CHAPTER IV

RESULTS AND DISCUSSION

1. Isolation and identification of bacteria

The bacterium, S9730, was isolated from an unidentified hydroid. This strain is gram-negative, rod sphere (0.5-0.8 × 1.8-2.5 μm) (Figure 1) and motile by polar flagella. It shows circular, smooth, yellow orange colony on marine agar (Figure 2). Morphological, cultural, physiological and biochemical characteristics of S9730 are shown in Table 7.

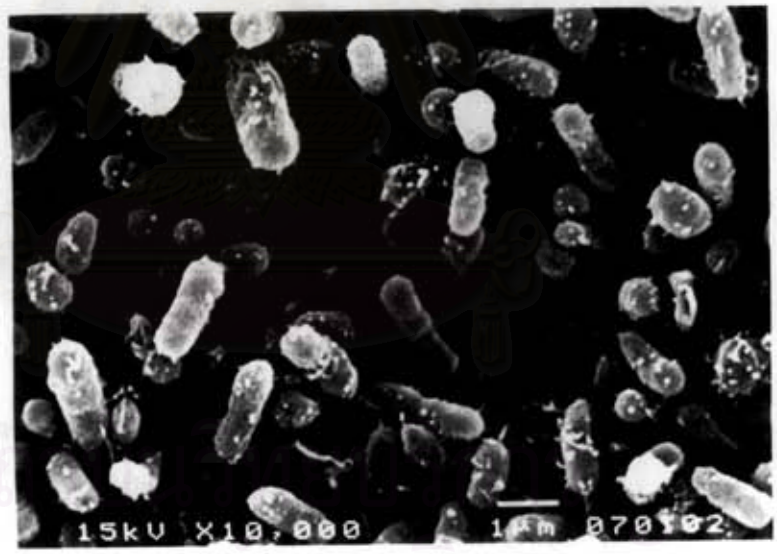

Figure 1. Scanning electron micrograph of S9730

Figure 2. The colonial appearance of S9730 on marine agar plate at 30 °C for 3 days.

Table 7. Characteristics of *Alteromonas* sp. S9730

Characteristic	S9730	Characteristic	S9730
Cell form	Straight rod	Acid formation from:	
Cell size (μm)	0.5-0.8×	D-glucose	−
	1.8-2.5	D-mannose	W
Flagella	+	D-galactose	−
Gram reaction	−	D-fructose	−
Pigmentation	+	Sucrose	+
Aerobiosis	+	Maltose	W
Growth at: 4 °C	−	Cellobiose	W
35 °C	+	Melibiose	+
40 °C	−	Lactose	−
Growth at least 0.1% NaCl	+	Salicin	+
Growth at 0.2% NaCl	+	D-manitol	W
0.5% NaCl	+	L-arginine	−
1.0% NaCl	+	Glycerol	+
2.0% NaCl	+	Utilization of : D-glucose	+
5.0% NaCl	+	D-mannose	W
10.0% NaCl	−	D-galactose	−
Growth at pH : 6	+	D-fructose	W
7	+	Sucrose	−
8	+	Maltose	+
9	+	Cellobiose	−
Reduction of nitrate	−	Melibiose	−
Hydrolysis of arginine	+	Lactose	−
Catalase	+	Salicin	−
Production of: amylase	+	Sorbitol	−
chitinase	−	D-manitol	−
gelatinase	+	L-arginine	W
		glycerol	−

+, positive reaction; W, weak reaction; −, negative reaction

The marine bacterium S9730 was identified as *Alteromonas* sp. (Holt *et al.*, 1994) by comparing with others related genera (Table 2). Afterward, the characteristics of S9730 (Table 7) was compared with Characteristics of the species of the genus *Alteromonas* (Table 3). S9730 has showed similar characteristic to *Alteromonas aurantia* but some characteristics are not similar with this species(Table 3). Therefore, marine bacterium S9730 was unidentified left in as any species.

2. Structure elucidation of isolated compounds

The white crystals of K002 (weight = 28 mg) were obtained from fraction F002 by crystallization using $CHCl_3$ (Scheme 2). K005 (weight = 51 mg) is the white crystals which were crytallized from fraction F005 by using $CHCl_3$. The crystallization technique was used to obtained the red crystals of K004 (weight = 111 mg) from fraction F004. The compounds were characterized according to their spectroscopic data including IR, UV, NMR and mass spectra.

2.1 Compound K005

EIMS : *m/z* (relative intensity) (Figure 3)

154 (16[M^+]), 126 (3), 111 (32), 98 (17), 83 (100), 70 (77), 68 (71), 55 (66)

Optical Rotation : $[\alpha]^{20}_{D}$, in MeOH

-125° (c = 0.29)

UV : λ_{max} nm (ε), in MeOH

274 (5421)

IR (KBr disc) : ν cm^{-1} (Figure 4)

3114, 1679, 1644

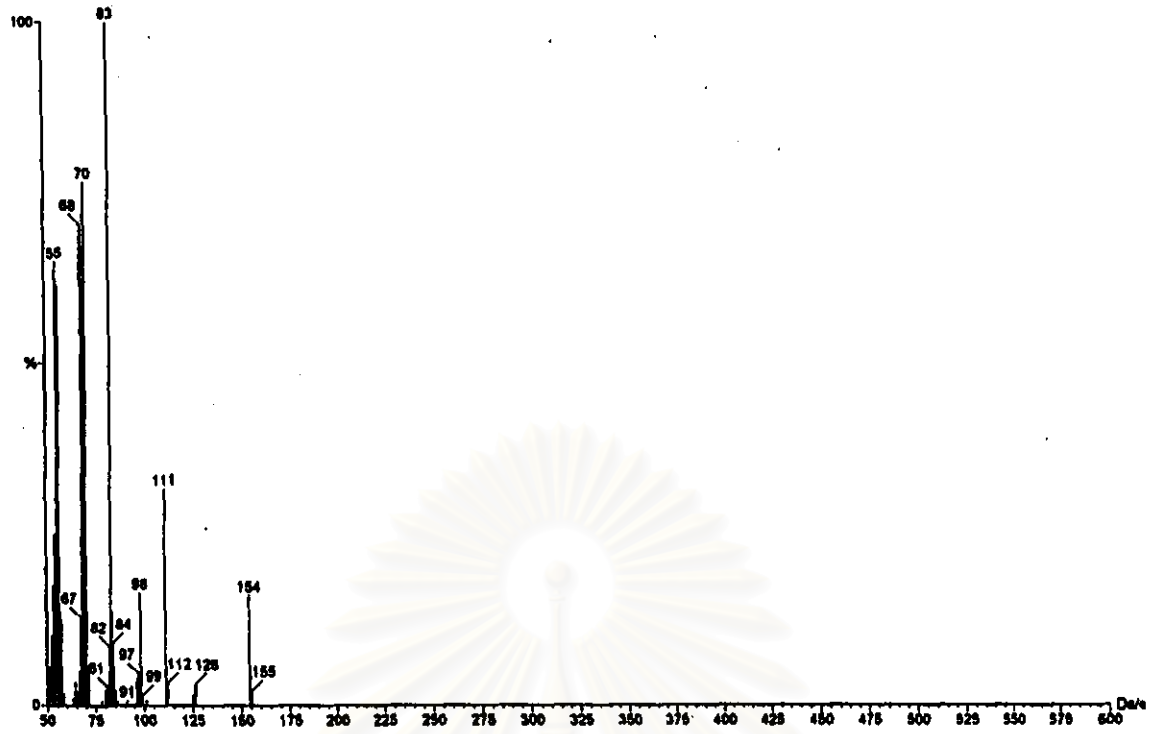

Figure 3. EI mass spectrum of compound K005

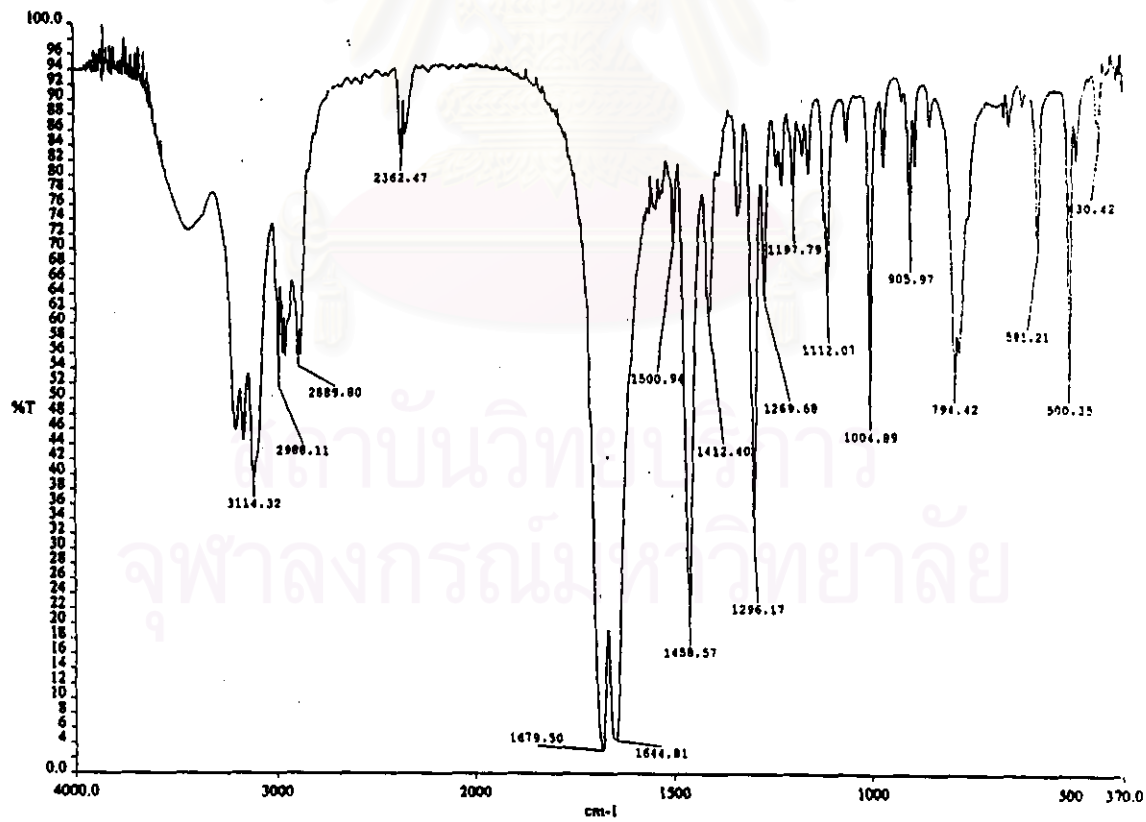

Figure 4. IR spectrum of compound K005

^{1}H-NMR (300 MHz, in $CDCl_3$) : δ in ppm, *J* in Hz (Figure 5)

6.34 (1H, br s; H-4), 4.11 (1H, d, *J* = 16.1 Hz; H-3), 4.08 (1H, s; H-6), 3.89 (1H, dd, J = 16.4, 4.4 Hz; H-3′), 3.52-3.64 (2H, m; H-9, H-9′), 2.19(1H, m; H-7), 1.97-2.07 (2H, m; H-8, H-7′), 1.91 (1H, m; H-8′)

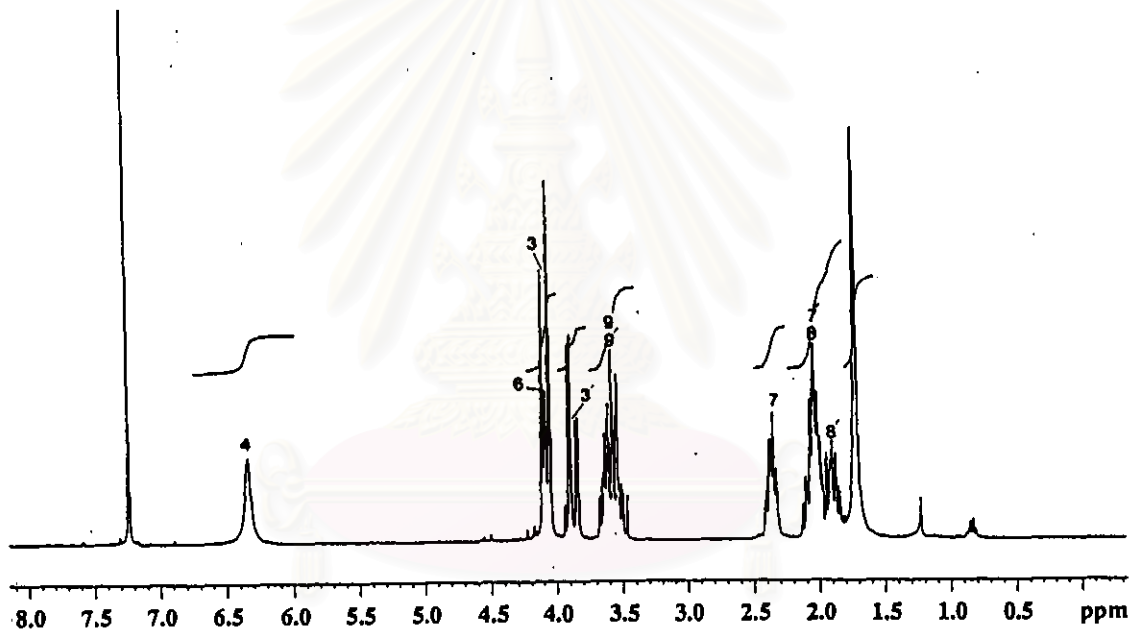

Figure 5. 300 MHz ^{1}H NMR spectrum of compound K005 ($CDCl_3$)

^{13}C-NMR (75 MHz, in $CDCl_3$) : δ in ppm (Figure 6)

169.7 (C-2), 163.4 (C-5), 58.5 (C-6), 46.6 (C-3), 45.3 (C-9), 28.5 (C-7), 22.4 (C-8)

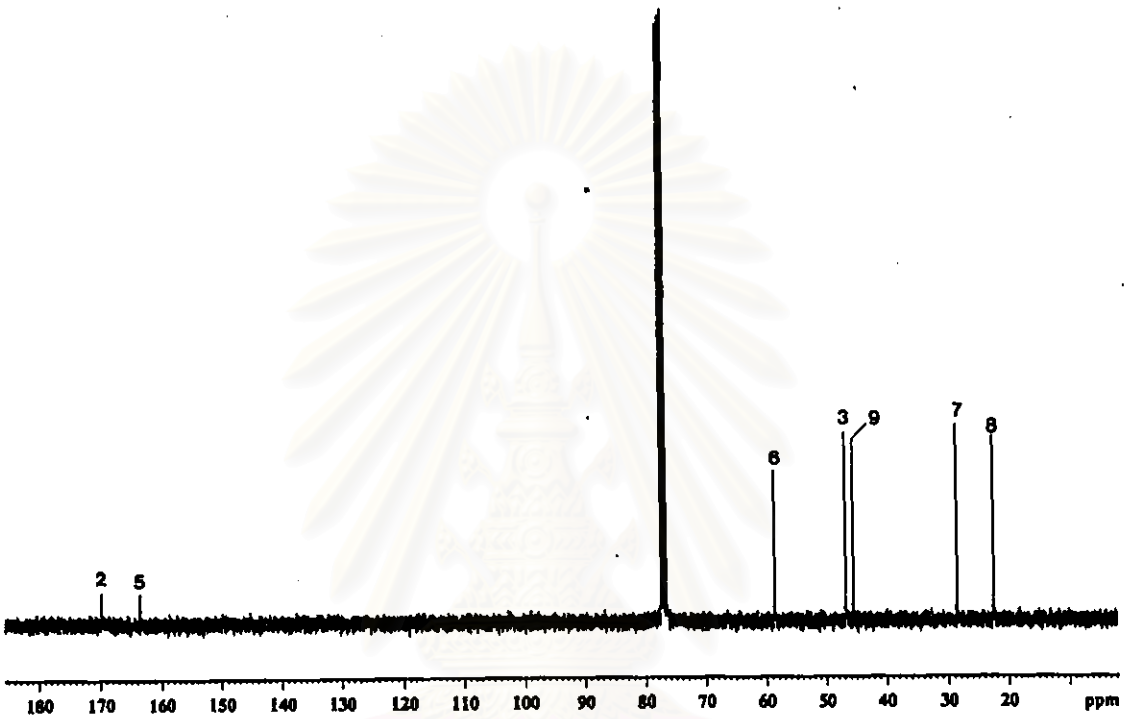

Figure 6. 75 MHz ^{13}C NMR spectrum of compound K005 ($CDCl_3$)

The mass spectrum of compound K005 (Figure 3) showed the molecular ion peak at *m/z* 154, suggesting a molecular formula of $C_7H_{10}O_2N_2$. The IR spectrum (Figure 4) revealed the maximum absorption bands at 1644 and 1679 cm^{-1} (carbonyl stretching), 3114 cm^{-1} (amide NH stretching) and 2889-2998 cm^{-1} (cycloalkane stretching).

The ^{1}H NMR spectrum of compound K005 (Figure 5) displayed 10 proton signals including four methylene proton signals, one methine and one amide proton signals. The ^{13}C NMR (Figure 6) and DEPT 135 (Figure 7) spectra provided 7 carbon signals for two amide carbonyl carbon signals, one methine carbon signal and four methylene carbon signals.

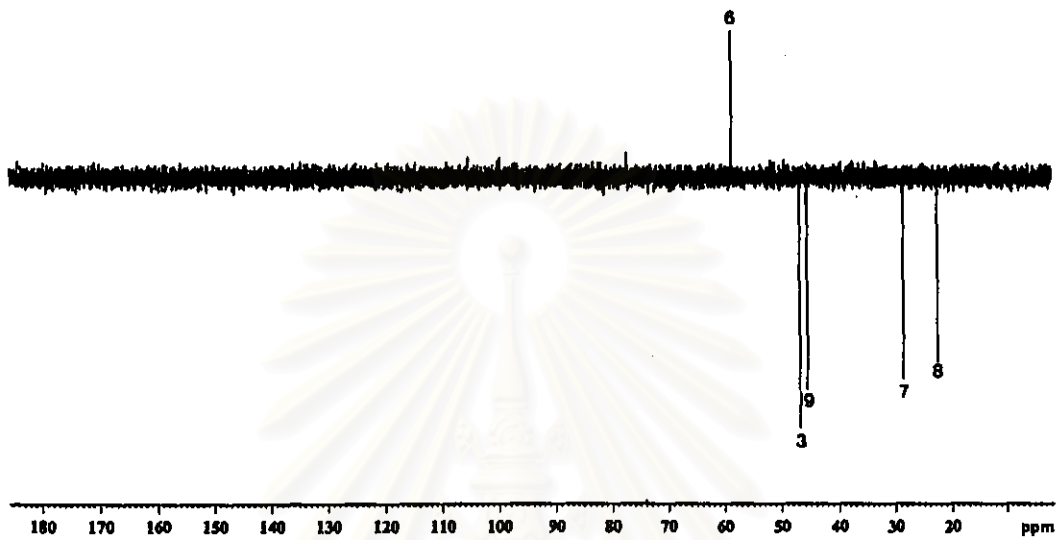

Figure 7. DEPT 135 spectrum of compound K005 ($CDCl_3$)

The ^1H-^1H COSY (Figure 8) spectrum revealed coupling protons though their correlations as following: H-6 to H-7 and H-7′, H-7 and H-7′ to H-8, and H-8′, H-8, and H-8′ to H-9, and H-9′,

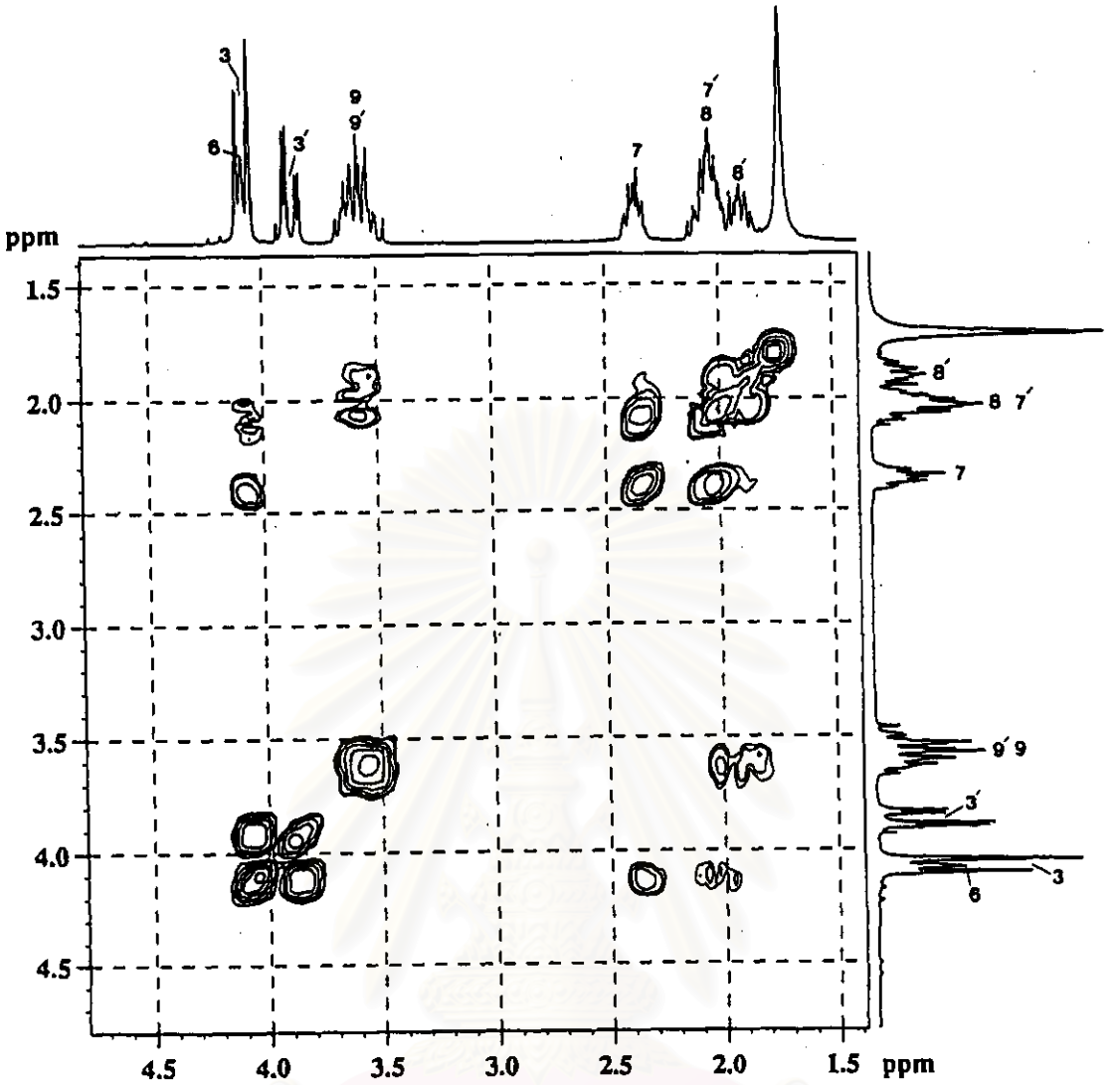

Figure 8. COSY 45 spectrum of compound K005 ($CDCl_3$)

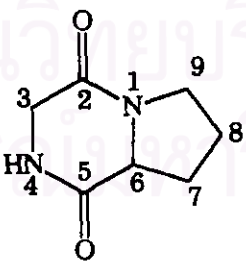

Figure 9. *Cyclo-(Gly-Pro)*

The spectral data of compound K005 was identical to those of a known compound (Figure 9), 3, 6-dioxo-hexahydro-pyrrole[1,2-a]-Pyrrazine or *cyclo-(Gly-Pro)*, which was previously isolated from the starfish, *Luidia clathrata*

(Pettit *et al.*, 1973) and from the sponge, *Geodia baretti* (Lidren, and Bohlin, 1986). It was therefore concluded that compound K005 was *cyclo*-(*Gly*-*Pro*).

2.2 Compound K002

EIMS : *m/z* (relative intensity) (Figure 10)

210 (3[M^+]), 154 (13), 129 (69), 110 (28), 94 (57), 82 (31), 70 (69), 55 (100)

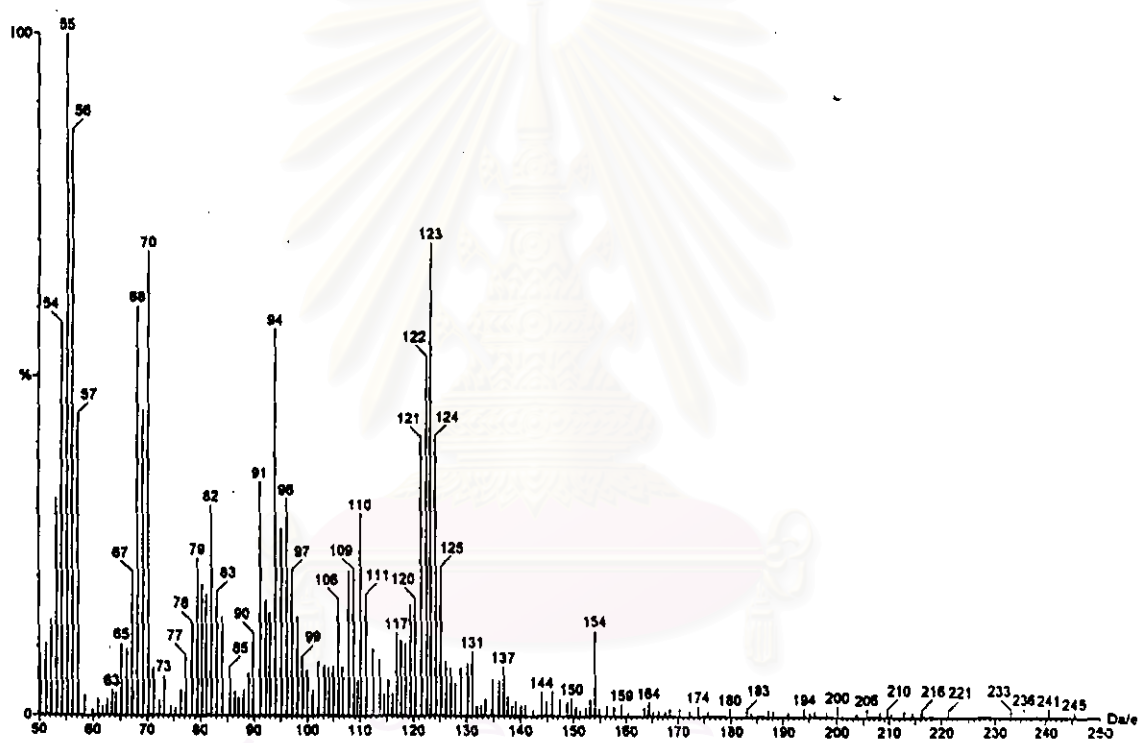

Figure 10. EI mass spectrum of compound K002

Optical Rotation : $[\alpha]^{20}_{D}$, in MeOH

-99° (c = 0.29)

UV : λ_{max} nm (ε), in MeOH

273 (1049)

IR (KBr disc) : ν cm^{-1} (Figure 11)

3261, 2951, 2880, 1670, 1634

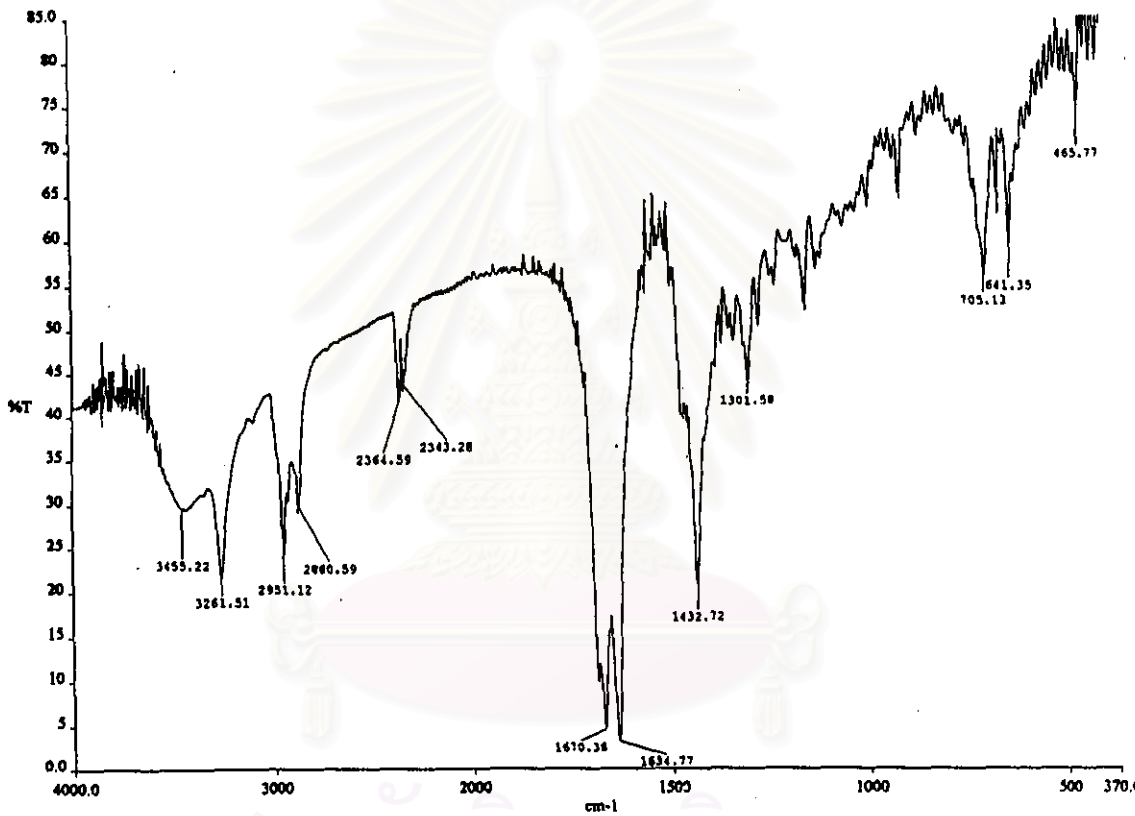

Figure 11. IR spectrum of compound K002

^{1}H-NMR (300 MHz, in $CDCl_3$) : δ in ppm, *J* in Hz (Figure 12)

5.89 (1H, br s; H-4), 4.09 (1H, t, *J* = 7.8 Hz; H-6), 3.99 (1H, dd, *J* = 9.7, 3.0 Hz; H-3), 2.77-3.59 (2H, m; H-9, H-9′), 2.32 (1H, m; H-7), 1.96-2.18 (3H, m; H-7′, H-8, H-10), 1.89 (1H, m; H-8′), 1.74 (1H, m; H-11), 1.50 (1H, m; H-10′), 0.99 (3H, d, *J* = 6.5 Hz; 12-CH_3), 0.94 (3H, d, *J* = 6.5 Hz; 13-CH_3)

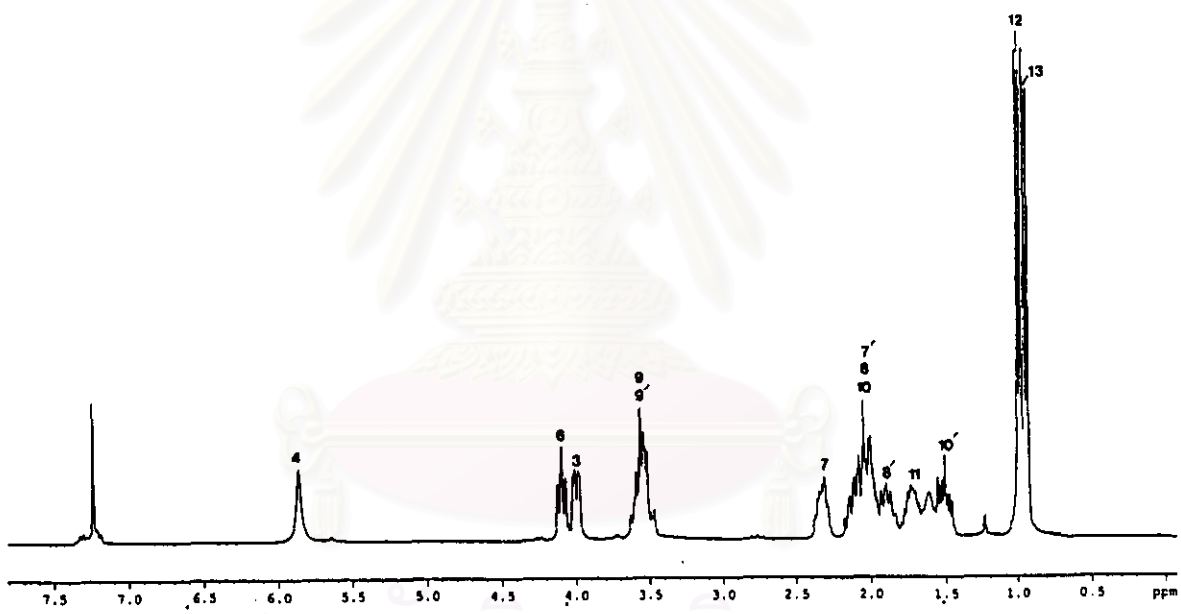

Figure 12. 300 MHz ^{1}H NMR spectrum of compound K002 ($CDCl_3$)

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

170.3 (C-2), 166.3 (C-5), 59.4 (C-6), 53.9 (C-3), 45.5 (C-9), 39.1 (C-10), 28.7 (C-7), 24.9 (C-11), 23.4 (C-8), 22.9 (C-12), 21.3 (C-13)

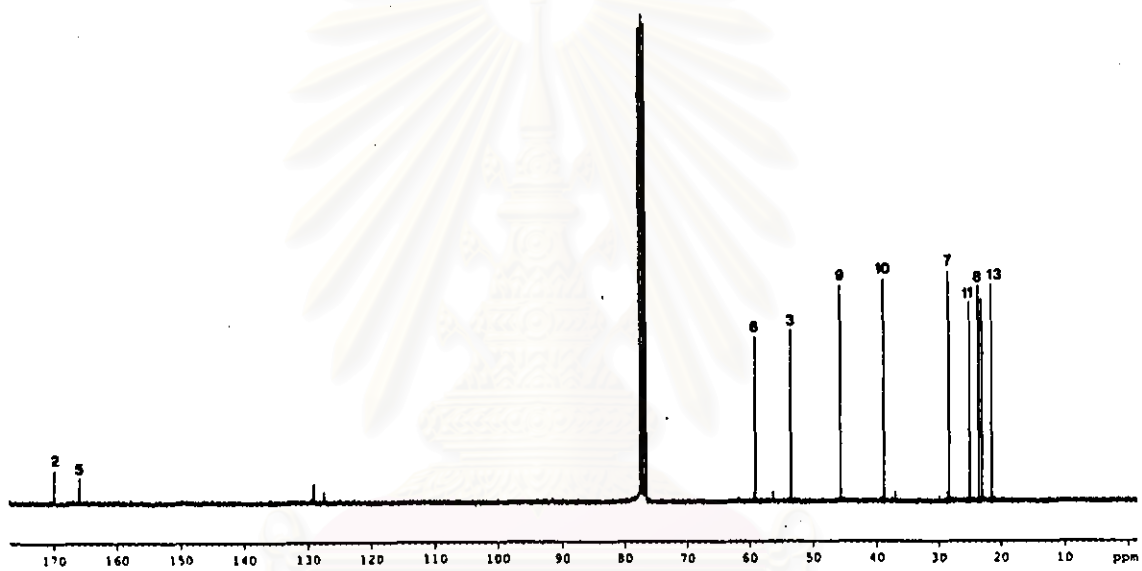

Figure 13. 75 MHz ^{13}C NMR spectrum of compound K002 ($CDCl_3$)

The mass spectrum of K002 (Figure 10) displayed a molecular ion peak at *m/z* 210 tentatively suggesting a molecular formular of $C_{11}H_{18}O_2N_2$. The IR spectrum (Figure 11) exhibited the maximum absorption bands at 3261 cm^{-1} (NH stretching), 2951-2880 cm^{-1} (cycloalkane stretching), and 1670 and 1634 cm^{-1} (carbonyl stretching).

As seen on the ^{1}H NMR spectrum (Figure 12), compound K002 contained one amide proton signal, three methine proton signals, four methylene, and two methyl proton signals. The ^{13}C NMR spectrum (Figure 13) showed 11 carbon signals which could be classified by DEPT spectra (Figures 14 and 15).

These spectral data exhibited two amide carbonyl carbon signals, two methyl carbon signals, four methylene carbon signals, and three methine carbon signals. The complete assignments of compound K002 were determined by ^{1}H-^{1}H COSY (Figure 16) and HMQC (Figure 17) spectra.

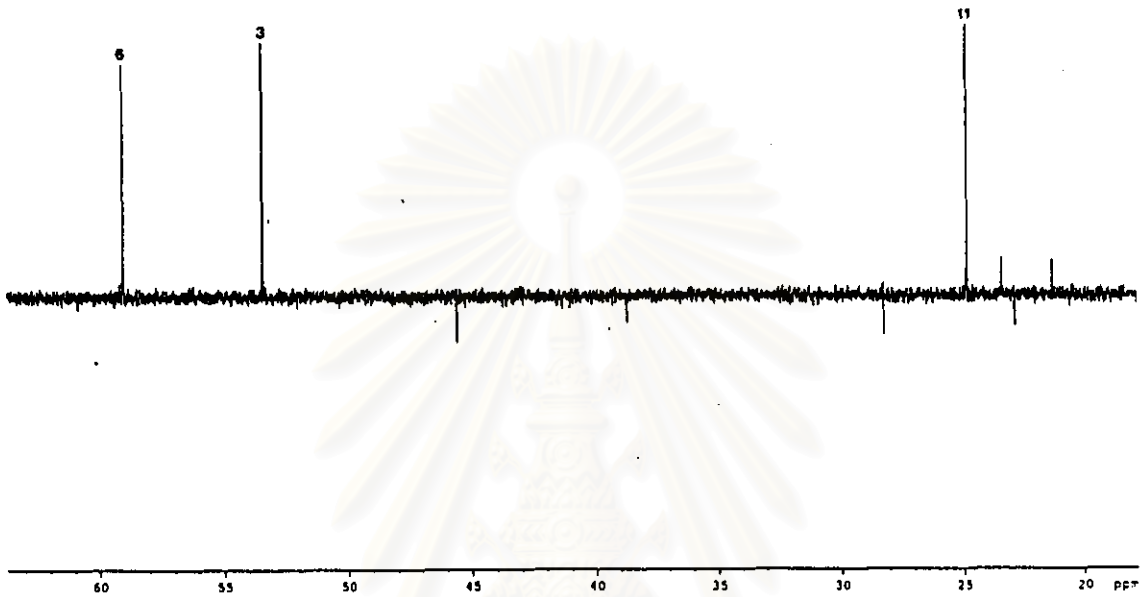

Figure 14. DEPT 90 spectrum of compound K002 ($CDCl_3$)

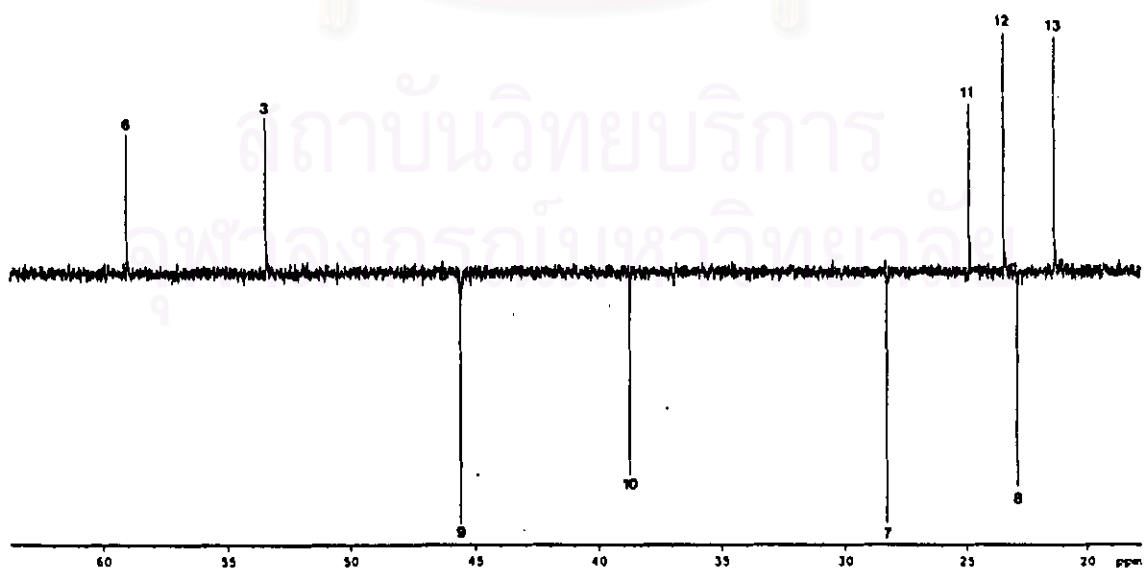

Figure 15. DEPT 135 spectrum of compound K002 ($CDCl_3$)

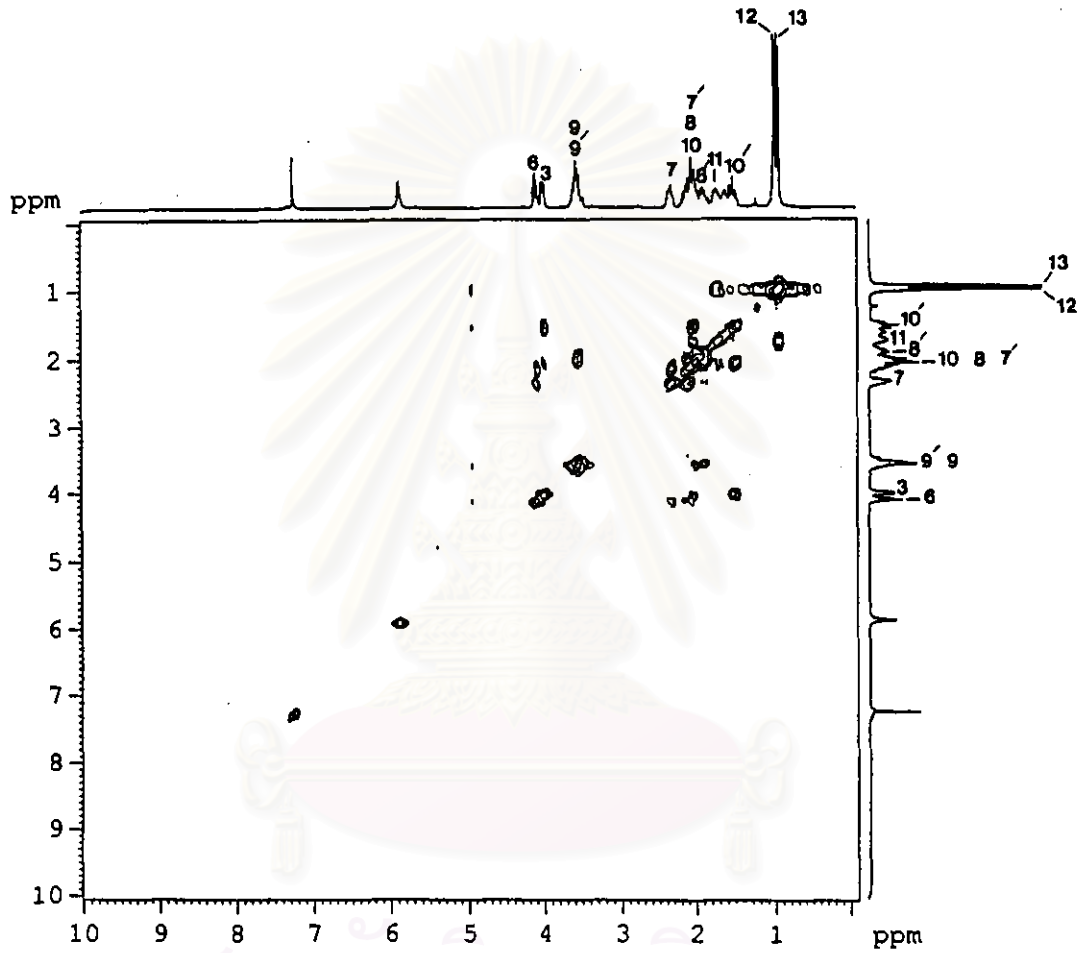

Figure 16. COSY 45 spectrum of compound K002 ($CDCl_3$)

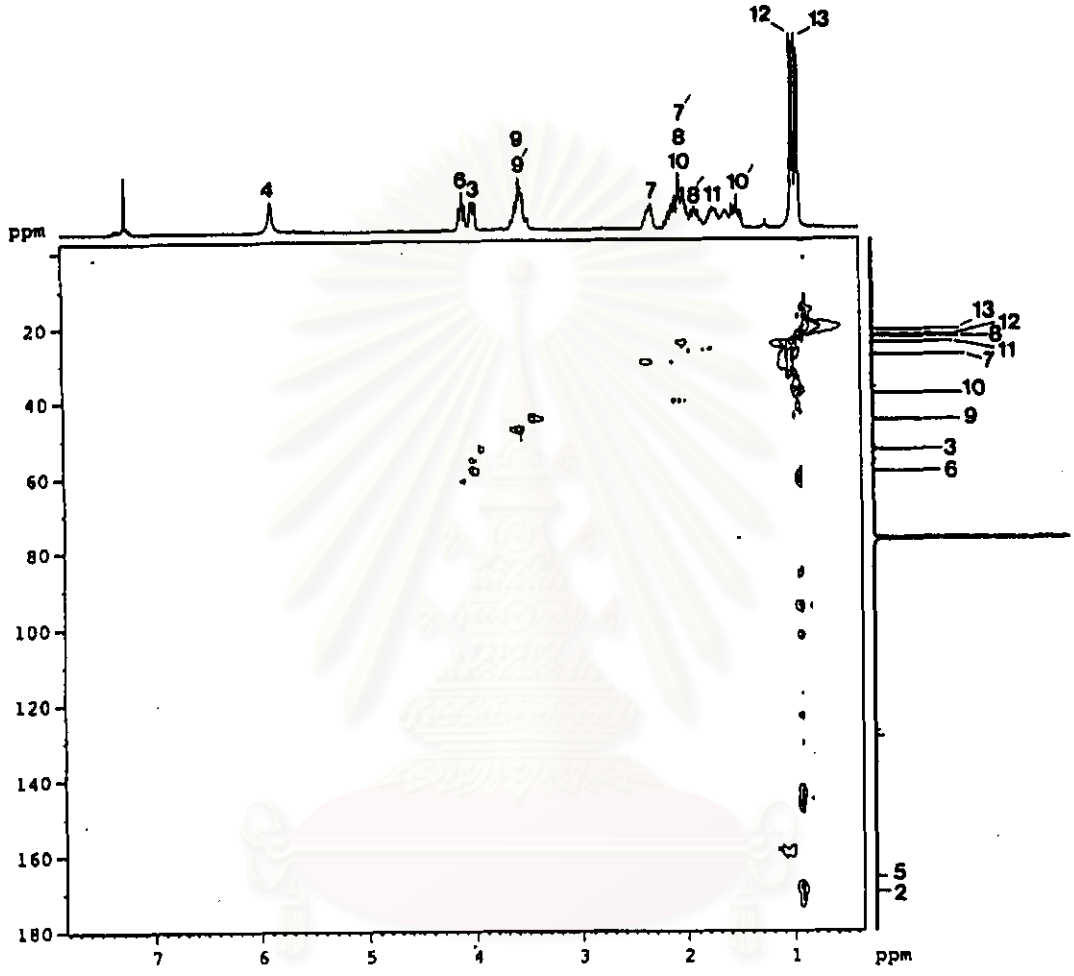

Figure 17. HMQC spectrum of compound K002 ($CDCl_3$)

The ^{1}H-^{1}H COSY spectral data revealed the correlations of both H-12 and H-13 to H-11; H-11 to H-10; H-3 to H-10; H-6 to H-7; H-7 to H-8; and H-8 to H-9 (Figure 16). ^{1}H and ^{13}C NMR data of K002 were identical to those of the known *cyclo (-Pro-Leu-)* (Table 8) as previously reported by Schmitz *et al.*, (1983): Steirle *et al.*, (1988): Adamczeski *et al.*, (1995). It was therefore concluded that K002 was the known *cyclo (-Pro-Leu-)*.

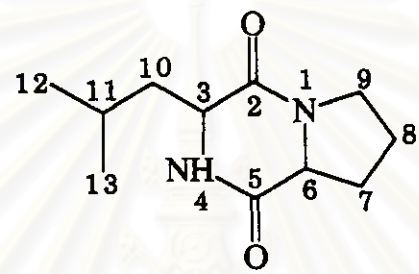

Figure 18. *Cyclo (-Pro-Leu-)*

Table 8. ^{1}H and ^{13}C NMR spectra of compound K002 (in $CDCl_3$) compared with *cyclo (-Pro-Leu-)* (in $CDCl_3$)

Position	δC		δH (number of H, multiplicity, *J*)	
	K002	*cyclo (-Pro-Leu-)*	K002	*cyclo (-Pro-Leu-)*
1	-	-	-	-
2	170.3	171.4	-	-
3	53.9	53.4	3.99 (1H, dd, *J* = 9.7, 3.0 Hz)	4.01 (1H, dd, *J* = 9.4, 3.4 Hz)
4	-	-	5.89 (1H, br s)	5.91 (1H, br s)
5	166.3	167.1	-	-
6	59.4	59.1	4.09 (1H, t, *J* = 7.8 Hz)	4.12 (1H, t, *J* = 8.1 Hz)
7	28.7	28.2	2.32 (1H, m), 1.96-2.18 (1H, m)	2.13 (1H, m) 2.33 (1H, m)
8	23.4	22.8	1.96-2.18 (1H, m), 1.89 (1H, m)	1.94-1.86 (1H, m)
9	45.5	45.6	2.77-3.59 (2H, m)	3.6 (2H, m)
10	39.1	38.2	1.96-2.18 (1H, m), 1.50 (1H, m)	2.01 (1H, m) 1.52 (1H, ddd, *J* = 14.5, 9.6, 4.9 Hz)
11	24.9	24.8	1.74 (1H, m)	1.76-1.69 (1H, m)
12	22.9	22.8	0.99 (3H, d, *J* = 6.54 Hz)	0.94 (3H, d, *J* = 6.3 Hz)
13	21.3	21.2	0.94(3H, d, *J* = 6.51 Hz)	1.0 (3H, d, *J* = 6.3 Hz)

*Cyclo (-Pro-Leu-) from Adamczeski *et al.*, (1995)

2.3 Compound K004

EIMS : *m/z* (relative intensity) (Figure 19)

147 (44[M^+]), 119 (100), 92 (100), 76 (22), 64 (68), 50 (37)

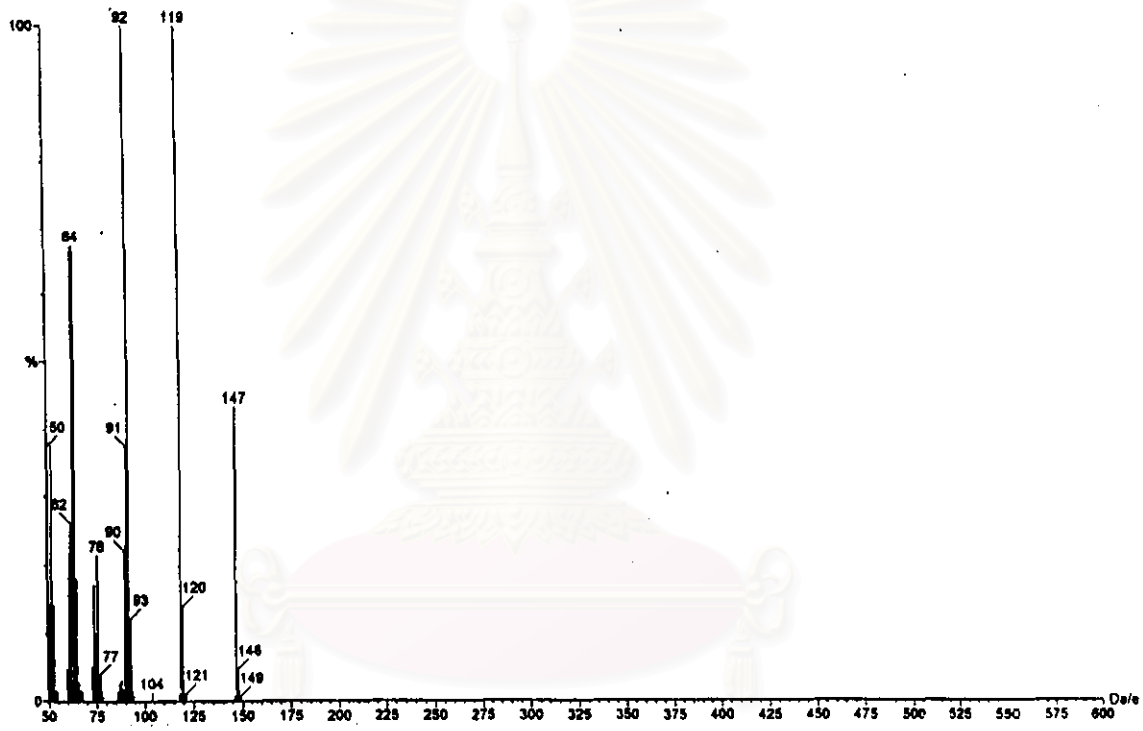

Figure 19. EI mass spectrum of compound K004

UV : λ_{max} nm (ε), in MeOH

242 (3980)

IR (KBr disc) : ν cm^{-1} (Figure 20)

3192, 1746, 1727

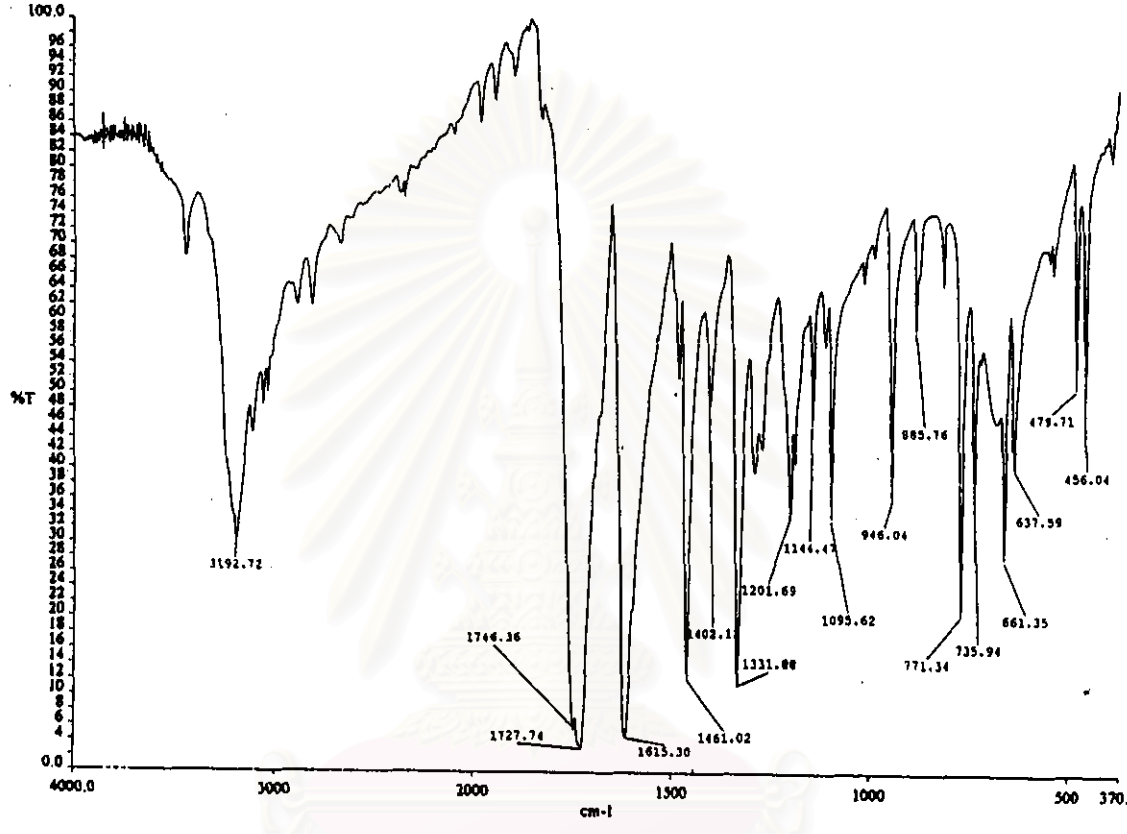

Figure 20. IR spectrum of compound K004

^{1}H-NMR (300 MHz, in DMSO-d_6) : δ in ppm, J in Hz (Figure 21)

11.03 (1H, br s; H-1), 7.57 (1H, t, J = 7.8 Hz; H-6), 7.50 (1H, d, J = 7.5 Hz; H-4), 7.05 (1H, t, J = 7.5 Hz, H-5), 6.91 (1H, d, J = 7.8 Hz; H-7)

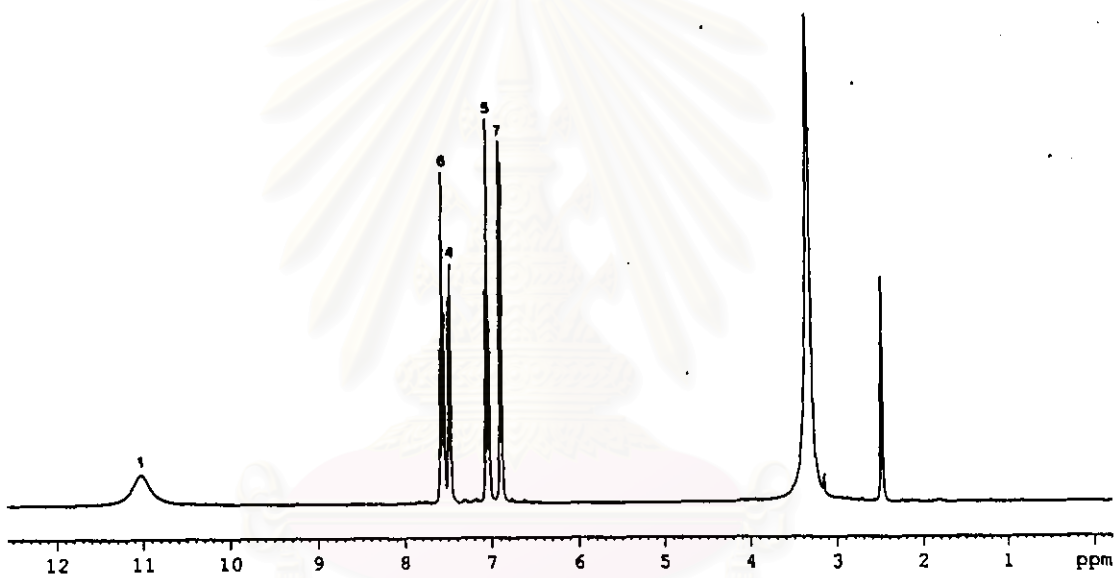

Figure 21. 300 MHz ^{1}H NMR spectrum of compound K004 (DMSO-d_6)

^{13}C-NMR (75 MHz, in DMSO-d_6) : δ in ppm (Figure 22)

184.2 (C-3), 159.2 (C-2), 150.6 (C-7a), 138.3 (C-4), 124.7 (C-6), 122.7 (C-5), 117.8 (C-3a), 112.2 (C-7)

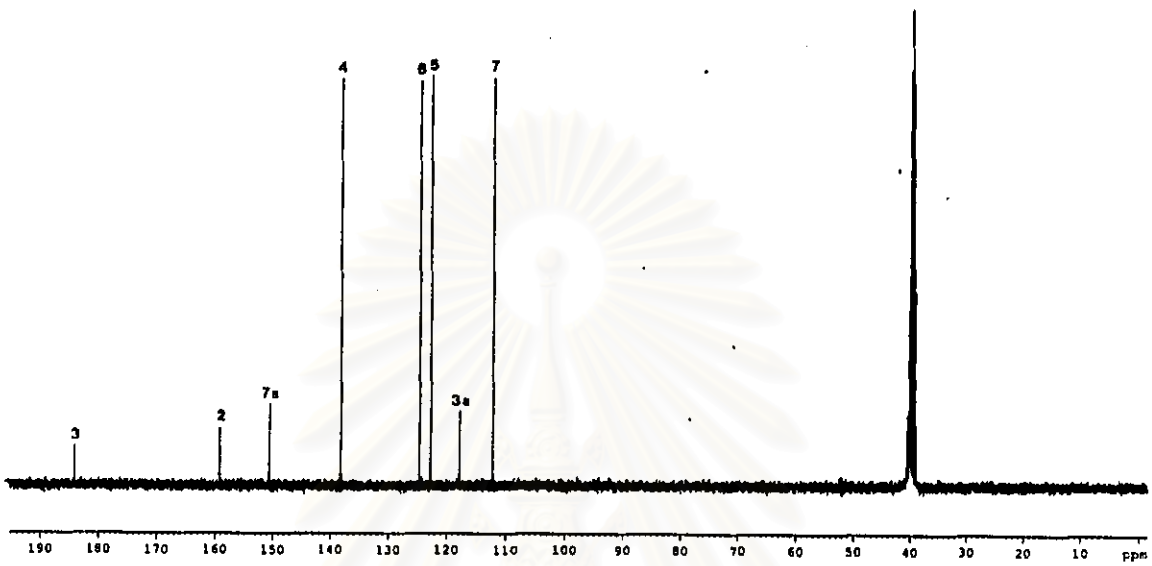

Figure 22. 75 MHz ^{13}C NMR spectrum of compound K004 (DMSO-d_6)

The mass spectrum of compound K004 (Figure 19) showed a molecular ion peak at *m/z* 147 implying a moleclar formular of $C_8H_5O_2N$. The IR spectrum (Figure 20) indicated the presence of an amide group at 3192 cm^{-1}, a carbonyl at 1746 and 1727 cm^{-1}, and an aromatic ring at 1615 cm^{-1}.

The ^1H NMR spectrum (Figure 21) showed 5 proton signals including one amide signal, and four aromatic methine proton signals. The ^{13}C NMR spectrum (Figure 22) revealed 8 carbon signals which could be classified by examination of the DEPT spectrum (Figure 23). These spectral data provided signals for two carbonyl carbons, two quaternary carbons, and four aromatic methine carbons.

The ^{1}H-^{1}H COSY spectral data allowed the assignment of proton connected to their respective carbons. The ^{1}H-^{1}H COSY spectrum (Figure 24) of K004 exhibited the correlation of aromatic protons; H-4 (at δ 7.50) to H-5 (at δ 7.05); H-5 (at δ 7.05) to H-6 (at δ 7.57); H-6 (at δ 7.57) to H-7 (at δ 6.91).

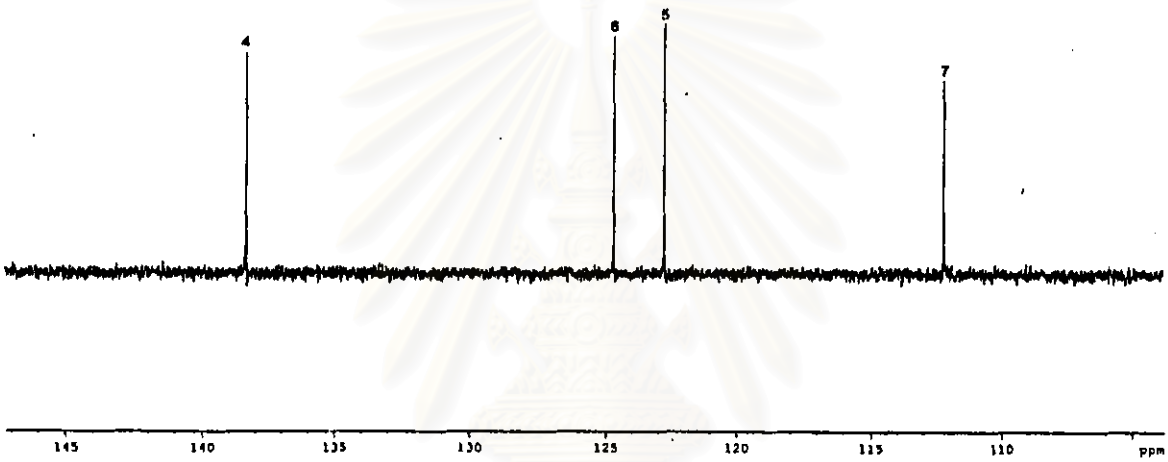

Figure 23. DEPT 135 spectrum of compound K004 (DMSO-d_6)

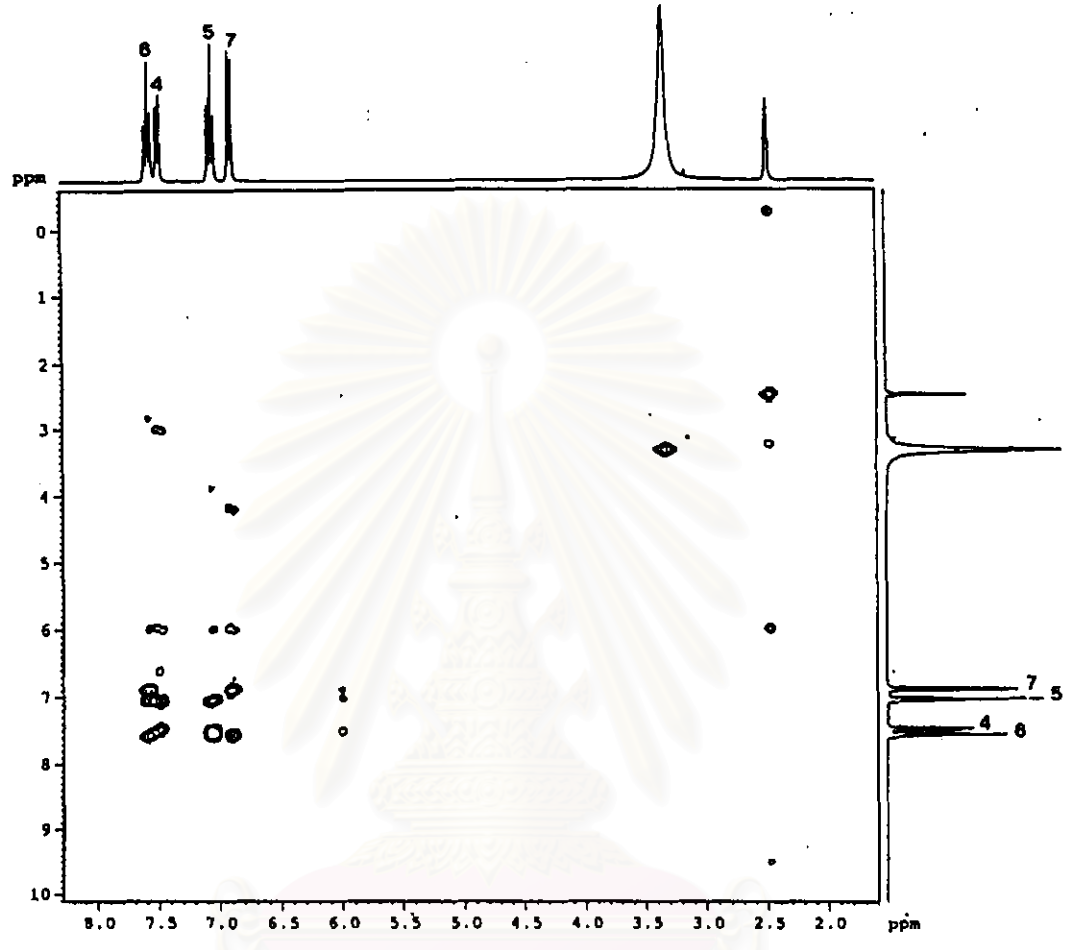

Figure 24. COSY 45 spectrum of compound K004 (DMSO-d_6)

Careful comparison ^{1}H and ^{13}C NMR data of K004 with those of reported data (Gassman, Cue, and Luh, 1977; Gil-Turnes, Hay, and Fenical, 1989), revealed that K004 had spectral data identical to those of the known compound 2,3-indolinedione (isatin) (Figure 26). And the results obtained from HETCOR (Figure 25) spectral data suggested that the assingment of C-5 and C-6 in the previous report (Gassman, Cue, and Luh, 1977) should be revised as in Table 9. The ^{1}H NMR and ^{13}C NMR spectral data of compound K004 and isatin are in Table 9.

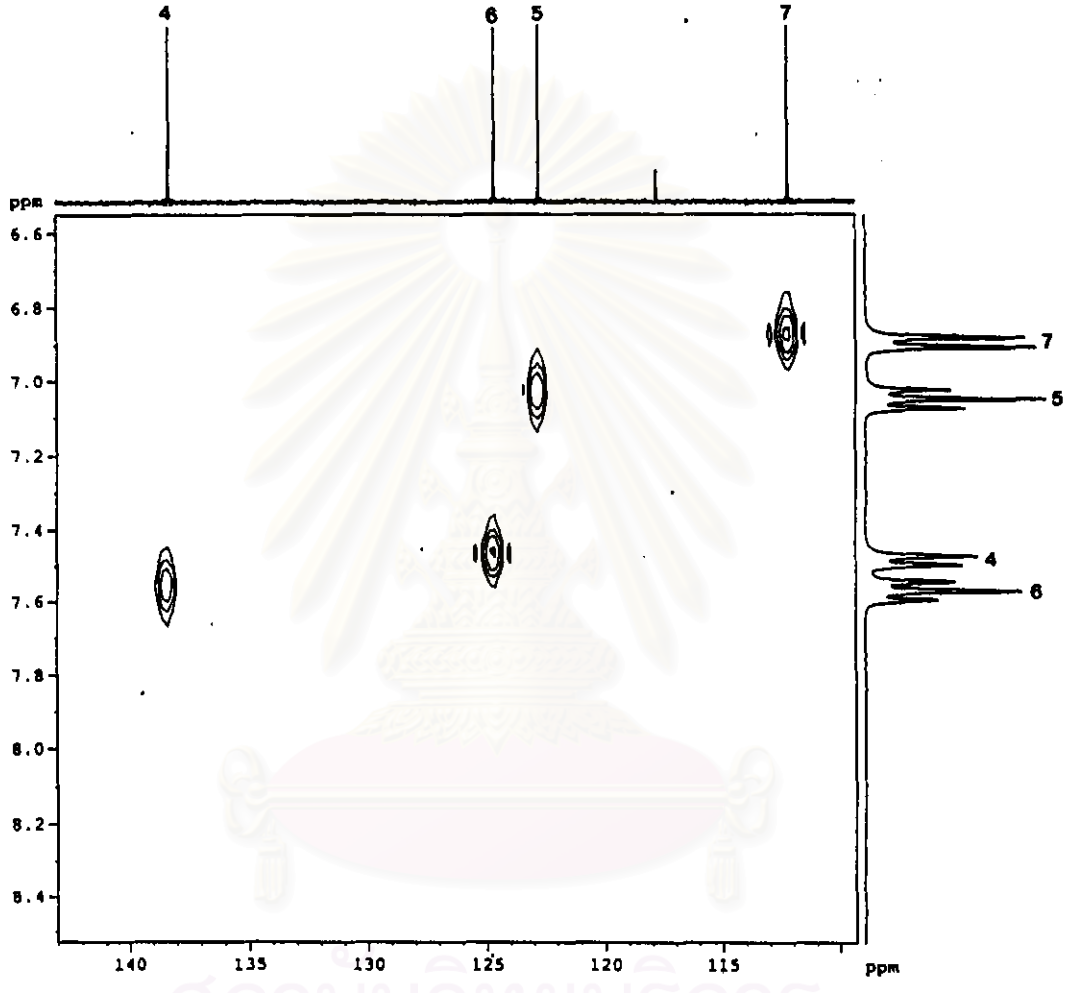

Figure 25. HETCOR NMR spectrum of compound K004 (DMSO-d_6)

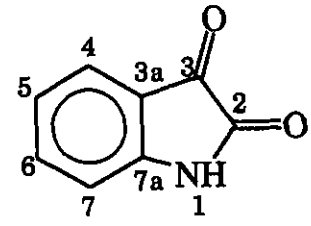

Figure 26. Isatin

Table 9. ^{1}H and ^{13}C NMR spectra of compound K004 (in DMSO-d_6) and isatin (in $CDCl_3$)

Position	δC		δH (number of H, multiplicity, *J*)	
	K004	Isatin*	K004	Isatin**
1	-		11.03 (1H, br s)	8.2 (1H, s)
2	159.2	159.5	-	-
3	184.2	184.6	-	-
3a	117.8	117.9	-	-
4	138.3	138.5	7.50 (1H, d, *J* = 7.5 Hz)	7.5 (1H, d, *J* = 7.8 Hz)
5	122.7	124.9	7.05 (1H, t, *J* = 7.5 Hz)	7.1 (1H, dd, *J* = 7.7 Hz)
6	124.7	122.8	7.57 (1H, t, *J* = 7.8 Hz)	7.6 (1H, dd, *J* = 7.7 Hz)
7	112.2	112.4	6.91 (1H, d, *J* = 7.8 Hz)	7.0 (1H, d, *J* =7.8 Hz)
7a	150.6	150.9	-	-

*The ^{13}C NMR data were from Gassman, Cue, and Luh (1977)

**and ^{1}H NMR data were from Gil-Turnes, Hay, and Fenical (1989).

3. Antibacterial activity of compounds K004 and K005

The crude CH_2Cl_2 extract exhibited antibacterial activity against both gram positive and gram negative bacteria, but it did not show the activity against fungi. Consequently, pure compounds, K004 and K005, obtained after the purification of crude extracts were further studied on the antibacterial activity against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 bacteria, as well as vibriosis in shrimp (*Vibrio* spp.). Compound K004 exhibited antibacterial activity, having the inhibition zone in the range of 19-26 mm at the concentration of 100 μg/disc. However, compound K005 did not show the activity (Table 10). Compound K004 exhibited moderate antibacterial activity with the MIC ranging from 31-62 μg/ml (Table 11).

Table 10. Antibacterial activity of K004 and K005 (100 μg/disc).

Bacteria	Inhibition zone (mm)	
	K004	K005
Escherichia coli ATCC 25922	21.8	0
Staphylococcus aureus ATCC 25923	22.4	0
Vibrio harveyi 94/55	20.8	0
Vibrio harveyi (Sombut)	24.5	0
Vibrio harveyi 97/17	26.4	0
Vibrio alginolyticus 96061	26.8	0
Vibrio alginolyticus 97032	24.3	0
Vibrio parahaemolyticus 94-60	21.8	0
Vibrio parahaemolyticus (string)	19.6	0
Vibrio vulnificus 94/4	22.2	0

Table 11. Minimum inhibition concentration of compound K004 (isatin)

Bacteria	MIC (μg/ml)
Escherichia coli ATCC 25922	62.5
Staphylococcus aureus ATCC 25923	62.5
Vibrio harveyi 94/55	31.3
Vibrio alginolyticus 97032	31.3
Vibrio vulnificus 94/4	31.3
Vibrio parahaemolyticus 94/60	31.3
Vibrio parahaemolyticus (string)	31.3

Table 12. The MIC values of chemotherapeutants used for the treatment of vibriosis in shrimp (Ruangpan, and Kitao, 1992).

Chemotherapautants	Inhibition concentration (μg/ml)
Sulphamonomethoxine	300
Cefazolin	>100
FR_{1881}	>100
Steptomycin	100
Erythromycin	37.5
Oxytetracycline	37.5
Trimethoprim	37.5
Furazolidone	18.8
Ampicillin	9.5
Chloramphenicol	9.5
Piromidic acid	9.5
Nalidixic acid	2.4
Amoxicillin	1.2

Table 12. (continued)

Chemotherapautants	Inhibition concentration (μg/ml)
Cephalexin	1.2
Flumequine	0.6
Oxolinic acid	0.6
Miloxacin	0.6
Coprofloxacin	0.6
VP_{2674} (Byer)	0.3

Compound K004 showed the activity against all strains of *Vibrio* sp. at the MIC of 31.3 μg/ml (Table 11). It was found that compound K004 had similar antibacterial activity to some commercial available antibiotics such as Erythromycin, Oxytetracycline and Trimethoprim, as shown in Table 12. Interestingly, compound K004, isatin, could inhibit growth of the virulent pathogenic bacteria *Vibrio* sp. that causes problems in shrimp farming from time to time. The emergence of drug resistant bacteria has gradually been increased (Inglis *et al.*, 1992). The application of using isatin producing bacteria in shrimp farming may be possible.

Isatin had been previously isolated from *Alteromonas* sp., which was found on the surface of embryos of the shrimp *Palaemon macrodactylus*. This bacterium was proved to protect the shrimp embryos from the pathogenic fungi *Lagenidium callinectes*. Isatin was responsible to this activity (Sofia, Hay, and Fenical, 1989). The isatin producing bacterium *Alteromonas* sp. could be possibly used for the control of fungi *Lagenidium* sp. infection. *Lagenidium* sp. is a common fungus which causes disease in shrimp hatcheries (Tonguthai, and Chanratchakool, 1992).

4. Production of isatin from *Alteromonas* sp. S9730

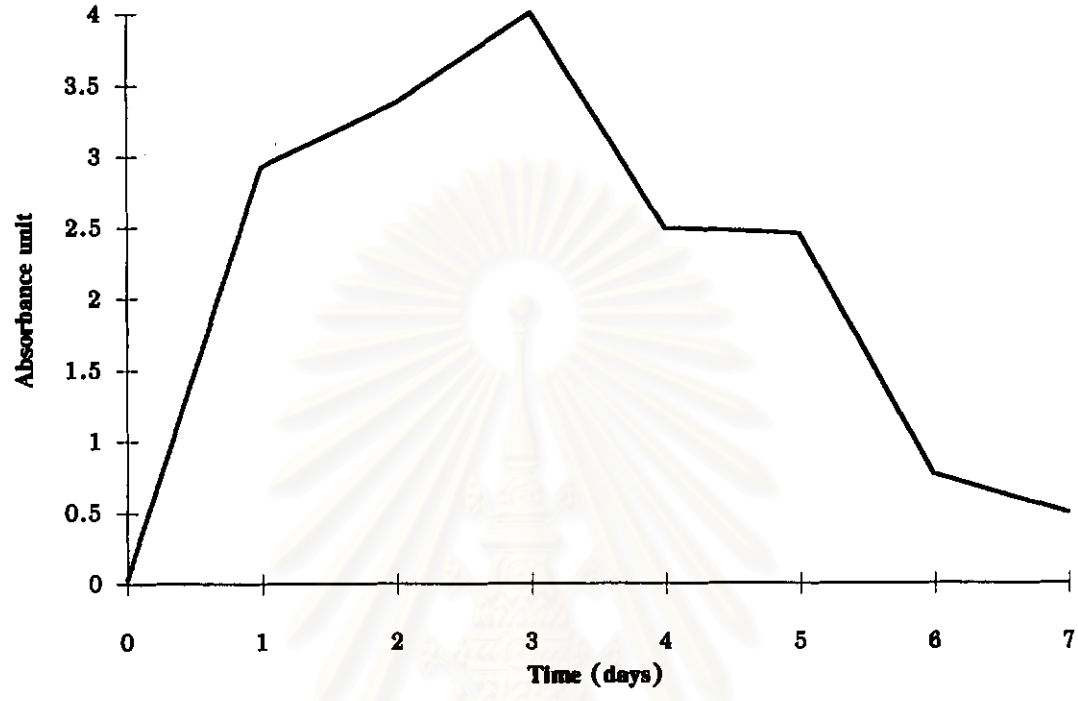

Figure 27. Growth curve of *Alteromonas* sp. S9730 in 7 days

From Figure 26, the bacterial cells increased rapidly in first 24 hours of the experiment. Thereafter, they grew slowly from the first day to the third day which was the highest cell density in this experiment, and they decreased slowly from the third day to the last day of experiment.

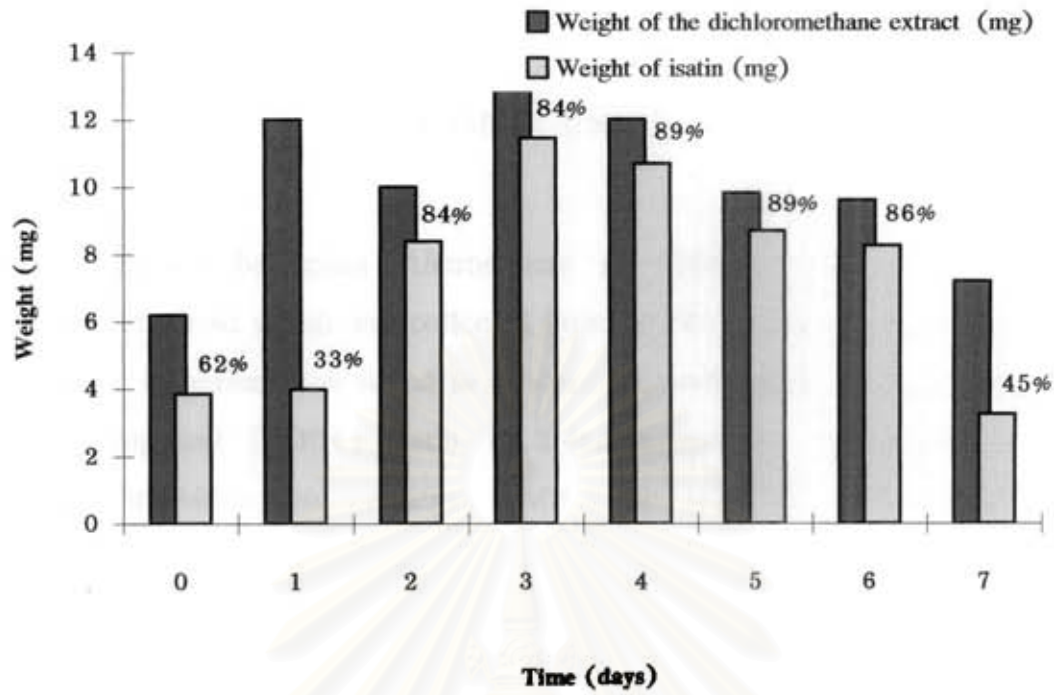

Figure 28. Weight of isatin in CH_2Cl_2 crude extract

Figure 27 showing a change of the total weight of CH_2Cl_2 crude extract pattern correlate with growth curve pattern (Figure 26). The composition of isatin in CH_2Cl_2 crude extract is not increase in the first day of experiment but the others composition increase correlatively with growth. It can suggest that they are primary metabolites which necessitate for growth.

The composition of isatin in CH_2Cl_2 crude extract increases from the first day to day 3 of experiment, which is the highest composition and weight by approximate 89% (11mg) of total CH_2Cl_2 crude extract. Afterward, it reduces though the seventh day of experiment.