CHAPTER III

EXPERIMENTAL

1. Microbial strains

A total of 12 actinomycete strains, which produced non-motile spores were isolated from soil samples collected from many provinces in Thailand (Table 5). The stock cultures were kept in cold room at 4 ^{o}C at the department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University. The isolates were cultivated on yeast malt extract agar (YMA) for working and stock culture. The type strains of all validly described species of *Micromonospora* except for *M. gallica* were obtained from Japan Collection of Microorganisms (JCM) as shown in Table 6. A yellow mutant of the fungus *M. kaoliang* KB20M10.2 was isolated and identified after Iizuka and Lin (1981) by Professor Dr. Busaba Yongsmith., and deposited (Registration No. KB20M10.2) at the Department of Microbiology, Faculty of Science, Kasetsart University.

Table 5. Sources, Locations, and isolate numbers of strains from soils.

Sources	Locations	Isolate no.
Marine soil	Kao Pee-Pee, Krabi	MA-1, MA-2
Marine soil	Hua-Hin, Prajuabkirikhun	JSM1-1, JSM1-3
Soil	Thepsathit, Chaiyaphume	MC5-1
Soil		MC7-1
Soil	psathit, Chaiyaphume	R1-1
Soil	Khao Kanjun, Ratchaburi	PNK1-3, PNK1-5
Soil	Tatien, Pattaloong	KN-6
Soil	Ratchaburi	TT2-9
Soil	Prutatien, Pattaloong	FLM-2
	Phalamee, Pisanuloak	

Table 6. Sources of the validly described *Micromonospora* species

Strain	Source	Reference
M. chersina JCM 9459^T	Soil, Gujarat State, India	Tomita *et al.*, 1992
M.coerulea JCM 3175^T	Soil, Mt. Heleakala, USA	Luedemann, 1971
M.purpurechromogenes JCM 3156^T	Adobe soil, California, USA	Luedemann, 1971
M. echinospora JCM 3073^T	Soil, Jamesville, New York, USA	Luedemann and Brodsky, 1964
M. carbonacea JCM 3139^T	Soil	Luedemann and Brodsky,1965
M. chalcea JCM 3031^T	Soil	Skerman *et al.*, 1980
M. inositola JCM6239^T	Forest soil, Hokkaido Pref, Japan	Kawamoto *et al.*, 1974
M. olivasterospora JCM 7348^T	Soil, Hiroshima Pref, Japan	Kawamoto *et al.*, 1983
M. nigra JCM 8973^T	Mud, New York, USA	Weinstein *et al.*, 1968
M. halophytica JCM 3125^T	Mud, New York, USA	Weinstein *et al.*, 1968
M. aurantiaca JCM 10878^T	Soil, USSR	Kudrina, 1969
M. rosaria JCM3159^T	Soil, San Jacinto, Texus, USA	Wagman *et al.*, 1972
M. matsumotoense JCM 9104^T	Soil, Matsumoto, Nagano Pref., Japan	Asano *et al.*, 1989
M. pallida JCM3133^T	Soil, Jamesville, New York, USA	Luedemann and Brodsky, 1964
M. aurantinigra JSM 12357^T	Peat, Thatien, Pattaloong, Thailand	Thawai *et al.*, 2004
M. eburnea JSM 12345^T	Soil, Laan Khway, Yala, Thailand	Thawai *et al.*, 2005
M. endolithica JSM 12677^T	Soil, endolithic, anthractic	Hirsch *et al.*, 2004

2. Preliminary antimicrobial activity screening of isolates for antibiotic production

The antimicrobial activity of the isolated fractions and pure compounds was examined by agar disc diffusion method (Lorian, 1980) against *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Micrococcus luteus* ATCC 9341, and *Candida albicans* ATCC 10231. All tested microorganisms were cultivated on Mueller-Hinton agar (MHA, Difco®) slant at 37 ^{o}C for 24 h, but the yeast strain was cultivated on Sabouraud's dextrose agar (SDA, Difco®) slant at 37 ^{o}C for 24 h. The cell cultures were washed from an agar surface and suspended with sterile normal saline solution (NSS), and standardized to match a 0.5 turbidity standard of MaFarland No. 1, providing approximately 1 x 10^{8} CFU/ml. Each of molten (25 ml) MHA and SDA was poured into 9 cm diameter Petri dish and allowed to solidify to form base layer. Each tested microorganisms was swabbed on the surface of MHA and SDA plates. All tested samples (1 mg/disc) were dissolved in methanol and then applied on a sterile paper disc (diameter 6 mm), using 20 μl methanol as a control solvent disc. These paper discs were left in a sterile Petri dish until the solvent was completely dried. The dried paper discs were place on the surface of the swabbed plates and incubated at 37 ^{o}C and 30 ^{o}C for bacterial strains and yeast strains, respectively, for 24 h. The diameters of inhibition zones were subsequently measured.

3. Identification methods

Morphological, cultural, physiological, and biochemical properties of microorganisms were determined by the methods as described in the International *Streptomyces* Project (ISP) (Shirling and Gottlieb, 1996; Arai, 1975).

3.1 Morphological and cultural characteristics

Morphological characteristics were determined using a simple inclined coverslip technique (Williams and Cross, 1971). This technique was used to determine the number of spores at the end of mature hyphae, form of the spore chain and spore bearing hyphae and other special morphological observations. For the scanning electron microscopic examination, the selected strains of actinomycete were grown on YMA plate (ISP medium no. 2). by crosshatch streak method (Shirling and Gottlieb, 1966) and incubated at 30 ^{o}C for 10-14 days. The spores and mycelia were observed with electron microscopy (Itoh *et al.*, 1989).

Cultural characteristics were studied on the colors of mature substrate mycelium, spore, and diffusible pigment using crosshatch streak (Shirling and Gottlieb, 1966). The selected strains were cultivated on eight different agar media (Appendix I), yeast extract-malt extract agar (ISP

medium no 2.), oatmeal agar (ISP medium no.3), inorganic salt-starch agar (ISP medium no.4), glycerol asparagines agar (ISP medium no. 5), peptone-yeast extract ion agar (ISP medium no. 6), tyrosine agar (ISP medium no. 7), glucose asparagines agar, and nutrient agar, and all were incubated at 30 °C for 7-14 days. The color of the reverse (under) side of the mass growth of substrate mycelium on eight media, spore color, and the cultural characteristics were observed.

3.2 Physiological and Biochemical characteristics

3.2.1 Carbon utilization

Basal agar medium, (Shirling and Gottlieb, 1966), (Appendix I) supplement with 0.3% Casamino acid was prepared and a carbon source was added to give concentration of approximately 1%. After autoclave at 110 °C for 10 min, the mixture was agitated, and 25 ml of this mixture was poured into 9 cm petridish.

Carbon source and controls required for the test was repeated belowed :

No carbon source	(negative control)
D-glucose	(positive control)
D-mannitol	D-ribose
Lactose	D-fructose
L-rhamnose	D-melibiose
L-arabinose	D-galactose
Raffinose	Cellubiose
Inositol	Salicin

The uninoculated plates were dried by leaving them at room temperature. A loopful of washed culture was inoculated on the agar surface by streaking straight across the dish. Plates were inoculated in duplicate and incubated at 28-30 °C for 10-14 days. Examination by comparing growth or a given carbon source with two controls, growth on basal medium alone, and growth on basal medium plus glucose was performed.

Results were recorded as follow :

1. Strongly positive utilization (++), when growth on tested carbon in basal medium is equal to or greater than growth on basal medium plus glucose.

2. Positive utilization (+), when growth on tested carbon is significantly better than on basal medium without carbon, but somewhere less than on basal medium plus glucose.

3. Utilization doubtful (+/-), when growth on tested carbon is only slightly better than on basal medium without carbon and significantly less than on basal medium plus glucose.

4. Utilization negative (-), when growth is similar to or less than growth on basal medium without carbon (Utilization is always recorded as negative if growth is not better than no carbon control).

3.2.2 Strach hydrolysis

All selected strains were streaked on the surface of inorganic salts-starch agar plate (ISP-4) (Shirling and Gottlieb, 1966, Appendix I) and incubated at 28-30 ^{o}C for 10 days. After inocubation was complete, Gram's iodine solution was poured on the surface of the agar plate. If starch hydrolysis was present, a dark blue color did not appear.

3.2.3 Gelatin liquefaction

All selected strains were inoculated into test tube of Bouillon gelatin broth (Arai, 1975, Appendix I) and incubated at 28-30 ^{o}C for 21 days. The inoculated tube was compared with uninoculate control when placed both tubes at 20 ^{o}C for 30 min. If the gelatin was hydrolyzed, it became liquid, not solidify.

3.2.4 Nitrate reduction

All selected strains were inoculated into Peptone KNO_3 broth (Arai, 1975, Appendix I) and incubated at 28-30 ^{o}C for 4-6 days. On forth day, 1 ml of the culture was transferred into a test tube and two drops of sulfanilic acid reagent, and then by three drops of *N,N*-dimethyl-1-naphthylamine solution were added. If nitrites were present, the mixture would become pink to red.

3.2.5 Milk coagulation and milk peptonization

All selected strains were inoculated in tube of sterile 10% skim milk (Appendix I) in distilled water and incubated at 28-30 ^{o}C for 7-14 days. If milk was peptonized, milk would be converted to clear solution. If milk was coagulated, milk would precipitate.

3.2.6 NaCl tolerance

All selected strains were streaked on YMA plates (ISP medium no 2.) to which NaCl concentrations were added to give concentrations of 0, 1.5, 3, 4, 5, 6, and 7%. The plates were incubated at 28-30 ^{o}C for 7-14 days. Observed maximum concentrations for which the culture growths were recorded.

3.2.7 Temperature tolerance

All selected strains were streaked on YMA plates (ISP medium no. 2.). The plates were incubated at 10, 15, 30, 40, 45, and 50 ^{o}C, for 7-14 days. Observed maximum temperatures for which the culture growths were recorded.

3.2.8 pH tolerance

All selected strains were streaked on YMA plates (ISP medium no. 2) that separately adjust pH at 4, 4.5, 5, 6, 7, 8, and 9. The plates were incubated at 30 ^{o}C for 7-14 days. Observed minimum pH for which the culture growths were recorded.

3.3 Chemotaxonomic characteristics

3.3.1 Cell wall acyl type

Dried cells (10 mg) were hydrolyzed with 100 μl of 6N HCl at 100 ^{o}C for 2 h. The hydrolyzed solution was then loaded into Dowex (CH_3COO^- form) column (5 cm in height). The column was eluted with 400 μl distilled water and twice of 1 ml distilled water and 0.5 N HCl. The final fraction was added with DON reagent (Appendix II) and 2 N H_2SO_4 and measured O.D. at 530 nm. The sample that contained N-glycolylmuramic acid in peptidoglycan, the value of $O.D._{530}$ is higher than 10 nM.

3.3.2 Whole-cell sugar analysis

Dried cells (50 mg) were hydrolyzed with 1 N H_2SO_4 at 100 ^{o}C for 2 h. The pH of hydrolyzed solution was adjusted with $Ba(OH)_2$ into pH 5.2-5.5. The solution was then centrifuged, and the supernatant was evaporated and 400 μl of distilled water was added into the dried sample. The sample was deionized with Dowex 1 (OH^- form) and Dowex 50 (H^+ form) and filtered. Finally, the deionized sample was analyzed by HPLC.

3.3.3 Diaminopimelic acid analysis

Dried cells (10 mg) were hydrolyzed with 6N HCl at 100 ^{o}C for 18 h. The hydrolyzed solution was filtrated and evaporated. The 400 μl of distilled water was added into the dried sample. The solution was loaded into cellulose HPTLC plate no. 5787 and developed with MeOH : H_2O : 6N HCl : pyridine (80:26:4:10). Finally, the cellulose HPTLC plate was sprayed with 0.5% ninhydrin in n-butanol for detection.

3.3.4 Amino acid composition of peptidoglycan

Wet cells (2 g) were suspended with phosphate buffer (pH 7.2) and sonicated for 45 min. The unbroken cells were removed by centrifugation at 4,000 rpm for 10 min. The broken cells were collected by centrifugation of the supernatant at 10,000 rpm for 1 h and treated with pronase

E solution for 2 h at 37 °C. The samples were then washed with phosphate buffer (pH 7.6) twice and treated with 5% TCA solution at 100 °C for 20 min. Finally, the samples were collected and washed with distilled water for three times, with ethanol and diethyl ether, and dried in the vacuum tray.

Cell walls (1 mg) were hydrolyzed with 6N HCl at 100 °C for 18 h. The samples were filtered and dried with a rotary evaporator. Finally, 100 μL of distilled water was added into the sample. For normal amino acid analysis, 10 μL of hydrolysate was added with 240 μL of 0.02 N HCl, and detected with amino acid analyzer.

3.3.5 Cellular fatty acid analysis

Saponification Dried cells (40 mg) were put into screw-cap tube and added with 1 ml of reagent 1 (Appendix II), and this suspension was shaken well. The suspension was then heated at 100 °C for 30 min and cooled to room temperature in water.

Methylation The reagent 2 (Appendix II) was added into the suspension and mixed for 5 to 10 sec with vortex mixer. The suspension was heated at 80 °C for 10 min and cooled to room temperature in water.

Extraction The suspension was added with reagent 3 (Appendix II) and mixed for 10 min and then transferred the upper layer to another tube.

Base wash The reagent 4 (Appendix II) was added into the suspension and mixed for 5 min, if it became to emulsion form, added the reagent 5 (Appendix II) into the suspension. The upper layer was transferred to vial for GC.

3.3.6 Polar lipid analysis

Extraction Dried cells (150-300 mg) were added with 3 ml of MeOH : 0.3% NaCl aq. (100:10) and 3 ml of petroleum ether and mixed them for 15 min. The lower layer was added with 1 ml of petroleum ether and mixed them for 2-5 min. The lower layer was heated at 100 °C for 5 min and cooled immediately at 37 °C for 5 min. The mixture of $CHCl_3$: MeOH : water (90 : 100 : 30) was added into the suspension and mixed for 1 h. The upper layer was transferred into another tube. The lower layer was extracted again with $CHCl_3$: MeOH : water (50 : 100 : 40) and the supernatant was transferred to the upper layer tube. The upper layer tube was added with 1.3 ml of chloroform and water. The final lower layer was dried with N_2 gas (< 37 °C).

Analysis of polar lipid The polar lipid fraction was dissolved with 60 µl of chloroform : MeOH (2 : 1) and applied to two-dimentional silica HPTLC no. 1.05633 developed with the 1st solvent system chloroform : MeOH : Water (65 : 25 : 4) and the 2nd solvent system chloroform : acetic acid : MeOH : water (40 : 7.5 : 6 : 2).

Detection Dittmer and Lester reagent (Appendix II) was used for the detection of all phospholipids (Blue spot). Ninhydrin reagent (Appendix II) was used for detection of phosphatidylethanolamine (PE) and its derivatives (lyso-PE, OH-PE and methyl-PE). Heat at 110 °C for 10 min after spraying. Anisaldehyde reagent (Appendix II) was used for detection of glycolipids (green-yellow spot) and other lipid (blue spot). Heat at 110 °C for 10 min after spraying. Dragendroff reagent (Appendix II) was used for detection of choline-containing phospholipids (phosphatidylcholine).

3.3.7 Mycolic acid analysis

Dried cells (50-200 mg) were hydrolyzed with 10% KOH-MeOH at 100 °C for 2 h. The hydrolyzed suspension was added with 6 N HCl and extracted with n-hexane (or petroleum ether) twice. The upper layer was dried with N_2 gas. The dried sample was then added with benzene : MeOH : H_2SO_4 (10 : 20 : 1) and heated at 100 °C for 2 h (methylation step). After cooling, the suspension was added with water and n-hexane for the extraction. The upper layer was transferred to another tube and extracted again with n-hexane and the applied to Silica gel TLC. The TLC plate was developed by n-hexane : diethylether (4 : 1) and detected the spot with I_2 vapor or H_2SO_4 reagent (heat at 110 °C for 10 min after spraying).

3.3.8 Menaquinone analysis

Dried cells (100-500 mg) were extracted with chloroform : MeOH (2 : 1) overnight. The suspension was then filtered and dried under rotary evaporator. The dried sample was dissolved with a small amount of acetone and applied onto a silica gel TLC (Merck no. 1.05744). The applied TLC was then developed by 100% benzene and the band of menaquinone was detected by using a UV lamp (254 nm). The menaquinone band was scraped and dissolved with acetone (HPLC grade). The suspension was filtered and dried it up with N_2 gas. The menaquinone sample was analyzed by HPLC.

3.3.9 Analysis of DNA base composition

Chromosomal DNA was isolated from cells grown in yeast extract-malt extract broth for 4-5 days according to the method of Tamaoka (1994) with minor modification. Cells were

harvested and suspended in 10 ml of saline-EDTA buffer pH 8.0 (Appendix II). The cell suspension was inoculated with 20 mg of lysozyme at 37 ^{o}C for 30 min followed by incubation period of 10 min at 50 ^{o}C with 1.0 ml of 10% SDS. The phenol extraction was then carried out by adding an equal volume of phenol : chloroform (1 : 1) (Appendix II) to the sample for removal of protein and other debris. The upper layer of the mixture was collected after centrifugation at 10,000 rpm for 20 min. Chromosomal DNA was precipitated with two volumes of ice cold absolute ethanol. DNA was dissolved with 0.1xSSC (Appendix II) and treated with RNase A, RNase T_1 and proteinase K solution (Appendix II) at 37 ^{o}C for 1 h for removal of RNA and protein, respectively. Chromosomal DNA was stored in 0.1xSSC at 4 ^{o}C.

The 10 µL of heated DNA (1 mg/ml) was hydrolyzed with 10 µl nuclease P_1 at 50 ^{o}C for 1 h and followed by incubation period of 1 h at 37 ^{o}C with 10 µl of alkaline phosphatase. The hydrolyzed DNA was determined using HPLC method of Tamaoka & Komagata (1984). An equimolar mixture of nucleotides (Yamasa Shoyu, Choshi, Japan) was used as the quantitative standard for analysis of DNA base composition.

3.4 DNA-DNA hybridization

Chromosomal DNA for DNA-DNA hybridization was purified by repeated phenol extraction to remove RNA and others. DNA was precipitated and dissolved in 0.1xSSC. Crude DNA was treated with RNase A until completely cleaned RNA, and phenol : chloroform extraction was repeated twice for cleaning protien. DNA was then precipitated by adding 2 volumes of cold absolute ethanol and was carefully washed with 70% and 95% ethanol, respectively and dried. DNA was dissolved with 0.1xSSC and stored at 4 ^{o}C.

The spectrophotometric method for DNA quantitation was used to determine both concentration and relative purity of DNA in a solution. Two absorption spectra (A_{260} and A_{280}) were used. The DNA is suitable for DNA-DNA hybridization if the ratio of A_{260}/A_{280} is between 0.56-0.59.

3.4.1 DNA labeling probe with photobiotin

A 10 µl of DNA solution (1 mg/ml) and 15 µl of photobiotin solution (1 mg/ml) were mixed in an Eppendorf tube and then irradiated with sunlamp for 30 min on ice. After irradiation, free photobiotin was removed by adding 100 µl of 0.1 M Tris-HCl buffer pH 9.0, and 100 µl of n-butanol into biotinylated DNA solution. The solution was then mixed and centrifuged at 12,000 rpm for 20 sec. The upper layer was removed. Again, 100 µl of n-butanol was added; and the

solution mixed, centrifuged and the upper layer was removed. After centrifugation at 12,000 rpm for 20 sec, the upper layer was removed. The biotinylated DNA solution was boiled for 15 min and immediately cooled in ice. The solution was sonicated for 3 min and dissolved in 10 ml of hybridization solution (Appendix II).

3.4.2 Photobiotin labeling DNA-DNA hybridization

The photobiotin labeling DNA-DNA hybridization was performed by the method described by Ezaki, Hashimoto, and Yabuuchi (1989). DNA (10μg) of an unknown strain, type strain and reference DNA (calf thymus) were boiled for 10 min and immediately cooled in ice. Then, 500 μl of 2xPBS (Appendix II), 100 μl of 0.1 $MgCl_2$ and sterile distilled water were added to make a total volume of 1 ml and mixed well. 100 μl of a heat denatured DNA solution was added to microdilution wells (Nunc-Immuno™ plate : MaxiSorp™ surface, Nunc GmbH & Co. KG) and fixed by incubation at 37 °C for 2 h. After incubation, the DNA solution was removed, and 200 μl of a prehybridization (Appendix II) was added to microdilution wells. Microdilution plate was incubated at 53-55 °C (hybridization temperature) for 1-2 h. The prehybridization solution was removed from the wells and replaced with 100 μl of a hybridization mixture containing biotinylated DNA. Microdilution plate was incubated at 53-55 °C (hybridization temperature) for 15-18 h.

3.4.3 Detection of biotin-containing hybrids

After hybridization the microdilution wells were washed three times with 200 μl of 0.2xSSC buffer. Then 100 μl of Streptavidin-beta-D-galactosidase solution (Appendix II) was added to microdilution wells and incubated at 30 °C for 10 min. Solution I was removed from the wells and replaced with 100 μl of solution II (Appendix II). Microdilution plate was incubated at 37 °C for 30 min. After incubation, microdilution plate was washed three times with 200 μl of PBS. 100 μl of solution III (Appendix II) was added, and the plate was incubated at 37 °C for 10 min. The fluorescence intensity was measures with a MicroFlour reader (Dynatech Laboratories, Inc., Alexandria, Va.) at a wavelength of 360 nm for excitation and 450 nm for emission.

3.5 16S rDNA analysis

3.5.1 16S rDNA amplification by PCR

The PCR was performed in a total volume of 50 μl containing 1 μl of DNA sample, 0.25 μl of Taq DNA polymerase, 5 μl of 10 x polymerase buffer, 4 μl of dNTP mixture, 2.5 μl of 10 μM forward and reverse primers (Appendix III) and 34.75 μl of Millique water. A DNA Thermal

Cycler (Gene Amp® PCR System 2400; Perkin Elmer) was used with a temperature profile of 3 min at 95 ^{o}C followed by 30 cycles of 30 sec at 95 ^{o}C (denaturing of DNA), 15 sec at 55 ^{o}C (primer annealing), and 1 min at 72 ^{o}C (polymerization) and a final extension for 5 min at 72 ^{o}C. The PCR amplified products were analyzed by running 5 µl of the reaction mixture on a 1% agarose gel in Tris-acetate EDTA buffer (Appendix II). Agarose gel was stained in an ethidium bromide solution (0.5 mg/ml) and examined under UV-transilluminator (UVP Inc.) to visualize the amplified 16S rDNA band.

3.5.2 16S rDNA sequencing

The amplified 16S rDNA was used as templates for sequencing with big dye terminator sequencing Kit (Perkin Elmer) and analyzed by AB1377 automated DNA sequencer (Perkin Elmer). The sequencing reaction for each sample was performed in a DNA Thermal Cycler (Gene amp® PCR System 2400; Perkin Elmer) with a temperature profile of 30 sec at 96 ^{o}C followed by 25 cycles of 10 sec at 96 ^{o}C (denaturing of DNA), 5 sec at 50 ^{o}C (primer annealing), and 4 min at 60 ^{o}C (polymerization). Sequencing for each sample is carried out in both forward and reverse directions (Appendix III).

3.5.3 16S rDNA sequence analysis and phylogenetic tree construction

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/ against previously reported sequence at the GenBank/EMBL/DDBJ database. The sequence was multiply aligned with selected sequences obtained from the three main databases by using the CLUSTAL W version 1.81. The alignment was manually verified and adjusted prior to the construction of a phylogenetic tree. The phylogenetic tree was constructed by using the neighbor-joining (Saitou and Nei, 1987) and maximum parsimony methods (Kluge and Farris, 1969) in the MEGA program version 2.1 (Kumar *et al.*, 2001). The confidence values of branches of the phylogenetic tree were determined using the bootstrap analyses (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 X program (Thompson *et al.*, 1997). Gaps and ambiguous nucleotides were eliminated from the calculations.

4. Fermentation of the selected *Streptomyces* strain for antibiotic production

A loopful of PNK1-3 was inoculated into 50 ml of seed medium consisting of glucose (0.4%), yeast extract (0.4%), malt extract (1.0%), pH 7.3 (in a 250 ml Erlenmeyer flask). The flask was incubated on a rotary shaker at 200 rpm at room temperature for 4 days.

The vegetative seed (2 ml) was transferred into a 500 ml of Erlenmeyer flask containing 200 ml of production medium which comprised glucose (0.4%), yeast extract (0.4%), malt extract (1.0%), and $CaCO_3$ (0.1%), pH 7.3. The flask was incubated on a rotary shaker at 200 rpm at room temperature for 7 days.

5. Chromatographic techniques

5.1 Analytical thin-layer chromatography

Every fractions of Sephadex LH-20 purified PNK1-3 was spotted on analytical thin-layer chromatography by one dimension ascending technique.

Absorbent : Silica gel GF_{254} coated on an aluminium sheet (E. merck)

Layer thickness : 250 µm

Distance : 5 cm

Temperature : Laboratory temperature (30-35 ^{o}C)

Detection :
1. Visual detection under daylight
2. Visual detection under ultraviolet light at wavelengths of 254 and 365 nm.
3. Spraying with anisaldehyde reagent and heated until colors developed
4. Visual detection in an iodine vapor

5.2 Column chromatography

5.2.1 Flash column chromatography

The crude sample of EtOAc extract PNK1-3 further investigated using Flash column chromatography.

Adsorbent : Silica gel 60 (No. 7734), particle size 0.063-0.200 nm (70-230 mesh ASTM) (E. Merck)

Packing method : The adsorbent was suspended in an eluent. The adsorbent slurry was poured into a column and allowed to settle overnight.

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

Detection : Fractions were examined by TLC technique in the same manner as described in the Section 5.1.

5.2.2 Gel filtration chromatography

The crude sample of EtOAc extract PNK1-3 and CH_2Cl_2 extract red yeast rice further investigated using Flash column chromatography

Adsorbent : Sephadex LH-20 (Amersham Biosciences)

Packing method : Sephadex LH-20 gel was suspended in an eluant and left standing overnight to swell prior to use, then poured into the column and allowed to settle.

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

Detection : Fractions were examined by TLC technique in the same manner as described in the Section 5.1.

6. Spectroscopy

6.1 Ultraviolet (UV) absorption spectroscopy

The pure samples were measured for maximum absorbancy by UV spectrophotometer. The UV spectra (in methanol and chloroform) were recorded on a Cary 1E UV-visible spectrophotometer UVIDEC-650 (The National Center for Genetic Engineering and Biotechnology (BIOTEC).

6.2 Infrared (IR) absorption spectroscopy

The pure samples were measured for transmitant by IR spectrophotometer. IR spectra (KBr disc and neat film) were recorded on a Perkin Elmer FT-IR 1760X spectrometer (Scientific and Technological Research Equipment Center, Chulalongkorn University).

6.3 Mass spectrometry

The pure samples were measured the mass by mass spectrometer. Electrospray ionization-time of flight mass spectra (ESI-TOF MS) were recorded on a Micromass LCT mass spectrometer (The National Center for Genetic Engineering and Biotechnology (BIOTEC). The mixture of MeCN : H_2O (50 : 50) containing 0.02% of formic acid was used as a solvent.

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

^{1}H and ^{13}C NMR, DEPT 90 and 135, HMQC, HMBC, NOESY, and ^{1}H -^{1}H COSY spectra were obtained from a Bruker DRX-400 NMR spectrometer, operated at 400 MHz for protons and 100 MHz for carbons (The National Center for Genetic Engineering and Biotechnology (BIOTEC).

6.5 Optical rotations

The pure samples in methanol and chloroform were measured by turn plane polarized light by optical rotations with Sodium D line (589 nm) on a Perkin Elmer Polarimeter 341 (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

7. Solvents

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

8. Bioassay

Bioassays were conducted at the BIOTEC by the Bioassay Research Facility, which carried out antimalarial activity, cytotoxicity and antitubercular activity. Brief methods were provided below.

8.1 Antimalarial activity

The parasite *P. falciparum* (K1, multidrug resistant strain) was cultured continuously according to the method of Trager and Jensen (1976). 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). Briefly, a mixture of 200 μL of 1.5% of erythrocytes with 1% parasitemia at the early ring stage was pre-exposed to 25 μL of the medium containing a test sample dissolved in DMSO (0.1% final concentration) for 24 h employing the incubation conditions described above. Subsequently, 25 μL of [^{3}H] hypoxanthine (Amersham, USA) in culture medium (10 μCi) was added to each well, and plates were incubated for an additional 24 h. Levels of incorporated radioactively labeled hypoxanthine indicating parasite growth were determined using the Top Count microplate scintillation counter (Packard, USA). An IC_{50} value of 1.2 $\pm$ 0.02 μg/ml (n = 3) was observed for the standard compound, dihydroartemisinin.

8.2 Cytotoxicity

The cytotoxic assay employed the colorimetric method reported by Skehan *et al* (1990). KB (human epidermoid carcinoma of cavity, ATCC CCL-17) and BC (breast cancer cell line) cells were determined by colorimetric cytotoxicity assay that measured cell growth from cellular protein content according to Skehan *et al* (1990). Elliptine was used as positive control and DMSO (10%) was used as negative control. Briefly, cells at a logarithimic growth phase were harvested and diluted to 10^5 cells/ml with fresh medium and gently mixed. Testing compound was dissolved in DMSO (concentration at 20 mg/ml), and this solution was then diluted with distilled water to obtain a stock solution at 0.4 mg/ml (with 10% DMSO). The stock solution (10 µL) and cell suspension (190 µL) were transferred into microtiter plates (concentration at 20 mg/ml with 0.05% DMSO). If the compound is active at 20 µg/ml, a series of solutions were prepared by twofold dilution of the stock solution (diluted with 10% DMSO solution), and exposed to cells as mentioned above, in order to obtain IC_{50} value. Plates were incubated at 37 °C under 5% CO_2 atmosphere for 72 h. After incubation period, cells were fixed by 50% trichloroacetic acid. The plates were incubated at 4 °C for 30 min, washed with water, and air-dried at room temperature. The plates were stained with 0.05% sulforhodamine B (SRB) dissolved in 1% acetic for 30 min. After staining period, SRB was removed with 1% acetic acid. Plates were air-dried before bound dye was solubilized with 10 mM Tris base for 5 min on shaker. Optical density was read in a microtiter plate reader at wavelength 510 nm. Ellipticine, the reference substance, exhibited activity toward BC and KB cell lines, both with the IC_{50} of 0.3 µg/ml.

8.3 Antitubercular activity

The antitubercular activity was assessed against *M. tuberculosis* H37Ra using the Microplate Alamar Blue Assay (MABA) (Collins and Franzblau, 1997). *M. tuberculosis* H37Ra was grown in 100 ml of 7H9GC (Difco) containing 0.005% Tween 80. Cultures were incubated in a 500-ml plastic flask on a rotary shaker at 200 rpm and 37 °C until they reached an optical density of 0.4–0.5 at 550 nm. Bacteria were washed and suspended in 20 ml of phosphate-buffered saline and passed through an 8 µm pore-size filter to eliminate clumps. The filtrates were aliquoted and stored at -80 °C. Antimicrobial susceptibility testing was performed in 96-well microplates. Outer perimeter wells were filled with sterile water to prevent dehydration. Initial screened-sample dilutions were prepared in either DMSO or distilled deionized water. The

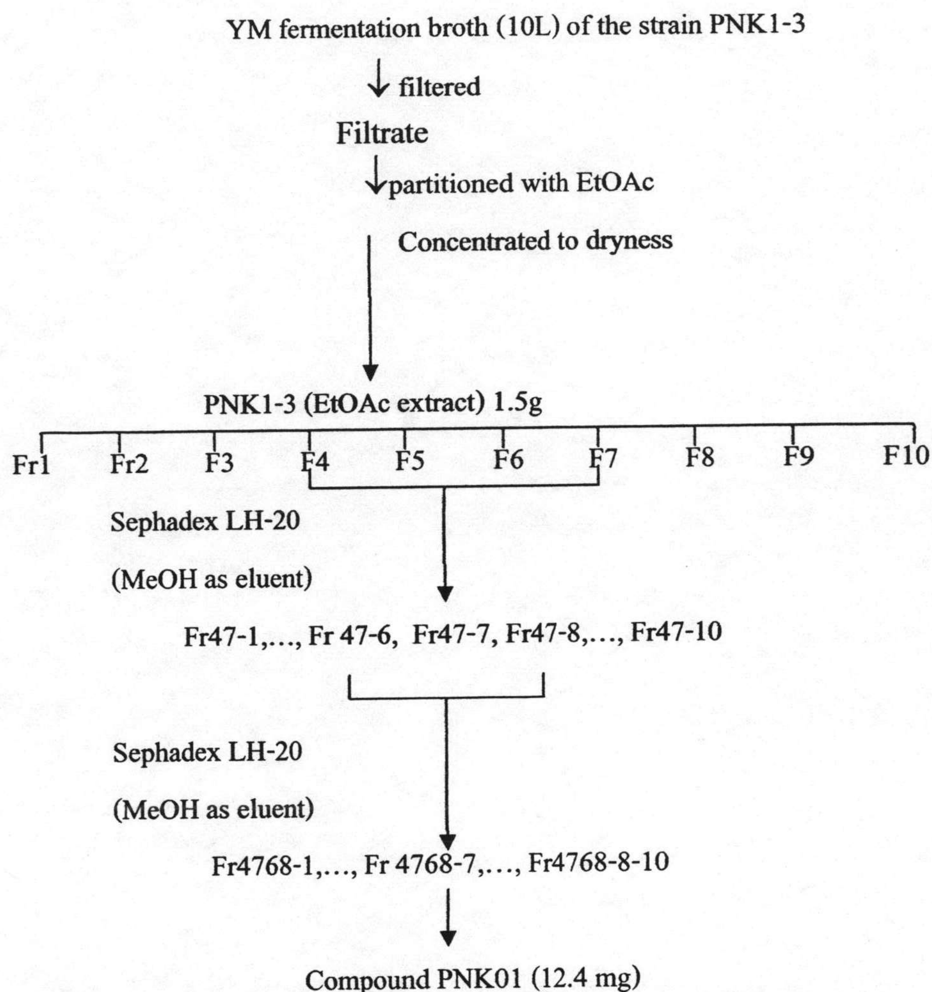
dissolved-screened samples were then diluted by Middlebrook 7H9 media containing 0.2% v/v glycerol and 1.0 g/L casitone (7H9GC), and subsequent twofold dilutions were performed in 0.1 ml of 7H9GC in the microplates. Frozen inocula were diluted 1:100 in 7H9GC. Addition of 0.1 ml of inocula to the well resulted in final bacterial titers of about $5x10^4$ CFU/ml. Wells containing sample only were used to determine whether the test-samples themselves could reduce the dye or not. Additional control wells consisting of only bacteria (B) or only medium (M) were also included. Plates were incubated at 37°C. Starting at day six of incubation, 20 μL of Alamar Blue solution and 12.5 μL of 20% Tween 80 were added to B and M wells, and plates were re-incubated at 37°C. Wells were observed at 24 h for a colour change from blue to pink. If the B wells became pink by 24 h, Alamar Blue solution was added to all testing plates. However, if a colour (blue) of M and B wells did not change, both wells were tested daily until a colour of B wells change from blue to pink. After the change of B well colour, Alamar Blue solution was subsequently added to all remaining wells. Plates were then incubated at 37°C for 24 h, and the results were recorded with a fluorescence multi-well reader (Cyto Fluor, Series 4000) at the excitation and emission wavelengths of 530 and 590 nm, respectively. The standard drugs, isoniazid and kanamycin sulfate, showed respective MIC values of 0.040–0.090 and 2.0– 5.0 μg/ml.

9. Extraction and isolation of PNK01 from PNK1-3

The YM fermentation broth (10L) (Difco) of PNK1-3 was filtered through a Buchner funnel packed with Kieselguhr. The filtrate was partitioned with ethyl acetate (10L x 3). The ethyl acetate layer was collected and concentrated under reduced pressure at 40°C to yield 1.5 g of ethyl acetate extract (dark yellow powder) as shown in Scheme 1.

The ethyl acetate extract of PNK1-3 was examined for antimicrobial activity using the method described in the Section 2. The crude ethyl acetate extract (1.5 g) of PNK1-3 was fractionated by Sephadex LH-20 (3 cm inner diameter and 16 cm long), MeOH as eluent to give ten fractions (Fr1-Fr10). Fractions (25 ml) were collected. Fractions Fr4-Fr7 were pooled and further purified by Sephadex LH-20 column, also using MeOH as eluent. The fractions Fr476-Fr478 fractions were pooled and purified by Sephadex LH-20 column, also using MeOH as eluent to yield ten fractions (Fr47681-Fr476810). Fraction Fr47687 is geldanamycin (PNK01).

PNK01 was obtained as orange-yellow powder (53.0 mg, 3.5% of ethyl acetate of YM broth). It was a major product in fermentation broth.



Scheme 1 Extraction of YM fermentation of the strain PNK1-3

10. Extraction and isolation of Compounds from *M. kaoliang*

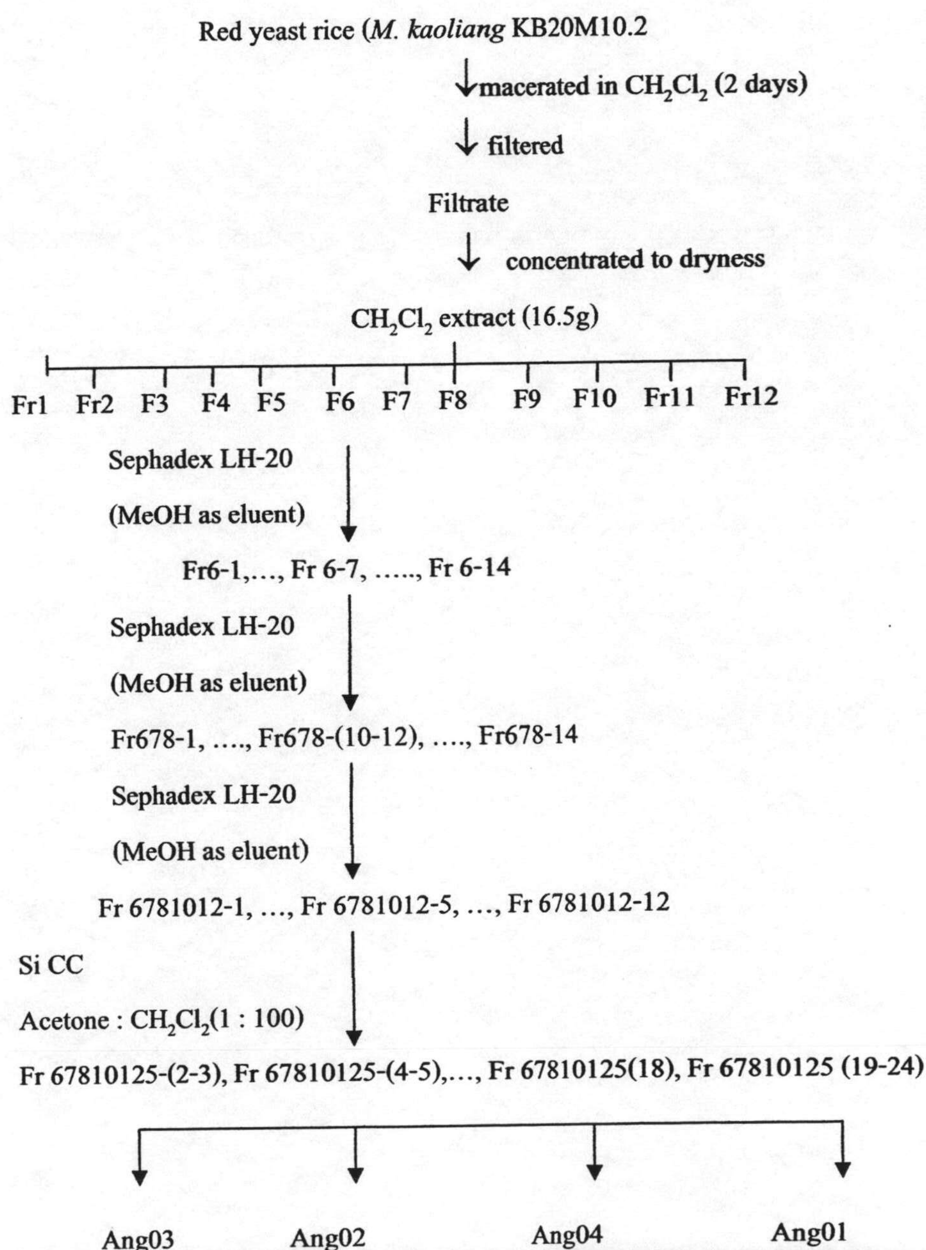
10.1 Extraction of Compounds from *M. kaoliang*

The *M. kaoliang* KB 20M 10.2 grown on rice (1kg) was soaked in CH_2Cl_2 (4 l) for two days. The filtrate of the extract was evaporated under reduced pressure, to give dark browny gummy crude extract (16.5g).

10.2 Isolation of Compounds from CH_2Cl_2 Extract

The CH_2Cl_2 extract was dissolved in MeOH, filtered through filter paper, and then applied on the top of Sephadex LH-20 column. The fractions were collected from gel

filtration chromatography on Sephadex LH-20 using MeOH as eluent. Twelve fractions (50 ml each) were collected. Fraction 6 was repeatedly chromatographed (on Sephadex LH-20), yielding 14 fractions. Fractions 7-8 were combined and further isolated by Sephadex LH-20, (MeOH as eluent), to give fourteen fractions. Fractions 10-12 were pooled and subjected to Sephadex LH-20 also using MeOH as eluent, to give twelve fractions. Fraction 5 was purified by silica gel 60 (No. 7734) as adsorbent, 1% acetone in CH_2Cl_2 as mobile phase to furnish compounds Ang 01 (80 mg), Ang 02 (2.8 mg), Ang 03 (20 mg), and Ang 04 (4.2 mg).



Scheme 2 Extraction of CH_2Cl_2 extract of *M. kaoliang* KB20M10.2 grown on rice