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M.Sc. (Chemistry)
GRADE / DEGREE

Department of Chemistry
FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

Part A: Investigation of a newly identified plant *Hernandia Didymantha Donn,* Synthesis of Phenethyl Cinnamide.
Part B: Selective removal of ascaridol from the essential oil of *Chenopodium Ambrosioides var. Ambrosioides.*
Part C: Synthesis of canophyllol analogues

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Part A: Investigation of a newly identified plant *Hernandia Didymantha Donn*, Synthesis of Phenethyl Cinnamide. Part B: Selective removal of ascaridol from the essential oil of *Chenopodium Ambrosioides var. Ambrosioides*. Part C: Synthesis of canophyllol analogues.

Lynn Marguerite Primeau, B.Sc.

A thesis submitted to the
School of Graduate Studies and Research
University of Ottawa
in partial fulfillment of the requirements for the degree of
Master of Science in Chemistry
in the
Ottawa-Carleton Chemistry Institute
September 2006

These soumise a la
Faculte des Etudes Superieures et Postdoctorales
Universite d'Ottawa
en vue de l'obtention de la
Maitrise des Sciences
a
L'Institue de Chimie d'Ottawa-Carleton

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Your file Votre référence
ISBN: 978-0-494-59879-5
Our file Notre référence
ISBN: 978-0-494-59879-5

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Chapter 3: Synthesis of canophyllol analogues.

The stars, Earth, stones, life of all kinds, form a whole in relation to each other and so close is this relationship that we cannot understand a stone without some understanding of the great sun. No matter what we touch, an atom, or a cell, we cannot explain it without knowledge of the universe. The laws governing the universe can be made interesting and wonderful to children, more interesting than things themselves, and then begin to ask:

What am I?

What is the task of humanity in this wonderful universe?

-Maria Montessori, *To Educate the Human Potential*

The Greek Myth of Gaia

Mother Earth
The mother of us all,
the oldest of all,
hard,
splendid as rock
Whatever there is that is of the land
it is she
who nourishes it,
It is the Earth
that I sing.

-Homer

A land of wheat and barley, vines,
fig trees and pomegranates,
A land of olives and honey;
A land wherein thou shalt eat
bread without scarceness.

- Deuteronomy 8:8-9

Acknowledgements

I have been so blessed during the course of my life. So many people have added to my growth, spirituality, and just plain love of life itself; some are still around me and some have left, each one of them contributed to who I am.

First and foremost to my supervisor, Dr. Tony Durst, I am very grateful to you for accepting me in your research group, you are a tremendous mentor, positive role model, and teacher, it has been an honor Sir. In good and in bad times you have always allowed me to be myself. To Dr. John Thor Arnason for all your help, knowledge and wonderful stories

To all of my past and present lab colleagues: Eva, Helmi, Hari, Martin, Asim, Philippe, Sasmita, Ranga, Gordanna, Karol, Venu, Andrew, Virginie, Herbert, Yara, Doris, and Alison thank you for your time, knowledge and help. To Dr. Clem Kazakoff for all your help for my mass spectroscopy and GC.

To my friends Lee, Karen, Lynn the Red, Soha, Rick, Marie, Gabrielle, Sylvie and all the rest... who understood that weekends were not always for partying, but for studying and working. A special thank you to my proofreaders Jim, Laurie, Charlie.

To my family, Grant, Erik, Veronica, Marcia, ma tante Paulyn, Mimi, Josee for understanding why I've had to be away for so long.

Lastly, to my mother Mimi, someone I could always look up to and would never let me down no matter what. Whenever things around us crumbled... you never did. This thesis is dedicated to you mom!

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ABBREVIATION AND SYMBOLS

Ac	acetyl
Ac_2O	acetic anhydride
br	broad
br s	broad singlet
°C	degree celcius
^{13}C NMR	carbon-13 nuclear magnetic resonance
CH_2Cl_2	methelyne chloride
CH_2N_2	diazomethane
cm-1	wavenumber
δ	chemical shift
dd	doublet of doublets
DMAP	4-Dimethylaminopyridine
dt	doublet of triplets
EC_{50}	effective concentration for 50% mortality
EI	electron ionization
equiv.	equivalents
Et	ethyl
Et_3N	triethylamine
Et_2O	diethyl ether
EtOAc	ethyl acetate
EtOH	ethanol

GC/MS	gas chromotography/mass spectroscopy
HRMS	high resolution mass spectroscopy
h	hours
^{1}H NMR	proton nuclear magnetic resonance
Hz	hertz
IC_{50}	concentration to inhibit growth by 50%
J	coupling constant
liq.	liquid
m	multiplet
$[M]^+$	molecular ion
Me	methyl
m/z	mass to charge ratio
MeOH	methanol
min	minutes
mL	milliliter
m.p.	melting point
MS	low resolution mass spectroscopy
NMR	nuclear magnetic resonance
OAc	acetoxy
Ph	phenyl
ppm	parts per million
q	quartet

rt	room temperature
s	singlet
t	triplet
THF	tetrahydrofuran
TLC	thin layer chromatography

ABSTRACT

Part A.

A number of constituents isolated from the ethanol extracts of the leaves of the neotropical species *Hernandia Didymantha Donn*. These include series of ethyl esters of common C14, C16, and C18 saturated and unsaturated fatty acides. The presence of the ethyl ester of the C17 fatty acid was indicated by GC-MS. The major non polar component was shown to be nerolidol, **4**, an open chain sesquiterpene. Ethnobotanical reports indicate that this sesquiterpene can be used as a natural pesticide and as a transdermal carrier. It is also reported to have anti-malarial activity. The major more polar component was shown to be the tryptamine derivative phenethyl cinnamide, **1**. The structure identification was based on comparison of literature data and a two step synthesis (80%) from cinnamic acid and tryptamine.

Part B.

Chenopodium ambrosioides has been known for centuries as an antifungal and vermifuge remedie. As an infusion, *Chenopodium ambrosioides* is safe and an effective treatment. The essential oil has been investigated as an organic insecticide by Codena Inc, (St Charles sur Richelieu, Quebec). Registration was initially rejected by the U.S. Environmental Protection Agency because of the known toxicity of one of the components, the monoterpene endoperoxide ascaridol, compound **26**. The selective removal of ascaridol was achieved without affecting the other constituents present in the essential oil using either the reaction with zinc in acetic acid or Lindlar Catalyst poisoned with quinoline and hydrogen at room temperature under several atmospheres of pressure. In each case the reduction product is the diol **27**. The treated oil still showed excellent insecticidal properties and was approved for use as an organic pesticide by the U.S. Environmental Protection Agency.

Considerable efforts were made to optimize the zinc/acetic acid. Unfortunately the optimized procedure still required considerable excess amounts of both reagents. This procedure, although successful from a chemistry and safety point of view, was not economically viable.

The second method using the poisoned Lindlar Catalyst and H_2 a gave clean reduction of ascaridol without causing reduction of any of the many olefinic components present in the oil. We showed that the catalyst could be recycled at least five times without significant loss of activity. Finally to give a reduced essential oil free of any quinoline, we prepared catalyst in hexane and removed the excess quinoline by washing several times with hexanes. Metallic contaminants in the oil such as lead and palladium which are components of the Lindlar catalyst were effectively removed by stirring the final product with 1% silica gel followed by filtration. This method appears to be inexpensive and thus suitable for commercial production of the safe "Codena Oil".

Part C.

Literature reports have shown that ursolic acid, a triterpene acid has a positive effect on the elimination of biofilms without any damage to the bacteria function. Since ursolic acid and canophyllol are both pentacyclic triterpenes a series of analogues of canophyllol have been synthesized and characterized by 1H NMR, 13C NMR, MS and their melting points. These compounds in which we made relatively simple changes in the functionality at C-3 and C-28 of the canophyllol molecule, together with a variety of similar betulinic acid compounds are now available for testing as potential controls of biofilm formation. These test will be carried out by the research group of Dr. R. Sattar of the University of Ottawa, Faculty of Medicine. Canophyllol was obtained as the major triterpene component in the bark of *Ruptilliocarpon caracolito* collected in the Osa Penninsula, Costa Rica.

CHAPTER 1

Chapter 1.

Background

The plant family Hernandiaceae has been used in traditional medicine by indigenous peoples in the subtropical and tropical zones[1]. This small family of trees consists of four genera and over 50 species. Phytochemical investigations of this family reveal the presence of alkaloids (bioactive hetercocyclic nitrogen compounds), lignans (dimers of cinnamic acid derivatives) and ethereal oils. Pharmacological studies have shown tha *Hernandia ovigera* contains several lignans that inhibit Epstein-Barr virus[2] while *Hernandia nymphaeifolia*[3], *Hernandia ovigera*[4] and *Hernandia Sonora*[4] contain lignans with growth inhibitory properties on cancer cells . *Hernandia peltata*[5] contains an anti-malarial alkaloid.

The fossil record of Hernandiaceae and its sister counterpart, Menimiancene, reaches back to the Paleocene epoch some 43 million years ago. The evolution of the Laurales order, to which the Hernandiaceae belong , dates back to the Creataceous/Late Tertiary period (136-65 million years ago)[6].

Because the Hernandiaceae are rare and poorly studied, they may yield novel structures with useful biological properties. *Hernandia dydimantha* Donn is a neo-tropical tree which was targeted for study by our research group because it is a relatively rare species from lowland tropical forests. It is now listed as a "vulnerable", species by the International Union for Conservation of Nature[7]. . *Hernandia Didymantha* Donn, is distributed in the area from Columbia to Honduras in lowland semi-evergreen tropical forest.

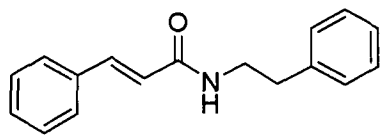
A more detailed view of the phytochemical relationships of the orders in the same subclass is shown in Table 1.1.

Table 1.1. Phytochemistry of the Magnoliidae[8,9,10].

Orders and representative family:			
Order: Piperales	Order: Rutales	Order: Laurales	Order: Asterales
Family: Piperaceae	Family: Rutaceae	Family: Hernandiaceae	Family: Asteraceae
Characteristic phytochemicals:			
Alkaloids	alkaloids	alkaloids	alkaloids
Amides	amides		amides
Lignans		lignans	lignans
Neolignans	furocoumarins	anthroquinones	sesquiterpenes
Terpenes, steroids	limonoids		polyacetylenes
	coumarins		

When comparing the four orders from Table 1, we see that only alkaloids are present in all of them. Amides and lignans are found in three orders and the other compounds are found in only 1-2 orders. phenethyl cinnamide, **1**.

One compound of interest in this thesis, phenethyl cinnamide, **1**. is found in plants representing 3 orders. These are *Clausena India*(Rutales), *Spilanthes ocymifolia* (Asterales) and the species reported here, *Hernandia dydimantha* (Laurales), produce the same compound, This simple tryptamine-derived amide was first reported in 1984[11]. It was isolated, along with two other compounds, from the leaves of the Salvadorian species *Spilanthes ocymifolia* that is used in folk medicine as a remedy against toothache and rabies. The second report of this amide showed that is had also been isolated from *Clausena india* found in the semi-evergreen forests and the central mountain rainforest of Sri Lanka in 1996[12]. As shown later in this chapter this compound is also found in *Hernandia didymantha.*



(1)

RESULTS AND DISCUSSION
CHAPTER 1

RESULTS AND DISCUSSION

Leaf material of *Hernandia* was collected in Tortuguero, Costa Rica in the Atlantic swamp forest almost at sea level by Pablo Sanchez of Universidad Nacional at Heredia, Costa Rica and Dr. Thor Arnason from the Biology Department of the University of Ottawa in 1999. It was placed in 1L Nalgene bottles and immediately covered with 95% ethanol for preservation and subsequent transport. Once in Ottawa it was stored in a refrigerator at 5°C. A voucher specimen is kept at the herbarium of the Universidad Nacional, Costa Rica.

The leaves were macerated in a food processor with 95% ethanol, left overnight and then filtered. This procedure was repeated twice more. The filtrate was then evaporated under reduced pressure to yield a green, gummy substance. A total of 32g of crude dried plant material extract and 137g of dry, insoluble material were obtained.

The total crude extract was then extracted by stirring with 300mL of each of the following solvents: petroleum ether, dichloromethane, ethyl acetate, acetone, and finally methanol.

After each extraction the mixture was filtered and the filtrate was evaporated. The yields of extracts with the different solvents are shown in Table 1.

Table 2 –Yields of extracts

Solvent used	Amount of crude extract (g)
Pet. Ether	13.0
Dichloromethane	5.7
Ethyl Acetate	4.3
Acetone	4.5
Methanol	1.3

Each fraction was initially investigated by analytical tlc, followed by silica gel column chromatography.

Chromatography of the petroleum ether extracts gave 1.02 g of very non-polar compounds. The NMR spectrum of this material suggested that it consisted of ethyl esters of mainly unsaturated fatty acids. The major components were identified by GC-MS, **Figure 1.3.1.**

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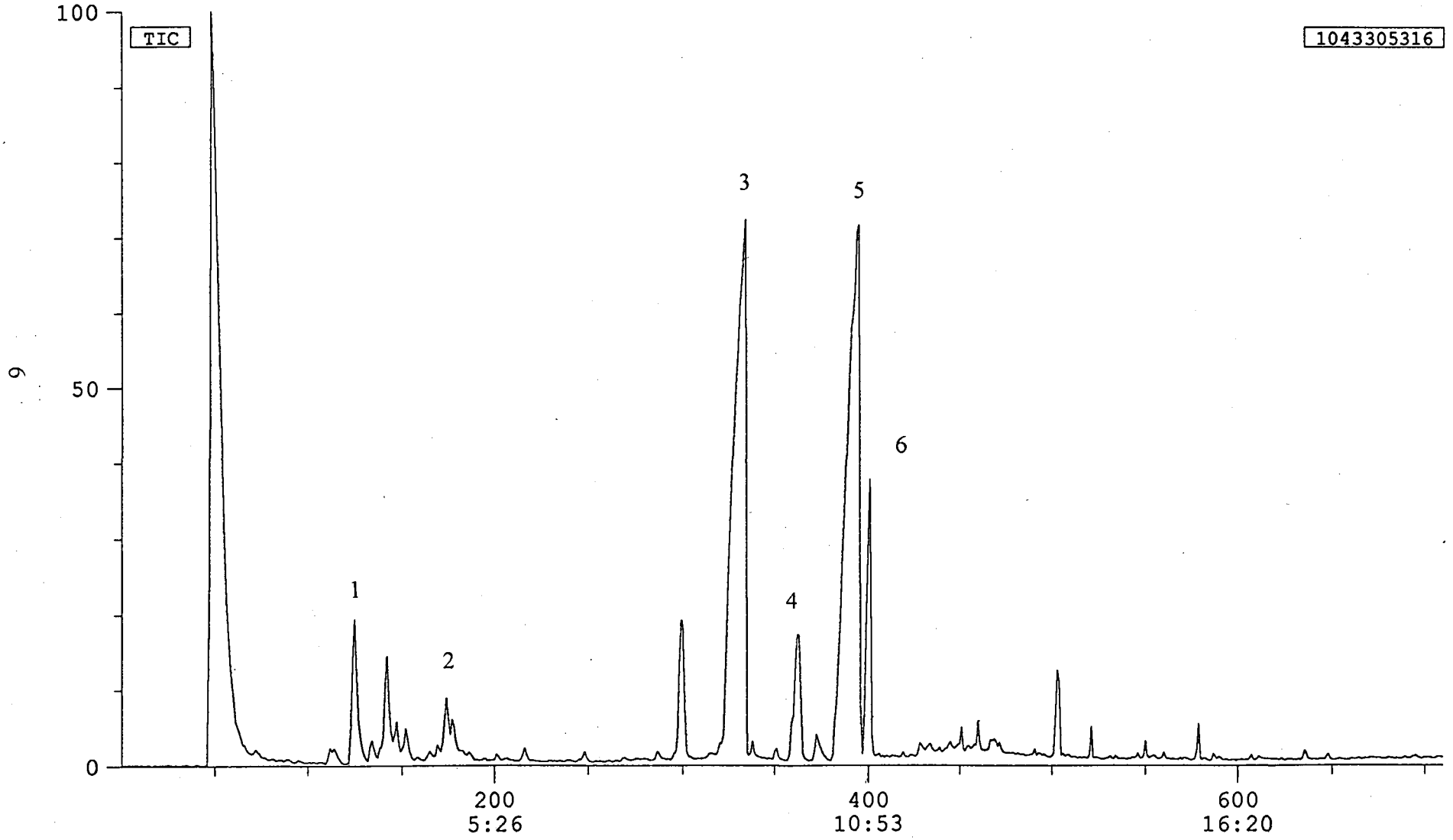
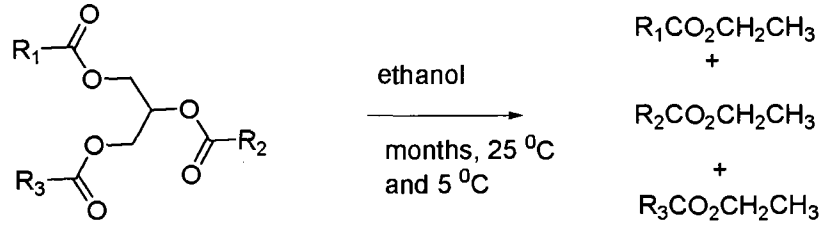


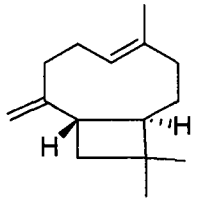
Figure 1.3.1. GC of the Fatty Esters in the Petroleum Ether Fraction

The MS of the peaks **1** through **6** gave molecular ion peaks at m/e = 270, 284 , 298, 308 310 and 312. These peaks are consistent with the calculated molecular weights of the ethyl esters of myristic (14:0), palmitic (16:0), heptadecanoic (17:0), linoleic (18:2, 9c,12c), linolenic (18:3) and oleic (18:1, 9c), and stearic(18:0)acids, respectively. In each case a significant M- 45 peak was observed, as expected for ethyl esters. We were surprised to find a significant amount of the C_{17} saturated ester since most fatty acid mixtures consist of only an even number of carbon atoms. Normally, fatty acids are stored in plants and animals as glycerides. We presume that the long storage of the material in ethanol resulted in trans-esterification.

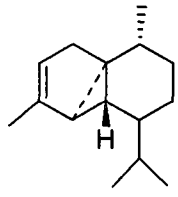


Scheme 1.4.1 Trans esterification of glycerides

The GC showed the presence of a number of other components that were eluted more rapidly than the fatty acid esters. The peaks labeled 6 and 7 each showed a molecular ion at m/e=204 ($C_{15}H_{24}$). Peak matching of the fragmentation patterns with the MS database strongly suggested (99% similarity) that these compounds were the sesquiterpenes caryophyllene, **2**, and cubebene, **3**. In literature, both of these compounds are reported to have good insecticidal properties[13, 14].



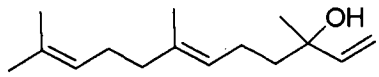
β-caryophyllene
2



α-cubebene
3

Continued elution of the petroleum ether extracts with solvents of increasing polarity gave 3.2g of yellow orange oil. The MS of this material showed a MW of 222

consistent with $C_{15}H_{26}O$. A search of the database stored in our MS computer showed a nearly 100% correspondence of this materials' fragmentation pattern to that of nerolidol, **4**,



4, nerolidol

The ^{1}H NMR spectrum, **Figure 1.3.2**, of this material was consistent with this structure assignment. It showed the required number of alkene hydrogens, including a doublet of doublets (J=16 and 8Hz) due to the methine hydrogen of the terminal vinyl group. Finally, a comparison of the spectrum of the yellowish oil completely matched the structure of authentic nerolidol, as published by the Aldrich Co[15]. We have not yet verified whether this compound is the R or the S isomer of nerolidol.

The abundance of this compound, which represents 10% of the total ethanol extracts is explained by the fact that the collection of this plant was carried out at the end of the dry season in Costa Rica. Lopes et al.[16] found that plants produced different compounds during different seasons. Usually fatty acids are produced preferentially during the rainy season while sesquiterpenes, such as nerolidol, are produced during the dry season, indicating that it acts as a protection for the plant. In addition to its use as a natural pesticide[17] this sesquiterpene is found in other plants where it is used to attract a red "bodyguard" mite for protection from an herbivour spider mite[18]. Nerolidol is currently under investigation as a skin penetration enhancer for the transdermal delivery of therapeutic drugs[19]. It appears to enhance bacterial permeability and susceptibility to exogenous antimicrobial compounds[20]; it is also part of an essential oil treatment of malaria by the Waiapi Indigenous people living in the Amazon[16].

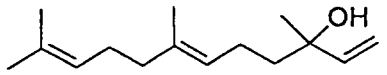
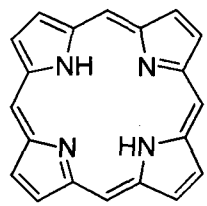


Figure 1.3.2. ^{1}H NMR of Nerolidol

Further elution afforded 15mg of a greenish oil. The NMR spectrum of this material was reminiscent of that of a porphyrin, **5**, isolated a number of years ago in our laboratory by Eva Punianin. The exact structure of both the Puniani compound and the one isolated here are not known. The unusual features of the spectrum were several singlets between 7 and 9 ppm. A comparison of the two spectra is shown in **Figure 1.3.3**. Porphyrins are aromatic heterocyclic macrocycles made from 4 pyrrole subunits linked on opposite sides through 4 methine bridges. They are components of chlorophyll, the light-harvesting system of plants.



5

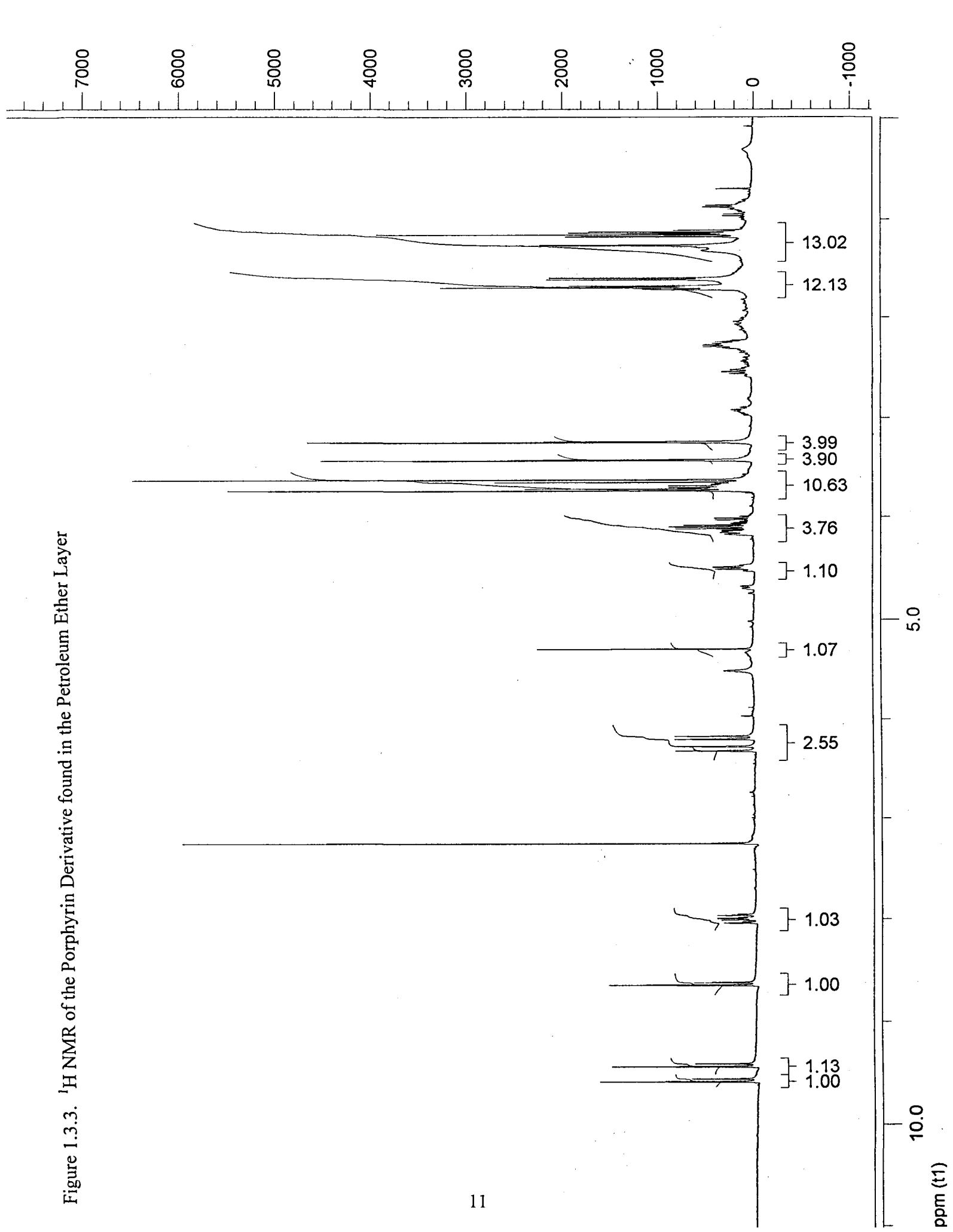
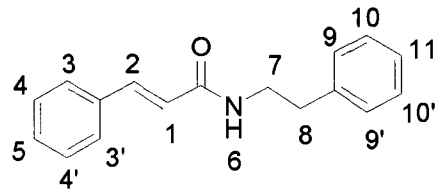


Figure 1.3.3. ^{1}H NMR of the Porphyrin Derivative found in the Petroleum Ether Layer

The crude dichloromethane extracts (5.7g) were placed on a silica gel column. In the initial elution with mainly hexane gave1.2g of a mixture of ethyl esters of fatty acids, similar to that obtained from the petroleum ether fraction. Further elution gave 2.8g of white crystals with a melting range of 125-127°C. This material was identified as phenethyl cinnamide, **1,** based on both its proton and carbon NMR and eventual synthesis. Since this compound had already been reported twice before from other species, we were able to compare our spectroscopic data to that reported in the literature, **Table 3**. This is the first report of the compound in this species.

Table 3 – Table of comparison ^{1}H NMR and ^{13}C NMR

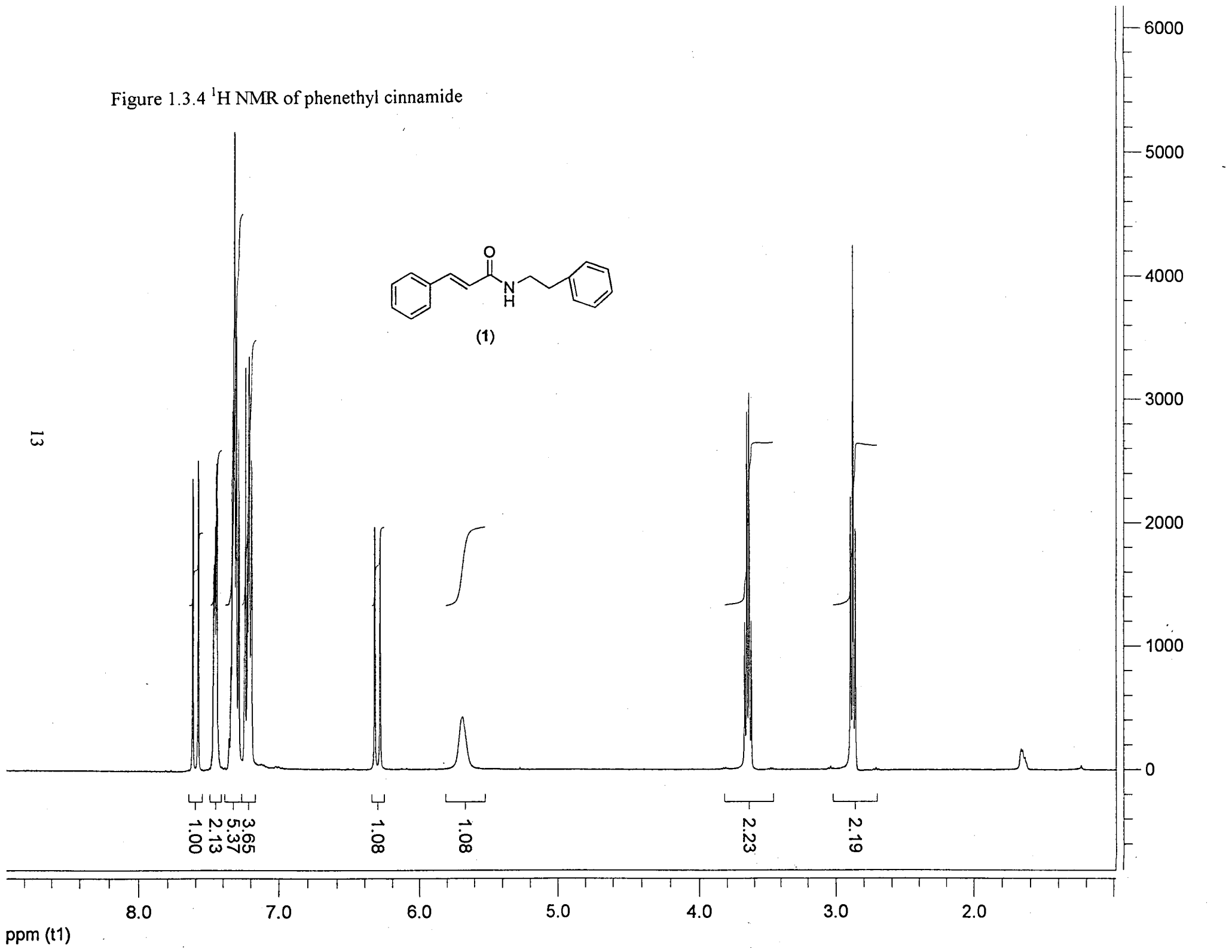


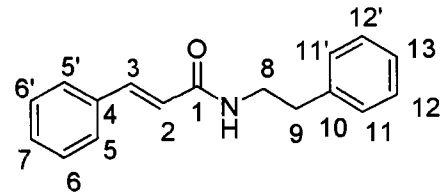
1

1984 – phenethyl cinnamide	1997 – phenethyl cinnamide	Hernandia Didymantha
1 7.62, dd, J=9Hz*	1 7.62, d, J=15.6 Hz	1 7.58, d, J=15.6Hz
2 6.43, d, J=9Hz*	2 6.31, d, J=15.6Hz	2 6.34, d, J=15.6Hz
3-3'	3-3' 7.48,dd=7.7, 1.8Hz	3-3'
4-4' 7.53 – 7.0	4-4' 7.38-7.31	4-4' 7.47-7.19
5	5	5
6 5.66, br.	6 5.60,br,t, J=6.6Hz	6 5.68, br
7 3.65, dt, J=8,7 Hz	7 3.67, q, J=6.6Hz	7 3.64, q, J=6.8Hz
8 2.88, t, J=8Hz	8 2.90, J=6.6Hz	8 2.87, t, J=6.9Hz
9- 9'	9-9'	9-9'
10-10'7.53-7.0	10-10' 7.38-7.31	10-10' 7.47-7.19
11	11	11

* this result is inaccurate as a trans coupling constant should be 15Hz

Figure 1.3.4 ^{1}H NMR of phenethyl cinnamide





1

1984 – phenethyl cinnamide from the *Spinlanthes ocymfolia* plant		2003 – phenethyl cinnamide from the *Hernandia dydimantha Donn* plant	
^{13}C NMR - data		^{13}C NMR - data	
1	165.6	1	165.9.
2	120.5	2	120.6
3	140.8	3	141.0
4	134.6	4	134.5
5-5'	128.6	5-5'	126.2
6 –6'	128.6	6-6'	128.7
7	129.4	7	129.6
8	35.6	8	35.7
9	40.7	9	40.7
10	138.6	10	138.9
11-11'	127.6	11-11'	127.8
12-12'	128.5	12-12'	128.8
13	126.3	13	126.5

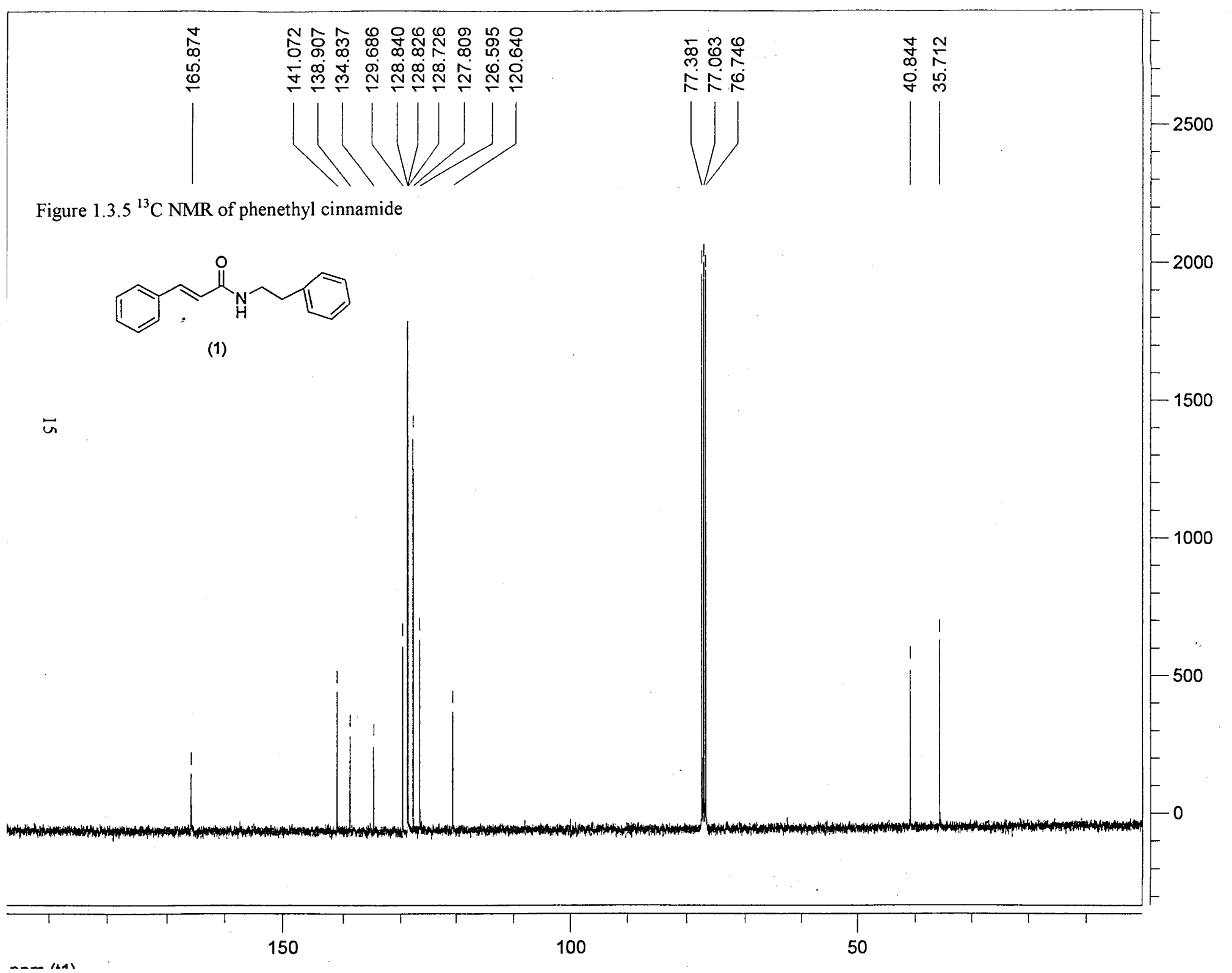
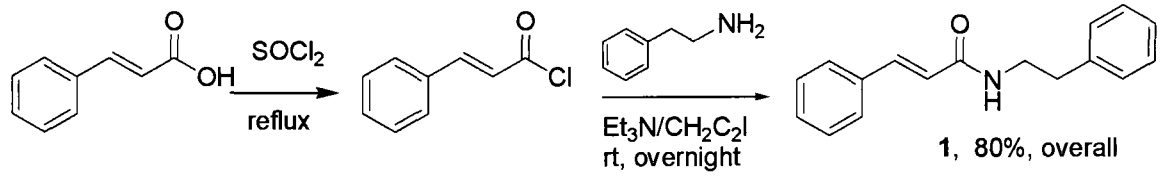


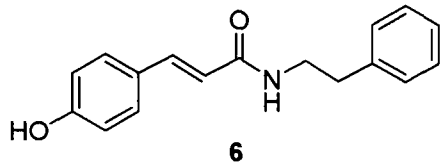
Figure 1.3.5 ^{13}C NMR of phenethyl cinnamide

The melting point range of 125-127°C of this compound was almost identical with the other two values of 125-126°C and 126-127°C respectively reported earlier. The first reported synthesis of phenethyl cinnamide was a one pot, one-step reaction where cinnamic acid was heated with phenethylamine[22]. This method only afforded a very small yield. Our synthesis involved initial activation of cinnamic acid as its acid chloride followed by addition of phenethylamine (with triethylamine to neutralize the liberated HCl). This two step procedure resulted in an overall yield of 80% compared to the 25% reported earlier[22], Scheme 1.4.2.



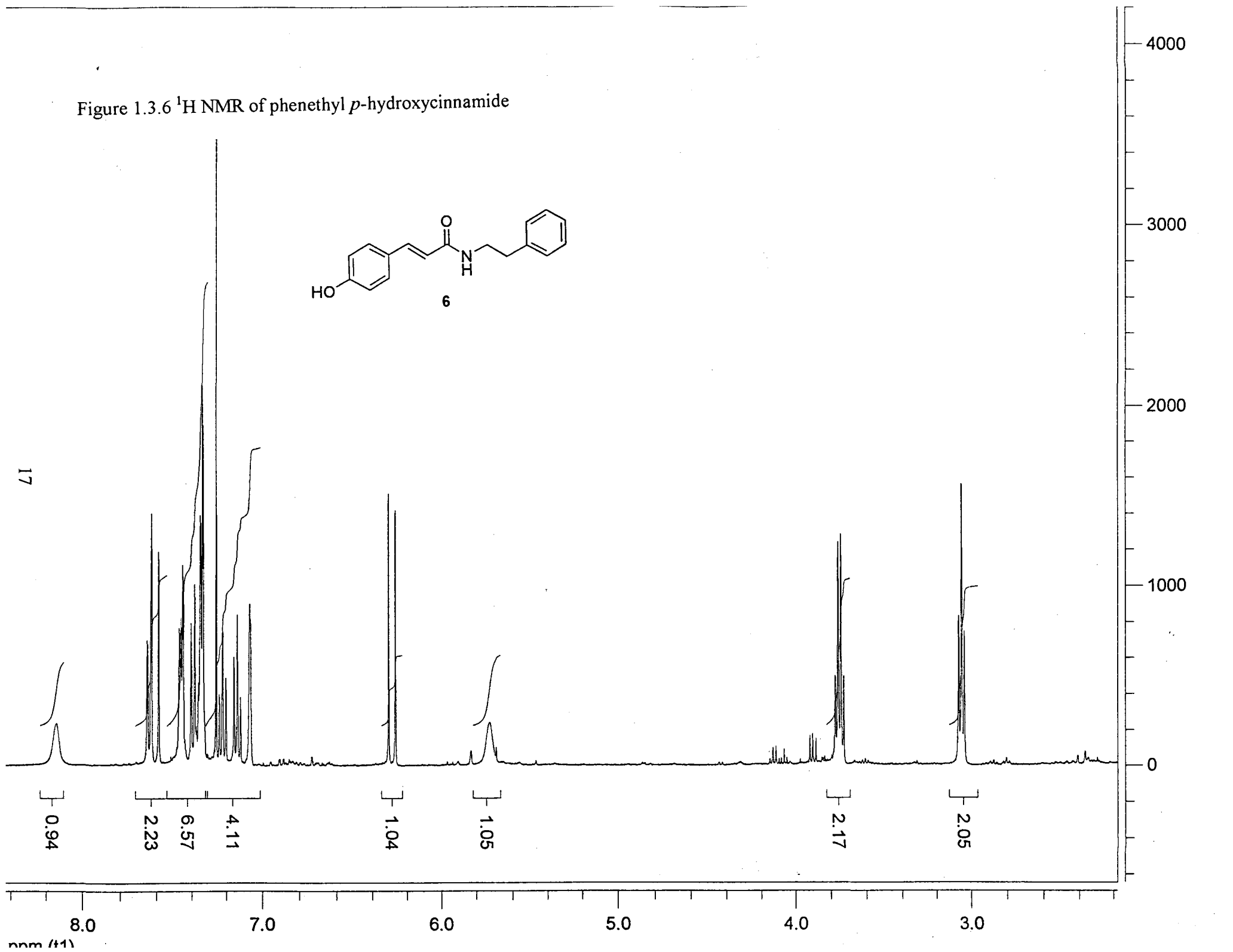
Scheme 1.4.2. Two step synthesis of phenethyl cinnamide

Continued chromatography of the dichloromethane extracts allowed us to isolate one addition compound. This crystalline solid showed a ^{1}H NMR which was very similar to phenethyl cinnamide with the exception of a phenolic hydroxy group at δ 8.81ppm and a more complex aromatic region integrating to 9 protons, **Figure 1.3.7**. This material was identified as phenethyl *p*-hydroxycinamide, **6**.

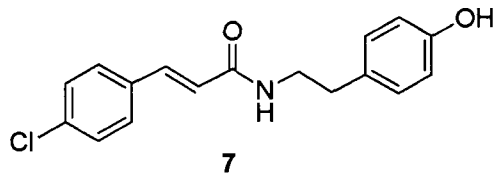


6

Figure 1.3.6 ^{1}H NMR of phenethyl *p*-hydroxycinnamide



In a published report this compound, along with other substituted cinnamides, were prepared and assayed for inhibition of three subtypes of NMDA receptors[20]. NMDA receptors (NMDAR) are ionotropic receptors for glutamate (NMDA is the name of its selective specific agonist). NMDARs plays a critical role in synaptic plasticity mechanisms and thus are necessary for several types of learning and memory. These receptors are over stimulated during ischemic stroke, head trauma and other neurodegenerative conditions[21]. This excitotoxicity process often leads to cell damage or death. The authors found that N-(2-(4-hydroxyphenyl)ethyl)-4-chlorocinnamide, **7**, showed good potential and selective antagonist activity of the NR1A/2B receptor subtype. Because it is found in large concentrations, the compound likely acts as a neurotoxin to herbivores that ingest large amounts of the plant.



7

Both the analytical tlcs and the ^{1}H NMR spectra of the crude ethyl acetate, acetone and methanol extracts showed the presence of the ethyl esters of fatty acids and phenethyl cinnamide, **1**, in addition to other more polar components. These fractions were not further investigated.

Conclusions

Three major constituents were found in the ethanol extracts of the leaves of *Hernandia Didymantha* Donn. These are esters of fatty acids, sesquiterpene nerolidol and phenethyl cinnamide. The latter two compounds constituted about 10 % of the extracts or 2% of the dried plant material. Both compounds have been reported to have potentially useful biological properties and appear to have plant defense properties. Only leaves were studied in this report, the bark and wood should also be investigated for the presence of alkaloids and lignans.

EXPERIMENTAL
CHAPTER 1

Experimental section:

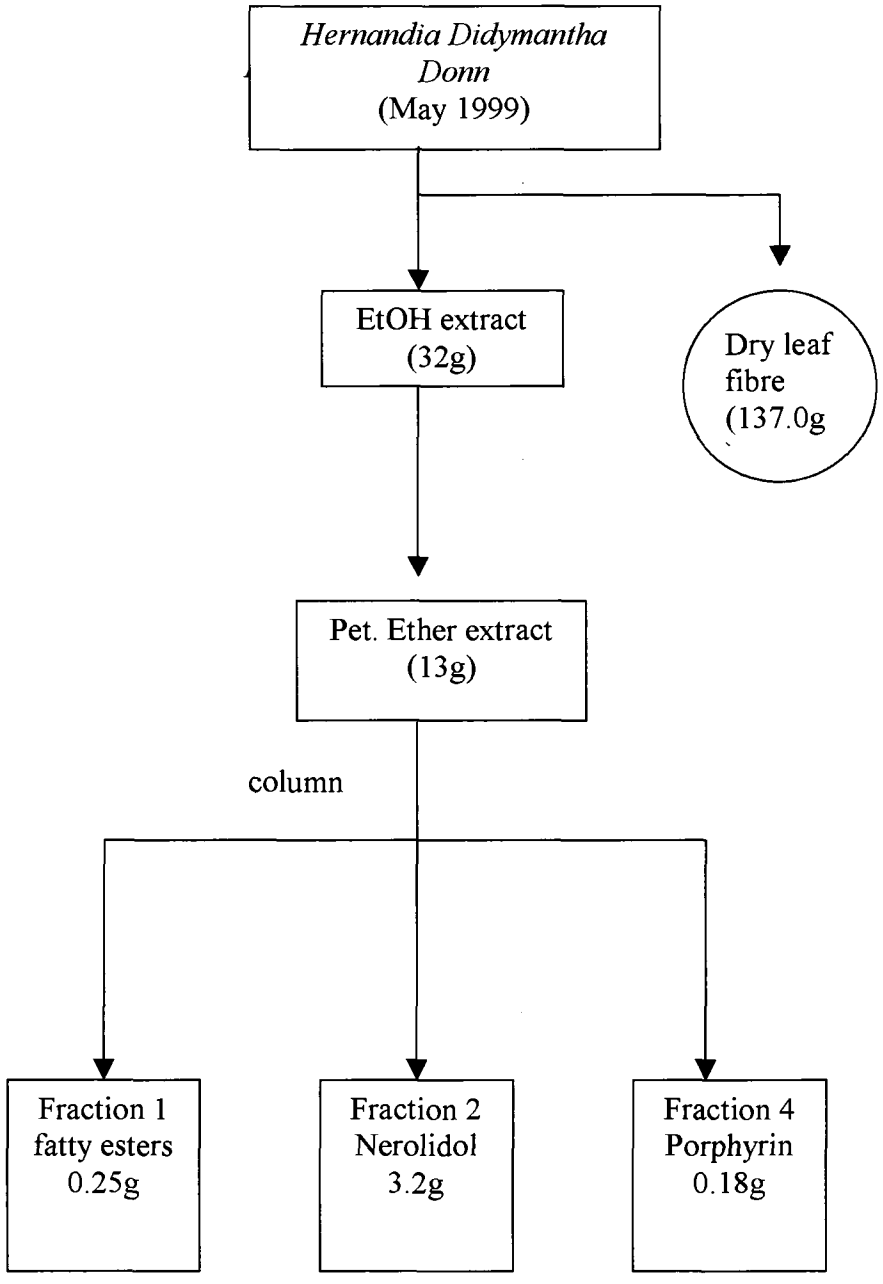
GENERAL: Mass spectra were obtained by means of a Kratos Concept II 2H and the GC were done on a Kratos Concept II 2H. ^{1}H NMR and ^{13}C NMR were recorded on a Bruker AMX-400, Bruker AMX-300 spectrometers with shifts reported in ppm. The multiplicities of the NMR signals were reported using the following abbreviations, singly or in combinations: singlets (s), broad singlet (br s), doublet (d), triplet (t), quartet (q) and multiplet (m).

Thin layer chromatography (tlc) were carried out using Kieselgel 60 F_{254} precoated 0.25mm plates. Visualization of the spots was facilitated by UV irradiation followed by charring of a tlc which has been dipped in a molybdate (2.5g) and cerric sulphate hydrate (1.0g) in a solution of sulfuric acid (10mL) in distilled water (90mL). Silica gel 270-400 Mesh was used for flash chromatography.

Preparation of EtOH extracts from *Hernandia Didymantha Donn.*

Leaves of Hernandia Didymantha Donn (collected May 1999) and the 95% EtOH were blended in a conventional kitchen blender and additional 95% EtOH was added when required to facilitate the blending process. The blended material was left to stand for 1 day at room temperature (22°C) and then filtered. The insoluble dried fiber weighed 137.0 g. The filtrate was concentrated under diminished pressure and dried under high vacuum for 1 day to afford a dark green gum of the EtOH extract (32g). Several *Hernandia* plant extracts were prepared in this way in order to permit the isolation of plant metabolites and with the ultimate goal of identifying the biologically active principle(s). This is shown in the flow chart below.

plant metabolites and with the ultimate goal of identifying the biologically active principle(s). This is shown in the flow chart below.

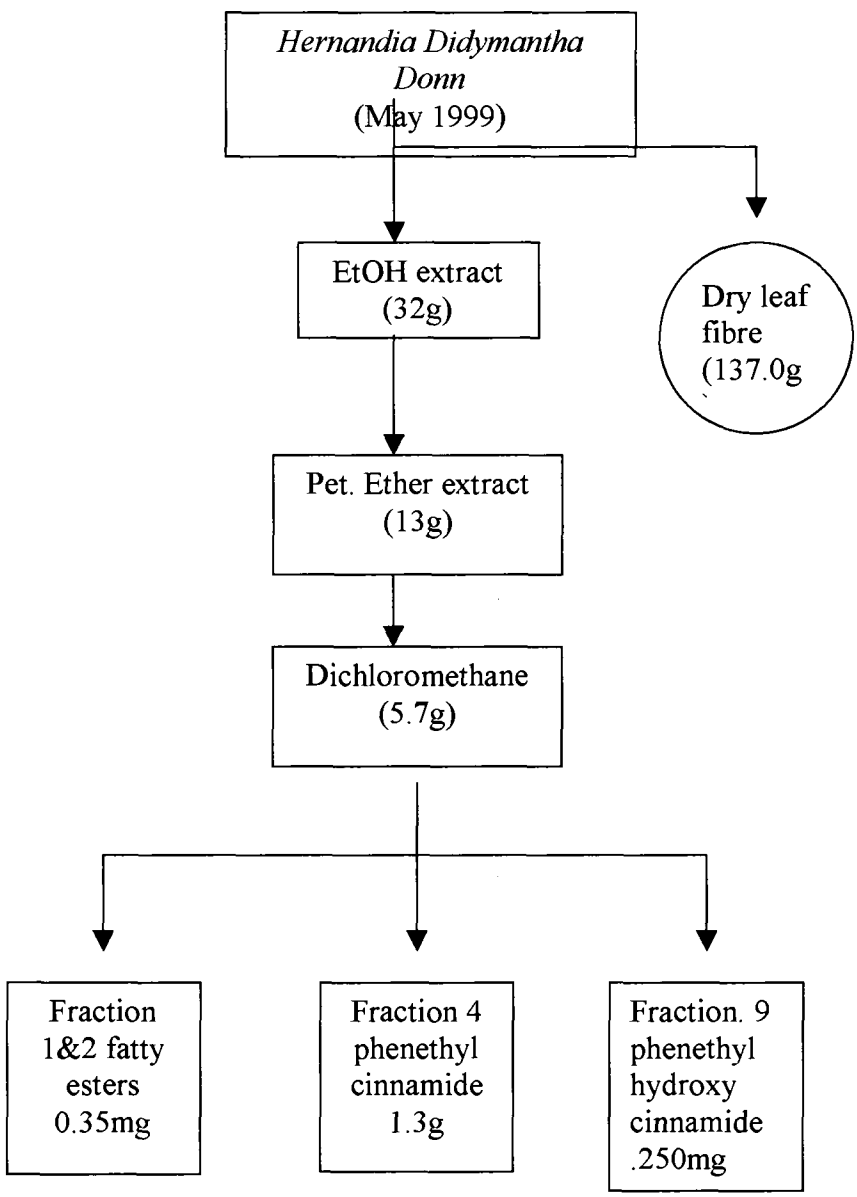


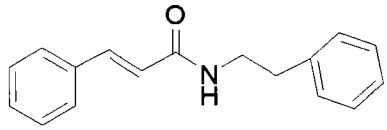
Isolation of plant metabolites from *Hernandia Didymantha Donn.*

The EtOH extracts, obtained as describes above were stirred with 300mL of pet. ether at room temperature overnight (15h) and then filtered. This procedure was repeated.

twice more. The combined pet. ether extracts were concentrated under reduced pressure to furnish a dark green pet ether extract (13g). This material was chromatographed over silica gel beginning with hexanes-EtOAc mixtures and increasing the polarity to afford three major fractions.The fractions are reported in order of increasing polarity.

Fraction 1 (0.20g), was a yellow oil of inseparable mixtures of fatty esters, **2**, **3**, **4** ,**5**. Fraction 2 (3.02g), yielded Nerolidol, **6**. Fraction 3 (0.18g), was re-chromatographed over silica gel (hexanes-EtOAc solvent gradient) to give, after recrystallisation from hexanes/CH_2Cl_2, the porphyrin, 7, as a dark green compound.





(1)

Ten equivalents of thionyl chloride, ($6.75x10^{-3}$mole, 0.6 mL) were added to one equivalent of cinammic acid ($6.75x10^{-4}$ mole, 100mg) at 0°C. This mixture was then refluxed neat at 80°C for 6 hours. Tlc analysis showed no starting material remained (eluent 70:30 Hexane:EtOAc). In a flame dry 50mL round bottom flask, phenethylamine ($7.37x10^{-4}$mole, 0.1mL) was dissolved in 5mL of dry dichloromethane and brought to 0°C in an ice bath. Triethylamine ($7.37x10^{-4}$mole, 0.1mL) was then added. This mixture was brought to room temperature and let stir for about 1 hour. The deprotonated amine was then carefully canulated into the crude cinnamic acyl chloride. Once the amine was added, the mixture was stirred and left to react overnight. To the crude product was then added 5mL of water and the solvent was evaporated. The mixture was then washed 3x5mL of dichloromethane, dried over $MgSO_4$ and concentrated to afford a white solid in the amount of 134mg, 79.3% yield.

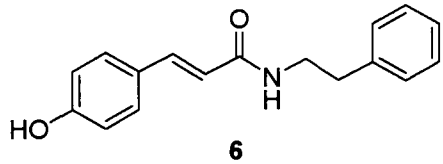
m.p. 125-127°C

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.58 (d, J=15.6Hz, 1H), 6.34 (d, J=15.6Hz, 1H), 7.47-7.19 (m), 5.69 (br. s, 1H), 3.64 (q, J-6.8Hz, 2H), 2.87 (t, J=6.9Hz, 3H).

^{13}C NMR (400 MHz, $CDCl_3$): δ 140.92, 129.62, 128.79, 127.76, 122.33, 122.11, 120.75, 119.63, 118.82, 113.11, 111.26, 69.35, 39.92, 25.35

MS (EI) m/z (rel. int.): 251 $[M]^+$ (27.3), 182.1 (0.6), 146.1 (9.6), 131.1 (100.0), 104.1 (18.8), 77.0 (8.1), 51.0 (2.0), 31.0 (0.7

HRMS: Calculated for $C_{17}H_{17}NO$, 251.1310, found 251.13265.



6

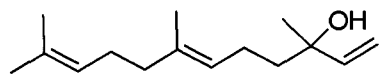
m.p. °C

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.03 (br, s, 1H), 7.60 (d, J=15.6Hz, 1H), 6.26 (d, J=15.6Hz, 1H), 7.45-7.06 (m), 5.65 (br. s, 1H), 3.73 (q, J-6.5Hz, 2H), 3.04 (t, J=6.5Hz, 3H).

^{13}C NMR (400 MHz, $CDCl_3$): δ 140.92, 129.62, 128.79, 127.76, 122.33, 122.11, 120.75, 119.63, 118.82, 113.11, 111.26, 39.92, 25.35

MS (EI) m/z (rel. int.): 210 $[M]^+$ 210, 177, 150, 135, 123, 108, 95, 84, 69, 55, 43

HRMS: Calculated for $C_{17}H_{17}NO_2$, 267.1259.



4, nerolidol

^{1}H NMR (400 MHz, $CDCl_3$): δ 5.8 (dd, J=17.3,17.3Hz,1H), 5.5-4.95 (m, 4H H_6 and H_{10} and AB of ABX $CH=CH_2$), 2.3-1.85 (m, 6H), 1.75-1.4 (m, 3H), 1.71 (br. s, C_{11} (Z)-Me), 1.63 & 1.61 (2 br. s, 3H each, C_7-Me and C_{11}(E)-Me), 1.29 (s, 3H C_3-Me)

^{13}C NMR (400 MHz, $CDCl_3$): δ 145.02, 135.53, 131.20, 124.21, 124.10, 73.47, 42.02, 39.70, 27.86, 26.66, 22.70, 17.67, 15.99

MS (EI) m/z (rel. int.): 204 $[M]^+$, 189, 161, 148, 136, 107, 93, 81, 69, 55, 41

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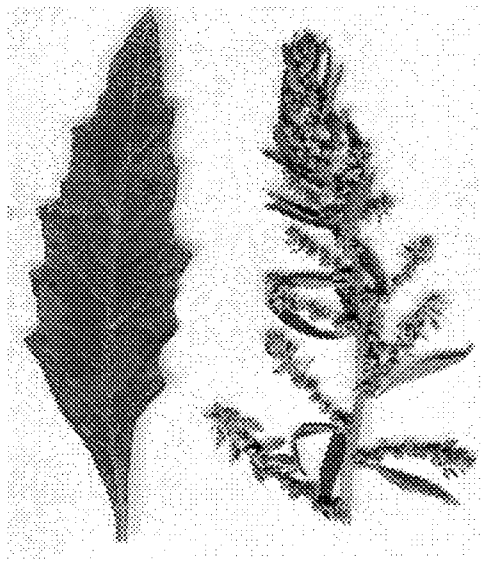
CHAPTER 2

Chapter 2.

Background

Figure 2.1.1 ***Chenopodium ambrosioides var. ambrosioides***



The plant, *Chenopodium ambrosioides*, known in English as Wormseed and in Central and South America as Epizote, in Peru as Paico is native to Mexico and Central America though it is now grown extensively in parts of the United States.

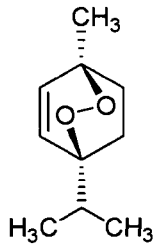
It has long been used as a medical treatment by indigenous American peoples. The people of the Yucatan used it to treat intestinal parasites, asthma, excessive mucus, chorea and other nervous afflictions, the Ticuna in the Amazon used it to expel intestinal worms[1] and as a sedative[2], the Siona-Secoya and Kofan of South America for intestinal worms, the Wayapi for stomach upsets and internal hemorrhages from injury. In Cameroon herbal medicine[3] it is considered an important remedy for hookworms, roundworms and tapeworms and for coughs, asthma, bronchitis, angina, to relieve

intestinal gas. In Piura a leaf decoction is used to expel intestinal gas, as a mild laxative, as an insecticide, and as a natural remedy for cramps.

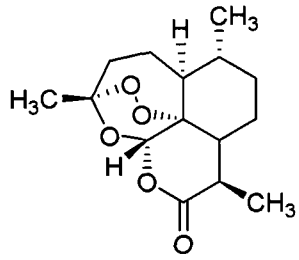
Epizote is rich in monoterpenes. The seed and fruit contain a large amount of the essential oil whose main active ingredient is ascaridol[4]. The chemical was first isolated in 1895 by a German pharmacist living in Brazil and it has been attributed with most of the worm-expelling and pain relieving properties of the plant as well as the antifungal[5] effects.

The essential oil extracted from the seed 'Oil of Chenopodium' was once listed in the U.S. Pharmacopoeia as a drug used against amoebas, roundworms and hookworms. It was once called Baltimore oil because of the large production facility in Baltimore that specialized in extracting oil from the plant. The essential oil, in doses slightly above the therapeutic levels has toxic side effects including cardiac disturbances, convulsions, respiratory disturbances, sleepiness, vomiting and weakness and even death[6]. Therefore it fell out of favour as an internal remedy.

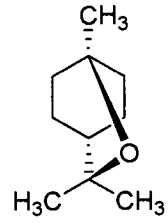
In 1924 Smillie and Pessoa showed that the ascaridol in the essential oil, **27**, not only gave the anti-helmitic property, but was responsible for the above mentioned fatalities[7]. In one of the many tests done with ascaridol, Okuyama et al.[8], observed decreases body weight, hypothermia and decreased locomotory activity in mice when ascaridol was administered at a dose of 100mg/Kg. The tripling of this dose caused death. A few decades later, in 1990, Pollack et al.[9], observed that ascaridol inhibited the growth of *Plasmodium falciparium in vitro* as did artemisin, **29**, where the similar compound cineol, **30** did not. This lead to the conclusion that the endoperoxide in ascaridol gave the oil its antimalarial activity as in artemisinin. Unfortunately, due to the known toxicity of ascaridol the anti-malarial activity could not be exploited further.



26, ascaridol



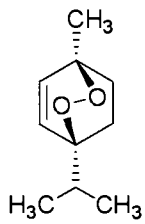
29, artimisinin



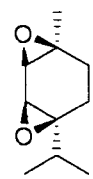
30, cineole

Johnson and Croteau reported in 1984[10] that the essential oil of *Chenopodium ambrosioides* contained between 16 and 61% ascaridol, 2-10% *p*-cymene, 7-53% α-terpinene, 0.3-0.7% γ-terpinene, 5-48% limonene, 1-8% iso-ascaridol, 0.2-1% trans-diol and 0.1-4% cis-diol derived from ascaridol, **Figure 2.1.2**. Helene Chiasson of Codena Inc. in private communication carried out similar studies on the oil obtained from this weed and found the same major component present somewhat similar ranges: 8.8 to 29% ascaridol, 5.5-14% *p*-cymene, 40-70% α-terpene, 0.3-0.5% γ-terpinene, 2.5-10% limonene, 0.0-0.6% iso-ascaridol.. The variations can be explained by variables such as genotype, the season of harvest, soil, area and growth conditions.

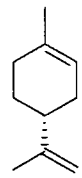
Figure 2.1.2. Major compounds found in the essential oil of *Chenopodium ambrosioides*.



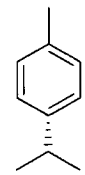
26, ascaridol



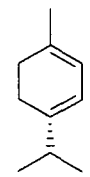
isoacaridol



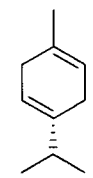
limonene



p-cymene



α-terpinene



γ-terpinene

As mentioned above several tribes used an infusion of the weed to rid the stomach of unwanted parasites. Sorger et al. in 2004[11], from McMaster University, showed that an infusion of *Chenopodium ambrosioides* had of a nematocidal activity, which was due to a hydrophillic component different from ascaridol. These authors found that after

extracting the aqueous layer of the infusion with hexanes close to 90% of the nematocidal activity remained in the aqueous layer and that the hexane containing ascaridol layer accounted for about 10% of the bioactivity.

In 1993, Codena Inc, a small company, situated in Saint-Charles-sur-Richelieu, Quebec, began to develop biologically based methods of pest control and started to investigate and develop the use of the essential oil from *Chenopodium ambrosioides* for it's effectiveness against greenhouse parasites. The need for biological agents is now well established in vegetable crops since such control is within the definition of organic produce. Such methods of parasite control are now also being considered in floriculture because of health and environmental concerns of growers and consumers. The use of pest control on plants before introduction in greenhouses is needed since new pests are constantly being introduced in greenhouses because of the use of new plant hosts or plant stock originating from different geographical areas.

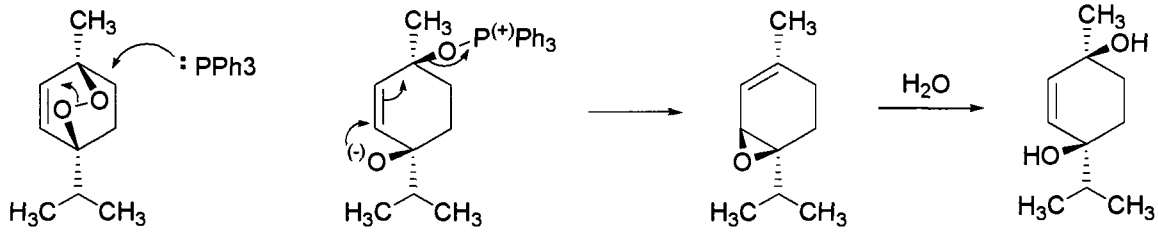
Codena researchers were able to show that the essential oil from *Chenopodium ambrosioides* provided effective control of a variety of plant pest including green peach aphid, *Myzus percicae* (Sulzer) (Homoptera; Aphididae), western flower thrips, *Frankliniella occidentalis* (pergande), (Thysanoptera: Thripidae), and greenhouse whitefly, *Trialeurodes vaporariorium* (Westwood), (Homoptera: Aleyrodidae). Codena's marketing plan called for three formulations,

- an emulsifiable concentrate(EC) with a 25% concentration of active ingredient, produced in 4 – L containers for commercial greenhouse use,
- a microemulsion (ME) for use against lawn insects and diseases,
- a ready-to-use (RTU) formulation, at a concentration of 0.5 % distributed in 750 ml spayers for domestic use on indoor plants in homes, buildings and public areas.

The challenge was to remove the toxic ascaridol selectively and economically thereby maintaining the requirements of production in commercial quantities.

The Durst research group was approached by Codena and asked to develop a method for selective removal of ascaridol from the oil. Selectivity was of primary importance since a chemical method of removing the material that changed other components was likely to have a negative effect on the biological activity of the oil. Removal of the oil by a physical method, such as distillation, was not considered feasible since many of the other components would have boiling points similar to ascaridol and would likely be removed with ascaridol during the most careful distillations. No investigations were carried out in this direction.

A review of the literature dealing with the reduction of ascaridol itself showed that the peroxide bond had been cleaved using several different methods. One of these methods used triphenylphosphine[12]. This method was not considered since it uses the rather expensive reagent in molar quantities. This procedure also produces triphenylphosphine oxide, which is often very difficult to separate from other organic products. Finally, a mixture of the allylic epoxide **31** and the diol **32** are given as products. The mechanism of this reduction is given below, Scheme 2.1.1.

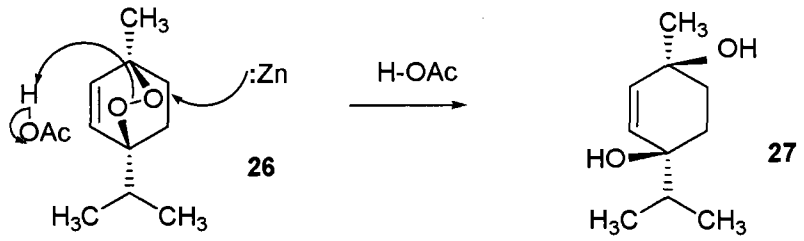


26, ascaridol **31** **32**

Scheme 2.4.1. Mechanism of the reaction of triphenylphosphine reaction with ascaridol.

Other known methods of reducing peroxides were also considered. These include dimethyl sulfide and sodium dithionite. The dimethyl sulfide approach was eliminated for esthetic reasons since it was assumed that such treatment would leave behind a small amount of residual foul smelling dimethyl sulfide or dimethyl sulfoxide. The reduction of ascaridol with sodium dithionite in a two phase, water-ethyl acetate system was attempted but was not successful.

Treatment the essential oil containing the ascaridol with Zn and acetic acid[13] was investigated next. It was anticipated that such a reduction, patterned after the reduction of ozonides, obtained form the ozonolysis of alkenes[14] would be selective for the peroxide in ascaridol, Scheme 2.1.3. It was expected that the other major components found in the *Chenopodium ambrosioides* oil would not be affected by such a treatment, Scheme 2.1.2.



Scheme 2.4.2. Mechanism of the reduction of ascaridol to it's diol using Zn and Acetic Acid

Scheme 2.4.3. Reduction of the ozonide and cleavage results in carbonyl fragments.

Reduction of ascaridol with Zn and acetic acid at 35-40°C occurred cleanly with pure ascaridol in about 4 hours. The only product obtained was the cis diol **27**. Dr. Helmi Hussain then carried out in the Zn/acetic acid procedure on the *Chenopodium ambrosioides* oil which was renamed "Codena Oil" which contained about 7% of ascaridol. Gas chromatography analysis of the oil showed that the ascaridol concentration had been reduced to less that 0.01% of the original content and that a new peak appeared having the same retention time as the pure cis 1-4 diol, **27**. Importantly, the GC analysis showed that the other components present in the Codena Oil had not

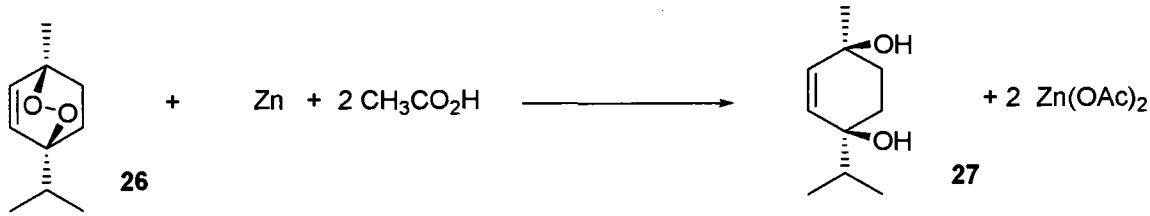
changed. A small amount of more polar material appeared at higher retention time appeared in the GC.

A 100 g sample of Codena Oil was subsequently reduced and sent to Codena for bioassays. We were gratified to find that that the "reduced oil" retained more than 95% of the activity of the original oil.

In 2003, Hussain scaled up this procedure, to reduce a 2L batch of Codena Oil. GC analysis confirmed that the product was the same quality as that prepared in small scale runs. His work had also been repeated successfully by Josee Philippe, a research associate during summer of 2004 on two 1L and one1.5L batches.

The reduction of the Codena Oil was successful. It produced a product that retained more than 95% of the activity of the native insecticide. Also, since it was now free of ascaridol, the reduced oil met the regulations of the Canadian and US Food and Agriculture agencies. It could therefore be registered as a safe organic insecticide. The major concern regarding commercialization that remained was the cost of the reduction both in terms of reagents and the need to dispose of the waste products. Thus Codena asked if it would be possible to reduce the amount of reagents and solvents that were used in the reduction process.

The stoichiometry of this reaction requires that one mole of zinc and two moles of acetic acid are consumed in the process. The diol, **27,** and one equivalent of $Zn(OAc)_2$ are produced. Excess zinc was removed by filtration. The zinc diacetate and any excess acetic acid were removed, according to the Hussain procedure by diluting the reaction mixture with ethyl acetate and, washing with 10% NaOH solution and finally with water. Each of these manipulations carries with it a cost in terms of solvents or reagents and also time. It was therefore agreed that, the Durst group would set out to optimize this procedure, ideally by using not much more than the stoichiometric amount of zinc and acetic acid. If successful this would also lower the cost of the workup and recovery of the reduced Codena Oil. We also planned to investigate the use of higher temperature and thus shorter reaction times. Optimization of all these factors was expected lead to a substantial reduction in the cost of producing the environmentally acceptable pesticide.



Scheme 2.4.4 Stoichiometry of the reduction of ascaridol.

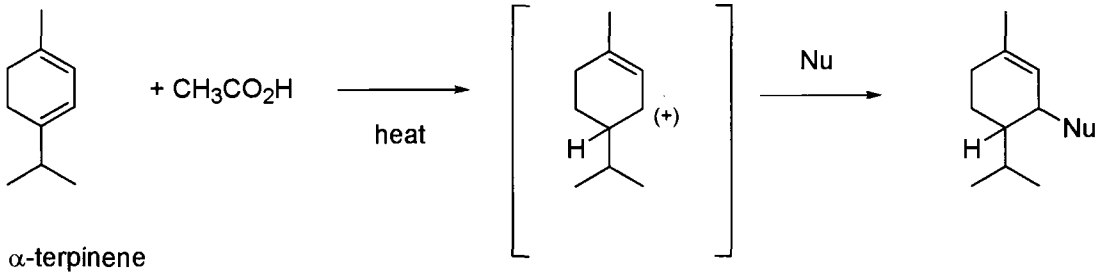
As mentioned above the native Codena Oil" contains approximately 7% by weight of ascardiol, so, for the sake of argument, and to be on the conservative side, let us assume a maximum of 10% [almost 50% extra] of ascaridol per L of "Codena Oil". The density of the oil is about 0.9g/mL, thus the estimate that each L of oil contains a maximum of 90g of ascaridol.

Calculations and Analysis.

90g of ascaridol represents 90/168=0.54 moles. The minimum amount of zinc required to reduce 90g of ascaridol: 65 x 0.54 = 35g. The minimum amount of acetic acid needed is 2 x 60 = 120 x 0.54 = 65g.

Hussain's procedure called for 100g of zinc dust and 750mL {785g} of acetic acid to reduce 500mL of essential oil. This compares to the minimum calculated amount of 32g of acetic acid and 17g of zinc. In other words 20 times excess of acetic acid and almost 6 times excess zinc was employed by Hussain.

The above analysis of the quantities of acetic acid and zinc used in the reduction suggests the opportunity of reducing the amount of these chemicals while achieving the desired result. The purpose of these experiments were to determine the minimum amounts of these reagents that can be used that would still give essentially complete removal of the ascaridol. It was also important that the rate of reaction not be slowed down significantly relative to the time prescribed by Hussain or that significantly higher reaction temperatures would be needed to complete the reduction in a reasonable time since higher temperatures may initiate a number of acetic acid catalyzed reaction of other components in the mixture, for example α-terpenine



α-terpinene

Scheme 2.4.5. Acid catalyzed reaction on α-terpinene.

It was anticipated that reducing the zinc and acetic acid levels to the stoichiometric amounts might not be feasible, however even modes reductions would have a significant impact on the overall cost of the process.

For example, even a four-fold reduction in the amount of acetic acid compared to that used by Hussain [still almost a 6 fold excess!] would result in a saving of about 1.2L of acetic acid per L of "Codena Oil" to be reduced. A 50% reduction in the amount of zinc used [3 times the required amount] would save 100g of zinc per L of oil to be reduced.

RESULTS AND DISCUSSION
CHAPTER 2

RESULTS AND DISCUSSION

In order to optimize the method for eliminating ascaridol from the Codena Oil we began first by showing that we could successfully repeat the Hussain experiments. Once this was established a variety of experiments were planned in which we would systematically reduce either the zinc or acetic acid concentration, or both. The effect of temperature on the reduction reaction would also be investigated. The result of each experiment was analyzed by G.C.

2.2.1 Reduction of ascaridol 26 to 27.

In order to do this we needed samples of the pure ascaridol and the reduction product **27**. A small sample of ascaridol was supplied by Codena. The reduction or the peroxide bridge in **26** was carried out following the Hussain procedure. The isolated yield of pure **27** was more than 85%. The ^{1}H NMR of both ascaridol, **26** (**Figure 2.2.1**), and the diol, **27** (**Figure 2.2.2**), are reproduced on the following pages. The key, easily visible change, was the shifting of the AB pattern for the alkene hydrogens from $\delta = 6.5$ in **26** to $\delta = 5.6$ in **27**.

The Original Codena Oil was first put through the GC in order to determine which constituent were present in the oil. Four major peaks were observed: α-terpinene, *p*-cymene, limonene and ascaridol, **Figure 2.2.3 & Figure 2.2.3a**. Their retention times are 8:80, 10:20, 10:45 and 22.23 respectively.

Figure 2.2.1 ^{1}H NMR of ascaridol, **26**

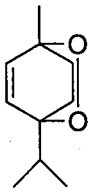
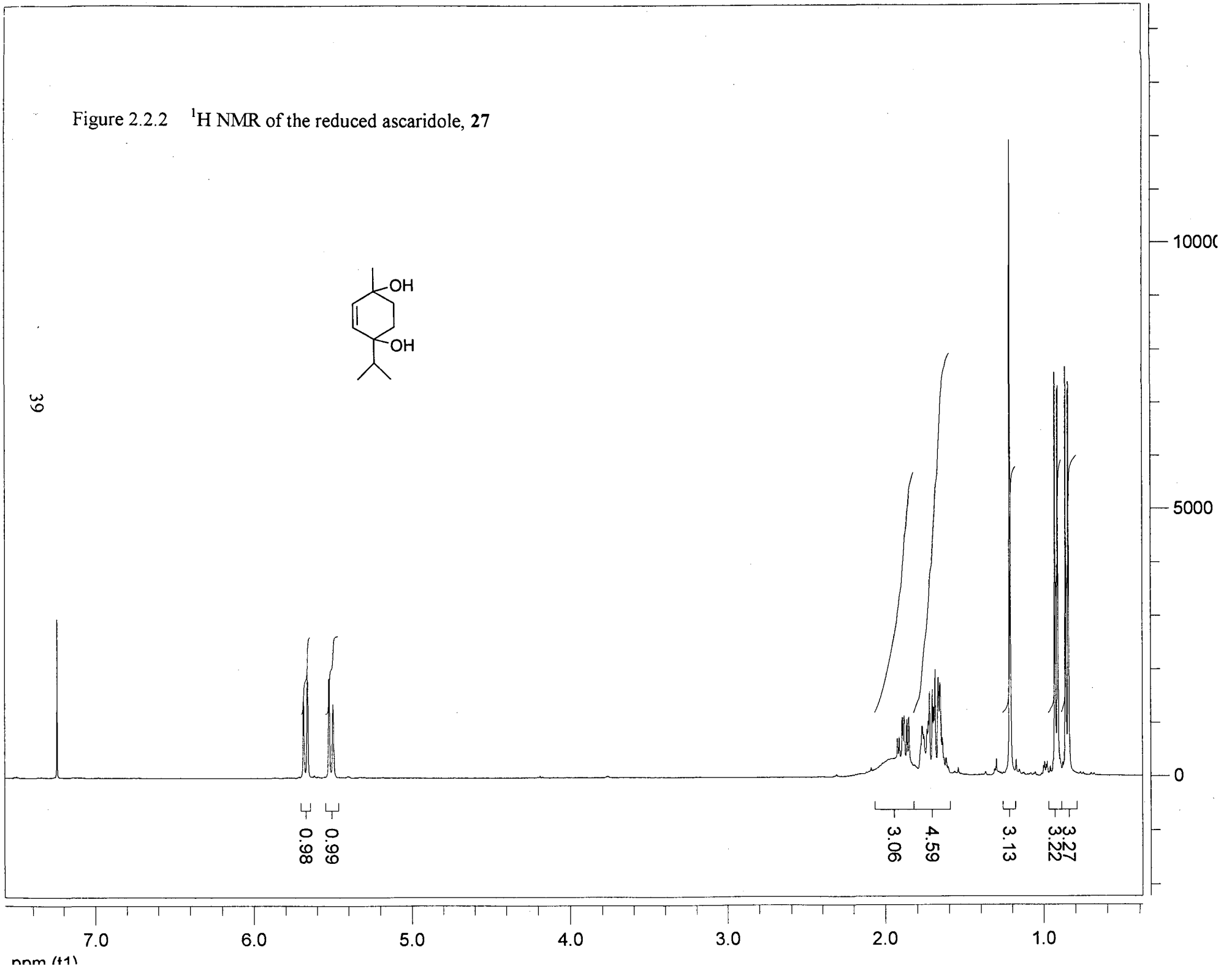


Figure 2.2.2 ^{1}H NMR of the reduced ascaridole, **27**



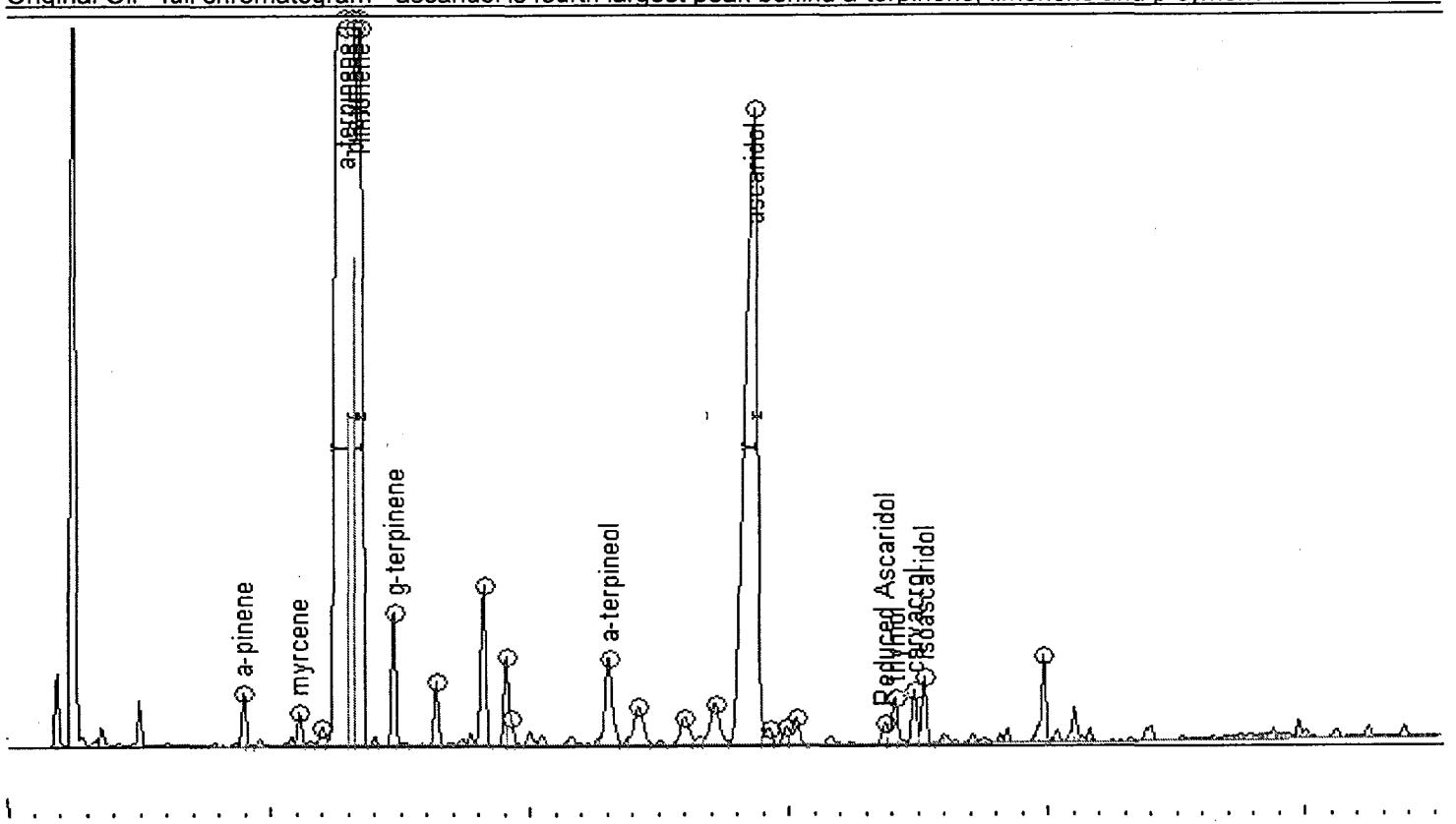


Figure 2.2.3. GC of full chromatogram of Original Codena Oil

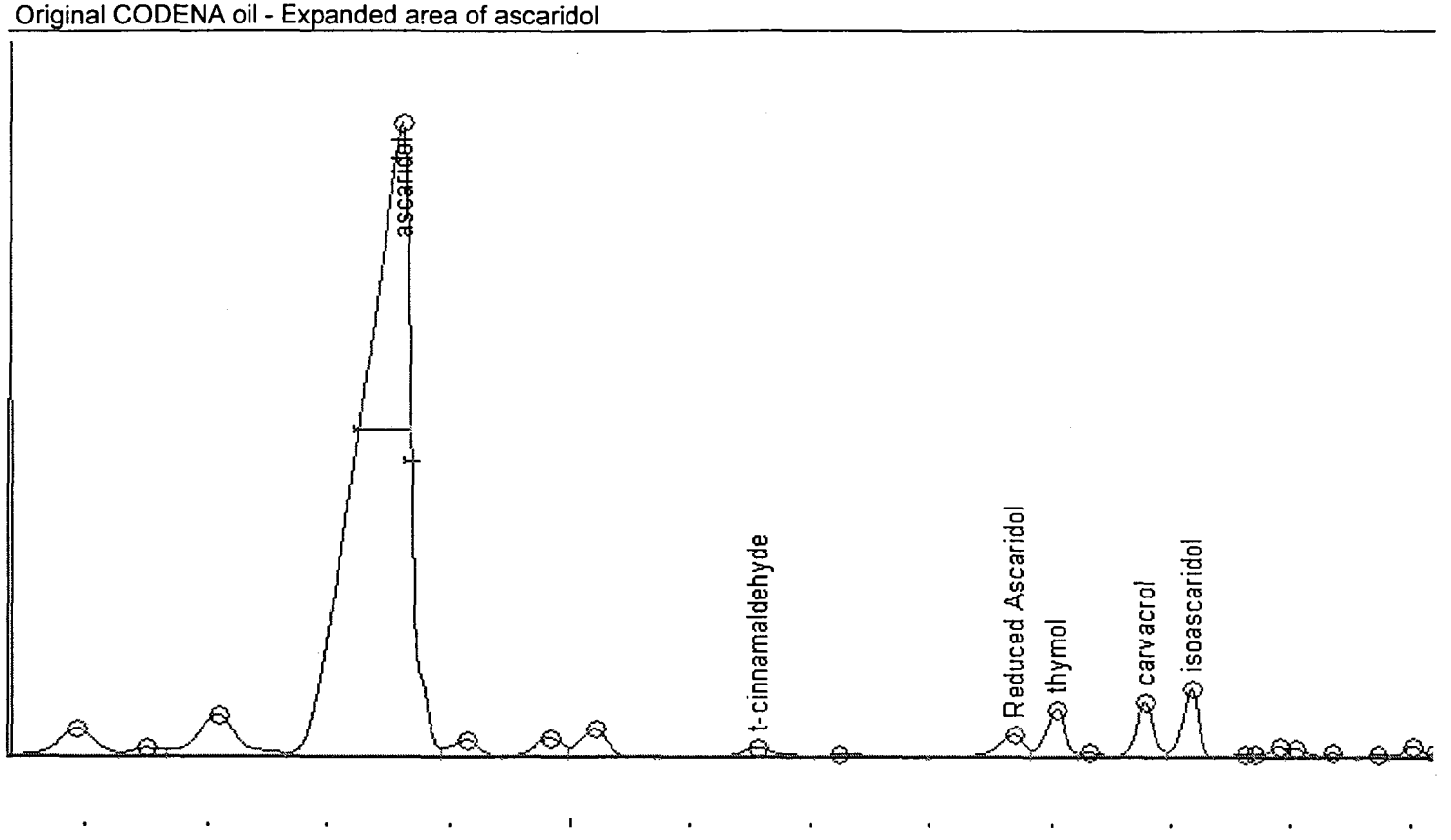


Figure 2.2.3a. GC of Original Codena Oil with expansion of the ascaridol area.

Once the tlc showed that ascaridol was reduced, a sample of the oil was put through the GC. The Codena Oil had been completely reduced due to the disappearance of the ascaridol peak and a new peak eluting at 33.25, that of the diol, **27**, **Figure 2.2.4 & Figure 2.2.4a**.

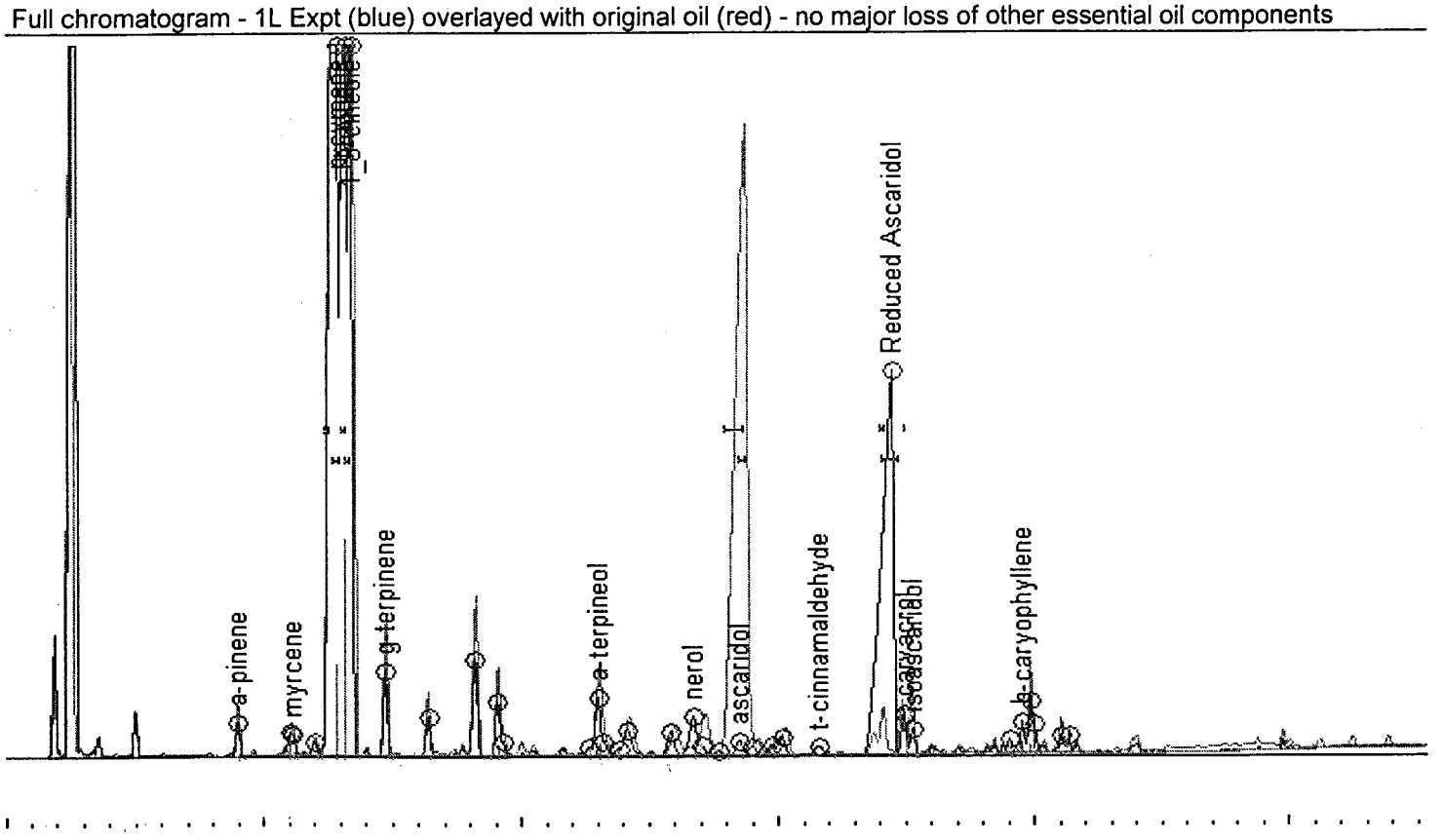


Figure 2.2.4 – GC of the Reduced Codena Oil showing a new peak of **27** and elimination of **26**.

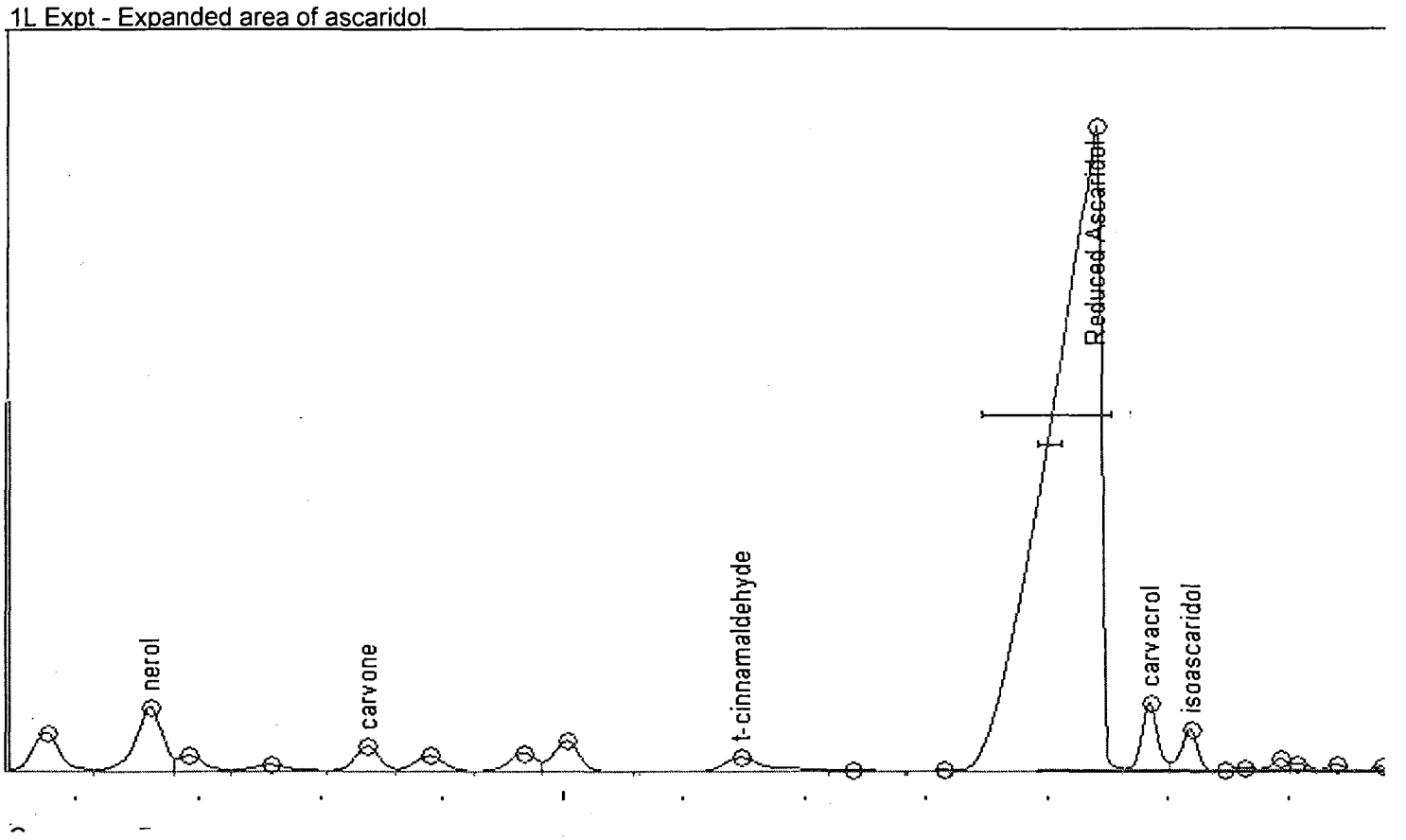


Figure 2.2.4a – Expanded are of the Reduced Codena Oil showing a new peak of **27** and elimination of **26**

The essentially complete reduction of **26** in the Codena Oil could also be verified qualitatively by examining the ^{1}H NMR of the reduction product, **Figure 2.2.5**. The disappearance of the alkene AB doublet at δ 6.5 signaled the disappearance of **26**. The alkene peaks of **27** overlapped with those of other alkene components, such as limonene and the terpenenes in the mixture. Finally, analytical TLC also gave a good indication concerning the status of the reduction of **26** since **26** stained heavily in a unique spot on the plate. Thus the disappearance of that spot signaled that ascaridol was no longer a component of the oil, **Figure 2.2.6**.

Figure 2.2.5. ^{1}H NMR of Original Codena Oil. AB doublet at δ6.6ppm corresponds to **26**.

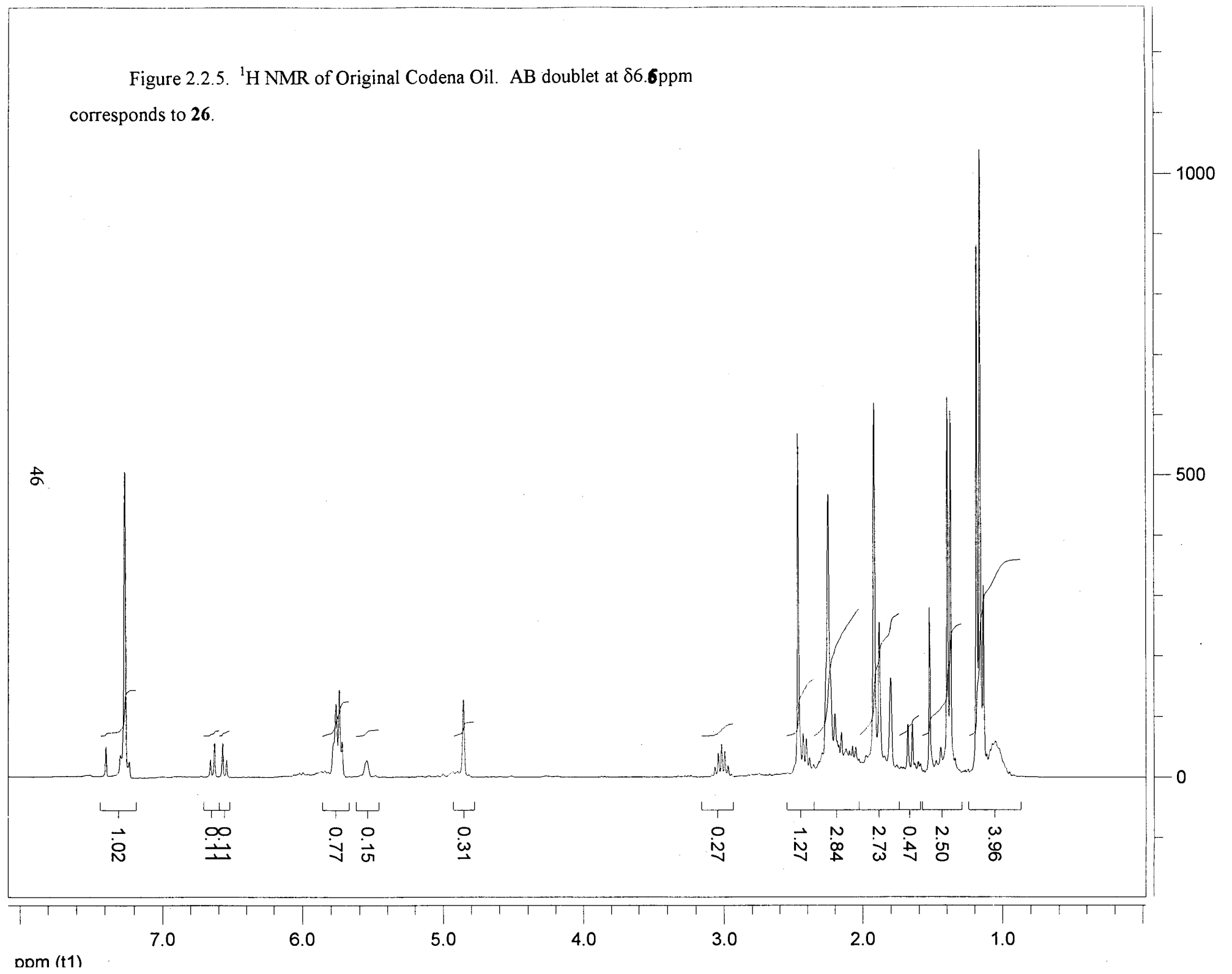
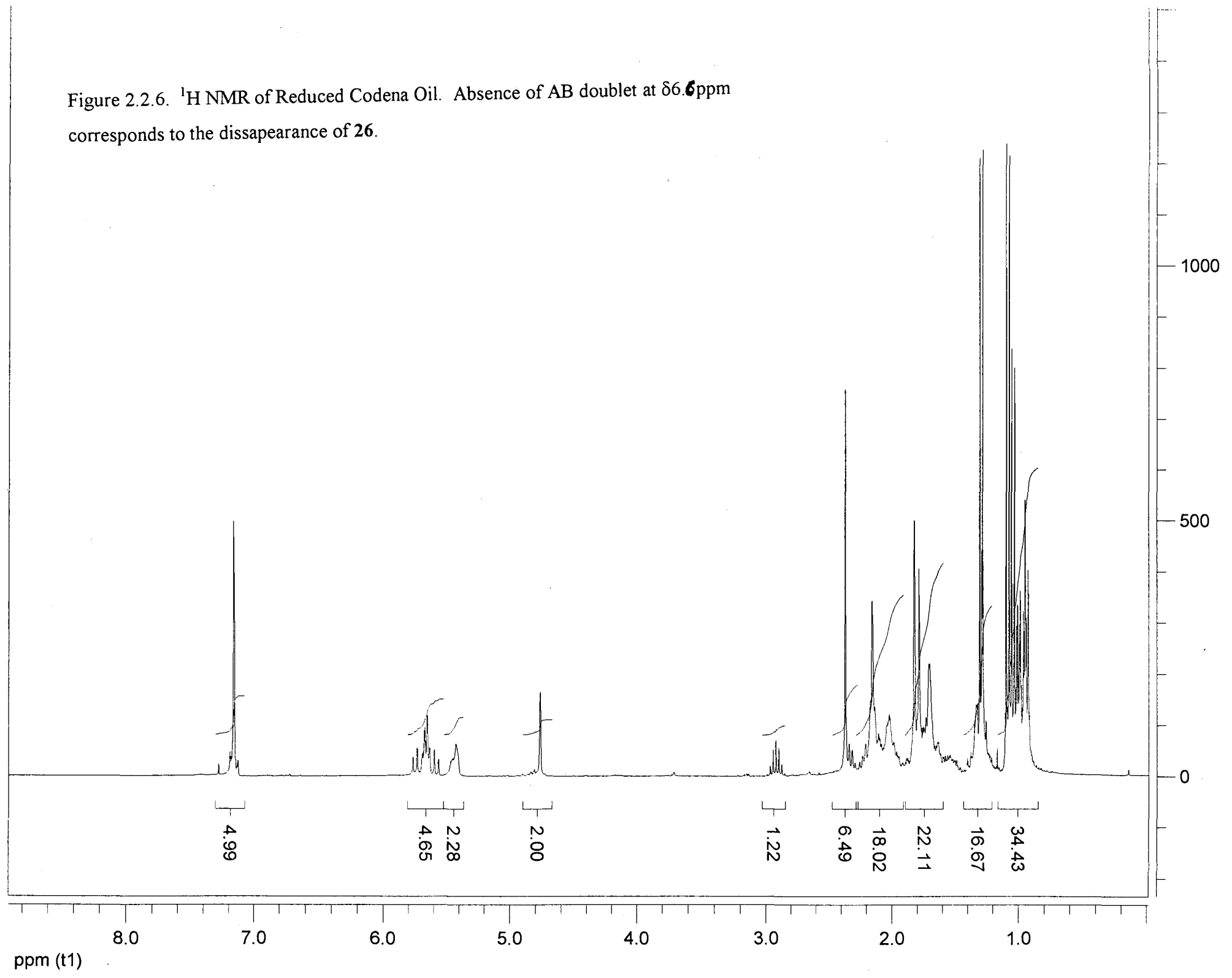


Figure 2.2.6. ^{1}H NMR of Reduced Codena Oil. Absence of AB doublet at δ6.6ppm corresponds to the dissapearance of **26**.



2.2.2 Condition changes to the Hussain procedure starting with quantities of acetic acid and temperature.

Twenty-four experiments were performed in which we varied to the ratio of Codena Oil to acetic acid, Codena Oil to zinc, and then both. We also varied the temperature and tested the question of whether a stronger acid, such as sulfuric acid or p-toluene sulfonic acid would catalyze efficiently the peroxide reduction and water could be used in place of acetic acid as the proton donor. The results pertaining to the variance in acetic acid and temperature are shown in Table 1.

Table 1. Effect of various quantities of acetic acid and temperature on the reduction of Codena Oil with zinc.

Trial	Essential Oil (g)	Zinc dust powder (g)	Glacial acetic acid (mL)	Temp (°C)	Time (15hrs)	GC – Ascardol left (%)
1	54.71	10.64	75	35-40	1.5	0
2	53.87	9.84	30	42	15	0.01
3	50.26	10.16	15	35-40	15	3.9
4	50.26	10.07	7.5	35.40	15	4.7
5	53.18	10.14	15	35-40	15	4.0
6	1L	100.07	750	42	15	0
7	52.18	9.96	15	50	15	0.3
8	50.04	9.96	15	50	12	0.4
9	51.56	10.09	15	90	3	0
10	49.87	10.06	15	60	7	0

The original Hussain protocol utilized 750mL of acetic acid and 100g of zinc for 500mL of Codena Oil, that is a 1:1. ratio of Codena Oil to acetic acid. The reaction was carried out with vigorous stirring at room temperature to 42°C for 15h for complete ascaridol removal. After completion of the reaction the mixture was dilutes with 1 L of ethyl acetate and washed with several times with 10% NaOH solution. The mixture was then dried over anhydrous sodium sulfate and the ethyl acetate was removed under reduced pressure. The weight of recovered oil was greater 90%. The product was analyzed by GC. The disappearance of ascaridol was carefully monitored. The "%

ascaridol remaining" in **Table 1** refers to the % of the original ascaridol present in the Codena Oil. Thus The GC of Trial 2 showed that 4.7 % of the original ascaridol remained.

The optimization experiments were carried out at about the one-tenth scale with the same results (**Table 1**, Exp .1). When the weight ratio of acetic acid to Codena Oil was changed from the above 1.5 to 1 to about 0.6 to 1 (Exp.2) while keeping the other variables constant. GC analysis of the product showed the ascaridol content in the oil had decreased to an acceptable 0.01%. Further decrease of the weight to weight ratio of acetic acid to Codena Oil to 0.3 and 0.15 resulted in 3.9 and 4.7% ascaridol content after 15 hours of reaction time. A further decrease of the ratio to 0.3 and 0.15, Exp 4 and 5 respectively resulted in 3.9%.

We then investigated whether an increase in reaction temperature would result in complete removal of the ascaridol within the 15 hours reaction time frame using a 1 to 0.3 ratio of Codena Oil to glacial acetic acid. When the zinc / acetic acid treatment was carried out at 50°C the ascaridol content in the oil was reduced to 0.4% after 12hours and 0.3% after 15 hours of reaction time, Experiments 5 and 6. A further increase in reaction temperatures to 60°C and 90°C, (Experiment 7 and 8) resulted in removal of all the ascaridol in 7 and 3 hours, respectively.

Unfortunately, the GC of the product when the reaction temperature was either 60 or 90 ^{0}C showed the formation of a significantly larger amount of material with longer retention time components than present in the native Codena Oil itself and in the lower temperature reduction. The formation of these higher molecular weight impurities significantly changed the composition of the Oil, which might also change its insecticidal properties. Should such a process be used, Codena would need to show the efficacy of the "new oil" in a new series of bioassays. Additionally they would need to identify the structures of the new components and show that they are non-toxic in order to get approval for use from Agriculture Canada or the U.S. Environmental Protection Agency.

Thus the use of temperatures beyond 40-45°C in this process was considered as not acceptable.

At this time we were asked by Codena to scale up our best procedure and produce 1L of reduced Codena Oil for field-testing purposes. Based on the above results we conservatively chose a 0.75:1 ratio of acetic acid to Codena Oil and successfully produced almost 1L of product whose GC analysis confirmed the complete removal of ascaridol (Exp. 4).

2.2.3. Condition changes to the Hussain procedure, varying ratios of zinc to oil.

At this point we felt that we had established the most favorable ratio of acetic acid to oil that gave essentially complete removal of ascaridol in less than 15 hours at temperatures below 42 ^{0}C in order to avoid undesirable reactions involving other components in the Codena Oil. We then began to investigate the possibility that we could reduce significantly the ratio of the zinc powder to the essential oil. The results of these experiments are shown in **Table 2**.

Table 2. Reductions of the Codena Oil with varying ratios of zinc to oil.

Trial	Essential Oil (g)	Zinc dust powder (g)	Glacial acetic acid (mL)	Temp (°C)	Ratio CodenaOil to zinc	GC – Ascardol left (%)
12	50.	3.7	37.5	31	14	3.1
13	50	5	37.5	31	10	2.6
14	50	6.0	45	35	8.3	0.9
15	1L	80	750	35-40	12.5	0.6

When the ratio of Codena Oil to zinc was changed from the 5 : 1 ratio to 14 to 1 (Expt. 12) and as with the Expt. 6, keeping the other variables constant, the GC analysis showed that the ascaridol content in the oil had only reduced to 3.1% even after 15 hours.

Since Experiment 12 showed that 3.1% ascaridol, it prompted us to decrease the Codena Oil to zinc 10: 1 (Exp 13). After our 15 hours period and even with the change to the ratio of zinc to oil, ascaridol still remains at am unacceptable high level of 2.6%.

Once more we needed to increase the amount of zinc, this time we decided on a ratio of 8.3 to 1 Codena Oil vs zinc (Exp 14). After our 15 hour period, the GC analysis showed that 0.9% of ascaridole still remained, which though a much improved result, is over maximum limits.

Once again, we were asked by Codena to produce a further 1L of reduced Codena Oil. Based on the above result we presumed it would require further increases to reduce the ascaridole to acceptable levels. We therefore chose a ratio Codena Oil to zinc of 12.5 to 1 (Exp 15). After a 15h reaction time, tlc indicated that all the ascaridol had been converted, however GC analysis showed a residual amount of 0.6% ascaridol. The oil was sent to Codena for further analysis and to also see if the level of ascaridol in the oil was acceptable for their experimentation.

2.2.4. Condition changes to the Hussain procedure, varying acid catalyst solutions.

Our final set of experiments was to see if adding a catalytic amount of a strong acid such as HCl would help with our time factor, we also wanted to see if a milder acid such as sulfonic acid would also be feasible to use. Both concentrated and different dilutions of acid were investigated with the following results in **Table 3**.

Table 3 Reduction of Codena Oil with strong acid catalysis.

Trial	Acid used (1 mL)	Temp. (°C)	Ascaridol remaining (%)
16	Conc. HCl	30-35	0
17	10 %HCl	30-35	0
18	5 % HCl	30-35	0
19	Conc. HCl	0-5	1.1
20	10% HCl	20	0
21	5% HCl	0-5	1.3
22	1% HCl	0-5	0
23	0.5% HCl	20-30	0
24	TsOH	20-30	0

In each experiment an additional acid catalyst was added to a rapidly stirred mixture of 1 g of zinc powder and 10 mL of Codena Oil, dissolved in 7.5 mL of acetic acid at the indicated temperature for 1 hour. In the first experiment in this series 1 mL of conc. HCl (Exp16), after 1 hour a tlc was done and showed no ascardol remaining. We were pleased when the GC analysis confirmed that no ascaridol remained however late eluting peaks were showing and even more problematic was that less of the diol compound was being formed, and higher peaks for other, early eluting compounds, were observed. These were not identified

Seeing the results of experiment 16 with concentrated HCl, we then decided to use a diluted 10% (Exp. 17) and 5% concentration of HCl (Exp. 18) solution. Interestingly the GC analysis confirmed that after 1 hour of reaction time, no ascaridol remained in either experiment. Substantial amounts of late eluting peaks were observed for both experiments. The reduction of ascaridol was clearly greatly accelerated by the stronger acid, but his was accompanied by the late eluting by-products. Since the HCl catalyzed reductions were complete in less than one hour at 30-35°C, we decided to carry out the reduction at 0°C hoping that the lower reaction temperature might avoid the side reactions. A number of reactions were attempted using 1 mL of concentrated HCl, then 10 %, 5%, 1% and 0.5% HCl per 50mL of Codena Oil., 37.5 mL of acetic and 10 g of zinc powder. For each of these experiments GC analysis showed that virtually all of the

ascaridol had been reduced within one hour of reaction time. Unfortunately, the late eluting peaks continued to be present to essentially the same extent.

We had carried out a number of experiments with both concentrated and different dilutions of HCl and variations in the temperature with results consistently showing late eluting peaks. We decided to change our focus to the somewhat weaker acid, p-toluenesulphonic acid. In Experiment 20, we added 100mg of this acid to the typical reaction mixture. Even though the GC analysis showed no ascaridol remaining there was a formation of a new unidentified peak at 14 min., little or insignificant amounts of the diol is produced, probably due to subsequent dehydration and the late eluting peaks are again present.

At this point a decision needed to be made whether to continue the optimization experiments. For example, we considered the use of acids such as chloroacetic and trichloroacetic acid with acidity intermediate between acetic and hydrochloric acid or toluenesulfonic acid to catalyze the reduction but perhaps not the formation of the higher boiling or more polar by-products.

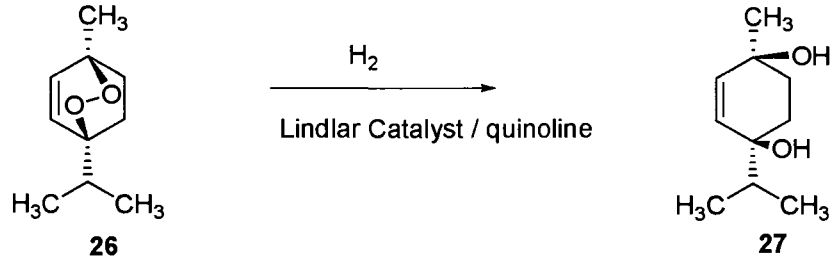
Overall, the now optimized procedure on a scale of 50g of Codena Oil, required only 15 mL of acetic acid, 10g of zinc with temperature of 30-40°C and a reaction time of 7 hours. We had been able to reduce the cost only with respect to the amount of acetic acid and processing time. This information was sent to Codena for field-testing. Additionally, more than 1L of Codena Oil was reduced using this protocol and sent to Codena for continued field-testing.

The decision was made to abandon this approach since this careful evaluation of the 'optimized' process by Codena and its consultants indicated that it could not produce material at an economically viable cost.

2.5.5 Catalytic reduction of the Codena Oil with a Poisoned Lindlar Catalyst.

The chemical literature contains many examples of the reduction of carbon-carbon triple bonds to cis alkenes using a Lindlar catalyst poisoned with quinoline.[15] Even typical Introductory Organic texts[16] describe the use of this catalyst to prepare a

variety of cis-disubstituted alkenes starting with acetylenic precursors. Interestingly, a search of the literature did not give examples involving the reduction of endoperoxides such as in ascaridol with such a catalyst system. Nevertheless, it was considered highly probable that this "poisoned" catalyst could be useful for converting the ascaridole, **26**, in the Codena Oil to the diol, **27**, Scheme 2.1.6. Furthermore, since this catalyst did not seem to reduce cis -alkenes to alkanes, it was expected that the various alkenes and dienes presents in the Codena Oil would be left unchanged. Also, since there are no reports of the reduction of carbonyl groups with this catalyst we were quite certain that any compounds containing this functionality would remain unchanged.



Scheme 2.4.6. Lindlar catalyst reduction of ascaridol.

The concept of a catalytic reduction, if successful, could offer many important economic advantages. The consumable reagent required in this reaction is hydrogen. No solvents would be involved since reduction would be carried out on the neat Codena Oil. It was also anticipated that the catalyst could be recovered and recycled. The equipment required would consist of a vessel that could withstand a reasonable amount of pressure. Such equipment is available in typical laboratories as a small scale Paar Hydrogenation Apparatus. Since hydrogenations are routinely carried out on a large scale in industry, it should not be difficult to find a supplier capable of carrying out a catalytic hydrogenation on the 100 -500 L batch scale eventually envisaged by Codena.

RESULTS AND DISCUSSION

An initial experiment using 80mg of pure ascaridole in 3 mL of hexanes with 2mg of Lindlar (Palladium on calcium carbonate, loading: 5wt. %, poisoned with lead from the Aldrich Company) catalyst poisoned with 1 drop of quinoline showed that reduction of **26** to **27** occurred at room temperature, under 5 and 15 atmosphere pressure of H_2. Importantly, the NMR of the crude product showed that there was no over-reduction, that is the double bond in **27** had remained intact. As mentioned above it was important to verify this since the Codena Oil contains many components with double bonds and such compounds need to be unchanged during the ascaridol removal so that the activity of the reduced Codena Oil is not compromised.

Based on the above results, we reduced 10g of Codena Oil with 150mg of Lindlar catalyst and 0.6*u*L of quinoline at atmospheric pressure. We were able to carry out the reduction on the neat Codena Oil and thus eliminate the solvent hexane whose subsequent removal *in vacuo*, may also remove portions of the more volatile components of the oil. The complete reductive cleavage required reaction time of 48h. A G.C. analysis, traces appended, showed that the reduced oil was essentially equivalent to the reduced oil except for the disappearance of ascaridol and the appearance of a peak due to the diol **27**.

2.2.6 Recycling of the Catalyst.

Two sets of experiments have been carried out, each on approximately 10g of Codena Oil, and approximately 200mg of Lindlar catalyst poisoned with 0.6*u*L of quinoline. When the hydrogenation was carried out at 5 atmosphere of hydrogen pressure (75 psi), the reaction time was approximately 24 hours (**Table 4**). The catalyst was recovered by decanting about 95% of the reduced Codena Oil. A new charge of 10 mL of Codena Oil was added and the reduction was repeated. The catalyst was re-used four times. There appears to be a small loss of activity in the fourth run as judged by the increased reaction time and the observation that a trace of ascaridol remained in the mixture. It is however not certain if this was due to the aging of the catalyst or a different

level of stirring and agitation during the reduction process. The product of each experiment was analyzed by GC to ensure the integrity of the reduced product.

Table 4. Results for the Lindlar catalyst procedure for experiment 4.

Trial	Essential Oil (g)	Lindlar catalyst (g)	Quinoline (μL)	Time (hrs)	Atmosphere H_2	GC – Unreacted ascaridol (%)
3	10.00	.150	0.6	48	5	0.0
4i)	10.28	.200	0.6	24	5	0.0
4ii)	8.79			24	5	0.0
4iii)	8.90			24	5	0.0
4iv)	9.55			48	5	0.2

A second set of reductions was carried out at 15 atmosphere of hydrogen pressure (225psi). The higher pressure reduced the reaction time to 4 hours (**Table 5**). There was no loss of robustness of the catalyst even after five runs with the same catalyst. The goal of reducing process time was achieved by increasing atmospheric pressure. Again, the 1H NMR and the GC of the product obtained indicated that no over-reduction of 5 or any of the alkene components of the Codena Oil had occurred.

Table 5. Results for the Lindlar catalyst procedure for experiment 5.

Trial	Essential Oil (g)	Lindlar catalyst (g)	Quinoline (μL)	Time (hrs)	Atmosphere H_2	GC – Unreacted ascaridol (%)
5i)	10.10	.180	0.6	4	15	0.0
5ii)	8.99			4	15	0.0
5iii)	9.18			4	15	0.0
5iv)	8.70			4	15	0.0
5v)	10.10			4	15	0.0

A sample of Codenal Oil prepared by this procedure was submitted to Codena for analysis. Since the Lindlar catalyst contained lead carbonate, there was obvious concern that the product might contain unacceptably high levels of lead or palladium . The Codena analysts reported the presence of measurable quantities of lead in the Oil. Additionally, they isolated from one of the batches a quantity of quinoline. The presence of both these contaminants was considered problematic since it might create problems with the regulatory agencies and prevent the commercialization of the Codena Oil produced by this process.

The following changes in the preparation if the catalyst allowed us to eliminate the presence of quinoline in the product. The commercial Lindlar catalyst was suspended in hexane, 10 mL per 100 mg of catalyst. About 200 μL of quinoline was added and the mixture was stirred for 15 min. The solid catalyst was allowed to settle and the hexane was carefully decanted. The hexane washing was repeated twice more prior to the use of the catalyst. The hexane treatment effectively removed any quinoline that was not bound to the catalyst. Fortunately, the activity of the catalyst was not compromised by this treatment.

We reasoned that any suspended or even dissolved catalyst might be removed from the decanted, reduced Codena Oil by either stirring it with 100 mg of the filter aid Celite, or with 100 mg silica gel and then filtering to remove the Celite or silica and any adhered material.

These procedures were carried on the usual small batch of Codena Oil. As expected the Oil produced in this manner contained no quinoline and only minute traces of lead and palladium. The silica gel treatment was found to be superior to the Celite for the lowering of the lead and palladium content in the reduced oil.

CONCLUSIONS

The removal of ascaridole, **26**, from the Codena Oil can be carried out effectively either with finely ground zinc in acetic acid or with Lindlar catalyst poisoned with small amounts of quinoline. Both procedures produce only the diol **27**. Neither procedure appears, based on GC analysis, to have an effect on any of the other components in the Codena Oil. Greater than 2L quantities of material produced by the zinc/acetic acid method were shipped to Codena for formulation and evaluation as an insecticide in greenhouses. This material has been approved for use by both the Canadian and US authorities for the use as an organic pesticide.

These reductions of the Codena Oil with the Lindlar catalyst are economically superior to that involving zinc and acetic acid since it uses hydrogen and the recyclable Lindlar catalyst. Most importantly this procedure does not require the use of other solvents and reagents and thus generates a minimum amount of chemical waste. Codena Inc. has been taken over by AgraWest of Davis California in the past year. The latter company continues to be interested in the commercialization of this product and is planning to produce as much as 5000-10,000 L of native Codena Oil from fields located in the province of Quebec.

EXPERIMENTAL
CHAPTER 2

GENERAL: Mass spectra were obtained by means of a Kratos Concept II 2H and the GC were done on a Kratos Concept II 2H. ^{1}H NMR and ^{13}C NMR were recorded on a Bruker AMX-400, Bruker AMX-300 spectrometers with shifts reported in ppm. The multiplicities of the NMR signals were reported using the following abbreviations, singly or in combinations: singlets (s), broad singlet (br s), doublet (d), triplet (t), quartet (q) and multiplet (m).

Thin layer chromatography (tlc) were carried out using Kieselgel 60 F_{254} precoated 0.25mm plates. Visualization of the spots was facilitated by UV irradiation followed by charring of a tlc which has been dipped in a molybdate (2.5g) and cerric sulphate hydrate (1.0g) in a solution of sulfuric acid (10mL) in distilled water (90mL). Silica gel 270-400 Mesh was used for flash chromatography.

All experiments are done with:

Glacial acetic acid – EDM™ (2.5L)
Zinc dust, <10 micron, 98+% - Aldrich Chemicals

GC – 5890 Hewitt Packard
Column DB-5 60MX0.53mm
Max temp 300°C prolonged
Heat carrier set at 20psi, oven 75°C
Auto-injector 6890 series:
2 micron injector (240°C)
Detector (250°C)
Temperature gradient 75°C augmenting 2°C /min to 110°C held for 15 minutes then augmenting 10°C /min to 300°C (5 minutes

Treatment of native Codena Oil with zinc and acetic acid:

Zinc powder was added slowly at ambient temperature to a solution of the essential oil and acetic acid contained in a 3L three-neck flask equipped with a mechanical stirrer and thermometer. The reaction is somewhat exothermic and the temperature of the mixture was maintained between 32-35°C by adjusting the rate of the addition. After all of the zinc had been added in small quantities, the temperature was

allowed to cool to room temperature and the mixture was stirred overnight. GC analysis showed a retention time identical to the diol (**27**). No other significant changes were seen in the GC of the product when compared to that of the starting material.

The mixture was filtered to remove the excess of zinc and the filtrate was washed with a cold 10% NaOH solution and the product was extracted with ethyl acetate. The combined ethyl acetate fractions were dried with anhydrous $MgSO_4$. The solvent was removed on a rotary evaporator under reduced pressure to give a yellow oil.

Optimization experiments:

These experiments were carried out as above using 50 g of native Codena Oil. The variations in the amount of acetic acid, zinc and the addition of other acid catalysts is given in the text and the various Tables.

The Lindlar catalyst reduction.

Experiments were done neat and under the described conditions all samples were put through GC and ^{1}HNMR.

Experiment 4:

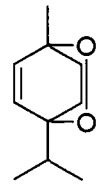
The first set of experiments was done using a 10g sample of Codena Oil, 150mg of the Lindlar catalyst, 6 μL of quinoline stirred under a 5 atmosphere pressure of H_2 (75 psi) in a Paar Hydrogenation Apparatus. After 1 hour a sample of the Codena Oil was analysed by tlc, then by GC. Once GC showed no ascaridol peak.

Workup consisted of filtering the Lindlar catalyst using Whatman® 70mm filter paper (allowing at least 1-2mL of the oil to keep the catalyst covered). Once the filtering was done the catalyst was re-used in a proceding experiment until GC showed a peak were the ascaridol is. ^{1}H NMR was performed on all samples.

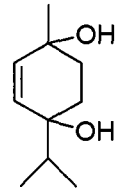
Experiment 5:

The second set of experiments was done using a 10g sample of Codena Oil, 150mg of the Lindlar catalyst, 6 μL of quinoline stirred under a 15 atmosphere pressure of H_2 (225psi) in a Paar Hydrogenation Apparatus. After 1 hour a sample of the Codena Oil was analysed by tlc, then by GC. Once GC showed no ascaridol peak.

Workup consisted of filtering the Lindlar catalyst using Whatman® 70mm filter paper (allowing at least 1-2mL of the oil to keep the catalyst covered). Once the filtering was done the catalyst was re-used in a proceeding experiment until GC showed a peak were the ascaridol is. ^{1}H NMR was performed on all samples.



^{1}H NMR: (400HMz, $CDCl_3$): δ 6.46 (d, J=10Hz, 1H), 6.38 (d, J=10Hz, 1H), 2.09-1.87 (m, 4H), 1.6 (d, J=10Hz, 2H), 1.34 (s, 3H), 0.97 (d, J=7Hz, 6H)
^{13}C NMR: (400HMz, CDCl3): δ 136.40, 133.056, 79.81, 74.38, 32.14, 29.57, 25.61, 21.42, 17.26, 17.18



^{1}H NMR: (400HMz, $CDCl_3$): δ 5.67 (d, J=10Hz, 1H), 5.50 (d, J=10Hz, 1H), 1.921.84-1.87 (m, 3H), 1.76-1.65 (m. 4H), 1.21 (s, 3H), 0.89 (d, J=7Hz, 6H)
^{13}C NMR: (400HMz, CDCl3):
δ 137.13, 131.90, 71.827, 69.44, 37.12, 34.71, 28.93, 26.98, 17.49, 16.33

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CHAPTER 3

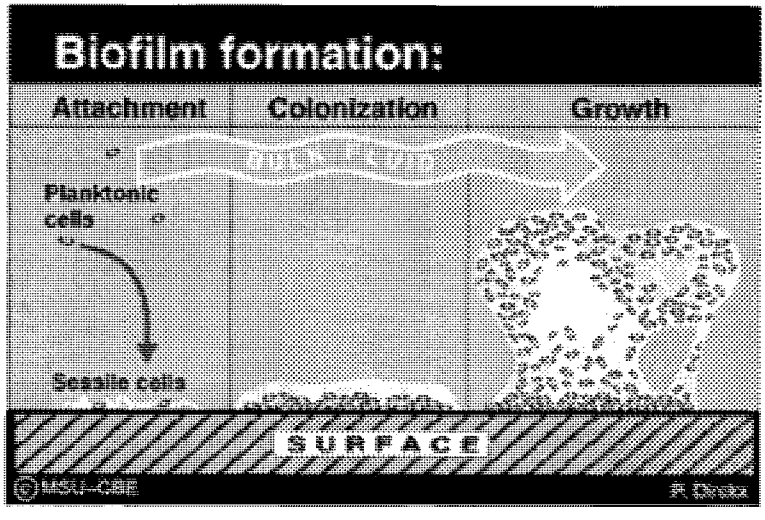
Chapter 3.

Background

It appears that, at some point in time, we have all had an encounter with biofilms. Common examples of biofilms are the plaque that forms on your teeth and causes them to decay, a gram-positive bacteria *Streptococcus pneumoniae*[1] or even that "gunk" that clogs your drain. If you are a nature enthusiast and have swam in a river or lake you may have encountered biofilms by slipping on those "slimy" rocks.

Biofilm forms when bacteria adhere to surfaces in aqueous environments and begin to excrete a slimy, glue-like substance that can anchor them to all kinds of material – such as metals, plastics, soil particles, medical implant materials, and tissue. A biofilm can be formed by a single bacterial species, but more often, biofilms consist of many species of bacteria, as well as fungi, algae, protozoa, debris and corrosion products. Essentially, biofilm may form on any surface exposed to bacteria and some amount of water. Once anchored to a surface, biofilm microorganisms carry out a variety of detrimental or even beneficial reactions (by human standards), depending on the surroundings environments conditions, **Figure 3.1.1**.

Figure 3.1.1 Biofilm formation.



Microbial biofilm on surfaces cost millions of dollars annually in equipment damage, product contamination, energy loss and medical infections such as prostatitis, biliary tract infections, and urinary catheter cystitis. Conventional ways of destroying the bacteria (antibiotics or disinfectant) are often ineffective against bacteria biofilm. The huge dose of antimicrobial required to remove the biofilm is usually environmentally unfriendly and medically impractical, as the dose of the antibiotic used to kill the bacteria could also kill the patient. Research must now look towards new strategies based on better understanding of how bacteria attach, grow and detach themselves to their hosts. Recently, there has been a tremendous interest in biofilm research where the ultimate aim is to prevent, control or even eradicate.

Medical Applications

Research has found that 40% of the cell wall proteins of the bacteria in biofilm are different from the planktonic cell[2]. This becomes problematic since antibiotics are primarily effective on dividing cells. It appears that bacterial cells in biofilm are embedded in a matrix of polysaccharides. This gives them an anaerobic advantage and along with low metabolic activity and the ability to extend time without division protects them from the effects of antibiotics[3].

Understanding the language of bacteria

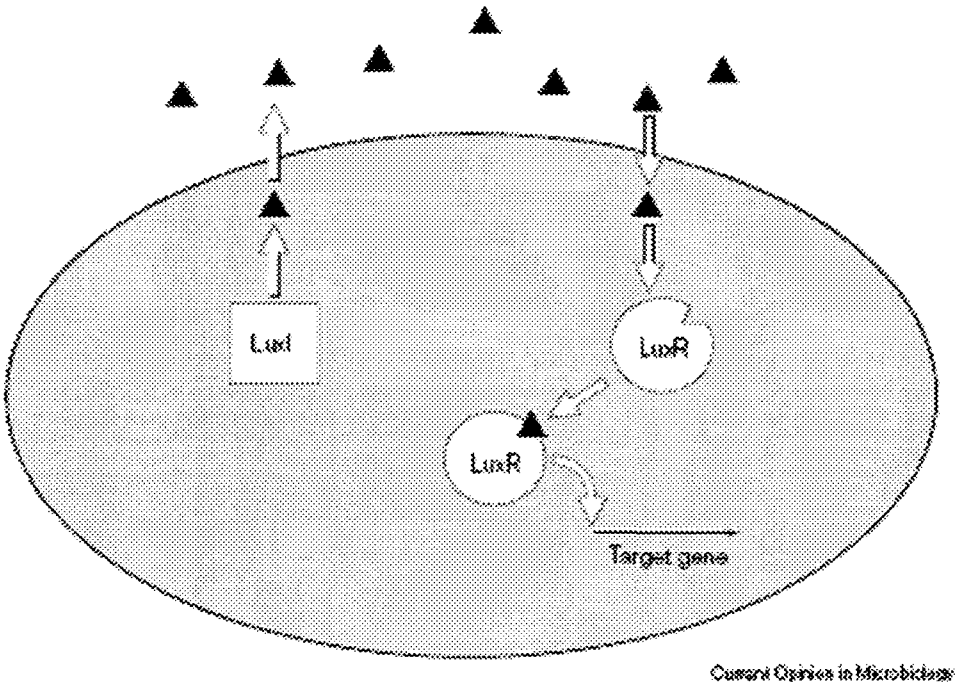
Bacteria produce and release chemical signals, such as pheromones or autoinducers, in search of similar cells in their close surroundings. This is also called "cell-cell communication." Other bacteria release the same autoinducers in response. One-cell organisms in effect become multi-cellular organisms and can act together. People or animals may have bacteria that cause some serious infectious diseases and yet not be infected unless the number of bacteria is large enough; that is to say, unless the quorum has been reached[4]. Bacteria use quorum sensing to regulate some form of gene expression by sensing their population density via signaling compounds, which are then excreted into the environment. Quorum sensing plays a role in enabling bacteria to build

complex community structures. Both Gram-negative and Gram-positive bacteria use this quorum sensing method in response to cell density, however they do so in different manners.

Gram-negative vs Gram-positive bacteria

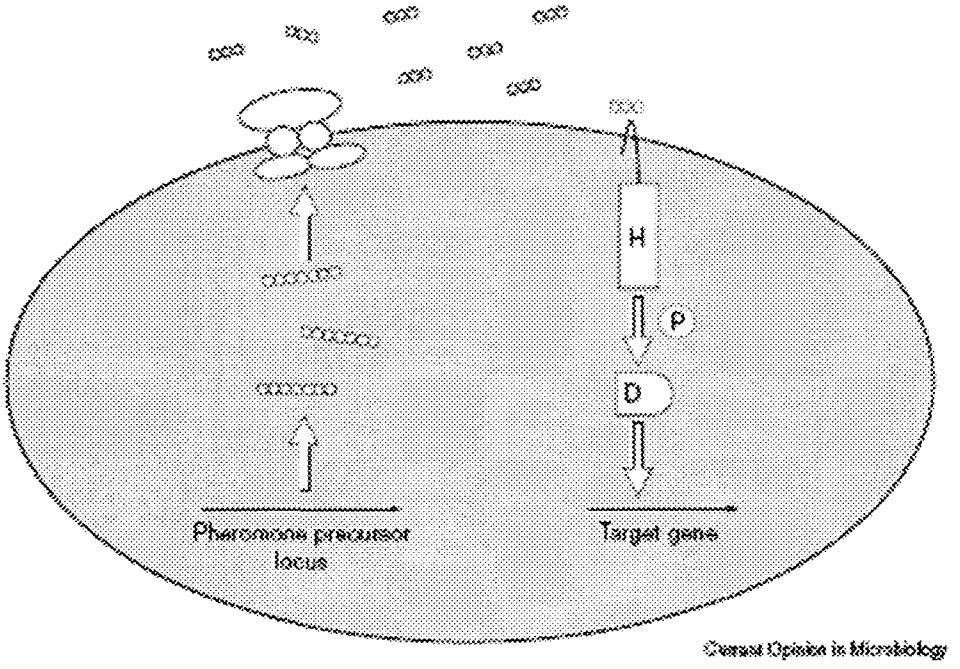
Gram-negative bacteria is the biological term for a bacteria that do not stain dark blue or violet by the Gram staining process; instead this organism will appear more of a red or pink. Many species of Gram-negative bacteria cause disease in a host organism. This pathogenic capability is usually associated with certain components of Gram-negative cell walls, in particular the lipopolysacharides[5]. Bassler from Princeton explains that several species[6] of Gram-negative bacteria use a LuxI/LuxR quorum sensing system[7]. LuxI-like autoinducers synthase are responsible for production of specific homoserine lactone (HSL) autoinducers where they are then freely diffused across the bacterial membrane. When HSL reach a specific concentration, it binds itself to the LuxR-like protein and together they activate transcription of the target genes, **Figure 3.1.2**.

Figure 3.1.2 LuxI/LuxR quorum sensing



Gram-positive bacteria, on the other hand, contain an extra cell membrane rendering them blue when tested using Gram's method. The difference in classification is largely based on a difference in the bacteria's cell wall structure's use of a peptide. These bacteria rely on a different autoinducer, such as a pheromone. Extracellular pheromones are recognized by membrane bound two-component sensor kinase proteins. These sensor proteins autophosphorylate on a histidine residue and subsequently transfer the phosphoryl group to responding regulators such as aspartate residues. Once the phosporylation done a response regulator protein either activates or represses transcription of specific target genes, **Figure 3.1.3**.

Figure 3.1.3. Peptide quorum sensing.

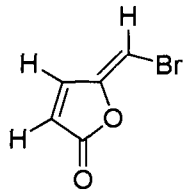


These two examples are important features of biofilms as it indicates that bacteria increased their chances of survival and proliferation by virtue of communal living.

Better understanding of the genetic basis of biofilm phenotype(s) formation allow new strategies in research and development on how to prevent and/or inhibit this microbial formation phenomenon from occurring. Since both Gram-negative and Gram-positive bacteria use quorum sensing, it would seem logical to look towards developing new compounds to prevent quorum sensing mechanism from occurring.

Preliminary work on the biofilm problem.

An important focus of biofilm research is the discovery of natural compounds that inhibit biofilm formation without affecting the natural growth of the cell. Furanone compounds produced by the Australian macroalga *D. Pulchra* have been shown to possess inhibitory properties as well as interfering with complex surface dependent phenomena such as swarming motility and biofilm formation of *Serratia liquefaciens*[8]. Natural furanone compounds have a rather limited effect on *P. aerugunosa*[9] when tested individually, however, natural quorum sensing inhibitor compounds can be further modified by means of combinatorial chemistry which is a highly efficient method of generating a large number of analogues for screening purposes. One such synthetic furanone compound is (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone[10]. This halogenated furanone was shown to inhibit quorum sensing in *pseudomonas aerugionasa*[11]and in another study[12] was found that at a low concentration of 60*u*g ml^{-1} it inhibited the biofilm formation of *E. coli* by decreasing it's thickness by more than half[13]. It appears to do so by the suppression of the autoninducer circuit[14].



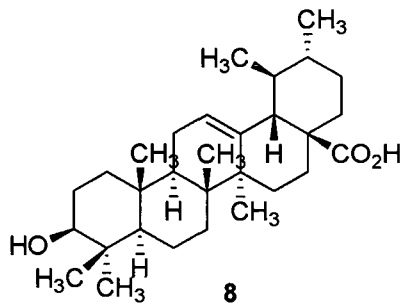
7

The same authors showed that ursolic acid (3β-hydroxy-urs-12-en-28-oic acid), a triterpenoid compound[15] found in the *Diospyros dendo* genus, was considered an important lead because of its relatively low toxicity. The *dendo*, commonly called the

Gabon Ebony and recently found in Costa Rica, has a height of approximately 50 feet; its sapwood is light yellowish-white and the heartwood is uniformly jet black and sometimes streaked with greenish-black markings. This is the black ebony used for at least 5,000 years in making furniture and small decorative items, including musical instruments, piano keys, and billiard cues.

For a long time, ursolic acid was considered to be pharmacologically inactive. Also since ursolic acid is a member of the triterpenoid acid family this suggested that this rather large family of natural products may yield other, possibly more active compounds[16]. In the past its alkali salts (e.g. potassium or sodium ursolates) were exclusively used as emulsifying agent in pharmaceuticals, cosmetics, and food preparation. However, upon closer examination, ursolic acid was found to be medically active both topically and internally. It has anti-inflammatory, anti-tumor (cancer) and anti-microbial properties.

Ursolic acid and its derivatives are constituents of numerous plants which have diversified phylogenetic origins and taxonomic positions. It has been isolated from a protective wax-like coating on apples, pears, cranberries, prunes, and other fruits as well as seaweed.



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Although the exact mechanism of inhibition is not clear, it is suggested that ursolic acid's induction of chemotaxis and motility genes function as a signal which tell the genes to remain too motile for adequate biofilm formation. Chemotaxis is a kind of responsive movement in which body cells, i.e. single cells, bacteria or multicellular organisms, direct their movements according to a certain chemicals in their environment. The critical stage in survival development of bacteria is heavily relied on in either finding

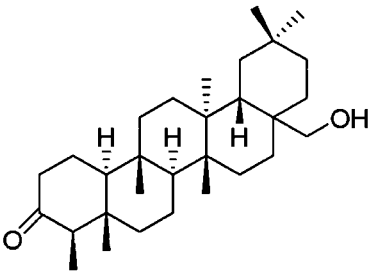
food by swimming towards the highest concentration of food molecules or by fleeing from toxins. The induction of chemotaxis and motility genes during the wrong stage of biofilm development may destabilize the biofilm and prevent the formation of mature biofilm. Even though the results also indicate that motility is not necessary for normal biofilm formation in E.coli in the presence of a conjugation plasmid, it would appear that ursolic acid and furanones such as 7 inhibit biofilm formation by controlling the chemotaxis gene in an oppostite manner and therefore affecting different stages of biolfilm formation.

It is not clear whether the plants produce ursolic acid constitutively or only after infection by a pathogen. Given that ursolic acid induces chemotaxis genes, ursolic acid may be a defense response of the host after the infection of the pathogen. Further study of the surface concentration of ursolic acid on plants and the effect of environmental conditions on its production may be informative.

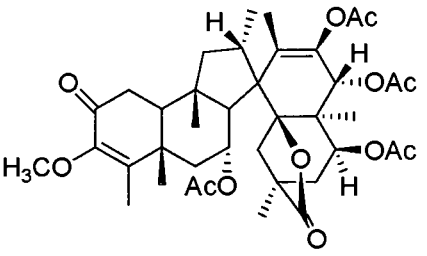
RESULTS AND DISCUSSION
CHAPTER 3

DISCUSSION AND RESULTS

S. Mackinnon reported in her PhD thesis[17] that the bark of *ruptiliocarpon caracoliton*, a rather rare tree endemic to the southwestern part of Costa Rica, contained significant amounts of canophyllol, **9**, a friedelin triterpenoid, in addition to a novel family of triterpenes called spirocaracolitones, **10**.



9, canophyllol

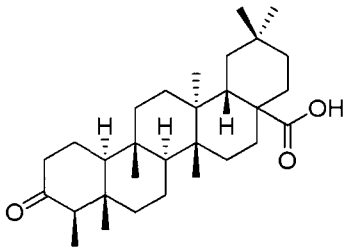


10, a spirocarocolitone

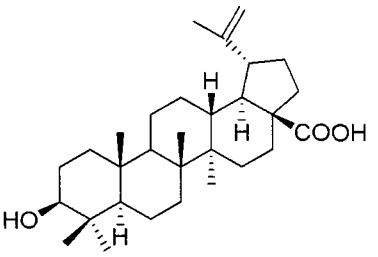
Indeed in subsequent collections canophyllol was found to be the major component constituting as much as 2% of the weight of the bark. Canophyllol is also reported as a constituent of other species such as *Calophylum calaba, Garcinia soicata* from Sri Lanka[18], *Euonymus revelutus*[19], *Maytenus diversifolia*[20,21] and *Triptergium wilfordii*[22].

3-Oxo-canophyllic acid, **11**, a known natural product can easily be prepared by oxidation of the primary alcohol function in canophyllol. Since we had access to a relatively large supply of canophyllol we envisaged creating a small library of 3-oxo-canophyllic acid derivatives for screening as potential biofilm inhibitors and comparing these to the related ursolic acid derivatives[23]. 3-Oxo-canophyllic acid has the basic friedelin structure and a somewhat different shape than ursolic acid. One might argue that it would be unlikely that the 3-oxo-canophyllic acid derivatives would have similar biological activity as the ursolic acid compounds. However if they did then a new family of active compounds would be identified. Earlier work in the group resulted in the

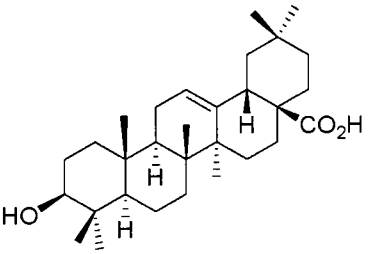
preparation of about 15-20 derivatives of betulinic acid, **12**[24]. Also our group also had access to derivatives of oleanolic acid, **13**. The screening of this library of triterpene acids would allow us to determine whether ursolic acid was unique in its biofilm inhibition activity.



11, 3-oxo-canophillic acid



12, betulinic acid



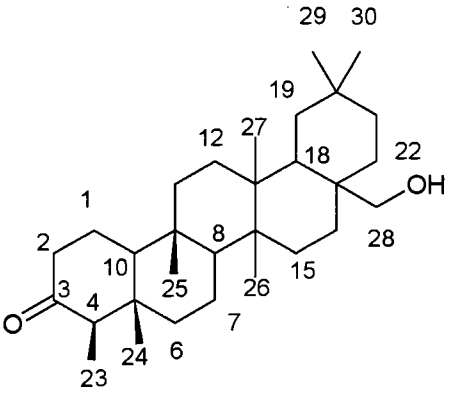
13, oleanolic acid

3.3.1 Canophyllol – Isolation and Purification.

Since canophyllol (**9**) is found in nature in more that one source, we were able to compare the characteristics of our material with what was reported in the literature[19.18.20.21.22]. The EIMS of (**9**) displayed a $[M]^+$ at m/z 442 which is in accordance with the molecular formula $C_{30}H_{50}O_2$. This was established by HRMS. The melting point range was found to be 291-293°C compared to literature value 311°C. Both ^1H and ^{13}C NMR data of (**9**) were in agreement with data given in the McKinnon thesis[17] and in the literature[24].

The ^1H NMR spectrum of (**9**) showed a sharp peak at δ 3.67 (s, 2H) due to the CH_2OH group. The geminal methyl groups of the D ring appeared at 0.66 (s, 3H-29) and 0.72 (s, 3H-30), while the five tertiary methyl singlets from the remaining rings were observed at 0.84 (s, H-23), 0.86 (s, H-24), 0.95 (s, H-25), 0.98 (s, H-27), 1.08 (s, H-26).

The ^{13}C NMR of (**9**) displayed diagnostic signals for the ketone of ring A at δ 213.37 (C-3) as well as the primary hydroxyl function of ring D at δ68.30 (C-28). The numbering in canphyllol is shown below.



3.3.2 Synthesis of canophyllol derivatives.

The C-3 oxo group on ring A and the C-28 hydroxyl group in canophyllol can be modified. It was envisioned that simple chemical modifications at these two positions could provide important, and even novel derivatives of canophyllol, with potential anti-biofilm formation activity.

A series of simple derivatives was synthesized. Thus, the C-28 hydroxyl group function of canophyllol was oxidized to the aldehyde giving canophyllal and 3-oxo-canophyllic acid which, in turn, was then modified by esterification and amide formation at C-28 and acetylation C-3. Other potential changes are shown below, **Figure 3.1.4**. At this stage, the chemistry was deliberately kept simple since we wanted to establish potential bioactivity in the biofilm area. More complex transformations could be undertaken based on the feedback from the bioassays.

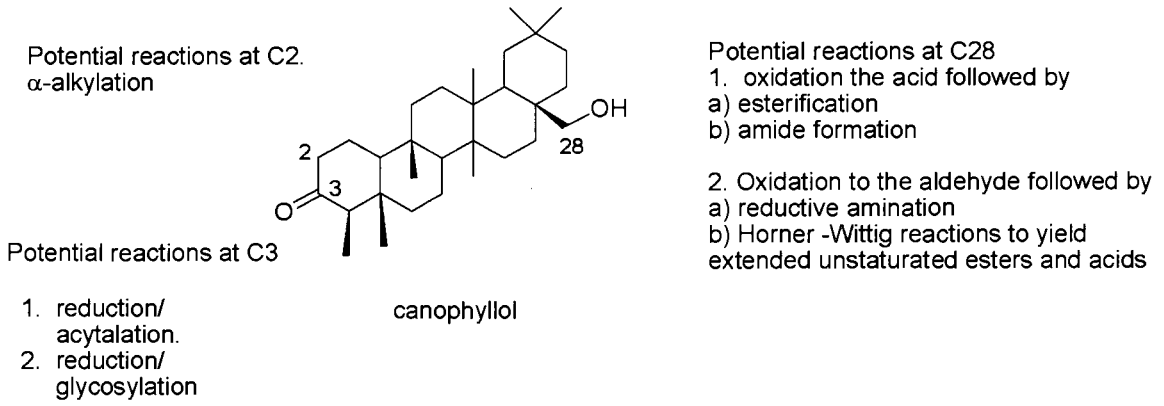
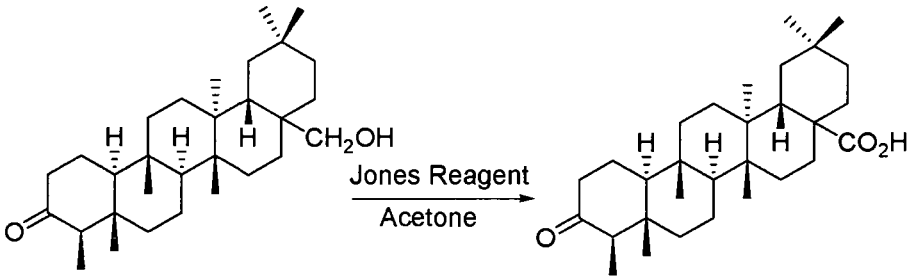


Figure 3.1.4. Derivitazation of canophyllol

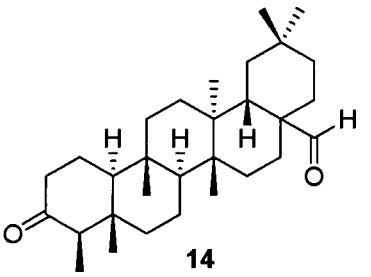
3.3.3 Oxidation of canophyllol.

The key derivative was 3-oxo-canophillic acid, **14**. We anticipated a one-pot preparation using Jones' reagent following the procedure used to convert betulin to betulonic acid[24].



Scheme 3.4.1. Oxidation of canophyllol

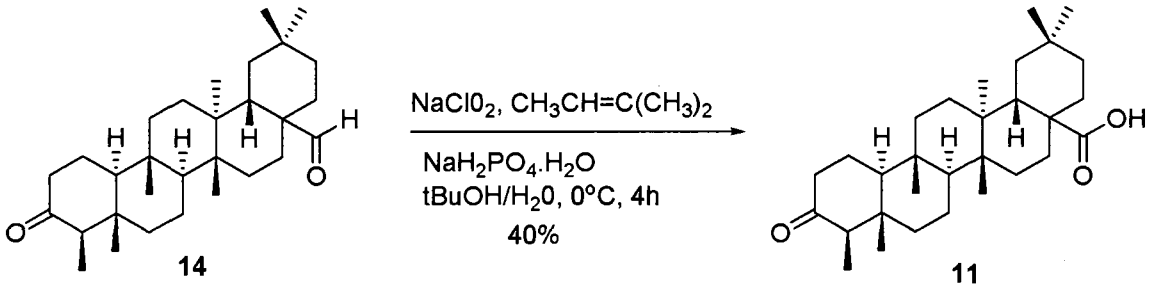
This experiment was first attempted on 100mg of canophyllol. Unfortunately the solubility of canophyllol in acetone is very low and the above quantity failed to dissolve completely in 40mL of acetone. Nevertheless excess Jones' reagent was added and the mixture was stirred vigourously for 15hours. TLC of the mixture showed that the product had contained a considerable amount of starting material remaining. We were able to isolate only 45mg (45%yield) of the partial oxidation product, canophyllal, **14**.



14

Canophyllal (**14**) is also found in nature in more that one source. Once again, we were able to compare characteristics of our product with what was found in literature[18,19,20,21,22]. The EIMS of (**14**) displayed a $[M]^+$ at m/z 440 which is in accordance with the molecular formula $C_{30}H_{48}O_2$. This was established by HRMS. The melting point range was found to be 258-260°C compared to literature value 262°C. Both ^1H and ^{13}C NMR data of (**14**) were in agreement with literature data. The ^1H NMR spectrum of (**14**) revealed the aldehyde proton with a sharp peak at δ 9.67 (s, 1H). The ^{13}C NMR of (**14**) displayed diagnostic signals for the ketone of ring A at δ 213.37 (C-3) as well as the aldehyde function at δ220 (C-27).

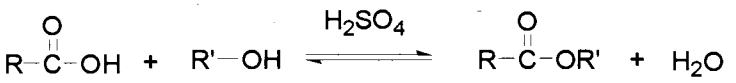
The aldehyde, **14**, was converted to **11** in a 40% yield (130mg) by using sulfamic acid and sodium chlorite as the oxidizing agent. This compound is also found in nature, again in more that one source. Once again, we were able to compare characteristics with what was found in literature. The EIMS of (**11**) displayed a $[M]^+$ at m/z 456 which is in accordance with the molecular formula $C_{30}H_{48}O_3$. This was established by HRMS. The melting point range was found to be 265-268°C compared to literature value 262°C. Both ^1H and ^{13}C NMR data of (**11**) show good agreement with literature data. The ^1H NMR spectrum of (**11**) did not reveal the carboxylic acid proton usually seen between δ10-12 ppm, however the ^{13}C NMR did show evidence of the carboxylic group at δ180 (C-28).



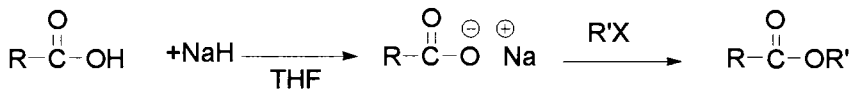
Scheme 3.4.2 Oxidation to canophyllal

3.3.4 Esterification of 3-oxo-canophyllic acid

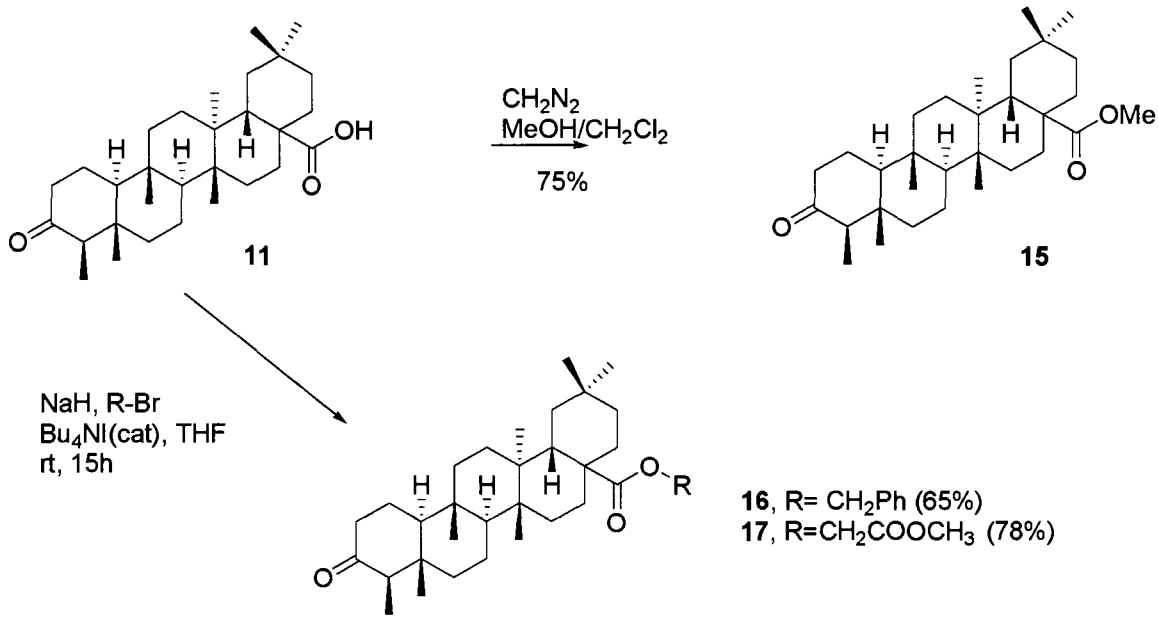
Esterification of acids are traditionally carried out by heating an acid with an alcohol in the presence of a strong mineral acid



They can also be carried out under basic conditions in which the carboxylate is reacted with an alkyl halide. Such reactions are typically carried out in aprotic solvents. We chose to use the latter sequence based on the experience of Eva Punianni[24] that butilinic acid was not readily converted into esters under the acid catalized conditions. In the special case of the methyl esters we used diazomethane.

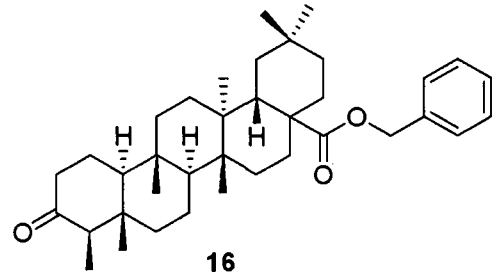


The esterifications under basic conditions were carried out in THF at 0°C using about 1.5 equivalents of NaH per canophyllic acid. After 30 min. the alkyl halide was added and the solution was kept at room temperature overnight. The purified yields of the benzyl ester **16** and the methyl acetoxy ester **17** were 60 and 75 %, respectively. The methyl ester **15** was obtained in 70.5 % isolated yield upon reaction of **11** with diazomethane.



Scheme 3.4.3 Preparation of the ester derivatives of 3-oxo-canophyllic acid

The EIMS of the methyl ester (**15**) displayed a $[M]^+$ at m/z 470 which is in accordance with the molecular formula $C_{31}H_{50}O_3$ which was verified by HRMS. The melting point range was found to be 242-244°C. Diagnostic features of both the ^1H and ^{13}C NMR spectra are the methoxy hydrogens of the C-28 ester at δ3.11 ppm and the carbon-13 of the ketone function in ring A at δ 213.37 (C-3). The methyl ester carbons were found at δ178, and δ51.2

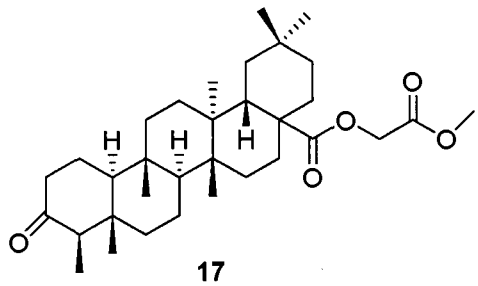


16

The benzyl ester was obtained as a white solid in a 60% yield following the general procedure. The EIMS of (**16**) displayed a $[M]^+$ at m/z 546 which is in accordance with the molecular formula $C_{37}H_{54}O_3$. This was established by HRMS. The melting point range was found to be 202-204°C. Both ^1H and ^{13}C NMR data of (**8**) are stated below.

The ^1H NMR spectrum of (**16**) revealed an AB pattern for the diastereostopic benzylic hydrogens of C-31 at δ5.01 and 5.03 ppm respectively.

The ^{13}C NMR of (**16**) displayed diagnostic signals for the ketone of ring A at δ 213.37 (C-3) and well as the carboxy function of C-28 at δ178, along with the benzylic carbon at δ109.2



17

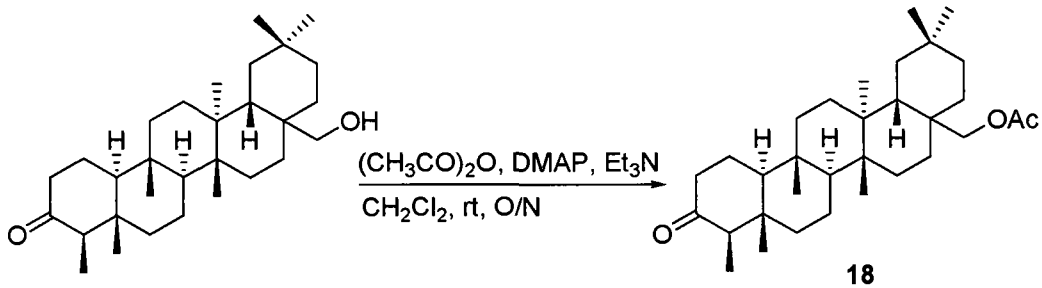
The reaction of 3-oxo-canophyllic acid with methyl bromoacetate under the same reaction conditions described for (**16**) resulted in the formation of methyl acetoxy canophyllate (**17**) as a white compound in a 75% yield with the mp of 178-180°C.

The EIMS of (**17**) displayed a $[M]^+$ at m/z 528 which is in accordance with the molecular formula $C_{33}H_{52}O_5$. This was established by HRMS. The melting point range was found to be 265-268°C. Both ^1H and ^{13}C NMR data of (**17**) are stated below.

The ^1H NMR spectrum of (**17**) revealed a large singlet integrating to three protons at d3.73 (s, 3H) attributable to the methylenic protons signal and a doublet at δ5.55 signaling the diastereotopic protons of the CO_2-CH_2-CO_2 part of the ester moiety.

The ^{13}C NMR of (**17**) displayed diagnostic signals for the ketone of ring A at δ 213.11 (C-3) and well as the carboxy function of C-28 at δ178.1 and C-31 carboxy function at δ168.51.

3.3.5 Synthesis of the acetoxy derivative of canophyllol (18)

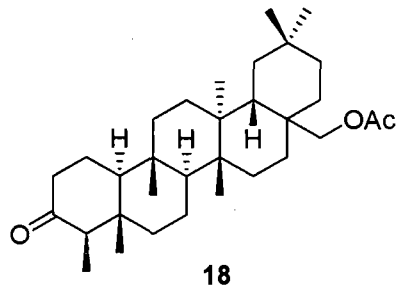


18

Scheme 3.4.4 Preparation of canophyll-28-acetoxy

The reaction of canophyllol with acetic anhydride, DMAP, Et_3N in CH_2Cl_2 at room temperature resulted in the formation canophyll-28-acetoxy (**18**) as a white compound in a yield of 67% with the mp of 158-160°C.

The EIMS of (**18**) displayed a $[M]^+$ at m/z 484 which is in accordance with the molecular formula $C_{32}H_{52}O_3$. This was established by HRMS. The melting point range was found to be 158-160°C. This is a new compound and both ^1H and ^{13}C NMR data of (**18**) are stated below.

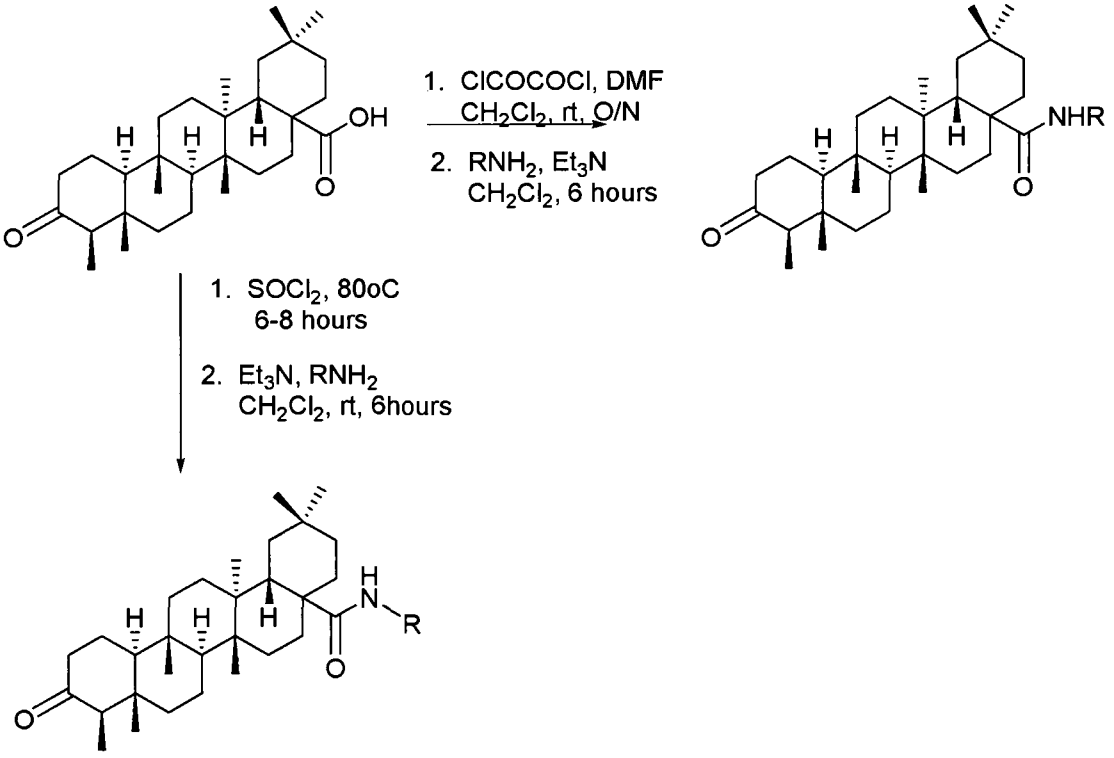


18

The ^1H NMR spectrum of (**18**) revealed a large singlet integrating to three protons at δ2.03 (s, 3H) attributable to the methelenic protons signal and a doublet of doublets at δ4.01 (dd, J=13Hz, 13Hz, 2H) signaling the diastereotopic protons of the CH_2OCOCH_3 part of the compound.

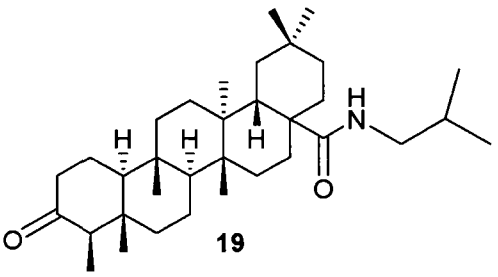
The ^{13}C NMR of (**18**) displayed diagnostic signals for the ketone of ring A at δ 213.11 (C-3) and well as the carboxy function of C-31 at δ178.1 and C-28 acetoxy function at δ168.51.

3.3.6 Preparation of amides of 3-oxo-canophyllic acid



Scheme 3.4.5 Preparation of amides of 3-oxo-canophyllic acid

Two methods were used to synthesize the amides of 3-oxo-canophyllic acid in good yields. Our first method involved treating (**11**) with oxalyl chloride and a few drops of DMF in CH_2Cl_2 to afford the 3-oxo-canophyl-28-oyl chloride, which was then coupled with the appropriate amine in the presence of Et_3N in CH_2Cl_2 at rt, after work up furnished the corresponding amide.



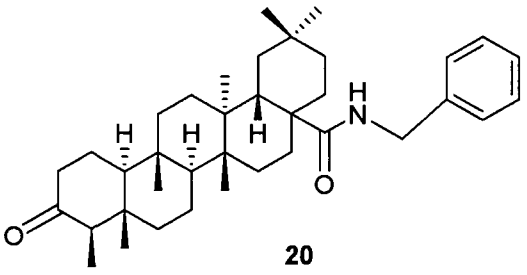
19

The amide was synthesized by reacting 3-oxo-canophyl-28-oyl chloride with isobutylamine under the same reaction procedure described above to furnish N[3-oxo-canophyl-28-oyl]-isobutylamine as a white solid, m.p. 208-210°C in 50% yield after silica gel column chromatography.

The EIMS of (**19**) displayed a $[M]^+$ at m/z 511 which is in accordance with the molecular formula $C_{34}H_{57}NO_2$. This was established by HRMS. This is a new compound and both ^1H and ^{13}C NMR data of (**19**) are stated below.

The ^1H NMR spectrum of (**19**) displays a triplet at δ5.91 (t, J=5.8Hz, 1H) characteristic of the CONH moiety. Also displayed is the diastereotopic protons of $NHCH_2CH(CH_3)_2$ which show two multiplets at δ3.07 (dd, J=16Hz, 7Hz, 1H)and δ3.00 (dd, J=16Hs, 7Hz, 1H).

The ^{13}C NMR of (**19**) displayed diagnostic signals for the ketone of ring A at δ 213.11 (C-3) and well as the amide function of C-28 at δ177. 6



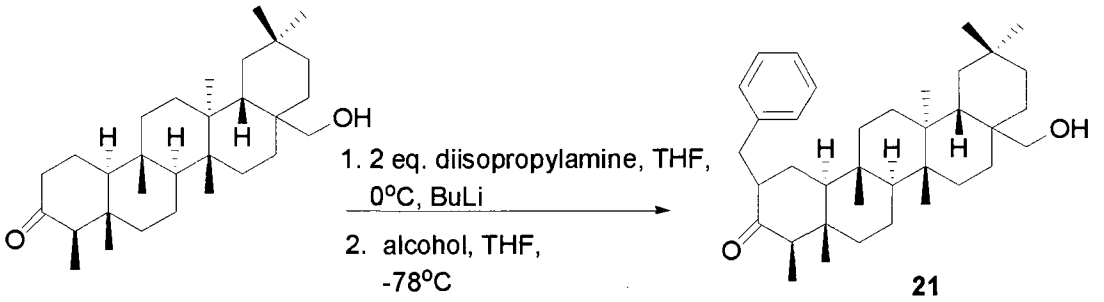
20

After two attempts to synthesize N[3-oxo-canophyl-28-oyl]-benzylamine (**20**) from 3-oxo-canophyllic acid, we decided to use the simpler second route. It consisted of reacting (**20**) with thionyl chloride to produce the 3-oxo-canophyl-28-oyl chloride, which was then coupled with the benzylamine in the presence of Et_3N in CH_2Cl_2 at rt.

The EIMS of (**20**) displayed a $[M]^+$ at m/z 545 which is in accordance with the molecular formula $C_{37}H_{55}NO_2$. This was established by HRMS. The melting point range was found to be 265-268°C. This is a new compound, however, gave 8mg of a mixture in a 13.5% yield, eventhough the 1H NMR is not clean, peaks relating to the amide moiety are visible. ^1H NMR data of (**20**) is stated below.

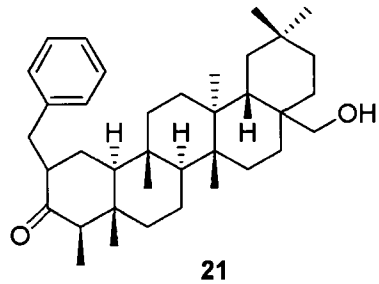
The ^1H NMR spectrum of (**20**) displays a broad singlet peak at δ6.04 characteristic of the CONH moiety. Also displayed is the diastereotopic protons of $NHCH_2$ which show a doublet at δ4.4 (d, J=4.6, 2H).

3.3.7 Preparation of alkylated canophyllol derivatives at position C-4.



Scheme 3.4.6 Alkylation of position C-4 of canophyllol

We now focused on the C3-C4 position of canophyllol in order to synthesize new derivatives, 4-benzyl-canophyllol. The thermodynamic compound, 4-benzyl-canophyllol, **21**, was prepared using the standard procedure of diisopropylamine, THF, BuLi, the alcohol being (**9**), alkyl halide along with the reaction condition of –78°C.



21

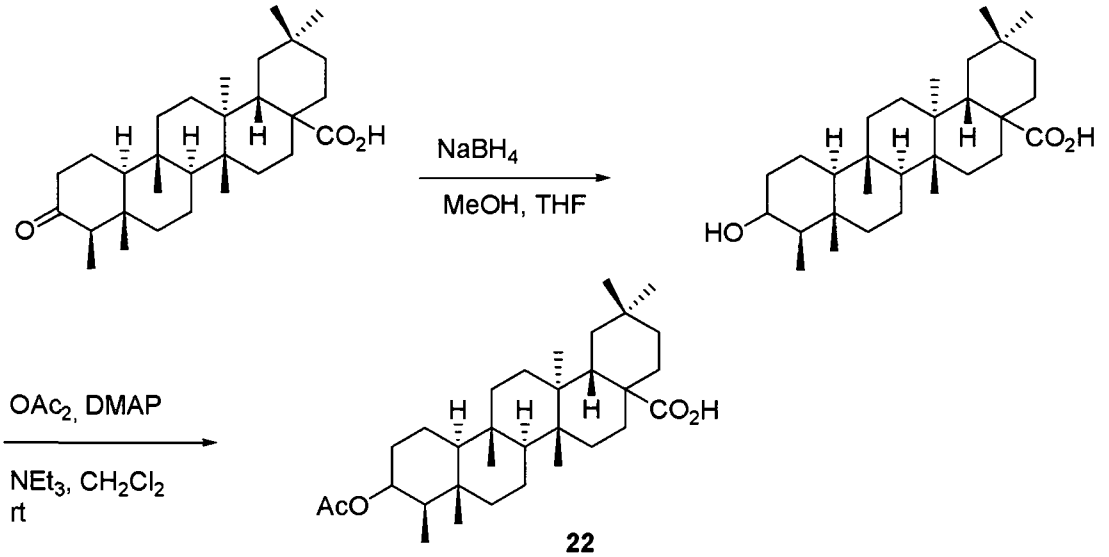
The alkylated alcohol (**21**) was synthesized from the described procedure to afford a white fluffy powder, m.p. 238-240°C in 34% yield (54mg) after silica gel column chromatography.

The EIMS of (**21**) displayed a $[M]^+$ at m/z 532 which is in accordance with the molecular formula $C_{36}H_{53}O_1$ and established by HRMS. However our compound's $[M]^+$ m/z should be 532 as the molecular weight of 532.84g with the molecular formula of $C_{37}H_{56}O_2$. The difference of [M-1]=32 corresponds to the CH_2OH group at the C-28 position and looking at the peak at m/z 273.2 gives us a good analysis that this group seems to "fly off" when it gets hit by the beam of electrons before analysis information starts. The melting point range was found to be 238-240°C. This is a new compound and both ^1H and ^{13}C NMR data of (**11**) are stated below.

The ^1H NMR spectrum of (**21**) displays a singlet at δ3.61 (2H), representing the methelene hydrogens of the benzylic group. Also displayed is a multiplet between d7.3-7.1 representing the benzylic group itself.

The ^{13}C NMR of (**21**) displayed diagnostic signals for the ketone of ring A at δ 213.26 (C-3), the aromatic peaks between d139.15-126.48 and well as the hydroxy group of C-28 at δ68.01.

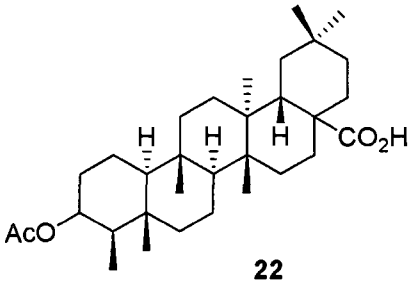
3.3.8 Acetylation of the 3-oxo-canophyllic acid



Scheme 3.4.7 Acetylation of 3-oxo-canophyllic acid

The acetylation of canophillic acid was synthesized from the described procedure to afford 30mg of a white fluffy powder, in 27% yield after silica gel column chromatography.

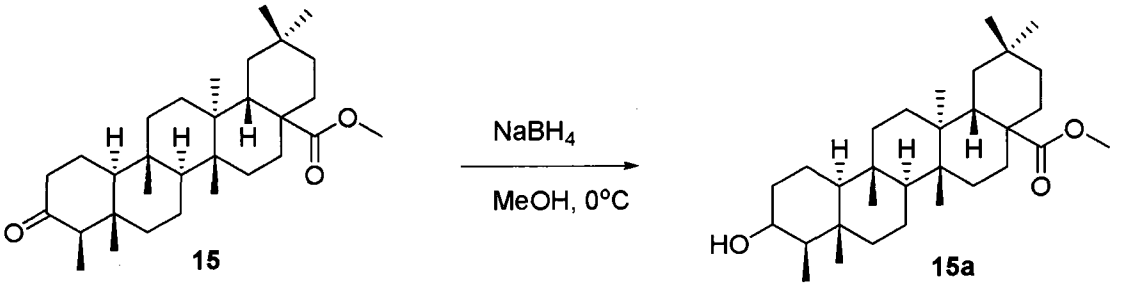
The EIMS of (**22**) displayed a $[M]^+$ at m/z 542 which is in accordance with the molecular formula $C_{35}H_{58}O_4$ and established by HRMS. The melting point range was found to be 282-284°C. This is a new compound and both ^1H and ^{13}C NMR data of (**22**) are stated below.



The ^1H NMR spectrum of (**22**) displays a doublet at δ4.8 (d, J=2.5Hz, 1H) from the hydrogen at C-3 and a singlet at δ2.01 (s, 3H) corresponding to the methoxy group of the acetoxy group at position C-3.

The ^{13}C NMR of (**22**) displayed diagnostic signals for the carboxylic group at C-28 of the D ring at δ 184.97, the C=O function at δ170.91 of the acetoxy group at position C-3 as well as the C-3 carbon at δ74.56

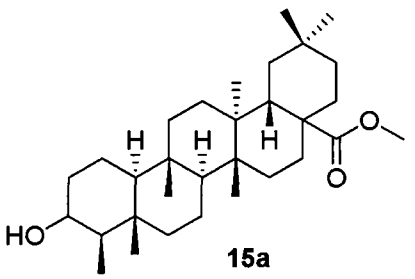
3.3.9 Reduction of the methyl ester analogue



Scheme 3.4.8 Reduction of the Methyl Ester analogue

The reduction of the methyl ester was synthesized from the described procedure to afford 20mg of a white fluffy powder, in 40% yield after silica gel column chromatography.

The EIMS of **15a** displayed a $[M]^+$ at m/z 472 which is in accordance with the molecular formula $C_{31}H_{55}O_3$ and established by HRMS. The melting point range was found to be 287-289°C. This is a new compound and both ^1H and ^{13}C NMR data of **15a** are stated below.

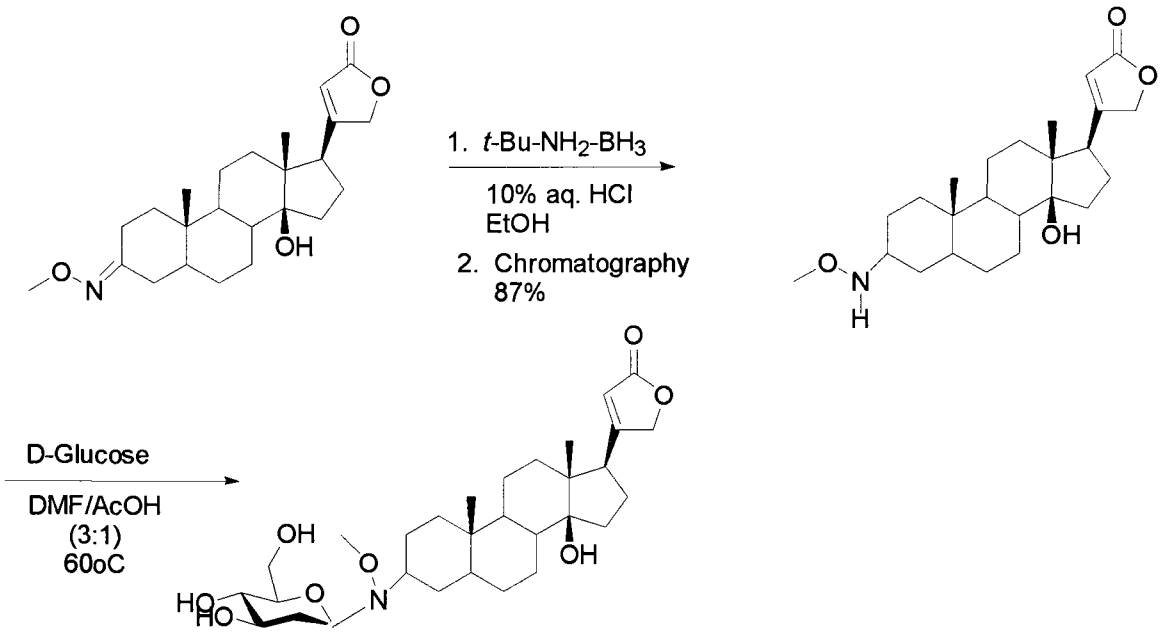


The ^1H NMR spectrum of **15a** displays a singlet at δ3.72 representing the hydroxy group of C-3 and a singlet at d3.6 representing the methoxy group of the ester at C-28 from the hydrogen at C-3 and a singlet at δ2.01 (s, 3H) corresponding to the methoxy group of the acetoxy group at position C-3.

The ^{13}C NMR of **15a** displayed diagnostic signals for the carboxylic group at C-28 of the D ring at δ 179.97 and at the hydroxy group at C-3 δ72.68.

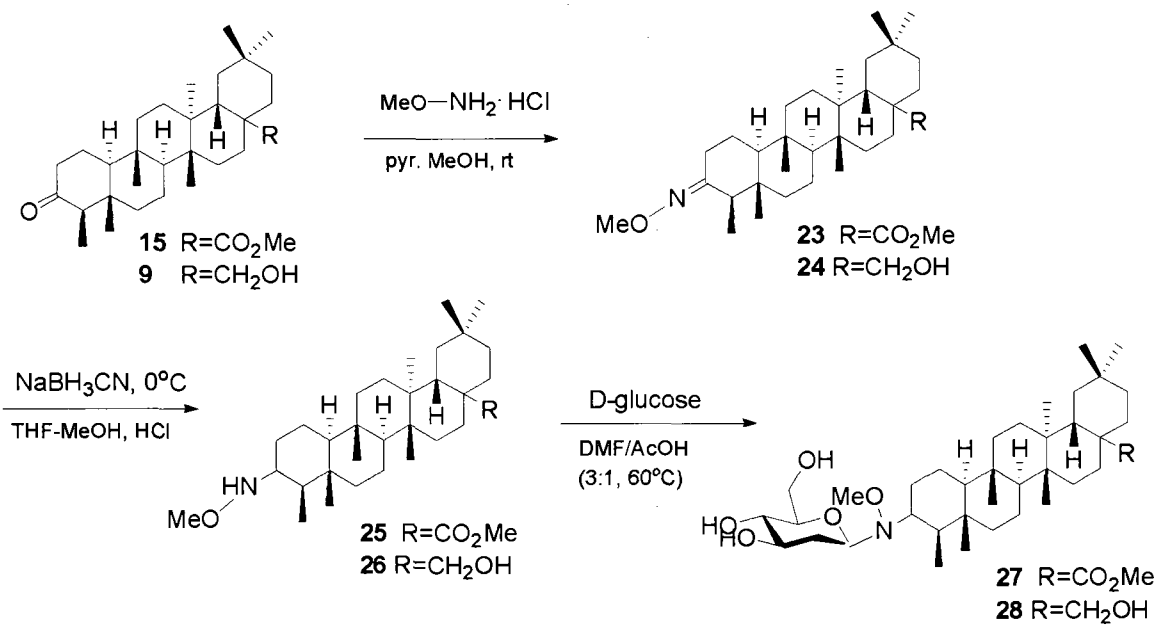
3.3.10 Preparation of C3-Oxime canophyllol and Methyl Ester analogues en route to glycosylation.

Glycosylation of natural products have been shown to be a reliable platform for the development of many front-line drugs. Our compounds have very low solubility in water and organic solvents, which could mean inefficient biological efficacy. Langenhan et al.[25], showed a procedure to make cardiac glycosides by neoglycorandomization in scheme 3.4.9.



Scheme 3.4.9. Synthesis of glycosides by neoglycoramdomization

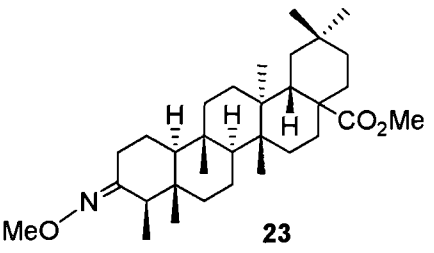
We, therefore, anticipated that glycosylation would improve aqueous solubility. The goal was to introduce a sugar at position C-3 by preparing oxime analogues, reducing and finally the addition of D-glucose.



Scheme 3.4.10 Glycosylation of compounds **15**, **9**

Both alcohol and methyl ester oximes gave yields of 70 and 77% respectively. Even though good yields were also achieved for the reduced products. After three unsuccessful trials and lack of time, we were unable to add the glucose to compounds **25** and **26**.

a) The oxime methyl ester, **23**, was synthesized from the described procedure to afford a white fluffy powder, in 77% yield after silica gel column chromatography.



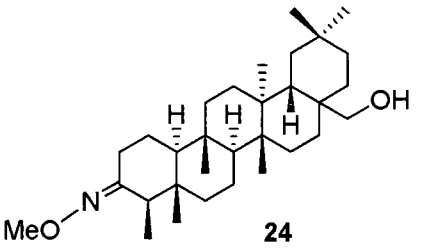
23

The EIMS of (**23**) displayed a $[M]^+$ at m/z 499 which is in accordance with the molecular formula $C_{32}H_{53}NO_3$ and established by HRMS. The melting point range was found to be 214-216°C. This is a new compound and both ^1H and ^{13}C NMR data of (**23**) are stated below.

The ^{1}H NMR spectrum of (**23**) displays two singlets at δ3.78 (s, 3H) and δ3.26 (s, 3H) corresponding to the methoxy group of the oxime group at C-3 along with the methoxy group of the methyl ester at C-28. Also displayed 3.26 (ddd, J=14.0 Hz, J=4.6 Hz, J=1.8 Hz, 1H, H-2), 2.36 (dd, J=13.4 Hz, J=4.2 Hz, 1H, H-2).

The ^{13}C NMR of (**23**) displayed diagnostic signals for the ketone of ring A at δ 179.0 (C-3) as well as the C=N function at δ160.7 and the C-28 C=O function at δ216.1

The oxime alcohol, **24**, was synthesized from the described procedure to afford a white fluffy powder, in 70% yield after silica gel column chromatography.



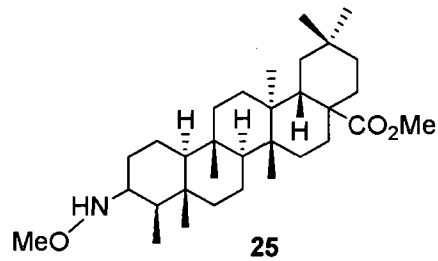
24

The EIMS of (**24**) displayed a $[M]^{+}$ at m/z 471 which is in accordance with the molecular formula $C_{31}H_{53}NO_2$ and established by HRMS. The melting point range was found to be 238-240°C. This is a new compound and both ^{1}H and ^{13}C NMR data of (**24**) are stated below.

The ^{1}H NMR spectrum of (**24a**) displays a singlet at δ3.80 (s, 3H) corresponding to the methoxy group of the oxime group at C-3 along a singlet representing the hydrogens of the alcohol moiety of C-28 at δ3.60.

The ^{13}C NMR of (**24**) displayed diagnostic signals for the ketone of ring A at δ 179.0 (C-3) as well as the C=N function at δ160.9 and the C-28 function at δ67.9

b) The reduced oxime methyl ester, **25**, was synthesized using sodium cyanoborohydride and afforded a white fluffy powder, in 82% yield after silica gel column chromatography.



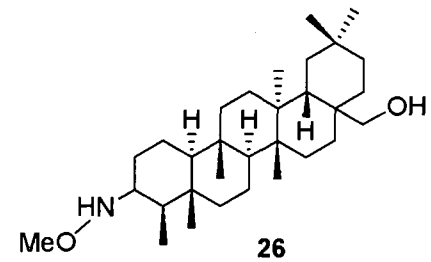
25

The EIMS of (**25**) displayed a $[M]^+$ at m/z 501 which is in accordance with the molecular formula $C_{32}H_{55}NO_3$ and established by HRMS. The melting point range was found to be 215-216°C. This is a new compound and both ^1H and ^{13}C NMR data of (**25**) are stated below.

The ^1H NMR spectrum of (**25**) displays two singlets at δ3.63 (s, 3H) and δ3.51 (s, 3H) corresponding to the methoxy group of the oxime group at C-3 along with the methoxy group of the methyl ester at C-28. Also displayed 2.93 (m, 1H, H-2), 2.39 (dd, J=13.4 Hz, J=4.2 Hz, 1H, H-2).

The ^{13}C NMR of (**25**) displayed diagnostic signals for the ketone of ring A at δ 179.0 (C-3) as well as the reduced C-N function at δ62.3. and the C-28 C=O function at δ216.1

The reduced oxime alcohol, **26**, was synthesized using sodium cyanoborohydride and afforded a white fluffy powder, in 99% yield after silica gel column chromatography.



26

The EIMS of (**26**) displayed a $[M]^+$ at m/z 473 which is in accordance with the molecular formula $C_{31}H_{55}NO_2$ and established by HRMS. The melting point range was found to be 218-220°C. This is a new compound and both ^1H and ^{13}C NMR data of (**26**) are stated below.

The ^{1}H NMR spectrum of (**26**) displays a singlet at δ3.**66** (s, 3H) corresponding to the methoxy group of the oxime group at C-3 with the hydrogens of the C-28 alcohol at d3.61.

The ^{13}C NMR of (**26**) displayed diagnostic signals for the ketone of ring D at δ 179.0 (C-3) as well as the reduced C-N function at δ62.3.

EXPERIMENTAL
CHAPTER 3

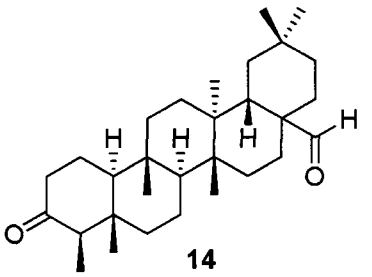
GENERAL: Mass spectra were obtained by means of a Kratos Concept II 2H and the GC were done on a Kratos Concept II 2H. ^{1}H NMR and ^{13}C NMR were recorded on a Bruker AMX-400, Bruker AMX-300 spectrometers with shifts reported in ppm. The multiplicities of the NMR signals were reported using the following abbreviations, singly or in combinations: singlets (s), broad singlet (br s), doublet (d), triplet (t), quartet (q) and multiplet (m).

Thin layer chromatography (tlc) were carried out using Kieselgel 60 F_{254} precoated 0.25mm plates. Visualization of the spots was facilitated by UV irradiation followed by charring of a tlc which has been dipped in a molybdate (2.5g) and cerric sulphate hydrate (1.0g) in a solution of sulfuric acid (10mL) in distilled water (90mL). Silica gel 270-400 Mesh was used for flash chromatography.

Procedure for the synthesis of canophyllal and 3-oxo-canophyllic acid (11-14): Jones'reagent (2.5mL) was added to a solution of canophyllol (.500g, $1.13x10^{-3}$moles) in acetone (20mL) at 0°C until the solution remained persistently a reddish-orange. After a few hours the reaction was quenched with MeOH (5mL) followed by water (3mL) and the organic solvent was evaporated under reduce pressure. More water was added (3mL) the residue was extracted with chloroform (5 x 5mL). The combined chloroform extract was then washed successively with water (2mL), brine (2mL) and water (2mL). The organic layers were combined, dried over $MgSO_4$ and concentrated *in vacuo* to afford 380mg of crude canophyllal/3-oxo-canophyllic acid as pale yellow crude.

The crude keto-aldehyde mixture (380mg) is placed in a round bottom flask with THF (30mL) and H_2O (5mL) and the solution is stirred until dissolution. The mixture is cooled to –10°C, in an ice bath, and stirred. Sulfamic acid (0.6g, 1.5e, mole) dissolved in 2mL of water is added to the stirred solution, followed by the addition of sodium chlorite (0.25g, 2 equiv, mole) dissolved in 2mL of water. The reaction is monitored by tlc with 7:3 hexane:EtOAc as eluent. Once all signs of the aldehyde have been oxidized, the reaction is quenched with water (10mL) and the organic solvent evaporated on the rotary evaporator. The aqueous later is then extracted with chloroform (4 x 15mL), the combined organic layers are then washed with water (5mL), brine (5mL), water (5mL),

dried over $MgSO_4$ and then concentrated *in vacuo*. The crude product was purified by silica gel column chromatography eluting with hexane-EtOAc gradient system to furnish canophyllal (**16**) (190mg, 45%)along with 3-oxo-canophyllic acid (**11**) (130mg, 40%) both as white solids.



14

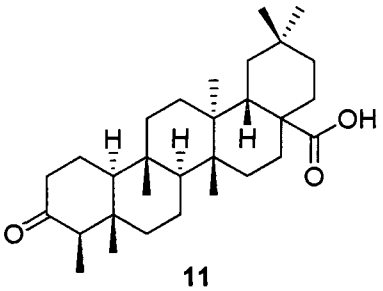
m.p. 265-268°C

^{1}H NMR (500 MHz, $CDCl_3$): δ 9.63 (s, 1H), 2.28-1.87 (m, 7H), 1.78-1.14 (m, 23H), 1.04 (s, 3H), 1.02 (s, 3H), .09 (s, 3H), 0.82 (s, 3H), 0.64 (s, 3H), 0.60 (s, 3H).

^{13}C NMR (500 MHz, $CDCl_3$): δ 213.0 (C=O of the aldehyde), 209.09 (C=O of the ketone), 76.7, 59.2, 58.2, 47.7, 42.0, 41.5, 41.1, 38.7,37.7, 37.1, 36.4, 35.4, 34.9, 34.5, 33.4, 32.4, 32.3, 30.6, 29.4, 28.3, 28.0, 22.3, 20.0, 18.8, 18.0, 17.2, 14.6, 6.8

MS (EI) m/z (rel. int.): 440 $[M]^+$ (2.8), 411 (43.6), 395 (4.2), 355 (4.4), 301 (5.2), 273 (53.3), 259 (12.8), 231 (13.4), 191 (18.2), 163 (36.6), 149 (28.7), 137 (100.0), 123 (44.3), 95 (59.5), 81 (50.7), 69 (55.5), 55 (60.1), 41 (43.7).

HRMS: Calculated *for $C_{29}H_{48}O2$, 440.36,* found 440.36418.



11

m.p. 258-260°C

^{1}H NMR (400 MHz, $CDCl_3$): δ 2.39-2.189 (m, 6H), 2.02-1.89 (m, 2H), 1.75-1.54 (m 4H), 1.53-1.07 (m 28H), 1.01 (s, 3H), 0.92 (s, 3H), 0.84 (s, 6H), 0.78 (s, 3H), 0.61 (s, H)

^{13}C NMR (400 MHz, $CDCl_3$): δ 213.4 (C=O ketone), 185.0 (C=O carboxylic),67.8, 59.4, 58.3, 53.1, 44.8, 42.2, 39.1, 37.9, 36.0, 34.6, 32.4, 31.3, 29.6, 28.4, 23.9, 22.3, 20.6, 17.5, 14.7, 6.8.

MS (EI) m/z (rel. int.): 456 $[M]^+$ (5.7), 423 (8.3), 395 (23.2), 371 (5.9), 339 (2.1), 303 (5.5), 273 (35.8), 250 (14.1), 231 (37.8), 217 (10.2), 205 (24.5), 189 (37.7), 175 (63.8), 163 (100.0), 135 (27.8), 121 (39.0), 109 (52.8), 95 (75.9), 69 (48.0), 41 (39.7).

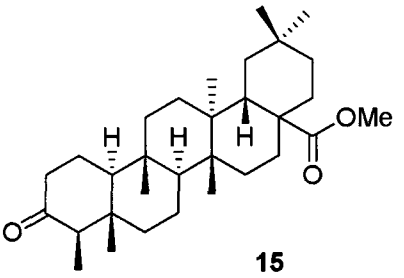
HRMS: Calculated *for $C_{30}H_{48}O_3$, 456.3603,* found *456.35871.*

General Procedure for the synthesis of esters (15-17): To a solution of 3-oxo-canophyllic acid (100mg) in THF (10mL) at room temperature (22°C) was added NaH (3 mole equivalent). The mixture was stirred for 30 mins before the appropriate alkyl halide (2.5 mole equivalent) was added, followed by 10 mole % of Bu_4NI. The solution was left to stir overnight (15h) before it was quenched with water (20mL). THF was removed by rotary evaporation before it was extracted with chloroform (3 x 15mL). The combined chloroform extracts were washed with water (10mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by silica gel chromatography using a hexane-EtOAc gradient as eluant to furnish the desired ester.

Methyl canophyllate

The yellow ethereal diazomethane solution [prepared by adding 250mL of nitroso methyl urea to a solution of 20% KOH (1mL) in ether (5mL) at 0°C] was added dropwise to a stirred solution of 3-oxo-canophyllic acid (50mg, $1.09x10^{-4}$ mole) dissolved in CH_2Cl_2 (30mL) and 5 drops of MeOH until the reaction mixture remained permanently yellow. The excess CH_2N_2 was left to evaporate in the fumehood over the weekend affording a clear solution. The solvent was removed *in vacuo* and the residue was re-dissolved in chloroform (20mL) and washed successively with H_2O (10mL), brine (10mL) and H_2O (10mL). The organic layer was dried over MgSO4 and the reaction

mixture was concentrated in vacuo. The residue was purified by silica gel chromatography using a hexane-EtOAc gradient as eluant to furnish (**15**) as a white solid (36mg, 70.5%).



15

m.p. 242-244°C

^{1}H NMR (400 MHz, $CDCl_3$): δ 3.64 (s, 3H, OMe), 2.49-2.19 (m, 6H), 1.94-1.74 (m, 2H), 1.62-1.14 (m, 25H), 1.13 (s, 3H), 1.01 (s, 3H), 0.92 (s, 3H), 0.82 (s, 3H), 0.70 (s, 3H)

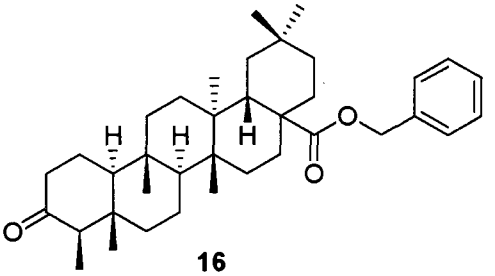
^{13}C NMR (400 MHz, $CDCl_3$): δ 213.1 (C=O ketone), 179.4 (C=O methyl ester), 59.3, 58.2, 53.0, 51.8, 44.9, 42.0, 41.5, 41.1, 38.9, 38.1, 37.6, 37.6, 35.9, 35.4, 34.8, 34.5, 32.7, 32.6, 31.0, 29.7, 29.4, 28.4, 22.3, 20.3, 18.5, 18.1, 17.5, 14.7, 6.8.

MS (EI) m/z (rel. int.): 470 $[M]^+$ (10.5), 455 (10.4), 423 (71.5), 379 (17.1), 319 (38.1), 273 (69.5), 231.2 (27.1), 189 (65.7), 163 (88.1), 95 (100.0), 69 (89.7).

HRMS: Calculated *for $C_{31}H_{50}O_3$, 470.3760,* found *470.37548.*

Benzyl canophyllate

The reaction of 3-oxo-canophyllic acid (50mg, $1.09x10^{-4}$ mole) in THF (3mL) at 22°C with NaH (0.02g, $1.13x10^{-3}$ mol), benzyl bromide (0.07mL, $5.65x10^{-4}$ mole) and 10 mole % of Bu_4NI (0.04mg) for 15 hours according to the general procedure, furnished (**16**) as a white fluffy solid (42mg, 60% yield), after the same general workup and purification by silica gel chromatography of hexanes-EtOAc gradient.



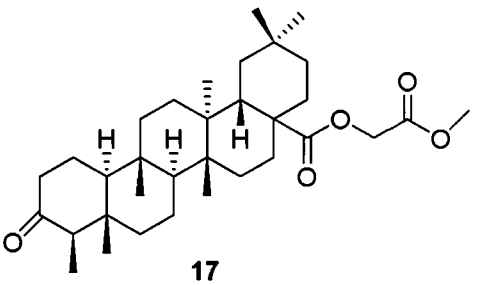
16

m.p. ℃

***^{1}H NMR** (400 MHz, $CDCl_3$): δ*

Methyl acetoxy canophyllate

The reaction of 3-oxo-canophyllic acid (50mg, $1.09x10^{-4}$ mol) in THF (3mL) at 22°C with NaH (0.02g, $1.13x10^{-3}$ mol), methyl bromoacetate (0.07mL, $5.65x10^{-4}$ mole) and 10 mole % of Bu4NI (0.04mg) for 15 hours according to the general procedure, furnished a white fluffy solid (43mg, 75%), after the same general workup and purification by silica gel chromatography of hexanes-EtOAc gradient.



17

m.p. 178-180°C

^{1}H NMR (400 MHz, $CDCl_3$): δ 4.58 (dd, J-15.8, 9.4Hz, 2H), 3.73 (s, 3H, OMe), 2.43-2.21 (m, 6H), 1.96-1.80 (m, 2H), 1.73-1.18 (m, 25H), 1.15 (s, 3H), 1.02 (s, 3H), 0.91 (s, 3H), 0.84 (s, 3H), 0.69 (s, 3H)

^{13}C NMR (400 MHz, $CDCl_3$): δ 213.11 (C=O ketone), 178.11 (C=O carbonyl), 168.51 (C=O of the acetoxy), 60.83, 59.30, 58.23, 52.99, 52.07, 45.05, 42.07, 41.52, 41.12,

38.96, 38.01, 37.68, 37.61, 35.79, 35.49, 34.86, 34.57, 32.72, 32.34, 31.06, 29.72, 29.36, 28.38, 22.27, 20.50, 18.59, 18.11, 17.48, 14.66, 6.8

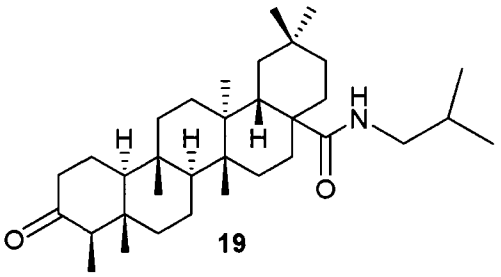
MS (EI) m/z (rel. int.): 528 $[M]^+$ (14.6), 510.4 (30.0), 438.3 (20.4), 411.4 (36.5), 322.2 (15.8), 273.2 (48.5), 231.2 (23.9), 189.2 (60.1), 163.1 (100.0), 138.1 (49.8), 95.1 *(78.5**HRMS**:* Calculated *for $C_{33}H_{52}O_5$, 528.3815,* found *528.3806.*

Amides of 3-oxo-canophyllic acid

General procedure for synthesizing the amides of 3-oxo-canophyllic acid:
Oxalyl chloride (1.5 mole equivalent) and 1 drop of DMF was added drop wise to a solution of 3-oxo-canophyllic acid (50mg, $1.09x10^{-4}$ mol) in CH_2Cl_2 (5mL) and stirred overnight (15hrs) at room temperature (22°C). The solvent and the DMF were removed *in vacuo*, the residue was re-dissolved in CH_2Cl_2 (2mL) and added drop wise to another solution containing the appropriate amine (1.1 mole equivalent) and triethylamine (Et_3N), (1.1 mole equivalent) in CH_2Cl_2 (5mL) at 0°C. Stirring was continued for a few hours. The reaction mixture was washed successively with water (5 mL), 1% HCl (5mL), water (5mL), dried over $MgSO_4$, filtered and concentrated *in vacuo* and chromatographed over silica gel chromatography using hexane-EtOAc gradient as eluant to afford the desired amides.

3-oxo-canophyllic isopropyl amide

The amide was isolated as a white solid (25mg, 50% yield), after reacting (50 mg, $1.09x10^{-4}$ mole) in CH_2Cl_2 (5mL) with oxalyl chloride (0.04mL, $1.64x10^{-4}$ mole), 1 drop of DMF, isobutylamine (0.02mL, $1.2x10^{-4}$mole) in the presence of Et_3N (0.04mL, $1.2x10^{-4}$mole) in CH_2Cl_2 (5mL) according to the general procedure and work up. Purification of the crude product was achieved by silica gel column chromatography using a hexane-EtOAc gradient.



19

m.p. 208-210°C

^{1}H NMR (400 MHz, $CDCl_3$): δ 5.9 (br,s, 1H), 3.13 (m, 1H), 2.91(m, 1H), 2.6 (dd, J=13.2, 9Hz, 1H), 0.99 (s, 3H, H-26), 0.95 (s, 3H, H-27), 0.88 (s, 3H, H-25), 0.87 (d, J=7.6 Hz, 3H, H-23), 0.75 (s, 3H, H-24), 0.66 (s, 3H, H-29), 0.64 (s, 3H, H-30).

^{13}C NMR (400 MHz, $CDCl_3$): δ 213.14 (C=O ketone), 177.65 (C=O of carbonyl),59.39, 58.23, 53.03, 47.46, 44.72, 42.10, 41.52, 41.17, 39.07, 37.76, 37.67, 37.24, 35.55. 35.15, 34.57, 32.88, 32.42, 31.65, 29.78, 28.44, 28.28, 22.26, 20.42, 20.28, 18.69, 18.17, 17.72, 14.65, 6.7

MS (EI) m/z (rel. int.): 511 $[M]^+$ (7.7), 496.4 (20.1), 439.4 (36.3), 411.4 (10.7), 379.3 (2.4), 273.2 (16.4), 209.2 (12.6), 166.1 (9.3), 137.1 (33.4), 109.1 (22.6), 74.1 (100.0), 57.1 (30.4).

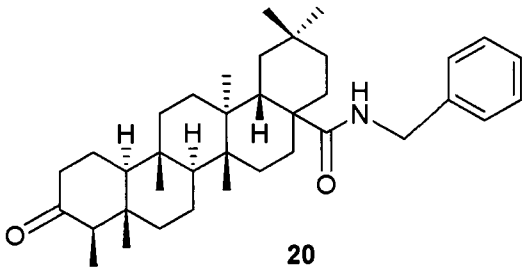
HRMS: Calculated *for $C_{34}H_{57}NO_2$, 511.4389,* found *511.4402.*

3-oxo-canophyllic benzyl amide

The procedure for preparing benzyl amide failed twice. We therefore altered this procedure by first forming the acetyl chloride produce using $SOCl_2$ (10 equivalents) along with the 3-oxo-canophyllic (50mg, $1.09x10^{-4}$ mole) acid and refluxed this mixture at 80°C for a period of 6-8 hours. To the residue, CH_2Cl_2 (5mL) was added then washed with water (2mL), 5% NaOH (2mL), water (2mL), dried over $MgSO_4$ and concentrated *in vacuo.*

The residue was re-dissolved in CH_2Cl_2 (2mL) and added drop wise to another solution containing the benzyl amine (1.1 mole equivalent) and triethylamine, (1.1 mole equivalent) in CH_2Cl_2 (5mL) at 0°C. Stirring was continued for a few hours. The

reaction mixture was washed successively with water (5 mL), 1% HCl (5mL), water (5mL), dried over $MgSO_4$, filtered and concentrated *in vacuo* and chromatographed over silica gel chromatography using hexane-EtOAc gradient as eluant to afford the desired amine (73%).



20

m.p. °C

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.36-7.06 (m, 5H), 5.05 (br. s, 1H, NH), 3.24 (dd, J= 15.0, 9.4Hz, 2H), 2.43-2.21 (m, 6H), 1.96-1.80 (m, 2H), 1.73-1.18 (m, 25H), 1.15 (s, 3H), 1.02 (s, 3H), 0.91 (s, 3H), 0.84 (s, 3H), 0.69 (s, 3H)

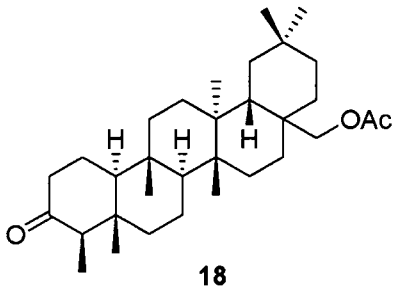
^{13}C NMR (400 MHz, $CDCl_3$): δ

MS (EI) m/z (rel. int.): 499 $[M]^+$ (37), 469 (40), 468 (100), 440 (18), 250 (23), 236 (17), 190 (18), 189 (60), 168 (77), 154 (39), 126 (34), 114 (65), 101 (28), 95 (23), 81 (26), 69 (24), 55 (20), 41 (21).

HRMS: Calculated *for $C_{32}H_{53}NO_3$, 499.4025,* found *499.4041.*

Preparation of Canophyll-28-acetoxy:

A solution of 3-oxo-canophyllic acid (50mg, mole), Et_3N (0.4mL, mole) and DMAP (5mg) in CH_2Cl_2 (30mL) at 22°C was stirred for 10 min. before acetic anhydride (250mg, 0.30mL) was added and stirring was continued overnight (15hrs). The reaction mixture was washed successively with water (10mL), 5% HCl (10mL), water (10mL), dried over $MgSO_4$, filtered then concentrated *in vacuo*. The crude product was purified by silica gel chromatography eluting with hexane-EtOAc gradient to furnish 30mg **18** in a yield of 67%.



18

m.p. 158-160°C

^{1}H NMR (400 MHz, $CDCl_3$): δ 4.12 (dd, J=15.7, 7.5Hz, 2H), 2.43-2.21 (m, 6H), 1.96-1.80 (m, 2H), 1.73-1.18 (m, 25H), 1.15 (s, 3H), 1.02 (s, 3H), 0.91 (s, 3H), 0.84 (s, 3H), 0.69 (s, 3H)

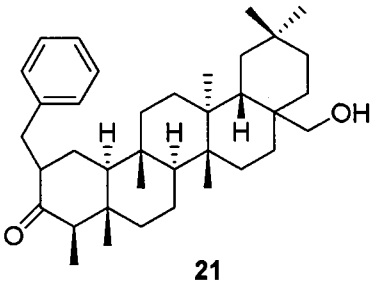
^{13}C NMR (400 MHz, $CDCl_3$): δ 213.11 (C=O ketone), 171.48 (C=O carbonyl), 76.71, 69.14, 59.47, 58.22, 52.50, 42.09, 41.50, 41.24, 39.38, 39.18, 38.10, 37.44, 35.40, 34.41, 34.33, 33.72, 33.07, 32.70, 32.18, 31.34, 30.16, 29.61, 28.04, 22.25, 21.09, 19.21, 18.22, 18.08, 14.67, 6.8

MS (EI) m/z (rel. int.): 484 $[M]^+$ (0.9), 469 (1.3), 424 (9.1), 411 (24.7), 393 (1.1), 367 (0.3), 339 (2.0), 301 (3.8), 273 (1.0), 247 (10.3), 231 (23.6), 218 (11.1), 203 (24.2), 189 (38.2), 177 (67.0), 163 (27.4), 149 (39.2), 123 (73.2), 95 (69.8), 69 (63.1), 43 (100).

HRMS: Calculated *for $C_{32}H_{52}O_3$, 484.3916,* found *484.*

Preparatiom of 4-benzyl-canophyllol: Under nitrogen atmosphere. A solution of Diisopropylamine (0.15mL, moles) in THF (5mL) was brought down to 0°C in an ice bath to which BuLi (0.4mL, moles) was then added drop wise and stirred until a pale yellow solution was observed. The deprotonated base was then brought down to –78°C and a solution of canophyllol (150mg, $3.3x10^{-4}$ mole) in THF (5mL) slowly added. The reaction mixture was allowed to react and finally the benzyl bromide (0.15mL, $8.22x10^{-4}$ mole) was added and let stir overnight. THF (5mL) was added and the reaction mixture was washed with sat. NH_4Cl (5mL), H_2O (5mL), organic layers were combined and dried over $MgSO_4$, filtered then concentrated *in vacuo*. The crude product was purified by

silica gel chromatography eluting with hexane-EtOAc gradient to furnish 54mg of **21** the desired products in yield of 30%.



21

m.p. 238-240°C

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.13 (m, 5H), 3.60 (s, 2H), 2.76-2.48 (m, 6H), 2.2-2.14 (m, 2H), 1.99-1.20 (m, 25H), 1.15 (s, 3H), 1.09 (s, 3H), 0.95 (s, 3H), 0.79 (s, 3H), 0.68 (s, 3H)

^{13}C NMR (400 MHz, $CDCl_3$): δ 213.32 (C=O), 139.20, 129.24, 126.46, 68.09, 59.52, 58.36, 55.12, 53.93, 52.57, 51.42, 42.15, 41.23, 39.83, 38.45, 34.97, 33.58, 31.50, 30.11, 29.18, 28.02, 24.78, 22.00, 19.50, 14.59, 6.71

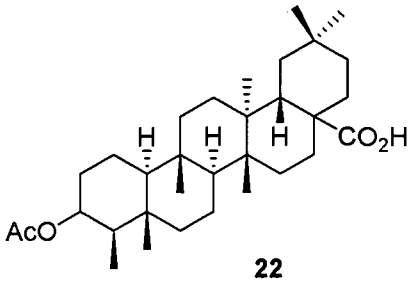
MS (EI) m/z (rel. int.): 501 $[M]^+$ (16.4), 411.4 (69.8), 363.3 (10.2), 301.2 (5.7), 273.2 (52.9), 247.4 (9.2), 163.2 (15.7), 137.1 (100), 109 (35.4), 55.1 (22.1).

HRMS: Calculated *for $C_{32}H_{53}NO_3$, 532.4280,* found *501.4 (due to CH_2OH missing)*.

Preparation for the Acetylation of the canophyllic acid(22):

Dissolved **11** (0.100mg, $2.19x10^{-4}$mole) in a 12:4mL of methanol/THF followed by the addition of sodium borohydride (0.03mg, $8.77x10^{-4}$mole). The mixture was then stirred for 2 hours at room temperature. Tlc analysis showed that there was no more starting material. The reaction mixture was then quenched with a saturated solution of NH_4Cl and the organic solvent was then evaporated. The residue was extracted 3X5mL of EtOAc and washed with 1mL of water then 1mL of brine. The combined organic layers were dried over $MgSO_4$, then concentrated *in vacuo*. The crude product was purified by silica gel chromatography eluting with hexane-EtOAc gradient to furnish 70mg of canophillic acid in a yield of 70%. The canophillic acid was then dissolved in 25mL CH_2Cl_2. To the dissolved acid triethylamine (0.064mL, $1.53x10^{-4}$ mole), DMAP

($7.65x10^{-6}$mole) and acetic anhydride (0.043mL, $4.58x10^{-4}$ mole). The reaction mixture was then stirred for 4 hours at room temperature. The residue was extracted 3X5mL of CH_2Cl_2 and washed with 1mL of brine. The combined organic layers were dried over $MgSO_4$, then concentrated *in vacuo*. The crude product was purified by silica gel chromatography eluting with hexane-EtOAc gradient to furnish 30mg of acetylated canophillic acid in a yield of 27%.



22

m.p 282-284°C

^{1}H NMR (400 MHz, $CDCl_3$): δ 4.87 (s, 1H), 2.40-2.25 (m, 2H), 2.07 (s, 3H), 1.89-1.85 (dd, J= 15.7, 7,8Hz 1H), 1.75-1.59 (m, 2H), 1.51-1.03 (m., 25H), 1.01 (s, 3H),0.97 (s, 3H), 0.91 (s, 3H), 0.83 (s, 3H), 0.77 (s, 3H)

^{13}C NMR (400 MHz, $CDCl_3$): δ 185.2 (C=O of carbonyl), 170.5 (C=O of acetoxy), 73.95, 60.11, 53.20, 47.39, 44.72, 41.55, 38.85, 37.87, 37.37, 35.93, 34.86, 34.52, 32.58, 32.19, 31.15, 29.65, 29.40, 28.39, 21.72, 20.52, 18.59, 17.78, 17.51, 15.72, 10.42.

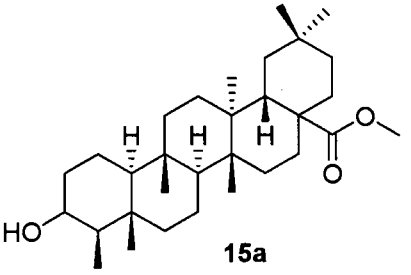
MS (EI) m/z (rel. int.): 500 $[M]^+$ (6.7), 467.3 (29.7), 440.4 (61.3), 395.4 (4.9), 372.3 (13.4), 275.2 (38.7), 231.2 (56.8), 191.2 (70.2), 147.1 (65.1), 95.1 (100).

HRMS: Calculated *for $C_{32}H_{52}O_4$, 500.753,* found *500.38398.*

Preparation of the reduced methyl ester(15a)

Dissolved **15** (0.30mg, $6.37x10^{-4}$mole) in 25mL of methanol in an ice bath (0°C) followed by the addition of sodium borohydride (0.09mg, $2.55x10^{-3}$mole). The mixture was then stirred for 2 hours at room temperature. Tlc analysis showed that there was no more starting material. The residue was extracted 3X5mL of ether and washed with 1mL

of water then 1mL of brine. The combined organic layers were dried over $MgSO_4$, then concentrated *in vacuo*. The crude product was purified by silica gel chromatography eluting with hexane-EtOAc gradient to furnish 20mg of the reduced methyl ester in a yield of 40%.



15a

m.p. 268-270°C

^{1}H NMR (400 MHz, $CDCl_3$): δ 3.70 (s, 1H), 3.62 (s, 3H, OMe), 2.40-2.20 (m, 2H), 1.86(dd, J=15.3, 9.2Hz, 1H), 1.71-1.10 (m., 26H), 1.02 (s, 3H),0.97 (s, 3H), 0.96 (s, 3H), 0.81 (s, 3H), 0.67 (s, 3H)

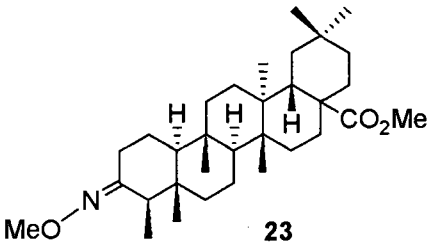
^{13}C NMR (400 MHz, $CDCl_3$): δ 179.0 (C=O),72.67, 61.16, 53.08, 51.80, 49.15, 45.00, 41.51, 38.87, 38.12, 37.76, 37.64, 37.32, 35.9, 35.40, 35.16, 34.85, 34.50, 32.61, 31.21, 29.65, 29.43, 28.44, 20.16, 18.53, 17.70, 17.35, 16.39,10.62.

MS (EI) m/z (rel. int.): 472 $[M]^+$ (3.2), 440.4 (51.7), 374.0 (4.0), 332.0 *(20.2), 275.2 (26.2), 231.2 (16.9), 189.2 (51.5), 162.0 (100), 95.1 (38.5), 31.0 (25.5).*

HRMS: *Calculated* for $C_{31}H_{52}O_3$, 472.743, *found.*

3-Oxime canophyllol methyl ester (23)

3-Oxocanophyllol methyl ester **23** (130 mg, 2.85 x 10^{-4} mol) was dissolved in methanol (200 mL) and pyridine (0.34 mL) at room temperature. Methoxylamine hydrochloride (238 mg, 1.42 x 10^{-2} mol) was added and the solution was stirred overnight, then concentrated. The resulting residue was re-dissolved in EtOAc (200 mL) and washed with 1M HCl (30 mL) and brine (30 mL), dried over $MgSO_4$, filtered and concentrated in vacuo. The desired oxime **23** was obtained as a white solid (110 mg, 77%) after purification with SiO_2 column chromatography, eluting with EtOAc/hexane gradient.



23

m.p. 214-216°C

^{1}H NMR (400 MHz, $CDCl_3$): δ 3.78 (s, 3H, OMe), 3.62 (s, 3H, OMe), 3.26 (ddd, J=14.0 Hz, J=4.6 Hz, J=1.8 Hz, 1H, H-2), 2.36 (dd, J=13.4 Hz, J=4.2 Hz, 1H, H-2), 2.28 (dd, J=15.0 Hz, J=9.4 Hz, 1H, H-18), 1.91 (q, J=6.7Hz, 1H, H-4), 0.99 (s, 3H, H-26), 0.95 (s, 3H, H-27), 0.88 (s, 3H, H-25), 0.87 (d, J=7.6 Hz, 3H, H-23), 0.75 (s, 3H, H-24), 0.66 (s, 3H, H-29), 0.64 (s, 3H, H-30).

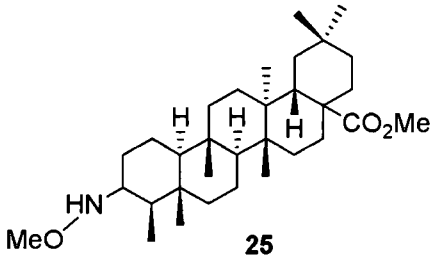
^{13}C NMR (400 MHz, $CDCl_3$): δ 179.0 (C=O), 160.7 (C=N), 60.7, 59.5, 52.7, 51.5, 50.5, 44.6, 40.6, 39.7, 38.5, 37.7, 37.2, 37.1, 35.6, 35.1, 34.5, 34.2, 32.4, 32.3, 30.8, 29.3, 29.0, 28.1, 24.8, 20.2, 19.9, 18.2, 17.8, 17.1, 13.8, 8.0.

MS (EI) m/z (rel. int.): 499 $[M]^+$ (37), 469 (40), 468 (100), 440 (18), 250 (23), 236 (17), 190 (18), 189 (60), 168 (77), 154 (39), 126 (34), 114 (65), 101 (28), 95 (23), 81 (26), 69 (24), 55 (20), 41 (21).

HRMS: Calculated *for $C_{32}H_{53}NO_3$, 499.4025,* found *499.4041.*

Reduction of 3-Oxime canophyllol methyl ester (23) to (25)

Oxime **23** (110 mg, 2.2 x 10^{-4} mol) was suspended in MeOH-THF (1:1, 40 mL) at 0°C. Sodium cyanoborohydride (138.5 mg, 2.20 x 10^{-2} mol) dissolved in THF (5 mL) was added, followed by dropwise addition of 1M HCl in MeOH (2 mL). After 20 minutes the reaction was quenched with $NaHCO_3$ (20 mL). The organic solvents were evaporated in vacuo, before the aqueous layer was extracted with EtOAc (2 x 50 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated. The crude reaction mixture was purified by SiO_2 column chromatography eluting with EtOAc/hexane gradient to afford compound **25** (90 mg, 82%) as a white solid.



25

m.p. 215-216°C

^{1}H NMR (400 MHz, $CDCl_3$): δ 3.63 (s, 3H, OMe), 3.51 (s, 3H, OMe), 2.93 (m, 1H, H-2), 2.39 (dd, J=13.4 Hz, J=4.2 Hz, 1H, H-2), 2.31 (dd, J=14.8 Hz, J=9.6 Hz, 1H, H-18), 1.03 (s, 3H, H-26), 0.97 (s, 3H, H-27), 0.91 (s, 3H, H-25), 0.88 (d, J=7.6 Hz, 3H, H-23), 0.81 (s, 6H, H-29, H-30), 0.67 (s, 3H, H-24).

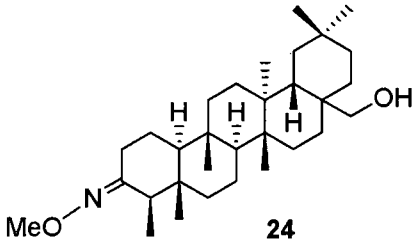
^{13}C NMR (400 MHz, $CDCl_3$): δ 179.4 (C=O), 62.3 (C-N), 62.2, 61.6, 53.1, 51.8, 48.2, 45.0, 41.5, 38.9, 38.1, 37.8, 37.6, 37.3, 36.0, 35.4, 34.8, 34.5, 32.6, 31.2, 30.5, 29.6, 29.4, 28.4, 20.1, 18.5, 17.8, 17.6, 16.5, 15.9, 11.9.

MS (EI) m/z (rel. int.): 501 $[M]^+$ (20), 470 (17), 203 (12), 189 (26), 109 (12), 95 (17), 86 (100), 85 (47), 83 (72), 81 (15), 69 (18), 55 (18), 46 (11), 43 (10), 41 (14).

HRMS: Calculated *for $C_{32}H_{55}NO_3$, 501.4182,* found *501.4200.*

3-Oxime canophyllol (24)

3-Oxocanophyllol (200 mg, 4.52 x 10^{-4} mol) was dissolved in methanol (200 mL) and pyridine (0.37 mL) at room temperature. Methoxylamine hydrochloride (189 mg, 2.26 x 10^{-3} mol) was added and the solution was stirred overnight, then concentrated. The resulting residue was re-dissolved in EtOAc (200 mL) and washed with 1M HCl (30 mL) and brine (30 mL), dried over $MgSO_4$, filtered and concentrated in vacuo. Compound **24** was obtained as a white solid (150 mg, 70%) after purification with SiO_2 column chromatography, using an EtOAc/hexane solvent gradient.



24

m.p. 238-240°C

^{1}H NMR (400 MHz, $CDCl_3$): δ 3.80 (s, 3H, OMe), 3.60 (s, 2H, H-28), 3.30 (ddd, J=14.0 Hz, J=4.4 Hz, J=1.7 Hz, H-2), 1.96 (q, J=6.7 Hz, H-4), 1.09 (s, 3H, H-26), 0.97 (s, 3H, H-27), 0.96 (s, 3H, H-25), 0.0.92 (d, J=6.8 Hz, 3H, H-23), 0.88 (s, 3H, H-24), 0.81 (s, 3H, H-29), 0.71 (s, 3H, H-30).

^{13}C NMR (400 MHz, $CDCl_3$): δ 160.9 (C=N), 67.9 (C-28), 61.0, 60.0, 52.4, 50.8, 41.1, 40.1, 39.4, 38.1, 37.2, 35.3, 35.1, 34.4, 34.2, 33.3, 32.8, 31.3, 31.2, 30.1, 29.1, 28.1, 25.1, 20.5, 19.1, 19.0, 18.3, 18.0, 14.1, 8.3.

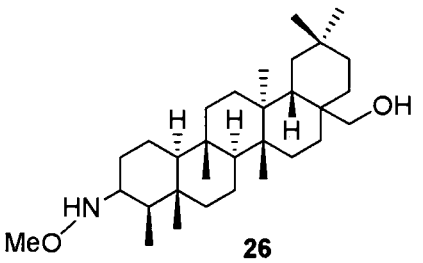
MS (EI) m/z (rel. int.): 471 $[M]^+$ (18), 441 (33), 440 (83), 410 (29), 302 (18), 221 (18), 208 (15), 203 (27), 191 (18), 189 (17), 168 (100), 163 (18), 154 (33), 149 (17), 140 (17), 137 (27), 135 (16), 126 (42), 123 (20), 121 (16), 114 (69), 109 (34), 107 (23), 101 (41), 95 (41), 81 (36), 69 (32), 67 (20), 55 (37), 41 (30)..

HRMS: Calculated *for $C_{31}H_{53}NO_2$, 471.4076,* found *471.4074*

Reduction of 3-Oxime canophyllol (24) to (26)

Oxime **24** (50 mg, 1.06 x 10^{-4} mol) was suspended in MeOH-THF (1:1, 30 mL) at 0°C. Sodium cyanoborohydride (66.7 mg, 5.31 x 10^{-4} mol) dissolved in THF (2 mL) was added, followed by dropwise addition of 1M HCl in MeOH.(1 mL). After 1h the reaction was quenched with $NaHCO_3$ (20 mL). The organic solvents were evaporated in vacuo, before the aqueous layer was extracted with EtOAc (2 x 50mL). The organic layer was dried over $MgSO_4$, filtered and concentrated. The crude reaction mixture was purified by SiO_2 column chromatography eluting with EtOAc/hexane gradient to afford compound

26 (50 mg, 99%) as a white solid. Note: The desired product ran together with the starting material on TLC.



26

m.p. 218-220°C

^{1}H NMR (400 MHz, $CDCl_3$): δ 3.66 (s, 2H, H-28), 3.61 (s, 2H, H-30), 3.03 (m, H-2), 1.08 (s, 3H, H-26), 0.98 (s, 3H, H-27), 0.97 (s, 3H, H-25), 0.90 ((s, 3H, H-24), 0.89 (d, J=7.2 Hz, 3H, H-23), 0.83 (s, 3H, H-29), 0.82 (s, 3H, H-30).

^{13}C NMR (400 MHz, $CDCl_3$): δ 67.9 (C-28), 62.2 (C-N), 62.0, 61.4, 52.4, 47.7, 41.2, 39.1, 38.9, 37.9, 36.8, 34.9, 34.8, 34.1, 33.9, 33.0, 32.5, 30.9, 30.8, 29.9, 29.8, 28.8, 18.8, 18.6, 18.0, 17.5, 16.1, 15.7, 11.6.

MS (EI) m/z (rel. int.): 473 $[M]^+$ (18), 442 (24), 161 (11), 123 (15), 121 (11), 109 (19), 107 (11), 95 (24), 95 (41), 86 (100), 84 (30), 82 (51), 69 (20), 55 (22), 41 (15).

HRMS: Calculated *for $C_{31}H_{55}NO_2$, 473.4323,* found *473.4248.*

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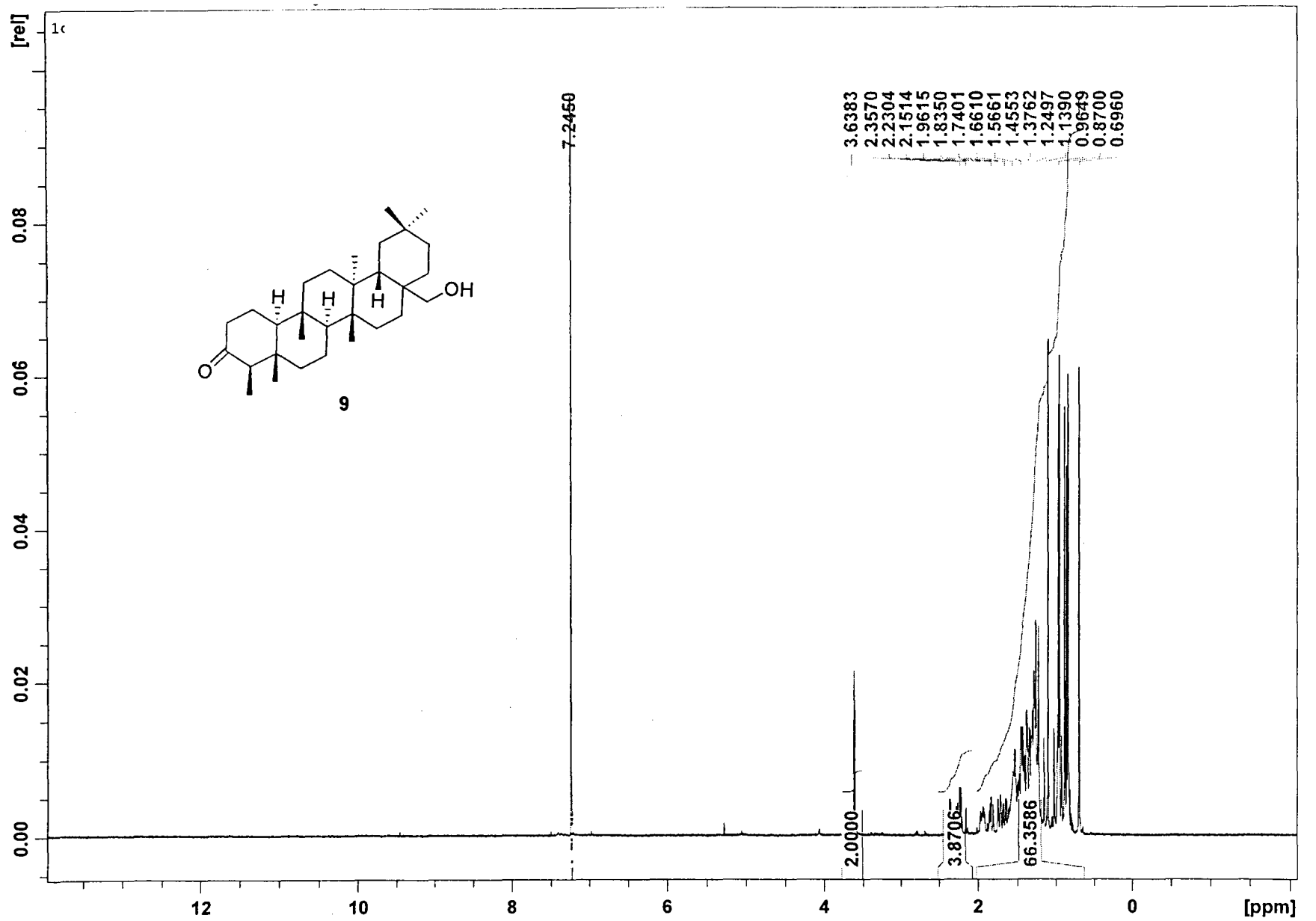
APPENDIX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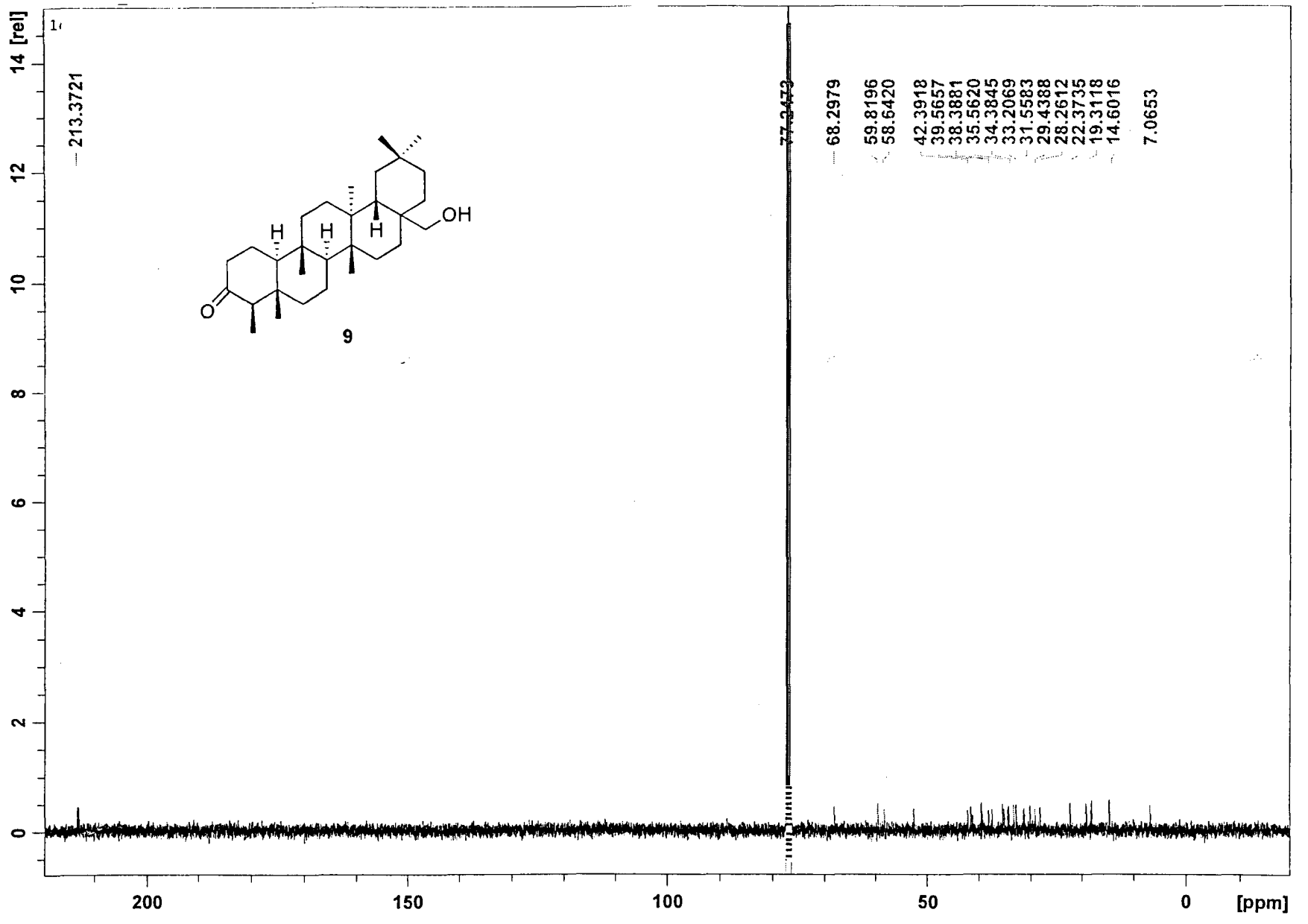
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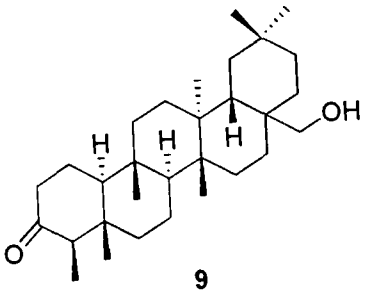

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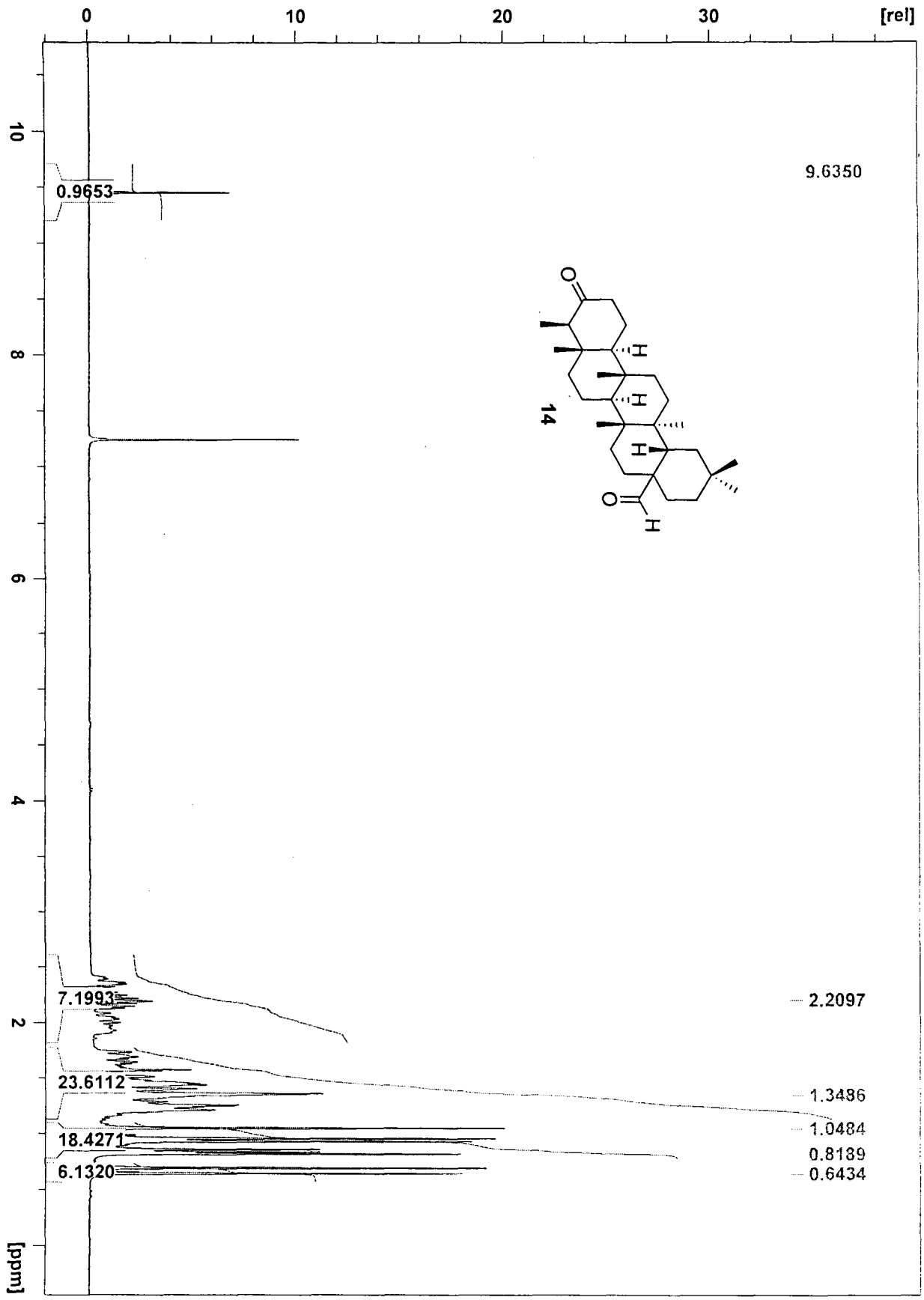
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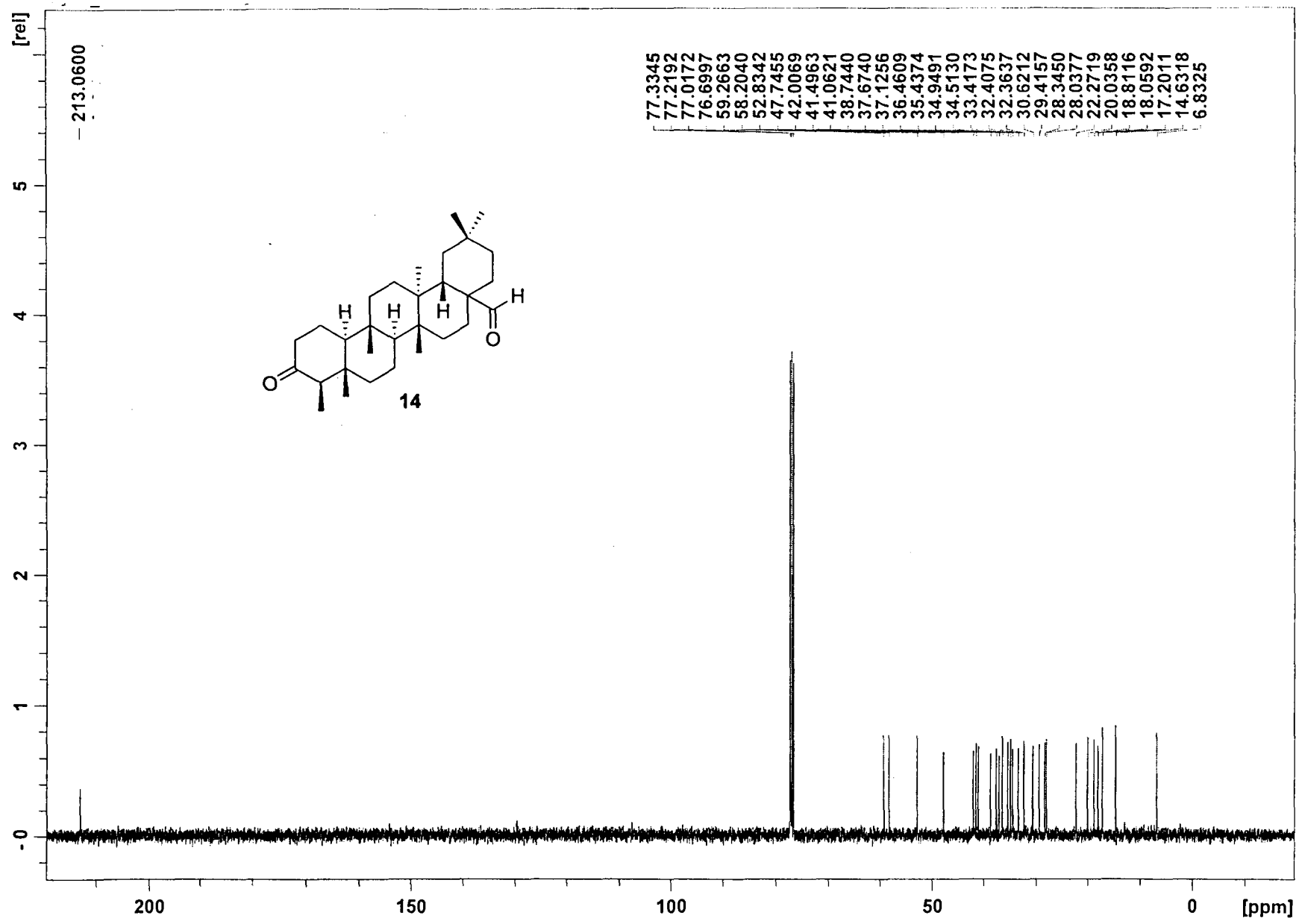
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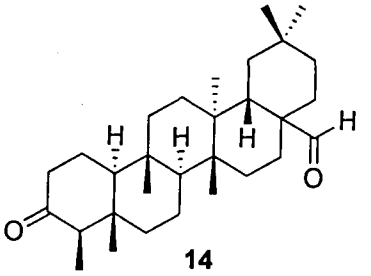

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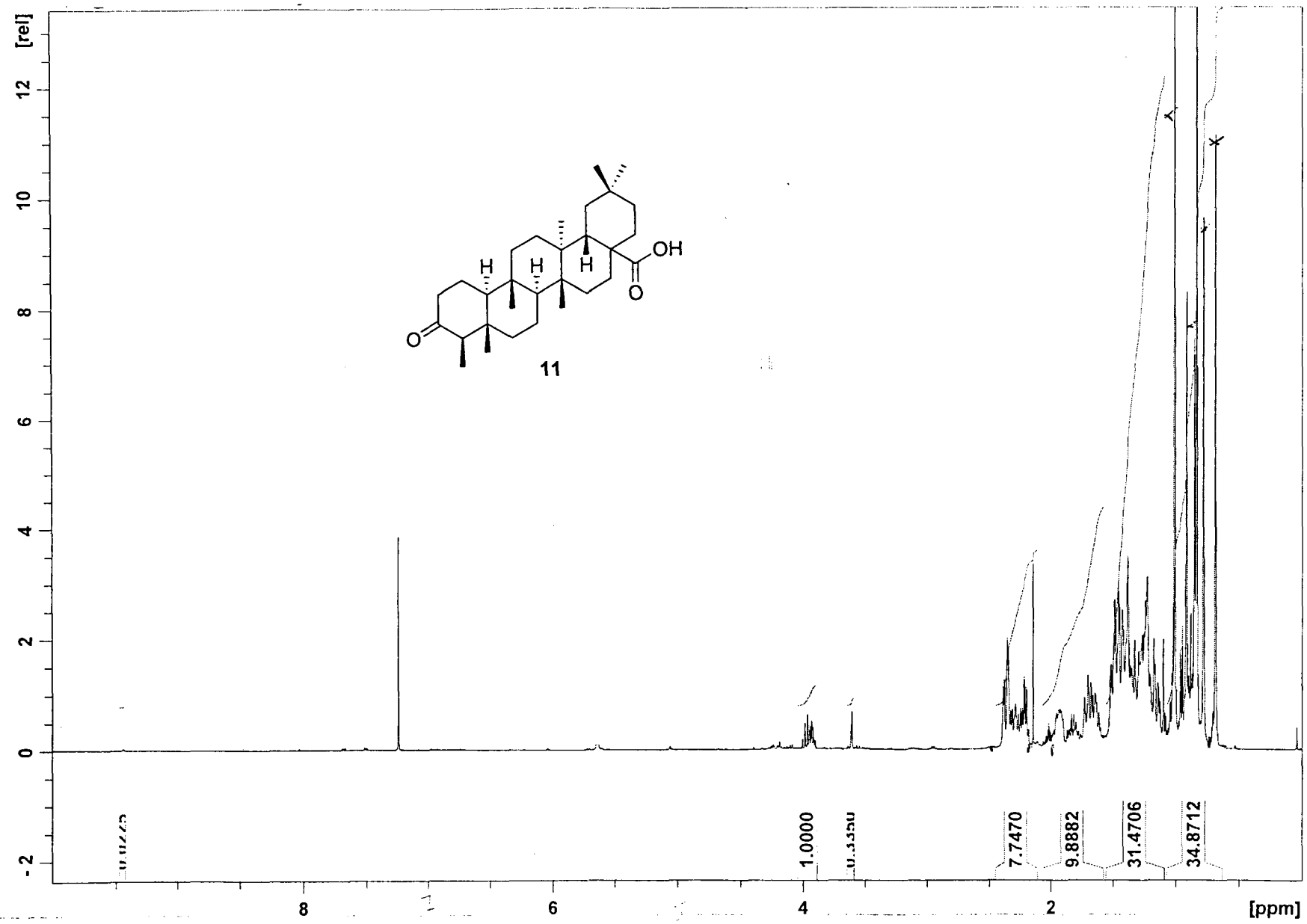
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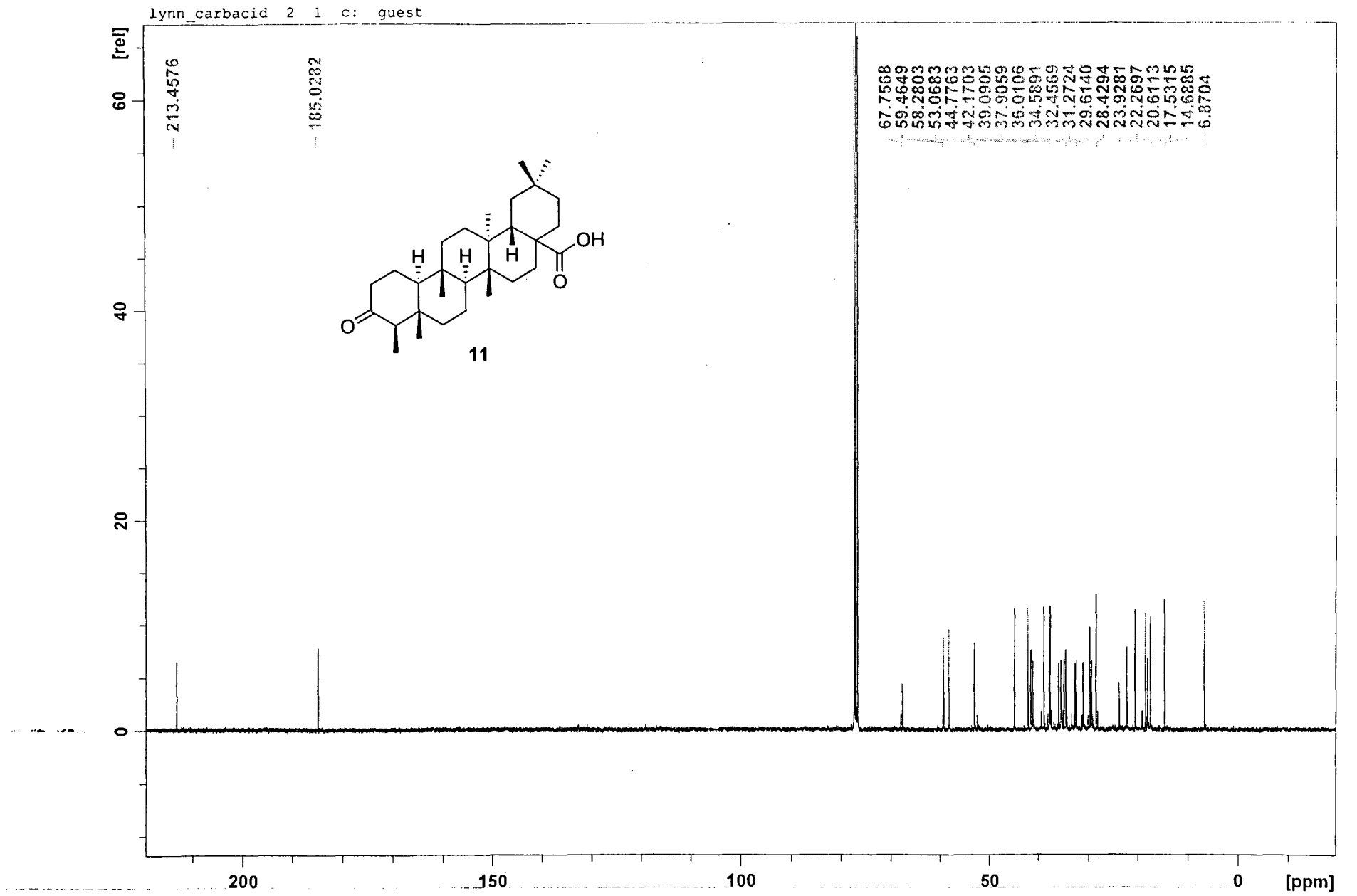
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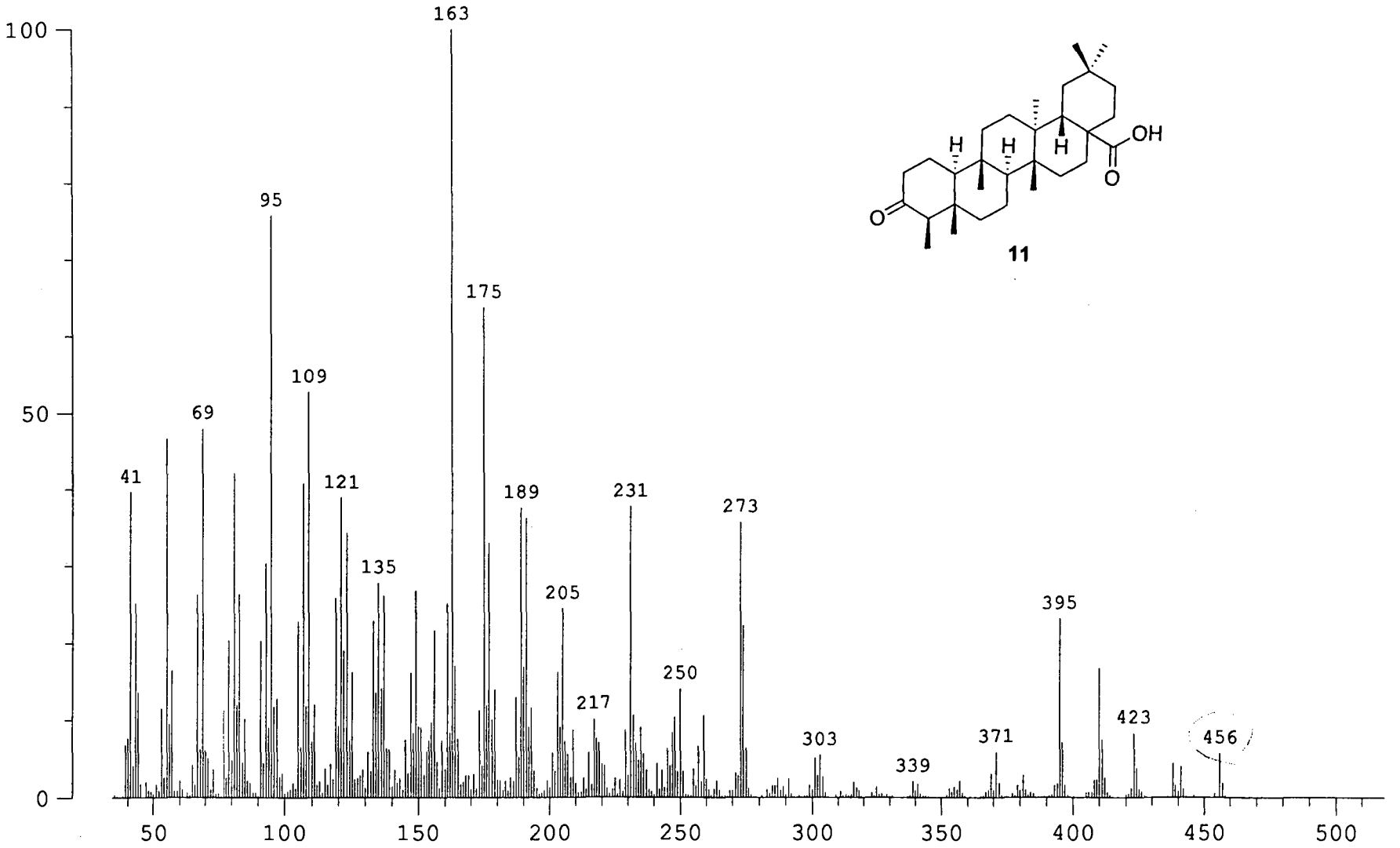

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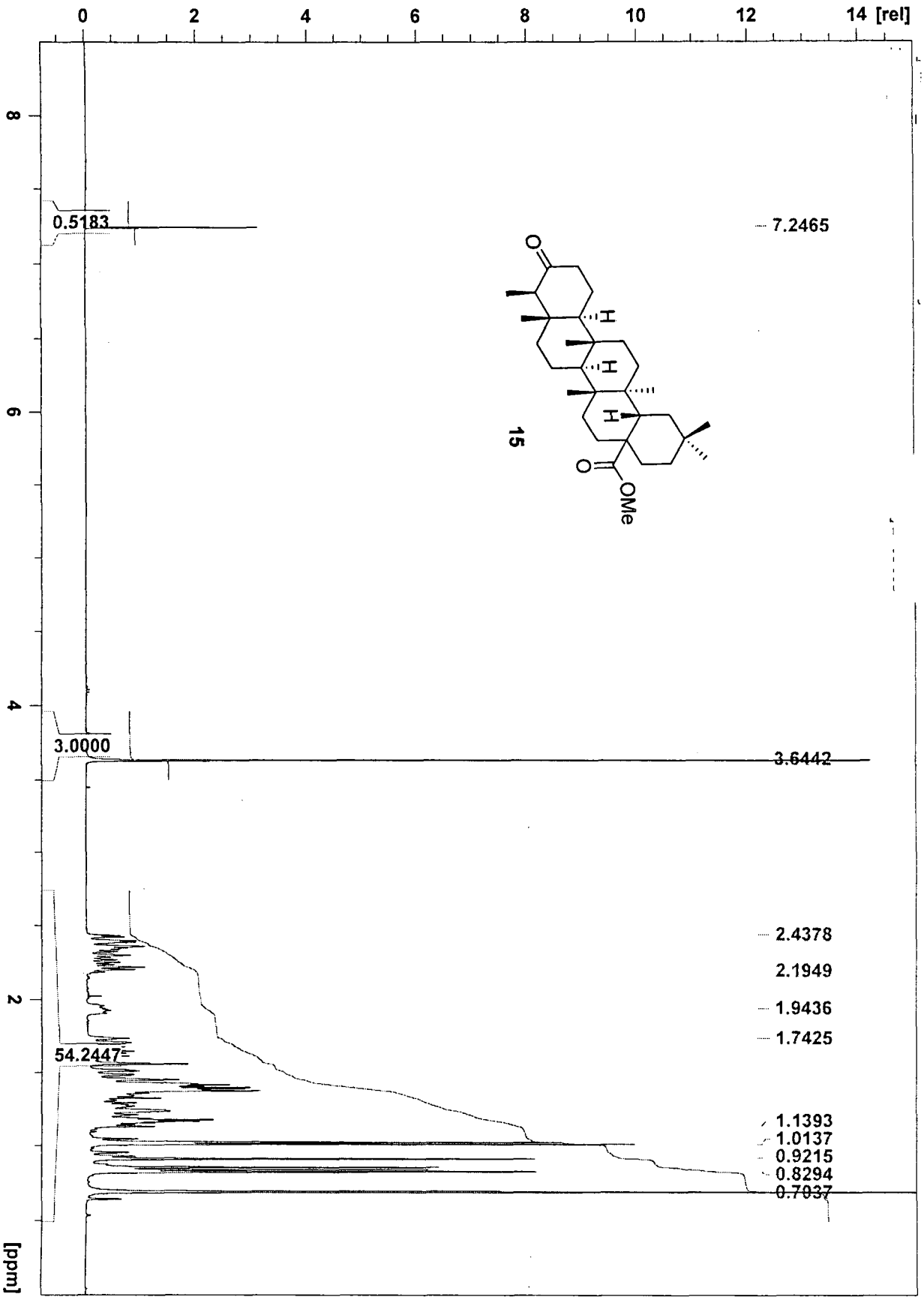
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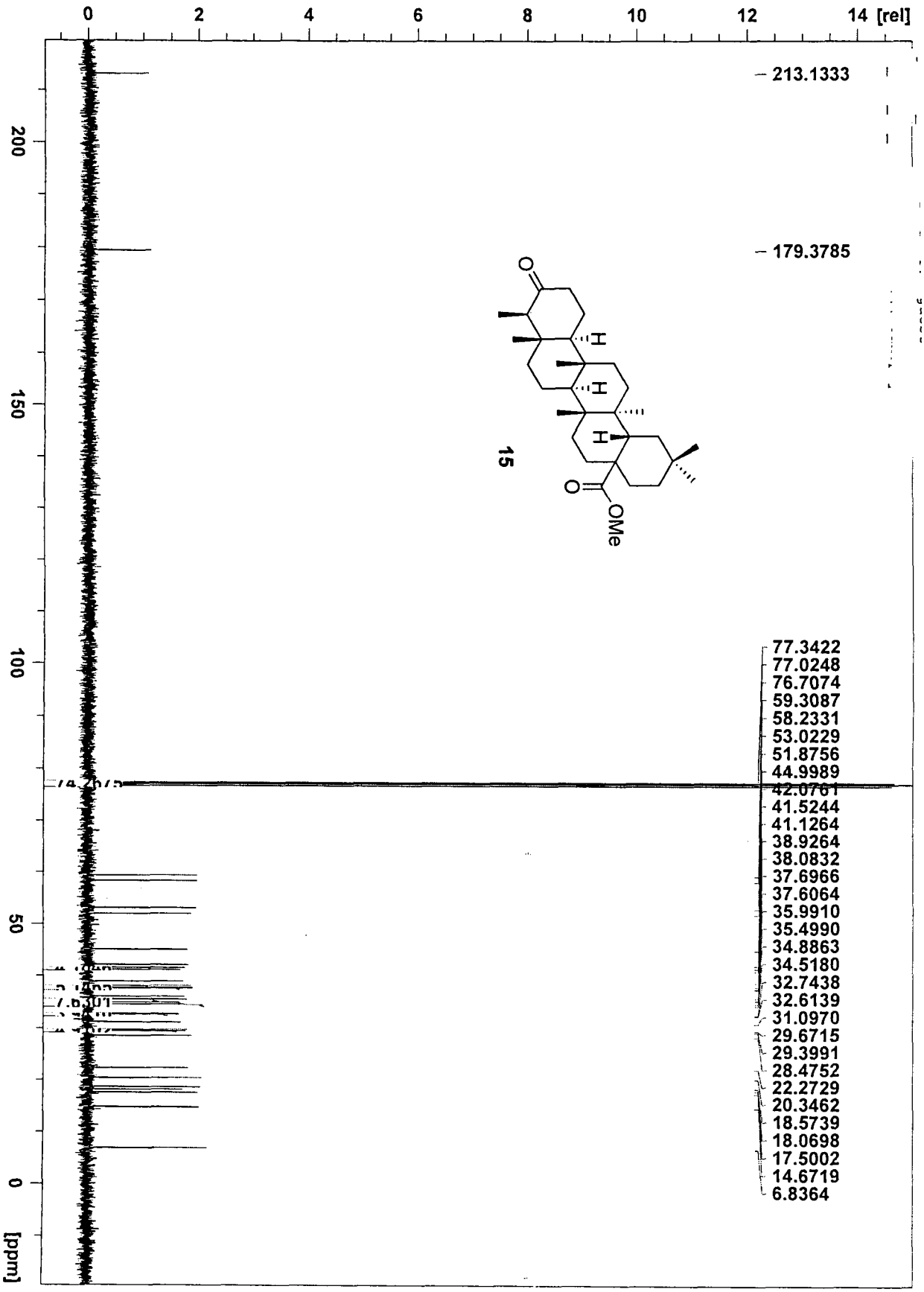
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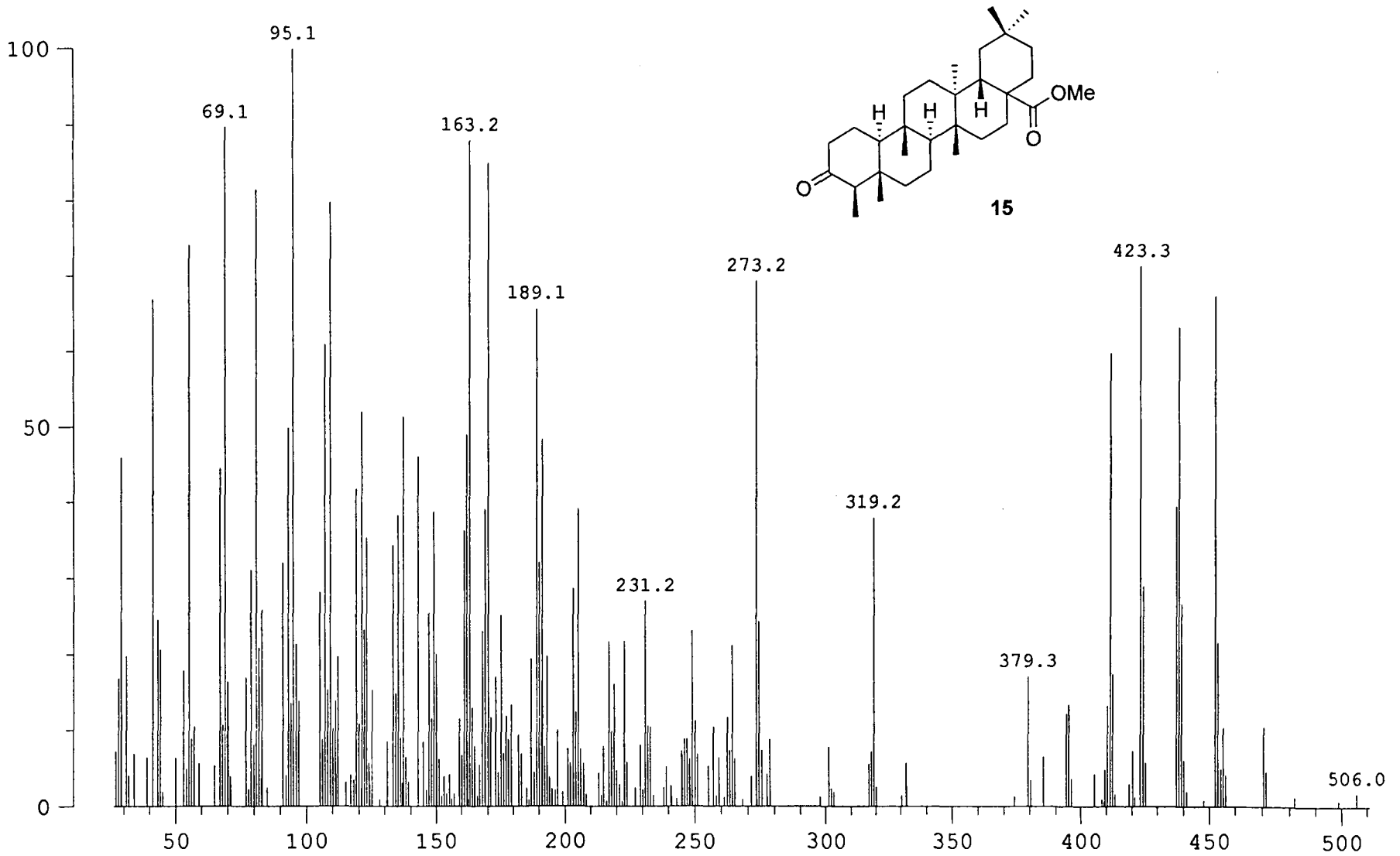

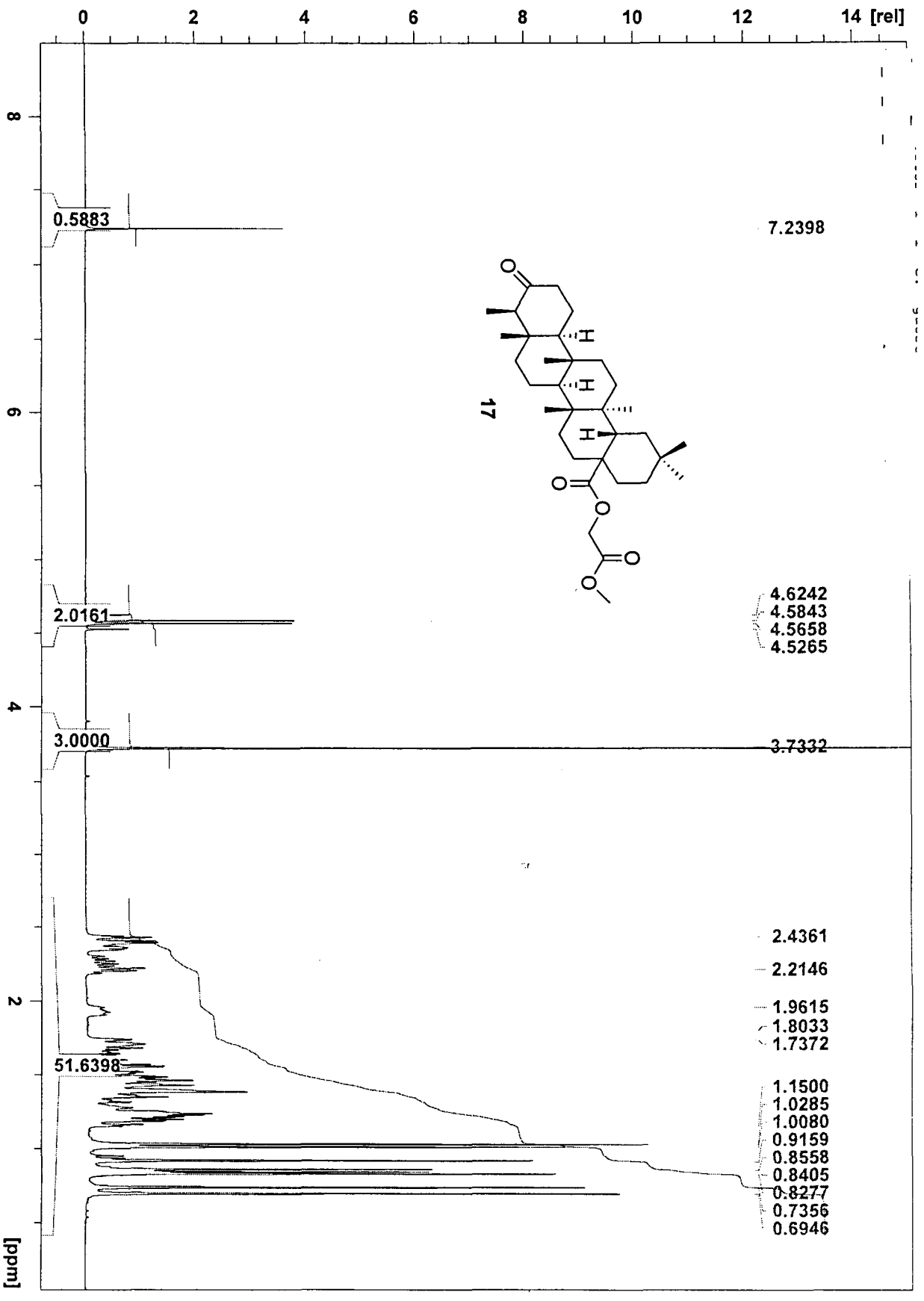
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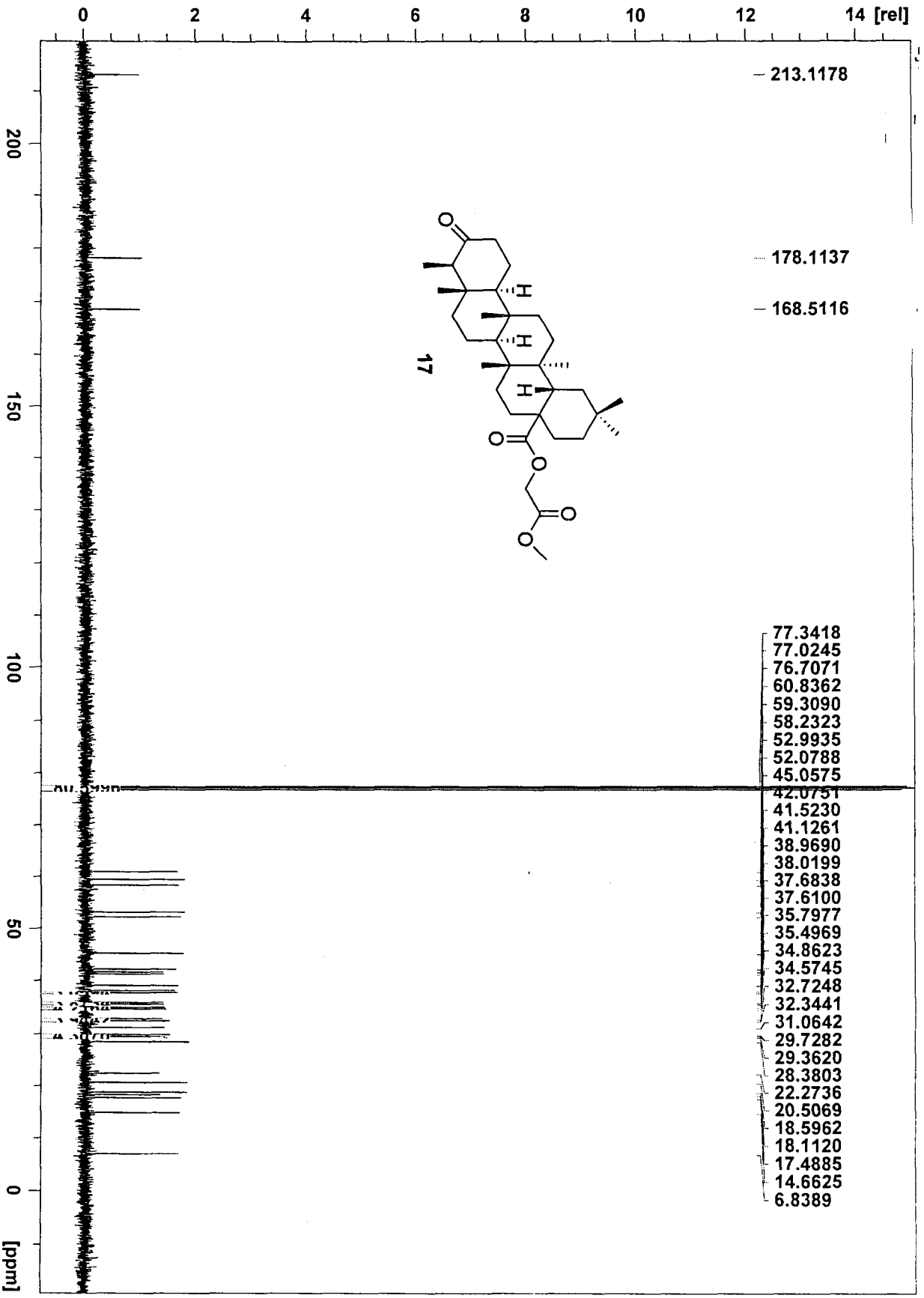
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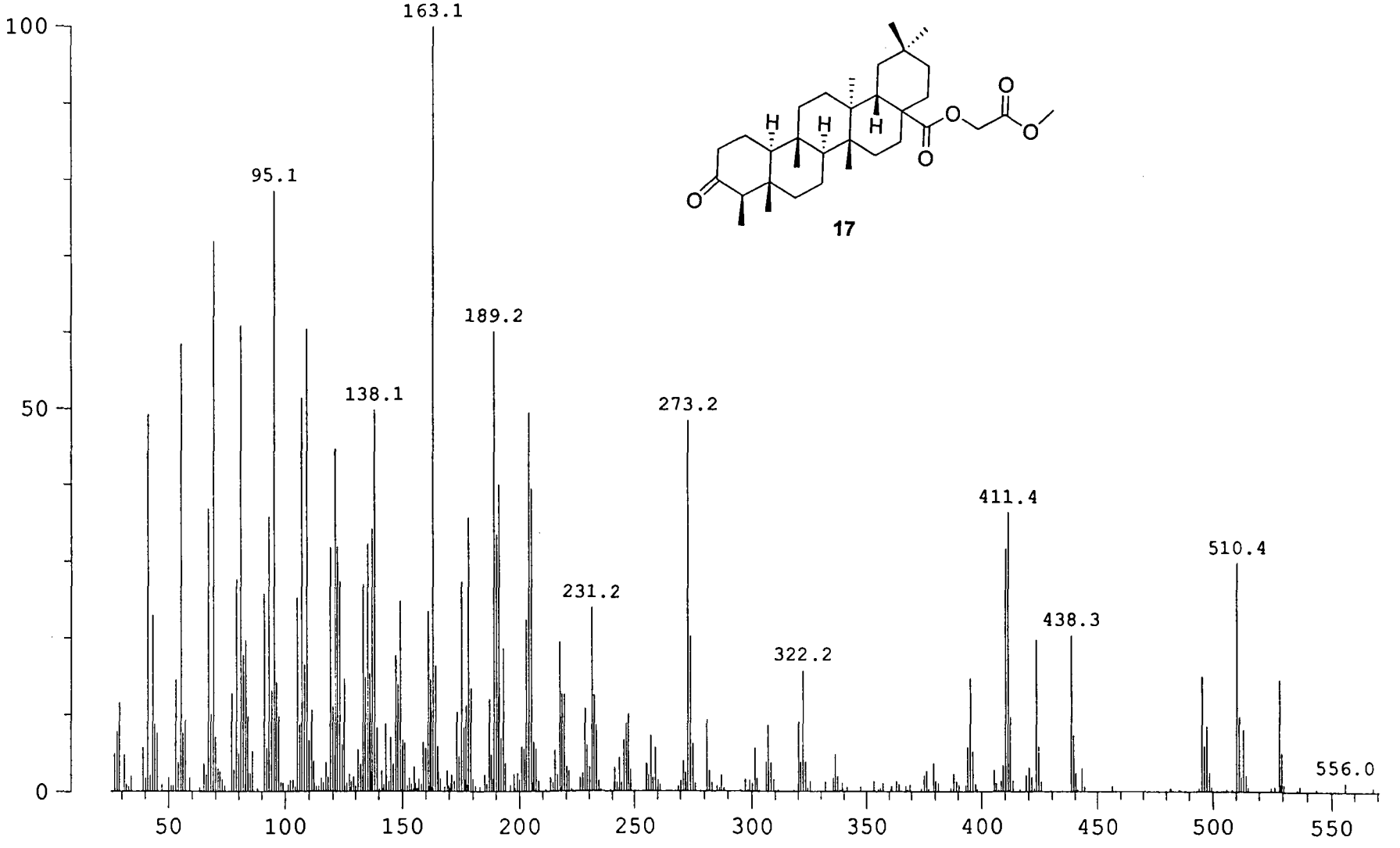
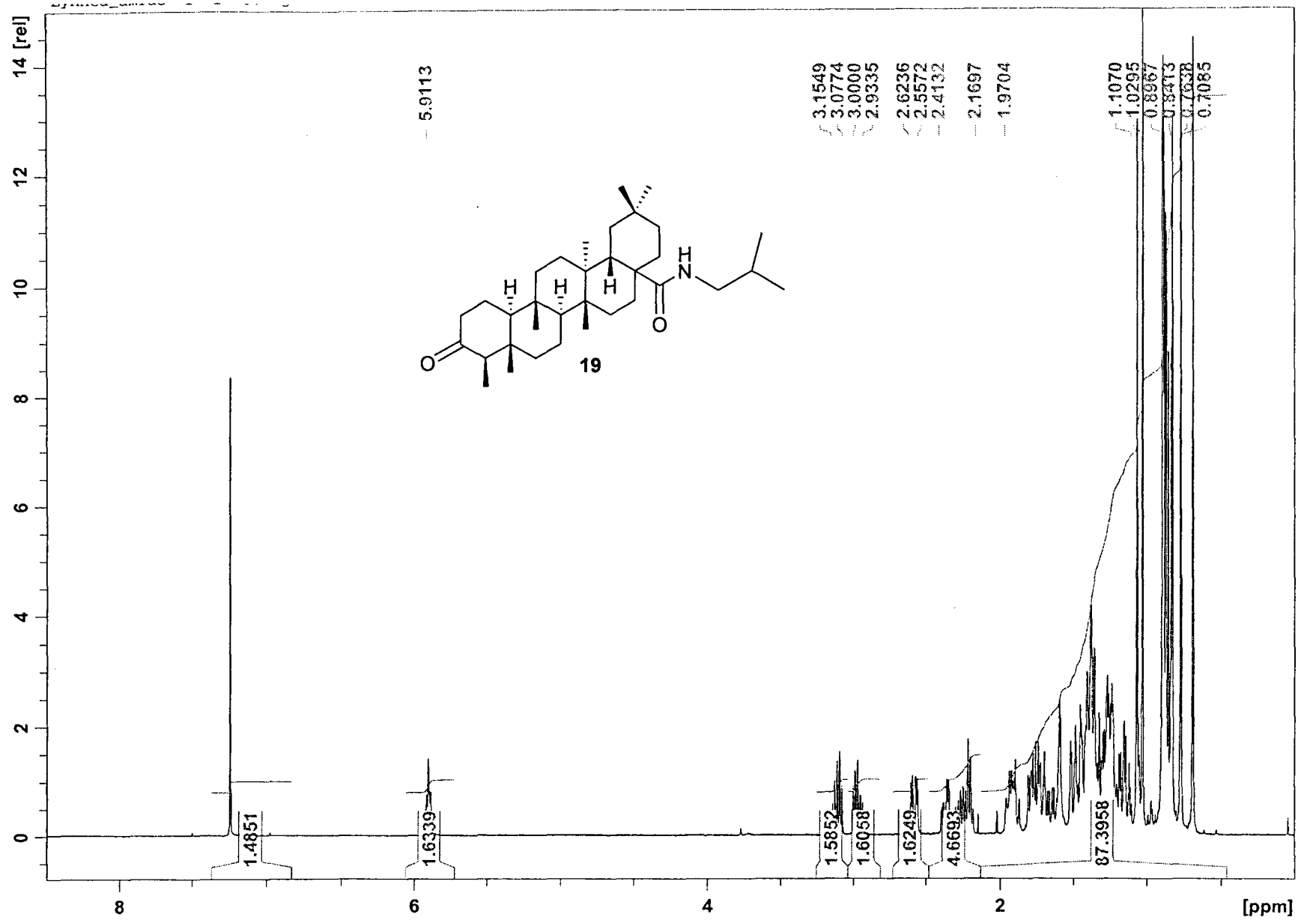
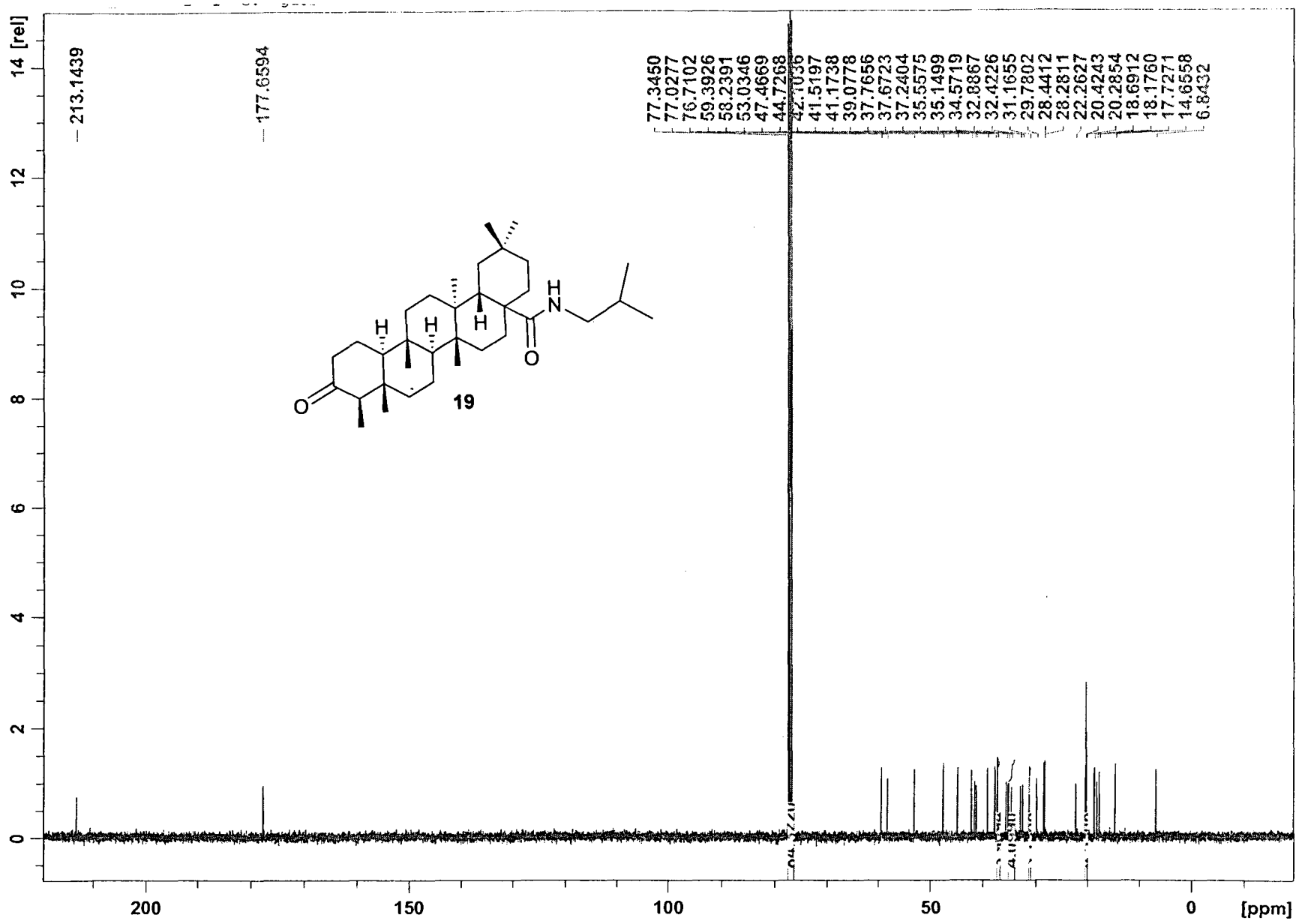
52.9935
52.0788
45.0575
42.0751
41.5230
41.1261
38.9690
38.0199
37.6838
37.6100
35.7977
35.4969
34.8623
34.5745
32.7248
32.3441
31.0642
29.7282
29.3620
28.3803
22.2736
20.5069
18.5962
18.1120
17.4885
14.6625
6.8389
200
150
100
50
0
[ppm]

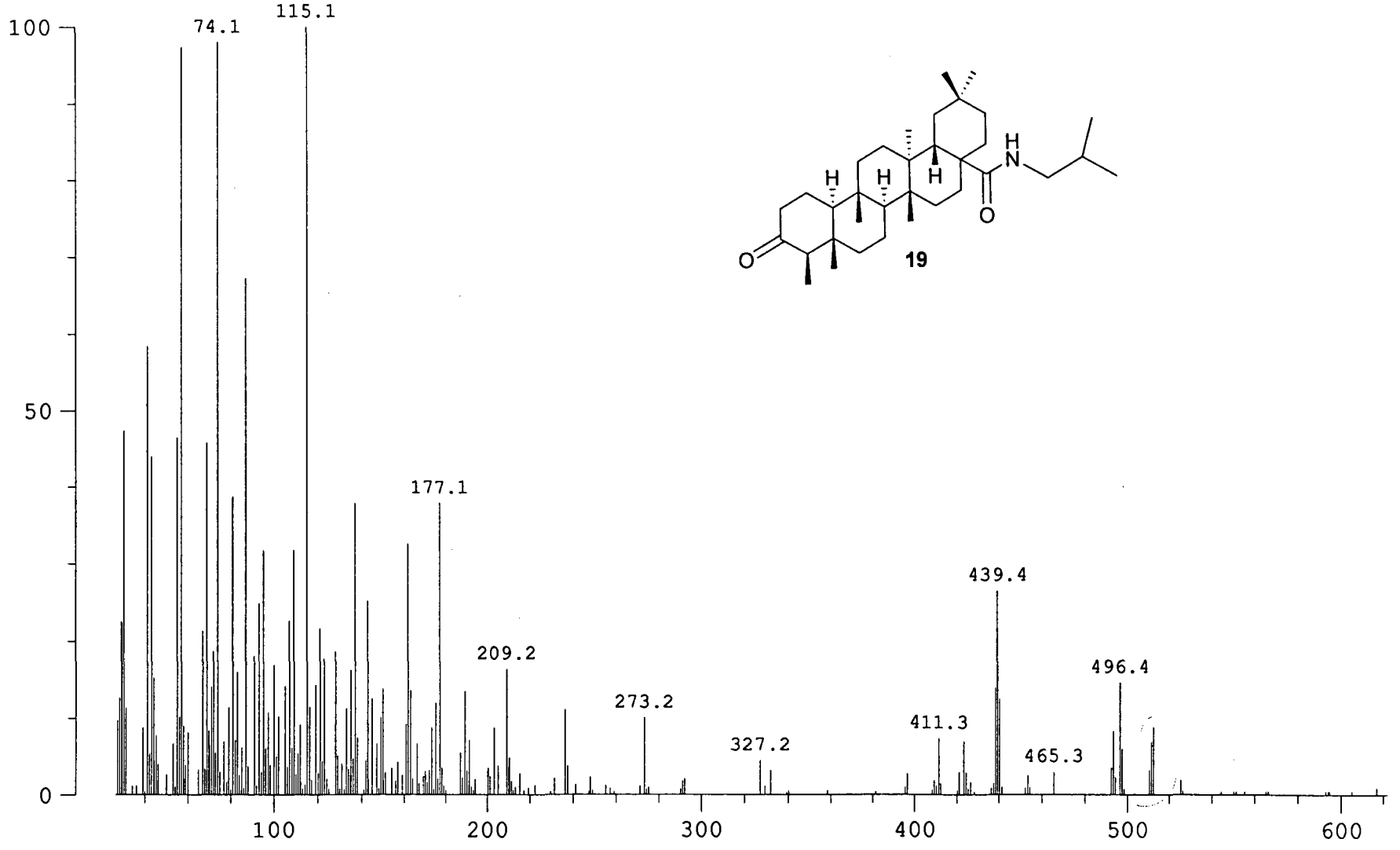
2b7599 Scan 6 RT=1:05 100%=52433 mv 21-Jun-2006 10:40
HRP +EI

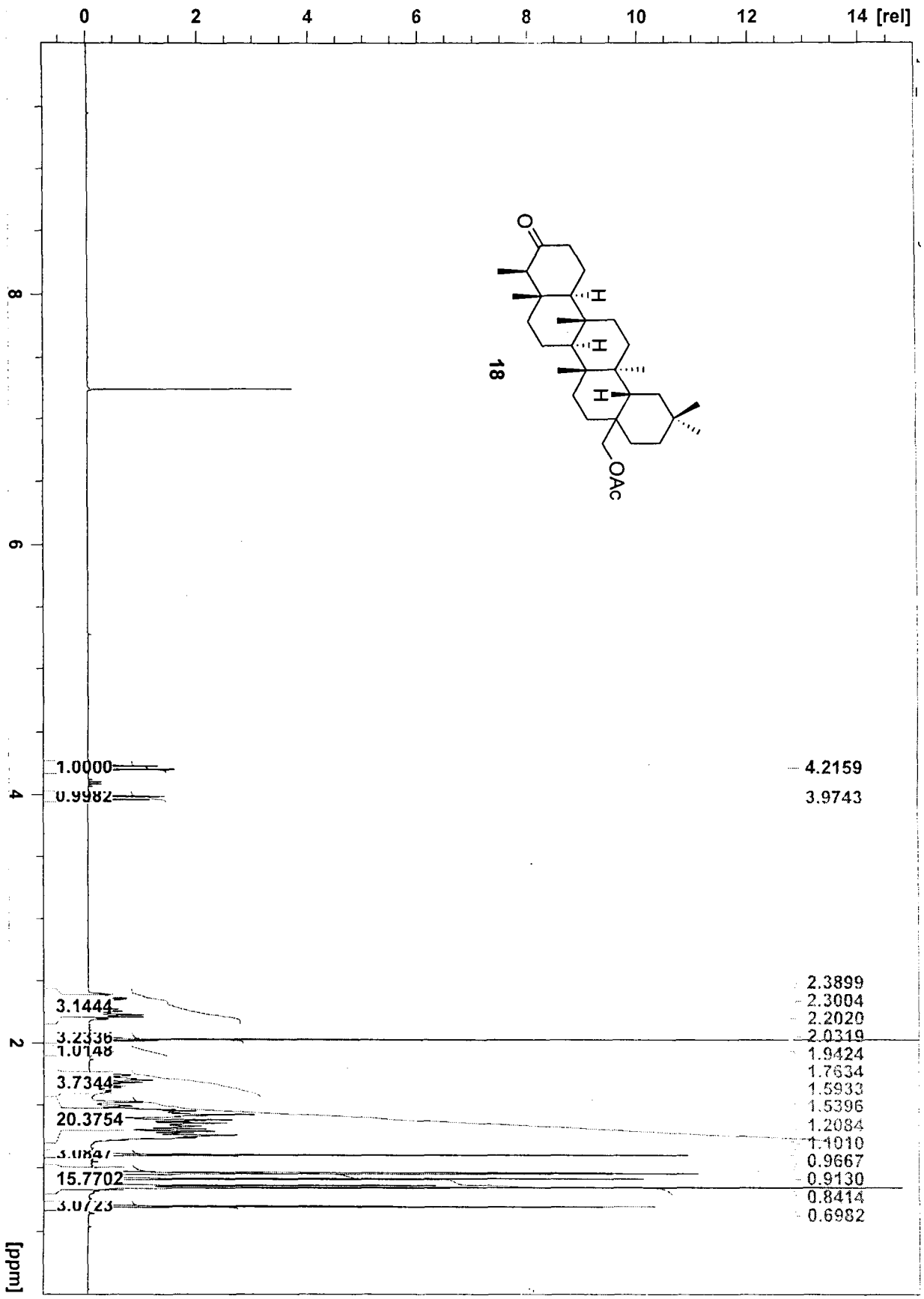


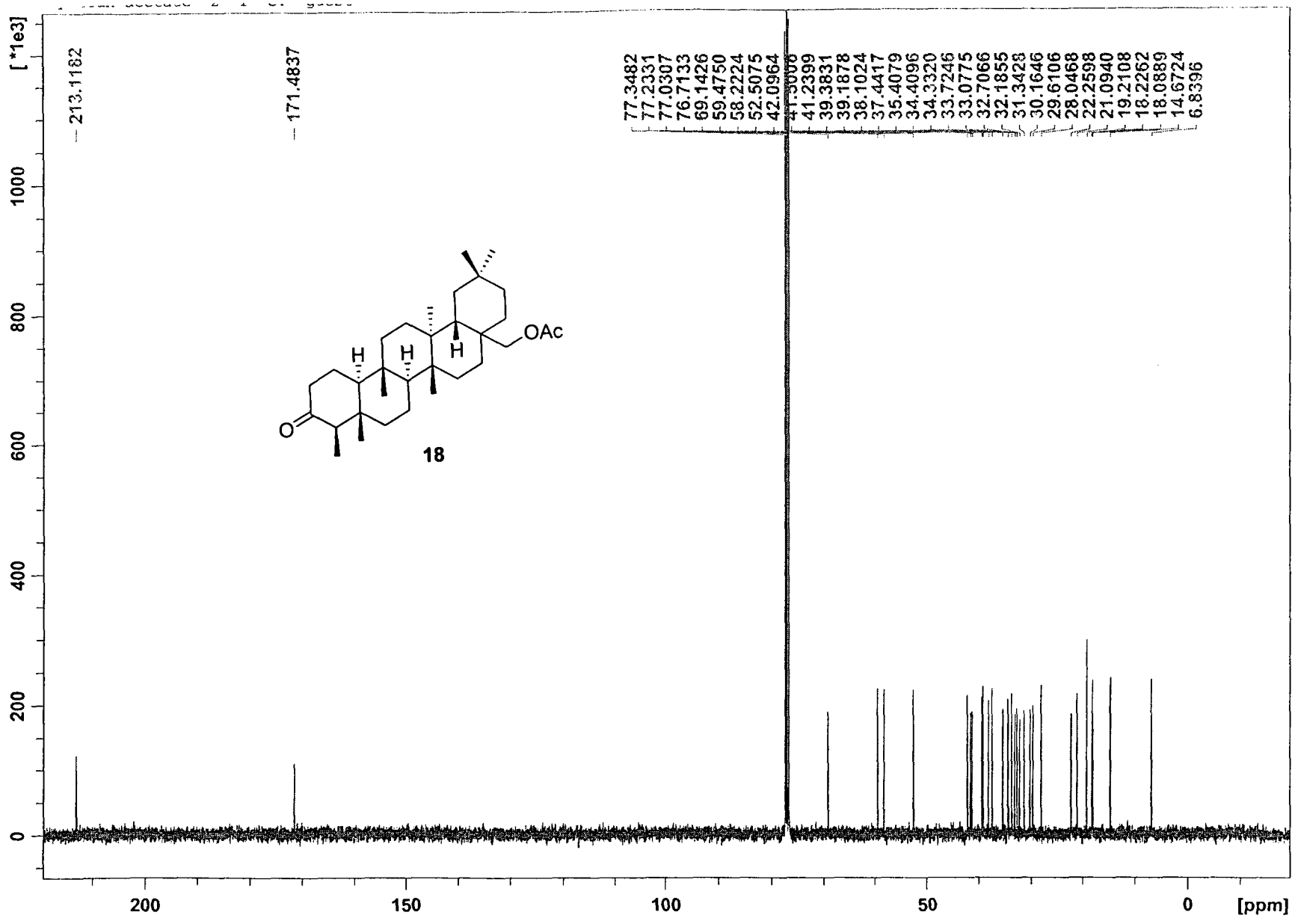

14 [rel]
12
10
8
6
4
2
0
8
6
4
2
[ppm]
5.9113
3.1549
3.0774
3.0000
2.9335
2.6236
2.5572
2.4132
2.1697
1.9704
1.1070
1.0295
0.8967
0.8413
0.7638
0.7085
1.4851
1.6339
1.5852
1.6058
1.6249
4.6693
87.3958
19


213.1439
177.6594
77.3450
77.0277
76.7102
59.3926
58.2391
53.0346
47.4669
44.7268
42.1036
41.5197
41.1738
39.0778
37.7656
37.6723
37.2404
35.5575
35.1499
34.5719
32.8867
32.4226
31.1655
29.7802
28.4412
28.2811
22.2627
20.4243
20.2854
18.6912
18.1760
17.7271
14.6558
6.8432
14 [rel]
12
10
8
6
4
2
0
200
150
100
50
0
[ppm]
H
H
H
N
H
O
O
19

2b7635 Scan 12 RT=2:17 100%=21619 mv 04-Jul-2006 08:24
HRP +EI

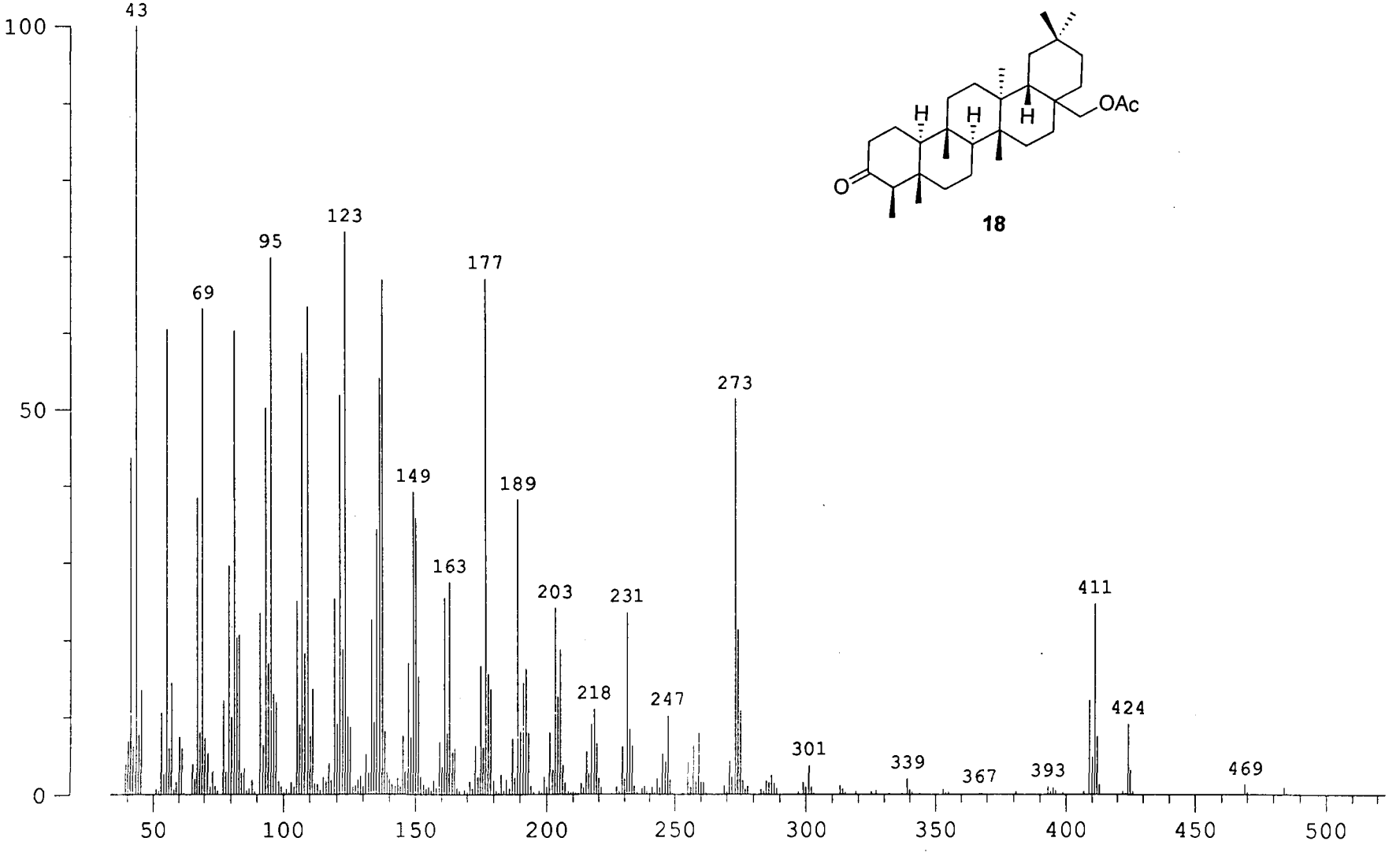



0
2
4
6
8
10
12
14 [rel]
8
6
4
2
[ppm]
18
O
H
H
H
OAc
1.0000
0.9982
3.1444
3.2336
1.0148
3.7344
20.3754
15.7702
4.2159
3.9743
2.3899
2.3004
2.2020
2.0319
1.9424
1.7634
1.5933
1.5396
1.2084
1.1010
0.9667
0.9130
0.8414
0.6982

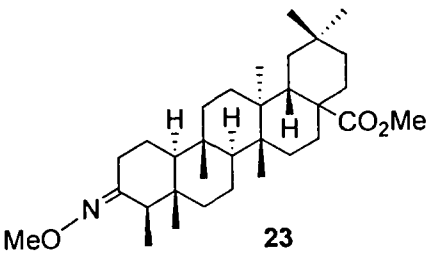

213.1182
171.4837
77.3482
77.2331
77.0307
76.7133
69.1426
59.4750
58.2224
52.5075
42.0964
41.3008
41.2399
39.3831
39.1878
38.1024
37.4417
35.4079
34.4096
34.3320
33.7246
33.0775
32.7066
32.1855
31.3423
30.1646
29.6106
28.0468
22.2598
21.0940
19.2108
18.2262
18.0889
14.6724
6.8396
[*1e3]
1000
800
600
400
200
0
200
150
100
50
0
[ppm]
OAc
O
H
H
H

18

1h3475 Scan 27 RT=0:43 100%=134501 mv 10-Mar-2006 10:38
LRP +EI

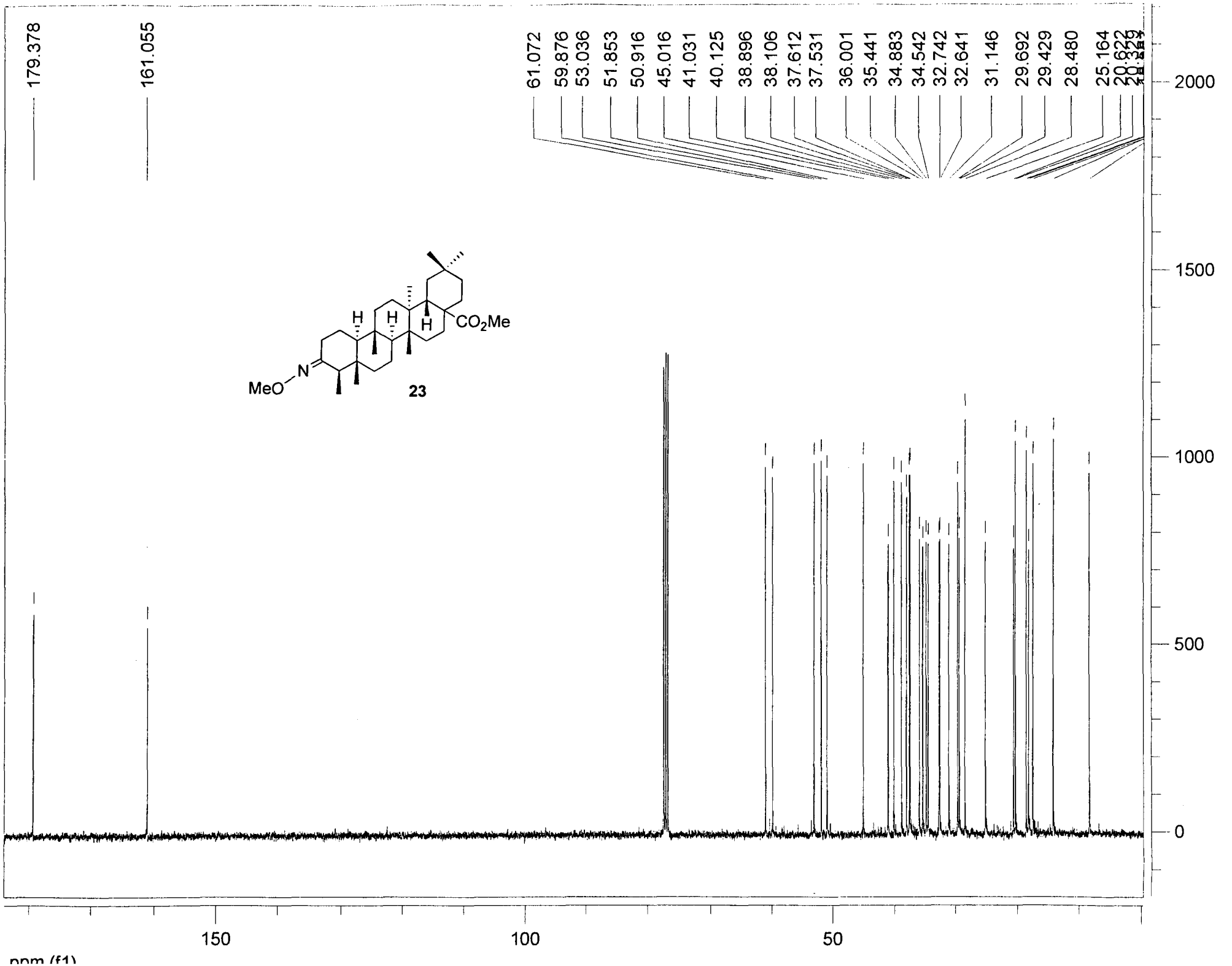


18

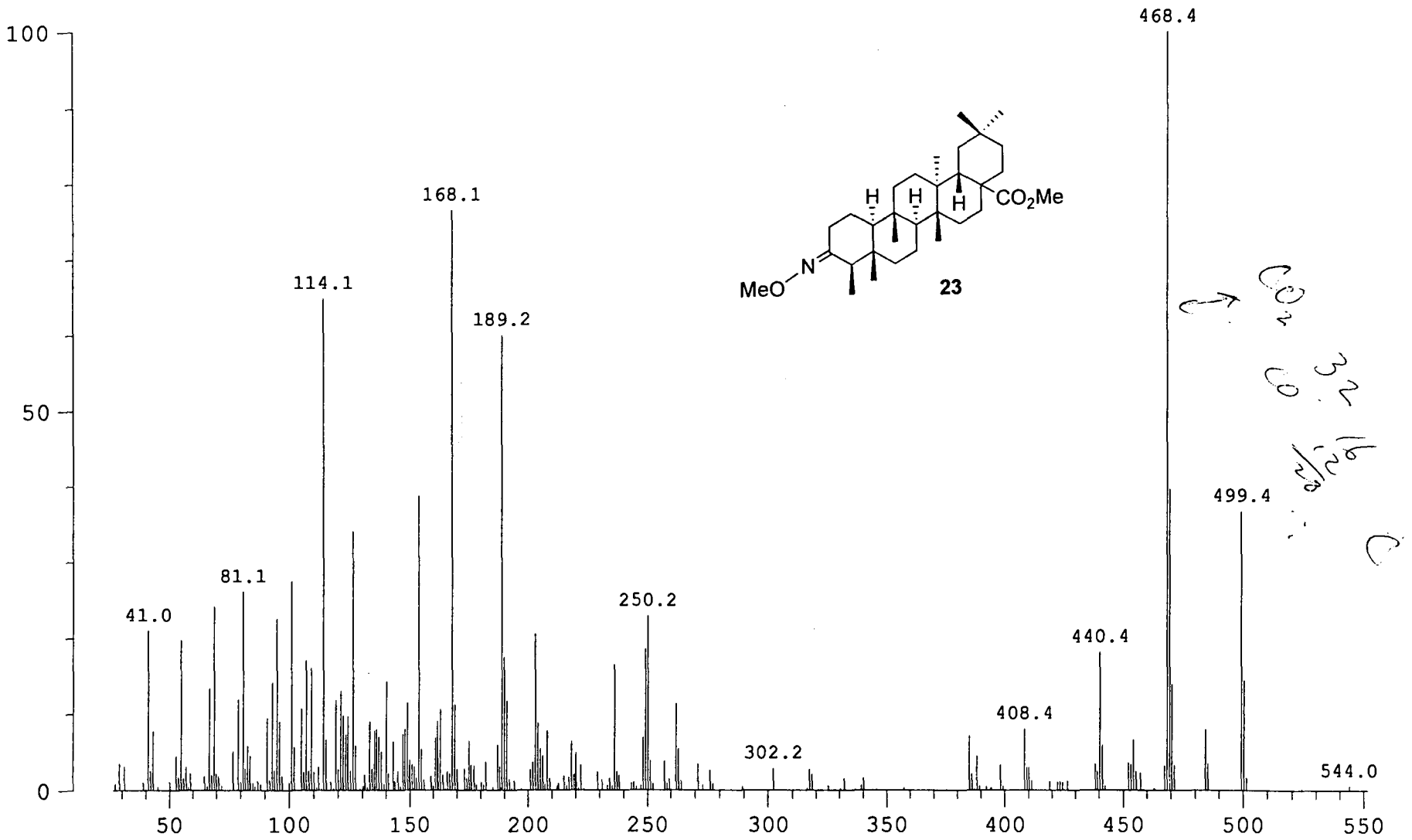

H
H
H
CO2Me
MeO
N

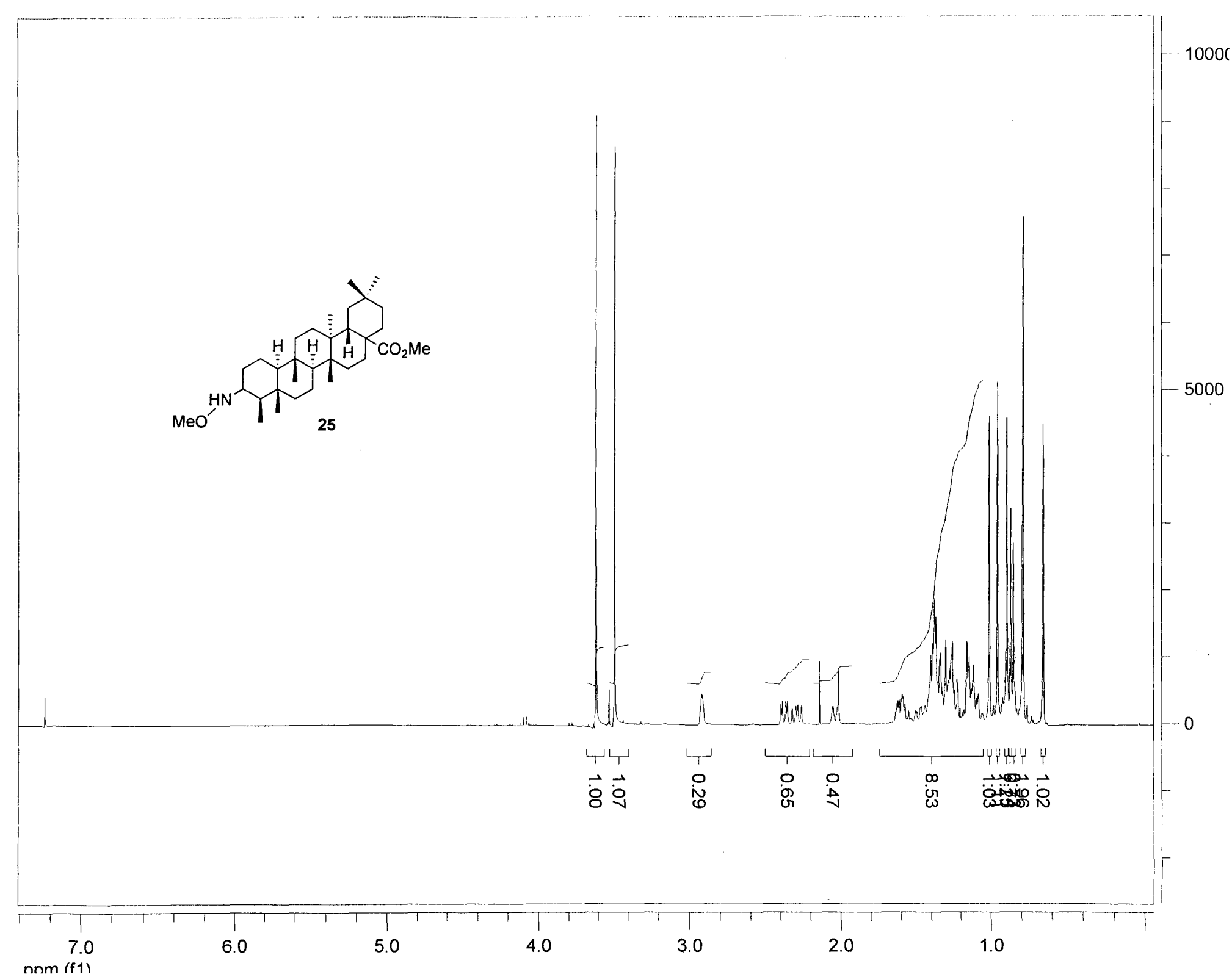
23

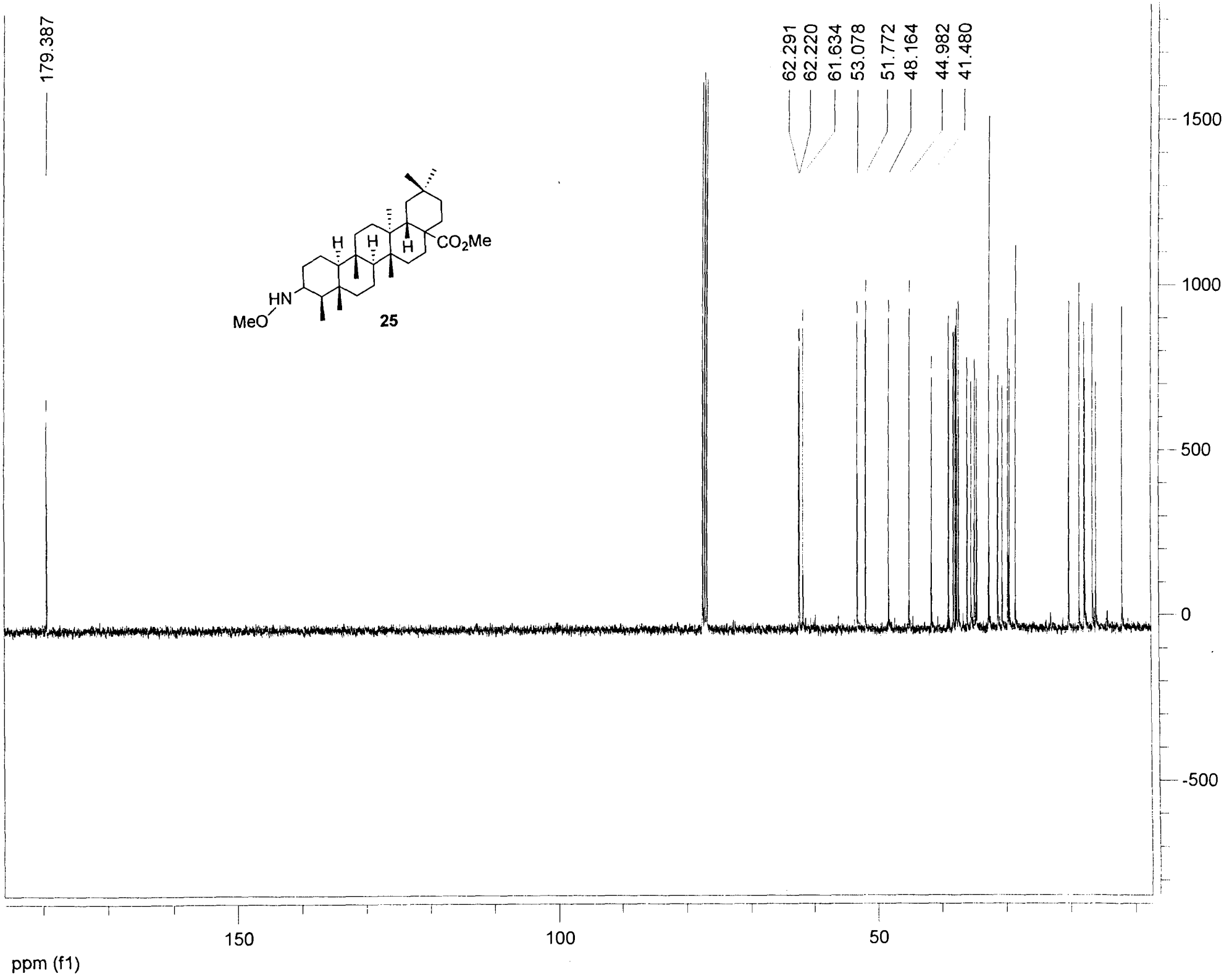
10000
5000
0
1.00
1.00
0.31
0.67
0.04
0.39
8.16
2.10
2.24
1.21
2.08
7.0
6.0
5.0
4.0
3.0
2.0
1.0
ppm (f1)


179.378
161.055
61.072
59.876
53.036
51.853
50.916
45.016
41.031
40.125
38.896
38.106
37.612
37.531
36.001
35.441
34.883
34.542
32.742
32.641
31.146
29.692
29.429
28.480
25.164
2000
1500
1000
500
0
150
100
50
ppm (f1)
MeO
N
H
H
H
CO2Me
23

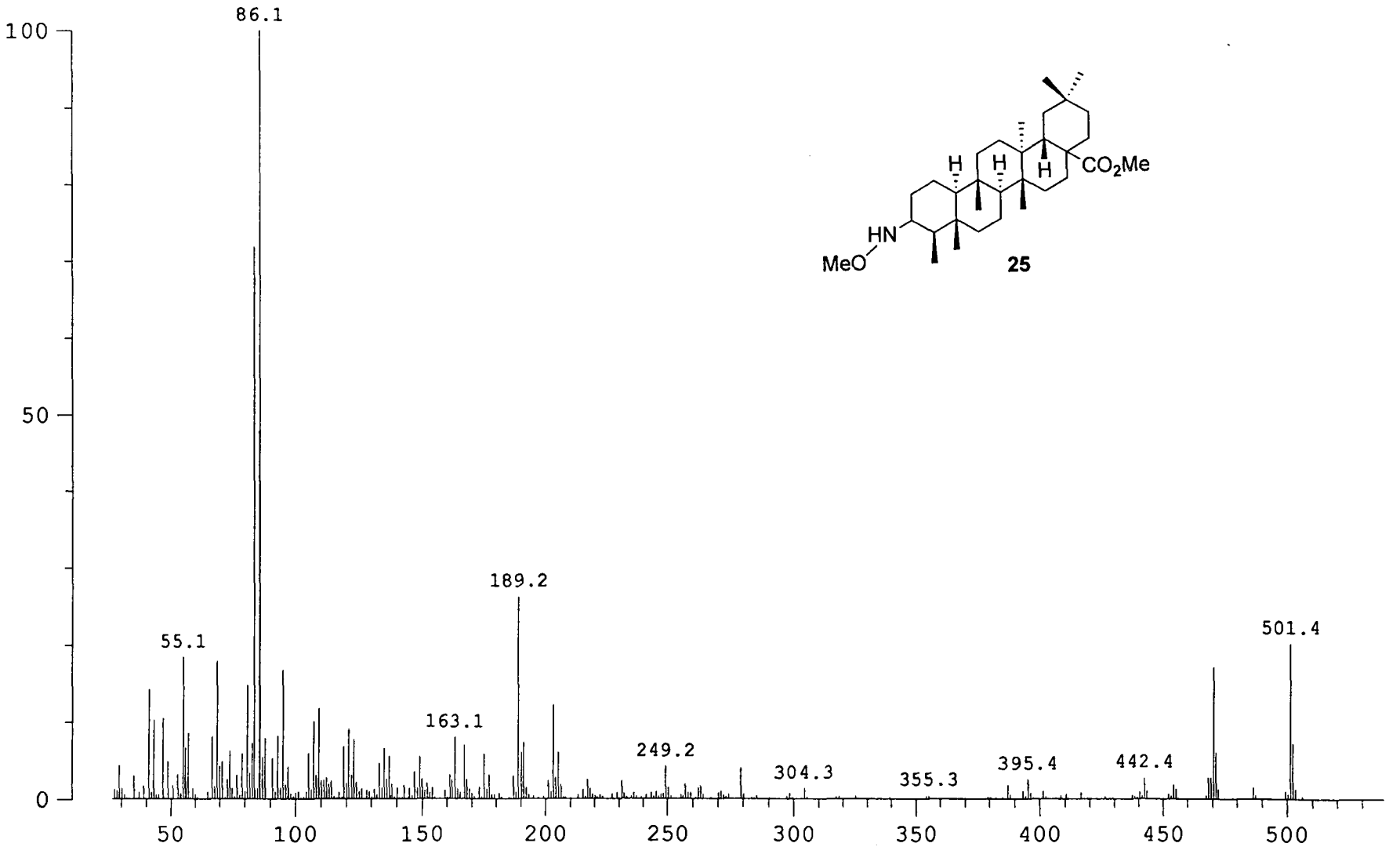
2b7819 Scan 10 RT=1:54 100%=45006 mv 03-Aug-2006 08:12
HRP +EI can3



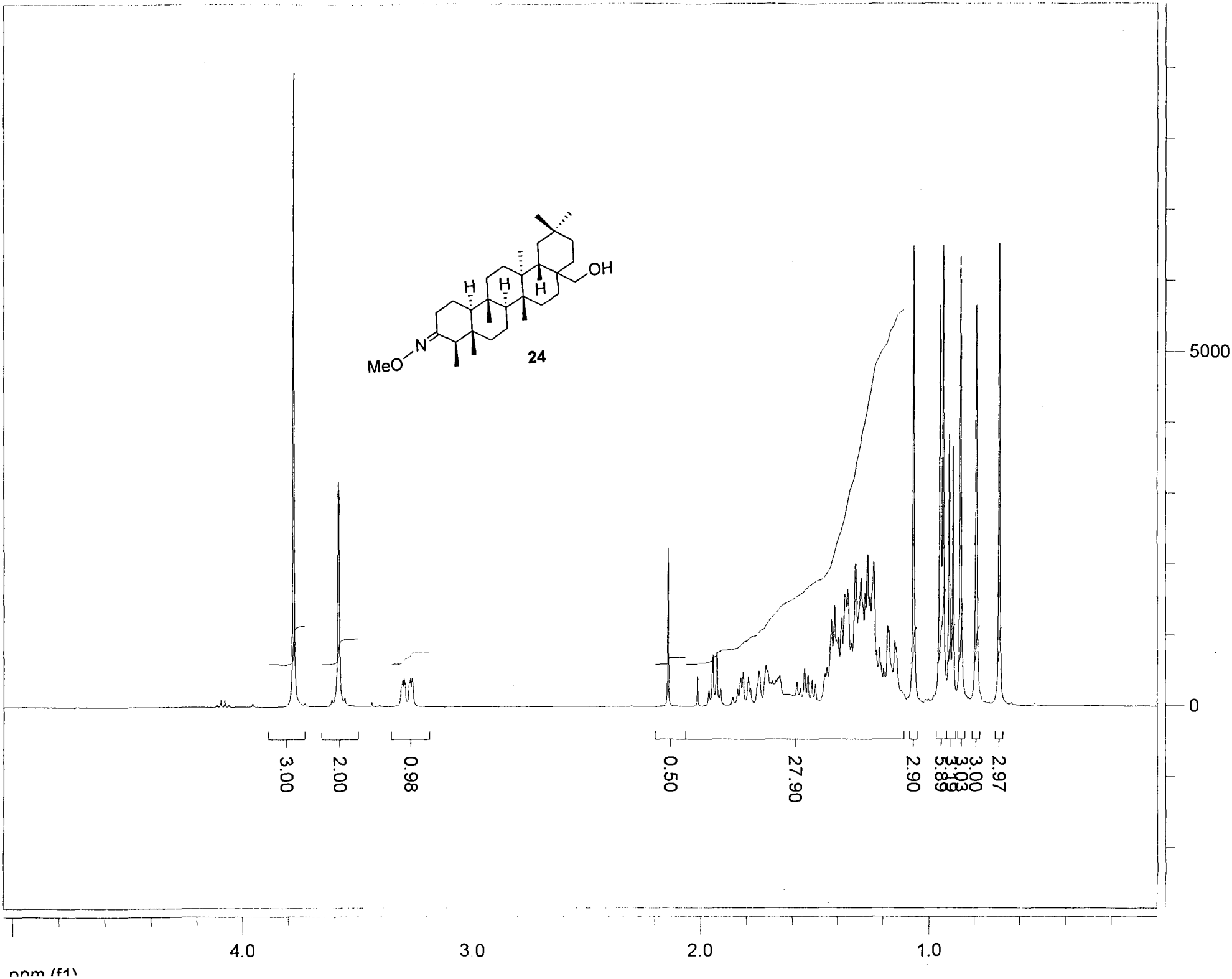

HN
MeO
H
H
H
CO2Me
25
10000
5000
0
1.00
1.07
0.29
0.65
0.47
8.53
1.03
1.02
7.0
6.0
5.0
4.0
3.0
2.0
1.0
ppm (f1)

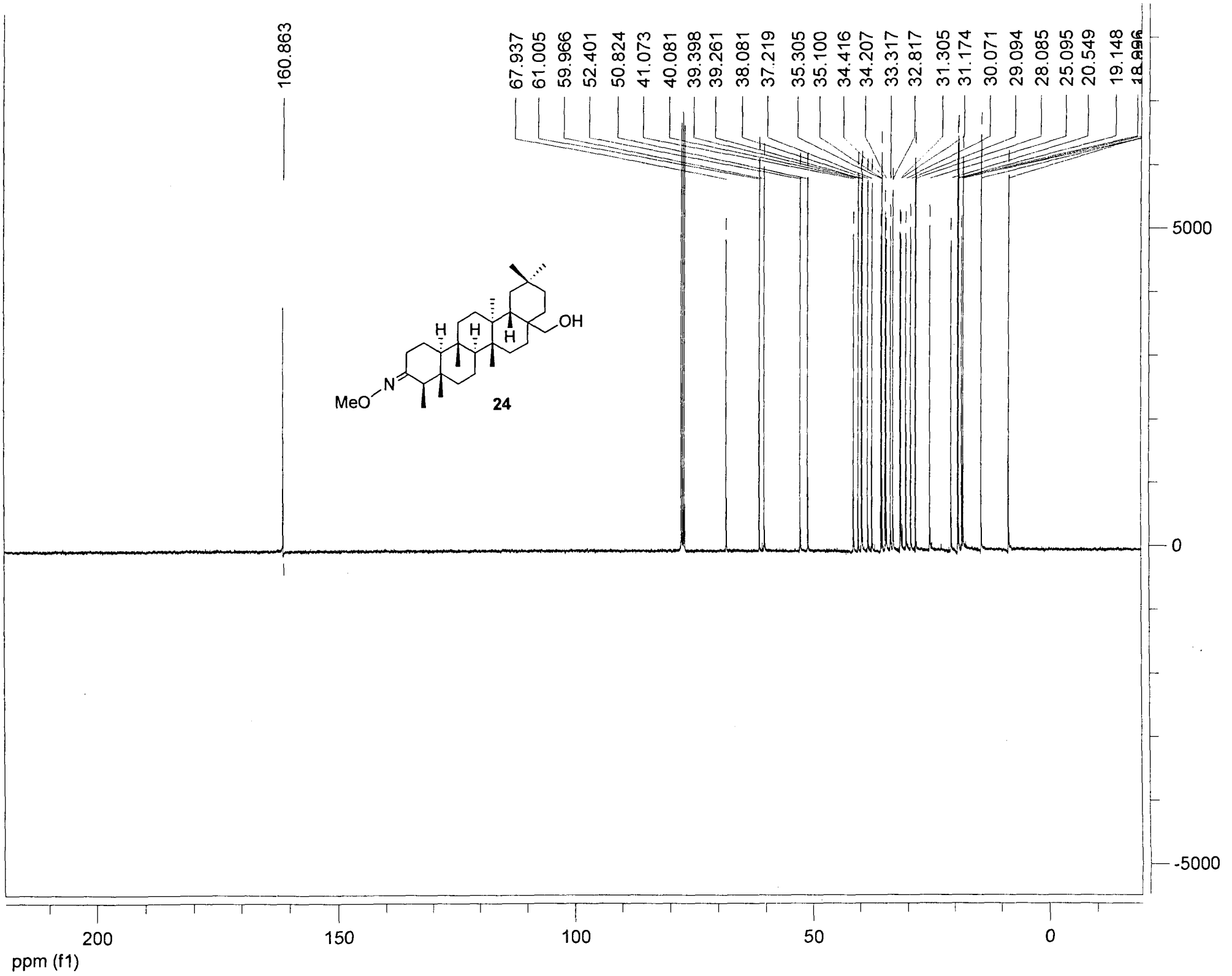

179.387
62.291
62.220
61.634
53.078
51.772
48.164
44.982
41.480
1500
1000
500
0
-500
150
100
50
ppm (f1)
HN
MeO
CO2Me
25

2b7890 Scan 4 RT=0:39 100%=153561 mv 31-Aug-2006 08:51
HRP +EI can2bor1



25


OH
MeO
N
24
5000
0
3.00
2.00
0.98
0.50
27.90
2.90
5.89
3.03
3.00
2.97
4.0
3.0
2.0
1.0
ppm (f1)


160.863
67.937
61.005
59.966
52.401
50.824
41.073
40.081
39.398
39.261
38.081
37.219
35.305
35.100
34.416
34.207
33.317
32.817
31.305
31.174
30.071
29.094
28.085
25.095
20.549
19.148
5000
0
-5000
200
150
100
50
0
ppm (f1)
OH
H
H
H
N
MeO

24

2b7781 Scan 8 RT=1:24 100%=44932 mv 31-Jul-2006 08:25
HRP +EI can01