectrum of pheophytin a (**A9**)



HMBC spectrum of pheophytin a (**A9**)



Expanded HMBC spectrum of pheophytin a (**A9**)



Expanded HMBC spectrum of pheophytin a (**A9**)

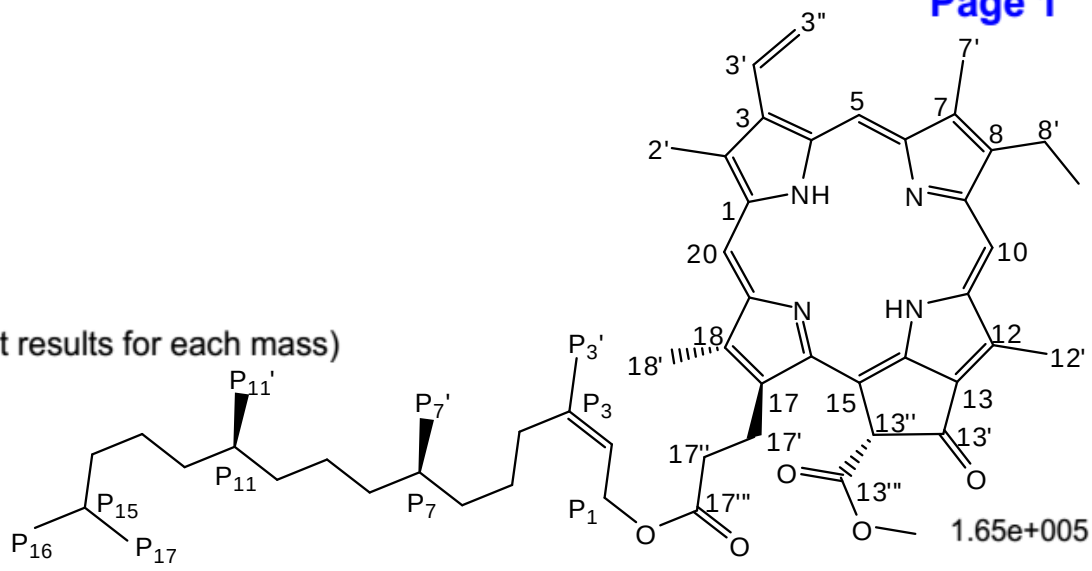
Elemental Composition Report

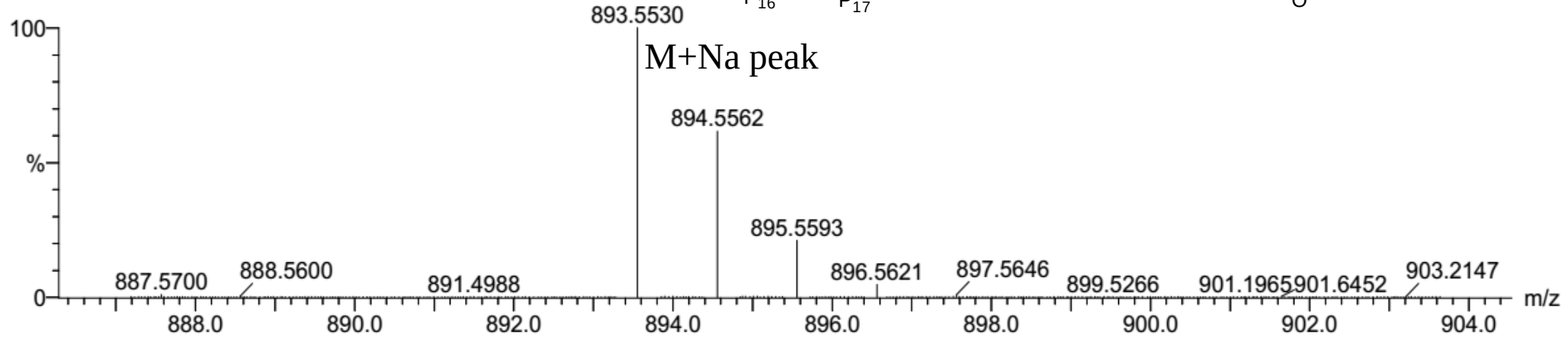
Single Mass Analysis

Tolerance = 5.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
13 formula(e) evaluated with 1 results within limits (up to 20 closest results for each mass)
Elements Used:
C: 50-55 H: 70-75 N: 0-5 O: 0-5 Na: 0-1

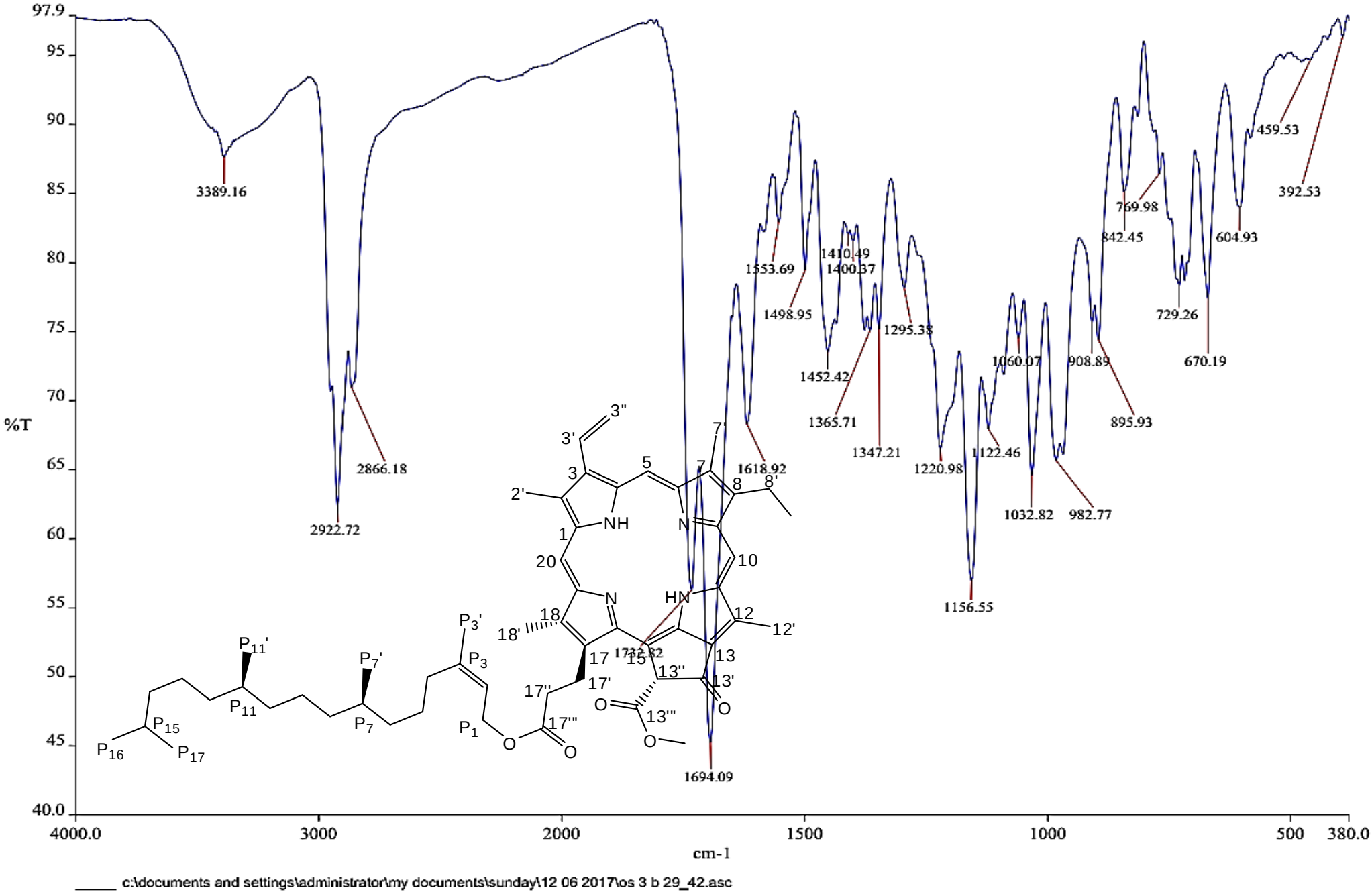
05-3-B-29-42 53 (1.755) Cm (1:61)
TOF MS ES+



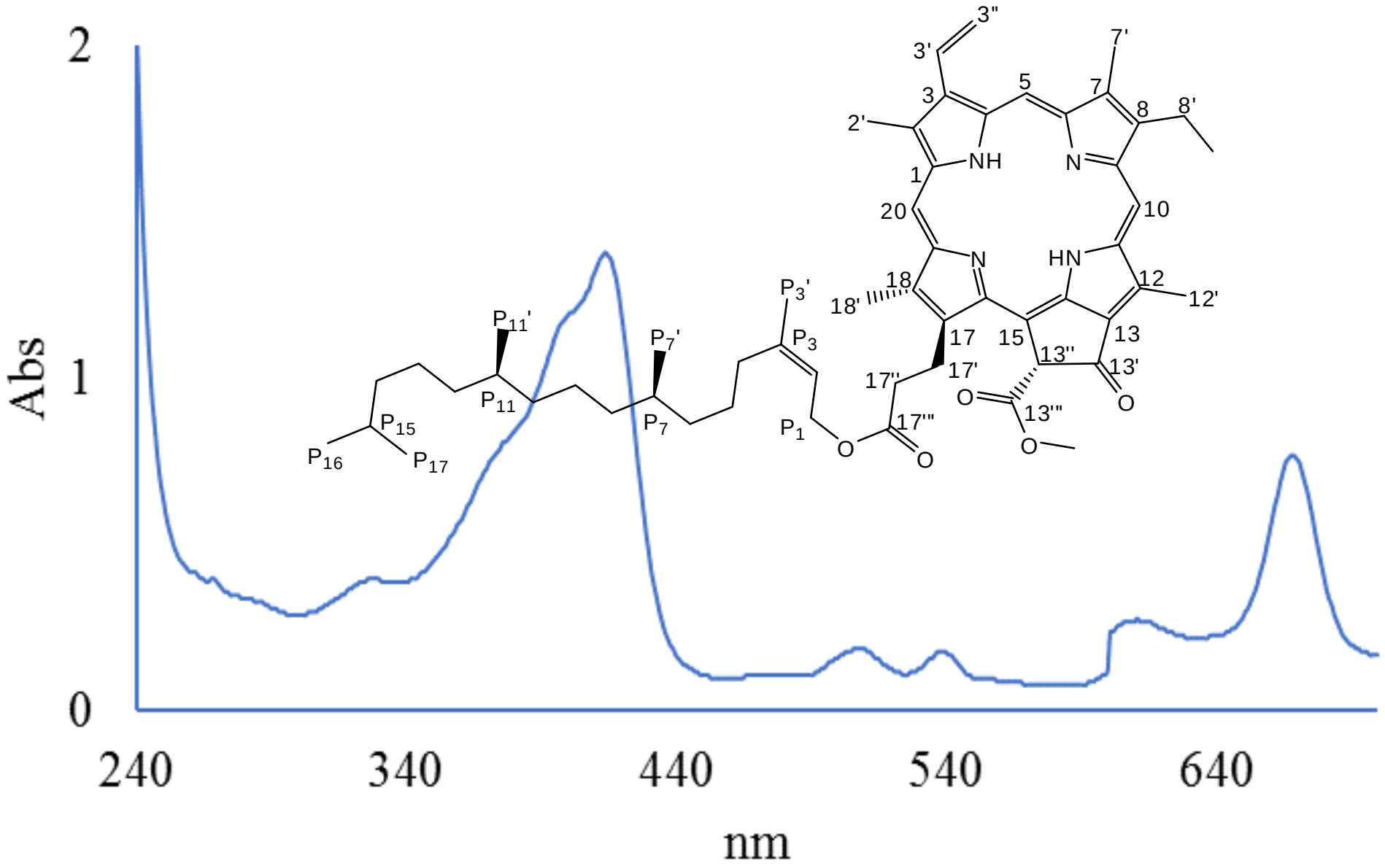


Minimum:				-1.5			
Maximum:		5.0	5.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
893.5530	893.5557	-2.7	-3.0	20.5	361.3	0.0	C55 H74 N4 O5 Na

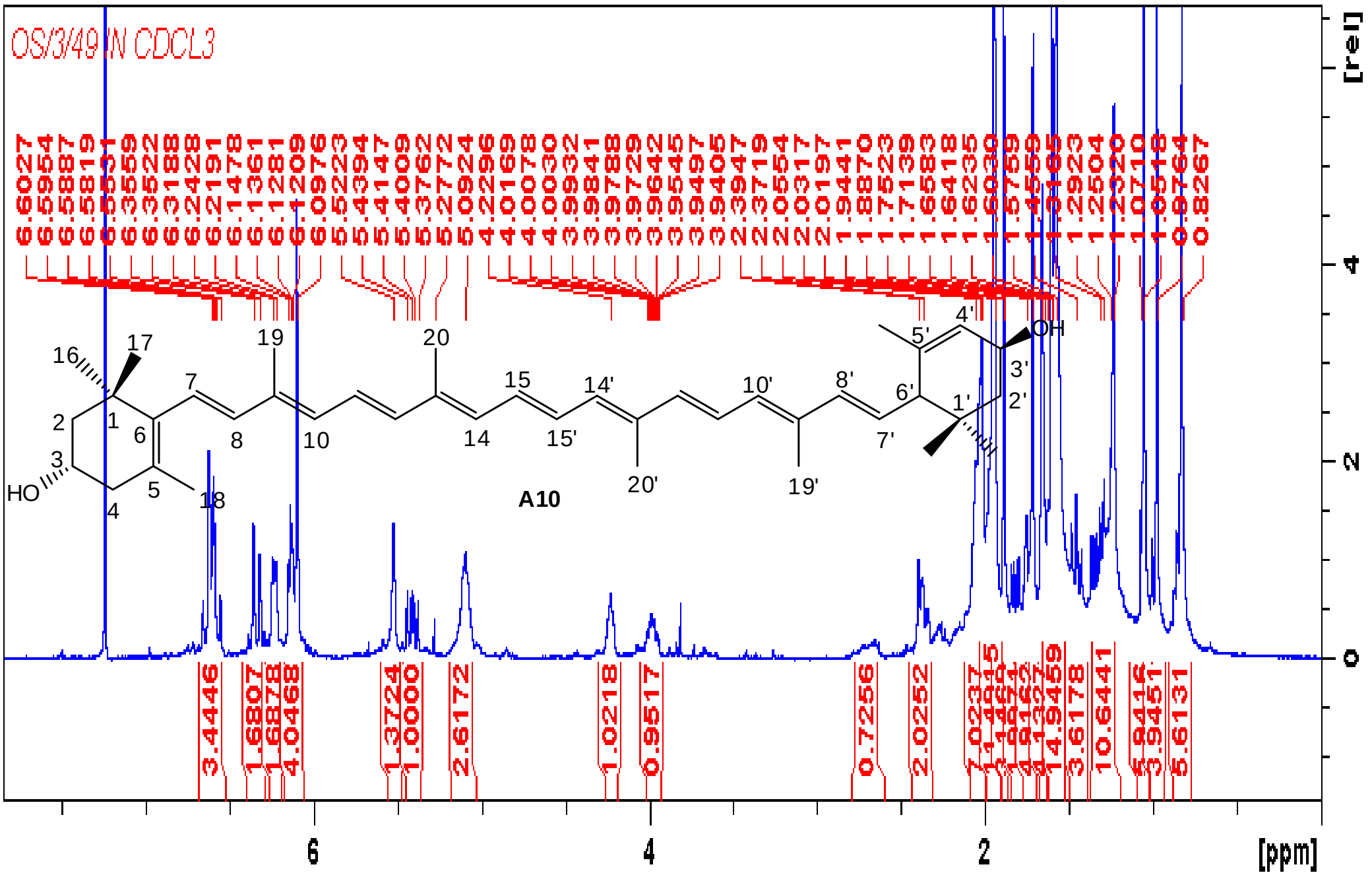
Mass spectrum of pheophytin a (**A9**)



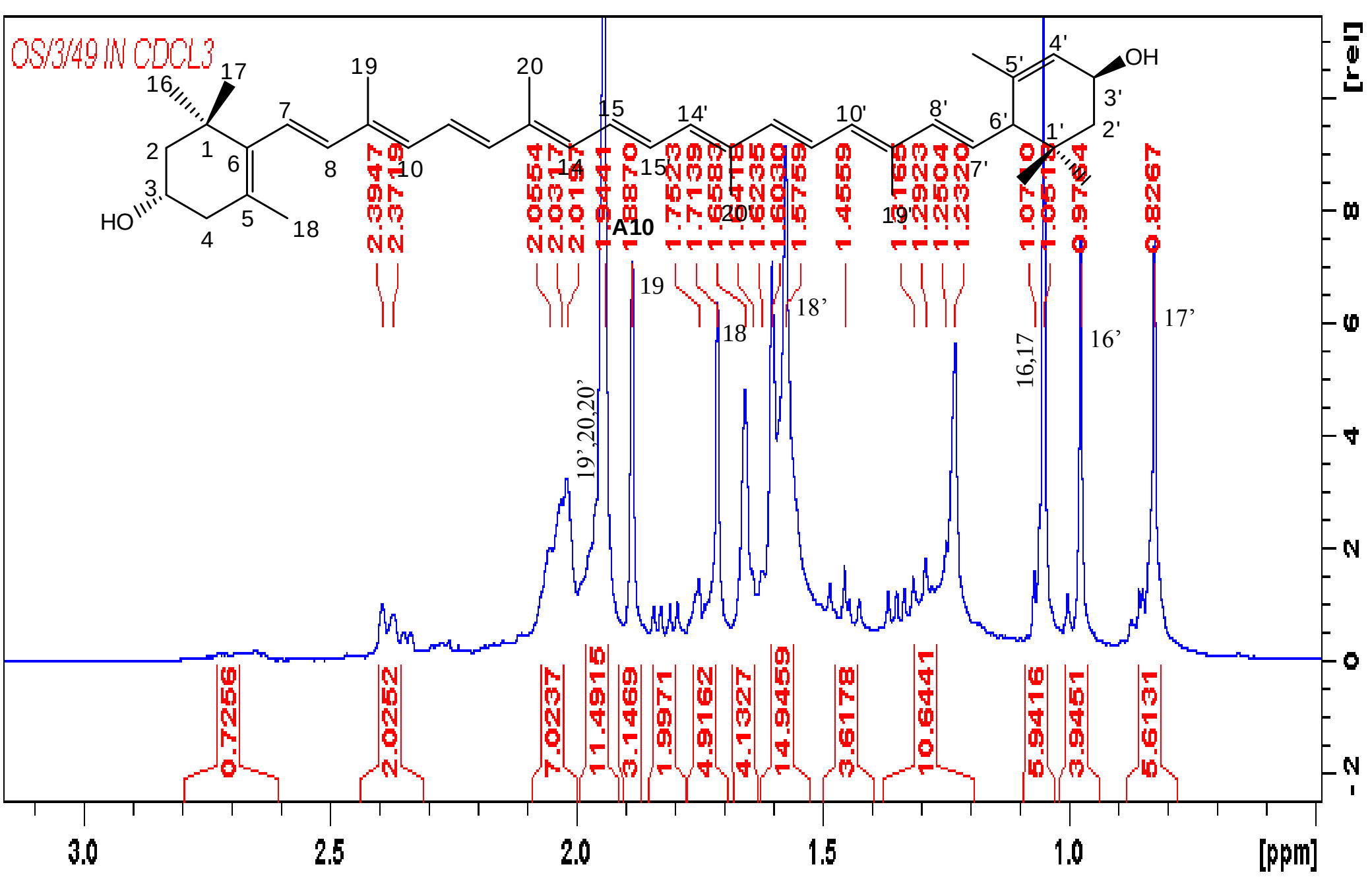
IR spectrum of pheophytin a (**A9**)



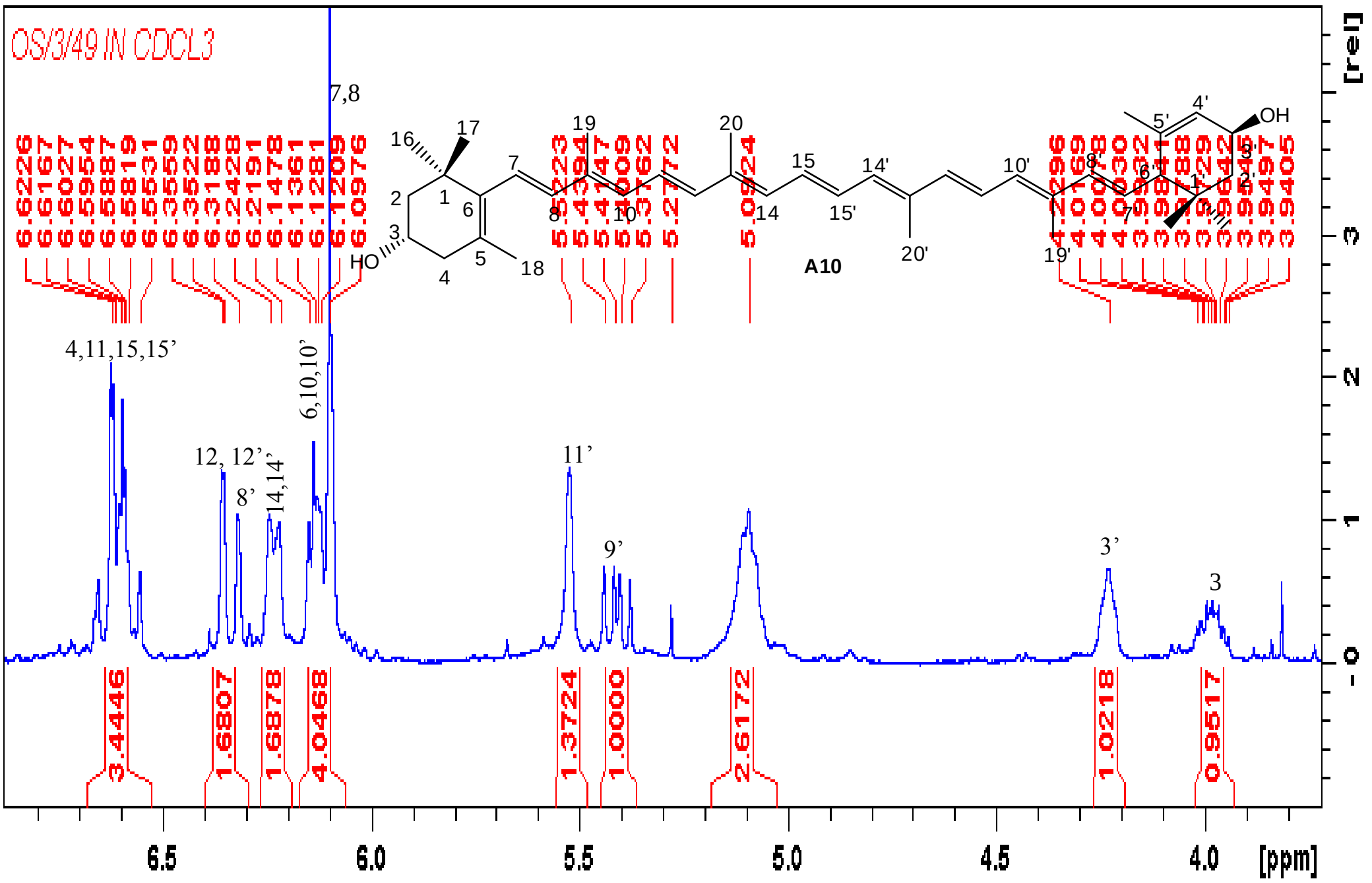
UV spectrum of pheophytin a (**A9**)



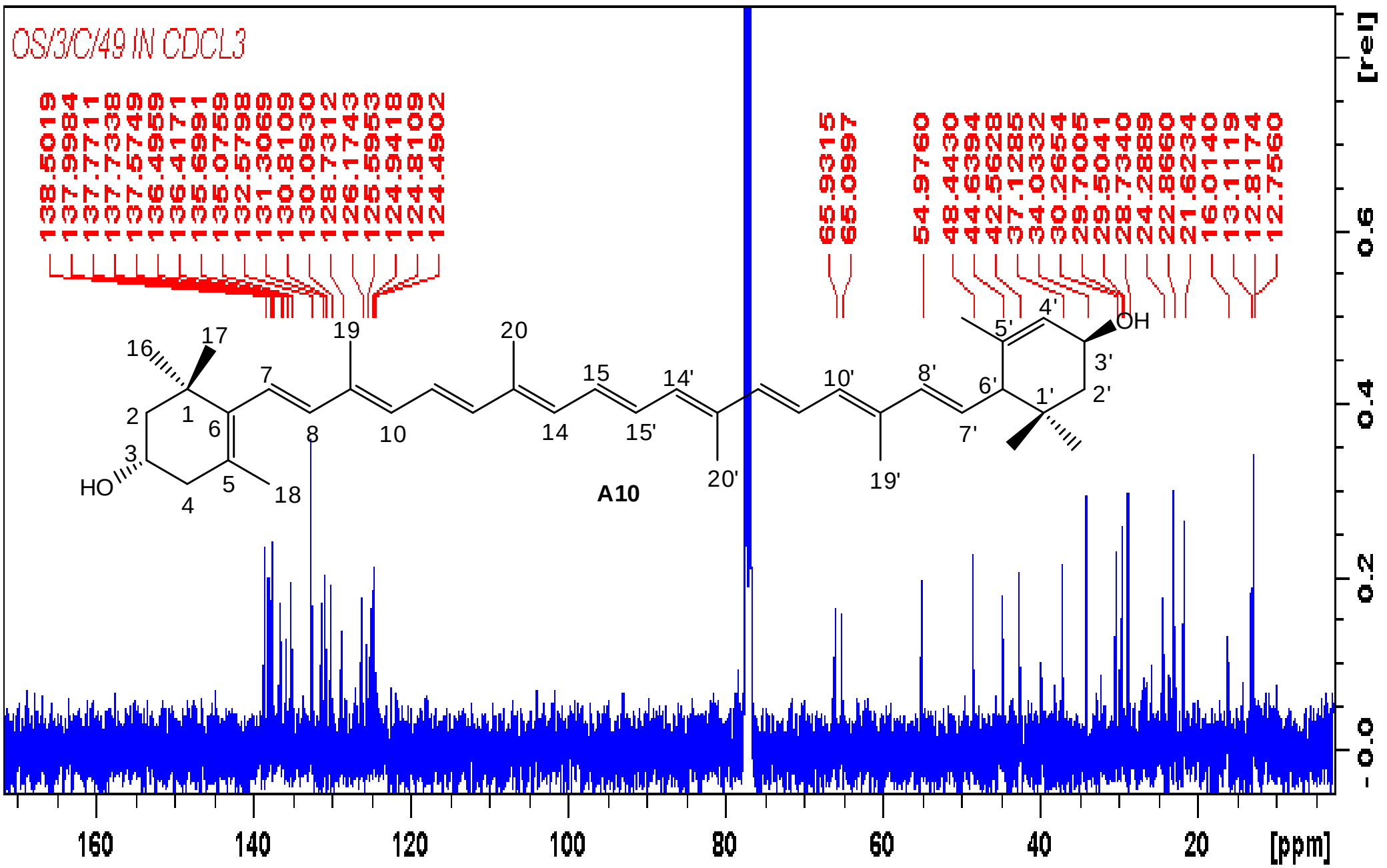
^{1}H NMR spectrum of lutein (**A10**)



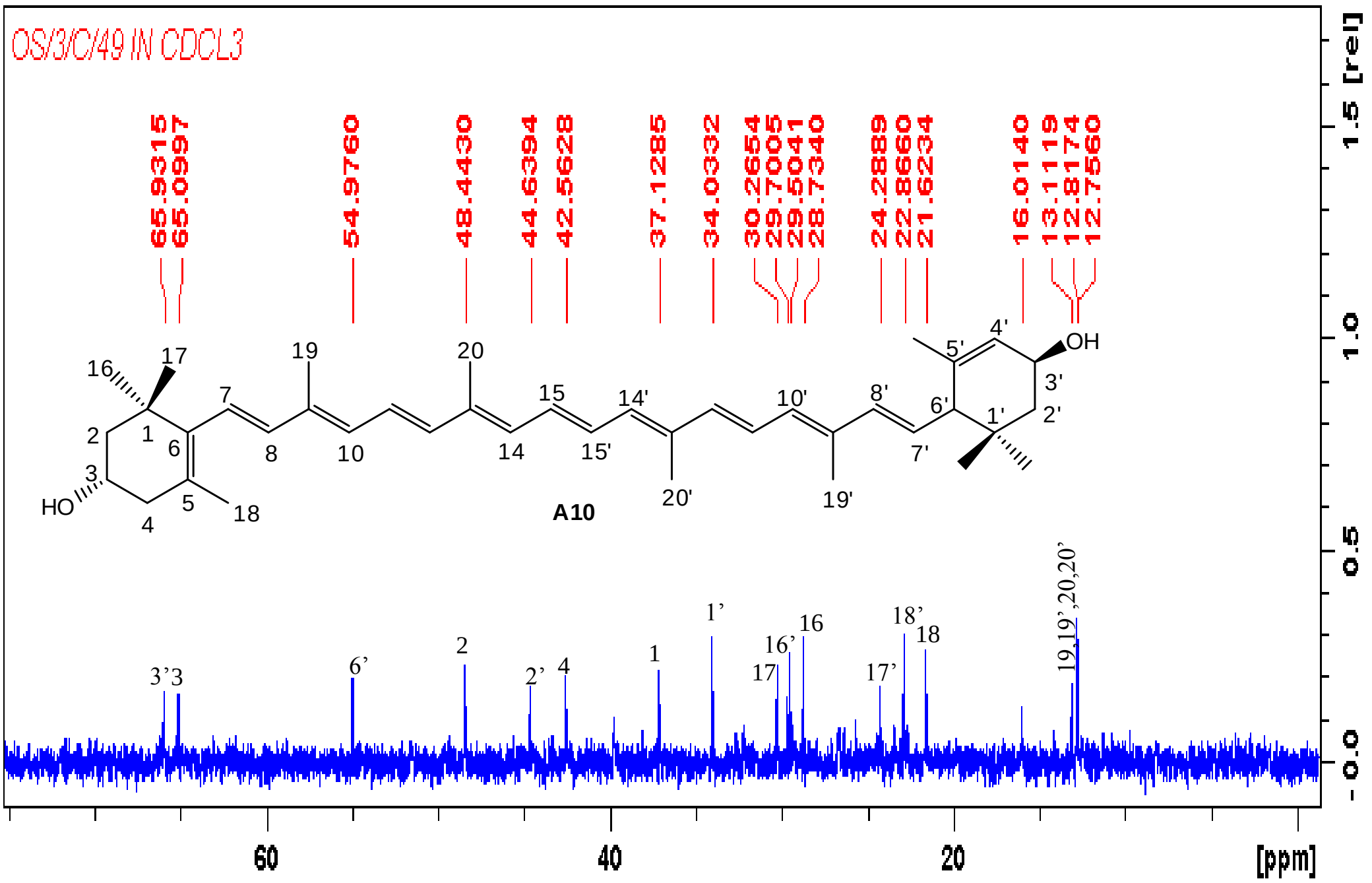
Expanded ^{1}H NMR spectrum of lutein (**A10**)



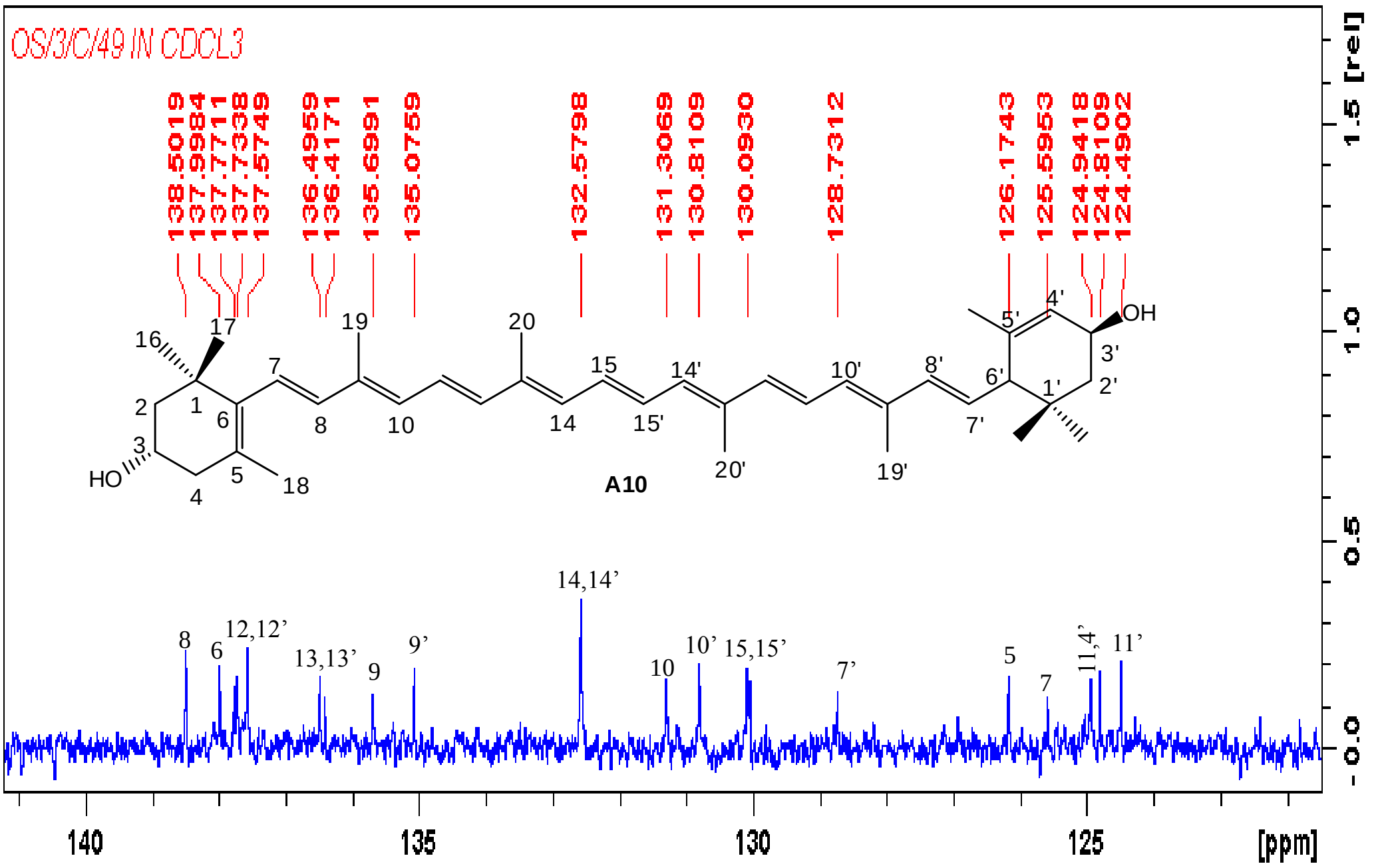
Expanded ^{1}H NMR spectrum of lutein (**A10**)



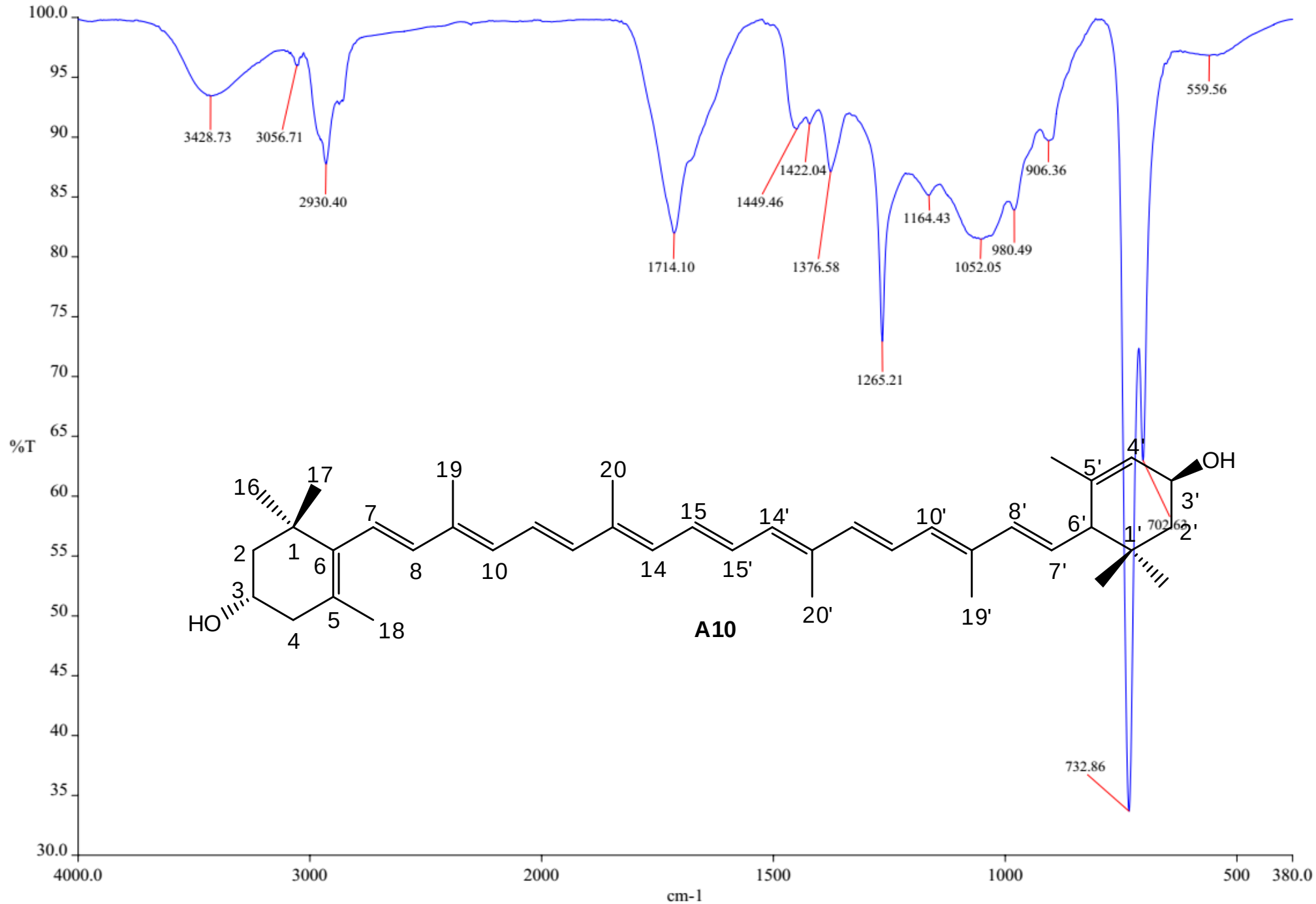
^{13}C NMR spectrum of lutein (**A10**)



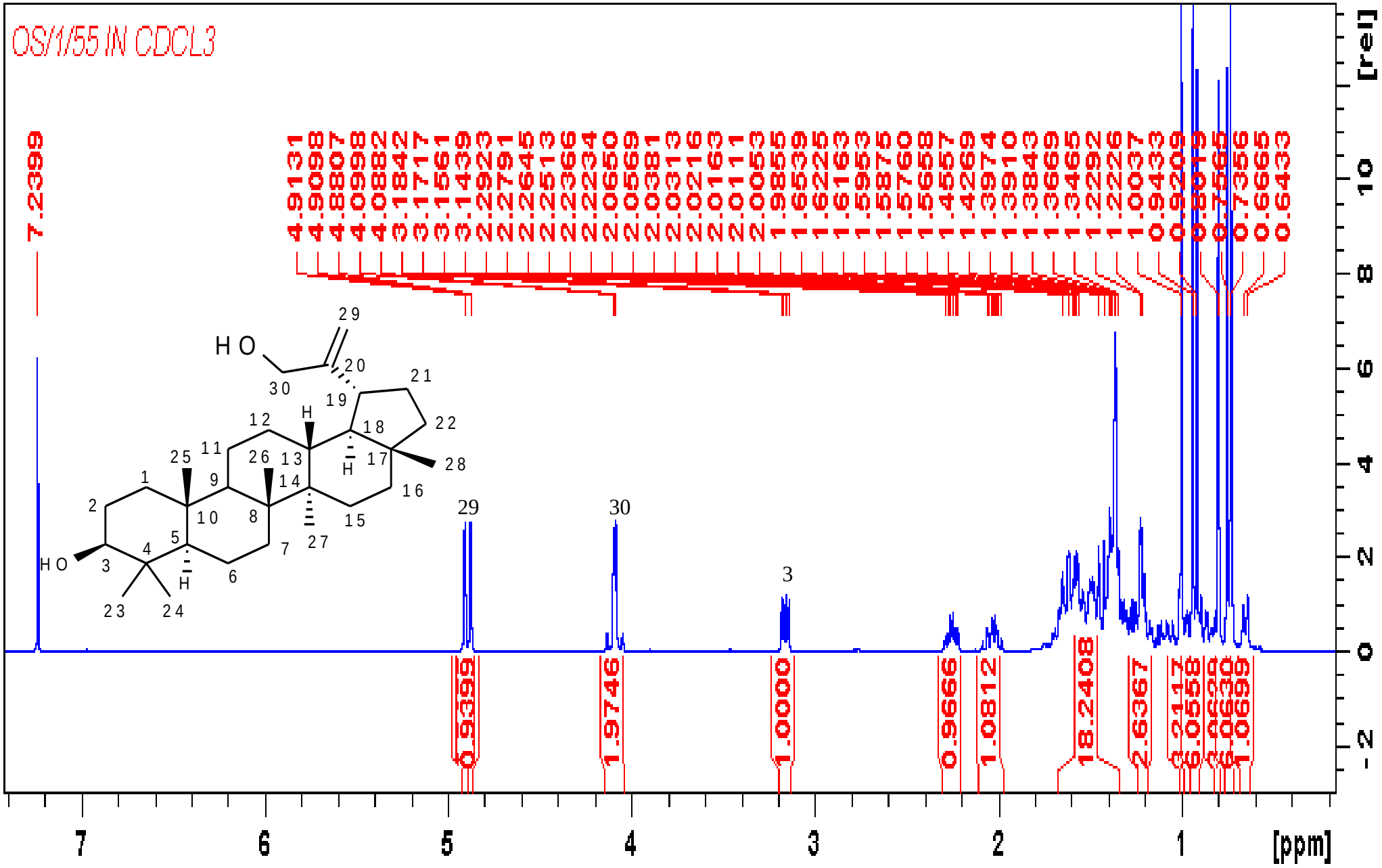
Expanded ^{13}C NMR spectrum of lutein (**A10**)



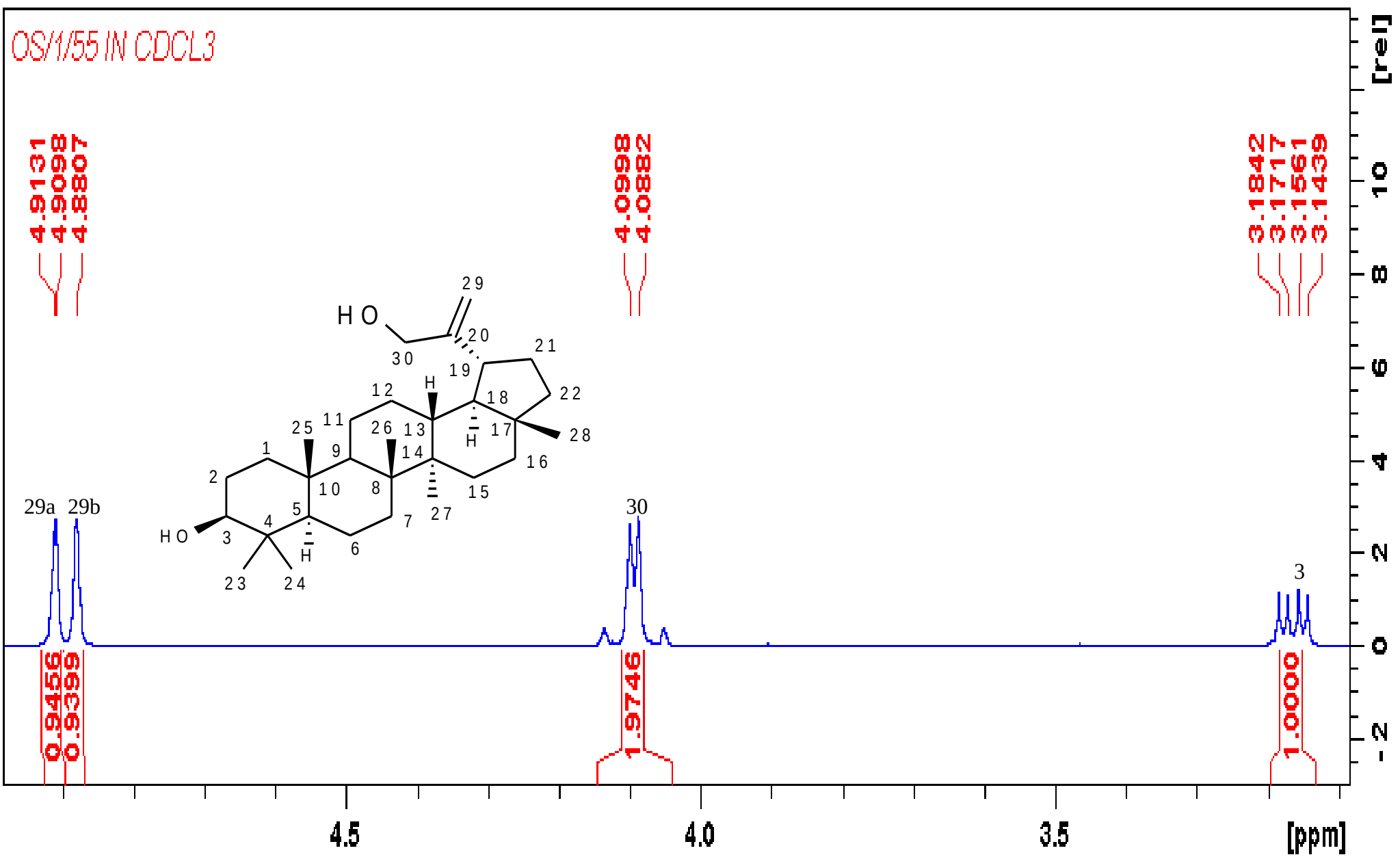
Expanded ^{13}C NMR spectrum of lutein (**A10**)



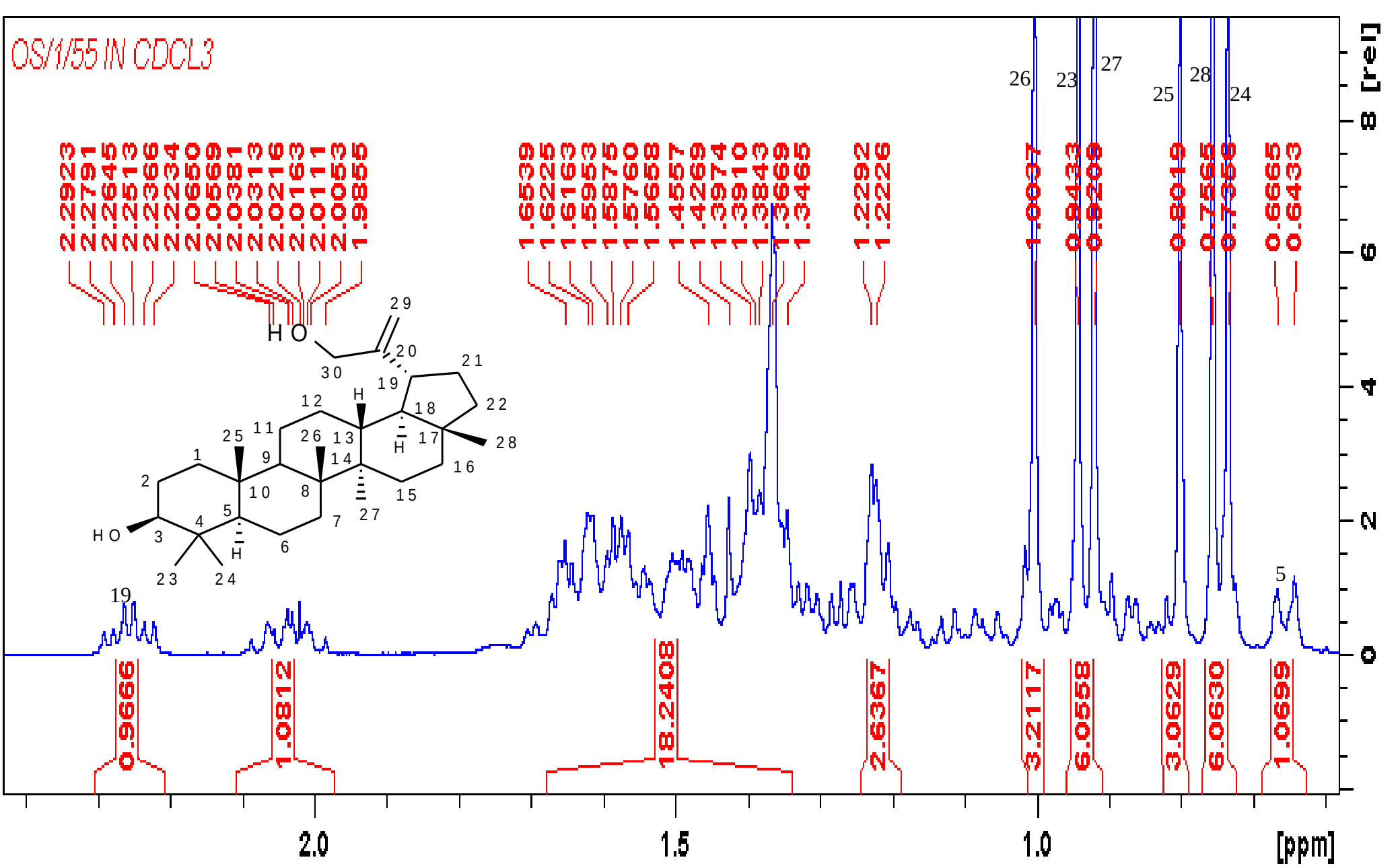
IR spectrum of lutein (**A10**)



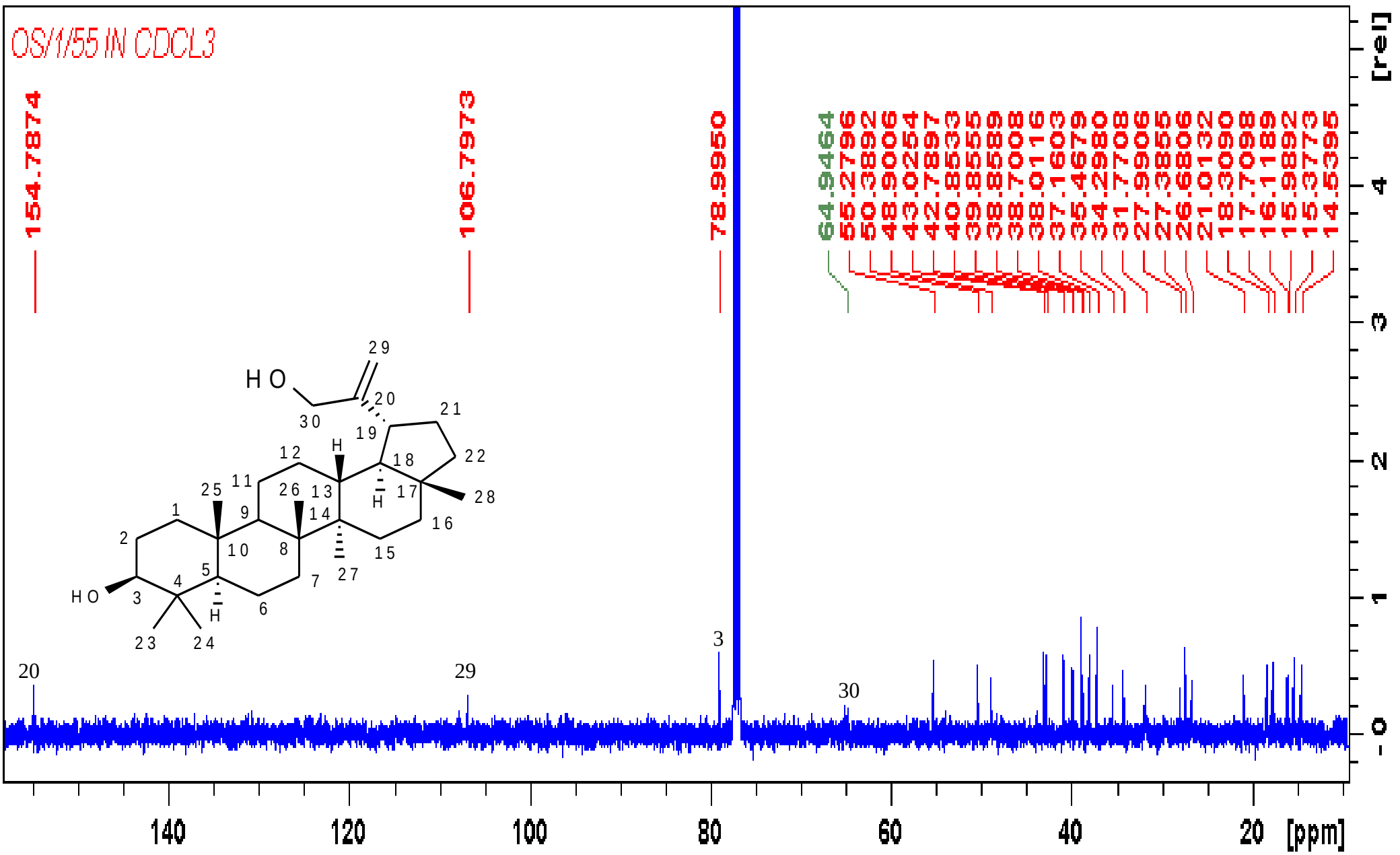
^{1}H NMR spectrum of 30-hydroxylup-20(29)-en-3β-ol (**B1**)



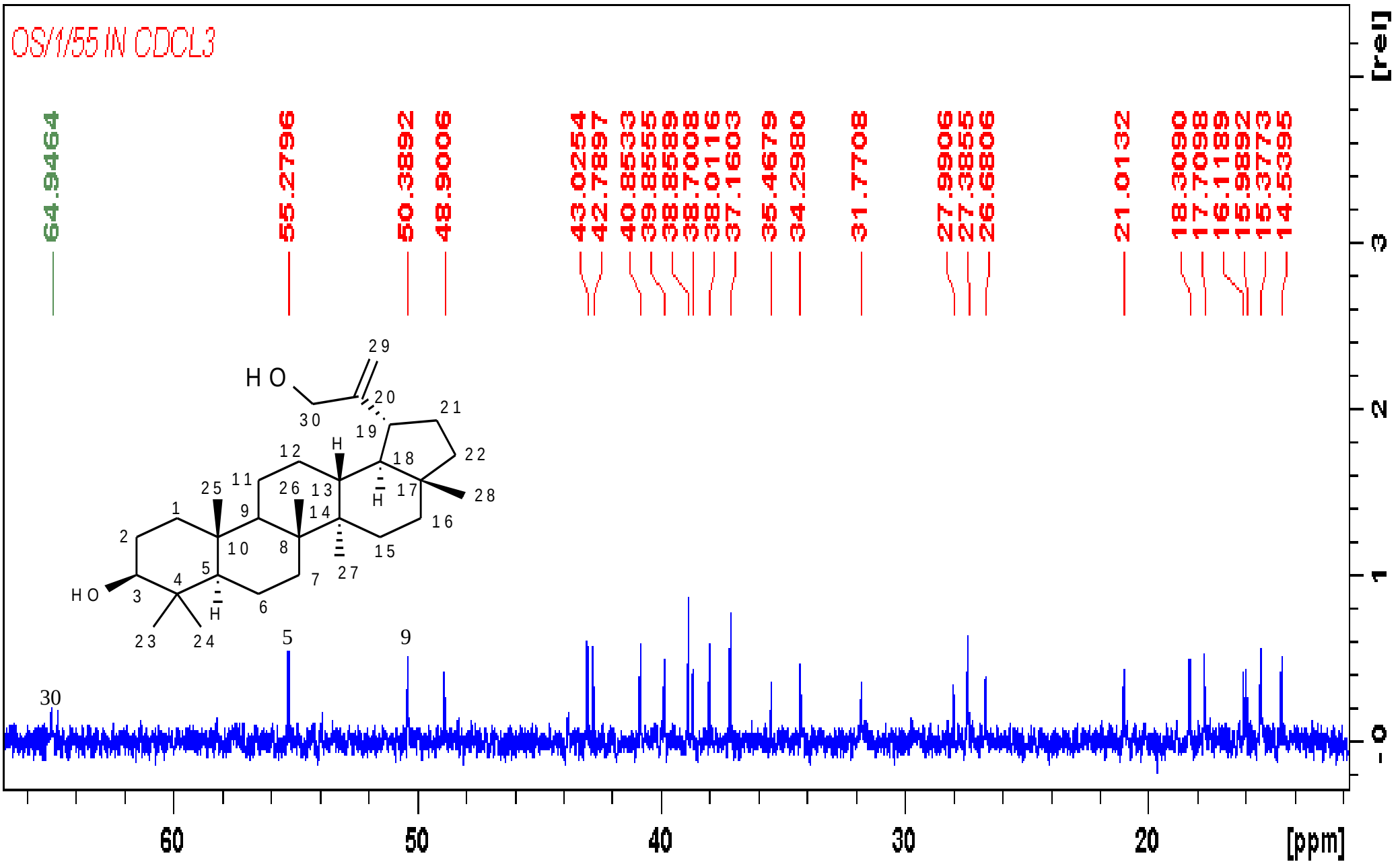
Expanded ^{1}H NMR spectrum 30-hydroxylup-20(29)-en-3β-ol (**B1**)



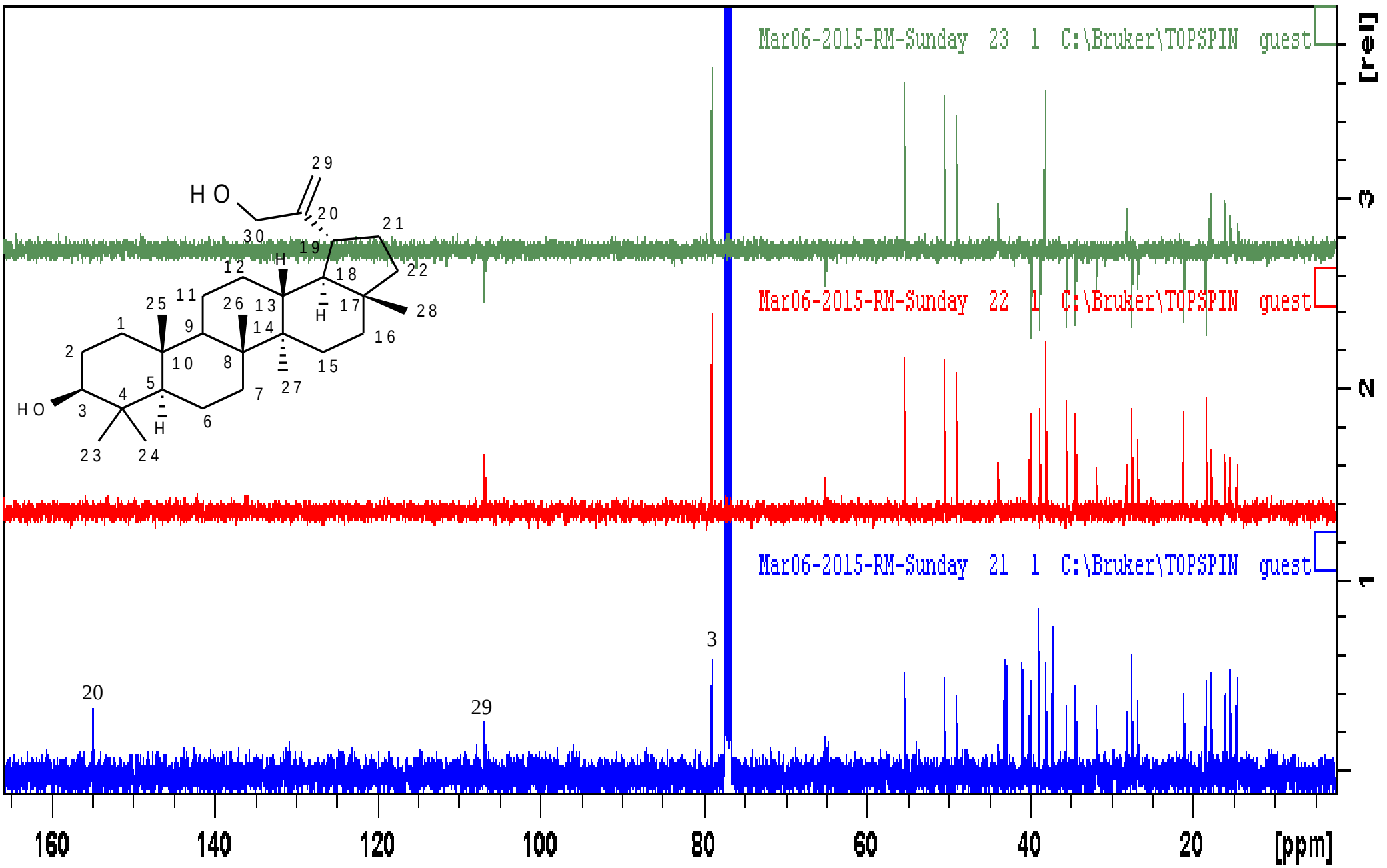
Expanded ^{1}H NMR spectrum of 30-hydroxylup-20(29)-en-3β-ol (**B1**)



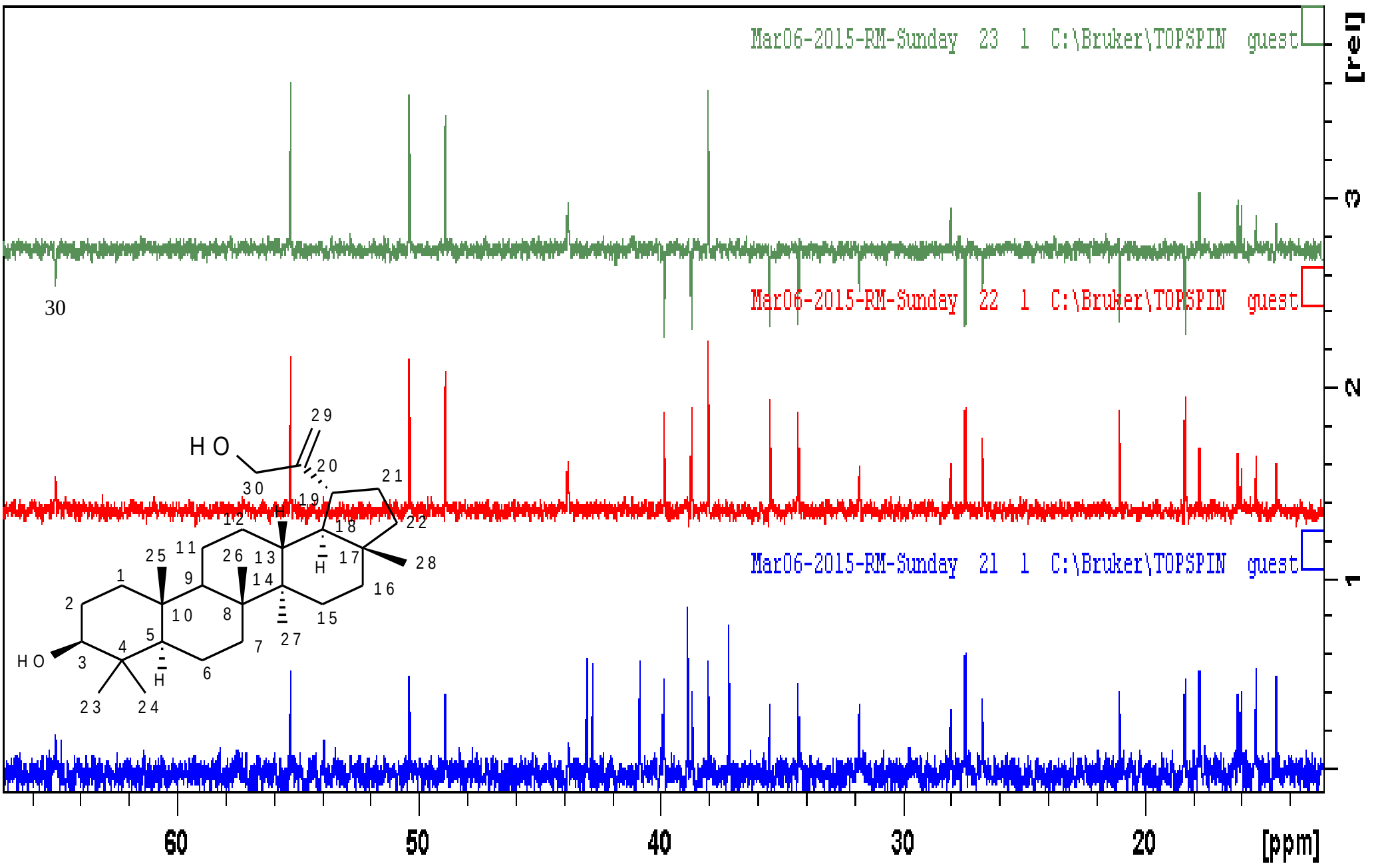
^{13}C NMR spectrum of 30-hydroxylup-20(29)-en-3β-ol (**B1**)



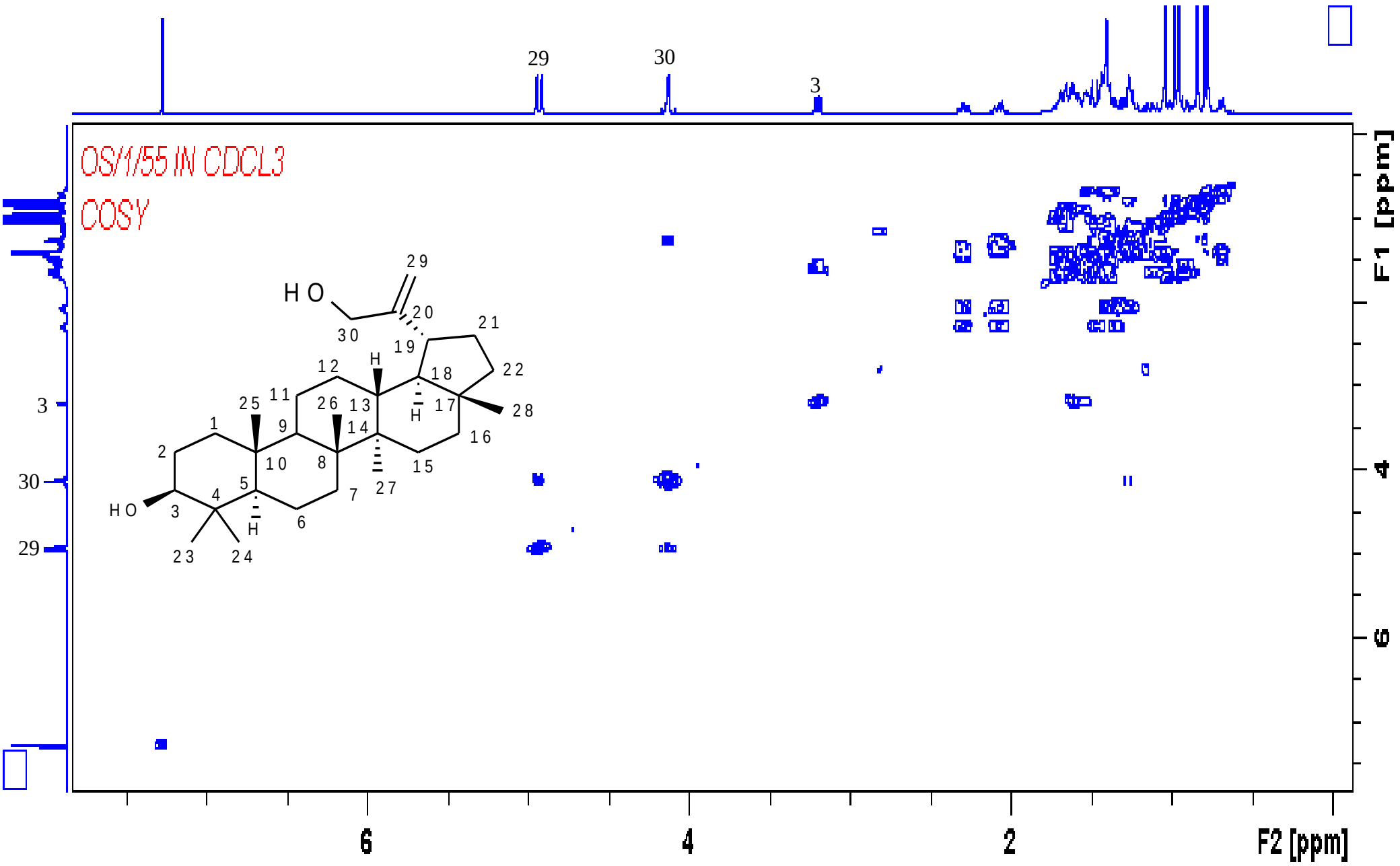
Expanded ^{13}C NMR spectrum of 30-hydroxylup-20(29)-en-3β-ol (**B1**)



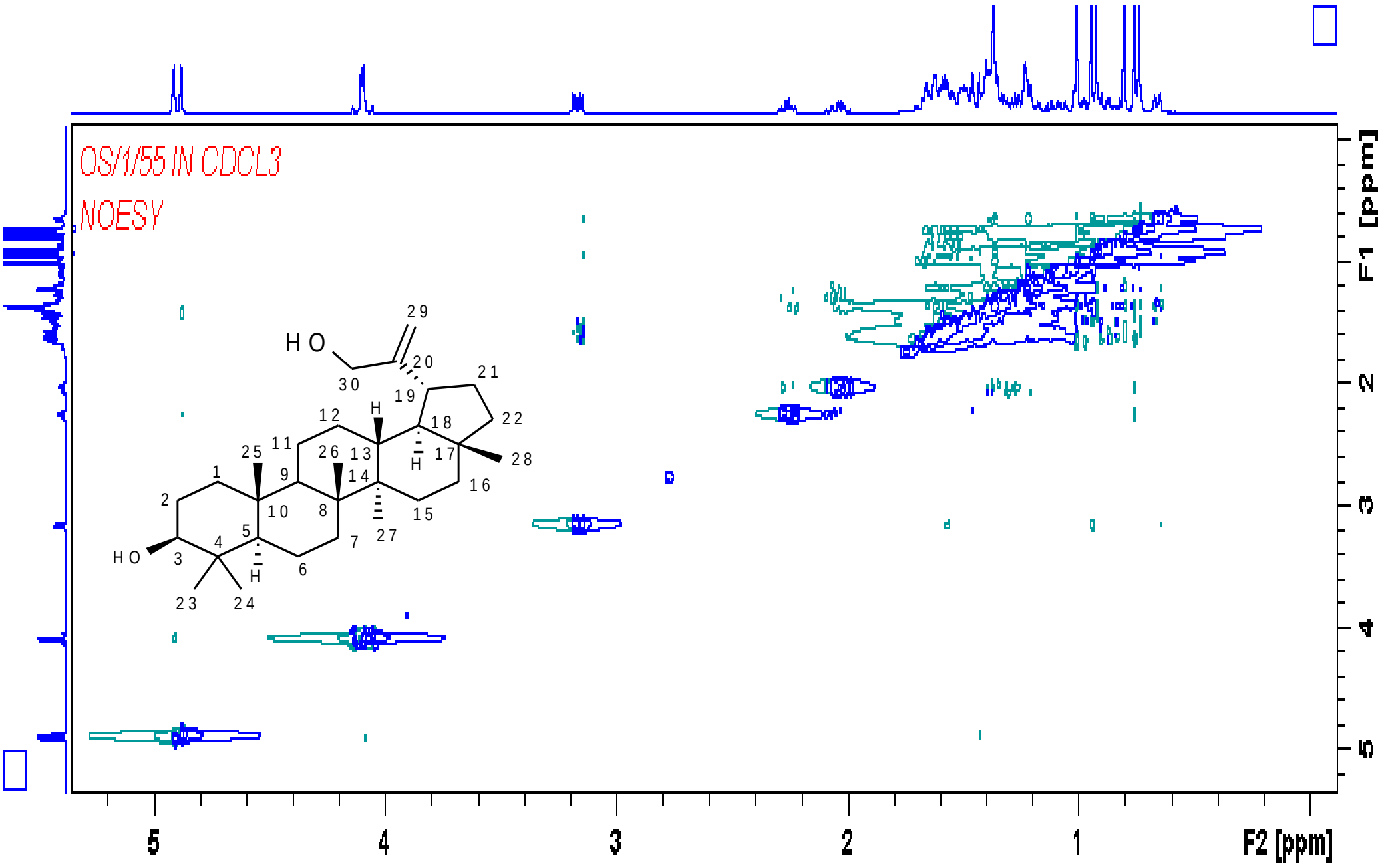
DEPT spectrum of 30-hydroxylup-20(29)-en-3β-ol (**B1**)



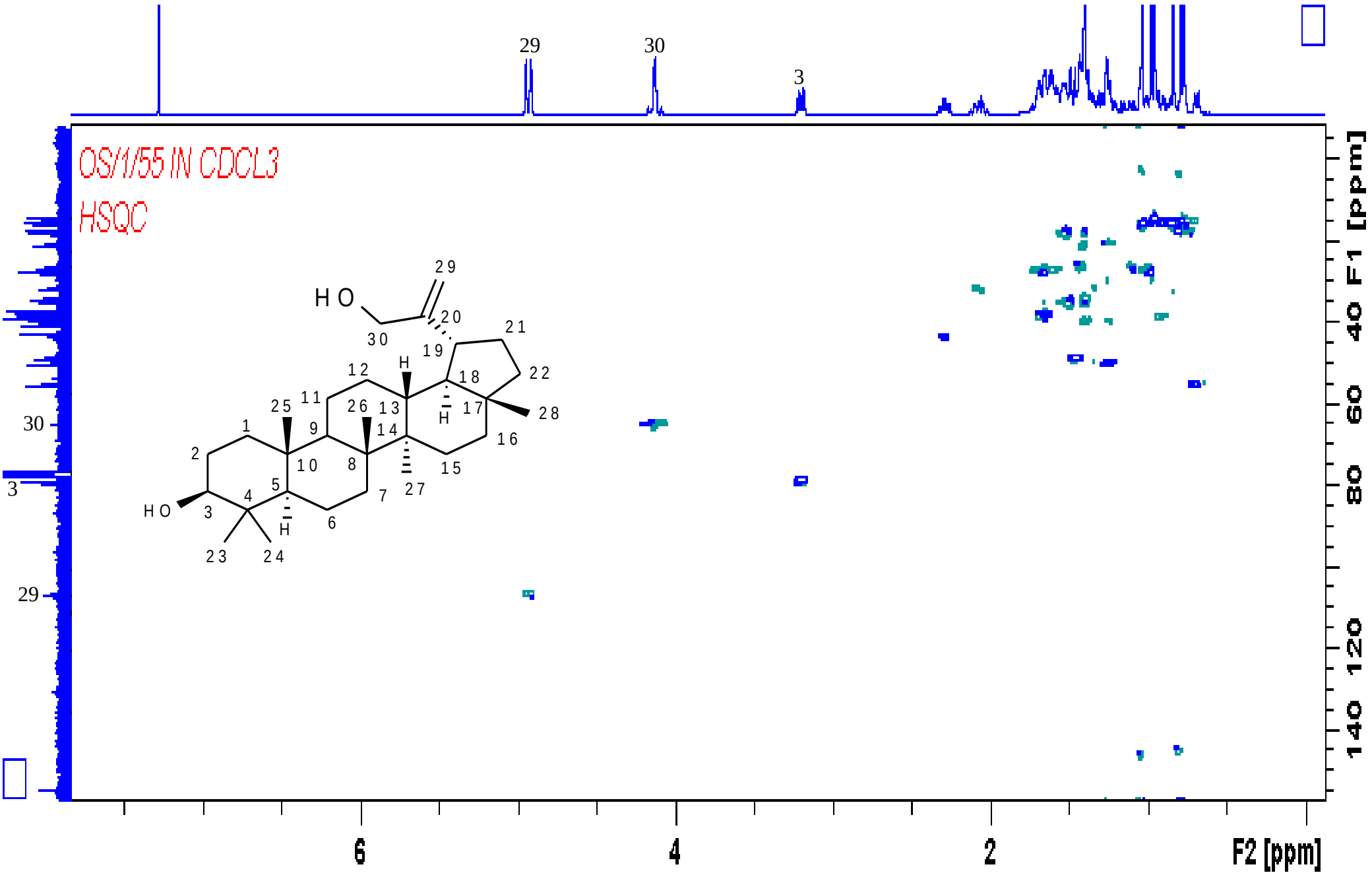
Expanded DEPT spectrum of 30-hydroxylup-20(29)-en-3β-ol (**B1**)



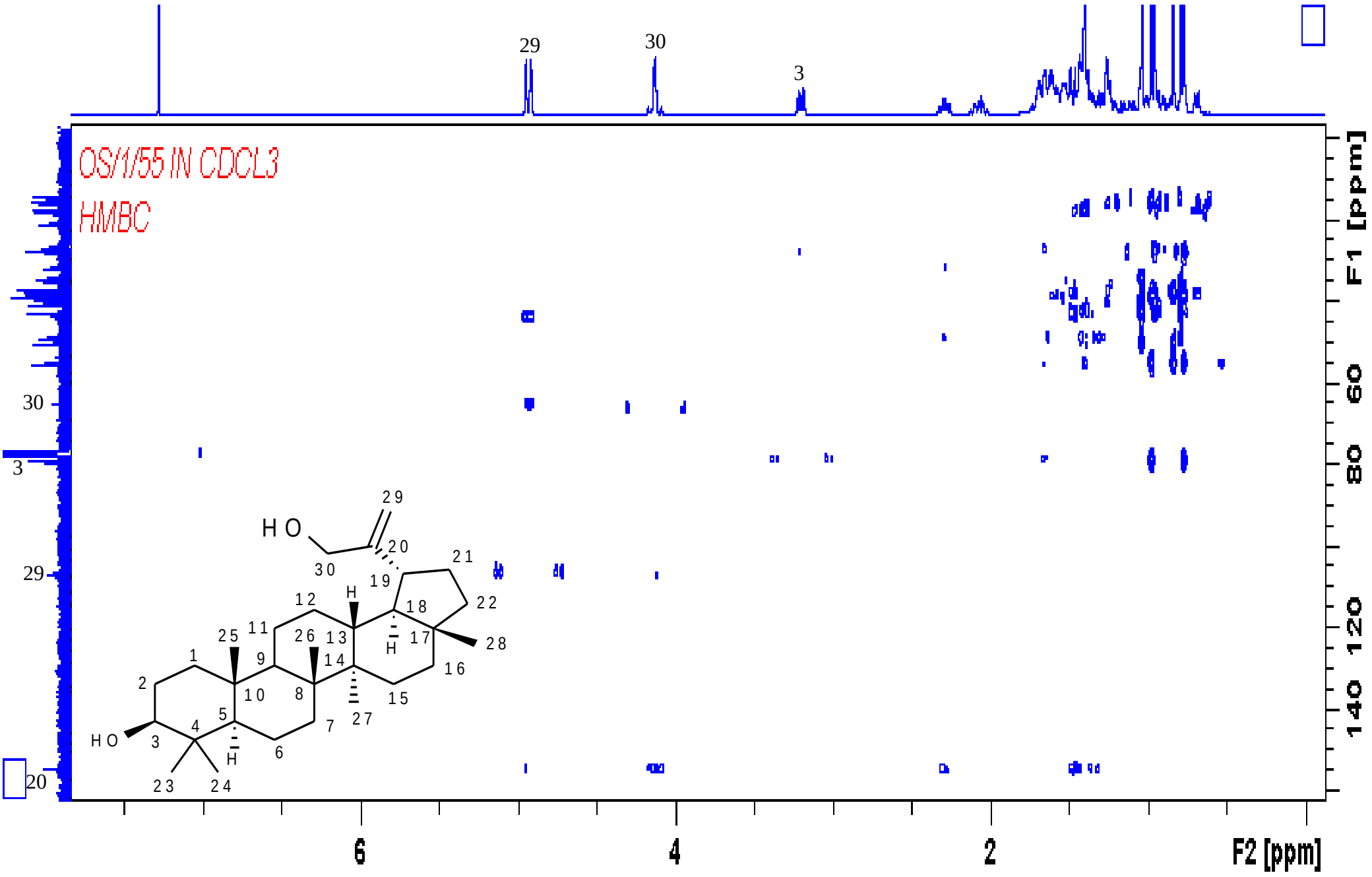
COSY spectrum of 30-hydroxylup-20(29)-en-3*β*-ol (**B1**)



NOESY spectrum of 30-hydroxylup-20(29)-en-3*β*-ol (**B1**)



HSQC spectrum of 30-hydroxylup-20(29)-en-3*β*-ol (**B1**)



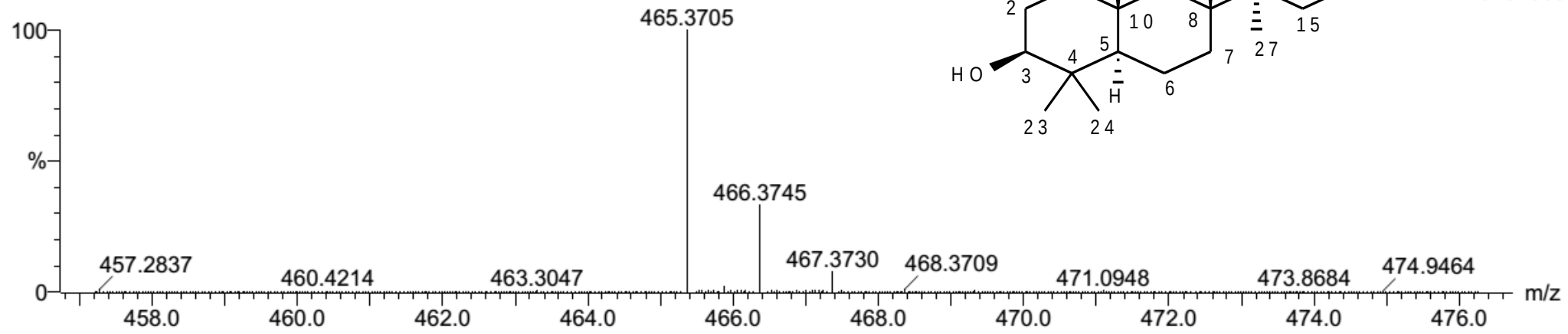
HMBC spectrum of 30-hydroxylup-20(29)-en-3β-ol (**B1**)

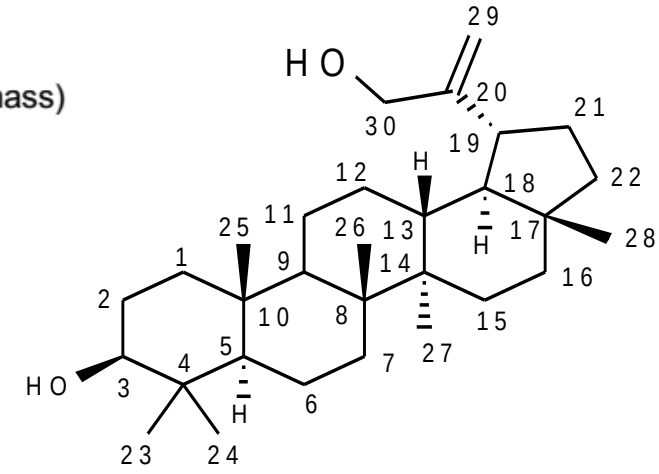
Single Mass Analysis

Tolerance = 5.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
8 formula(e) evaluated with 1 results within limits (up to 20 closest results for each mass)
Elements Used:
C: 20-35 H: 40-55 O: 0-5 Na: 1-1

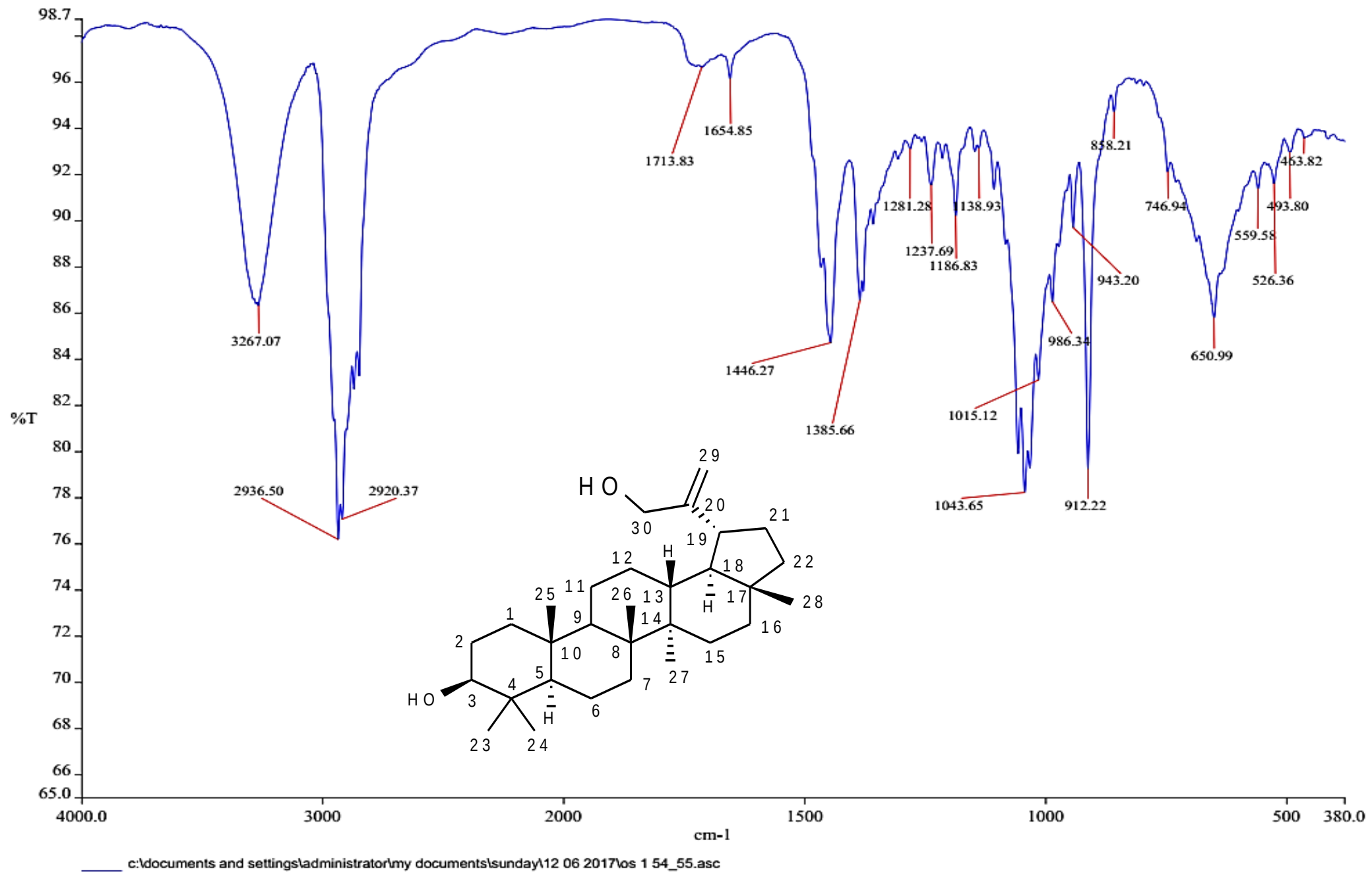
05-1-55 17 (0.573) Cm (1:60)
TOF MS ES+



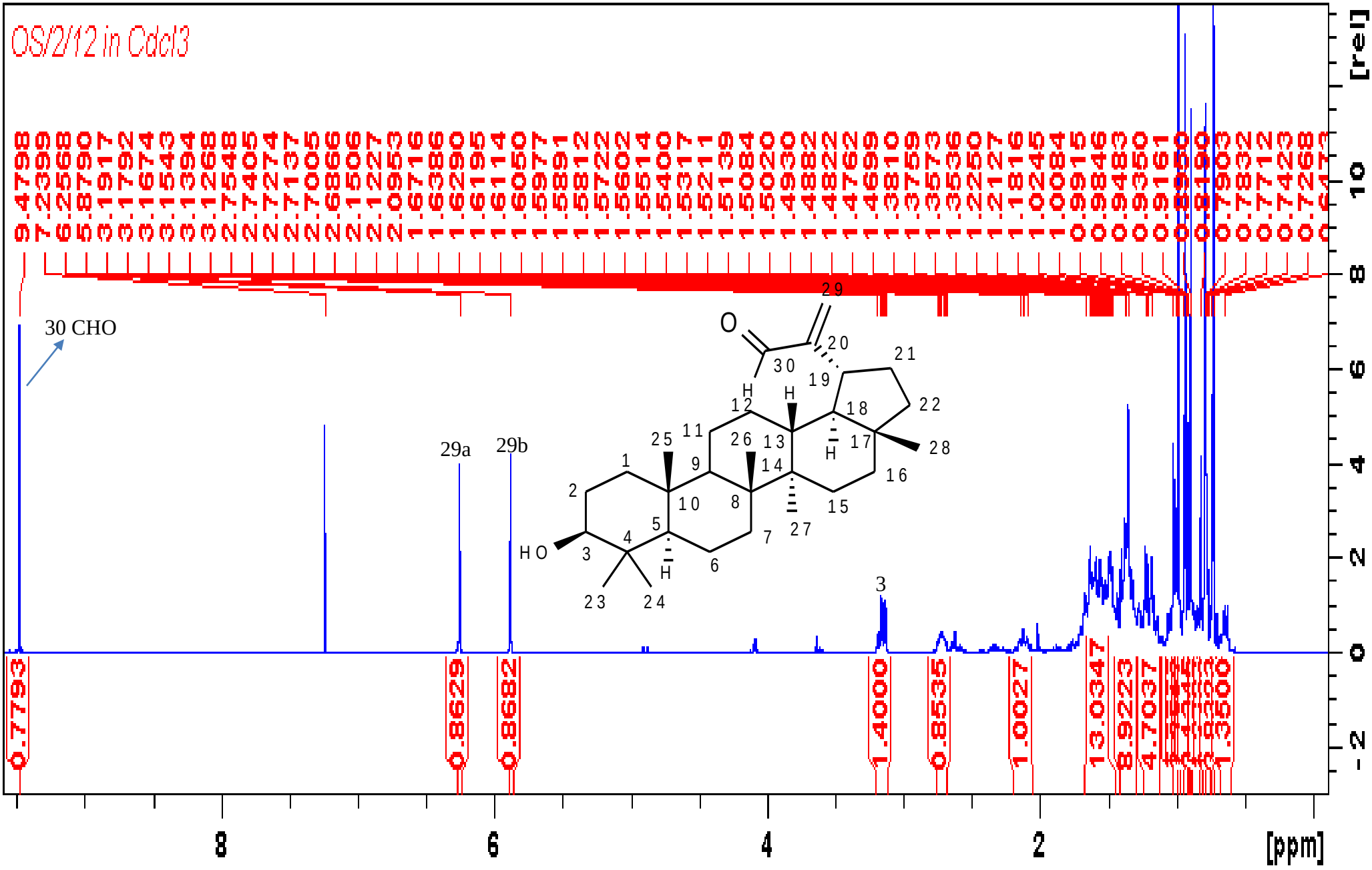


Minimum:				-1.5			
Maximum:		5.0	5.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
465.3705	465.3709	-0.4	-0.9	5.5	565.1	0.0	C30 H50 O2 Na

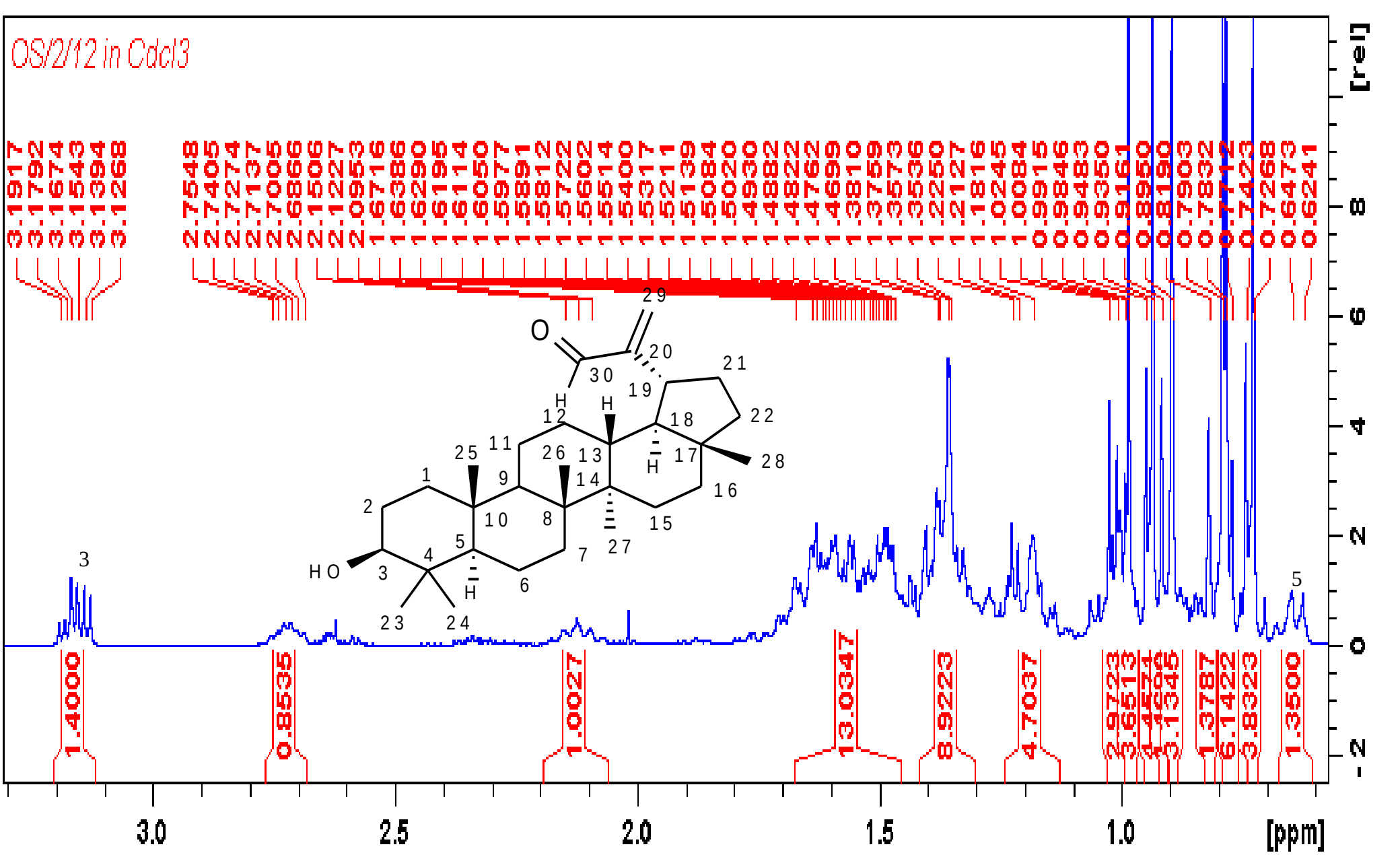
Mass Spectrum of 30-hydroxylup-20(29)-en-3β-ol (**B1**)



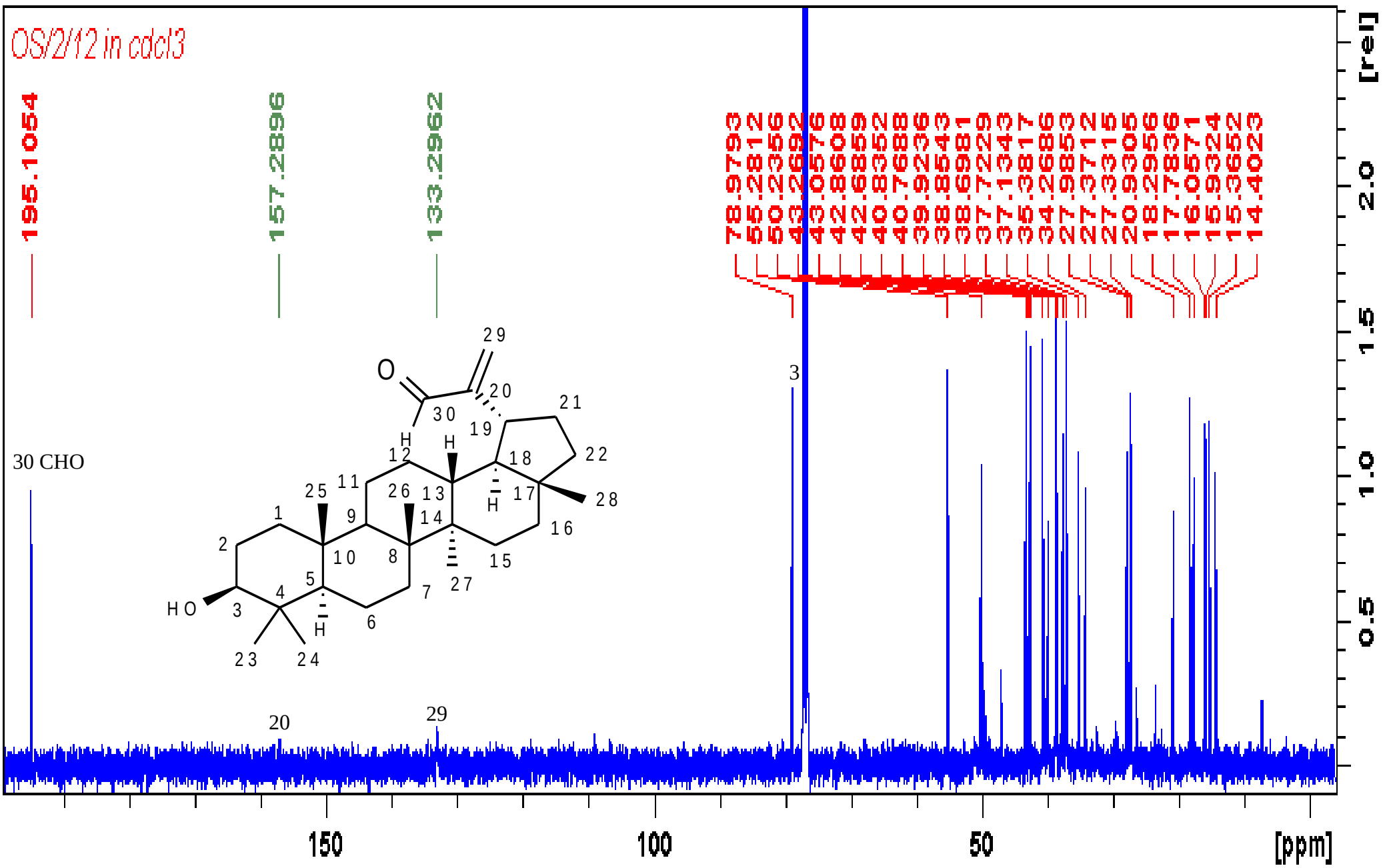
IR Spectrum of 30-hydroxylup-20(29)-en-3β-ol (**B1**)



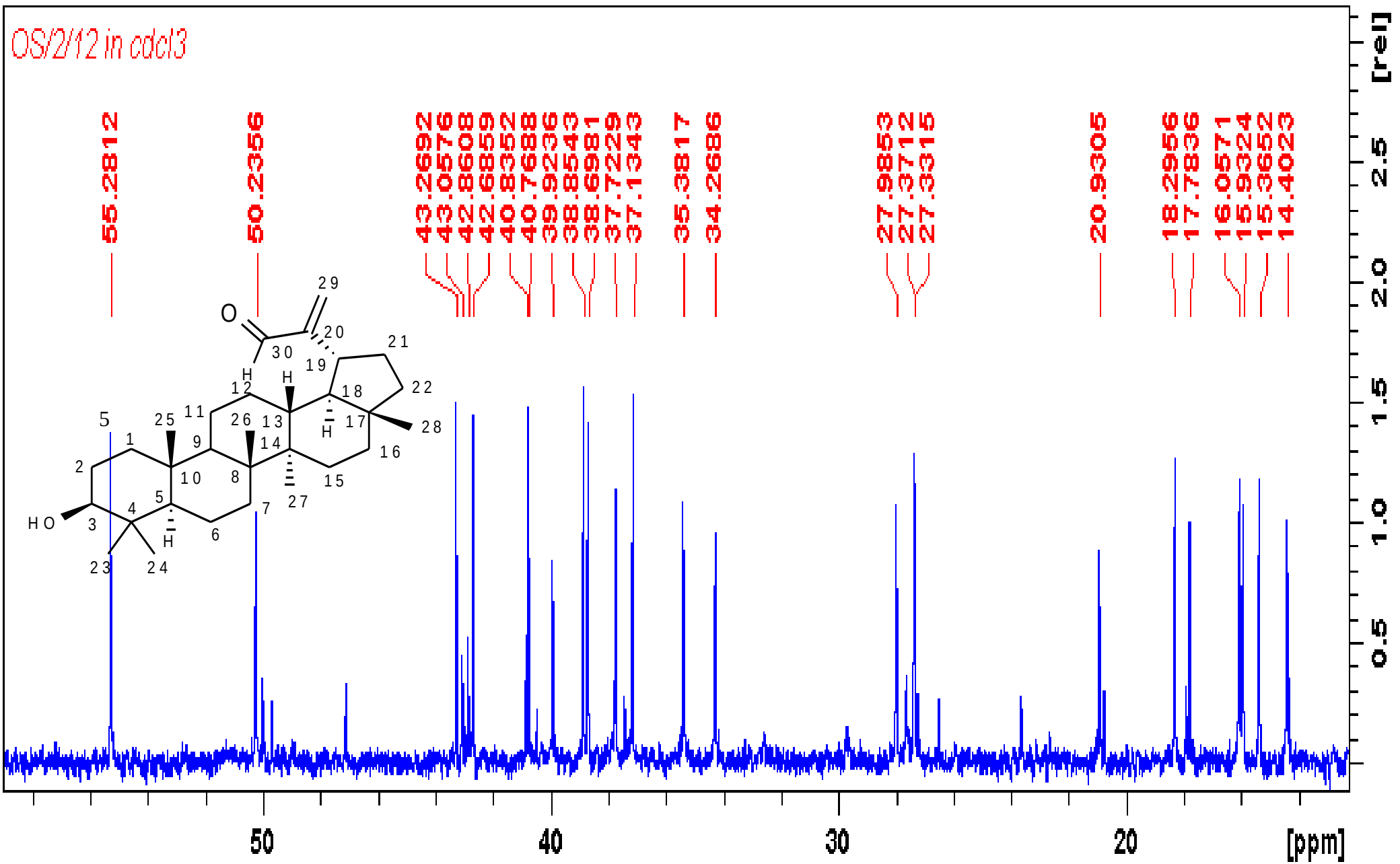
^{1}H NMR spectrum of 3β-hydroxy-20(29)-en-lupan-30-al (**B2**)



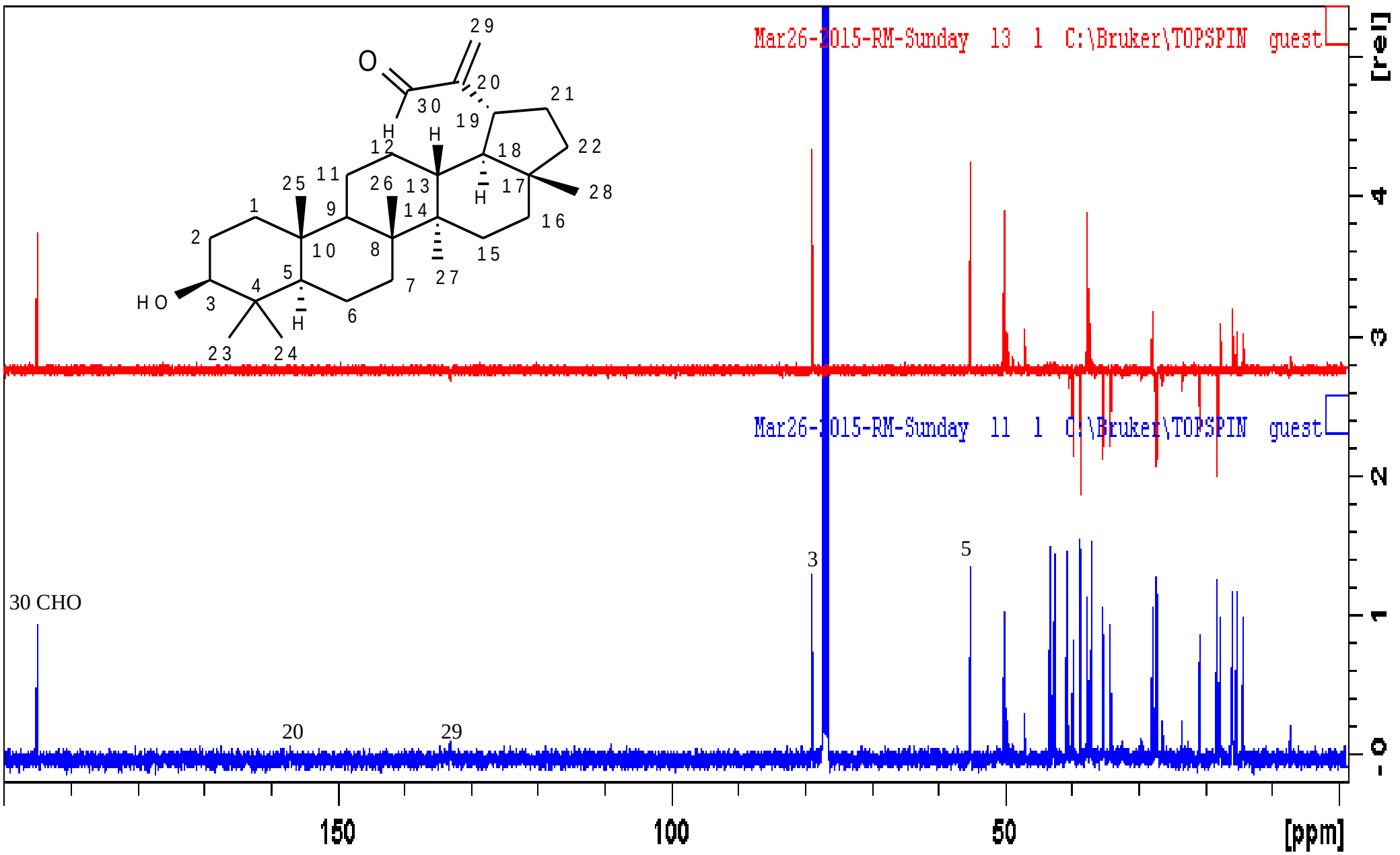
Expanded ^{1}H NMR spectrum of 3β-hydroxy-20(29)-en-lupan-30-al (**B2**)



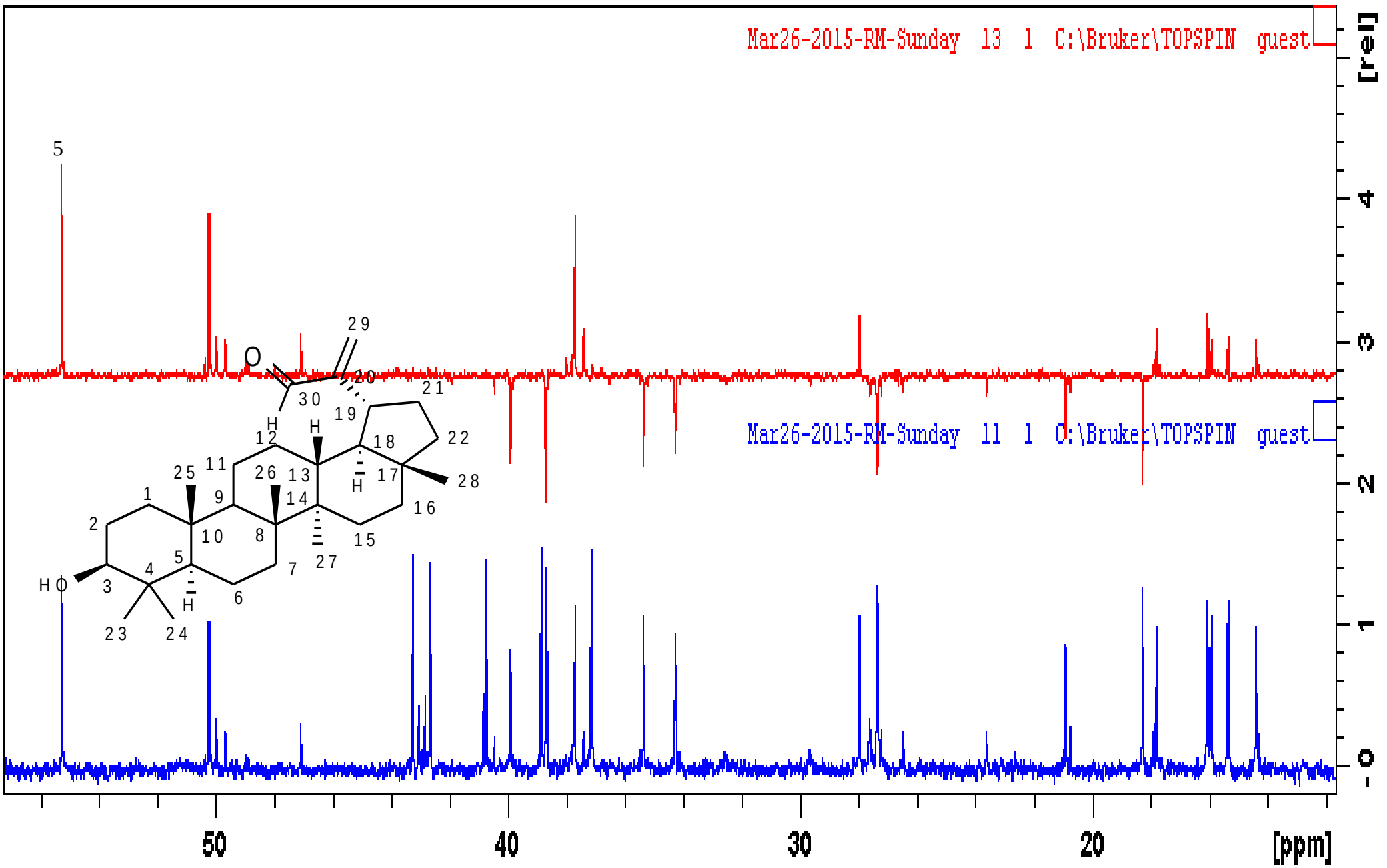
^{13}C NMR spectrum of 3β-hydroxy-20(29)-en-lupan-30-al (**B2**)



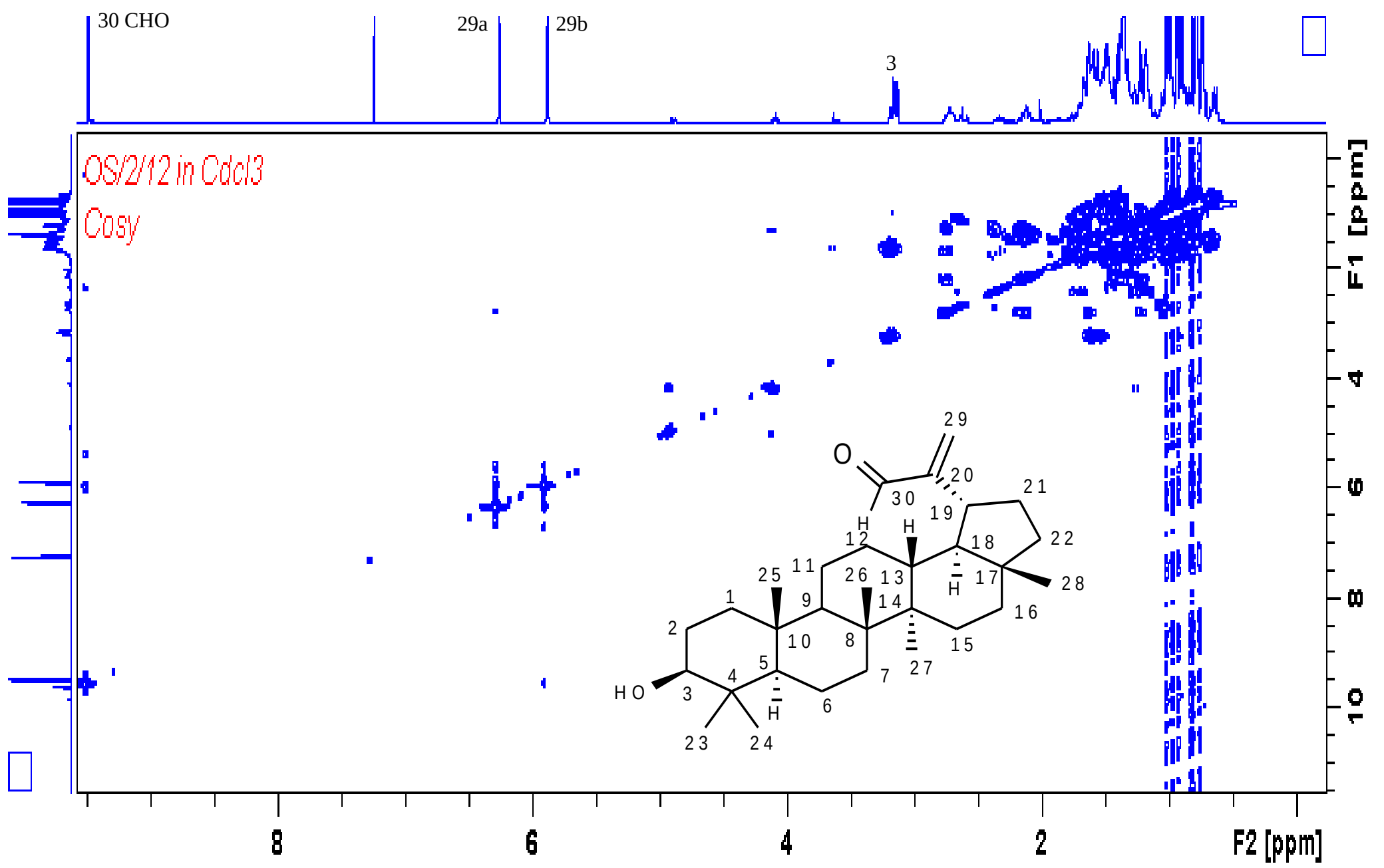
Expanded ^{13}C NMR spectrum of 3β-hydroxy-20(29)-en-lupan-30-al (**B2**)



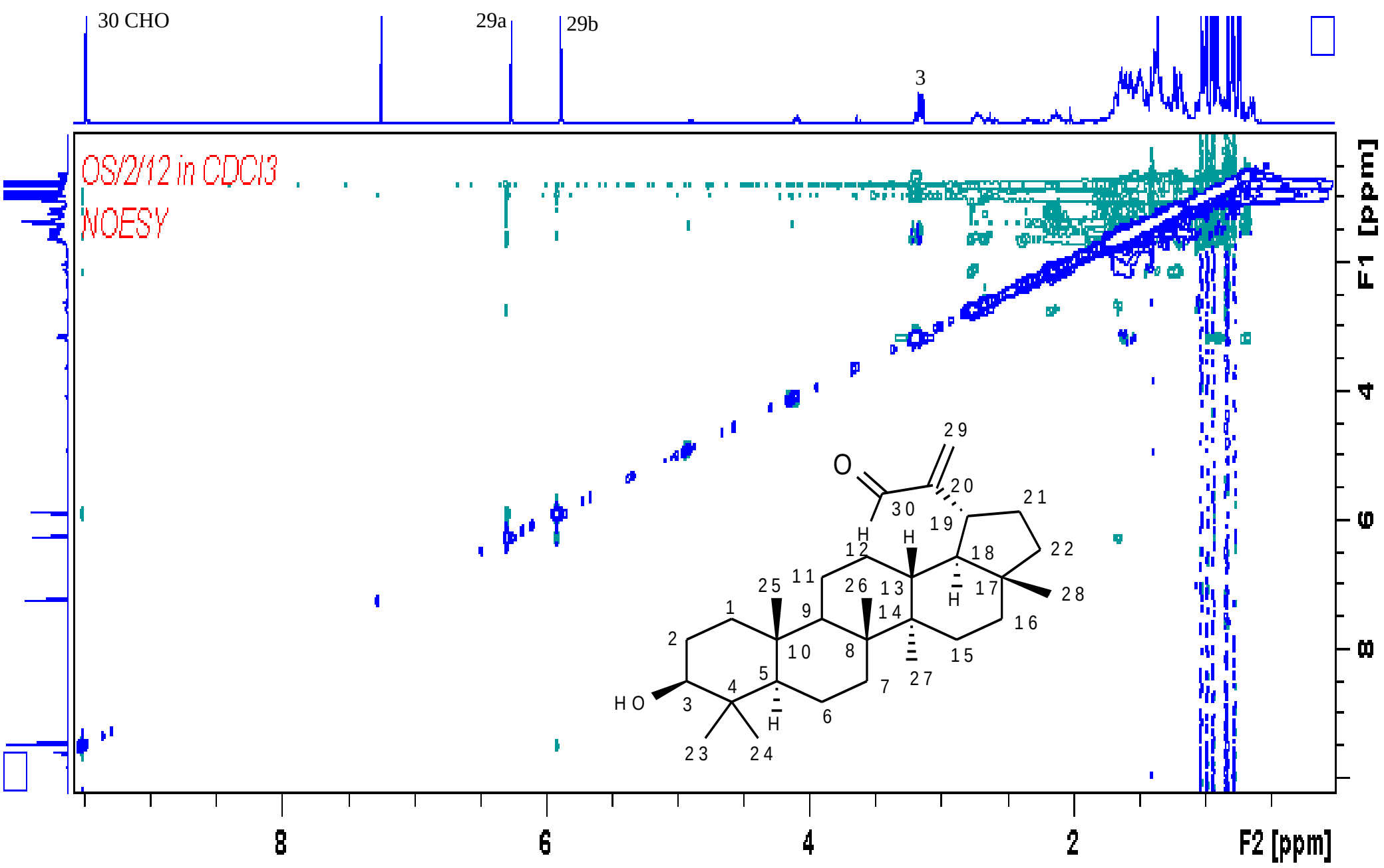
DEPT spectrum of 3β-hydroxy-20(29)-en-lupan-30-al (**B2**)



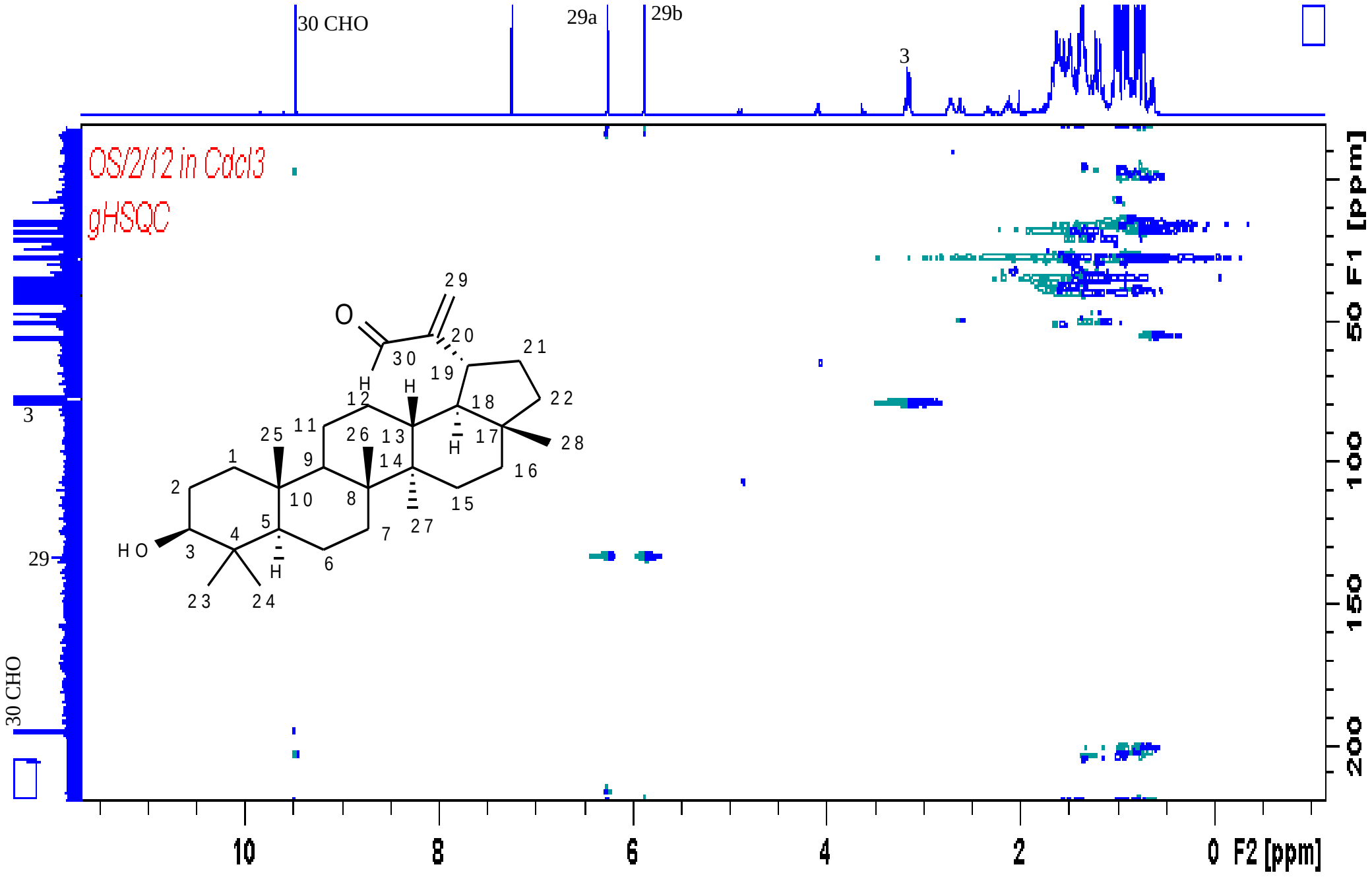
Expanded DEPT spectrum of 3β-hydroxy-20(29)-en-lupan-30-al (**B2**)



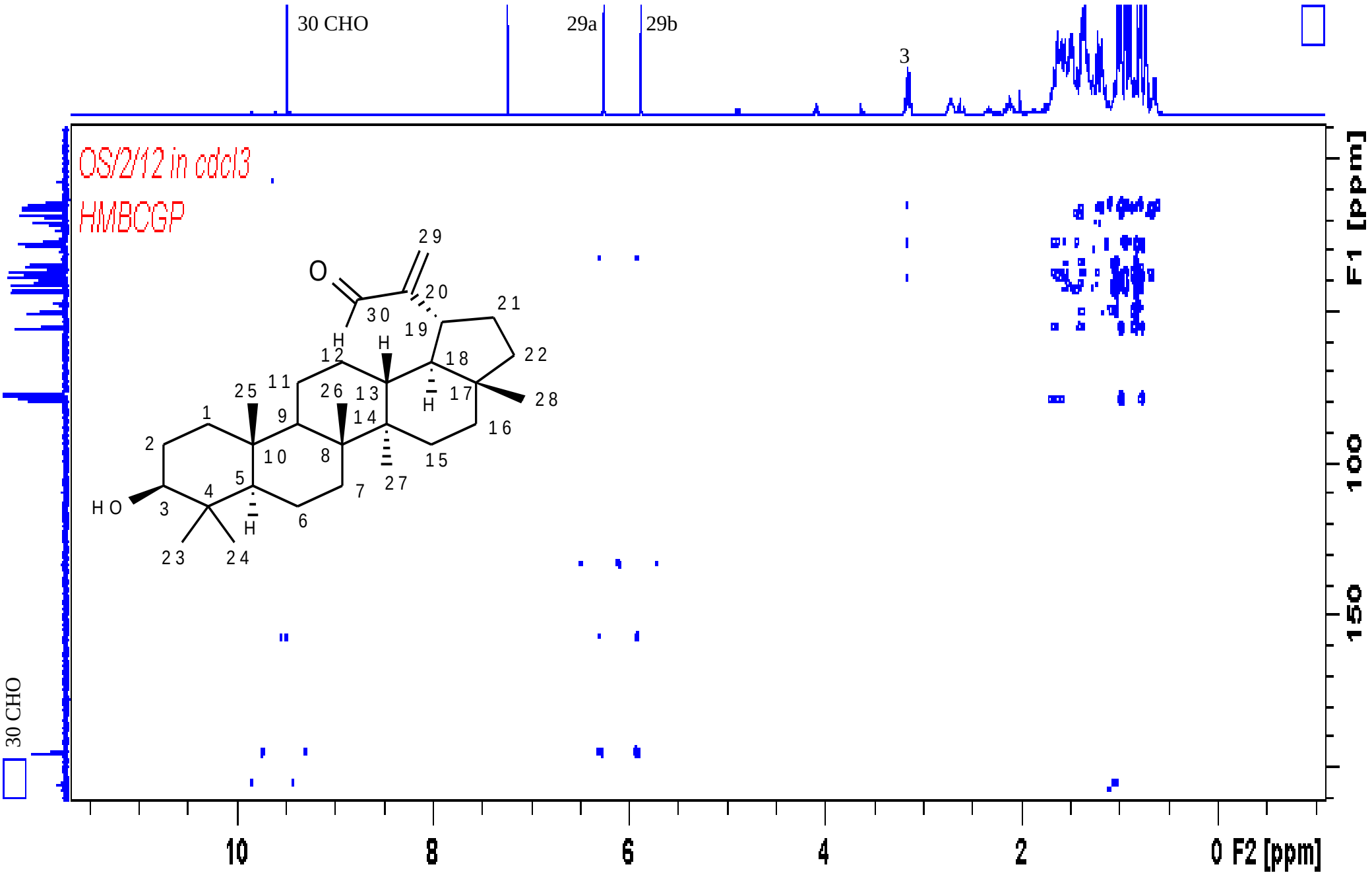
COSY spectrum of 3β-hydroxy-20(29)-en-lupan-30-al (**B2**)



NOESY spectrum of 3β-hydroxy-20(29)-en-lupan-30-al (**B2**)



HSQC spectrum of 3*β*-hydroxy-20(29)-en-lupan-30-al (**B2**)



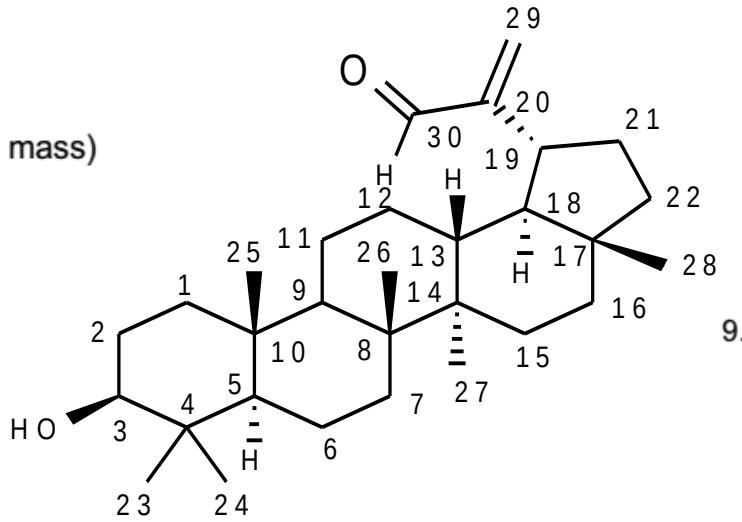
HMBC spectrum of 3β-hydroxy-20(29)-en-lupan-30-al (**B2**)

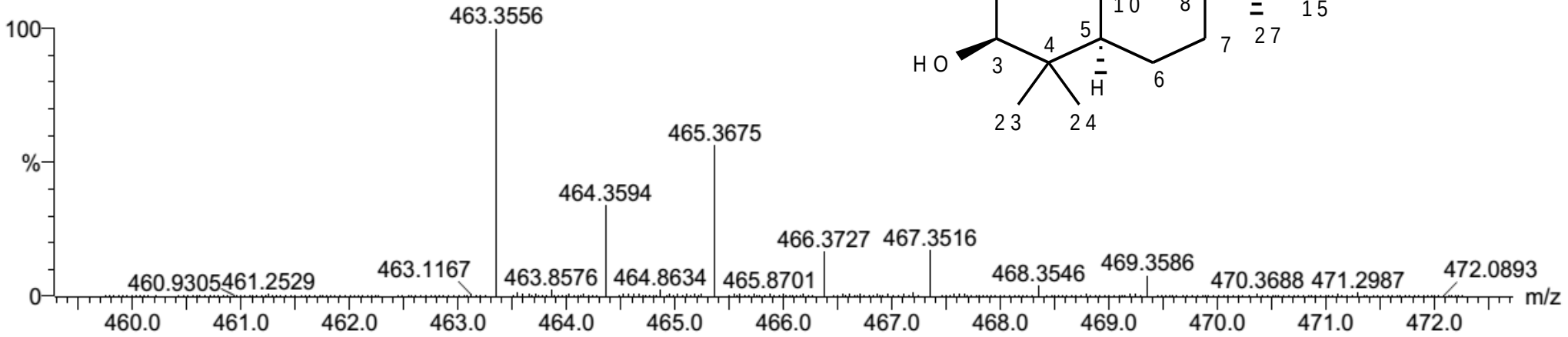
Single Mass Analysis

Tolerance = 5.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
8 formula(e) evaluated with 1 results within limits (up to 20 closest results for each mass)
Elements Used:
C: 20-35 H: 40-55 O: 0-5 Na: 1-1

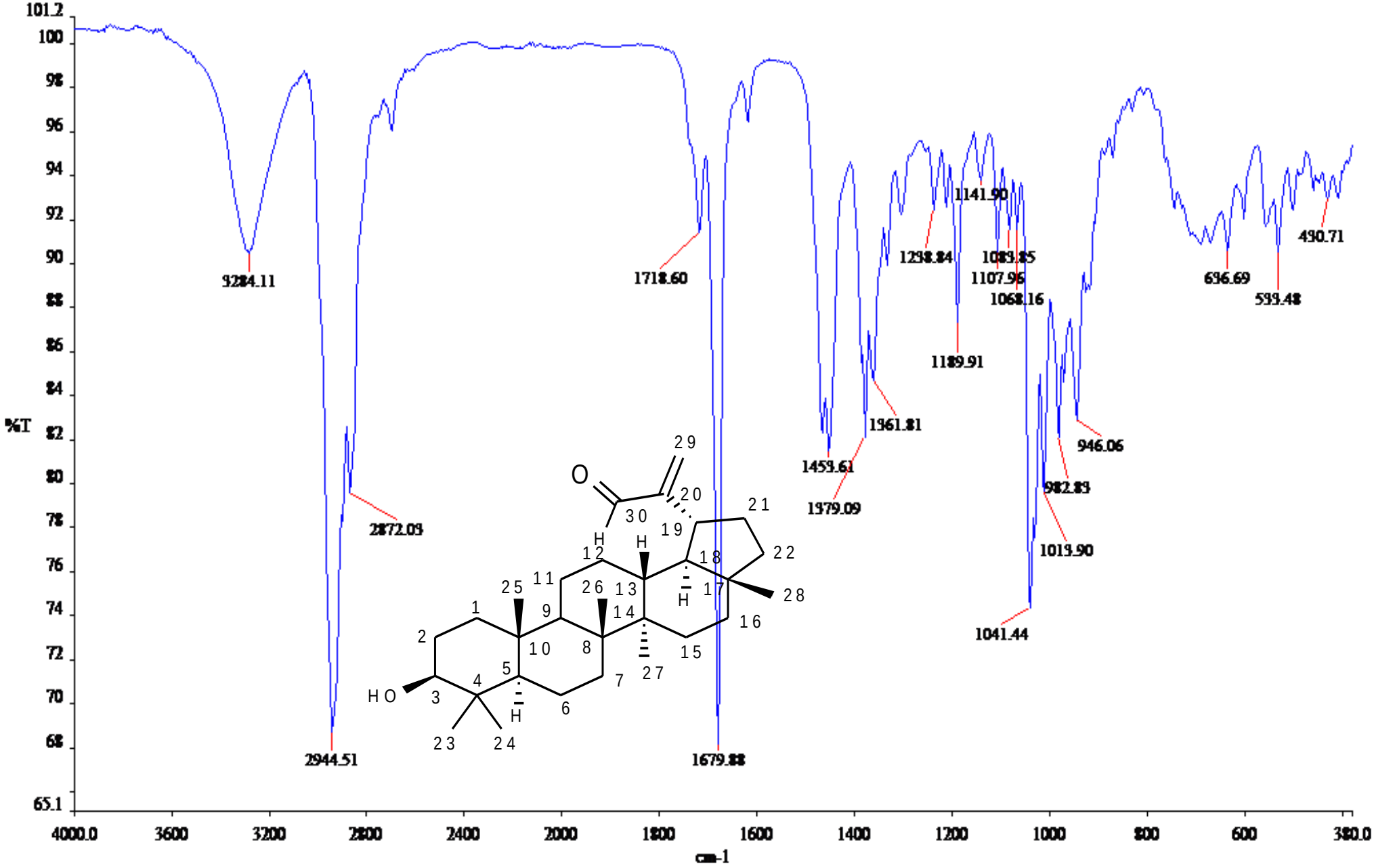
05-2-12 53 (1.755) Cm (1:61)
TOF MS ES+



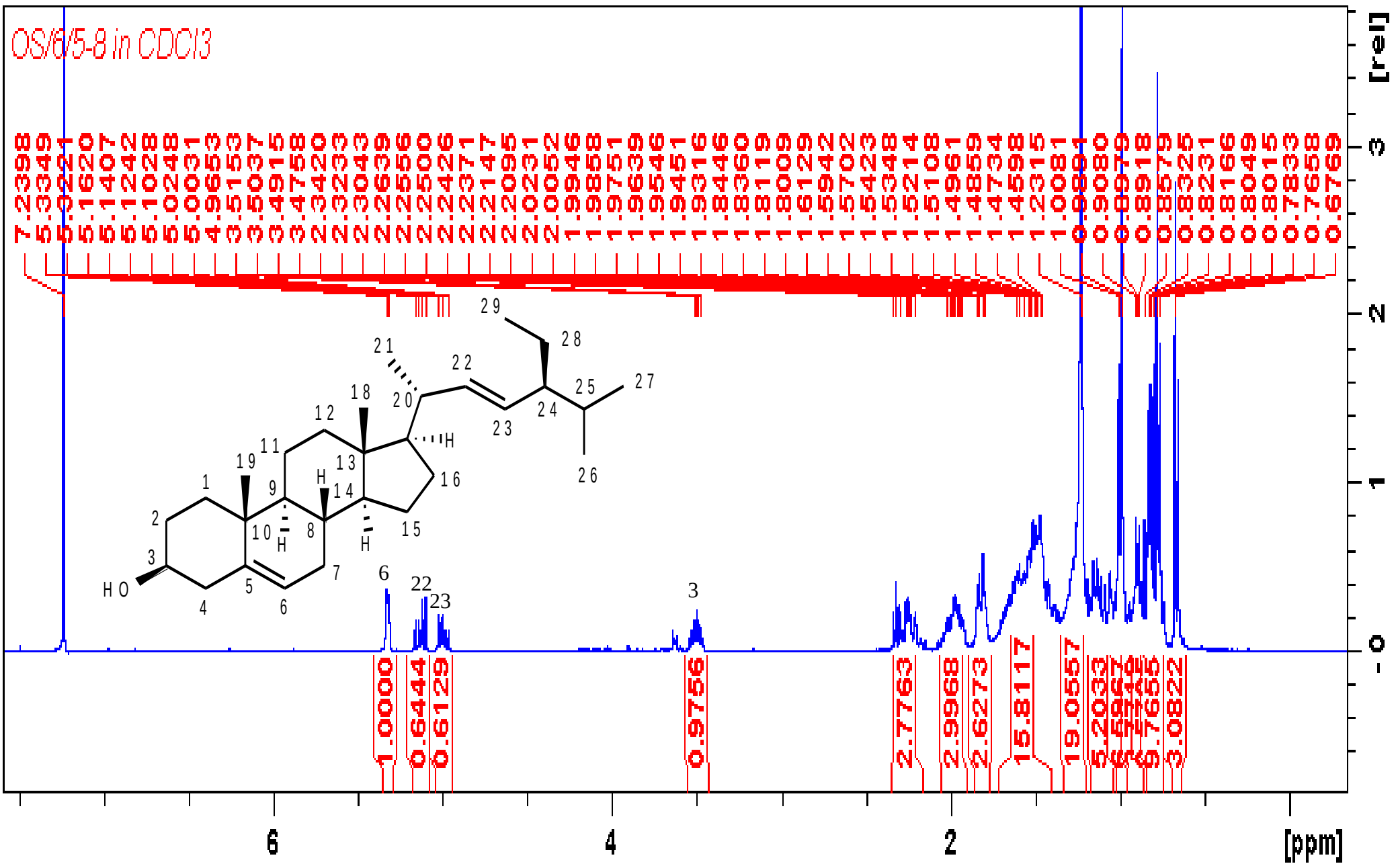


Minimum:				-1.5			
Maximum:		5.0	5.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
463.3556	463.3552	0.4	0.9	6.5	597.7	0.0	C30 H48 O2 Na

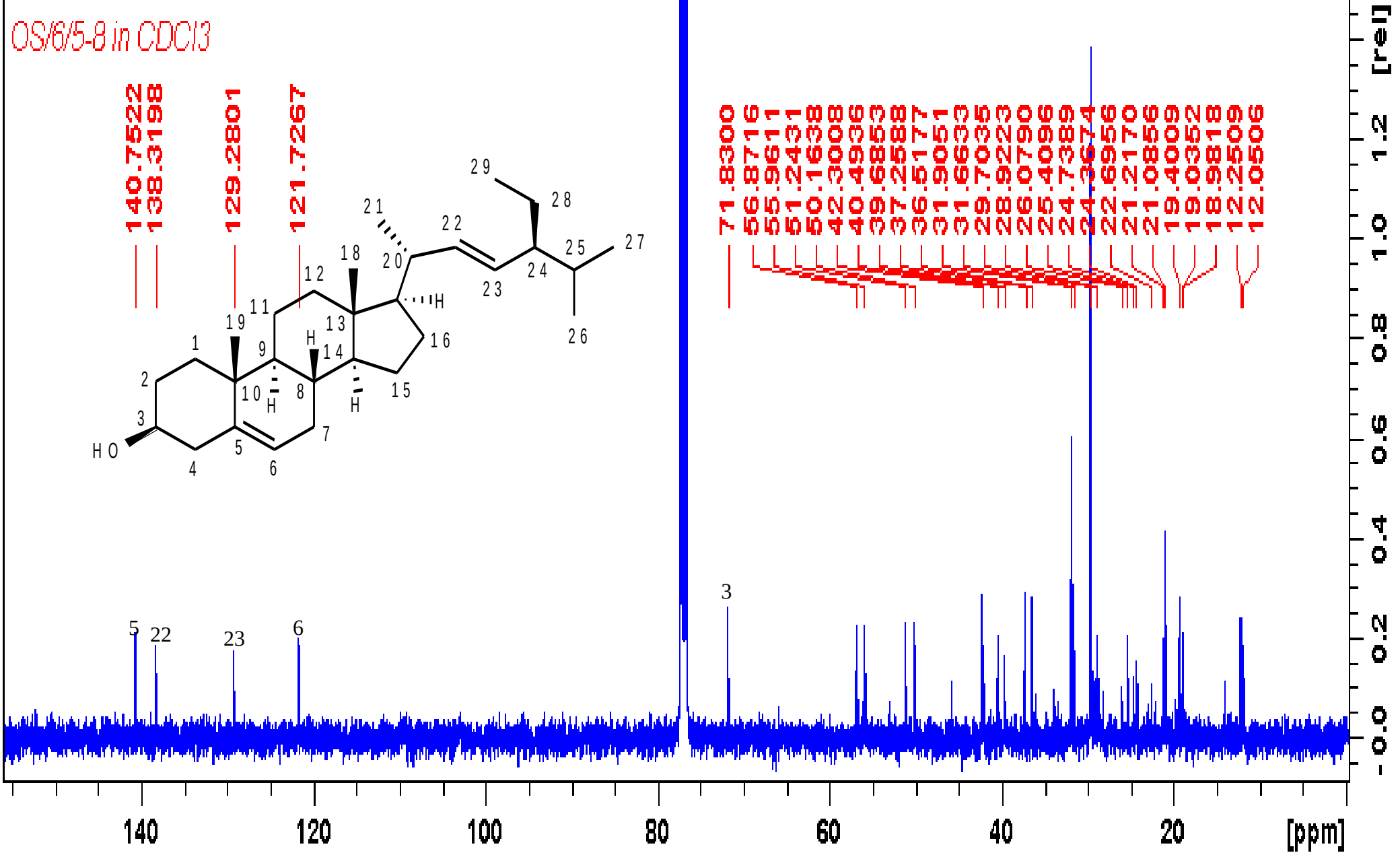
Mass Spectrum of 3β-hydroxy-20(29)-en-lupan-30-al (**B2**)



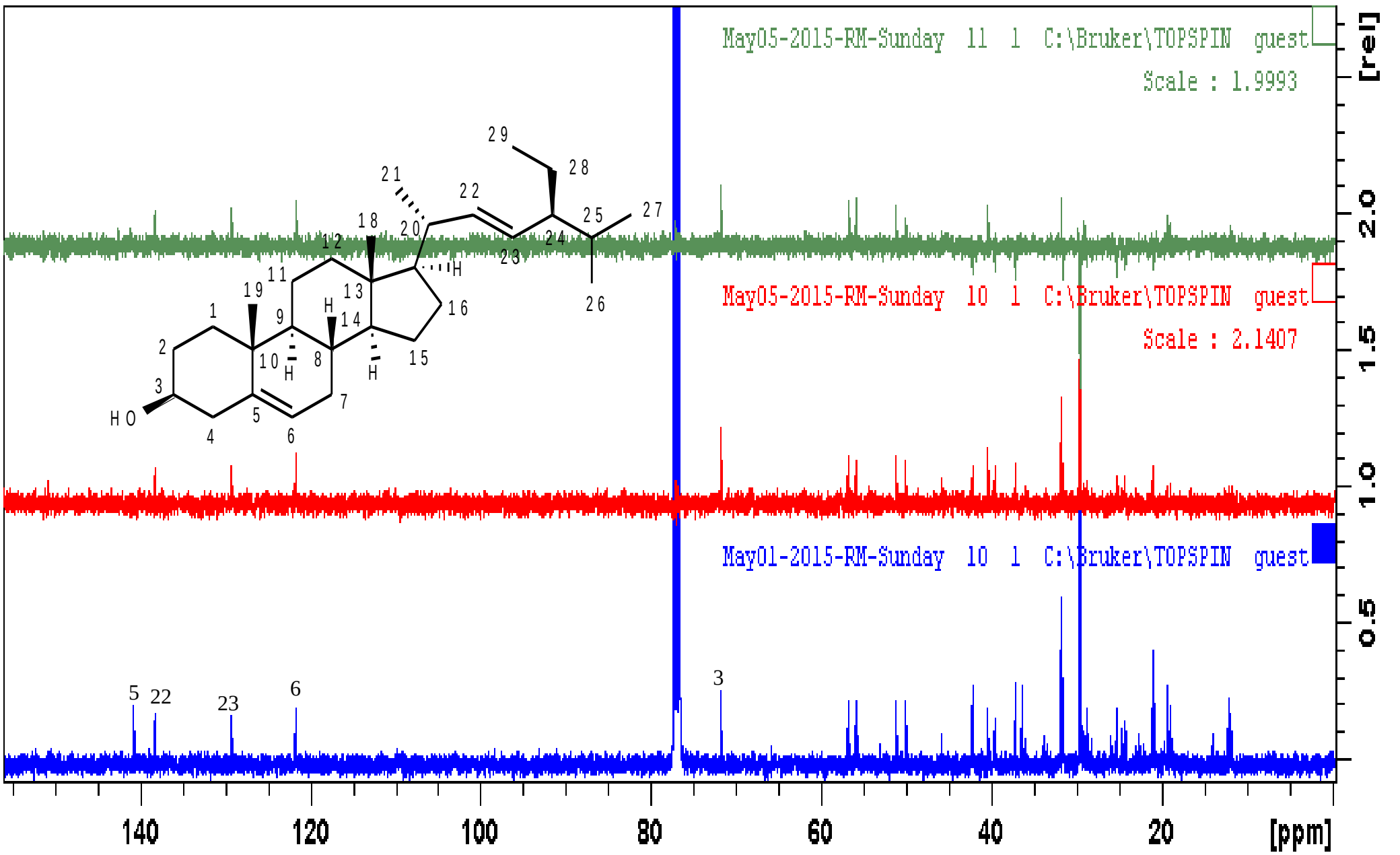
IR spectrum of 3β-hydroxy-20(29)-en-lupan-30-al (**B2**)



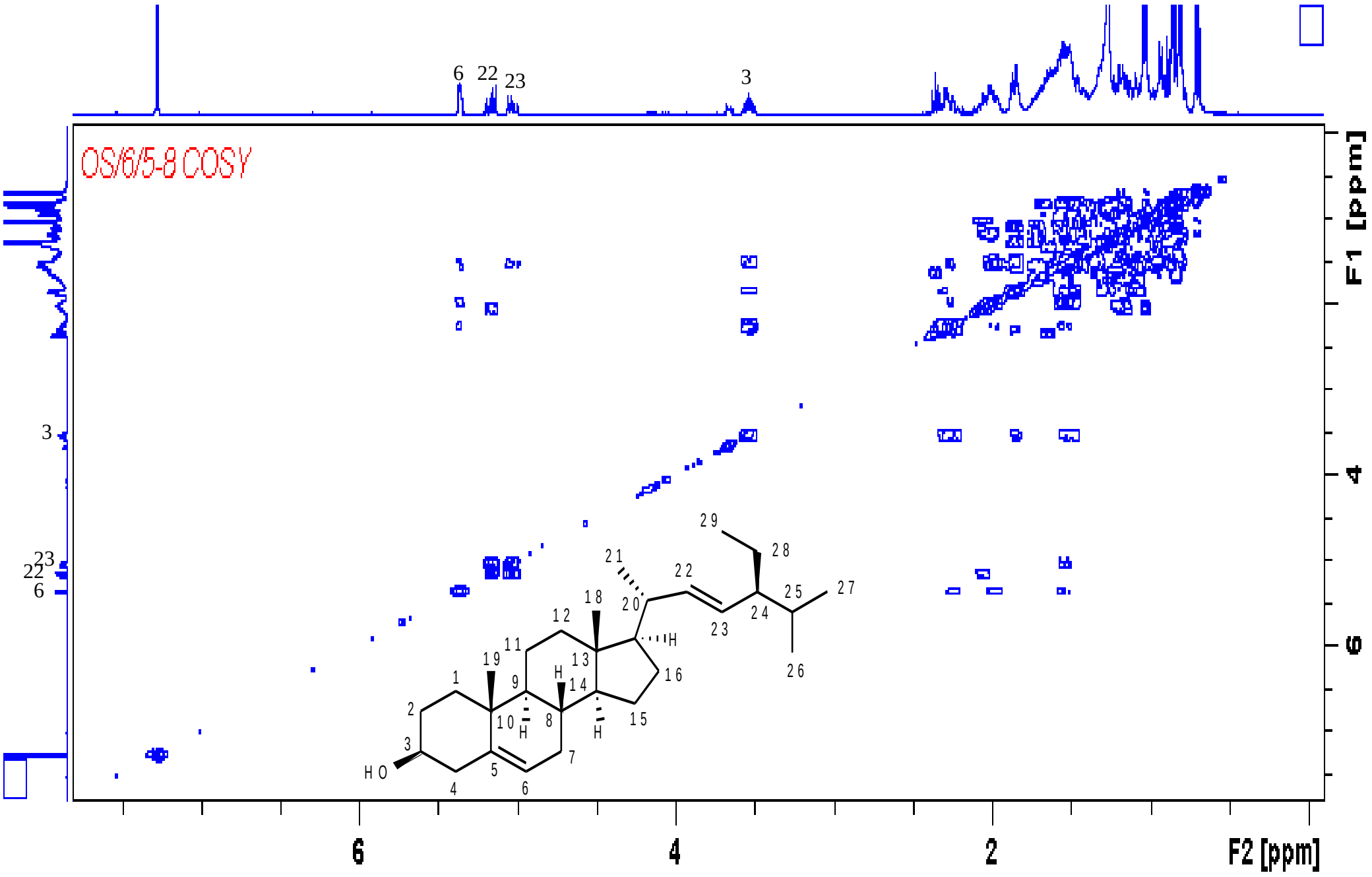
^{1}H NMR spectrum of stigmasterol (**B4**)



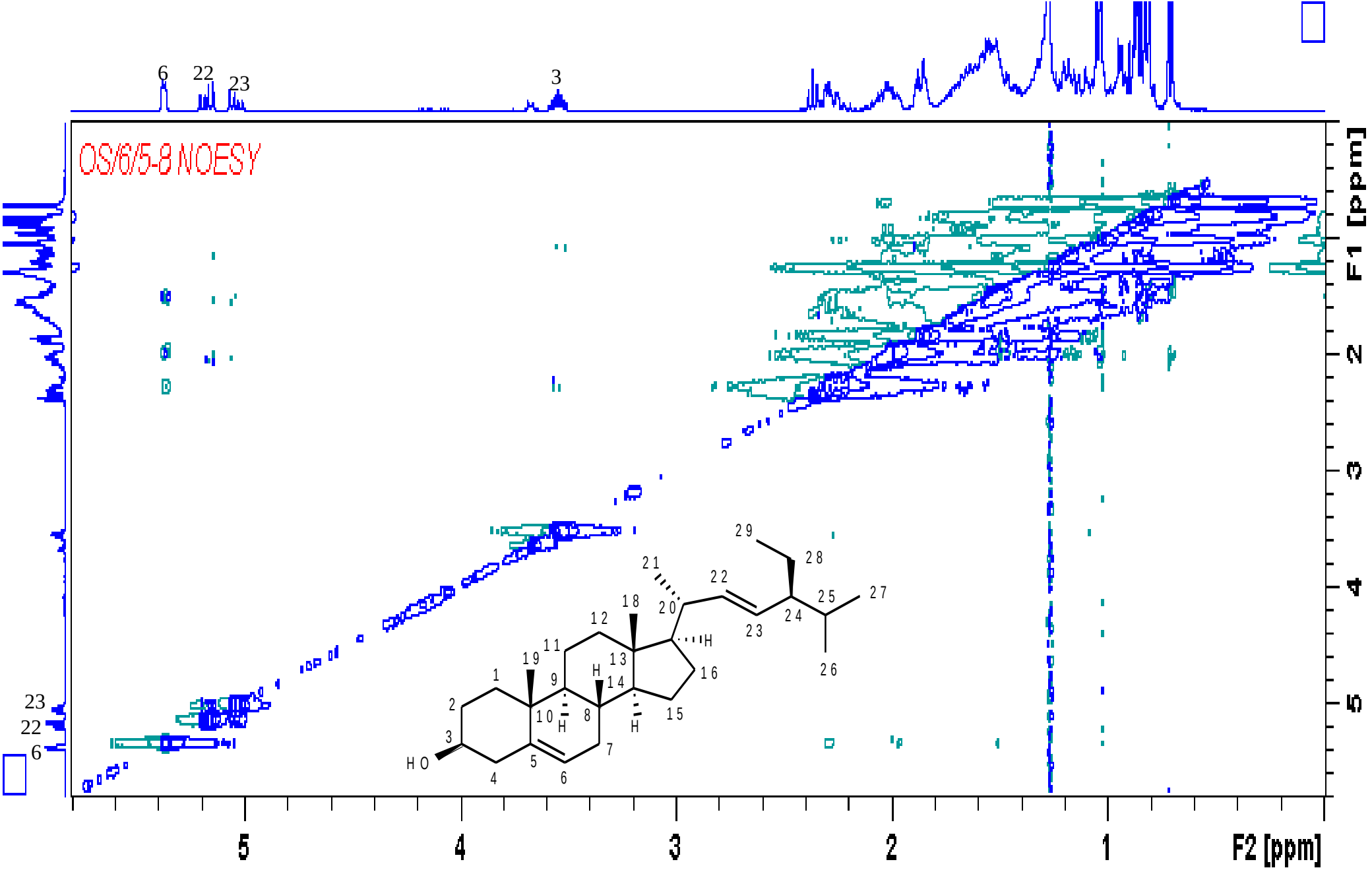
^{13}C NMR spectrum of stigmasterol (**B4**)



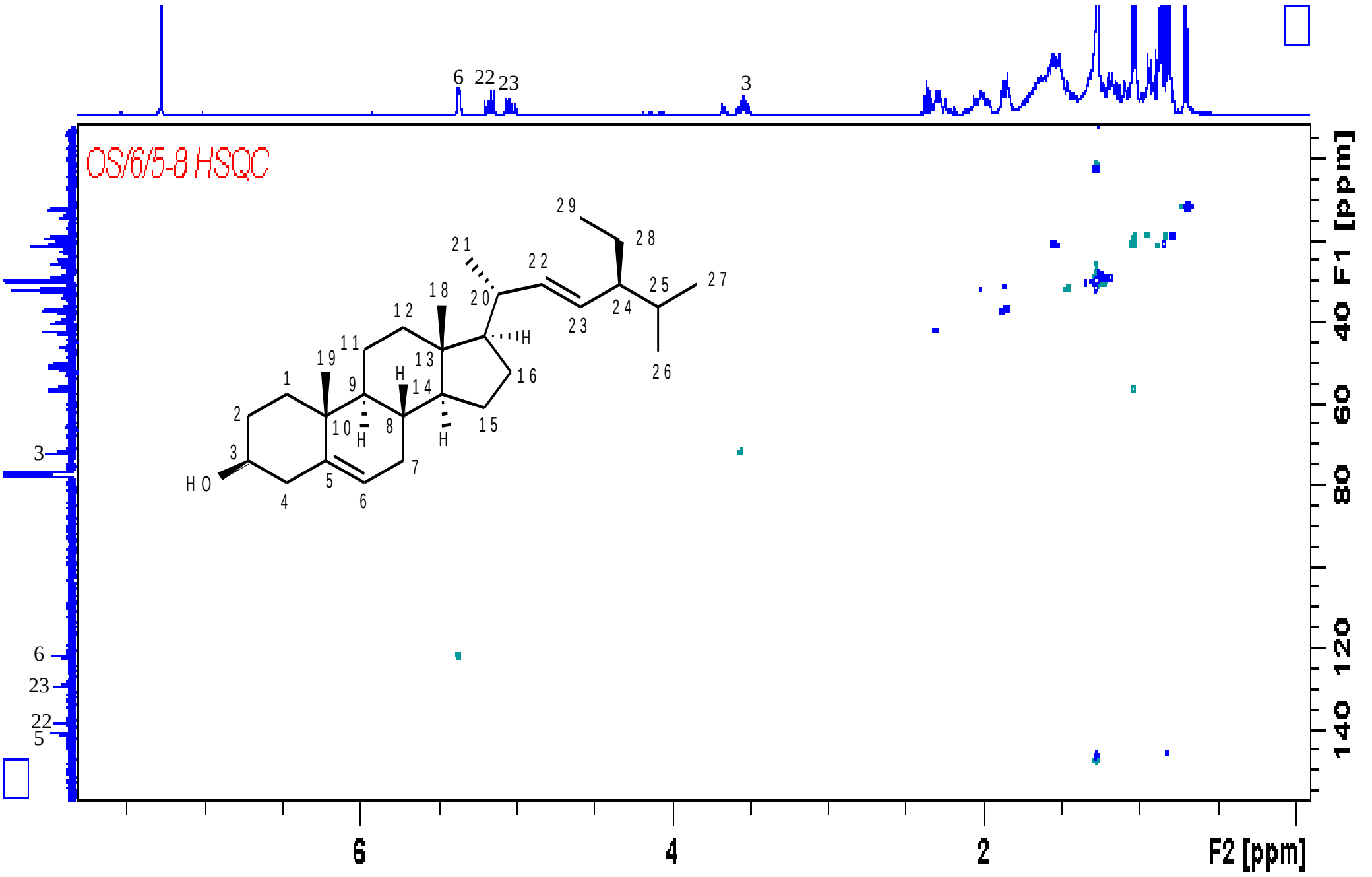
DEPT spectrum of stigmasterol (**B4**)



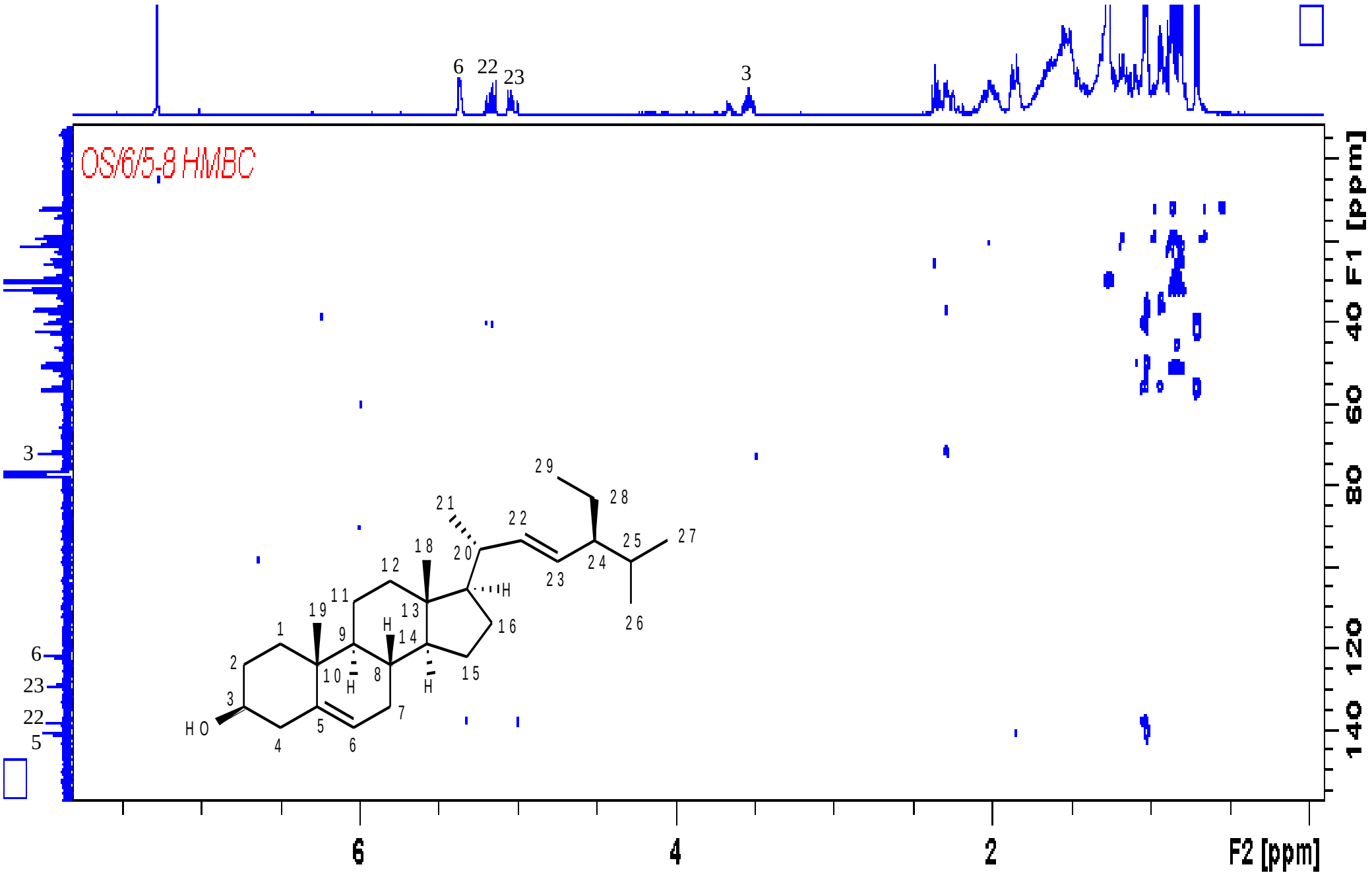
COSY spectrum of stigmasterol (**B4**)



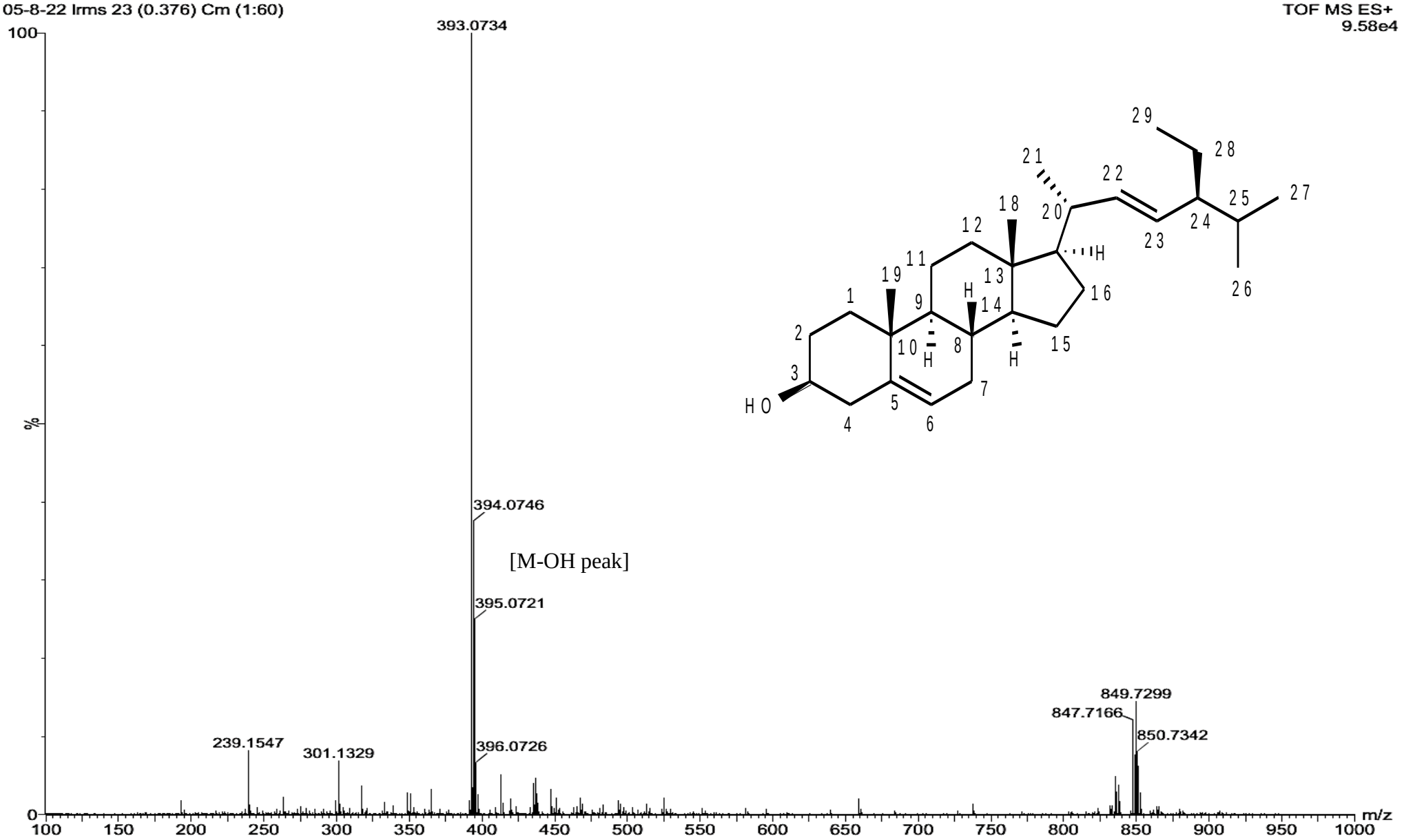
NOESY spectrum of stigmasterol (**B4**)



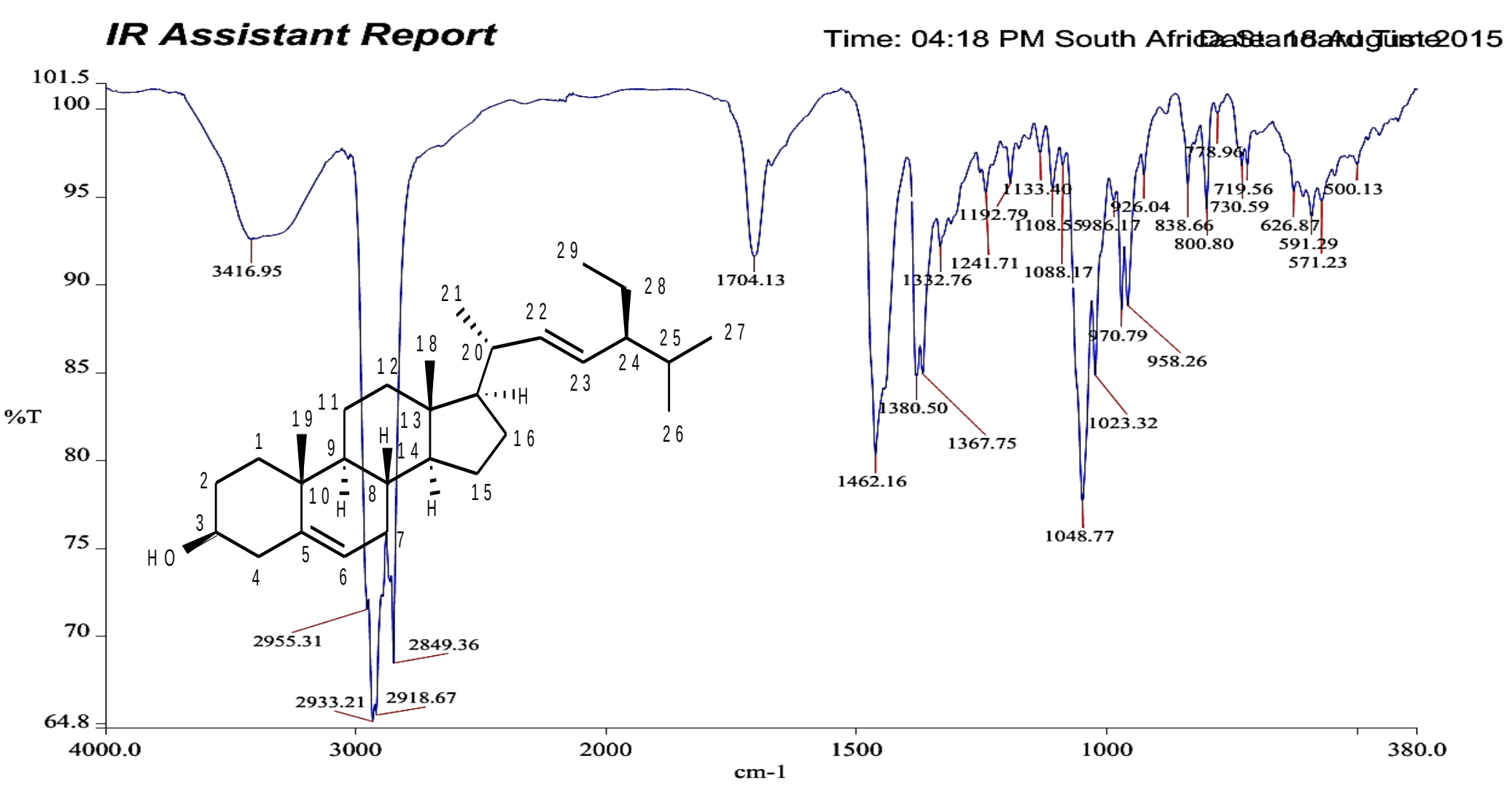
HSQC spectrum of stigmasterol (**B4**)



HMBC spectrum of stigmasterol (**B4**)



Mass Spectrum of stigmasterol (**B4**)

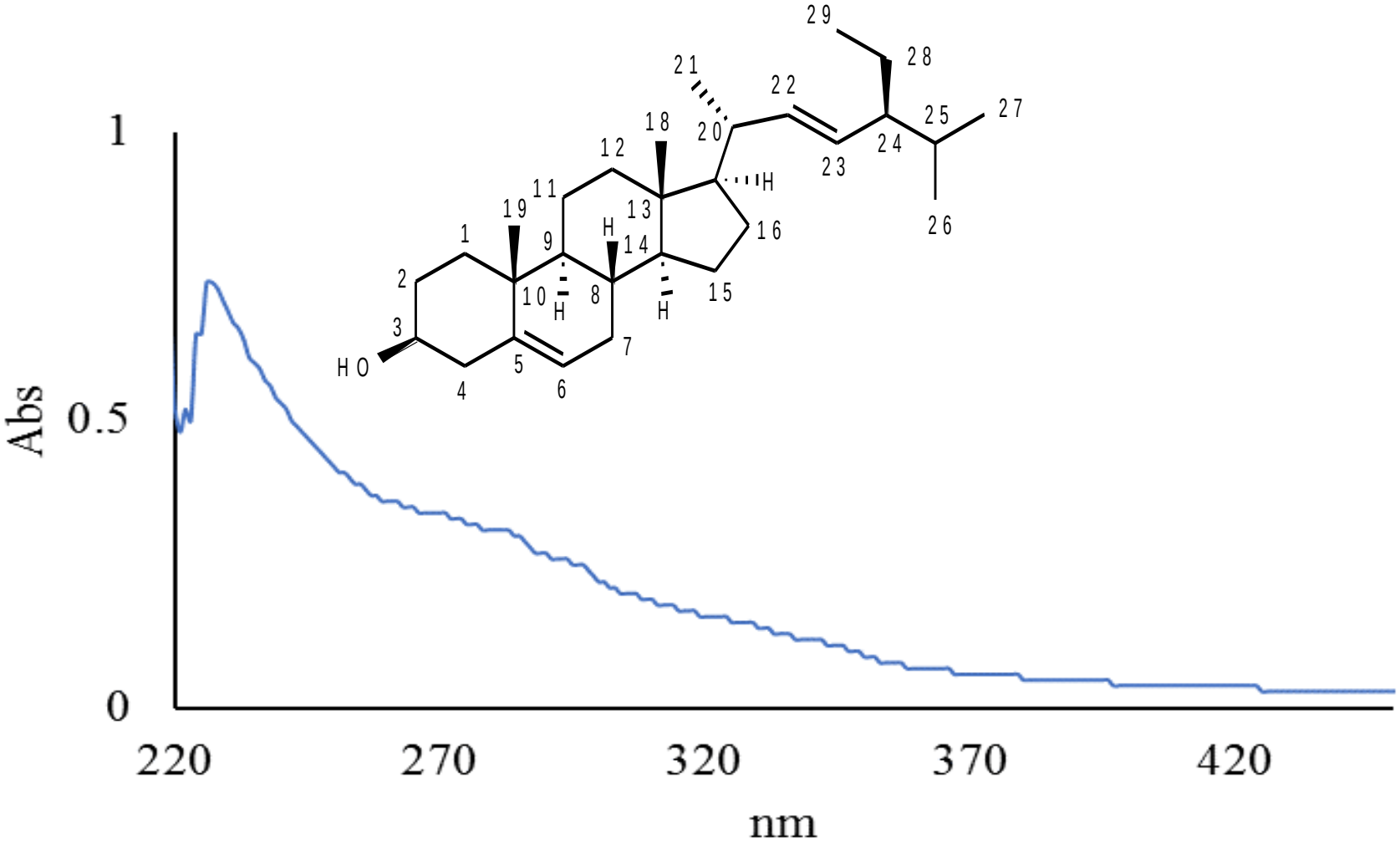


Analyst: Analyst

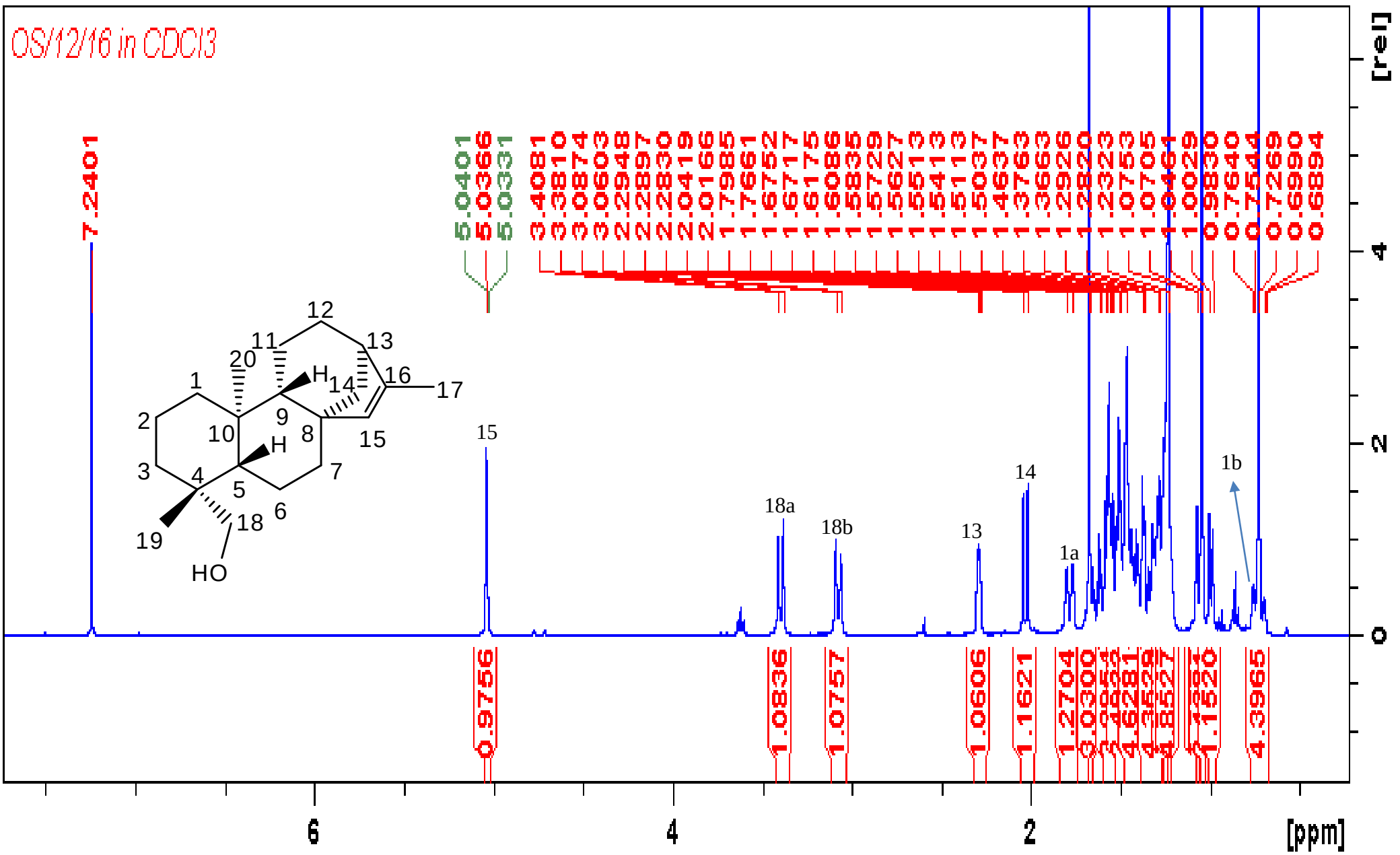
Description:

Spectrum Pathname: C:\pel_data\spectra\OS 8 21 24.003

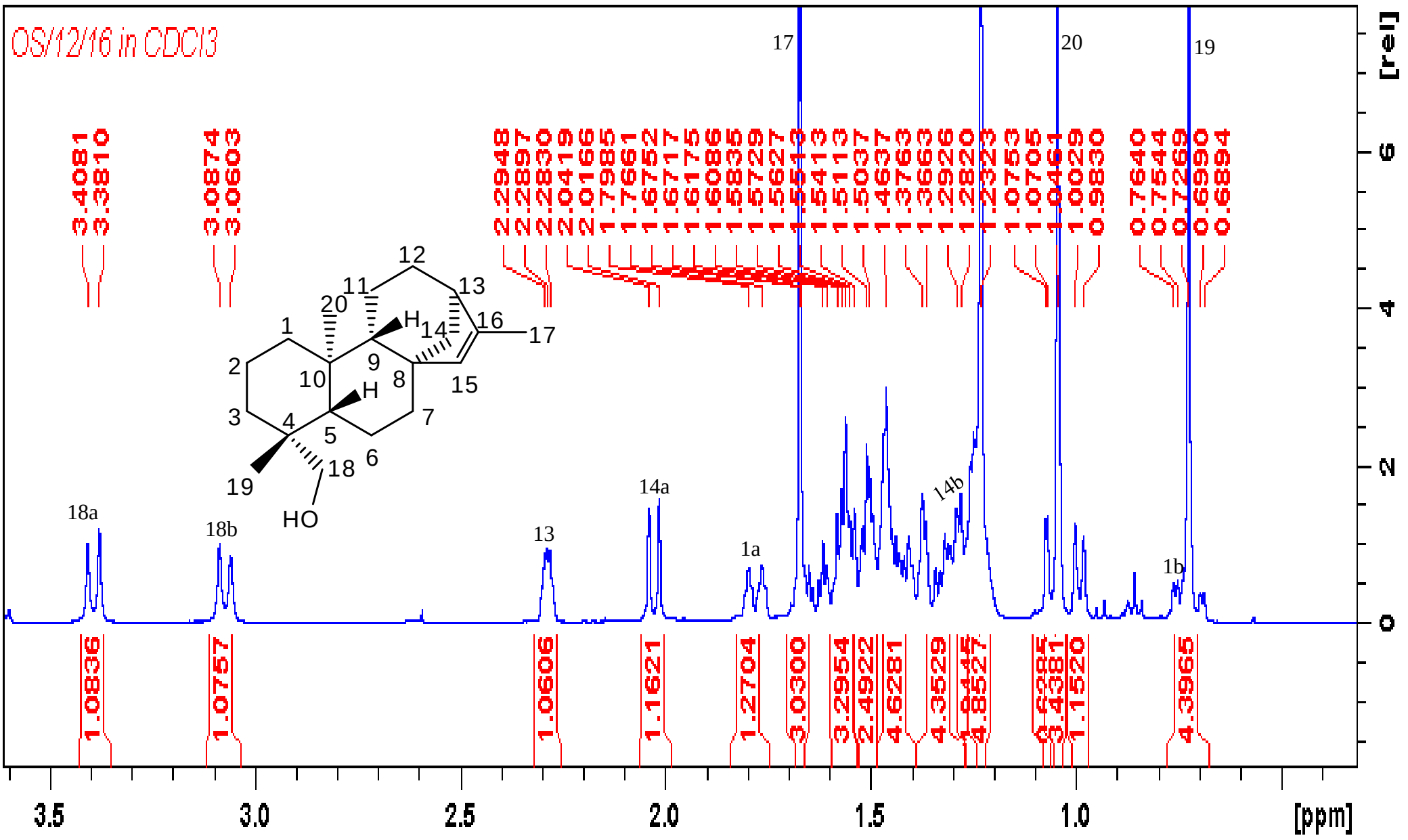
IR spectrum of stigmasterol (**B4**)



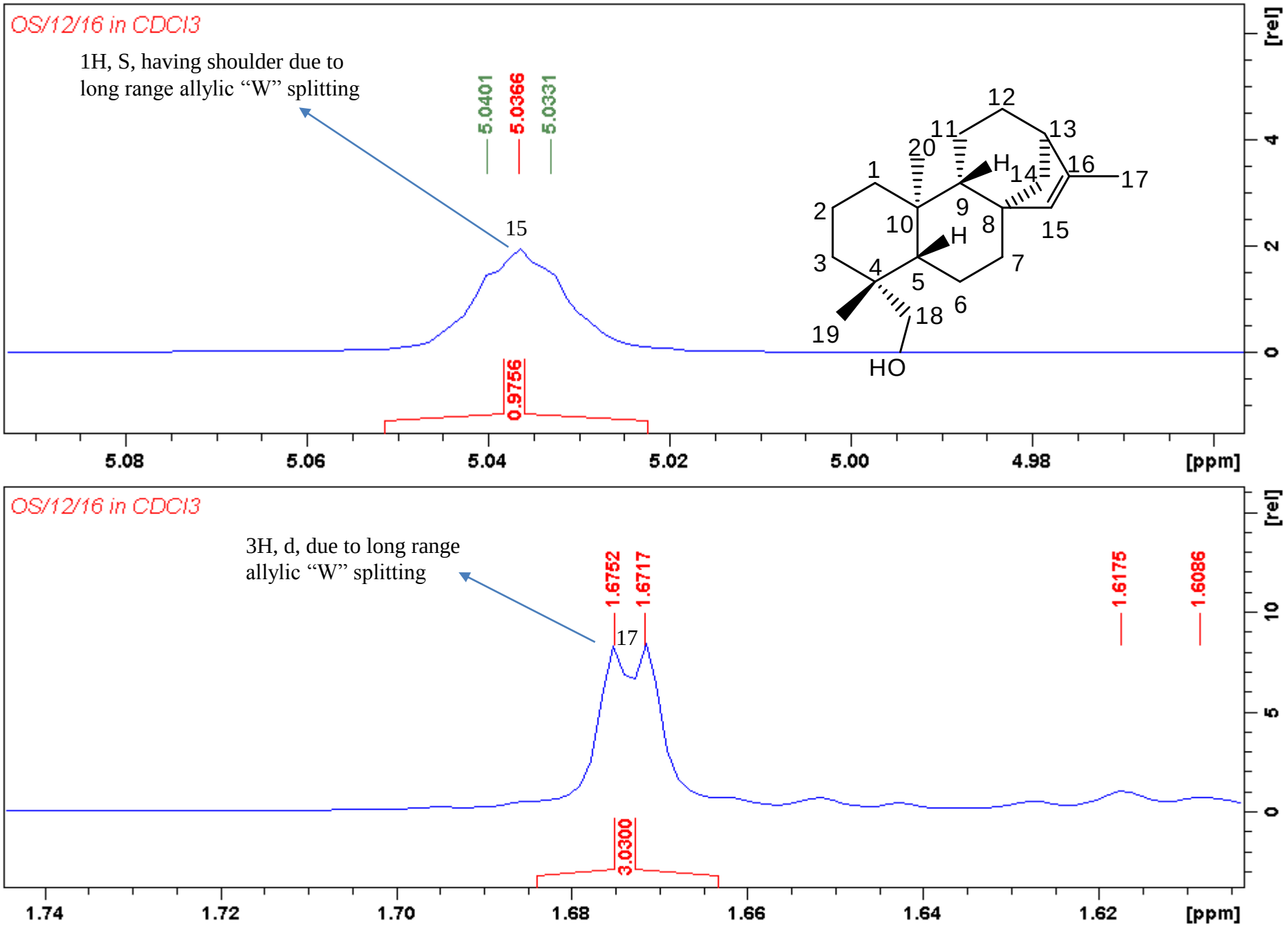
UV spectrum of stigmasterol (**B4**)



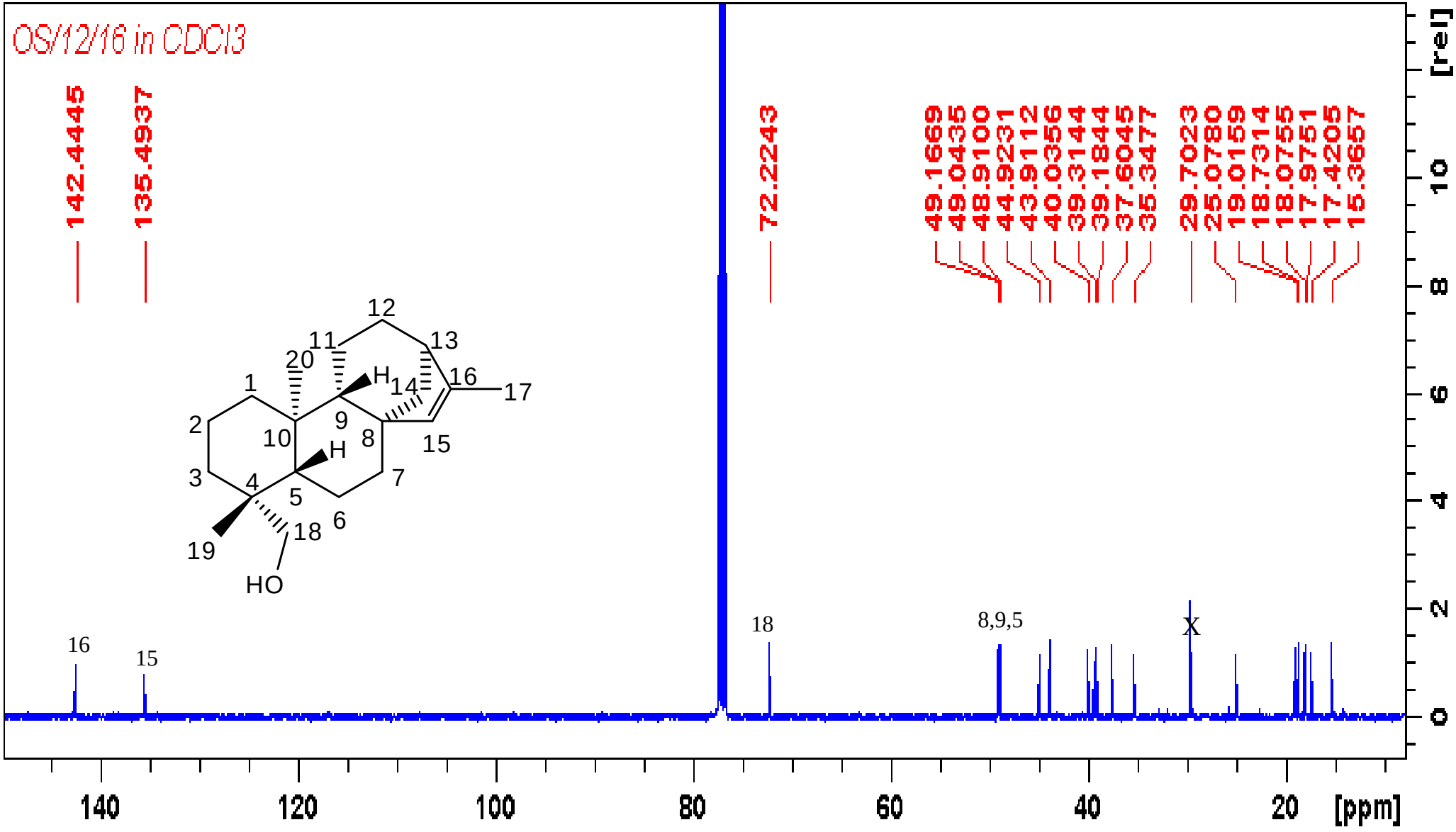
^{1}H NMR spectrum of *ent*-kaur-15-en-18-ol (**B5**)



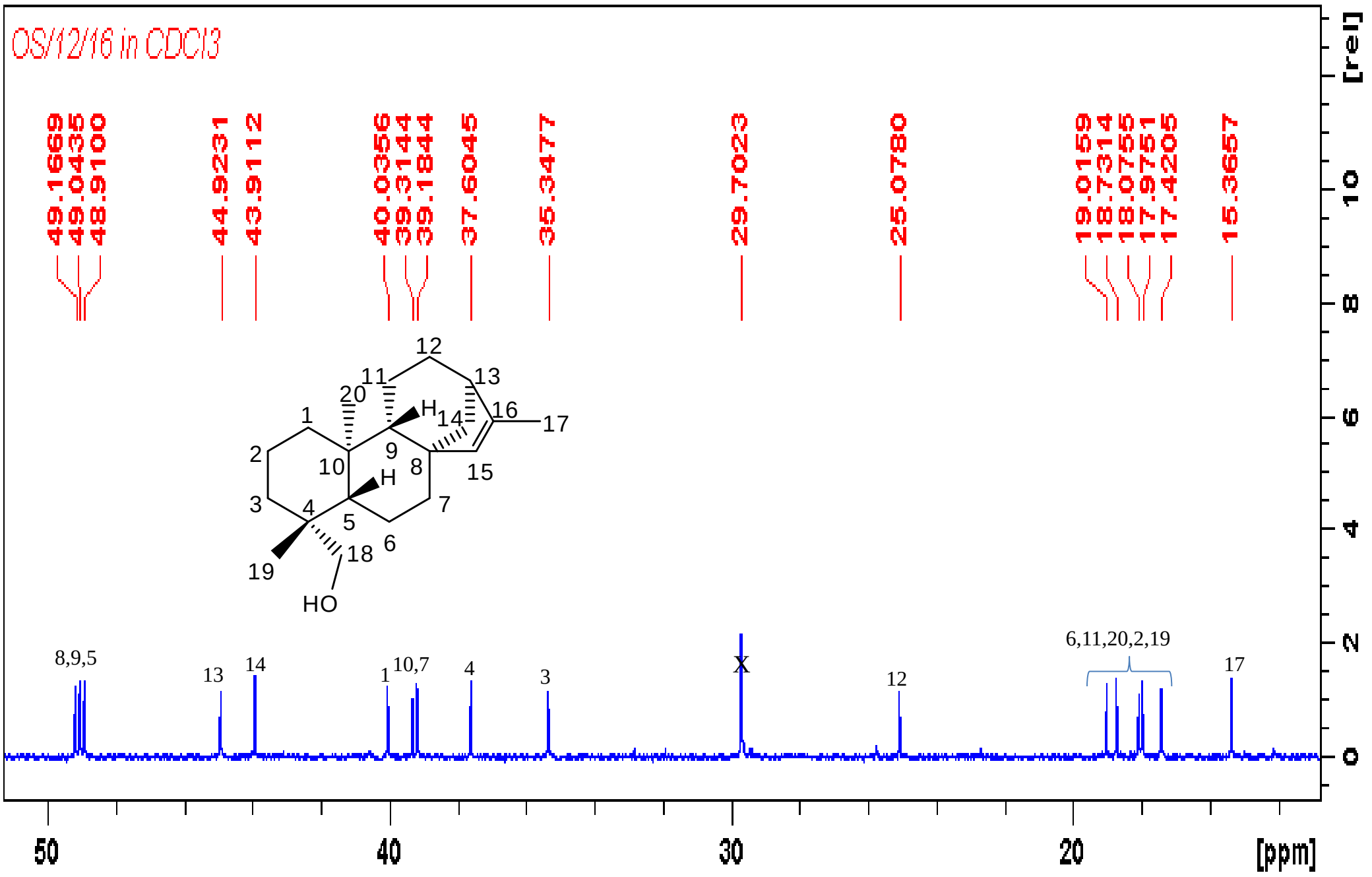
Expanded ^{1}H NMR spectrum of *ent*-kaur-15-en-18-ol (**B5**)



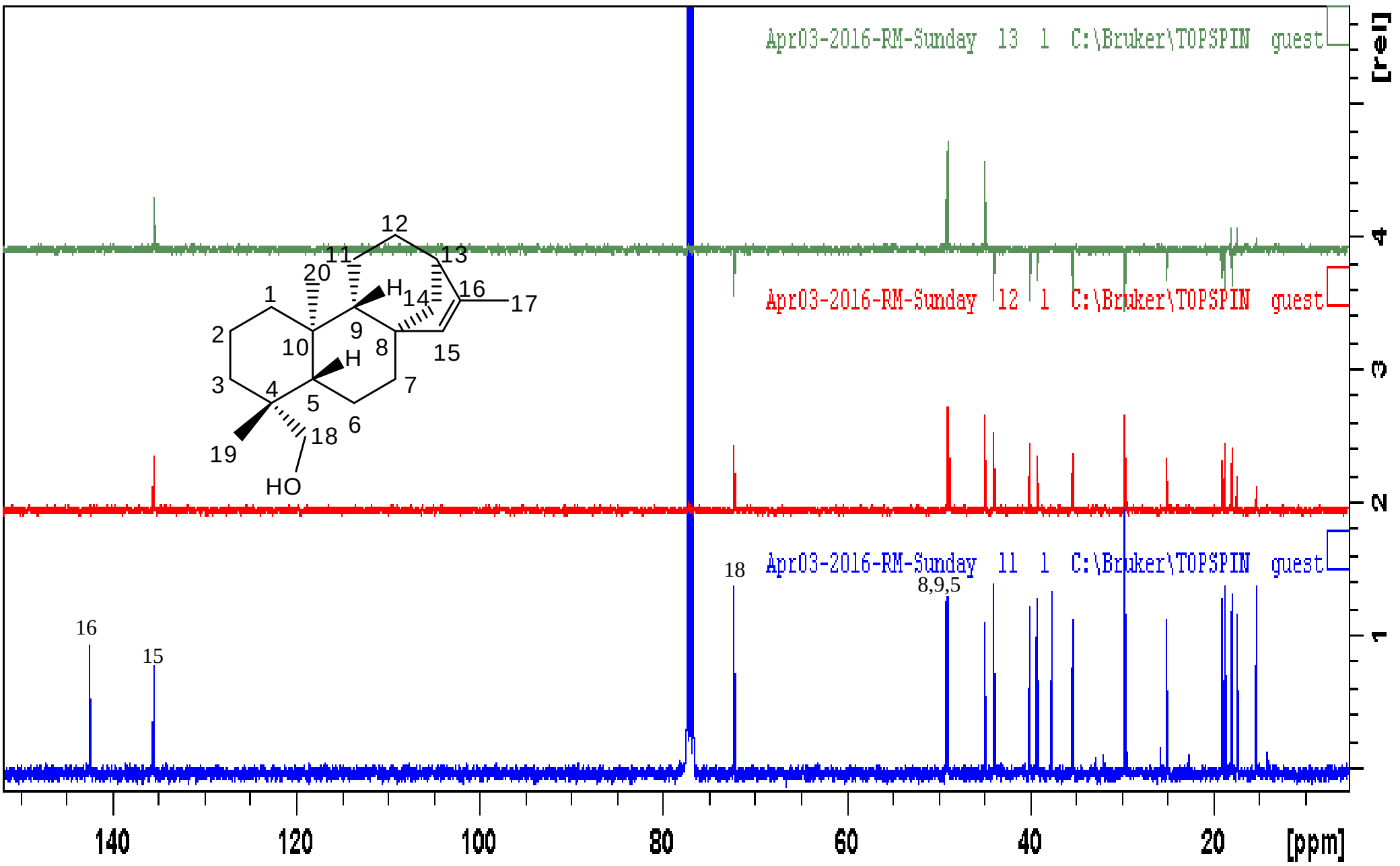
Expanded ^{1}H NMR spectrum of *ent*-kaur-15-en-18-ol (**B5**)



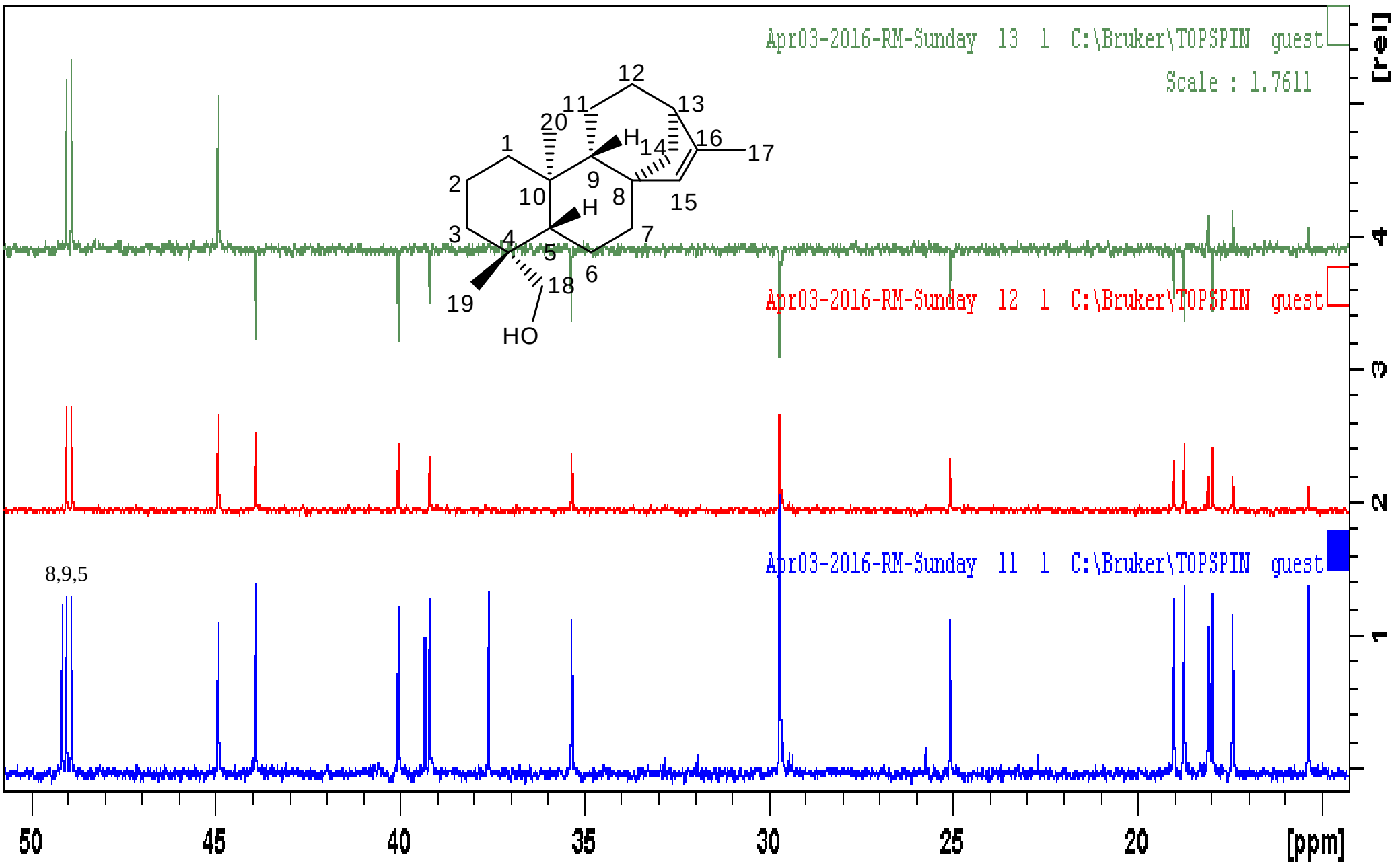
^{13}C NMR spectrum of *ent*-kaur-15-en-18-ol (**B5**)



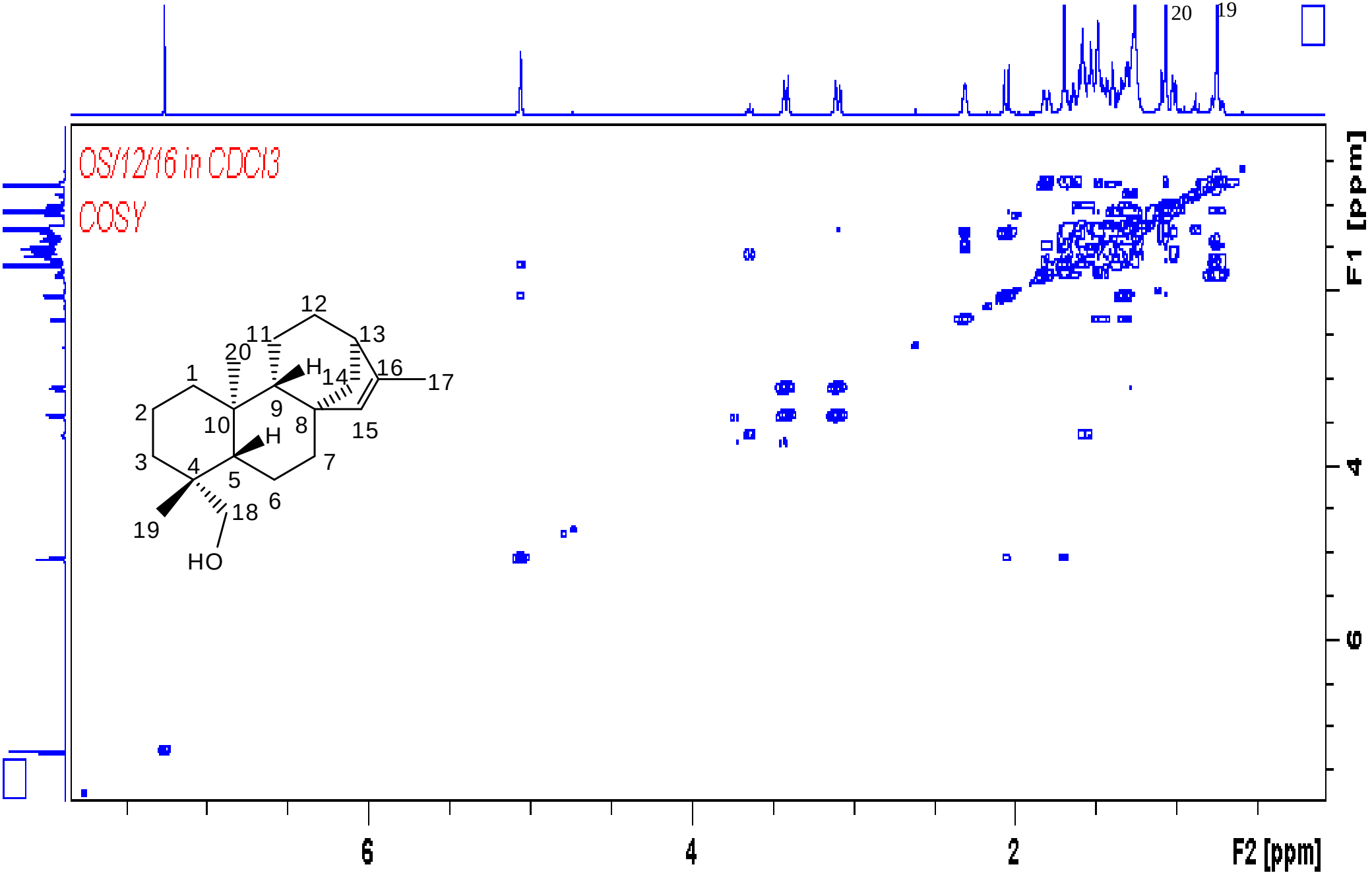
Expanded ^{13}C NMR spectrum of *ent*-kaur-15-en-18-ol (**B5**)



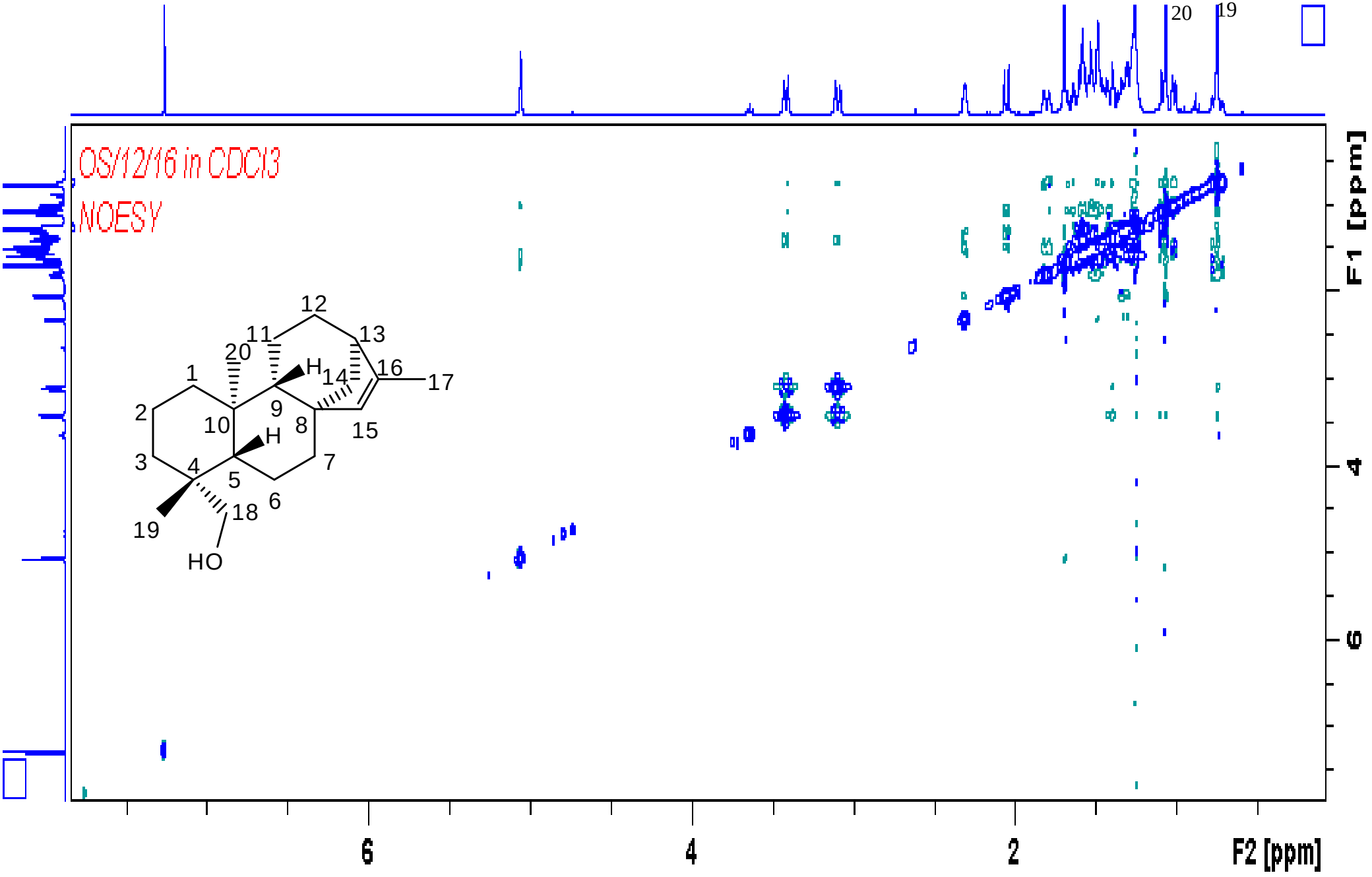
DEPT spectrum of *ent*-kaur-15-en-18-ol (**B5**)



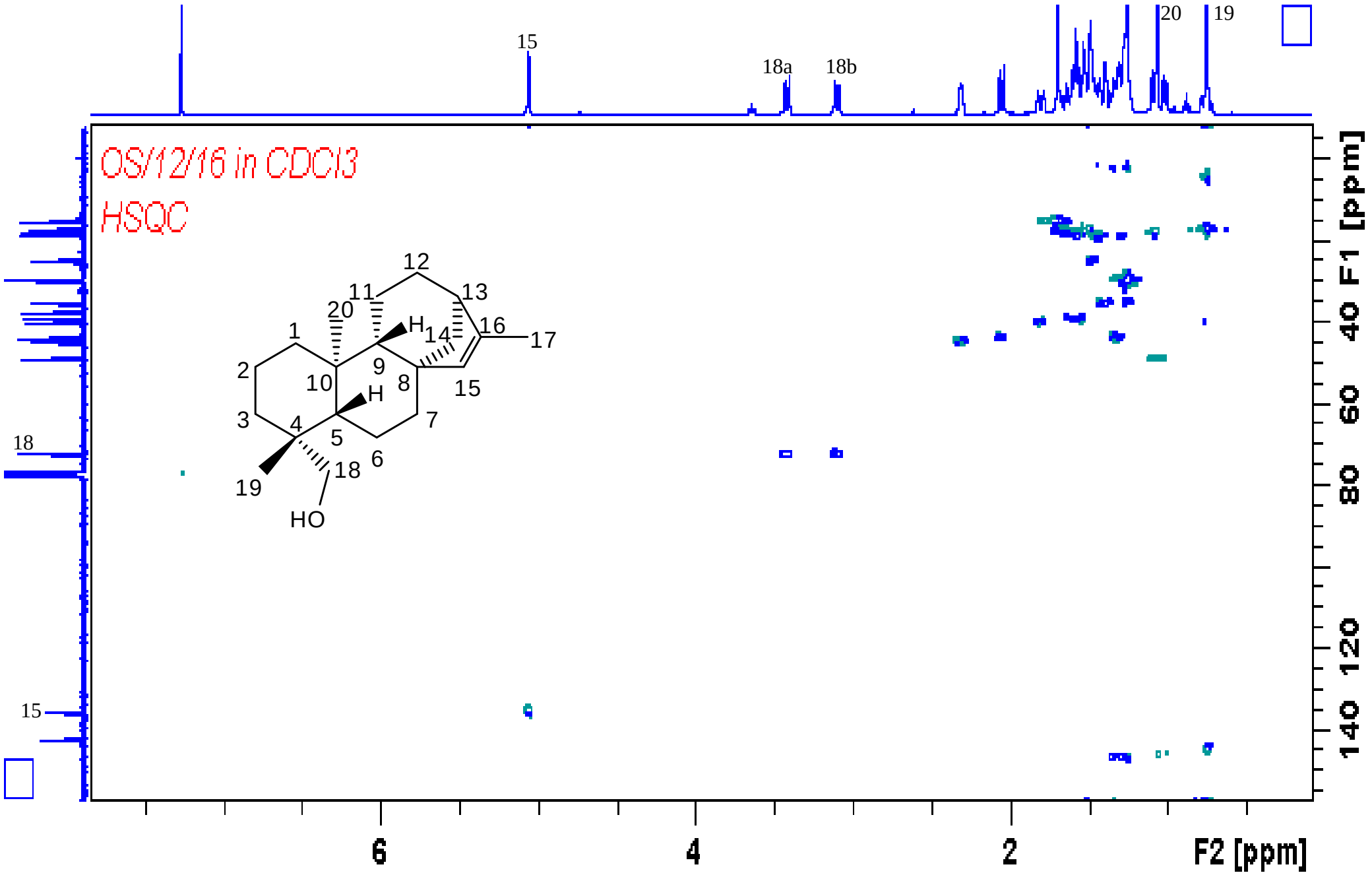
Expanded DEPT spectrum of *ent*-kaur-15-en-18-ol (**B5**)



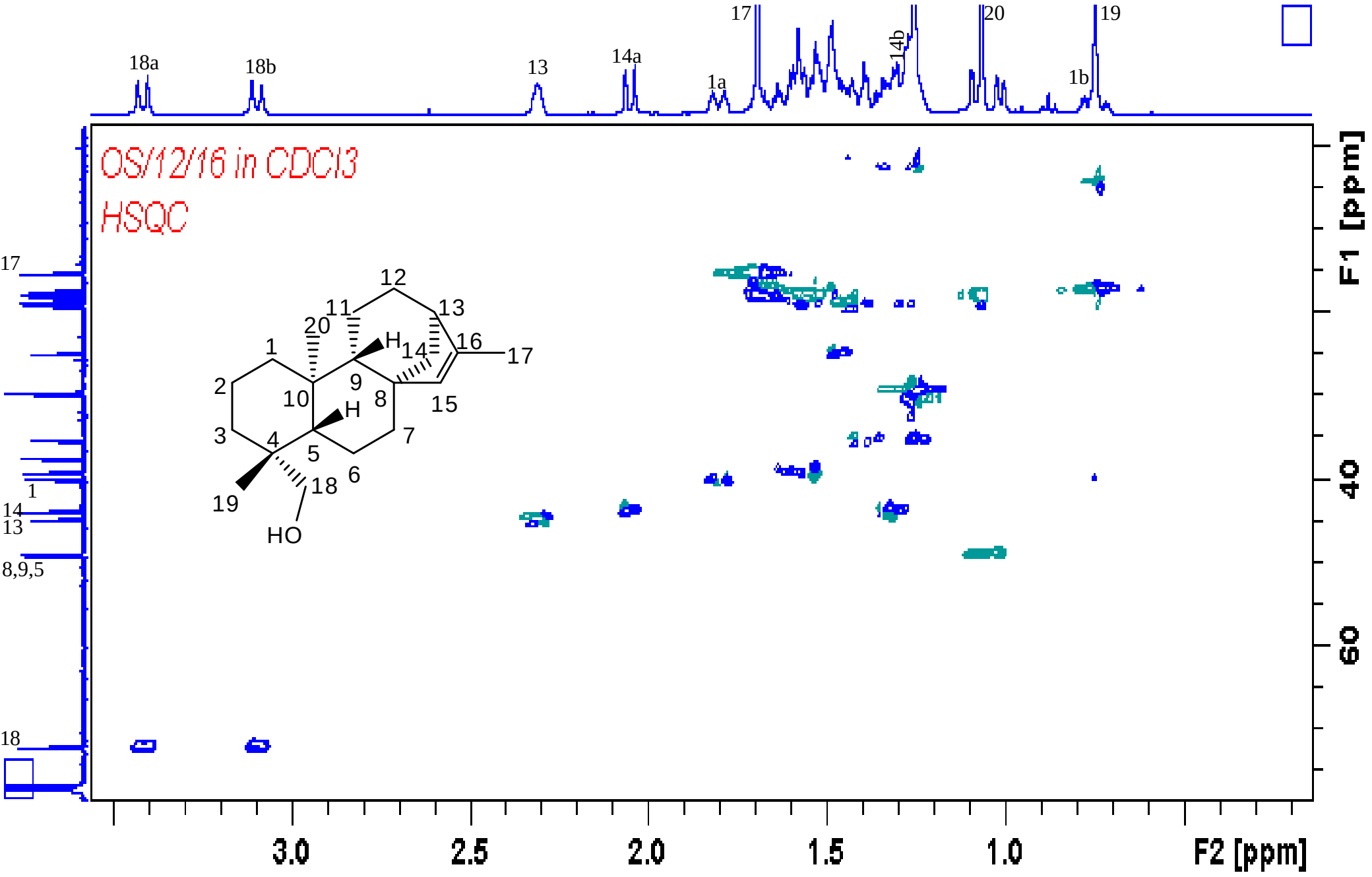
COSY spectrum of *ent*-kaur-15-en-18-ol (**B5**)



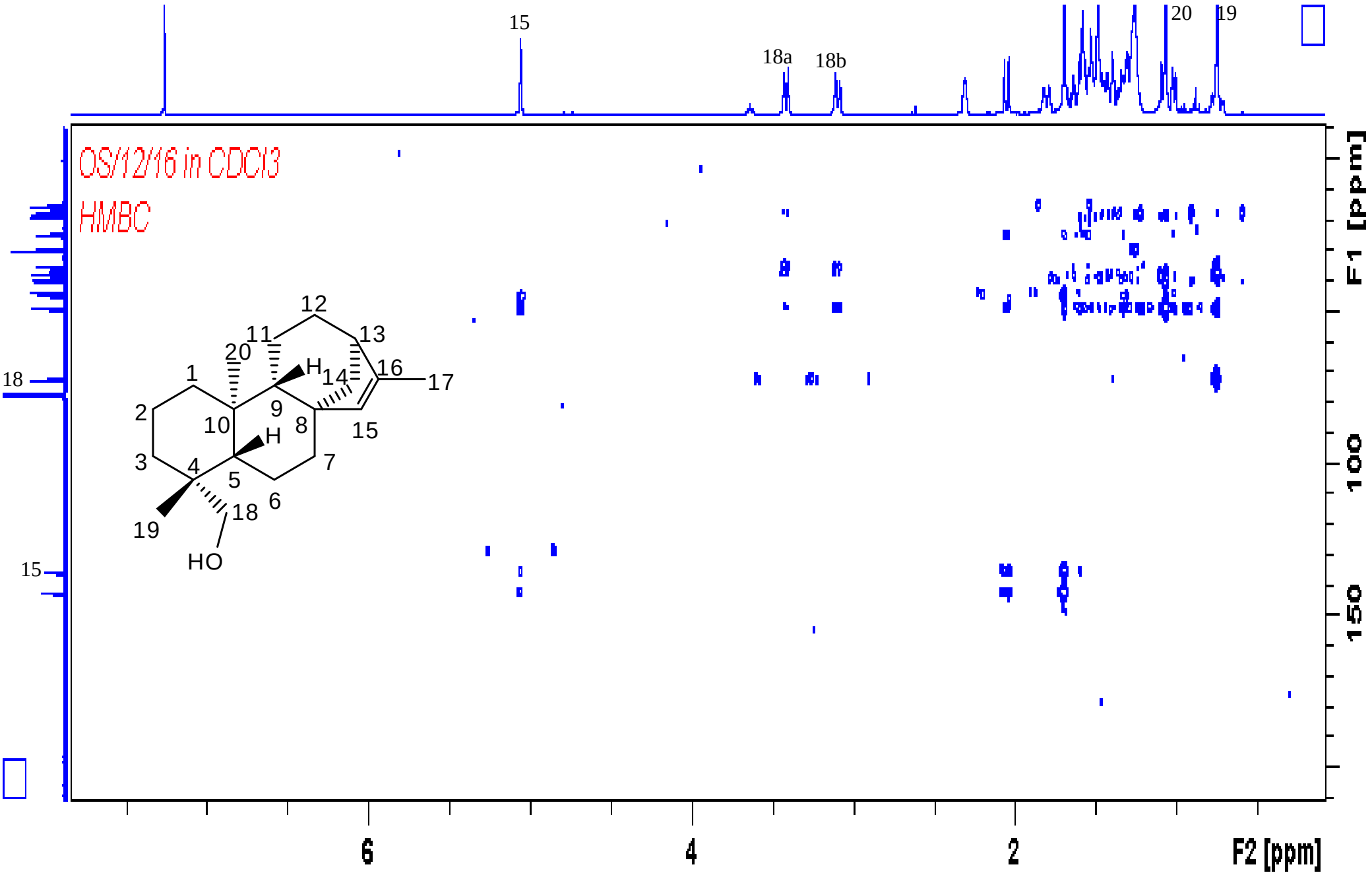
NOESY spectrum of *ent*-kaur-15-en-18-ol (**B5**)



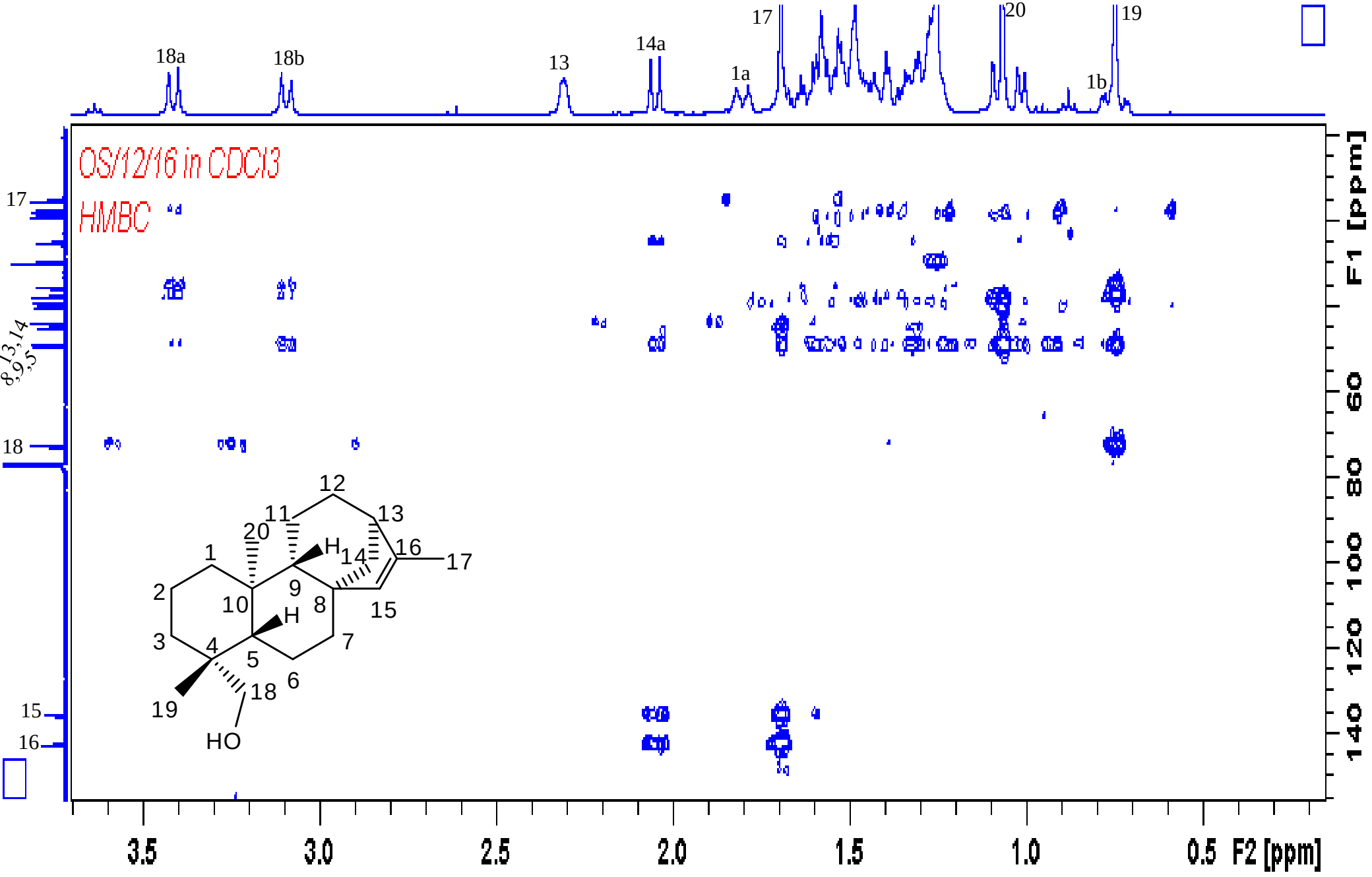
HSQC spectrum of *ent*-kaur-15-en-18-ol (**B5**)



Expanded HSQC spectrum of *ent*-kaur-15-en-18-ol (**B5**)



HMBC spectrum of *ent*-kaur-15-en-18-ol (**B5**)



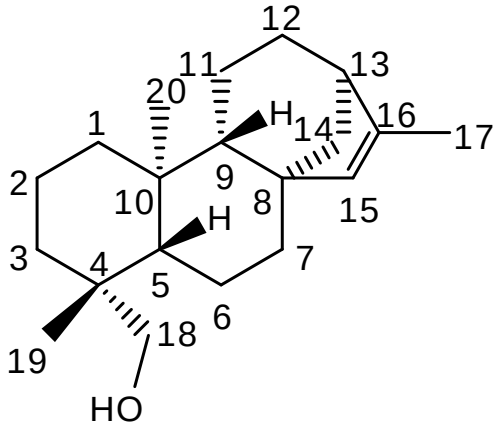
Expanded HMBC spectrum of *ent*-kaur-15-en-18-ol (**B5**)

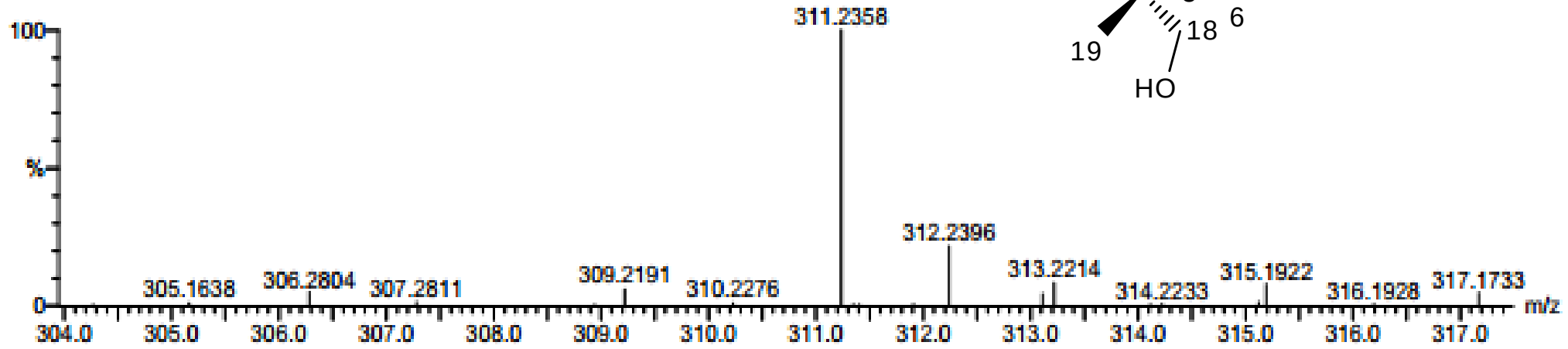
Single Mass Analysis

Tolerance = 5.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 2

Monoisotopic Mass, Even Electron Ions
6 formula(e) evaluated with 1 results within limits (up to 20 best isotopic matches for each mass)
Elements Used:
C: 20-25 H: 30-35 O: 0-5 Na: 0-1

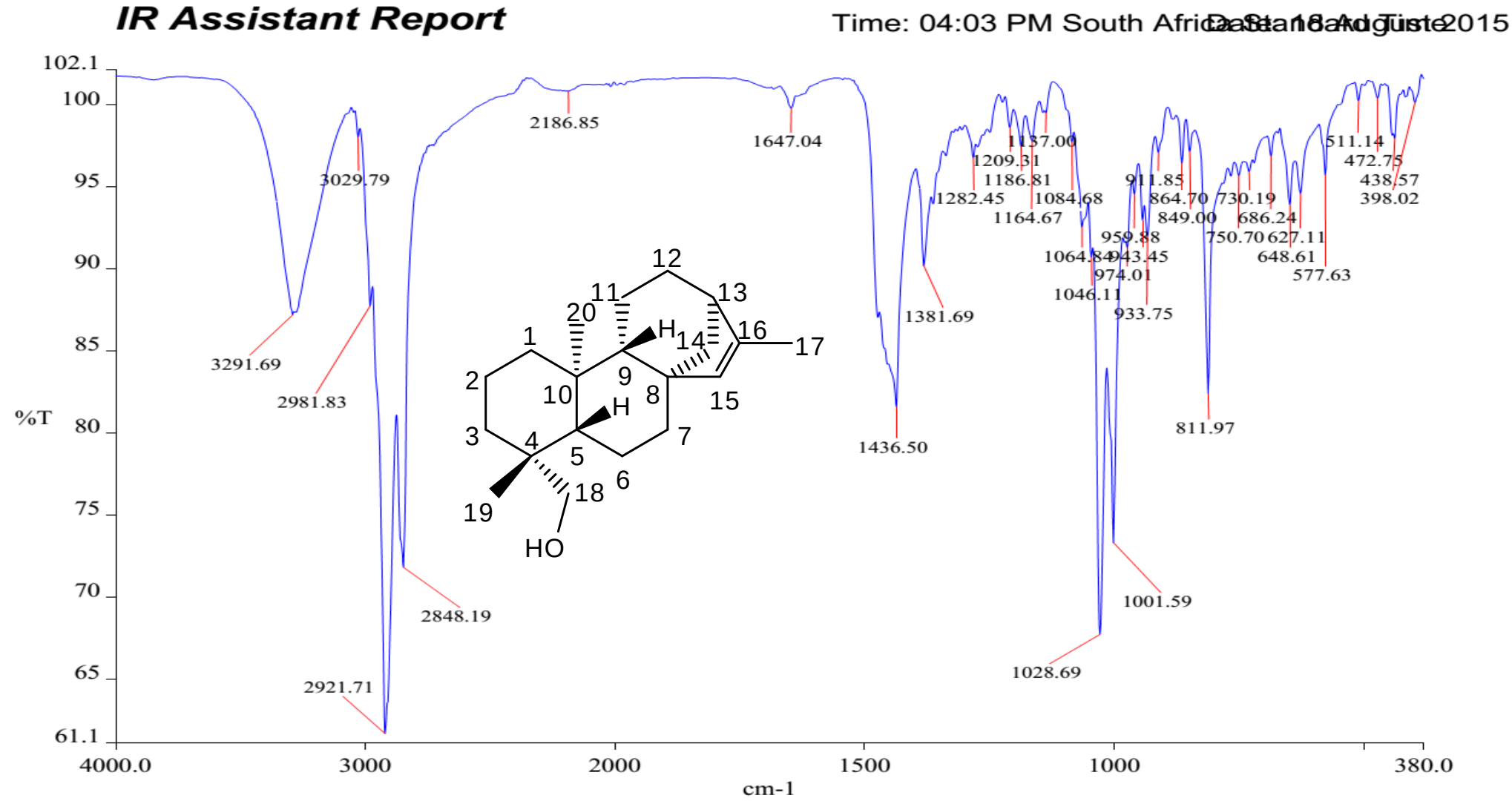
05-12-16 5 (0.168) Cm (1:60)
TOF MS ES+





Minimum:				-1.5			
Maximum:		5.0	5.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
311.2358	311.2351	0.7	2.2	4.5	55.9	0.0	C20 H32 O Na

Mass spectrum of *ent*-kaur-15-en-18-ol (**B5**)

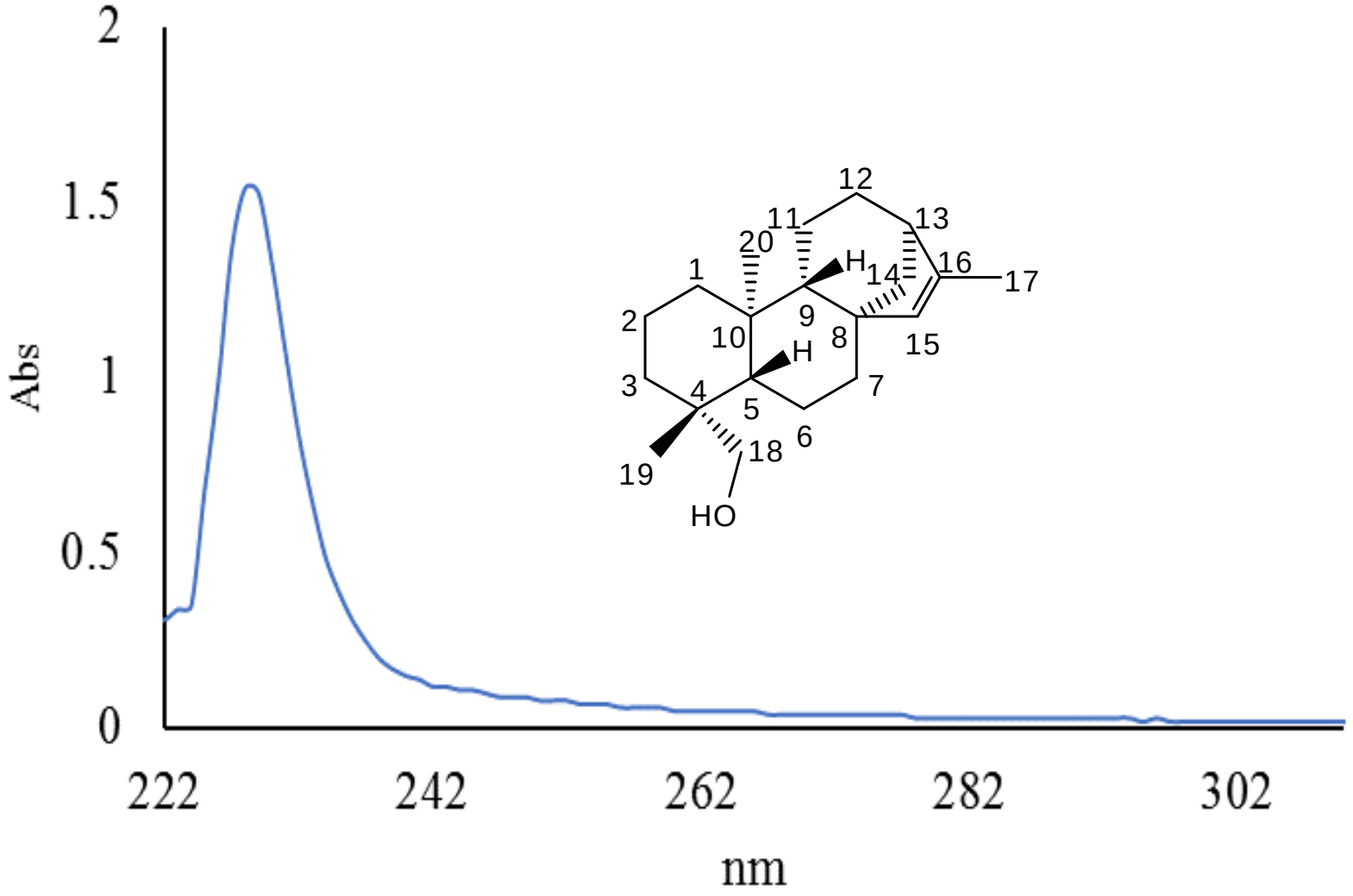


Analyst: Analyst

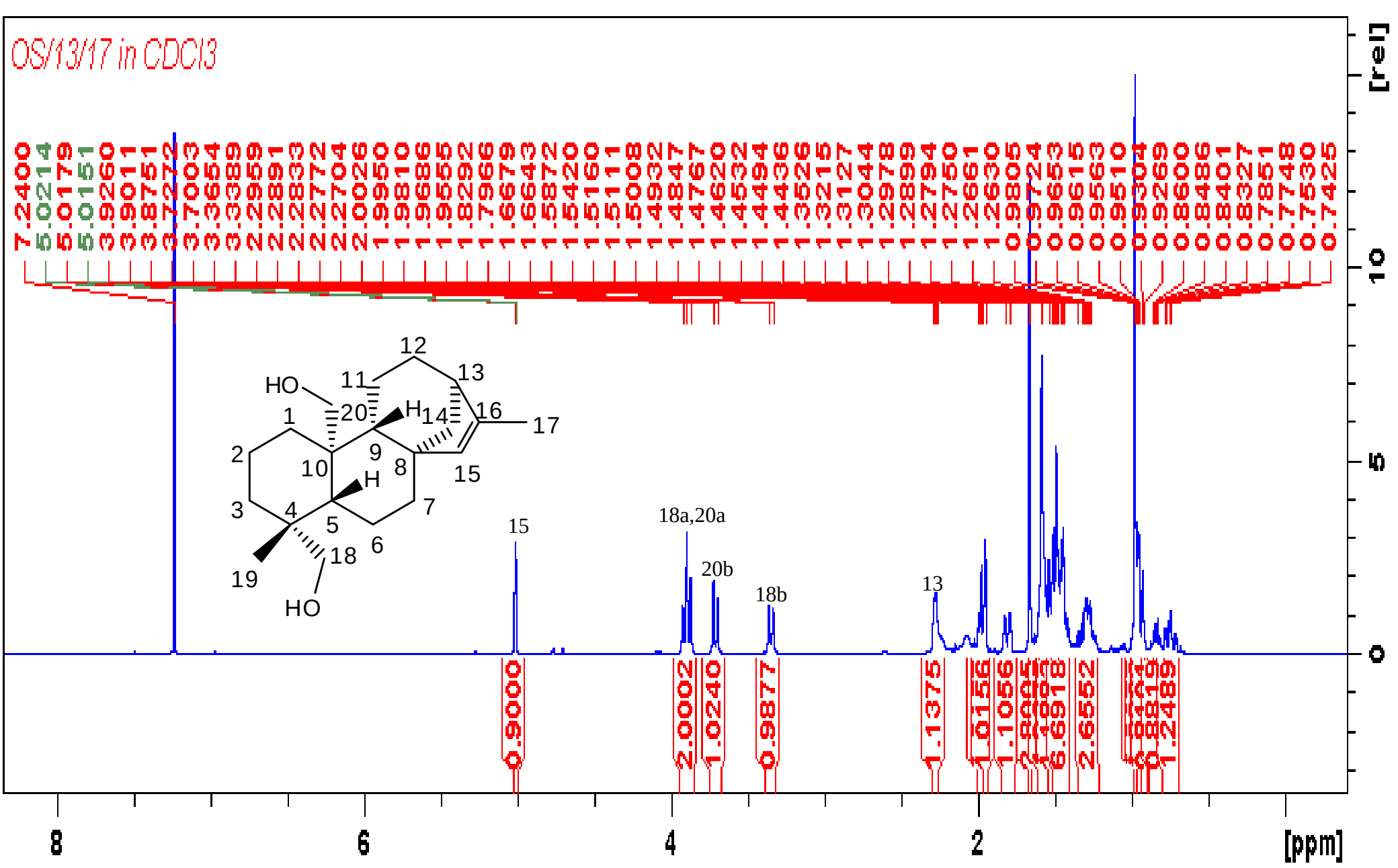
Description:

Spectrum Pathname: C:\pel_data\spectra\OS 12 16.003

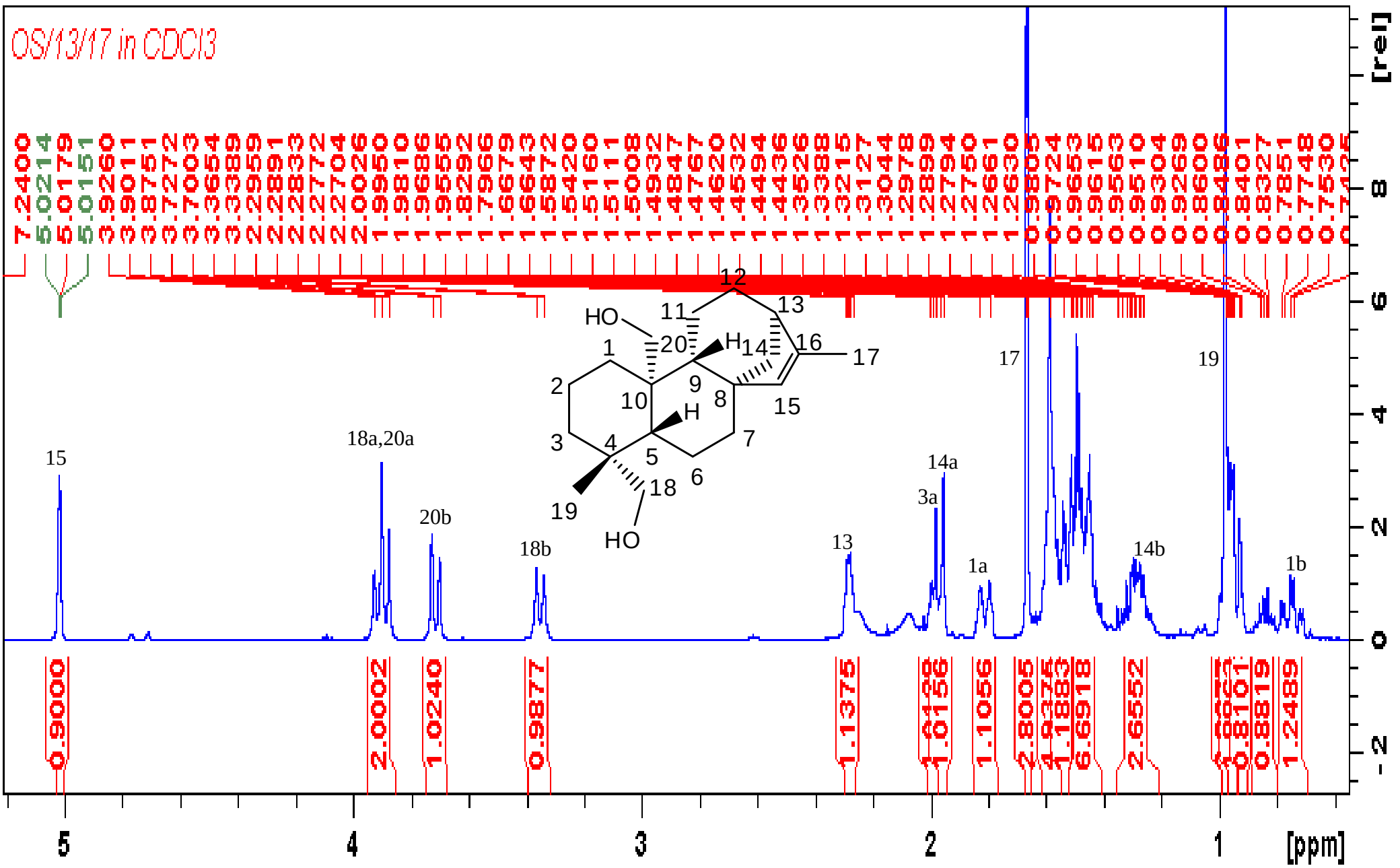
IR spectrum of *ent*-kaur-15-en-18-ol (**B5**)



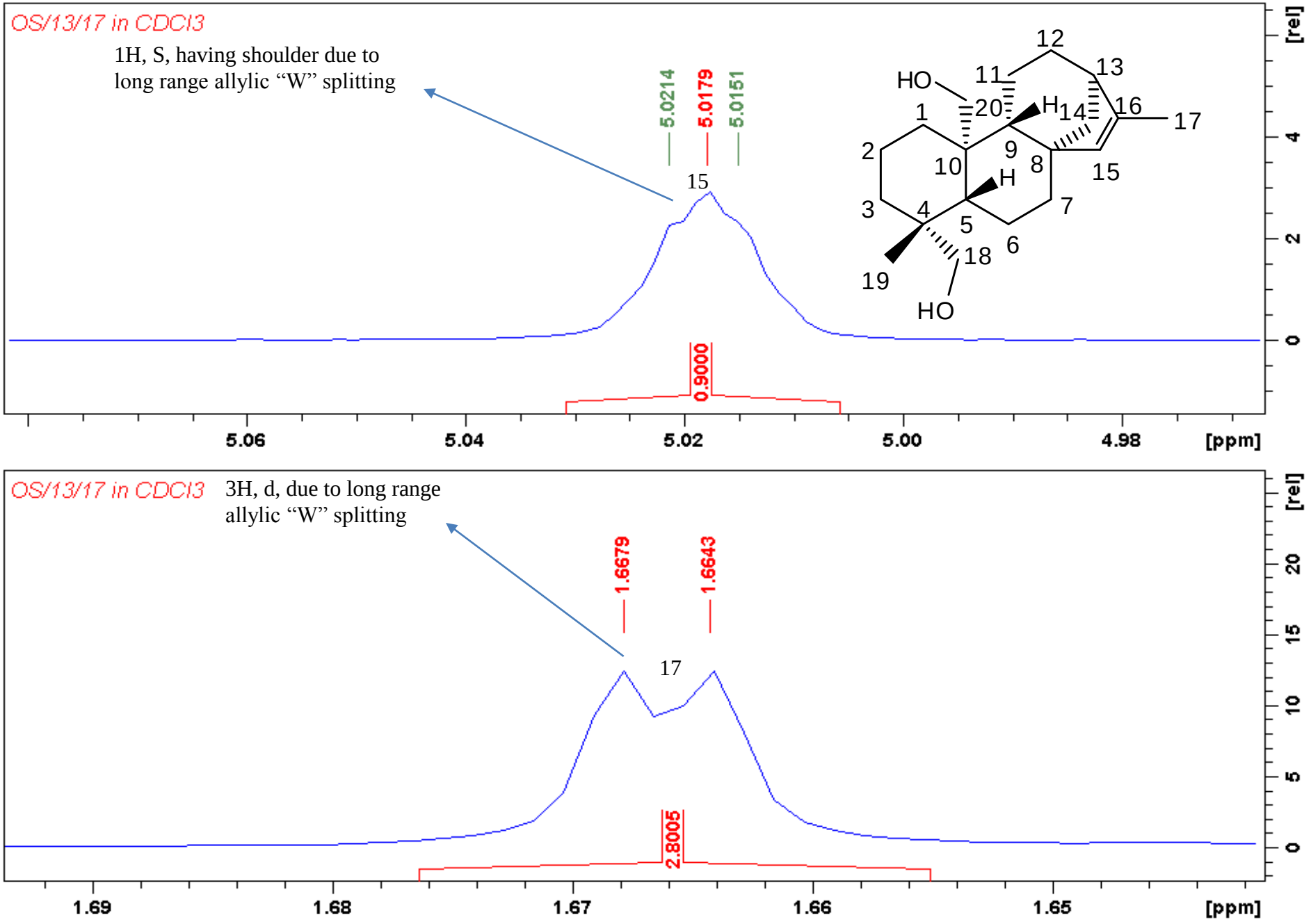
UV spectrum of *ent*-kaur-15-en-18-ol (**B5**)



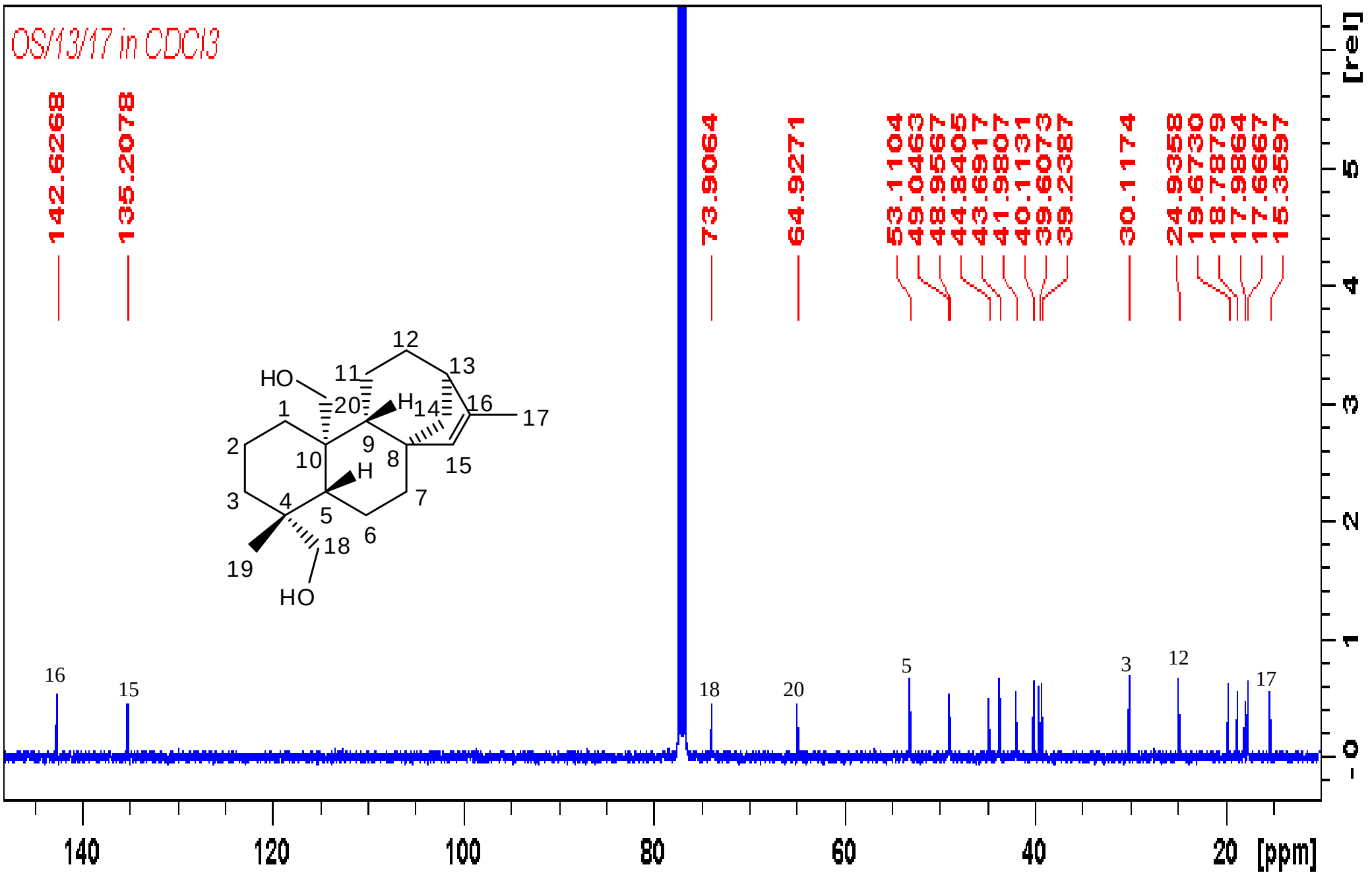
^{1}H NMR spectrum of *ent*-kaur-15-en-18,20-diol (**B6**)



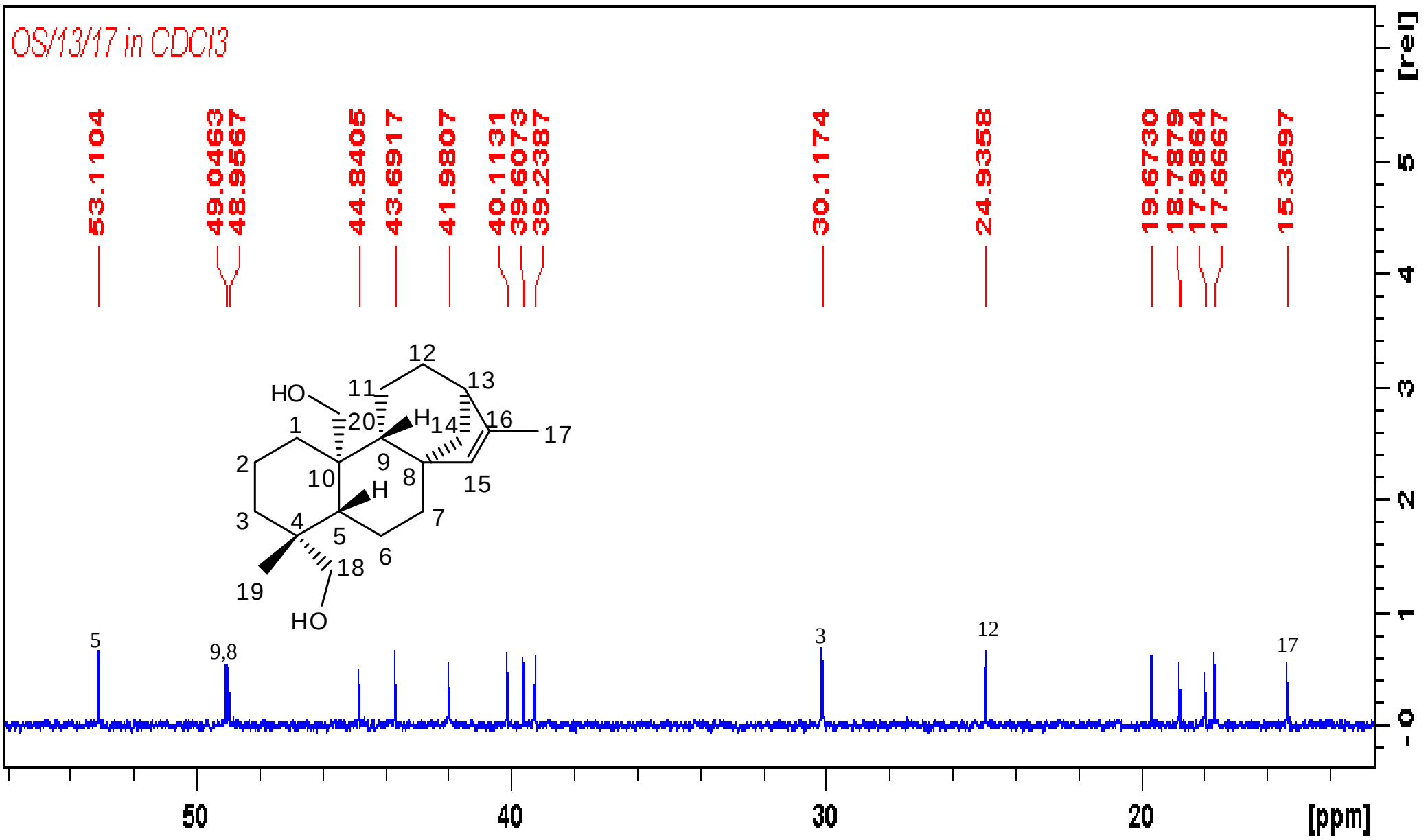
Expanded ^{1}H NMR spectrum of *ent*-kaur-15-en-18,20-diol (**B6**)



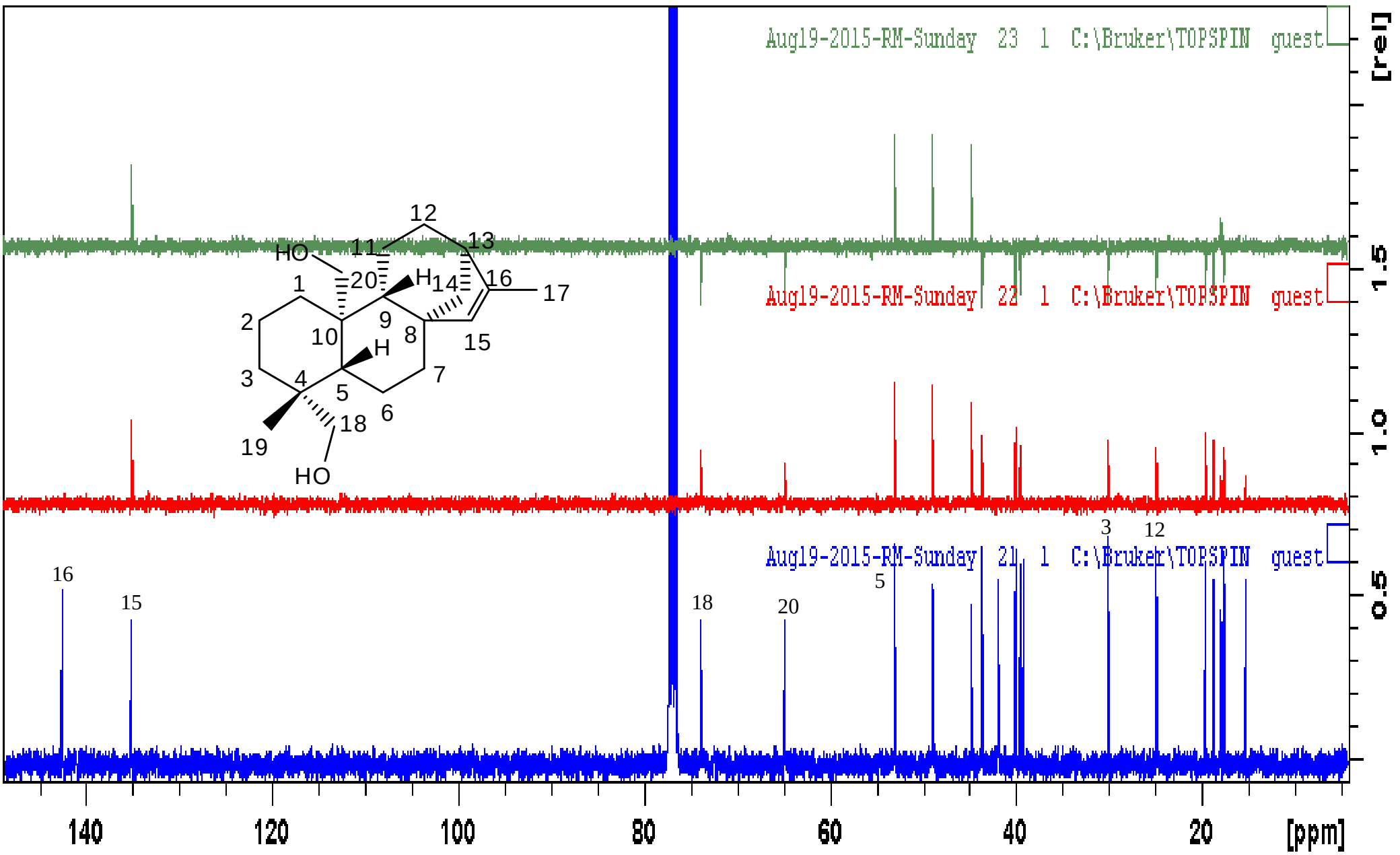
Expanded ^{1}H NMR spectrum of *ent*-kaur-15-en-18,20-diol (**B6**)



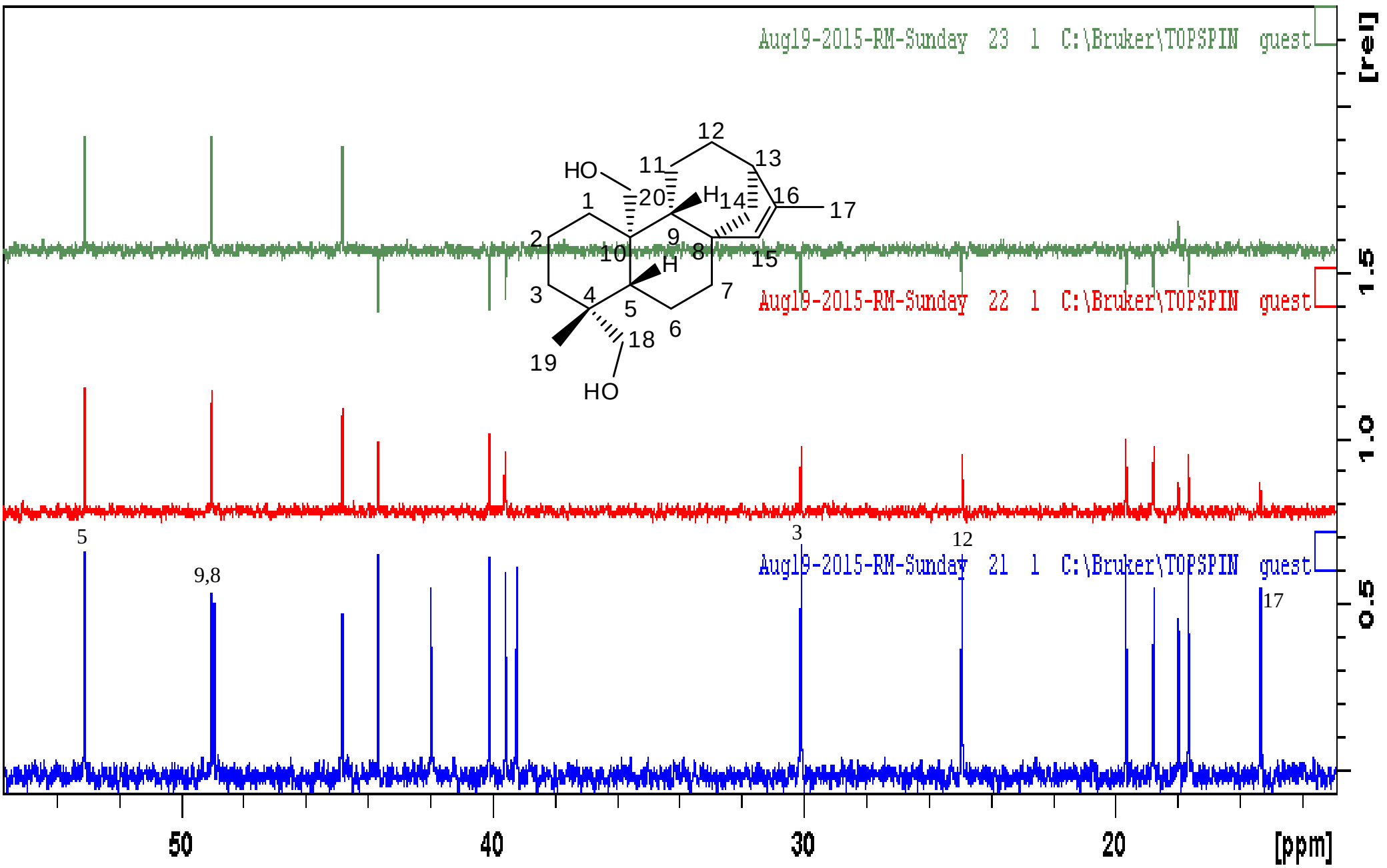
^{13}C NMR spectrum of *ent*-kaur-15-en-18,20-diol (**B6**)



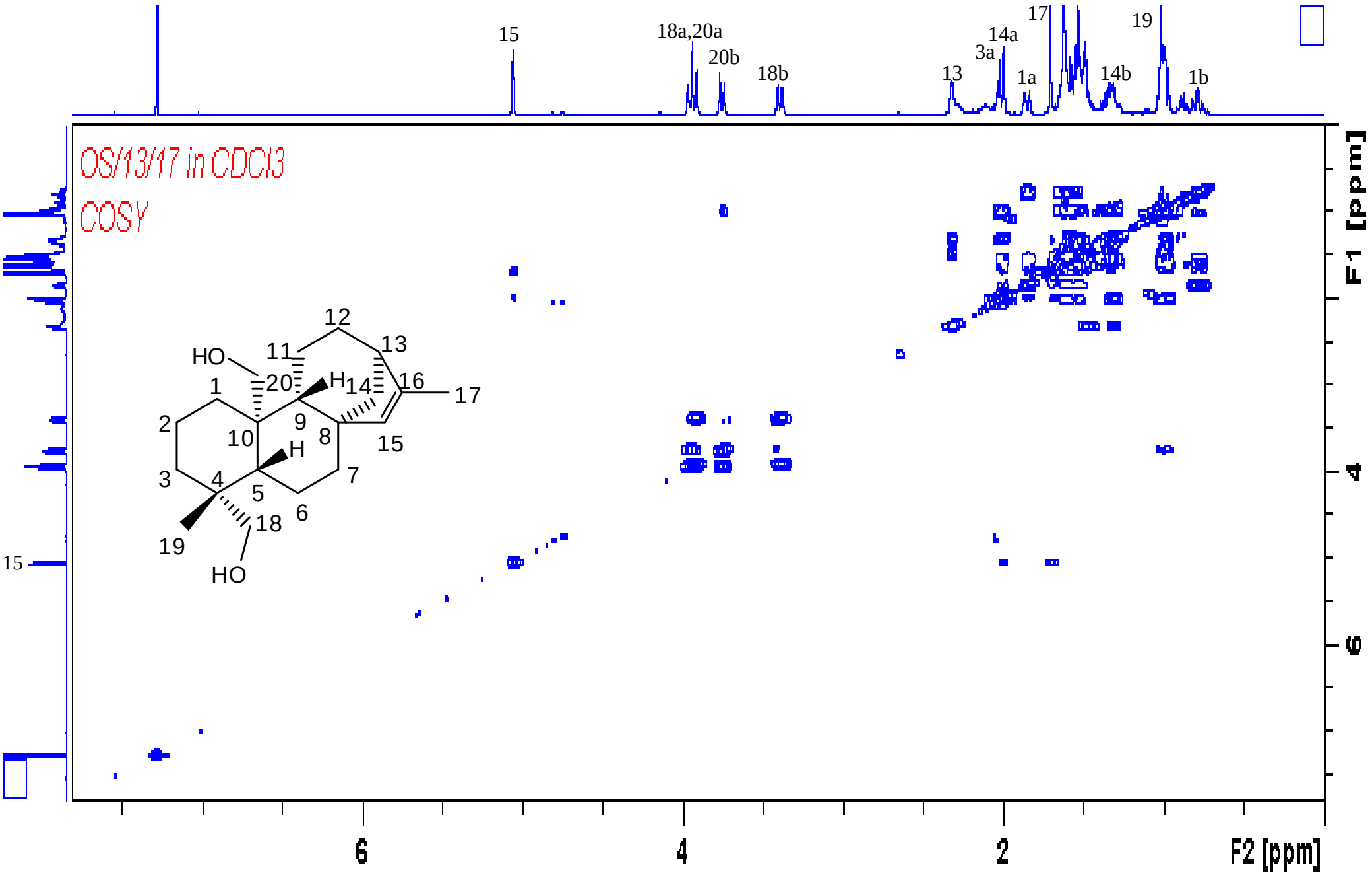
Expanded ^{13}C NMR spectrum of *ent*-kaur-15-en-18,20-diol (**B6**)



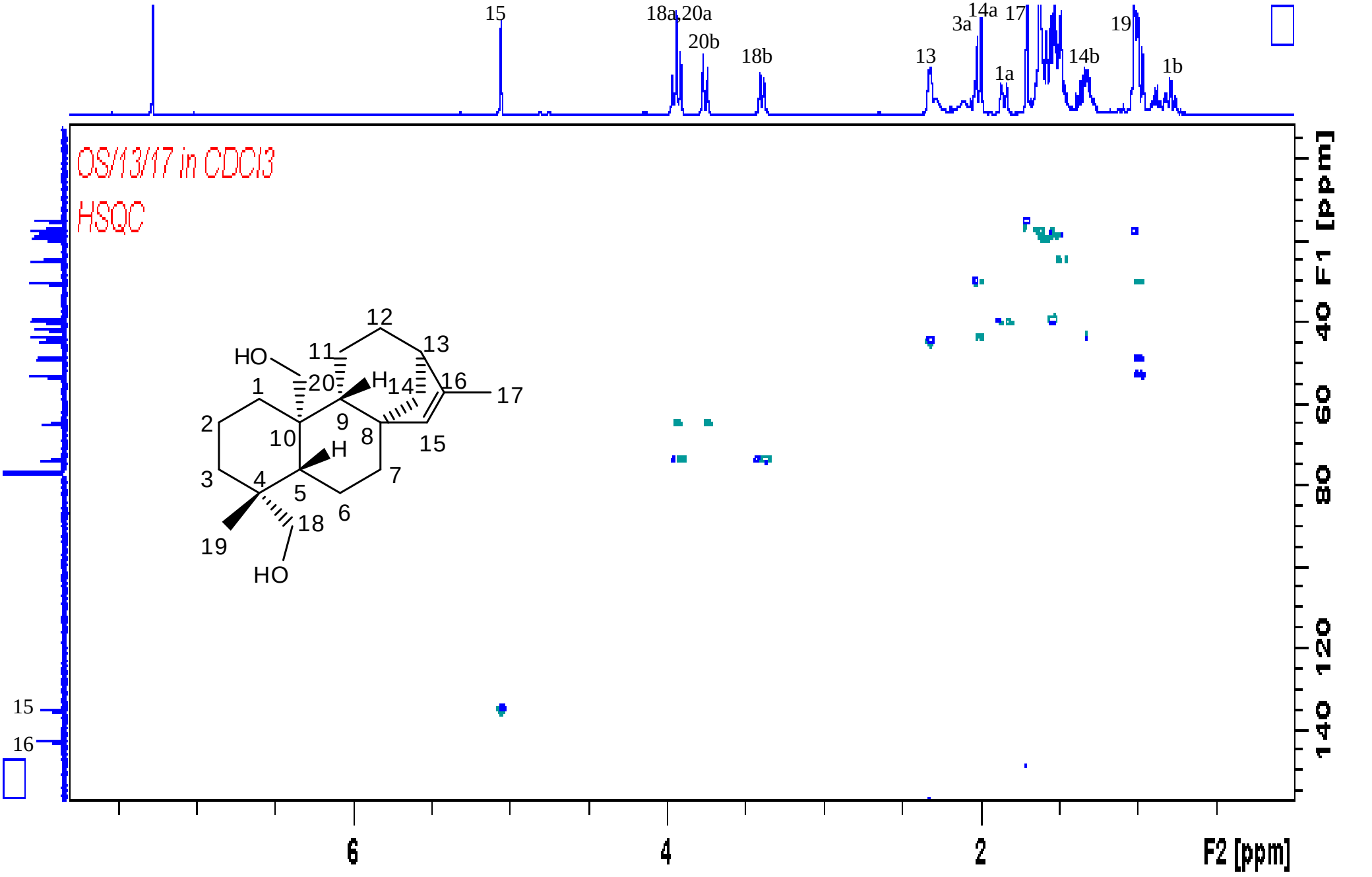
DEPT spectrum of *ent*-kaur-15-en-18,20-diol (**B6**)



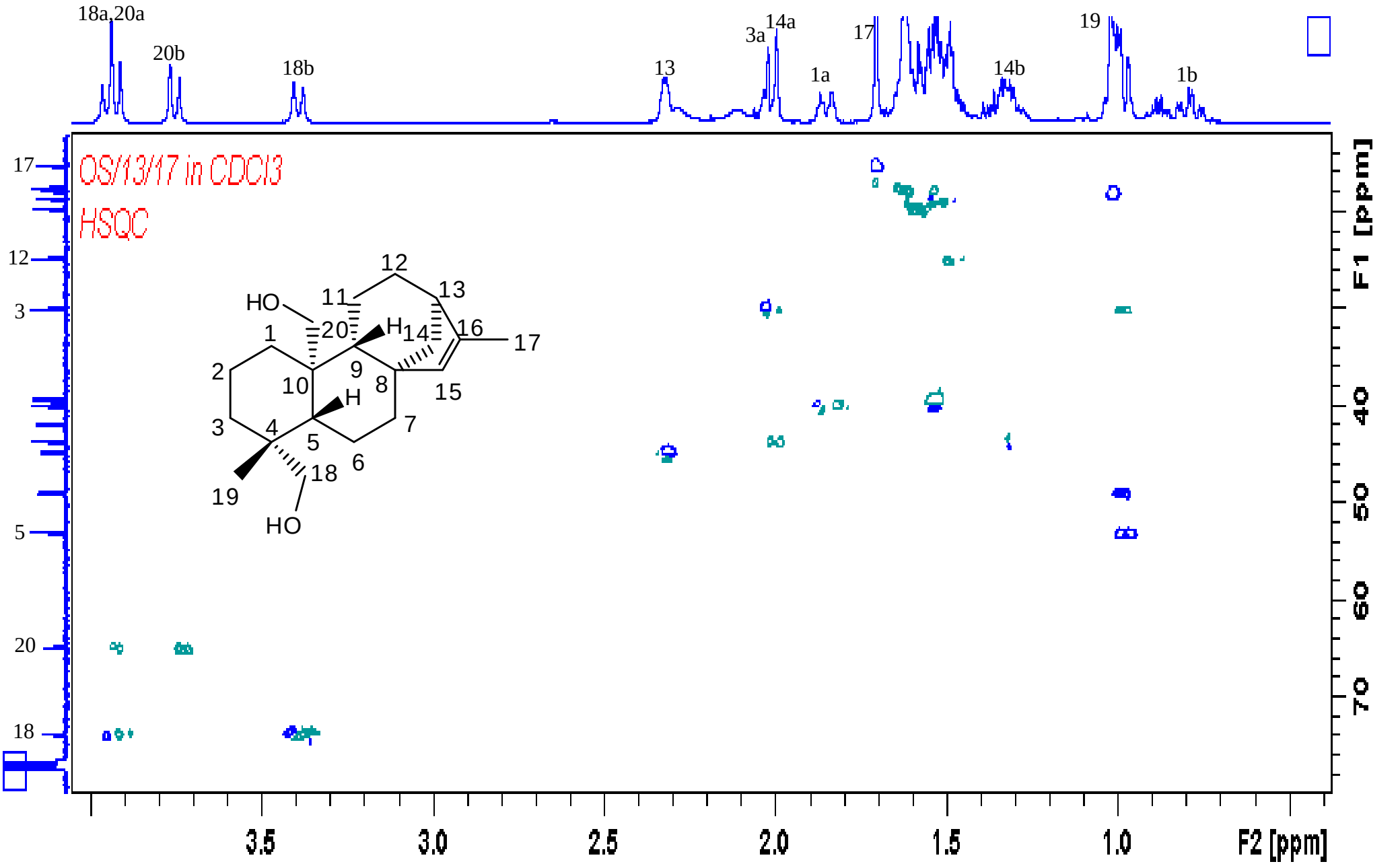
Expanded DEPT spectrum of *ent*-kaur-15-en-18,20-diol (**B6**)



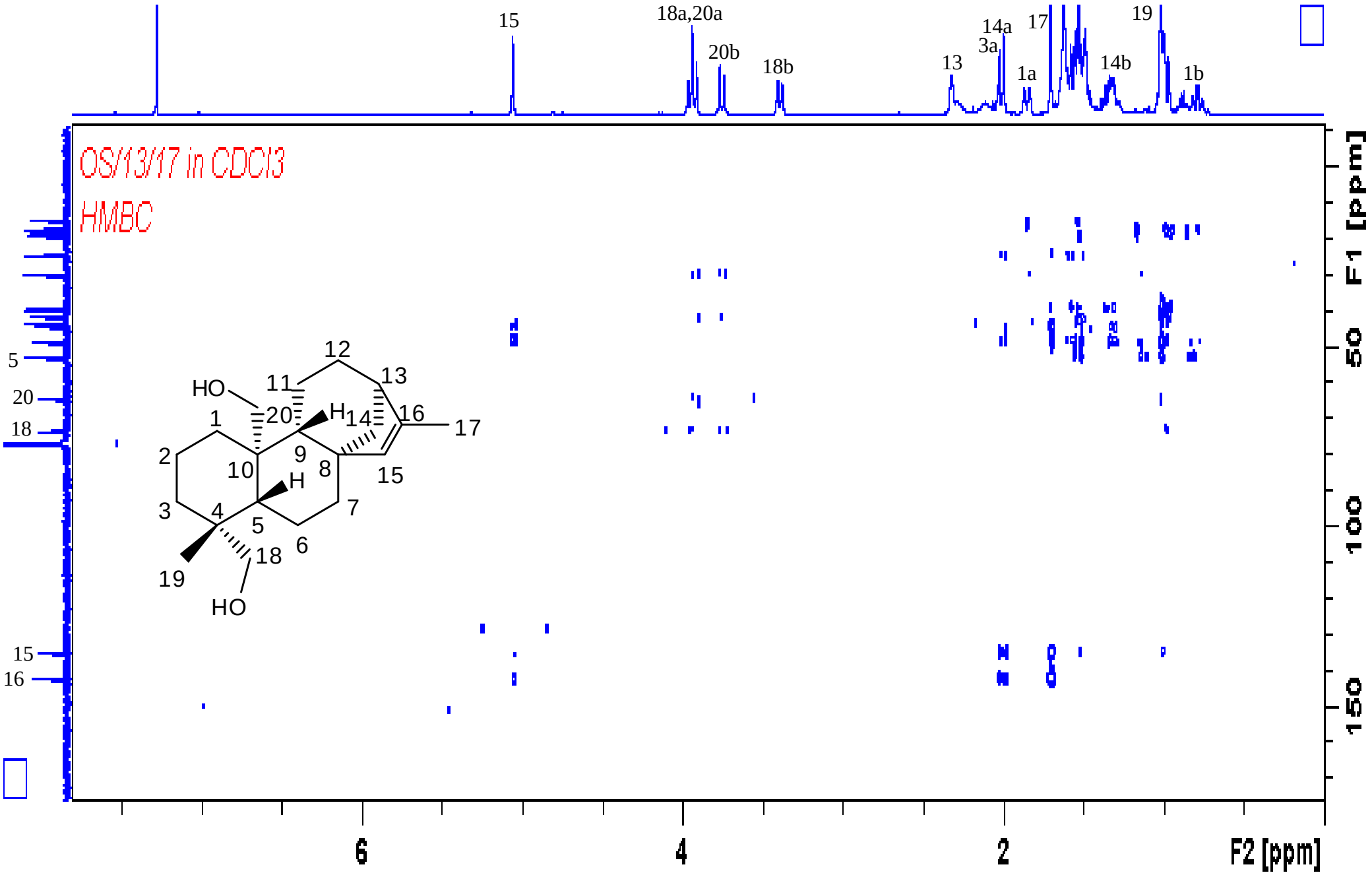
COSY spectrum of *ent*-kaur-15-en-18,20-diol (**B6**)



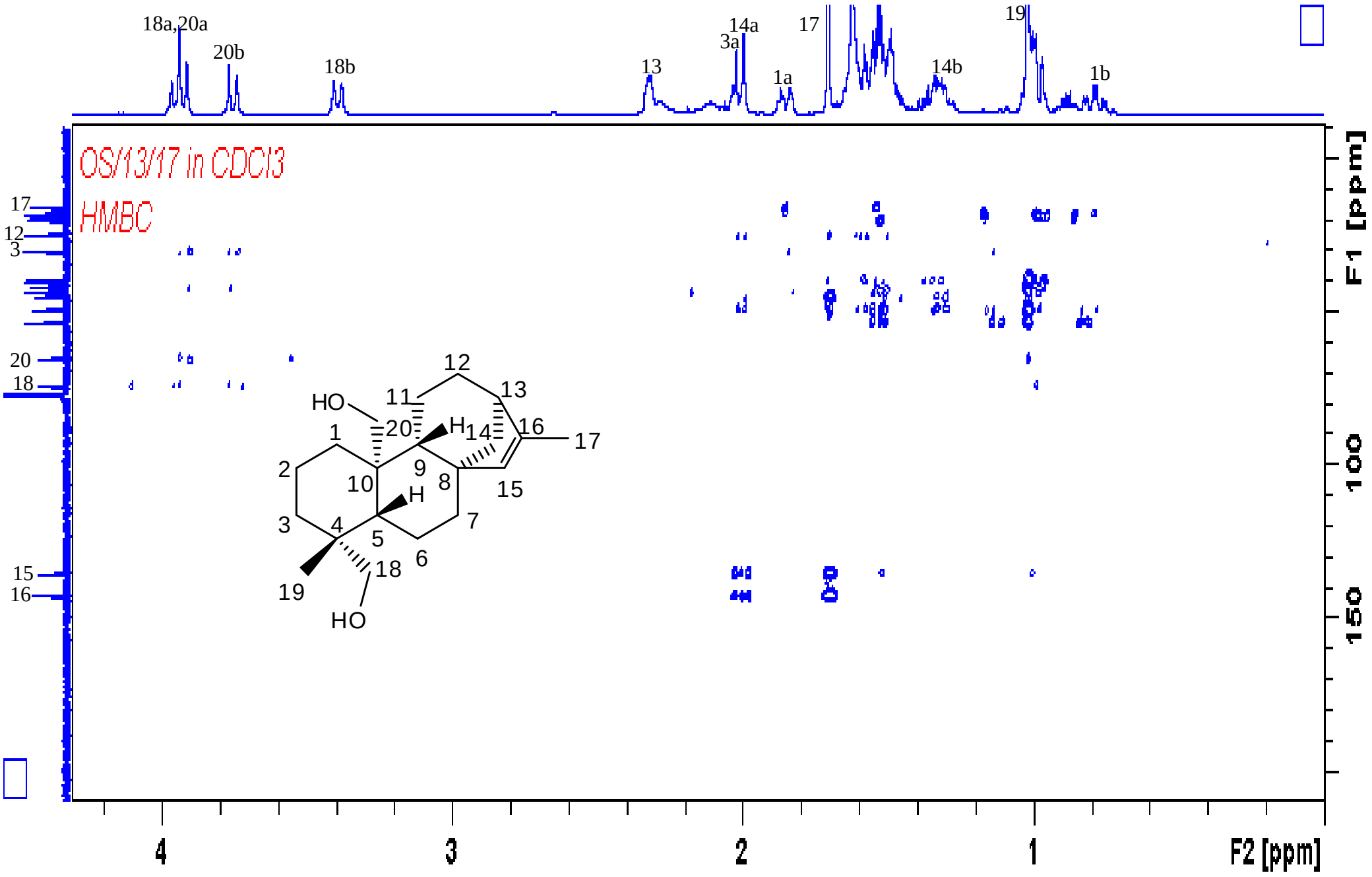
HSQC spectrum of *ent*-kaur-15-en-18,20-diol (**B6**)



Expanded HSQC spectrum of *ent*-kaur-15-en-18,20-diol (**B6**)



HMBC spectrum of *ent*-kaur-15-en-18,20-diol (**B6**)



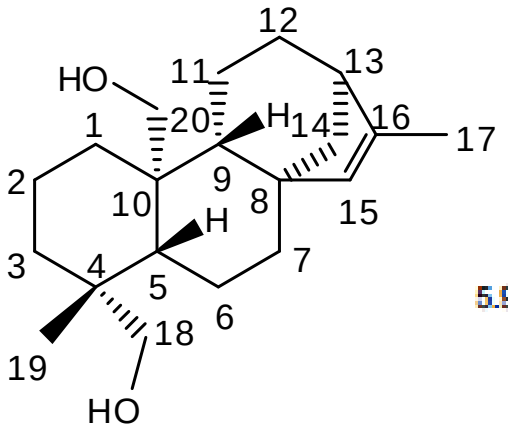
Expanded HMBC spectrum of *ent*-kaur-15-en-18,20-diol (**B6**)

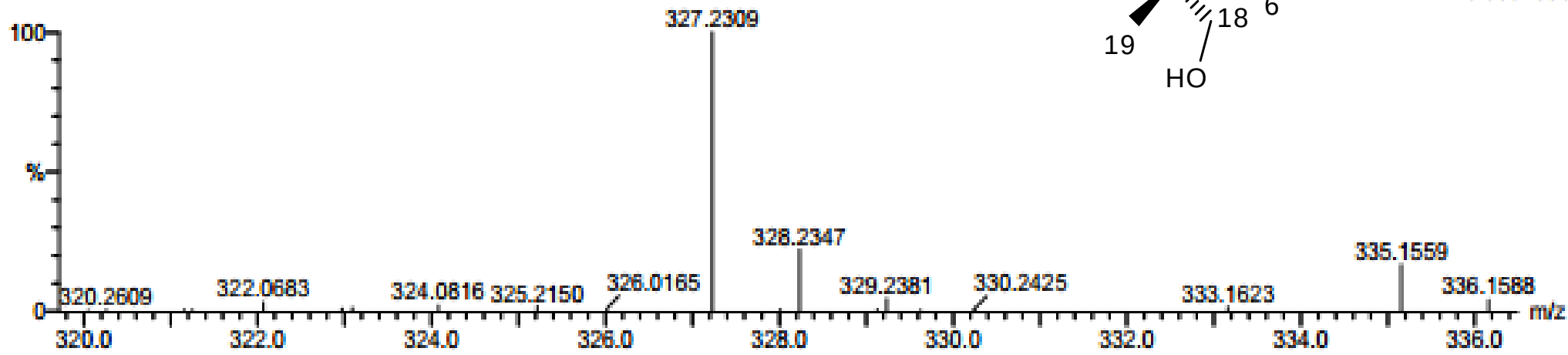
Single Mass Analysis

Tolerance = 5.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 2

Monoisotopic Mass, Even Electron Ions
2 formula(e) evaluated with 1 results within limits (up to 20 best isotopic matches for each mass)
Elements Used:
C: 20-25 H: 30-35 O: 0-5 Na: 1-1

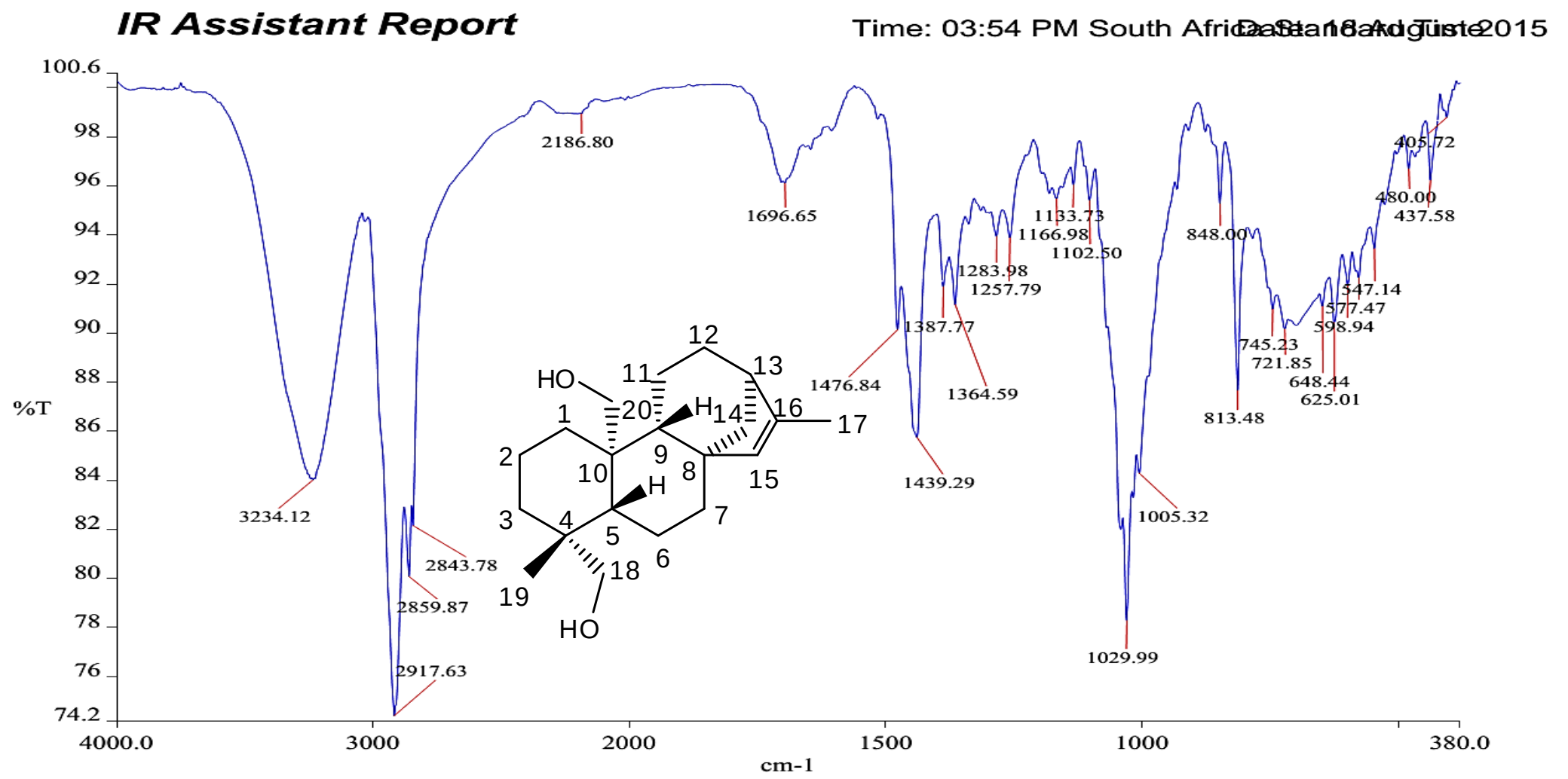
05-13-17 36 (1.181) Cm (1:61)
TOF MS ES+



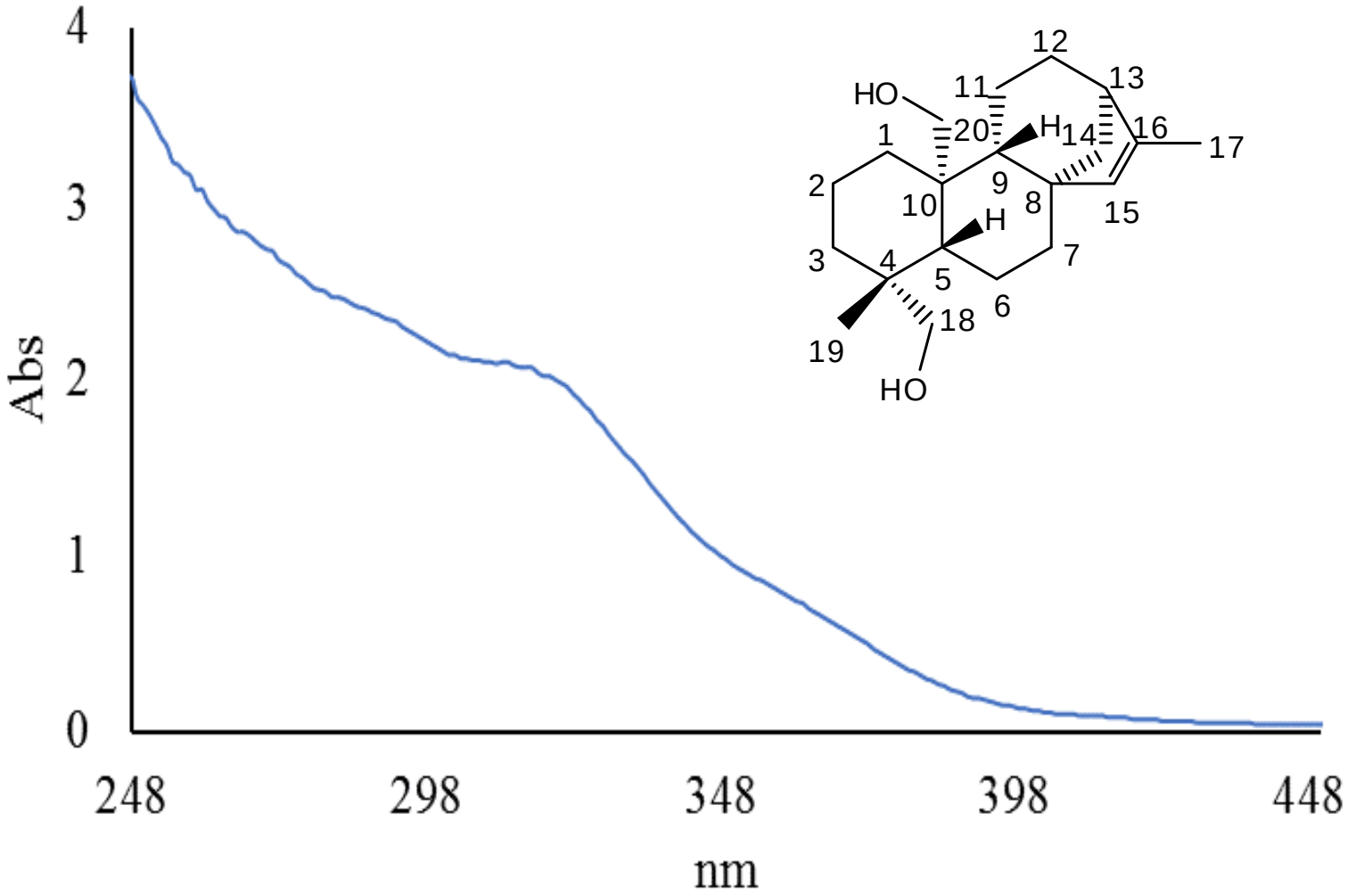


Minimum:				-1.5			
Maximum:		5.0	5.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
327.2309	327.2300	0.9	2.8	4.5	31.6	0.0	C20 H32 O2 Na

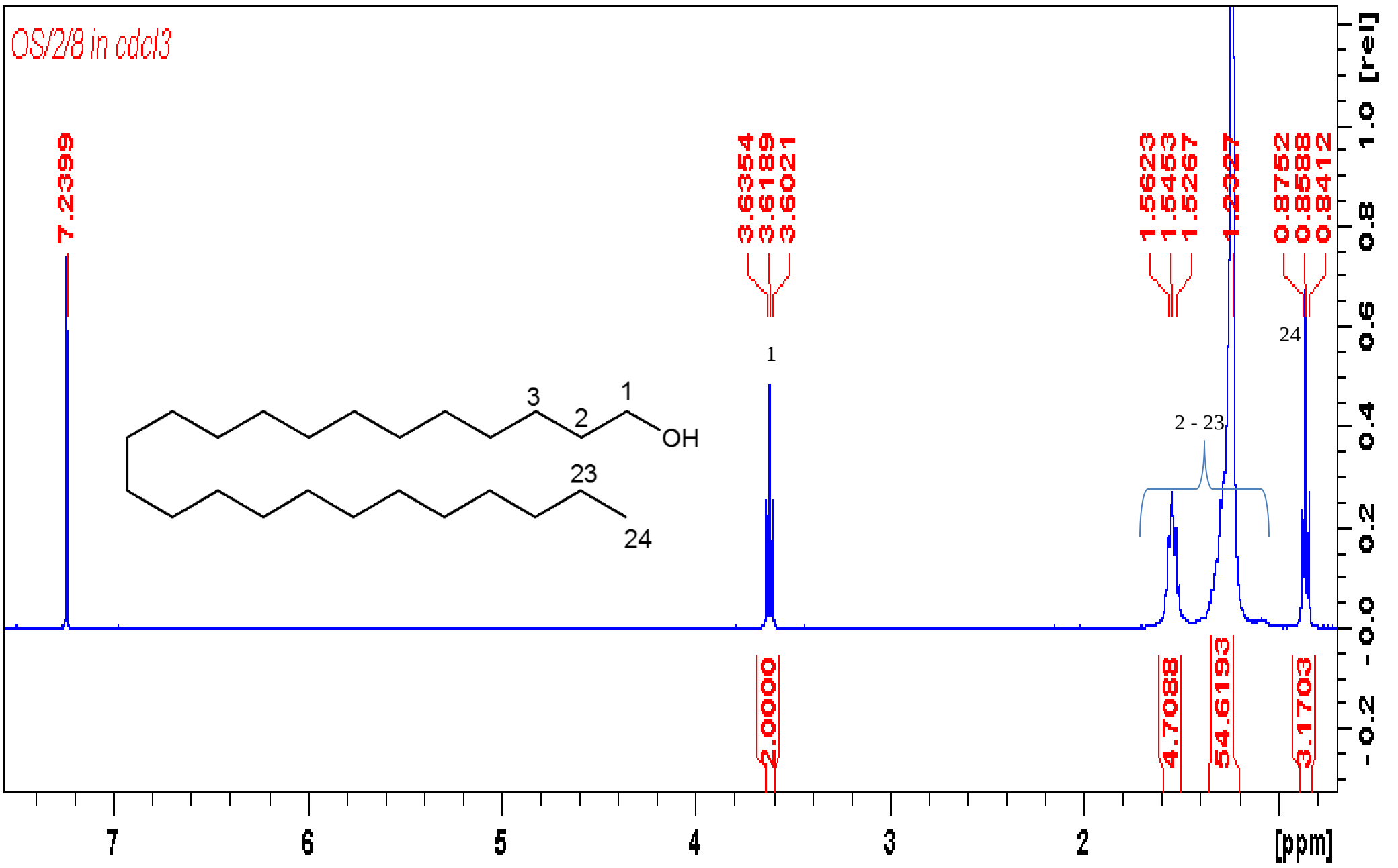
Mass spectrum of *ent*-kaur-15-en-18,20-diol (**B6**)



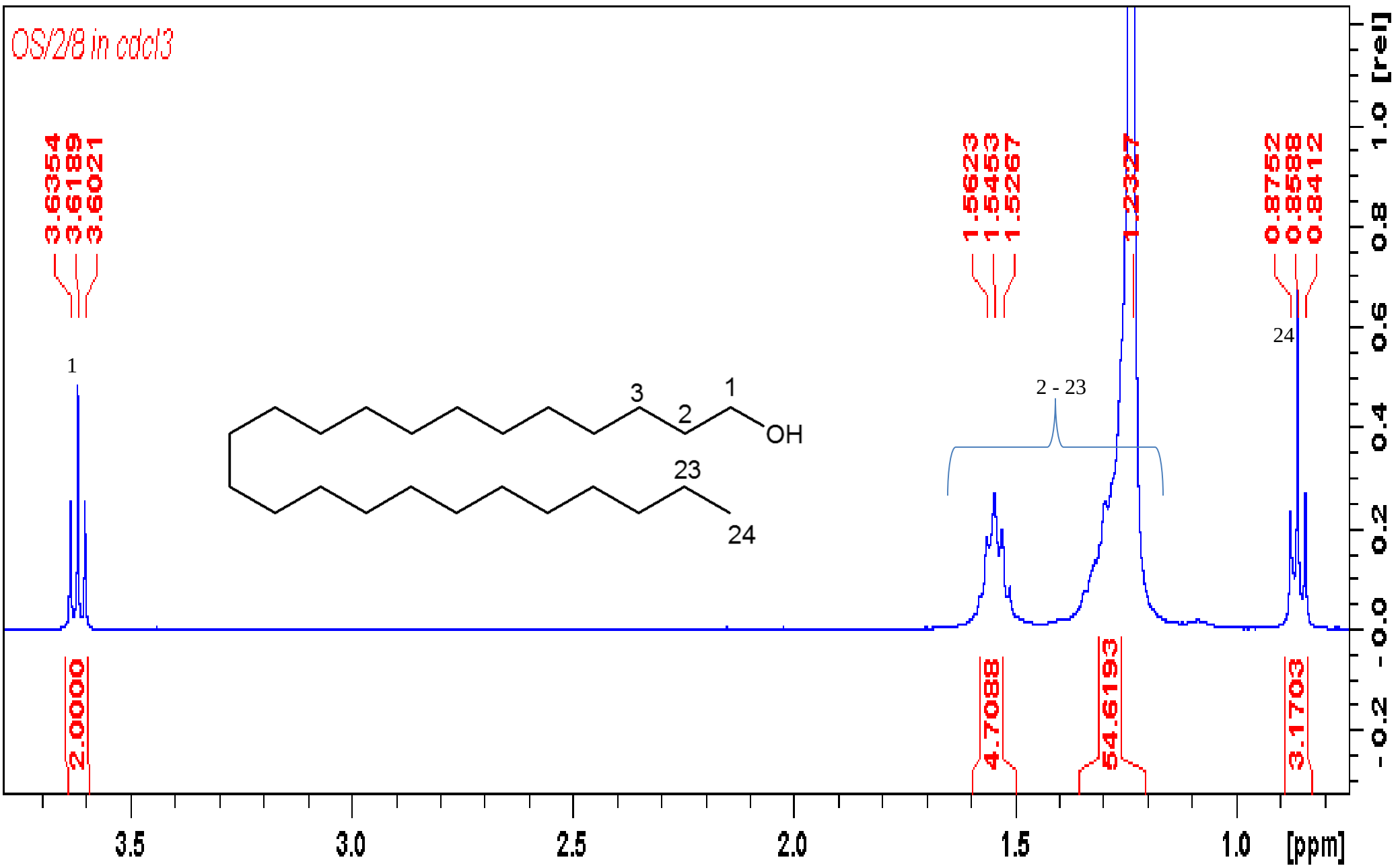
IR spectrum of *ent*-kaur-15-en-18,20-diol (**B6**)



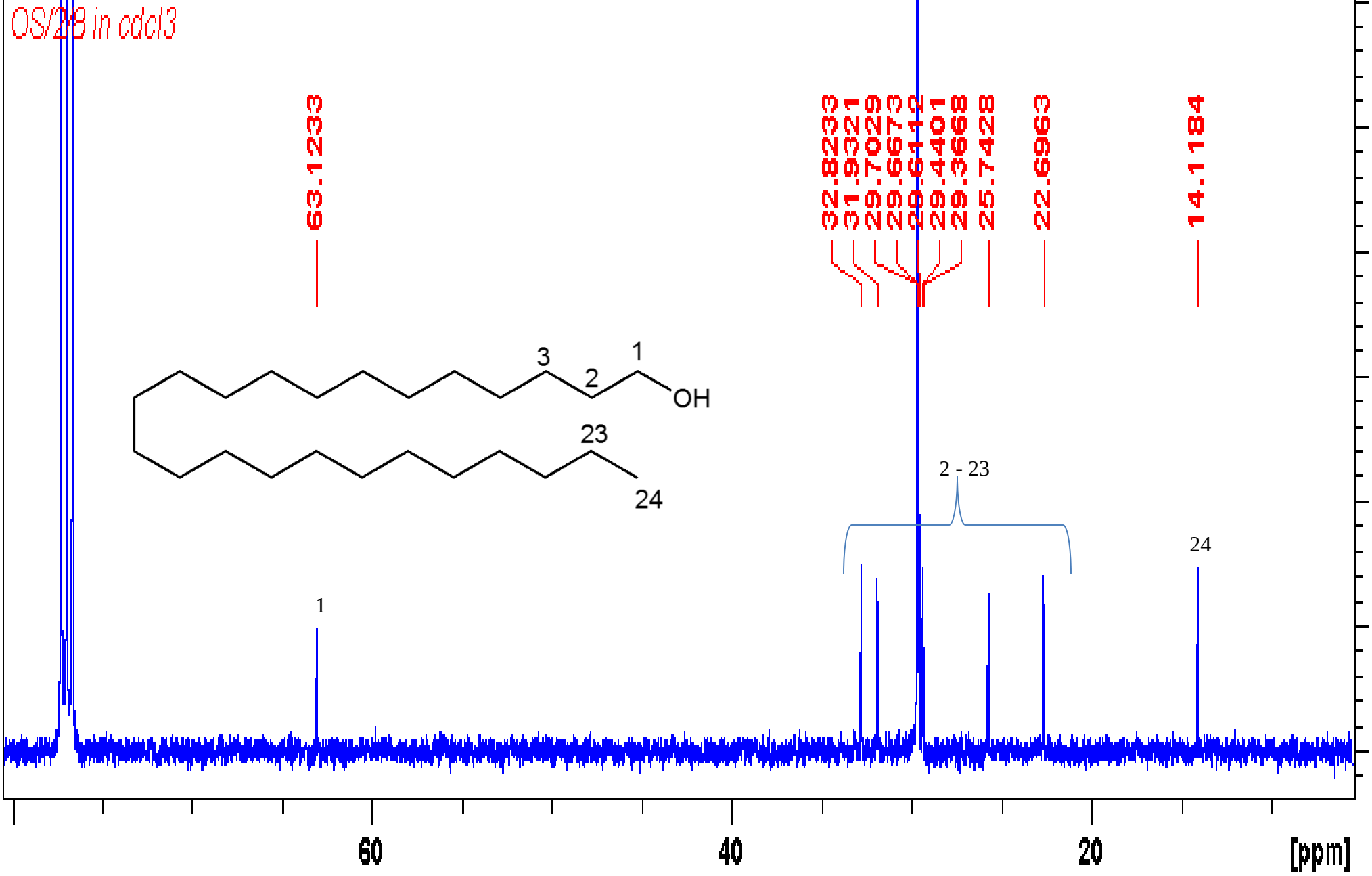
UV spectrum of *ent*-kaur-15-en-18,20-diol (**B6**)



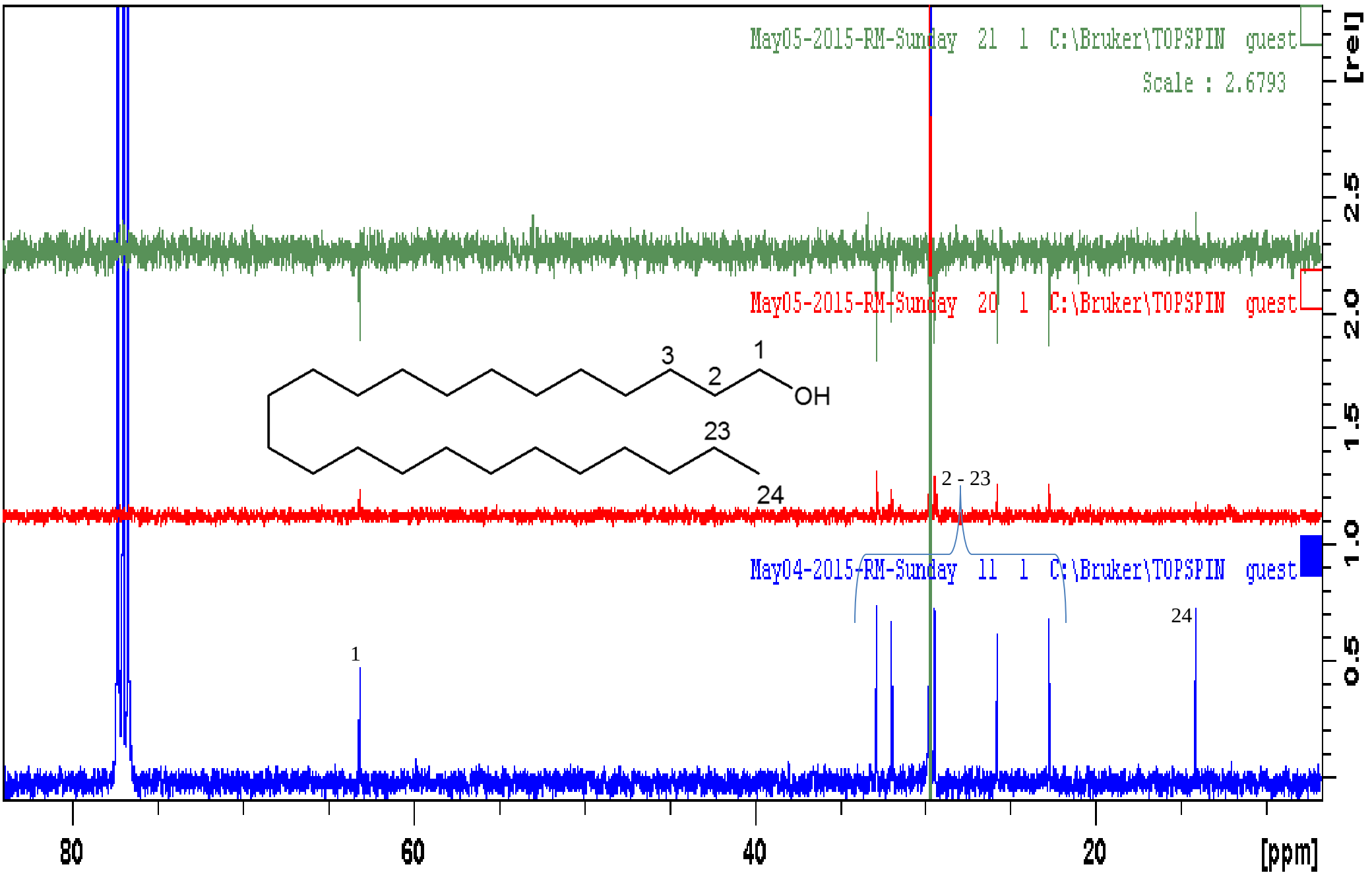
^{1}H NMR spectrum of tetracosan-1-ol (**B7**)



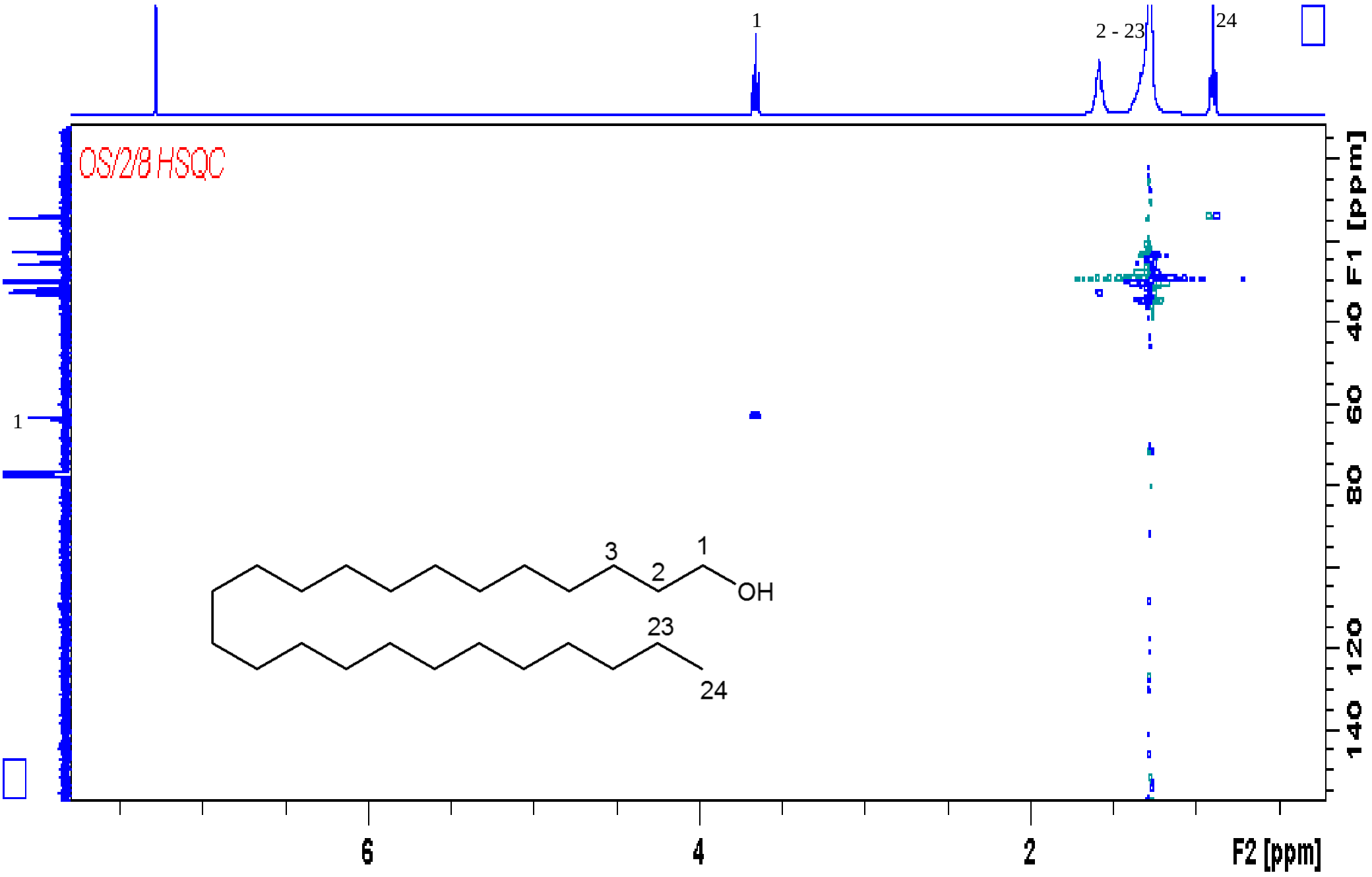
Expanded ^{1}H NMR spectrum of tetracosan-1-ol (**B7**)



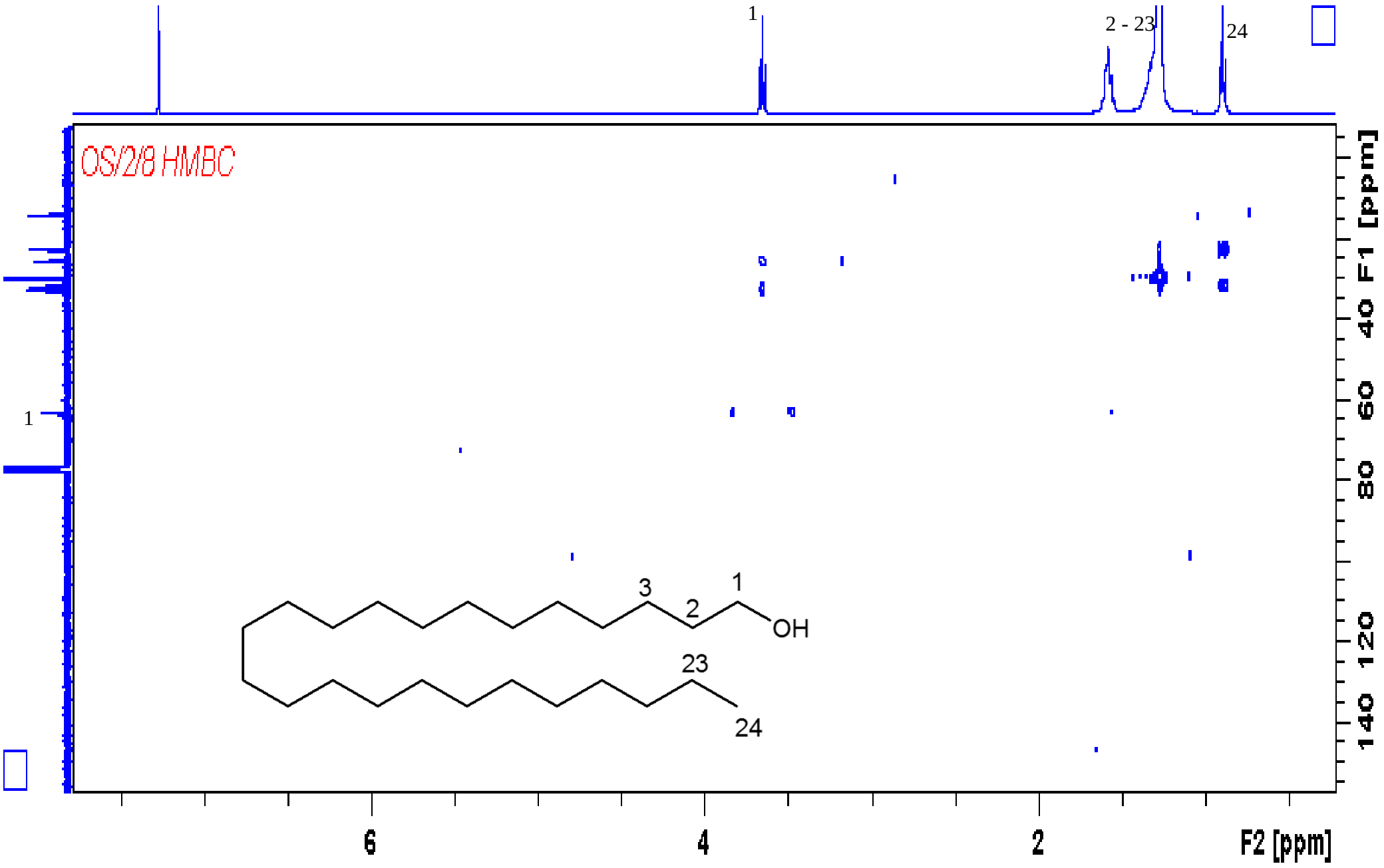
^{13}C NMR spectrum of tetracosan-1-ol (**B7**)



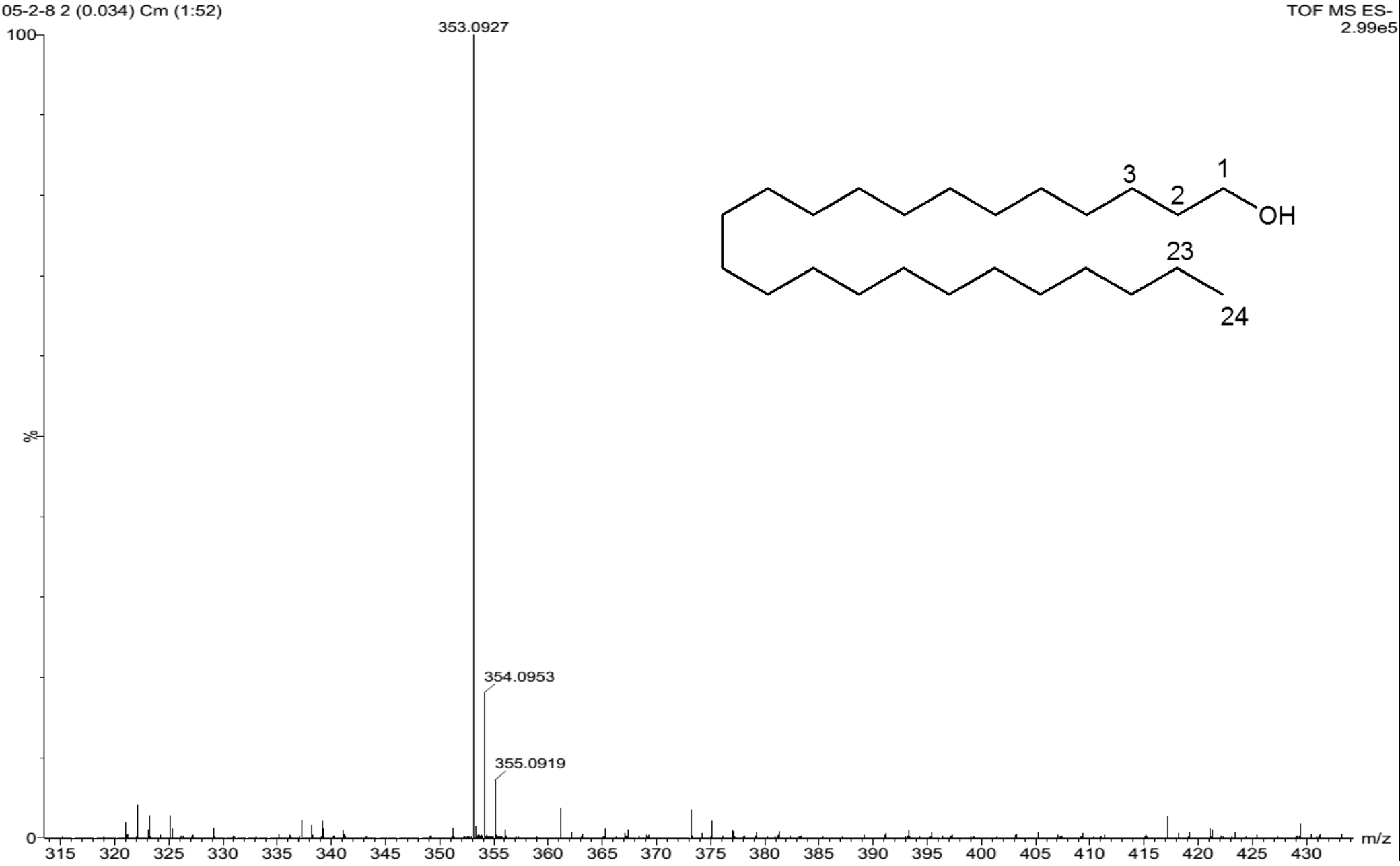
DEPT spectrum of tetracosan-1-ol (**B7**)



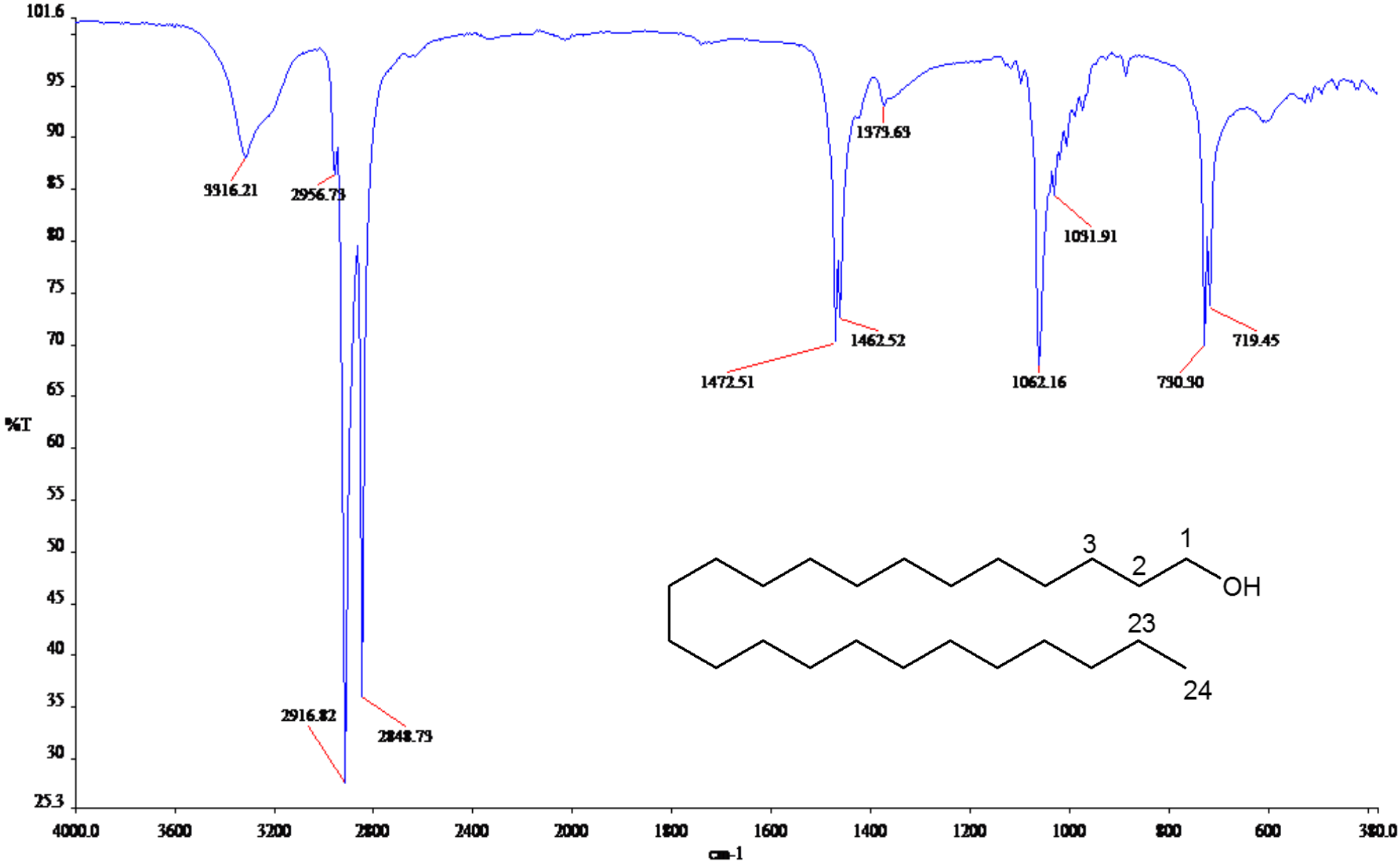
HSQC spectrum of tetracosan-1-ol (**B7**)



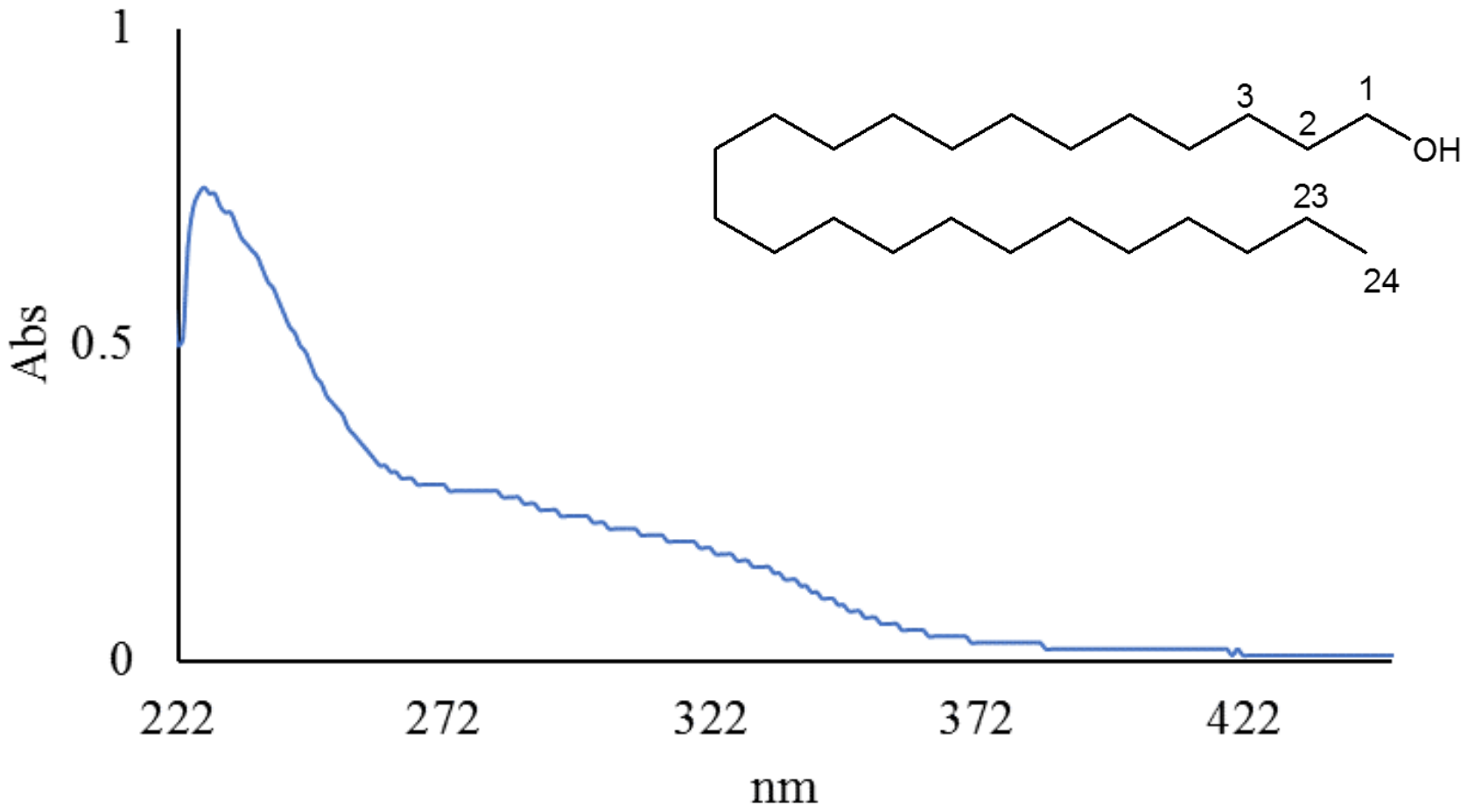
HMBC spectrum of tetracosan-1-ol (**B7**)



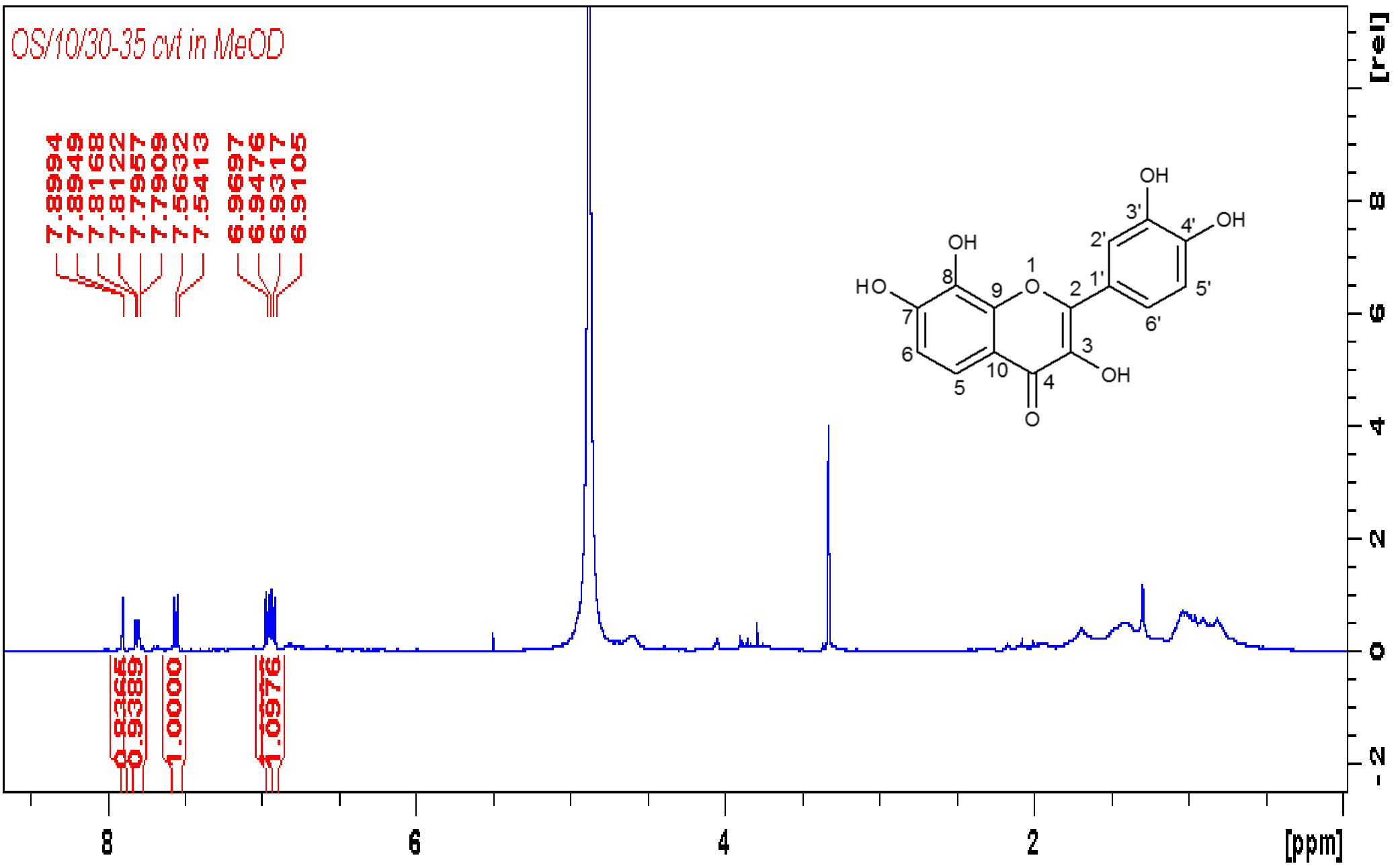
Mass spectrum of tetracosan-1-ol (**B7**)



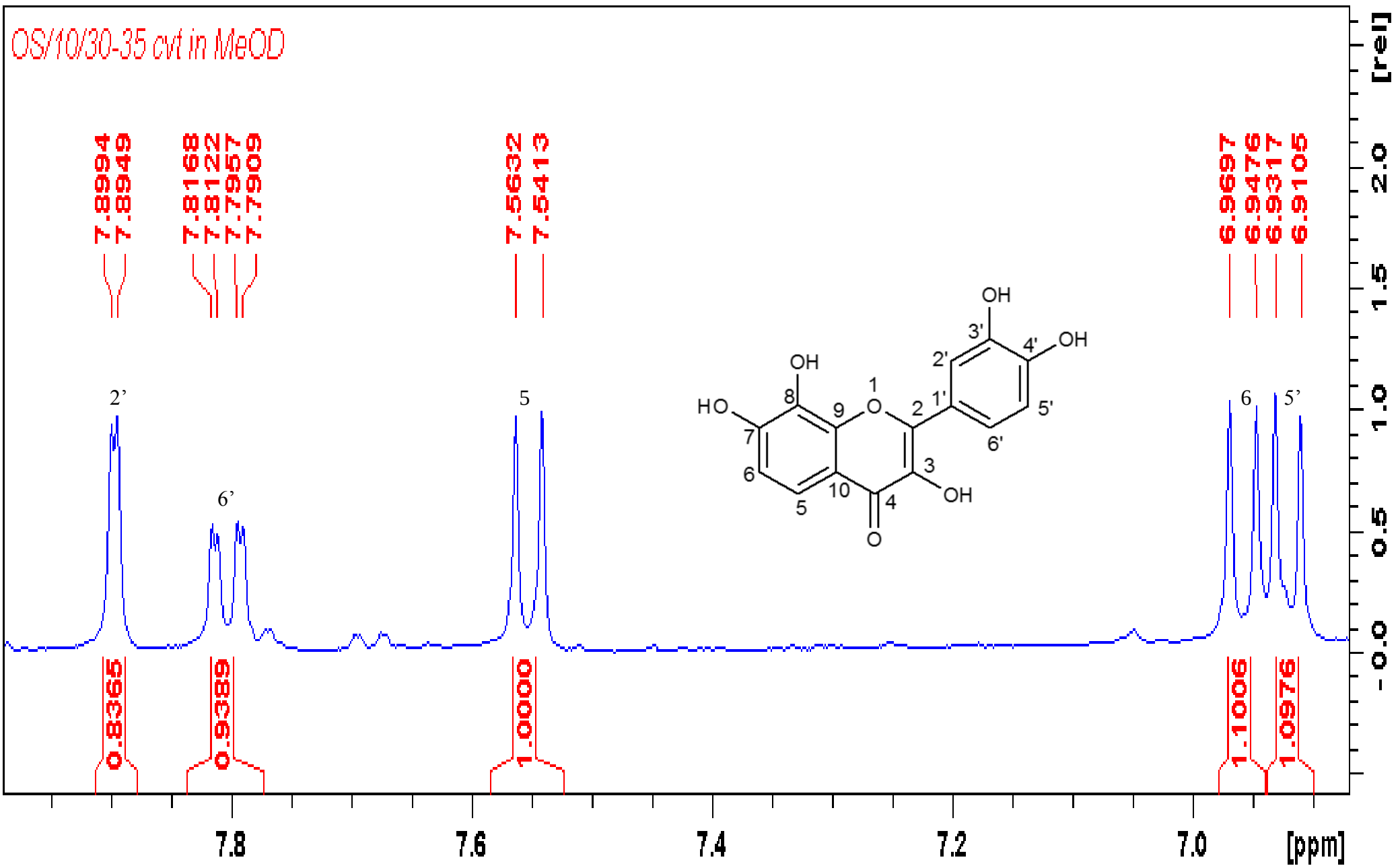
IR spectrum of tetracosan-1-ol (**B7**)



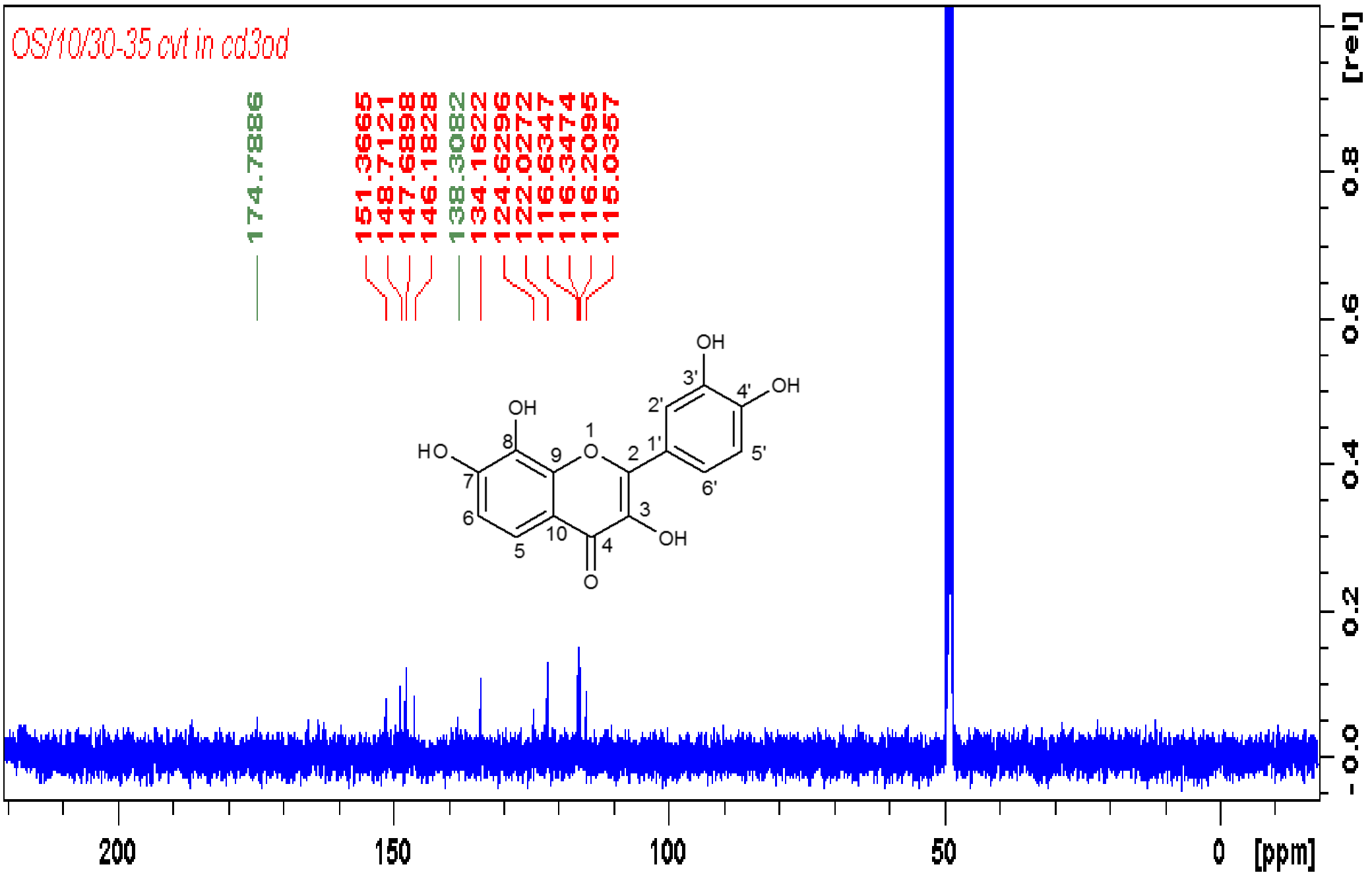
UV spectrum of tetracosan-1-ol (**B7**)



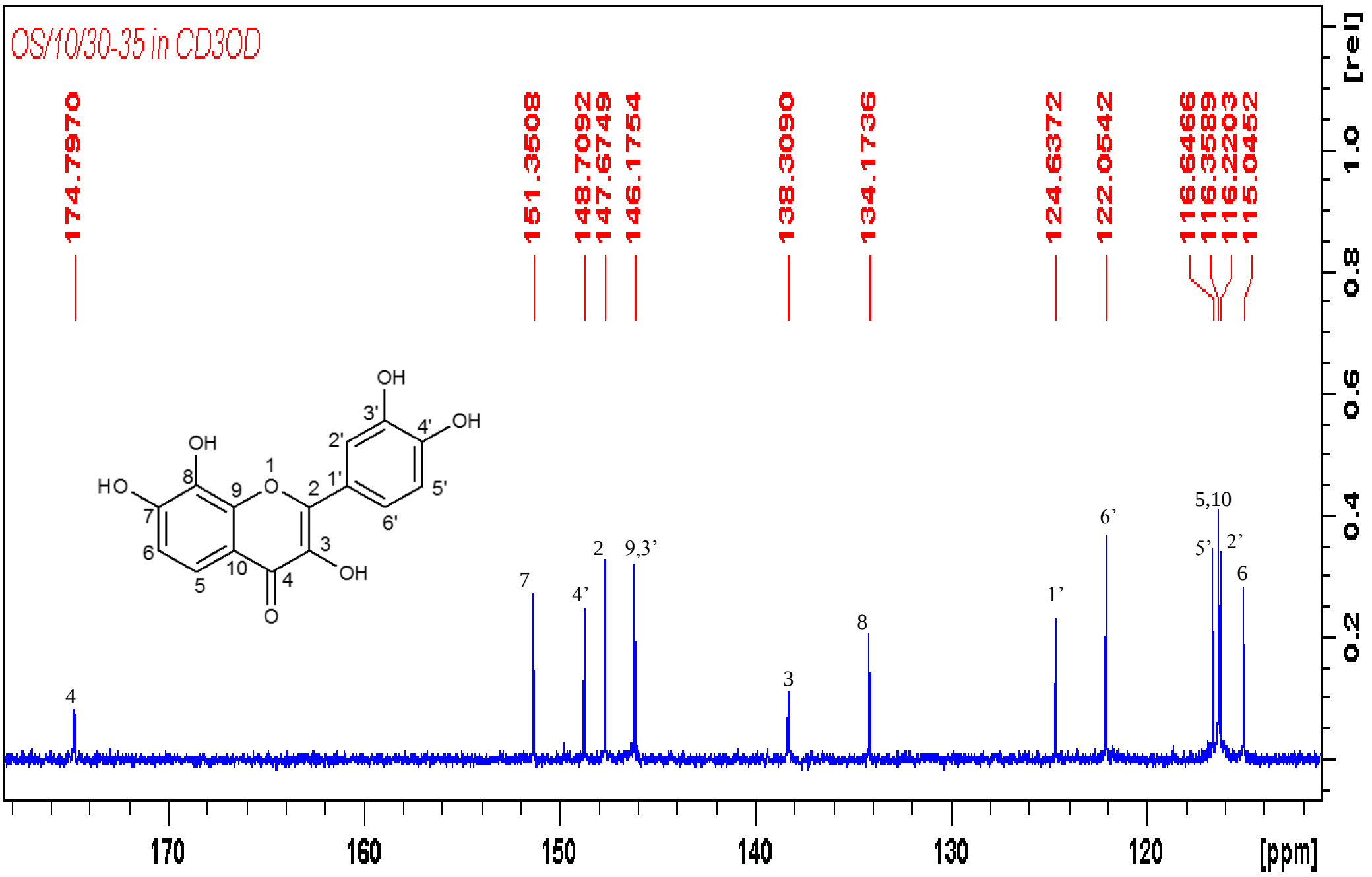
^{1}H NMR spectrum of melanoxetin (**B8**)



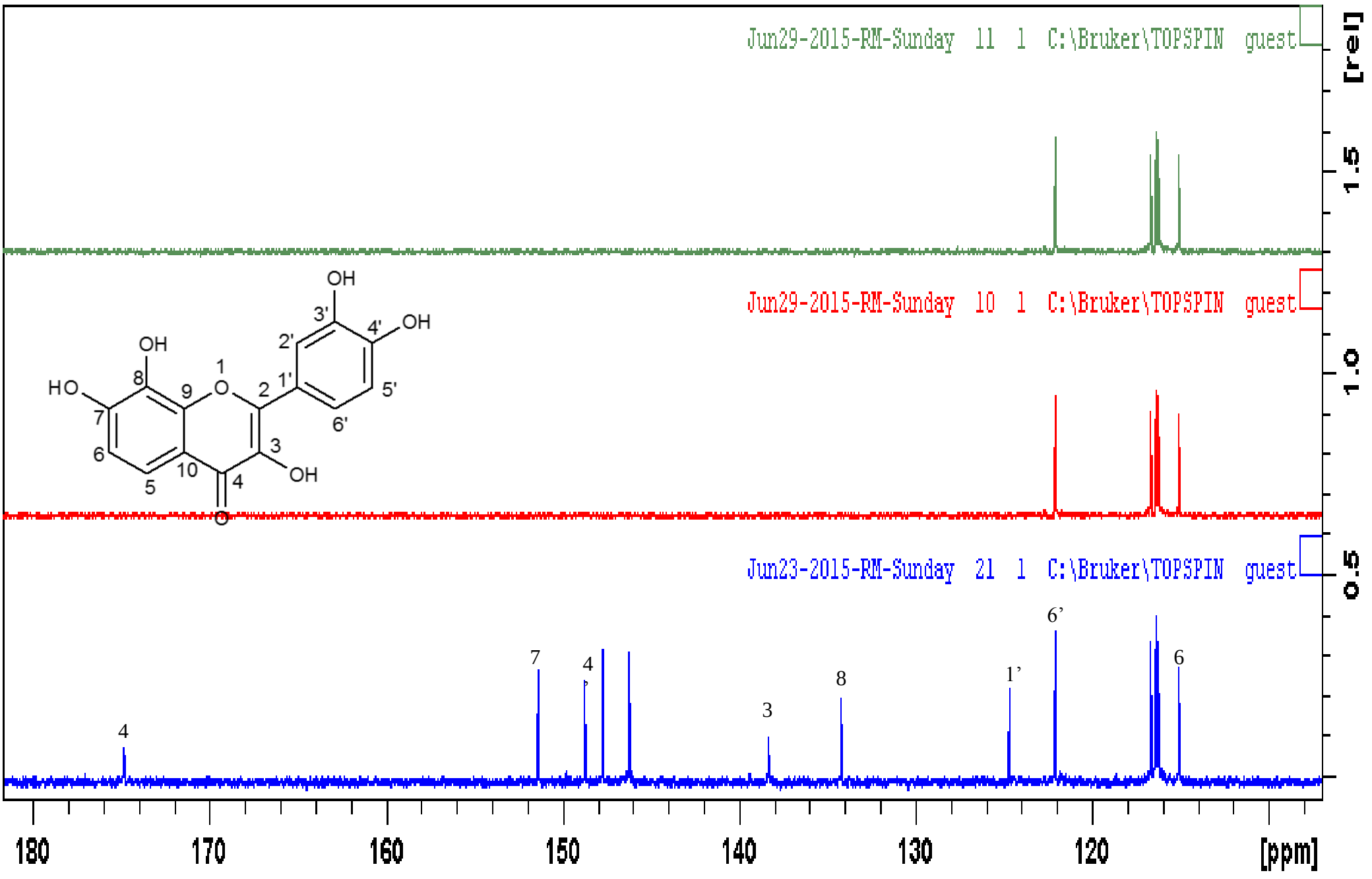
Expanded ^{1}H NMR spectrum of melanoxetin (**B8**)



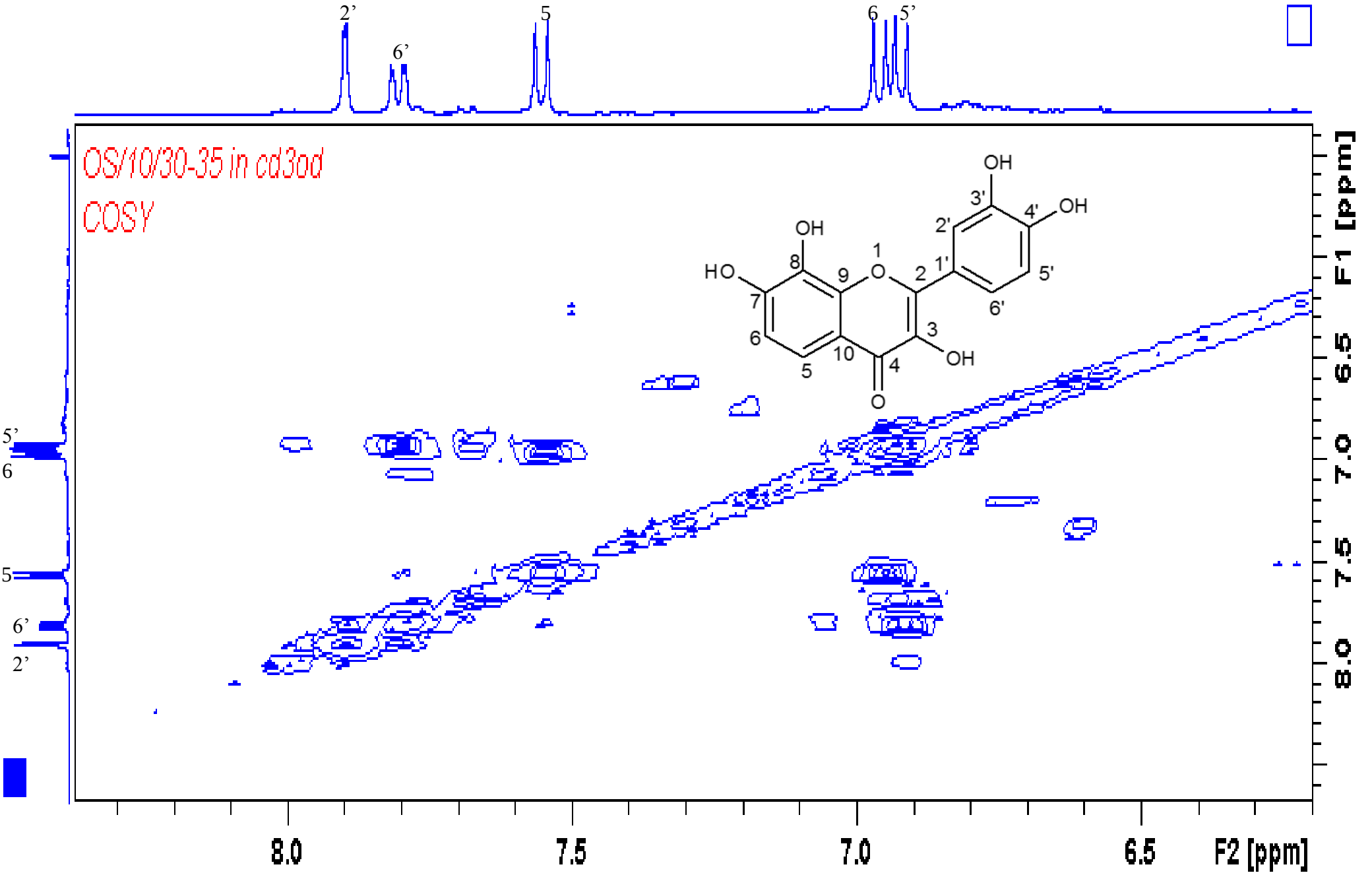
^{13}C NMR spectrum of melanoxetin (**B8**)



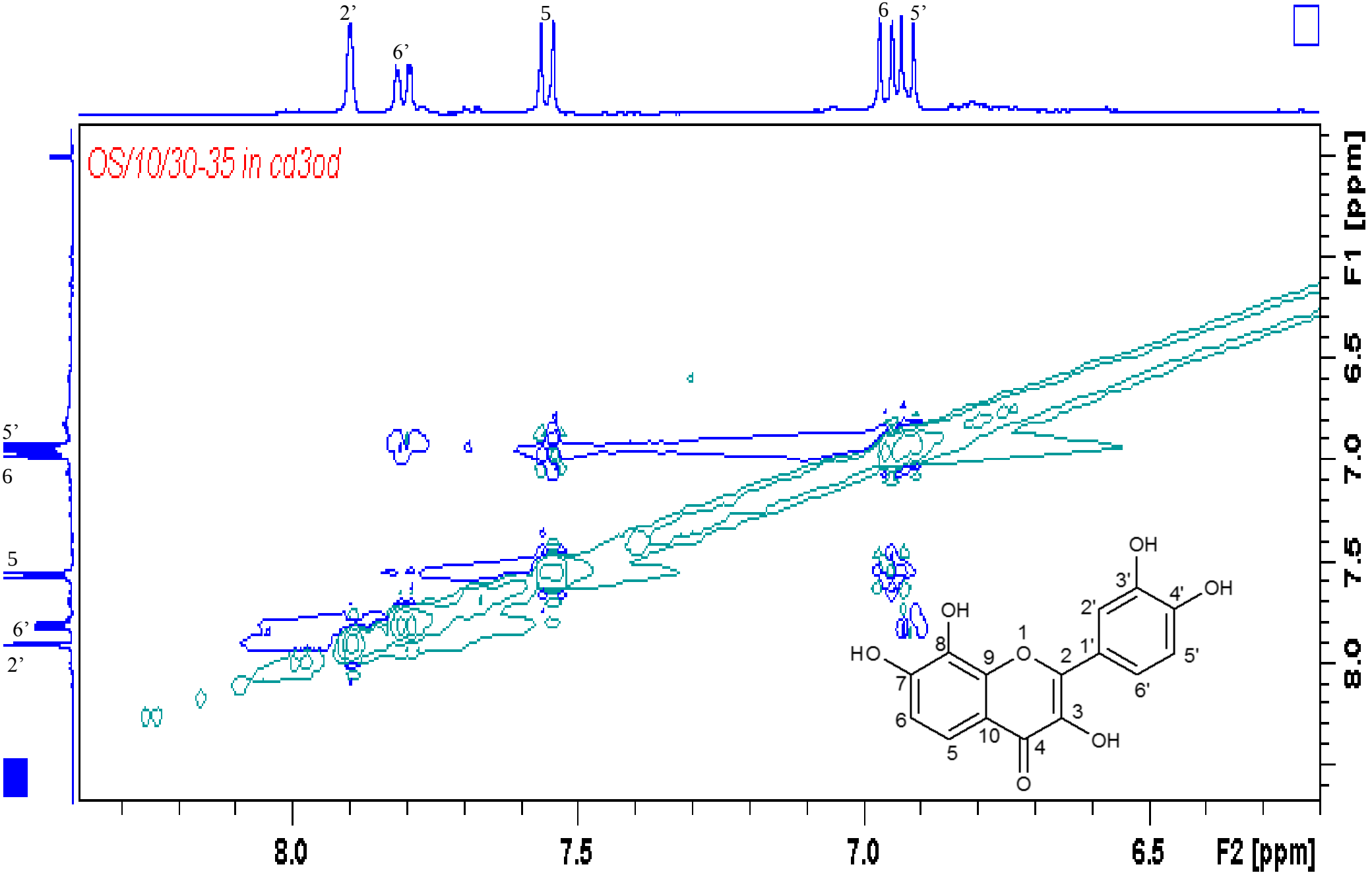
Expanded ^{13}C NMR spectrum of melanoxetin (**B8**)



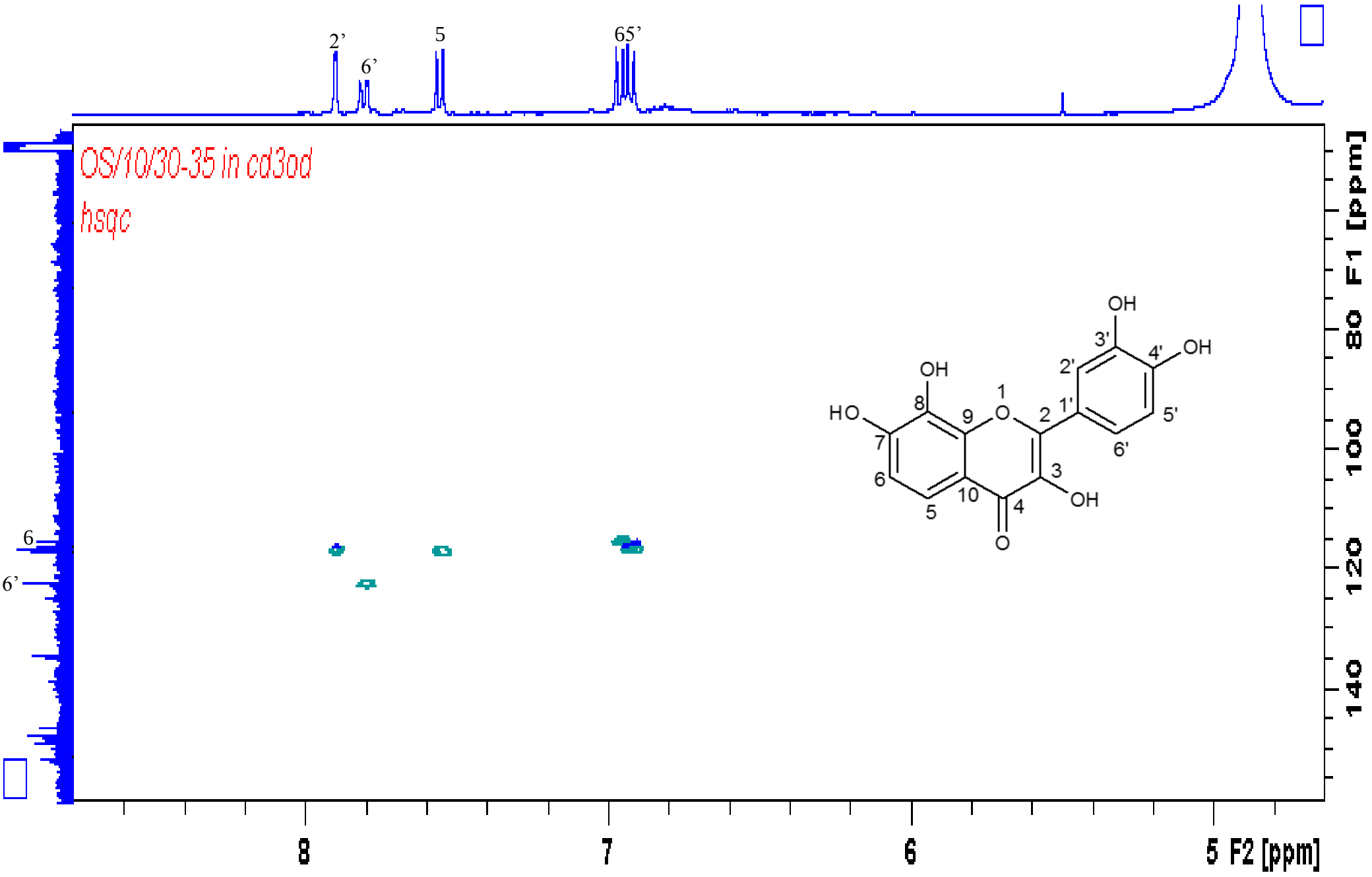
DEPT spectrum of melanoxetin (**B8**)



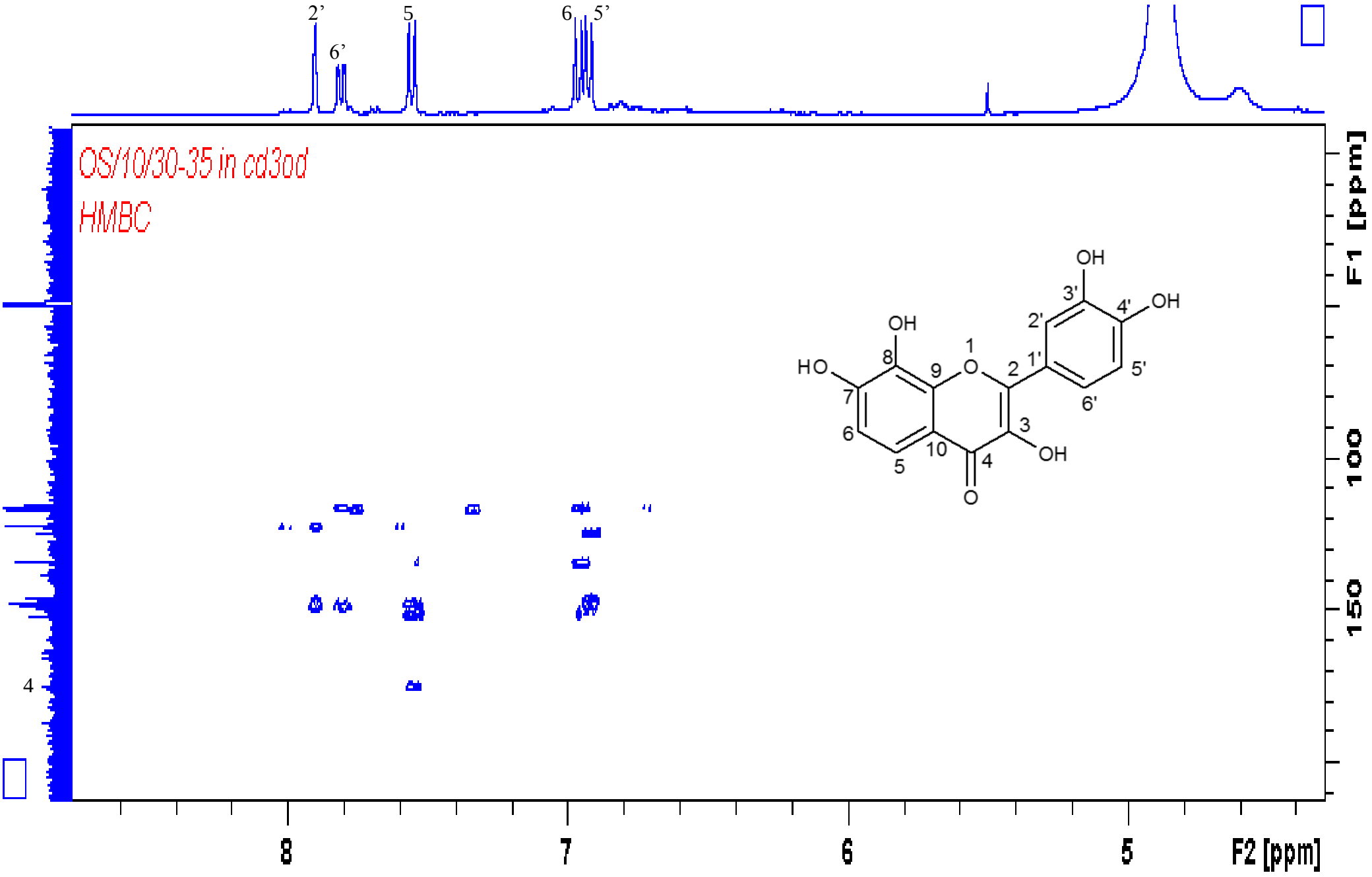
COSY spectrum of melanoxetin (**B8**)



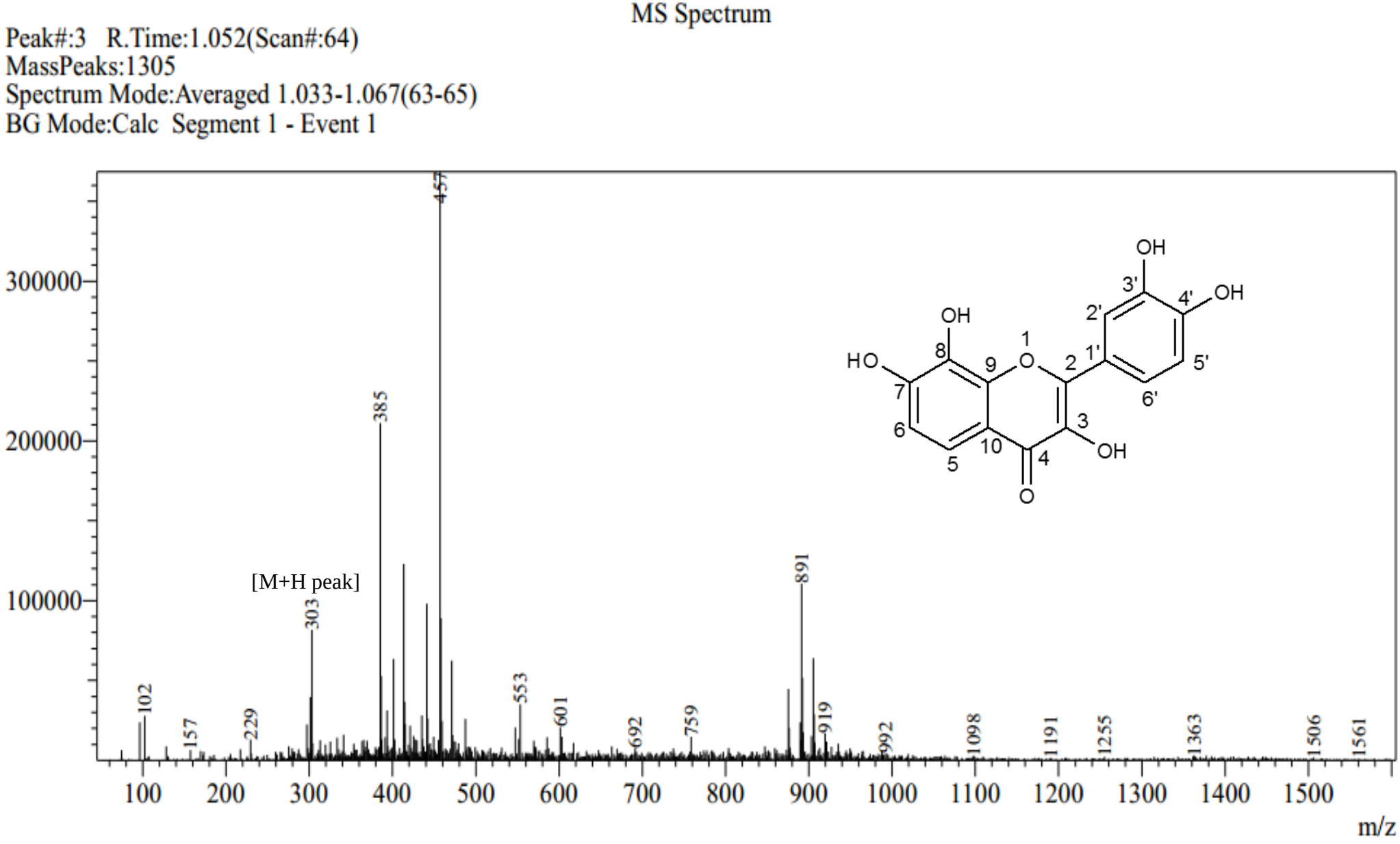
NOESY spectrum of melanoxetin (**B8**)



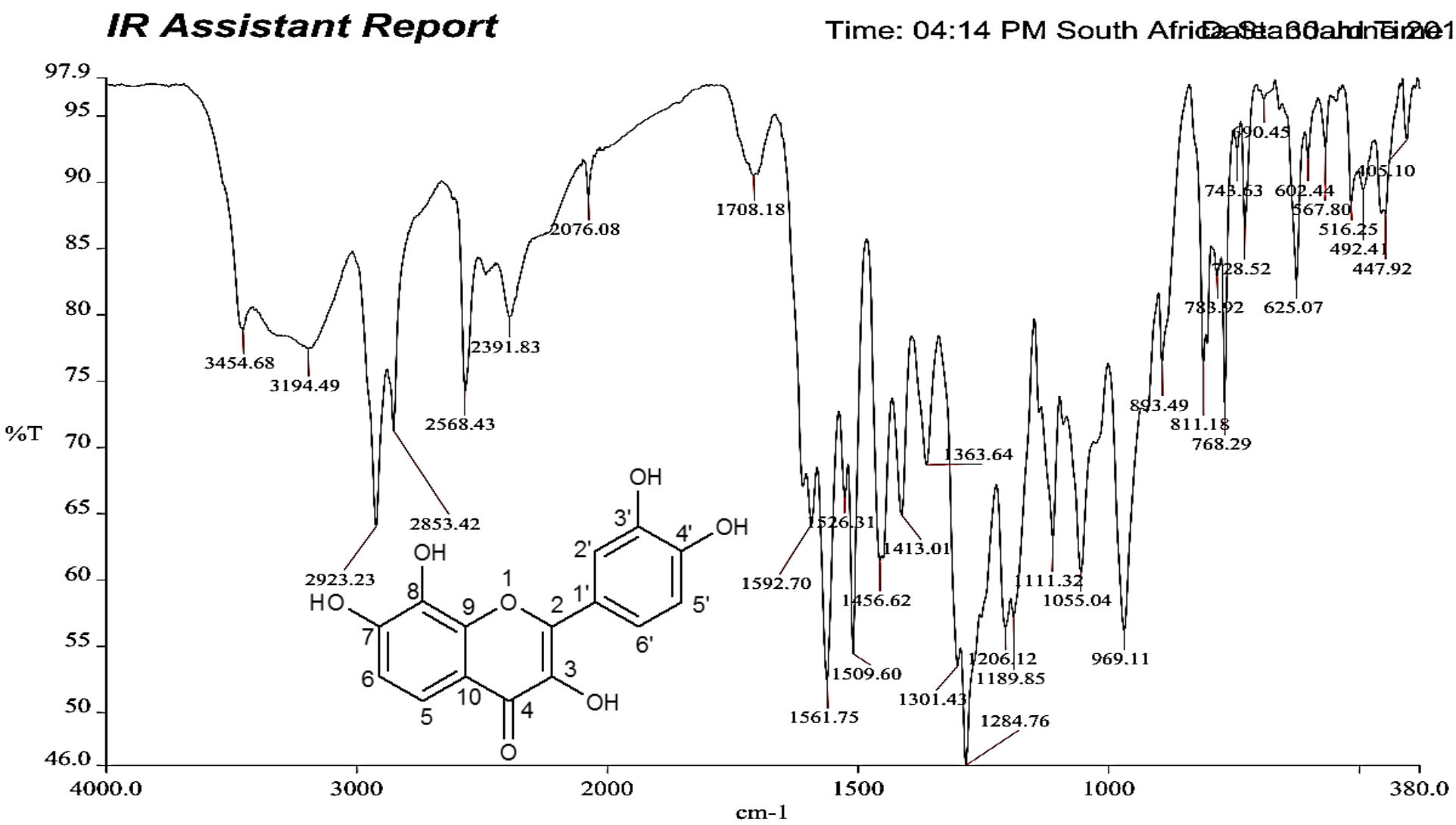
HSQC spectrum of melanoxetin (**B8**)



HMBC spectrum of melanoxetin (**B8**)



Mass spectrum of melanoxetin (**B8**)

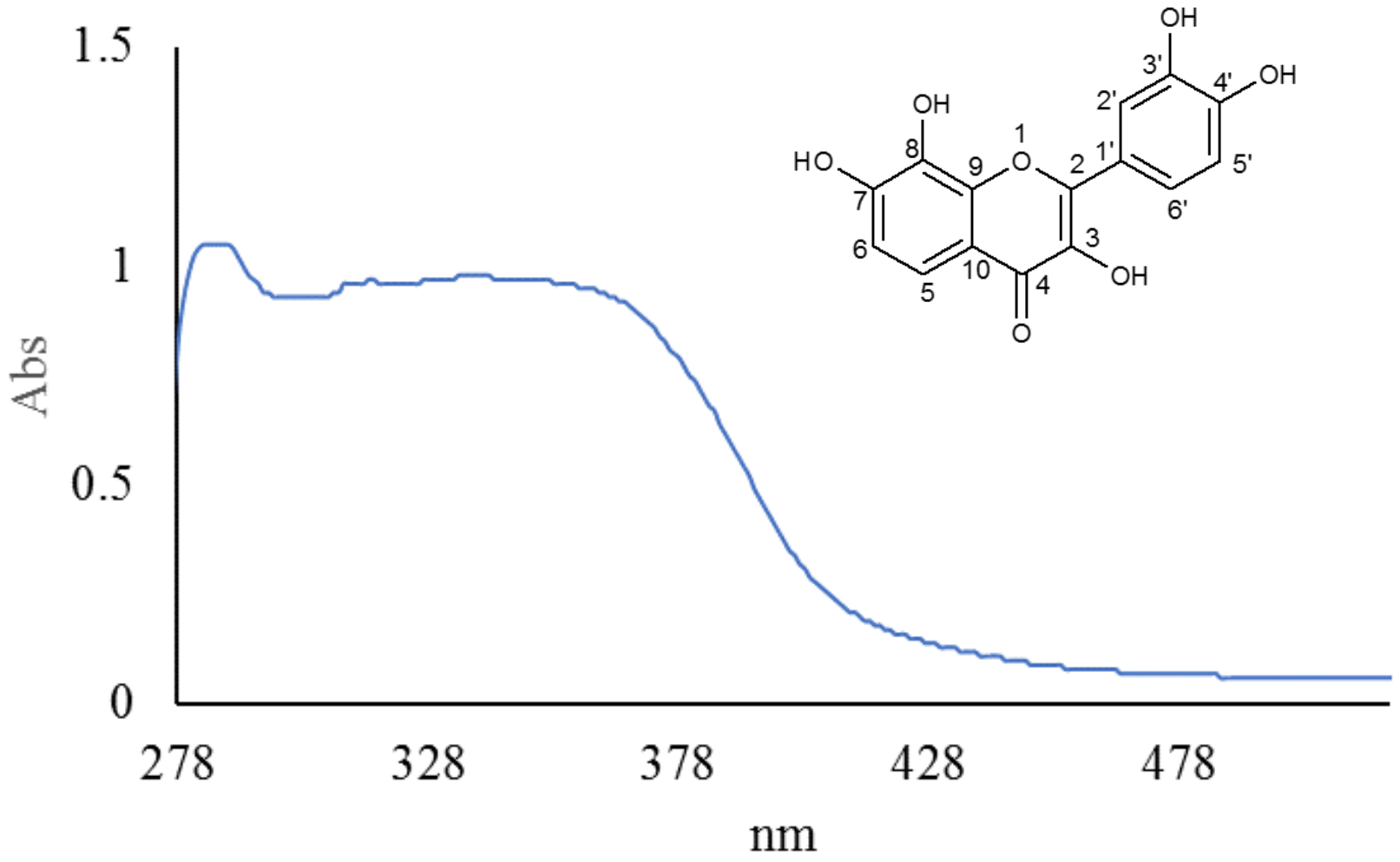


Analyst: Analyst

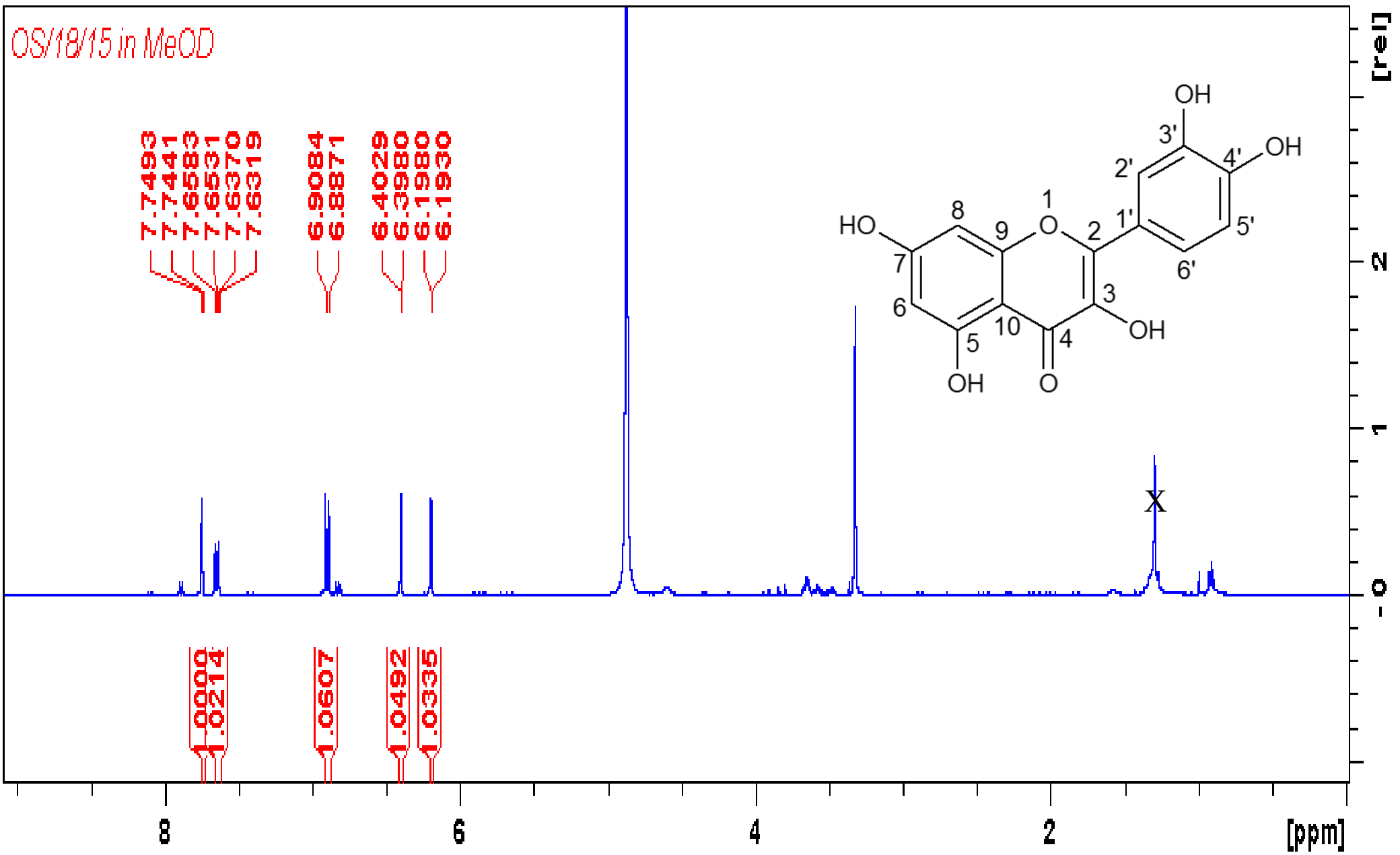
Description:

Spectrum Pathname: C:\pel_data\spectra\OS 10 30-35.002

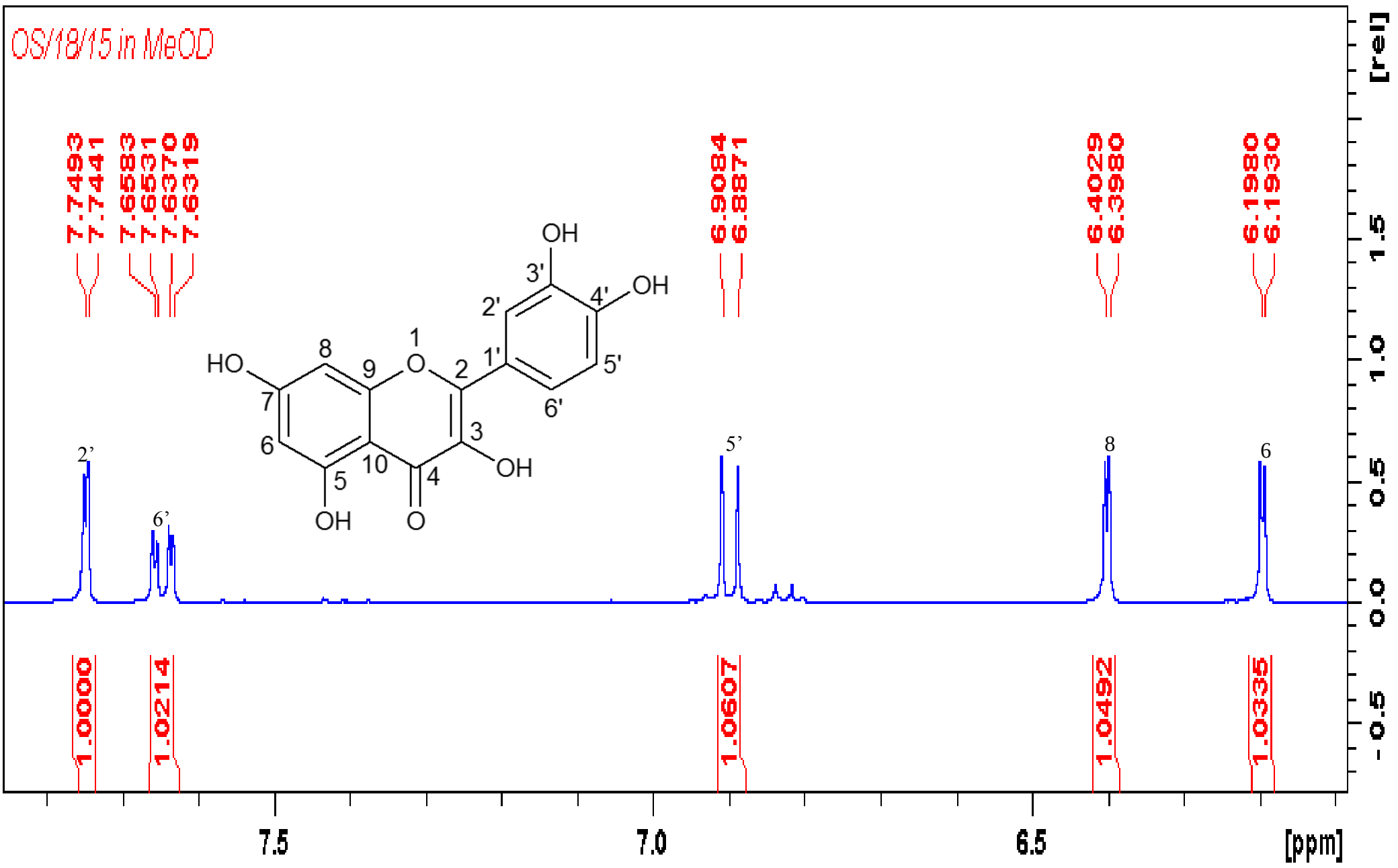
IR spectrum of melanoxetin (**B8**)



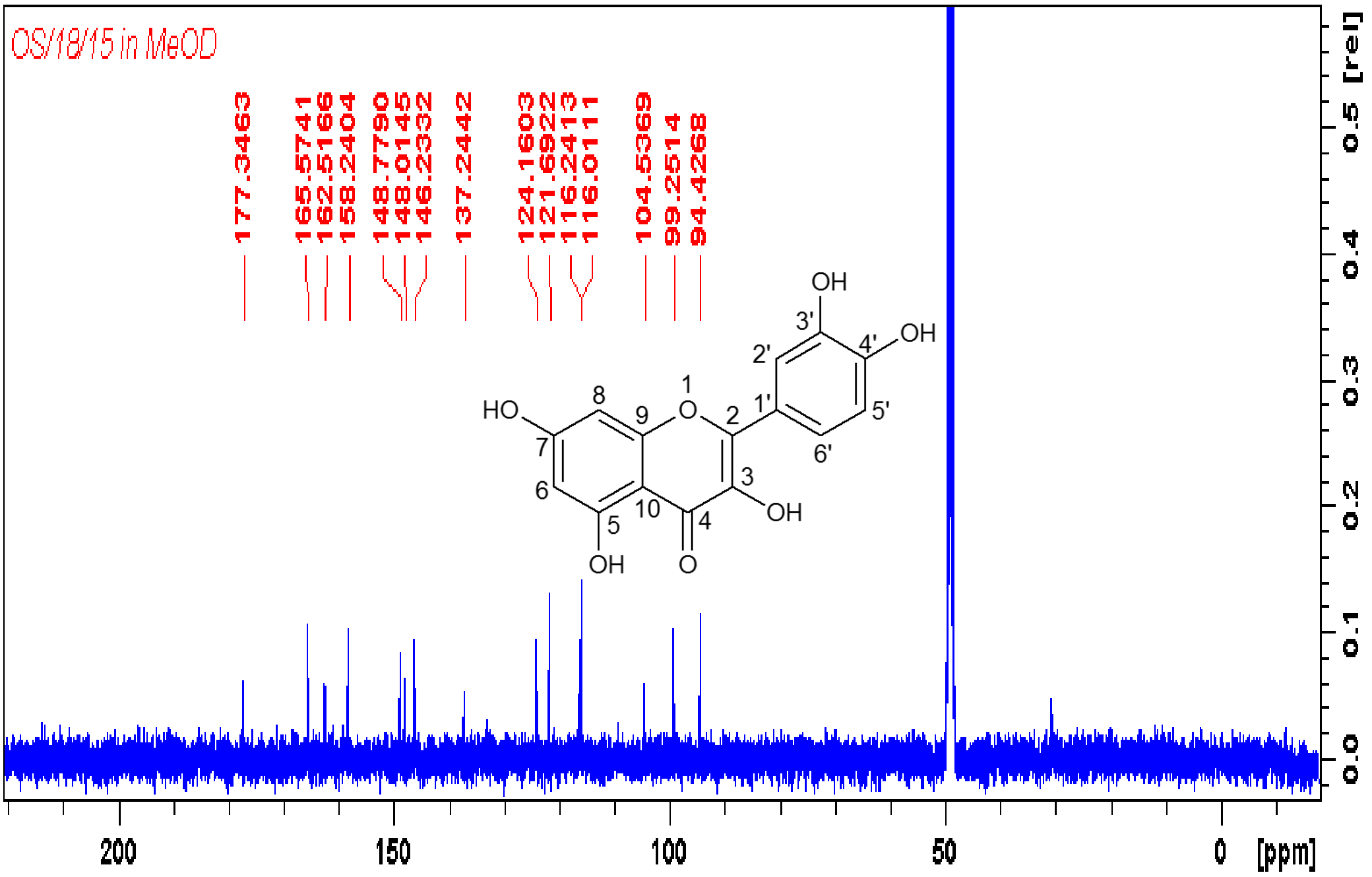
UV spectrum of melanoxetin (**B8**)



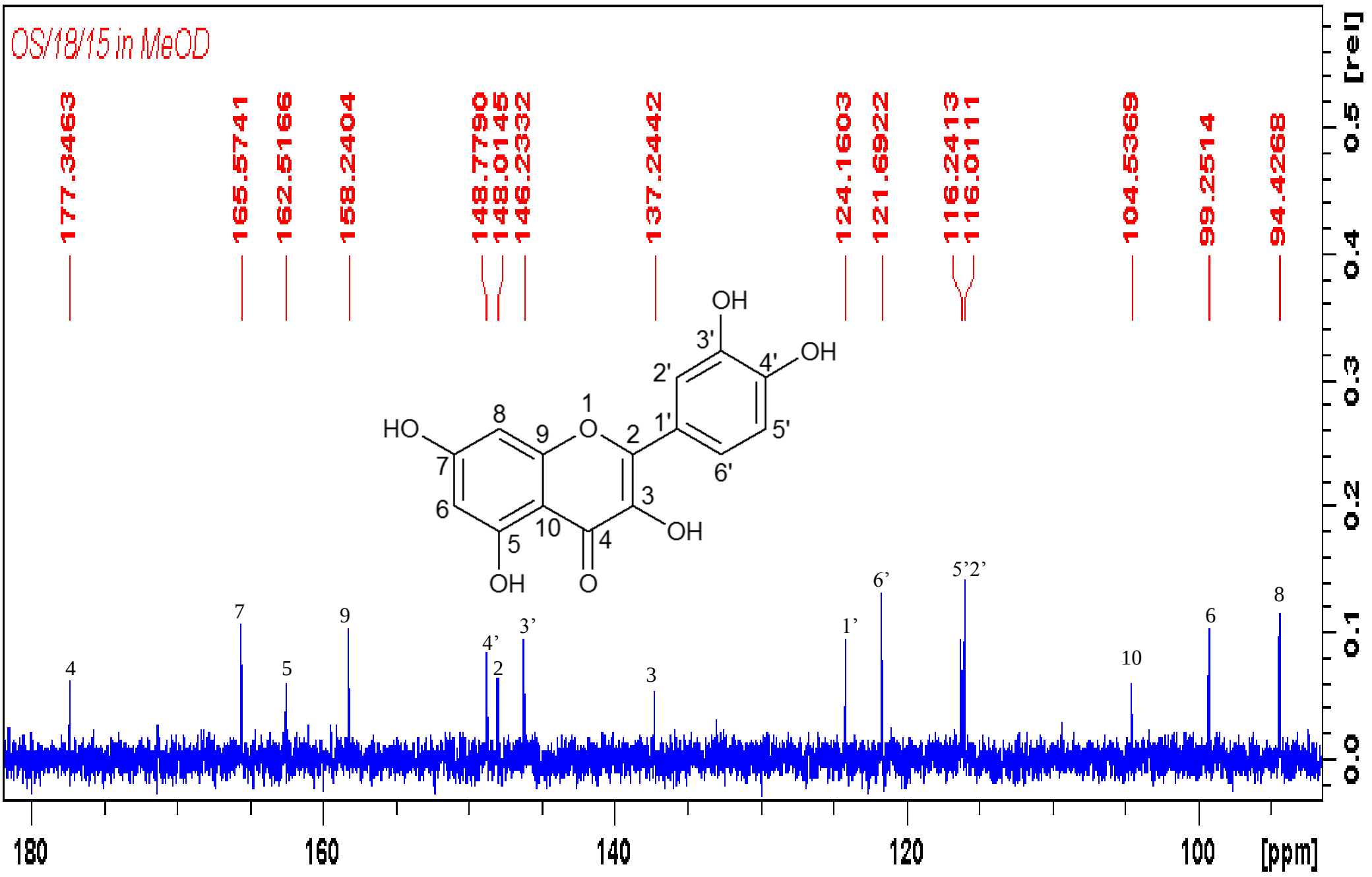
^{1}H NMR spectrum of quercetin (**B9**)



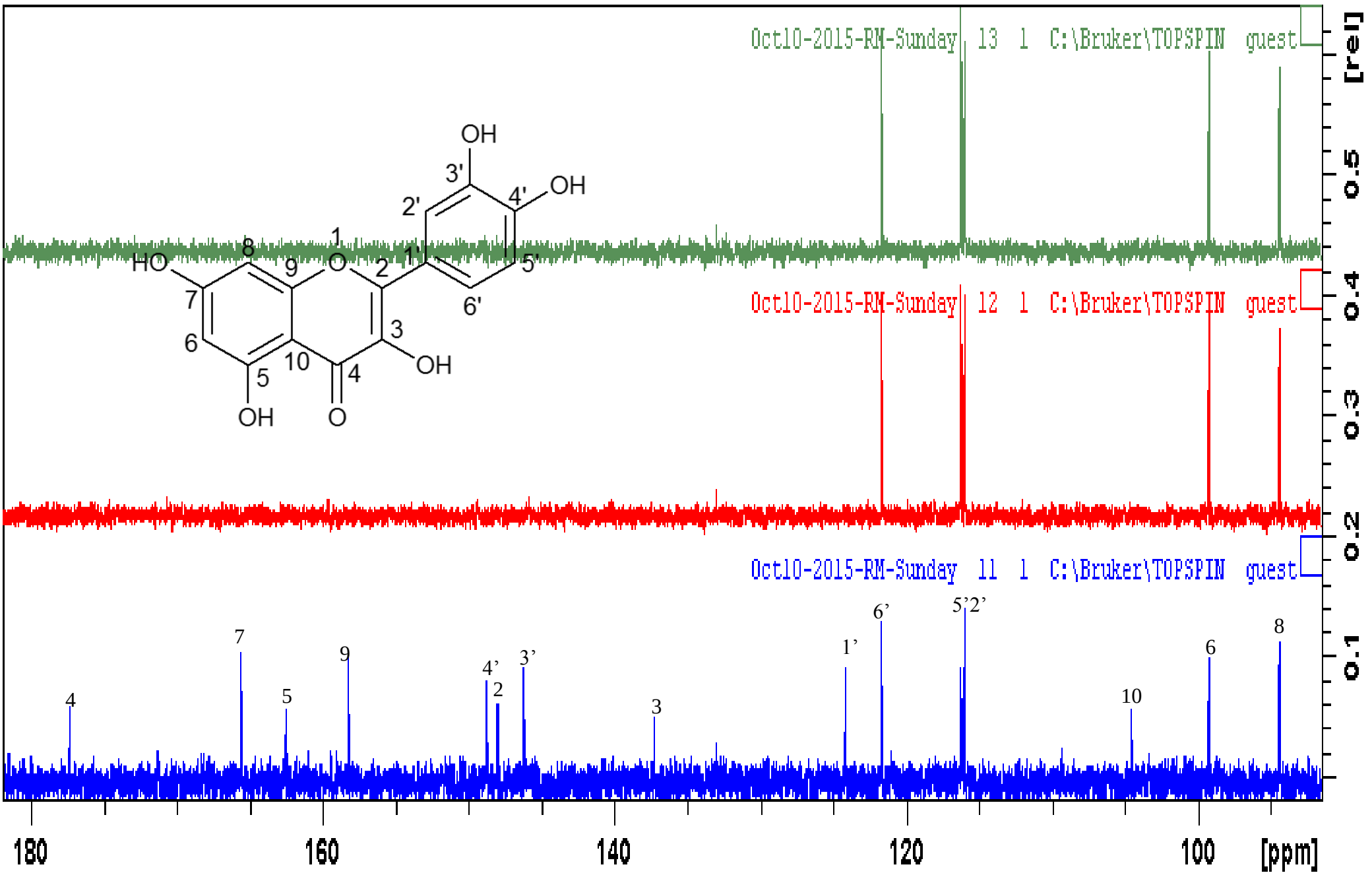
Expanded ^{1}H NMR spectrum of quercetin (**B9**)



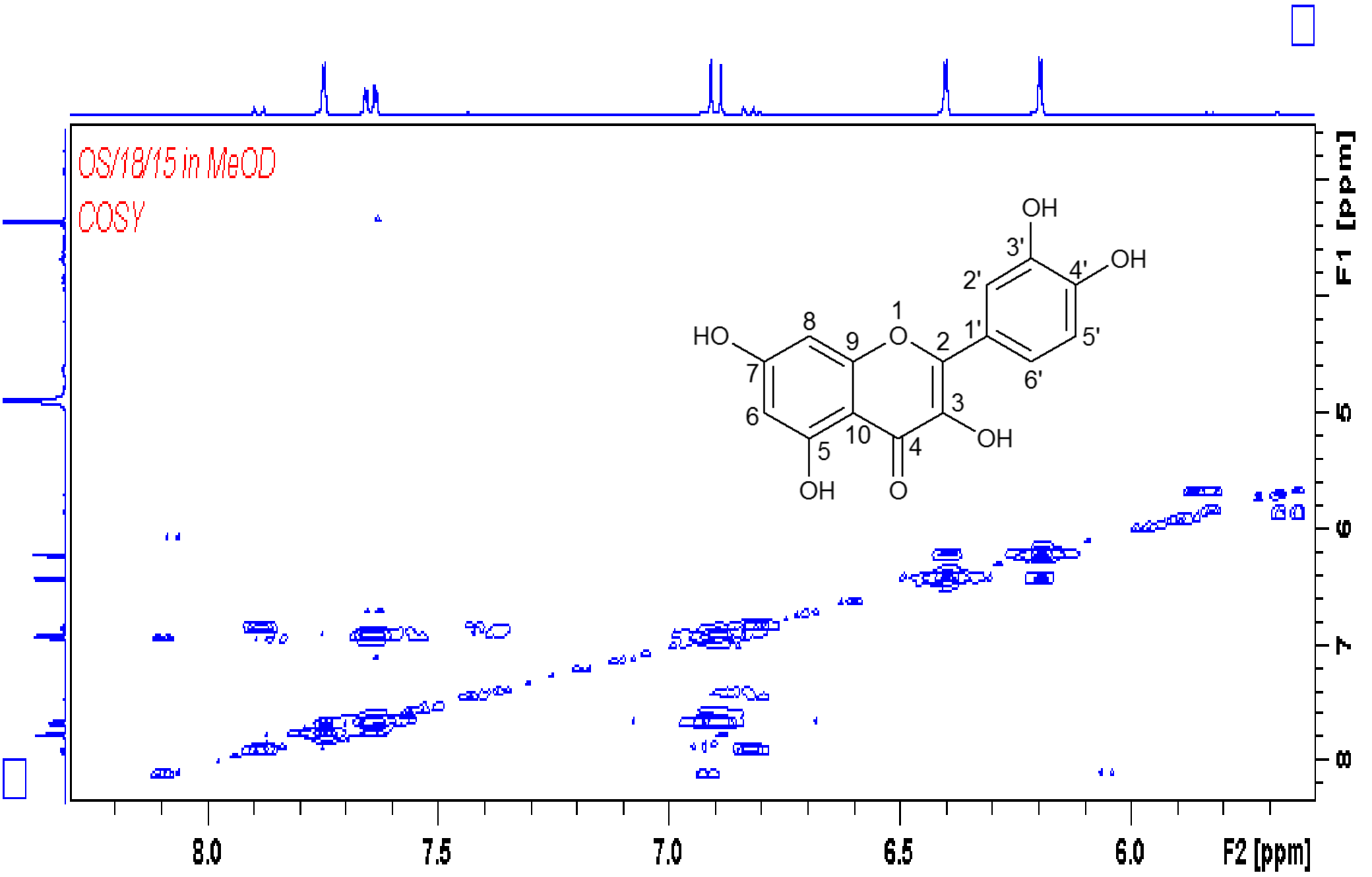
^{13}C NMR spectrum of quercetin (**B9**)



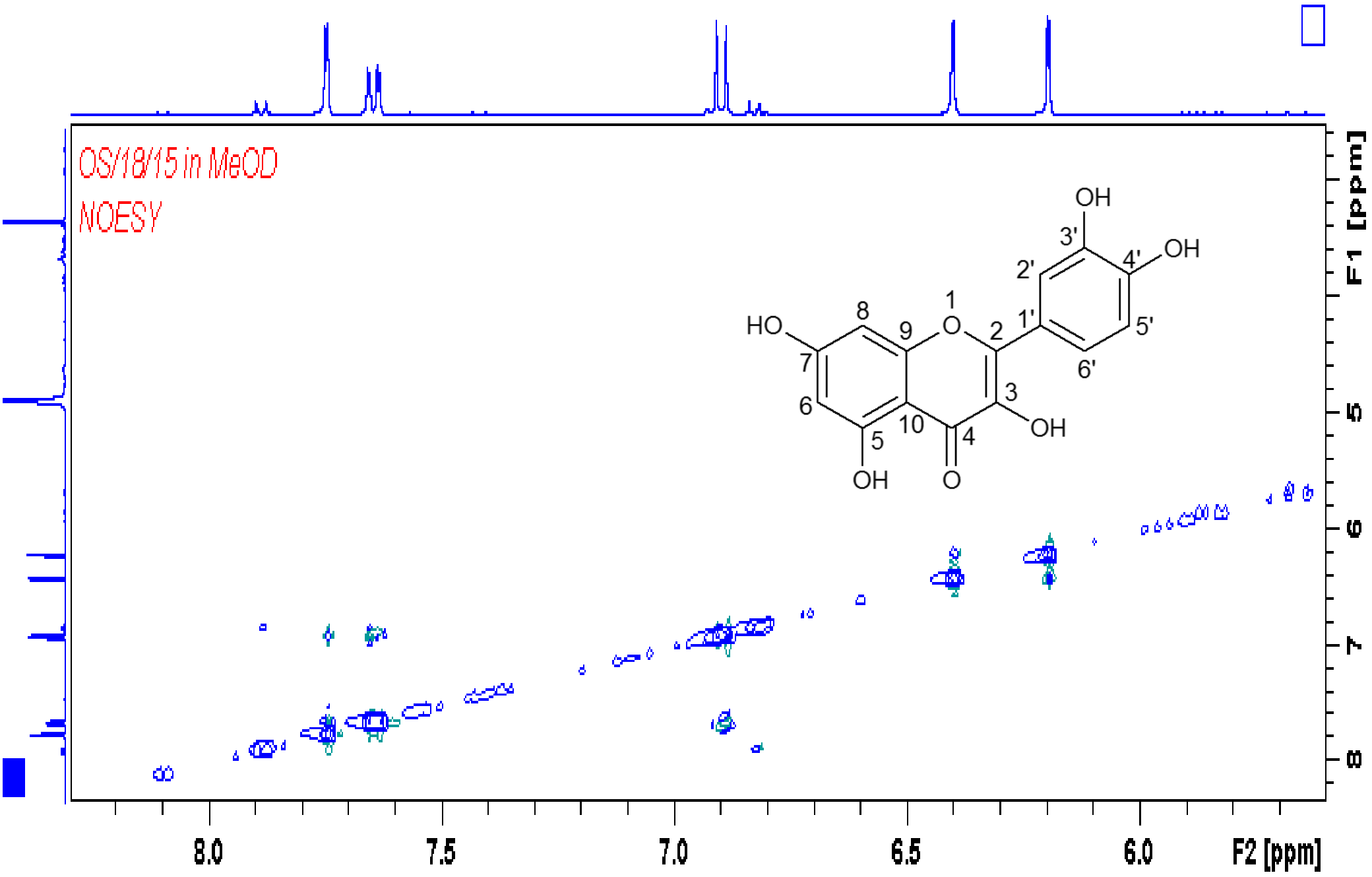
Expanded ^{13}C NMR spectrum of quercetin (**B9**)



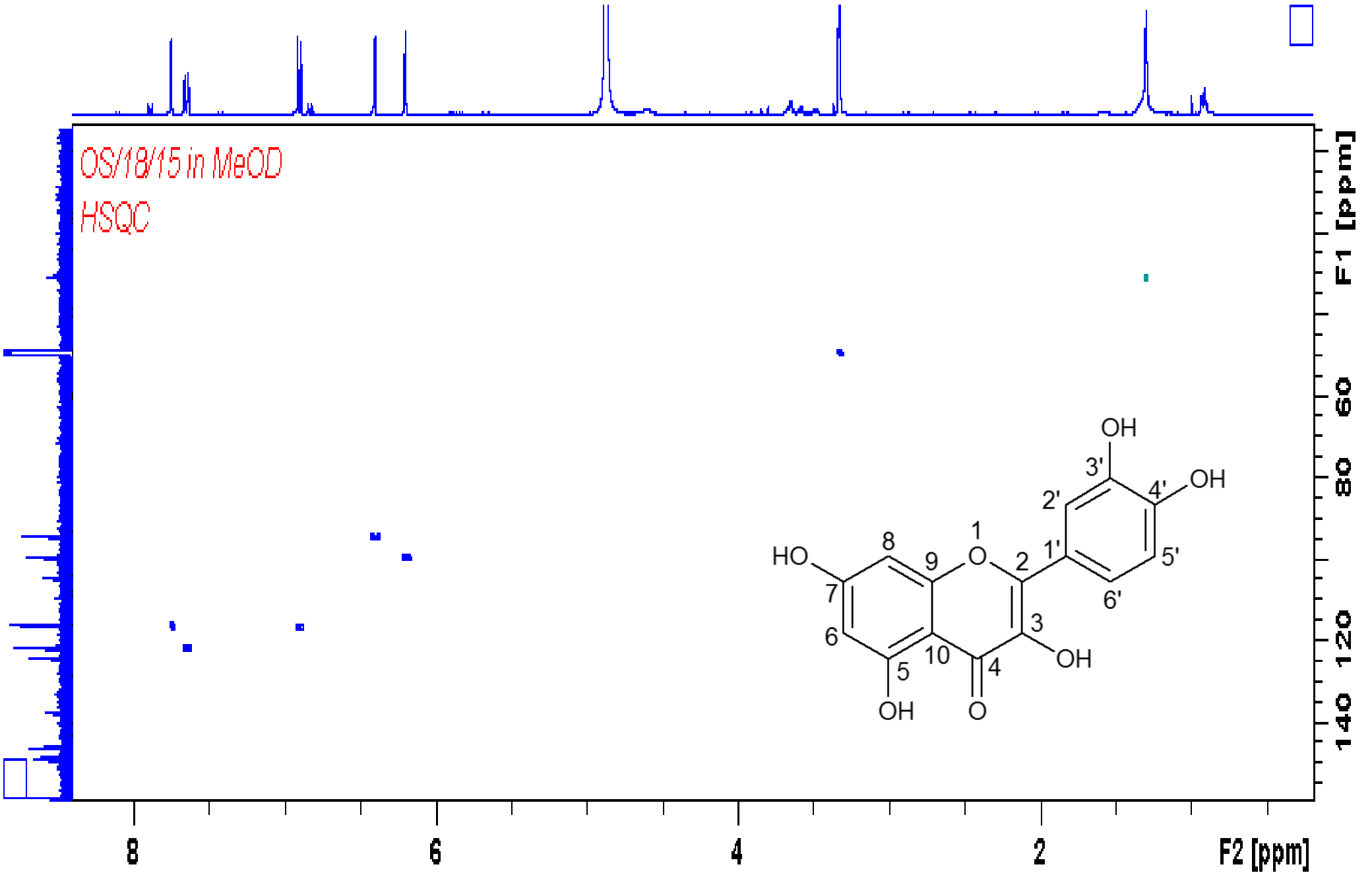
DEPT spectrum of quercetin (**B9**)



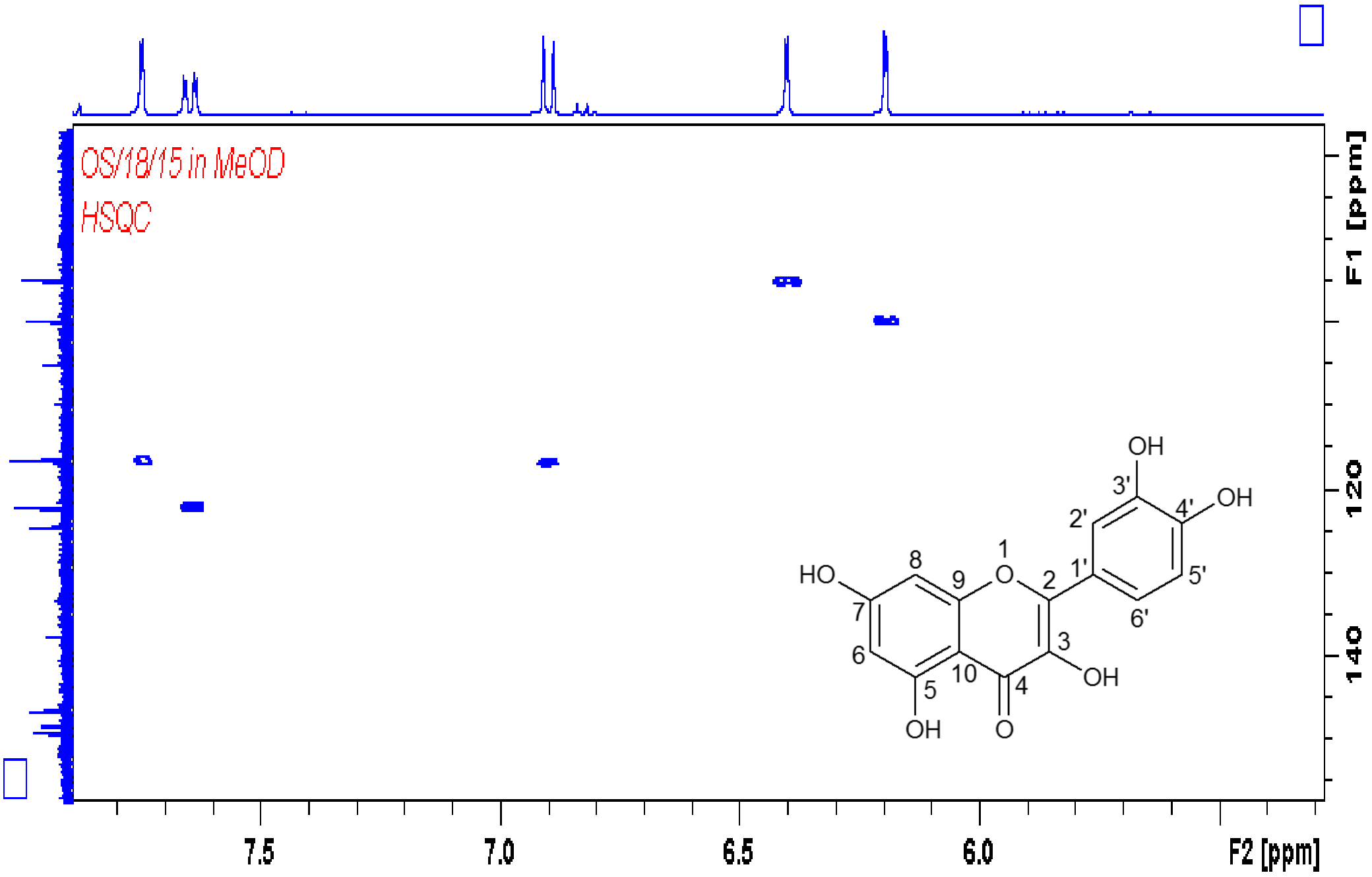
COSY spectrum of quercetin (**B9**)



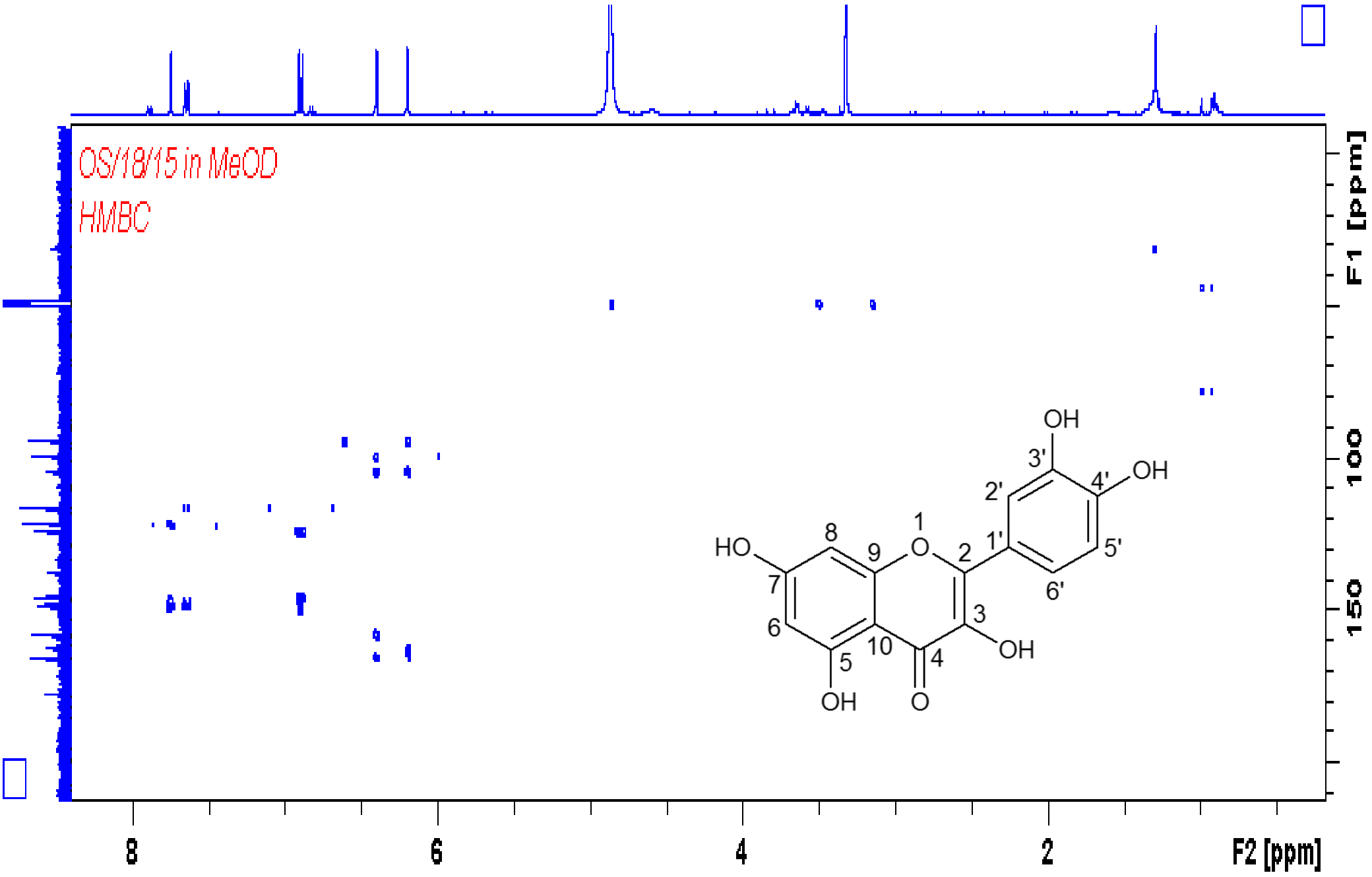
NOESY spectrum of quercetin (**B9**)



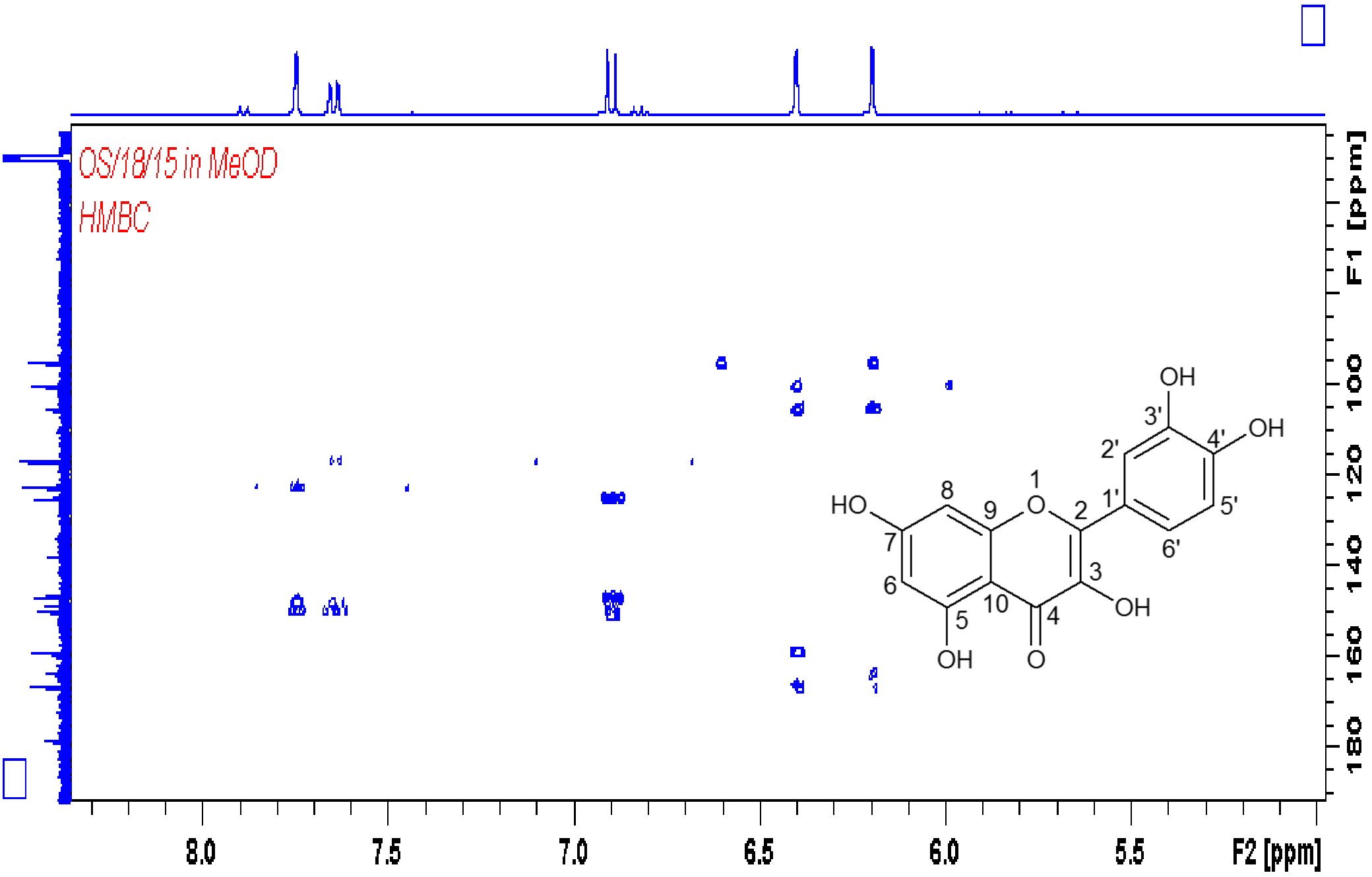
HSQC spectrum of quercetin (**B9**)



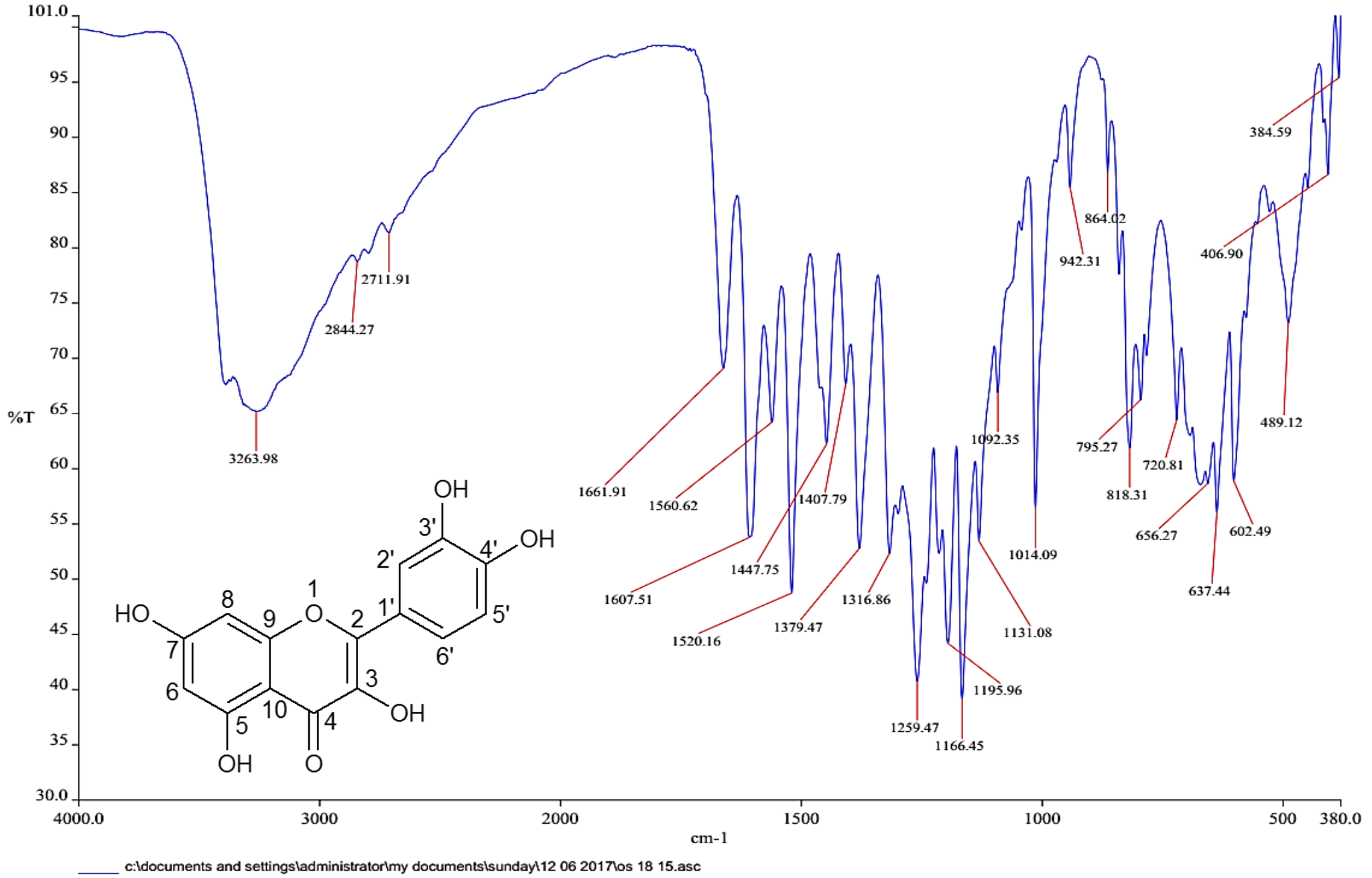
Expanded HSQC spectrum of quercetin (**B9**)



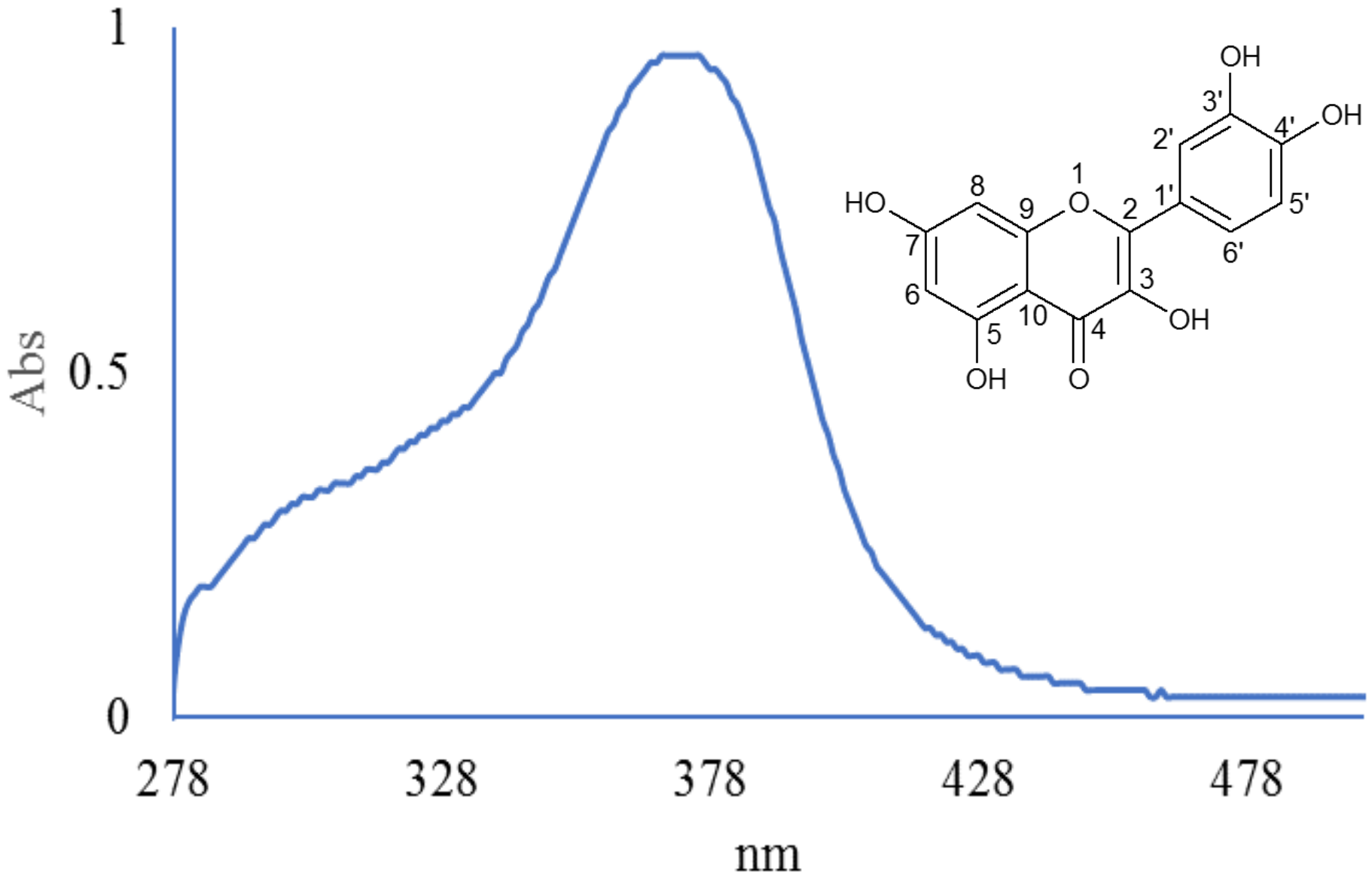
HMBC spectrum of quercetin (**B9**)



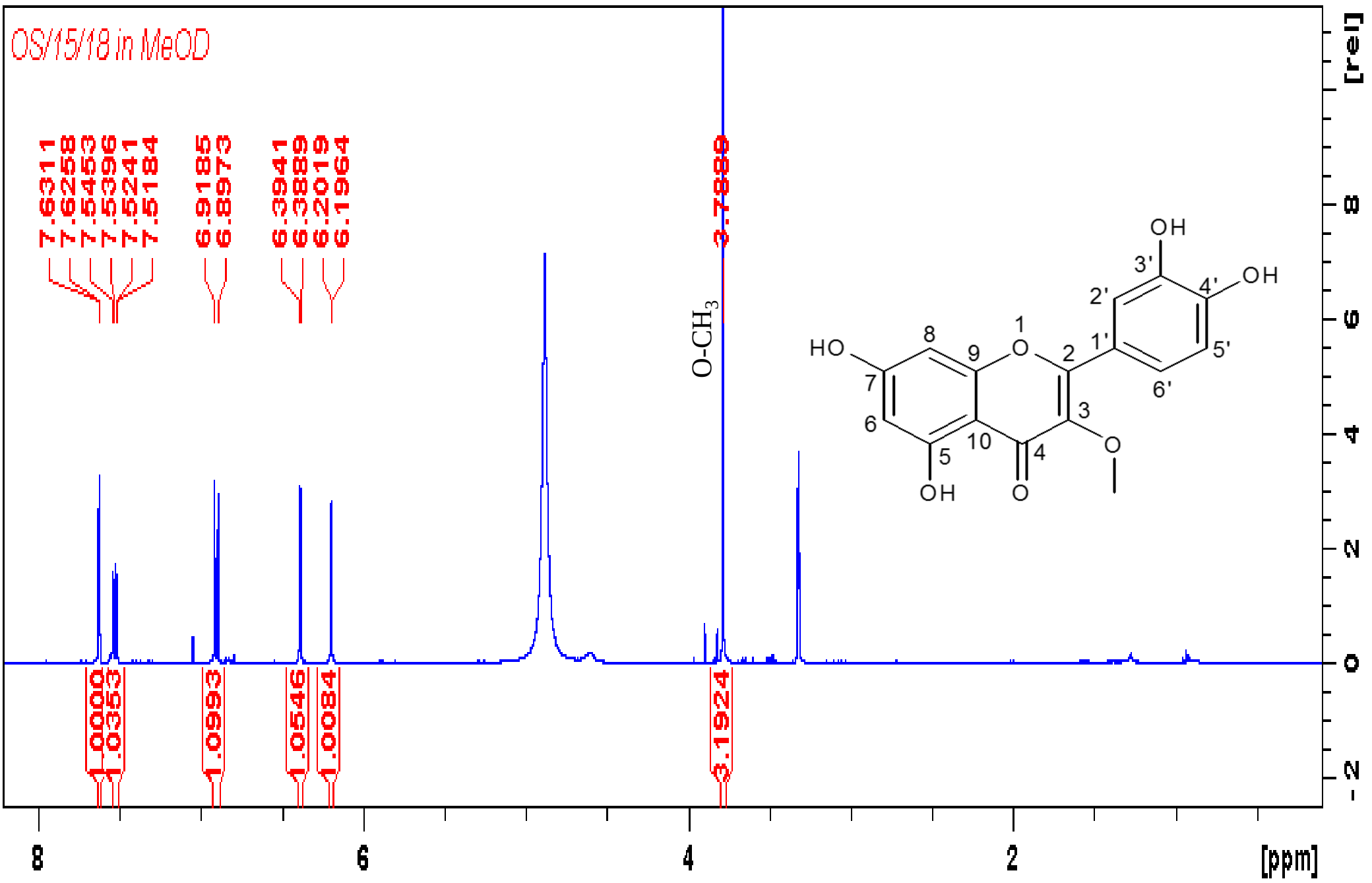
Expanded HMBC spectrum of quercetin (**B9**)



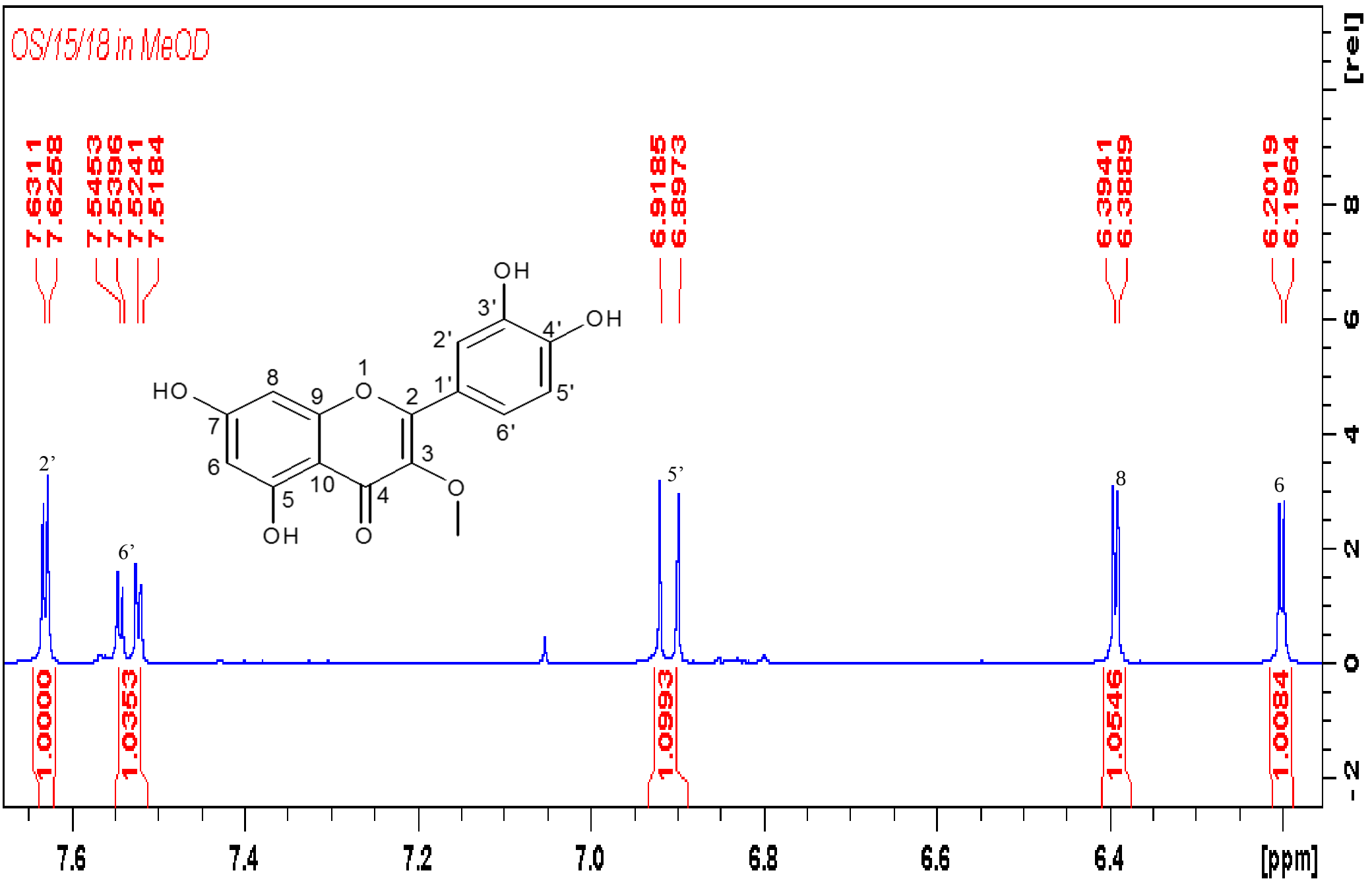
IR spectrum of quercetin (**B9**)



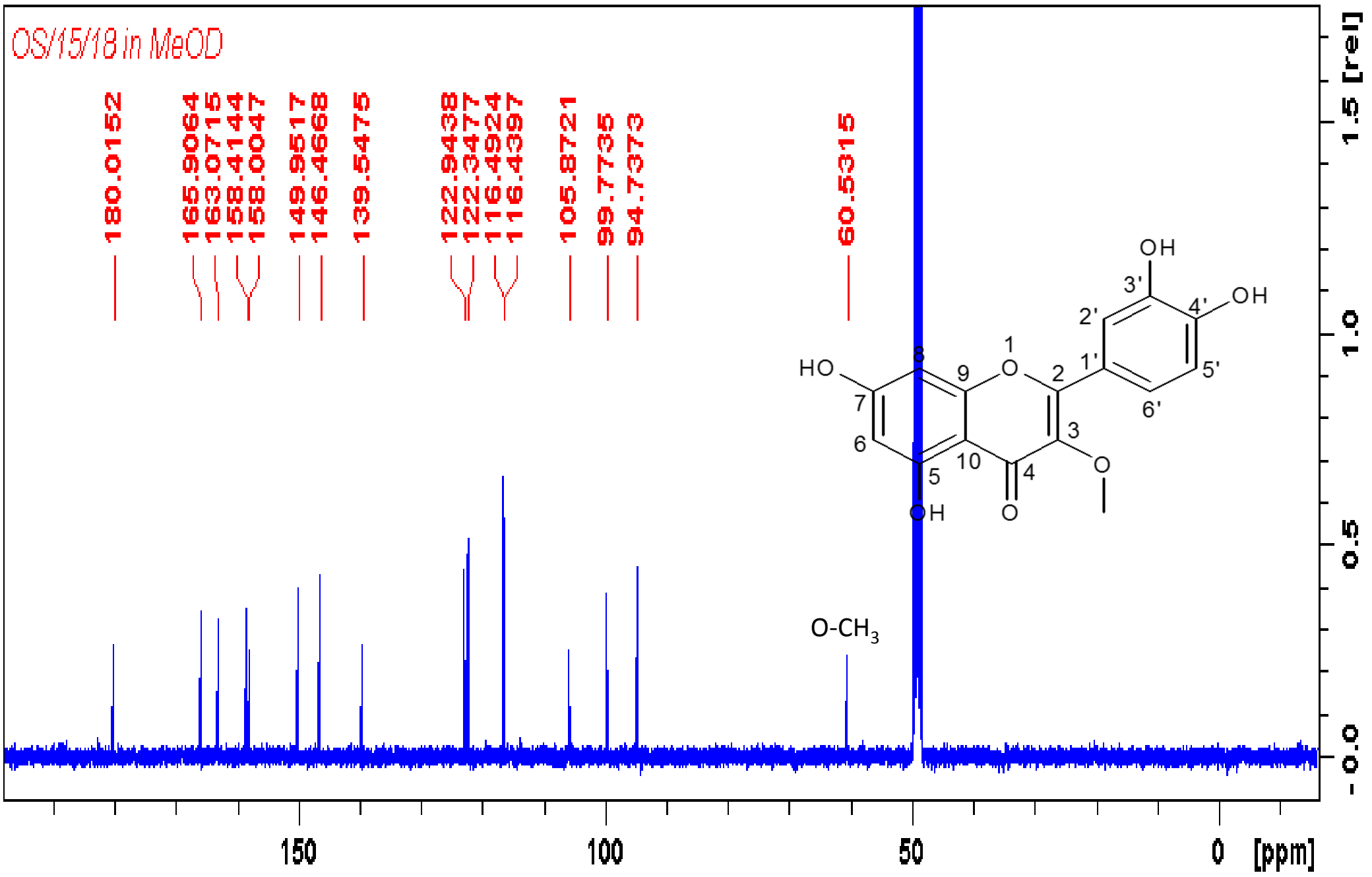
UV spectrum of quercetin (**B9**)



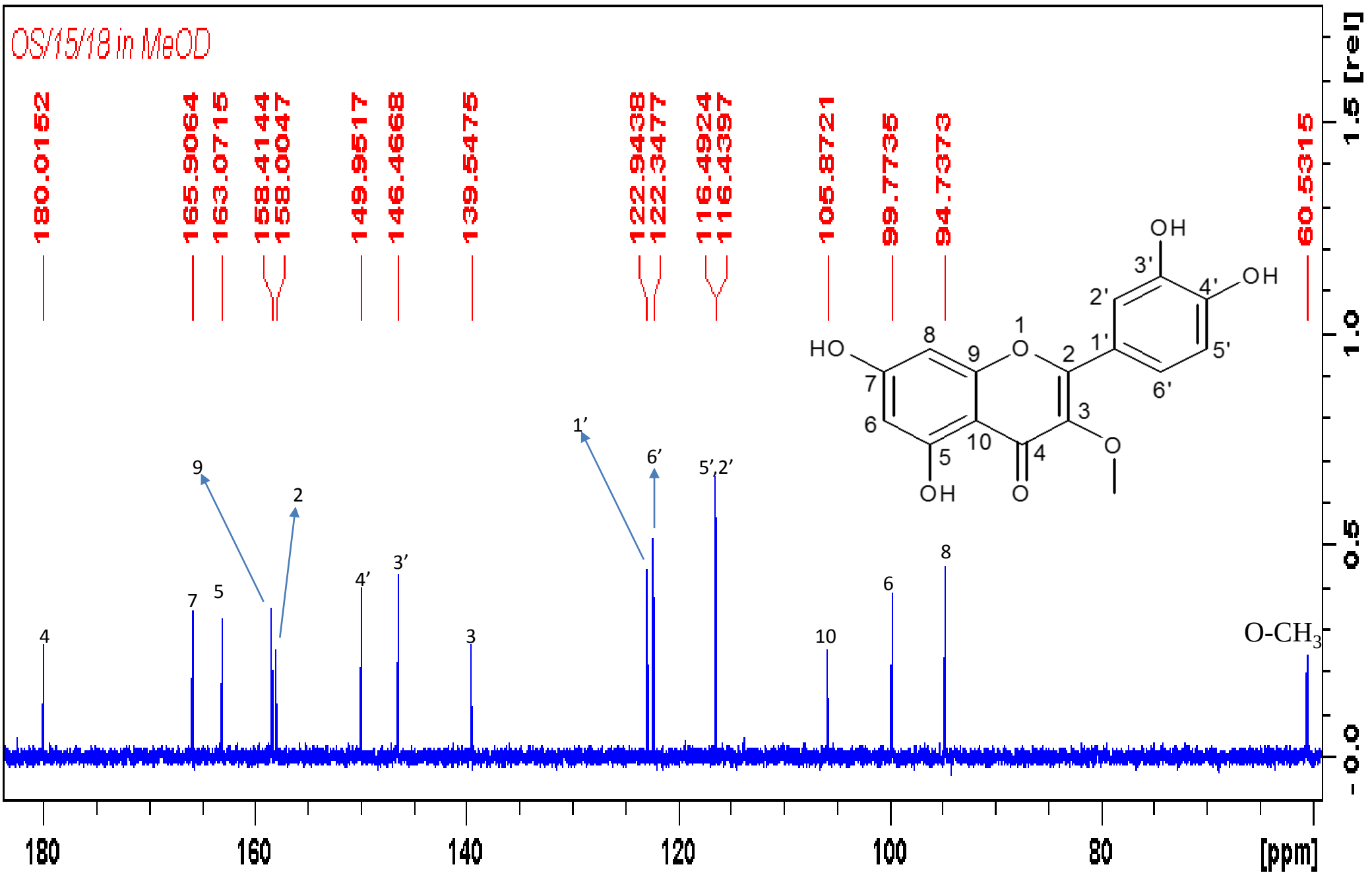
^{1}H NMR spectrum of quercetin-3-*O*-methyl ether (**B10**)



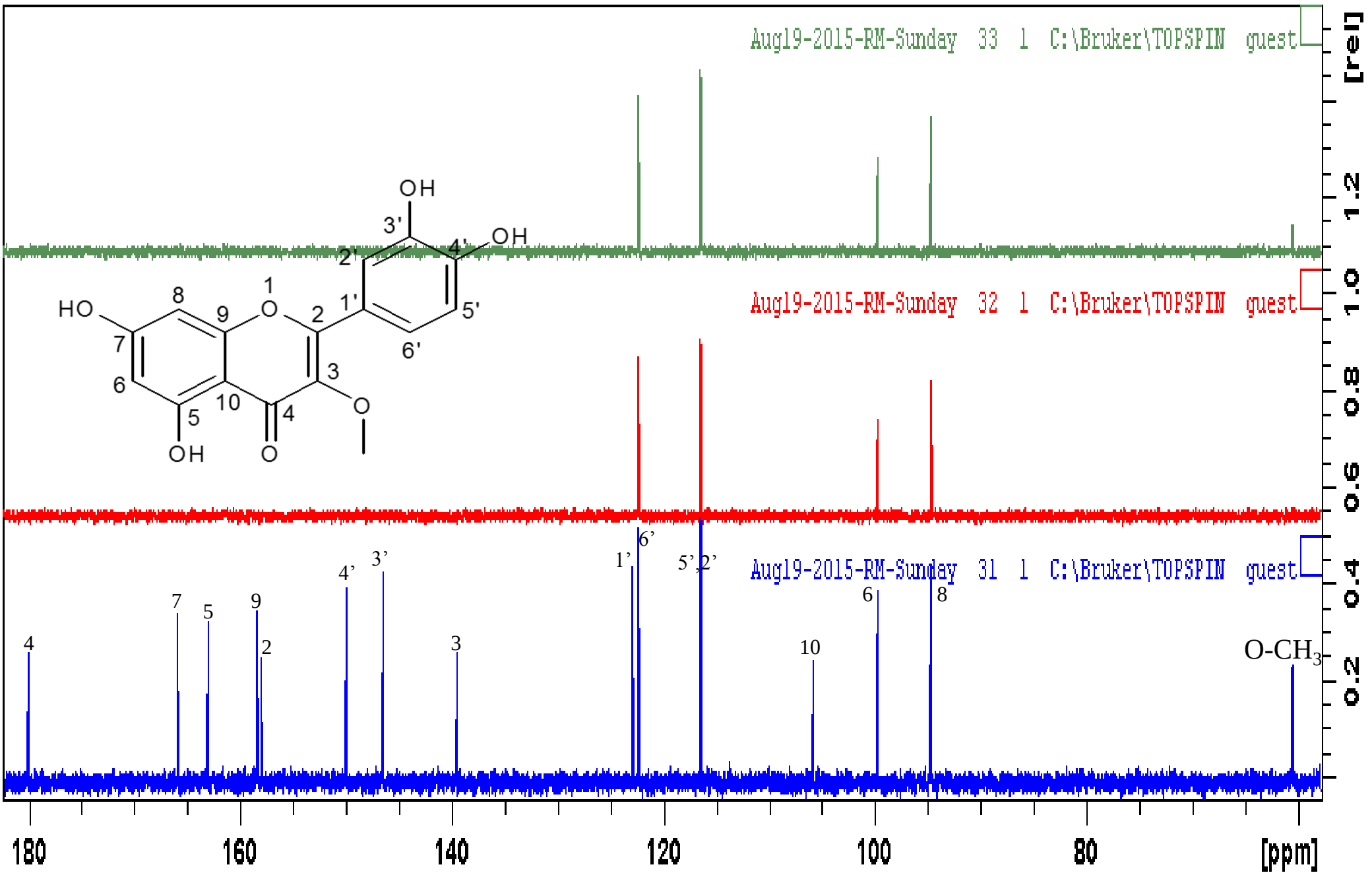
Expanded ^{1}H NMR spectrum of quercetin-3-*O*-methyl ether (**B10**)



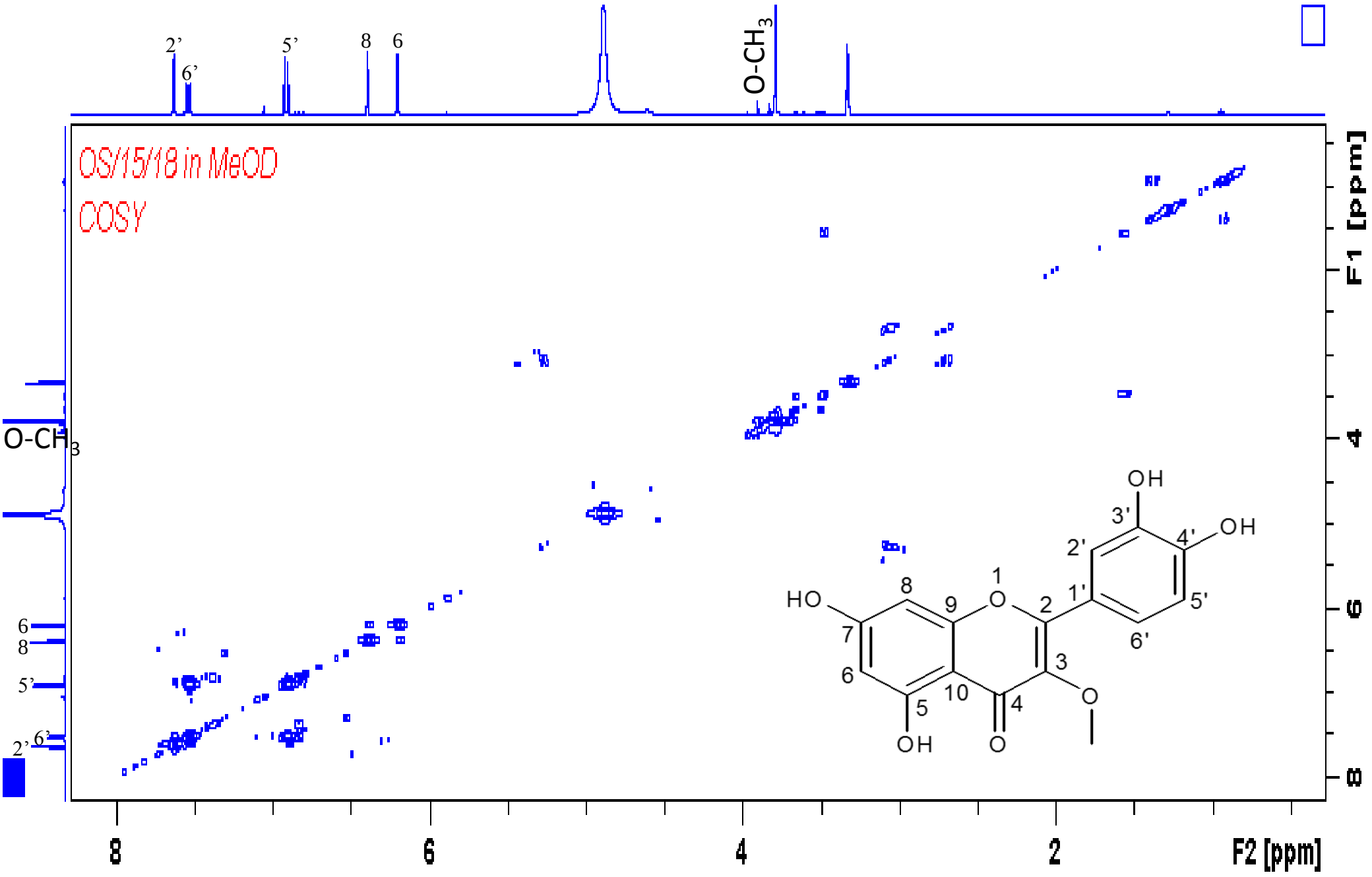
^{13}C NMR spectrum of quercetin-3-*O*-methyl ether (**B10**)



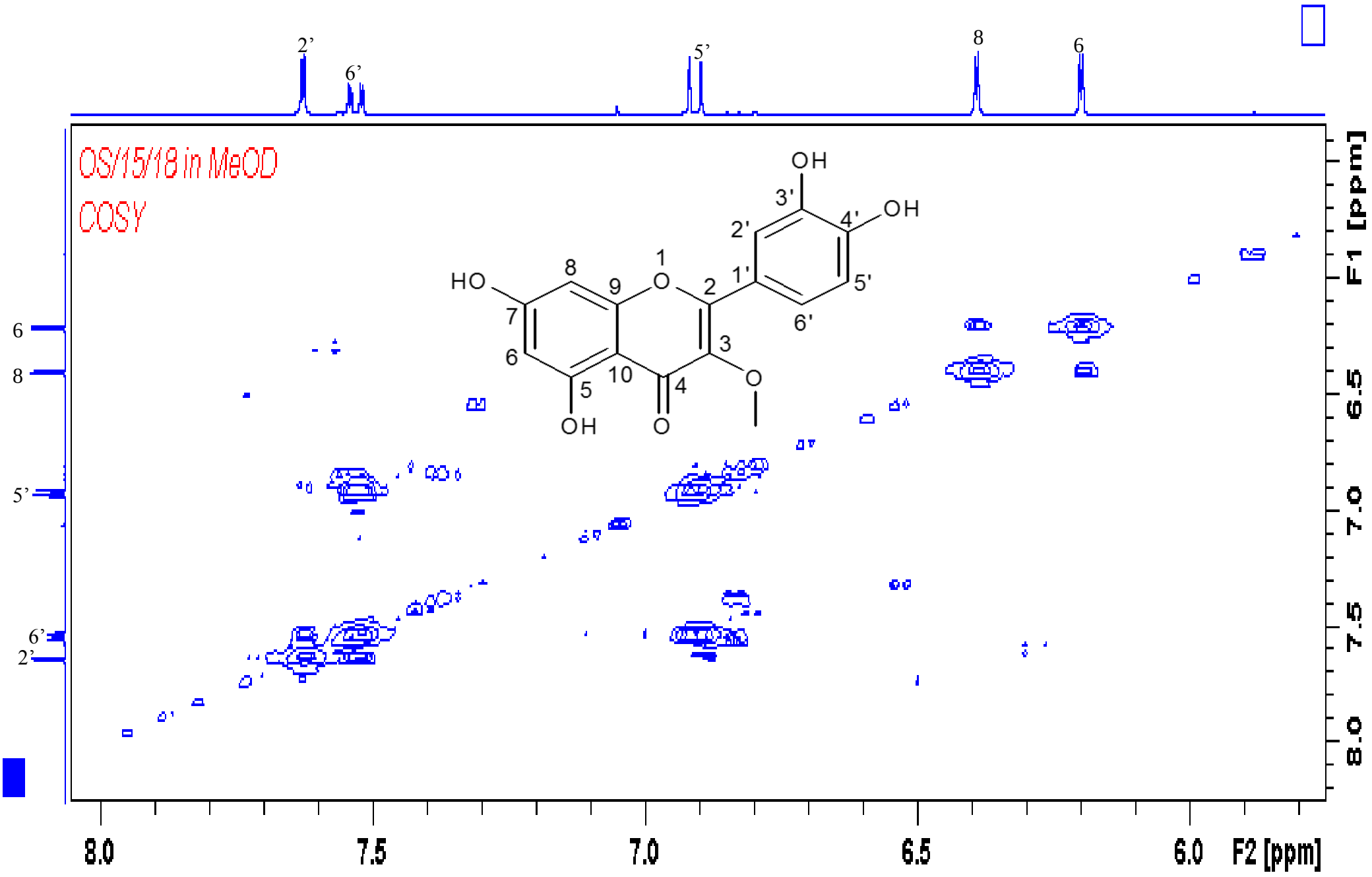
Expanded ^{13}C NMR spectrum of quercetin-3-*O*-methyl ether (**B10**)



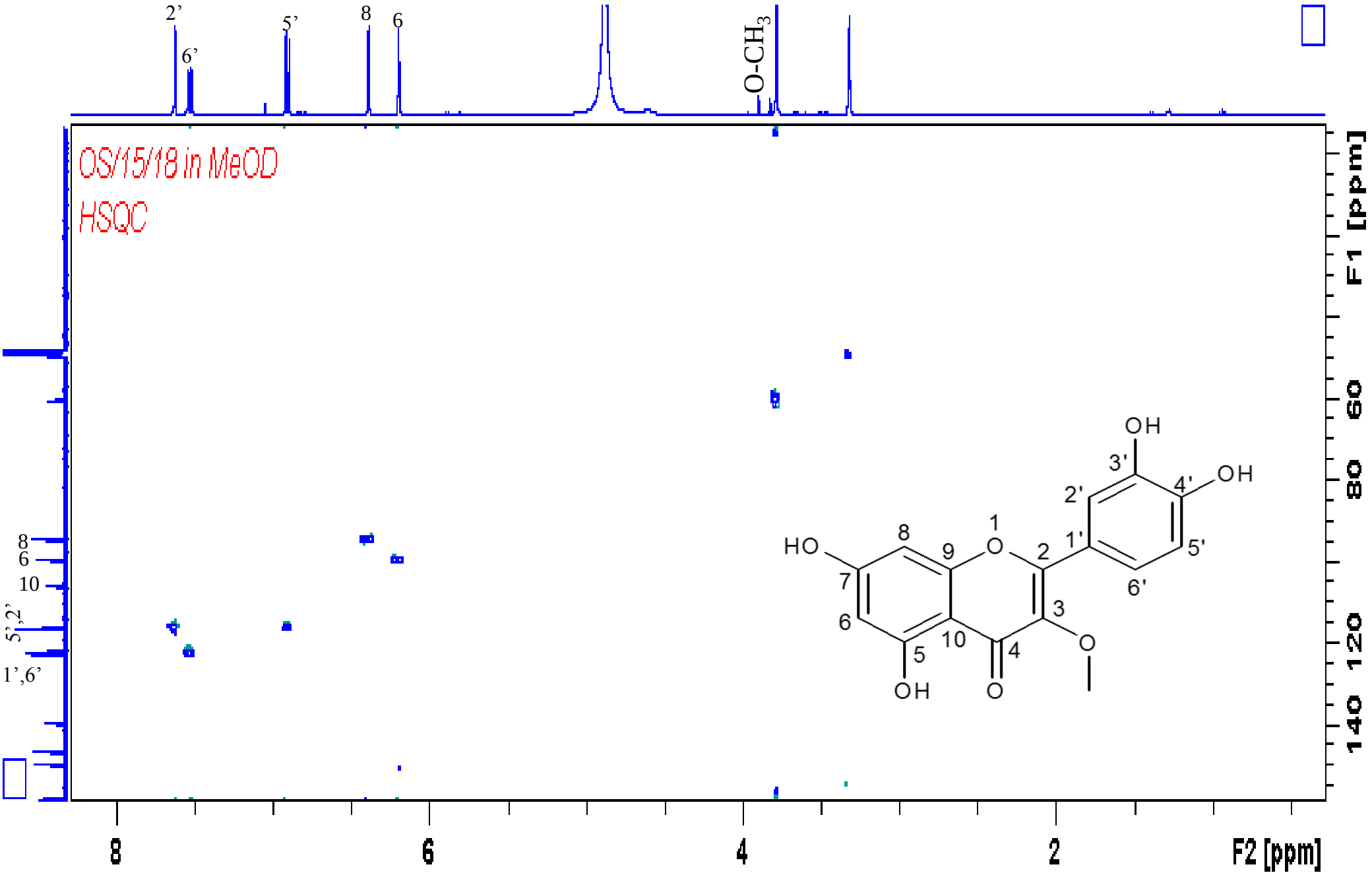
DEPT spectrum of quercetin-3-*O*-methyl ether (**B10**)



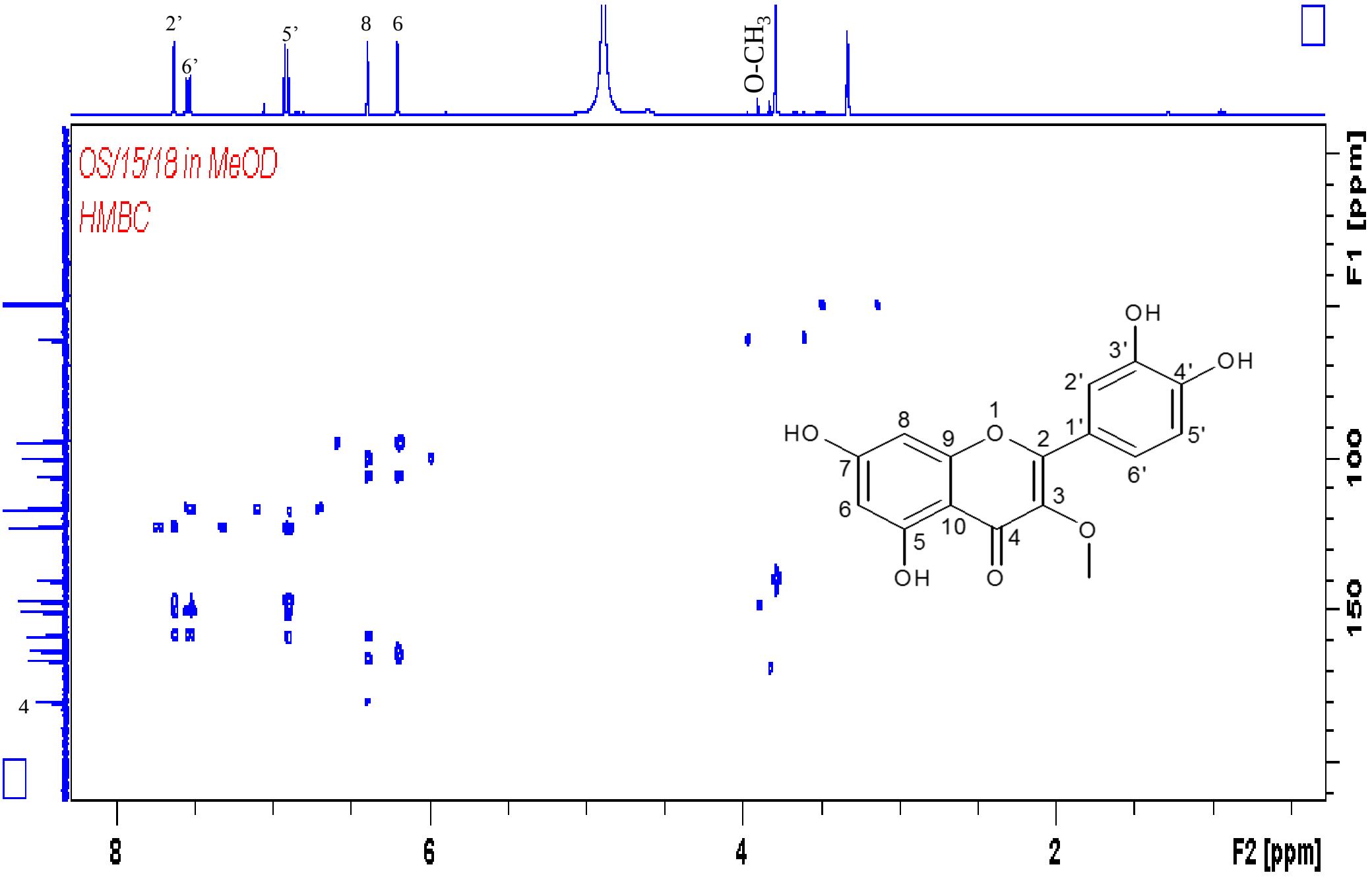
COSY spectrum of quercetin-3-*O*-methyl ether (**B10**)



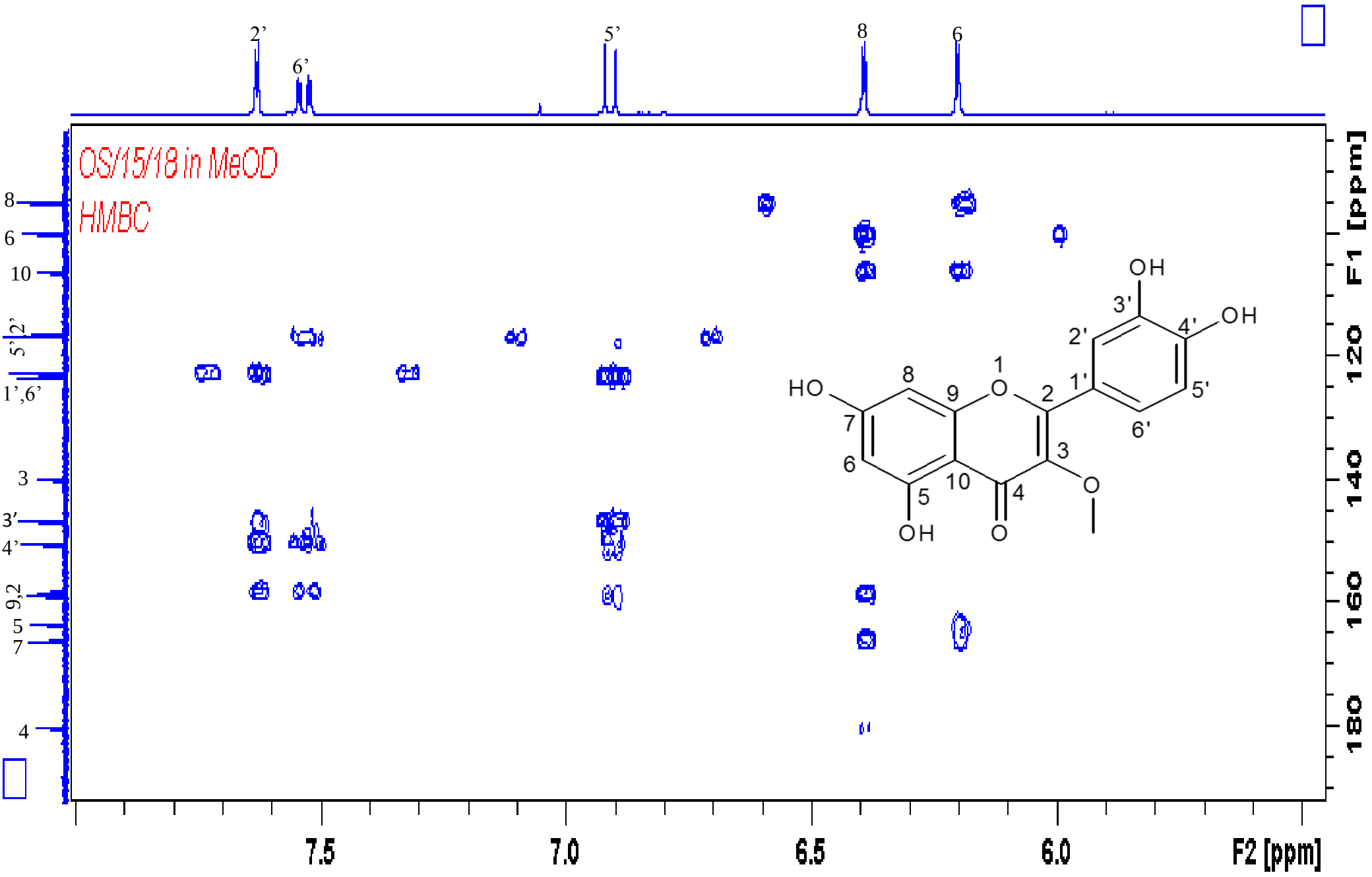
Expanded COSY spectrum of quercetin-3-*O*-methyl ether (**B10**)



HSQC spectrum of quercetin-3-*O*-methyl ether (**B10**)



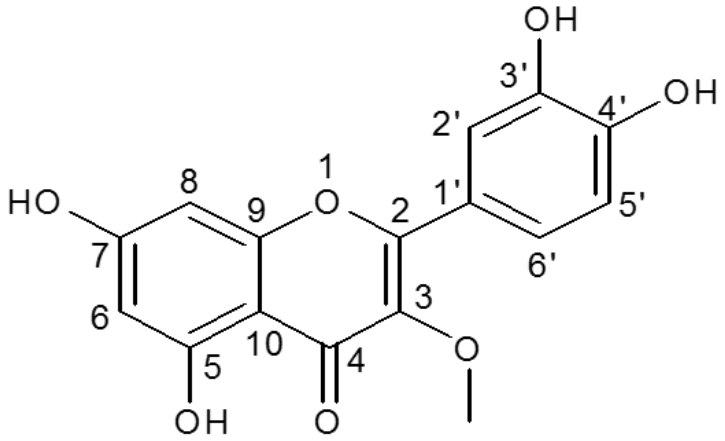
HMBC spectrum of quercetin-3-*O*-methyl ether (**B10**)



Expanded HMBC spectrum of quercetin-3-*O*-methyl ether (**B10**)

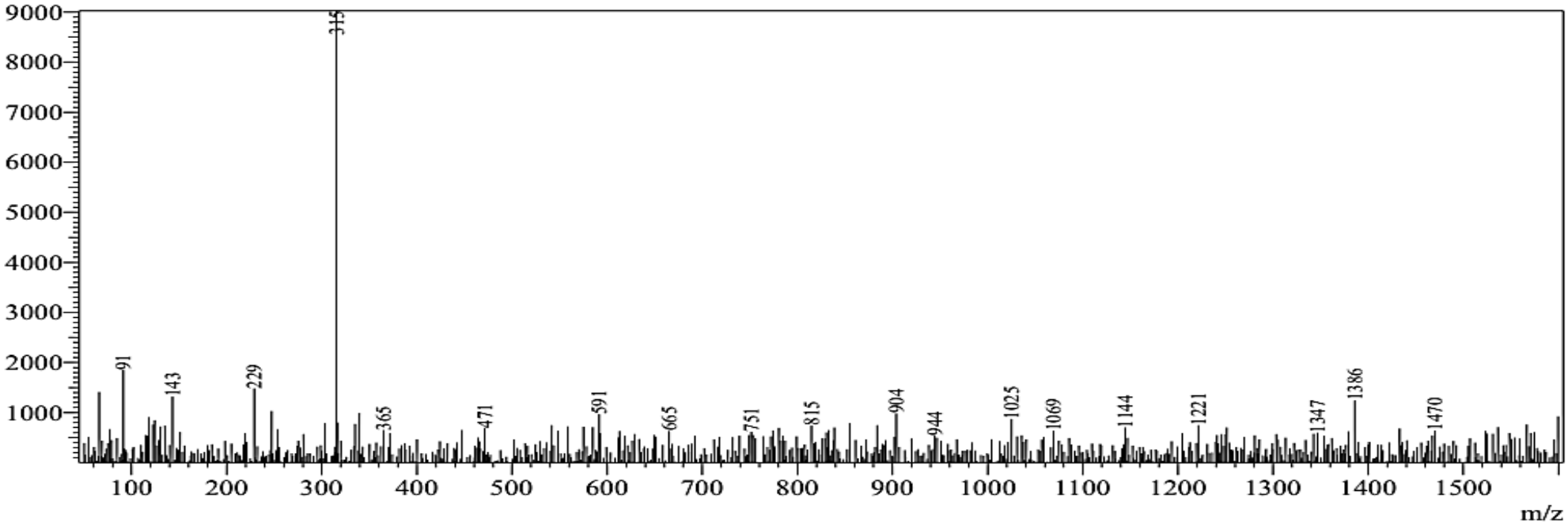
Sample Information

Date Acquired	: 2016/04/14 03:09:34 PM
Sample Type	: Unknown
Sample Name	: 53
Sample ID	:
Data File	: 53a.lcd
Method File	: no column run.lcm
Report Format File	: mass spectrum.lsr
Tuning File	: 03022016.lct

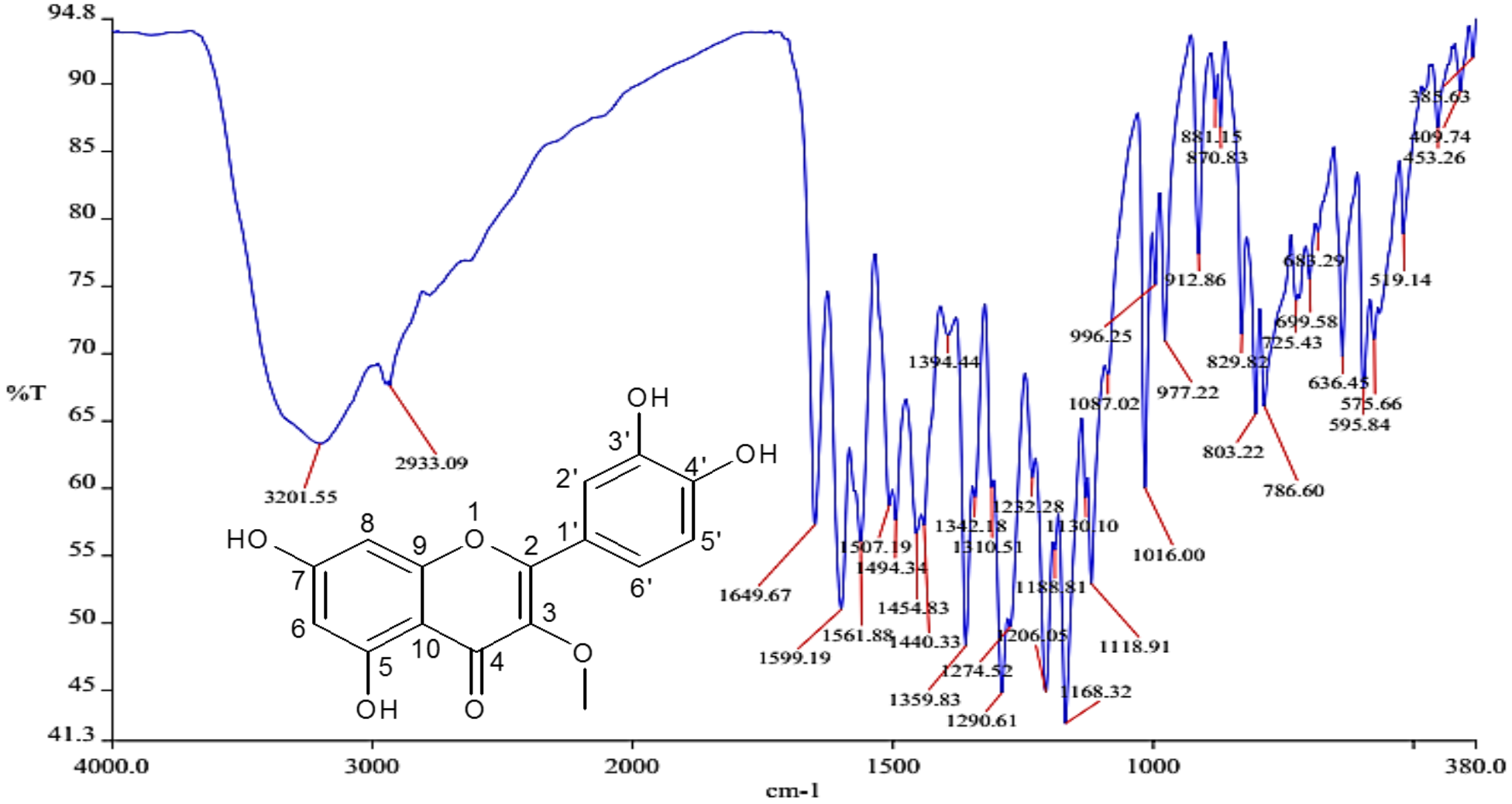


MS Spectrum

Peak#:1 R.Time:1.000(Scan#:61)
MassPeaks:781
Spectrum Mode:Averaged 0.983-1.017(60-62)
BG Mode:Calc Segment 1 - Event 1



Mass spectrum of quercetin-3-*O*-methyl ether (**B10**)

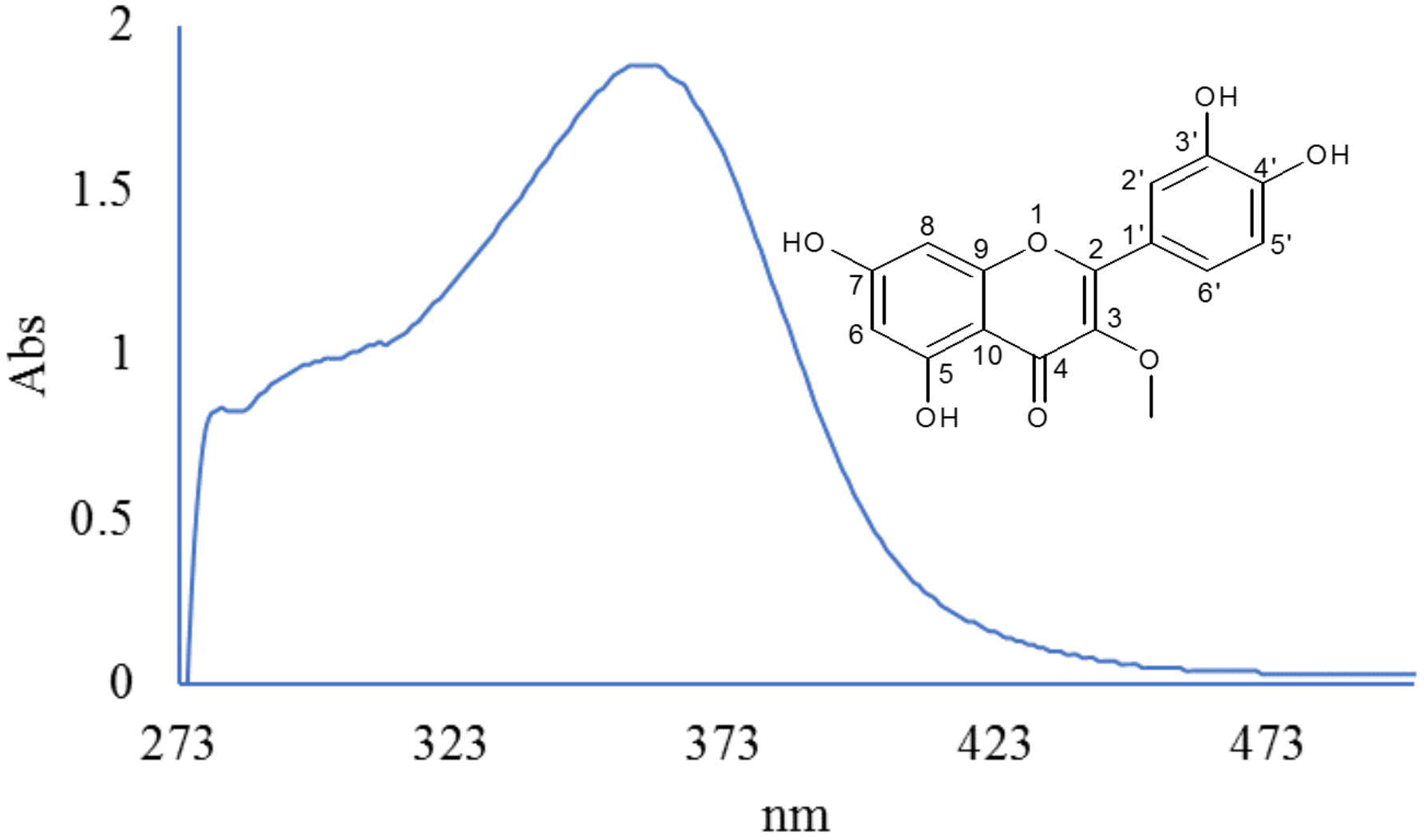


Analyst: Analyst

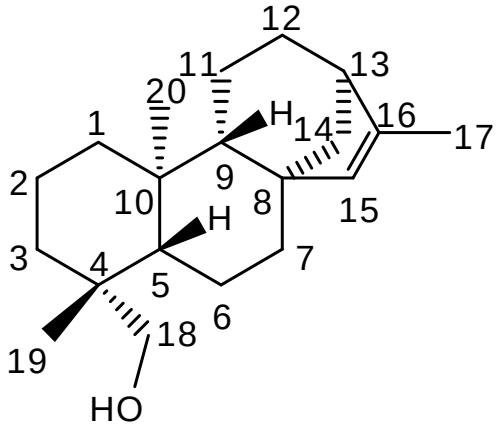
Description:

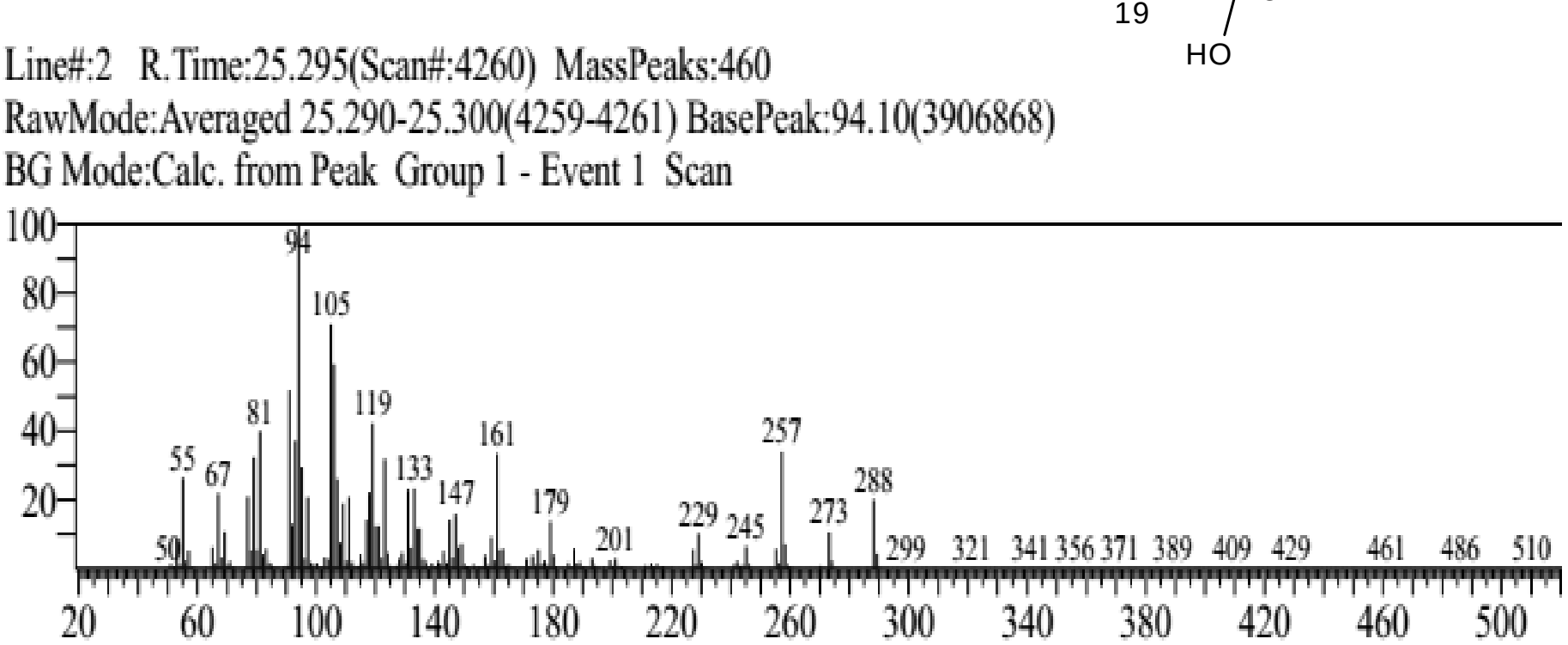
Spectrum Pathname: C:\pel_data\spectra\OS 15 18.003

IR spectrum of quercetin-3-*O*-methyl ether (**B10**)

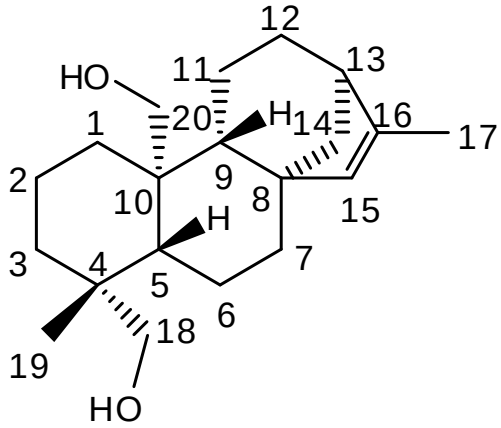


UV spectrum of quercetin-3-*O*-methyl ether (**B10**)

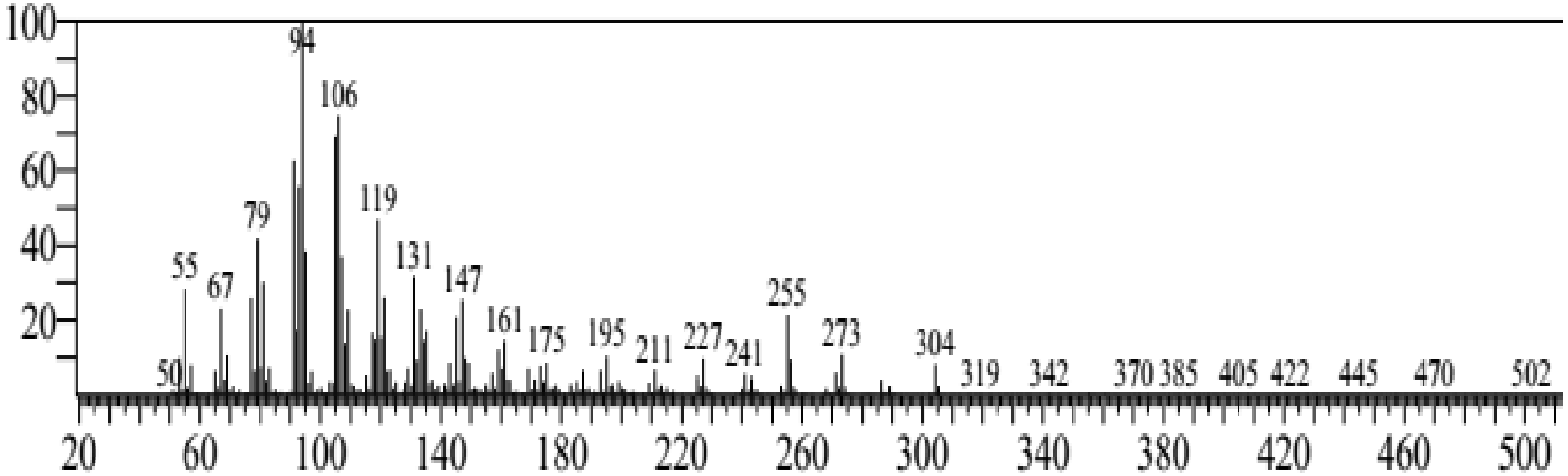




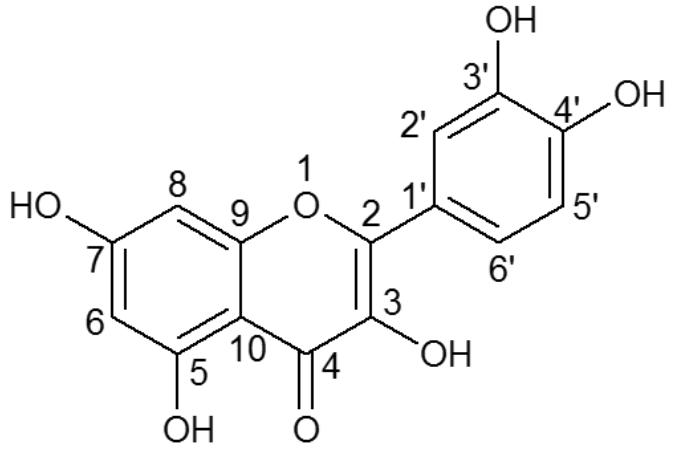
GCMS fragmentation pattern of *ent*-kaur-15-en-18-ol



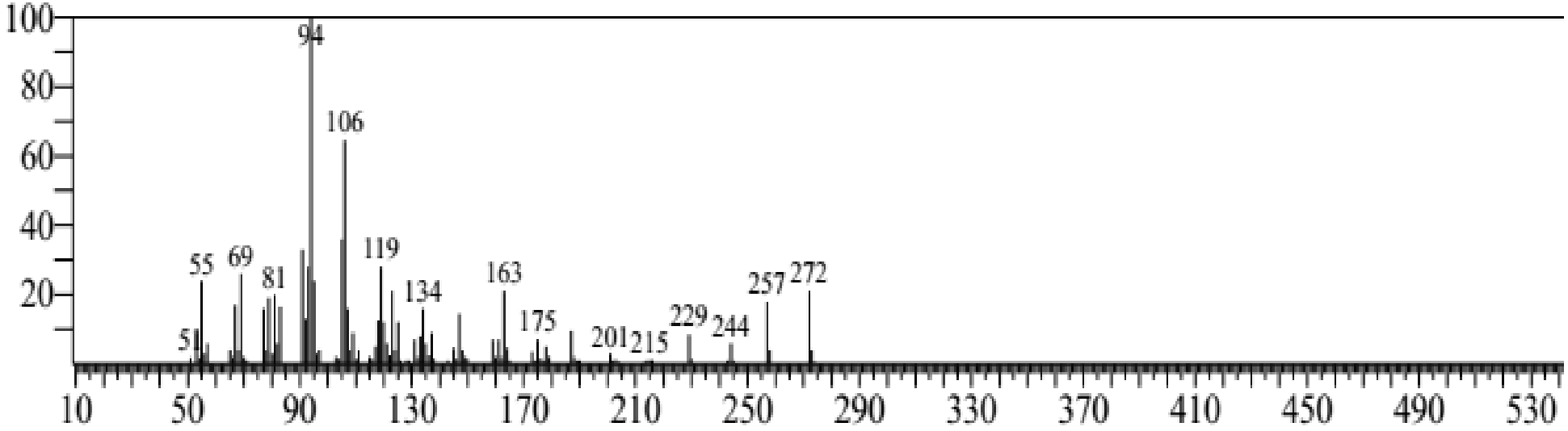
Line#:5 R.Time:29.775(Scan#:5156) MassPeaks:450
RawMode:Averaged 29.770-29.780(5155-5157) BasePeak:94.05(80488)
BG Mode:Calc. from Peak Group 1 - Event 1 Scan



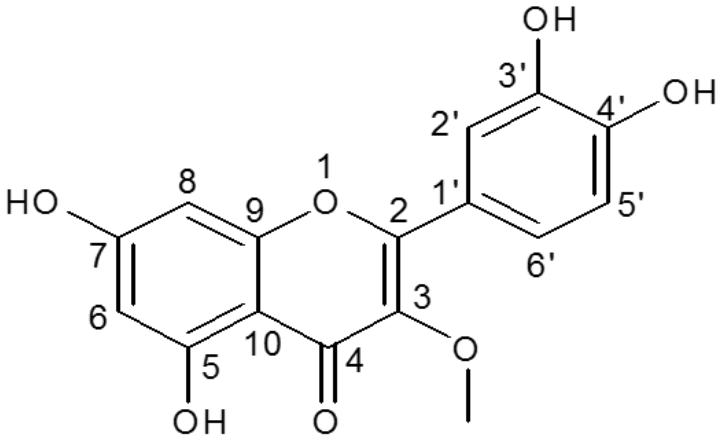
GCMS fragmentation pattern of *ent*-kaur-15-en-18,20-diol (**B6**)



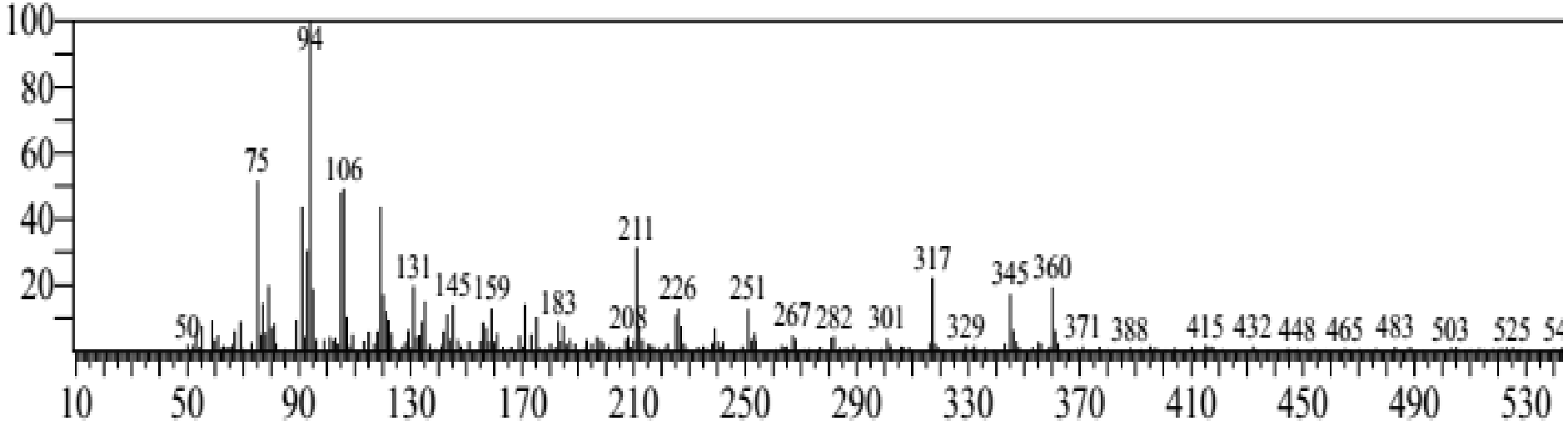
Hit#:4 Entry:96995 Library:NIST11.lib
SI:67 Formula:C20H32 CAS:5947-50-2 MolWeight:272 RetIndex:1794
CompName:Kaur-15-ene $$ (-)-Isokaurene $$ Ent-15-Kaurene $$ Isokauren $$ Isokaurene $$ Kryptomeren $$ 15-



GCMS fragmentation patterns of quercetin



Line#:1 R.Time:27.335(Scan#:4668) MassPeaks:409
RawMode:Averaged 27.330-27.340(4667-4669) BasePeak:94.05(3539)
BG Mode:Calc. from Peak Group 1 - Event 1 Scan



GCMS fragmentation pattern of quercetin-3-*O*-methyl ether