CHAPTER III

EXPERIMENTAL

Source and authentification of the tunicate

Green colonies of the tunicate, *Didemnum sp.*, were collected by hands from Phi-Phi Island, Krabi Province, Thailand. The tunicate voucher specimens were preserved in 10% formalin in seawater and kept at the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University. The remaining samples were frozen at -20 °C prior to extraction.

General techniques

1. Chromatography

1.1 Analytical thin layer chromatography

Adsorbent	: Silica gel 60 F-254 precoated plate (E. Merck No. 1.0715)
	: Silica gel C-18 F-254 precoated plate (E. Merck)
Layer thickness	: 250 μm
Technique	: One way, ascending
Distance	: 5 cm
Temperature	: Room temperature 25-30 °C
Detection	: 1). Visual detection under daylight
	2). Ultraviolet light at the wavelengths of 254 and 365 nm
	3). Spraying with anisaldehyde-sulphuric acid solution and heating at 100-110 °C for a few minutes

1.2 Column chromatography

1.2.1 Gel filtration chromatography

Adsorbent : Sephadex LH-20

Packing : The adsorbent was suspended in the eluent and left to swell for 24 hrs. before using, then poured into the column and allowed to settle properly.

Sample loading : The sample was dissolved in a small volume of the eluent and loaded onto the top of the column.

Examination of eluates

: Fractions were examined by TLC under ultraviolet light at wavelengths of 254 and 365 nm and by exposing to iodine vapour and anisaldehyde-sulphuric acid solution, respectively.

1.2.2 Flash column chromatography

Adsorbent : Polystyrene (MCI Gel)
High Porous Polymer CHP20P (75~150μ) Lot No. 4F511
Mitsubishi Chemical Corporation

Packing : The adsorbent was wet-packed after being suspended in 30% methanol/water. The slurry of adsorbent was poured into the column, tapped and pressed down under air pump, then allowed to settle overnight.

Sample loading : The sample was dissolved in a small volume of the eluent, triturated with adsorbent, air dried and loaded onto the top of the column.

Examination of eluates

: Fractions were examined in the same manner as described in section 1.2.1.

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

Column : LiChrospher® 100 RP-18 (10μm)
LiChroCART® 250-10 Cat. 50853

Flow rate : 1.3 ml/min

Mobile phase : methanol - water (1:1)

Sample preparation

: Dissolve sample in methanol - CH_3CN (7:3) and filter through Toyopak® ODS before injection.

Injection volume : 200 μl

Pump : LC-10AD (Shimadzu Corporation)

Detector : SPD-10A (Shimadzu Corporation)

Recorder : C-R6A Chromatopac (Shimadzu Corporation)

Temperature : 20 °C

2. Spectroscopy

2.1 Mass spectra (MS)

The mass spectra were obtained on a Finnigan MAT 700 mass spectrometer Equipped with Finnigan Electrospray Source operating at -31 eV CID voltage with capillary temperature of 230 °C.

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

The nmr spectra were obtained on a JEOL LMN.α series NMR spectrometer (500 MHz for ^{1}H nmr and 125 MHz for ^{13}C NMR) (The Scientific and Technological Research Equipment Center, Chulalongkorn University).

2.3 Ultraviolet - visible (UV) absorption spectra

Ultraviolet - visible (UV) absorption spectra were obtained on Milton Roy spectronic 3000 Diode Array spectrometer.

2.4 Infrared (IR) absorption spectra

The spectra were obtained on Perkin Elmer FT-IR Spectrum 2000 Spectrometer .

2.5 Optical rotation

The optical rotation were obtained on Perkin Elmer Polarimeter 341.

3. Solvent

All of organic solvents used in this work, excluding the deuterated solvents for nmr spectra and HPLC grade solvents for semipreparative HPLC, were commercial grade that had to be redistilled prior to use.

Bioactivity determination

1. Antimicrobial activity

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

1.1 Microorganisms

The representative microorganisms were kindly provided by Assistant Professor Sathaporn Sirotamarat of the Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University. Three species of microorganisms were employed in the determination :

1. *Staphylococcus aureus* ATCC6538 representing gram positive bacteria.
2. *Escherichia coli* ATCC6633 representing gram negative bacteria.
3. *Candida albicans* representing fungi.

All of these microorganisms were subcultured in suitable media prior to use in order to intensify their activities. The incubation was carried out at 37 °C, 24 hrs for bacteria and 48 hrs for fungi.

1.2 Media

- Trypticase soy agar (TSA)

The medium used was BBL® Trypticase Soy Agar (Becton-Dickinson 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	

The formula powder (40 g) was dispersed in 1 liter of purified water and stirred until well-suspended. The agar suspension, then, was heated to complete dissolution. The medium was sterilized by autoclaving at 121 °C for 15 minutes. This medium was used for inoculating the bacteria during the assay.

- Sabouraud dextrose agar (SDA)

The medium used was BBL® Sabouraud Dextrose Agar (Becton-Dickinson 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	

The formula powder (65 g) was dispersed in purified water and throughly mixed. The suspension was then boiled to completely dissolve the ingredients. This medium was used in subculturing and inoculating of yeast during the assay.

1.3 Sample preparation

The sample was dissolved in methanol and diluted to the concentration of 5 mg/ml. Twenty microliters of this solution was tranferred to a 6 mm disc (Whatman® AA disc for antibiotic assay) and allowed to dry. Thus, final concentration was 100 µg/disc. The determination was made in triplicate.

1.4 Bioassay

The bioassay was carried out by using aseptic technique. Prior to use, all glasswares and materials had to be sterilized by autoclaving at 100 °C for 20 minutes. Each microorganism was suspended in sterilized 0.85% saline solution and spreaded in three dimensions on the surface of suitable medium plate.

The sample disc were put on the microbe-spreaded medium plates and, then, incubated at 37 °C for 24 hrs for bacteria, or 48 hrs for yeast. After incubation, if the test sample had antimicrobial activity, the clear zone would appeared around the disc. The diameter of inhibition zone was measured and reported in the scale of millimeter.

2. Brine shrimp lethality activity

The bioassay was applied from the microwell cytotoxicity assay method (Solis *et al.*, 1993) and as shown in Scheme 1. This method is recognized as a simple bioassay for natural product researches with the advantages of being rapid, inexpensive and simple (no aseptic techniques are required). Activities of broad range of known active compounds are manifested as toxicity to the brine shrimp, a tiny crustacean. Brine shrimps have been utilized in various bioassay system, for example, analysis of pesticide residues, mycotoxins, stream pollutants, anaesthetics, dinoflagellate toxins, morphine-like compounds, toxicity of oil dispersants, cocarcinogenicity of phorbol esters and toxicity in marine environments (Meyer *et al.*, 1982).

2.1 Brine shrimp

The eggs of brine shrimp (*Artemia salina* LEACH)(Aquarium product® , USA) were hatched in a shallow rectangular box filled with artificial sea water. The box was divided into two compartments by a septum which had a few 2-mm holes. One compartment containing the brine shrimp's eggs was darken, while another one was illuminated by a tungsten lamp. After 24 hrs, the nauplii of brine shrimp would

hatch and moved directly to the bright side. A small quantity of yeast was added into this as their nutrient.

2.2 Artificial sea water

Salt (Instant Ocean, Aquarium system Inc., 38 g) was dissolved in 1 liter of purified water and the solution was filtered through a filter paper (Whatman® filter paper No. 1)

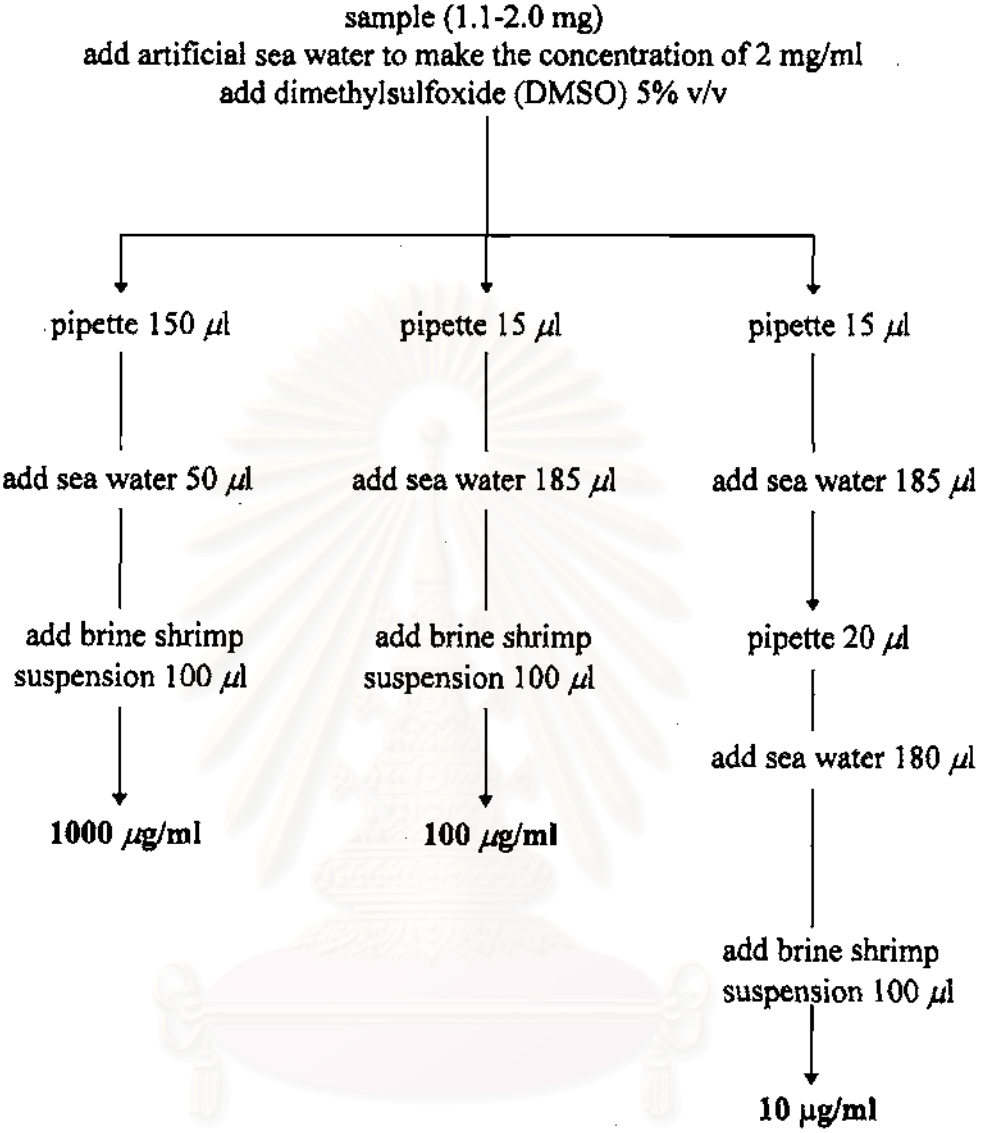
2.3 Sample preparation

The test sample was dissolved in DMSO (5% of total volume of stock solution) and diluted with sea water to make a concentration of 2 mg/ml. The sample stock solution was diluted with a proper volume of artificial sea water and tranferred to the microwells. The samples were tested at concentrations of 10, 100, 1000 μg/ml. The final volume of each well (300 μl) contains 7.5, 0.75 and 0.075 μl of DMSO, respectively. If most of the brine shrimps were dead at the concentration of 10 μg/ml, the sample concentration would be further decreased to 1, 10, 100 μg/ml or 0.1, 1, 10 μg/ml. All determinations were made in triplicate so that the total number of brine shrimps in each concentration was about 30. Control microwells were prepared by using only DMSO and then treated in the same manner as the test sample.

2.4 Bioassay

About ten brine shrimps (in 100 μl of brine suspension) were transferred to each wells with a micropipette. The microwells were maintained under illumination. After 24 hrs, the dead and the survivors were counted under stereomicroscope.

The number of dead brine shrimps in the test wells of each concentration were subtracted from those in the control wells, then calculated in term of LD_{50} by using Finney equation program. The LD_{50} values and 95% confidence intervals were determined from the 24-hour counts using the probit analysis method



Scheme 1. Brine shrimp lethality test at the concentrations of 1000, 100, 10 µg/ml.

described by Finney. In case where data were insufficient for this technique, the dose response data were transformed into a straight line by means of a logic transformation and the LD_{50} was derived from the best fit line obtained by linear regression analysis.

3. Cytotoxic activity

The cytotoxicity of the test sample was evaluated against some human tumor cell lines and cultured tumor cells from test animal. The assay result was helpfully supplied by Dr. Lola Gracia Gravalos, Pharma Mar S.A., Spain.

3.1 Target cells

The cell lines utilized in this bioassay were

P-388 (a methylcholanthrene-induced lymphoid neoplasm in a DBA/z mouse, a non-anchorage dependent cell line),

A-549 (human non-small cell lung carcinoma),

HT-29 (human colon adenocarcinoma, moderately well differentiated).

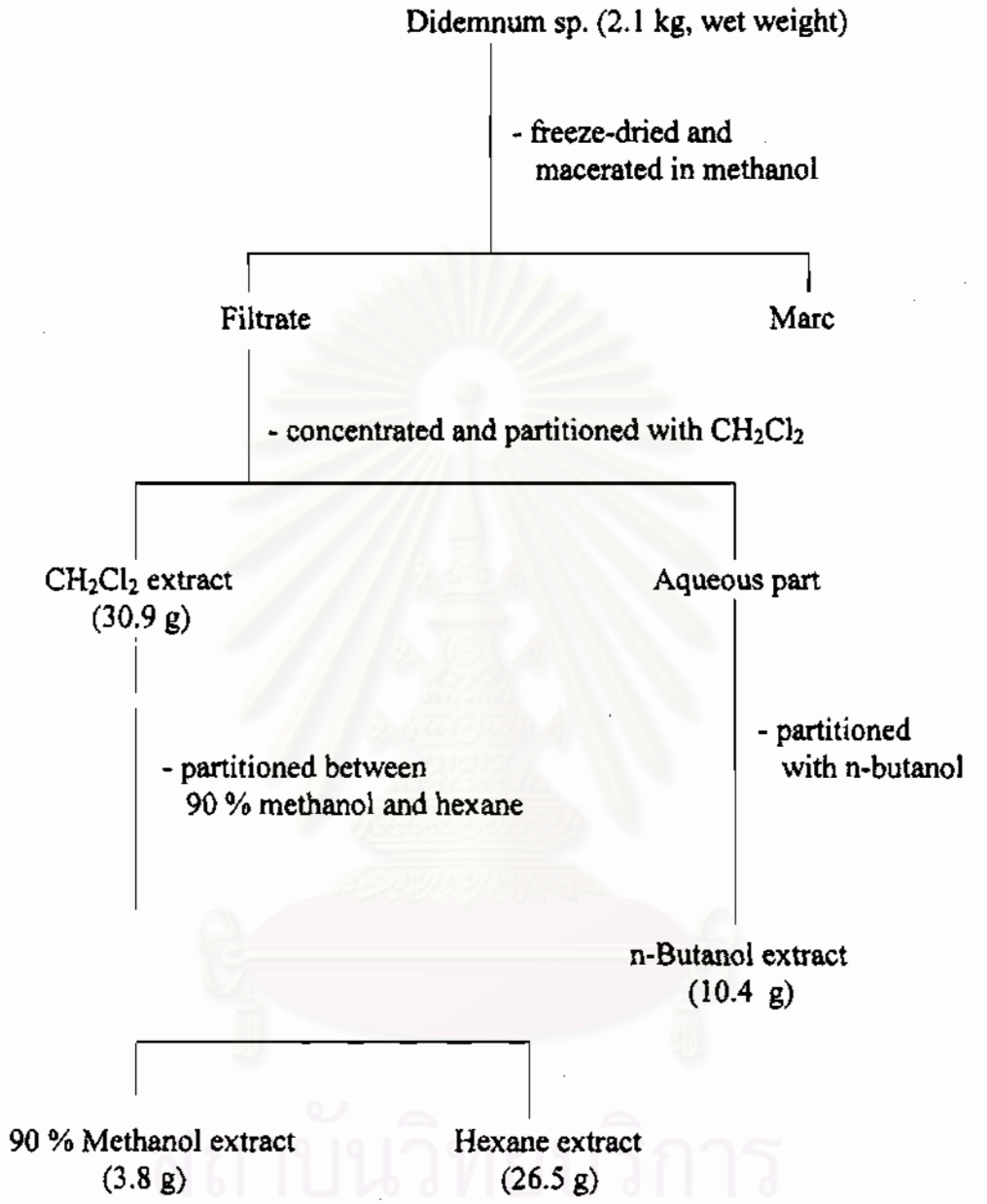
3.2 Bioassay

For P-388 cell line, the cells were incubated in the presence of the test sample for 48 hrs. Cell growth was measured by counting the cells with the electronic counting device. The result was obtained as the ED_{50} of each test sample. In human tumor assay, 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-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), a formazan dye, which would be cleaved by active mitocondria to produce a blue colour. Optical density which was directly proportional to cell number was measured by spectrometer (Mosmann, 1983).

Extraction

The tunicates, *Didemnum sp.* (2.1 kg wet weight), were freez-dried. Dried tunicates (440 g) were repeatedly macerated three times, two days each, in two liters of methanol. The filtrate was evaporated under reduced pressure at temperature not exceeding 50 °C. The syrupy mass was diluted with 600 ml of 10 % methanol in water and then partitioned with 200 ml of CH_2Cl_2 15 times. The lower CH_2Cl_2 layer was separated and evaporated under reduced pressure to yield 30.9 g of a syrupy mass (1.47 % of wet weight). A small portion of this fraction was kept as a reference and also for bioactivity screening. The upper aqueous layer was evaporated to remove the remaining CH_2Cl_2 and then partitioned with n-butanol. The n-butanol extract was evaporated under reduced pressure to yield 10.4 g of a syrupy mass (0.50 % of wet weight).

The CH_2Cl_2 extract was dissolved in 180 ml of methanol and diluted with purified water to make 90 % methanol solution. It was partitioned with 100 ml of hexane 15 times. Both extracts were evaporated under reduced pressure to yield 26.5 g of hexane and 3.8 g methanol extract (1.26 % and 0.18 % of wet weight, respectively). A small amount of these extracts were also reserved as references and for bioactivity screening. The extraction of the tunicate is shown in Scheme 2.



Scheme 2. Extraction scheme of the tunicates, *Didemnum sp.*

Isolation of chemical constituents from the tunicate, *Didemnum sp.*

From the bioactivity screening tests, the extracts from these tunicates exhibited antiviral, cytotoxic and brine shrimp lethality activities. The results are presented in Table 5.

Table 5. Bioactivity of the extracts from the tunicate, *Didemnum sp.*

	Cytotoxic activity ED_{50} (μg/ml)			Brine shrimp lethality activity	Anti HIV-I activity
	P-388	A-549	HT-29	LD_{50} (μg/ml)	(% Inhibition)
CH_2Cl_2	2.5	2.5	2.5	99	14 % at 0.021 μg
hexane	2.5	2.5	2.5	>200	9 % at 2 μg
90% methanol	2	1	1	30	toxic*
n-butanol	>20	>20	>20	>500	-

toxic* : killed all host cells

1. Isolation of compounds KMF006-1 and KMF006-2

The methanol extract (3.8 g) was fractionated by gel filtration flash column chromatography (2.5 x 90 cm) eluted with methanol. Each 25 ml fraction was collected and combined according to TLC patterns to give 12 fractions, MF001-MF012. The antiviral activity of these fractions is shown in Table 6.

Table 6. Combined fractions from the methanol extract and antiviral activity.

Number of eluate	Fraction	Anti-HIV ED_{50} (μg/ml)	Weight (mg)
1-6	MF001	-	335.6
7	MF002	2.0	538.7
8	MF003	1.5	374.5
9-10	MF004	> 5.0	575.1
11	MF005	-	367.4
12	MF006	-	345.6
13	MF007	-	350.1
14	MF008	-	69.2
15-16	MF009	-	31.7
17-18	MF010	-	16.2
19-20	MF011	-	7.8
21-30	MF012	-	2.6

Fraction MF006 (345.6 mg) yielded crystals in $CHCl_3$ - MeOH (1:1) at room temperature. The mother liquor was thus removed and the crystals were further recrystallized in $CHCl_3$ - MeOH (1:1) to give white needles (19 mg, 9.0×10^{-4} % of wet weight), codenamed as KMF006. KMF006 was found to be a mixture of two compounds, codenamed as KMF006-1 and KMF006-2.

2. Isolation of compounds MF042 and MF043

Fraction MF002 (538.7 mg) was fractionated by gel filtration flash column chromatography (2.5 x 80 cm) eluted with hexane - $CHCl_3$ (1:1). Each 20 ml fraction was collected and combined according to TLC patterns to give ten fractions, MF013-MF022. The antiviral activity of these fractions is shown in Table 7.

Table 7. Combined fractions from the fraction MF002 and antiviral activity.

Number of eluate	Fraction	Activity (% inhibition)	Weight (mg)
1-7	MF013	38 % at 0.6 μg	48.4
8-9	MF014	38 % at 0.3 μg	89.3
10	MF015	-	12.7
11-15	MF016	70 % at 4.5 μg	53.3
16-17	MF017	-	11.1
18-21	MF018	-	18.5
22-28	MF019	-	74.2
29-31	MF020	-	42.8
32-35	MF021	-	50.7
36-46	MF022	-	159.9

Base on their activities, the fractions MF013, MF014, MF015 and MF016 were combined and dissolved in a small volume of methanol - water (7:3) and triturated with polystyrene (300 mg). The mixture was dried and then fractionated by a reverse phase flash column chromatography using polystyrene (2.5 x 20 cm) as the adsorbent. Each 50 ml fraction was obtained by using the eluents in the order as shown in Table 8. The fractions were combined according to their TLC patterns to give seven fractions, MF029-MF035.

Table 8. Combined fractions from the fractions MF013-MF016.

Number of eluate	Eluent	Fraction	Weight (mg)
01-04	MeOH - H_2O (7:3)	MF029	152.9
05-08	MeOH - H_2O (8:2)	MF030	14.5
09-12	MeOH - H_2O (9:1)	MF031	65.4
13-17	MeOH		
18-22	MeOH - Acetone (9:1)	MF032	18.8
23-30	MeOH - Acetone (8:2)	MF033	9.4
31-38	MeOH - Acetone (7:3)	MF034	32.7
39-46	MeOH - Acetone (6:4)		
47-54	MeOH - Acetone (5:5)		
55-70	Acetone		
71-78	1 % AcOH in Acetone	MF035	219.7
79-86	1 % AcOH in MeoH		
87-94	1 % NH_4OH in MeOH		

Fraction MF029 (152.9 mg) was dissolved in eight ml of methanol - CH_3CN (7:3). A portion (200 μl) of the solution was injected in to a semipreparative HPLC column and detected at λ 204 nm. The column was eluted with methanol - water (1:1). The 200 μl injection were repeated 40 times. The fractions were collected at different retention time as shown in Table 9.

Table 9. Combined fractions from the fraction MF029 and their retention time.

Fraction	Retention time (min)	Weight (mg)
MF037	11.2	123.7
MF038	13.3	1.4
MF039	17.8	2.5
MF040	21.2	4.1
MF041	26.1	0.9
MF042	28.6	1.7
MF043	32.3	2.5
MF044	38.1	1.8
MF045	48.4	5.5

Fractions MF042 (1.7 mg, 8.0 x 10^{-5} % of wet weight) and MF043 (2.5 mg, 1.1 x 10^{-4} % of wet weight), both brown powder, were subjected to identification of their chemical components.

Spectral data of the isolated compounds

The compounds were characterized according to their spectroscopic data.

1. Compound KMF006-1

^{1}H nmr (500 MHz, in DMSO-d_6) : δ ppm (Figures 12-14, Pages 91-94)

8.10 (1H, s), 7.59 (1H, s), 6.64 (2H, br s), 5.59 (1H, d, J = 5.5 Hz), 5.29 (1H, d, J = 5.5 Hz), 5.07 (1H, d, J = 5.5 Hz), 4.38 (1H, q, J = 5.5 Hz), 3.88 (1H, p, J = 5.5 Hz), 3.83 (1H, q, J = 5.5 Hz), 1.26 (3H, d, J = 6.4 Hz)

^{13}C nmr (125 MHz, in DMSO-d_6) : δ ppm (Figure 15, Page 95)

157.1, 152.0, 150.3, 126.8, 103.1, 86.9, 79.1, 74.5, 73.3, 52.2, 19.0

ms : m/z (% relative intensity) (Figures 23-24, Pages 103-104)

377 (100,$[M+H]^+$), 261 (50), 134 (8), 117 (12)

uv : λ_{max} nm (Figure 27, Page 107)

210, 283 (MeOH)

ir (KBr disc) : ν cm^{-1} (Figure 28, Page108)

3476, 3295, 1630

2. Compound KMF006-2

^{1}H nmr (500 MHz, in DMSO-d_6) : δ ppm (Figures 12-14, Pages 92-94)

8.10 (1H, s), 7.58 (1H, s), 6.64 (2H, br s), 6.01 (1H, d, J = 5.5 Hz), 5.31 (1H, d, J = 6.1 Hz), 5.08 (1H, d, J = 5.5 Hz), 4.37 (1H, q, J = 5.5 Hz), 3.89 (1H, p, J = 5.5 Hz), 3.83 (1H, q, J = 5.5 Hz), 1.26 (3H, d, J = 6.4 Hz)

^{13}C nmr (125 MHz, in DMSO-d_6) : δ ppm (Figure 15, Page 95)

156.9, 152.5, 149.8, 121.6, 100.9, 87.1, 87.0, 79.2, 74.5, 73.3, 19.0

ms : m/z (% relative intensity) (Figure 25-26, Pages 105-106)

331 (98, $[(M+2)H]^+$), 329 (100, $[M+H]^+$), 215 (4), 213 (8), 134, 117

uv : λ_{max} nm (Figure 27, Page 107)

210, 283 (MeOH)

ir (KBr disc) : ν cm^{-1} (Figure 28, Page 108)

3476, 3295, 1630

3. Compound MF043

^{1}H nmr (500 MHz, in DMSO-d_6) : δ ppm (Figures 30-31, Pages 110-111)

8.10 (1H, s), 7.60 (1H, s), 6.65 (2H, br s), 5.95 (1H, d, J = 1.5 Hz), 4.20 (1H, dq, J = 6.4, 3.4 Hz), 4.13 (1H, br s), 3.81 (1H, br d, J = 3.4 Hz), 1.20 (3H, d, J = 6.4 Hz)

^{13}C nmr (125 MHz, in DMSO-d_6) : δ ppm (Figure 32, Page 112)

157.2, 151.9, 149.5, 128.0, 105.0, 89.3, 81.2, 77.9, 76.5, 51.3, 13.9

ms : m/z (% relative intensity) (Figures 39-40, Pages 119-120)

377 (100,$[M+H]^+$), 261 (50), 134 (6), 117 (3)

uv : λ_{max} nm (log ε) (Figure 41, Page 121)

209 (3.94), 283 (3.42) (MeOH)

Optical Rotation : $[\alpha]^{20}_{D}$ = -11° (c = 0.0007, MeOH)

4. Compound MF042

^{1}H nmr (500 MHz, in DMSO-d_6) : δ ppm (Figures 42-43, Pages 122-123)

8.10 (1H, s), 7.54 (1H, s), 5.98 (1H, d, J = 1.5 Hz), 4.21 (1H, dq, J = 6.4, 3.4 Hz), 4.13 (1H, br s), 3.82 (1H, br d, J = 3.4 Hz), 1.21 (3H, d, J = 6.4 Hz)

ms : m/z (% relative intensity) (Figures 44-45, Pages 124-125)

331 (100, $[(M+2)H]^+$), 329 (92, $[M+H]^+$), 215 (8), 213 (10), 134, 117

uv : λ_{max} nm (log ε) (Figure 46, Page 126)

208 (3.78), 273 (3.18) (MeOH)

Optical Rotation : $[\alpha]^{20}_{D} = 2^{\circ}$ (c = 0.0005, MeOH)