# CHAPTER III

# EXPERIMENTAL

1. Source of Marine Organism

The specimen of a Thai marine sponge, *Petrosia* sp., was collected by SCUBA diving (-10 m) from Sichang Island area, Chonburi province, Thailand in March, 1992. The sponge voucher specimen was preserved in 70 % v/v ethanolic solution and collected at Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University for subseqeuent confirmation of its taxonomy. The remaining were frozen at -20 ° C until they were extracted.

2. General Techniques

2.1 Analytical Thin-layer Chromatography (TLC)

Technique : One way, ascending.

Adsorbent : Silica gel 60 F254 (E. Merck) precoated plate.

Plate size : 5 × 8 cm.

Layer thickness : 0.2 mm.

Solvent systems :

a.) chloroform: ethyl acetate (2:1)

b.) n-hexane: ethyl acetate (9:1).

Distance : 6 cm.

Temperature : 25-30 ° C.

Detection : Ultraviolet light. The compounds which contain unsaturated bonds become visible as quenching spots (254 nm).

2.2 Column chromatography

Column size : 2.5 × 15 cm, 5.0 × 15 cm.

Technique: Flash column chromatography using nitrogen gas pressure to control flow rate of 2 ml/min.

Adsorbent : Silica gel G 0.040-0.063 mm (E. Merck).

Packing : Adsorbent (30 g for 2.5 × 15 cm and 60 g for 5.0 × 15 cm column) poured as a suspension in chloroform into the column.

Sample loading : The portion of crude extract was dissolved in small amount of organic solvent and then, added slowly onto the top of the column.

Temperature : 25-30 ° C.

Mobile Solvents:

a.) n-hexane: ethyl acetate (9:1).

b.) chloroform: ethyl acetate (2:1)

Collection of eluate : Each fraction of 25 ml was collected.

Fractionation : Those fractions giving quenching spot by exposure to short wavelength ultraviolet light (254 nm) were examined by thin-layer chromatography. The same pattern quenching spots of each fraction were combined and evaporated under reduced pressure to dryness.

2.3 Semi-preparative High-performance Liquid Chromatography (HPLC)

Controller : Waters 600E multisolvent delivery system .

Column : Stainless steel column Bondaclone 10 C18 (7.8 × 300 mm).

Guard column : C-18 perisorb C (1.2 × 4.5 mm).

Flow rate : 2.5 ml/minute.

Mobile phase : Acetonitrile : water 75:25.

Sample preparation: Dissolve 10 mg of sample in 1 ml of methanol and filter through Toyopak® ODS before injection.

Injection volume : 200 µl.

Detector : Ultraviolet photometer (Waters 484) set at 235 nm.

Temperature : 20-25 ° C.

Recorder : Waters 746.

2.4 Analytical High-performance Liquid Chromatography (HPLC)

Controller : Waters 600E multisolvent delivery system .

Column : Stainless steel column S10 ODS 2 Phase Sep (4.6 × 25 mm).

Guard column : C-18 perisorb C (1.2 × 4.5 mm).

Flow rate : 1 ml/min.

Mobile phase : Acetonitrile : Water 80:20.

Sample Preparation: Dissolve 1 mg of sample in 2 ml of methanol (c = 0.5 mg/ml), except H-1 diluted to have a final concentration of 0.25 mg/ml. Filter through Toyopak® ODS before injection.

Injection volume : 6, 4, 10 µl (**H-1**, **H-2**, **H-3**, respectively).

Detector : Ultraviolet photometer (Waters 484) set at 235 nm.

Temperature : 20-25 ° C.

Recorder : Waters 746.

2.5 Spectroscopy

2.5.1 Ultraviolet (UV) absorption spectra. The ultraviolet absorption spectra were obtained on a Perkin Elmer Lambda 15 UV/VIS Spectrophotometry in chloroform (Division of Drug Analysis, Department of Medical Sciences).

2.5.2 Infrared (IR) absorption spectra. The infrared absorption spectra were obtained on a Shimadzu IR-440 infrared spectrometer. The materials were dissolved in chloroform and examined in Potassium Bromide cell, then subtract the spectrum of solvent (The Scientific and Technological Research Equipment Center, Chulalongkorn University).

2.5.3 Nuclear magnetic resonance (NMR) spectra. The nuclear magnetic resonance spectra were obtained on a JEOL JNM-GSX series (500 MHz for $^{1}$H-NMR and 125 MHz for $^{13}$C-NMR) (Faculty of Pharmaceutical Sciences, University of Tokyo and the Scientific and Technological Research Equipment Center, Chulalongkorn University) or Bruker BZH-200 spectrometer (200 MHz for $^{1}$H-NMR and 50 MHz for $^{13}$C-NMR) (Department of Chemistry, Faculty of Sciences, Chulalongkorn University).

2.5.4 Mass spectra were determined on a JEOL FX 3000 double focusing spectrometer for EIMS which was operated at 70 ev with inlet temperature 150 - 240 °C (The Scientific and Technological Research Equipment Center, Chulalongkorn University).

2.6 Solvents

2.6.1 For Column Chromatography and Thin-layer Chromatography (TLC), all organic solvents were redistilled before use.

2.6.2 For High-performance Liquid Chromatography (HPLC), use analytical grade solvents and triple distilled water.

2.7 Biological Assay

2.7.1 Cytotoxic Activity Test

In this work, the cytotoxicity test was performed against human tumor cell lines and cultured tumor cells from test animal. The assay procedure was helpfully carried out by Professor Tatsuo Higa and Dr. Jun-ichi Tanaka of Department of Marine Sciences, University of the Ryukyus, Japan. The method of assay was briefly described as below (Mossman, 1983).

2.7.1.1 Target cells

The cell lines in this assay were *P-388* ( A methyl-cholanthrene-induced lymphoid neoplasm in a DBA/2 mouse. A non-anchorage dependent cell line), *A-549* (Human non-small cell lung carcinoma) and *HT-29* (Human colon adenocarcinoma, moderately well differentiated).

2.7.1.2 Method of Bioassay

For *P-388* cell lines, cells were incubated in the presence of test sample for 48 hours. Cell growth was determined by counting cells using an electronic counting device. The result was obtained by determining the $ED_{50}$ of a test sample.

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

2.7.2 Brine Shrimp Lethality Bioassay

This method, utilizing brine shrimp (*Artemia salina* Leach), was proposed as a simple bioassay for natural product research. It was selected because it could detect a braod spectrum of pharmacological activities and it was simply to guide phytochemical screening and fractionation. The test could be employed in laboratory at low cost and rapid. The method was described as below(Meyer et al., 1982).

2.7.2.1 Hatching the Shrimp

Brine shrimp eggs (Aquarium Products, U.S.A.) were hatched in a shallow rectangular dish filled with artificial sea water which was prepared with a commercial salt mixture (Instant Oceans, Aquarium Systems Inc.) and deionized water (c = 38 g/l). A plastic divider with several 2 mm holes was jointed in the dish to make two unequal compartments. The egg (ca.50 mg) were sprinkled into the layer compartment which was darkened, while the smaller compartment was illuminated. After 36 hours the phototropic nauplii were collected by pipette from the lighted side, having been separated by the divider from their shells.

2.7.2.2 Sample Preaparation

Samples were prepared by dissolving 2 mg of sample in 2 ml of methanol (solution A). Solution B was prepared by diluting 0.05 ml of solution A to 0.5 ml with methanol. Appropriate amounts of solution (500 µl A, 50 µl A, and 100 µl B for 100,10,1 µg/ml, respectively) were transfered to glass vials ( 2 × 5.5 cm). The vials were allowed to dry in air overnight and, then added with 5 ml of artificial sea water which was prepared with a commercial salt mixture (Instant Oceans, Aquarium Systems Inc.) and deionized water (c = 38 g/l). The control was prepared using only methanol. Three replicates were prepared for each dose level.

2.7.2.3 Bioassay

Ten shrimps were transferred to each sample vial using a disposable pipette. The nauplii could be counted macroscopically in the stem of the pipette against a lighted background. A drop of dry yeast suspension (3 mg in 5 ml artificial sea water) was added as food to each vial. The vials were maintained under illumination. Survivors were counted after 24 hours, and the percent deaths at each dose and control were determined. In cases where control deaths occurred, the data were corrected using Abbott's formula : % deaths = [(test-control)/control] × 100.

2.7.2.4 $LD_{50}$ Determinations

$LD_{50}$ 's and 95 % confidence intervals were determined from the 24 hours counts using the probit analysis method described by Finney Programme. In cases where data were insufficient for this technique, the dose-response data were transformed into a straight line by means of a logit transformation; the $LD_{50}$ was derived from the best fit line obtained by linear regression analysis.

2.7.3 Microbiological Assay

This assay was kindly supported by Assistant Professor Sathaporn Sirotamarat of Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University. The details of assay were described as below.

2.7.3.1 Test Microorganisms

The microorganisms using in this test were

- *Staphylococcus aureus* ATCC 6538 P
- *Bacillus subtilis* ATCC 6633
- *Escherichia coli* ATCC 25922

- *Pseudomonas aeruginosa* ATCC 29336
- *Candida albicans.*

2.7.3.2 Media

The media required for the preparations of inocula and for the tests were descibed as below.

A. Nurient agar (NA)

Formula per liter of Purified Water

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

Final pH 7.4

Suspended all ingredients in purified water 1 liter and mixed throughoutly. Heated with frequent agitation and boiled for 1 minute to completely dissolve the ingredient. Sterilized by autoclaving at 121° C for 15 minutes.

This medium was used to make the NA slants which were used in subculturing the bacteria at 37 ° C for 24 hours before use.

B. Trypticase Soy Agar (TSA)

The medium was BBL® Trypticase Soy Agar supplied by Becton-Dickenson Microbiology System.

Formula per liter 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

Suspended 40 g of powder in 1 liter of purified water and mixed throughoutly. Heated with frequent agitation and boiled for 1 minute to completely dissolve the powder. Sterilized by autoclaving at 121° C for 15 minutes.

This medium was used for culturing the bacteria in the period of assay.

C. Sabouraud Dextrose Agar (SDA)

The medium was BBL® Sabouraud Dextrose Agar supplied by Becton-Dickenson Microbiology System.

Formula per liter 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

Suspended all ingredients in 1 liter of purified water and mixed throughoutly. Heated with frequent agitation and boiled for 1 minute to completely dissolve the powder. Sterilized by autoclaving at 121° C for 15 minutes.

This medium was used to be the SDA slant for subculturing the fungus at 37 ° C for 24 hours before use and for culturing the fungus in period of assay.

2.7.3.3 Sample Preparation

Samples were prepared by dissolving 5 mg of compound or extract in 1 ml of methanol (solution A). Solution B was prepared by diluting 0.1 ml of solution A to 1.0 ml with methanol. Pipetted 20 µl of solution A and 20 µl of solution B for 100 and 10 µg/disc, respectively, to 6-mm discs for antibiotic assay (Whatman® AA) in glass petri-dish (20 × 100 mm). The discs were dried in air. Two replicates were prepared for each dose level.

2.7.3.4 Microbiological Assays

A. Inoculum

Transferred the test microorganisms subcultured for 24 hours at 37 ° C from the NA slant (for bacteria) or the SDA slant (for fungus) to a 0.9 % w/v solution of saline (NSS) in test tubes. Adjusted the turbidity by compared with Mac Faulan suspension No. 1 (equivalent to 300,000,000 cell/ml).

B. Microbial assay procedure

The Microbiological agar diffusion method was used. First prepared the media by warming the agar until they melt , and allowed to cool at temperature about 50 ° C. Poured the media to the plate with aseptic technique, then allowed to cool at room temperature until they were solidify. Swabed the broth and streaked in three dimensions on the agar surface with aseptic technique. The discs were placed on the surface of the inoculated medium with aseptic technique. The plates are incubated at 37 ° C for 24 hours ( for bacteria) and 48 hours (for fungus). Measured the diameter of clear zone in mm with vernia.

3. Extraction and Isolation

3.1 Extraction

The small pieces of frozen sponges (12.7 kg) was macerated with methanol (24 liters). It was allowed to macerate for three-day period and filtered. The marc was macerated with three successive portions of methanol (20 liters) and filtered. The combined methanol filtrate was concentrated under reduced pressure to give the least volume of the residue (300 ml). The residue was partitioned with dichloromethane (2 × 300 ml).

The dichloromethane layer was separated and concentrated under reduced pressure to yield 50 g of syrupy mass of the dichloromethane extract (0.4 %w/w). The extract was dissolved in suitable amount of methanol and further added with distilled water to give the 90% methanol in water solution (600 ml). This solution was then partitioned with n-hexane (3 × 500 ml) The two layers were separated by using separating funnel and evaporated under reduced pressure to yield syrupy mass of the hexane extract (30 g, 0.24 % w/w) and the 90% methanol extract (20 g, 0.16 %w/w).

The aqueous layer was partitioned by n-butanol (3 × 200 ml). The butanol extract was separated from the aqueous layer and concentrated under reduced pressure to yield 7 g of syrupy mass (0.05 % w/w).

3.2 Isolation

Since the crude 90 % methanol extract has shown the best activity in the brine shrimp lethality assay, it was selected to be investigated for its bioactive constituents. The crude 90 % methanol extract was first purified by silica gel flash column chromatography using chloroform and ethyl acetate (2:1) as an eluent and finally with

methanol until no quenching spots could be detected by TLC. Fractions of 25 ml were collected and examined by TLC. Those fractions of similar pattern were combined and evaporated under reduced pressure to give the following fractions:

(1) fractions 1-2 afforded pool A (145 mg)

(2) fractions 3-10 afforded pool B (1.2 g)

(3) fractions 11-18 afforded pool C (383 mg)

(4) fractions 19-22 afforded pool D (158 mg)

(5) fractions 23-50 afforded pool E (1.1 g)

Pools A, B, C, D, E were tested for brine shrimp lethality activity ($LD_{50}$ = 5, 3, 3, 4, 30 μg/ml, respectively). Pool B was further purified by silica gel flash column chromatography using a mixture of chloroform and ethyl acetate (2:1) as an eluent and finally with methanol. Fractions of 25 ml were collected and examined by TLC. Fractions containing homogeneous pattern were combined and evaporated under reduced pressure as the followings:

(1) fractions 1-6 afforded pool B-1 (88 mg)

(2) fractions 7-10 afforded pool B-2 (562 mg)

(3) fractions 11-17 afforded pool B-3 (253 mg)

(4) fractions 18-25 afforded pool B-4 (82 mg)

(5) fractions 26-34 afforded pool B-5 (176 mg)

Pools B-1, B-2 , B-3, and B-4 were tested for brine shrimp lethality activity ($LD_{50}$ = 4.5, 14, 2.7, 1.3 μg/ml, respectively). All of these pools were readily degraded by oxidation. Pool B-3 showed good activity for brine shrimp lethality activity, thus it was selected to be further purified. However, the isolation of pool B-3 was not successful although the semi-preparative HPLC technique was used. Due to the maximum yield of pool B-2, it was interesting to be further investigated after the failure in isolating pool B-3. The infrared spectrum of a pool B-2 showed the carboxylic acid functional group at $\nu_{max}$ 3,425 $cm^{-1}$ and 1,709 $cm^{-1}$ (Figure 7). Consequently, the esterification of pool B-2 by using diazomethane was more easily

isolated. The methyl ester of pool B-2 (300 mg) was further purified by silica gel flash column chromatography using n-hexane and ethyl acetate (9:1) as eluent. Fractions of 25 ml were collected and examined by TLC. Fractions of similar quenching spots were combined and evaporated under reduced pressure to give the following fractions:

(1) fractions 1-7 afforded pool B-21 (176 mg)

(2) fractions 8-12 afforded pool B-22 (96 mg)

(3) fractions 13-16 afforded pool B-23 (6.2 mg)

(4) fractions 17-32 afforded pool B-24 (61.5 mg)

Pool B-22 was further purified by semi-praparative HPLC. The chromatographic procedure was carried out using (a) a stainless steel column (7.8 × 300 mm) packed with 10 μm octadecylsilane chemically bonded to porous silica (Bondaclone 10C18), (b) Mobile phase : Acetonitrile/water 75/25 at a flow rate of 2.5 ml/min and (c) an ultraviolet photometer set at 235 nm. The injection volume was indicated in general techniques. Fractions of similar chromatogram pattern were combined and evaporated under reduced pressure to dryness by filling a few volume of toluene. The isolated chemical substances were designated as follows (Figure 8):

(1) the retention time at 17 minute afforded compound H-1 (9.7 mg)

(2) the retention time at 20 minute afforded compound H-2 (6.8 mg)

(3) the retention time at 24 minute afforded compound H-3 (7.5 mg)

Later, the isolated compounds were analyzed by HPLC. The procedure was carried out using acetonitrile /water 80/20 as mobile phase at a flow rate of 1 ml/min and S10ODS2 Phase Sep (4.6 × 250 mm) column as stationary phase and detected at 235 nm. The chromatograms of each compound were shown in Figure 9.

4. Esterification of Pool B-2

The IR spectrum of pool B-2 showed the carboxylic acid functional group at $\nu_{max}$ = 3,425 and 1,709 $cm^{-1}$ (Figure 7). Pool B-2 should contain a mixture of unstable acids which were difficult to be obtained in pure form. Thus, methylation by

diazomethane of pool B-2 was prepared. The preparations of diazomethane and esterification were described as the followings:

4.1 Preparation of Nitrosomethylurea

In a 1-liter erlenmeyer flask, gently heated a mixture of acetamide (15 g) in bromine (22 g) on a steam bath to dissolve acetamide completely. Then, dropwised, (by separatory funnel) with stirring by a glass rod, a solution of sodium hydroxide (10 g in 40 ml of water ). The mixture was heated on a steam bath until effervescence set in, after which heating was continued for an additional 3 minutes. Cooled the mixture in an ice bath for 1 hour. Filtered the solution and washed the precipitate with $2 \times 10$ ml of cold water. Crude acetyl methylurea is white powder (14.5 g). Then, dissolved acetyl methylurea (9.8 g) with concentrated hydrochloric acid (10 ml) in 125-ml erlenmeyer flask, and heated with stirring by a glass rod on a steam bath for 10 minutes. Then, added water (10 ml) cautiously. The mixture was cooled in an ice bath below 5 $^{\circ}$ C. Slowly added a cold (5$^{\circ}$ C) saturated solution of sodium nitrite into the mixture, and swirled in the cold for 30 minutes. Filtered and washed the nitrosomethylurea with cold water ($2 \times 2$ ml). Dried in a vacuum desiccator for 1 hour. Stored the product in a brown bottle in the fridge. Crude nitrosomethylurea is white powder (6.2 g).

4.2 Preparation of Diazomethane in Ether Solution

Placed 50 ml anhydrous diethylether in a 125-ml erlenmeyer flask with a very clean teflon stirring bar, added 17.5 ml cold 50 % aqueous solution of potassium hydroxide. Cooled to 5 $^{\circ}$ C in an ice water bath. With a very slow stirring, added 5.15 g of N-nitrosomethylurea in small portions over a time period of 1 hour. Allowed to stir slowly until evolution of gas ceased. Poured into a clean 125-ml erlenmeyer flask over a layer of potassium hydroxide pellets. Corked and stored in freezer for 2 hours before using.

4.3 Esterification with Diazomethane

Dissolved residue B-2 (320 mg green mixture) in anhydrous diethylether (1 ml) and cooled to 0$^{\circ}$ C on an ice bath . Added the ethereal solution of diazomethane in 1-ml portions, (allowed nitrogen evolution to cease between additions), until the esterification was completed (checked by TLC; the solvent system was hexane: ethyl acetate 9:1). After addition was complete (20 minutes), the solution was allowed to stir for an additional 5 minutes at 0$^{\circ}$ C. Any precipitated solids were filtered off. The

solution was warmed slowly to room temperature and the ether was removed by using nitrogen gas. The methy ester of a pool B-2 was dried in a vaccuum desiccator. It is a mixture of yellow oil and solid (300 mg).

5. Biological Activity

5.1 Cytotoxicity

The dichloromethane extract and th ebutanol extract from *Petrosia* sp.were tested. The dichloromethane extract showed cytotoxic activities against *P-388* (mouse leukemia), *A-549* (Human non-small cell lung carcinoma) and *HT-29* (Human colon adenocarcinoma) at $ED_{50}$ 5 μg/ml. The butanol extract was negative in the cytotoxic activity test.

5.2 Brine Shrimp Lethality Bioassay

The dichloromethane extract showed more potent activity for brine shrimp lethality bioassay than the butanol extract. All of the fractions were assayed for this test to investigate the active constituents. The conclusion of the brine shrimp activity of each fraction was shown in Table 5.

Table 5 Brine shrimp lethality bioassay-directed isolation

| First step | Fraction and activity ( μg/ml) | Second step | Fraction and activity ( μg/ml) |
|---|---|---|---|
| Original Dichloro-methane extract | $LD_{50} = 2.4$ | | |
| n-Butanol extract | $LD_{50} > 100$ | | |
| n-Hexane extract | $LD_{50} = 6$ | | |
| 90% Methanol extract | $LD_{50} = 2$ | | |
| $CHCl_3$/EtOAc (2:1) | A. $LD_{50} = 5$<br>B. $LD_{50} = 3$<br>C. $LD_{50} = 3$<br>D. $LD_{50} = 4$<br>E. $LD_{50} = 30$ | $CHCl_3$/EtOAc (2:1) | B-1. $LD_{50} = 4.5$<br>B-2. $LD_{50} = 13.9$<br>B-3. $LD_{50} = 2.7$<br>B-4. $LD_{50} = 1.3$ |

Pool B-2 was esterified by diazomethane and further purified by silica gel column chromatography to investigate the active fractions. Pool B-22 was purified again by means of reversed-phase HPLC. This procedure led us to isolate three compounds, **H-1**, **H-2**, and **H-3**. The $LD_{50}$ activities of brine shrimp lethality assay of the isolated compounds were shown in Table 6.

Table 6 Brine shrimp lethality activity of the isolated compounds

| Compound | Brine Shrimp Lethality Bioassay Activity ($LD_{50}$ , μg/ml)) |
|---|---|
| H-1 | 0.8 |
| H-2 | 0.5 |
| H-3 | 2.8 |

5.3. Antimicrobial activity

The butanol extract showed the antimicrobial activity against *Bacillus subtilis* : diameter inhibition zone 4 mm at the concentration 100 µg/disc. In the other hand, the dichloromethane extract did not show the antimicrobial activity against any test organisms in this assay.

6. Characterized of the Isolated Compounds

The isolated compounds were characterized by the spectroscopic data of ultraviolet , infrared , nuclear magnetic resonance and mass spectra.

6.1 Characterization of **H-1**

H-1 was a mixture of two compounds (**H-1A** and **H-1B**) obtained as colorless oil. It was soluble in chloroform and methanol. The compound was unstable, particularly when exposed to air. Thus, it was kept under nitrogen atmosphere.

Molecular weight:

360 (proposed molecular weight from EIMS)

IR spectrum (Figure 10):

$\nu_{max}$ ($cm^{-1}$) : 2,931, 2,361, 1,734

UV spectrum in chloroform (Figure 11):

$\lambda_{max}$ ($\varepsilon_{max}$) = 284 (17,084); 269 (19,735); 254 (17,313) nm

EI Mass spectrum (Figure 12):

*m/e* (%, relative intensity) : 347 (0.29), 345 (0.31), 331 (0.42), 329 (0.37), 314 (0.18), 303 (0.35), 301 (0.34), 281 (100), 249 (0.70), 221 (13.96), 207 (32.41), 193 (31.29), 191 (25.23), 179 (71.12), 165 (68.42), 153 (23.22), 128 (23.98), 115 (45.28),

6.1.1 Characterization of **H-1A**

500 MHz $^{1}$H-NMR spectrum in $CDCl_3$ : δ (ppm) ( multiplicity, *J* Hz) (Figure 13)

1.53(m), 1.85(quintet , *7.2*), 2.14(qd, *7, 1.5*), 2.26(brt, *7*), 2.34(brt , *7.2*), 2.45(t , *7.2*), 3.68(s), 5.56(dq, *15.9, 2.2*), 6.17(dt , *15.9, 7*), 6.30(dd, *14.1, 2.2*), 6.64(d, *14.1*)

125 MHz $^{13}$C NMR spectrum in CDCl3 : δ (ppm) (Figure 24)

18.67, 18.98, 23.50, 27.60, 27.65, 30.08, 32.71, 51.62, 65.46, 66.11, 76.15, 77.47, 84.64, 90.49, 109.61, 117.64, 117.71, 145.25, 173.37

6.1.2 Characterization of **H-1B**

500 MHz $^{1}$H-NMR spectrum in $CDCl_3$ : δ (ppm) (multiplicity, *J* Hz) (Figure 25)

1.53(m), 1.88(quintet , *7*), 2.30(td, *7, 2*), 2.36(qd, *7, 1.2*), 2.42 (td, *7, 0.9*), 2.46 (t , *7*), 3.70 (s), 5.49(dqt, *10.8, 1.2*), 6.03(dt, *10.8, 7*), 6.18(dt, *14.0, 2*), 6.58(d, *14.0* )

125 MHz $^{13}$C NMR spectrum in $CDCl_3$ : δ (ppm) (Figure 36 )

19.07, 19.22, 23.45, 27.72, 27.87, 29.69, 32.63, 51.60, 66.00, 72.27, 77.32, 78.16, 83.42, 92.78, 108.56, 117.07, 117.98, 147.18, 173.33

6.2 Characterization of **H-2**

H-2 was obtained as colorless oil. It was soluble in chloroform and methanol. The compound was unstable, particularly when exposed to air. Thus, it was kept under nitrogen atmosphere to prevent oxidation.

Molecular weight:

362, 364 (EIMS)

IR spectrum: (Figure 37)

$\nu_{max}$ ($cm^{-1}$) : 1,732, 2,355 , 2,934

UV spectrum in chloroform : (Figure 38)

$\lambda_{max}$ ($\varepsilon_{max}$): 242 (15,698) nm.

EI Mass spectrum : (Figure 39)

*m/e* (%, relative intensity): 364(4.3), 362(4.25), 333(3.59), 332 (3.82), 283 (19.47), 251 (8.92), 223 (28.54), 209 (65.03), 197 (37.82), 195 (35.24), 181 (70.6), 167 (100.0), 155 (71.07), 141 (68.88), 129 (56.69), 115 (47.43), 91 (93.34), 77 (67.61)

500 MHz $^{1}$H-NMR spectrum in $CDCl_3$ : δ (ppm) ( multiplicity, *J* Hz) (Figure 40 )

1.38 (4H, m), 1.52 (4H, broad quintet, *7*), 1.84 (2H, quintet, *7*), 2.24 (2H, brt, *7*), 2.25 (2H, td, *7*, *2*), 2.33 (2H, brt , *7*), 2.44 (2H, t , *7*), 3.67 (3H, s), 6.16 (1H, dt , *14*, *2*), 6.56 (1H, d, *14* )

50 MHz $^{13}$C NMR spectrum in $CDCl_3$ : δ (ppm) (Figure 44)

18.65, 19.10, 19.34, 23.49, 28.07, 28.15, 28.25(2 signals), 32.69, 51.60, 65.20, 66.14, 75.99, 77.32, 77.72, 92.98,117.02, 117.97, 173.38