Acetylcholinesterase Inhibitors from the Thai Sponge Corallium sp.

Rosanne Langjea

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of
Master of Pharmacy in Pharmaceutical Sciences
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การแยกตัวสารควบคู่ไปกับการทดสอบอุทธิ้ด้านบนไซโอมอร์ซิลิโอสีทอลเรซาร์จากการสกัดจากฝอยน้ำไทยชนิดหนึ่งในสกุล *Corticium* ทำให้สามารถแยกสารประกอบกลุ่ม steroidal alkaloids ชนิดใหม่ได้ 1 ชนิด คือ 4-acetoxy-plakinamine B และสารประกอบ trihydroxy sterol 1 ชนิด ซึ่งยังไม่สามารถวิเคราะห์โครงสร้างที่แน่นอนได้ การวิเคราะห์ทางสุนทรียศาสตร์ใช้วิธีทางสเปクトโตรโฟโต ได้แก่ UV, IR, NMR และ MS spectroscopy จากการทดสอบอุทธิ้ด้านบนไซโอมอร์ซิลิโอสีทอลเรซาร์และอุทธิ้ความเป็นพิษต่อเซลล์ของ 4-acetoxy-plakinamine B พบว่าสารดังกล่าวมีอุทธิ์ด้านบนไซโอมอร์ซิลิโอสีทอลเรซาร์ที่ (IC₅₀ ที่กับ 3.75±1.69 μM) แต่ไม่แสดงอุทธิ์ความเป็นพิษต่อเซลล์มีเร็ว และจากการวิเคราะห์จุดสิ้นสุดของการอับซิปกิจกรรมของเอนไซม์ซิลิโอสีทอลเรซาร์พบว่าเป็นแบบคันเล็กคับหรือ ผลการวิเคราะห์ค่า Vₘₐₓ และ Kₘ ในการปรับย่อเอนไซม์ซิลิโอสีทอลเรซาร์ พบว่า 4-acetoxy-plakinamine B ยับยั้งเอนไซม์แบบผสม (mixed-competitive inhibition)
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, $V_{\text{max}}$ and $K_m$ was measured to reveal that the compound inhibited the targeted enzyme in a mixed-competitive manner.
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<td>4</td>
<td>$^{13}$C NMR spectrum of 102 (125 MHz, C$_6$D$_6$)</td>
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<td>5</td>
<td>$^1$H NMR spectrum of 102 (500 MHz, C$_6$D$_6$)</td>
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</tr>
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<td>46</td>
<td></td>
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| Isolation protocol for the sponge, *Corticium sp.* |
LIST OF ABBREVIATIONS AND SYMBOLS

$[\alpha]_D$ specific rotation

$\delta$ chemical shift (in ppm)

$\varepsilon$ molar extinction coefficient

$\lambda_{\text{max}}$ maximum wavelength

$\nu_{\text{max}}$ 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 distortioneness 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
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>J</td>
<td>coupling constant</td>
</tr>
<tr>
<td>$K_m$</td>
<td>Michaelis constant</td>
</tr>
<tr>
<td>m</td>
<td>multiplet (for NMR signals)</td>
</tr>
<tr>
<td>$m/z$</td>
<td>mass-over-charge ratio</td>
</tr>
<tr>
<td>MIC</td>
<td>minimum inhibitory concentration</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectroscopy</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>QSAR</td>
<td>quantitative structure-activity relationship</td>
</tr>
<tr>
<td>s</td>
<td>singlet (for NMR signals)</td>
</tr>
<tr>
<td>SE</td>
<td>standard error</td>
</tr>
<tr>
<td>SRB</td>
<td>sulphorhodamine B</td>
</tr>
<tr>
<td>t</td>
<td>triplet (for NMR signals)</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>$t_k$</td>
<td>retention time</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet-visible</td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>maximum velocity</td>
</tr>
<tr>
<td>w/v</td>
<td>weight by volume</td>
</tr>
</tbody>
</table>
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 Tethyidea). Two of these, spongothermidine 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).

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)

<table>
<thead>
<tr>
<th>Name</th>
<th>Source</th>
<th>Status (disease)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ziconotide (Prialt&lt;sup&gt;TM&lt;/sup&gt;)</td>
<td><em>Conus magus</em></td>
<td>Approved (neuropathic pain)</td>
</tr>
<tr>
<td>Ecteinascidin 743</td>
<td><em>Ecteinascidia turbinata</em></td>
<td>Phase III (cancer)</td>
</tr>
<tr>
<td>(Yondelis&lt;sup&gt;TM&lt;/sup&gt;)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AE-941 (neovastat)</td>
<td>Shark</td>
<td>Phase III (antiasthmatic)</td>
</tr>
<tr>
<td>Dehydrodendemin B</td>
<td><em>Aplidium albicans</em></td>
<td>Phase II (cancer)</td>
</tr>
<tr>
<td>(Aplidine&lt;sup&gt;TM&lt;/sup&gt;)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bryostatin 1</td>
<td><em>Bugula neritina</em></td>
<td>Phase II (cancer)</td>
</tr>
<tr>
<td>Soblidotin (TZT-1027)</td>
<td><em>Dolabella auricularia</em></td>
<td>Phase II (cancer)</td>
</tr>
<tr>
<td>Synthatadin (ILX 651)</td>
<td><em>Dolabella auricularia</em></td>
<td>Phase II (cancer)</td>
</tr>
<tr>
<td>Kahalamide F</td>
<td><em>Elysiarufescens/Bryopsis sp.</em></td>
<td>Phase II (cancer)</td>
</tr>
<tr>
<td>HTI-286 (hemiasterlin</td>
<td><em>Cymbastella sp.</em></td>
<td>Phase II (cancer)</td>
</tr>
<tr>
<td>derivative)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Squalamine</td>
<td><em>Squalus acanthias</em></td>
<td>Phase II (cancer)</td>
</tr>
<tr>
<td>PM00104 (jorumycin</td>
<td><em>Jorunna funebris</em></td>
<td>Phase I (cancer)</td>
</tr>
<tr>
<td>derivative; Zalypsy&lt;sup&gt;TM&lt;/sup&gt;)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E7389 (halicondrin B</td>
<td><em>Lissodendoryx sp.</em></td>
<td>Phase I (cancer)</td>
</tr>
<tr>
<td>derivative)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ES-285 (spisulosine)</td>
<td><em>Spisula polynyma</em></td>
<td>Phase I (cancer)</td>
</tr>
<tr>
<td>Discodermolide</td>
<td><em>Discodermia dissoluta</em></td>
<td>Phase I (cancer)</td>
</tr>
<tr>
<td>KRN-7000</td>
<td><em>Agelas mauritianus</em></td>
<td>Phase I (cancer)</td>
</tr>
<tr>
<td>GTS-21 (anabaseine</td>
<td><em>Paranemertes peregrina</em></td>
<td>Phase I (Alzheimer’s)</td>
</tr>
<tr>
<td>derivative)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CGX-1160 and CGX-1007</td>
<td><em>Conus geographus</em></td>
<td>Phase I (pain)</td>
</tr>
</tbody>
</table>
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 entorhinal 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 completely 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).

![Chemical structures of tacrine, donepezil, rivastigmine, and galantamine](image)

**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 ophthalmic 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).

\[
\begin{align*}
3 & \quad \begin{array}{c}
\text{H}_3\text{C} \\
\text{N} \\
\text{O} \\
\text{O} \\
\text{H}_3\text{C} \\
\text{N} \\
\text{H} \\
\text{N} \\
\text{CH}_3 \\
\text{CH}_3 \\
\end{array} \\
& \quad 4 \\
& \quad \begin{array}{c}
\text{H}_3\text{C} \\
\text{N} \\
\text{O} \\
\text{O} \\
\text{H}_3\text{C} \\
\text{N} \\
\text{CH}_3 \\
\text{N} \\
\text{CH}_3 \\
\end{array} \\
& \quad 5 \\
& \quad \begin{array}{c}
\text{H}_3\text{C} \\
\text{N} \\
\text{O} \\
\text{CH}_3 \\
\text{CH}_3 \\
\text{N} \\
\text{CH}_3 \\
\end{array}
\end{align*}
\]

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 advantages 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 Amaryllidaceae 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).

![Chemical structures of crinine (7) and related alkaloids](image)

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).
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 IC₅₀'s of 1.71 and 4.25 μM, respectively (Atta-ur-Rahman et al., 2002a).

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 IC₅₀'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 IC₅₀ of 37.8 μM (Park et al., 1996).

![Structures of alkaloids 13, 14, 15, 16, and 17](image)

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).
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<sub>50</sub>s 1.0 and 7.0 µM, respectively). The plant was also known in Chinese medicines for its calming effects, and there is evidence showing the neurodegenerative-protecting activity in its root extract (Houghton and Howes, 2005; Viegas et al., 2005).

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>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>$IC_{50}$ (µM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>salignenamide C (22)</td>
<td><img src="image1" alt="Image" /></td>
<td>61.3</td>
<td>38.4</td>
</tr>
<tr>
<td>salignenamide D (23)</td>
<td><img src="image2" alt="Image" /></td>
<td>185.2</td>
<td>23.8</td>
</tr>
<tr>
<td>2β-hydroxyepipachysamine D (24)</td>
<td><img src="image3" alt="Image" /></td>
<td>78.2</td>
<td>29.0</td>
</tr>
<tr>
<td>Reference</td>
<td>IC$_{50}$ (nM)</td>
<td>BChe</td>
<td>AChE</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>---------------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>Atta-ur-Rahman et al., 2002b</td>
<td>3.7</td>
<td>6.2</td>
<td>6.4</td>
</tr>
<tr>
<td>Atta-ur-Rahman et al., 2002b</td>
<td>4.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atta-ur-Rahman et al., 2002b</td>
<td>18.0</td>
<td></td>
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</table>

Table 2 Steroidal alkaloids as cholinesterase inhibitors (cont.)

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>IC$_{50}$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>suligenamide E (25)</td>
<td><img src="image" alt="Suligenamide E" /></td>
<td>25</td>
</tr>
<tr>
<td>suligenamide F (26)</td>
<td><img src="image" alt="Suligenamide F" /></td>
<td>26</td>
</tr>
<tr>
<td>axillarine C (27)</td>
<td><img src="image" alt="Axillarine C" /></td>
<td>27</td>
</tr>
<tr>
<td>Name</td>
<td>Reference</td>
<td>Structure</td>
</tr>
<tr>
<td>---------------</td>
<td>----------------------------------------</td>
<td>------------------------------------------------------------------</td>
</tr>
<tr>
<td>axillarine F</td>
<td>Attar-ur-Rahman et al., 2002b</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>sarcoine</td>
<td>Attar-ur-Rahman et al., 2002b</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>3-N-demethylsarcoine (30)</td>
<td>Attar-ur-Rahman et al., 2002b</td>
<td><img src="image" alt="Structure" /></td>
</tr>
</tbody>
</table>

Table 2: Steroidal alkaloids as cholinesterase inhibitors (cont.)
<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>IC$_{50}$ (μM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>saligcinnamid (31)</td>
<td><img src="31.png" alt="Image" /></td>
<td>20.0  4.8</td>
<td>Atta-ur-Rahman et al., 2002b</td>
</tr>
<tr>
<td>salignenamide A (32)</td>
<td><img src="32.png" alt="Image" /></td>
<td>50.6  4.6</td>
<td>Atta-ur-Rahman et al., 2002b</td>
</tr>
<tr>
<td>vaganine A (33)</td>
<td><img src="33.png" alt="Image" /></td>
<td>8.6   2.3</td>
<td>Atta-ur-Rahman et al., 2002b</td>
</tr>
<tr>
<td>Name</td>
<td>Structure</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; (µM) AChE</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; (µM) BChE</td>
</tr>
<tr>
<td>--------------------</td>
<td>-----------</td>
<td>---------------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>axillaridine A (34)</td>
<td><img src="image" alt="Structure of axillaridine A (34)" /></td>
<td>5.2</td>
<td>2.5</td>
</tr>
<tr>
<td>sarsaligone (35)</td>
<td><img src="image" alt="Structure of sarsaligone (35)" /></td>
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<td>2.2</td>
</tr>
<tr>
<td>sarsaligone (36)</td>
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<td>4.3</td>
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Table 2: Steroidal alkaloids as cholinesterase inhibitors (cont.)
<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
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<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>epoxynepapakistamine-A (37)</td>
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<tr>
<td>funtumafrine (38)</td>
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<td>6.6</td>
</tr>
<tr>
<td>N-methylfuntumine (39)</td>
<td><img src="image" alt="Structure" /></td>
<td>97.6</td>
<td>12.7</td>
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</table>
Table 2 Steroidal alkaloids as cholinesterase inhibitors (cont.)

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>IC₅₀ (µM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AChE</td>
<td>BChE</td>
</tr>
<tr>
<td>isosarcodine (40)</td>
<td><img src="image1" alt="Structure" /></td>
<td>10.3</td>
<td>1.9</td>
</tr>
<tr>
<td>sarcodine (41)</td>
<td><img src="image2" alt="Structure" /></td>
<td>49.8</td>
<td>18.3</td>
</tr>
<tr>
<td>sarcocine (42)</td>
<td><img src="image3" alt="Structure" /></td>
<td>20.0</td>
<td>3.9</td>
</tr>
<tr>
<td>Name</td>
<td>Structure</td>
<td>IC$_{50}$ (μM)</td>
<td>Reference</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>-----------</td>
<td>----------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>alkaloid-C (43)</td>
<td><img src="image" alt="Structure of alkaloid-C" /></td>
<td>42.2</td>
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<tr>
<td>5,14-dehydro-3-N-demethylsaracodine (44)</td>
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<td>&gt;200.0</td>
<td>25.0</td>
</tr>
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<td>14-dehydro-3-N-demethylsaracodine (45)</td>
<td><img src="image" alt="Structure of 14-dehydro-3-N-demethylsaracodine" /></td>
<td>183.1</td>
<td>10.1</td>
</tr>
<tr>
<td>Name</td>
<td>Structure</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>-------------------------</td>
<td>-----------</td>
<td>----------------------------------------</td>
<td></td>
</tr>
<tr>
<td>16-dehydroasarcorne (46)</td>
<td><img src="46.png" alt="Structure" /></td>
<td>Atta-ur-Rahman et al., 2004a</td>
<td></td>
</tr>
<tr>
<td>2,3-dehydroasarginine (47)</td>
<td><img src="47.png" alt="Structure" /></td>
<td>Atta-ur-Rahman et al., 2004a</td>
<td></td>
</tr>
<tr>
<td>sarcoyagine-C (48)</td>
<td><img src="48.png" alt="Structure" /></td>
<td>Atta-ur-Rahman et al., 2004a</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>IC₅₀ (µM)</th>
<th>AChE</th>
<th>BChE</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.5</td>
<td>7.0</td>
<td>187.8</td>
</tr>
</tbody>
</table>

Table 2: Steroidal alkaloids as cholinesterase inhibitors (cont.)
<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>IC$_{50}$ (nM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>salignarine-C (49)</td>
<td><img src="image" alt="Structure of salignarine-C" /></td>
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<tr>
<td>2-hydroxysalignarine-E (50)</td>
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<td>16.0</td>
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</tr>
<tr>
<td>5,6-dihydroasaroneidine (51)</td>
<td><img src="image" alt="Structure of 5,6-dihydroasaroneidine" /></td>
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<td>Atta-ur-Rahman et al., 2004b</td>
</tr>
<tr>
<td>Name</td>
<td>Structure</td>
<td>IC₅₀ (µM)</td>
<td>Reference</td>
</tr>
<tr>
<td>-----------------------</td>
<td>--------------------</td>
<td>----------</td>
<td>-------------------------------------</td>
</tr>
<tr>
<td>Salignamine (52)</td>
<td><img src="image" alt="Structure" /></td>
<td>249.0</td>
<td>Atta-ur-Rahman et al., 2004bb</td>
</tr>
<tr>
<td>2-hydroxysalignamine (53)</td>
<td><img src="image" alt="Structure" /></td>
<td>82.5</td>
<td>Atta-ur-Rahman et al., 2004b</td>
</tr>
<tr>
<td>Salignarine-F (54)</td>
<td><img src="image" alt="Structure" /></td>
<td>30.2</td>
<td>Atta-ur-Rahman et al., 2004b</td>
</tr>
<tr>
<td>Name</td>
<td>Structure</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; (μM)</td>
<td>Reference</td>
</tr>
<tr>
<td>---------------------</td>
<td>----------------------</td>
<td>-----------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>salanine-C (55)</td>
<td>![Structure 55]</td>
<td>7.8</td>
<td>Attar-ur-Rahman et al., 2004b</td>
</tr>
<tr>
<td>(N)-[formyl (methyl)amino] salanine-B (56)</td>
<td>![Structure 56]</td>
<td>48.6</td>
<td>Attar-ur-Rahman et al., 2004b</td>
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<tr>
<td>dictyopholine (57)</td>
<td>![Structure 57]</td>
<td>3.7</td>
<td>Attar-ur-Rahman et al., 2004b</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Reference</th>
<th>BChE (μM)</th>
<th>AChE (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Attar-ur-Rahman et al., 2004b</td>
<td>32.2</td>
<td>10.5</td>
</tr>
<tr>
<td></td>
<td>48.6</td>
<td>6.2</td>
</tr>
</tbody>
</table>

Table 2: Steroidal alkaloids as cholinesterase inhibitors (cont.)
<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>( IC_{50} ) (( \mu \text{M} ))</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>epipachysamine-D (58)</strong></td>
<td><img src="image" alt="Structure" /></td>
<td>28.9</td>
<td>Atta-ur-Rahman et al., 2004b</td>
</tr>
<tr>
<td><strong>saracose (59)</strong></td>
<td><img src="image" alt="Structure" /></td>
<td>20.0</td>
<td>Atta-ur-Rahman et al., 2004b</td>
</tr>
<tr>
<td><strong>iso-N-formylchonemorphine (60)</strong></td>
<td><img src="image" alt="Structure" /></td>
<td>6.4</td>
<td>Atta-ur-Rahman et al., 2004b</td>
</tr>
<tr>
<td>Name</td>
<td>Structure</td>
<td>Reference</td>
<td>(IC_{50} (\mu M))</td>
</tr>
<tr>
<td>------------------</td>
<td>-----------</td>
<td>--------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>Sarcoxin (61)</td>
<td><img src="image" alt="Structure of Sarcoxin (61)" /></td>
<td>Atta-ur-Rahman et al., 2004a</td>
<td>12.5</td>
</tr>
<tr>
<td>Hookerianamide A (62)</td>
<td><img src="image" alt="Structure of Hookerianamide A (62)" /></td>
<td>Choudhary et al., 2004</td>
<td>200.0</td>
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<tr>
<td>Hookerianamide B (63)</td>
<td><img src="image" alt="Structure of Hookerianamide B (63)" /></td>
<td>Choudhary et al., 2004</td>
<td>0.8</td>
</tr>
</tbody>
</table>

**Table 2**: Steroidal alkaloids as cholinesterase inhibitors (cont.)
<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>hoekermanamide C (64)</td>
<td>![Structure of hoekermanamide C (64)]</td>
<td>Choudhary et al., 2004</td>
</tr>
<tr>
<td>hoekermanamine A (65)</td>
<td>![Structure of hoekermanamine A (65)]</td>
<td>Choudhary et al., 2004</td>
</tr>
<tr>
<td>philchowkiamide A (66)</td>
<td>![Structure of philchowkiamide A (66)]</td>
<td>Choudhary et al., 2004</td>
</tr>
</tbody>
</table>

**Table 2** Steroidal alkaloids as cholinesterase inhibitors (cont.)

<table>
<thead>
<tr>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>BChE</th>
<th>AChE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.6</td>
<td>0.9</td>
<td>0.4</td>
</tr>
<tr>
<td>23.2</td>
<td>18.9</td>
<td>0.5</td>
</tr>
</tbody>
</table>

- IC<sub>50</sub> = 50% inhibitory concentration
- BChE = butyrylcholinesterase
- AChE = acetylcholinesterase
<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th><strong>IC_{50} (µM)</strong></th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>hookerianamide-D (67)</td>
<td><img src="67.png" alt="Structure" /></td>
<td>59.0 100.2</td>
<td>Choudhary <em>et al.</em>, 2005</td>
</tr>
<tr>
<td>hookerianamide-E (68)</td>
<td><img src="68.png" alt="Structure" /></td>
<td>15.9 6.0</td>
<td>Choudhary <em>et al.</em>, 2005</td>
</tr>
<tr>
<td>hookerianamide-F (69)</td>
<td><img src="69.png" alt="Structure" /></td>
<td>1.6 7.2</td>
<td>Choudhary <em>et al.</em>, 2005</td>
</tr>
<tr>
<td>Name</td>
<td>Structure</td>
<td>IC_{50} (μM)</td>
<td>Reference</td>
</tr>
<tr>
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</tr>
<tr>
<td>hookerianamide-G (70)</td>
<td><img src="image" alt="Structure" /></td>
<td>11.4</td>
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<td>terminaline (71)</td>
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<td>6-O-buxafurandiene (72)</td>
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<td>-</td>
</tr>
<tr>
<td>Reference</td>
<td>IC$_{50}$ (µM)</td>
<td>Structure</td>
<td>Name</td>
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<td>-----------</td>
<td>----------------</td>
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<td>------</td>
</tr>
<tr>
<td>Babar et al., 2006</td>
<td>-</td>
<td><img src="image1" alt="Structure" /></td>
<td>7-deoxy-6-O-buxanuridine (73)</td>
</tr>
<tr>
<td>Babar et al., 2006</td>
<td>-</td>
<td><img src="image2" alt="Structure" /></td>
<td>benzoylbufoxetidene (74)</td>
</tr>
<tr>
<td>Babar et al., 2006</td>
<td>-</td>
<td><img src="image3" alt="Structure" /></td>
<td>buxapapilline (75)</td>
</tr>
</tbody>
</table>

Table 2: Steroidal alkaloids as cholinesterase inhibitors (cont.)
<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>IC$_{50}$ (µM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>buxaquamine (76)</td>
<td><img src="image" alt="Structure of buxaquamine" /></td>
<td>76.0</td>
<td>Babar et al., 2006</td>
</tr>
<tr>
<td>irehine (77)</td>
<td><img src="image" alt="Structure of irehine" /></td>
<td>100.0</td>
<td>Babar et al., 2006</td>
</tr>
</tbody>
</table>
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, although 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>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>Activities</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>meridine (78)</td>
<td><img src="image1" alt="Structure" /></td>
<td>antifungal (<em>Candida albicans</em>; MIC 0.2 µg/mL and <em>Cryptococcus neoformans</em>; MIC 0.8 µg/mL)</td>
<td>McCarthy et al., 1992</td>
</tr>
<tr>
<td>lokysterolamine A (79)</td>
<td><img src="image2" alt="Structure" /></td>
<td>cytotoxicity (P-388, IC&lt;sub&gt;50&lt;/sub&gt; 0.5 µg/mL; A-549, IC&lt;sub&gt;50&lt;/sub&gt; 0.5 µg/mL; HT-29, IC&lt;sub&gt;50&lt;/sub&gt; 1.0 µg/mL; MEL-28, IC&lt;sub&gt;50&lt;/sub&gt; 5 µg/mL); antimicrobial (<em>B. subtilis</em>, 19 mm; 50 µg/disc); antifungal (<em>C. albicans</em>, 11 mm; 50 µg/disc)</td>
<td>Jurek et al., 1994</td>
</tr>
<tr>
<td>lokysterolamine B (80)</td>
<td><img src="image3" alt="Structure" /></td>
<td>cytotoxicity (P-388, IC&lt;sub&gt;50&lt;/sub&gt; 1.0 µg/mL; A-549, IC&lt;sub&gt;50&lt;/sub&gt; 0.5 µg/mL; HT-29, IC&lt;sub&gt;50&lt;/sub&gt; 1.0 µg/mL; MEL-28, IC&lt;sub&gt;50&lt;/sub&gt; &gt;2 µg/mL); antimicrobial (<em>B. subtilis</em>, 8 mm; 50 µg/disc)</td>
<td>Jurek et al., 1994</td>
</tr>
<tr>
<td>Name</td>
<td>Structure</td>
<td>Activities</td>
<td>Reference</td>
</tr>
<tr>
<td>------</td>
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<td>------------</td>
<td>-----------</td>
</tr>
<tr>
<td>3α-amino-23, 29-imino-β (9a)-homo-19-nor-5α-stigmasta-1(10),7,9,(11), 23(Δ)-tetraene (81)</td>
<td><img src="image1" alt="Structure 81" /></td>
<td>no reported activity available</td>
<td>De Marino et al., 1998</td>
</tr>
<tr>
<td>3α-amino-23,29-imino-β (9a)-homo-19-nor-5α-stigmasta-1(10),7,23,(11), 23(Δ)-triene (82)</td>
<td><img src="image2" alt="Structure 82" /></td>
<td>no reported activity available</td>
<td>De Marino et al., 1998</td>
</tr>
<tr>
<td>plakinamine C (83)</td>
<td><img src="image3" alt="Structure 83" /></td>
<td>anti-HIV (inhibit syncytia formation after HIV infection of MT4 cell line at 0.1μg/mL)</td>
<td>De Marino et al., 1999</td>
</tr>
<tr>
<td>Name</td>
<td>Structure</td>
<td>Activities</td>
<td>Reference</td>
</tr>
<tr>
<td>-----------------------------</td>
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</tr>
<tr>
<td>plakinamine D (84)</td>
<td><img src="84.png" alt="Structure" /></td>
<td>cytotoxicity (NSCLC-N6, IC50 3.3 μg/mL)</td>
<td>De Marino et al., 1999</td>
</tr>
<tr>
<td>N, N'-dimethyl-4-oxo-3-epi-plakinamine B (85)</td>
<td><img src="85.png" alt="Structure" /></td>
<td>cytotoxicity (NSCLC-N6, IC50 3.6 μg/mL)</td>
<td>De Marino et al., 1999</td>
</tr>
<tr>
<td>25,26-dihydro-plakinamine A (86)</td>
<td><img src="86.png" alt="Structure" /></td>
<td>anti-HIV activity (inhibit syncytia formation after HIV infection of MT4 cell line at 0.05 μg/mL); cytotoxicity (NSCLC-N6, IC50 5.7 μg/mL)</td>
<td>De Marino et al., 1999</td>
</tr>
<tr>
<td>Name</td>
<td>Structure</td>
<td>Activities</td>
<td>Reference</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>23-(N-methyl)-plakinamine A (87)</td>
<td><img src="image1" alt="Structure 1" /></td>
<td>anti-HIV activity (inhibit syncytia formation after HIV infection of MT4 cell line at 0.1 μg/mL); cytotoxicity (NSCLC-N6, IC₅₀ = 4.9 μg/mL)</td>
<td>De Marino et al., 1999</td>
</tr>
<tr>
<td>plakinamine E (88)</td>
<td><img src="image2" alt="Structure 2" /></td>
<td></td>
<td></td>
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<tr>
<td>plakinamine F (89)</td>
<td><img src="image3" alt="Structure 3" /></td>
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<td>Name</td>
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</tr>
<tr>
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<td>------------------------------------------------</td>
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<td>------------------------------------------------</td>
</tr>
<tr>
<td>plakinamine G (90)</td>
<td><img src="image1.png" alt="Structure" /></td>
<td>Borbone et al., 2002</td>
<td>cytotoxicity (C6, IC&lt;sub&gt;50&lt;/sub&gt; 6.8 μg/mL)</td>
</tr>
<tr>
<td>plakinamine H (91)</td>
<td><img src="image2.png" alt="Structure" /></td>
<td></td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; 6.1 μg/mL</td>
</tr>
<tr>
<td>4α-hydroxydemethyl-</td>
<td><img src="image3.png" alt="Structure" /></td>
<td></td>
<td></td>
</tr>
<tr>
<td>plakinamine B (92)</td>
<td><img src="image4.png" alt="Structure" /></td>
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</table>

Table 3: Compounds isolated from sponges of the genus Cnoria (cont.)
<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>Activities</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>tetrahydroplakinamine A</td>
<td><img src="93.png" alt="Structure" /></td>
<td>cytotoxicity (C6, IC&lt;sub&gt;50&lt;/sub&gt; 1.4 µg/mL)</td>
<td>Borbone et al., 2002</td>
</tr>
<tr>
<td>(93)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>plakinamine I (94)</td>
<td><img src="94.png" alt="Structure" /></td>
<td>cytotoxicity (HCT-116, IC&lt;sub&gt;50&lt;/sub&gt; 10.6 µM)</td>
<td>Ridley and Faulkner, 2003</td>
</tr>
<tr>
<td>plakinamine J (95)</td>
<td><img src="95.png" alt="Structure" /></td>
<td>cytotoxicity (HCT-116, IC&lt;sub&gt;50&lt;/sub&gt; 6.1 µM)</td>
<td>Ridley and Faulkner, 2003</td>
</tr>
<tr>
<td>Name</td>
<td>Structure</td>
<td>Activities</td>
<td>Reference</td>
</tr>
<tr>
<td>-----------------------------</td>
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</tr>
<tr>
<td>plakinamine K (96)</td>
<td><img src="image" alt="Structure 96" /></td>
<td>cytotoxicity (HCT-116, IC&lt;sub&gt;50&lt;/sub&gt; 1.4 μM)</td>
<td>Ridley and Faulkner, 2003</td>
</tr>
<tr>
<td>24,25-dihydroplakinamine K (97)</td>
<td><img src="image" alt="Structure 97" /></td>
<td>cytotoxicity (HCT-116, IC&lt;sub&gt;50&lt;/sub&gt; 1.4 μM)</td>
<td>Ridley and Faulkner, 2003</td>
</tr>
<tr>
<td>cortistatin A (98)</td>
<td><img src="image" alt="Structure 98" /></td>
<td>anti-proliferative activity (HUVECs, IC&lt;sub&gt;50&lt;/sub&gt; 0.0018 μM)</td>
<td>Aoki et al., 2006</td>
</tr>
<tr>
<td>Name</td>
<td>Structure</td>
<td>Activities</td>
<td>Reference</td>
</tr>
<tr>
<td>-----------------</td>
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</tr>
<tr>
<td>cortistatin B</td>
<td><img src="image" alt="Structure 99" /></td>
<td>anti-proliferative activity (HUVECs, IC₅₀ 1.1 μM)</td>
<td>Aoki et al., 2006</td>
</tr>
<tr>
<td>(99)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cortistatin C</td>
<td><img src="image" alt="Structure 100" /></td>
<td>anti-proliferative activity (HUVECs, IC₅₀ 0.019 μM)</td>
<td>Aoki et al., 2006</td>
</tr>
<tr>
<td>(100)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cortistatin D</td>
<td><img src="image" alt="Structure 101" /></td>
<td>anti-proliferative activity (HUVECs, IC₅₀ 0.15 μM)</td>
<td>Aoki et al., 2006</td>
</tr>
<tr>
<td>(101)</td>
<td></td>
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</tr>
</tbody>
</table>
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 CH₂Cl₂-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 CH₂Cl₂-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 Packard® 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$_6$H$_5$D$_3$ for $^1$H NMR; and 128 ppm, C$_6$D$_6$ 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 acetylthioiiodide (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<sub>50</sub>, K<sub>m</sub> and V<sub>max</sub> 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 (2×10⁴ 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$_2$Cl$_2$ 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$_2$Cl$_2$-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 (Econsol® 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$_2$Cl$_2$ 1.5:1:7.5; eluted with 20% CH$_2$Cl$_2$ in MeOH). Compound 103 was obtained and identified as 4-acetoxy-plakanamine B (2.8 mg).

2.5 Physical properties of isolated compound

4-acetoxy-plakanamine B (103): viscous yellow liquid; [α]$_D^0$ +21.9$^\circ$ (c 0.0014, MeOH); IR (thin film) $\nu_{max}$ 3400, 2925, 1740, 1240 cm$^{-1}$; UV (MeOH) $\lambda_{max}$ (log ε) 242 (4.29) nm; $^1$H and $^{13}$C NMR (500 MHz for $^1$H and 125 MHz for $^{13}$C, C$_6$D$_6$) 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$_2$N$_2$, 508.4029).
Lyophilized sponge (263 g)

<table>
<thead>
<tr>
<th>Hexane-extract</th>
<th>CH$_2$Cl$_2$-extract</th>
<th>MeOH-extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1.9 g; 22% inhibition)*</td>
<td>(1.4 g; 86% inhibition)*</td>
<td>(21.2 g; 95% inhibition)*</td>
</tr>
</tbody>
</table>

1) Sephadex LH-20  
   (20% EtOAc in MeOH)

2) SiO$_2$ column  
   (20% acetone in hexane)

3) Normal phase HPLC  
   (Econosil silica/gradient)

   Compound 102  
   (1.4 mg)

1) Sephadex LH-20  
   (20% EtOAc in MeOH)

2) SiO$_2$ column  
   (10% MeOH in EtOAc)

3) RP-C$_{18}$ column (40% aq. MeCN with 0.1% diethylamine)

4) Prep. TLC (repeated)  
   (MeOH:acetone:CH$_2$Cl$_2$
   1.5:1:7.5)

Compound 103  
(2.8 mg)

* % inhibition of AChE at 0.1 mg/mL

**Scheme 1** Isolation protocol for the sponge, *Corticum* 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 *Corticism*, was subjected to the further chemical investigation for the bioactive components. The bioassay-guided purification of the Thai sponge *Corticism* sp. led to the isolation of a new steroidal alkaloid (102), 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 *Corticism* sp.

The sponge *Corticism* 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$_2$Cl$_2$, and to MeOH. The CH$_2$Cl$_2$-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), SiO₂ column (10% MeOH in EtOAc), SiO₂-bonded phase C-18 column (40% aq. MeCN with 0.1% diethylamine), and repeated preparative SiO₂ TLC (MeOH:acetone:CH₂Cl₂ 1.5:1:7.5).

Compound 103 has a molecular formula of C₃₅H₅₂N₂O₂ as established by means of the El mass spectrum, which shows a molecular peak at m/z 508 ([M]+), and of its 33 carbon signals observable in the ¹³C NMR spectrum (125 MHz, C₆D₆; Figure 2). This molecular formula was supported by the HR-EIMS spectrum, which showed a molecular peak at m/z 508.4001 (calcld for C₃₅H₅₂N₂O₂ 508.4029). The proposed molecular formula requires the unsaturation degrees of 9. The ¹³C 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 νₘₐₓ 1740 cm⁻¹, confirming the presence of the carbonyl functionality. The UV spectrum showed the maximal absorption at λₘₐₓ 242 nm.

The ¹H NMR spectrum of 103 (500 MHz, C₆D₆; 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 $^{13}\text{C}$ NMR spectrum of 103 (125 MHz, C$_6$D$_6$)
Figure 3 $^1$H NMR spectrum of 103 (500 MHz, C$_6$D$_6$)
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 $\delta$ 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 $\delta$ 1.20 (m, H-5), 2.10 (m, H-6) and 5.23 (br s, H-7); fragment C at $\delta$ 1.74 (m, H-9), 1.83 (m, H-11) and 2.00 (m, H-12); fragment D at $\delta$ 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 $\delta$ 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.

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 $\delta_H$ 2.25 and $\delta_H$ 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.
Table 4 NMR data of 103 (500 MHz for $^1$H and 125 MHz for $^{13}$C; CD$_2$OD)

| Position | $\delta_{H}$ (mult.; $J$ in Hz) | $\delta_{C}$ (mult.) | HMBC correlation  
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<td>2.62 (br d; 2.6, 1H)</td>
<td>57.9 (CH)</td>
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<td>-</td>
<td>127.4 (C)</td>
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Table 4 (cont.)

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<thead>
<tr>
<th>Position</th>
<th>$\delta_H$ (mult.; $J$ in Hz)</th>
<th>$\delta_C$ (mult.)</th>
<th>HMBC correlation (C $\rightarrow$ H)</th>
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<td>H-23, H-29</td>
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<td>29</td>
<td>2.43 (m, 2H)</td>
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<tr>
<td>N-CH$_2$-27</td>
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</tr>
<tr>
<td>OCOCH$_3$-4</td>
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<td>OCOCH$_3$-4</td>
<td>1.72 (s, 3H)</td>
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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 ($\delta_C$ 12.3 and 15.1, and $\delta_H$ 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$_2$Cl$_2$-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$_6$D$_6$; Figure 5) suggested that 102 was a steroid derivative, with typical methine and methylene signals at $\delta$ 1.0-2.0. The major functionalities as deducible in the $^1$H NMR spectrum include a hydroxy group ($\delta$ 3.96), and an $E$-
Figure 5 $^1$H NMR spectrum of 102 (500 MHz, C$_6$D$_6$)
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$_6$D$_6$, 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.
### Table 5 NMR data of 102 (500 MHz for $^1$H and 125 MHz for $^{13}$C; C₆D₆)

<table>
<thead>
<tr>
<th>Position</th>
<th>$\delta_\text{H}(\text{mult.}; J \text{ in Hz})$</th>
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<th>HMBC correlation (C $\rightarrow$ H)</th>
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<td>130.5 (CH)</td>
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<td>-</td>
<td>78.8 (C)</td>
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<td>H-17, H-18</td>
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### 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

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cytotoxicity</th>
<th>AChE inhibition activity (IC₅₀; μM)</th>
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<tr>
<td></td>
<td>MCF-7</td>
<td>Hela</td>
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<td>103 (% inhibition at 5 μg/mL)</td>
<td>27.75</td>
<td>-48.09</td>
</tr>
<tr>
<td>galantamine</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>camptothecin (IC₅₀; μg/mL)</td>
<td>0.8×10⁻⁴</td>
<td>2.9×10⁻⁴</td>
</tr>
</tbody>
</table>

The IC₅₀ 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

<table>
<thead>
<tr>
<th>AChE</th>
<th>V_max (dmA/min±SE)</th>
<th>K_m (μM±SE)</th>
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<tr>
<td>without inhibitors</td>
<td>108.5±4.0</td>
<td>729±107.9</td>
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<tr>
<td>with 103 (7.0 μM)</td>
<td>47.5±3.2</td>
<td>3805±591.9</td>
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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 saligenenamide 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 evidently 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, axillaridrine A (34) and sarsaligenenone (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 SiO₂ 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.
REFERENCES


APPENDIX
IR spectrum of 103
COSY spectrum of 103 (500 MHz, C₆D₆)
HMBC spectrum of 103 (500 MHz, C₆D₆)
DEPT 135 spectrum of 103 (125 MHz, CD$_3$OD)
HMOC spectrum of 102 (500 MHz, C₆D₆)
HMBC spectrum of 102 (500 MHz, C₆D₆)
VITAE

Name Miss Roosanee Langjai
Student ID 4752009

Education Attainment

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List of Publication and Proceeding


R. Langjai, 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.