CHAPTER 3

EXPERIMENT

3.1 Biological Material

Cyanobacterium was a *Lyngbya majuscula* (voucher specimen is available from Dr. Namthip Sitachitta as collection number NS042101-4) strain collected from Ratchamonkol Beach (depth 2-3 ft), Trung Province, Thailand, in April 2002. The sample was immediately preserved material in 2-propanol and keep at –40 °C until extraction.

3.2 Equipments

Nuclear magnetic resonance (NMR) spectra were recorded on Varian Unity Inova 500 instrument operating at 500.115 MHz for ^{1}H NMR and at 125.766 MHz for ^{13}C NMR. ^{1}H and ^{13}C NMR chemical shifts were referenced to residue solvent peaks: δ_H 7.26 and δ_C 77.0 for $CDCl_3$. The HSQC experiments were optimized for $^{1}J_{CH}$ = 140 and 250 Hz, and the HMBC experiments for $^{n}J_{CH}$ = 7 or 4 Hz. Optical rotations were determined using a Perkin Elmer model 341 polarimeter. UV and IR spectra were measured on HP4853 UV-Visible spectrophotometers, and Nicolet Fourier Transform Infrared Spectrophotometer: Impact 410, respectively. LR-FAB mass spectra were recorded VG-70SE mass spectrometer and HR-FAB mass spectra were performed with a JOEL JMS-700 spectrometer. The isolation of **27** to **29** was performed on Waters™ Model 662, a Waters™ Model 486MS variable-wavelength UV detector, and a Waters™ Model 600S controller.

3.3 Chemicals

Most solvents used in this research were commercial grade and were distilled prior to use. For HPLC, reagent grade solvents were used. Absorbents such silica gel

60 Merck (0.063-0.200 mm), sephadex LH-20 and alltech C18 500 mg 2.8 ml were used for chromatography techniques. TLC was performed on precoated Merck silica gel 60 F_{254} plates.

3.4 Marfey's Analysis

Hydrolysis of compound A (0.3 mg) was achieved in 0.5 mL of 6 N HCl placed in sealed ampoule at 105 °C for 12 hours. Trace HCl was removed under N_2 stream. The resulting hydrolysate was resuspended in 50 μL of 0.1% of 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide (FDAA, Marfey's reagent) solution in acetone and 100 μL of 0.1 N $NaHCO_3$ were added. The reaction mixture was heated at 80 °C for 3 min. The solution was cooled to room temperature, neutralized with 50 μL of 0.2 N HCl and diluted with 100 μL of MeCN:H_2O:TFA (50:50:0.05).

This solution was analyzed by reversed phase HPLC with two isocratic solvent systems (Alltech™ Econosil C18; solvent I: 40% MeCN + 0.05 % TFA, solvent system II: 37.5% MeCN + 0.05 % TFA) in 20 min at 1 mL/min (UV detection at λ 340 nm). Analyses in solvent I established the residue L-Val (8.31 min; D-Val, 11.54 min), L- N-MeVal (11.04 min; D-N-MeVal, 13.54 min), and L-Pro (5.65 min; D-Pro, 6.06 min). The remaining residue, L-allo-Ile (19.79 min; D-allo-Ile, 31.45 min; L-Ile, 19.97 min; D-Ile, 32.09 min) was analyzed in solvent II.

3.5 Bioassay Procedures

3.5.1 Brine Shrimp Toxicity Assay

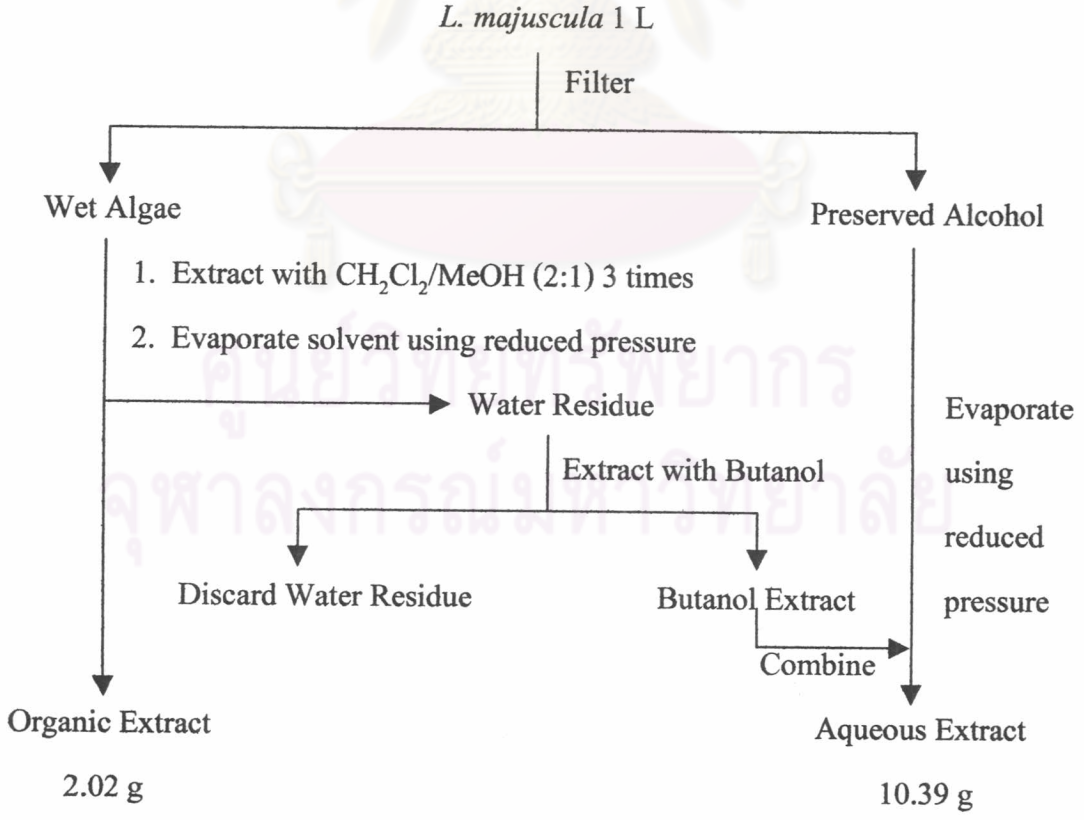
The method used is slightly modified from the original description.[44] About 5 newly hatched brine shrimp (*Artemia salina*) in ca. 0.5 mL artificial sea water were added to each well in a 24-well plate containing different concentrations of the sample in 50 μL DMSO and 4.5 mL artificial sea water to make a total volume of ca. 5 mL. Samples and controls were run in duplicate. After 24 hours at 30 °C, the brine shrimp were observed, and the number dead and alive were counted to generate LD_{100} values.

3.5.2 Ichthyotoxicity Assay

In the modified method[35], the tested extraction or pure compound was prepared by dissoved with 0.06 ml DMSO and added 40 mL distilled water in 100 mL beakers. Subsequently, a fish was introduced into the beaker and observed for 60 min. At this time, the fish was recorded for 'death', 'sublethal toxicity' or 'lack of effect'. The experiment was tested for two times and compared with controls that were prepared by dissoved with 0.06 ml DMSO and added 40 mL distilled water in 100 mL beakers and introduced a fish into the beaker.

3.6 Extraction

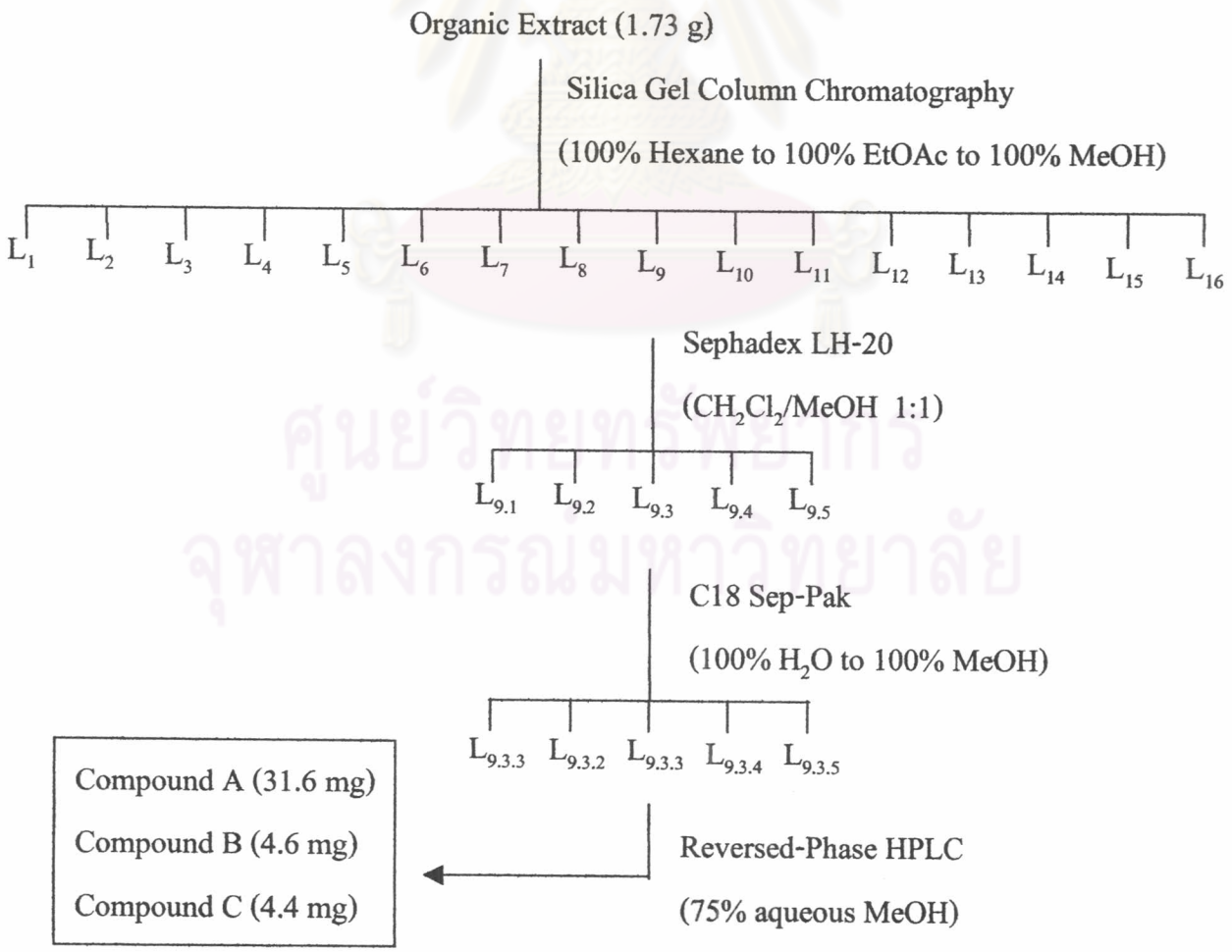
Approximately 1 L wet wt of preserved alga was extracted with CH_2Cl_2/MeOH (2:1) three times to give 2.02 g of crude organic extract and 168.6 g dry wt of extracted algal material. The procedures and results of the extractions were summarized in Scheme 3.1.



Scheme 3.1 Extraction Procedure of *Lyngbya majuscula*

3.7 Isolation and Purification

A portion of the organic extract (1.73 g) was subjected to Si gel column chromatography with a stepped gradient elution from 100% hexane to 100% EtOAc to 100% MeOH in EtOAc, giving sixteen distinctive fractions. Fraction 9 was purified by size exclusion chromatography Sephadex LH-20 using CH_2Cl_2/MeOH (1:1) as eluent to provide five major fractions (fraction 9-1 to 9-5). Fraction 9-3 was further separated by C18 Sep-Pak using stepped-wise gradient elution starting from 100% H_2O to 100% MeOH. Five fractions (fraction 9-3-1 to 9-3-5) were collected and concentrate to dryness. Reversed-phase HPLC (Alltech^{TM} Econosil C18, 5 μm, 4.6x250 mm; 75% aqueous MeOH in 110 min at 1 mL/min, and monitoring at 220 nm) of fraction 9-3-3 afforded compound A (31.6 mg, t_r = 28.0 min), compound B (4.6 mg, t_r = 56.5 min) and compound C (4.4 mg, t_r = 80.9 min). The procedures and results of the extractions were summarized in Scheme 3.2.



Scheme 3.2 Isolation Procedure of Organic Extract