# CHAPTER III

## EXPERIMENTAL

### 1. Sample collection and isolation of marine bacteria

The two marine *Bacillus* strains, Sc018 and Sc026 including a marine bacterium, Sc004 were isolated from marine sediments and seawater, respectively, and were collected at 15-25 m depth around Sichang Island (an island in the East coast of the Gulf of Thailand), Chonburi province, Thailand, in October 1997. In order to isolate marine bacteria, 0.5 ml of seawater or 0.5 g of each sediment was suspended in sterile seawater (4.5 ml) and was subsequently diluted to 1:100, 1:1,000 and 1:10,000 with sterile water. The marine bacteria were isolated by the spread plate technique (Brock, *et al.*, 1993) on the marine agar (MA) plates. Each dilution (0.1 ml) was spreaded on the surface of MA plates containing 0.50 % Bactopeptone (Difco), 0.10 % yeast extract (Difco), 0.01 % ammonium iron (III) citrate, and 1.80 % agar in 100 ml seawater, and adjusted pH to 7.6. Plates were incubated at 30°C for 3 days. The circular, raised with entired edge and white, cream, yellow or pale brown colonies of *Bacillus* species were picked up and streaked for purification on MA plates and were incubated at 30°C for 3 days. Colonies were transferred to cultivate on MA slants and incubated at 30°C for 3 days. Stock cultures were kept in cold room at 4°C and were deposited at the Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand.

**2. Identification of bacteria** (Barrow and Feltham, 1993)

**2.1 Morphological and cultural characteristics**

Cell form, cell size, cell arrangement, and colonial appearance were observed on cell grown on MA. Motility may be inferred by observing the spreading growth in a semisolid agar. The modified Leifson stain method (Forbes, 1981) using fuchsin and tannic acid was used to determine the flagella morphologies and arrangements. Cell form, spore form, spore surface, and flagella were observed by scanning electron microscope (SEM) using the method of De man, *et al* (1986).

**2.2 Biochemical characteristics**

The presence of catalase was indicated by the evolution of gas quickly after dropping 3 % $H_2O_2$ on the surface of culture. Oxidase (cytochrome c oxidase) appeared as a dark purple color after dropping Kovac's reagent on the filter paper streaked with culture. Urease activity was tested on Christensen's urea medium (yellow color). The urease producing microorganisms gave an alkali production (red color). The methyl red (MR) test and the Voges-Proskauer (VP) test for acetylmethylcarbinol (acetoin) were carried out with the same MR-VP culture broths. The positive MR test was indicated by a red color of the overnight culture broth after dropping methyl red. The VP test could be carried out by adding creatine solution, and mixture of α-naphthol and KOH, repectively, and a positive reaction was indicated by a red color. Consumption on glucose was monitored by oxidation or by fermentation using the oxidation-fermentation (OF) test, oxidizers showed acid production in the open tube only; fermenters produced acid in the paraffin-covered tube, starting from the bottom in the open tube. The test of the microorganism's ability to use casein, hippurate and starch as sources of carbon as well as its ability to hydrolyse them were performed. The positive hydrolysis reactions were

indicated by clear zone, pink color (alkali production), and colorless around the culture growth for the hydrolysis of casein, hippurate, and starch, respectively. The positive citrate utilization was indicated by culture growth on a Simmon's citrate medium and the color of medium turned blue. The culture producing α or β hemolysin presented green color or clear zone, respectively, on a human blood agar. Indole production was examined by adding Kovac's indole reagent in a culture broth and a positive reaction was a red color in the reagent layer. Nitrate reduction allowed the disappearance of nitrate and the appearance of nitrite as a reduction product. If nitrite was present, the culture broth became pink to red color after adding sulfanilic acid solution and dimethyl-α-naphthylamine solution, respectively. Acid production from sugars could be detected by a phenol red (pH indicator) in the medium. The sugars required for the test were L-arabinose, D-(+)-cellobiose, D-(-)-fructose, D-galactose, D-glucose, inositol, lactose, maltose, D-mannitol, D-(+)-mannose, melibiose, melezitose, L-rhamnose, raffinose, D-(-)-ribose, salicin, sorbitol, sorbose, sucrose, D-(+)-trehalose. $2H_2O$, and D-(+)-xylose.

**2.3 Physiological characteristics**

Temperature ranges for growth of microorganisms could be examined by the culture growth on MA slants incubated at different temperatures from 0.6°C to 50°C for 1-5 days. The ability of a culture to grow at particular temperature was confirmed by growth at 30°C after subculture. The NaCl tolerance (salt tolerance) could be examined by the culture growth on MA slants containing various NaCl concentrations of 0 %, 2 %, 4 %, 6 %, 8 %, and 10 % incubated at 30°C for 1-5 days.

**2.4 Determination of DNA base composition**

The isolation of DNA and determination of DNA base composition as described previously by Tamaoka, J. (1994) were used. The cultured cells at middle or late phase of logarithmic growth was hydrolyzed by lytic enzymes and subsequently denatured proteins and removed polysaccharides by phenol and chloroform, respectively. After washing with 99% (v/v) ethanol, the DNA pellet was further hydrolyzed by RNase A and proteinase K solutions for hydrolysis of residual RNA and proteins. The partially purified DNA was repeatedly treated with phenol and chloroform, respectively. The purified DNA was hydrolyzed into nucleosides by using nuclease P1 and alkaline phosphatase solutions and deoxyribonucleosides was measured by HPLC. The mole% G+C content of DNA was calculated by area under the curve of HPLC chromatogram.

**3. Fermentation conditions**

All cultures, Sc018, Sc026, and Sc004 were streaked on the surface of MA plates and incubated at 30°C for 1 day. A heavy inoculum of each culture was transferred into a 500-ml Erlenmeyer flask containing 250 ml of MB (marine broth) and incubated on a rotatory shaker (200 rpm) at 30°C for 3 days.

**4. Chromatographic techniques**

**4.1 Analytical thin layer chromatography (TLC)**

Technique : One dimension, ascending

Adsorbent : Silica gel F254 or C-18 reversed-phase plate (E. Merck)

Layer thickness : 250 μm

Distance : 5 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 (0.5% anisaldehyde, 5% sulfuric acid, and 10% glacial acetic acid in MeOH) and heated until colors developed

**4.2 Column chromatography**

**4.2.1 Gel filtration chromatography**

Gel filter : Sephadex LH-20 (Pharmacia Biotech AB)

Packing method : Sephadex gel was suspended in the eluant and left standing overnight to swell prior to use. It was then poured into the column and allowed to settle.

Sample loading : The sample was dissolved in a small volume of the eluant and applied on top of the 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 : Silica gel 60 (No. 9385), particle size 40-63 μm (230-400 mesh ASTM) (E. Merck) or C-18 reversed-phase gel, particle size 40-63 μm (230-400 mesh ASTM) (E.Merck)

Packing method : Adsorbent was suspended in the eluant. The adsorbent was slurry poured into the column, flowed down by an air pump and then allowed to settle overnight.

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

Detection : Fractions were examined by the TLC technique in the same manner as described in Section 4.1.

### 4.2.3 High performance liquid chromatography

Column : Lichrosorb C-18 reversed-phase (10 x 250 mm, 7 μm)

Flow rate : 2.0 ml/min

Solvent system : Gradient elution of $CH_3CN$:0.1 % TFA in $H_2O$ from 10:90 to 90:10.

Sample preparation : The sample was dissolved in $H_2O$:$CH_3CN$ (50:50) and filtered through cotton before injection.

Detection wavelength : 220 nm

Injection volume : 150 μl

Detector : SpectroMetric 4100 UV spectrometer

Recorder : Linear

Temperature : 27-30°C

### 4.3 Crystallization technique

Compounds P056 WC and P193 were recrystallized from a mixture of $CHCl_3$ and MeOH. Each compound was dissolved in MeOH until saturation and subsequently cold $CHCl_3$ was added. The solution was left standing at -32°C until crystals were formed.

## 5. Spectroscopy

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

$^1H$ and $^{13}C$ NMR, DEPT 135, HETCOR, HMQC, COSY, TOCSY, and HMBC spectra were recorded on a Bruker AVANCE DPX-300 FT-NMR spectrometer, operating at 300 MHz for protons and 75 MHz for carbons, and chemical shifts (ppm) of the residual undeuterated solvents were used as references. Proton detected heteronuclear correlations were measured using HMQC (optimized for $^1J_{HC}$ = 145 Hz) and HMBC (optimized for $^nJ_{HC}$ = 3, 4, and 8 Hz) pulse sequences. Some NMR experiments were also recorded on a Bruker AVANCE DPX-400 FT-NMR spectrometer and a JEOL JNM-A500 NMR spectrometer.

### 5.2 Mass spectra (MS)

Electron impact mass spectra (EIMS) and chemical ionization mass spectra (CIMS) were recorded with a Micromass (VG Platform II, Fisons Instrument) mass spectrometer with ionization energy at 30 or 70 eV and with methane gas, respectively. The HRFABMS spectra were obtained from a JEOL JMS-HX 110 double focusing mass spectrometer of EBE arrangement with JMS-DA 7000 data system, 10 kV acceleration voltage, fast-atom xenon gas accelerated at a voltage of 3 kV. Glycerol or

m-nitrobenzyl alcohol (mNBA) or magic bullet were used as the matrix, and NaCl was used as alkali metal cation source.

**5.3 Ultraviolet (UV) absorption spectra**

UV (in methanol) spectra were obtained from a Milton Roy Spectronic 3000 Array spectrophotometer.

**5.4 Infrared (IR) absorption spectra**

IR spectra were obtained from a Perkin-Elmer 2000 FT-IR spectrophotometer. The compounds were examined as dry film on NaCl cell.

**5.5 Optical rotation**

Optical rotations were measured on a Perkin-Elmer 341 polarimeter using a sodium lamp operating at 589 nm.

The measurements of MS, UV, IR, NMR, and optical rotation properties were performed at the Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University; The Scientific and Technological Research Equipment Center (STREC), Chulalongkorn University; the National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA); and the Institute of Molecular and Cellular Biosciences, the University of Tokyo.

**6. Melting point**

Melting temperatures were determined on a Gallenkamp melting point apparatus.

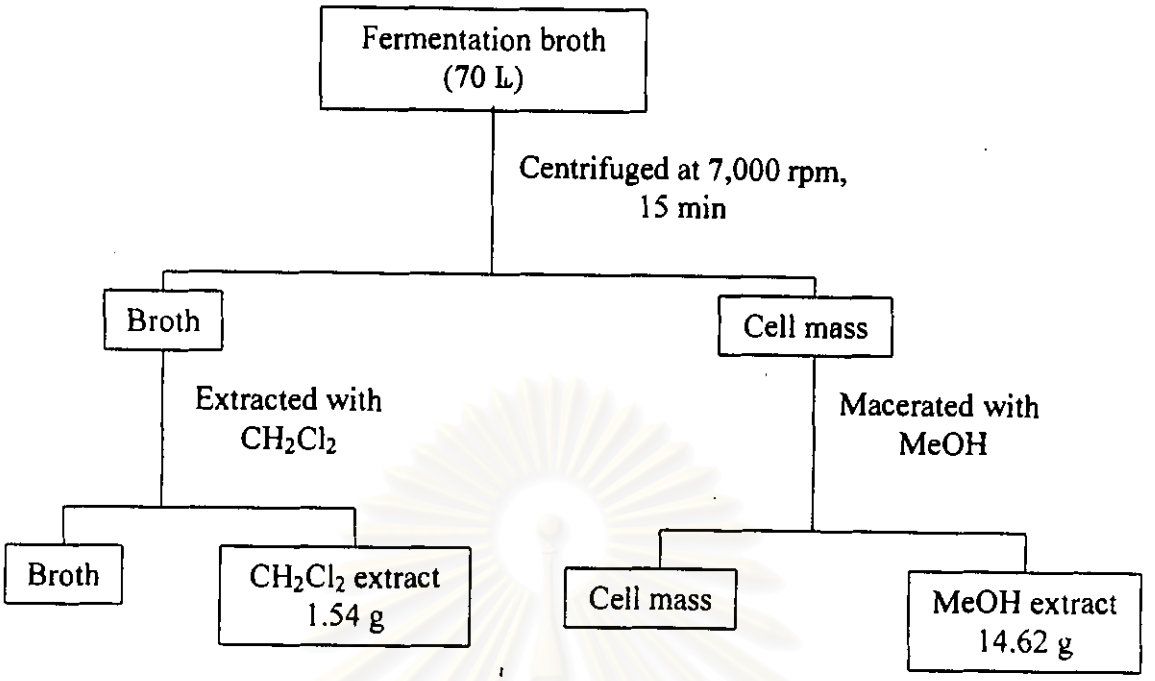
**7. Solvents**

All commercial grade solvents were redistilled prior to use. HPLC grade and analytical grade solvents were used for the mobile phase of HPLC and TLC, respectively.

**8. Extraction**

**8.1 Extraction of the marine *Bacillus* sp. Sc018 culture broth**

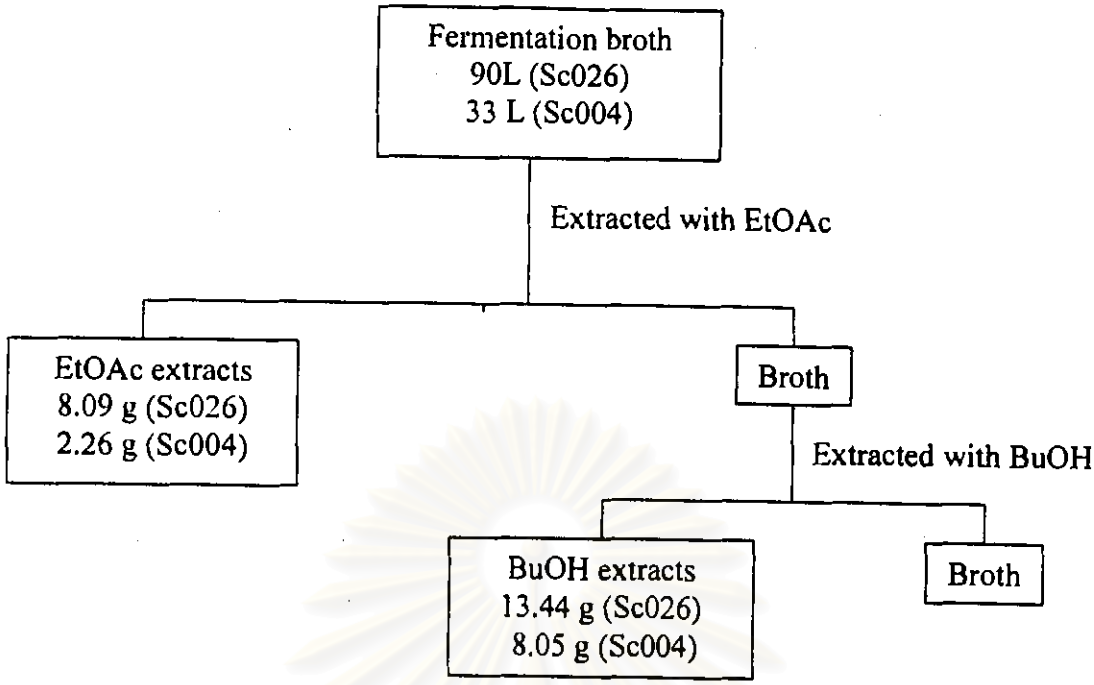
The culture broth of *Bacillus* sp. Sc018 (70 L) was centrifuged at 7,000 rpm for 15 minutes to separate broth from bacterial cells. The broth was extracted repeatedly three times with $CH_2Cl_2$. The $CH_2Cl_2$ fractions were combined and subsequently evaporated under reduced pressure at temperature not exceeding 50°C until dryness. A crude $CH_2Cl_2$ extract (1.54 g) was then obtained (Scheme 1). The bacterial cells were macerated repeatedly three times in MeOH each, for 1 day and then filtered. The MeOH filtrates were combined and subsequently evaporated under reduced pressure at temperature not exceeding 50°C to give a MeOH extract (14.62 g). The $CH_2Cl_2$ and the MeOH extracts were subjected to antiviral assay against *Herpes simplex* virus type I and type II, using the modified colorimetric method (Section 12.2).




**Scheme 1**. Extraction of the marine *Bacillus* sp. Sc018 culture broth

**8.2 Extraction of the marine *Bacillus* sp. Sc026 culture broth and the marine bacterium Sc004 culture broth**

Each culture broth of *Bacillus* sp. Sc026 (90 L) and the bacterium Sc004 (33 L) was partitioned repeatedly three times with EtOAc. The EtOAc fractions from each culture broth were combined and subsequently evaporated under reduced pressure at temperature not exceeding 50°C to yield 8.09 g (Sc026) and 2.26 g (Sc004) of EtOAc extracts, respectively (Scheme 2). The aqueous fractions were subsequently extracted with BuOH repeatedly three times. Each of the BuOH fractions was evaporated separately under reduced pressure at temperature not exceeding 50°C. During evaporation, each BuOH fraction was added with $H_2O$ until dryness. The crude BuOH extracts obtained from Sc026 and Sc004 were 13.44 g and 8.05 g, respectively. All of the EtOAc and the BuOH extracts were subjected to antimicrobial assay using an agar diffusion method (Section 12.1).



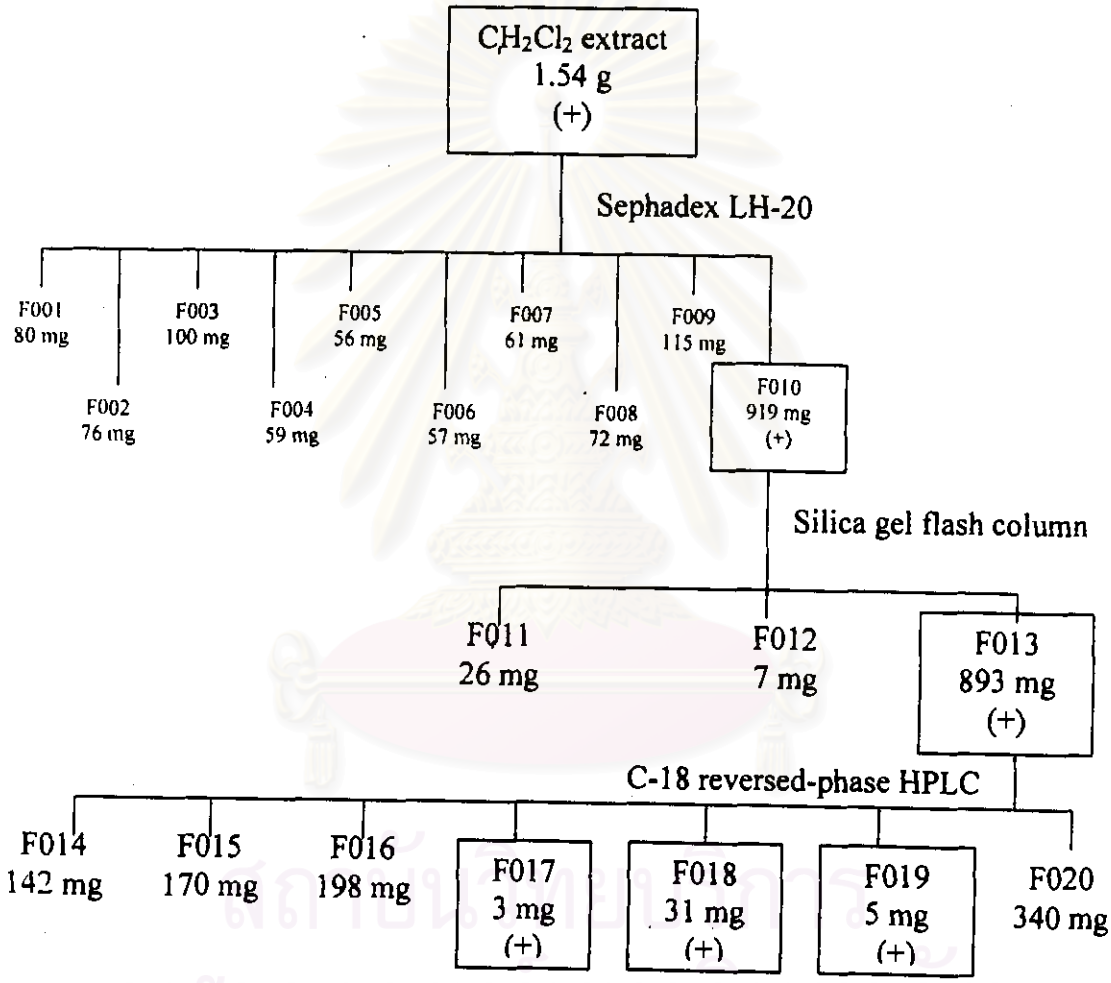

**Scheme 2**. Extraction of the marine *Bacillus* sp. Sc026 culture broth and the marine bacterium Sc004 culture broth

**9. Isolation and purification of crude extracts**

**9.1 Isolation and purification of the $CH_2Cl_2$ extract obtained from the marine *Bacillus* sp. Sc018**

The $CH_2Cl_2$ extract (1.54 g) exhibiting moderate anti-HSV activity was dissolved in a small amount of a mixture of $CH_2Cl_2$:MeOH:hexane (75:20:5) and then fractionated by a Sephadex LH-20 gel filtration column. The column (2.5 cm inner diameter and 100 cm long) was eluted with an isocratic elution of $CH_2Cl_2$:MeOH:hexane (75:20:5). Fractions (30 ml, each) were collected. The combined fractions were guided by the TLC technique (section 4.1, $CHCl_3$:MeOH, 9:1) to give 8 fractions (F003-F010). The F010 fraction (919 mg) was further purified by a silica gel flash column (2.0 cm inner diameter and 18 cm long), using a gradient elution of hexane, $CHCl_3$ and MeOH from Hexane:$CHCl_3$ (9:1) to $CHCl_3$:MeOH (7:3) to yield 3 fractions (F011-F013). The

F013 fraction (893 mg), eluted with $CHCl_3$:MeOH (7:3), was rechromatographed over a C-18 reversed-phase HPLC column using a gradient elution of $CH_3CN$:0.1 % TFA in $H_2O$ from 10:90 to 90:10, to yield 7 fractions (F014-F020). Fractions F017 (3 mg), F018 (31 mg), and F019 (5 mg) were eluted with $CH_3CN$:0.1 % TFA in $H_2O$ at 22:78, 23:77, and 25:75, respectively. The HPLC chromatogram was shown in Figure 1.




*Results of the anti-HSV activity are in parentheses.

**Scheme 3.** Isolation and purification of the $CH_2Cl_2$ extract obtained from the marine *Bacillus* sp. Sc018

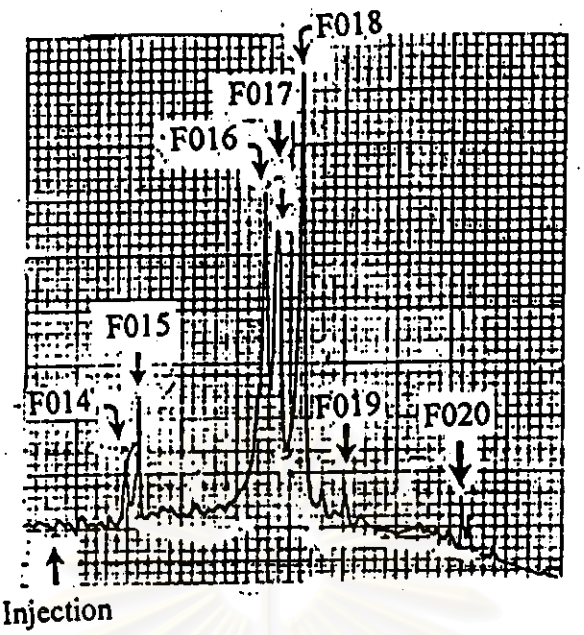
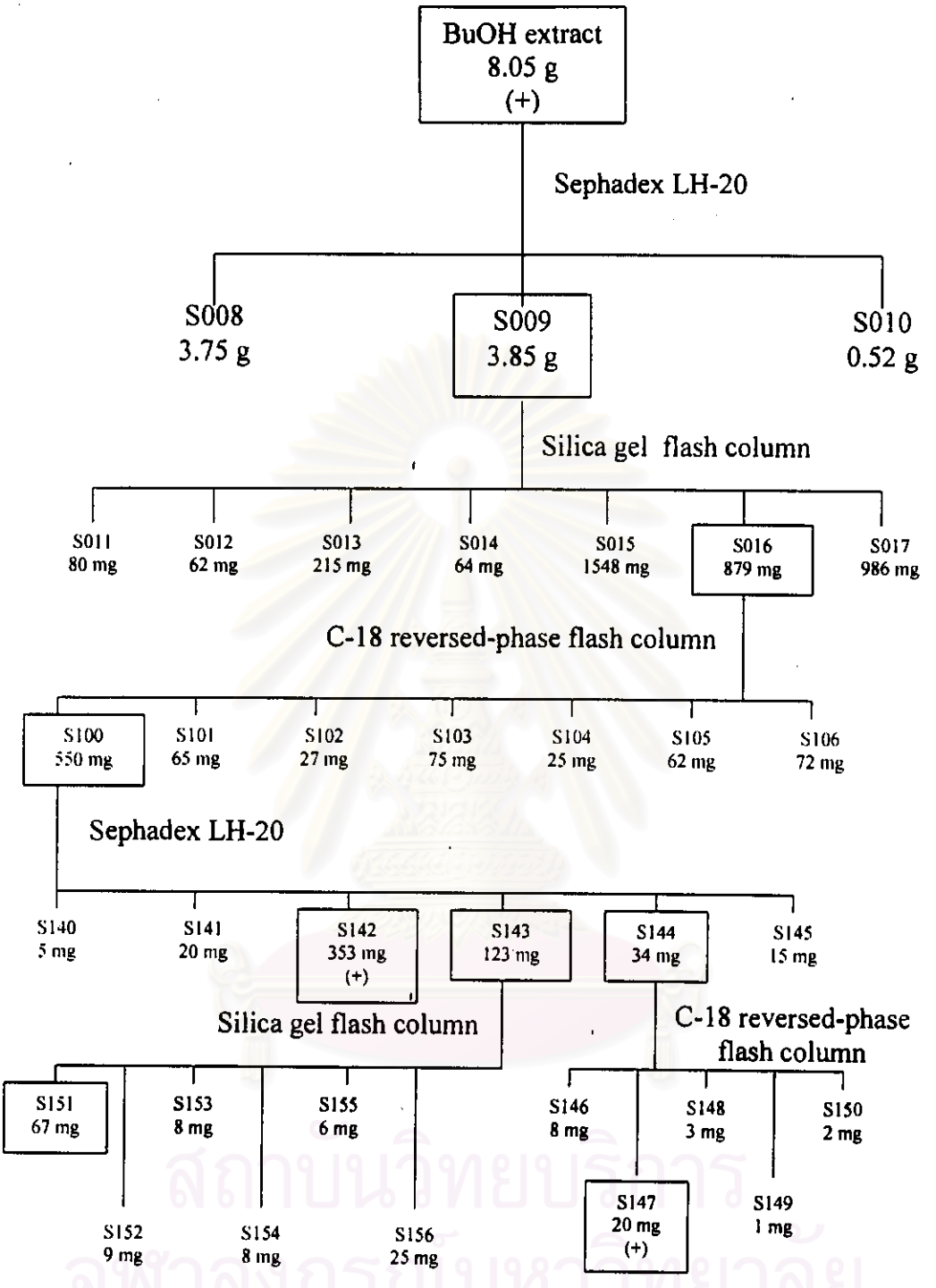



**Figure 1.** HPLC chromatogram of *cyclo*-(D-prolyl-isoleucyl) (F017), *cyclo*-(*trans*-4-hydroxy-L-prolyl-L-phenylalanyl) (F018), and *cyclo*-(D-prolyl-leucyl) (F019)

**9.2 Isolation and purification of the BuOH extract obtained from the marine bacterium Sc004**

The BuOH extract (8.05 g), which exhibited anti-HSV activity, was subjected to gel filtration on a Sephadex LH-20 column (3 cm inner diameter and 80 cm long), and eluted with an isocratic elution of $CH_2Cl_2$:MeOH:hexane (75:20:5) to give 3 fractions (S008-S010) (Scheme 4). The S009 fraction (3.85 g) was further purified by a silica gel flash column (5 cm inner diameter and 15 cm long), using a gradient elution from 100 % EtOAc followed by EtOAc:MeOH (60:40) to yield 7 fractions (S011-S017). The S016 fraction (879 mg) was rechromatographed over a C-18 reversed-phase flash column (1.5 cm inner diameter and 30 cm long), using a gradient elution of the mixture of MeOH:$H_2O$ (20:80 to 70:30) to yield 7 fractions (S100-S106). The S100 fraction (550 mg) was further purified by a Sephadex LH-20 column (1.5 cm inner diameter and 100 cm long), using an isocratic elution of $CH_2Cl_2$:MeOH:hexane (75:20:5) to give 6 fractions

(S140-S145). The S143 fraction (123 mg) was rechromatographed over a silica gel flash column (3 cm inner diameter and 25 cm long), using a gradient elution of $CHCl_3$:MeOH (90:10 to 50:50), to yield 6 fractions (S151-S156). The S142 fraction (353 mg) showed an interesting pure spot on TLC plate and was also similar to the S151 fraction (67 mg). The S144 fraction (34 mg) was rechromatographed over a C-18 reversed-phase flash column (2 cm inner diameter and 30 cm long), using an isocratic elution of MeOH:$H_2O$ (50:50) to yield 5 fractions (S146-S150). The S147 fraction (20 mg) was a pure compound.

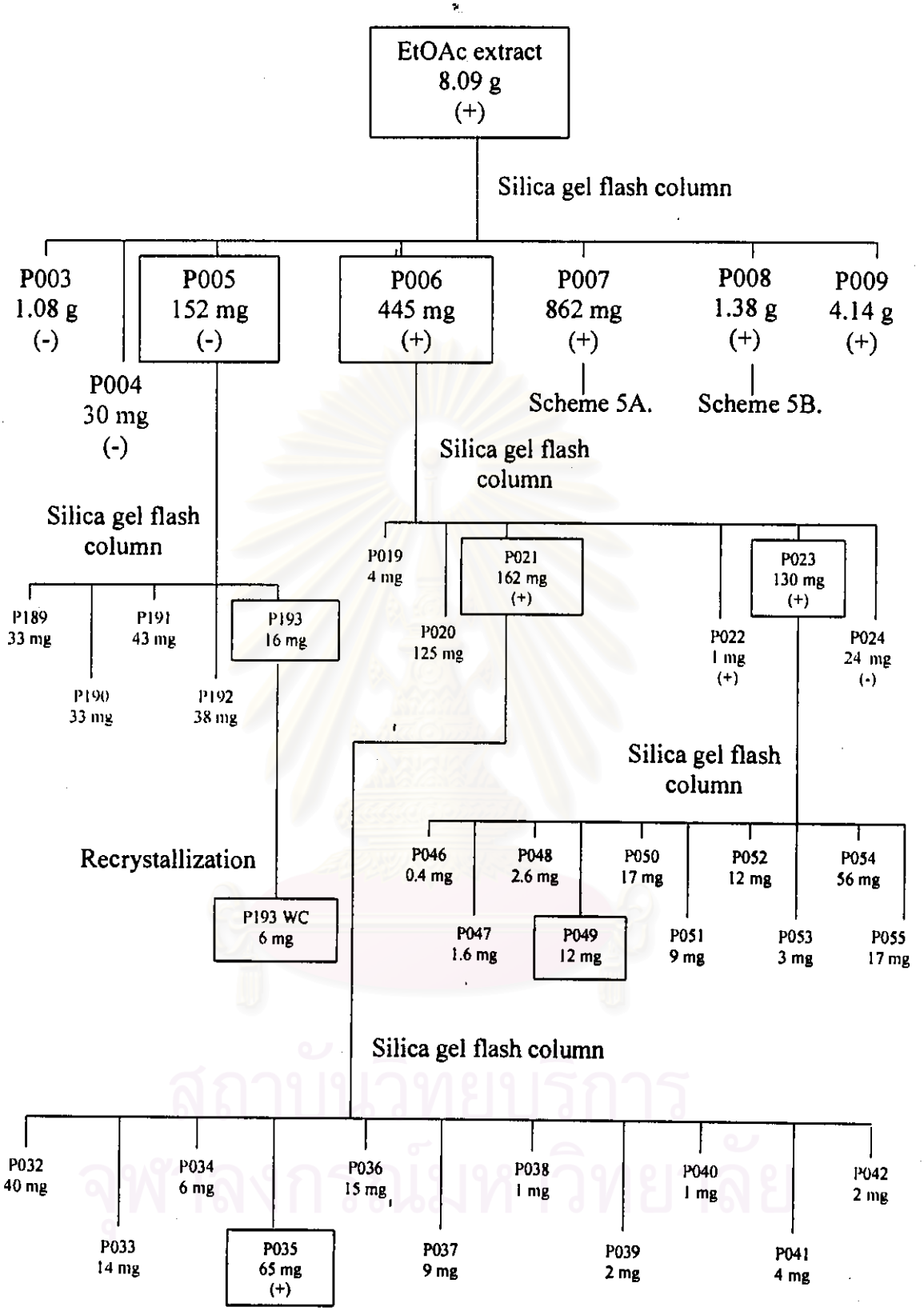



*Results of the anti-HSV activity are in parentheses.

**Scheme 4.** Isolation and purification of the BuOH extract obtained from the marine bacterium Sc004

**9.3 Isolation and purification of the EtOAc extract obtained from the marine *Bacillus* sp. Sc026**

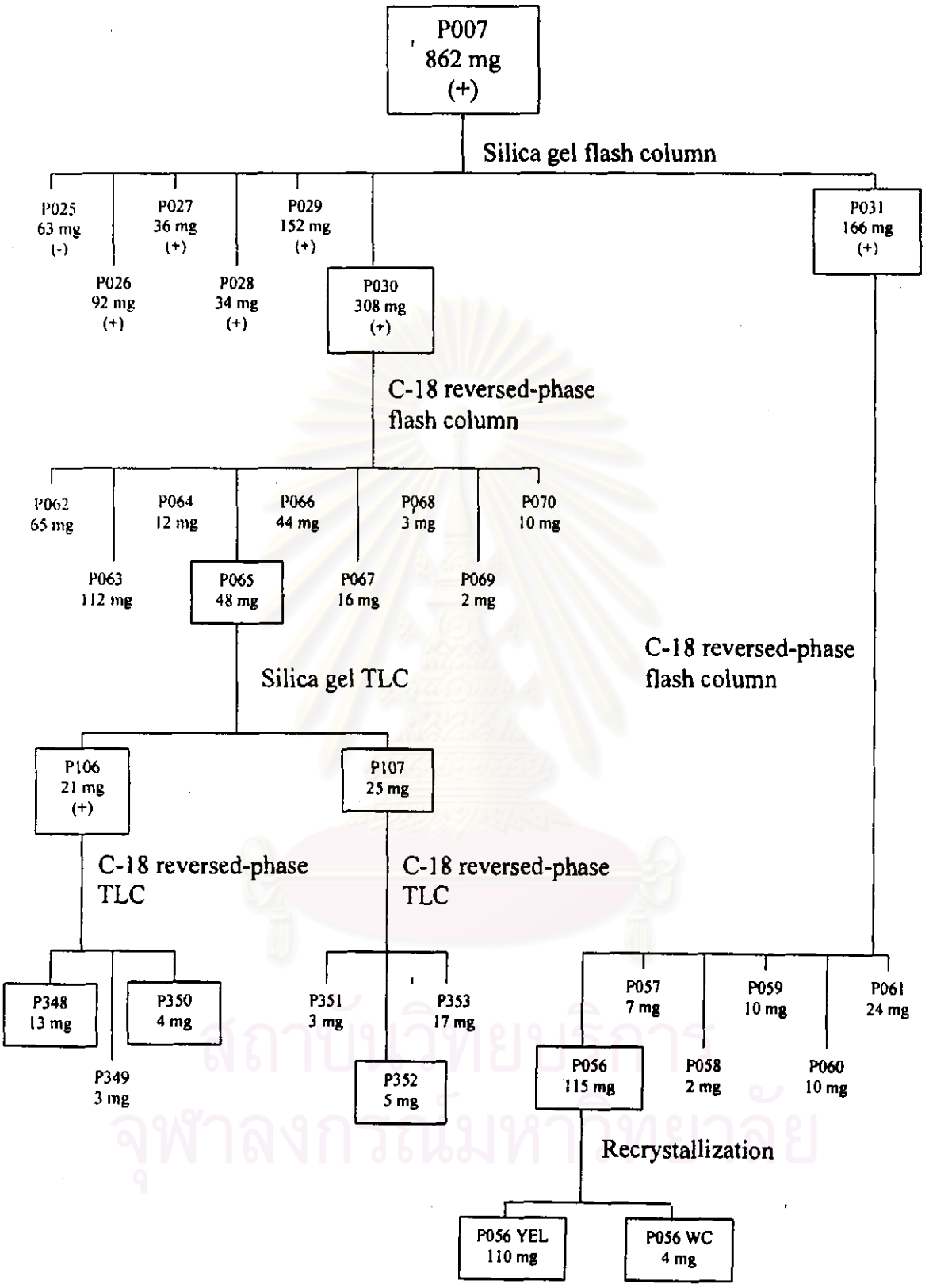
The EtOAc extract (8.09 g) exhibiting antibacterial activity against *B. subtilis* and *S. aureus* at the dose of 1 mg/disk, was fractionated by a silica gel flash column (7.5 cm inner diameter and 17 cm long), using a gradient elution of $CHCl_3$:MeOH (100 % $CHCl_3$ to 100 % MeOH), to afford 7 fractions (P003-P009) (Scheme 5). The P005 fraction (152 mg, eluted with 100 % $CHCl_3$) was rechromatographed over a silica gel flash column (2 cm inner diameter and 15 cm long), using an isocratic elution of $CHCl_3$:EtOAc (50:50) to give 5 fractions (P189-P193). The P193 fraction was recrystallized from a mixture of $CHCl_3$ and MeOH to yield a compound, P193 WC (6 mg). The P006 fraction (445 mg), eluted with 10 % MeOH in $CHCl_3$, was rechromatographed over a silica gel flash column (4 cm inner diameter and 45 cm long), using a gradient elution from $CHCl_3$:EtOAc (90:10) to EtOAc:MeOH (20:80), to yield 6 fractions (P019-P024). The P021 fraction (162 mg), eluted with 100 % EtOAc, was further purified by a silica gel flash column (3 cm inner diameter and 25 cm long), using a gradient elution of $CHCl_3$, EtOAc, and $(CH_3)_2CO$ from $CHCl_3$:EtOAc:$(CH_3)_2CO$ (82:6:12) to EtOAc : $(CH_3)_2CO$ (50:50), yielding 11 fractions (P032-P042). The P035 fraction (65 mg), eluted with $CHCl_3$:EtOAc:$(CH_3)_2CO$ (82:6:12), showed positive black color after spraying with anisaldehyde reagent. The P023 fraction (130 mg), eluted with 10-40 % MeOH in $CHCl_3$, was further subjected to a silica gel flash column (4 cm inner diameter and 25 cm long), using a gradient elution of $CHCl_3$:$(CH_3)_2CO$ from 80:20 to 50:50, yielding 10 fractions (P046-055). The P049 fraction (12 mg), eluted with $CHCl_3$: $(CH_3)_2CO$ (70:30), was similar to the P193WC fraction.




*Results of the antimicrobial activity are in parentheses.

**Scheme 5.** Isolation and purification of the EtOAc extract obtained from the marine *Bacillus* sp. Sc026

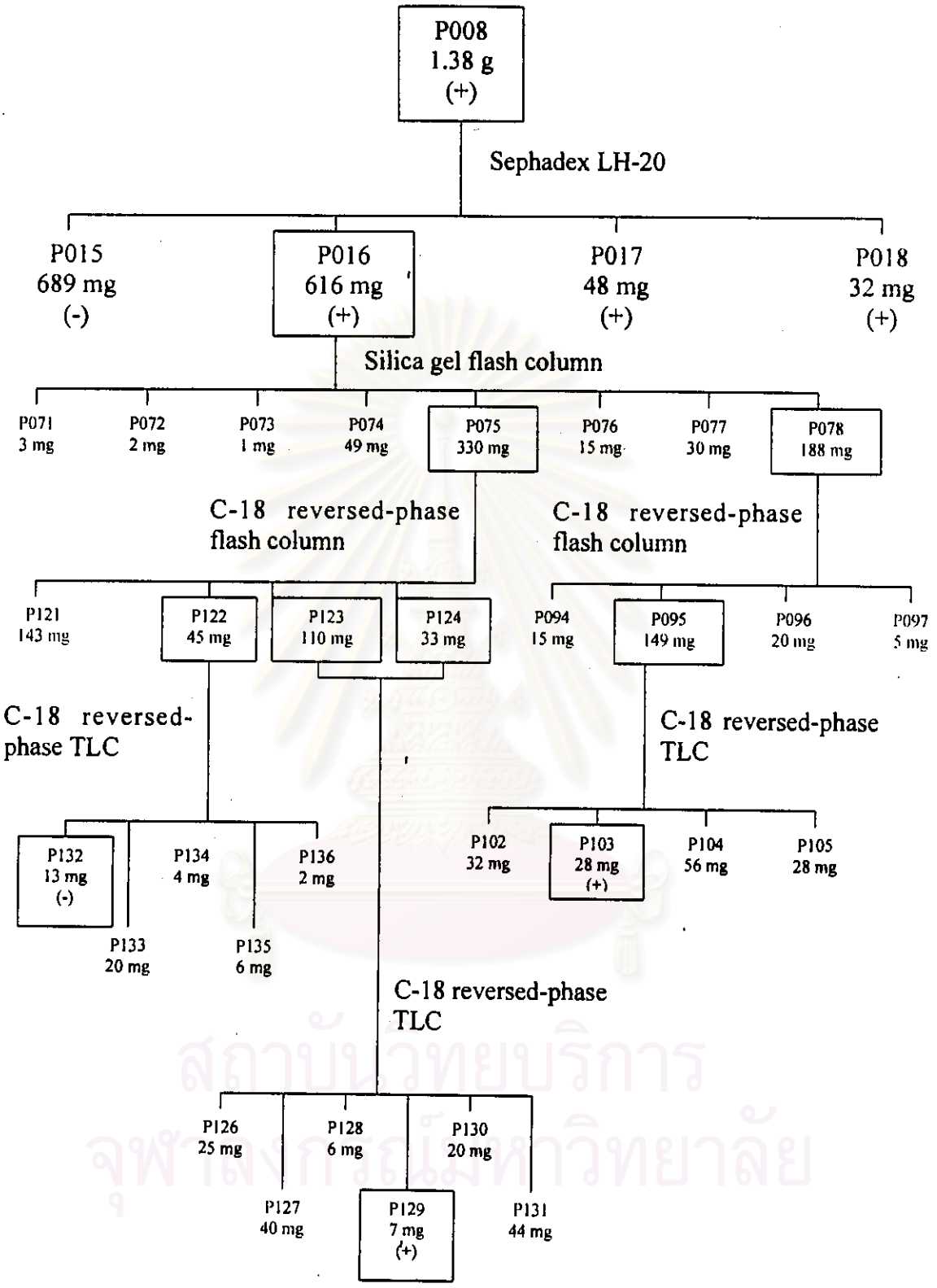
The P007 fraction (862 mg), eluted with 20% MeOH in $CHCl_3$, was rechromatographed over a silica gel flash column (4 cm inner diameter and 20 cm long), using a gradient elution from $CHCl_3$:EtOAc (40:60) to EtOAc:MeOH (20:80), yielding 7 fractions (P025-P031) (Scheme 5A). The P030 fraction (308 mg) was rechromatographed over a C-18 reversed-phase flash column (2 cm inner diameter and 15 cm long), using a gradient elution of MeOH:$H_2O$ from 50:50 to 90:10, yielding 9 fractions (P062-P070). The P065 fraction (48 mg), eluted with MeOH:$H_2O$ (50:50), was fractionated by a silica gel TLC developed with a solvent system of $CHCl_3$:$(CH_3)_2CO$ (50:50), yielding 2 fractions (P106-P107). The P106 fraction (21 mg, higher $R_f$ value) was further purified by C-18 reversed-phase TLC plates developed with a solvent system of THF:$H_2O$ (30:70), yielding 3 fractions (P348-P350). The P348 fraction (13 mg, higher $R_f$ value) and the P350 fraction (4 mg, lower $R_f$ value) showed negative and positive pink color after spraying with anisaldehyde reagent, repectively. The P107 fraction (25 mg, lower $R_f$ value) was further purified by C-18 reversed-phase TLC plates developed with a solvent system of THF:$H_2O$ (30:70), yielding 3 fractions (P351-P353). The P352 fraction (5 mg) showed positive red color after spraying with anisaldehyde reagent. The P031 fraction (166 mg), eluted with 50-80 % MeOH in EtOAc, was rechromatographed over a C-18 reversed-phase flash column (1.5 cm inner diameter, 15 cm long), using a gradient elution of MeOH:$H_2O$ from 50:50 to 100 % MeOH, yielding 6 fractions (P056-P061). The P056 fraction (115 mg), eluted with 50 % MeOH in $H_2O$, was recrystallized from $CHCl_3$ and MeOH to afford the fraction P056 WC (4 mg) and the P056 YEL fraction (110 mg).




*Results of the antimicrobial activity are in parentheses.

**Scheme 5A.** Isolation and purification of fraction P007 obtained from the marine *Bacillus* sp. Sc026

The P008 fraction (1.38 g), eluted with 40 % MeOH in $CHCl_3$, was rechromatographed over gel filtration on a Sephadex LH-20 column (3 cm inner diameter and 100 cm long), using an isocratic elution of $CHCl_3$:MeOH:hexane (75:20:5) to afford 4 fractions (P015-018) (Scheme 5B). The P016 fraction (616 mg) was rechromatographed over a silica gel flash column (3 cm inner diameter and 22 cm long), using a gradient elution of $CHCl_3$:MeOH from 95:5 to 20:80, yielding 8 fractions (P071-P078). The P078 fraction (188 mg), eluted with 80 % MeOH in $CHCl_3$, was fractionated by a C-18 reversed-phase flash column (3 cm inner diameter and 15 cm long), using a gradient elution of MeOH:$H_2O$ from 80:20 to 90:10, yielding 4 fractions (P094-P097). The P095 fraction (149 mg), eluted with 80 % MeOH in $H_2O$, was further purified with a C-18 reversed-phase TLC developed with a solvent system of MeOH:$H_2O$ (80:20), yielding 4 fractions (P102-P105). The P103 fraction (28 mg) showed positive blue color after spraying with anisaldehyde reagent. The P075 fraction (330 mg, eluted with 15 % MeOH in $CHCl_3$) was fractionated by a C-18 reversed-phase flash column (1.5 cm inner diameter and 15 cm long), using a gradient elution of MeOH:$H_2O$ from 50:50 to 100 % MeOH, yielding 5 fractions (P121-P125). The P123 fraction (110 mg) and the P124 fraction (33 mg) were combined and were further purified with a C-18 reversed-phase TLC plates developed with a solvent system of MeOH:$H_2O$ (80:20), yielding 6 fractions (P126-P131). The P129 fraction (7 mg) showed positive black color after spraying with anisaldehyde reagent. The P122 fraction (45 mg), eluted with 50 % MeOH in $H_2O$, was further purified with C-18 reversed-phase TLC plates developed with a solvent system of MeOH:$H_2O$ (80:20), yielding 5 fractions (P132-P136). The P132 fraction (13 mg) showed positive pink color after spraying with anisaldehyde reagent.




*Results of the antimicrobial activity are in parentheses.

**Scheme 5B.** Isolation and purification of fraction P008 obtained from the marine *Bacillus* sp. Sc026

**10. Acetylation of compounds P035 and P132**

The IR and mass spectral data indicated the presence of hydroxyl group(s) in compounds P035 and P132, so, acetylation of compounds P035 and P132 was performed to confirm the secondary nature of the alcohol group(s). The sample (1 mg) was put into a 5-ml round-bottom flask with a magnetic bar inside. Anhydrous pyridine and acetic anhydride, 0.2 ml each, were added. The flask was sealed with a rubber septum and the air inside replaced by nitrogen gas. The mixture was stirred at room temperature and the reaction monitored by TLC (EtOAc:MeOH, 9:1) every 6 hours. The reactions were completed within 43 and 26 hours for compounds P035 and P132, respectively.

**11. Synthesis and purification of *cyclo*-(L-prolyl-L-Phenylalanyl)**

**11.1 Preparation of L-phenylalanine methyl ester hydrochloride** (Bondanszky and Bodanszky, 1994)

L-phenylalanine (3.60 g, 21.8 mmol) was suspended in anhydrous methanol (20 ml), and then thionyl chloride (2 ml) was added. The mixture was cooled and stirred in an ice-water bath for 10 minutes, and then refluxed at 70°C for 2 hours. After refluxing, the excess thionyl chloride and methanol were removed in vacuo and the residue was washed with n-hexane to afford L-phenylalanine methyl ester hydrochloride (4.03 g, 86 % yield), m.p. 158-160°C.

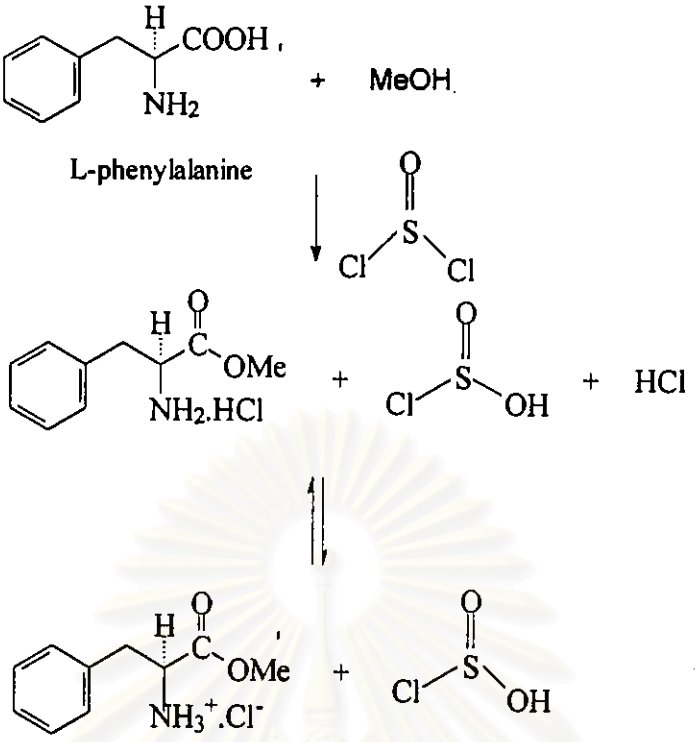



**Figure 2.** Chemical reaction of the formation of L-phenylalanine methyl ester hydrochloride

**11.2 Preparation of *tert*-butyloxycarbonyl-L-proline** (Bondanszky and Bodanszky, 1994)

L-proline (1.15 g, 10 mmol) in a mixture of dioxane (20 ml), water (10 ml), and 1 N NaOH (10 ml) was stirred and cooled in an ice-water bath. Di-*tert*-butylpyrocarbonate (2.4 g, 11 mmol) was then added and stirred at room temperature for 20 hours. The solution was concentrated *in vacuo* to about 10 to 15 ml, cooled in an ice-water bath, covered with a layer of EtOAc (30 ml) and acidified with a dilute solution of HCl to pH 2-3. The aqueous phase was repeatedly extracted with EtOAc (3x45 ml). The obtained EtOAc extracts were pooled, washed with water (2x30 ml), dried over

anhydrous $Na_2SO_4$ and evaporated *in vacuo*. The crystal was washed with cold n-hexane yielding *tert*-butyloxycarbonyl-L-proline (1.54 g, 72 % yield), m.p. 133-135°C.

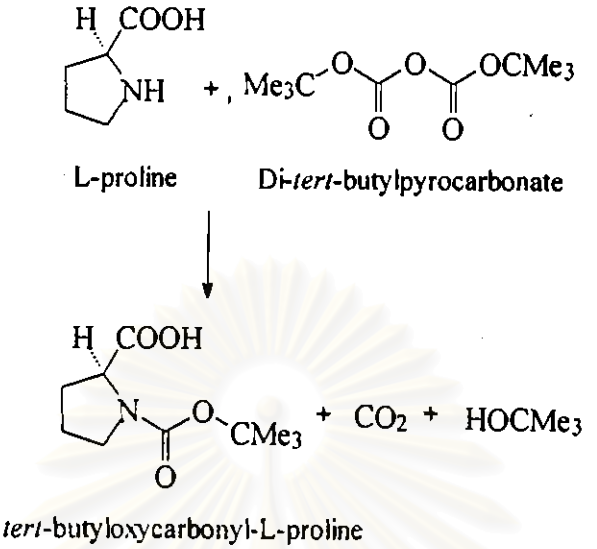



**Figure 3.** Chemical reaction of the formation of *tert*-butyloxycarbonyl-L-proline

**11.3 Preparation of *tert*-butyloxycarbonyl-L-prolyl-L-phenylalanine methyl ester** (Bondanszky and Bodanszky, 1994)

L-phenylalanine methyl ester hydrochloride (220 mg, 1 mmol) was dissolved in $CH_2Cl_2$ (1.5 ml). Triethylamine (TEA) (104 mg, 1 mmol) was added. The mixture was cooled and stirred in an ice-water bath for 30 minutes. The solution of *tert*-butyloxycarbonyl-L-proline (215 mg, 1 mmol) in $CH_2Cl_2$ (1.5 ml) was added. The mixture was stirred in an ice-water bath for 30 minutes. The solution of dicyclohexylcarbodiimide (DCC) (206 mg, 1 mmol) in $CH_2Cl_2$ (1 ml) was then added. The mixture was stirred in an ice-water bath for 30 minutes. The reaction mixture was stirred at room temperature for 22 hours, and then was evaporated to dryness *in vacuo* to yield a crude dipeptide (1.60 g). The crude dipeptide was purified by silica gel flash chromatography with an isocratic elution of hexane: EtOAc (1:1), yielding 4 fractions

(Dip1-4). The first fraction (Dip1) was a pure *tert*-butyloxycarbonyl-L-prolyl-L-phenylalanine methyl ester (301 mg, 80% yield).

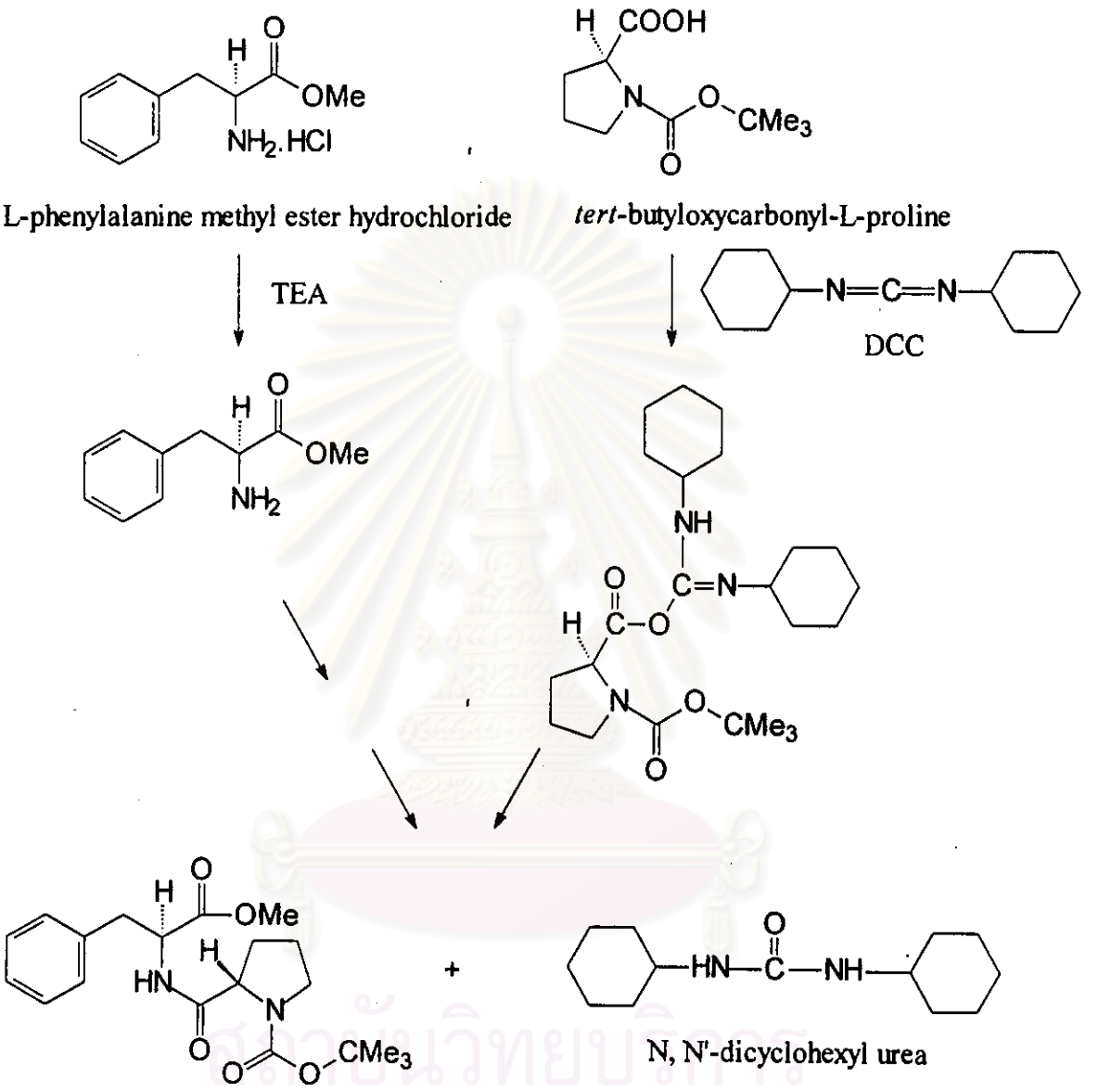
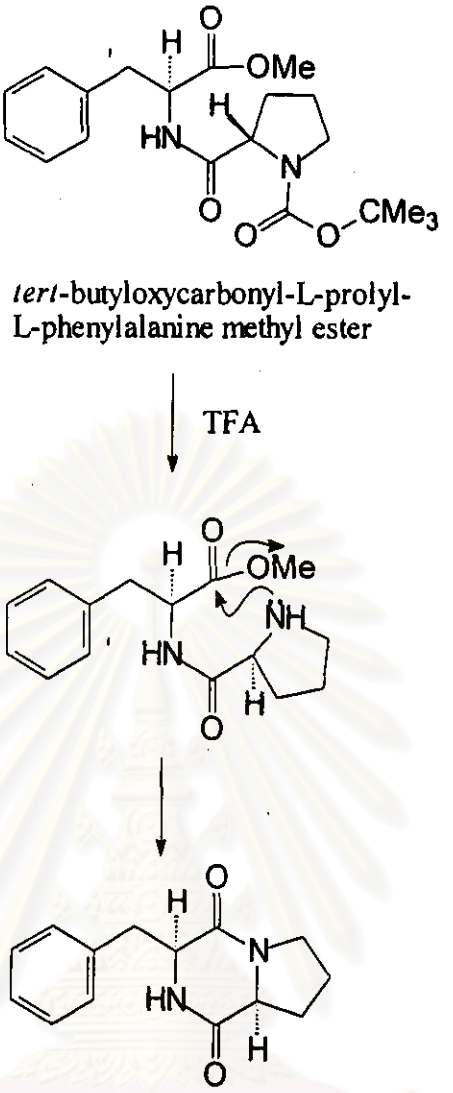



**Figure 4.** Chemical reaction of the formation of *tert*-butyloxycarbonyl-L-prolyl-L-phenylalanine methyl ester

**11.4 Formation of *cyclo*-(L-prolyl-L-phenylalanyl)** (Nitecki, Halpern, and Westley, 1968)

*tert*-Butyloxycarbonyl-L-prolyl-L-phenylalanine methyl ester (50 mg, 0.13 mmol) was dissolved in TFA (1 ml) and the solution was stirred at room temperature for 3 hours. After removal of excess TFA under a stream of $N_2$ gas, the residue containing the crude L-proline-L-phenylalanine methyl ester was dissolved in n-butanol (3 ml) and toluene (1 ml). The solution was refluxed at 120°C for 15 hours with the solvent volume maintained by addition of fresh n-butanol, and then was evaporated to dryness in vacuo to yield a crude diketopiperazine (62 mg). The crude *cyclo*-(L-prolyl-L-phenylalanyl) was purified by silica gel flash chromatography using a gradient elution from $CHCl_3$:hexane (1:2) to $CHCl_3$:MeOH (1:1), yielding 5 fractions (dkp24-28). The fourth fraction (dkp27) was a pure *cyclo*-(L-prolyl-L-phenylalanyl) (24 mg, 75 % yield).




*tert*-butyloxycarbonyl-L-prolyl-L-phenylalanine methyl ester

*cyclo*-(L-prolyl-L-phenylalanyl)

**Figure 5.** Mechanism of the cyclization of *cyclo*-(L-prolyl-L-phenylalanyl)

## 12. Biological activity

### 12.1 Antimicrobial activity

Antimicrobial activity was assessed using an agar diffusion method and autobiography (Lorian, 1980). Activity was performed against *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, and *Candida albicans* ATCC 10231. All tested bacteria were cultivated overnight on Trypticase soy agar (TSA) slant at 37°C, and the fungal *C. albicans* was cultivated on Sabouraud

dextrose agar (SDA) slant at 37°C for two days. The cell cultures were washed from the agar surface with sterile normal saline solution (NSS). The culture was then standardized to match a 0.5 turbidity standard of MacFarland No.1, which provided approximately $1x10^8$ CFU (colony forming unit)/ml. Each of molten (20 ml) TSA and SDA was separately poured into 9-cm diameter petri dishes and allowed to solidify to form base layer. A loopful of each tested microorganisms was spread on the surface of TSA and SDA plates. All crude extracts were dissolved in $CHCl_3$ and/or MeOH and the concentration adjusted to 50 mg/ml. Each solution (20 µl) was applied on a sterile paper disk (5 mm diameter) or a Silica gel TLC aluminum sheet (0.5 cm x 6.5 cm). These paper disks (1mg/disk) or TLC plates (1 mg/plate) were left in each sterile petridish until the solvents were completely dry. The dried paper disks or TLC plates were placed on the surface of the inoculated agar plates already spreaded with tested microorganisms. The plates spreaded with tested bacteria or fungi were incubated at 37°C for 18 hours, or 48 hours, respectively. The diameters and the $R_f$ values of inhibition zones were measured. The crude extracts expressed good antimicrobial activity were subsequently selected for further study.

**Media**

**Trypticase soy agar (TSA)**

The medium was Difco® tryptic soy agar (Becton Dickinson Microbiology System).

Formula per liter of purified water

| | |
|---|---|
| Tryptone peptone (pancreatic digest of casien | 15.0 g |
| Soytone peptone (papaic digest of soybean meal) | 5.0 g |
| Sodium chloride | 5.0 g |

| | |
|---|---|
| Agar | 15.0 g |

Final pH 7.30±0.2

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

**Sabouraud dextrose agar (SDA)**

The medium was Difco® Sabouraud dextrose agar (Becton Dickinson Microbiology System).

Formula per liter of purified water

| | |
|---|---|
| Neopeptone | 10.0 g |
| Dextrose | 40.0 g |
| Agar | 15.0 g |

Final pH 5.60±0.2

The formula powder (65 g) was dispersed in 1 liter of distilled water and boiled to dissolve completely. The medium was sterilized by autoclaving at 121°C for 15 minutes. This medium was used for cultivation of yeast during the assay.

**12.2 Anti-herpes simplex virus activity**

**12.2.1 Anti-HSV activity using sulforhodamine B colorimetric method**

For testing anti-HSV activity, the modified colorimetric method which has been described previously by Skehan *et al.* (1990) was used. The suspension of Vero cell line was added to 96-well microtiter plate (190 µl/well) and then incubated in a $CO_2$ incubator at 37°C for 30 minutes. The sample solution dissolved in 10 µl of 100 %

dimethyl sulfoxide (DMSO) and *Herpes simplex* viruses (30 plaque forming unit, PFU/well) were added to 96-well microtiter plate before incubating in $CO_2$ incubator at the same condition for 3 days. After incubation period, cultures were fixed by adding 100 µl/well of cold 50 % aqueous trichloroacetic acid (TCA) and further incubated at 4°C for 30 minutes. Before washing four times with tap water and air-drying at room temperature, cultures fixed with TCA were stained for 30 minutes with 100 µl/well of 0.4 % (*w/v*) sulforhodamine B (SRB) dissolved in 1 % acetic acid. Unbound dye was removed by four washes with 1 % acetic acid and protein-bound dye was extracted with 10 mM unbuffered Tris base (pH 10) for determination of optical density at 515 nm using an ELISA plate reader. $ED_{50}$ (µg/ml) values were calculated by nonlinear regression analysis (percent survival versus concentration). In each experiment, acyclovir was used as a positive control.

### 12.2.2 Anti-HSV activity using plaque reduction method

Cell-free HSV (30 plaque forming unit, PFU/well) was mixed with the maintenance medium containing various concentrations of sample and incubated at 37°C for 1 hour. After incubation, the mixtures were inoculated into monolayer Vero cell culture in 96-well microtiter plates and incubated at 37°C for 2 days in $CO_2$ incubator. The cells were stained with 1% crystal violet in 10% formalin for 1 hour. The plaques were counted under an inverted-microscope.

### 12.3 Cytotoxic activity

Cytotoxic activity against a breast cancer cell line (BC), a human epidermoid carcinoma cell line of the narsopharynx (KB), and a Vero cell line was performed by sulforhodamine B (SRB) colorimetric method

**12.4 Antimalarial activity**

Antimalarial assay was performed according to the method of Trager and Jensen (1976) using continuous cultures (*in vitro*) of asexual erythrocytic stages of *Plasmodium falciparum* (K1, multidrug resistant strain). Quantitative assessment of antimalarial activity (*in vitro*) was determined by means of the microculture radioisotope technique based upon the method described by Desjardins *et al.*, 1979. Effective concentration ($EC_{50}$) represents the concentration which causes 50% reduction in parasite growth as indicated by the *in vitro* uptake of [$^3$H]-hypoxanthine by *P. falciparum*. An $EC_{50}$ value of 0.16 μg/ml (3.1 μM) was observed for the standard sample, chloroquine diphosphate, in the same test system.