CHAPTER 2

EXPERIMENTAL

2.1 General

Unless stated otherwise, all chemicals and chromatographic packing materials were used as purchased. The solvents used for general purposes were all commercial grade, and were re-distilled prior to use. All HPLC solvents were HPLC grade, and were filtered though a 0.45 μm membrane filter and degassed in ultrasonic bath (30 min) prior to use. Analytical TLC was performed on silica gel 60 F 254 on aluminum supports (0.02 mm layer thickness; Merck®). Visualization was detected by means of UV absorption (254 nm), and of spraying with anisaldehyde reagent followed by heating. The size exclusion chromatography was conducted on a column of Sephadex™ LH-20 (GE Healthcare®), which was allowed to be saturated with eluting solvents as indicated for 24 hours prior to use. Flash chromatography was carried out on silica gel 60 (particle size 0.04-0.06 mm; Scharlau®).

The preparative HPLC was performed on a Water® 600E multisolvent delivery system. This was connected to a Water® 484 tunable detector and to a Rheodyne® 7125 injector port. Optical rotation was measured on a Perkin-Elmer 341 polarimeter (Germany). X-ray crystallography was operated on a Bruker-Nonius kappa CCD diffractometer (Germany). UV spectra were recorded on a Hewlett Packard® 8452A diode array spectrophotometer (France). IR spectra were recorded on a Jasco® IR-810 infared spectrophotometer (Japan). Mass spectra were operated on an MAT 95 XL mass spectrometer (Germany). NMR spectra were recorded on an FT-NMR Varian® Unity Inova 500 spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C) (Germany). The chemical shifts were reported in the δ scale relative to the solvent signals; i.e., benzene-d_6 (7.15 ppm of residual C_6HD_5 for ^{1}H NMR and 128.0 ppm for ^{13}C NMR) and chloroform-*d* (7.24 ppm of residual $CHCl_3$ for ^{1}H NMR and 77.0 ppm for ^{13}C NMR).

2.2 Sponge material

The sponge *Ciocalapata* sp. was collected from Koh-Tao, Surat-Thani Province, Thailand (10°07.569′ N; 99°48.665′ E) at the depth of 15-20 m. The specimens were kept in the

refrigerator at -20°C until the time of separation. The sponge appeared to be lumpy mass with porous texture and rough surface. The color of this sponge was pale khaki, with dark brown to green spots on its outer surface. The sponge was identified by Dr. Somchai Bussarawit of Phuket Marine Biological Center, Phuket, Thailand, to belong to the genus *Ciocalapata* (class Demospongiae, order Halichondrida, family Halichondriidae). The voucher specimen (PMBC 24607) was deposited at Phuket Marine Biological Center, Phuket, Thailand.

2.3 Bioactivity determination

2.3.1 Antimalarial activity

The antimalarial activity determination was performed at Bioassay Laboratory (BIOTEC central research unit, BIOTEC, Thailand), according to the microculture radioisotope technique as described by Desjadins et al (1979) and modified by Jongrungruangchok et al (2004). The tested parasites were *Plasmodium falciparum* K1 strain (chloroquine- and pyrimethamine-resistant), continuously cultured in RPMI 1640 medium containing 20 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid), 32 mM $NaHCO_3$, and 10% heat activated human serum, according to Trager and Jensen (1976). Briefly described, the mixture of 1.5% erythrocytes with 1% parasitemia at early ring stage (200 µL) was added into RPMI 1640 medium containing samples (25 µL) for 24 hours at 37°C in humidity air tight box, previously flushed with the mixture of O_2 (5%), CO_2 (5%) and N_2 (90%). [^{3}H]-Hypoxanthine in culture medium 25 µL was then added, and the culture was incubated for 24 hours. The level of incorporated radioisotope hypoxanthine, indicating the growth of parasites, was detected using TopCount microplate scintillation counter. The activity was reported in IC_{50} using dihydro-artemisinin as standard reference (IC_{50} = 4.4 nM).

2.3.2 Cytotoxic activity

The cytotoxicity determination was kindly supported by Assist. Prof. Dr. Supreeya Yuenyongsawad of Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University. The cell line used in this experiment was MCF-7 (breast adenocarcinoma). The cytotoxic activity was determined using SRB assayed according to a protocol adopted from Skehan et al (1990).

In brief, the monolayer culture of the targeted tumor cells in a 96-well microtiter plate was treated with a serial dilution of tested sample (five concentrations) in culture medium EMEM with glutamine, supplemented with 10% heat-inactivated new born calf serum, 50 IU/mL penicillin G sodium, 50 μg/mL streptomycin sulphate, and 0.125 μg/mL amphotericin B. The plate was incubated for 6 days at 37°C (5% CO_2 and 95% humidity), at the middle of which time the medium was refreshed. At the end of incubation, cells were then fixed with 100 μL of iced-cold 40% trichloroacetic acid (Aldrich Chemical) for 1 hour at 4°C, washed with distilled water (five times), and stained with 0.4% SRB in 1% acetic acid (Sigma®) for 30 minutes. The dye was rinsed and the plate was allowed to dry for an overnight. Tris base (Sigma®, 100 μL, 10 mM) was added. The plate was shaked on gyrator shaker for 20 minutes. The survival percentage of the cells was determined according to the intensity of the resulting pink color at 492 nm on Power Wave X plate reader (Bio-TEK instrument, Inc.). The IC_{50}'s were evaluated according to the survival-percentage plot, and were referred to standard camptothecin (Aldrich, IC_{50} towards MCF-7 0.0016 ± 0.0001 μM).

2.4 Isolation and purification

The freeze-dried sponge (279 g) was consecutively extracted with a series of solvents to yield hexane-, CH_2Cl_2-, and CH_3OH-extracts (7, 6, and 63 g, respectively). The hexane-extract (IC_{50} against *Plasmodium falciparum* K1 strain 0.05 μg/mL) was selected for the further fractionation using chromatographic techniques, beginning with vacuum chromatography over a SiO_2 column, eluted with step-gradient solvents starting from hexane to 50% CH_3OH in CH_2Cl_2. Compound **4** (101 mg) were obtained along with a series of fractional pools. Two major fractions were selected for the further isolation according to the potent antimalarial activity and their obtainable masses.

The less polar fraction I (IC_{50} 0.27 μg/mL) was separated using flash chromatographic technique over a SiO_2 column (2% EtOAc in hexane), followed by HPLC RP-C18 column (Phenomenex®, 10 μm, 250×10 mm; 10% aq CH_3CN, flow rate 4.5 mL/min) and compounds **2**, **3**, and **1** (4, 7, and 6 mg, respectively) were obtained (t_R's 20.8, 22.6, and 24.0 min, respectively).

The more polar fraction II (IC_{50} 0.45 μg/mL) was separated consecutively with Sephadex LH-20 (50% EtOAc in hexane), SiO_2 (50% EtOAc in hexane; and 9:18:73 THF:EtOAc:hexane), and HPLC RP-C18 (Phenomenex®, 10 μm, 250×10 mm, 5% aq CH_3CN, flow rate 3 mL/min) and compounds **5** and **6** were obtained (3 and 4 mg, respectively) at t_R's 21.1 and 33.8 min, respectively. All the isolation protocols are summarized in Scheme 1.

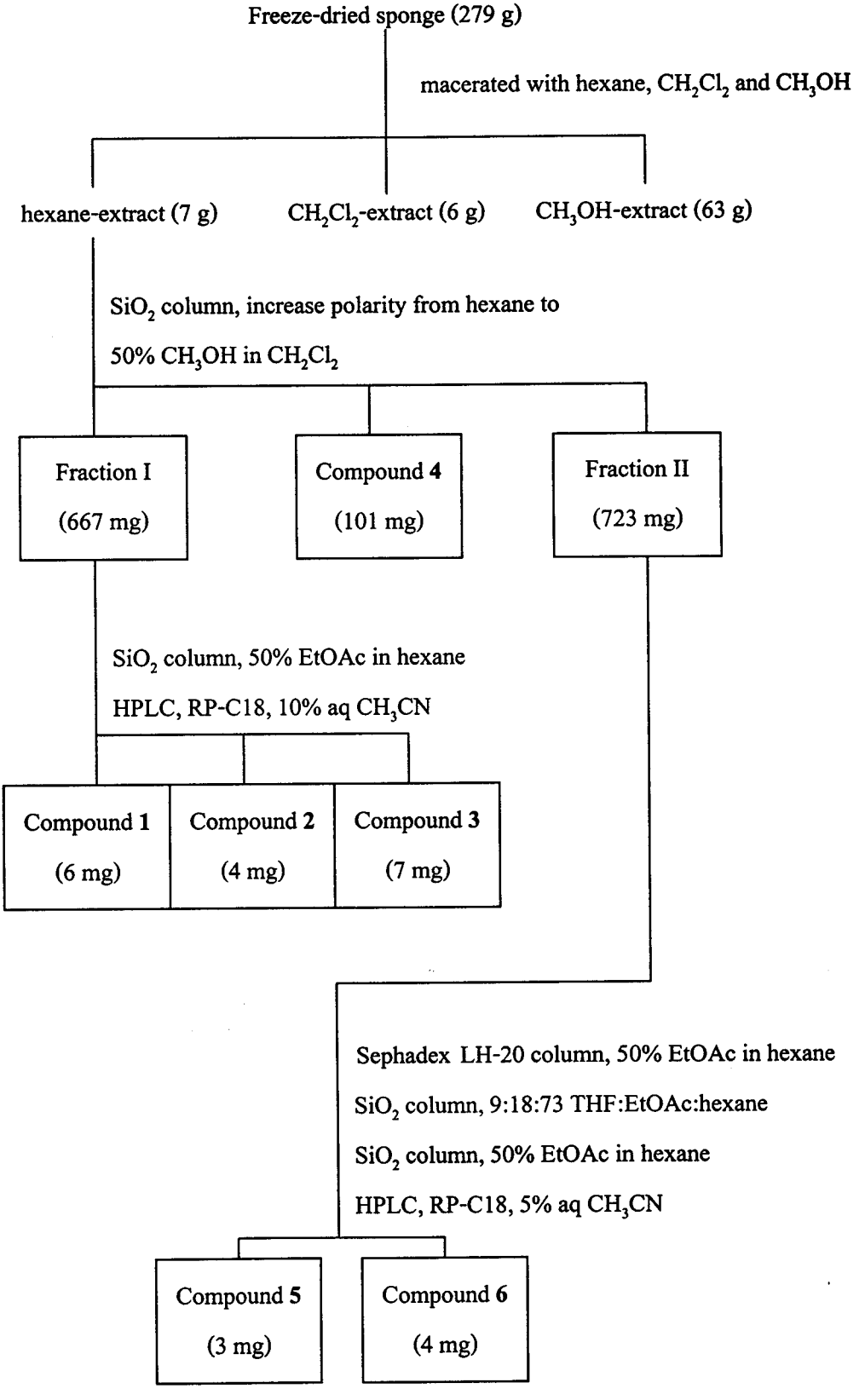
2.5 Physical properties of isolated compounds

8-Isocyanoamphilecta-11(20),15-diene (1): white amorphous solid; $[\alpha]_D$ -51 (*c* 0.33; $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 244 (2.38) nm; IR (thin film) ν_{max} 2950, 2150, 895 cm^{-1}; ^1H and ^{13}C NMR (500 MHz for ^1H and 125 MHz for ^{13}C) see Table 4; ESIMS *m/z* (% relative intensity) 320 ($[M+Na]^+$, 100). HRESIMS *m/z* 320.2338 (calcd for $C_{21}H_{31}NNa$, 320.2348).

7-Isocyanoamphilecta-11(20),15-diene (2): white amorphous solid; $[\alpha]_D$ +63 (*c* 0.25; $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 244 (2.70) nm; IR (thin film) ν_{max} 2950, 2125, 880 cm^{-1}; ^1H and ^{13}C NMR (500 MHz for ^1H and 125 MHz for ^{13}C) see Table 5; ESIMS *m/z* (% relative intensity) 320 ($[M+Na]^+$, 100), 271 (9).

8-Isocyanoamphilecta-11(20),14-diene (3): white amorphous solid; $[\alpha]_D$ -27 (*c* 0.38; $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 244 (2.57) nm; IR (thin film) ν_{max} 2925, 2125 cm^{-1}; ^1H and ^{13}C NMR (500 MHz for ^1H and 125 MHz for ^{13}C) see Table 6; ESIMS *m/z* (% relative intensity) 320 ($[M+Na]^+$, 100), 271 (18).

8,15-Diisocyano-11(20)-amphilectene (4): white needles (hexane); $[\alpha]_D$ -56 (*c* 0.28; $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 244 (2.41) nm; IR (thin film) ν_{max} 2925, 2150 cm^{-1}; ^1H and ^{13}C NMR (500 MHz for ^1H and 125 MHz for ^{13}C) see Table 7; ESIMS *m/z* (% relative intensity) 347 ($[M+Na]^+$, 8), 271 (30), 212 (100).



Scheme 1 Isolation scheme of the sponge *Ciocalapata* sp.

5,9-*epi*-Dioxyergost-6,22-dien-3,8,14-triol (5): white amorphous solid; $[\alpha]_D$ +15 (*c* 0.04; $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 244 (2.86) nm; IR (thin film) ν_{max} 3450, 3250, 2975, 2875, 1460, 1350 cm^{-1}; ^1H and ^{13}C NMR (500 MHz for ^1H and 125 MHz for ^{13}C) see Table 8; EIMS *m/z* (% relative intensity) 442 ($[M-H_2O]^+$, 4), 317 (100), 107 (77); HREIMS *m/z* 442.3085 (calcd for $C_{28}H_{42}O_4$, 442.3083).

5,8-*epi*-Dioxyergosta-6,22-dien-3-ol (6): white amorphous solid; $[\alpha]_D$ -16 (*c* 0.38; $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 244 (2.68) nm; IR (thin film) ν_{max} 3500, 3200, 2975, 2925 cm^{-1}; ^1H and ^{13}C NMR (500 MHz for ^1H and 125 MHz for ^{13}C) see Table 9; EIMS *m/z* (% relative intensity) 428 ($[M]^+$, 5), 396 (100), 149 (57).