CHAPTER IV

RESULTS AND DISCUSSION

1. Sample collection and isolation of actinomycetes

The actinomycete strain TRA 9875-2 was isolated from a rotten bark sample collected from mangrove forest along the Andaman coast, Trang Province, Thailand, in October 1998 and was isolated by spread plate technique on PCA medium (Brock *et al.*, 1993).

2. Identification and characterization of actinomycetes

Identification and characterization for the strain of isolated actinomycete were performed by using the method described in International *Streptomyces* Project (ISP) (Shirling and Gottlieb, 1966) and the characteristic described in Bergey's Manual of Systematic Bacteriology (Cross, 1994).

2.1 Morphological and cultural characteristics

2.1.1 Morphological characteristics

The vegetative mycelium of the strain TRA 9875-2 growing on YMA medium was initially white and turned into dark-gray during the course of incubation (30 days) and became darken with the time of incubation as shown in Figure 1.

The scanning electron micrographs revealed the unbranced aerial mycelia and the spiral in dense cluster spore chain as shown in Figure 2.

Figure 1 The colonial appearance of *Streptomyces* sp. TRA 9875-2 on YMA medium (30 days).

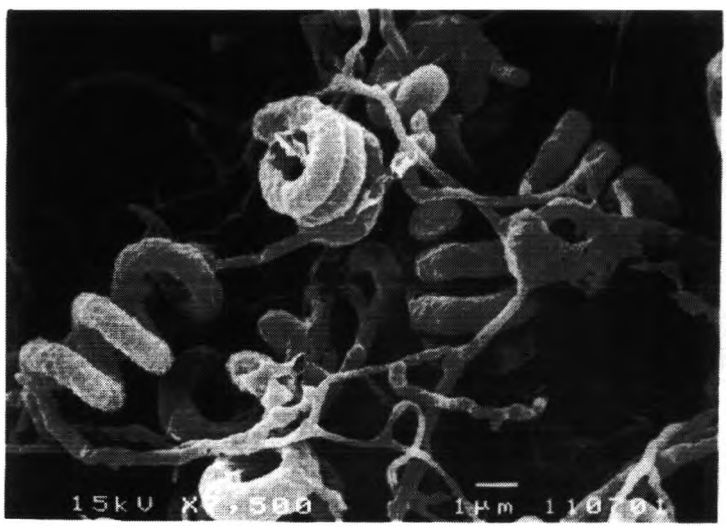

Figure 2 Scanning electron micrograph of spore-bearing substrate mycelium of *Streptomyces* sp. TRA 9875-2 on 10% YMA medium (20 days).

2.1.2 Cultural characteristics

Cultural characteristics of the strain TRA 9875-2 cultured on various media at room temperature for 14 days are shown in Table 4.1. The color determination of the strain was based on the Jacal Color Card L2200, Japan Color Research Institute.

Table 4.1 Cultural characteristics of *Streptomyces* sp. TRA 9875-2.

Medium	**Growth appearance**	**Color of reversed colony**	**Color of aerial mycelium**	**Color of spore**	**Color of soluble pigment**
Yeast extract - Malt extract agar	Good, smooth powdery	Light reddish yellow	White	Blackish gray	None
Oatmeal agar	Good, wrinkled	Pale yellow	Pale yellow	Black	None
Inorganic salt-Starch agar	Good, smooth powdery	Pale yellow	Grayish white	None	None
Glycerol-Asparagine agar	Good, smooth powdery	Pale yellow	Grayish yellow	Blackish gray	None
Tyrosine agar	Good, wrinkled	Grayish yellow	Light gray	Blackish gray	Brown

2.2 Physiological and biochemical characteristics

The physiological and biochemical characteristics of the strain TRA 9875-2 are shown in Table 4.2 and the utilization of carbon sources are shown in Table 4.3.

Table 4.2 Physiological and biochemical characteristics of *Streptomyces* sp. TRA 9875-2.

Characteristics	**Reaction**
Optimum temperature for growth	20 – 30°C
Nitrate reduction	Positive
Gelatin liquefaction	Positive
Starch hydrolysis	Positive
Coagulation of milk	Positive
Peptonization of milk	Positive
Melanin formation	Positive*
Tyrosinase reaction	Positive
Cellulolytic activity	Negative
Maximum NaCl tolerance	5%
Range of pH tolerance	5 – 11

* On Tyrosine agar.

Table 4.3 Utilization of carbon sources of *Streptomyces* sp. TRA 9875-2.

Sources of carbon	**Growth**
No carbon sources	Negative
D-Glucose, Raffinose, Rhamnose	Positive (+ + +)
L-Arabinose, D-Fructose, L-Inositol, D-Mannitol, Sucrose, D-Xylose	Positive (+ + + +)

2.3 Cell wall analysis

The chemical analysis of cell wall diaminopimelic acid (DAP) isomers revealed that the strain TRA 9875-2 contained L-isomer of diaminopimelic acid, indicating the type I cell wall.

The actinomycete strain TRA 9875-2 was identified as *Streptomyces* based on its morphological and cultural characteristics, including the cell wall chemical analysis.

3. Structure elucidation of the isolated compounds

Three compounds were isolated from the ethyl acetate extract of GPM and YM fermentation broths of *Streptomyces* sp. TRA 9875-2 after several chromatographic techniques. The ethyl acetate extract of GPM fermentation broth of the strain TRA 9875-2 gave two known compounds, geldanamycin (KTR 75001k, 174 mg, 1.2% of ethyl acetate extract, and 3.87×10^{-4}% w/v of GPM fermentation broth), 17-*O*-demethylgeldanamycin (KTR 75008k, 6 mg, 0.04% of ethyl acetate extract, and 1.33×10^{-5}% w/v of GPM fermentation broth) and a new compound, 17-*O*-demethyl-dihydrogeldanamycin (KTR 75010, 6 mg, 0.04% of ethyl acetate extract, and 1.33×10^{-5}% w/v of GPM fermentation broth) while that of YM fermentation broth of this strain gave only two known compounds, geldanamycin (358 mg, 14.6% of ethyl acetate extract, and 1.79×10^{-3}% w/v of YM fermentation broth) and 17-*O*-demethylgeldanamycin (16 mg, 0.65% of ethyl acetate extract, and 8×10^{-5}% w/v of YM fermentation broth). The yield percentage of these three compounds is based on the ethyl acetate extract (15.0 g of ethyl acetate extract of GPM fermentation broth and 2.46 g of ethyl acetate extract of YM fermentation broth) and volume of the fermentation broth (45 L of GPM fermentation broth and 20 L of YM fermentation broth), respectively. The chemical structures of these isolated compounds were determined by analyses of their spectroscopic data including UV, IR, MS, and NMR spectral data.

3.1 Structure elucidation of geldanamycin (KTR75001k)

Compound KTR75001k was obtained as a yellow amorphous powder showing optical rotation $[\alpha]^{25}_{D}$ +46.0° (c, 0.500, $CHCl_3$). The IR absorption spectrum (Figure 12) displayed characteristic bands at 3,452 cm^{-1} (O-H stretching), 1,692 cm^{-1} (C=O stretching) and 1,651 cm^{-1} (C=O stretching, amide I band). The UV spectrum in MeOH (Figure 11) of compound KTR75001k exhibited λ_{max} (ϵ) at 256 (10,201), 304 (11,870) and a broad weak shoulder at 400 (560) nm. The HRFABMS of this compound exhibited the pseudomolecular ion peak $[M+Na]^+$ at *m/z* 583.2587 (calculated for $C_{29}H_{40}N_2O_9Na$ at 583.2632). However, the FABMS (Figure 13) of this compound showed a peak at *m/z* 563 as the most abundant mass ion. Since the

NMR spectroscopic data suggested that this compound contained a quinone moiety, and it is known that some quinones could yield apparent $M^+ + 2$ peaks in the ion source of the mass spectrometer (Sasaki *et al.*, 1970; Morisaki *et al.*, 1996), therefore the molecular formula of this compound should be $C_{29}H_{40}N_2O_9$ with the MW 560.

The 300 MHz ^{1}H-NMR spectrum of compound KTR75001k in $CDCl_3$ (Figure 14) revealed that this compound contained four methyl proton signals at δ 0.92, 0.93, 1.75, and 1.98 ppm; three methoxy proton signals at δ 3.25, 3.31, and 4.08 ppm; two methylene proton signals at δ 1.73 and 2.41 ppm; eleven methine proton signals at δ 1.93, 2.73, 3.35, 3.48, 4.29, 5.14, 5.77, 5.83, 6.53, 6.90, and 7.22 ppm; and three exchangeable proton signals at δ 3.01, 4.80, and 8.67 ppm. The 75 MHz ^{13}C-NMR spectrum of compound KTR75001k in $CDCl_3$ (Figure 15) gave twenty-nine carbon signals, consistent with the molecular formula. The carbon signals were classified by the DEPT 135 spectrum (Figure 16) and HMQC spectrum (Figures 17-19) as four methyl carbon signals at δ 12.50, 12.66, 13.01, and 23.06 ppm; three methoxy carbon signals at δ 56.77, 57.31, and 61.71 ppm; two methylene carbon signals at δ 32.85 and 34.76 ppm; eleven methine carbon signals at δ 27.99, 32.28, 72.62, 80.93, 81.23, 81.64, 11.63, 126.18, 127.07, 132.97, and 136.25 ppm; and nine quaternary carbon signals at δ 127.42, 133.11, 134.66, 137.86, 155.77, 156.75, 167.99, 183.88, and 184.70 ppm. Analysis of the ^{13}C-NMR spectrum (Table 4.4) indicated the presence of two quinone carbonyl carbons at δ 183.88 and 184.70 ppm, one amide carbonyl carbon at δ 167.99 ppm, and one carbamate carbon at δ 155.77 ppm.

Analyses of the ^{1}H-^{1}H COSY (Figure 20) and TOCSY (Figure 21) spectra of KTR75001k recorded in $CDCl_3$ established the partial structures from H-3 to H-7, H-9 to H-13 and 14-Me to H-15. There was no correlation between H-13 to H-14 from the spectra in $CDCl_3$, however the correlation could be observed in pyridine-d_5 as shown in Figure 22 and summarized in Figure 3.

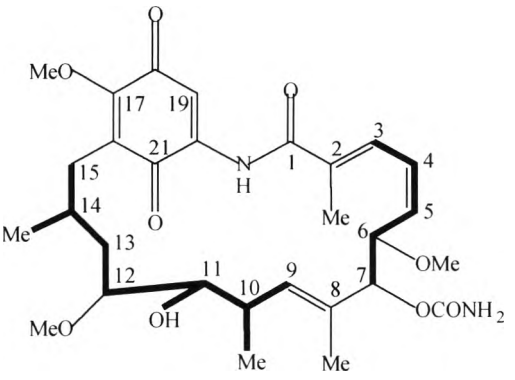

Figure 3 The ^{1}H-^{1}H correlations (bold line) in the ^{1}H-^{1}H COSY and TOCSY spectra of KTR75001k.

The complete ^{13}C assignments of KTR75001k were obtained from the HMBC spectra ($^{n}J_{HC}$ = 8 Hz and 4 Hz) (Figures 23-24) showing the following long-range correlations; NH (δ 8.67) to amide carbonyl carbon C-1 (δ 167.99) and quinone carbonyl carbon C-21 (δ 184.70); 2-Me (δ 1.98) to C-1 (δ 167.99), C-2 (δ 134.66), and C-3 (δ 127.07); 8-Me (δ 1.75) to C-7 (δ 81.64), and C-8 (δ 133.11); 6-OMe (δ 3.25) to C-6 (δ 81.23); 12-OMe (δ 3.31) to C-12 (δ 80.93); 17-OMe (δ 4.08) to C-17 (δ 156.75); H-7 (δ 5.14) to carbamate carbonyl carbon (δ 155.77), C-8 (δ 133.11), and C-8-Me (δ 13.01); H-10 (δ 2.73) to C-8 (δ 133.11); H-12 (δ 3.35) to C-14 (δ 27.99); 2H-15 (δ 2.41) to C-17 (δ 156.75), and quinone carbonyl carbon C-18 (δ 183.88); and H-19 (δ 7.22) to C-17 (δ 156.75), C-20 (δ 137.86), and C-21 (δ 184.70). The ^{1}H-^{13}C long-range correlations from the HMBC spectrum of KTR75001k in $CDCl_3$ are shown in Figure 4 and summarized in Table 4.4.

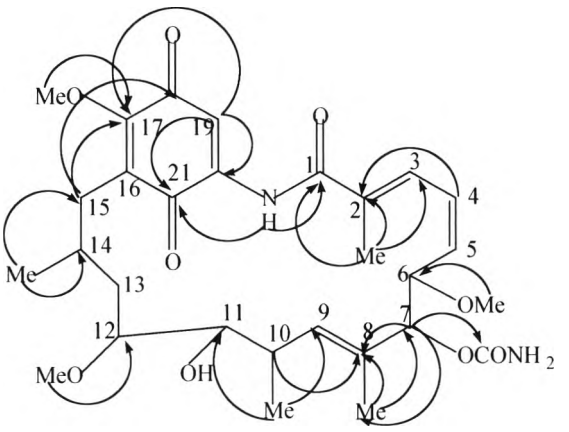

Figure 4 The important ^{1}H-^{13}C long-range correlations in the HMBC spectrum of KTR75001k in $CDCl_3$.

Table 4.4 The ^{1}H, ^{13}C-NMR and HMBC spectral data of KTR75001k in $CDCl_3$.

Position	KTR75001k			
	δ_C (ppm)	δ_H (ppm), mult, (*J* in Hz)	Long-range correlations in HMBC spectra	
			$^{n}J_{HC}$ = 8 Hz	$^{n}J_{HC}$ = 4 Hz
1	167.99	-	-	-
2	134.66	-	-	-
2-Me	12.50	1.98, 3H, s	C-1, C-2, C-3	C-1, C-2, C-3
3	127.07	6.90, d, (12)	-	-
4	126.18	6.53, t, (12, 11)	-	C-2, C-4
5	136.25	5.83, t, (11, 10)	-	-
6	81.23	4.29, d, (10)	-	-
6-OMe	57.31	3.25, 3H, s	C-6	C-6
7	81.64	5.14, brs	7-O<u>C</u>O, C-8	7-O<u>C</u>O, C-8, C-8-Me
7-O<u>C</u>O<u>NH</u>$_2$	155.77	4.80, NH_2, brs	-	-
8	133.11	-	-	-
8-Me	13.01	1.75, 3H, s	C-7, C-8	C-7, C-8
9	132.97	5.77, d, (10)	-	-
10	32.28	2.73, m	-	C-8
10-Me	12.66	0.93, 3H, d, (7)	C-9, C-11	C-9, C-11
11	72.62	3.48, m	-	-
11-OH	-	3.01, OH, brs	-	-
12	80.93	3.35, m	-	C-14
12-OMe	56.77	3.31, 3H, s	C-12	C-12
13	34.76	1.73, 2H, m	-	-
14	27.99	1.63, brs	-	-
14-Me	23.06	0.92, 3H, d, (6)	C-14, C-15	C-14, C-15
15	32.85	2.41, 2H, m	-	C-14, C-17, C-18
16	127.42	-	-	-
17	156.75	-	-	-
17-OMe	61.71	4.08, 3H, s	C-17	C-17
18	183.88	-	-	-
19	111.63	7.22, s	C-17, C-21	C-20, C-21
20	137.86	-	-	-
21	184.70	-	-	-
NH	-	8.67, NH, brs	-	C-1, C-21

Table 4.5 Comparison of ^{1}H and ^{13}C-NMR spectral data of KTR75001k with that of geldanamycin.

Position	KTR75001k[a]		Geldanamycin	
	δ_C (ppm)	δ_H (ppm),mult,(*J* in Hz)	δ_C (ppm)[b]	δ_H (ppm), mult[c]
1	167.99	-	169.1	-
2	134.66	-	133.2	-
2-Me	12.50	1.98, 3H, s	12.2	1.91, 3H
3	127.07	6.90, d, (12)	128.4	6.95, d
4	126.18	6.53, t, (12, 11)	125.7	6.56, t
5	136.25	5.83, t, (11, 10)	137.8	5.80, t
6	81.23	4.29, d, (10)	81.6	4.34, d
6-OMe	57.31	3.25, 3H, s	56.0	3.22, 3H, s*
7	81.64	5.14, brs	80.6	4.86, brs
7-$OCONH_2$	155.77	4.80, NH_2, brs	156.0	6.45, HH_2, brs
8	133.11	-	132.6	-
8-Me	13.01	1.75, 3H, s	12.5	1.61, 3H, brs
9	132.97	5.77, d, (10)	131.9	5.51, d
10	32.28	2.73, m	32.1	3.61
10-Me	12.66	0.93, 3H, d, (7)	23.3	0.97, 3H, d#
11	72.62	3.48, m	71.9	3.29**
11-OH	-	3.01, OH, brs	-	-
12	80.93	3.35, m	80.2	3.07**
12-OMe	56.77	3.31, 3H, s	56.5	3.23, 3H, s*
13	34.76	1.73, 2H, m	31.0	1.45, 2H, brs
14	27.99	1.63, brs	26.6	1.91
14-Me	23.06	0.92, 3H, d, (6)	13.0	0.76, 3H#
15	32.85	2.41, 2H, m	31.7	2.42, 2H, m
16	127.42	-	128.1	2.42, m
17	156.75	-	156.4	-
17-OMe	61.71	4.08, 3H, s	61.0	3.93, 3H, s
18	183.88	-	183.6	-
19	111.63	7.22, s	110.9	7.02, s
20	137.86	-	139.6	-
21	184.70	-	183.1	-
NH	-	8.67, NH, brs	-	9.14, NH, brs

[a] observed in $CDCl_3$ and recorded at 300 MHz (^{1}H) and 75 MHz (^{13}C).

[b] observed in DMSO-d_6 (Omura *et al.*, 1979).

[c] observed in DMSO-d_6 (Rinehart, Jr. and Shield, 1976).

*, **, # interchangeable signals.

The presence of a hydroxyl group in the structure of KTR75001k was confirmed by acetylation which produced an acetylated derivative. The 300 MHz ^{1}H-NMR spectrum of this acetylated derivative in $CDCl_3$+benzene-d_6 is shown in Figure 27. The proton resonance at C-11 position was shifted downfield approximately 1.5 ppm. The FABMS (Figure 28) of this compound revealed the pseudomolecular ion peak $[M+2+H]^+$ at *m/z* 605. On the basis of these spectral data, compound KTR75001k has only one hydroxyl group in the molecule at position C-11.

The geometries of double bonds at C-2=C-3, C-4=C-5, and C-8=C-9 were determined by NOESY experiment (Figures 25-26). The NOESY spectral data of compound KTR75001k was observed in pyridine-d_5. The present correlations of 2-Me to H-4, H-4 to H-5, H-3 to H-6 and H-7, and H-9 to H-7 and the absent correlations of H-3 to 2-Me and H-9 to 8-Me suggested that geometries of double bonds at C-2=C-3 and C-8=C-9 are *E* and C-4=C-5 is *Z* and the preferred conformation is shown in Figure 5.

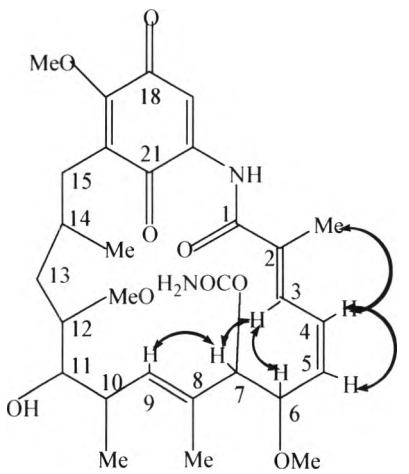

Figure 5 The chemical structure of geldanamycin (KTR75001k).

Comparison the spectral data of compound KTR75001k to those reported in the literatures (Rinehart, Jr. and Shield, 1976; Omura *et. al.*, 1979) confirmed that it is the known compound, geldanamycin (Table 4.5). Geldanamycin was obtained from the culture broth of *Streptomyces hygroscopicus* var. *geldanus* in 1970 (DeBoer *et al.*, 1970). However, based upon the HMBC spectra, the ^{13}C

chemical shifts of C-10-Me and C-14-Me for this compound should be revised (at δ 12.66 ppm for C-10-Me and 23.06 ppm for C-14-Me).

3.2 Structure elucidation of 17-*O*-demethylgeldanamycin (KTR75008k)

Compound KTR75008k was obtained as orange needles with optical rotation $[\alpha]^{25}_{D}$ +15.5° (c, 0.100, MeOH). The HRFABMS established the molecular formula of this compound as $C_{28}H_{38}N_2O_9$, showing the pseudomolecular ion peak $[M+Na]^+$ at *m/z* 569.2479 (calculated for $C_{28}H_{38}N_2O_9Na$ at 569.2475). However, the FABMS (Figure 33) also showed the pseudomolecular ion peak $[M+2+H]^+$ at *m/z* 549. The molecular formula of this compound should be $C_{28}H_{38}N_2O_9$ with MW 546. The IR absorption spectrum (Figure 32) showed characteristic bands at 3,431 cm^{-1} (O-H stretching), 1,689 cm^{-1} (C=O stretching) and 1,639 cm^{-1} (C=O stretching, amide I band). The UV absorption spectrum in MeOH of compound KTR75008k (Figure 31) showed maxima absorption (ϵ) at 235 nm (20,219) and 328 nm (24,590).

The 300 MHz ^{1}H-NMR spectrum of KTR75008k in $CDCl_3$ + DMSO-d_6 (Figure 34) presented four methyl proton signals at δ 0.81, 0.91, 1.67, and 1.92 ppm; two methoxy proton signals at δ 3.20 and 3.24 ppm; two methylene proton signals at 1.60 and 2.31 ppm; eleven methine proton signals at 1.77, 2.69, 3.33, 3.49, 4.23, 5.01, 5.69, 5.80, 6.46, 6.91, and 7.17 ppm; and two exchangeable proton signals at 5.43 and 8.90 ppm. The 75 MHz ^{13}C-NMR spectrum in $CDCl_3$ + DMSO – d_6 (Figure 35) showed twenty five carbon signals which were classified by the DEPT 135 (Figure 36) as three methyl signals at δ 12.19, 12.60, and 23.22 ppm; two methoxy carbon signals at δ 56.29 and 56.85 ppm; two methylene carbon signals at δ 32.06 and 33.09 ppm; eleven methine carbon signals at δ 27.31, 31.87, 71.99, 80.53, 80.97, 81.48, 108.18, 125.48, 127.28, 132.28, and 136.78 ppm; and seven quaternary carbon signals at δ117.26, 132.74, 133.62, 139.50, 155.77, 167.82, and 183.16 ppm. Analyses of the HMQC spectrum (Figure 37) indicated that this compound contained two equivalent methyl carbon signals at δ 12.19 ppm (C-2-Me and C-10-Me), two equivalent quaternary carbon signals at δ 155.77 of which one is identified as carbamate carbonyl carbon (C-7-$OCONH_2$) and the other is an ordinary quaternary carbon (C-

17), and two equivalent quinone carbonyl carbons at δ 183.16 ppm (C-18 and C-21). This compound also contained one amide carbonyl carbon at δ 167.82 ppm (C-1).

The ^{1}H-^{1}H COSY spectrum of KTR75008k (Figure 38) revealed the similarity of the lactam ring of its chemical structure to that of geldanamycin (KTR75001k). The assignments of the partial structures from H-3 to H-7, H-9 to H-13, and 14-Me to H-15 are shown in Figure 6.

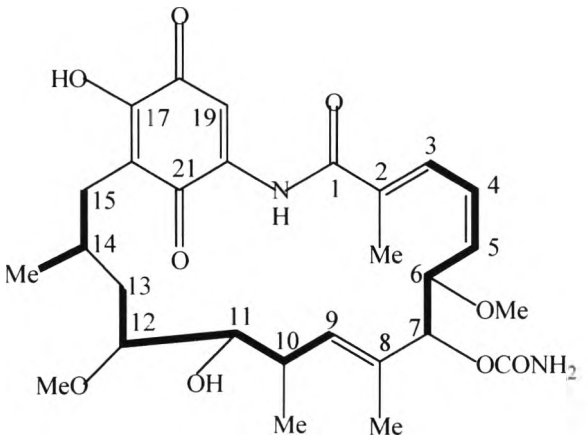

Figure 6 The ^{1}H-^{1}H correlations (bold line) in the ^{1}H-^{1}H COSY spectrum of KTR75008k in $CDCl_3$+DMSO-d_6.

The absence of 17-OMe signal in the ^{1}H and ^{13}C-NMR spectra of this compound was the main difference from those of geldanamycin (KTR75001k). The observation suggested that it was 17-*O*-demethyl derivative of geldanamycin (KTR75001k).

The HMBC spectrum ($^{n}J_{HC}$ = 8 Hz) of KTR75008k (Figures 39-42) exhibited long-range correlations of 2-Me (δ 1.92) to C-1 (δ 167.82), C-2 (δ 133.62), and C-3 (δ 127.28); 8-Me (δ 1.67) to C-7 (δ 80.97), and C-8 (δ 132.74); 6-OMe (δ 3.20) to C-6 (δ 56.85); 12-OMe (δ 3.24) to C-12 (δ 80.53); H-7 (δ 5.01) to carbamate carbonyl carbon (δ 155.77), and C-9 (δ 132.28); 14-Me (δ 0.91) to C-13 (δ 33.09); and H-19 (δ 7.17) to C-17 (δ 155.77), C-18 (δ 183.16), C-20 (δ 139.50), and C-21 (δ 183.16). The ^{1}H-^{13}C long-range correlations from the HMBC spectrum of KTR75008k are shown in Figure 7 and summarized in Table 4.6.

Table 4.6 The ^{1}H, ^{13}C-NMR and HMBC spectral data of KTR75008k in $CDCl_3$+DMSO-d_6.

Position	KTR75008k		
	δ_C (ppm)	δ_H (ppm), mult, (*J* in Hz)	Long-range correlations in HMBC spectrum ($^{n}J_{HC}$=8 Hz)
1	167.82	-	-
2	133.62	-	-
2-Me	12.19	1.92, 3H, s	C-1, C-2, C-3
3	127.28	6.91, d, (12)	-
4	125.48	6.46, t, (12, 11)	-
5	136.78	5.80, t, (11, 9)	-
6	81.48	4.23, d, (9)	-
6-OMe	56.85	3.20, 3H, s	C-6
7	80.97	5.01, brs	7-OCO, C-9
7-OCONH$_2$	155.77	5.43, NH$_2$, brs	-
8	132.74	-	-
8-Me	12.60	1.67, 3H, s	C-7, C-8
9	132.28	5.69, d, (9)	-
10	31.87	2.69, m	-
10-Me	12.19	0.81, 3H, d, (7)	C-9, C-10, C-11
11	71.99	3.49, m	-
12	80.53	3.33, m	-
12-OMe	56.29	3.24, 3H, s	C-12
13	33.09	1.60, 2H, brs	-
14	27.31	1.77, m	-
14-Me	23.22	0.91, 3H, d, (6)	C-13, C-14, C-15
15	32.06	2.31, 2H, m	-
16	117.26	-	-
17	155.77	-	-
18	183.16	-	-
19	108.18	7.17, s	C-17, C-18, C-20, C-21
20	139.50	-	-
21	183.16	-	-
NH	-	8.90, NH, brs	-

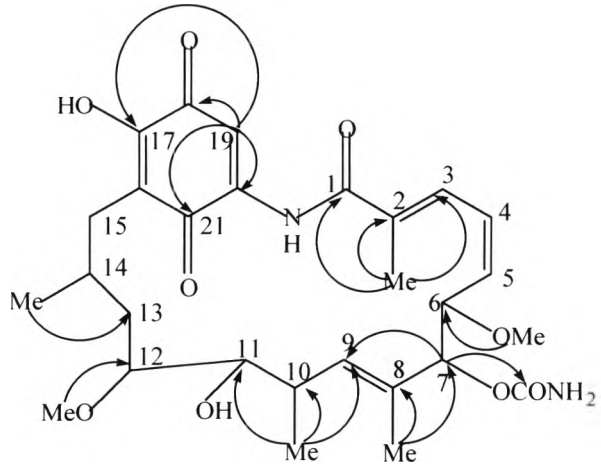

Figure 7 The important ^{1}H-^{13}C long-range correlations in the HMBC spectrum of KTR75008k in $CDCl_3$+DMSO-d_6.

The geometries of double bonds at C-2=C-3, C-4=C-5, and C-8=C-9 were determined by NOESY experiment (Figures 41-42). The NOESY spectral data of compound KTR75008k was observed in $CDCl_3$+DMSO-d_6. The present correlations of 2-Me to H-4, H-4 to H-5, H-3 to H-6, and H-9 to H-7 and the absent correlations of H-3 to 2-Me and H-9 to 8-Me suggested that geometries of double bonds at C-2=C-3 and C-8=C-9 are *E* and C-4=C-5 is *Z* and its preferred conformation is shown in Figure 8.

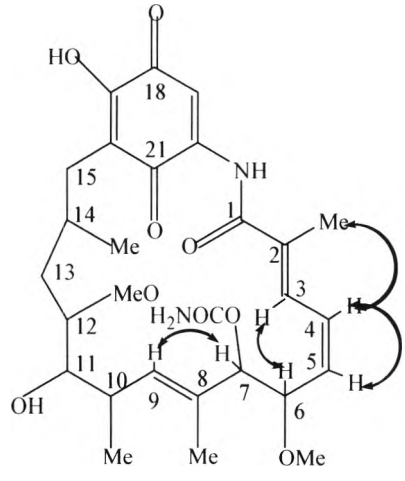

Figure 8 The chemical structure of 17-*O*-demethylgeldanamycin (KTR75008k).

The ^{1}H and ^{13}C-NMR spectral data of KTR75008k as summarized in Table 4.8 indicated that this compound is structurally similar to geldanamycin. The

absence of 17-OMe of this compound was identical to 17-*O*-demethylgeldanamycin which was previously reported as a demethylation product of geldanamycin by the reaction with barium hydroxide ($Ba(OH)_2$) (Rinehart, Jr. and Shield, 1976). Compound KTR75008k is therefore identified as the known compound, 17-*O*-demethyl-geldanamycin, which is found for the first time as a naturally occurring substance produced by the mangrove *Streptomyces* sp. TRA 9875-2. Moreover, the present work successfully established the assignments of protons and carbons in 17-*O*-demethyl-geldanamycin.

3.3 Structure elucidation of 17-*O*-demetyldihydrogeldanamycin (KTR 75010)

Compound KTR 75010 was obtained as violet amorphous solid with optical rotation $[\alpha]^{25}_{D}$ +20.5° (c, 0.100, MeOH). The UV absorption spectrum of KTR 75010 obtained from methanol solution revealed maxima absorption (ϵ) at 326 nm (15,288) and weak absorption at 596 nm (575) (Figure 45). The IR absorption spectrum presented characteristic bands at 3,423 cm^{-1} (O-H stretching), 1,719 cm^{-1} (C=O stretching, amide I band) and 1,636 cm^{-1} (C=O stretching, lactam ring) (Figure 46). The ESI TOFMS (Figure 47) showed the pseudomolecular ion peak $[M+Na]^+$ at *m/z* 571, and the based peak at *m/z* 569 $[M-2+Na]^+$. The HR ESI TOFMS showed the pseudomolecular ion peak $[M+Na]^+$ at *m/z* 571.2649 (calculated for $C_{28}H_{40}N_2O_9Na$ at 571.2632).

The 300 MHz ^{1}H-NMR spectrum of compound KTR75010 in $CDCl_3$ + DMSO-d_6 (Figure 48) revealed four methyl proton signals at δ 0.81, 0.83, 1.66, and 1.92 ppm; two methoxy proton signals at δ 3.14 and 3.22 ppm; two methylene proton signals at δ 0.99 and 2.24 ppm; eleven methine proton signals at δ 1.70, 2.59, 3.23, 3.41, 4.25, 4.93, 5.73, 5.77, 6.51, 6.89, and 6.98 ppm; and two exchangeable proton signals at δ 6.09 and 9.58 ppm. The 75 MHz ^{13}C-NMR spectrum in $CDCl_3$ + DMSO – d_6 (Figure 49) showed twenty three carbon signals which were classified by the DEPT 135 spectrum (Figure 50) as three methyl carbon signals at δ 12.43, 12.84, and 23.68 ppm; two methoxy carbon signals at δ 56.21 and 56.39 ppm; two methylene carbon signals at δ 29.30 and 33.22 ppm; ten methine carbon signals at δ 27.73, 31.97, 71.76, 80.51, 81.38, 106.36, 125.98, 126.82, 132.47, and 136.05 ppm; and six quaternary carbon signals at δ 113.57, 132.84, 134.00, 143.82, 156.18, and 167.81 ppm. Analyses of the HMQC

spectrum (Figures 51-55) indicated that this compound contained two equivalent methyl carbon signals at δ 12.43 ppm (C-2-Me and C-10-Me), two equivalent methine carbon signals at δ 81.38 ppm (C-6 and C-12).

The ^{1}H-^{1}H COSY spectrum of KTR75010 (Figures 56-57) gave the similar proton-proton connections of the chemical structure of KTR75010 to that of 17-*O*-demethylgeldanamycin (KTR75008k). The partial structures of KTR75010 from H-3 to H-7, H-9 to H-13 and 14-Me to H-15 are shown in Figure 9.

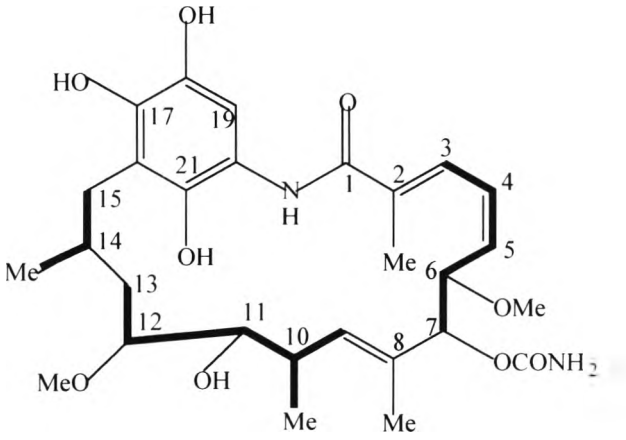

Figure 9 The ^{1}H-^{1}H correlations (bold line) in the ^{1}H-^{1}H COSY spectrum of KTR75010 in $CDCl_3$+DMSO-d_6.

The complete assignments of KTR75010 were achieved by analyses of the HMBC ($^{n}J_{HC}$ = 8 Hz and 4 Hz) spectra, which demonstrated long-range correlations of 2-Me (δ 1.92) to C-1 (δ 167.81), C-2 (δ 134.00), C-3 (δ 122.29), and C-4 (δ 125.98); 8-Me (δ 1.67) to C-7 (δ 80.51) and C-8 (δ 132.84); 10-Me (δ 0.81) to C-9 (δ 132.47), C-10 (δ 31.91), and C-11 (δ 71.76); 14-Me (δ 0.83) to C-13 (δ 29.30) and C-15 (δ 33.22); 6-OMe (δ 3.14) to C-6 (δ 81.38); 12-OMe (δ 3.22) to C-12 (δ 81.38); H-19 (δ 6.89) to C-17 (δ 143.82), C-18 (δ 156.18), C-20 (δ132.84), and C-21 (δ 143.82); H-7 (δ 4.93) to carbamate carbonyl carbon (δ 156.81), C-8 (δ 132.84), and C-9 (δ 132.47); and NH (δ 9.58) to C-21 (δ 143.82). The HMBC correlations finally revealed two equivalent quaternary carbon signals at δ 132.84 ppm (C-8 and C-20), two equivalent quaternary carbon signals at δ 143.82 ppm (C-17 and C-21), and two equivalent quaternary signals at δ 156.18 ppm (C-7-$OCONH_2$, C-18). The carbon signal

Table 4.7 The ^{1}H, ^{13}C-NMR and HMBC spectral data of KTR75010 in $CDCl_3$+DMSO-d_6.

Position	KTR75010			
	δ_C (ppm)	δ_H (ppm),mult,(*J* in Hz)	Long-range correlations in HMBC spectra	
			$^{n}J_{HC}$=8 Hz	$^{n}J_{HC}$=4 Hz
1	167.81	-	-	-
2	134.00	-	-	-
2-Me	12.43	1.92, 3H, s	C-1, C-2, C-3	C-1, C-2, C-4
3	126.82	6.98, d, (11)	-	-
4	125.98	6.51, t, (11, 11)	-	-
5	136.05	5.77, t, (11, 10)	-	-
6	81.38	4.25, d, (10)	-	-
6-OMe	56.39	3.14, 3H, s	C-6	C-6
7	80.51	4.93, brs	7-OCO, C-8, C-9	7-OCO, C-9
7-OCONH$_2$	156.18	6.09, NH$_2$, brs	-	-
8	132.84	-	-	-
8-Me	12.84	1.66, 3H, s	C-7, C-8	C-7, C-8
9	132.47	5.73, d, (9)	-	-
10	31.91	2.59, m	-	-
10-Me	12.43	0.81, 3H, d, (7)	C-9, C-11	C-9, C-10
11	71.76	3.41	-	-
12	81.38	3.23	-	-
12-OMe	56.21	3.22, 3H, s	C-12	C-12
13	29.30	0.99, 2H, brs	-	-
14	27.73	1.70	-	-
14-Me	23.68	0.83, 3H, d, (7)	C-13	C-15
15	33.22	2.24, 2H, m	-	-
16	113.57	-	-	-
17	143.82	-	-	-
18	156.18	-	-	-
19	106.36	6.89, s	C-17, C-21	C-18, C-20
20	132.84	-	-	-
21	143.82	-	-	-
NH	-	9.58, NH, brs	C-21	-

Table 4.8 The 300 MHz ^{1}H-NMR and 75 MHz ^{13}C-NMR spectral data of geldanamycin, 17-*O*-demethylgeldanamycin, and 17-*O*-demethyldihydrogeldanamycin.

Position	Geldanamycin[a]		17-*O*-demethylgeldanamycin[b]		17-*O*-demethyldihydro-geldanamycin[b]	
	δ_C(ppm)	δ_H(ppm),mult, (*J* in Hz)	δ_C(ppm)	δ_H(ppm),mult, (*J* in Hz)	δ_C(ppm)	δ_H(ppm),mult, (*J* in Hz)
1	167.99	-	167.82	-	167.81	-
2	134.66	-	133.62	-	134.00	-
2-Me	12.50	1.98, 3H ,s	12.19	1.92, 3H, s	12.43	1.92, 3H, s
3	127.07	6.90, d, (12)	127.28	6.91, d, (12)	126.82	6.98, d, (11)
4	126.18	6.53, t, (12, 11)	125.48	6.46, t, (12, 11)	125.98	6.51, t, (11, 11)
5	136.25	5.83, t, (11, 10)	136.78	5.80, t, (11, 9)	136.05	5.77, t, (11, 10)
6	81.23	4.29, d, (10)	81.48	4.23, d, (9)	81.38	4.25, d, (10)
6-OMe	57.31	3.25, 3H, s	56.85	3.20, 3H, s	56.39	3.14, 3H, s
7	81.64	5.14, brs	80.97	5.01, brs	80.51	4.93, brs
7-OCONH$_2$	155.77	4.80, NH$_2$, brs	155.77	5.43, NH$_2$, brs	156.18	6.09, NH$_2$, brs
8	133011	-	132.74	-	132.84	-
8-Me	13.01	1.75, 3H, s	12.60	1.67, 3H, s	12.84	1.66, 3H, s
9	132.97	5.77, d, (10)	132.28	5.69, d, (9)	132.47	5.73, d, (9)
10	32.28	2.73, m	31.87	2.69, m	31.91	2.59, m
10-Me	12.66	0.93, 3H, d, (7)	12.19	0.81, 3H, d, (7)	12.43	0.81, 3H, d, (7)
11	72.62	3.48, m	71.99	3.49, m	71.76	3.41
12	80.93	3.35, m	80.53	3.33, m	81.38	3.23
12-OMe	56.77	3.31, 3H, s	56.29	3.24, 3H, s	56.21	3.22, 3H, s
13	34.76	1.73, 2H, m	33.09	1.60, 2H, brs	29.30	0.99, 2H, brs
14	27.99	1.63, brs	27.31	1.77, m	27.73	1.70
14-Me	23.06	0.92, 3H, d, (6)	23.22	0.91, 3H, d, (6)	23.68	0.83, 3H, d, (7)
15	32.85	2.41, 2H, m	32.06	2.31, 2H, m	33.22	2.24, 2H, m
16	127.42	-	117.26	-	113.57	-
17	156.75	-	155.77	-	143.82	-
17-OMe	61.71	4.08, 3H, s	-	-	-	-
18	184.70	-	183.16	-	156.18	-
19	111.63	7.22, s	108.18	7.17, s	106.36	6.89, s
20	137.86	-	139.50	-	132.84	-
21	183.88	-	183.16	-	143.82	-
NH	-	8.67, NH, brs	-	8.90, NH, brs	-	9.58, NH, brs

[a]Data were observed in $CDCl_3$.

[b]Data were observed in CDCl3+DMSO-d_6.

at δ 167.81 ppm was identified as the amide carbonyl carbon (C-1). The ^{1}H and ^{13}C-NMR spectral data are summarized in Table 4.7. The long-range correlations between protons-carbons of KTR75010 by the HMBC spectra ($^{n}J_{HC}$ = 8 Hz and 4 Hz) (Figures 58-62) are summarized in Table 4.7 and shown in Figure 10.

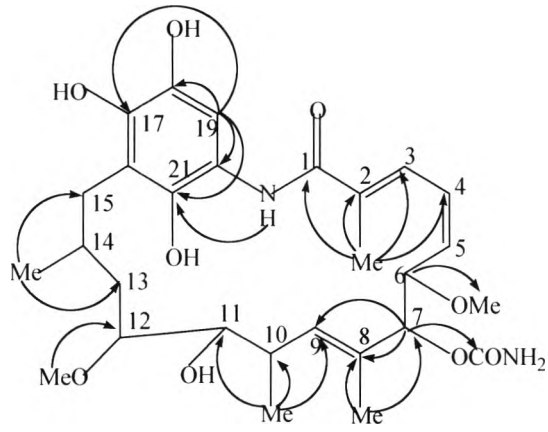

Figure 10 The important ^{1}H-^{13}C long-range correlations in the HMBC spectrum of KTR75010 in $CDCl_3$+DMSO-d_6.

Most of proton and carbon resonances in the ^{1}H and ^{13}C-NMR spectra of 17-*O*-demethylgeldanamycin and KTR75010 were similar. The upfield shift of H-19 from δ 7.17 of 17-*O*-demethylgeldanamycin to 6.89 of KTR75010 and the downfield shift of the amide proton from δ 8.90 of 17-*O*-demethylgeldanamycin to 9.58 of KTR75010 were the main ^{1}H-NMR spectral differences between KTR75010 and 17-*O*-demethylgeldanamycin (KTR75008k). The ^{13}C-NMR spectrum of this compound also showed major different signals from that of 17-*O*-demethylgeldanamycin (KTR75008k) as follows; the absence of the quinone carbonyl carbons at δ 183.16 ppm (C-18 and C-21) and the presence of the two oxygenated aromatic carbons at δ 143.82 ppm (C-21), and δ 156.18 ppm (C-18). These spectral data were the support evidence of the replacement of the hydroquinone part to the quinone part in compound KTR75010. Therefore, KTR75010 was identified as a new hydroquinone derivative, 17-*O*-demethyldihydro-geldanamycin.

Several hydroquinoid ansamycins, for example, ansatrienin B **[17]** produced by *Streptomyces collinus* (Damberg, Russ, and Zeeck, 1982), dihydro-herbimycins A-C **[25-27]** produced by *S. hygroscopicus* AM-3672 (Lin, Blasko, and

Cordell, 1988), and mecbecin II **[29]** produced by *Nocardia* sp. No. C-14919 (Muroi *et al.*, 1980; 1981) have been previously reported. Ansatienin B **[17]** and its benzoquinone derivative, ansatrienin A **[14]** were active against fungi (Damberg, Russ, and Zeeck, 1982; Lazar *et al.*, 1983). Dihydroherbimycins A-C **[25-27]** and their benzoquinone derivatives, herbimycins A-C **[22-24]** exhibited cytotoxic activity against P-388 and KB lymphocytic leukemia cell lines (Lin, Blasko, and Cordell, 1988). Macbecin II **[29]** and its benzoquinone derivative, macbecin I **[28]** displayed antitumor, antibacterial, antifungal, and antiprotozoal activities (Muroi *et al.*, 1980; 1981).

4. Biological Activities

Due to the screening test for antimicrobial activity of the crude ethyl acetate extract obtained from the GPM fermentation broth of *Streptomyces* sp. TRA 9875-2 showed antimicrobial activity against *Candida albicans* ATCC 10231 and *Staphylococcus aureus* ATCC 25923, the isolation and purification for the bioactive compound(s) produced by this strain was performed. Two known compounds, geldanamycin and 17-*O*-demethylgeldanamycin were isolated together with a new compound, 17-*O*-demethyldihydrogeldanamycin.

Geldanamycin displayed significant antifungal activity against *C. albicans* ATCC 10231 with an inhibition zone of 16.2 mm at concentration of 100 μg/disc, antimalarial activity against *Plasmodium falciparum* (K1, multidrug resistant strain) at EC_{50} of 0.063 μg/ml, and cytotoxic activity against KB and BC cell lines at ED_{50} of 1.1 μg/ml and 0.33 μg/ml, respectively, 11-*O*-methylgeldanamycin, obtained from methylation of geldanamycin showed antibacterial activity against *S. aureus* ATCC 25923 with an inhibition zone of 8.7 mm, while 17-*O*-demethylgeldanamycin, 17-*O*-demethyldihydrogeldanamycin, and 11-*O*-acetylgeldanamycin, obtained from acetylation of geldanamycin exhibited no antimicrobial activity at the same concentration as geldanamycin. However, 17-*O*-demethylgeldanamycin displayed weak cytotoxic activity against KB and BC cell lines at ED_{50} of 10.4 μg/ml and 3.1 μg/ml, respectively, 11-*O*-methylgeldanamycin exhibited antimalarial activity against *P. falciparum* at EC_{50} of 7.1 μg/ml and cytotoxic activity against BC cell line at ED_{50}

of 6.8 μg/ml but exhibited no cytotoxic activity against KB cell line, and 11-*O*-acetylgeldanamycin exhibited antimalarial activity against *P. falciparum* at EC_{50} of 11.7 μg/ml and cytotoxic activity against BC cell line at ED_{50} of 2.1 μg/ml. Due to the limited amount of sample available, 17-*O*-demethyl-dihydrogeldanamycin was not subjected to further biological activity study.

The results of antimicrobial activity of fractions and pure compounds obtained from *Streptomyces* sp. TRA 9875-2 are shown in Table 4.9, while the antimalarial activity and cytotoxic activity of the pure compounds are summarized in Table 4.10.

Table 4.9 Antimicrobial activity of fractions and the pure compounds obtained from *Streptomyces* sp. TRA 9875-2.

Fractions or compounds	Concentrations (μg/disc)	Inhibition Zone (mm)	
		C. albicans ATCC 10231	*S. aureus* ATCC 25923
EtOAc extract	1.000	17.5	10.1
CKTR 9875-2	1.000	17.1	-
Hexane extract	1.000	-	10.4
ME 9875-2	1.000	12.5	8.7
ME75001	1.000	-	13.2
ME75002	1.000	15.8	7.6
ME75003	1.000	-	-
ME75004	1.000	-	-
ME75005	100	16.0	6.4
ME75006	100	-	-
ME75007	100	-	-
ME75008	100	-	-
ME75009	100	-	-
ME75010	100	16.5	6.6
ME75011	100	-	-
ME75012	100	-	9.1
ME75013	100	-	7.8
KTR75001	ND	ND	ND
KTR75002	100	-	-
KTR75003	100	-	-
KTR75004	100	-	6.7
KTR75005	ND	ND	ND
KTR75006	ND	ND	ND
KTR75007	ND	ND	ND

Table 4.9 (continued)

Fractions or compounds	Concentrations (μg/disc)	Inhibition Zone (mm)	
		C. albicans ATCC 10231	*S. aureus* ATCC 25923
KTR75008	ND	ND	ND
KTR75009	ND	ND	ND
KTR75011	ND	ND	ND
YMTR 9875-2	1.000	16.6	22.3
YMTR75001	1.000	-	29.3
YMTR75002	1.000	23.2	19.0
YMTR75003	1.000	19.4	6.3
YMTR75004	1.000	17.2	7.8
YMTR75005	1.000	-	6.5
YMTR75006	ND	ND	ND
YMTR75007	1.000	17.2	-
YMTR75008	ND	ND	ND
YMTR75009	ND	ND	ND
YMTR75010	100	13.4	-
YMTR75011	ND	ND	ND
YMTR75012	ND	ND	ND
YMTR75013	100	-	-
YMTR75014	ND	ND	ND
YMTR75015	ND	ND	ND
YMTR75016	ND	ND	ND
YMTR75018	ND	ND	ND
YMTR75019	100	-	-
YMTR75020	100	-	-
YMTR75021	100	-	-
YMTR75022	100	-	-
YMTR75023	100	-	-
YMTR75024	100	-	-
Geldanamycin	100	16.2	-
17-*O*-demethylgeldanamycin	100	-	-
17-*O*-demethyldihydro-geldanamycin	100	-	-
11-*O*-methylgeldanamycin	100	-	8.7
11-*O*-acetylgeldanamycin	100	-	-

ND. Not determined

Table 4.10 Antimalarial and cytotoxic activities of the pure compounds from *Streptomyces* sp. TRA 9875-2.

Compounds	Cytotoxic activity ED_{50} (μg/ml)		Antimalarial activity EC_{50} (μg/ml)
	KB cell line	BC cell line	
geldanamycin	1.1	0.33	0.063
11-*O*-methylgeldanamycin	-	6.8	7.1
11-*O*-acetylgeldanamycin	ND	2.1	11.7
17-*O*-demethylgeldanamycin	10.4	3.1	ND
17-*O*-demthyldihydrogeldanamycin	ND	ND	ND

ND, Not determined

According to the biological activities of geldanamycin and its derivative, suggest that geldanamycin is the most active compound of these five compounds and the functional groups at C-11 and C-17 positions are important for their biological activities. The replacement of a methoxyl group for a hydroxyl group at C-11 position resulted in antibacterial activity instead of antifungal activity, while the replacement of an acetoxyl group for a hydroxyl group at C-11 position exhibited no antimicrobial activity. The replacement of a methoxyl group or an acetoxyl group for a hydroxyl group at C-11 position resulted in decreasing of antimalarial and cytotoxic activities. The replacement of hydroxyl group for methoxyl group at C-17 position led to decrease in cytotoxic activity and established no antimicrobial activity.

Geldanamycin (KTR75001k) has been previously isolated from the terrestrial *Streptomyces hygroscopicus* var. *geldanus* in 1970 as moderately active *in vitro* against protozoa, bacteria, and fungi. It is active against L-1210 and KB cells growing in culture and against the parasite *Syphacia oblevata*, *in vivo* (DeBoer *et al.*, 1970). It is also reported as a potent antitumor (Supko *et al.*, 1995), an inhibitor of proto-oncogenic protein kinases, such as erbB2 (Chavany *et al.*, 1996), EGF receptor tyrosine kinase and non-receptor tyrosine kinases such as v-src (Whitesell and Cook, 1996). In addition, it is a potent inhibitor of the nuclear hormone receptor family (Stebbins *et al.*, 1997). Geldanamycin also binds specifically to the heat shock protein Hsp 90 (Chavany *et al.*, 1996; Whitesell *et al.*, 1994) and to its endoplasmic

recticulum homologue GP96 (Chavany *et al.*, 1996), and thus interferes with conformational maturation of proteins (Whitesell *et al.*, 1994) and the cellular stress response (Schneider *et al.*, 1996).

Since there was a report about the hydroquinones, dihydroherbimycins A-C **[25-27]** showed weaker cytotoxic activity against P-388 and KB lymphocytic leukemia cell lines than their benzoquinone derivatives, herbimycins A-C **[22-24]** (Lin, Blasko, and Cordell, 1988). The activity of 17-*O*-demethyldihydro-geldanamycin was suggested to be weaker than its benzoquinone derivatives, geldanamycin and 17-*O*-demethylgeldanamycin.

The fractions showed antibacterial activity against *Staphylococcus aureus* ATCC 25923 are on further investigation for the active compound(s).