CHAPTER II

EXPERIMENTAL AND RESULTS

2.1 Starting materials and purification of solvents

2.1.1 Starting materials

Chemical used in this research work were obtained from the following sources :

Tetrahydrofuran (THF), triethylamine, chloroform, methanol, dimethyl sulfoxide (DMSO) were obtained from Fluka Company.

The buffer, HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethane-sulfonic acid) was purchased from Sigma Chemical Company.

Ethyl chloroformate, N-octylamine, dodecylamine, decylamine, 1-tetradecylamine, succinic anhydride, t-BOC-L-Alanine (t-Butyl-oxy-carbonyl-L-alanine), t-BOC-L-Valine (t-Butyl-oxy-carbonyl-L-valine), t-BOC-L-Alanine-ONP (BAN : t-Butyl-oxy-carbonyl-L-alanine-p-nitro-phenyl ester), trypsin (EC 3.4.21.4), α-chymotrypsin (EC. 3.4.21.1) were obtained from Professor Dr.Bela Ternai of La Trobe University in Australia.

2.1.2 Purification of solvents

1. Tetrahydrofuran (THF) 2.5 litres was distilled over 10 g. of cuprous chloride and stored over a type 5A molecular sieves (17).

2. Triethylamine 200 mls. was distilled over sodium pellets and stored over molecular sieves (18).

3. Chloroform was stabilised to light by the addition of 1% of ethanol. To remove ethanol, it was shaken for one day with anhydrous calcium chloride, filtered and distilled, neglecting the turbid first runnings. Purified chloroform was used immediately (19,20).

4. methanol : The small residual amount of water was removed by treatment with magnesium methoxide. In a two litres flask fitted with a reflux condenser and a drying tube was placed 5 g. of magnesium (metal), 0.5 g. of iodine and 50 mls. of methanol. The mixture was warmed on a steam bath until the iodine color disappeared and vigorous evolution of hydrogen occurred. After the methoxide has been formed ; 1 lit. of methanol was added, and the mixture was heated to reflux for 30 minutes, the alcohol was then distilled, with precaution taken to prevent entry of moisture (21).

2.2 Instruments used

The melting points were determined with a cover glass sample holder electrothermal melting point apparatus.

pH M-83 Radiometer pH meter were used for pH measurements.

Microanalyses were carried out by the technical staff at the Scientific and Technological Research Equipment Centre (STREC) of Chulalongkorn University.

The following instruments were used to obtain the UV, IR, NMR and Elemental Analysis respectively :-

UV-visible spectrophotometer, UV-240 Shimadzu with 10 mm.-matched quartz cells- and was fitted with a thermostatic cell compartment, operating at 37 ± 0.4°C

Infrared spectrophotometer, IR-440 Shimadzu

Fourier Transform NMR spectrometer, JNM-FX 90 Q JEOL at 21.1 tesla.

Elemental Analyzer, 240C Perkin-Elmer.

2.3 Syntheses

2.3.1 General Synthetic Procedure

A -5°C solution of triethylamine (12.5 mmoles) and an N-protected amino acid (12.5 mmoles) in 25 ml. dry THF was added to a solution of ethylchloroformate (25 mmoles) in dry THF at -10°C. The solution was stirred for 8 minutes. A -10°C solution of an amino compound (13.8 mmloes) in dry THF (20 ml.) was added quickly with stirring. The solution was allowed to stir at room temperature for 10 hours. The solvent was removed under reduced pressure and the residue was taken up in ethyl acetate (180 ml.). The organic layer was successively washed with 5% $NaHCO_3$ (65 ml.), water (65 ml.), 1M. HCl (65 ml.) respectively. It was dried by using a dehydrating agents. The solvent was removed under reduced pressure to yield a white solid or oil. The product was purified by either recrystallization from a mixed solvents of ethyl acetate and hexane or column chromatography using silica gel as absorbent and 50%MeOH in CH_2Cl_2 as an eluent. The yields were varied from 44% to 80%. All products and intermediates had the expected ^1H NMR spectra and correct microanalysis.

2.3.2 Specific Procedures

2.3.2.1 Preparation of t-BOC-A-NH-C_8H_{17} (Compound 11) [22]

The solution of t-BOC-A-OH (0.95 g., 5 mmoles) in dry THF (12.5 ml.) and triethylamine (0.7 ml., 5 mmoles) was cooled to -10°C before ethyl chloroformate (0.94 ml., 10 mmoles) was added. The solution was then stirred for 8 minutes (activation time) at -10°C before a solution of octylamine (2.8 g., 15 mmoles) dissolved in 20 ml. THF and triethylamine (0.7 ml., 5 mmoles) was added dropwise.

After allowing the reaction mixture to stir at the room temperature for 10 hrs., the solvent was removed under reduced pressure. To the aqueous solution was mixed with 75 ml. ethyl acetate and 5% $NaHCO_3$ (25 ml.) followed by shaking for extraction. Then the ethyl acetate layer was separated and washed with water (25 ml.). 1M. HCl (25 ml.) and water (25 ml.) respectively. After drying over anhydrous Na_2SO_4 , evaporation of the ethyl acetate, to yield a light yellow oil. (1.05 g., 70%) b.p. = 264-267°C. TLC : R_f = 0.68 ($CHCl_3$/MeOH/AcOH, 85:10:5, v/v)

2.3.2.2 Preparation of t-BOC-A-NH-$C_{10}H_{21}$ (Compound 12)

A solution of ethyl chloroformate (0.94 ml., 10 mmoles) in THF (25 ml.) was stirred and cooled to -10°C. To this was added dropwise over 3 minutes, a previously cooled (-10°C) solution of triethylamine (0.7 ml., 5 mmoles) and t-BOC-Ala-OH (0.95 f., 5 mmoles) in THF (12.5 ml.). The mixture was stirred at -10°C for 8 minutes (activation time) before a solution of decylamine (2.36 g., 15 mmoles) in THF (30 ml.) was added. The solution was then stirred at the room temperature for 10 hrs., the solvent was removed under vacuum. To the white crystalline solid was added ethyl acetate (75 ml.) and 5% $NaHCO_3$ (25 ml.).

Following thorough shaking and then separation, the ethyl acetate layer was washed with water (25 ml.), 1M.HCl (25 ml.) and finally with water (25 ml.). After drying over anhydrous Na_2SO_4 evaporation of the ethyl acetate, yield a white crystalline solid (0.98 g., 60%), m.p.= 53-56°C, recrystallizing solvent : ethyl acetate : Hexane (1:3). TLC : R_f = 0.70 ($CHCl_3$/MeOH/AcOH, 85:10:5,v/v)

2.3.2.3 Preparation of t-BOC-A-NH-$C_{14}H_{29}$ (Compound 13)

A solution of ethyl chloroformate (0.94 ml., 10 mmoles) in THF (25 ml.) was stirred and cooled to -10°C. To this was added dropwise over 3 minutes, a previously cooled (-10°C) solution of triethylamine (0.7 ml., 5 mmoles) and t-BOC-Ala-OH (0.95 g., 5 mmoles) in THF (12.5 ml.). The mixture was stirred at -10°C for 8 minutes (activation time) before a solution of tetradecylamine (3.2 g., 15 mmoles) in THF (30 ml.) was added.

After allowing solution to stir at the room temperature for 10 hrs., the solvent was removed under vacuum. To the white crystalline solid was added ethyl acetate (75 ml.) and 5% $NaHCO_3$ (25 ml.)

Following thorough shaking and then separation, the ethyl acetate layer was washed with water (25 ml.), 1M.HCl (25 ml.) and finally with water (25 ml.). After drying over anhydrous Na_2SO_4 evaporation of the ethyl acetate, yield a white crystalline solid (1.25 g., 65%) m.p. = 72-74°C. TLC : R_f = 0.75 ($CHCl_3$/MeOH/AcOH, 85:10:5, v/v)

2.3.2.4 Preparation of t-BOC-V-NH-$C_{12}H_{25}$ (Compound 14)

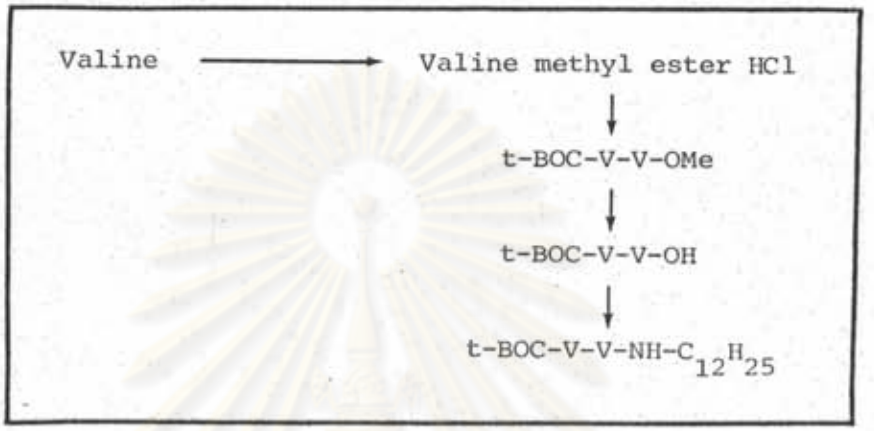
A solution of ethyl chloroformate (0.94 ml., 10 mmoles) in THF (25 ml.) was stirred and cooled to -10°C. To this was added dropwise over 3 minutes, a previously cooled (-10°C) solution of triethylamine (0.7 ml., 5 mmoles) and t-BOC-Val-OH (1.085 g., 5 mmoles) in THF (12.5 ml.). The mixture was stirred at -10°C for 8 minutes (activation time) before a solution of dodecylamine (2.8 g., 15 mmoles) in THF (30 ml.) was added.

After allowing solution to stir at the room temperature for 10 hrs., the solvent was removed under vacuum. To the white crystalline solid was added ethyl acetate (75 ml.) and 5% $NaHCO_3$ (25 ml.)

Following thorough shaking and then separation, the ethyl acetate layer was washed with water (25 ml.), 1MHCl (25 ml.) and finally with water (25 ml.). After drying over anhydrous Na_2SO_4 evaporation of the ethyl acetate, yield a white crystalline solid (1.35 g., 70%) m.p. = 73-76°C. TLC : R_f = 0.84 ($CHCl_3$/MeOH/AcOH, 85:10:5, v/v)

2.3.2.5 Preparation of t-BOC-V-V-NH-$C_{12}H_{25}$ (Compound 15)[23]

There are four steps of reaction involved in this preparation. The method of this preparation is schematically described below :



Step 1 : Preparation of Valine methyl ester HCl

Into a stirred suspension of valine (12 g., 0.1 mole) in dry methanol (120 ml.) a dry HCl gas was bubbled through until it was saturated,keeping the solution cool to -5°C or lower at all time. The saturated HCl solution was then stoppered and allowed to stand for 2 days at below 0°C. The solvent was removed under reduced pressure. Any precipitated salt was dissolved in dry methanol and the solvent was removed under reduced pressure. The hygroscopic solid of the hydrochloride salt of valine methyl ester (11.9 g., 90%, m.p.= 170-172°C) was obtained and stored at below 0°C.

Step 2 : Preparation of t-BOC-V-V-OMe

The solution of t-BOC-V-OH (4.34 g., 20 mmoles) in 20 ml. dry THF and triethylamine (2.8 ml., 20 mmoles) was cooled to -10°C with stirring before the addition of ethyl chloroformate (1.9 ml., 20 mmoles). The mixture was further kept at -10°C for 20 min. with continuous stirring. To this solution was added dropwise a solution

of valine methyl ester HCl from step 1 (4.64 g., 20 mmoles) in 15 ml. THF, 2 ml. water and triethylamine (2.8 ml., 20 mmoles). The solution was stirred continuously for 15 hrs. at room temperature. The solution was then placed in a separating funnel and 70 ml. ethyl acetate and 25 ml. water were added. The organic layer was collected and the aqueous layer was extracted with a further 160 ml. of ethyl acetate. The combined organic layers were then washed with 30 ml. of 1M.HCl, 5% $NaHCO_3$ (60 ml.) and water (60 ml.) respectively and then dried with $MgSO_4$. After filtration the solvent was removed under reduced pressure to give a waxy white solid of t-BOC-V-V-OMe (5.14 g., 78%), m.p.=131-133°C.

Step 3 : Preparation of t-BOC-V-V-OH

A solution of t-BOC-V-V-OMe (3.3 g., 0.01 mole) previously made from step 2, in dry methanol (40 ml.) was cooled to 0°C, 1M.NaOH (22 ml.) was added, and the mixture was then stirred for 5 hrs. at room temperature. The gelatinous precipitate was formed and was acidified with 1M.HCl under cooling condition. The mixture was kept at 4°C overnight, and extracted three times with a mixture of ethyl acetate (75 ml.), 10% $NaHCO_3$ (25 ml.) and dried with Na_2SO_4. The ethyl acetate layer was separated and then the solvent was removed under reduced pressure. A white powder was obtained (1.85 g., 60%) m.p. = 145-147°C.

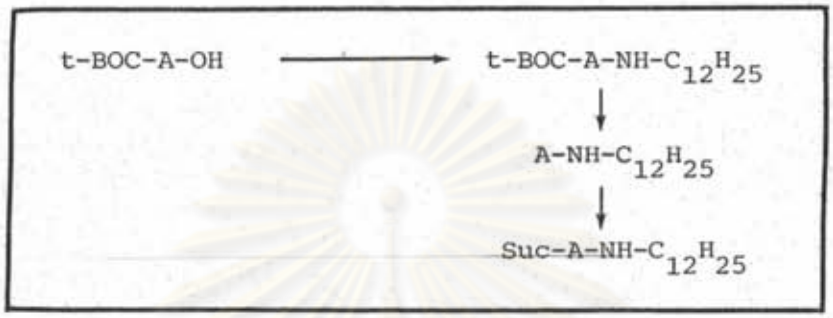
Step 4 : Preparation of t-BOC-V-V-NH-$C_{12}H_{25}$

The ethyl chloroformate (0.94 ml., 10 mmoles) in THF (25 ml.) was stirred and cooled to -10°C, and added to a solution of triethylamine (0.7 ml., 5 mmoles) and t-BOC-V-V-OH ; was made from step 3, (0.82 g., 2.6 mmoles) in THF 12.5 ml. The mixture was stirred at -10°C for 8 minutes (activation time) before a solution of

dodecylamine (1.4 g., 7.8 mmoles) in THF (30 mls.) was added. After allowing the solution to stir at the room temperature for 10 hrs., the solvent was removed under reduced pressure. To the white crystalline solid was added ethyl acetate (75 ml.) and 5%$NaHCO_3$ (25 ml.) and the mixture was shaken thoroughly a few times. The ethyl acetate layer was collected and washed with H_2O (25 ml.), 1M.HCl (25 ml.) and finally with H_2O (25 ml.) After drying over anhydrous Na_2SO_4, the ethyl acetate was evaporated and the solid was recrystallised from ethyl acetate : hexane (1:3) to form white crystals (1.27 g., 78%) m.p. = 107-110°C. TLC : R_f = 0.87 ($CHCl_3$/ MeOH/AcOH, 85:10:5, v/v)

2.3.2.6 Preparation of Suc-A-NH-$C_{12}H_{25}$ (Compound 16) [24]

There are three steps of reaction involved in this preparation. The method of this preparation is schematically decribed below:



Step 1 : Preparation of t-BOC-A-NH-$C_{12}H_{25}$ (Compound 9)

A solution of ethyl chloroformate (0.94 ml., 10 mmoles) in THF (25 ml.) was stirred and cooled to -10°C. To this was added dropwise over 3 minutes, a previously cooled (-10°C) solution of triethylamine (0.7 ml., 5 mmoles) and t-BOC-A-OH (0.95 g., 5 mmoles) in THF (12.5 ml.). The mixture was stirred at -10°C for 8 minutes (activation time) then a solution of dodecylamine (2.8 g., 15 mmoles) in THF (30 ml.) was added. After allowing the solution to stir at the room temperature for 10 hrs., the solvent was removed under reduced pressure to form a white crystalline solid. It was then added ethyl acetate (75 ml.) and 5% $NaHCO_3$ (25 ml.) and should thoroughly a few times.The ethyl acetate layer was collected and washed with water (25 ml.), 1M.HCl (25 ml.), and finally with water (25 ml.). After drying over anhydrous Na_2SO_4 evaporation of the ethyl acetate, yielded a white crystalline solid (1.18 g., 66%) m.p. = 67-69°C.

Step 2 : Removal of t-BOC from t-BOC-A-NH-$C_{12}H_{25}$

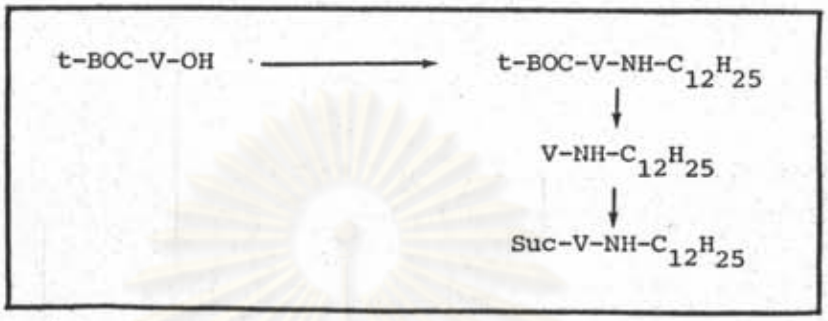
t-BOC-A-NH-$C_{12}H_{25}$ from step 1 (0.6 g., 1.76 mmoles) was dissolved in a saturated solution of HCl in ethyl acetate (8 ml.). After 2 hrs. of stirring at 25°C, the solvent was removed under reduced pressure and the residue was dehydrated in a dessicator over NaOH pellets for 24 hrs. A white crystalline solid of A-NH-$C_{12}H_{25}$ (0.4 g., 89%) m.p. 78-81°C was obtained. The product was confirmed by proton NMR with the disappearance of the t-CH_3's proton peak δ1.44-1.5.

Step 3 : Succinylation of A-NH-$C_{12}H_{25}$

A mixture of A-NH-$C_{12}H_{25}$ (0.38 g., 1.3 mmoles) was made from step 2 and succinic anhydride (0.5 g., 5 mmoles) in 250 ml. $CHCl_3$ was stirred for 20 hrs., followed by evaporation of the solvent. After thorough trituration of the residue with 400 ml. water for 5 hrs., the product remained as white crystalline powder (0.4 g., 80%) m.p.=100-103°C. TLC : R_f = 0.42 ($CHCl_3$/MeOH/AcOH, 85:10:5,v/v)

2.3.2.7 Preparation of Suc-V-NH-$C_{12}H_{25}$ (Compound 17)[24]

There are three steps of reaction involved in this preparation. The method of this preparation is schematically decribed below:



Step 1 : Preparation of t-BOC-V-NH-$C_{12}H_{25}$

In this preparation, as described in 2.3.2.4 (Preparation of compound 14).

Step 2 : Removal of t-BOC from t-BOC-V-NH-$C_{12}H_{25}$

t-BOC-V-NH-$C_{12}H_{25}$ from step 1 (0.675 g., 1.76 mmoles) was treated with a saturated solution of HCl in ethyl acetate (8 ml.). After 2 hrs. of stirring at 25°C, the solvent was removed under reduced pressure and the residue was dehydrated in a dessicator over NaOH pellets for 24 hrs., to give a white crystalline solid (0.45 g., 80%), m.p.=96-99°C. This product was confirmed by proton NMR with the disappearance of the t-CH_3's proton peak δ 1.44-1.5

Step 3 : Succinylation of V-NH-$C_{12}H_{25}$

A mixture of V-NH-$C_{12}H_{25}$ (0.42 g., 1.3 mmoles) was made from step 2 and succinic anhydride (0.5 g., 5 mmoles) in 250 ml. $CHCl_3$ was stirred for 20 hrs. Then the solvent was removed by evaporation, after thorough trituration of the residue with 400 ml. water for 5 hrs., the product was remained as the white crystalline powder (0.53 g., 72%) m.p.=180-182°C. TLC : R_f = 0.45 ($CHCl_3$/MeOH/AcOH, 85:10:5, v/v)

2.4 Purity Verification of the synthetic peptides

The synthetic peptides were readily purified by recrystallisation several time from various solvents until their sharp melting points were obtained. The purity of the synthetic peptides was further verified by thin-layer chromatography and elemental analysis. The experiments and results are described in the following section.

2.4.1 Thin-layer chromatography of the synthetic peptides

A thin-layer chromatography (25, 26, 27) was employed to determine the purity of both the starting materials and the synthetic peptides by using a plastic TLC plate which coated with silica gel (60F Merck) and the thickness was approximately 0.2 mm. Spot on the TLC plates approximately 1 µl. each of the starting materials and the synthetic peptides in ethyl acetate which was equivalent to about 0.001 g/µl. The TLC plate was then developed several times on different solvents with various ratio of the solvents. By comparisons the differences between the R_f values of the starting materials and their corresponding synthetic peptides under the same TLC system, it was ascertained that the synthetic peptides were purely formed since each of them showed a single regular round spot without tailing when exposed to UV radiation.

2.4.2 Elemental Analysis of the synthetic peptides

Purities of the synthetic peptides can also be verified by the method of elemental analysis. Percentages of carbon, hydrogen, nitrogen and oxygen content in these substances were analysed and compared with those calculated ones. The resulting were shown in Table 2.1.

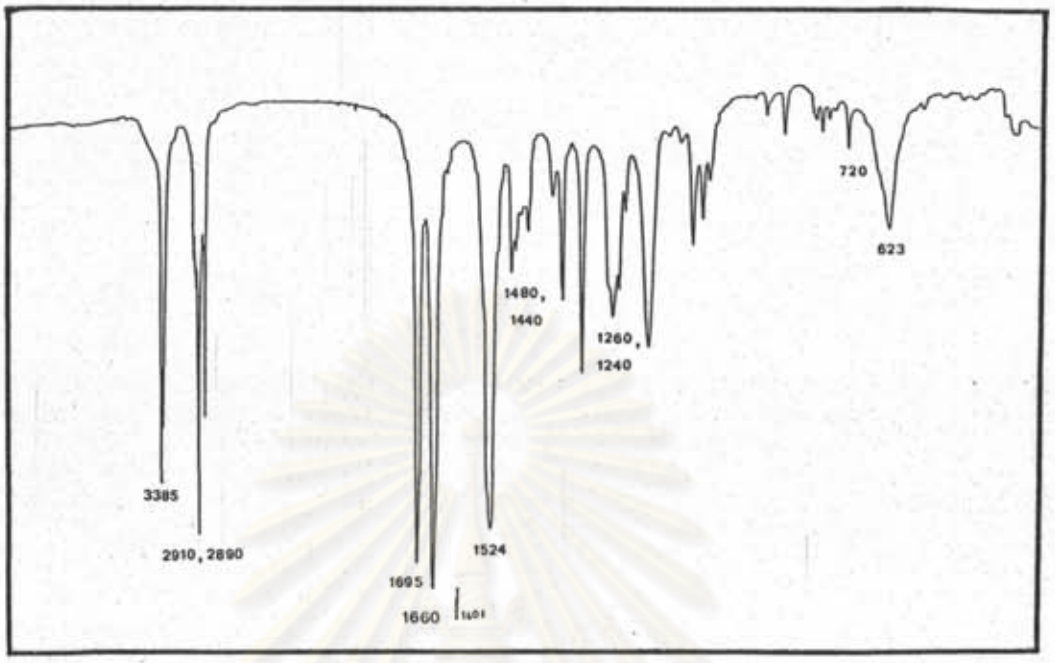
Table 2.1 The elemental analysis of synthesised peptides

No.	Compound	Calc. and Found	Elemental Analysis (%)			
			C	H	N	O
11.	t-BOC-A-NH-C_8	Calc. for $C_{16}H_{32}O_3N_2$	64.00	10.67	9.33	16.00
		Found	64.02	10.70	9.31	16.08
12.	t-BOC-A-NH-C_{10}	Calc. for $C_{18}H_{36}O_3N_2$	65.85	10.98	8.54	14.63
		Found	65.74	10.95	8.59	14.52
13.	t-BOC-A-NH-C_{14}	Calc. for $C_{22}H_{44}O_3N_2$	68.75	11.46	7.29	12.50
		Found	68.69	11.38	7.31	12.48
14.	t-BOC-V-NH-C_{12}	Calc. for $C_{22}H_{44}O_3N_2$	68.75	11.46	7.29	12.50
		Found	68.70	11.42	7.25	12.43
15.	t-BOC-V-V-NH-C_{12}	Calc. for $C_{27}H_{53}O_4N_3$	67.08	10.97	8.69	13.25
		Found	67.19	10.89	8.72	13.09
16.	Suc-A-NH-C_{12}	Calc. for $C_{19}H_{36}O_4N_2$	64.04	10.11	7.86	17.97
		Found	64.12	10.06	7.79	17.64
17.	Suc-V-NH-C_{12}	Calc. for $C_{21}H_{40}O_4N_2$	65.62	10.42	7.29	16.67
		Found	65.58	10.40	7.25	16.56

2.5 Structural Elucidation of the synthetic peptides

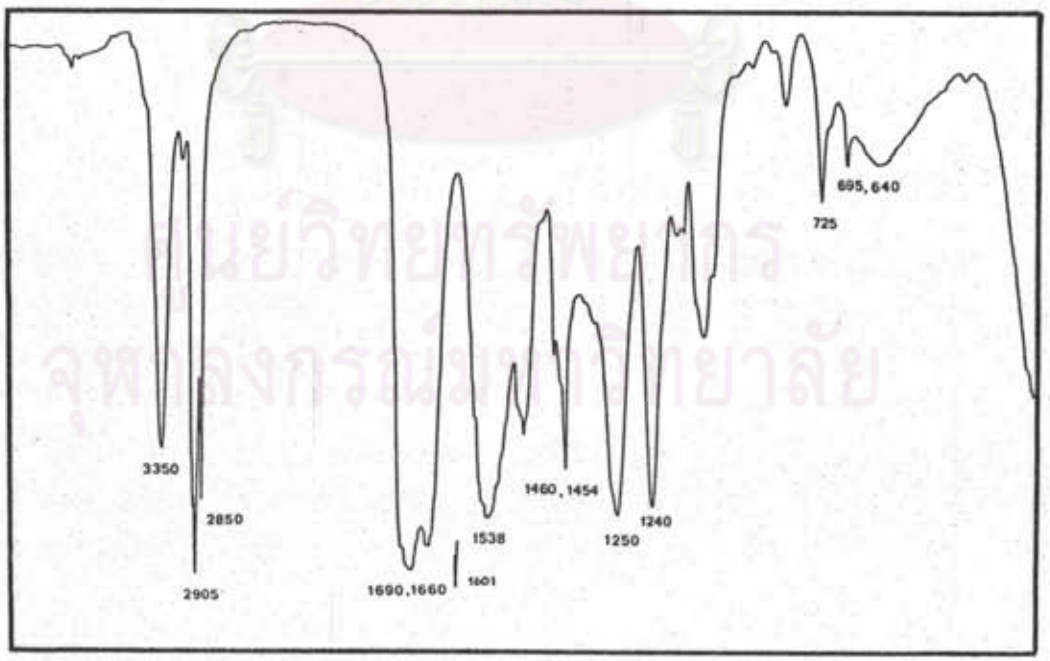
2.5.1 By Infrared Spectroscopy

Infrared spectra are useful to interprete empirically the structure of the compounds by observing their functional group frequencies and finger prints. The spectra of these synthetic peptides were compared with spectra of their similar skeletoned known compounds. The IR spectra of these compounds and their assignments of various important bands were given as follows. These spectra were recorded on a Shimadzu IR-440 infrared spectrophotometer by using pure liquid or solid samples in KBr cell or KBr disc accordingly. The IR spectra are shown in Fig.2.1 to Fig.2.8 and their corresponding assignments of various bands in each of the synthetic peptides are described in Table 2.2 to Table 2.5.



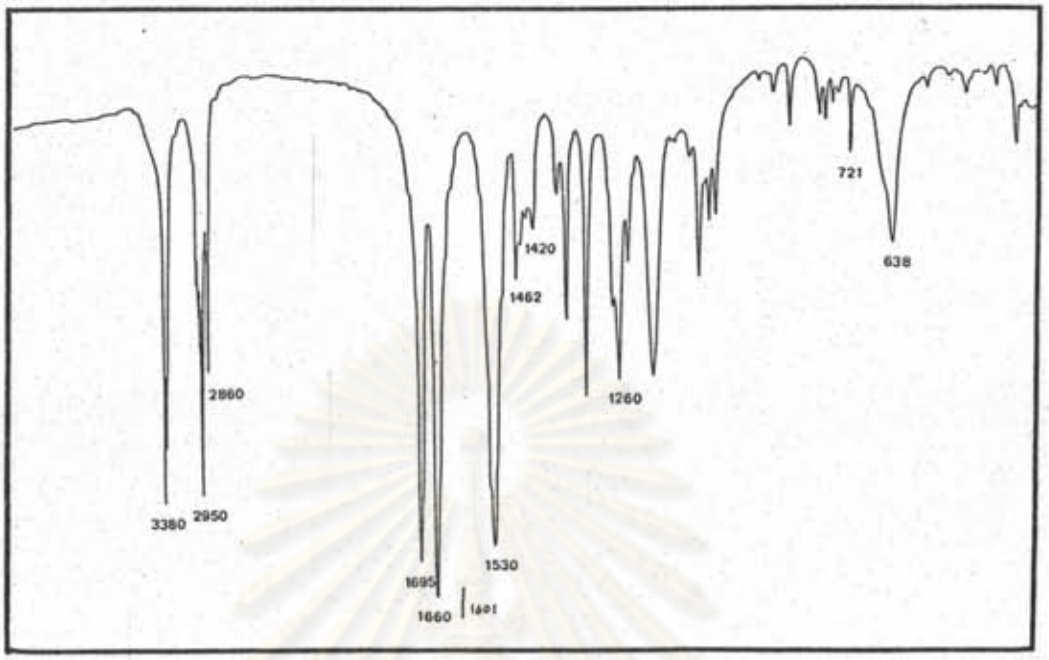
wavenumber (cm^{-1})

Fig. 2.1 IR Spectrum of compound 9 in KBr disc.



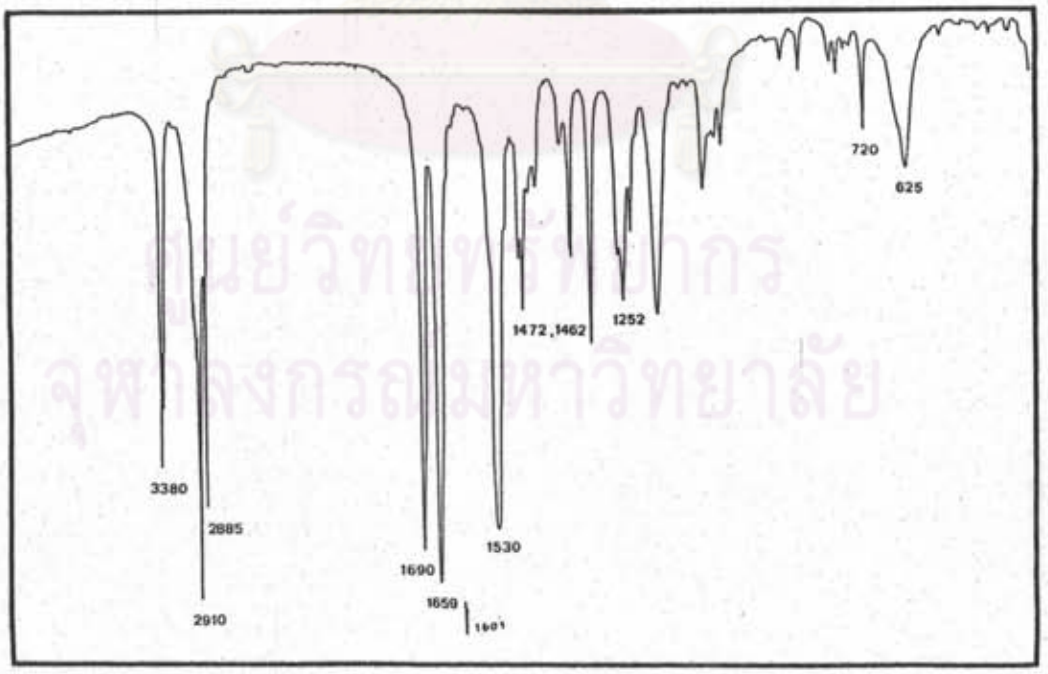
wavenumber (cm^{-1})

Fig. 2.2 IR spectrum of compound 11 (liquid film on KBr cell)



wavenumber (cm^{-1})

Fig. 2.3 IR Spectrum of compound 12 in KBr disc.

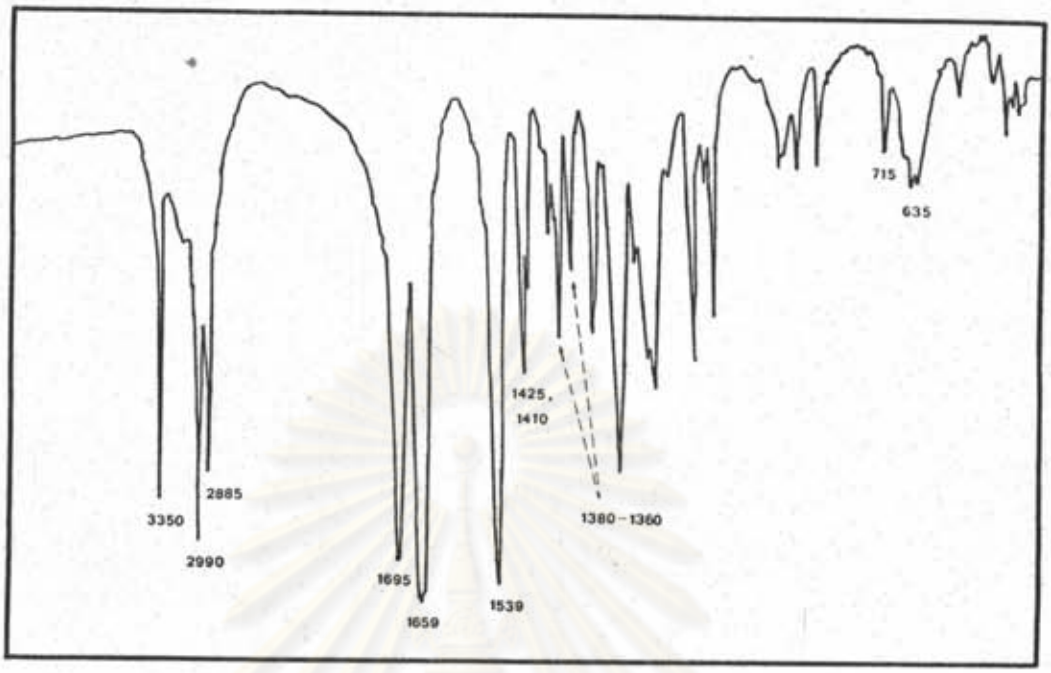


wavenumber (cm^{-1})

Fig. 2.4 IR Spectrum of compound 13 in KBr disc.

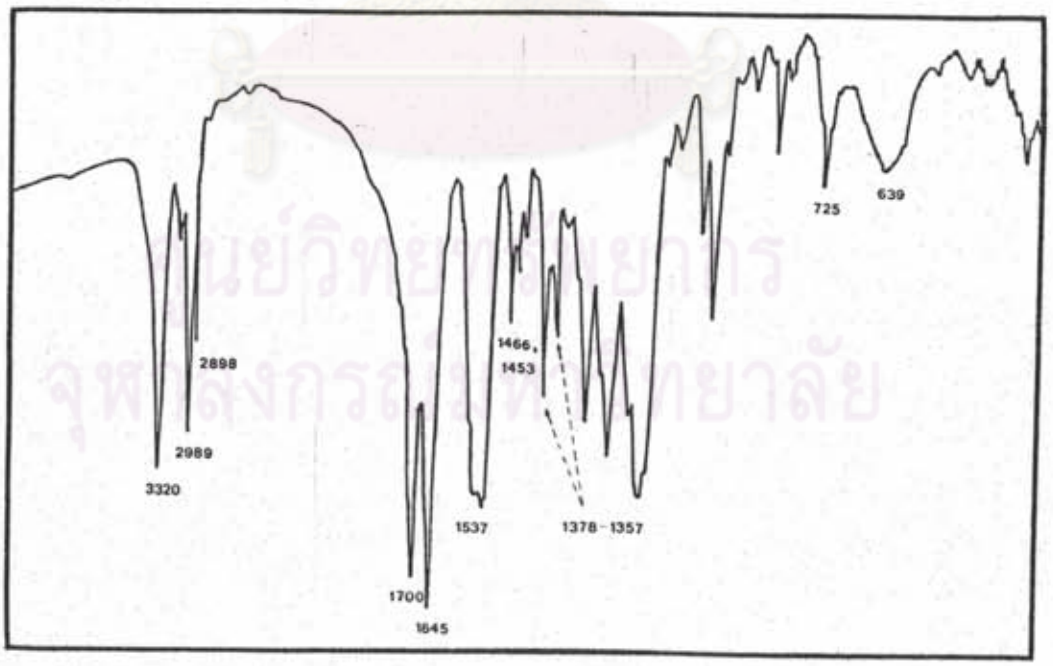
Table 2.2 Assignment of the various important bands in the IR spectrum of compound 9,11,12 and 13

No.	Short formula	Absorption peaks max. ($cm.^{-1}$)						
		3385-3350	2950-2850	1695-1659	1538-1524	1480-1420	1260-1238	725-625
9.	t-BOC-A-NH-C_{12}	3385	2910,2890	1695,1660	1524	1480,1440	1260,1240	720-623
11.	t-BOC-A-NH-C_8	3350	2905,2850	1690,1660	1538	1460,1454	1250,1240	725-640
12.	t-BOC-A-NH-C_{10}	3380	2950,2860	1695,1660	1530	1462,1420	1260,1240	720-638
13.	t-BOC-A-NH-C_{14}	3380	2910,2885	1690,1659	1530	1472,1462	1252,1238	720-625
Assignment		antisym. N-H str. and sym. N-H str. (N-H str. of secondary amine group)	antisym. C-H str. and Sym. C-H str.	C=O str. (amide I band) was coupling with N-H bending (amide II band), and N-H in plane bending (amide II band)	N-H in plane bending (amide II band)	C-N str. and N-H bending (amide III band)	C-N str. and N-H bending (amide III band)	N-H out of plane bending (amide V band) and O-C-N bending (amide IV,VI band), and CH_2-chain more than 4 carbons



wavenumber (cm^{-1})

Fig. 2.5 IR Spectrum of compound 14 in KBr disc.



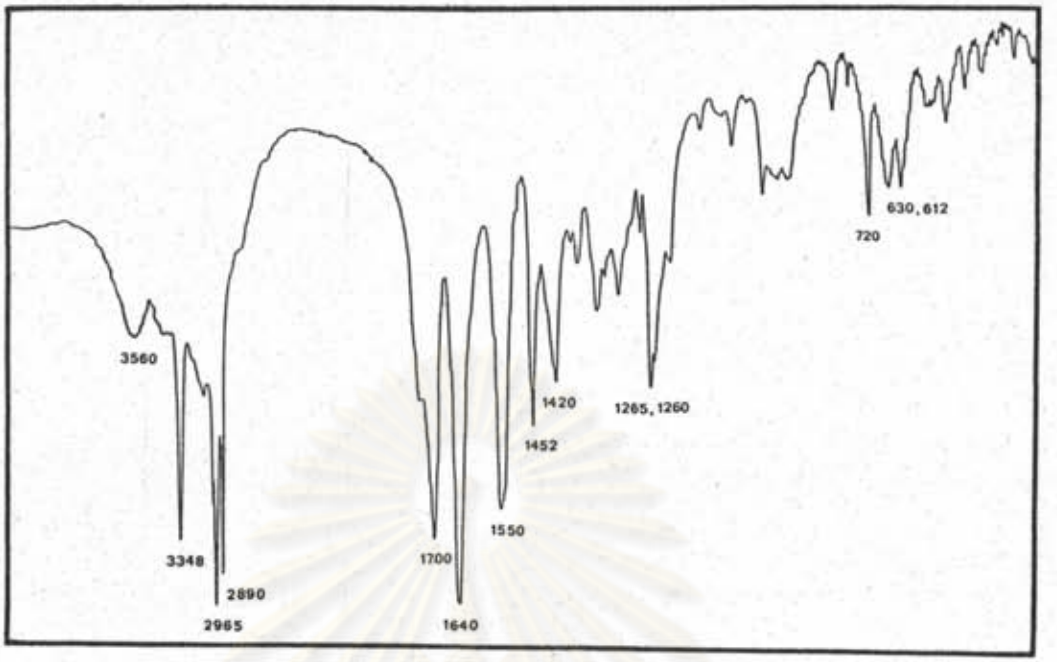
wavenumber (cm^{-1})

Fig. 2.6 IR spectrum of compound 15 in KBr disc.

Table 2.3 Assignment of the various important bands in the IR spectrum of compound 14 and 15

No.	Short formula	Absorption peaks max. ($cm.^{-1}$)						
		3350-3320	2990-2885	1700-1645	1539-1537	1466-1410	1380-1357	725-635
14.	t-BOC-V-NH-C_{12}	3350	2990,2885	1695,1659	1539	1425,1410	1380,1360	715-635
15.	t-BOC-V-V-NH-C_{12}	3320	2989,2898	1700,1645	1537	1466,1453	1378,1357	725-639
Assignment		antisym. N-H str. and sym. N-H str. (N-H str. of secondary amine group)	antisym. C-H str. and sym. C-H str.	C=O str.(amide I band) was coupling with N-H bending (amide II band) and N-H in plane bending (amide II band)	N-H in plane bending (amide II band)	C-N str. and N-H bending (amide III band)	C-H bending bands characteristic of CH_3- and = CH	N-H out of plane bending (amide V band) and O-C-N bending (amide IV,VI band), and CH_2-chain more than 4 carbons

Fig 2.7 IR Spectrum of compound 16 in KBr disc.

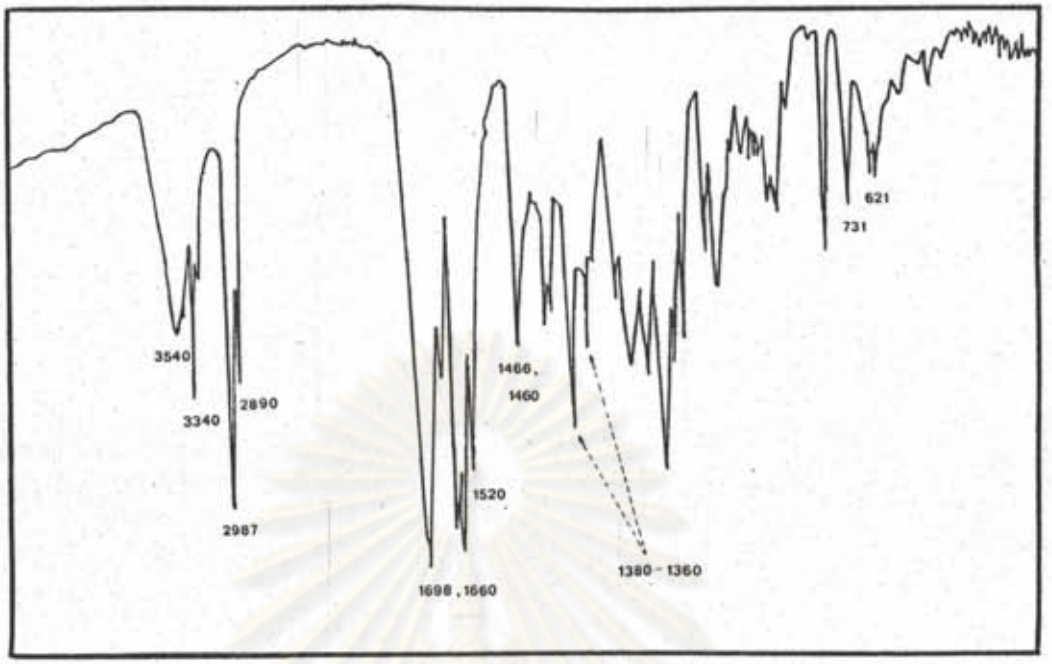


wavenumber ($cm.^{-1}$)

Table 2.4 Assignment of the various important bands in the IR spectrum of Suc-A-NH-C_{12} (No.16)

Band ($cm.^{-1}$)	Assignment
3560-2890	O-H stretching
3348	antisymmetric N-H stretching and symmetric N-H stretching (N-H stretching of secondaty amine group)
2965,2890	antisymmetric C-H stretching and symmetric C-H stretching
1700,1640	C=O stretching (amide I band) was coupling with N-H bending (amide II band), and N-H in plane bending (amide II band)
1550	N-H in plane bending (amide II band)
1452,1420	C-N stretching and N-H bending (amide III band)
1265,1260	C-N stretching and N-H bending (amide III band)
720-612	N-H out of plane bending (amide V band), and O-C-N bending (amide IV,VI band), and CH_2-chain more than 4 carbons

Fig 2.8 IR Spectrum of compound 17 in KBr disc.



wavenumber ($cm.^{-1}$)

Table 2.5 Assignment of the various important bands in the IR spectrum of Suc-V-NH-C_{12} (No.17)

Band ($cm.^{-1}$)	Assignment
3540-2890	O-H stretching
3340	antisymmetric N-H stretching and symmetric N-H stretching (N-H stretching of secondary amine group)
2987,2890	antisymmetric C-H stretching and symmetric C-H stretching
1698,1660	C=O stretching (amide I band) was coupling with N-H bending (amide II band), and N-H in plane bending (amide II band)
1520	N-H in plane bending (amide II band)
1466,1460	C-N stretching and N-H bending (amide III band)
1380,1360	C-H bending bands characteristic of CH_3- and CH
731-621	N-H out of plane bending (amide V band), and O-C-N bending (amide IV, VI band), and CH_2-chain more than 4 carbons

2.5.2 By Nuclear Magnetic Resonance Spectroscopy

Structures of the synthetic peptides can be elucidated by using the proton and carbon 13 NMR spectroscopy. The sample was dissolved in a suitable solvent prior to the operations. The proton NMR is able to characterize the aliphatic and aromatic proton patterns complement while the C-13 NMR indicates the number of carbon atoms and their positions. These spectra were obtained from a FX 90 Q Jeol Fourier transform NMR spectrometer. The result with their corresponding assignments of each synthetic peptides are presented both of the proton and carbon 13 NMR, in Fig. 2.9 to Fig. 2.24 and Table 2.6 to Table 2.3 respectively.

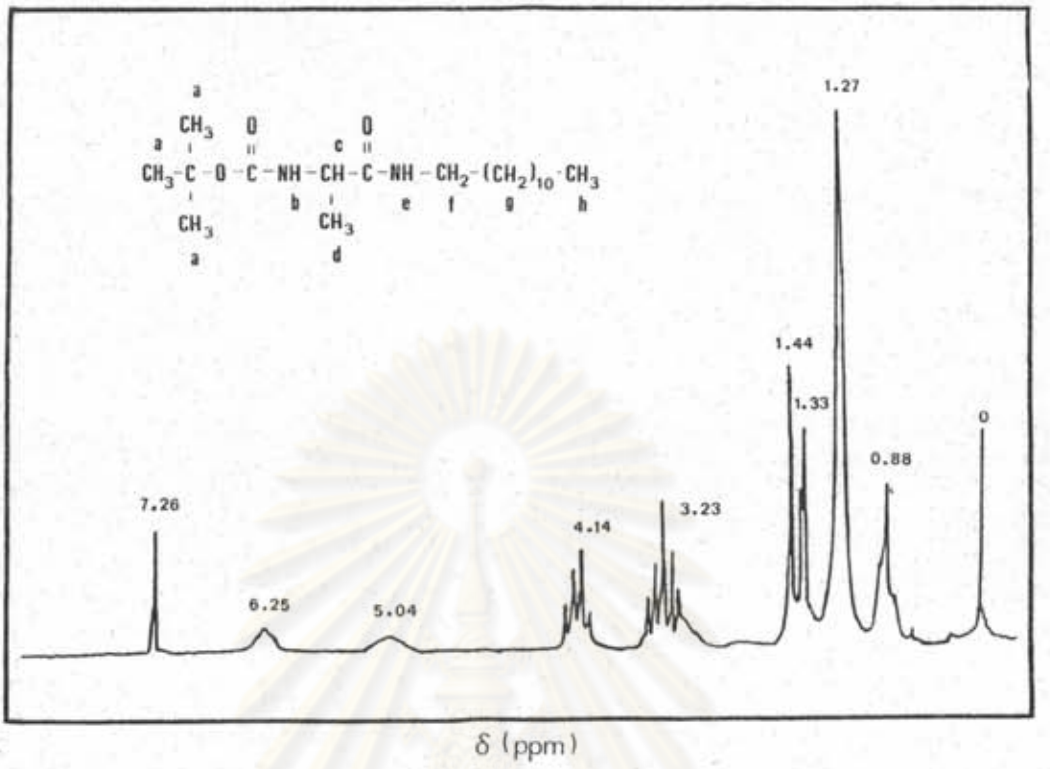


Fig. 2.9 ^{1}H NMR spectrum of compound 9 in $CDCl_3$

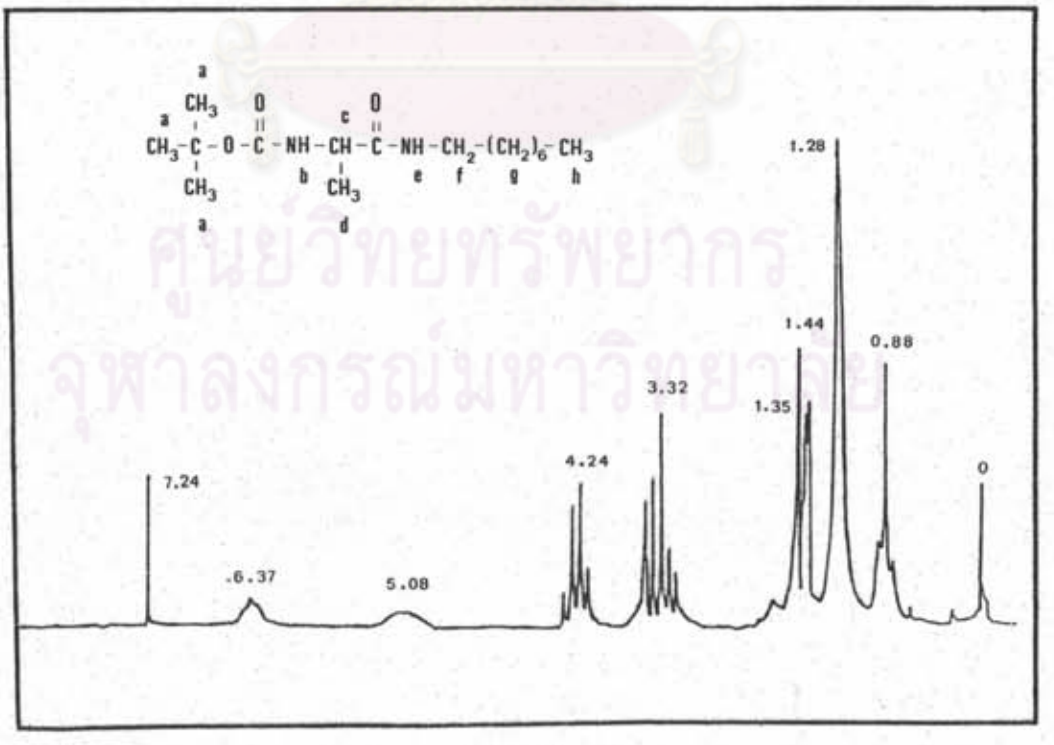


Fig. 2.10 ^{1}H NMR spectrum of compound 11 in $CDCl_3$

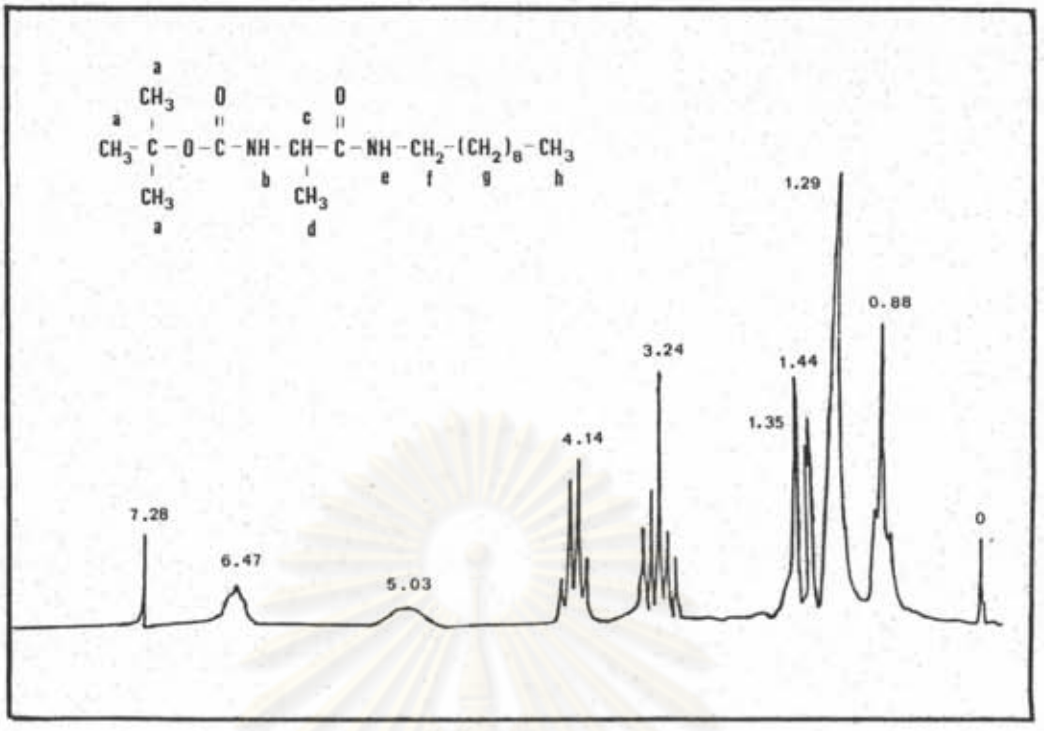


Fig. 2.11 ^{1}H NMR spectrum of compound 12 in $CDCl_3$

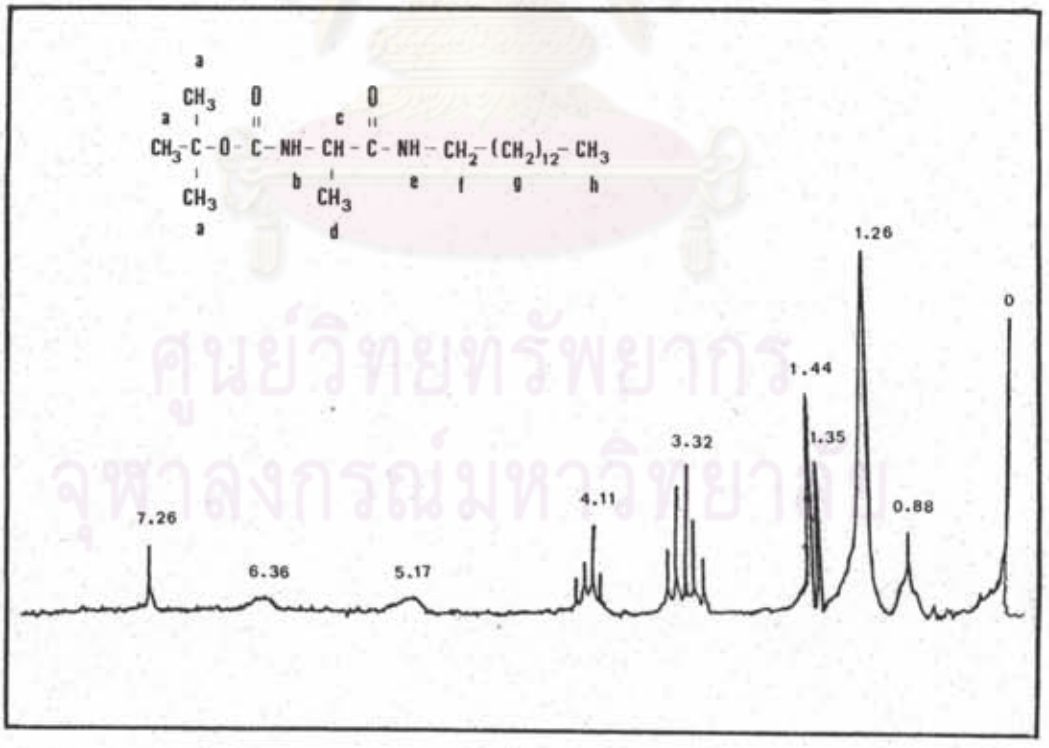
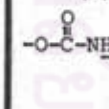
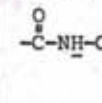
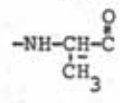
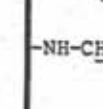
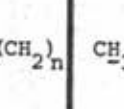
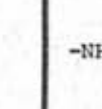
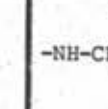
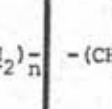


Fig. 2.12 ^{1}H NMR spectrum of compound 13 in $CDCl_3$

Table 2.6 Assignment of the proton NMR signals of compound 9,11,12 and 13

($CDCl_3$ as solvent)

Compound No.	Chemical Shift (ppm)							
	6.25-6.47(br)*	5.03-5.17(br)*	4.11-4.24(g)	3.23-3.32(m)	1.44(s)	1.33-1.35(d)	1.26-1.27(br.s)	0.88(br.t)
9.	6.25	5.04	4.14	3.23	1.44	1.33	1.27	0.88
11.	6.37	5.08	4.24	3.32	1.44	1.35	1.28	0.88
12.	6.47	5.03	4.14	3.24	1.44	1.35	1.29	0.88
13.	6.36	5.17	4.11	3.32	1.44	1.35	1.26	0.88
Tentative Assignments	(b) 	(e) 	(c) 	(f) 	(a) 	(d) 	(g)  n=10,6,8,12 respectively	(h) 

* disappear when drop D_2O

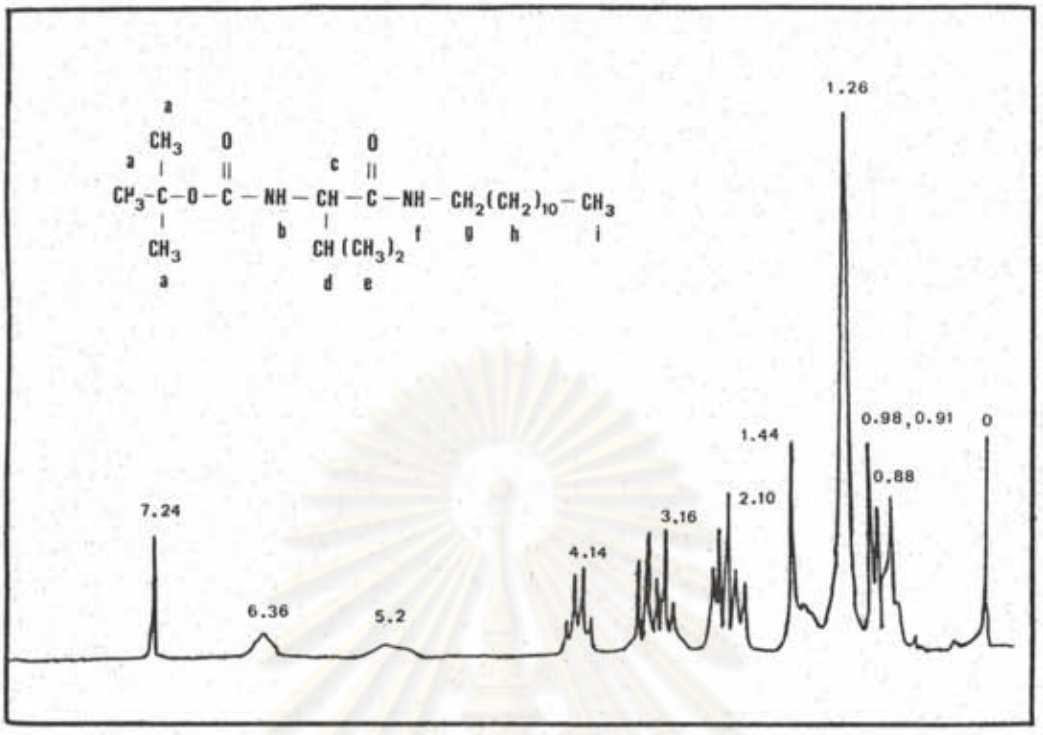


Fig. 2.13 ^{1}H NMR spectrum of compound 14 in $CDCl_3$

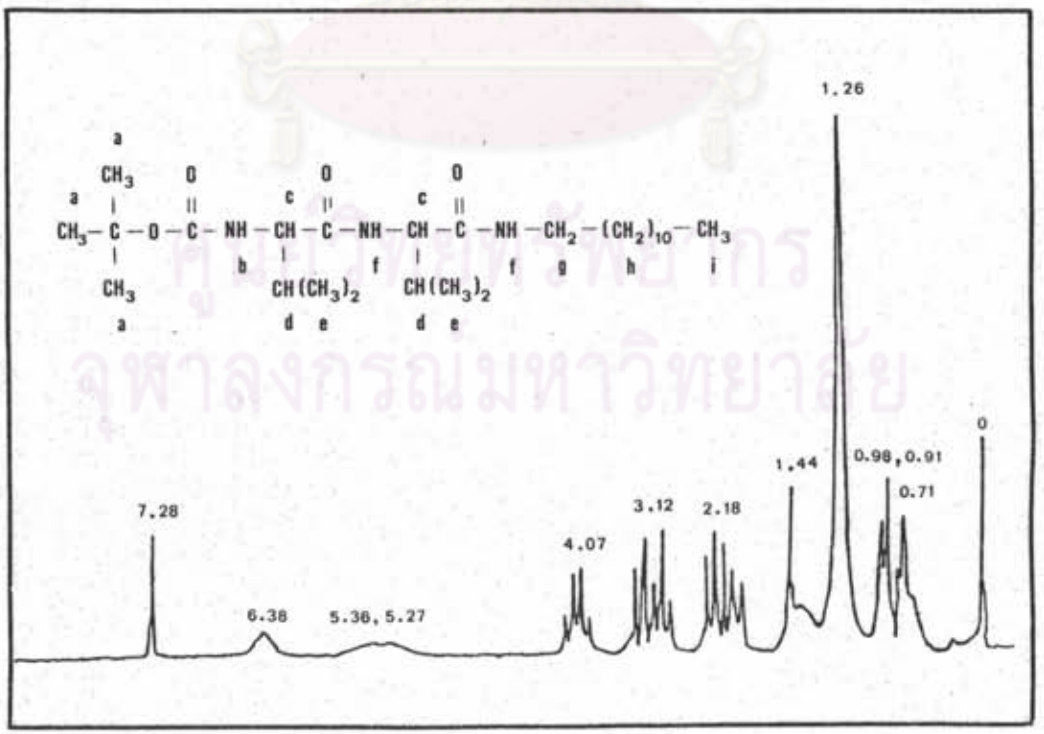
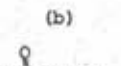
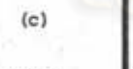
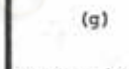
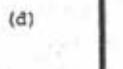


Fig. 2.14 ^{1}H NMR spectrum of compound 15 in $CDCl_3$

Table 2.7 Assignment of the proton NMR signals of compound 14 and 15 ($CDCl_3$ as solvent)

Compound No.	Chemical Shift (ppm)								
	6.36-6.28 (br)*	5.20-5.36 (br)*	4.07-4.14 (q)	3.12-3.16 (m)	2.10-2.18 (m)	1.44 (s)	1.26 (br.s)	0.91-0.98 (d)	0.71-0.88 (t)
14.	6.36	5.20	4.14	3.16	2.10	1.44	1.26	0.98,0.91	0.88
15.	6.38	5.36,5.27	4.07	3.12	2.18	1.44	1.26	0.98,0.91	0.71
Tentative Assignments	(b)	(f)	(c) 	(g) 	(d) 	(a) 	(h) 	(e)	(i) 

* disappear when drop D_2O

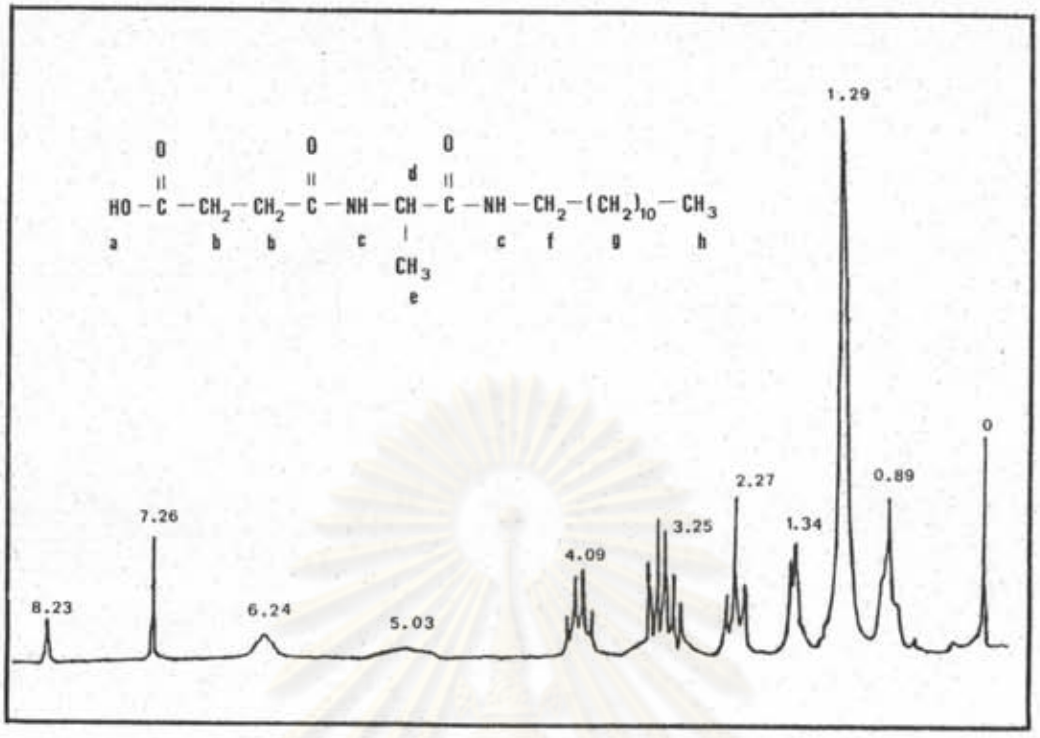


Fig. 2.15 ^{1}H NMR spectrum of compound 16 in $CDCl_3$

Table 2.8 Assignments of proton NMR signals of compound 16 ($CDCl_3$ as solvent)

Chemical Shift (ppm)	Multiplicity	Tentative Assignments
8.23	singlet *	(a) HO-C(=O)-CH2-
6.24,5.03	br. singlet *	(c) -C(=O)-NH-CH(CH3)-C(=O)-NH-CH2-
4.09	quartet	(d) -NH-CH(CH3)-C(=O)-
3.25	multiplet	(f) -NH-CH2-(CH2)10-
2.27	triplet	(b) -C(=O)-CH2-CH2-C(=O)-
1.34	doublet	(e) -NH-CH(CH3)-C(=O)-
1.29	br. singlet	(g) -NH-CH2-(CH2)10-
0.89	br. triplet	(h) -(CH2)10-CH3

* disappear when drop D_2O

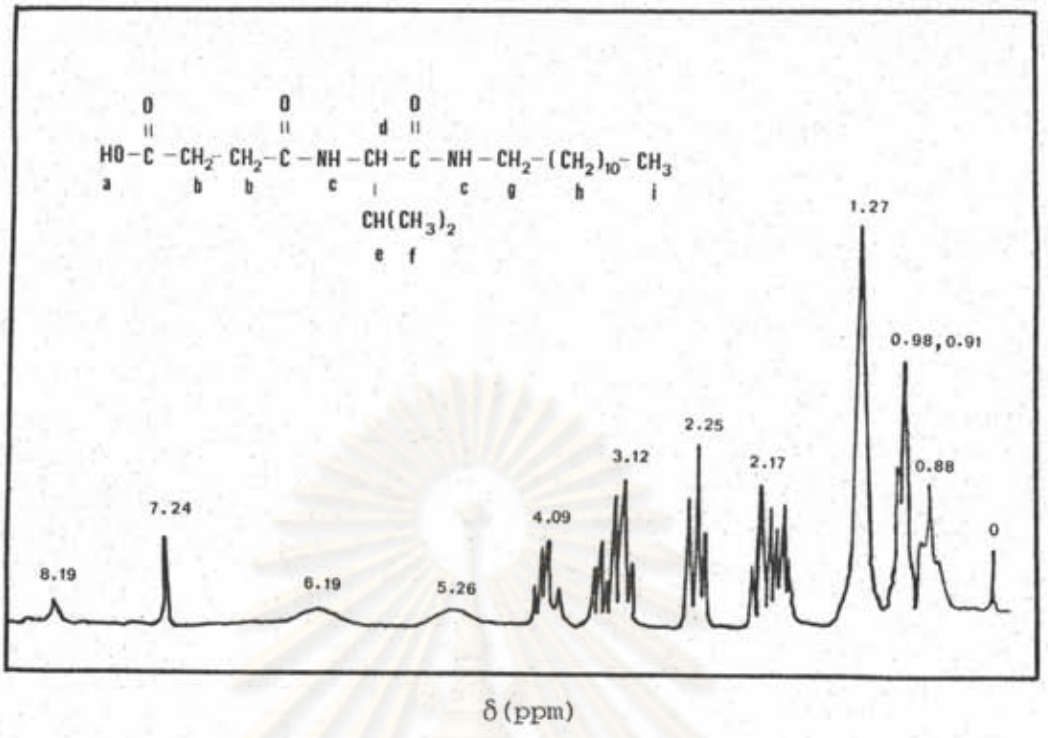


Fig. 2.16 ^{1}H NMR spectrum of compound 17 in $CDCl_3$

Table 2.9 Assignments of proton NMR signals of compound 17 ($CDCl_3$ as solvent)

Chemical Shift (ppm)	Multiplicity	Tentative Assignments
8.19	singlet *	(a) HO-C(=O)-CH2-
6.19,5.26	br. singlet *	(c) -C(=O)-NH-CH(CH(CH3)2)-C(=O)-NH-CH2-
4.09	quartet	(d) -NH-CH(CH(CH3)2)-C(=O)-
3.12	multiplet	(g) -NH-CH2-(CH2)10-
2.25	triplet	(b) -C(=O)-CH2-CH2-C(=O)-
2.17	multiplet	(e) -NH-CH(CH(CH3)2)-C(=O)-
1.27	br. singlet	(h) -NH-CH2-(CH2)10-
0.98-0.91	doublet	(f) -NH-CH(CH(CH3)2)-C(=O)-
0.88	br. triplet	(i) -(CH2)10-CH3

* disappear when drop D_2O

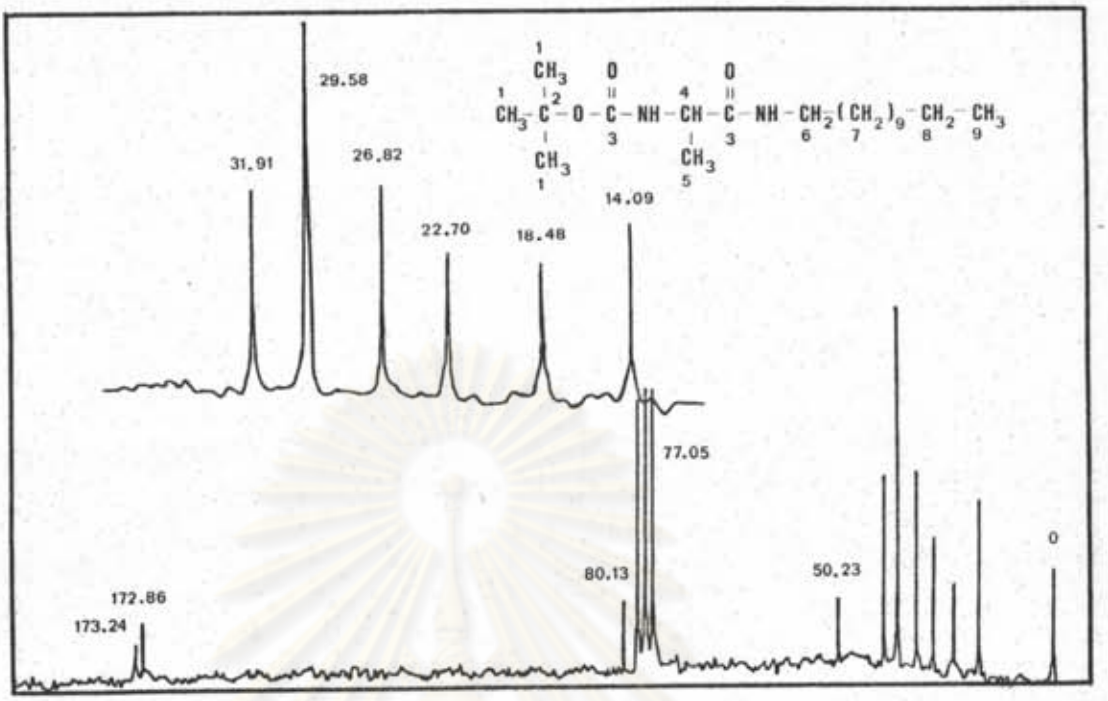


Fig. 2.17 ^{13}C NMR spectrum of compound 9 in $CDCl_3$

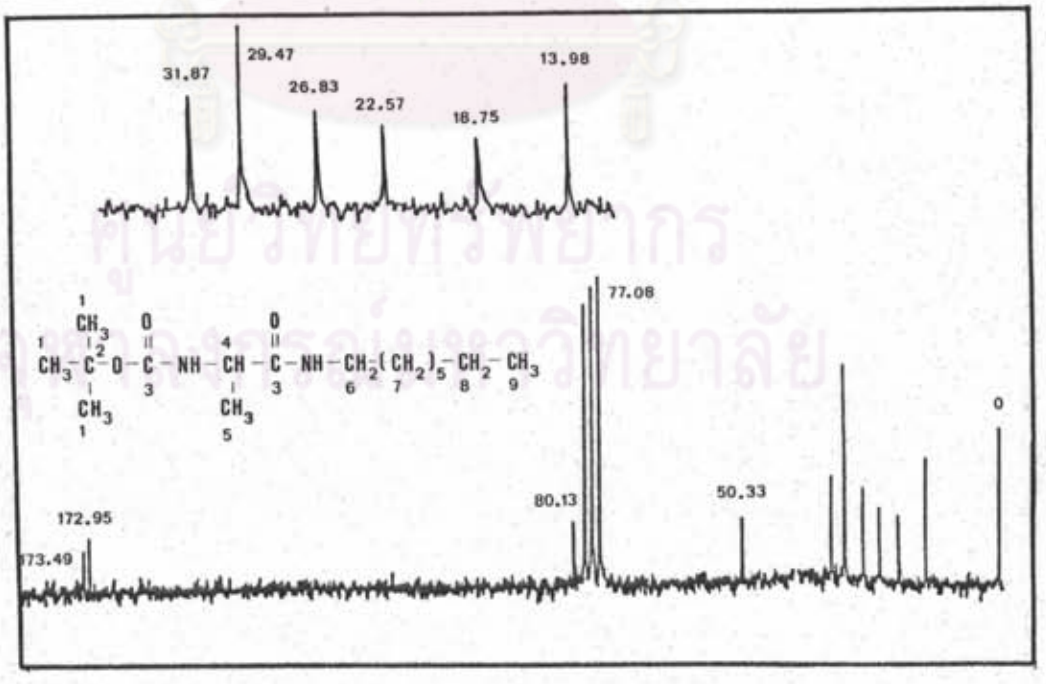


Fig. 2.18 ^{13}C NMR spectrum of compound 11 in $CDCl_3$

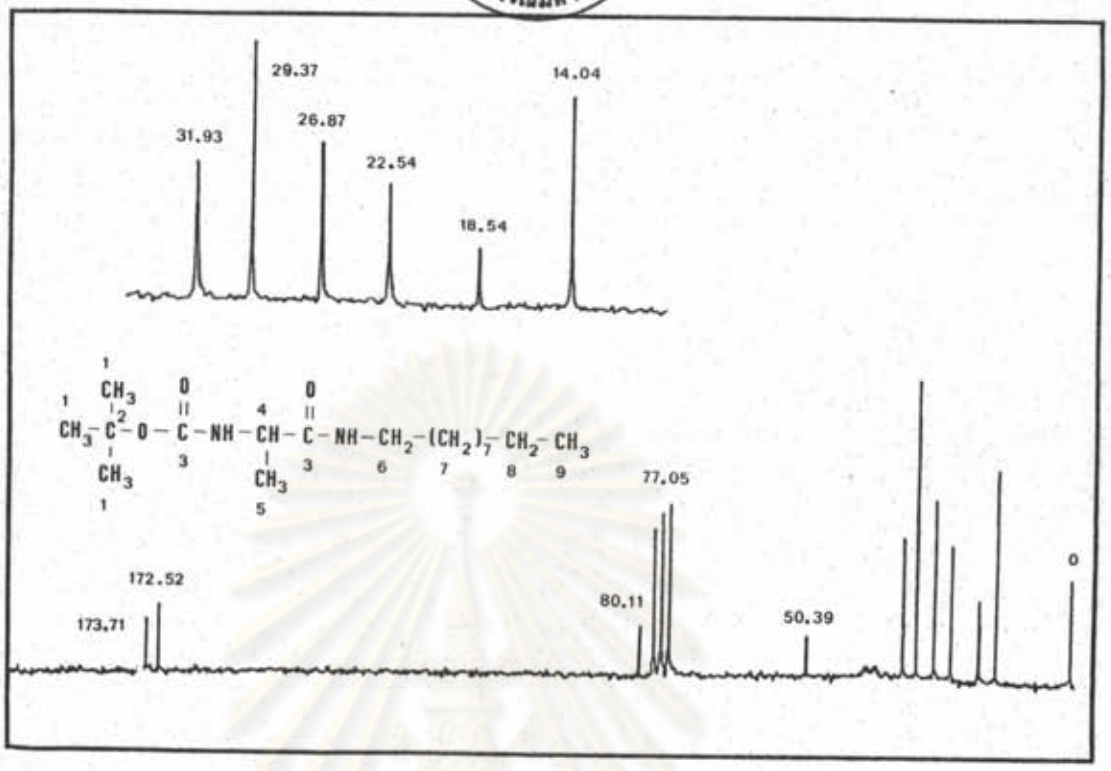


Fig. 2.19 ^{13}C NMR spectrum of compound 12 in $CDCl_3$

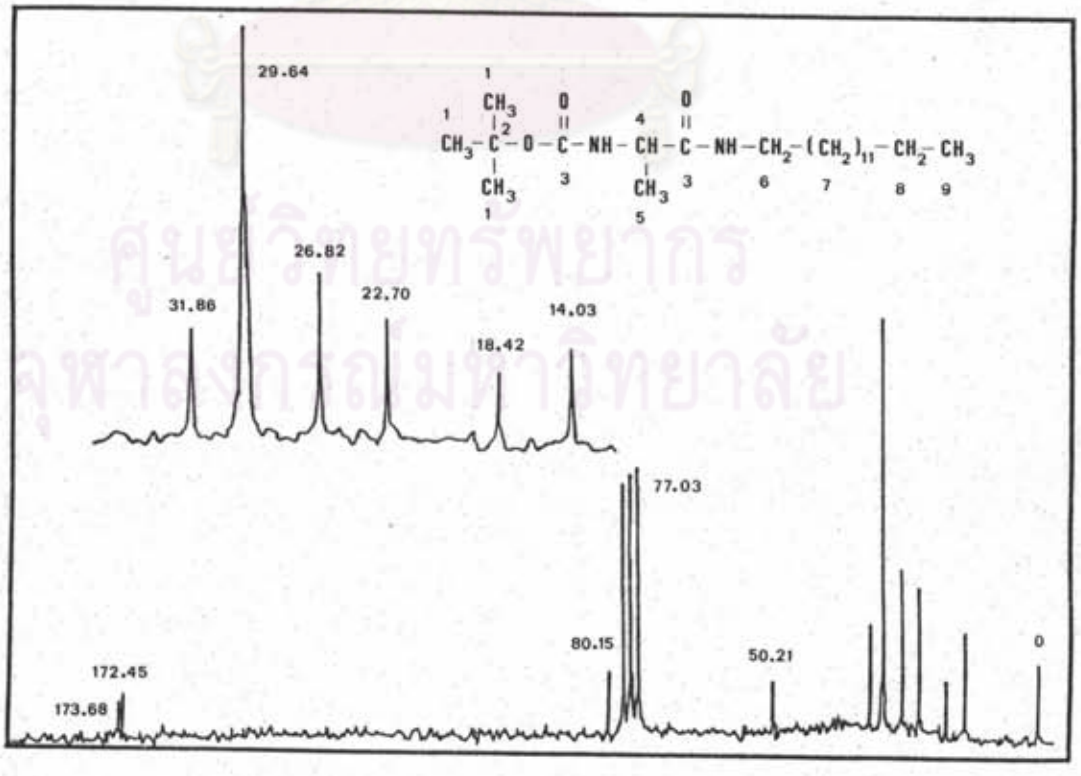


Fig. 2.20 ^{13}C NMR spectrum of compound 13 in $CDCl_3$

Table 2.10 Assignment of carbon 13 NMR signals of compound 9,11,12 and 13

($CDCl_3$ as solvent)

Compound No.	Chemical Shift (ppm)								
	173.71-172.45	80.11-80.15	50.21-50.39	31.86-31.93	29.37-29.64	26.82-26.87	22.54-22.70	18.42-18.75	13.98-14.09
9.	173.24,172.86	80.13	50.23	31.91	29.58	26.82	22.70	18.48	14.09
11.	173.49,172.95	80.13	50.33	31.87	29.47	26.83	22.57	18.75	13.98
12.	173.71,172.52	80.11	50.39	31.93	29.37	26.87	22.54	18.54	14.04
13.	173.68,172.45	80.15	50.21	31.86	29.64	26.82	22.70	18.42	14.03
Tentative Assignments	C-3	C-2	C-4	C-6	C-7	C-1	C-8	C-5	C-9

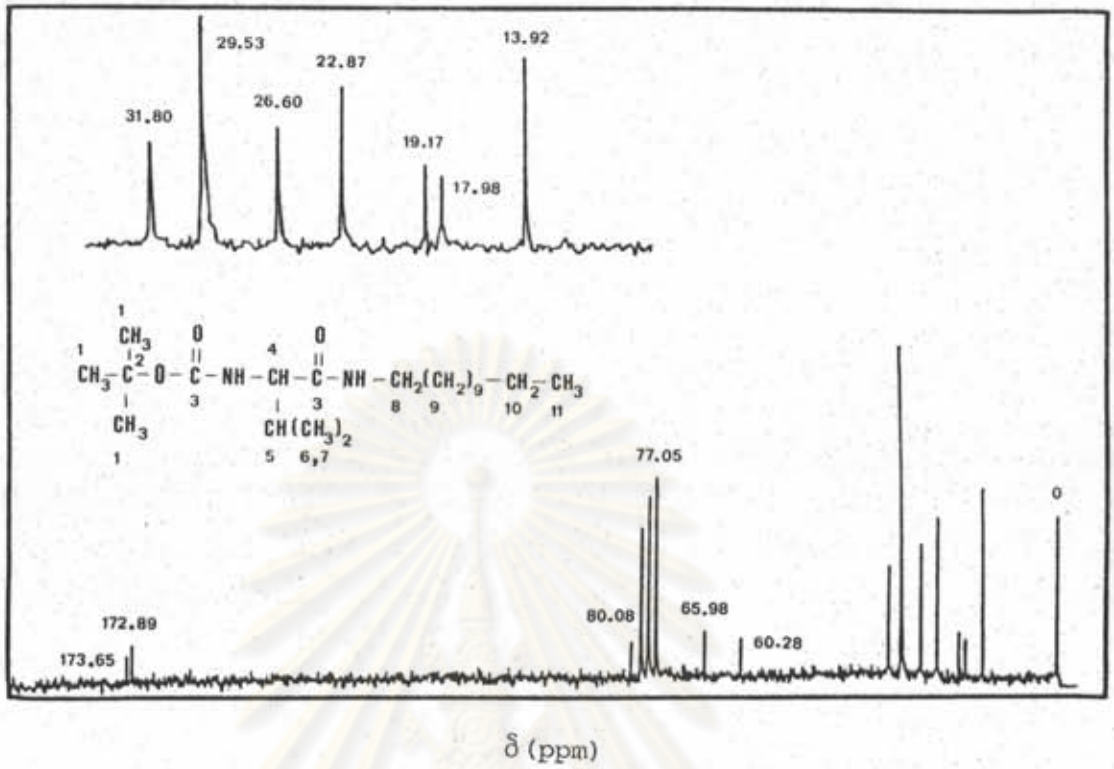


Fig. 2.21 ^{13}C NMR spectrum of compound 14 in $CDCl_3$

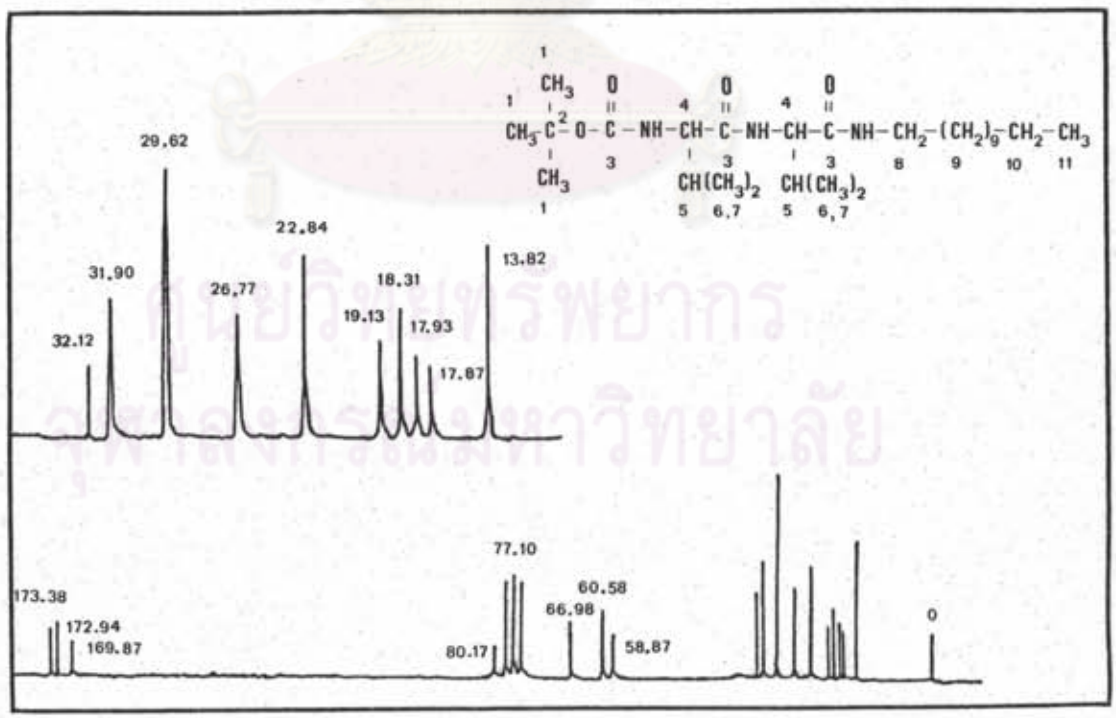
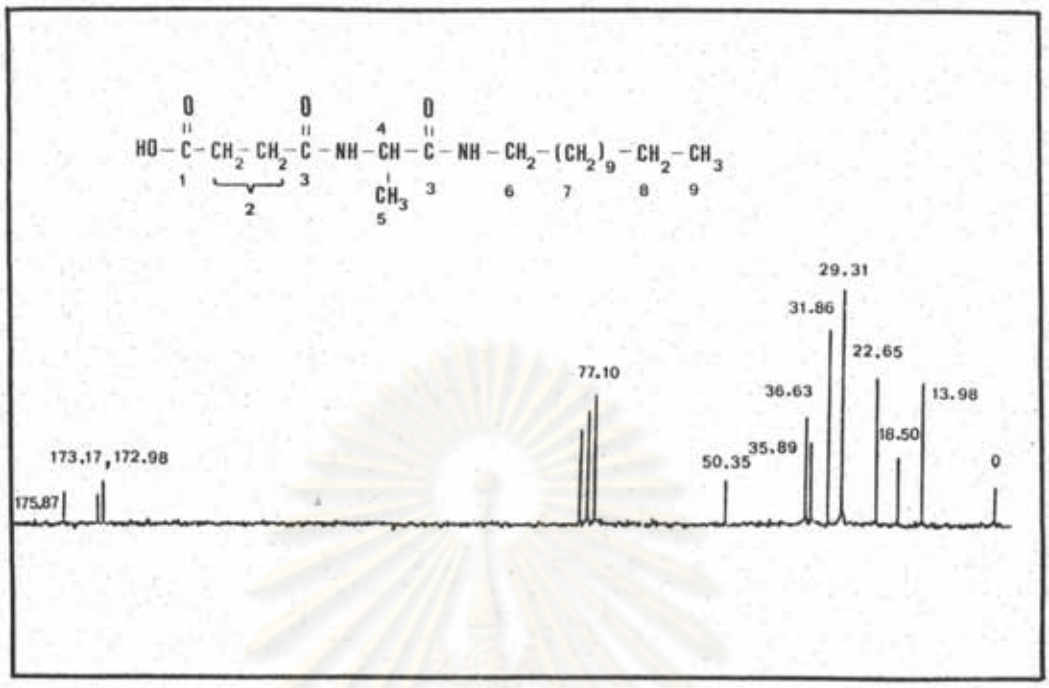


Fig. 2.22 ^{13}C NMR spectrum of compound 15 in $CDCl_3$

Table 2.11 Assignment of carbon 13 NMR signals of compound 14,15

($CDCl_3$ as solvent)

Compound No.	Chemical Shift (ppm)									
	169.87-173.65	80.08-80.17	65.98-66.98	58.87-60.58	31.80-32.12	29.53-29.62	26.60-26.77	22.84-22.87	17.87-19.17	13.82-13.92
14.	173.65,172.89	80.08	65.98	60.28	31.80	29.53	26.60	22.87	19.17,17.98	13.92
15.	173.38,172.94, 169.87	80.17	66.98	60.58,58.87	32.21,31.90	29.62	26.77	22.84	17.87,17.93 18.31,19.13	13.82
Tentative Assignments	C-3	C-2	C-8	C-4	C-5	C-9	C-1	C-10	C-6,C-7	C-11



δ (ppm)

Fig. 2.23 ^{13}C NMR spectrum of compound 16 in $CDCl_3$

Table 2.12 Assignment of carbon 13 NMR signals of compound 16 ($CDCl_3$ as solvent)

Chemical Shift (ppm)	Tentative Assignments
175.87	C-1
173.17,172.98	C-3
50.35	C-4
36.63, 35.89	C-2
31.86	C-6
29.31	C-7
22.65	C-8
18.50	C-5
13.98	C-10

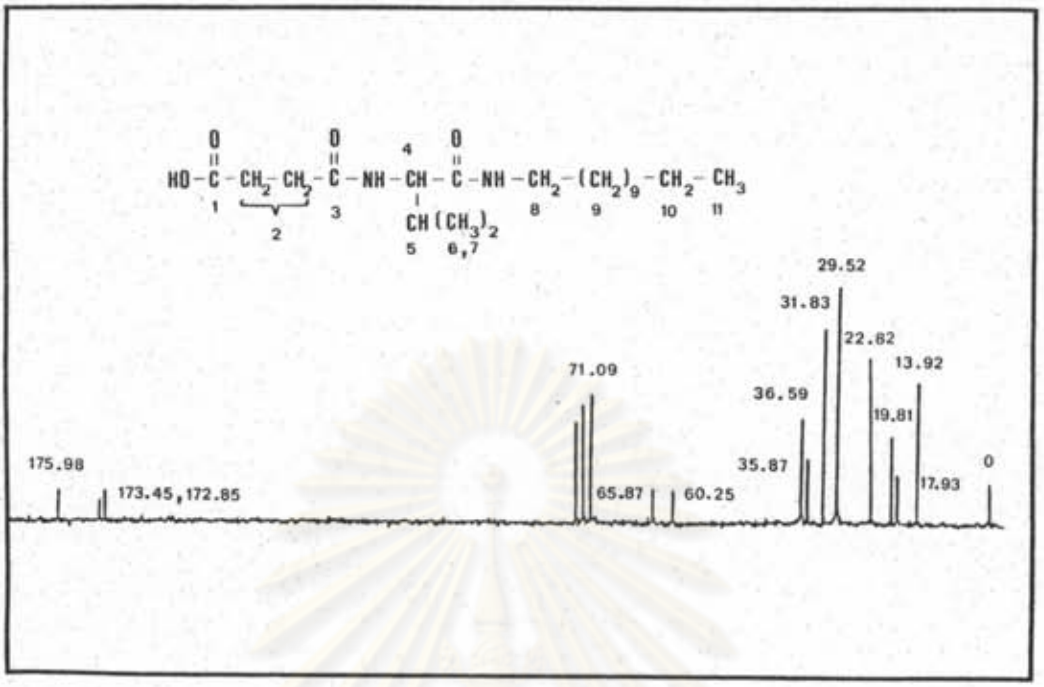


Fig. 2.24 ^{13}C NMR spectrum of compound 17 in $CDCl_3$

Table 2.13 Assignment of carbon 13 NMR signals of compound 17 ($CDCl_3$ as solvent)

Chemical Shift (ppm)	Tentative Assignments
175.98	C-1
173.45,172.85	C-3
65.87	C-8
60.35	C-4
35.59,35.87	C-2
31.83	C-5
29.52	C-9
19.18,17.93	C-6,C-7
13.92	C-11

2.6 Enzyme Kinetic Experiments

2.6.1 Enzymatic Assays

The spectrophotometric method in these enzymatic assays was basically similar to that for trypsin and chymotrypsin. The hydrolysis of BAN was followed by the decrease in absorbance at 400 nm.

All the solutions were maintained at 37°C during these assays which were carried out by using a UV-240 Shimadzu with 10 mm. matched quartz-cells, recording spectrophotometer equipped with a thermostatic cell compartment. The enzyme was assayed according to the method of Visser and Blout (28). To a cuvette was added 30-150 µl. of BAN (2 mM in Spectrograde DMSO) and HEPES buffer (pH7.5) to make a final volume of 3000 µl. The cuvette was thermally equilibrate in the spectrophotometer for at least 5 minutes followed by addition of BAN. After vigorous mixing and incubation for 20 minutes, the rate of production of p-nitrophenol, which is followed by measurement of the increase in absorbances at 400 nm. ($\Delta \epsilon_{400}$ 19,000) was recorded.

2.6.2 Effect of enzyme concentration

The initial rate of an enzyme reaction is the rate after earliest time that the reaction can be measured after mixing the reactants. It is used for all enzyme assays, and all calculations concerning enzyme kinetics. In order for any enzyme reaction to proceed, on whatever scale, certain fundamental conditions apply. For all enzyme processes, assuming the correct temperature, length of reaction time relationship, a medium at the pH optimum and constant substrate concentration, the rate of enzyme reaction increases linearly with increasing the amount of enzyme as long as the

concentration of enzyme is much less than that of the substrate.

The determination of the effect of trypsin and chymotrypsin concentration was carried out by the following procedures.

(a) Enzyme stock solution An enzyme concentration (0.2 mg./ml. for trypsin and 0.1 mg./ml. for chymotrypsin) was dissolved in 0.1 M HEPES buffer pH 7.0, containing 10% DMSO, 0.05 M NaCl. The enzyme praparation should be in an ice water bath.

(b) Substrate stock solution A 2 mM of BAN, the elastase substrate was prepared in DMSO solution.

The following enzyme solutions were prepared in Table 2.14

Table 2.14 Initial velocities as the function of varying enzyme concentration

No.	HEPES (µl)	H_2O (µl)	Enzyme (µl)	DMSO (µl)	BAN (µl)
1.	2 x 500	1450	100	300	150
2.	2 x 500	1350	200	300	150
3.	2 x 500	1250	300	300	150
4.	2 x 500	1150	400	300	150
5.	2 x 500	950	600	300	150
6.	2 x 500	750	800	300	150
7.	2 x 500	550	1000	300	150

(c) Assay Method

The sample cell contained enzyme, while the reference cell had no enzyme present. The total volume in each sample was 3.0 ml. An incubation time of approximately 5 minutes at 37°C was allowed after the addition of substrate in DMSO to both the reference and sample cells. The initial rate of product of p-nitrophenol was followed spectrophotometrically at 400 nm. for 20 minutes.

The results of trypsin and chymotrypsin concentrations are shown in Table 2.15 and Table 2.16 respectively. They were tabulated in Fig. 2.25. The results appeared in Fig. 2.25 showed the initial velocity of each enzyme against the increasing amount of the enzyme. The first part of the curve showed a linear relationship between the reaction velocity and increasing amount of the enzyme. The maximum and constant initial velocities were obtained above 500 µl. of the added enzyme. Hence the addition of 500 µl. of each enzyme was selected as the optimun enzyme concentration for the entire experiments.

Table 2.15 Initial velocities as a function of varying the trypsin concentration

Amount of trypsin (µl)	Initial Velocities (Δ Abs/min)			
	(1)	(2)	(3)	average
100	0.021	0.017	0.016	0.018
200	0.034	0.033	0.035	0.034
300	0.060	0.054	0.060	0.058
400	0.085	0.080	0.081	0.082
600	0.113	0.108	0.109	0.110
800	0.119	0.123	0.118	0.120
1000	0.128	0.125	0.131	0.128

Table 2.16 Initial velocities as a function of varying the chymotrypsin concentration

Amount of chymotrypsin (µl)	Initial Velocities (Δ Abs/min)			
	(1)	(2)	(3)	average
100	0.030	0.026	0.028	0.028
200	0.060	0.065	0.061	0.062
300	0.093	0.094	0.095	0.094
400	0.123	0.123	0.126	0.124
600	0.171	0.175	0.170	0.172
800	0.189	0.190	0.185	0.188
1000	0.194	0.190	0.192	0.192

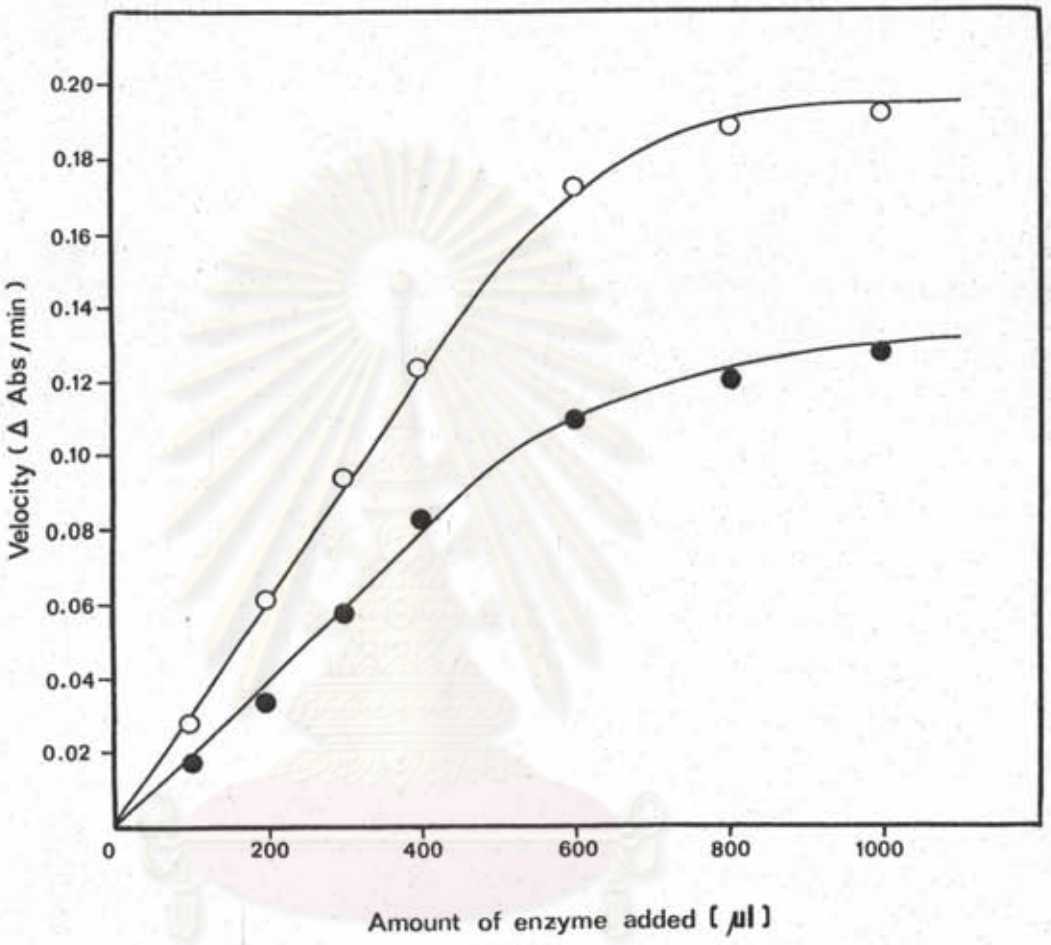


Fig. 2.25 The effect of enzyme concentration on the initial velocities

● Trypsin (0.2 mg./ml.)

O Chymotrypsin (0.1 mg./ml.)

2.6.3 Effect of substrate concentration

The initial velocity is also dependent on the concentration of substrate in the medium but the dependency observed is not as straight forward as that found on changing enzyme concentration. For a given amount of enzyme under standard conditions, the initial reaction velocity varies with an increase of initial substrate concentration. However the rate cannot be increased indefinitely by raising the amount of substrate levels.

The study of the effect of substrate concentration on trypsin and chymotrypsin activities were carried out by varying the substrate concentration (Table 2.17).

Table 2.17 Initial velocities as the function of varying substrate concentration

No.	HEPES (µl)	H_2O (µl)	Enzyme (µl)	DMSO (µl)	BAN (µl)
1.	2 x 500	2 x 525	500	445	5
2.	2 x 500	2 x 525	500	440	10
3.	2 x 500	2 x 525	500	430	20
4.	2 x 500	2 x 525	500	420	30
5.	2 x 500	2 x 525	500	380	70
6.	2 x 500	2 x 525	500	340	110
7.	2 x 500	2 x 525	500	300	150
8.	2 x 500	2 x 525	500	260	190
9.	2 x 500	2 x 525	500	220	230

The assay procedure was carried out as described for the assay of enzyme concentrations. The results of the effect of substrate concentration on trypsin and chymotrypsin activities are displayed in Table 2.18 and Table 2.19 respectively. The corresponding plots appeared in Fig. 2.26. The reasoning can be simplified as follows : while free enzyme is available, an increase in substrate concentration will result in a higher rate, but when all enzyme molecules are saturated with substrate their total effect will be exerted, giving the maximun velocities. The result appeared in Fig. 2.26 showed the plot of initial velocities of each enzyme against the added amount of the substrate. The maximum and constant velocities were obtained above 30 µl. of the substrate solution. Since the velocities did not increase on addition of an excess volume of the substrate solution, 150 µl. of 2 mM. of substrate solution was adopted throughout this experiment.

Table 2.18 The effect of various substrate concentrations on the initial velocities of trypsin

Amount of substrate(µl)	Initial Velocities (Δ Abs/min)			
	(1)	(2)	(3)	average
5	0.027	0.029	0.028	0.028
10	0.055	0.052	0.052	0.053
20	0.089	0.084	0.085	0.086
30	0.101	0.099	0.103	0.101
70	0.104	0.118	0.113	0.115
110	0.118	0.123	0.122	0.120
150	0.124	0.129	0.125	0.126
190	0.128	0.129	0.124	0.127
230	0.127	0.125	0.126	0.126

Table 2.19 The effect of various substrate concentrations on the initial velocities of chymotrypsin

Amount of substrate(µl)	Initial Velocities (Δ Abs/min)			
	(1)	(2)	(3)	average
5	0.046	0.042	0.047	0.045
10	0.076	0.072	0.074	0.074
20	0.112	0.107	0.108	0.109
30	0.131	0.135	0.130	0.132
70	0.144	0.139	0.137	0.140
110	0.152	0.150	0.148	0.150
150	0.154	0.157	0.154	0.155
190	0.155	0.156	0.157	0.156
230	0.158	0.158	0.155	0.157

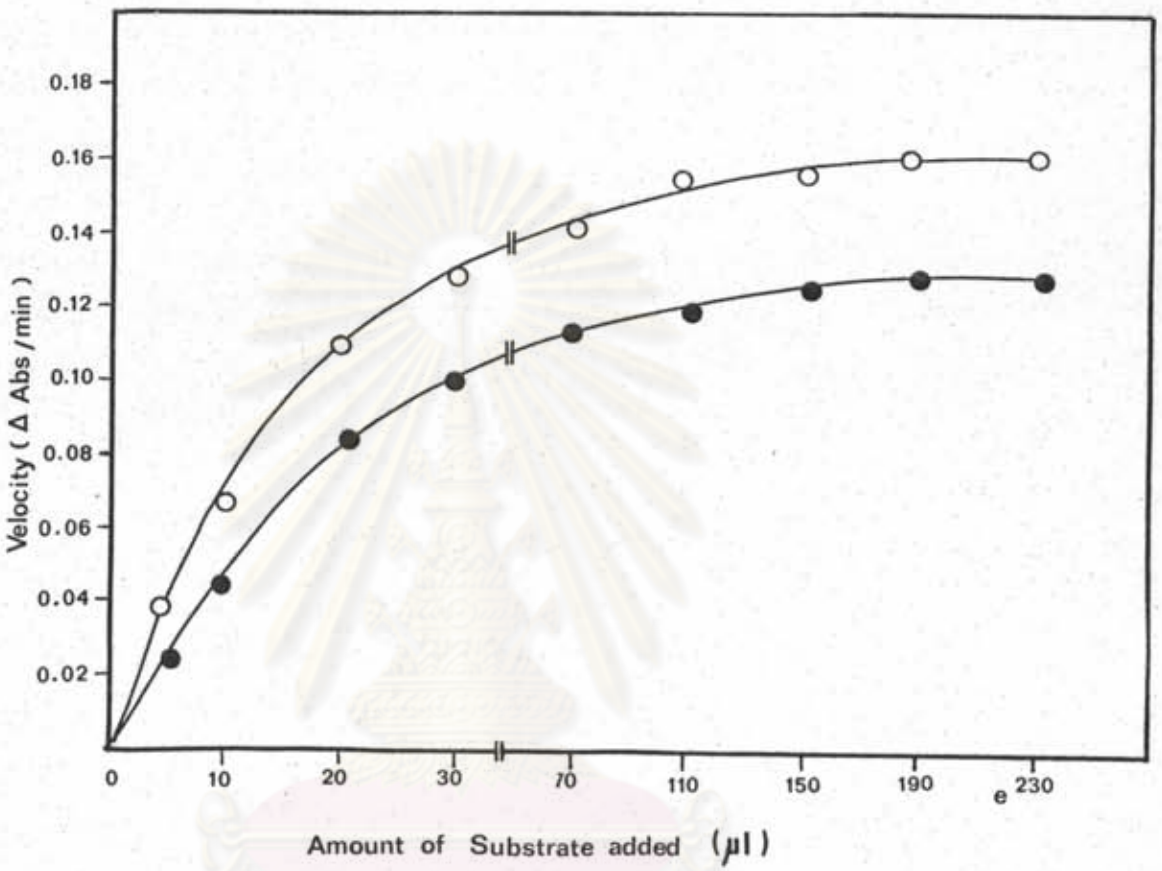


Fig. 2.26 The effect of substrate concentration (2 mM.)

on the initial velocities of enzymes :

● trypsin

○ chymotrypsin

2.6.4 The optimum pH of the enzyme activity

The pH dependence of the trypsin and chymotrypsin activities for studying the optimum pH of the enzymatic reaction was determined as follows :

(a) Enzyme stock solution : trypsin and chymotrypsin were assayed at 0.2 mg./ml. and 0.1 mg./ml. respectively. (using the optimal amount of enzyme obtained in 2.6.2)

(b) Substrate stock solution : The elastase substrate BAN 2 mM was prepared in DMSO.

(c) Buffer 0.1 M HEPES buffer of different pH values were used containing 10% DMSO, 0.05 M.NaCl at 37°C and the pH adjustment was done by 0.1 M.NaOH with pH ranging 5.00-9.00.

For the determination of the optimum pH, the following solutions were prepared.

	Sample(µl)	Reference(µl)
HEPES	2 x 500	3 x 500
H_2O	2 x 525	2 x 525
Enzyme	500	-
DMSO	300	300
BAN	150	150

The assay procedure was carried out as described for the assay of enzyme concentrations. The results obtained are shown in Table 2.20 and Table 2.21. The corresponding plots are presented in Fig. 2.27. The maximum and constant initial velocities were obtained in the range of pH 6.7-7.2 (max. 7.0) for trypsin, and of pH 7.4-8.05

(max. 7.5) for chymotrypsin. In this research, the optimum pH of each enzyme as indicated were therefore employed in all the entire experiments.

Table 2.20 Determination of the optimum pH for trypsin

Observed pH	Initial Velocities(ΔAbs/min)			
	(1)	(2)	(3)	average
5.20	0.028	0.023	0.024	0.025
6.01	0.039	0.038	0.034	0.037
6.51	0.051	0.050	0.049	0.050
7.04	0.114	0.118	0.116	0.116
7.52	0.098	0.099	0.097	0.098
8.05	0.085	0.089	0.084	0.086
8.56	0.040	0.041	0.045	0.042
9.03	0.019	0.015	0.014	0.016

Table 2.21 Determination of the optimum pH for chymotrypsin

Observed pH	Initial Velocities(ΔAbs/min)			
	(1)	(2)	(3)	average
5.20	0.046	0.043	0.045	0.045
6.01	0.077	0.078	0.073	0.076
6.51	0.103	0.107	0.102	0.104
7.04	0.162	0.159	0.159	0.160
7.52	0.198	0.195	0.195	0.196
8.05	0.135	0.132	0.135	0.134
8.56	0.116	0.113	0.113	0.114
9.03	0.043	0.038	0.039	0.040

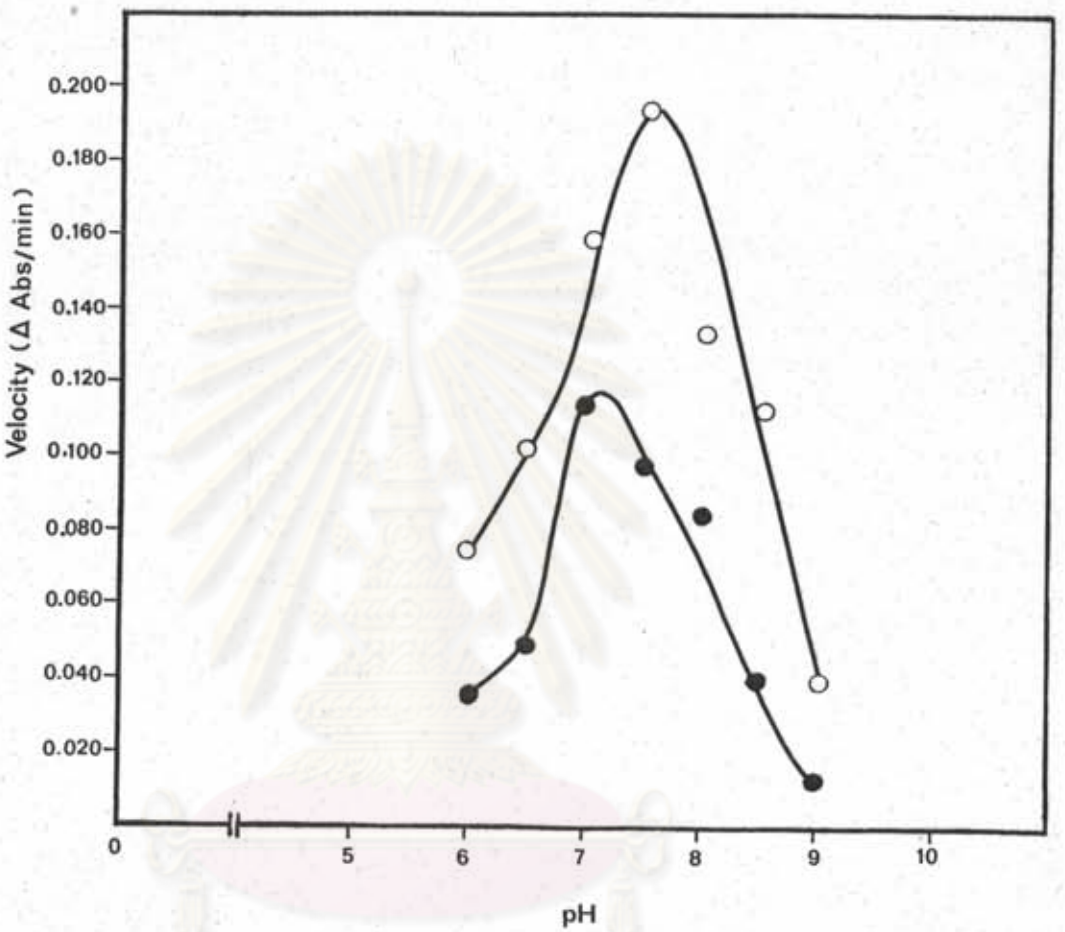


Fig. 2.27 Determination of the activity of (●)trypsin and (○)chymotrypsin as a function of pH

2.6.5 The optimum temperature of the enzyme activity

For the investigation of the effects of temperature on trypsin and chymotrypsin activities, the assay procedure was essentially the same as described for the assay of enzyme concentrations and the optimum pH for each enzyme. The temperature was varied from 12.75 to 70.03°C in both enzymes system.

The optimum temperature for trypsin and chymotrypsin are presented in Table 2.22 and Table 2.23 with their curves were plotted in Fig. 2.28. It was shown that the optimum temperature of both enzymes was essentially the same at 37°C. This optimum temperature was used for all subsequent studies.

Table 2.22 Determination of the optimum temperature for trypsin

Observed Temperature (°C)	Initial Velocity (ΔAbs/min)			
	(1)	(2)	(3)	average
12.75	0.019	0.016	0.020	0.018
20.34	0.028	0.033	0.030	0.030
30.01	0.063	0.062	0.065	0.063
35.07	0.097	0.092	0.099	0.096
38.10	0.112	0.109	0.109	0.110
40.03	0.097	0.103	0.107	0.102
45.04	0.098	0.093	0.094	0.095
50.01	0.079	0.075	0.074	0.076
60.05	0.054	0.050	0.052	0.052
70.03	0.004	0.003	0.005	0.004

Table 2.23 Determination of the optimum temperature for chymotrypsin

Observed Temperature (°C)	Initial Velocity (Δ Abs/min)			
	(1)	(2)	(3)	average
12.75	0.022	0.019	0.024	0.022
20.34	0.038	0.036	0.041	0.038
30.01	0.094	0.089	0.090	0.091
35.07	0.141	0.142	0.137	0.140
38.10	0.148	0.150	0.146	0.148
40.03	0.142	0.143	0.145	0.143
45.04	0.139	0.138	0.137	0.138
50.01	0.137	0.132	0.133	0.134
60.05	0.115	0.111	0.110	0.112
70.03	0.055	0.050	0.054	0.053

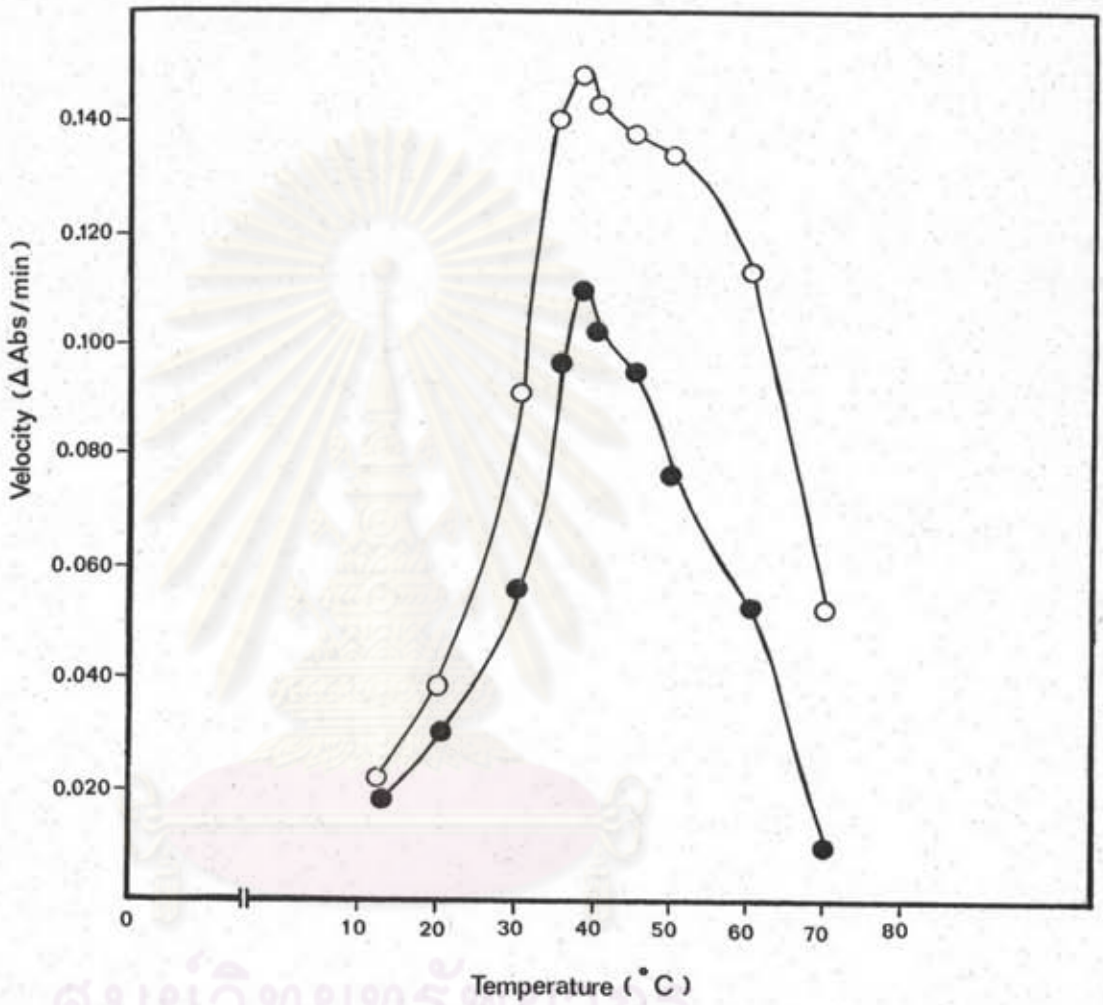


Fig. 2.28 Determination of the activity of (●) trypsin and (O) chymotrypsin as a function of temperature.

2.6.6 Determination of the percentage of inhibition of synthetic inhibitors

The experimental conditions described in this section were followed by the optimum conditions obtained in the previous section. The elastase substrate BAN 2 mM was dissolved in DMSO. Buffer, HEPES 0.1 M was used containing 10% DMSO, 0.05 M.NaCl at 37°C. The production of p-nitrophenol was examined to follow up the activities of trypsin and chymotrypsin at 0.2 mg./ml. and 0.1 mg./ml. respectively. These enzymes were kept in an ice water bath during the experiments. The synthetic inhibitors, 10 mM were dissolved in DMSO, and the total concentration was varied to determine the ID_{50}.

For the determination of percentage inhibition, the following solutions were prepared.

Control Run :

	Sample(µl)	Reference(µl)
HEPES	2 x 500	3 x 500
H_2O	2 x 525	2 x 525
Enzyme	500	-
Inhibitor	-	-
DMSO	300	300
BAN	150	150

Inhibitor Run :

	Sample(µl)	Reference(µl)
HEPES	2 x 500	3 x 500
H_2O	2 x 525	2 x 525
Enzyme	500	-
Inhibitor	300	300
BAN	150	150

control sample has no inhibitor present.

control reference has no inhibitor or enzyme present.

The sample cell contained enzyme plus inhibitor, while the reference cell contained only buffer and inhibitor without enzyme. An incubation time of approximately 5 minutes was allowed after the addition of substrate in DMSO to both the reference and sample cells.

Percentage inhibition was calculated by comparing the rate of the release of absorbing species produced in the samples with and without inhibitors. The results were exhibited in Table 2.24.

Table 2.24 Percentage inhibition of synthetic inhibitors

No.	Inhibitor	Enzyme (a)	% Inhibition								
			$10x10^{-3}M$	$5x10^{-3}M$	$2.5x10^{-3}M$	$10^{-3}M$	$5x10^{-4}M$	$10^{-4}M$	$5x10^{-5}M$	$2.5x10^{-5}M$	$10^{-5}M$
1.	Z-V-NH-C_{12}	HLE	—	—	—	—	—	—	37	—	—
		PPE	—	—	—	—	—	—	14	—	—
		chymotrypsin	—	—	—	62.56	47.17	26.77	15.99	11.71	0.49
		trypsin	—	—	—	65.14	11.32	6.25	0	—	—
2.	Z-V-V-NH-C_{12}	HLE	—	—	—	—	—	—	62	41	—
		PPE	—	—	—	—	—	—	20	—	—
		chymotrypsin	—	—	—	66.27	53.14	22.69	22.09	0	—
		trypsin	—	—	—	23.38	16.38	0.89	—	—	—
3.	C_{14}-A-V-OMe	HLE	—	—	—	—	—	—	35	5	—
		PPE	—	—	—	—	—	—	30	0	—
		chymotrypsin	—	—	—	—	—	23.78	14.18	11.94	—
		trypsin	—	—	—	—	—	8.03	0	—	—
4.	C_{14}-V-V-OMe	HLE	—	—	—	—	—	—	56	58	—
		PPE	—	—	—	—	—	—	0	0	—
		chymotrypsin	—	—	—	83.56	—	67.92	52.41	35.17	15.65
		trypsin	—	—	—	43.52	21.88	5.88	0	—	—

Table 2.24 continued

No.	Inhibitor	Enzyme[a]	% Inhibition								
			$10x10^{-3}M$	$5x10^{-3}M$	$2.5x10^{-3}M$	$10^{-3}M$	$5x10^{-4}M$	$10^{-4}M$	$5x10^{-5}M$	$2.5x10^{-5}M$	$10^{-5}M$
5.	C_{14}-V-A-OEt	HLE	—	—	—	—	—	—	43	0	—
		PPE	—	—	—	—	—	—	44	0	—
		chymotrypsin	—	—	—	88.37	62.69	36.71	16.99	0	—
		trypsin	—	—	—	18.01	11.44	0	—	—	—
6.	C_{14}-V-V-A-OEt	HLE	—	—	—	—	—	—	46	0	—
		PPE	—	—	—	—	—	—	21	0	—
		chymotrypsin	—	97.77	—	89.61	56.18	44.14	10.28	7.52	0.78
		trypsin	—	—	—	44.56	17.73	0	—	—	—
7.	C_{12}-V-V-OMe	HLE	—	—	—	—	—	—	50	—	—
		PPE	—	—	—	—	—	—	48	—	—
		chymotrypsin	—	—	—	94.75	80.83	25.96	13.20	—	0
		trypsin	—	—	—	—	75.89	54.39	23.03	—	15.69
8.	NH_2-A-NH-C_{12}	HLE	—	—	—	—	—	—	15	—	—
		PPE	—	—	—	—	—	—	28	—	—
		chymotrypsin	85.56	62.70	19.38	11.93	5.91	—	0	—	—
		trypsin	—	—	61.42	7.70	0.31	—	0	—	—

Table 2.24 continued

No.	Inhibitor	Enzyme[a]	% Inhibition								
			$10x10^{-3}$M	$5x10^{-3}$M	$2.5x10^{-3}$M	10^{-3}M	$5x10^{-4}$M	10^{-4}M	$5x10^{-5}$M	$2.5x10^{-5}$M	10^{-5}M
9.	t-BOC-A-NH-C_{12}	HLE	—	—	—	—	—	84	40	—	5
		PPE	—	—	—	—	—	10	0	0	—
		chymotrypsin	—	—	77.33	36.91	28.20	—	3.12	—	—
		trypsin	—	—	74.28	32.43	25.17	—	0	—	—
10.	C_{14}-V-V-V-OMe	HLE	—	—	—	—	—	—	67	—	—
		PPE	—	—	—	—	—	—	62	—	—
		chymotrypsin	—	93.39	85.98	74.75	43.42	23.58	22.58	1.60	0
		trypsin	—	—	93.85	56.57	31.50	20.42	11.02	1.85	—
11.	t-BOC-A-NH-C_8	HLE	—	—	—	—	—	84	40	—	0
		PPE	—	—	—	—	—	10	—	—	—
		chymotrypsin	—	—	84.11	71.01	54.11	37.40	—	0	—
		trypsin	—	—	84.86	46.63	35.45	20.14	—	—	—
12.	t-BOC-A-NH-C_{10}	HLE	—	—	—	—	—	96	37	—	7
		PPE	—	—	—	—	—	0	—	—	—
		chymotrypsin	—	—	81.24	55.18	43.79	35.34	13.81	0	—
		trypsin	—	—	75.56	22.14	—	12.82	—	—	—

Table 2.24 continued

No.	Inhibitor	Enzyme (a)	% Inhibition								
			$10x10^{-3}$M	$5x10^{-3}$M	$2.5x10^{-3}$M	10^{-3}M	$5x10^{-4}$M	10^{-4}M	$5x10^{-5}$M	$2.5x10^{-5}$M	10^{-5}M
13.	t-BOC-A-NH-C_{14}	HLE	—	—	—	—	—	1	—	—	—
		PPE	—	—	—	—	—	2	—	—	—
		chymotrypsin	—	—	69.77	30.31	14.79	2.68	0	—	—
		trypsin	—	—	73.39	53.30	21.21	0	—	—	—
14.	t-BOC-V-NH-C_{12}	HLE	—	—	—	—	—	100	64	—	12
		PPE	—	—	—	—	—	4	0	—	—
		chymotrypsin	—	—	—	72.95	60.89	42.66	7.44	3.14	0
		trypsin	—	—	—	50.26	26.05	10.10	0	—	—
15.	t-BOC-V-V-NH-C_{12}	HLE	—	—	—	—	—	100	100	—	85
		PPE	—	—	—	—	—	0	0	—	—
		chymotrypsin	—	—	83.07	70.16	56.50	37.50	13.92	0.86	—
		trypsin	—	—	91.32	54.98	35.79	23.84	5.40	0.98	—
16.	Suc-A-NH-C_{12}	HLE	—	—	—	—	—	0	—	—	—
		PPE	—	—	—	—	—	4	—	—	—
		chymotrypsin	—	—	84.84	71.56	55.73	39.41	23.58	13.98	1.09
		trypsin	—	—	—	66.28	47.54	17.42	10.86	0.54	—
17.	Suc-V-NH-C_{12}	HLE	—	—	—	—	—	0	—	—	—
		PPE	—	—	—	—	—	3	—	—	—
		chymotrypsin	—	—	—	93.36	76.64	66.23	25.41	18.03	2.26
		trypsin	—	—	—	86.34	71.93	50.61	21.22	16.91	1.46

(a) The inhibitions of HLE and PPE were obtained from Australia.

\- means not determined.

2.6.7 Determination of the type of inhibition and the values of kinetic parameters.

The type of inhibition was determined by a Lineweaver-Burk plot. Initial velocities were determined by varying substrate concentrations (0.02-0.1 mM.) at 3 fixed concentrations of the inhibitors. Samples were made up the same way as for trypsin and chymotrypsin inhibitions studies.

The value of K_m was determined by using a plot of 1/V against 1/[S] in a Lineweaver-Burk plot. Where as the slope of each of these line (K_m/V_{max}) against concentration of inhibitors being used, gave the value of K_i by extrapolation back to the [I] abscissa. The interception points on the 1/V axis of the Lineweaver-Burk plot against the concentrations of inhibitors being used, gave the K_I by extrapolation back to [I] abscissa. A computer programma was used to calculate the values of K_m, K_i and K_I by a least squares method.

The assay procedure was carried out similarly to that described in the section 2.6.2. The data involved in the determination of the types of inhibition are presented in Appendix III. The results of Lineweaver-Burk data of the synthetic inhibitors No. 1-17 upon the enzymes, trypsin and chymotrypsin were presented in Table III.1 to Table III.35. The data were plotted as exhibited in Fig. 2.9 to Fig. 2.62 accordingly.

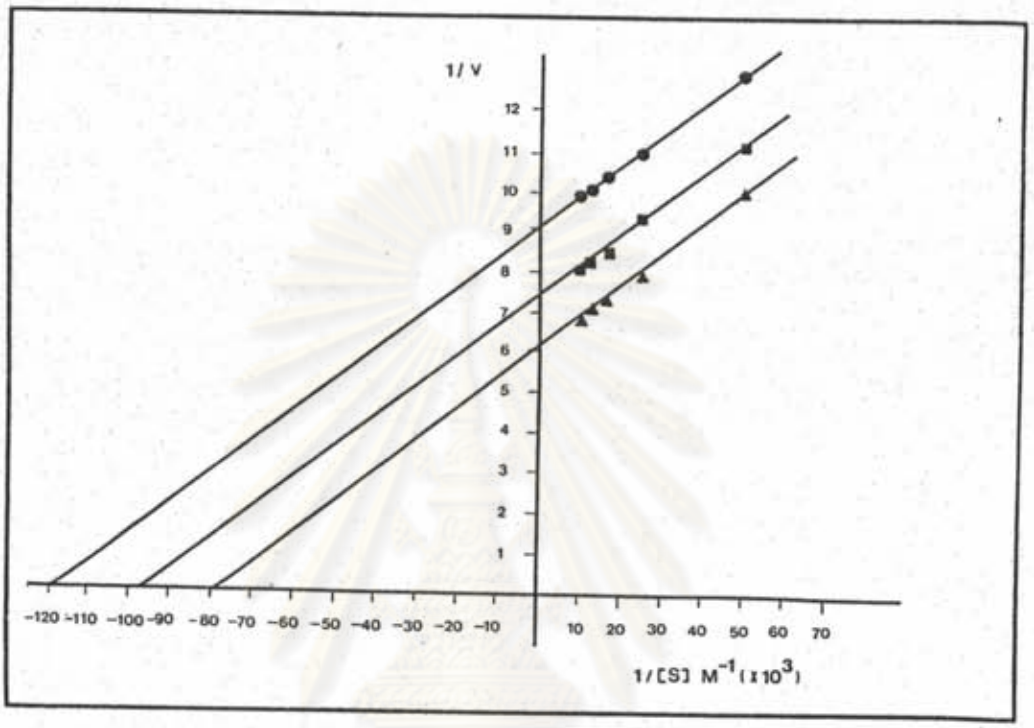


Fig. 2.29 Lineweaver-Burk double - reciprocal plots of kinetic data of compound 1 by Trypsin (The initial velocities are given as the increase in the absorbance at 400 nm. per minute. Each of point represents the mean of four experiments.)

▲—▲ no inhibitor, ■—■ inhibitor 150 µl.

●—● inhibitor 200 µl. Experiments were performed in 0.1 M.HEPES buffer and 10% DMSO, 0.05 M.NaCl at pH 7 and 37°C.

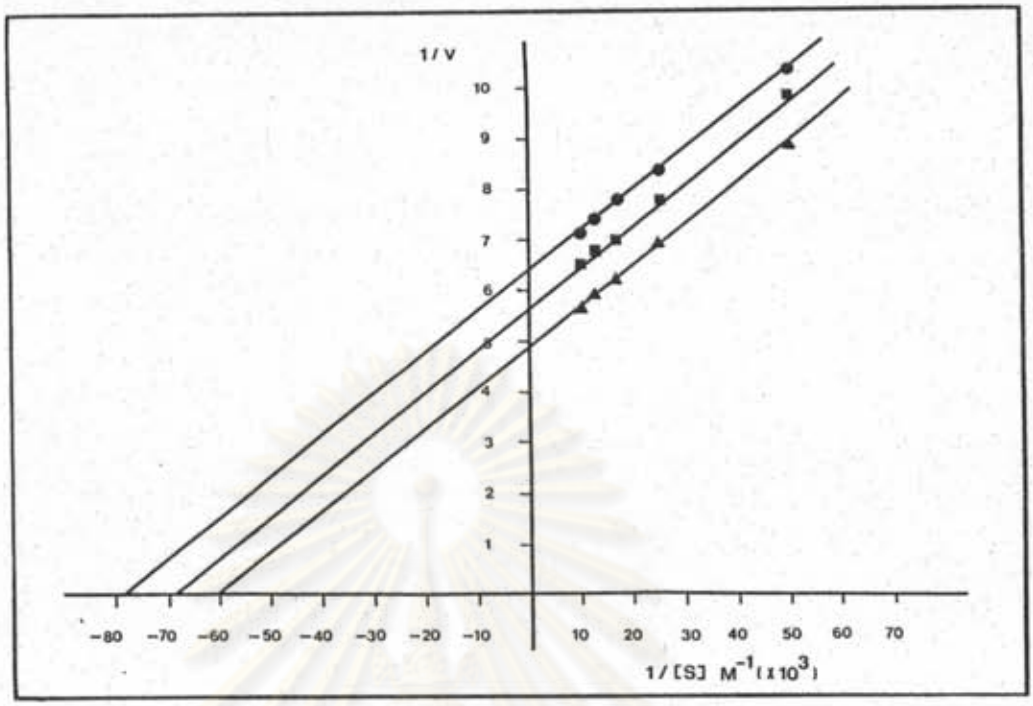


Fig. 2.30 Lineweaver-Burk double - reciprocal plots of kinetic data of compound 2 by trypsin

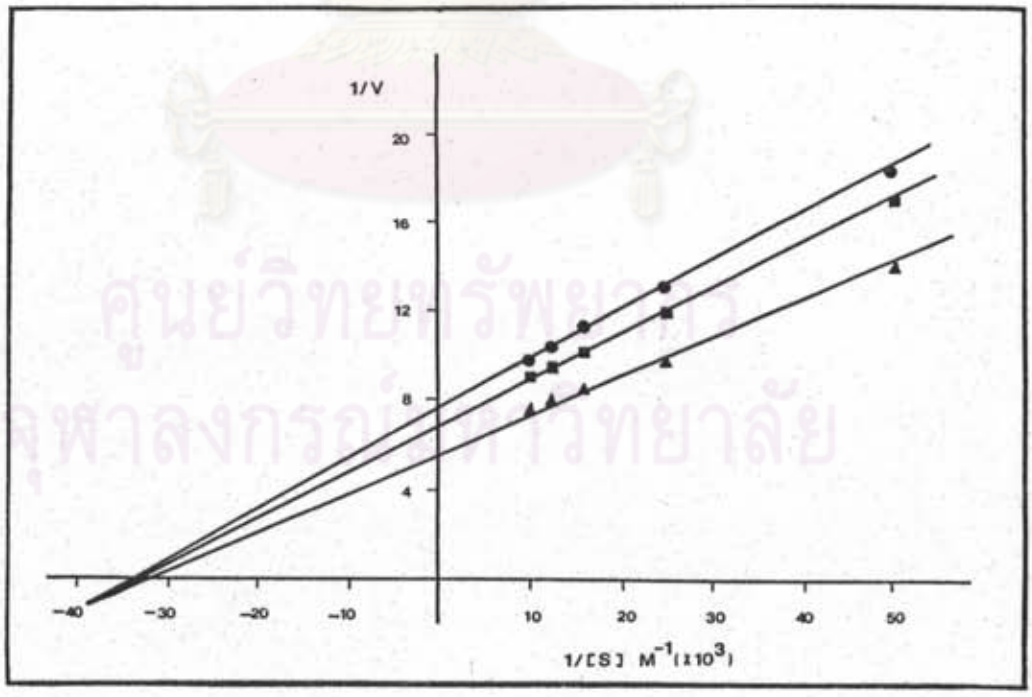


Fig. 2.31 Lineweaver-Burk double - reciprocal plots of kinetic data of compound 3 by trypsin

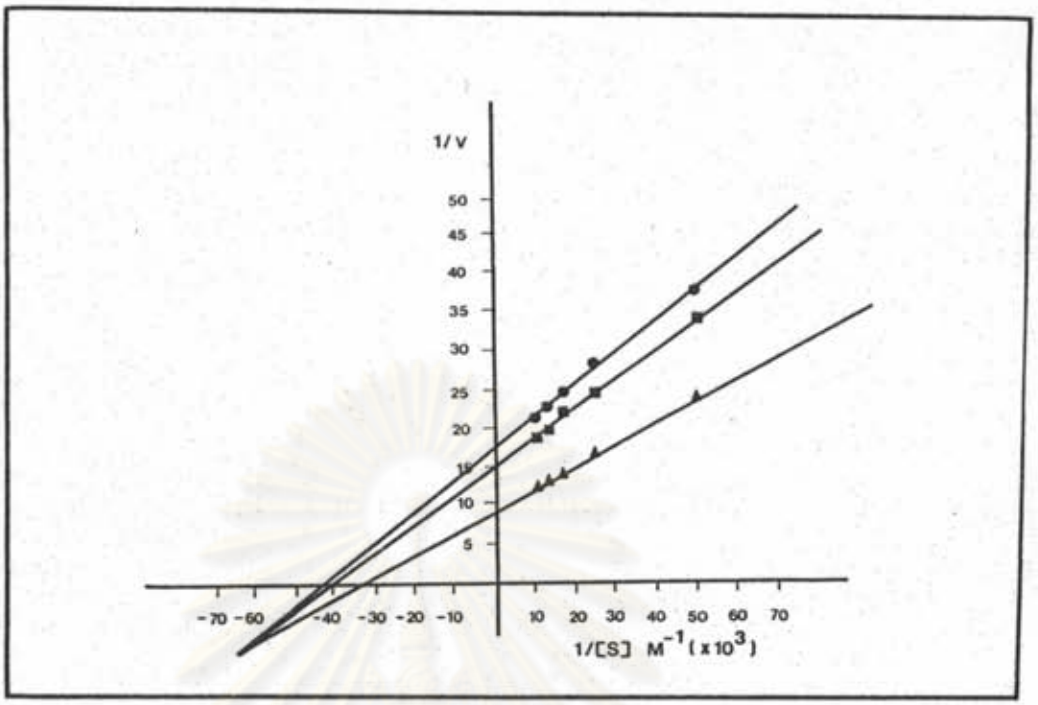


Fig. 2.32 Lineweaver-Burk double reciprocal plots of kinetic data of compound 4 by trypsin

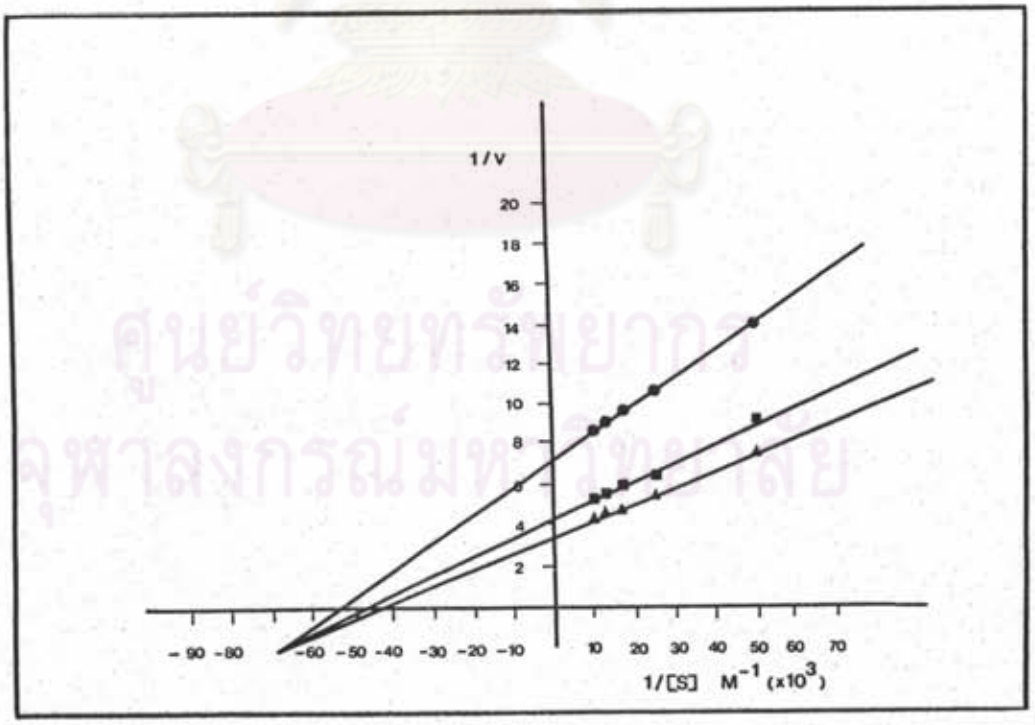


Fig. 2.33 Lineweaver-Burk double reciprocal plots of kinetic data of compound 5 by trypsin

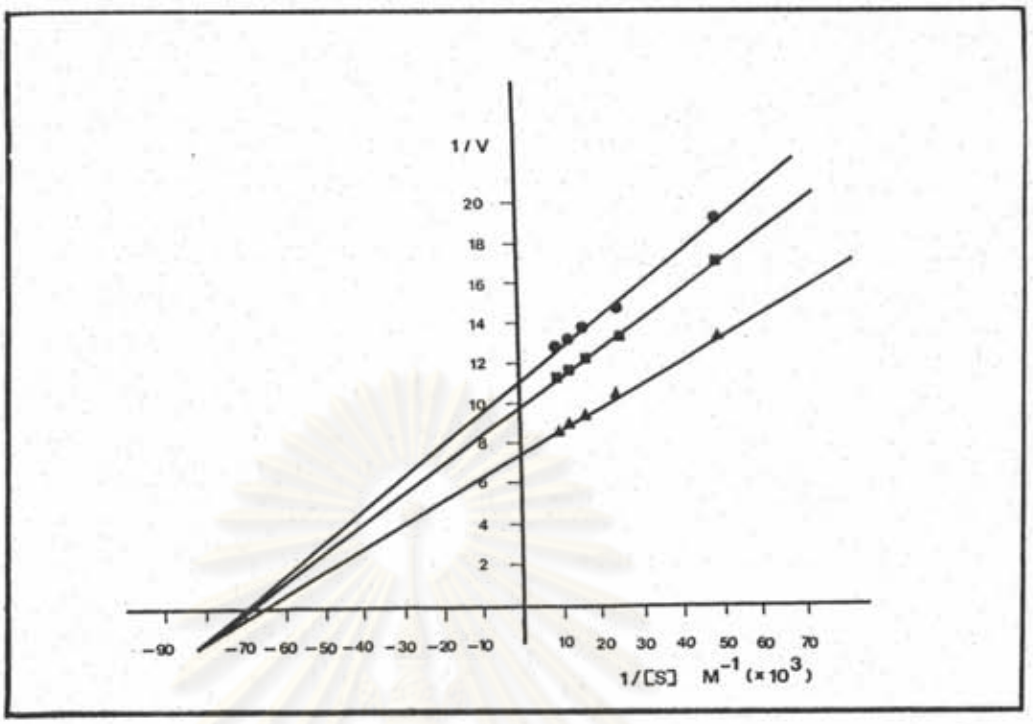


Fig. 2.34 Lineweaver-Burk double reciprocal plots of kinetic data of compound 6 by trypsin

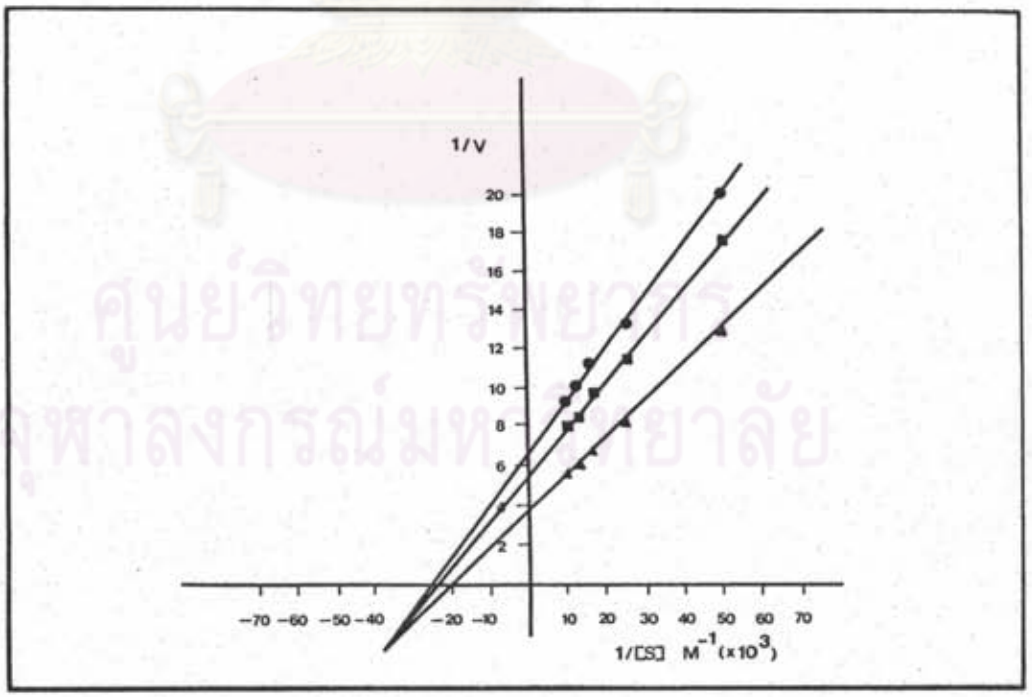


Fig. 2.35 Lineweaver-Burk double reciprocal plots of kinetic data of compound 7 by trypsin

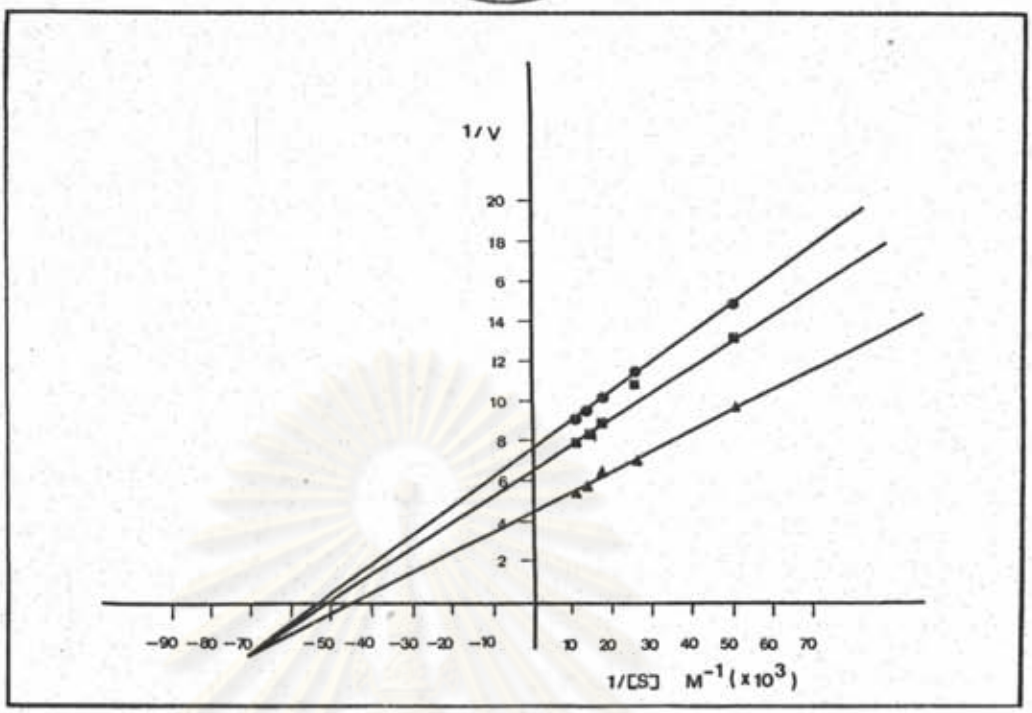


Fig. 2.36 Lineweaver-Burk double reciprocal plots of kinetic data of compound 8 by trypsin

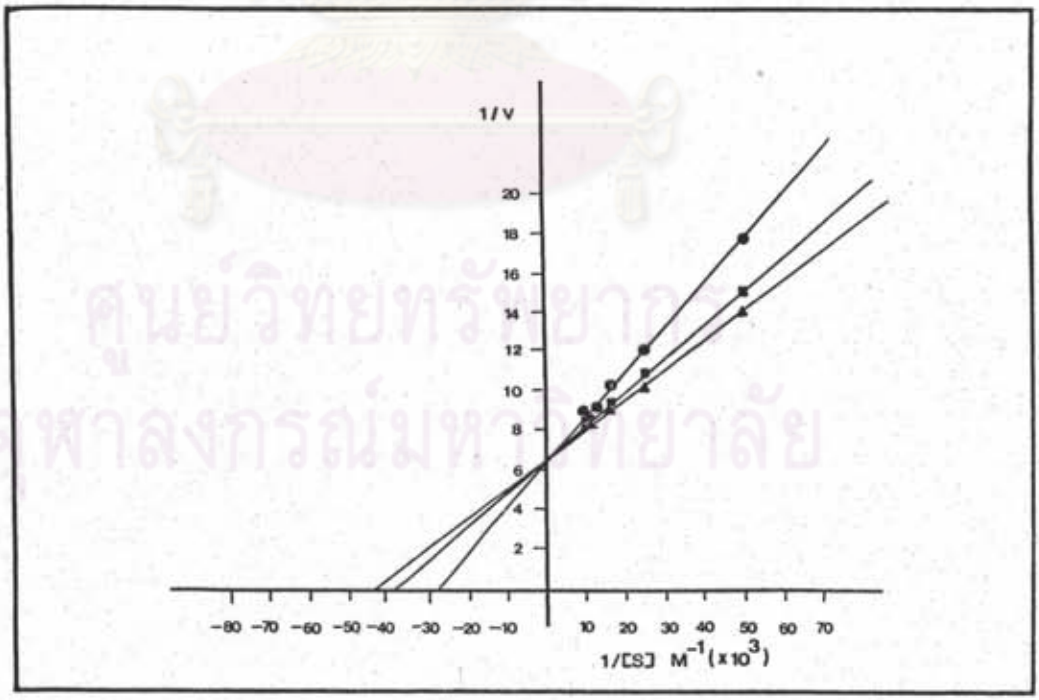


Fig. 2.37 Lineweaver-Burk double reciprocal plots of kinetic data of compound 9 by trypsin

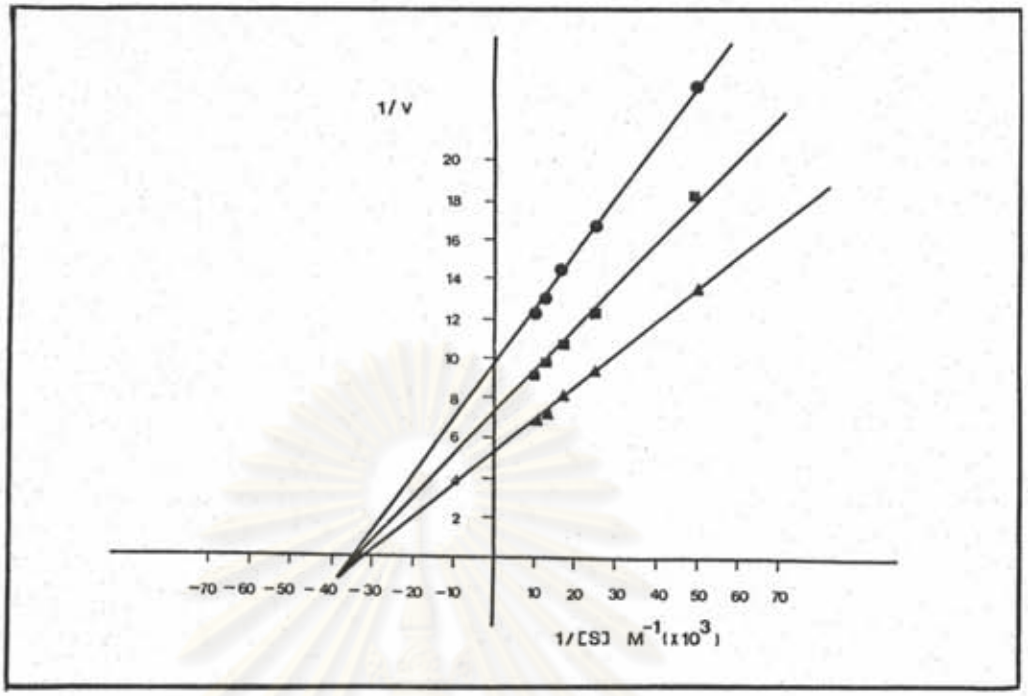


Fig. 2.38 Lineweaver-Burk double reciprocal plots of kinetic data of compound 10 by trypsin

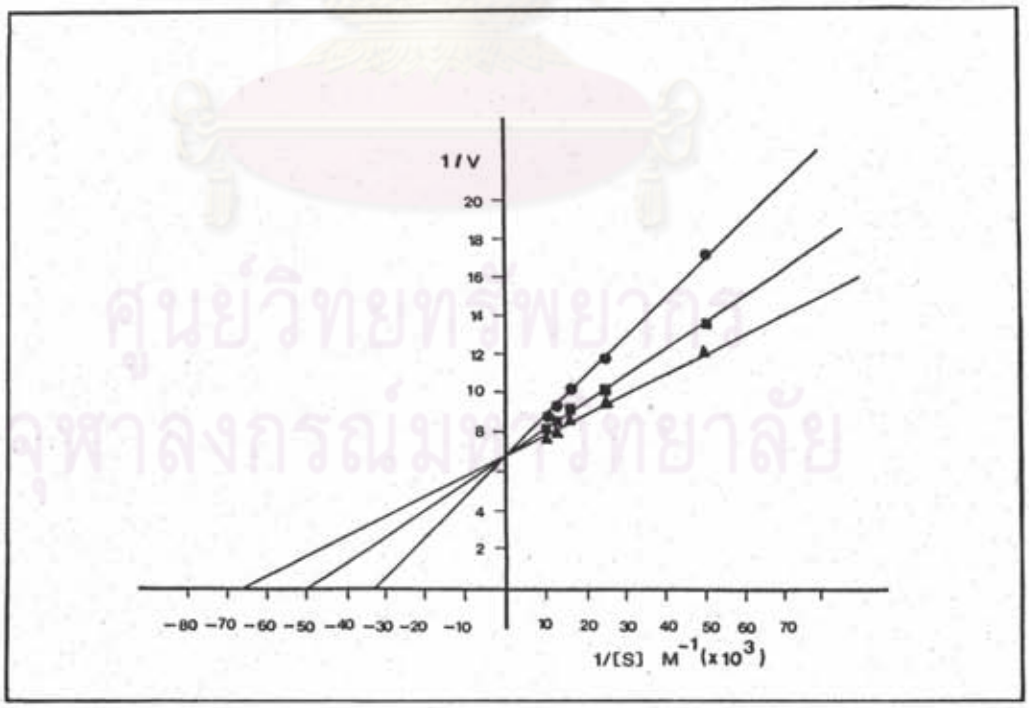


Fig. 2.39 Lineweaver-Burk double reciprocal plots of kinetic data of compound 11 by trypsin

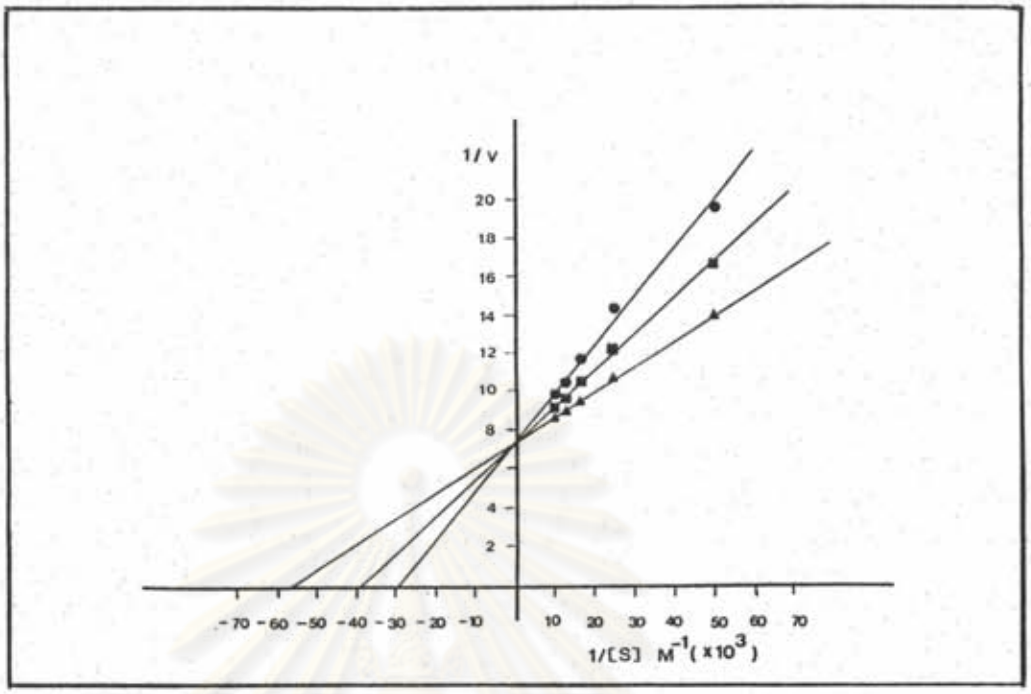


Fig. 2.40 Lineweaver-Burk double reciprocal plots of kinetic

kinetic data of compound 12 by trypsin

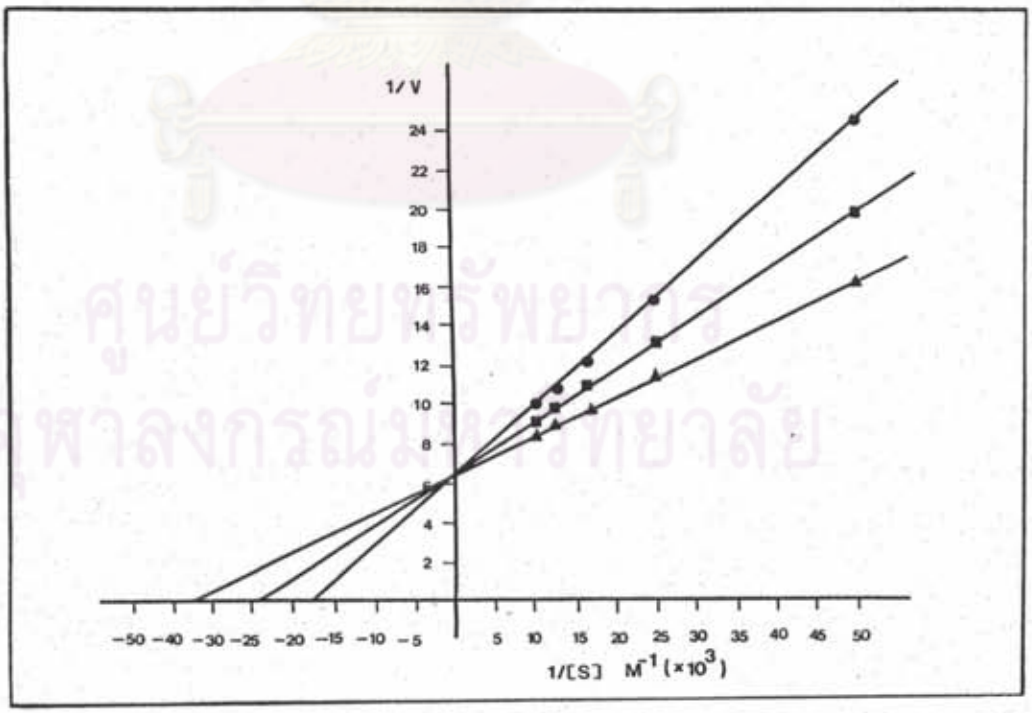


Fig. 2.41 Lineweaver-Burk double reciprocal plots of

kinetic data of compound 13 by trypsin

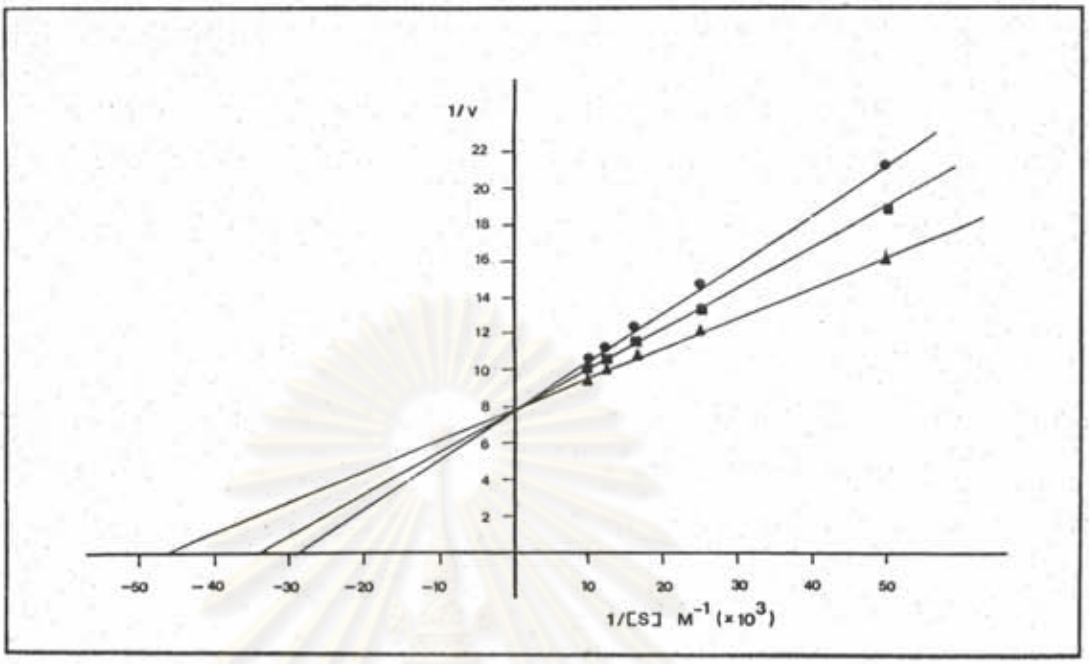


Fig. 2.42 Lineweaver-Burk double reciprocal plots of kinetic data of compound 14 by trypsin

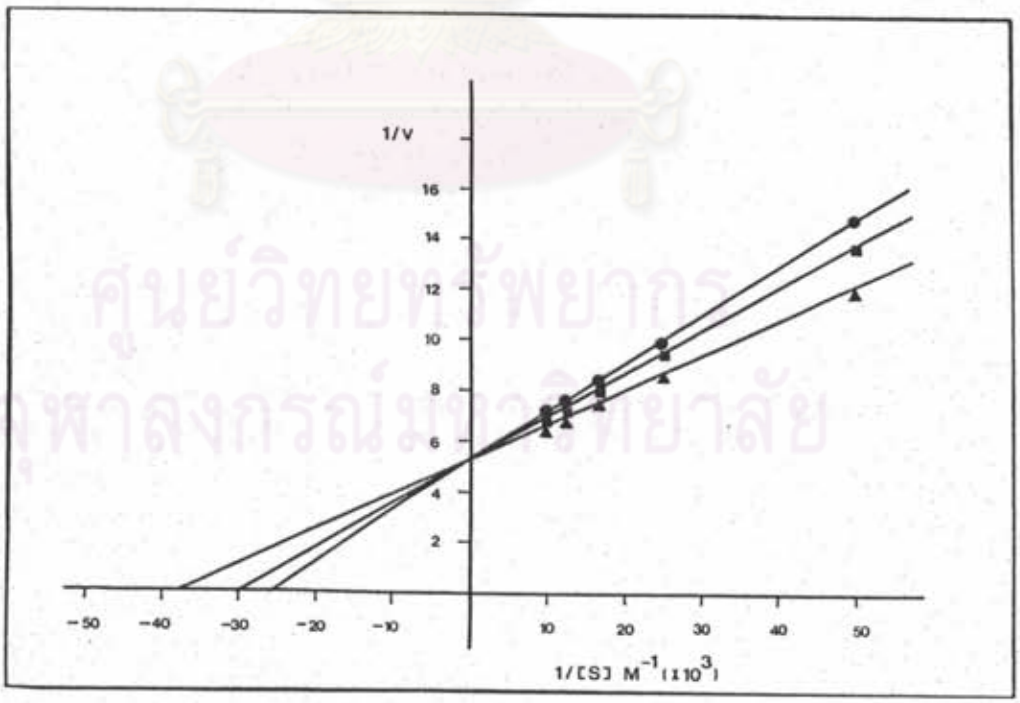


Fig. 2.43 Lineweaver-Burk double reciprocal plots of kinetic data of compound 15 by trypsin

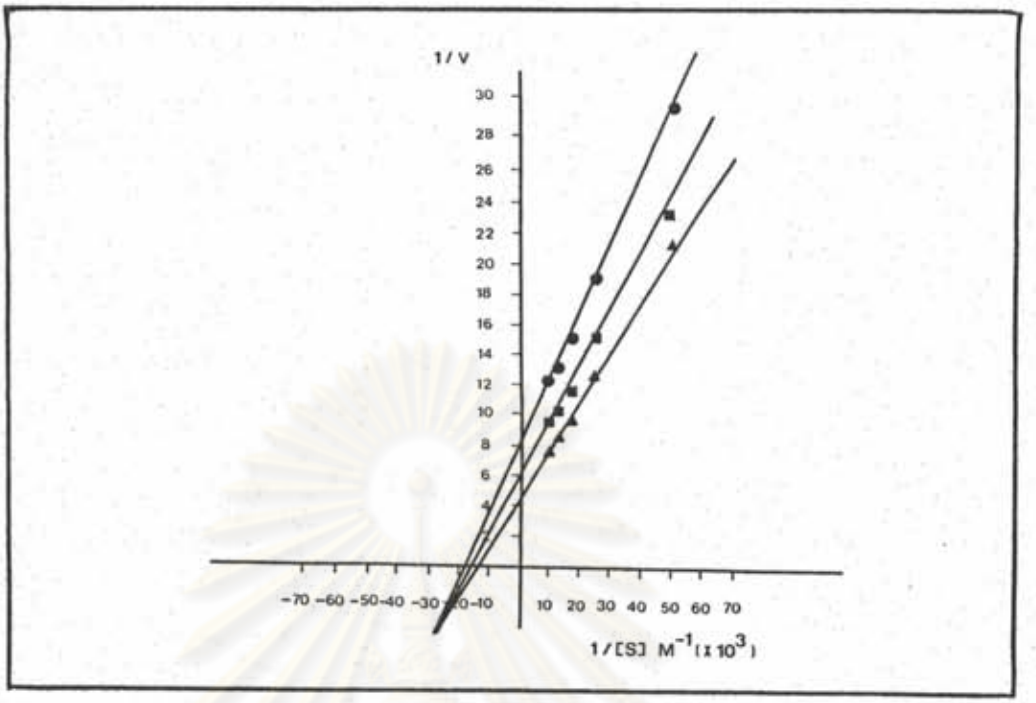


Fig. 2.44 Lineweaver-Burk double reciprocal plots of kinetic data of compound 16 by trypsin

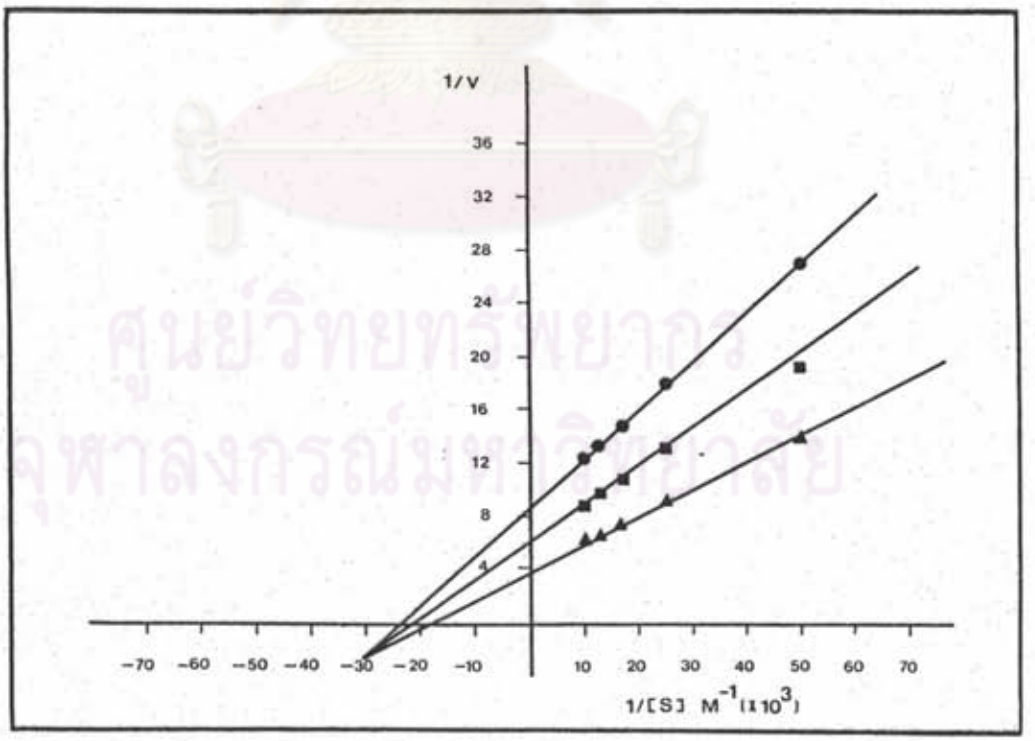


Fig. 2.45 Lineweaver-Burk double reciprocal plots of kinetic data of compound 17 by trypsin

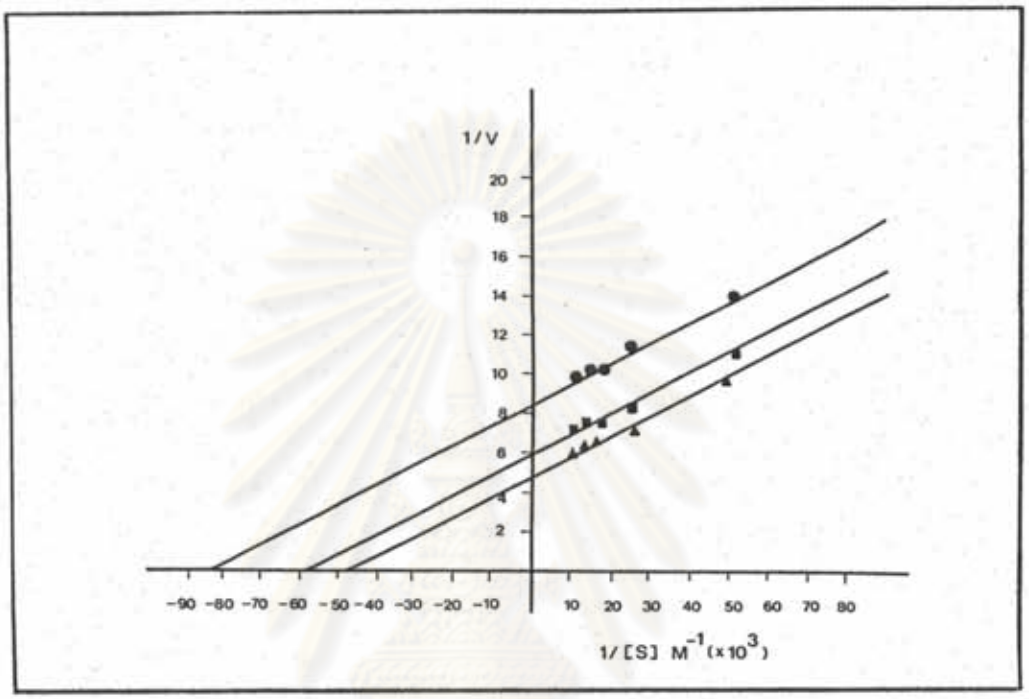


Fig. 2.46 Lineweaver-Burk double - reciprocal plots of kinetic data of compound 1 by chymotrypsin (The initial velocities are given as the increase in the absorbance at 400 nm. per minute. Each of point represents the mean of four experiments.) ▲—▲ no inhibitor ; ■—■ inhibitor 150 µl. ; ●—● inhibitor 200 µl. Experiments were performed in 0.1 M.HEPES buffer and 10% DMSO, 0.05 M.NaCl at pH 7.5 and 37°C

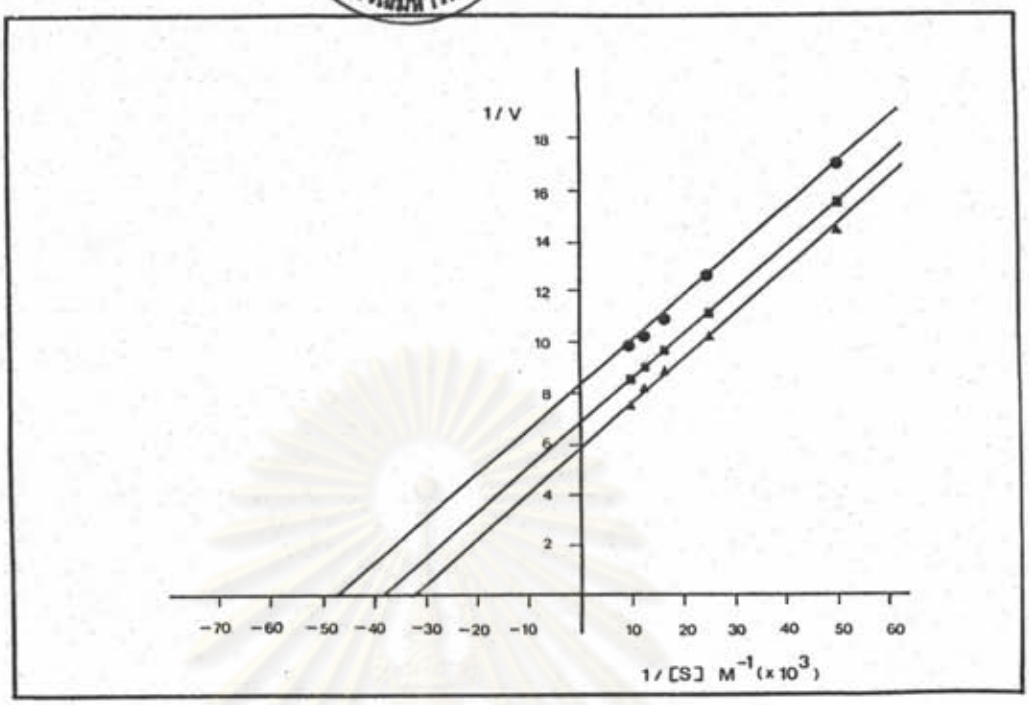


Fig. 2.47 Lineweaver-Burk double - reciprocal plots of kinetic data of compound 2 by chymotrypsin

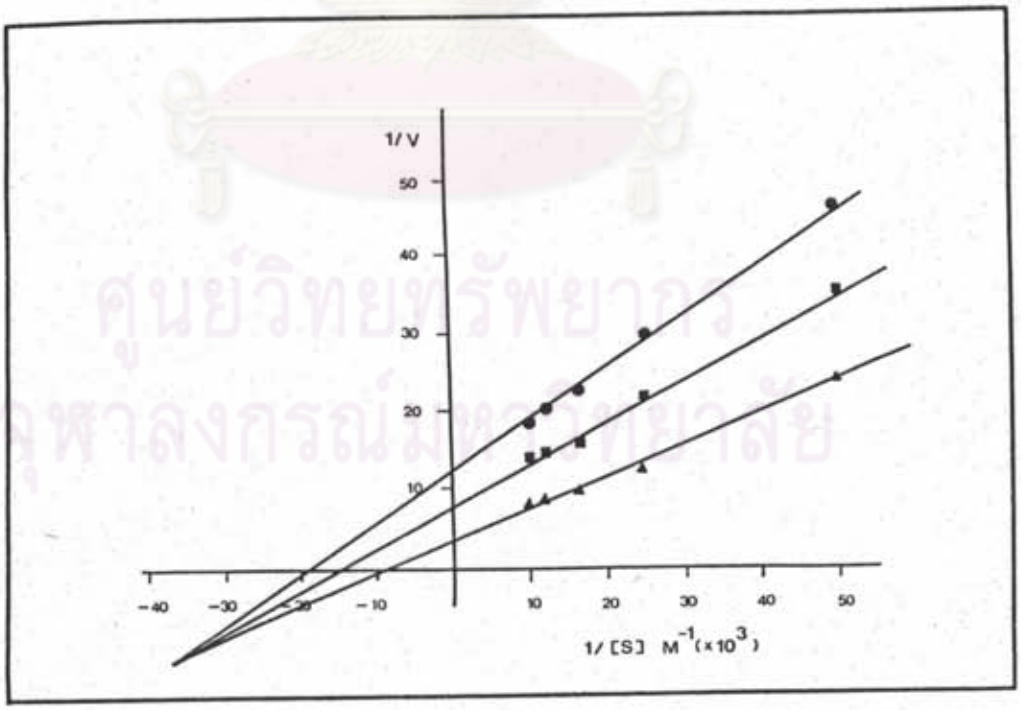


Fig. 2.48 Lineweaver-Burk double - reciprocal plots of kinetic data of compound 3 by chymotrypsin

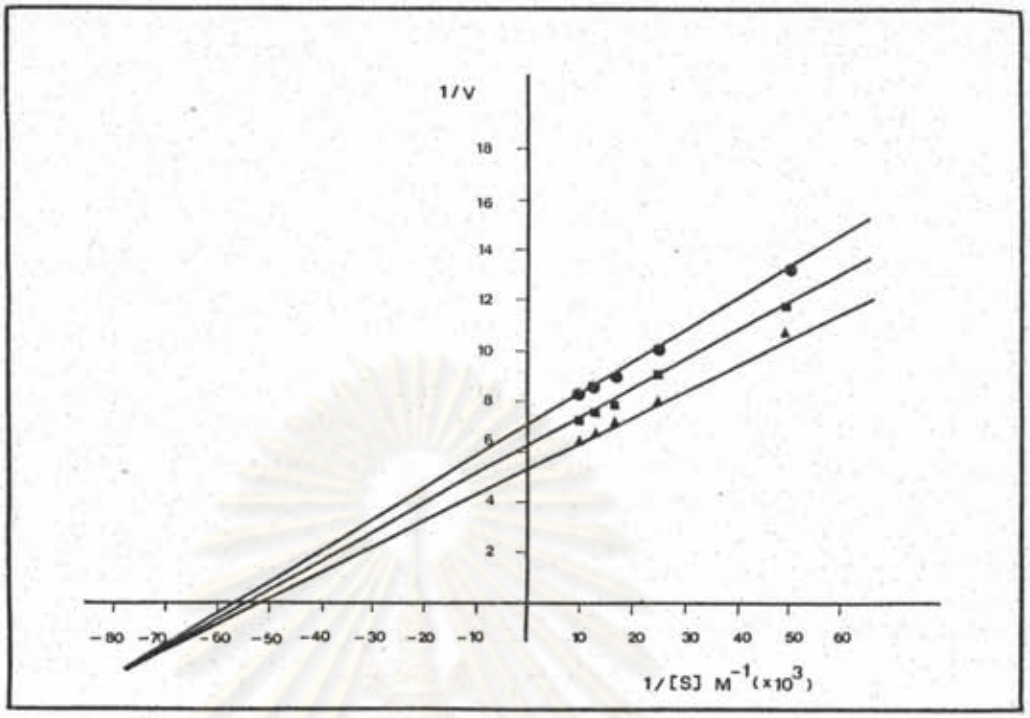


Fig. 2.49 Lineweaver-Burk double reciprocal plots of kinetic data of compound 4 by chymotrypsin

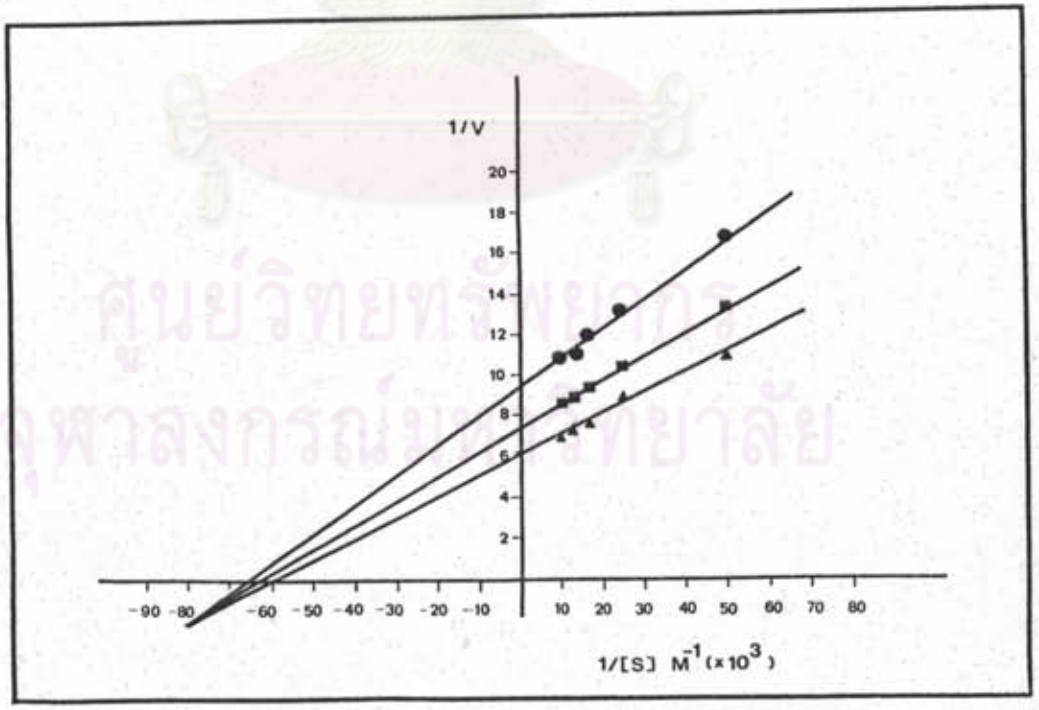


Fig. 2.50 Lineweaver-Burk double reciprocal plots of kinetic data of compound 5 by chymotrypsin

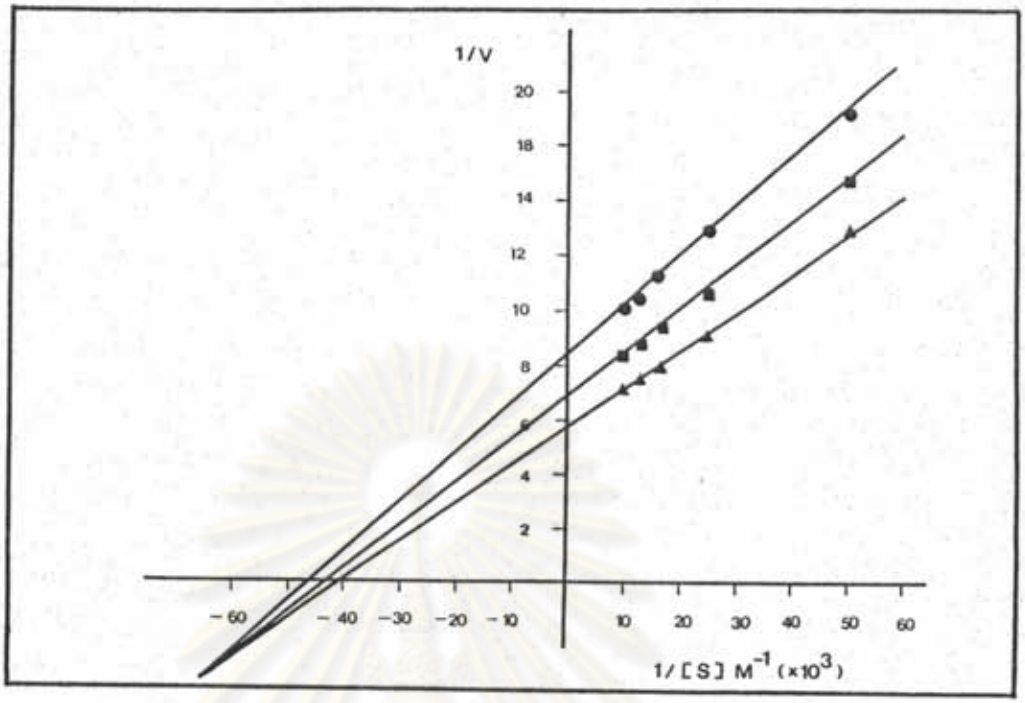


Fig. 2.51 Lineweaver-Burk double reciprocal plots of kinetic data of compound 6 by chymotrypsin

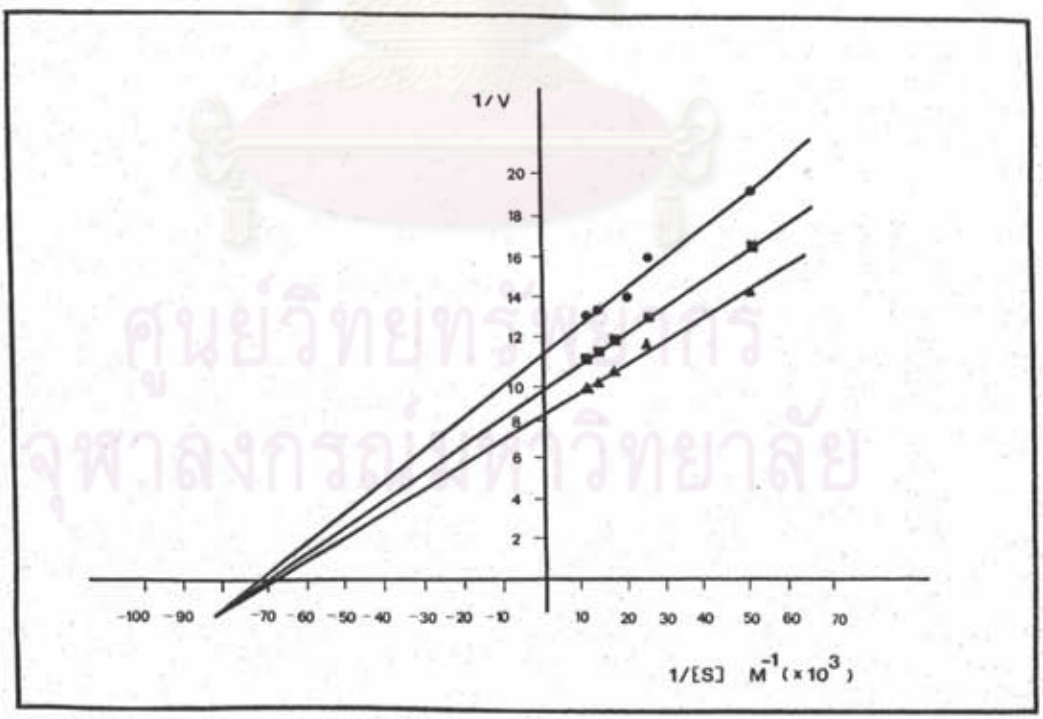


Fig. 2.52 Lineweaver-Burk double reciprocal plots of kinetic data of compound 7 by chymotrypsin

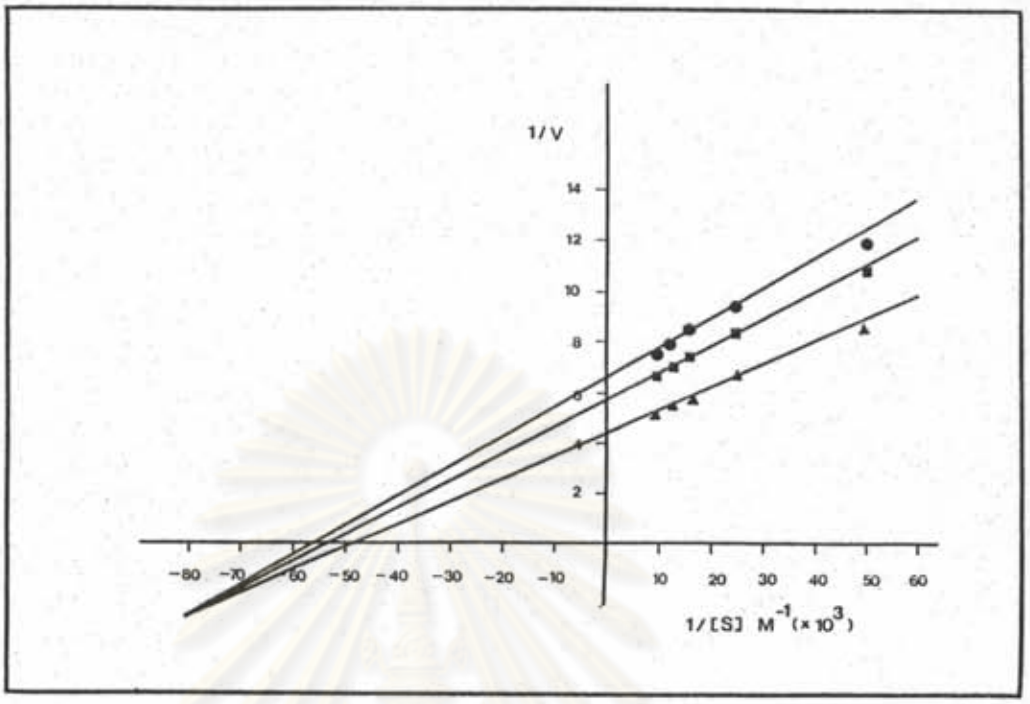


Fig. 2.53 Lineweaver-Burk double reciprocal plots of kinetic data of compound 8 by chymotrypsin

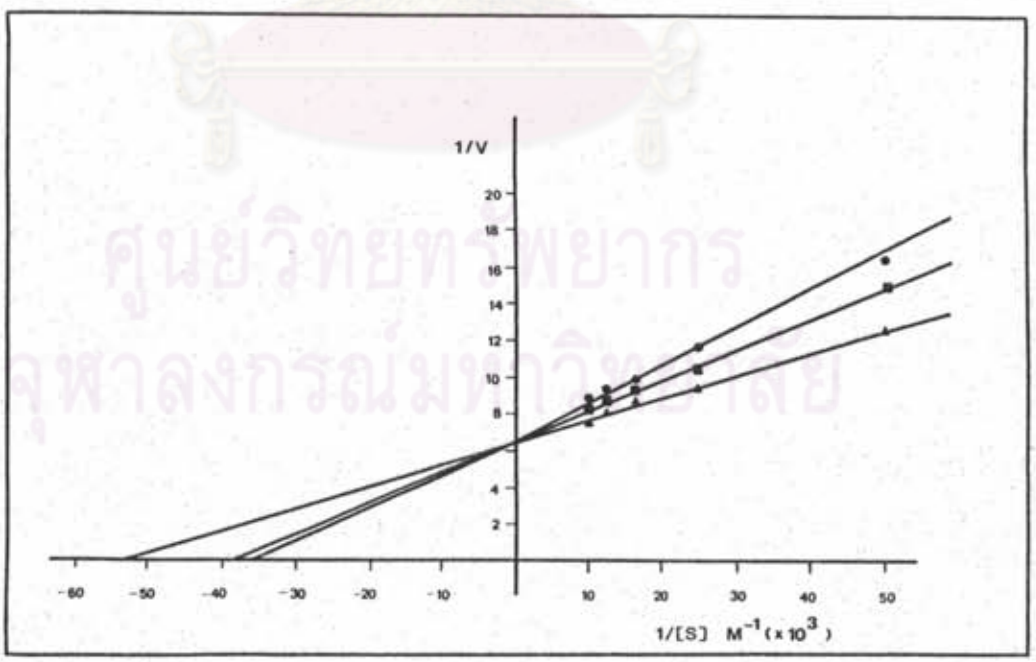


Fig. 2.54 Lineweaver-Burk double reciprocal plots of kinetic data of compound 9 by chymotrypsin

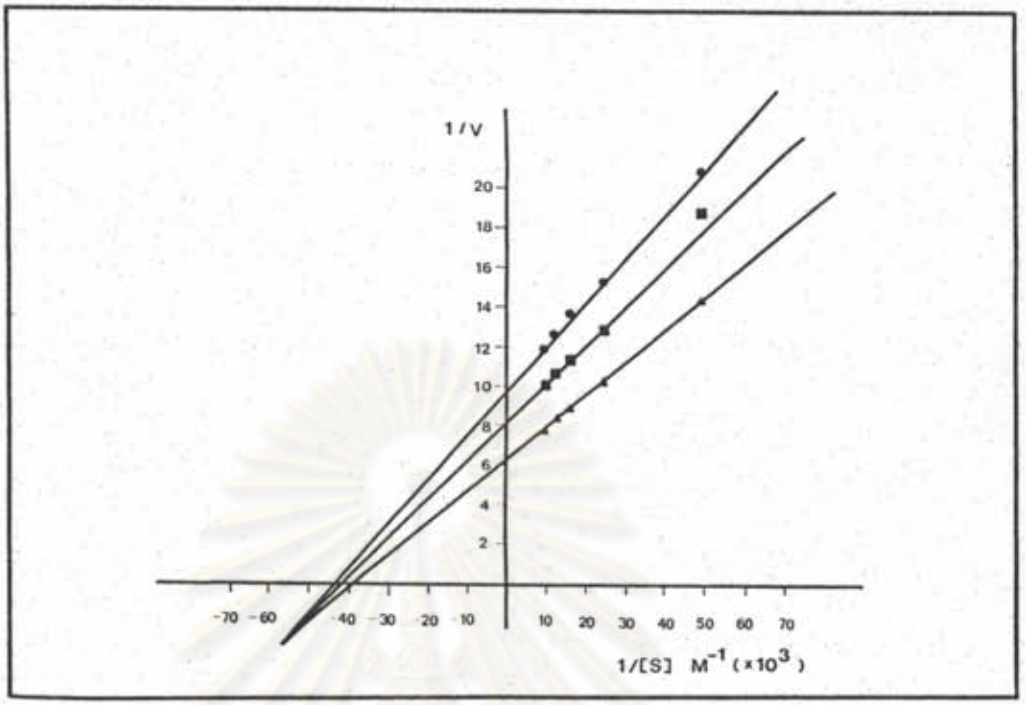


Fig. 2.55 Lineweaver-Burk double reciprocal plots of kinetic data of compound 10 by chymotrypsin

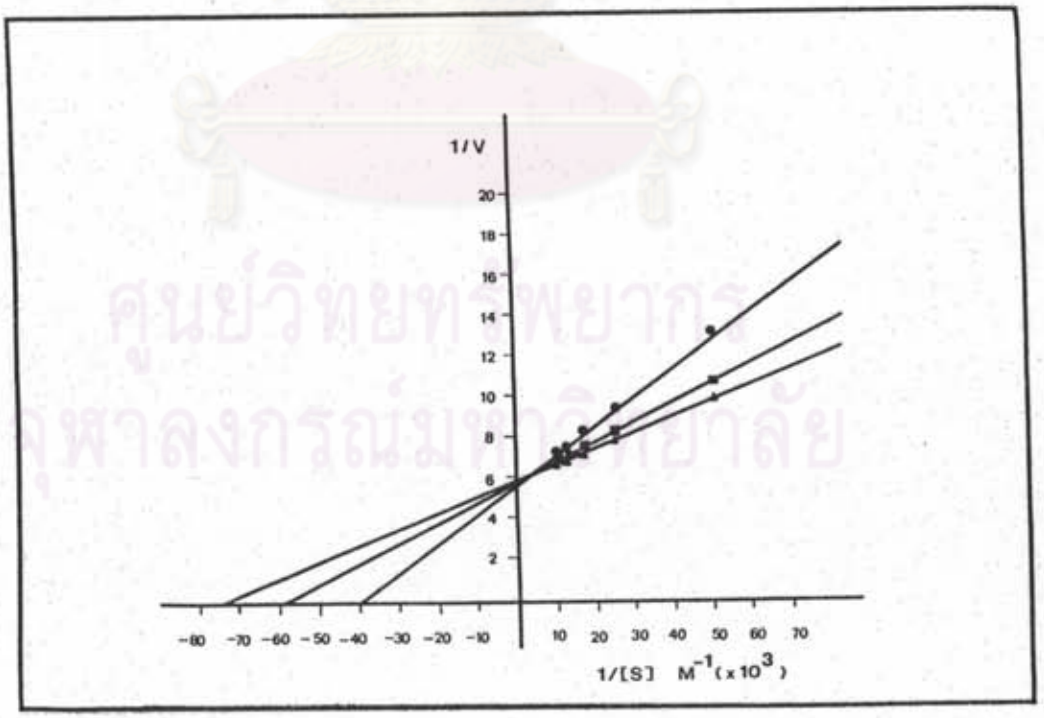


Fig. 2.56 Lineweaver-Burk double reciprocal plots of kinetic data of compound 11 by chymotrypsin

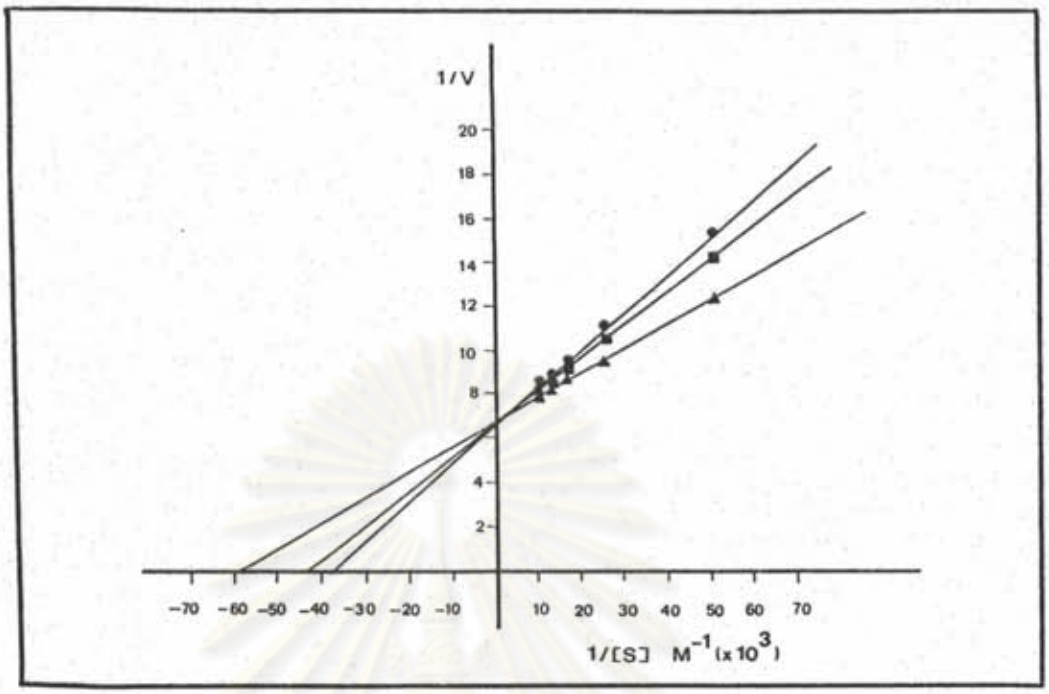


Fig. 2.57 Lineweaver-Burk double reciprocal plots of kinetic data of compound 12 by chymotrypsin

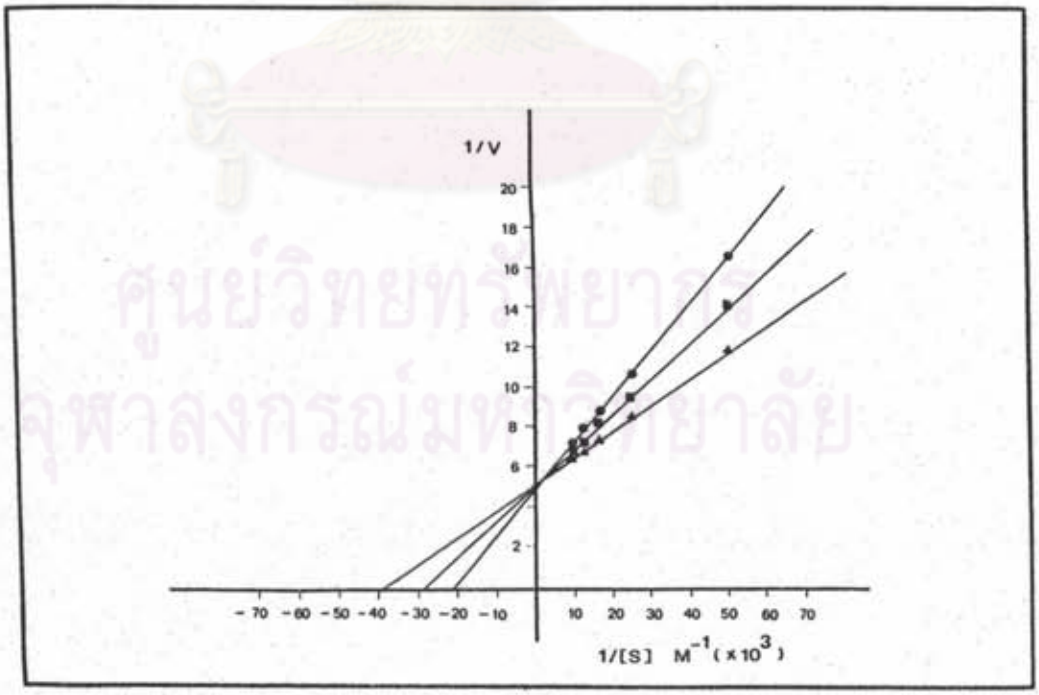


Fig. 2.58 Lineweaver-Burk double reciprocal plots of kinetic data of compound 13 by chymotrypsin

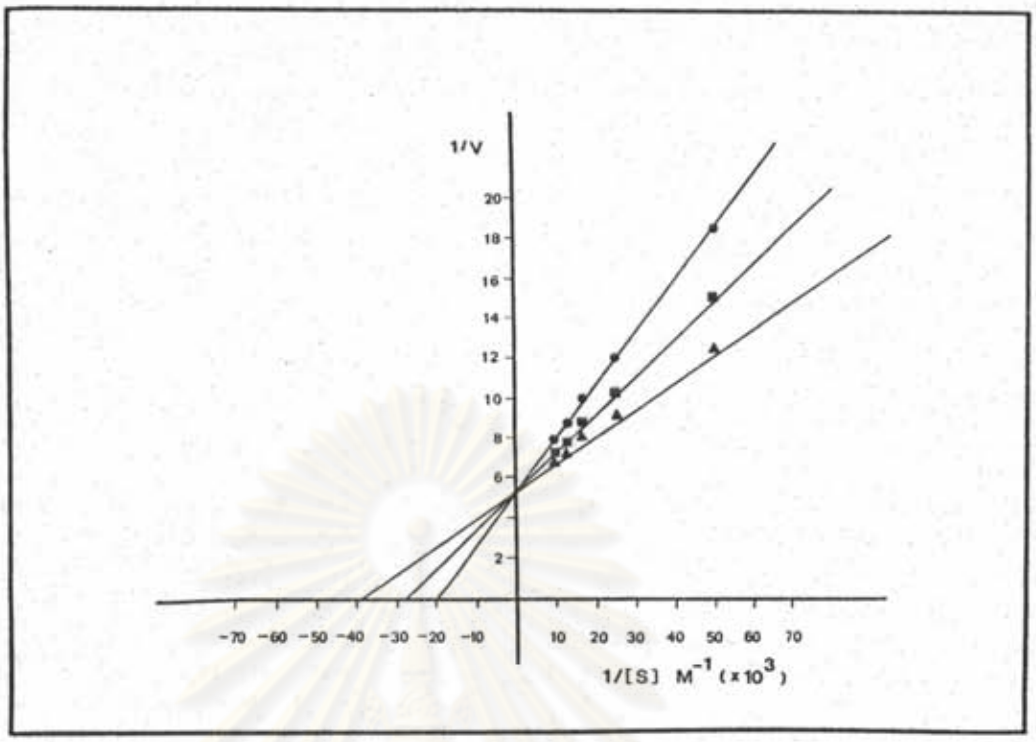


Fig. 2.59 Lineweaver-Burk double reciprocal plots of kinetic data of compound 14 by chymotrypsin

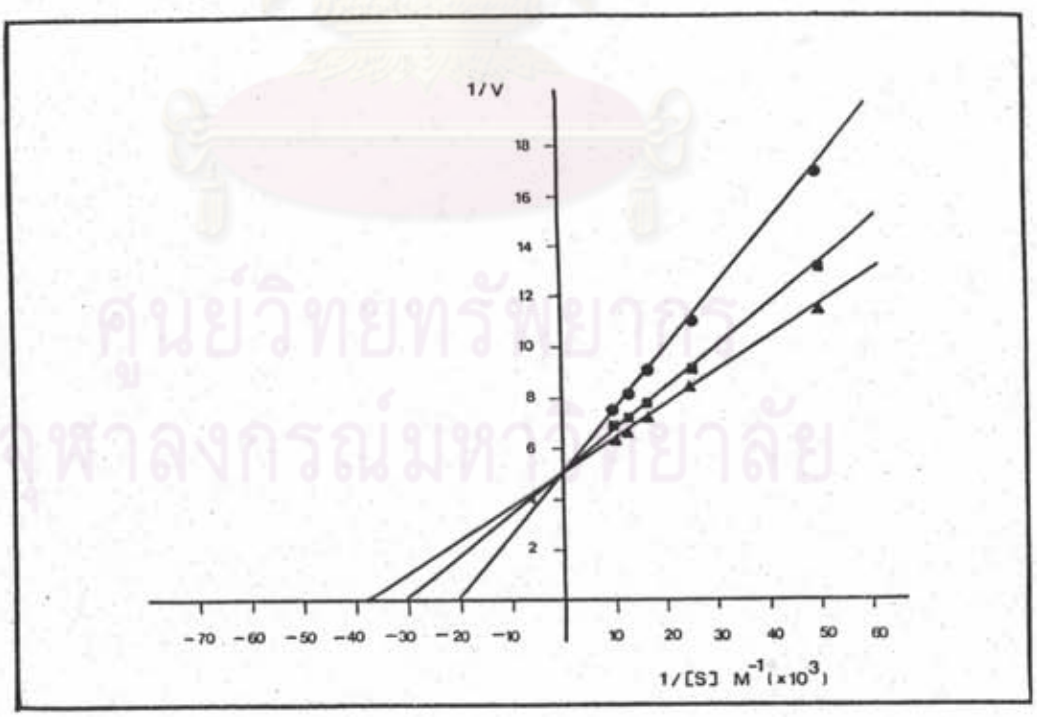


Fig. 2.60 Lineweaver-Burk double reciprocal plots of kinetic data of compound 15 by chymotrypsin

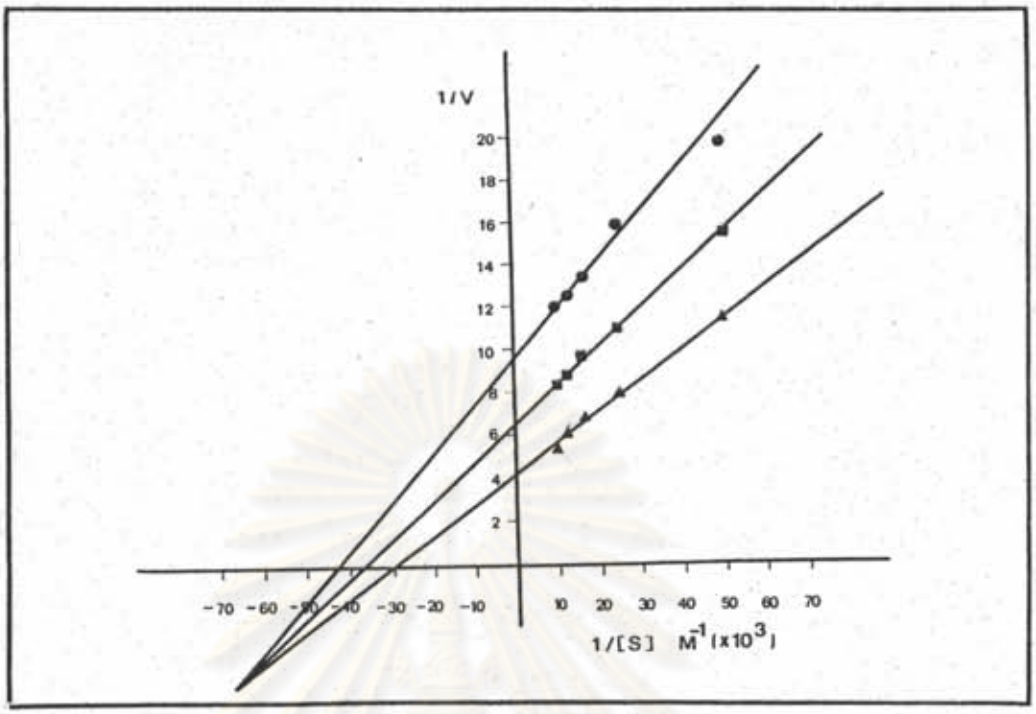


Fig. 2.61 Lineweaver-Burk double reciprocal plots of kinetic data of compound 16 by chymotrypsin

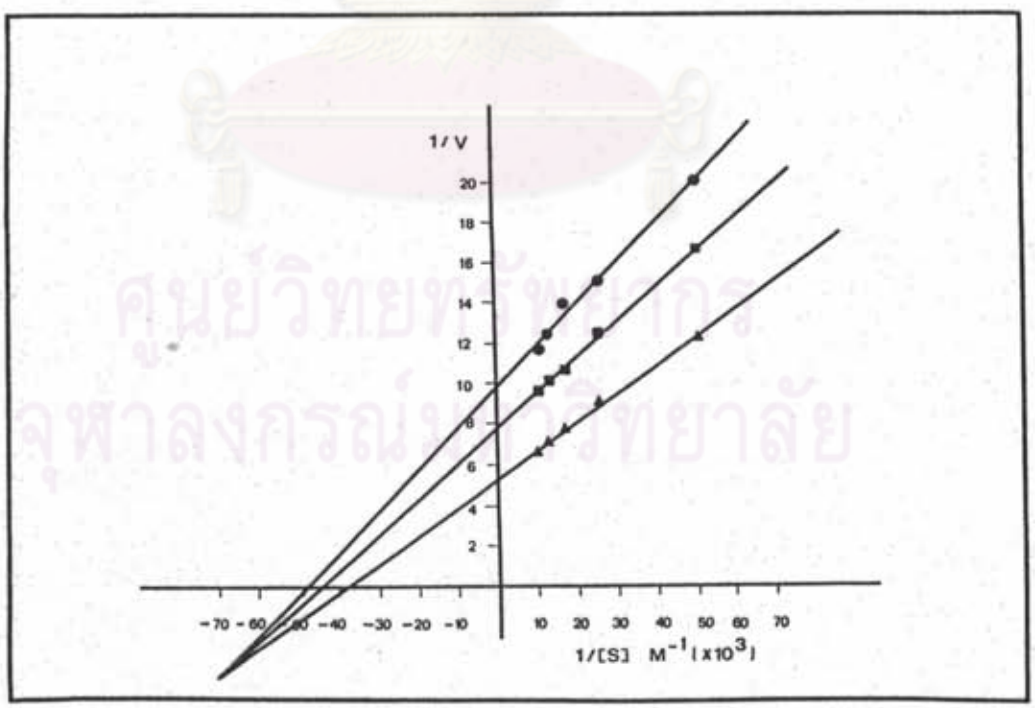


Fig. 2.62 Lineweaver-Burk double reciprocal plots of kinetic data of compound 17 by chymotrypsin