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.

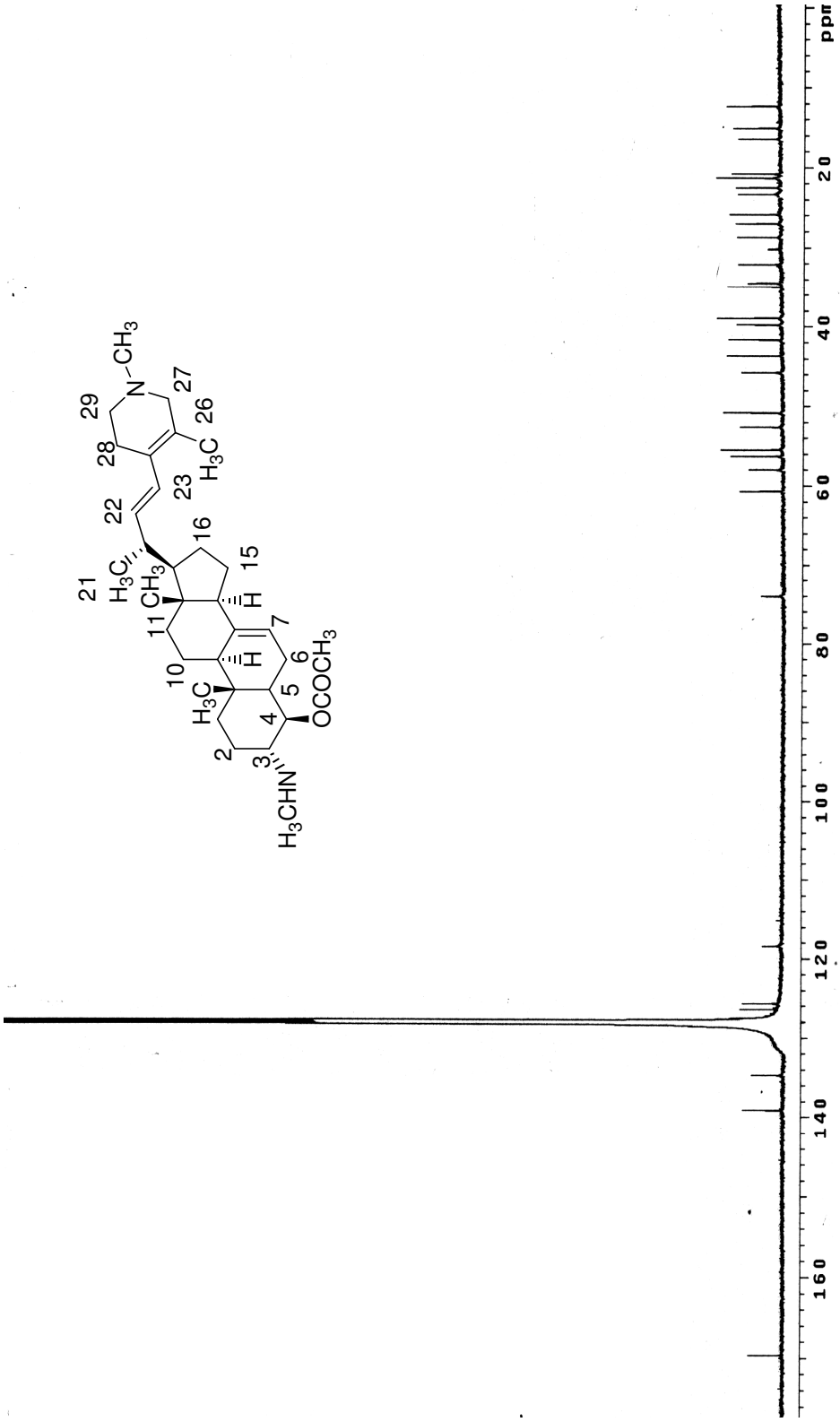


Figure 2 ^{13}C NMR spectrum of **103** (125 MHz, C_6D_6)

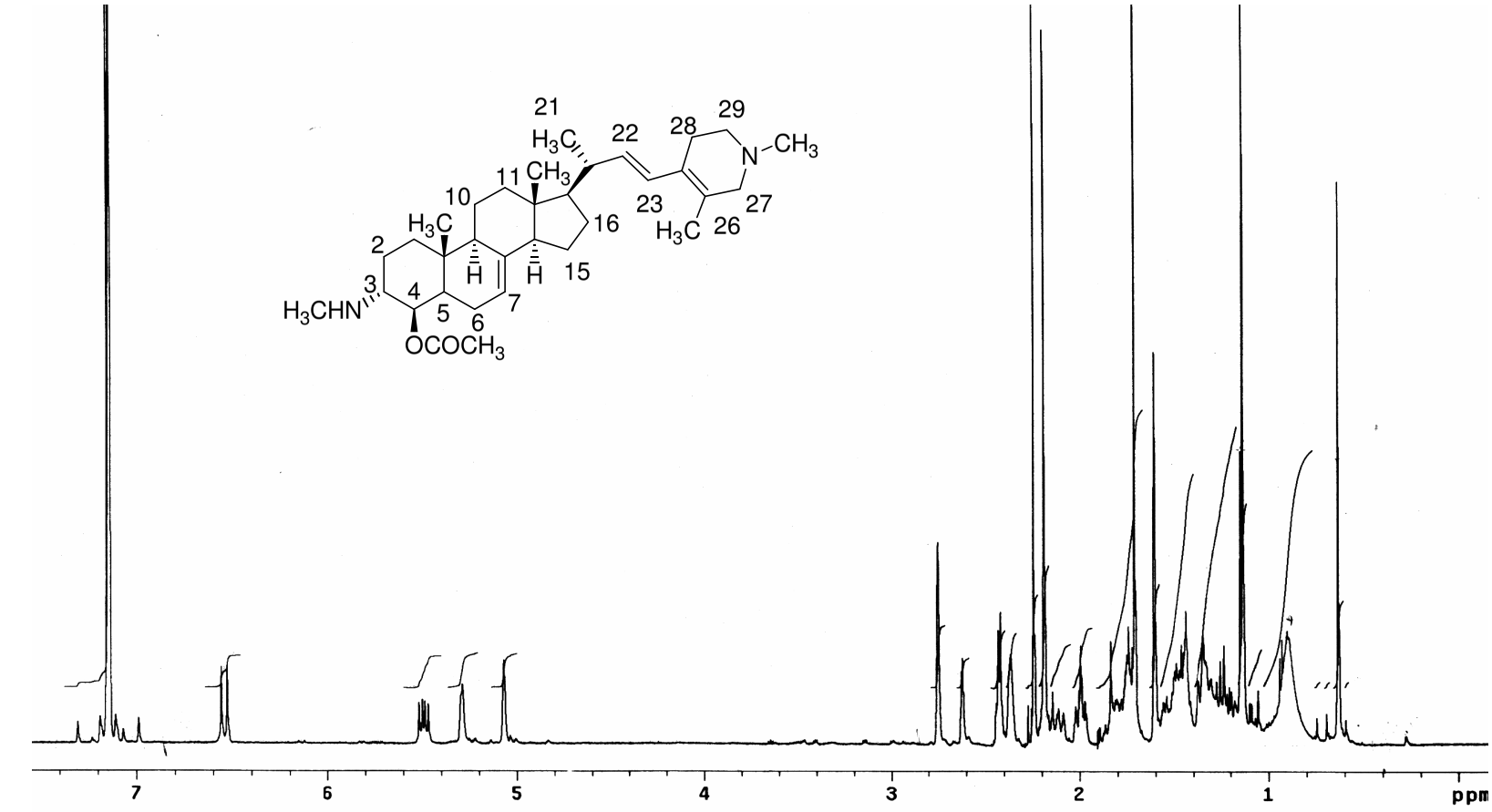
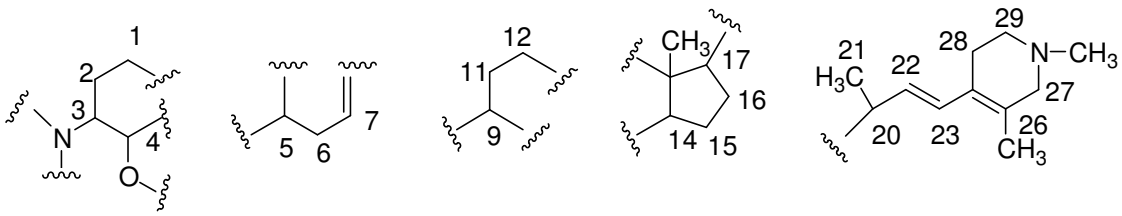


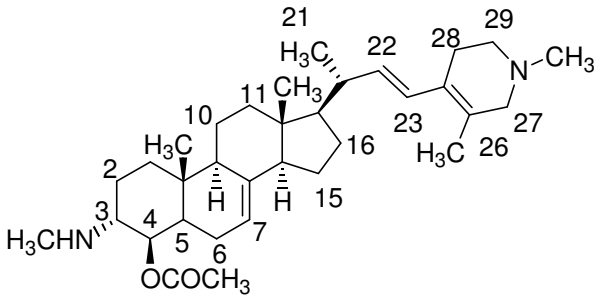
Figure 3 ^{1}H NMR spectrum of **103** (500 MHz, C_6D_6)

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 (δ 3.96), and an *E*-

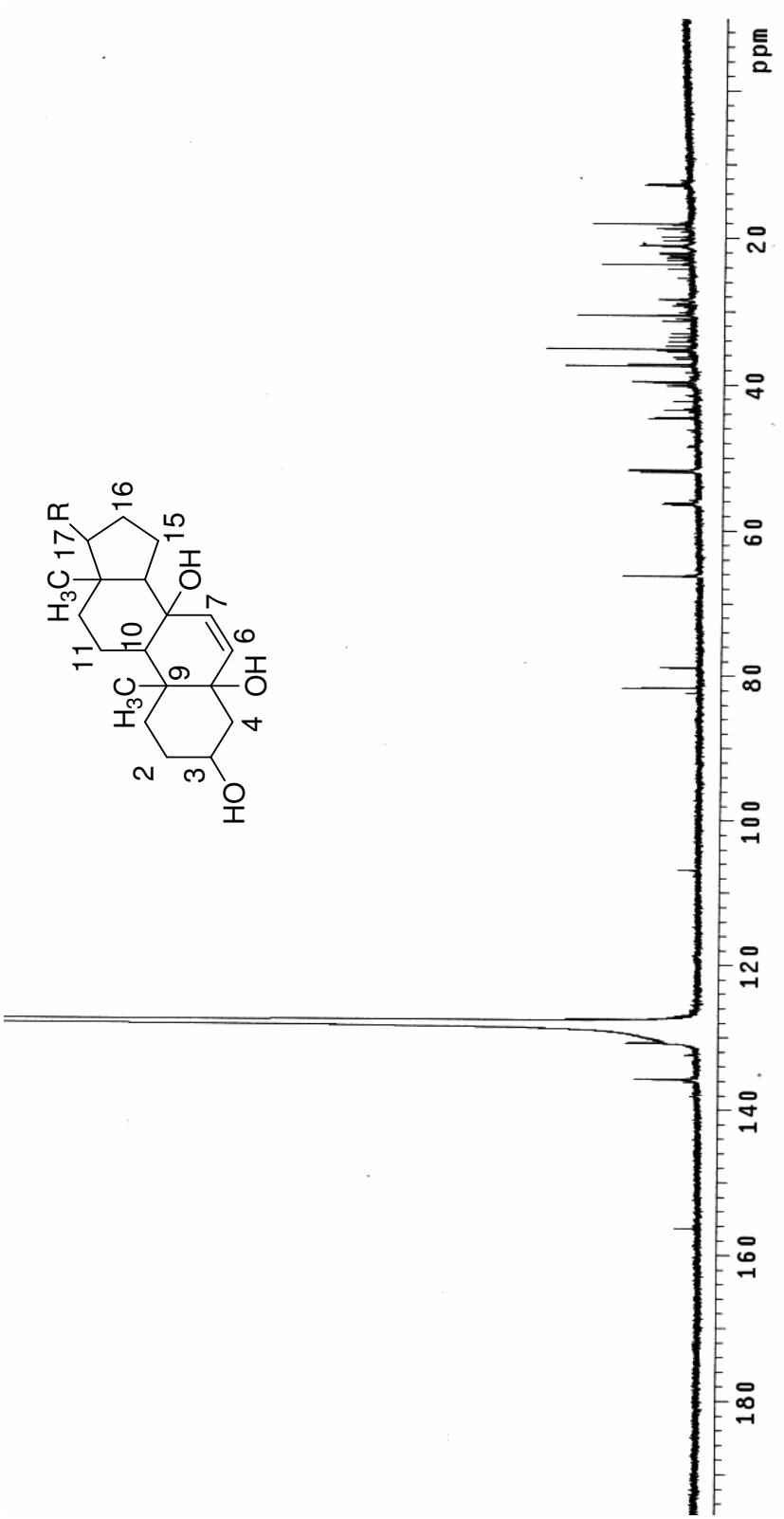


Figure 4 ^{13}C NMR spectrum of **102** (125 MHz, C_6D_6)

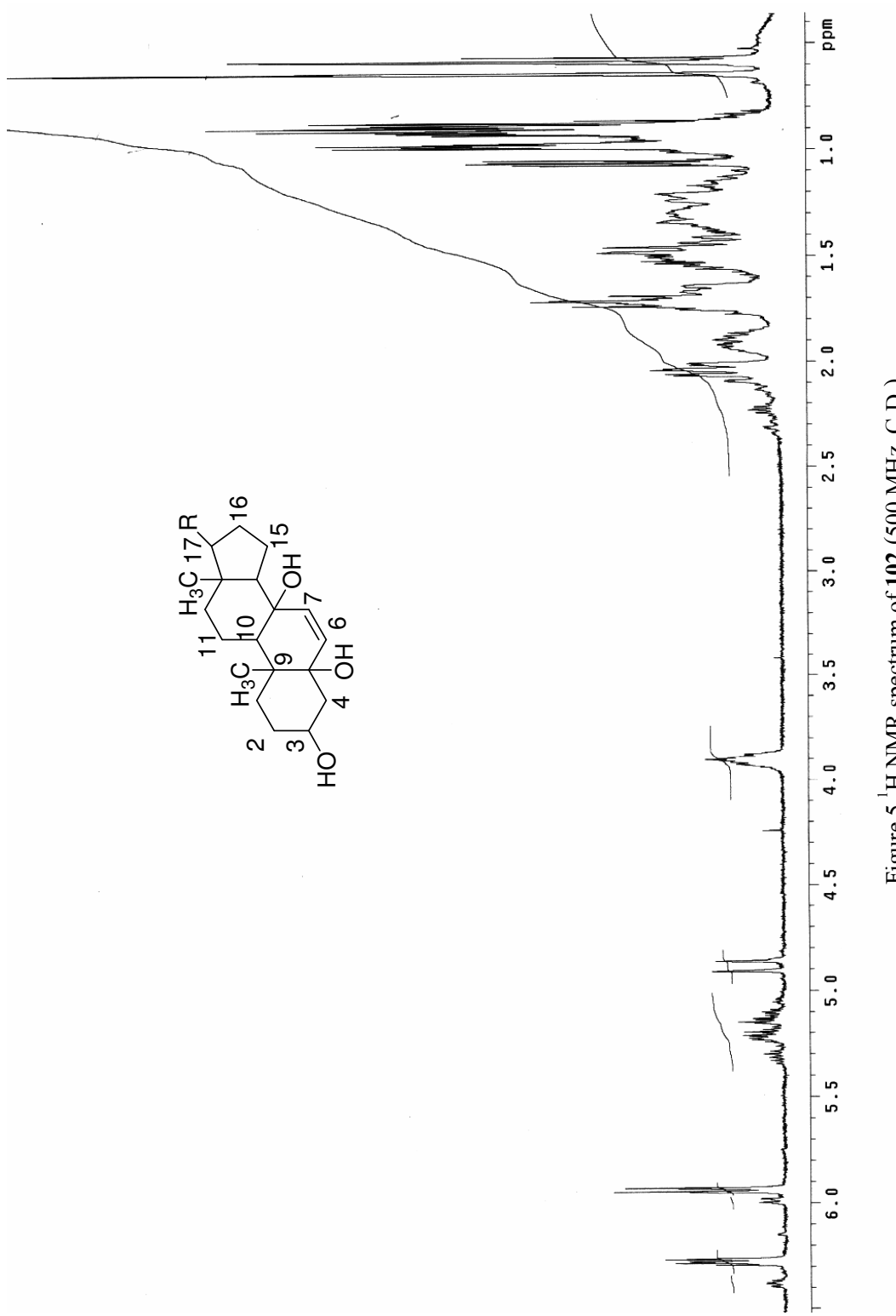
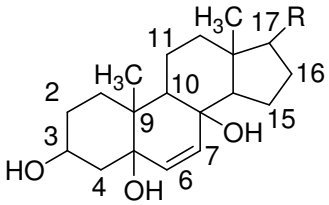


Figure 5 ^{1}H NMR spectrum of **102** (500 MHz, C_6D_6)

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.