CHAPTER III

MATERIALS AND METHODS

1. Sample collection and isolation

Bacterial sample (S9730) was collected by scuba diving at Si-Chang Island, Chon-Buri province in October 1997. The bacterium was associated with an unidentifed hydroid growing on an artificial reef at 40 feet depth. The sample was diluted in steriled sea water and was isolated by spread plate technique(Brock, *et al.*, 1993) on a marine agar plate (section 8.3.4).

2. Identification of bacteria

Morphological and cultural characteristics were studies on cell form, cell size, cell arrangement, colonial appearance, and motility. Cell form was observed and scanning electron microscope by the method of De man, De man, and Gupta (1986). Physiological and biochemical characteristics on catalase; nitrate reduction; hydrolysis of arginine; production of amylase, chitinase and gelatinase; acid formation and carbon utilization were determined by the methods described in Cowan and Steel's Manual for identification of medical bacteria (Borrow and Feltham, 1993). The effect of temperature (4, 35 and 40 °C) and different concentrations of NaCl (0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 %) were tested by using marine broth (section 8.3.4).

3. Fermentation conditions

The strain S9730 was grown on a marine agar slant for a day at room temperature and was used as the inoculum. Eight of each 250 ml of inoculated marine broth in 500 ml Erlenmeyer flask were incubated on a rotary shaker (200 rpm) for 3 days at room temperature. It was cultivated repeatedly for 34 liters until 1 g of the crude dichloromethane extract was collected.

4. Chromatography techniques

4.1 Analytical thin-layer Chromatography

Technique	:	One dimension ascending
Adsorbent	:	Siliga gel F_{254} or siliga gel C_{18} reversed phase plate (E. Merck).
Layer thickness	:	250 μm
Distance	:	6 cm
Temperature	:	Laboratory temperature (25-36 °C)
Detection	:	1. Visual detection under daylight. 2. Ultraviolet light at wavelengths of 254 and 365 nm 3. Spraying with anisaldehyde reagent and heated until colors developed.

4.2 Column chromatography

4.2.1 Gel Filtration Chromatography

Gel filter	:	Sephadex LH-20
Packing method	:	Gel filter was suspended in an eluent and left standing to swell for 24 hours prior to use. It was then poured into a 2.5 cm diameter column and allowed to settle tightly (77 cm height).
Sample loading	:	The sample was dissolved in a small volume of an eluent and loaded on top of a column.
Detection	:	Fractions were examined by TLC technique in the same manner as described in section 4.1

4.2.2 Flash Column Chromatography

Adsorbent	:	Siliga gel 60 (No. 9385) particle size 0.040-0.063 nm (230-400 mesh ASTM) (E. Merck)
Packing method	:	Adsorbent was suspended in an eluent. The slurry of adsorbent was poured into a 1.5 cm diameter column. Adsorbent was flowed down by an air pump, then allowed to settle overnight (17 cm height).
Sample loading	:	The sample was dissolved in a small volume of an eluent and loaded on top of a column.
Detection	:	Fractions were examined by TLC technique in the same manner as described in section 4.1

4.2.3 High Performance Liquid Chromatography

Column	:	LiChrospher® 100RP-18 (10μm) LiChroCART® 125-4 Cat. 50853
Flow rate	:	0.5 ml/min
Solvent system	:	Gradient elution from H_2O : THF (95:5) to H_2O : THF (60:40).
Sample preparation	:	Sample was dissolved in THF and filtered through cotton before injection.
Detection wavelength	:	234 nm
Injection volume	:	200 μl
Pump	:	Consta Metric 4100
Detector	:	Spectro Metric 4100
Recorder	:	Linear
Temperature	:	27-30 °C

4.3 Crystallization technique

Compounds K002, K004 and K005 were recrystallized in chloroform. Each compound was dissolved in chloroform until saturation. The saturated solution was left standing at room temperature until crystals were obtained.

5. Spectroscopy

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

^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were obtained with a Bruker Avance DPX-300 FT-NMR Spectrometer, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

5.2 Mass Spectra (MS)

Electron Impact Mass Spectra (EIMS) of K004 and K005 were performed with a Micromass (VG Platform II, Fisons Instrument) Mass Spectrometer (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

5.3 Ultraviolet (UV) Absorption Spectra

UV(in methanol) spectra were obtained from a Milton Roy Spectronic 3000 Array spectrometer (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

5.4 Infrared (IR) Absorption Spectra

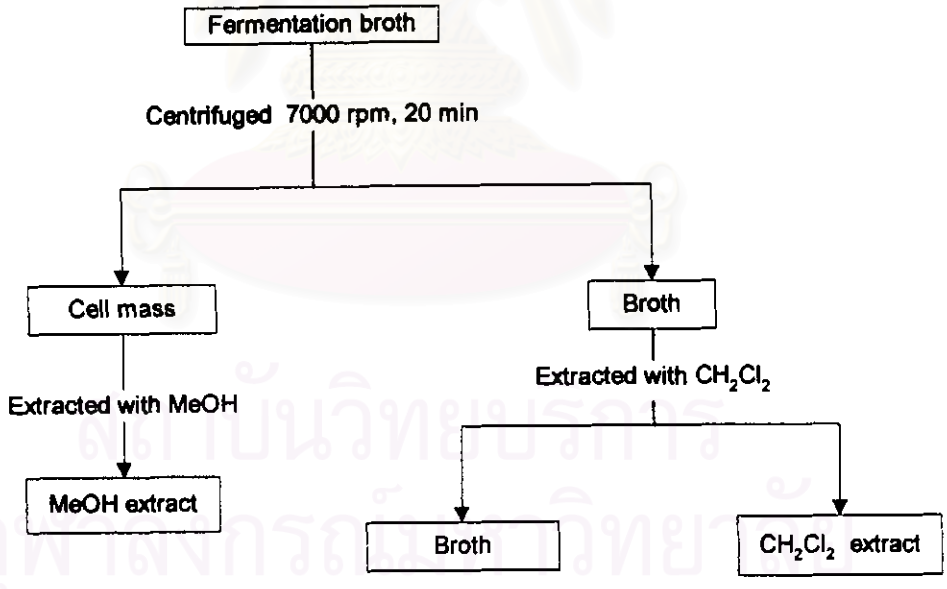
IR (KBr disc and film) spectra were obtained from a Perkin Elmer FT-IR 1760X spectrometer (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

5.5 Optical Rotation

Optical rotation were measured on a Perkin-Elmer Polarimeter model 341 (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

6. Extraction

The culture broth of the bacterium, *Alteromonas* sp. S9730 (34 l) was centrifuged at 7,000 rpm for 20 minutes to separate broth from bacterial cells. The broth was extracted repeatedly three times with dichloromethane (CH_2Cl_2). The CH_2Cl_2 fraction was separated and evaporated under reduced pressure at the temperature not exceeding 40 °C until it dried. Crude CH_2Cl_2 extract (1 g) was obtained (Scheme 1).



Scheme 1. Seperation of *Alteromonas* sp. S9730

Bacterial cells were macerated in methanol for 1 day and then filtered. This methanol fraction was evaporated under reduced pressure at the temperature not exceeding 40 °C to give a methanol extract. Both extracts were determined for antibiotic activity using a agar disc diffusion method (section 8.1).

7. Isolation and purification of the extract

Only the CH_2Cl_2 extract (1 g) exhibited antibacterial activity. It was dissolved in a small amount of $CHCl_3$ and then fractionated by Sephadex LH-20 column chromatography. A column (2.5 cm inner diameter and 77 cm long) was eluted with chloroform. Fractions (20 ml, each) were collected.

Table 5. Purification of the CH_2Cl_2 extract by a Sephadex LH-20 column

Fraction code	Number of elution	Volume (ml)	Weight (mg)
F001	1-5	100	9
F002	6-11	120	77
F003	12-23	250	156
F004	24-59	400	198
F005	60-61	40	145
F006	62-63	40	214
F007	64	250	38

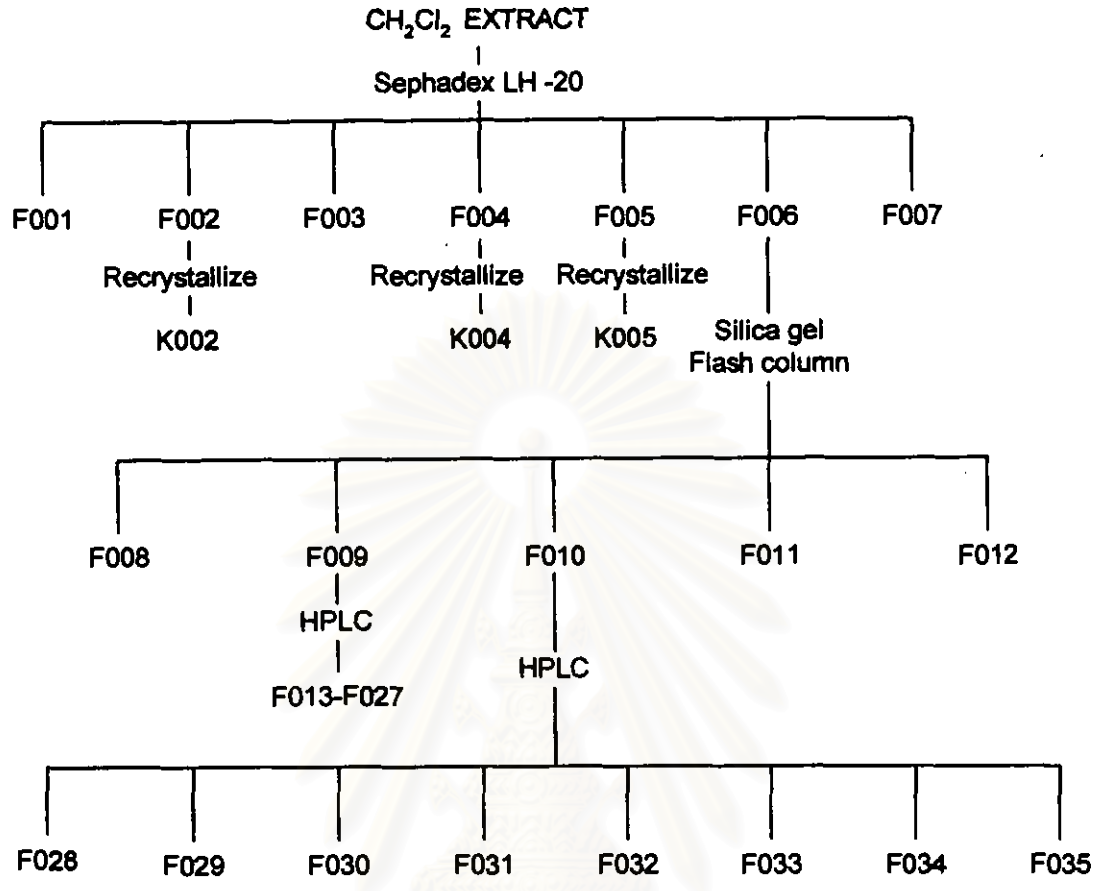
The combination of fractions was guided by a TLC technique (section 4.1) to give 7 fractions (as shown in Table 5). White crystals of K002 (28 mg, 4.75 % yield of the crude CH_2Cl_2 extracts), orange crystals of K004 (111 mg, 10.34 % yield of the crude CH_2Cl_2 extracts), and white crystals of K005 (51 mg, 2.61 % yield of the crude CH_2Cl_2 extracts) were obtained from the recrystallization (section 4.3) of fractions F002, F004, and F005, respectively (Scheme 2).

The fraction F006 was further purified by a silica gel flash column (2 cm inner diameter and 16 cm long). $CHCl_3$ and MeOH were used as eluents (Table 6).

Table 6. Purification of F006 by flash column chromatography.

Fraction code	Number of elution	Volume (ml)		Weight (mg)
		$CHCl_3$	MeOH	
F008	1-10	100	-	9
	11-12	38	2	
F009	13-20	152	8	25
	21	18	2	
F010	22-40	342	38	125
F011	41-51	180	20	25
	52-81	480	120	
F012	82	-	100	17

Each fraction (20 ml) was collected and combined according to their TLC patterns, giving 5 fractions. The fractions F009 and F010 were further fractionated by HPLC (section 4.2.3). F010 yielded a pure compound F028. However, a chemical structure of F028 could not be identified due to the limited amount of sample.



Scheme 2. Isolation and purification of CH_2Cl_2 extract

8. Antimicrobial test

8.1 Agar disc diffusion method

The agar disc diffusion method was used for antibacterial activity test.

8.1.1 Preparation of inoculum

The bacterial strains used for antibacterial test were as follows:

1. *Escherichia coli* ATCC 25922
2. *Staphylococcus aureus* ATCC 25923
3. *Vibrio harveyi* 94/55
4. *Vibrio harveyi* (Sombut)

5. *Vibrio harveyi* 97/17
6. *Vibrio alginolyticus* 96061
7. *Vibrio alginolyticus* 97032
8. *Vibrio parahaemolyticus* 94/60
9. *Vibrio parahaemolyticus* (string)
10. *Vibrio vulnificus* 94/4

All strains of *Vibrio* sp. were wild type which were isolated from vibriosis pathogenic shrimp (kindly provided by Dr. Pikul Jirwanidpisan, Marine Biotechnology Research Unit at Chulalongkorn University).

The *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were cultured on a trypticase soy agar (TSA) slant at 37 °C (for 1day). All strains of *Vibrio* sp. were cultured on a marine agar slant at room temperature (for 1 day). Each bacterial strain was suspended in a normal saline solution. The turbidity of suspension was adjusted to a 0.5 turbidity (standard of McFarland No. 1). Both *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were spreaded on the surface agar of a trypticase soy agar plate, and all strains of *Vibrio* sp. were spreaded on the surface agar of a marine agar.

8.1.2 Sample preparation for antimicrobial test

Compounds K004 and K005 were dissolved in acetone and adjusted the concentration to 500 μg/ml. The solution (200 μl) was dropped on a steriled paper disc (5 mm diameter). This paper disc was left in a steriled petridish for an hour or until the paper was completely dried.

8.1.3 Antimicrobial assay

A dried paper disc was placed onto the surface of the inoculated agar plates, which was spreaded with either *Escherichia coli* ATCC 25922 or *Staphylococcus aureus* ATCC 25923 incubated at 37 °C for 18 h, whereas the plate inoculated with *Vibrio* sp. was incubated at room temperature for 18 h.

The areas of inhibition zones were measured. The compound, which displayed good antibacterial activity, was subsequently selected for further study.

8.2 Determination of Minimum Inhibition Concentration (MIC)

The compound isatin was found to have good antimicrobial activity. The determination of MIC of isatin was carried out by a broth microdilution test (Woods and Washington, 1995).

8.2.1 Preparation of the bacteria

The bacterial strains used for the MIC determinations are the same strains as those in an antibacterial test (8.1.1).

The *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and Vibrio sp. were cultured in the same manner as that in 8.1.1. The colonies of *E. coli* and *S. aureus* were suspended in Mueller Hinton broth and incubated at 37°C for 6 h. The colonies of *Vibrio* sp. were placed in mueller hinton broth containing 0.85% of solution NaCl, and incubated (for 6 h) at room temperature. The turbidity was made to 0.5 turbidity compared with the standard of Mcfarland No.1, which provided approximately 1×10^{8} CFU/ml. The inoculum was further diluted to 5×10^{5} CFU/well in Mueller Hinton broth.

8.2.2 Antibacterial assay for the determination of MIC

The isatin was dissolved in methanol and diluted with methanol to give the concentrations ranging from 0.9 μg to 50 μg. Solution of isatin (containing different concentrations) were individually added to the corresponding well of a sterile multiwell microplate (96 Flat-shaped wells). This plate was left in the laminar flow until methanol was completely dried. The dilute inoculum (200μl) was poured into each well. The plate was incubated at 25 °C for 18 h. Each bacterial strain was done in duplicate. The lowest concentration of sample that showed growth inhibition was considered as the MIC by measuring culture turbidity. The turbidity was obtained from a microplate reader, Bio-Rad model 450 (Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

8.3 Media

8.3.1 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-dissolved. The agar solution was heated until all materials dissolved in water. The medium was sterilized by autoclaving at 121 °C for 15 minutes. This medium was used for bacterial cultivation during the assay.

8.3.2 Sabouraud dextrose agar (SDA)

The medium 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 added in purified water and throughly mixed. The solution was then boiled so as to completely dissolve the ingredients. This medium was used for cultivation of yeast cultures during the assay.

8.3.3 Mueller Hinton Broth (MHB)

The medium was Difco® Mueller Hinton Broth.

Formula per liter of purified water.

Beef, Infusion from bacto casamino acids	300 g
Technical	17.5 g
Starch	1.5 g

Final pH 7.3 ± 0.1

The formula powder (38 g) was suspended in 1 liter distilled or deionized water and boiled to dissolve completely. The medium was sterilized at 121 °C for 15 minutes. This medium was used for cultivation of bacteria during the assay.

8.3.4 Marine Broth (MB)

Formula per liter of sea water(salinity = 35 ppt).

Difco Bacto-peptone (Difco)	5 g
Difco Bacto-Yeast extract (Difco)	1 g
Glucose	5 g
Ferric ammonium citrate	0.1 g

adjust pH to 7.2

The medium powder was suspended in 1 liter sea water. The medium was sterilized at 121 °C for 15 minutes. This medium was used for cultivation of working culture. Marine agar was prepared by adding 1.8% agar and was used for stock culture madium.

9. Determination of bacterial growth

Growth was determined by measuring the OD (Optimum Density), using a Shimadzu UV-160A, UV-Visible recording spectrometer at 625 nm (Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University). On the first day of experiment, growth was measured for every 3 h, but, from day 2 - day 7, growth was measured for every 24 h.

10. Determination of yield of bioactive compounds

HPLC was employed for the determination of secondary metabolites compositions in a culture broth of *Alteromonas* sp. S9730

HPLC conditions were as follows:

Column	:	LiChrospher® 100 RP-18 (5 μm) 250×4 mm Merck 50983
Flow rate	:	0.3 ml/min
Mobile phase	:	Isocratic water: CH_3CN (7:3)
Sample preparation	:	Sample was dissolved in CH_3CN and filtered through cotton before injection.
Detection wavelength	:	242 nm
Injection volume	:	50 μl
Pump	:	Shimadzu LC-10AD
Detector	:	Shimadzu SPD-10A
Recorder	:	Shimadzu C-R6A
Temperature	:	27-30 °C