Acetylcholinesterase Inhibitors from the Thai Sponge *Corticium* sp.

Roosanee Langjae

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Pharmacy in Pharmaceutical Sciences

Prince of Songkla University

2007

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Major Program Pharmaceutical Sciences

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The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Master of Pharmacy Degree in Pharmaceutical Sciences

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(Assoc. Prof. Dr. Krerkchai Thongnoo)

Dean of Graduate School

ชื่อวิทยานิพนธ์	สารที่มีฤทธิ์ต้านเอนไซม์อะซีติลโคลีนเอสเทอเรสจากฟองน้ำไทยในสกุล *Corticium*
ผู้เขียน	นางสาวรุสนี เล่งเจ๊ะ
สาขาวิชา	เภสัชศาสตร์
ปีการศึกษา	2550

บทคัดย่อ

การแยกสกัดสารควบคู่ไปกับการทดสอบฤทธิ์ต้านเอนไซม์อะซีติลโคลีนเอสเทอเรสจากสารสกัดจากฟองน้ำไทยชนิดหนึ่งในสกุล *Corticium* ทำให้สามารถแยกสารประกอบกลุ่ม steroidal alkaloids ชนิดใหม่ได้ 1 ชนิด คือ 4-acetoxy-plakinamine B และสารประกอบ trihydroxy sterol 1 ชนิด ซึ่งยังไม่สามารถวิเคราะห์โครงสร้างที่แน่นอนได้ การวิเคราะห์หาสูตรโครงสร้างใช้วิธีทางสเปคโตรสโคปี ได้แก่ UV, IR, NMR และ MS spectroscopy จากการทดสอบฤทธิ์ต้านเอนไซม์อะซีติลโคลีนเอสเทอเรสและฤทธิ์ความเป็นพิษต่อเซลล์ของ 4-acetoxy-plakinamine B พบว่าสารตัวอย่างแสดงฤทธิ์ต้านเอนไซม์อะซีติลโคลีนเอสเทอเรสที่ดี (IC_{50} เท่ากับ 3.75±1.69 μM) แต่ไม่แสดงฤทธิ์ความเป็นพิษต่อเซลล์มะเร็ง และจากการวิเคราะห์จลนศาสตร์ของการยับยั้งกิจกรรมของเอนไซม์อะซีติลโคลีนเอสเทอเรสพบว่าเป็นแบบผันกลับได้ ผลการวิเคราะห์ค่า V_{max} และ K_m ในการยับยั้งเอนไซม์อะซีติลโคลีนเอสเทอเรส พบว่า 4-acetoxy-plakinamine B ยับยั้งเอนไซม์แบบผสม (mixed-competitive inhibition)

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ABSTRACT

The bioassay-guided fractionation of the Thai sponge *Corticium* sp. led to the isolation of a new steroidal alkaloid, 4-acetoxy-plakinamine B, along with an unidentified trihydroxy sterol. The structure elucidation was achieved by means of spectroscopic analyses, including UV, IR, NMR and mass spectra. 4-Acetoxy-plakinamine B showed potent acetylcholinesterase-inhibiting activity (IC_{50} 3.75±1.69 μM), with no significant cytotoxicity observed. The enzyme inhibition of 4-acetoxy-plakinamine B against acetylcholinesterase was reversible. In order to determine the kinetics of enzyme inhibition, V_{max} and K_m was measured to reveal that the compound inhibited the targeted enzyme in a mixed-competitive manner.

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Roosanee Langjae

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LIST OF ABBREVIATIONS AND SYMBOLS

$[\alpha]_D$	specific rotation
δ	chemical shift (in ppm)
ε	molar extinction coefficient
λ_{max}	maximum wavelength
ν_{max}	maximum wave number
ACh	acetylcholine
AChE	acetylcholinesterase
AChE-I's	acetylcholinesterase inhibitors
AD	Alzheimer's disease
ATCI	acetylthiocholine iodide
BChE	butyrylcholinesterase
br	broad (for NMR signals)
c	concentration
CoMFA	comparative molecular field analysis
CoMSIA	comparative molecular similarity indices analysis
COSY	correlation spectroscopy
d	doublet (for NMR signals)
DEPT	distortioness enhancement by polarization transfer
dmA	delta milliabsorption
DTNB	5,5'-dithiobis[2-nitrobenzoic acid]
EIMS	electron-impact mass spectroscopy
ESIMS	electro-sprayed ionization mass spectroscopy
HMBC	heteronuclear multiple-bond multiple-quantum coherence
HMQC	heteronuclear multiple-quantum coherence
HPLC	high pressure liquid chromatography
HREIMS	high-resolution electron-impact mass spectroscopy
IC_{50}	inhibitory concentration at 50% of tested subject
IR	infrared

LIST OF ABBREVIATIONS AND SYMBOLS (cont.)

J	coupling constant
K_m	Michaelis constant
m	multiplet (for NMR signals)
m/z	mass-over-charge ratio
MIC	minimum inhibitory concentration
MS	mass spectroscopy
NMR	nuclear magnetic resonance
QSAR	quantitative structure-activity relationship
s	singlet (for NMR signals)
SE	standard error
SRB	sulphorhodamine B
t	triplet (for NMR signals)
TLC	thin layer chromatography
t_R	retention time
UV	ultraviolet-visible
V_{max}	maximum velocity
w/v	weight by volume

CHAPTER 1

INTRODUCTION

1.1 Marine natural products and drug development

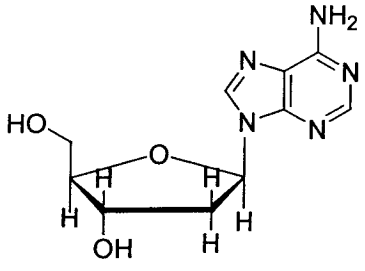
In the area of drug discovery, nature is considered the most attractive source of the therapeutic candidates as the tremendous chemical diversity is found in millions of species of plants, animals and microorganisms. For most of currently used medicines, natural products are starting points for drug discovery and development. As the results, natural products and their derivatives represent more than 50% of all the drugs in clinical use.

Over the past decades, conventional searches for bioactive natural products have relied heavily on terrestrial plants as primary sources. Also, soil-derived microbes were found to be another excellent source of biologically active compounds. However, the continual search for new sources of possible drugs eventually led researchers to look to the ocean. Oceanic marine organisms are of scientific interest for two major reasons. To begin with, marine organisms constitute a major share of the Earth's biological resources. Secondly, marine organisms often possess unique anatomical structures, metabolic pathways, reproductive systems, and sensory and chemical defense mechanisms (Pawlik, 1993), due to the adaptation to a wide range of environmental conditions. The range of marine habitats encompass the frigid cold polar Arctic and Antarctic seas, to the warm and bright shallow waters of the tropics, and to the great pressures of the deep ocean floor. Recent improvements in underwater life-support systems have extensively facilitated the collection of marine organisms from largely unexplored, yet harsh, regions of the oceans. As the results, many bioactive chemicals from the marine organisms have been isolated and characterized over the past 40 years, and some even holds great promise for useful biotechnological application towards a wide range of pharmaceutical compounds, medical research materials, agricultural products, novel energy sources and bioremediation techniques (Faulkner, 2002).

1.1.1 Marine-derived natural products in clinical development

The field of marine natural products has produced a plethora of chemically interesting and important bioactive natural products. During the 1950s, Bergmann's group at

Yale isolated several nucleosides from the Caribbean sponge *Cryptotethya crypta* (family Tethylidea). Two of these, spongothymidine and spongouridine, contained the rare arabinose sugar rather than ribose, which is a quite ubiquitous sugar in nucleosides. This discovery led researchers to synthesize the analogues, ara-A (vidarabine) **(1)**, and ara-C (cytarabine) **(2)**. The two compounds are currently used as antiviral and antileukemic agents, respectively (Guyot, 2000).



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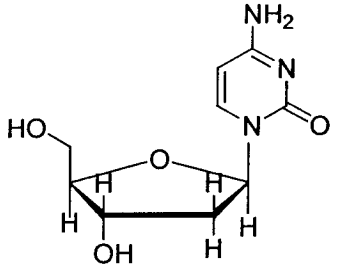

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Since then, the field of marine natural products has grown substantially with numerous natural and synthetically-derived compounds evaluated as clinical drug candidates. Whereas it might be claimed that this is resulted from generous funding by the U.S. National Cancer Institute (NCI), there is also an underlying preponderance of anti-tumor agents produced by marine organisms. Although most have failed due to ineffectiveness or toxicity problems, a fair number of marine derived agents have been passing into clinical trials. Shown in Table 1 are a few selected agents, either as approved drugs or agents currently under clinical investigation.

Table 1 Marine-derived natural products currently approved or in clinical trials (Newman and Cragg, 2004)

Name	Source	Status (disease)
Ziconotide (Prialt™)	*Conus magus*	Approved (neuropathic pain)
Ecteinascidin 743 (Yondelis™)	*Ecteinascidia turbinata*	Phase III (cancer)
Æ-941(neovastat)	Shark	Phase III (antiasthmatic)
Dehydrodidemnin B (Aplidine™)	*Aplidium albicans*	Phase II (cancer)
Bryostatin 1	*Bugula neritina*	Phase II (cancer)
Soblidotin (TZT-1027)	*Dolabella auricularia*	Phase II (cancer)
Synthatodin (ILX 651)	*Dolabella auricularia*	Phase II (cancer)
Kahalamide F	*Elysia rufescens/Bryopsis* sp.	Phase II (cancer)
HTI-286 (hemiasterlin derivative)	*Cymbastella* sp.	Phase II (cancer)
Squalamine	*Squalus acanthias*	Phase II (cancer)
PM00104 (jorumycin derivative; Zalypsis™)	*Jorunna funebris*	Phase I (cancer)
E7389 (halicondrin B derivative)	*Lissodendoryx* sp.	Phase I (cancer)
ES-285 (spisulosine)	*Spisula polynyma*	Phase I (cancer)
Discodermolide	*Discodermia dissoluta*	Phase I (cancer)
KRN-7000	*Agelas mauritianus*	Phase I (cancer)
GTS-21(anabaseine derivative)	*Paranemertes peregrina*	Phase I (Alzheimer's)
CGX-1160 and CGX-1007	*Conus geographus*	Phase I (pain)

It is not quite surprising to find that most marine-derived drug candidates as seen in Table 1 are anticancer agents, considering that most of the metabolites are in fact produced as toxic agents for chemical defense. However, certain number are otherwise applicable in some other remote diseases, including the analgesic ziconotide and the famous antiaging pseudopterosins. In the remaining section of this review, the use of another group of marine-derived natural products as acetylcholinesterase inhibitors, i.e., promising candidates for the treatment of Alzheimer's disease, is introduced. Such application extends marine natural products research into other disease areas, and suggests its potential as one of the leading branches of research in drug development.

1.2 Alzheimer's disease and cholinesterase inhibitors

1.2.1 Pathophysiology of Alzheimer's disease (AD)

Neuroimaging of the patients with AD or other dementias may reveal atrophy of the brain, such as enlarged ventricles and sulci and narrowed gyri, although these features are not always present. Neuronal loss is the main neuropathologic feature underlying the symptoms of AD. Microscopically, AD is characterized by the presence of amyloid plaques and neurofibrillary tangles. Amyloid plaques contain deposits of β-amyloid, which is a 40- to 42-amino acid peptide derived from amyloid precursor protein. Neurofibrillary tangles are a hyperphosphorylated τ-protein, which forms paired helical filaments. AD is also associated with a loss of cholinergic neurons, which project from the basal forebrain to the cerebral cortex and the hippocampus. The loss of cholinergic neurons is progressive and results in profound memory disturbances (Akhondradeh and Abbasi, 2006).

1.2.2 Cholinergic hypothesis

The first neurotransmitter defect commonly found in AD patients involved acetylcholine (ACh). Because cholinergic function is required for short-term memory function, it has been known that cholinergic deficit in AD patients is also responsible for much of short-term memory deficit. Markers for the cholinergic neurons such as choline acetyltransferase and acetylcholinesterase, which are enzymes responsible for synthesis and degradation of ACh, respectively, decrease in the cortex and hippocampus. The earliest loss of neurons occurs in the nucleus basalis and the entorhinol cortex, where cholinergic neurons are preferentially affected.

One of the most prominent features of AD is a significant deficit in cholinergic transmission in this certain brain area. It was found that concentrations of ACh decrease by nearly 90% in patients with AD in the early illness. The decrease in ACh-dependent neurotransmission is thought to lead to the functional deficits of AD patients (Francis *et al.*, 1999; Akhondradeh and Abbasi, 2006).

Clinical drug trials in patients with AD have focused on drugs that augment the levels of ACh in the brain to compensate such losses of cholinergic functions. These drugs include ACh precursors, muscarinic agonists, nicotinic agonists, and cholinesterase inhibitors. The current focus of AD treatment is the use of agents that increase the availability of intrinsic ACh by inhibiting the enzyme acetylcholinesterase (AChE). This may restore the cholinergic functions in the brain and significantly reduce the severity of dementia. As the cognitive dysfunction and other features of AD are mediated by the loss of function at cholinergic synapses in the neocortex and hippocampus, agents that replace the lost cholinergic functions have been suggested to be useful in the management of disease (Hoe *et al.*, 2002).

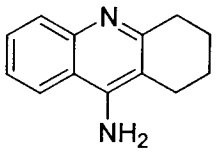
1.2.3 Acetylcholinesterase inhibitors (AChE-I's)

Due to obscure and unknown nature of the disease principle, there are no long-term remedies that are entirely accepted as perfect treatment for AD. Several approaches that are employed by physicians and practitioners include the use of antipsychotic drugs to relieve the symptoms of dementia (Aupperle, 2006). Also, for the patients suffering from mild symptoms of early-stage AD, the use of medicinal plant, *Gingko biloba*, is also acceptable among certain physicians (Eslami *et al.*, 2003; Akhondradeh and Abbasi, 2006). However, the best direct approach that targets one of the causes of disease is possibly the use of AChE-I's. Whereas such approach is still controversial for the beneficial effects which normally last no longer than one year, it is still among the best approaches to improve the patients' quality of life (Bullock, 2002; Mukherjee *et al.*, 2007).

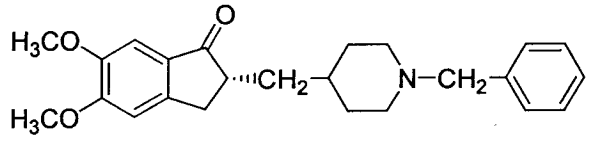
AChE-I's enhance the cholinergic transmission by reducing the enzymatic degradation of ACh. Since cholinergic dysfunction is considered a primary cause of AD, and the degree of cognitive improvement in AD patients are reportedly correlated to central cholinergic

deficiency, elevation of ACh level therefore is thought to be helpful, especially in improving the symptoms of cognitive deficits (Coyle *et al.*, 1983; Chemnitius *et al.*, 1996).

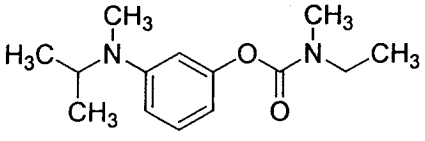
To date, only four AChE-I's have been approved by USA-FDA (Zarotsky *et al.*, 2003). The first drug approved for general clinical use in AD was tacrine. Three new AChE-I's, donepezil (Aricept®), rivastigmine (Exelon®), and galantamine (Reminyl®), are also currently available (Eslami *et al.*, 2003). Neither of the four AChE-I's are completly effective, however, especially in the case of severe AD. Furthermore, several side effects have also been reported. In most cases, the adverse effects, mainly gastrointestinal in nature, are mild to moderate, and are reported by 25-46% of patients (Alwahhabi, 2005).



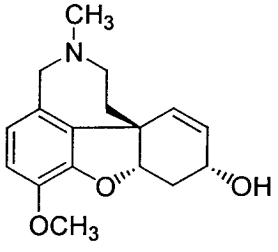
tacrine



donepezil



rivastigmine



galantamine

Figure 1 FDA-approved drugs for AD

As mentioned earlier, even though AChE-I's may provide effective temporary relief of symptoms in some patients, there are currently no cures for AD (Hecker and Snellgrove, 2003). However, with only approach acceptable and fairly efficient for the treatment of such desperate disease, drug research and development are still based primarily on the cholinergic hypothesis that supports the cognition improvement by regulation of the synthesis and release of ACh in the brain.

1.3 Cholinesterase inhibitors derived from natural products

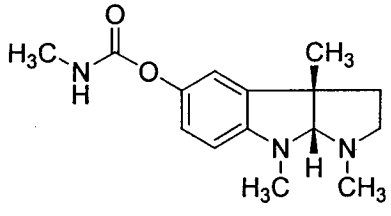
To date, various groups of natural products and their synthetic analogues have been reported to exhibit cholinesterase inhibitory activities to an interesting extent. Among these,

alkaloids constitute a large proportion of enzyme inhibitors. The observation is not surprising considering the fact that for AChE active site, positively charged nitrogens are among the required elements of compounds that can bind to a similar region to that of ACh. Nevertheless, a series of non-nitrogenous compounds, namely terpenoids, have been reported with significant inhibiting potency (Mukherjee *et al.*, 2007). The lack of positively-charged moiety among these molecules suggested the possibility of allosteric binding sites, although a thorough investigation regarding such interaction is yet to be explored.

1.3.1 Alkaloids

1.3.1.1 Physostigmine

Physostigmine **(3)** or eserine was isolated from the calabar bean, the seed of *Physostigma venenosum* Balf., in the nineteenth century in studies stimulated by the use of the seeds as an ordeal poison. The early applications of physostigmine were limited to opthalmic preparations and the treatment of myasthenia gravis. However, the realization of its cognitive benefits in both animal models and human subjects led to the development of synthetic analogues bearing the carbamoyl moiety, including neostigmine **(4)** and rivastigmine **(5)**. The latter, as mentioned earlier, has become an approved drug used in patients with early-state AD (Houghton and Howes, 2005).



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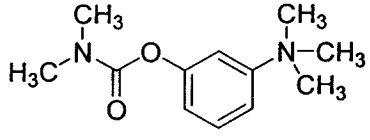

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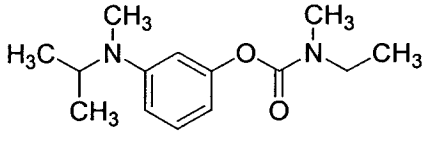

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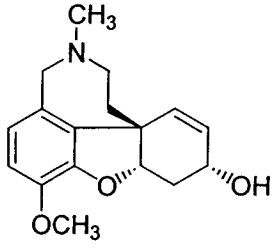
1.3.1.2 Galantamine and related Amaryllidaceous alkaloids

Galantamine **(6)** was found in several members of the Amaryllidaceae such as the Chinese medicinal herb, *Lycoris radiata* Herb. and the European *Galanthus nivalis* L. and

Narcissus spp. Its properties were first exploited in Bulgaria in the mid-twentieth century for the treatment of polio victims, but it only came into prominence as a treatment for AD during the 1990's. Galantamine has been licensed in Europe for AD treatment since 2001. Among several adventages of galantamine over other anti-Alzheimer's drugs include the longer benefit on cognitive functions, which reportedly last for at least 3 years (Eslami *et al.*, 2003; Houghton and Howes, 2005).

Other related alkaloids isolated from other Amaryllidaceous species includes crinine **(7)** and its dihydroisoquinoline analogues. Major sources of these alkaloids include plants of the genus *Crinum*. Most of these alkaloids express the AChE inhibiting activity with IC_{50}'s of 213–490 μM (Viegas *et al.*, 2005).



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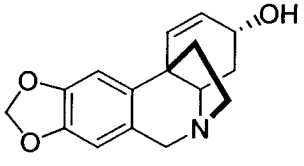

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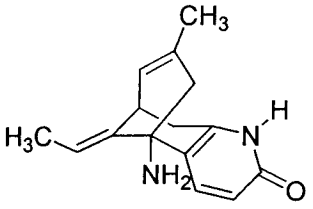
1.3.1.3 Huperzine A and *Lycopodium* alkaloids

Huperzine A **(8)**, isolated from clubmoss *Huperzia serrata* (Thunb. ex Murray) Trevis (syn. *Lycopodium serratum* Thunb.), is a potent, highly specific and reversible inhibitor of AChE (Wang and Tang, 1998). The compound was found to reverse or attenuate cognitive deficits in a broad range of animal models. Clinical trials in China have demonstrated that huperzine A significantly relieves memory deficits in aged subjects, patients with benign senescent forgetfulness, AD and vascular dementia (VD), with minimal peripheral cholinergic side effects (Wang and Tang, 2005).

The discovery of huperzine derivatives from *Huperzia* sp. also led to the investigation in several species of *Lycopodium* mosses, most of which have been long known among Chinese medicinal herbs. Various alkaloids presumably derived from related quinolizidine precursors, such as carinatumins A **(9)** and B **(10)**, were reported to exhibit AChE inhibitory activity in a various extent (Choo *et al.*, 2007).



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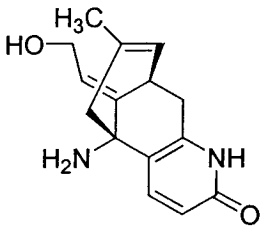

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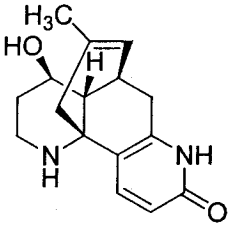

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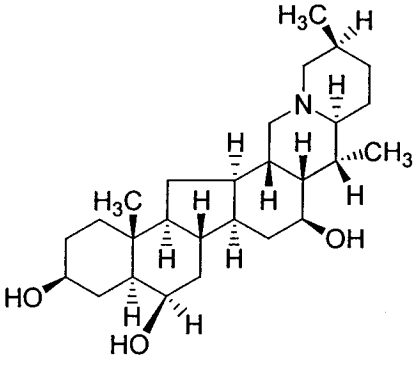
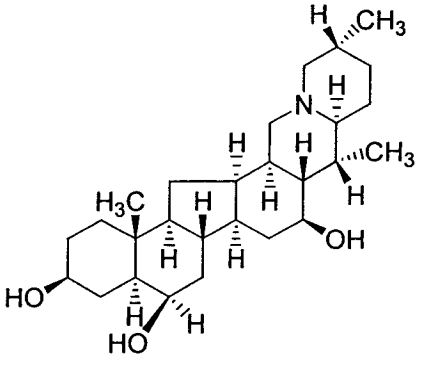
1.3.1.4 Steroidal alkaloids and alkaloids with terpenoid skeletons

Apart from the well-known Solanaceous steroidal alkaloids, some of which were also reported active as AChE inhibitors, most of steroidal alkaloids that showed potent AChE inhibitory activity were isolated from medicinal plants of the family Buxaceae, especially those from the genus *Sarcococca* (Kalauni *et al.*, 2002; Choudhary *et al.*, 2003; Babar *et al.*, 2006). Due to the close relation with the main focus of this thesis, the major review on *Sarcococca* alkaloids and related analogues will be re-addressed in a more elaborated detail in section 1.3.3.

Although remotely related in core structures, certain nitrogenated terpenoids also coincidentally exhibited cholinesterase-inhibiting activity. The examples include delavine **(11)** and persicanidine **(12)** from the bulbs of plant in genus *Fritillaria*, which show butyrylcholinesterase-inhibiting activity with IC_{50}'s of 1.71 and 4.25 μM, respectively (Atta-ur-Rahman *et al.*, 2002a).



11

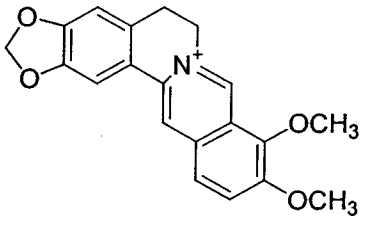


12

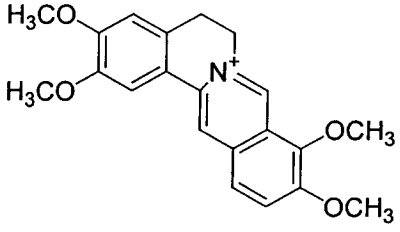
1.3.1.5 Miscellaneous alkaloids

Although it is not an intention of this review to compile all the AChE inhibitors completely, it is worth exemplifying here certain interesting alkaloids that exhibit a cholinesterase

inhibitory activity in an interesting extent. Of particular interest were those with protoberberine and indole moieties. These include berberine **(13),** palmatine **(14),** and protopine **(15)** from *Corydalis speciosa* Maxim., which showed cholinesterase-inhibiting activity with IC_{50}'s of 3.3, 5.8, and 16.1 μM, respectively (Kim *et al.*, 2004). For indole alkaloids, the prototypes included rutaecarpine **(16)** and dehydroevodiamine **(17),** both of which were isolated from *Evodia rutaecarpa* (Juss) Benth. Compound **17** showed AChE-inhibiting activity with IC_{50} of 37.8 μM (Park *et al.*, 1996).



13



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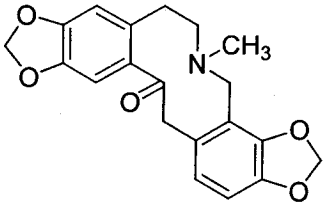
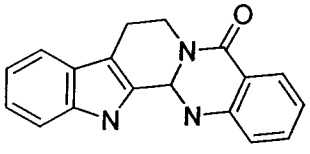
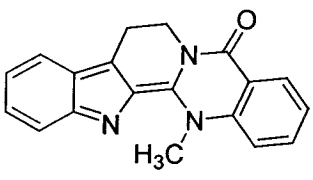


15



16

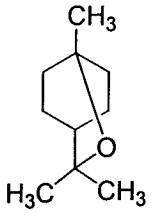


17

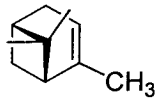
1.3.2 Terpenoids

As mentioned earlier, despite the extensive studies on the AChE binding sites that suggested a requirement of positively-charged moieties, certain oxygenated and lipophilic terpenoids, i.e., non-positive compounds, were reported highly active in cholinesterase-inhibiting assays. Of particular interest were volatile and small-molecule terpenoids, which were actually good for the memory as recorded in their history.

One such group of plants was the various European species of *Salvia* (family Labiatae). An ethanolic extract and oil of *S. officinalis* L. and *S. lavandulaefolia* Vahl. were investigated for AChE-inhibiting activity. Whereas the isolated single components such as 1,8-cineole **(18)** and α-pinene **(19),** from the both *Salvia* species, were virtually inactive, the total volatile oils were found active in animal models (Houghton and Howes, 2005).

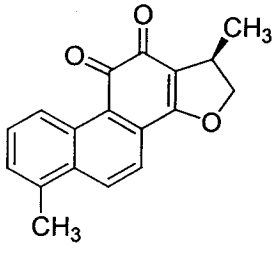


18

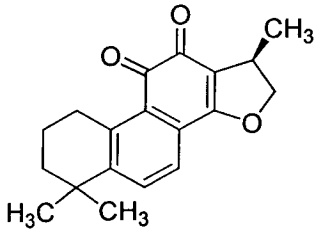


19

Larger-size terpenoids such as the norditerpenes, dihydrotanshinone **(20)** and cryptotanshinone **(21)** from root of *S. miltiorrhiza* Bunge, were also among AChE-inhibiting terpenes. Compounds **20** and **21** showed high AChE inhibitory activity (IC_{50}'s 1.0 and 7.0 μM, respectively). The plant was also known in Chinese medicines for its calmative effects, and there is evidence showing the neurodegenerative-protecting activity in its root extract (Houghton and Howes, 2005; Viegas *et al.*, 2005).



20



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1.3.3 Buxaceous steroidal alkaloids

The chemical structures of steroidal alkaloids found in natural products exhibit a wide variety both in the core steroid skeletons and nitrogenous substituent groups. However, as mentioned in section 1.3.1.4, most of steroidal alkaloids that were reported active as AChE inhibitors were predominantly isolated from medicinal plants of the family Buxaceae, especially those from the genera *Sarcococca* and *Buxus* (Babar *et al.*, 2006). The chemical structures and potency towards the AChE inhibition of Buxaceous steroidal alkaloids and related compounds are shown in Table 2.

Primarily, the core structures of the AChE-inhibiting steroidal alkaloids from Buxaceous plants are based on pregnane-type steroid skeleton, with nitrogenated substituted groups on C-3 and C-20. Although the molecular docking study suggested influence from either

of the nitrogens, the QSAR study indicated the positive effects from C-3 amino or amide nitrogen. Surprisingly, the remote nitrogen on C-20 was found irrelevant to the potency. In addition, the negatively-charged functional groups surrounding rings A and B (other than on C-3 and C-4) posted negative influences on the enzyme-inhibiting activity (Zaheer-ul-Huq *et al.*, 2003b; Khalid *et al.*, 2004a).

Table 2 Steroidal alkaloids as cholinesterase inhibitors

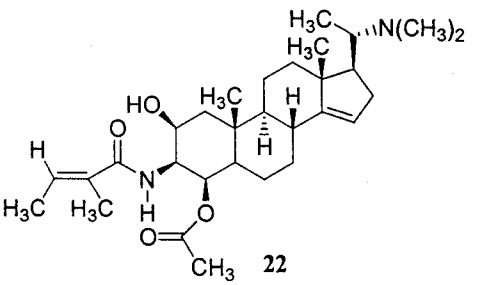
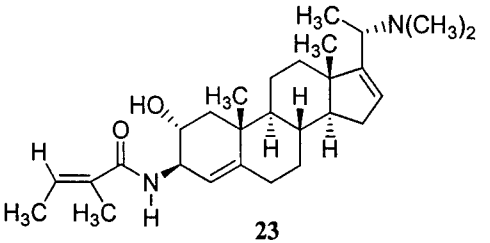
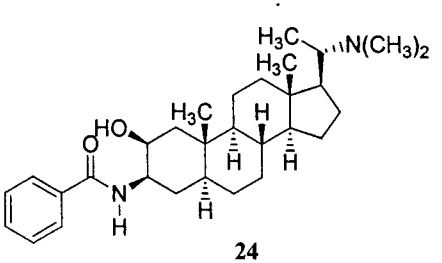
Name	Structure	IC_{50} (μM)		Reference
		AChE	BChE	
salignenamide C **(22)**	 **22**	61.3	38.4	Atta-ur-Rahman *et al.*, 2002b
salignenamide D **(23)**	 **23**	185.2	23.8	Atta-ur-Rahman *et al.*, 2002b
2β-hydroxyepipachysamine D **(24)**	 **24**	78.2	29.0	Atta-ur-Rahman *et al.*, 2002b

Table 2 Steroidal alkaloids as cholinesterase inhibitors (cont.)

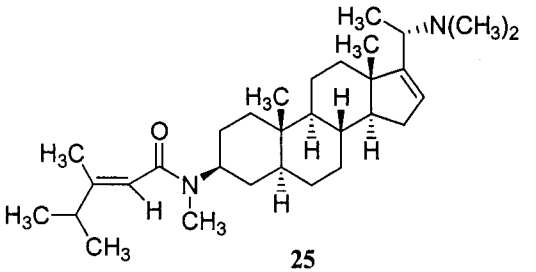
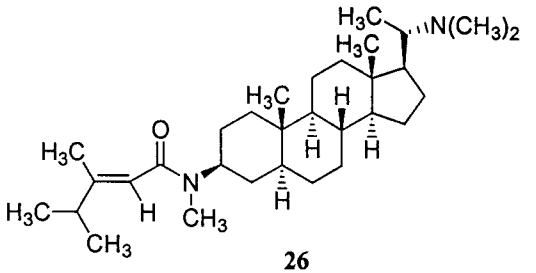
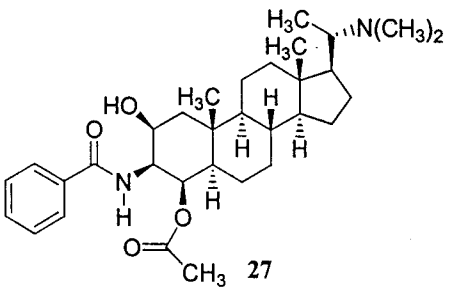
Name	Structure	IC_{50} (μM)		Reference
		AChE	BChE	
salignenamide E **(25)**	 **25**	6.2	3.7	Atta-ur-Rahman *et al.*, 2002b
salignenamide F **(26)**	 **26**	6.4	4.1	Atta-ur-Rahman *et al.*, 2002b
axillarine C **(27)**	 **27**	227.9	18.0	Atta-ur-Rahman *et al.*, 2002b

Table 2 Steroidal alkaloids as cholinesterase inhibitors (cont.)

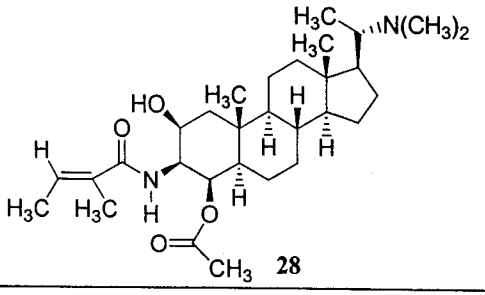
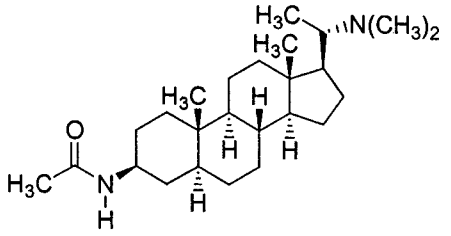
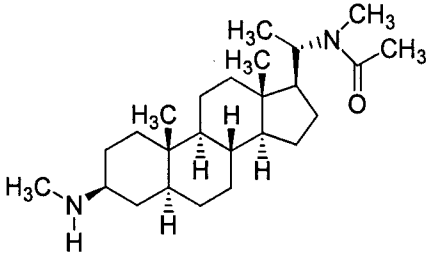
Name	Structure	IC_{50} (μM)		Reference
		AChE	BChE	
axillarine F **(28)**	 **28**	182.4	18.2	Atta-ur-Rahman *et al.*, 2002b
sarcorine **(29)**	 **29**	70.0	10.3	Atta-ur-Rahman *et al.*, 2002b
3-*N*-demethylsarcodine **(30)**	 **30**	204.2	16.6	Atta-ur-Rahman *et al.*, 2002b

Table 2 Steroidal alkaloids as cholinesterase inhibitors (cont.)

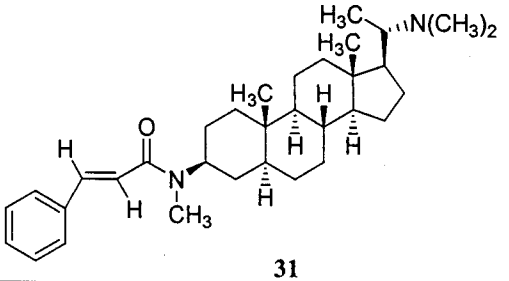
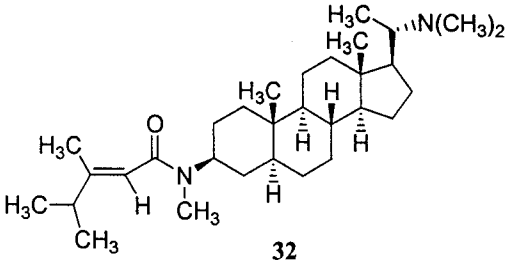
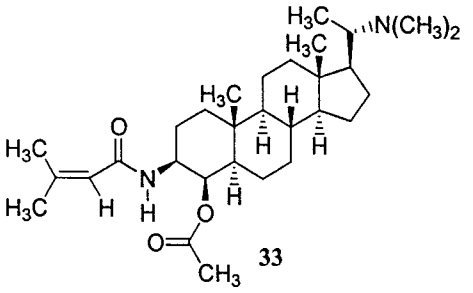
Name	Structure	IC_{50} (μM)		Reference
		AChE	BChE	
saligcinnamide **(31)**	 **31**	20.0	4.8	Atta-ur-Rahman *et al.*, 2002b
salignenamide A **(32)**	 **32**	50.6	4.6	Atta-ur-Rahman *et al.*, 2002b
vaganine A **(33)**	 **33**	8.6	2.3	Atta-ur-Rahman *et al.*, 2002b

Table 2 Steroidal alkaloids as cholinesterase inhibitors (cont.)

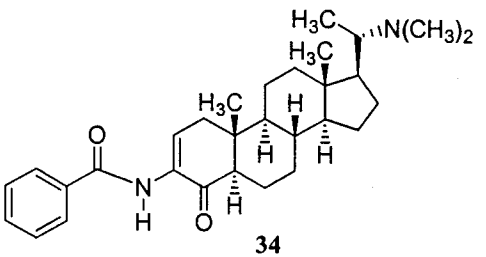
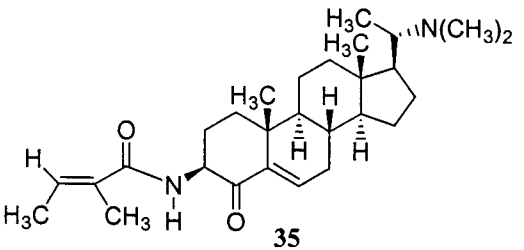
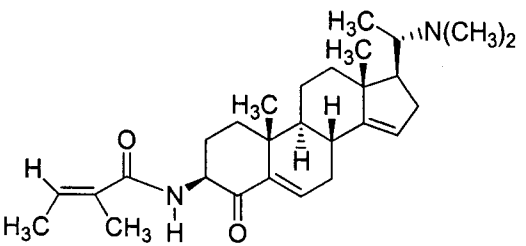
Name	Structure	IC_{50} (μM)		Reference
		AChE	BChE	
axillaridine A **(34)**	 **34**	5.2	2.5	Atta-ur-Rahman *et al.*, 2002b
sarsalignone **(35)**	 **35**	7.0	2.2	Atta-ur-Rahman *et al.*, 2002b
sarsalignenone **(36)**	 **36**	5.8	4.3	Atta-ur-Rahman *et al.*, 2002b

Table 2 Steroidal alkaloids as cholinesterase inhibitors (cont.)

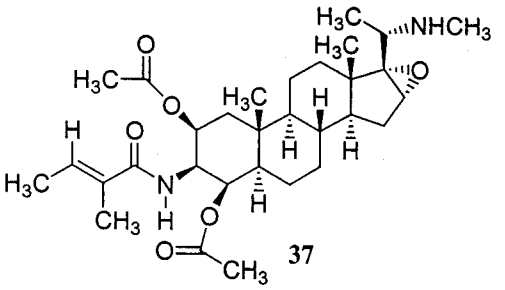
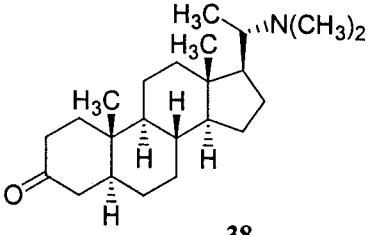
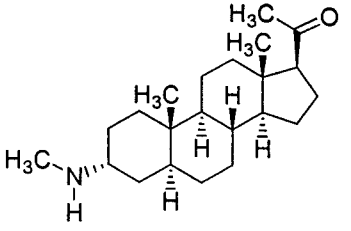
Name	Structure	IC_{50} (μM)		Reference
		AChE	BChE	
epoxynepapakistamine-A **(37)**	 **37**	>200.0	77.4	Kalauni *et al.*, 2002
funtumafrine **(38)**	 **38**	45.8	6.6	Kalauni *et al.*, 2002
N-methylfuntumine **(39)**	 **39**	97.6	12.7	Kalauni *et al.*, 2002

Table 2 Steroidal alkaloids as cholinesterase inhibitors (cont.)

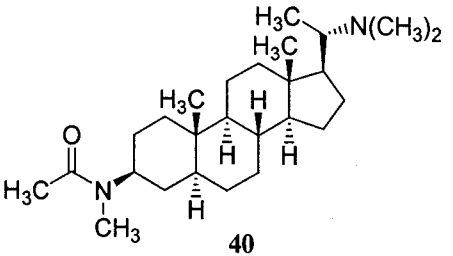
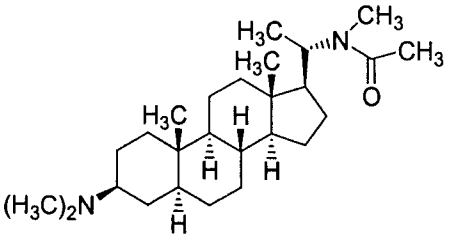
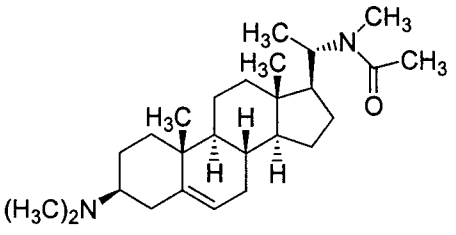
Name	Structure	IC_{50} (μM)		Reference
		AChE	BChE	
isosarcodine **(40)**	 **40**	10.3	1.9	Khalid *et al.*,2004b
sarcodine **(41)**	 **41**	49.8	18.3	Khalid *et al.*,2004b
sarcocine **(42)**	 **42**	20.0	3.9	Khalid *et al.*,2004b

Table 2 Steroidal alkaloids as cholinesterase inhibitors (cont.)

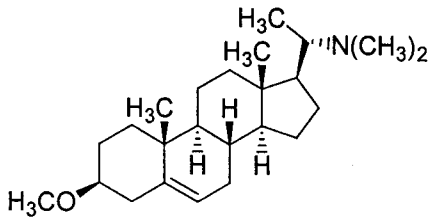
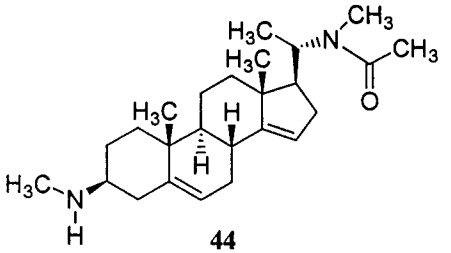
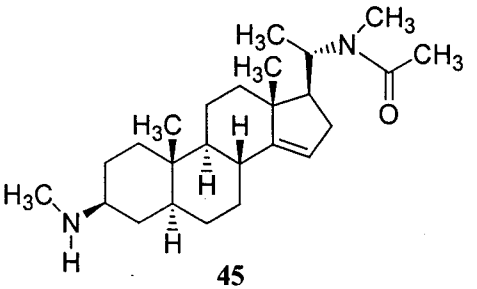
Name	Structure	IC_{50} (μM)		Reference
		AChE	BChE	
alkaloid-C **(43)**	 **43**	42.2	22.1	Khalid *et al.*,2004b
5,14-dehydro-3-*N*-demethylsaracodine **(44)**	 **44**	>200.0	25.0	Atta-ur-Rahman *et al.*, 2004a
14-dehydro-3-*N*-demethylsaracodine **(45)**	 **45**	183.1	10.1	Atta-ur-Rahman *et al.*, 2004a

Table 2 Steroidal alkaloids as cholinesterase inhibitors (cont.)

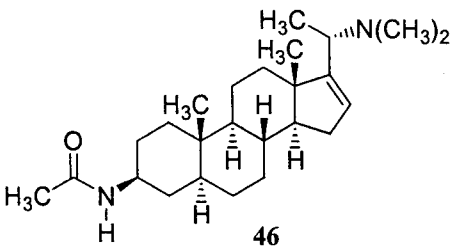
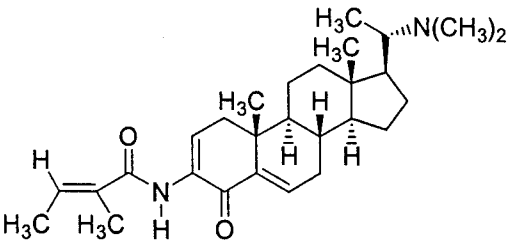
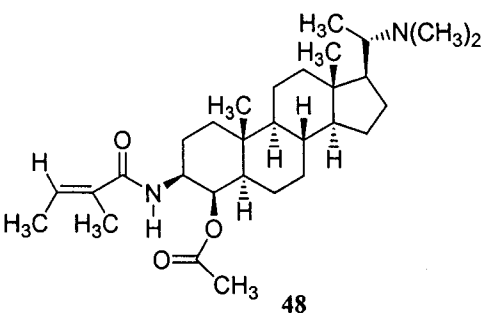
Name	Structure	IC_{50} (μM)		Reference
		AChE	BChE	
16-dehydrosarcorine **(46)**	 **46**	12.5	4.0	Atta-ur-Rahman *et al.*, 2004a
2,3-dehydrosarsalignone **(47)**	 **47**	7.0	32.2	Atta-ur-Rahman *et al.*, 2004a
sarcovagenine-C **(48)**	 **48**	187.8	1.5	Atta-ur-Rahman *et al.*, 2004a

Table 2 Steroidal alkaloids as cholinesterase inhibitors (cont.)

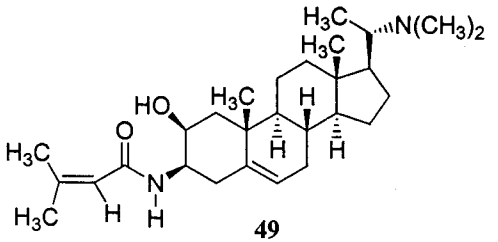
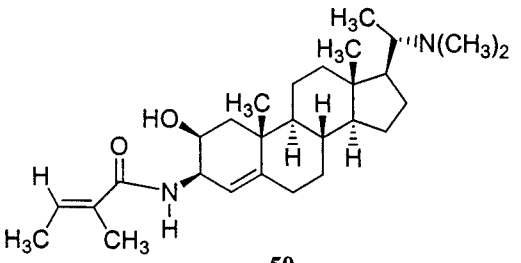
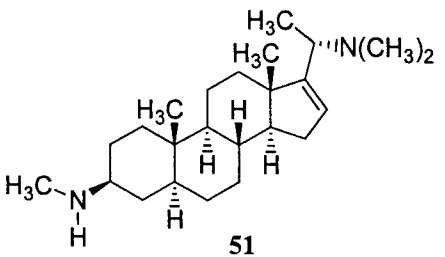
Name	Structure	IC_{50} (μM)		Reference
		AChE	BChE	
salignarine-C **(49)**	 **49**	19.7	1.3	Atta-ur-Rahman *et al.*, 2004a
2-hydroxysalignarine-E **(50)**	 **50**	16.0	6.9	Atta-ur-Rahman *et al.*, 2004b
5,6-dihydrosarconidine **(51)**	 **51**	20.3	1.9	Atta-ur-Rahman *et al.*, 2004b

Table 2 Steroidal alkaloids as cholinesterase inhibitors (cont.)

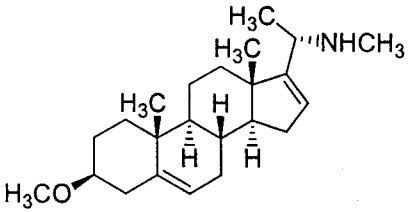
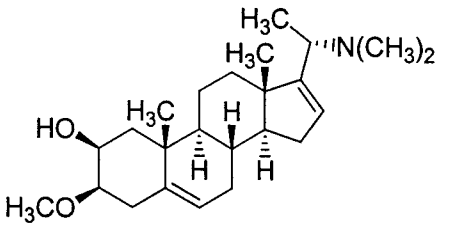
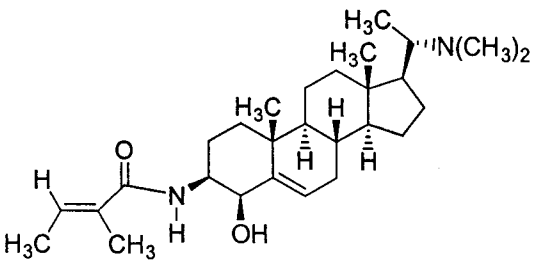
Name	Structure	IC_{50} (μM)		Reference
		AChE	BChE	
salignamine **(52)**	 **52**	249.0	25.7	Atta-ur-Rahman *et al.*, 2004bb
2-hydroxysalignamine **(53)**	 **53**	82.5	21.0	Atta-ur-Rahman *et al.*, 2004b
salignarine-F **(54)**	 **54**	30.2	1.9	Atta-ur-Rahman *et al.*, 2004b

Table 2 Steroidal alkaloids as cholinesterase inhibitors (cont.)

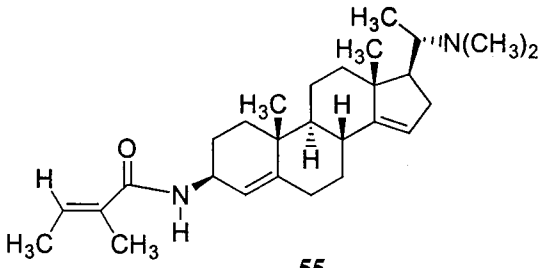
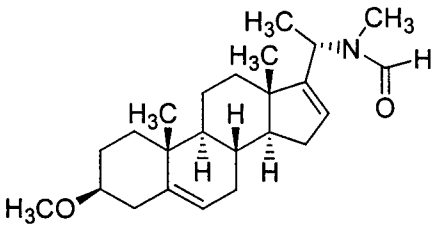
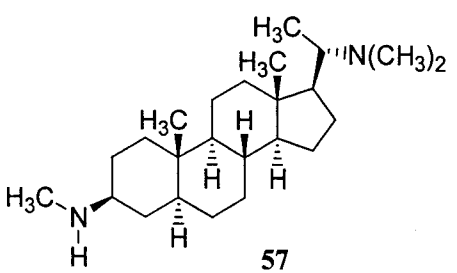
Name	Structure	IC_{50} (μM)		Reference
		AChE	BChE	
salonine-C **(55)**	 **55**	7.8	32.2	Atta-ur-Rahman *et al.*, 2004b
N-[formyl (methyl)amino] salonine-B **(56)**	 **56**	48.6	10.5	Atta-ur-Rahman *et al.*, 2004b
dictyophlebine **(57)**	 **57**	6.2	3.7	Atta-ur-Rahman *et al.*, 2004b

Table 2 Steroidal alkaloids as cholinesterase inhibitors (cont.)

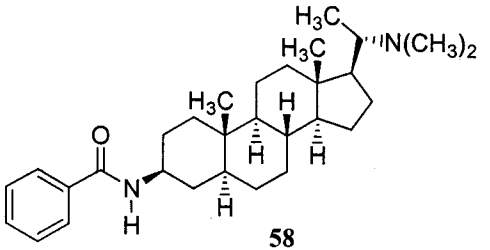
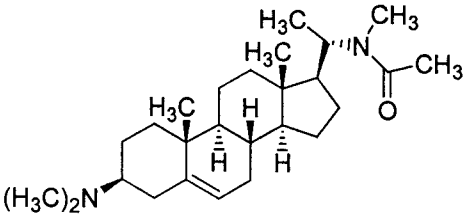
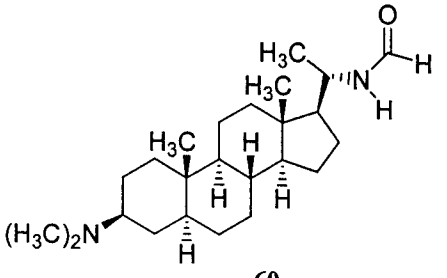
Name	Structure	IC_{50} (μM)		Reference
		AChE	BChE	
epipachysamine-D **(58)**	 **58**	28.9	2.8	Atta-ur-Rahman *et al.*, 2004b
saracosine **(59)**	 **59**	20.0	3.9	Atta-ur-Rahman *et al.*, 2004b
iso-*N*-formylchonemorphine **(60)**	 **60**	6.4	4.1	Atta-ur-Rahman *et al.*, 2004b

Table 2 Steroidal alkaloids as cholinesterase inhibitors (cont.)

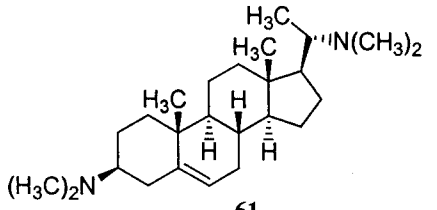
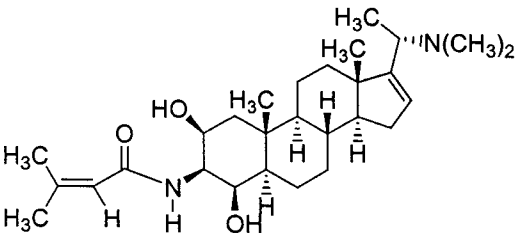
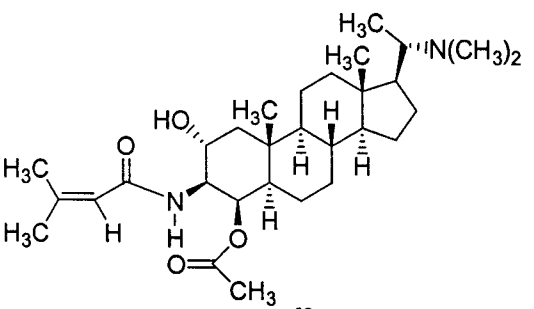
Name	Structure	IC_{50} (μM)		Reference
		AChE	BChE	
sarcodinine **(61)**	 **61**	40.0	12.5	Atta-ur-Rahman *et al.*, 2004b
hookerianamide A **(62)**	 **62**	82.7	200.0	Choudhary *et al.*, 2004
hookerianamide B **(63)**	 **63**	26.4	0.8	Choudhary *et al.*, 2004

Table 2 Steroidal alkaloids as cholinesterase inhibitors (cont.)

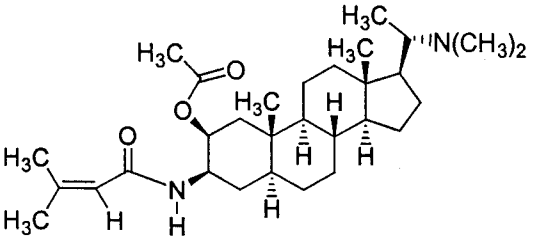
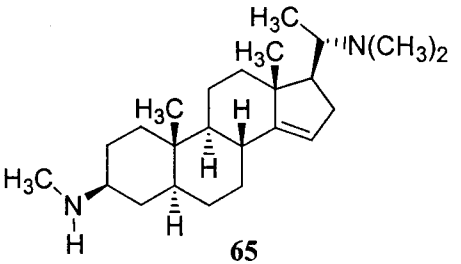
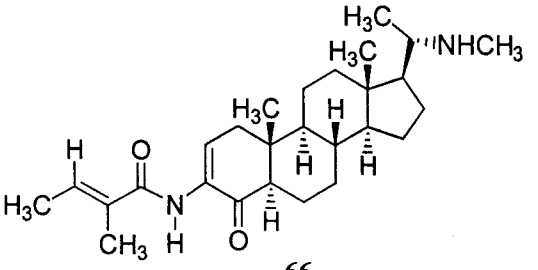
Name	Structure	IC_{50} (μM)		Reference
		AChE	BChE	
hookerianamide C **(64)**	 **64**	23.2	0.6	Choudhary *et al.*, 2004
hookerianamine A **(65)**	 **65**	18.9	0.9	Choudhary *et al.*, 2004
phulchowkiamide A **(66)**	 **66**	0.5	0.4	Choudhary *et al.*, 2004

Table 2 Steroidal alkaloids as cholinesterase inhibitors (cont.)

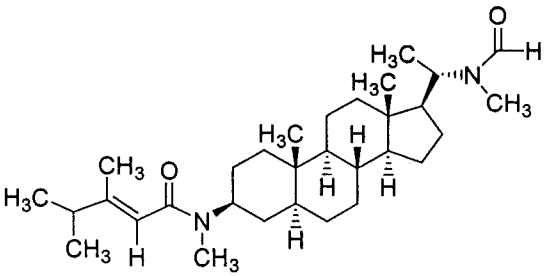
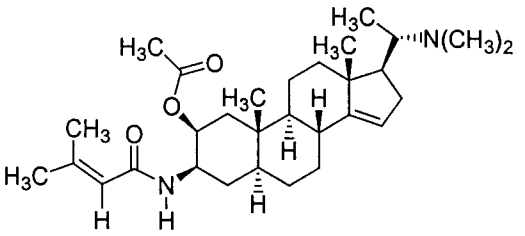
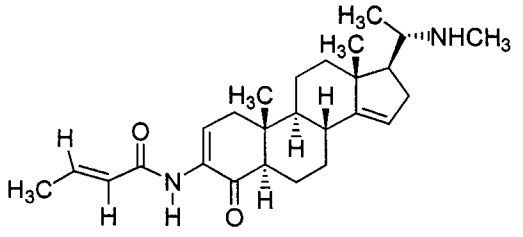
Name	Structure	IC_{50} (μM)		Reference
		AChE	BChE	
hookerianamide-D **(67)**	 **67**	59.0	100.2	Choudhary *et al.*, 2005
hookerianamide-E **(68)**	 **68**	15.9	6.0	Choudhary *et al.*, 2005
hookerianamide-F **(69)**	 **69**	1.6	7.2	Choudhary *et al.*, 2005

Table 2 Steroidal alkaloids as cholinesterase inhibitors (cont.)

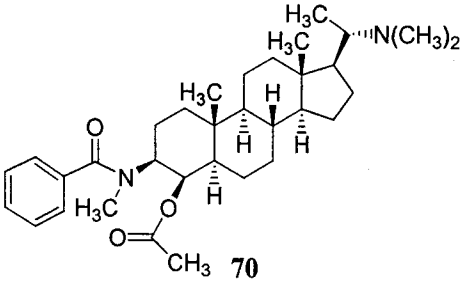
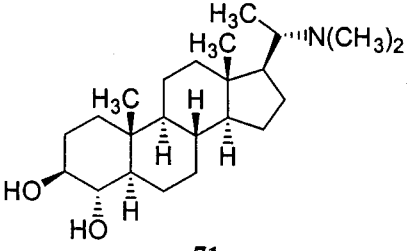
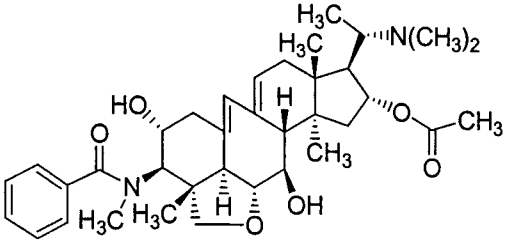
Name	Structure	IC_{50} (μM)		Reference
		AChE	BChE	
hookerianamide-G (**70**)	 **70**	11.4	1.5	Choudhary *et al.*, 2005
terminaline (**71**)	 **71**	113.1	0.6	Choudhary *et al.*, 2005
6-*O*-buxafurandiene (**72**)	 **72**	17.0	-	Babar *et al.*, 2006

Table 2 Steroidal alkaloids as cholinesterase inhibitors (cont.)

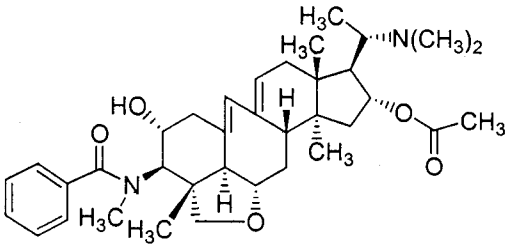
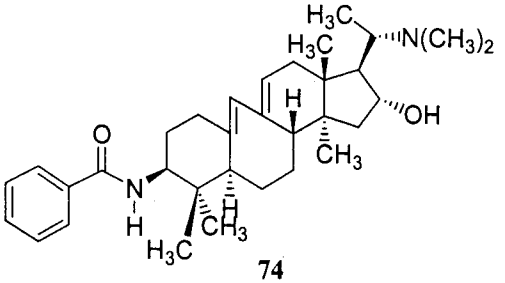
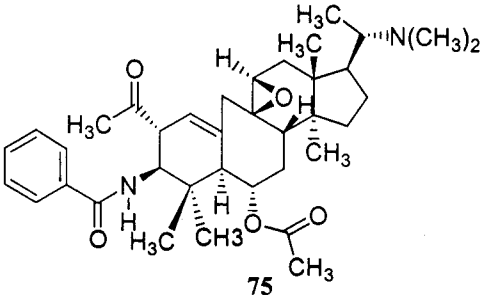
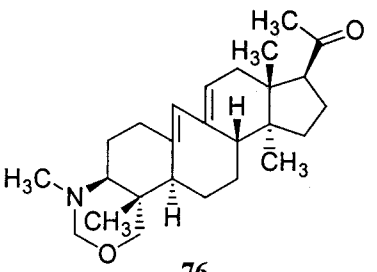
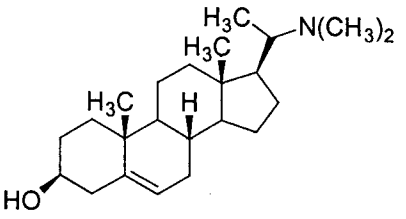
Name	Structure	IC_{50} (μM)		Reference
		AChE	BChE	
7-deoxy-6-*O*-buxafurandiene **(73)**	 **73**	13.0	-	Babar *et al.*, 2006
benzoylbuxidienine **(74)**	 **74**	35.0	-	Babar *et al.*, 2006
buxapapillnine **(75)**	 **75**	80.0	-	Babar *et al.*, 2006

Table 2 Steroidal alkaloids as cholinesterase inhibitors (cont.)

Name	Structure	IC_{50} (μM)		Reference
		AChE	BChE	
buxaquamarine **(76)**	 **76**	76.0	-	Babar *et al.*, 2006
irehine **(77)**	 **77**	100.0	-	Babar *et al.*, 2006

1.4 The sponge *Corticium* sp.

1.4.1 Taxonomy of *Corticium* sp.

The sponge *Corticium* sp. belongs to the family Plakinidae. The characterization of this genus are as followed; thinly encrusting, contractile surface; spiculation exclusively tetractines of single size and candellabras, althrough spicules occasionally absent completely; aphodal choanocyte chambers (Hooper, 2000).

1.4.2 Compounds associated with sponges from the genus *Corticium*

Only handful of chemical investigations were carried out with the sponges of the genus *Corticium*. To our knowledge, only two major groups of secondary metabolites were reported. Apart from the pyridoacridine meridine (**78**, entry 1, Table 3), most compounds associated with the *Corticium* sponges are steroidal alkaloids of plakinamine family. The core structures of most steroidal alkaloids isolated from *Corticium* sponges are based on the stigmastane-type steroids, with certain exceptional cases, most of which possess two nitrogen atoms substituted primarily at C-3 and C-26. The chemical structures and biological activities of all the compounds from the *Corticium* are summarized in Table 3.

Table 3 Compounds isolated from sponges of the genus *Corticium*

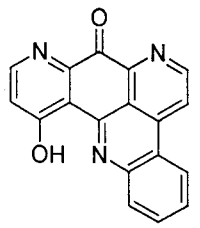
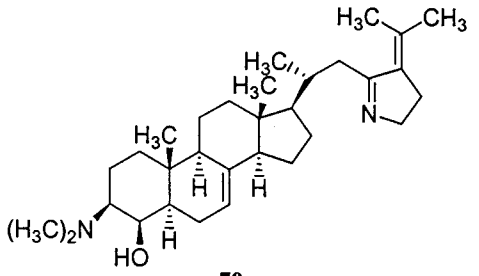
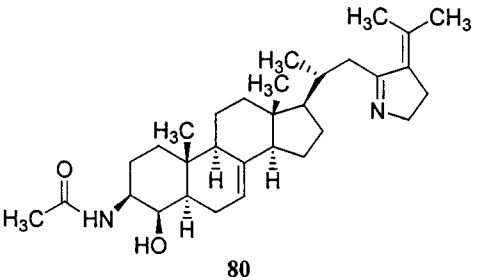
Name	Structure	Activities	Reference
meridine **(78)**	 **78**	antifungal (*Candida albicans*; MIC 0.2 μg/mL and *Cryptococcus neoformans*; MIC 0.8 μg/mL)	McCarthy *et al.*, 1992
lokysterolamine A **(79)**	 **79**	cytotoxicity (P- 388, IC_{50} 0.5 μg/mL; A-549, IC_{50} 0.5 μg/mL; HT-29, IC_{50} 1.0 μg/mL; MEL-28, IC_{50} 5 μg/mL); antimicrobial (*B. subtilis*, 19 mm; 50 μg/disc); antifungal (*C. albicans*, 11 mm; 50 μg/disc)	Jurek *et al.*, 1994
lokysterolamine B **(80)**	 **80**	cytotoxicity (P- 388, IC_{50} 1.0 μg/mL; A-549, IC_{50} 0.5 μg/mL; HT-29, IC_{50} 1.0 μg/mL; MEL-28, IC_{50} >2 μg/mL); antimicrobial (*B. subtilis*, 8 mm; 50 μg/disc)	Jurek *et al.*, 1994

Table 3 Compounds isolated from sponges of the genus *Corticium* (cont.)

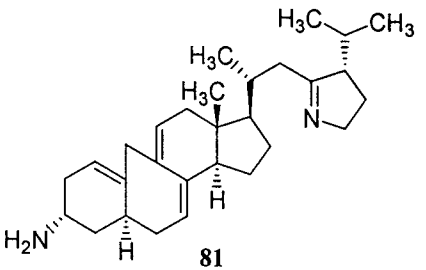
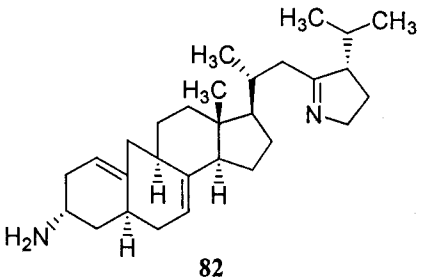
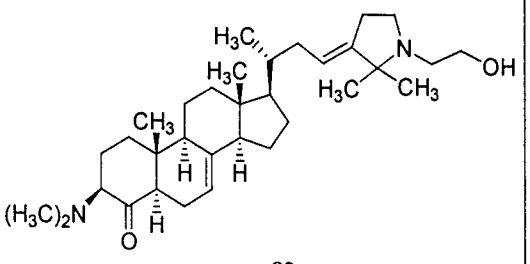
Name	Structure	Activities	Reference
3α-amino-23, 29-imino-β (9a)-homo-19-nor-5α-stigmasta-1(10),7,9,(11), 23(*N*)-tetraene **(81)**	 **81**	no reported activity available	De Marino *et al.*, 1998
3α-amino-23,29-imino-β (9a)-homo-19-nor-5α-stigmasta-1(10),7,23,(11), 23(*N*)-triene **(82)**	 **82**	no reported activity available	De Marino *et al.*, 1998
plakinamine C **(83)**	 **83**	anti-HIV (inhibit *syncytia* formation after HIV infection of MT_4 cell line at 0.1μg/mL)	De Marino *et al.*, 1999

Table 3 Compounds isolated from sponges of the genus *Corticium* (cont.)

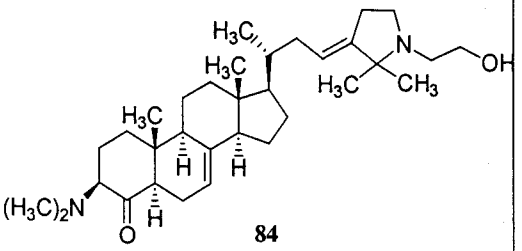
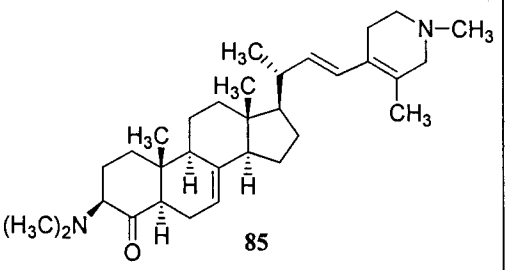
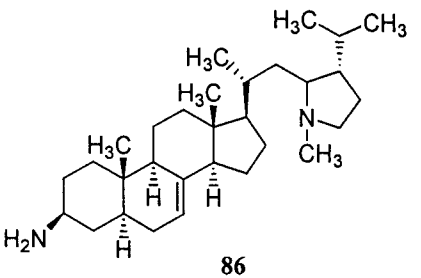
Name	Structure	Activities	Reference
plakinamine D **(84)**	 **84**	cytotoxicity (NSCLC-N6, IC_{50} 3.3 μg/mL)	De Marino *et al.*, 1999
N, *N*- dimethyl-4-oxo-3-*epi*-plakinamine B **(85)**	 **85**	cytotoxicity (NSCLC-N6, IC_{50} 3.6 μg/mL)	De Marino *et al.*, 1999
25,26-dihydro-plakinamine A **(86)**	 **86**	anti-HIV activity (inhibit *syncytia* formation after HIV infection of MT_4 cell line at 0.05 μg/mL); cytotoxicity (NSCLC-N6, IC_{50} 5.7 μg/mL)	De Marino *et al.*, 1999

Table 3 Compounds isolated from sponges of the genus *Corticium* (cont.)

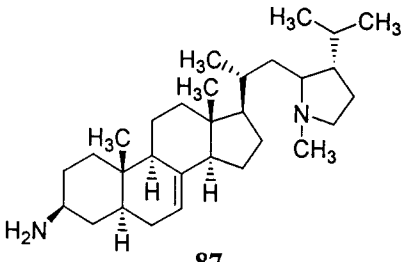
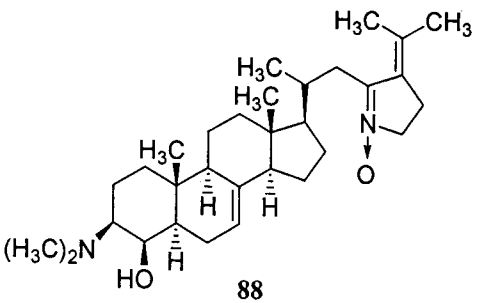
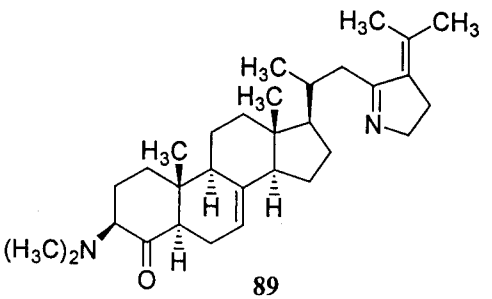
Name	Structure	Activities	Reference
23-(*N*-methyl)-plakinamine **A (87)**	 **87**	anti-HIV activity (inhibit *syncytia* formation after HIV infection of MT_4 cell line at 0.1 μg/mL); cytotoxicity (NSCLC-N6, IC_{50} 4.9 μg/mL)	De Marino *et al.*, 1999
plakinamine E **(88)**	 **88**	cytotoxicity (K562, IC_{50} 0.2 μg/mL); antifungal (*C. albicans*, 12 mm; 25 μg/disc); DNA- and RNA- cleaving activities at 10 μg/20mL	Lee *et al.*, 2001
plakinamine F **(89)**	 **89**	cytotoxicity (K562, IC_{50} 1.3 μg/mL); antifungal (*C. albicans*, 8 mm; 25 μg/disc)	Lee *et al.*, 2001

Table 3 Compounds isolated from sponges of the genus *Corticium* (cont.)

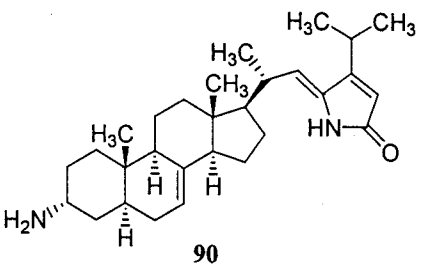
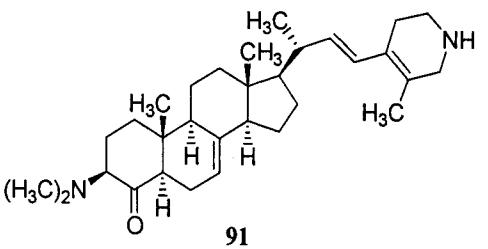
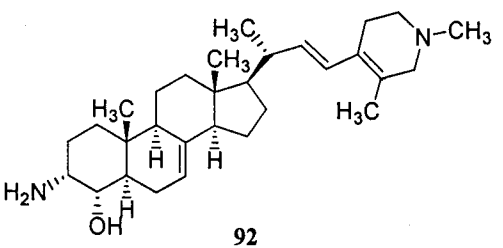
Name	Structure	Activities	Reference
plakinamine G **(90)**	 **90**	cytotoxicity (C6, IC_{50} 6.8 µg/mL)	Borbone *et al.*, 2002
plakinamine H **(91)**	 **91**	cytotoxicity (C6, IC_{50} 9.0 µg/mL; RAW 264, IC_{50} 61.0 µg/mL)	Borbone *et al.*, 2002
4α-hydroxydemethyl-plakinamine B **(92)**	 **92**	cytotoxicity (C6, IC_{50} 26.1 µg/mL; RAW 264, IC_{50} 16.2 µg/mL)	Borbone *et al.*, 2002

Table 3 Compounds isolated from sponges of the genus *Corticium* (cont.)

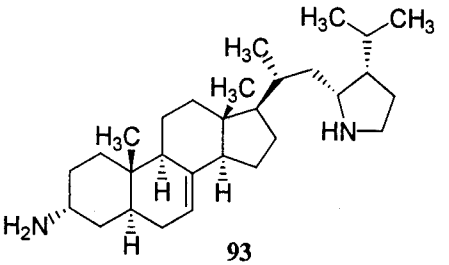
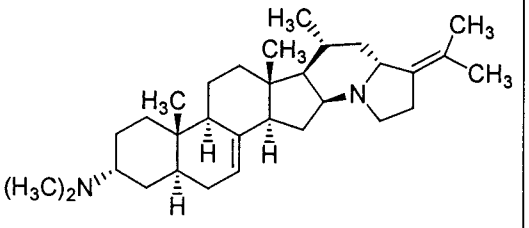
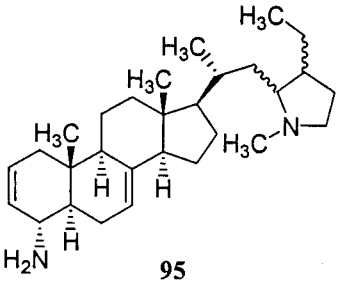
Name	Structure	Activities	Reference
tetrahydroplakinamine A **(93)**	 **93**	cytotoxicity (C6, IC_{50} 1.4 μg/mL)	Borbone *et al.*, 2002
plakinamine I **(94)**	 **94**	cytotoxicity (HCT-116, IC_{50} 10.6 μM)	Ridley and Faulkner, 2003
plakinamine J **(95)**	 **95**	cytotoxicity (HCT-116, IC_{50} 6.1 μM)	Ridley and Faulkner, 2003

Table 3 Compounds isolated from sponges of the genus *Corticium* (cont.)

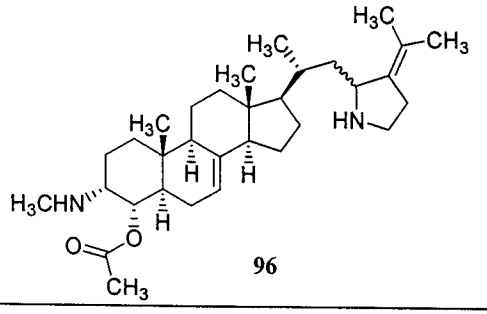
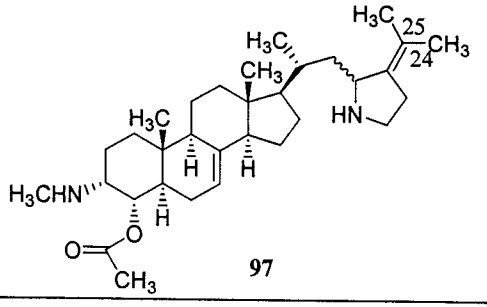
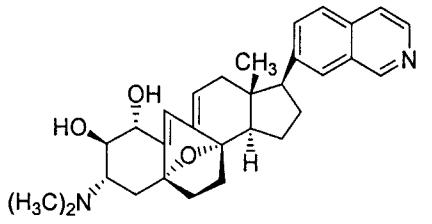
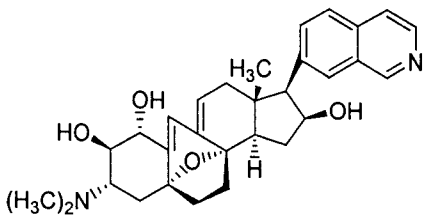
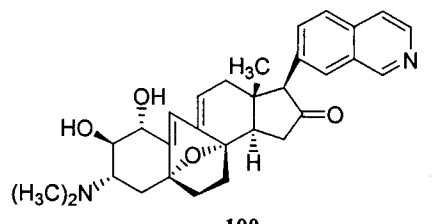
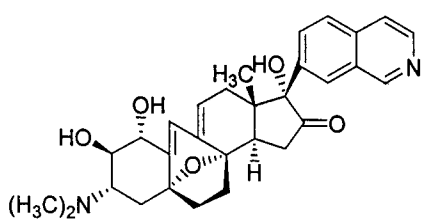
Name	Structure	Activities	Reference
plakinamine K **(96)**	 **96**	cytotoxicity (HCT-116, IC_{50} 1.4 μM)	Ridley and Faulkner, 2003
24,25-dihydroplakinamine K **(97)**	 **97**	cytotoxicity (HCT-116, IC_{50} 1.4 μM)	Ridley and Faulkner, 2003
cortistatin A **(98)**	 **98**	anti-proliferative activity (HUVECs, IC_{50} 0.0018 μM)	Aoki *et al.*, 2006

Table 3 Compounds isolated from sponges of the genus *Corticium* (cont.)

Name	Structure	Activities	Reference
cortistatin B **(99)**	 **99**	anti-proliferative activity (HUVECs, IC_{50} 1.1 μM)	Aoki *et al.*, 2006
cortistatin C **(100)**	 **100**	anti-proliferative activity (HUVECs, IC_{50} 0.019 μM)	Aoki *et al.*, 2006
cortistatin D **(101)**	 **101**	anti-proliferative activity (HUVECs, IC_{50} 0.15 μM)	Aoki *et al.*, 2006

1.5 Objectives

From our expedition to Koh-Tao, Surat Thani, we found that the crude extract of the Thai sponge, *Corticium* sp., showed a potent inhibitory activity against AChE. The MeOH- and CH_2Cl_2-extracts (0.1 mg/mL) showed strong inhibition against AChE at 95 and 86%, respectively.

The objectives of this project focus on the following:

1) to isolate the AChE-inhibitors from the MeOH- and CH_2Cl_2-extracts of the sponge, *Corticium* sp., using bioassay-guided fractionation,
2) to elucidate the structure of the isolated compounds, and
3) to determine the AChE inhibitory activity of the isolated compounds.

Especially, along with the ultimate goal to finding effective drugs for the treatment of AD that can complement the currently used remedy, we also wish to expand the results as one of the most effective exploitations of marine bio-resources from Thai territory waters.

CHAPTER 2

EXPERIMENTAL

2.1 General

Unless otherwise noted, all solvents for general purposes were commercial grade and were re-distilled prior to use. All preparative HPLC solvents were HPLC grade and were filtered through a 0.45-μm nylon membrane. This was degassed by submerging in an ultrasonic bath prior to use, then continually purged with helium throughout the operation. Thin-layer chromatography (TLC) was performed on Merck® pre-coated silica gel 60 F254 plates (0.20-mm thickness). Visualization was done by observation under UV light (254 nm), and by Dragendorff spraying reagent (orange spot on yellow background). Preparative TLC was carried out using in-house silica gel 60 GF254 plates (Merck®, 0.25-mm thickness). The size-exclusion chromatography was conducted on a column of Sephadex LH-20 (Pharmacia®), which was allowed to be saturated with eluting solvents as indicated for an overnight prior to use. Flash chromatography was carried out using Merck® silica gel 60 (particle size 0.04-0.06 mm, 230-400 mesh ASTM). HPLC was performed on a Water® 600E multisolvent delivery system, equipped with a Water® 484 tunable absorbance detector. This was connected to a Rheodyne® 7125 injector port.

Optical rotation was measured on a Perkin-Elmer 341 polarimeter. UV spectra were recorded on a Hewlett Peckard® 8452A diode array spectrophotometer (France). IR spectra were recorded on Jasco® IR-810 infrared spectrophotometer (Japan). LR and HR mass spectra were obtained from a Thermofinnigan® MAT 95 mass spectrometer (Germany). NMR spectra were recorded on an FT-NMR Varian Unity® Inova 500 spectrometer (Germany), at 500 MHz (for ^{1}H) and 125 MHz (for ^{13}C). The chemical shifts were reported on the δ scale relative to the solvent signals (7.15 ppm, residual C_6HD_5 for ^{1}H NMR; and 128 ppm, C_6D_6 for ^{13}C NMR).

2.2 Sponge material

The sponge *Corticium* sp. were collected using SCUBA at the depth of 18-20 m, from Koh-Tao, Surat Thani, Thailand (10°, 07.569′N, 99°, 48.665 ′E), in April 2003, and in April 2004. The specimens were all preserved in ice chest (0°C) immediately upon surfacing, then at -20°C once returned to laboratory until further investigation. Upon surfacing, the specimen appeared as a small flat colonial sponge (3- to 15-cm wide, 0.2- to 0.4-cm thick), with a leathery texture. The outer color was dark brownish grey, with paler grey color inside. The taxonomic identification was carried out by Dr. Somchai Bussarawit of Phuket Marine Biological Center, Phuket, Thailand, which belonged to the genus *Corticium* (Family Plakinidae, Order Homosclerophorida). The voucher specimen (PMBC21360) was deposited at Phuket Marine Biology Center, Phuket.

2.3 Bioactivity determination

2.3.1 Acetylcholinesterase inhibition activity

2.3.1.1 Microplate assay

The activity determination was kindly supported by Assoc. Prof. Dr. Kornkanok Ingkaninan of Department of Pharmaceutical Chemistry and Pharmacognosy, Faculty of Pharmaceutical Sciences, Naresuan University. The AChE inhibitory activity was measured by a protocol developed by Ellman *et al.* (1961; modified by Ingkaninan *et al.*, 2006). In brief, 125 µL of 3 mM 5,5'-dithiobis[2-nitrobenzoic acid] (DTNB) (Sigma®), 25 µL of 15 mM acetylthioiodide (ATCI) (Sigma®), 50 µL of buffer (Tris-HCl pH 8.0), and 25 µL of sample (triplicate) dissolved in buffer, supplemented with not more than 10% methanol (final concentration), were added to each well, followed by 25 µL of 0.28 U/mL AChE (electric eel, type VI-S, E.3.1.1.7, Sigma®). The microplate was then read at 405 nm every 5 s for 2 min using a CERES UV 900C microplate reader (Bio-Tek Instrument, USA). Enzyme activity was calculated as a percentage of the reaction velocities compared to that of the assay using buffer as negative control. Inhibitory activity was calculated from 100 subtracted by the percentage of enzyme activity. The IC_{50}, K_m and V_{max} were analyzed using the software package Prism (Graph Pad Inc, San Diego, CA, USA). The inhibitory activity was referred to that of galantamine (Sigma®) as positive standard.

2.3.1.2 Thin-layer chromatography (TLC) assay

The bioassay-detected TLC for AChE inhibition was modified from Rhee *et al.* (2001). The TLC protocol was performed as readily stated. Upon developing the tested sample-applied TLC, the plate was dried at an ambient temperature, then sprayed with 30 mM ATCI followed by 20 mM DTNB. The plate was dried at an ambient temperature for 45 min, then sprayed with 10.17 U/mL AChE. After 20 min, the plate was observed under day light. A positive result was referred to a colorless spot on the yellow background.

2.3.2 Cytotoxic activity

The determination was kindly supported by Assist. Prof. Supreeya Yuenyongsawad of Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University. The targeted cell lines were MCF-7 (breast adenocarcinoma), Hela (human cervical cancer), HT-29 (colon cancer) and KB (human oral cancer). The sulphorhodamine B (SRB) assay protocol was modified from Skehan *et al.* (1990).

Briefly described, 100 μL of monolayered culture of each cell line in a 96-well microliter plate (2×10^3 cells/well) was treated with an appropriate dilution of tested sample, each dissolved in culture medium (10% newborn calf serum; Biowhittaker), supplemented with penicillin (100 U), streptomycin (100 μg/mL) and amphotericin B (25 μg/mL). The plates were incubated for 72 hours. At the end of each exposure time, the medium was removed. The wells were washed with medium, and 200 μL of fresh medium were added to each well. The plates were incubated for an additional 72-hour period, after which time cells were fixed with 100 μL of ice-cold 40% trichloroacetic acid (Aldrich Chemical). After a 1-hour incubation (4°C), each well was washed five times with tap water. SRB solution (0.4% w/v in 1% acetic acid, 50 μL, Sigma®) was added, and left in contact with the cells for 30 min. After removing the dye, the plate was dried. 100 μL of 10 mM Tris base (Sigma®) was then added, the plates were shaken gently for 20 minutes on a gyratory shaker. The resulting pink color was detected at 492 nm on (Bio-TEK Instrument, USA). The activity was reported as cell mortality percentage at an indicated concentration, and was referred to that of standard camptothecin (Aldrich®).

2.4 Isolation and purification

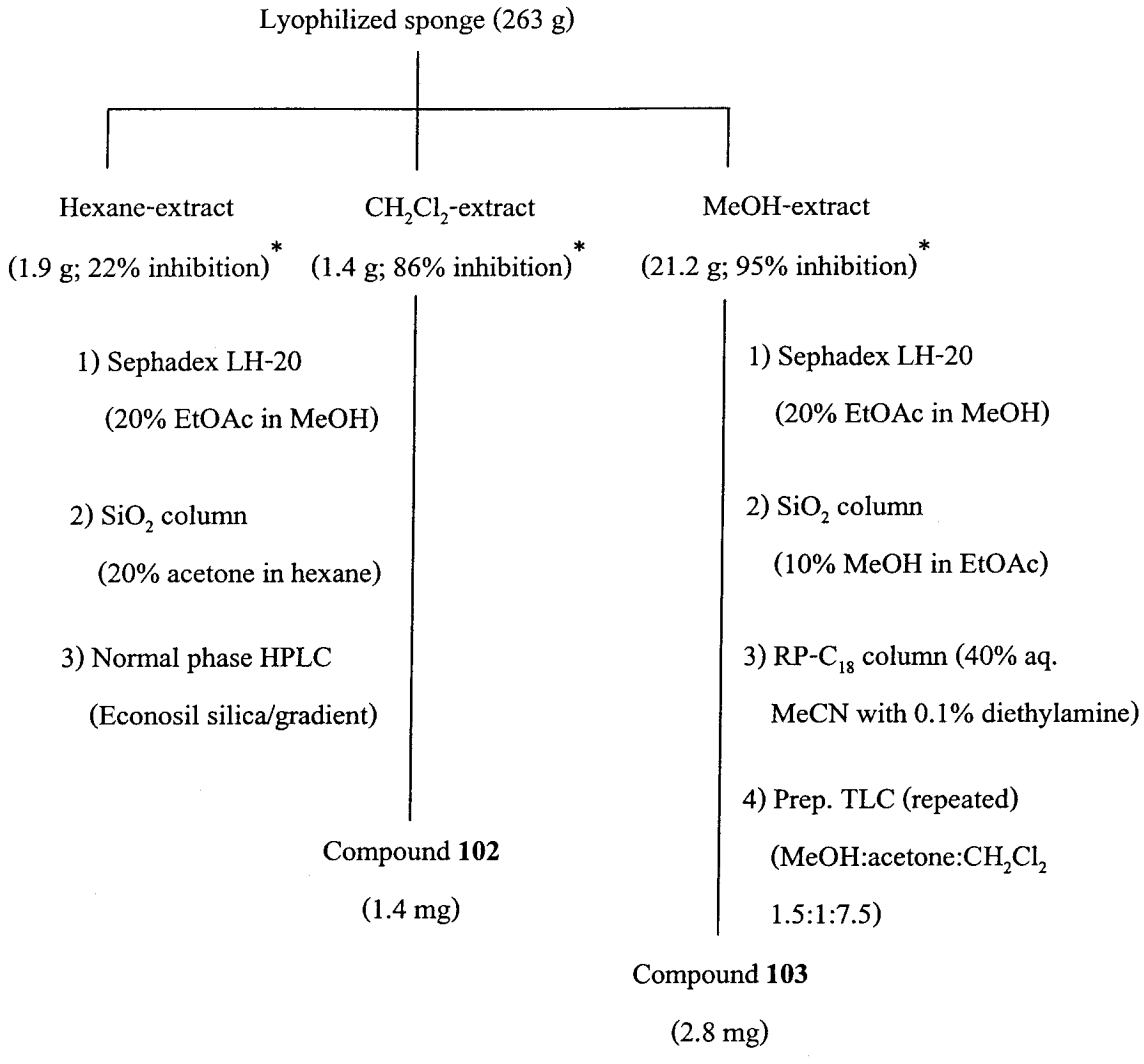
The sponge *Corticium* sp. was collected in April 2003 and April 2004. The freeze-dried specimens (263 g) were consecutively and exhaustively extracted with hexane, CH_2Cl_2 and MeOH (3×2 L, each) to yield the corresponding extracts (1.9 g, 0.7%; 1.4 g, 0.5%; 21.2 g; 8.1%, respectively). The isolation protocol as followed are summarized in Scheme 1.

The CH_2Cl_2-extract (86% inhibition against AChE, 0.1 mg/mL) was subjected to a Sephadex LH-20 column (120×2.54 cm, 20% EtOAc in MeOH, 800 mL). After fractional pool, three major active fractions (79%, 82% and 92% inhibition against AChE, 0.1 mg/mL) was obtained. The last active fraction (181 mg) was further purified over a SiO_2 column (15×6.35 cm, 20% acetone in hexane, 1 L), followed by a SiO_2 HPLC column (Econosil® semi-preparative, 10 μ, 250×7.0 mm; gradient 5 to 10% *i*-PrOH in hexane in 20 min, 1.5 mL/min, 254 nm). Compound **102** (1.4 mg) eluted at t_R of 17 min. It was identified as a trihydroxy sterol.

The MeOH-extract was fractionated with the chromatographic technique using a Sephadex LH-20 column (120×2.54 cm, 20% EtOAc in MeOH, 2 L). An active fraction (1.6 g), monitored by AChE inhibitory assay (96% inhibition at 0.1 mg/mL) was further purified as followed, SiO_2 (15×6.35 cm, 10% MeOH in EtOAc, 600 mL), RP-C_{18}column (15×2.54 cm, 40% aq. MeCN with 0.1% diethylamine, 1 L), repeated preparative TLC (MeOH:acetone:CH_2Cl_2 1.5:1:7.5; eluted with 20% CH_2Cl_2 in MeOH). Compound **103** was obtained and identified as 4-acetoxy-plakinamine B (2.8 mg).

2.5 Physical properties of isolated compound

4-acetoxy-plakinamine B (103): viscous yellow liquid; $[\alpha]_D$ +21.9 ° (*c* 0.0014, MeOH); IR (thin film) ν_{max} 3400, 2925, 1740, 1240 cm^{-1}; UV (MeOH) λ_{max} (log ε) 242 (4.29) nm; ^1H and ^{13}C NMR (500 MHz for ^1H and 125 MHz for ^{13}C, C_6D_6) see Table 2.2; EIMS *m/z* (relative intensity) 508 ($[M]^+$, 63), 493 (100), 433 (10), 164 (36), 136 (41); HREIMS *m/z* 508.4001 (calcd for $C_{33}H_{52}O_2N_2$, 508.4029).



* % inhibition of AChE at 0.1 mg/mL

Scheme 1 Isolation protocol for the sponge, *Corticium* sp.

CHAPTER 3

RESULTS AND DISCUSSION

As part of our ongoing investigation of bioactive metabolites from Thai marine invertebrates, the MeOH-extract of a sponge collected from the vicinity of Koh Tao, Surat-Thani, was found to exhibit potent AChE inhibiting activity at 0.1 mg/mL (>95% inhibition). The sponge, later identified to belong to the genus *Corticium*, was subjected to the further chemical investigation for the bioactive components. The bioassay-guided purification of the Thai sponge *Corticium* sp. led to the isolation of a new steroidal alkaloid **(103)**, along with an unidentified sterol **(102)**. Compound **103** was submitted to the AChE inhibiting activity determination, and a potent enzyme-inhibiting activity (IC_{50} 3.75±1.69 μM) was observed.

3.1 Isolation of the acetylcholinesterase-inhibiting compounds from the sponge *Corticium* sp.

The sponge *Corticium* sp. was collected at the depth of 18-20 m from Koh-Tao, Surat Thani, Thailand, in April 2003 and in April 2004. The lyophilized sponge (263 g) was consecutively and exhaustively macerated in a series of solvents, started from hexane, to CH_2Cl_2, and to MeOH. The CH_2Cl_2-extract (86% inhibition against AChE, 0.1 mg/mL) was fractionated and purified by chromatographic technique, and a trihydroxy sterol **(102)** was obtained (1.4 mg). The MeOH-extract, which showed the most potent AChE inhibiting activity (95% inhibition against AChE, 0.1 mg/mL), was subjected to the further chromatographic separation, and a new active compound (IC_{50} 3.75±1.69 μM), later identified as 4-acetoxy-plakinamine B **(103)**, was obtained (2.8 mg).

It should be mentioned here that, in fact, more minor active components, as observable by means of TLC-enzyme inhibiting assay, are still present in the extract. However, due to the peculiarity in the solubility and interaction between the active compounds and chromatographic packing materials, most of the active components were unable to be obtained. Presumably, the interaction between acidic SiO_2 and basic amino nitrogen caused the strong entrapment of compounds, thus leading to the major loss. For the remaining fractions, although

certain components were isolated, most were obtained in an amount so small that the further structure elucidation was unable to be performed.

3.2 The structure elucidation of the isolated compounds

The isolation of sponge *Corticium* sp. yielded two steroidal compounds, an unidentified trihydroxy sterol and a new steroidal alkaloid. This section of the report will first discuss the elucidation for the steroidal alkaloid **(103)**, followed by that for the trihydroxy sterol **(102)**.

3.2.1 The structure elucidation of compound 103

Compound **103** was obtained as a viscous yellow liquid (2.8 mg) from the MeOH-extract using chromatographic isolation, including Sephadex LH-20 (20% EtOAc in MeOH), SiO_2 column (10% MeOH in EtOAc), SiO_2-bonded phase C-18 column (40% aq. MeCN with 0.1% diethylamine), and repeated preparative SiO_2 TLC (MeOH:acetone:CH_2Cl_2 1.5:1:7.5).

Compound **103** has a molecular formula of $C_{33}H_{52}N_2O_2$ as established by means of the EI mass spectrum, which shows a molecular peak at *m/z* 508 ($[M]^+$), and of its 33 carbon signals observable in the ^{13}C NMR spectrum (125 MHz, C_6D_6; Figure 2). This molecular formula was supported by the HR-EIMS spectrum, which showed a molecular peak at *m/z* 508.4001 (calcd for $C_{33}H_{52}N_2O_2$ 508.4029). The proposed molecular formula requ ires the unsaturation degrees of 9. The ^{13}C NMR spectrum indicated the presence of one carbonyl carbon and three double bonds; five ring systems were therefore required for **103**. The IR spectrum showed an absorption at ν_{max} 1740 cm^{-1}, confirming the presence of the carbonyl functionality. The UV spectrum showed the maximal absorption at λ_{max} 242 nm.

The ^1H NMR spectrum of **103** (500 MHz, C_6D_6; Figure 3) showed three singlet methyls (δ 0.64, H-18; 1.14, H-19; and 1.60, H-26), one doublet methyl (δ 1.15, H-21), and a series of overlapped multiplet methylenes and methines (δ 1.0-2.0), all of which are characteristic to the steroid nucleus. This corresponded well with a series of methine and methylene aliphatic carbons resonating in a high-field region (δ 20-55) as observed in DEPT spectra.

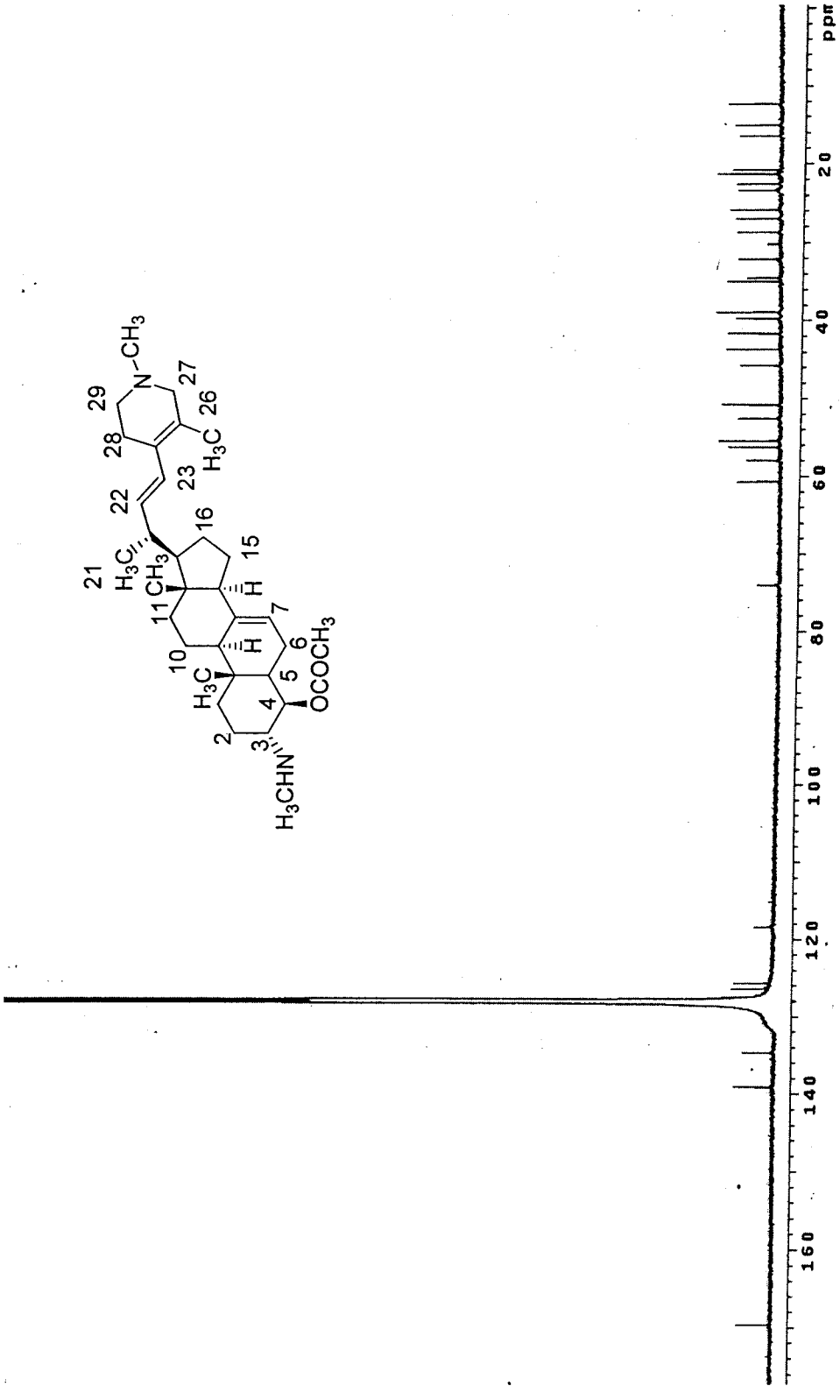


Figure 2 ^{13}C NMR spectrum of **103** (125 MHz, C_6D_6)

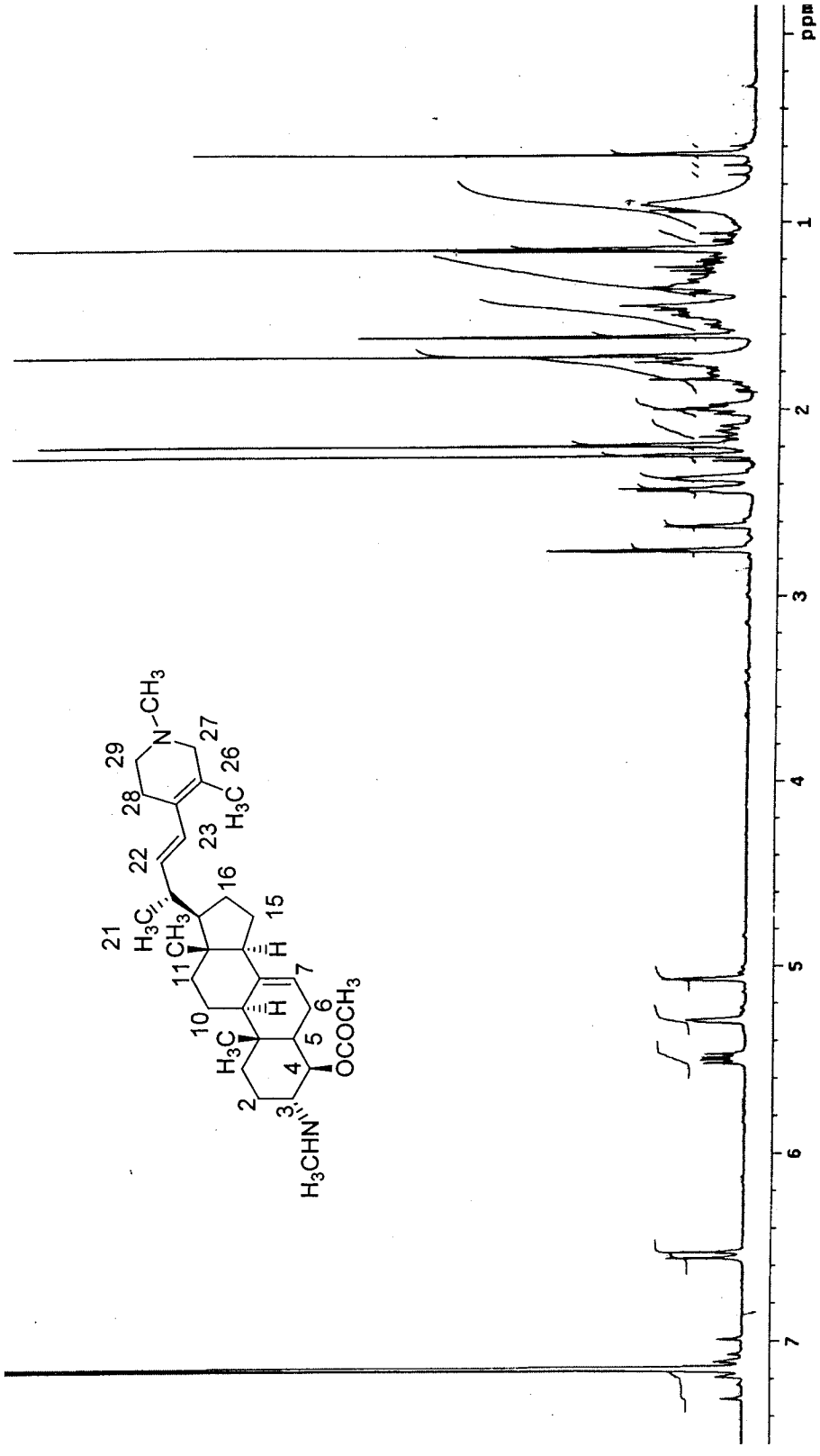
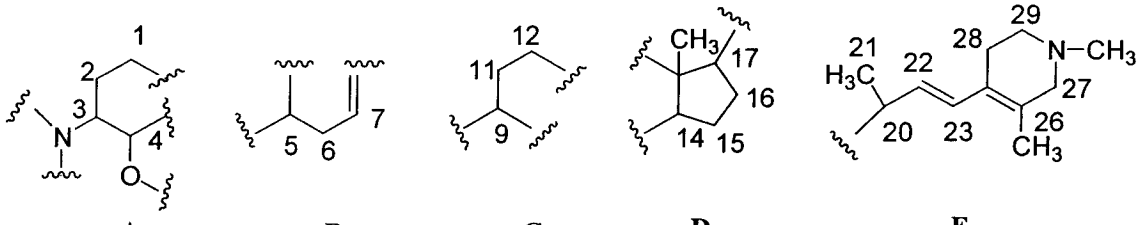


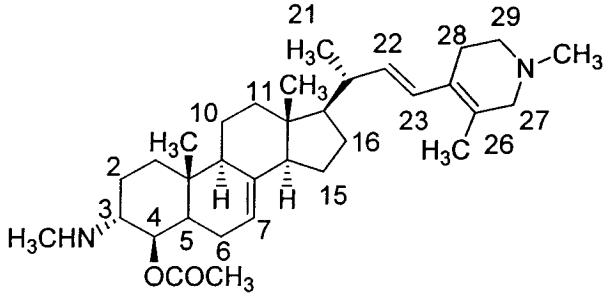
Figure 3 ^{1}H NMR spectrum of **103** (500 MHz, C_6D_6)

Interpretation of the ^{1}H-^{1}H correlations observable in the ^{1}H,^{1}H-COSY spectrum led to five partial structures of the steroid skeleton. These included fragment A at δ 1.45 (m, H-1), 1.36 (m, H-2), 2.62 (br d, J = 2.6 Hz, H-3), and 5.07 (br s, H-4); fragment B at δ 1.20 (m, H-5), 2.10 (m, H-6) and 5.23 (br s, H-7); fragment C at δ 1.74 (m, H-9), 1.83 (m, H-11) and 2.00 (m, H-12); fragment D at δ 1.80 (m, H-14), 1.78 (m, H-15), 1.50 (m, H-16) and 1.25 (m, H-17); and fragment E at δ 2.18 (m, H-20), 1.15 (d, J = 5.8 Hz, H-21), 5.50 (dd, J = 15.3, 8.9 Hz, H-22), 6.53 (d, J = 15.3 Hz, H-23), 1.60 (s, H-26), 2.75 (br s, H-27), 2.37 (br s, H-28) and 2.43 (m, H-29) as shown.



A B C D E

Connecting the five fragments by means of HMBC spectral analysis (Table 4) led to a steroid skeleton of a stigmastane type possessing an olefinic moiety on C-7, and a tetrahydropyridinyl group as terminal end on the C-17 side chain. Two additional singlet methyls at δ_H 2.25 and δ_H 1.72 were assigned to belong to a methyl amino and an acetoxy groups on C-3 and C-4, respectively, according to their corresponding HMBC correlations. The structure of **103** was therefore proposed as a new acetoxy analogue of stigmastane-type steroidal alkaloids, designated as 4-acetoxy-plakinamine B. The NMR spectral data of **103** were summarized in Table 4.



103

Table 4 NMR data of **103** (500 MHz for ^{1}H and 125 MHz for ^{13}C; C_6D_6)

Position	δ_H (mult.; *J* in Hz)	δ_C (mult.)	HMBC correlation (C ⟶ H)
1	1.45 (m, 2H)	32.1 (CH_2)	H-19
2	1.36 (m, 2H)	22.5 (CH_2)	H-1
3	2.62 (br d; 2.6, 1H)	57.9 (CH)	
4	5.07 (br s, 1H)	74.0 (CH)	
5	1.20 (m, 1H)	38.8 (CH)	H-19
6	2.10 (m, 2H)	25.8 (CH_2)	H-7
7	5.23 (br s, 1H)	118.4 (CH)	
8	-	139.1 (C)	
9	1.74 (m, 1H)	50.8 (CH)	H-7, H-19
10	-	34.4 (C)	H-19
11	1.83 (m, 2H)	23.3 (CH_2)	
12	2.00 (m, 2H)	39.7 (CH_2)	H-18
13	-	43.6 (C)	H-16, H-18
14	1.80 (m, 1H)	55.4 (CH)	H-18
15	1.78 (m, 2H)	28.8 (CH_2)	H-17
16	1.50 (m, 2H)	21.2 (CH_2)	
17	1.25 (m, 1H)	56.2 (CH)	H-21
18	0.64 (s, 3H)	12.3 (CH_3)	
19	1.14 (s, 3H)	15.1 (CH_3)	
20	2.18 (m, 1H)	41.6 (CH)	H-21, H-22, H-23
21	1.15 (d; 5.8, 3H)	21.3 (CH_3)	
22	5.50 (dd; 15.3, 8.9, 1H)	134.6 (CH)	H-21, H-27
23	6.53 (d; 15.3, 1H)	125.6 (CH)	
24	-	126.3 (C)	H-22, H-23, H-26, H-27, H-29
25	-	127.4 (C)	H-23, H-26, H-27

Table 4 (cont.)

Position	δ_H (mult.; J in Hz)	δ_C (mult.)	HMBC correlation (C ⟶ H)
26	1.60 (s, 3H)	16.4 (CH_3)	
27	2.75 (br s, 2H)	60.7 (CH_2)	H-26, H-29, N- CH_3
28	2.37 (br s, 2H)	27.0 (CH_2)	H-23, H-29
29	2.43 (m, 2H)	52.6 (CH_2)	H-27, H-28, N- CH_3-27
NH-CH_3-3	2.25 (s, 3H)	34.9 (CH_3)	H-4
N-CH_3-27	2.19 (s, 3H)	45.7 (CH_3)	
O*C*OCH_3-4	-	169.6 (C)	H-4, OCO*C*H_3-4
OCO*C*H_3-4	1.72 (s, 3H)	20.8 (CH_3)	

The relative configuration on rings A-D was proposed according to the key chemical shifts and coupling constants affected by the orientation of the substituted groups. The amino group on C-3 and acetoxy one on C-4 were both proposed to adopt an axial orientation due to the minute coupling constant (J = 2.6 Hz) between H-3 and H-4. The typical chemical shifts of C-18 and C-19 (δ_C 12.3 and 15.1, and δ_H 0.64 and 1.14, respectively) indicated the axial orientation of the two methyls, thus suggesting the all-*trans* conformation of the steroid ring system (Keyzers *et al.*, 2002). Similar rationale was applied to the chemical shift of H-17 at 1.25 ppm; thus a similar orientation of the C-17 side chain to those of other steroids in the plakinamine family was proposed as shown (De Marino *et al.*, 1998).

3.2.2 The structure elucidation of compound 102

Compound **102** was obtained as a viscous white compound (1.4 mg) from the CH_2Cl_2-extract by successive chromatographic techniques using Sephadex LH-20 (20% EtOAc in MeOH), SiO_2 column (20% acetone in hexane), and SiO_2 HPLC column (gradient 5 to 10% *i*-PrOH in hexane in 20 min).

The ^{1}H NMR spectrum of **102** (500 MHz, C_6D_6: Figure 5) suggested that **102** was a steroid derivative, with typical methine and methylene signals at δ 1.0-2.0. The major functionalities as deducible in the ^{1}H NMR spectrum include a hydroxy group (δ3.96), and an *E*-

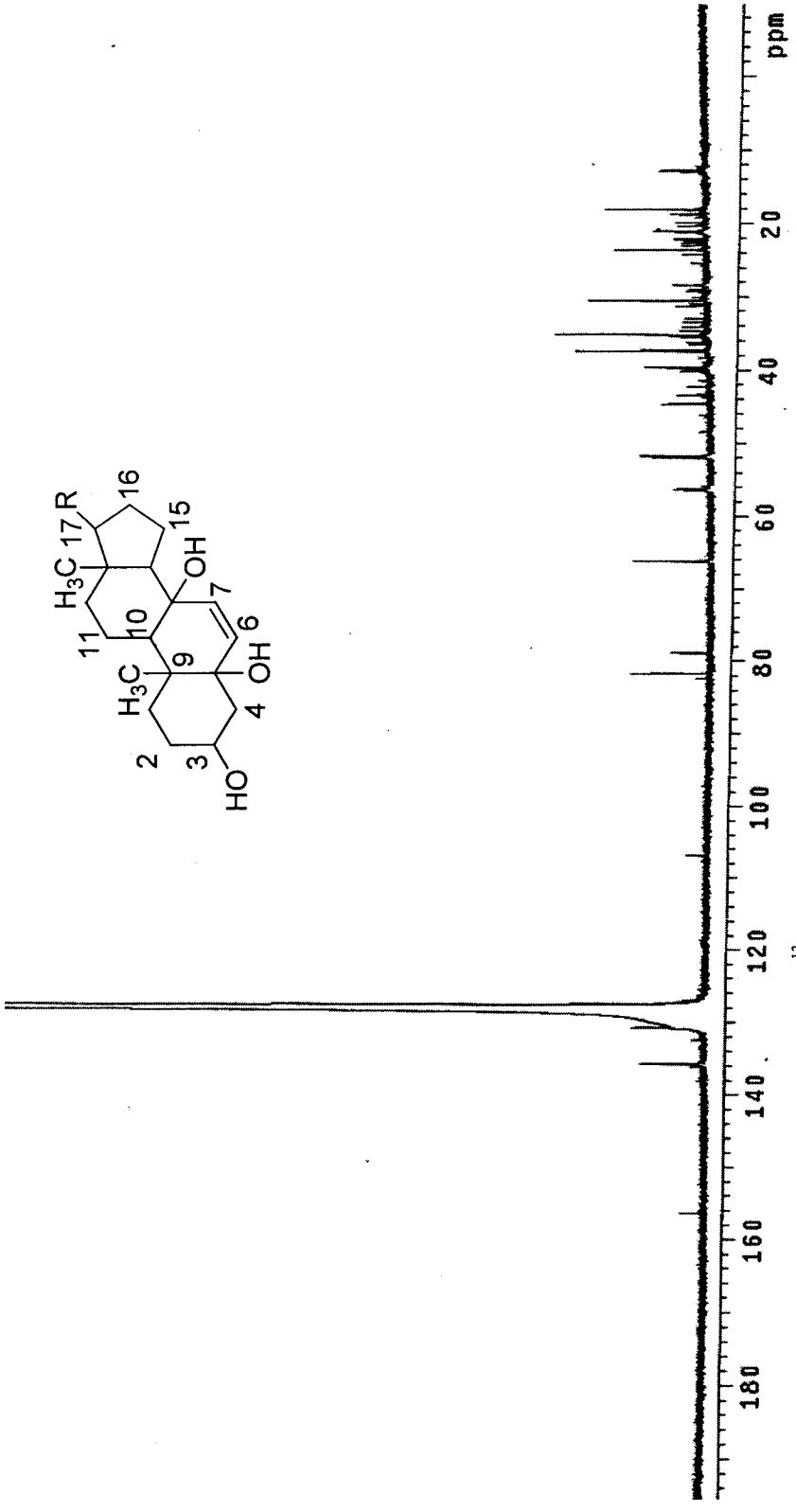


Figure 4 ^{13}C NMR spectrum of **102** (125 MHz, C_6D_6)

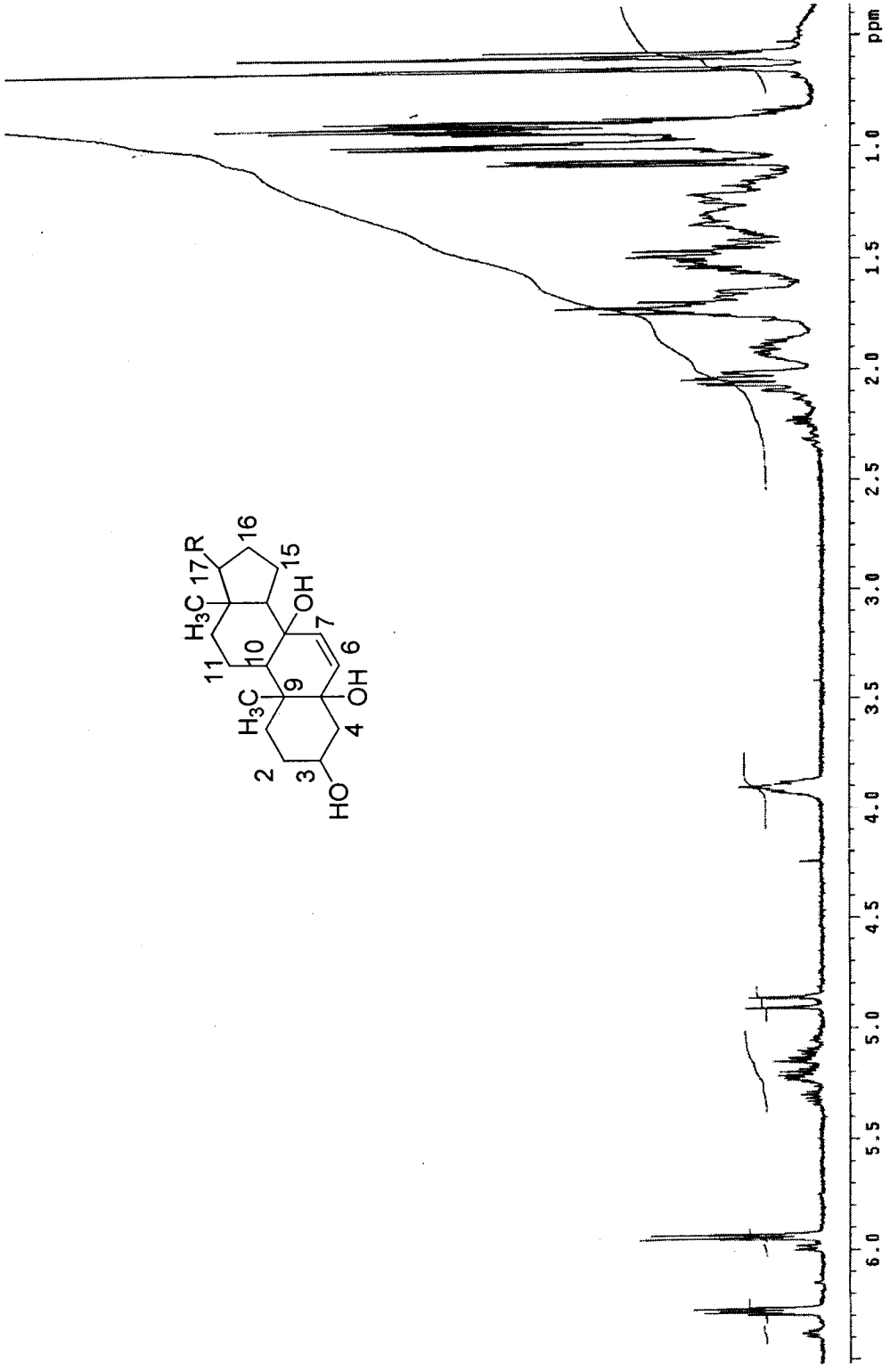
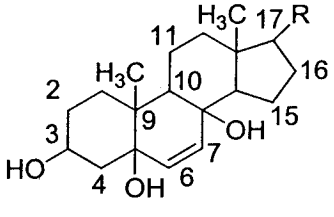


Figure 5 ^{1}H NMR spectrum of **102** (500 MHz, C_6D_6)

olefin (δ 5.99 and 6.33), presumably located on C-3 and C-6, respectively. Two additional hydroxy groups as observable in the ^{13}C NMR spectrum (125 Hz, C_6D_6, Figure 4) at δ 81.7 and 78.8, suggested that **102** is a tri-hydroxyl sterol.

The impurity contaminating the NMR spectra of **102**, however, prohibited a full structure determination of **102**. Such contaminants also interfered the mass spectra of **102** (both in EI and ESI modes) in such way that the precise molecular mass was unable to be deduced. Whereas the number of terminal methyl groups might be presumed to be two groups, thus suggesting an isopropyl moiety, the clarity of signals was again too skeptical. For the current moment, the plausible structure of **102** was therefore proposed as a 3,5,8-trihydroxy sterol, with no further side chain structure elucidated. Table 5 showed below summarized NMR data of only the core steroid structure of **102**.



102

Table 5 NMR data of **102** (500 MHz for ^{1}H and 125 MHz for ^{13}C; C_6D_6)

Position	δ_H (mult.; *J* in Hz)	δ_C (mult.)	HMBC correlation (C ⟶ H)
1	1.50 (m, 2H)	35.1 (CH_2)	H-19
2	1.78 (m, 2H)	36.5 (CH_2)	H-4
3	3.96 (m, 1H)	66.3 (CH)	H-1, H-2, H-4
4	2.05 (m, 2H)	37.4 (CH_2)	
5	-	81.7 (C)	H-1, H-4, H-6, H-7, H-19
6	5.99 (d; 8.7, 1H)	135.7 (CH)	
7	6.33 (dd; 8.7, 5.0, 1H)	130.5 (CH)	H-9
8	-	78.8 (C)	H-6, H-7, H-9
9	1.58 (m, 1H)	51.7 (CH)	H-19
10	-	44.6 (C)	H-9
11	1.77 (m, 2H)	30.5 (CH_2)	H-12
12	2.10 (m, 2H)	36.3 (CH_2)	
13	-	39.6 (C)	H-17, H-18
14	0.90 (m, 1H)	56.5 (CH)	
15	1.52 (m, 2H)	33.5 (CH_2)	H-17
16	1.25 (m, 2H)	23.6 (CH_2)	H-15
17	1.01 (m, 1H)	56.3 (CH)	H-18
18	0.62 (s, 3H)	12.7 (CH_3)	
19	0.68 (s, 3H)	12.7 (CH_3)	

3.3 Biological activities of compound 103

Compound **103** was assessed for the AChE inhibitory activity using the microplate reader assay (Ellman *et al.*, 1961; modified by Ingkaninan *et al.*, 2006), and for cytotoxic activity against four cancer cell lines (MCF-7, Hela, HT-29, and KB) using SRB assay (Skehan *et al.*, 1990). The inhibitory activities in both assays were shown in Table 6.

Table 6 The inhibitory activities of compound **103**

Compound	Cytotoxicity				AChE inhibition activity (IC_{50}; μM)
	MCF-7	Hela	HT-29	KB	
103 (% inhibition at 5 μg/mL)	27.75	-48.09	34.28	24.36	3.75±1.69
galantamine	-	-	-	-	0.59±0.14
camptothecin (IC_{50}; μg/mL)	0.8×10^{-4}	2.9×10^{-4}	$<0.1\times10^{-4}$	4.7×10^{-4}	-

The IC_{50} of **103** in the AChE inhibitory activity assay (3.75±1.69 μM) was in a good range as compared to that of the standard galantamine. The inhibition of **103** against AChE was independent from the incubation time (up to 60 min, data not shown), thus suggesting that compound **103** inhibit AChE reversibly. In order to determine the inhibitory mode of compound **103**, kinetics analysis of enzyme inhibition was conducted, and V_{max} and K_m were calculated from a nonlinear regression using a software Prism (Table 7). Upon addition of **103** (7.0 μM), V_{max} of AChE toward the hydrolysis of acetylthiocholine iodide decreased approximately two fold. On the other hand, K_m of the enzyme significantly increased when **103** (7.0 μM) was added. Such contrasted changes in K_m and V_{max} indicated that **103** inhibited the targeted enzyme in a mixed-competitive manner, i.e., combination between competitive and noncompetitive inhibition.

Table 7 V_{max} and K_m of AChE with and without inhibitors

AChE	V_{max} (dmA/min±SE)	K_m (μM±SE)
without inhibitors	108.5±4.0	729±107.9
with **103** (7.0 μM)	47.5±3.2	3805±591.9

To date, the primary group of steroid derivatives reported to possess AChE inhibiting activity has been the pregnane-type steroidal alkaloids from medicinal plants of the

families Buxaceae and Apocynaceae, especially those from the genus *Sarcococca* (Endress *et al.*, 1990; Atta-ur-Rahman and Choudhary, 1999). A series of alkaloids from *Sarcococca* and related genera have been readily exemplified in section 1.3.3 of this thesis. The potency of AChE inhibition of the *Sarcococca* alkaloids ranged from 5.2 to 227.9 μM as already mentioned (see Table 3).

The AChE inhibitory activity of the *Sarcococca* alkaloids in fact have been extensively studied. It was found that the inhibition kinetics of most pregnane-type steroidal alkaloids fell into a noncompetitive mode with certain analogs such as salignenamide A that showed a so-called "mixed-linear" competitive fashion (Khalid *et al.*, 2004a). Regarding the structure activity relationship, a primary in silico enzyme-docking study by Zaheer-ul-Haq *et al.* (2003a) suggested that either, or both, nitrogens on C-3 and C-20 exerted a strong influence in the enzyme binding activity. However, such argument was not strongly confirmative for an unambiguous linear relationship among the binding energy and potency was not met. The 3-D QSAR studies based on CoMFA and CoMSIA models by the same research group did however point the most influential functional groups to the negative functionalities surrounding ring A; i.e., the amino or amide nitrogen on C-3 (Zaheer-ul-Haq *et al.*, 2003b).

The resemblance between the plakinamines and *Sarcococca* alkaloids are evidentially recognizable; i.e., the core steroidal structures possessing nitrogenous functionalities on C-3. It is therefore not quite surprising that such strong AChE inhibitory activity can be observed with compound **103**. The potency of **103** indeed was comparable to, or even stronger than, that of most *Sarcococca* alkaloids. For examples, axillaridine A **(34)** and sarsalignenone **(36)**, representing the most potent alkaloids in their series, showed the AChE inhibiting activity with IC_{50}'s of 5.2 and 5.8 μM (referred to IC_{50} of galantamine 0.45 μM), respectively (Khalid *et al.*, 2004a), as compared to the IC_{50} of **103** at 3.75 μM (Table 6).

The structural difference on C-17 side chains, on the other hand, also supported the 3-D QSAR observations as mentioned above (Zaheer-ul-Haq *et al.*, 2003b) that the remote nitrogen on C-17 side chain expressed less influence on the enzyme inhibition activity. Also, the enzyme inhibition kinetics between the two groups were different. Most *Sarcococca* alkaloids were noncompetitive inhibitors, whereas the inhibition mode of **103** was mixed one. Although the mere single result from compound **103** should not be used to make an exclusive conclusion, it

is reasonable to speculate that the steroidal side chains exert certain close relationship on the inhibition modes of such compound.

CHAPTER 4

CONCLUSION

Similar to several other marine organisms, the sponge *Corticium* sp. is among the prolific sources of unusual metabolites never found associated with terrestrial plants and animals. The stigmastane-type steroidal alkaloids, the major chemical constituents of the *Corticium* sponges, were reported active in wide range of biological systems, from cytotoxicity to antifungal and anti-HIV (De Marino *et al.*, 1999; Lee *et al.*, 2001). Here, the isolation and structure elucidation of 4-acetoxy-plakinamine B **(103)** were reported. The compound is a new member of plakinamine-type alkaloids, which are common among several species of *Corticium*. The AChE-inhibiting activity of **103** is also reported here for the first time.

As already discussed, the potency of **103** in AChE-inhibiting assay was in a comparable range to that of galantamine (approximately fivefold less active). The activity was also in a same range to, or better than, that of most AChE-inhibiting steroidal alkaloids (Zaheer-ul-Haq *et al.*, 2003b; Khalid *et al.*, 2004a). The compound **103** was virtually non-toxic against cancer cell lines, implying potential for further development. It is therefore interesting to explore further on other derivatives in the same family of compounds.

The difficulty encountered throughout this investigation laid primarily on the isolation of compounds with hydrophobic core structures, yet highly polar due to hydrophilic and basic nitrogenated functionalities. The loss of minor components due to the basic entrapment onto acidic SiO_2 may be overcome by uses of rather neutral or basic chromatographic packing materials, such as alumina, or by that of bonded-phase chromatography specific for basic functional groups.

Overall, this work has demonstrated that Thai marine organisms are among the potential sources for biologically active compounds. The observation made earlier in Chapter 1 regarding the majority of marine natural products as cytotoxicity has been extended to other branch here, i.e., the enzyme-inhibiting activity. Extensive studies on the exploitation of such magnificent bioresources will lead to a better management policy on the marine bioresource utilization towards the prosperity and sustainability.

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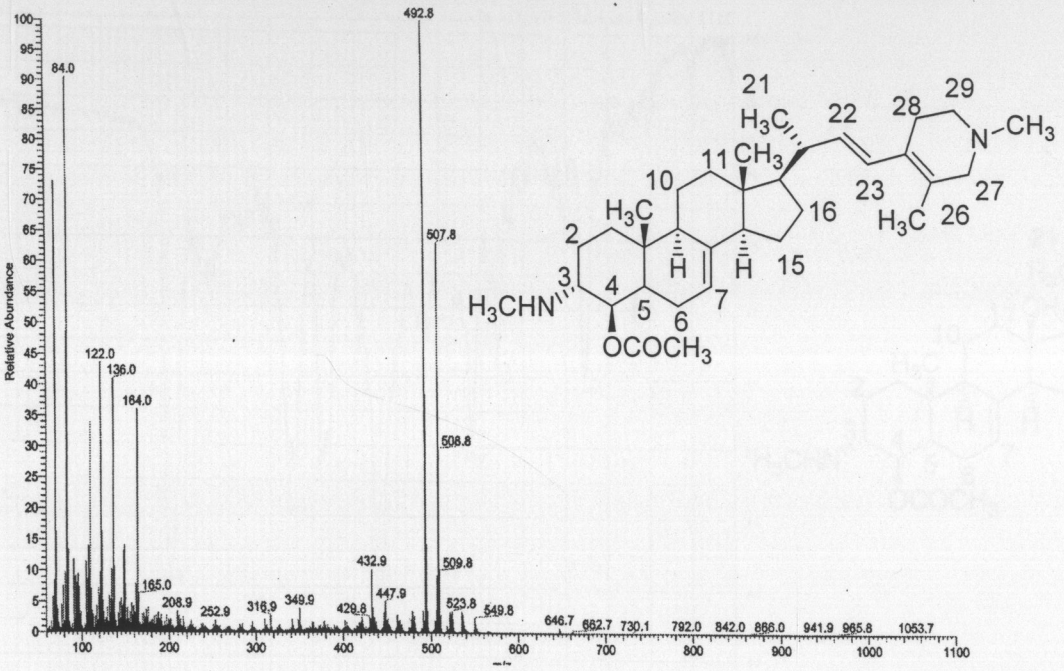
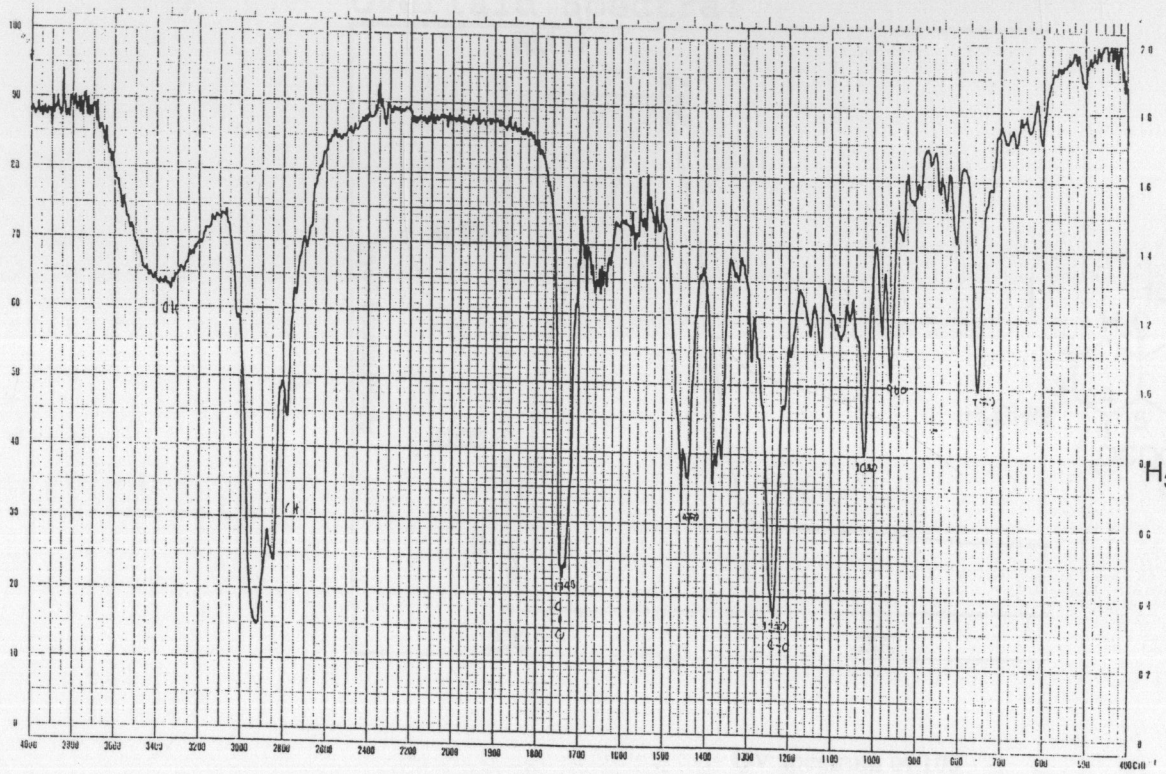
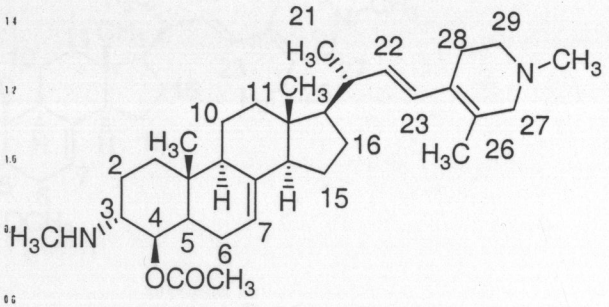
APPENDIX

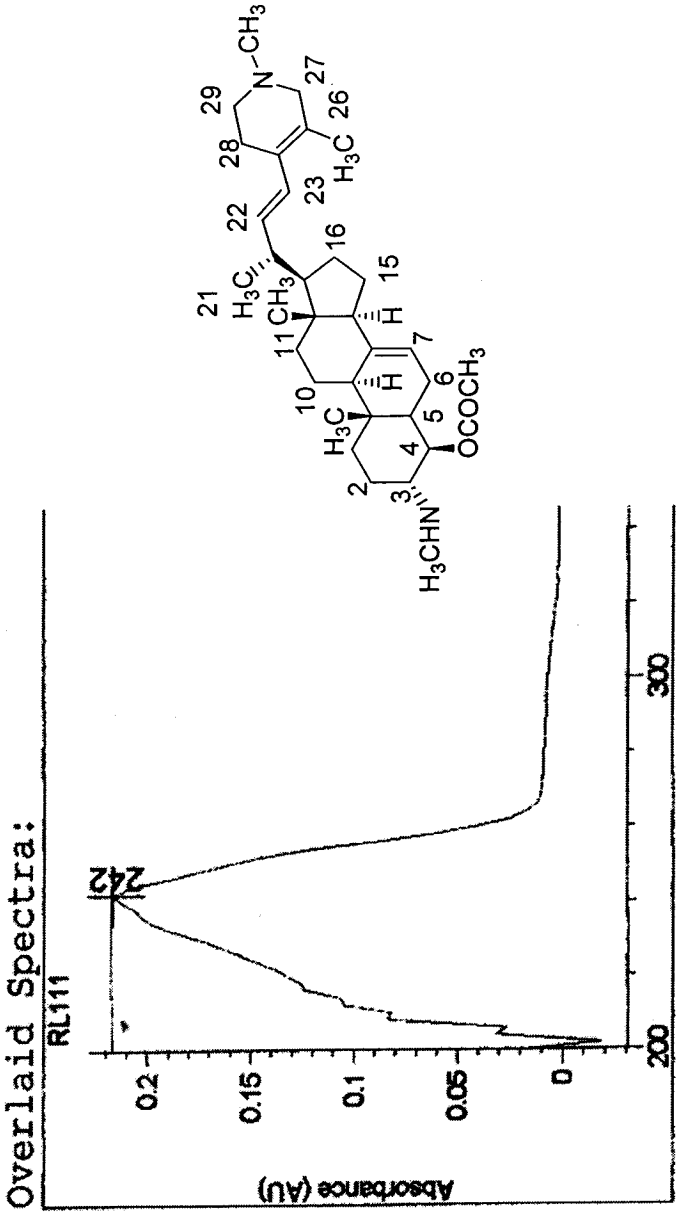


MS spectrum of **103**





IR spectrum of **103**



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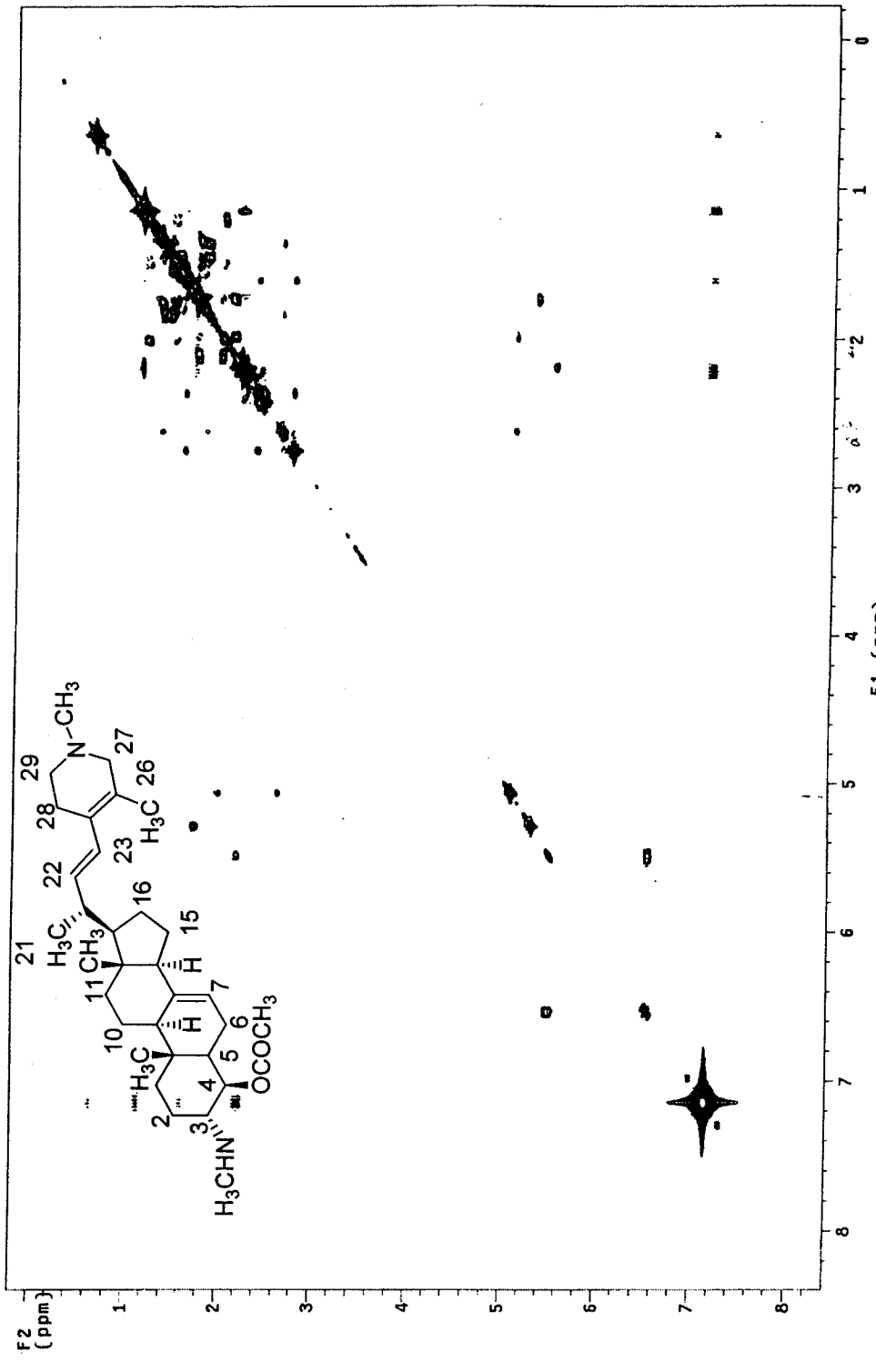
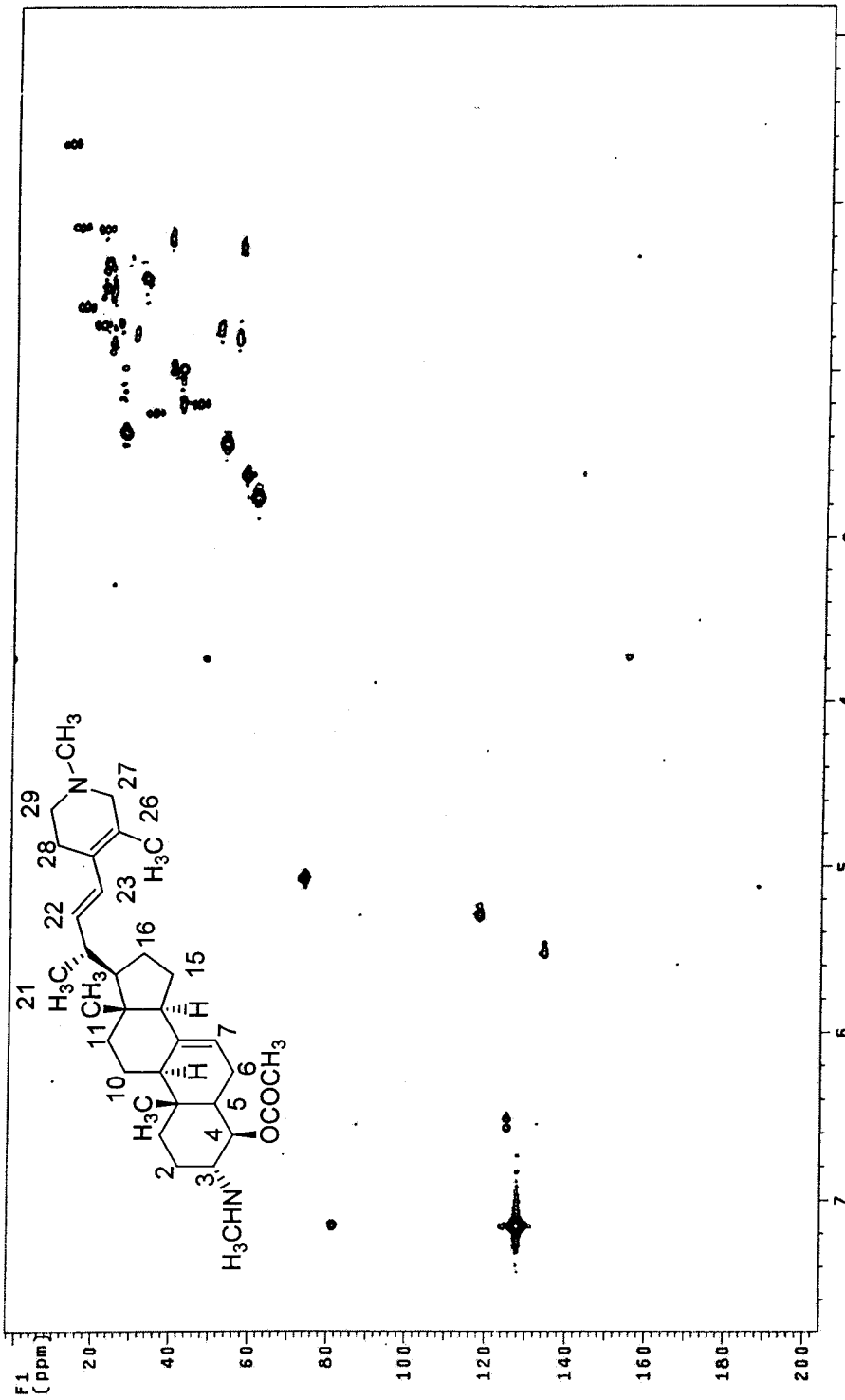
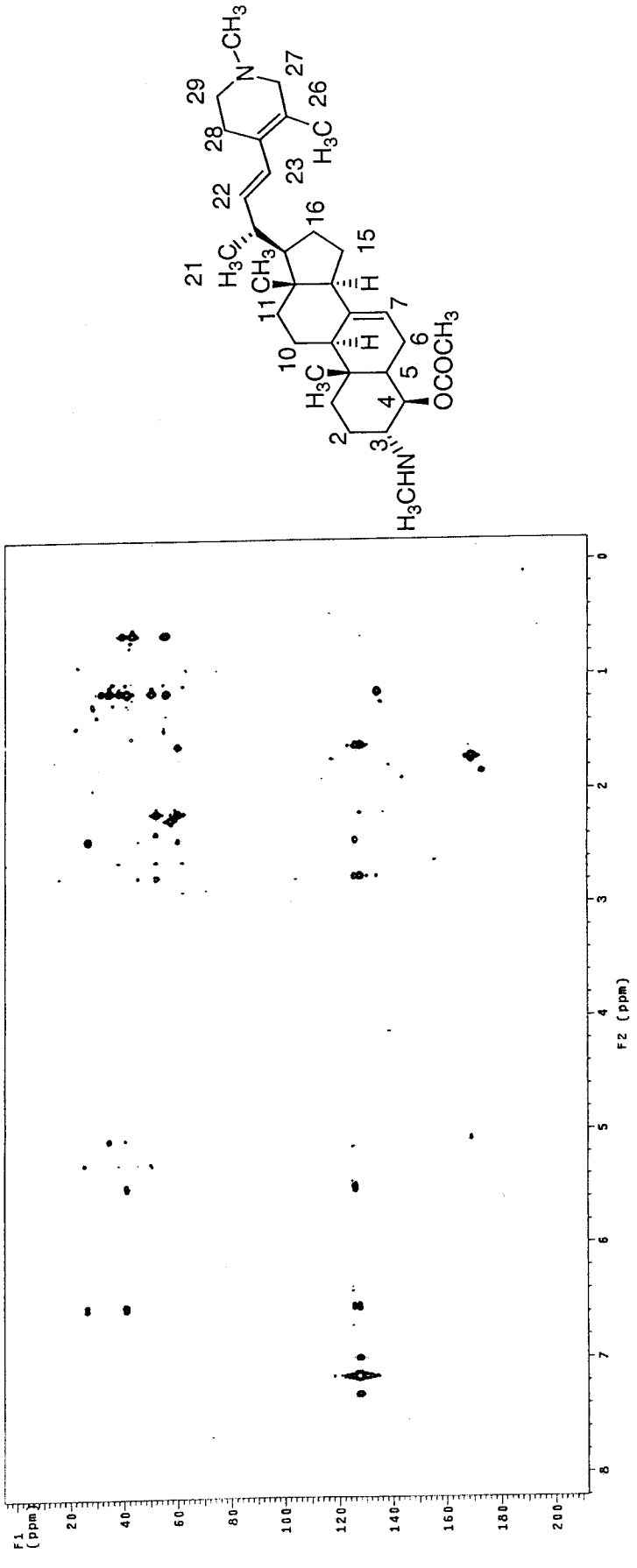


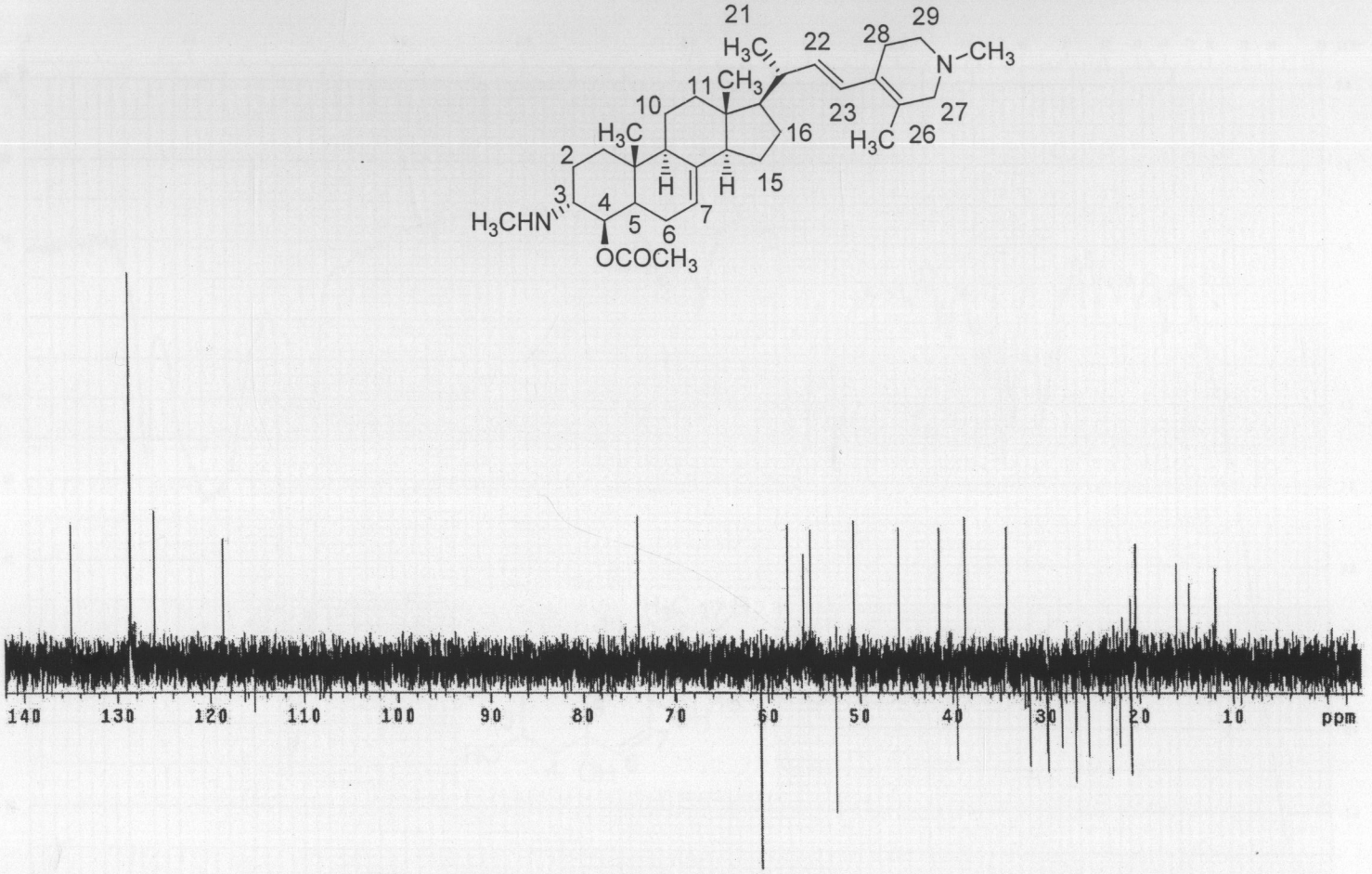
COSY spectrum of **103** (500 MHz, C_6D_6)



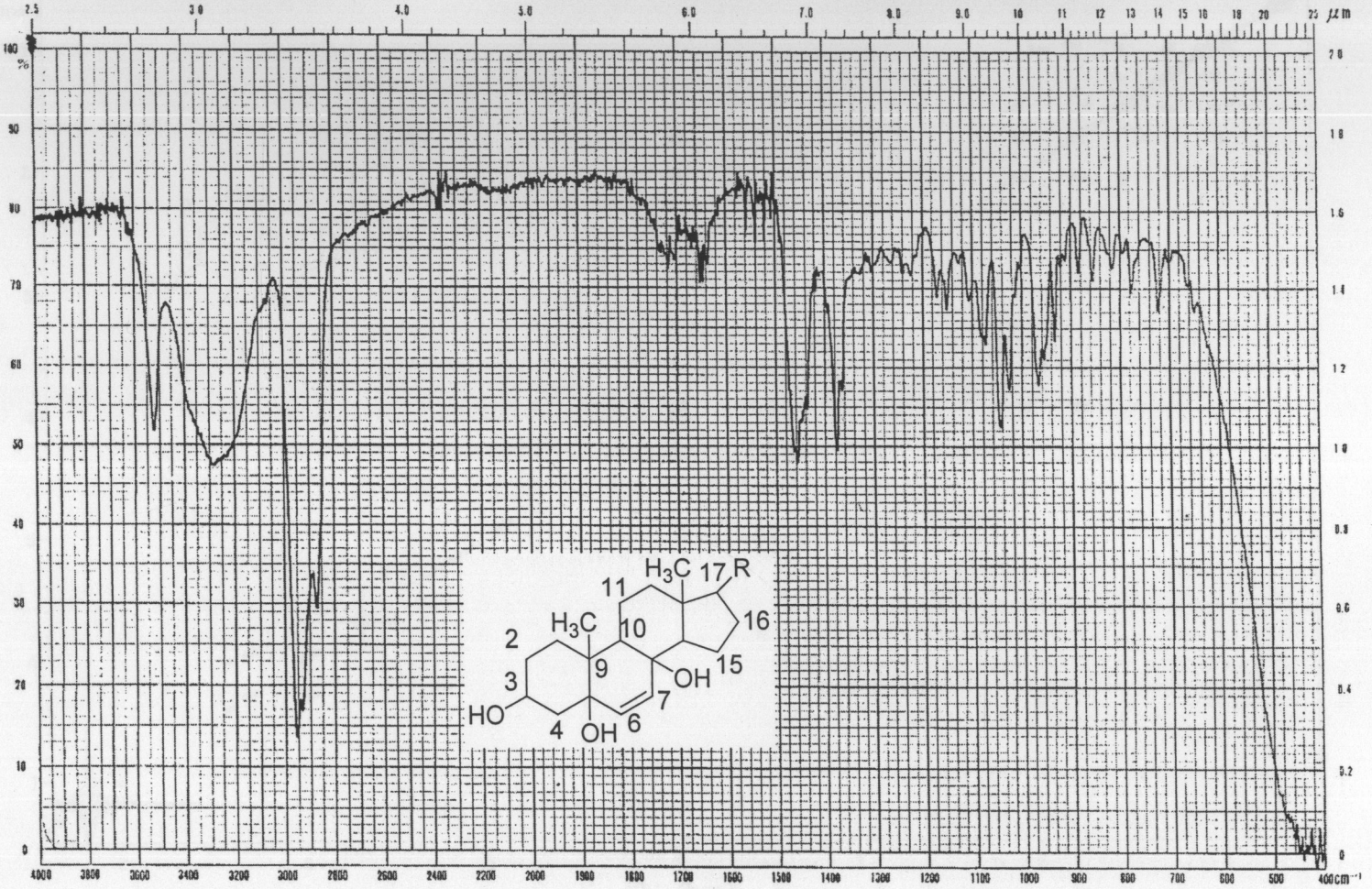
HMQC spectrum of **103** (500 MHz, C_6D_6)



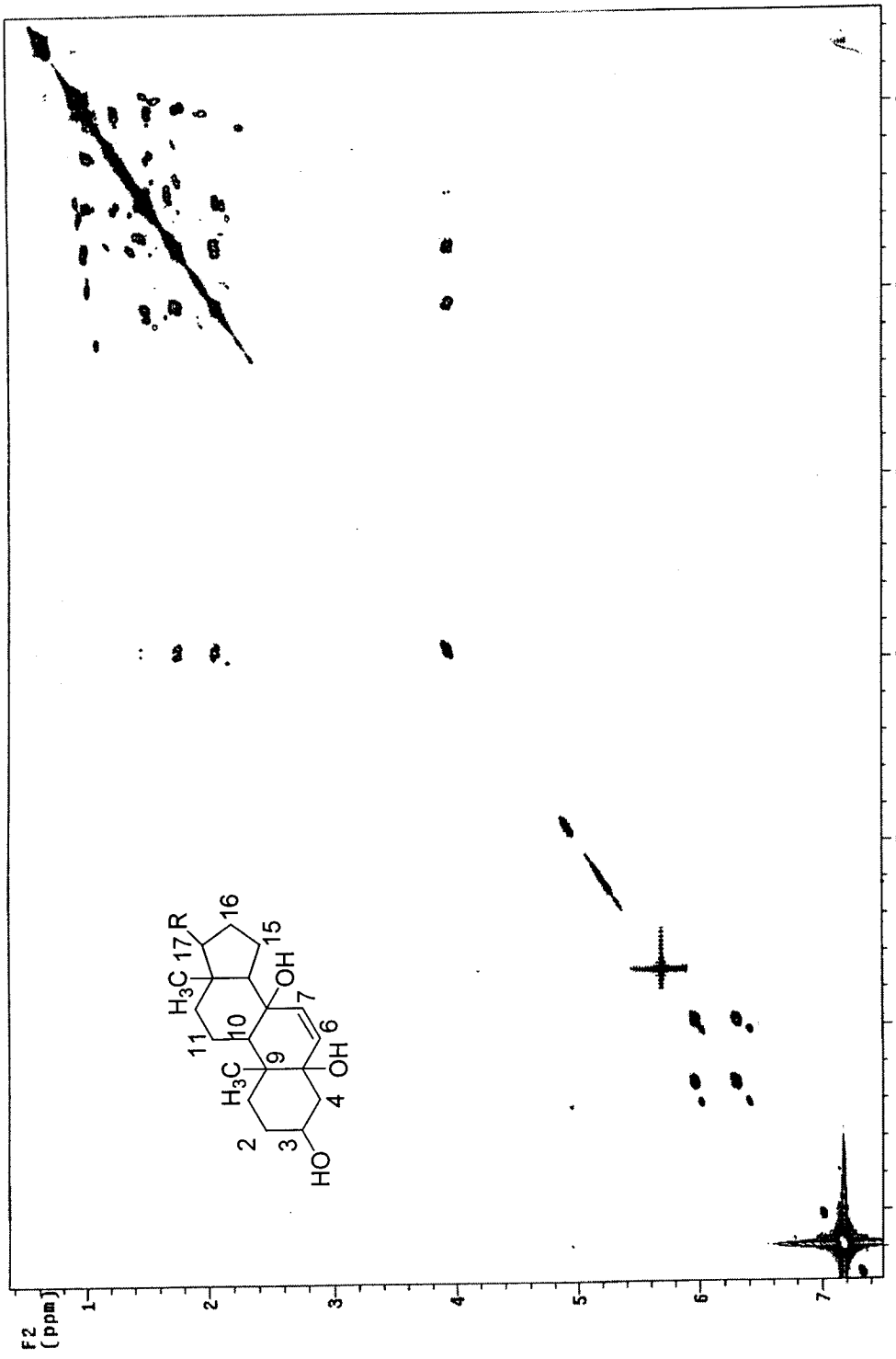
HMBC spectrum of **103** (500 MHz, C_6D_6)



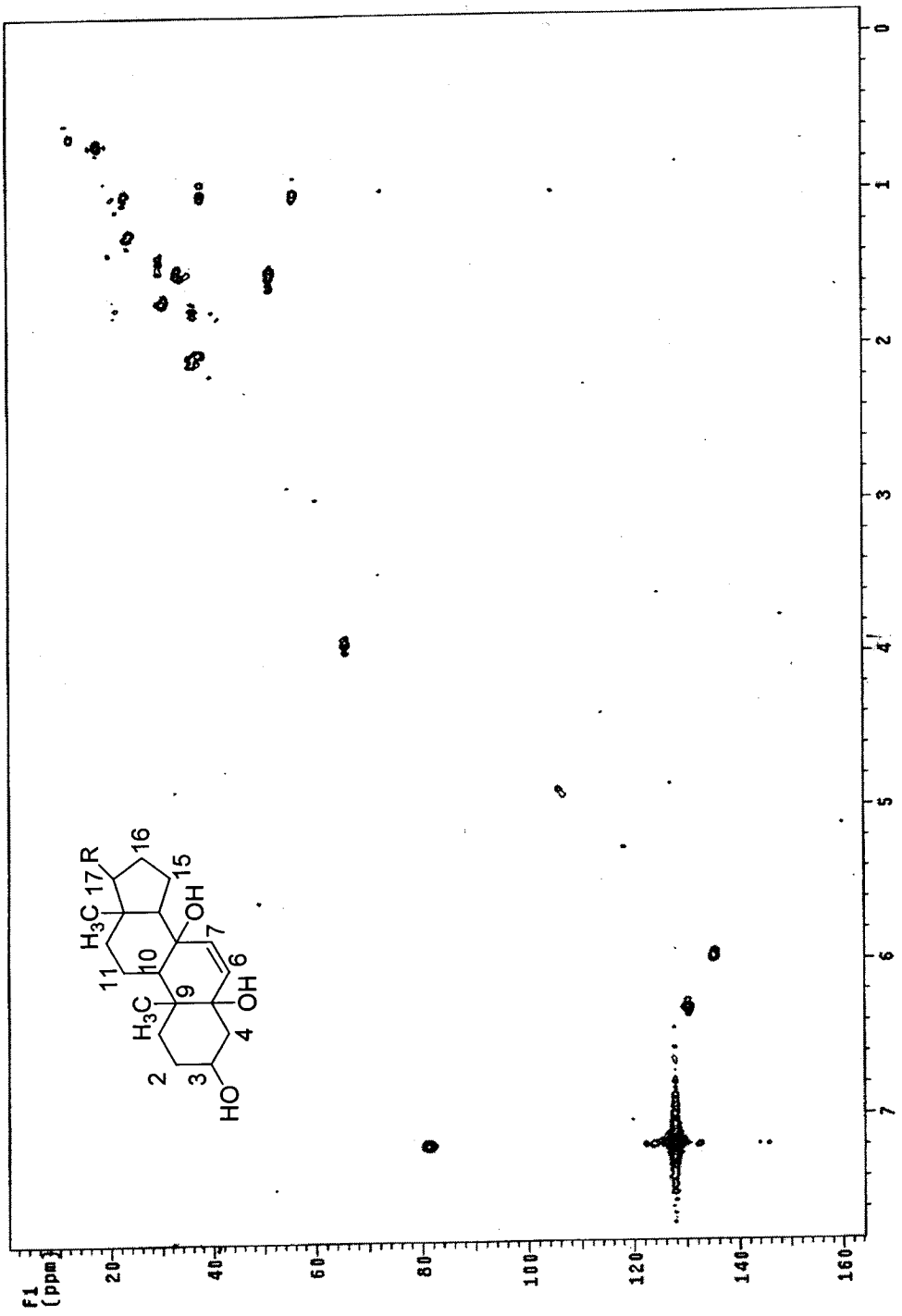
DEPT 135 spectrum of **103** (125 MHz, C_6D_6)



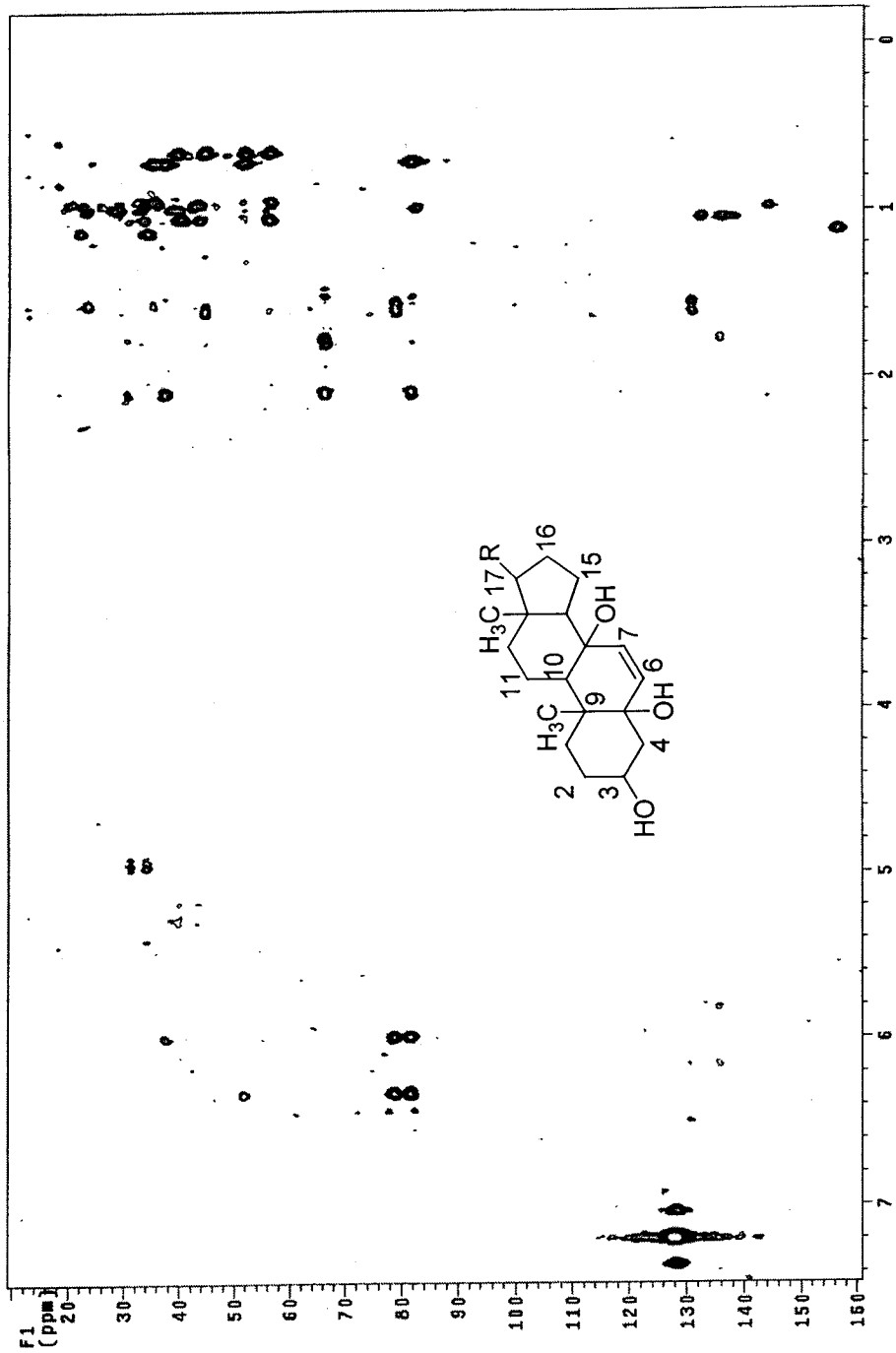
IR spectrum of **102**



COSY spectrum of **102** (500 MHz, C_6D_6)



HMQC spectrum of **102** (500 MHz, C_6D_6)



HMBC spectrum of **102** (500 MHz, C_6D_6)

VITAE

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Education Attainment

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List of Publication and Proceeding

R. Langjae, S. Bussarawit, S. Yuenyongsawad, K. Ingkaninan, A. Plubrukarn. 2007. Acetylcholinesterase-inhibiting steroidal alkaloid from the sponge, *Corticium* sp. Steroids (in press).

R. Langjae, S. Bussarawit, S. Yuenyongsawad, K. Ingkaninan, A. Plubrukarn. 2007. An acetylcholinesterase-inhibiting steroidal alkaloids from the sponge, *Corticium* sp. In 12th international symposium on marine natural products, Queenstown, New Zealand.

R. Langjae, S. Bussarawit, S. Yuenyongsawad, K. Ingkaninan, A. Plubrukarn. 2007. Acetylcholinesterase-inhibiting steroidal alkaloid from the sponge, *Corticium* sp. In 7th national graduate research conference, Prince of Songkla University, Surat Thani campus.