CHAPTER III

EXPERIMENT

1. Sample collection and isolation of actinomycetes

A rotten bark sample was collected from a mangrove forest on the west coast of Trang province, Thailand, in October 1998. The sample (0.5 g) was suspended in 5.0 ml of sterile seawater and was diluted to 1:10 in sterile seawater. The sample suspension was heated on a waterbath at 60° C for 5 minutes, then 0.1 ml of the dilution was spreaded on potato carrot agar (PCA) and sodium casienate agar (SCA) plates added with antibiotics (Brock *et al.*, 1993). The plates were incubated at room temperature for 7-14 days until white powdery colonies appeared. A powdery colony was picked up and streaked on yeast extract – malt extract agar (YMA). A single colony was transferred to YMA slants and incubated at room temperature for 7 – 14 days.

It was found that the isolated actinomycete strain TRA 9875-2 produced abundant white aerial mycelium, which afterward transformed into moist and hygroscopic dark-gray mycelia and produced dark spores. A stock culture of the strain TRA 9875-2 was kept on YMA slant in a refrigerator at the Department of Pharmacognosy and the Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

2. Identification and characterization of actinomycetes

The characterization of the actinomycete strain TRA 9875-2 was carried out by the methods described in the International *Streptomyces* Project (ISP) (Shirling and Gottlieb, 1966) and in Bergey's Manual of Systematic Bacteriology (Cross, 1994).

2.1 Morphological and cultural characteristics

2.1.1 Determination of morphological characteristics

Morphological characteristics, spore bearing hyphae, spore chain, number of spores, spore morphology and spore surface were studied by using simple inclined coverslip technique (William and Cross, 1971). The culture on YMA medium was incubated at room temperature for 20 and 30 days until mature spores bearing hyphae were shown. For the scanning electron microscopic (SEM) examination, the selected strain was grown on YMA plate by crosshatch streak technique (Shirling and Gottlieb, 1966) and incubated at room temperature for 30 days. The spores and mycelia were observed with a scanning electron microscope (SEM) using the method of De man, De man and Gupta (1986).

2.1.2 Culture characteristics

Culture characteristics were studied on the colors of mature aerial mycelium, substrate mycelium, spore and diffusible soluble pigment using crosshatch streak (Shirling and Gottlieb, 1966). The strain was cultivated on five different agar media, inorganic salt-starch agar, glycerol-asparagine agar, tyrosine agar, yeast extract-malt extract agar, and oatmeal agar, and all were incubated at room temperature for 7-14 days.

The color of the reverse (under) side of the mass growth of substrate mycelium on five media, the spore color, and the cultural characteristics were observed.

2.2 Physiological and biochemical characteristics

2.2.1 Melanin formation

The strain TRA 9875-2 was cultivated on a tyrosine agar plate and incubated at room temperature, which was then compared with uninoculated plate (negative control). The colony forming a greenish brown to brown or to black

diffusible pigment or a distinct brown pigment modified by other colors were reported as positive (+). Absence of brown to black colors or total absence of diffusible pigment was reported as negative (-) for melanin formation.

2.2.2 Carbon utilization

The carbon sources used in this study were D-glucose as positive control, L-arabinose, sucrose, D-xylose, L-inositol, D-mannitol, D-fructose, rhamnose, raffinose, and no carbon source as negative control. Carbon source was added into basal agar medium to give approximately 1% concentration. The growth of the strain TRA 9875-2 in these carbon sources was observed after 10-14 days and compared with the positive and negative controls.

2.2.3 NaCl tolerance test

The strain TRA 9875-2 was cultivated on YMA plates contained 0-12% of NaCl and incubated at room temperature. The cell growth was observed after incubation for 10-14 days.

2.2.4 pH tolerance test

The strain TRA 9875-2 was tested on YMA plates adjusted pH from pH 3 to pH 14 and incubated at room temperature. The cell growth was observed after incubation for 10-14 days.

2.2.5 Nitrate reduction

The strain TRA 9875-2 was inoculated in peptone nitrate broth and incubated at room temperature for 10 days. On the tenth day, three drops of sulfanilic acid reagent and two drops of dimethyl-α-napthylamine solution were added into 1 ml of the culture broth in the test tube. If the bacterium could reduce nitrate into nitrite, the mixture would become pink to red.

2.2.6 Starch hydrolysis

The strain TRA 9875-2 was grown on an inorganic salt-starch agar plate and incubated at room temperature for 10-14 days. On the fourteenth day after incubation, Gram's iodine solution was dropped on the surface of the agar plate. If the bacterium could hydrolyse starch, a dark blue color would not appear on the plate.

2.2.7 Tyrosine hydrolysis

The strain TRA 9875-2 was cultivated on a tyrosine agar plate and incubated at room temperature for 10-14 days. If the bacterium could produce tyrosinase enzyme, a clear zone around the colony would be present.

2.2.8 Gelatin liquefaction

The strain TRA 9875-2 was inoculated into a test tube of nutrient gelatin broth and incubated at room temperature for 21 days compared with uninoculated tube after incubating both tubes at 20°C for 30 minutes. The gelatin appear to be liquid when the bacterium could hydrolyse it.

2.2.9 Milk coagulation and milk peptonization

The strain TRA 9875-2 was inoculated in a tube of skim milk broth and incubated at room temperature for 21 days. If the bacterium could produce proteolytic enzyme the skim milk converted to clear solution or the skim milk was precipitated.

2.2.10 Cellulose decomposition

The strain TRA 9875-2 was inoculated in a tube of cellulose decomposition medium and incubated at room temperature for 30 days. If the bacterium could produce cellulase enzyme, the filter paper (Whatman No.1) was digested.

2.3 Cell wall analysis

The chemical analyses of cell wall diaminopimelic acid (DAP) isomers were done as described by Komagata and Suzuki (1987). Dried whole-cell of the strain TRA 9875-2 was hydrolyzed. DAP isomers were separated by thin layer chromatography (TLC) on a cellulose plate (Merck No. 5577). The standard of DL-DAP (0.01M) and the hydrolysate of two known strains that contained meso- and L-DAP were applied for reference purposes. Methanol-water-6 N hydrochloric acid-pyridine (80:26:4:10, V/V) was used as solvent system to develop the TLC. The spots were visualized by spraying with 0.2% ninhydrin solution in water-saturated n-butanol followed by heating at 100°C for 5 minutes. DAP isomers appear as dark-green spots with R_f value 0.29 (L-isomer) and 0.24 (meso- and DL- isomer). Spots will disappear in a few minutes.

3. Fermentation method

A loopful of the strain TRA 9875-2 cultivated on YMA slants for 7 days was inoculated into a 100-ml of the seed medium (glucose beef extract, GBP) in a 500-ml Erlenmeyer flask and was shaken on a rotary shaker (200 rpm) at room temperature for 3 days. Two ml of seed culture was inoculated into 200 ml of the production medium (glycerol peptone medium, GPM and yeast extract-malt extract medium, YMB) in a 500-ml Erlenmeyer flask incubated on a rotary shaker (200 rpm) at room temperature for 5 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)

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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

4.2 Column chromatography

4.2.1 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 slurried 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

4.2.2 Gel filtration chromatography

Gel filter : 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

5. Crystallization technique

Compound KTR75001k was crystallized by dissolving in $CHCl_3$ until saturated and then MeOH was added. The solution was left standing at room temperature until yellow amorphous powder was formed.

Compound KTR75008k was crystallized from a mixture of $CHCl_3$: MeOH : Hexane (75:20:5). Each compound was dissolved in the mixture until saturated and left standing at room temperature until the orange-needle crystals were formed.

6. Spectroscopy

6.1 Ultraviolet (UV) absorption spectroscopy

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

6.2 Infrared (IR) absorption spectroscopy

IR spectrum of KTR75001k (KBr disc) was obtained from a Perkin Elmer FT-IR 1760 X Spectrometer (The Scientific and Technological Research Equipment Center (STREC), Chulalongkorn University).

IR spectra of other compounds were obtained from a Perkin Elmer 2000 FT-IR spectrometer (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

6.3 Mass spectroscopy (MS)

The FABMS and HRFABMS spectra were obtained from a JEOL JMS-HX 110 double focusing mass spectrometer of EBE arrangement with JMA-DA 7000 data system, 10 kV acceleration voltage, and fast-atom xenon gas accelerated at voltage of 3 kV. Glycerol or m-nitrobenzyl alcohol (mNBA) were used as the matrix, and NaCl

was used as alkali metal cation source (The Institue of Molecular and Cellular Biosciences, the University of Tokyo).

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

^{1}H and ^{13}C-MNR, DEPT 135, COSY, TOCSY, HMQC, HMBC and NOESY spectra were obtained from a Bruker AVANCE DPX-300 FT-NMR spectrometer operating at 300 MHz for protons and 75 MHz for carbons. Proton detected heteronuclear correlations were measured using HMQC (optimized for $^nJ_{HC}$ = 145 Hz) and HMBC (optimized for $^nJ_{HC}$ = 4 or 8 Hz) pulse sequences (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

Deuterated solvents; chloroform-*d* ($CDCl_3$), dimethylsulfoxide-d_6 (CD_3SOCD_3), benzene-d_6 (C_6D_6), methanol-d_4 (CD_3OD), and pyridine-d_5 (C_5D_5N) 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 (^{13}C) for $CDCl_3$, 2.49 ppm (^{1}H) and 39.7 ppm (^{13}C) for CD_3SOCD_3, 7.15 ppm (^{1}H) and 128.0 ppm (^{13}C) for C_6D_6, 3.30 ppm (^{1}H) and 49.0 ppm (^{13}C) for CD_3OD and 8.71, 7.55 and 7.19 ppm (^{1}H) and 123.5, and 135.5 and 149.2 ppm (^{13}C) for C_5D_5N.

6.5 Optical rotation

Optical rotation was measured on a Perkin-Elmer 341 polarimeter using a sodium lamp operating at 589 nm (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

7. Solvents

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

8. Biological activity

8.1 Antimicrobial activity

Antimicrobial activity of the fractions and pure compounds were tested by using the agar disc diffusion method (Lorian, 1980). Activity was performed against *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Candida albicans* ATCC 10231. All tested bacteria were cultivated on tryptic soy agar slant, TSA (Difco®), and the yeast, *Candida albicans* ATCC 10231 was cultivated on Sabouraud dextrose agar slant, SDA (Difco®) at 37 °C for 24 hours. The cell cultures were washed from the agar surface and suspended with steriled normal saline solution (NSS), and standardized to match a 0.5 turbidity standard of MacFarland No. 1, provided approximately 1×10^8 CFU (colony forming unit/ml). Each of molten (20 ml) TSA and SDA was separated and poured into 9 cm diameter petri dish and allowed to solidify to form base layer. A loopful of each tested microorganisms was swabbed on the surface of TSA and SDA plates. All tested samples were dissolved in the suitable solvent and then applied on steriled paper disc for disc diffusion assay. These paper discs were left in steriled petri dish until the solvent was completed dried. The dried paper discs were placed on the surface of the swabbed plates and incubated at 37 °C for 24 hours. The diameters of inhibition zones were measured. Fractions exhibited good antimicrobial activity were subsequently selected for further study.

8.2 Antimalarial activity

Plasmodium falciparum (K1, multidrug resistant strain) was cultured according to the method of Trager and Jensen (1976) using continuous cultures (in vitro) 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. 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.

8.3 Cytotoxic activity

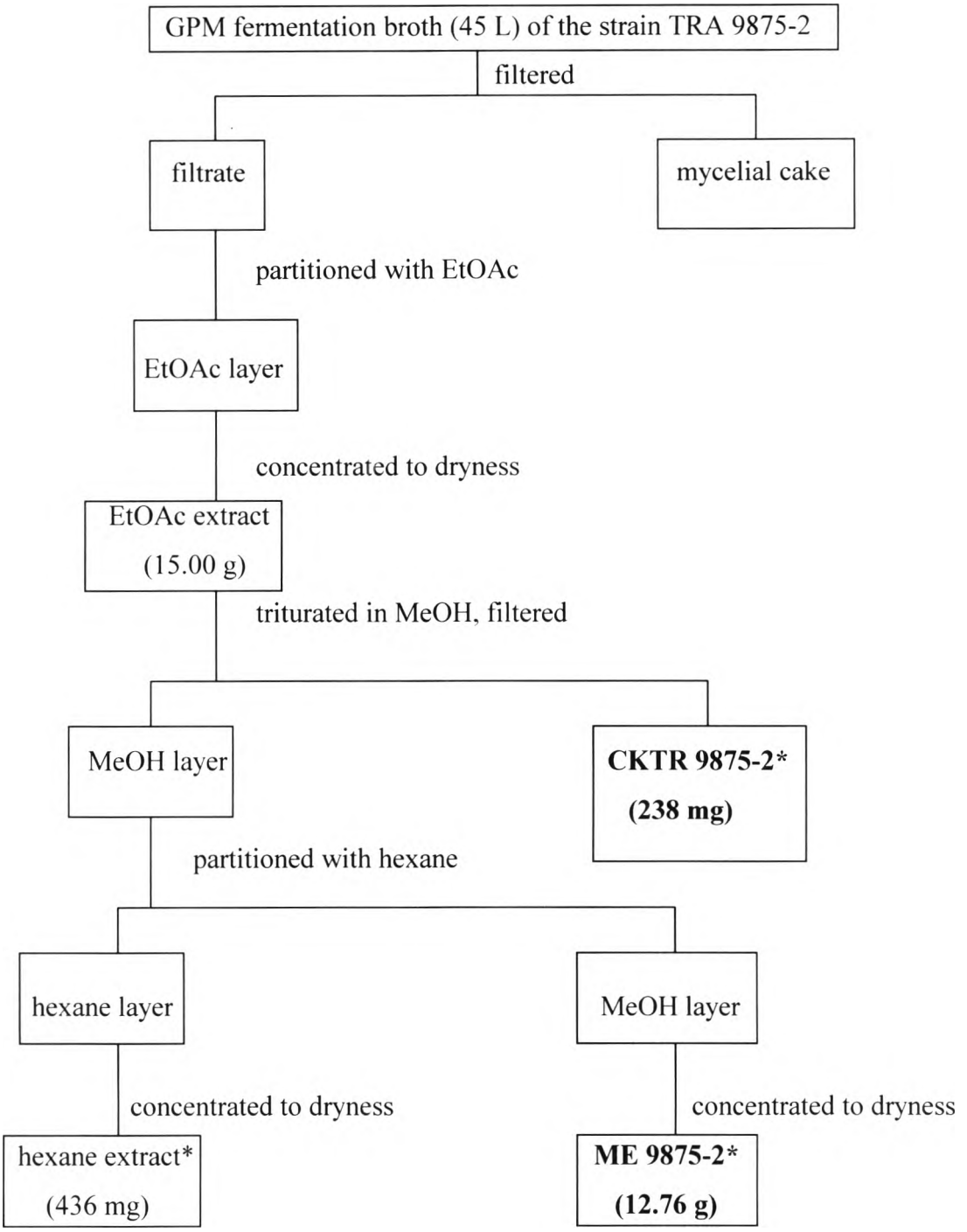
Cytotoxic activity against a breast cancer cell line (BC), a human epidermoid carcinoma cell line of the nasopharynx (KB), and a Vero cell line (African monkey kidney cell line) was performed by sulforhodamine B (SRB) colorimetric method (Skehan *et al.*, 1990).

9. Extraction

The GPM fermentation broth (45 L) was filtered through a Buchner funnel packed with Kieselguhr (diatomaceous earth or diatomite or bacillarieae earth). The filtrate was partitioned with ethyl acetate (30 L × 3). The ethyl acetate layer was collected and concentrated under reduced pressure at 45 °C to yield 15 g of ethyl acetate extract (dark brown oily liquid). The ethyl acetate extract was triturated with 50 ml MeOH and filtered through sinterglass funnel to yield 238 mg of orange-yellow amorphous powder (CKTR 9875-2). The MeOH solution was partitioned with hexane (25 ml × 3). The MeOH layer and the hexane layer were concentrated under reduced pressure at 45 °C to yield 12.67 g of the MeOH extract (ME 9875-2, dark brown oily liquid) and 436 mg of the hexane extract (brown oily liquid) as shown in Scheme 1.

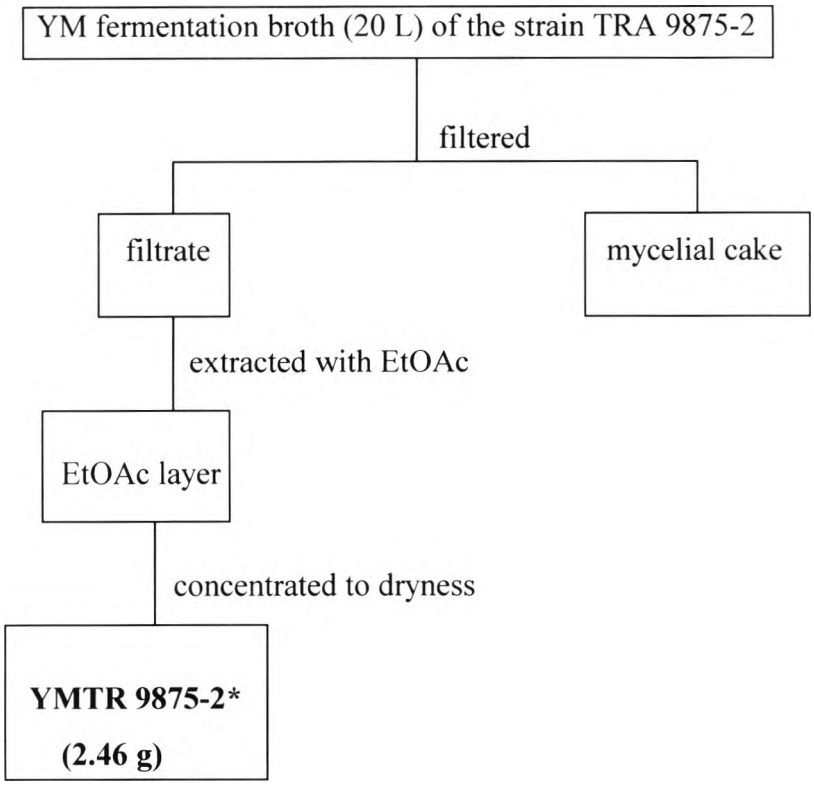
The YM fermentation broth (20 L) was filtered through a Buchner funnel packed with Kieselguhr. The filtrate was partitioned with ethyl acetate (15 L × 3), the ethyl acetate layer was concentrated under reduced pressure at 45 °C to yield 2.46 g of orange-yellow amorphous powder (YMTR 9875-2) as shown in Scheme 2.

All extracts were examined for antimicrobail activity by the method as described in Section 8.



*positive antimicrobial activity

Scheme 1 Extraction of the GPM fermentation broth of the strain TRA 9875-2.



*positive antimicrobial activity

Scheme 2 Extraction of YM fermentation broth of the strain TRA 9875-2.

10. Isolation

The CKTR 9875-2, the ME 9875-2 and the YMTR 9875-2 showed the yellow and purple spots on the TLC (Si Gel, 10% MeOH in $CHCl_3$, R_f = 0.50 and 0.28, respectively) as the major products. Then the main yellow and purple spots were isolated by several chromatographic techniques to obtain the pure compounds.

10.1 Isolation of geldanamycin (KTR75001k)

The CKTR 9875-2 (238 mg) was purified by flash column chromatography using Si Gel as adsorbent (column 1.5 × 15 cm) as shown in Scheme 3. The gradient of MeOH in $CHCl_3$ was used as mobile phase to give 4 fractions (KTR75001-KTR75004) after combination of fractions which similar chromatographic patterns (Si Gel, 10% MeOH in $CHCl_3$) as shown in Table 3.1. Fifteen ml fractions were collected.

Table 3.1 Fractions obtained from CKTR 9875-2.

Fraction Code	Fraction	Percentage of MeOH in $CHCl_3$	Volume of Mobile Phase (ml)	Total Weight (mg)
KTR75001	1-3	5	45	144
KTR75002	4-8	5	75	8
	9-11	8	45	
KTR75003	12-14	8	45	8
KTR75004	15-24	8	150	47
	25-28	10	60	
	29	30	15	
	30	50	15	
	31-35	100	75	

Fraction KTR75001 (144 mg) was crystallized by a mixture of $CHCl_3$ and MeOH to yield a yellow amorphous powder of KTR75001k (108 mg) which later was identified as geldanamycin (Section 3.1, Chapter IV).

The ME 9875-2 (12.76 g) was fractionated on a Sephadex LH 20 column (column 2.5 × 80 cm) as shown in Scheme 4. The column was eluted with MeOH. The fractions were collected 25 ml each and then combined based on the similar TLC chromatographic patterns (Si Gel, 10% MeOH in $CHCl_3$) to yield 4 fractions of ME75001-ME75004 as shown in Table 3.2.

Table 3.2 Fractions obtained from ME 9875-2.

Fraction Code	Fraction	Total Volume (ml)	Total Weight (g)
ME75001	1-6	750	1.50
ME75002	7-8	250	5.80
ME75003	9-16	1000	7.11
ME75004	17-25	1125	0.08

Fraction ME75002 (5.80 g) was separated on a Si Gel flash column (column 2.5 × 15 cm). The gradient of MeOH in $CHCl_3$ was used as mobile phase. Twenty-five ml fractions were collected. The TLC technique (Si Gel, 10% MeOH in $CHCl_3$) was used to combine fractions with similar chromatographic patterns to yield 4 fractions of ME75005-ME75008 as shown in Table 3.3.

Table 3.3 Fractions obtained from ME75002.

Fraction Code	Fraction	Percentage of MeOH in $CHCl_3$	Volume of Mobile Phase (ml)	Total Weight (g)
ME75005	1-6	0	750	0.63
	7-28	1	2750	
	29-33	3	625	
ME75006	34-42	5	1125	0.27
ME75007	43-48	5	750	1.37
	49-55	10	875	
ME75008	56-58	20	375	2.19
	59-65	30	875	
	66-69	50	500	
	70-80	100	1375	

Fraction ME75005 (630 mg) showed antimicrobial activity. It was

separated on a Si Gel flash column (column 2.5 × 13 cm). The gradient MeOH in $CHCl_3$ was again used as mobile phase. Twenty-five ml fractions were collected. The TLC technique (Si Gel, 10% MeOH in $CHCl_3$) was used to combine the similar chromatographic patterns to yield 5 fractions of ME75009-ME70013 as shown in Table 3.4.

Table 3.4 Fractions obtained from ME75005.

Fraction Code	Fraction	Percentage of MeOH in $CHCl_3$	Volume of Mobile Phase (ml)	Total Weight (mg)
ME75009	1-6	1	150	200
	7-20	2	350	
ME75010	21-31	2	275	158
ME75011	32-45	3	350	81
ME75012	46-51	5	150	74
	52-58	8	175	
	59-64	10	150	
	65-68	20	100	
	69-71	30	75	
	72-74	50	75	
ME75013	75-79	50	125	22
	80-88	100	225	

Fraction ME75010 (158 mg) was gradually crystallized from a small amount of $CHCl_3$ in MeOH to yield 66 mg of a yellow amorphous powder (KTR75001k) that was later identified as geldanamycin, which is identical to CKTR 9875-2.

The extract YMTR 9875-2 (2.46 g) was purified on a Sephadex LH 20 column (column 2.54 × 92 cm) as shown in Scheme 5. A mixture of $CHCl_3$: MeOH: hexane (75:20:5) was used as eluant. Fifteen ml fractions were collected. After combination of the similar chromatographic pattern fractions by using TLC (Si Gel, 10% MeOH in $CHCl_3$), 5 fractions of YMTR75001-YMTR75005 were obtained as shown in Table 3.5.

Table 3.5 Fractions obtained from YMTR 9875-2.

Fraction Code	Fraction	Volume of Eluent (ml)	Total Weight (mg)
YMTR75001	1-3	90	230
YMTR75002	4	30	316
YMTR75003	5-6	60	1480
YMTR75004	7	30	260
YMTR75005	8-15	240	202

Fraction YMTR75003 (1.48 g) was separated again on a Sephadex LH 20 column using a mixture of $CHCl_3$: MeOH: hexane (75:20:5) as eluant. Ten ml fractions were collected. The TLC technique (Si Gel, 10% MeOH in $CHCl_3$) was used to combine the similar chromatographic pattern fractions, to give 3 fractions of YMTR75006-YMTR75008 as shown in Table 3.6.

Table 3.6 Fractions obtained from YMTR75003.

Fraction Code	Fraction	Volume of Eluent (ml)	Total Weight (mg)
YMTR75006	1-2	20	17
YMTR75007	3-9	70	1400
YMTR75008	10-13	40	162

Fraction YMTR75007 (1.40 g) was separated on a Si Gel flash column (column 3.5 × 10 cm) using the gradient of MeOH in $CHCl_3$ as eluent. The 50-ml fractions were collected. Combination of the similar chromatographic pattern fractions by TLC (Si Gel, 10% MeOH in $CHCl_3$), 6 fractions of YMTR75009-YMTR75014 were obtained as shown in Table 3.7.

Fraction YMTR 75010 (976 mg) was applied on top of a Sephadex LH 20 column (column 2.54 × 98 cm) using a mixture of $CHCl_3$: MeOH (1:1) as mobile phase. Twenty-five ml fractions were collected. The TLC technique (Si Gel, 10% MeOH in $CHCl_3$) was used to combine the similar chromatographic pattern fractions to yield 4 fractions of YMTR75015-YMTR75018 as shown in Table 3.8.

Table 3.7 Fractions obtained from YMTR75007.

Fraction Code	Fraction	Percentage of MeOH in $CHCl_3$	Volume of Eluent (ml)	Total Weight (mg)
YMTR75009	1-2	1	100	11
YMTR75010	3-34	1	160	976
	35-38	2	200	
YMTR75011	39-40	3	100	31
	41-42	5	100	
YMTR75012	43-45	10	150	241
YMTR75013	46-57	10	600	162
	58-59	15	100	
	60-63	20	200	
	64-65	30	100	
	66-68	50	150	
YMTR75014	69-70	100	100	29

Table 3.8 Fractions obtained from YMTR75010.

Fraction Code	Fraction	Volume of Mobile Phase (ml)	Total Weight (mg)
YMTR75015	1-2	50	21
YMTR75016	3	25	539
KTR75001k	4	25	358
YMTR75018	5-6	50	29

Fraction YMTR 75016 (539 mg) showed the main yellow spot (geldanamycin) on the TLC (Si Gel, 10% MeOH in $CHCl_3$, R_f = 0.50) similarly to the fraction KTR75001k (358 mg).

10.2 Isolation of 17-*O*-demethylgeldanamycin (KTR75008k) and 17-*O*-demethyldihydrogeldanamycin (KTR75010)

Fraction KTR75004 (47 mg) showed a purple spot (R_f = 0.28) 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 × 60 cm) as shown in Scheme 3. A mixture of $CHCl_3$: MeOH: hexane (75:20:5) was used as eluant. Twenty ml fractions were collected. After

combination of the similar chromatographic pattern fractions by using TLC (Si Gel, 10% MeOH in $CHCl_3$), 3 fractions of KTR75005-KTR75007 were obtained as shown in Table 3.9.

Table 3.9 Fractions obtained from KTR75004.

Fraction Code	Fraction	Volume of Eluent (ml)	Total Weight (mg)
KTR 75005	1-2	40	8
KTR75006	3-5	60	10
KTR75007	6-29	480	28

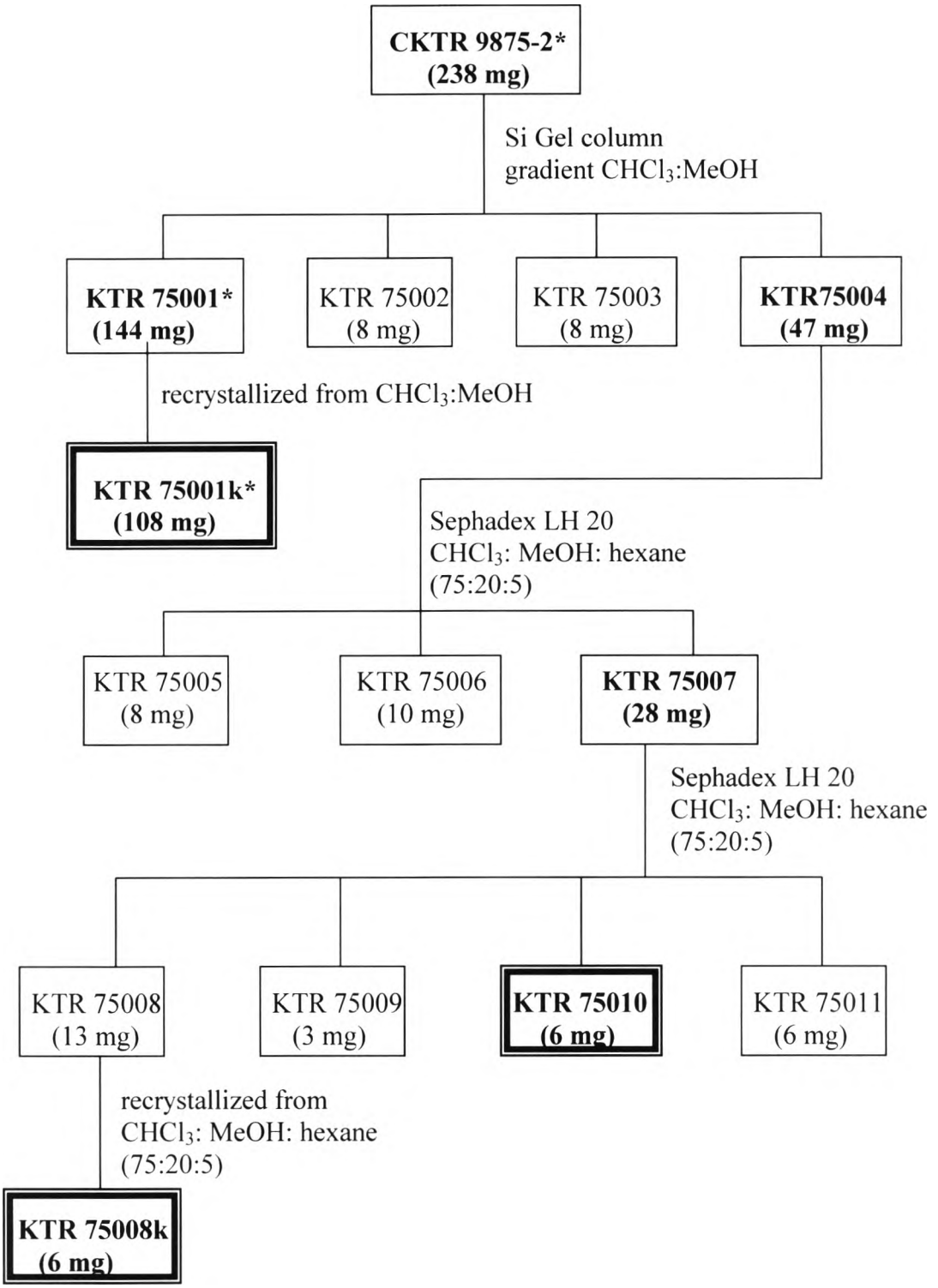
Fraction KTR75007 (28 mg) showed only one tailing purple spot on the TLC (Si Gel, 10% MeOH in $CHCl_3$). Gel filtration chromatographic technique was applied to separate this fraction. A mixture of $CHCl_3$: MeOH: hexane (75:20:5) was used as eluant. Fractions were collected on the basis of the color bands on a Sephadex LH 20 column to yield 4 fractions of KTR75008-KTR75011 as shown in Table 3.10.

Table 3.10 Fractions obtained from KTR75007.

Fraction Code	Band Color	Volume of Eluent (ml)	Total Weight (mg)
KTR75008	Yellow-brown	80	13
KTR75009	Purple-brown	60	3
KTR75010	Violet	120	6
KTR75011	Pale violet	575	6

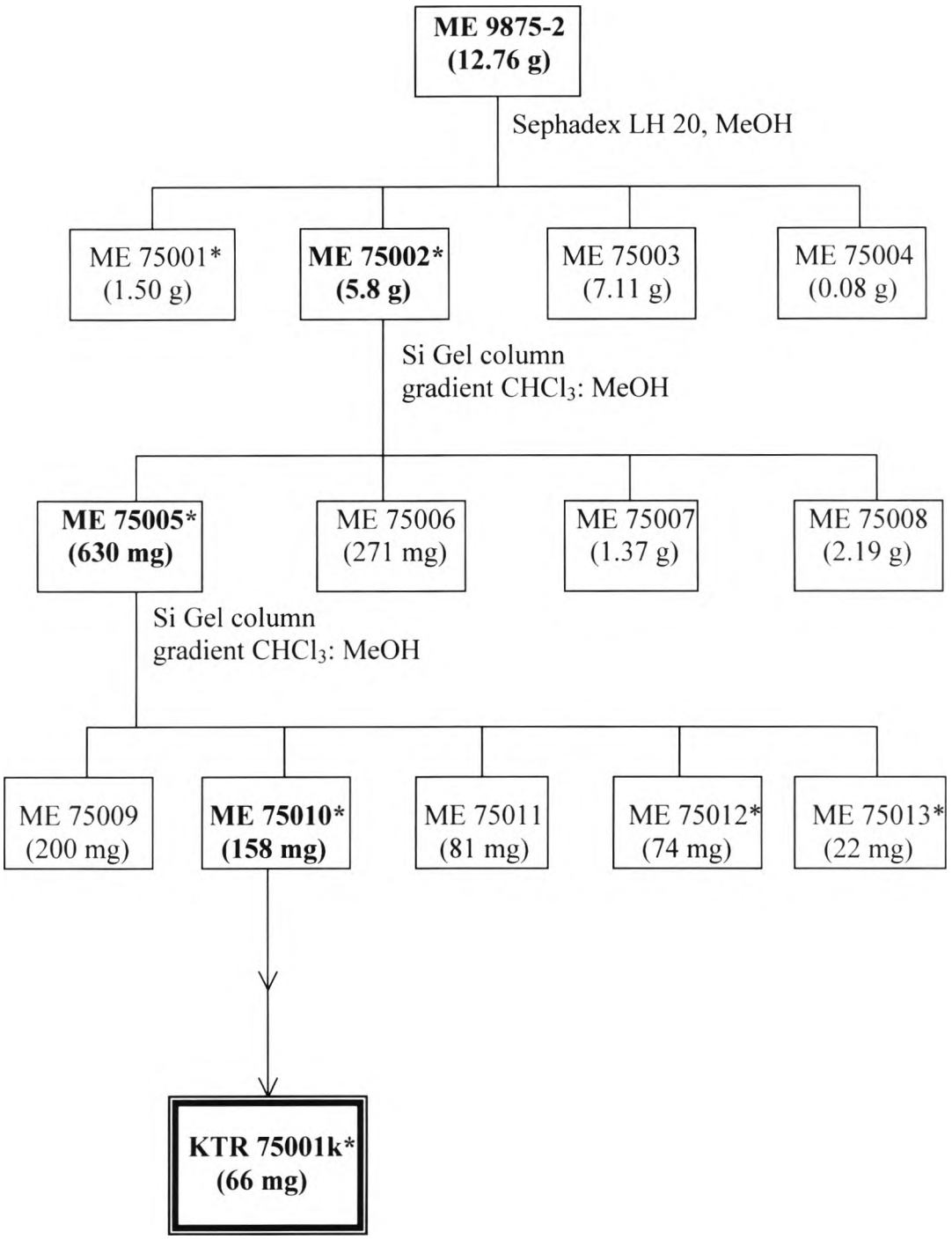
Fraction KTR75008 (13 mg) was crystallized from a mixture of $CHCl_3$: MeOH: hexane (75:20:5) to yield 6 mg of the orange needle crystals (KTR75008k), which was later identified as 17-*O*-demethylgeldanamycin (Section 3.2, Chapter IV).

Fraction KTR75010 (6 mg) was obtained as a light violet substance. This fraction showed only one clear spot that was later identified as a new derivative of geldanamycin, 17-*O*-demethyldihydrogeldanamycin (Section 3.3, Chapter IV).



*positive antimicrobial activity

Scheme 3 Fractionation of CKTR 9875-2 of the strain TRA 9875-2.



*positive antimicrobial activity

Scheme 4 Fractionation of ME 9875-2 of the strain TRA 9875-2.

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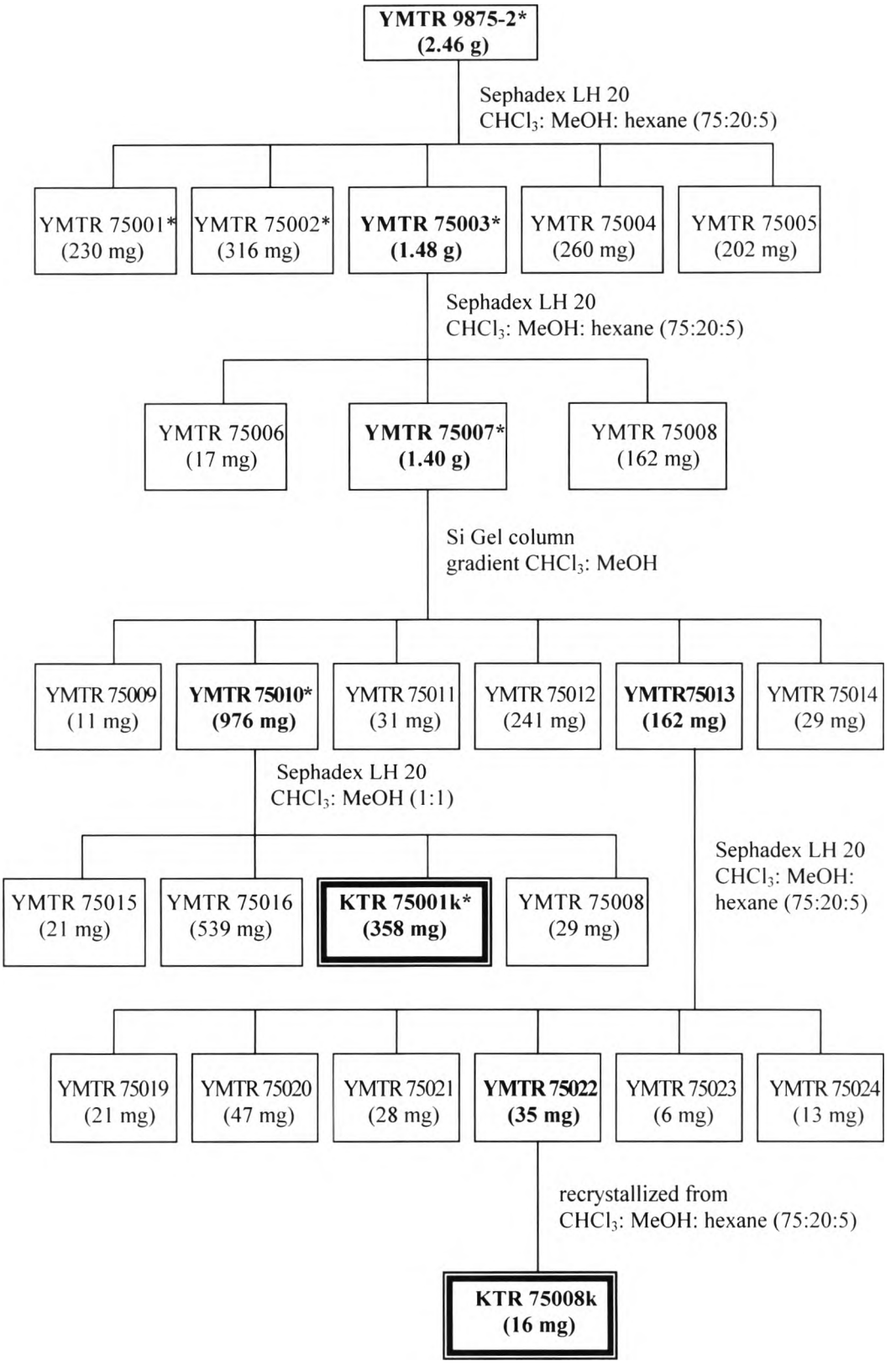

*positive antimicrobial activity

Scheme 5 Fractionation of YMTR 9875-2 of the strain TRA 9875-2.

In a similar fashion to that of CKTR 9875-2, fraction YMTR75013 (162 mg) showed a main purple spot on the TLC (Si Gel, 10% MeOH in $CHCl_3$, R_f = 0.28). Fraction YMTR75013 was purified on a Sephadex LH 20 column (column 2.54 × 92 cm) as shown in Scheme 5. A mixture of $CHCl_3$: MeOH: hexane (75:20:5) was used as eluant. Fractions were collected on the basis of the color bands on a Sephadex LH 20 column to yield 6 fractions of YMTR75019-YMTR75024 as shown in Table 3.11.

Table 3.11 Fractions obtained fromYMTR75013.

Fraction Code	Band Color	Volume of Solvent (ml)	Total Weight (mg)
YMTR75019	Pale brown	225	21
YMTR75020	Orange-brown	25	47
YMTR75021	Yellow-brown	25	28
YMTR75022	Pale orange	1000	35
YMTR75023	No color	1000	6
YMTR75024	No color	100	13

Fraction YMTR75022 (35 mg) was crystallized from a mixture of $CHCl_3$: MeOH: hexane (75:20:5) to yield 16 mg of the small orange needles (KTR75008k) which was later identified as 17-*O*-demethylgeldanamycin.

The others bioactive fractions obtained from the strain TRA 9875-2 was kept in the refrigerator (–30 °C) for further investigation.

11. Physical and chemical properties of the isolated compounds

11.1 Geldanamycin (KTR75001k)

$[\alpha]^{25}_{D}$: +46.0° (c, 0.500, $CHCl_3$)

UV : λ_{max} nm (ϵ), in methanol; Figure 11
256 (10202), 304 (11870), 400 (560)

IR : ν_{max} cm^{-1}; Figure 12
3,452, 1,692, 1,651

FABMS : *m/z*; Figure 13

563 $[M+2+H]^+$

HRFABMS : *m/z* 583.2587 (found)

583.2632 (calculated for $C_{29}H_{40}N_2O_9Na$)

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

see Figure 14 and Table 4.4 (Section 3.1,Chapter IV)

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

see Figure 15 and Table 4.4 (Section 3.1,Chapter IV)

11.2 17-*O*-demethylgeldanamycin (KTR75008k)

$[\alpha]^{25}_D$: +15.5° (c, 0.100, MeOH)

UV : λ_{max} nm (ϵ), in methanol; Figure 31

235 (20219), 328 (24590)

IR : ν_{max} cm^{-1}; Figure 32

3,431, 1,689, 1,639

FABMS : *m/z*; Figure 33

549 $[M+2+H]^+$

HRFABMS : *m/z* 569.2479 (found)

569.2475 (calculated for $C_{29}H_{38}N_2O_9Na$)

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

see Figure 34 and Table 4.7 (Section 3.2,Chapter IV)

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

see Figure 35 and Table 4.7 (Section 3.2,Chapter IV)

11.3 17-*O*-demethyldihydrogeldanamycin (KTR75010)

$[\alpha]^{25}_D$: +20.5° (c, 0.100, MeOH)

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

214 (32240), 321 (3825)

IR : ν_{max} cm^{-1}; Figure 46

3,423, 1,719, 1,636

ESITOFMS : *m/z*; Figure 47

571 $[M+Na]^+$, 569 $[M-2+Na]^+$

HRESITOFMS: *m/z* 571.2649 (found)

571.2632 (calculated for $C_{29}H_{40}N_2O_9Na$)

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

see Figure 48 and Table 4.7 (Section 3.3,Chapter IV)

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

see Figure 49 and Table 4.7 (Section 3.3,Chapter IV)

12. Acetylation of geldanamycin (KTR75001k)

The IR spectral data indicated the presence of hydroxyl group(s) in compound KTR75001k, acetylation of this compound was performed to confirm the presence of hydroxyl group(s).

Sample (15 mg) was put into a 5-ml round bottom flask with a magnetic bar inside. Anhydrous pyridine (2 ml) and acetic anhydride (2 ml) were added. The flask was sealed with a rubber septum and the air inside was replaced by nitrogen gas. The mixture was stirred at room temperature overnight. The reaction was monitored by TLC (Si Gel, 10% MeOH in $CHCl_3$) and completed within 24 hours. Extraction with $CHCl_3$ and purification by preparative TLC (Si Gel, 10% MeOH in $CHCl_3$) resulted in 7 mg of 11-*O*-acetylgeldanamycin.

11-*O*-acetylgeldanamycin: yellow needle crystals; molecular formula, $C_{31}H_{42}N_2O_{10}$; FABMS *m/z* 605 $[M+2+H]^+$, Figure 28; ^{1}H-NMR data ($CDCl_3$+benzene-d_6), 2-Me (δ 1.78, 3H, s); H-3 (δ 7.39, d, 14); H-4 (δ 6.25, t, 11, 11); H-5 (δ 5.71, t, 10, 10); H-6 (δ 4.33, d, 10); 6-OMe (δ 3.11, 3H, s); H-7 (δ 5.12, brs); 7-OCONH$_2$ (δ 4.33, NH$_2$, brs); 8-Me (δ 1.50, 3H, s); H-9 (δ 5.11, d, 10); H-10 (δ 3.02, m); 10-Me (δ 0.86, 3H, d, 7); H-11 (δ 4.92); 11-OAc (δ 1.83, 3H, s); H-12 (δ 3.21); 12-OMe (δ 3.22, 3H, s); H-13 (δ 1.26, 2H, brs); H-14 (δ 1.93, m); 14-Me (δ 1.05, 3H, d, 6); H-15 (δ 2.36, 2.20, 2H, m); 17-OMe (δ 3.78, 3H, s); H-19 (δ 7.37, s), Figure 27.

13. Methylation of geldanamycin (KTR75001k)

Sample (15 mg) was put into a 5-ml round bottom flask with a magnetic bar inside. Anhydrous potassium carbonate (15 mg), methyl iodide (2 drops), and DMF (2 ml) were added. The flask was sealed with a rubber septum and the air inside was replaced by nitrogen gas. The mixture was stirred at room temperature overnight. The reaction was monitored by TLC (Si Gel, 10% MeOH in $CHCl_3$) and completed within 24 hours. Extraction with $CHCl_3$ and purification by preparative TLC (Si Gel, 10% MeOH in $CHCl_3$) resulted in 7 mg of 11-*O*-methylgeldanamycin.

11-*O*-methylgeldanamycin: yellow substance; molecular formula, $C_{30}H_{42}N_2O_9$; FABMS *m/z* 577 $[M+2+H]^+$, Figure 30; ^{1}H-NMR data ($CDCl_3$), 2-Me (δ 1.95, 3H, s); H-3 (δ 6.30, d, 11); H-4 (δ 6.26, t, 13, 13); H-5 (δ 5.21, t, 10, 10); H-6 (δ 4.00, t, 10, 10); 6-OMe (δ 3.11, 3H, s); H-7 (δ 4.99, d, 10); 7-OCONH$_2$ (δ 4.58, NH_2, brs); 8-Me (δ 1.23, 3H, s); H-9 (δ 5.19, d, 10); H-10 (δ 2.35, brs); 10-Me (δ 1.03, 3H, d, 6); H-11 (δ 3.60); 11-OMe (δ 3.34, 3H, s); H-12 (δ 2.85); 12-OMe (δ 3.30, 3H, s); H-13 (δ 1.62, 2H, m); H-14 (δ 2.17, m); 14-Me (δ 0.59, 3H, d, 7); H-15 (δ 2.51, 2.59, 2H, m); 17-OMe (δ 4.07, 3H, s); H-19 (δ 6.30, s), Figure 29.