# CHAPTER III

# EXPERIMENTAL

## SOURCES AND AUTHENTICATION OF SPONGES

The colonies of Thai sponge, *Biemna fortis* (Topsent), distribute in inter tidal area of Si-Chang Island, Chonburi, Thailand. They were collected from gravel or crevice between stones when it was low tide condition in October 1993. The sponge voucher specimen was preserved in 70% ethanolic solution and deposited at the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University. The remaining samples were chopped into small pieces and frozen at -20 $^{o}$C until they were extracted.

## GENERAL TECHNIQUES

### 1. Thin Layer Chromatography (TLC)

Adsorbent : The TLC plates for routine works were

(i) precoated TLC plates of silica gel G60 F-254 (E. Merck)

(ii) precoated TLC plates of silica gel RP2 F-254 (E. Merck)

(iii) precoated TLC plates of silica gel RP8 F-254 (E. Merck)

(iv) precoated TLC plates of silica gel RP18 F-254 (E. Merck)

(v) preparative TLC plates of silica gel 60 F-254 (E. Merck)

Layer thickness : 250 μm for precoated TLC plate

1 mm for preparative TLC plate

Technique : One way, ascending

Distance : 5.5 cm

Temperature : Laboratory temperature (29-35°C)

Detection : 1) Visual detection under day light

2) Visual detection under ultraviolet light at the wavelengths of 254 and 365 nm

3) Visual detection by spraying with 0.5% Anisaldehyde - sulfuric acid and glacial acetic acid reagent and heating at 110°C for 5 minutes

4) Iodine Vapor ;

Green and brown spots developed after treating the plate in Iodine vapor for 2-5 minutes.

Plate size : 6.7 x 5.0 cm for precoated TLC plates, 10 x 20 cm for preparative TLC plates

**2. Column Chromatography**

2.1 Gel Filtration Chromatography

Adsorbent : Sephadex LH-20

Packing : Adsorbent was suspended in the eluent and left for saturation in eluent for approximate 24 hours. The suspension was poured into a column and allowed to be settled tightly.

Addition of the sample extract :

The extract was dissolved in a small volume of eluent and carefully loaded, avoided disturbing gel surface, on the top of the column.

Examination of eluates:

Fractions were examined by TLC using visual detection under day light, ultraviolet light at wavelengths of 254 and 365 nm, iodine reaction and anisaldehyde - sulfuric acid spraying reagent, respectively. The similar fractions were combined and evaporated to dryness under reduced pressure.

Solvent : Hexane, Chloroform and Methanol

2.2 Flash chromatography

Adsorbent : Silica gel 60 (number 9385) particle size 0.040-0.063 mm (230-400 mesh ASTM)

Packing : adsorbent was wet packed. The slurry of adsorbent in the diluent (hexane) was poured into the column and allowed to be settled tightly under the pressure by the air pump.

Addition of the sample extract :

The extract was dissolved in a small volume of eluent and carefully loaded, avoided disturbing the surface of gel, on the top of the column.

Examination of eluates :

Fractions were examined in the same manner as described in section 2.2.1

Solvent : Hexane, Chloroform and Methanol

2.3 High Pressure Liquid Chromatography (HPLC)

The chromatogram of HPLC was received from the series apparatus of Quaternary Solvent Delivery System (constaMetric 4100 and constaMetric 4100 Bio), Solvent Delivery System (constaMetric 4100), Automatic Sample Injection System (autoMetric 4100), Photo Diode Array Detector (spectroMonitor 5000) and controlled all processes with program LCtalk window function, version 2.02

Adsorbent : Silica gel 60, Reversed phase RP-18

Particle size : 5 μm

Column length : 125 mm (analytical), 250 mm (semipreparative)

Internal diameter : 4.0 mm

Addition of the sample extract :

The sample was accurately weighed and dissolved with suitable amount of methanol to 1 mg/ml concentration and injected 10 μl in each loading. Fractionation depended on sample's peaks which were exhibited on chromatogram.

**3. Crystallization technique**

The compounds were crystallized in a suitable solvents that rarely dissolved compounds whereas extremely dissolved impure compounds, and in a suitable condition (low or room temperature).

**4. Acetylation reaction**

Flask of 5 ml contained 2 mg of sample and magnetic bar was loose closed with rubber stopper and then the flask was put in vacuum desiccator dried sample in approximately 30 minutes. Subsequently, the flask was tightly closed to protect moisture. Dry pyridine approximately 500 µl was instilled through rubber stopper for dissolve sample since this process until finished this reaction, mixed sample and reagents were stirred by magnetic stirrer and was flushed by $N_2$ gas. Added acetic anhydride 500 µl - 1 ml to form acetylated compound and left overnight for complete reaction. The reaction was checked by TLC technique compared with initial sample at begining after added acetic anhydride, and at every 6 hrs. later. After complete reaction that TLC technique checking disappeared of initial sample but showed acetylated compound instead, dry pyridine and acetic acid anhydride were taken out by evaporation. Acetylated compound was further absolutely dried by vacuum pump in desiccator.

**5. Spectroscopy**

5.1 Ultraviolet (UV) Absorption Spectra

Ultraviolet spectra were obtained with a Shimadsu double beam spectrometer (Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University). The samples were dissolved and adjusted the concentrations using chloroform (analytical grade, E. Merck) as solvent.

Sample Preparation : Accurately weighed 1 mg of sample dissolved and adjusted with methanol in 10 ml volumetric flask. The dilution can be made in order to obtain a certain concentration for calculate absorbtivity.

5.2 Infrared Spectra (IR)

Infrared spectra were obtained with a Shimadsu IR-440 infrared spectrometer (The Scientific and Technological Research Equipment Center, Chulalongkorn University), using potassium bromide discs or sodium chloride discs to determine the spectra.

5.3 Mass Spectra (MS)

The Electron Impact Mass Spectra (EIMS) were obtained with a Fisons VG Trio 2000 quadrupole mass spectrometer (which was operated at 70 eV at the Department of Chemistry, Faculty of Sciences, Chulalongkorn University) and Finigan Mat Incos 50, quadrupole mass spectrometer (which was operated at 70 eV at the Department of Chemistry, Faculty of Sciences, Mahedol University). The fast atom bombardment mass spectra (FAB MS) were obtained from the departmetn of Marine Sciences, University of the Ryukue.

5.4 Proton and Carbon-13 Nuclear Magnetic Resonance ($^1$H and $^{13}$C NMR)

The 500 MHz $^1$H NMR spectra, 125 MHz. $^{13}$C NMR spectra and 2D NMR spectra were obtained with a JEOL JMN-A500 spectrometer (The Scientific and Technological Research Equipment Center (STREC), Chulalongkorn University).

The chemical shifts were reported in ppm scale using the chemical shift of trimethylsilane (TMS) at 0 ppm as the reference signal. Whereas deuterated chloroform ($CDCl_3$) + 0.05% TMS (V/V) and deuterated methanol ($CD_3OD$) were used as operating solvents.

**6. Solvents**

All organic solvents, which were used throughout this work, were commercial grade and had to be redistillated prior to use, excluding the solvents for HPLC were analytical grade and also filtered through filter paper (Nyloaflo$^{R}$, diameter 47 mm, pore size 0.45 μm, Nylon Membrane filters of Gelman Sciences) before used.

## BIOACTIVITY DETERMINATION

**1. Cytotoxic activity**

The study for bioactive compounds, cytotoxic activity against cell lines, represent solid tumor, are very importance and very interesting. The sample were tested against some human tumor cell lines and cultured tumor cells from the test animals. The determination was helpfully supported by Professor Lola Garcia Gravaloss, Screening Department, Pharma Mar, S.R., SPAIN.

1.1 Target Cells

The cell lines utilized as the target cells in this test were

(i) P388 (a methylcholanthrene-induced lymphoid neoplasm in a DBA/2 mouse, a non-anchorage dependent cell lines)

(ii) A-549 (monolayer culture of a human lung carcinoma)

(iii) HT-29 (monolayer culture of human colon adreno-carcinoma, moderately well differentiated)

It is noted that the P388 was the induced tumor cell lines in mouse. While the A-549 and HT-29 cell lines were the human tumor cell lines.

1.2 Bioassay

In P388 cell line testing, the cells were incubated in the presence of the test sample for 48 hours. The growth cells were examined by counting cell using the

electronic counting device. The results of each sample was expressed by the $IC_{50}$ in μg/ml, it means the concentration of sample that inhibit 50% the growth of the indicate cell lines. The degree of cytotoxicity is high ($IC_{50}$ : <1 μg/ml), medium ($IC_{50}$ : 1-5 μg/ml) and small ($IC_{50}$ : 5-20 μg/ml).

In human tumor assay, the cells were plated overnight in 96-well microtiter plates. Serial dilutions of the test sample were added and cells were incubated for 4-6 days. The measurement of cell growth was performed by a colorimetric assay using 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), a formazan dye, which world be cleaved by active mitochondria to produce a blue color. Optical density which was directly proportional to cell number was measured by spectrometer (Mosmann, 1983).

**2 Sea Urchin egg Lethality Test**

This bioassay was applied from the Embryology study of Echinoderm. Whereas the development of embryo in the period of fertilized egg to cleavage is very rapid just like in tumor cells. Thus, they can demonstrate to represent anti tumor activity of test sample if the test sample can inhibit the cleavage of fertilized eggs and the characteristic of Oligolecithal egg, small egg yolk, which can easy to observe cleavage process. In addition to reported the result in term of $LD_{50}$, the result of, this bioassay method also could demonstrate to predict the mechanism of activity such as; DNA synthesis inhibitors, RNA synthesis inhibitors, Micro tubule assembly inhibitors or Protein synthesis inhibitors which induced to research cytotoxic activities (Fusetani, 1988).

2.1 Sea Urchin Egg Preparation

Sea Urchin in reproductive cycle. Both mature female and male were eviscerated the grinders and ovary (female) out of their body. Then, their body were light shake in sea water in order to release sperms or mature eggs. It is considered that released sperms in sea water were not diluted before fertilize with eggs. In some

cases, female would excited to release eggs by inoculate 0.5 Molar KCl or sea water in intracoelomic (Giudice, 1973).

Mixed mature eggs and more amount of sperms for fertilization. It is noted that if excess sperms were mixed, polyspermy and failure fertilization would appear. After fertilization for 5-10 minutes, fertilized eggs would be randomed to check under microscope (100-400x), Jelly coat was observed of mature eggs were not fertilized. In the other hand fertilization membrane was observed if mature eggs were fertilized.

2.2 Sample Preparation

The test sample was dissolved in ethanol and diluted to be concentration of 1 mg/100 ml. 100 ml of this stock solution was filled with 900 ml of sea water to make concentration of 100 mg/ml and 10 mg of stock solution was filled with 990 ml of sea water to be concentration of 10 mg/ml. Both concentration, 100 and 10 mg/ml, were prepared in triplicate. Only sea water in petridisk was prepared to be control, triplicate, and carried out in the same fashion as test sample.

2.3 Bioassay

100 ml of each concentration in petridisk, triplicate, was added with fertilized eggs (approximate 300 eggs/disk) 6, 12 and 24 hours subsequently, the development of fertilized eggs were detected, compared with control disc which embryo would pass through cleavage period to blastula and gastrulation period in finally, and counted division and undivision cells by microscope. The results were reported in term of $LD_{50}$ which indicated the strength of cytotoxic activity while the development of fertilized eggs observed were indicated to mechanism of their activity.

**3 Brine Shrimp Lethality Activity Test**

The bioassay was applied from the method of Meyer et al. (1982)

3.1 Brine Shrimp

The eggs of *Artemia salina* Leach (Aquarium product$^{R}$) were hatched in a shallow rectangular dish which was divided into 2 sections by a plastic divider bearing a few of 2 mm holes. One dark section contains the shrimp's eggs, while another one was illuminated by a tungsten lamp. This disc was filled with artificial sea water and approximate 6 mg/l dried yeast to be food for nauplii of the shrimp. After 48 hours at 25-29$^{o}$C, the nauplii of the shrimp had been allowed to be mature and they would move to the lighted part whereas their shells were left to float in dark part, divided by plastic divider.

3.2 Artificial Sea Water

The commercial salt mixture (Instant Ocean, Aquarium System) 38 g was dissolved in 1 litre of purified water and then filtered through filter paper (Whatman$^{R}$ filter paper No. 1). This artificial sea water would give 1 U.S gallon (38 grams/litre).

3.3 Sample Preparation

The test sample was prepared in three concentrations 0.1, 0.01 and 0.001 mg/ml of artificial sea water, respectively. Each concentration was made in triplicate. Methanol was used to dissolve the test sample and dilute to proper concentrations. When the sample rarely dissolved it artificial sea water, the sonication was able to be obtained to help dissolving. Methanol vials were prepared to be control vials, triplicate, and carried out in the same fashion as test sample.

3.4 Bioassay

Ten mature nauplii were transferred to each vial with disposable pipette by counting them in the stem of the pipette against the tungsten light background. Subsequently, one drop of yeast suspension (3 mg of dry yeast in 5 ml of artificial sea water) was added into each vial as food for the nauplii. Then, the vials were maintained under illumination. After 24 hours, the dead nauplii in each vial were counted and subtracted with the number of dead nauplii in control vials. Finally, the value of concentration, the number of initial nauplii in each triplicate concentration and the number of dead nauplii were key to computer. After that $LD_{50}$ value would calculate and report.

**4 Antimicrobial Activity**

The disc method was executed in this determination of antimicrobial activity.

4.1 Microorganisms

4 types of microorganisms were used in this test. Throughout this test were kindly supplied by Assistant Professor Sathaporn Sirotamarat of Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University. These microorganisms are

- Staphylococcus aureus ATCC 6538 P
- Bacillus subtilis ATCC 6638
- Escherrichia coli ATCC 25922
- Candida albicans

All of these microorganisms were subcultured in suitable media prior to use in order to intensify their activities. The inoculation was carried out at 37°C, 18-24 hours for bacteria or 48 hours for yeast.

4.2 Media

The medium used 3 formula. One was prepared for NA slants which were used in subculturing bacteria. The other was prepared for TSA for inoculating the bacteria during the assay. The last one was prepared for SDA slants for subculturing yeast and inoculating yeast during the assay.

(i) Nutrient agar (NA)

Formula per litre of purified water:

| | |
|---|---|
| Beef extract | 3.0 g |
| Peptone | 5.0 g |
| Agar | 20.0 g |

Final pH 7.4 ± 0.2

All ingredients were suspended in purified water 1 litre and thoroughly mixed. It was heated with frequent agitation and boiled for 1 minute to completely dissolve. The medium was sterilized by autoclaving at 121°C for 15 minutes.

(ii) Trypticas soy agar (TSA)

The Medium used was BBL$^{R}$ Trypticase$^{R}$ Soy Agar (Becton-Dickinlon Microbiology system).

Formula per litre of purified water:

| | |
|---|---|
| Pancreatic digest of casein | 15.0 g |
| Papaic digest of casein | 5.0 g |
| Sodium chloride | 5.0 g |
| Agar | 15.0 g |

Final pH 7.3 ± 0.2

Powder 40 g was suspended in purified water 1 litre and thoroughly mixed. It was heated with frequent agitation and boiled for 1 minute to complete dissolve. The medium was sterilized by autoclaving at 121°C for 15 minutes.

(iii) Sabouraud dextrose agar (SDA)

The medium used was BBL$^{R}$ Sabouraud Dextrose Agar (Becton-Dickinson Microbiology System).

| Formula per litre of purified water: | |
|---|---|
| Pancreatic digest of casein | 5.0 g |
| Peptic digest of animal tissue | 5.0 g |
| Dextrose | 40.0 g |
| Agar | 15.0 g |
| Final pH 5.6 ± 0.2 | |

Powder 65 g was suspended in purified water 1 litre and thoroughly mixed. It was heated with frequent agitation and boiled for 1 minute to completely dissolve. The medium was sterilized by autoclaving at 121°C for 15 minutes.

4.3 Sample Preparation

The test sample was dissolved in methanol and diluted to the concentration of 5 mg/ml. 0.02 ml of this solution was transferred to a 6.0 mm disc (Whatman$^{R}$ AA disc for antibiotic assay) and allowed to dry. The final concentration was 0.10 mg/disc. The determination was made in duplicate.

4.4 Bioassay

Throughout procedures in this bioassay were carried out by the aseptic technique. All glasswares and materials had to be sterilized before use with hot air oven at 180°C for 1 hour.

Each microorganism was suspended in sterilized normal saline solution. The turbidity of the suspensions can adjusted to be equal to that of Mac Farland suspension No.1 (equivalent to 300,000,000 cell/ml). Each microorganism suspension, then, was three-dimensionally spreaded on the surface of suitable medium plate.

The sample discs were put on the microbe-spreaded medium plates. These plates, then, were incubated at 37°C for 18-24 hours for bacteria, or for 48 hours for yeast. After inoculation, the clear zone would appear if was measured and reported in scale of millimeter.

**THE EXTRACTIONS**

155 Kg wet weight of Thai sponges, *Biemna fortis* (Topsent), were chopped into small pieces and repeatedly macerated for 3 times in methanol (each, for 125 litre and 2 days). Recovered methanol was re-used in the second and the third maceration. Filtration with kieselguhr and paper filter (Whatman® No.1) were processed after maceration. The filtrate of each maceration was carefully concentrated to remove some methanol under reduced pressure at approximate 50°C because the nature of this filtrate was easy to float so, it could bump in this condition. The concentrated filtrate was partitioned with dichloromethane (60 litre). The extract was separated in dichloromethane extracts (F042) and aqueous extracts (F043). F042 was evaporated to dryness under reduced pressure to yield 28.5 g of dark-brown mass (0.01425% based on wet weight of sponge). Approximate 60 mg was reserved for a reference sample and bioactivity screening.

Fraction F042 was diluted in methanol (360 ml) and added water (40 ml) to make 90% methanol in water. This solution was partitioned with hexane (400 ml). Both extracts were dryness by evaporated under reduced pressure and absorb moisture with silica gel in vacuum. The yield of hexane extract (F044) and methanol extract (F045) were 7.446 g and 20.377 g (0.0048% and 0.01315% based on wet weight of

sponge), respectively. It is considered that every fractions were reserved in a little amount for references and bioactivity screening.

After these liquid-liquid extraction technique, isolation processes were gone on by chromatography technique as shown in scheme I which was isolated by consideration of constituent' quantities, observed by analytical TLC. On the other hand, Scheme II showed isolation scheme of the bioactive constituent followed the bioassay of cytotoxic activity and $^1$H-NMR spectra of fraction's survey.

## ISOLATION FOR BIOACTIVE COMPOUNDS AND STEROIDS

### 1. Bioassay-Directed and $^1$H-NMR-Directed Isolation of Bioactive Constituents (K201) from the Thai Sponge, *Biemna fortis* (Topsent)

Several interesting bioactivities were preliminarily screened for the organic extracts of *Biemna fortis* (Topsent). It was found that the dichloromethane extract (F042) and the butanol extract (F046) showed high cytotoxicity against cell lines P-388, A-549 and HT-29. Moreover, F042 inhibited cell division of fertilized sea urchin egg. On the other hand, the two fractions did not show both brine shrimp lethal activity and antimicrobial activity.

The isolation of bioactive compounds was summarized in Scheme II, F045 (20.38 g) was subjected to fractionate by sephadex LH-20 column chromatography technique using column size 2.5 x 25 cm and 10% methanol in chloroform as the eluent. The eluants were collected based on color bands showing on the column as shown in Table 1 and were examined by TLC using 10% methanol in chloroform as the developing solvent. The fractionation of F045 yielded 4 fractions (F047 - F050).

F048 which comprised large amount of sample and showed interesting quenching spots by UV 254 nm detection was considered for further fractionation. Two portions of F048 were divided for silica gel flash column chromatography (3.24 g

of sample, 4 x 15 cm, gradient MeOH in $CHCl_3$) and sephadex LH-20 column chromatography (7.39 g of sample, 2.5 x 90 cm, $CHCl_3$) to yield F055 - F060 and F051 - F054, respectively.

F051 from sephadex LH-20 column exhibited significant cytotoxicity ($IC_{50}$ = 0.2 mg/ml) comparing with F048. Sephadex LH-20 column (column size 2.5 x 90 cm) with 20% hexane in chloroform was successively used to concentrate the active fraction (F140) from F051 which was then repeatedly fractionated by sephadex LH-20 with 80% hexane in chloroform. Fractions from F140 were combined based on $^1$H-NMR spectral data (Figure 2-11) to yield F167 - F171.

Table 1. The fractionations of F045 (F047-F050) and F048 (F051-F054, F055-F060)

| Fraction No. | Color band | Weight (g) |
|---|---|---|
| 047 | dark brown | 2.8 |
| 048* | brown | 11.38 |
| 049 | green | 0.61 |
| 050 | green yellow | 2.12 |
| 051* | dark brown | 1.25 |
| 052 | brown | 2.7 |
| 053 | green | 2.8 |
| 054 | green yellow | 3.0 |
| 055 | pale yellow | 0.14 |
| 056 | yellow | 2.35 |
| 057 | orange | 0.60 |
| 058 | yellow | 0.40 |
| 059 | pale yellow | 42.3 |
| 060 | dark green | 0.31 |

F169 (250 mg) which comprised interesting and distinguished peaks in $^1$H-NMR spectrum and showed the highest interesting cytotoxicity ($IC_{50}$ = 0.25 μg/ml) was further isolated by sephadex LH-20 column chromatography with 100% hexane as eluent, column size 2 x 42 cm, to yield F183 -F188. Subsequently, F187 (40 mg) the most active fraction from F169 was subjected to isolate by means of sephadex LH-20 column using a longer column (column size 3 x 60 cm) with hexane to give F193 -

F196. These fractions were combined based on color bands on the column and similar TLC pattern on RP-2 plate using 20% water in methanol as the developing solvent.

Table 2. The fractionations of F140 and F169 to receive F167 - F171 and F183 - F188

| Fraction No. | Color band | Weight (mg) |
|---|---|---|
| 167 | light brown | 5 |
| 168 | black | 754 |
| 169* | deep brown | 257 |
| 170 | light brown | 84 |
| 171 | (MeOH eluate) | 103 |
| 183 | brown | 52 |
| 184 | yellow | 54 |
| 185 | light yellow | 42 |
| 186 | deep yellow (1) | 18 |
| 187* | deep yellow (2) | 46 |
| 188 | bright yellow | 3 |

Table 3. The fractionation of F187

| Fraction No. | Color band | Weight (mg) |
|---|---|---|
| 193 | yellow (1) | 3 |
| 194 | yellow (2) | 8 |
| 195* | brown | 26 |
| 196 | yellow (3) | 2 |

F195 exhibited the same interesting positive Iodine-vapor spot on RP-2 TLC pattern as F187. F195 (26 mg) was repeatedly isolated by means of shorter packing of Sephadex LH-20 column (column size 2 x 20 cm) with hexane following by 50% hexane in chloroform to collect yellow band. F197 - F200 were collected and the interesting spot on RP-2 TLC pattern was concentrated in F198 (the yellow band on column chromatography).

Table 4. The fractionation of F195

| Fraction No. | Color band | Weight (mg) |
|---|---|---|
| 197 | (clear) | 2 |
| 198* | yellow | 15 |
| 199 | white - yellow | 3 |
| 200 | brown | 6 |

Because the amount of bioactive compounds in F198 could be increased by isolation from the remained F045. Therefore, the remained F045 (1.9 g) was fractionated by sephadex LH-20 column chromatography (column size 2.5 x 50 cm, 20% hexane in chloroform, 3 times) to give F149 - F155 (254, 204, 198, 72, 62, 23 and 527 mg, respectively). The eluates were examined by TLC technique and the fractions that showed similar TLC were combined. Fortunately, bioactivity was concentrated in F150 (32 mg) and F151 (70 mg). Subsequently, both fractions were isolated by means of sephadex LH-20, 1 x 60 cm, 2.5 x 25 cm, in hexane and followed by 50% hexane in chloroform to yield F189 - F192 and F227 - F231, respectively as shown in Table 5. It was found that the interesting spot on RP-2 TLC pattern was in F190.

Table 5. The fractionations of F150 and F151

| Fraction No. | Color band | Weight (mg) |
|---|---|---|
| 189 | yellow | 4 |
| 190* | clear | 13 |
| 191 | deep yellow | 4 |
| 192 | light yellow | 7 |
| 227 | pale yellow | 2 |
| 228 | yellow | 3 |
| 229* | light yellow | 2 |
| 230 | deep yellow | 27 |
| 231 | green yellow | 17 |

Based on the bioactivity and $^{1}$H-NMR - directed isolation, F198 and F190 fractions should contain bioactive compounds. F190 (13 mg) and F198 (15 mg) were

subsequently purified by RP-2 TLC (20 x 20 cm) technique with 20% $H_2O$ in MeOH. to yield F201 (3.4 mg) and F209 (1.2 mg), respectively.

The mass spectra data (Figure 12 - 15 ) indicated that there were more than one compound and proposed to be the isomerism compounds. Subsequently, HPLC technique (semipreparative RP-18 column) was tried for F201 (from F150) and F229 (from F151) 15% $H_2O$ in MeOH as eluent to give fractions A001 - A005 and A006 - A010, respectively. Fractions A004 and A007 were collected based on the main peak of HPLC chromatogram (Figure 16) which showed the same retention time and the same isogram of UV scan compared with the main peak on HPLC chromatogram and isogram of UV scan of F201.

In addition, A004 and A007 also showed the same interesting peak of $^1$H-NMR pattern as F201.

Table 6. The preparative RP-2 TLC technique fractionations of F190 and F198

| Fraction No. | $R_f$ | $I_2$ Vapor Test | UV Detection $\lambda_{max}$ (nm) | | visible | weight (mg) |
|---|---|---|---|---|---|---|
| | | | 254 | 365 | | |
| 201 | 0.63 | +ve | Quenching | - | yellow | 3.4 |
| 202 | 0.33 | -ve | - | green | - | 0.2 |
| 203 | 0.32 | -ve | - | blue | - | 0.5 |
| 209 | 0.62 | +ve | Quenching | - | yellow | 1.2 |
| 210 | 0.56 | +ve | Quenching | - | yellow | 0.5 |
| 211 | 0.54 | -ve | - | green | - | 0.5 |
| 212 | 0.50 | -ve | - | blue | - | 0.1 |
| 213 | 0.46 | -ve | - | blue | - | 0.3 |
| 214 | 0.43 | -ve | - | green | - | 0.2 |

## 2 The Isolation For Steroid Compounds

### 2.1 The isolation for K057

From analytical TLC of F048 expressed one major violet spot, when anisaldehyde in $H_2SO_4$ was sprayed on TLC plate and then heated for 5 minutes, it was observed under ultraviolet light at 254 nm by showing quenching spot. Divided amount of F048 (3.28 g) was isolated by flash column chromatography (4 x 15 cm) using gradient chloroform in methanol. The eluates were collected based on the color bands. The series of eluents were used as follow:

| | | |
|---|---|---|
| 4% MeOH in $CHCl_3$ | 1000 ml | Fractions # 1 - 16 |
| 8% MeOH in $CHCl_3$ | 800 ml | Fractions # 17 - 35 |
| 20% MeOH in $CHCl_3$ | 600 ml | Fractions # 36 - 44 |
| 30% MeOH in $CHCl_3$ | 400 ml | Fractions # 45 - 49 |
| 100% MeOH | 400 ml | Fractions # 50 - 63 |

The eluates were checked by TLC using 10% methanol in chloroform as developing solvent and combined eluates which gave similar chromatographic pattern as shown in Table 7. 2 mg of combined fractions were sent to test cytotoxicity.

Table 7. The combined fractions from F048

| Fraction | Number of eluates | Weights (mg) | color band |
|---|---|---|---|
| 055 | 1 - 3 | 138 | yellow |
| 056 | 4 - 16 | 250 | orange |
| 057 | 17 - 35 | 300 | yellow |
| 058 | 36 - 44 | 400 | light yellow |
| 059 | 45 - 49 | 42 | green |
| 060 | 60 - 63 | 313.6 | dark brown |

One major violet spot, positive anisaldehyde in sulfuric acid test, distinguished on analytical TLC of F057. When this fraction was evaporated for dryness, white needle crystals were observed. After recrystallization with methanol for

3 times, it yielded 22.9 mg ($1.47 \times 10^{-5}$ % based on wet weight of sponge) and named K057. The identify of K057 mainly used NMR technique both 1D and 2D NMR as discussed in next chapter IV.

2.2 The isolation for K068

In the same analytical TLC screening process of F048 as mentioned in 2.1 the interesting positive Iodine detected spot in chloroform to be solvent system was observed and this spot was observed again in F057 after fractionation of F048 by flash column chromatography, the same process to received F057 and K057 finally. Divided F056 (1.89 g), one of fractions from F048 as stated in section 2.1 was isolated by means of flash column chromatography (2.5 x 14 cm, 2 times) with gradient chloroform and methanol as an eluent in the order as shown below:

| | | |
|---|---|---|
| 5% MeOH in $CHCl_3$ | 120 ml | fraction # 1 - 8 |
| 20% MeOH in $CHCl_3$ | 45 ml | fraction # 9 - 11 |
| 50% MeOH in $CHCl_3$ | 45 ml | fraction # 12 - 13 |
| 100% MeOH | 50 ml | fraction # 14 |

Approximate 15 ml of eluates were collected based on the color bands and methanol 50 ml for fraction # 14 was used to exhausively wash the column until the eluates were diluted and clear comparing to the former ones. The eluates were examined by TLC using two developing system as follow:

$CHCl_3$ for fraction # 1 - 4

10% MeOH in $CHCl_3$ for fraction # 5 - 14

Thus, the fractions giving similar chromatographic patterns were combined and designated as shown in Table 8.

It was found that the interested positive Iodine vapour tested spot was shown in F063, so F063 was reprocess of isolating by means of silica gel column chromatography (2.5 x 11 cm) with gradient $CHCl_3$:MeOH as eluant in the order that

| | | |
|---|---|---|
| $CHCl_3$ | 150 ml | . fraction # 1 - 9 |

| | | |
|---|---|---|
| 3% MeOH in $CHCl_3$ | 75 ml | fraction # 10 - 14 |
| 10% MeOH in $CHCl_3$ | 70 ml | fraction # 15 - 18 |
| 100% MeOH | 50 ml | fraction # 19 |

Table 8. The combined fractions from F056

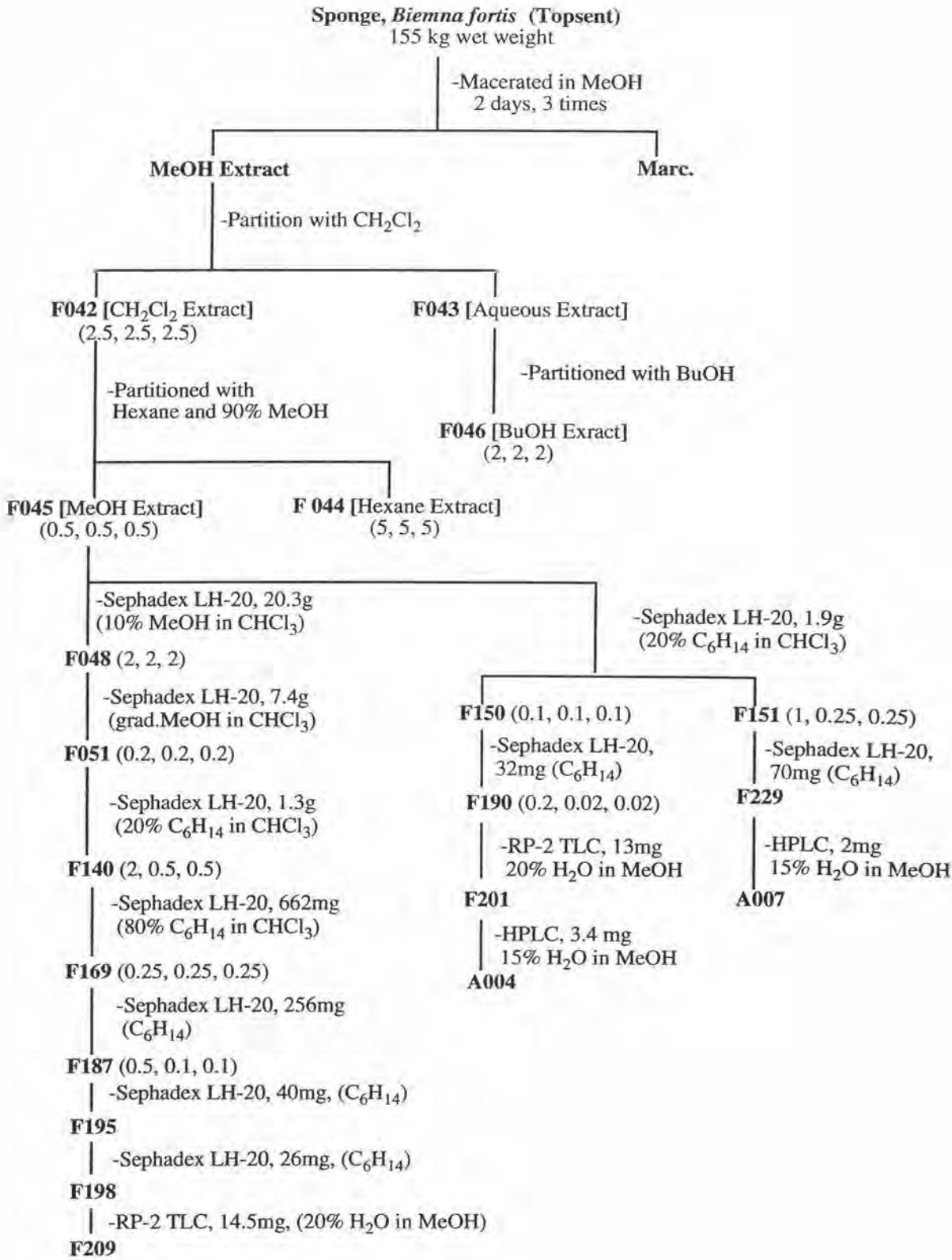
| Fraction | Number of eluates | weight (mg) | color band |
|---|---|---|---|
| 061 | 1 | 4.2 | yellow |
| 062 | 2 - 3 | 256.2 | orange |
| 063 | 4 | 397.8 | brown - green |
| 064 | 5 - 7 | 275.7 | light - yellow |
| 065 | 8 - 11 | 48.8 | orange |
| 066 | 12 - 13 | 11.2 | orange |

MeOH 50 ml eluted fraction # 19 in order to wash the column until the eluates were diluted and clear comparing to the former ones . Other fractions were collected approximate 15 ml based on color bands. Subsequently, the eluates were examined by TLC using two developing system that chloroform for fraction 1 - 7 and 5% methanol in chloroform for the others. The fractions were combined and designated based on chromatography pattern. Fraction F068 was received and K068 was crystallized in MeOH. For mother liquor of F068 was purified by preparative TLC plates of silica gel G 60 F 254 with suitable developing solvent, 5% ethylacetate in chloroform. The chromatographic pattern on TLC showed that there were at least 4 compounds. One of these was distinguish in Iodine-vapour detection and named as K218 to yield 9.9 mg ($6.38 \times 10^{-6}$ % based on wet weight of sponge). Other compounds were light blue in UV 365 nm detection. According to $^1$H-NMR data of K218, it was confermed that K218 was the same compound as K068 by the identical $^1$H-NMR data of K068 and K218 so they were combined and identified by both 1D and 2D NMR to be the main technique as discussed in Chapter IV.

2.3 The isolation for K084

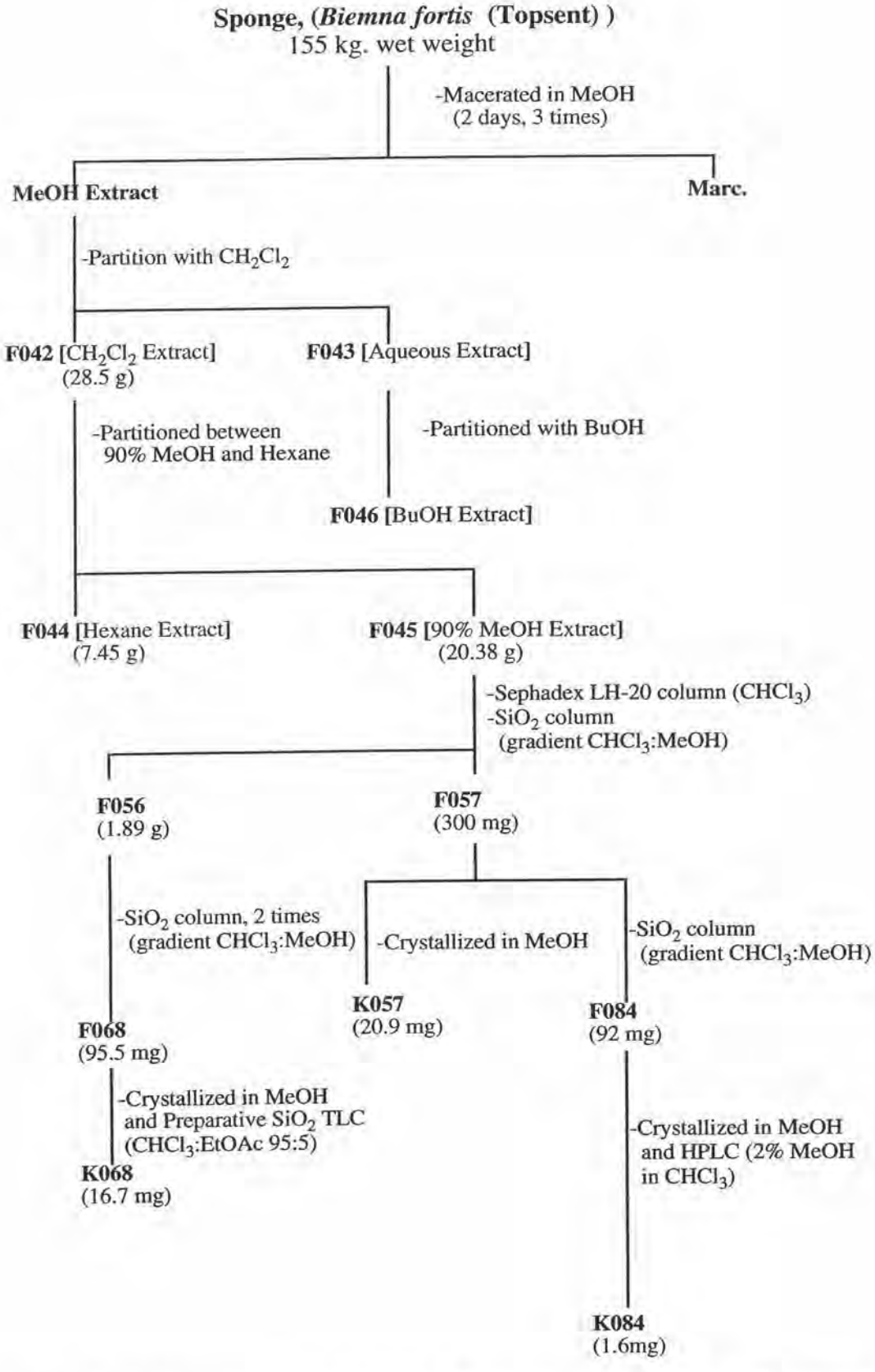
F064, F074, F076 and mother liquor of F057 were gathered in account of likely chromatographic pattern TLC and required spot. Then gathered fractions (391 mg) were further isolated by Sephadex LH-20 column with chloroform as eluent and collected 30 ml of each fraction.

The eluates were examined by TLC using 10% MeOH in $CHCl_3$ as developing system and combined eluates which gave similar chromatographic pattern in four fractions. One of these was F054 which white compound was crystallized from and further purified by means of recrystallize in methanol. White needle crystal, designed as K068, yielded 1.6 mg ($1.03 \times 10^{-6}$ % based on wet weight of sponge).




Scheme I. Isolation of bioactive constituents from *Biemna fortis* (Topsent) based on cytotoxicity bioassay

-The results of cytotoxicity (μg/ml) are shown in parenthesis of each fraction




Scheme II. Isolation of steroids from the Thai spong, *Biemna fortis* (Topsent)

## CHARACTERIZATION OF THE ISOLATED COMPOUNDS

### 1. Characterization of K201

K201 was obtained as yellow syrupy mass and soluble in chloroform.

EIMS ; *m/e* (relative intensity), Figure 10 - 13

peak 1) 658.3(4), 482.4(9), 438.8(32), 398(27), 354.4(100), 310.6 (98), 266.8(11), 232.9(5), 204.9(10), 161.0(27), 133.0(41), 89.1(60), 44.9(76)

peak 2) 613.2(2), 569.6(15), 526.0(39), 485.4(16), 441.8(94), 398.0 (70), 354.4(11), 310.6(6), 248.8(8), 204.9(18), 161.0(36), 133.0(69), 891(86), 44.9 (100)

peak 3) 656.3(10), 613.0(28), 569.6(22), 528.9(46), 485.3(44), 441.8 (26), 398.0(10), 354.4(7), 310.6(5), 248.8(10), 204.9(19), 177.0(29), 161.0(32), 133.0(72), 89.1(84), 44.9(100)

UV ; $\lambda_{max}$ nm (ε) , in chloroform; Figure 26

223.4, 276.0

IR ; υ $cm^{-1}$, KBr disc; Figure 25

2990, 2850 (high), 2000 - 1650 (weak), 1100, 1010(high), 801 (high).

$^{1}H$ NMR ; δ ppm, 500 MHz, in chloroform-d; Figure 9

7.23 (1H, dd, J = 8.85, 10.69 Hz), 7.16 (1H, dd, J = 7.02, 8.8 Hz), 6.83 (3H, dd, J = 6.71, 4.88 Hz), 6.82 (3H, d, J = 8.54 Hz), 4.16 (5H, dt, J = 4.88 Hz), 3.84 (5H, dt, J = 4.88 Hz), 3.72 (1H, t, J = 4.8 Hz), 3.62( 1H, t, J = 4.8 Hz), 3.68 (t, J = 4.8 Hz), 3.65 (br.s), 1.26(d, J = 10.98 Hz), 1.25 (s), 1.2 (2H, d, J = 5.8 Hz)

$^{13}C$-NMR ; δ ppm, 125 Mhz, in chloroform-d; Figure 16

163.28, 159.68, 156.42, 156.31, 142.93, 142.29, 127.61, 127.58, 127.39, 127.01, 126.91, 126.73, 113.99, 113.91, 113.88, 113.80, 113.68, 77.47, 72.59, 70.80, 70.63, 70.57, 70.54, 70.51, 70.29, 69.85, 69.83, 67.37, 67.34, 67.28, 63.57, 61.72, 51.95, 44.85, 40.26, 38.01, 37.41, 36.97, 32.83, 32.64, 32.51, 31.45, 29.67,

**2. Characterization of K057**

K057 was crystallized as white needle in methanol and soluble in 30% methanol in chloroform.

EIMS ; *m/e* (relative intensity); Figure 30

412(2), 397(10), 376(38), 361(6), 369(5) 269(7), 251(100), 224(14), 209(32)

UV ; λ max nm (ε), in chloroform; Figure 28

252(2211.4), 356(3.07).

IR ; υ $cm^{-1}$, KBr disc; Figure 27

3442(broad), 2990, 2850(high), 1052(medium), 990(medium).

$^1$H-NMR ; δ ppm, 500 MHz, in 30% methanol-$d_4$ in chloroform-d; Figure 31-33

0.61 (3H, s, $H_3$-18), 0.82 (3H, d, J = 6.7 Hz, $H_3$-26 or $H_3$-27), 0.85 (3 H, d, J = 6.7 Hz, $H_3$-26 or $H_3$-27), 0.92 (3H, d, J = 7 Hz, $H_3$-28),1.03 (3H, J = 6.7 Hz, $H_3$-21), 1.06 (3H, s, $H_3$-16), 1.3 (1H, m, H-17), 1.31 (1H, m, H-16), 1.34 (2H, m, $H_2$-12), 1.4-1.64 (8H, overlapping, H-2, H-15, H-25, H-11, H-1). 1,67 (1H, ddd, J = 2.5, 5, 13.3 Hz, $H_{eq}$-4), 1.75 (1H, m, H-16), 1.83 (1H, m, $H_{eq}$-2), 1.86 (1H,dq, J = 7.7 Hz, H-24), 1.92 (1H, m, H-14), 1.99 (1H, m, H-9), 2.05 (1H, m, H-20), 2.07 (1H, dd, J = 11.1, 13.3 Hz, $H_{ax}$-4), 3.57 (1H, ddd, J = 5.2 Hz, H-6), 3.9 (1H, tt, J = 11.1, 13.3 Hz, H-3), 5.17 (1H, dd, 7.6, 15.3, H-22), 5.23 (1H, dd, J = 7.4, 15.3 Hz, H-23), 5.31 (1H, ddd, J = 2, 2.2, 5.2 Hz, H-7).

$^{13}$C NMR ; δ ppm, 125 MHz, in 30% methanol-$d_4$ in chloroform-d; Figure 34

12.35, 17.69, 18.41, 19.71, 20.02, 21.21, 22.14, 23.08, 28.12, 30.60, 32.95, 33.26, 37.20, 39.10, 39.48, 40.59, 43.02, 43.35, 43.86, 56.12, 56.20, 67.45, 73.1, 76.12, 117.69, 132.27, 135.69, 143.52

**3. Characterization of K068**

K068 was crystalized as white amorphous crystal in methanol and soluble in chloroform.

EIMS ; *m/e* (relative intensity); Figure 60

398(13), 380(12), 273(10), 365(5), 255(36), 231(7), 228(5), 213(16)

UV ; $\lambda_{max}$ nm (ε), in chloroform; Figure 59

241(394.9)

IR ; υ $cm^{-1}$, KBr disc; Figure 58

3450(broad), 2990, 2850(high), 1052(medium), 980(medium).

$^1$H-NMR ; δ ppm, 500 MHz, in 30% methanol-$d_4$ in chloroform-d; Figure 61-63

0.69 (3H, s, $H_3$-18), 0.82 (3H, d, J = 6.7 Hz, $H_3$-27), 0.85 (3H, d, J = 6.7 Hz, $H_3$-26), 0.91 (3H, d, J = 6.7 Hz, $H_3$-28), 1.00 (3H, s, $H_3$-19), 1.01 (3H, d, J = 6.4 Hz, $H_3$-21), 1.45 (1H, m, H-25), 1.50 (1H, m, $H_{ax}$-2), 1.56 (1H, m, H-8), 1.69 (1 H, tt, J = 4, 9.6 Hz, H-16), 1.81 (1H, m, $H_{eq}$-2), 1.83 (1H, m, H-1), 1.85 (1H, m, H-24), 1.86 (1H, m, H-1), 1.96 (1H, m, H-7), 1.99 (1H, m, H-7), 2.02 (1H, m, H-20), 2.29 (H, dddd, J = 2.44, 5, 11.39, 13.12 Hz, $H_{ax}$-4), 3.52 (1H, tt, J = 4.8, 11.29 Hz, H-3), 5.16 (1H, dd, J = 7.63, 15.26 Hz, H-22), 5.21 (H, dd, J = 15.26, 7.02 Hz, H-23), 5.35 (1H, dt, J = 1.83, 5.19 Hz, H-6)

$^{13}$C-NMR ; δ ppm, 125 MHz, in chloroform-d, Figure 64

140.78, 135.85, 131.75, 121.69, 71.82, 56.86, 56.04, 50.18, 42.81, 42.34, 42.25, 40.13, 39.71, 37.27, 36.53, 53.11, 31.92, 31.92, 31.69, 28.52, 24.29, 21.08, 20.97, 19.95, 19.63, 19.40, 17.61, 12.08

**4 Characterization of K084**

K084 was crystalized as white amorphous crystal in methanol and soluble in chloroform.

EIMS ; *m/e* (relative intensity); Figure 79

428(1.29), 410(9.29), 392(16.14), 285(4.044),267(5.95), 258(2.92), 243(3.91), 240(3.82), 225(5.24), 173(15.43).

UV ; $\lambda_{max}$ nm (ε), in chloroform; Figure 78

256(527.6), 323(41.2).

IR ; υ $cm^{-1}$, KBr disc; Figure 77

3450, 3200(broad), 2990, 2850(medium), 1675(high), 1627(weak), 990, 890(medium).

$^{1}$H-NMR ; δ ppm, 500 MHz, in chloroform-d; Figure 80 - 83

0.59 (3H, m, $H_3$-18), 0.82 (6H, d, J = 0.82 Hz, $H_3$-26 or 27), 0.9 (3H, d, J = 7.0 Hz, $H_3$-28), 0.93 (3H, s, $H_3$-19), 1.01 (1H, d, J = 6.4 Hz, $H_3$-21), 1.36 (m, H-17), 1.42 (m, Hb-1), 1.45 (m, $H_{ax}$-4), 1.48 (m, H-25), 1.60 (m, Hb-11), 1.62 (m, Ha-1), 1.73 (m, Hb-12), 1.78 (m, Ha-11), 1.8 (m, Ha-16, $H_{ax}$-2), 1.86 (m, H-24), 1.89 (m, $H_{eq}$-2), 2.02 (q, J = 8.2 Hz, H-20), 2.09 (m, Ha-12), 2.11 (m, $H_{eq}$-4), 2.13 (m, H-14), 2.50 (ddd, J = 2.14, 7.1, 11.9 Hz, H-9), 4.01 (1H, tdd, J = 5.5, 11.6, 15.0 Hz, H-3), 5.14 (1H, dd, J = 8.2, 15.3 Hz, H-22), 5.21 (1H, dd, J = 7.6, 15.3 Hz, H-23), 5.64 (1H, dd, J = 2.14, 2.14 Hz, H-7)

$^{13}$C-NMR ; δ ppm, 125 MHz, in chloroform-d, Figure 84

198.1, 165.1, 134.9, 132.5, 119.7, 77.8, 67.4, 56.0, 55.7, 44.7, 43.9, 42.8, 40.4, 40.2, 38.8, 36.5, 33.1, 30.3, 30.2, 22.4, 27.8, 21.9, 21.1, 19.9, 19.5, 17.5, 16.4, 12.6