Phytochemical Studies on *Anthemis tigreensis*

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By

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Addis Ababa University

Office of Research and Graduate Programme

Phytochemical Studies on *Anthemis tigreensis*

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Dedicated to

My Mother

&

Father

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Abstract

In this thesis, a phytochemical study of *Anthemis tigreensis* has been undertaken and chemical investigation on the essential oil and solvent extract of the plant has been conducted. There are no reports on the phytochemical study of the plant prior to this work.

A. tigreensis in the family Asteraceae, in the genuse *Anthemis* is the only species found in Ethiopia. There are 20 species in the genus *Anthemis* distributed in Southern Europe, South West Asia, and North Africa. *A. tigreensis* has medicinal value and is important in traditional medicine like the other species in the genus.

The essential oil obtained by hydrodistillation was investigated by GC, GC-MS, and NMR spectroscopy. Ten compounds constituting 66% of the oil were identified, with bornyl acetate as the major component. To the best of our knowledge, bornyl acetate (48%) is reported here for the first time as a major component of the oil of *Anthemis* species.

Three sesquiterpene lactones **At-8** (2E,6E)-8-(tetrahydro-4-methylene-5-oxofuran-3-yl)-2,6-dimethylocta-2,6-dienal, **At-11** Antheindurolide A, and **At-7** dihydro-5-((2-(hydroxymethyl)-6-methyl-3-methylene-7-oxa-bicyclo[4.1.0]hept-4-en-1-yl)methyl)-3-methylfuran-2(3H)-one were isolated and characterized from the solvent extract of the plant. 1D and 2D-NMR, UV, IR and MS spectroscopic techniques were used for characterization of the compounds. To the best our knowledge, the sesquiterpene lactone, **At-8** is reported for the first time. **At-11** was identified as Antheindurolide A, a sesquiterpene lactone isolated previously from *A. pseeudocotula*.

1. Introduction

1.1 Importance of Natural products

The term 'natural product' is commonly used for those organic compounds of natural origin that are unique to one organism, or common to small number of closely related organisms. Natural products are secondary metabolites of an organism. In most instances, they appear to be non-essential to the plant, insect, or microorganism producing them. This feature can be put as a marked contrast to the other organic compounds in nature, i.e.; sugars, amino acids, nucleotides, and the polymers derived from them, which are both essential and ubiquitous.[1]

1.2 Essential oils

Essential oils are one of the main classified parts of natural products. Essential oils are a mixture of fragrant, volatile compounds, named after the aromatic plant material of a single type. All pure essential oils are both antiseptic and volatile. The most appealing uses are in aromatherapy and in luxury perfumery. They are also used in food, beverage and confectionary industries.[2]

The use of essential-oil containing plants for their pleasant fragrance is as old as human civilization. Incense and myrrh are the oldest known aromatic materials; they are mentioned in Assyrian clay tablets.[1] Aromatic materials were wide spread in Pharaonic Egypt as fragrance materials and to embalm corpses. Their role in embalming is well documented and the compounds used can be detected even today. The early use of fragrance materials is also well documented in the Indian Vedic literature. Hundreds of aromatic substances and their uses (mainly religious and medicinal) have been listed in other classical sources such as the Gilgamesh Epic, the Bible and by Greek authors such as Herodotus and Hippocrates. Theophrastus (372 – 287 BC) gave the first detailed description of the procedure for preparing perfumes using extraction with fat. It is to Paracelsus (1493 – 1541) that we owe the term 'essential' in essential oil. He expounded the theory of the 'quinta essential', believing that this quintessence was the truly effective element in a medical preparation; essential oil isolation is an important goal of pharmaceutical science to this day (Teisseire, 1994).[1]

1.2.1 Methods of Producing Essential Oils

Three distinct processes are used to produce essential oils: solvent extraction, expression and distillation. In addition to these, now a days Supercritical Fluid Extraction (SFE) is used to extract the essential oils.

Solvent extraction is an industrial process that is used for fragile flowers, where the heat and pressure used in the distillation process would destroy the essential oil. Highly purified, volatile hydrocarbons are used to dissolve aroma compounds from plant material, followed by the removal of the solvent by distillation.

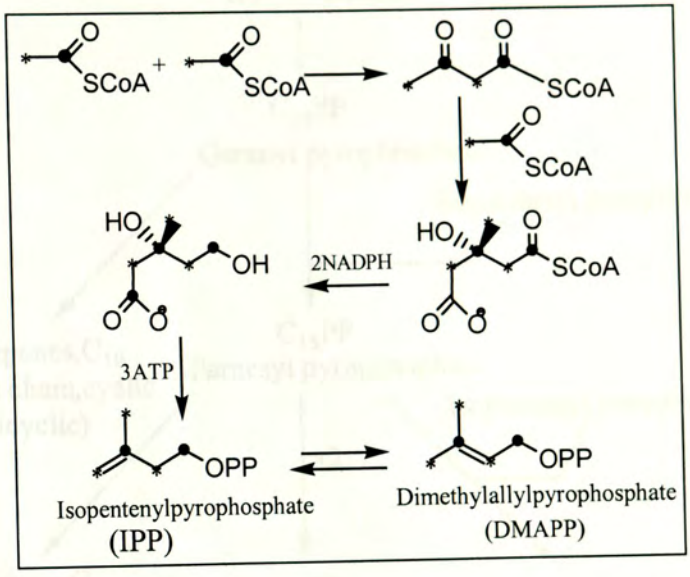
Expression is used to obtain the essential oils from the peel of citrus fruits. In expression method, the fruit peel is compressed, lacerated or abraded to rupture the oil cells in the exocarp and release the essential oil.

Several forms of distillation are applied to produce essential oils, the most important being water distillation, steam distillation and hydro diffusion. Water distillation or hydrodistillation is a very old process for the production of essential oil and has undergone centuries of improvement. The principle in water distillation is to boil and vaporize a suspension of aromatic plant material and water in a vat so that its vapors can be condensed and collected. The essential oil, which is immiscible with water, is then separated by gravity in a 'Florentine flask'. Steam distillation is a similar process, but hot steam is forced through the plant material to extract the essential oil. Hydrodiffusion is a recent process in which low-temperature, low-pressure steam is used to extract the essential oils.

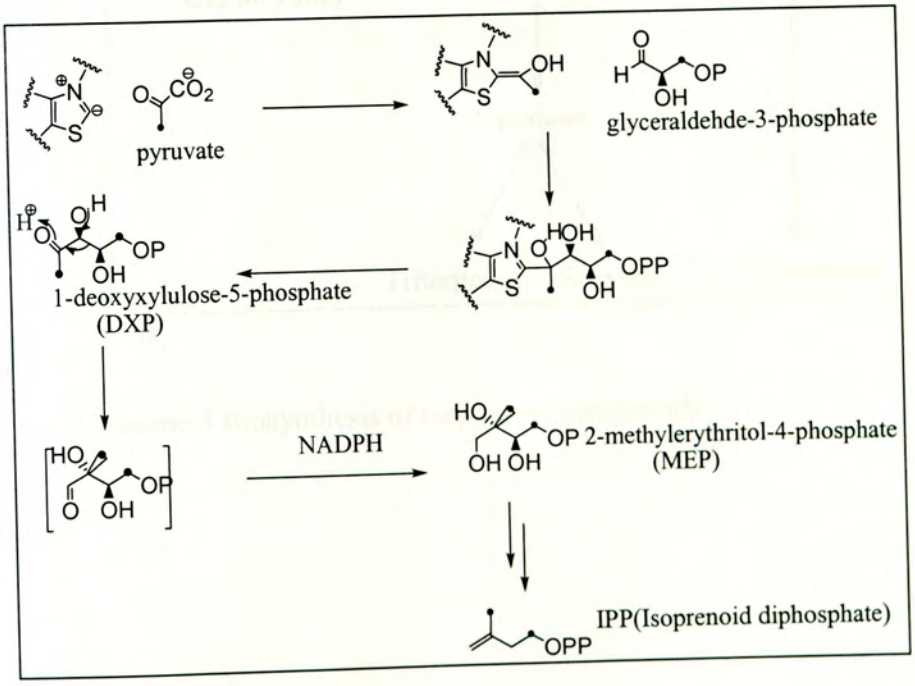
Extraction of the essential oils using supercritical fluid extraction (SFE) has gained increasing attention. Supercritical fluid, as its called, is heavy like liquid but with penetration power of gas. These qualities make supercritical fluids effective and selective solvents for the extraction of essential oils.

1.3 Biosynthesis of terpenoids

Isoprenoid diphosphate (IPP), the universal building block for all isoprenoids is formed by two different biosynthetic routes: the well known acetate/mevalonate pathway (Scheme 1) and the mevalonate-independent pathway starting from pyruvate and glyceraldehydes-3-phosphate (Scheme 2).[3] These five carbon intermediates are responsible for the formation of all the terpenes.

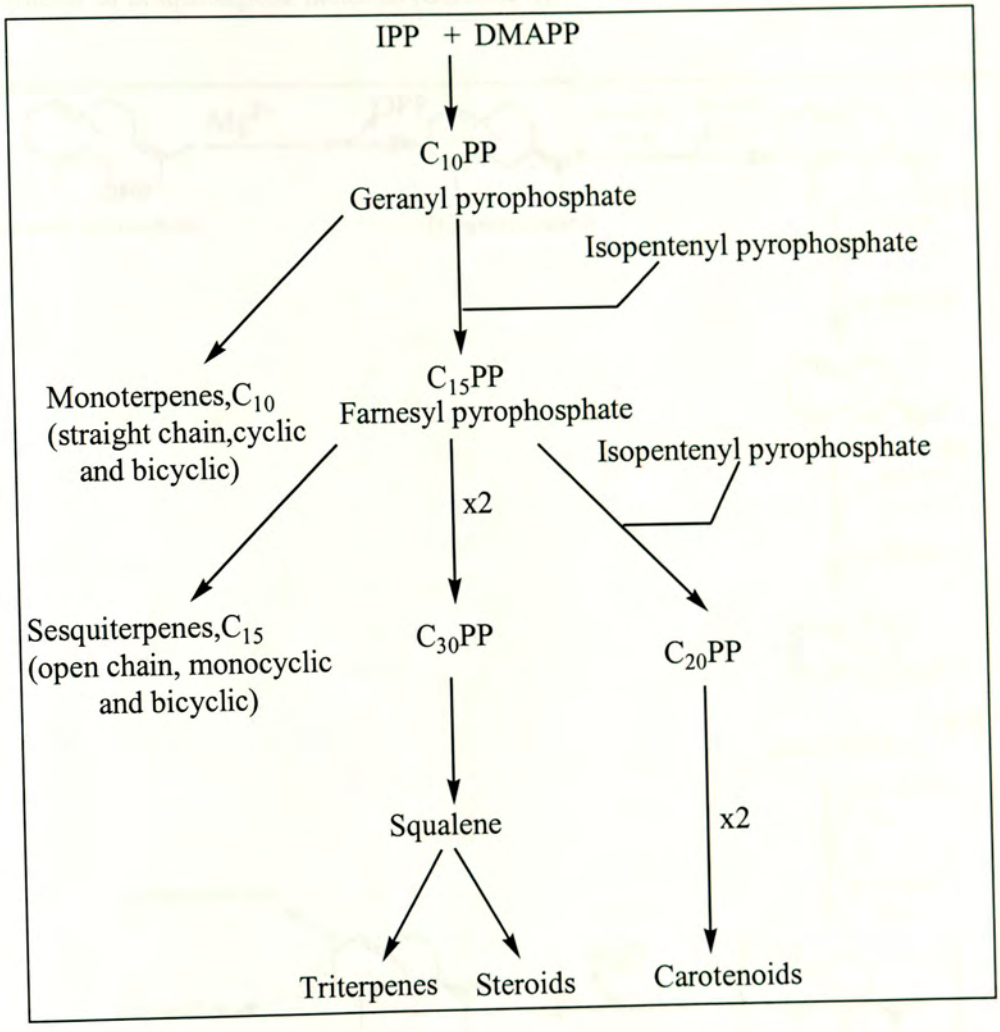


Scheme 1. Mevalonate pathway for the biosynthesis of isoprenoids



Scheme 2. Mevalonate-independent glyceraldehydes-3-phosphate/pyruvate pathway for isoprenoid biosynthesis

In the biosynthesis of the terpenoids, isopentenyl pyrophosphate (IPP) is condensed to the IPP isomer, dimethylallylpyrophosphate (DMAPP), to form prenyl diphosphates of different chain lengths (Scheme 3).



Scheme 3 Biosynthesis of isoprenoid compounds

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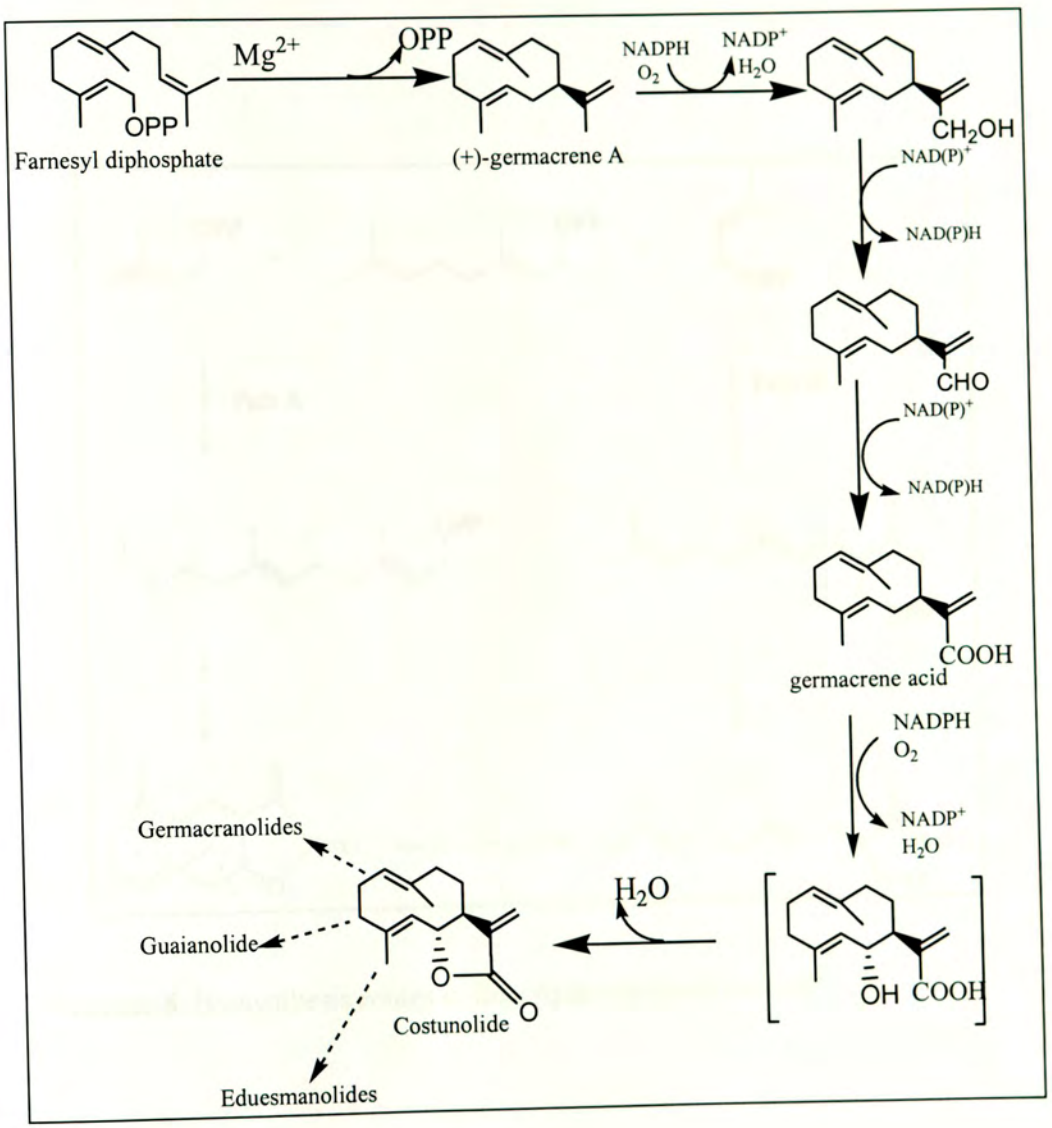
1.3.1 Sesquiterpene lactones

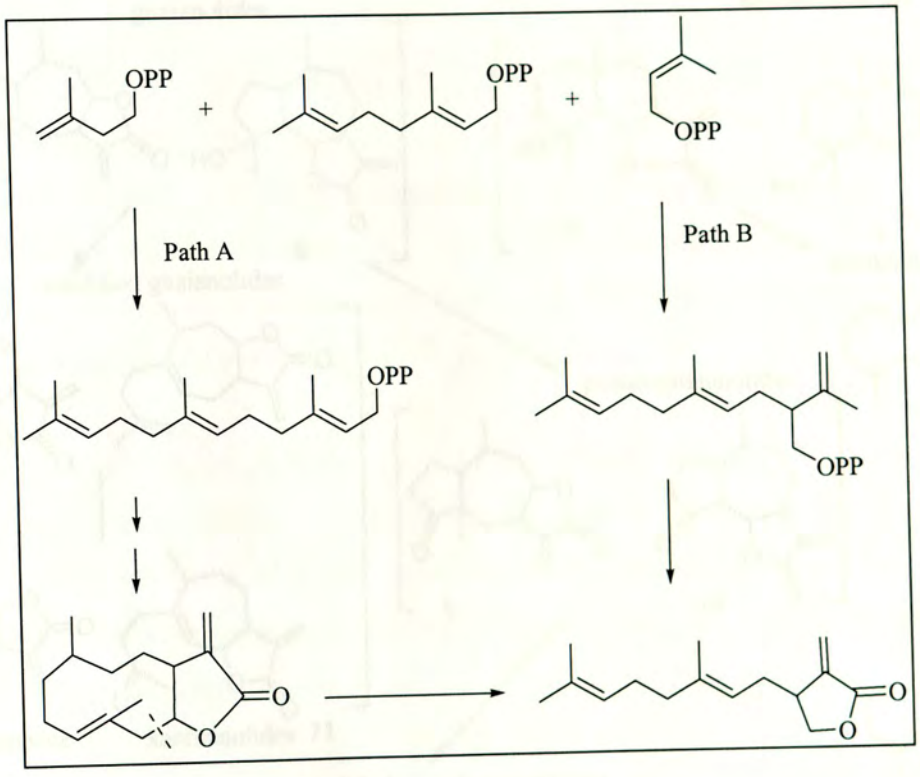
Sesquiterpenes are characteristic constituents of the Asteraceae family. These secondary metabolites are primarily classified on the basis of their carbocyclic skeletons into germacranolide (**1, 2, 3, 4**), guaianolides (**5, 6**), eudesmanolides (**7, 8**), pseudoguaianolides (**9, 10**) and xanthanolides **11** (Scheme 6).[4] (+)-Costunolide is considered to be a branching point in the biosynthesis of sesquiterpene lactones (Scheme 4).



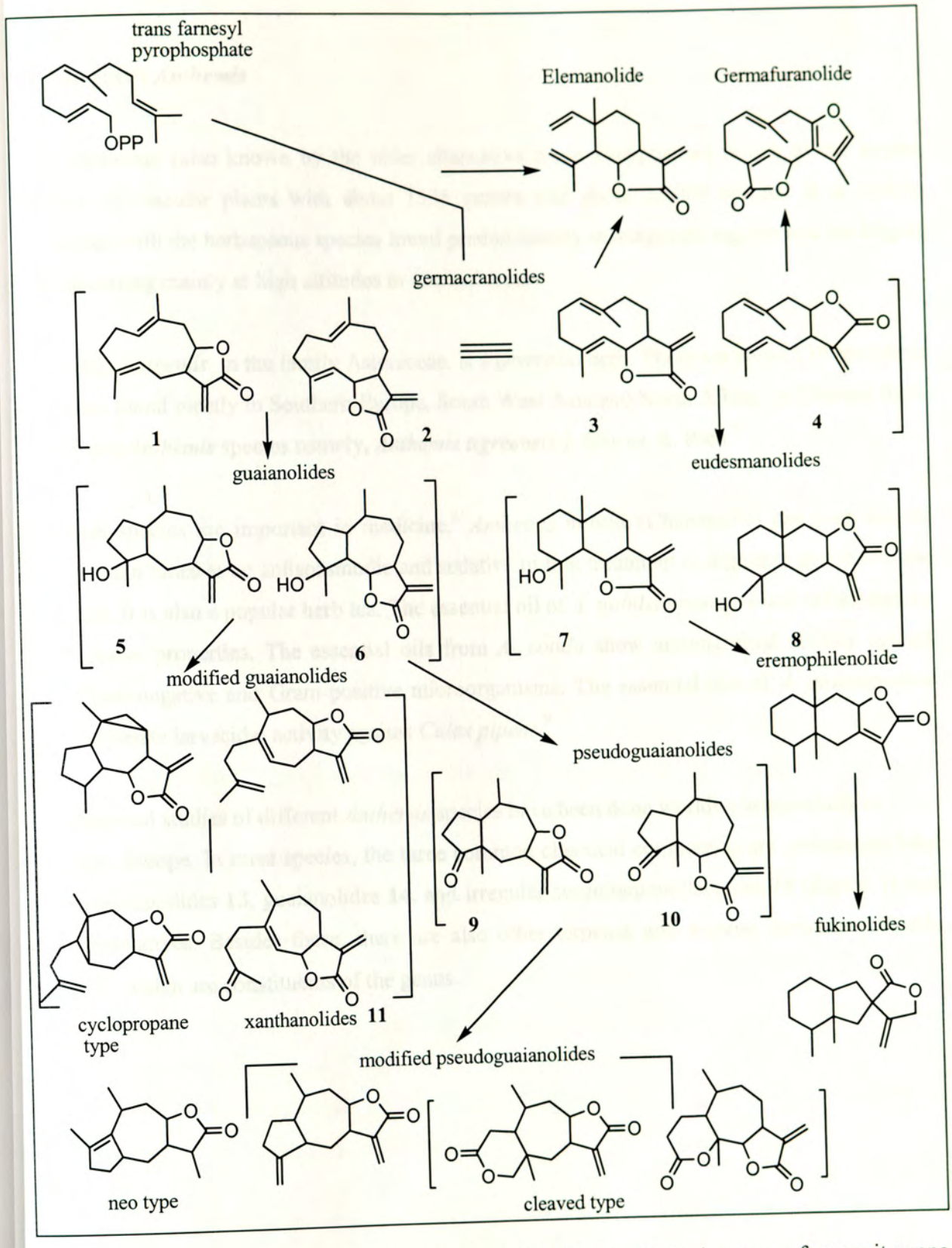
Scheme 4. Biosynthesis of Costunolide

1.3.1 Possible biosynthesis routes to linear sesquiterpene lactone

The described sesquiterpene lactones are members of a small group of linear sesquiterpenoids the biosynthesis of which is proposed to follow two different pathways (Scheme 5). The first involves the conventional mechanism for the biosynthesis of the germacrane skeleton followed by cleavage (path A). The alternative path B was very recently established by feeding of *A. cotula* with isotopically labeled glucose precursors.[5]



Scheme 5. Biosynthesis routes to linear sesquiterpene lactone



Scheme 6. Possible biogenetic relationships of different skeletal types of sesquiterpene lactones.

1.4 The genus *Anthemis*

The Asteraceae (also known by the older alternative name Compositae) is one of the largest families of vascular plants with about 1535 genera and about 23,000 species. It is widely distributed with the herbaceous species found predominantly in temperate regions and the largest trees occurring mainly at high altitudes in tropical areas.

The genus *Anthemis*, in the family Asteraceae, is a perennial herb. There are about 210 species in the genus found mostly in Southern Europe, South West Asia and North Africa. In Ethiopia there is only one *Anthemis* species namely, *Anthemis tigreensis* J. Gay ex A. Rich.[6]

Anthemis species are important in medicine.[8] *Anthemis nobilis* (Chamomile) has been known from Roman times as an antispasmodic and sedative in folk treatment of digestive and rheumatic disorders. It is also a popular herb tea. The essential oil of *A. nobilis* possesses anti-inflammatory and sedative properties. The essential oils from *A. cotula* show antimicrobial activity against both Gram-negative and Gram-positive microorganisms. The essential oils of *A. melampodina* has a moderate larvacidal activity against *Culex pipens*.[9]

The chemical studies of different *Anthemis* species have been done worldwide especially in Southern Europe. In most species, the three common chemical compounds are germacranolides **12**, eudesmanolides **13**, guaianolides **14,** and irregular sesquiterpene lactones **15** (Figure 1) and their derevatives. Besides these, there are also other terpenes and terpene derivatives (Table 1),[10, 11] which are constituents of the genus.

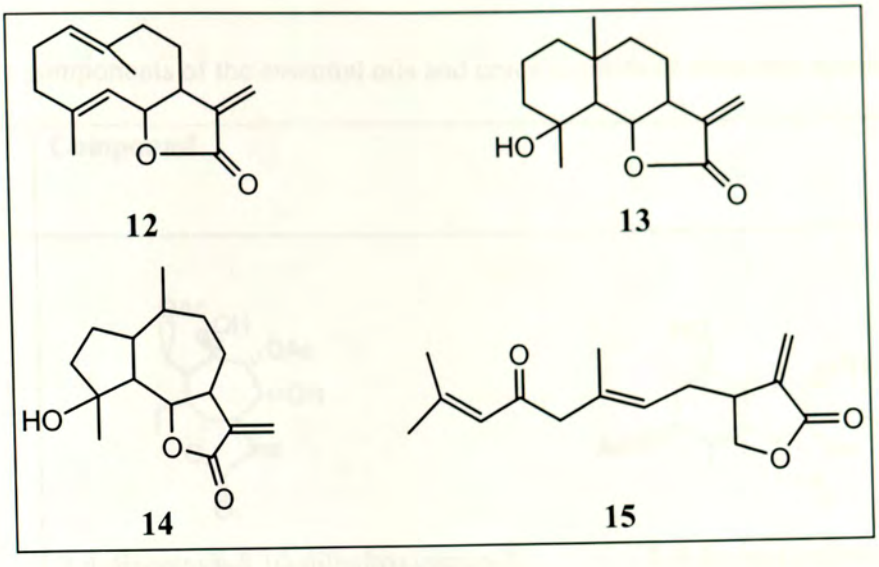


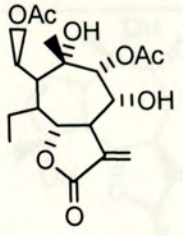
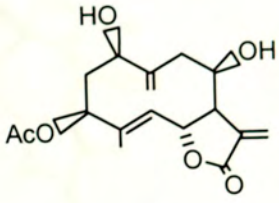
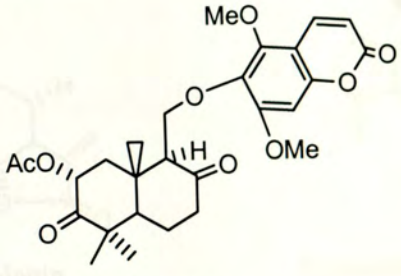
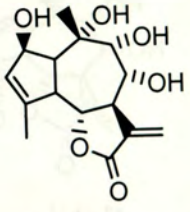
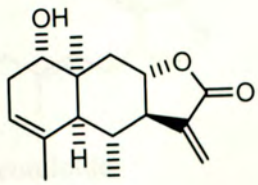
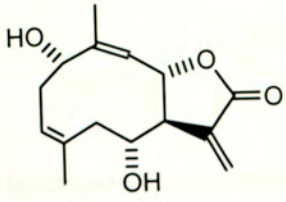
Figure 1: Some sesquiterpene lactone types from *Anthemis* sp.

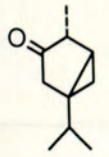
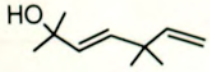
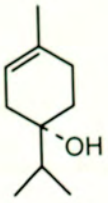
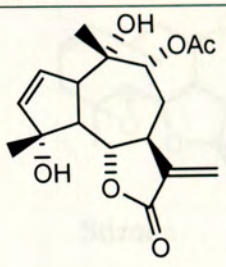
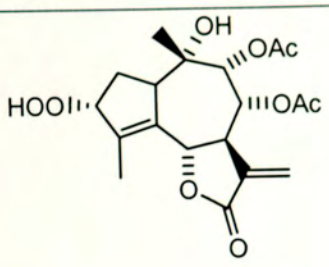
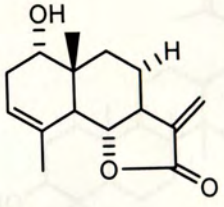
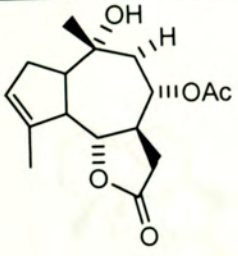
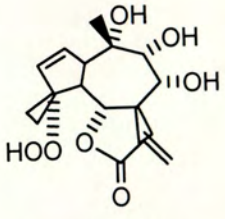
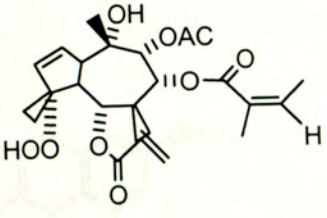
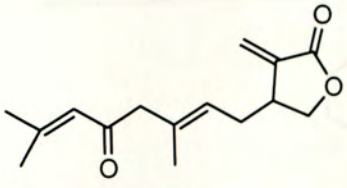
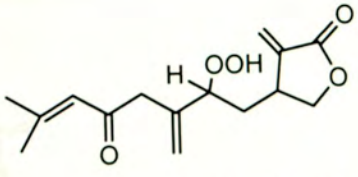
1.5 *Anthemis tigreensis*

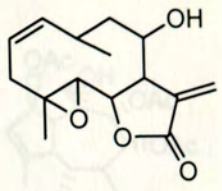
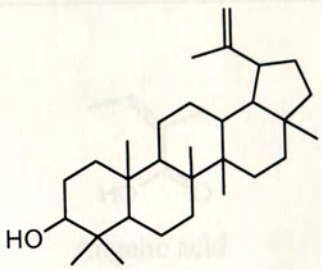
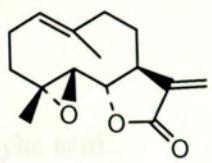
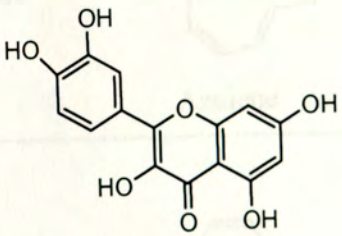
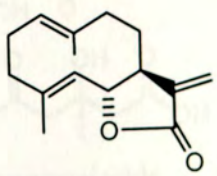
A. tigreensis, vernacular name 'embaha' (in Tigreegna), is a prostrate or sometimes erect annual herb growing to 5-60 cm high. Its leaves are gray-green, alternate and divided into rounded segments. Its flower heads are white, attractive on long stalks. It is a common plant of higher altitudes and sometimes locally dominant in fallow fields. It grows on degraded area, mountain slopes, in grassland and seasonally water logged soils, at altitudes between 1800 – 4620 m.

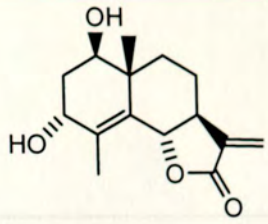
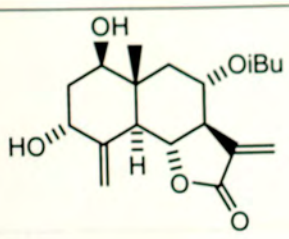
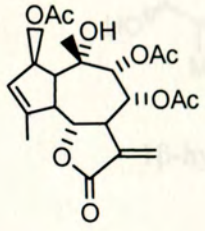
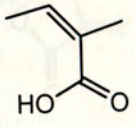
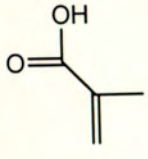
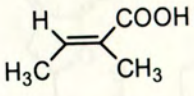
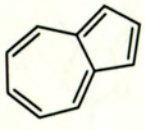
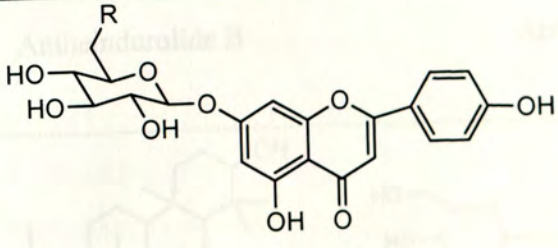
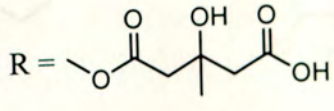
In Ethiopia, *A. tigreensis* is found in Tigray, Gojjam, Gonder, Wollo, Shoa, Arsi, Harrer, and Bale, extending to Kenya, Uganda, and Tanzania.[6] It flowers through out the year, but especially in masses after the short rains. Honeybees collect pollen and nectar from the flowers. In local medicine, the roots are used against wet eczema.[7] A literature survey indicated that there are no published reports on the chemistry of the plant.

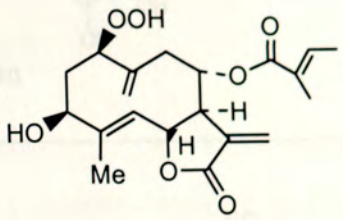
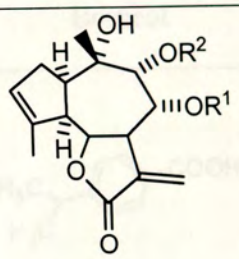
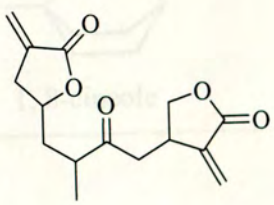
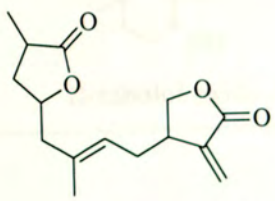
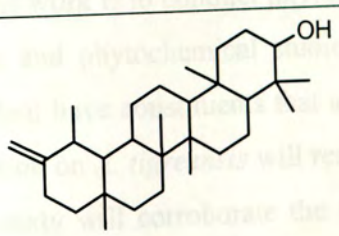
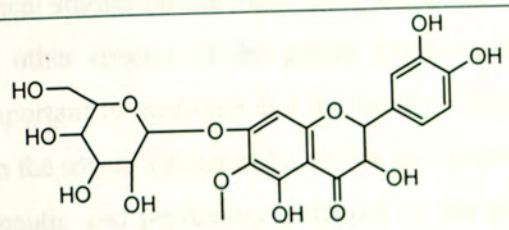
Table 1. Some components of the essential oils and crude extracts of *Anthemis* species

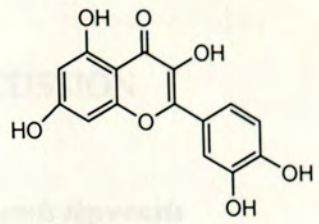
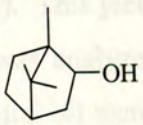
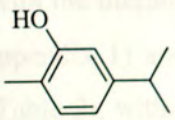
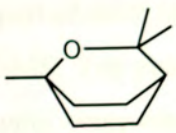
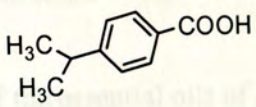
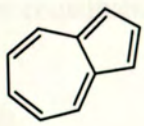
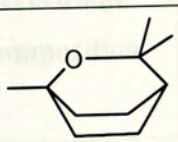
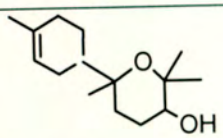
Species	Compound	Ref.
A. aetnensis	 2,9-diacetoxy-8,10-dihydroxyguaia-3, 11(13)-diene-6,12-olide  1-β-hydroxyarbysculin  7-acetoxypectanone	12
A. alpestris	 2,8,9,10-Tetrahydroxyguia-3,11(13)-dien-6,12-olide	13
A. altissima	 Altissin  Tatridin-A	14

A. carpatica	 α-thujone  Yomogi alcohol  Terpine-4-ol	15
	 Anthemolide A  Anthemolid E	16
	 Douglanin  Cumambrin A	17
A. cretica	 Anthemolide B  8-O-angeloyl-9-O-acetyl-anthemolide B	18
A. cotula (Mayweed or stinking chamomile)	 Anthecotuloide  5-hydroperoxy-6,13-dehydro-5,6-dihydro-anthecotuloide	19

A. cupaniana	 Stizolin	20
	 Lupeol  Parthenolide	21
	 Quercetin	22
	 Costunolide	23

A. fuscata	 Armefolin  3α-hydroxy-8α-isobutyryloxyreynosin	24
A. hydruntina	 Hydruntinolides B	25
A. noblis (Roman or English chamomile)	 Angelic acid  Methacrylic acid  Tiglic acid  Azulene	26
	  Chamaemeloside	27

	 1β-hydroperoxyisonobilin	28
A. plutonia	 9α-acetoxycmmambrin B	29
A. pseduocotula	 Antheindurolide B  Antheindurolide A	30
A. tinctoria	 Taraxasterol  Patulitrin	31

	 Quercetin	32
A. xylopoda	 Borneol  Carvacrol  1,8-Cineole	33
Matricaria recutita (German or Hungarian chamomile)	 Cuminic acid  Azulene	26
	 1, 8-cineole  Bisabolol oxide A	34

1.6 Objectives of the work

The objective of this work is to conduct phytochemical studies on the plant since it has not been investigated before and phytochemical studies on other species of the genus *Anthemis* have revealed that the plant have constituents that are important in medicine and perfumery. Similar chemical investigation on *A. tigreensis* will result in the identification of bioactive and fragrance constituents. The study will corroborate the therapeutic and perfumery potential of the plant. This will lead to the domestication and conservation of the plant in the world. Therefore, in these studies the specific objectives are to isolate and characterize the constituents of the

essential oils obtained from the plant, and to isolate and characterize the constituents of the petroleum ether and chloroform extracts of the plant.

2. RESULTS and DISCUSSION

2.1 Essential oils of *Anthemis tigreensis*

Hydrodistillation of the fresh aerial parts of *A. tigreensis* gave a pale yellow aromatic essential oil with a yield of 0.12% (v/w). This yield compared with the literature report of other species is low. [15, 20, 21, and 22] The oil was analyzed by GC (Appendix 1) and GC-MS. Ten compounds constituting 66% of the essential oil were identified (Table 2), with the major component being bornyl acetate (48%). Literature survey on the essential oils of other species [15, 20, 21, and 22] of the genus indicated that the composition of the essential oils of *A. tigreensis* is different from the oils of the other species in that it has bornyl acetate as a major constituent.

Table 2. Constituents of the essential oils of A. *tigreensis*

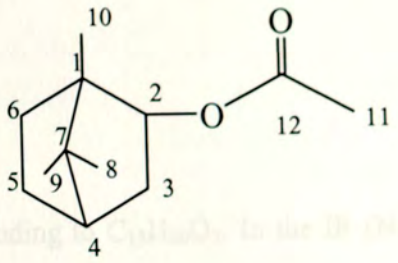
Peak no.	Compound	Percentage composition	Method of identification
1	α-Pinene	0.12	GC-MS, PE*
2	Camphene	0.67	GC-MS
3	3-carene	0.44	GC-MS
4	1,8 cineol	2.33	GC-MS, PE
5	Borneol	10.82	GC-MS
6	Bornyl acetate	48.42	GC-MS, NMR
7	β-Caryophllene	0.11	GC-MS, PE
8	γ-Cadinene	1.16	GC-MS
9	Caryophyllene oxide	1.30	GC-MS,
10	Cubenol	1.03	GC-MS

* PE = Peak enhancement

The crude oil was also analyzed by NMR. The presence of the main constituent of the essential oil, bornyl acetate **16**, was also confirmed from the ^{1}H NMR (Appendix 1), ^{13}C NMR spectra and DEPT-135 NMR (Table 3, Appendix 2). The ^{13}C data obtained for bornyl acetate is in agreement with the data reported in the literature (Table 3).[35]

Table 3. ^{13}C NMR data of bornyl acetate **16**

Carbon number	^{13}C (ppm)	Literature ^{13}C (ppm)
1	48.17	47.76
2	80.32	79.36
3	37.16	36.96
4	45.32	45.11
5	27.48	27.25
6	28.43	28.18
7	49.10	48.75
8	20.11	19.59
9	19.23	18.63
10	13.86	13.46
11	21.67	20.57
12	171.90	170.08



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2.2 Solvent extraction of *Anthemis tigreensis*

The dried and powdered plant was extracted with petroleum ether and then chloroform. The compounds isolated were characterized from the 1D- and 2D-NMR, UV, IR, and MS spectra data. The extraction of the plant and isolation of compounds is described in detail in the experimental part.

2.2.1 Characterization of At-8

Molecular ion peak (EIMS) at m/z 248 corresponding to $C_{15}H_{20}O_3$. In the IR (NaCl) spectrum (Appendix 4), the absorption band at 2925 cm^{-1} and 2854 cm^{-1} showed the presence of a methylene group. Absorption bands at 1764 cm^{-1}, 1268 cm^{-1}, and 1115 cm^{-1} demonstrated the presence of a five membered lacton, C=O, and O-C-C stretch respectively. Absorption band at 1683 cm^{-1} showed the presence of a conjugated aldehyde. The signal at 1415 cm^{-1} indicated the presence of terminal alkene. A weak absorption band at 1643 cm^{-1} showed the presence of C=C stretching. The UV spectrum displayed absorption band at λ_{max} (in $CHCl_3$) 309 nm indicating the presence of a conjugated carbonyl chromophore.

The ^1H spectrum (Appendix 5, Table 4), showed peaks at δ 1.59 (3H, s), and 1.67 (3H, s) integrating for two methyl protons. Methylene signals appear at δ 2.15 (2H, t, J 7Hz), 2.31 (1H, m, J 7Hz) & 2.24 (1H, m, J 6.5 Hz), and 2.40 (2H, q, J 7Hz). One broad multiplet peak appears at δ 3.07 integrating for one hydrogen. Peaks at δ 3.90 (1H, t, J 5Hz) & δ 4.34 (1H, dd, J 8Hz) each integrating for one hydrogen correspond to proton attached to a carbon, which is also connected to an electronegative atom such as oxygen. Vinylic protons appeared at δ 5.10 (1H, dd, J 7Hz & 6.5Hz), 6.38 (1H, t, J 7Hz), 5.57 (1H, d), and 6.18 (1H, d). An aldehyde proton appeared at δ 9.31.

Table 4. ^{1}H and ^{1}H-^{1}H COSY spectra data of compound **At-8**

Hydrogen on Carbon no.	**^{1}H δ (ppm)**	**COSY**
1	9.31(s)	-
3	6.38(t)	$H^3 \leftrightarrow H^4$, $H^3 \leftrightarrow H^5$
4	2.40(q)	$H^4 \leftrightarrow H^3$
5	2.15(t)	$H^5 \leftrightarrow H^{14}$, $H^5 \leftrightarrow H^7$
7	5.10(t)	$H^7 \leftrightarrow H^9$, $H^7 \leftrightarrow H^{14}$
8	2.31(m), 2.24(m)	$H^8 \leftrightarrow H^7$, $H^8 \leftrightarrow H^9$
9	3.07(m)	$H^9 \leftrightarrow H^8$, $H^9 \leftrightarrow H^{12}$, $H^9 \leftrightarrow H^{13}$
12	3.90(q), 4.34(t)	$H^{12} \leftrightarrow H^9$
13	6.18(d), 5.58(d)	$H^{13} \leftrightarrow H^9$
14	1.67(s)	$H^{14} \leftrightarrow H^5$
15	1.60(s)	$H^{15} \leftrightarrow H^3$

The ^{13}C NMR and DEPT-135 spectra (Appendix 6, Table 5) indicated that **At-8** has 15 carbon atoms. The spectra showed two methyl carbons at δ 9.5 and 16.6, three methylene carbons at δ 27.6, 32.4, 38.4, one oxymethylene carbon atom at δ 70.9, a terminal methylene at δ 120.8, three vinylic quaternary carbons at δ 137.9, 138.5, and 139.9, three methine carbon at δ 39.1, 120.8, and 154.1, one aldehyde carbon at δ 195.4, and one ester carbon at δ 171.1.

Table 5. ^{13}C and HMBC spectra data of compound **At-8**

Carbon no.	^{13}C (ppm)	DEPT	HMBC
1	195.43	CH	$H^1 \rightarrow C^3, C^{15}, C^2$
2	138.50	-	-
3	154.07	CH	$H^3 \rightarrow C^{15}, C^1, C^4, C^5$
4	27.61	CH_2	$H^4 \rightarrow C^5, C^2, C^3$
5	38.39	CH_2	$H^5 \rightarrow C^{14}, C^4, C^7, C^6, C^3$
6	137.95	-	-
7	120.81	CH	$H^7 \rightarrow C^{14}, C^8, C^5$
8	32.45	CH_2	$H^8 \rightarrow C^9, C^7, C^{12}, C^{10}, C^6$
9	39.08	CH	$C^9 \rightarrow H^{12}, H^8, H^{13}$
10	139.89	-	-
11	171.08	-	-
12	70.87	CH_2	$H^{12} \rightarrow C^8, C^9, C^{10}, C^{11}$
13	122.36	CH_2	$H^{13} \rightarrow C^9, C^{10}, C^{11}$,
14	16.61	CH_3	$H^{14} \rightarrow C^6, C^7, C^5$
15	9.56	CH_3	$H^{15} \rightarrow C^2, C^3, C^1$

In the HMBC spectrum (Appendix 7, Table 5), aldehyde proton correlated with the methyl group C-15 at δ 9.56, vinylic quaternary carbon C-2, and the conjugated alkene C-3. Proton of C-3 correlated with methyl group C-15, carbonyl carbon of aldehyde C-1, two methylene groups C-4 and C-5. These observations together with HSQC (Appendix 8) led to partial structure **I** (Figure 2). Protons of C-5 correlated with C-4, C-14, one of the vinylic quaternary carbon, and C-7. These observation resulted in fragment unit **II.** Carbonyl carbon of ester C-11 correlated with diasterotopic protons of C-12, which is attached with oxygen; C-11 also correlated with other diasterotopic protons of terminal alkene C-13. Protons of C-13 correlated with vinylic quaternary carbon C-10, and methine C-9. Protons of C-12 also correlated with methine C-9. These analyses gave fragment unit **III.** Diasterotopic protons of C-8 correlated with C-6, C-7, C-9, C-12, and with quaternary carbons. These correlation gives fragment **IV**.

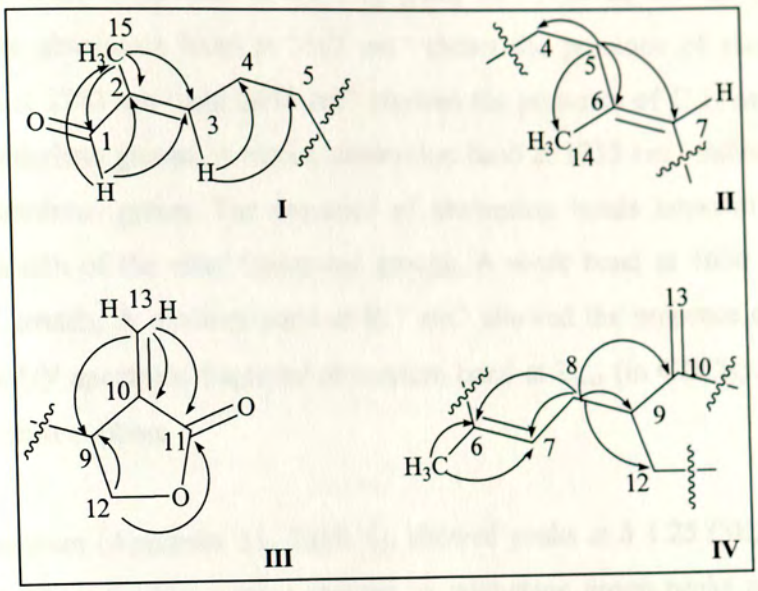
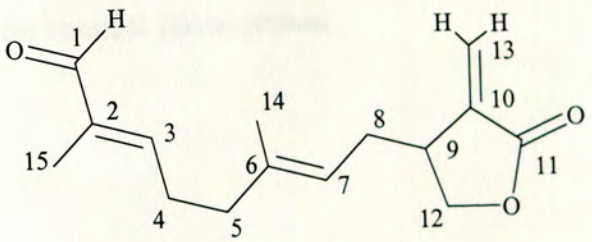


Figure 2. The proposed fragments of At-8 with HMBC correlation.

Combination of fragment **I, II, III,** and **IV** (Figure 2) give the proposed structure **17** for compound **At-8**. To the best of our knowledge, **At-8** is not reported in the literature. The numbering of structure **17** is arbitrary.



17

(2E,6E)-8-(tetrahydro-4-methylene-5-oxofuran-3-yl)-2,6-dimethylocta-2,6-dienal

2.2.2 Characterization of At-11

At-11 is a white solid compound of melting point 58-59°C. In the IR (NaCl) spectrum (Appendix 10), the absorption band at 3102 cm^{-1} shows the presence of alkene C-H stretch. Absorption bands at 2983 cm^{-1} and 2938 cm^{-1} showed the presence of C-H asymmetric stretch for methyl and methylene groups. A strong absorption band at 1755 cm^{-1} indicated the presence of a γ-lactone functional group. The presence of absorption bands between 1300-1000 cm^{-1} illustrated C-O stretch of the ester functional groups. A weak band at 1656 cm^{-1} showed the presence of C=C stretch. A medium band at 817 cm^{-1} showed the presence of a trisubstituted double bond. The UV spectrum displayed absorption band at λ_{max} (in $CDCl_3$) 240 nm indicated the presence of a chromophore.

The ^1H NMR spectrum (Appendix 11, Table 6), showed peaks at δ 1.25 (3H, d, J 7Hz), and δ 1.64 (3H, s) integrating for two methyl protons. A methylene group peaks appeared at δ 1.95 (1H, m, J 7.6Hz & 5Hz) and δ 2.08 (1H, m, J 4Hz) each integrating for one proton. Other methylene signals appear at δ 2.23 (2H, br m) and 2.36 (2H, br m). Peaks at δ 2.65 (1H, m, J 7.5Hz), and 3.09 (1H, br m) correspond to methine protons. Other methine group attached to an electronegative atom appears at δ 4.58 (1H, m, J 5.5Hz & 7.5Hz). Peaks at δ 3.95 (1H, dd, J 5Hz) and 4.30 (1H, dd, J 9Hz) correspond to a hydrogen attached to an electronegative atoms. Vinylic proton appeared at δ 5.08 (1H, brdd, J 7Hz & 6Hz). Peaks at δ 5.63 (1H, d, J 2.3Hz) and 6.21 (1H, d, J 2.6Hz) stand for terminal alkene protons.

Table 6. ^{1}H and ^{1}H-^{1}H COSY spectral data of compound **At-11**

Hydrogen on Carbon no.	^{1}H δ (ppm)	COSY
1	1.95(m), 2.08(m)	H-1→H-10, H-2
2	4.58(m)	H-2→H-1, H-3
3	2.23(br m), 2.36(br m)	H-3→H-15, H-2
4	-	-
5	5.10(m)	H-5→H-15, H-6
6	2.23(br m), 2.36(br m)	H-6→H-7, H-5
7	3.09 (br m)	H-7→H-6, H-8, H-13
8	3.95 (dd), 4.30 (dd)	H-8→H-7
9	-	-
10	2.65 (m)	H-10→H-14, H-1
11	-	-
12	-	-
13	5.63 (d), 6.21 (d)	H-13→H-7
14	1.23 (d)	H-14→H-10
15	1.64 (s)	H-15→H-3, H-5

The ^{13}C NMR and DEPT-135 (Appendix 12, Table 7) indicated that **At-11** has 15 carbons. The spectra shows two methyl carbons at δ 15.7 and 16.7. Three methylene signals appear at δ 32.1, 34.9, and 45.2. One methylene group attached to an electronegative atom appears at δ 70.4. A terminal methylene is attested by the signal at δ 122.2. Peak at δ 33.7 and 38.5 represented for methine groups. Methine group attached to an electronegative atom appeared at δ 76.3. Vinylic methine showed at δ 123.1. Two vinylic quaternary carbons appeared at δ 134.1 and 137.8. The presence of ester functional group was shown by the peaks at δ 170.5 and 179.6.

Table 7. ^{13}C and HMBC spectra data of compound **At-11**

Carbon No.	^{13}C δ (ppm)	DEPT	HMBC
1	34.9	CH_2	H-1↔ C-2, C-3, C-9, C-10, C-14
2	76.7	CH	H-2↔C-9
3	45.2	CH_2	H-3↔C-1, C-2, C- 4, C- 15
4	134.1	-	-
5	123.1	CH	H-5↔C-3, C-6, C-15
6	32.1	CH_2	H-6↔C-5, C-7, C-8, C-11
7	38.5	CH	-
8	70.4	CH_2	H-8↔C-6, C-7, C-11, C-12
9	179.6	-	-
10	33.7	CH	H-10↔ C-1, C-9, C-14
11	137.8	-	-
12	170.5	-	-
13	122.2	CH_2	H-13↔C-7, C-11, C-12
14	15.7	CH_3	H-14↔ C-1, C-9, C-10
15	16.7	CH_3	H-15↔C-3, C-4, C-5

In the HMBC spectrum (Appendix 13, Table 7), the proton on methyl C-14 correlate with the carbonyl C-9, methin C-10, and methylene C-1. Proton of methylene C-1 correlates with methine C-10, and methyl C-14, carbony C-9, methine carbon C-2, and methylene C-3. The methine proton H-2 correlates with carbonyl C-9. Protons of methylene H-3 also correlate with methine C-2, and methylene C-1. These observations together with HSQC (Appendix 14) lead to fragment **I** (Figure 3). Protons of C-3 correlate with quaternary carbon C-4, and methyl C-15. Methyl protons of C-15 correlate with vinylic quaternary carbon atoms C-4, C-5, and methylene C-3. A vinylic proton on C-5 correlates with C-15, C-6, and C-3. Protons of C-6 correlate with C-5. These observation lead to fragment **II**. Protons of C-6 correlate with a vinylic quaternary C-11, methine C-7, and methylene C-8. Methylene protons at C-8 correlate with ester carbonyl C-

12, vinylic quaternary C-11, methylene C-6, and methine C-7. The terminal alkene C-13 protons correlate with C-7, C-11, and with ester carbonyl C-12. These analyses lead to fragment **III.**

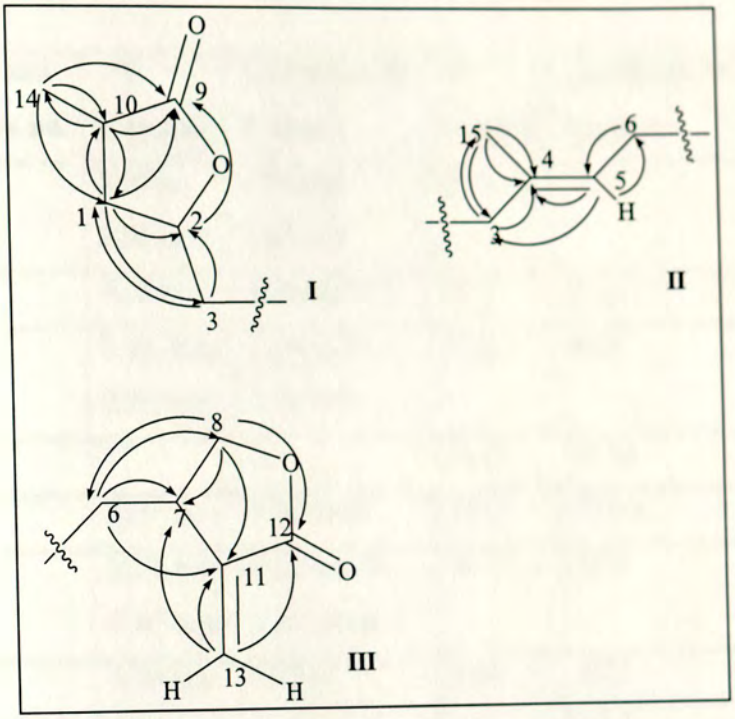
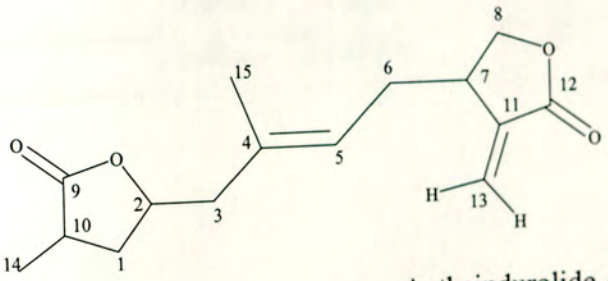


Figure 3. The proposed fragments for **At-11** with HMBC correlation

Combination of fragments **I, II, III** (Figure 3) result in the proposed structure **18** for compound **At-11**. The ^{1}H and ^{13}C data obtained for **At-11** are in agreement with those reported for Antheindurolide A from *A. pseudocotula* (Table 8).[18] **At-8** is the precursor of **At-11.** It can be converted through oxidation of the aldehyde functional group in to cyclic ester.



Antheindurolide A (**18**)

Table 8. ^{1}H and ^{13}C spectra data of **At-11** compared with literature report.

Carbon and hydrogen no.	^{1}H δ (ppm)	Literature ^{1}H δ (ppm)	^{13}C δ (ppm)	Literature ^{13}C δ (ppm)
1	1.95 m 2.08 m	1.91ddd 2.05 ddd	34.9	34.6
2	4.58 m	4.55 dddd	76.7	76.2
3	2.23 brm 2.36 brm	2.18 brdd 2.32 brdd	45.2	44.9
4	-	-	134.1	137.6
5	5.10 m	5.14 brdd	123.1	123.0
6	2.23 brm 2.36 brm	2.22 brddd 2.31 brddd	32.1	31.8
7	3.09 brm	3.05 m	38.5	38.2
8	3.95 dd 4.30 dd	3.89 dd 4.34 dd	70.4	70.3
9	-	-	179.6	179.7
10	2.65 m	2.6 ddq	33.7	33.5
11	-	-	137.8	134.0
12	-	-	170.5	170.6
13	5.6 3 d 6.21 d	5.60 d 6.14 d	122.2	122.1
14	1.23 d	1.19 d	15.7	15.5
15	1.64 s	1.69 brs	16.7	16.4

2.2.3 Characterization of At-7

In the IR (NaCl) spectrum (Appendix 16), the absorption band at 3465 cm^{-1} showed the presence of alcohol functional group. Absorption band at 3097 cm^{-1} indicated the presence of alkene C-H stretch. A strong band at 1763 cm^{-1} and a weak band at 1662 cm^{-1} demonstrated the presence of γ-lactone functional group and C=C stretch respectively. The band at 1050 cm^{-1} shows the presence of C-O stretch of primary alcohol. The three bands that found between 1300 - 1000 cm^{-1}, i.e. 1267 cm^{-1}, 1191 cm^{-1}, and 1118 cm^{-1} showed the presence of C-O stretch of ester functional group.

The H ^{1}NMR spectrum (Appendix 17, Table 9), showed peaks at δ 1.28 (3H, d), and δ 1.35 (3H, s) represent methyl protons. Signals appear at δ 2.64 (1H, m), and δ 3.7 (1H, m) are for methine protons. Other oxymethine proton showed at δ 4.63 (1H, m). A vinylic protons appeared at δ 5.72 and δ 5.74 each integrating for one protons. Methylene protons appeared at δ 1.78 and 1.90 each integrating for one proton. Other methylene signals appeared at δ 1.99 and δ 2.07 each integrating for one proton. Oxymethlene showed at δ 4.00 (1H, t) and δ 4.50 (1H, t) each integrating for one hydrogen. Terminal alkene protons appeared at δ 5.58 and δ 6.31 each integrating for one hydrogen.

The ^{13}C NMR (Appendix 18) and DEPT-135 (Appendix 19) indicated that **At-7** has 15 carbons. The spectra show two methyl carbons at δ 15.9 and at δ 29.2. Two methylene carbons appear at δ 36.6 and δ 47.4. One oxymethylene appear at δ 70.4 and one terminal methylene showed at δ 123.4. Methine carbons showed at δ 33.8, δ 75.6, and δ 42.56. Other vinylic methine carbons appeared at δ 125.7 and δ 140.9. Two signals at δ 72.3 and δ 77.3 are represent quaternary carbon atoms that attached with oxygen atom. One carbonyl carbon showed at δ 179.2. One vinylic quaternary carbon appears at δ 137.4.

Table 9. ^{1}H and ^{13}C spectra data of compound **At-7**

Carbon and hydrogen no.	^{1}H δ (ppm)	^{13}C δ (ppm)	DEPT
1	-	179.1	-
2	2.67 m	33.8	CH
3	1.90 m 1.95 m	36.6	CH_2
4	4.63 m	75.6	CH
5	2.04 m 2.10 m	47.4	CH_2
6	-	77.3	-
7	-	72.3	-
8	5.72	140.9	CH
9	5.74	125.7	CH
10	-	137.4	-
11	3.70	42.6	CH
12	4.00 t 4.50 t	70.4	CH_2
13	5.58 d 6.31 d	123.4	CH_2
14	1.35 s	29.2	CH_3
15	1.28 d	15.9	CH_3

The ^{1}H and ^{13}C spectra data for fragment **I** are very similar to the same kind of fragment found in compound **At-11** (Table 6 and 7). The second fragment **II** (Figure 4) of the structure is the most probable structure as deduced from ^{1}H, ^{13}C and DEPT spectra data. From the two fragments, the following structure **19** is proposed for compound **At-7**.

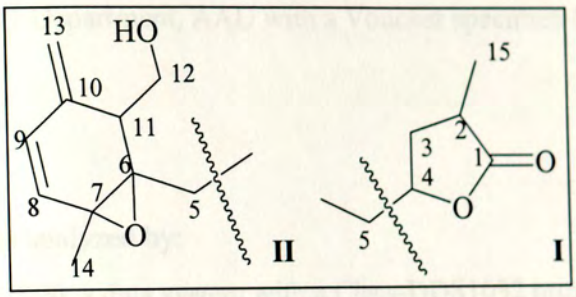
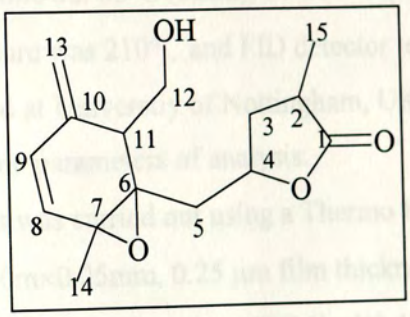


Figure 4. The proposed fragments of **At-7**



19

dihydro-5-((2-(hydroxymethyl)-6-methyl-3-methylene-7-oxa-bicyclo[4.1.0]hept-4-en-1-yl)methyl)-3-methylfuran-2(3H)-one

3. EXPERIMENTAL

A. tigreensis was collected in July 2004 and in October 2004 from Bale zone, Bale Mountains National Park (BMNP), Sannate Plateau. The plant was authenticated by Prof. Sebsibe Demissew of the Biology Department, Addis Ababa University, and deposited in The National Herbarium, Biology Department, AAU with a Voucher specimen No. 287.

3.1 General

The oil sample was analyzed by:

- DANI GC-1000, a data system with a Class-DDS1032 program chromatograph equipped with DB-5 column (30m × 320mm i.d., 0.25 μm), with N_2 as the carrier gas at a flow rate of 2ml/min and a split ratio of 1:7. Oven temperature was programmed as follows: 60°C (5min), 60-210°C (5°C /min), 240°C (10min); injector temperature was 210°C and FID detector temperature was 260°C. The oil was also analyzed at Universtiy of Nottingham, UK, using a Shimadzu GC-2010 applying the same parameters of analysis.
- GC- MS analysis was carried out using a Thermo Finnigan Polaris – Q FC-MS. A DB-5 column (30m×0.25mm, 0.25 μm film thickness) fused silica column was used with oven temperature programme 40°C (1min) 40°C – 220°C at 10°C/min, and 220°C (6 min), Helium was used as a carrier gas. GC-MS was run at the School of Chemistry, University of Nottingham, Nottingham, UK.
  Characterization of compounds was done by computerized matching of the acquired mass spectra with library spectra, by peak enhancement and NMR.

The isolated pure compounds were analyzed by using NMR Bruker Avance 400 MHz in $CDCl_3$ for NMR spectra, GENESY'S 2PC spectrometer (200-600 nm) at room temperature for UV spectra, Perkin- Elmer BX spectrometer (400-4000cm^{-1}) in NaCl pellets for IR spectra. δ value are given in ppm relative to TMS as internal standards. Melting point was determined on electrothermal digital melting point apparatus.

The extract constituents on TLC plates were detected by UV fluorescence at 254 nm and 366 nm and by spraying with 0.5% vanillin in Conc. H_2SO_4. Column chromatography was performed using silica gel column (32-63 µm).

3.2 Isolation of essential oils

The oil was isolated from the aerial part of the plant by hydrodistillation at atmospheric pressure using Clevenger type apparatus for 3 hours. The obtained oil was dried over anhydrous sodium sulphate and stored at 4-6°C.

3.3 Extraction and Isolation

The whole plant was dried and powdered. 585 g of the powdered plant was soaked in 1.5 L petroleum ether (40-60°C) at room temperature for 24 hrs. The extract was filtered and then concentrated under reduced pressure (35°C) to yield 7.74 g (1.32%). The marc was then extracted with chloroform twice. The first extract yielded 11.44 g (1.94%) and the second extract yielded 8.2 g (1.40%).

The petroleum ether extract was applied on a column packed with 200 g silica gel. Isolation was carried out using the solvents hexane, ethyl acetate, and methanol in increasing polarity. A total of 22 fractions were obtained. Fractions that showed the same R_f value and the same characteristic colour on TLC were combined (Table 9).

Table 10. Petroleum ether extract fractions.

Solvent system	Ratio	Fractions	Volume (ml)	Fractions combined	Code
Hexane	10:0	1-2	300	1-2	PE1
Hexane- ethyl acetate	9:1	3-4	100	3-13	PE2
,,	8:2	5	100		
,,	7:3	6-7	150		
,,	6:4	8-9	150		
,,	5:5	10-11	150		
,,	6:4	12-13	150		
,,	3:7	14	100	14	PE3
,,	2:8	15	100	15-17	PE4
,,	1:9	16	75		
,,	0:10	17	100		
Ethyl acetate- methanol	5 : 5	18-19	100	18-19	PE5
,,	4:6	20-21	100	20-21	PE6
,,	3:7	22	100	21	PE7

PE= petroleum extract

The chloroform extract (11.44 g) was applied on a column packed with 300 g silica gel. The column was eluted using chloroform and methanol solvents of increasing polarity. A total of seven fractions were collected, with each amounting to 150 ml. Fractions that showed the same R_f value and the same characteristic colour on TLC were combined (Table 10).

Table 11. Chloroform extract fractions

Solvent system	Ratio	Fractions	Volume (ml)	Combined fractions	Code
$CHCl_3$	10:0	1	1501		CE1
$CHCl_3$ - MeOH	9:1	2	150		CE2
>>	8:2	3	150		CE3
>>	7:3	4	150		CE4
>>	6:4	5	150	5-6	CE5
>>	5:5	6	150		
>>	0:10	7	150		CE6

CE = chloroform extract

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The second chloroform extract (8.2 g) was applied on a silica gel column and was eluted with mixture of chloroform and methanol solvents of increasing polarity. 11 Fractions were collected and combined into 5 parts based on their R_f value and the same characteristic colour on TLC analysis (Table 11).

Table 12: The second chloroform extract fractions

Solvent system	**Ratio**	**No. of fractions**	**Collected volume (ml)**	**Combined fractions**	Code
$CHCl_3$:MeOH	10:1	1	100		2^{nd}CE1
	9:1	2, 3	100		2^{nd}CE2
	8:2	4, 5, 6	100	3-5	2^{nd}CE3
	7:3	7, 8, 9	150	6-10	2^{nd}CE4
	5:5	10, 11	150	11-13	2^{nd}CE5

3.3.1 Isolation of At-8

PE4 was concentrated under reduced pressure (on a rotavapor) to yield 284 mg and was applied on CC silica gel (25 g) and fractionated further into 10 fractions of 15 ml each. Fraction 8-10 (**At-8**) were combined based on TLC (developed in 4:2:1 ratio of hexane, chloroform, and ethyl acetate) that showed R_f value of 0.5 in this solvent system.

At-8 is a reddish oily substance. IR V_{max} cm^{-1} 1764, 1683, 1643, 2925, 1268, 1415. ^1H NMR (400MHz, $CDCl_3$) δ 9.31 (1H, s, H-1); 6.38 (1H, t, 3H); 2.40 (1H, q, H-4); 2.15 (2H,t,H-5); 5.10 (1H, t, H-7); 2.31 (1H, m, H-8); 2.24 (1H, m, H-8); 3.07 (1H, m, H-9); 3.90(1H, q, H-12); 4.34 (1H, t, H-12); 6.18 (1H, d, H-13); 5.58 (1H, d, H-13); 1.67 (3H, s, H-14); 1.60 (3H, s, H-15). UV λ_{max} ($CDCl_3$) 309nm. The ^{13}C NMR data and spectrum are shown in Table 5 and Appendix 6 respectively.

3.3.2 Isolation of At-11

CE2 (1.4 g) was applied on 40 g silica gel column and eluted using chloroform and methanol solvent of increasing polarity. Nine fractions were collected. Based on TLC analysis, fractions that showed the same characteristic were combined.

Table 12. Fractions of CE2

Solvent system	Ratio	Fractions	Volume (ml)	Combined fractions	Code
$CHCl_3$:MeOH	10:1	1-6	250		CE2-1
>>	9:1	7	30	2-7	CE2-2
>>	8:2	8	30		CE2-3
>>	7:3	9	30		CE2-4

Fraction CE2-2 was concentrated on a rotavapor, and was rinsed with diethyl ether to give a white crystal. Repeated rinsing of this fraction with diethyl ether and TLC analysis in different solvent systems resulted in 90 mg of pure compound **At-11.** Fraction CE3 (1.1 g) was concentrated on a rotavapor and was rinsed with diethyl ether to yield 198 mg of **At-11.**

At-11 is a white solid (mp 57-59°C). IR V_{max} cm-^{1} 1755, 1656, 3102, 2983, 1458, 1164, 925, 817. ^{1}H NMR (400MHz,$CDCl_3$) δ 2.53 (1H, m, H-2); 1.80 (1H, m,H-3); 1.98 (1H, m, H-3); 4.48 (1H, m, H-4); 2.14 (1H, brm, H-5); 2.24 (1H, brm, H-5); 5.08 (1H, brdd, H-7); 2.14 (1H, brm, H-8); 2.24 (1H, brm, H-8); 2.99 (1H, brm, H-9); 3.84 (1H, dd, H-10); 4.28 (1H, dd, H-10); 5.53 (1H, d, H-13); 6.12 (1H, dd, H-13); 1.15 (3H, d, H-14); 1.54 (3H, s, H-15). UV λ_{max} ($CDCl_3$) 240 nm. The ^{13}C NMR data and spectrum are shown in Table 7 and Appendix 12.

3.3.3 Isolation of At-7

CE5 (1.8 g) was applied on a silica gel column and eluted with mixture of chloroform, acetone, and methanol solvents of increasing polarity. 22 fractions were collected. Each fraction amount in average 25 ml. the fractions were combined according to their R_f value and same color on TLC analysis.

Fraction 12-14 (84 mg) was applied on 13 g silica gel and eluted with diethyl ether and chloroform solvents of increasing polarity. **At-7** was isolated along with other compound that have the same R_f value in different solvent systems, but slightly different R_f value in a ratio 4.5:0.5 diethyl ether and chloroform solvent mixture.

They have similar color of spot when the TLC was sprayed with 0.5% vanillin in H_2SO_4. 12.6 mg of the two components was applied on a preparative TLC (1mm layer thickness) and developed in 4.5:0.5 diethyl ether and chloroform solvent mixture to afford **At-7** (6.2 mg).

To increase the amount of **At-7**, the marc left from the first chloroform extract was extracted again with chloroform and further isolation was conducted. The second chloroform was applied on a sephadex CC. After more purification of the chlorophyll free part elutant on a silica gel CC, it was applied on a PTLC and developed in 1:3 hexane and diethyl ether solvent mixture to yield **At-7** (2.2mg).

AT-7 is an oily and unstable compound. It showed a pink color when it was sprayed with 0.5% vanillin in H_2SO_4. Its ^1H and ^{13}C spectra data are presented in Table 9.

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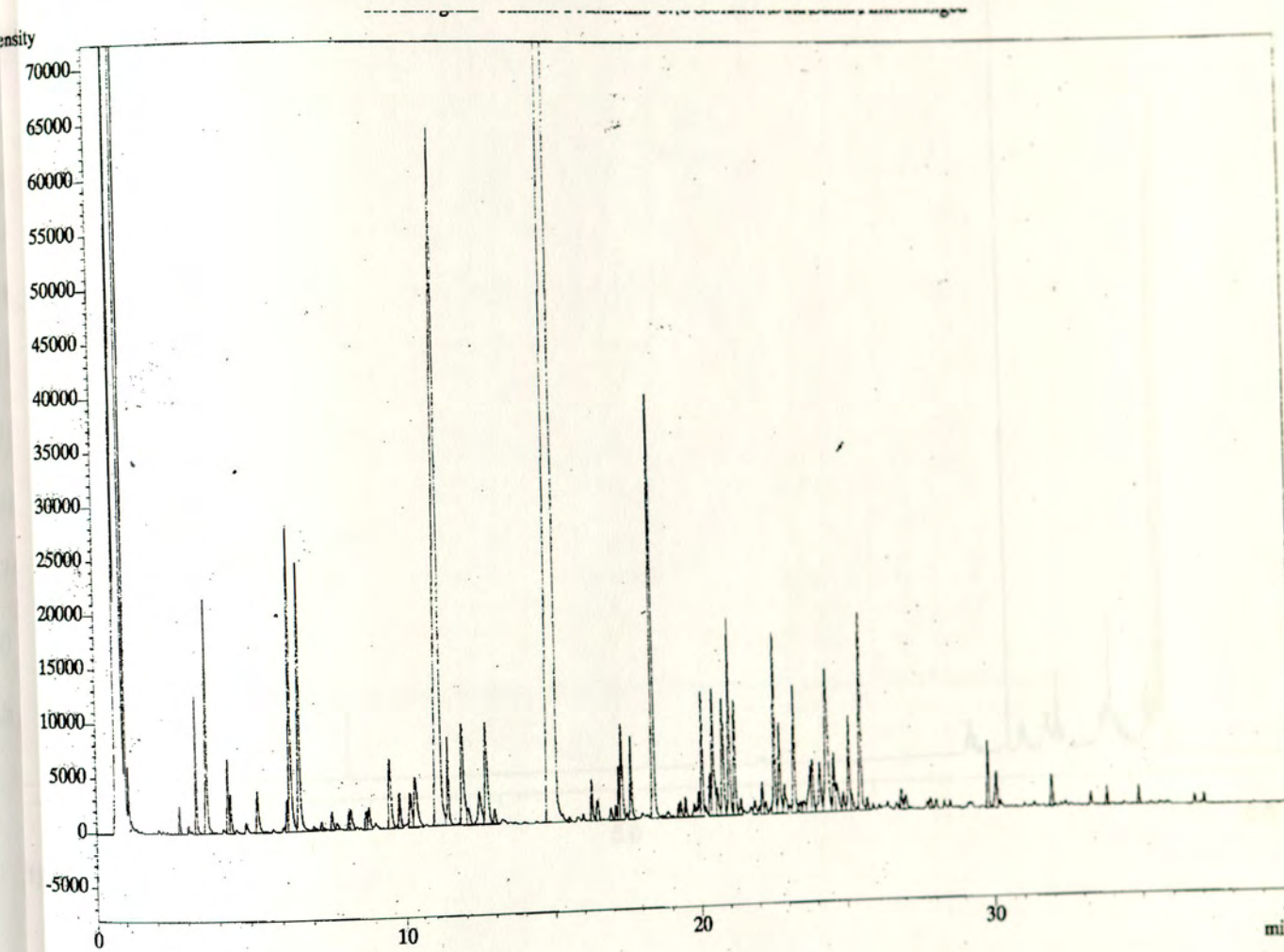
Appendix 1: GC chromatogram of the essential oil of *Anthemis tigreensis*



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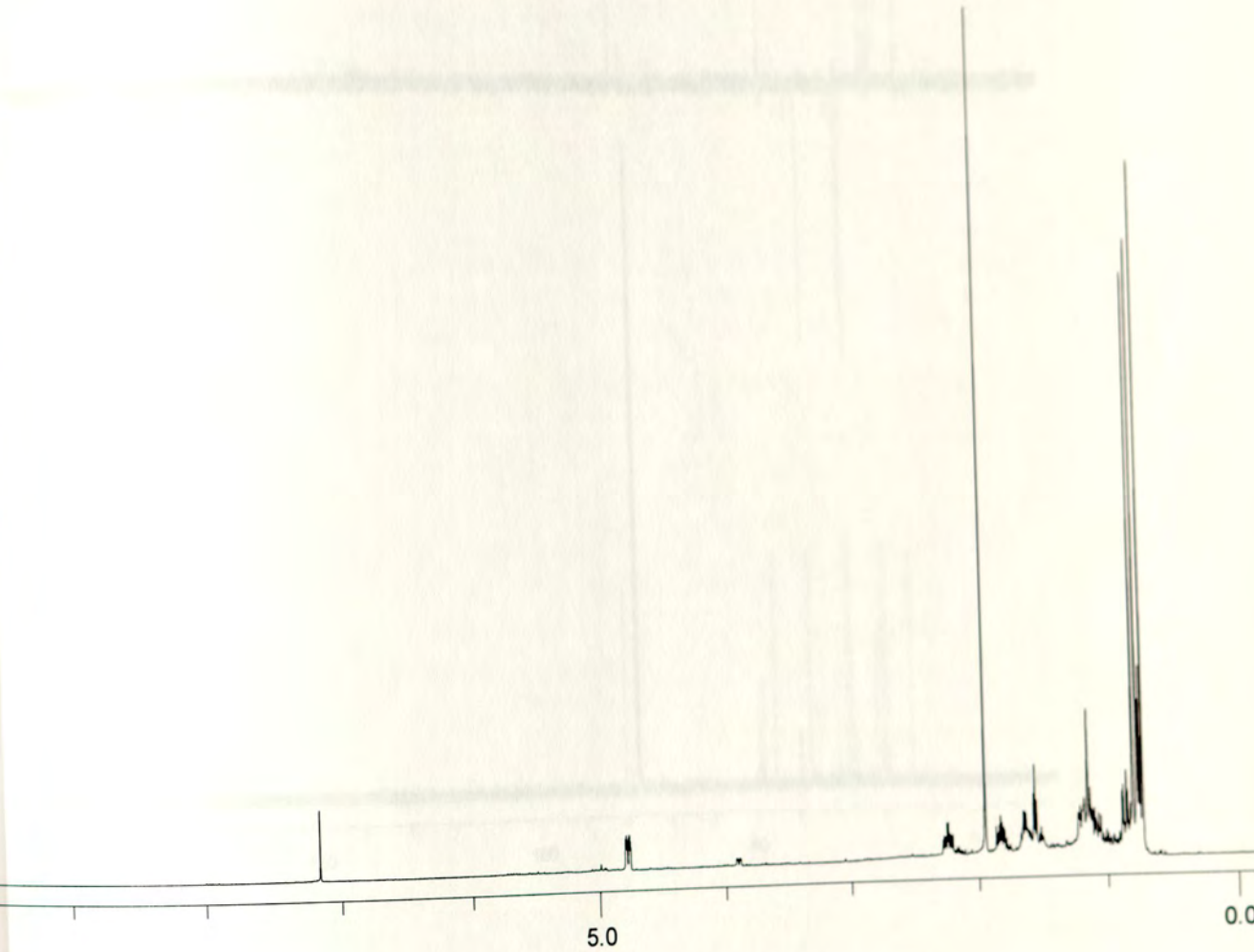
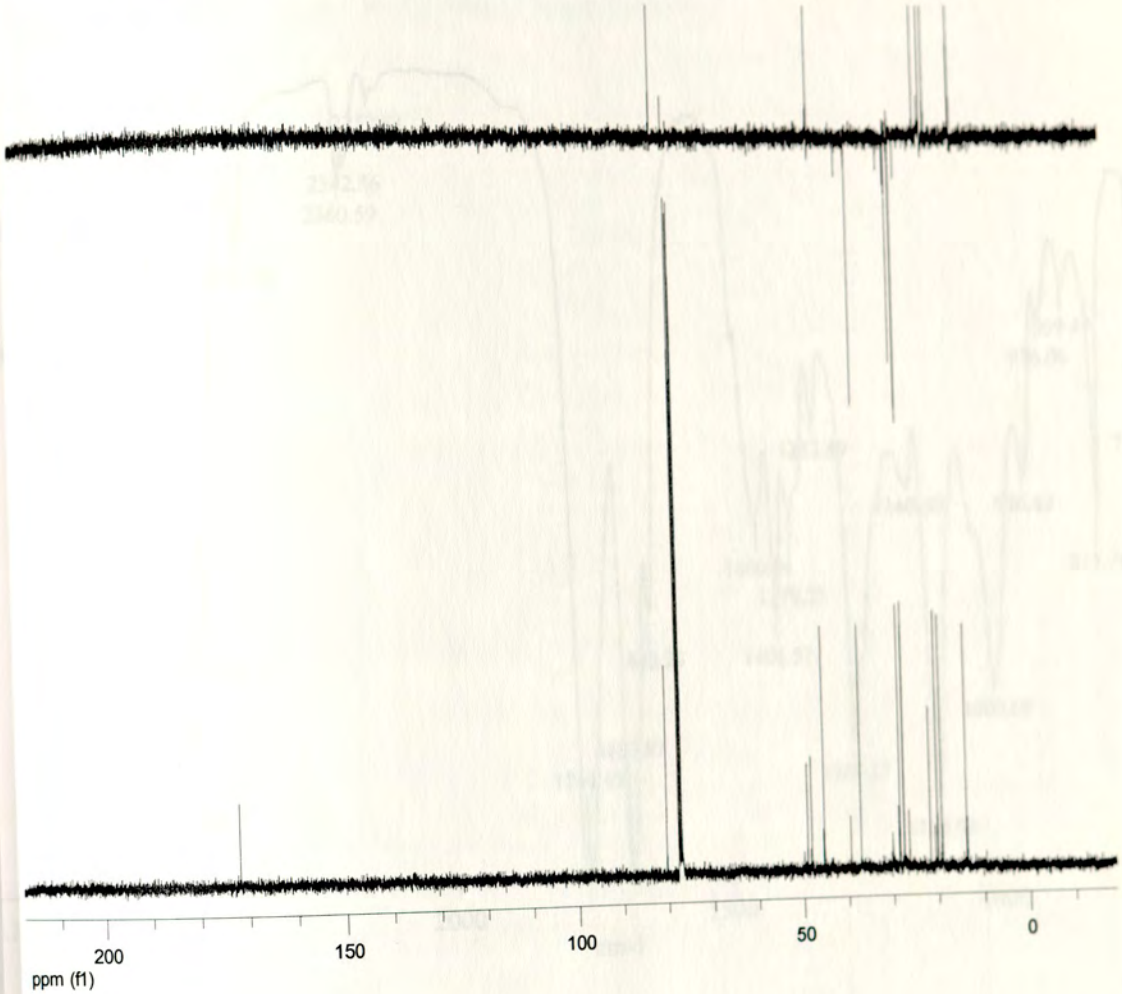
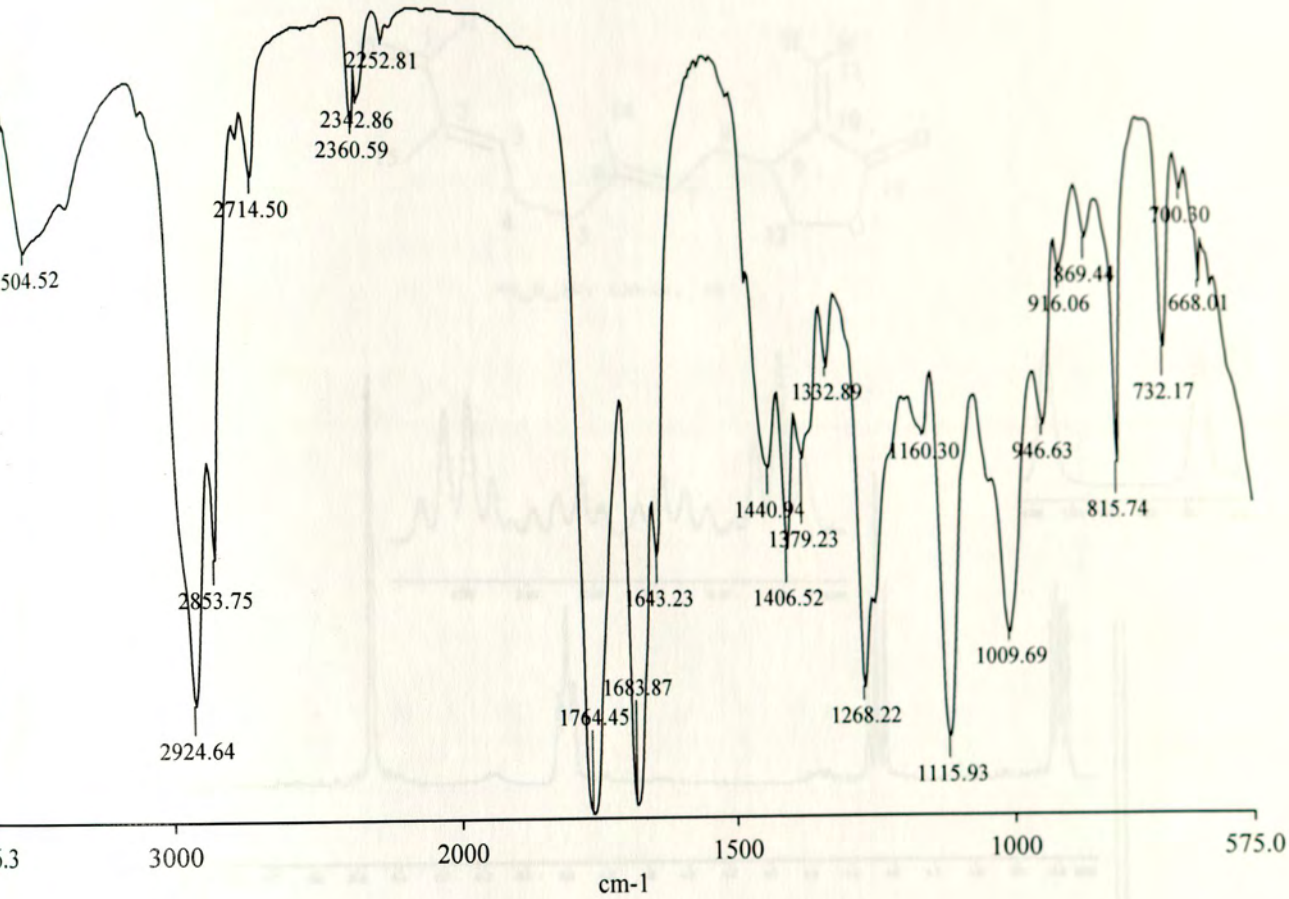
Appendix 2: ^{1}H NMR spectrum of the essential oil



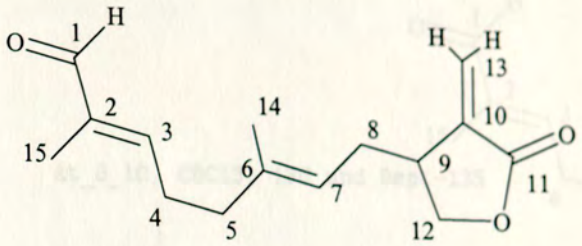
Appendix 3: ^{13}C and DEPT-135 NMR spectrum of the essential oil

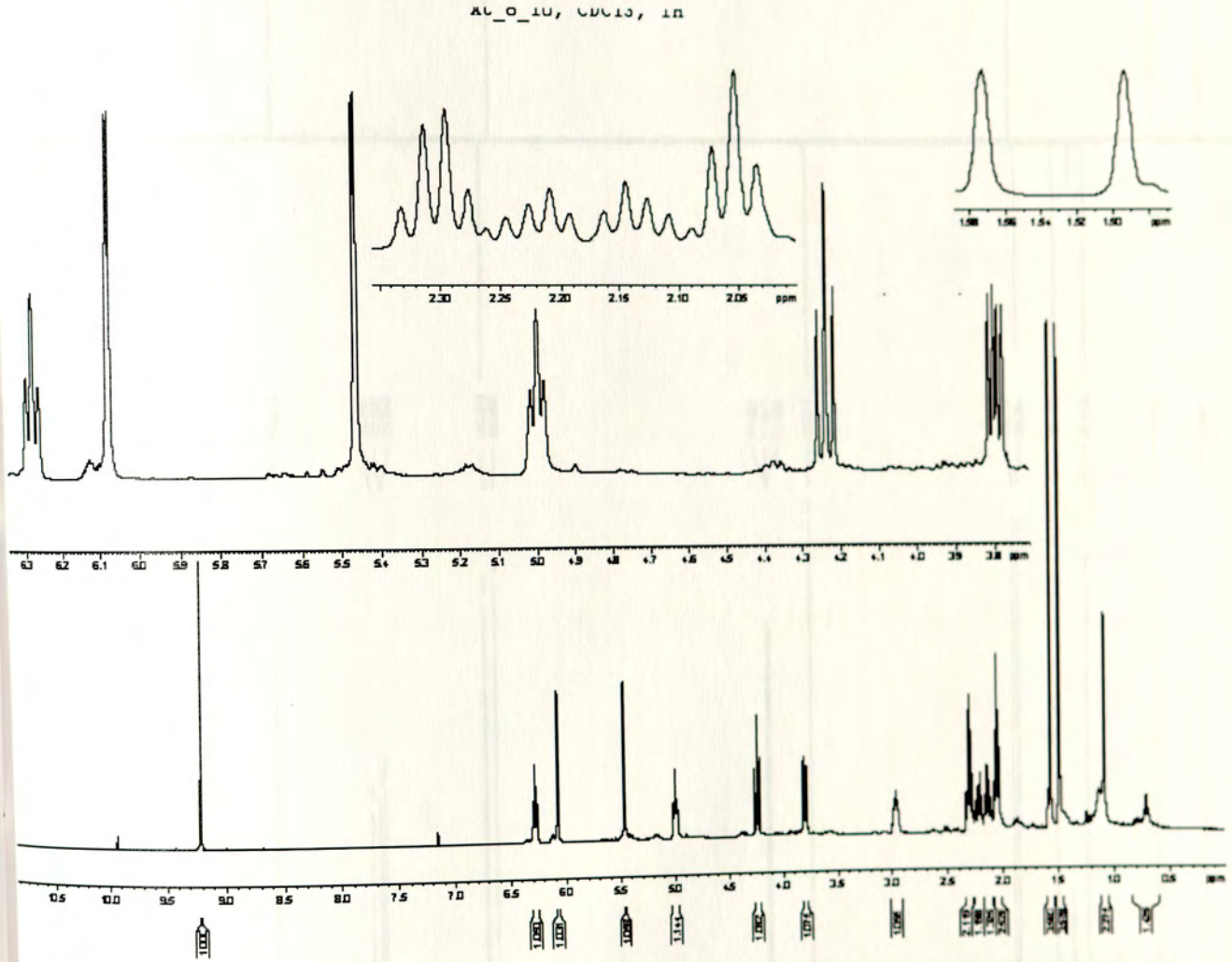


Appendix 4: IR spectrum of **At-8 (17)**

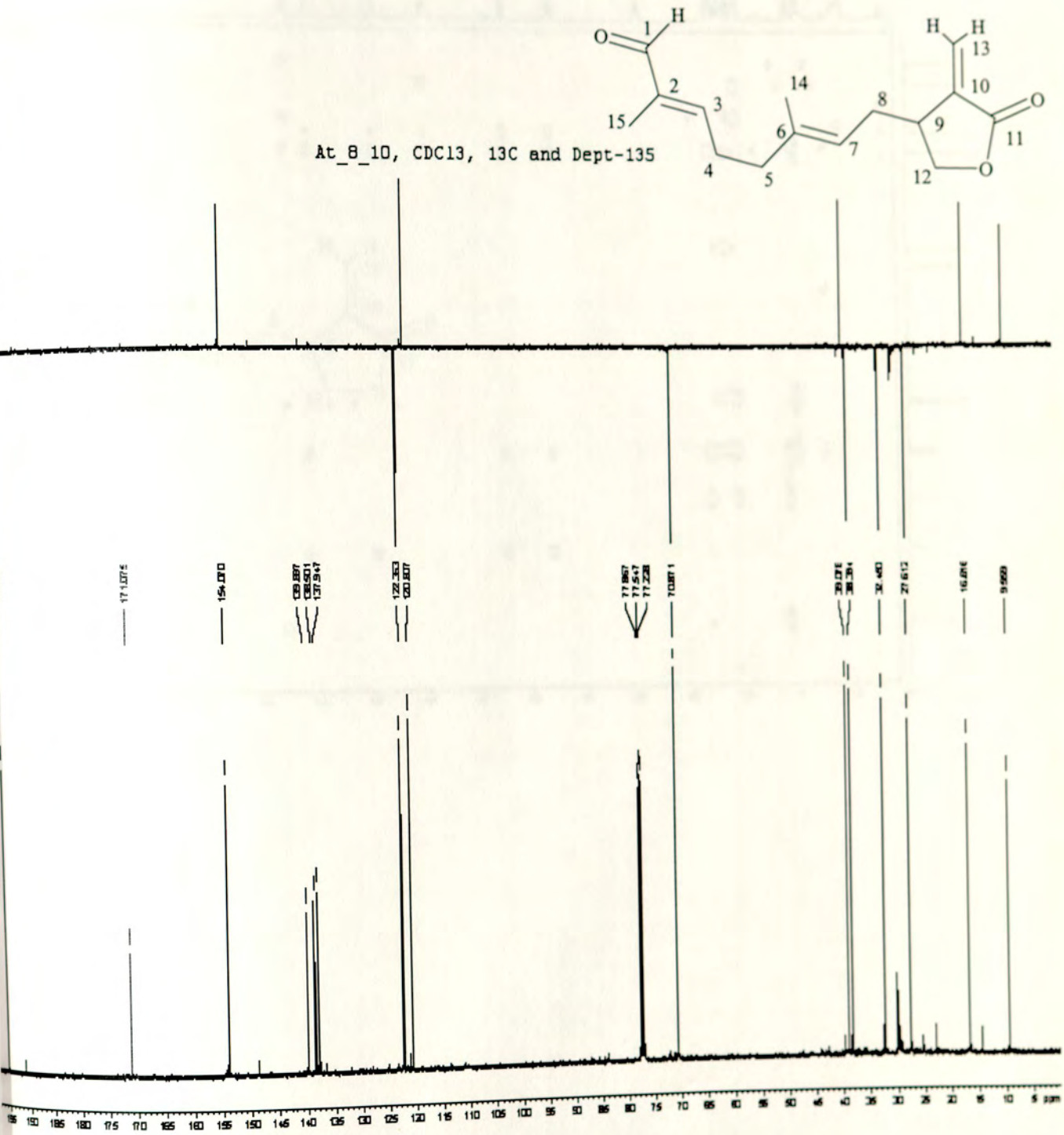


Appendix 5: ^{1}H NMR spectrum of **At-8 (17)** see Table 4

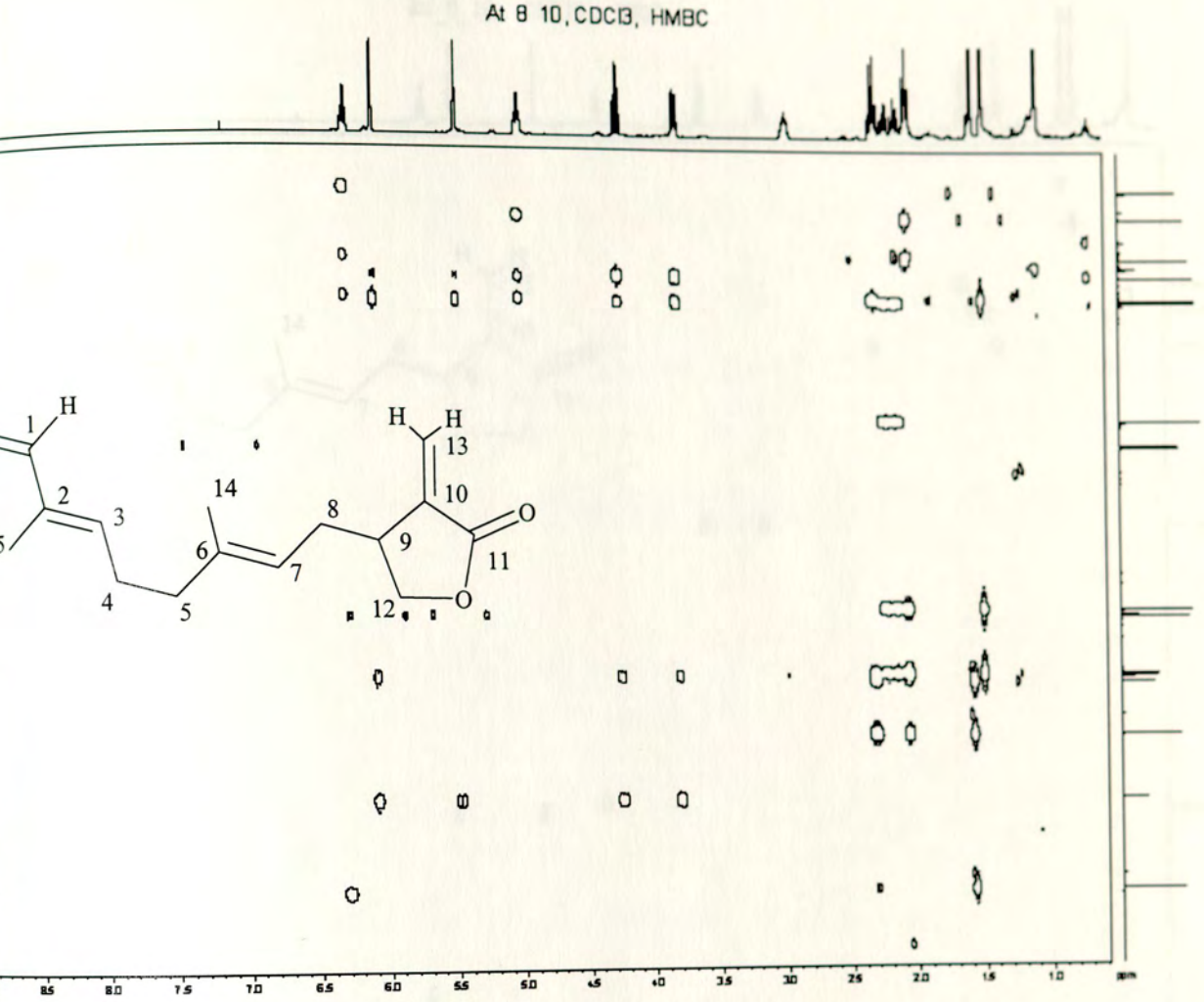




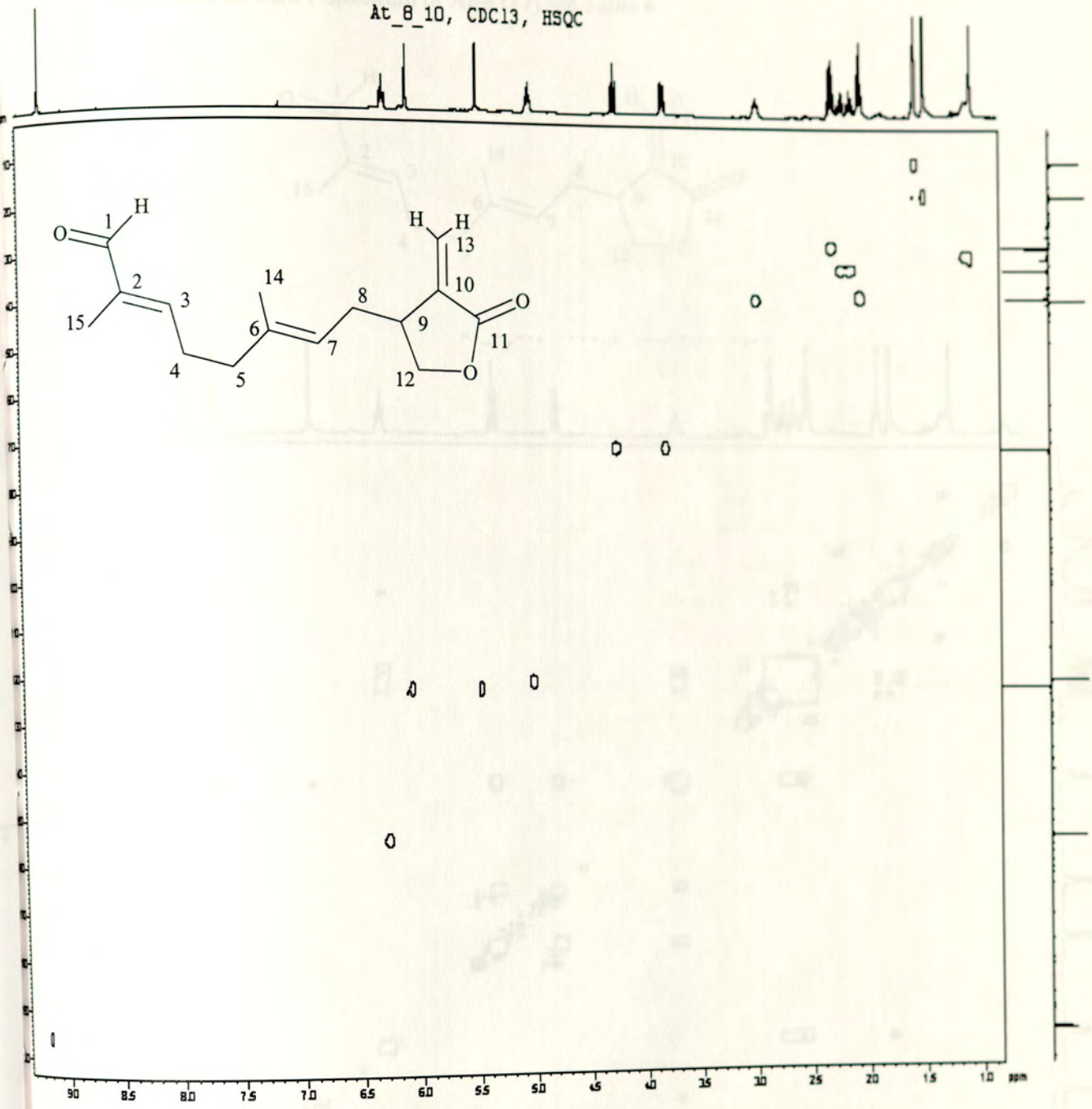
Appendix 6: ^{13}C NMR spectrum of **At-8** (**17**) see Table 5



Appendix 7: HMBC spectrum of **At-8 (17)** see Table 5



Appendix 8: HSQC spectrum of **At-8 (17)**



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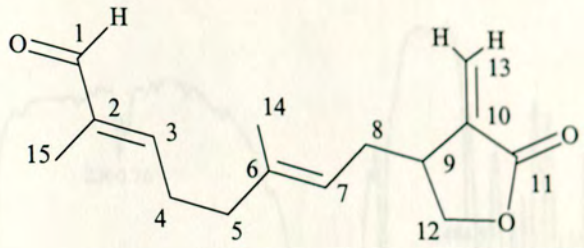
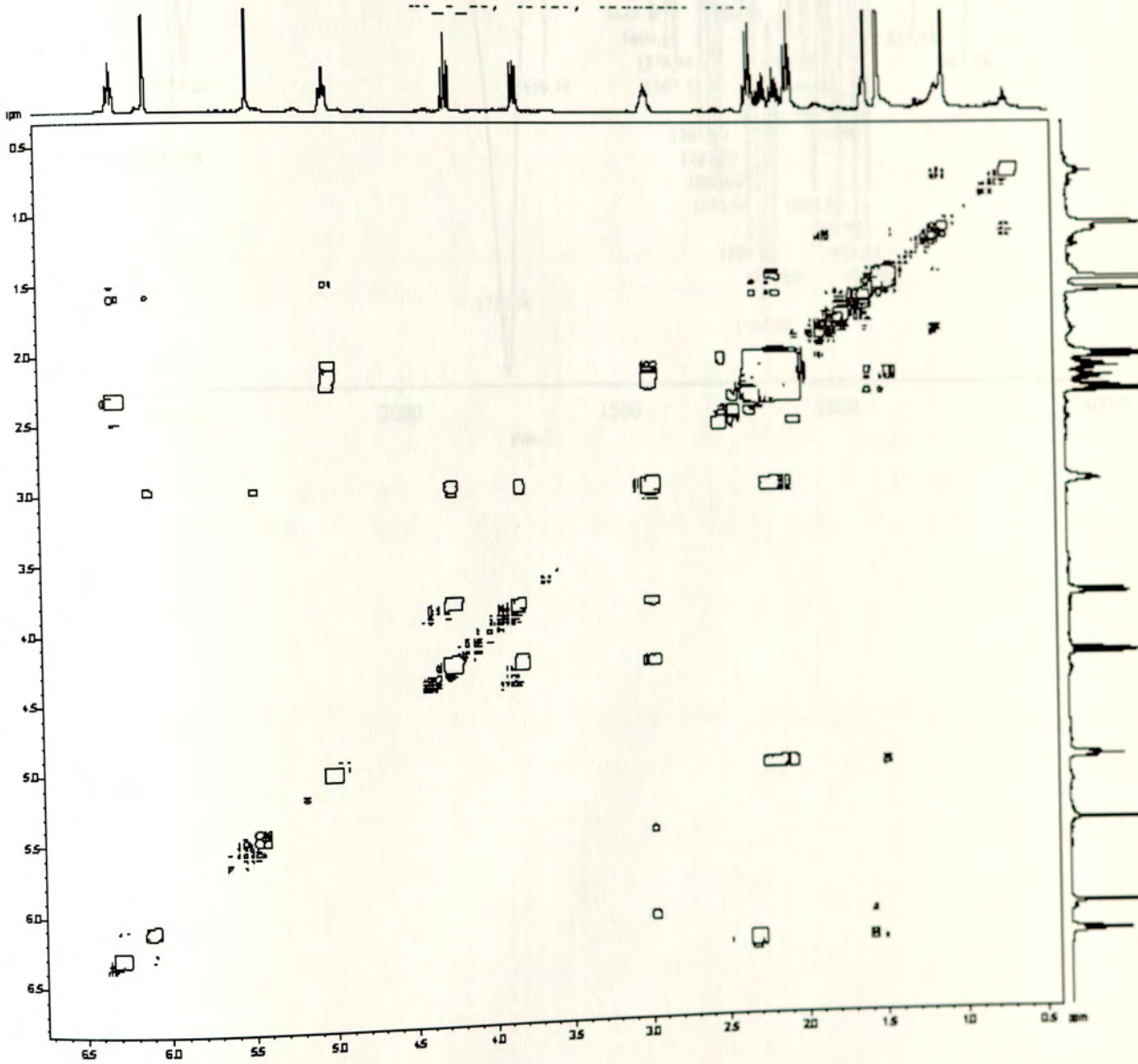
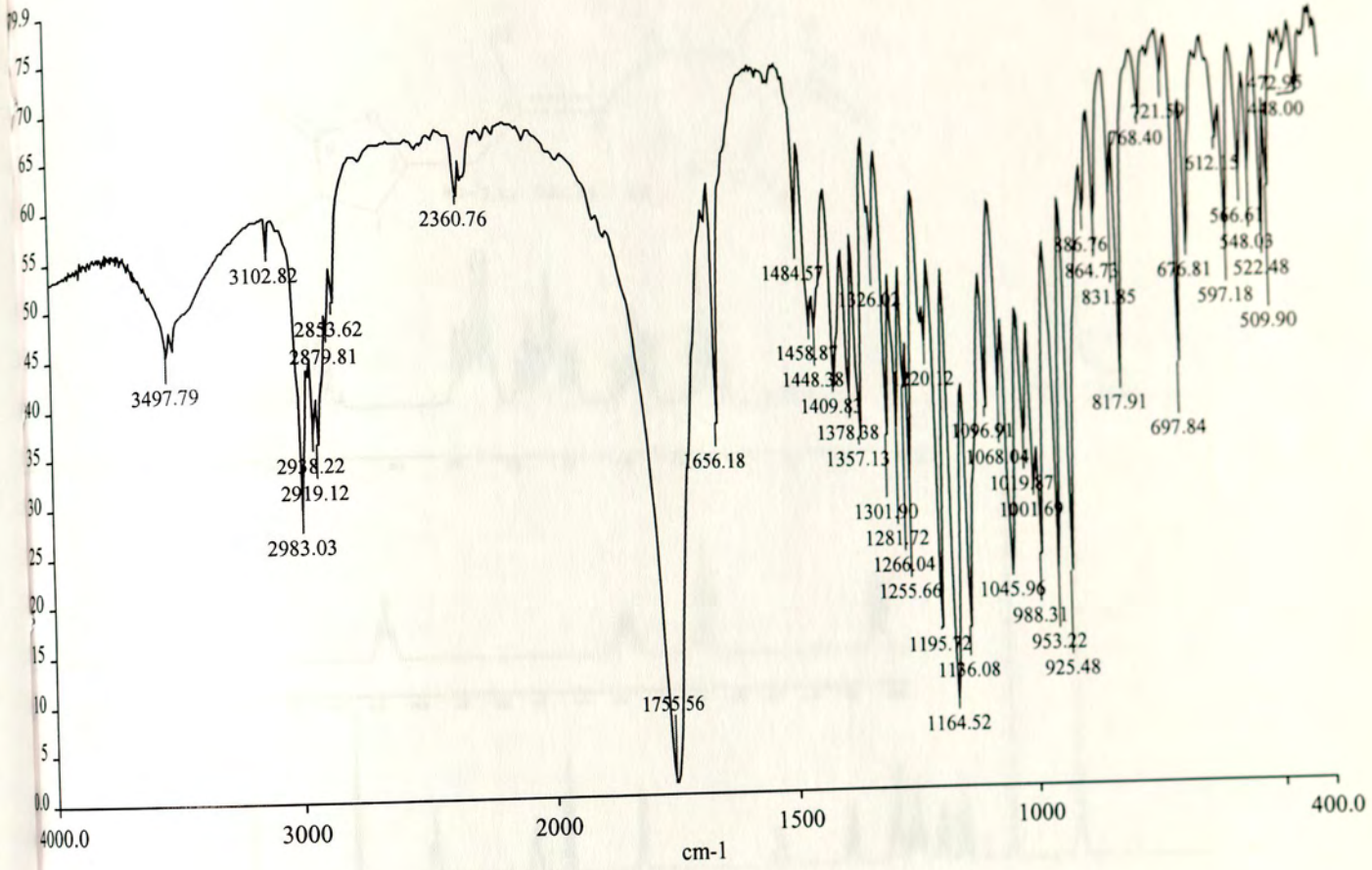
Appendix 9: COSY spectrum of **At-8 (17)** see Table 4

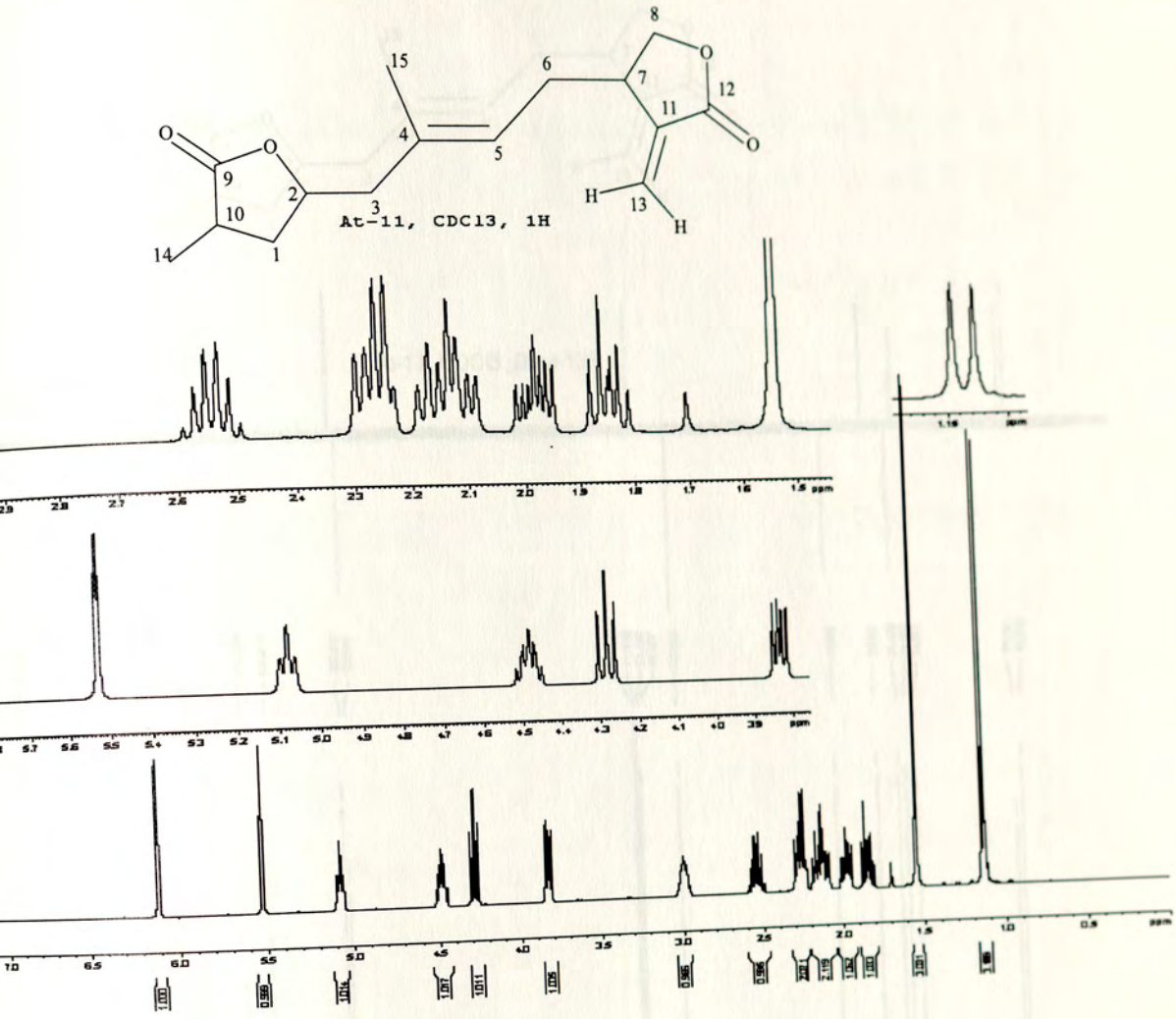




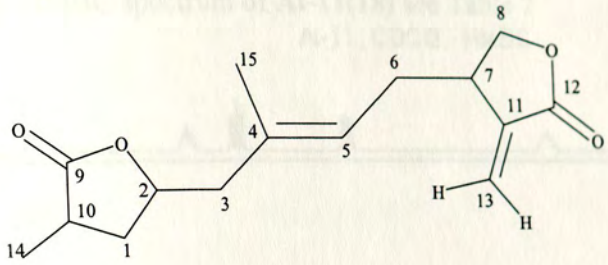
Appendix 10: IR spectrum of **At-11 (18)**

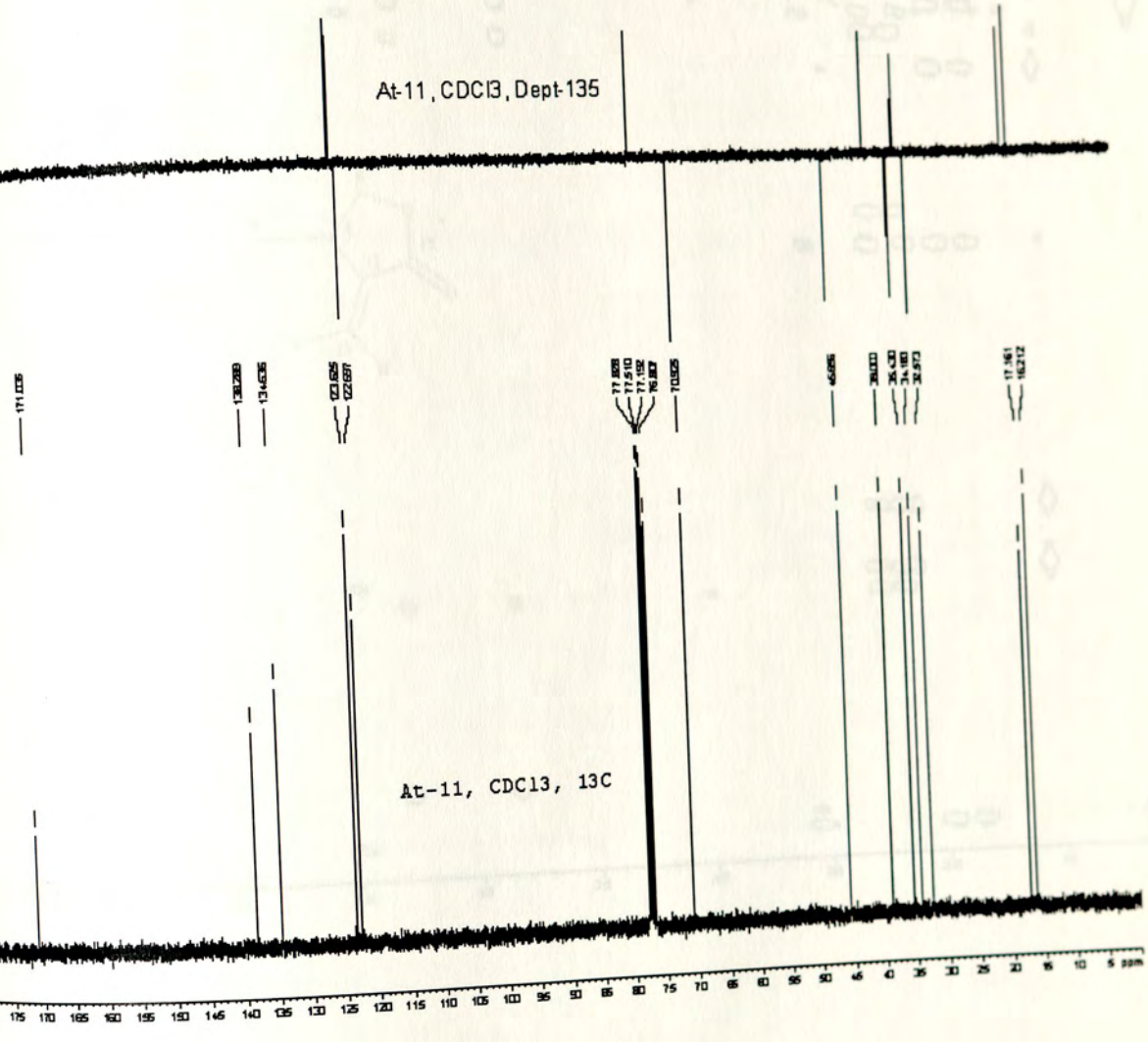


Appendix 11: ^{1}H NMR spectrum of **At-11 (18)** see Table 6

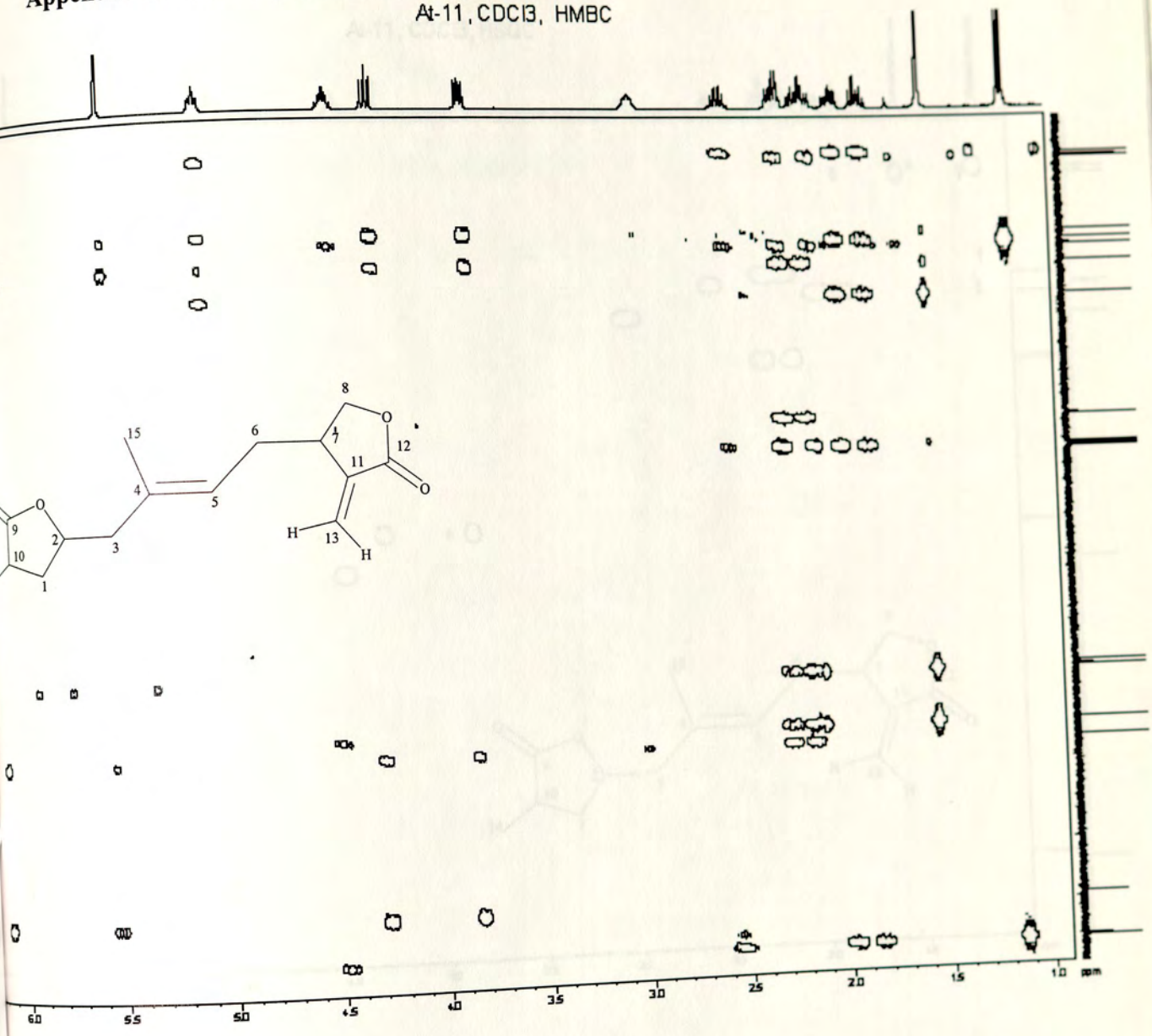


Appendix 12: ^{13}C NMR and DEPT-135 spectrum of **At-11 (18)** see Table 7

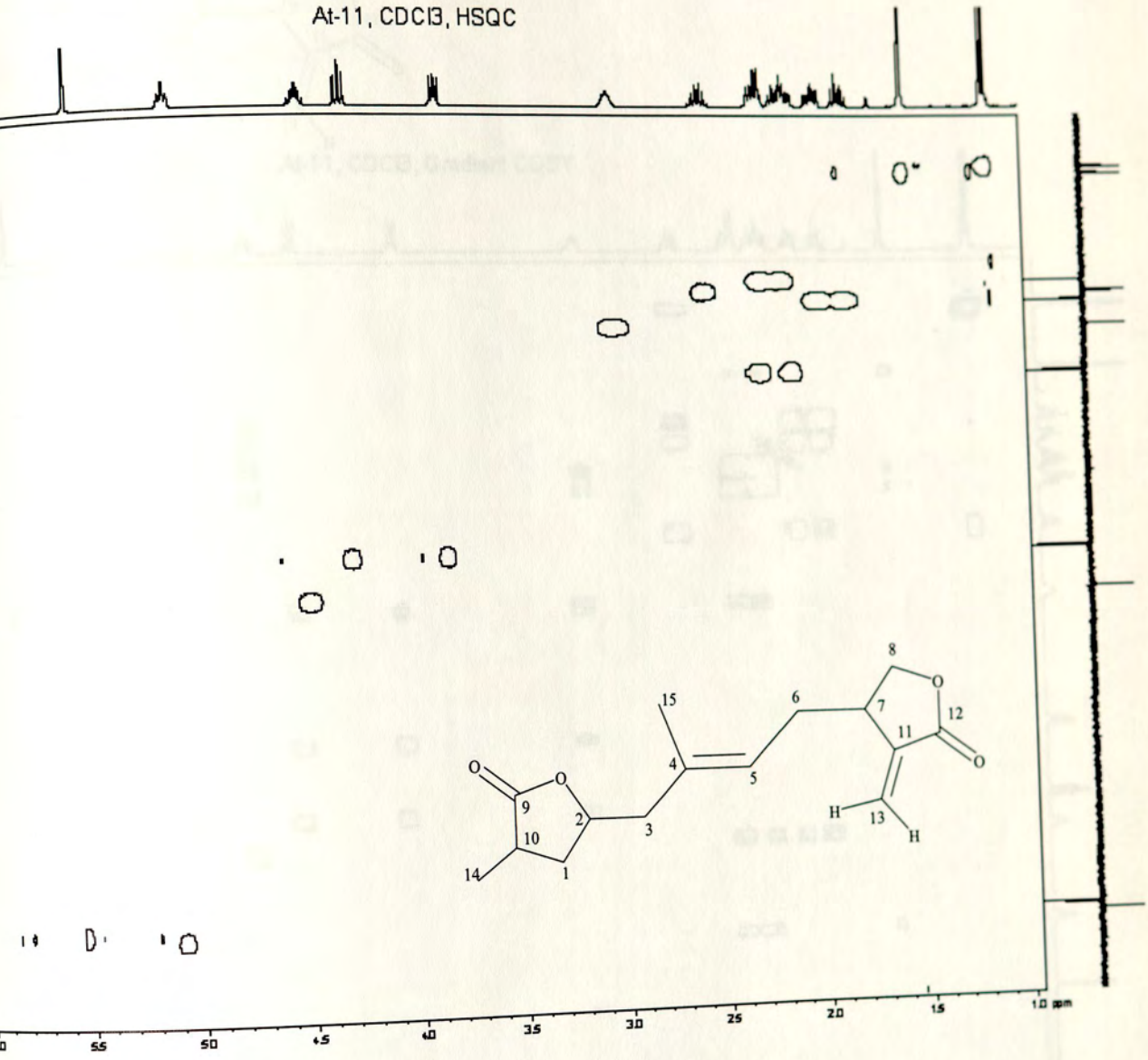




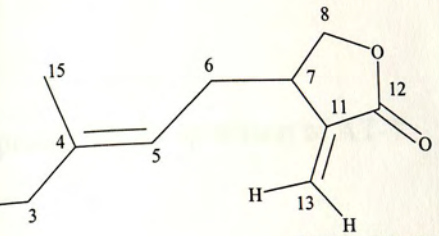
Appendix 13: HMBC spectrum of **At-11(18)** see Table 7

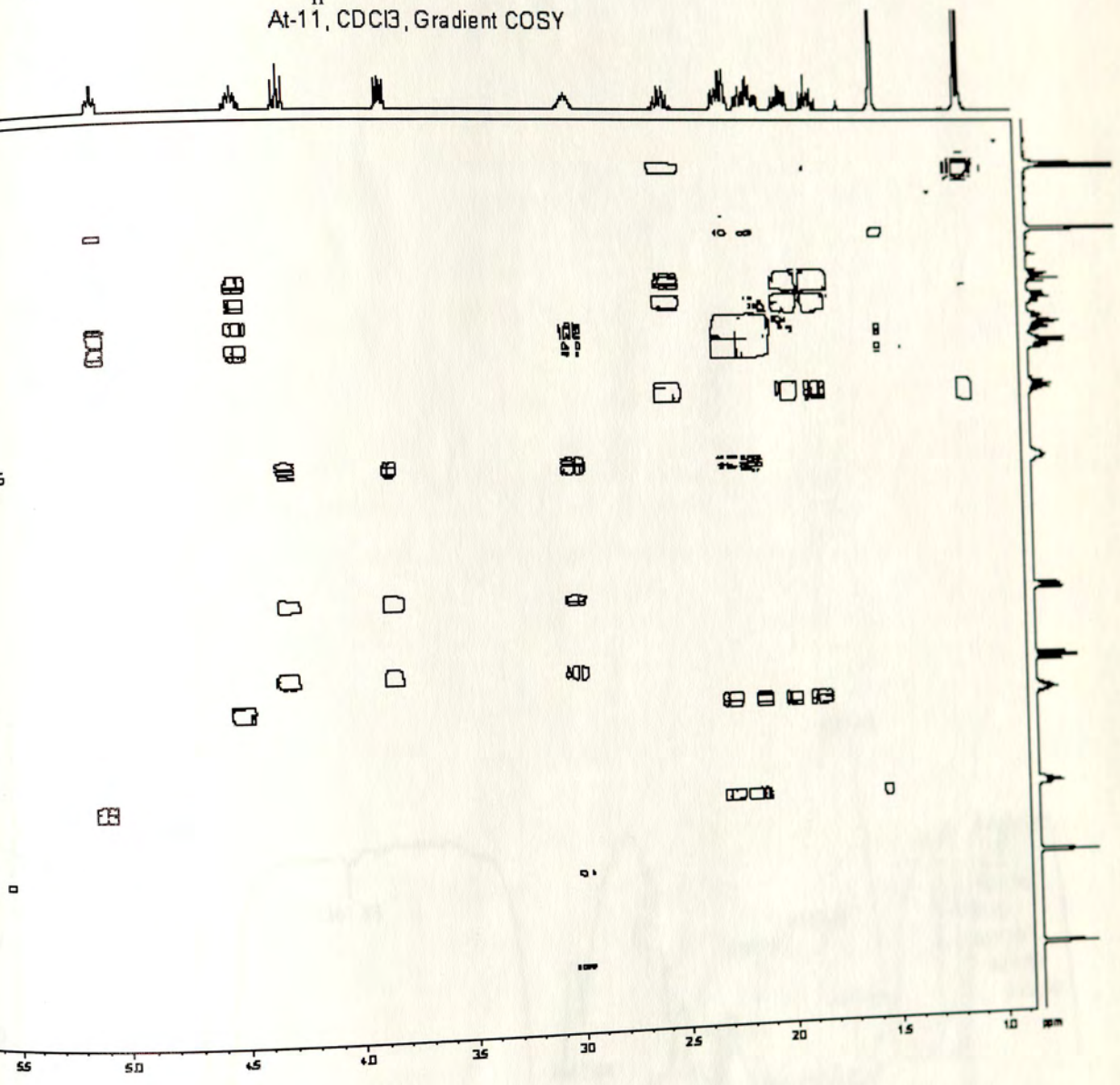


ppendix 14: HSQC spectrum of **At-11(18)**

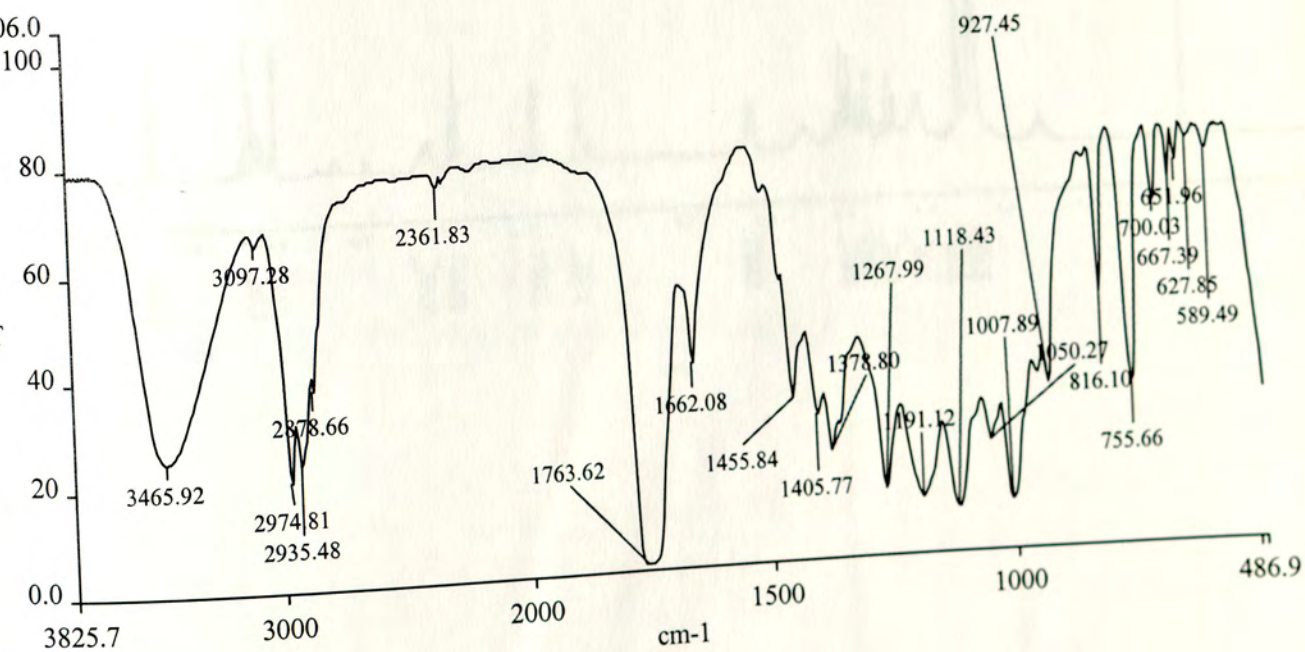


ppendix 15: COSY spectrum of **At-11 (18)** see Table 6

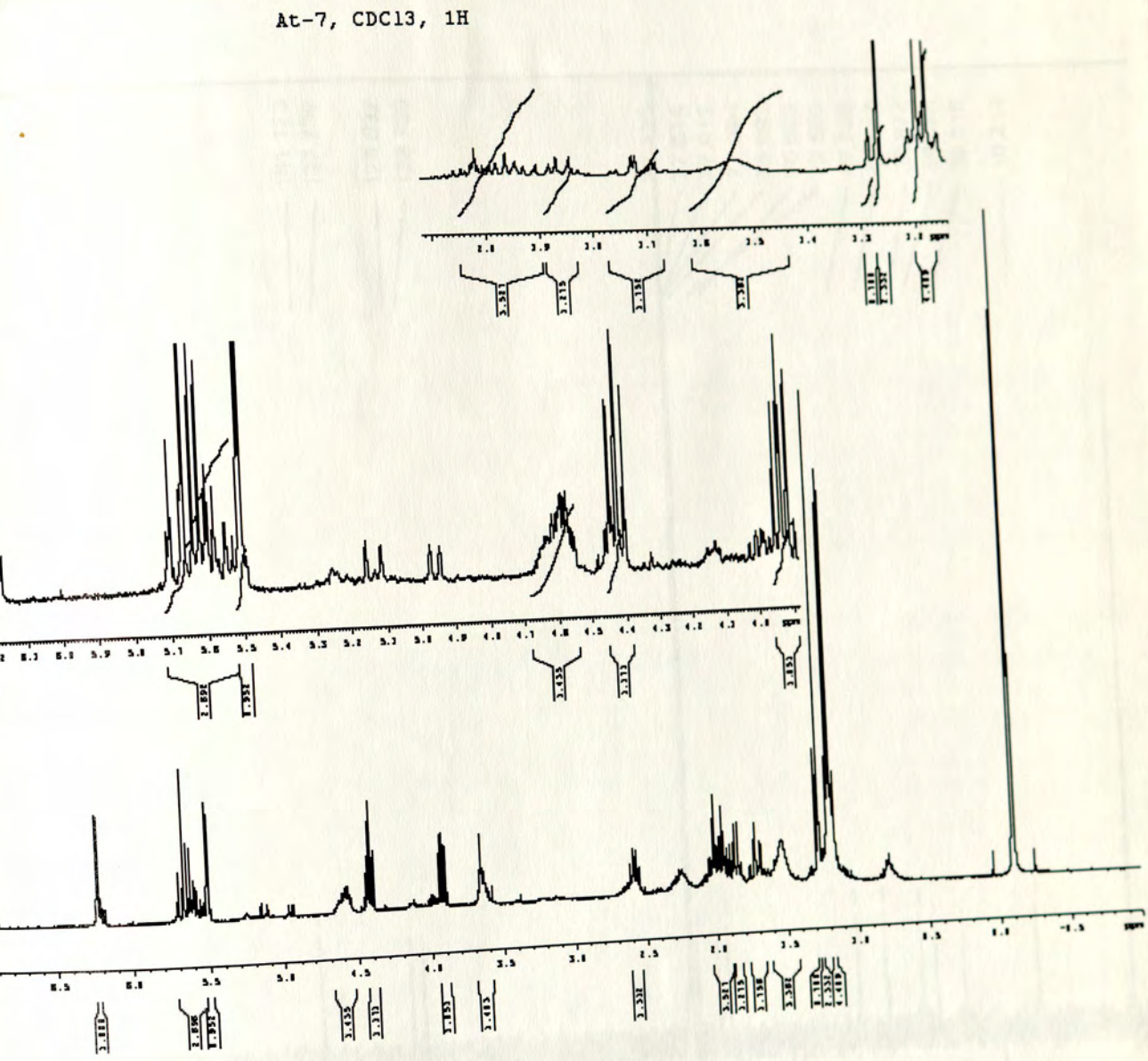




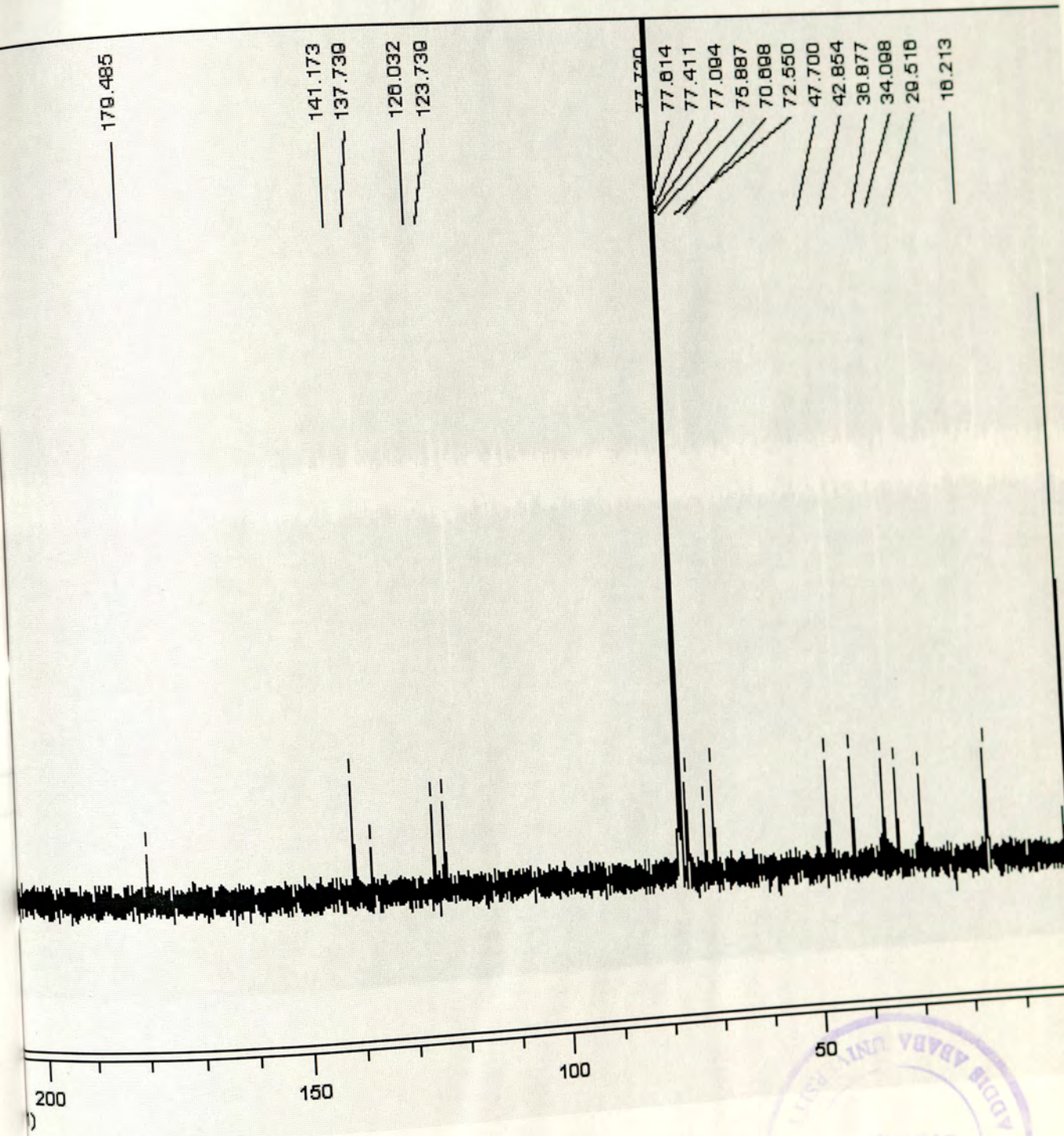
Appendix 16. IR spectrum of **AT-7**



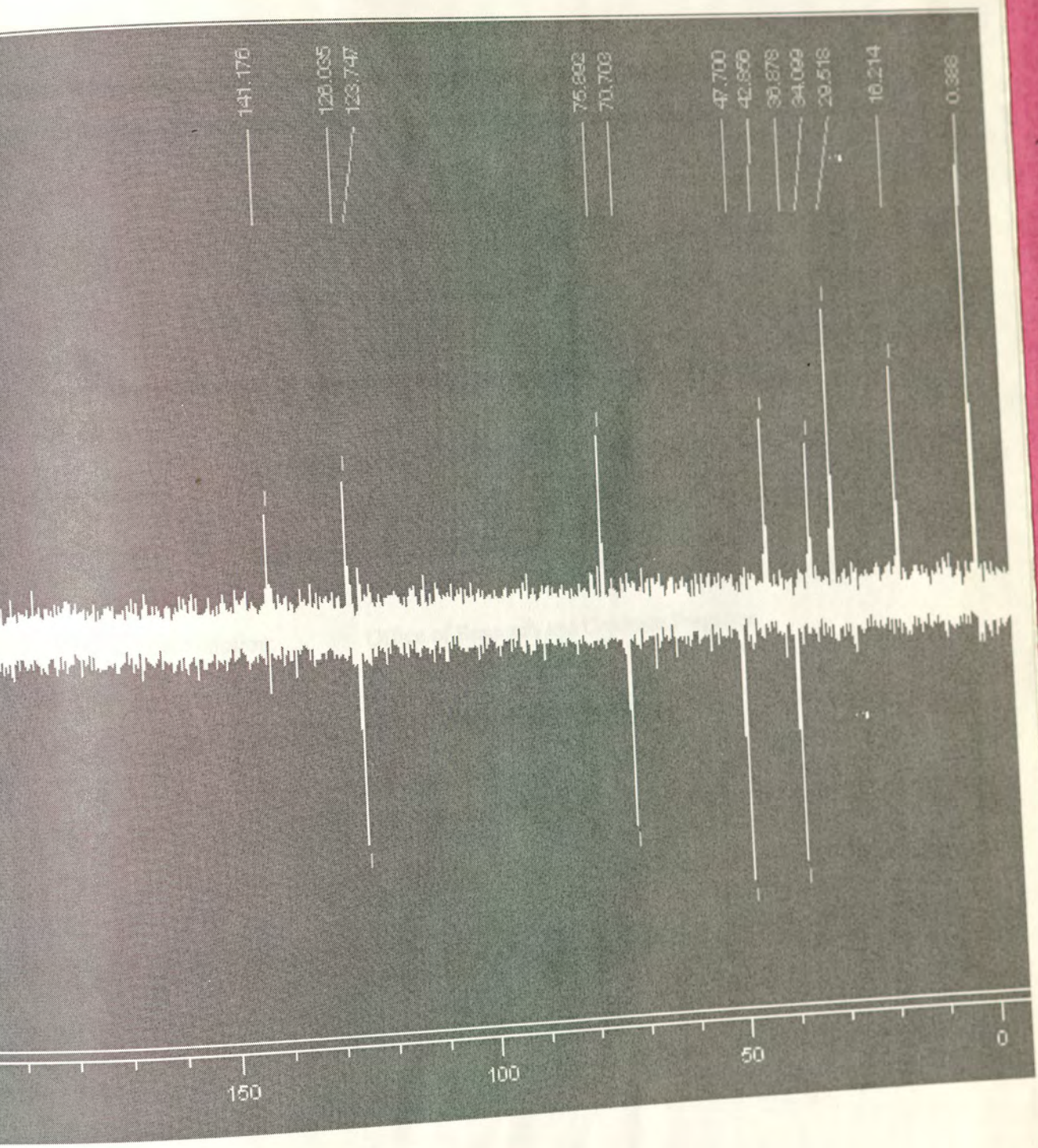
ɐendix 17. ^{1}H NMR spectrum of At-7



Appendix 18. ^{13}C NMR spectrum of At-7



ndix 19. DEPT-135 spectrum of At-7



Declaration

The thesis is my original work, has not been presented for a degree in this and other university and that all sources of material used for the thesis have been duly acknowledged.

Name: Sinedu Dejene Tefera

Signature: .....

The thesis has been submitted for examination with my approval as university advisor;

Dr Nigist Asfaw

Signature: .....

Place and data of submission: Office of Research and Graduate Programs

Addis Ababa University

June 2005