Phytochemical studies on *Buxus hyrcana* and *Barleria prionitis*

By

Sarfraz Akhter

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Abstract

The research work embodied in this thesis describes the phytochemical studies on two medicinally important plants, *Buxus hyrcana* and *Barleria prionitis* Linn. Chemical investigations on the methanolic extract of the leaves of *B. hyrcana* have led to the isolation of one non-nitrogenous triterpene, arbora-1,9(11)-dien-3-one (**73**) and seven known steroidal alkaloids, cyclobuxoviridine (**74**), *E*-buxenone (**75**), *Z*-buxenone (**76**), moenjodaramine (**77**), homomoenjodaramine (**78**), buxamine B (**79**), 31-hydroxybuxamine B (**80**). These natural products were found to be active in acetylcholinestrase (AChE) and glutathion *S*-transferase (GST) inhibition assays.

Phytochemical studies on the ethanolic extract of aerial parts of *Barleria prionitis* Linn resulted in the isolation and identification of one known triterpene, lup-20(29)-ene-3β-ol (lupeol) (**87**) that it displayed moderate AChE and GST inhibitory activity. Compound (**87**) was isolated in a quantity sufficient to prepare three derivatives, and to evaluate them for GST and AChE inhibitory activities. It was decided to synthesize 3-acetyl-lupeol (**88**), 20-29 epoxylupeol (**89**) and 29-amino-20-hydroxylupeol (**90**) by using compound (**87**) as a main precursor. Compound **89** showed moderate AChE activity and compound **88** exhibited weak GST activity. The structures of all of the above mentioned compounds were elucidated with the help of extensive spectroscopic techniques such as UV, IR, MS, 1D-NMR and 2D-NMR.

Dedicated to my family

For their patience and endurance

CONTENTS

LIST OF ABBREVIATIONS

Abbreviation	Name
2° CC	Secondary column chromatography
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChEI	Acetylcholinesterase inhibitor
AD	Alzheimer disease
AIDS	Acquired immune deficiency syndrome
BChE	Butyrylcholinesterase
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
$CDCl_3$	Deuterated Chloroform
CDNB	1-chloro-2, 4-dinitrobenzene
CIMS	Chemical ionization mass spectrum
CLL	Chronic lymphocytic leukemia
CNS	Central nervous system
COSY	Correlation Spectroscopy
DEPT	Distorsionless Enhancement by Polarization Transfer
DMAPP	Dimethyl allyl pyrophosphate
DNA	Deoxyribonucleic acid
DSS	Differential smart screen
DTNB	5-5-dithio-bis (2-nitrobenzoic acid)
EIMS	Electron impact mass spectrum
GSH	Glutathione

GST	Glutathione *S*-Transferase
HCS	High content screening
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HMBC	Heteronuclear Multiple-Bond Correlation
HPLC	High performance liquid chromatography
HSQC	Heteronuclear Single Quantum Correlation
HTPS	High-throughput pharmacological screening
IC_{50}	Inhibition concentration by 50 %
IPP	Isopentenyle pyrophosphate
IR	Infrared
LC-MS	Liquid chromatography-mass spectrometry
MS	Mass Spectroscopy
NMR	Nuclear magnetic resonance
PAS	Peripheral anionic site
RSV	Respiratory Syncytial Virus
SARS	Severe Acute Respiratory Syndrome
TLC	Thin layer chromatography
UV	Ultra violet

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CHAPTER 1

General Introduction

1.0 Introduction

Life, disease and death are complementary to each other in every organism's life. Nature has created a super organism, "human being", who has the ability to get benefit from natural sources including terrestrial and marine organisms. Plants are important for mankind's survival because they are used as a vital source of nutrition, shelter, clothing, means of transportation as well as fertilizers, flavors, fragrances, and medicines etc. Plants produce a large number of secondary metabolites because of abiotic and biotic stress[1]. These secondary metabolites are also known as natural products and are used as competing plant and insect deterrents. These secondary metabolites are important for the ultimate survival of living organisms as they play ecologically significant roles of their interaction with their surroundings. Secondary metabolites include alkaloids, steroids, coumarins, lignans, and flavonoids as well as many other chemical classes. Living organisms synthesize secondary metabolites from primary metabolites. The primary metabolites are the building blocks of life and examples include proteins, fats, carbohydrates and lipids, which are necessary for growth and reproduction. We as human being use these natural products (secondary metabolites) to cure and treat various ailments. For instance, taxol purified from pacific yew tree, *Taxus brevifolia*, is a drug of choice for the treatment of breast, ovarian and lung cancers[2].

1.1 Natural products in historical prospective

In primitive ages various natural materials such as different parts of plants, herbs and animal products were used as medicines for the treatment of different diseases. For

example, oils of cedar (*Cedrus* species), cypress (*Cupressus sempervirens*), poppy juice (*Papaver somniferum*), licorice (*Glycyrrhiza glabra*) and myrrh (*Commiphora* species) were, and remain to be used for the treatment of various diseases like influenza, cough, inflammations, and parasitic infections etc[3]. The effective treatments were recorded and documented, which led to the foundation of early ethnopharmacopia (the use of herbs to treat various ailments). This provided knowledge regarding the history of drugs, which is long lasting. Furthermore, Chinese Materia Medica "Shen Nung Ben Tsao Jing" describes about 6000 drugs out of which 4800 are of plant origin. These included *Panax ginseng*, *Ganoderma japonicum*, *Lioyd* (reishi mushroom) and *Chrysanthemum morifolium ramat* (Chrysanthemum). These herbs are still popular and used today as they were previously[4]. Sun Simiao has also described the use of herbal medicine in his book[5] "Qian Jin Yi Fang" whereas Li Shi Zhen has recorded 1898 herbal drugs and 8160 prescriptions in his book from the year 1596[6-7].

The Greeks took lead in the use of herbal drugs and the European healing system is considered to have originated with Hippocrates (460–377 BC) and Aristotle (384–322 BC), who were impressed by ancient Indian and Egyptian health systems. Theophratus (300 BC), the philosopher and natural scientist, has described the medicinal properties of herbs and methods to change their characteristics through cultivation in his famous book "History of Plants". Dioscorides, a Greek physician (100 AD), recorded the collection, storage and use of medicinal herbs during his stay with the Roman Army. "De Materia Medica", written by Dioscorides is one of the most famous books, which provided the base for most of the later knowledge of herbal medicine. It is generally accepted to be the first European herbal system and was the standard reference in Europe for more than

1000 years. Galen (130-200AD) was the first Roman pharmacist who introduced complex formulations of various drugs with multiple ingredients. He was the author of more than two-dozen books about herbs and their uses in medicine [8-10].

Arabic traditional writing on the use of traditional medicine is considered the oldest since the earliest written information about medicinal plants comes from ancient Shanidar IV records. In these documents, the medicinal uses of pollens of different species of plants including *Centaures solstitialis* (Asteraceae), *Ephedra altissima* (Ephedraceae), *Althea* species (Malvaceae) have been described[11]. The King of Babylon (ca. 1700BC) commissioned a comprehensive set of civil laws and in the light of these laws, Assyrians and Sumerians listed hundreds of herbal formulations on clay tablets. The Egyptians are also known to have recorded medical and pharmaceutical information on papyrus and in wall paintings on tombs dating from the Old Kingdom. The Ebers Papyrus, which originates from about 1500 BC, contains ancient medicinal knowledge from before 3000 BC and it discusses various diseases and their possible forms of treatment [12].

Ancient Hindu records of medicinal plants and their use do not refer to any foreign medicinal system although Greek and Middle Eastern writings refer to ideas and drugs of Indian origin. In East India, the beginning of systemized medicine is considered to have started with Ayurveda, which is an experimental and holistic set of guidelines to maintain balance and harmony in the human body. The origin of Ayurveda is believed to be a divine revelation of the ancient Indian creator God Lord Brahma[13] and this knowledge was transferred directly to Daksha Prajapati by Lord Brahma in the form of shloka sung[14]. *Azadirachta indica* (Neem), *Centella asiatica* (Gotu Kola), *Cinnamomum*

camphora (Camphor) and *Withania somnifera* (Aswargandha) [15] are commonly used Ayurvedic medicinal plants.

In the United States, early settlers selected herbal remedies on the basis of Native American practices and later on their experience-based knowledge, which then became the basis of the pharmacopoeia. *Echinacea purpurea* (Echinacea) and *Hydrastis canadensis* (Goldenseal) are most commonly used medicinal plants of the United States[16].

1.2 Natural products—Source of drug discovery

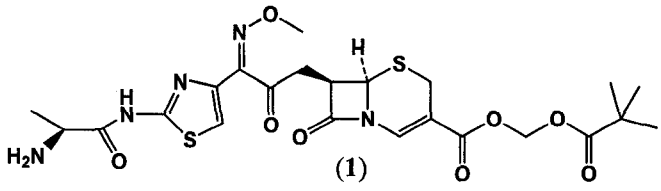
According to World Health Organization survey, 80% of the world's population relies on traditional medicines for their health care[17]. Moreover, 74% of the 119 currently most important drugs in the USA contain active ingredients from traditionally used medicinal plants while 25% of all prescriptions dispensed presently contain an active ingredient derived from plants[18].

As a result of 1993's National Prescription Audit of the United States, it was observed that 150 of the most prescribed drugs were based on 99 compounds, which were directly derived from natural products and approximately 55% of the prescribed drugs were either natural products or had structures based on natural product pharmacophores[19]. In the last two-decades, records of natural products as a source of new drugs show that 16.4% of newly launched drugs are derived directly from natural products and 12% have been designed based on a natural product[20].

In present circumstances, the pharmaceutical market is facing some major challenges, including the high cost and time of new drug discovery, drug development and marketing of new drugs. This has resulted in a decrease in the number of new medicines introduced

into the world market. Although some success has been made over recent years with natural products, pharmaceutical companies have generally lost their interest in natural products as a platform for drug discovery due to the introduction of molecular biology and combinatorial chemistry. Combinatorial chemistry can provide libraries of thousands of compounds in a short period of time. Although natural product screening and isolation provide fewer compounds over a longer period, it can lead to novel molecular structures, which is seen very rarely through combinatorial chemistry[21]. The degree of chemical diversity found in natural products is vaster and broader when compared to any synthetic source[22, 23]. It is hoped that interest in natural products will continue to exist and grow to become an even more valuable source of new drugs. With the development of rapid bioassay directed isolation techniques, it is now possible to isolate and characterize bioactive natural products present in trace amounts. Thus natural sources can be explored more efficiently for structurally complex biologically active molecules.

The continued investigation of new sources has led to the discovery of many biomolecules that are now being used for the treatment of various diseases. For example, β-lactam antibacterial drugs are produced by semi-synthesis from a natural product template. At present, two broad-spectrum antibiotics of this class, which are both derived from natural sources, ceftizoxime-alapivoxil (**1**) and ceftobiprole (**2**) are in clinical trials[24]. Both drugs are very effective against both Gram positive and negative bacteria but ceftobiprole (**2**) is found to be active against methicillin resistant *Staphylococcus aureus* and penicillin resistant *Streptococcus pneumoniae.*



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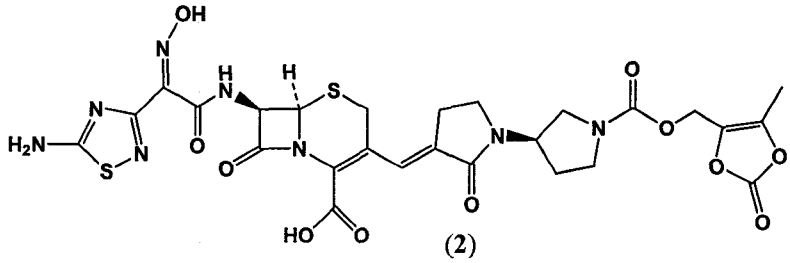
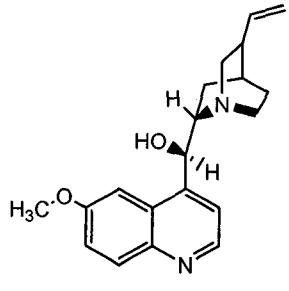


(2)

One of the well known natural products was Quinine (**3**), isolated from bark of the South American tree "Cinchona". It is a naturally occurring alkaloid that saved millions of lives in 17th and 18th century because of its antimalarial activity[25]. It also led to the development of other antimalarial drugs such as chloroquine (**4**) [26]. Artemisinin (**5**) is a peroxy bridge containing compound, originally isolated from Chinese herb "*Artemisia annua*" that has been used for over 2000 years by the Chinese to cure malaria[27]. It is a sesquiterpenoid which is effective against cerebral malaria as well as both chloroquinine-resistant and chloroquinine-sensitive strains of *Plasmodium falciparum*[28].



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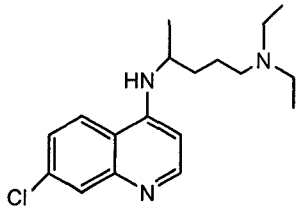

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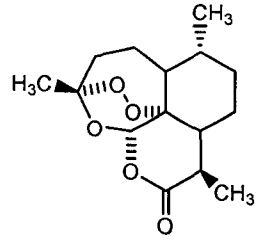
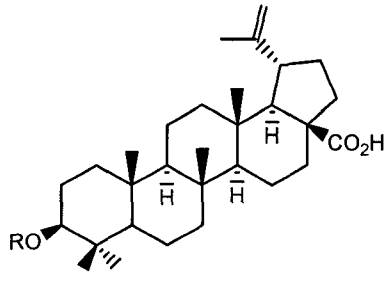


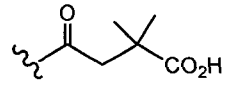
(5)

Viral diseases like HIV, hepatitis B and C (HCV), influenza, Ebola, dengue fever, Severe Acute Respiratory Syndrome (SARS) [29], and yellow fever are all emerging threats to human life. Great effort has been made to discover antiviral drugs, especially in the field

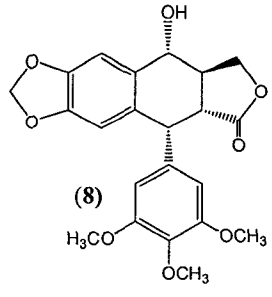
of HIV and hepatitis. One of the most promising compounds so far evaluated for the treatment of HIV is a semi-synthetic derivative of the plant triterpenoid, betulinic acid (**6**)[30], which has been found to be a weak inhibitor of HIV replication. Lee and co-workers at the University of North Carolina has identified a semi-synthetic derivative (**7**) as the promising agent for the treatment of HIV[31].



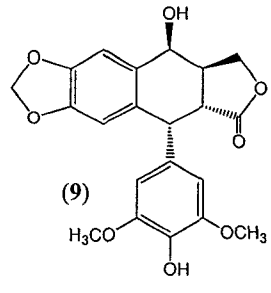
(**6**) R=H

(**7**) R= 

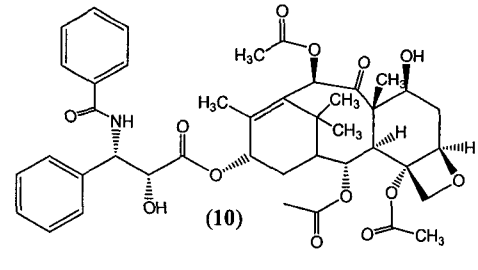
The history of natural products as anticancer compounds began in 1947 with podophyllotoxin (**8**), which was first isolated from *Podophyllum peltatum* [32]. However, due to its cytotoxity it is only used in the topical treatment of genital warts[33]. Podophyllotoxin acts by preventing the polymerization of tubulin into microtubules where as 4-demethylepipodophyllotoxin (**9**) analog inhibits topoisomerase II, preventing replication of DNA[34]. Taxol (**10**), isolated from the yew tree, *Taxus brevifolia*, is the drug of choice for the treatment of breast, ovarian and lung cancers, as well as AIDS-related Kaposi's sarcoma. Because of its outstanding anticancer activity and relatively low toxicity compared with other anticancer drugs taxol is widely used for chemotherapy[2].



(**8**)

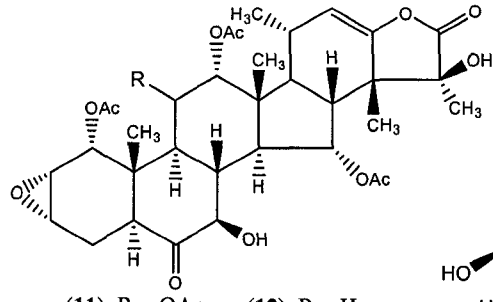


(**9**)

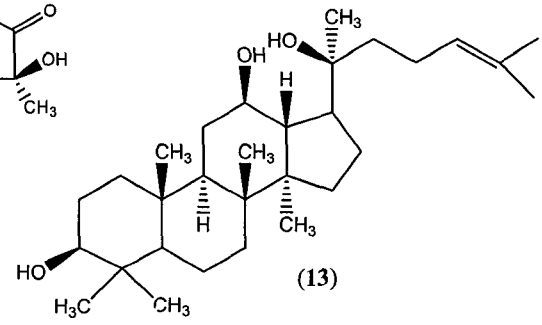


(**10**)

The plant steroids, taccalonolides A (**11**) and E (**12**) found in different *Tacca* species is comprised of taccalonolides A–V. Taccalonolide A, an antiproliferative agent used against mouse p388 tumor cells, was reported in 1987 from Chinese medicinal plant *Tacca plantagine*[35]. Taccalonolides A and E, isolated from *Tacca chantrieri* were reported to be the first microtubule-stabilizing agents of plant origin[36] since the discovery of taxol and protopanaxadiol (**13**), isolated from *Panax ginseng*, was reported to be cytotoxic against multidrug resistant tumors[37].

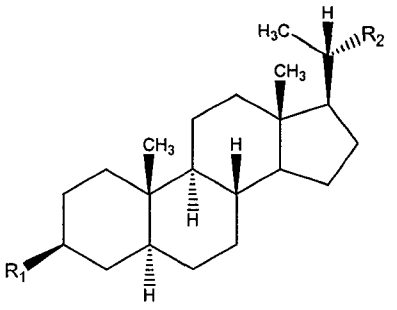


(**11**) R = OAc (**12**) R = H



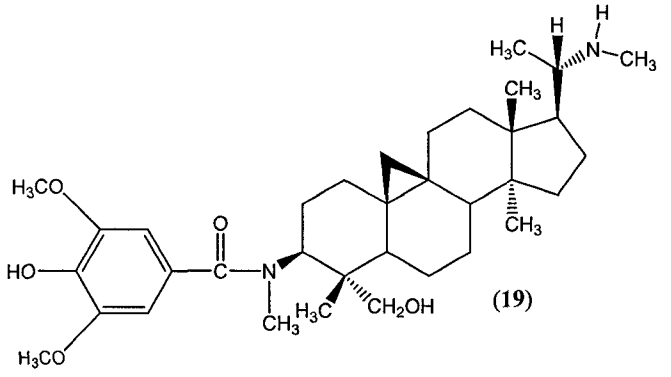
(**13**)

In the last decade biologically active steroidal alkaloids have been reported with different activities such as anti-HIV, anti-malarial, anti-bacterial, anti-AChE and anti-BChE but they are found in a relatively small number of families[38-39]. Buxaceae family is the most prominent in this respect. For example, isosarcodine (**14**), sarcorine (**15**), sarcodine (**16**), sarcocine (**17**) and alkaloid-C (**18**) isolated from *Sarcococca saligna* (Buxaceae), were reported as inhibitors of acetylcholinestrase (IC_{50} = 10.31, 69.99, 49.77, 20.0 and 42.2μM, respectively) and butyrylcholinestrase (IC_{50} = 1.89, 10.33, 18.31, 3.86 and 22.13μM, respectively). The compounds (**15-18**) reported dose-dependent spasmolytic activity in the rabbit jejunum intestinal preparations and also relaxed the high K^+-induced contraction, indicative of a calcium channel-blocking mechanism[39].



	R_1	R_2	Unsaturation
(14)	$N CH_3Ac$	$N(CH_3)_2$	
(15)	$N HAc$	$N(CH_3)_2$	
(16)	$N(CH_3)_2$	$N CH_3Ac$	
(17)	$N(CH_3)_2$	$N CH_3Ac$	$\Delta^{5,6}$
(18)	OCH_3	$N(CH_3)_2$	$\Delta^{5,6}$

Some other steroidal bases (**19-24**) isolated from *Buxus papillosa*, have been reported to exhibit AChE and BChE inhibitory activities. All of these compounds (**19-24**) have been shown to have weak to moderate anti-AChE and anti-BChE activities with none having IC_{50} greater than 235 and 2.73μM, respectively[40].



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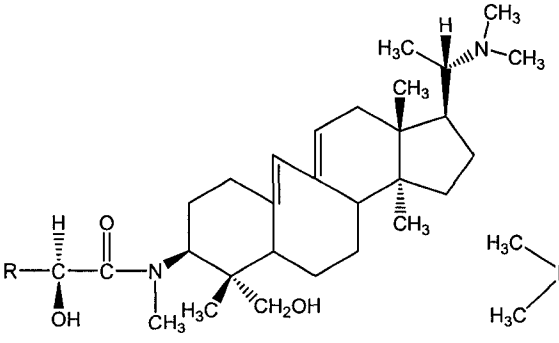
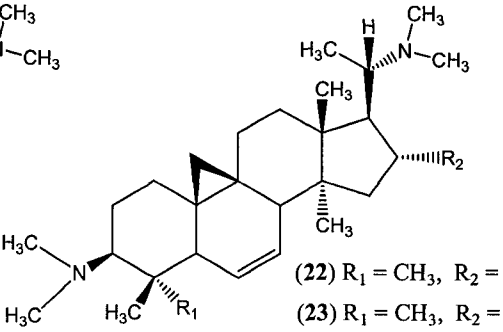
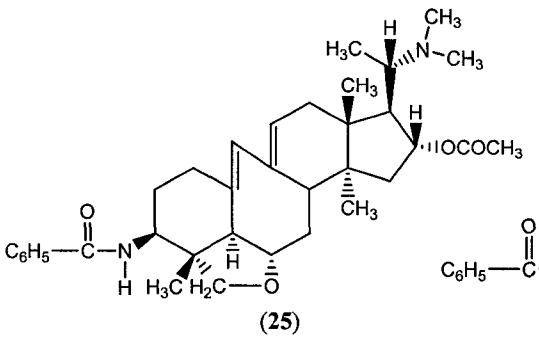


(20) R = $CH(CH_3)_2$ **(21)** R = $CH_3CHC_2H_5$



(22) $R_1 = CH_3$, $R_2 = H$
(23) $R_1 = CH_3$, $R_2 = OH$
(24) $R_1 = CH_2OH$, $R_2 = OH$

Four antibacterial steroidal alkaloids, cyclovirobuxeine F (**25**), *N*-benzoyl-*O*-acetylbuxalongifoline (**26**), buxasamarine (**27**) and cyclobuxamidine (**28**) were purified from *Buxus longifolia*. Alkaloids **25-27** showed antibacterial activity against *Salmonella typhi*, *Shigella flexneri* and *Pseudomonas aeruginosa*, while **28** was active against *S. typhi* and *Escherichia coli* [41].



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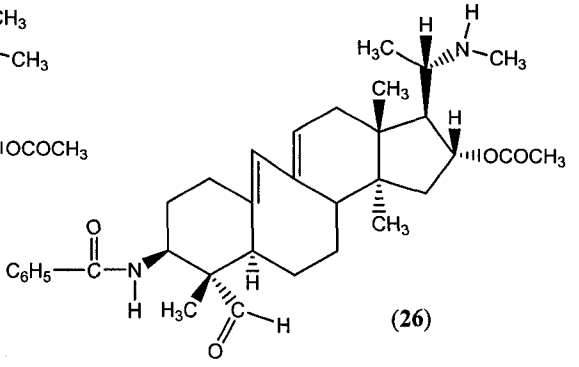

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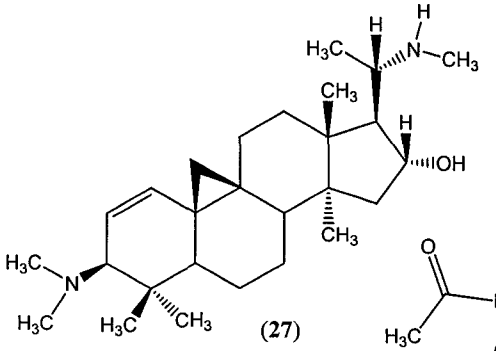

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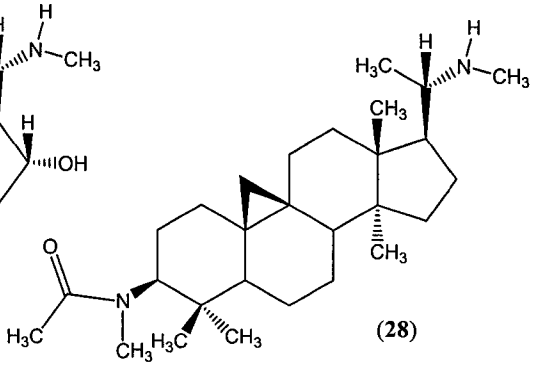
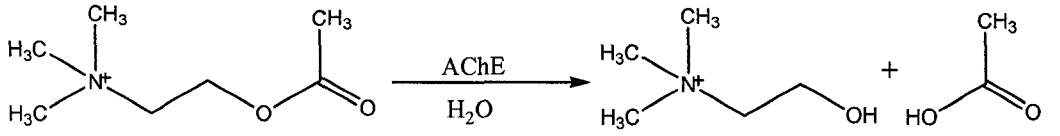


(28)

1.3 Acetylcholinestrase as Potential Targets in Alzheimer's disease

Acetylcholinestrase (AChE) is an extrinsic membrane bound enzyme that terminates the signal transduction of the cationic neurotransmitter acetylcholine (ACh). AChE functions in the central and peripheral nervous system along with ACh receptors. AChE activates and starts its function as soon as a presynaptic nerve process releases ACh, which readily diffuses across the synapse and stimulates its receptors. This results in a series of

reactions, which triggers the action potential in the postsynaptic cell. AChE terminates this process by hydrolytic cleavage of ACh as shown in Figure **1**.

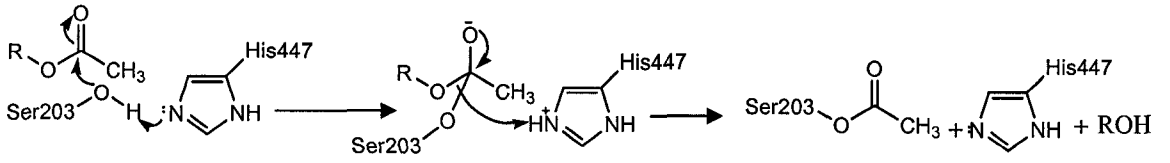


Acetylcholine Choline Acetic acid

Figure 1 Breakdown of ACh into choline and acetic acid

The breakdown of ACh into choline and acetic acid by acetylcholinestrase proceeds in two successive stages, acylation and deacylation, as shown in Figure **2**.

Acylation



Deacylation

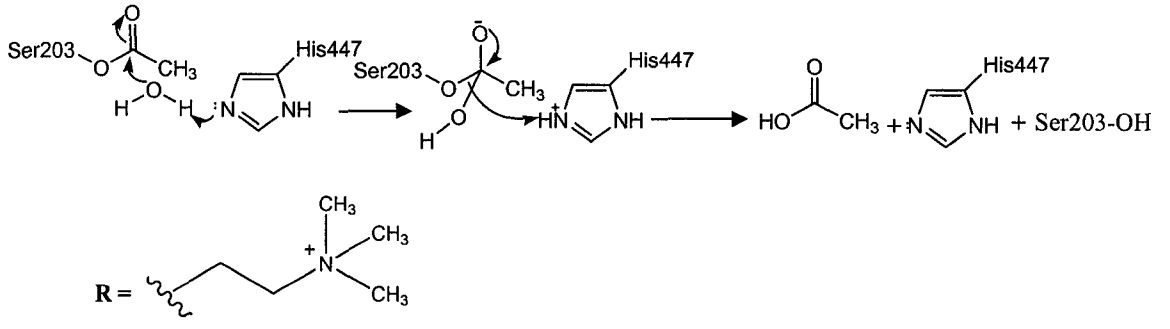


Figure 2 Reaction mechanism of the hydrolysis of ACh catalyzed by AChE.

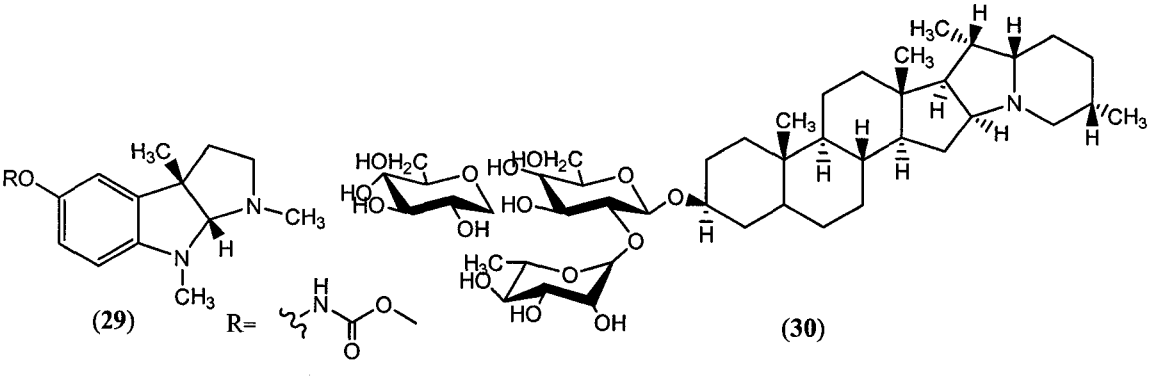
AChE has also been found to be involved in many other functions including roles as an adhesion protein, a bone matrix protein, in neurite growth and in the production of amyloid fibrils, which are found in the brain cell of patients with Alzheimer's disease (AD). The common cause of AD is accepted to be a deficiency in cholinergic

neurotransmission. The rational therapeutic approach to treat AD is to enhance the ACh level in the brain. This can be achieved by using AChE inhibitors[42].

1.3.1 Alkaloids as AChE inhibitors

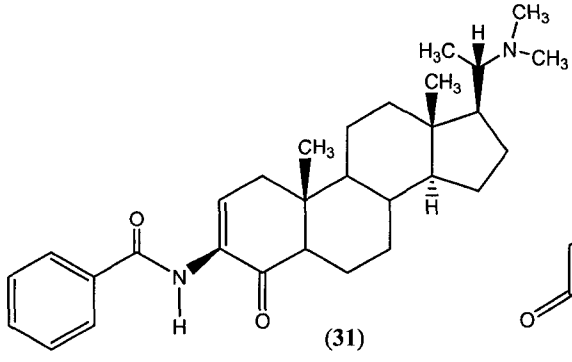
AChE and inhibitor interactions have been investigated throughly due to its pharmaceutical and pesticidal importance[43]. AChE inhibitors can be divided into two major groups, those bind to the active site at the bottom of the gorge in the enzyme structure and those that bind to the peripheral anionic site (PAS). Alkaloidal inhibitors bind to oxyanion hole of the active site at the bottom of the gorge and the important features of an inhibitor appear to be a positively-charged nitrogen[44].

Physostigmine (**29**) is a prototype acetylcholinesterase inhibitor that was isolated in 1864 from *Physostigma venenosum* (Papilionaceae). Subsequently, a number of pharmacological studies were carried out, but the importance of **29** was recognized in 1926 with the discovery of its cholinergic effects due to its dose-dependent inhibitory activity of AChE with IC_{50} value of 0.25 μM [45]. The steroidal alkaloids occur in a variety of structures but they are found in relatively few plant families. Solanaceae is one of the prominent families in this respect. The toxicity of members of *Solanum* and related species is considered to be caused by the presence of compounds like α-Solanine (**30**), reported to have moderate anti-AChE effect[46].

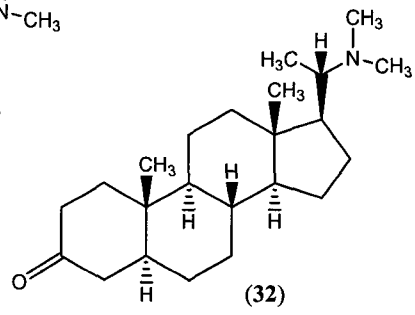


(29) (30)

Buxaceae is another family known as a rich source of steroidal alkaloids[47-50] which is considered the reason for toxicity in this family. Many reports have been published for the investigation of cholinesterase inhibitors from Buxaceae. For example axillaridine-A (**31**), purified from the *Sarcococca saligna* along with twenty two other steroidal alkaloids was found to be the best AChE inhibitor with IC50 value of 5.21μM[47]. Funtumafrine-C (**32**), isolated from *Sarcococca coriacea* has been shown to have anti-AChE activity with an IC_{50} value of 45.8 μM [48].

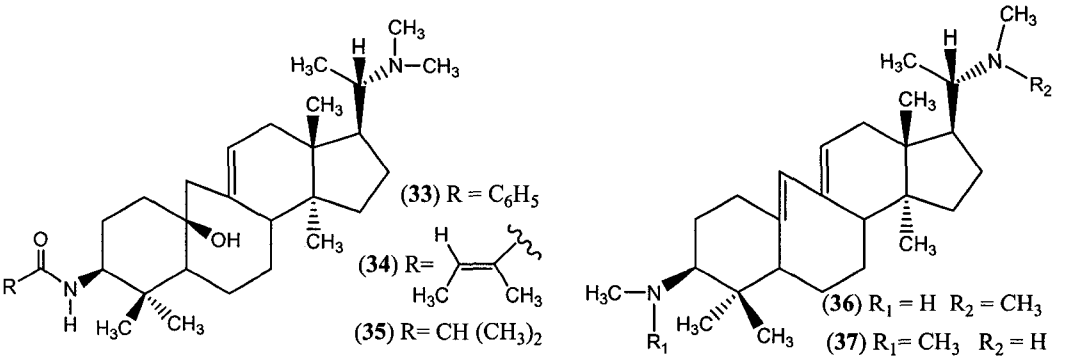


(31)



(32)

In 2003 Choudhary and his coworkers reported three *N*-acyl analogues of buxahyrcanine (**33-35**) from *Buxus hyrcana* with AChE and BChE inhibitory activities all below an IC_{50} value of 440 μM [49]. The alkaloids buxamines B (**36**) and C (**37**), isolated from *B. papillosa* and *B. hyrcana* are reported to inhibit AChE concentration dependently and non-competitively with IC_{50} values of 74 and 7.5 μM, respectively[50]. The difference in anticholinestrase activity was explained by docking studies, which revealed that **37** penetrated deeper into the AChE gorge than **36**, and that the positioning of the C-3 tertiary amino group resembles the quaternary ammonium group of ACh better than the secondary amino group of **36**[50].



Currently, reminyl (galanthamine) is used as an AChE inhibitor in the treatment of AD and it is reported to be safer in regards to side effects compared with other commercially available AChE inhibitors such as cognex (tacrine), Aricept (donepezil) and Exelon (rivastigmine) [51]. However, these inhibitors can only be used to treat mild to moderate levels of AD. There is still a need to discover more efficient drugs for AD.

1.4 Glutathione *S*-Transferase (GST) and its role in drug resistance

Glutathione *S*-Transferases (GSTs) are a group of enzymes that catalyze the conjugation of glutathione to a variety of hydrophobic and electrophilic compounds. These enzymes play an important role in the detoxification and metabolism of potential carcinogenic molecules that can damage genetic material (DNA). GSTs are also known to protect the body against potential alkylating agents, which unfortunately includes useful drugs. They are also responsible for the elimination of xenobiotics and endobiotics through glutathione adduct formation which makes the products more water-soluble to assist in subsequent excretion[52]. A general reaction catalyzed by GST is shown below (Figure **3**).

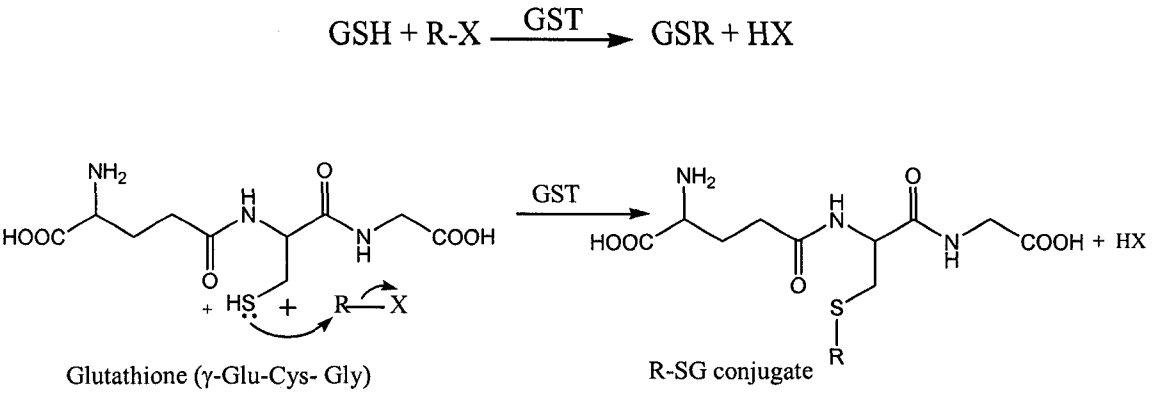


Figure 3 A general reaction catalyzed by GST; R-X = electrophilic substrate

A number of GST isozymes, especially GST π and μ, become over-expressed in a wide range of malignancies like cancer of the lung, colon, kidney[53], ovary[54], esophagus and stomach[55] due to an adaptive cellular response which protects cellular nucleophiles from drug-induced damage. Many findings lead to an established correlation between GST overexpression and clinical drug resistance[56]. For example, a 2-fold increase in GST activity was determined in lymphocytes from chronic lymphocytic leukemia patients (CLL), who were resistant to chlorambucil (anticancer drug), compared to lymphocytes from untreated CLL patients[57]. Such over expression of GSTs may be used as a prognostic marker in cancer patients under chemotherapy. GSTs have been implicated to metabolize anticancer agents and conjugate to GSH through thioester bond formation by utilizing detoxification mechanisms. Resultantly, they biotransform anticancer agents into more hydrophilic compounds to ease in their elimination from the body and this consequently reduces the effects of chemotherapy[58] (Figure **4**).

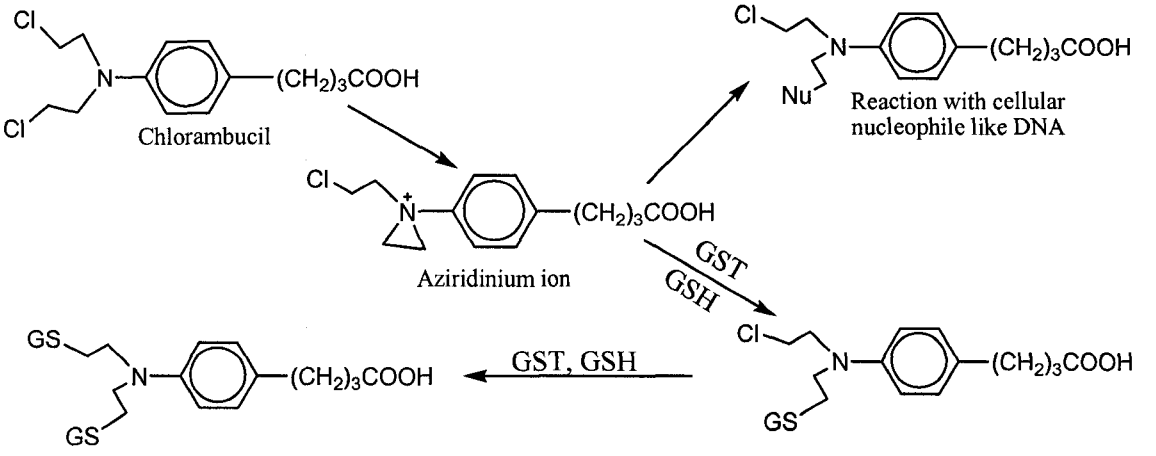
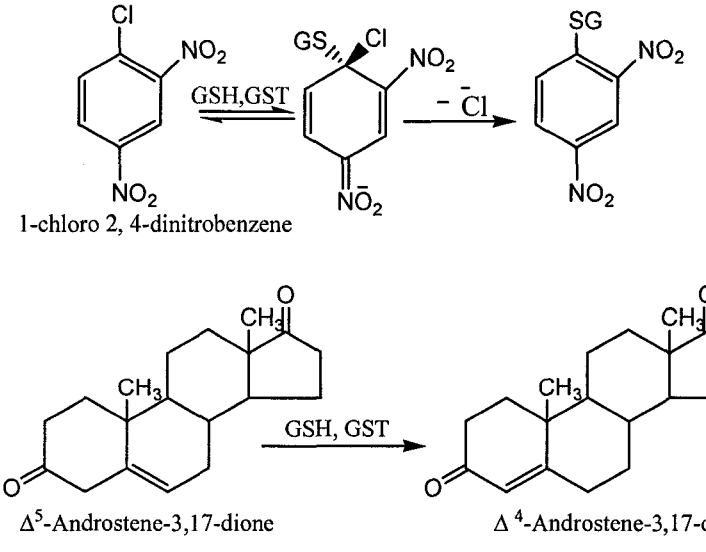
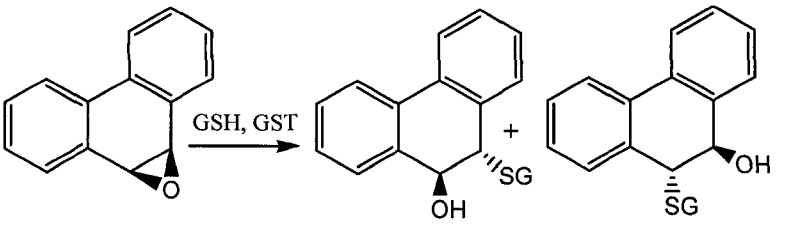


Figure 4 Function of GST and GSH in the removal of chlorambucil, an anticancer drug.

A number of alkylating agents and their metabolites have been probed as GST substrates, including chlorambucil[59] (as shown in Figure **4**), cyclophosphamide[60], aldophosphamide[61], melphalan[62], mechlorethamine[63], tris(1-aziridinyl) phosphine sulfide (thiotepa), tris(1-aziridinyl) phosphine oxide (tepa)[64], 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU)[65] and acrolein[66]. Few more examples of typical GST catalyzed reactions are shown in Figure **5**.

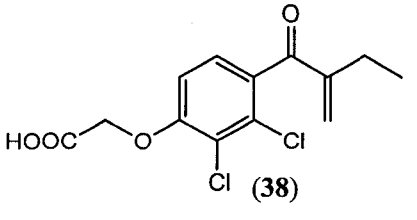




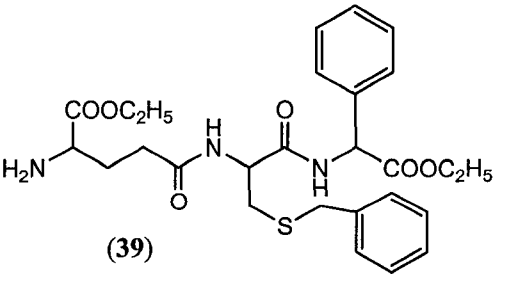
phenanthrene 9,10-oxide (9S, 10S)-GS-Phen. (9R, 10R)-GS-Phen.

Figure 5 GST catalyzed reactions

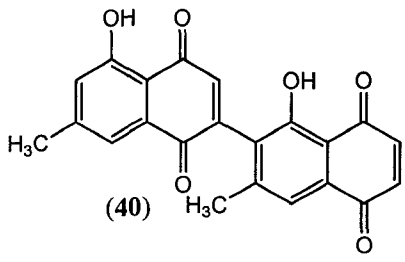
Several mechanisms have been reported regarding the removal of glutathione conjugates from the cell[67]. The link between drug response and GST mechanism of action provides an alternative target to improve the effect of therapeutic agents. The use of selective inhibitors of GST as adjuvant in chemotherapy within therapeutic limitations can provide an alternative approach for improved chemotherapy. In this respect, many competitive and non-competitive GST inhibitors have been investigated. Examples include, ethacrynic acid[68] (**38**), TER199 (γ-glutamyl *S*-benzyl cysteinyl phenyl glycine diethyl ester) [69] (**39**), diospyrin[70] (**40**), quercetin[71] (**41**) and β-estradiol 3, 17-disulfate[72] (**42**). Due to therapeutic limitations and different mode of action of GSTs slight success has been achieved so for. It is therefore highly desirable to explore new GST inhibitors that can be used as adjuvant to enhance the effect of chemotherapeutics.



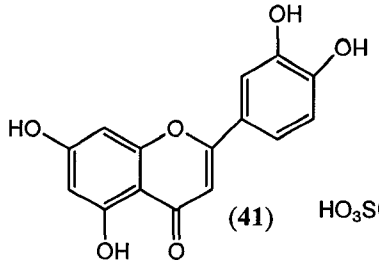
(**38**)



(**39**)



(40)



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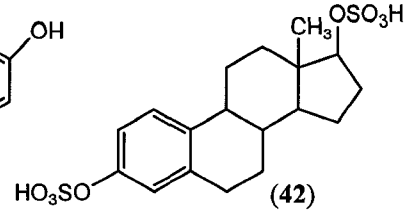

(42)

Though a little effort has been directed toward the screening of natural products which can be used as potential drug candidates to cure various diseases, but with the growing number of patients and diseases it is highly desirable to screen every single natural product in different bioassays to evaluate its medicinal importance. Natural products offer a wide range of structural diversity with characteristic features. Because of this, compounds isolated from *Buxus hyrcana* and *Barleria prionitis* were evaluated for their GST and AChE inhibitory activities. Chapter **2** and **3** explain the characterization of these purified compounds and investigational results in terms of AChE and GST inhibitory activities.

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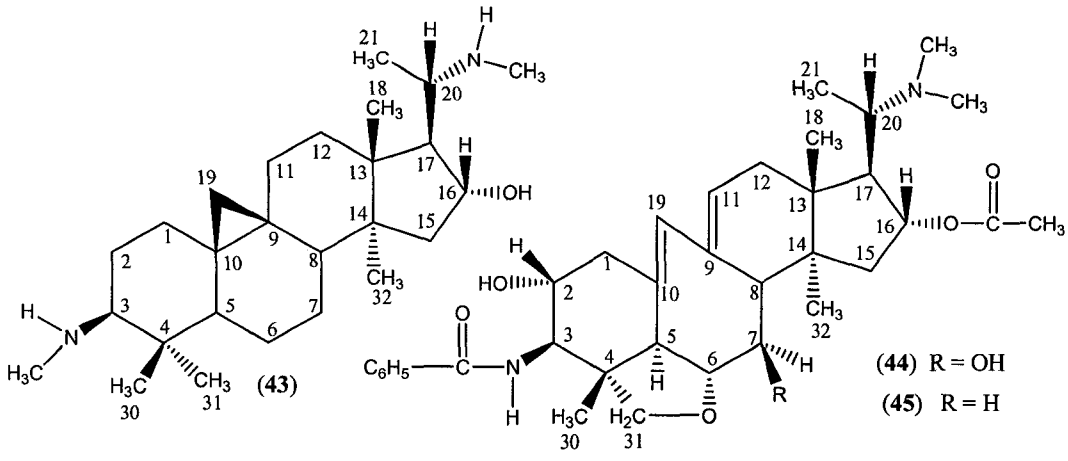
CHAPTER 2

Phytochemical studies on the leaves of *Buxus hyrcana*

2.1 Introduction

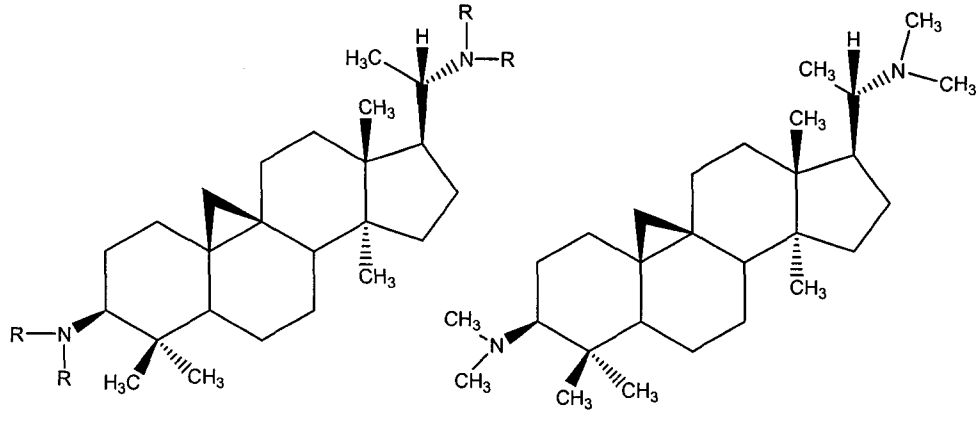
Genus *Buxus* is a rich source of steroidal alkaloids. This genus has several species including *B. semipervirens, B. papillosa, B. microphylla, B. hildebrandtii,* and *B. hyrcana*[1]. The crude extracts of the aforementioned plants are used in the indigenous (India, Iran, Australia) system of medicine to treat various ailments including fatigue, rheumatism, malaria, tuberculosis, depression and skin diseases[2]. The ethanolic extract of *B. semipervirens* containing cycloartenol, lanosterol, cyclobuxine-D and buxamine has been reported to be active against the Human Immunodeficiency Virus (HIV)[3]. It has further been reported that this extract delayed the progression of HIV disease in HIV-infected asymptomatic patients[4]. *Buxus* alkaloids have also shown interesting biological activities including anti-bacterial, anti-mycobacterial, anti-HIV and anti-malarial activities[2,4-7]. For instance, cyclovirobuxine-D (**43**) has shown activity against heart disorders[8]. Similarly, O^6-buxafurandiene (**44**) and 7-deoxy-O^6-buxafurandiene (**45**) exhibit acetylcholinestrase inhibitory activity with IC_{50} values of 17 and 13μM respectively[9].



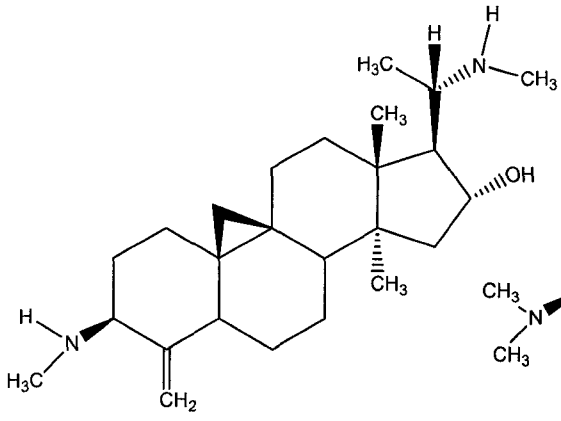
(43) (44) R = OH (45) R = H

These alkaloids have a unique triterpenoid-steroidal pregnane type skeleton with C-4 methyl groups, a 9β, 10β-cycloartenol system, and a degraded C-20 side chain. Previous phytochemical studies on various *Buxus* species have resulted in the isolation of over 200 steriodal bases[10, 11]. Structurally *Buxus* alkaloids can be divided into two main classes.

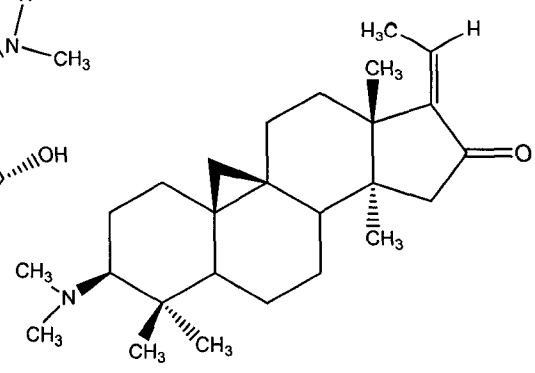
A: Derivatives of 9β, 10β-cyclo-4, 4, 14α-trimethyl-5α-pregnane system (**46**). Cycloprotobuxine-A (**47**)[12], cyclobuxine-D (**48**)[13], cyclobuxophylline-K (**49**)[14] are representative examples of this class.



(**46**) **R**=H or CH_3 (**47**)

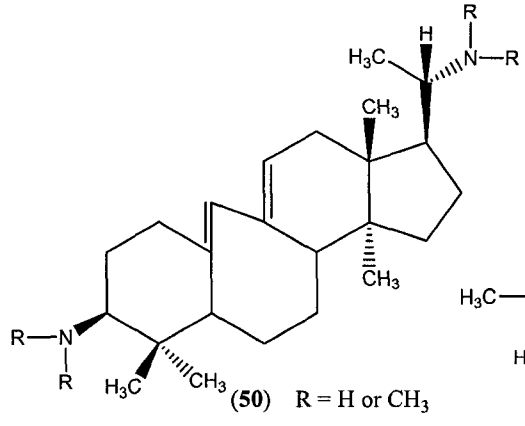


(48)

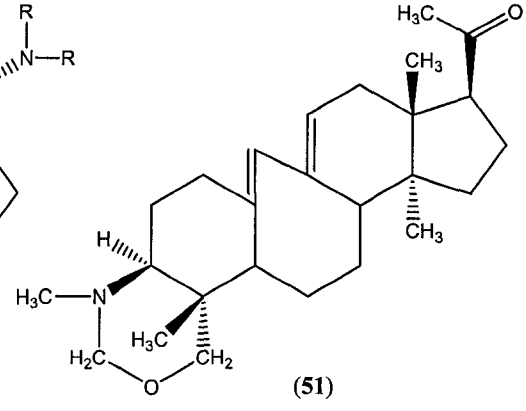


(49)

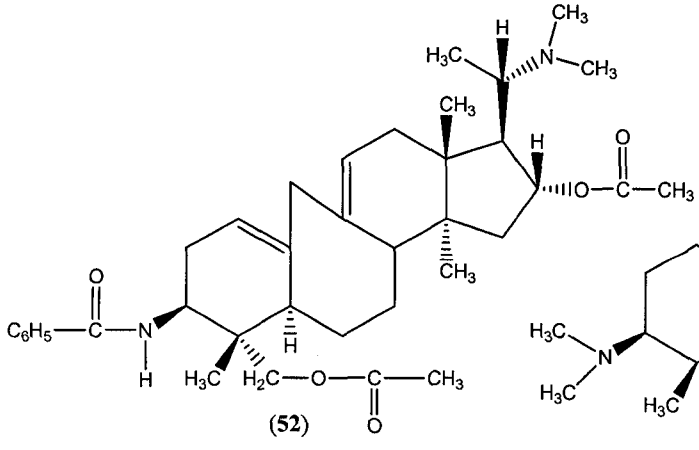
B: Derivatives of *abeo* 9(10→19) 4, 4, 14α-trimethyl-5α-pregnane system (**50**). Buxaquamarine (**51**), 16α, 31-diacetylbuxadine (**52**)[14], and buxaminol-E (**53**)[15] belong to this class of *Buxus* alkaloids.



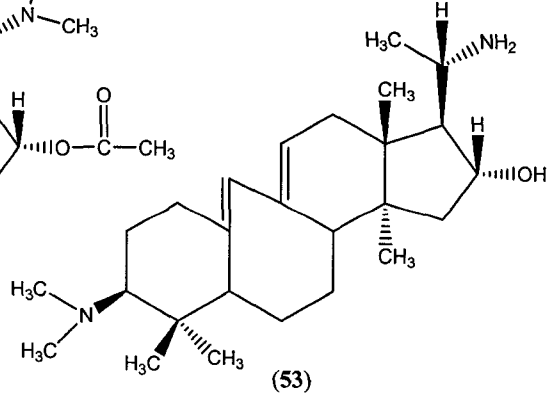
(**50**) R = H or CH_3



(**51**)



(**52**)



(**53**)

B. hyrcana is a tree like shurb[16] that is abundant in Iran[17].The aqueous extract of *B. hyrcana* is used for the treatment of malaria, rheumatism, venereal diseases and skin infections. The methanolic extract of *B. hyrcana* exhibited AChE inhibitory activity with IC_{50} value[9] of 45µg/ml. Previous chemical studies on this plant have yielded 26 steroidal bases. Their names and biological activities (if any) are listed in Table **1.**

Based on reported importance of *Buxus* alkaloids in the literature, it was decided to carry out phytochemical studies on the methanolic extract of leaves of *B. hyrcana* of Iranian origin. These chemical investigations have yielded one known non-nitrogenous triterpenoid, arbora-1,9(11)-dien-3-one (**73**) along with seven known nitrogenous triterpenoids, cyclobuxoviridine (**74**), *E*-buxenone (**75**), *Z*-buxenone (**76**), moenjodaramine (**77**), homomoenjodaramine (**78**), buxamine-B (**79**), 31-hydroxybuxamine-B (**80**). This thesis is the first report of (**73**) being isolated from *B. hyrcana* where as previously it was reported as a natural product from *Glycosmis arborea*[18]. Structures of all of these compounds were established with the aid of extensive spectroscopic studies. This chapter describes the isolation and structure elucidation of these compounds (**73-80**) as well as their AChE and GST inhibitory data.

Table 1 Steroidal alkaloids reported from *B. hyrcana* with their bioactivity

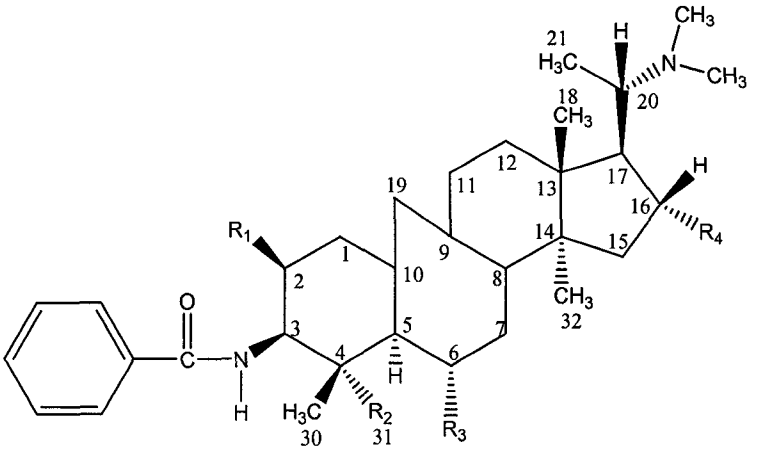
Name of compound	**Biological activity**		
	ǂAChE (IC_{50} µM)	₸BChE (IC_{50} µM)	†Cytotoxic activity (IC_{50} µM)
N-Benzoylbuxahyrcanine (**33**) [11]	>1000	310.6±0.1	----------
N-tigloylbuxahyrcanine (**34**) [11]	443.0±15.1	31.2±3.0	----------
N-isobutyroylbuxahyrcanine (**35**) [11]	>1000	53.7>10003.7	----------
Buxamine-B (**36**) [19]	74.0	----------	----------
Buxamine-C (**37**) [19]	7.50	----------	----------
O^6-buxafurandiene (**44**) [9]	17.0	----------	----------
7-deoxy-O^6-buxafurandiene (**45**) [9]	13.0	----------	----------
Hyrcanone (**54**)[20]	145.0±11.5	20.0±1.9	24.8±3.4
Hyrcanol (**55**) [20]	----------	----------	>178
Hyrcatriene (**56**) [20]	----------	1.71±0.02	>206
Buxabenzacinine (**57**) [20]	468.0±5.0	350.0±20.2	33.6±9.7
2α,16β,31-triacetylbuxiran (**58**)[22]	----------	----------	----------
2α,16β,31-triacetyl-9-11-dihydrobuxiran (**59**) [22]	----------	----------	----------
Benzoylbuxidienine (**60**)[9]	35.0	----------	----------
N_b-dimethylcycloxobuxoviricine(**61**) [20]	310.0±22.1	1.12±0.1	194±9.8
Hyrcamine (**62**) [20]	83.0±1.3	----------	----------
Buxidine (**63**) [20]	210.6±8.3	58.6±2.1	>192
Buxandrine (**64**) [20]	175.4±11.0	37.1±0.1	>178
Buxippine-K (**65**) [20]	>1500	210.0±23.0	84.6±23.1
E-buxenone (**66**) [20]	----------	----------	133.8±33.0
Hyrcanine (**67**) [21]	----------	----------	----------
Homomoenjodaramine (**68**)[21]	19.2±0.32	----------	----------
Menjodaramine (**69**) [21]	50.8±0.812	----------	----------
Buxaquamarine (**70**) [9]	76.0	----------	----------
Buxapapillinine (**71**) [9]	80.0	----------	----------
Irehine(**72**) [9]	100.0	----------	----------

ǂAChE = Acetylcholinestrase inhibition activity

₸BChE = Butyrylcholinestrase inhibition activity

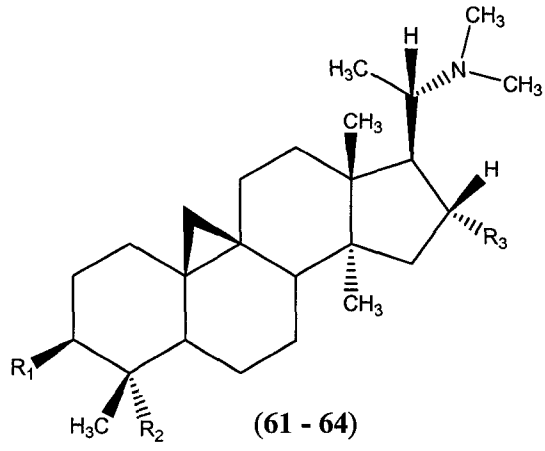
† Cytotoxic activity against fibroblast cell line

Structures of compounds **54-72** listed in table **1**.



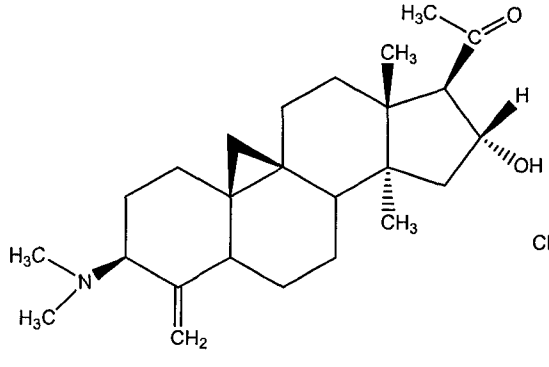
(54 - 60)

	R_1	R_2	R_3	R_4	Unsaturation site
(54)	H	CH_3	H	H	$\Delta^{1,10}$, 11-C=O
(55)	OH	CH_3	AcO	H	$\Delta^{1,10}$, $\Delta^{9,11}$
(56)	H	CH_3	H	H	$\Delta^{1,2}$, $\Delta^{9,11}$ $\Delta^{10,19}$
(57)	H	$AcOCH_2$	H	OH	$\Delta^{1,10}$, $\Delta^{9,11}$
(58)	AcO	$AcOCH_2$	H	AcO	$\Delta^{1,10}$, $\Delta^{9,11}$
(59)	AcO	$AcOCH_2$	H	AcO	$\Delta^{1,10}$
(60)	H	CH_3	H	OH	$\Delta^{10,19}$, $\Delta^{9,11}$

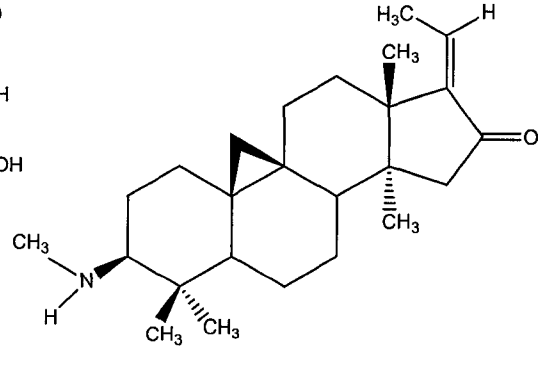


(61 - 64)

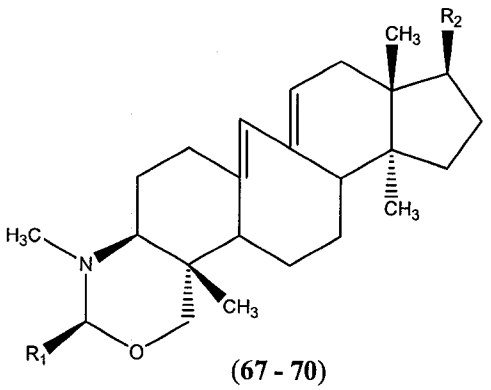
	R_1	R_2	R_3	Unsaturation site
(61)	C=O	CH_3	OH	$\Delta^{1,2}$
(62)	NH-tigloyl	$HOCH_2$	AcO	-------
(63)	NH-benzoyl	$HOCH_2$	OH	$\Delta^{6,7}$
(64)	NH-benzoyl	$HOCH_2$	AcO	$\Delta^{6,7}$



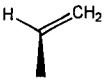
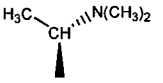
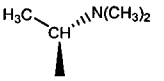
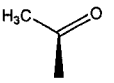
(65)

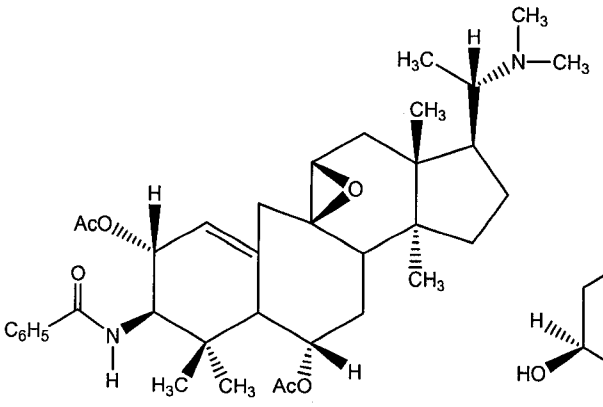


(66)

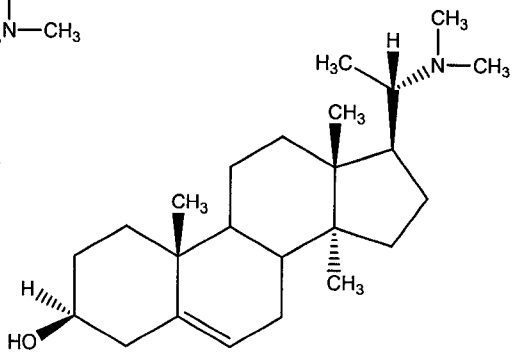


(67 - 70)

	R_1	R_2
(67)	H	
(68)	CH_3	
(69)	H	
(70)	H	



(71)

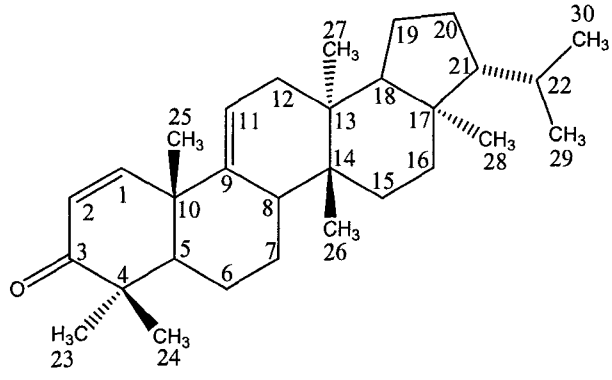


(72)

2. 2 Results

2.2.1 Arbora-1,9(11)-dien-3-one (73)

Arbora-1,9(11)-dien-3-one (**73**) was purified from fraction F_2 as a white amorphous solid by preparative TLC, using petroleum ether-tetrahydrofurane-diethylamine (95:5:1) as a mobile phase (see experimental section for detail).



(73)

The UV spectrum of **73** showed a maximum absorption at 228 nm indicating the presence of an α, β-unsaturated carbonyl chromophore[23].

The electron impact mass spectrum (EIMS) of **73** showed a molecular ion peak (M^+) at *m/z* 422 and chemical ionization mass spectrum (CIMS) showed a quasimolecular ion $[M+1]^+$ peak at *m/z* 423. In EIMS, an ion at *m/z* 407 was observed due to the loss of a methyl group from the molecular ion. A careful interpretation of MS and ^{13}C-NMR data suggested a molecular formula of $C_{30}H_{46}O$ for **73**.

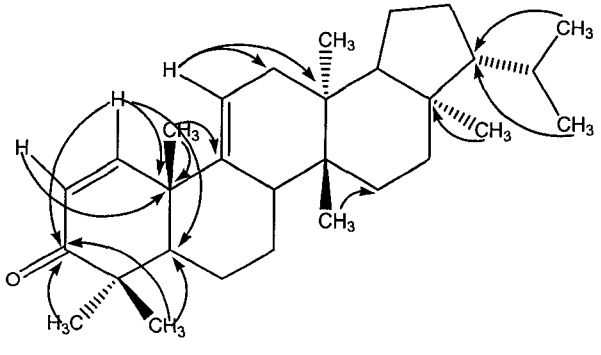
The ^{1}H-NMR spectrum ($CDCl_3$, 300MHz) displayed six singlets at δ 0.75, 0.95, 1.03, 1.09, 1.15, and 1.25 due to the protons of C-28, C-27, C-25, C-24, C-23 and C-26 methyl groups, respectively. A six-proton doublet at δ 1.12 (J = 6.5 Hz) was due to the methyl protons of C-29 and C-30. The C-11 olefinic proton resonated as a broad singlet at δ 4.89.

Two doublets, integrating for one-proton each, appeared at δ 7.19 and 5.84 ($J_{1,2}$ = 10.2 Hz) were ascribed to the C-1 and C-2 vinylic protons, respectively.

The COSY-45° spectrum was used to assign the ^{1}H-NMR chemical shift assignments. The vinylic protons of C-1 (δ 7.19) and C-2 (δ 5.84) showed vicinal coupling with each other while the C-11 olefinic proton (δ 4.89) showed cross-peaks with the C-12 methylene protons (δ 1.32 and 2.31).

The ^{13}C-NMR ($CDCl_3$, 50MHz) spectrum of **73** showed the resonance of all thirty carbons. Multiplicity of each carbon signal was determined by DEPT experiment. The HSQC spectrum was also recorded in order to determine the ^{1}H/^{13}C one-bond shift correlations of all protonated carbon atoms. The complete ^{1}H and ^{13}C-NMR chemical shift assignments of **73** are shown in Table **2**.

The HMBC spectrum of **73** exhibited long range correlations of H-1 (δ 7.19) with the C-3 (205.7), C-5 (δ 45.1) and C-10 (δ 39.6), while the H_3-23 (δ 1.15) and H_3-24 (δ 1.09) displayed HMBC interactions with the C-3 (δ 205.7) and C-5 (δ 45.1). Similarly, the H-11 (δ 4.89) showed a cross peak with the C-12 (δ 34.3) and C-13 (δ 37.6). The important HMBC interactions are shown around structure **73**, below.



Important HMBC interactions of **73**

These spectral data suggested that compound **73** was a member of pentacyclic triterpenoid of hopane series as the ^{1}H and ^{13}C-NMR spectral data were found to be identical to those compounds of this series, reported in the literature[24-27]. This is the first report of the isolation of this compound from *B. hyrcana.*

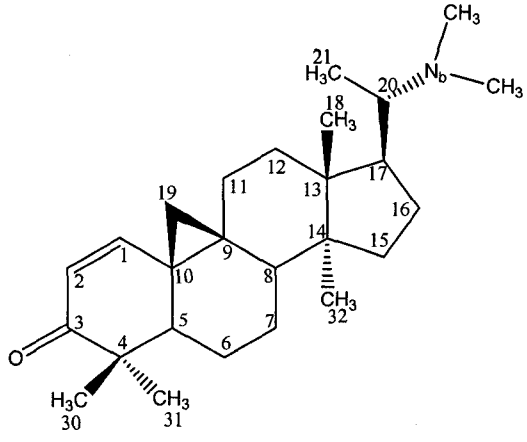
Table 2 ^{1}H and ^{13}C-NMR chemical shift assignments of **73**

Carbon No.	^{1}H-NMR (δ)	^{13}C-NMR (δ)
1	7.19 d, J = 10.2 Hz	160.2
2	5.84 d, J = 10.2 Hz	125.2
3	----	205.7
4	----	33.9
5	1.52 m	45.1
6	1.07 and 1.38 m	21.4
7	1.48 and 1.74 m	18.9
8	1.41*m	38.6
9	----	142.3
10	----	39.6
11	4.89 br s	129.9
12	1.32 and 2.31 br s	34.3
13	---	37.6
14	---	41.6
15	0.95* and 1.34 m	29.7
16	1.36 and 2.36 br s	37.3
17	---	43.5
18	1.55* m	53.7
19	1.14 and 1.84 m	26.1
20	1.11 and 1.76 m	27.5
21	1.55* m	53.7
22	1.41* m	38.6
23	1.15 s	16.7
24	1.09 s	21.4
25	1.03 s	25.3
26	1.25 s	29.1
27	0.95* s	31.3
28	0.75 s	14.4
29	1.12 d, J = 6.5 Hz	19.9
30	1.12 d, J = 6.5 Hz	27.8

Solvent: $CDCl_3$
* Overlapping signls

2.2.2 Cyclobuxoviridine (74)

Cyclobuxoviridine (**74**) was isolated as a white amorphous solid from fraction F_a, which was obtained by eluting a column of silica gel with hexane-acetone (90:10) and was found to contain one major compound on analytical TLC. This major compound was purified by preparative TLC using hexane-acetone-diethylamine (95:5:1), as a developing solvent.



(74)

The UV spectrum of **74** showed absorption maxima at 268 nm, indicating the presence of an α, β unsaturated carbonyl group adjacent to the cyclopropyl ring [28].

The EIMS of **74** showed the molecular ion peak at *m/z* 383 where as the CIMS showed the quasimolecular ion $[M+1]^+$ peak at *m/z* 384. An ion at *m/z* 368 resulted from the loss of a methyl group from the molecular ion in EIMS of **74**. The base peak at *m/z* 72 was also observed in the mass spectrum of this compound. Another ion at *m/z* 85 was attributed to the cleavage of ring D. The combination of ^{13}C-NMR and mass spectral data helped to suggest a molecular formula $C_{26}H_{41}NO$ for **74**.

The ^{1}H-NMR ($CDCl_3$, 200 MHz) spectrum of **74** showed two three-proton singlets at δ 0.92 and 1.09, corresponding to C-18 and C-31 methyl protons respectively. A singlet of

six-protons at δ 1.26 was due to C-30 and C-32 methyls respectively. The C-21 secondary methyl group resonated as a doublet at δ 0.99 ($J_{21,\ 20}$ = 6.0 Hz), while neighboring C-20 methine proton appeared as a multiplet at δ 2.05. A six-proton singlet resonating at δ 2.50 was assigned to *N, N*-dimethyl protons. Two sets of AB doublets resonating at δ 0.75 and 0.81 ($J_{19\alpha,\ 19\beta}$ = 5.07 Hz) were assigned to the C-19 cyclopropyl methylene protons. The downfield shift from the usual chemical shift values[29] was due to the deshielding effect of the neighbouring olefinic functionality[30]. Two doublets integrating for one proton each at δ 6.75 ($J_{1,\ 2}$ = 10.1 Hz) and 5.94 ($J_{1,\ 2}$ =10.1 Hz) were ascribed to the C-1 and C-2 olefinic protons, respectively.

The ^{1}H-^{1}H COSY spectrum was used to establish the vicinal and geminal couplings. Two doublets resonating at δ 6.75 and δ 5.94 due to H-1 and H-2 methine protons showed cross peaks with each other. A methine proton of H-17 resonating at δ 1.62 showed vicinal couplings with H-20 at δ 2.05. The latter showed cross peak with methyl protons of H_3-21 at δ 0.99. Moreover, methylene protons of H_2-6 resonating at δ 1.53 and δ 0.90 showed vicinal couplings with H-5 at δ 1.72 and H_2-7 at δ 1.36. The H_2-7 displayed a cross peak with H-8 at δ 1.54.

The ^{13}C-NMR spectrum ($CDCl_3$, 50 MHz) of **74** showed the resonance of all twenty-six carbons. The C-18, C-30, C-32 and C-31 methyl carbons resonated at δ17.1, 18.6, 21.4 and 23.2, respectively. The olefinic C-1 and C-2 appeared at δ 153.5 and δ 126.9, respectively. The complete assignments to all carbon atoms of **74** are shown in Table **3**.

The UV, ^{1}H, ^{13}C-NMR, and mass spectral data of compound **74** were identical to those of cyclobuxoviridine, reported in the literature[28, 30, 31] and this characterized compound **74** as

cyclobuxoviridine. This compound was previously isolated from *B. papillosa*[28] and *B. microphylla*[30, 31]. This is the first report of the isolation of **74** from *B. hyrcana*.

Table 3 ^{13}C-NMR chemical shift assignments of compound **74**.

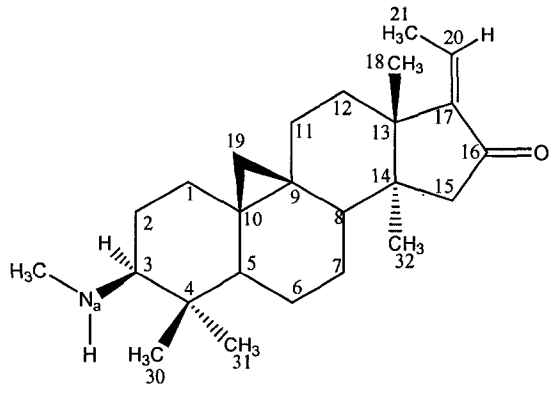
Carbon No.	^{13}C (δ)
1	153. 5
2	126.9
3	205.0
4	45.9
5	49.0
6	24.4
7	27.6
8	44.2
9	19.1
10	43.3
11	26.8
12	34.4
13	43.1
14	49.3
15	32.1
16	28.9
17	49.3
18	17.1
19	19.4
20	63.1
21	22.7
30	18.6
31	23.2
32	21.4
N(Me)$_2$	29.7

Solvent: $CDCl_3$

2.2.3 *E*-buxenone (75)

E-buxenone (**75**) was obtained as a colorless gummy material from fraction F_b by eluting the silica gel column with hexane-acetone (65:35), which was further purified by preparative TLC using hexane-acetone-diethylamine (90:10:1), as a mobile phase.

The UV spectrum of **75** showed absorption maxima at 243 nm, suggesting the presence of an α, β-unsaturated carbonyl system[32].



(**75**)

The EIMS and CIMS of **75** showed the molecular ion peak at *m/z* 369 and at *m/z* 370 $[M+1]^+$, respectively. A combination of mass and ^{13}C-NMR led us to derive molecular formula, $C_{25}H_{39}NO$. An ion at *m/z* 354 arose due to the loss of a methyl group from the molecular ion and another fragment at *m/z* 326 resulted from the loss of a carbon monoxide (CO) molecule from the M^+-15 ion, indicating the presence of carbonyl functionality in this compound. The base peak at *m/z* 57 resulted from the cleavage of ring A that suggested the presence of *N*-methyl functionality at C-3.

The ^{1}H-NMR spectrum ($CDCl_3$, 200 MHz) of **75** contained a set of two AB doublets at δ 0.42 and 0.63 ($J_{19\alpha,\ 19\beta}$ = 4.5 Hz) due to C-19 cyclopropyl methylene protons. Four three-proton singlets at δ 0.93, 1.10, 1.26, and 1.32 were ascribed to the four methyl protons attached to quaternary carbons atoms. A three-proton doublet at δ 1.82 ($J_{21,\ 20}$ = 7.4 Hz)

was due to the C-21 methyl protons. A one-proton quartet at δ 6.55 ($J_{21,20}$ = 7.4 Hz) was assigned to the C-20 olefinic proton. The C-3 methine proton resonated at δ 3.67 as a multiplet, while the *N*-methyl protons appeared as a three-proton singlet at δ 2.61.

The COSY-45° spectrum was also recorded to confirm the ^{1}H-NMR chemical shift assignments. The C-21 methyl protons (δ 1.82) showed cross peak with C-20 methine (δ 6.55) proton. The C-19 methylene protons (δ 0.42 and 0.63) also exhibited geminal coupling between them.

The ^{13}C-NMR spectrum ($CDCl_3$, 50 MHz) showed the resonance of all 25-carbons. Two downfield signals at δ146.0 and 129.7 were due to the vinylic C-17 and C-20, respectively. The *N*-CH_3 appeared at δ 29.7 while the C-16 carbonyl carbon resonated at δ 206.1. The complete ^{13}C-NMR chemical shift assignments of **75** are listed in Table **4**.

The UV, ^{1}H, ^{13}C-NMR, and mass spectral data of compound **75** were found to be identical to those of *E*-buxenone reported in the literature[19, 30, 32, 33] that helped to identify compound **75** as *E*-buxenone. This compound was previously purified from *B. hyrcana* [19], *B. microphyla* [30] and *B. sempervirens* [32, 33].

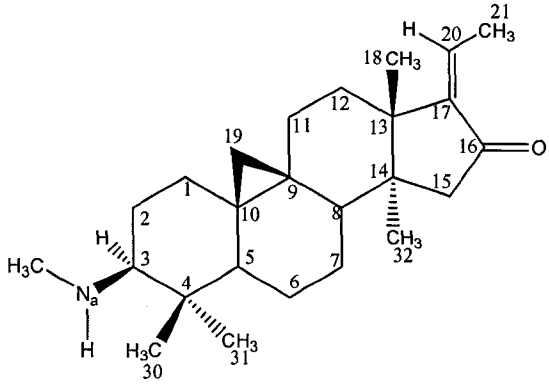
Table 4 ^{13}C- NMR chemical shift assignments of **75**

Carbon No.	$\delta^{13}C$
1	31.5
2	23.5
3	68.8
4	48.8
5	46.1
6	23.7
7	25.6
8	47.6
9	20.3
10	25.9
11	28.7
12	33.7
13	39.0
14	41.8
15	45.1
16	206.1
17	146.0
18	14.6
19	29.1
20	129.7
21	25.2
30	18.9
31	25.3
32	12.7
N_a-Me	29.7

Solvent $CDCl_3$

2.2.4 *Z*-buxenone (76)

The procedure described for the isolation of *E*-buxenone **75** also yielded *Z*-buxenone **(76)** as a colorless gummy material.



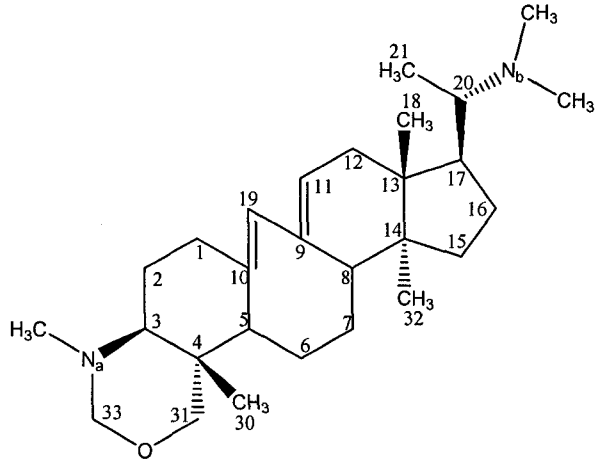
(76)

The UV and mass spectral data of **76** were similar to those of compound **75**, discussed previously. The ^{1}H-NMR and COSY-45° of **76** was found to be nearly the same as those discussed for compound **75**, with the exception of resonance for a doublet of C-21 methyl (δ 2.05) ($J_{21, 20}$ = 7.5 Hz) and a quartet of C-20 (δ 5.65) methine protons. This downfield resonance of C-21 methyl protons from δ 1.82 to 2.05 and up field resonance of C-20 methine proton from δ 6.55 to 5.65 compared with **75** suggested that compound **76** was a *Z*-isomer of **75**.

These spectral data led to propose structure **76** for this reported steroidal base. This compound was previously separated from *B. sempervirens*[34]. However, this is a first report of compound **76** from *B. hyrcana*.

2.2.5 Moenjodaramine (77)

Two steroidal alkaloids, moenjodaramine (**77**) and homomoenjodaramine (**78**) were isolated as a white crystalline solid by the preparative TLC of fraction F_c that was obtained by eluting the column with hexane-acetone (10:90), which were further purified by preparative TLC using ethylacetate-methanol-diethylamine (80:20:1) as mobile phase. The UV spectrum of **77** showed the absorption maxima at 236 and 245 nm, characteristics for the 9 (10→19) *abeo*-diene system[7].



(**77**)

The EIMS and CIMS data of **77** exhibited the molecular ion peak at *m/z* 426 and its combination with ^{13}C-NMR data proposed the molecular formula $C_{28}H_{46}N_2O$. The ion at *m/z* 411 resulted from the loss of a methyl group from the molecular ion. The EIMS of compound **77** showed ions at *m/z* 85 and 71. The base peak at *m/z* 58 was also observed in the mass spectrum of this compound. These fragments are found in the *Buxus* alkaloids having tetrahydrooxazine moiety at ring A[35].

The ^{1}H-NMR ($CDCl_3$, 200 MHz) showed three singlets, each integrating for three-proton at δ 0.70, 0.74, and 1.01 for the three-methyl groups due to C-18, C-32 and C-31, respectively. The C-21 methyl protons resonated as a doublet at δ 0.86 ($J_{21,20}$ = 6.0 HZ).

A three-proton singlet at δ 2.10 and six-proton singlet at δ 2.22 were assigned to *N*-*N*-dimethyl group attached to C-20 and *N*-methyl group at C-3 respectively. A set of two AB doublets resonating at δ 3.24 and δ 3.82 were due to C-31 methylene protons (J_{AB} = 10.4 Hz), while another set of two AB doublets centered at δ 3.57 and δ 4.42 (J_{AB} = 7.4 Hz) were attributed to C-33 methylene protons of the tetrahydrooxazine ring respectively. A singlet at δ 5.98 was ascribed to the isolated olefinic proton at C-19 while a multiplet at δ 5.54 was assigned to the C-11 olefinic proton.

The ^{13}C-NMR spectrum ($CDCl_3$, 50 MHz) showed four signals at δ 13.9, 14.2, 15.8, and 17.1 that were assigned to C-18, C-21, C-32, and C-30 methyl carbons, respectively. The olefinic carbon atoms C-19, C-11, C-10 and C-9 appeared at δ 129.9, 130.1, 134.1, and 138.1, respectively. The carbons of methyl groups attached to nitrogen at C-3 and C-20 were found to resonate at δ 36.4 and δ 29.7 respectively. The complete ^{13}C-NMR chemical shift assignments of **77** are shown in Table **5**.

The UV, MS, ^{1}H, and ^{13}C-NMR spectral data of **77** were identical with those of moenjodaramine reported in the literature [20, 35, 36]. Based on these spectral data, compound **77** was identified as moenjodaramine. This compound has been previously isolated from *B. hyrcana*[20], *B. papillosa*[35] and *B. hildebrandtii* [36].

Table 5 ^{13}C- NMR chemical shift assignments of Compound **77**

Carbon No.	$\delta\ ^{13}C$
1	39.2
2	26.9
3	71.2
4	42.9
5	48.4
6	25.4
7	25.9
8	49.1
9	138.1
10	134.1
11	130.1
12	38.5
13	39.8
14	48.6
15	32.9
16	28.9
17	49.7
18	13.9
19	129.9
20	60.4
21	14.2
30	17.1
31	88.5
32	15.8
33	97.6
N_a-Me	36.4
N_b-$(Me)_2$	29.7

Solvent: $CDCl_3$

2.2.6 Homomoenjodaramine (78)

The procedure described for the isolation of moenjodaramine **77** also yielded homomoenjodaramine (**78**) as a white crystalline solid.

The UV spectrum of homomoenjodaramine **78** was identical to that of **77** indicating the presence of similar chromophore as described for moenjodaramine (**77**).

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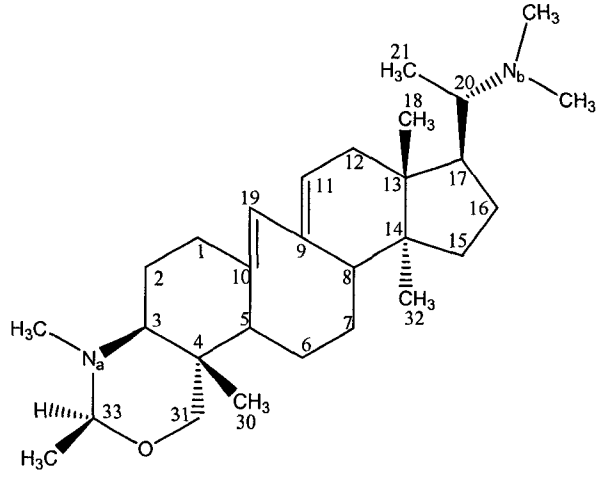


(78)

The EIMS and CIMS experiments were performed to determine the molecular ion peak. The EIMS displayed the molecular ion peak at *m/z* 440 where as the CIMS exhibited the quesimolecular ion $[M+1]^+$ at *m/z* 441. The combination of MS and ^{13}C-NMR data helped to propose the molecular formula $C_{29}H_{48}N_2O$. An ion at *m/z* 425 was observed due to the loss of a methyl group from the molecular ion. The base peak at *m/z* 72 was also observed due to the loss of trimethyliminium ion in the mass spectrum of this compound. The EIMS showed another ion at *m/z* 58, which is commonly observed in *Buxus* alkaloids having *N*-methyl or *N, N*-dimethyl group at C-20 of ring D[37].

The ^{1}H-NMR spectrum ($CDCl_3$, 200 MHz) of **78** was similar to that of compound **77** except for the C-33 methine proton appeared as a quartet at δ 3.62 ($J_{33\ \alpha,\ Me}$ = 5.4 HZ) and a three-proton doublet resonated at δ 1.33 ($J_{Me,\ 33\ \alpha}$ = 5.4 HZ). This suggested the substitution of a methyl group at C-33. For the correct identification of the methyl group at C-33 COSY 45° experiment was performed. The COSY 45° spectrum displayed vicinal coupling of C-33 methine proton (δ 3.62) with the methyl protons (δ 1.33).

The ^{13}C-NMR spectrum ($CDCl_3$, 50 MHz) also showed the resonance of all 29 carbons. Multiplicity of each carbon was established by using APT spectrum. It revealed the presence of eight methine, eight methylene, eight methyl and five quaternary carbons. Complete chemical shifts of **78** are listed in Table **6.**

The UV, ^{1}H, ^{13}C-NMR, and mass spectral data of compound **78** were distinctly identical to those of homomoenjodaramine, reported in the literature[20], and this characterized compound **78** as homomoenjodaramine. This compound has been previously isolated from *B. hyrcana*[20].

Table 6 ^{13}C- NMR chemical shift assignments of Compound **78**

Carbon No.	δ ^{13}C-NMR	†Multiplicity
1	39.1	CH_2
2	25.4	CH_2
3	70.7	CH
4	39.5	C
5	48.5	CH
6	33.0	CH_2
7	26.5	CH_2
8	49.6	CH
9	138.2	C
10	134.3	C
11	129.7	CH
12	38.4	CH_2
13	43.2	C
14	48.5	C
15	29.7	CH_2
16	26.9	CH_2
17	48.8	CH
18	17.1	CH_3
19	129.2	CH
20	64.5	CH
21	10.1	CH_3
30	13.9	CH_3
31	77.9	CH_2
32	15.8	CH_3
33	92.0	CH
N_a-CH3	38.5	CH_3
N_b-$(CH_3)_2$	34.9	CH_3
N_a-CH-$\underline{CH_3}$-O	20.8	CH_3

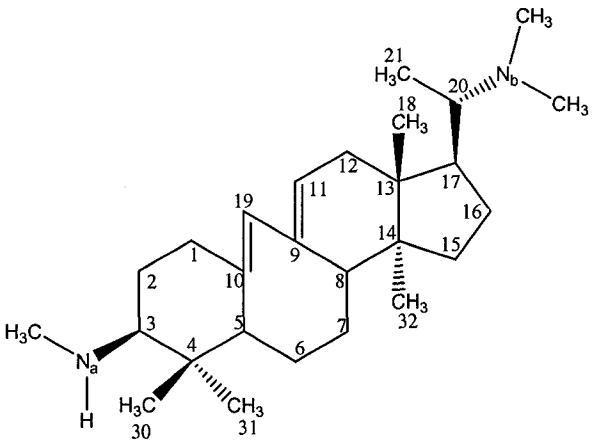
Solvent: $CDCl_3$

† Multiplicity was determined from APT experiment.

2.2.7 Buxamine-B (79)

Buxamine-B (**79**) was isolated as a colorless amorphous solid from fraction F_d, that was obtained by eluting the column with acetone-methanol (90:10). The analytical TLC analysis showed that it contains one major compound, which was purified by preparative TLC using ethylacetate-isopropanol-diethylamine (80:20:1) as the developing solvent.

The UV spectrum of buxamine-B **79** was similar to those of **77** and **78** indicating the presence of similar chromophore as described for moenjodaramine (**77**) and homomoenjodaramine (**78**).



(**79**)

The EIMS and CIMS spectra showed that compound (**79**) exhibited the molecular ion at *m/z* 398 and quasimolecular ion $[M+1]^+$ at *m/z* 399 respectively. The combination of ^{13}C-NMR and MS spectral data indicated the molecular formula of **79** as $C_{27}H_{46}N_2$. An ion at *m/z* 383 resulted due to the loss of a methyl group from the molecular ion. The EIMS of **79** showed two ions at *m/z* 43 and at *m/z* 57 due to the cleavage of ring A along with the nitrogen containing side chain at C-3. The base peak at *m/z* 72 was also observed due to the loss of trimethylliminium ion from ring D. This fragmentation pattern is found to be identical with those of reported MS data in the literature[38].

The ^{1}H-NMR spectrum ($CDCl_3$, 200 MHz) of **79** revealed the presence of four methyl groups attached to quaternary carbon atoms, which appeared as two three-proton singlets at δ 0.75 and 1.01 for C-32 and C-31 respectively. A six-proton singlet at δ 0.71 was attributed to C-18 and C-30 methyl groups. A three-proton doublet at δ 0.86 ($J_{21,20} = 6.5$ HZ) was due to the C-21 secondary methyl protons. A six-proton singlet at δ 2.24 and a three-proton singlet at δ 2.48 were attributed to the C-20 *N*, *N*-dimethyl and C-3 *N*-methyl protons respectively. A singlet at δ 5.98 was ascribed to the C-19 olefinic proton while a multiplet appeared at δ 5.51 was assigned to the C-11 olefinic proton.

The ^{13}C-NMR spectrum ($CDCl_3$, 50 MHz) of **79** showed the presence of twenty-seven carbon atoms in the molecule. The multiplicity of the carbon signals was established by using APT experiment. The olefinic carbons C-9, C-10, C-11and C-19 resonated at δ 138.5, 134.8, 128.9 and 129.1, respectively. The ^{13}C-NMR chemical shifts assignments of all the carbons with their multiplicity of this compound are shown in Table **7**.

The UV, MS, ^{1}H, and ^{13}C-NMR spectral data of **79** were found to be identical with those of buxamine-B reported in the literature [6, 22, 38, 39]. This compound has been previously isolated from *B. semipervirene*[6], *B. hyrcana*[22], *B. papillosa*[38] and *B. microphylla* [39].

Table 7 ^{13}C- NMR chemical shift assignments of Compound **79**

Carbon No	δ ^{13}C-NMR	†Multiplicity
1	38.5	CH_2
2	29.7	CH_2
3	68.3	CH
4	42.9	C
5	49.1	CH
6	25.6	CH_2
7	28.9	CH_2
8	51.3	CH
9	138.5	C
10	134.8	C
11	128.9	CH
12	40.3	CH_2
13	41.1	C
14	48.6	C
15	39.9	CH_2
16	26.9	CH_2
17	49.6	CH
18	15.7	CH_3
19	129.1	CH
20	61.6	CH
21	17.1	CH_3
30	14.7	CH_3
31	24.7	CH_2
32	14.1	CH_3
N_a-CH3	35.6	CH_3
N_b-$(CH_3)_2$	32.9 & 30.2	CH_3

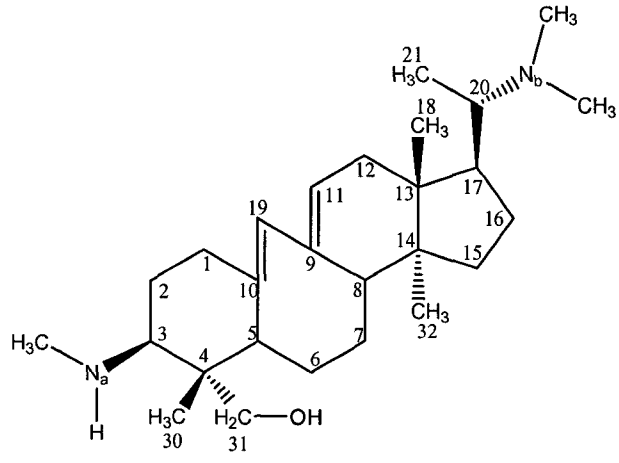
Solvent: $CDCl_3$

† Multiplicity was determined from APT experiment.

2.2.8 31-Hydroxybuxamine-B (80)

31-Hydroxybuxamine-B (**80**) was purified as a white amorphous solid from acetone-methnol (80:20 %) column chromatographic fraction F_e by preparative TLC using ethyl acetate-isopropanol-diethylamine (80:20:1) as mobile phase.

The UV spectrum of **80** was recorded that found to be similar to those of **77**, **78** and **79** indicating the presence of same chromophore in this compound.



(80)

The EIMS spectral studies were performed to deduce the molecular ion peak, which was observed at *m/z* 414. The comparison of ^{1}H-NMR and MS spectral data (fragmentation pattern) with reported literature[36] was consistent with the molecular formula $C_{27}H_{46}N_2O$. A peak at *m/z* 399 was due to the loss of a methyl group from the molecular ion. Compound (**80**) showed the base peak at *m/z* 72 which arose by cleavage of the ring D nitrogen containing side chain. The peak at *m/z* 57 was due to the cleavage of ring A along with the nitrogen containing side chain at C-3. Moreover a loss of $[M-18]^+$ at *m/z* 396 indicated the presence of alcoholic functionality in this compound.

The ^{1}H-NMR spectrum ($CDCl_3$, 200 MHz) revealed the presence of three methyl groups bonded to quaternary carbons appeared as three-proton singlets at δ 0.64, 0.70 and 0.95

for C-18, C-30 and C-32 respectively. A three-proton doublet at δ 1.18 ($J_{21,20}$ = 6.4 HZ) was due to the C-21 secondary methyl group while the C-20 methine proton appeared as a multiplet at δ 2.35. A six-proton singlet at δ 2.38 and a three-proton singlet at δ 2.29 were attributed to the *N*, *N*-dimethyl protons at C-20 and *N*-methyl protons at C-3 respectively. A singlet at δ 5.88 was ascribed to the C-19 olefinic proton while a multiplet appeared at δ 5.45 was assigned to the C-11 olefinic proton. The C-3 α-proton appeared as a multiplet at δ 3.50. A set of two AB doublets resonated at δ 3.35 and 3.75 (J_{AB} =10.4Hz) were due to the geminal methylene protons at C-31.

The COSY-45° spectrum was also recorded to confirm the ^{1}H-NMR chemical shift assignments. The C-11 olefinic proton (δ 5.45) showed COSY 45° interactions with the C-12 methylene protons (δ 1.84 and 2.0). The C-19 methine proton (δ 5.88) exhibited allylic interactions with the C-5 (δ 2.10) methine proton. Moreover, C-5 methine proton showed vicinal couplings with the C-6 (δ 1.36 and 1.68) which in turn showed cross peak with the C-7 (δ 1.98 and 1.45) methylene protons. The later displayed cross peaks with C-8 (δ 2.35) methine proton. A set of two AB doublets appeared at δ 3.35 and 3.75 were due to the geminal methylene protons at C-31 also showed cross peak with each other.

The EIMS, ^{1}H-NMR and two-dimensional ^{1}H-^{1}H COSY spectroscopic techniques were very informative regarding the presence and location of the hydroxyl group at C-31. As described above mass spectrum showed a characteristic loss of M^+-18 at *m/z* 396 indicating the presence of hydroxyl group. A deshielded two AB doublets for the C-31 methylene protons at δ 3.35 and 3.75 (J_{AB} = 10.4 Hz) further suggested the presence of the hydroxyl group. The absence of one three-proton signal for the two-methyl groups in the ^{1}H-NMR spectrum, which are normally substituted at C-4 in *Buxus* alkaloids,

confirmed the presence of hydroxyl methylene group at C-4 (this was indicative of the existence of hydroxyl group at C-31). It has been reported earlier that α-methyl substituted at C-4 under goes oxidation preferably compared with β-methyl, therefore α-stereochemistry was assigned to hydroxymethylene substituted at C-4[40, 41]. Based on the above spectral data (UV, Mass, ^{1}H-NMR, ^{1}H-^{1}H COSY) and its comparison with those reported in the literature[36], compound (**80**) was identified as 31-hydroxybuxamine-B. This is the first report of **80** from *B. hyrcana* although it has been previously reported from *B. hildebrandtii*[36].

2.3 Bioactivity of the chemical constituents of *Buxus hyrcana*

Enzyme inhibition is an important area of pharmaceutical research that has led to the discovery of a wide variety of drugs used to cure various ailments. Specific inhibitors interact with enzymes to block or decrease their activity towards their corresponding natural substrates. The importance of enzyme inhibitors as drugs is enormous since these molecules have been used for treating a number of physiological conditions. Because of the manifold of traditional activities of the plant *Buxus hyrcana*, we screened its constituents for new biological activities. The constituents from this plant were found to be active against acetylcholinesterease (AChE) and glutathione *S*-transferase (GST) enzymes. All of the compounds isolated from *B. hyrcana* showed AChE inhibitory activities. Compounds **74** to **80** were not tested for GST inhibitory activities due to their limited quantities. The IC_{50} values of all compounds are shown in Table **8**.

Table 8 AChE and GST inhibitory activities (IC_{50} = μM±SEM) of compounds **73** to **80**

Compounds	**AChE ± SEM**	**GST ± SEM**
Arbora-1,9(11)-dien-3-one **(73)**	47.89 ±1.87	74.23 ± 1.17
Cyclobuxoviridine **(74)**	179.68 ± 1.65	---
E-buxenone **(75)**	71.04 ± 2.90	---
Z-buxenone **(76)**	87.38 ± 2.40	---
Moenjodaramine **(77)**	57.88 ± 1.93	---
Homomoenjodaramine **(78)**	19.46 ± 0.34	---
Buxamine-B **(79)**	79.68 ± 1.34	---
31-hydroxybuxamine-B **(80)**	61.27 ± 0.67	---

SEM = Standard error of mean of three assays

The steroidal alkaloid, homomoenjodaramine (**78**) displayed the best AChE inhibitory activity while its structural analog moenjodaramine (**77**) showed 2-fold less anti-AChE activity which may be due to the absence of methyl group at C-33. The structural analysis and anti-AChE activity data of all of these purified compounds indicated that the presence of amino group at C-20 is not important for enzyme inhibition. However the presence of amino functionality at C-3 of these types of compounds seems necessary for the better interaction of enzyme and compounds. This hypothesis is evident from the anti-AChE activities of *E*-buxenone (**75**) (IC_{50} 71.04 μM) and *Z*-buxenone (**76**) (IC_{50} 87.38 μM) as both have amino group at C-3, where as cyclobuxoviridine (**74**) (IC_{50} 179.68 μM) has relatively 3-fold lower anti-AChE activity, which may be due to the absence of amino group at C-3. The anti-AChE activity of all of these compounds was very weak compared with reported AChE inhibitory activities of presently used AChE inhibitors like eserine (IC_{50} 0.041 μM), tacrine (IC_{50} 0.021 μM) and galanthamine (IC_{50} 0.45 μM) for the treatment of AD[42]. The pentacyclic triterpenoidal compound, Arbora-1,9(11)-dien-3-one (**73**) was also assayed for GST inhibitory activity (IC_{50} 74.23 μM). This GST inhibitory activity was excellent compared with standard triterpene sodium taurocholate (IC_{50} 398 μM) [43] and found to be weak compared with previously used chemosensitizer, ethacrynic acid (IC_{50} 16.0 μM) [44].

2.4 Discussion

Buxus hyrcana produces steroidal bases as revealed by the results obtained from previous phytochemical studies by our research group[9]. A few of the isolated compounds showed acetylcholinestrase (AChE) inhibition activity. It was decided to perform phytochemical studies on the crude extract (350gm) in order to isolate more steroidal alkaloids and to evaluate them for AChE inhibitory activity. These studies resulted in the isolation of eight more triterpenoids: arbora-1,9(11)-dien-3-one (**73**), cyclobuxoviridine (**74**), *E*-buxenone (**75**), *Z*-buxenone (**76**), moenjodaramine (**77**), homomoenjodaramine (**78**), buxamine-B (**79**), and 31-hydroxybuxamine-B (**80**). Quantities of each and their % yields are listed in Table **9**.

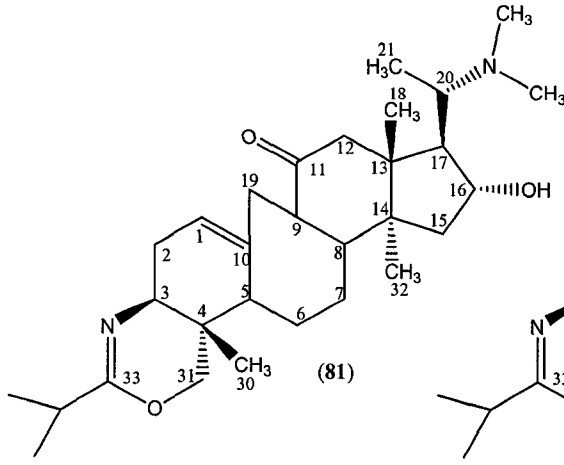
Table 9 Yields of eight triterpenoids isolated from *B. hyrcana*

Compound	Yield (mg)	% Yield
102	6.0	0.0017
103	3.2	0.0009
104	5.5	0.0015
105	3.2	0.0009
106	2.4	0.0007
107	3.3	0.0009
108	3.6	0.0010
109	1.5	0.0004

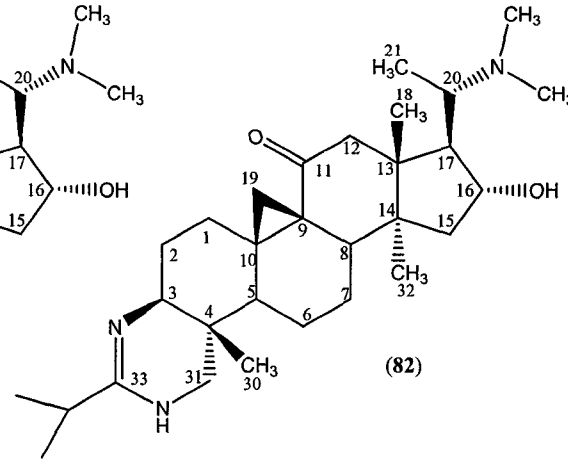
During the isolation procedures, analytical and preparative TLC were used extensively to establish the proper solvent system in order to purify these compounds. By performing analytical TLC, this might have contributed to a decreased final yield of these

compounds. It can therefore be concluded that to achieve an acceptable yield of these compounds, a proper HPLC/LC-MS method needs to be developed for the purification of these alkaloids. However, the R_f values and %yield calculated during these studies will help in isolating these compounds for future works.

Many plants of the family Buxaceae have been explored for the identification of acetylcholinestrase inhibitors[45]. Buxaminol-E, a pregnane type steroidal alkaloid from *B. sempervirens* has been reported as a good inhibitor of the enzyme[46]. Inhibition of AChE is considered as a potential target for the treatment of Alzheimer's disease along with possible therapeutic use in the treatment of Parkinson's disease, ageing and myasthenia gravis[47]. In the light of this literature evidence, it was decided that all the purified compounds from *B. hyrcana* should be investigated for anti-AChE effect in order to evaluate them for their medicinal importance. The results of these anti-AChE studies are summarized in Table **8**. All compounds (**73-80**) displayed AChE inhibitory activity in a dose-dependent manner. Homomoenjodaramine (**78**) showed excellent anti-AChE activity compared to other isolates (**73-77, 79-80**). This may be due to the presence of tetrahydrooxazine ring at C-3/C-4. This hypothesis seems true from the recent investigations of its structural analogs (**81-82**) which displayed AChE inhibitory activities with IC_{50} value of 0.029 and 0.018μM, respectively[48]. In the study it was also investigated that steroidal alkaloid having oxazine and pyrimidines moieties at C-3/C-4 positions are more active AChE inhibitors. Furthermore the presence of cyclopropane ring also increases the AChE inhibition activity in the oxazine and pyrimidine series.



(81)



(82)

In addition to this substitutent at C-33 in oxazines can also effect the inhibition of AChE. Since in case of homomoenjodaramine **(78)**, cyclopropane ring is absent while in case of moenjodaramine (**77**) cyclopropane ring as well as substitutent at C-33 both are absent, that may be the reason of its inefficient interaction with AChE, as a result has weaker AChE inhibitory effect. Galanthamine (reminyl) is an AChE inhibitor and is used to treat the symptoms of mild to moderate AD. The concentration of the inhibitor yielding 50 % inhibition of enzyme activity i.e. IC_{50} of galanthamine has been documented as 0.45μM *in vitro*[42]. In the present study AChE inhibitory data compared with galanthamine, homomoenjodaramine **(78)** displayed moderate inhibitory activity of AChE where as moenjodaramine (**77**) and all other purified compounds from *B. hyrcana* showed weaker inhibitory activity. The structural comparison of rest of the alkaloids (**74-76, 79-80**) with respect to their anti-AChE activity showed that secondary or tertiary amino group at C-3 is important for the enzyme inhibitory activity. This is also evident from the inhibitory activity of cyclobuxoviridine (**74**), which is about 2 to 3-fold less active compared to **75**, **76**, **79** and **80** due to the absence of amino group functionality at C-3.

Arbora-1,9(11)-dien-3-one (**73**) is a pentacyclic triterpene which showed anti-AChE activity with IC_{50} value of 47.89 μM. Based on intensive literature search, it is the first report from *B. hyrcana.* It needs more experimentation in order to investigate the reason of its relatively better anti-AChE activity. Arbora-1,9(11)-dien-3-one (**73**) was also assayed for glutathione *S*-transferase inhibitory activity (IC_{50} 74.23μM). This enzyme inhibitory activity was about 4-fold higher compared with standard steroidal inhibitor sodium taurocholate (IC_{50} 398 μM) [43]. This GST inhibitory activity of **73** possibly due to Michael type addition reaction on the endocyclic methylene group and resultantly formation of GSH adduct during the assay period.

Biogenetically non-nitrogenous and nitrogenous triterpenes are synthesized by plants. Natural synthesis of all terpenoids is based on the isoprene rule, which was proposed by Ruzica in 1938 [49, 50]. Isoprene (2-methyl buta-1,3-diene) is a monomeric unit which is formed by acetyl coenzyme-A as biogenetic precursor. Biochemically isoprene units are actually diphosphate esters termed as isopentenyl pyrophosphate (IPP) and dimethyl allyl pyrophosphate (DMAPP) as shown n Figure **6**.

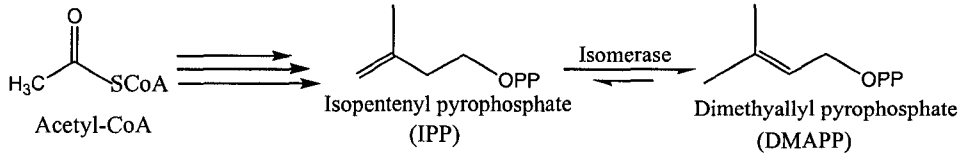


Figure 6 Formation of IPP from acetyl-CoA through mevalonate parthway.

These biochemically active isoprene units condense in a head to tail fashion to form pre-squalene which undergoes rearrangement to form squalene as a result of different catalytic actions of the squalene synthase enzyme. The cyclization of squalene is promoted by either oxidative or non-oxidative agents resulting in either lanosterol or cycloartenol type triterpenes. Lanosterol is distributed in vertebrates where as

cycloartenol is widespread in higher plants, serving as a biosynthetic precursor in the biosynthesis of triterpenoids[51].

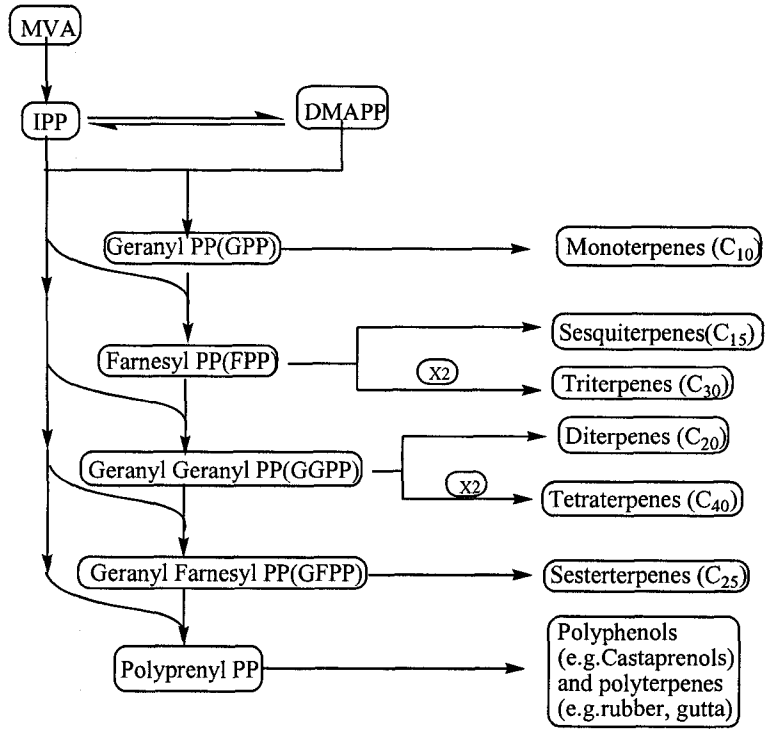


Figure 7 General biosynthesis of terpenoids; DMAPP = Dimethylallyl pyrophosphate, PP = Pyrophosphate, X2 = Dimerization

Buxus alkaloids are considered to be the derivatives of cycloartenol[2, 52, 53]. Though the mechanism for this has not yet been proven experimentally, one hypothesis asserts that this transformation takes place with 20-ketosteroid intermediates by oxidative cleavage of the C-20 side chain. In addition to this, oxidation of C-3 hydroxyl group may lead to the formation of 3,20-diketosteroids. Thus the *Buxus* alkaloids may have been derived by the reductive amination of mono or diketosteroids (Figure **8**). This hypothesis is also supported by the fact that most of the aminosteroids, 20-amino-3-ketosteroids, 3-amino-20-ketosteroids and 3,20-diaminosteroids have been reported from plants[2, 54].

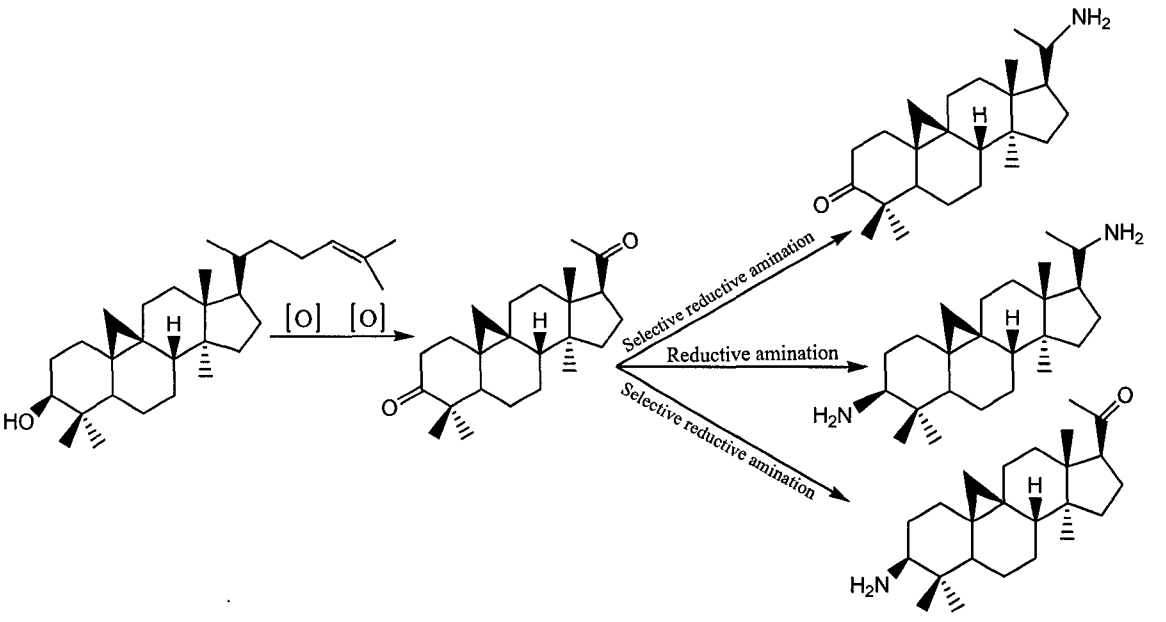


Figure 8 Biosynthesis of mono and diamino-ketosteroids.

In this way, *Buxus* alkaloids with a cyclopropane ring may directly form from cycloartenol and most of the structural diversity of *Buxus* alkaloids may arise due to the opening of cyclopropane ring. Herlem-Gaulier *et al* [53] proposed a possible mechanism for the generation of *abeo* 9(10→19)-diene system (conjugated or non-conjugated) by a decyclization processes (Figure **9**).

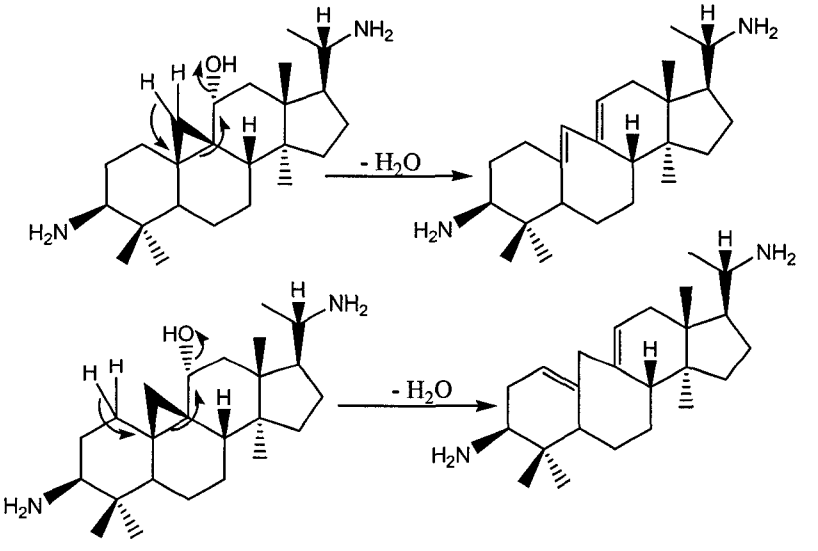


Figure 9 Biosynthesis of *abeo* 9(10→19)-diene conjugated and non-conjugated system

A characteristics feature of some Buxus alkaloids is the presence of a tetrahydrooxazine or methyl substituted tetrahydrooxazine ring[55], which may proceed in nature by the condensation of formaldehyde or acetaldehyde with the C-3 amino group. The attack of C-4 α-hydroxymethylene group[41] on the corresponding ketimine can yield tetrahydrooxazine ring. This process is summarized in Figure **10**.

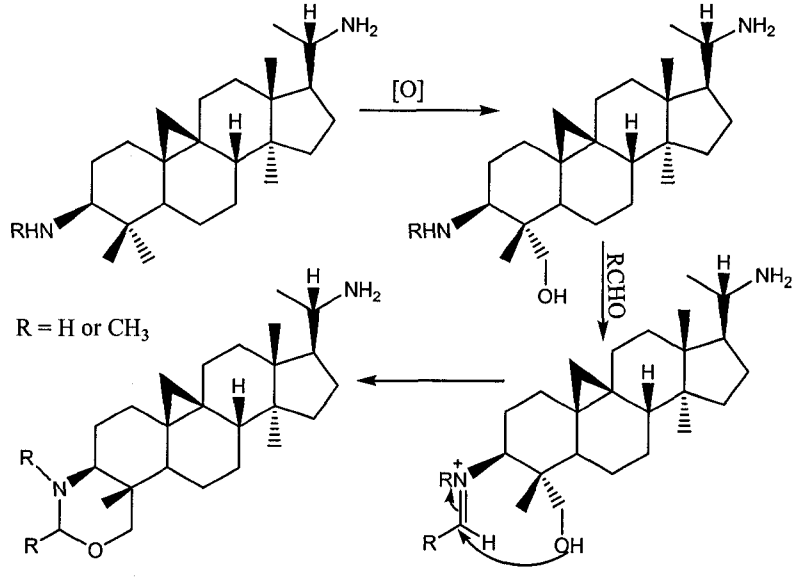


Figure 10 Biosynthesis of *Buxus* alkaloids having tetrahydrooxazine ring.

2.5 EXPERIMENTAL

2.5.1 General experimental conditions

2.5.1.1 Spectroscopy

Ultra violet (UV) spectra were recorded in methanol, dichloromethane and tetrahydrofurane (THF) on a Shimadzu UV-VIS-240 spectrophotometer. Infrared (IR) spectra were recorded on Michelson Bomem Hartmann and Braun-MB-series spectrophotometer in KBr. EIMS and CIMS data were obtained on a Hewlett Packard 5970 series II (mass selective detector) spectrometer using direct insertion probe method. Proton magnetic resonance (^{1}H-NMR) spectra were recorded in $CDCl_3$ on a Varian spectrometer at 200 MHz. The ^{13}C-NMR spectra were recorded at 50 MHz on the same instrument. All 2D-NMR spectra were recorded in $CDCl_3$ on Bruker 300 MHz at the University of Manitoba. Chemical shifts were recorded in (δ), ppm and referenced to solvents.

2.5.1.2 Chromatography

Column chromatography was carried out on silica gel (200-400 mesh type-60A°) purchased from Sigma-Aldrich. The purity of samples was checked on precoated silica gel GF 254 alumina backed plates (20 X 20 cm) (Merck Kieselgel).

2.5.1.3 Detection of triterpenes and alkaloids on TLC plates

An ultra violet lamp of wavelength 254 and 366 nm was used to view compounds. Triterpenes were visualized with the help of 10% H_2SO_4 spraying agent. The alkaloids were detected with the help of Dragendorff's spraying reagent. The alkaloids gave positive Dragendorff's test with orange-red colored spots. All ACS grade solvents

(methanol, ethyl acetate, dichloromethane, chloroform, hexane, petroleum ether and THF) were purchased from Fisher Scientific.

2.5.2 Plant material

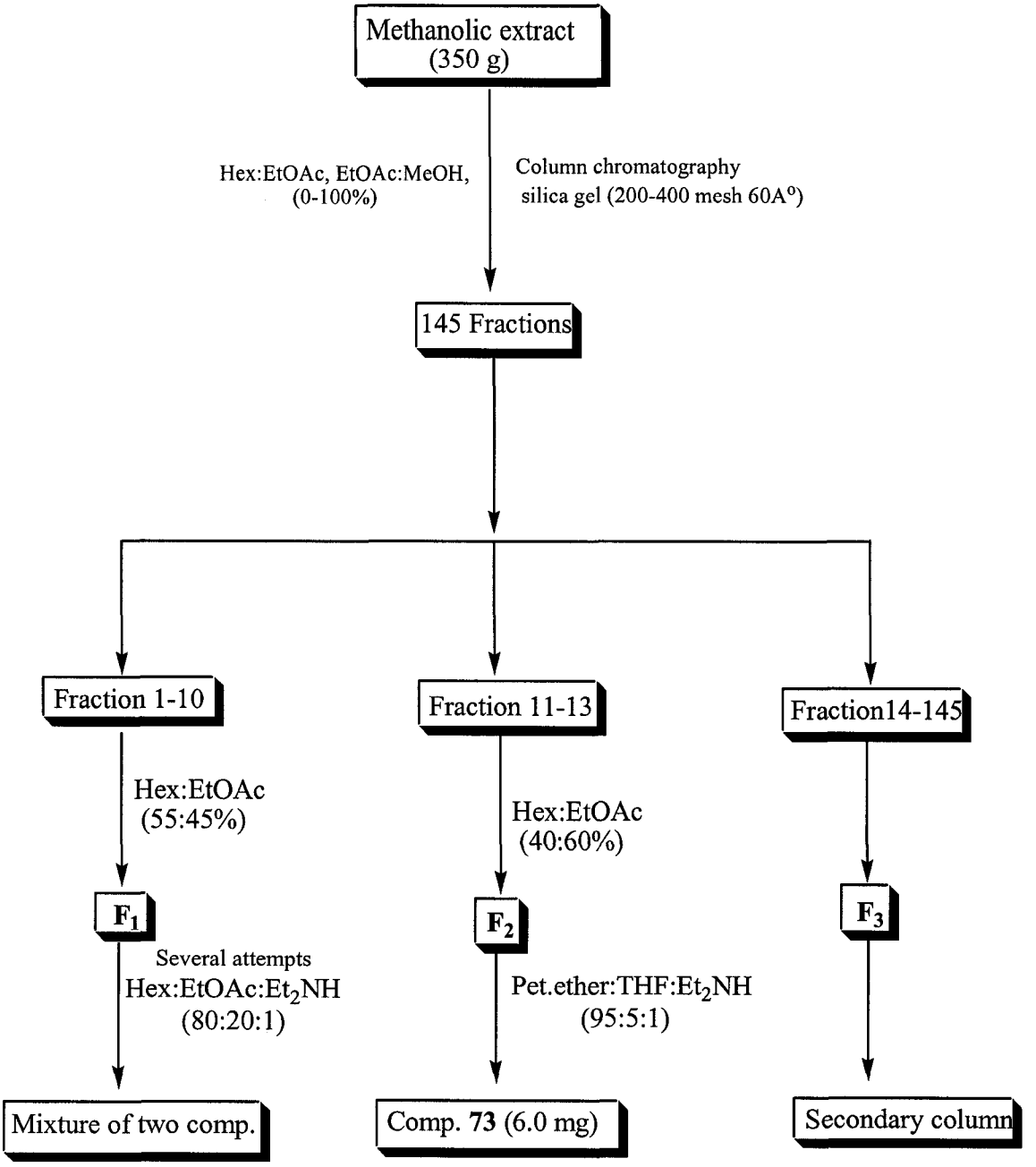
Dr. M. H. Meshkatalsadat, Department of Chemistry, University of Lorestan, Iran, collected the leaves of *B. hyrcana*. The plant was identified by Dr. Jahad Sazandegi, Mazandaran State, Iran, and a voucher specimen (No. B-530) was deposited in the herbarium of the Shaheed Beheshti University, Tehran, Iran. The crude methanolic extract of the leaves of *B. hyrcana* was shipped to us under a collaborative program between Department of Chemistry, University of Lorestan, Iran and the Department of Chemistry, University of Winnipeg, Manitoba, Canada.

2.5.3 Extraction and Isolation

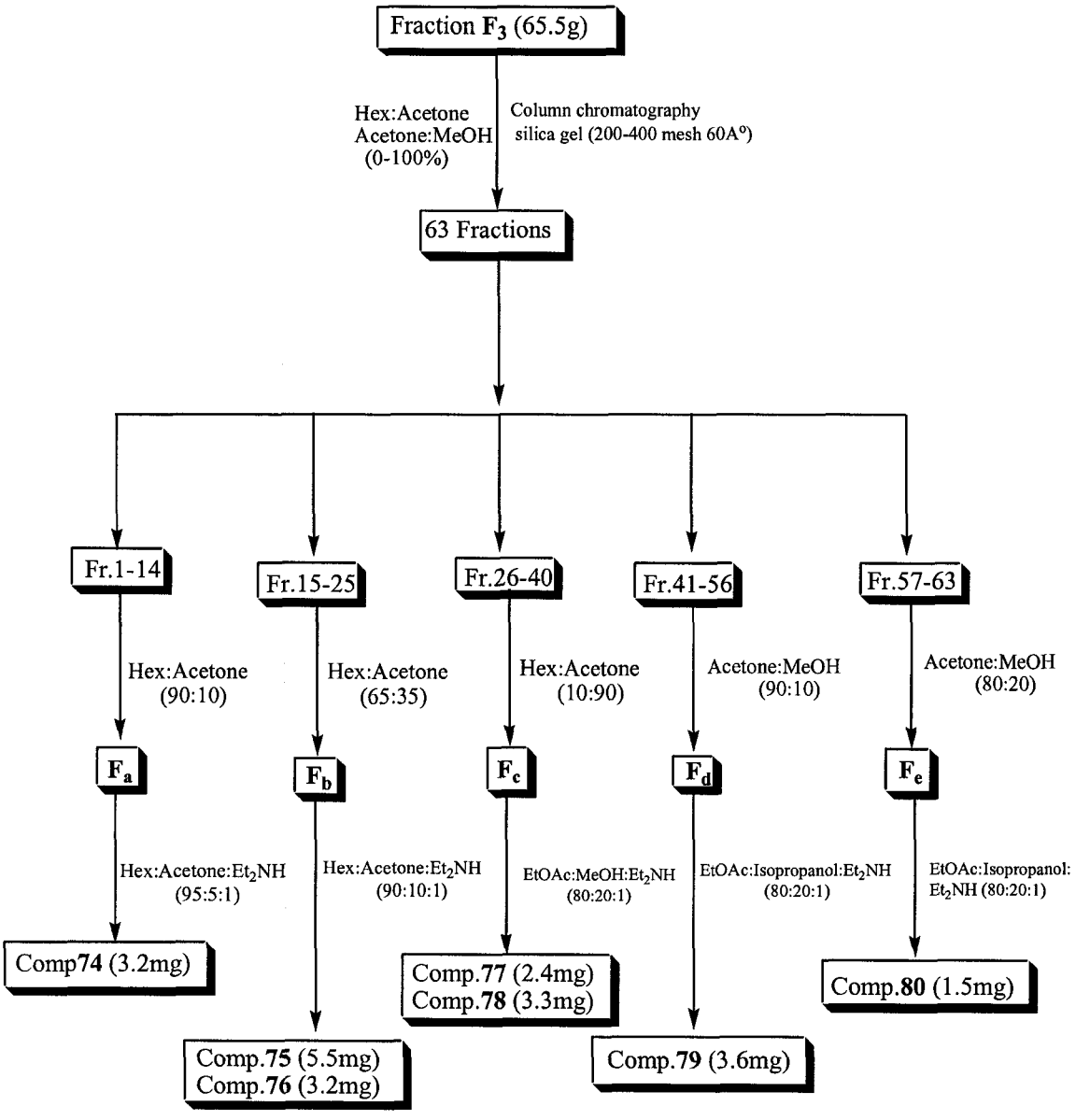
The crude methanolic extract (350 g) of *B. hyrcana*, was loaded onto a silica gel column. The column was eluted with *n*-hexane-ethyl acetate (0-100%), ethyl acetate-methanol (0-100%) to afford one hundred and forty five fractions. Similar fractions were combined on the bases of same R_f values. This gave three main fractions F_1, F_2, F_3. Fractions F_1 contained a mixture of two compounds revealed by analytical TLC, but several attempts to purify this mixture were not successful. The purification of fractions F_2 by preparative TLC using pet.ether: THF: Et_2NH (95:5:1) as mobil phase yielded one non-nitrogenous triterpene arbora-1,9(11)-dien-3-one (**73**) (6.0 mg, 0.0017 % yield, R_f 0.58). This non-nitrogenous triterpene has been isolated for the first time from *B. hyrcana* (scheme # **1**). Impure fraction F_3 (65.5g) from primary column was again fractioned by gradient elution of silica gel column. The column was eluted with *n*-hexane-acetone (0-100%), acetone-methanol (0-100%), to afford sixty-three fractions, which were again concentrated and

pooled together based on similar R_f values. It yielded five sub-fractions F_a to F_e which after successive column chromatography and preprative TLC yielded seven steroidal alkaloids, cyclobuxoviridine (**74**), *E*-buxenone (**75**), *Z*-buxenone (**76**), moenjodaramine (**77**), homomoenjodaramine (**78**), buxamine-B (**79**), 31-hydroxybuxamine-B (**80**), isolated from the leaves of *B. hyrcana*.

Compound (**74**) (3.2 mg, 0.0009 % yield, R_f = 0.72) was isolated as white amorphous solid from secondary column chromatographic fraction F_a by using Hex: Acetone: Et_2NH (95:5:1) as mobile phase. Compound (**75**) (5.5 mg, 0.0015 % yield, R_f = 0.30) and Compound (**76**) (3.2 mg, 0.0009 % yield, R_f = 0.42) were isolated as colorless gummy materials from 2° CC fractions F_b by subjecting on preparative TLC using Hex: Acetone: Et_2NH (90:10:1). Preparative TLC on 2° CC fraction F_c that was obtained on elution with Hex: Acetone (10:90) using EtOAc: MeOH: Et_2NH (80:20:1) yielded two compounds (**77**) (2.4 mg, 0.0007 % yield, R_f = 0.73) and compound (**78**) (3.3 mg, 0.0009 % yield, R_f = 0.49) as white crystalline solid. Compound (**79**) (3.6 mg, 0.001 % yield, R_f = 0.77) was purified from fraction F_d as colorless amorphous solid by using EtOAc: Isopropanol: Et_2NH (80:20:1). The last compound (**80**) (1.5 mg, 0.0004 % yield, R_f = 0.23) was also isolated from 2° CC fraction F_e (Acetone: MeOH) (80:20) that was subsequently purified by preparative TLC by using the same solvent system as used for compound (**79**) (scheme # **2**).



Scheme 1 Isolation procedure for compound **73**



Scheme 2 Isolation procedure for compounds **74-80**

2.5.4 Spectral data of arbora-1,9(11)-dien-3-one (73)

White amorphous solid

UV λ_{max} (CH_2Cl_2): 228 nm

^{1}H-NMR ($CDCl_3$, 300 MHz) (δppm): See Table-2

^{13}C-NMR ($CDCl_3$, 50 MHz) (δppm): See Table-2

EIMS ***m/z*** (rel. int. %): 422 $[M]^+$ (6), 407 $[M-CH_3]^+$ (6), 218 (8), 204 (12), 69 (100).

2.5.5 Spectral data of cyclobuxoviridine (74)

White amorphous solid

UV λ_{max} (MeOH): 268 nm

^{1}H-NMR ($CDCl_3$, 200 MHz) (δppm): δ0.75 and 0.81(2H, dd, $J_{19\alpha,\ 19\beta}$ = 5.07Hz, C-19 CH_2), 0.92 (3H, s , 18-Me), 1.09 (3H, s, 31-Me), 1.26 (6H, s, 30 and 32-Me), 0.99 (3H, d, $J_{21,\ 20}$ = 6.0 Hz, 21-Me), 2.50 (6H, s, *N*-$(Me)_2$), 2.05 (1H, m, $J_{21,\ 20}$= 6.0 Hz, $J_{20,\ 17}$ = 10.2 Hz, H-20), 5.94(1H, d, $J_{2,\ 1}$= 10.1Hz, H-2), 6.75 (1H, d, $J_{1,2}$ = 10.1Hz, H-1).

^{13}C-NMR ($CDCl_3$, 50 MHz) (δppm): See Table-3

EIMS ***m/z*** (rel. int. %): 383 $[M]^+$ (19), 368 $[M-CH_3]^+$ (12), 354 (20), 339 (14), 312 (12), 85 (20), 72 (100).

2.5.6 Spectral data of *E*-buxenone (75)

Colorless gummy material

UV λ_{max} (MeOH): 243 nm

^{1}H-NMR ($CDCl_3$, 200 MHz) (δppm): δ 0.42 (1H, d, $J_{19\alpha,\ 19\beta}$ = 4.5 Hz, H-19α), 0.63 (1H, d, $J_{19\ \beta,\ 19\ \alpha}$ = 4.5 Hz, H-19 β), 0.93 (3H, s , 18-Me), 1.10 (3H, s, 30-Me), 1.26 (3H, s, 31-

Me), 1.32 (3H, s, 32-Me), 1.82 (3H, d, $J_{21,20}$ = 7.4 Hz, 21-Me), 2.61 (3H, s, *N*-Me), 3.67 (1H, m, H-3), 6.55 (1H, q, , $J_{21,20}$ = 7.4 Hz, H-20).

^{13}C-NMR ($CDCl_3$, 50 MHz) (δppm): See Table-4

EIMS *m/z* (rel. int. %): 369 $[M]^+$ (25), 354 $[M\text{-}CH_3]^+$ (20), 83 (30), 71 (45), 57 (100).

2.5.7 Spectral data of *Z*-buxenone (76)

Colorless gummy material

UV λ_{max} (MeOH): λ_{max} 236, 243 nm

^{1}H-NMR ($CDCl_3$, 200 MHz) (δppm): δ 0.38 (1H, d, $J_{19\alpha,\ 19\beta}$ = 4.5 Hz, H-19α), 0.64 (1H, d, $J_{19\beta,\ 19\alpha}$ = 4.5 Hz, H-19 β), 0.80 (3H, s , 18-Me), 0.87 (3H, s, 30-Me), 0.92 (3H, s, 31-Me), 0.99 (3H, s, 32-Me), 2.05 (3H, d, $J_{21,20}$ = 7.50 Hz, 21-Me), 2.45 (3H, s, N-Me), 3.65 (1H, m, H-3), 5.65 (1H, q, , $J_{21,20}$ = 7.50 Hz, H-20).

EIMS *m/z* (rel. int. %): 369 $[M]^+$ (42), 354 $[M\text{-}CH_3]^+$ (35), 83 (30), 71 (45), 57 (100).

2.5.8 Spectral data of moenjodaramine (77)

White crystalline solid

UV (MeOH): λ_{max} 236, 245 nm, λ_{min} 203, 254 nm.

^{1}H-NMR ($CDCl_3$, 200 MHz) (δppm): 0.70 (3H, s , 18-Me), 0.74 (3H, s, 32-Me), 1.01 (3H, s, 30-Me), 0.86 (3H, d, $J_{21,20}$ = 6.0 Hz, 21-Me), 2.1 (3H, s, N_a-Me), 2.22 (6H, s, N_b-$(Me)_2$, 3.24, 3.82 (2H, dd, ($J_{31\alpha\ ,31\ \beta}$ = 10.4 Hz, H-31), 3.57, 4.42 (2H,dd, ($J_{33\alpha\ ,33\ \beta}$ = 7.4 Hz, H-33), 5.54 (1H, m, H-11), 5.98 (1H, s, H-19).

^{13}C-NMR ($CDCl_3$, 50 MHz) (δppm): See Table-5

EIMS *m/z* (rel. int. %): 426 $[M]^+$ (1.05), 411 $[M\text{-}CH_3]^+$ (2), 85 (15), 72 (70), 71 (45), 58 (30), 57(100).

2.5.9 Spectral data of homomoenjodaramine (78)

White crystalline solid

UV (MeOH): λ_{max} 238, 245 nm,

^{1}H-NMR ($CDCl_3$, 200 MHz) (δppm): 0.70 (3H, s , 18-Me), 0.76 (3H, s, 32-Me), 1.02 (3H, s, 30-Me), 0.95 (3H, d, $J_{21,20}$=6.4 Hz, 21-Me), 2.14 (3H, s, N_a-Me), 2.34 (6H, s, N_b $(Me)_2$, 3.32, 3.81 (2H, dd, ($J_{31\alpha\,,31\,\beta}$ =10.4 Hz, H-31), 3.62, (1H,q, ($J_{33\alpha\,,CH3}$ = 5.4 Hz, H-33), 1.33 (3H, d, $J_{CH3,33\alpha}$ = 5.4 Hz), 5.54 (1H, m, H-11), 5.98 (1H, s, H-19).

^{13}C-NMR ($CDCl_3$, 50 MHz) (δppm): See Table-6

EIMS ***m/z*** (rel. int. %): 440 $[M]^+$ (6), 425 $[M\text{-}CH_3]^+$ (5), 141(9), 85 (45), 72 (100), 58 (84).

2.5.10 Spectral data of buxamine-B (79)

Colorless amorphous solid

UV (MeOH): λ_{max} 237, 244 nm,

^{1}H-NMR ($CDCl_3$, 200 MHz) (δppm): 0.75 (3H, s , 32-Me), 1.01 (3H, s, 31-Me), 0.71 (6H, s, C-18 and 30-Me), 0.86 (3H, d, $J_{21,20}$ = 6.5 Hz, 21-Me), 2.48 (3H, s, N_a-Me), 2.24 (6H, s, N_b-$(Me)_2$, 5.51 (1H, m, H-11), 5.98 (1H, s, H-19).

^{13}C-NMR ($CDCl_3$, 50 MHz) (δppm): See Table-7

EIMS ***m/z*** (rel. int. %): 398 $[M]^+$ (1.5), 425 $[M\text{-}CH_3]^+$ (1.5), 85 (14), 72 (100), 58 (41).

2.5.11 Spectral data of 31-hydroxybuxamine-B (80)

White amorphous solid

UV (MeOH): λ_{max} 237, 244 nm,

^{1}H-NMR ($CDCl_3$, 200 MHz) (δppm): 0.64 (3H, s, 18-Me), 0.70 (3H, s, 31-Me), 0.95 (3H, s, 32-Me), 1.18 (3H, d, $J_{21,20}$ = 6.4 Hz, 21-Me), 2.29 (3H, s, N_a-Me), 2.38 (6H, s, N_b-$(Me)_2$, 5.45 (1H, m, H-11), 5.88 (1H, s, H-19)l, 3.50 (1H, m, H-3), 3.35, 3.75 (2H, dd, ($J_{30\alpha,30\beta}$ =10.4 Hz, H-30).

EIMS *m/z* (rel. int. %): 414 $[M]^+$ (15), 399 $[M\text{-}CH_3]^+$ (3), 129 (7), 115 (5), 85 (15), 72 (100), 58 (61).

2.6 Enzyme Inhibition Assays

2.6.1 Acetylcholinesterease Assay

Acetylcholinesterease inhibition was determined spectrophotometrically by using acetylthiocholine as substrate with slight modifications in the Ellman's method[56]. The reaction was carried in 100 mM sodium phosphate buffer at pH 7.8 at room temperature. In a typical assay, 126μL buffer, 50μL of 0.01M DTNB [5-5-dithio-bis (2-nitrobenzoic acid)], 2μL enzyme and 2μL test compound solution were mixed and incubated for 30 minutes. The reaction was then initiated by the addition of 20μL of 0.075M acetylthiocholine. Hydrolysis of acetylthiocholine was monitored by the formation of yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine at a wavelength of 406 nm. All assays were carried out in triplicate in 96-well microplate on KC-4 Bio-TEK microplate reader.

2.6.2 Glutathione *S*-Transferase Inhibition Assay

The inhibitory activity of GST was measured by following the Habig spectrophotometer method[57]. The assay was carried out after incubating the specific concentrations of the compounds with the enzyme at 25 °C for 10 minute. The final assay mixture contained

5mM GSH, 1mM CDNB, 100mM phosphate buffer (pH 6.5) in a 200μL solution and GST (with initial effective assay activity of 0.12106 UmL^{-1}). The assay measured the activity of GST in conjugating CDNB to GSH, and the product of conjugation was measured at 340 nm using KC-4 Bio-TEK microplate reader. The inhibitory activity of GST was calculated with reference to a control assay. Under these reaction conditions, basal coupling between the substrates was found to be insignificant. All assays were carried out in triplicates.

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CHAPTER 3

3.0 Phytochemical studies on the aerial parts of *Barleria prionitis*

3.1 Introduction

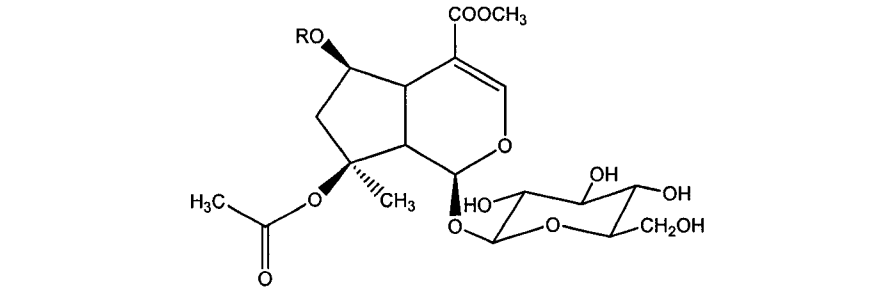
Barleria prionitis Linn is a member of the family Acanthaceae. It is commonly referred to as "Vajradanti" in Hindi, and "Karunta" in Sanskrit, and is abundant in tropical Asia, Africa, India, and Pacific[1,2].

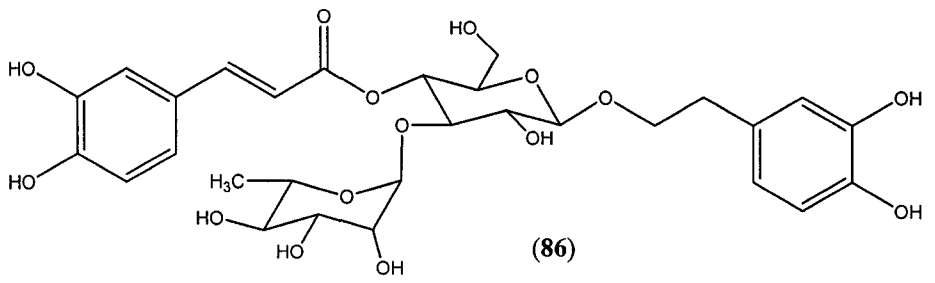
B. prionitis is an annual, prickly shrub that grows to approximately 1-3 feet high. Its brown and smooth branches are covered with oval shaped leaves with pointed tips ending in a short spine. The plant is armed with 5-20 mm long spines in the leaf axils. It produces flowers in the early dry season. The yellow, tubular flowers, 3 to 4 cm long with long, projecting stamens occur in an upright spike at the top of the plant[3].

B. prionitis is well known for various pharmaceutical applications in folk medicine of Asia[4]. For example, in Thailand, the extracts of its leaves and roots are taken orally to cure fever. The crude plant extract has been reported to exhibit antiviral activity[5]. In India, different parts of this plant are used for different purposes in traditional Indian medicine. For example, the hot aqueous extracts of leaves are used to promote healing of unhealthy wounds and to relieve joint pains and toothache[6]. Hot aqueous extracts of leaves and flowers are used to treat fever[7] while the green shoots of this plant are taken orally to treat whooping cough and asthma in infants and children[8]. Additionally, a hot-water extract of the dried leaves and roots of *B. cristata* is used orally to cure bronchitis and coughs[9,10]. The respiratory syncytial virus (RSV) infection of the lungs is one of the most common causes of "asthma-like" symptoms in infants. This indicates that the above mentioned hot aqueous extracts may have the ability to destroy viruses. Moreover, the

extracts of whole plant have also been reported to suppress the growth of the fungi, *Trichophyton mentagrophytes*, *in vitro*[11]. The aqueous bioactive fractions of *B. prionitis* have been reported to contain hepatoprotective, anti-stress and immunorestorative properties[12]. This plant has also been reported as an anti-arthritic, anti-inflammatory [13] and anti-fertility agent[14].

Previous chemical studies on *B. prionitis* resulted in the isolation of iridoids, iridoid glycosides and unsaponified materials [2,13, 15-18.] The iridoid glycosides **83**, **84**, barlerin **85**, and verbascoside **86,** from the whole-plant extracts of *B. prionitis* have been reported to exhibit potent activity against respiratory syncytial virus *in vitro*[19]. The antiviral activity of these pure compounds isolated from the genus *Barleria* provides a complete justification for the traditional use of different species of this genus to cure infectious diseases.





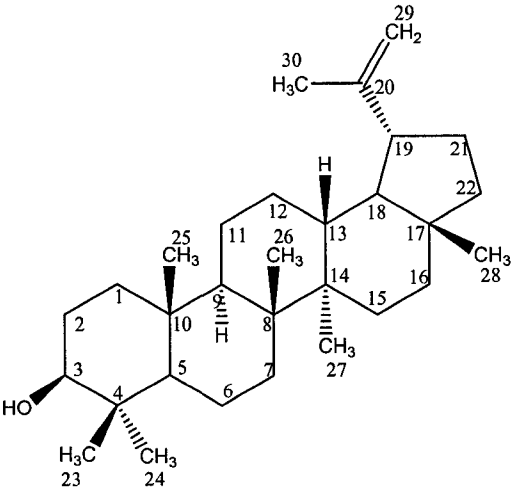
(86)

Considering the aforementioned medicinal importance of this plant, it was decided to isolate natural products from the ethanolic extract of *B. prionitis* and to evaluate them for AChE and GST inhibitory activities. These studies resulted in the isolation of a triterpenoidal natural product, lupeol (**87**). Compound **87** was isolated in a quantity sufficient to prepare three derivatives and evaluate them for GST and AChE inhibitory activities. It was decided to synthesize 3-acetyl-lupeol (**88**), 20-29 epoxylupeol (**89**), and 29-amino-20-hydroxylupeol (**90**) by using compound (**87**) as a synthetic precursor. Their structures were elucidated with the help of NMR spectroscopic studies.

3.2 Results

3.2.1 Lup-20(29)-ene-3β-ol (Lupeol) (87)

Phytochemical investigations on the ethanolic extract of *B. prionitis* have resulted in the isolation of a pentacyclic triterpenoid lupeol (**87**), as a white crystalline solid (see experimental section for detailed isolation procedure).



(87)

The UV spectrum of compound **87** displayed terminal absorption (λ_{max} 227 nm), indicating the absence of a conjugated π system. The IR spectrum (KBr) revealed intense absorptions at 3420 (OH), 2944 (C-H), and at 3065, 1637, 880 cm^{-1} for a terminal double bond[20].

The molecular formula, $C_{30}H_{50}O$, was established by the combination of ^{13}C-NMR and EIMS spectral data. Compound **87** showed molecular ion peak at *m/z* 426 in EIMS. A fragment at *m/z* 411 was due to the loss of a methyl group from the molecular ion. Another ion at *m/z* 393 showed the loss of a methyl group with water molecule (CH_3-H_2O) from the molecular ion.

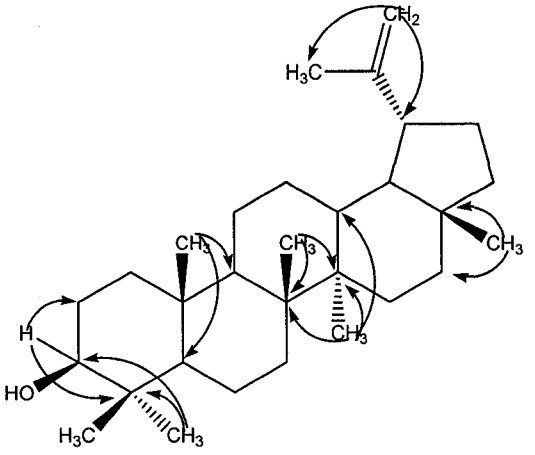
The ^{1}H-NMR spectrum ($CDCl_3$, 200 MHz) of **87** displayed seven tertiary methyl signals at δ 0.75, 0.77, 0.82, 0.94, 0.96, 1.02 and 1.68 due to C-23, C-24, C-25, C-26, C-27, C-28 and C-30 methyl protons, respectively. Two broad singlets, integrating for one-proton each, resonated at δ 4.56 and 4.68 were ascribed to C-29 methylene protons. Another one-proton double doublet at δ 3.20 (dd, $J_{1,2}$ = 5.6 and $J_{1,3}$ = 10.7 Hz) was assigned to C-3 methine proton. A one-proton multiplet at δ 2.38 was due to the C-19 methine proton.

The COSY-45° spectrum was also recorded to confirm the ^{1}H-NMR chemical shift assignments. In COSY-45° spectrum, the C-3 methine proton (δ 3.20) showed cross peak with C-2 methylene protons (δ 1.54 and 0.95), which in turn showed cross peaks with the C-1 methylene protons (δ1.67 and 0.90). The C-9 methine proton (δ 1.27) exhibited cross peaks with the C-11 methylene protons (δ 1.24 and 1.41). The latter showed cross peaks with the C-12 methylene protons (δ1.07 and 1.65), which in turn exhibited a cross peak with the C-13 methine proton (1.66). The C-19 methine proton (δ 2.38) showed cross peaks with the C-18 methine (δ 1.37) and C-21 methylene protons (δ 1.34 and 1.93), which in turn showed cross peaks with the C-22 methylene protons (δ 1.20 and 1.42).

The ^{13}C-NMR spectrum ($CDCl_3$, 50 MHz) of **87** showed that it contains 30 carbons. The DEPT experiment was used to establish the multiplicities of these carbon atoms and this showed the presence of seven methyl, eleven methylene, six methine, and six quaternary carbon atoms in the molecule. The complete ^{13}C-NMR and DEPT multiplicities assignments of the molecule **87** are presented in Table **10**.

The HSQC spectrum was helpful in determining the ^{1}H/^{13}C one-bond shift correlations of all protonated carbons.

The structural formula of **87** was also confirmed by HMBC spectrum. The C-3 methine proton (δ 3.20) showed long-range connectivities with the C-2 (δ 27.4) and C-4 (δ 38.7) carbons. The C-24 methyl protons (δ 0.77) showed connectivity to the C-3 (δ 79.0) and C-4 (δ 38.7) while the C-25 methyl protons (δ 0.82) showed cross peaks with C-5 (δ 55.2) and C-9 (δ 50.4) carbon atoms. The C-28 methyl protons (δ 1.02) exhibited cross peaks with C-16 (δ 35.5) and C-17 (δ 42.9) carbons atoms. The C-29 methylene protons (δ 4.56 and 4.68) also showed connectivity with the C-30 (δ 19.3) methyl and C-19 (δ 47.9) methine protons. Important HMBC interactions are shown in the figure below.



Long-range correlation of **87** determined through HMBC

The UV, ^{1}H, ^{13}C-NMR, and mass spectral data of compound **87** were distinctly identical to those of lupeol, reported in literature [20-25] and this led to the identification of compound **87** as lupeol. This compound was previously isolated from *Pyrus communis*[20], *Maytenus cuzcoina*[21], *Campanula lactiflora* [22], *Cirsium pascuarense* [23], *Rhus taitensis* [24] and *Ixeris chinensis* [25]. To the best of our knowledge, this is the first report of a naturally occurring triterpene (lupeol) from *B. prionitis.*

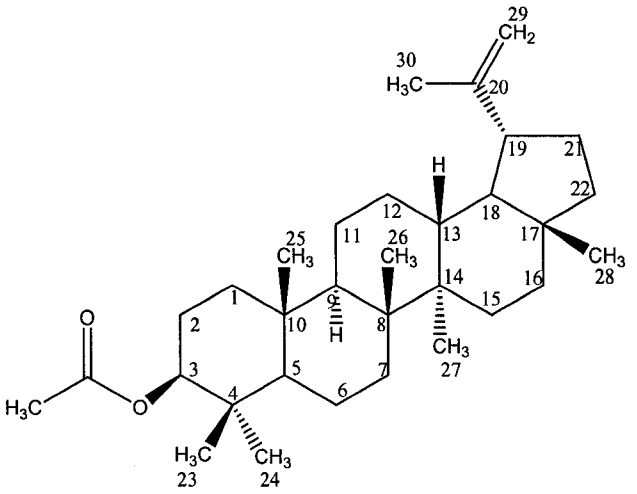
Table 10 ^{13}C- NMR chemical shift assignments of **87** with their multiplicities

Carbon No	δ^{13}C-NMR	†Multiplicity
1	38.8	CH_2
2	27.4	CH_2
3	79.0	CH
4	38.7	C
5	55.2	CH
6	18.3	CH_2
7	34.2	CH_2
8	40.8	C
9	50.4	CH
10	37.1	C
11	20.9	CH_2
12	29.7	CH_2
13	38.0	CH
14	42.8	C
15	25.1	CH_2
16	35.5	CH_2
17	42.9	C
18	48.3	CH
19	47.9	CH
20	150.9	C
21	29.8	CH_2
22	39.9	CH_2
23	15.3	CH_3
24	27.9	CH_3
25	16.1	CH_3
26	14.5	CH_3
27	15.9	CH_3
28	17.9	CH_3
29	109.3	CH_2
30	19.3	CH_3

†Multiplicity was determined from DEPT experiment. Solvent: $CDCl_3$

3.2.2Acetylation of lup-20(29)-ene-3β-ol (lupeol) (88)

Acetylation of lupeol **87** was performed using acetic anhydride/pyridine to yield acetylated lupeol (**88**) as white crystalline solid[26].



(88)

The UV spectrum of compound **88** showed maximum absorption at 229 nm. The IR spectrum (KBr) revealed intense absorption at 1735 cm^{-1} for the presence of carbonyl group.

The molecular formula, $C_{32}H_{52}O_2$, of the acetylated product **88** was established through ^{13}C-NMR and EIMS spectral data, which showed molecular ion peak at *m/z* 468 where as the parent compound (**87**) showed molecular ion peak at *m/z* 426.

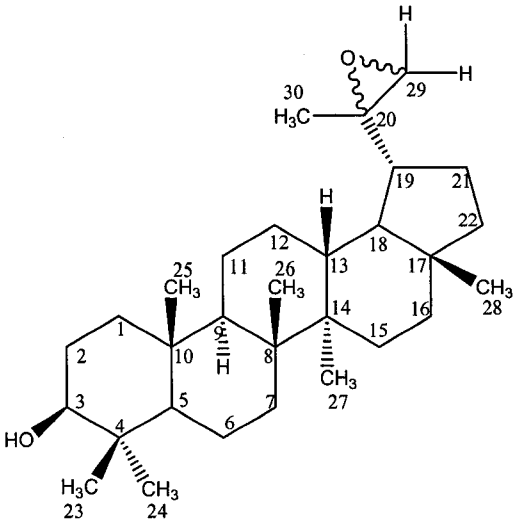
The ^{1}H-NMR spectrum ($CDCl_3$, 200 MHz) of the acetylated product **88** showed a downfield shift of the C-3 methine proton from δ 3.20 to 4.5. An additional three-proton singlet appeared at δ 2.01 due to the C-3 acetyl methyl protons. The rest of the ^{1}H-NMR spectrum was similar to that of the parent alcohol **87**.

The ^{13}C-NMR spectrum ($CDCl_3$, 50 MHz) of the acetylated product **88** showed the resonance of 32 carbons instead of 30 carbons of parent alcohol. The acetylated product

(**88**) showed a downfield shift of the C-3 carbon atom from δ 79.0 to 80.9. It also showed a signal at δ 171.1, indicated the presence of an ester carbonyl carbon in **88**. An additional signal at δ 21.3 was due to methyl carbon of the acetylated group. The rest of the ^{13}C-NMR spectrum was similar to that of the parent alcohol (**87**).

3.2.3 Epoxidation of side chain of lup-20(29)-ene-3β-ol (89)

Oxidation of the isopropenyl side chain of lupeol with *m*-chloroperbenzoic acid furnished epoxide[27] (**89**) as a white opaque solid.



(**89**)

The UV spectrum of compound **89** showed absorption maxima at 224 nm. The IR spectrum (KBr) revealed intense absorptions at 3519 (OH), 2925 (C-H) and at 1168 cm^{-1} for C-O-C group.

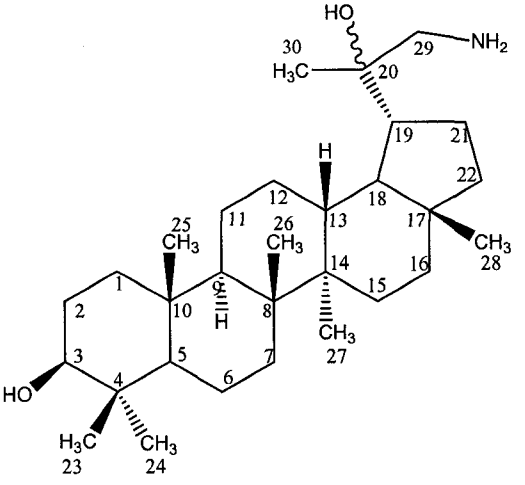
The CIMS data of **89** exhibited the quasimolecular ion $[M+1]^+$ at *m/z* 443. This mass spectral data in combination with the ^{13}C-NMR data provided molecular formula, $C_{30}H_{50}O_2$.

The ^{1}H-NMR spectrum ($CDCl_3$, 200 MHz) of the oxidized product (**89**) showed an upfield shift of the C-30 methyl protons from δ 1.68 to 1.25. In addition to this, two broad singlets of one-proton each corresponding to C-29 resonated at δ 4.56 and 4.68 were absent. The rest of the ^{1}H-NMR spectrum was similar to that of the parent alcohol **87**. The ^{13}C-NMR spectrum ($CDCl_3$, 50 MHz) of the oxidized product **89** showed two signals at δ 57.5 and 60.4 instead of signals at 109.3, 150.9 for C-29 and C-20, suggesting the

presence of an epoxide moiety at C-20/C-29. The rest of the ^{13}C-NMR spectrum was similar to that of the parent alcohol.

3.2.4 Preparation of amino-alcohol side chain of lup-20 (29)-ene-3β-ol (90)

First epoxidation of the isopropenyl side chain of lupeol with *m*-chloroperbenzoic acid was carried out to prepare an epoxide followed by the procedure described in the literature[27]. Then 29-amino-20-hydroxylupeol was synthesized by reacting compound **89** with 33% NH_3 using microwave radiations[28].



(90)

The formation of **90** was confirmed by recording the ^1H and ^{13}C NMR spectra. The ^1H-NMR spectrum of **90** showed the resonance of C-29 methylene protons at δ 2.18 as sharp singlet. The ^{13}C-NMR spectrum of **90** showed two signals at δ 60.4 and 75.8 instead of signals at δ 57.5 and 60.4 for C-29 and C-20 indicated the formation of 29-amino-20-hydroxylupeol. The rest of the ^{13}C-NMR spectrum was similar to that of the parent alcohol **89**.

3.3 Bioactivity of the chemical constituents of B. prionitis and its derivatives.

Due to the reported folk medicinal properties of *B. prionitis*, lupeol and its derivatives were evaluated in AChE and GST inhibition assays. All of these compounds were found to have inhibitory activities against acetylcholinesterease and glutathione *S*-transferase enzymes. The IC_{50} values of isolated compound **87** and its derivatives are shown in Table **11**.

Table 11 AChE and GST inhibitory activities (IC_{50} = μM ± SEM) of compounds **87** to **90**

Compounds	AChE ± SEM	GST ± SEM
Lupeol (**87**)	89.16 ± 1.70	60.85 ±1.25
3-acetyl-lupeol (**88**)	70.61 ± 1.98	147.41 ±1.35
20-29 epoxylupeol (**89**)	38.61 ± 0.36	103.59 ± 1.00
29-amino-20-hydroxylupeol (**90**)	67.60 ± 1.13	44.86 ± 1.87

SEM = Standard error of mean of three assays

Lupeol (**87**) displayed 89.16 μM anti-AChE activity. Among all three derivatives 20-29 epoxylupeol (**89**) was found to be the best AChE inhibitor (IC_{50} 38.61 μM) which indicate that epoxy functionality at C-20/C-29 is an important structural feature that provides improved interaction with the enzyme compared with parent compound. The rest of the derivatives, 3-acetyl-lupeol (**88**) and 29-amino-20-hydroxylupeol (**90**) displayed almost same AChE activity (IC_{50} values of 70.61 and 67.60 μM, respectively). Comparison of the AChE values of lupeol and its derivatives with that of reported inhibitory activities of standard AChE inhibitors, eserine (IC_{50} 0.041 μM), tacrine (IC_{50}

0.021 μM), and galanthamine (IC_{50} 0.45 μM) showed that compounds **87-90** are very weak AChE inhibitors[29].

Lupeol and its derivatives were also assayed against GST spectrophotometrically. Compound **87** and its derivatives **88-90** displayed variable GST inhibitory activities ranged from 44.86 to 147.41μM. These concentration dependent GST inhibitory activities were found to be excellent compared with reported steroidal type GST inhibitor sodium taurocholate (IC_{50} 398 μM) [30]. However concentration dependent GST inhibitory activity of 29-amino-20-hydroxylupeol (**90**) was found to be moderate compared with previously reported GST inhibitor ethacrynic acid (IC_{50} 16.0 μM) [31].

3.4 Discussion

From the ethanolic extract of *B. prionitis* a pentacyclic triterpenoid lupeol (**87**) was purified. It was a known triterpenoid that has been reported from a number of plant species of different genera[20-25]. Lupeol is a member of lupane triterpenoidal series. Most of the members of this class have different kinds of biological activities including anti HIV[32], antimicrobial and cytotoxic activity[21, 33]. The amount of this compound (135 mg), and aforementioned biological activities, encouraged us to modify this molecule into different derivatives and evaluate all of these derivatives along with the parent compound for acetylcholinestrase (AChE) and glutathione *S*-transferase (GST) inhibitory activity using inhouse available facilities. The results of these bioassays have been tabulated in Table **11**.

The AChE and GST inhibition bioassay results indicated that lupeol has variable inhibitory activity against both enzymes. The GST inhibitory results showed that acetylated lupeol (**88**) and 20,29-epoxylupeol (**89**), both have decreased inhibitory activity against GST compared with the parent compound. The decreased activity might be due to both olefinic functionality at C-20/C-29 and hydroxyl functionality at C-3, which might be necessary for the improved conjugation of GST with compounds of lupane series. On the other hand the results of GST inhibitory activity of derivative **90** showed that amino functionality at C-29 and hydroxyl functionality at C-3 and C-20 enhance the inhibitory activity of this molecule compared with the parent compound.

The AChE inhibitory data of the different derivatives of lupeol showed that epoxide ring at C-20/C-29 (**89**) enhances the AChE inhibitory activity. This might occur due to the improved interaction of this derivative with an active site of acetylcholinestrase

compared with the parent compound. On the other hand the openings of epoxide ring, as in the case of amino derivative (**90**) again decreased the AChE inhibitory activity. This indicated that the epoxide ring at C-20/C-29 was necessary for the improved interaction of this compound with the enzyme. However all of these compounds were weakly active against AChE, where as moderately active against GST compared with standard inhibitors of respective enzymes.

Terpenoidal natural products are widely distributed in nature having interesting chemical groups in their structures. The biogenetic isoprene rule describes the formation of different types of tetra and pentacyclic triterpenoids according to the conformation that squalene or 2,3-epoxy squalene adopts at the enzyme surface prior to its folding. Each leads to a particular cyclization product stereospecifically. Lupeol, which is a member of pentacyclic triterpenes may originate in nature by the folding of squalene oxide to a chair-chair-chair-boat conformation leading to the formation of dammarenyl cation. As a result of Wagner-Meerwein rearrangement dammarenyl cation is transformed into baccharenyl cation. Now a pentacyclic ring system can be formed by the cyclization of double bond giving a tertiary lupenyl cation. Lastly, a loss of proton from lupenyl cation gives lupeol[34] (Figure **11**).

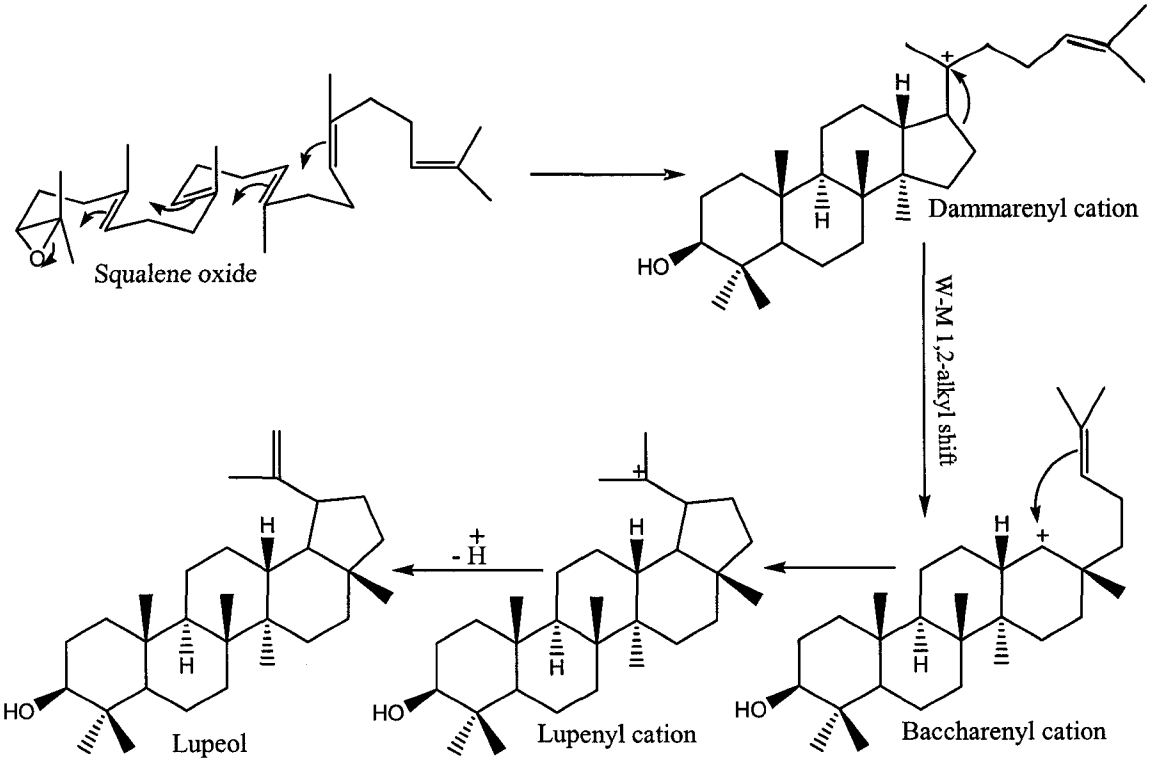


Figure 11 Biosynthesis of lupeol

3.5 EXPERIMENTAL

3.5.1 General experimental conditions

General experimental conditions for this part of research were the same as those described in chapter-**2** (page # **65**).

3.5.2 Plant material

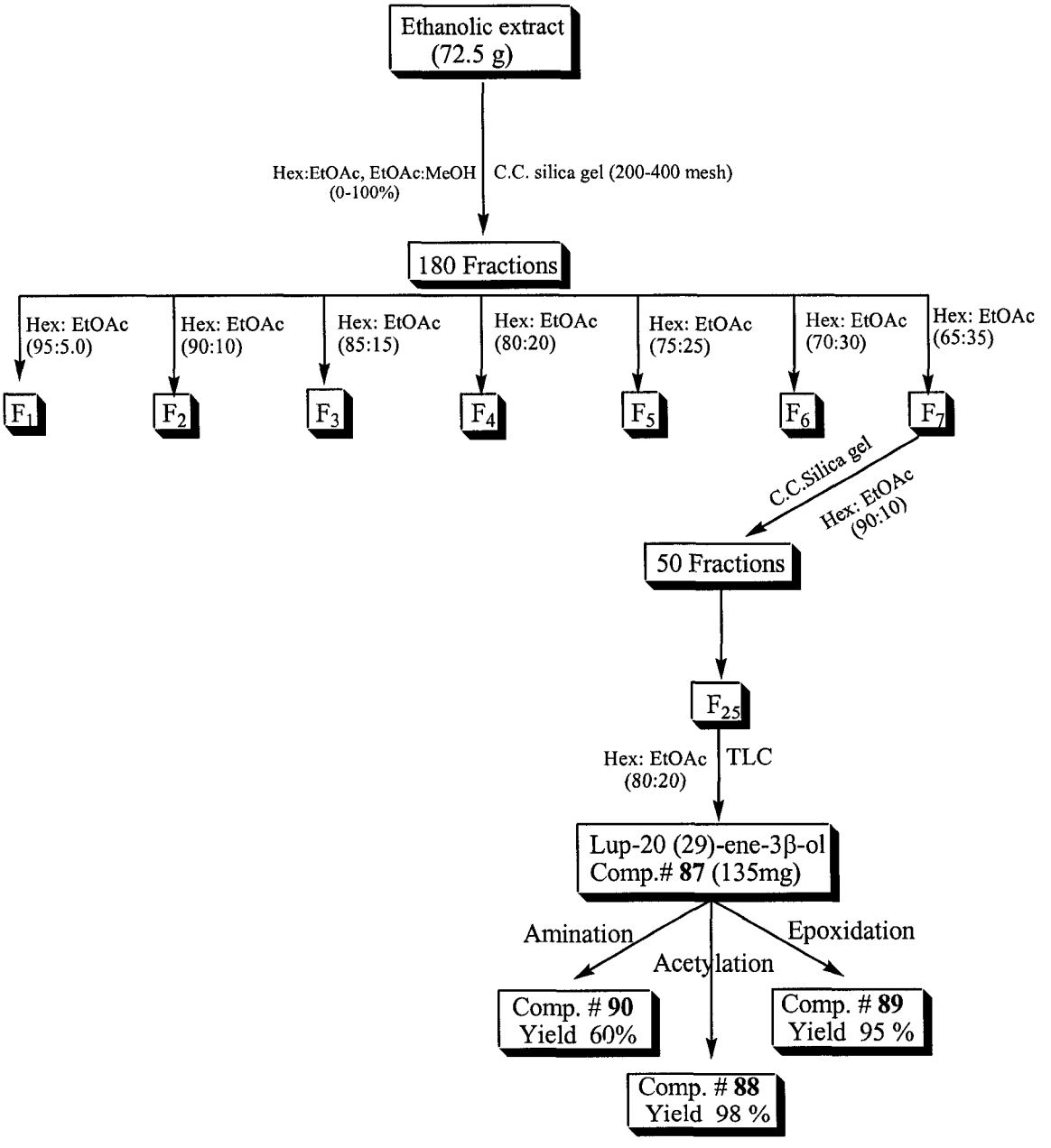
The aerial parts of *B. prionitis Linn* were collected from Gampaha, Western province of Sri Lanka in September 2004. Dr. Radhika Samarasakera identified the plant, and a voucher specimen was deposited with the Natural Product Development Group at Industrial Technology Institute, Colombo, Sri Lanka.

3.5.3 Extraction and Isolation

Our collaborator, Dr. Radhika Samarasakera from Gampaha, Western province of Sri Lanka in September 2004, collected the aerial parts of *B. prionitis* L. The crude ethanolic extract of the leaves of *B. prionitis* was shipped to us under a collaborative program between Natural Products Development Group at Industrial Technology Institute, Colombo, Sri Lanka and the Department of Chemistry, University of Winnipeg, Manitoba, Canada.

This extract (72.5 g) was fractionated by column chromatography over silica gel (200-400 mesh) using n-hexane-ethyl acetate (0-100 %), ethyl acetate-methanol (0-100 %), to afford 180 fractions. Similar fractions were combined together based on similar R_f values on analytical TLC. The primary fraction F_7 obtained at 65:35% (Hex: EtOAc) was again loaded on another silical gel column. The column was eluted with n-hexane-ethyl acetate (90:10 %) to afford fifty fractions. The sub-fraction F_{25} was subjected to preparative TLC

using n-hexane-ethyl acetate (80:20) as mobile phase to afford lupeol **(87)** as white crystalline solid (135 mg, 0.18 % yield, R_f = 0.50).



Scheme 3 Isolation procedure for compound **87**

3.5.4 Spectral data of lup-20(29)-ene-3β-ol (87)

White crystalline solid

UV λ_{max} (CH_2Cl_2): 227 nm

IR (KBr) ν_{max} cm^{-1}: 3420 (OH), 2944(C-H).

^{1}H-NMR ($CDCl_3$, 200 MHz) (δppm): 0.75 (3H, s , 23-Me), 0.77 (3H, s , 24-Me), 0.82 (3H, s , 25-Me), 0.94 (3H, s , 26-Me), 0.96 (3H, s , 27-Me), 1.02 (3H, s , 28-Me), 1.68 (3H, s , 30-Me), 3.20 (dd, $J_{1,2}$ = 5.6 and $J_{1,3}$ = 10.7 Hz , H-3), 4.56 (1H, brs, H-29), 4.68 (1H, brs, H-29),

^{13}C-NMR ($CDCl_3$, 50 MHz) (δppm): See Table-10

EIMS ***m/z*** (rel. int. %): 426 $[M]^+$ (18), 411 $[M-CH_3]^+$ (15), 393 (7), 218 (65), 204 (25), 189 (100),

3.5.5 Synthesis of 3-acetylupeol (88)

5 mg of lupeol **87** was dissolved in pyridine (0.5mL) in a 20 mL round bottom flask and then 1.0 mL of acetic anhydride was added to the mixture[26]. The flask was covered with stopper and it was stirred at room temperature for 18 hours. The completion of reaction was tested on TLC and stopped by adding distilled water (10mL) to the reaction flask. The aqueous layer was extracted with dichloromethane (3x10 mL). The dichloromethane layer was separated, dried over anhydrous sodium sulphate and evaporated under vacuum. The acetylated product was purified with the help of preparatory TLC using hexane: ethyl acetate (90:10) to afford 3-acetoxy-lupeol (**88**) (4.9 mg, yield 98%) as white crystalline solid.

Spectral data

UV λ_{max} (CH_2Cl_2): 229 nm

IR (KBr) ν_{max} cm^{-1}: 1735 (C=O) , 2939 (C-H).

^{1}H-NMR ($CDCl_3$, 200 MHz) (δppm): 4.5 (dd, $J_{1,2}$ = 5.6 and $J_{1,3}$ = 10.7 Hz , H-3), 4.56 (1H, brs, H-29), 4.68 (1H, brs, H-29), 2.01(3H, s , $\underline{Me}CO_2$), 0.75 (3H, s , 23-Me), 0.77 (3H, s , 24-Me), 0.82 (3H, s , 25-Me), 0.94 (3H, s , 26-Me), 0.96 (3H, s , 27-Me), 1.02 (3H, s , 28-Me), 1.68 (3H, s , 30-Me),).

^{13}C-NMR ($CDCl_3$, 50 MHz) (δppm): 37.9 (CH_2, C-1), 27.4 (CH_2, C-2), 80.9 (CH, C-3), 38.3 (-C-, C-4), 55.3 (CH, C-5), 18.2 (CH_2, C-6), 34.2 (CH_2, C-7), 40.8 (-C-, C-8), 50.3 (CH, C-9), 37.0 (-C-, C-10), 20.9 (CH_2, C-11), 29.7 (CH_2, C-12), 37.8 (CH, C-13), 42.8 (-C-, C-14), 25.0 (CH_2, C-15), 35.5 (CH_2, C-16), 42.9 (-C-, C-17), 48.2 (CH, C-18), 47.9 (CH, C-19), 150.9 (-C-, C-20), 29.8 (CH_2, C-21), 39.9 (CH_2, C-22), 16.5 (CH_3, C-23), 27.9 (CH_3, C-24), 16.2 (CH_3, C-25), 14.5 (CH_3, C-26), 15.9 (CH_3, C-27), 17.9 (CH_3, C-28), 109.3 (CH_2, C-29), 19.3 (CH_3, C-30), 21.3 (CH_3, $\underline{Me}CO_2$), 171.1 (-C-, $Me\underline{C}O_2$).

EIMS *m/z* (rel. int. %): 468 $[M]^+$ (18), 453 $[M-CH_3]^+$ (15), 393 (15), 218 (65), 204 (90), 189 (100).

3.5.6 Synthesis of 20 (29)-epoxylupeol (89)

10 mg of lupeol **87** was dissolved in 15 mL of dichloromethane in 30 mL round bottom flask [27]. The reaction mixture was cooled on ice bath to chill the solution. Then 8.04 mg of meta-chloroperbenzoic acid was added portion wise and the reaction mixture was allowed to stir for 8 hours while maintaining the temperature between 0° - 5 °C. The reaction was monitored by using analytical TLC. After the completion of the reaction, the reaction mixture was washed with 10% aqueous sodium bicarbonate solution (3x10 mL). The organic layer was washed with water, dried over anhydrous sodium sulphate and evaporated under reduced pressure. The product was purified by preparatory TLC using

hexane:ethyl acetate (75:25) to afford 20(29) epoxylupeol **(89)** (9.5 mg, yield 95%) as white opaque solid.

Spectral data

UV λ_{max} (CH_2Cl_2): 224 nm

IR (KBr) ν_{max} cm^{-1}: 3519 (OH), 2925 (C-H), 1168 (C-O-C).

^{1}H-NMR ($CDCl_3$, 200 MHz) (δppm): 3.20 (dd, $J_{1,2}$ = 5.6 and $J_{1,3}$ = 10.7 Hz , H-3), 0.75 (3H, s , 23-Me), 0.77 (3H, s , 24-Me), 0.82 (3H, s , 25-Me), 0.94 (3H, s , 26-Me), 0.96 (3H, s , 27-Me), 1.02 (3H, s , 28-Me), 1.25 (3H, s , 30-Me).

^{13}C-NMR ($CDCl_3$, 50 MHz) (δppm): 38.8 (CH_2, C-1), 27.3 (CH_2, C-2), 78.9 (CH, C-3), (38.7) (-C-, C-4), 55.2 (CH, C-5), 18.3 (CH_2, C-6), 34.2 (CH_2, C-7), 40.8 (-C-, C-8), 50.2 (CH, C-9), 37.2 (-C-, C-10), 21.0 (CH_2, C-11), 29.7 (CH_2, C-12), 38.0 (CH, C-13), 42.8 (-C-, C-14), 25.9 (CH_2, C-15), 35.4 (CH_2, C-16), 43.4 (-C-, C-17), 49.4 (CH, C-18), 46.4 (CH, C-19), 60.4 (-C-, C-20), 27.1 (CH_2, C-21), 39.7 (CH_2, C-22), 15.3 (CH_3, C-23), 27.9 (CH_3, C-24), 16.1 (CH_3, C-25), 14.4 (CH_3, C-26), 15.9 (CH_3, C-27), 17.9 (CH_3, C-28), 57.5 (CH_2, C-29), 18.1 (CH_3, C-30).

EIMS *m/z* (rel. int. %): 442 $[M]^+$ (18), 427 $[M-CH_3]^+$ (15), 384 (75), 218 (6), 207 (90), 189 (100).

3.5.7 Synthesis of 29-amino-20-hydroxylupeol (90)

Epoxide **89** (5 mg) and 5 mL of 33% NH_3 were placed in a 20 mL closed vial [28]. The vial was put in a standard microwave oven at a chosen power (1200W). The reaction was carried out for 5 minutes and 10 seconds with 5 sec. intervals. After cooling, the solution was extracted with ethyl acetate and the organic layer separated and dried over anhydrous

sodium sulphate. The solvent was evaporated under vacuum. The product 29-amino-20-hydroxylupeol (**90**) was obtained as a colorless solid (3.0 mg, yield 60%).

Spectral data

UV λ_{max} (CH_2Cl_2): 227 nm

^{1}H-NMR ($CDCl_3$, 200 MHz) (δppm): 3.20 (dd, $J_{1,2}$ = 5.6 and $J_{1,3}$ = 10.7 Hz , H-3), 2.18 (2H, s, C-29), 0.75 (3H, s , 23-Me), 0.77 (3H, s , 24-Me), 0.82 (3H, s , 25-Me), 0.94 (3H, s , 26-Me), 0.96 (3H, s , 27-Me), 1.02 (3H, s , 28-Me), 1.25 (3H, s , 30-Me).

^{13}C-NMR ($CDCl_3$, 50 MHz) (δ ppm): 38.8 (CH_2, C-1), 27.3 (CH_2, C-2), 77.6 (CH, C-3), 38.7 (-C-, C-4), 55.2 (CH, C-5), 18.3 (CH_2, C-6), 34.2 (CH_2, C-7), 40.8 (-C-, C-8), 50.2 (CH, C-9), 37.2 (-C-, C-10), 20.9 (CH_2, C-11), 29.7 (CH_2, C-12), 37.1 (CH, C-13), 43.3 (-C-, C-14), 25.9 (CH_2, C-15), 35.4 (CH_2, C-16), 43.4 (-C-, C-17), 49.4 (CH, C-18), 46.4 (CH, C-19), 75.8 (-C-, C-20), 27.1 (CH_2, C-21), 39.7 (CH_2, C-22), 15.3 (CH_3, C-23), 27.9 (CH_3, C-24), 16.0 (CH_3, C-25), 14.1 (CH_3, C-26), 15.9 (CH_3, C-27), 18.1 (CH_3, C-28), 60.4 (CH_2, C-29), 18.1 (CH_3, C-30).

3.6 Enzyme Inhibition Assays

3.6.1 Acetylcholinestrase Inhibition Assay

General and typical assay conditions for Acetylcholinestrase Inhibition Assay were same as those described in chapter-**2** (page # **73**).

3.6.2: Glutathione *S*-Transferase Inhibition Assay

General and typical assay conditions for Glutathione *S*-Transferase Inhibition Assay were same as those described in chapter-**2** (page # **73**).

Conclusions

The phytochemical investigation of *B. hyrcana* (Buxaceae) has resulted in the isolation of eight compounds, one tetracyclic triterpene and seven steroidal alkaloids. All of these compounds were characterized with the help of spectroscopic techniques. The compounds isolated and characterized from *B. hyrcana* were arbora-1,9(11)-dien-3-one (**73**), cyclobuxoviridine (**74**), *E*-buxenone (**75**), *Z*-buxenone (**76**), moenjodaramine (**77**), homomoenjodaramine (**78**), buxamine-B (**79**), 31-hydroxybuxamine-B (**80**). The compounds **73**, **74**, **76** and **80** were characterized for the first time from *B. hyrcana.* All of these compounds were evaluated for their inhibitory activity against AChE, an enzyme responsible for the hydrolytic cleavage of neurotransmitter ACh. Homomoenjodaramine (**78**) was found to be the best AChE inhibitor amongst all of the purified compounds from *B. hyrcana.* Based on intensive literature search and from the structural comparison of all of these purified *Buxus* alkaloids, it was inferred that tetrahydrooxazine moiety at C-3/C-4 is necessary for the best anti-AChE activity of homomoenjodaramine among all the isolated compounds in this study. But comparison of this AChE inhibitory activity results with inhibitory activity of galanthamine, eserine, and tacrine showed that these purified compounds have weaker anti-AChE activity. The compound (**73**) was also assayed against GST, an enzyme responsible for resistance of cancer cells in response to chemotherapeutic agents. This compound was found to be weakly active against GST compared with previously used chemosensitizer, ethacrynic acid.

The crude ethanolic extract of *B. prionitis* was also chemically investigated which resulted in the isolation of pentacyclic triterpene, lupeol (**87**). This compound was found to be weakly active against equine liver GST compared with ethacrynic acid. Chemical

modification of lupeol resulted into three derivatives. All of these derivatives displayed varying degrees of GST and AChE inhibitory activities. The derivative 20-29 epoxylupeol (**89**) showed the best anti-AChE activity (IC_{50} 38.61 μM) while the derivative 29-amino-20-hydroxylupeol (**90**) was found to be the best GST inhibitor (IC_{50} 44.86 μM) among all the derivatives. Compound **87** (lupeol) and its derivatives **88-90** along with AChE and GST inhibitory activities have been reported for the first time from *B. prionitis*. As a result of these investigations, the objective of this study has been achieved successfully with regards to the isolation and identification of the biologically active compounds from *B. hyrcana* and *B. prionitis*.

3.7 References

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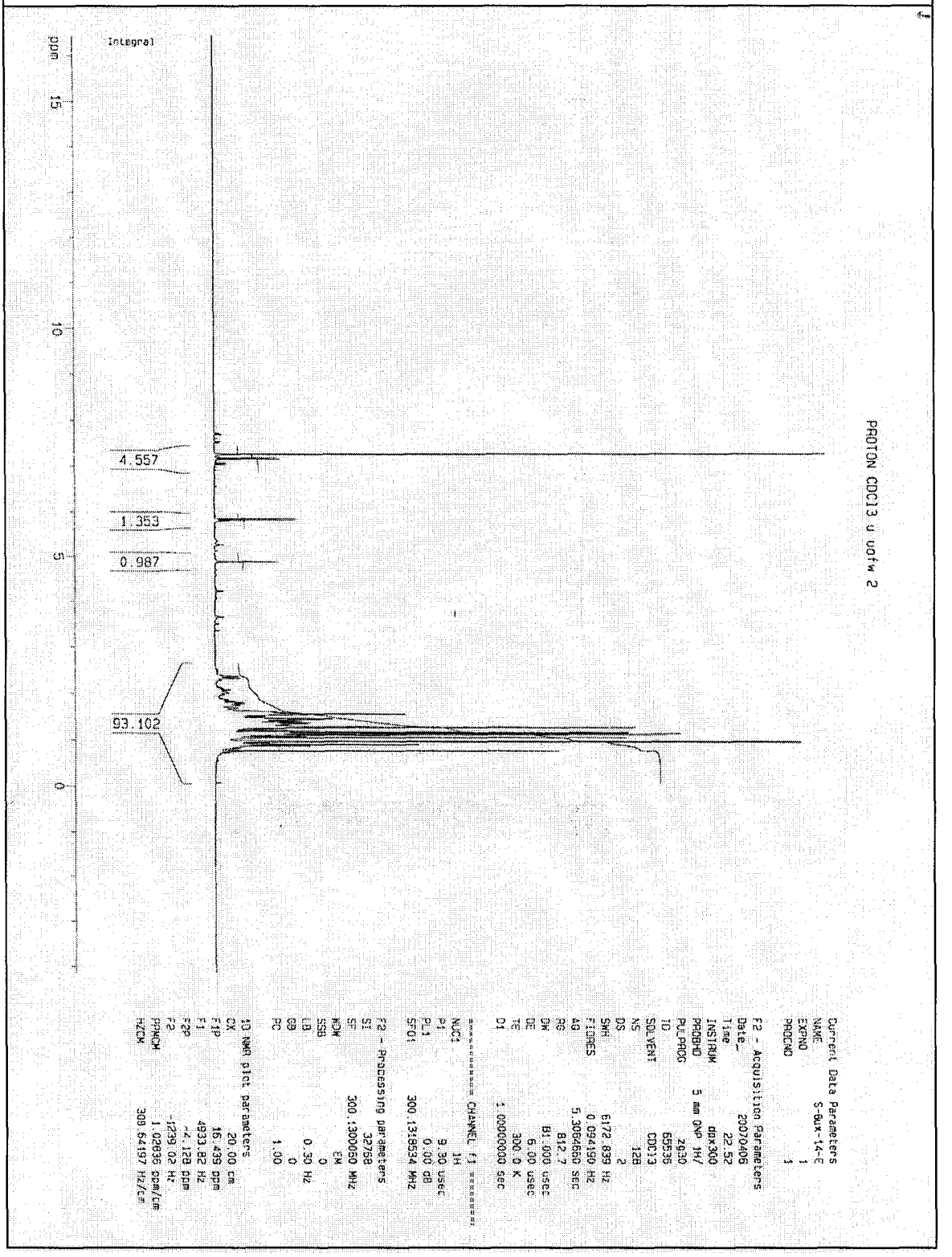
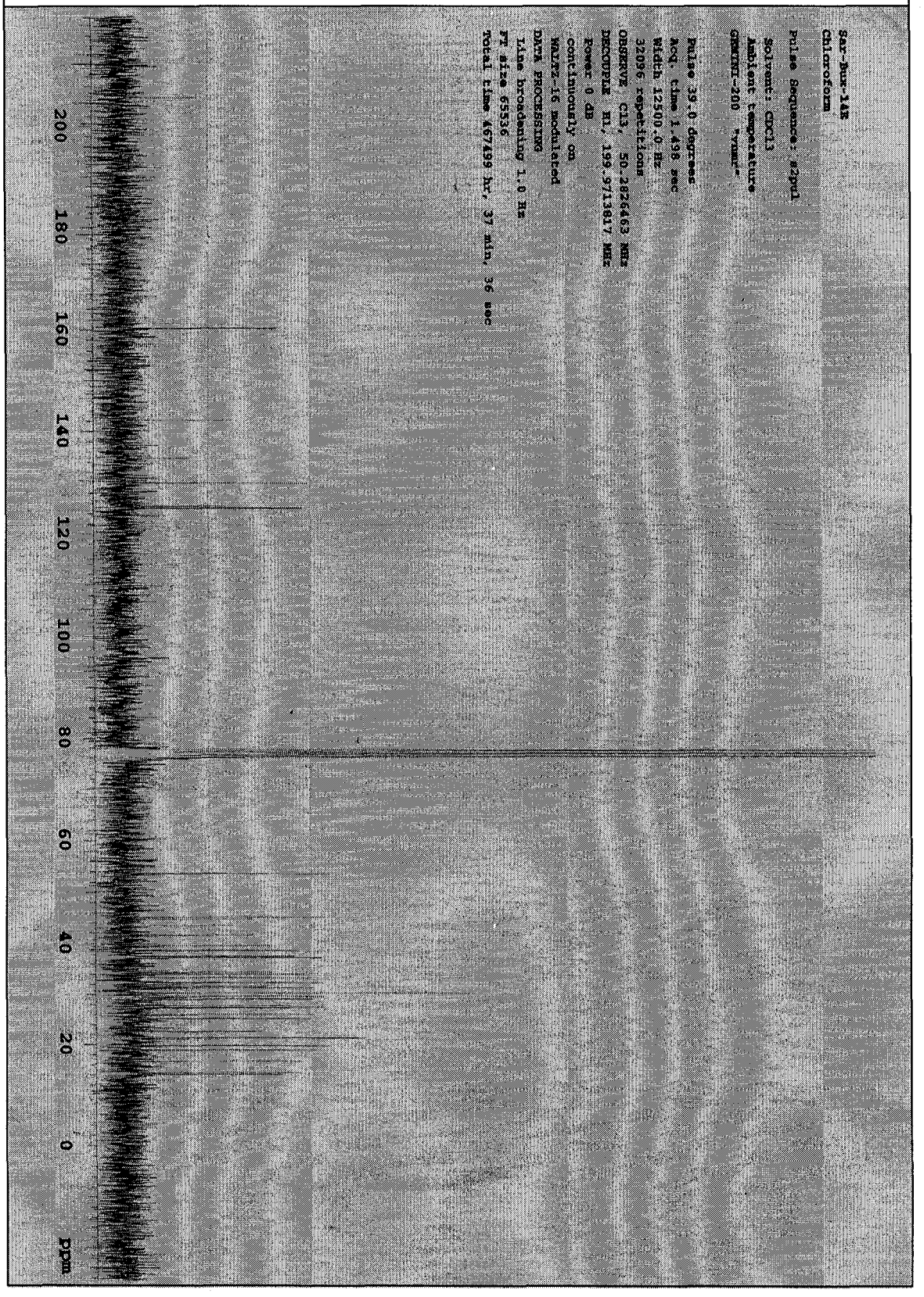
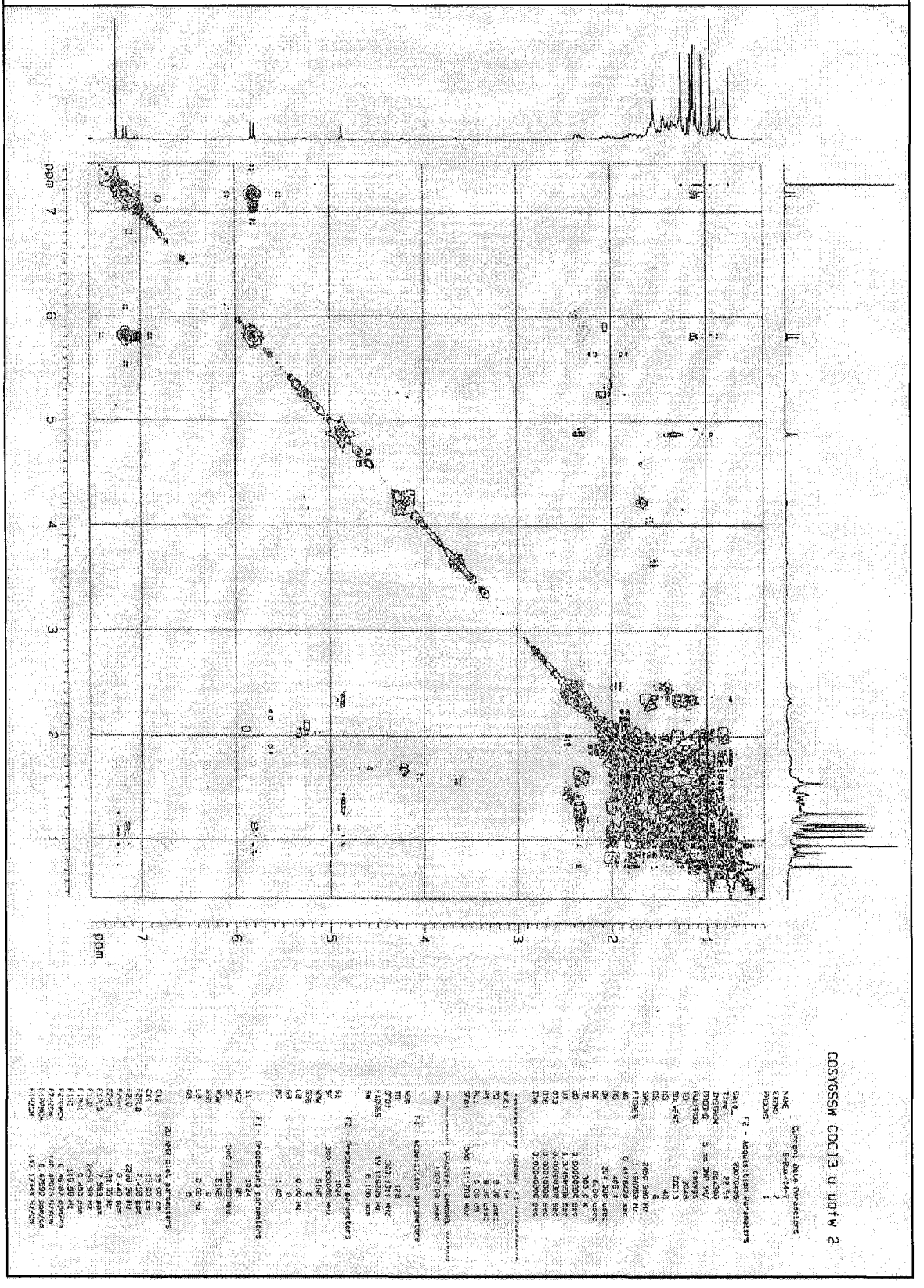
[1]H-NMR spectrum of arbora-1,9(11)-dien-3-one



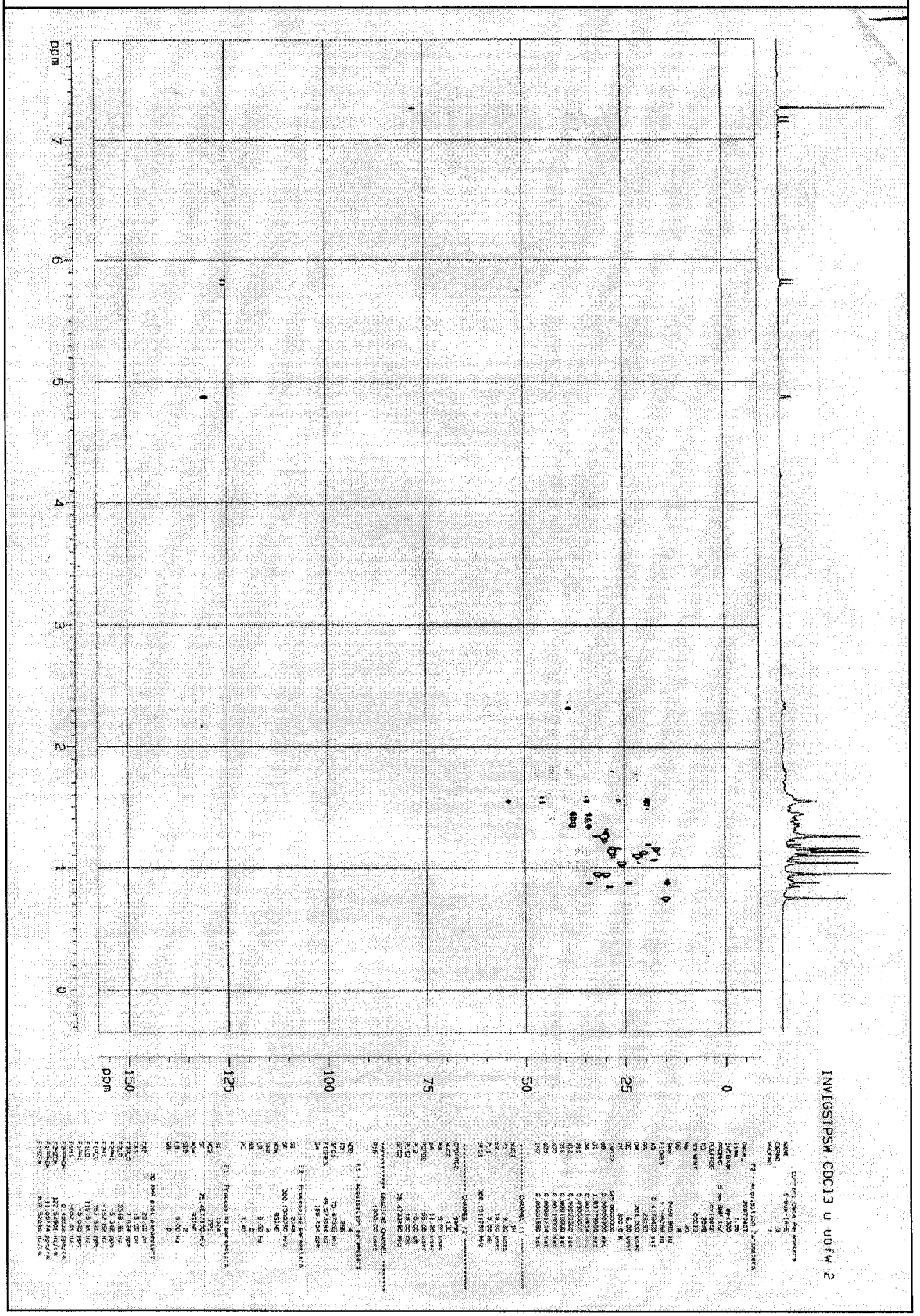
^{13}C-NMR spectrum of arbora-1,9(11)-dien-3-one



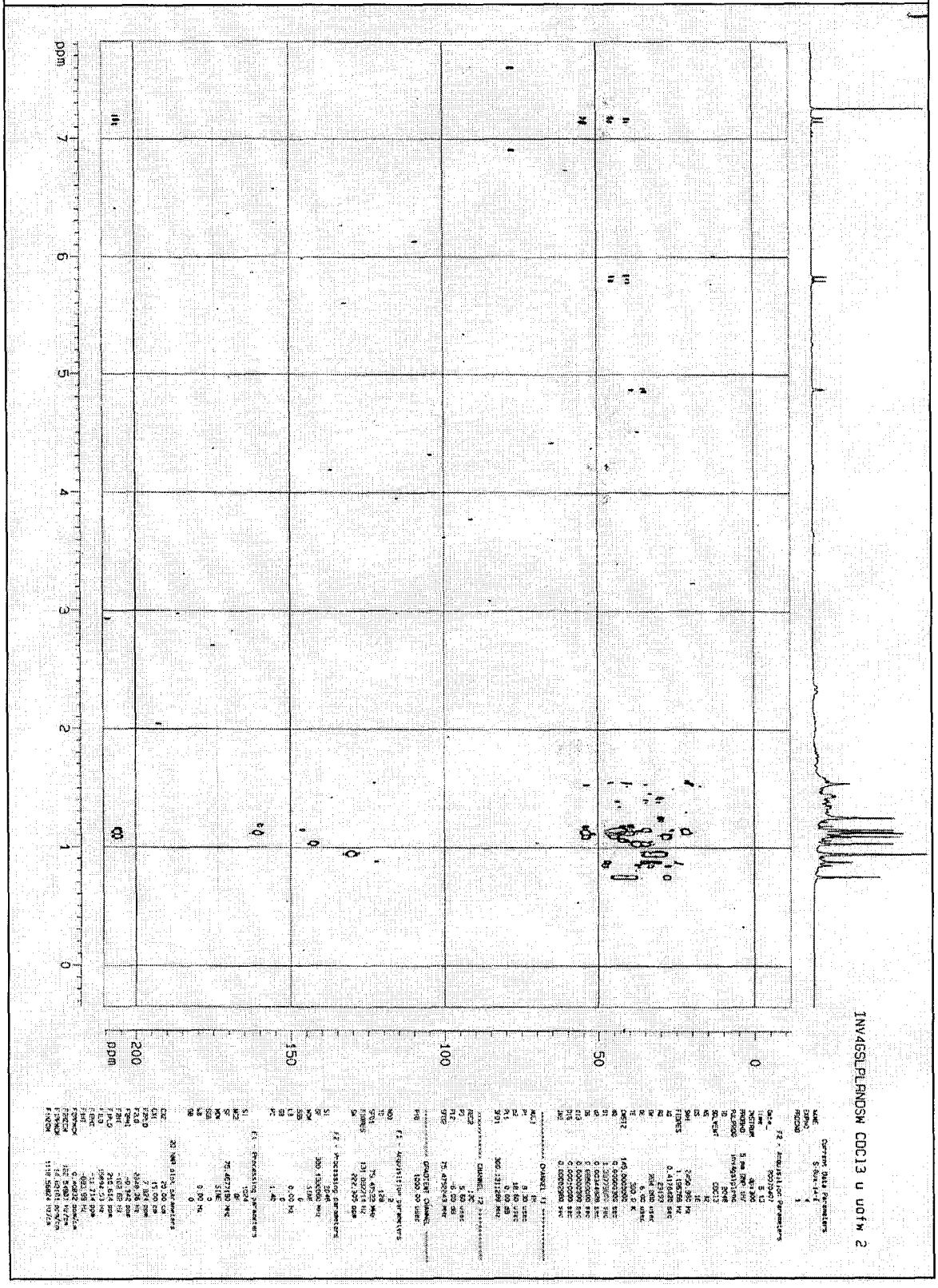
COSY-45° spectrum of arbora-1,9(11)-dien-3-one



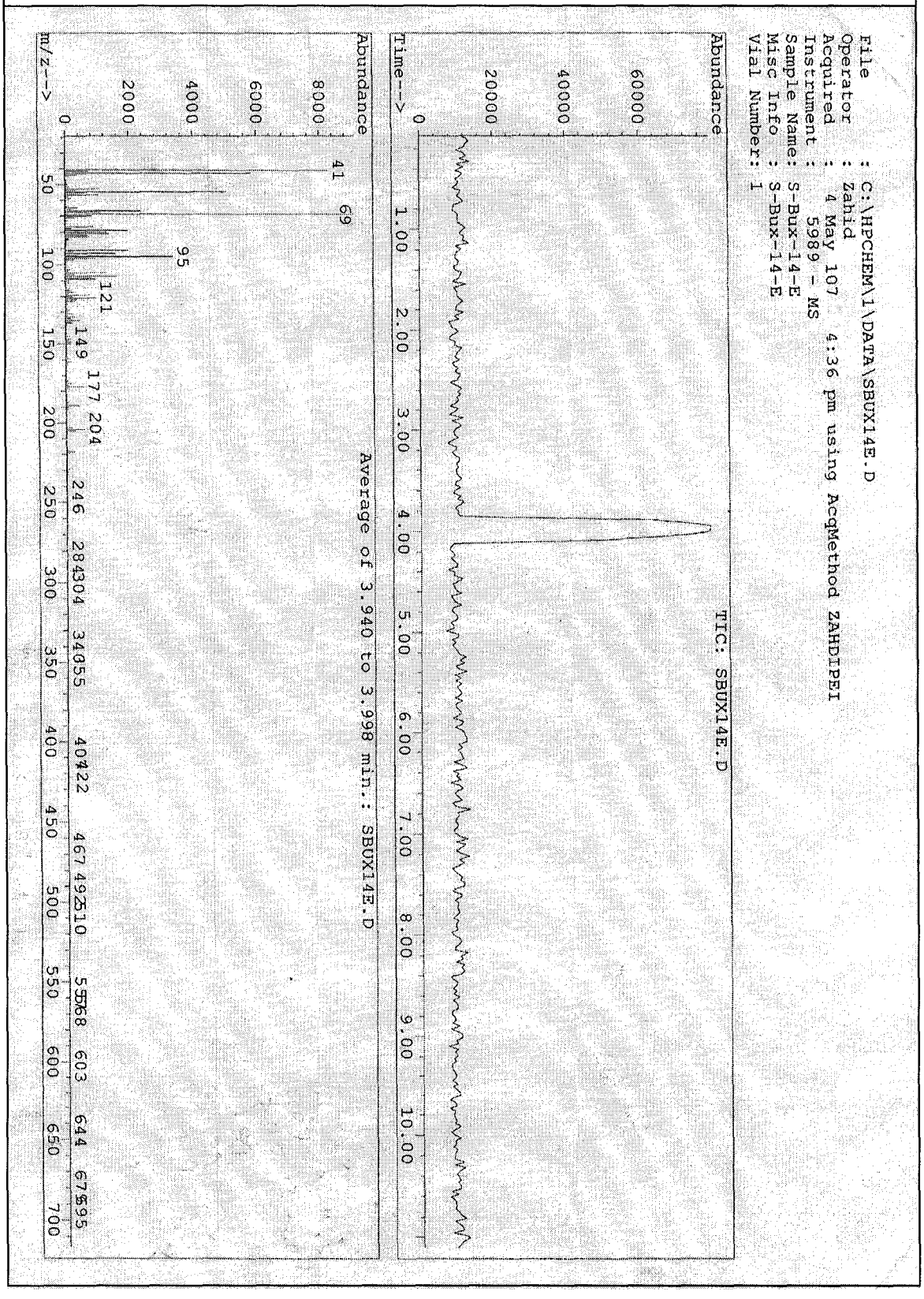
HSQC spectrum of arbora-1,9(11)-dien-3-one



HMBC spectrum of arbora-1,9(11)-dien-3-one

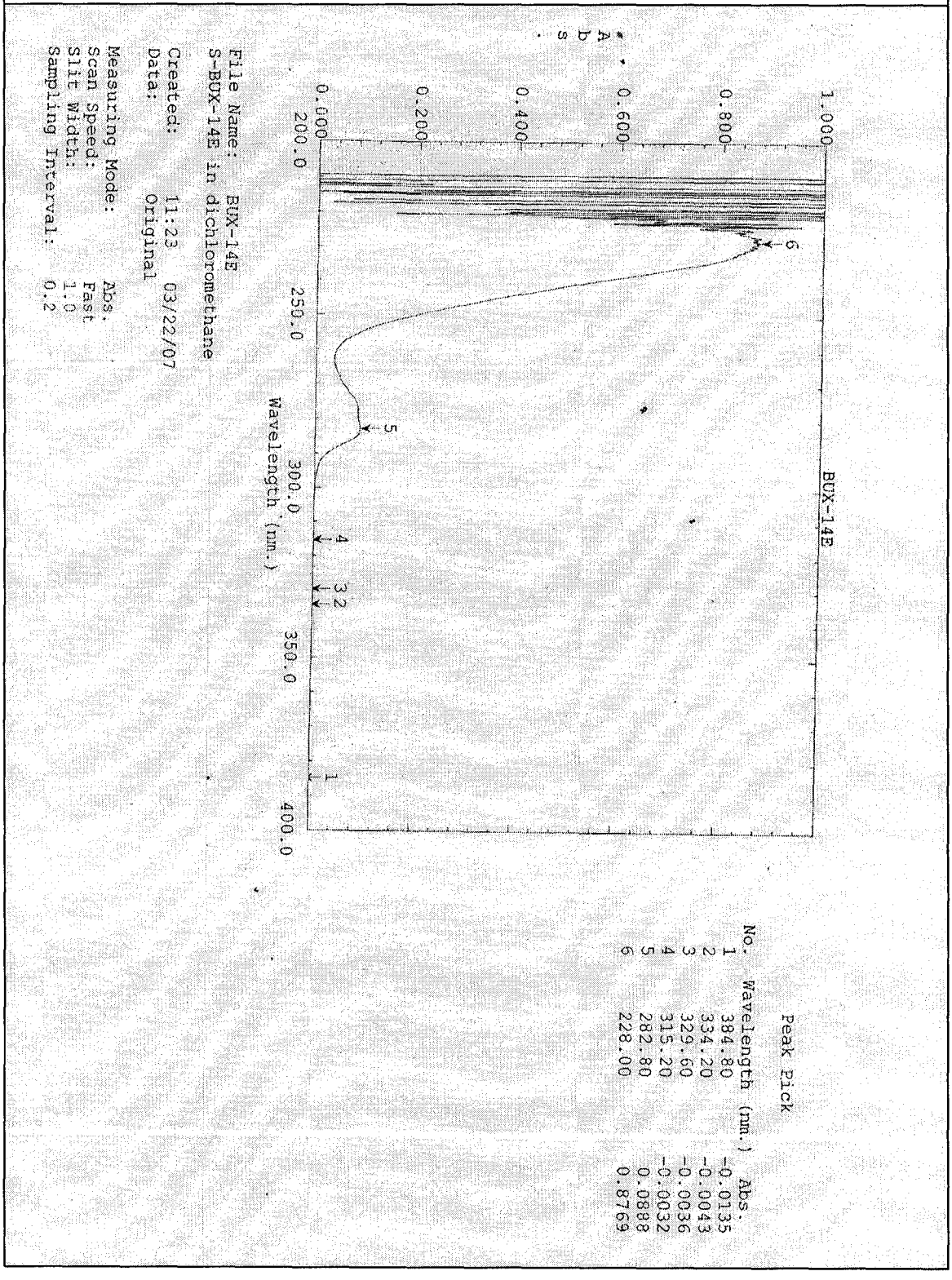


EIMS spectrum of arbora-1,9(11)-dien-3-one



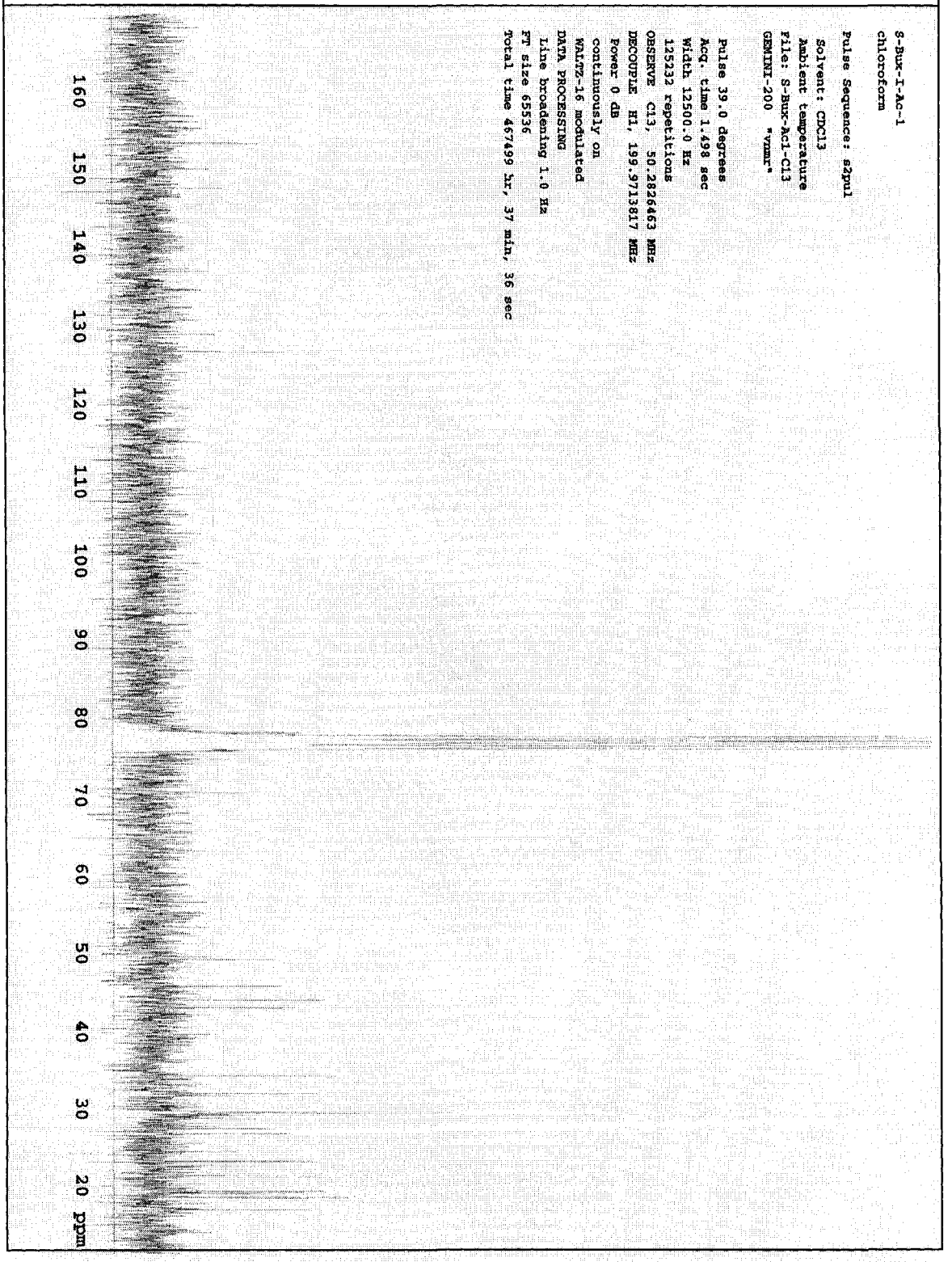
CIMS spectrum of arbora-1,9(11)-dien-3-one

UV spectrum of arbora-1,9(11)-dien-3-one

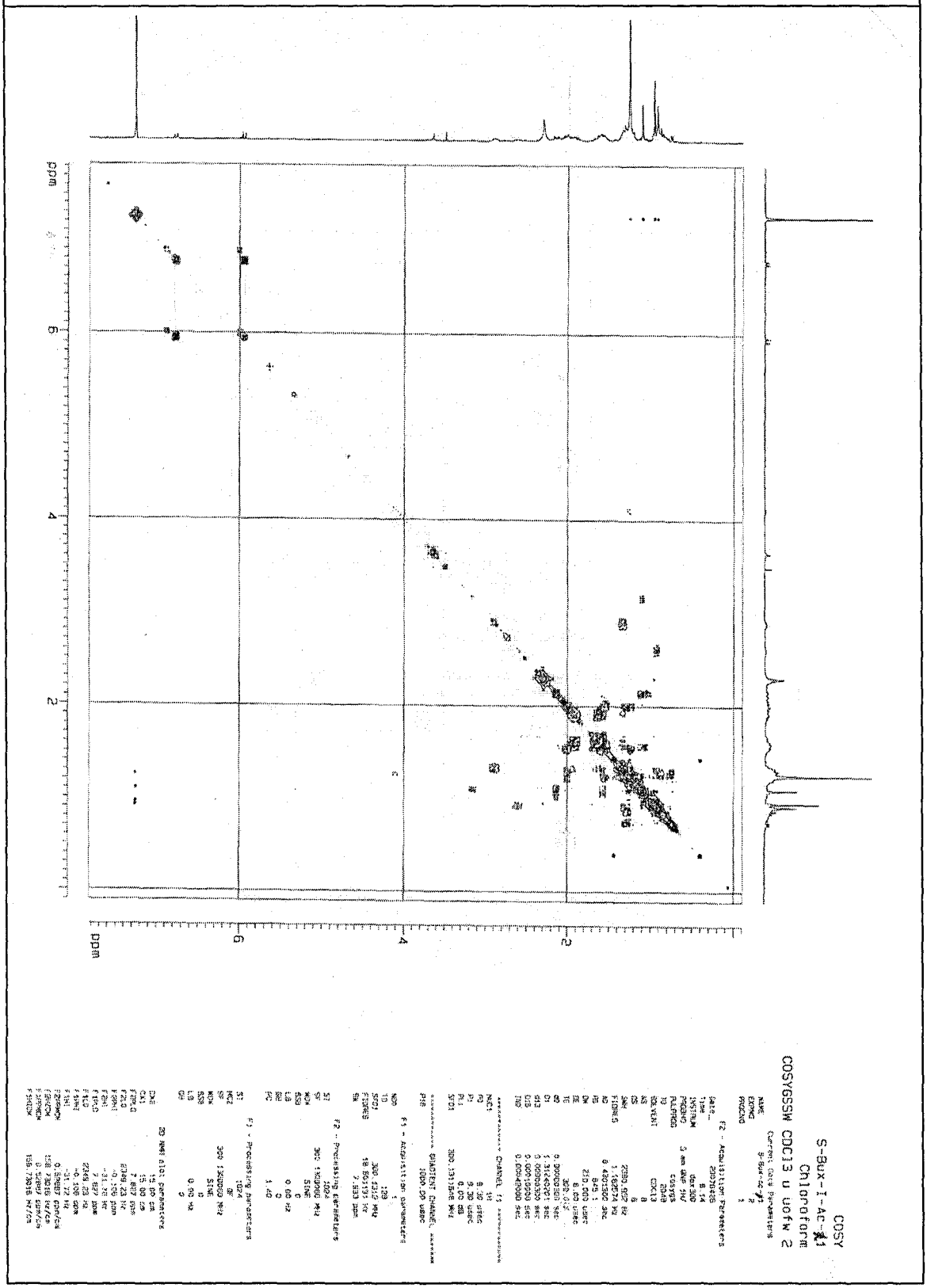


^{1}H-NMR spectrum of cyclobuxoviridine

^{13}C-NMR spectrum of cyclobuxoviridine



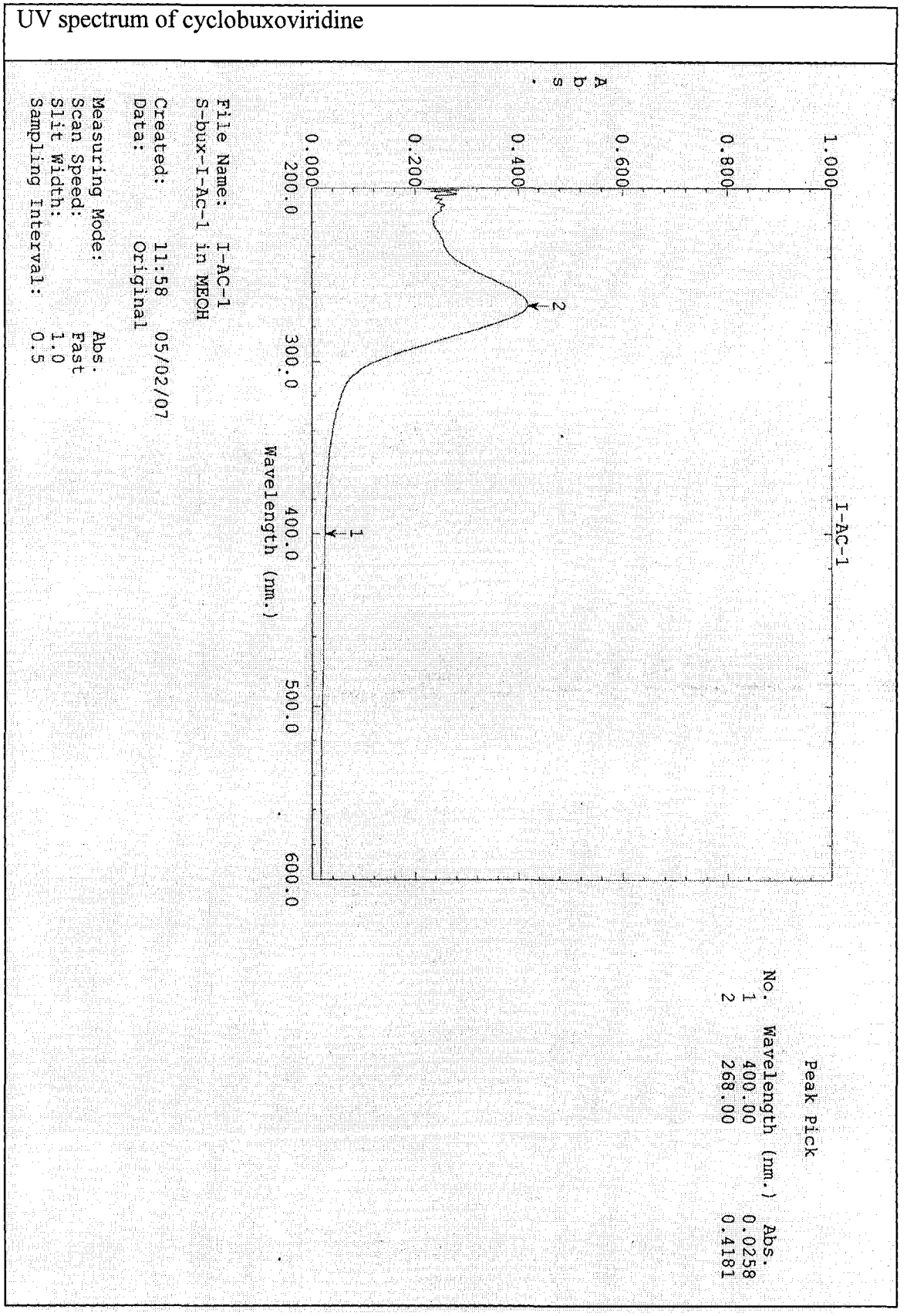
COSY-45° spectrum of cyclobuxoviridine



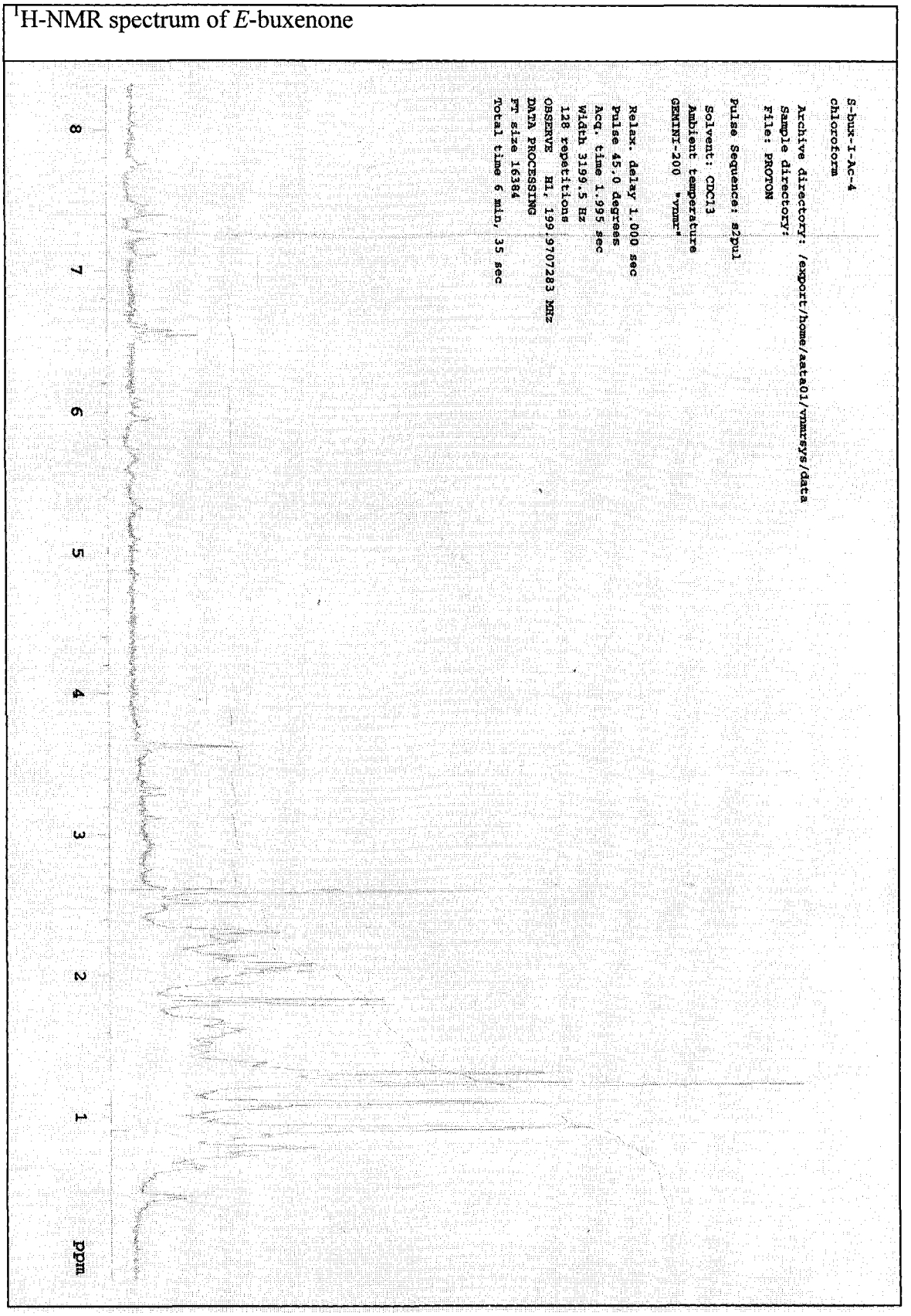
EIMS spectrum of cyclobuxoviridine

CIMS spectrum of cyclobuxoviridine

UV spectrum of cyclobuxoviridine



^{1}H-NMR spectrum of *E*-buxenone



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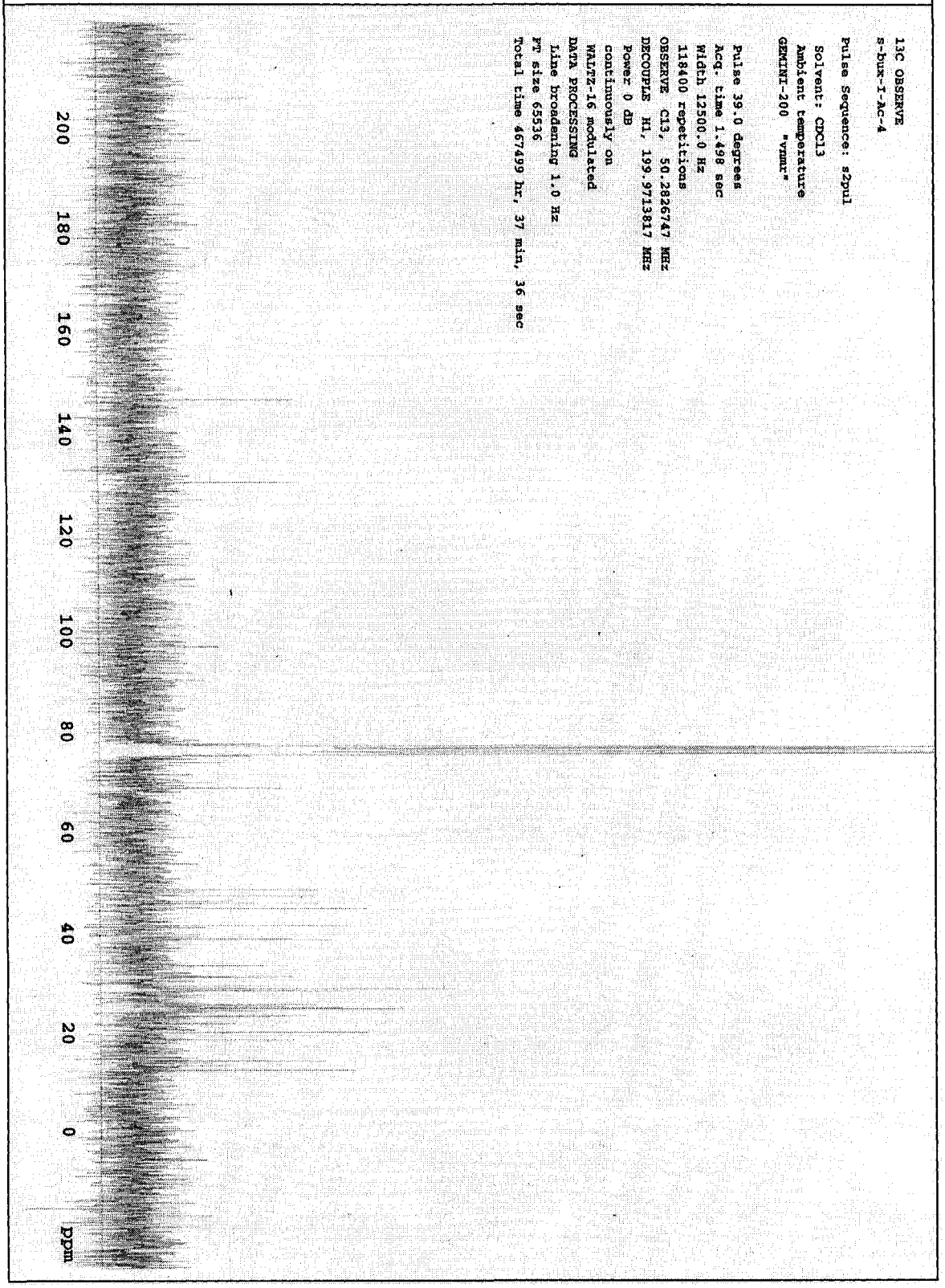

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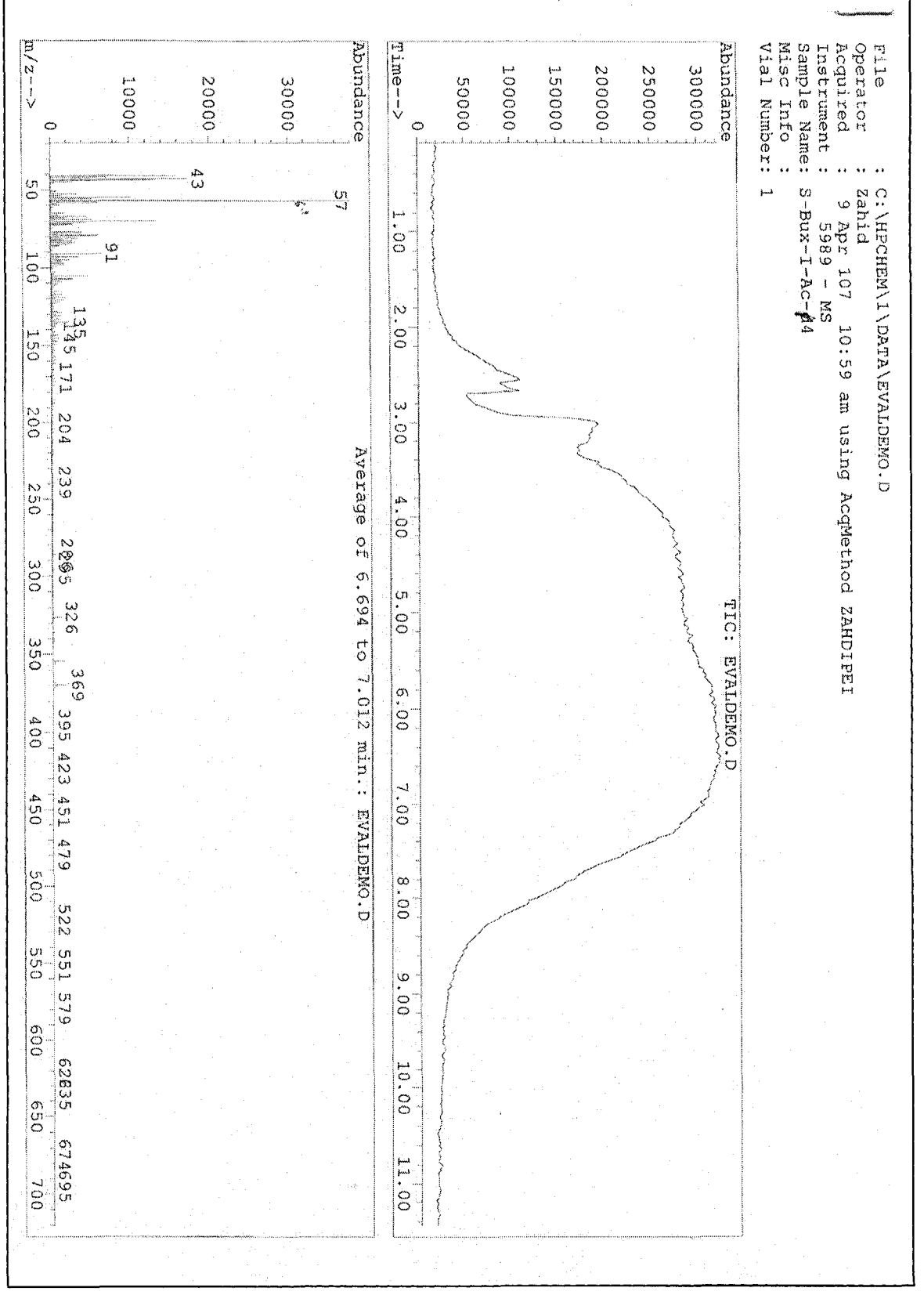

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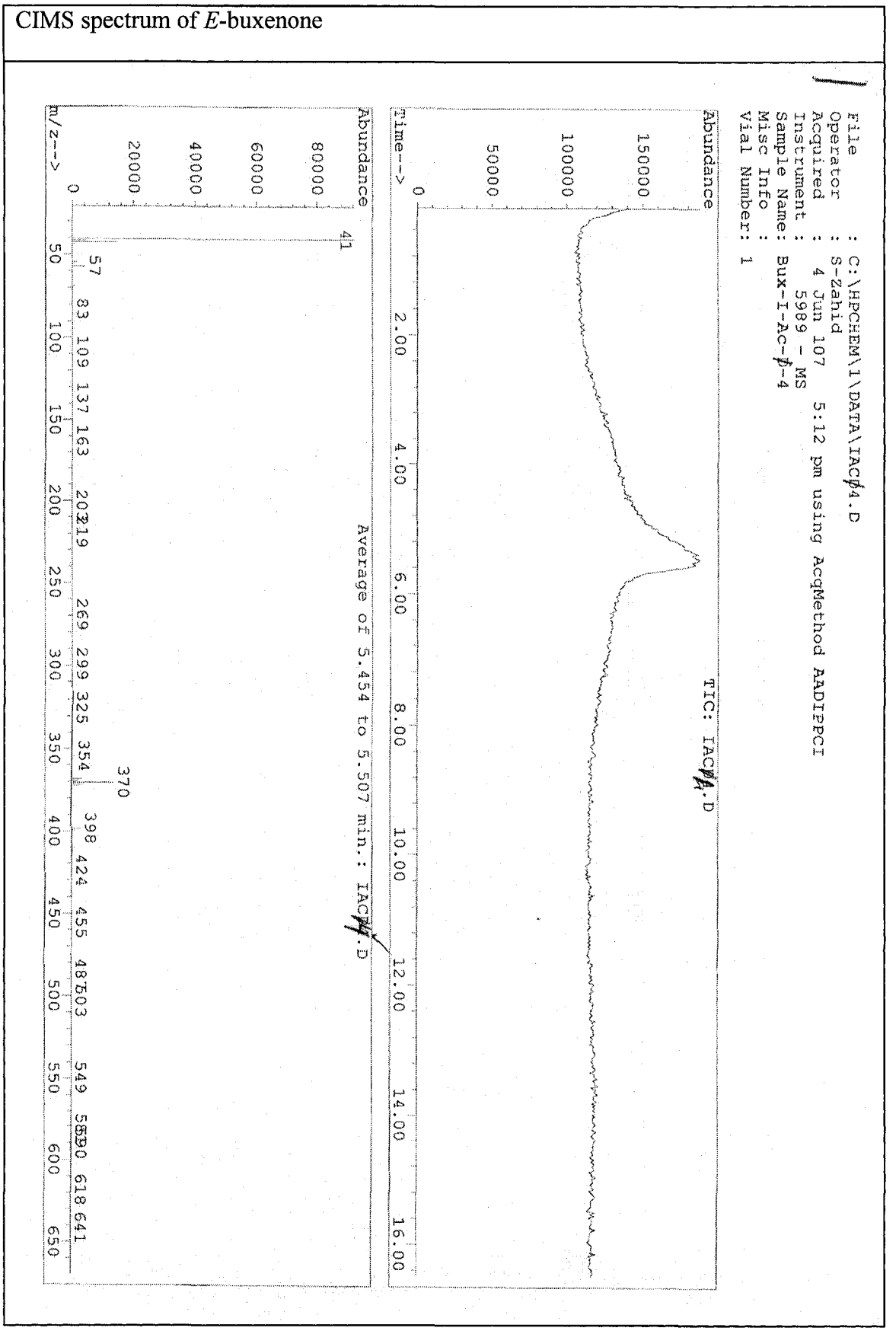
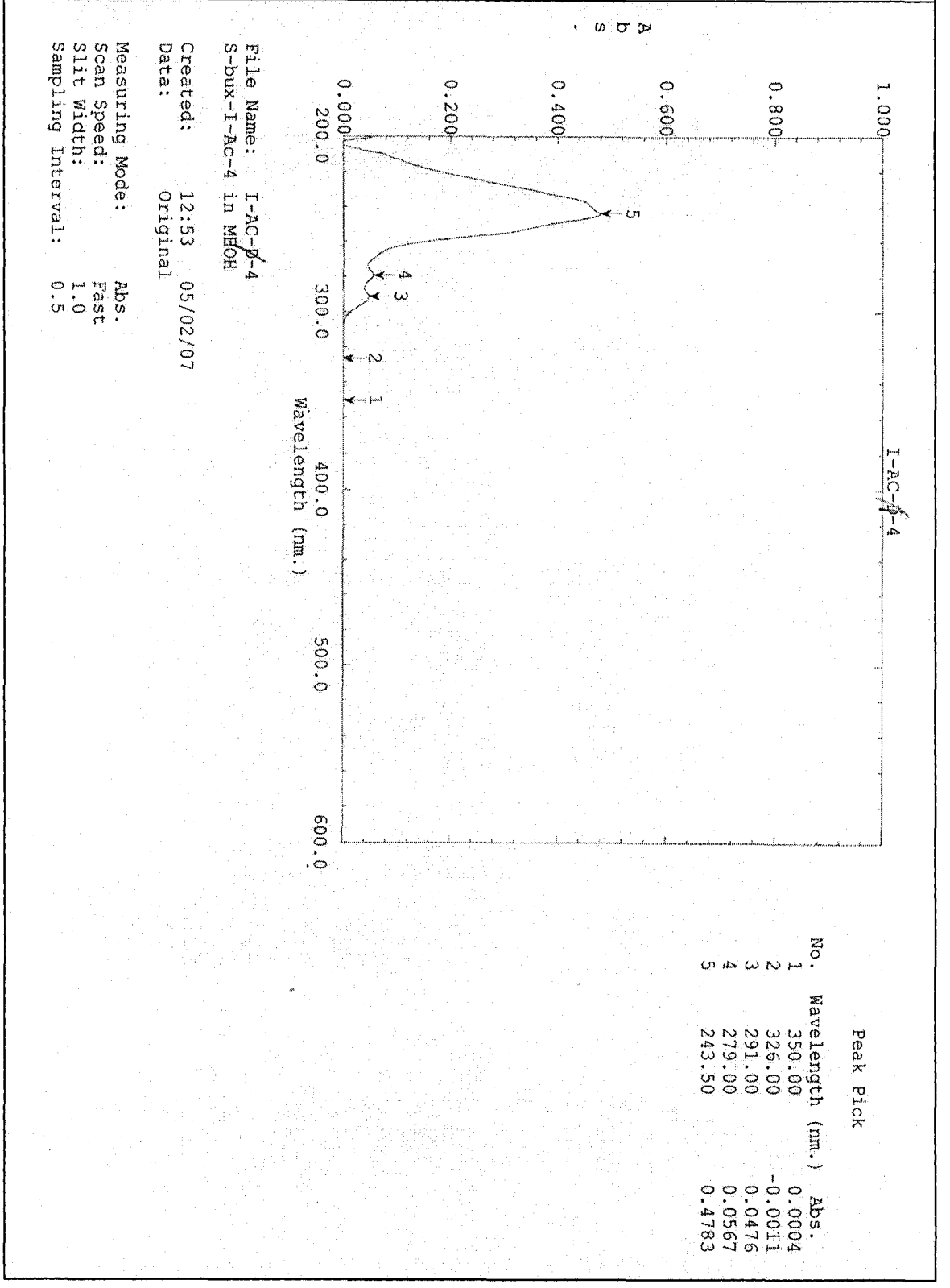


UV spectrum of *E*-buxenone



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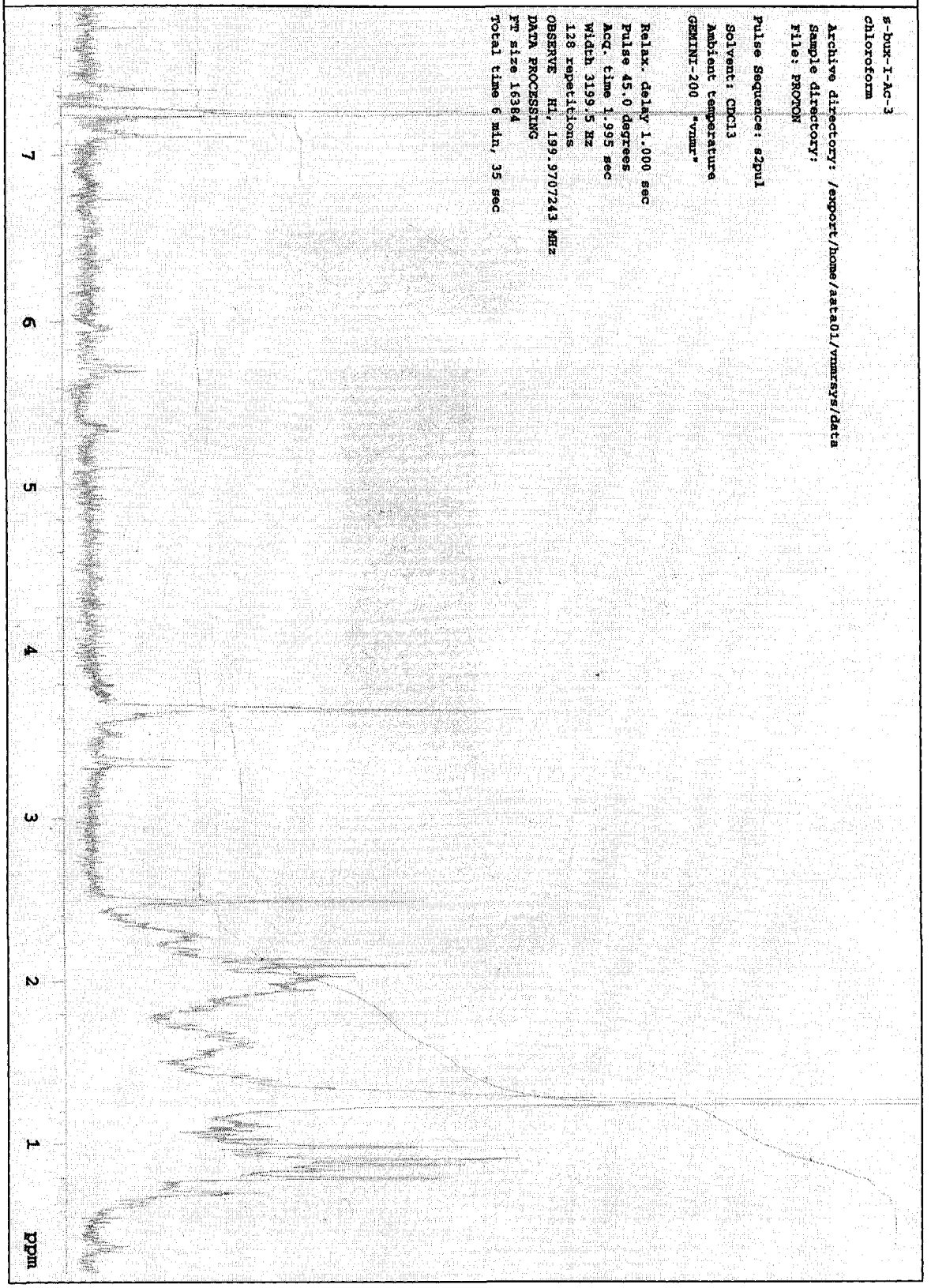

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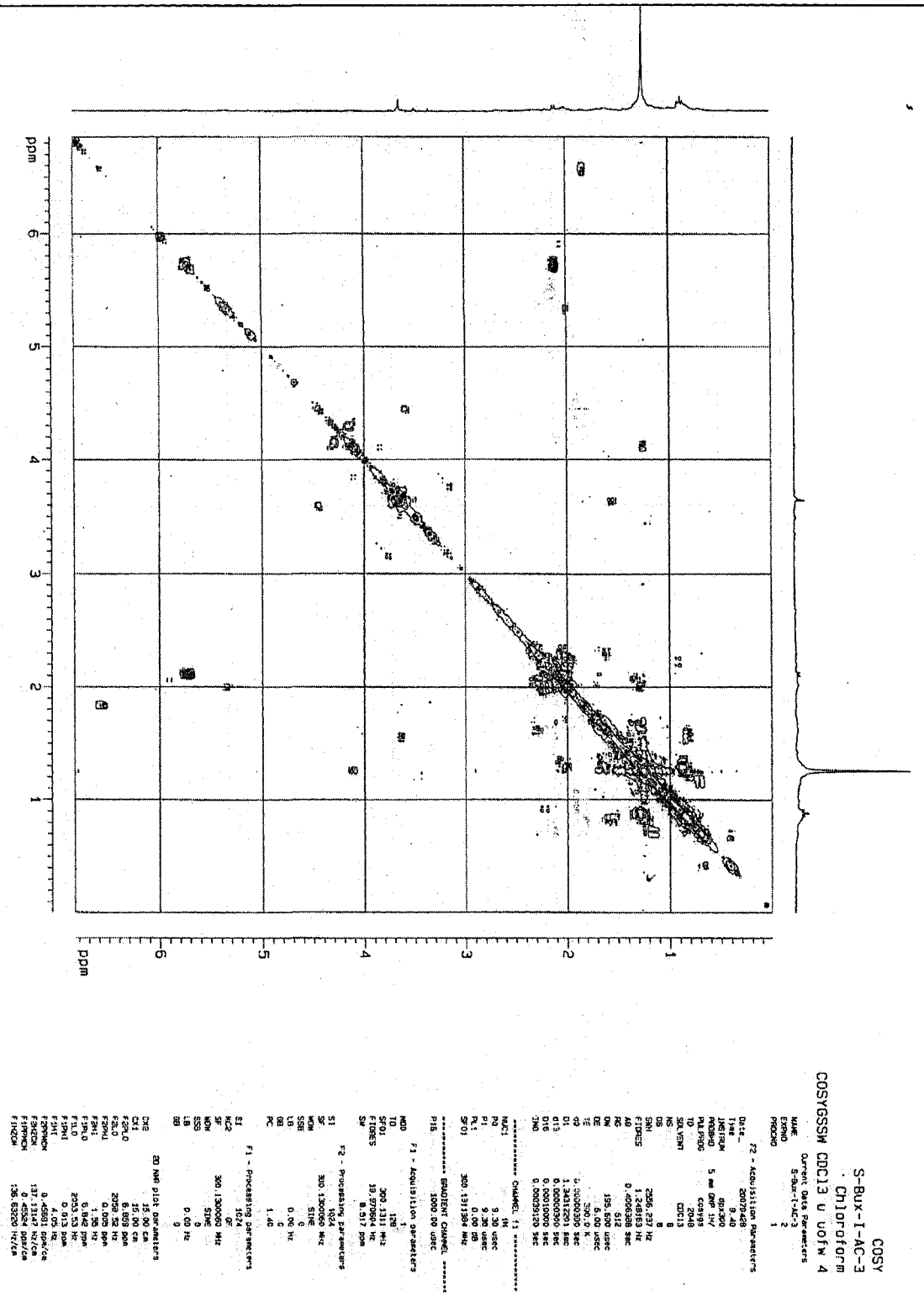
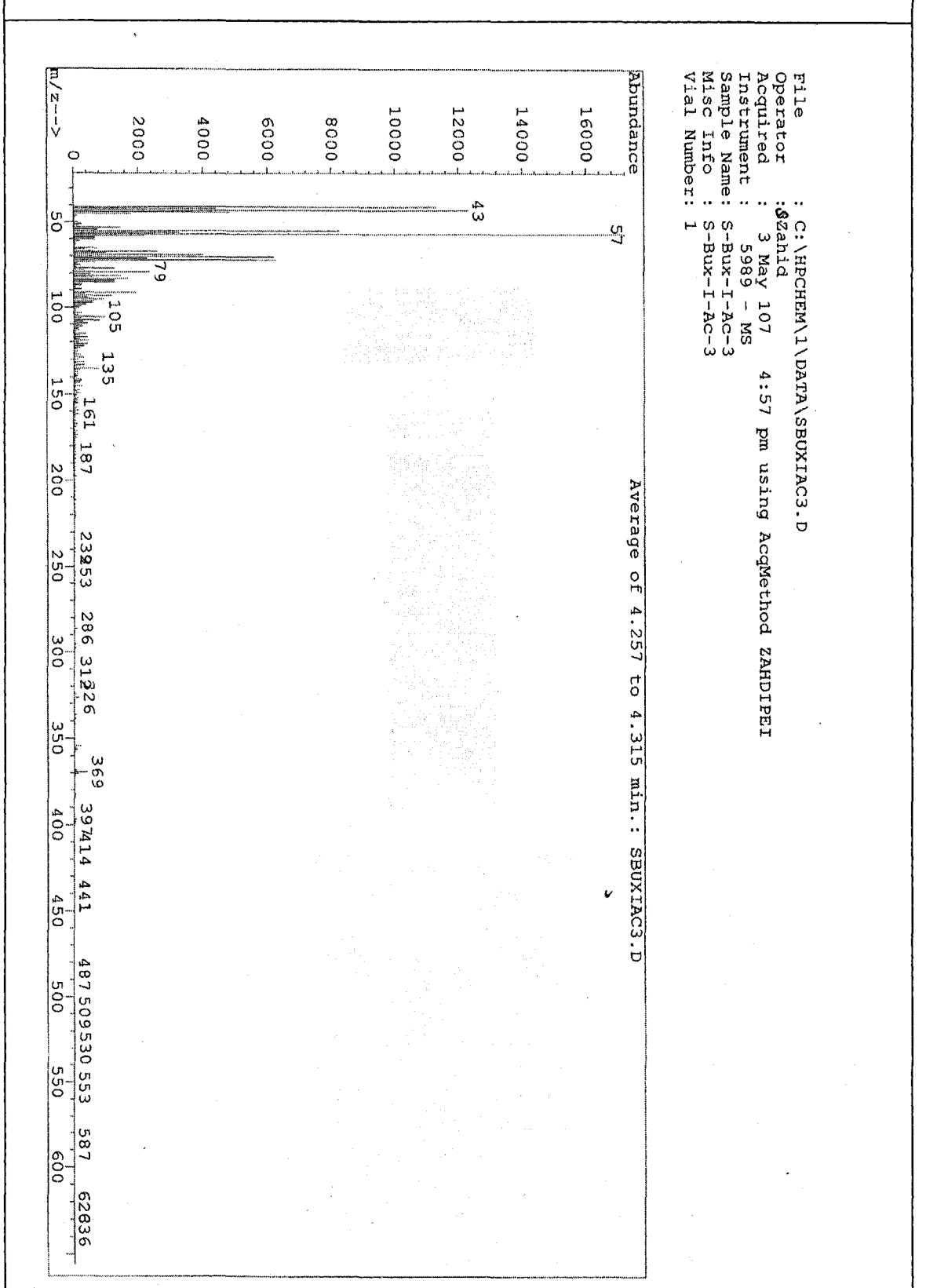
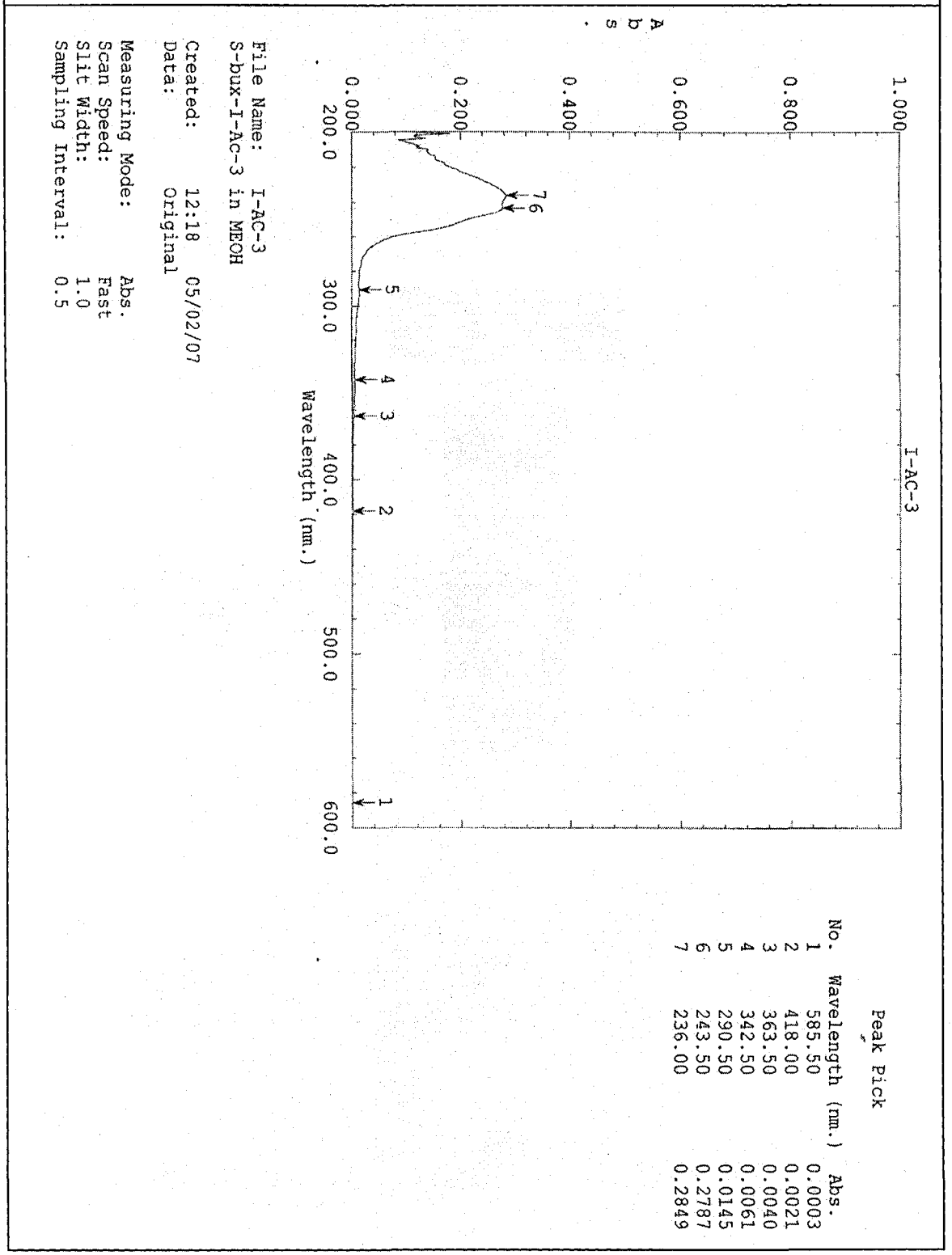


EIMS spectrum of *Z*- buxenone

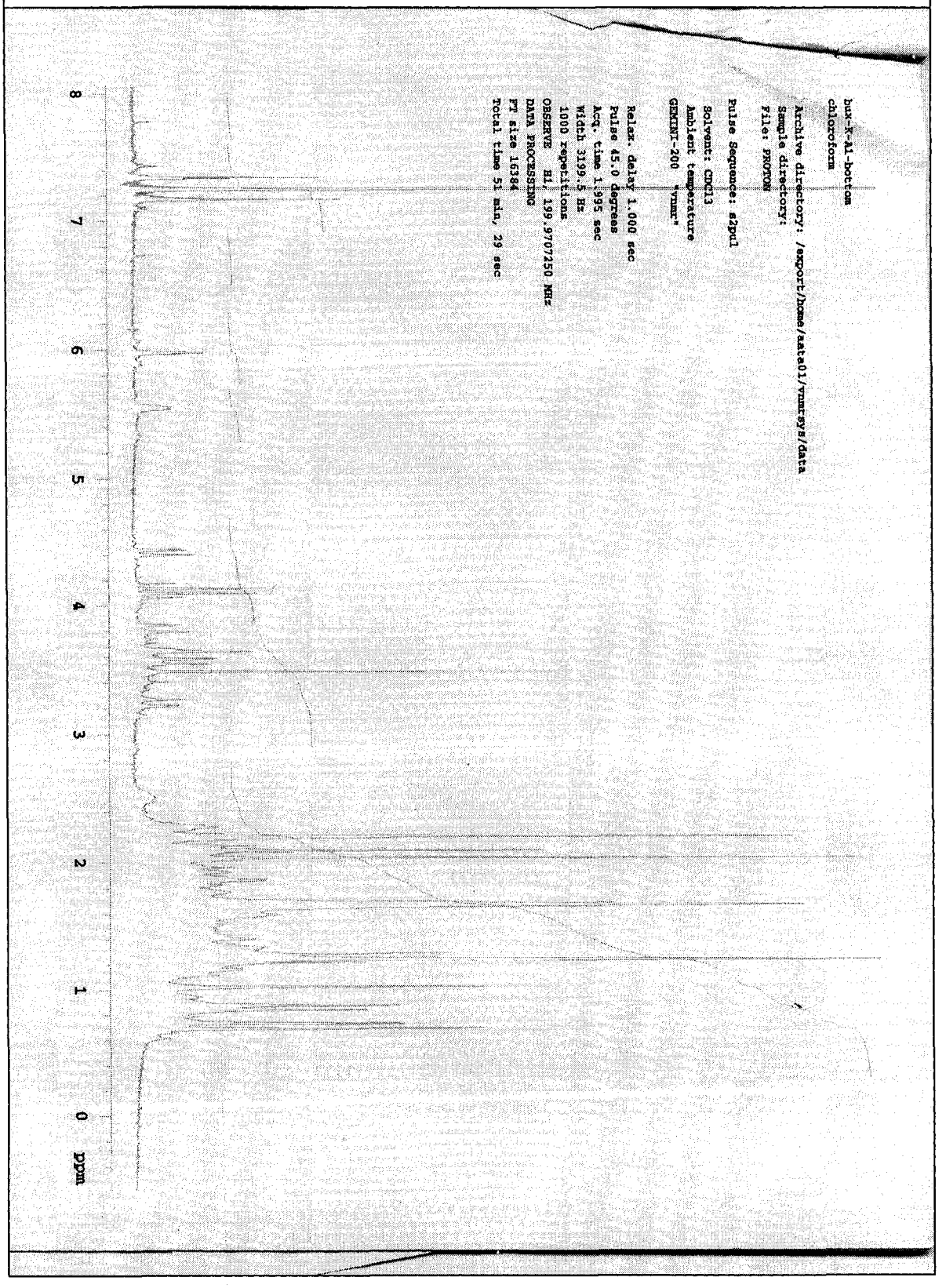


CIMS spectrum of *Z*- buxenone

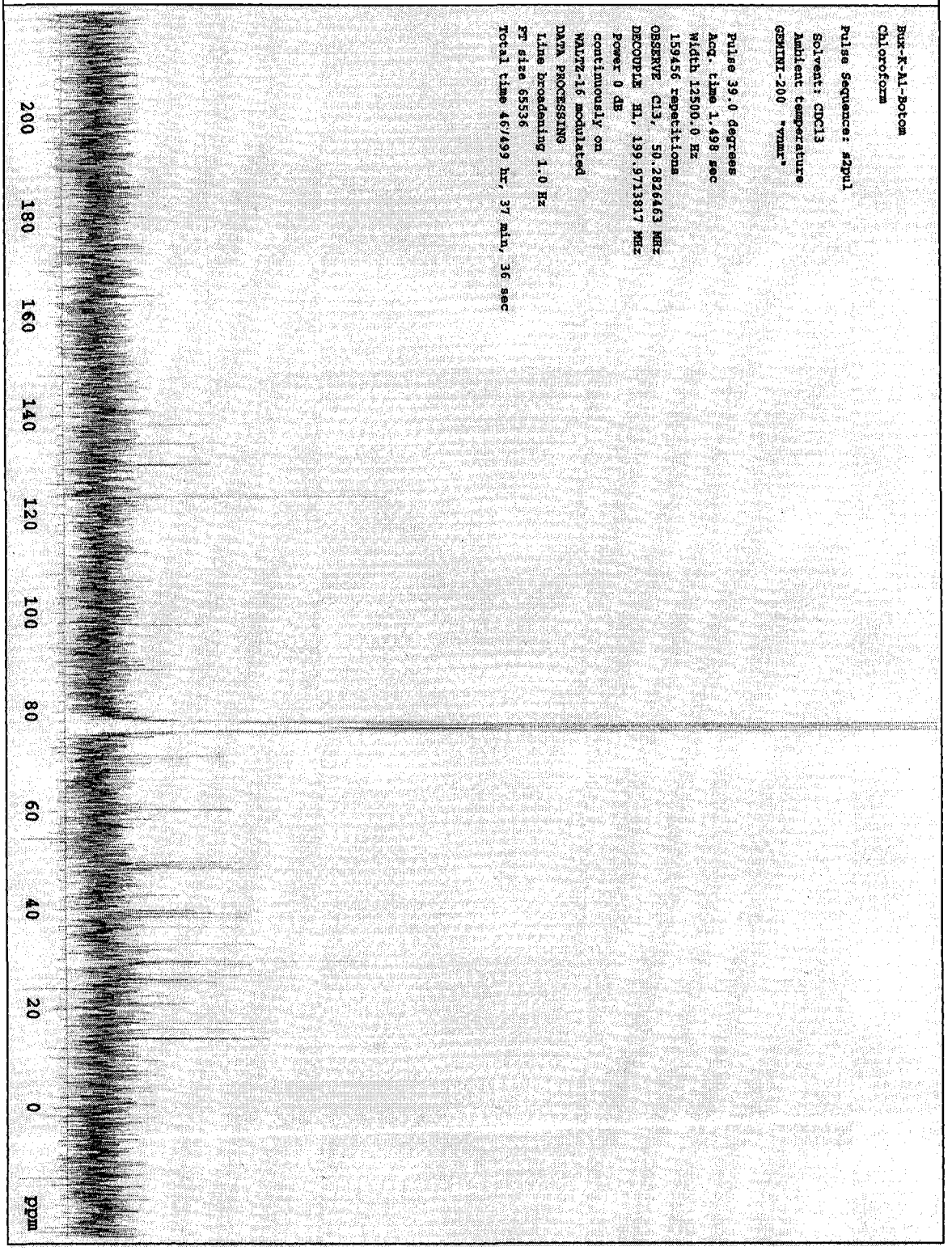
UV spectrum of *Z*- buxenone



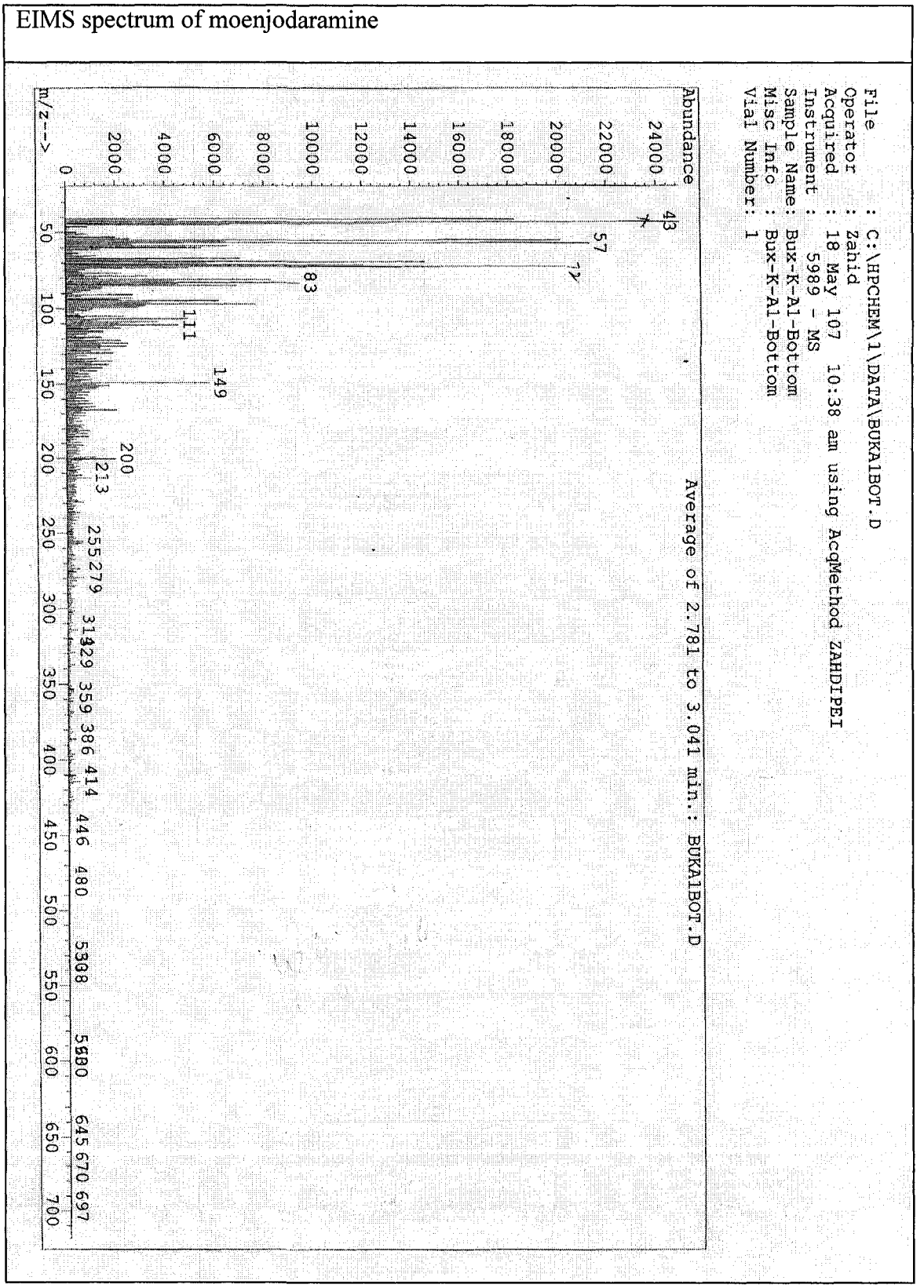
^{1}H-NMR spectrum of moenjodaramine



^{13}C-NMR spectrum of moenjodaramine

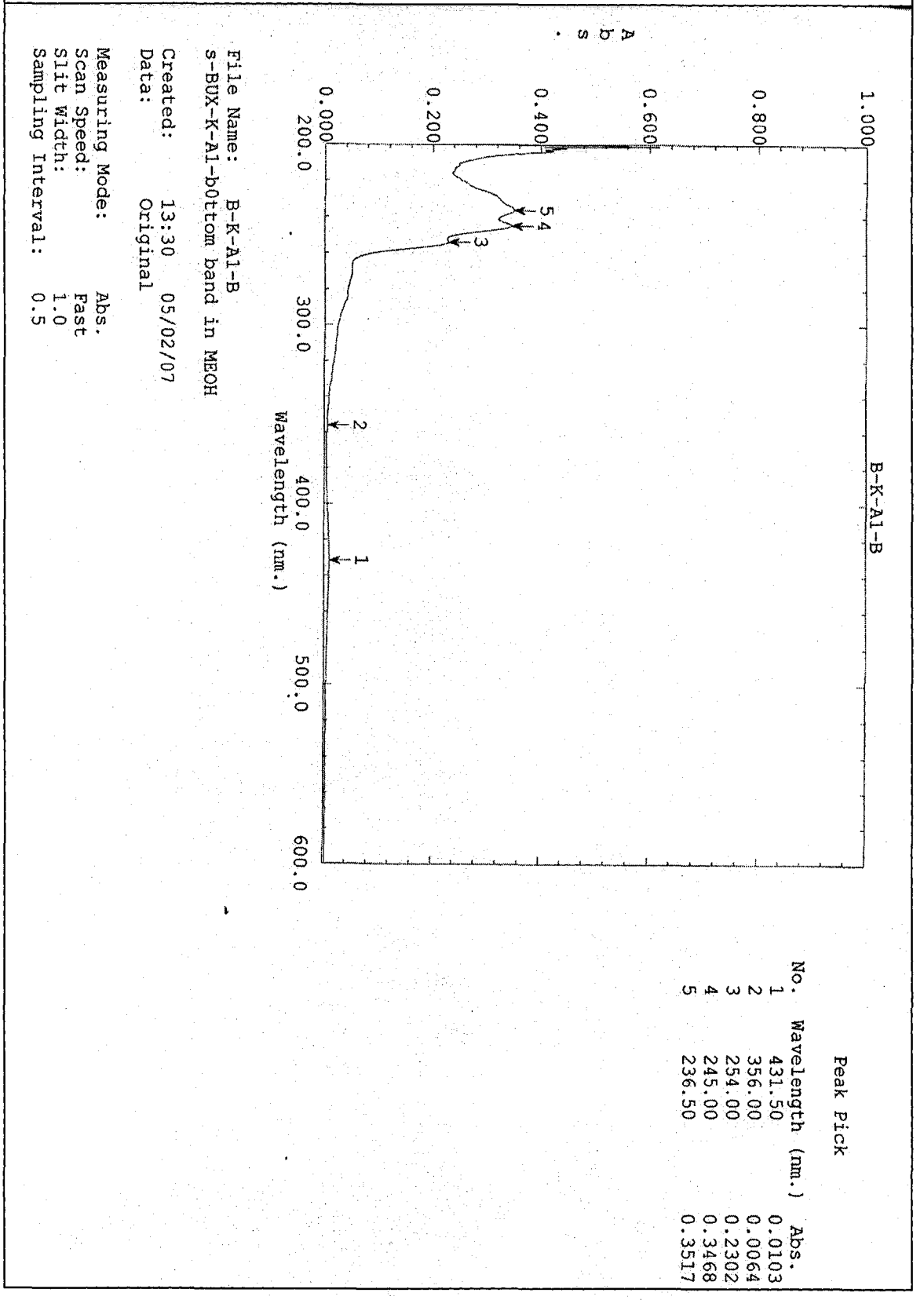


EIMS spectrum of moenjodaramine

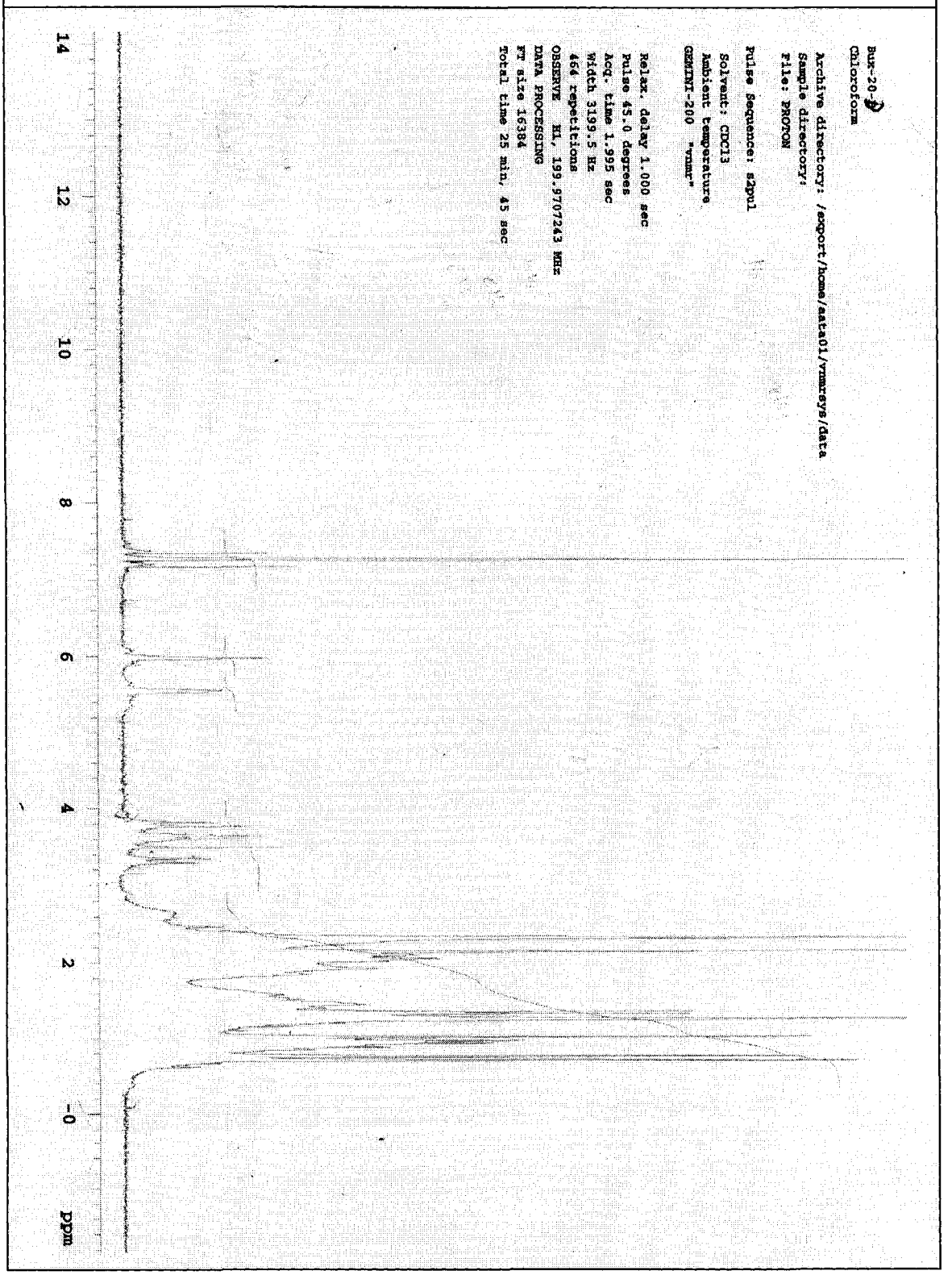


CIMS spectrum of moenjodaramine

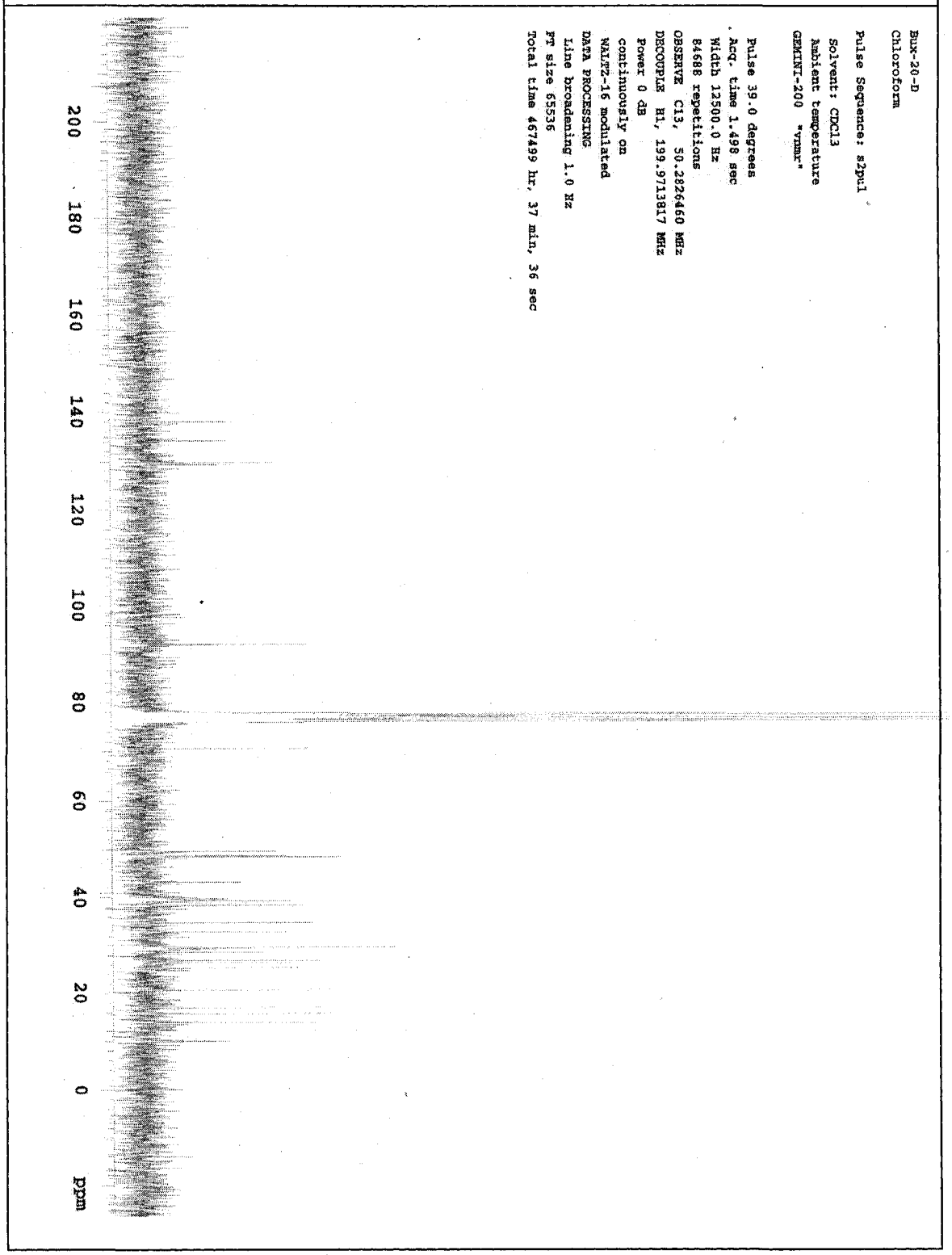
UV spectrum of moenjodaramine



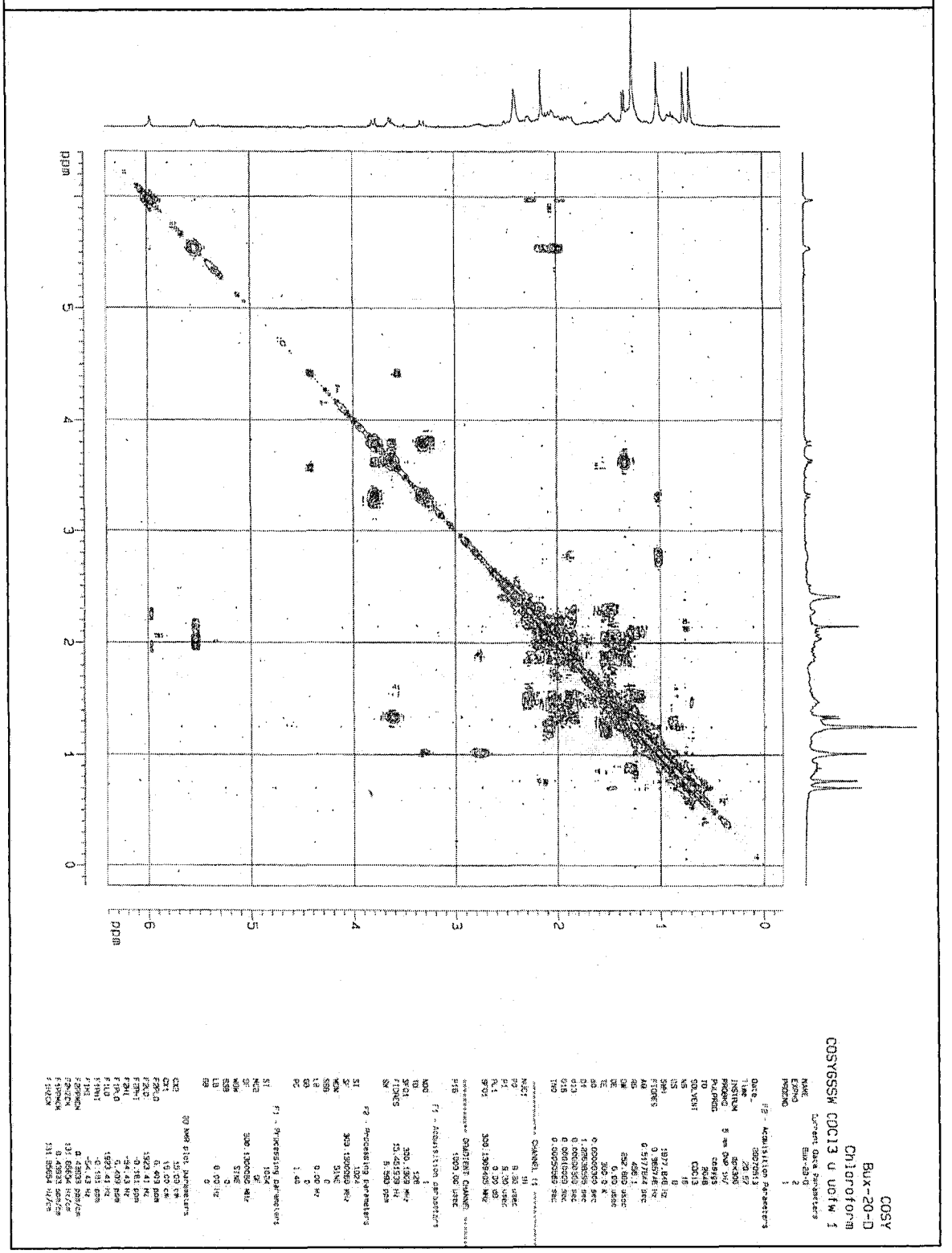
^{1}H-NMR spectrum of homomoenjodaramine



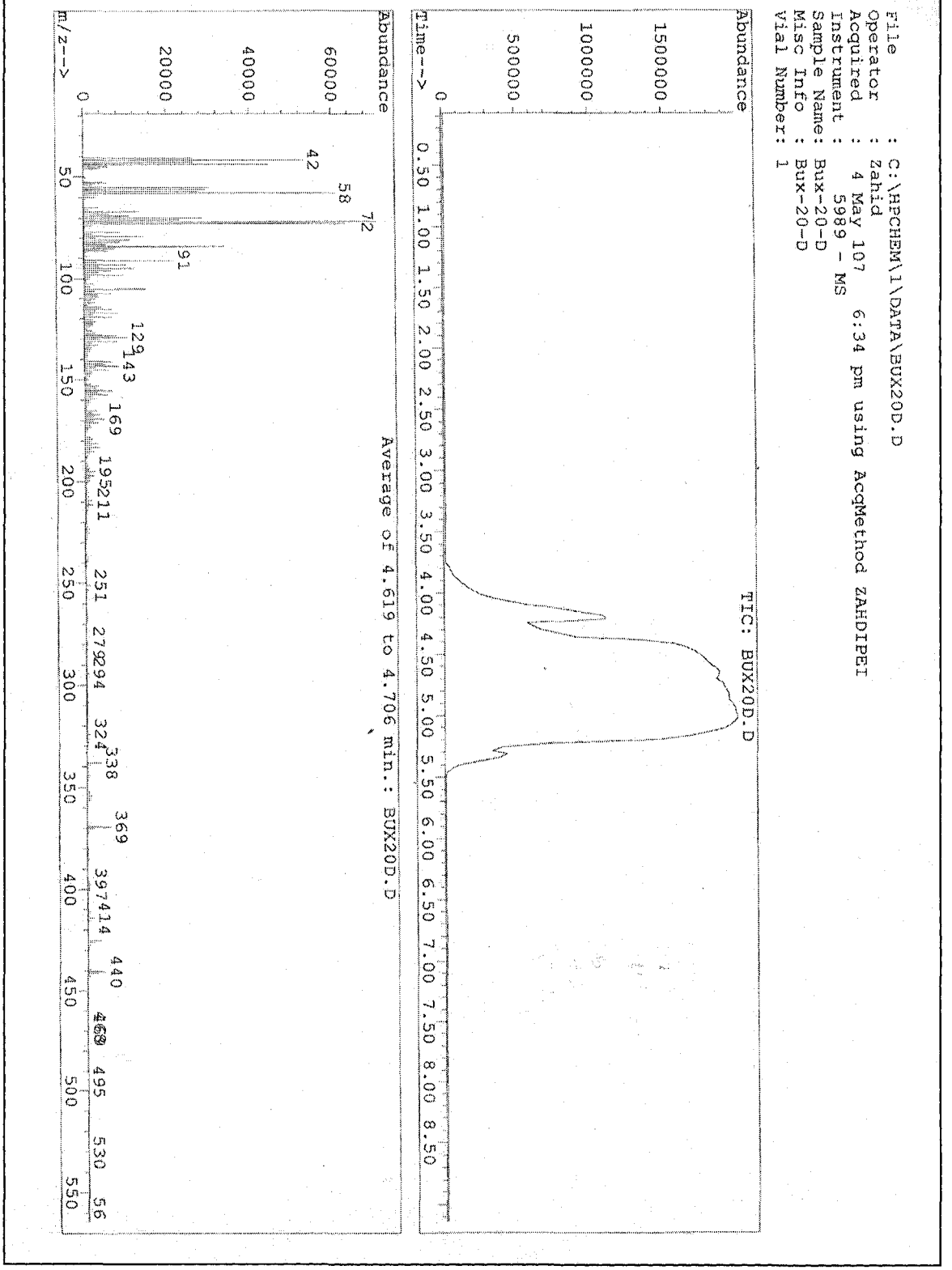
^{13}C-NMR spectrum of homomoenjodaramine



COSY-45° spectrum of homomoenjodaramine

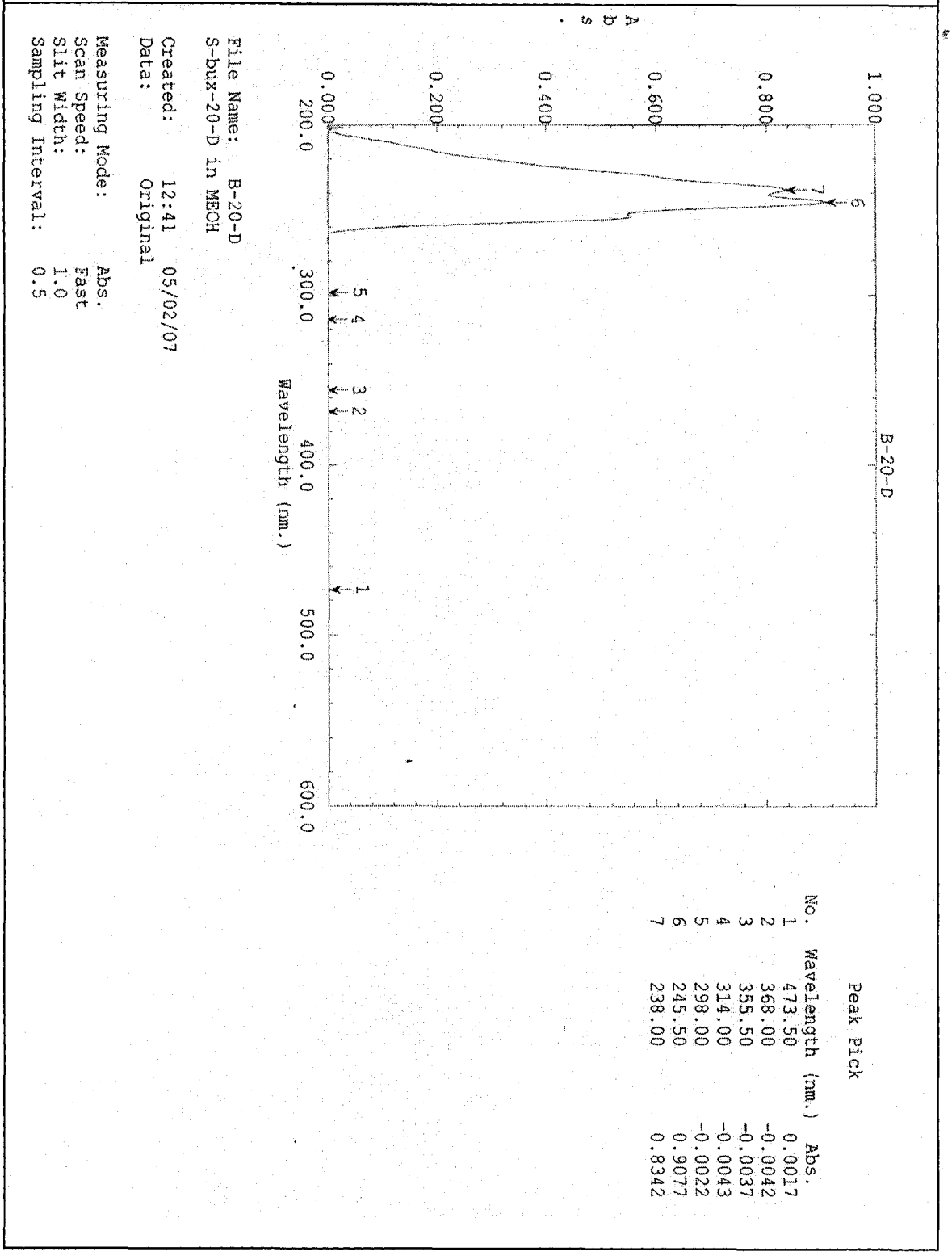


EIMS spectrum of homomoenjodaramine

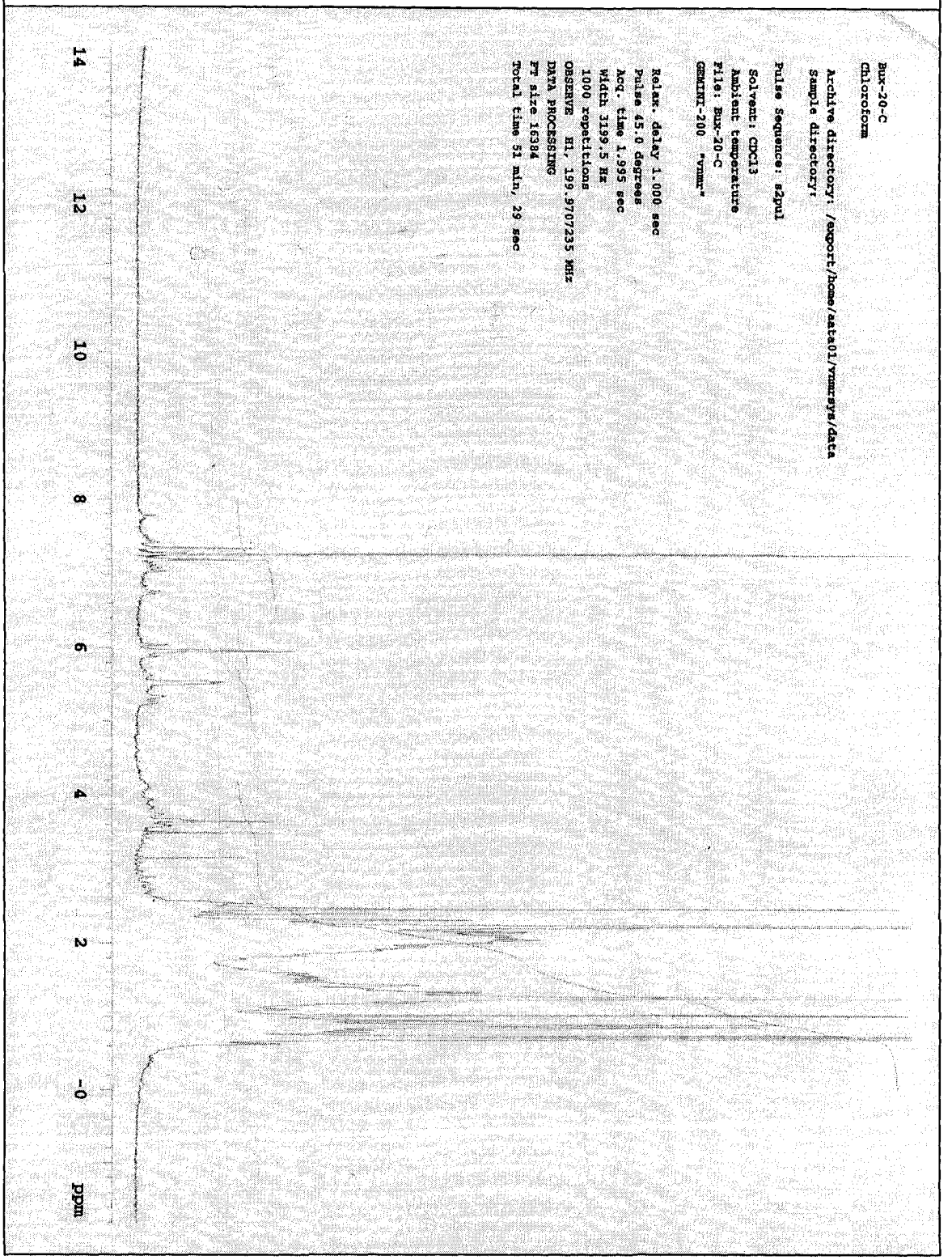


CIMS spectrum of homomoenjodaramine

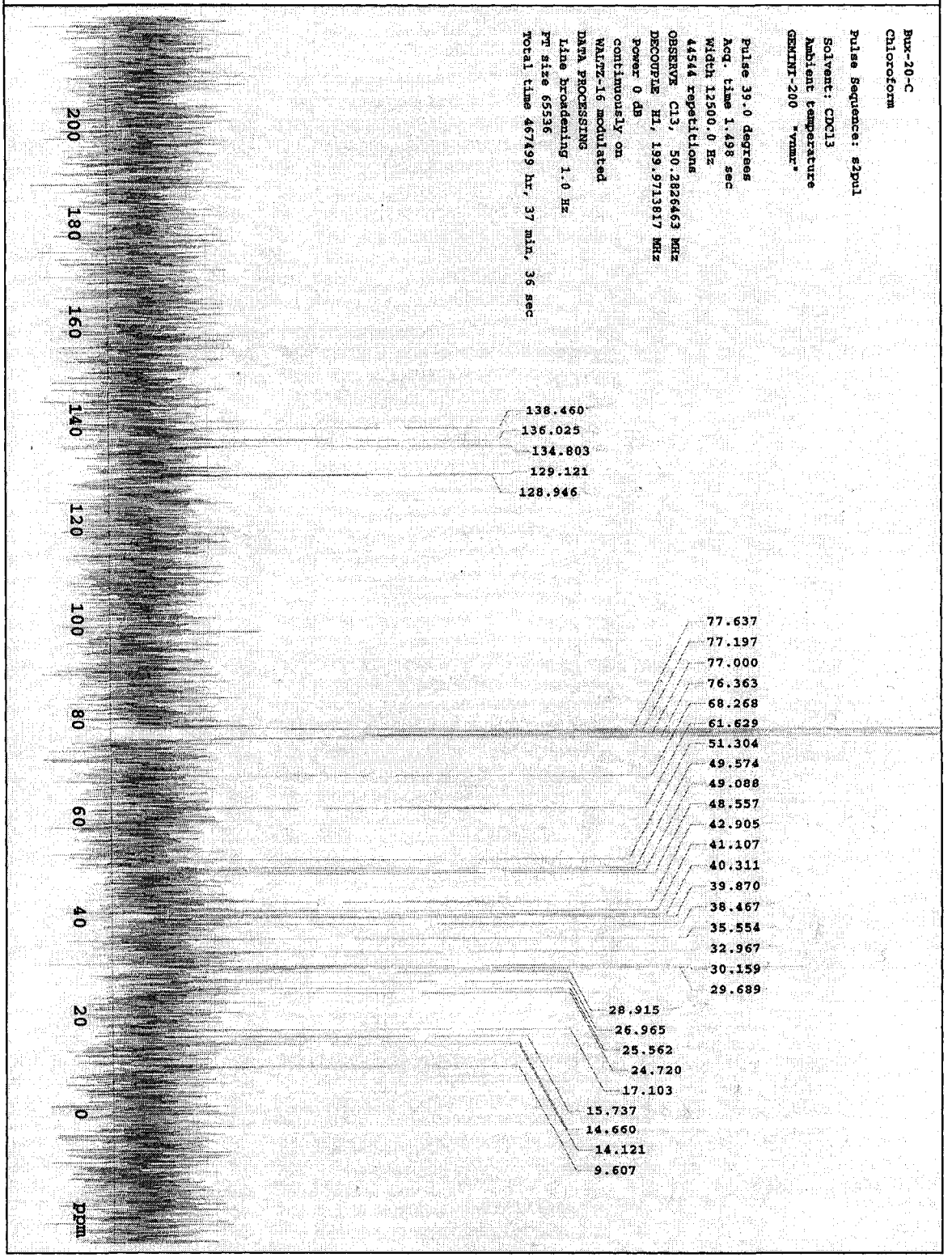
UV spectrum of homomoenjodaramine



^{1}H-NMR spectrum of buxamine-B

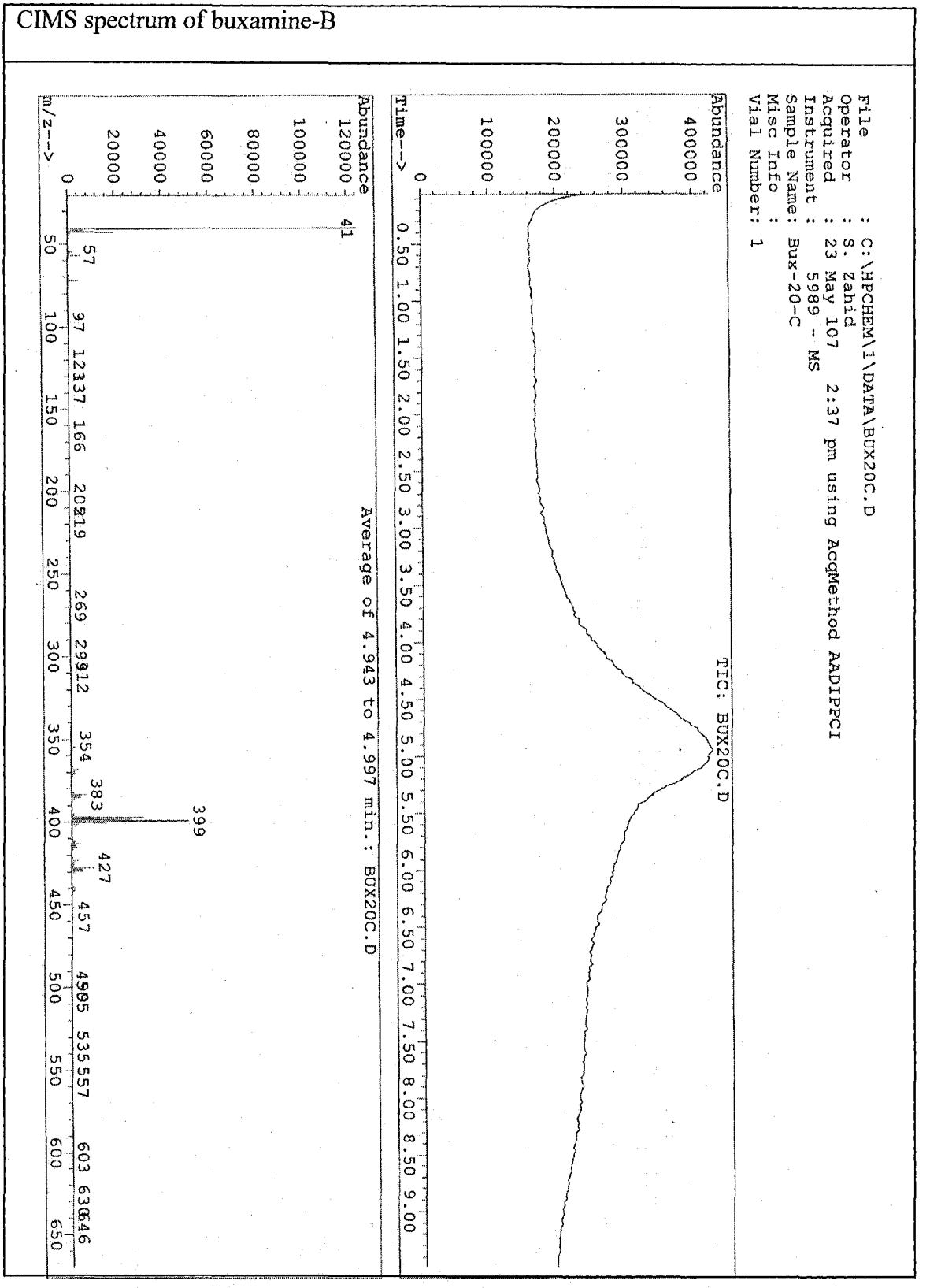


^{13}C-NMR spectrum of buxamine-B

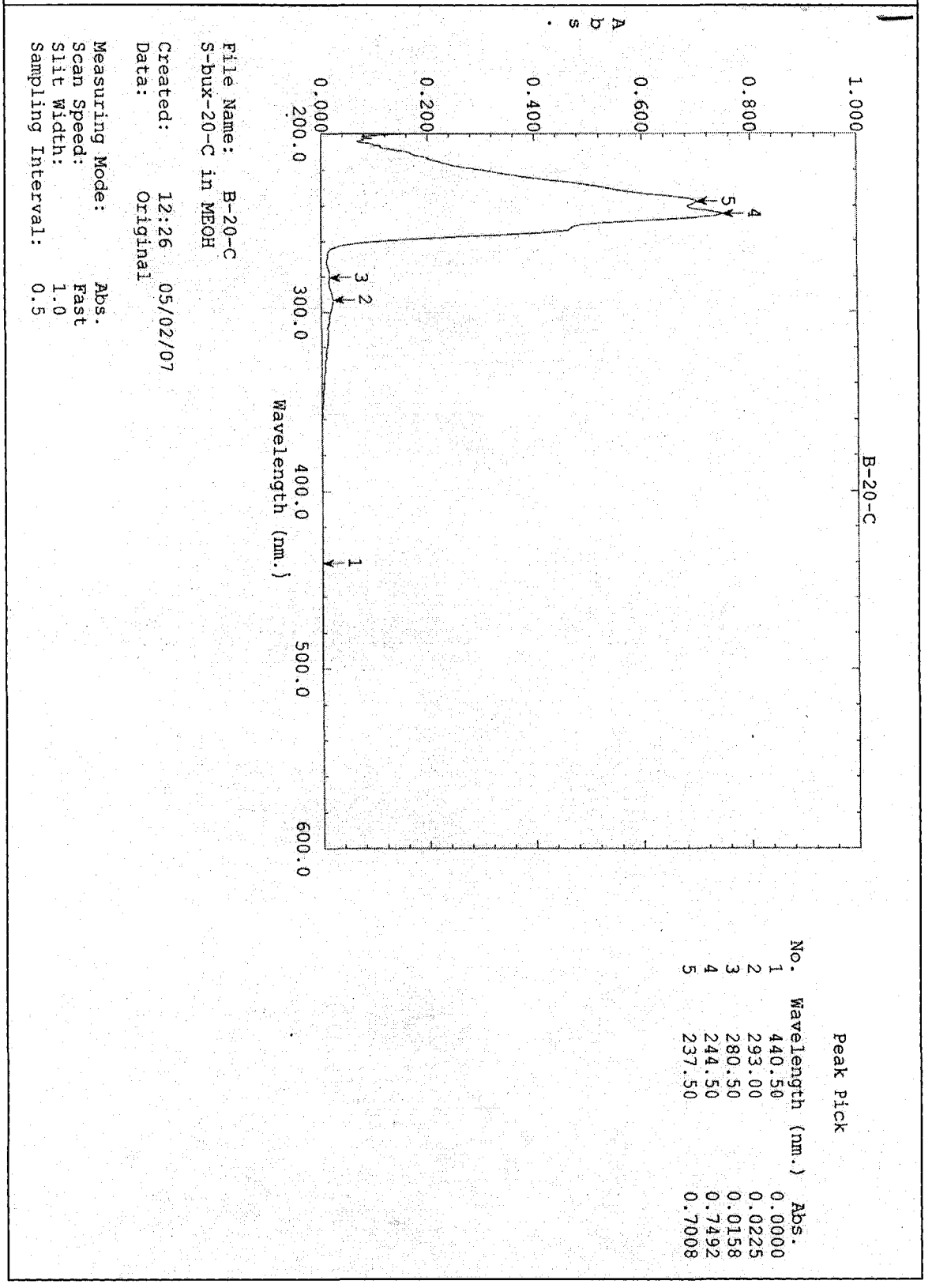


EIMS spectrum of buxamine-B

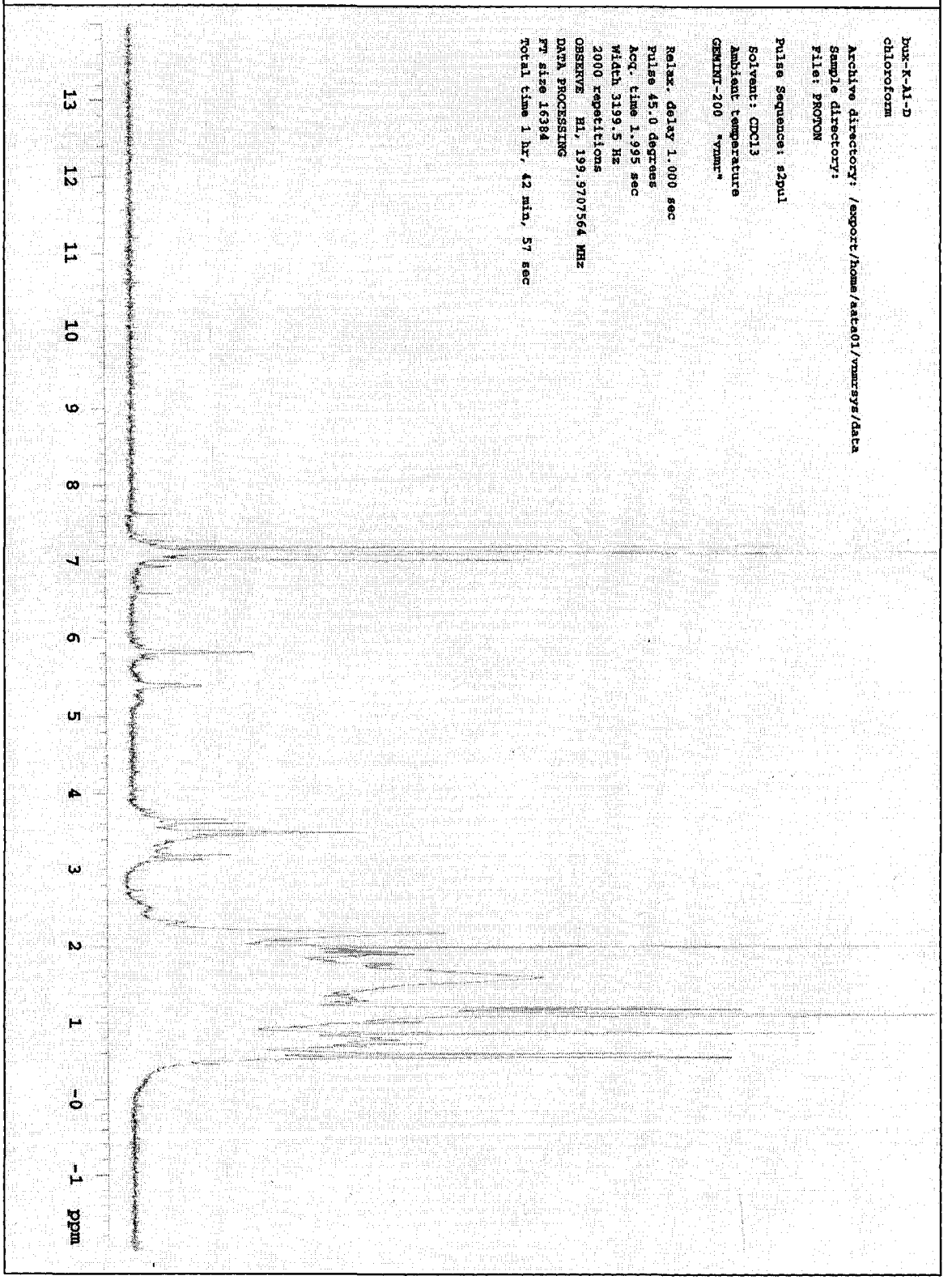
CIMS spectrum of buxamine-B



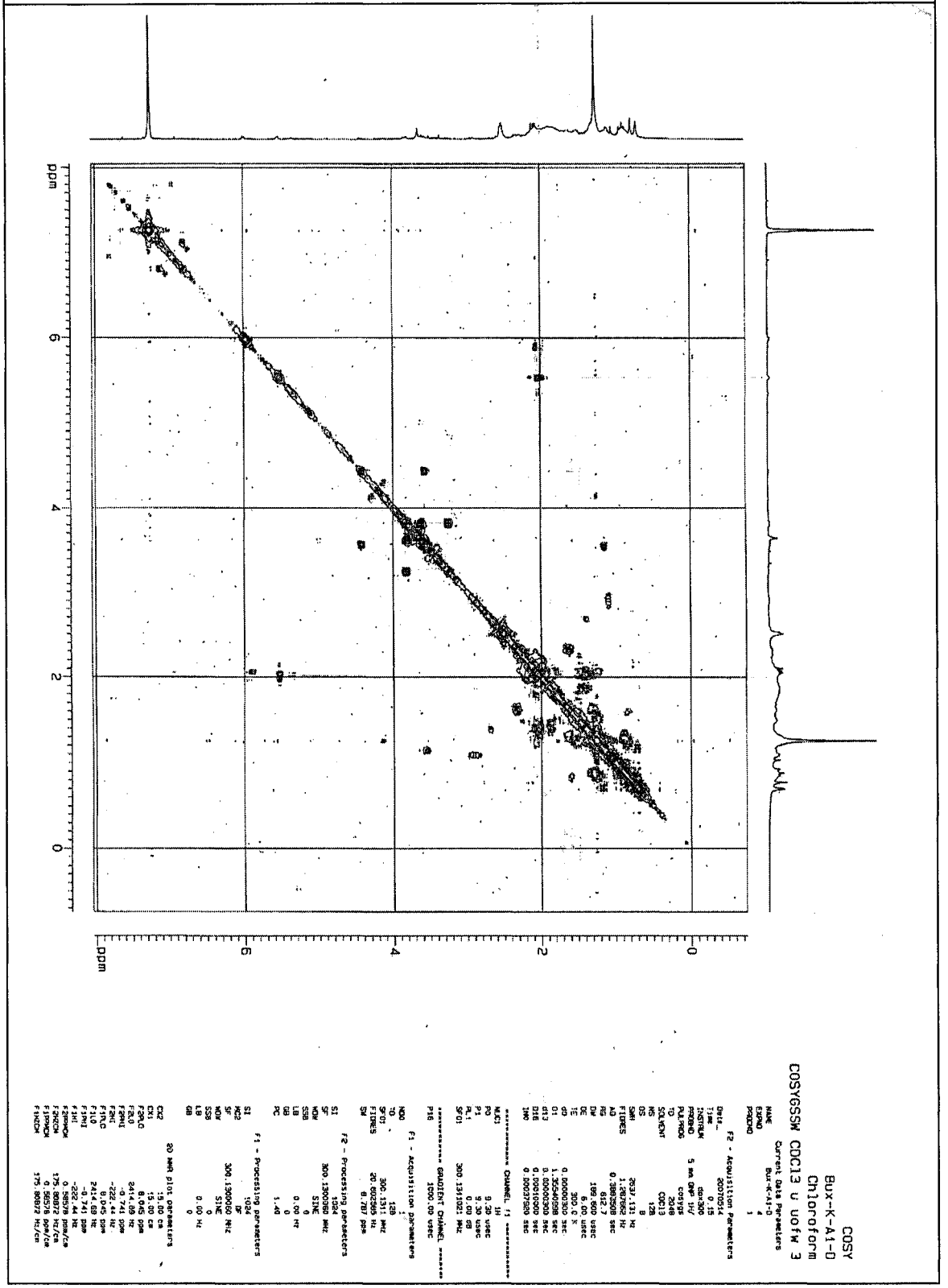
UV spectrum of buxamine-B



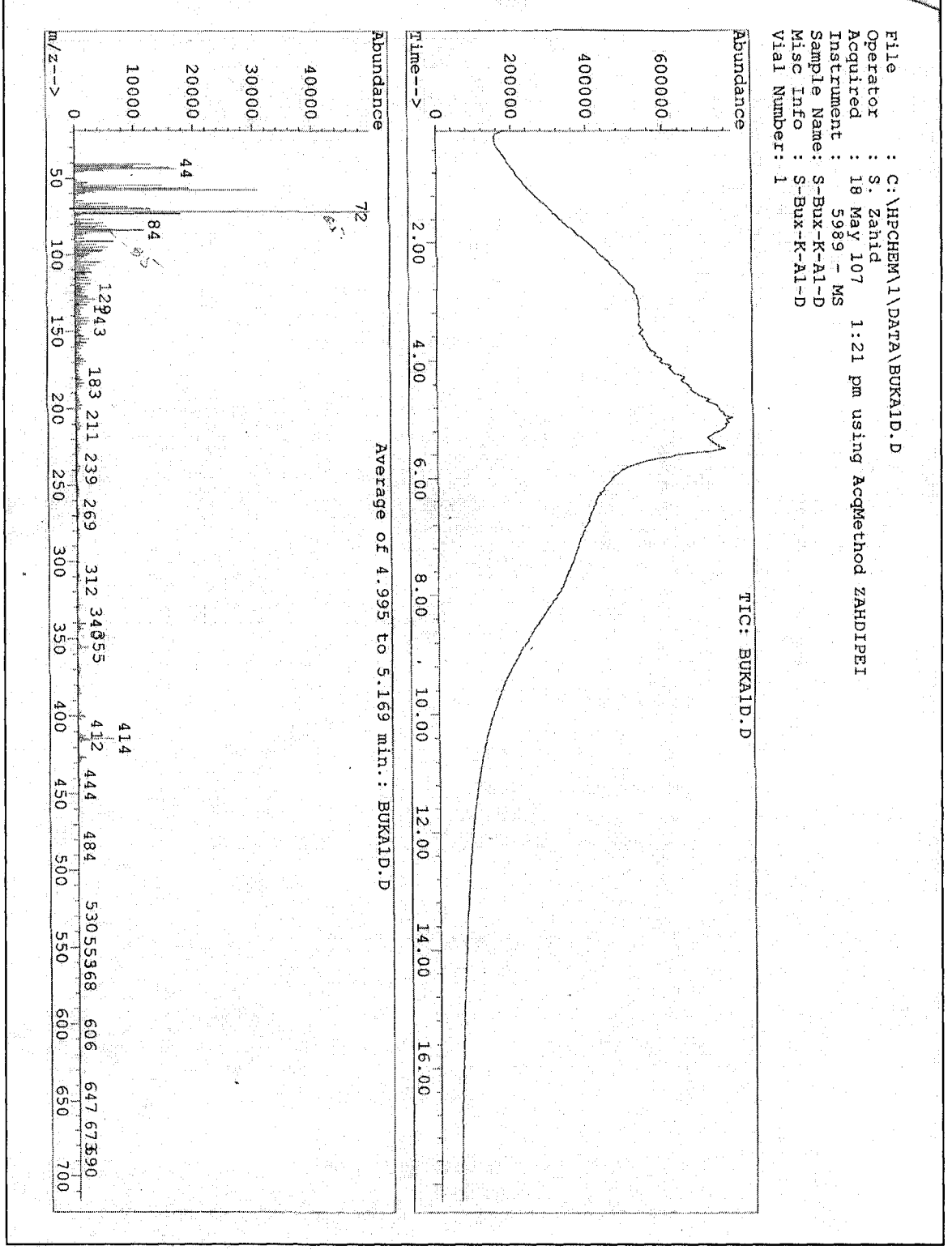
^{1}H-NMR spectrum of 31-hydroxybuxamine-B



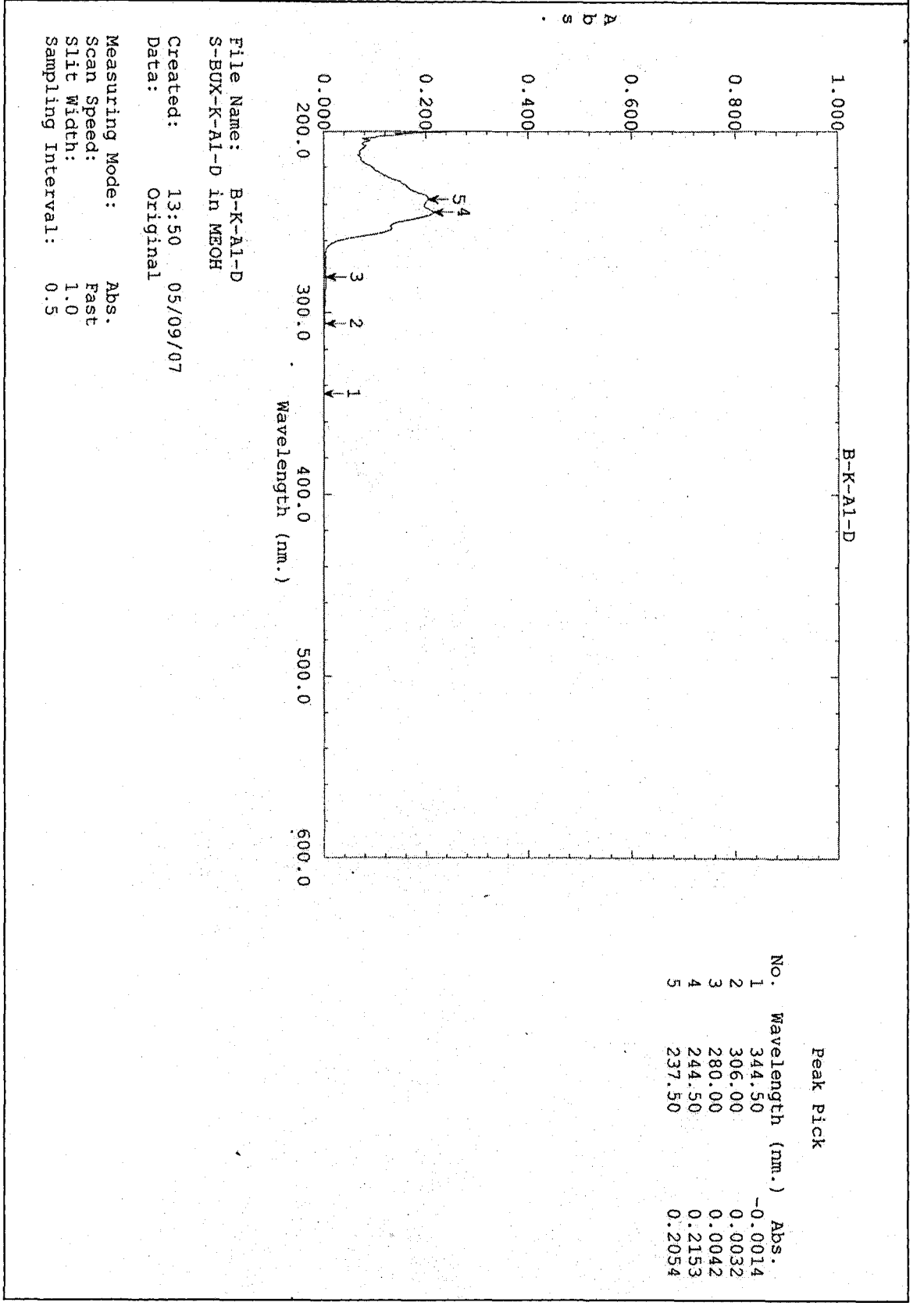
COSY-45° spectrum of 31-hydroxybuxamine-B



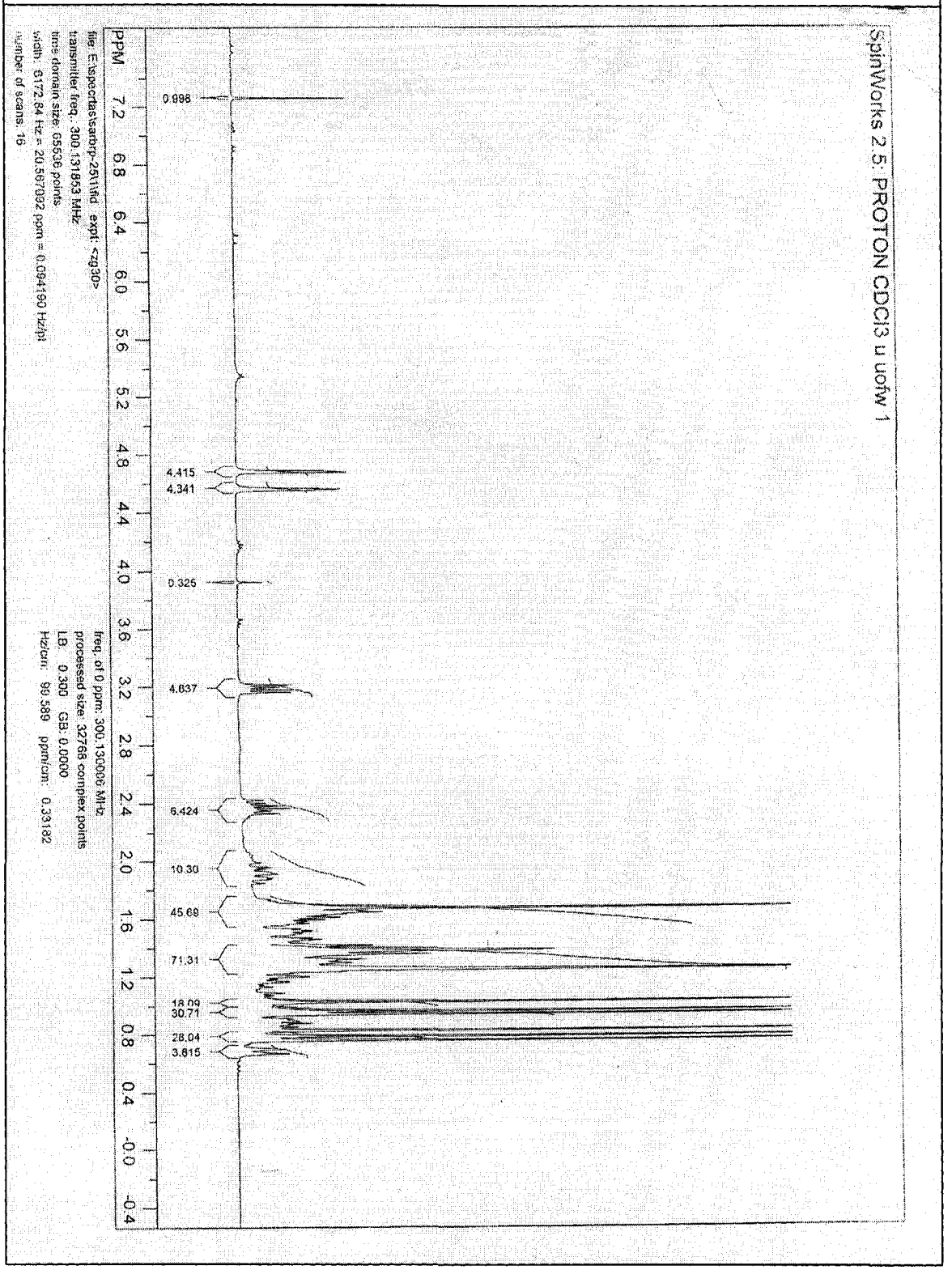
EIMS spectrum of 31-hydroxybuxamine-B



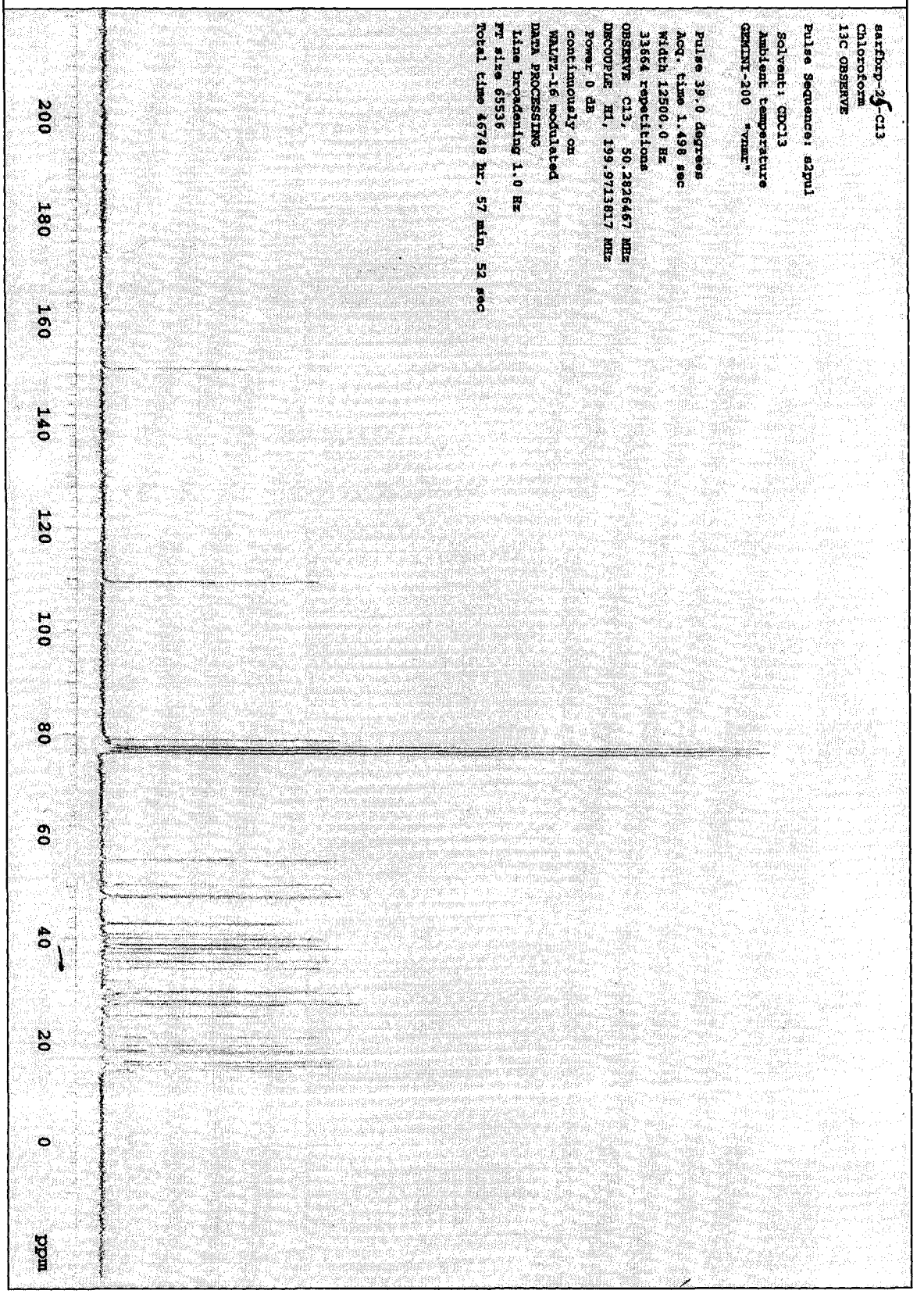
UV spectrum of 31-hydroxybuxamine-B



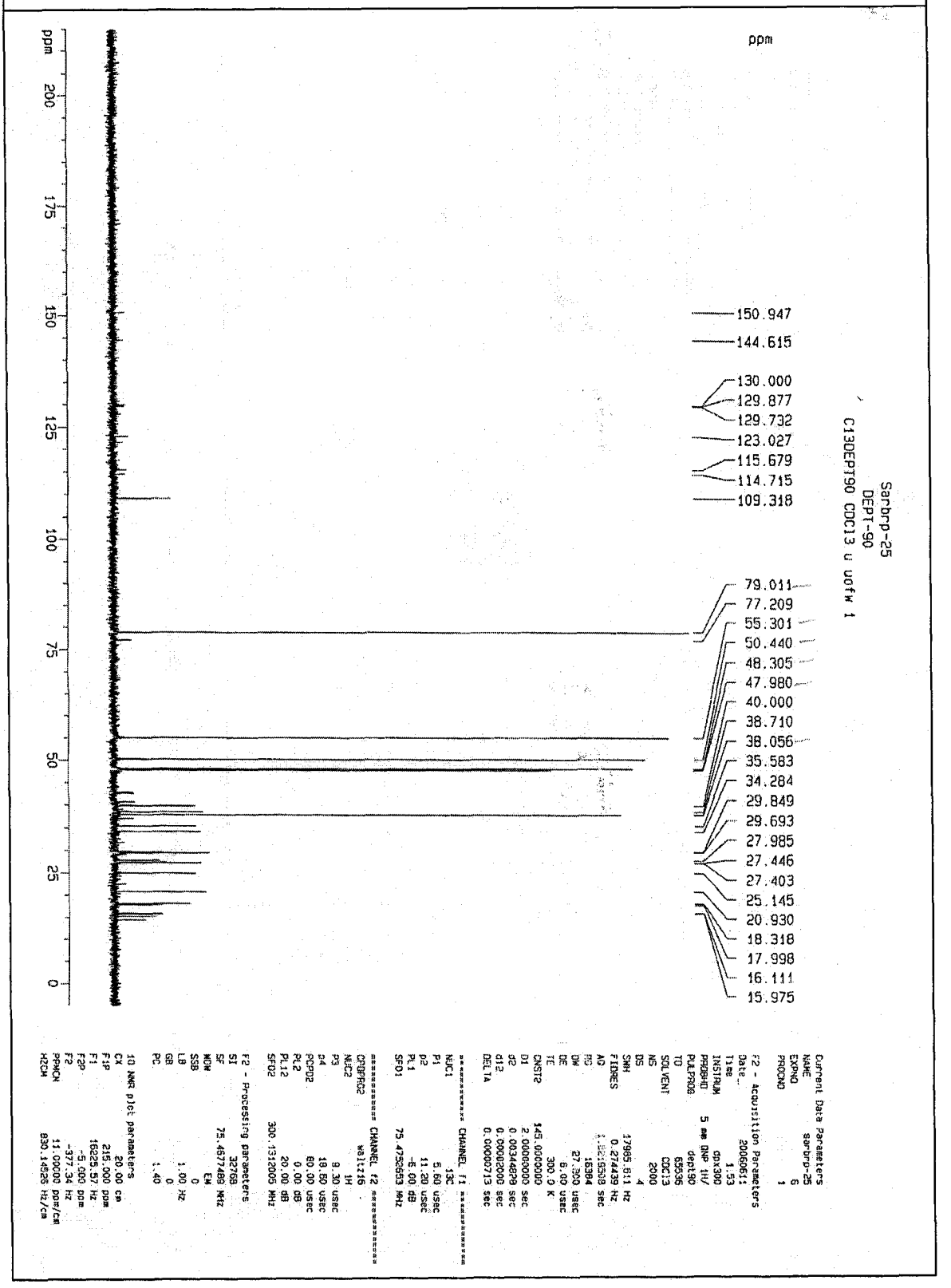
^{1}H-NMR spectrum of lupeol



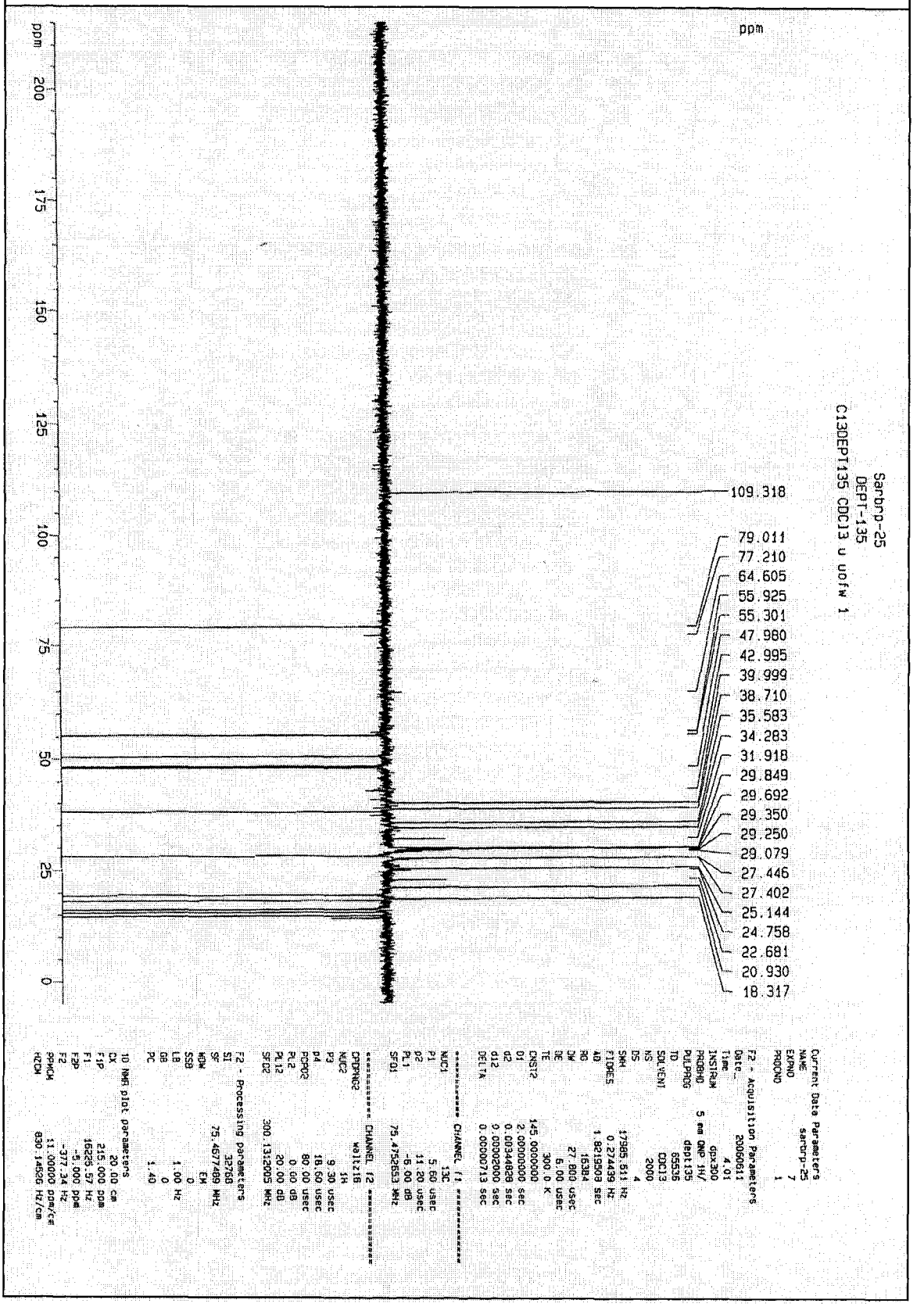
^{13}C-NMR spectrum of lupeol



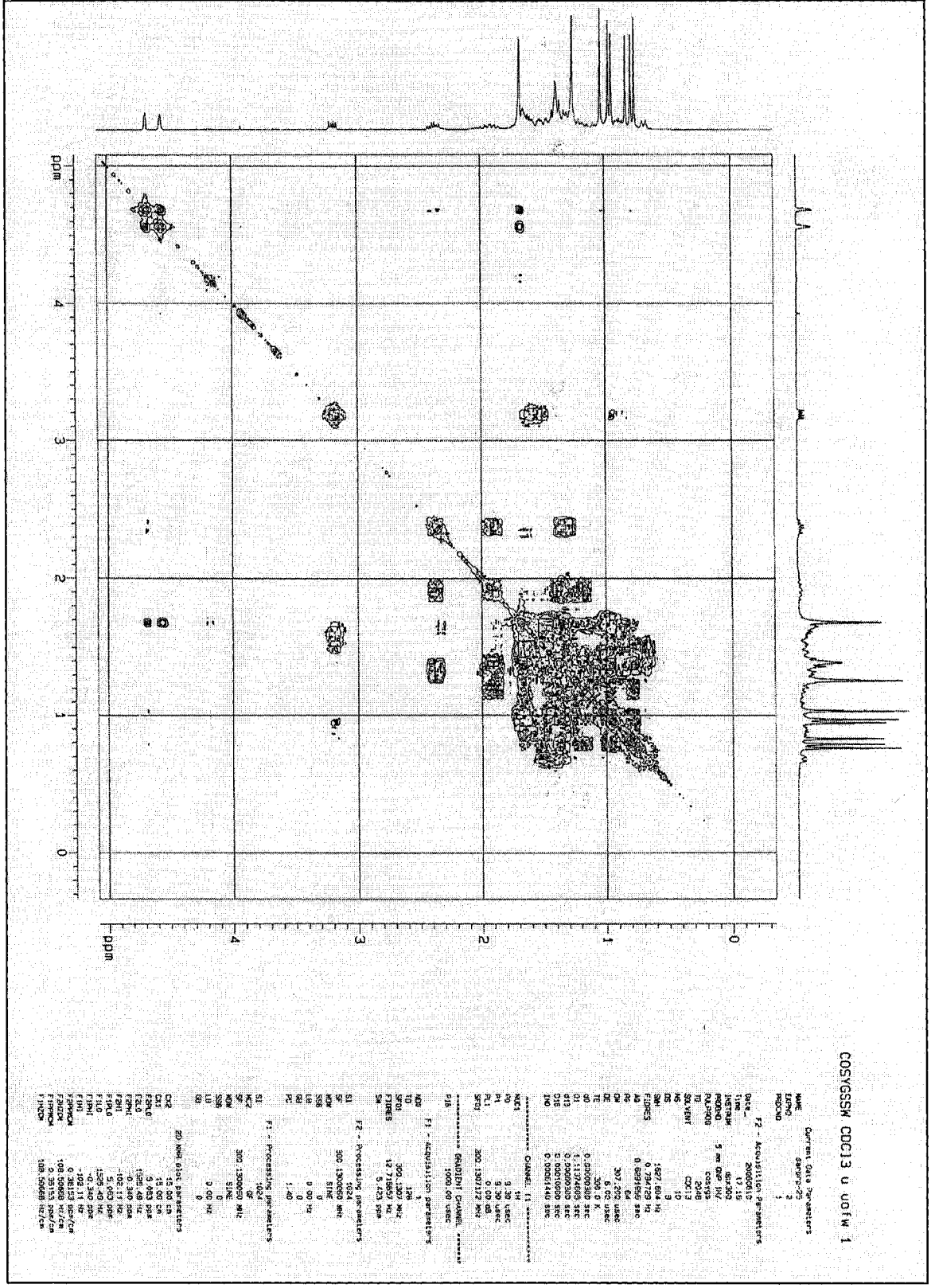
DEPT 90° spectrum of lupeol



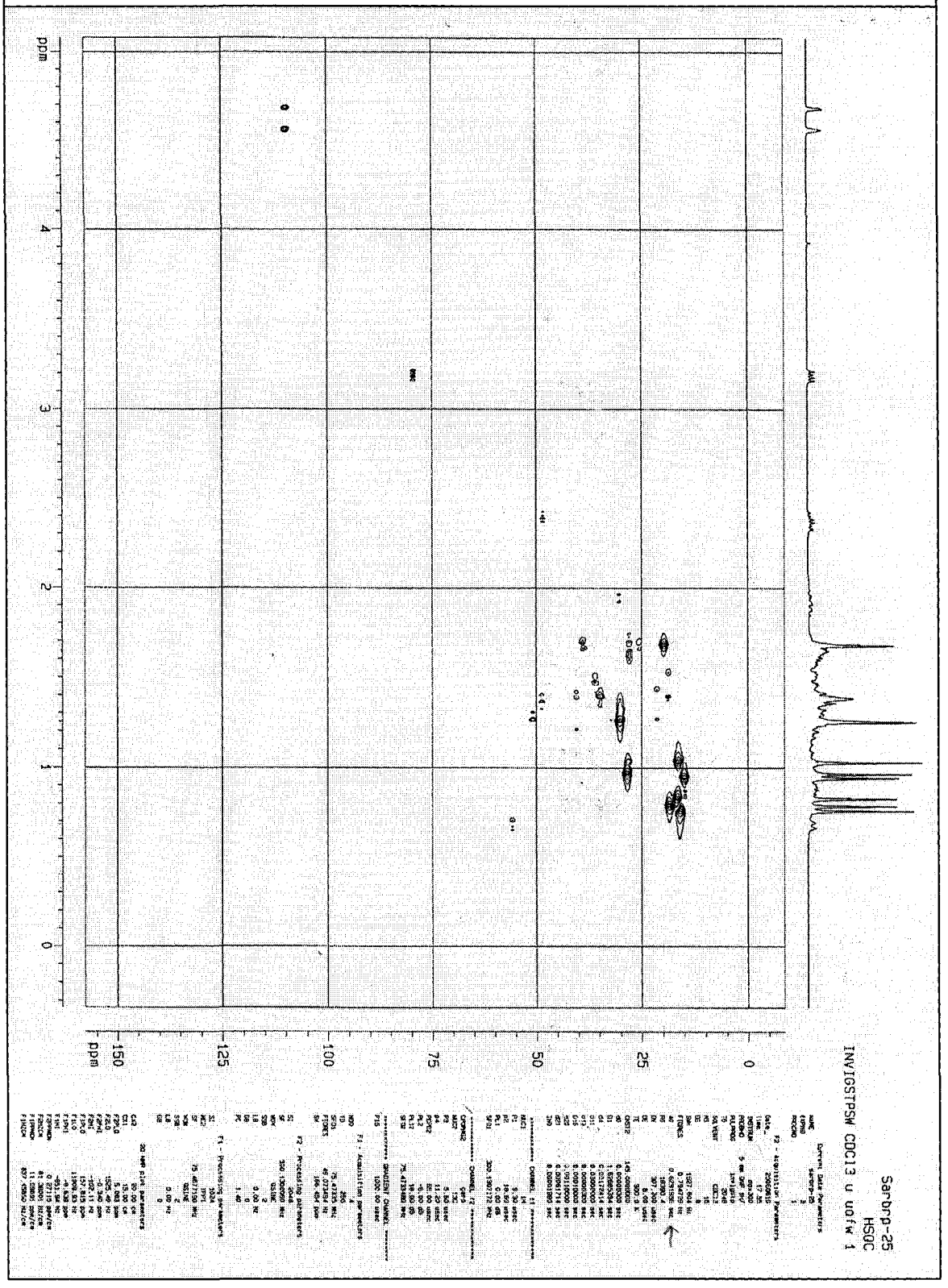
DEPT 135° spectrum of lupeol



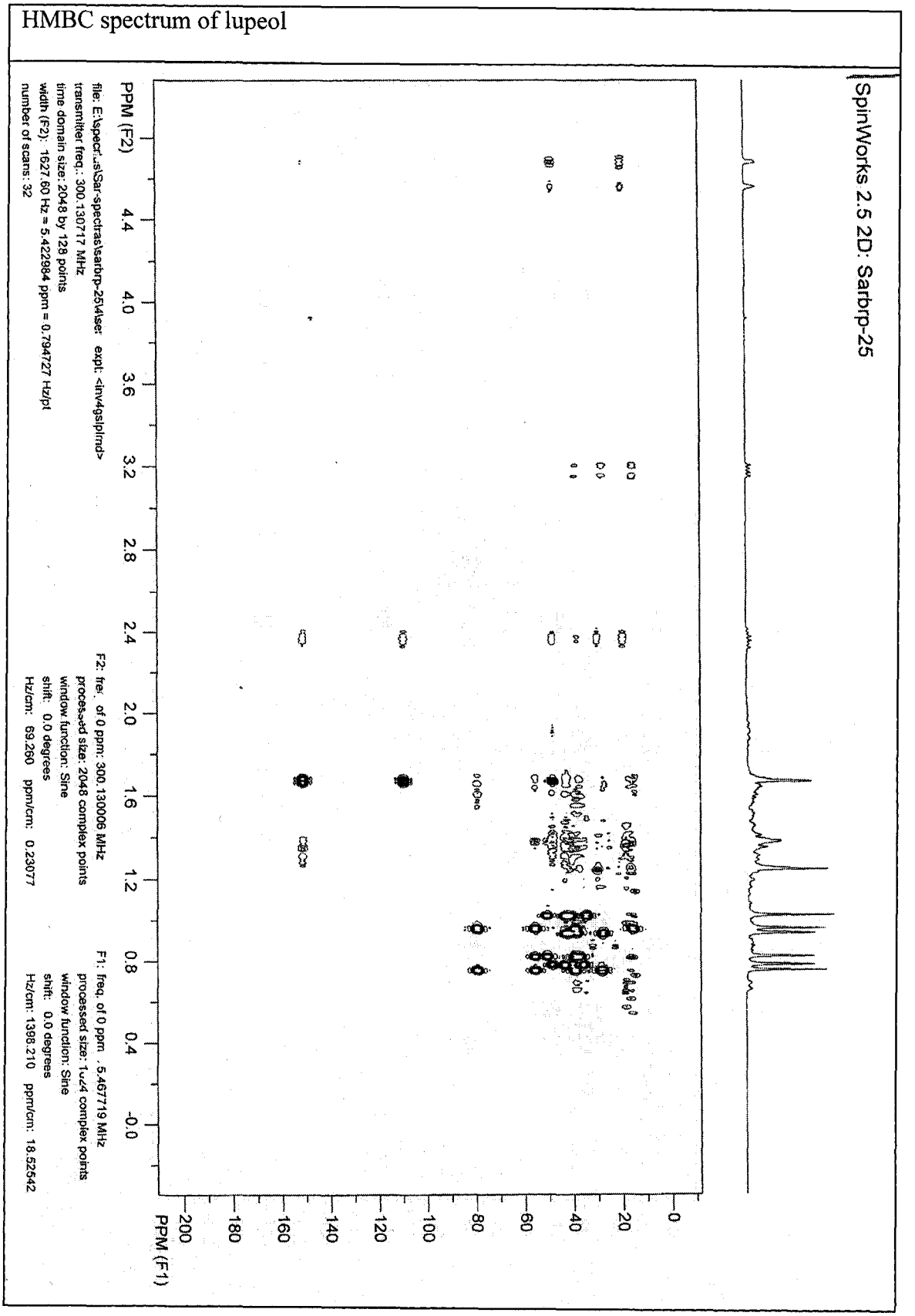
COSY-45° spectrum of lupeol



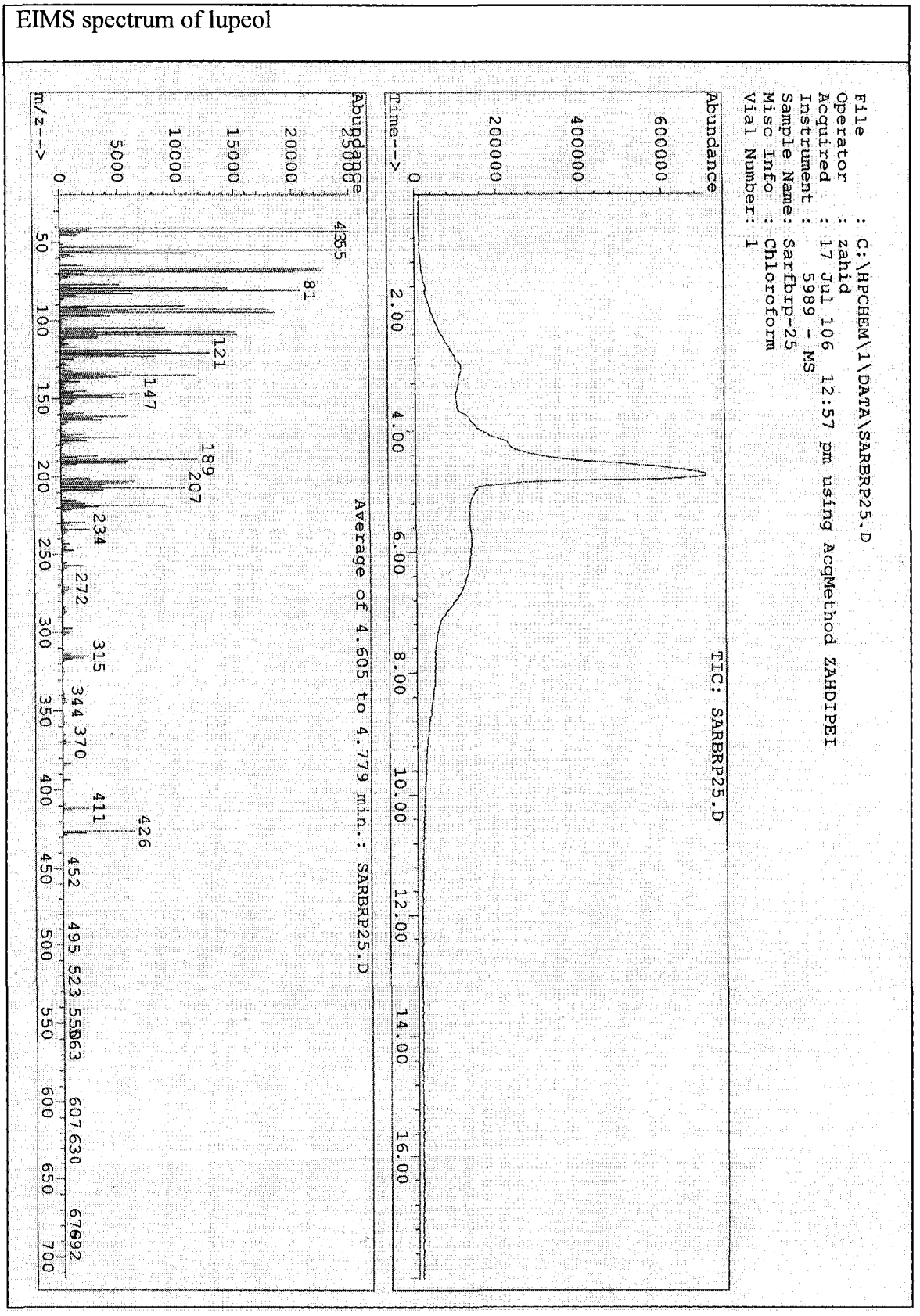
HSQC spectrum of lupeol



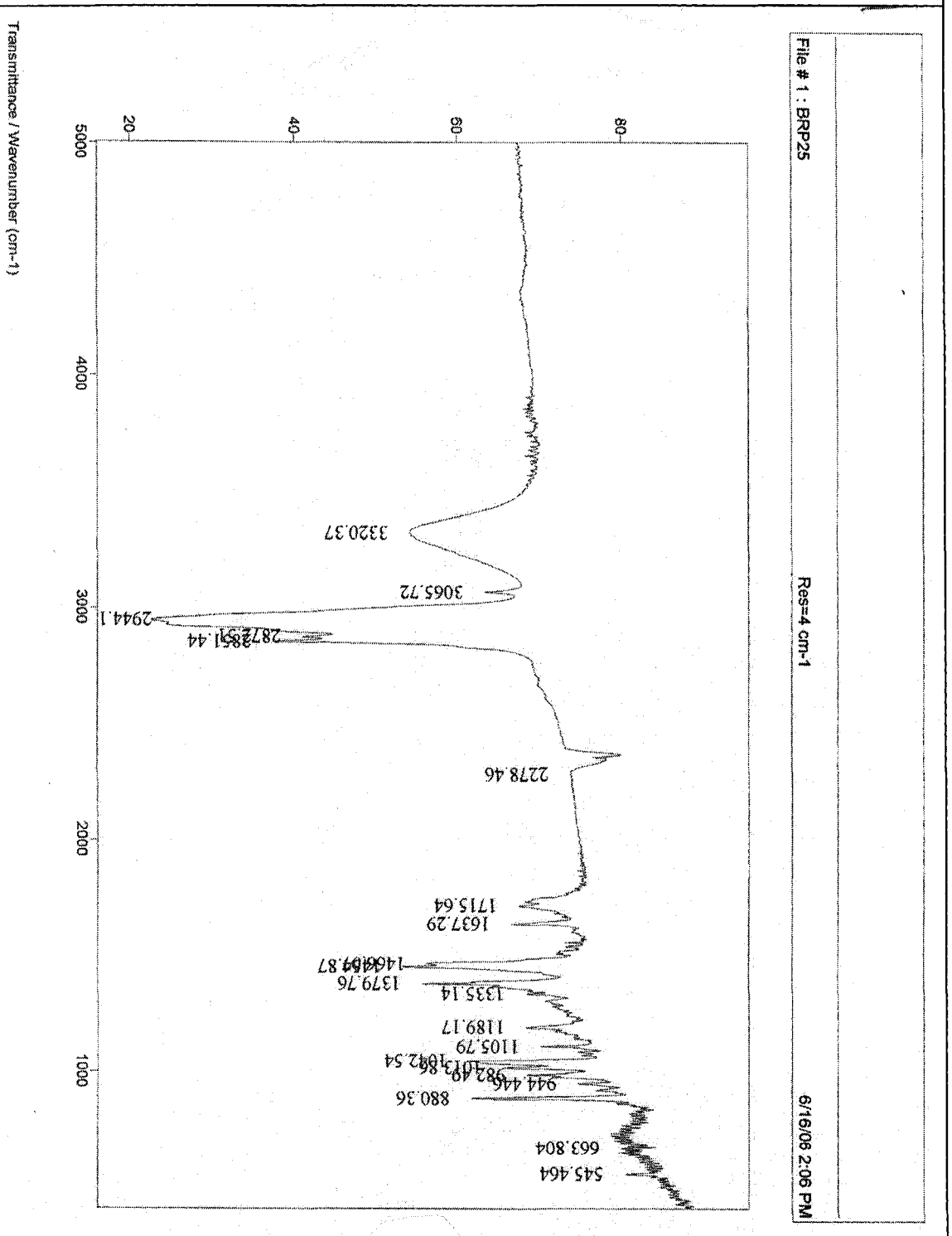
HMBC spectrum of lupeol



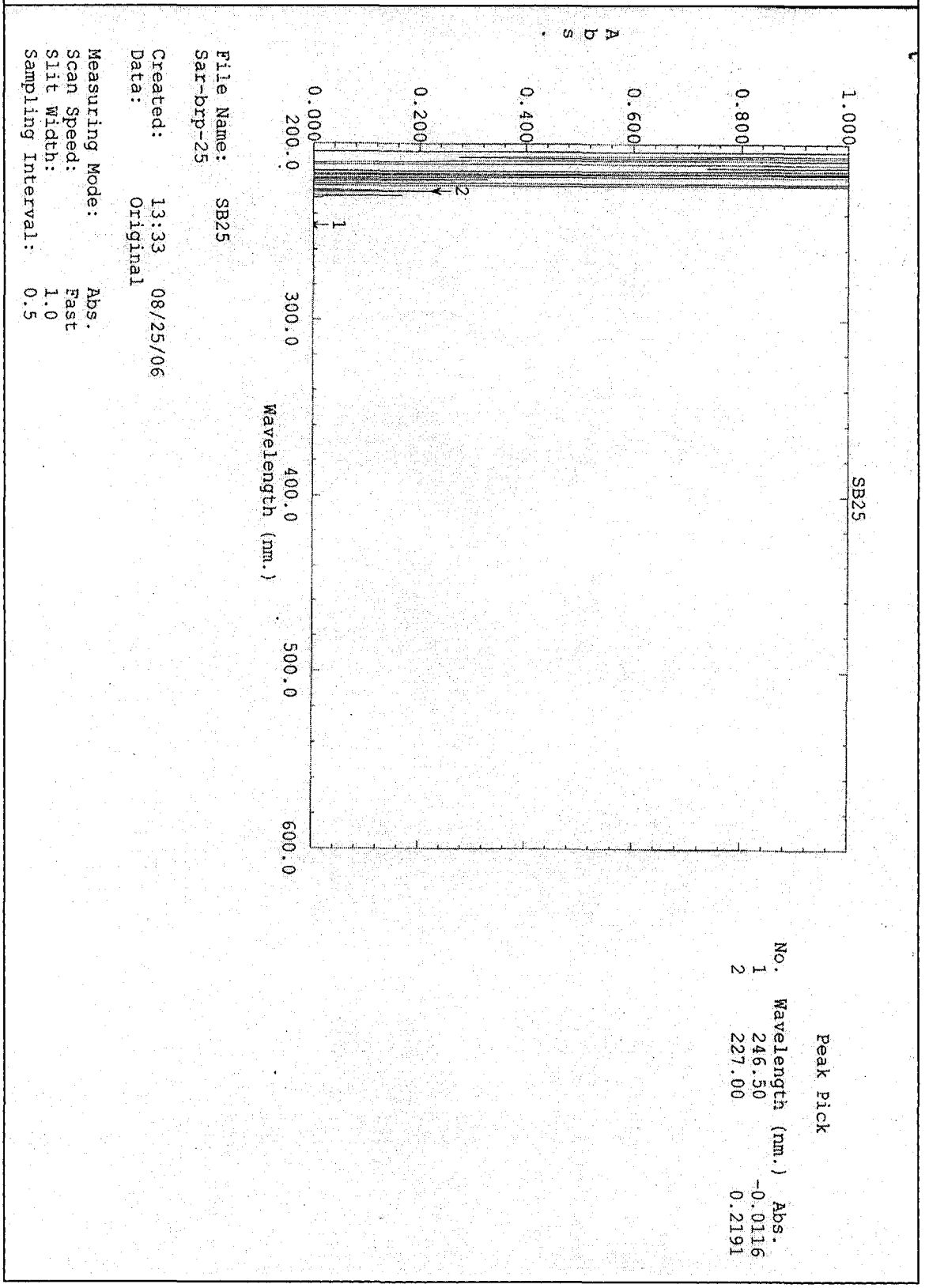
EIMS spectrum of lupeol



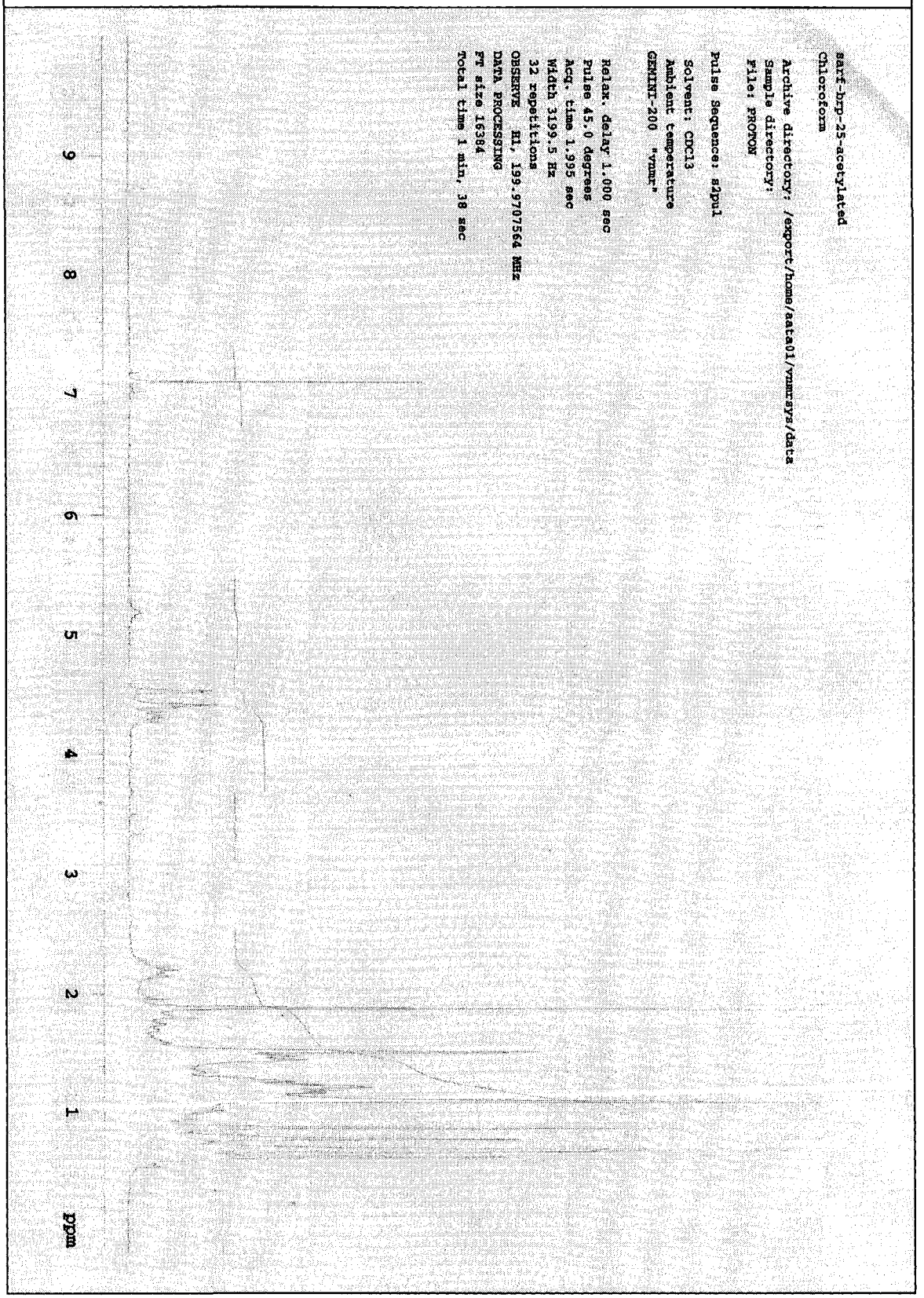
IR spectrum of lupeol



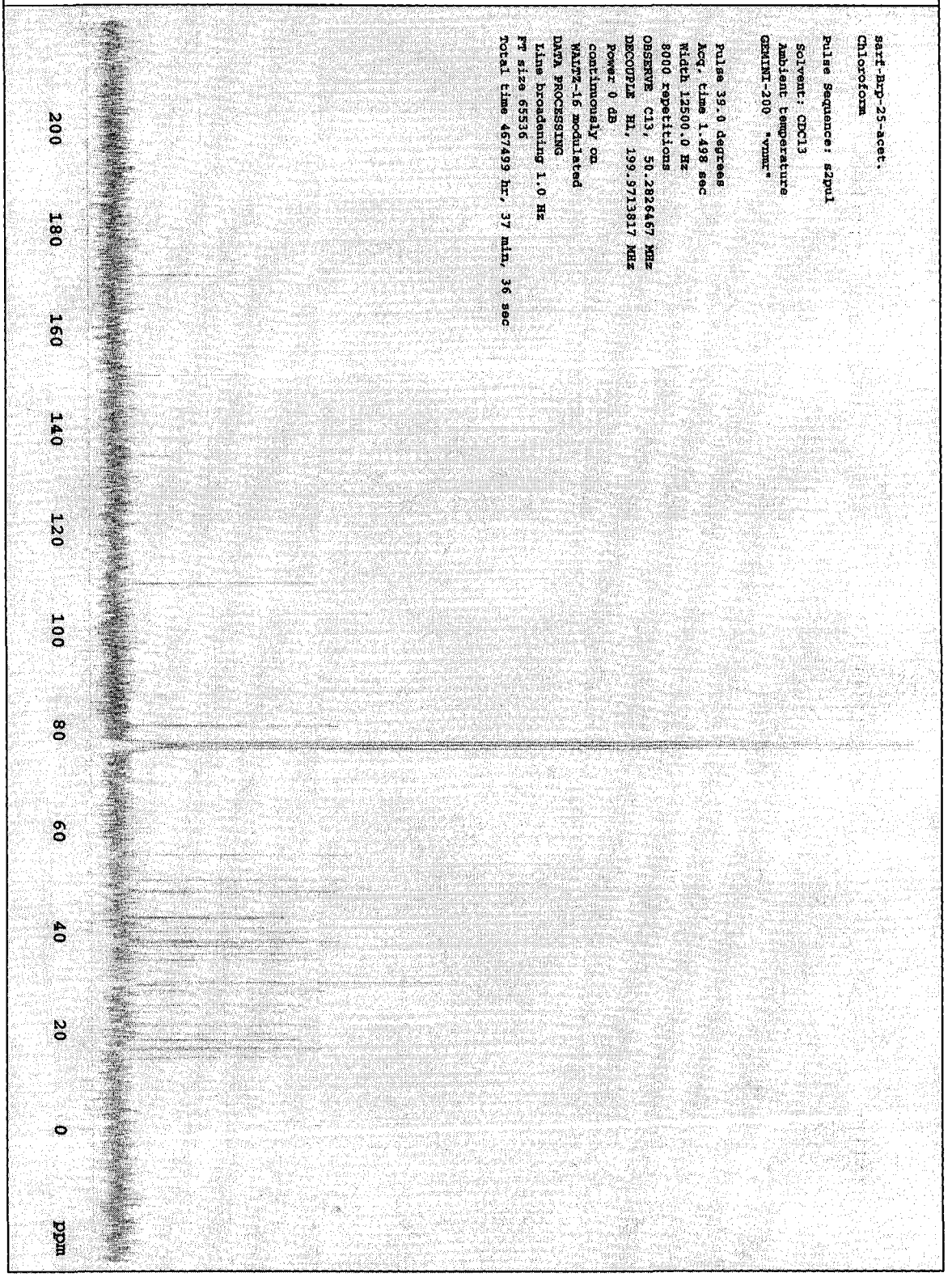
UV spectrum of lupeol



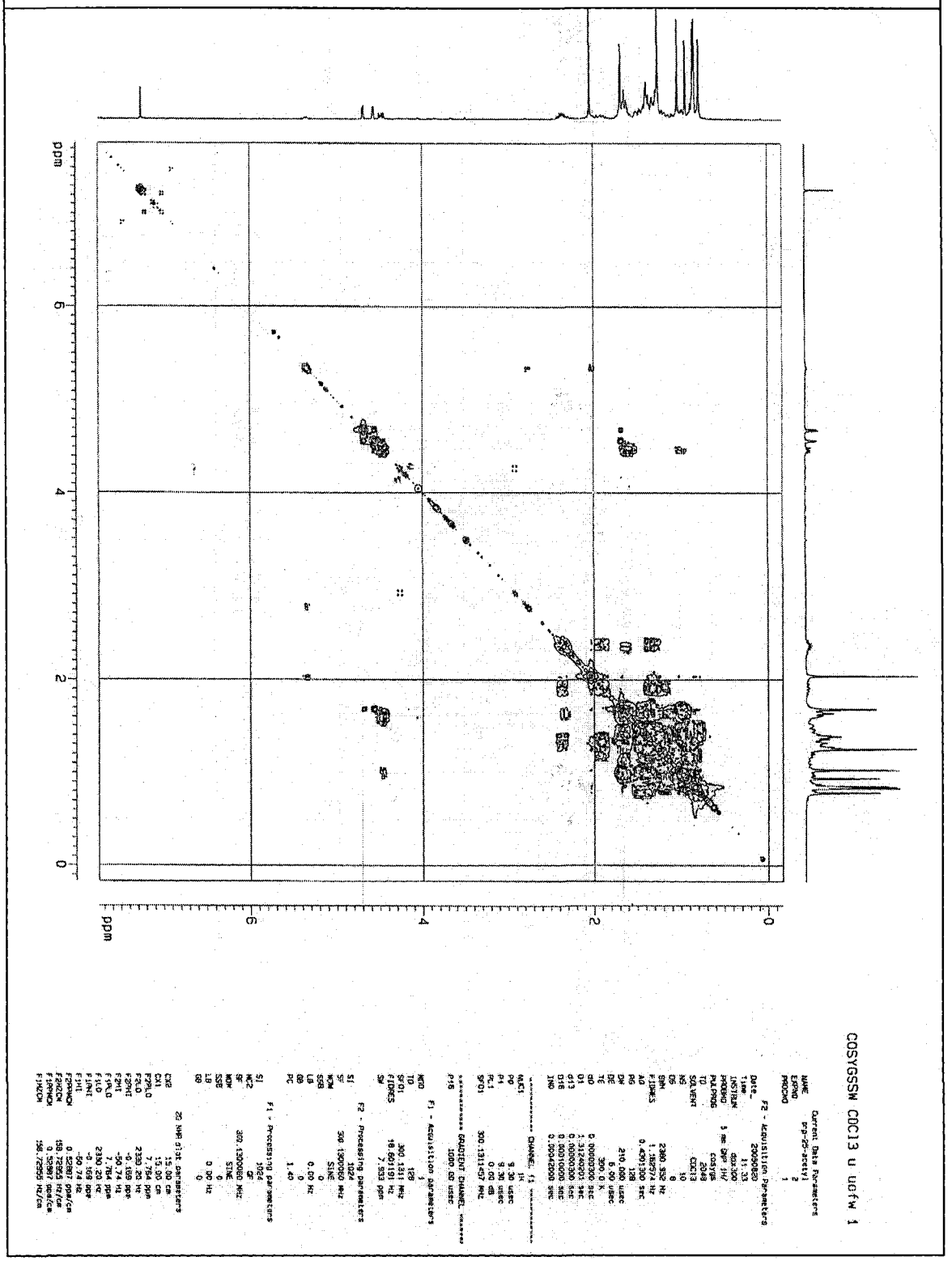
^{1}H-NMR spectrum of 3-acetyl lupeol



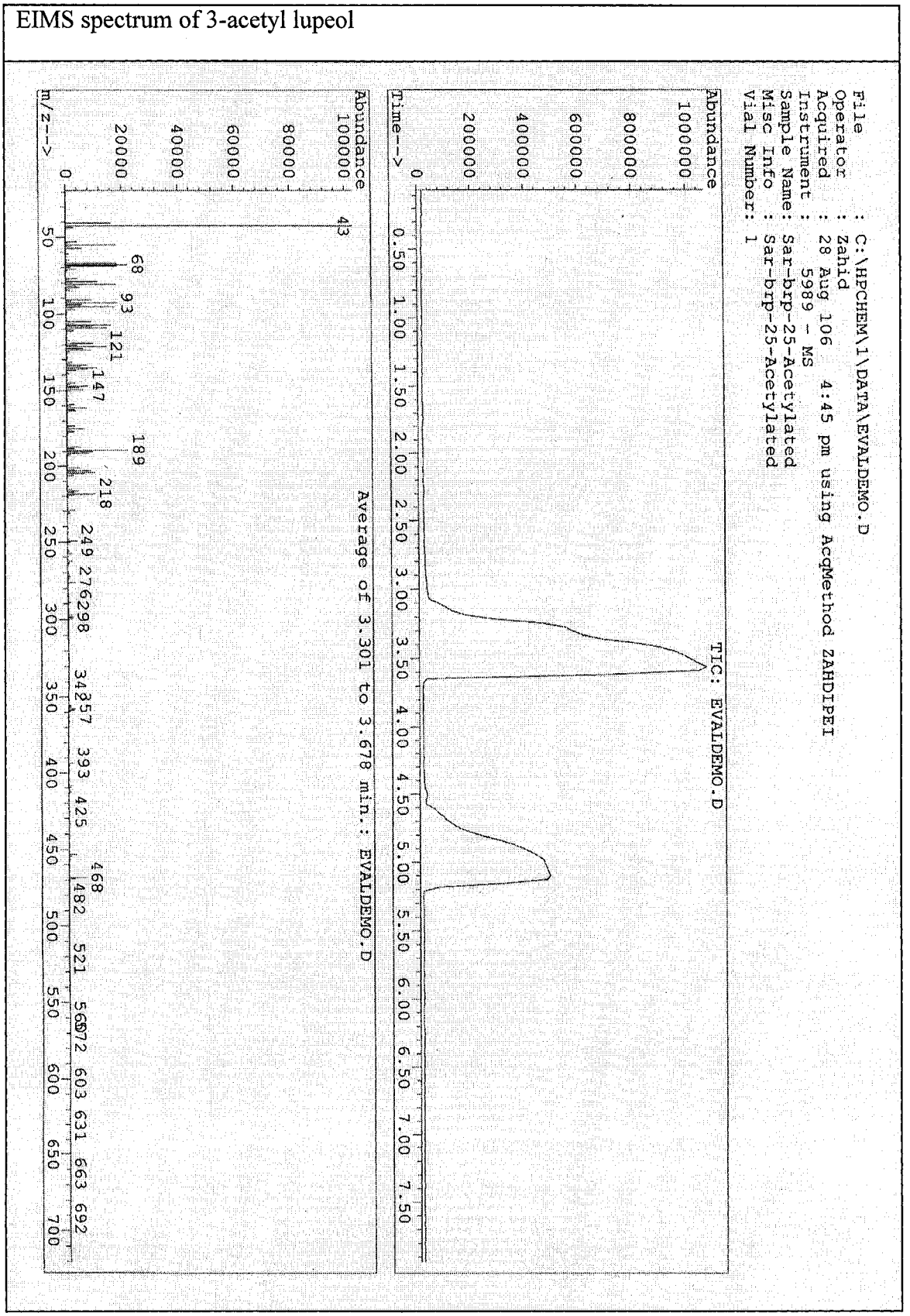
^{13}C-NMR spectrum of 3-acetyl lupeol



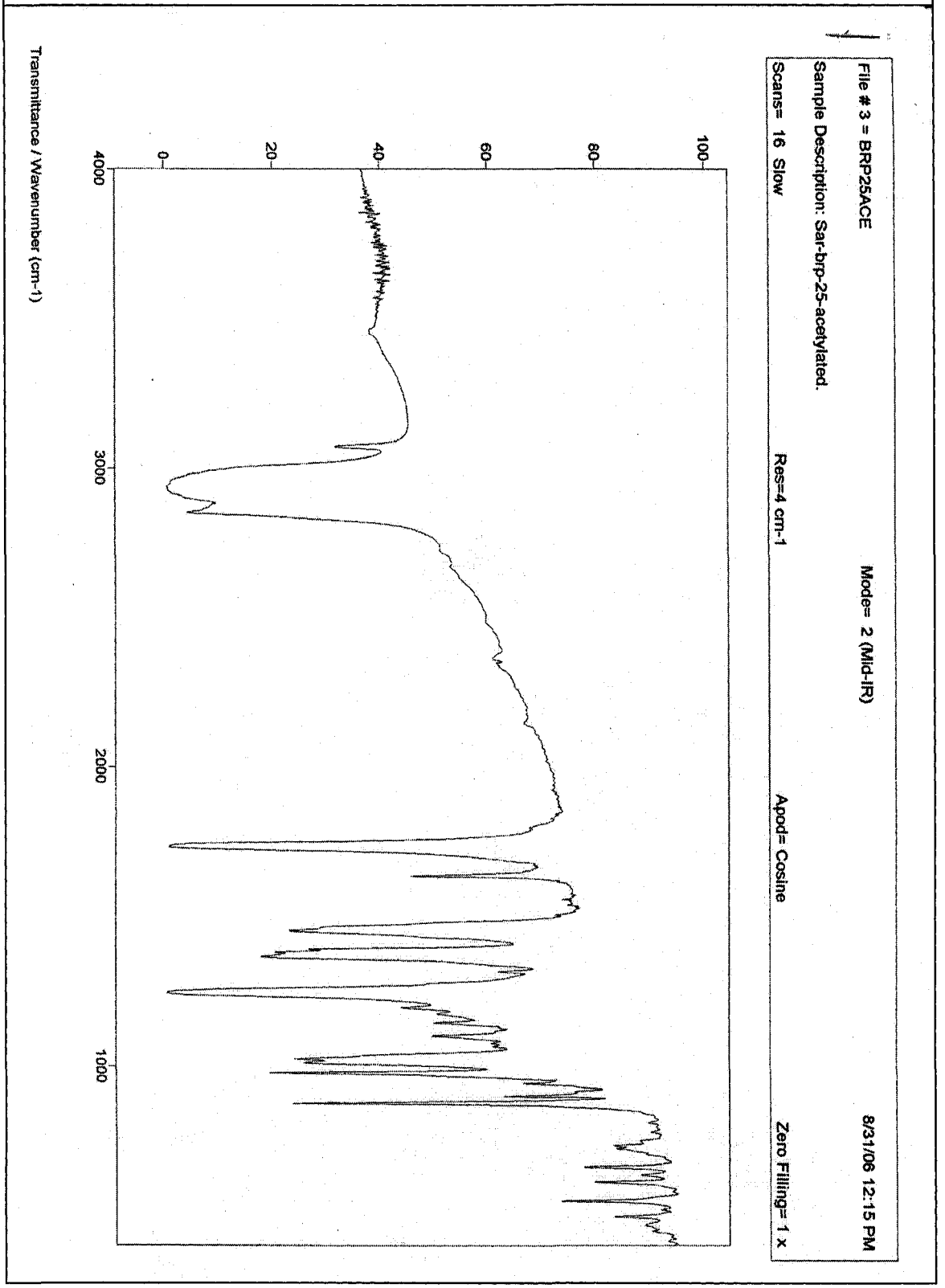
COSY-45° spectrum of 3-acetyl lupeol



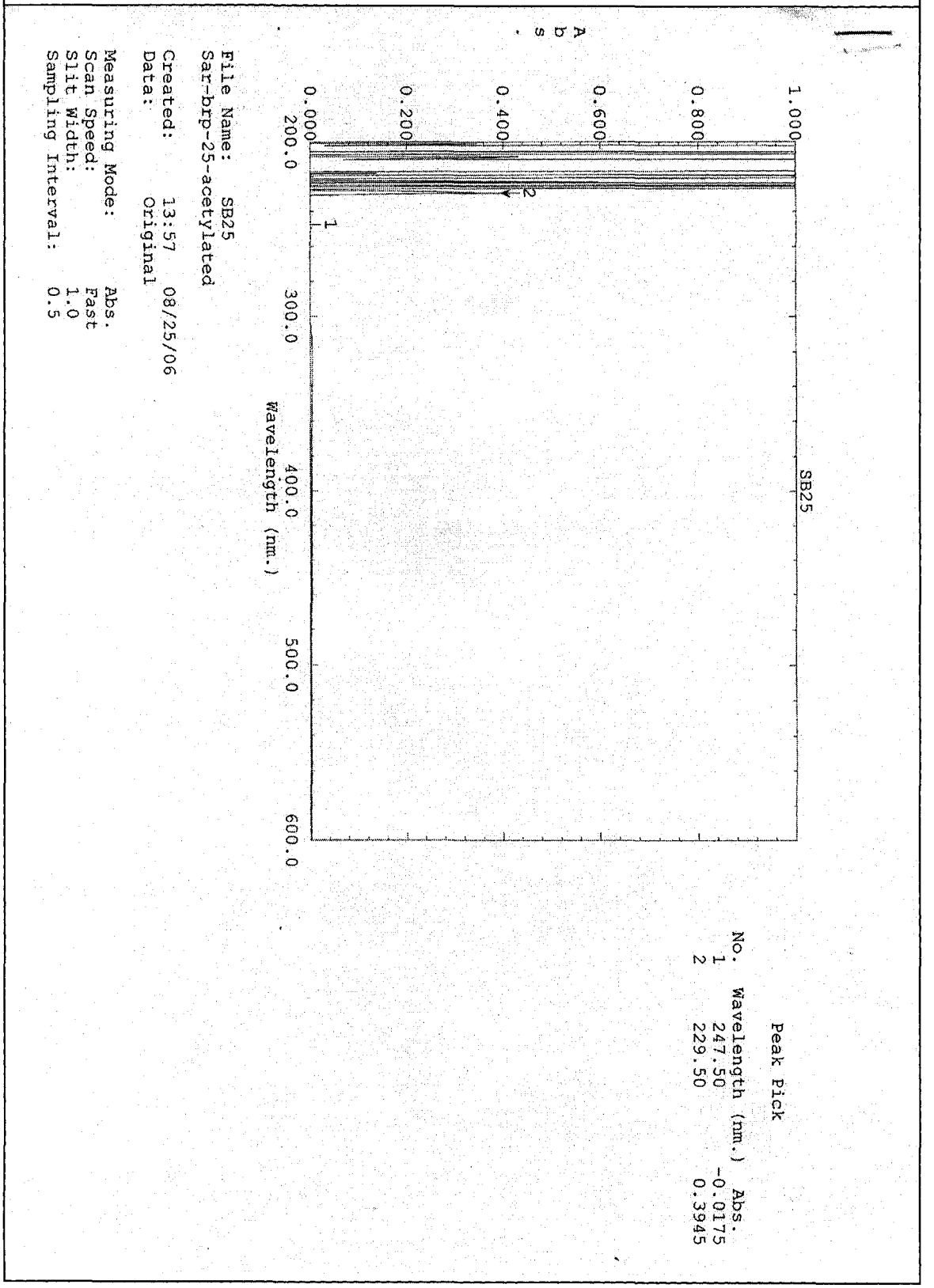
EIMS spectrum of 3-acetyl lupeol



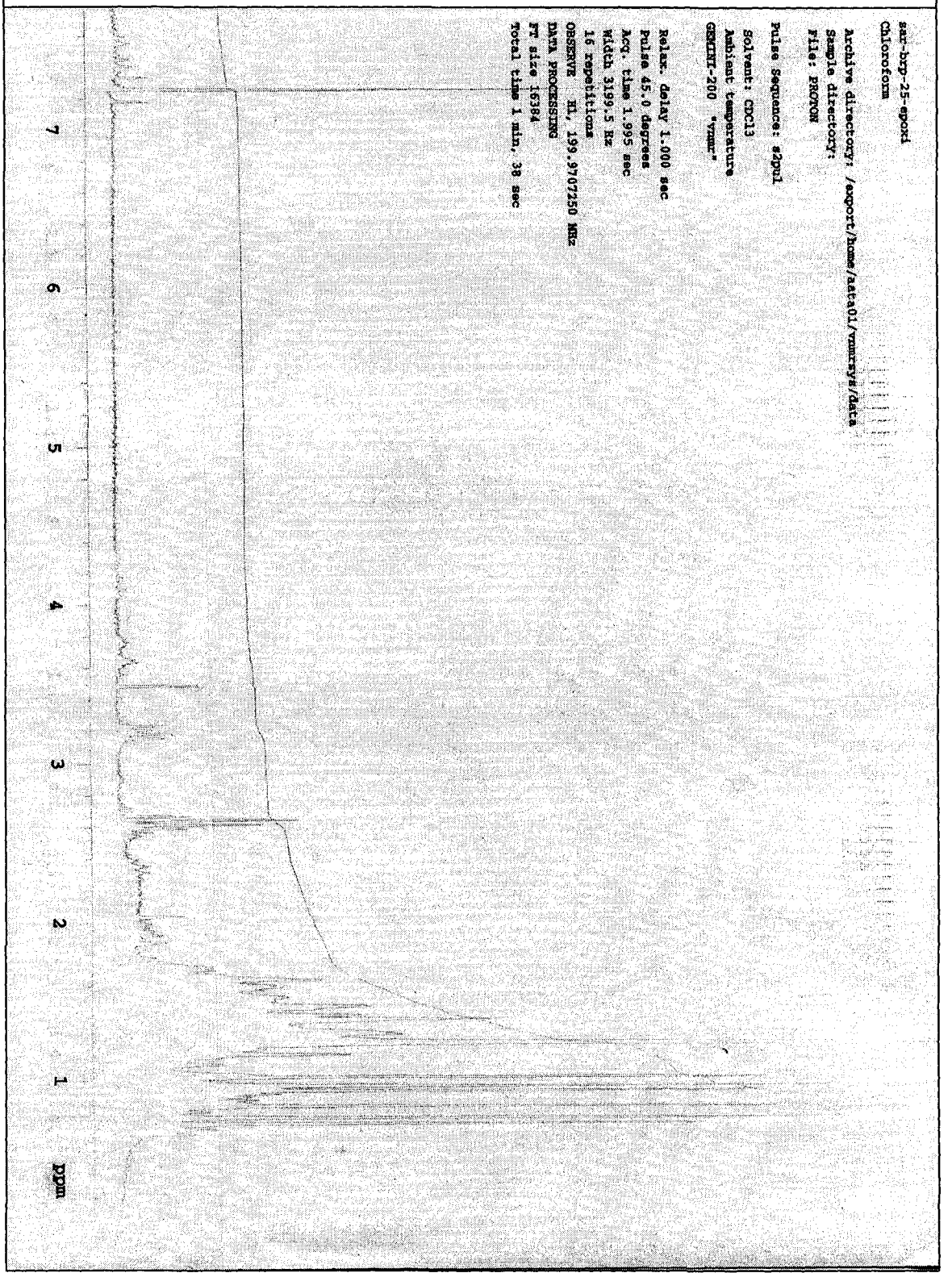
IR spectrum of 3-acetyl lupeol



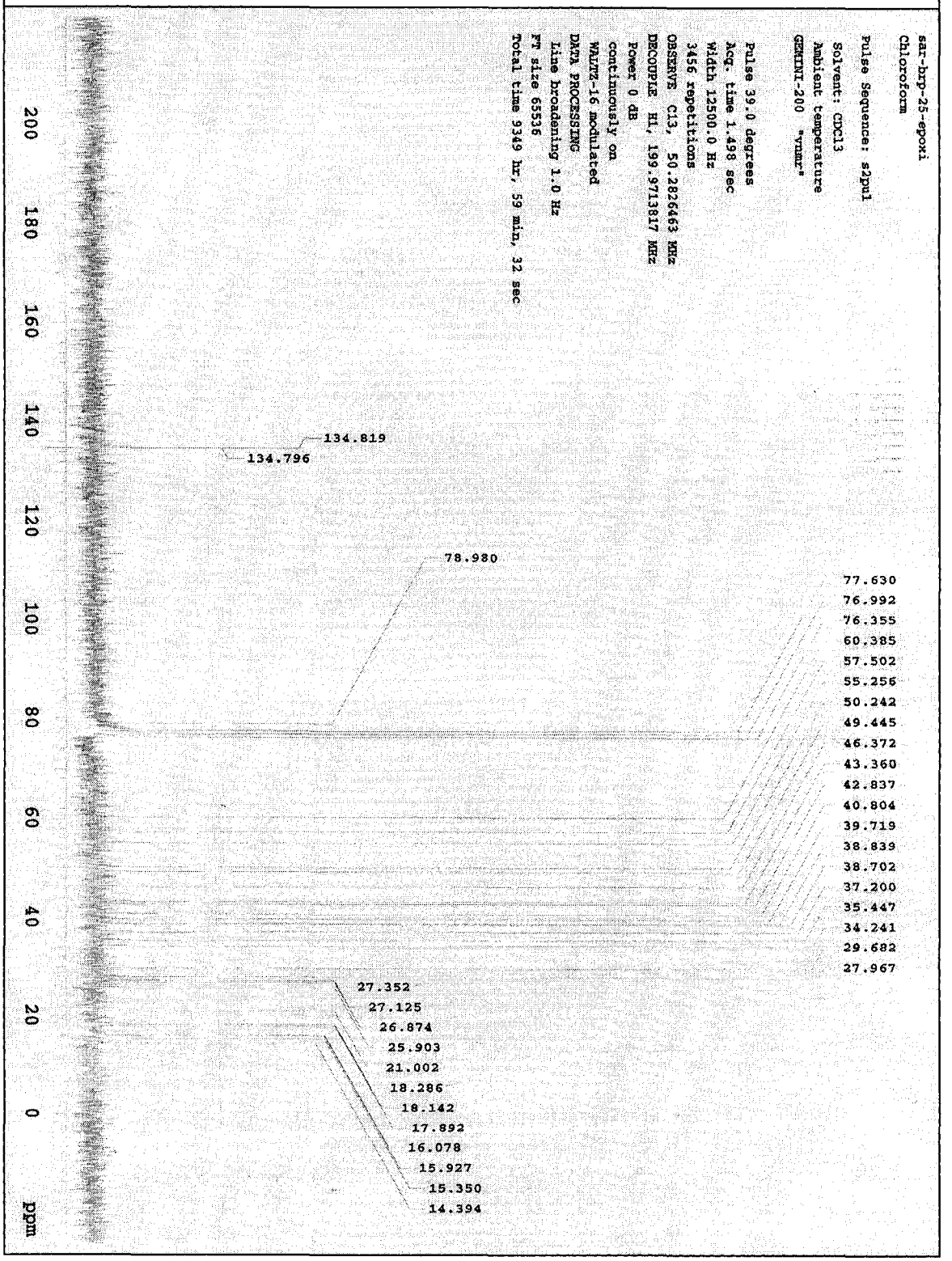
UV spectrum of 3-acetyl lupeol



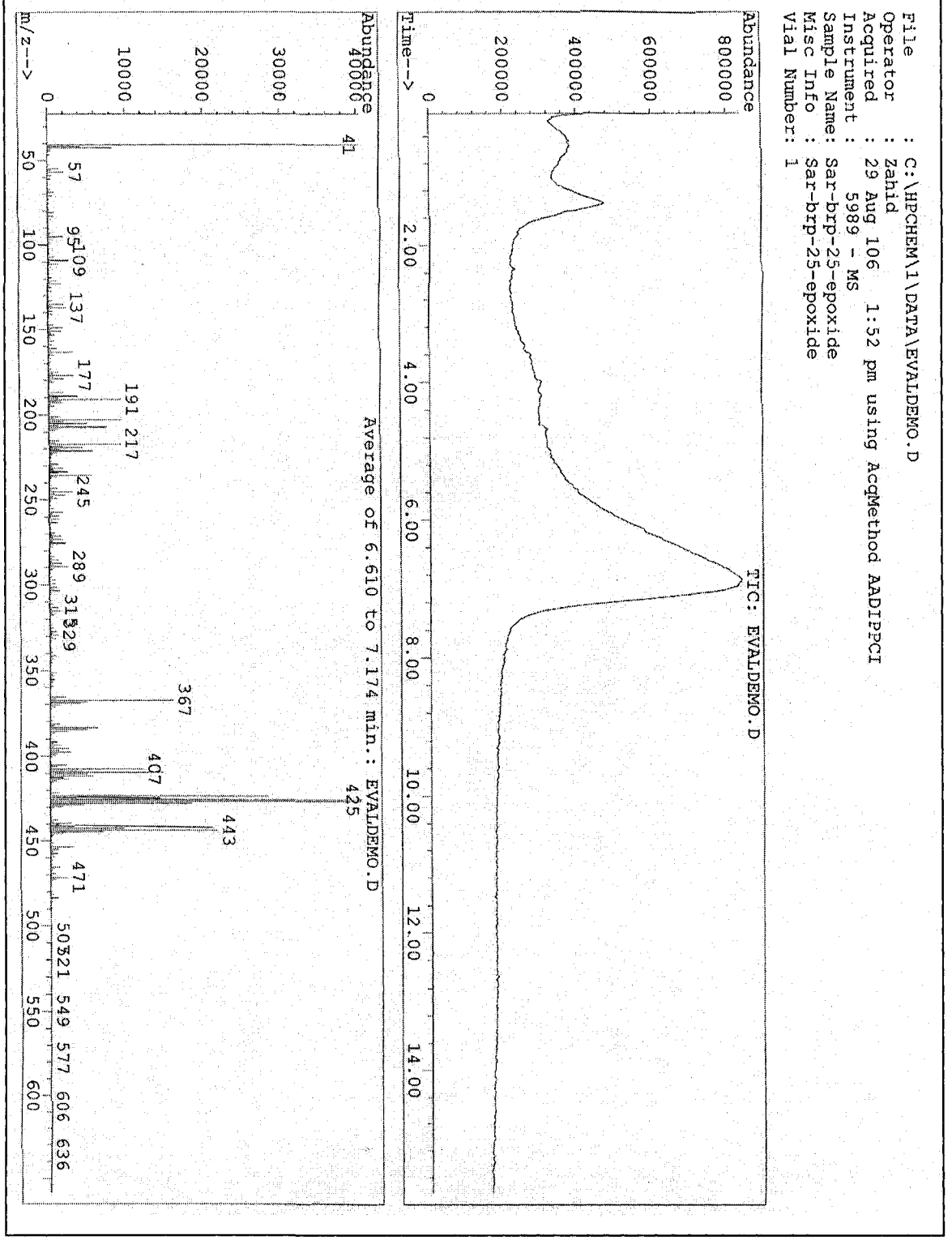
^{1}H-NMR spectrum of 20(29)-epoxylupeol



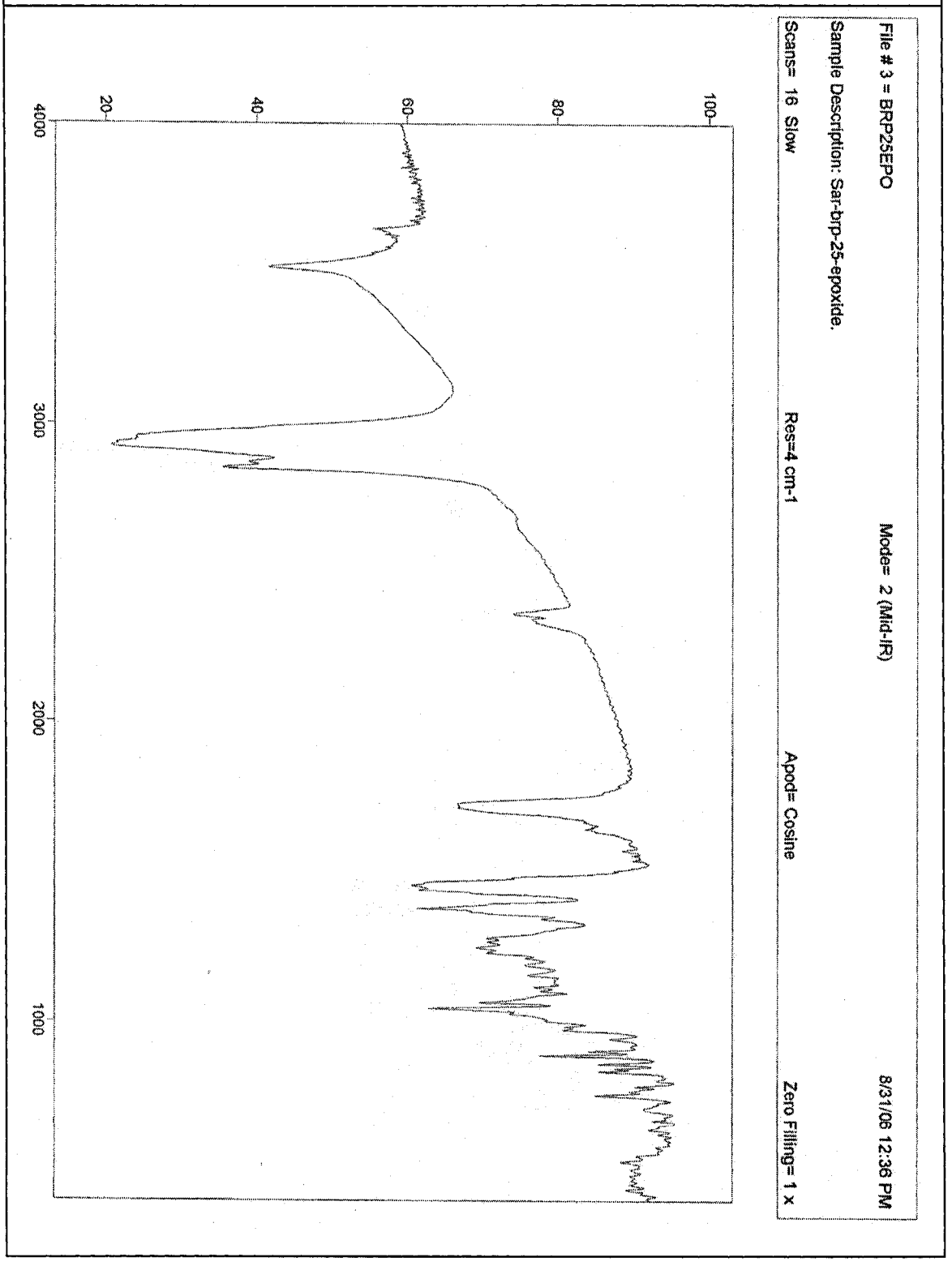
^{13}C-NMR spectrum of 20(29)-epoxylupeol



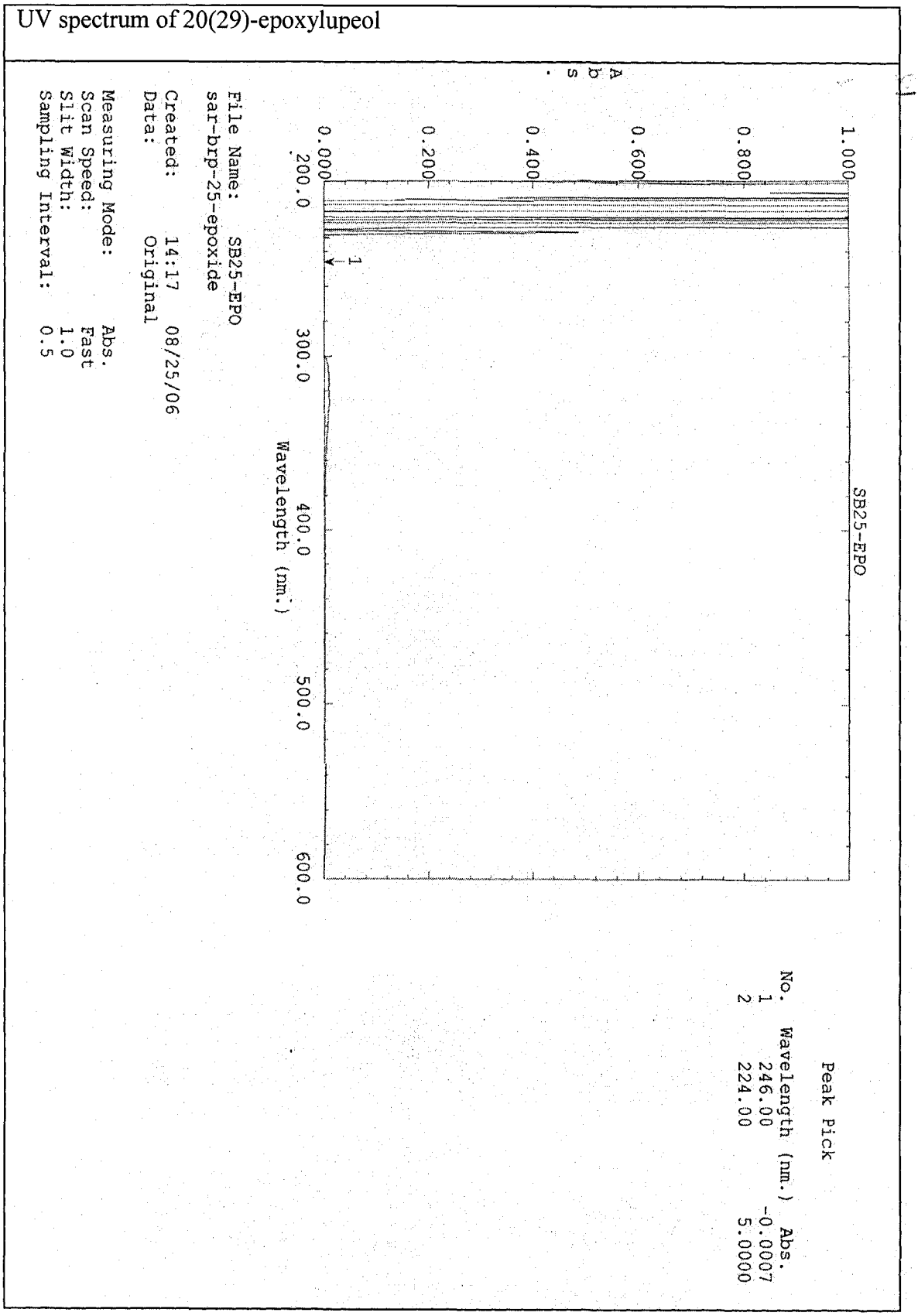
CIMS spectrum of 20(29)-epoxylupeol



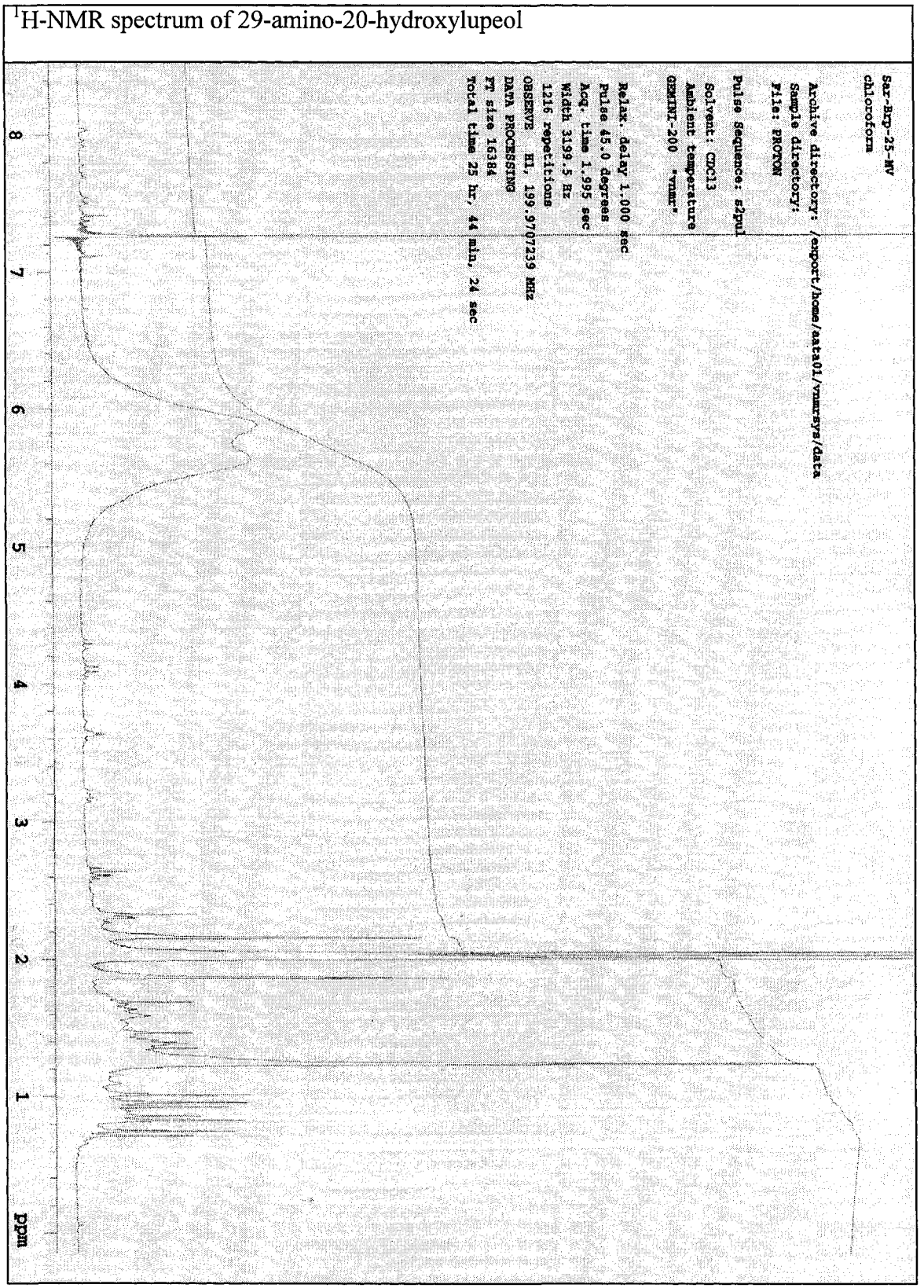
IR spectrum of 20(29)-epoxylupeol



UV spectrum of 20(29)-epoxylupeol



^{1}H-NMR spectrum of 29-amino-20-hydroxylupeol



^{13}C-NMR spectrum of 29-amino-20-hydroxylupeol