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APPENDIX I

A. Michaelis - Menten Equation

For many enzymes, the rate of catalysis V varies with the substrate concentration [S]. At a fixed concentration of the enzyme, V is nearly linearly proportional to [S], when the substrate concentration is small. At high substrate concentration, V is nearly independent of substrate concentration. In 1913, Leonor Michaelis and Maud Menten, proposed a simple model to account for these kinetic observations. The Michaelis-Menten equation is;

$$V = \frac{V_{max}[S]}{K_m + [S]} \qquad (1)$$

where V = The initial velocity of the reaction

V_{max} = The maximal velocity obtained at high substrate concentration

$[S]$ = Initial substrate concentration

K_m = The Michaelis-Menten constant

The Michaelis-Menten constant (K_m) is the substrate concentration at half its maximal velocity. K_m can also be expressed as the equilibrium constant of the reversible combination of an enzyme with its substrate.

It is convenient to transform the Michaelis-Menten equation into one that gives a straight line plot. This can be done by taking the reciprocal of both sides of equation (1), to give,

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \frac{x \quad 1}{[S]} \qquad (2)$$

This equation is known as the Lineweaver-Burk plot.

A plot of 1/V versus 1/[S], yields a straight line, with a slope of K_m/V_{max} and with an intercept on the y-axis of $1/V_{max}$ and an intercept on the x-axis of $-1/K_m$ (Fig. 1).

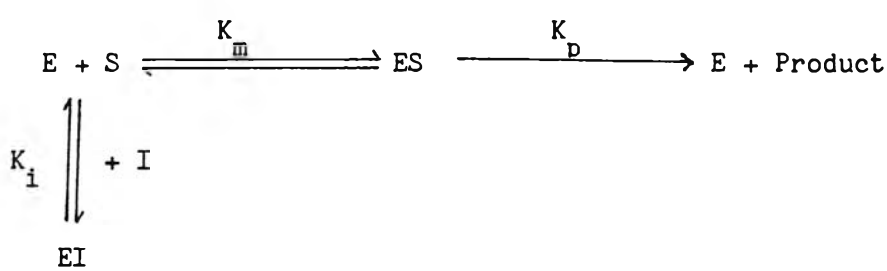
B. Determination of Enzyme-Inhibitor Dissociation Constants

The inhibition of an enzyme can provide an insight into the mechanism of enzyme activity. Enzyme inhibition may be either a reversible or irreversible process. In irreversible inhibition, the inhibitor is covalently bound to the enzyme or bound so tightly that its dissociation from the enzyme is very slow. Reversible inhibition on the other hand is characterized by a rapid equilibrium of the inhibitor and enzyme. Only reversible inhibition will be discussed here.

There are three simple types of inhibition mechanism; competitive, non-competitive and uncompetitive.

a. Competitive Inhibition

This inhibition assumes that both substrate and inhibitor compete for the same active site of the enzyme. The reactions involved are expressed by,



where K_i is the dissociation constant of the enzyme-inhibitor complex, EI.

K_p is the specific rate of chemical reaction of ES

There are various forms of competitive inhibition;

1. Classical model where substrate (S) and inhibitor (I) compete for the same binding site. In this situation, I must resemble S structurally.

2. I and S are mutually exclusive because of steric hindrance.

3. I and S share a common binding region on the enzyme.

4. The binding site for I and S are distinct, but overlapping.

5. Binding of I to a distinct inhibitor site causes a conformational change in the enzyme that distorts or masks the substrate binding site.

The Michaelis-Menten equation for competitve inhibition becomes;

$$V = \frac{V_{max}\,[S]}{K_m(1+[I]/K_i) + [S]} \qquad (3)$$

based on the Lineweaver-Burk plot, equation 3, is transformed to;

$$\frac{1}{V} = \frac{K_m}{V_{max}} \times \frac{(1 + [I]/K_i)}{[S]} + \frac{1}{V_{max}} \qquad (4)$$

A plot of 1/V versus 1/[S] (Fig. 2) shows that V_{max} is essentially constant and K_m steadily increases. The increase in K_m is because at any concentration of inhibitor, a portion of the enzyme is in the

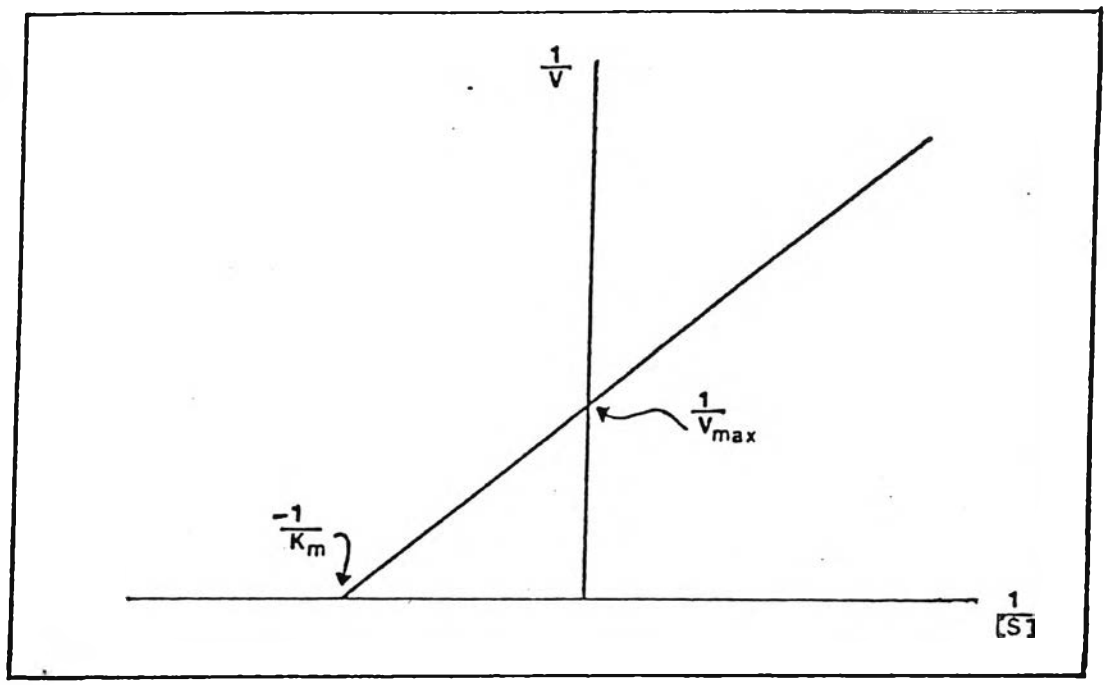


Fig.I.1 Lineweaver-Burk plot.

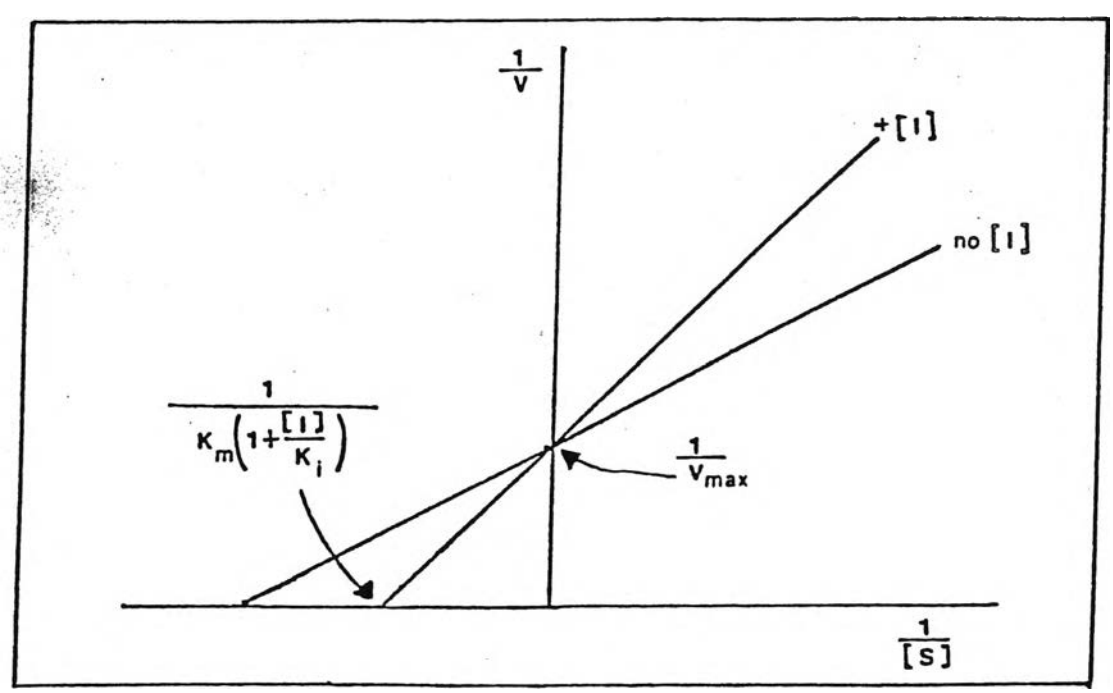


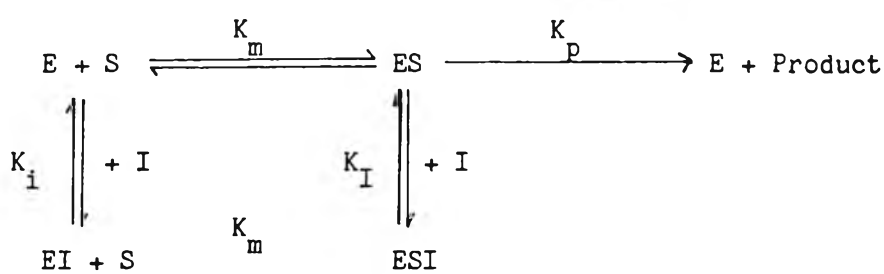
Fig.I.2 Lineweaver-Burk plots of competitive inhibition.

inactive EI form which has no affinity for S. V_{max} remains constant because all the enzyme is converted to the ES form.

The dissociation constant (K_i) of the enzyme-inhibitor complex can be determined from secondary plots, of the primary double reciprocal plot versus inhibitor concentration (Fig. 3). A straight line is obtained which extrapolates to the x-axis. The intercept on the x-axis is the K_i value of the inhibitor. K_i is expressed in concentration units.

b. Non-Competitive Inhibition

A second type of inhibition which is identified on the basis of kinetic analysis is non-competitive inhibition. A classical non-competitive inhibitor has no effect on substrate binding. The inhibitor and substrate bind reversibly, randomly and independently at different sites on the enzyme. All of the reactions taking place are summarized by,



For the sake of simplicity, it is assumed that K_i and K_I are the dissociation of inhibitor from EI and ESI respectively. The Michaelis-Menten equation which describes the velocity of this reaction is

$$V = \frac{(V_{max}[S])/(1 + [I]/K_i)}{K_m + [S]} \qquad (5)$$

The Lineweaver-Burk transformation of this equation is;

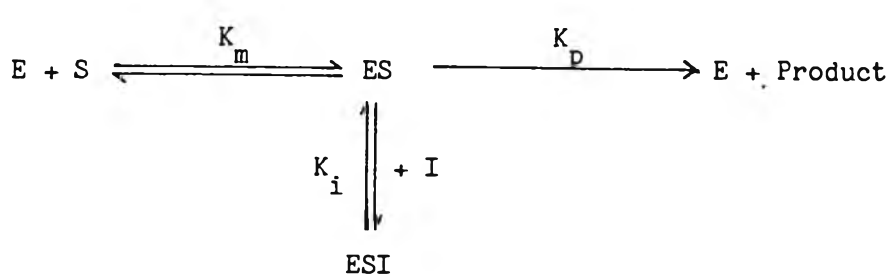
$$\frac{1}{V} = \frac{K_m(1 + [I]/K_i)}{V_{max} \times [S]} + \frac{(1 + [I]/K_i)}{V_{max}} \qquad (6)$$

A plot of 1/V versus 1/[S] (Fig. 4), shows that V_{max} is reduced. This is because the ESI complex is inactive, hence some of the enzyme is tied up as the inactive form. The independent binding of I and S results in an unchanged K_m.

The dissociation constant of the enzyme-inhibitor complex for non-competitive inhibitors can be determined from the previously described secondary plot for competitive inhibitors (Fig.3). Another method of determining the dissociation constant for non-competitive inhibitors is from a secondary plot, of the intercept of the primary double reciprocal plot (y-axis) versus inhibitor concentration (Fig. 5).

c. Uncompetitive Inhibition

The third type of inhibition is uncompetitive. A classical uncompetitive inhibitor reversibly binds only to the ES complex, resulting in an inactive ESI complex. The reactions which take place are,



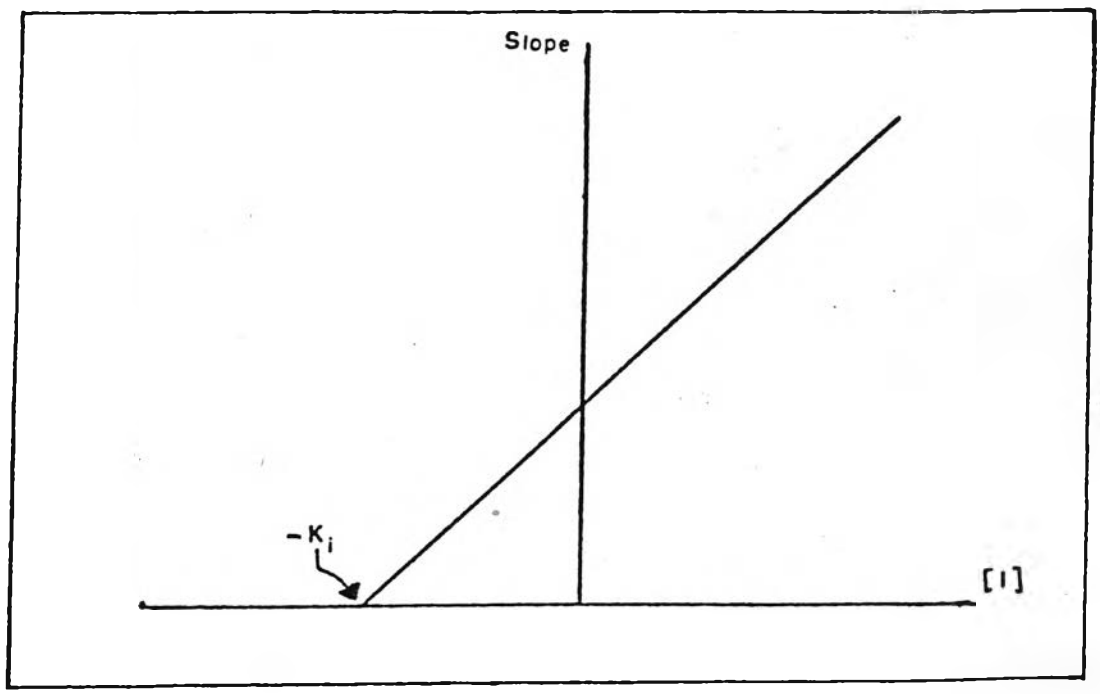


Fig.I.3 Plot of slope (from Lineweaver-Burk plot) versus inhibitor concentration.

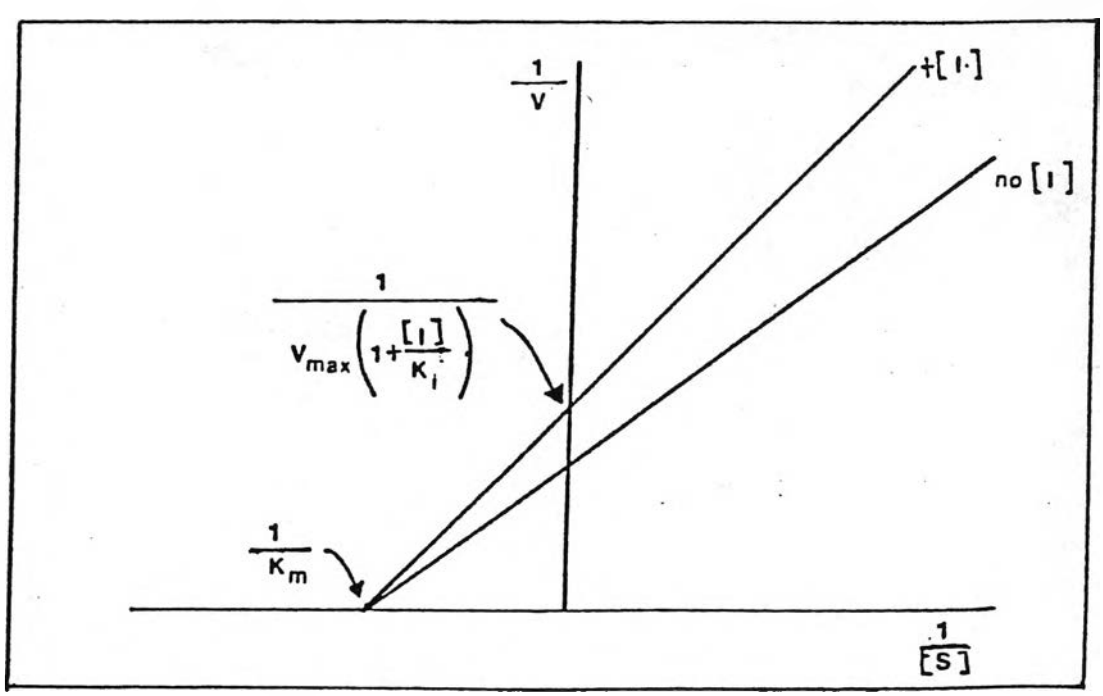


Fig.I.4 Lineweaver-Burk plots of non-competitive inhibition.

The Michaelis-Menten equation for uncompetitive inhibition is;

$$V = \frac{V_{max}[S]/(1 + [I]/K_i)}{K_m/ (1 + [I]/K_i) + [S]} \qquad (7)$$

The Lineweaver-Burk transformation of this equation is;

$$\frac{1}{V} = \frac{K_m (1/[S])}{V_{max}} + \frac{(1 + [I]/K_i)}{V_{max}} \qquad (8)$$

A plot of 1/V versus 1/[S], for uncompetitive inhibition, gives parallel lines (Fig. 6). From the plot, it is evident that both V_{max} and K_m are effected.

The dissociation constant of the enzyme-inhibitor complex (K_i) for uncompetitive inhibitors can be determined from the secondary plots of the intercept of the primary double reciprocal plot (y-axis) versus inhibitor concentration (Fig. 5), previously described for non-competitive inhibition.

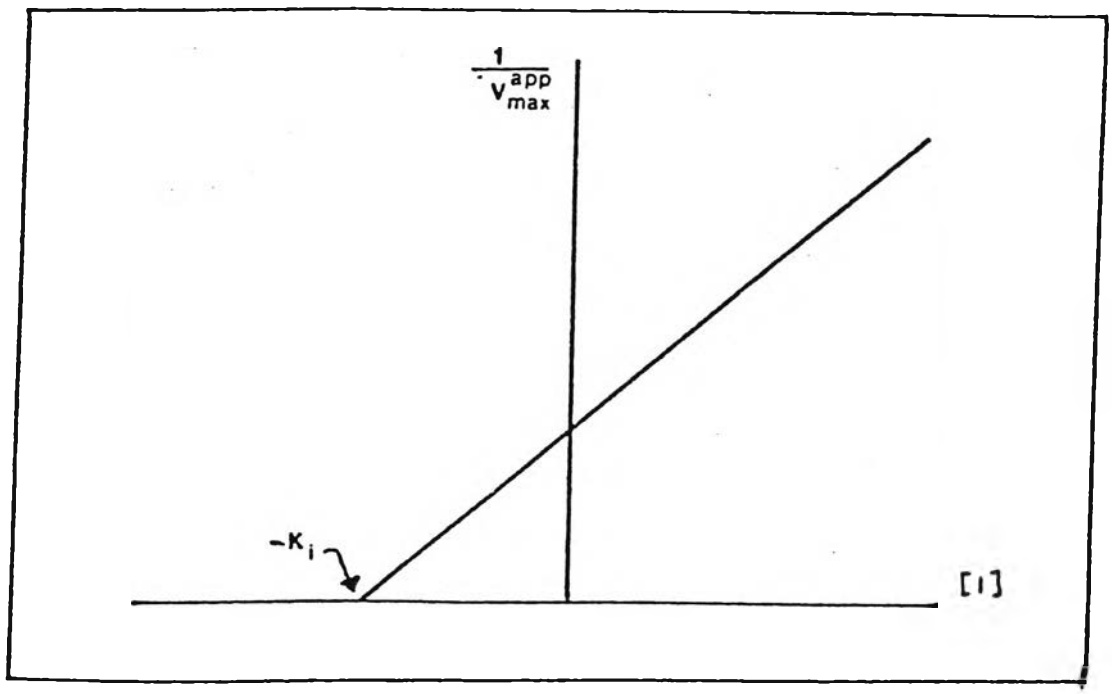


Fig.I.5 Plot of $1/V_{max}$ (from Lineweaver-Burk plot) versus inhibitor concentration.

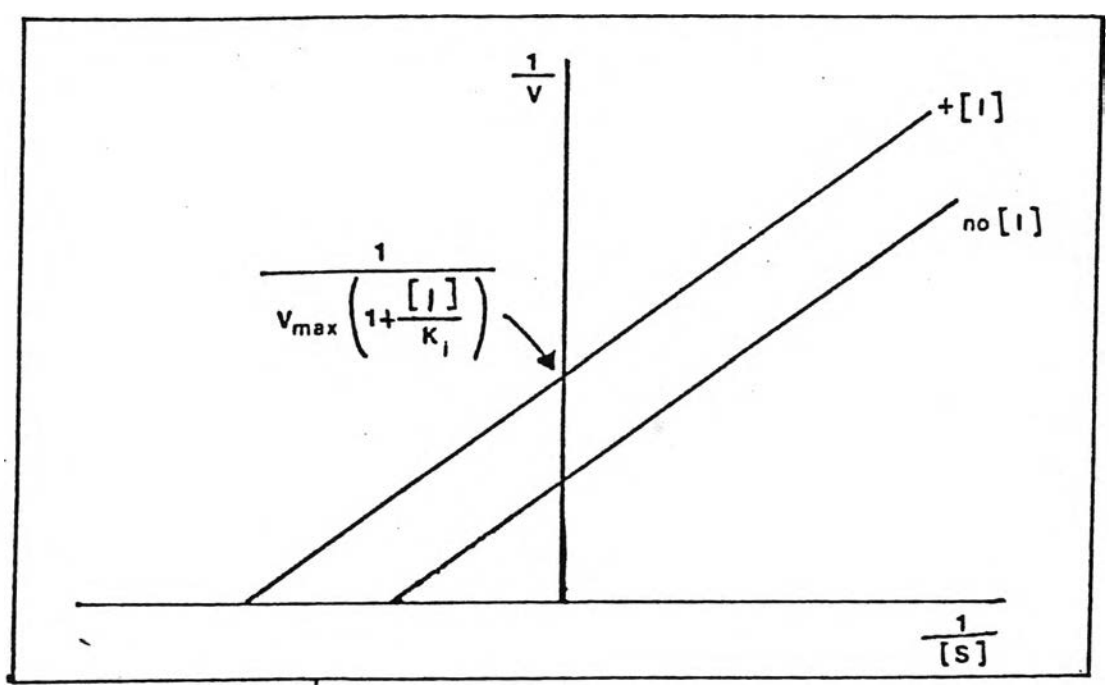


Fig.I.6 Lineweaver-Burk plots of uncompetitive inhibition.

APPENDIX II

DATA FOR THE DETERMINATION OF TYPES OF INHIBITION

Table II.1 Lineweaver-Burk data of compound I (chymotrypsin)

[S] mM	1/[S]	V	1/V	Regression analysis
[I] = 0				
0.10	10.00	0.0627	15.95	slope (K_m/V_{max}) = 0.7369
0.08	12.50	0.0577	17.33	x = -11.8539
0.06	16.67	0.0458	21.83	y = 8.7353
0.04	25.00	0.0367	27.25	correlation coefficient = 0.9991
0.02	50.00	0.0220	45.45	
[I] = 7 μM				
0.10	10.00	0.0500	20.00	slope (K_m/V_{max}) = 1.1200
0.08	12.50	0.0461	21.69	x = -7.1758
0.06	16.67	0.0384	26.04	y = 8.0371
0.04	25.00	0.0276	36.23	correlation coefficient = 0.9996
0.02	50.00	0.0156	64.10	
[I] = 8.4 μM				
0.10	10.00	0.0484	20.67	slope (K_m/V_{max}) = 1.2205
0.08	12.50	0.0416	24.04	x = -6.8759
0.06	16.67	0.0356	28.09	y = 8.3918
0.04	25.00	0.0256	39.06	correlation coefficient = 0.9998
0.02	50.00	0.0144	69.44	

Table II.2 Lineweaver-Burk data of compound II (chymotrypsin)

[I] = 0				
[S] mM	1/[S]	V	1/V	Regression analysis
0.10	10.00	0.0615	16.26	slope (K_m/V_{max}) = 0.7978
0.08	12.50	0.0510	19.61	x = -11.5574
0.06	16.67	0.0429	23.31	y = 9.2205
0.04	25.00	0.0345	28.99	correlation coefficient = 0.9987
0.02	50.00	0.0204	49.02	
[I] = 3 μM				
0.10	10.00	0.0549	18.21	slope (K_m/V_{max}) =0.9824
0.08	12.50	0.0489	20.45	x = -8.7575
0.06	16.67	0.0381	26.25	y = 8.6034
0.04	25.00	0.0308	32.47	correlation coefficient = 0.9989
0.02	50.00	0.0173	57.80	
[I] = 3.6 μM				
0.10	10.00	0.0512	19.53	slope (K_m/V_{max}) = 1.0412
0.08	12.50	0.0480	20.83	x = -8.5073
0.06	16.67	0.0364	27.47	y = 8.8578
0.04	25.00	0.0291	34.36	correlation coefficient = 0.9987
0.02	50.00	0.0164	60.97	

Table II.3 Lineweaver-Burk data of compound III (chymotrypsin)

[I] = 0				
[S] mM	1/[S]	V	1/V	Regression anylysis
0.10	10.00	0.0592	16.89	slope (K_m/V_{max}) = 0.9364
0.08	12.50	0.0469	21.32	x = -9.1114
0.06	16.67	0.0433	23.09	y = 8.5319
0.04	25.00	0.0300	33.33	correlation coefficient = 0.9971
0.02	50.00	0.0182	54.94	
[I] = 5 μM				
0.10	10.00	0.0379	26.38	slope (K_m/V_{max}) = 1.5787
0.08	12.50	0.0316	31.64	x = -6.8446
0.06	16.67	0.0241	36.90	y = 10.8055
0.04	25.00	0.0203	49.26	correlation coefficient = 0.9995
0.02	50.00	0.0111	90.09	
[I] = 6 μM				
0.10	10.00	0.0346	28.90	slope (K_m/V_{max}) = 1.9342
0.08	12.50	0.0295	33.90	x = -4.5791
0.06	16.67	0.0244	40.98	y = 8.8568
0.04	25.00	0.0182	54.95	correlation coefficient = 0.9991
0.02	50.00	0.0094	106.38	

Table II.4 Lineweaver-Burk data of compound IV (chymotrypsin)

[I] = 0				
[S] mM	1/[S]	V	1/V	Regression analysis
0.10	10.00	0.0566	17.67	slope (K_m/V_{max}) = 0.7399
0.08	12.50	0.0500	20.00	x = -14.1483
0.06	16.67	0.0431	23.20	y = 10.4683
0.04	25.00	0.0353	28.33	correlation coefficient = 0.9994
0.02	50.00	0.0210	47.62	
[I] = 4 μM				
0.10	10.00	0.0433	23.09	slope (K_m/V_{max}) = 1.2494
0.08	12.50	0.0400	25.00	x = -7.9041
0.06	16.67	0.0328	30.49	y = 9.8754
0.04	25.00	0.0244	40.98	correlation coefficient = 0.9997
0.02	50.00	0.0138	72.46	
[I] = 4.8 μM				
0.10	10.00	0.0386	25.91	slope (K_m/V_{max}) = 1.5263
0.08	12.50	0.0327	30.58	x = -6.9354
0.06	16.67	0.0288	34.72	y = 10.5858
0.04	25.00	0.0204	49.02	correlation coefficient = 0.9995
0.02	50.00	0.0115	86.96	

Table II.5 Lineweaver-Burk data of compound V (chymotrypsin)

[I] = 0				
[S] mM	1/[S]	V	1/V	Regression analysis
0.01	10.00	0.0677	14.99	slope (K_m/V_{max}) = 0.8052
0.08	12.50	0.0579	17.27	x = -8.7692
0.06	16.67	0.0483	20.70	y = 7.0610
0.04	25.00	0.0372	26.88	correlation coefficient = 0.9999
0.02	50.00	0.0211	47.39	
[I] = 3 μM				
0.10	10.00	0.0520	19.23	slope (K_m/V_{max}) = 1.2145
0.08	12.50	0.0448	22.32	x = -6.2500
0.06	16.67	0.0350	28.57	y = 7.5906
0.04	25.00	0.0260	38.46	correlation coefficient = 0.9996
0.02	50.00	0.0147	68.03	
[I] = 3.6 μM				
0.10	10.00	0.0451	22.17	slope (K_m/V_{max}) = 1.4952
0.08	12.50	0.0390	25.64	x = -4.9646
0.06	16.67	0.0305	32.79	y = 7.4231
0.04	25.00	0.0221	45.25	correlation coefficient = 0.9998
0.02	50.00	0.0122	81.97	

Table II.6 Lineweaver-Burk data of compound VI (chymotrypsin)

[I] = 0				
[S] mM	1/[S]	V	1/V	Regression analysis
0.10	10.00	0.0692	14.45	slope (K_m/V_{max}) = 0.6999
0.08	12.50	0.0567	17.64	x = -12.5592
0.06	16.67	0.0471	21.23	y = 8.7902
0.04	25.00	0.0367	27.25	correlation coefficient = 0.9966
0.02	50.00	0.0231	43.29	
[I] = 4.5 μM				
0.10	10.00	0.0570	17.54	slope (K_m/V_{max}) = 0.9857
0.08	12.50	0.0480	20.83	x = -8.2741
0.06	16.67	0.0400	25.00	y = 8.1554
0.04	25.00	0.0308	32.47	correlation coefficient = 0.9997
0.02	50.00	0.0174	57.47	
[I] = 5.4 μM				
0.10	10.00	0.0508	19.69	slope (K_m/V_{max}) = 1.0362
0.08	12.50	0.0438	22.83	x = -9.0856
0.06	16.67	0.0377	26.53	y = 9.4145
0.04	25.00	0.0286	34.97	correlation coefficient = 0.9998
0.02	50.00	0.0163	61.35	

Table II.7 Lineweaver-Burk data of compound VII (chymotrypsin)

[I] = 0				
[S] mM	1/[S]	V	1/V	Regression analysis
0.10	10.00	0.0549	18.21	slope (K_m/V_{max}) = 0.9194
0.08	12.50	0.0500	20.00	x = -9.4481
0.06	16.67	0.0407	24.57	y = 8.6866
0.04	25.00	0.0326	30.67	correlation coefficient = 0.9991
0.02	50.00	0.0182	54.95	
[I] = 0.6 μM				
0.10	10.00	0.0490	20.41	slope (K_m/V_{max}) = 1.0843
0.08	12.50	0.0416	24.04	x = -9.2205
0.06	16.67	0.0360	27.78	y = 9.9978
0.04	25.00	0.0267	37.45	correlation coefficient = 0.9997
0.02	50.00	0.0156	64.10	
[I] = 0.72 μM				
0.10	10.00	0.0457	21.88	slope (K_m/V_{max}) = 1.1948
0.08	12.50	0.0390	25.64	x = -8.3958
0.06	16.67	0.0338	29.59	y = 10.0313
0.04	25.00	0.0253	39.53	correlation coefficient = 0.9997
0.02	50.00	0.0143	69.93	

Table II.8 Lineweaver-Burk data of compound VIII (chymotrypsin)

[I] = 0				
[S] mM	1/[S]	V	1/V	Regression analysis
0.10	10.00	0.0465	21.51	slope (K_m/V_{max}) = 1.2023
0.08	12.50	0.0409	24.45	x = -8.2968
0.06	16.67	0.0320	31.25	y = 9.9753
0.04	25.00	0.0250	40.00	correlation coefficient = 0.9993
0.02	50.00	0.0143	69.93	
[I] = 0.5 μM				
0.10	10.00	0.0390	25.64	slope (K_m/V_{max}) = 1.4849
0.08	12.50	0.0360	27.78	x = -6.5819
0.06	16.67	0.0298	33.56	y = 9.7735
0.04	25.00	0.0211	47.39	correlation coefficient = 0.9995
0.02	50.00	0.0119	84.03	
[I] = 0.6 μM				
0.10	10.00	0.0378	26.46	slope (K_m/V_{max}) = 1.5852
0.08	12.50	0.0343	29.15	x = -6.156
0.06	16.67	0.0277	36.10	y = 9.7585
0.04	25.00	0.0205	48.78	correlation coefficient = 0.9997
0.02	50.00	0.0112	89.29	

Table II.9 Lineweaver-Burk data of compound IX (chymotrypsin)

[I] = 0				
[S] mM	1/[S]	V	1/V	Regression analysis
0.10	10.00	0.0683	14.64	slope (K_m/V_{max}) = 0.8183
0.08	12.50	0.0588	17.01	x = -8.7120
0.06	16.67	0.0471	21.23	y = 7.1290
0.04	25.00	0.0350	28.57	correlation coefficient = 0.9986
0.02	50.00	0.0210	47.62	
[I] = 25 μM				
0.10	10.00	0.0582	17.18	slope (K_m/V_{max}) = 0.9936
0.08	12.50	0.0521	19.19	x = -7.4335
0.06	16.67	0.0410	24.39	y = 7.3859
0.04	25.00	0.0305	32.79	correlation coefficient = 0.9995
0.02	50.00	0.0176	56.82	
[I] = 30 μM				
0.10	10.00	0.0571	17.51	slope (K_m/V_{max}) = 1.0975
0.08	12.50	0.0484	20.66	x = -6.2312
0.06	16.67	0.0391	25.58	y = 6.8387
0.04	25.00	0.0294	34.01	correlation coefficient = 0.9999
0.02	50.00	0.0162	61.73	

Table II.10 Lineweaver-Burk data of compound X (chymotrypsin)

[I] = 0				
[S] mM	1/[S]	V	1/V	Regression analysis
0.10	10.00	0.0667	14.99	slope (K_m/V_{max}) = 0.8052
0.08	12.50	0.0579	17.27	x = -8.7692
0.06	16.67	0.0483	20.70	y = 7.0610
0.04	25.00	0.0372	26.88	correlation coefficient = 0.9999
0.02	50.00	0.0211	47.39	
[I] = 40 μM				
0.10	10.00	0.0545	18.35	slope (K_m/V_{max}) = 1.1527
0.08	12.50	0.0447	22.37	x = -6.4691
0.06	16.67	0.0376	26.60	y = 7.4569
0.04	25.00	0.0273	36.63	correlation coefficient = 0.9997
0.02	50.00	0.0154	64.94	
[I] = 48 μM				
0.10	10.00	0.0468	21.37	slope (K_m/V_{max}) = 1.4155
0.08	12.50	0.0390	25.64	x = -5.3386
0.06	16.67	0.0327	30.58	y = 7.5568
0.04	25.00	0.0229	43.67	correlation coefficient = 0.9997
0.02	50.00	0.0128	78.13	

APPENDIX III

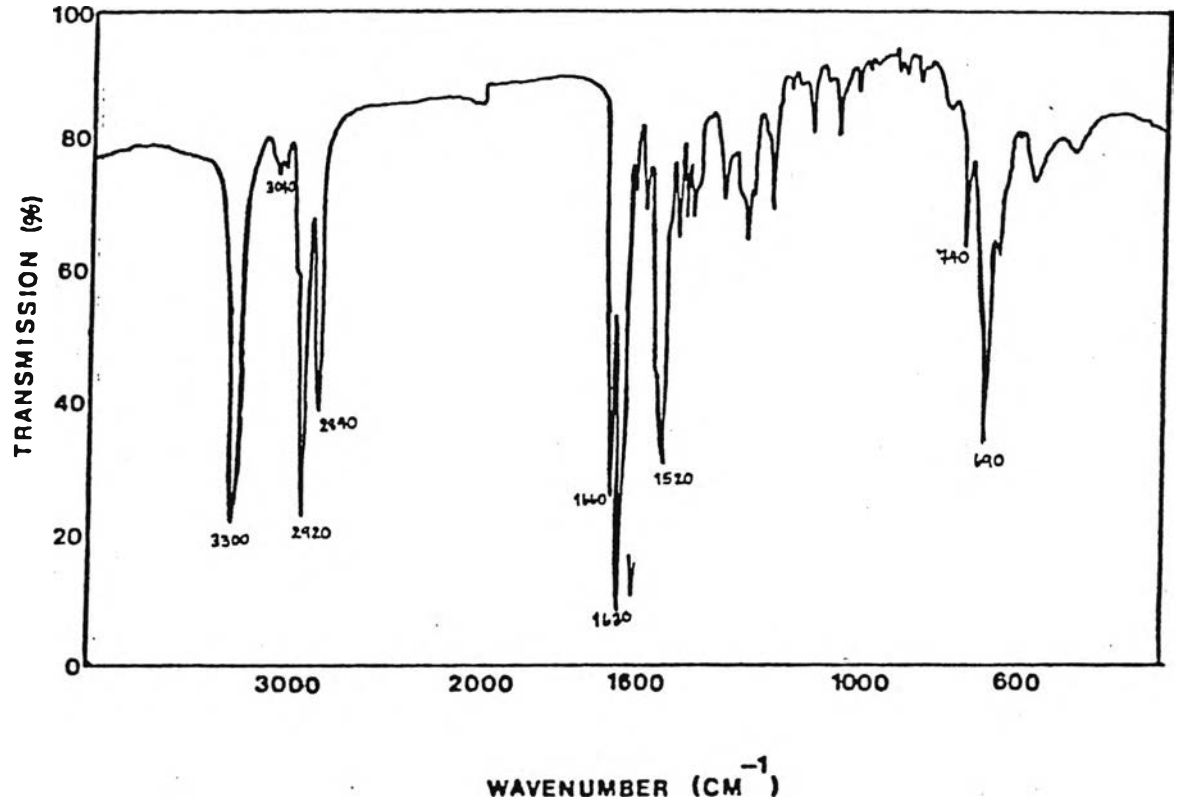


Fig.III.1 IR spectrum of compound I in KBr disc.

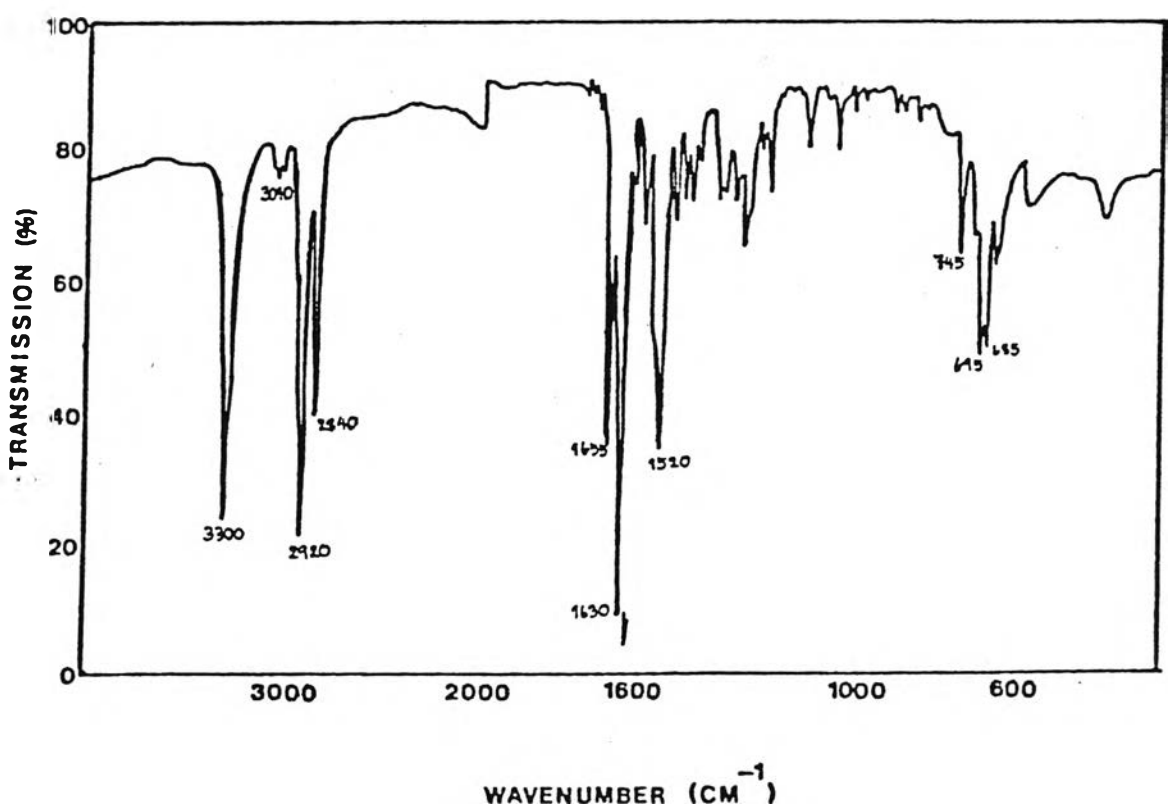


Fig.III.2 IR spectrum of compound II in KBr disc.

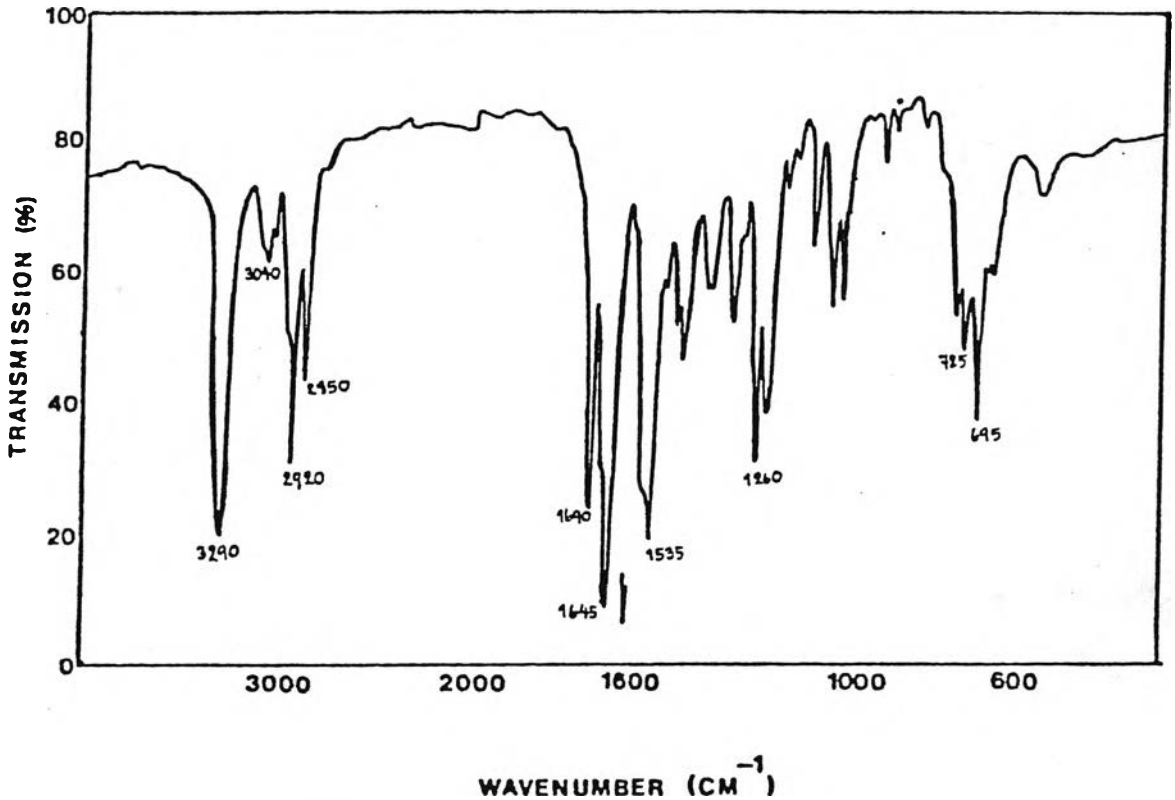


Fig.III.3 IR spectrum of compound III in KBr disc.

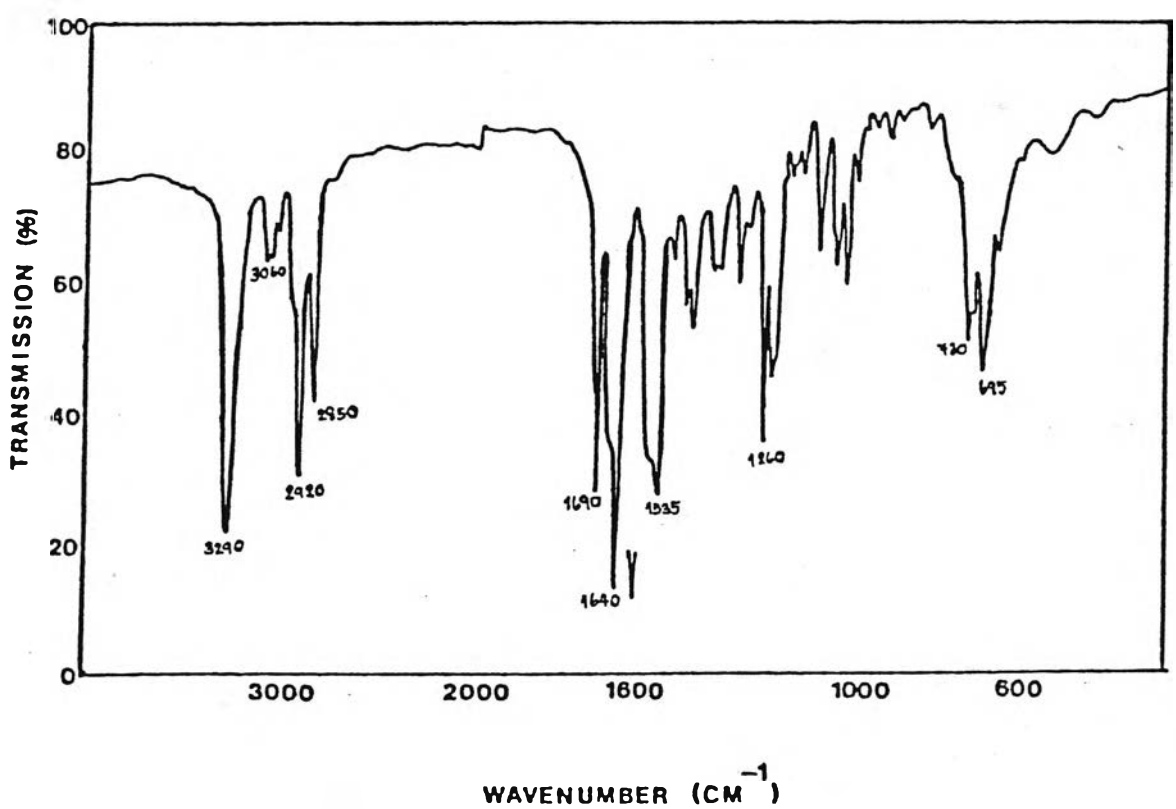


Fig.III.4 IR spectrum of compound IV in KBr disc.

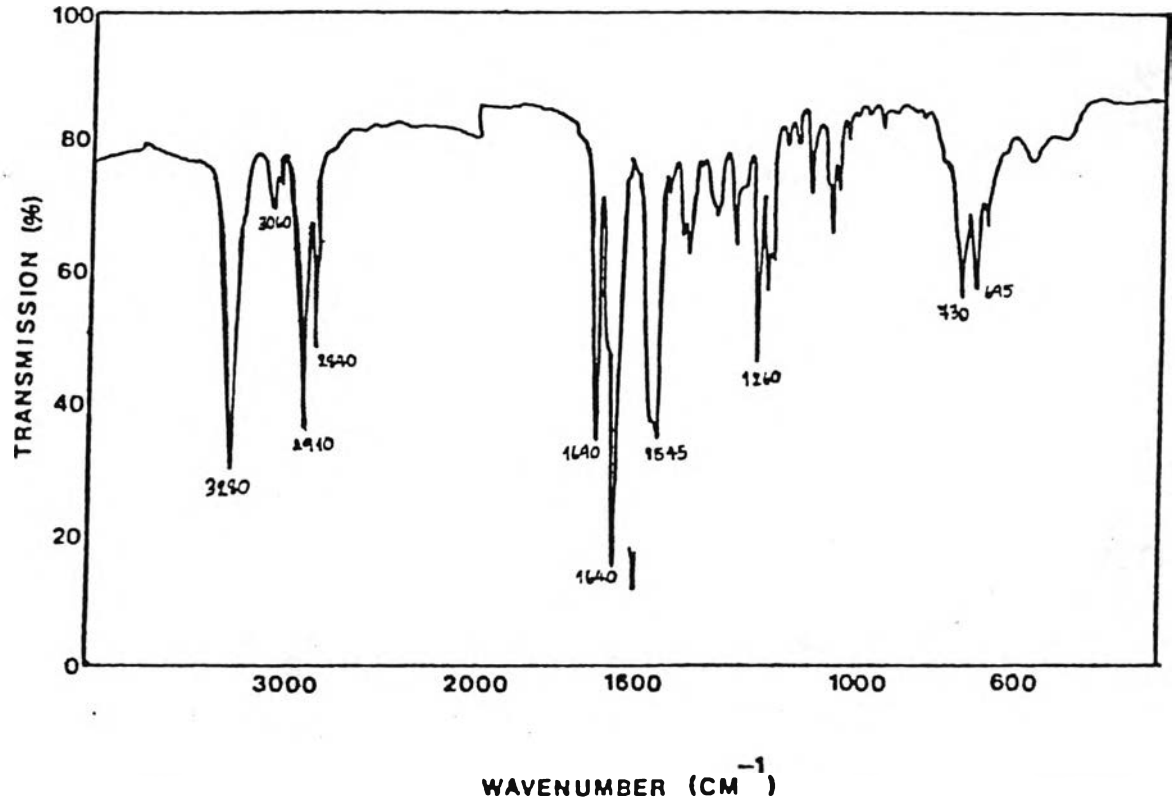


Fig.III.5 IR spectrum of compound V in KBr disc.

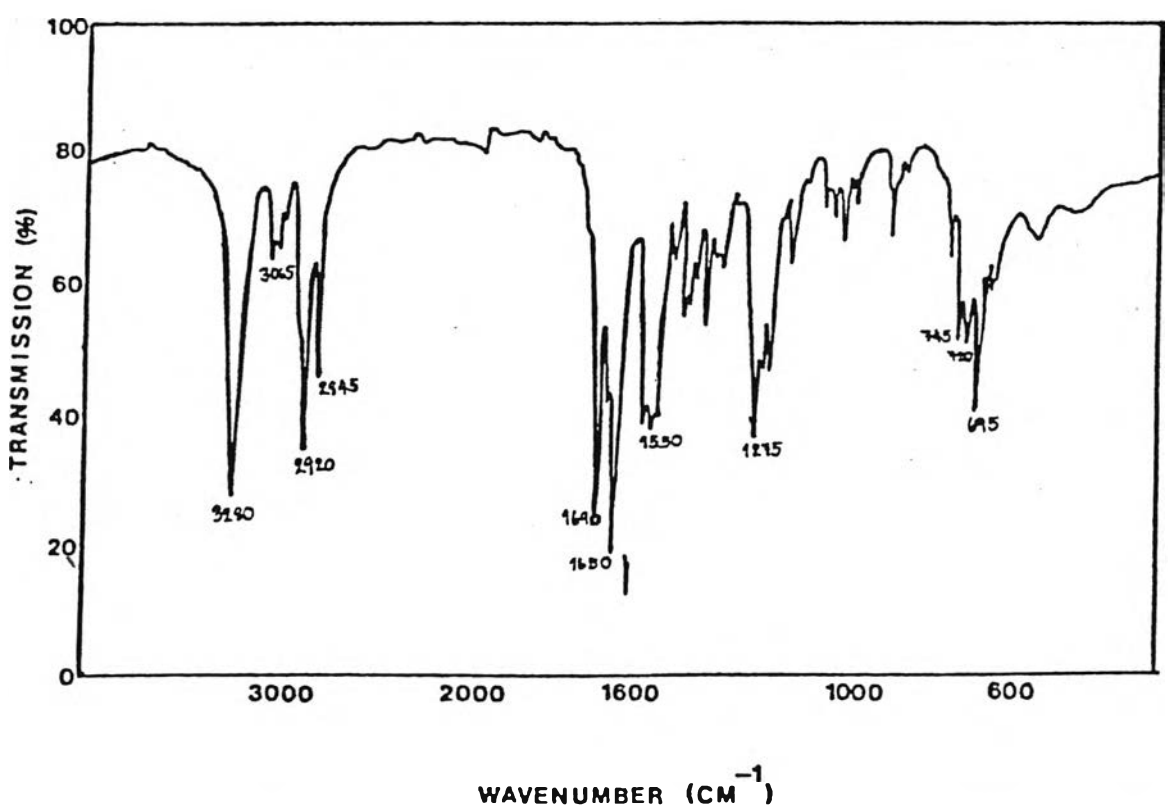


Fig.III.6 IR spectrum of compound VI in KBr disc.

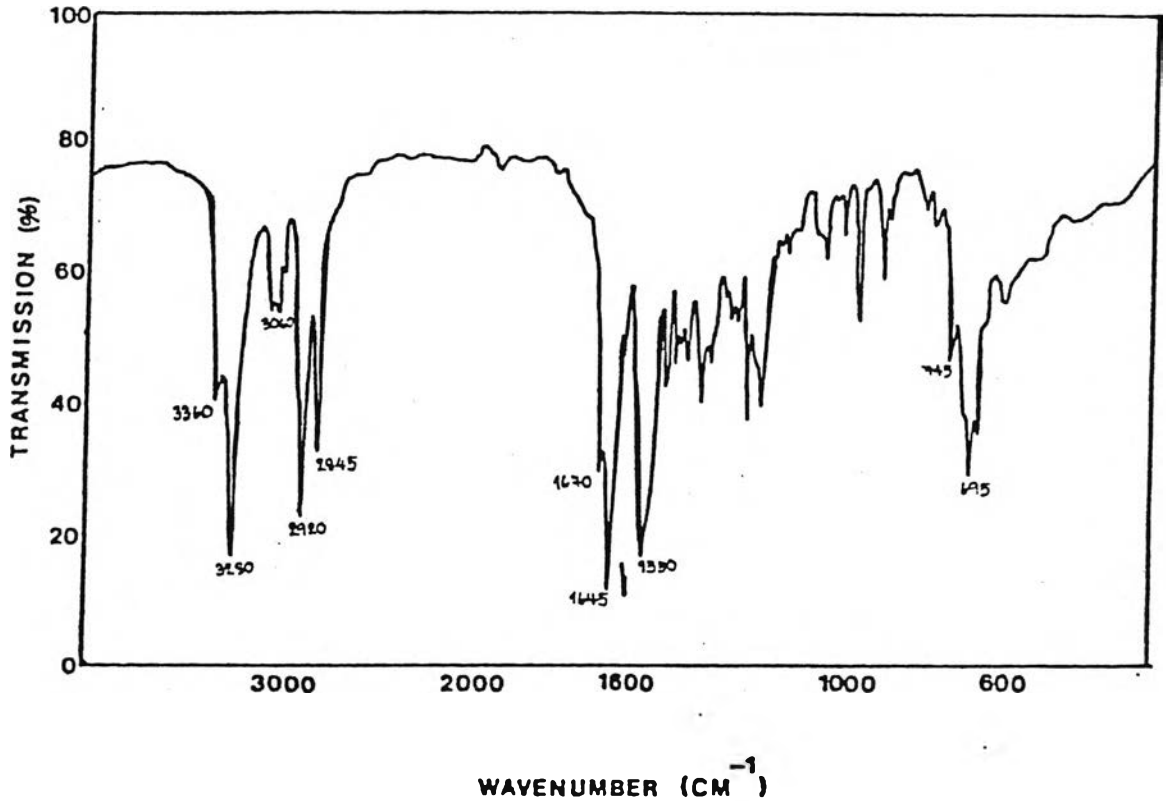


Fig.III.7 IR spectrum of compound VII in KBr disc.

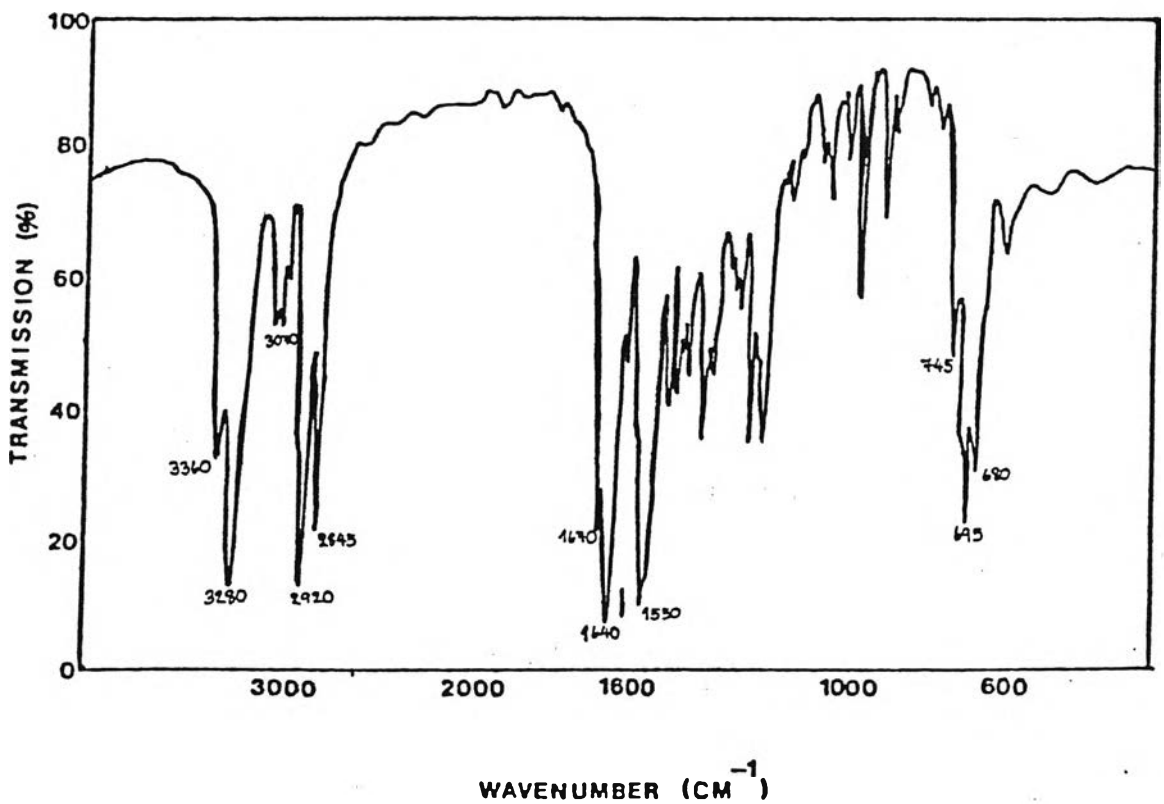


Fig.III.8 IR spectrum of compound VIII in KBr disc.

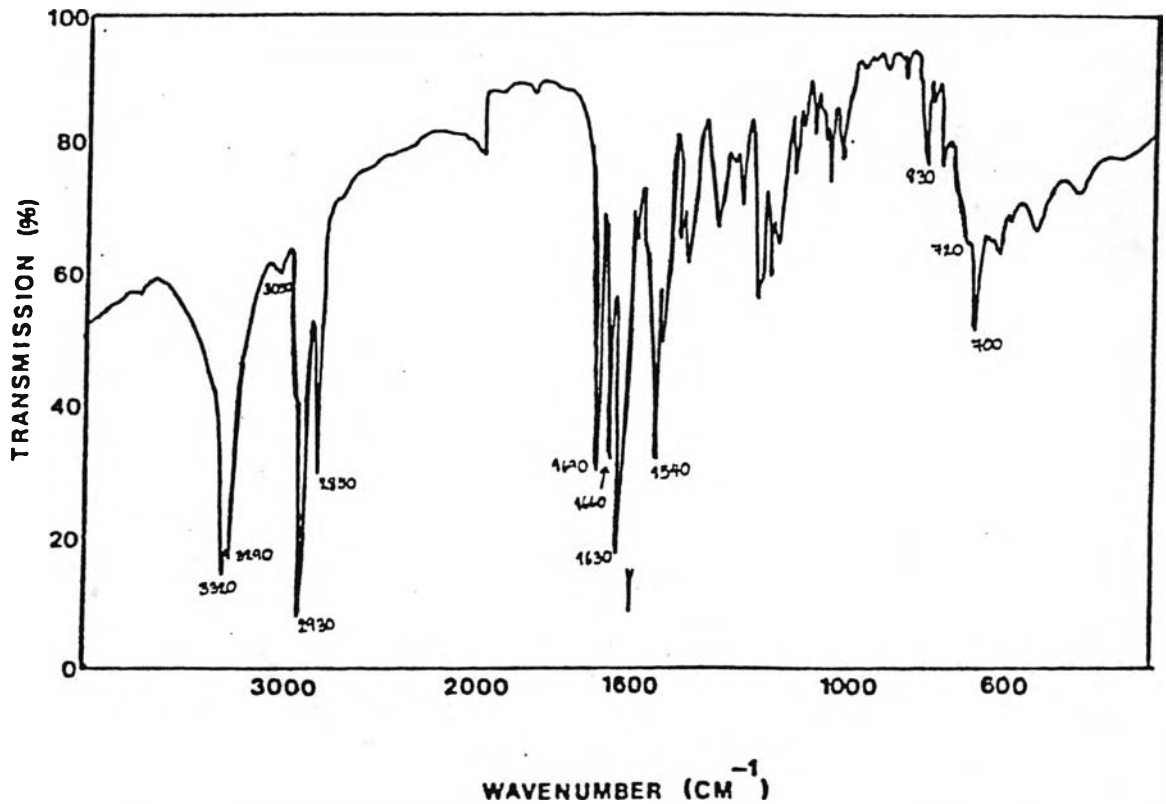


Fig.III.9 IR spectrum of compound IX in KBr disc.

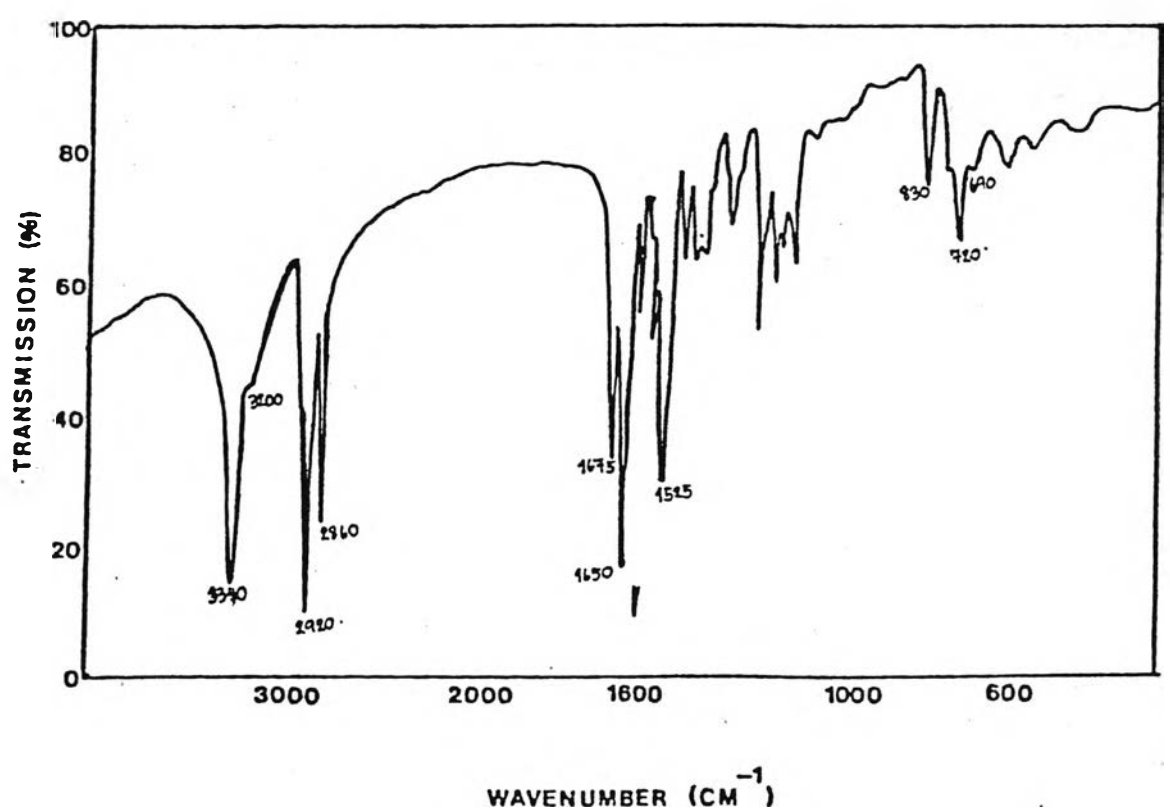


Fig.III.10 IR spectrum of compound X in KBr disc.

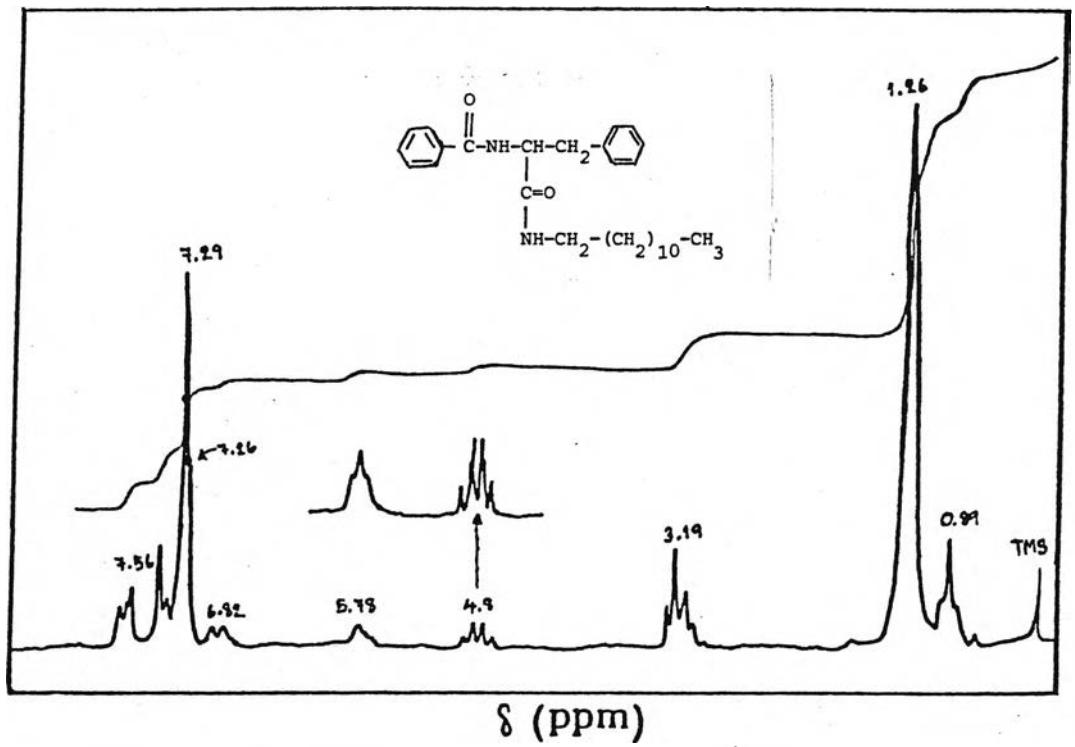


Fig.III.11 ^{1}H NMR spectrum of compound I in $CDCl_3$.

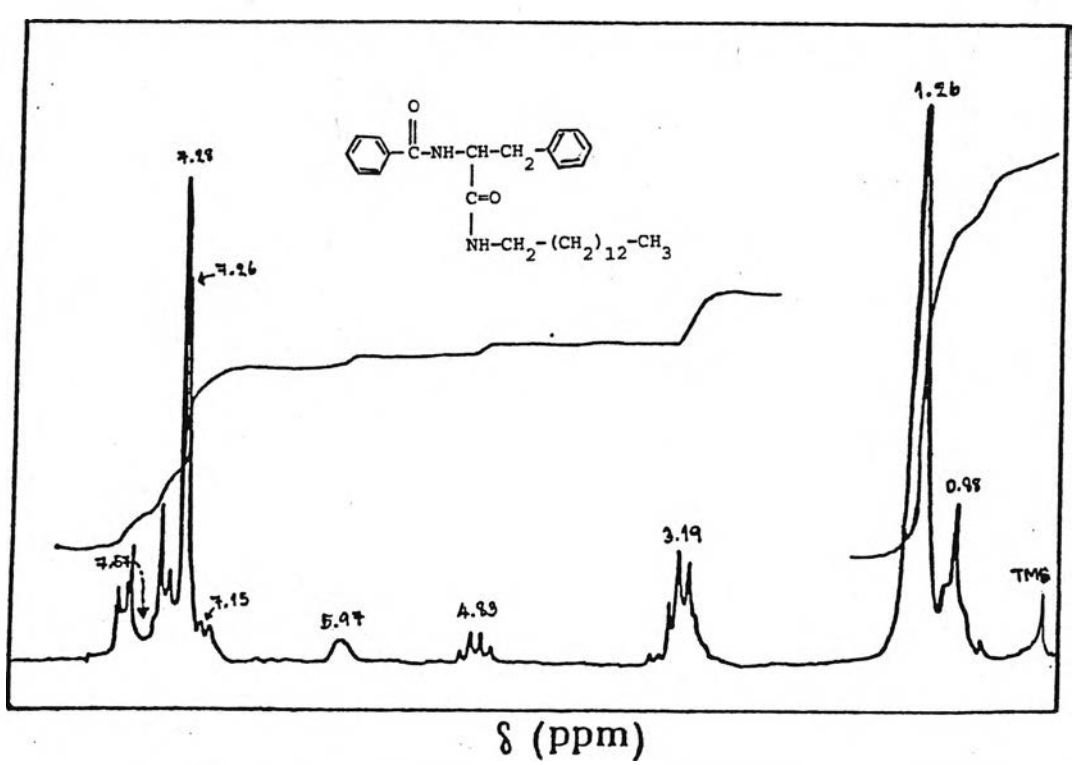


Fig.III.12 ^{1}H NMR spectrum of compound II in $CDCl_3$.

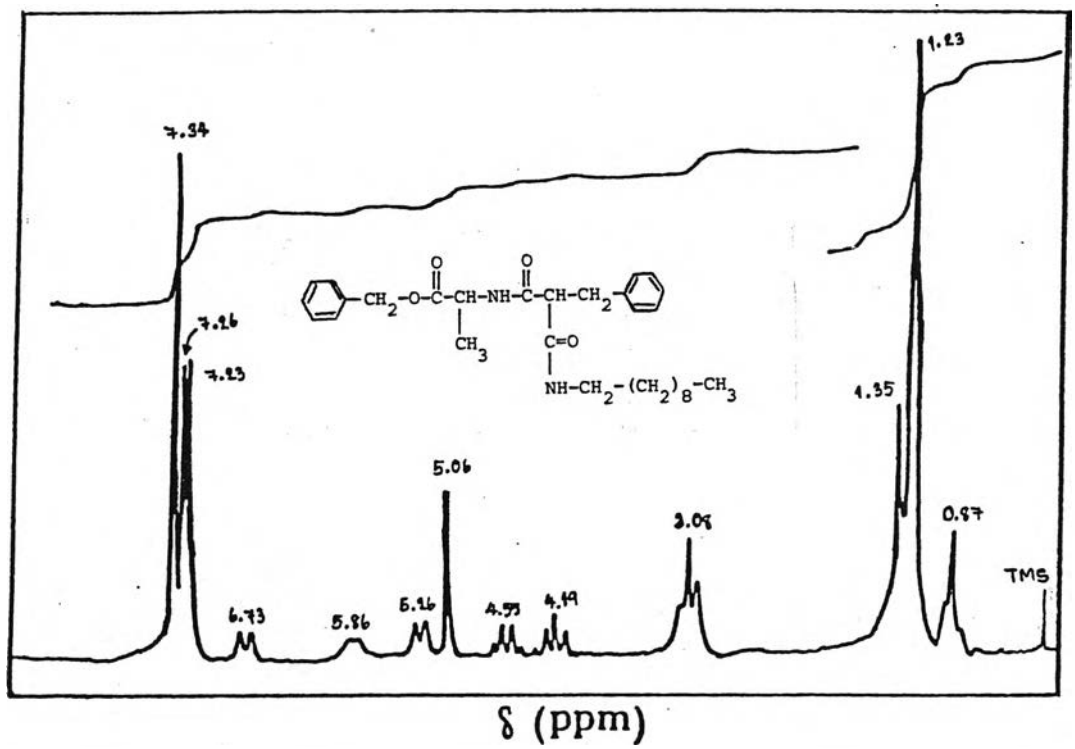


Fig.III.13 ^{1}H NMR spectrum of compound III in $CDCl_3$.

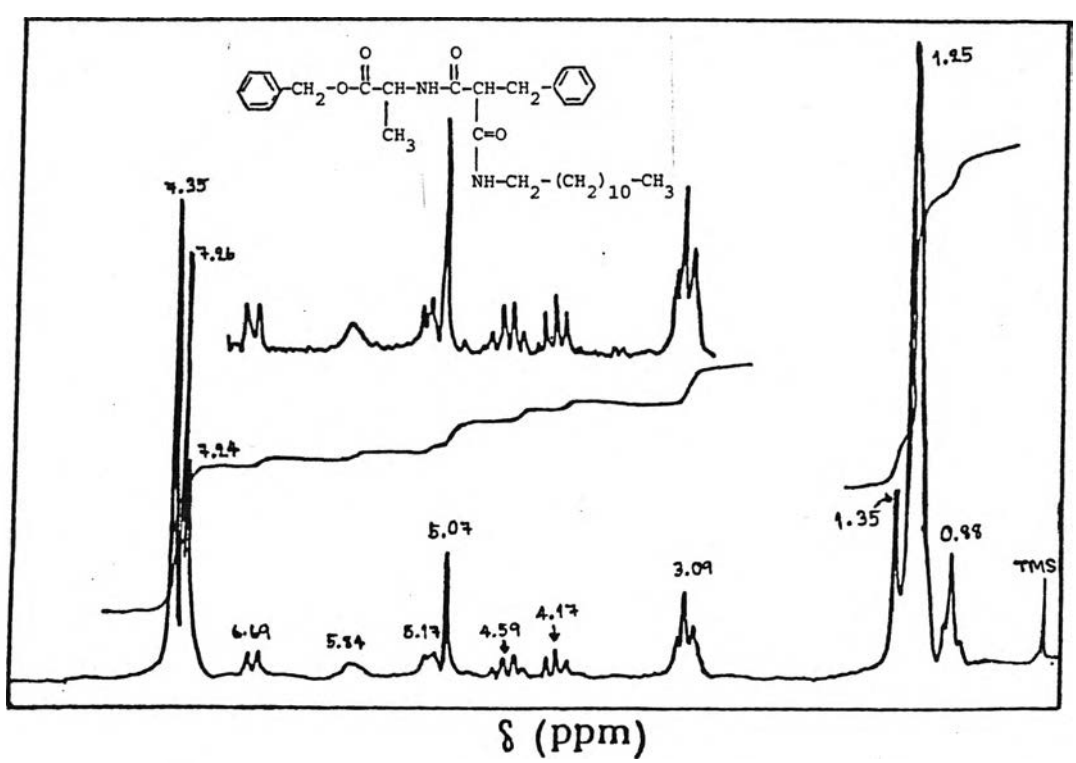


Fig.III.14 ^{1}H NMR spectrum of compound IV in $CDCl_3$.

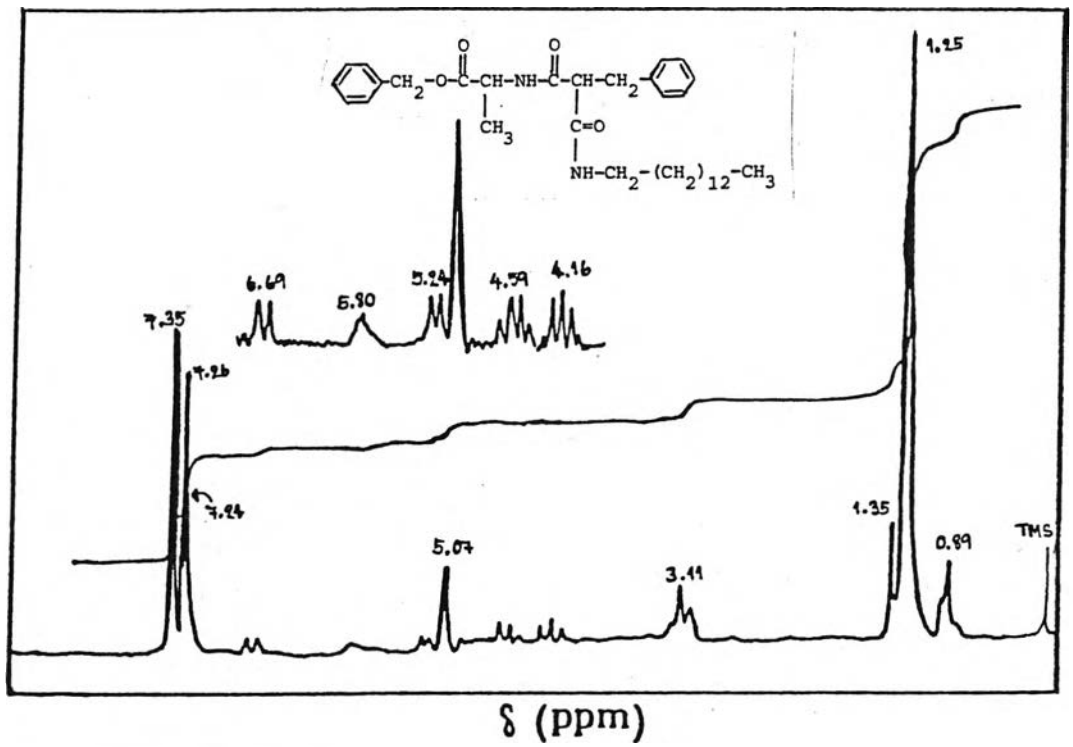


Fig.III.15 ^{1}H NMR spectrum of compound V in $CDCl_3$.

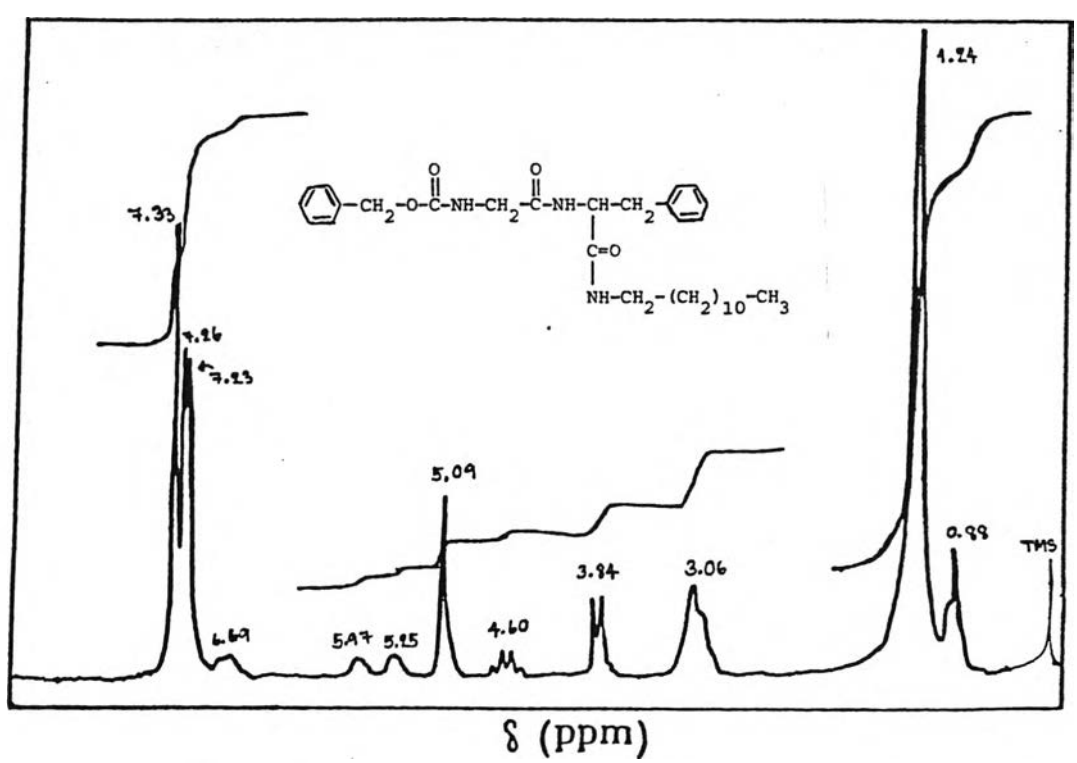


Fig.III.16 ^{1}H NMR spectrum of compound VI in $CDCl_3$.

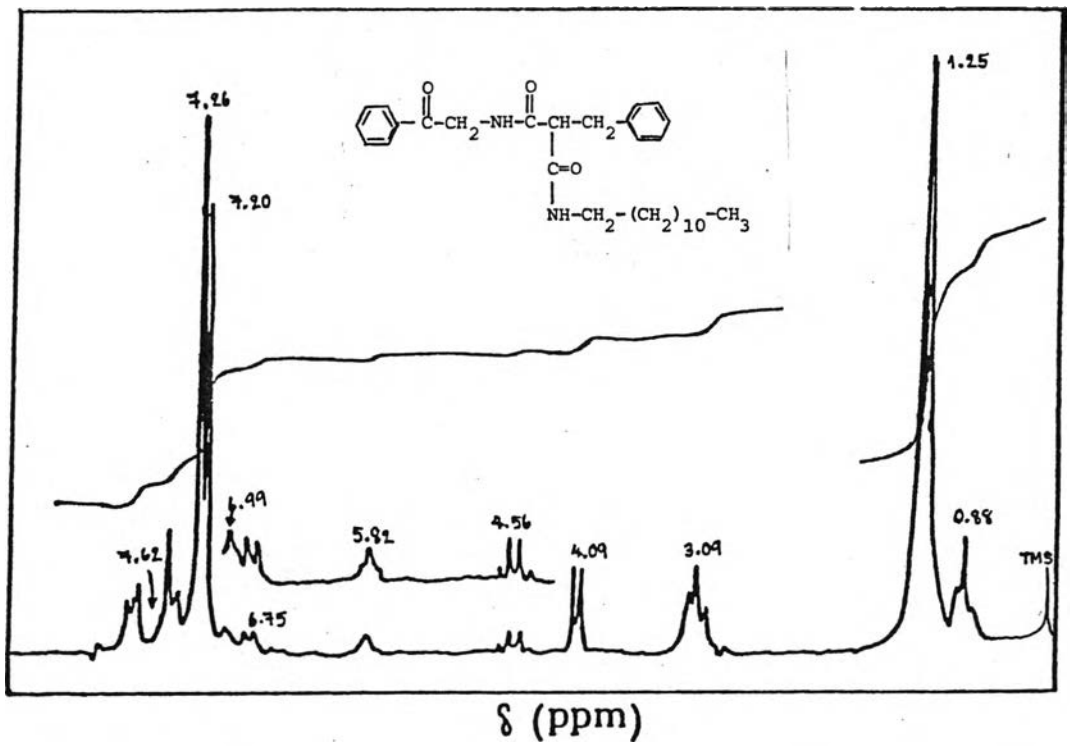


Fig.III.17 ^{1}H NMR spectrum of compound VII in $CDCl_3$.

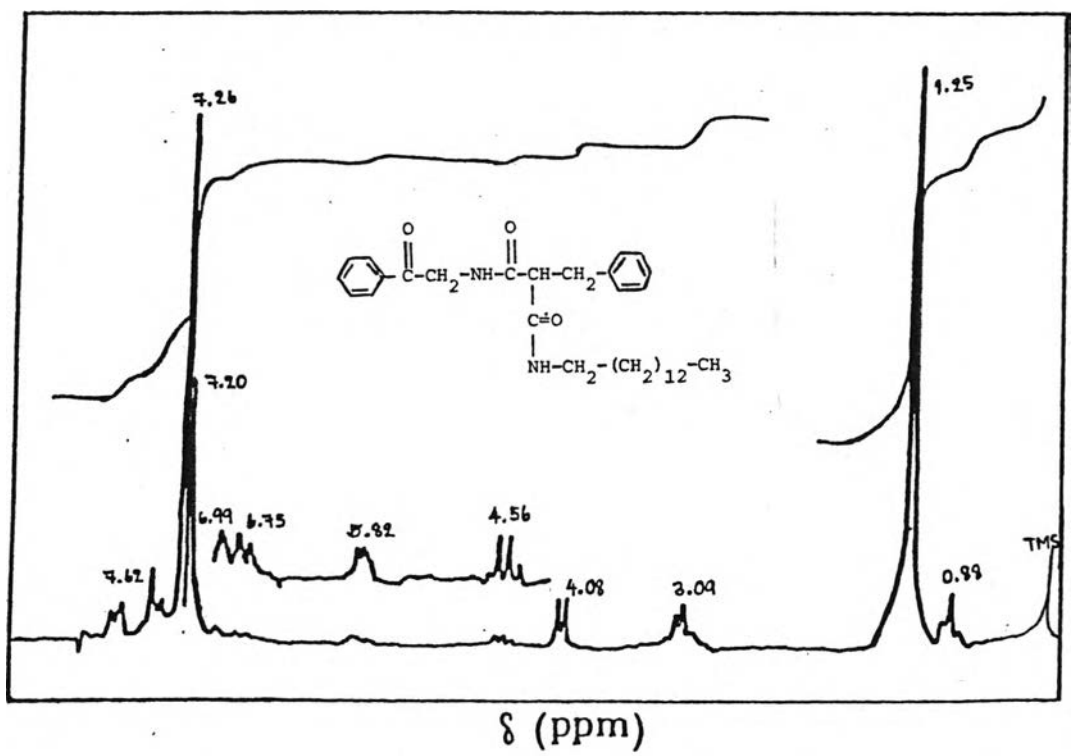


Fig.III.18 ^{1}H NMR spectrum of compound VIII in $CDCl_3$.

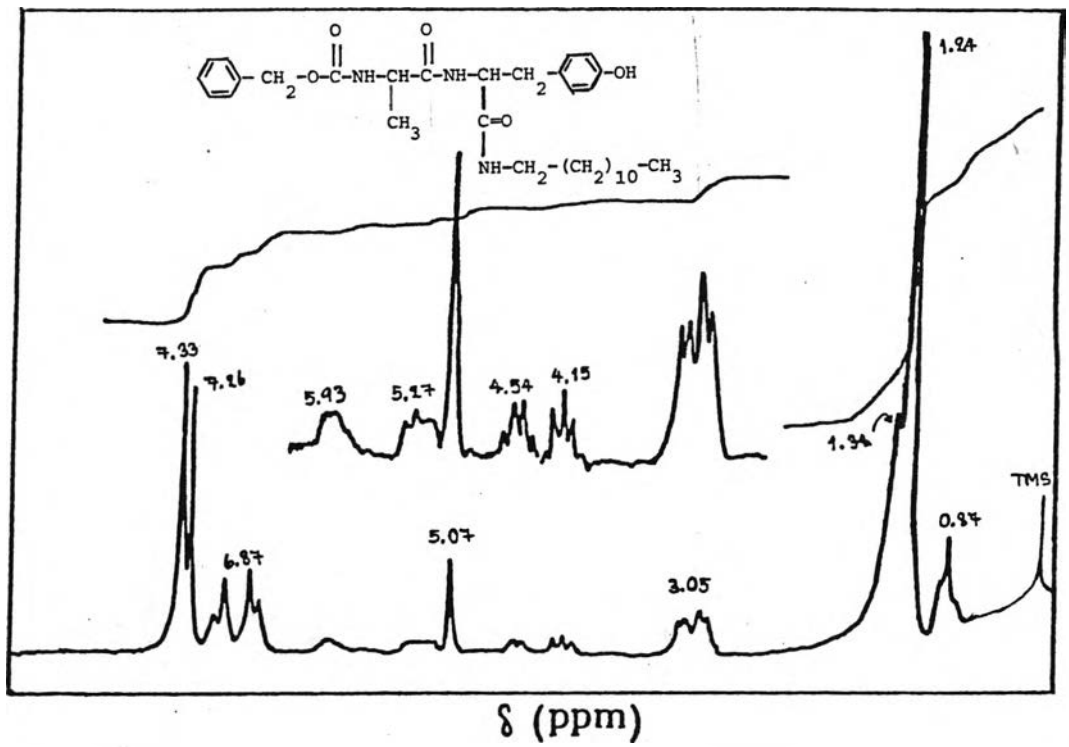


Fig.III.19 ^{1}H NMR spectrum of compound IX in $CDCl_3$.

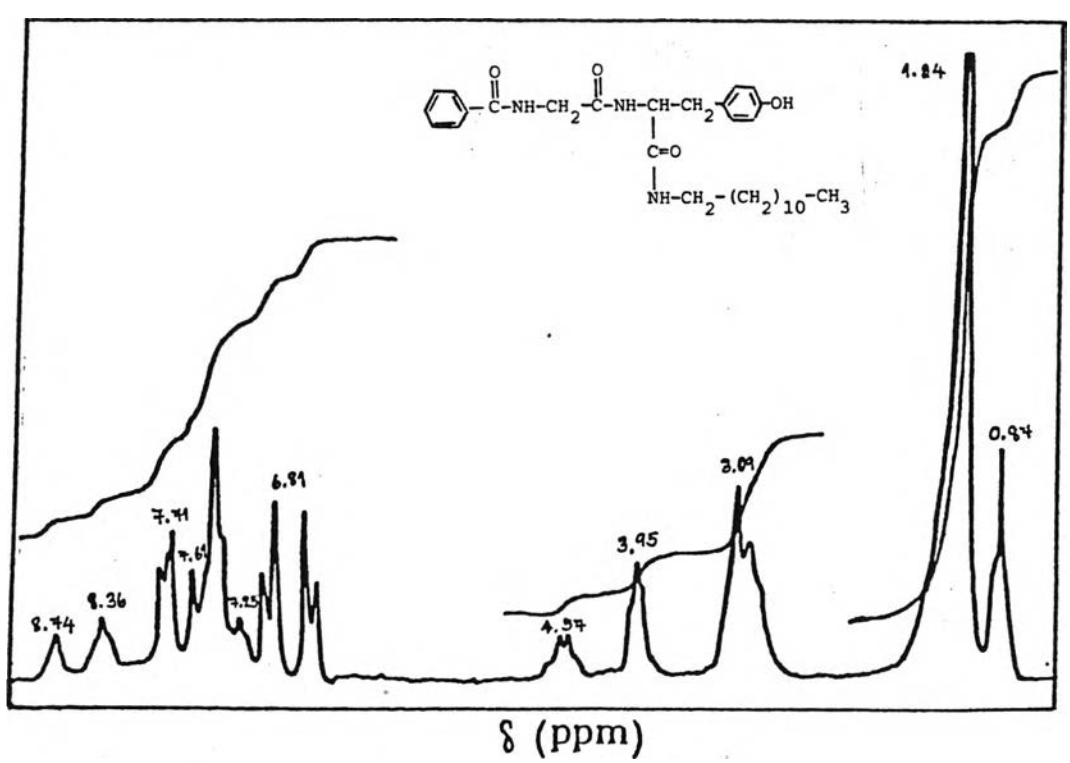


Fig.III.20 ^{1}H NMR spectrum of compound X in $CDCl_3$+DMSO-d_6.

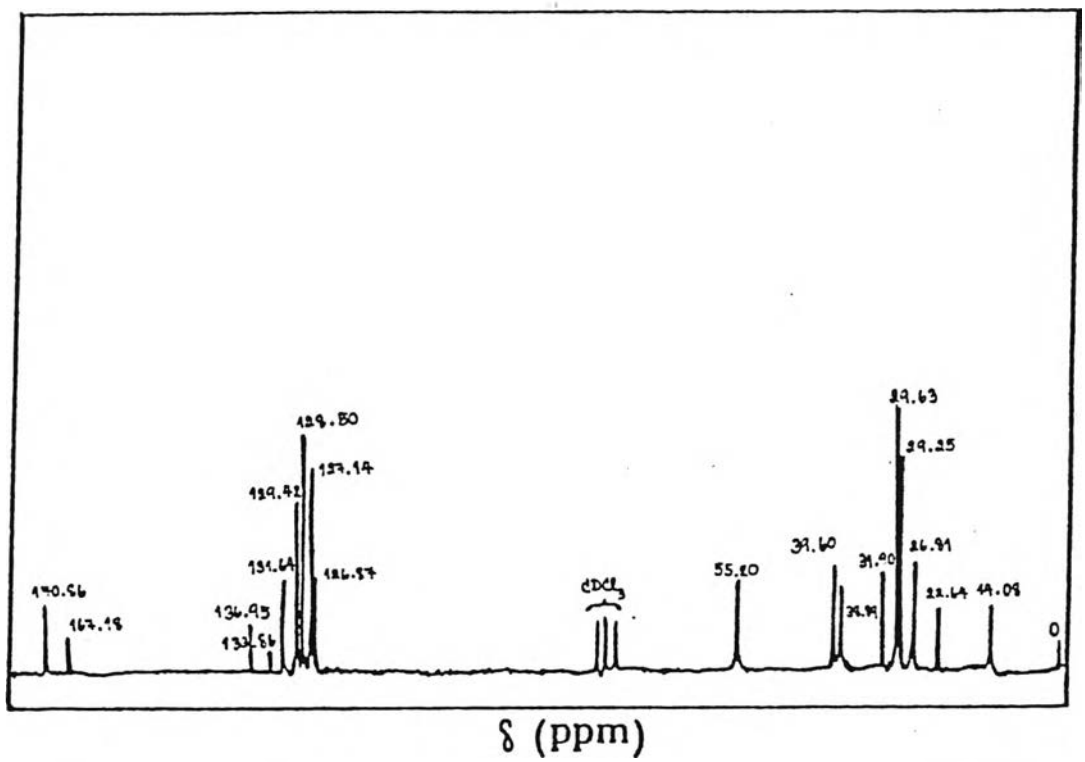


Fig.III.21 ^{13}C NMR spectrum of compound I in $CDCl_3$.

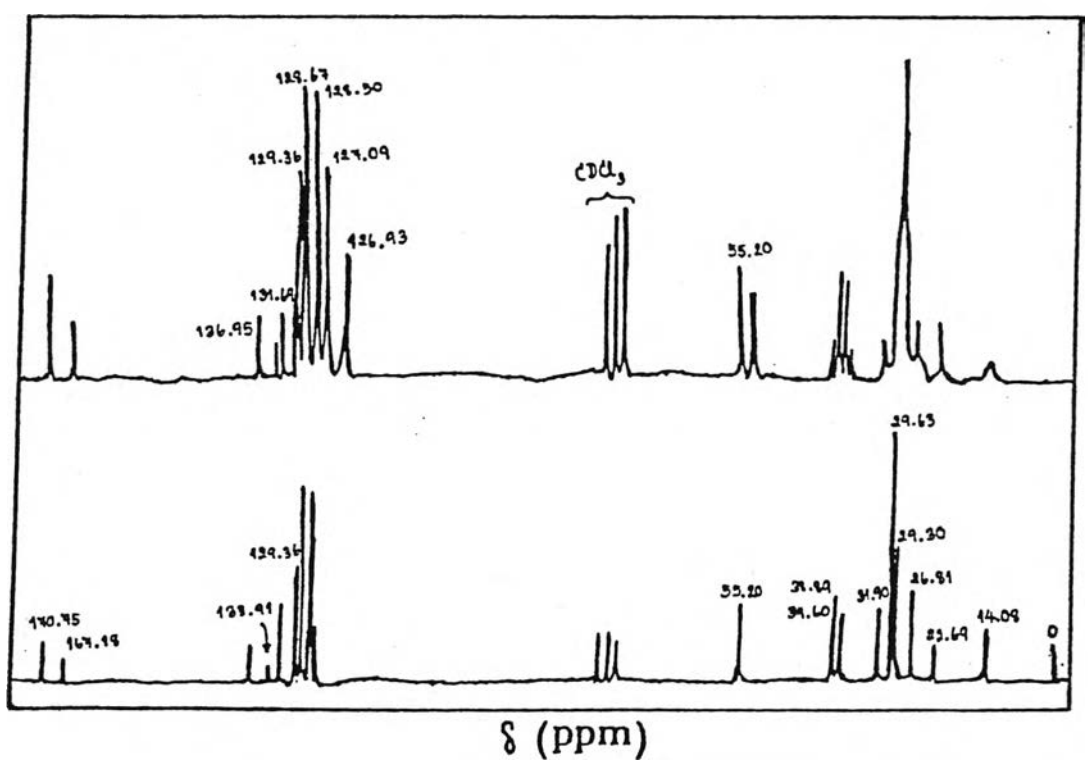


Fig.III.22 ^{13}C NMR spectrum of compound II in $CDCl_3$.

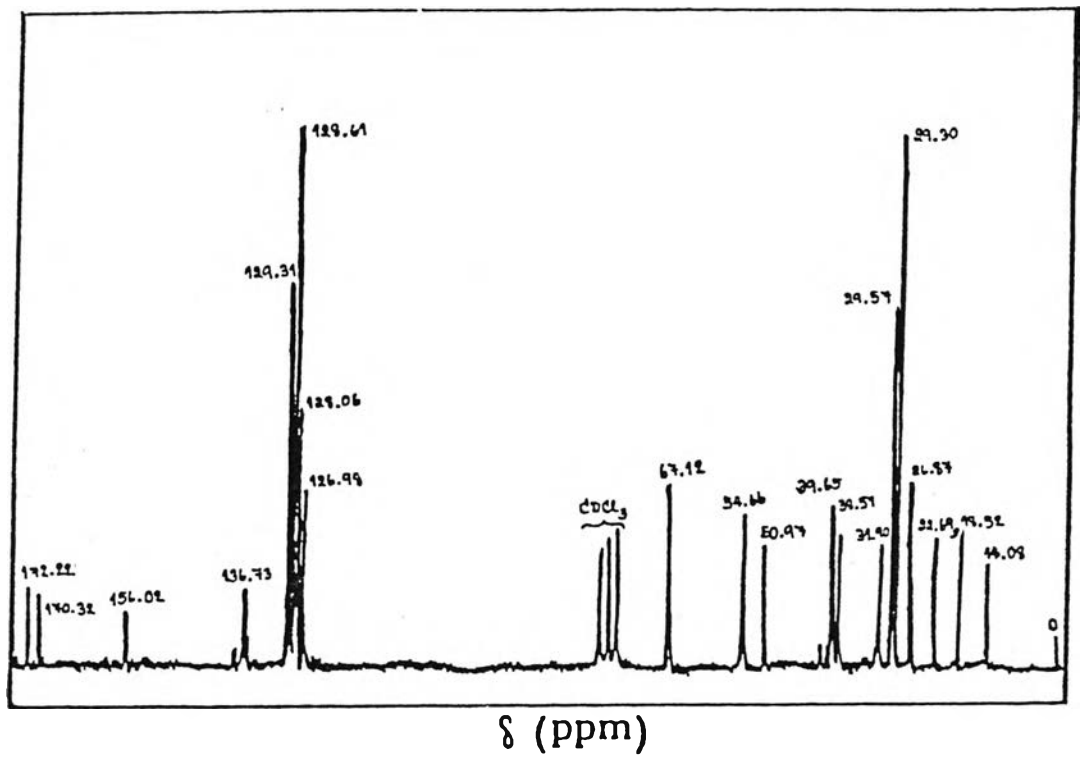


Fig.III.23 ^{13}C NMR spectrum of compound III in $CDCl_3$.

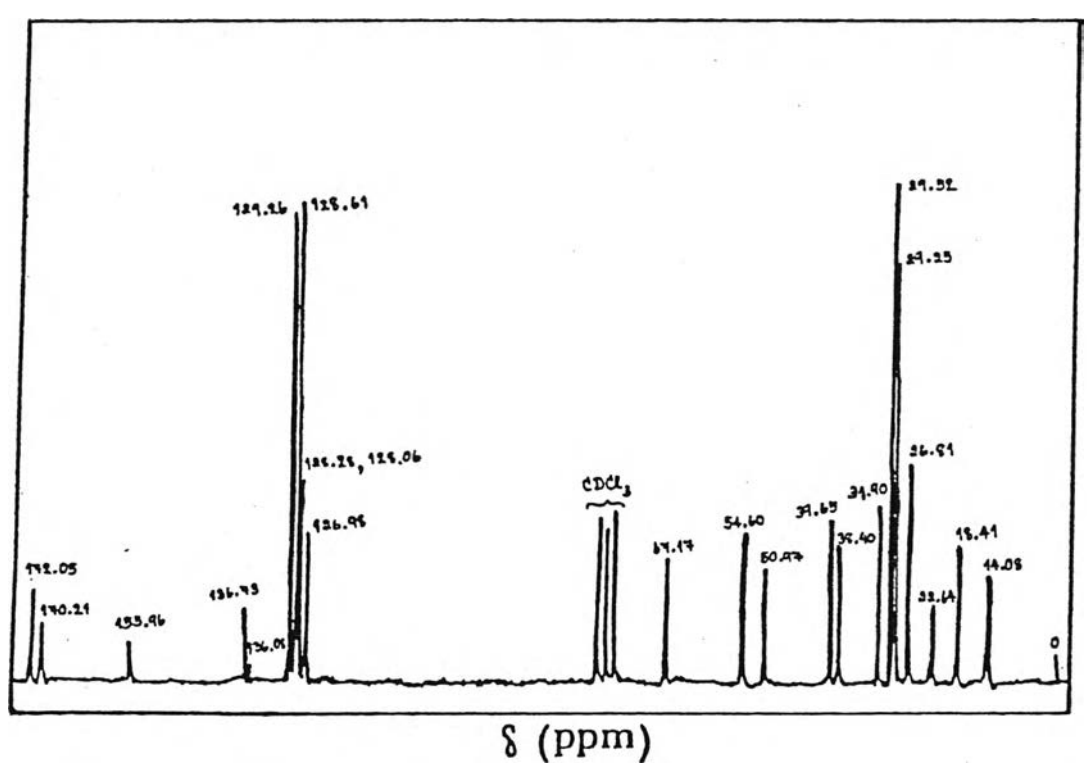


Fig.III.24 ^{13}C NMR spectrum of compound IV in $CDCl_3$.

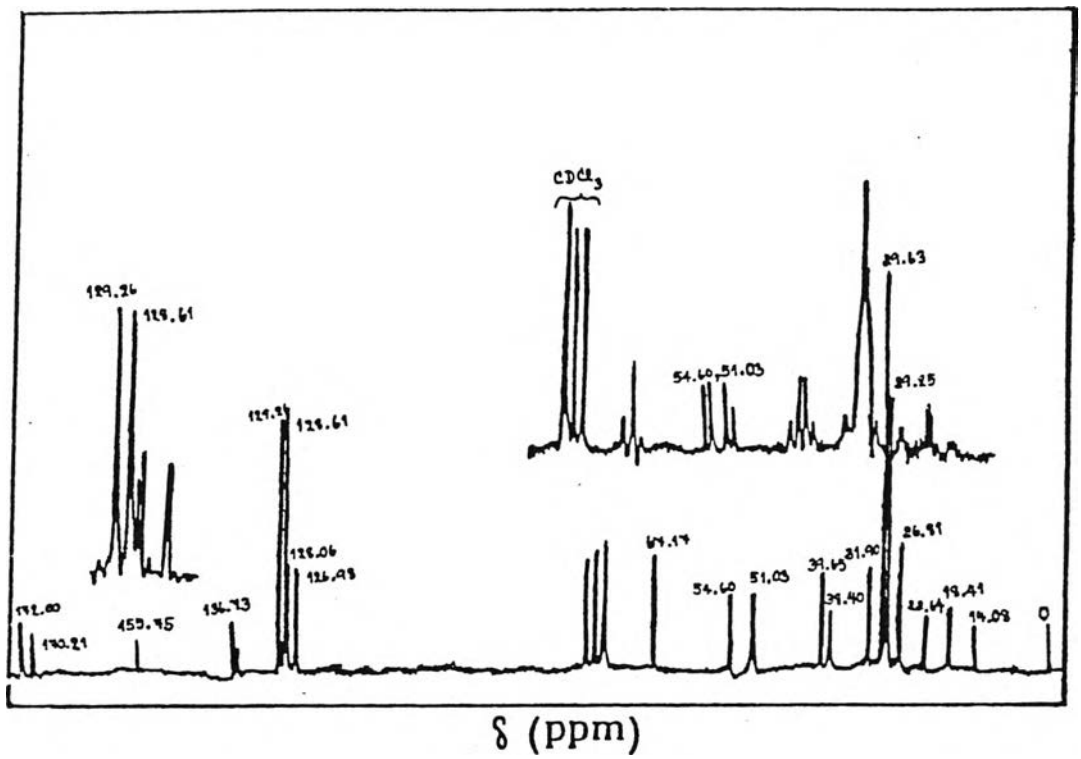


Fig.III.25 ^{13}C NMR spectrum of compound V in $CDCl_3$.

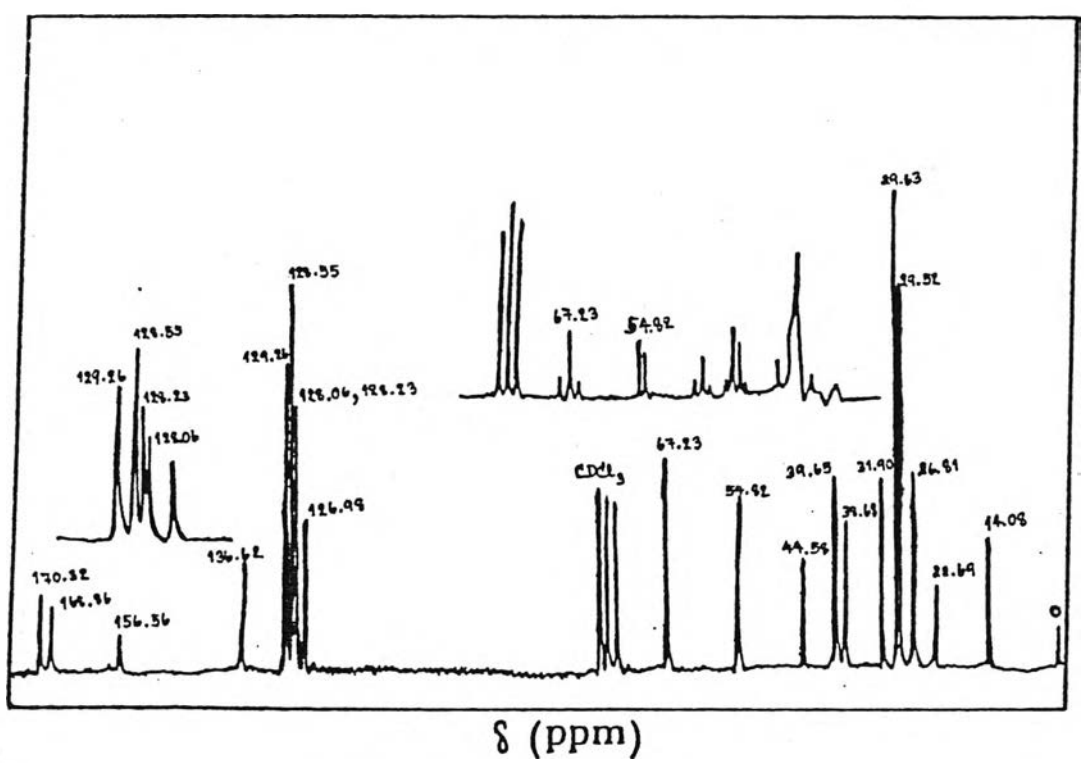


Fig.III.26 ^{13}C NMR spectrum of compound VI in $CDCl_3$.

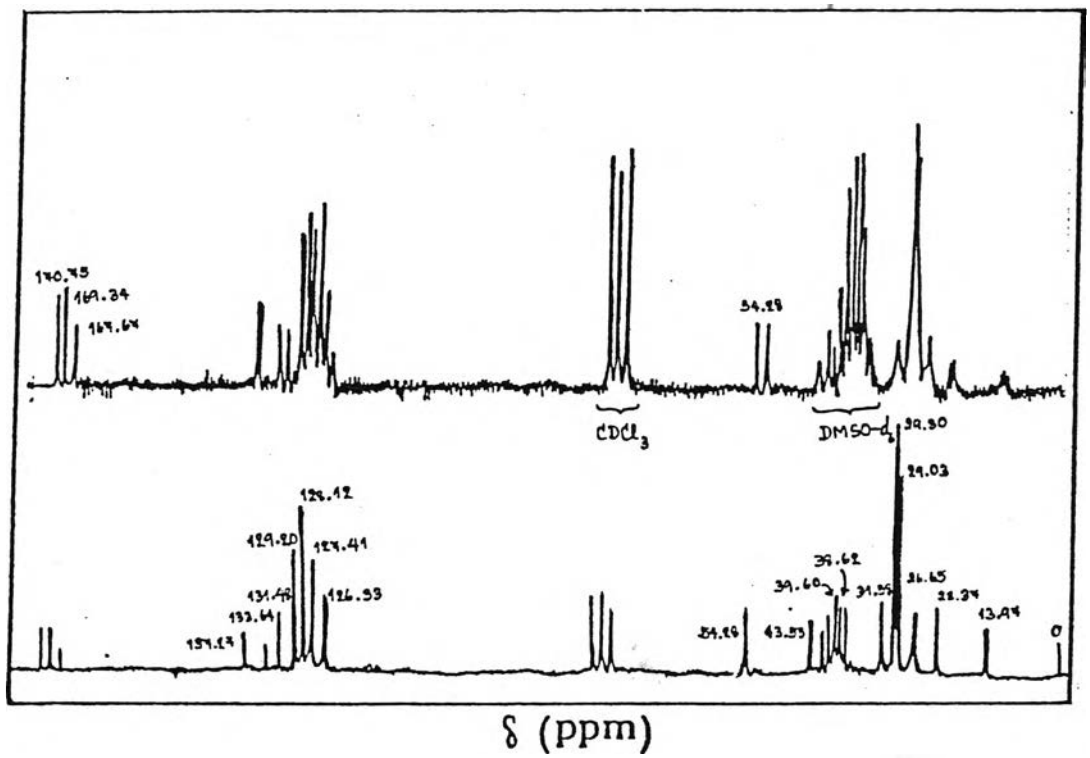


Fig.III.27 ^{13}C NMR spectrum of compound VII in $CDCl_3$+DMSO-d_6.

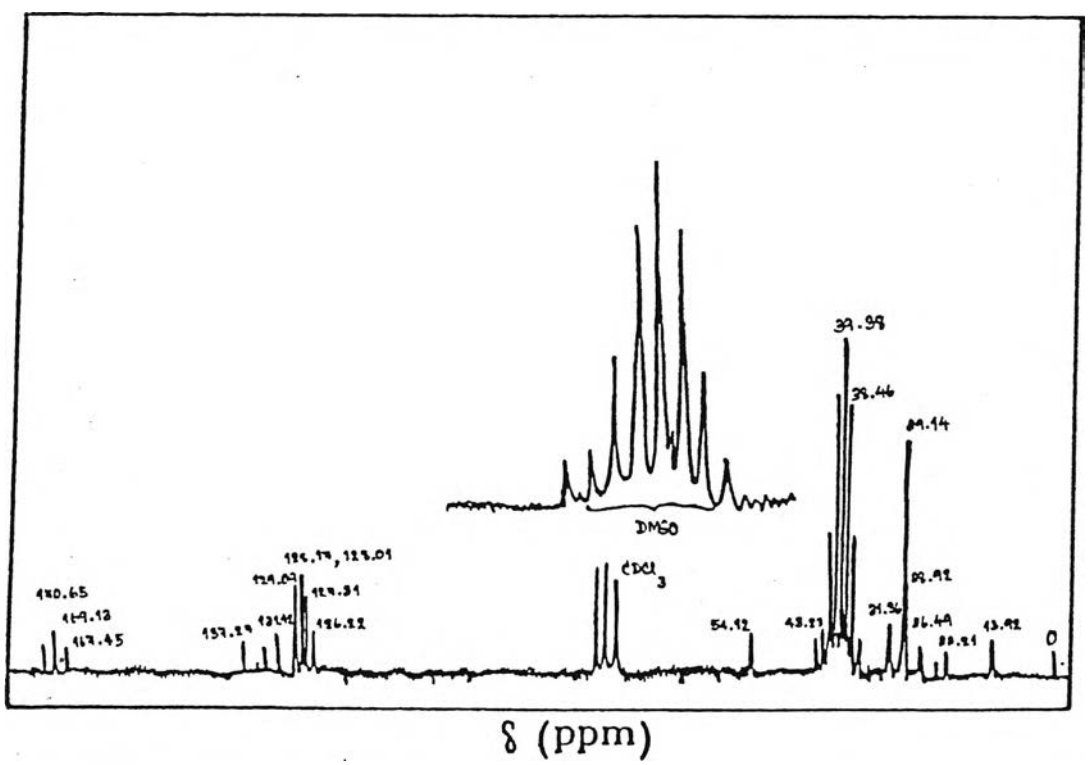


Fig.III.28 ^{13}C NMR spectrum of compound VIII in $CDCl_3$+DMSO-d_6.

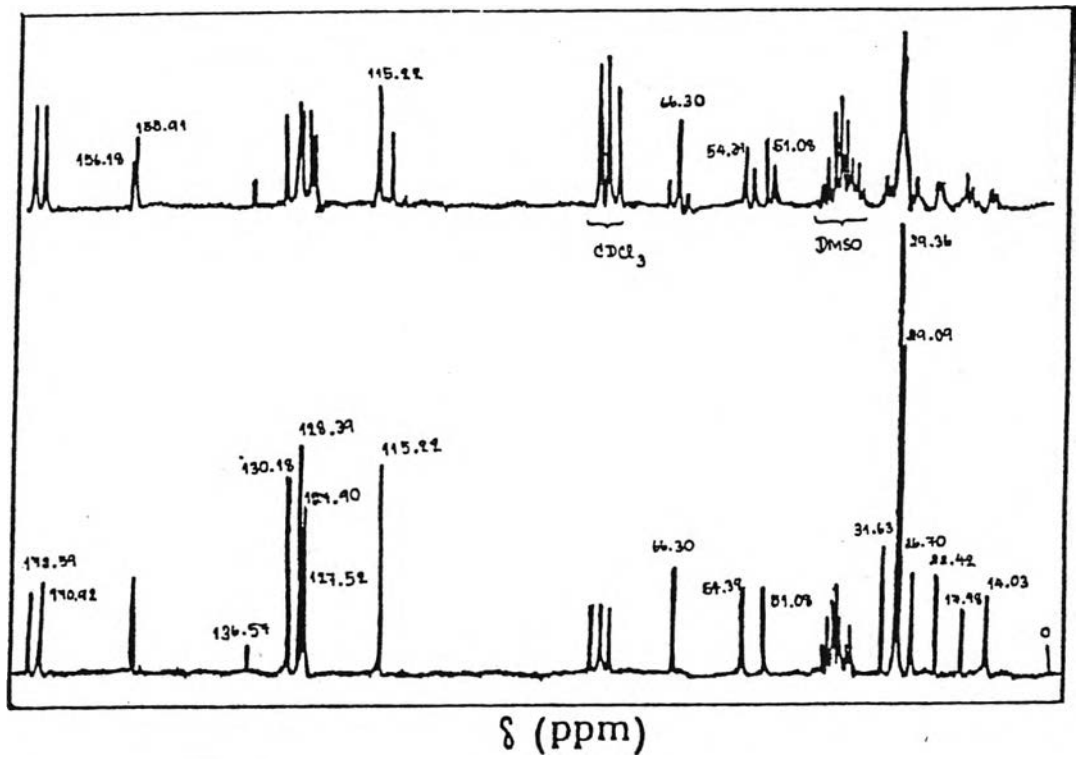


Fig.III.29 ^{13}C NMR spectrum of compound IX in $CDCl_3$+DMSO-d_6.

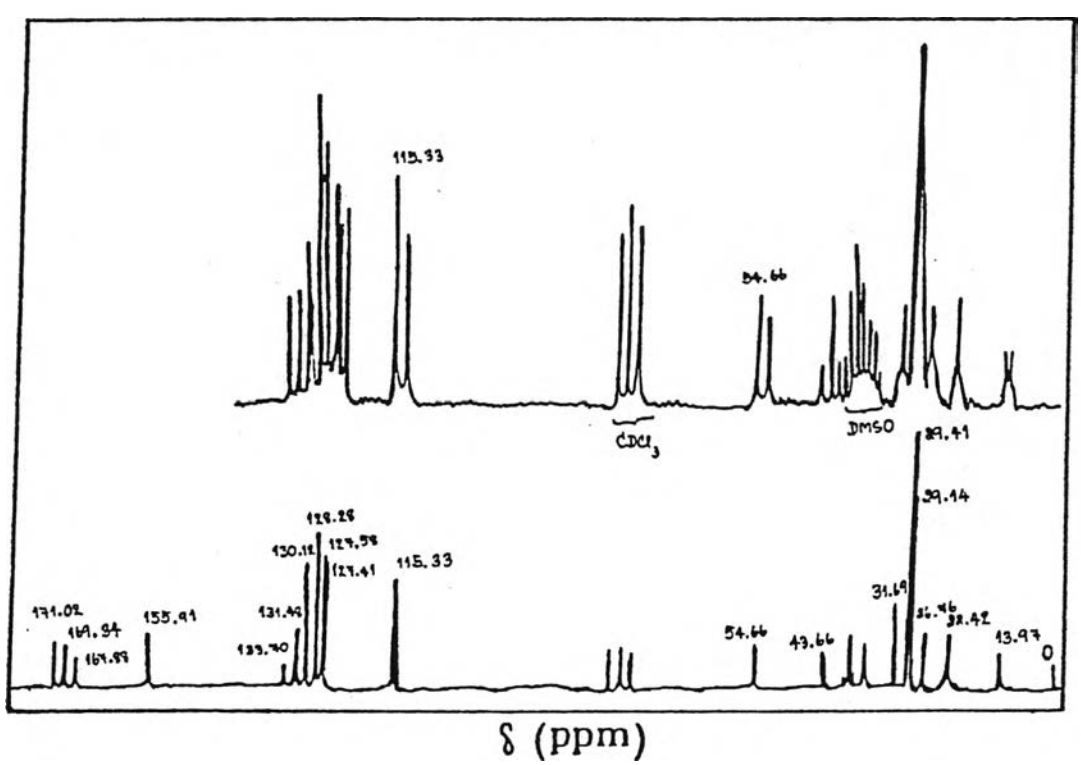


Fig.III.30 ^{13}C NMR spectrum of compound X in $CDCl_3$+DMSO-d_6.

VITA

Miss Wimon Pornsawatchai was born on December 16th, 1963 in Bangkok, Thailand. She has two brothers and one sister, and she is the oldest of her family. In 1986, she received her Bachelor degree of Science in the branch of chemistry from Chulalongkorn university. Since then she has been a graduate student, taking organic chemistry as her major course, in the Department of Chemistry, Faculty of Science, Chulalongkorn university. Her present address is 9/167 Asok-Dindaeng Road, Huaikhwang, Bangkok 10310.