CHAPTER III
EXPERIMENT

1. Sample collection, isolation and primary screening of actinomycetes

1.1 Sample collection and isolation of the isolates

Thirty-five soil samples collected from Samed Island, Rayong Province (Table 9) were preserved at 4°C until the process of isolation. A 0.5 g of each soil sample was suspended in sterile distilled water (4.5 ml.), heated at 55°C for 6 min. and was diluted to 1:100, 1:1,000 and 1:10,000 dilutions. Each dilution (0.1 ml.) was spreaded on surface of Starch-Casein Nitrate agar (SCA) plates (plus antibiotics, appendix). Plates were incubated at 30°C for 14-21 days depended on growth of each isolate. The powdery, blue, gray, green, red, violet, white and yellow colonies of *Streptomyces* species and the moist, pale yellow, orange, red brown, brown, black, blue green or purple colonies of *Micromonospora* species were pick up and streaked for purification on Yeast extract-Malt extract agar plates (YMA, ISP-2, appendix) incubated at 30°C for 7-14 days. Colonies were transferred to cultivate on YMA slants incubated at 30°C for 14 days. Stock cultures on slant were kept in cold room at 4°C.

1.2 Primary screening of antimicrobial activity of the isolates

All of the isolates were screened by streaking on the edge of YMA plates, incubated at 30°C for 14 days. Then five test organisms, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 on nutrient agar (NA) slant and *Candida albicans* ATCC 10231 on Sabouraud dextrose agar (SDA) were vertical streaked from the edges of each isolate. The plates were incubated 24 hr at 37°C. The clear inhibition distances of each test organism were observed.

2. Identification and characterization of actinomycetes

2.1 Morphological and cultural characteristics

All isolates were grown on YMA plate by crosshatches streak technique (Shirling and Gottlieb, 1966) and incubated at 30°C for 10-14 days. Spore bearing hyphae, spore chain, and spore morphology of them were studied by using simple inclined coverslip technique (William and Cross, 1971). The selected strain that showing good antimicrobial activity was cultivated on YMA medium for 14 days until mature spores bearing hyphae was shown and was examined using scanning electron microscopic (SEM) that recommended by Deman, and Gupta (1986). Cultural characteristics of strains were observed on various agar media at 30°C for the 14 days (Table 13). The Jacal Color Card L2200 (Japan Color Research Institute) was used in the determination of the color designations of colonies. Thirty isolates showing antimicrobial activity were selected for future study.

2.2 Physiological and biochemical characteristics

The temperatures (10°C, 28°C, 45°C) and pH (4.0, 5.0, 7.0, 9.0 and 10.0) effects on the growth of the 30 isolates showing antimicrobial activity were determined in Yeast extract-malt extract agar. Carbon sources utilization from a basal medium containing 1% of the carbon source was tested as described by Shirling, and Gottlieb (1966). Halotolerance were tested by the use of yeast extract-malt extract agar containing 2%, 4% or 6% NaCl. Gelatin liquefaction, peptonization and coagulation of milk, nitrate production, cellulose decomposition, chitin and starch hydrolysis were determined by cultivation on the media described by Arai (1975) and William and Cross (1971). The melanin formation and hydrogen sulfide production were examined in tyrosine agar and peptone iron agar supplemented with 0.1% (w/v) yeast extract incubated for 21 days, respectively.

2.3 Cell wall analysis

The chemical analysis of cell wall diaminopimelic acid (DAP) isomers was carried out by the method of Komagata and Suzuki (1987). Dried cell of the selected strain was hydrolyzed by whole-cell analysis. DAP isomers were separated by thin layer chromatography on cellulose plate (Merck No.5577). The standard of DL-DAP (0.01 M) and the hydrolysate of two known strains that contained *meso*- and L-DAP were applied for reference purposes. TLC was developed with the solvent system methanol-water-6 N hydrochloric acid-pyridine (80:26:4:10,v/v). The spots were visualized by spraying with 0.2% ninhydrin solution in water-saturated n-butanol followed by heating at 100°C for minutes. DAP isomers appear as dark-green spots with R_f 0.29 (L-isomer) and 0.24 (*meso*- and DL-isomer). Spots will gradually disappear in a few minutes.

2.4 16S rDNA sequence analysis and phylogenetic tree construction

2.4.1 DNA isolation and purification

DNA of the strain PC 4-3 was isolated from cells grown in YMB + 0.2% of glycine (Yamada, and Komagata, 1970) and purified by using phenol-chloroform (1:1,v/v) as described previously (Saito and Miura, 1963; Tamaoka, 1994). The spectrophotometric method of DNA quantitative (A_{260} and A_{280}) was used to determine both the concentration and relative purify of DNA in a solution.

2.4.2 16S rDNA sequence analysis and phylogenetic tree construction

16S rDNA of the strain PC 4-3 was amplified using the PCR method with a *Taq* DNA polymerase and primer 9F (5'GAGTTTGATCCTGGCTCAG'3, *Escherichia coli* numbering) and 1541R (5'AAGGAGGTGATCCAGCC'3). Amplification was carried out using a DNA thermal cycle (GeneAmp PCR System 2400; Applied Systems) according to the following program: 94°C for 5 min, followed by 35 cycles consisting of

denaturation at 94°C for 1 min, primer annealing at 50°C for 1 min and primer extension at 72°C for 2 min. At the end of the cycle, the reaction mixture was kept at 72°C for 5 min and then cooled to 4°C. The 1.5 kp amplified 16S rDNA fragment was separated by agarose gel electrophoresis and purified by a prep-A-Gene DNA purification kit (Bio-rad Laboratories, Hercules, Calif). The purified fragment was ligated into p-GEM-T-EASY vector (Promega) and then introduced into *Escherichia coli* strain DH-10B. The double-stranded DNA was sequenced by an ABI PRISM BigDye Terminator Cycle sequencing Ready Reaction kit (Applied Biosystems), according to the manufacturer's protocol, with the following primer: universal vector primer (T7 and SP6), 339F (5'CTCCTACGGGAG GCAGCAG'3), 357R (5'CTGCTGCCTCCCGTAG'3), 785F (5'GGATTAGATACCCTGGTA GTC3') 802R (5'TAC CAGGGTATCTAATCC'3), 1099F (5'GCAACGAGCGCAACCC'3) and 1115R (5'AGGGTTGC GCTCGTTG'3). The conditions for thermal cycling were as followed 96°C for 30 s, followed by 30 cycles of denaturation at 96°C for 10 s, primer annealing at 50°C for 10 s and primer extension at 60°C for 3 min. The products were analyzed with an ABI PRISM 377 Genetic Analyser (Applied Biosystems) at DNA Technology Laboratory (BIOTEC), Kasetsart University, Kamphaengsaen, Nakornpathom.

The sequence homology search was performed using the standard BLAST sequence similarity searching program version 2.2.1 from the web server http://www.ncbi.nlm.nih.gov/BLAST/; the sequence was multiply aligned with selected sequence obtained from the GenBank/EMBL/DDBJ database by using the CLUSTAL W version 1.81 program available from the web server http://workbench.sdsc.ed/; the alignment was manually verified and adjusted prior to the construction of a phylogenetic tree. Phylogenetic tree was constructed by using the neighbor-joining method (Saito and Nei, 1987) in the programs of MEGA version 2.1 (Kumar, *et. al.* 2001). The confidence values of branches of the phylogenetic tree were determined using bootstrap analysis (Felsenstein, 1985) based on 1000 resamplings. The values for sequence similarity among the closest strains were calculated manually after pairwise

alignments obtained using the CLUSTAL W version 1.81. Gaps and ambiguous nucleotides were eliminated from the calculations (Fitch, 1967).

3. Fermentation and antimicrobial activity test

3.1 Fermentation of the 30 isolates showing antimicrobial activity

Thirty isolates showing antimicrobial activity were inoculated into 250 ml Erlenmeyer flask containing 60 ml of seed medium (glucose-beef extract-peptone broth; GBP) and incubated on a rotary shaker (150 rpm) at RT for 4 days. Then 5 ml of the seed medium was transferred into a 500 ml Erlenmeyer flask containing 200 ml of production medium (yeast extract-malt extract-$CaCO_3$ broth) on rotary shaker (200 rpm) at RT 10 days. The fermentation broth was filtered through Whatman filter paper and then the broth filtrate was extracted with ethyl acetate. The antimicrobial activity of the isolate extracts was examined by an agar disc diffusion method (Lorian, 1980) as shown in Scheme 1. Evaluation of the antimicrobial activity was performed against *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 on nutrient agar (NA) slant and *Candida albicans* ATCC 10231. All tested bacteria were cultivated on Nutrient agar, NA (Difco®), whereas the yeast strain was cultivated on Sabouraud dextrose agar, SDA (Difco®) at 37°C for 24 hours. The cultures were washed from agar by normal saline solution. The cell suspensions were adjusted with standard of McFarland NO 0.5, provided approximately $1x10^8$ CFU (colony forming unit/ml). Each 20 ml of molten was separately poured into 9 cm diameter petri dishes and allowed to solidify to form base agar. A loopful of each tested microorganism was spread on the surface of each plate. Test samples were dissolved in suitable solvent and then were applied on sterile paper disc for disc diffusion assay or Silica gel TLC aluminium sheet for autobiography assay. These paper disc or TLC plates were left in each sterile petri dish until the solvent was completely dry. Then disc or plate was placed on the surface of the agar plate already spreaded with tested microorganisms and were incubated at 37°C, 24 hours for bacteria and 48 hours for

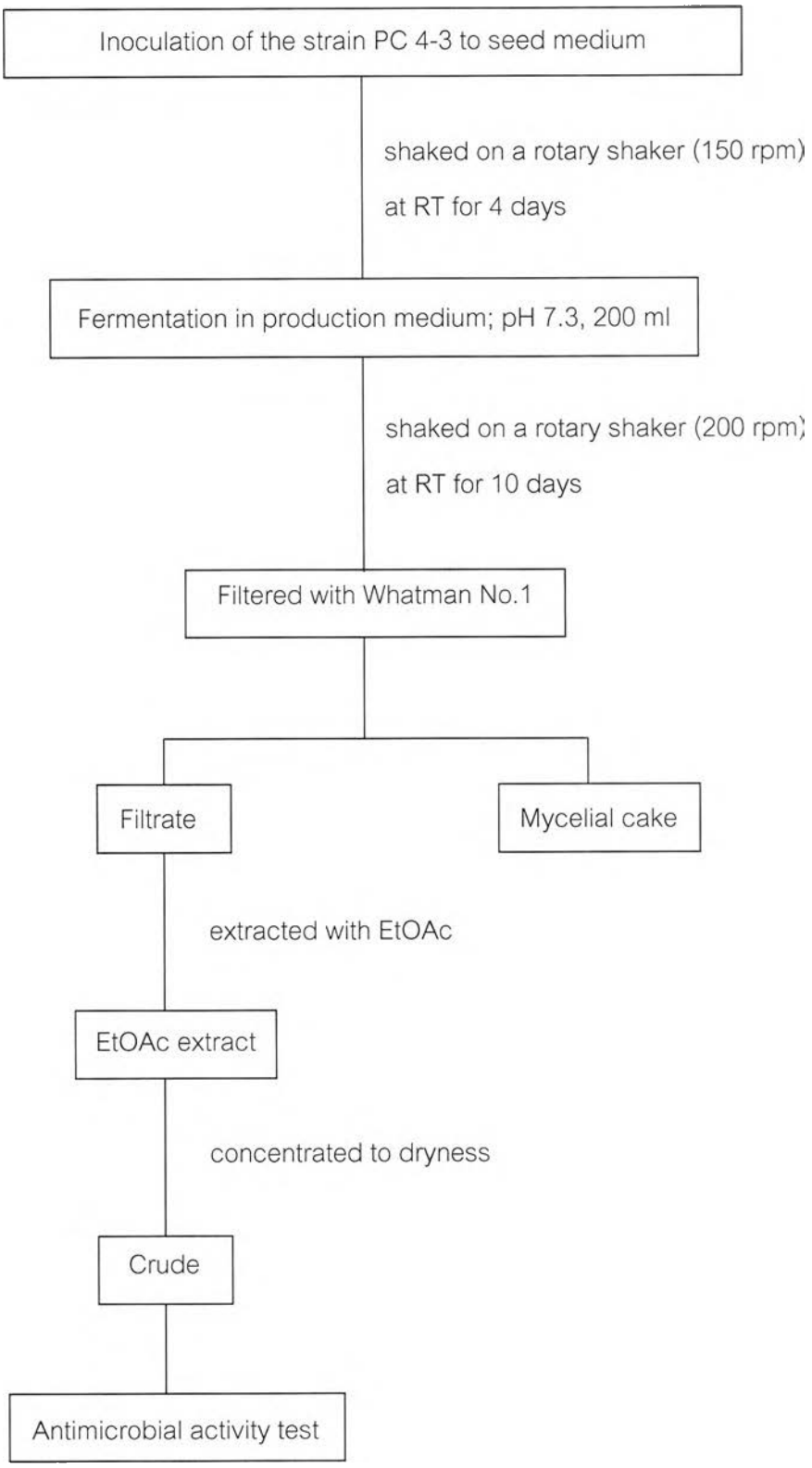
yeast. The diameter and R_f value of inhibition zones were measured. Fractions showing antimicrobial activity were subsequently selected for further study. The culture of the strain PC 4-3, showing the highest antimicrobial activity was selected for secondary metabolite fermentation.

3.2 Effect of media composition

The strain PC 4-3 was inoculated into 250 ml Erlenmeyer flask containing 60 ml of seed medium (GBP) and incubated on a rotary shaker (150 rpm) at RT for 4 days. Then 5 ml of the seed medium was transferred into 500 ml Erlenmeyer flasks containing 200 ml of each production medium; glucose-beef extract-peptone broth (GBP), peptone-yeast extract broth (PY), yeast extract-malt extract broth (YMB), and glucose-beef extract-yeast extract-tryptone broth (GBY) and incubated on a rotary shaker at 200 rpm at RT for 10 days. The fermentation broth was filtered through Whatman No. 1 filter paper and then the broth filtrate was extracted with ethyl acetate. Antimicrobial activity of the isolated extracts was examined by an agar disc diffusion method. The production medium that showing the highest antimicrobial activity was selected for secondary metabolite fermentation.

3.3 Effect of pH

The production medium selected in Section 3.2 (200 ml in 500 ml erlenmeyer flask) was adjusted to pH 6.0, 7.0 and 8.0 respectively and was inoculated with seed culture (GBP) of the strain PC 4-3. The flask was inoculated on a rotary shaker (200 rpm) at RT for 10 days. The pH of the fermentation broth was measured and the antimicrobial production during fermentation was monitored by agar diffusion method.



Scheme 1 Fermentation of the strain PC 4-3

4. General Techniques for isolation and identification of the pure compounds

4.1 Thin Layer Chromatography

4.1.1 Analytical Thin Layer Chromatography

Technique : one dimension ascending

Adsorbent : silica gel F_{254} coated on aluminium sheet (E. Merck)

Layer thickness : 250 μm

Distance : 5 cm

Temperature : laboratory temperature (25-30°C)

Detection : 1. Visual detection under daylight

2. Visual detection under ultraviolet light at wavelengths of 254 and 365 nm

3. Visual detection in iodine vapour

4. Visual detection under daylight after spraying with anisaldehyde reagent and heated until color developed

4.1.2 Preperative Thin Layer Chromatography

Technique : one dimension ascending

Adsorbent : silica gel F_{254} coated on aluminium sheet (E. Merck)

Layer thickness : 250 μm

Distance : 17 cm

Temperature : laboratory temperature (25-30°C)

Detection : 1. Visual detection under daylight

2. Visual detection under ultraviolet light at wavelengths of 254 and 365 nm

4.2 Column Chromatography

4.2.1 Quick Column Chromatography

Adsorbent	: silica gel 60 (No. 7734), particle size 0.063-0.200 ηm (70-230 mesh ASTM) (E. Merck)
Packing method	: The adsorbent was dry packed
Sample loading	: The sample was triturated with silica gel, dried and loaded 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. 7734), particle size 0.063-0.200 ηm (70-230 mesh ASTM) (E. Merck)
Packing method	: The adsorbent was slurred in the eluant and poured into a column and then allowed to settle overnight
Sample loading	: The sample was dissolved in a small volume of the eluant and loaded on top of the column
Detection	: Fractions were examined by TLC technique in the same manner as described in Section 4.1

4.2.3 Gel filtration Chromatography

Adsorbent	: Sephadex LH-20 (Pharmacia Biotech AB)
Packing method	: Sephadex gel was suspended in the eluant and left overnight 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 loaded on top of the column
Detection	: Fractions were examined by TLC technique in the same manner as described in Section 4.1

4.3 Spectroscopy

4.3.1 Ultraviolet (UV) absorption spectroscopy

UV (in MeOH) spectra were obtained from a HITACHI 220A Spectrometer (The Scientific and Technological Research Equipment Center (STREC), Chulalongkorn University).

4.3.2 Infrared (IR) absorption spectroscopy

IR spectra (KBr disc and NaCl cell) were obtained from a Perkin Elmer FT-IR 1760X spectrometer (The Scientific and Technological Research Equipment Center (STREC), Chulalongkorn University).

4.3.3 Mass spectroscopy (MS)

The Electrospray Ionisation-Time of Flight mass spectra (ESI-TOF MS) were recorded on a Micromass LCT mass spectrometer. MeCN:H_2O (50:50) containing 0.02% of formic acid was used as a solvent (The National Center for Genetic Engineering and Biotechnology, BIOTEC)

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

^1H and ^{13}C-NMR spectra were obtained from a Bruker AVANCE DPX-300 FT-NMR spectrometer operating at 300 MHz for protons and 75 MHz for carbons. (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

4.4 Solvents

Throughout this work, all commercial grade organic solvents were redistilled prior to use.

5. Extraction

The YM fermentation broth of PC 4-3 (56.8 L) was filtered through a glass funnel packed Whatman No.1, then the filtrate was partitioned with ethyl acetate and the aqueous layer was discarded. The ethyl acetate layer was collected and concentrated under reduced pressure at temperature not exceeding 50°C to give yield 5.51 g of mother liquor (YMPC 4-3; dark brown oily liquid) and 286.1 mg of orange-yellow precipitate (YMCPC) 4-3 as shown in Scheme 2. All of extracts were examined for antimicrobial activity by agar diffusion method.

6. Isolation and purification of the extracts

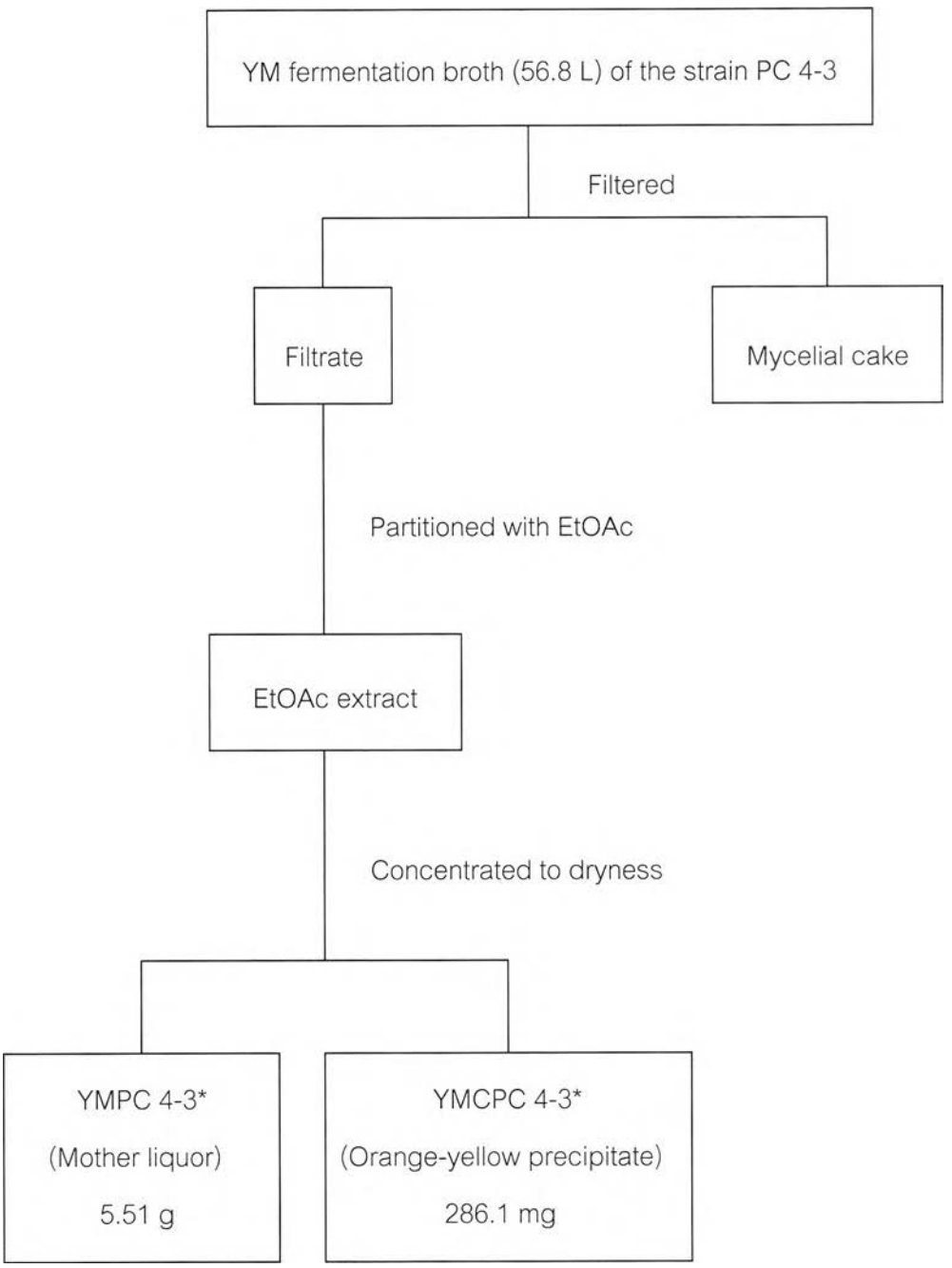
Mother liquor (YMPC 4-3) and precipitate (YMCPC 4-3) showed the yellow and purple spots on the TLC (Si Gel, 10% MeOH in $CHCl_3$, R_f = 0.54 and 0.27, respectively) as the major products. The main yellow and purple spots were isolated by several chromatographic techniques to obtain the pure compounds.

6.1 Isolation of geldanamycin (FPC43001a)

Precipitate (YMCPC 4-3; 286.1 mg) was purified by flash column chromatography using Si Gel as adsorbent (column 1.5 x 15 cm) as shown in Scheme 3. The gradient of MeOH in $CHCl_3$ was used as mobile phase to give 3 fractions (FPC43001-FPC43003) after combination of fractions with similar chromatographic patterns (Si Gel, 5% MeOH in $CHCl_3$) as shown in Table 7. Twenty milliliters of fractions were collected.

Table 7 Fractions obtained from YMCPC 4-3

Fraction code	Fraction	Percentage of MeOH in $CHCl_3$	Volume of mobile phase (ml)	Total weight (mg)
FPC43001	1-5	5	100	175
FPC43002	6-12	8	140	16
	13-15	10	60	
FPC43003	16-25	30	200	62
	26-30	50	100	
	31-35	100	100	



*positive antimicrobial activity

Scheme 2 Extraction of YM fermentation broth of the strain PC 4-3.

Fraction FPC43001 (175 mg) was purified by preparative TLC to give 2 bands, yellow and purple brown on the TLC (Si Gel 0.5% MeOH in $CHCl_3$, R_f = 0.50 and 0.25, respectively). Scratched the yellow band and crystallized by a mixture of $CHCl_3$ and MeOH to yield a yellow amorphous powder of FPC43001a (130 mg) and examined for antimicrobial activity by agar diffusion method.

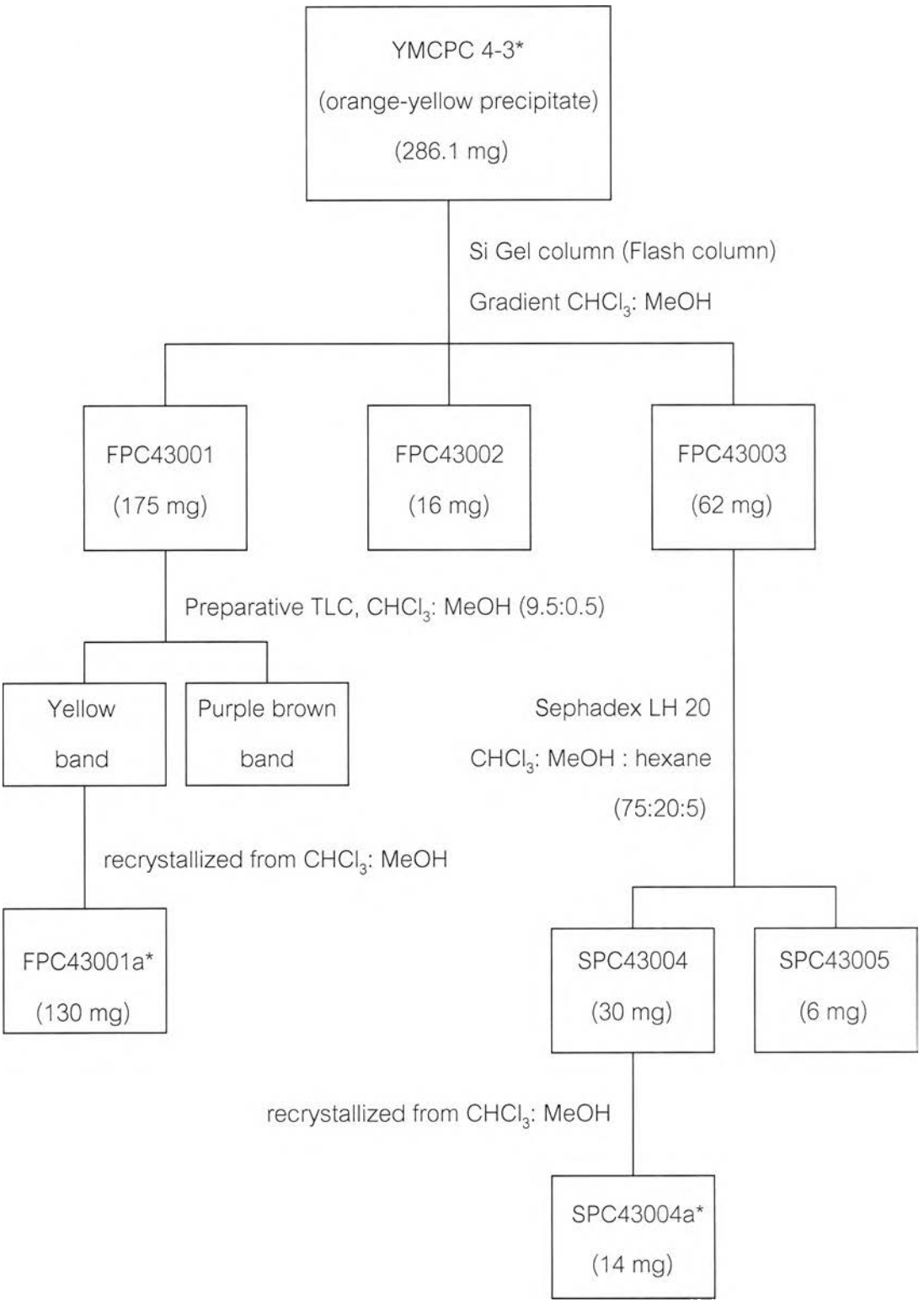
6.2 Isolation of 17-*O*-demethylgeldanamycin (SPC43004a)

Fraction FPC43003 (62 mg) showed a purple spot (R_f= 0.27) as the main spot on the TLC (Si Gel, 10% MeOH in $CHCl_3$). It was separated on a Sephadex LH 20 column (column 1.5 x 60 cm) as shown in Scheme 3. A mixture of $CHCl_3$: MeOH : hexane (75:20:5) was used as eluant. Fractions were collected on the basis of color bands on a Sephadex LH 20 column to yield 2 fractions as shown in Table 8

Table 8 Fractions obtained from FPC43003

Fraction code	Band color	Volume of mobile phase (ml)	Total weight (mg)
SPC43004	Yellowish brown	80	30
SPC43005	Purple brown	60	6

Fraction SPC43004 (30 mg) was crystallized from a mixture of $CHCl_3$: MeOH : hexane (75:20:5) to yield 14 mg of the orange needle crystals (SPC43004a) and examined for antimicrobial activity by agar diffusion method.



*positive antimicrobial activity

Scheme 3 Fractionation of the YMCPC 4-3 from the strain PC 4-3

7. Spectral data of the isolated compounds

7.1 Geldanamycin (FPC43001a)

$[\alpha]^{25}_{D}$: +11.32° (c, 0.100, $CHCl_3$)

UV : λ_{max} nm (ε), in methanol; Figure 6

260 (10360), 310 (12104), 410 (574)

IR : V_{max} cm^{-1}; Figure 7

3441, 1704, 1655

ESI-TOF MS : *m/z* 583.29 $[M+Na]^+$ Figure 8

^{1}H-NMR : δ_H (ppm), 300 MHz, in $CDCl_3$

see Figure 9 and Table 22 (Section 4.1, Chapter IV)

^{13}C-NMR : δ_C (ppm), 75 MHz, in $CDCl_3$

see Figure 10 and Table 22 (Section 4.1, Chapter IV)

7.2 17-*O*-demethylgeldanamycin (SPC43004a)

$[\alpha]^{25}_{D}$: +5.34° (c, 0.100, MeOH)

UV : λ_{max} nm (ε), in methanol; Figure 12

235 (20219), 315 (23615)

IR : V_{max} cm^{-1}; Figure 13

3441, 1697, 1651

ESI-TOF MS : *m/z* 569.29 $[M+Na]^+$ Figure 14

^{1}H-NMR : δ_H (ppm), 300 MHz, in $CDCl_3$+ DMSO-d_6

see Figure 15 and Table 23 (Section 4.2, Chapter IV)

^{13}C-NMR : δ_C (ppm), 75 MHz, in $CDCl_3$+ DMSO-d_6

see Figure 16 and Table 23 (Section 4.2, Chapter IV)