CHAPTER III

EXPERIMENTAL

Source of Sponges

The blue mucous sponges were collected by scuba diving at a depth of 5 m. from Si-Chang Island, Chonburi, Thailand, in March, 1993. The sponge voucher specimens were preserved in 70% ethanolic solution and kept at the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University. The remaining specimens were frozen at -20°C until they were extracted.

General Techniques

1. Thin-layer chromatography (TLC)

Technique : one way, ascending

Adsorbent : silica gel 60 F_{254} (E. Merck), precoated

Layer thickness : 0.25 mm

Distance : 5 cm.

Temperature : laboratory temperature (28-34°C)

Detection : a) ultraviolet light at wavelength 254 and 365 nm.
b) anisaldehyde sulphuric acid spraying reagent
c) iodine vapor

2. Column chromatography

2.1 Quick column chromatography

Adsorbent : a) silica gel 60 (number 9385) particle size 0.040-0.063 mm (230-400 mesh ASTM)
b) silica gel 60 (number 7734) particle size 0.063-0.200 mm (70-230 mesh ASTM)

Packing : a) wet-packed, the slurry of adsorbent in chloroform was poured into the column. The solvent was sucked out, and the adsorbent was pressed tightly.
b) dry-packed, adsorbent was poured into the column and pressed tightly.

Sample loading : The extract was dissolved in a small volume of solvent, mixed with sufficient quantity of kieselguhr, air dried, dried under the vaccum and added onto the top of a dry column.

Collection of eluate

: Fractions of 250 ml or 50 ml were collected.

Examination of eluate

: Fractions were examined by thin layer chromatography using ultraviolet absorption and anisaldehyde sulphuric acid spray reagent:

2.2 Flash column chromatography

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

b) silica gel 60 (number 7734) particle size 0.063-0.200 mm (70-230 mesh ASTM)

Packing : Adsorbent was dry-packed. The adsorbent was poured into the column, allowed to settle, and pressed tightly.

Simple loading : The extract was dissolved in a small volume of solvent, mixed with sufficent quantity of kieselguhr, air dried, dried under vaccum and added onto the top of a dry column.

Collection of eluate

: Fractions of 50 ml, 30 ml or 20 ml were collected.

Examination of eluate

: Fractions were examined by thin layer chromatography using ultraviolet absorption, anisaldehyde sulpuric acid spray reagent and iodine vapor.

2.3 Gel filtration chromatography

Packaging material : Sephadex LH-20

Packing Adsorbent was suspended in the eluant and left standing to swell prior to use for 24 hours. The suspension was poured into the column and allowed to settle tightly.

Sample loading : The extract was dissolved in a small volume of eluant and put on the top of the column.

Solvent : chloroform : methanol (4:1)

chloroform : methanol (3:1)

Collection of eluate

: Fractions of 20 ml or 15 ml were collected.

Examination of eluate

: Fractions were examined in the same manner as described in section 2.1

2.4 Semi-preparative high-performance liquid chromatography (HPLC)

Controller : Compaq Prolinea 4/33

Column : stainless steel column Bondclone 10 C-18 (7.8 x 300 mm.)

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

Flow rate : 1.5 ml/minute

Mobile phase : methanol : water (90:10)

Sample preparation: Dissolve 6 mg of sample in 2 ml of methanol and filter through Toyopak^{R} ODS before injection.

Injector : autometric sample injector (auto Metric 4100 R)

Injection volume : 100 µl.

Detector : photodiode array detector (spectro Monitor 5000 R) set at 242 nm.

Recorder : Epson LQ-1170

Temperature : 20-25°C

3. Spectroscopy

3.1 Ultraviolet visible (UV) absortion spectra

Ultraviolet-visible absorption spectra were obtained on Milton Roy spectronic 3000 Array (Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

3.2 Infrared (IR) absorption spectra

The spectra were obtained on Shimadzu IR-440 infrared spectrometer (The Scientific and Technological Research Equipment Center, Chulalongkorn University).

3.3 Mass spectra (MS)

Mass spectra were recorded on a Fisons VG Trio 2000 Mass Spectrometer for EIMS operating at 70 ev with in-let temperature 150°-240°C (Department of Chemistry, Faculty of Science, Chulalongkorn University).

3.4 Proton and carbon-13 nuclear magnetic resonance (^{1}H and ^{13}C NMR) spectra

The NMR spectra were obtained on a Jeol JMN.GSX spectrometer (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR), (The Scientific and Technological Research Equipment Center, Chulalongkorn University).

4. Solvents

For column chromatography and thin layer chromatography (TLC), all organic solvents (commercial grade) were redistilled before use.

For semipreparative hplc, hplc grade solvents

Bioactivity Determination

1. Brine shrimp lethality activity

This method, utilizing brine shrimp (*Artemia salina* Leach) was proposed as a simple bioassay for natural product research. It had the advantages of being rapid, inexpensive, and simple. The method is described as below (Meyer *et al.*, 1982).

1.1 Artificial sea water preparation

38 g of Commercial salt mixture (Instant Oceans, Aquarium System Inc.) was dissolved with purified water 1 liter. The solution was filtered.

1.2.Hatching the shrimp

Brine Shrimp eggs (Aquarium Products, U.S.A.) were hatched in a shallow rectangular dish filled with artificial sea water. A plastic divider with several 2 mm holes was jointed in the dish to make two unequal compartments. The eggs were sprinkled into the larger compartment which was darkened, while the smaller compartment was illuminated. After 48 hours the phototropic nauplii were collected by pipette from the lighted side.

1.3 Sample preparation

Sample 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 vial (2 x 5.5 cm). The vials were allowed to air dry overnight and then added with 5 ml of artificial sea water. Three replicates were prepared for each dose level.

1.4 Bioassay

Ten shrimp were transferred to each sample vial, and artificial sea water were added to make 5 ml. The nauplii can be counted macroscopically in the stem of the pipette against a bright background.

For control, ten shrimps were transferred to vial containing 5 ml. of artificial sea water. A drop of dry yeast suspension (3 mg in 5 ml of artificial sea water) was added as food to each vial. The vials were maintained under illumination. Survivors were counted after 24 hr.

1.5 LC_{50} determinations

LC_{50}'s and 95% confidence intervals were determined from the 24 hour counts using the probit analysis method described by Finney. The LD_{50} was derived from the best fit line obtained by linear regression analysis.

2. Antimicrobial activity

This assay was kindly supported by Assistance Professor Sathaporn Sirotammarat (Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

2.1 Technique

The determination of antimicrobial activity was determined by the disc method.

2.2 Test microorganisms

The microorganisms using in this test were

- *Staphylococcus aureus* ATCC 6538p
- *Bacillus subtilis* ATCC 6633
- *Escherichia coli* ATCC 25922
- *Candida albicans*

2.3 Media

- Nutrient agar (NA), The medium was used to make NA slants, which were used in subculturing the bacteria at 37°C for 24 hour befor use.

- Trypticase Soy Agar (TSA), The medium was BBL^{R} Trypticase Soy Agar supplied by Becton-Dickenson Microbiology System.

- Sabouraud Dextrose Agar (SDA), BBL^{R} supplied by Becton Dickenson Microbiology System was used for culturing the bacteria

Sterilized by autoclaving at 121°C for 15 minute. This medium was as the SDA slant for subculturing the fungus at 37°C for 48 hours before use and for culturing the fungus in period of assay.

2.4 Sample preparation

Sample was prepared by dissolving 5 mg of sample 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 disc for antibiotic assay (Watman ^{R} AA) in glass petri-dish (20 x 100 mm). The discs were dried in air. Two replicated were prepared for each dose level.

2.5 Bioassay

- 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 0.85 % 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).

- bioassay procedure

Prepared the media by warming the agar until 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 became solid. Swabed the broth and spreaded 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 were 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. Cytotoxic activity

In this work, the cytotoxicity test was performed against human tumor cell lines and cultured tumor cells from test animal. The method of assay was briefly described as below (Moss man, 1983).

3.1 Target cells

The cell lines in this assay were

- P-388 (A-methyl cholanthrene-induced lymphoid neoplasm in a DBA/Z mouse; a non-anchoroge dependent cell lines
- A-549 (Human non-small cell lung carcinoma)
- HT-29 (Human colon adenocarcinoma moderately well differentiated.)

3.2 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 microtiter plates. Serial dilutions of the test sample were added and cell were incubated for 4-6 days cell growth was measured by a colorimetic assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), a formazan dye, which was directly proportional to cell number (Mossman, 1983).

Extraction and Isolation

1. Extraction

The small pieces of frozen sponges (56 kg, wet weight) were macerated with acetone (36 liters), three times repeated and then filtered. The combined acetone filtrate was concentrated under reduced pressure at temperature not over 50°C. Remaining volume of this fraction was approximately 3.6 l. This fraction was partitioned with dichloromethane (3.6 l). The dichloromethane extract was separated and concentrated to dryness under reduced pressure to yield 112 g of mass (0.20% based on wet weight of sponge). The remaining from acetone extract after partitioning with dichloromethane was aqueous fraction. The aqueous fraction was concentrated under reduced pressure to yield 472 g of mass (0.84% based on wet weight of sponge).

2. Isolation of chemical constituents from the dichloromethane extract

Crude dichloromethane extract 112 g was fractionated by flash column chromatography to ten fractions as followed

Fraction	Eluants	Weight (g)
M-001	Hexane	15.10
M-002	Hexane	0.79
M-003	Hexane:EtOAc (20:1)	13.56
M-004	Hexane:EtOAc (10:1)	15.83
M-005	Hexane:EtOAc (5:1)	16.69
M-006	Hexane:EtOAc (2:1)	15.75
M-007	Hexane:EtOAc (1:1)	5.49
M-008	EtOAc	3.19
M-009	EtOAc:MeOH (10:1)	3.78
M-010	MeOH	6.63

Fractions M-004-M-006 contain known compounds, mycaperoxide A and mycaperoxide B. Fraction M-001-M-003 and M-007-M-010 were sent to test cytotoxicity. Results were shown as below.

	IC_{50} µg/ml		
Fractions	P-388	A-549	HT-29
M-001	10.0	10.0	10.0
M-002	10.0	10.0	10.0
M-003	5.0	5.0	10.0
M-007	0.2	0.2	0.2
M-008	1.0	1.0	1.0
M-009	5.0	10.0	10.0
M-010	5.0	10.0	20.0

Fraction M-007 was the best active fraction

2.1 Isolations of chemical constituents from fraction M-007

Fraction M-007 (4 g) was dissolved with a small volume of ethyl acetate and triturated with kieselguhr (5 g). The mixture was dried under vacuum. It was

fractionated by flash column chromotography, using a silica gel column (120 g, 4.2 x 17 cm.). The column was eluted with 20 % ethyl acetate in hexane. The eluates were collected 75 ml per fraction and examined by TLC using 50 % ethyl acetate in hexane as a developing solvent. The fractions with similar chromatographic pattern were combined.

Fractions	Number of eluates	Weight (mg)
M-045	1-8	723.3
M-046	9-13	83.2
M-047	14-23	42.5
M-048	24-34	134.5
M-049	35-36	284.0
M-050	57-72	138.5
M-051	73-90	66.7
M-052	91-106	952.5
M-053	107-113	805.0

2.1.1 Isolation for compounds M-059 and M-060

Fraction M-047 (42.5 mg) was fractionated by gel filtration technique, using a column of sephadex LH-20 (3 x 58 cm.) with 10 % methanol in chloroform as a eluant. M-047 was dissolved with eluant and added onto the top of column. The eluants were examine by thin layer chromatography. Fractions 6-8 were combined and repeatedly fractionated by gel filtration technique in order to isolate the mycaperoxides. Fractions 6-8 were combined and dried under reduce pressure. They were separated by flash column chromatographic technique, using silica gel column (20 g, 2.0 x 16.0 cm). The column was eluted with 1 % methanol in chloroform. The eluates were collected 15 ml./ fraction and examined by TLC, using 10 % methanol in chloroform as a developing solvent. Fractions 17-21 were combined and concentrated under reduced pressure. A white compound was crystallized from these fractions. The crystal was washed with small volume of cold methanol and dried. The crystal was recrystallized in methanol. The white crystal yield 7.2 mg. and names as M-055

6 mg of fraction M-055 was diluted with methanol 2 ml. 100 µl of solution was injected to semipreparative hplc column, detected at λ 242 nm. The column was eluted with 10 % of water in methanol. The 14 times of 100 µl injection were repeated. The eluate was collected at retention time as below.

Retention times (min.)	Fractions	Weight (μg)
21.23	M-056	< 100
24.18	M-057	< 100
30.00	M-058	500
37.89	M-059	400
47.69	M-060	200

HPLC chromatogram is shown in Figure 11. Fraction M-056 and fraction M-057 were very little amounts and M-058 were not pure. Fraction M-059 and fraction M-060 were assigned.

3. Isolation of chemical constituents from the aqueous extract

Crude aqueous extract 462 g was equally divided into two parts (2x231 g) each part was fractionated by the quick column chromatographic technique. The 231 g of mass was dissolved with a small-volume of methanol and triturated with kieselgulr (172 g). This mixture was dried under the vaccum. It was fractionated by the quick column chromatographic technique, using a sintered glass filter column of silica gel (600 g, 5x19.8 cm). The eluants were used in the order as shown below :

- Chloroform : methanol	(9.5 : 0.5)	1,000 ml	fractions 1-4
- Chloroform : methanol	(9.0 : 1.0)	6,000 ml	fractions 5-16
- Chloroform : methanol	(8.0 : 2.0)	11,000 ml	fractions 17-60
- Chloroform : methanol	(7.0 : 3.0)	2,250 ml	fractions 61-69
- Chloroform : methanol	(6.0 : 4.0)	1,750 ml	fractions 70-76
- Chloroform : methanol	(1.0 : 1.0)	2,750 ml	fractions 77-87
- Chloroform : methanol	(0.5 : 9.5)	3,000 ml	fractions 88-99

The eluates were collected 250 ml per fraction.and examined by TLC using 10% methanol in chloroform as developing solvent. The fraction showing similar chromatographic pattern were combined.

Fractions	Number of eluates	Weight (g)
A-001	1-9	1.0 g
A-002	10-18	1.4 g
A-003	19-27	1.3 g
A-004	28-70	20.0 g
A-005	71-90	119.3 g
A-006	91-99	143.0 g

3.1 The isolations of chemical constituents from fraction A-004

Fraction A-004 (9 g) was fractionated by gel filtration technique using a column of sephadex LH-20 (2.5 cm x 80 cm) with 20% methanol in chloroform as eluant. Each 0.6 g of fractions A-004 was dissolved with eluant and added onto the top of the column. The eluates were collected approximately 20 ml per fraction. The eluates were examined by TLC using 10% methanol in chloroform as developing solvent. The fractions showing similar chromatographic pattern were combined to yield three fractions namely, A-007, A-008 and A-009.

Fractions	Weights (g)
A-007	1.735
A-008	2.898
A-009	1.385

Fraction A-009 (1.103 g) was fractionated by flash column chromatographic techniques using a silica gel column (3 cm x 18.5 cm). The sample 1.103 g was divided in two parts. Each part was dissolved with small volume of methanol and triturated with kieselguhr (4 g) then dried under vaccum. The mixtures were added onto the top of the column. The column was eluted with 8 % methanol in chloroform. The eluates were collected 50 ml per fractions. The eluates were examined by TLC. Using 10% methanol in chloroform as a developing solvent. The fractions showing similar chromatographic pattern were combined to yield ten fractions, A-031-A-039.

Fractions	Weights (mg)
A-031	150
A-032	25
A-033	25
A-034	4
A-035	50
A-036	30
A-037	40
A-038	18
A-039	150

3.1.1 Isolation for compound A-044 and A-046

The TLC chromatogram of fraction A-033 showed that there was two quenching spots under ultraviolet light at wave length 254 nm. These two spots were negative with anisaldehyde sulphuric acid reagent. Fraction A-033 (50 mg) was isolated by the flash column chromatography using silica gel column (4.3 x 15.5 cm). Fraction A-033 was dissolved with small volume of methanol and triturated with kieselguhr (3 g). The mixture was dried under vaccum and added onto the top of the column. The column was eluted with 5% methanol in chloroform. The eluate was collected 30 ml of each fraction. The fractions were combined after examined with TLC. Fractions 16-27 were combined and concentrated under reduced pressure. A white compound was precipitated from this fractions. The precipitate was dissolved with 50% methanol in chloroform and recrystallized. It yielded 8 mg of white crystals and named as A-044. This compound was identified as thymine.

Fractions 38-49 were combined and concentrated under reduced pressure. A yellow pale compound was precipitated from these fractions. The precipitate was dissolved with 50% methanol in chloroform and recrystallized. It yield 10 mg of white crystals and named as A046. This compound was identified as uracil.

3.1.2 Isolation for compound A-047 and A-049

The TLC chromatogram of fraction A-037 showed two quenching spots under ultraviolet light at wave length 254 nm. These two spots were given violet color after spraying with anisaldehyde sulphuric acid reagent and heated. Fraction A-037 was fractionated by gel filtration technique, using sephadex LH-20 column (2.3 cm x 70 cm). The sample was dissolved with small volume of eluant and added onto the

top of the column. The column was eluted with 20% methanol in chloroform. The eluate was collected 15 ml of each fraction. Each fraction was determined by chromatographic TLC pattern. Fractions 9-12 were combined and concentrated under reduced pressure. A white compound was crystallized from this fraction. The crystal was washed with small volume of chloroform and dried under vaccum. It yielded 17 mg named as A-047. This compound was identified as thymidine.

Fractions 13-15 were combined to yield A-048. Fraction A-048 contained still two quenching spots under ultraviolet light at wave length 254 nm, thus fraction A-048 was repeatedly isolated by gel filtration technique as the same manner of fraction A-037. TLC patterns of fractions 6-8 were identically with TLC pattern of compound A-047 thus compound from this fraction was also thymidine. Fractions 11-14 from A-048 were combined and concentrated under reduced pressure. A white compound was crystallized from this fraction to yield 20 mg and named as A-049. This compound was identified as 2'-deoxyuridine.

Spectral Data of Isolated Compounds

1. Compound M-059

eims : m/z (% relative intensity); Figure 57
414 (16.3), 399 (1.5), 270 (3.1), 245 (4.6), 227 (24.5), 152, (93.7)

uv : λ_{max} nm (ε), in chloroform; Figure 40
243 (8,238)

ir : ν cm^{-1}, KBr (film); Figure 41
3,506, 1,676, 1,036

^{1}H nmr : δ ppm, 500 MHz, in chloroform-d; Figures 42-45
5.82 (1H, s), 4.35 (1H, t, J = 2.8 Hz), 2.52 (1H, ddd, J = 17.3, 15.0, 5.0 Hz), 2.38 (1H), 2.05 (1H), 2.04 (1H), 2.00 (1H), 1.95 (1H), 1.86 (1H), 1.70 (1H), 1.62 (1H), 1.53, (1H), 1.47-1.54 (2H), 1.37 (3H, s), 1.36 (1H), 1.31 (1H), 1.30 (1H), 1.24 (1H),1.16(1H), 1.14 (1H), 1.14 (1H), 1.13 (1H), 1.06 (1H), 1.02 (1H), 0.92 (3H, d, J = 6.7 Hz), 0.91 (1H), 0.85 (3H, d, J = 6.8 Hz),0.81(3H, d, J = 6.7 Hz), 0.78 (3H,d, J = 7.0 Hz), 0.78 (1H), 0.74 (3H, s)

^{13}C nmr : δ ppm, 125 MHz, in chloroform-d; Figures 46-47
200.33, 168.37, 126.39, 73.36, 56.16, 55.93, 53.66, 42.55, 39.65, 38.87, 38.61, 38.02, 37.16, 35.87, 34.29, 33.68, 32.45,

30.33, 29.76, 28.19, 24.18, 21.01, 20.21, 19.54, 18.68, 18.28, 15.42, 12.04

2. Compound M-060

eims : m/z (% relative intensity); Figure 39
428 (10.6, M^+), 413 (2.0), 386 (0.1), 287 (1.9), 269 (3.0), 245 (4.4), 227 (21.5), 152 (100)

uv : λ_{max} nm (ε), in chloroform; Figure 13
243 (8,238)

ir : ν cm^{-1}, KBr (film); Figure 14
3,431, 2,918, 1,617, 1,645, 1,037

^1H nmr : δ ppm, 500 MHz, in chloroform-d; Figures 15-18
5.82 (1H, s), 4.34 (1H, t, J = 2.9 Hz), 2.52 (1H, ddd, J = 17.2, 14.9, 5.0 Hz), 2.38 (1H, dddd, J = 17.2, 4.5, 2.7, 1 0 Hz), 2.05 (1H), 2.04 (1H), 2.00 (1H), 1.95 (1H), 1.86 (1H), 1.71 (1H), 1.68 (1H),1.62, (1H), 1.49-1.54 (2H), 1.38 (1H), 1.38 (3H, s), 1.36 (1H), 1.33 (1H), 1.32 (1H), 1.31 (1H), 1.23 (1H), 1.17 (1H), 1.15 (1H), 1.14 (1H), 1.13 (1H), 1.04 (1H), 1.01 (1H), 0.98 (1H), 0.94 (1H), 0.93 (3H, d, J = 6.6 Hz), 0.91 (1H), 0.86 (3H, t, J = 7.4 Hz) , 0.83 (3H, d, J = 6.8 Hz), 0.81 (3H, d, J = 7.0 Hz), 0.74 (3H, s)

^{13}C nmr : δ ppm, 125 MHz, in chloroform-d; Figures 19-20
200.44, 168.49, 126.36, 73.21, 56.08, 55.92, 53.66, 46.10, 42.54, 39.63, 38.54, 38.02, 37.13, 36.26, 34.28, 33.91, 29.76, 29.00, 28.18, 26.44, 24.18, 23.06, 21.01, 19.61, 19.53, 19.00, 18.80, 12.32, 12.03

3. Compound A-044

eims : m/z (% relative intensity) ; Figure 62
126 (78.5, M^+), 83 (8.6), 82 (8.6), 55 (100)

uv : λ_{max} nm (ε), in methanol; Figure 58
264.5 (9,529)

ir : ν cm^{-1}, KBr disc ; Figure 59
3,210, 1,739, 1,676

^1H nmr : δ ppm, 500 MHz, in methanol-d_4 ; Figure 60
7.12 (1H, q, J = 1.2 Hz), 1.75 (3H, d, J = 1.2 Hz)

^{13}C nmr : δ ppm, 125 MHz, in methanol-d_4 ; Figure 61
167.46, 153.73, 139.14 , 110.46, 12.08

4. Compound A-046

eims ; m/z (% relative intensity) ; Figure 67
112 (100 M^+), 69 (60.9), 68 (20.5), 42 (39.1), 41 (21.7)

uv ; λ_{max} nm (ε), in methanol; Figure 63
259.3 (6,916)

ir ; ν cm^{-1}, KBr disc ; Figure 64
3,093, 2,928, 1,734, 1,645

^1H nmr ; δ ppm, 500 MHz, in methanol-d4 ; Figure 65
7.24 (1H, d, J = 7.6 Hz), 5.52 (1H, d, J = 7.6 Hz)

^{13}C nmr ; δ ppm, 125 MHz, in methanol-d4 ; Figure 66
167.31, 153.52, 143.49, 101.73

5. Compound A-047

eims ; m/z (% relative intensity) ; Figure 78
242 (2.1, M^+), 212 (< 1), 153 (5.5), 126 (80.3), 117 (95.0), 99 (29.4), 83 (11.6), 81 (38.6), 55 (100)

cims ; m/z ; Figure 79
335 $(M+glycerol)^+$

uv ; λ_{max} nm (ε), in methanol ; Figure 68
266.4 (10,841)

ir ; ν cm^{-1}, KBr disc ; Figure 69
3,322, 1,702, 1,659

^1H nmr ; δ ppm, 500 MHz, in methanol-d_4 ; Figure 70
7.81 (1H, q, J = 1.2 Hz), 6.27 (1H, dd, J = 6.7, 6.5), 4.39, (1H, dt, J = 6.1, 3.3), 3.90 (1H, q, J = 3.4 Hz), 3.80 (1H, dd, J = 12.1, 3.2 Hz), 3.73 (1H, dd, 11.9, 3.6 Hz), 2.25 (1H,ddd, 13.2, 6.3, 3.8), 2.20 (1H,ddd, 13.7, 7,4, 6.1), 1.88(3H, d,J = 1.2 Hz)

^{13}C nmr ; δ ppm, 125 MHz, in methanol-d_4 ; Figure 71
166.45, 152.42, 138.21, 111.57, 88.87, 86.30, 72.25, 62.89, 41.24, 12.47

6. Compound A-049

eims ; m/z (% relative intensity) ; Figure 85
228 (< 1.0, M^+), 198 (< 1), 139 (5.5), 117 (100), 112 (30.6), 99 (11.6), 81 (9.8), 69 (44.1), 68 (21.5)

cims ; m/z ; Figure 86
321 $(M+glycerol)^+$

uv ; λ_{max} nm (ε), in methanol ; Figure 80
262.3 (9,450)

ir ; ν cm^{-1}, KBr disc ; Figure 81
3,269, 1,701, 1,674

^{1}H nmr ; δ ppm, 500 MHz, in methanol-d_4 ; Figure 82
7.97 (1H, d, J = 8.2 Hz), 6.26 (1H, dd, J = 6.7, 6.7 Hz), 5.69 (1H, d, J = 8.2 Hz), 4.38 (1H, dt, J = 6.1, 3.3 Hz), 3.92 (1H, q, J 3.4 Hz), 3.78 (1H,dd, 11.9, 3.4 Hz), 3.72 (1H, dd, 11.9, 3.6 Hz), 2.29 (ddd, J = 13.4, 6.3, 3.4 Hz), 2.20 (1H, ddd, J = 13.6, 7.3, 6.1 Hz)

^{13}C nmr ; δ ppm, 125 MHz, in methanol-d_4 ; Figure 83
166.27, 152.26, 142.55, 102.69, 89.03, 86.68, 72.29, 62.89, 41.41