Study on Acetylcholinesterase Inhibitory, Anti-inflammatory and Antioxidant Activities of *Holarrhena antidysenterica* Bark

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Thesis Title	Study on acetylcholinesterase inhibitory, anti-inflammatory and antioxidant activities of *Holarrhena antidysenterica* bark
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ชื่อวิทยานิพนธ์	การศึกษาฤทธิ์ต้านเอนไซม์อะซิติลโคลีนเอสเทอเรส ฤทธิ์ต้านการอักเสบ และฤทธิ์ต้านอนุมูลอิสระของเปลือกต้นโมกหลวง
ผู้เขียน	นายสุนทร มู่เก็ม
สาขาวิชา	วิทยาศาสตร์สมุนไพร (นานาชาติ)
ปีการศึกษา	2554

บทคัดย่อ

การศึกษาองค์ประกอบทางเคมีจากเปลือกต้นโมกหลวง (*Holarrhena antidysenterica*) วงศ์ Apocynaceae สามารถแยกสารจากส่วนเปลือกของพืช ประกอบด้วย steroidal alkaloids 4 ชนิด ได้แก่ conessine (**1**), isoconessimine (**2**), conessimine (**3**) และ N-3-methylholarrhimine (**4**) สารกลุ่ม coumarin 1 ชนิด คือ scopoletin (**5**) จากการศึกษาฤทธิ์ต้านเอนไซม์อะซิติลโคลีนเอสเทอเรส พบว่า สารกลุ่ม steroidal alkaloids มีค่า IC_{50} ในช่วง 0.12-20.08 μM และกลุ่ม coumarin มีค่า IC_{50} เท่ากับ 73.50 μM จากการศึกษาจลนพลศาสตร์ของการยับยั้งเอนไซม์อะซิติลโคลีนเอสเทอเรส พบว่า steroidal alkaloid มีฤทธิ์การยับยั้งแบบแข่งขัน การศึกษาความสัมพันธ์ระหว่างสูตรโครงสร้างและการออกฤทธิ์พบว่า **3** ออกฤทธิ์ดีที่สุด โดยมีค่า IC_{50} 0.12±0.03 μM ซึ่งจากการศึกษาในการทำ molecular docking พบว่า **3** มีแนวโน้มการเกิด H-bond กับ GLY 118 ของเอนไซม์ ทำให้การจับเกาะกับเอนไซม์ทำได้ดีและออกฤทธิ์ดีด้วย จากการศึกษาฤทธิ์ต้านการอักเสบโดยการวัดปริมาณไนตริกออกไซด์ที่หลั่งจากเซลล์เม็ดเลือดขาว (RAW 264.7) พบ ว่า **2** ที่ความเข้มข้น 30 μM สามารถยับยั้งการหลั่งไนตริกออกไซด์ได้ 43.6 % อย่างไรก็ตาม **2** มีความเป็นพิษต่อเซลล์ที่ความเข้มข้น 100 μM สารทั้ง 5 ชนิดไม่มีฤทธิ์ต้านอนุมูลอิสระ

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ABSTRACT

The investigation of chemical constituents of *Holarrhena antidysenterica* bark, family Apocynaceae led to the isolation of four steroidal alkaloids including, conessine (**1**), isoconessimine (**2**), conessimine (**3**), and N-3-methylholarrhimine (**4**) and one coumarin, scopoletin (**5**). The steroidal alkaloids and coumarin were subjected to acetylchlolinesterase inhibitory activity showed higher inhibitory activity with IC_{50} values in the range of 0.12-20.08 μM and a coumarin with an IC_{50} 73.50 μM. The kinetic analyses of acetylchlolinesterase inhibitory activity of steroidal alkaloids suggested the steroidal alkaloid inhibit in competitive manner. Among steroidal alkaloid, **3** showed the best activity with IC_{50} of 0.12 ± 0.03 μM. The molecular docking of **3** found that it can form hydrogen bonding with Gly-118. This may answer why **3** is more active. Furthermore, all compounds were studied on anti-inflammatory activity using NO production assay in murine macrophage like RAW 264.7. The results found that **2** at concentration 30 μM showed inhibition of NO production 43.6 %, however it has cytotoxicity in murine macrophage at concentration of 100 μM. All these compounds have no antioxidant activity.

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Sunthorn Mukem

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LIST OF ABBREVIATIONS AND SYMBOLS

ι	chemical shift in ppm
κ	molar extinction coefficient
ς_{max}	maximum wavelength
τ_{max}	wave number
COSY	correlation spectroscopy
d	doublet (for NMR signals)
DEPT	distortion less enhancement by polarization transfer
ED_{50}	effective dose at 50 percent
EIMS	electron-impact mass spectroscopy
HMBC	heteronuclear multiple bond correlation
HMQC	heteronuclear multiple-quantum coherence
IC_{50}	inhibitory concentration at 50 percent of tested subject
IR	infrared
J	coupling constant
$[M]^+$	molecular ion
m	multiplet (for NMR signals)
m/z	mass-over-charge ratio
nOe	nuclear Överhauser effect
MMR	nuclear magnetic resonance
ppm	part per million
s	singlet (for NMR signals)
t	triplet (for NMR signals)
*Tc*AChE	*Torpedo californica* acetylcholinesterase
U	unit
UV	ultraviolet

CHAPTER 1

INTRODUCTION

1.1 Alzheimer's disease (AD)

AD is a group of disease characterized by an age-dependent loss of memory and destroys of multiple cognitive functions. This incurable, degenerative, and terminal disease were first described by German psychiatrist and neuropathologist Alois Alzheimer in 1906 which was named after his name (Berchtold and Cotman, 1998). There are two major factors that are related to patient with AD. First, the cause of AD is associated with extracellular deposit of amyloid plaques. β-amyloid plaque is generated by aggregation of β-amyloid isoforms ranking from 40- to 42 residues amino acid peptide synthesized from amyloid precursor protein (Hardy and Selkoe, 2002). A second, intracellular neurofibrillary tangle is made of hyper-phosphorylated tau proteins by kinase phosphatase enzymes, and it is modified to form paired helical filaments (Akhondradeh and Abbasi, 2006). Therefore, these factors have been affecting to decrease of cholinergic neurotransmission, increase of oxidant species or oxidative stress and brain inflammation (Anekonda and Reddy, 2005).

1.2 Treatment of Alzheimer's disease

1.2.1 Cholinergic supportive agents

The cholinergic hypothesis was suggested that the number of acetylcholine (Ach) containing neurons in the brain is decrease affecting to cognitive function impairment, resulting mainly in a loss of memory, with neurological and neuropsychiatric disorders, observed in those with elderly people and AD. Based on the cholinergic hypothesis, this factor has served as the basis for the majority of treatment strategies and drug development approaches for patients with AD. Furthermore, scientist studies of the brains of patients who had mild cognitive

impairment led some to challenge the validity of the hypothesis as well as the rationale for using anticholinesterase drugs in the treatment of disorder, particularly in the earlier stages (Hostettmann *et al.*, 2006).

1.2.2 Anti-inflammatory agents

The relationships between inflammation and cognitive function or AD have received a great deal of attention in recent years with numerous reports linking inflammatory proteins to cognitive dysfunction or decline as well as with the development of AD (Alley *et al.*, 2008). Brain inflammation is associated with the synthesis and secretion mediated through cytokines and other secretary products (specifically, nitric oxide (NO), glutamate, and ACh) from activated glial cells, of a number of neuroactive molecules, including cytokines, reactive oxygen-and nitrogen-free radicals, complement proteins, excitatory amino acids, proteases and protease inhibitors. Among these, cytokines in particular are likely to contribute to the development of cognitive dysfunction given their ability to modulate neurotransmitter and second-messenger signal transduction systems which are known to subserve certain cognitive processes. Cytokines have been shown to affect noradrenaline, serotonin, acetylcholine, glutamate, NO and dopamine neurotransmission etc. NO is a gas molecule which is expressed in a variety of cells in both the peripheral nervous system and in the central nervous system. The neurotransmitter role of NO may be relevant to inflammatory neuromodulation. The lack of positively-charged moiety among these molecules suggested the possibility of allosteric binding site, although investigation regarding such interaction is yet to be found out. Macrophage/microglia-type inducible NOS, is expressed in microglia and astrocytes at membrane-bound and intracellular sites (Gahtan and Overmier, 1999). Current pharmacological therapy of AD use non steroidal anti-inflammatory drugs such as ibuprofen aspirin, indomethacin, diclofenac, naproxen, meloxicam, celecoxib and rofecoxib (Moore *et al*., 2010).

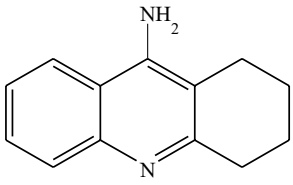
1.2.3 Antioxidant agents

Oxidative stress is a part of major factor involved in the development and progression of AD and other forms of dementia. A large body of data suggests that free radical oxidative damage particularly of neuronal lipids, proteins and nucleic acids, is extensive in the brains of AD patients (Anekonda and Reddy, 2005). The aggregation of βA induced to oxidative stress by lipid per-oxidation, and then it can be generated 4-hydroxynonenal (4HNE) in cell membrane. 4HNE involves with reactive oxygen species and neurofibrillary tangles formation. Oxidative stress can make dysregulation of Ca^{2+} homeostasis, and the result found that it is impairment of the electron transport chain, increased production of superoxide anion radical and decreased production of ATP. Superoxide anion is converted to H_2O_2 by the activity of superoxide dismutases and superoxide can interact with nitric oxide (NO) via nitric oxide synthase to produce pro-oxidant peroxynitrite ($ONOO^-$). Interaction of H_2O_2 with Fe^{2+} or Cu^+ generates the hydroxyl radical ($OH^{\bullet}$), a highly reactive oxyradical and potent inducer of membrane-associated oxidative stress and inflammatory that contributes to the dysfunction of the endoplasmic reticulum in patient AD. Current pharmacological therapy approaches related to AD treatment including antioxidant therapy such as vitamin E (α-tocopherol), selegeline, and *Ginkgo biloba* extract (Pratico, 2008).

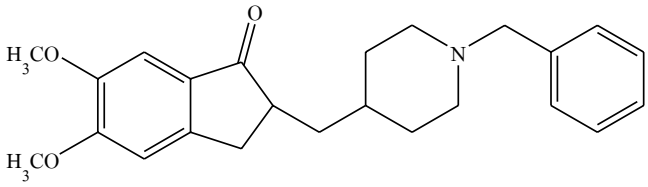
1.2.4 Anticholinesterase drugs

Acetylcholinesterase inhibitors (AChE-I's) are important drugs for treating and preventing of acetylcholine levels in patients with AD. Therefore, there are four AChE-I's have been reported and approved by U.S. Food and Drug Administration (USA-FDA) (Zarotsky *et al*., 2003) that cure the symptoms of AD. The first drug to be approved for use in the treatment of AD was tacrine (Cognex®). Currently, it is consider as hepatotoxic. There are three new AChE-I's including, donepezil (Aricept®) rivastigmine (Exelon®) and galantamine (Reminyl®). They are efficient for the treatment of patients with AD for mild to moderate stages and are available on the market (Delfilippi and Crismon, 2003). However, there are several side effects reported from

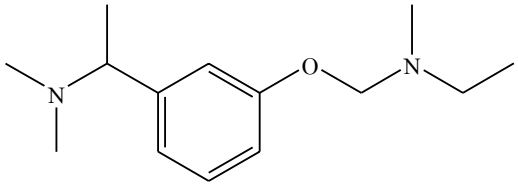
AChE-I's mainly gastrointestinal complaints. The incident is about 25-46% of patents (Alwahhabi, 2005). These AChE-I's are shown in Figure 1.



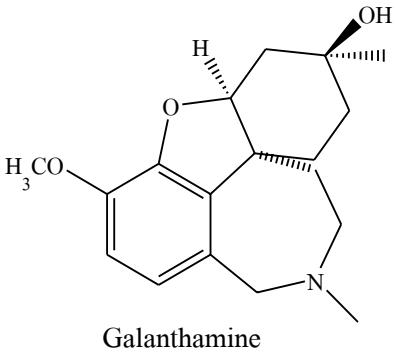
Tacrine



Donepezil



Rivastigmine



Galanthamine

Figure 1 The structure of AChE inhibitors

Among them, galanthamine was an inhibitor from natural products. *Galanthus* species have been used traditionally in Bulgaria and Turkey to treat painful neurological conditions by topical application. The galanthamine was first isolated in the 1950 from *Galanthus nivalis* (Amaryllidaceae). It appears also in other genera of the Amaryllidaceae family compose of *Narcissus*, *Lycoris* and *Leucojum*. This alkaloid is reported to be more selective for acetylcholinesterase (AChE) than butyrylcholinesterase (BuChE). Galanthamine has a dual mode of action. As an inhibitor of AChE, it can blocks the degradation of acetylcholine and thereby increases the concentration of available transmitter. A recent study also mentioned neuroprotective effects by induction of an anti-apoptotic protein (Hotettmann *et al*., 2006).

1.2.5 Cholinesterase inhibitors derived from natural products

To date, there are many natural products and their synthesized analogues have been studied on cholinesterase inhibitory mechanisms. Alkaloid is a major group of interesting. As mentioned before, galanthamine is an approved drug and is also natural alkaloid. So, the several alkaloids have been reported to show AChE inhibitory activity, and they are used for the symptomatic treatment of AD. The observation have no surprising considering the fact that for AChE active site, positively charged nitrogen which common found in alkaloid are among the require elements of compounds that can be bound to as the same region to that of ACh. The group of non-alkaloidal inhibitors such as terpenoid is also good candidate for treatment AD. This inhibitor can be avoided the side effect or toxic that found in alkaloid (Chaiyana *et al.*, 2010).

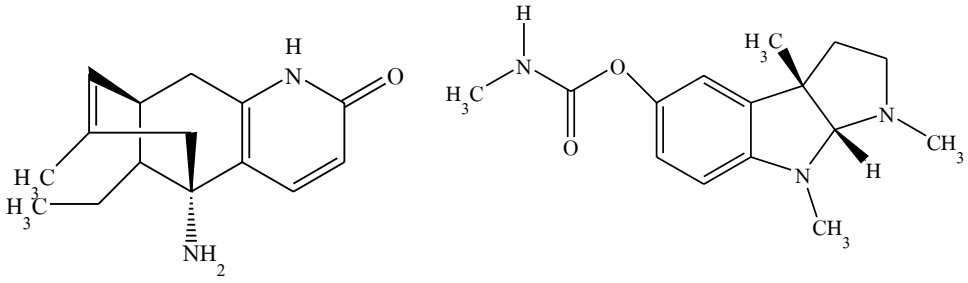
Huperzine A

An extract from the club moss *Huperzia serrata* and *Lycopodium derratum*, has been used for centuries in Chinese herbal medicine to treat ailments varying from confusion in Alzheimer's disease to schizophrenia. The extract contains alkaloid called huperzine A (Figure. 2)**,** which acts as an anticholinesterase agents. Binding site is very specific and therefore the drug can be used in a small dose, thus minimizing the risk of side effects. It has been undergoing clinical trials in China and has been shown to have memory-enhancing effects. It is also a promising lead structure for the development of the next generation of AD drug (Hotettmann *et al*., 2006).

Physostigmine

Physostigmine or eserine (Figure 2) is a compound isolated from a natural product which was discovered in 1864 as a product of the poisonous calabar bean, the seed of *Physostigma venenosum* Blaf., from West Africa. The crude extract of this bean was fed to criminals to assess whether they were guilty or innocent. Death indicated a guilty verdict (Patrick, 2005). Physostigmine (Synapton®) has limited medicinal use because of serious side effects,

short-half-life and non-selective inhibition. However, it has only been used in the treatment of glaucoma or as an antidote for atropine poisoning. Furthermore, it can be used in the treatment of myasthenia gravis. Therefore, this compound can be modified to be candidate for treatment of AD by the development of synthetic analogous bearing the cabamoyl moiety, including neostigmine, revastigmine and pyridostigmine (Anekonda and Reddy, 2005).

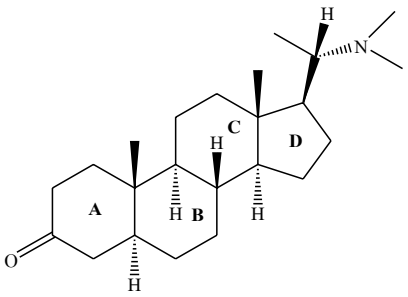


Huperzine A Physostigmine

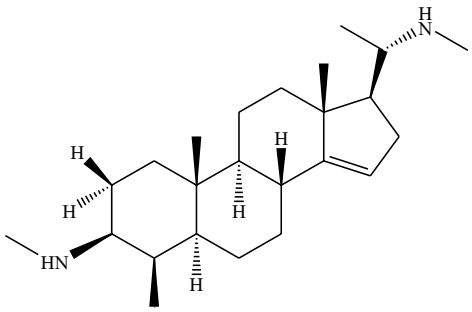
Figure 2 The structures of alkaloid AChE inhibitors

Steroidal alkaloids

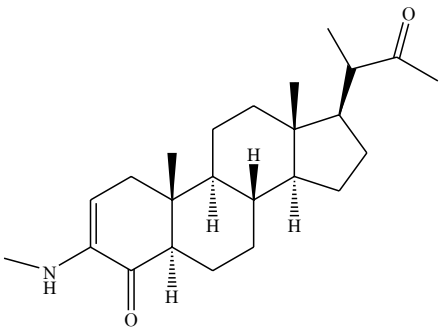
Sarcococca species are the one of the major sources of steroidal alkaloids. The steroidal alkaloids are the major compound of *Sarcococca* species belonging to the Buxaceae family and some of them demonstrate interesting biological activities such as antibacterial, antitumor, antiulcer or anti-AChE activities. Several species were studied to find new active alkaloids. For example, three compounds consist of epoxynepapakistamine A, funtumafrine C and N-methylfuntumine were isolated from leave of *Sarcococca coriacea*, All these compounds are more selective inhibitors against BuChE than AChE (Kalauni *et al.*, 2002). These AChE-I's are shown Figure 3.



Funtumafrine C



Epoxynepapakistamine A



N-methylfuntumine

Figure 3 AChE inhibitors from *Sarcococca coriacea*

1.3 *Holarrhena antidysenterica* (L.) Wall.

1.3.1 Taxonomy characteristics

H. antidysenterica is belonging to the Apocynaceae family, commonly known in Thai as "Mok luang" (Smitinand, 2001) and commonly known as Kurchi in commerce. Characteristic of morphology of this plant are a deciduous lactiferous medicinal shrub the small tree, 9-10 meters in height and the bark pale, grayish in color. The leaves are consisting of 9-18 cm long and 4-8 cm broad, broadly ovate to elliptic. Flowers in terminal are corymbs cymes; white, slightly fragrant (Figure 4). The fruit pods that have size 20 to 40 cm long, in pairs, cylindrical and narrow. The seeds are linear-oblong, 1 cm long. 25-30 seeds per pod, smoky in color (Pratrick, 2005; Satyavati *et al*., 1987). Chemical constituents from *H. antidysenteriga* are shown in table 1.

Figure 4 *Holarrhena antidysenterica* (L.) Wall.

1.3.2 Biological activities of *H. antidysenterica*

In the past, this plant has been used in the folk medicine for various purposes. Also, it is the most popular preparations used in the treatment of diarrhea, dysentery, asthma, bronchopneumonia and malaria (Kumar *et al*., 2007). The clinical controlled trials have proved that *H. antidysenterica* to be the best for amoebicidal. The efficacy of this herb in intestinal amoebiasis and giardiasis is nearly 70%. The alkaloid fraction of *H. antidysenterica* bark extract also showed significant activity against *Staphylococcus aureus, Staphylococcus epidermidis* and *Streptococcus faecalis* with MIC in the rank of 95-170 µg/ml (Chakraborty and Brantner, 1999). In micro broth dilution assay, the extract exhibited MIC of 500 µg/ml against *Mycobacterium avium* and >500 µg/ml for *Mycobacterium smegmatis* (Newton *et al*., 2002).

The methanolic extract of seeds was evaluated the hypoglycemic effect in normal (Normoglycemic) and in streptozotocin-induced diabetic Wistar rats. Oral administration of methanolic extract (250 mg/kg) for 18 days resulted in decrease in blood glucose level compared to the diabetic control. In addition, oral administration of the extract significantly

decreased serum total cholesterol, triglyceride levels and at the same time markedly increased liver glycogen, thus proving the potent anti-diabetic property of the plant (Mana *et al*., 2010).

On the other hand, the powder from stem barks of this plant given to the patients in a dose of 4 gram twice a day for two weeks. The results found that it has significant efficacy of the drug in the symptom stoppage of bleeding in bleeding piles (Pal *et al*., 2009).

1.4 Objectives

The aims of this study are

(1) to investigate AChE inhibitory, anti-inflammatory and antioxidant activities of *H. antidysenterica* and

(2) to study and isolation chemical constituents from *H. antidysenterica* that response for AChE inhibitory, anti-inflammatory and antioxidant activities.

Table 1 chemical constituent from *H. antidysenteriga*

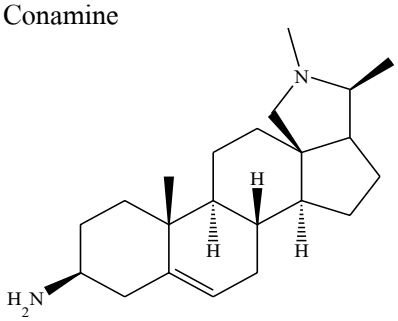
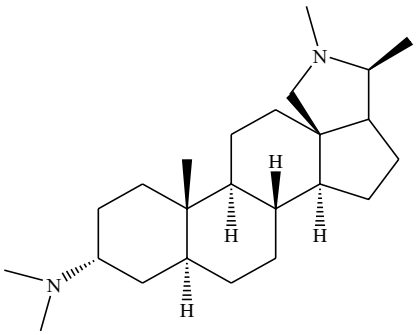
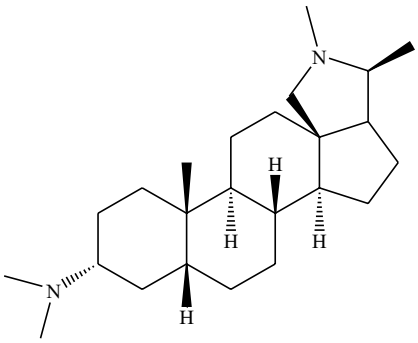
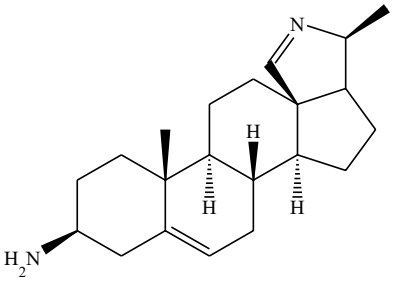
Compound	Part of plant	Reference
Conamine 	Bark	(Siddiqui, 1936)
Concuressine 	Bark	(Siddiqui, 1936)
Dihydroconcuressine 	Bark	(Labler and Sorm, 1963)
Conessidine 	Bark	(Tschesche and Roy, 1956)

Table 1 (cont.)

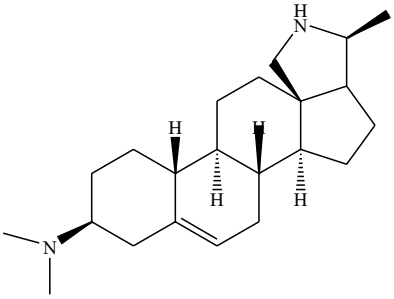
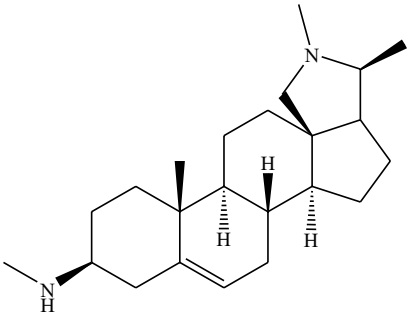
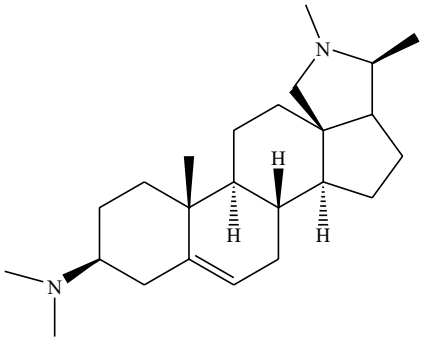
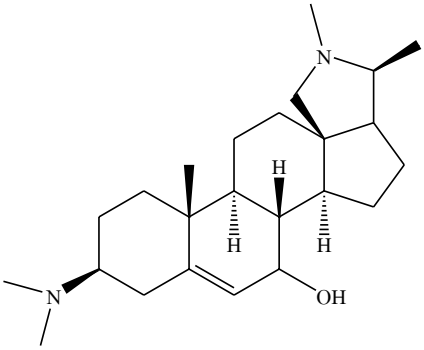
Compound	Part of plant	Reference
Conessimine 	Bark	(Tran *et al.*, 2006)
Isoconessimine 	Bark	(Zirihi *et al.*, 2005)
Conessine 	Bark	(Zirihi et al., 2005)
7-alpha-hydroxyconessine 	Bark	(Tschesche and Ockenfels, 1964)

Table 1 (cont.)

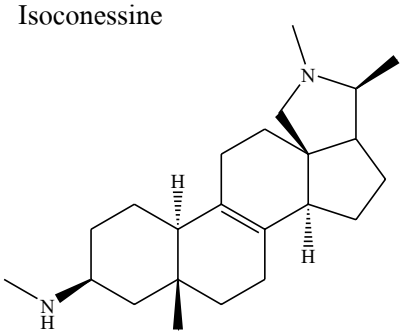
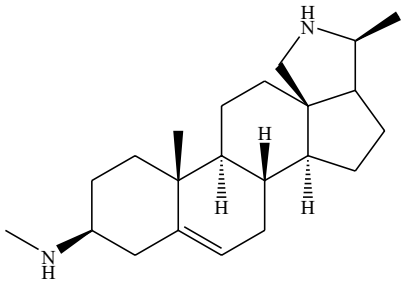
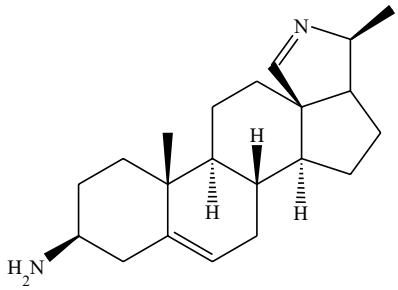
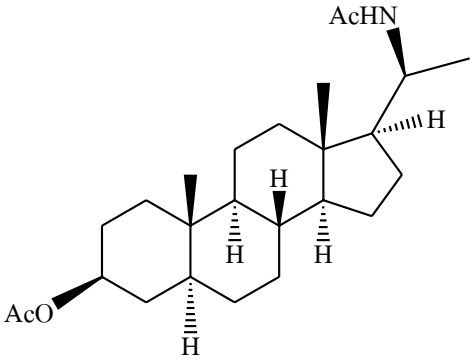
Compound	Part of plant	Reference
Isoconessine 	Bark	(Siddiqui, 1935)
Conimine 	Bark	(Tran *et al.*, 2006)
Conkurchine 	Bark	(Shanker and Basu, 1961)
Holacetine 	Bark	(Rej *et al.*, 1976)

Table 1 (cont.)

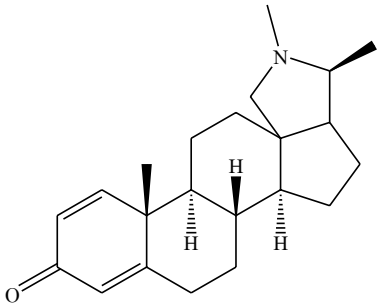
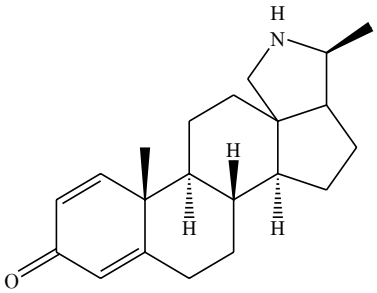
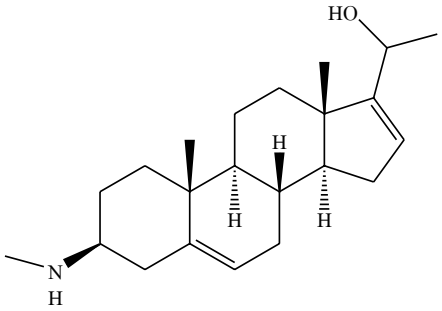
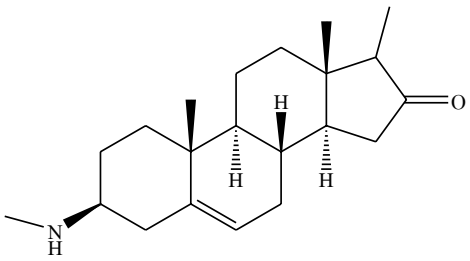
Compound	Part of plant	Reference
Holadiene 	Bark	(Siddiqui *et al.*, 1993)
Norholadiene 	Bark	(Begum *et al.*, 1994a)
Holadysamine 	Leaves	(Janot *et al.*, 1966)
Holadysine 	Leaves	(Janot *et al.*, 1966)

Table 1 (cont.)

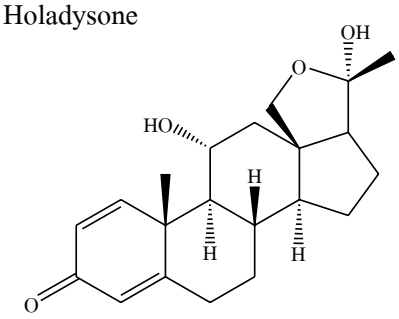
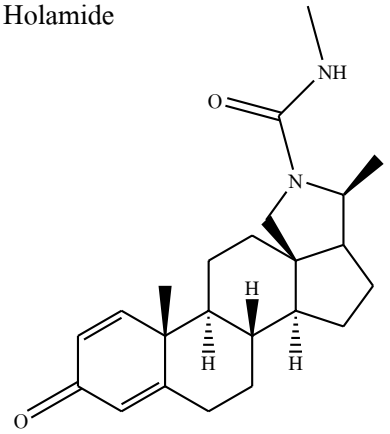
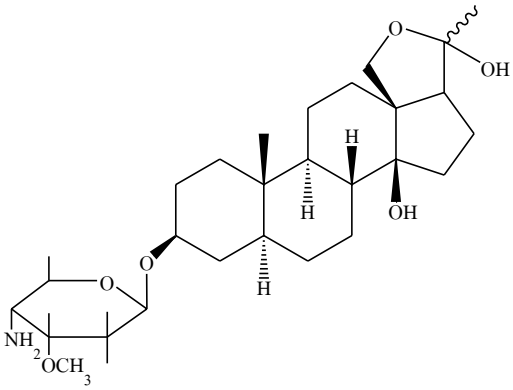
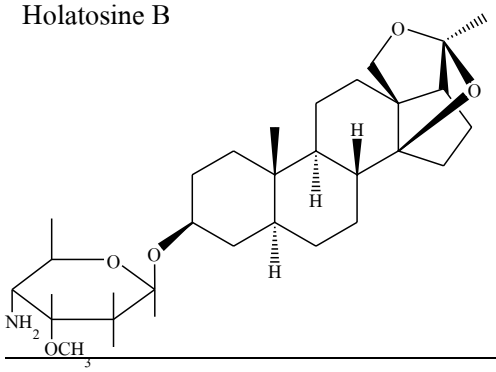
Compound	Part of plant	Reference
Holadysone 	Bark	(Siddiqui *et al.*, 1994)
Holamide 	Bark	(Siddiqui *et al*., 1994)
Holatosine A 	Leaves	(Janot *et al.*, 1970)
Holatosine B 	Leaves	(Janot *et al.*, 1970)

Table 1 (cont.)

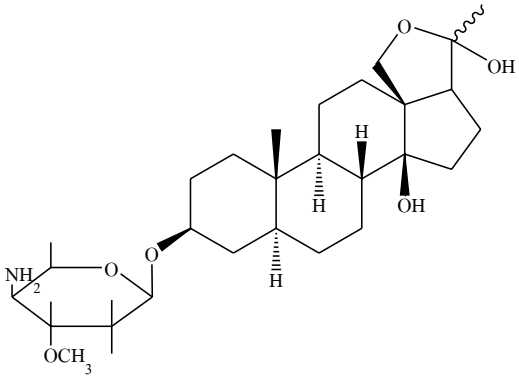
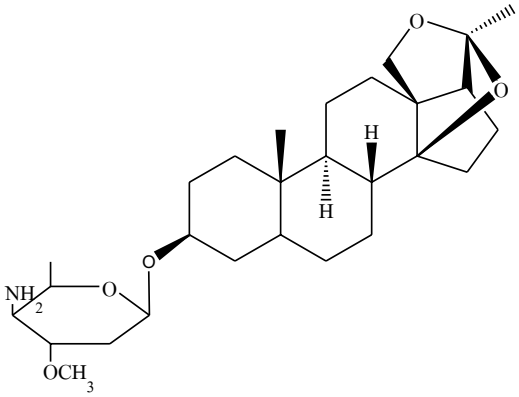
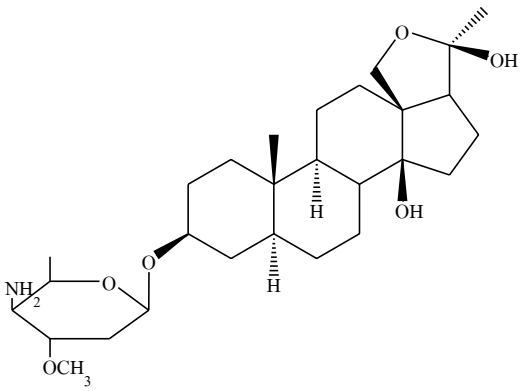
Compound	Part of plant	Reference
Holatosine C	Leaves	(Khuong Huu *et al.*, 1971)
		
Holatosine D	Leaves	Khuong Huu *et al.*, 1971
		
Holatosine E	Leaves	(Goutarel *et al.*, 1972)
		

Table 1 (cont.)

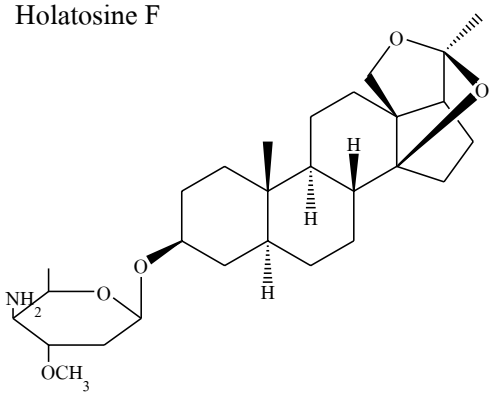
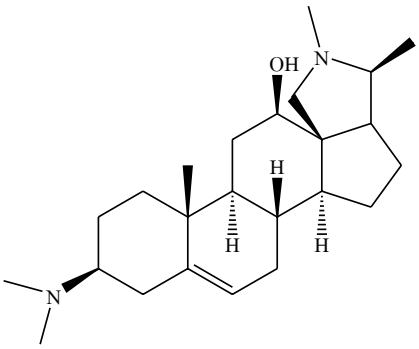
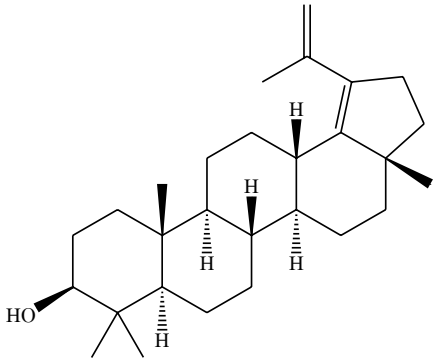
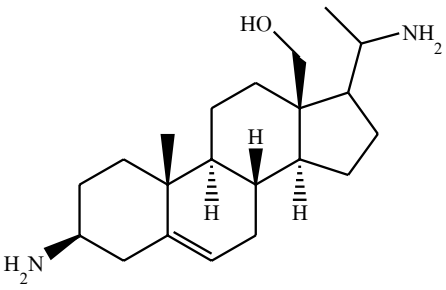
Compound	Part of plant	Reference
Holatosine F 	Leaves	(Goutarel *et al.*, 1972)
Holarrhenine 	Bark	(Tschesche and Wiensz, 1958)
Holarrhenol 	Bark	(Ali and Gupta, 1994)
Holarrimine 	Seed	(Siddiqui and Siddiqui, 1981)

Table 1 (cont.)

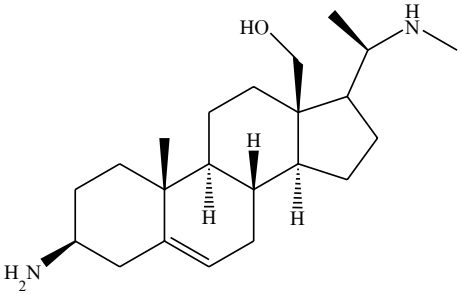
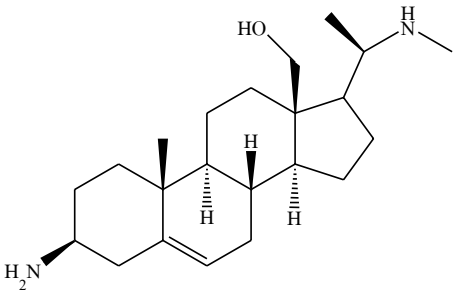
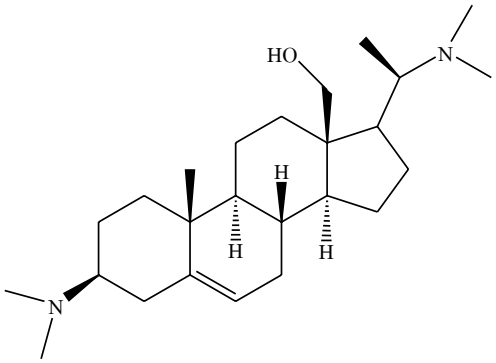
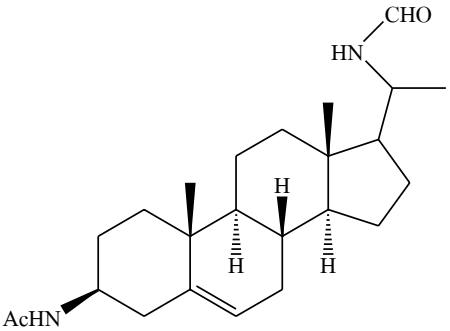
Compound	Part of plant	Reference
20-N-methylholarhimine 	Bark	(Tschesche and Wiensz, 1958)
3-N-methylholarhimine 	Bark	(Tschesche and Wiensz, 1958)
Tetramethylhorrharimine 	Bark	(Willaman and Li, 1970)
Holarrhicine 	Seed	(Siddiqui and Siddiqui, 1981)

Table 1 (cont.)

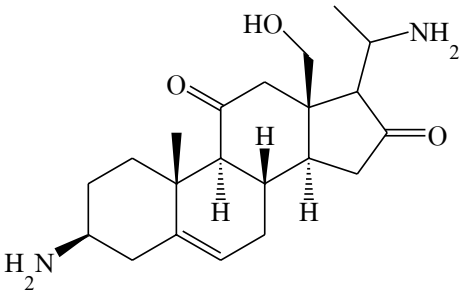
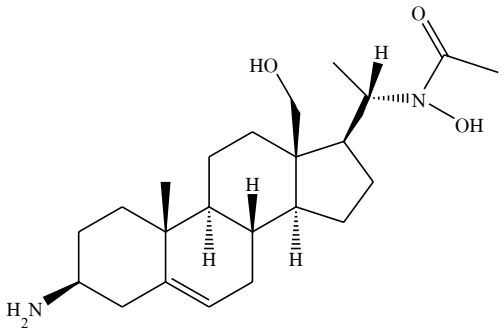
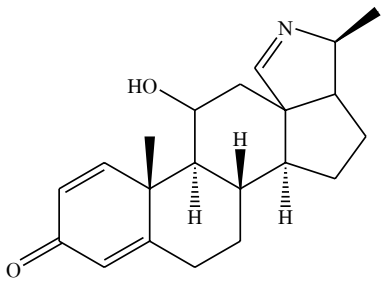
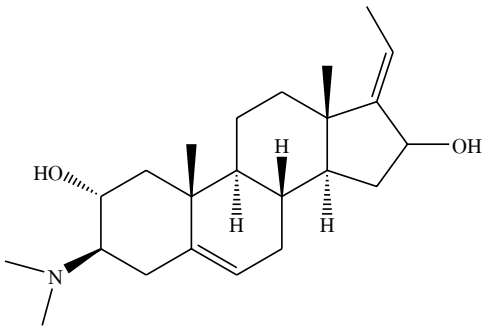
Compound	Part of plant	Reference
Holarrifine 	Bark	(Siddiqui and Shamsuddin, 1989)
Holadysenterine 	Bark	(Kumar *et al.*, 2007)
Holonamine 	Bark	(Tschesche and Ockenfels, 1964)
Kurchaline 	Leaves	(Janot *et al.*, 1966)

Table 1 (cont.)

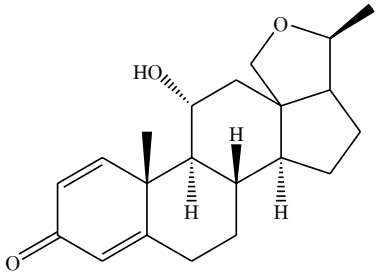
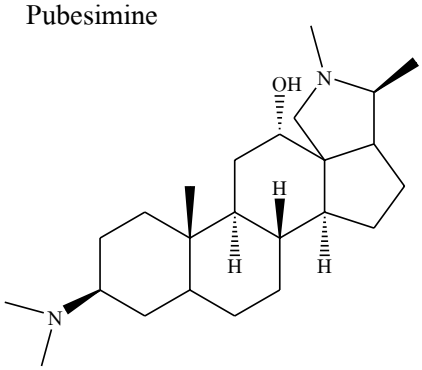
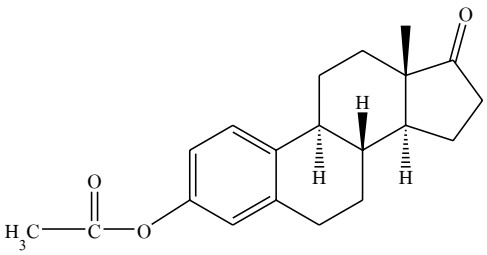
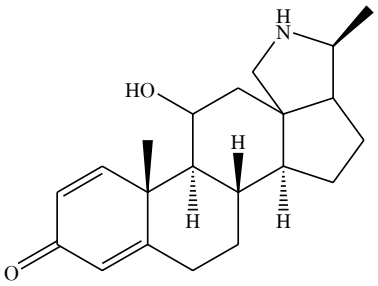
Compounds	Part of plant	Reference
Pubadysone 	Bark	(Siddiqui *et al.*, 2001)
Pubesimine 	Bark	(Begum *et al.*, 1994b)
Puboestrene 	Bark	(Siddiqui *et al.*, 2001)
Regholarrhenine A 	Bark	(Bhutani *et al.*, 1988)

Table 1 (cont.)

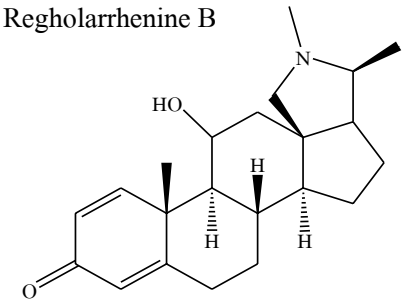
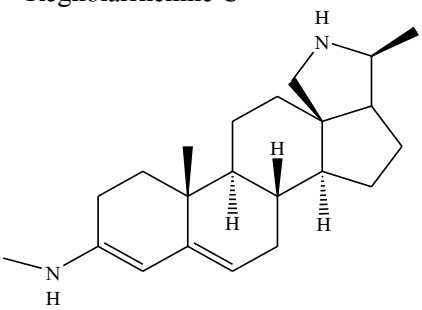
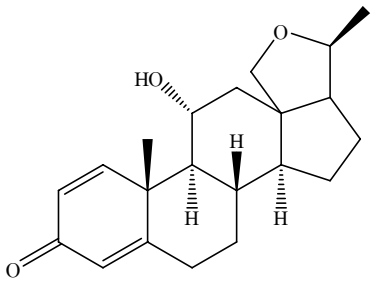
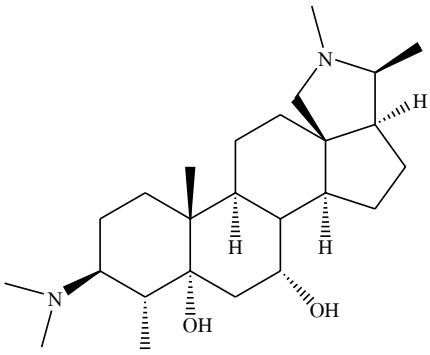
Compounds	Part of plant	Reference
Regholarrhenine B 	Bark	(Bhutani *et al.*, 1988)
Regholarrhenine C 	Bark	(Bhutani *et al.*, 1988)
Regholarrhenine D 	Bark	(Bhutani *et al.*, 1990)
Regholarrhenine E 	Bark	(Bhutani *et al.*, 1990)

Table 1 (cont.)

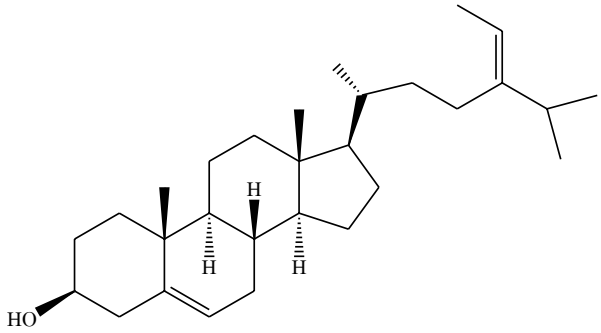
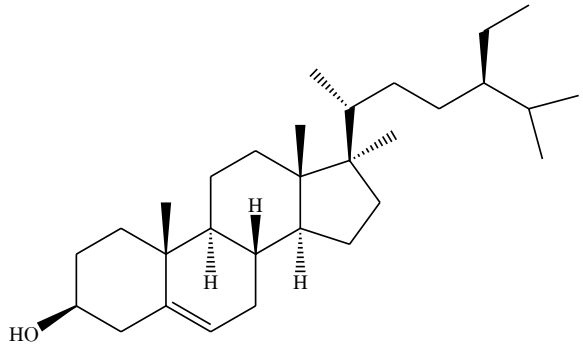
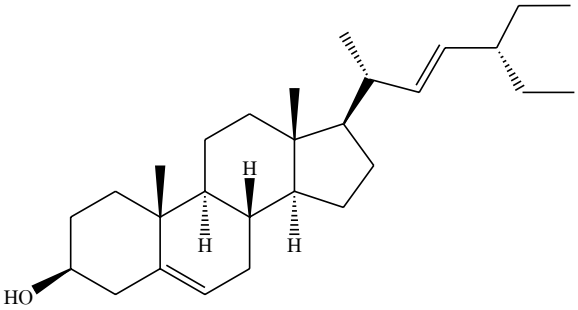
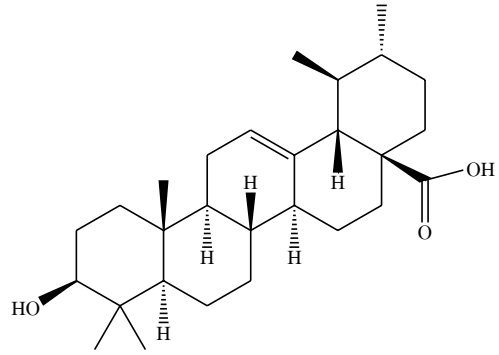
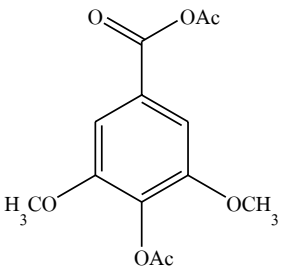
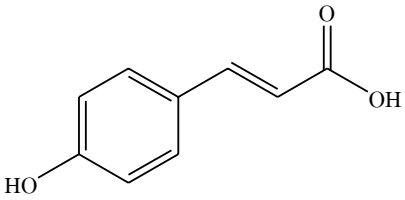
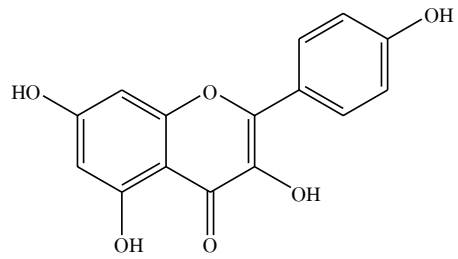
Compounds	Part of plant	Reference
28-isofucosterol	Bark	(Heble *et al.*, 1976)
		
Beta-sitosterol	Seed	(Kumar and Ali, 2001)
		
Stigmasterol	Seed	(Sanjib *et al.*, 2009)
		

Table 1 (cont.)

Compounds	Part of plant	Reference
Ursolic acid 	Leaves	(Daniel and Sabnis, 1978)
Alpha-resoclyclic 	Leaves	(Daniel and Sabnis, 1978)
Para-coumaric acid 	Leaves	(Daniel and Sabnis, 1978)
Kaempferol 	Leaves	(Daniel and Sabnis, 1978)

CHAPTER 2

EXPERIMENTAL

2.1 General experiment procedures

^{1}H and ^{13}C NMR spectra were recorded on a Varian Inova 500 MHz or Bruker Avance 300 MHz. The chemical shifts (ppm) were referred to the solvent peaks (chloroform-*d*; δ_H 7.25 for residual $CDCl_3$, and δ_C 77.0 and methanol-*d*; δ_H 3.30 for residual CD_3OD, and δ_C48.3). Mass spectra were measured on a Waters 2690-LCT Alliance-Micromass and Thermofinnigan MAT 95 XT mass spectrometers. IR spectra were recorded on a Jasco IR-810 infrared spectrometer. UV spectra were obtained in ethanol on a Hewlett Packard 8452A diode array spectrophotometer. Basic alumina, Silica gel 60 (Merck, 40-63 μm) and Sephadex®LH-20 (Sigma–Aldrich, 25-100 μM, Germany) were use for column chromatography, while thin-layer chromatography (TLC) was performed on Merck silica gel F_{254} aluminium sheet (250 μM thickness) and spots were detected by UV absorption and spraying with dragendorff's reagent for detection of alkaloids.

2.2 Plant material

The bark of *H. antidysenterica* was collected from the Botany Garden of the Faculty of Pharmaceutical Sciences. A voucher specimen number SKP013080101 of the plant material was deposited at the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai, Songkhla, Thailand.

2.3 Preparation of plant extracts

The fresh bark (3.5 kg) was washed and dried at 50 °C for two days and then ground into powder. The dried bark powder (1.3 kg) of *H. antidysenterica* was extracted by

maceration with ethanol (3.5 L×3). The ethanol extract was dried under reduced pressure by rotary evaporator to give a crude extract (130 g).

2.4 *In vitro* assay for biological activity

2.4.1 Microplate assay for AChE activity

The assay for measuring AChE activity was modified from the assay described by Ellman *et al*. (1961) and Ingkaninan *et al.* (2003). The AChE used in the assay from electric eel (type VI-S, EC 3.1.1.7; Sigma, USA). Briefly, 125 µl of 3 mM 5,5-dithiobis-(2-nitrobenzoic acid) (Sigma, USA), 25 µl of 15 mM acetylthiocholine iodide (Sigma, USA), and 50 µl of buffer, 25 µl of sample dissolved in buffer containing not more than 10% ethanol were added to the wells, followed by 25 µl of 0.28 U/ml AChE. The absorbance was detected at 405 nm every 10s for 2 min by a PowerWaveX microplate reader (Bio-Tek Instrument, USA). The velocities of the reactions were measured. Enzyme activity was calculated as a percentage of the velocities compared to that of the assay using buffer without any inhibitor. Inhibitory activity was calculated from 100 subtracted by the percentage of an enzyme activity. The concentration of *H. antidysenterica* and galanthamine is tested for triplicate to determine 50% enzymatic inhibitory activity (IC_{50}) using the software package Prism (Graph Pad Inc., San Diego, USA). The activity was used galanthamine (Sigma, USA) as standard reference.

2.4.2 Evaluation of anti-inflammatory activity

Inhibitory effect on NO production by murine macrophage like RAW 264.7 (ATCC No. TIB-71) cells are evaluated using a modified method from that previously reported (Tewtrakul and Itharat, 2007). The RAW264.7 cells [purchased from Cell Lines Service (CLS)] are cultured in Roswell Park Memorial Institute (RPMI) (Gibco, USA) medium supplemented with 0.1% sodium bicarbonate, 2 mM glutamine, penicillin G (100 U/ml) (Invitrogen, USA), streptomycin (100 µg/ml) (Invitrogen, USA) and 10% fetal calf serum (Gibco, USA). The cells are harvested with trypsin-EDTA (Gibco, USA) and diluted to a suspension in fresh medium. The

cells are seeded in 96-well plates with $1x10^5$ cells/well and allow adhering for 1 h at 37 °C in a humidified atmosphere containing 5% CO_2. After that the medium is replaced with fresh medium containing 100 µg/ml of lipopolysaccharide (Gibco, USA) together with test samples at various concentrations and then incubated for 48 h. NO production is determined by measuring the accumulation of nitrite in the culture supernatant using the greiss reagent. The activity was reported in IC_{50} using indomethacin (Sigma, USA) as standard reference.

2.4.3 Cytotoxicity test

Cytotoxicity is determined using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Kumamoto, Japan) colorimetric method (Dong *et al*., 2001). Briefly, after 48 h incubation with test samples, MTT solution (10 µl, 5 mg/ml in phosphate buffered saline) is added to the wells. After 4 h incubation, the medium is removed, and isopropanol containing 0.04 M HCl is then added to dissolve the formazan production in the cells. The absorbance of formazan solution is measured with a microplate reader at 570 nm. The test compounds are considered to be cytotoxic when the absorbance at 570 nm of the sample-treated group is less than 80% of that in the control (vehicle-treated) group. Indomethacin is used as a positive control. The stock solution of each test sample is dissolved in DMSO, and the solution is added to the RPMI medium (final DMSO is 1%). Inhibition (%) is calculated using the following equation and IC_{50} values are calculated from the graph plotted of % inhibition against standard compound concentrations.

$$\text{Inhibition (\%)} = [A - B/A - C] \times 100$$

A – C: NO_2– concentration (µM) [A: LPS (+), sample (-); B: LPS (+), sample (+); C: LPS (-), sample (-)]. For statistical analysis, the values are expressed as mean ± S.E.M of four determinations.

2.4.4 Antioxidant activity test

To determine antioxidant activity of *H. antidysenterica* extract was evaluated by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay (Blois, 1953), the dried residue obtained from

extraction was dissolve in absolute ethanol (1 mg/ml) as stock solution, diluted each sample for at least 5 concentrations in absolute ethanol (two-fold dilutions), transferred 100 µL of each sample solution into an eppendorf tube.

DPPH (Fluka, Germany) solution ($6x10^{-5}$ M) was prepared in absolute ethanol, and it transferred (100 µL) to mix with sample solution. The mixture was shaken and stood at the room temperature for 30 minutes in the dark. The mixture was then measured the absorbance at 520 nm, using a mixture of 100 µL sample solution and 100 µL absolute ethanol as blank. The control solution in each experiment was prepared as follows:

- **Control ethanol:** mix of absolute ethanol (100 µL) with $6x10^{-5}$ M DPPH in absolute ethanol (100 µL); **blank:** absolute ethanol. Calculation of % inhibition with equation as follows :

$$\% \text{ Inhibition} = (A_{control} - A_{sample})/A_{control} \times 100$$

Where $A_{control}$ is the absorbance of DPPH solution with out sample and A_{sample} is the absorbance of DPPH solution with sample solution.

Dose-response curve was plotted between % inhibition and concentrations. Linear regression analysis was carried out for calculating the effective concentration of sample required to scavenge DPPH radical by 50 % (ED_{50} value). In each experiment, standard butylated hydroxytoluene (BHT) (Sigma–Aldrich, Germany) is used as a positive control. Dissolve the standard compounds with absolute ethanol. Dilute each standard compound for at least 5 concentrations in absolute ethanol.

2.5 Molecular docking study

The crystal structure of TcAChE X-ray complexes with donepezil code ID: 1EVE (Kryger *et al*., 1999) was used for steroidal alkaloid docking, and complexes with tacrine code ID: 1ACJ (Harel et *al*., 1993) using for scopoletin docking. They were received from the Protein Data Bank (PDB) (RCSB PDB, 2011).

The protein and ligand analysis were prepared by using Discovery Studio 2.5 program. For protein, the ligand and water molecules were removed from each structure. All

hydrogen atoms were added into the protein structure in order to set the complex to be the neutral molecule. For donepezil and tacrine, the atom types were assigned and hydrogen atoms were added.

The structures of ligands consist of steroidal alkaloid derivatives and scopoletin were created by assigning atom types based on GAUSSIAN program.

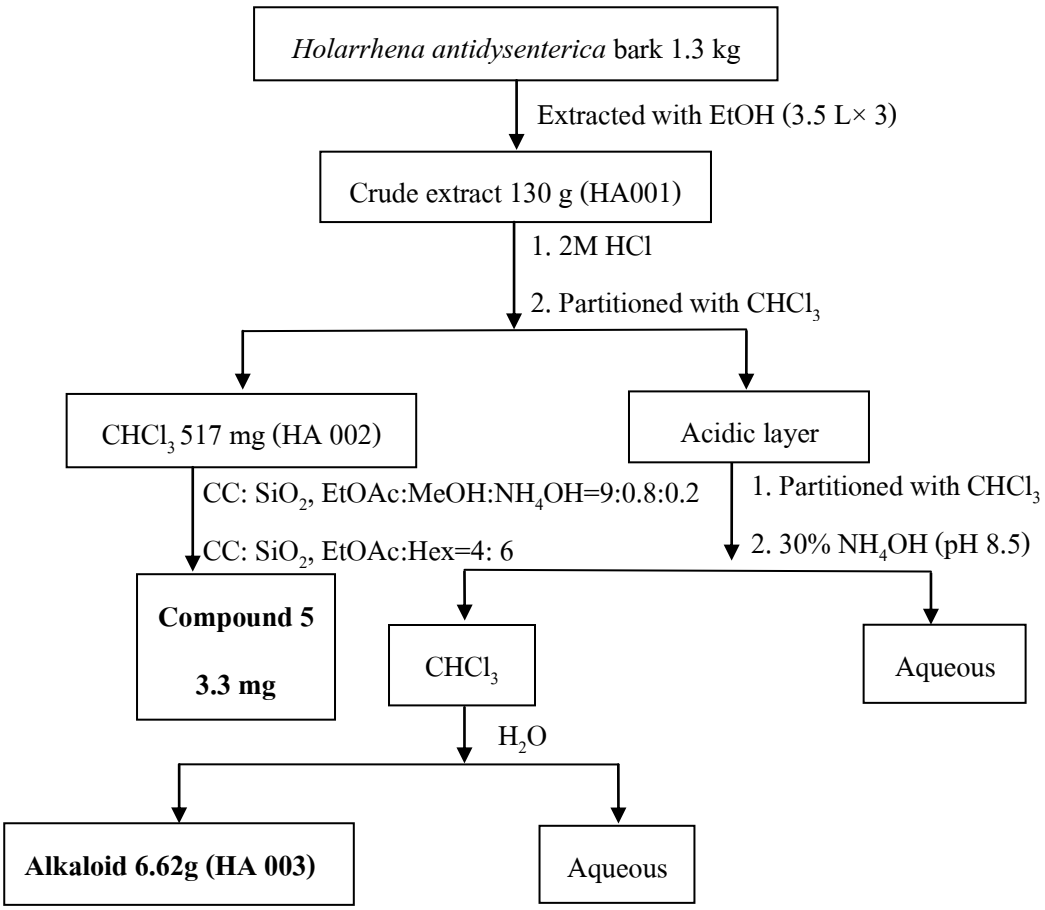
The Gold program was employed to generate a set of docked conformations for each ligand. This program uses a genetic algorithm to explore conformational spaces and ligand binding modes. Then, steroidal alkaloids were docked into PDB code 1EVE and scopoletin was docked into PDB code1ACJ. The GoldScore fitness functions were used to analyze the binding in each docked compound. Fifteen genetic algorithm runs were setting. The orientation of each docked compound with GoldScore was selected to analyze the interaction of steroidal alkaloids and coumarin in AChE binding pocket or aromatic gorge.

2.6 Isolation and purification

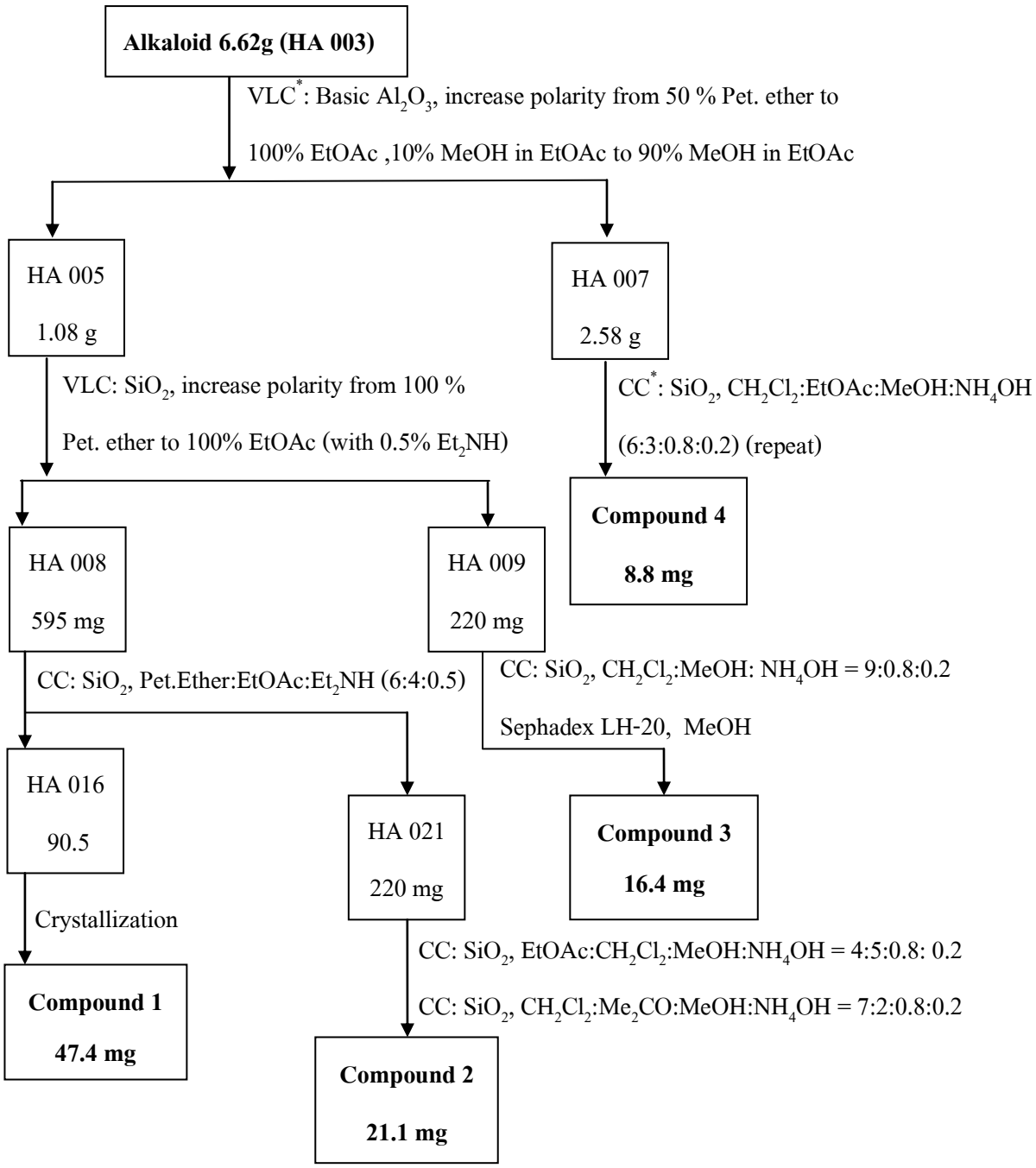
2 M HCl 300 ml was added to the ethanolic extract (130 g) then extracted with $CHCl_3$ to remove neutral component (517 mg). The aqueous acid layer was then made alkaline (pH 8.5) with NH_4OH (30%) solution and repeatedly extracted with $CHCl_3$. The combined CH_3Cl extracts were washed with H_2O, dried and evaporated *in vacuo* to obtain crude alkaloids (6.62 g) (Scheme 1).

The crude alkaloid extract was subjected to column chromatography on basic alumina using Pet. ether-EtOAc and EtOAc–MeOH (gradient) to give fractions number 4-7. Fraction numbers (Fr. No.) 5 and 7 were further purified on silica gel column. Fractionation of Fr. No. 5, the silica gel column was eluted with Pet. Ether and EtOAc (gradient) and alkalinize with 0.5% Et_2NH to give Fr. No. 8-10. Fractionation of Fr. No. 8, the silica gel column was eluted with 60 % Pet. ether in EtOAc and basicified with Et_2NH 0.5% to give Fr. No. 11-18. Fraction No. 12 was crystallized in acetone to give compound **1** (47.4 mg, 0.0036%). Fractionation of Fr. No. 15, the silica gel column was eluted with EtOAc: CH_2Cl_2: MeOH: NH_4OH (4:5:0.8:0.2) and it was repeated isolation by silica gel column eluted with CH_2Cl_2: Me_2CO: MeOH: NH_4OH (7:2: 0.8:0.2) to give compound **2** (21.1 mg, 0.0016%). Fractionation of Fr. No. 9, the silica gel column

was eluted with CH_2Cl_2: MeOH: NH_4OH (9:0.8:0.2) and repeat isolation on Sephadex LH-20 eluted with MeOH to give compound **3** (16.4 mg, 0.0013%). Fractionation of Fr. No. 7, the silica gel column was eluted with CH_2Cl_2: EtOAc: MeOH: NH_4OH (6:3:0.8:0.2) and repeat isolation using the same condition to give compound **4** (8.8 mg, 0.00068%). Fraction from neutral component $CHCl_3$ layer was separated on silica gel column and eluted with EtOAc: MeOH: NH_4OH (9:0.8:0.2) and further isolated on silica gel column eluted with EtOAc: hexane (4:6) to give compound **5** (3.3 mg, 0.00025%). Isolation diagrams of *H. antidysenterica* bark are show in Scheme 1 and Scheme 2.



Scheme 1 Isolation diagram of crude alkaloid from *H. antidysenterica* bark



VLC* = Vacuum liquid chromatography

CC* = Column chromatography

Scheme 2 Isolation diagram of compounds from *H. antidysenterica* bark

2.7 Physical properties and spectroscopic data of isolated compounds

Conessine (1): white solid: IR (KBr) τ_{max} 3437, 2939, 2760, 1451, 1346, 1206, 1174, 1147, 1040, 997, 888,831, 799 cm^{-1}.; UV (EtOH) ς_{max} (log κ) 202 nm. (3.85); ^{1}H and ^{13}C NMR see table 5; EIMS *m/z* 356 (5), 341 (7), 84 (100), 71 (7).

Isoconessimine (2): white solid: IR (neat) τ_{max} 3429, 2949, 1558, 1404, 1102, 1053, 1013, 867, 835, 796, 651 cm^{-1}.; UV (EtOH) ς_{max} (log κ) 202 nm. (3.9); ^{1}H and ^{13}C see NMR table 6; EIMS *m/z* (relative intensity) 342 [M^{+}] (3), 326 (5), 257 (100), 70 (72).

Conessimine (3): white solid: IR (KBr) τ_{max} 3430 (NH), 2937, 2780, 1558, 1380, 1152, 1034, 828, 802, cm^{-1}.; UV (EtOH) ς_{max} (log κ) 202 nm. (3.09); ^{1}H and ^{13}C NMR see table 7; EIMS *m/z* (relative intensity) 342 [M^{+}] (5), 84 (100), 61 (8).

N-3-methylholarrhimine (4): white solid: IR (neat) τ_{max} 3390, 2938, 1557, 1405, 1260, 1045, 945, 800, 655 cm^{-1}.; UV (EtOH) ς_{max} (log κ) 202 nm. (3.26); ^{1}H and ^{13}C NMR see table 4; EIMS *m/z* (relative intensity) 346 [M^{+}] (3), 303 (11), 277 (25), 84 (25), 70 (100).

Scopoletin (5): white needles: IR (KBr) τ_{max} 3338, 1705, 1608, 1566, 1510, 1435, 1291, 1262, 1140, 1019, 922, 861, 820, 745, 663, 591 cm^{-1}; UV ς_{max} (log κ) 205 (3.4), 229 (3.0), 298 (2.6), 346 nm. (2.9); ^{1}H and ^{13}C NMR see table 8; EIMS *m/z* (relative intensity) 192 [M^{+}] (100), 177 (54), 164 (22), 149 (40), 121 (13), 79 (72), 69 (24).

CHAPTER 3

RESULTS AND DICUSSION

3.1 AChE inhibitory, anti-inflammatory and antioxidant activities of *H. antidysenterica* bark extract

Bark extract of *H. antidysenterica* was evaluated for AChE inhibitory, anti-inflammatory and antioxidant activities and IC_{50} values were calculated. Further separation of bark extract obtains neutral and alkaloid crude extract (Table 2). Both extract evaluated for biological activities (Table 3).

Table 2 Results from alkaloid preparation

Crude	Weight	(%w/w) yield	Ethanol (L)
Fresh bark	3.5 kg	-	10
Dried Bark	130 g	3.7	-
Alkaloid	6.62 g	0.19	-
Neutral	517 mg	0.01	-

3.2 The isolation of active compounds

The ethanolic extract of bark was tested on AChE inhibitory, anti-inflammatory and antioxidant activities. Then, it was extracted by acid-base technique to obtain alkaloid crude and neutral crude, and they were tested on these activities. The results found that alkaloid and

neutral had high activity against AChE activity more than other activities, therefore these fractions were subjected to the chromatographic isolation to obtain the active compounds (**1**-**5**).

Table 3 The activity of AChE inhibitory, anti-inflammatory and antioxidant activities of *H. antidysenterica* bark extract

Sample	**AChE inhibitory** IC_{50} **(µg/mL)**	**Anti-inflammatory** IC_{50} **(µg/mL)**	**Antioxidant** ED_{50} **(µg/mL)**
Bark extract	2.99± 0.57	40.52	10.19
Neutral crude extract	5.60± 1.77	4.19*	13.06
Alkaloid crude extract	1.44± 0.96	31.31	>100
Galanthamine	0.21±0.11	-	-
Indomethacin	-	8.93	-
BHT	-	-	1.70

*Cytotoxicity was observed at 100, 30, 10, µg/mL.

3.3 The structure elucidation of isolated compounds

3.3.1 N-3-methylholarrhimine (compound 4)

Compound **4** was obtained as white solid. The molecular formula of **4** was suggested as $C_{22}H_{38}N_2O$ by the $[M]^+$ peak at 346 *m/z* in EIMS spectrum (Figure 7). Five degree of unsaturations were assigned for four rings and one olefin. The absorption band in the IR spectrum at 3390 cm^{-1} was suggested the OH in molecular. The UV spectrum shows the maximal absorption at ς 202 nm. (log κ3.26). This compound was giving orange color with Dragendorff's reagent.

The ^1H NMR spectrum (Figure 5) displayed two singlet methyl signals at ι 1.04, ι 2.38 and one doublet methyl signal at ι 1.24 suggested three methyl groups correspond to steroidal alkaloid moiety. The olefinic protons at ι 5.32 (d; 3.1) suggested H-6, aliphatic protons between ι 0.92 and ι 2.44 typical for a steroidal skeleton (Table 4).

The ^1H-^1H COSY spectrums direct correlation between hydroxymethylene protons at## #3.45 (d; 11.7 Hz) and 3.63 (d; 11.7 Hz) correspondent to H-18, signals of H-6 (ι 5.32) showed correlation with H-7 (ι #2.00), N-methyl group (ι 2.38) at C-3 showed correlation long-range interaction with ι 0.90 (H-4). A multiplet proton at ι 3.25 (H-20) showed correlation with the H-21 methyl group at ι 1.24, confirming substituent of methyl group on C-20).

The ^{13}C NMR spectrum (Figure 6) of compound **4** revealed 22 resonances consist of three methyls, nine methylenes, seven methines and three quaternaries. The carbon signals of methyl group resonance at ι 19.8, 23.7, and 33.2 were suggested to C-19, C-21, C-22, respectively. Two olefinic carbons at ι 142.3 and 122.1 were suggested to C-5 and C-6 on the basis of these observations, respectively. The attachment of amino substituent groups at ι 60.9 was C-3 amino substituent (Table 4).

From the HMBC spectrum, the olefinic proton H-6 showed correlation with C-10 (ι 38.3) and C-8 (ι 33.2), while H-3 (ι 2.32) methine proton exhibited correlation with C-4 (ι 34.9), C-5 (ι 142.3) and C-6 (ι 122.1). The hydroxymethylene protons showed HMBC correlation with C-17 (ι 57.8), C-20 (ι 47.6).

The structure of compound **4** has been assigned to be N-3-methylholarrhimine which confirm based on spectral data from that previously reported (Kasal *et al.*, 1963).

Table 4 NMR data of compound **4** (500 MHz for ^{1}H and 125 MHz for C^{13} in CD_3OD)

Position	Type	$\delta^{13}C$#	$\delta^{1}H$, mult. (*J* in Hz)#	HMBC correlation (H→C)
1	CH_2	39.0	1.10 (m)	-
			1.82 (m)	-
2	CH_2	24.7	1.38 (m)	-
			1.68 (m)	-
3	CH	60.9	2.32 (m)	C-4, C-5, C-6
4	CH_2	34.9	0.90 (m)	-
			1.94 (m)	-
5	C	142.3	-	C-8, C-10
6	CH	122.1	5.32 (d; 3.1)	-
7	CH_2	33.2	2.00 (m)	-
			2.33 (m)	-
8	CH	33.2	1.50 (m)	C-5, C-6
9	CH	52.1	1.10 (m)	-
10	C	38.3	-	-
11	CH_2	21.3	1.18 (m)	C-19
12	CH_2	39.2	2.08 (m)	-
			2.36 (m)	-
13	C	47.5	-	-
14	CH	57.8	1.50 (m)	-
15	CH_2	22.5	1.18 (m)	-
			1.75 (m)	-

Table 4 (cont.)

Position	Type	ι ^{13}C#	ι ^{1}H, mult. (*J* in Hz)#	HMBC correlation (H→C)
16	CH_2	28.7	1.39 (m)	C-15
17	CH	57.8	1.20 (m)	C-15
18	CH_2	58.5	3.45 (d; 11.7)	C-13, C-17, C-20
			3.63 (d; 11.7)	C-17,
19	CH_3	19.8	1.04 (s)	C-5, C-9, C-10
20	CH	47.6	3.25 (m)	-
21	CH_3	23.7	1.24 (d; 6.6)	C-20, C-17
22	CH_3	33.2	2.38 (s)	C-3

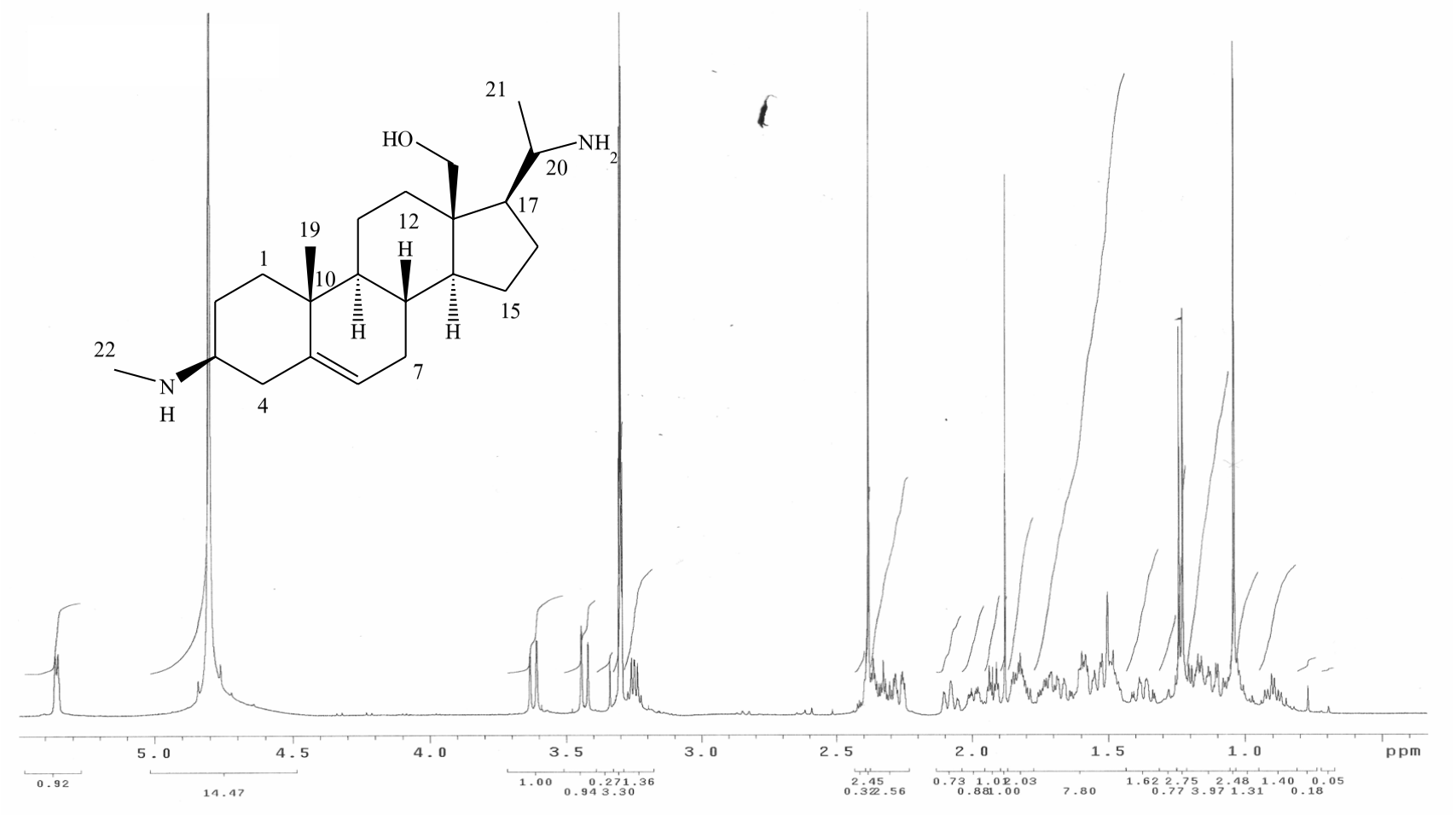


Figure 5 The structure and ^{1}H NMR spectrum of compound **4** (500 MHz in CD_3OD)

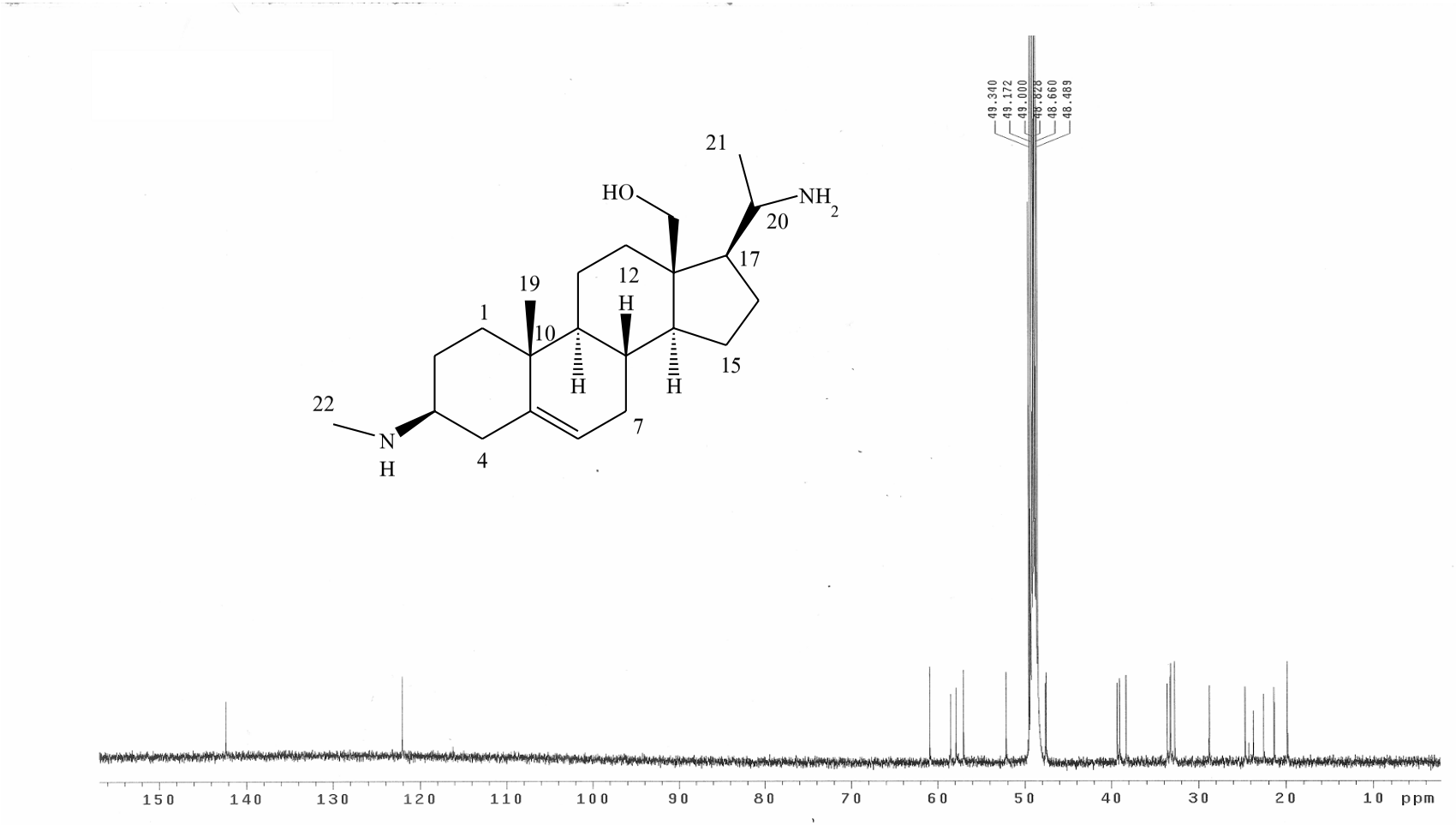


Figure 6 The structure and ^{13}C NMR spectrum of compound **4** (125 MHz in CD_3OD)

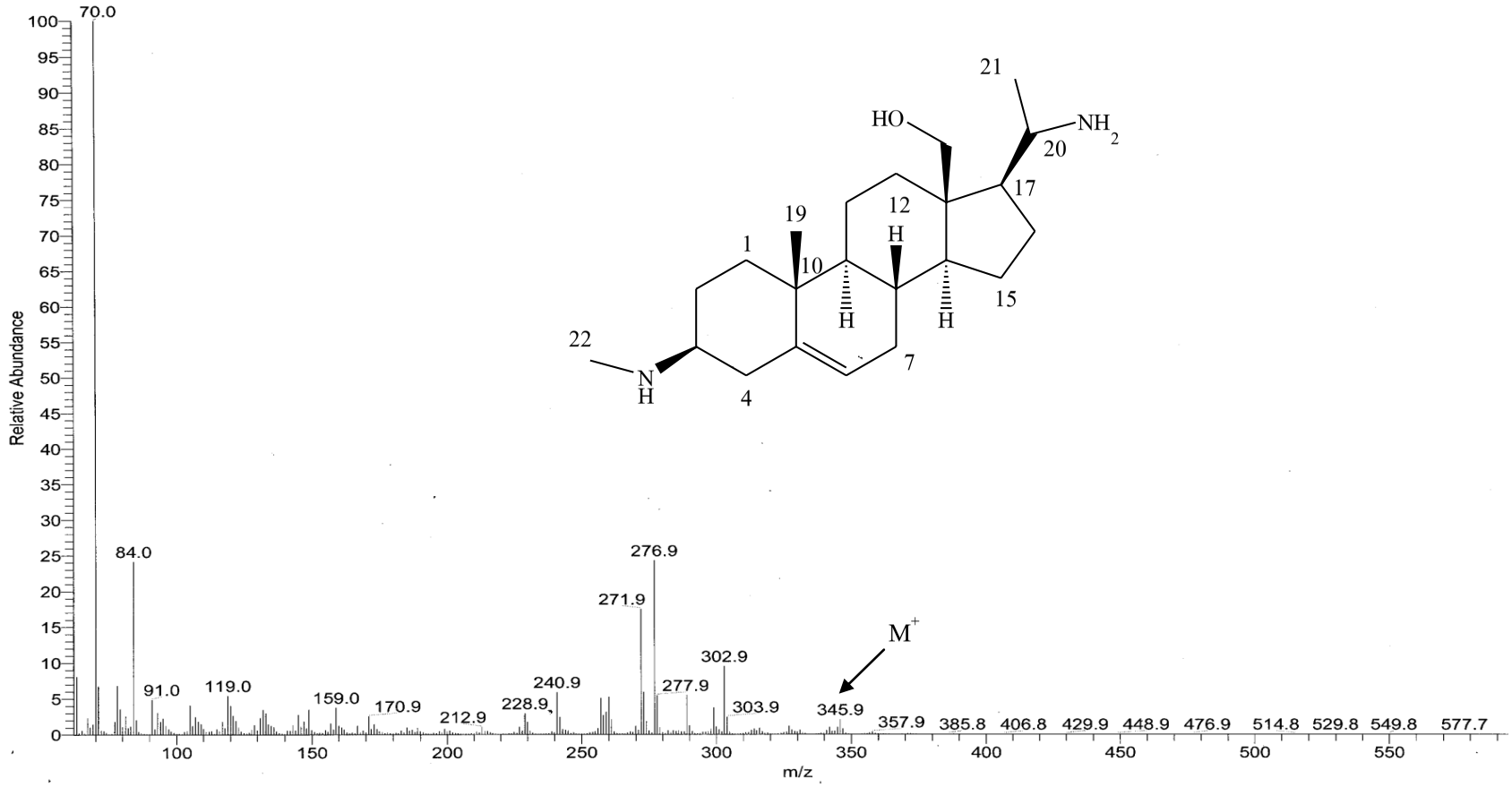


Figure 7 EIMS spectrum of compound **4**

3.3.2 Conessine (compound 1)

Compound **1** was obtained as a white amorphous solid. Its low-resolution mass spectrum in EIMS showed $[M]^+$356 (Figure 10) corresponded to the molecular of $C_{24}H_{40}N_2$, indicating six degrees of unsaturation in the molecule. These were accounted for five rings, and one double bond. The IR spectrum showed presence of NH group absorption band at 3437 (NH) cm^{-1}. The UV spectrum shows the maximal absorption at ς 202 nm. (log κ3.85). This compound was giving orange color with Dragendorff's reagent.

The ^{1}H NMR spectrum data (Table 5, Figure 8) of **1** differed from **4** by lacking of OH functional group and containing of pyrrolidine moiety. Its NMR ^{1}H spectrum data (Table 3, Figure 5) exhibited three singlet signals at ι 0.93 (H-19), ι 2.25 (H-22), ι 2.33 (H-23) and (H-24) and one doublet at 1.08 (H-21) suggested characteristic of four methyl groups correspond to steroidal alkaloid moiety. The olefinic protons at ι 5.32 assigned to H-6, aliphatic protons between ι 0.92 and ι 2.44 typical for a steroidal skeleton. The multiplets signals at ι 2.18 and ι 2.44 were assigned to H-3 and H-20, respectively.

The ^{13}C NMR spectrum (Figure 9) of compound **1** revealed 23 resonances consist of four methyls, nine methylenes and seven methines. The carbon signals of methyl groups resonance at ι 18.9, 14.1, 40.7 and 41.0 were assigned to C-19, C-21, C-22, C-23 and C-24 (C-23 and C-24 show at the same chemical shift), respectively. Two olefinic carbons at ι 141.0 and 120.5 were assigned to C-5 and C-6 on the basis of these observations, respectively. The attachment of amino substituent groups at ι 64.5 and ι 62.9 were C-3 and C-21 amino substituent respectively. The C-3 position has dimethyl amino groups, and C-21 has monomethyl group. Therefore two methyl groups at H-19 and H-21 are represented a main major of methyl in core structure of this series steroidal alkaloid from *H. antidysenterica*. A structure compound **1** has been assigned to be conessine. The structure data was confirmed based on that previously reported (Zirihi *et al*., 2005).

Table 5 NMR data of compound **1** (300 MHz for ^{1}H and 75 MHz for ^{13}C in $CDCl_3$)

		Compound 1		**Conessine (Zirihi *et al.*, 2005)**	
Position	**Type**	**$\delta^{13}C$**	**$\delta^{1}H$ (mult; *J* in Hz)**	**$\delta^{13}C$**	**$\delta^{1}H$ (mult; *J* in Hz)**
1	CH_2	38.1	1.00 (m)	38.2	1.03 (m)
			1.80 (m)		1.83 (m)
2	CH_2	24.4	1.41 (m)	24.8	1.40 (m)
			1.77 (m)		1.73 (m)
3	CH	64.5	2.16 (m)	64.9	2.18 (m)
4	CH_2	34.5	2.16 (m)	34.8	2.18 (m)
5	C	141.0	-	141.4	-
6	CH	120.5	5.36 (d; 4.5)	121.1	5.42 (d; 4.1)
7	CH_2	31.5	2.06 (m)	31.9	2.03 (td)
8	CH	33.0	1.30 (m)	33.4	1.31 (m)
9	CH	49.5	0.90 (m)	49.9	0.92 (m)
10	C	36.4	-	36.9	-
11	CH_2	21.5	1.13 (m)	22.0	1.11 (m)
			1.54 (m)		1.56 (m)
12	CH	38.1	1.31 (m)	38.6	1.31 (m)
			1.78 (m)		1.73 (m)
13	C	50.0	-	50.5	-
14	CH_2	55.3	1.22 (m)	55.8	1.11 (m)
15	CH_2	24.4	1.31 (m)	24.3	1.31 (m)
			1.78 (m)		1.73 (m)
16	CH_2	27.0	1.21 (m)	27.4	1.20 (m)
			1.47 (m)		1.56 (m)

Table 5 (cont.)

		Compound 1		Conessine (Zirihi *et al.*, 2005)	
Position	**Type**	**δ ^{13}C**	**δ ^{1}H (mult; *J* in Hz)**	**δ ^{13}C**	**δ ^{1}H (mult; *J* in Hz)**
17	CH	53.1	1.85 (m)	53.5	1.83 (m)
18	CH_2	63.9	1.93 (m)	64.3	1.93 (m)
			3.05 (d; 10.2)		3.03 (d; 10.7)
19	CH_3	18.9	0.93 (s)	19.4	0.90 (s)
20	CH	62.9	2.44 (m)	63.4	2.44 (m)
21	CH_3	14.1	1.08 (d; 6.3)	14.6	1.07 (d; 6.3)
22	CH_3	40.7	2.25 (s)	41.1	2.25 (s)
23	CH_3	41.0	2.33 (s)	41.3	2.33 (s)
24	CH_3	41.0	2.33 (s)	41.3	2.33 (s)

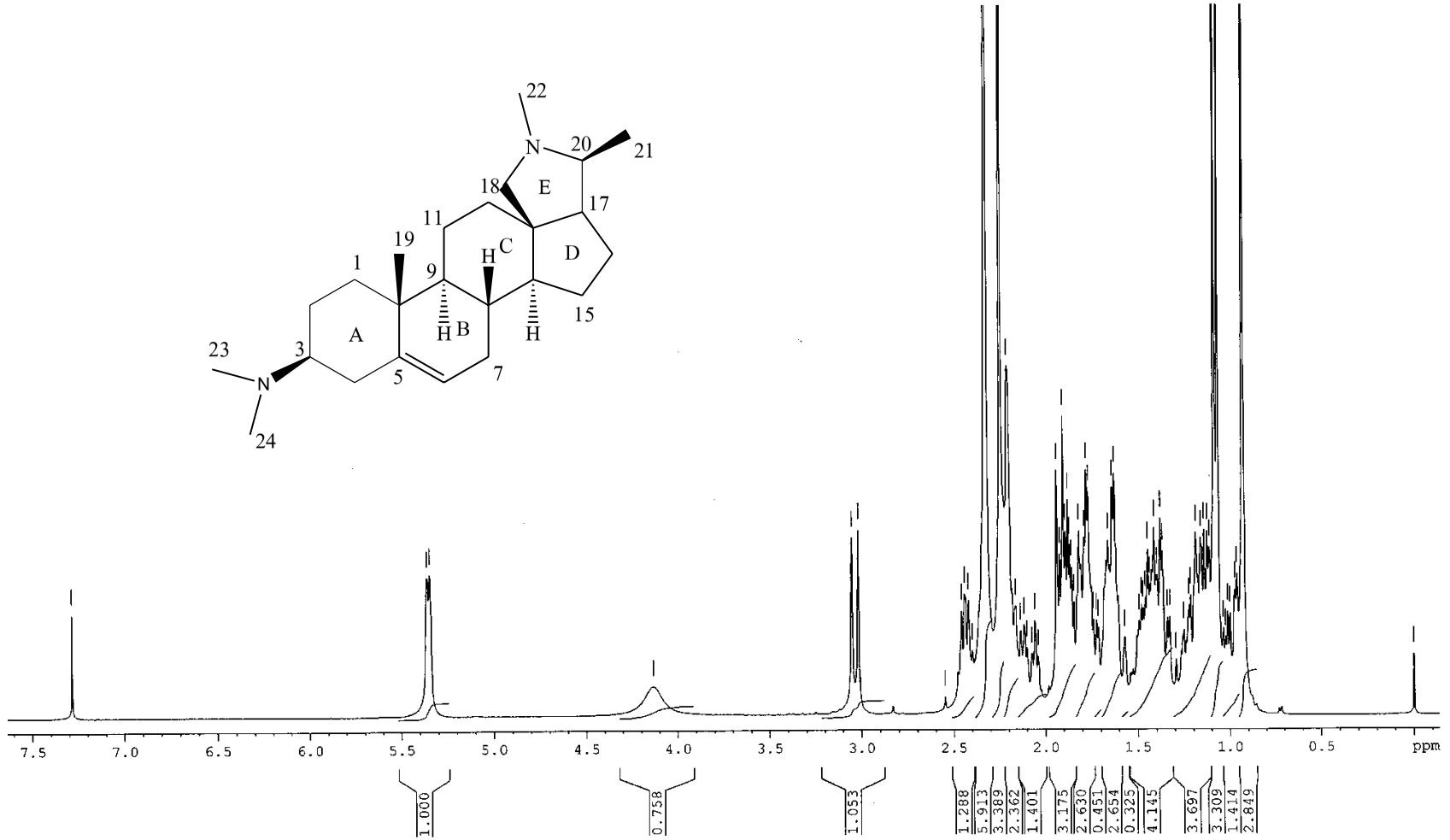


Figure 8 The structure and ^{1}H NMR spectrum of compound **1** (300 MHz in $CDCl_3$)

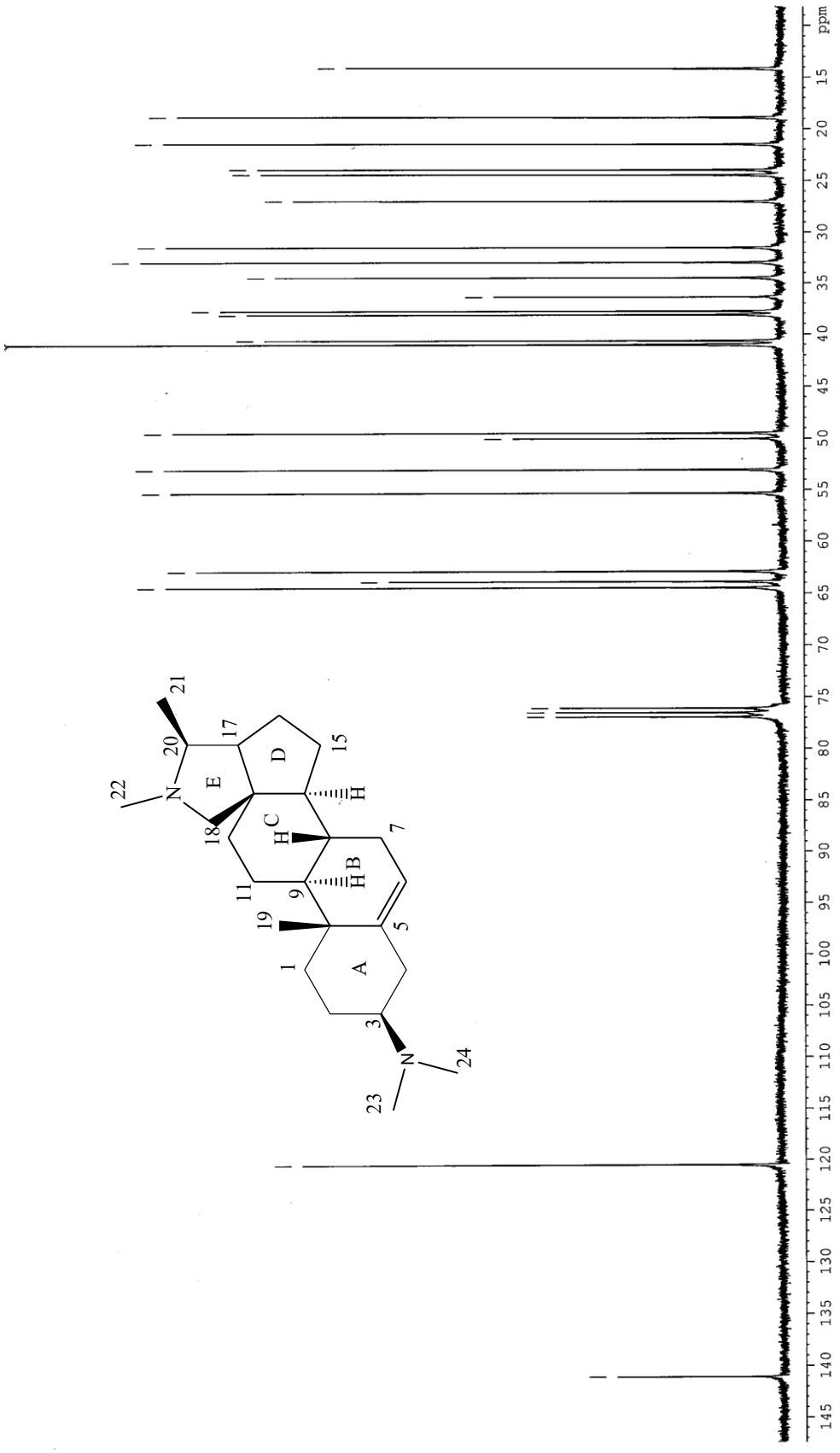


Figure 9 The structure and ^{13}C NMR spectrum of compound **1** (75 MHz in $CDCl_3$)

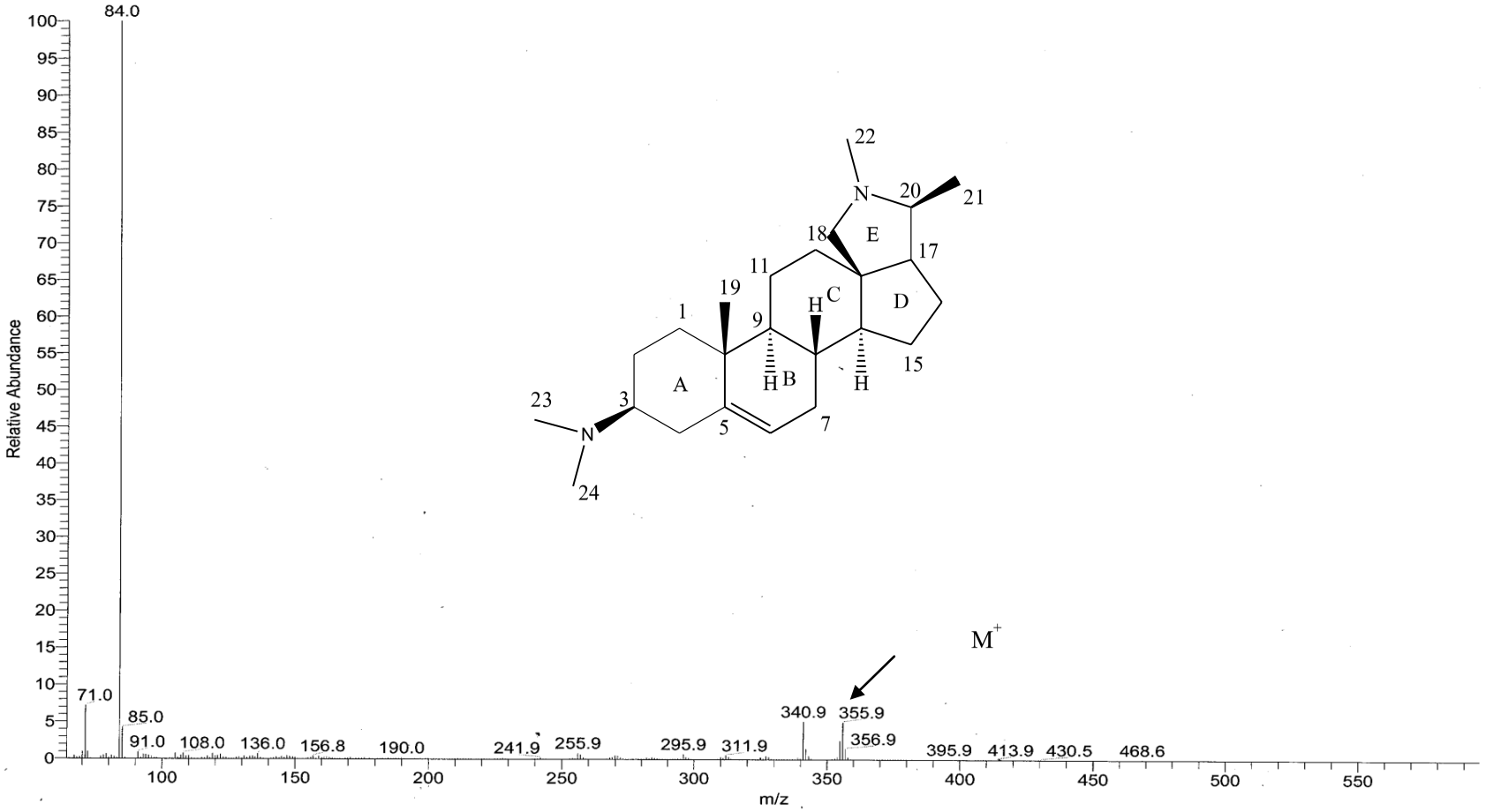


Figure 10 EIMS spectrum of compound **1**

3.3.3 Isoconessimine (compound 2)

Compound **2** was isolated as white solid. The molecular formula of **2** was established as $C_{23}H_{39}N_2$ by showing the $[M]^+$ peak at 342 *m/z* in EIMS spectrum (Figure 13). This compound was giving orange color with Dragendorff's reagent. Almost identical to those compound **1**, the spectral data of **2** (Table 6) including the ^{1}H NMR (Figure 11) and ^{13}C NMR (Figure 12) spectra, indicated that the two compounds were closely related. The major differences among **1** and **2** were observable in the ^{1}H NMR spectrum is N-methyl singlet at ι#2.33 which correspond to 3H instead of 6H in **1**. The structure compound **2** has been assigned to isoconessimine. The structure data was confirmed based on that previously reported (Zirihi *et al*., 2005).

Table 6 NMR data of compound **2** (300 MHz for ^{1}H and 75 MHz for ^{13}C in $CDCl_3$)

		Compound 2		**Isoconessimine (Zirihi *et al.*, 2005)**	
Position	**Type**	**ι ^{13}C**	**ι ^{1}H (mult; *J* in Hz)**	**ι ^{13}C**	**ι ^{1}H (mult; *J* in Hz)**
1	CH_2	37.5	1.15 (m)	38.7	1.10 (m)
			1.85 (m)		1.80 (m)
2	CH_2	27.4	1.65 (m)	28.8	1.67 (m)
			1.77 (m)		1.73 (m)
3	CH	58.3	2.48 (m)	59.8	2.59 (m)
4	CH_2	36.7	1.15 (m)	37.9	1.21 (m)
			1.90 (m)		1.42 (m)
5	C	139.7	-	141.3	-
6	CH	122.1	5.36 (d; 3.9)	120.8	5.40 (d; 3.9)
7	CH_2	32.0	1.65 (m)	32.0	1.60 (m)
			2.10 (m)		2.02 (m)
8	CH	33.4	1.15 (m)	33.2	1.21 (m)
9	CH	49.9	1.00 (m)	50.0	0.94 (m)

Table 6 (cont.)

		Compound 2		Isoconessimine (Zirihi *et al*., 2005)	
Position	**Type**	**δ ^{13}C**	**δ ^{1}H (mult; *J* in Hz)**	**δ ^{13}C**	**δ ^{1}H (mult; *J* in Hz)**
10	C	36.5	-	37.1	-
11	CH_2	22.0	1.20 (m)	22.0	1.18 (m)
			1.70 (m)		1.67 (m)
12	CH_2	38.5	1.40 (m)	39.3	1.32 (m)
			1.80 (m)		1.75 (m)
13	C	50.5	-	50.4	-
14	CH	55.7	1.20 (m)	55.9	1.21 (m)
15	CH_2	24.3	1.20 (m)	24.5	1.23 (m)
			1.60 (m)		1.55 (m)
16	CH_2	27.4	1.50 (m)	26.7	1.49 (m)
			1.75 (m)		1.70 (m)
17	CH	53.1	1.85 (m)	53.6	1.97 (m)
18	CH_2	64.1	2.35 (m)	64.6	2.30 (m)
			3.03 (d; 10.5)		2.79 (d; 10.7)
19	CH_3	18.9	0.95 (s)	19.4	0.92 (s)
20	CH	63.5	2.35 (m)	63.2	2.32 (m)
21	CH	14.5	1.09 (d; 6.3)	14.8	1.04 (d; 6.4)
22	CH_3	41.1	2.25 (s)	41.1	2.20 (s)
23	CH_3	33.4	2.33 (s)	33.4	2.43 (s)

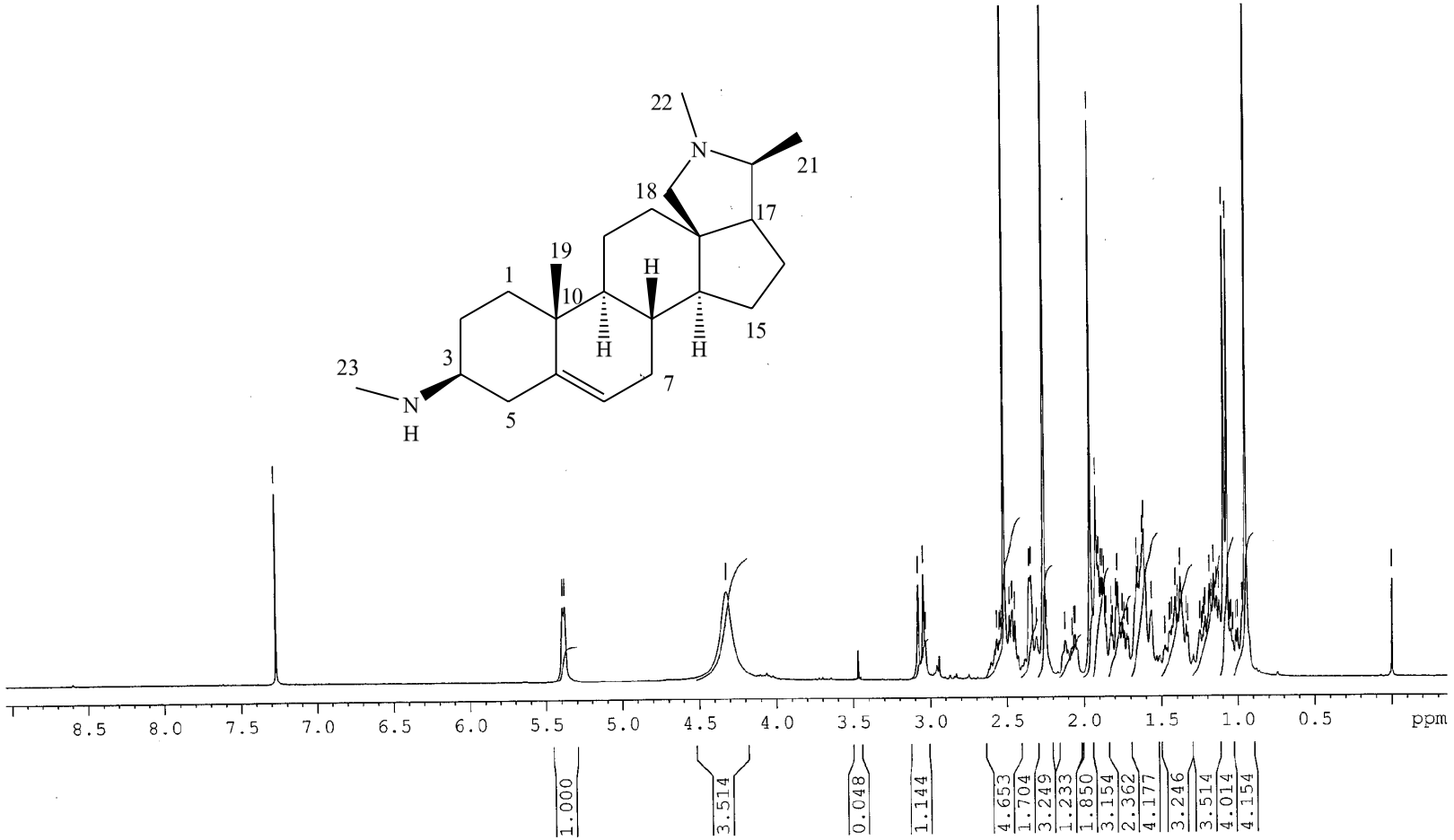


Figure 11 The structure and ^{1}H NMR spectrum of compound **2** (300 MHz in $CDCl_3$)

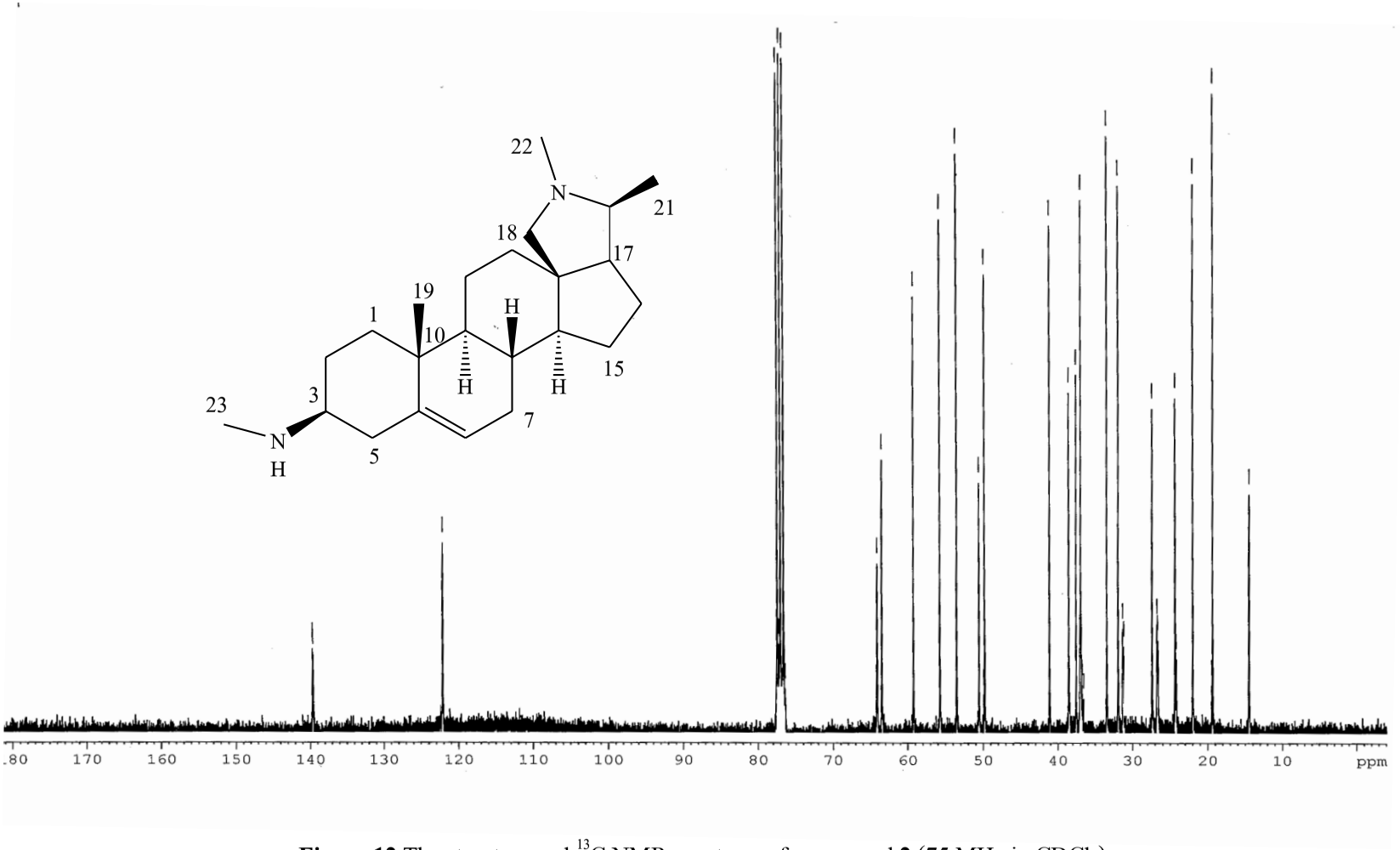


Figure 12 The structure and ^{13}C NMR spectrum of compound **2** (75 MHz in $CDCl_3$)

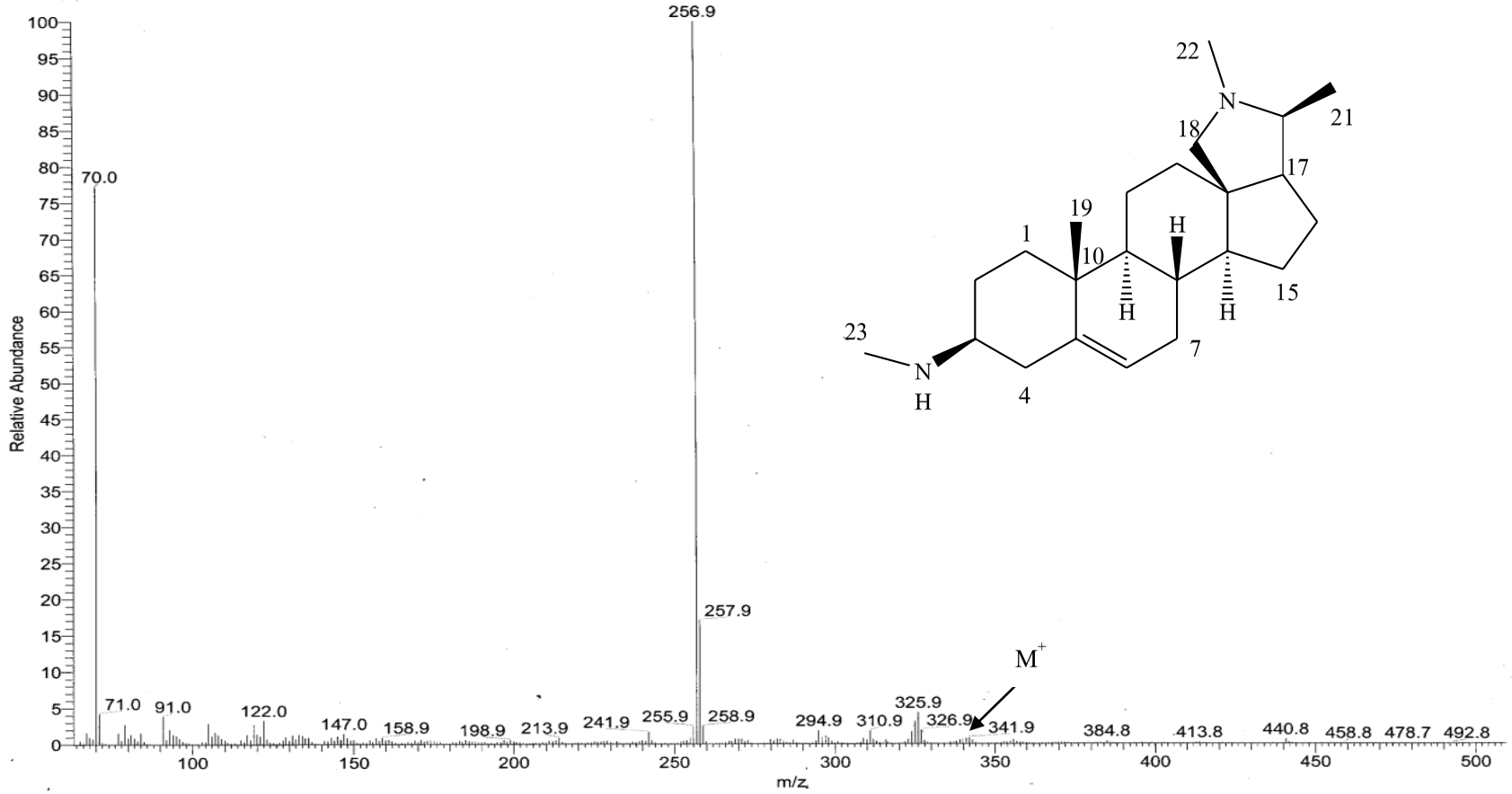


Figure 13 EIMS spectrum of compound **2**

3.3.4 Conessimine (compound 3)

Compound **3** was obtained as a white solid. The molecular formula of **3** was established as $C_{23}H_{39}N_2$ by the $[M]^+$ peak at 342 *m/z* in EIMS spectrum (Figure 16). This compound was giving orange color with Dragendorff's reagent. Almost identical to those **1,** the spectral data of **3** (Table 7**)** including the ^{1}H NMR spectra (Figure 14) and ^{13}C NMR (Figure 15) and, indicated that the two compounds were closely related. The major differences among **1** and **3** were observable in the ^{1}H NMR spectrum which has no methyl on amino group singlet at ring E. The structure compound **3** has been assigned to conessimine which confirm based on spectral data from a previous report (Tran *et al.*, 2006).

Table 7 NMR data of compound **3** (300 MHz for ^{1}H and 75 MHz for ^{13}C in $CDCl_3$)

Position	**Type**	**ι ^{13}C**	**ι ^{1}H (mult; *J* in Hz)**
1	CH_2	38.2	1.01 (m)
			1.95 (m)
2	CH_2	24.9	1.46 (m)
			1.77 (m)
3	CH	64.8	2.10 (m)
4	CH_2	35.1	2.17 (m)
5	C	141.8	-
6	CH	120.3	5.36, (d; 4.5)
7	CH_2	31.8	1.77 (m)
			1.91 (m)
8	CH	33.7	1.43 (m)
9	CH	49.7	0.93 (m)
10	C	36.8	-
11	CH_2	21.9	1.27 (m)
			1.52 (m)
12	CH_2	26.6	1.31 (m)

Table 7 (cont.)

Position	Type	$\delta^{13}C$	$\delta^{1}H$ (mult; *J* in Hz)
			1.78 (m)
13	C	50.2	-
14	CH	55.2	1.13 (m)
15	CH_2	22.5	1.31 (m)
			1.74 (m)
16	CH_2	36.5	1.40 (m)
			1.77 (m)
17	CH	52.9	1.87 (m)
18	CH_2	63.9	2.77 (d; 12.3)
			2.95 (d; 12.3)
19	CH_3	19.4	0.89 (s)
20	CH	56.6	3.46 (m)
21	CH	14.9	1.28 (d; 6.6)
22	CH_3	41.6	2.29 (s)
23	CH_3	41.6	2.29 (s)

Figure 14 The structure and ^{1}H NMR spectrum of compound **3** (300 MHz in $CDCl_3$)

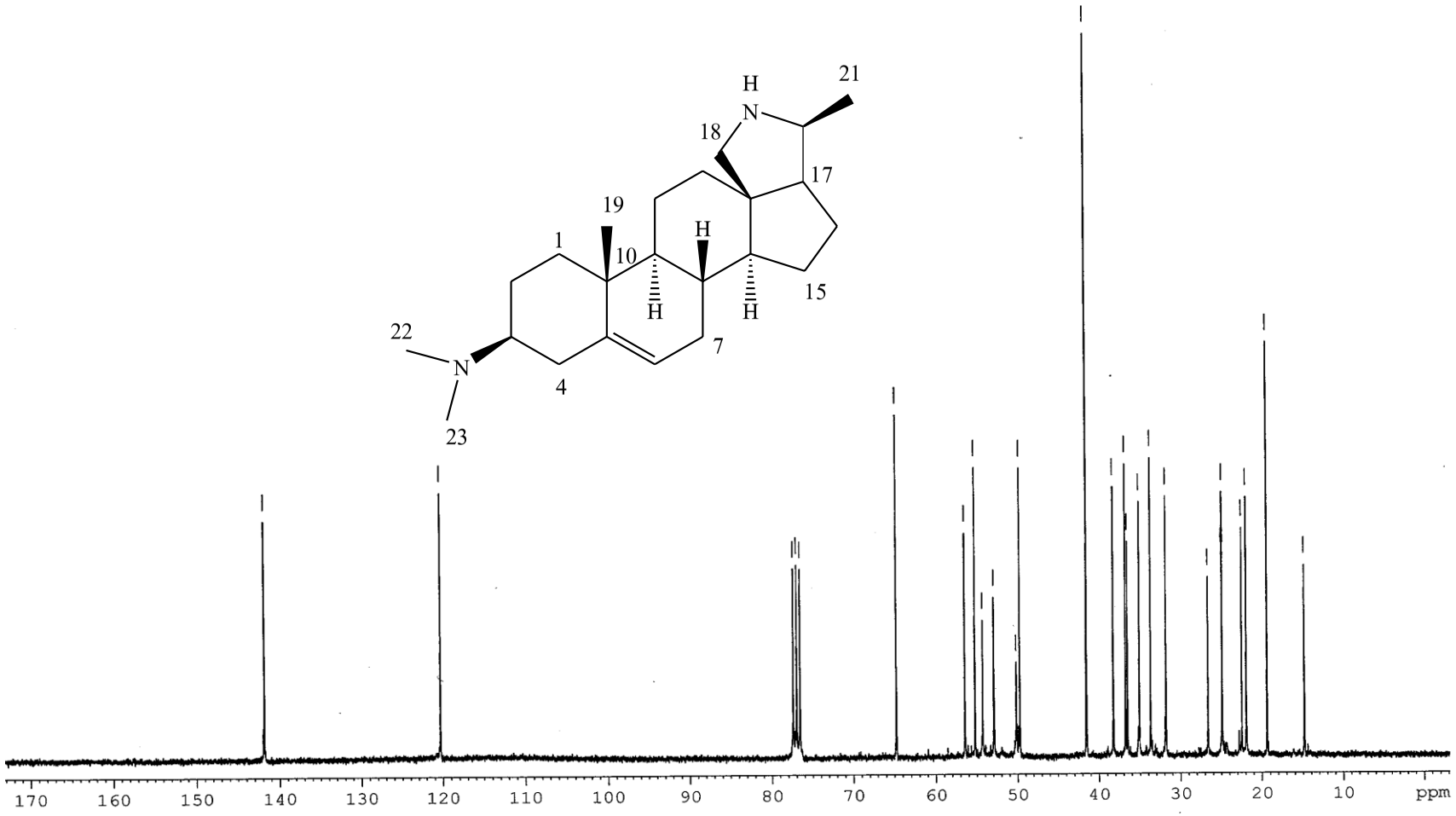


Figure 15 The structure and ^{13}C NMR spectrum of compound **3** (75 MHz in $CDCl_3$)

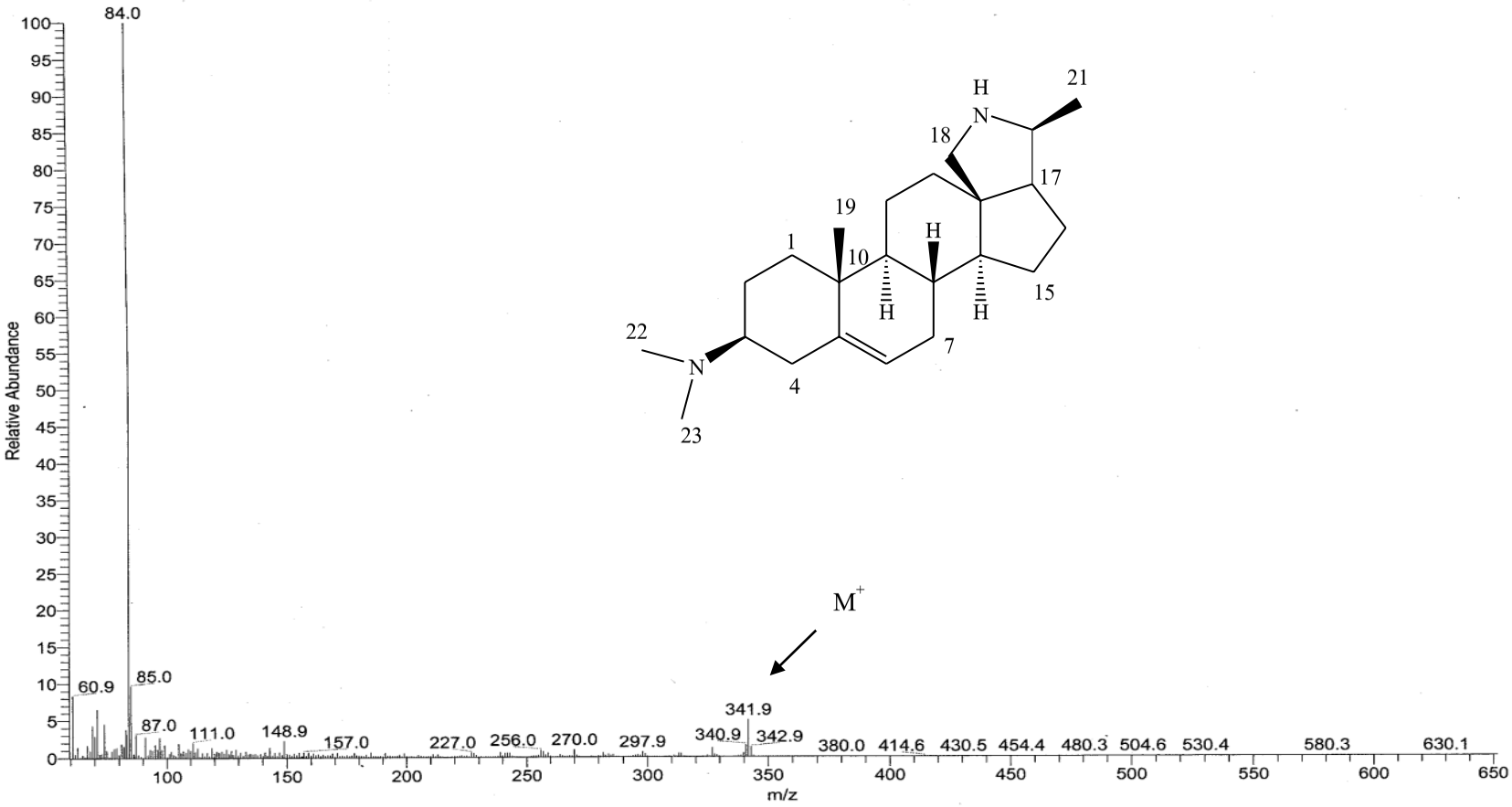


Figure 16 EIMS spectrum of compound **3**

3.3.5 Scopoletin (compound 5)

Compound **5** was isolated as a yellow solid. The molecular formula of this compound was established as $C_{10}H_8O_4$ according to the $[M]^+$ peak at *m/z* 192 in low resolution EIMS (Figure 19) indicating seven degrees of unsaturation in the molecule including one benzene ring, one cyclic ring and two double bond. The IR spectrum of this compound displayed absorption bands characteristic of carbonyl group (1700 cm^{-1}, C=O) and hydroxyl group (3338 cm^{-1}). The UV spectrum shows the maximal absorption at UV λ_{max} (log ε) 205 (3.4), 229(3.0), 298 (2.6), 346 (2.9).

Its ^{1}H NMR spectrum (Figure 17) and ^{13}C NMR (Figure 18) revealed the presence of two doublets at δ 7.58 (H-4, J = 9.4 Hz) and 6.25 (H-3, J = 9.4 Hz), these two protons are coupled together and resonated and different chemical shift because of alpha beta unsaturated ketone effect. The two singlet signals appeared at δ 6.83 and δ 6.93 were assigned for the two protons H-5 and H-8, respectively (Table 8). A nOe experiment (Figure 20) found the enhancement of proton signals at δ 7.58 (H-4) and at δ 3.94 (OCH_3) due to the irradiation at δ 6.83 were observed, suggested OCH_3 substitute at C-6 on structure. Therefore, this compound was identified as scopoletin which confirm based on spectral data from that previously reported (Xu *et al.*, 2008).

Table 8 NMR data of compound **5** (500 MHz for ^{1}H and 125 MHz for ^{13}C in $CDCl_3$)

Position	**Type**	**$\delta^{13}C$#**	**$\delta^{1}H$ (mult; *J* in Hz)#**
2	C	161.4	-
3	CH	113.4	6.25 (d; 9.4)
4	CH	143.3	7.58 (d; 9.4)
5	CH	107.5	6.83 (s)
6	C	143.9	-
7	C	150.2	-
8	CH	103.2	6.90 (s)
9	C	150.2	-
10	C	111.5	-
OCH_3	CH_3	56.4	3.94 (s)

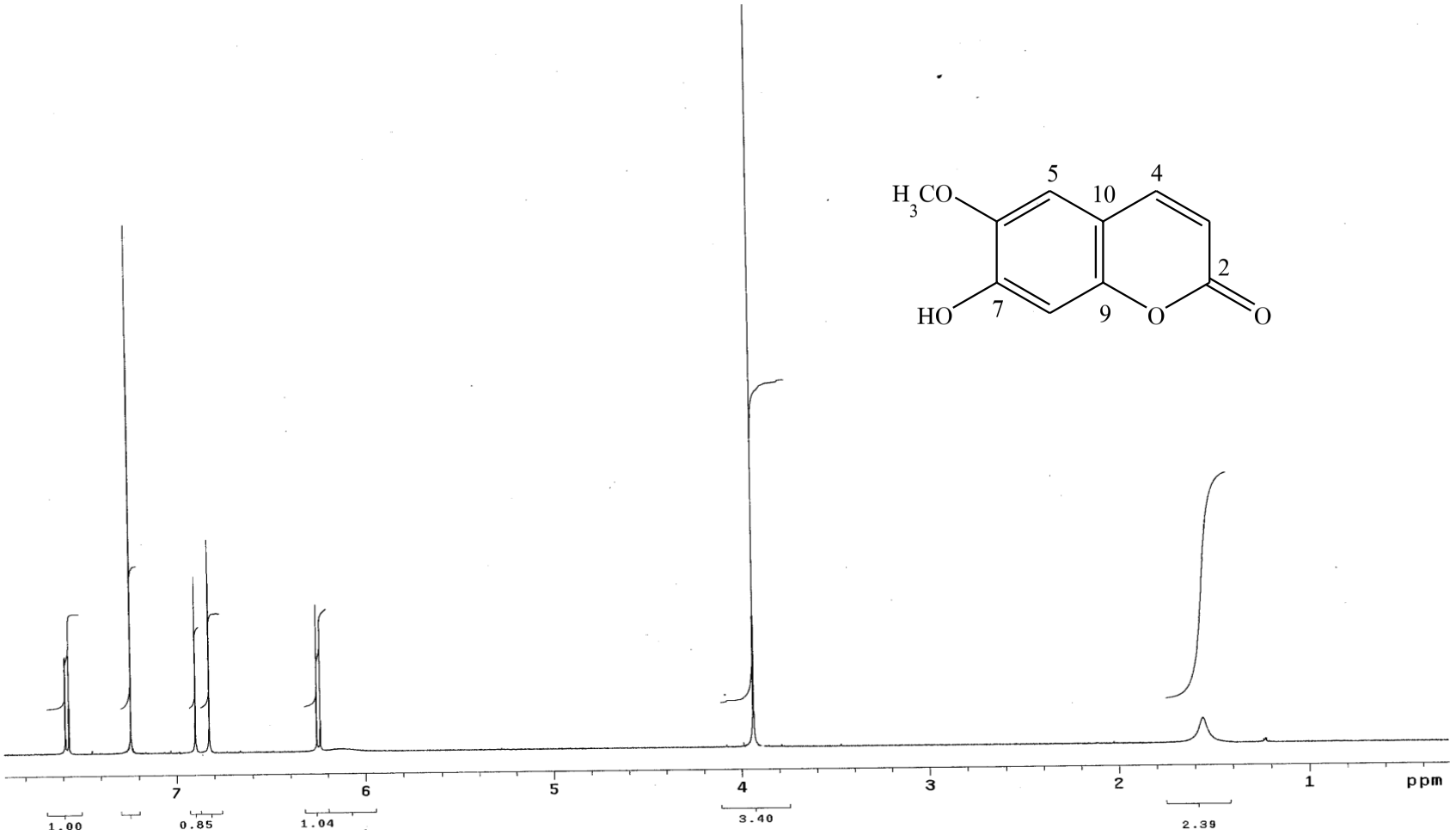


Figure 17 The structure and ^{1}H NMR spectrum of compound **5** (500 MHz in $CDCl_3$)

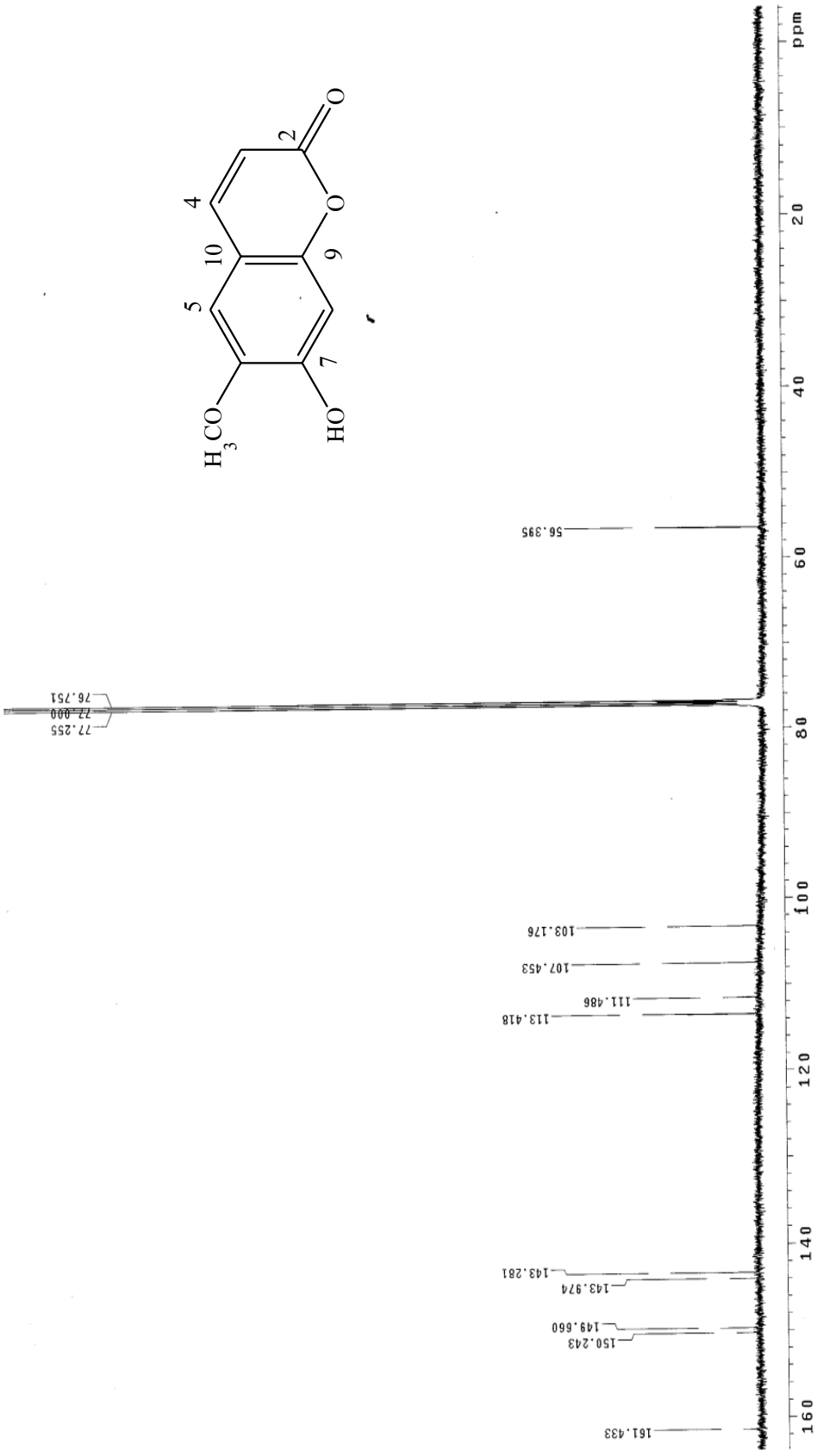


Figure 18 The structure and ^{13}C NMR spectrum of compound **5** (125 MHz in $CDCl_3$)

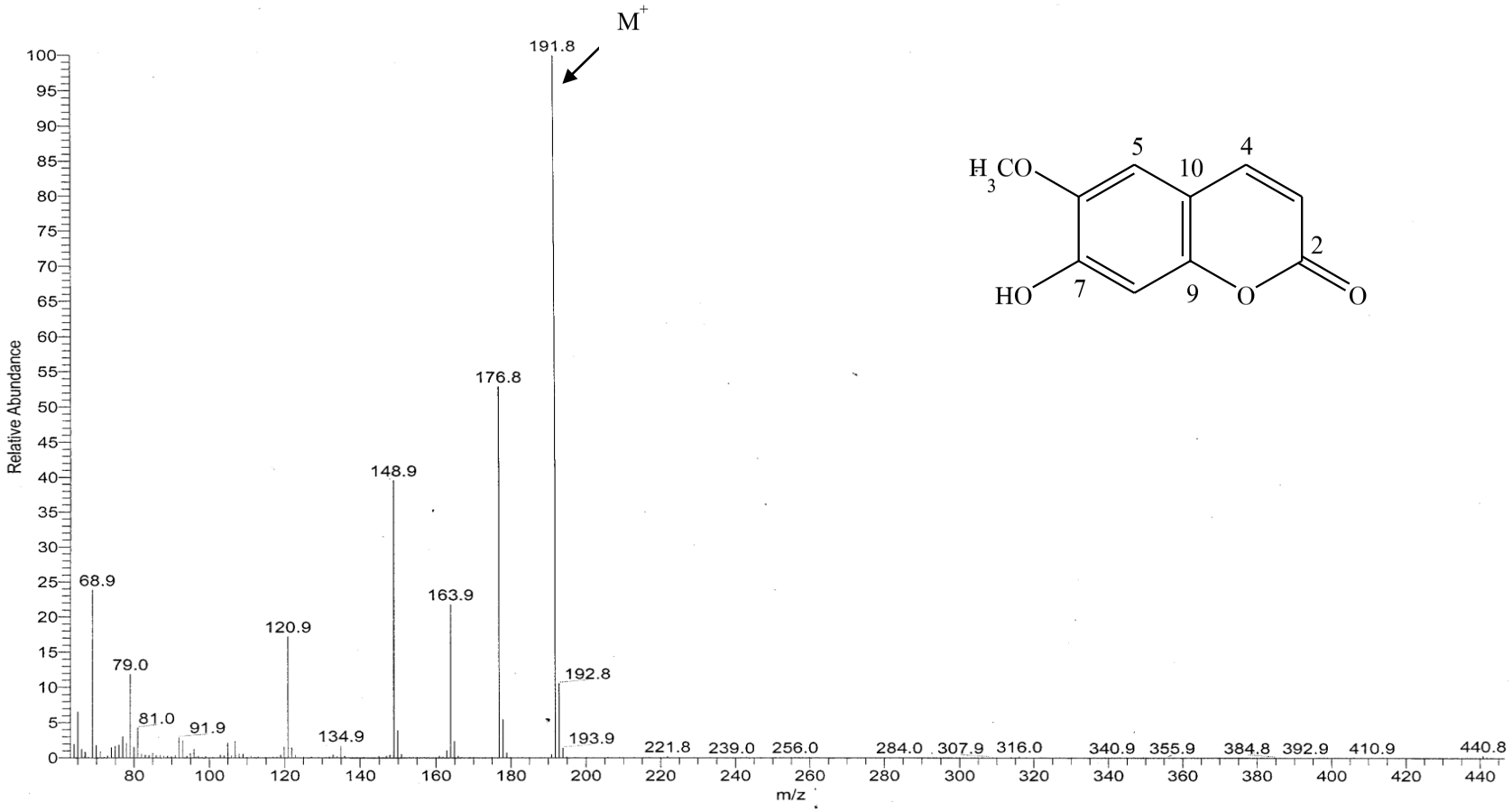


Figure 19 EIMS spectrum of compound **5**

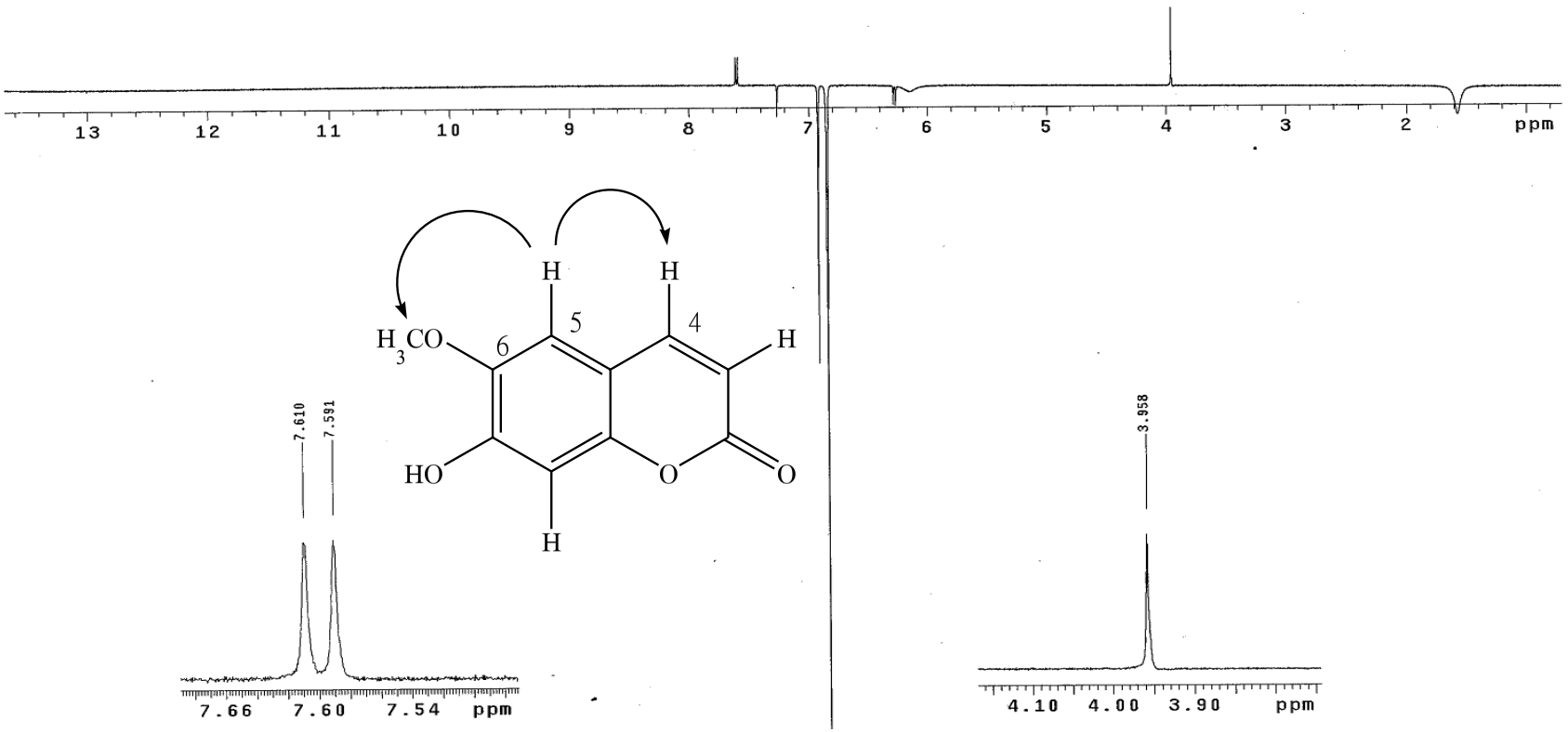


Figure 20 Selected nOe correlations of compound **5**

3.4 AChE inhibitory, anti-inflammatory and antioxidant activities of isolated compounds

Four steroidal alkaloids and one coumarin were subjected to test on acetylcholinesterase inhibitory, NO production by murine macrophage like RAW 264.7 cells for anti-inflammatory activity, and antioxidant activity using DPPH assay. The results are shown in Table 9.

Table 9 The biological activities of isolated compounds

Compounds	**AChE inhibitory** IC_{50} **(μM)**	**Anti- inflammatory % inhibition (μM)**	**Antioxidant** ED_{50} **(μM)**
Conessine (**1**)	1.50±0.90	-	>100
Isoconessimine (**2**)	20.08±12.96	43.6 (30 μM)	>100
Conessimine (**3**)	0.12±0.03	-	>100
N-3-methylholarrhimine (**4**)	17.82±5.95	-	>100
Scopoletin (**5**)	73.50±3.45	-	>100
Galanthamine	0.74±0.11	-	-
Indomethacin	-	60.5 (30 μM)	-
BHT	-	-	7.72

These results indicated that all of isolated compounds showed AChE inhibitory activity in the range of 0.12-73.5 μM. Compound **3** is the most active and the inhibition activity is higher than galanthamine. From the observation, the numbers of methyl groups substituted on -N that are connected to the C-3 of the steroid nucleus obviously play important roles in the inhibitory action on AChE. The two methyl groups substituent have higher activity than one

methyl group. Compound **5** also showed potent inhibition on AChE with an IC_{50} value of 73.5±3.45 μM.

The activity of anti-inflammation found that only compound **2**, has activity at 30 μM with % inhibition 43.6, however it has cytotoxicity in murine macrophage at concentration of 100 μM. It is exhibited that these five compounds did not have antioxidant activity.

The kinetic analysis of the AChE inhibitory activity of steroidal alkaloid is shown in Table 9. The K_m and V_{max} value were calculated from nonlinear regression using software pack as Prism. The V_{max} value of AChE against ACTI was relatively unchanged. In contrast, the K_m value was significantly increased. These results indicated that all steroidal alkaloids in this study inhibited AChE in a competitive manner.

Table 10 The AChE inhibition kinetics of isolated steroidal alkaloids

AChE inhibition kinetics	**V_{max}(dmA. min^{-1})**	**K_m(μM)**	**Type of inhibition**
No inhibitor	150.4	987.9	-
Conessine (**1**)	173.0	1652.0	Competitive
Isoconessimine (**2**)	167.8	1448	Competitive
Conessimine (**3**)	129.9	1252	Competitive
N-3-methylholarrhimine (**4**)	149.3	1119	Competitive

3.5 Molecular docking study

Structures of the AChE in complexed with donepezil and tacrine are shown in Figure 21A and 23A, respectively. The GOLD program was used for docking donepezil and tacrine into the binding pocket of AChE. The root mean square deviation (rmsd) of docking conformation were 1.63 for donepezil (E2020, PDB code 1EVE) and 0.70 for tacrine (THA 999, 1ACJ) comparing to X-ray structure, respectively (Table 11). GoldScore fitness functions of docked donepezil and tacrine were 62.57 and 50.99, respectively. The orientations of x-ray structure and docked donepezil and tacrine with GoldScore are shown in Figure 21B and 23B, respectively.

The GOLD program is directly identifying site of ligand-receptor binding. RMSD value of re-docked molecule that obtained by this package was less than 2 Å. Comparison of the different docking results of four steroidal alkaloids and one coumarin ligands which are shown in table 10. This similar binding mode is not surprising since steroidal alkaloid compounds contain almost identical structures with minor differences only at side chains C-3 and/or C-20 or by substitution of methyl groups. They penetrate the aromatic gorge ring A in front part of molecule. Thus, ring A is placed in the bottom of the gorge, which might be due to the apparently greater hydrophobicity of ring A in comparison with that of the five-member ring D.

Table 11 GoldScore and RMSD of E2020 and THA999 and GoldScore of isolated compounds

Compound	**Gold score**	**RMSD**
Donepezil (E2020)	62.57	1.63
Conessine (**1**)	53.38	-
Isoconessimine (**2**)	55.83	-
Conessimine (**3**)	60.49	-
N-3-methylholarrhimine (**4**)	57.64	-
Tacrine (THA999)	50.99	0.70
Scopoletin (**5**)	49.37	-

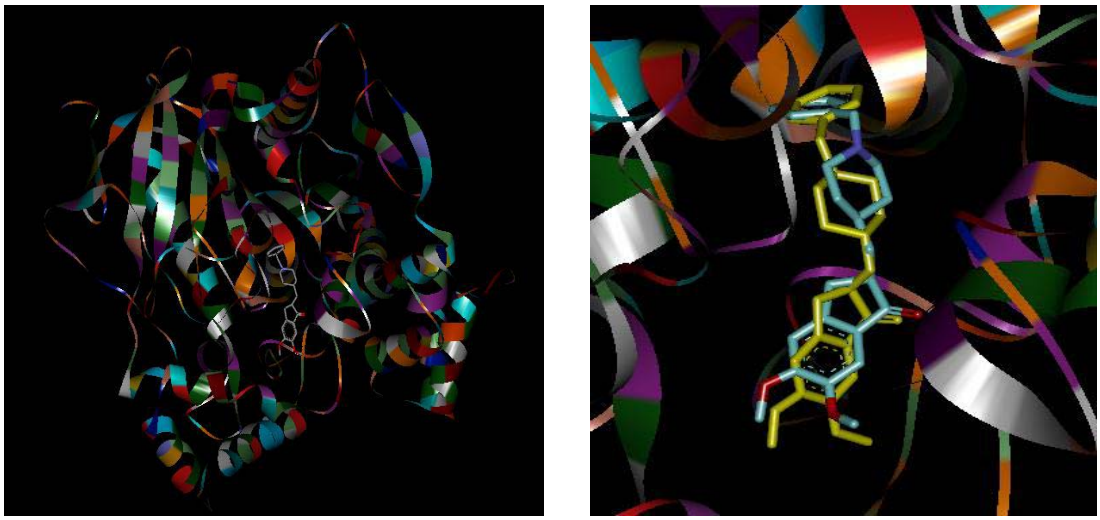
Table 12 The interaction of isolated compounds with *Tc*AChE X-ray

Proteins (PDB code)	**Ligands**	**Type of Interaction (Protien-Ligand)**	**Residue**	**Distance (A∀**	**Hydrophobic interaction with residue**
1EVE	Conessine (**1**)	-	-	-	Asp-72, Trp-84, Gly-117, Gly-118, Try-121, Try-130, Glu-199, 330, Trp-279, Phe-331, and His-440
	Isoconessimine (**2**)	-	-	-	Asp-72, Trp-84, Gly-117, Gly-118, Try-121, Try-130, Glu-199, 330,Trp-279, Phe-331, and His-440
	Conessimine (**3**)	H bond (NH-N)	GLY 118	3.06	Asp-72, Trp-84, Gly-117, Gly-118, Try-121, Try-130, Glu-199, Phe-330, Phe-331, and His-440
	N-3-Methyl-holarrhimine (**4**)	-	-	-	Asp-72, Trp-84, Gly-117, Gly-118, Try-121, Try-130, Glu-199, 330, Phe-331, and His-440
1ACJ	Scopoletin (**5**)	H bond (OH-O)	TRY 84	2.59	Glu-99, Phe-330,Tyr- 334-Trp-432, Ile-439, His- 440, Gly-441,
		ϕ-ϕ (benzene-benzene), (indole-benzene) (benzene -lactone ring) (indole -lactone ring)	TRP 84	3.60, 3.02 5.30, 4.05	

The main hydrophobic interactions between the hydrocarbon skeleton of steroidal alkaloid inhibitors and the protein were observed with the residues Asp-72, Trp-84, Gly-117, Gly-118, Try-121, Try-130, Glu-199, 330, Phe-331, and His-440 of the aromatic gorge (Figure 22) (Table 12).

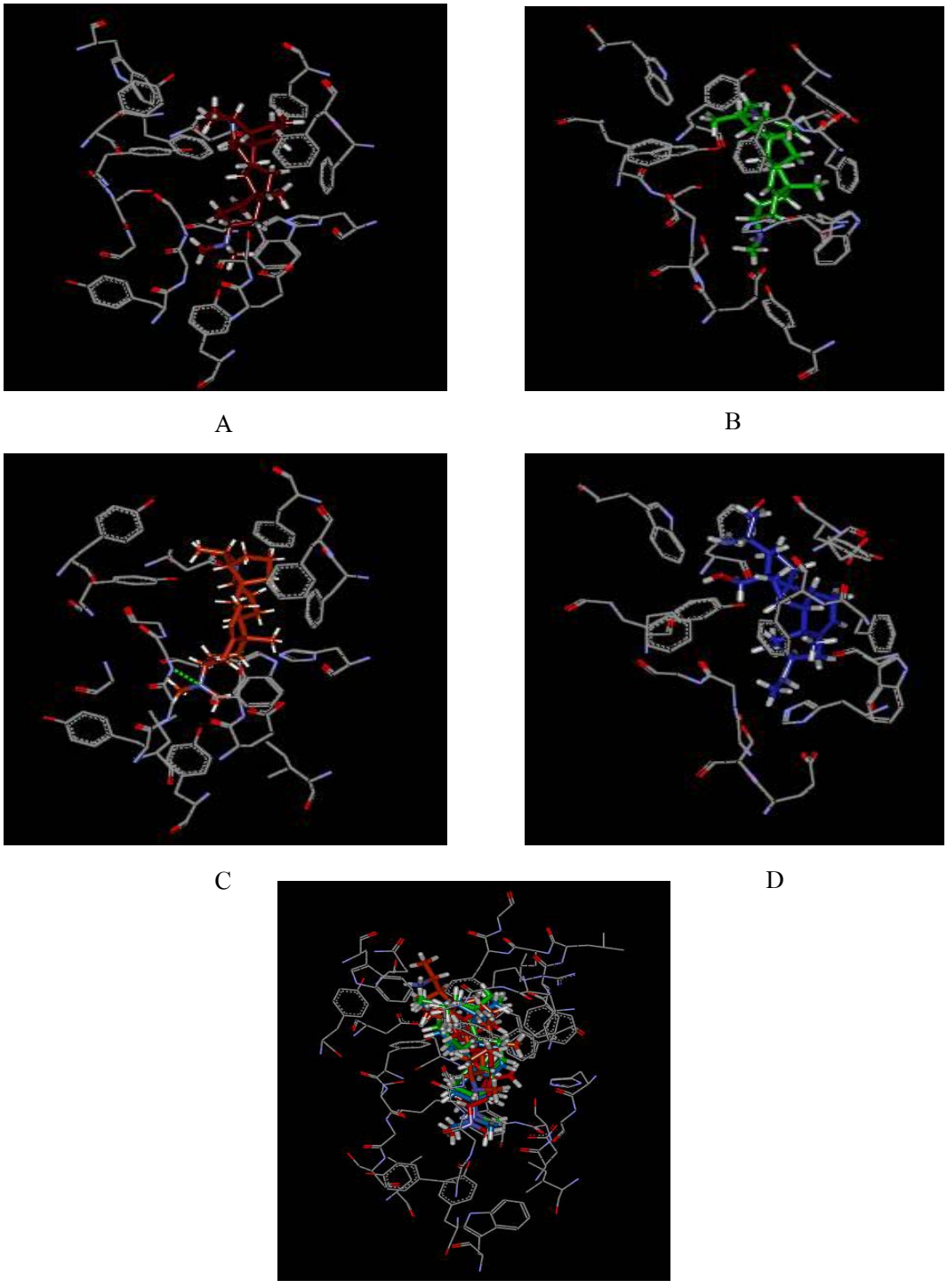
There is hydrogen bonding between compound **3** and Gly-118 is 3.06 Å, and other steroidal alkaloids have not shown any hydrogen bonding. This may answer why compound **3** is more active because of the interaction with the active site by H-bond (Figure 22 C).

For compound **5** it penetrates the aromatic gorge by placing the benzene ring in the bottom of the AChE gorge. The protein was observed with the residues are shown H bond (2.59 Å) with Trp-84, and ϕ-ϕ interaction between benzene ring of **5** and benzene ring of Trp-84 (3.60 Å), between benzene ring of **5** and indole ring of Trp-84 (3.02 Å), between lactone ring of 5 and benzene ring of Trp-84 (5.30) and between lactone ring of **5** and indole ring of Trp-84 (4.05) (Figure 24).



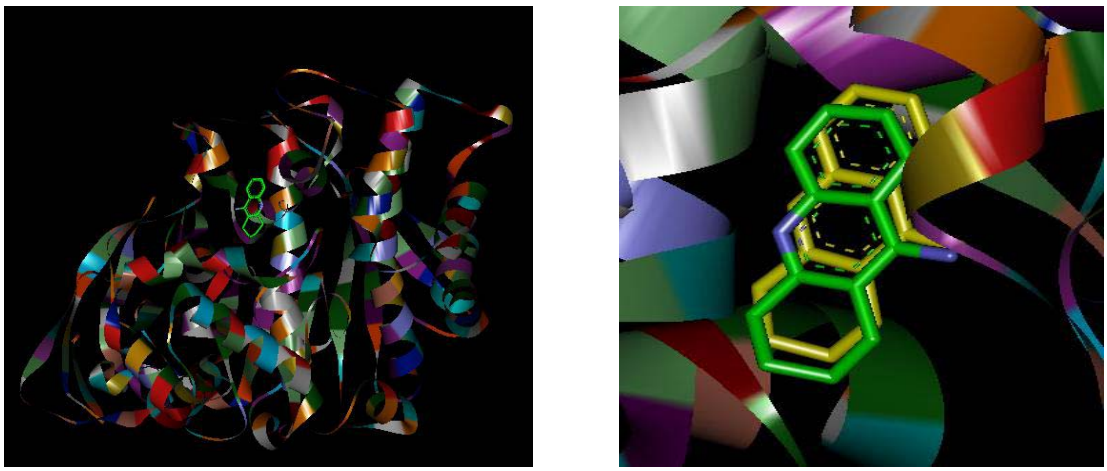
A B

Figure 21 The structure and orientation of donepezil in AChE binding pocket complex: (A) AChE crystal structure complexed with donepezil (atom-type color) (PDB code 1EVE), (B) Geometries obtained from X-ray (yellow color) and docking atom-type color) using GoldScore fitness functions



A B C D E

Figure 22 Docking of steroidal alkloids in the aromatic gorge of AChE: conesssine (A), isoconessimine (B), conessimine (C), N-3-methylholarrhimine (D) and superimposition of all steroidal alkaloids in the aromatic gorge (E).



A B

Figure 23 The structure and orientation of tacrine in AChE binding pocket complex (PDB code 1ACJ): (A) The acetylcholinesterase crystal structrure complexed with tacrine. (B) Orientation of tacrine in AChE binding pocket and geometries obtained from X-ray (yellow color) and docking (atom-type color) using GoldScore fitness function.

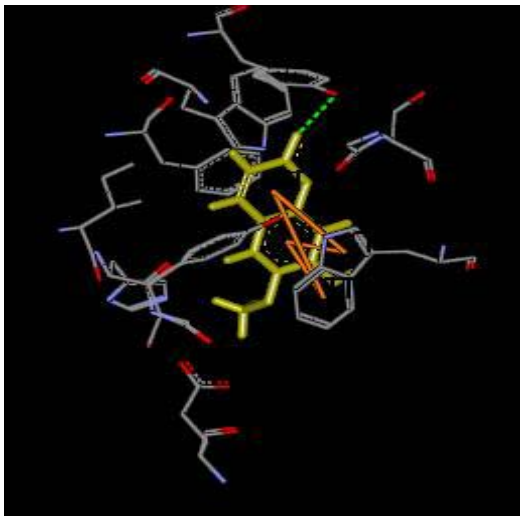


Figure 24 Docking of scopoletin in the aromatic gorge of AChE

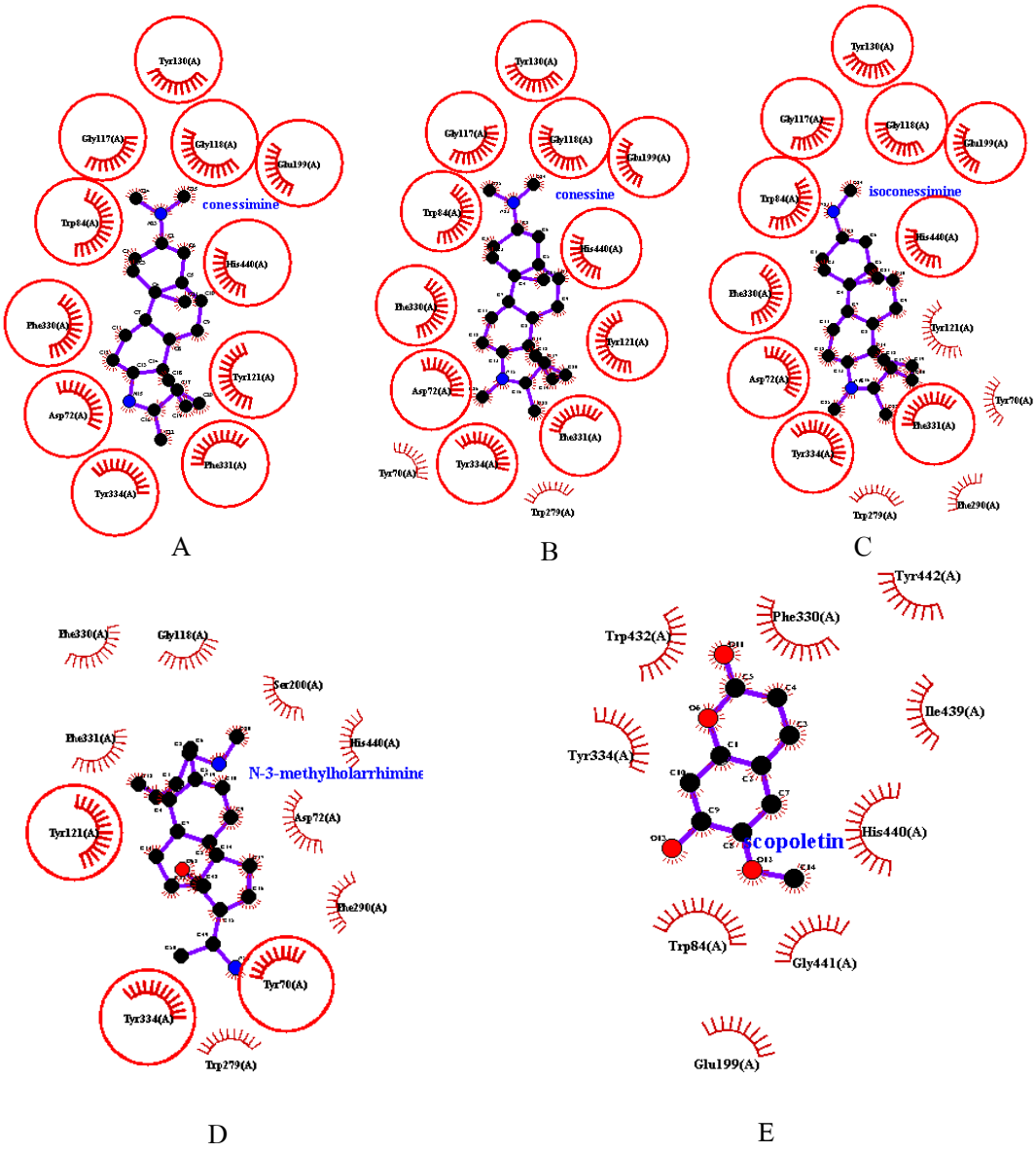


Figure 25 Schematic diagram obtained by LIGPLOT: conesssine (A), isoconessimine (B), conessimine (C), N-3-methylholarrhimine (D) and scopoletin (E)

CHAPTER 4

CONCLUSION

The investigation of AChE inhibitory, anti-inflammatory and antoxidant activity from *H. antidysenterica*, five compounds were isolated from the bark including four steroidal alkaloids; conessine (**1**), isoconessimine (**2**), conessimine (**3**), and N-3- methylholarrhimine (**4**) and one coumarin; scopoletin (**5**).

This is the first report of AChE inhibitors from *H.antidysenterica. In vitro* study found that those compounds presented a high activity, and compound **3** is considered as the highest. The AChE inhibitory kinetic of steroidal alkaloids were found to be competitive. The molecular docking suggested that the major interaction of the enzyme-inhibitor complexes is due to hydrophobic interaction inside the aromatic gorge of this cholinesterase. The other interaction, H-bond is found to be response for the activity of compound **3**. For compound **5**, H-bond and ϕ-ϕ interaction are found to be response for AChE inhibitory effect.

Compound **2** showed anti-inflammatory activity, however also exhibited toxic to macrophage like RAW 264.7 cells at concentration of 100 μM.

H. antidysenterica have been a source of clinically useful compounds, leads for synthetic modification and tools for mechanistic studies. Steroidal alkaloids are interested for medicinal chemists to study structure-activity relationships as AChE inhibitors to find interaction between ligand or inhibitor and enzyme. The compound which shows potent and selective against AChE enzyme was considered to be developed as a new therapeutic agent for AD.

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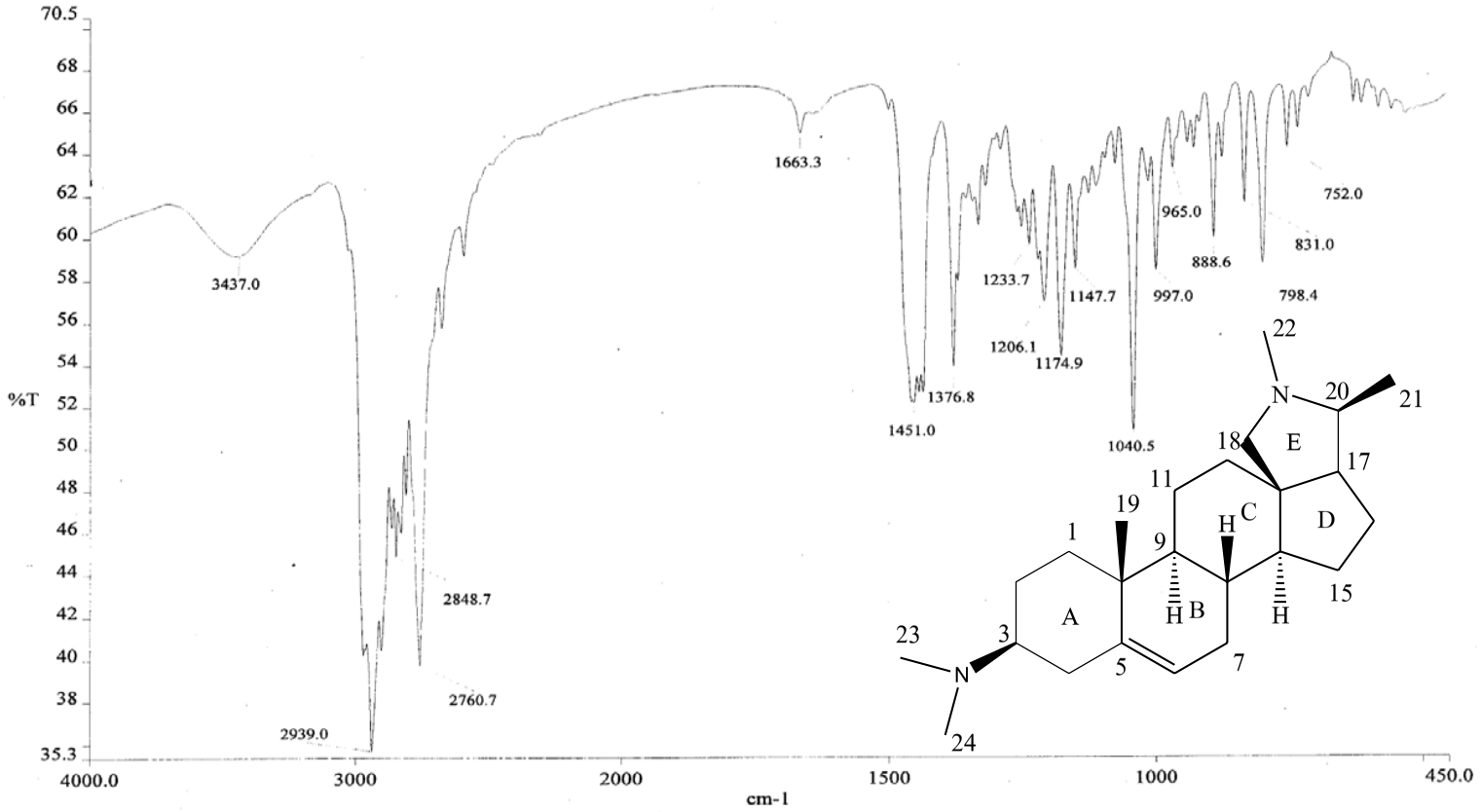
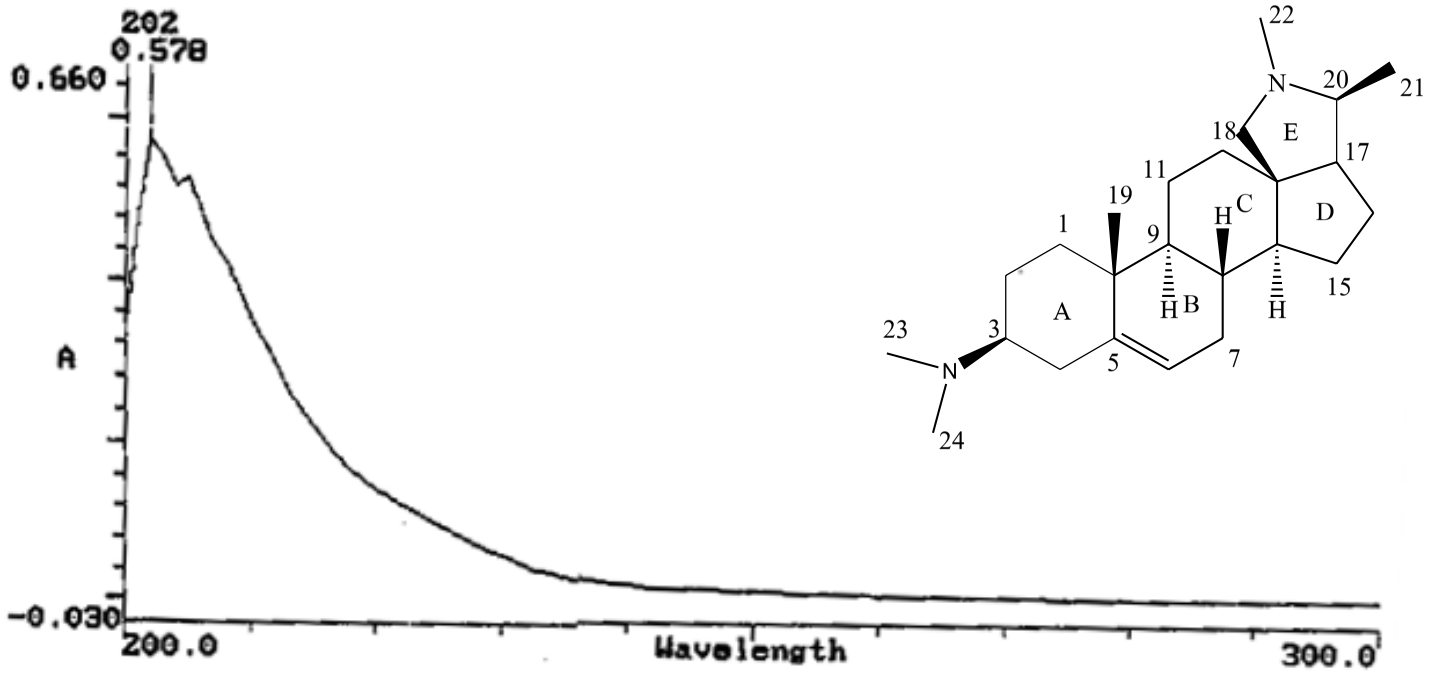
APPENDIX



IR spectrum of compound **1**



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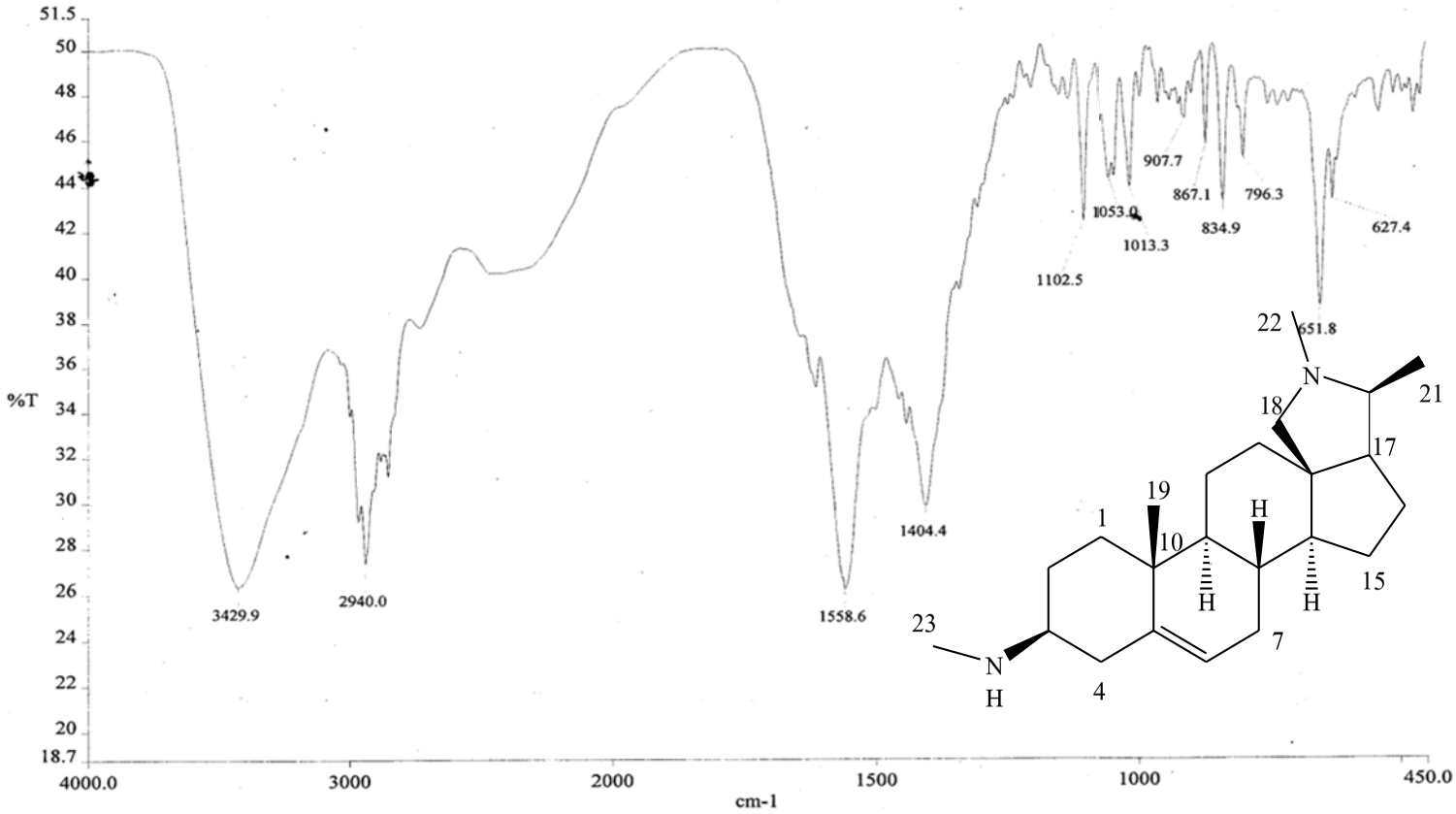

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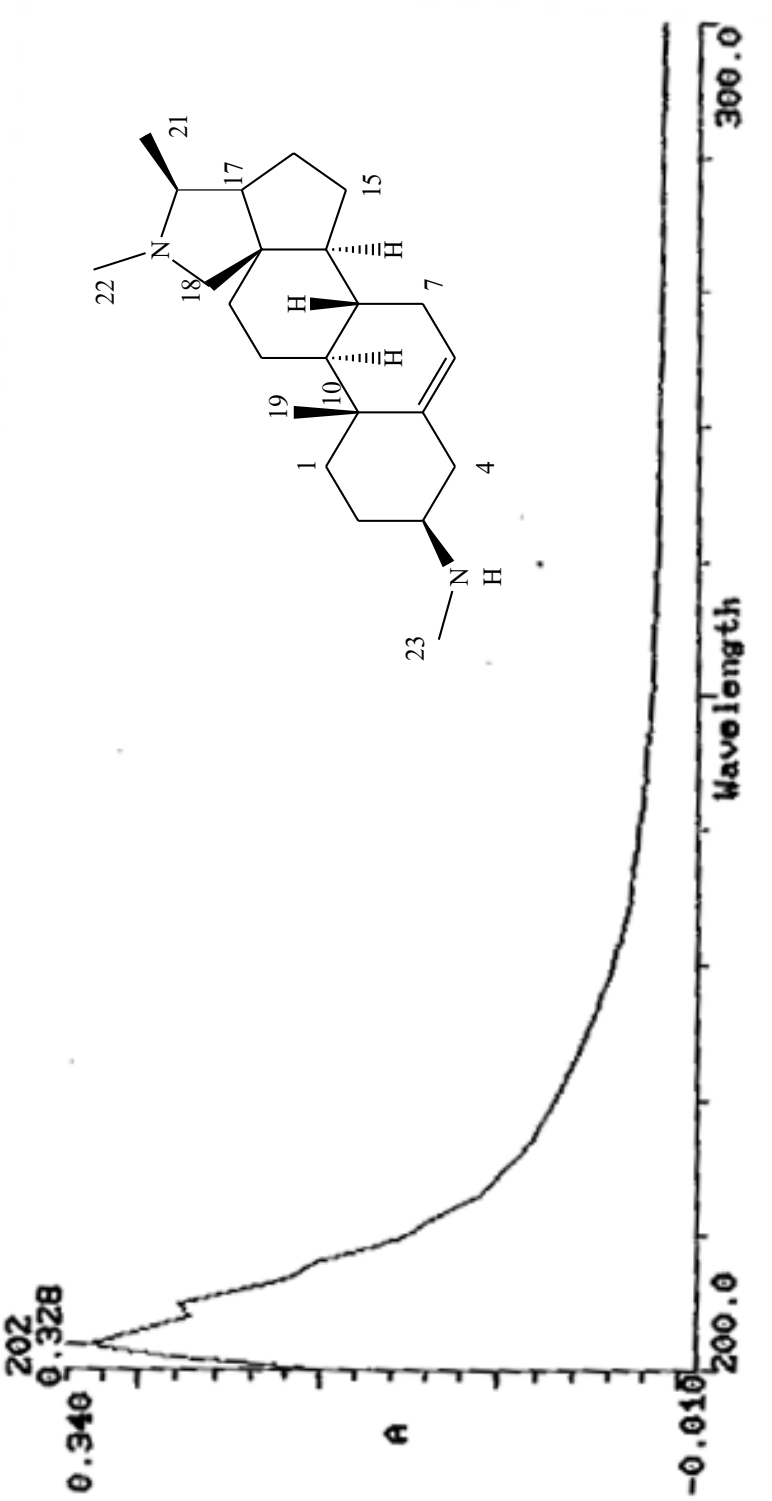

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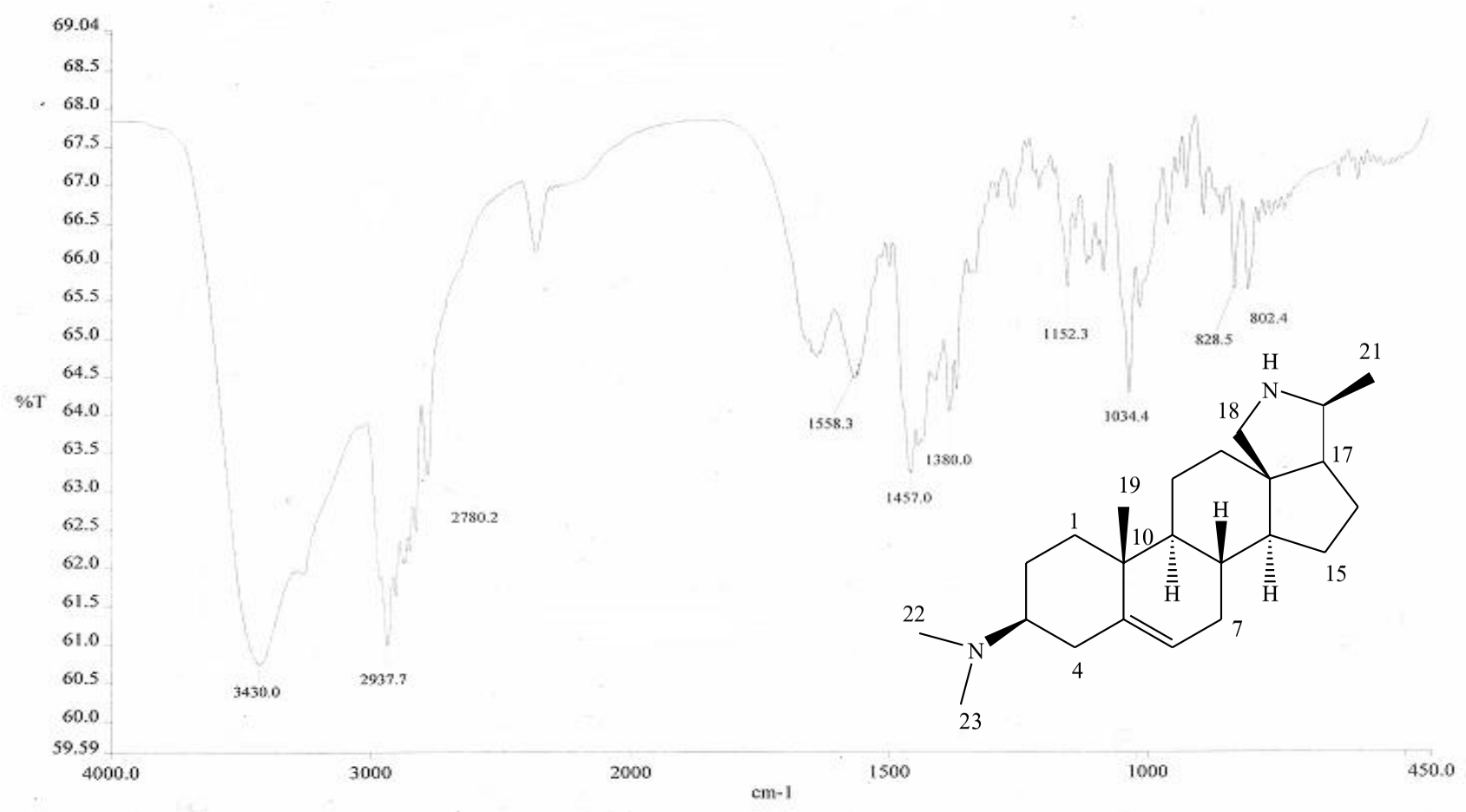

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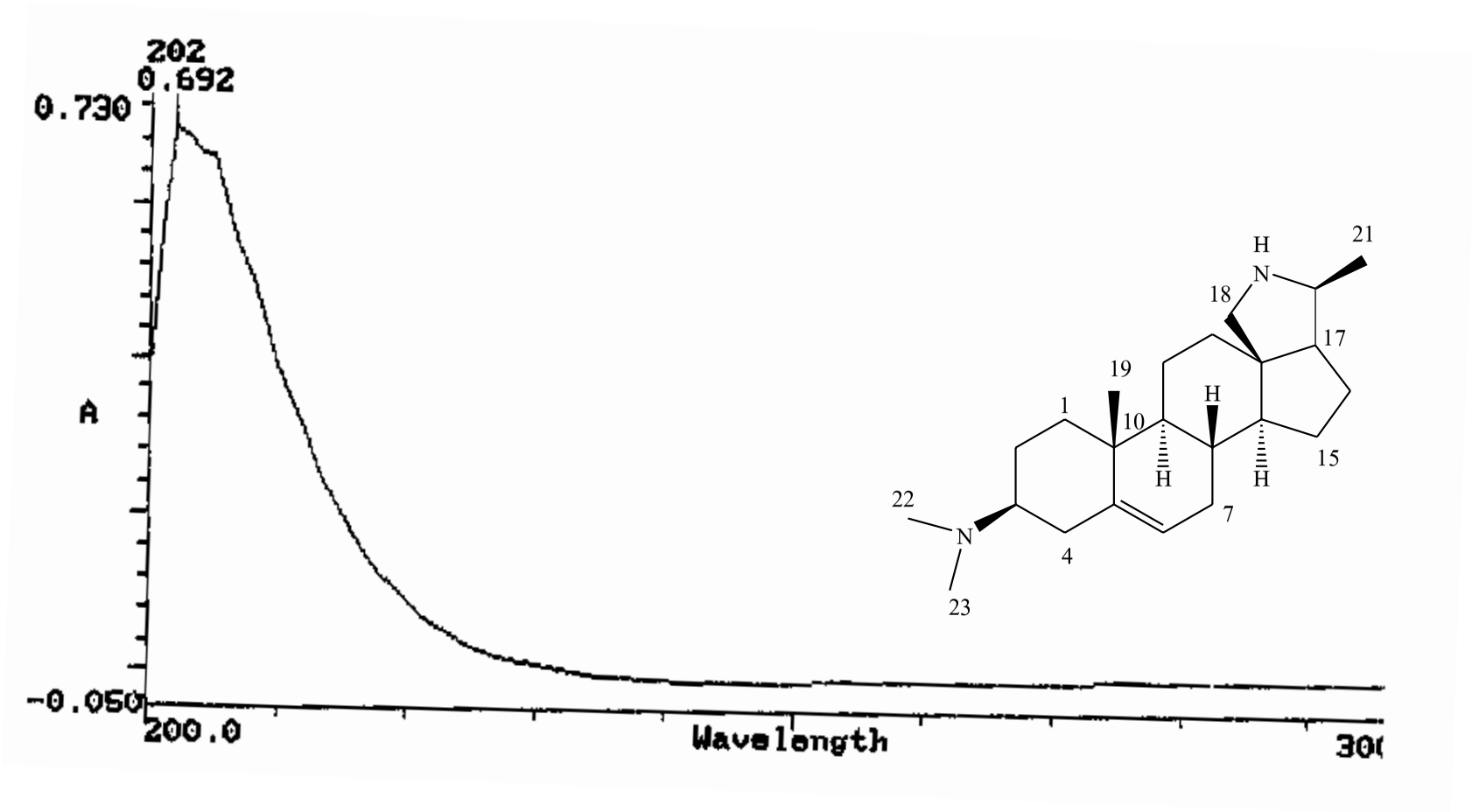

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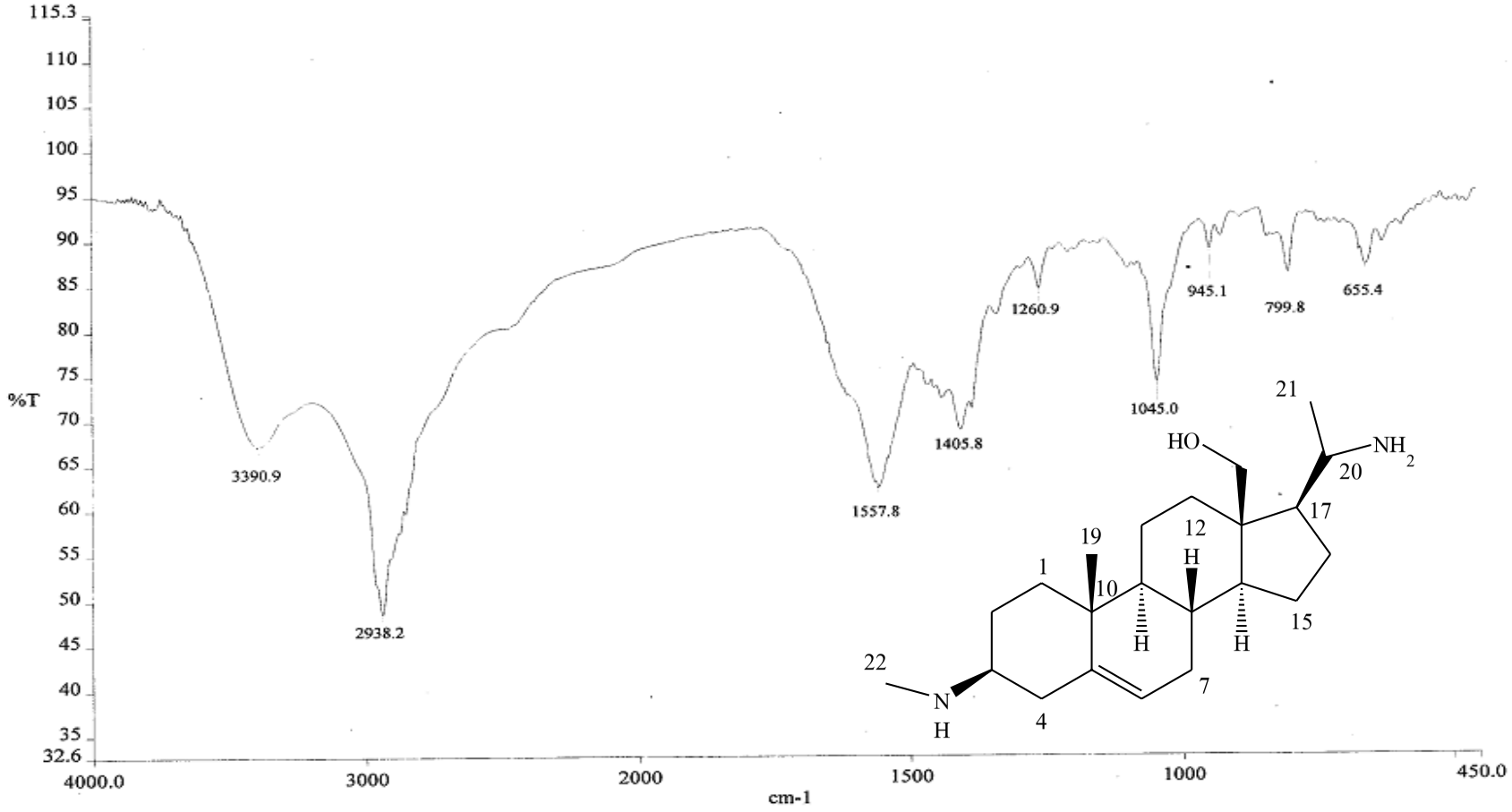

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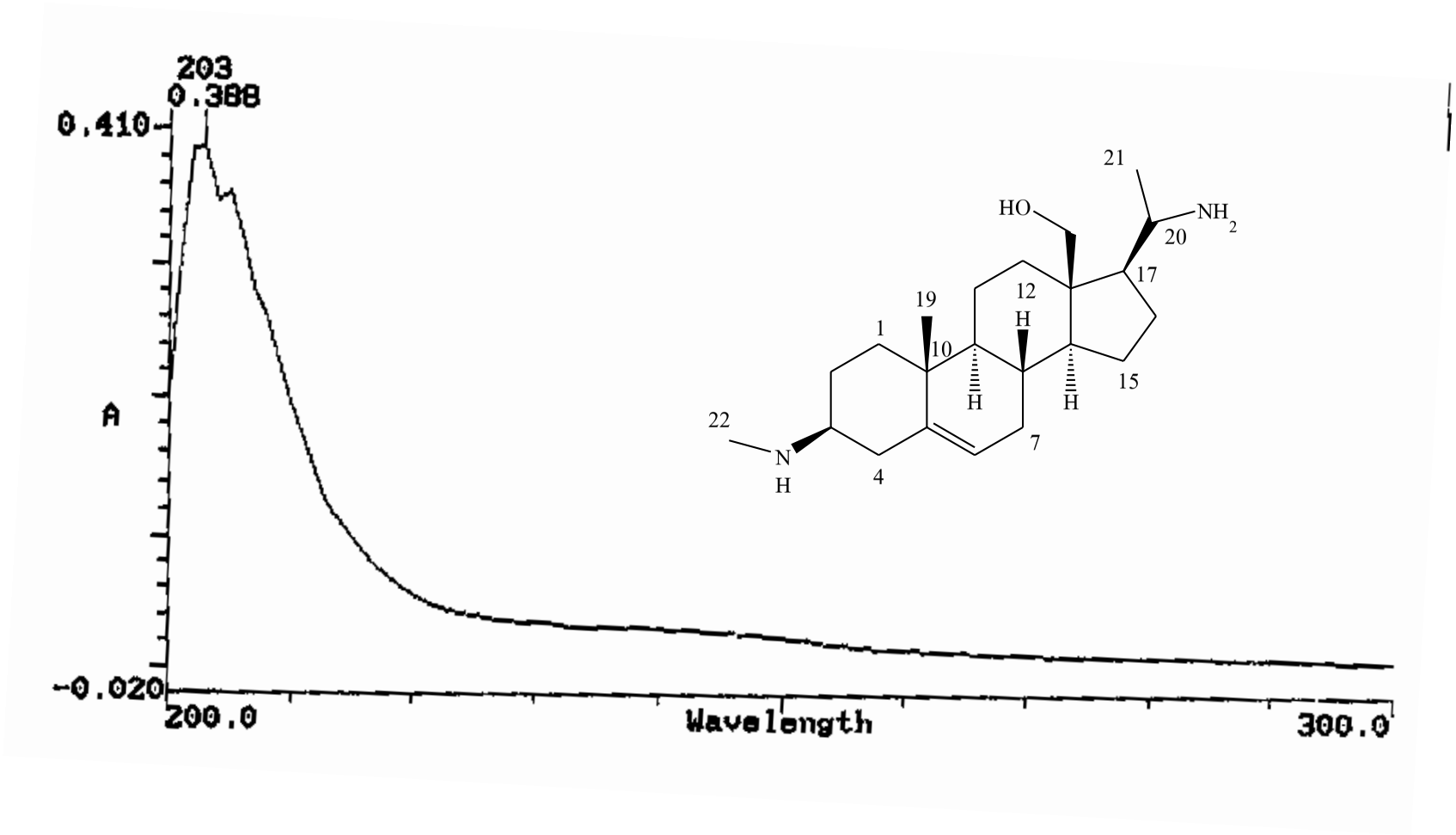

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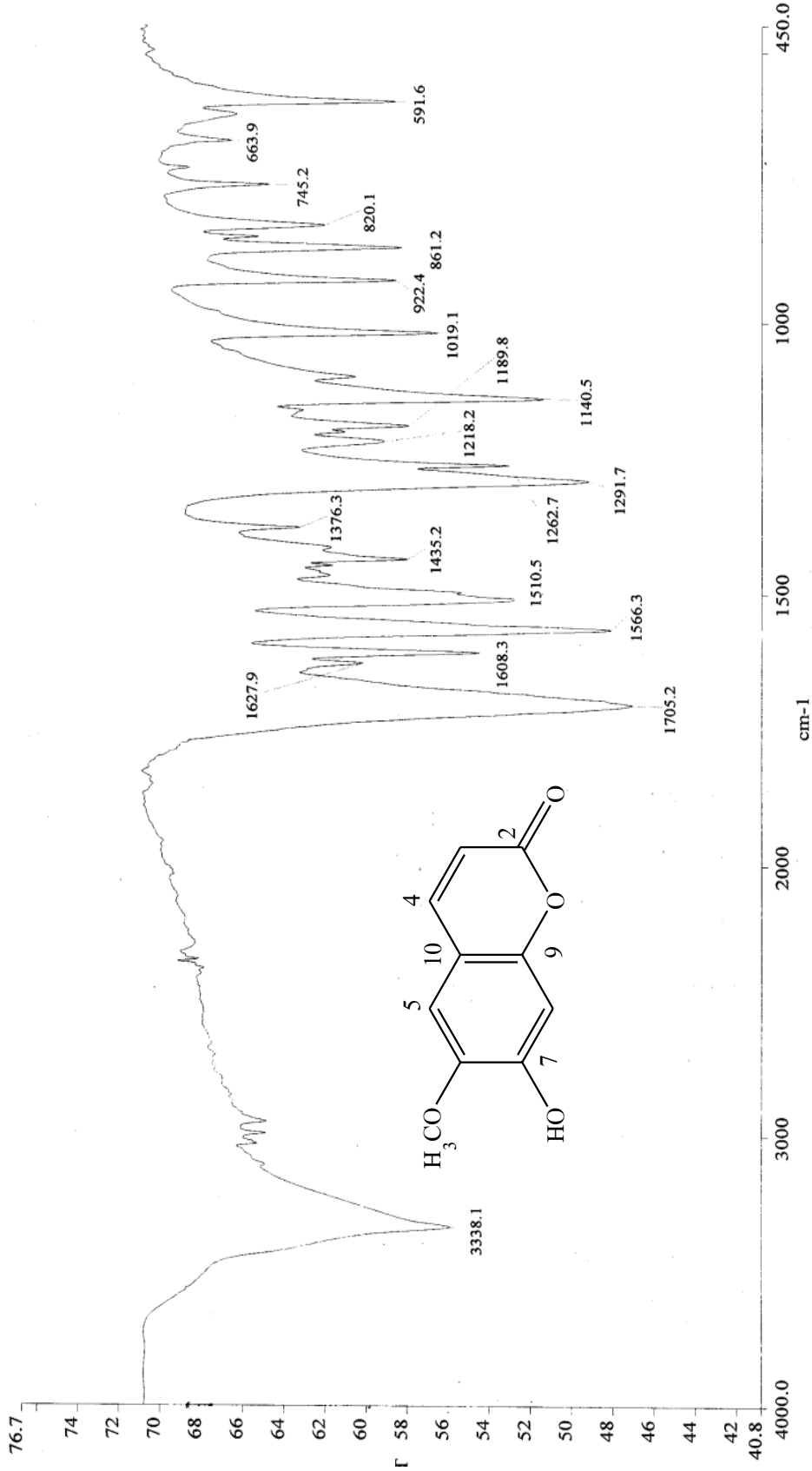

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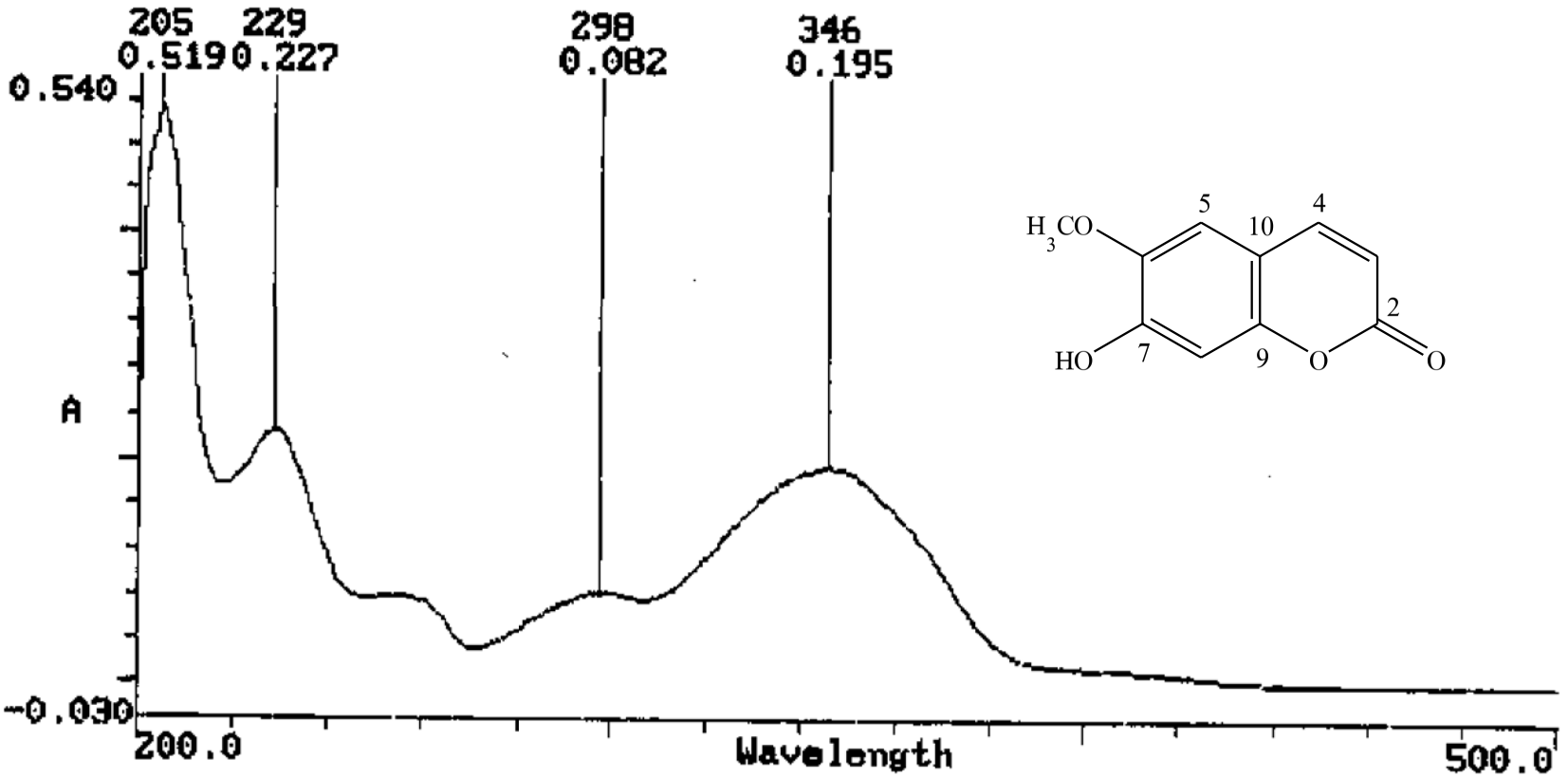

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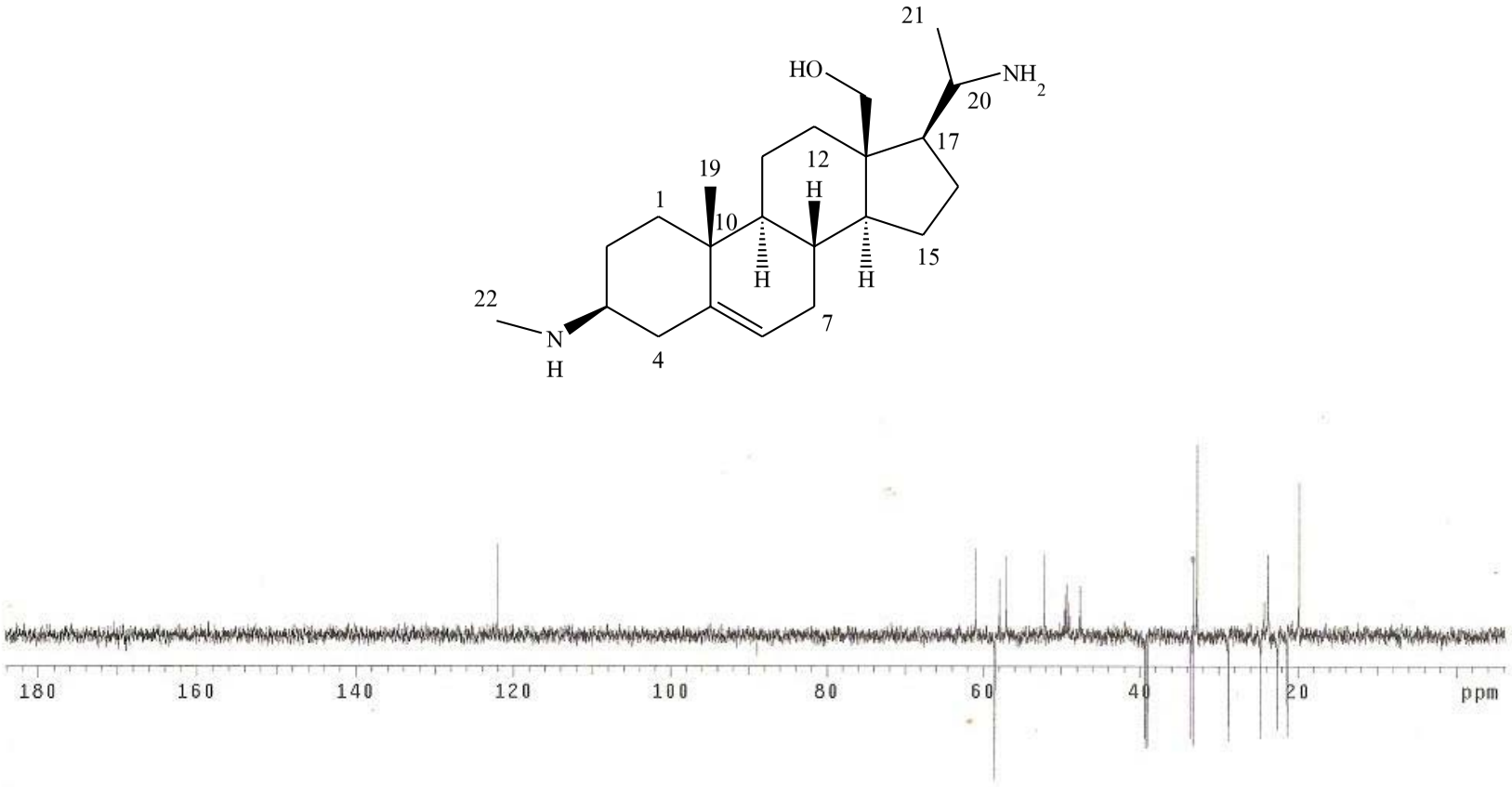
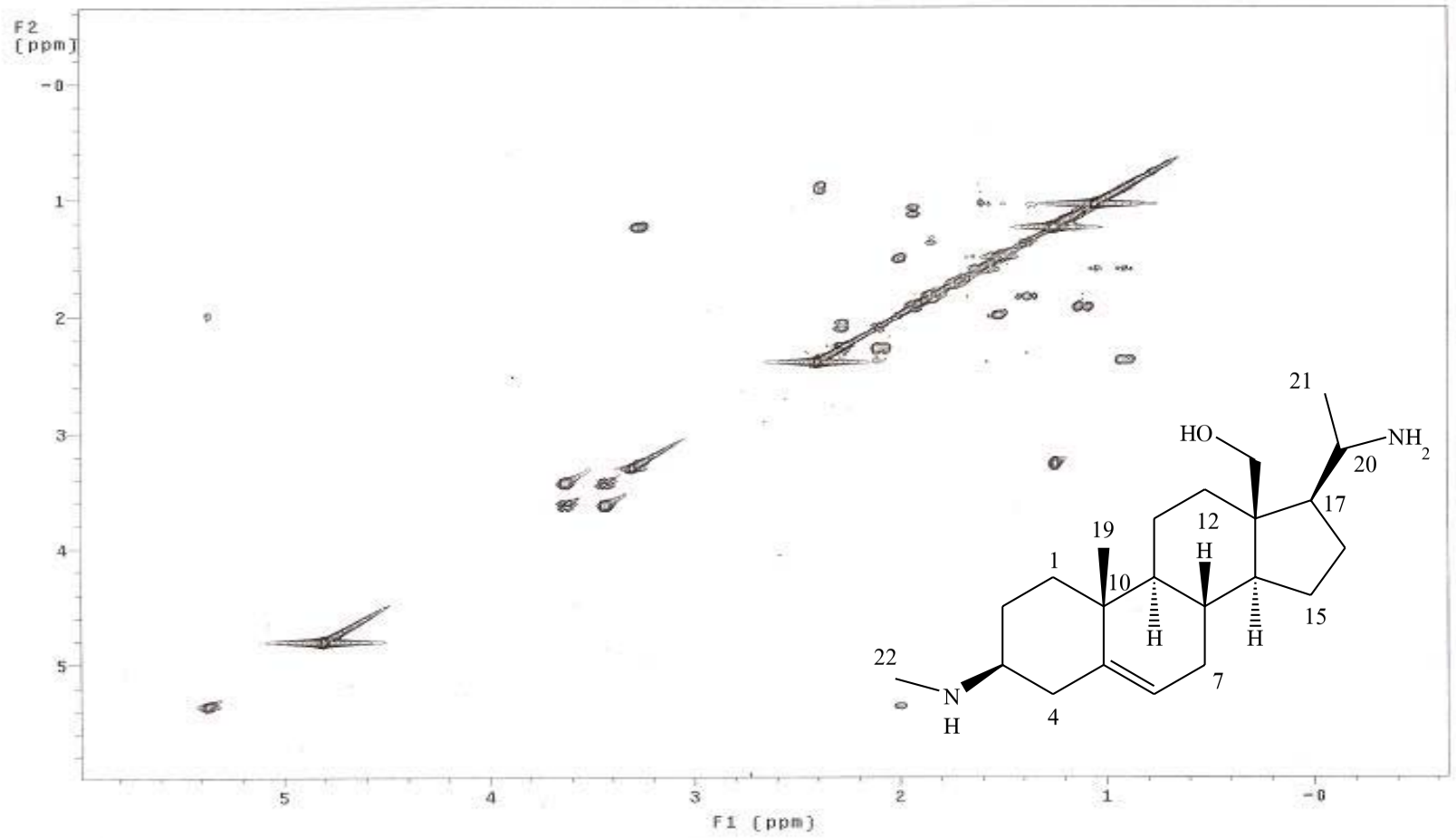
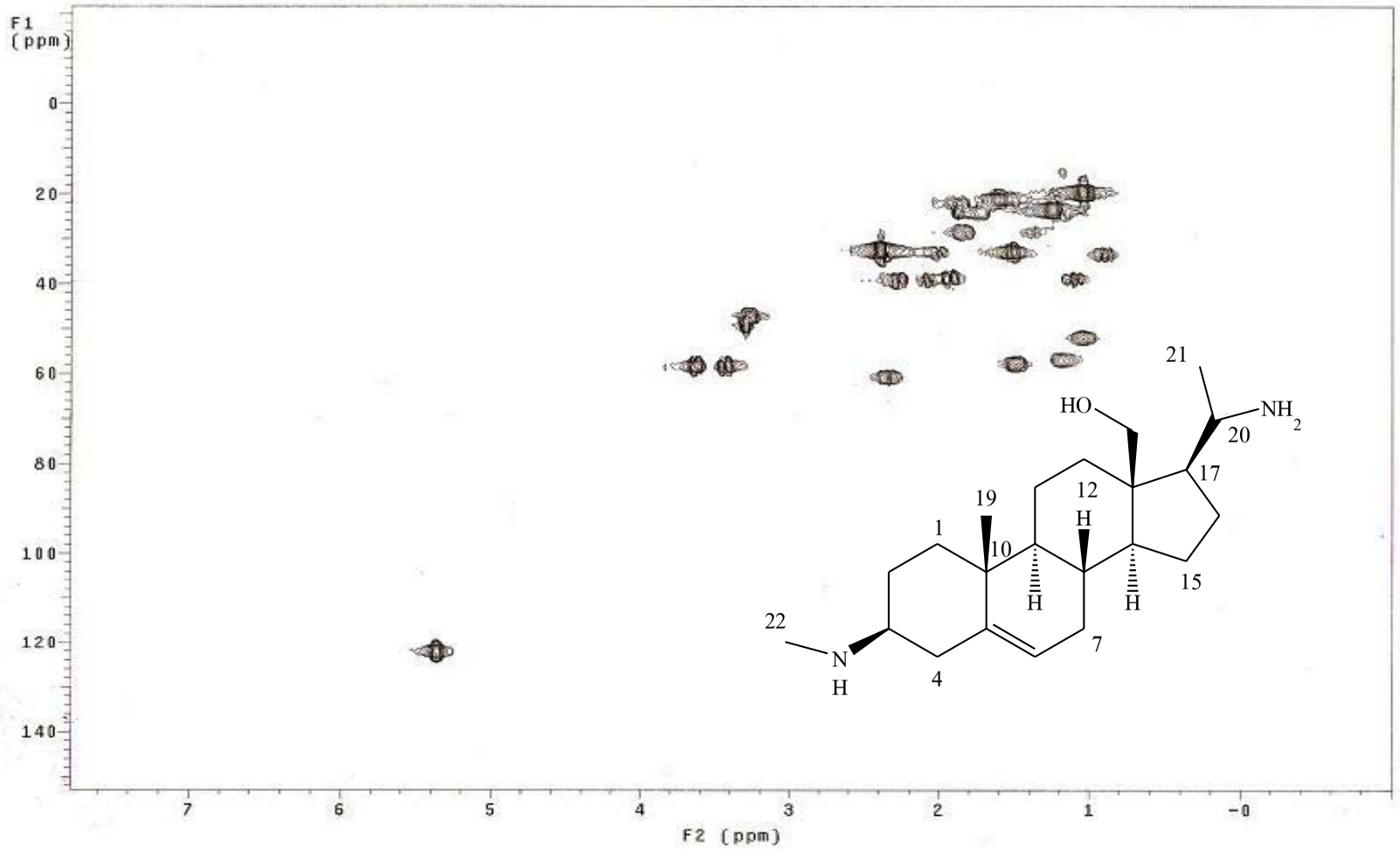


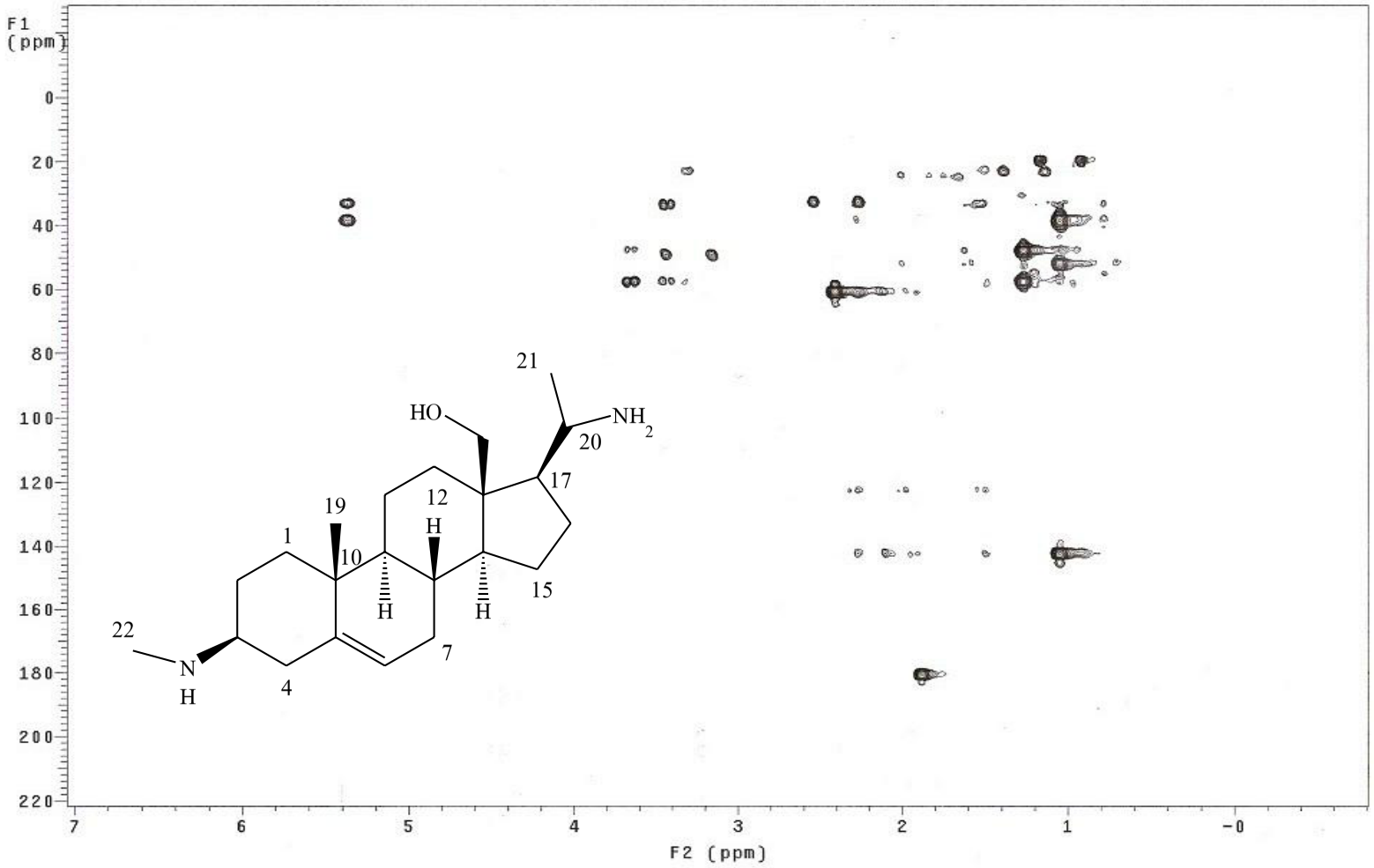
Dept 135 spectrum of compound **4**



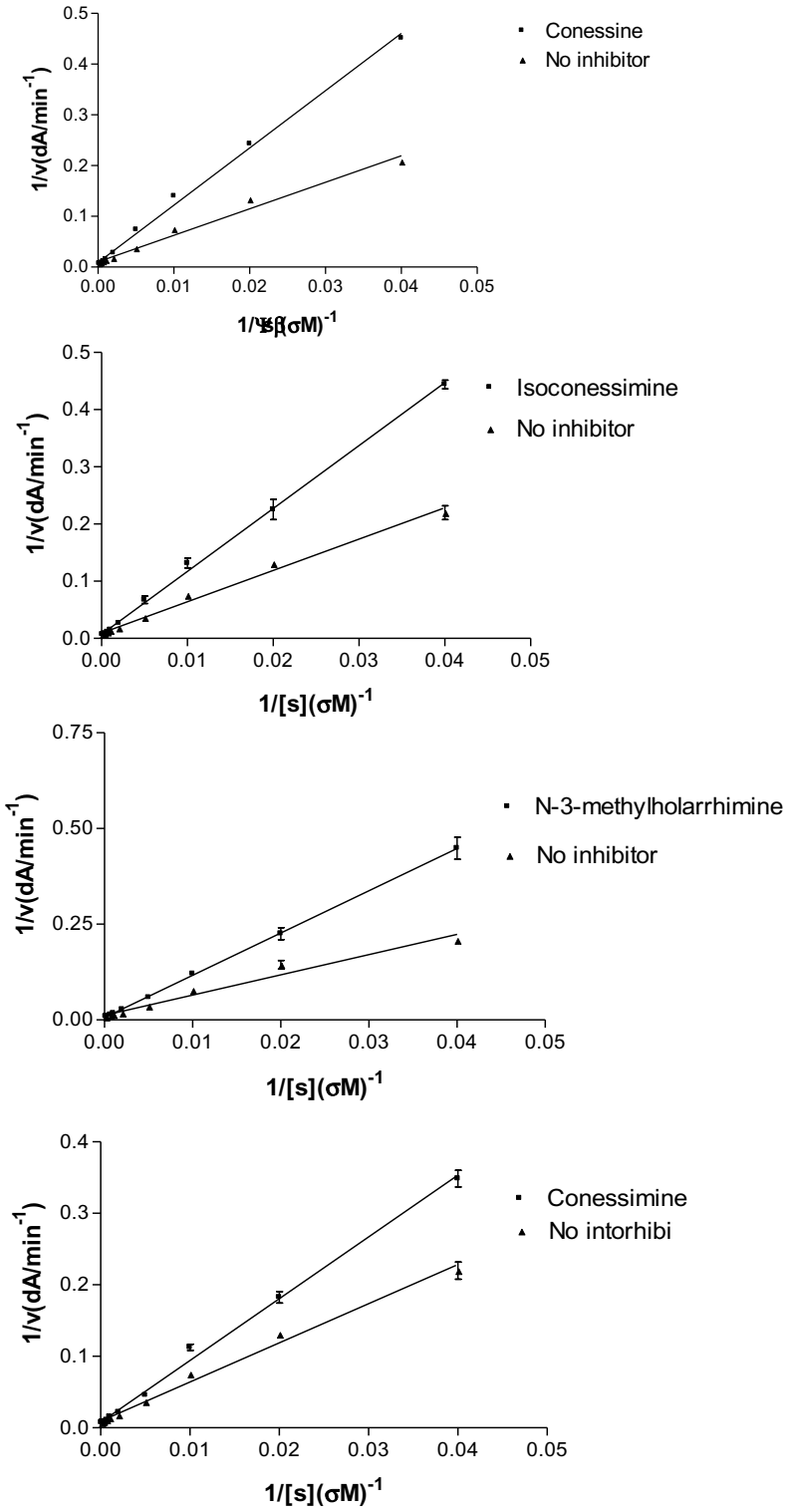
^{1}H-^{1}H COSY spectrum of compound **4** (500 MHz, CD_3OD)



HMQC spectrum of compounds **4** (500 MHz, CD_3OD)



HMBC spectrum of compounds **4** (500 MHz, CD_3OD)



Lineweaver-Burk plots of AChE activity over a range of substrate concentrations (25 µM to 10 mM) of steroidal alkaloids

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