CHAPTER III

EXPERIMENTAL

1. Sample collection and isolation of the endophytic fungus ARE-1

Healthy leafs of *Annona reticulata* L. (Annonaceae) were collected from the botanical garden of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand, in May 2000.

Leaf of *A. reticulata* was cleaned and dried. The surface of leaf was sterilized with 70% ethanol for 1 min, sodium hypochlorite solution (6% available chlorine) for 5 min and then washed 2 times in sterilized water for 1 min (Schulz *et al.*, 1993). Then the middle rib and lamina were cut into small pieces (8x8 mm) and placed on water agar. The water agar plates were incubated at 30°C and examined for fungal germination everyday. Aseptically, hyphal tip was isolated and transferred onto potato dextrose agar (PDA) by cutting and picking with steriled pasture pipette. The PDA plates were incubated for 7 days at 30 °C. Purity of the fungus was determined by colony morphology. The fungus isolates were collected for further study.

2. Classification of the endophytic fungus isolate ARE-1

2.1 Conventional method basing on morphology

2.1.1 Macroscopic morphology

Colony morphology of endophytic fungus isolate ARE-1 was determined by growing the fungus at 30 °C for 7 days on 5 different media (in Appendix C), *i.e.* Czapek yeast autolysate agar (CzYA), malt Czapek agar (MCzA), malt extract agar (MEA), Sabouraud's dextrose agar (SDA) and yeast extract sucrose agar (YES).

2.1.2 Microscopic morphology

Endophytic fungus isolate ARE-1 was grown on PDA and banana leaf agar at 30 °C for 2 months under the condition of 12 h darkness/12 h black light (near UV light). The fungal culture was examined for fruiting body production under a stereomicroscope. Wet mount of the fungal culture with lactophenol cottton blue was examined for conidia production under a light microscope.

2.2 Molecular method basing on ribosomal RNA gene sequence

2.2.1 DNA extraction

Endophytic fungus isolate ARE-1 was grown on patato dextrose broth at 30 °C for 5 days. The mycelium was harvested by centrifugation and washed 3 times with sterile distilled water. The pellet was lyophilized and then ground into fine powder using a mortar and pestle. The ground mycelium was filled upto one third of a 1.5-ml microfuge tube and subjected to DNA extraction according to Lee and Taylor (1990). A 400-µl volume of lysis buffer (in Appendix C) was added and the mixture was mixed with vortex until being homogeneous. The tube was then incubated at 65 °C for 1 h. A 400-µl volume of chloroform : phenol (in Appendix C) was added to the mixture and the tube was inverted several times. The mixture was centrifuged at 10,000xg for 15 min at room temperature. The aqueous (top) phase containing the DNA was transferred to a new tube. Then, 10 µl of 3M sodium acetate was added to the aqueous phase followed by 0.54 volume of cold isopropanol. The tube was inverted gently before DNA precipitate was spun down at room temperature as previously for 2 min. The pellet was washed once with cold 70% ethanol before leaving dry. The DNA pellet was resuspended in 100 µl TE (10mM Tris HCl, 0.1 mM EDTA) buffer.

2.2.2 Polymerase chain reaction (PCR)

ITS1-5.8S-ITS2 regions of ribosomal DNA (rDNA) were amplified by PCR using the forward primer ITS5 and the reverse primer ITS4 as described by White *et al.*, 1990. The primer sequences were shown in Table 2. Oligonucleotide primers were synthesized using ABI PRISM™, DNA/RNA synthesizer model 392, Perkin Elmer, by Bioservice Unit (BSU) at the National Science and Technology Development Agency (NSTDA), Bangkok, Thailand. The reaction mixture was prepared on ice. The amplification reaction was performed in the total volume of 50 µl consisting of 5 ng of template DNA, 0.5 µM of each primer, 50 µM of individual dNTP, 2.5 nM of $MgCl_2$, 50 mM KCl, 10 mM of Tris-HCl at pH 8.3 and 0.5 U of *Taq* DNA polymerase. For each test a negative control was included without template DNA. Ice-cold PCR reaction tubes were transferred to an Eppendrof Mastercycler Gradient PCR machine.

The amplification process consisted of a pre-denaturation step at 95 °C for 3 min and 29 consecutive cycles of 95 °C for 50 sec (denaturation), 45 °C for 40 sec (annealing) and 72 °C for 40 sec (extension), with a final 72 °C for 10 min for extension in the last cycle.

Amplified products were analyzed by electrophoresis in 2 % agarose gel prepared in Tris-acetate-EDTA (TAE) buffer under 100 voltage of electrical power. The agarose gel was then stained in 0.5 µg/ml ethidium bromide solution and destained. The DNA was visualized under UV-transluminator at 312 nm. The molecular size of amplified products was determined by comparison with the standard marker λDNA digested with *Pst*I restriction enzyme.

2.2.3 DNA sequencing and phylogenetic analysis

Purified PCR product was sequenced in an automated sequencer (ABI PRISM™ model 377, Perkin Elmer). Primers ITS2, ITS3, ITS4 and ITS5 (Table 2) were used in the sequencing reactions in order to sequence both DNA strands. This was done by the Bioservice Unit (BSU) at the National Science and Technology Agency (NSTDA), Bangkok, Thailand.

The 5.8S sequence of isolate ARE-1 was used as the query sequence to search for similar sequences from GenBank by using BLASTN2.2.4 (Altschul *et al.*, 1997). Species having 98-100 % identity sequences and having complete data of ITS1-5.8S-ITS2 sequences were used as reference taxa for subsequent phylogenetic analysis. The ITS1-5.8S-ITS2 sequences of isolate ARE-1 were also used as the query sequences. Species having complete sequences with high percentage identity were included in reference taxa. The ITS1-5.8S-ITS2 regions of all selected taxa were aligned with Clustal W (Thomson *et al.*, 1994) and the results were adjusted manually by BioEdit Version 4.7.8 to maximize alignment. The alignment data were subsequently used for maximum-parsimony analysis using the heuristic search with tree bisection-reconnection branch swapping in PAUP 4.0b10 (Swofford, 2002). For each search, 10 replications of random stepwise sequence addition were performed. Statistical support for the internal branches was estimated by bootstrap analysis with 1,000 replications. *Chaenothecopsis pusilla* was used as an outgroup taxon.

Table 2 Primers for amplification and sequencing of complete ITS1, 5.8S and ITS2 sequences of rRNA gene.

rRNA	Gene primer	Product size (bp)	Tm (°C)
ITS5	GGAAGTAAAAGTCGTAACAAGG	315	63
ITS2 *	GCTGCGTTCTTCATCGATGC		62
ITS3 *	GCATCGATGAAGAACGCAGC	330	62
ITS4	TCCTCCGCTTATTGATATGC		58

* primer for DNA sequencing only.

2.3 Preservation of the endophytic fungus ARE-1

Endophytic fungus isolate ARE-1 was kept at the Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University. The cultures grown on yeast extract sucrose (YES) agar slants and corn meal agar slants were kept under 3-times autoclaved liquid paraffin at room temperature. The culture on Sabouraud's dextrose agar (SDA) block was kept in 10 % glycerol and freezed at -70 °C. They were kept in duplicate.

3. Fermentation

Fungal endophyte isolate ARE-1 was cultivated on YES agar at 30 °C for 1 week. The agar culture was then cut into 1x1-cm pieces by a steriled sharp blade. Six pieces of agar culture were inoculated into a 1-L Erlenmeyer flasks containing 200 ml of yeast extract sucrose broth. The flasks were incubated under standstill condition at 30 °C for 21 days.

4. Chromatographic techniques

4.1 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 or 10% H_2SO_4 reagent and heat until color developed

4.2 Column chromatography

4.2.1 Gel filtration chromatography

Gel filter : Sephadex LH-20 (Pharmacia)

Packing method : Sephadex gel was suspended in the eluent 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 amount of eluent, then applied gently on the top of the column.

Detection : Fractions were examined by ^1H NMR (400 MHz) spectroscopy.

4.2.2 High Performance Liquid Chromatography

Adsorbent : Reversed-phase column (LichroCART RP C_{18}, 10 μM, 250 x 10 mm)

Sample loading : The sample was dissolved in a small amount of eluent (MeCN and Water) then injected into the loop of the column.

Flow rate : 4.5 ml/min

Detection : UV-photodiode array detector

5. Spectroscopy

5.1 Ultraviolet (UV) spectroscopy

UV (in MeOH) spectra were obtained from a CARY 1 E UV- Visible spectrophotometer at the National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Science Park, Bangkok, Thailand.

5.2 Infrared (IR) absorption spectroscopy

IR spectra of pure compounds (film technique) were obtained from a Bruker Vector 22 FT-IR spectrophotometer at the National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Science Park, Bangkok, Thailand.

5.3 Mass spectroscopy (MS)

Electronspray ionization time of flight mass spectra (ESI-TOF MS) were obtained on a Micromass LCT mass spectrometer at the National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Science Park, Bangkok, Thailand.

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

^{1}H NMR (500 MHz) and ^{13}C-NMR (125 MHz) DEPT 135, COSY, HMQC, HMBC and NOESY spectra were obtained from a Bruker ADVANCE DPX-500 FT-NMR spectrometer at the National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Science Park, Bangkok, Thailand.

Deuterated solvents; chloroform-*d* ($CDCl_3$), methanol-*d*4 (CD_3OD) and acetone-*d*6 were used in NMR experiments. Reference signals were the signals of residual

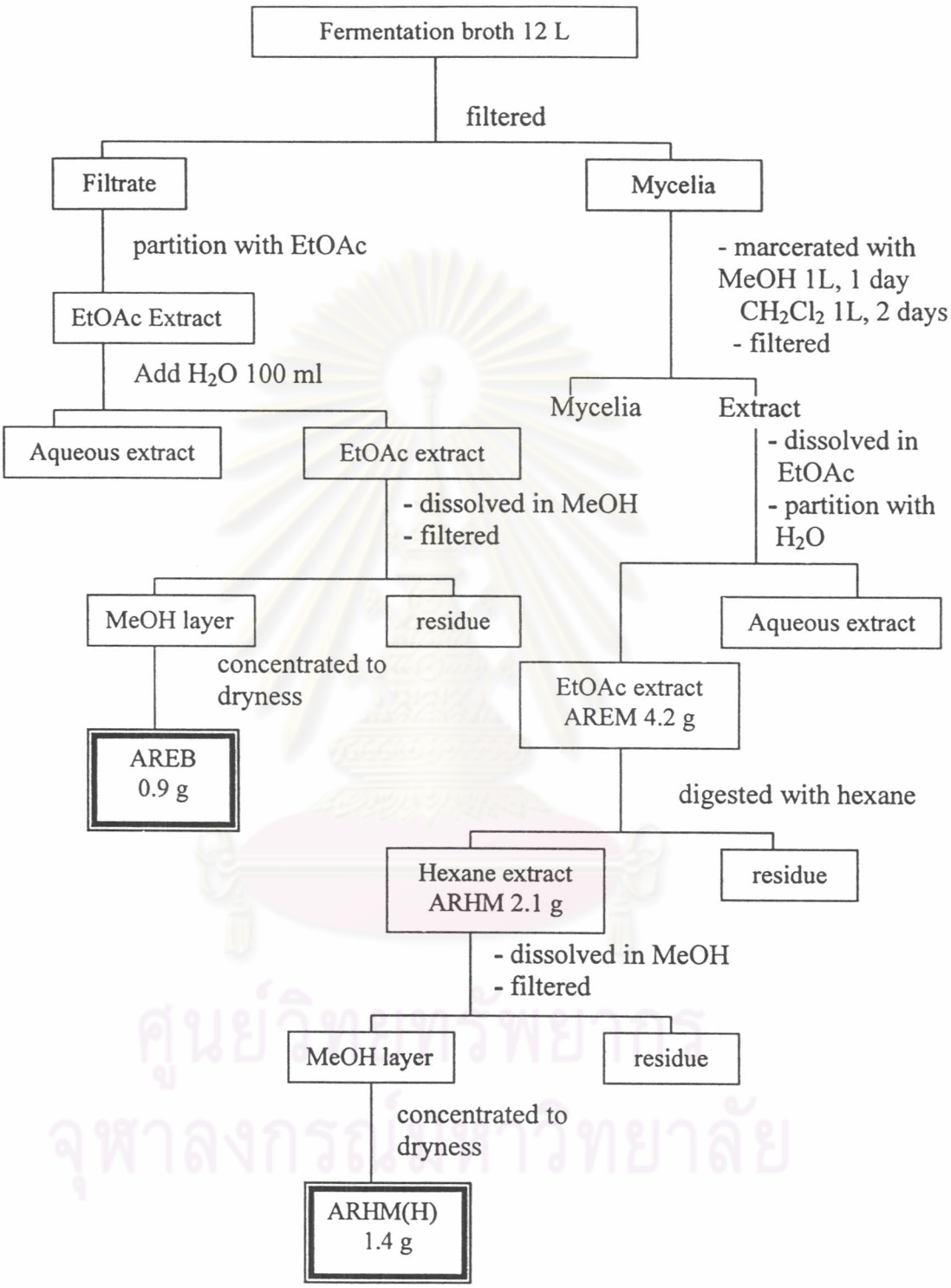
undeuterated solvents at δ 7.24 ppm (^{1}H) and 77.0 ppm *t* (^{13}C) for $CDCl_3$, 3.35 ppm (^{1}H) and 49.0 ppm *sept* (^{13}C) for CD_3OD and 2.05 ppm (^{1}H) and 29.8 ppm *sept* (^{13}C) and 206.0 ppm *s* (^{13}C) for acetone-*d*6.

6. Solvent

Throughout this work, all organic solvents were of analytical grade.

7. Extraction

The fermentation broth (12 L) was filtered through a layer of cotton gauze and exhaustively pressed. The filtrate was partitioned with an equal volume of ethyl acetate (EtOAc) 3 times. The EtOAc layer was collected and concentrated under reduced pressure at 40 °C to yield 0.9 g of EtOAc extract (dark brown oily liquid). For the mycelia, they were extracted with MeOH (1L,1 day) and CH_2Cl_2 (1L, 2 days). The extract of mycelia (AREM) was digested with EtOAc and hexane to yield extracts of 4.2 g and 2.1 g, respectively. The extraction of the fermentation broth and mycelia of the endophytic fungus isolate ARE-1 is shown in Scheme I.



Scheme I Extraction of the fermentation broth and mycelia of the endophytic fungus isolate ARE-1.

8. Isolation of AREB

The AREB 0.9 g was purified by gel filtration chromatography using Sephadex LH-20 (column 2.5 x 80 cm), and eluted with MeOH. Seven fractions were obtained and assigned as AREB 1 (2.0 mg), AREB 2 (67.0 mg), AREB 3 (345.0 mg), AREB 4 (222.0 mg), AREB 5 (55.0 mg), AREB 6 (25.0 mg) and AREB 7 (20.0 mg).

8.1 Isolation of AREB 3575 HP 22

Fractions were combined based on patterns of ^{1}H NMR spectral data. AREB 3 fraction (345.0 mg) possessed high yield and exhibited interesting ^{1}H NMR pattern. It was subjected to Sephadex LH-20 column using MeOH as mobile phase. Fractions (20 ml) were collected. Fractions with similar ^{1}H NMR pattern were combined to give seven major fractions, AREB 31, AREB 32, AREB 33, AREB 34, AREB 35, AREB 36 and AREB 37, as shown in Table 3

Table 3 Fractions obtained from AREB 3

Fraction code	Weight (mg)
AREB 31	1.0
AREB 32	2.0
AREB 33	7.0
AREB 34	18.0
AREB 35	238.0
AREB 36	76.0
AREB 37	2.0

Fraction AREB 35 (238.0 mg) possessed high yield and exhibited interesting ^{1}H NMR pattern so it was seperated on Sephadex LH20 column (1cm x 50 cm) using MeOH as mobile phase. Eight fractions (10 ml) were collected and assigned as AREB 351, AREB 352, AREB 353, AREB 354, AREB 355, AREB 356, AREB 357 and AREB 358, as shown in Table 3.1

Table 3.1 Fractions obtained from AREB 35

Fraction code	Weight (mg)
AREB 351	0.5
AREB 352	2.0
AREB 353	2.0
AREB 354	4.4
AREB 355	17.1
AREB 356	98.0
AREB 357	99.3
AREB 358	15.1

Fraction AREB 357 (99.3 mg) was further purified on Sephadex LH20 column (1 cm x 40 cm). MeOH was used as a mobile phase. Fractions (8 ml) were collected and fractions with similar ^{1}H NMR pattern were combined. Seven fractions, AREB 3571, AREB 3572, AREB 3573, AREB 3574, AREB 3575, AREB 3576 and AREB 3577 were obtained, as shown in Table 3.2

Table 3.2 Fractions obtained from AREB 357

Fraction code	Weight (mg)
AREB 3571	0.7
AREB 3572	1.2
AREB 3573	1.5
AREB 3574	10.8
AREB 3575	64.4
AREB 3576	14.3
AREB 3577	6.2

Fraction AREB 3575 (64.4 mg) exhibited interesting ^1H NMR pattern. It was subjected to semi-preparative HPLC using a reversed-phase column (Lichrocart HR C_{18}, 10 μM, 250 x 10 mm) with MeCN/H_2O (40/60) as an eluent (a flow rate of 4.5 ml/min) to obtain five fractions, as shown in Table 3.3

Table 3.3 Fractions obtained from AREB 3575

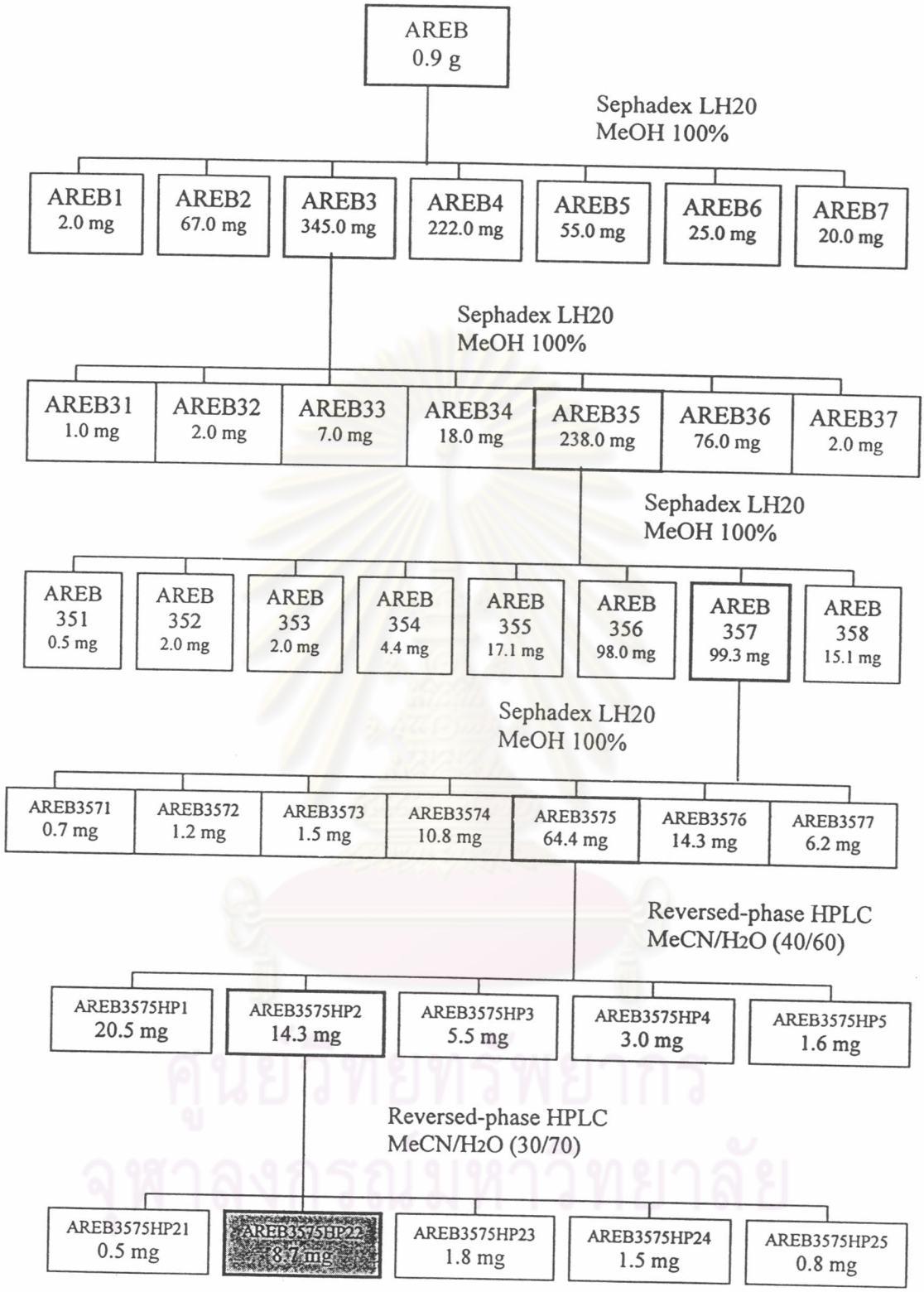
Fraction code	Tr (min)	Weight (mg)
AREB 3575 HP1	1.5	20.5
AREB 3575 HP2	3.5	14.3
AREB 3575 HP3	4.2	5.5
AREB 3575 HP4	8.6	3.0
AREB 3575 HP5	13.0	1.6

Fraction AREB 3575 HP2 (14.3 mg) was subjected to seperation by repeated semi-preparative HPLC using a reversed-phase column (Lichrocart HR C_{18}, 10 μM, 250 x 10 mm). Five major fractions were obtained (Table 3.4).

Table 3.4 Fractions obtained from AREB 3575 HP2

Fraction code	Tr (min)	Weight (mg)
AREB 3575 HP21	2.5	0.5
AREB 3575 HP22	3.7	8.7
AREB 3575 HP23	5.0	1.8
AREB 3575 HP24	6.5	1.5
AREB 3575 HP25	10.5	0.8

Fraction AREB 3575 HP22 (8.7 mg) was yellow oil and identified as succinic acid monoethyl ester. Isolation of AREB 3575 HP22 is shown in Scheme II.



Scheme II Isolation of compound AREB 3575 HP22.

8.2 Isolation of AREB 485 HP4 and AREB 485 HP5

AREB 4 fraction (222.0 mg) showed interesting ^{1}H NMR pattern, and it was seperated on Sephadex LH20 column (1.5 cm x 60 cm) using MeOH as mobile phase. Fractions (20 ml) were collected. Fractions with similar ^{1}H NMR pattern were combined to give nine fractions, AREB 41-AREB 49, as shown in Table 4.

Table 4 Fractions obtained from AREB 4

Fraction code	Weight (mg)
AREB 41	0.5
AREB 42	1.2
AREB 43	2.8
AREB 44	8.3
AREB 45	12.4
AREB 46	34.7
AREB 47	56.6
AREB 48	93.0
AREB 49	8.1

Fraction AREB 48 (93.0 mg) was further purified by Sephadex LH20 column (1 x 60 cm) using MeOH as eluent. Fractions (10 ml) were collected, and nine fractions were obtained, as shown in Table 4.1

Table 4.1 Fractions obtained from AREB 48

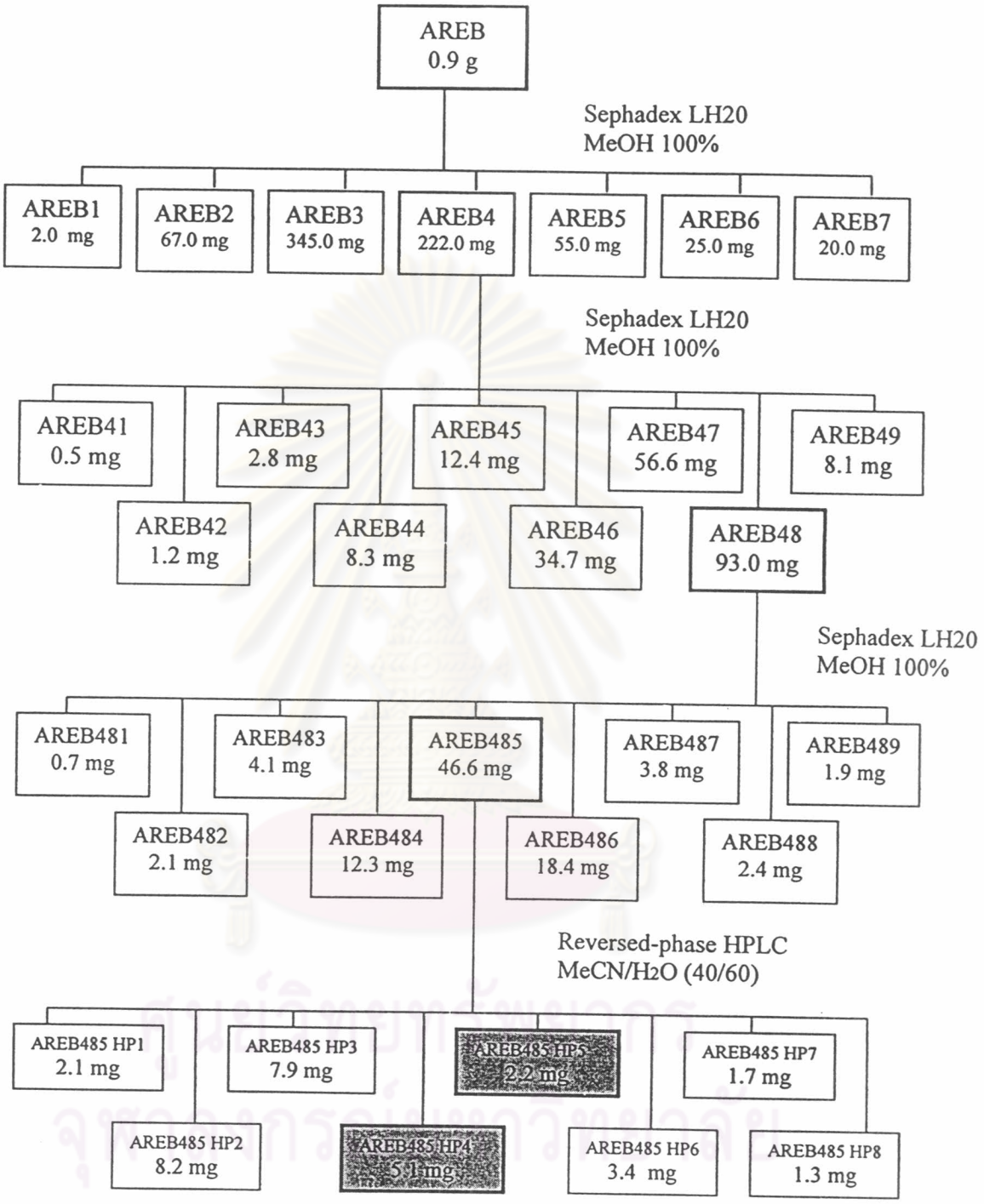
Fraction code	Weight (mg)
AREB 481	0.7
AREB 482	2.1
AREB 483	4.1
AREB 484	12.3
AREB 485	46.6
AREB 486	18.4
AREB 487	3.8
AREB 488	2.4
AREB 489	1.9

Fraction AREB 485 (46.6 mg) was purified by semi-preparative HPLC using a reversed-phase column (Lichrocart HR C_{18}, 10μM, 250 x 10 mm) with MeCN/H_2O (40/60) as an eluent (a flow rate of 4.5 ml/min) to obtain eight fractions, as shown in Table 4.2

Table 4.2 Fractions obtained from AREB 485

Fraction code	Tr (min)	Weight (mg)
AREB 485 HP1	2.2	2.1
AREB 485 HP2	2.8	8.2
AREB 485 HP3	4.0	7.9
AREB 485 HP4	5.8	5.1
AREB 485 HP5	6.4	2.2
AREB 485 HP6	8.4	3.4
AREB 485 HP7	12.0	1.7
AREB 485 HP8	28.0	1.3

Fraction AREB 485 HP4 (5.1 mg) was pure compound, and identified as phenyl acetic acid, while fraction AREB 485 HP5 (2.2 mg) was identified as 2(4′-hydroxy-phenyl) ethyl acetate. Isolation of compounds AREB 485 HP4 and AREB 485 HP 5 (Scheme III).



Scheme III Isolation of compounds AREB 485 HP4 and AREB 485 HP5.

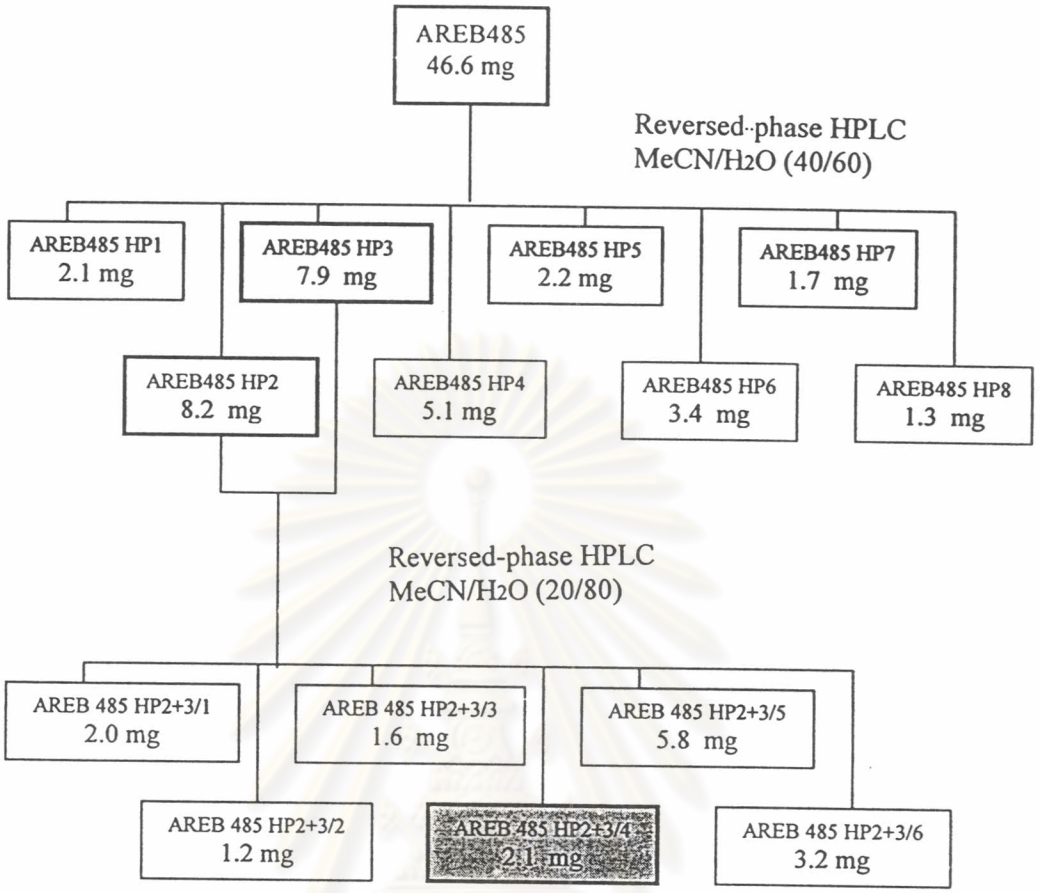
8.3 Isolation of AREB 485 HP2+3/4

Fractions AREB 485 HP2 (8.2 mg) and AREB 485 HP3 (7.9 mg) showed similar patterns of ^{1}H NMR, and they were combined, and purified by semi-preparative HPLC using a reversed-phase column (Lichrocart HR C_{18}, 10μM, 250 x 10 mm). HPLC mobile phase was MeCN/H_2O (20/80), and the flow rate was at 4.5 ml/min. Six fractions were obtained (Table 4.3), and fraction AREB 485 HP2+3/4 was pure and identified as 4-hydroxyphenethyl alcohol or tyrosol.

Table 4.3 Fractions obtained from AREB 485 HP2+3

Fraction code	T*r* (min)	Weight (mg)
AREB 485 HP2+3/1	3.0	2.0
AREB 485 HP2+3/2	3.8	1.2
AREB 485 HP2+3/3	4.2	1.6
AREB 485 HP2+3/4	6.0	2.1
AREB 485 HP2+3/5	7.8	5.8
AREB 485 HP2+3/6	10.2	3.2

Isolation of compound AREB 485 HP2+3/4, as shown in Scheme IV.



Scheme IV Isolation of compound AREB 485 HP2+3/4.

8.4 Isolation of ARHM(H) 76

ARHM(H) fraction (1.4 g) obtained from hexane extract of mycelia was purified by a Sephadex LH20 column (2 cm x 80 cm) using MeOH as mobile phase. Ten fractions were obtained (Table 5).

Table 5 Fractions obtained from ARHM(H)

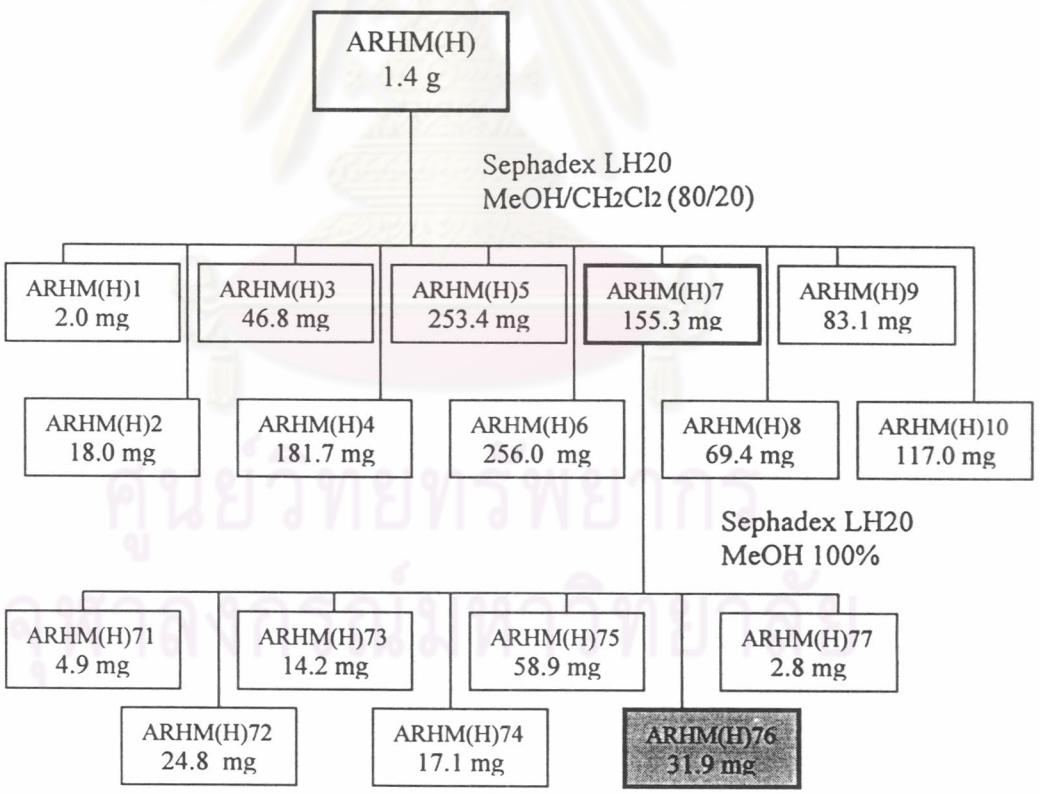
Fraction code	Weight (mg)
ARHM(H) 1	2.0
ARHM(H) 2	18.0
ARHM(H) 3	46.8
ARHM(H) 4	181.7
ARHM(H) 5	253.4
ARHM(H) 6	256.0
ARHM(H) 7	155.3
ARHM(H) 8	69.4
ARHM(H) 9	83.1
ARHM(H) 10	117.0

Fraction ARHM(H) 7 (155.3 mg) exhibited interesting ^{1}H NMR pattern, and was further purified by Sephadex LH20 column (1 cm x 50 cm) using MeOH as mobile phase. Fractions (10 ml) were collected to yield ARHM(H) 71- ARHM(H) 79 (Table 5.1).

Table 5.1 Fractions obtained from ARHM(H) 7

Fraction code	Weight (mg)
ARHM(H) 71	4.9
ARHM(H) 72	24.8
ARHM(H) 73	14.2
ARHM(H) 74	17.1
ARHM(H) 75	58.9
ARHM(H) 76	31.9
ARHM(H) 77	2.8

Fraction ARHM(H) 76 was a pure compound and identified as ergosterol.



Scheme V Isolation of compound ARHM(H)76.

9. Physical Properties of isolated compounds

9.1 AREB 3575 HP22

UV : λ_{max} nm(∈) in methanol ; Figure 8

: 203 (3957)

IR : ν max cm^{-1} ; Figure 7

: 3420, 2926, 1732, 1653, 1457, 1272

ESI-TOF MS : *m/z* ; Figure 9

m/z 169.0471 (found)

169.0477 (calculated for $C_6H_{10}O_4Na$)

^{1}H NMR : δH (ppm), 500 MHz, in $CDCl_3$

see Figure 10 and Table 6

^{13}C NMR : δC (ppm), 125 MHz, in $CDCl_3$

see Figure 11 and Table 6

9.2 AREB 485 HP4

UV : λ_{max} nm(∈) in methanol ; Figure 20

: 205 (8850), 258 (578)

IR : ν max cm^{-1} ; Figure 19

: 3420, 2915, 1715, 1517, 1236, 700

ESI-TOF MS : *m/z* ; Figure 21

m/z 159.0429 (found)

159.0422 (calculated for $C_8H_8O_2Na$)

^{1}H NMR : δH (ppm), 500 MHz, in $CDCl_3$

see Figure 22 and Table 7

^{13}C NMR : δC (ppm), 125 MHz, in $CDCl_3$

see Figure 23 and Table 7

9.3 AREB 485 HP5

UV : λ_{max} nm(∈) in methanol ; Figure 30

: 202 (8462), 222 (6126), 277 (1799), 284 (1608)

IR : ν max cm^{-1} ; Figure 29

: 3420, 2924, 1717, 1558, 1238

ESI-TOF MS : *m/z* ; Figure 31

m/z 203.0684 (found)

203.0684 (calculated for $C_{10}H_{12}O_3Na$)

^{1}H NMR : δH (ppm), 500 MHz, in $CDCl_3$

see Figure 32 and Table 9

^{13}C NMR : δC (ppm), 125 MHz, in $CDCl_3$

see Figure 33 and Table 9

9.4 AREB 485 HP2+3/4

UV : λ_{max} nm(∈) in methanol ; Figure 45

: 202 (7545), 222 (6643), 279 (1702)

IR : ν max cm^{-1} ; Figure 44

: 3387, 2926, 1515, 1242, 1050, 817

ESI-TOF MS : *m/z* ; Figure 46

m/z 161.0588 (found)

161.0578 (calculated for $C_8H_{10}O_2Na$)

^{1}H NMR : δH (ppm), 500 MHz, in acetone-*d*6 and $CDCl_3$

see Figure 47 and Table 10

^{13}C NMR : δC (ppm), 125 MHz, in acetone-*d*6 and $CDCl_3$

see Figure 48 and Table 10

9.5 ARHM(H) 76

UV	:	λ_{max} nm(ϵ) in methanol ; Figure 58
	:	204 (19115), 260 (11257), 271 (15655), 281 (16231) 292 (10031)
IR	:	ν max cm^{-1} ; Figure 57
	:	3424, 2954, 1658, 1458, 1368, 1037
^{1}H NMR	:	δH (ppm), 500 MHz, in $CDCl_3$ see Figure 59
^{13}C NMR	:	δC (ppm), 125 MHz, in $CDCl_3$ see Figure 60 and Table 12

10. Determination of biological activities

Determinations of antimalarial and antituberculosis activities were conducted by Bioassay Research Facility at the National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Science Park, Bangkok, Thailand.

10.1 Antimalarial activity

Plasmodium falciparum (K1, multidrug resistant strain) was cultured according to the method of Trager and Jenson (1976) using continuous cultures of asexual erythrocytic stages. Quantitative assessment of antimalarial activity (*in vitro*) was determined by mean of the microculture radioisotope technique based upon the method described by Desjardins *et al.*, 1979. IC_{50} represents the concentration of extract that inhibited parasite growth by 50 % as indicated by the *in vitro* uptake of [^{3} H]-hypoxanthine by *P. falciparum*. An IC_{50} value of 0.16 µg/ml (3.1 µM) was observed for the standard sample, chloroquine diphosphate.

10.2 Antituberculosis activity

Antituberculosis activity was done by the Microplate Alamar Blue Assay (MABA) (Collins and Franzblau, 1997). The compounds were tested against *Mycobacterium tuberculosis* H37Ra strain in the volume of 200 µl in 96 well microplate. In each well, a standard inoculum of the bacteria (of about 10^5 cells/ml) is incubated with the compounds for one week. The control well contains bacteria, but without any drugs.