CHAPTER III

EXPERIMENTAL

Source and Authentification of the Soft Coral

The colonies of the soft coral *Cladiella tuberosa* Tixier Durivault were collected by SCUBA diving at the depth of 3 - 4 m from Si-chang Island, Chonburi, Thailand. The soft coral was identified as *Cladiella tuberosa* Tixier Durivault by Professor Yehuda Benayahu, Tel-Aviv University, Israel. The soft coral voucher specimens were preserved in 70% ethanolic solution 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). Visual detection after exposed to iodine vapour.
   4). Spraying with anisaldehyde-sulphuric acid solution and heating at 100 - 110 °C for a few minutes.

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1.2 Column chromatography

1.2.1 Quick column chromatography

Adsorbent : Silica gel 60 (No. 9385) particle size 0.040 - 0.063 mm (230 - 400 mesh ASTM).
Silica gel for TLC plate.

Packing : Adsorbent was dry - packed into the column. The column was tapped and pressed down from the top of the adsorbent to insure tightly packing. The final height of the adsorbent was 5 - 7 cm.

Sample loading : The sample was dissolved in a small volume of dichloromethane and triturated with sufficient quantity of the adsorbent. The mixture was dried and put onto the top of 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 : Silica gel 60 (No. 9385) particle size 0.040-0.063 mm (230 - 400 mesh ASTM).

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

Addition of the sample : 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 in the same manner as described in section 2.1.2.1.

1.2.3 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 tightly.

Addition of the sample : 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 in the same manner as described in section 2.1.2.1.

2. Spectroscopy

2.1 Infrared (IR) absorption spectra

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

2.2 Mass spectra (MS)

The electron impact mass spectrum (eims) of CHF028 was obtained on a Finnigan MAT mass spectrometer with INCOS 50 data system (Department of Chemistry, Faculty of Sciences, Mahidol University). The eims of CMF0201, CMF035 and CMF0361 were obtained on a Fisons VG TRIO 2000 mass spectrometer (Department of Chemistry, Faculty of Sciences, Chulalongkorn University). The FABMS of CMF0361 was obtained on a JEOL SX102A mass spectrometer (Department of Marine Sciences, University of the Ryukyus, Japan).

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

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

3. Solvent

All of organic solvents used in this work, excluding the deuterated solvents for nmr spectra, 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. *Escherrichia 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 hours for fungi.

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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 $\pm$ 0.2	

The formular 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 $\pm$ 0.2	

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The formula powder (65 g) was dispersed in purified water and throughly mixed. The suspension was then boiled to completely dissolve the ingredients. The 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 transferred 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 duplicate.

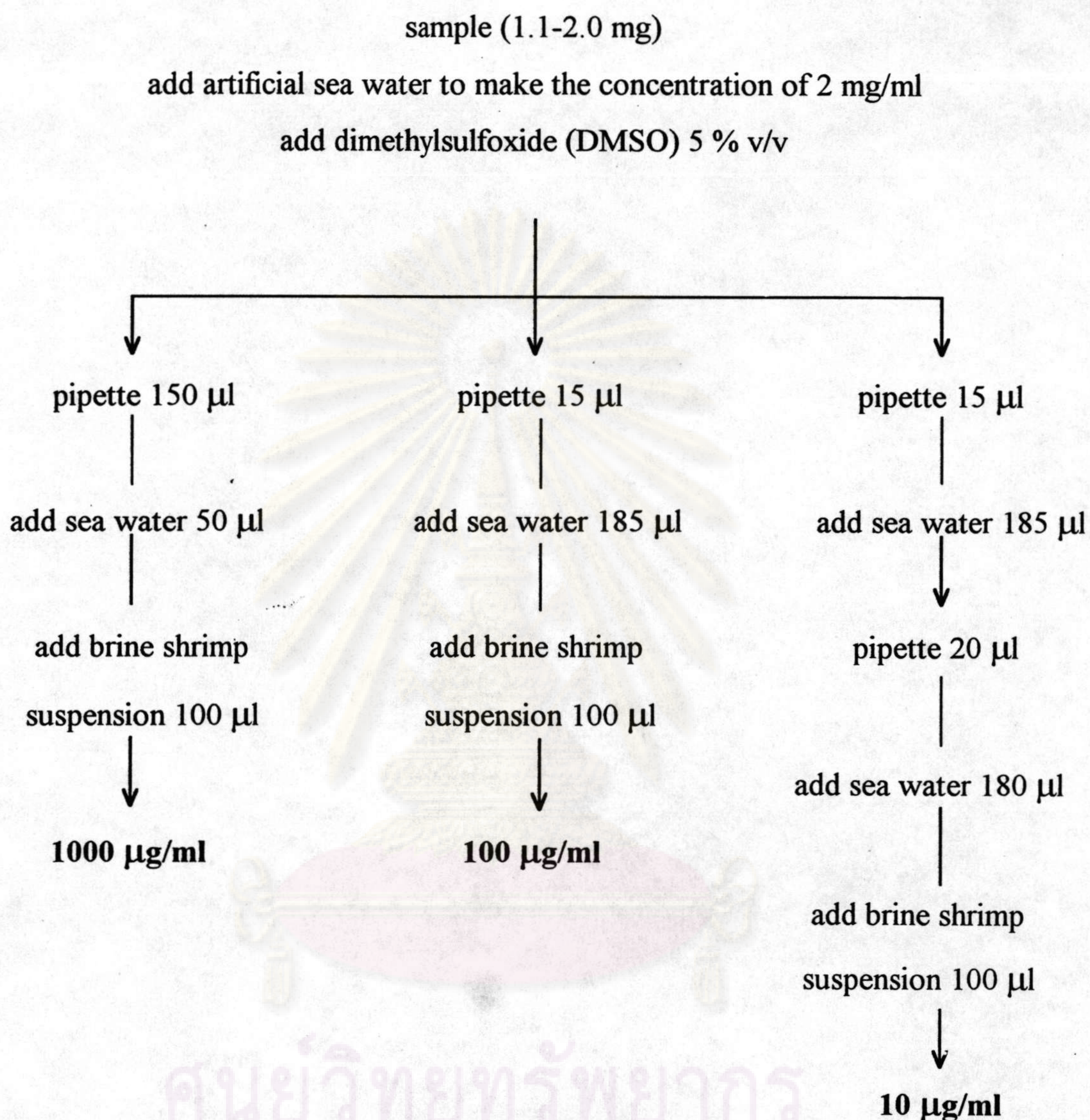
1.4 Bioassay

This bioassay was carried out by using aseptic technique. All glasswares and materials had to be sterilized before use with autoclave 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 hours for bacteria, or 48 hours 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, 1993) and as shown in the Scheme 6. 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 systems, for example, analysis of pesticide residues, mycotoxins, stream pollutants, anaethetics, dinoflagellate toxins, morphine-like compounds, toxicity of oil dispersants, cocarcinogenicity of phorbol esters and toxicity in marine environments (Meyer *et al.*, 1982).



Scheme 6. Brine shrimp lethality test at the concentrations of 1000, 100 ,10 µg/ml. The final volume of each well (300 µl) contains 7.5, 0.75, and 0.075 µl of DMSO, respectively.

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 shimp'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 side 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 transferred to the microwells. The samples were tested at concentrations of 10, 100, 1000 μg/ml. 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 described by Finney. 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 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 Garcia 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

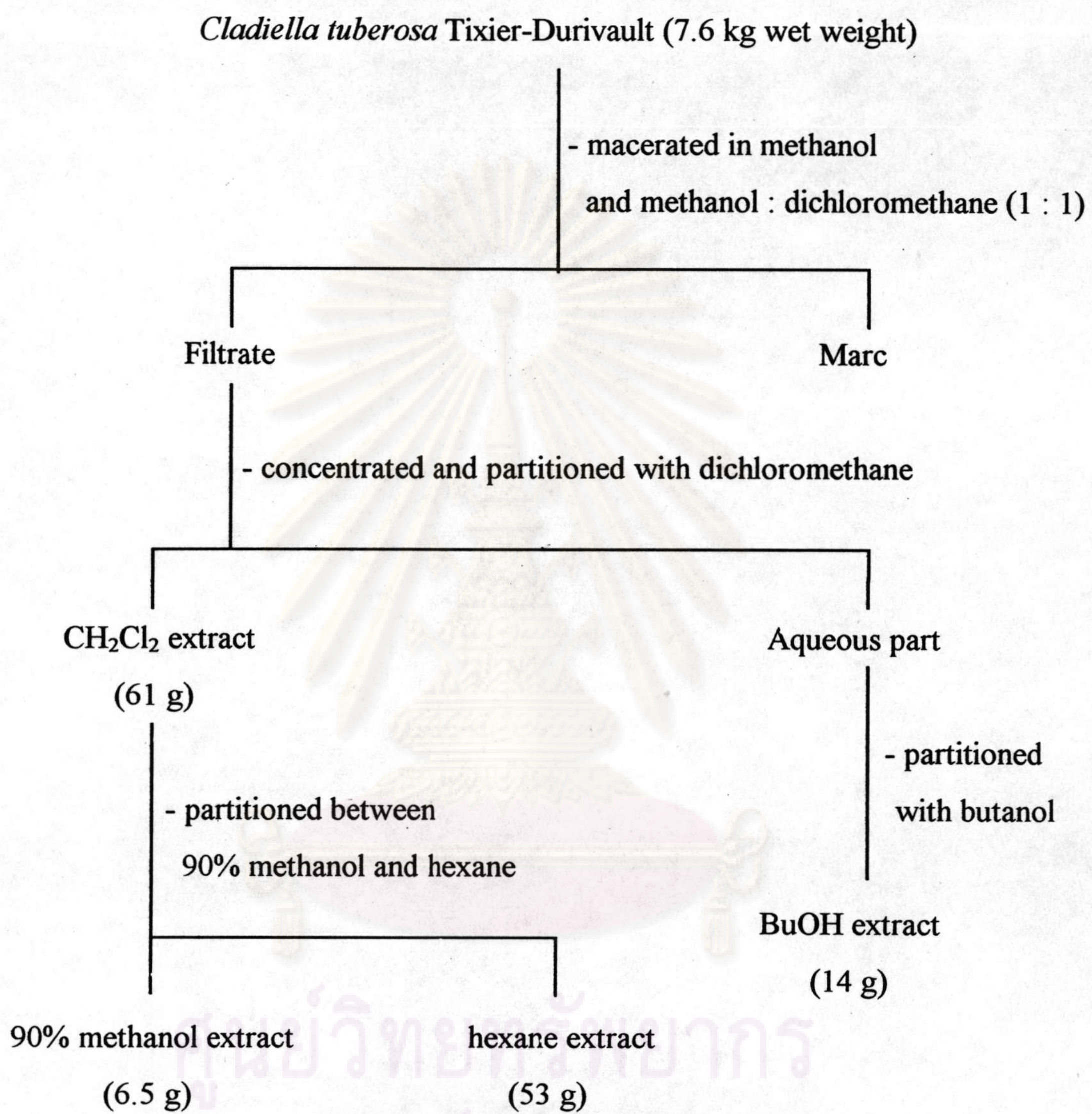
For P-388 cell line, the cells were incubated in the presence of the test sample for 48 hours. Cell growth was measured by counting the cells with the electronic counting device. The result was obtained as the ED_{50} of each test sample. In human tumor assay, cells were plated overnight in 96-well microtiter 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 mitochondria to produce a blue colour. Optical density

which was directly proportional to cell number was measured by spectrometer (Mosmann, 1983).

Extraction

The soft coral, *Cladiella tuberosa* Tixier Durivault (7.6 kg wet weight) were chopped into small pieces. They were repeatedly macerated three times (2 days each) in methanol (8 liters) and once in methanol - CH_2Cl_2 (1 : 1), (8 liters) to increase the solubilization of non-polar compounds. The filtrate of each maceration was evaporated under reduced pressure at temperature not exceeding 50 °C. The concentrated filtrate (approximately 2 l) was partitioned with 500 ml of CH_2Cl_2 10 times. The lower CH_2Cl_2 layer was separated and evaporated under reduced pressure to yield 61 g of syrupy mass (0.80 % of soft coral wet weight). A small portion of this fraction was kept as a reference and also for bioactivity screening. The upper aqueous layer, after partition with CH_2Cl_2, was evaporated to remove the remaining CH_2Cl_2 and then partitioned with butanol. The butanol extract was evaporated under reduced pressure to yield 14 g of dry extract (0.18 % of soft coral wet weight).

The CH_2Cl_2 extract was dissolved in methanol (100 ml) and diluted with purified water to make 90% methanol solution. It was then partitioned with hexane (5 x 300 ml). Both extracts were evaporated under reduced pressure to yield 53 g of hexane and 6.5 g of methanol extract (0.69 % and 0.08 % wet weight, respectively). A small amount of these extracts were also reserved as references and for bioactivity screening. The extraction of the soft coral was shown in Scheme 7.



Scheme 7. Extraction and isolation scheme of the soft coral *Cladiella tuberosa* Tixier-Durivault

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Isolation of Chemical Constituents from the Soft Coral *Cladiella tuberosa* Tixier-Durivault

From the bioactivity screening tests, the extracts from this soft coral exhibited both the cytotoxic and brine shrimp lethality activities. The results were present in Table 2.

Table 2. Bioactivities of the extracts from the soft coral *Cladiella tuberosa* Tixier-Durivault

extract	Cytotoxic activities IC_{50} (µg/ml)			Brine shrimp lethality activities LD_{50} (µg/ml)
	P-388	A-549	HT-29	
hexane	2	2	5	3
methanol	2.5	2.5	5	20
butanol	10	10	10	65

According to these results, the brine shrimp lethality test correlated well with the cytotoxicity activities. Hence, the isolation of bioactive compounds from this soft coral was guided by using the brine shrimp lethality test and examining their TLC patterns.

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1. Isolation of Compound CHF028

The hexane extract (17 g) was dissolved in a small volume of dichloromethane and triturated with silica gel (20 g). The mixture was dried and then fractionated by quick column chromatography using sintered glass filter column (Pyrex no. 2) of silica gel (200 g, 9.0 x 6.5 cm.). Group of fractions (3 x 200 ml for each fraction) were obtained by using the eluent in the order as shown below.

fractions	# 1-2	hexane
	# 3-8	hexane - chloroform (9 : 1)
	# 9-14	hexane - chloroform (7 : 3)

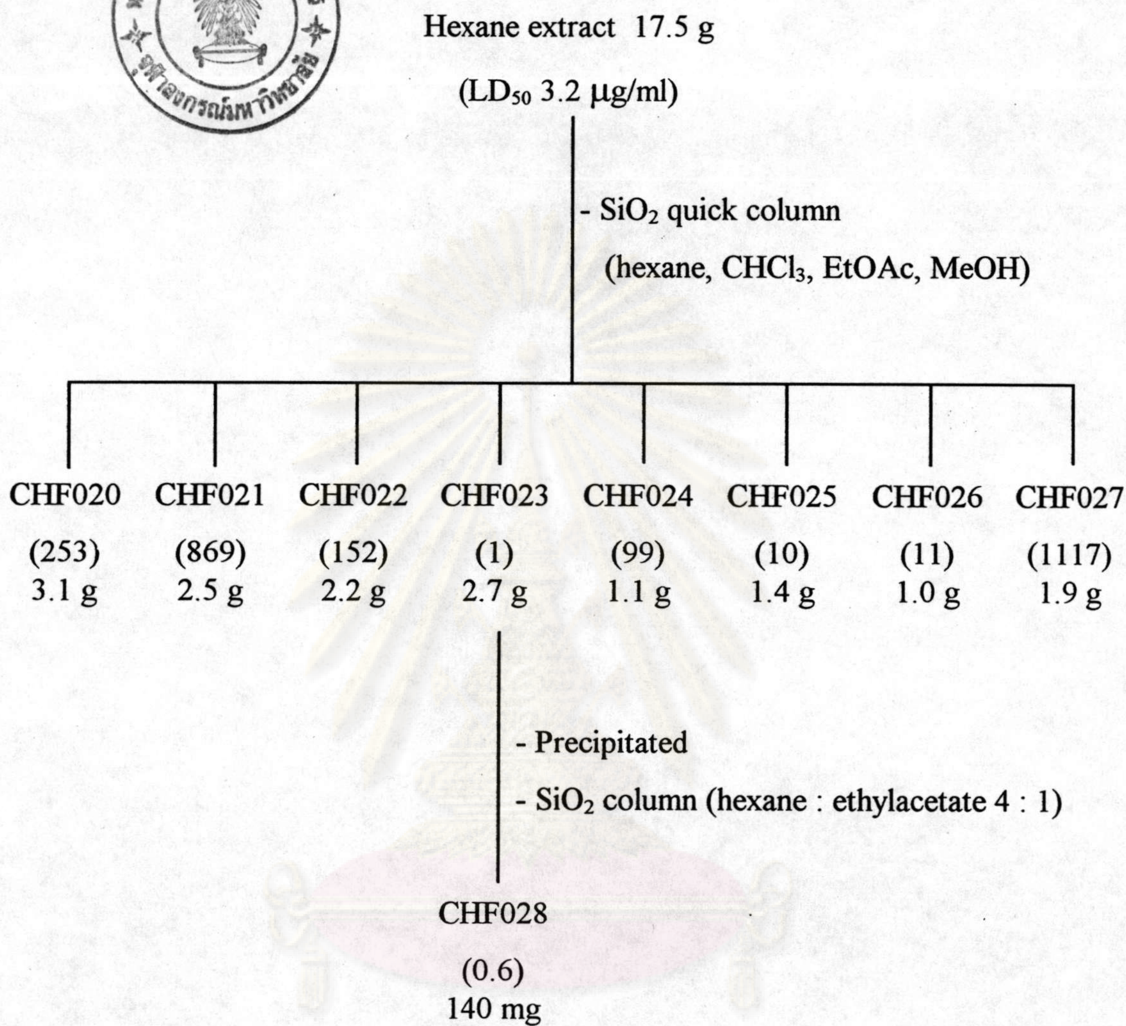
fractions	# 15-21	hexane - chloroform (1 : 1)
	# 22-24	chloroform
	# 25-26	ethyl acetate
	# 27-29	ethyl acetate - methanol (8 : 2)
	# 30-31	ethyl acetate - methanol (1 : 1)

The eluates were evaporated and combined according to their TLC patterns, brine shrimp lethality and cytotoxic activities giving eight fractions, CHF020 - CHF027. The results were shown in Table 3 and the isolation of compound CHF028 from the hexane extract was present in Scheme 8.

Table 3. The combined fractions from the hexane extract and their brine shrimp lethality activities (LD_{50}).

number of eluates	fractions	LD_{50} (μg/ml)	weight (g)
1-3	CHF020	253	3.1
4-8	CHF021	869	2.5
9-10	CHF022	152	2.2
11-14	CHF023	1	2.7
15-20	CHF024	99	1.1
21-25	CHF025-6	10	2.4
26-31	CHF027	1117	1.9

Based on the test result, fraction CHF023 was retested at lower concentration range and found to produce lethality with the same LD_{50}. Fraction CHF023 (2.7 g) yielded crystals in hexane - ethyl acetate (4 : 1) at room temperature. The mother liquor was thus removed and the crystals (160 mg) were further purified by flash column chromatography, using silica gel (25 g, 2 x 20 cm) and 5% ethyl acetate in hexane as eluent. The eluates were evaporated and then combined after TLC verification to afford a pure compound with the R_f of 0.64 in hexane - ethyl acetate (4 : 1). It was recrystallized in hexane : ethyl acetate (4 : 1) to give colourless needles, (140 mg, 5.5 x 10^{-3} % of wet weight) and was codenamed CHF028.



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Isolation scheme of compound CHF028 from the hexane extract of the soft coral

The methanol extract (4.5 g) was equally divided into two portions and further fractionated by column chromatography using silica gel (100 g, 4.0 x 14 cm.) as adsorbent. The sample (2.2 g each) was dissolved in a small volume of chloroform - ethyl acetate (1 : 1) mixture, and loaded onto the top of the column. Fifty ml of the eluates were collected per fraction. The eluents were used in the order as shown below.

fractions # 1-16	chloroform - ethyl acetate (1 : 1)
# 17-26	ethyl acetate
# 27-38	ethyl acetate - methanol (98 : 2)
# 39-48	ethyl acetate - methanol (90 : 10)
# 49	ethyl acetate - methanol (1 : 1)

The fractions showing the same TLC patterns were combined. These pooled fractions were tested for their brine shrimp lethality activity and cytotoxicity. The results are shown in Table 4. The isolation of compounds from the methanol extract were present in Scheme 9.

Table 4. The combined fractions from the methanol extract and their activities

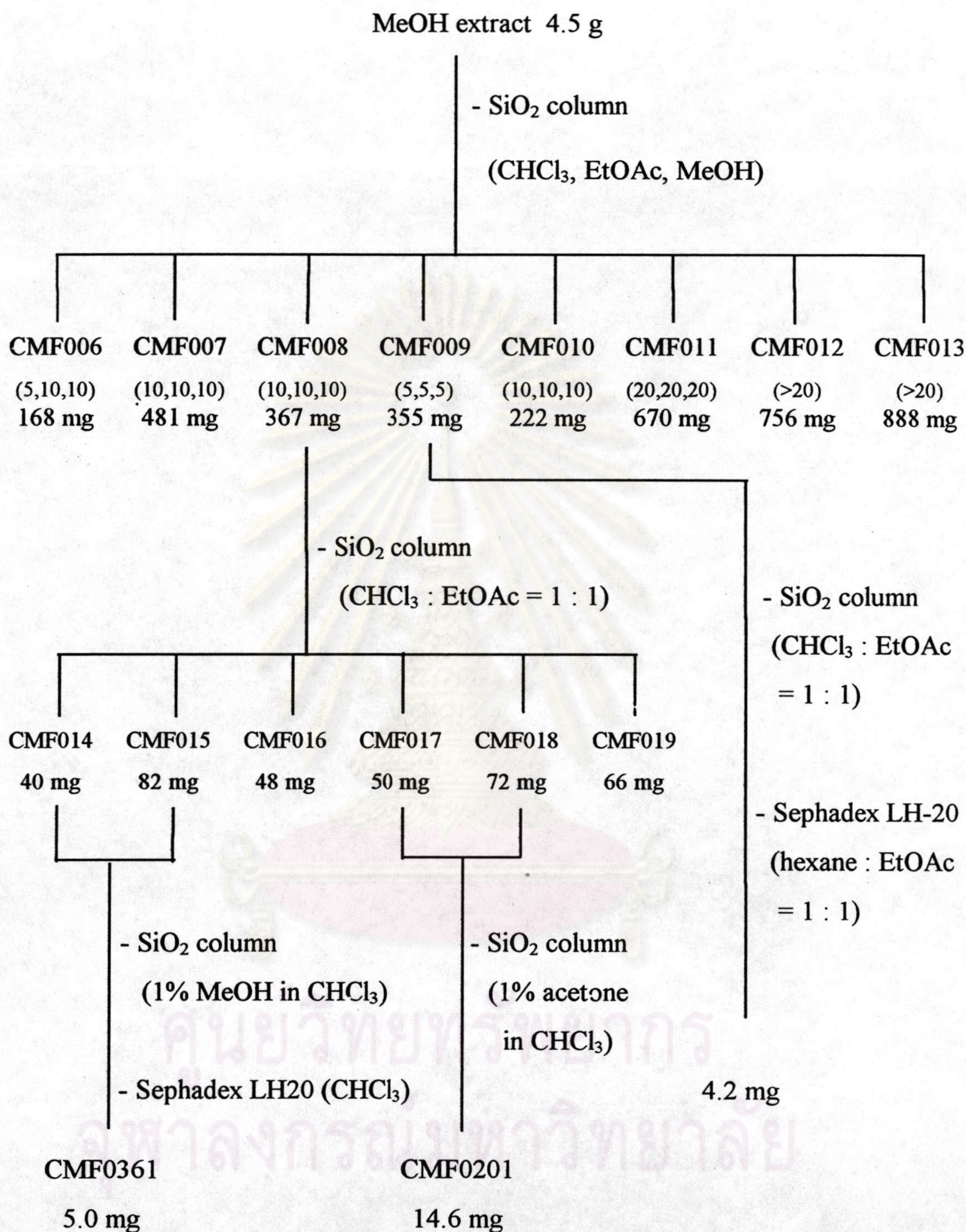
number of eluates	fractions	IC_{50} (μg/ml)			LD_{50} (μg/ml)	weight (mg)
		P-388	A-549	HT-29		
1-3	CMF006	5	10	10	65	168
4-6	CMF007	10	10	10	100	481
7-14	CMF008	10	10	10	38	367
15-21	CMF009	5	5	5	21	355
22-31	CMF010	10	10	10	10	222
32-38	CMF011	20	20	20	>1000	670
39-40	CMF012	>20	>20	>20	>1000	756
41-49	CMF013	>20	>20	>20	>1000	888

2. Isolation of Compound CMF035

Fraction CMF009 (355 mg) was fractionated by flash column chromatography using silica gel column (55g, 2 x 30 cm) with chloroform - ethyl acetate gradient as eluent. Each 25 ml fraction was collected and combined according to TLC patterns. The fractions which showed a spot visible by iodine vapour with the R_f value of 0.44 ethyl acetate - chloroform (3 : 1) were combined into another fraction, codenamed as CMF028 (105 mg). This fraction was further purified using a column of silica gel (20 g, 2.5 x 15 cm) with 2% methanol in chloroform as eluent and three fractions (approximately 25 ml each) were collected : CMF032 (70 mg), CMF033 (20 mg), and CMF034 (14 mg). The iodine-positive spot was found mainly in fraction CMF033, so this fraction was further purified by a Sephadex LH-20 column (1.5 x 45 cm) with 5% methanol in chloroform as eluent. Each 15 ml of the eluates were collected. The fourth and fifth fractions, both with a positive spot to I_2 vapour and anisaldehyde-sulphuric acid solution (producing brown colour) were combined and recrystallized in hexane - acetone (1 : 1) to afford colourless needles (4.2 mg, 8.0 x 10^{-5} % of wet weight) codenamed CMF035.

3. Isolation of Compound CMF0201

The TLC chromatogram in ethyl acetate - chloroform (3 : 1) of fraction CMF008 exhibited two distinguishable spots which were positive to I_2 vapour. The minor and more polar compound was the already isolated CMF035, therefore the major and less polar compound would be isolated. The fraction CMF008 (367 mg) was fractionated by using silica gel column (35 g, 2.5 x 15 cm) with a gradient of chloroform - ethyl acetate as an eluent and 25-ml fractions were collected. The eluates were examined by TLC and then combined to give six fractions, CMF014 - CMF019. The fractions CMF017 (50 mg) and CMF018 (72 mg) were further purified by flash column chromatography using silica gel (10 g, 1.0 x 25 cm) with 1% acetone in chloroform as eluent. Fifteen-ml fractions were collected, examined, then combined to



Isolation scheme of compound CMF0201, CMF0361, and CMF035 from the methanol extract of the soft coral

note : The values in parentheses are IC_{50} (μg/ml) for the cytotoxicity assay

afford fraction CMF020. This fraction was recrystallized in hexane - acetone (1 : 1) to give colourless needles (14.6 mg, 2.8 x 10^{-4} % of wet weight) and was codenamed as CMF0201.

4. Isolation of Compound CMF0361

From fraction CMF008, fractions CMF014 (40 mg) and CMF015 (97 mg), both of which were yellow solid that gave a positive spot to I_2 vapour and anisaldehyde - sulphuric acid solution with the R_f value of 0.64 in ethyl acetate - chloroform (3 : 1), were isolated using columns of silica gel (10 g, 1.0 x 25 cm), eluted with 1%methanol in chloroform. Each 25-ml fraction was collected. A white compound found in fractions 3 and 4 was further purified by using a Sephadex LH-20 column (1 x 45 cm) eluted with chloroform. About 15 ml of the eluates was collected per fraction and then inspected by TLC. A white powder from fractions 4-5 was recrystallized in hexane - acetone (3 : 1) to give colourless needles (5.0 mg, 9.4 x 10^{-5} % of wet weight) and codenamed as CMF0361.

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Acetylation of CMF0201

The NMR, IR and mass spectral data indicated the presence of hydroxy group in compound CMF0201, so acetylation was performed to confirm the secondary nature of the alcohol group. The sample (1 mg) was put into a 5-ml round-bottom flask with a magnetic bar inside. Pyridine and acetic anhydride, 0.5 ml each, were added. The flask was sealed with rubber septum and the air inside replaced by nitrogen gas. The mixture was stirred at room temperature and the reaction monitored by TLC every 2 hrs. The reaction was completed within 6 hrs.

The Mosher's method

The Mosher's method was applied in the elucidation of the absolute configuration of secondary alcohols and primary amines by using high-field ^{1}H nmr spectroscopy (Ohtani, 1991). The most important factor is the difference in steric bulkiness between the phenyl group of the α-methoxy-α-trifluoromethyl-α-phenylacetic acid (MTPA) and the substitutions on both β carbons between (*S*)-MTPA ester and (*R*)-MTPA ester on the MTPA plane (Figure 3).

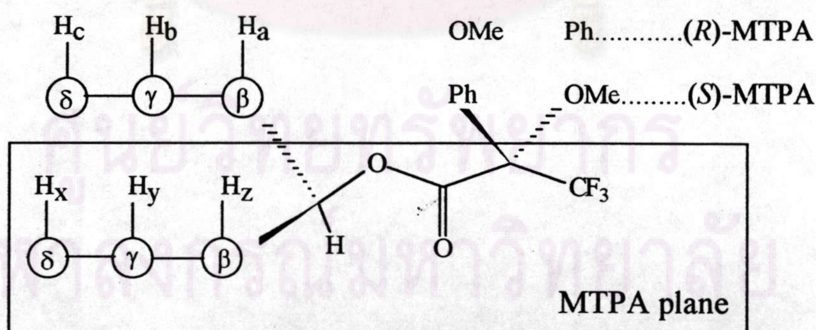

Figure 3. The MTPA plane

Due to the diamagnetic effect of the benzene ring, the Ha,b,c...nmr signals of the *(R)*-MTPA ester should appear upfield relative to those of the *(S)*-MTPA ester.

The reverse should hold true for Hx,y,z. Therefore, when $\Delta\delta = \delta_S - \delta_R$, protons on the right side of MTPA plane (Figure 3) must have positive value ($\Delta\delta > 0$) and protons on the left side of the plane must have negative value ($\Delta\delta < 0$). This can be illustrated as followed (Figure 4) :

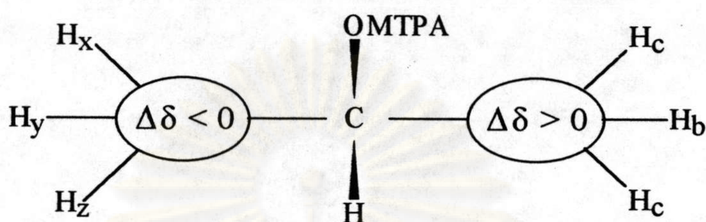

Figure 4. Illustration of the configuration of MTPA derivative

The Mosher's method can be extended as follows:

1). Assign as many proton signals as possible with respect to each of the *(R)*- and *(S)*-MTPA esters.

2). Obtain $\Delta\delta$ values for the protons.

3). Put the protons with positive $\Delta\delta$ on the right side and those with negative $\Delta\delta$ on the left side of Figure 4.

4). Construct a molecular model of the compound and confirm that all the assigned protons with positive and negative $\Delta\delta$ values are actually found on the right and left sides of the MTPA plane, respectively.

The absolute values of $\Delta\delta$ must be proportional to the distance from the MTPA moiety. When these conditions are all satisfied, model of Figure 4 will indicate the absolute configuration of the compound.

Experimental section

1. Materials.

    1.1 Commercially available (*R*)- and (*S*)-MTPA acid

    1.2 Oxalyl chloride

    1.3 Pyridine

    1.4 Dichloromethane

2. Procedures.

2.1 The sample was weighed and calculated its amount in term of millimole (mM). Usually 1-10 mg of secondary alcohol was used in the MTPA ester preparation.

2.2 The amount of *(R)*- and *(S)*-MTPA acid was calculated in term of mM and used at least ten times more than the sample. Their amounts were then converted to milligram (mg) term.

2.3 Each MTPA acid was weighed and then transferred to a round bottom flask with a magnetic bar inside. The acid was put under a stream of N_2 gas and then dried under vacuum.

2.4 Both *(R)*- and *(S)*-MTPA acids were converted to the acid chloride by using oxalyl chloride containing dimethylformamide. The reagent was injected via syringe through rubber septum into the flask containing the acid. In this step, the dryness was achieved by connecting the flask to vacuum pump and performing the reaction under N_2 gas. The reaction was allowed to proceed for about 1 hr with constant stirring.

2.5 The sample was dissolved in a small amount of dichloromethane and injected through rubber septum into the flask under N_2 gas.

2.6 The amount of pyridine was calculated in term of mM (the same amount as MTPA acid) and injected into the flask.

2.7 The reaction mixture was then allowed to stand at room temperature and monitored by TLC after 12, 24 and 48 hours. The reaction with primary amines would be completed within a few minutes; unhindered secondery alcohols, such as 2-propanol, in less than 10 minutes while most other secondary alcohols would take 12 hours or less. Phenyl-*tert*-butyl-carbinol reaction was completed in approximately 48 hours.

2.8 The MTPA ester was purified by column chromatography using chloroform - ethyl acetate (1 : 1) as eluent.

2.9 The chemical shift differences were examined for as many protons as assignable by NMR techniques of which 2D-HH COSY or decoupling experiment were sometimes required for more reliability.

Spectral Data of the Isolated Compounds

The isolated compounds were characterized by the spectroscopic data including IR, NMR and mass spectra.

1. Compound CHF028

eims : m/z (% relative intensity); (Figure 15, page 104)

304(5.0, M^+), 286(3.3), 261(2.2), 243(3.8), 219(7 6), 179(57.0), 107(61.1), 93(100), 81(95.7)

ir (KBr disc) : ν cm^{-1} ; (Figure 16, page 105)

3400, 3080, 1660, 1040, 900

^{1}H nmr (500 MHz, in chloroform-d) : δ ppm; (Figure 17-19, page 106-108)

5.55 (1H, br d, J=8.5 Hz), 4.74 (1H, t, J=2.0 Hz), 4.67 (1H, t, J= 1.6 Hz), 3.98 (1H, dd, J = 8.2, 5.3 Hz), 3.70 (1H, br s), 2.67 (1H, dd, J= 8.2, 7.6 Hz), 2.42 (1H, dd, J= 14.0, 5.3 Hz), 2.38 (1H, tdd, J= 12.7, 8.5, 4.5 Hz), 2.22 (1H, dt, J= 13.4, 3.3 Hz), 2.10 (1H, m), 2.08 (1H, dd, J = 11.8, 7.2 Hz), 2.08 (1H, m), 1.99 (1H, d, J =14.0 Hz), 1.84 (3H, s), 1.80 (1H, m), 1.78

(1H, m), 1.69 (1H, dq, J = 12.8, 3.3 Hz), 1.64 (1H, ddd, J = 14.0, 6.4, 4.5 Hz), 1.29 (1H, tt, J = 12.2, 3.3 Hz), 1.14 (3H, s), 1.00 (1H, qd, J = 12.8, 3.3 Hz), 0.93 (3H, d, J = 6.8 Hz), 0.73 (3H, d, J = 6.8 Hz)

^{13}C nmr (125 MHz, in chloroform-d) : δ ppm; (Figure 20, page 109)

147.0(s), 132.5(d), 124.5(s), 110.1(t), 92.2(d), 80.5(d), 75.1(s), 47.9(d), 46.1(d), 42.6(d), 39.0(t), 38.5(t), 31.5(t), 29.5(q), 28.0(d), 25.2(t), 24.3 (t), 22.0(q), 20.5(q), 15.5(q)

2. Compound CMF035

eims : m/z (% relative intensity); (Figure 33, page 122)

320(5.0, M^+), 302(4.5), 277(8.2), 262(9.0)

ir (film) : ν cm^{-1} ; (Figure 34, page 123)

3425, 3080, 1650, 1070, 900

^{1}H nmr (500 MHz, in chloroform-d) : δ ppm; (Figure 35-36, page 124-125)

4.67 (1H, s), 4.64 (1H, s), 4.57 (1H, br d, J=5.8 Hz), 4.13 (1H, ddd, J = 11.6, 7.0, 3.6 Hz), 3.63 (1H, s), 2.97 (1H, t, J = 7.0 Hz), 2.26 (1H, dd, J = 15.0, 11.6 Hz), 2.25 (1H, dt, J = 13.7, 3.6 Hz), 2.17 (1H, ddd, J = 11.0, 7.3, 1.3 Hz), 2.04 (1H, br t, J = 13.7 Hz), 1.99 (1H, m), 1.86 (1H, ddd, J = 14.6, 10.4, 1.8 Hz), 1.80 (1H, ddd, J = 14.6, 8.8, 1.5 Hz), 1.74 (1H, m), 1.73 (1H, dd, J = 15.0, 3.6 Hz), 1.70 (1H, dq, J = 12.8, 3.6 Hz), 1.29 (1H, br t, J = 11.0 Hz), 1.20 (3H, s), 1.16 (3H, s), 1.06 (1H, qd, J = 12.8, 3.0 Hz), 0.97 (3H, d, J = 6.7 Hz), 0.80 (3H, d, J = 6.7 Hz)

^{13}C nmr (125 MHz, in chloroform-d) : δ ppm; (Figure 37, page 126)

147.9(s), 109.2(t), 90.5(d), 80.1(d), 78.1(d), 77.0(s), 74.9(s), 53.0(d), 45.3(t), 45.1(d), 43.7(d), 39.9(t), 31.6(t), 30.3(q), 29.3(t), 29.1(d), 24.8(t), 23.0(q), 22.0(q), 15.9(q)

3. Compound CMF0201

eims : m/z (% relative intensity); (Figure 45, page 134)

302(60.0, $[M - H_2O]^+$), 284(6.0), 259(17.5), 241(17.0), 217(20.0), 179(100), 161(14.1), 133(24.0), 93(45.8), 43(43.0)

ir (KBr disc) : ν cm^{-1} ; (Figure 46, page 135)

3500, 1650, 1070, 910

^1H nmr (500 MHz, in chloroform-d) : δ ppm; (Figure 47-49, page 136-138)

5.52 (1H, br s), 5.14 (1H, br s), 4.80 (1H, t, *J* = 2.0 Hz), 4.67 (1H, t, *J* = 2.0 Hz), 4.40 (1H, dd, *J* = 11.0, 4.0 Hz), 4.12 (1H, dd, *J* = 10.3, 5.5 Hz), 3.66 (1H, s), 2.96 (1H, dd, *J* = 10.5, 7.6 Hz), 2.79 (1H, ddd, *J* = 13.5, 5.5, 1.0 Hz), 2.28 (1H, br d, *J* = 13.7 Hz), 2.27 (1H, dt, *J* = 13.5, 3.3 Hz), 2.22 (1H, dd, *J* = 11.5, 7.6 Hz), 2.08 (1H, tdd, *J* = 11.0, 4.4, 4.0 Hz), 2.05 (1H, br t, *J* = 13.0 Hz), 1.87 (1H, tdd, *J* = 11.0, 6.8, 3.6 Hz), 1.81 (1H, m), 1.79 (1H, ddd, *J* = 15.0, 11.0, 3.6 Hz), 1.73 (1H, dq, *J* = 12.8, 3.3 Hz), 1.62 (1H, ddd, *J* = 15.0, 6.8, 4.0 Hz), 1.29 (1H, tt, *J* = 12.8, 3.0 Hz), 1.23 (3H, s), 1.04 (1H, qd, *J* = 12.8, 3.3 Hz), 0.96 (3H, d, *J* = 6.7 Hz), 0.74 (1H, d, *J* = 6.7 Hz)

^{13}C nmr (125 MHz, in chloroform-d) : δ ppm; (Figure 50, page 139)

152.2(s), 146.3(s), 116.6(t), 111.2(t), 91.9(d), 79.7(d), 74.1(s), 72.9(d), 47.7(d), 44.4(d), 44.1(d), 39.3(t), 35.6(t), 35.1(t), 31.8(t), 27.9(d), 27.0(q), 25.3(t), 22.0(q), 15.2(q)

acetylated derivative

^1H nmr (500 MHz, in chloroform-d) : δ ppm; (Figure 58, page 147)

5.44 (1H, br s), 5.29 (1H, dd, *J* = 11.3, 4.1 Hz), 5.12 (1H, br s), 4.79 (1H, t, *J* = 2.0 Hz), 4.65 (1H, t, *J* = 2.0 Hz), 4.12 (1H, dd, *J* = 10.3, 5.5, Hz), 3.66 (1H, brs), 3.03 (1H, ddd, *J* = 13.5, 5.5, 1.2 Hz), 2.93 (1H, dd, *J* = 10.3, 7.9 Hz), 2.27 (1H, dt, *J* = 13.2, 3.3 Hz), 2.22 (1H, br d, *J* = 13.5 Hz), 2.21 (1H, dd, *J* = 12.2, 7.6 Hz), 2.05 (1H, br t, *J* = 13.0 Hz), 2.02 (1H, m),

1.99 (3H, s), 1.94 (1H, tdd, J = 11.0, 7.0, 3.6 Hz), 1.83 (1H, ddd, J = 15.0, 11.2, 3.6 Hz), 1.81 (1H, m), 1.74 (1H, dq, J = 12.8, 3.4 Hz), 1.60 (1H, ddd, J = 15.0, 6.7, 4.0 Hz), 1.30 (1H, tt, J = 12.8, 3.0 Hz), 1.26 (3H, s), 1.04 (1H, qd, J = 12.8, 3.3 Hz), 0.96 (3H, d, J = 6.7 Hz), 0.74 (3H, d, J = 6.7 Hz)

(*S*)-MTPA derivative

^{1}H nmr (500 MHz, in chloroform-d) : δ ppm; (Figure 61-63, page 150-152)

5.503 (1H, dd, J = 11.0, 4.0 Hz), 5.441 (1H, br s), 5.195 (1H, br s), 4.785 (1H, t, J = 2.0 Hz), 4.633 (1H, t, J = 2.0 Hz), 4.120 (1H, dd, J = 10.3, 5.5 Hz), 3.651 (1H, br s), 3.499 (3H, s), 3.064 (1H, dd, J = 13.5, 5.5 Hz), 2.892 (1H, dd, J = 10.3, 7.6 Hz), 2.265 (1H, br d, J = 13.5 Hz), 2.260 (1H, dt, J = 13.0, 3.3 Hz), 2.203 (1H, dd, J = 12.2, 7.6 Hz), 2.040 (1H, br t, J = 13.0 Hz), 1.968 (1H, tdd, J = 11.0, 4.4, 4.0 Hz), 1.945 (1H, tdd, J = 11.0, 7.0, 3.6 Hz), 1.815 (1H, ddd, J = 15.0, 11.0, 3.6 Hz), 1.793 (1H, m), 1.728 (1H, dq, J = 12.8, 3.3 Hz), 1.560 (1H, m), 1.285 (1H, tt, J = 12.8, 3.0 Hz), 1.228 (3H, s), 1.040 (1H, dq, J = 12.8, 3.3 Hz), 0.955 (3H, d, J = 6.7 Hz), 0.748 (3H, d, J = 6.7 Hz)

(*R*)-MTPA derivative

^{1}H nmr (500 MHz, in chloroform-d) : δ ppm; (Figure 64-66, page 153-155)

5.463 (1H, dd, J = 11.0, 4.0 Hz), 5.253 (1H, br s), 5.100 (1H, br s), 4.780 (1H, t, J = 2.0 Hz), 4.622 (1H, t, J = 2.0 Hz), 4.110 (1H, dd, J = 10.3, 5.5 Hz), 3.652 (1H, br s), 3.519 (3H, s), 3.102 (1H, dd, J = 13.5, 5.5 Hz), 2.878 (1H, dd, J = 10.3, 7.6 Hz), 2.260 (1H, dt, J = 13.0, 3.3 Hz), 2.259 (1H, br d, J = 13.5 Hz), 2.198 (1H, dd, J = 12.2, 7.6 Hz), 2.082 (1H, tdd, J = 11.0, 4.4, 4.0 Hz), 2.040 (1H, br t, J = 13.0 Hz), 1.982 (1H, tdd, 11.0, 7.0, 3.6 Hz), 1.826 (1H, ddd, J = 15.0, 11.0, 3.6 Hz), 1.798 (1H, m), 1.722 (1H, dq, J = 12.8, 3.3 Hz), 1.590 (1H, ddd, J = 15.0, 7.0, 4.0 Hz), 1.290 (1H, tt, J = 12.8, 3.0 Hz), 1.230 (3H, s), 1.040 (1H, dq, J = 12.8, 3.3 Hz), 0.955 (3H, J = 6.7 Hz), 0.748 (3H, J = 6.7 Hz)

4. <u>Compound CMF0361</u>

hrfabms : m/z (M+H); (Figure 67, page 156)

observed : 413.2916, calculated : 413.2904

eims : m/z (relative intensity); (Figure 68, page 157)

394(1.5%, $[M - H_2O]^+$), 334(4.5%), 302(4.0%), 291(4.0%), 259(7.0%), 237(60%), 179(45%), 115(100%)

ir (film) : ν cm^{-1} ; (Figure 69, page 158)

3450, 1726, 1458, 1370, 1085, 756

^{1}H nmr (500 MHz, in chloroform-d) : δ ppm; (Figure 70-71, page 159-160)

4.08 (1H, dd, J = 11.0, 3.6), 4.06 (1H, ddd, J = 11.0, 7.5, 3.6 Hz), 3.55 (1H, br s), 3.36 (1H, br s), 2.82 (1H, t, J = 7.5 Hz), 2.40 (1H, br d, J = 11.6 Hz), 2.30 (1H, ddd, J = 15.0, 11.0, 1.2 Hz), 2.18 (1H, dd, J = 10.4, 7.5 Hz), 1.99 (3H, s), 1.97 (1H, m), 1.85 (1H, ddd, J = 14.6, 8.0, 2.0 Hz), 1.80 (1H, ddd, J = 14.6, 10.3, 2.0 Hz), 1.74 (1H, dd, J = 15.0, 3.6 Hz), 1.71 (1H, m), 1.46 (3H, s), 1.41 (1H, m), 1.40 (1H, br t, J = 11.6), 1.22 (1H, m), 1.20 (1H, m), 1.16 (3H, s), 1.16 (3H, s), 0.95 (3H, d, J = 6.7 Hz), and 0.82 (3H, d, J = 6.7 Hz)

^{13}C nmr (125 MHz, in chloroform-d) : δ ppm; (Figure 72, page 161)

170.2(s), 90.8(d), 90.8(d), 82.9(s), 76.5(s), 75.6(d), 74.6(s), 57.1(d), 54.4(d), 46.4(t), 42.6(d), 42.0(d), 41.0(t), 30.6(t), 30.3(q), 29.1(d), 25.7(t), 24.5(q), 24.0(q), 22.6(q), 21.8(q), 17.8(t) and 15.4(q)

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