

CHAPTER III



EXPERIMENTAL

PIGMENTS PRODUCTION

Zymomonas mobilis CM 141 isolated from Department of Biological, Faculty of Sciences, Chiang Mai University was inoculated on the YGP agar medium (contained 1% yeast extract, 1% peptone, 5% glucose and 1.5% agar) and incubated at 30° C for 48 hours in the aerobic conditions. The colonies were found to be very small. The plate was left in an incubator at 30° C for 24 hours. Very good growth and pigments production were observed.

GENERAL TECHNIQUES

1. Thin Layer Chromatography (TLC)

1.1 Analytical Thin Layer Chromatography

Adsorbent : Precoated TLC plates of silica gel G60 F- 254
(E. Merck).

- Layer thickness : 250 μm .
- Plate size : 10 x 5 cm.
- Technique : One way, ascending.
- Distance : 8.5 cm.
- Temperature : Laboratory temperature (29° C - 35° C).
- Solvent systems : Various solvent systems, depending on the separated compounds.
- Detection : 1. Visual detection under day light.
2. Visual detection under ultraviolet light at the wavelength of 254 and 365 nm.
3. TLC scanner detection under ultraviolet light at the wavelength of 534 nm.
4. Visual detection by spraying with 0.5% p-methylaminobenzaldehyde in ethanol (Ehrlich-Reagent) and placed in an atmosphere of fuming HCL for 10-15 minutes.
Deep red and pink bands developed for the pyrrolic compounds.
5. Visual detection by the exposure to iodine vapours for 2-5 minutes.

1.2 Preparative Thin-Layer Chromatography

Adsorbent : preparative TLC plates of silica gel 60 F-254 (E. Merck).

Layer thickness : 1 mm.

- Plate size : 20 x 20 cm.
Technique : One way, ascending.
Distance : 18.5 cm.
Temperature : Laboratory temperature (29° C - 35° C).
Solvent systems : Various solvent systems, depending on the separated compounds.

Loading of the Sample Extract :

The concentrated extract was applied as a narrow band TLC plates.

- Detection : 1. Visual detection under day light.
2. Visual detection under ultraviolet light at the wavelength of 254 and 365 nm.

Collection of the Red Pigment :

The isolated band was scraped off the plate and eluted immediately solvents. The solvent was evaporated on a rotary evaporator under reduced pressure.

2. Column Chromatography

2.1 Quick Column Chromatography

- Absorbent : Silica gel 60 (number 7734), particle size 0.063 - 0.200 mm (70-230 mesh ASTM).
Column size : Glass column 2-4 inches diameter.
Packing : Dried packing.

- Sample loading : Carefully loaded the small amount of dried silica gel which adsorbed the sample extract, avoided disturbing the surface of the gel.
- Solvent : Various solvent systems, depending on the separated compounds.
- Collection the eluate :
Collect and combine the eluates of each color band from the column.

2.2 Flash Column Chromatography

- Adsorbent : Silica gel 60 (number 7729), particle size under 0.063 mm (230 mesh ASTM).
- Column size : Glass column, 3/4-2 inches diameter.
- Packing : Dried packing.
- Sample loading : Carefully load the small amount of dried silica gel which adsorb the sample extract, avoided disturbing the surface of the gel.

- Examination of eluates :
The separation of similar pigments from the column was monitored by thin-layer chromatography. Fractions were examined by TLC using visual detection under day light, ultraviolet light at the wavelengths of 254 and 365 nm, iodine reaction and Ehrlich - reagent, respectively.
The similar fractions were combined and

evaporated to dryness.

Solvent : Various solvent systems, depending on the separated compounds.

Collection the eluate :

Collect and combine the eluates of each color band from the column.

2.3 Gel Filtration Chromatography

Adsorbent : Sephadex LH-20.

Column size : Glass column, 3/4-2 inches diameter.

Packing : The adsorbent was suspended in the eluent and left for the saturation for approximate 24 hours. The suspension was poured into a column and allowed to be settled uniformly.

Sample loading : The extract was dissolved in a small volume of eluent and carefully loaded, avoided disturbing gel surface.

Examination of eluates :

The separation of similar pigments from the column was monitored by thin-layer chromatography.

Fractions were examined by TLC using visual detection under day light, ultraviolet light at the wavelenghts of 254 and 365 nm, iodine reaction and Ehrlich - reagent, respectively.

The similar fractions were combined and

evaporated to dryness.

Solvent : Various solvent systems, depending on the separated compounds.

Collection the eluate :

Collect and combine the eluates of each color band from the column.

3. Crystallization technique

The compounds were crystallized in suitable solvents that rarely dissolved compounds whereas extremely dissolved impurities at low or room temperatures.

4. Spectroscopy

4.1 Ultraviolet (UV) Absorption spectra

Ultraviolet spectra were obtained from a Shimadzu double beam spectrometer (Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University). The samples were dissolved and the concentrations were adjusted using acetone, benzene, chloroform, ethanol, ethyl acetate, hexane, and methanol as solvent.

Sample Preparation : Accurately weighed 1 mg of sample dissolved and adjusted with chloroform in 10 ml volumetric flask. The dilution

could be made in order to obtain a certain concentration for the calculation of absorptivity.

Acid and alkali conditions for spectral analysis were obtained by adding 1 ml of 1 N hydrochloric acid or 1 ml of 1 N sodium hydroxide solution, respectively, to 10 ml of the ethanol solution.

4.2 Fluorescence spectra

Fluorescence spectra were obtained with a Jasco FP-777 Spectrofluorometer (Department of pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

The samples were dissolved and the concentrations were adjusted using chloroform as solvent.

4.3 Infrared Spectra (IR)

Infrared spectra were obtained with a Shimadzu IR-440 infrared spectrometer (The Scientific and Technological Research Equipment Center, Chulalongkorn University), using thin film on sodium chloride window to determine the spectra.

4.4 Mass Spectra (MS)

The Electron Impact Mass Spectra (EIMS) were obtained from Finigan Mat Incos 50, quadrupole mass spectrometer which was operated at 70 eV (Department of Chemistry, Faculty of Sciences, Mahidol University).

A few mcg of sample was introduced directly into the ionization chamber using sample probe. The sample was heated and the mass was scanned. The number of scan was selected and recorded as a mass spectrum.

4.5 Proton and Carbon-13 Nuclear Magnetic Resonances Spectra (^1H and ^{13}C NMR Spectra)

The 500 MHz ^1H NMR spectra, 125 MHz ^{13}C NMR spectra and 2D NMR spectra were obtained with a JEOL JMN-A500 spectrometer (The Scientific and Technological Research Equipment Center, Chulalongkorn University).

The Chemical shifts were reported in ppm scale using the chemical shift of trimethylsilane (TMS) at 0 ppm as the reference signal. Whereas deuterated chloroform (CDCl_3) + 0.05% TMS (V/V) and deuterated methanol (CD_3OD) were used as operating solvents.

30 - 50 mg of sample was dissolved in 1 - 2 ml of deuterated solvent, filtered, and transferred to a 5 mm NMR tube. Push the sample tube into a spinner then insert the sample tube and spinner into the sample depth gauge. The tube was adjusted until the round bottom was just below the bottom mark appropriate to the sample tube diameter. Insert the sample tube into the magnet, the sample tube would start spinning and signal was adjusted and locked be presented in a color display. Adjusted the "FIELD" in order to position the up and down sweep signals symmetrically about the center of the screen. After the field was locked, shimming was operated to produce maximum amplitude and decay time (ringing) of the observed signal. The parameters were adjusted to required experiments such as proton NMR, carbon-13 NMR, DEPT, H-H cosy, HMQC, HMBC, NOE and irradiation.

5. Melting Point

Melting points were obtained from a Buechi glass capillary apparatus (Electrothermal Melting Point Apparatus) (Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

A few mg of sample was ground in agate mortar and a finely powder was filled into capillary tube which was sealed at one end. The sample tube was put into the instrument and the temperature was raised at a rate of 4° C - 5° C per minute and 1° C per minute at the melting point range.

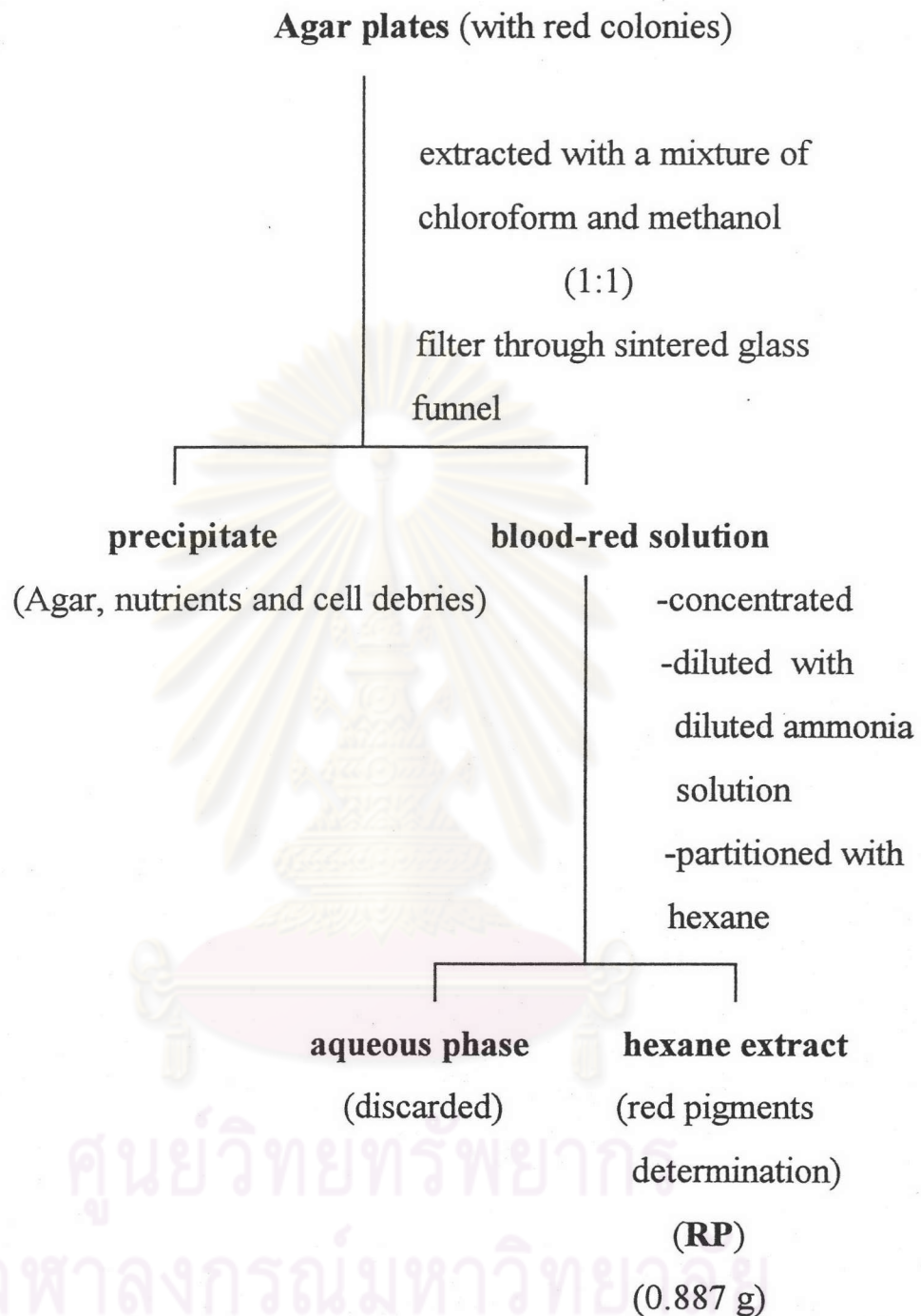
6. Solvents

All chemical were of analytical grade and solvents were purified by redistillation.

PIGMENTS EXTRACTION

The red pigments on the agar plates (100 plates) were extracted with the mixture of methanol: chloroform (1:1) solution (5 l.) and then filtered through sintered glass funnel. The filtrate was blood-red solution. The combined filtrates were concentrated under reduced pressure to about half of the original volume. The concentrated extract was basified with diluted ammonia solution and partitioned with hexane. Washed the combined hexane extract (5 l.) with diluted ammonia solution and the aqueous phase was discarded. Then the hexane extract was evaporated under reduced pressure to dryness (RP, 0.887 g) at low temperature (below 40° C) (Scheme 5.).

After the liquid-liquid extraction technique, isolation processes were performed using chromatography techniques.



Scheme 5. Red pigments extraction.

ISOLATION OF RED PIGMENTS

The dried extract (RP, 0.887mg) was deep red greasy semisolid. The red pigments were isolated firstly by quick column chromatography (adsorbent: silica gel 60 g) eluted with 20% ethyl acetate in hexane (discarded the first portion of eluate, 2 l., and other parts, 10 l. (orange-pink fluorescence color solution) were collected).

The solution was collected and passed through a second flash column chromatography (adsorbent : silica gel 60 g), gradiently eluted with the solvent system of ethyl acetate and hexane from 1% to 10%, by 1% increment. The eluates collected based on the color bands and were examined by TLC, using 50% ethylacetate in hexane and 15% ethanol in toluene as developing system. The most red pigments were separated with 8% ethyl acetate in hexane. The fraction was collected (about 10 l.) and evaporated to dryness (0.1885 g).

The dried sample was dissolved in a small volume of chloroform and passed it through a gel column chromatography, eluted with 20% methanol in chloroform. The eluate collected, based on the color bands and was examined by TLC using 50% ethylacetate in hexane and 15% ethanol in toluene as developing systems. These red pigments were separated in 5 colored band (not absolutely separated), purple brown, purple blue, red brown, deep red and pink. Deep red pigment (in 5 l. of solvent) was the most part and was almost pure. Other pigments were not

pure and the amounts were too small to be conducted in the experiments of structure elucidation.

Table 7. The portions of red pigments separated from gel filtration chromatography.

Color bands	weight (g)
purple brown	0.0514
purple blue	0.0226
red brown	0.0101
deep red	0.0916
pink	0.0056

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The deep red pigments (0.0916 g) was again dissolved in a small amount of methanol, basified with diluted ammonia solution and partitioned with hexane. The hexane extract was combined and concentrated. It was then separated again by preparative TLC. The concentrated extract (5ml) was applied as a narrow band on TLC plate (10 plates, each 0.5ml) and developed in 50% ethyl acetate in hexane. All TLC operations were performed in day light. The plates were dried at room temperature in a fume hood. The red color band (about 2 cm broad, distance 6.7-8.6 cm, solvent front 18 cm) appeared separately in two shades of red color (RP-1 (red) and RP-2 (orange-red), Rf 0.45 and 0.40, respectively, average Rf 0.425) were immediately separated from the plates (each 1 cm broad) by acetone (each, 2l.). There were another two bands, red brown color band (0.5 cm broad) at Rf 0.2 (RP-3) and pink color band (0.5 cm broad) at Rf 0.8 (RP-4), of which the amounts were very small and difficult to be eluted from the adsorbent, so it was not further investigated.

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Hexane Extract (0.887 g.)

Quick Column Chromatography:

- eluted with 20% ethyl acetate in hexane (10 l.)
- evaporated to dryness

Red Pigments

Flash Column Chromatography:

- gradient elution with mixture solution of ethyl acetate and hexane (1% to 10%, 1% increment)
- most red pigments were separated in 8% ethyl acetate in hexane (10 l.)
- evaporated to dryness

Red Pigments (0.1885 g)

Gel filtration Column

Chromatography:

- eluted with 20% methanol in chloroform.

- separated in five bands (not absolutely separation).
- collected the forth band (5 l.).
- evaporated to dryness.

Red Pigments (0.0916 g)

Preparative TLC

- developed with 50% ethylacetate in hexane
- collect the two red bands at Rf 0.45 and 0.40 as RP-1 and RP-2, respectively

RP-1 (0.027 g)

RP-2 (0.045 g)

Scheme 6. Isolation of the red pigments.

CHARACTERIZATION OF ISOLATED COMPOUNDS

1. Characteristics of RP-1

RP-1 was an amorphous red platelets with green metallic sheen. It was soluble in ethanol, chloroform, hexane.

EIMS : m/z (relative intensity);

324 (1.5%), 323 (5.0%), 266 (7.1%), 91 (100%), 57
(74.6%), 55 (72.2%)

(Figure 11.)

UV - VIS : maximum absorption wavelength, λ_{\max} , nm ($E^{1\%}$, 1cm)

In Ethanol; 534 (452×10^6) (Figure 12.)

In Chloroform; 536 (931×10^6) (Figure 13.)

In Acid ethanol; 534 (1095×10^6) (Figure 14.)

In Basic Ethanol; 532 (421×10^6) (Figure 15.)

IR : frequency, ν , cm^{-1} , Thin-Film on sodium chloride window;

3500-3100 (broad) (N-H stretching)

2925-2854 (strong) (C-H stretching)

1741 (medium) (C=N stretching)

1599 (medium) (C=C stretching)

1541, 1458 (medium) (C-H bending of CH₃)
 1231, 1073 (weak) (C-N stretching)
 (figure 16.)

¹H NMR : chemical shift, δ , ppm, 500 MHz, in chloroform-d₁;
 (relative to internal standard TMS.)

0.90
 1.25
 1.55 (2H, q, $J = 7.63$ Hz)
 2.39 (2H, t, $J = 7.63$ Hz)
 2.54 (3H, s)
 4.01 (3H, s)
 6.08 (1H, d, $J = 2.14$ Hz)
 6.35 (1H, ddd, $J = 2.14, 2.44, 4.02$ Hz)
 6.68 (1H, d, $J = 2.14$ Hz)
 6.92 (1H, ddd, $J = 1.50, 2.40, 4.02$ Hz)
 6.96 (1H, sb)
 7.23 (1H, ddd, $J = 1.50, 2.25, 2.44$ Hz)
 12.57 (1H, sb),
 12.75 (2H, sb)
 (Figure 17.)

^{13}C NMR : chemical shift, δ , ppm, 125 MHz, in chloroform- d_1 ;
(relative to internal standard TMS, multiplicities
determined by DEPT spectrum)

14.00	(Q)
14.91	(Q)
25.33	(T)
29.34	(T)
29.80	(T)
30.30	(T)
58.69	(Q)
92.81	(D)
111.72	(D)
116.06	(D)
117.00	(D)
120.74	(D)
122.29	(D)
125.19	(S)
126.97	(S)
128.43	(S)
128.55	(S)
147.09	(S)
147.75	(S)
165.79	(S)

(Figure 20.)

mp : 150° C - 152° C

2. Characteristics of RP-2

RP-2 was an amorphous red platelets with green metallic sheen. It was soluble in ethanol, chloroform, hexane.

EIMS : m/z (relative intensity);

324 (6.3%), 323 (22.3%), 266 (29.6%), 149 (50.8%)

91 (100%), 57 (74.6%), 55 (72.2%)

(Figure 23.)

UV - VIS : maximum absorption wavelength, λ_{\max} , nm ($E^{1\%}$ 1 cm)

In Ethanol; 469 (50.8×10^6),

535 (41.7×10^6)

(Figure 24.)

In Chloroform; 539 (157.7×10^6) (Figure 25.)

In Acid ethanol; 535 (131.3×10^6) (Figure 26.)

In Basic Ethanol; 468 (48.0×10^6),

533 (29.3×10^6)

(Figure 27.)

FLUORESCENCE : wavelength, λ , maximum nm;

Excitation : 380

Emission : 688

(Figure 28.)

IR : frequency, ν , cm^{-1} , Thin-Film on sodium chloride window; .

3500-3100 (broad) (N-H stretching)

2925, 2853 (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)

(Figure 29.)

^1H NMR : chemical shift, δ , ppm, 500 MHz, in chloroform- d_1 ;

(relative to internal standard TMS.)

0.92

1.25

1.44 (2H, q, $J = 7.63$)

1.82 (3H, s)

2.22 (2H, t, $J = 7.63$)

3.95 (3H, s)

6.04 (1H, sb)

6.15	(1H, <u>dd</u>)
6.33	(1H, <u>sb</u>)
6.66	(1H, <u>dd</u>)
6.68	(1H, <u>sb</u>)
6.79	(1H, <u>sb</u>)

(Figure 30.)

^{13}C NMR : chemical shift, δ , ppm, 125 MHz, in chloroform- d_1 ;
(relative to internal standard TMS, multiplicities
determined by DEPT spectrum)

10.55	(Q)
14.02	(Q)
22.50	(T)
25.51	(T)
30.20	(T)
31.64	(T)
58.00	(Q)
95.21	(D)
110.00	(D)
112.30	(D)
115.78	(D)
120.78	(D)
122.55	(D)
124.25	(S)
127.42	(S)

128.41 (S)

136.44 (S)

137.90 (S)

158.81 (S)

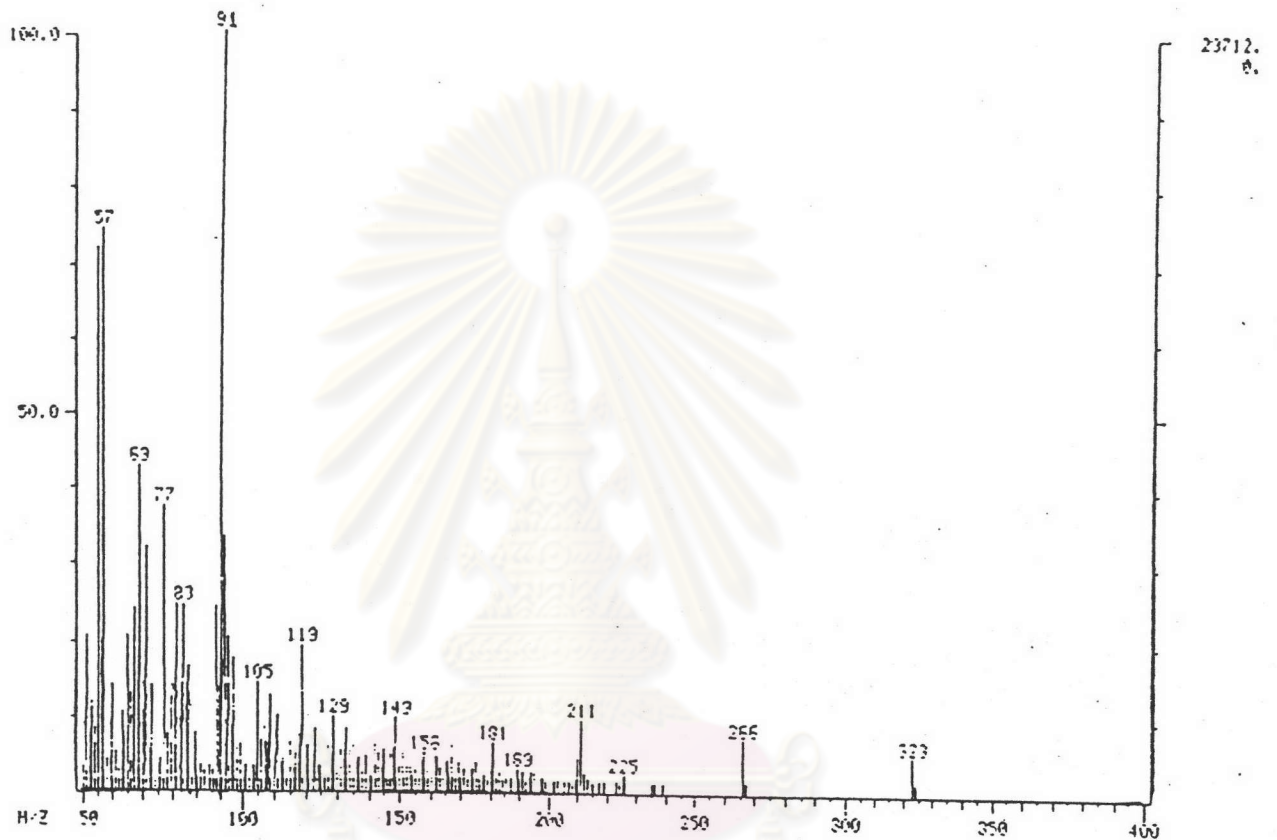
160.65 (S)

(Figure 33.)

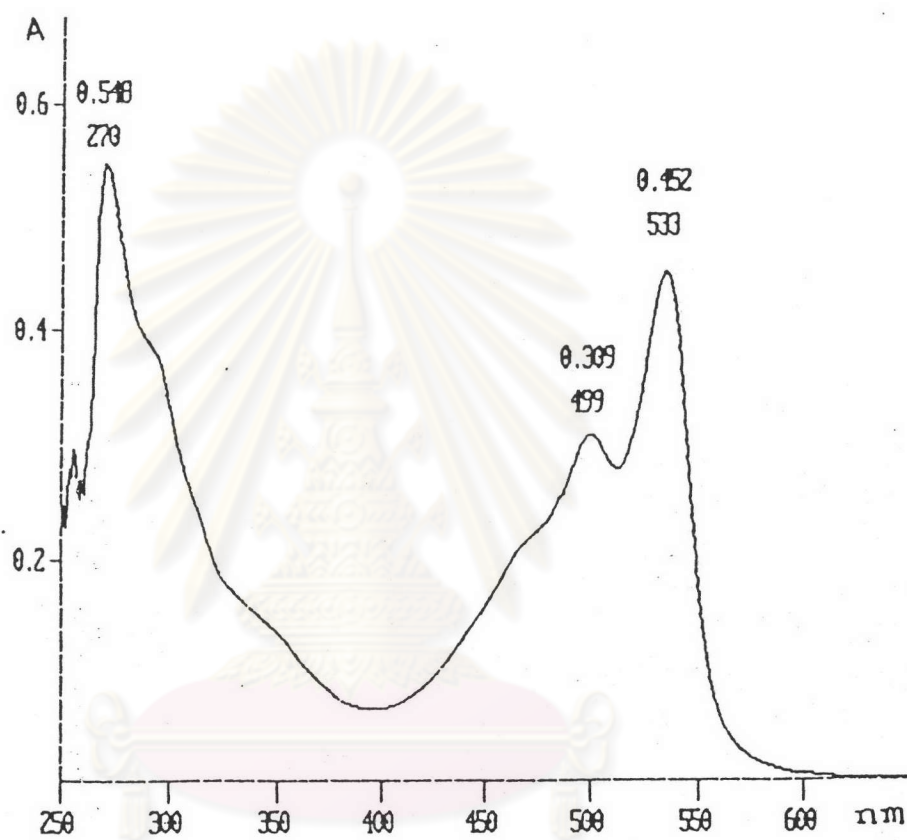
mp : 90° C - 92° C



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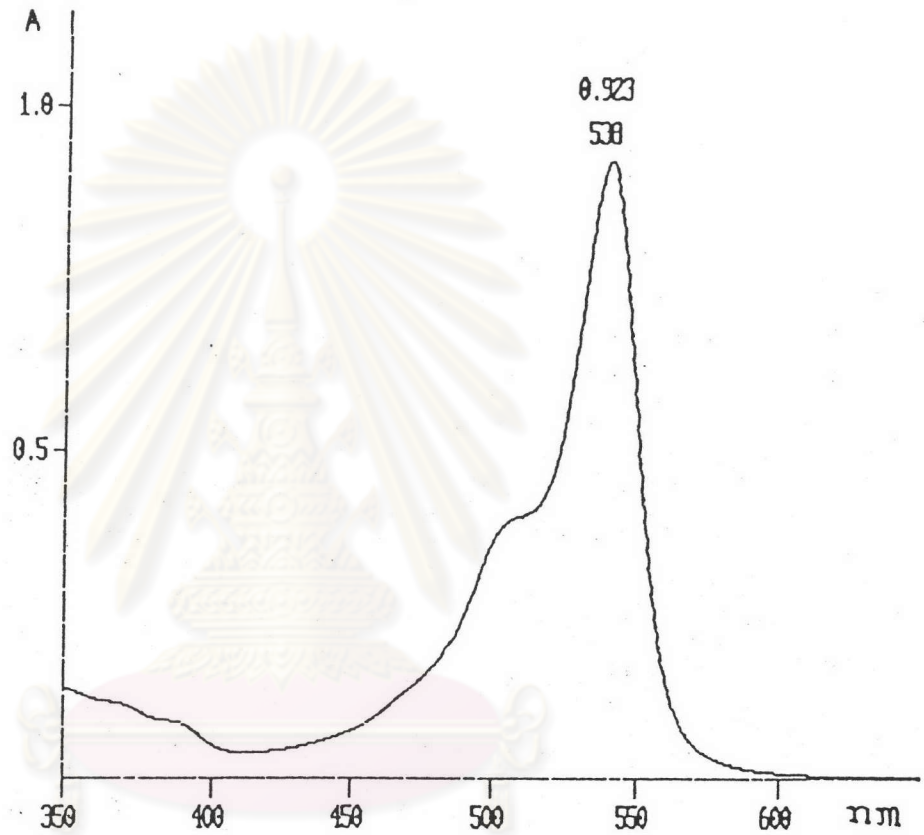


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Figure 11. The mass spectrum of RP-1.



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Figure 12. The UV-VIS spectrum of RP-1 in ethanol.



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Figure 13. The UV-VIS spectrum of RP-1 in chloroform.

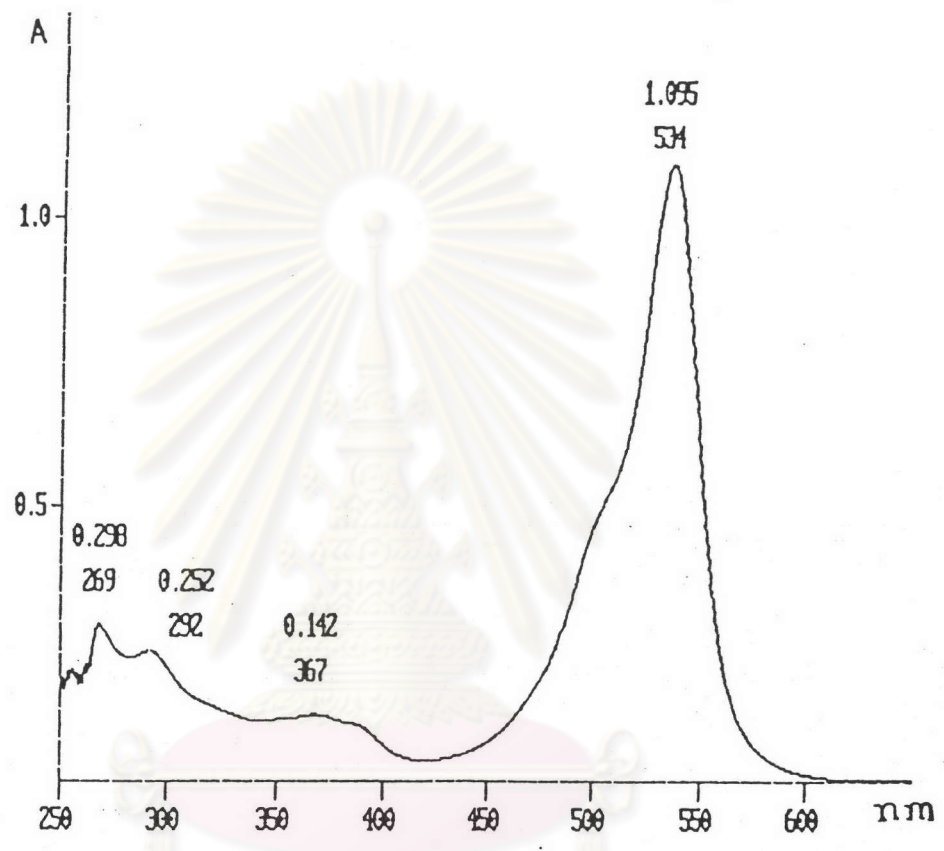
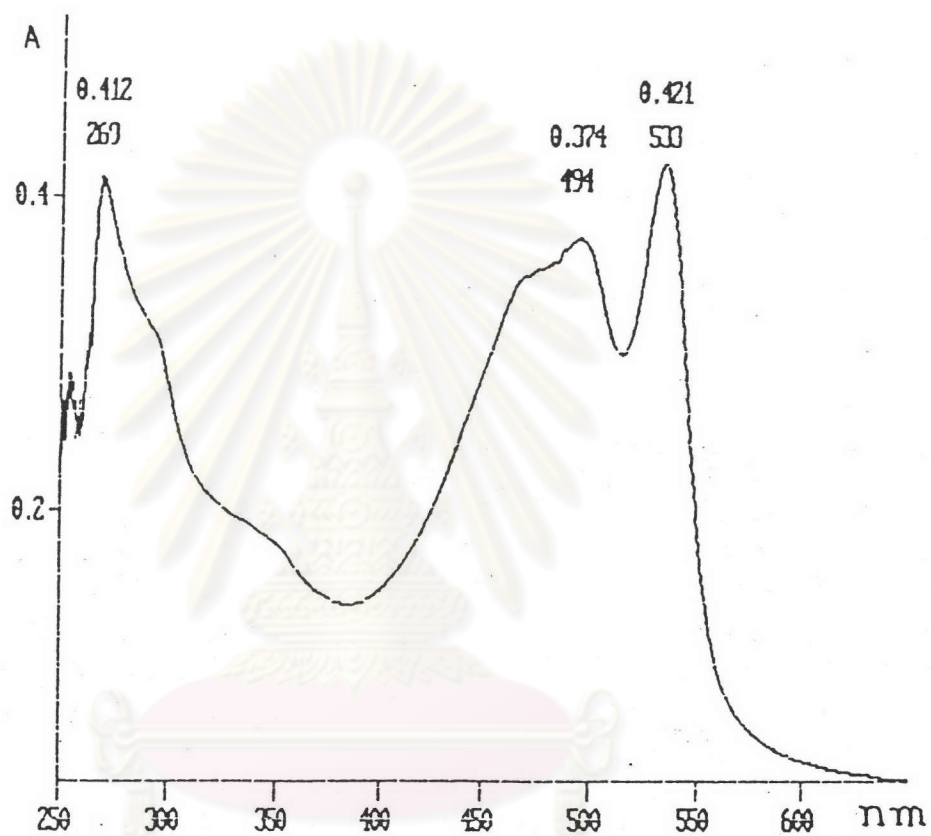
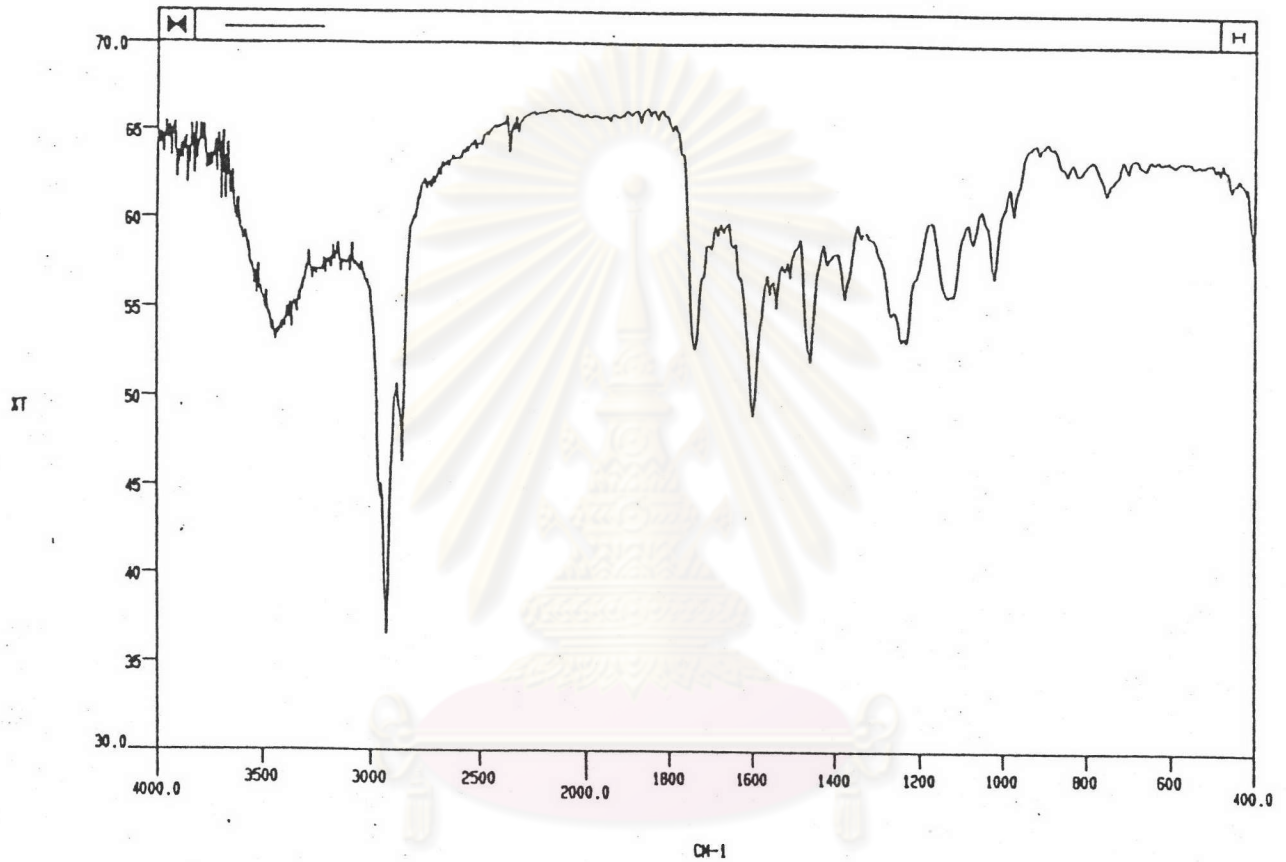


Figure 14. The UV-VIS spectrum of RP-1 in acid ethanol.

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Figure 15. The UV-VIS spectrum of RP-1 in basic ethanol.



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Figure 16. The Infra-red spectrum of RP-1.

Figure 17. The 500 Mhz ^1H NMR spectrum of RP-1 in deuterated chloroform.

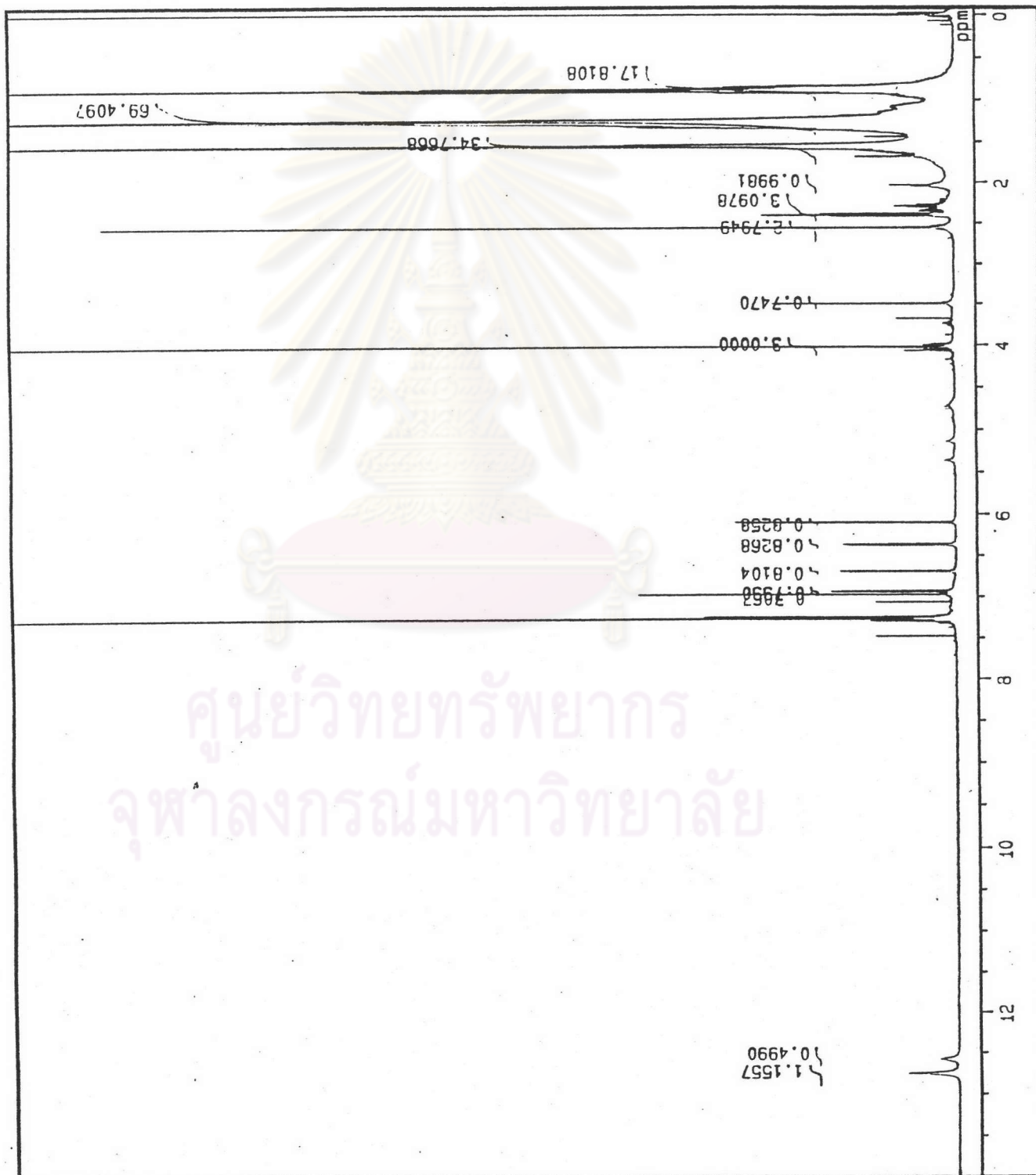
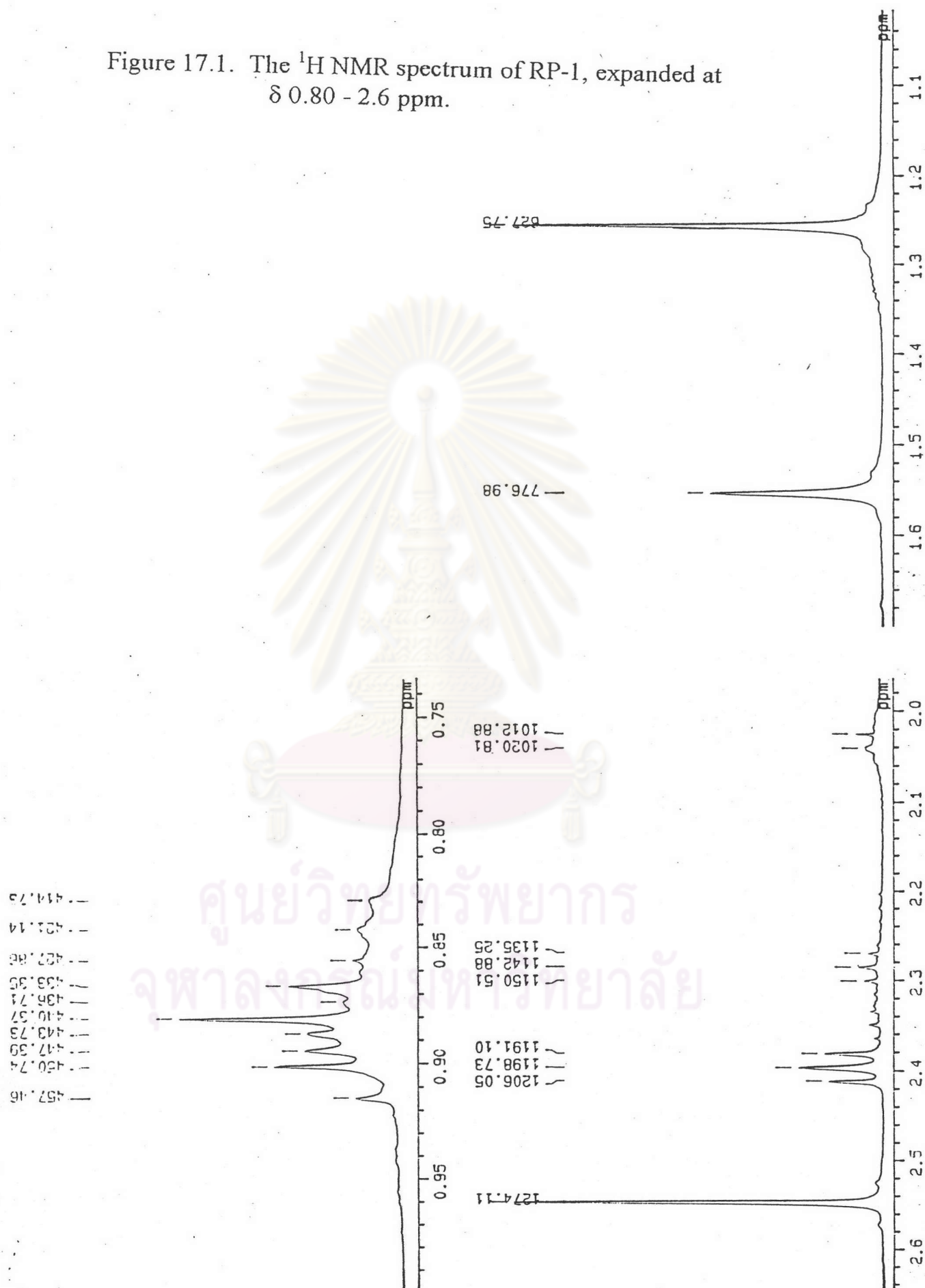


Figure 17.1. The ^1H NMR spectrum of RP-1, expanded at δ 0.80 - 2.6 ppm.



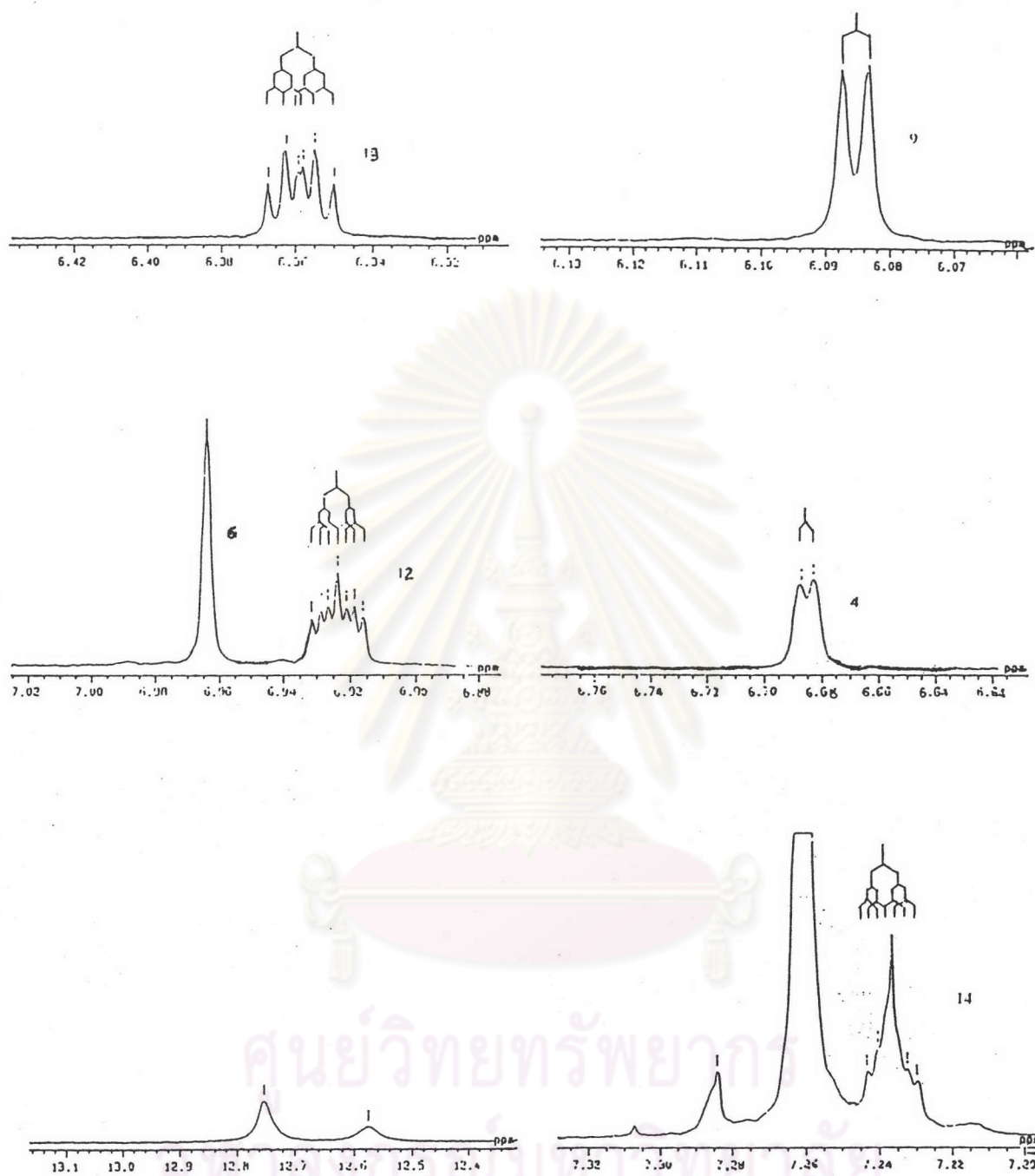


Figure 17.2. The ^1H NMR spectrum of RP-1, expanded at δ 6.07 - 7.32 ppm and 12.4 - 13.1 ppm.

Figure 17.3. The assignment of ^1H NMR spectrum of RP-1.

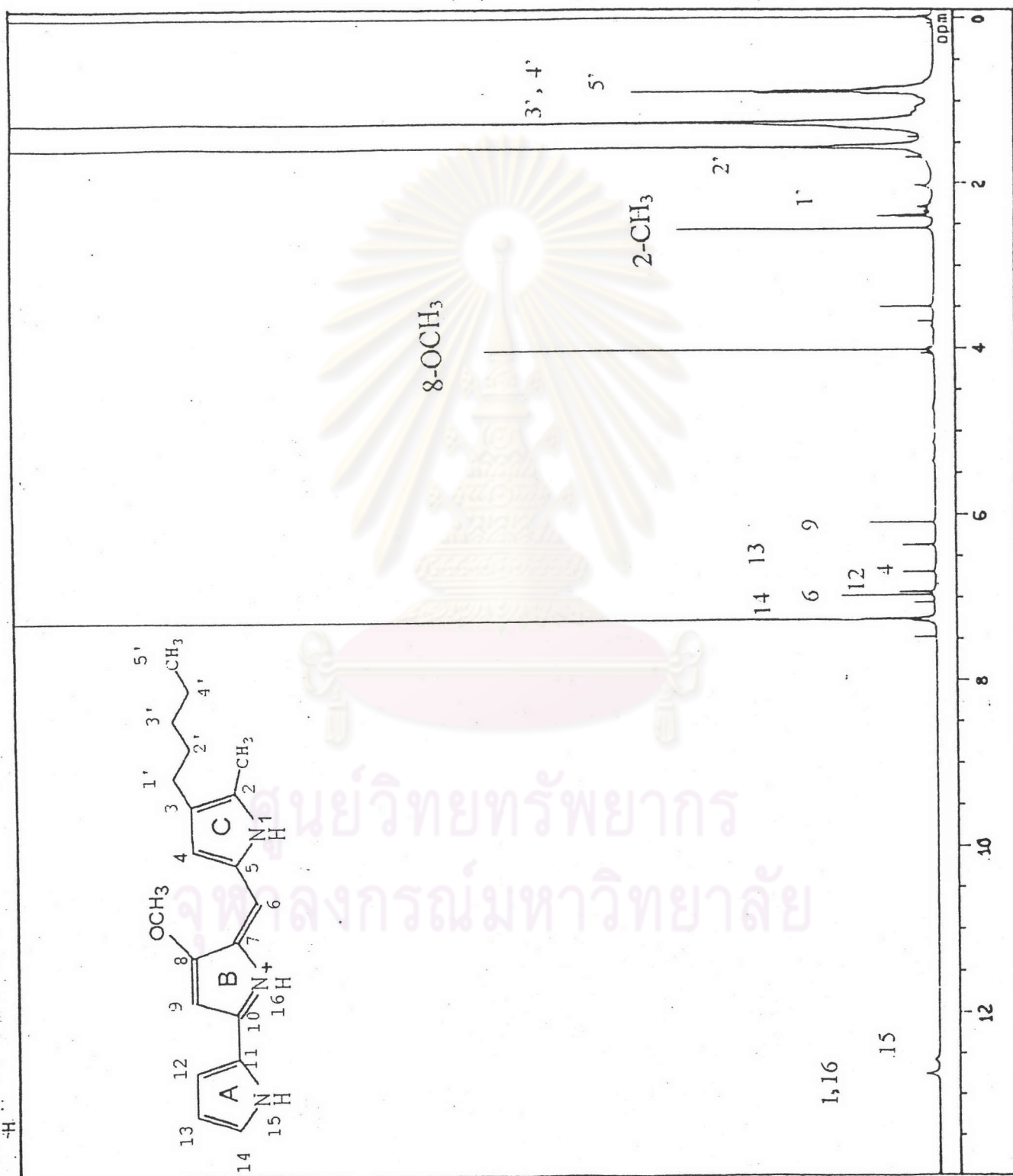


Figure 18. The H-H COSY spectrum of RP-1.

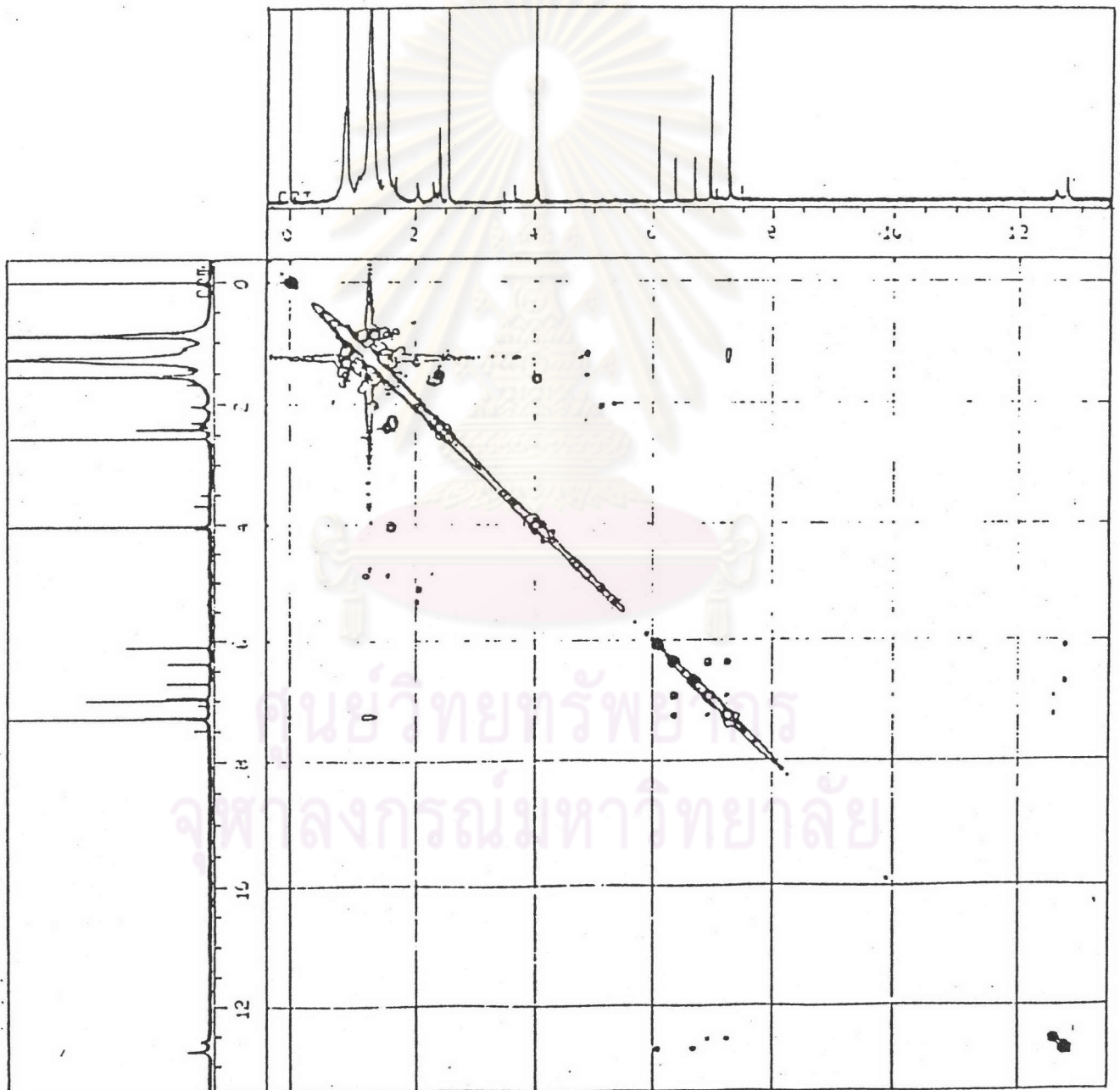


Figure 18.1. The H-H COSY expanded spectrum of RP-1 showing the coupling between signals at δ 1.55 and 2.39 ppm.

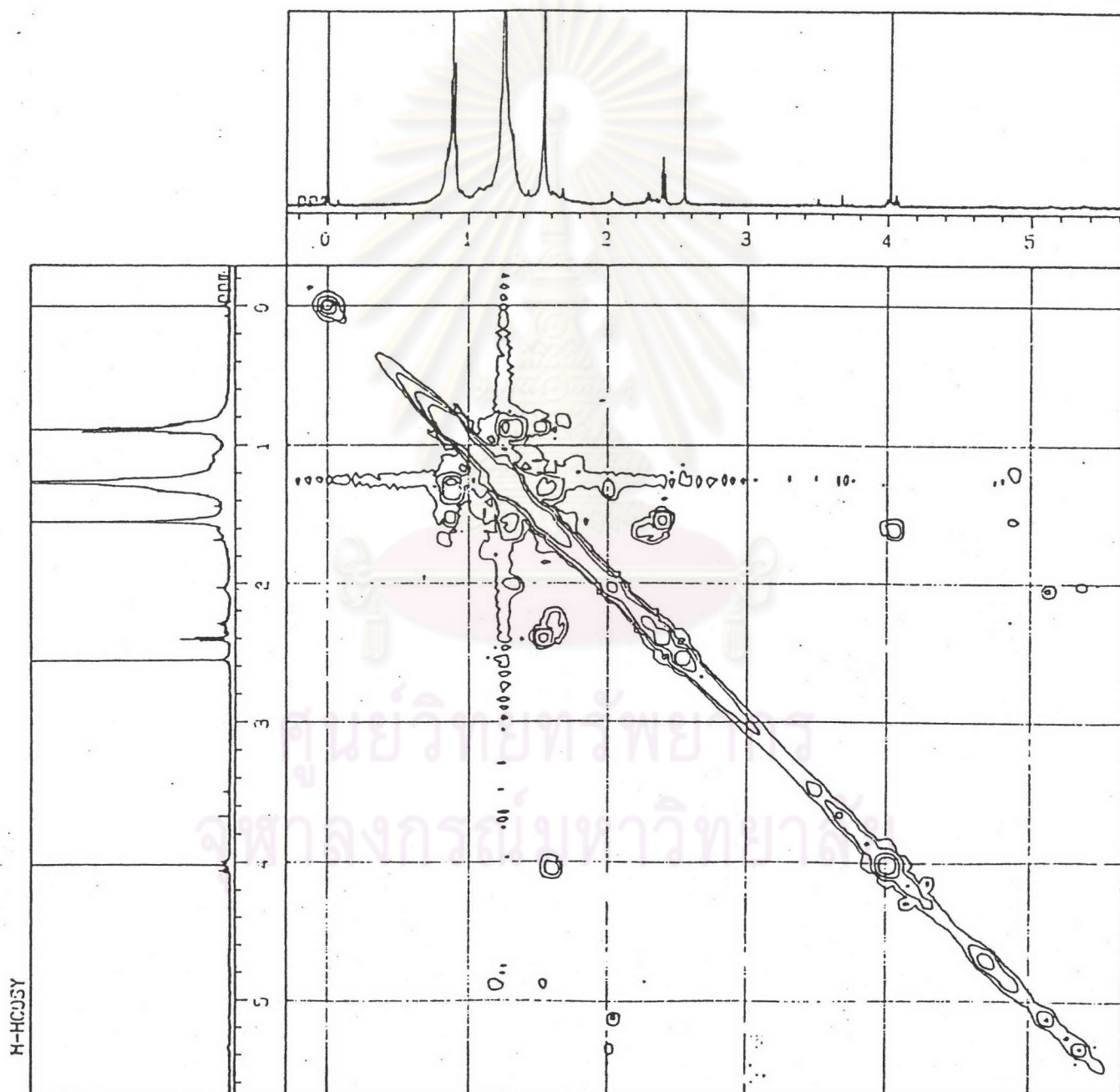


Figure 18.2. The H-H COSY expanded spectrum of RP-1 showing the long-range coupling of signals at δ 2.39, 1.55, and 6.68 ppm.

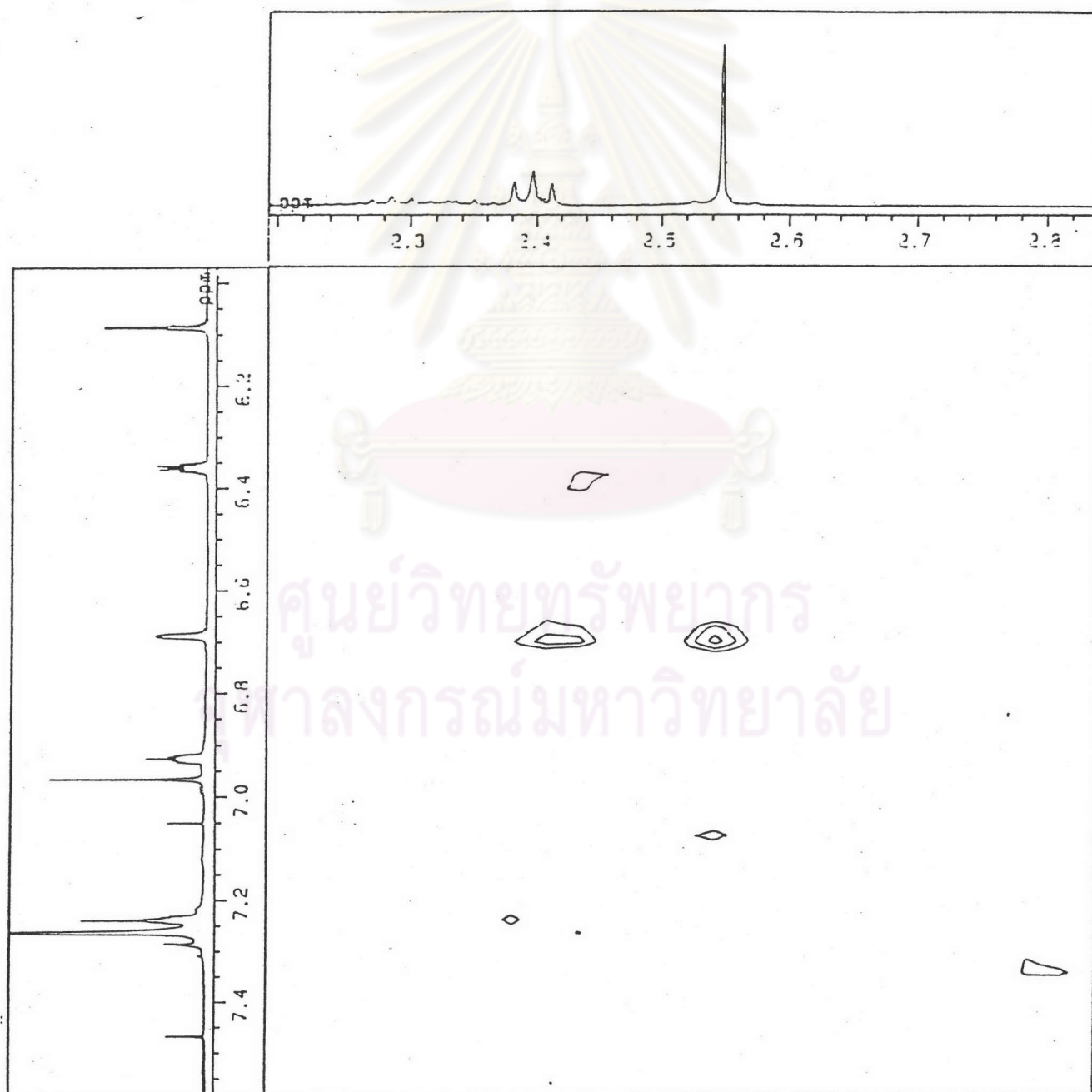


Figure 18.3. The H-H COSY expanded spectrum of RP-1 in the olefinic region showing the coupling between signals at δ 6.35, 6.92, and 7.23 ppm.

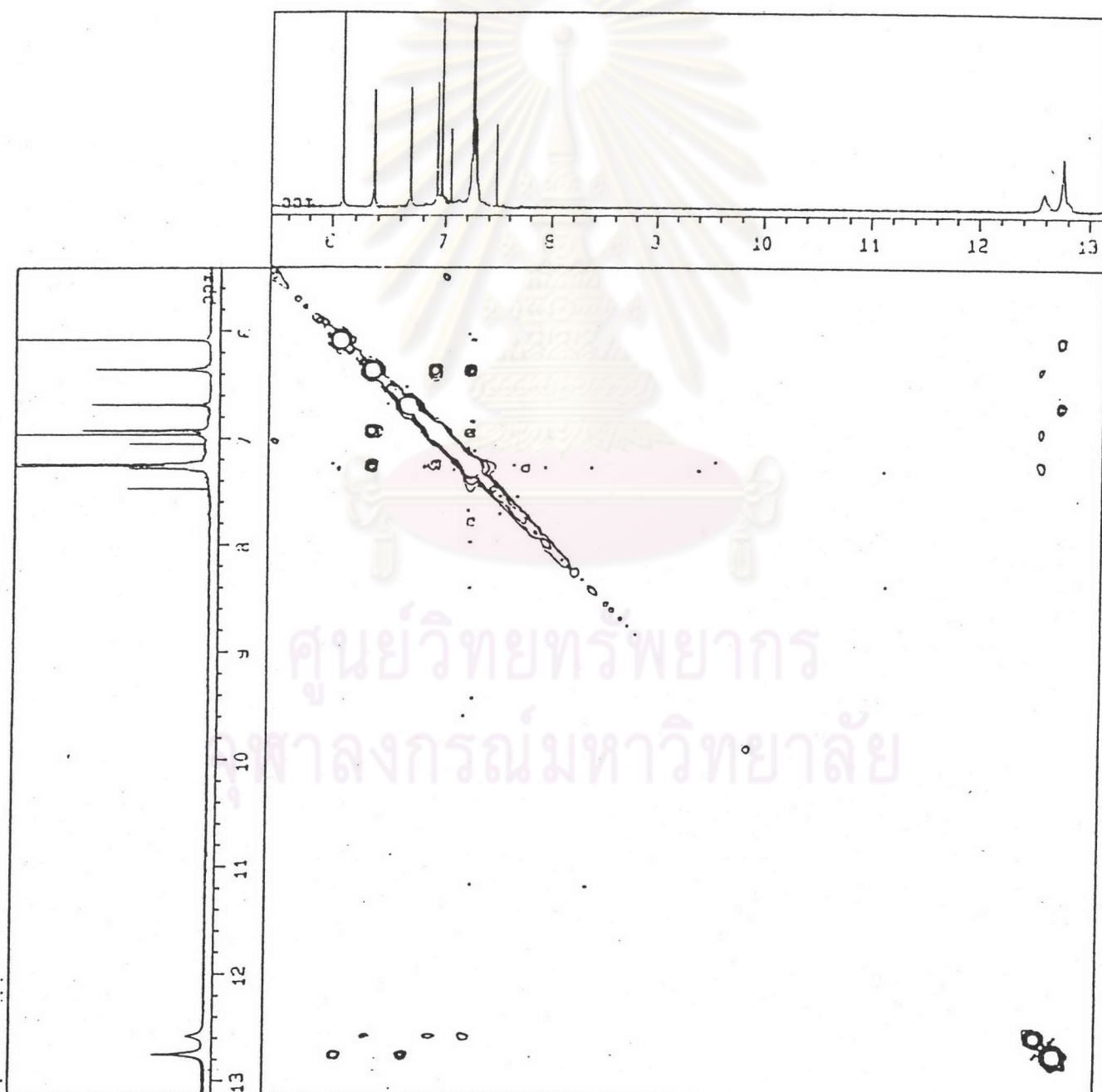


Figure 18.4. The H-H COSY expanded spectrum of RP-1 in the olefinic region showing the long-range coupling of signals at δ 6.35, 6.92, and 7.23 to the signal at δ 12.57 ppm and the long-range coupling between signals at δ 6.08 and 6.68 to the signal at δ 12.75 ppm.

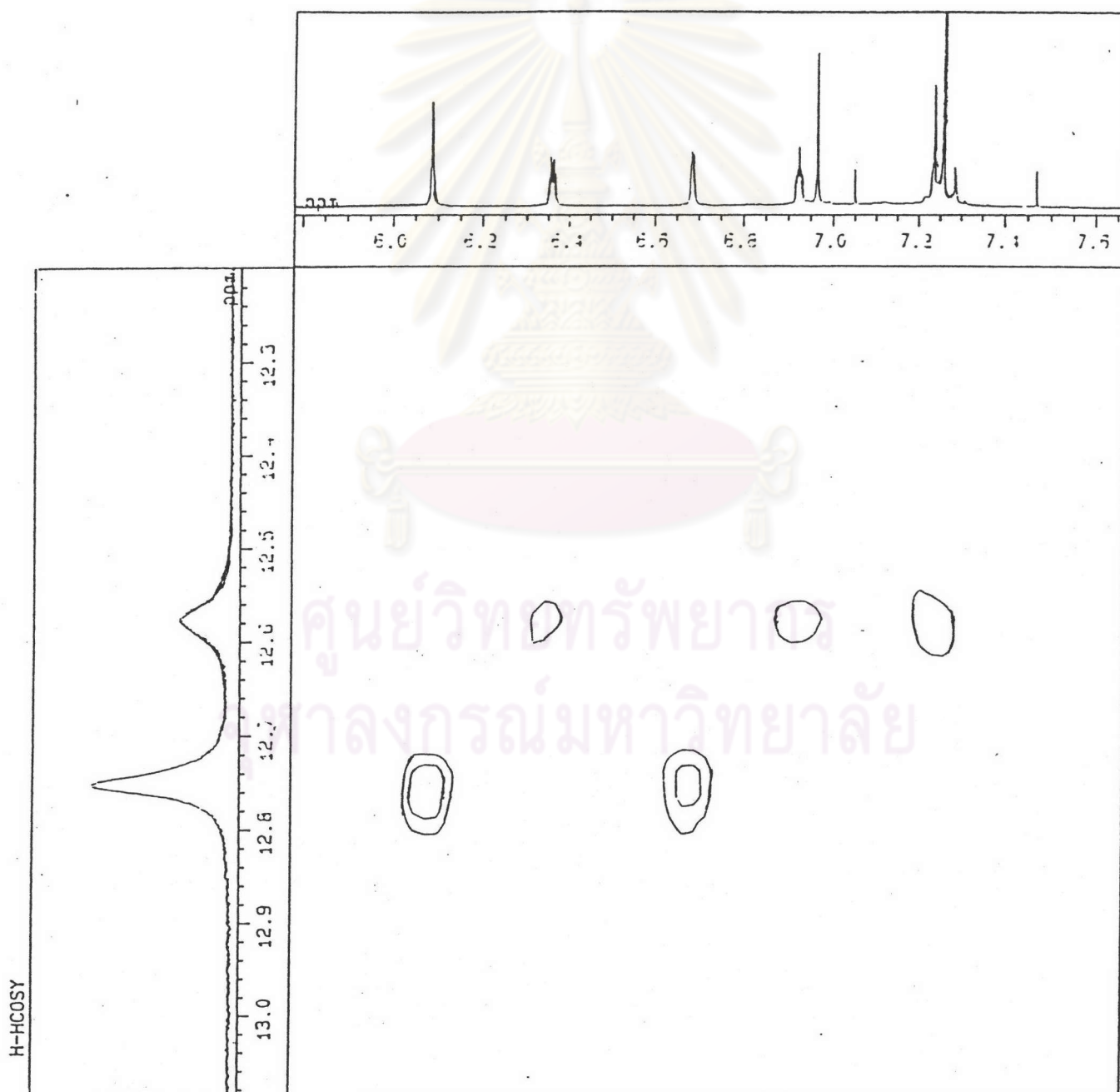


Figure 19. The ^1H NMR spectrum of RP-1 in olefinic region, irradiated at δ 6.923 ppm.

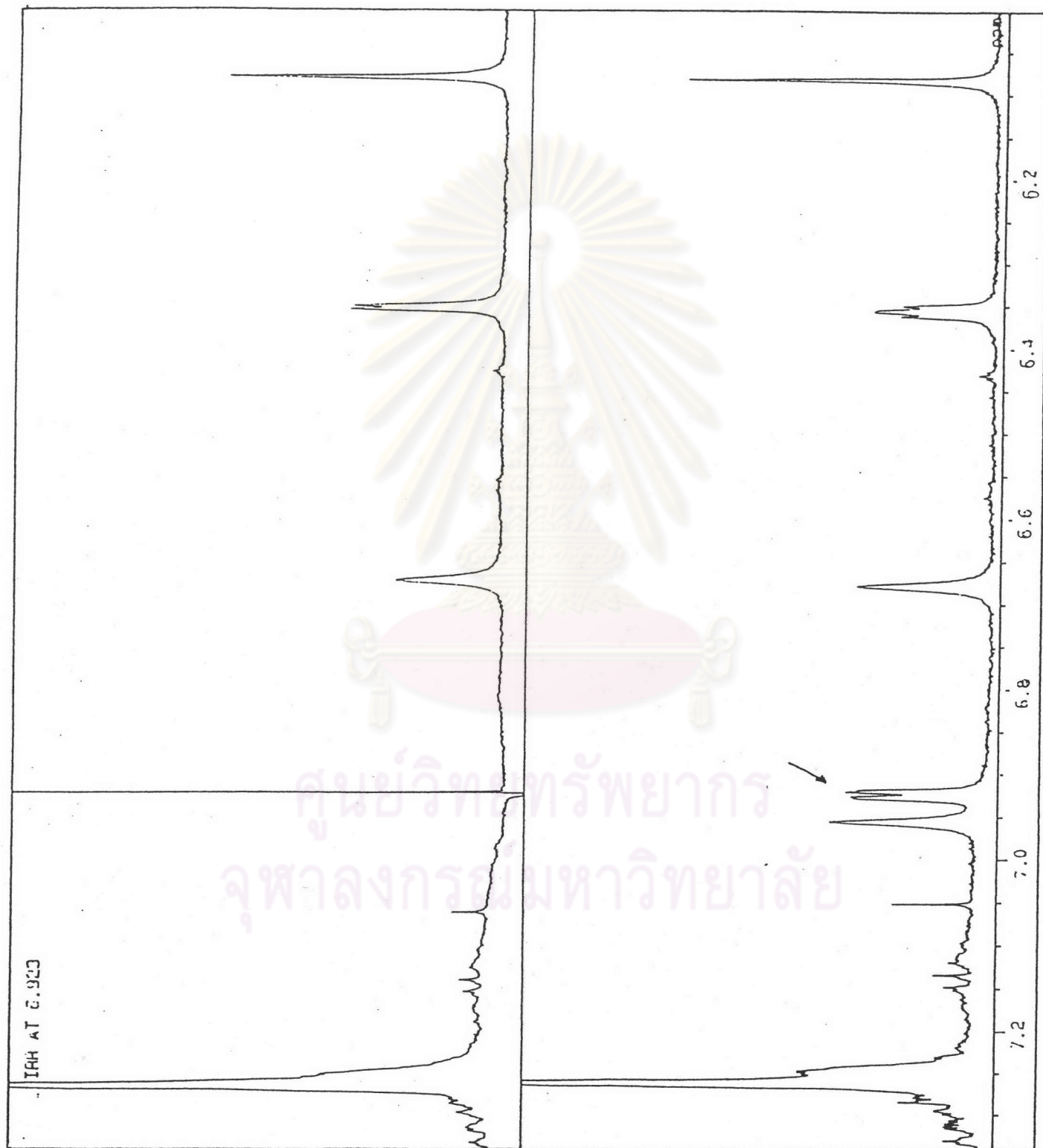


Figure 20. The 125 Mhz ^{13}C NMR spectrum of RP-1 in deuterated chloroform.

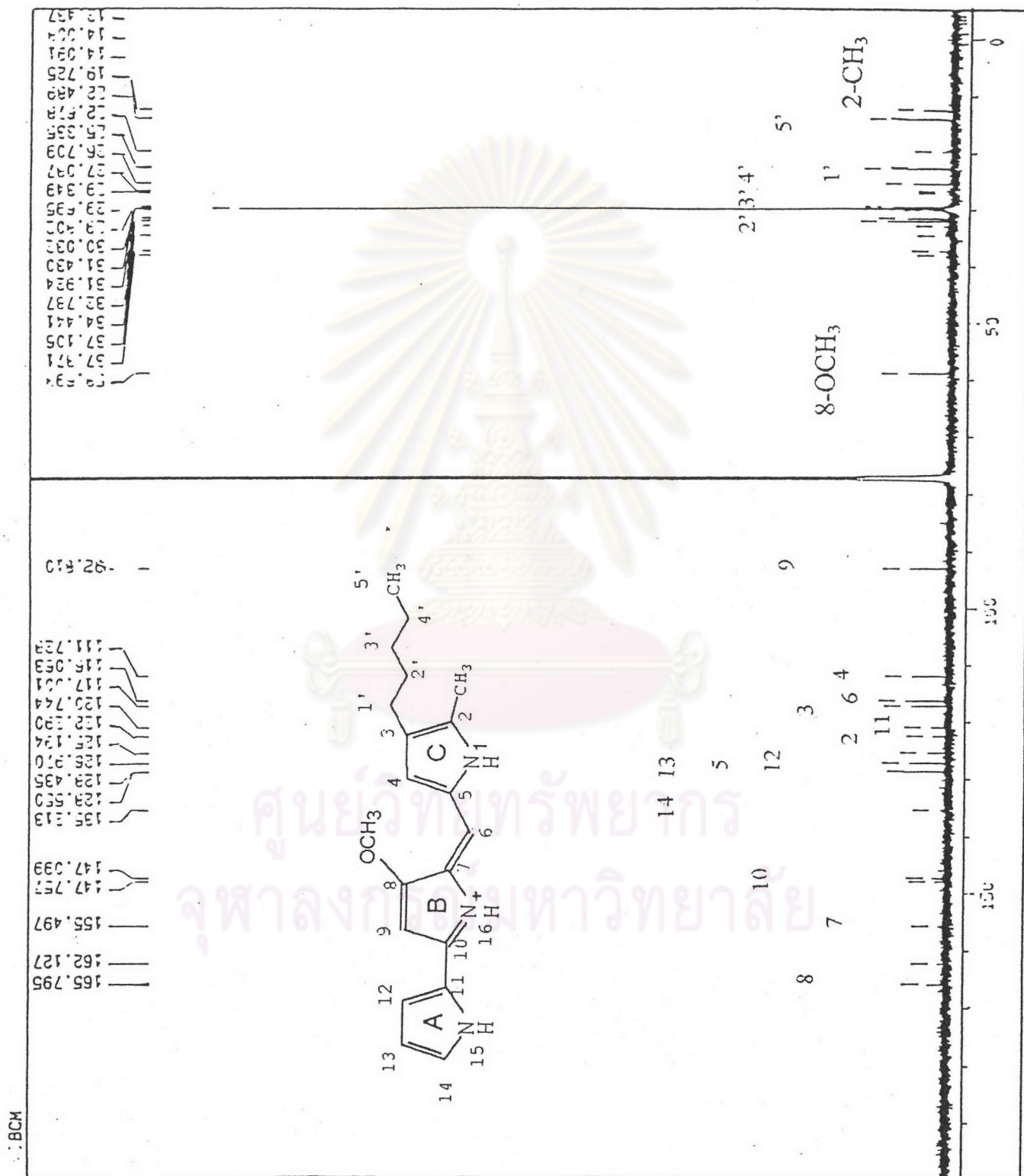


Figure 21. The DEPT spectrum of RP-1.

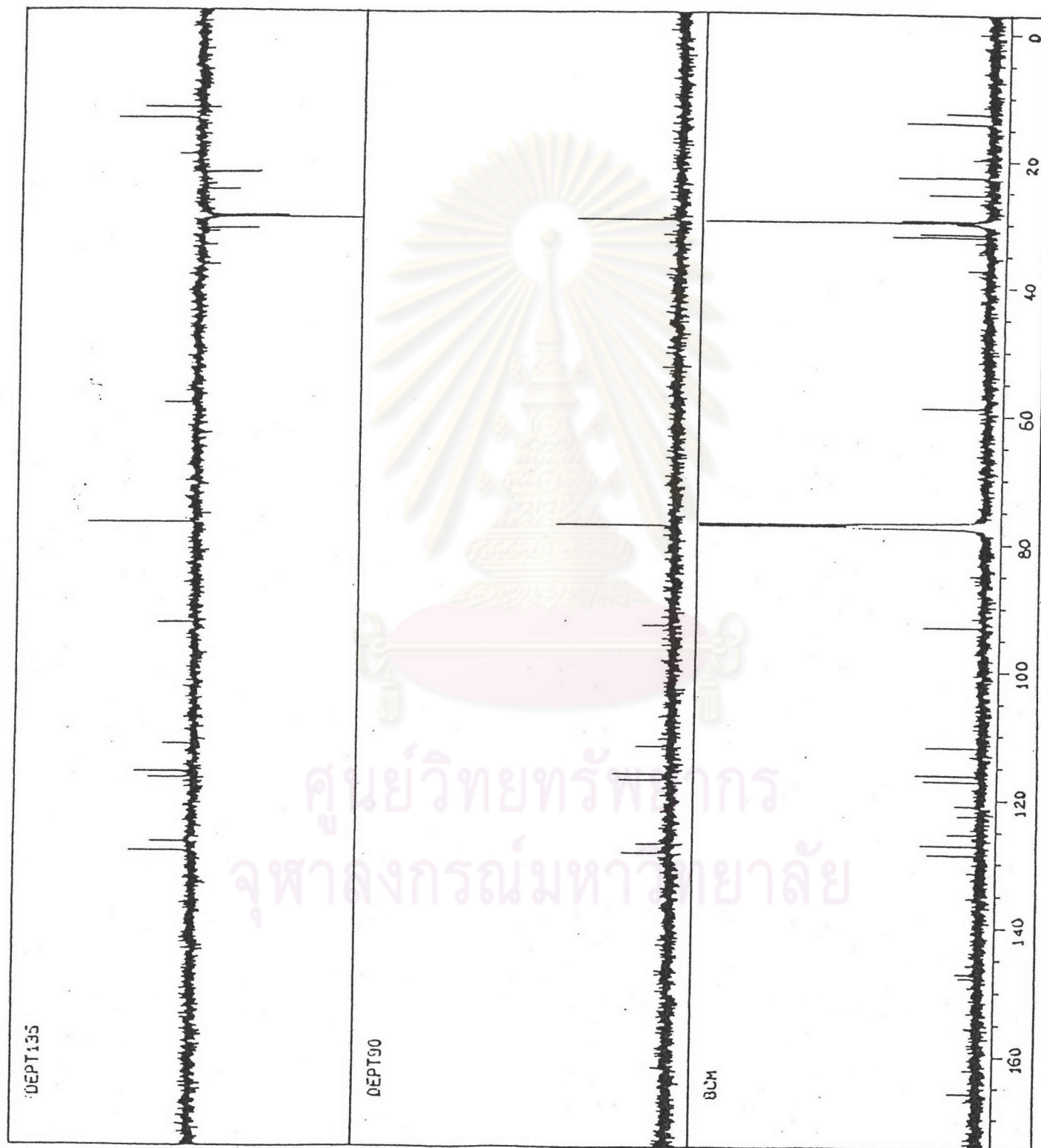
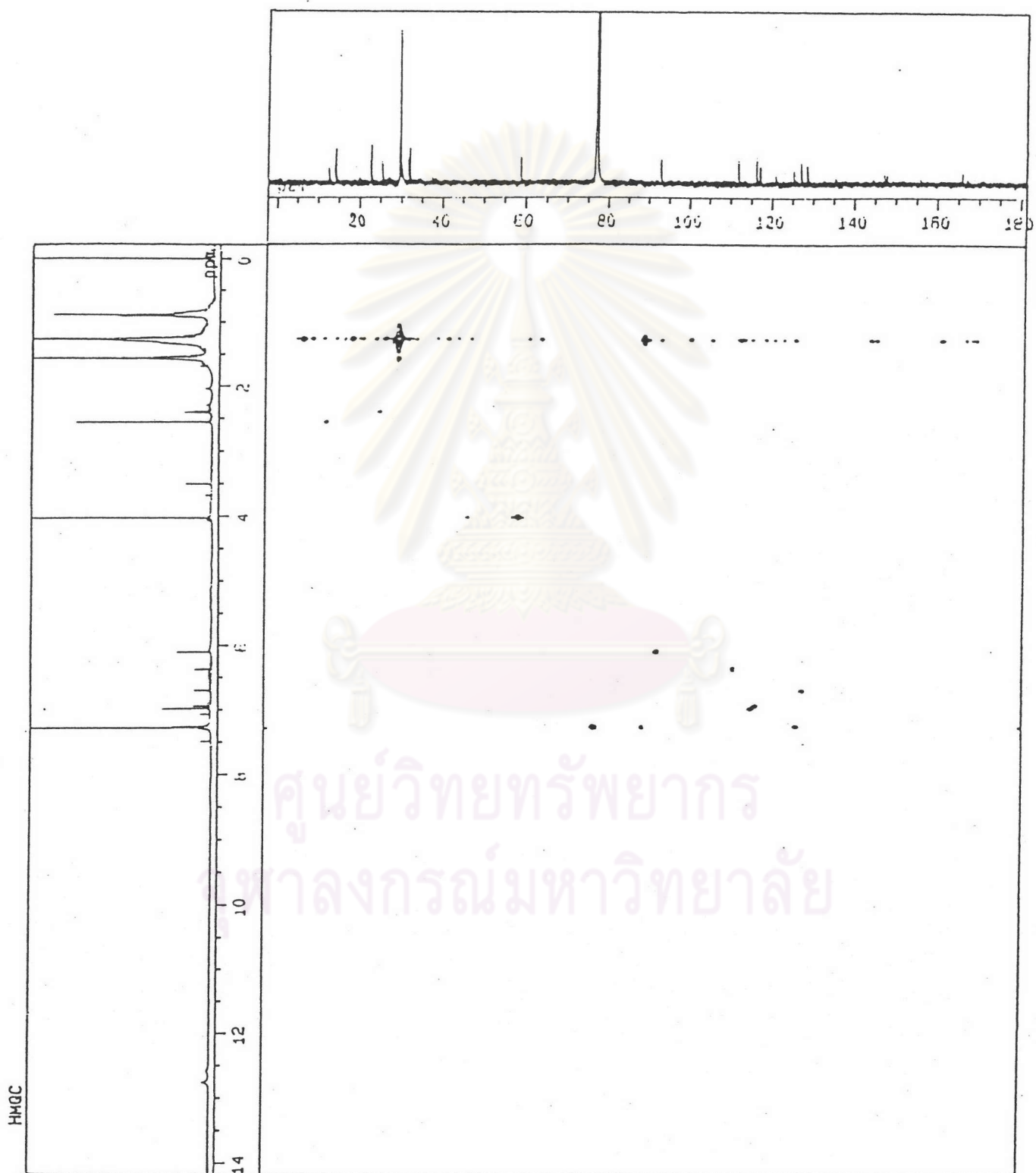


Figure 22. The C-H COSY spectrum of RP-1.



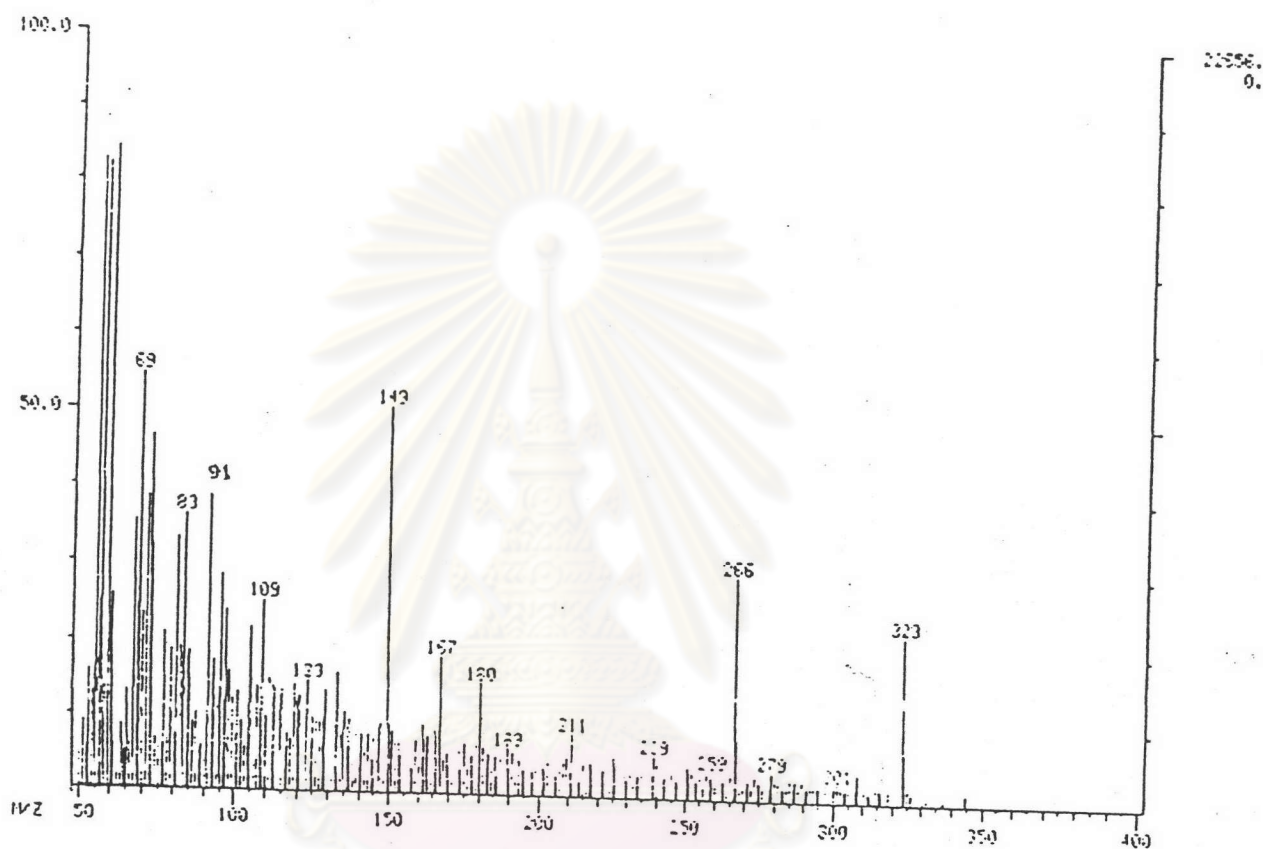


Figure 23. The Mass spectrum of RP-2.

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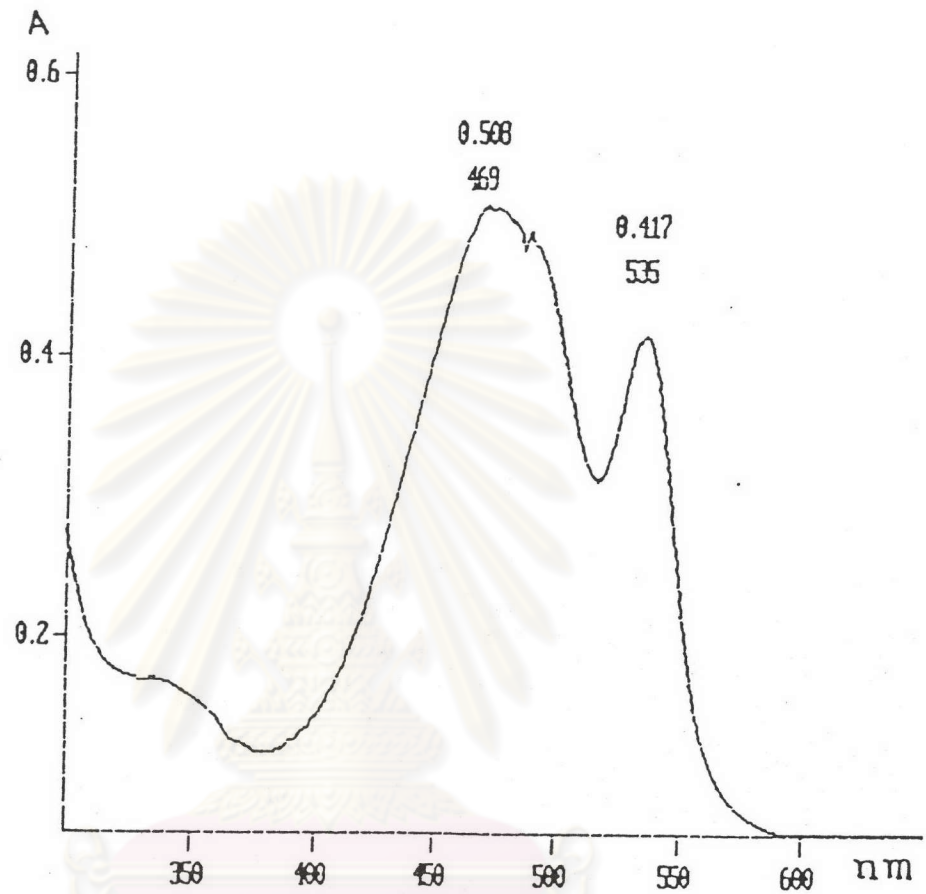


Figure 24. The UV-VIS spectrum of RP-2 in ethanol.

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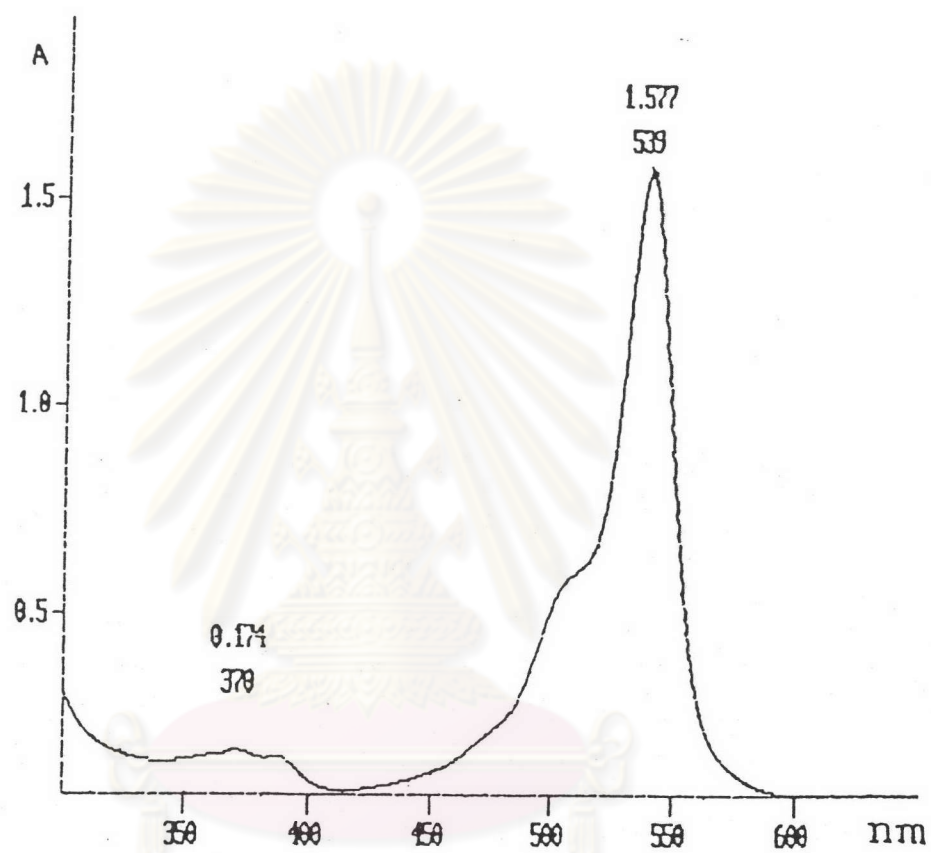


Figure 25. The UV-VIS spectrum of RP-2 in chloroform.

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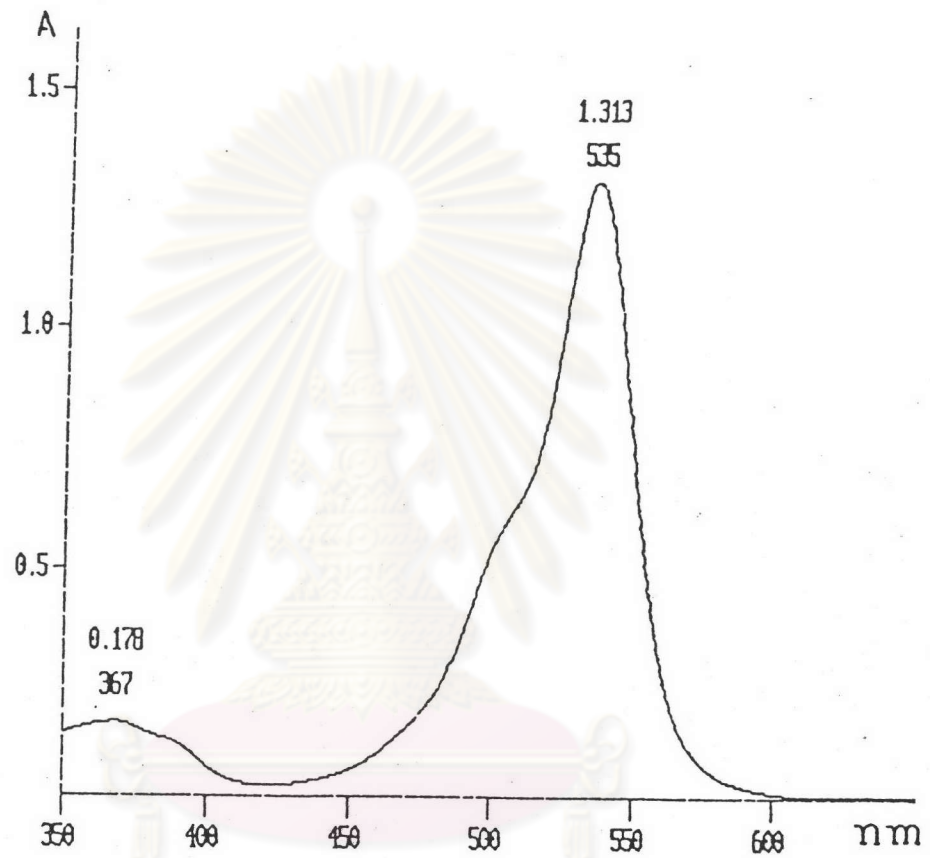


Figure 26. The UV-VIS spectrum of RP-2 in acid ethanol.

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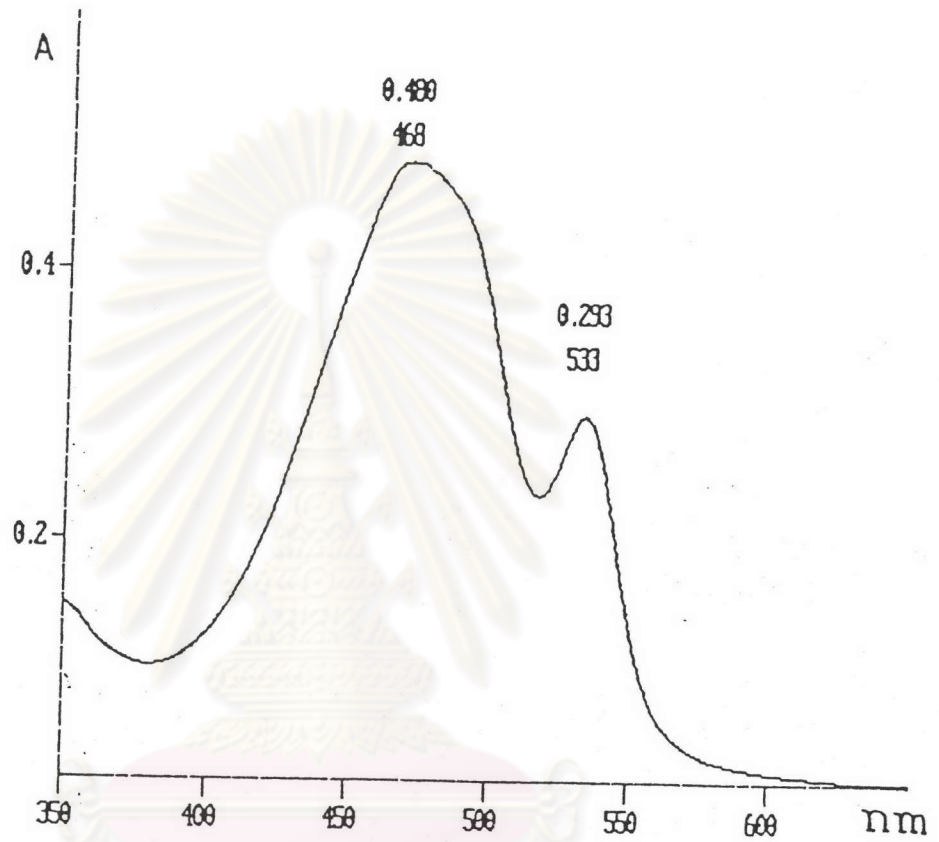


Figure 27. The UV-VIS spectrum of RP-2 in basic ethanol.

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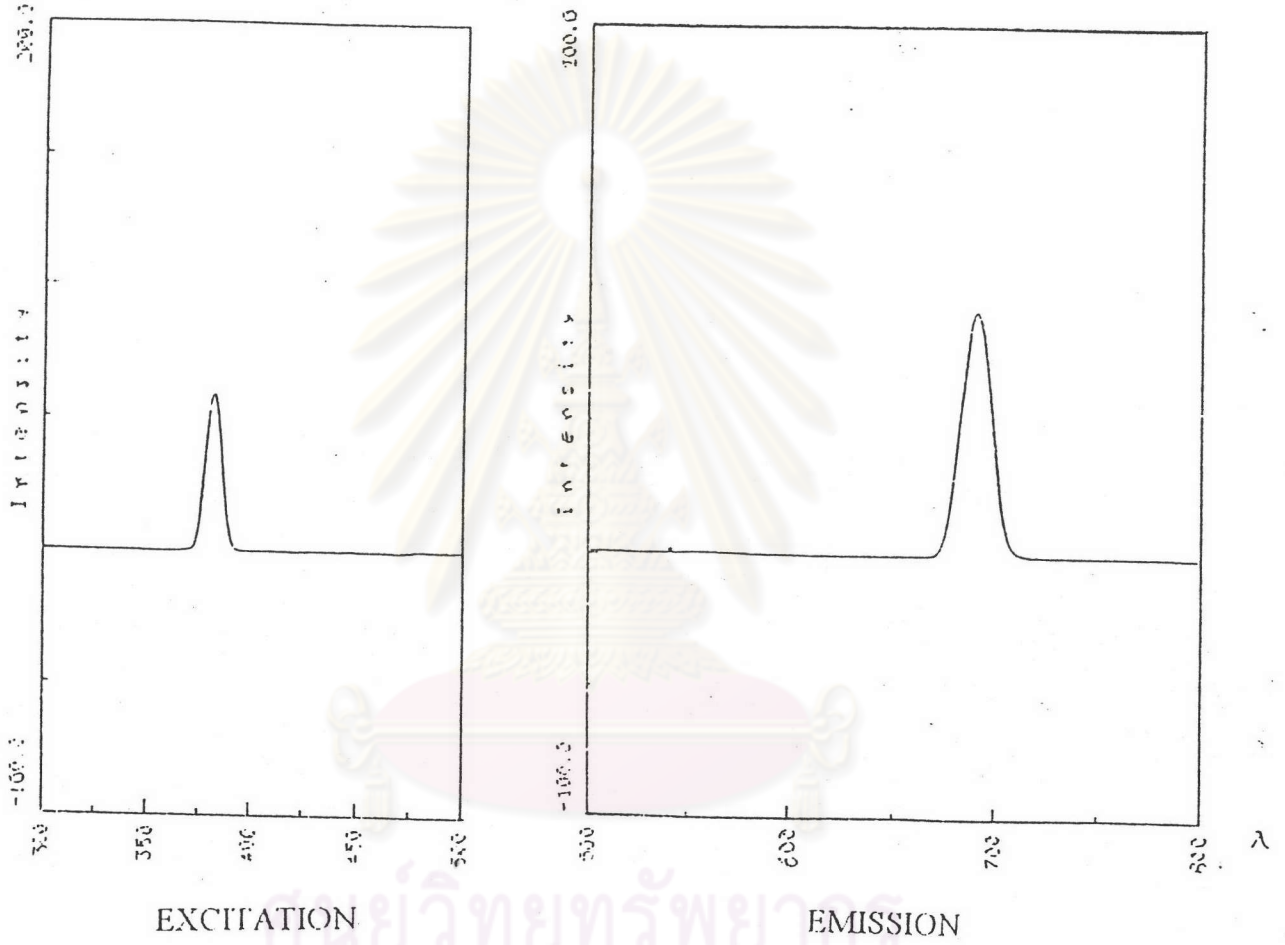


Figure 28. The Fluorescence spectrum of RP-2.

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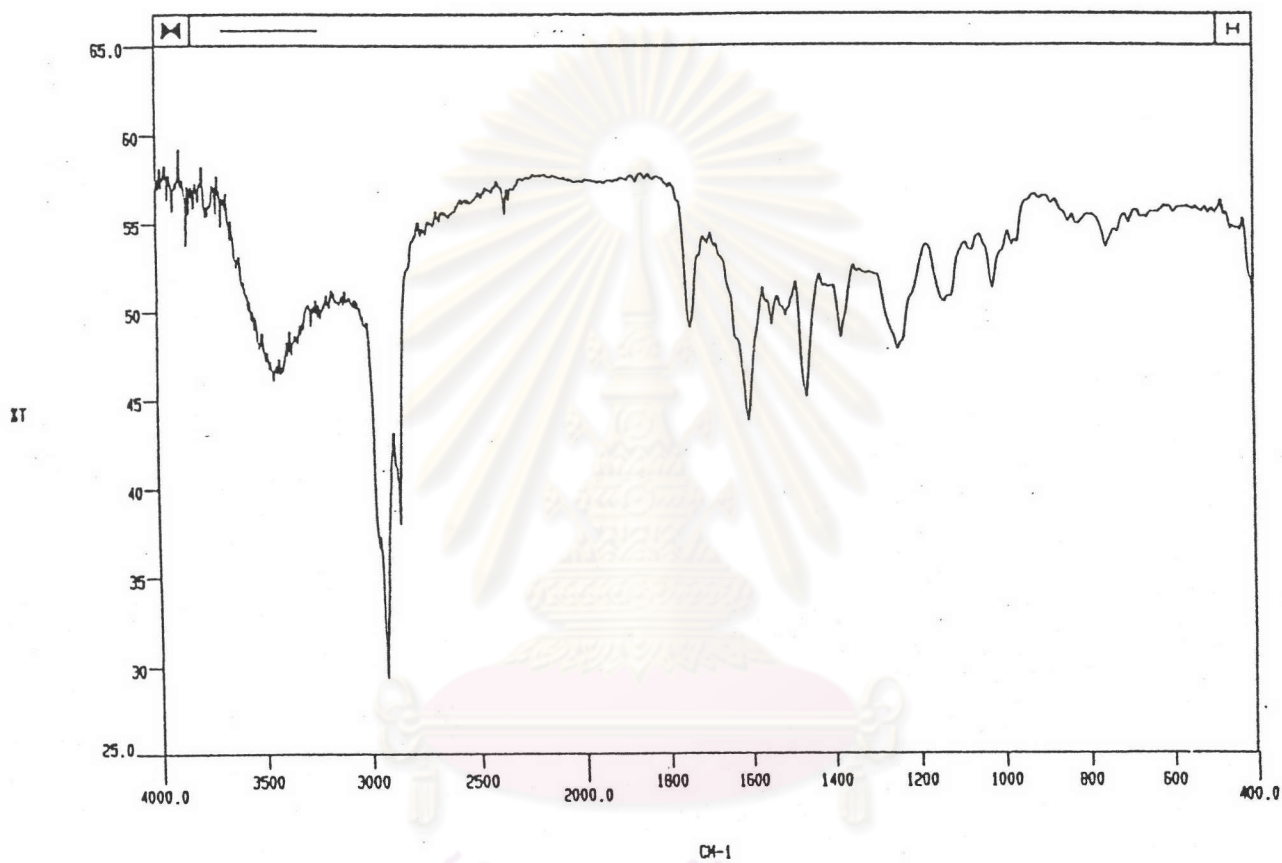


Figure 29. The Infra-red spectrum of RP-2.

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Figure 30. The 500 Mhz ^1H NMR spectrum of RP-2 in deuterated chloroform.

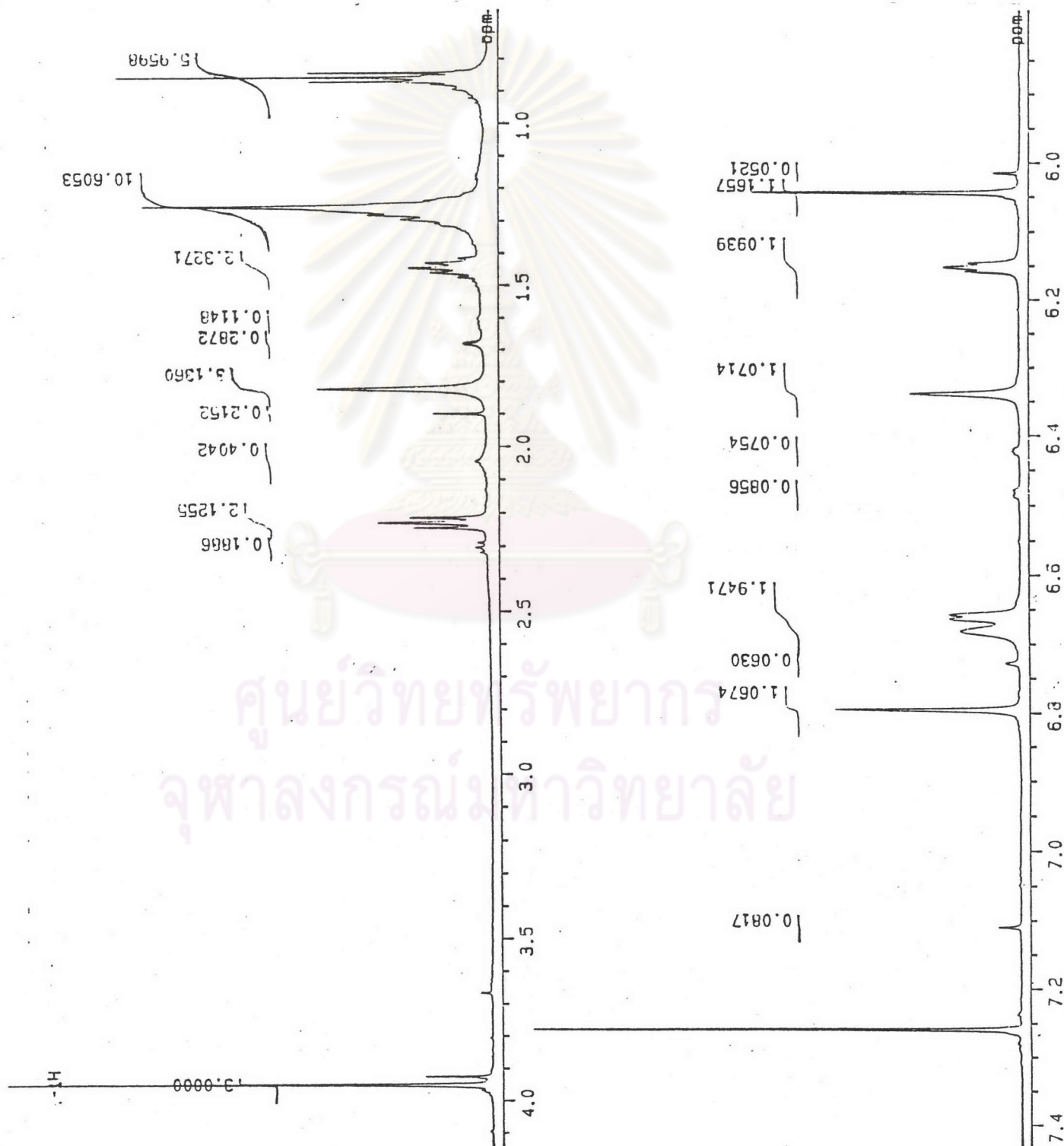
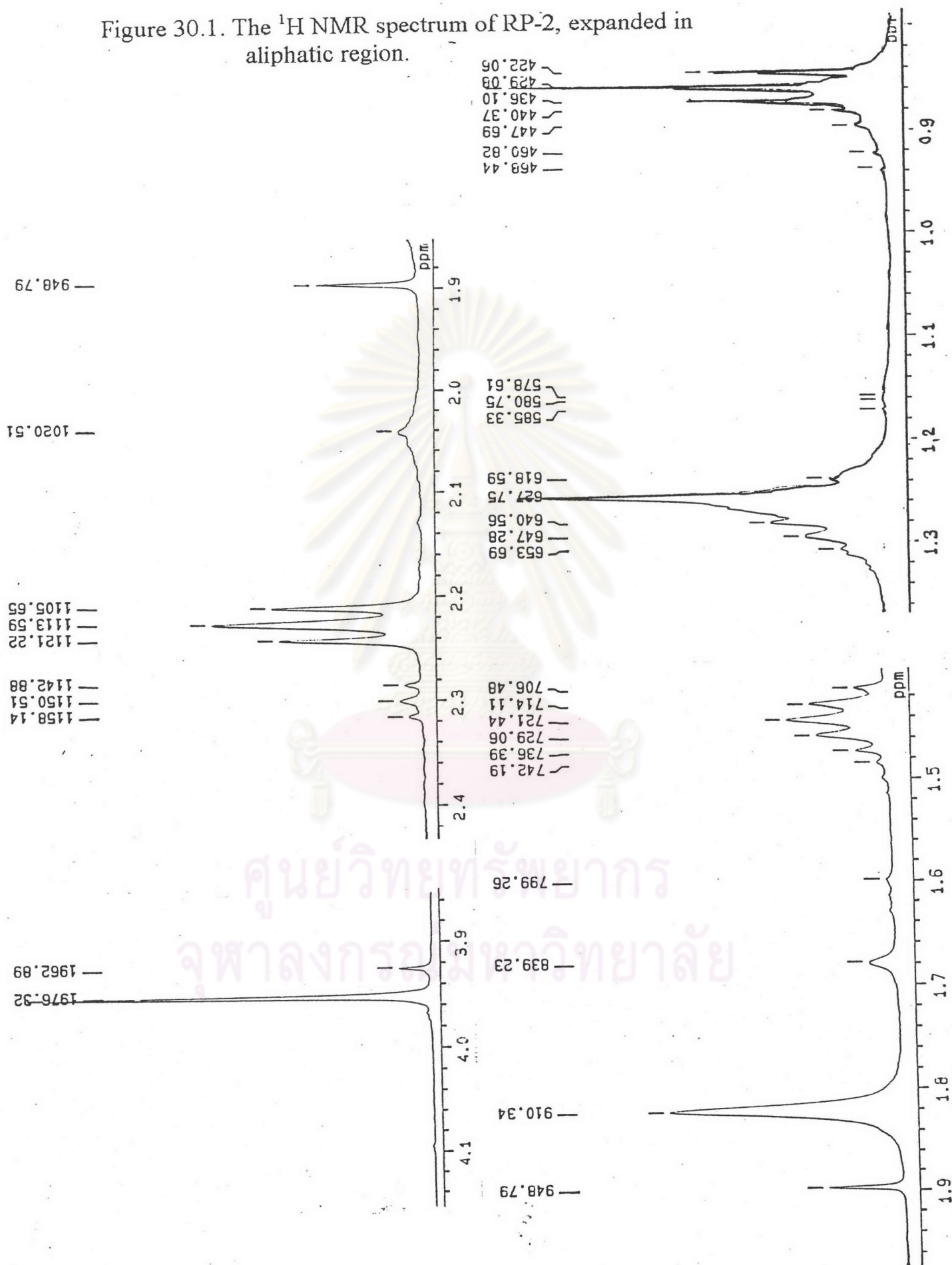


Figure 30.1. The ¹H NMR spectrum of RP-2, expanded in aliphatic region.



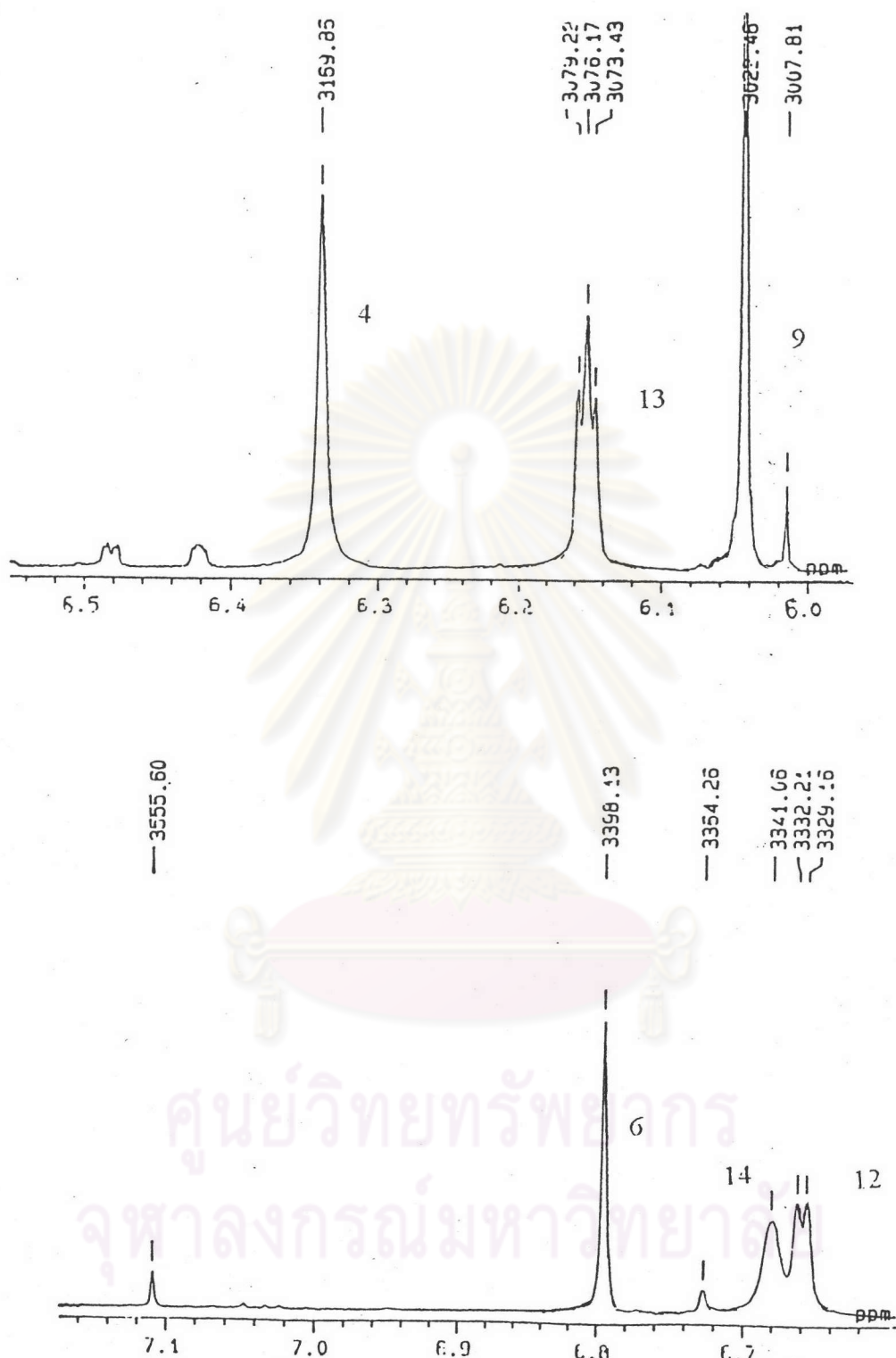


Figure 30.2. The ^1H NMR spectrum of RP-2, expanded in olefinic region.

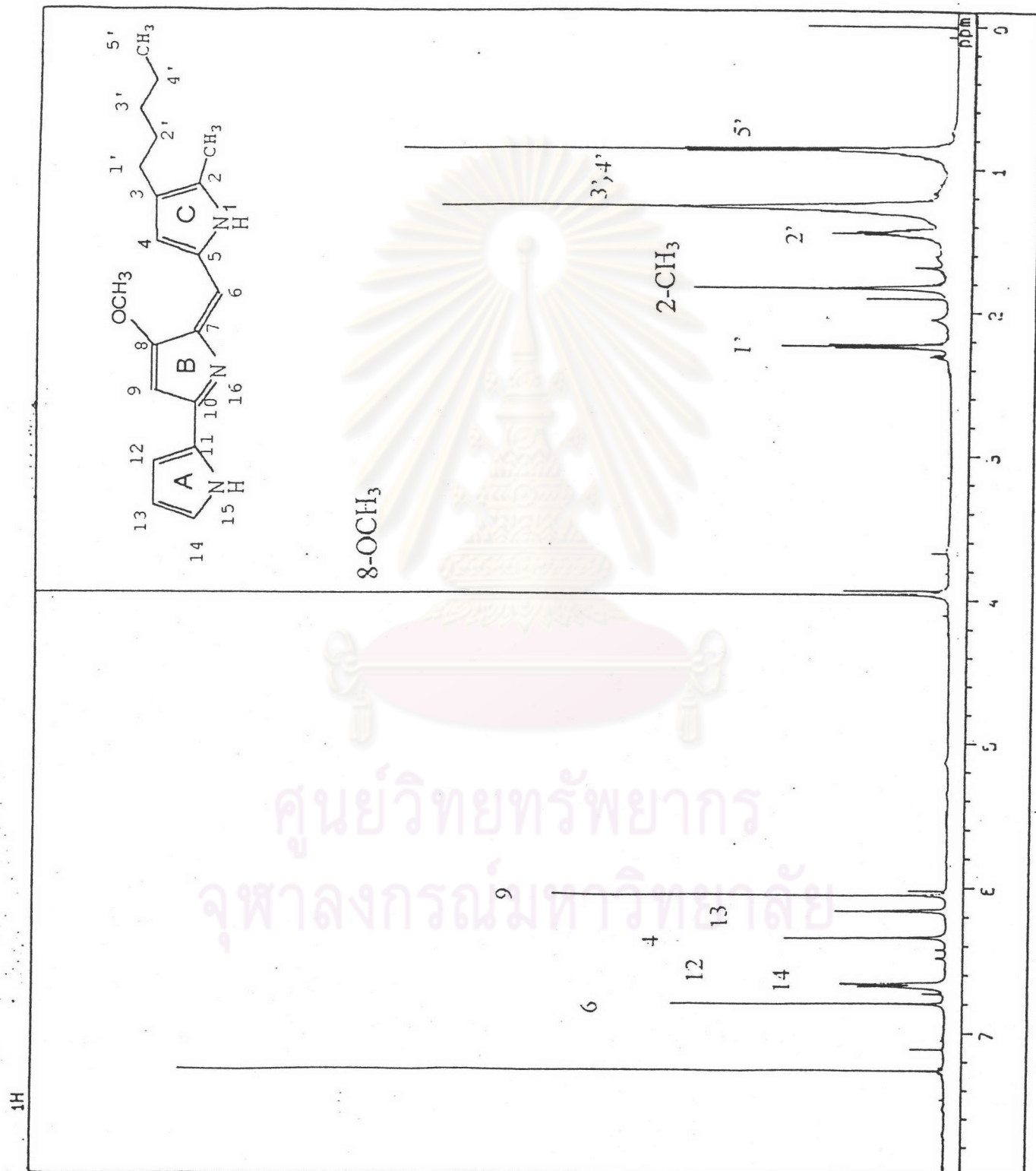
Figure 30.3. The assignment of ^1H NMR spectrum of RP-2.

Figure 31. The H-H COSY spectrum of RP-2.

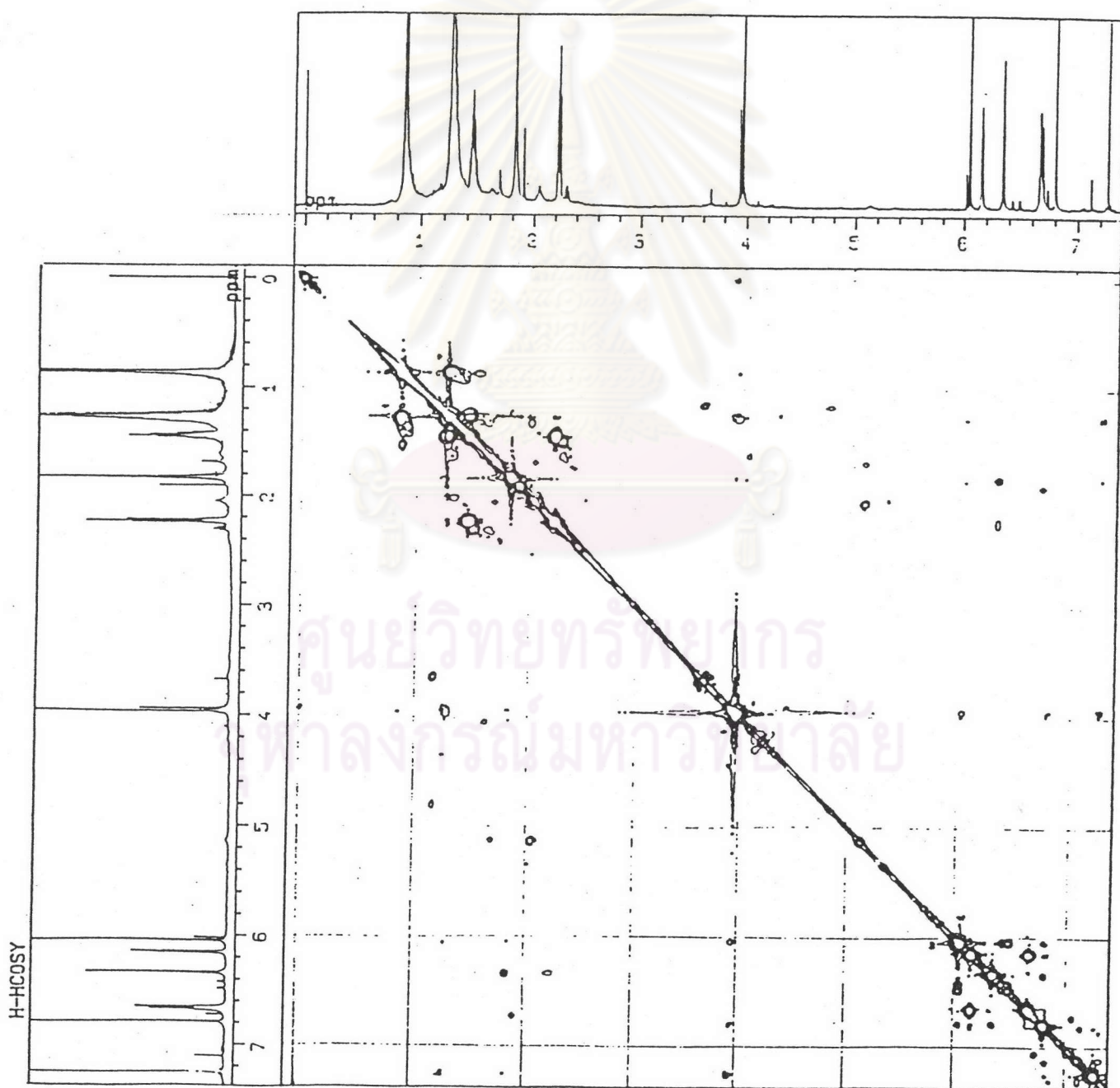


Figure 31.1. The H-H COSY expanded spectrum of RP-1 showing the coupling between signals at δ 1.44 and 2.22 ppm.

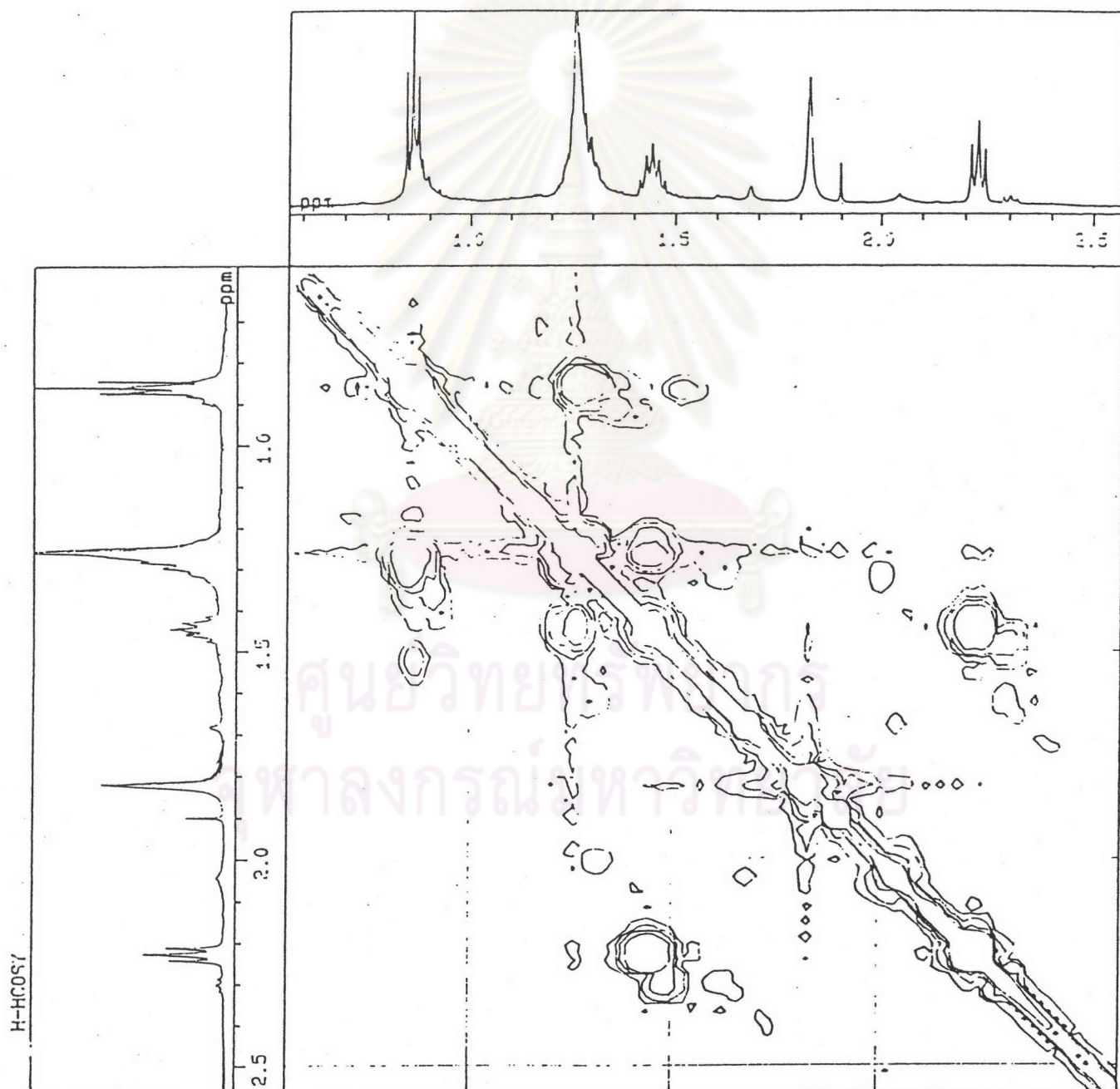


Figure 31.2. The H-H COSY spectrum of RP-2 expanded in the olefinic region.

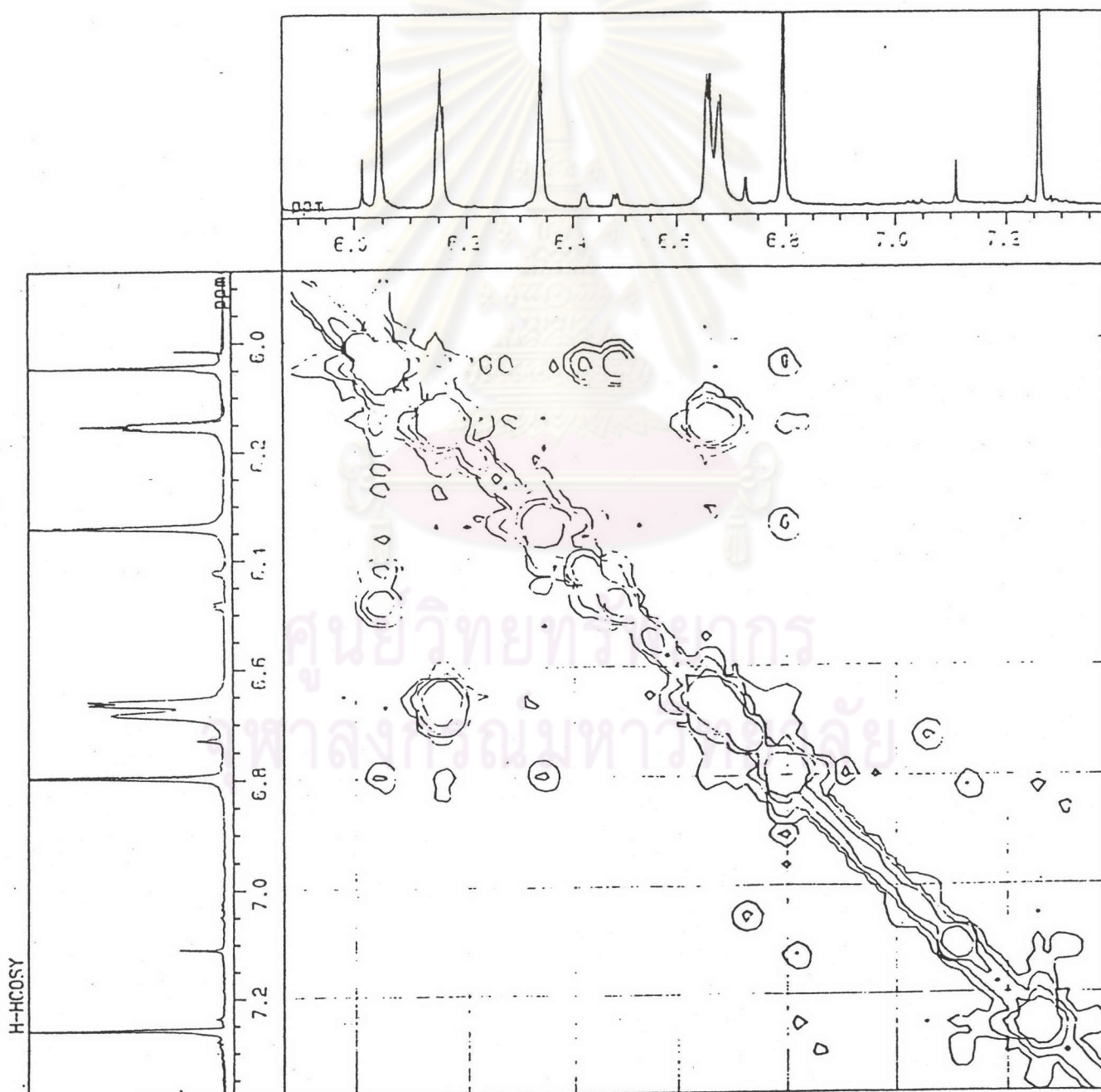


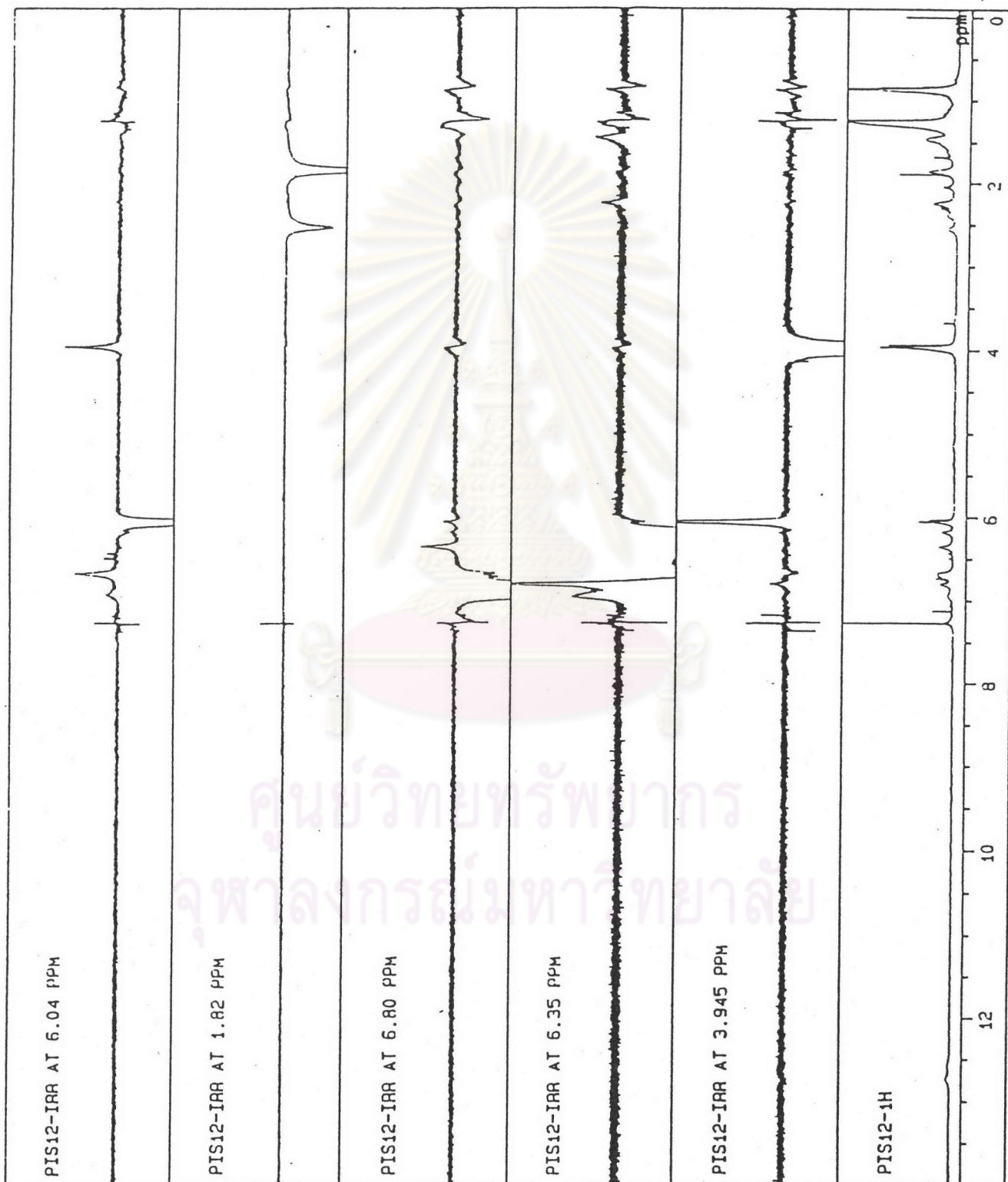
Figure 32. The ^1H NMR spectrum of RP-2 in NOE experiments.

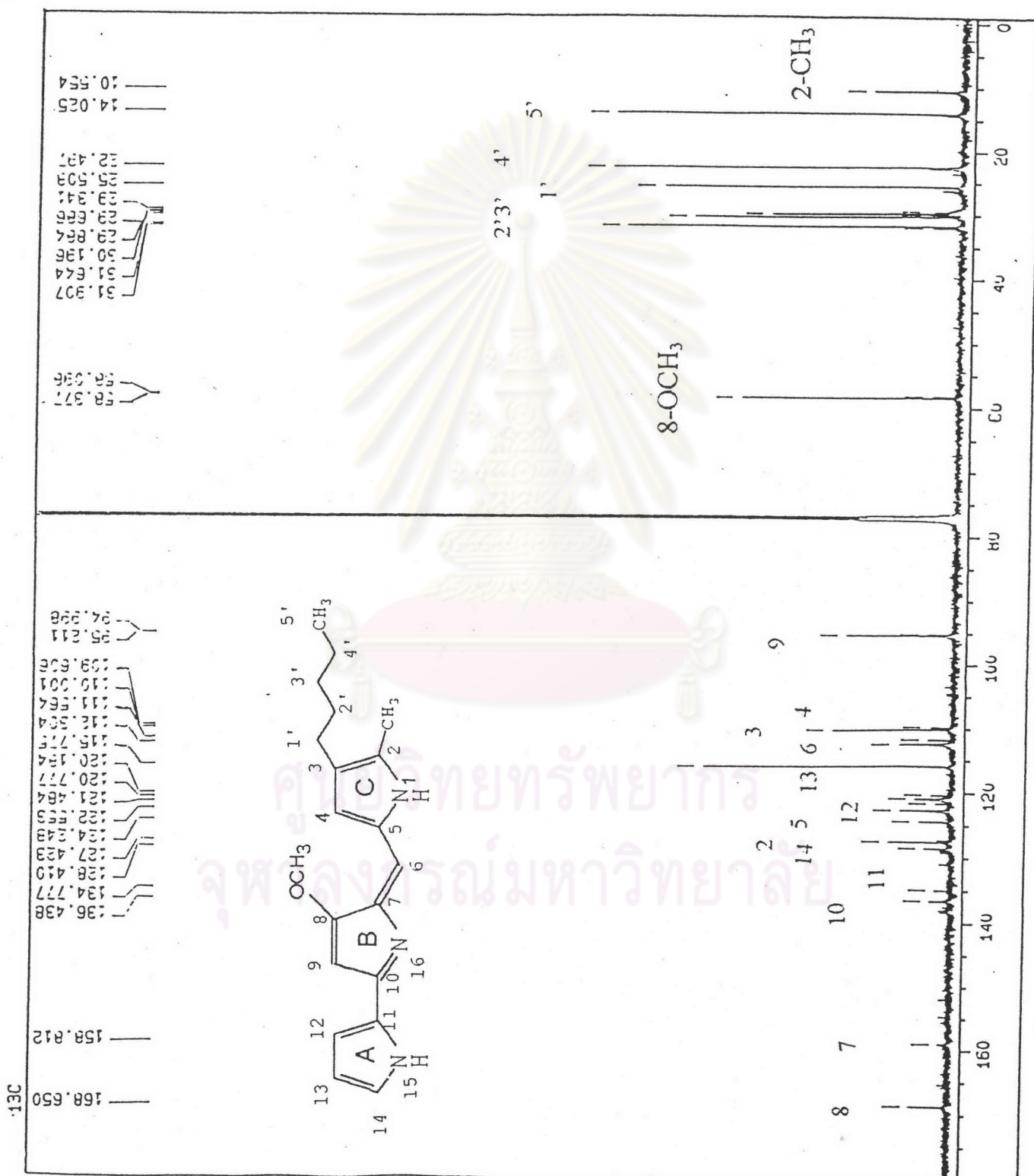
Figure 33. The 125 Mhz ^{13}C NMR spectrum of RP-2.

Figure 34. The DEPT spectrum of RP-2.

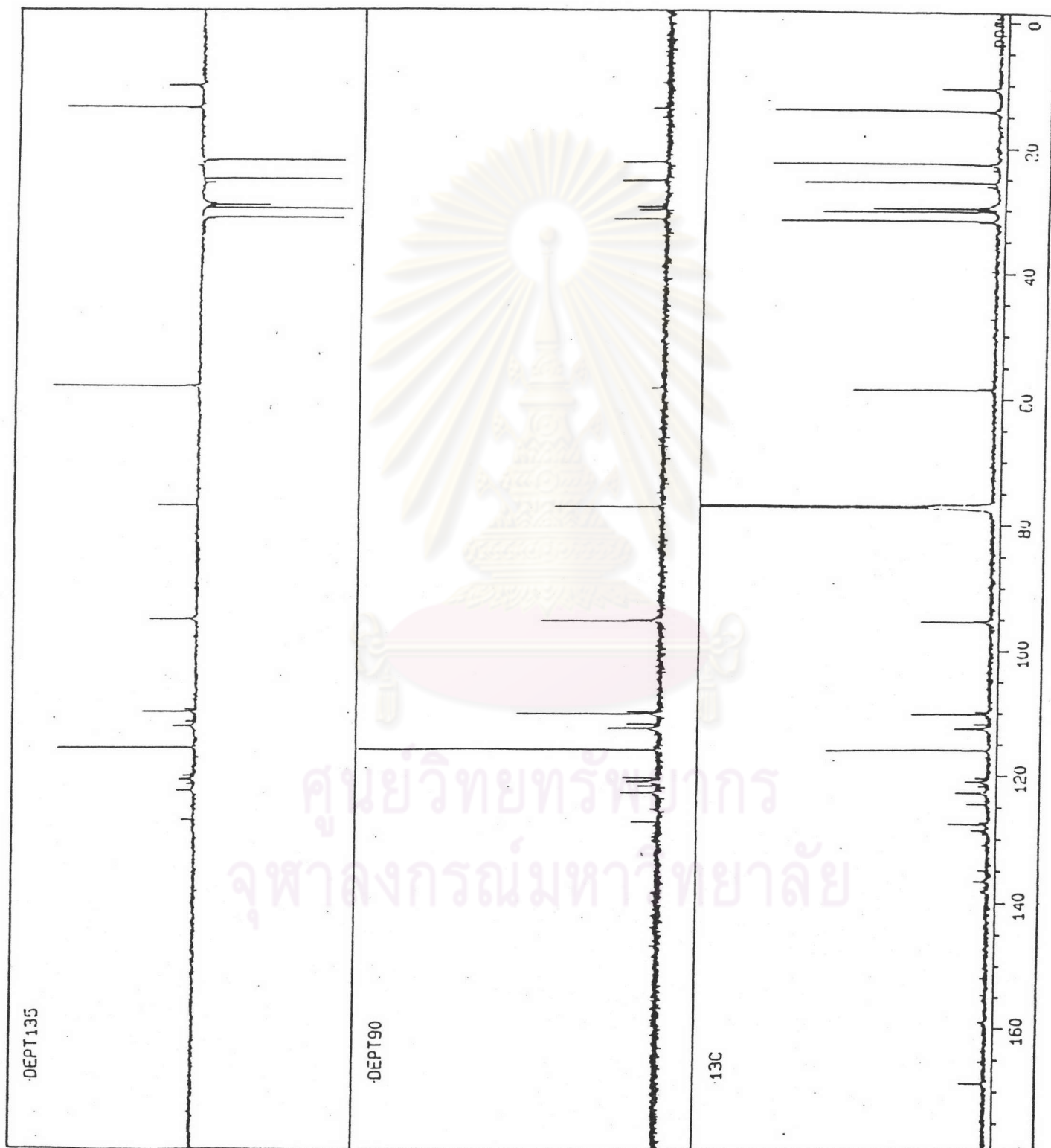


Figure 35. The C-H COSY spectrum of RP-2.

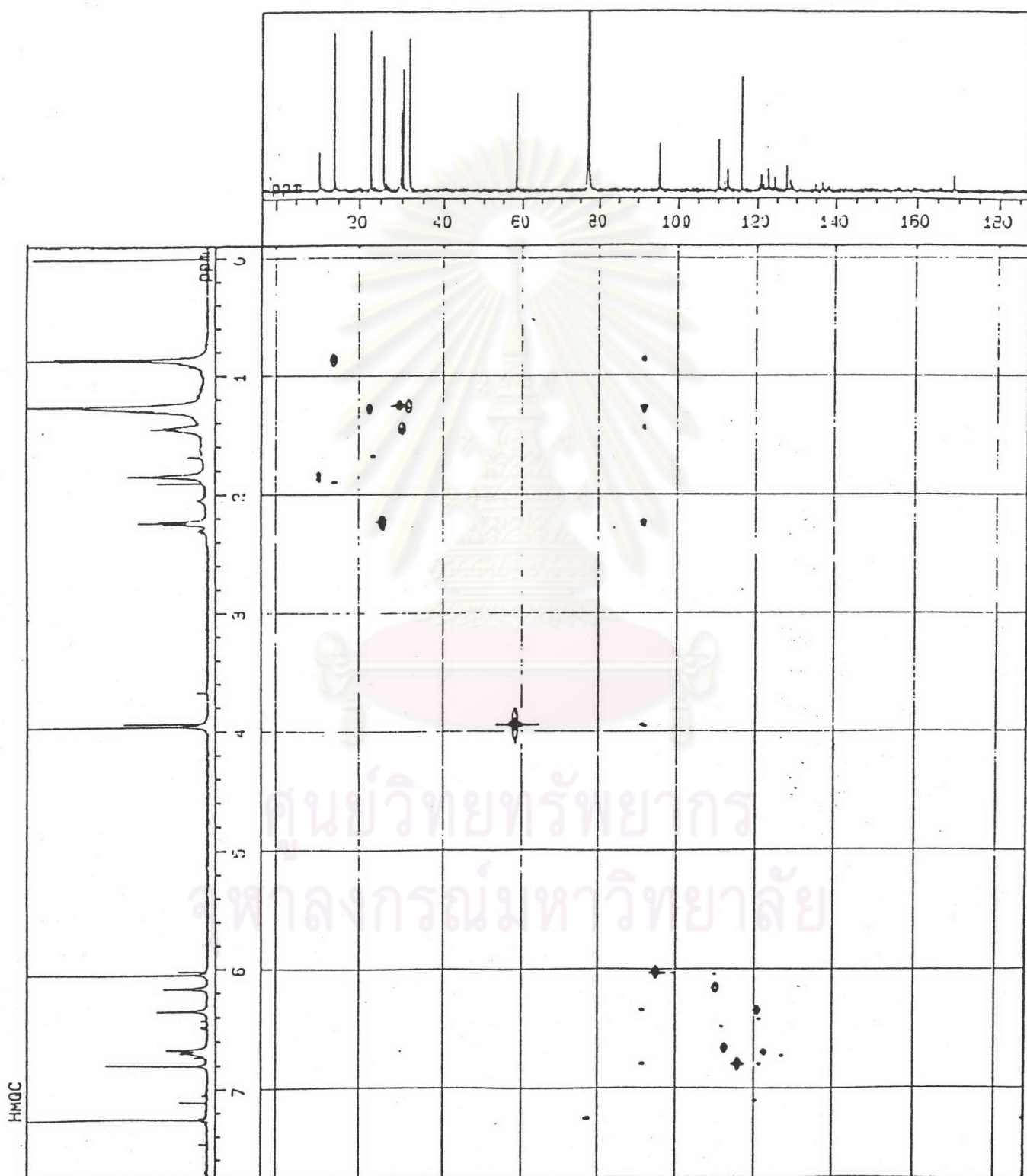


Figure 36. The HMBC spectrum of RP-2.

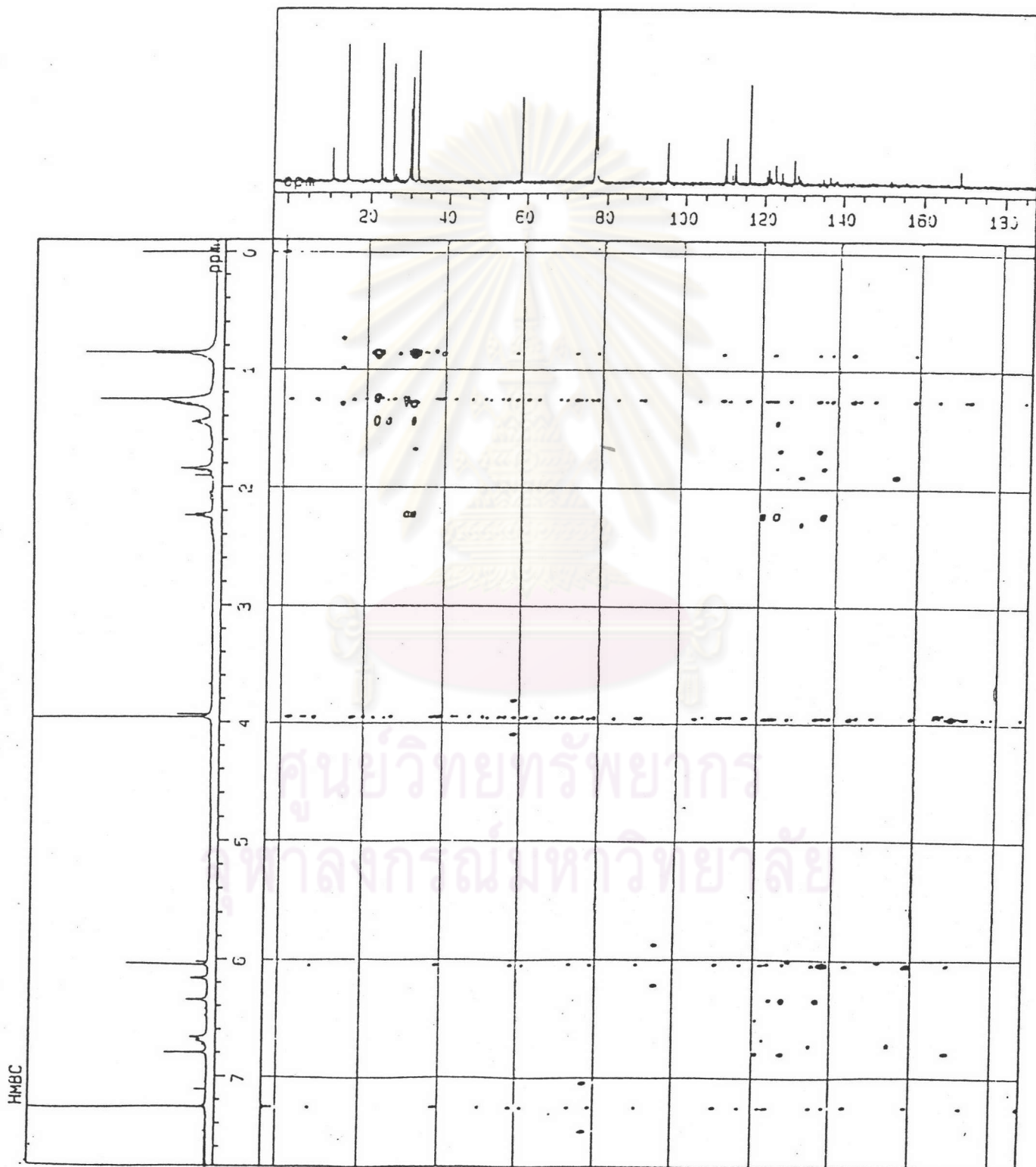


Figure 36.1. Assignment of carbon at 2-, 3- and 4- position in the prodigiosin molecule.

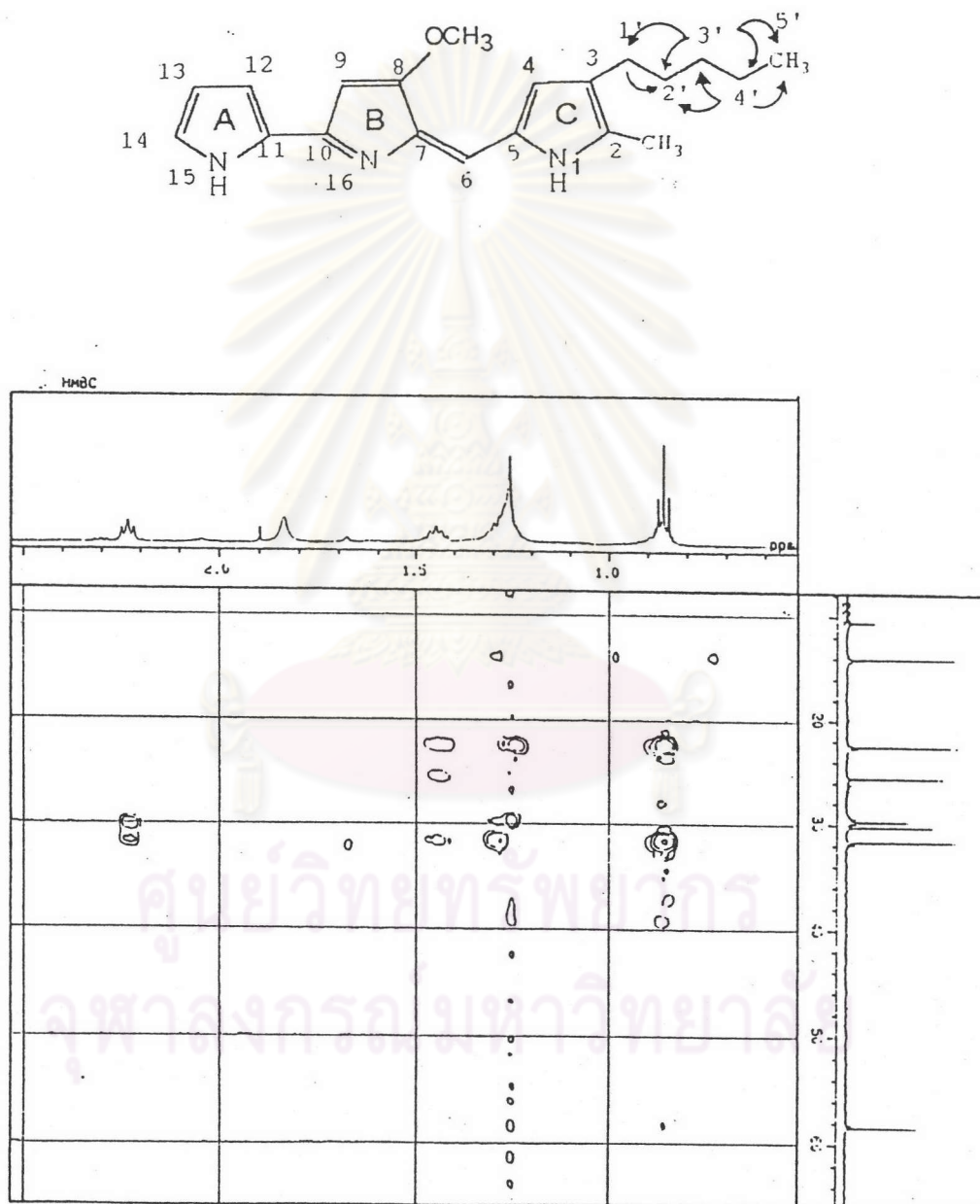


Figure 36.2. Assignment of quarternary carbon at 2-, 3- and 4-position in the prodigiosin molecule.

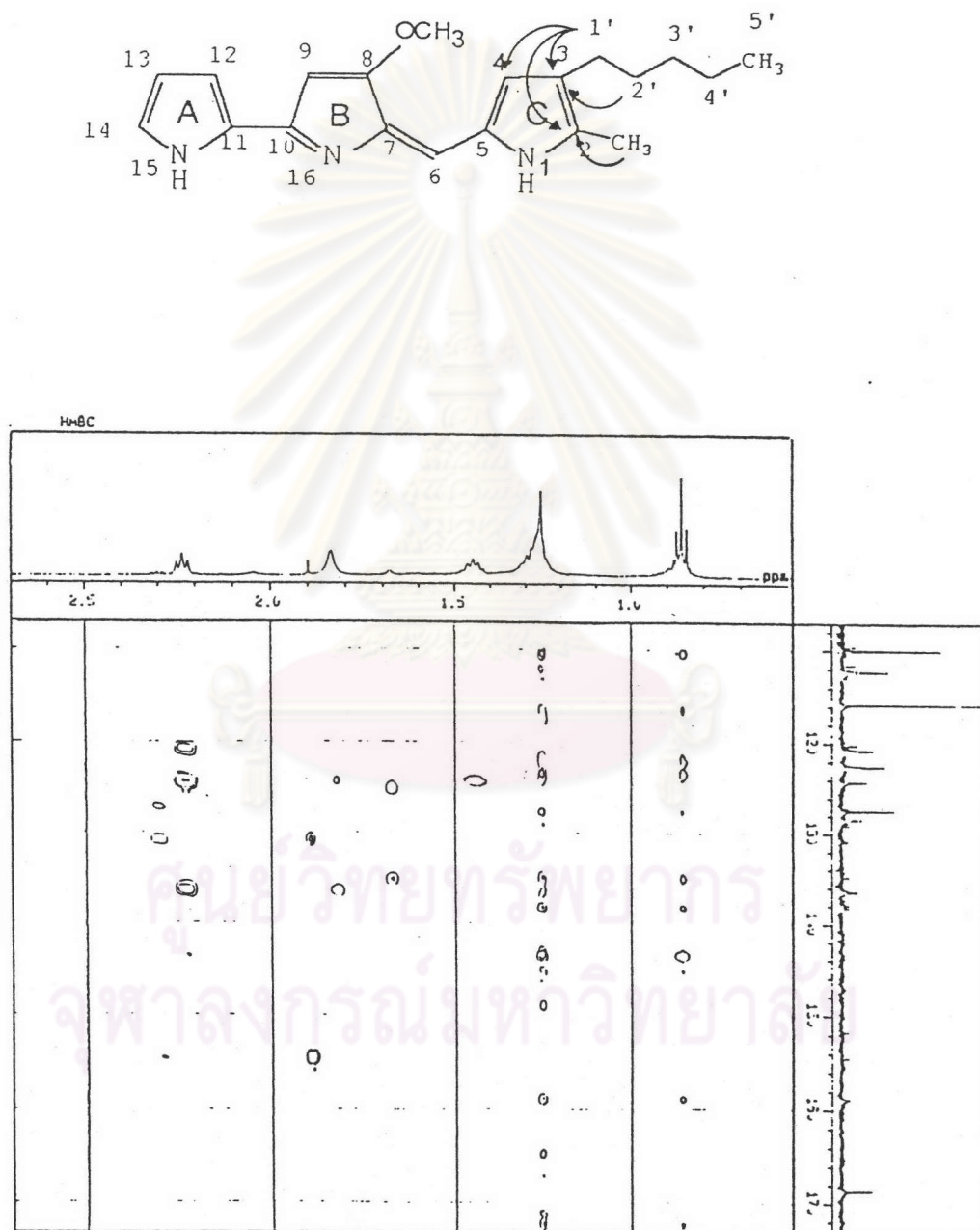


Figure 36.3. Assignment of quaternary carbon at 7-, 8-, and 10-position in the prodigiosin molecule.

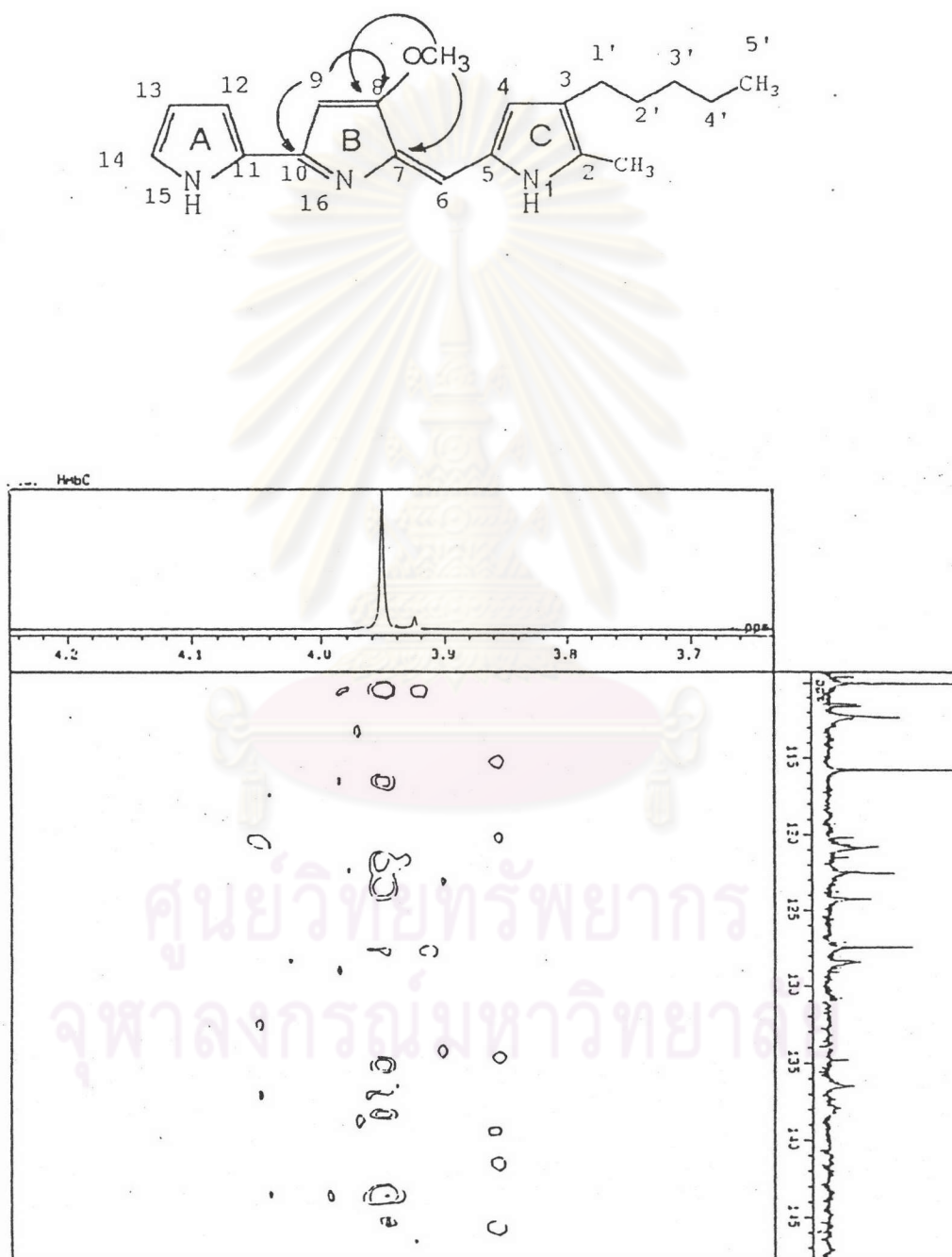


Figure 36.4. Assignment of quarternary carbon at 7- and 8-position in the prodigiosin molecule.

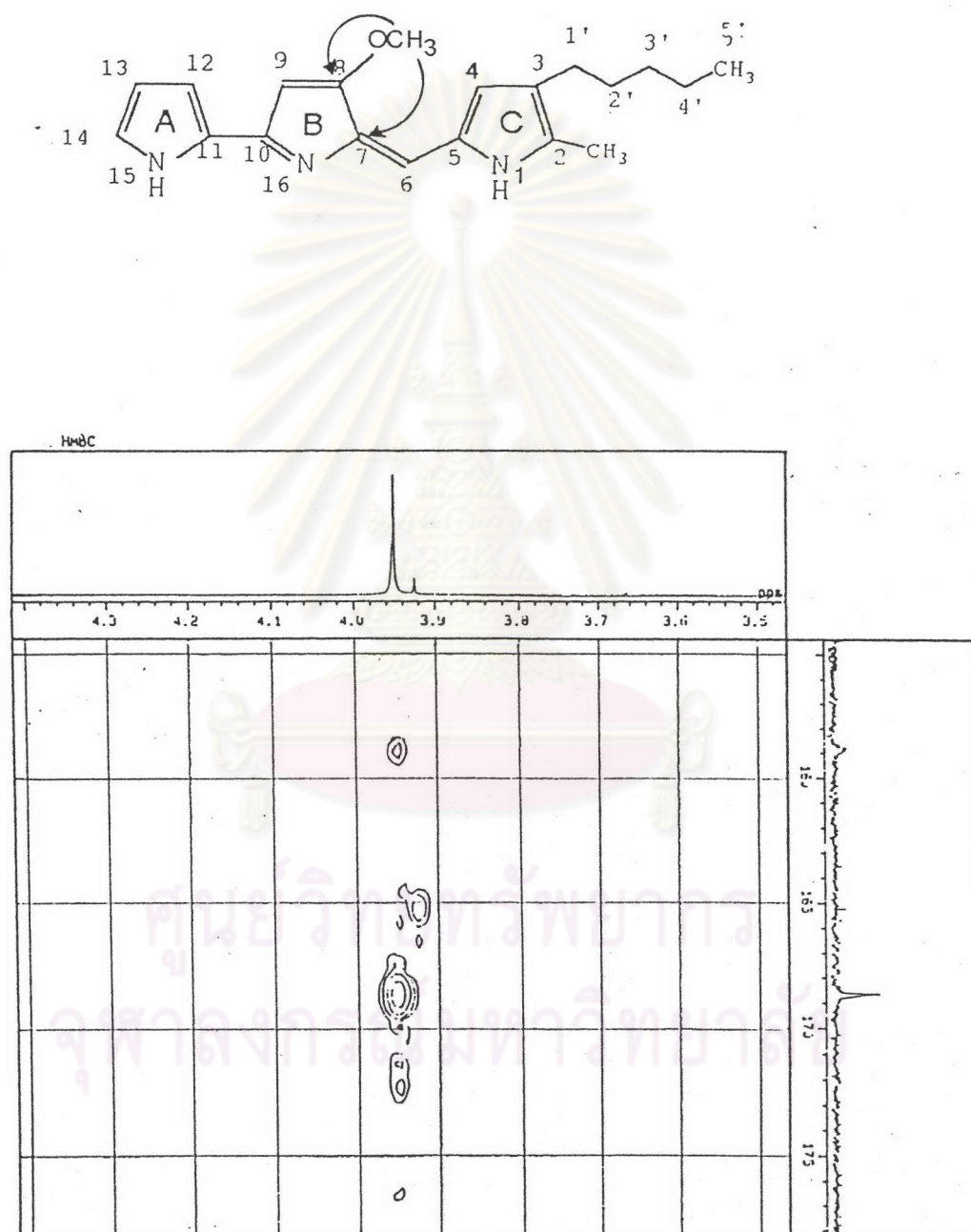
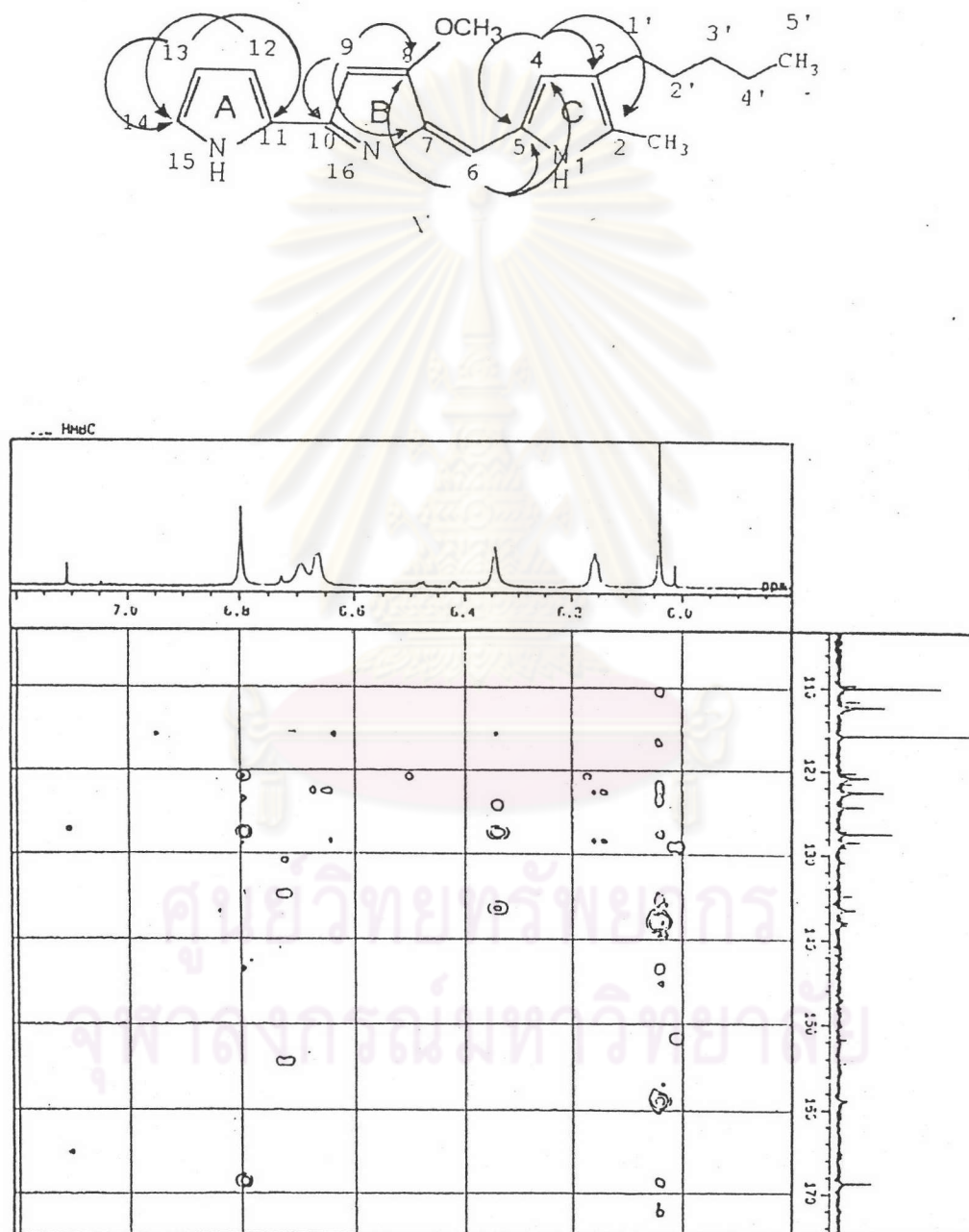


Figure 36.5. Assignment of quaternary carbon at 2-, 3-, 5-, 7-, 8-, 10-, and 11-position in the prodigiosin molecule.



MUTATION EXPERIMENT

Mutant strain of *Zymomonas mobilis* CM 141 producing red pigments was induced to the mutation for the purpose of producing larger amount of red pigments. The mutations were conducted by ultraviolet light and chemicals. An active (24 hours) broth culture of mutant strain of *Zymomonas mobilis* CM 141 was mixed with a mutagen and the reaction was stopped by diluting 1/100 into the YPG broth, incubated at 30° C for 24 hours. The mutants were streaked on the YPG agar and incubated at 30° C for suitable time, the pigments were produced within 24-72 hours under aerobic conditions.

1. Preparation of active broth culture

Pigmented strains of *Zymomonas mobilis* were inoculated on the YPG agar plates and incubated at 30° C for 24 hours. The pure colonies were isolated, inoculated in the YGP broth and incubated at 30° C for 24 hours. Again transferred one loop of the YGP broth culture to another YGP broth and incubated at 30° C for 24 hours. This was the active broth culture.

2. Mutation treatment

2.1 Treatment by ultraviolet light

Placed the active culture broth of the mutant strain of *Zymomonas mobilis* under ultraviolet lamp, stirred with magnetic stirrer all the time for 0, 2, 5, 10, 20 and 30 minutes. Serial ten-fold dilution was done to stop the reaction and the pure colonies were isolated. The mutants were cultured on the YGP agar for the pigments production.

2.2 Treatment by chemicals

2.2.1 N-methyl-N-nitro-N-nitrosoguanidine (NTG)

Mixed well the active culture broth of mutant strain of *Zymomonas mobilis* with NTG solution in the concentration 200 mcg/ml. After 10, 20 and 30 minutes, serial ten-fold dilution was done to stop the reaction and the pure colonies were isolated. The mutants were cultured on the YGP agar for the pigments production.

2.2.2 Hydroxylamine

Mixed well the active culture broth of mutant strain of *Zymomonas mobilis* with 1M hydroxylamine solution and 0.001M EDTA solution. After 10, 20 and 30 minutes, serial ten-fold dilution was done to

stop the reaction and pure colonies were isolated. The mutants were cultured on the YGP agar for the pigments production.

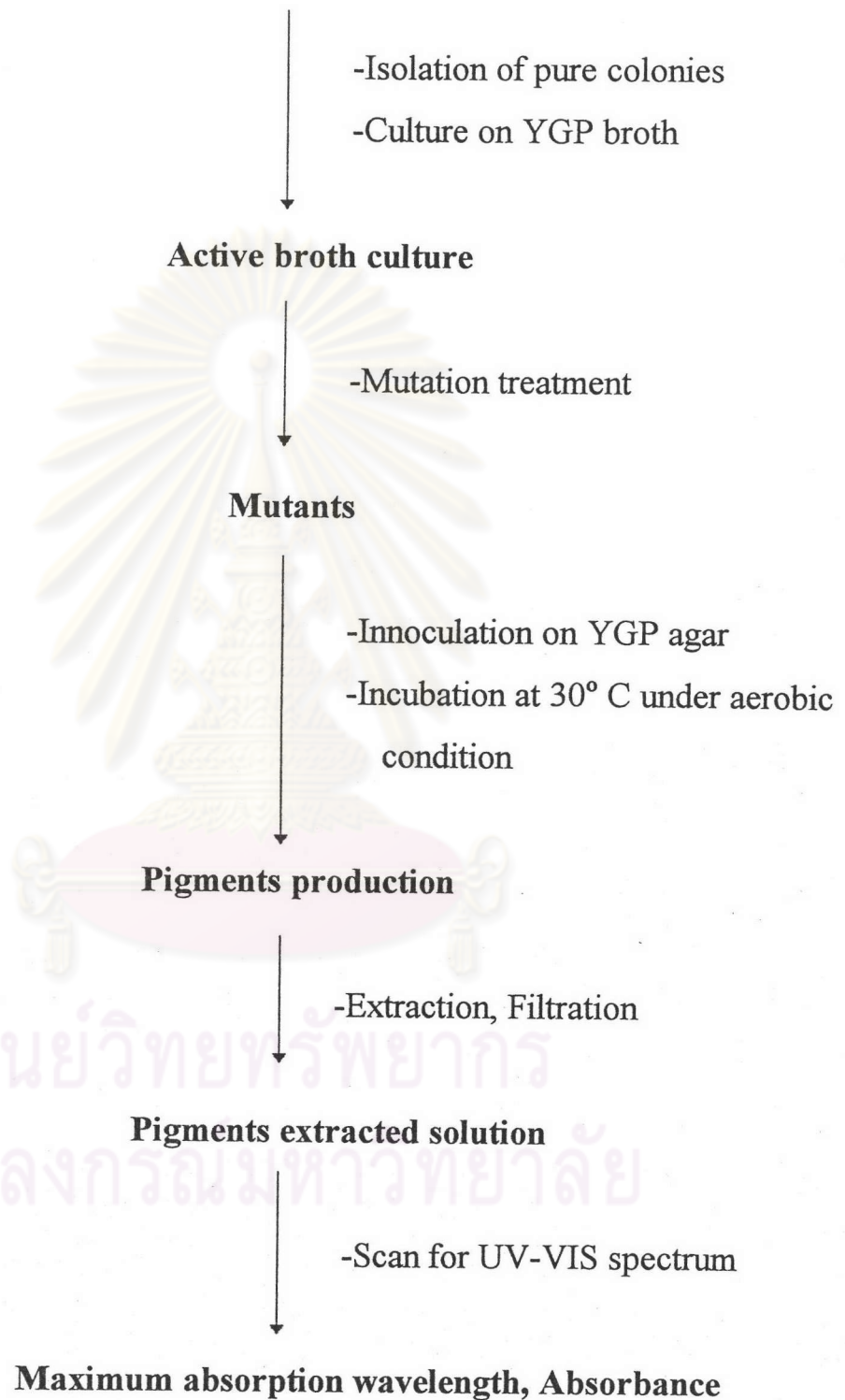
3. Pigment production

Mutant strains were inoculated on the YPG agar medium and incubated at 30° C in the aerobic condition for 24-72 hours. The pigments were produced.

4. Extraction of the pigments

The pigments on the agar plate were extracted with the mixture of methanol : chloroform (1:1) solution and then filtered through sintered glass funnel. The filtrates were scanned for UV-VIS spectrum (200-700 nm). The maximum absorption wavelength and absorbance were recorded.

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Pigmented strains of *Zymomonas mobilis*

Scheme 7. Mutation Experiment.

CHARACTERISTICS OF THE MUTANTS

The mutants produced pigments colonies of the following colors: yellow, pale-orange, deep orange, violet, red with green metallic sheen, pale green, pink and red. These were shown in Table 8.

Table 8. Mutation treatments and Characteristic of the mutant strains.

Treatments	Parent strain	Time of exposure (minutes)	Code	Culture time ^a (hours)	Characteristic size (diameter, mm)	color	
-	CM 141	-	-	24	4-5	red	
UV light	CM 141	10	A ₅	72	2-3	pale yellow	
NTG	CM 141	10	A ₉	72	1-2	dark orange	
			A ₁₁₈	24	4-5	red	
		20	A ₁₀	72	2-3	pale orange	
		A ₇₈	10	A ₁₃₈	48	4-5	violet
			A ₃₂	10	A ₁₄₁	24	4-5
A ₁₄₂	48	4-5			green-violet disappeared after 48 hrs		

Table 8 (cont.). Mutation treatments and characteristic of the mutant strains.

Treatments	Parent strain	Time of exposure (minutes)	Code	Culture time ^a (hours)	Characteristic size (diameter, mm)	color
			A ₁₄₄	24	4-5	pale pink
			A ₁₆₀	24	4-5	red with green metallic sheen
			A ₁₉₆	24	4-5	pale pink
			A ₁₉₈	24	4-5	pale pink
		20	A ₁₃₇	24	4-5	pale pink
			A ₁₄₅	24	4-5	pale red
			A ₁₄₉	48	4-5	violet
			A ₁₅₀	48	4-5	violet
			A ₁₅₁	24	4-5	red
		30	A ₁₄₀	48	4-5	pale green disappeared after 48 hrs
			A ₁₄₆	24	4-5	pink
			A ₁₄₈	24	4-5	pale pink

Table 8 (cont.). Mutation treatments and Characteristic of the mutant strains.

Treatments	Parent strain	Time of exposure (minutes)	Code	Culture time ^a (hours)	Characteristic	
					size (diameter, mm)	color
Hydroxylamine	CM141	10	A ₃₁	24	4-5	pink
			A ₇₇	24	4-5	red with metallic sheen
			A ₇₈	24	4-5	red with metallic sheen
		20	A ₄₈	24	4-5	deep red
			A ₄₉	24	4-5	red with metallic sheen
			A ₁₀₉	24	4-5	red
			A ₁₁₃	24	4-5	red
		30	A ₅₀	24	4-5	deep red
			A ₇₅	24	4-5	pink
			A ₁₀₇	24	4-5	red

Table 8 (cont.). Mutation treatments and Characteristic of the mutant strains.

Treatments	Parent strain	Time of exposure (minutes)	Code	Culture time ^a (hours)	Characteristic	
					size (diameter, mm)	color
A ₇₈		10	A ₁₈₆	24	4-5	red with metallic sheen
		30	A ₁₆₂	24	4-5	red
A ₃₂		10	A ₁₆₄	24	4-5	pale pink
			A ₁₇₂	48	4-5	orange
			A ₁₈₂	24	4-5	red
			A ₁₈₇	24	4-5	pale pink
		20	A ₁₆₉	24	3	pale yellow
			A ₁₈₃	24	4-5	red with metallic sheen
			A ₁₉₄	24	4-5	pale pink
			A ₁₉₅	24	4-5	pink
	30	A ₁₆₃	24	4-5	pale pink	
		A ₁₆₆	24	4-5	pale pink	

Table 8 (cont.) Mutation treatments and Characteristic of the mutant strains.

Treatments	Parent strain	Time of exposure (minutes)	Code	Culture time ^a (hours)	Characteristic size (diameter, mm)	color
			A ₁₇₆	24	4-5	red with metallic sheen
			A ₁₈₄	24	4-5	red
			A ₁₈₅	48	4-5	violet
			A ₁₈₈	24	4-5	pink
			A ₁₈₉	24	4-5	pale pink
			A ₁₉₁	24	4-5	pale pink
			A ₁₉₃	24	4-5	pale pink
Hydrxylamine ^b	A ₉	10	A ₃₅	72	2-3	deep orange
		20	A ₃₂	72	2-3	deep orange
			A ₃₄	72	2-3	deep orange
		30	A ₂₉	72	4-5	pale orange
		30	A ₃₃	72	2-3	deep orange

^a culture time for pigmentation

^b 0.5 M hydroxylamine solution

UV-VIS SPECTRUM OF EXTRACTED PIGMENT SOLUTION

The mutants produced pigments colonies of the following colors: yellow, pale-orange, deep orange, violet, red with green metallic sheen, pale green, pink and red. The pigments were extracted by solvent of methanol and chloroform (1:1) mixture. The extracted pigments solution were measured the absorbance at the maximum absorption wavelength with Spectrophotometer UV 2100 (Table 9.).

Table 9. Absorbance of the extracted pigment solution at maximum absorption wavelength.

Code	Maximun absorption wavelength (nm)	Absorbance
CM141	530	5.00
A ₅	290	0.75
A ₉	530	2.15
A ₁₀	290	0.60
A ₂₉	290	0.80
A ₃₁	530	4.45
A ₃₂	475	1.40
A ₃₃	280	6.50

Table 9 (cont.). Absorbance of the extracted pigment solution
at maximum absorption wavelength.

Code	Maximun absorption wavelength (nm)	Absorbance
A ₃₄	485	1.80
A ₃₅	290	1.10
A ₄₆	530	5.00
A ₄₉	522	4.80
A ₅₀	525	4.80
A ₇₅	522	4.50
A ₇₇	530	3.70
A ₇₈	535	2.95
A ₁₀₇	530	4.05
A ₁₀₉	500	6.50
A ₁₁₃	528	9.00
A ₁₁₆	525	5.00
A ₁₃₆	540	0.95
A ₁₃₇	287	0.40
A ₁₄₀	470	0.60
A ₁₄₁	535	3.95
A ₁₄₂	470	2.15
A ₁₄₄	290	0.80

Table 9 (cont.). Absorbance of the extracted pigment solution
at maximum absorption wavelength.

Code	Maximun absorption wavelength (nm)	Absorbance
A ₁₄₅	537	1.50
A ₁₄₆	290	0.95
A ₁₄₈	290	0.50
A ₁₄₉	290	1.40
A ₁₅₀	290	0.85
A ₁₅₁	537	2.62
A ₁₆₀	535	1.40
A ₁₆₂	530	4.50
A ₁₆₃	287	0.40
A ₁₆₄	287	0.50
A ₁₆₆	290	0.90
A ₁₆₉	290	0.75
A ₁₇₂	475	1.00
A ₁₇₆	535	3.40
A ₁₈₂	535	2.25
A ₁₈₃	530	6.50
A ₁₈₄	535	2.65
A ₁₈₅	290	0.45

Table 9 (cont.). Absorbance of the extracted pigment solution
at maximum absorption wavelength.

Code	Maximun absorption wavelength (nm)	Absorbance
A ₁₈₆	525	5.00
A ₁₈₇	535	1.10
A ₁₈₈	290	0.65
A ₁₈₉	285	0.60
A ₁₉₁	285	0.70
A ₁₉₃	285	0.45
A ₁₉₄	290	1.10
A ₁₉₅	525	5.00
A ₁₉₆	290	0.80
A ₁₉₈	290	1.50

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