CHAPTER IV

RESULT AND DISCUSSION

The mutant strain of *Zymomonas mobilis* CM 141 was innoculated on the YGP agar medium (contained 1% yeast extract, 1% peptone, 5% glucose and 1.5% agar) and incubated at 30° C for 72 hours in the aerobic condition. The red pigments on tha agar plates were extracted by the mixture of methanol : chloroform solution (1:1) and then filtered throught sinter glass funnel. The filtrate was blood-red solution.

1. STABILITY OF THE RED PIGMENTS IN THE CRUDE EXTRACT

The deep red solution was darken on standing. The color was changed to deep blue-violet color. The UV-VIS spectrum of the diluted solution (60 times in chloroform) and the prolonged standing solutions (in the tight, light resistance container at room temperature) at 2, 4, and 12 weeks were shown in Figure 37-40. and concluded in Table 10.

Table 10. The absorbance of the crude extract containing red pigments.

Time (weeks)	wavelength (nm)	Absorbance
0	539	0.510
2	539	0.317
4	541	0.213
12	597	0.035
	619	0.037

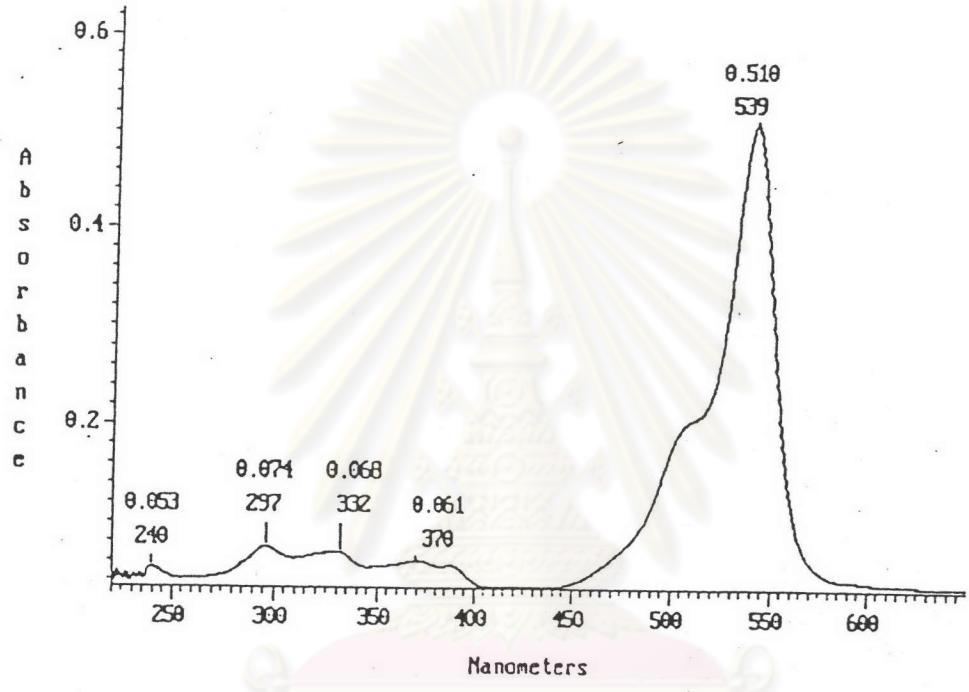


Figure 37. The UV-VIS spectrum of the red pigments in the crude extract at the begining (0 week).

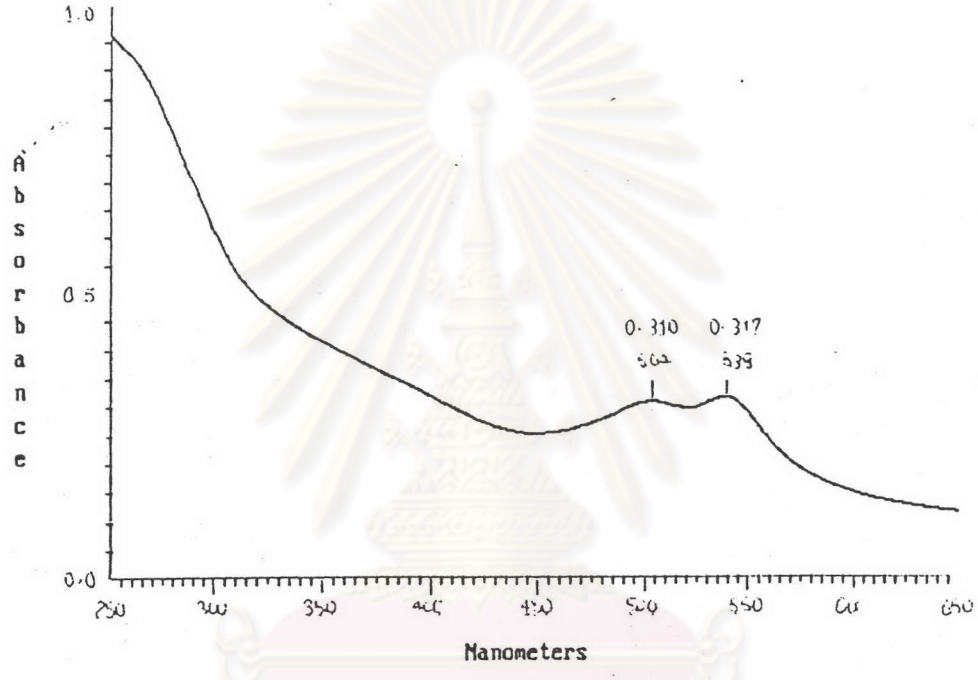


Figure 38. The UV-VIS spectrum of the red pigments in the crude extract after standing for two weeks.

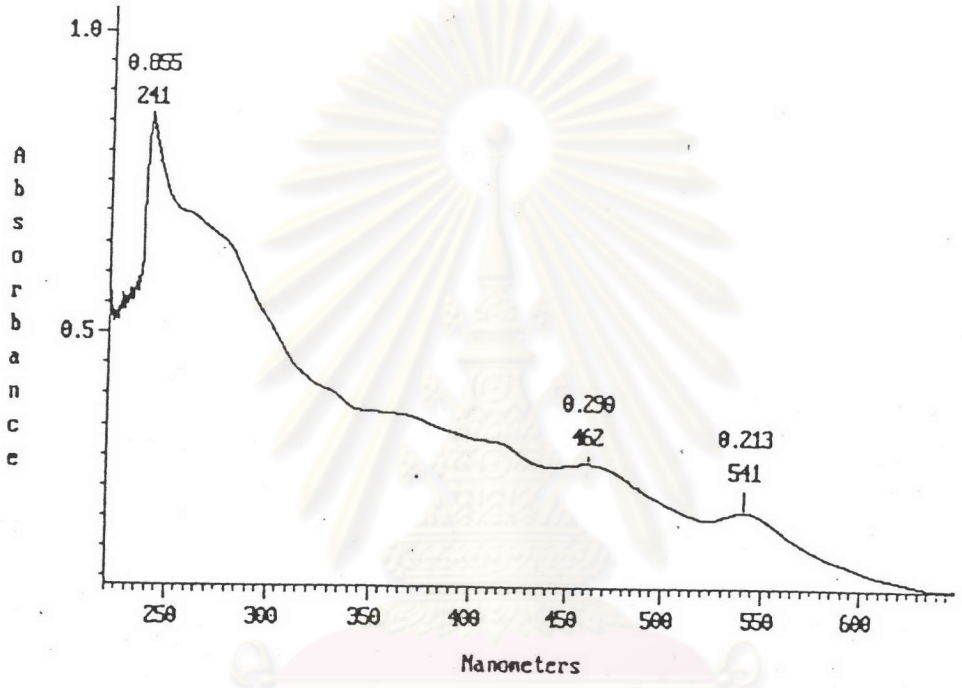


Figure 39. The UV-VIS spectrum of the red pigments in the crude extract after standing for four weeks.

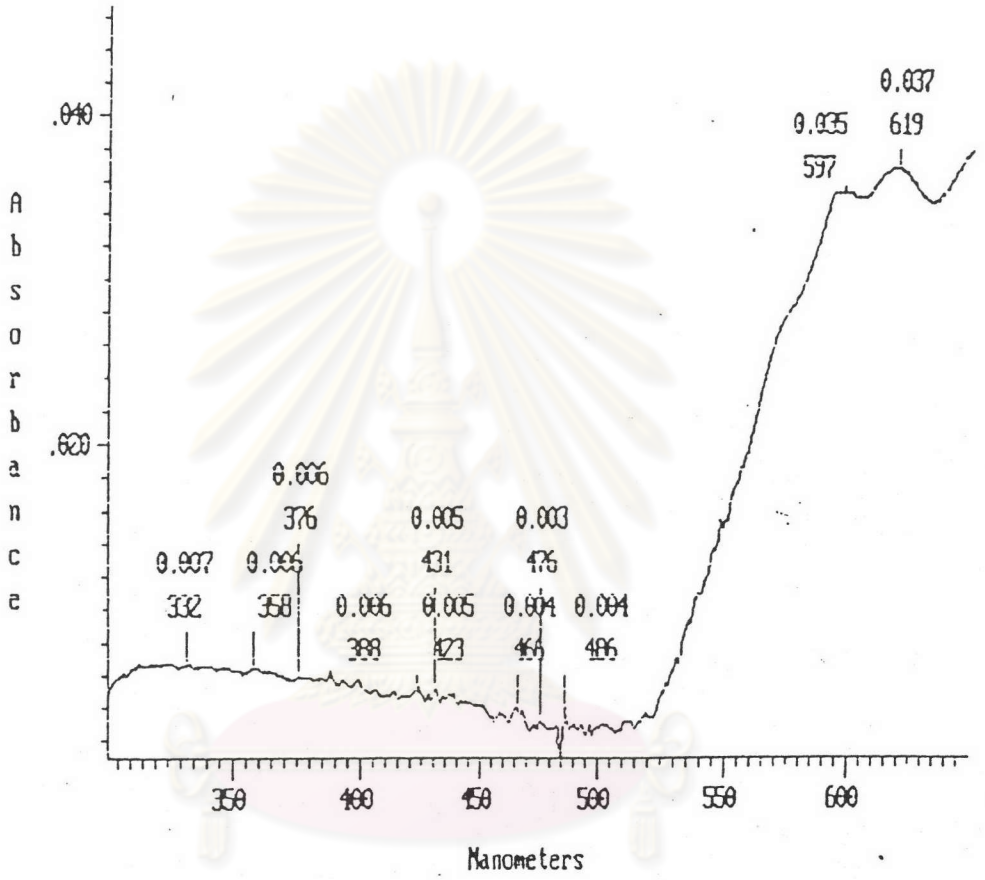


Figure 40. The UV-VIS spectrum of the red pigments in the crude extract after standing for twelve weeks.

At the maximum absorption wavelength (539 - 541nm), the absorbance of the solution was decreased from 0.510 to 0.317 and 0.213 when standing for 2 and 4 weeks, respectively. The maximum absorption wavelength was shifted from 539 nm to 597 and 619 nm with the absorbance 0.035 and 0.037 respectively when standing for 12 weeks. These were showed that the red pigments were not stable and would change their structure on prolong standing even in air and light protected condition.

The stability of the red pigments was the problem in the isolation and elucidation of pure compounds because the decomposition occured all the time. It took very long time in the separation procedure. Large volume of solvents were used to elute the red pigments (see Table 11.).

Because of the large volume of the nonpolar solvent used in the elution, the nonpolar compound espectially fatty acids which were the components of microorganisms (Bishop and Still, 1963) were co-eluted with the red pigments. And because of the stability, the red pigment fraction was shown one spot of red component on TLC when it was concentrated immediatly at low temperature (below 50° C) under reduce pressure and shown many spots even it was kept under nitrogen in the dark for overnight. The preparative TLC was used to purified in the final step and the pure compound must be conducted the further experiments immediately.

Table 11. Separation procedure of red pigments.

Procedure	Solvent	Volume (ml)	Weight (g)
1. Extraction	Methanol : chloroform (1:1)	5000	-
2. Liquid-liquid extraction	Hexane	5,000	0.887
3. Quick column chromatography	20% ethyl acetate in hexane	10.000	-
4. Flash column chromatography	8% ethyl acetate in hexane	10,000	0.1885
5. Gel filtration chromatography	20% methanol in chloroform	5,000	0.0916
6. Preparative TLC	Acetone	2,000	0.027 + 0.045

The RP-1 and RP-2 were separated from preparative TLC. The yield were 3.04 % and 5.07 %, respectively, base on the dry hexane extracted.

2. STRUCTURE ELUCIDATION OF RP-1.

Compound RP-1 was an amorphous, red platelets with green metallic sheen powder that gave positive result with Erlich-reagent. It melting point was 150° - 152° C. The diluted solution in solvents were pink color. The UV-VIS spectra of RP-1 in ethanol, chloroform, acid ethanol and basic ethanol (Figure 12-15.). Table 12 showed the maximum absorption wavelength in visible region which implied the conjugated double bond system.

Table 12. The UV-VIS maximum absorption of RP-1.

Solvents	Maximum Absorption Wavelength (nm)	$E^{1\%}$, 1cm
Ethanol	534	452×10^6
Chloroform	536	931×10^6
Acid Ethanol	534	1095×10^6
Basic Ethanol	532	421×10^6

The IR spectrum of RP-1 (Figure 16.) showed the pattern of the functional group as Table 13.

Table 13. The IR spectrum assignment of RP-1.

Range of absorption (cm^{-1})	Assignment
3500-3100 (broad)	N-H stretching
2925-2800 (strong)	C-H stretching
1741 (medium)	C=N stretching
1599 (medium)	C=C stretching
1460, 1380(medium)	C-H bending of CH_3
1231, 1073(weak)	C-N stretching

From these datas implied that RP-1 was the conjugated double bond pyrrolic compound.

The ^{1}H NMR spectrum of RP-1 showed six protons in the following region, i.e. (Figure 17.) at δ 6.08 (1H, d, J = 2.14 Hz), 6.35 (1 H, ddd, J = 2.14, 2.44, 4.02 Hz), 6.68 (1H, d, J = 2.14), 6.92 (1H, ddd, J = 1.50, 2.40, 4.02 Hz), 6.96 (1H, sb) and 7.23 (1H, ddd, J = 1.50, 2.25, 2,44 Hz). Three protons at δ 6.35, 6.92 and 7.23 ppm were coupled each other and showed the long-range coupling with the proton at δ 12.57 ppm in the H-H COSY spectrum (Figure 18.). This showed the pattern of three proton coupling (AB_2) in mono-substituded pyrrole ring at 2- or 5- position (Breitmaier, 1993). The signal at δ 6.08 ppm showed the coupling with the signal at δ 6.96 ppm and long -range coupling with the signal at δ 12.75 ppm and the signal at δ 6.68 ppm also showed the coupling with the signal at δ 6.96 ppm and long-range coupling with the signal at δ 12.75 ppm. These coupling patterns implied that these three protons were not in the same pyrrole ring. It would be in another two tri-substituted pyrrole rings linked with one methine proton. It was concluded that RP-1 was linear tripyrrole structure.

The natural compounds of linear tripyrrole structure was the red pigment found from the microorganism (Britton,1983) called **prodigiosins**. The common structure (parent nucleus) was 5-(2-pyrryl)-2,2 -dipyrrylmethene called prodigiosene (Wasserman et al., 1960).

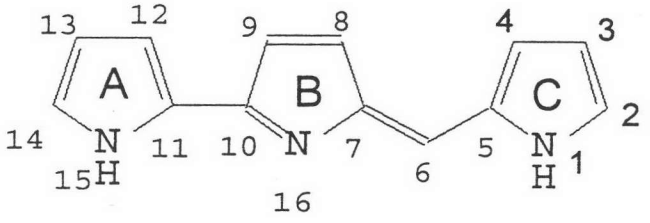


Figure 41. 5-(2-pyrryl)-2,2 -dipyrrylmethene (prodigiosene).

The numbering system chosen here for the prodigiosin series was more appropriate for systematic chemical and NMR discussion for which every carbon needed to be number (the system selected had close kinship to that in Chemical Abstracts Index Guide for the bile pigments) (Deol et al., 1974)

The ^{1}H NMR chemical shift in aromatic region of RP-1 compared to prodigiosin compounds reported in literature were shown in Table 14. The position of protons of RP-1 was assigned by H-HCOSY (Figure18.).

Table 14. The ^{1}H NMR chemical shift of RP-1 and prodigiosin-like compounds.

Position	Chemical shift (ppm), splitting pattern						
	RP-1	I^a	I^b	II	VI	VII	X
Aromatic region							
1	12.75sb	nr	nr	nr	12.67	11.75	nr
3	-	-	-	6.34m	6.52	6.6d	-
4	6.68db	6.69d	6.30s	6.55m	-	6.6d	-
6	6.96sb	6.97s	6.84s	6.97s	7.12	6.81s	6.9s
9	6.08db	6.09d	6.08s	6.20d	6.10	5.99s	6.1s
12	6.92ddd	6.94m	6.74m	6.90m	6.90	6.41d	6.7
13	6.35ddd	6.37m	6.15m	6.34m	6.34	5.77d	6.2
14	7.23ddd	7.25m	6.74m	7.23m	7.22	-	6.8
15	12.57sb	nr	nr	nr	12.71	11.71	nr
16	12.75sb	nr	nr	nr	12.58	nr	nr

Table 14 (cont.). The ^{1}H NMR chemical shift of RP-1 and prodigiosin-like compounds.

Position	Chemical shift (ppm), splitting pattern						
	RP-1	I^a	I^b	II	VI	VII	X
Aliphatic region							
8-OCH_3	4.01s	4.01s	3.96s	4.01s	4.02s	3.89s	3.98s
2-CH_3	2.54s	2.55s	2.26s	-	-	-	2.0s
3-CH_2-	2.39t	2.40m	1.68m	2.85t	2.54t	2.11t	2.35m
Al-CH_3	0.90t	0.90t	0.88t	1.24t	0.93t	0.87t	0.95t

I^a = Prodigiosin protonated form (Boger and Patel, 1988)

I^b = Prodigiosin free base form (Boger and Patel, 1988)

II = Undecylprodigiosin (Wasserman et al. 1966)

VI = Cycloundecylprodigiosin (Gerber, 1970)

VII = Cycloethylpropylprodigiosin (Gerber and Gauthier, 1979)

X = Butylcycloheptylprodigiosin (Laatsch et al., 1991)

(Figure 7.)

s = singlet

d = douplet

b = broad

nr = not reported

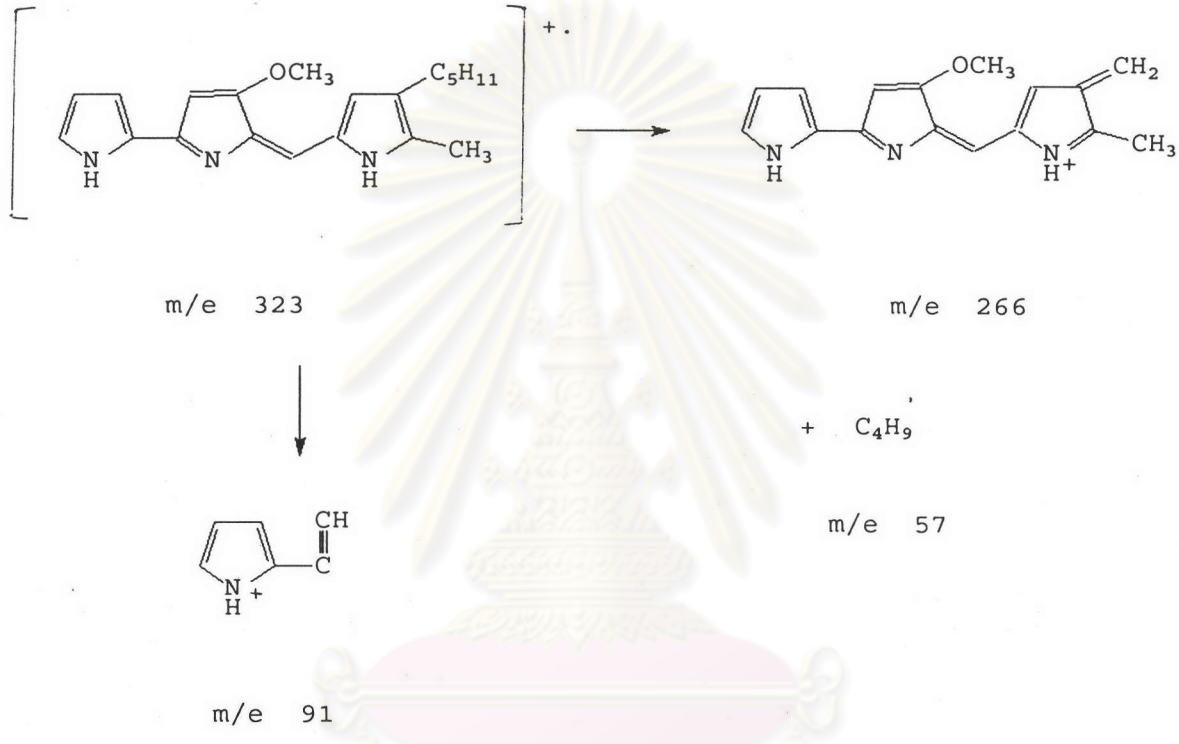
RP-1 was the prodigiosene nucleus compound, the aliphatic chemical shifts were almost identical with those of prodigiosin that was substituted at 2-, 3- and 8- position. The signal at δ 4.01 ppm (3H, s) was the methoxy group. The signal at δ 2.54 ppm (3H, s) was the methyl group substituted on pyrrole ring. The signal at δ 2.39 ppm (2H, t) was the methylene group substituted on pyrrole ring. The signal at δ 0.90 ppm (3 H, t) was the methyl group at the end of aliphatic side chain. There was a broad signal at δ 1.2 - 1.3 ppm, which belonged to the methylene groups at the aliphatic side chain. These implied that RP-1 was prodigiosin-like compound contained methyl at 2- position and aliphatic long chain substitution at 3-position on the prodigiosene nucleus.

The identification of alkyl homologues from prodigiosin by mass spectrometry should thus be relatively easy (Jackson and Kenner, 1967). It was already known that the predominant side chain cleavage in prodigiosin pigments was β to a pyrrole ring: 266 m.u. in prodigiosin (I), 255 m.u. in nonyprodigiosin (III) and undecylprodigiosin (II) (Gerber, 1973).

The mass spectrum of RP-1 (Figure 11.) showed the molecular peak ion (M^+) at 323 m.u. The base peak was 91 m.u. The other peaks were 266, 57, and 55 m.u. Since the 91 m.u. peak was predominant in the spectrum of 4-methoxy-2,2 -bipyrrole as well as all prodigiosin-like compounds it must represent a fragment of ring A and B, not C. Since 91 m.u. = C_6H_5N the fragment must contain the atom of ring A as

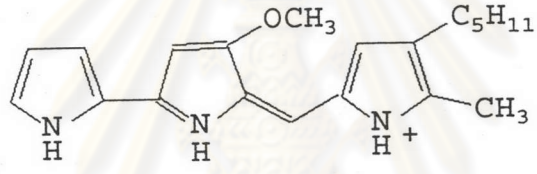
well as two carbons of ring B because fragmentations which would leave ring B intact were not favored (Jackson and Kenner, 1967). Other intense ion produced results from β-cleavage of the alkyl side-chain (metastable peak at 219). (A weak ion was also observed for the loss of 15 mass units from the parent ion, and this may be derived either by cleavage of the terminal methyl group of the alkyl side-chain or by cleavage of methyl from the methoxy group).

The fragment of RP-1 comprised characteristic fragment of prodigiosin (I). The peak at m/z 323 was the molecular peak of prodigiosin. The scheme 8. showed the fragmentation pathway of prodigiosin (I) (Jackson and Kenner, 1967).



Scheme 8. Fragmentation of prodigiosin.

From all the datas could be concluded that RP-1 was 2,2 -bipyrrole,4-methoxy-5-[(5-methyl-4-pentyl-2H-pyrrole-2-ylidene)methyl], the *Chemical Abstracts'* systemetic name for prodigiosin in the protonated form (Figure 42.) which found naturally (Rapoport and Holden, 1962).



RP-1 : 2,2'-bipyrrole,4-methoxy-
5-[(5-methyl-4-pentyl-2H-
pyrrole-2-ylidene)methyl]

Figure 42. Chemical structure of RP-1.

The ^{13}C NMR spectrum of RP-1 (Figure 20.) showed twenty signals of twenty carbons. They were divided into seven quaternary carbons (all of them were sp^2), six methine carbons (all of them were sp^2), four methylene carbons and three methyl carbons (DEPT, Figure 21.). The methyl signal at δ 58.69 ppm was characteristic of methoxy signal. The methyl signal at δ 14.00 ppm was characteristic of 2-substituted pyrrole. And the methyl signal at δ 14.91 ppm was the methyl at the end of aliphatic chain. The aliphatic side chain was pentyl (four methylene and one methyl). The seven quarternary and six methine carbons were in the prodigiosene nucleus. The HMQC (Figure 22.) were used in the assignment of carbon of RP-1 (Table 18.).

3. STRUCTURE ELUCIDATION OF RP-2.

Compound RP-2 was an amorphous, red platelets with green metallic sheen powder that gave positive result with Erlich-reagent. It melting point was 90° - 92° C. The diluted solution in solvents were orange-yellow color. The UV-VIS spectra of RP-2 in ethanol, chloroform, acid ethanol and basic ethanol (Figure 24-27., Table 15.). showed the maximum absorption wavelength in visible region which implied the conjugated double bond system.

Table 15. The UV-VIS maximum absorption of RP-2.

Solvents	Maximum Absorption Wavelength (nm)	$E^{1\%}$, 1cm
Ethanol	469	50.8×10^6
	535	41.7×10^6
Chloroform	539	157.7×10^6
Acid Ethanol	535	131.3×10^6
Basic Ethanol	468	48.0×10^6
	533	29.3×10^6

RP-2 in ethanol showed two maximum absorption wavelength at 469 nm and 535 nm with the $E^{1\%}$,1cm almost the same while there was one maximum absorption wavelength at 539 nm in chloroform. When it was in acid condition the absorption at 469 nm was disappeared and showed again in basic condition. These were implied that the chemical structure of RP-2 was effected by solvent and H^+ ion. RP-2 was in two forms; free form and protonated form.

The IR spectrum of RP-2 (Figure 29.) showed the pattern of the functional group as Table 16.

Table 16. The IR spectrum assignment of RP-2.

Range of absorption (cm^{-1})	Assignment
3500-3100 (broad)	N-H stretching
2925-2800 (strong)	C-H stretching
1741 (medium)	C=N stretching
1601 (medium)	C=C stretching
1541,1459 (medium)	C-H bending of CH_3
1244,1136,1020 (weak)	C-N stretching
974, 823 (weak)	C-H bending out of plane

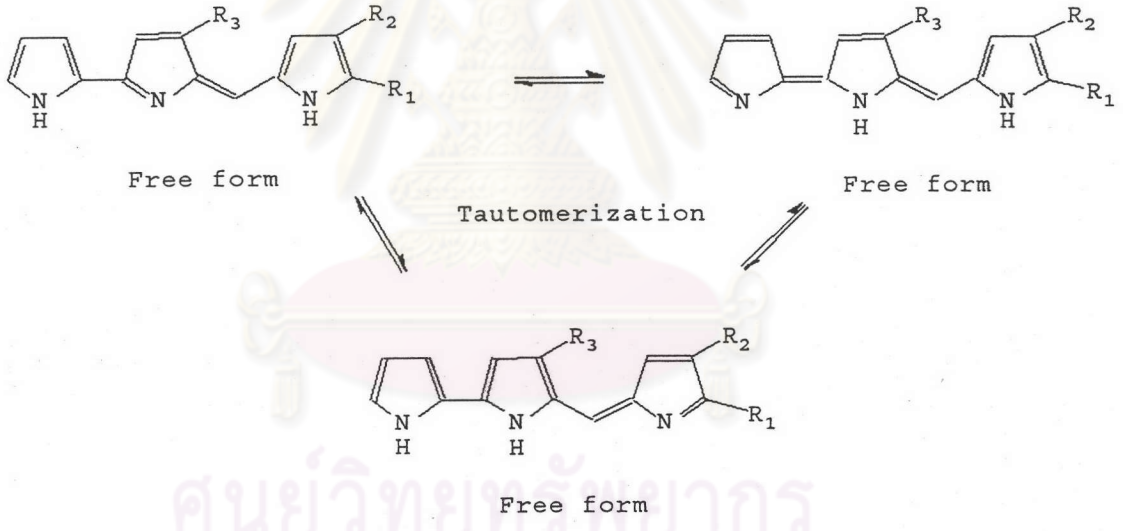
From these datas implied that RP-2 was the conjugated double bond pyrrolic compound.

The ^{1}H NMR spectrum of RP-2 showed six protons in the aromatic region (Figure 30.), almost identical with RP-1, at δ 6.04 (1H, sb), 6.15 (1H, dd), 6.33 (1H, sb), 6.66 (1H, dd), 6.68 (1H, sb) and 6.79 (1H, sb) ppm. But not like the ^{1}H NMR of RP-1 that all peaks were broad and not shown the sharp splitting pattern that made it impossible to determine the *J* value. From the H-H COSY spectrum showed in Figure 31., three protons at δ 6.15, 6.66 and 6.68 ppm were coupled each other. This showed the pattern of three proton (AB_2) in mono-substituded pyrrole ring at 2- or 5- position. The signal at δ 6.04 and 6.33 ppm showed the coupling with the signal at δ 6.79 ppm but no coupling to each other. Each signal showed singlet broad. It would be in another two tri-substituted pyrrole rings linked with one methene proton.

It was concluded that RP-2 was linear tripyrrole structure, prodigiosene. From the broad signals would imply that there was more than one form of chemical structure in the solution (tautomerization) (Scheme 9.)

Rapoport and Holden (1962) reported that prodigiosene free form was in two tautomers. Our ^{1}H NMR experiment, the two signal at the low

field (δ 12.57 and 12.75 ppm that showed in RP-1) were disappeared. This implied that the two protons on prodigiosene free form were shifted between 1-, 15-, and 16- position. One nitrogen of the three pyrrole rings was free, but not fixed. These would concluded that there were three tautomers mixture in the solution of prodigiosin free form.



Scheme 9. Tautomerization of prodigiosene.

However when prodigiosin was protonated, as it occured naturally and as it was found synthetically, any tautomers became indistinguistable.

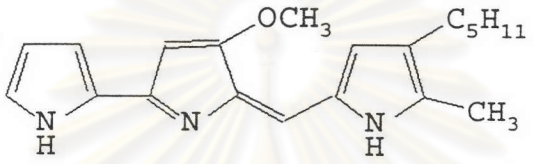
RP-2 was the prodigiosene nucleus compound, the aliphatic chemical shifts were almost identical with those of prodigiosin that was substituted at 2-, 3- and 8- position. The signal at δ 3.95 ppm (3H, s) was the methoxy group. The signal at δ 1.82 ppm (3H, s) was the methyl group substituted on pyrrole ring. The signal at δ 2.22 ppm (2H, t) was the methylene group substituted on pyrrole ring. The signal at δ 0.92 ppm (3 H, t) was the methyl group at the end of aliphatic side chain. There was a broad signal at δ 1.2 - 1.3 ppm which was the methylene groups in the aliphatic side chain. These implied that RP-2 was prodigiosin-like compound contained methyl at 2- position and aliphatic long chain substitution at 3-position on the prodigiosene nucleus.

The mass spectrum of RP-2 (Figure 23.) showed that the molecular peak ion (M^+) at 323 m.u. The base peak was 91 m.u. The other peaks were 266, 149, 57 and 55 m.u. These were used to identify the alkyl homolouges of prodigiosin-like compounds .

The fragment of RP-2 comprised characteristic fragment of prodigiosin (I) (Scheme 8.) The peak at m/z 323 was the molecular peak of prodigiosin.

The ^{13}C NMR spectrum of RP-2 (Figure 33.) showed twenty signals of twenty carbons. They were divided into seven quaternary carbons (all of them were sp^2), six methine carbons (all of them were sp^2), four methylene carbons and three methyl carbons (DEPT, Figure 34.). The methyl signal at δ 58.00 ppm was characteristic of methoxy signal. The methyl signal at δ 10.55 ppm was characteristic of 2-substituted pyrrole. And the methyl signal at δ 14.02 ppm was the methyl at the end of aliphatic chain. The aliphatic side chain was pentyl (four methylene and one methyl). The seven quarternary and six methine carbons were in the prodigiosene nucleus. The HMQC (Figure 35.), and HMBC (Figure 36.) were used in the assignment of carbon of RP-2 (Table 18.).

From all the datas could be concluded that RP-2 was 2,2 -bipyrrole,4-methoxy-5-[(5-methyl-4-pentyl-2H-pyrrole-2-ylidene)methyl], the *Chemical Abstracts'* syatemetic name for prodigiosin in the free form.



RP-2 : 2,2'-bipyrrole,4-methoxy-
5-[(5-methyl-4-pentyl-2H-
pyrrole-2-ylidene)methyl]

Figure 43. Chemical structure of RP-2.

Table 17. The 500 MHz ^{1}H NMR assignment of RP-1, RP-2 in deuterated chloroform and prodigiosin 2 forms [a].

Position	Chemical Shift, ppm			
	RP-1	Prodigiosin (protonated form)	RP-2	Prodigiosin (free form)
1	12.75	-	-	-
4	6.68	6.69	6.33	6.30
6	6.96	6.97	6.97	6.84
9	6.08	6.09	6.04	6.08
12	6.92	6.94	6.66	6.74
13	6.35	6.37	6.15	6.15
14	7.23	7.25	6.68	6.74
15	12.57	-	-	-
16	12.75	-	-	-
8-OCH_3	4.01	4.01	3.95	3.96
2-CH_3	2.54	2.55	1.82	2.26
1'	2.39	2.40	2.22	1.68
2'	1.55	1.55	1.44	1.46
3', 4'	1.25	1.34	1.25	1.28
5'	0.90	0.90	0.92	0.88

[a] From Boger and Patel, 1988.

Table 18. The 125 Mhz ^{13}C NMR assignment of RP-1, RP-2 in deuterated chloroform and prodigiosin 2 forms [a,b].

Position	Chemical Shift, ppm			
	RP-1	Prodigiosin (protonated form)	RP-2	Prodigiosin (free form)
2	122.29	124.7	128.41	131.4
3	117.00	119.8	112.30	115.3
4	111.72	114.3	110.00	112.6
5	126.97	129.0	125.55	125.4
6	116.06	111.2	115.78	118.7
7	155.49	123.3	158.81	140.7
8	165.79	168.3	160.65	171.9
9	92.81	95.5	95.21	98.2
10	147.75	150,2	137.90	162.4
11	120.74	148,8	136.44	139.6
12	125.19	130.8	124.25	126.9
13	128.43	130.8	120.78	123.8
14	128.55	127.6	127.42	130.1

Table 18 (cont.). The 125 Mhz ^{13}C NMR assignment of RP-1, RP-2 in deuterated chloroform and prodigiosin 2 forms [a,b].

Position	Chemical Shift, ppm			
	RP-1	Prodigiosin (protonated form)	RP-2	Prodigiosin (free form)
OCH_3	58.69	61.3	58.00	61.0
C_2-CH_3	12.43	14.8	10.55	12.7
1'	25.33	27.8	25.51	28.3
2'	29.80	32.2	31.64	32.9
3'	30.30	34.0	30.20	34.5
4'	29.34	25.0	22.50	25.2
5'	14.91	16.5	14.02	16.7

[a] From Cushley et al., 1975.

[b] Chemical shifts were given relative to HMDS and calculated from the solvent peak $CHCl_3$ (δ_c 80.0 ppm). Since chloroform did show solvent effects of several tenths of a part per million, all data had been rounded off to the nearest 0.1 ppm.

There were the chemical shifts difference between the ^{1}H and ^{13}C NMR spectrum of RP-1 and RP-2 when compared with the reported prodigiosin (Table 17. and Table 18.). These may be because of the different NMR instrument, solvent and internal standard. Finally the tautomerization of these compounds, showed many signals in the NMR spectrum and the different predominant species may be occurred.

Figure 44. The ^{1}NMR of RP-1 and RP-2 in comparison.

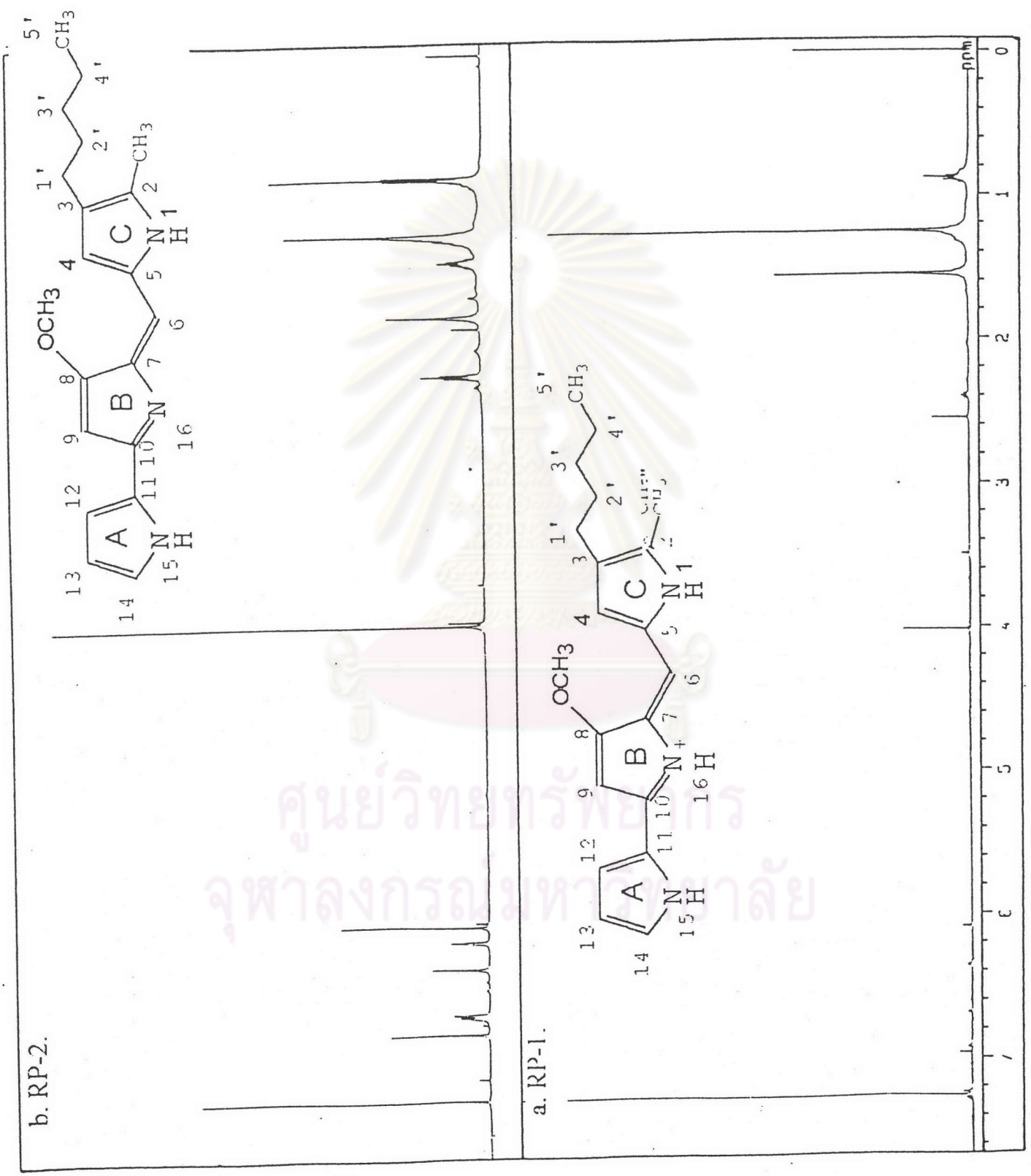


Figure 44.1. The ^{1}H-NMR spectra of RP-1 and RP-2, in comparison, expanded at δ 0.5 - 2.7 ppm.

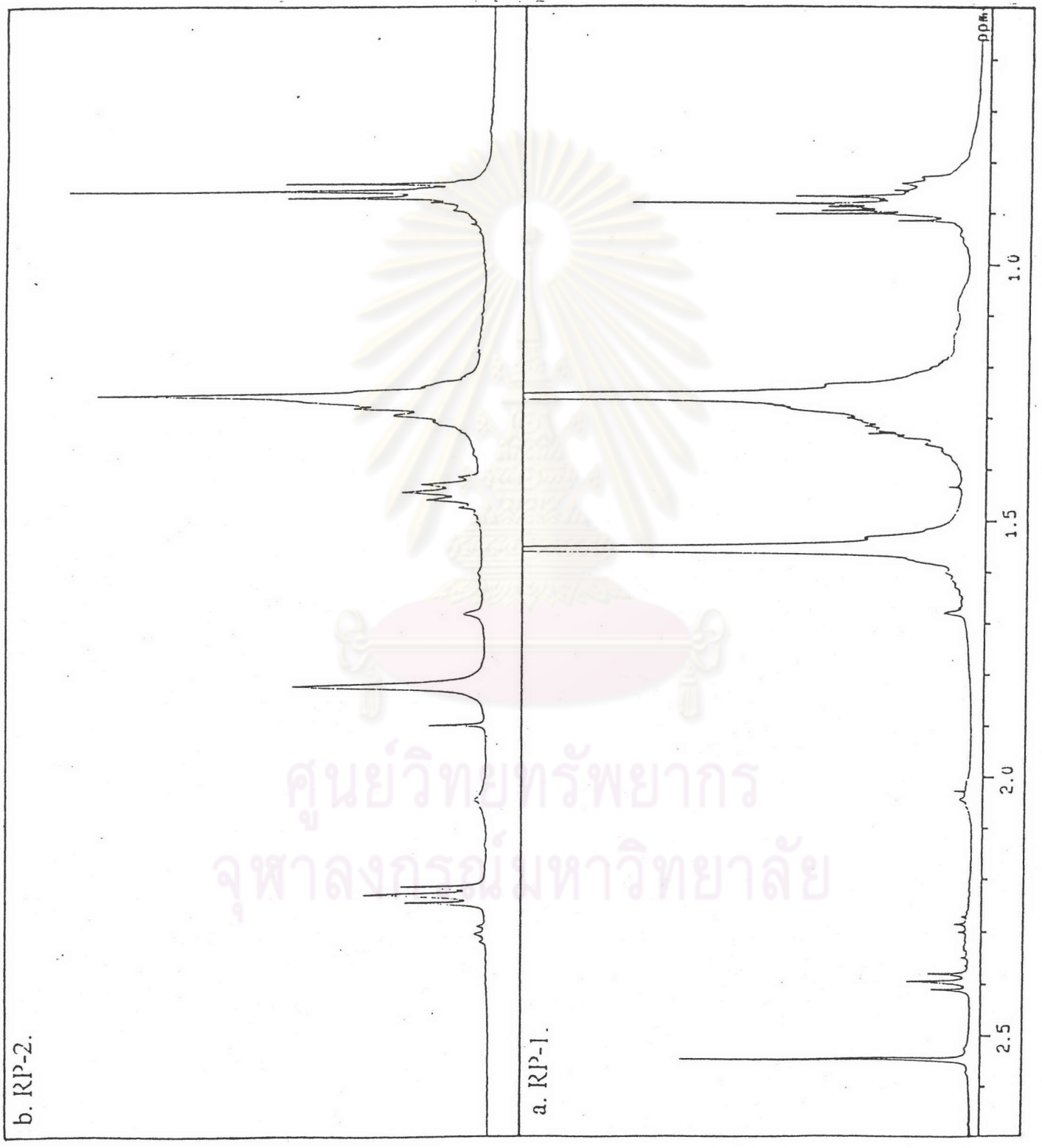
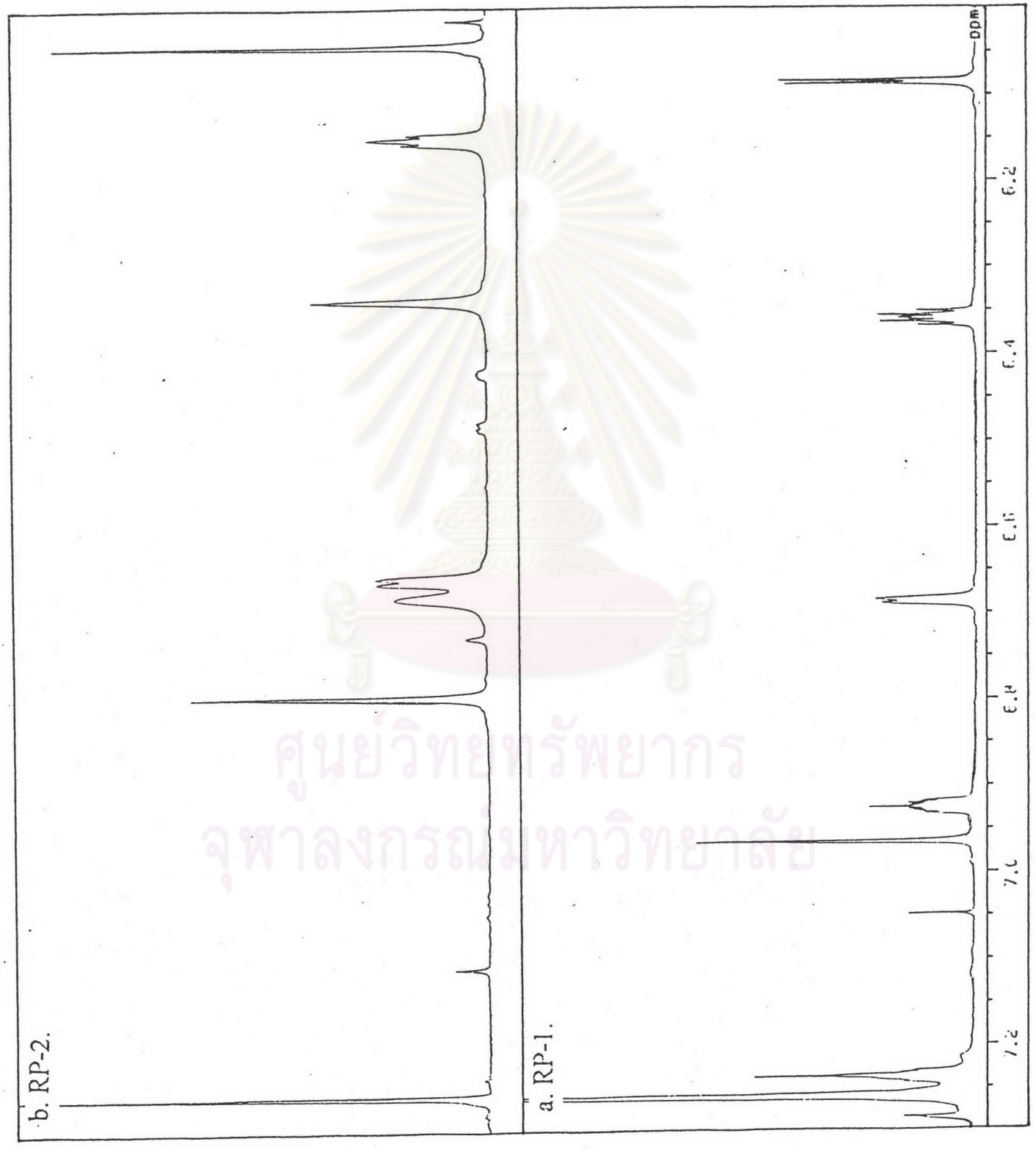


Figure 44.2. The ^{1}H-NMR spectra of RP-1 and RP-2, in comparison, expanded at δ 6.0 - 7.3 ppm



4. TRANSFORMATION OF RP-2 TO RP-1.

When RP-2 was stored in deuterated chloroform with air and light protection in refregirator for about two months, the water rich deuterated chloroform was added for dilution. The ^{1}H NMR signal was changed as shown in Figure 45. Every peaks were broaden and shift to down field. The signals at δ 12.58 ppm and 12.74 ppm were shown. The color of the solution was changed from orange to deep red-violet. From H-H COSY spectrum (Figure 46) showed the coupling pattern almost the same as before. This could implied that there was a little change in structure. The main structure was still the prodigiosene .

Because the red pigment was unstable as discuss before the RP-2 changed compound was purified by preparative TLC using 50 % ethyl acetate in hexane as solvent system. The band of red pigment was moved to Rf value 0.425 (average), very broad (about 2 cm) and splitted into two shades of red color (not absolutely separated) . The highest was red (even wet or dry) (1.5 cm broad).The lowest was orange-red when wet and red when dried (0.5 cm broad). And left the purple brown residue on the origin (could not move in this system). Eluted each band separately as RP-2A and RP-2B, respectively, with acetone and evaporated to dryness. The UV-VIS absorption of these fractions were shown in Table 19.

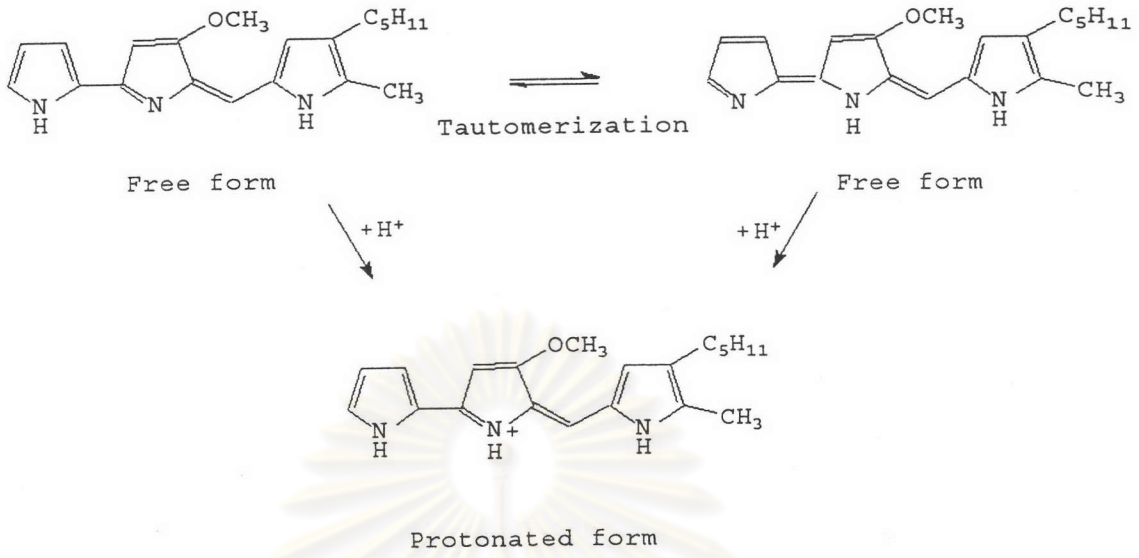
Table 19. The UV-VIS absorption of rechromatographed RP-2.

Solvent	RP-2 Fraction	Wavelength ($E^{1\%}$,1 cm)	
		λ1	λ2
Chloroform	A	s (500nm)	538 (931 X 10^6)
	B	s (500nm)	536 (1049 X 10^6)
Ethanol	A	499 (309 X 10^6)	533 (452 X 10^6)
	B	499 (320 X 10^6)	534 (475 X 10^6)
Acid ethanol	A	s (500nm)	535 (885 X 10^6)
	B	s (500nm)	535 (1042 X 10^6)
Basic ethanol	A	497 (301 X 10^6)	532 (397 X 10^6)
	B	s (500nm)	531 (470 X 10^6)

It could conclude that these two fractions were the same compound and were effected by the solvent but not the hydrogen ion.

The ^{1}H NMR spectrum of RP-2A (the most yield, < 5 mg) was shown in Figure 46. There were six olefinic protons at δ 6.08 (d), 6.35 (ddd), 6.68 (d), 6.92 (ddd), 6.96 (sb) and 7.23 (ddd) ppm and showed the splitting pattern the same as RP-1 (Figure 47). These were confirmed that RP-1 and RP-2 were the same compound in different forms. RP-1 was the protonated form of prodigiosin, which reported was the form occured in the nature and RP-2 was the free form of prodigiosin in tautomers mixture. When RP-2 was protonated, it changed to RP-1, the proton did the deshielding effect so every proton signals in the molecule were shifted to down field. The protonation was occured by the traces water in deuterated chloroform which would form when the chloroform was stored for a long time without stabilizer (Remington, 1995). Pure chloroform readily decomposed on keeping particularly if exposed to moisture and sunlight, resulting in formation of phosgene (carbonyl chloride [$COCl_2$]) and other products. In general, the presence of a small amount of alcohol in chloroform greatly retarded or prevented this decomposition. The stabilizer in deturated chloroform was silver foil. Chloroform was not effected by acids, but was decomposed by alkali hydroxide into alkali chloride and sodium formate :

$$CHCl_3 + 4NaOH \longrightarrow 3NaCl + HCOONa + 2H_2O$$



Scheme 10. The spontaneously transformation of prodigiosin base form to protonated form by the hydrogen ion.

Hubbard and Rimington (1950) had reported that prodigiosin in solution could exist in one of two distinct but readily interconvertible forms or as a mixture of these two forms, depending on the hydrogen ion concentration of the medium. There were the difference in the position of the absorption maxima as well as in the width and intensity of the absorption bands of the two forms. In acid solution, the pigment was red, due to asharp, high, narrow main band with a maximum at 535-540 nm and a slight shoulder on the low wavelength limb of the curve at about 500 nm. This hump was always present and was independent of the purity of the sample. In alkali solution, the pigment was orange-yellow due to a

broader, less intense, roughly symmetrical band centered at 470 nm. The secondary hump at 530 nm, was due to a trace of the acid form chromogen. The base form was also fluorescence with excitation maximum at 380 nm and emission maximum at 688 nm whiel the acid form was not. This also reported by Castro et al., 1959.

The pure 470 nm band could only be obtained in fairly alkali solvents.The pure 540 nm band is easily obtained in all non-alkaline organic solvents and seemed to be the more stable form as the 470 nm chromogen passes into the 540 nm chromogen spontaneously on prolonged standing.

Monk (1957) also reported of the 3 absorbing components, the orange one at 475 nm was very unstable and appeared to convert to a form absorbing at 535 nm. The 500 nm component apparently was not affected by pH changed, but in alkali conditions the 535 nm peak was shifted to 475 nm.

He reported that the 475 nm component was very unstable at room temperature and was altered in HCl so as to absorb at 535 nm. This shift was partially reversible, but the instability of the 475 nm component eventually produced a stable terminal spectra similar to that of the acidified fresh pigment and independent of pH.

Stefanye (1960) reported that protonated prodigiosin was about as weak an acid as hydrogen sulfide or hydrochlorous acid, but still considerably stronger than water or alcohol. The traces carbon dioxide in water (as carbonic acid) and acid impurities in the solvent could change the color from orange-yellow to red immediately. Merely shaking an aqueous acetone or alcohol solution of the orange basic form of prodigiosin would change the color to red because of carbon dioxide picked up from the air (Hearn, Castro and Elson, 1968). This meaned prodigiosin free form was very unstable and easily change to protonated form by carbon dioxide and moisture in air or traces hydrogen ions in solvents. And this was the reason why prodigiosin occurred naturally and as it was formed synthetically was in the protonated form.

Prodigiosin was a very weak base (pKa = 8.25 in aqueous ethanol). It could not protonated to form salt with weak acid. The salt form was done by shaking with strong acid such as hydrochloric and perchloric acid.

In our experiment showed that the protonated form of prodigiosin was happened by hydration. By the reason of the nearness of Rf value, the effect of hydrogen ion, the same IR spectrum and the same molecular weight in the mass spectrum. Also the absolutely dissappeared of the signal at δ 1.82 ppm after two weeks was confirmed the formation of hydrated prodigiosin form.

From the former experiments were not interpreted the change in spectrophotometric data chemically, only assumed that the spectral shift was due to the formation of a salt on one of the pyrrole nitrogen. Our experiments shown that the protonation was occured by hydration, not by acid.

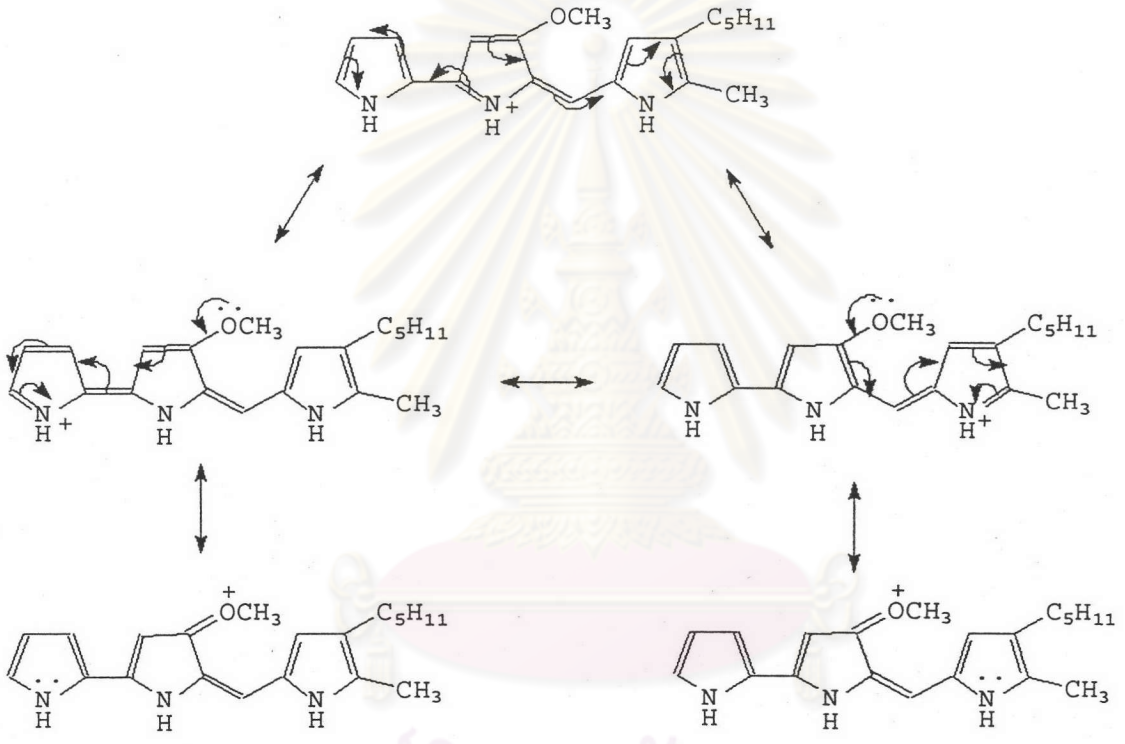
Free form prodigiosin was very unstable, it spontaneously transformed to protonated form by traces of water in solvents, adsorbent, and moisture in the air. The protonated form was more stable than free form, it was not effected by pH (Table 12.). In the purification process of liquid-liquid extraction which basified and extracted with hexane, the protonated form was transformed to free form. The extracted prodigiosin in acetone, ethylacetate and hexane were shown the two form of prodigiosin in the difference ratio, not only the free form (Table 20. and Figure 48-50.). The traces water in methanol and chloroform made prodigiosin showed only protonated form, maximum absorption at 533 and 538, respectively, with a limb at 500 nm (Figure 51 and 52.)

Table 20. The UV-VIS absorption of reextracted RP-2A.

solvent	Color	Wavelength (nm)		Ratio
		λ_1	λ_2	
Acetone	Yellow-orange	463	529	2.9:1
Ethylacetate	Yellow-orange	470	533	1.5:1
Hexane	Orange	463	537	1:1.1
Methanol	Orange	-	533	-
Chloroform	Pink	-	538	-

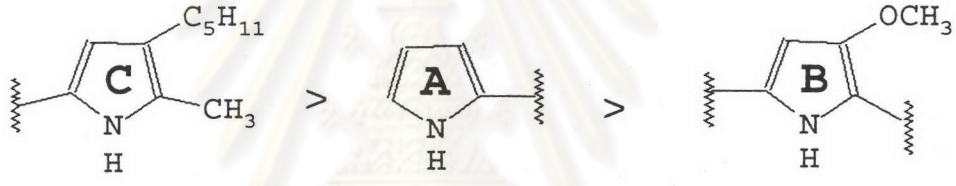
This experiment was shown the ^{1}H NMR spectrum changed during the transformation reaction of prodigiosin free form (orange-yellow color) (RP 2) to prodigiosin protonated form (red color) (RP 1) (Figure 46). The assignment of ^{1}H NMR and ^{13}C NMR of these two forms were reported (Table 17., 18.).

The protonation could be occured with the resonance of the electron in the molecule (scheme 11). The ^{1}H NMR spectrum showed the coupling of proton in the ring with the amine proton in all pyrrole rings. These were confirmed that there were three protons in the 1-, 15-, and 16-position (hydrated form).



Scheme 11. Protonation of the prodigiosin.

Consideration the basicity of the pyrrole ring A, B and C in prodigiosin molecule, ring C was more basic than ring B because the two alkyl substitution were electron donating groups and ring A was the least because the methoxy substitution was electron withdrawal group. So protonation at the 1-position in the ring C must be preferable.



Scheme 12. The basicity of the pyrrole rings in prodigiosin molecule.

Table 21. The 500 Mhz of ^{1}H NMR chemical shift difference of RP-1 and RP-2 in deuterated chloroform.

Position		Chemical Shift, ppm		
		RP-1	RP-2	Different
ring A,	12	6.92	6.66	0.26
	13	6.35	6.15	0.20
	14	7.23	6.68	0.55
	15	12.57	-	-
ring B,	9	6.08	6.04	0.04
	8-OCH_3	4.01	3.95	0.06
	16	12.75	-	-
ring C,	1	12.75	-	-
	2-CH_3	2.54	1.82	0.72
	3-CH_2-	2.39	2.22	0.17
	4	6.68	6.33	0.35

From the difference of the chemical shift of the two forms of prodigiosin, the ^{1}H NMR signals of protonated form were more down field than of free form because of the deshielding effect of protonation (Table 21.). The most difference value was at ring C, 2- CH_3 position (0.72 ppm). This would be concluded that the protonation was occured at 1- position more than another position and was the predominated form.

Boger and Patel (1988) reported that the prodigiosin salt form was protonated at 16- position. Our experiment showed that the protonation must be occured at 1- position as showed in Figure 42.

Figure 45. The 500 Mhz ^{1}H-NMR spectrum of RP-2 in water-rich deuterated chloroform, the spontaneously transformed to protonated form.

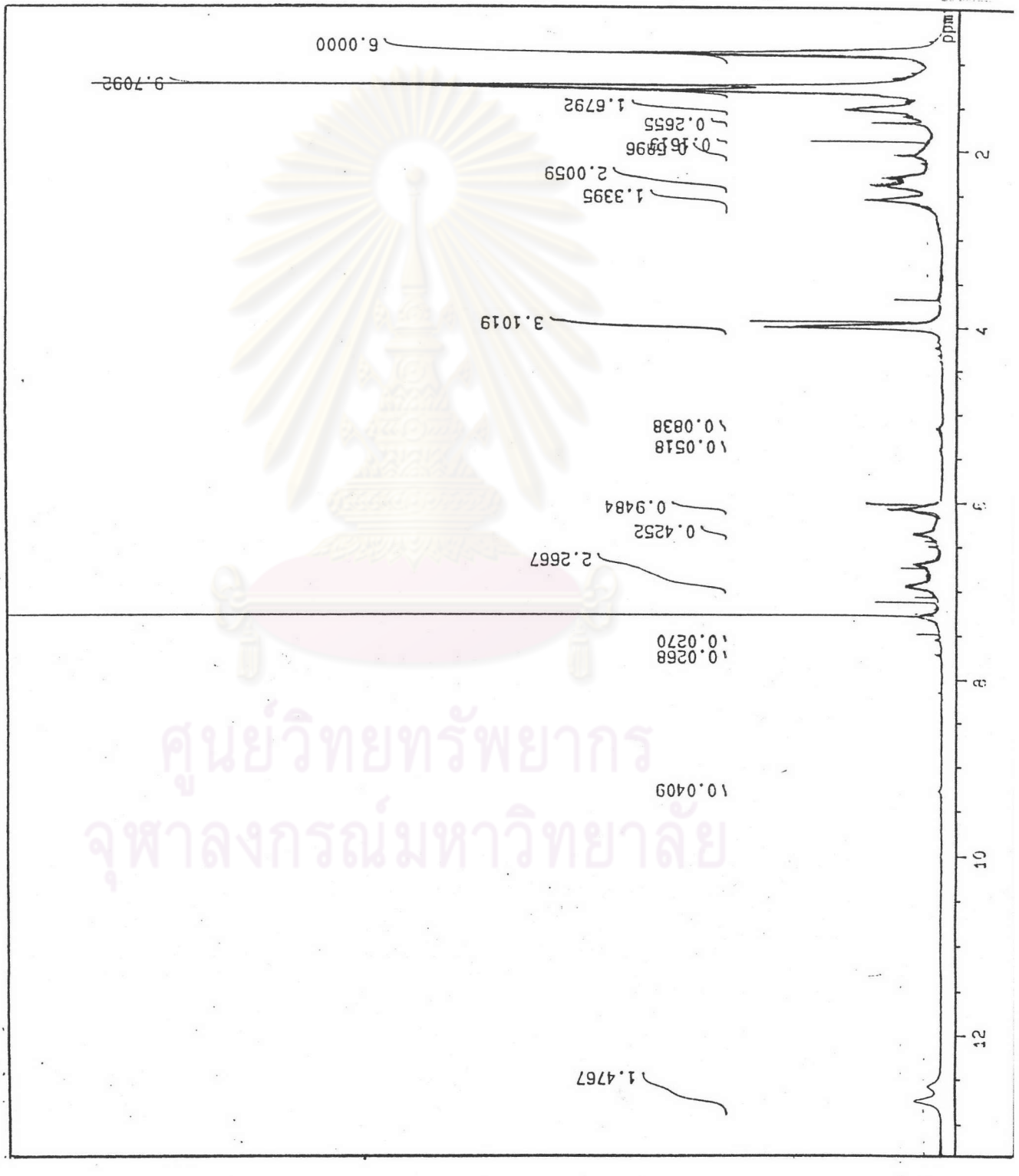


Figure 45.1. The ^{1}H-NMR spectrum of RP-2, the spontaneously transformed to protonated form in various time.

Figure 45.2. The ^{1}H-NMR spectrum of RP-2, the spontaneously transformed to protonated form, expanded at δ 0.5 - 3.0 ppm.

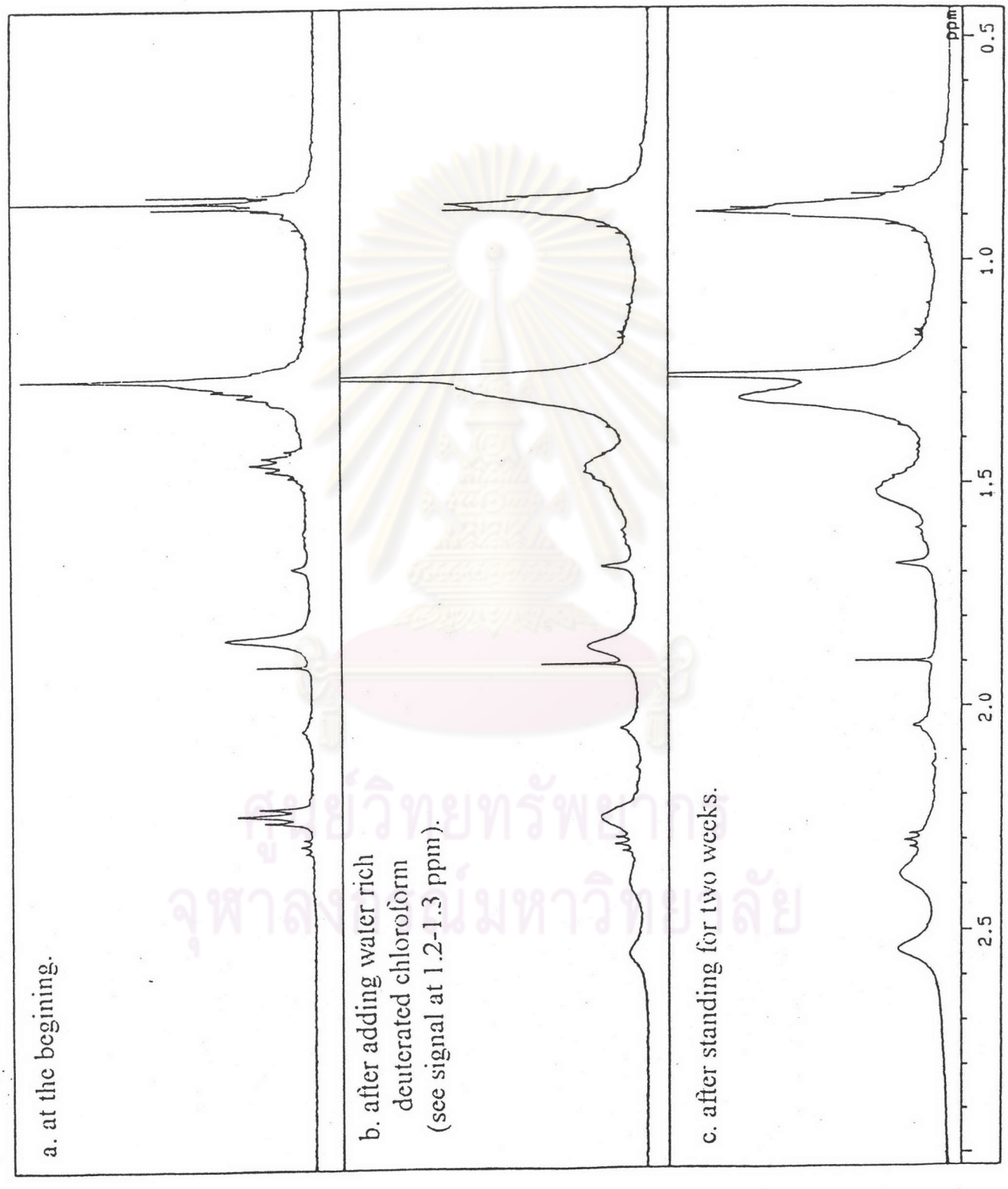


Figure 45.3. The ^{1}H NMR spectrum of RP-2, the spontaneously transformed to protonated form, expanded at δ 5.5 - 8.0 ppm

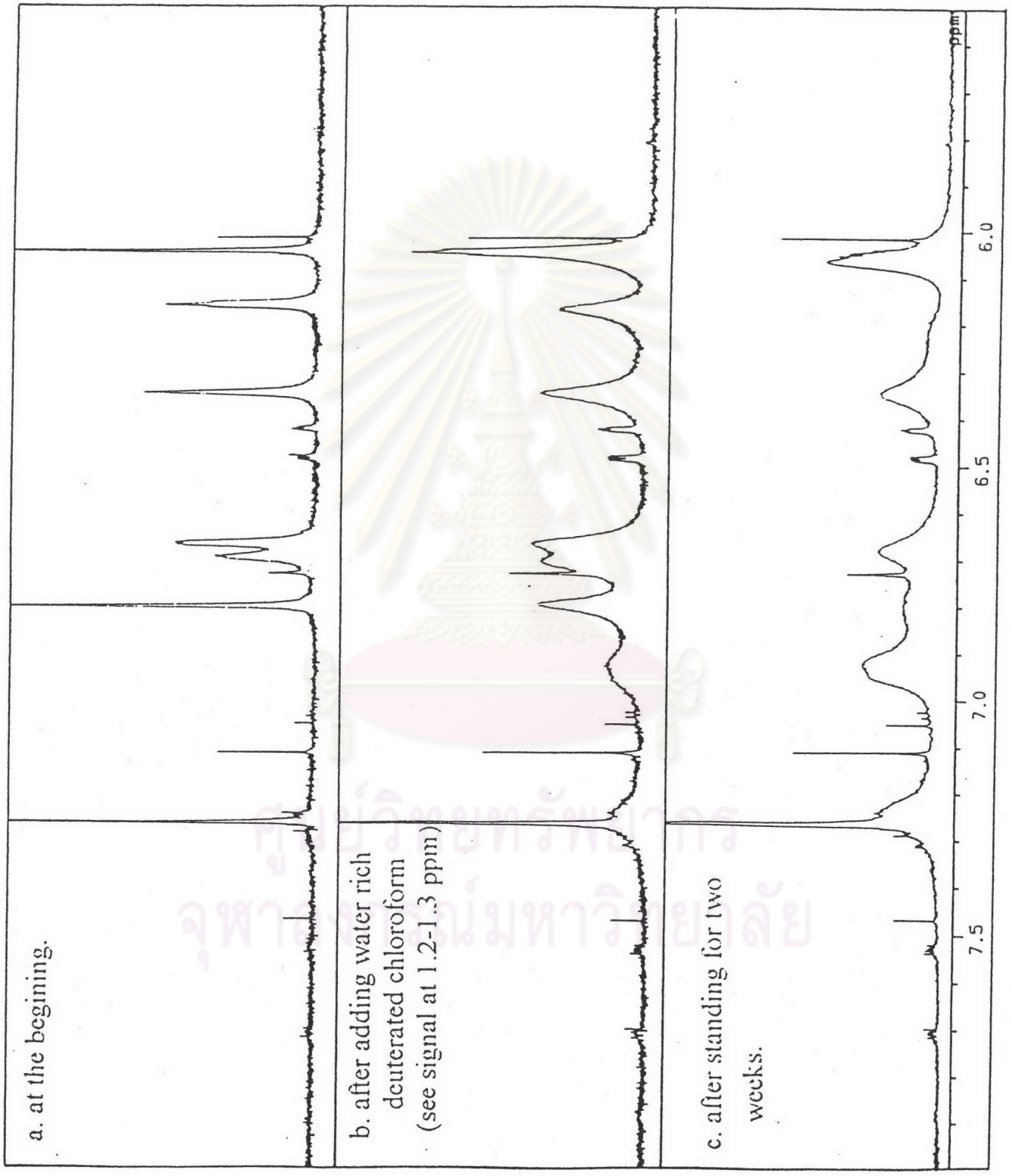
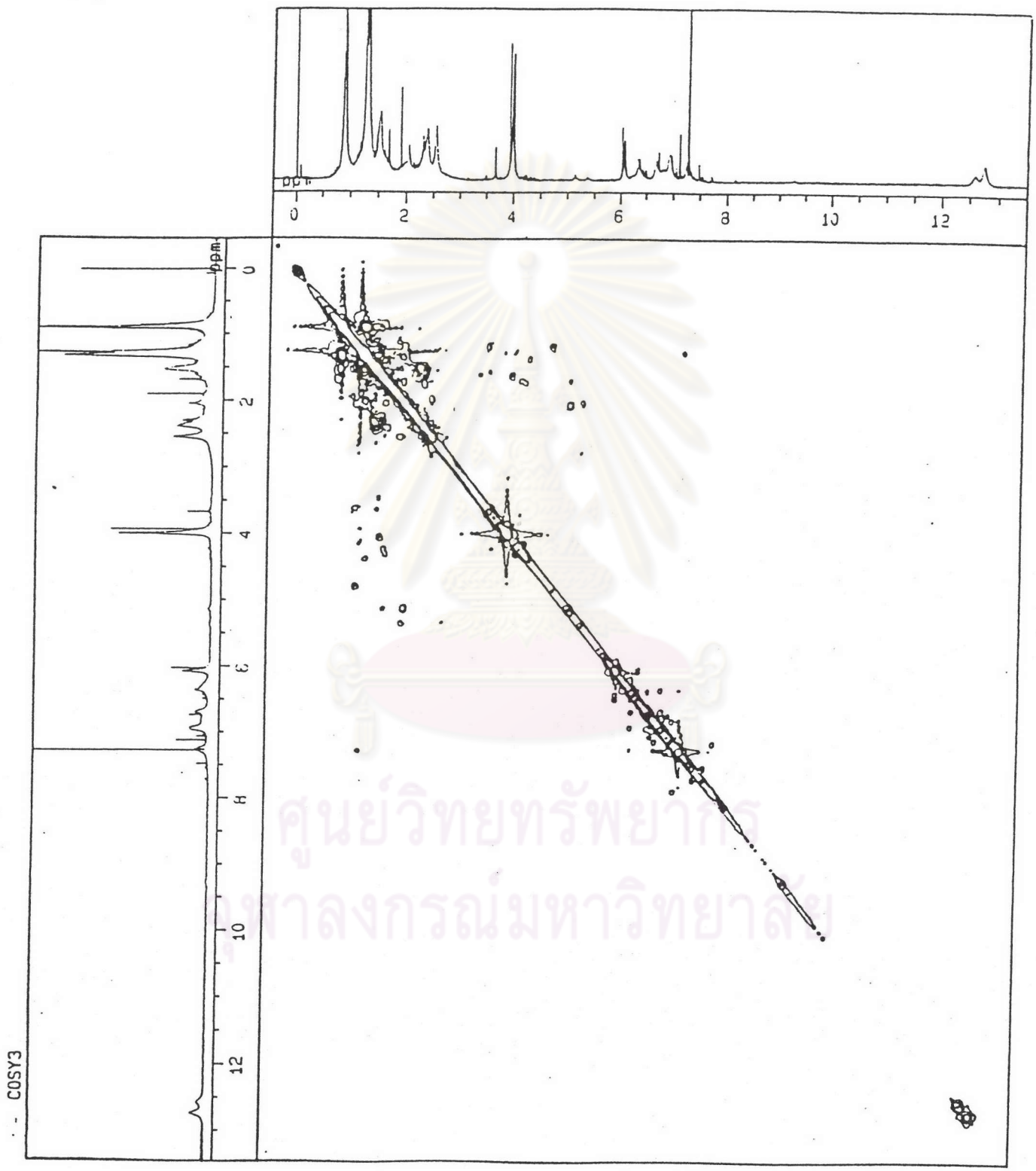


Figure 46. The H-H COSY spectrum of transformed RP-2.



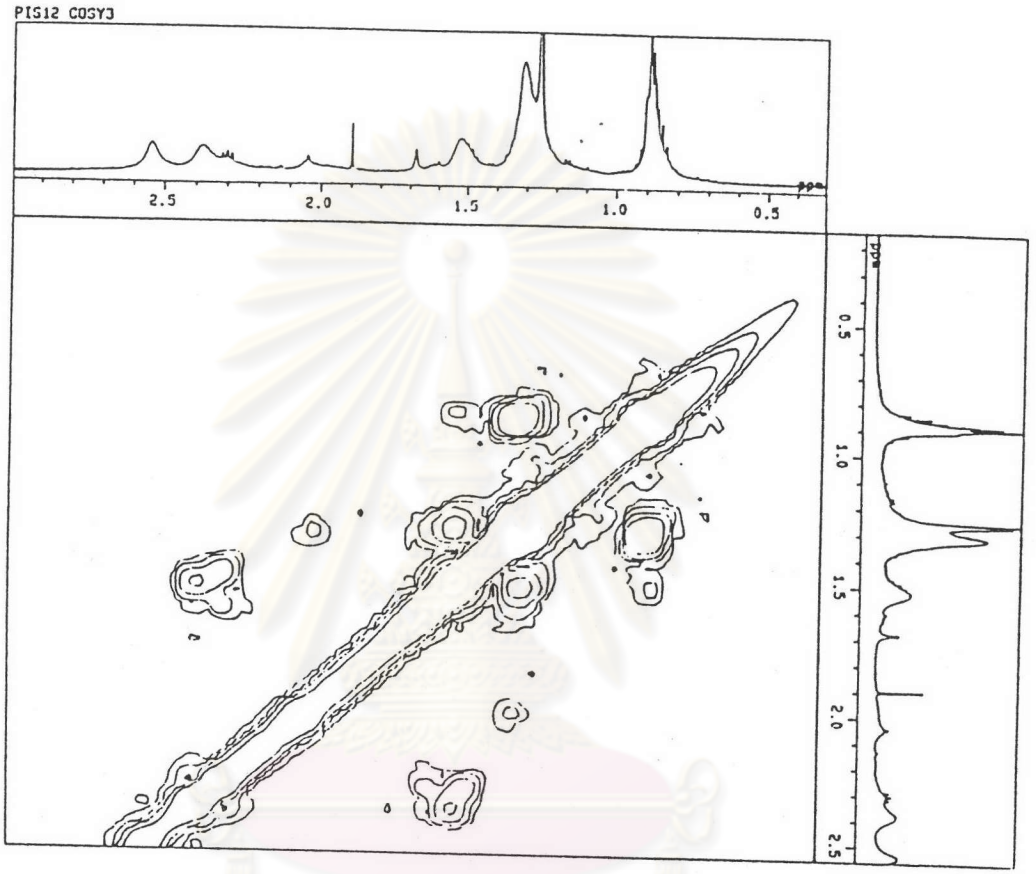


Figure 46.1. The H-H COSY spectrum of transformed RP-2, expanded at δ 0.5 - 3.0 ppm .

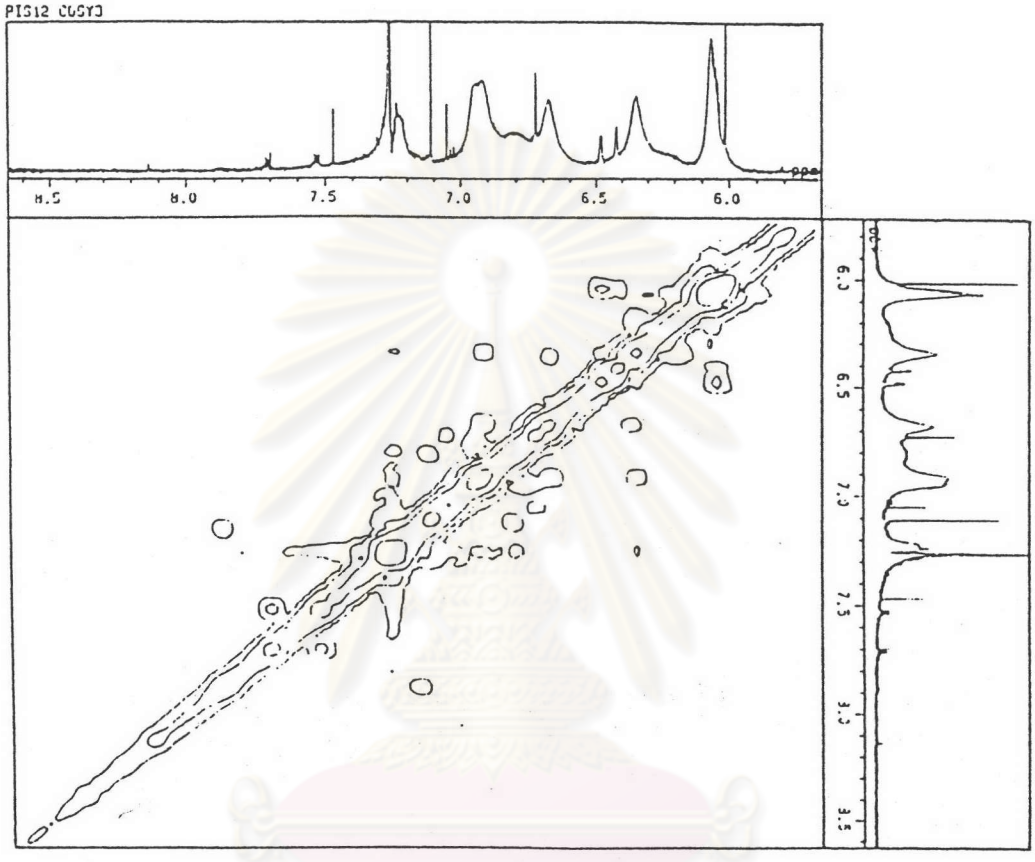


Figure 46.2. The H-H COSY spectrum of transformed RP-2, expanded at δ 5.5 8.5 ppm.

Figure 47. The 500 Mhz ^{1}H NMR spectra of RP-2A in deuterated chloroform, compared with RP-1 and RP-2.

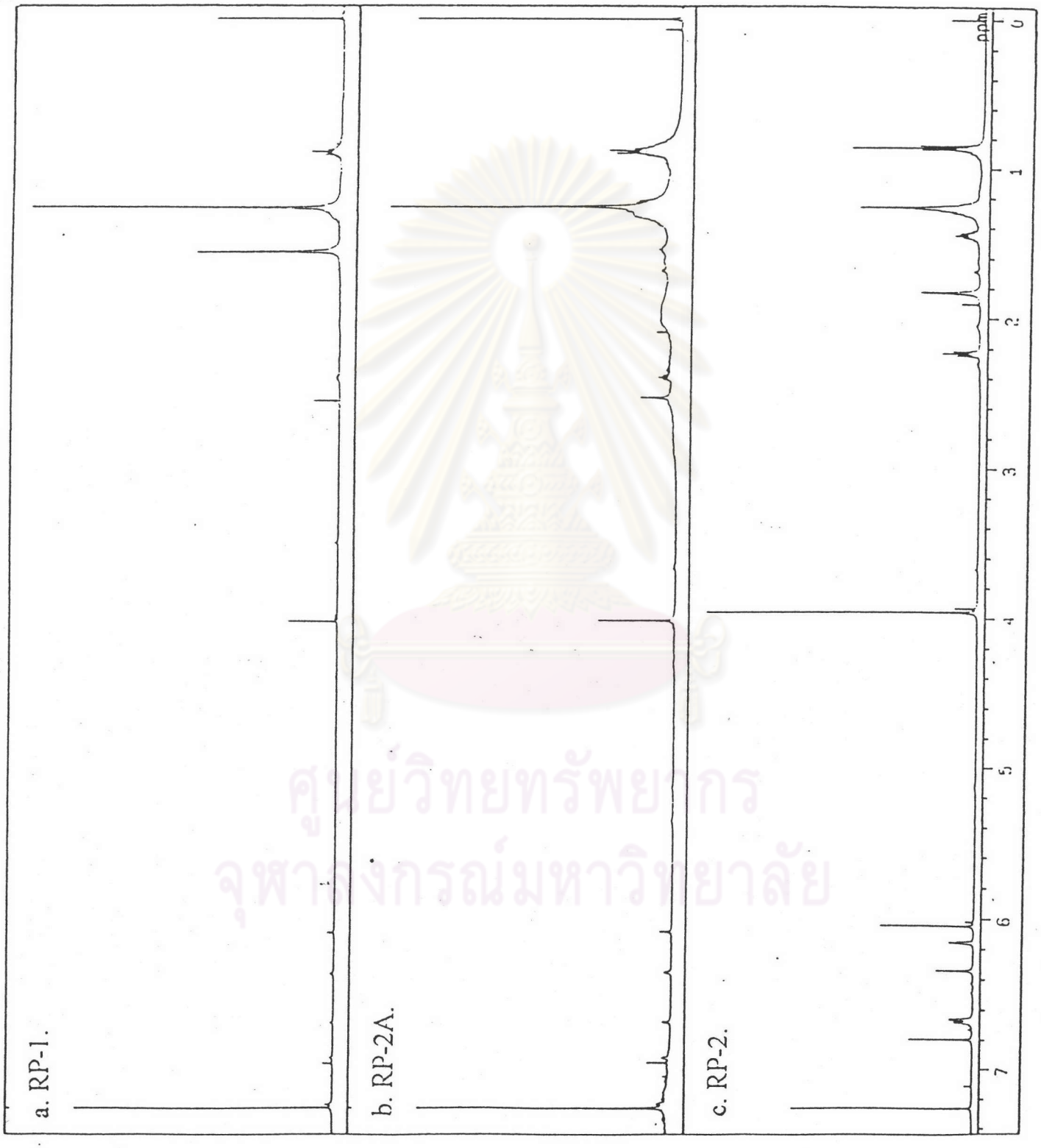


Figure 47.1. The ^{1}H NMR spectra of RP-2A, compared with RP-1 and RP-2, expanded at δ 0.8 - 2.8 ppm.

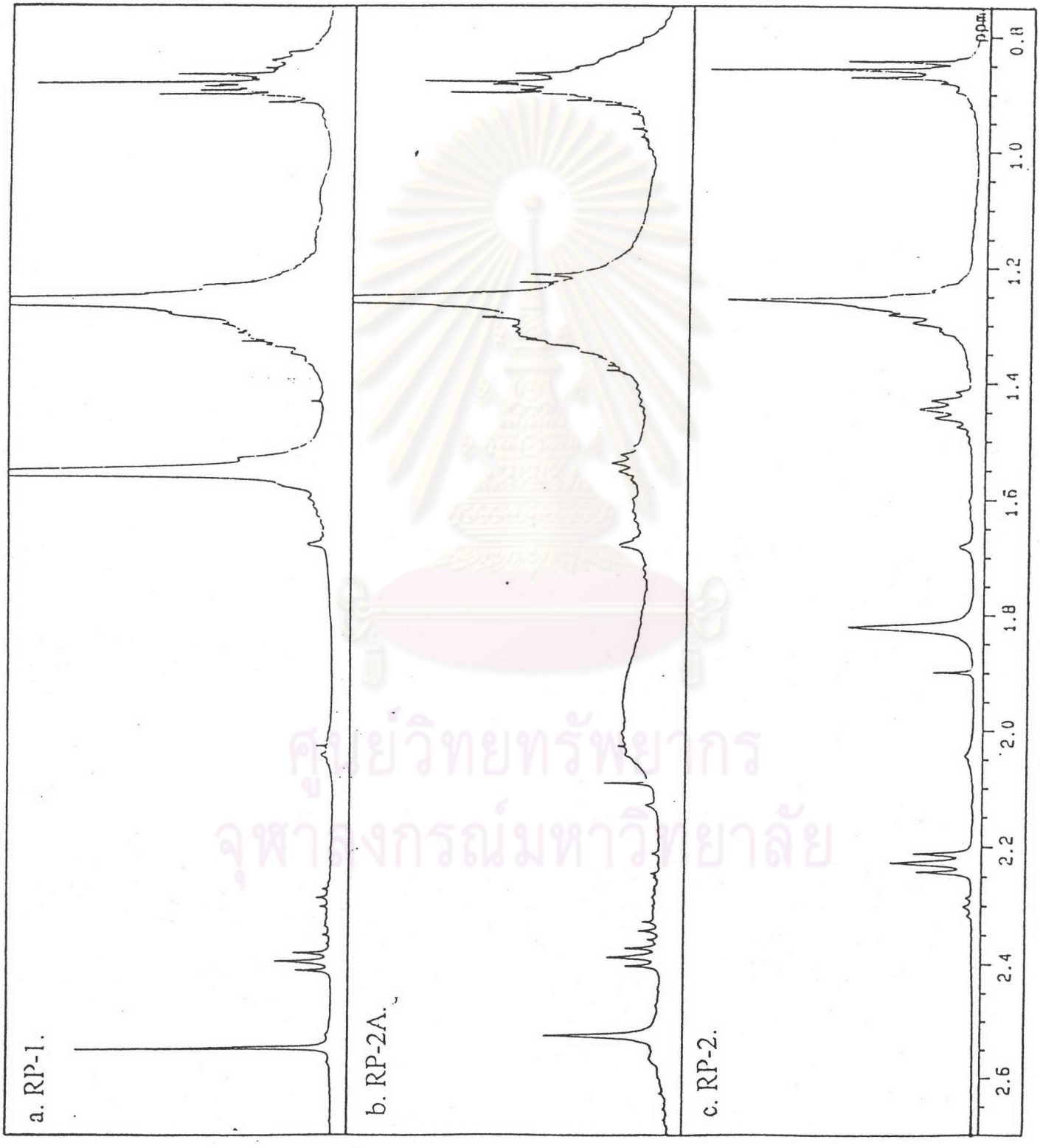
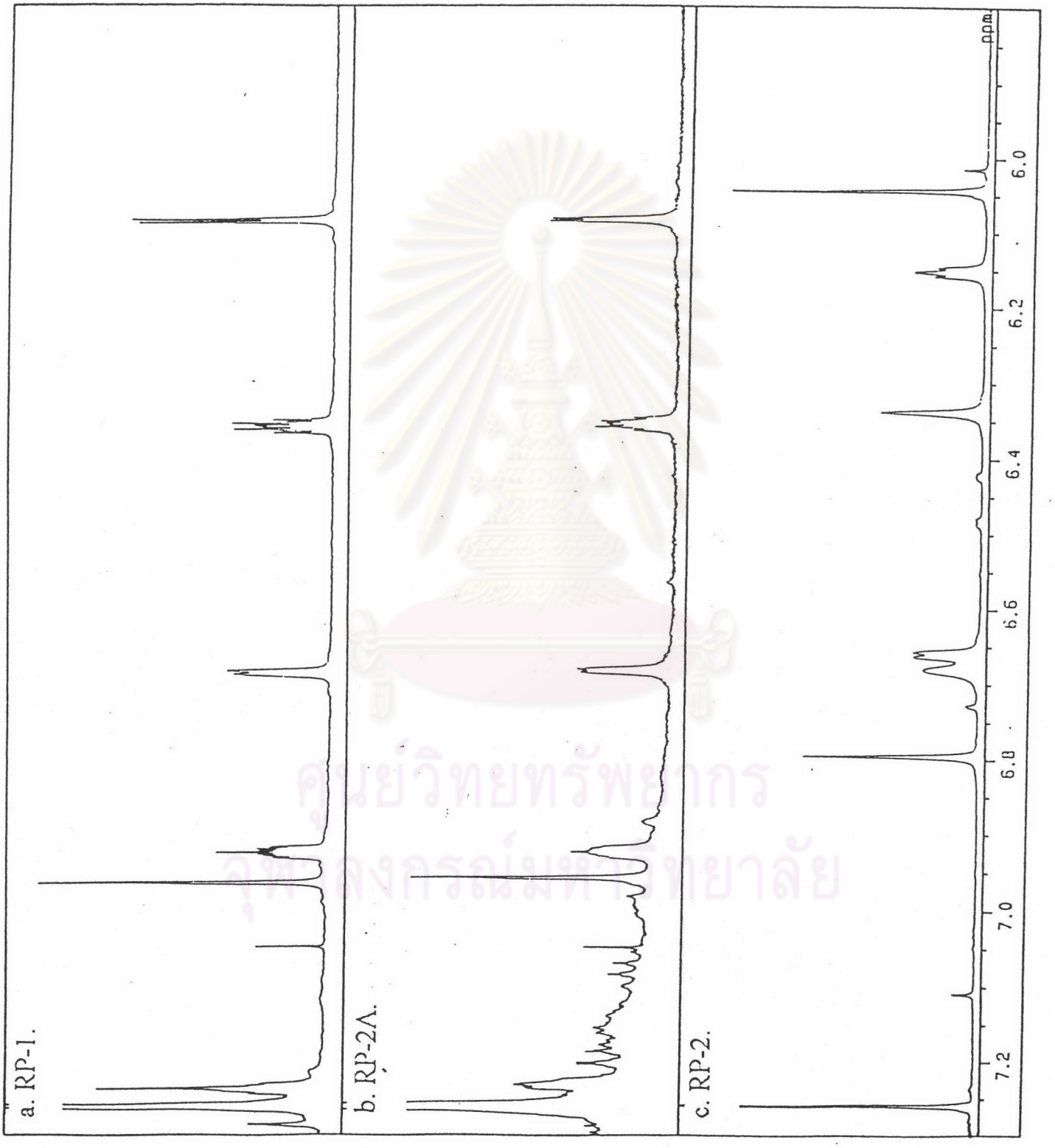


Figure 47.2. The ^{1}H NMR spectra of RP-2A, compared with RP-1 and RP-2, expanded at δ 5.5 - 7.4 ppm.



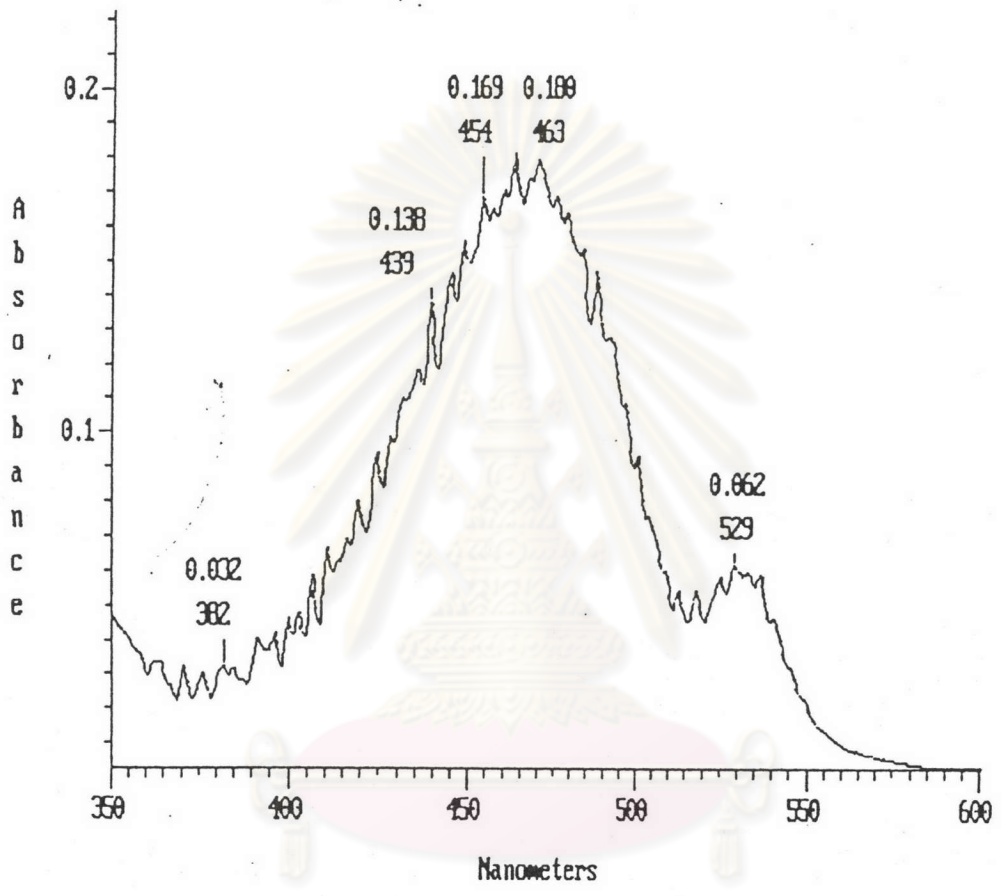


Figure 48. The UV-VIS spectrum of reextracted RP-2A in acetone.

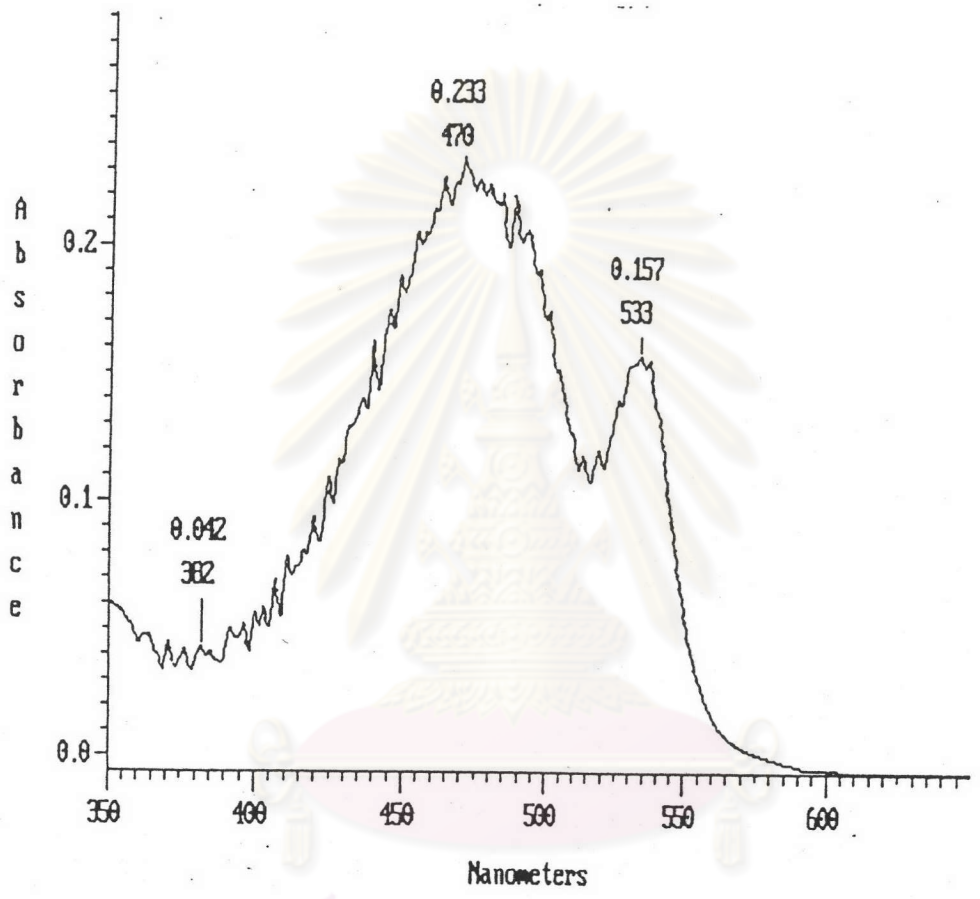


Figure 49. The UV-VIS spectrum of reextracted RP-2A in ethylacetate.

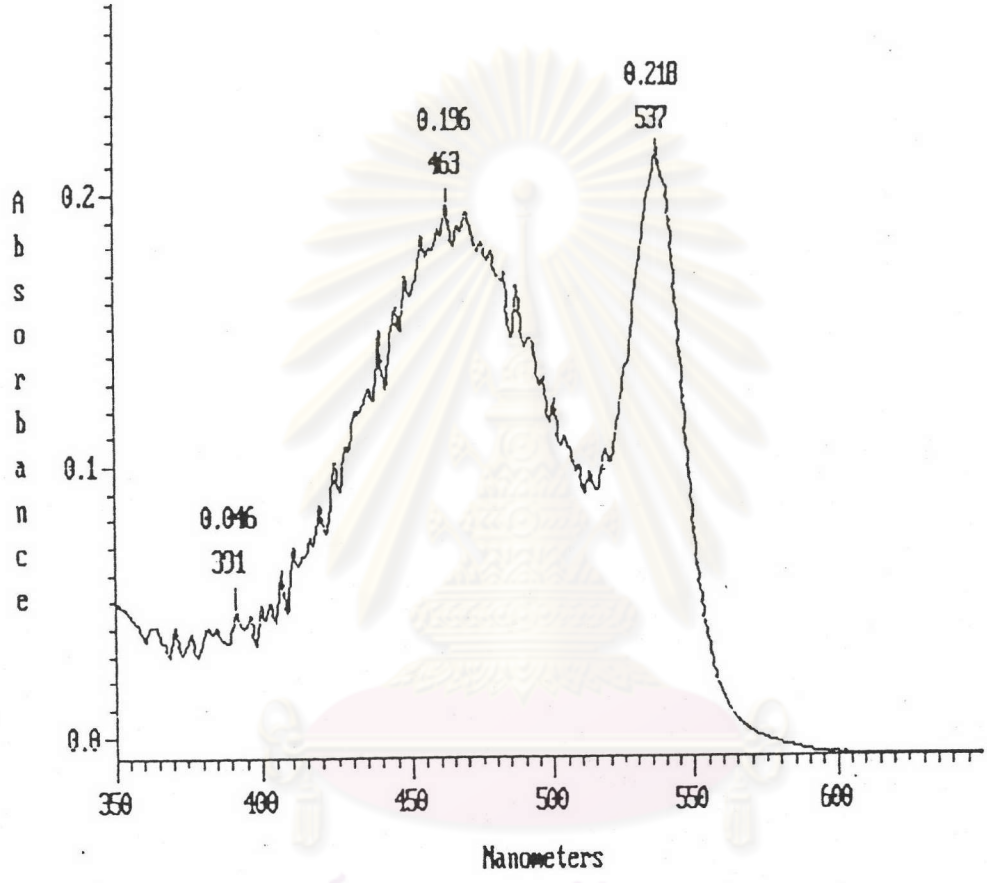


Figure 50. The UV-VIS spectrum of reextracted RP-2A in hexane.

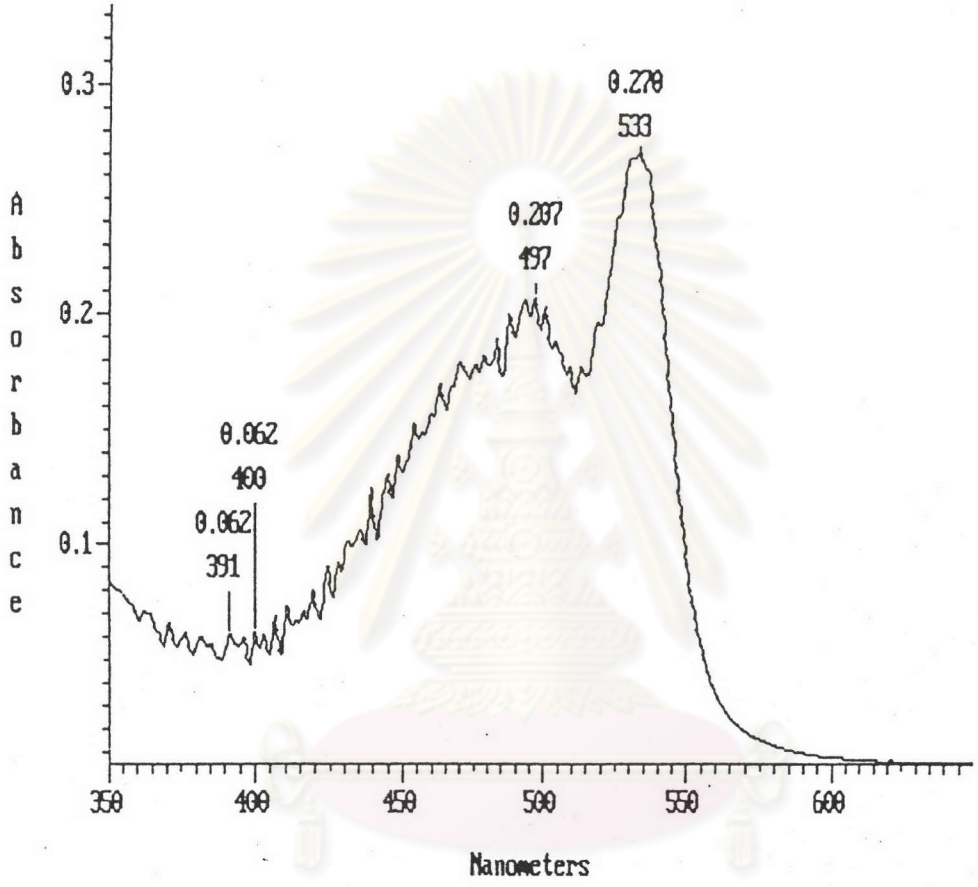


Figure 51. The UV-VIS spectrum of reextracted RP-2A in methanol.

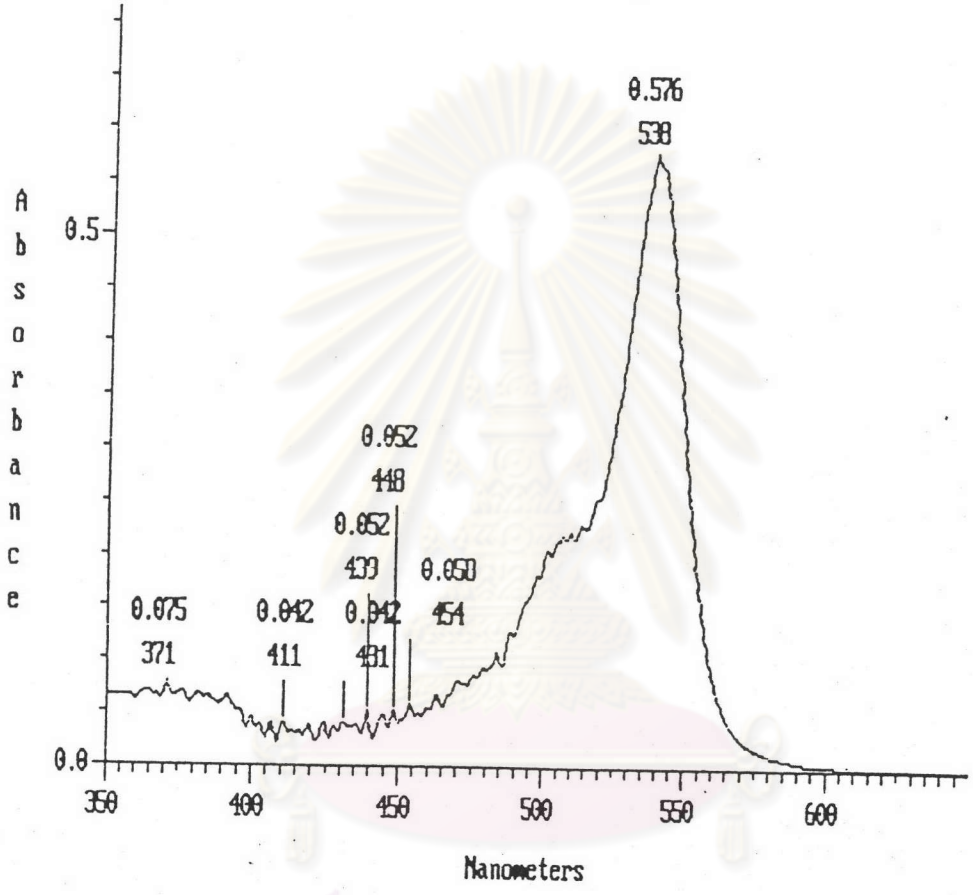
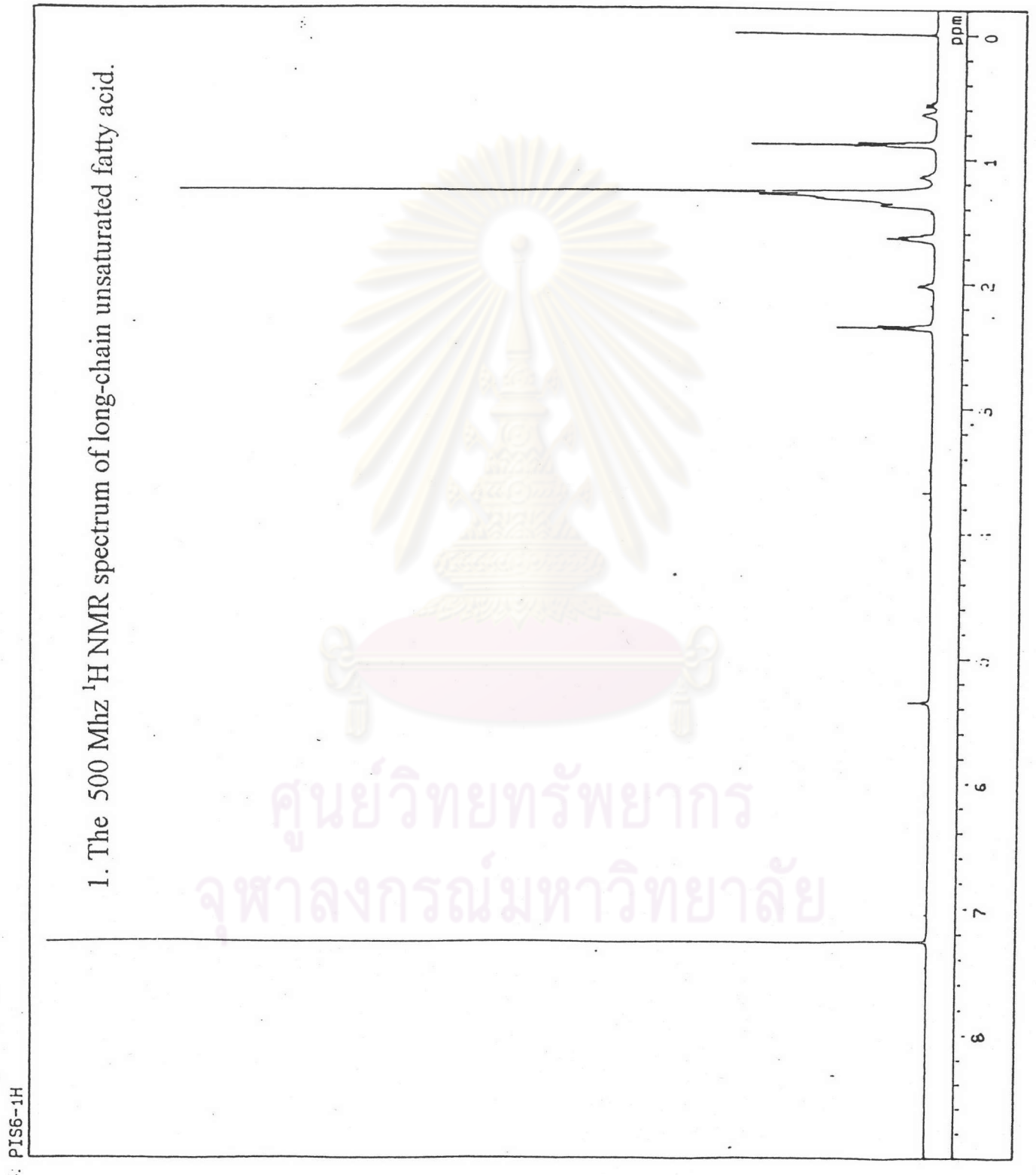


Figure 52. The UV-VIS spectrum of reextracted RP-2A in chloroform.

Figure 53. The long-chain unsaturated fatty acid found in the purification process, in the first column chromatography of the red pigments.

1. The 500 Mhz ^{1}H NMR spectrum of long-chain unsaturated fatty acid.
2. The 125 Mhz ^{13}C NMR spectrum of long-chain unsaturated fatty acid.



2. The 125 Mhz ^{13}C NMR spectrum of long-chain unsaturated fatty acid.

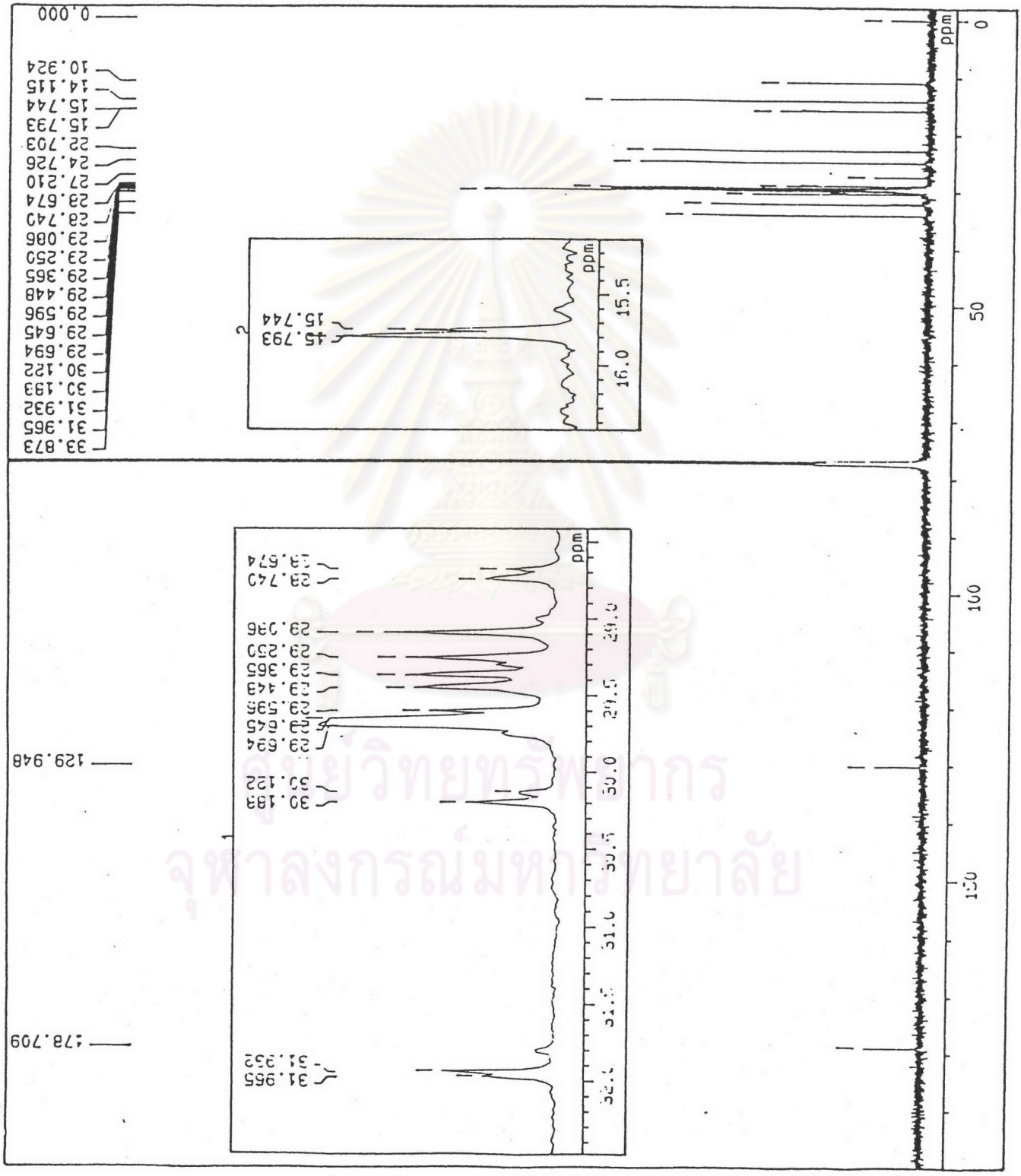


Figure 54. The 500 Mhz ^{1}H NMR spectrum of long-chain unsaturated fatty acid in mixture with pyrrolic compound, from the second column chromatography.

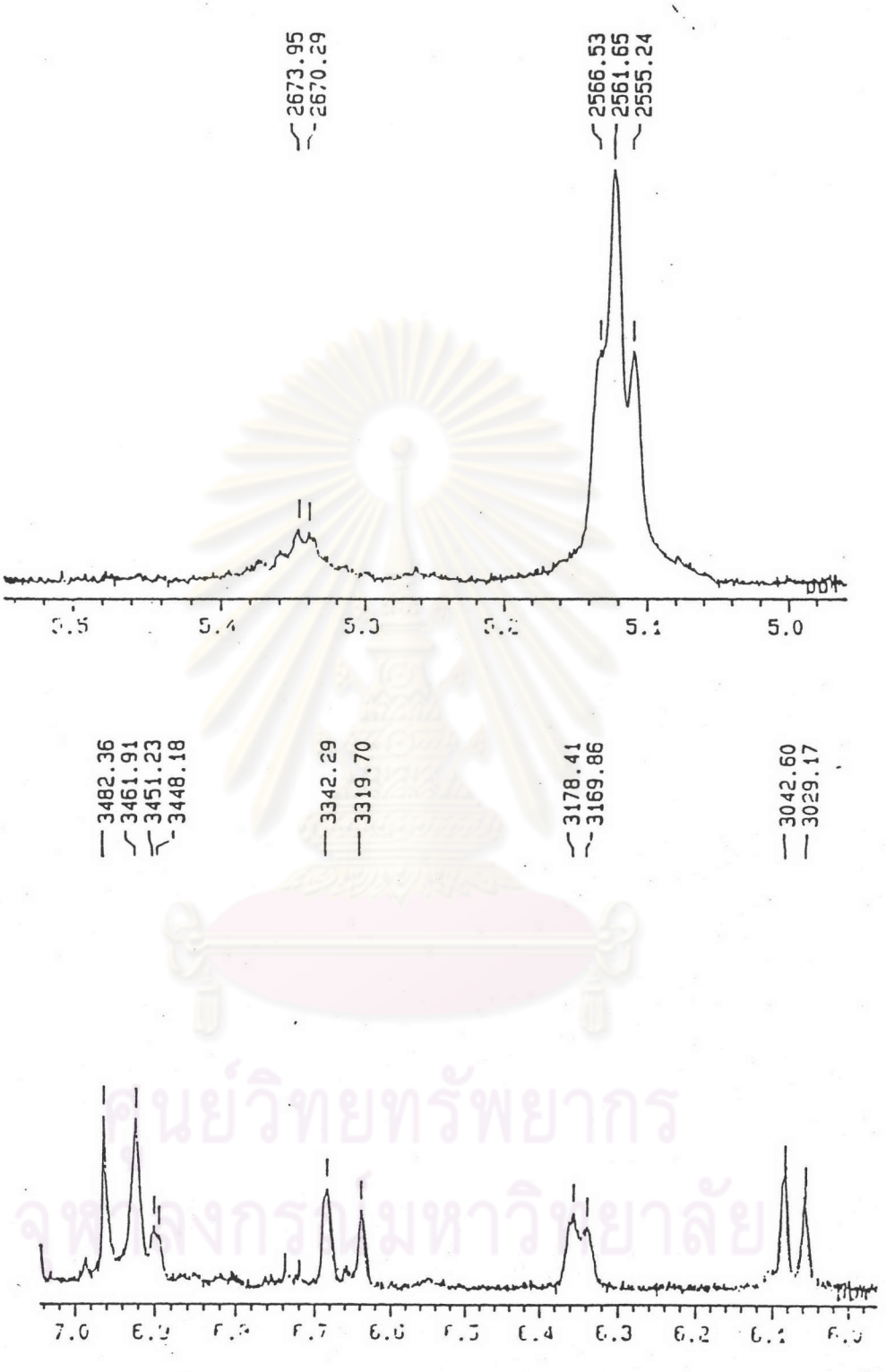
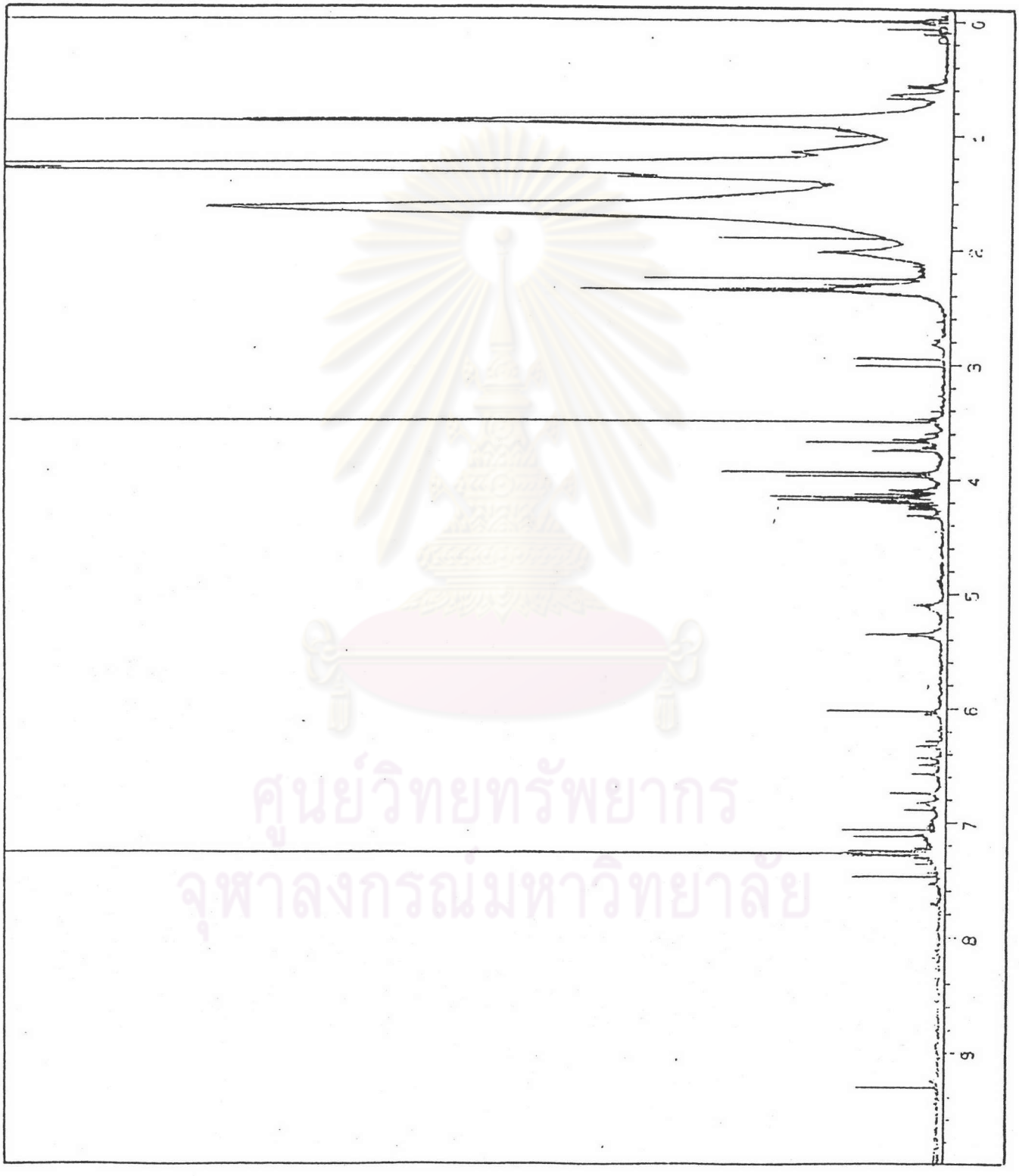


Figure 54.1. The 500 Mhz ^{1}H NMR spectrum of long-chain unsaturated fatty acid in mixture with pyrrolic compound, from the second column chromatography, expanded in olefinic region.

Figure 55. The 500 Mhz ^{1}H NMR spectrum of long-chain unsaturated fatty acid in mixture with pyrrolic compound, from the third column chromatography.

Figure 56. The 500 Mhz ^{1}H NMR spectrum of RP-3 in deuterated chloroform.



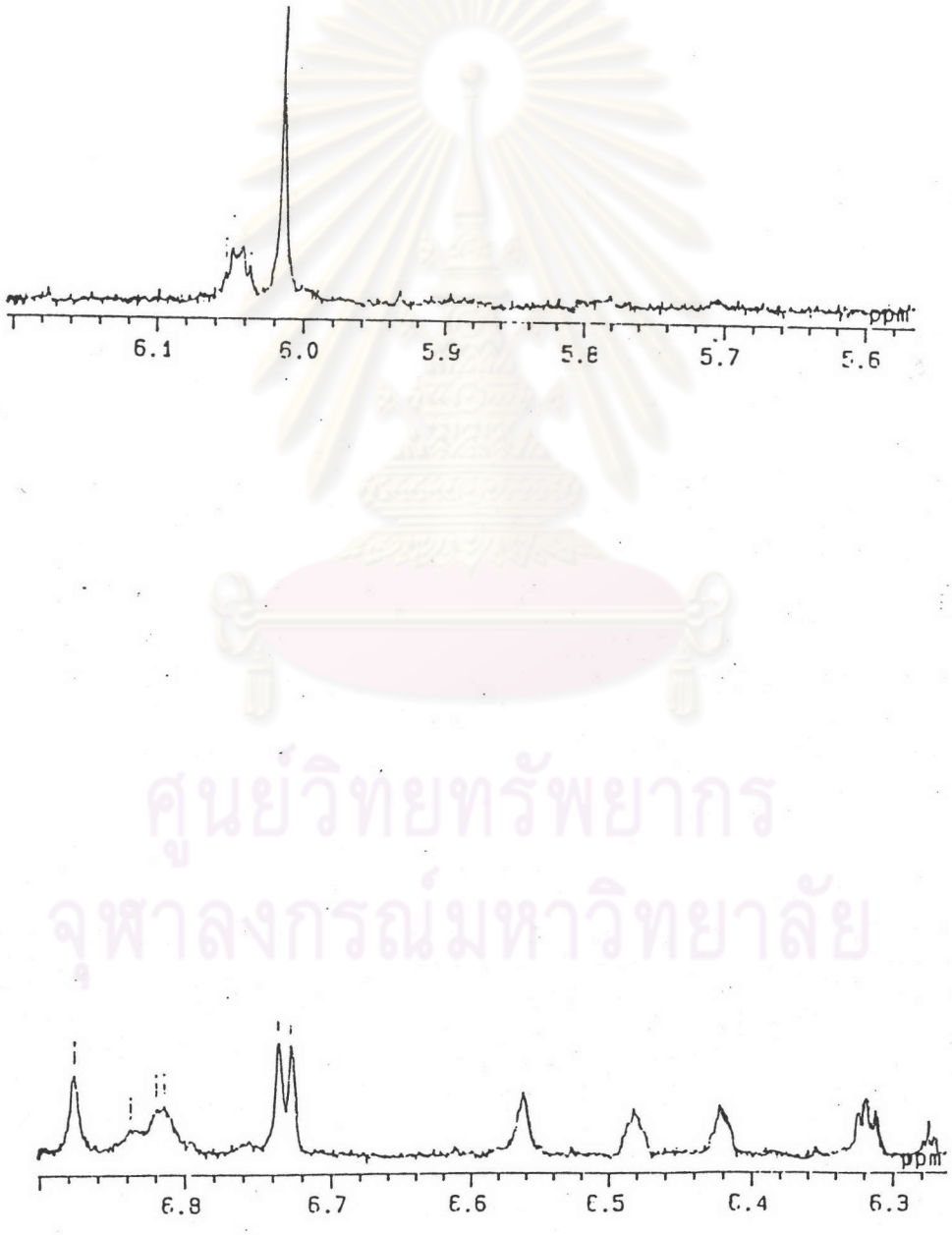


Figure 56.1. The 500 Mhz ^{1}H NMR spectrum of RP-3, expanded in olefinic region.

Figure 57. The 500 Mhz ^{1}H NMR spectrum of RP-4 in deuterated chloroform, compared with RP-1 and RP-2.

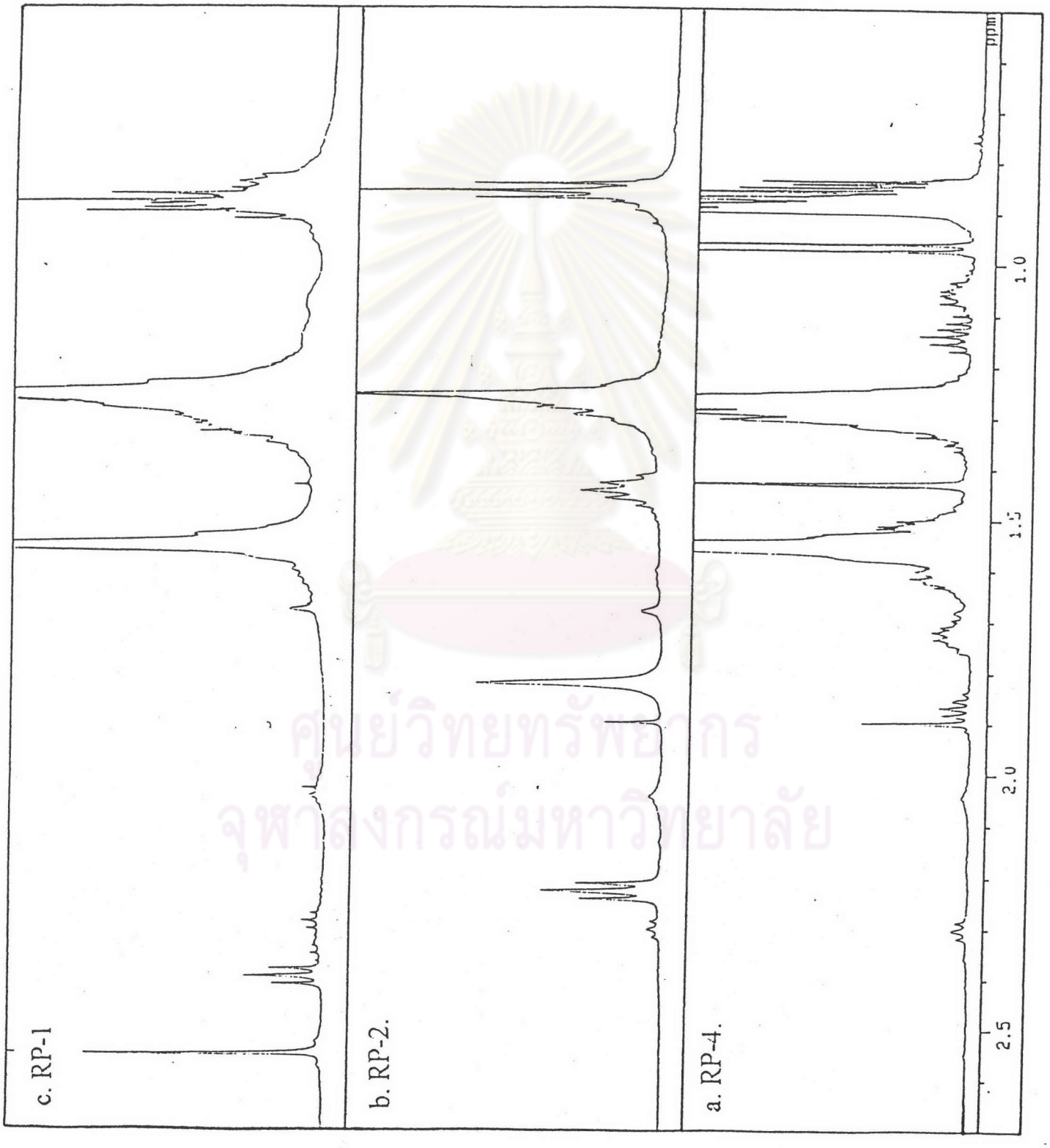


Figure 57 (cont.). The ^{1}H NMR spectrum of RP-4 (a) in comparison to RP-1 (c) and RP-2 (b).

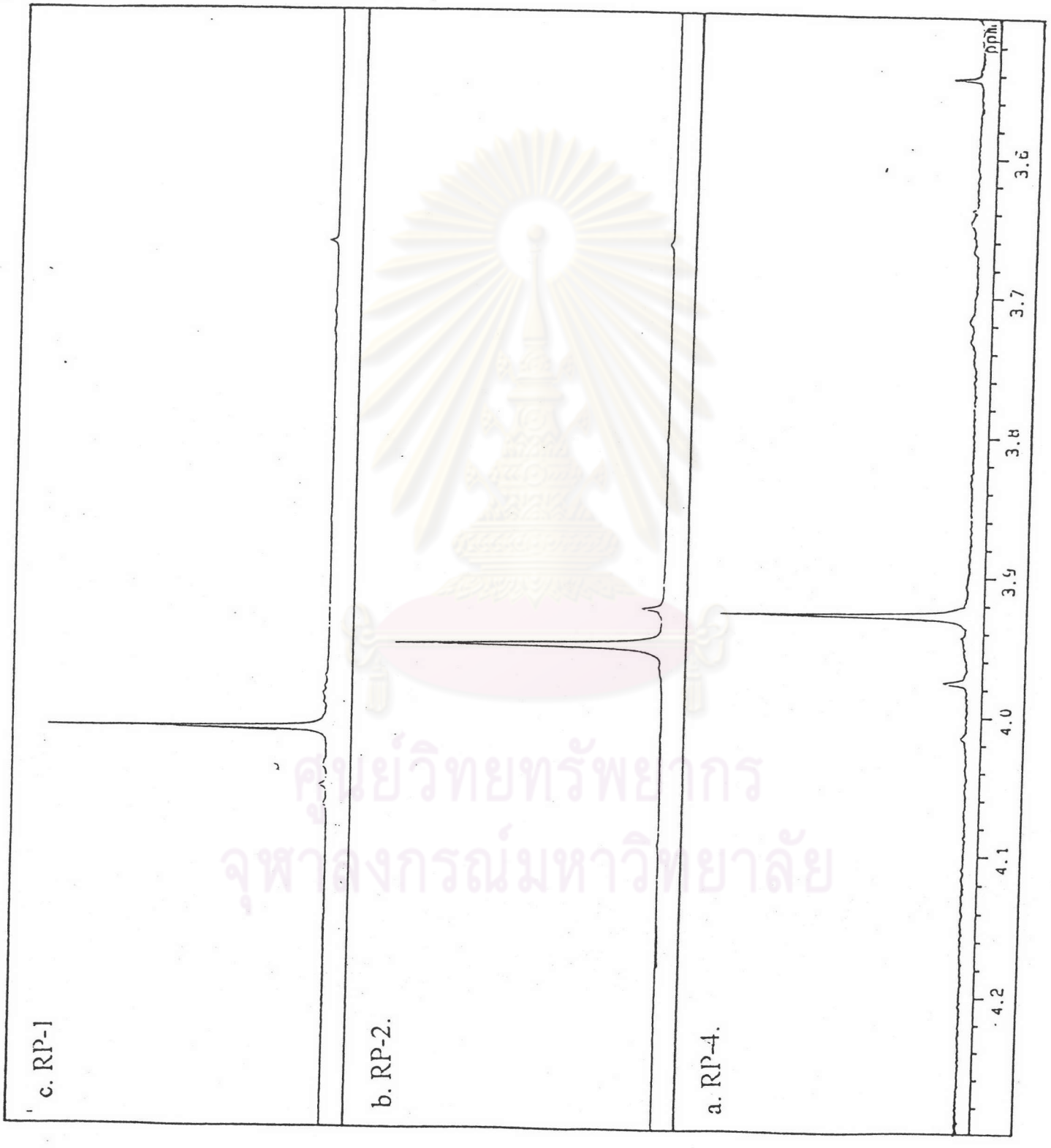
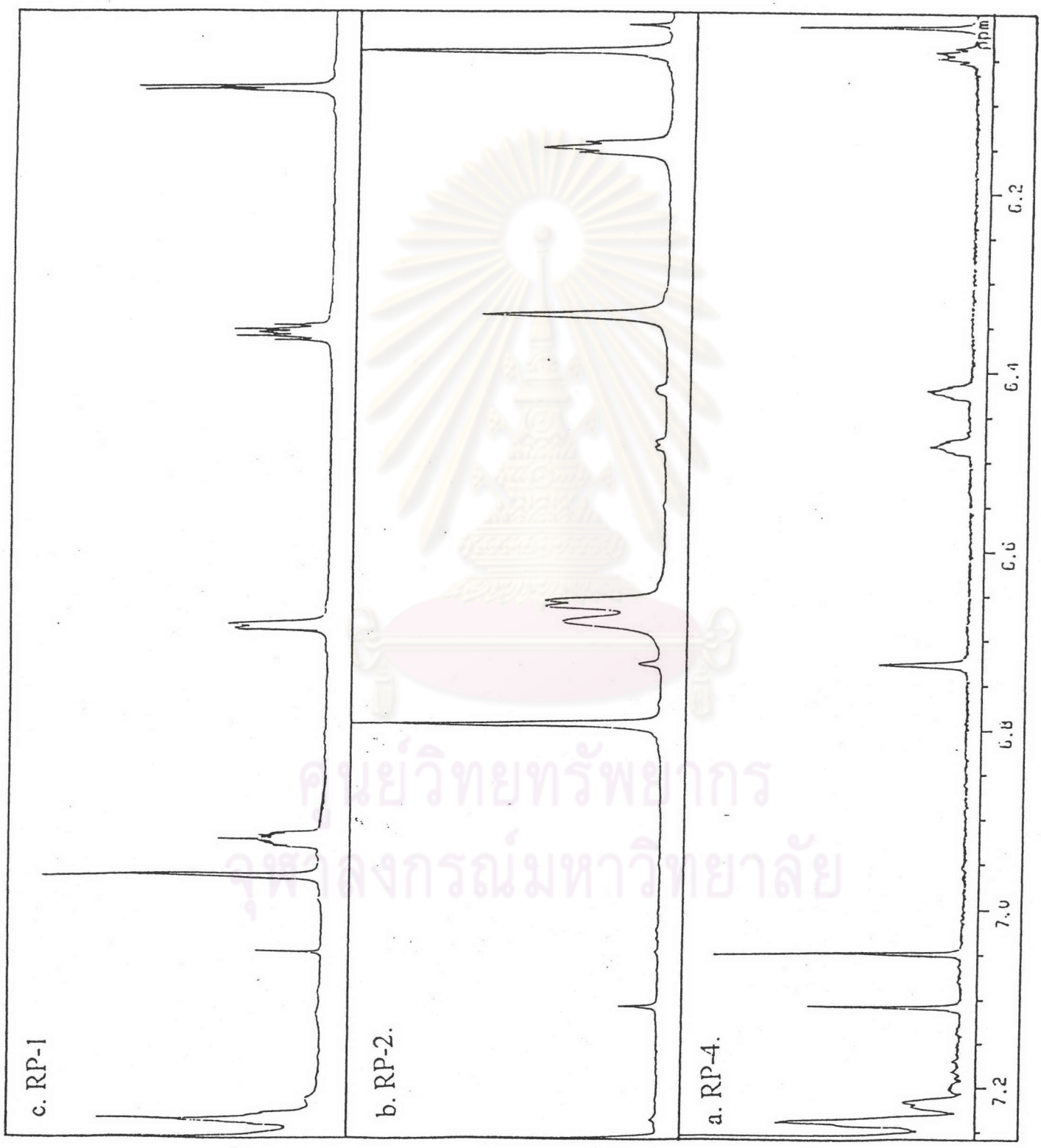


Figure 57 (cont.). The ^{1}H NMR spectrum of RP-4 (a) in comparison to RP-1 (c) and RP-2 (b).



5. PRODIGIOSIN : RED PIGMENT FROM *ZYMOMOMAS MOBILIS* CM 141

Prodigiosin was a water - soluble red antibiotic pigment and was a secondary metabolite (Williams, 1973). Maximal production occured after cellular multification had ceased. Culture of mutant of *Zymomonas mobilis* CM 141 was grown in 48 hours in the anaerobic condition and then the amount of red pigments were increased rapidly in aerobic condition and secreased on to the medium.

Solutions of prodigiosin free form (orange-yellow color) fluoresced when excited at 380 nm (λ_{max}) showed the emission at 688 nm (λ_{max}) (Figure 28), but the red solution did not, implied that it was in bound form. This was also reported by Castro et al. (1959).

The multiplicity of fractions in crude pigment extract may be partially the result of association of prodigiosin with different organic acid and partially of other natural pigments and artifacts. Prodigiosin probably occured primarily as a salt of fatty acid in close association with the lipid portion of cell membrane (Castro et al, 1959) When prodigiosin itself was chromatographed, especially after exposure to light, acid, alkali or oxidants, additional colored bands may appeared.

Hearn et al (1972) also reported that prodigiosin pigment always show a number of bands when chromatographed . However, highly

purified prodigiosin itself continued to show a multiplieity of minor bands on chromatography or CCD (countercurrent distribution).

When highly purified prodigiosin was chromatographed as the free base, the red and orange bands eluted seem to be the acid and base forms, the acid band arising from interaction of the base with some acid in the solvent, adsorbent, or atmosphere (Hearn, Castro and Elson, 1968). The magenta to blue bands at the top of the column appear to be decomposition products.

Because of the high absorptivity of prodigiosin in the visible region, solutions or chromatographic band could be highly colored and yet contained very small amout of pigment. Even minor compounds were easily visible because of the relatively high absorbance of prodigiosene derivatives. Consequently, minute traces of acid or basic in absorbents or solvents could produce detectable color changed.

The long-chain unsaturated fatty acids could be to stimulate growth and pigment prodigiosin production of *Serratia marcescens* (Bishop and Still, 1963). An analysis of the lipids of the bacterium *Serratia marcescens* had shown that palmitic acid and 9,10-methylenehexadecanoic acid constitute about 75% of the total fatty acids. Mono-unsaturated acids account for only about 10% of the total fatty acid and consist predominantly of 9,10-hexadecenoic acid (palmitoleic acid) and 11,12-octadecenoic acid (vaccenic acid).

Because of the high absorptivity and occured primarily as a salt of fatty acid in close association with the lipid portion of cell membrane, it was very difficult to purify the red pigment (prodigiosin) and always detected unsaturated long-chain fatty acid in every process of purification showed by ^{1}H NMR spectrum (large quantity of long-chain fatty acid and very low quantity of prodigiosin (but intense color)) (Figure 53., 54, and 55.). Fatty acids were removed by alkali treatment and rechromatography for many times and separated the prodigiosin by preparative TLC in the final step.

Zymomonas mobilis was an anaerobic Gram-negative bacterium that torelated oxygen had been observed to change its metabolism during the transition from anaerobiosis to aerobiosis. A swich from ethanol plus carbon dioxide to the formation of acetaldehyde, acetoin, and other by products occurs. Another product formed under aerobic conditions was acetate, which was produced via the oxidation of acetaldehyde (scheme 2.). The production of acetic acid increased in the presence of oxygen. In the presence of oxygen, ethanol and lactate could also serve as sources of reductant being oxidized in the process, suggesting that the enzymatic process involved in the formation of products in reversible.

Prodigiosin was biosynthesis from acetate, glycine, alanine, proline, serine and methionine (Figure 8.). In the aerobic condition *Zymomonas mobilis* increased in the acetate production and decreased the ethanol production. In this condition it could be produced prodigiosin as another secondary metabolites.

Prodigiosin was readily soluble in organic solvents, but poorly soluble in water. All prodigiosin pigments were red in the salt form and yellow in the free base form, the color of a TLC spot while the plate was running was a rough indication of the amount of pigment in each form. All spots were pink when dry. Neutral and alkali solutions were orange-yellow, turning red after acidification. Prodigiosin could exist in two distinct forms, depending upon the hydrogen ion concentration of the solution. In an acid medium the pigment was red and exhibited a sharp spectral peak at 535 nm (534-536 nm). The shoulder on the low wavelength limb of the acid curve at about 500 nm was persistent in the whole pigment. In an alkali medium the pigment was colored orange-yellow and prossessed a broader spectral curve centered at 468 nm and another small peak at 535 nm.

Red pigments portion from column chromatography showed three spots on TLC (system : 15% ethanol in toluidine) one red brown color (Rf 0.2) (RP-3), one red color (Rf 0.425) (prodigiosin, RP-1,RP-2), one pink color (Rf 0.8) (RP-4) and purple-brown residue. The ^{1}H NMR of RP-3 and RP-4 were shown in Figure 56 and 57. RP-3 (Figure 56.) was shown about ten to twevel protons in the olefinic regions (from δ 6.0 to 7.2 ppm). This was shown that RP-3 was pyrrolic compound may be more than one compounds. RP-4 was shown six olefinic protons (from δ 6.0 to 7.2 ppm). Like RP-1 and RP-2 (Figure 57.), there were three singlet broad signals at δ 6.01, 6.72 and 7.10 ppm and three multiplet signals at δ 6.04, 6.41 and 6.44 ppm. This may be conclude that RP-4 was prodigiosene

nucleus. The whole structure can not elucidate because of the very small amount of them. RP-3 and RP-4 would be the decomposition products.

Williams et al (1956) established that the prodigiosin was not a single substance, but was made up of at least four distinct components, one blue and three red. The red pigments were very unstable to the action of sunlight. The data from infrared and chromatographic analysis indicate that the red and blue pigments, although very similar, were not identical, and that the blue pigment was probably a dimer of the red pigment.

Hearn et al (1970) reported that 2-(2-pyrryl)-prodigiosene (Figure 58.) was produced when purified prodigiosin derivatives were allowed to stand in solution.

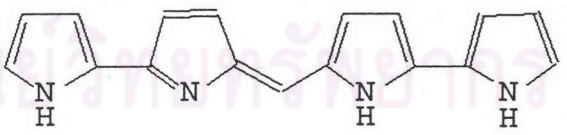


Figure 58. 2-(2-pyrryl)-prodigiosene.

They also reported that Prodigiosene derivatives with highly substituted monopyrrole rings showed a tendency to disproportionate to dipyrrylmethenes as well. By detection of the ions with m/e ratios expected for a alkyldipyrrylmethene were observed. The abundance of these ions increased with the temperature and time of the sample in the mass spectrometer.

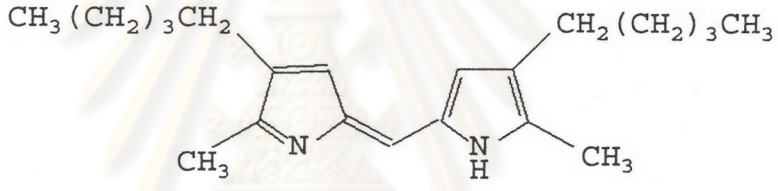


Figure 59. Dipyrrylmethene.

They concluded that when 6-methoxyprodigiosenes were being purified from natural sources, traces of additional pigments were almost invariably encountered, even late in the purification process. The type of pyrryl-bipyrryl exchange behavior observed obove may offer an

explanation for the presence of some of these pigments, particularly of those which appear to be artifacts.

Finally, even synthetic prodigiosene derivatives had been found to rearrange chemically under relatively mild conditions (Hearn et al., 1970).

From these experiments, it could be concluded that there were another prodigiosin-like compounds produced by *Zymomonas mobilis* CM141. The further studies were to development of improved red pigment producing strains of *Zymomonas mobilis* CM141.

6. MUTATION EXPERIMENT.

Mutant strain of *Zymomonas mobilis* CM 141 producing red pigment was induced mutation for development of improved red pigment-producing strains by mutation and selection (Primrose, 1987). The mutations were conducted by ultraviolet light and chemicals, N-methyl- N- nitro- N- nitrosoguanidine (NTG) (200 mcg/ml) and hydroxylamine (0.5 and 1 M), at 10, 20 and 30 minutes. The isolated mutant strains were cultured on YGP agar for pigment production. It was found that the mutants produced pigmented colonies that difference from the parent strain. Fifty-three isolated out of 200 isolates produced pigmented colonies of the following colors: yellow, pale orange, deep orange,violet, red with green metallic sheen, pale green, pink and red (Table 8). The quantity of the pigments were examined by spectrophotometry (Appendix). The pigments on the agar plate were extracted by the mixture of methanol : chloroform (1:1) solution and then filtered throught sinter glass funnel. The filtrates were scanned for UV-VIS spectrum (200-700 nm). The maximum absorption wavelength and absorbance were recorded (Table 9).

From the UV-VIS spectrum, it was found that twenty-five mutant strains were shown the maximum absorption wavelength at about 500-540 nm which were the red color solution. Five mutant strains were shown the maximum absorption wavelength at about 470-485 nm which were the orange color solution. Another (twenty-three mutant strains) were shown

the maximum absorption wavelength in the UV region (285-290 nm) which was the general absorption spectrum of organic compound.

All mutants isolated after ultraviolet light irradiation were nonpigmented. The extracted solution showed pale yellow color, maximum absorption wavelength at 290 nm with the absorbance 0.75 (Table 9).

Some mutant strains isolated after NTG treatment were pigmented, some were not (Table 9). The extracted solution of strain A_{10} showed pale yellow color, maximum absorption wavelength at 290 nm with the absorbance 0.6. Two mutant strains, A_9 and A_{116} showed deep orange and red color, maximum absorption wavelength at 530 nm with the absorbance 2.15 and 5.0, respectively (Table 21.). Strain A_9 took a very long time for pigment production (compared with the other strains) and gave low yield of red pigments, less than the parent strain. Strain A_{118} showed the properties the same as the parent strains.

Table 22. Pigment extracted from mutant strains mutated by N-methyl-N-nitro-N-nitrosoguanidine treatment of *Zymomonas mobilis* CM 141.

Strain no	Time of exposure) (minutes)	Culture time (hours)	Pigment color	Max. Absorption wavelength,nm (Absorbance)
A_9	10	72	deep orange	530 (2.15)
A_{116}	10	24	red	525 (5.00)

Ten red pigment produced mutant strains were isolated after hydroxylamine treatment of the *Zymomonas mobilis* CM 141. The extracted solution of these mutants were deep red color, showed the maximum absorption wavelength at 522-530 nm with the absorbance 2.95 - 9.0 (Table 23.). Strain A_{109} and A_{113} showed the more intense red color than parent strain (*Zymomonas mobilis* CM 141) especially strain A_{113} showed the absorbance intense more than parent strain 1.8 time. This strain was the expected strain.

Table 23. Pigment extracted from mutant strains mutated by hydroxylamine (1M) treatment of *Zymomonas mobilis* CM 141.

Strain no	Time of exposure) (minutes)	Culture time (hours)	Pigment color	Max. Absorption wavelength,nm (Absorbance)
A_{31}	10	24	red	530 (4.45)
A_{46}	20	24	red	530 (5.00)
A_{49}	10	24	red	522 (4.80)
A_{50}	10	24	red	525 (4.80)
A_{75}	10	24	red	522 (4.50)
A_{77}	10	24	red	530 (3.70)
A_{78}	10	24	red	535 (2.95)
A_{107}	10	24	red	530 (4.05)
A_{109}	10	24	red	520 (6.50)
A_{113}	20	24	red	528 (9.00)

Strain A_9 and A_{78} were conducted to mutate again by hydroxylamine treatment to find the better strains. Strain A_{32} , A_{34} and strain A_{162} , A_{186} were found from strain A_9 and strain A_{78}, respectively.

Strains A_{78} was conducted to mutate again by NTG treatment to find the better strains. Strain A_{138} was found and the extracted solution showed red-violet color which maximum absorption wavelength at 540 nm.

The culture time for pigmentation of the mutate A_{32} and A_{34} was as long as the parent (A_9) but the less of red pigments production (Table 24.). Also the strain A_{162} and A_{186} from A_{78} showed the same properties (culture time of pigmentation and absorbance) as the parent (Table 25.).

Table 24. Pigment extracted from mutant strains mutated by hydroxylamine (0.5M) treatment of strain A_9.

Strain no	Time of exposure) (minutes)	Culture time (hours)	Pigment color	Max. Absorption wavelength,nm (Absorbance)
A_{32}	20	72	deep orange	475 (1.40)
A_{34}	20	72	deep orange	485 (0.95)

Table 25. Pigment extracted from mutant strains mutated by hydroxylamine (1M) treatment of strain A_{78}.

Strain no	Time of exposure) (minutes)	Culture time (hours)	Pigment color	Max. Absorption wavelength,nm (Absorbance)
A_{162}	30	24	red	530 (4.50)
A_{186}	10	24	red	525 (5.00)

Strain A_{32} produced the differnt color pigments and maximum absorption wavelength. The mutation experiment were conducted to this strain by N-methyl-N-nitro-N-nitrosoguanidine 200 mcg/ml (Table 26.) and hydroxylamine 1M (Table 27.). All the mutant strains showed culture time for pigmentation shorter than the parent. Only the strain A_{172} showed the same color pigment and maximum absorption wavelength but less absorbance than the parent. The other strains showed the red pigments and maximum absorption wavelength at 525-535 nm. Strain A_{183} showed the absorbance at maximum absorption wavelength, 530 nm, more than that produced by *Zymomonas mobilis* CM 141.

Table 26. Pigment extracted from mutant strains mutated by N-methyl-N-nitro-N-nitrosoguanidine treatment of strain A_{32}.

Strain no	Time of exposure) (minutes)	Culture time (hours)	Pigment color	Max. Absorption wavelength,nm (Absorbance)
A_{140}	30	24	orange	470 (0.60)
A_{141}	10	24	red	535 (3.95)
A_{142}	10	24	orange	470 (2.15)
A_{145}	20	24	red	537 (1.50)
A_{151}	20	24	red	537 (2.62)
A_{160}	10	24	red	535 (1.4)

Table 27. Pigment extracted from mutant strains mutated by hydroxylamine (1M) treatment of strain A_{32}.

Strain no	Time of exposure) (minutes)	Culture time (hours)	Pigment color	Max. Absorption wavelength,nm (Absorbance)
A_{172}	10	48	orange	475 (1.00)
A_{176}	30	24	red	535 (3.40)
A_{182}	10	24	red	535 (2.25)
A_{183}	20	24	red	530 (6.50)
A_{184}	30	24	red	535 (2.65)
A_{187}	10	24	pink	535 (1.10)
A_{195}	20	24	pink	525 (5.00)

Fifty-three isolated out of 200 isolates produced color colonies. From these only thirty mutation strains that the pigment extracted solution showed the maximum absorption at 470-537 nm, those were the orange to red color solution. Finally, four mutant strains (A_{33}, A_{109}, A_{113}, and A_{183}) produced red pigment solution more intense than and took the culture time for pigmentation as long as *Zymomonas mobilis* CM 141 (24 hours) except strain A_{33} (72 hours). Of all strain A_{113} produced the most red pigment, 1.8 times more than *Zymomonas mobilis* CM 141, it was the best strain in these experiments.