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 Homosclero-phorida). 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

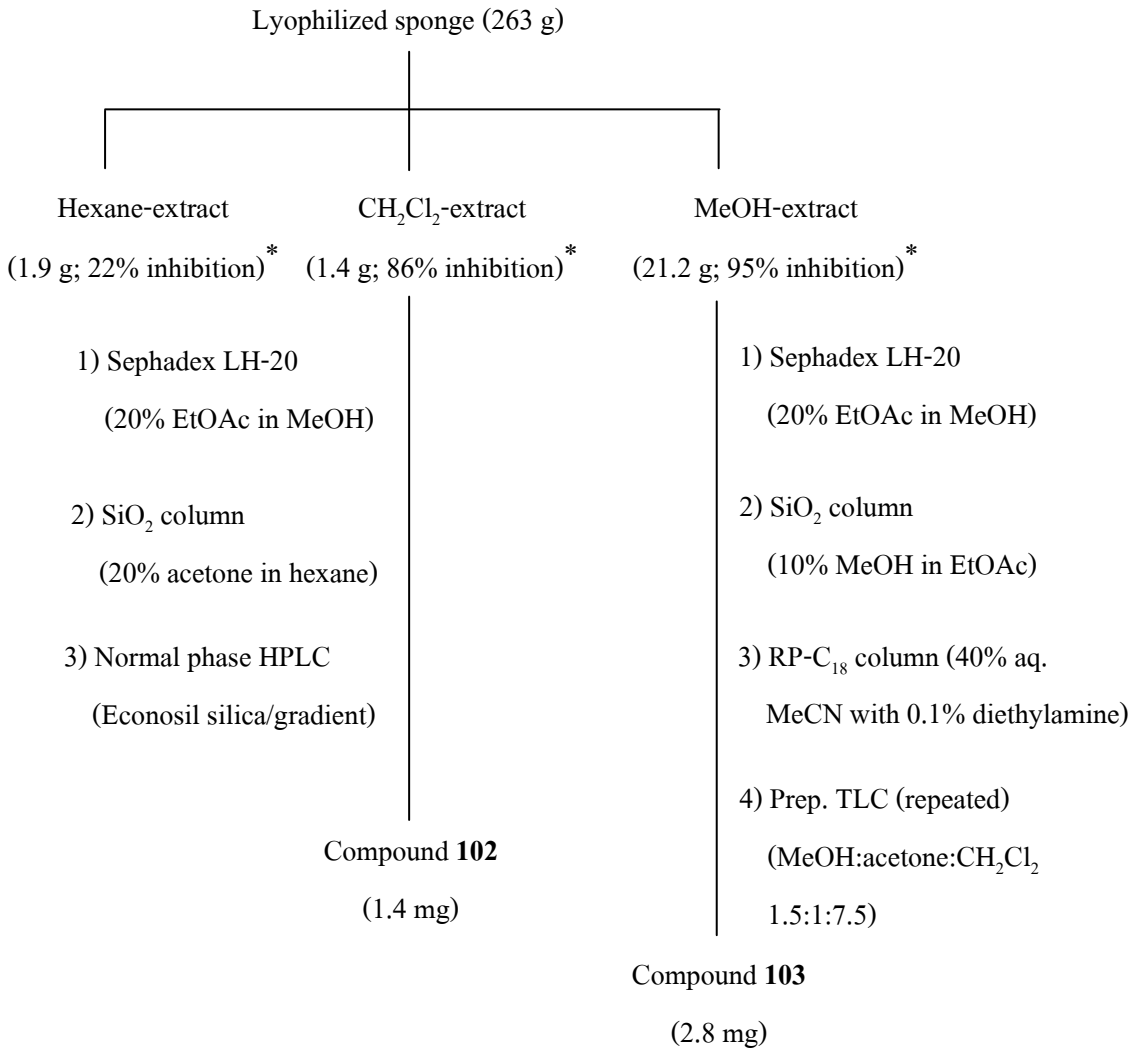
The sponge *Corticium* sp. was collected in April 2003 and April 2004. The freeze-dried specimens (263 g) were consecutively and exhaustively extracted with hexane, CH_2Cl_2 and MeOH (3×2 L, each) to yield the corresponding extracts (1.9 g, 0.7%; 1.4 g, 0.5%; 21.2 g; 8.1%, respectively). The isolation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