ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

DEPARTMENT OF CHEMISTRY

PHYTOCHEMICAL INVESTIGATION ON THE LEAVES OF ACHYRANTHES ASPERA

BY: G/MESKEL G/MICAEL

ADVISOR: Dr. ASEBIR FISEHA

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APPROVED BY THE EXAMINING BOARD:

Dr. Ashebir Fiseha

Advisor _____

Dr. Nigist Asfaw

Examiner _____

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LIST OF ABBREVIATION

TLC……..Thin Layer Chromatography

PTLC……Preparatory Thin Layer Chromatography

CC……Column chromatography

IR……..Infrared

UV……Ultra Violent

NMR…….....Nuclear Magnetic Resonance

DEPT……Distortion less Enhancement by Polarization Transfer

COSY……...Correlation Spectroscopy

HMQC……..Hetronuclear Multi Quantum Correlation

HMBC……..Hetronuclear Multiple Bond Correlation

δ……delta (symbol for chemical shift)

d……doublet

dd……..doublet of doublet

m……...multiplet

s…….singlet

PPM……..Parts Per Million

MHz……..Mega Hertz

mL…….milliliter

Km……Kilometer

RF……..Retention Factor

g…….gram

mg……..milligram

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Abstract

Achyranthes aspera is a well known medicinal plant in the family Amaranthaceae. The plant is locally known as “Telenge”. It is traditionally used in asthma and cough. The root of Achyranthes aspera is used for anti snake bite by giving the ground root with water until the patient vomits and regains consciousness while the crushed leaves are used to cure strained back. Phytochemical investigation on the leaves of this plant resulted in the isolation of two compounds (**AA-1**and **AA-2**). Compound AA-1 is a sesquiterpene while compound AA-2 is an insect molting ecdysteroid hormone namely:

1.) 4,4a,7,8-tetrahydro-4-hydroxy-4a,7,8-trimethyl-8-(tetrahydro-3,4,5-trihydroxy-6-(hydroxymethyl)-2H-pyran-2-yloxy)naphthalen-2(3H)-one
2.) 20-hydroxyecdysone.

Structural elucidations of those compounds are based on IR, UV, ^{1}D NMR (^{1}H, ^{13}C &DEPT) and ^{2}D NMR (COSY, HMQC &HMBC) spectroscopic data obtained and in comparison with similar compounds isolated from the plant.

To the best of my knowledge, there is no report for the isolation of the first compound (AA-1) from this plant and other families and species.

1. Introduction

1.1. Natural products

Plant-derived substances have recently become of great interest owing to their versatile applications [1]. Plants are used medicinally in different countries and are a source of many potent and powerful drugs [2]. Natural product chemistry is a science, which studies different products from living matter, animals or plants. These products are very important for mankind. According to their nature, natural products can be used for different purposes. For instance, humans use them for pharmaceutical purpose, for preparing foodstuffs, insecticides, antioxidants, coloring matters, flavors and fragrances, extraction of enzymes, pheromones and so on [3].

Natural product is a chemical compound or substance produced by a living organism. They may be extracted from tissues of terrestrial plants, marine organism or micro - organism fermentation. In that respect any biological molecule is a natural product, but in general the term is reserved for secondary metabolites (carotinoids, phytosterines, saponines, phenolic compounds, alkaloids, glycosinates, terpenes etc.), produced by an organism [4]. Secondary metabolites are chemical compounds derived from living organisms. The study of natural products involves isolation in a pure form of these compounds and investigation of their structure, formation, use, and purpose in the organism. Secondary metabolites appear to function primarily in defense against predators and pathogens and in providing reproductive advantage as intraspecific and interspecific attractants [5].

Natural products, such as plants extract, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug discoveries because of the unmatched availability of chemical diversity. According to the World Health Organization (WHO), more than 80% of the world's population relies on traditional medicine for their primary healthcare needs [4].
Medicinal plants have played a significant role in ancient traditional systems of medication in many countries. They are rich source of bioactive compounds and thus serve as important raw materials for drug production. Now-a-days multiple drug resistance has developed due to the indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious disease. This situation forced scientists to search for new antimicrobial substances [6].

1.2. The Genus Achyranthes

Amaranthaceae is a cosmopolitan family consisting of 64 genera and about 800 species, mostly abundant in tropical regions of America, Africa and India .The family, represented by herbs and few shrubs, contains most of the important allergic species. The family is an important component of arid and semi-arid floras, especially of Australia and North America, and often found in saline habitats [7].

The genus Achyranthes belongs to the family Amaranthaceae. It is perennial stiff erect herb, growing up to 1 m height. Stems are square, leaves elliptic, ovate or broadly rhombate. Some other species of the genus *Achyranthes* viz. *A. fauriei, A. bidentata, A. japonica, A. ferruginea* etc. have also been investigate for their active constituents and pharmacological potential [8].

1.3. Achyranthes aspera

1.3.1. Background of Achyranthes aspera

Achyranthes aspera Linn, Amaranthaceae commonly known as Latjeera in Hindi, Shikhari or Apamarga in Sanskrit, Prickly-chaff flower plant in English, is an erect or procumbent, annual or perennial herb, found on road sides, field boundaries and waste places as a weed throughout India up to an altitude of 2100 m [9]. Achyranthes aspera L., locally known as “Telenge or ambulale” is one of the traditionally used anti-fertility plants in the indigenous health care delivery system of Ethiopia [10].

Achyranthes aspera Linn, belonging to the family Amaranthaceace is an erect annual herb, 1-2 m. in height, often occurs with a woody base. It has angular stems, ribbed, simple or branched from the base, often tinged with reddish purple colour. The leaves are thick, opposite, velvety tomentose, ovate-elliptic or obviate-rounded variable in shape and size [11]. It bears bisexual, greenish-white flowers, numerous in auxiliary or terminal spikes up to 75 cm. long. The seed are sub cylindrical, truncate at the apex, rounded at the base, reddish brown [12]. The plant is considered a bitter acrid, carminative, astringent, pectoral, cardio tonic and diuretic [13].

Figure 1: Achyranthes aspera plant.

1.3.2. Cultivation and distribution of Achyranthes aspera

Achyranthes aspera Linn. (Amaranthaceae), a genus of herbs or small shrubs, is distributed throughout the tropical and subtropical regions. It is an erect, annual herb, commonly found in India, Baluchistan, Ceylon, Tropical Asia, Africa, Australia and America [14, 15]. In Philippines the plant is used to relieve toothache, dysentery and bowel complaint [16]. It is found on road sides, field boundaries and waste places as a weed throughout India up to an altitude of 2100 m and in South Andaman Islands [17].

1.4. Use of Achyranthes aspera

1.4.1. Traditional use of Achyranthes aspera

The plant is highly esteemed by traditional healers and used in treatment of asthma, bleeding, in facilitating delivery, boils, bronchitis, cold, cough, colic, debility, dropsy, dog bite, dysentery, ear complications, headache, leucoderma, pneumonia, renal complications, scorpion bite, snake bite and skin diseases . Traditional healers claim that addition of *A. aspera* would enhance the efficiency of any drug of plant origin [18].

It is pungent, ant phlegmatic, ant periodic, diuretic, purgative and laxative, useful in edema, dropsy and piles, boils and eruptions of skin etc. Crushed plant is boiled in water and is used in pneumonia. Infusion of the root is a mild astringent in bowel complaints. The flowering spikes or seeds, ground and made into a paste with water, are used as external application for bites of poisonous snakes and reptiles, used in night blindness and coetaneous diseases. For snake bites the ground root is given with water until the patient vomits and regains consciousness. Inhaling the fume of *Achyranthes aspera* mixed with *Smilax oval folia* roots is suggested to improve appetite and to cure various types of gastric disorders [17]. It is useful in hemorrhoids, leaves and seeds are emetic, hydrophobia, carminative, resolve swelling, digestive and expel phlegm. Ash of the plant is applied externally for ulcers and warts. The crushed leaves rubbed on aching back to cure strained back. Afresh piece of root is used as tooth brush [19].

1.4.2. Medicinal use of Achyranthes aspera

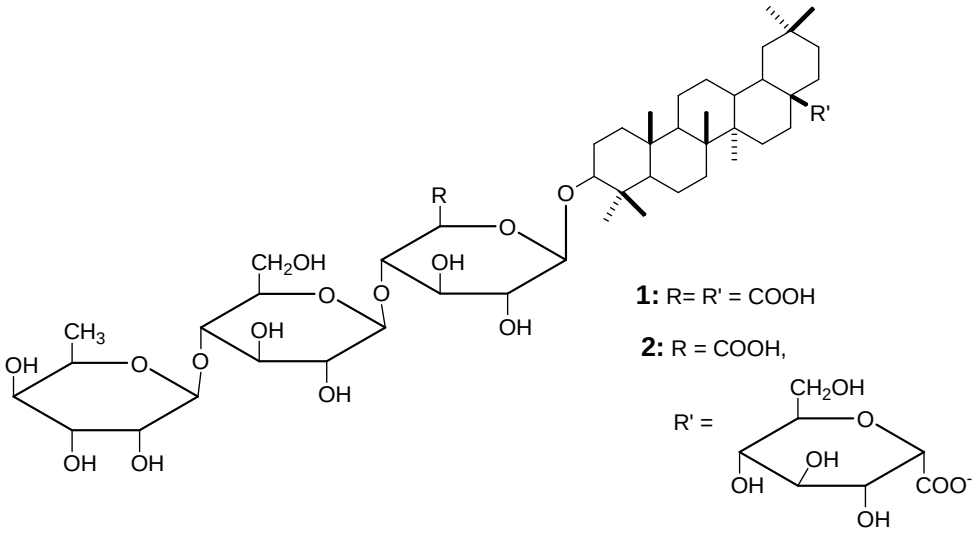
Medicinal plants have played a significant role in ancient traditional systems of medication in many countries. They are rich source of bioactive compounds and thus serve as important raw materials for drug production. *Achyranthes aspera* is one of the important medicinal plants having many theaurapetic uses as Odontalgic, Rheumatism, Bronchitis, skin disease and rabies [6]. The whole plant, the roots and the seeds possess the medicinal properties against many ailments. Ethno pharmacological studies depicted its use in dropsy, skin eruptions, colic, as a diuretic, astringent and purgative as an antidote for snake bite. The inflorescence is used in

cough. The seeds are employed as an emetic, purgative, and cathartic, in gonorrhea, whooping cough, as an anti-asthmatic and for insect bite [20]. The chloroform and ethanol extracts of roots of the *Achyranthes aspera* are reported to have anti-implantation & abortifacient activity. The ethanol extract of the root posses spermicidal activity. The aqueous and methanolic extracts of the whole plant have hypoglycemic effect [8, 21].

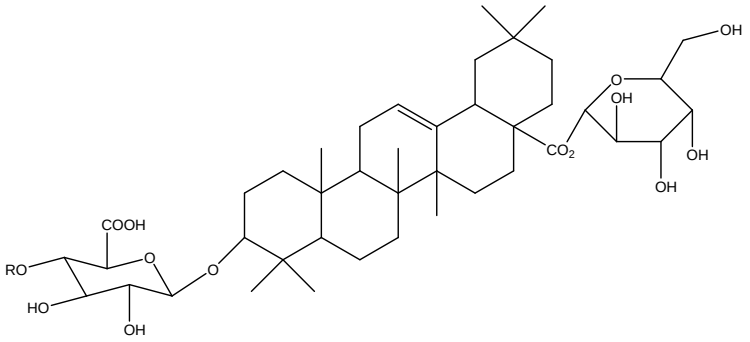
1.5. Chemical constituent of Achyranthes aspera

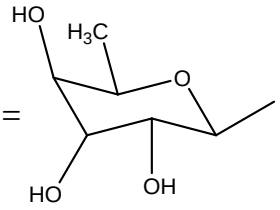
Phytochemistry is the study of phytochemicals produced in plants, describing the isolation, purification, identification, and structure of the large number of secondary metabolic compounds found in plants. Different compounds can be isolated from different part of the plant. Phytochemical investigation on Achyranthes aspera reported to have Alkaloids, flavonoids, saponins, steroids and terpenoids [17].

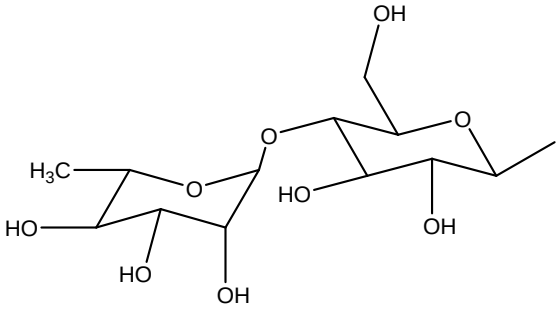
- Chemical investigations of the seeds of *Achyranthes aspera* resulted in the isolation & identification of Saponin A (**1**) and saponin B (**2**). They are glycosides of oleanolic acid. [22, 23].



- Two other saponins, Saponin C (**3**) & Saponin D (**4**) was also isolated from the unripe fruits of Achyranthes aspera. The saponins were found to be steroidal in nature [17, 24].

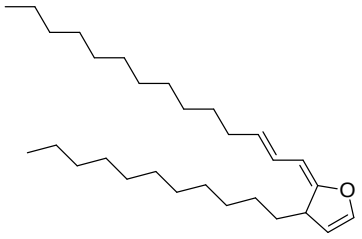


3: R = 

4: R = 

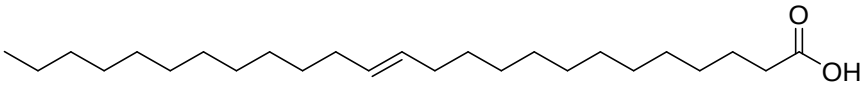
α-L-Rhamnopyranosyl α-L-Rhamnopyranosyl-(1→4)-β-Glucopyranosyl

- A new cyclic chain aliphatic fatty acid (**5**) was isolated from seeds of the plant [22].



5

- A new aliphatic acid n-hexacos-14-enoic acid (**6**) and Strigmasta-5, 22-dien-3-β-ol (**7**) was isolated from ethanol extract of the roots of Achyranthes aspera [25]



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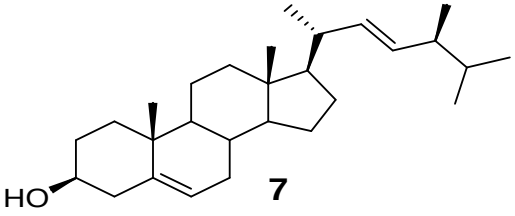

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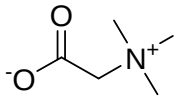
- Two water soluble alkaloids Betaine (**8**) and Achyranthine (**9**) were isolated from the whole part of the plant [26].



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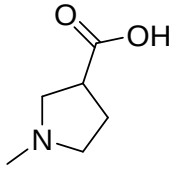

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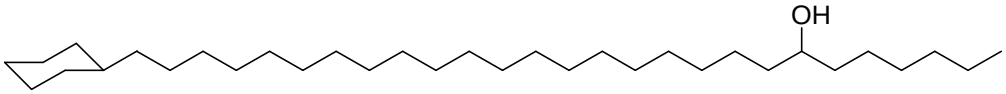
- Two long chain compounds 27- cyclohexylheptacosan-7-ol (**10**) and16-hydroxy-26-methylheptacosan-2-ones (**11**) were isolated from the shoots of Achyranthes aspera [24, 27].



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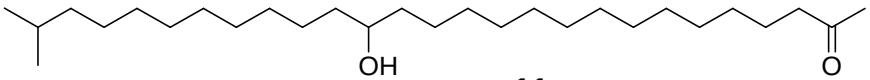
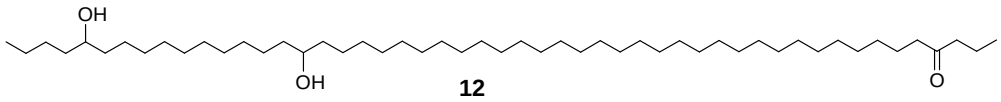


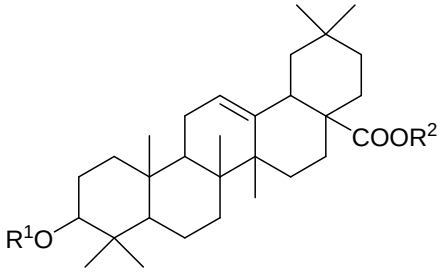
11

- A dihydroxy ketone from the shoots of Achyranthes aspera has been isolated and characterized as 36, 47- dihydroxy henpentacontan-4-one (**12**) [17].



12

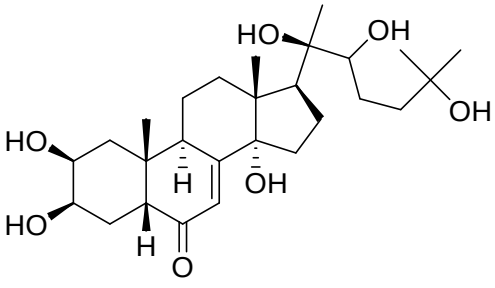
- Three bisdesmosidic saponins (**13-15**), 20-hydroxyecdysone (**16**), and quercetin-3-O-β -D-galactoside (**17**) were isolated from the methanol extract of the aerial parts of Achyranthes aspera [28].



NP-W R^1= alpha-L-Rhamno pyranosyl-(1-3)-beta-D-gluco pyranosyl Acid
R^2= beta-D-glyco pyranosyl

14 : R^1= beta-D-Galacto pyranosyl-(1-3)-beta-D-gluco pyranosyl Acid
R^2= beta-D-glyco pyranosyl

15 : R^1=beta-D-Glucuronic pyranosyl Acid
R^2= beta-D-glyco Pyranosyl



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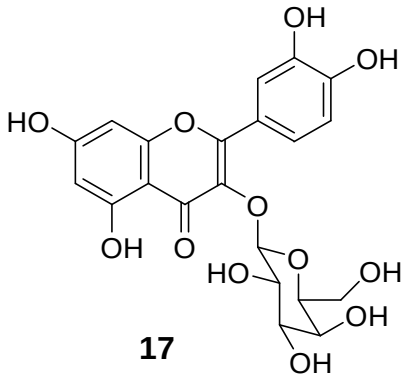
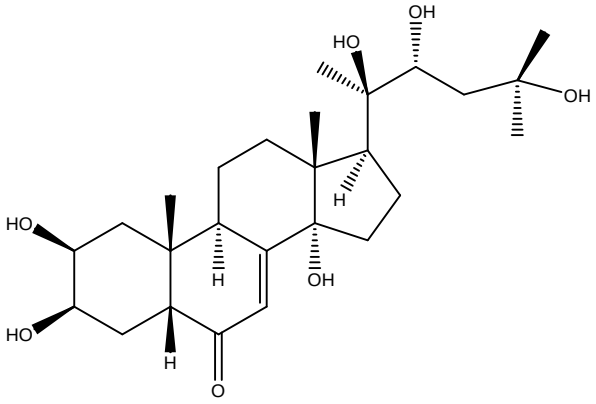


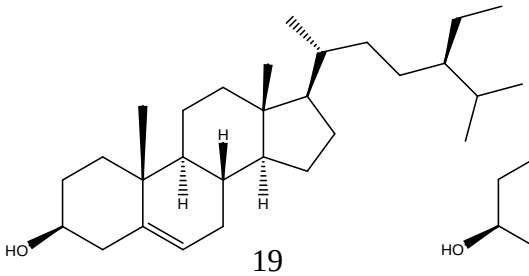
17

- An ecdysone (**18**) was isolated from the roots of Achyranthes aspera [22].



18

- Three compounds, beta-sitostrol (**19**), stigma sterol (**20**) and oleanolic acid (**21**) were also isolated from the ethyl acetate extract of aerial part of Achyranthe aspera [24].



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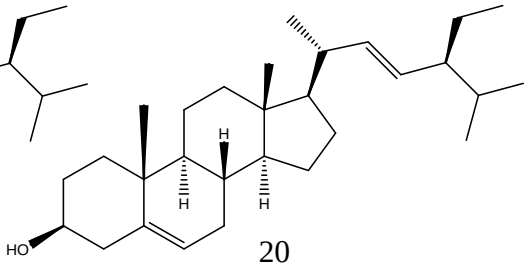

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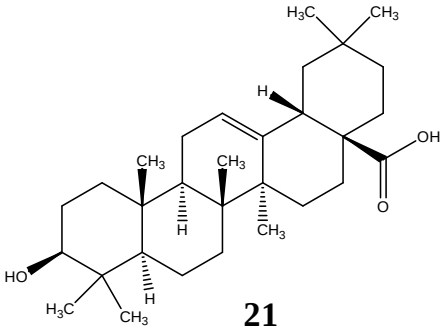

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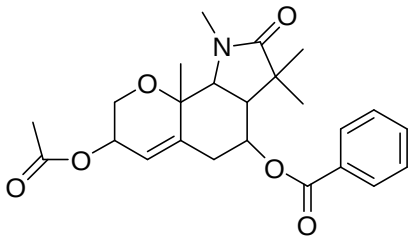
- 3-Acetoxy-6-benzoyloxyapangamide (**22**) has been isolated from an ethyl acetate extract of the stem of *Achyranthes aspera*. The extract was found to show mild antibacterial activity against Bacillus cereus [29].



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1.6. Terpenes

Terpenes are compounds that are built up from isoprene units. Their structures are divisible in to C_5 isoprene units (2-methylbuta-1, 3-diene) linked in a head-to- tail manner. This isoprene rule provided a useful guide in structure determination. Isoprenoids, also referred to as terpenes or terpenoids, are the most diverse class of natural products consisting of over 40,000 structurally different compounds, which have been isolated from animal and microbial species as well as a wide variety of plant organs . Terpenoids are classified by the number of these C_5 isoprene units that they contain (Table-1) [30].

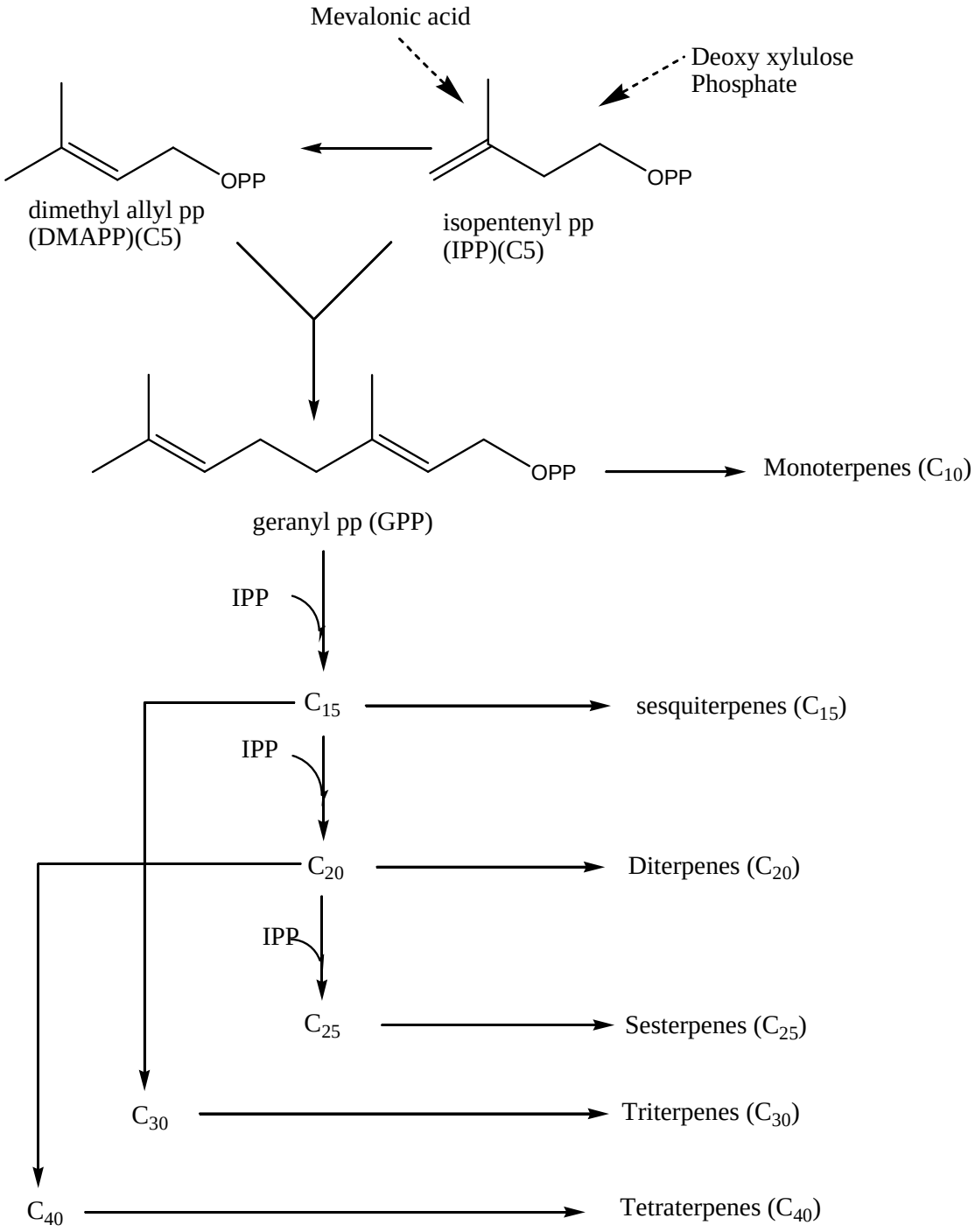
Table 1: Classification of Terpenoids

Name	Number of isoprene units	Number of carbon atoms
Hemi terpenoids	1	5
Mono terpenoids	2	10
Sesqui terpenoids	3	15
Di terpenoids	4	20
Sester terpenoids	5	25
Tri terpenoids	6	30
Tetra terpenoids	8	40
Poly isoprenoids	>8	>40

1.6.1. Biosynthesis of Terpenoids

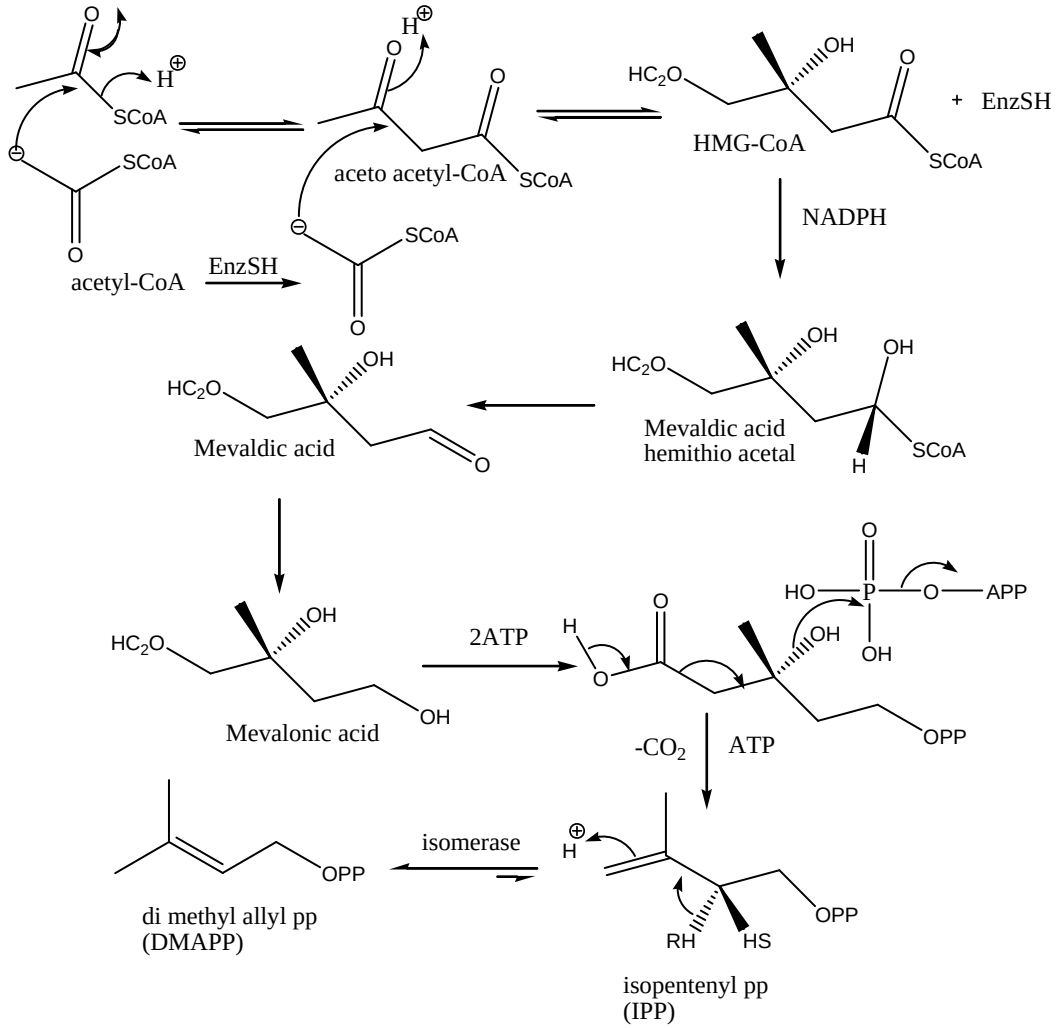
Terpenoid biosynthesis begins with the commitment of three molecules of acetate to the formation of isopentenyl diphosphate (which contains five carbon atoms: C_5). The subsequent key reaction in terpenoid biosynthesis is the isomerization of isopentenyl diphosphate (IPP) to dimethylallyl diphosphate (DMAPP). Condensation of these C_5 isomers by a prenyltransferase forms geranyl diphosphate (GPP) (C_{10}) as a precursor for monoterpenes. Subsequent addition of isopentenyl diphosphate results in the formation of farnesyl diphosphate (FPP) (C_{15}) as a precursor for sesquiterpenes, and geranyl geranyl diphosphate (GGPP) (C_{20}) as a precursor for diterpenes [31].

Isoprene itself had been characterized as a decomposition product from various natural cyclic hydrocarbons, and was suggested as the fundamental building block for these compounds. Isoprene is produced naturally but is not involved in the formation of these compounds, and the biologically active isoprene unit were identified as the diphosphate (pyrophosphate) esters dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate(IPP). In the biosynthesis of terpenoids IPP are condensed to DMAPP (head- tail addition) by various prenyltransferases to finally form prenyl diphosphates of different chain lengths (Scheme 1) [32].



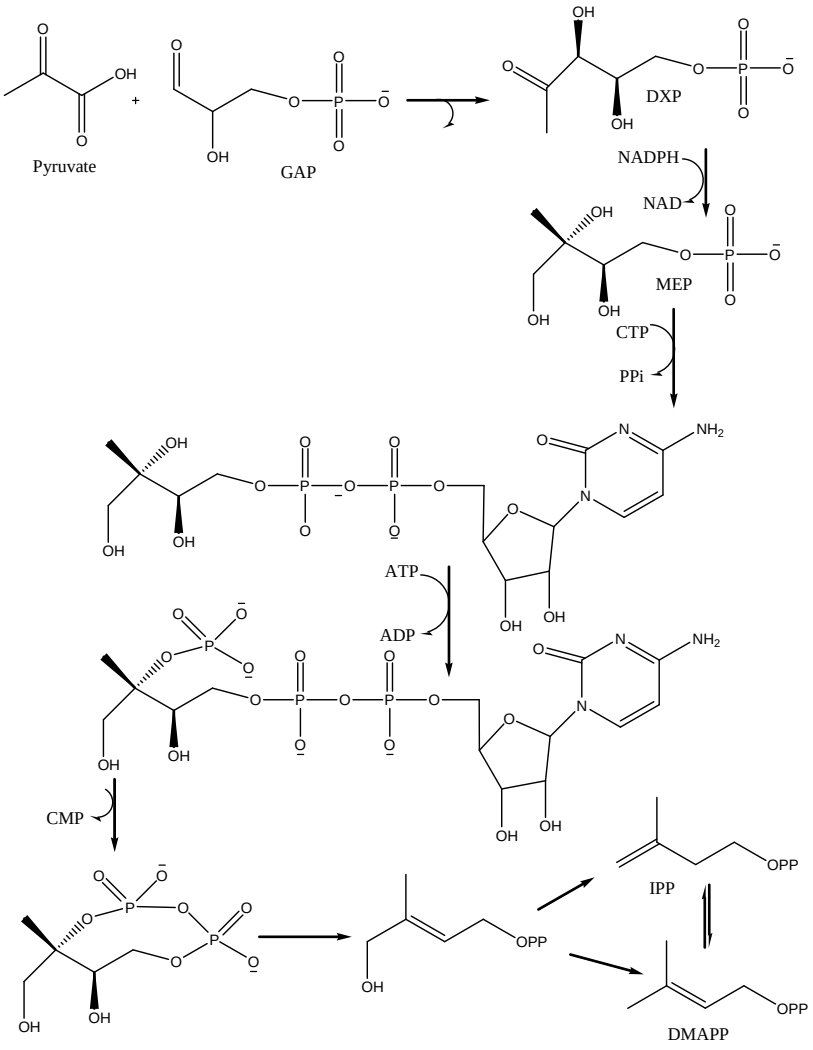
Scheme -1: Biosynthesis of terpenoids

The biochemical isoprene unit, IPP, may be derived by two pathways, by way of intermediates Mevalonic acid (MVA) or 1-deoxy D-xylose 5-phosphate (deoxy xylose phosphate:DXP) [31]. In the mevalonate pathway three molecules of acetyl CoA are used to form mevalonic acid. Two molecules combine initially in a Claisen condensation to give acetoacetyl CoA, and a third is incorporated via a stereospecific aldol addition giving the branched-chain ester β-hydroxy-β-methylglutaryl-CoA (HMG-Co A). Mevalonate is then transformed to IPP by phosphylation twice at C_5 followed by a decarboxylation step (scheme-2) [32, 33].



Scheme -2: Mevalonic acid pathway for the biosynthesis of isoprenoid

The second pathway begins with conversion of glucose into glyceraldehyde-3-phosphate (GAP) and pyruvate, followed by thiamine-mediated decarboxylation of pyruvate. Condensation with GAP generates 1-deoxy-D-xylulose-5-phosphate (DXP). DXP then undergoes a rearrangement and reduction to give 2-*C*-methyl-D-erythritol-4-phosphate (MEP). This step represents the first committed step of the non-MVA pathway, since DXP is used in other biosynthetic pathways. After several transformations, the cyclic diphosphate is made. At this point, the [4Fe-4S] $^{2+}$ metal cluster sequentially transfers two electrons to open the diphosphate and eliminate the inactivated secondary hydroxyl group. A similar iron-sulfur cluster performs a second two-electron transfer to yield an allylic anion that can give either IPP or DMAPP upon protonation (scheme-3) [32].

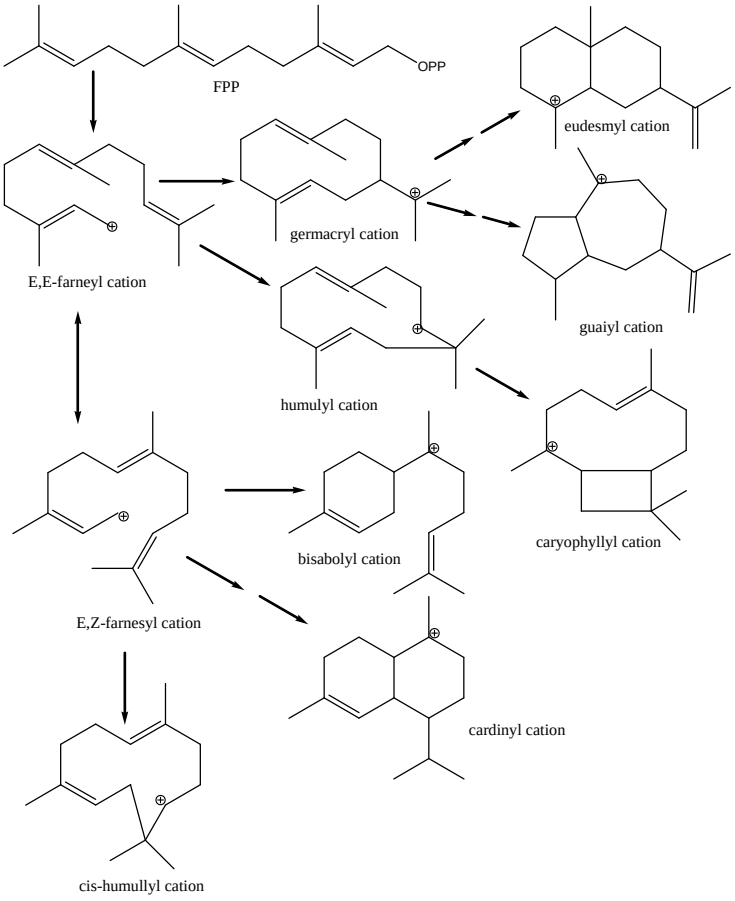


Scheme -3: The deoxy xylulose phosphate pathway for isoprenoid

1.6.2. Biosynthesis of Sequiterpenoids

Sesquiterpenoids are defined as the group of 15 carbon compounds derived by the assembly of 3 isoprenoid units and they are found mainly in higher plants but also in invertebrates. Sesquiterpenes, with monoterpenes, are an important constituent of essential oils in plants. They are the most diverse group of isoprenoids. In plants, they function as pheromones and juvenile hormones. Sesquiterpene structures present several acyclic, mono-, bi-, tri-, and tetracyclic systems [31].

Addition of further C_5 isoprene diphosphate (IPP) unit to geranyldiphosphate in an extension of the prenyl transferase reaction leads to the fundamental sesquiterpene precursor, farnesyl diphosphate (FPP). FPP can then give rise to linear and cyclic sesquiterpenes (Scheme- 4) [31].



Scheme -4: Biosynthesis of sesquiterpenes

2. **Objective of the study**

2.1. General objective

Achyranthes aspera is a medicinal plant throughout the world, which is used for many ailments. Traditionally it is used for anti snake bites, coughs, asthma etc. Many reports showed that the plant also have different pharmacological activities. The leaves of Achyranthes aspera reported to have diuretic, antinoceptive, anti bacterial and antifungal activities. However, there is no report for the isolation and characterization of the active compounds found in the leaves of Achyranthes aspera particularly from the species that exist in Ethiopia. Therefore, the main objective of this study is to isolate compounds from the leaves of Achyranthes aspera and characterized them through different spectroscopic techniques.

2.2. Specific objective

The specific objective of this study is to:

- Prepare extracts from leaves of *A. aspera.*
- Isolate major compounds from extract of *Achyranthes aspera* using different separation techniques.
- Identify and characterize the isolated compounds using a variety of spectroscopic techniques.

3. Result and discussion

Two compounds (**AA-1** and **AA-2**) were isolated from the methanol extract of leaves of Achyranthes aspera. Structure elucidations of the compounds were based on their spectroscopic data and in comparison with data obtained from literature. The characterizations of the two compounds are described below.

3.1. Characterization of AA-1

The compound **AA-1** was isolated as a yellowish semi solid with RF value 0.38 in $CHCl_3$: MeOH: EtOAc (6:2:2). In the IR spectrum (**Appendix- 7**) strong and sharp absorption band at 3411 cm^{-1} indicated the presence of OH group. An absorption band at 1654 cm^{-1} indicated the C=O stretch of α, β-unsaturated carbonyl group. The UV spectrum (**Appendix- 8**) displayed an absorption band at λ max 236 nm (in MeOH) shows the presence of α, β-unsaturated carbonyl chromophore.

The ^{1}H-NMR spectrum (**Appendix-1**) of the compound **AA-1** showed 1H doublet at δ 6.01 indicating a methine attached to carbon 9 and 1H singlet at δ 5.89 indicating a methine attached to the olefenic carbon 4. On the other hand, 1H a doublet of doublet at δ 5.75 indicating a methine attached to the olefenic carbon 8. A 1H triplet at δ 4.55 indicating the presence of methine attached to oxygen and sharp singlet peak at δ 3.32 indicating the presence of OH group. The spectrum also indicated that there two 3H singlet at δ 1.96 and 1.03 indicating the presence of two methyl group attached to quaternary carbons. A 3H doublet at δ 1.29 showed the presence of methyl group attached to methine.

By comparing the ^{13}C (**Appendix- 2**) and DEPT-135 (**Appendix- 3**) NMR spectra, there existed a quaternary carbon atom at δ 199.85, which indicated the presence of conjugated carbonyl group. The quaternary carbon peak at δ 165.70 indicated the olefenic carbon β to the carbonyl group. The peaks at δ 132.36, 132.30 and 125.72 indicated the existence of three olefinic carbon atoms. In addition there existed two quaternary carbon atoms at δ 78.61 and 41.03. The DEPT-

135 NMR displayed two downward peaks at δ 61.43 and 49.35, which showed the presence of two methylene groups, in which one is oxygenated carbon at δ 61.43. There were nine peaks left which were assigned as six methine (at δ 76.96, 76.80, 73.55, 73.23, 70.28 and 22.0) which are attached to oxygen and three methyl groups (at δ 23.30, 20.8 and 18.16). The ^{1}H, ^{13}C and DEPT NMR data of the compound is summarized in Table 2.

Table 2: ^{1}H, ^{13}C and DEPT NMR of compound **AA-1**

Carbon No	δ (^{1}H NMR)	δ (^{13}C NMR)	δ (DEPT-135 NMR)	Remark
1	4.55(1H, t)	73.2	73.2	CH
2	2.62 (1H, d) & 2.19 (1H, d)	49	49	CH_2
3	-	199.8	-	C
4	5.89 (1H, s)	125.7	125.7	CH
5	-	165	-	C
6	-	78	-	C
7	1.05 (1H, m)	22.0	22.0	CH
8	5.75 (1H,	132.30	132.30	CH
9	6.01 (1H, d)	132.36	132.36	CH
10	-	41	-	C
11	1.03 (3H, s)	23.3	23.3	CH_3
12	1.96 (3H, s)	18.0	18.0	CH_3
13	1.29 (3H, d)	20.6	20.6	CH_3
1’	4.29 (1H, d)	99.8	99.8	CH
2’	3.29(1H, m)	76.8	76.8	CH
3’	3.20(1H, m)	73.5	73.5	CH
4’	3.28(1H,m)	70.2	70.2	CH
5’	3.18(1H,m)	76.9	76.9	CH
6’	3.87 (1H, d) &3.65 (1H, d)	61.4	61.4	CH_2

The ^{2}D (COSY, HMQC and HMBC) NMR spectra of the compound **AA-1** also further supported in predicting the structure. From COSY spectrum **(Appendix-4)** (**Table-3),** the proton peak at δ 6.01(H-9) is correlated with the proton peak at δ 5.75(H-8).The spectrum also shows that the proton peak at δ 1.96 (H-12) is correlated with the proton peak at δ 1.29 (H-13), 1.05 (H-7) and 5.89 (H-4).

Table 3: COSY (1H ↔ 1H) correlation of AA-1

Carbon No	COSY(^{1}H ↔ ^{1}H)
C-9 (δ 132.36)	H-9 ↔ H-8
C-8 (δ 132.30)	H-8 ↔ H-9
C-4 (δ125.7)	H-4 ↔ H-12
C-1’ (δ 99.8)	H-1’ ↔ H-3’
C-5’ (δ 76.9)	H-5’ ↔ H-6’
C-2’ (δ 76.8)	H-2’ ↔ H-4’
C-3’ (δ 73.5)	H-3’ ↔ H-1’
C-1 (δ 73.2)	H-1 ↔ H-13,H-9
C-4’ (δ 70.2)	H-4’ ↔ H-2’
C-6’ (δ 61.4)	H-6’ ↔ H-5’,H-13
C-2 (δ 49.0)	H-2 ↔ H-7,H-11
C-11 (δ 23.3)	H-11 ↔ H-2,H-7
C-7 (δ 22.0)	H-7 ↔ H-11
C-13 (δ 20.6)	H-13 ↔ H-1,H-6’,H-12
C-12 (δ 18.0)	H-12 ↔ H-4,H-13

From HMQC spectrum **(Appendix-5)**, the protons at δ 6.01 (1H, d) and 5.75 (1H, dd) correlated with the carbon peaks at δ 132.36 and 132.30 respectively. And the proton peak at δ 4.55 (1H, t) correlated with the carbon peak at δ 73.2. The HMQC result is summarized in **Table-4**.

Table 4: HMQC correlation of **AA-1**

Carbon No	Hydrogen No	Remark
C-9 (δ 132.36)	δ 6.01(1H,d)	CH
C-8 (δ 132.30)	δ 5.75(1H,dd)	CH
C-4 (δ125.7)	δ 5.89(1H,s)	CH
C-1' (δ 99.8)	δ 4.29(1H,d)	CH
C-5' (δ 76.9)	δ 3.18(1H,m)	CH
C-2' (δ 76.8)	δ 3.29(1H,m)	CH
C-3' (δ 73.5)	δ 3.20(1H,m)	CH
C-1 (δ 73.2)	δ 4.55(1H,t)	CH
C-4' (δ 70.2)	δ 3.28(1H,m)	CH
C-6' (δ 61.4)	δ 3.87(1H,d) & 3.65(1H,d)	CH_2
C-2 (δ 49.0)	δ 2.62(2H,d) & 2.19(2H,d)	CH_2
C-11 (δ 23.3)	δ 1.03(3H,s)	CH_3
C-7 (δ 22.0)	δ 1.05(1H,m)	CH
C-13 (δ 20.6)	δ 1.29(3H,d)	CH_3
C-12 (δ 18.0)	δ 1.96(3H,s)	CH_3

The HMBC spectrum **(Appendix-6)** also helps in predicting the structure of compound **AA-1** (Table-5). From HMBC correlation of **AA-1,** the proton on C-2 has long range correlation with C-3, C-4, C-6 and C-10 as shown in **Figure-2**.

Table 5: HMBC Correlation of **AA-1.**

Proton No	Carbon's correlated
H-9	H-9 ↔ C-1,C-5,C-6
H-8	H-8 ↔ C-1,C-6,C-13
H-4	H-4 ↔ C-2,C-6,C-12
H-1'	H-1' ↔ C-2',C-5',C-6
H-5'	H-5' ↔ C-6'
H-2'	H-2' ↔ C-3'
H-3'	H-3' ↔ C-2',C-4'
H-1	H-1 ↔ C-9,C-8,C-13
H-4'	H-4' ↔ C-3',C-5'
H-6'	H-6' ↔ C-4',C-5'
H-2	H-2 ↔ C-3,C-6,C-4,C-10
H-11	H-11 ↔ C-2,C-3,C-7,C-10
H-7	H-7 ↔ C-6,C-10,C-11
H-13	H-13 ↔ C-1,C-8
H-12	H-12 ↔ C-4,C-5,C-6

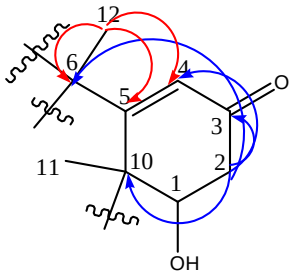


Figure 2: Partial structure I HMBC Correlation of compound **AA-1**

On the other hand, the proton on C-9 correlates with C-1, C-5, C-6 and the proton on C-8 also correlates with C-13, C-6 and C-1 as shown below (**Figure 3**).

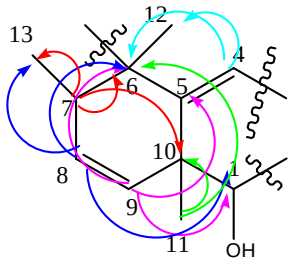


Figure 3: Partial structure II HMBC correlation of **AA-1**

Based on the spectroscopic data, the proposed structure of compound **AA-1** is given in Figure- 4 (4,4a, 7, 8-tetrahydro-4-hydroxy-4a, 7, 8-trimethyl-8-(tetrahydro-3, 4, and 5-trihydroxy-6 (hydroxymethyl)-2H-pyran-2 yloxy) naphthalen-2(3H)-one). To the best of my knowledge there is no report for the isolation of this compound from this plant.

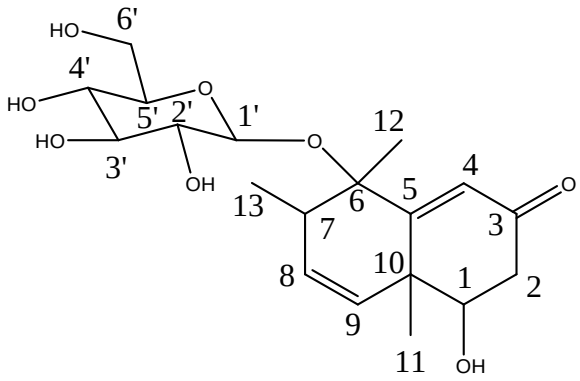


Figure 4: Structure of Compound **AA-1**

3.2. Characterization of AA-2

The compound **AA-2** was isolated as a yellowish solid with RF value of 0.44 in $CHCl_3$: MeOH: EtOAc (6: 2: 2). In the IR spectrum (**Appendix- 15**) absorption band at 3350 cm^{-1} indicated the presence of hydroxyl group. An absorption band at 1450 cm^{-1} indicated the C=O stretch of α, β-unsaturated carbonyl group.

^{1}H NMR spectrum (**Appendix-9**) of compound **AA-2** shows that 1H doublet at δ 5.83 indicates the methine attached to the C-7 and 1H triplet at δ 3.17 indicates methine attached to C-9.
A sharp singlet peak at δ 4.6 indicates the presence of OH group. The ^{1}H NMR spectrum also shows that there are five 3H singlet peak at δ 1.30, 1.22, 1.21, 0.95 and 0.90 which indicated that the presence of five methyl groups attached to the quaternary carbons**.**

^{13}C NMR spectrum (**Appendix-10**) indicated that compound **AA-2** has 27 carbon atoms. The peak at δ 206.5 indicates that the presence of ketone carbonyl group. The ^{13}C NMR also shows that the presence of two olefenic carbons at δ 168 and 122.1. DEPT-135 spectra (**Appendix-11**) indicates that there are seven quaternary, seven methine, eight down ward methtylene groups (δ 42.4, 37.3, 35.1, 32.5, 31.8, 30.8, 27.3, 23.7) and five quaternary methyl carbons, from these carbon groups six are resonated in the region corresponding to oxygenated carbons (δ 85.2, 78.4, 77.9, 71.3, 68.7, and 68.5). The ^{1}H NMR, ^{13}C NMR & DEPT spectra of compound AA-2 is given in Table 6.

Table 6: ^{1}H NMR, ^{13}C NMR & DEPT spectra of compound **AA-2**

Carbon No	δ ^{1}H NMR	δ ^{13}C NMR	δ DEPT-135	Remark
1	1.83(1H,d),1.78(1H,m)	37.4	37.4	CH_2
2	3.87(1H,m)	68.7	68.7	CH
3	3.97(1H,m)	68.5	68.5	CH
4	1.73(2H,m)	30.8	30.8	CH_2
5	2.38(1H,m)	51.8	51.8	CH
6	-	206.5	-	C
7	5.83(1H,s)	122.1	122.1	CH
8	-	168.0	-	C
9	3.17(1H,t)	35	35	CH
10	-	39.3	-	C
11	1.79(1H,m),1.64(1H,m)	21.5	21.5	CH_2
12	2.08(1H,m),1.82(1H,m)	32.5	32.5	CH_2
13	-	49*	-	C
14	-	85.2	-	C
15	1.95(1H,m),1.62(1H,m)	31.8	31.8	CH_2
16	1.99(2H,m)	23.7	23.7	CH_2
17	2.41(1H,t)	50.5	50.5	CH
18	0.90(3H,s)	18.0	18.0	CH_3
19	0.95(3H,s)	24.4	24.4	CH_3
20	-	77.9	-	C
21	1.30(3H,s)	21.0	21.0	CH_3
22	3.32(1H,t)	78.4	78.4	CH
23	1.59(2H,m)	27.3	27.3	CH_2
24	1.72(1H,m),1.45(1H,m)	42.4	42.4	CH_2
25	-	71.3	-	C
26	1.22(3H,s)	29.7	29.7	CH_3
27	1.21(3H,s)	28.9	28.9	CH_3

The ^{1}H and ^{13}C NMR data obtained for **AA-2** is comparable with the data obtained for the 20-hydroxyecdysone (**16)** (Table-7).

Table 7: Comparison ^{1}H &^{13}C NMR of **AA-2** with 20-hydroxyecdysone (**16**)

Carbon No	δ ^{13}C NMR		δ ^{1}H NMR	
	AA-2	**16**	**AA-2**	**16**
1	37.4	33.5	1.83(1H,d),1.78(1H,m)	1.83,1.46
2	68.7	68.1	3.87(1H,m)	3.88
3	68.5	66.8	3.97(1H,m)	3.9
4	30.8	28.4	1.73(2H,m)	1.78,174
5	51.8	50.6	2.38(1H,m)	2.42
6	206.5	201.9	-	-
7	122.1	121.6	5.83(1H,s)	5.8
8	168.0	165.0	-	-
9	35	33.3	3.17(1H,t)	3.2
10	39.3	37.5	-	-
11	21.5	20.5	1.79(1H,m),1.64(1H,m)	2.02,1.78
12	32.5	30.9	2.08(1H,m),1.82(1H,m)	2.18,1.98
13	49*	47.1	-	-
14	85.2	83.0	-	-
15	31.8	30.6	1.95(1H,m),1.62(1H,m)	2.02,1.64
16	23.7	20.5	1.99(2H,m)	1.86,1.76
17	50.5	49.4	2.41(1H,t)	2.43
18	18.0	16.8	0.90(3H,s)	0.94
19	24.4	22.9	0.95(3H,s)	1.02
20	77.9	75.4	-	-
21	21.0	19.4	1.30(3H,s)	1.24
22	78.4	79.7	3.32(1H,t)	3.36
23	27.3	25.2	1.59(2H,m)	1.71,1.32
24	42.4	40.7	1.72(1H,m),1.45(1H,m)	1.84,1.46
25	71.3	68.1	-	-

26	29.7	28.9	1.22(3H,s)	1.24
27	28.9	28.7	1.21(3H,s)	1.28

The symbol ***** indicates the peak that observed at δ 49 is overlapped by the solvent.

The above spectral data were further supported by a variety of ^{2}D (COSY, HMQC and HMBC) spectroscopic data. From COSY spectrum (**Appendix-12) (Table-8**) of compound **AA-2**, the proton peak at δ 5.83 correlated with the proton peak at δ (3.17 and 1.79). The spectrum also shows that the proton peak at δ 2.41 correlated with the proton peak at δ 2.08.

Table 8: COSY Correlation of **AA-2**

Carbon No	COSY(^{1}H ↔ ^{1}H) correlation
C-7(δ122.1)	H-7 ↔ H-9
C-22(δ78.4)	H-22 ↔ H-21
C-2(δ68.7)	H-2 ↔ H-4,H-3,H-1
C-3(δ68.5)	H-3 ↔ H-2,H-1
C-17(δ50.5)	H-17 ↔ H-15,H-12
C-1(δ37.4)	H-1 ↔ -3,H-2
C-9(δ35.1)	H-9 ↔ H-7,H-1
C-12(δ32.5)	H-12 ↔ H-17,H-11
C-15(δ31.8)	H-15 ↔ H-17,H-16
C-4(δ30.8)	H-4 ↔ H-2
C-16(δ27.3)	H-16 ↔ H-15
C-11(δ21.5)	H-11 ↔ H-12
C-21(δ21)	H-21 ↔ H-22,H-18
C-18(δ18)	H-18 ↔ H-21

From HMQC spectrum **(Appendix-13)**, the proton peak at δ 5.83 (1H, s) is correlated with carbon peak at δ 122.1 and the proton peak at δ 3.17 (1H, t) correlated with the carbon peaks at δ 35.1. The proton peak at δ 0.95(3H, t) and 0.90 (3H, t) correlated with the carbon peak at δ 24.4 and 18.0 respectively. The HMQC result is summarized in Table-9.

Table 9: HMQC Correlation of **AA-2**

Carbon No	Hydrogen No	Remark
C-7(δ122.1)	5.83(1H,s)	CH
C-22(δ 78.4)	3.32(1H,t)	CH
C-2(δ68.7)	3.87(1H,m)	CH
C-3(δ68.5)	3.97(1H,m)	CH
C-5(δ51.8)	2.38(1H,m)	CH
C-17(δ50.1)	2.41(1H,t)	CH
C-24(δ42.4)	1.72(1H,m,1.45(1H,m)	CH_2
C-1(δ37.4)	1.83(1d),1.78(1H,m)	CH_2
C-9(δ35.1)	3.17(1H,t)	CH
C-12(δ32.5)	2.08,1.82	CH_2
C-15(δ31.8)	1.95,1.62	CH_2
C-4(δ30.8)	1.73(2H,m)	CH_2
C-26(δ29.7)	1.22(3H,s)	CH_3
C-27(δ28.9)	1.21(3H,s)	CH_3
C-16(δ27.3)	1.99(2H,m)	CH_2
C-19(δ24.4)	0.95(3H,s)	CH_3
C-23(δ23.7)	1.59(2H,m)	CH_2
C-11(21.5)	1.79(1H,m),1.64(1H,m)	CH_2
C-21(δ21)	1.30(3H,s)	CH_3
C-18(δ18)	0.90(3H,s)	CH_3

HMBC spectrum **(Appendix-14) (Table-10**) of compound **AA-2** also shows correlation of some proton with the carbons. The correlation is given in Figure 5.

Table 10: HMBC Correlation of **AA-2**.

Proton No	Carbons correlated
H-27	C-24,C-25,C-22
H-19	C-5,C-1
H-18	C-14,C-13,C-12

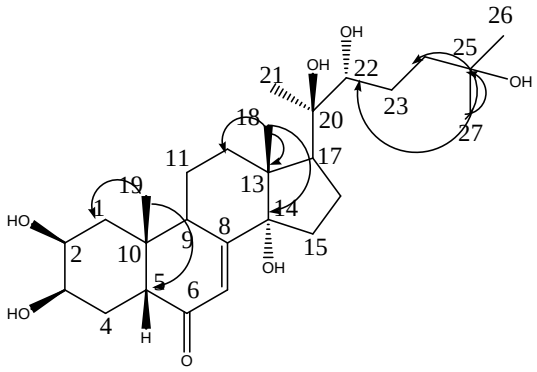


Figure 5: HMBC correlation of compound **AA-2**.

Based on the spectroscopic data obtained and in comparison with literature (**16)**, the structure of compound **AA-2** is proposed to be as 20-hydroxyecdysone (Figure 6)**.**

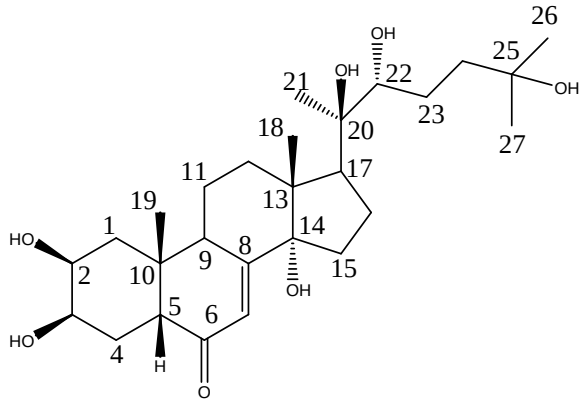


Figure 6: Structure of compound **AA-2.**

4. Conclusion

Achyranthes aspera is a medicinal plant found in the family of Amarantacea. Pharmacological studies on this plant reports to have anti bacterial, anti fungal, anti abortition and anti inflammation. Phytochemical investigation on this plant reports the plant is rich in terpenes, saponins, steroids etc. In this study, two compounds were isolated. Compound AA-1 is a sesquiterpene and namely: 4,4a, 7, 8-tetrahydro-4-hydroxy-4a, 7, 8-trimethyl-8-(tetrahydro-3, 4, and 5-trihydroxy-6 (hydroxymethyl)-2H-pyran-2 yloxy) naphthalen-2(3H)-one. The second compound (AA-2) is an ecdysteroid, insect moulting hormone and identified as 20-hydroxyecdysone. To the best of my knowledge there is no report for isolation of the first Compound (**AA-1)** from this plant.

5. Experimental

5.1. General

^{1}H, ^{13}C, and ^{2}D NMR spectra were recorded on a Bruker advance 400MHz spectrometer with TMS as internal started. The ultraviolet and visible (UV-Vis) spectra were taken on GENESY'S 2PC UV-Vis scanning spectrometer in the range 200-1000 cm^{-1}. Infrared (IR) spectra were obtained on Perkin-Elmer BX Infrared spectrometer using KBr and MeOH in the range 4000-400 cm^{-1}. Solvents were removed using the Buchi type rotavapor under reduced pressure at 30 ^{0}c. Mixtures of compounds were separated using chromatoron (model 79247), Column Chromatography and Preparatory TLC. TLC analyses were carried out on TLC plates 0.2 mm thick layer of Merck silica gel 60 F254 coated on aluminum foil. Compounds on TLC were detected using UV light on the wave length of 254&365 nm and spraying with 1% vanillin in sulfuric acid.

5.2. Collection and identification of the plant material

The fresh leaves and stem containing the fruits of the plant were collected from Mekelle**,** capital City of Tigray regional state of Ethiopia, which is 780 km from Addis Ababa, and identified by the botanist national herbarium of Ethiopia. The leaves and the stems were collected separately and the collected leaves are dried at room temperature.

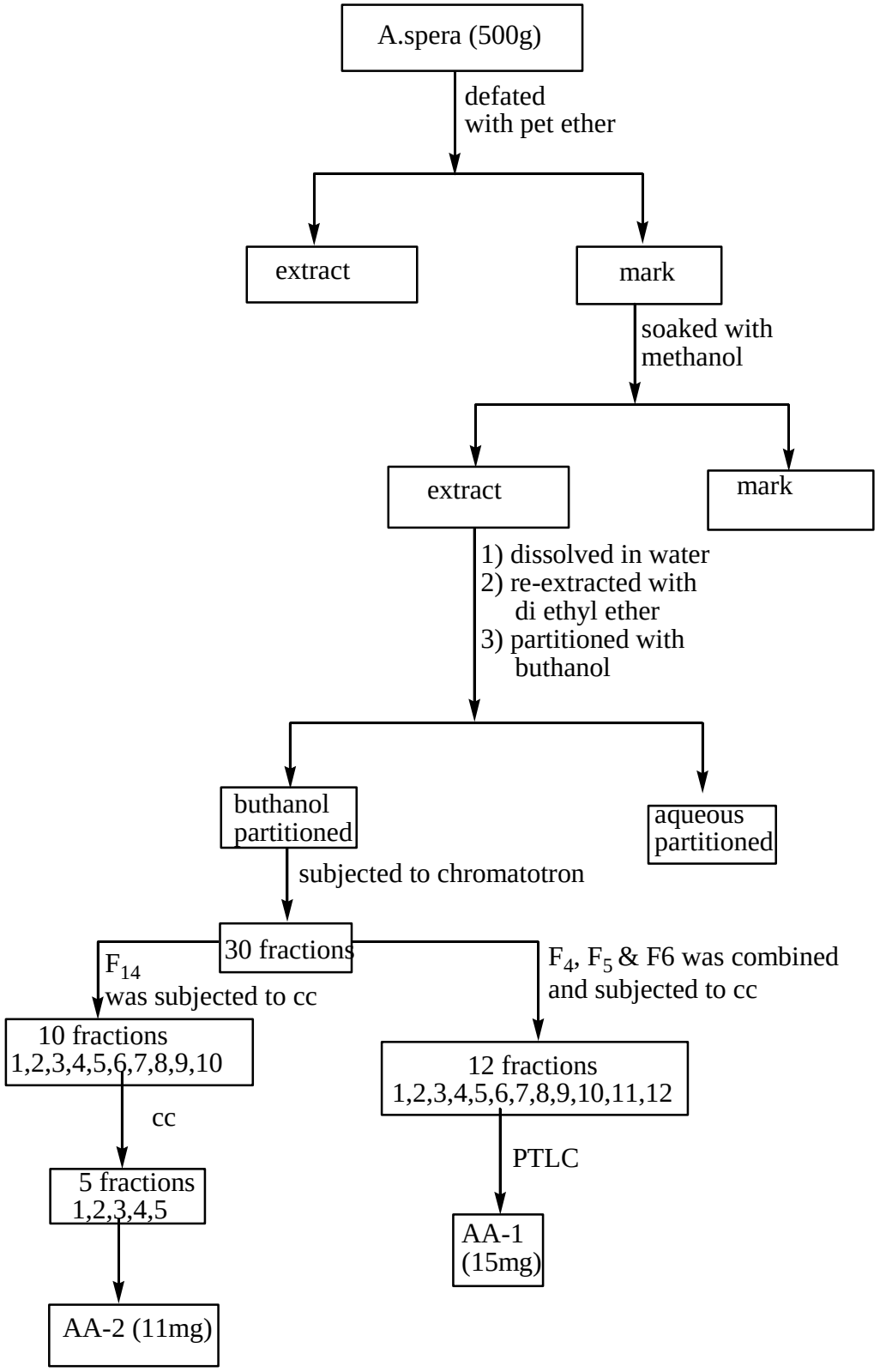
5.3. Isolation and analysis

Air dried and finely powdered leaves of the plant (500 g) were defatted with petroleum ether in a percolator at room temperature for 72h. After the extract was filtered, the solvent free powder (the marc) was exhaustively extracted with methanol in a percolator and the solvent was removed using Rotavapor to afford a greenish gum (45 g). The crude methanol extract was taken up in water and re-extracted with diethyl ether until all the chlorophyll pigments were removed. The aqueous phase was then partitioned with n-butanol saturated with water (300 ml) three times. The butanol partitioned was concentrated under reduced pressure using vaccum

distillation to give crude mixture (3 g). This mixture was dissolved in the solvent system of MeOH: $CHCl_3$ (8:2) and subjected to Chromatotron and 30 fractions were collected using a 100 ml Erlenmeyer flask, each 20ml.The first seventeen fractions (i.e F_1-F_{17}) were collected using MeOH: $CHCl_3$ (8:2) solvent system and each fractions were checked in TLC plate using $CHCl_3$: MeOH: EtOAc (6:2:2) solvent system to identify the components of the fractions collected. After TLC was checked fractions that had similar retention factor (RF) was combined together. Based on this F_4, F_5 and F_6 were mixed and the solvent was removed using rotavapor. The combined fractions are also subjected to Column chromatography (CC) on silica gel (20g) to afford 12 fractions collecting in a Test tube. Fraction 6 was subjected to Preparatory Thin Layer Chromatography (PTLC) to give compounds AA-1.

The TLC of compound AA-1 is run with $CHCl_3$: MeOH: EtOAc (6:2:2) solvent system and it is UV active and showed a purple color seeing in UV lamp at 254 nm. After spraying with 1% vanillin in H_2SO4 and heated in hotplate a purple color was observed. The compound obtained (AA-1) is yellowish semisolid with RF value 0.38.

The fourteenth fraction collecting above is subjected to CC on silica gel (15g) on gradient elution starting with chloroform to methanol and collecting another 10 fractions using Erlenmeyer flask, 20ml each. After the TLC of each fraction was checked, the sixth fraction is again subjected to column chromatography on small amount of silica gel for purifying with solvent system $CHCl_3$: MeOH (8:2) and five fractions were collected. Finally the third fraction affords 11mg of compound AA-2. Its TLC is run with $CHCl_3$: MeOH: EtOAc (6:2:2) showed yellow color spot after spraying with 1% vanillin in H_2SO_4**.** Relatively with the compound AA-1, it is less Polar and has RF value of 0.44. The method of isolation is summarized below (scheme-5).



Scheme-5: Method of isolation of components of A.aspera

6. Spectral data

The compound **AA-1** is yellowish semisolid with RF value 0.38.
IR: (KBr)V_{max}: 3411, 2928, 1654, 1454, 1377, 1258, 1078, 875, 644 cm^{-1}; UV-VIS spectrum (MeOH) λ_{maX} = 236 nm.

^{1}H NMR (400 MHz): δ 6.01(1H, d, H-9), 5.75 (1H, dd, H-8), 5.89 (1H, s, H-4),4.29 (1H, d, H-1'), 3.18 (1H, m, H-5'), 3.29 (1H, m, H-2'), 3.20 (1H, m, H-3'), 4.55 (1H, t, H-1), 3.28 (1H, m, H-4'), 3.87 (2H, d, H-6'), 2.62 (2H, d, H-2), 1.96 (3H, s, H-12), 1.29 (3H, s, H-13), 1.05 (1H, s, H-7), 1.03 (3H, s, H-11).

^{13}C NMR (100 MHz, MeOD): δ 199.8 (C-3), 165.7 (C-5), 132.36 (C-9), 132.30 (C-8), 125.7 (C-4), 99.8 (C-1'), 78.6 (C-6), 76.9 (C-5'), 76.8 (C-2'), 73.5 (C-3'), 73.2 (C-1), 70.2 (C-4'), 61.4 (C-6'), 49 (C-2), 41(C-10), 23.3 (C-11), 22 (C-7), 20.6 (C-13), 18 (C-12).

Compound **AA-2** is yellow colored solid with RF value 0.44.
IR (MeOH) V_{max}: 3350, 2945, 2522, 2045, 1450, 1028, 667 cm^{-1}

^{1}H NMR (400 MHz, MeOD): δ 5.83(1H, s, H-7), 3.97(1H, m, H-3), 3.87(1H, m, H-2), 3.32 (1H, t, H-22), 3.17 (1H, t, H-9), 2,41 (1H, t, H-17), 2.38 (1H, m, H-5), 2.08 (2H, m, H-12), 1.99 (2H, m, H-16), 1.95(2H, m, H-15), 1.83 (2H, m, H-1), 1.79 (2H, m, H-11), 1.73 (2H, m, H-4), 1.72 (2H, m, H-24),1.59 (2H, m, H-23), 1.30 (3H, s, H-21), 1.22 (3H, s, H-26), 1.21 (3H, s, H-27), 0.95 (3H, s, H-19), 0.90 (3H, s, H-18);

^{13}C NMR (100 MHz, MeOD): δ 205 (C-6), 168 (C-8), 122.1 (C-7), 85.2 (C-14), 78.4 (C-22), 77.9 (C-20), 71.3 (C-25), 68.7 (C-2), 68.5 (C-3), 51.8 (C-5), 50.5 (C-17), 49.0 (C-13), 42.4 (C-24), 39.2 (C-10), 37.3 (C-1), 35.1 (C-9), 32.5 (C-12), 31.8 (C-15), 30.4 (C-4), 29.7 (C-26), 28.9 (C-27), 27.3 (C-23), 24.4 (C-19), 23.7 (C-16), 21.5 (C-11), 21.0 (C-21), 18.0 (C-18).

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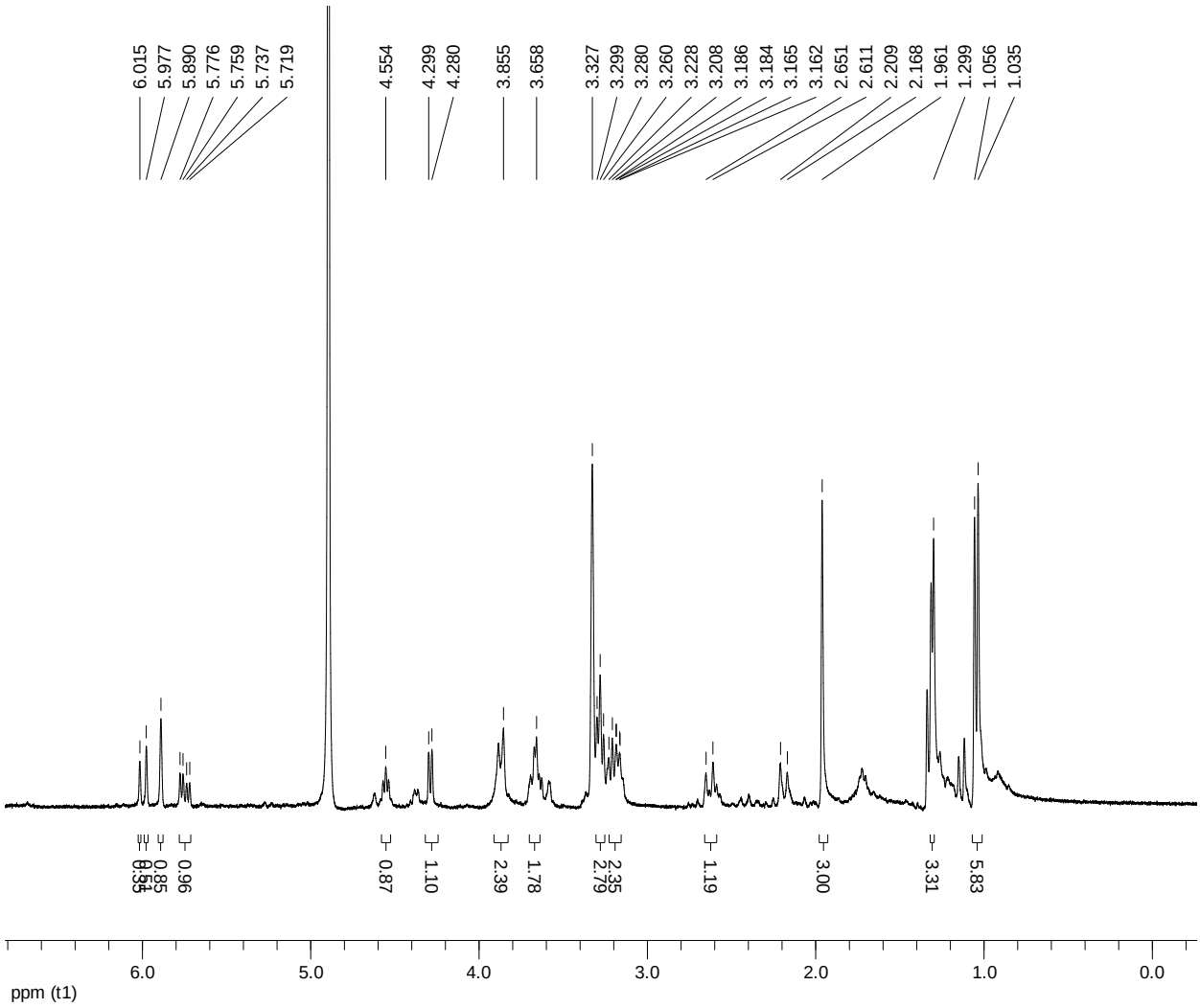
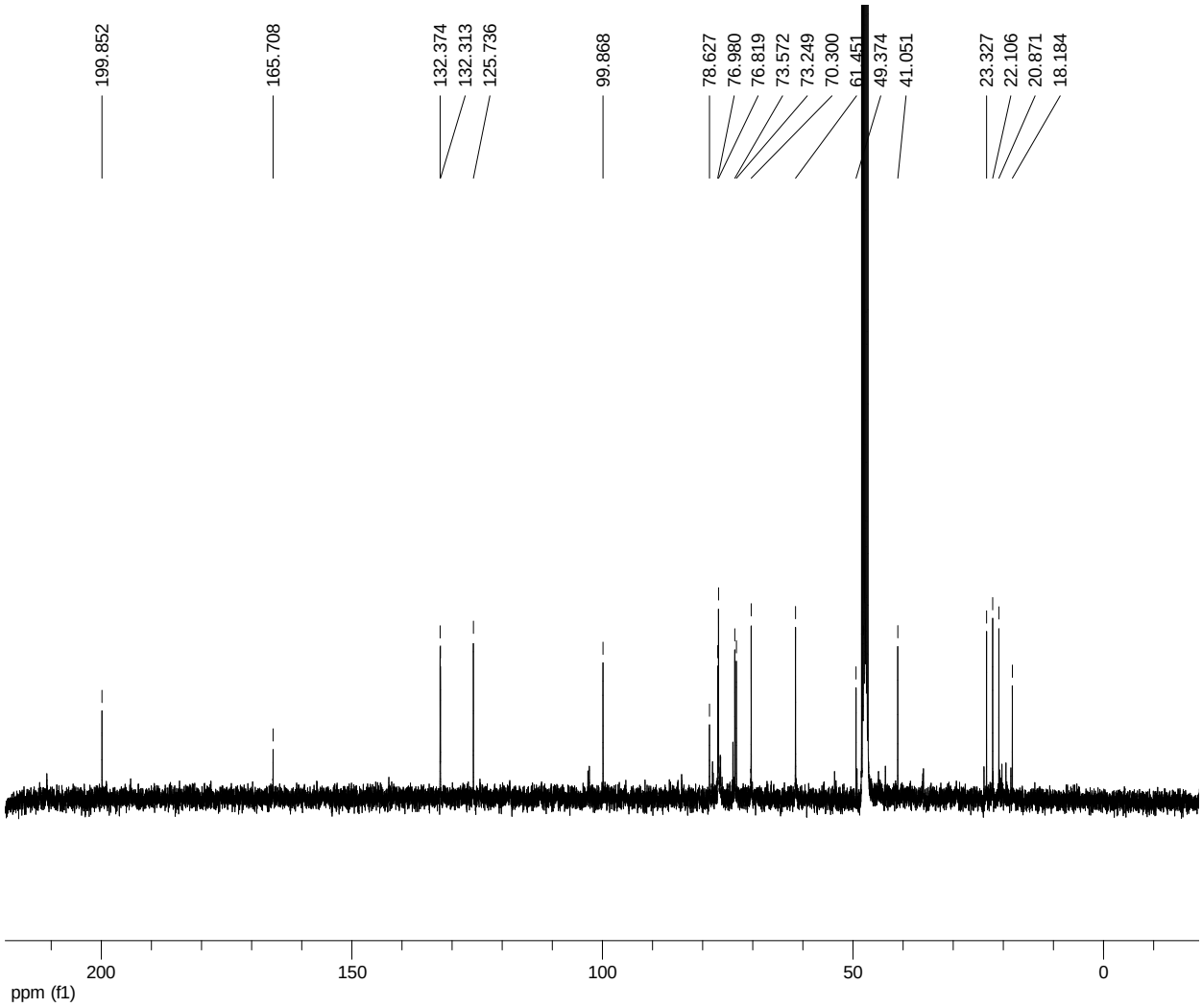
APPENDICES

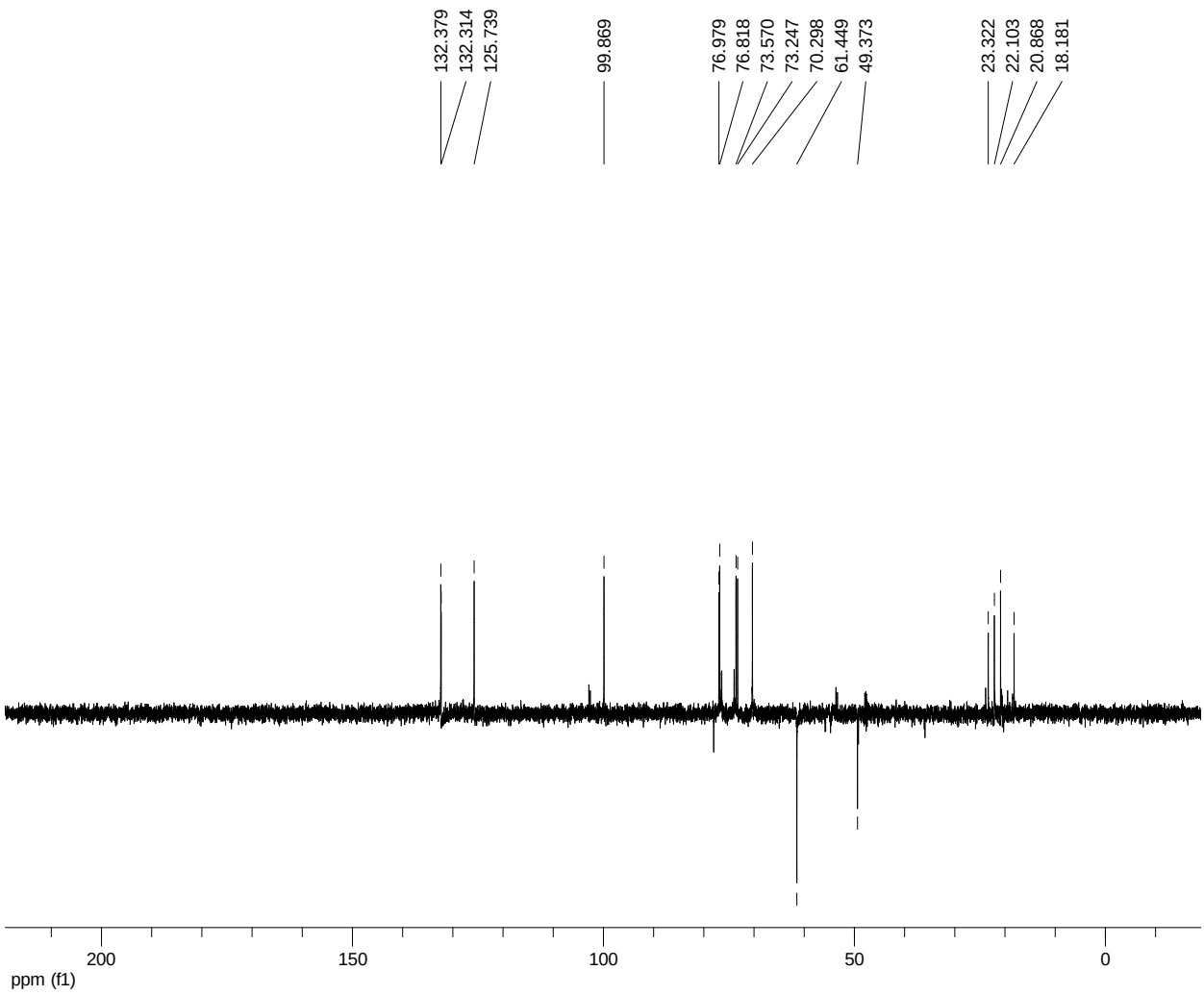
Appendix-1: ^{1}H NMR of AA-1



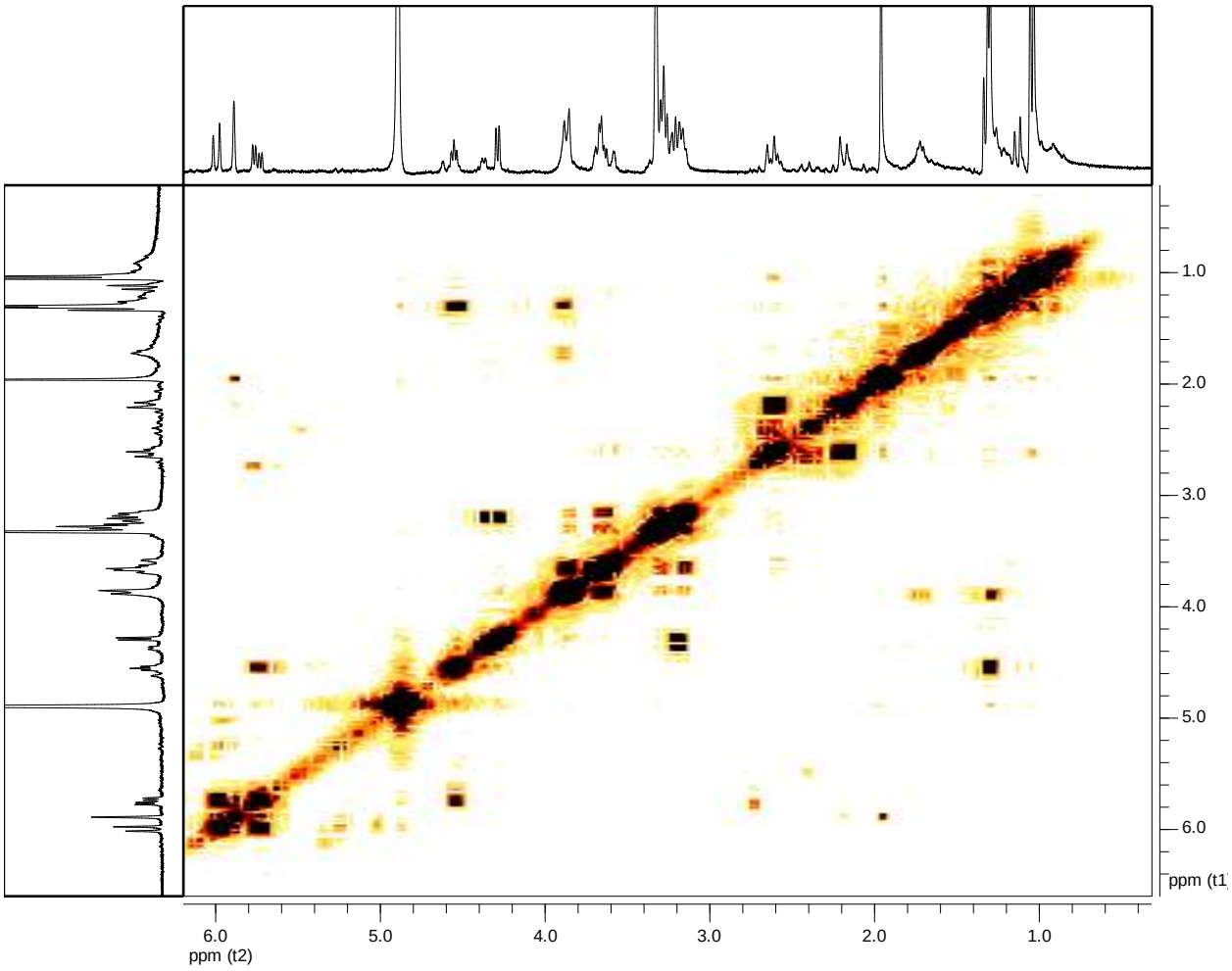
Appendix-2: ^{13}-C NMR of AA-1



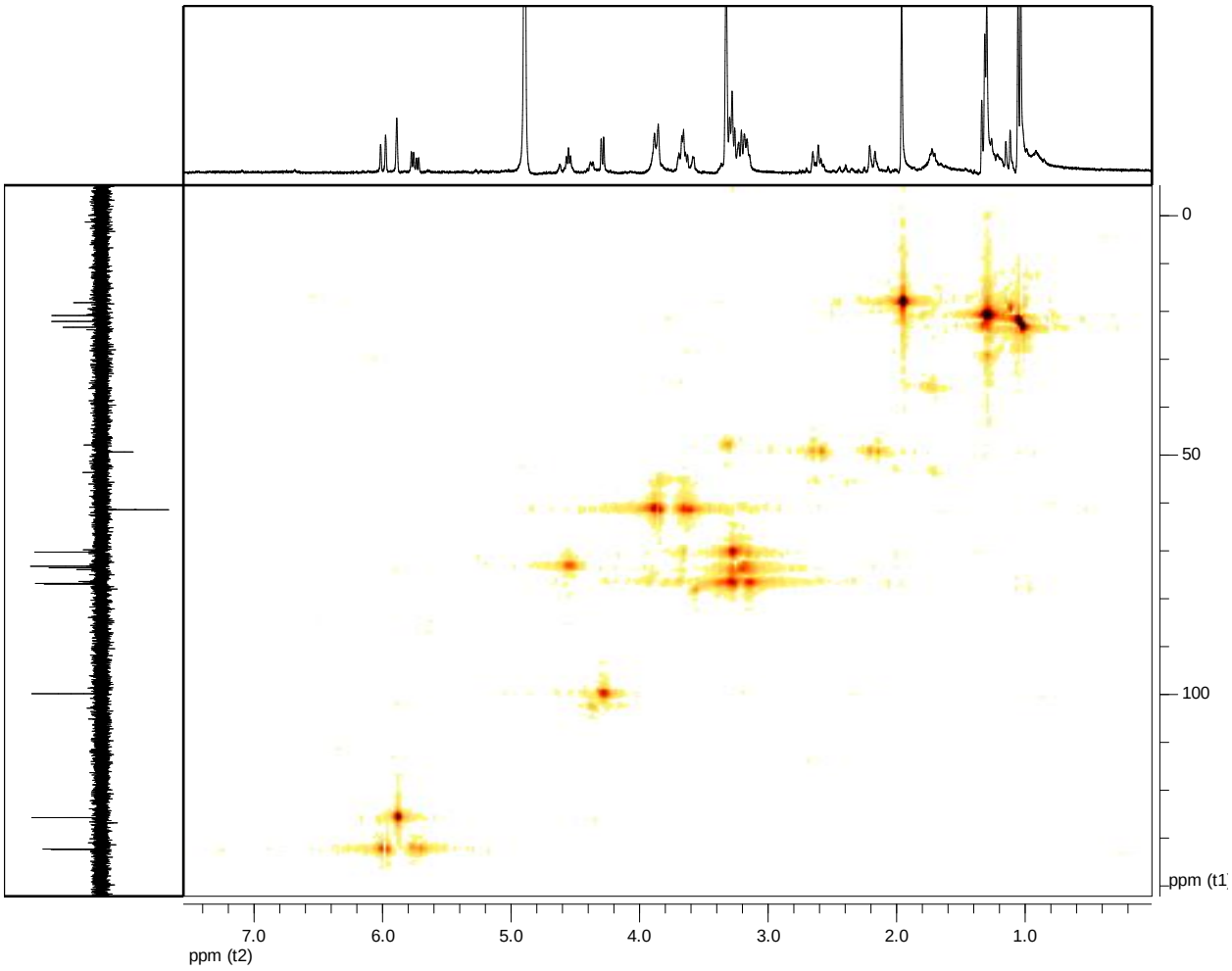
Appendix-3: DEPT -135 of AA-1



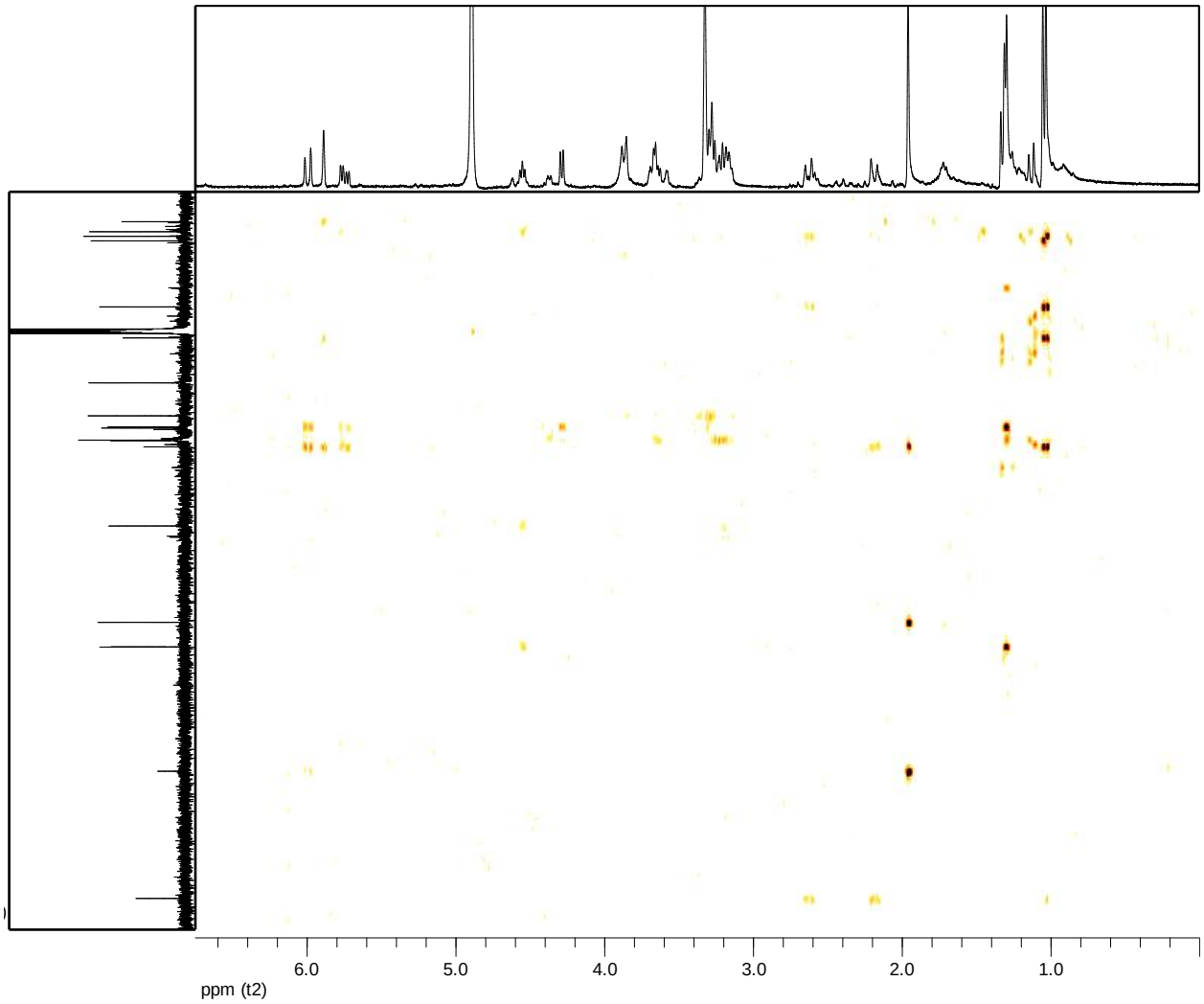
Appendix-4: COSY spectrem of AA-1



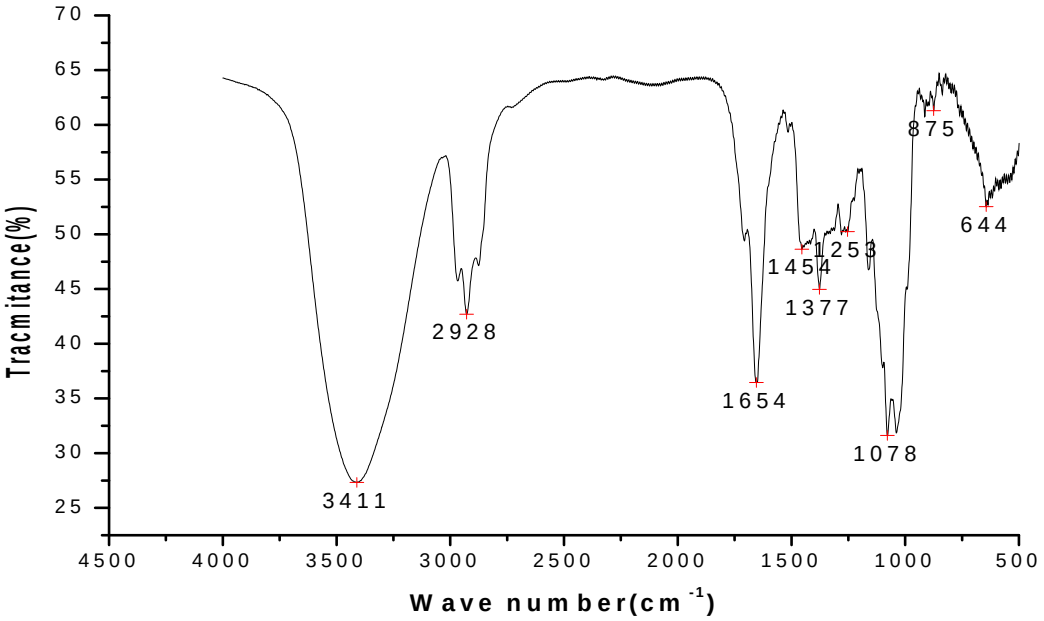
Appendix-5: HMQC spectrum of AA-1



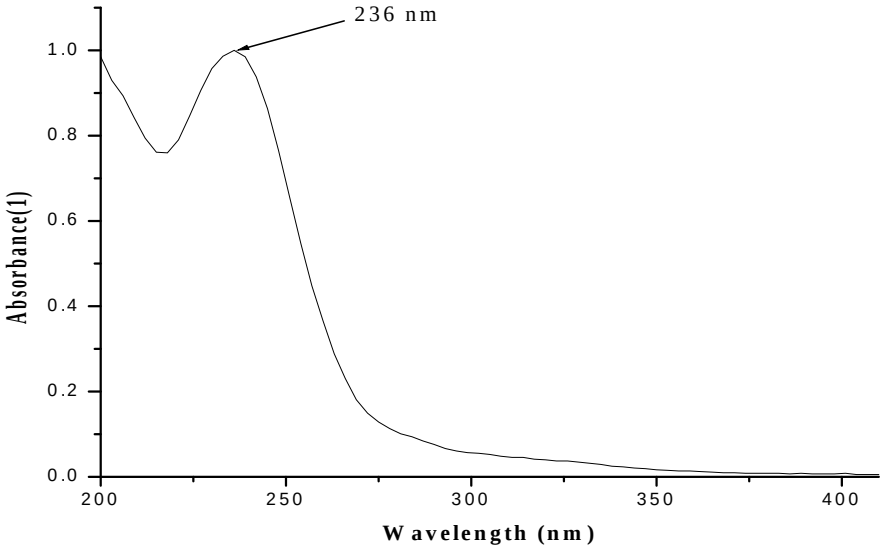
Appendix -6: HMBC spectrum of AA-1



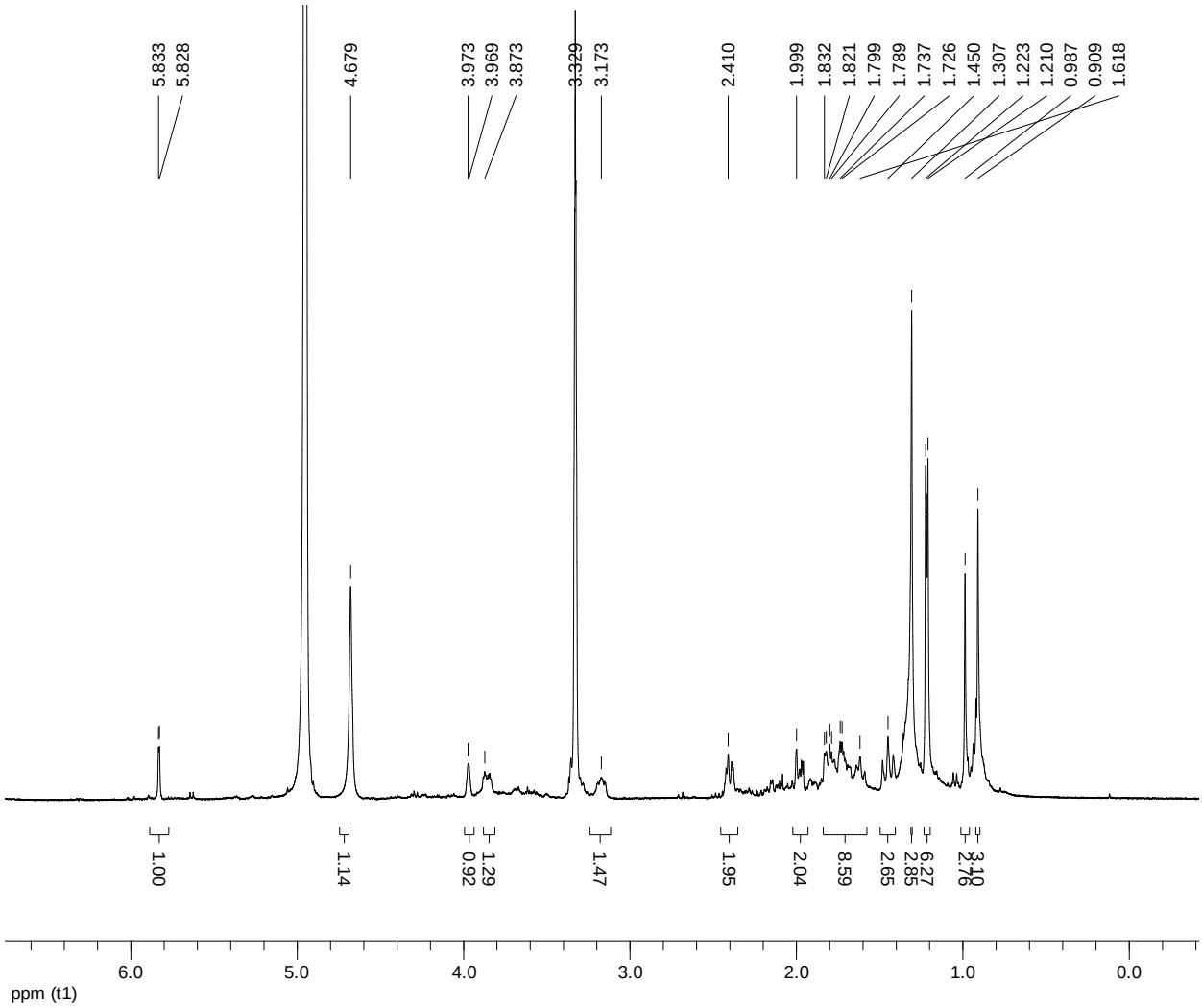
Appendix-7: IR spectra of AA-1



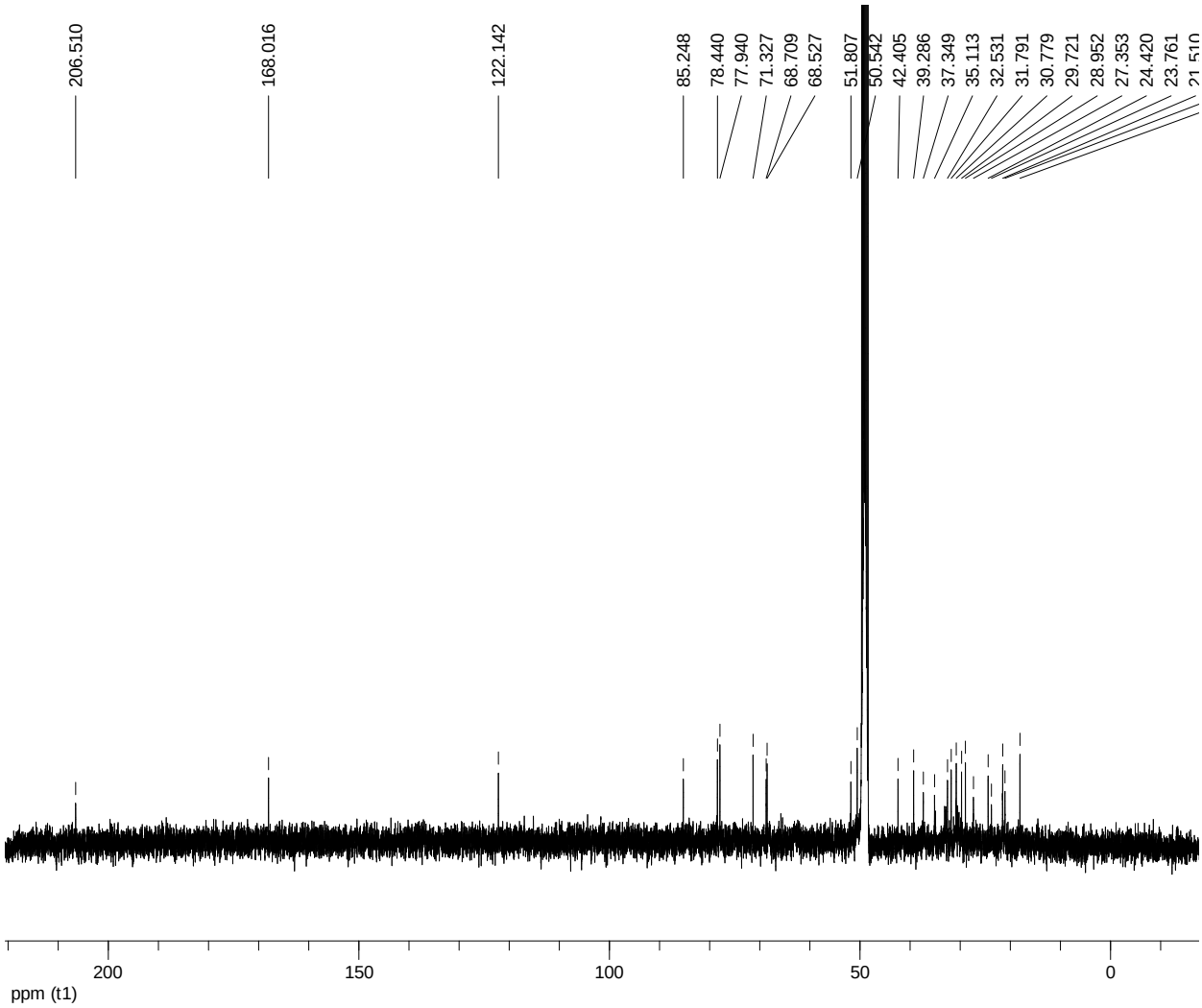
Appendix-8: UV spectra of AA-1



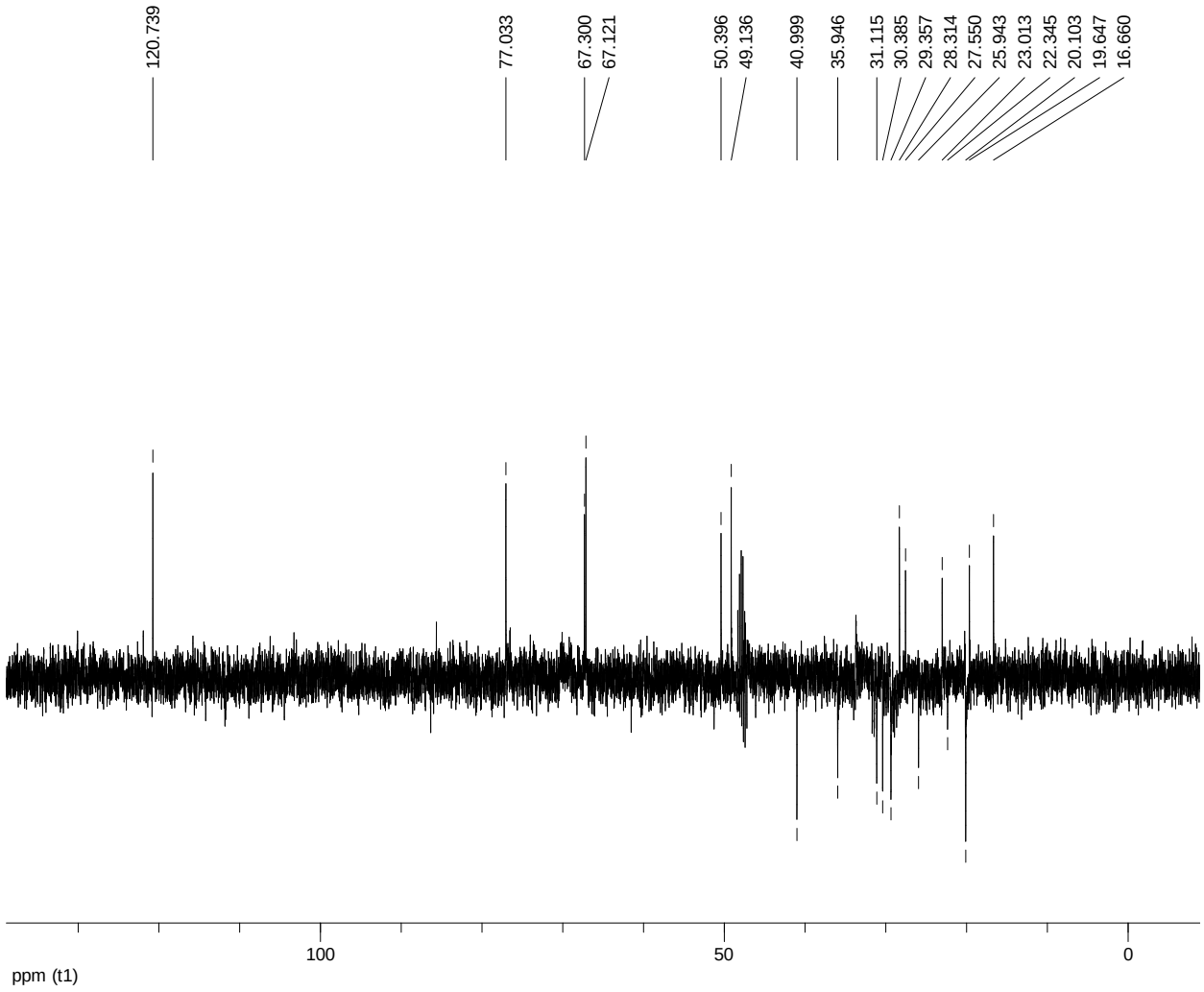
Appendix-9: ^{1}H NMR of AA-2



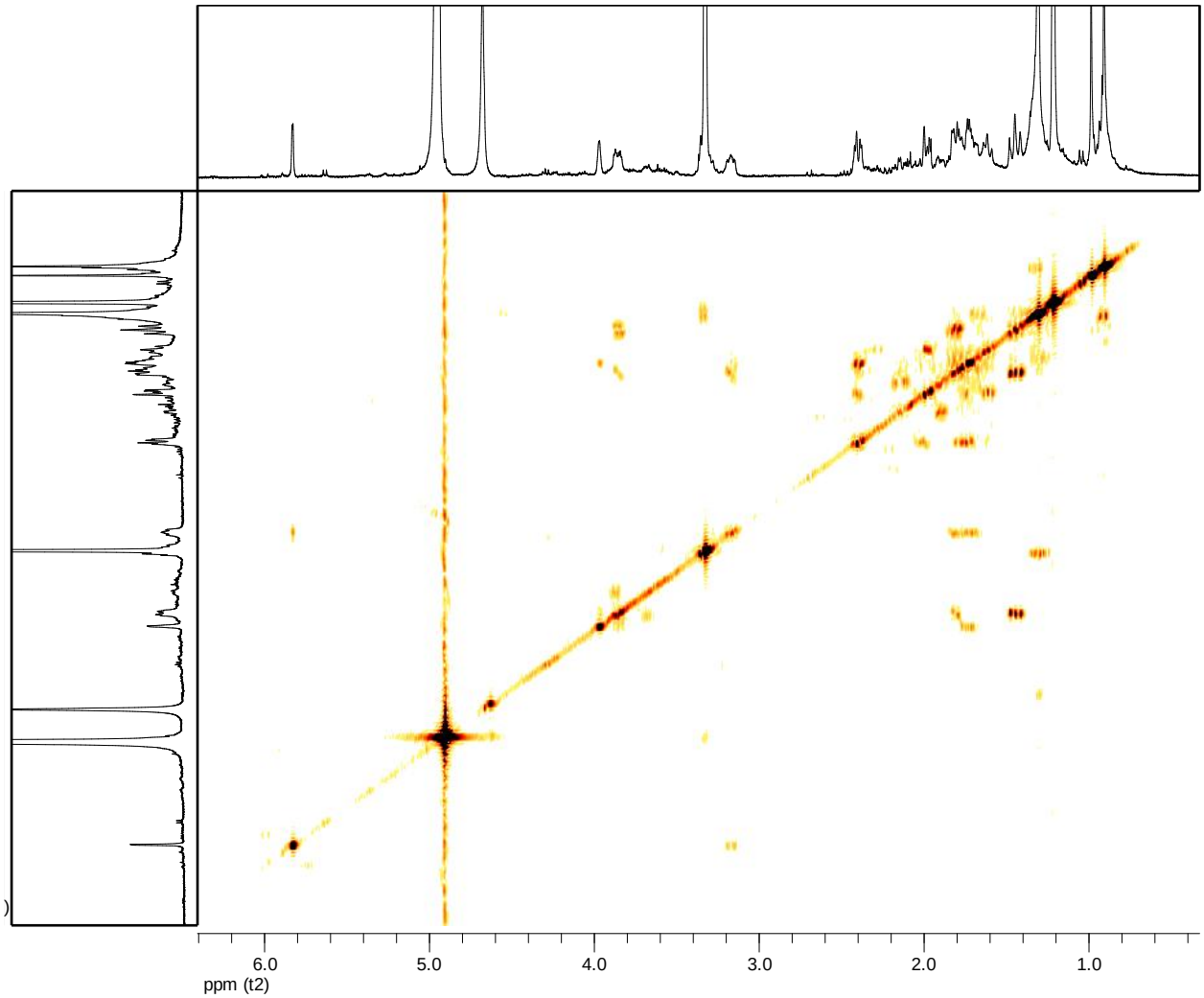
Appendix-10: ^{13}C NMR of AA-2



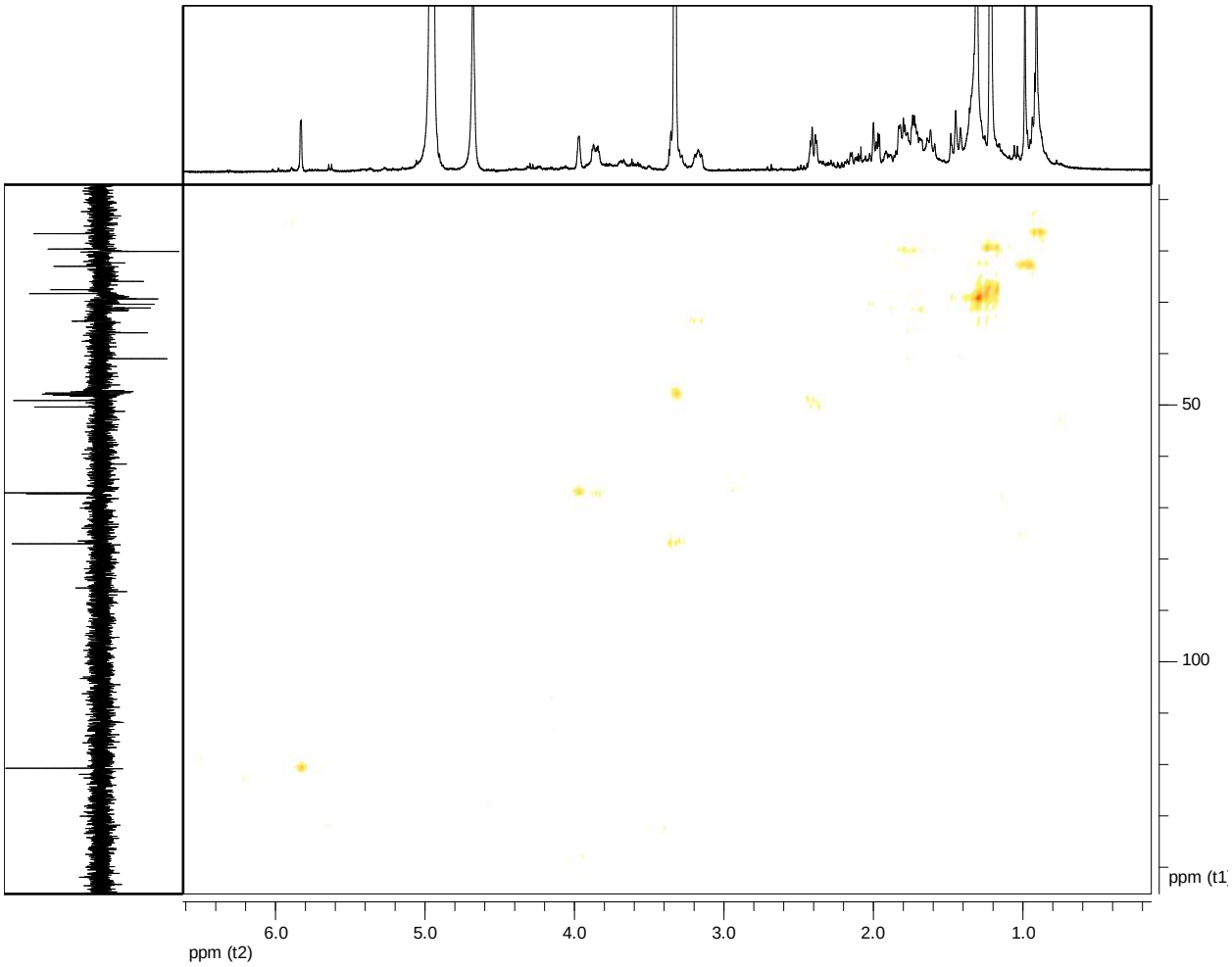
Appendix-11: DEPT-135 NMR of AA-2



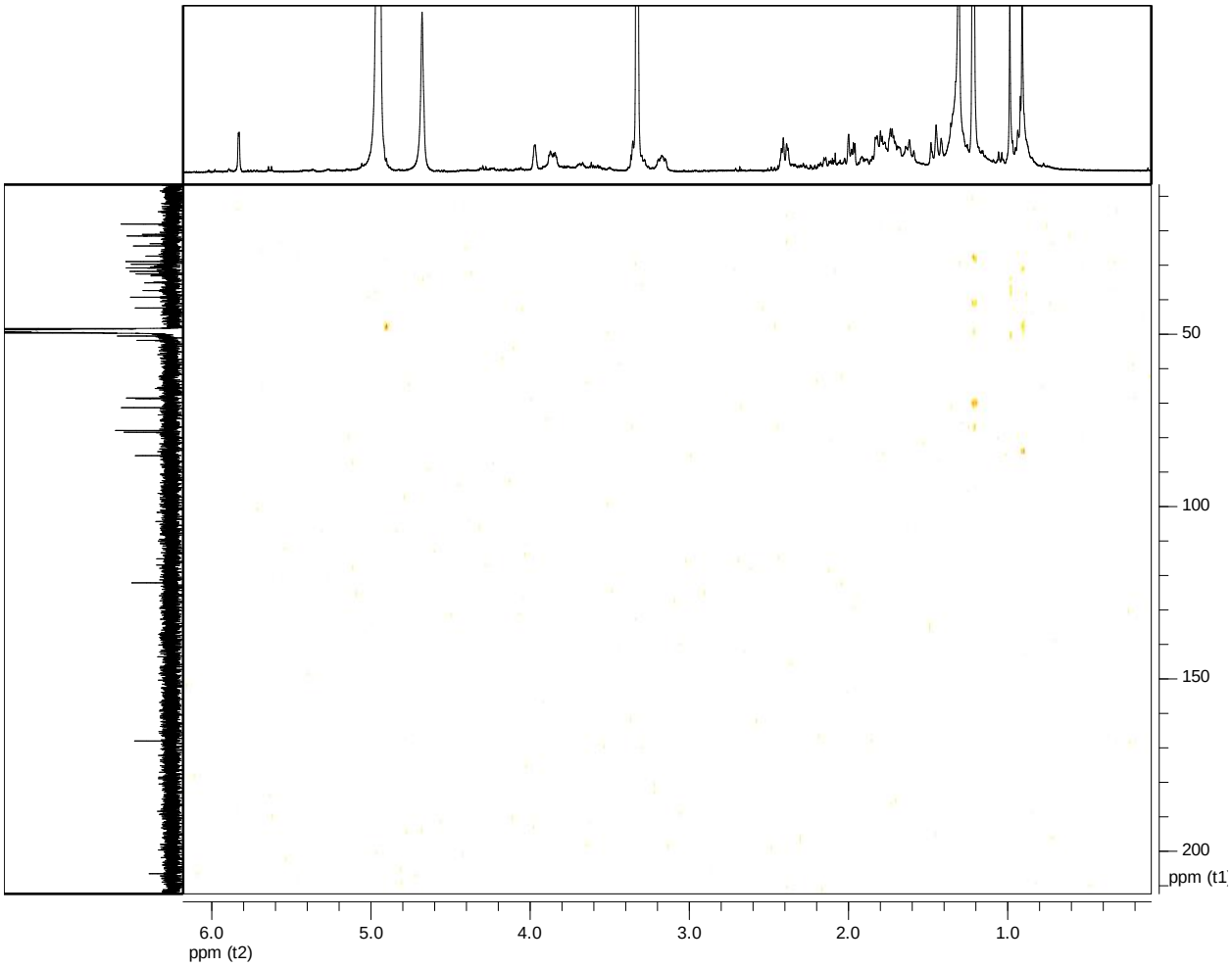
Appendix-12: COSY spectrum of AA-2



Appendix-13: HMQC spectrum of AA-2



Appendix-14: HMBC spectrum of AA-2



Appendix-15: IR spectrum of AA-2