

## CHAPTER III

### KINETIC METHODS AND EXPERIMENTAL

#### 3.1 Starting Materials and Purification of Solvents

##### 3.1.1 Starting Materials

Chemicals used in this research were obtained from the following sources:

Tetrahydrofuran (THF), ethyl chloroformate, triethylamine, benzoyl chloride, benzyl chloroformate, L-phenylalanine, L-tyrosine, glycine, N-benzoyl glycine, Z-L-alanine, Z-glycine, petroleum ether b.p. 40-60 °C, absolute methanol, diethyl ether, thionyl chloride, toluene, sodium chloride, dimethyl sulfoxide, hexane, ethyl acetate, chloroform were purchased from Fluka Company. All of them were A.R. grade except hexane, ethyl acetate and chloroform.

Trypsin (EC 3.4.21.4),  $\alpha$ -chymotrypsin (EC 3.4.21.1), Succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine p-nitroanilide (Suc-Ala-Ala-Pro-Phe-pNA),  $N_{\alpha}$ -benzoyl-dl-arginine p-nitroanilide (BAPNA) were purchased from Sigma Chemical Company.

Human cathepsin G (EC 3.4.21.20), the buffer: N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) were obtained from Professor Dr. Bela Ternai of La Trobe University, Australia.

##### 3.1.2 Purification of Solvents

Tetrahydrofuran was dried with sodium pellets and

was refluxed with  $\text{LiAlH}_4$ . The solvent was then distilled from  $\text{LiAlH}_4$  and stored over molecular sieves type 4A (70).

Triethylamine was distilled over potassium hydroxide and stored over molecular sieves type 4A (70).

Chloroform, hexane and ethyl acetate (commercial grade) were distilled before use.

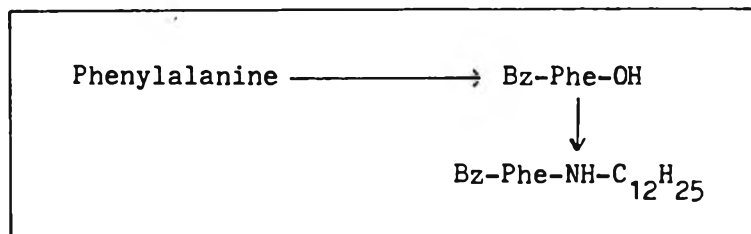
## 3.2 Syntheses

### 3.2.1 Preparation of Carbobenzoxyglycine (71)

Glycine ( 3.75 g, 0.05 moles ) was dissolved in 2 N sodium hydroxide (25 mL). The solution was stirred and cooled in an ice bath. Benzyl chloroformate ( 7 mL, 0.05 moles ) and 4 N sodium hydroxide (12.5 mL) was then added simultaneously to the vigorously stirred solution. The solution was continued to be stirred and cooled for 40 minutes. After that, the solution was extracted with toluene (100 mL) and the aqueous layer was extracted once with ether (50 mL). The aqueous solution was then cooled in an ice bath and acidified to Congo red with concentrated hydrochloric acid. The precipitate was filtered and washed with cold water and recrystallised from chloroform. A white crystalline solid was obtained (7.5 g, 72%) m.p. 119-120 °C (Lit [71], m.p. 119-120 °C).

### 3.2.2 Preparation of Bz-Phe-NH-C<sub>12</sub>H<sub>25</sub> (compound I)

There are two steps of reaction involved in this preparation which is schematically described below :



#### Step 1: Preparation of Bz-Phe-OH

L-Phenylalanine (1.65 g, 10 mmols) was dissolved in water (40 mL) containing sodium hydroxide (0.8 g, 20 mmols). The solution was cooled at 0 to 5°C before the addition of benzoyl chloride (1.2 mL, 10.3 mmols) and a solution of sodium hydroxide (0.4 g, 10 mmols) in water (20 mL). The mixture was cooled and stirred continuously for 3 hours. The solution was then acidified to Congo red with concentrated hydrochloric acid. The crude product was filtered off and washed with ice-cold water and recrystallised from boiling water. A white crystalline solid was obtained (1.6 g, 60%), m.p. 142-143°C (lit[72], m.p. 142-143°C).

#### Step 2: Preparation of Bz-Phe-NH-C<sub>12</sub>H<sub>25</sub>

Bz-Phe-OH (0.8 g, 3 mmols), prepared from step 1, was dissolved in dry THF (30 mL) and triethylamine (0.5 mL, 3.5 mmols). The solution was stirred and cooled at -5 to -10°C before the addition of ethyl chloroformate (0.7 mL, 7 mmols). After adding ethyl chloroformate, the mixture was stirred at the same temperature for 10 minutes before a solution of dodecylamine (1.7 g, 9 mmols) in dry THF (10 mL) was added. The solution was then stirred at

room temperature for 8 hours and the solvent was removed under reduced pressure. The residue was dissolved in the mixture of ethyl acetate-chloroform and washed with saturated  $\text{NaHCO}_3$  (25 mL). The organic layer was then separated and washed again with water (25 mL), 1 M HCl (25 mL) and water (25 mL) respectively. After drying over anhydrous  $\text{Na}_2\text{SO}_4$ , the ethyl acetate was evaporated and the solid was recrystallised from ethyl acetate to form a white powder (1.0 g, 77%) m.p. 138-140°C. TLC :  $R_f = 0.86$  (MeOH :  $\text{CHCl}_3 = 1:4$ )

Elemental Analysis for  $\text{C}_{28}\text{H}_{40}\text{N}_2\text{O}_2$ , calc'd: C 77.06, H 9.17, N 6.42

found : C 77.07, H 9.47, N 6.36

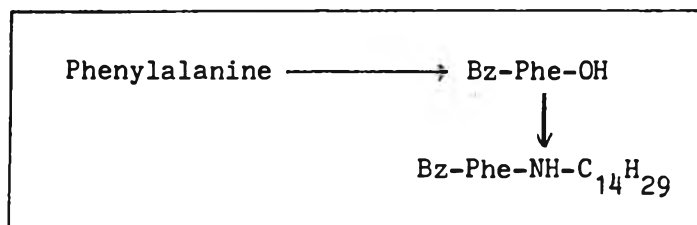
IR (KBr)  $\nu$  ( $\text{cm}^{-1}$ ) ( Fig. III.1 ) : 3300 (-NH stretching) ; 3060, 3020 (-CH aromatic); 2920, 2840 (-CH stretching); 1660, 1630 (HN-C=O); 1520 (-NH bending)

$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  (ppm) ( Fig. III.11 ) : 0.89 (t, 3H,  $\text{CH}_3$ ); 1.26 (s, 20H,  $\text{CH}_2$  's chain); 3.19 (m, 4H,  $-\text{CH}_2-\text{Ar}$ ,  $-\text{NH}-\text{CH}_2-$ ); 4.8 (q, 1H,  $\text{NH}-\text{CH}-\text{CH}_2$ ); 5.78 (br., 1H,  $-\text{NH}-\text{CH}_2$ ); 6.82 (br., 1H,  $-\text{NH}-\text{CH}-$ ); 7.29 (s, 5H,  $\text{Ar}-\text{CH}_2$ ); 7.56 (m, 5H,  $\text{Ar}-\text{C}=\text{O}$ )

$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  (ppm) ( Fig. III.21 ) : 14.08 ( $\text{CH}_3$ ); 22.64, 26.81, 29.25, 29.63, 31.90 ( $\text{CH}_2$  's chain); 38.89 ( $-\text{NH}-\text{CH}_2-$ ); 39.60 ( $-\text{CH}_2-\text{Ar}$ ); 55.20 ( $-\text{NH}-\text{CH}-\text{CH}_2-$ ); 126.87, 127.14, 128.50, 129.42, 131.64, 133.86, 136.95 ( $\text{Ar}-\text{C}=\text{O}$ ,  $-\text{CH}_2-\text{Ar}$ ); 167.18 ( $\text{O}=\text{C}-\text{NH}$ ); 170.86 ( $\text{Ar}-\text{C}=\text{O}$ )

### 3.2.3 Preparation of Bz-Phe-NH-C<sub>14</sub>H<sub>29</sub> (Compound II)

There are two steps of reaction involved in this preparation which is schematically described below:



#### Step 1: Preparation of Bz-Phe-OH

This product had already been prepared in the synthesis of Bz-Phe-NH-C<sub>12</sub>H<sub>25</sub> (Compound I)

#### Step 2: Preparation of Bz-Phe-NH-C<sub>14</sub>H<sub>29</sub>

This step used Bz-Phe-OH (0.8 g, 3 mmoles) prepared from step 1 and tetradecylamine (2 g, 9 mmoles). The reaction was carried out under the same reaction conditions described for the synthesis of Bz-Phe-NH-C<sub>12</sub>H<sub>25</sub>. The product remained as white solid and was recrystallised from ethyl acetate to yield a white solid (0.9 g, 65%) m.p. 132-133°C. TLC:R<sub>f</sub> = 0.89 (MeOH:CHCl<sub>3</sub> = 1:4)

Elemental analysis for C<sub>30</sub>H<sub>44</sub>N<sub>2</sub>O<sub>2</sub>, calc'd: C 77.59, H 9.48, N 6.03

found : C 77.48, H 9.75, N 5.87

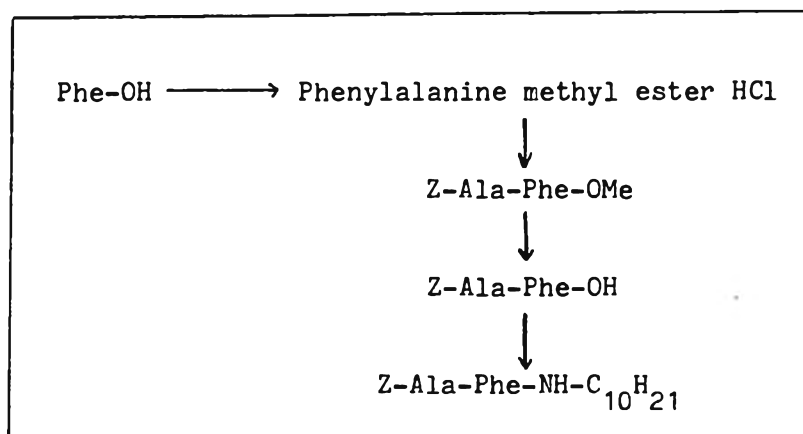
IR (KBr)  $\nu$  (cm<sup>-1</sup>) ( Fig. III.2 ): 3300 (-NH stretching); 3060, 3020 (-CH aromatic); 2920, 2840 (-CH stretching); 1655, 1630 (HN-C=O); 1520 (-NH bending)

<sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  (ppm) ( Fig. III.12 ): 0.88 (t, 3H, CH<sub>3</sub>); 1.26 (s, 24H, CH<sub>2</sub>'s chain); 3.19 (m, 4H, -CH<sub>2</sub>-Ar, -NH-CH<sub>2</sub>-); 4.83 (q, 1H, -NH-CH-CH<sub>2</sub>); 5.97 (br., 1H, -NH-CH<sub>2</sub>); 7.15 (br., 1H, NH-CH); 7.28 (s, 5H, Ar-CH<sub>2</sub>); 7.57 (m, 5H, Ar-C=O)

$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  (ppm) (Fig. III.22): 14.08 ( $\text{CH}_3$ ); 22.69, 26.81, 29.30, 29.63, 31.90 ( $\text{CH}_2$ 's chain); 38.89 ( $-\text{NH}-\underline{\text{CH}}_2-$ ); 39.60 ( $-\underline{\text{CH}}_2-\text{Ar}$ ); 55.20 ( $-\text{NH}-\underline{\text{CH}}-\text{CH}_2-$ ); 126.93, 127.09, 128.50, 129.36, 131.69, 133.91, 136.95 ( $\text{Ar}-\underline{\text{C}}=\text{O}$ ,  $\text{CH}_2-\underline{\text{Ar}}$ ); 167.18 ( $-\text{CH}-\underline{\text{C}}=\text{O}$ ); 170.75 ( $\text{Ar}-\underline{\text{C}}=\text{O}$ )

### 3.2.4 Preparation of Z-Ala-Phe-NH-C<sub>10</sub>H<sub>21</sub> (Compound III)

There are four steps of reaction involved in this preparation which is schematically described below :



Step 1: Preparation of Phenylalanine methyl ester HCl

Phenylalanine (1.65 g, 10 mmoles) was suspended in absolute methanol (10 mL). Thionyl chloride (0.9 mL, 12 mmoles) was added dropwise down the condenser and the solution was then refluxed for 2 hours. The solvent was then evaporated to yield a very viscous oil and the residue crystallised by adding absolute ether. The hygroscopic solid of the hydrochloride salt of phenylalanine methyl ester was obtained (2 g, 93%) m.p. 157-159 °C (lit [73] , m.p. 158-160 °C). This product was stored at below 0 °C before use.

Step 2: Preparation of Z-Ala-Phe-OMe

Z-alanine (2 g, 9 mmoles) was dissolved in dry THF (40 mL) and triethylamine (1.3 mL, 9 mmoles). The solution was cooled at -5 to -10°C with stirring before the addition of ethyl chloroformate (1 mL, 10 mmoles). The stirring was continued further at the same temperature for 15 minutes before the addition of a solution of phenylalanine methyl ester HCl from step 1 (1.94 g, 9 mmoles) in THF (10 mL), water (5 mL) and triethylamine (1.3 mL, 9 mmoles). The solution was stirred continuously for 8 hours at room temperature. After this time, the solution was placed in a separating funnel and ethyl acetate (75 mL) and water (25 mL) were added. The organic layer was collected and the aqueous layer was extracted with ethyl acetate (100 mL). The combined organic layers were washed with saturated  $\text{NaHCO}_3$  (25 mL), water (25 mL), 1 M HCl (25 mL) and water (25 mL) respectively and then dried with  $\text{Na}_2\text{SO}_4$ . The solvent was removed under reduced pressure and then the ethyl acetate-petroleum ether was added to the residue. A white crystalline solid was obtained (2.6 g, 76%), m.p. 100-101 °C (Lit [74], m.p. 100-101 °C).

Step 3: Preparation of Z-Ala-Phe-OH

Z-Ala-Phe-OMe (2.6 g, 6.8 mmoles) previously prepared from step 2 was dissolved in methanol (20 mL). With stirring at room temperature, 1 N NaOH (15 mL) was added dropwise. The solution was stirred for 5 hours at room temperature. The methanol was removed and the residue was then diluted with water (10 mL). The solution was acidified with 1 M HCl with cooling and extracted with ethyl acetate (100 mL). The ethyl acetate layer was separated and

dried with  $\text{Na}_2\text{SO}_4$  and then the solvent was removed under reduced pressure. After the residue had been dried in the air for several days, a white crystalline solid was obtained and washed with diethyl ether (1.9 g, 75%), m.p. 128-129°C.

Step 4: Preparation of Z-Ala-Phe-NH-C<sub>10</sub>H<sub>21</sub>

This step used Z-Ala-Phe-OH (0.6 g, 1.6 mmoles) previously prepared from step 3 and decylamine (0.7 g, 4.8 mmoles). The reaction was carried out under the same manner described for the synthesis of Bz-Phe-NH-C<sub>12</sub>H<sub>25</sub>. The product remained as a waxy white solid and the solid was recrystallised from ethyl acetate. After several hours, a waxy white solid had changed into a white powder (0.75 g, 92%), m.p. 161-163°C. TLC :  $R_f = 0.84$  (MeOH:CHCl<sub>3</sub> = 1:4)

Elemental analysis for C<sub>30</sub>H<sub>43</sub>N<sub>3</sub>O<sub>4</sub>, calc'd: C 70.73, H 8.45, N 8.25

found : C 70.52, H 8.56, N 8.03

IR (KBr)  $\nu$  (cm<sup>-1</sup>) ( Fig. III.3 ) : 3290(-NH stretching); 3060, 3020(-CH aromatic); 2920, 2850 (-CH stretching); 1690, 1660, 1645 (O=C-NH); 1535 (-NH bending)

<sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  (ppm) ( Fig. III.13 ) : 0.87 (t, 3H, CH<sub>3</sub>); 1.23 (s, 16H, CH<sub>2</sub>'s chain); 1.35 (d, 3H, -NH-CH-CH<sub>3</sub>); 3.08 (m, 4H, CH<sub>2</sub>-Ar, -NH-CH<sub>2</sub>-); 4.19 (m, 1H, -NH-CH-CH<sub>3</sub>); 4.55 (q, 1H, -NH-CH-CH<sub>2</sub>-); 5.06 (s, 2H, Ar-CH<sub>2</sub>-O-); 5.26 (br., 1H, -NH-CH); 5.86 (br., 1H, -NH-CH<sub>2</sub>); 6.73 (br., 1H, -NH-CH); 7.23 (s, 5H, Ar-CH<sub>2</sub>); 7.34 (s, 5H, Ar-CH<sub>2</sub>-O-)

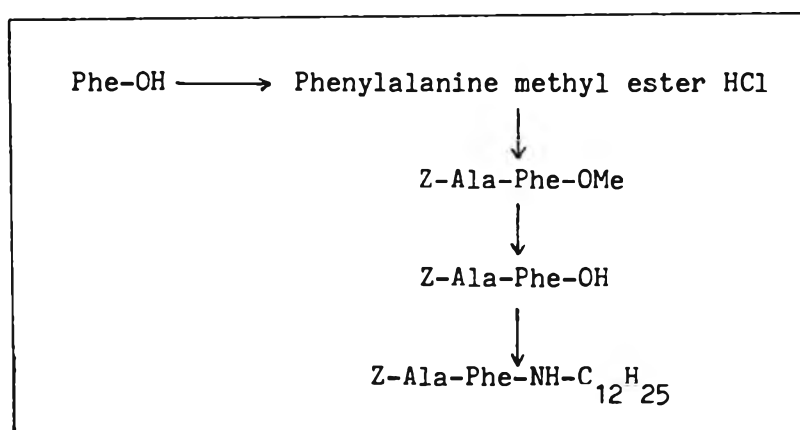
<sup>13</sup>C NMR (CDCl<sub>3</sub>)  $\delta$  (ppm) ( Fig. III.23 ) : 14.08 (CH<sub>3</sub>); 18.52 (CH-CH<sub>3</sub>); 22.69, 26.87, 29.30, 29.57, 31.90 (CH<sub>2</sub>'s chain); 38.51 (-NH-CH<sub>2</sub>-); 39.65 (-CH<sub>2</sub>-Ar); 50.97 (-NH-CH-CH<sub>3</sub>);



54.66 ( $-\text{NH}-\underline{\text{CH}}-\text{CH}_2$ ); 67.12 ( $\text{Ar}-\underline{\text{CH}}_2-\text{O}$ ); 126.98, 128.06, 128.28, 128.61, 129.31, 136.73 ( $\underline{\text{Ar}}-\text{CH}_2$ ,  $\underline{\text{Ar}}-\text{CH}_2-\text{O}$ ); 156.02 ( $\text{O}-\underline{\text{C}}=\text{O}$ ); 170.32 ( $\text{CH}_3-\text{CH}-\underline{\text{C}}=\text{O}$ ); 172.22 ( $-\text{CH}-\underline{\text{C}}=\text{O}$ )

### 3.2.5 Preparation of Z-Ala-Phe-NH-C<sub>12</sub>H<sub>25</sub> (Compound IV)

There are four steps of reaction involved in this preparation which is schematically described below :



Step 1,2 and 3 had already been made in the synthesis of Z-Ala-Phe-NH-C<sub>10</sub>H<sub>21</sub> (Compound III)

#### Step 4: Preparation of Z-Ala-Phe-NH-C<sub>12</sub>H<sub>25</sub>

This step used Z-Ala-Phe-OH (0.6 g, 1.6 mmoles) previously prepared from step 3 and dodecylamine (0.9 g, 4.8 mmoles). The reaction was carried out under the same reaction conditions described for the synthesis of Bz-Phe-NH-C<sub>12</sub>H<sub>25</sub>. The product remained as white solid and the solid was recrystallised from ethyl acetate to yield a white powder (0.76 g, 88 %), m.p. 148-150°C. TLC:  $R_f = 0.84$  (MeOH:CHCl<sub>3</sub> = 1:4)

Elemental analysis for C<sub>32</sub>H<sub>47</sub>N<sub>3</sub>O<sub>4</sub>, calc'd: C 71.51, H 8.75, N 7.82

found : C 71.62, H 9.04, N 7.77

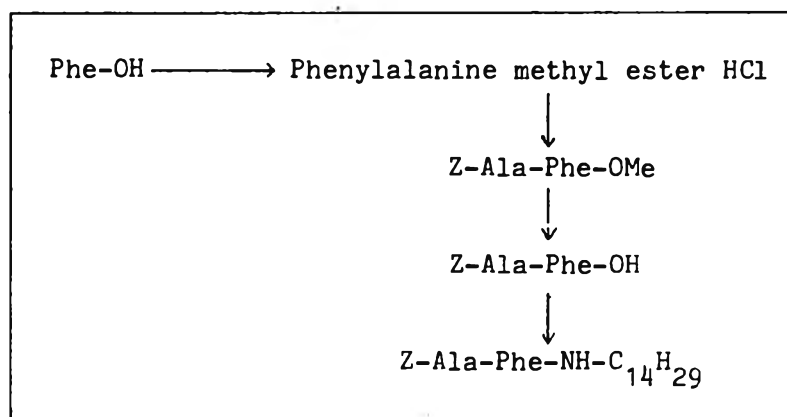
IR (KBr)  $\nu$  ( $\text{cm}^{-1}$ ) (Fig. III.4): 3290(-NH stretching); 3080,3025(-CH aromatic); 2920,2850 (-CH stretching); 1690,1660,1640 (O=C-NH); 1535 (-NH bending)

$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$ (ppm) ( Fig. III.14 ): 0.88 (t,3H, $\text{CH}_3$ ); 1.25 (s,20H,  $\text{CH}_2$  's chain) ; 1.35 (d,3H,-NH-CH- $\text{CH}_3$ ) ; 3.09 (m,4H, - $\text{CH}_2$ -Ar , -NH- $\text{CH}_2$ -) ; 4.17 (m, 1H, -NH- $\text{CH}$ - $\text{CH}_3$ ) ; 4.59 (q,1H,-NH- $\text{CH}$ - $\text{CH}_2$ ); 5.07 (s,2H, Ar- $\text{CH}_2$ -O-); 5.17 (br., 1H, -NH-CH) ; 5.84 (br., 1H,-NH- $\text{CH}_2$ ) ; 6.69 (br., 1H, -NH-CH); 7.24 (s, 5H, Ar- $\text{CH}_2$ ); 7.35 (s, 5H, Ar- $\text{CH}_2$ -O)

$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$ (ppm) ( Fig. III.24 ): 14.08 ( $\text{CH}_3$ ); 18.41 ( $\text{CH}-\text{CH}_3$ ); 22.64, 26.81,29.25,29.52, 29.63, 31.90 ( $\text{CH}_2$  's chain); 38.40 (-NH- $\text{CH}_2$ -) ; 39.65 (- $\text{CH}_2$ -Ar) ; 50.97 (-NH- $\text{CH}$ -); 54.60 (-NH- $\text{CH}$ - $\text{CH}_2$ ); 67.17 (Ar- $\text{CH}_2$ -O); 126.98, 128.06, 128,28, 128.61, 129.26, 136.08, 136.73 ( Ar- $\text{CH}_2$ -O- , - $\text{CH}_2$ -Ar ); 155.96 (-O-C=O); 170.21 (-CH-C=O); 172.05 (O=C-NH)

### 3.2.6 Preparation of Z-Ala-Phe-NH-C<sub>14</sub>H<sub>29</sub> (Compound V)

There are four steps of reaction involved in this preparation which is schematically described below :



Step 1,2 and 3 had already been made in the synthesis of Z-Ala-Phe-NH-C<sub>10</sub>H<sub>21</sub> (Compound III)

Step 4: Preparation of Z-Ala-Phe-NH-C<sub>14</sub>H<sub>29</sub>

This step used Z-Ala-Phe-OH (0.6 g, 1.6 mmoles) previously prepared from step 3 and tetradecylamine (1.0 g, 4.8 mmoles). The reaction was carried out under the reaction conditions described for the synthesis of Bz-Phe-NH-C<sub>12</sub>H<sub>25</sub>. The product remained as white solid and the solid was recrystallised from ethyl acetate to form a white powder (0.63 g, 69%), m.p. 145-147°C. TLC: R<sub>f</sub> = 0.87 (MeOH:CHCl<sub>3</sub> = 1:4)

Elemental analysis for C<sub>34</sub>H<sub>51</sub>N<sub>3</sub>O<sub>4</sub>, calc'd: C 72.21, H 9.03, N 7.43

found : C 72.29, H 9.27, N 7.23

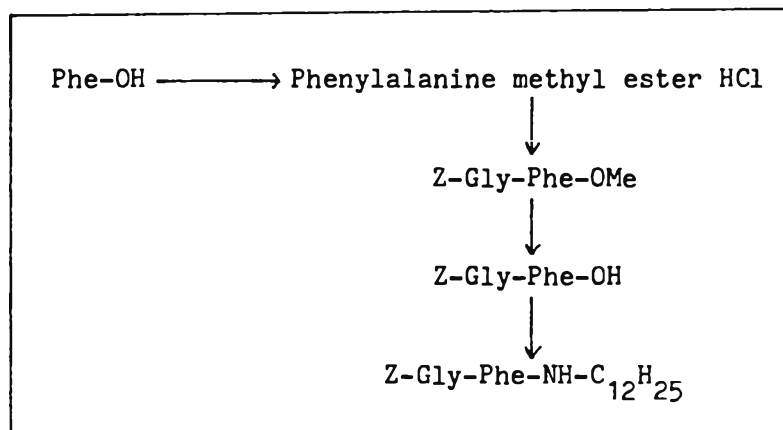
IR (KBr)  $\nu$  (cm<sup>-1</sup>) ( Fig. III.5 ): 3280(-NH stretching); 3070,3020(-CH aromatic); 2910,2840 (-CH stretching); 1690,1660,1640 (O=C-NH); 1545 (-NH bending)

<sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$ (ppm) ( Fig. III.15 ): 0.89 (t, 3H, CH<sub>3</sub>); 1.25 (s, 24H, CH<sub>2</sub>'s chain) ; 1.35 (d, 3H, -NH-CH-CH<sub>3</sub>) ; 3.11 (m, 4H, CH<sub>2</sub>-Ar , -NH-CH<sub>2</sub>); 4.16 (m, 1H, -NH-CH-CH<sub>3</sub>); 4.59 (q, 1H, -NH-CH-CH<sub>2</sub>); 5.07 (s, 2H, Ar-CH<sub>2</sub>-O-) ; 5.24 (br., 1H, O=C-NH); 5.80 (br., 1H, O=C-NH-CH<sub>2</sub>-) ; 6.69 (br., 1H, -NH-CH); 7.24 (s, 5H, Ar-CH<sub>2</sub>-) ; 7.35 (s, 5H, Ar-CH<sub>2</sub>-O-)

<sup>13</sup>C NMR (CDCl<sub>3</sub>)  $\delta$ (ppm) ( Fig. III.25 ): 14.08 (CH<sub>3</sub>); 18.41 (CH-CH<sub>3</sub>); 22.64, 26.81, 29.25, 29.52, 29.63, 31.90 (CH<sub>2</sub>'s chain); 38.40 (-CH<sub>2</sub>-(CH<sub>2</sub>)<sub>12</sub>-); 39.65 (Ar-CH<sub>2</sub>); 51.03 (-NH-CH-); 54.60 (NH-CH-CH<sub>2</sub>-); 67.17 (Ar-CH<sub>2</sub>-O-); 126.98, 128.06, 128.28, 128.61, 129.26, 136.73 (-CH<sub>2</sub>-Ar , Ar-CH<sub>2</sub>-O-); 155.75 (-O-C=O) ; 170.21 (-CH-C=O); 172.00 (O=C-NH-)

### 3.2.7 Preparation of Z-Gly-Phe-NH-C<sub>12</sub>H<sub>25</sub> (Compound VI)

There are four steps of reaction involved in this preparation which is schematically described below :



#### Step 1: Preparation of phenylalanine methyl ester

HCl

This product had already been made in step 1 of the synthesis of Z-Ala-Phe-NH-C<sub>10</sub>H<sub>21</sub> (Compound III)

#### Step 2: Preparation of Z-Gly-Phe-OMe

This step used Z-glycine (1.04 g, 5 mmoles) and phenylalanine methyl ester HCl (1.08 g, 5 mmoles). The reaction was carried out under the same reaction conditions described for the synthesis of Z-Ala-Phe-OMe, yielded an oil.

#### Step 3: Preparation of Z-Gly-Phe-OH

The oil previously prepared from step 2 was dissolved in methanol (10 mL). With stirring at room temperature, 1 N NaOH (10 mL) was added dropwise. The solution was stirred for 5 hours at room temperature. The methanol was removed and the residue was then diluted with water (20 mL). The solution was acidified to Congo red with 1 M HCl with cooling and extracted with ethyl acetate (100 mL).

The ethyl acetate layer was separated and dried with  $\text{Na}_2\text{SO}_4$  and then the solvent was removed under reduced pressure. After the residue had been dried in the air for several days, a white crystalline solid was obtained and washed with diethyl ether (1.17 g, 66%), m.p. 125-127°C (lit [74], m.p. 125-126°C).

Step 4: Preparation of Z-Gly-Phe-NH-C<sub>12</sub>H<sub>25</sub>

This step used Z-Gly-Phe-OH (1.0 g, 2.8 mmoles) prepared from step 3 and dodecylamine (1.55 g, 8.4 mmoles). The reaction was carried out under the reaction conditions described for the synthesis of Bz-Phe-NH-C<sub>12</sub>H<sub>25</sub>. The product remained as a waxy white solid and the solid was recrystallised from ethyl acetate. After several hours, the waxy white solid had changed into a white powder (1.2 g, 82%), m.p. 120-121°C. TLC:  $R_f = 0.79$  (MeOH:CHCl<sub>3</sub> = 1:4)

Elemental analysis for C<sub>31</sub>H<sub>45</sub>N<sub>3</sub>O<sub>4</sub>, calc'd: C 71.13, H 8.60, N 8.03

found: C 70.95, H 8.75, N 7.92

IR (KBr)  $\nu$  (cm<sup>-1</sup>) ( Fig. III.6 ): 3280 (-NH stretching); 3075, 3020 (-CH aromatic); 2920, 2845 (-CH stretching); 1690, 1670, 1650 (O=C-NH); 1570, 1550 (-NH bending)

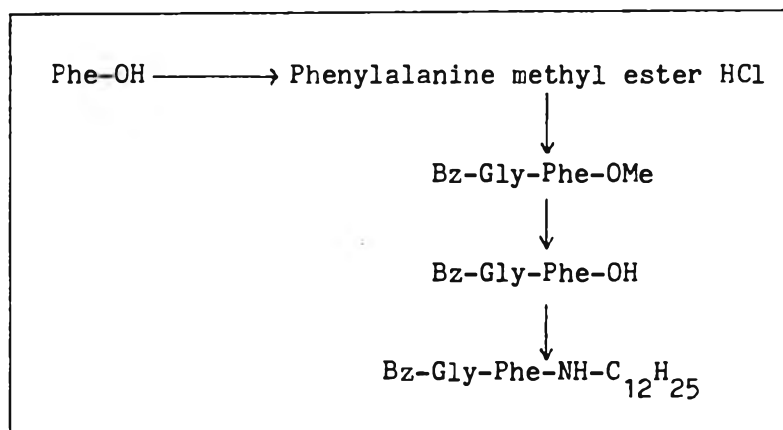
<sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  (ppm) ( Fig. III.16 ): 0.88 (t, 3H, CH<sub>3</sub>); 1.24 (s, 20H, CH<sub>2</sub>'s chain); 3.06 (m, 4H, -CH<sub>2</sub>-Ar, -NH-CH<sub>2</sub>-); 3.84 (d, 2H, NH-CH<sub>2</sub>); 4.60 (q, 1H, NH-CH-CH<sub>2</sub>); 5.09 (br., 1H, O=C-NH-CH<sub>2</sub>-C=O); 5.97 (br., 1H, O=C-NH-CH<sub>2</sub>); 6.69 (br., 1H, O=C-NH-CH); 7.23 (s, 5H, Ar-CH<sub>2</sub>-); 7.33 (s, 5H, Ar-CH<sub>2</sub>-O-C=O)

<sup>13</sup>C NMR (CDCl<sub>3</sub>)  $\delta$  (ppm) ( Fig. III.26 ): 14.08 (CH<sub>3</sub>); 22.69, 26.81, 29.25, 29.52, 29.63, 31.90 (CH<sub>2</sub>'s chain); 38.68

( $-\underline{\text{C}}\text{H}_2-(\text{CH}_2)_{10}-$ ); 39.65 ( $-\underline{\text{C}}\text{H}_2-\text{Ar}$ ); 44.58 ( $-\text{NH}-\underline{\text{C}}\text{H}_2-$ );  
 54.82 ( $-\text{NH}-\underline{\text{C}}\text{H}-\text{CH}_2-$ ); 67.23 ( $\text{O}-\underline{\text{C}}\text{H}_2-$ ); 126.98, 128.06,  
 128.23, 128.55, 129.26, 136.62 ( $\underline{\text{A}}\text{r}-\text{CH}_2-$ ,  $-\text{O}-\text{CH}_2-\underline{\text{A}}\text{r}$ );  
 156.56 ( $-\text{O}-\underline{\text{C}}=\text{O}$ ); 168.86 ( $\text{CH}_2-\underline{\text{C}}=\text{O}$ ); 170.32 ( $-\text{CH}-\underline{\text{C}}=\text{O}$ )

### 3.2.8 Preparation of Bz-Gly-Phe-NH-C<sub>12</sub>H<sub>25</sub> (Compound VII)

There are four steps of reaction involved in this preparation which is schemetically described below :



Step 1: Preparation of phenylalanine methyl ester HCl

This product had already been prepared in step 1 of the synthesis of Z-Ala-Phe-NH-C<sub>10</sub>H<sub>21</sub> (Compound III)

Step 2: Preparation of Bz-Gly-Phe-OMe

This step used Bz-Gly-OH (1.79 g, 10 mmoles) and phenylalanine methyl ester HCl (2.16 g, 10 mmoles). The reaction was carried out under the same reaction conditions described for the synthesis of Z-Ala-Phe-OMe, yielded white solid (2.14 g, 63%), m.p. 120-121°C.

Step 3: Preparation of Bz-Gly-Phe-OH

This step used Bz-Gly-Phe-OMe (2.14 g, 6.3 mmoles), methanol (15 mL) and 1 N NaOH (25 mL). The reaction was carried out under the reaction conditions described for the synthesis of Z-Ala-Phe-OH, yielded a white crystalline solid (1.72 g, 84%), m.p. 146-148 °C (lit [74], m.p. 148-150 °C).

Step 4: Preparation of Bz-Gly-Phe-NH-C<sub>12</sub>H<sub>25</sub>

This step used Bz-Gly-Phe-OH (0.8 g, 2.5 mmoles) and dodecylamine (1.38 g, 7.5 mmoles). The reaction was carried out under the same reaction conditions described for the synthesis of Bz-Phe-NH-C<sub>12</sub>H<sub>25</sub>. The product was recrystallised from ethyl acetate to form a white powder (0.88 g, 71 %), m.p. 188-190 °C. TLC:R<sub>f</sub> = 0.82 (MeOH:CHCl<sub>3</sub> = 1:4).

Elemental analysis for C<sub>30</sub>H<sub>43</sub>N<sub>3</sub>O<sub>3</sub>, calc'd: C 73.02, H 8.72, N 8.52

found : C 72.98, H 8.86, N 8.44

IR (KBr)  $\nu$  (cm<sup>-1</sup>) (Fig. III.7): 3360, 3280 (-NH stretching); 3075, 3020 (-CH aromatic); 2920, 2845 (-CH stretching); 1670, 1645 (O=C-NH); 1550 (-NH bending)

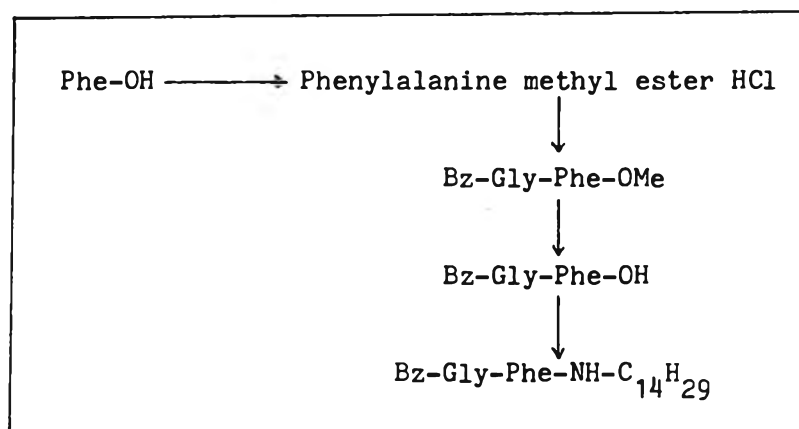
<sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  (ppm) (Fig. III.17): 0.88 (t, 3H, CH<sub>3</sub>); 1.25 (s, 20H, CH<sub>2</sub>'s chain); 3.09 (m, 4H, -CH<sub>2</sub>-Ar, -NH-CH<sub>2</sub>-); 4.09 (d, 2H, -NH-CH<sub>2</sub>-C=O); 4.56 (q, 1H, -NH-CH); 5.82 (br., 1H, -NH-CH<sub>2</sub>); 6.75 (br., 1H, O=C-NH-CH); 6.99 (br., 1H, O=C-NH); 7.20 (s, 5H, Ar-CH<sub>2</sub>); 7.62 (m, 5H, Ar-C=O)

<sup>13</sup>C NMR (CDCl<sub>3</sub>+DMSO-d<sub>6</sub>)  $\delta$  (ppm) (Fig. III.27): 13.97 (CH<sub>3</sub>); 22.37, 26.65, 29.03, 29.30, 31.58 (CH<sub>2</sub>'s chain); 38.62 (-HN-CH<sub>2</sub>-); 39.60 (-CH<sub>2</sub>-Ar); 43.55 (-HN-CH<sub>2</sub>-C=O); 54.28 (-NH-CH-); 126.33, 127.41, 128.12, 128.23, 129.20,

131.48, 133.64, 137.27 (  $\text{Ar}-\text{C}=\text{O}$ ,  $-\text{CH}_2-\text{Ar}$  ) ; 167.67  
 ( $\text{CH}_2-\text{C}=\text{O}$ ) ; 169.34 ( $\text{CH}-\text{C}=\text{O}$ ) ; 170.75 ( $\text{Ar}-\text{C}=\text{O}$ )

### 3.2.9 Preparation of Bz-Gly-Phe-NH-C<sub>14</sub>H<sub>29</sub> (Compound VIII)

There are four steps of reaction involved in this preparation which is schematically described below :



Step 1,2 and 3 had already been prepared in the synthesis of Bz-Gly-Phe-NH-C<sub>12</sub>H<sub>25</sub> (Compound VII)

#### Step 4: Preparation of Bz-Gly-Phe-NH-C<sub>14</sub>H<sub>29</sub>

This step used Bz-Gly-Phe-OH (0.8 g, 2.5 mmoles) and tetradecylamine (1.6 g, 7.5 mmoles). The reaction was carried out under the same reaction conditions described for the synthesis of Bz-Phe-NH-C<sub>12</sub>H<sub>25</sub>. The product was recrystallised from ethyl acetate to form a white powder (0.83 g, 64%), m.p. 193-195°C. TLC:R<sub>f</sub> = 0.87 (MeOH:CHCl<sub>3</sub> = 1:4)

Elemental analysis for C<sub>32</sub>H<sub>47</sub>N<sub>3</sub>O<sub>3</sub>, calc'd: C 73.42, H 8.99, N 8.03  
 found : C 73.69, H 9.12, N 8.05

IR (KBr)  $\nu$  (cm<sup>-1</sup>) (Fig.III.8): 3360, 3280 (-NH stretching); 3070, 3020  
 (-CH aromatic); 2920, 2845 (-CH stretching); 1670, 1640



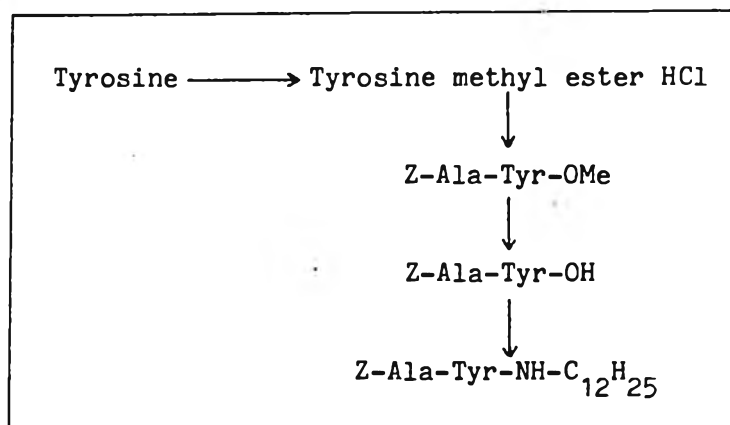
(O=C-NH); 1550 (-NH bending)

$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$ (ppm) (Fig. III.18): 0.88 (t, 3H,  $\text{CH}_3$ ); 1.25 (s, 24H,  $\text{CH}_2$ 's chain); 3.09 (m, 4H,  $-\text{CH}_2-\text{Ar}$ ,  $\text{NH}-\text{CH}_2-$ ); 4.08 (d, 2H,  $\text{NH}-\text{CH}_2-\text{C}=\text{O}$ ); 4.56 (q, 1H,  $\text{HN}-\text{CH}$ ); 5.82 (br., 1H,  $\text{NH}-\text{CH}_2$ ); 6.75 (br., 1H,  $\text{O}=\text{C}-\text{NH}-\text{CH}$ ); 6.99 (br., 1H,  $\text{O}=\text{C}-\text{NH}-$ ); 7.20 (s, 5H,  $\text{Ar}-\text{CH}_2-$ ); 7.62 (m, 5H,  $\text{Ar}-\text{C}=\text{O}$ )

$^{13}\text{C}$  NMR ( $\text{CDCl}_3+\text{DMSO}-d_6$ )  $\delta$ (ppm) (Fig. III.28): 13.92 ( $\text{CH}_3$ ); 22.21, 26.49, 28.92, 29.14, 31.36 ( $\text{CH}_2$ 's chain); 38.46 ( $-\text{HN}-\text{CH}_2-$ ); 39.38 ( $-\text{CH}_2-\text{Ar}$ ); 43.23 ( $-\text{HN}-\text{CH}_2-\text{C}=\text{O}$ ); 54.12 ( $\text{HN}-\text{CH}-\text{CH}_2-$ ); 126.22, 127.31, 128.01, 128.17, 129.09, 131.42 ( $\text{Ar}-\text{C}=\text{O}$ ,  $\text{CH}_2-\text{Ar}$ ); 167.45 ( $-\text{CH}_2-\text{C}=\text{O}$ ); 169.13 ( $-\text{CH}-\text{C}=\text{O}$ ); 170.65 ( $\text{Ar}-\text{C}=\text{O}$ )

### 3.2.10 Preparation of Z-Ala-Tyr-NH-C<sub>12</sub>H<sub>25</sub> (Compound IX)

There are four steps of reaction involved in this preparation which is schematically described below :



#### Step 1: Preparation of Tyrosine methyl ester HCl

This step used tyrosine (1.81 g, 10 mmoles), absolute methanol (10 mL) and thionyl chloride (0.9 g, 12 mmoles). The reaction was carried out under the reaction conditions described

for the synthesis of phenylalanine methyl ester HCl, yielded a white crystalline solid (2.08 g, 90 %), m.p. 190-192°C (Lit [74], m.p. 190°C).

Step 2: Preparation of Z-Ala-Tyr-OMe

Z-alanine (1.11 g, 5 mmoles) and tyrosine methyl ester HCl (1.16 g, 5 mmoles) were used. The reaction was carried out under the same manner described for the synthesis of Z-Ala-Phe-OMe. A white crystalline solid was obtained (1.42 g, 71%), m.p. 119-121°C.

Step 3: Preparation of Z-Ala-Tyr-OH

This step used Z-Ala-Tyr-OMe (1.42 g, 3.5 mmoles), methanol (10 mL) and 1 N NaOH (5 mL). The reaction was carried out under the same reaction conditions described for the synthesis of Z-Ala-Tyr-OH, yielded white crystalline solid (0.97 g, 72%), m.p. 153-154°C.


Step 4: Preparation of Z-Ala-Tyr-NH-C<sub>12</sub>H<sub>25</sub>


This step used Z-Ala-Tyr-OH (0.9 g, 2.3 mmoles) and dodecylamine (1.3 g, 6.9 mmoles). The reaction was carried out under the same reaction conditions described for the synthesis of Bz-Phe-NH-C<sub>12</sub>H<sub>25</sub>. The product was recrystallised from ethyl acetate to form a white powder (0.89 g, 70 %), m.p. 160-162°C. TLC:R<sub>f</sub> = 0.85 (MeOH:CHCl<sub>3</sub> = 1:4)

Elemental analysis for C<sub>32</sub>H<sub>47</sub>N<sub>3</sub>O<sub>5</sub>, calc'd: C 69.44, H 8.50, N 7.59

found : C 69.48, H 8.76, N 7.58

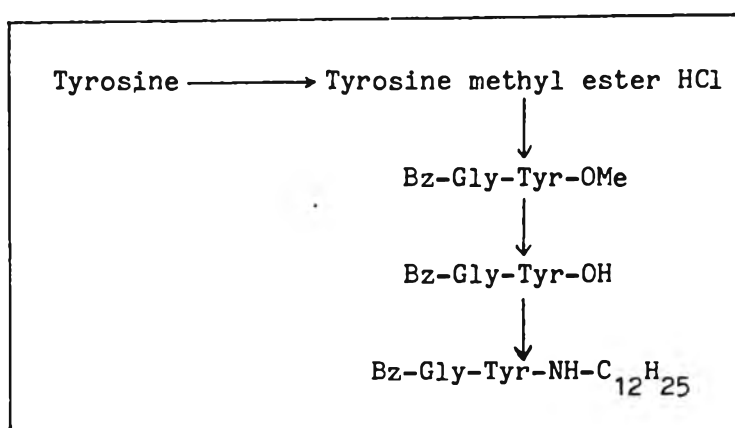
IR (KBr)  $\nu$  (cm<sup>-1</sup>) (Fig. III.9): 3320, 3290 (-NH bending); 3050 (-CH aromatic); 2930, 2850 (-CH stretching); 1690, 1660, 1630 (O=C-NH); 1540 (-NH bending)

$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  (ppm) (Fig. III.19): 0.87 (t, 3H,  $\text{CH}_3$ ); 1.24 (s, 20H,  $\text{CH}_2$  's chain); 1.34 (d, 3H,  $\text{CH}_3$ -CH); 3.05 (m, 4H,  $-\text{CH}_2$ -Ar, NH- $\text{CH}_2$ ); 4.15 (m, 1H, NH- $\text{CH}$ -); 4.54 (q, 1H, NH- $\text{CH}$ - $\text{CH}_2$ ); 5.07 (s, 2H,  $\text{CH}_2$ -O-); 5.27 (br., 1H, NH-CH); 5.93 (br., 1H, NH- $\text{CH}_2$ ); 6.87 (db. of db., 4H, ); 7.33 (s, 6H, Ar- $\text{CH}_2$ , -OH)

$^{13}\text{C}$  NMR ( $\text{CDCl}_3$  + DMSO- $d_6$ )  $\delta$  (ppm) (Fig. III.29): 14.03 ( $\text{CH}_3$ ); 17.98 ( $\text{CH}-\text{CH}_3$ ); 22.42, 26.70, 29.09, 29.36, 31.63 ( $\text{CH}_2$  's chain); 38.73 ( $-\text{CH}_2$ -NH-); 39.65 ( $-\text{CH}_2$ -Ar); 51.08 ( $-\text{CH}-\text{CH}_3$ ); 54.39 ( $-\text{NH}-\text{CH}$ -); 66.30 ( $\text{CH}_2$ -O-); 115.22, 127.52, 127.79, 127.90, 128.39, 130.18, 136.57, 155.91 ( $-\text{CH}_2$ -Ar, ); 156.18 ( $-\text{O}-\text{C}=\text{O}$ ); 170.92 ( $-\text{CH}-\text{C}=\text{O}$ ); 172.59 ( $\text{O}=\text{C}-\text{NH}-\text{CH}_2$ -)

### 3.2.11 Preparation of Bz-Gly-Tyr-NH-C<sub>12</sub>H<sub>25</sub> (Compound X)

There are four steps of reaction involved in this preparation which is schematically described below :



#### Step 1: Preparation of Tyrosine methyl ester HCl

This product had already been prepared in step 1 of the synthesis of Z-Ala-Tyr-NH-C<sub>12</sub>H<sub>25</sub> (Compound IX).

Step 2: Preparation of Bz-Gly-Tyr-OMe

Bz-Gly-OH (1.79 g, 10 mmol) and tyrosine methyl ester HCl (2.32 g, 10 mmol) were used. The reaction was carried out under the same reaction conditions described for the synthesis of Z-Ala-Phe-OMe. After evaporating ethyl acetate, the residue was added with petroleum ether and small portions of ethyl acetate. The mixture was stored below 0 °C for crystallisation. A white crystalline solid was obtained (1.32 g, 37%), m.p. 139-140 °C.

Step 3: Preparation of Bz-Gly-Tyr-OH


Bz-Gly-Tyr-OMe (1.32 g, 3.7 mmol) previously prepared from step 2 was dissolved in dioxane (10 mL). With stirring at room temperature, 1 N NaOH (6 mL) was added dropwise. The solution was stirred continuously for 5 hours at room temperature. The solution was then diluted with water (10 mL) and acidified with 1 M HCl with cooling. The solution was extracted with ethyl acetate (100 mL) and the organic layer was dried with Na<sub>2</sub>SO<sub>4</sub>. The solvent was then removed under reduced pressure. After the residue had been dried in the air for several days, a white crystalline solid was obtained and washed with diethyl ether (1.12 g, 89%), m.p. 203-204 °C.


Step 4: Preparation of Bz-Gly-Tyr-NH-C<sub>12</sub>H<sub>25</sub>

Bz-Gly-Tyr-OH (1.0 g, 2.9 mmol) and dodecylamine (1.6 g, 8.7 mmol) were used. The reaction was carried out under the same reaction conditions described for the synthesis of Bz-Phe-NH-C<sub>12</sub>H<sub>25</sub>. The product was recrystallised from ethyl acetate to form a white powder (1.18 g, 80%), m.p. 150-152 °C. TLC: R<sub>f</sub> = 0.83 (MeOH:CHCl<sub>3</sub> = 1:4).

Elemental analysis for  $C_{30}H_{43}N_3O_4$ , calc'd: C 70.73, H 8.45, N 8.25  
found : C 70.38, H 8.58, N 8.16

IR (KBr)  $\nu$  ( $cm^{-1}$ ) (Fig. III.10): 3340 (-NH stretching); 3050 (-CH aromatic); 2920, 2860 (-CH stretching); 1675, 1650 (O=C-NH); 1525 (-NH bending)

$^1H$  NMR ( $CDCl_3 + DMSO-d_6$ )  $\delta$  (ppm) (Fig. III.20): 0.87 (t, 3H,  $CH_3$ ); 1.24 (s, 20H,  $CH_2$ 's chain); 3.09 (m, 4H,  $-CH_2-Ar$ ,  $-NH-CH_2-$ ); 3.95 (br., 2H,  $-HN-CH_2-C=O$ ); 4.57 (q, 1H,  $HN-CH$ ); 6.81 (db. of db., 4H, ); 7.25 (br., 1H,  $NH-CH_2-(CH_2)_{10}$ ); 7.61 (s, 1H,  $-OH$ ); 7.71 (m, 5H,  $Ar-C=O$ ); 8.36 (br., 1H,  $O=C-NH-CH-$ ); 8.74 (br., 1H,  $O=C-NH-CH_2-$ )

$^{13}C$  NMR ( $CDCl_3 + DMSO-d_6$ )  $\delta$  (ppm) (Fig. III.30): 13.97 ( $CH_3$ ); 22.42, 26.76, 29.14, 29.41, 31.69 ( $CH_2$ 's chain); 38.78 ( $NH-CH_2$ ); 39.76 ( $-CH_2-Ar$ ); 43.66 ( $-NH-CH_2-$ ); 54.66 ( $NH-CH-C=O$ ); 115.33, 127.41, 127.58, 128.28, 130.12, 131.48, 133.70, 155.91 ( $Ar-C=O$ , ); 167.88 ( $-CH_2-C=O$ ); 169.34 ( $-CH-C=O$ ); 171.02 ( $Ar-C=O$ )

### 3.3 Enzyme Assays

#### 3.3.1 Preparation of Solutions

##### 3.3.1.1 Enzyme Stock Solution

Trypsin (1 mg) was dissolved in 1 mL 1 mM HCl. The enzyme solution was kept in an ice bath before the experiments.

Chymotrypsin (0.25 mg) was dissolved in 1 mL 1 mM HCl. Stock enzyme solution was then diluted with 100 mL 1 mM HCl before use. The enzyme solution was also kept in an ice bath

before the experiments.

HLC-G (175 mg) was dissolved in 1 mL NaOAc buffer. The enzyme solution was also kept in an ice bath.

#### 3.3.1.2 Substrate Stock Solution

A 2 mM of BAPNA, the trypsin substrate, and a 2 mM of Suc-Ala-Ala-Pro-Phe-pNA, the chymotrypsin and cathepsin G substrate, were prepared in DMSO.

#### 3.3.1.3 Buffer Solution

HEPES buffer was prepared by dissolving 0.1 M HEPES and 0.5 M NaCl in distilled water. The buffer solution was then adjusted to pH 7.5 by using 10 M NaOH.

NaOAc buffer was prepared by dissolving 50 mM NaOAc and 0.45 M NaCl, pH 5.5 in distilled water.

#### 3.3.1.4 Inhibitor Stock Solution

All inhibitors, (1 mM) were dissolved in DMSO, then aliquots of this solution were made up to 50  $\mu$ L.

### 3.3.2 Determination of The Percentage Inhibition of Synthetic Inhibitors

All solutions were maintained at 37 °C during these assays which were carried out by using a UV-240 Shimadzu spectrophotometer equipped with a 10 mm matched quartz-cell and thermostatic cell compartment. The enzyme was assayed according to the method of Visser and Blout (75). The enzyme was preincubated with the inhibitor or without the inhibitor for 5 minutes at 37 °C before the addition of substrate. An incubation time of approximately 5 minutes was allowed after the addition of

substrate to both the reference and sample cells. The initial rate of the production of p-nitroaniline was followed by measurement of the increase in absorbance at 405 nm . The increase in absorbance was linear in all cases during the measurement.

The synthetic inhibitors, were used at different concentrations to determine the percentage inhibition of each inhibitor.

Percent inhibition was calculated as follows:

$$\% \text{ inhibition} = \frac{a-b}{a} \times 100$$

where:            a = rate of the release of absorbing species in the absence of inhibitor

                  b = rate of the release of absorbing species in the presence of inhibitor

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

Control Run:

	Sample cell ( $\mu\text{L}$ )	Reference cell ( $\mu\text{L}$ )
HEPES	800	800
Enzyme	50	-
NaOAc buffer or 1 mM HCl	-	50
Inhibitor	-	-
DMSO	100	100
Substrate	50	50

Inhibitor Run:

	Sample cell ( $\mu\text{L}$ )	Reference cell ( $\mu\text{L}$ )
HEPES	800	800
Enzyme	50	-
NaOAc buffer or 1 mM HCl	-	50
Inhibitor	50-100	50-100
DMSO	50-100	50-100
Substrate	50	50

Control sample cell had no inhibitor present.

Control reference cell had no inhibitor or enzyme present.

The total volume of each cell is 1000  $\mu\text{L}$ .

1 mM HCl used for testing trypsin and chymotrypsin, but NaOAc buffer used for testing cathepsin G.

The results of determination of the percentage of inhibition are shown in Table 3.1



Table 3.1 Percentage inhibition of synthetic inhibitors.

No	Inhibitor	Enzyme (a)	% Inhibition				
			50 $\mu$ M	10 $\mu$ M	5 $\mu$ M	1 $\mu$ M	0.5 $\mu$ M
I	Bz-Phe-NH- C <sub>12</sub> H <sub>25</sub>	trypsin	7.50	0	-	-	-
		chymotrypsin	72.92	38.46	10.34	0	-
		cathepsin G	I	-	-	-	-
II	Bz-Phe-NH- C <sub>14</sub> H <sub>29</sub>	trypsin	10.61	0	-	-	-
		chymotrypsin	78.72	41.23	20.60	2.08	0
		cathepsin G	I	-	-	-	-
III	Z-Ala-Phe-NH- C <sub>10</sub> H <sub>21</sub>	trypsin	0	-	-	-	-
		chymotrypsin	89.58	56.14	35.98	0	-
		cathepsin G	I	-	-	-	-
IV	Z-Ala-Phe-NH- C <sub>12</sub> H <sub>25</sub>	trypsin	0	-	-	-	-
		chymotrypsin	90.38	73.08	38.46	3.85	0
		cathepsin G	I	-	-	-	-
V	Z-Ala-Phe-NH- C <sub>14</sub> H <sub>29</sub>	trypsin	0	-	-	-	-
		chymotrypsin	94.64	82.14	48.21	0	-
		cathepsin G	I	-	-	-	-
VI	Z-Gly-Phe-NH- C <sub>12</sub> H <sub>25</sub>	trypsin	0	-	-	-	-
		chymotrypsin	87.93	47.53	24.14	1.72	-
		cathepsin G	I	-	-	-	-

Table 3.1 Continued.

No	Inhibitor	Enzyme <sup>(a)</sup>	% Inhibition				
			50 $\mu\text{M}$	10 $\mu\text{M}$	5 $\mu\text{M}$	1 $\mu\text{M}$	0.5 $\mu\text{M}$
VII	Bz-Gly-Phe-NH- $\text{C}_{12}\text{H}_{25}$	trypsin	17.46	0	-	-	-
		chymotrypsin	-	89.39	68.18	22.00	0
		cathepsin G	I	-	-	-	-
VIII	Bz-Gly-Phe-NH- $\text{C}_{14}\text{H}_{29}$	trypsin	13.89	0	-	-	-
		chymotrypsin	-	-	72.73	25.76	0
		cathepsin G	I	-	-	-	-
IX	Z-Ala-Tyr-NH- $\text{C}_{12}\text{H}_{25}$	trypsin	5.55	-	-	-	-
		chymotrypsin	28.04	4.05	0	-	-
		cathepsin G	I	-	-	-	-
X	Bz-Gly-Tyr-NH- $\text{C}_{12}\text{H}_{25}$	trypsin	7.04	-	-	-	-
		chymotrypsin	34.18	8.55	-	-	-
		cathepsin G	I	-	-	-	-

(a) The percentage inhibition of synthetic inhibitors for cathepsin G were determined at the Department of Chemistry, La Trobe University, Australia.

- not measured.

I inactive

### 3.3.3 Determination of The Type of Inhibition

The type of inhibition was determined by a Lineweaver-Burk plot. Initial velocities were determined by varying substrate concentrations (0.02-0.01 mM) at 3 fixed concentration of the inhibitors.

The assay procedure was carried out similarly to that described in section 3.3.2. The data involved in the determination of the types of inhibition are presented in Appendix II. The results of Lineweaver-Burk data of the synthetic inhibitors No. I-X of chymotrypsin are presented in Tables II.1 to Table II.10. The data were plotted as shown in Fig. 3.1 to Fig.3.10 respectively.

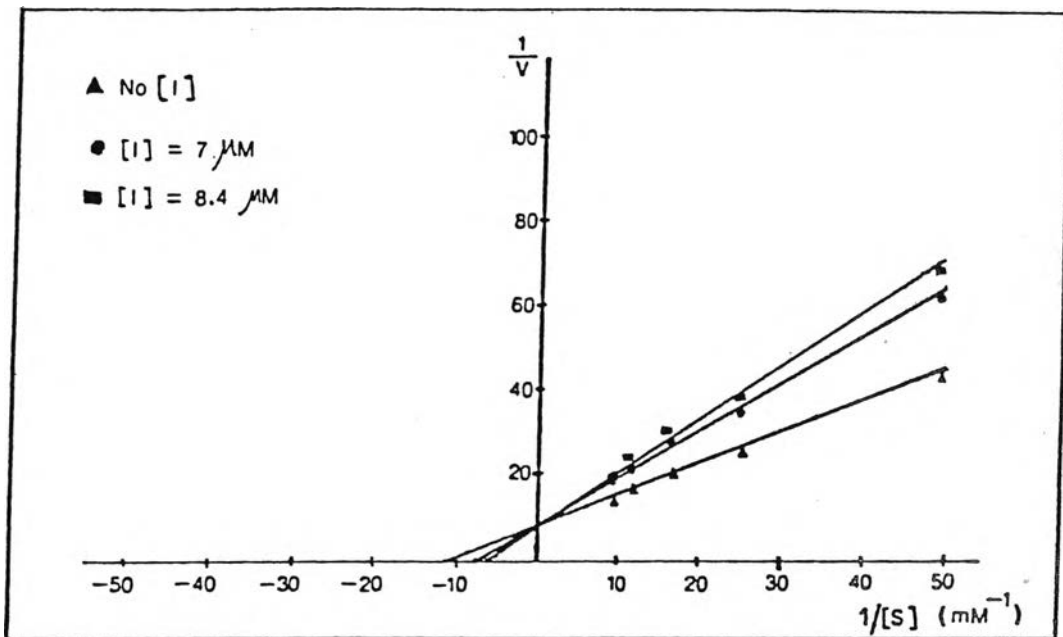


Fig.3.1 Lineweaver-Burk double-reciprocal plots of kinetic data of compound I by chymotrypsin.

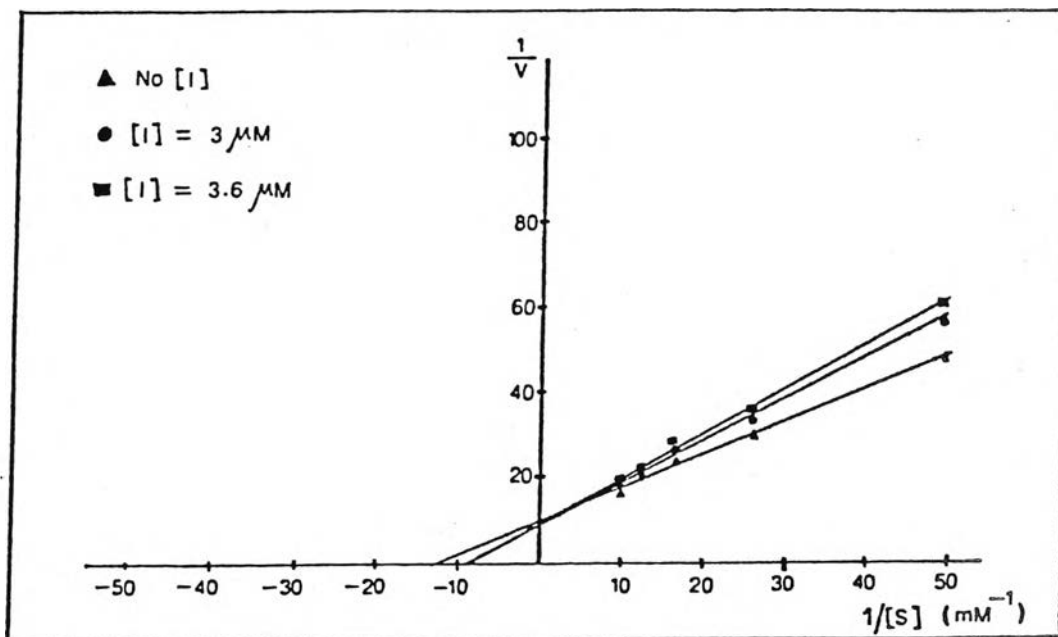


Fig.3.2 Lineweaver-Burk double-reciprocal plots of kinetic data of compound II by chymotrypsin.

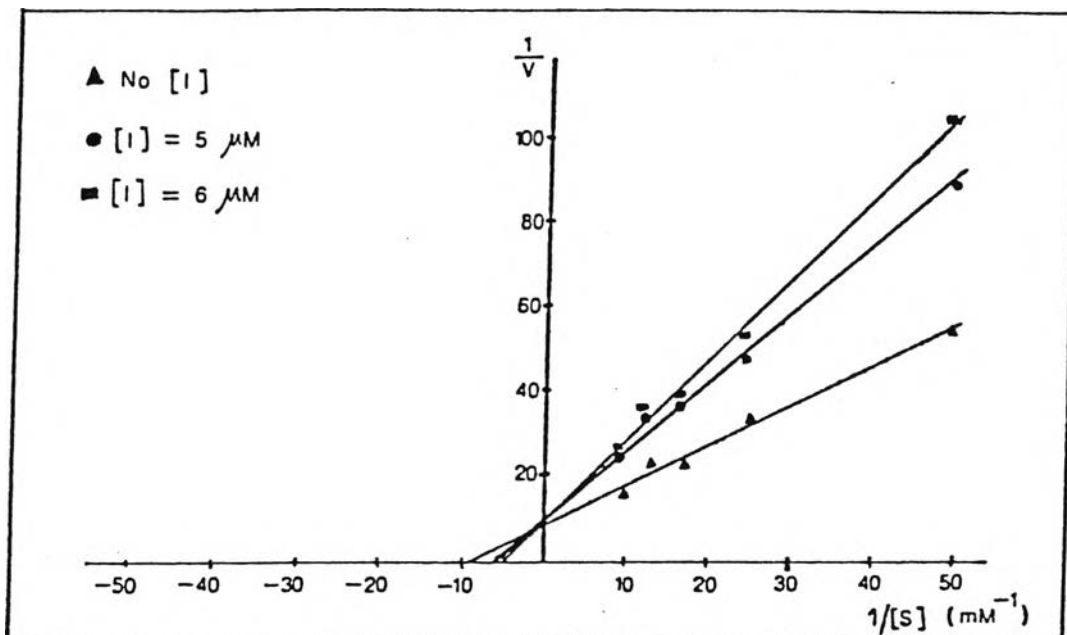


Fig.3.3 Lineweaver-Burk double-reciprocal plots of kinetic data of compound III by chymotrypsin.

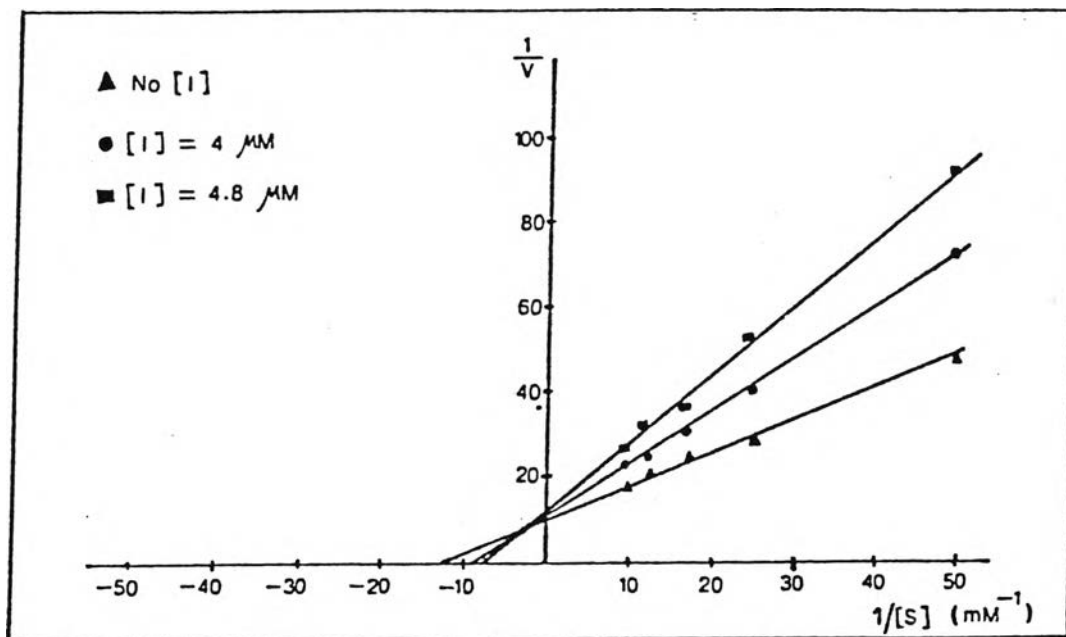


Fig.3.4 Lineweaver-Burk double-reciprocal plots of kinetic data of compound IV by chymotrypsin.

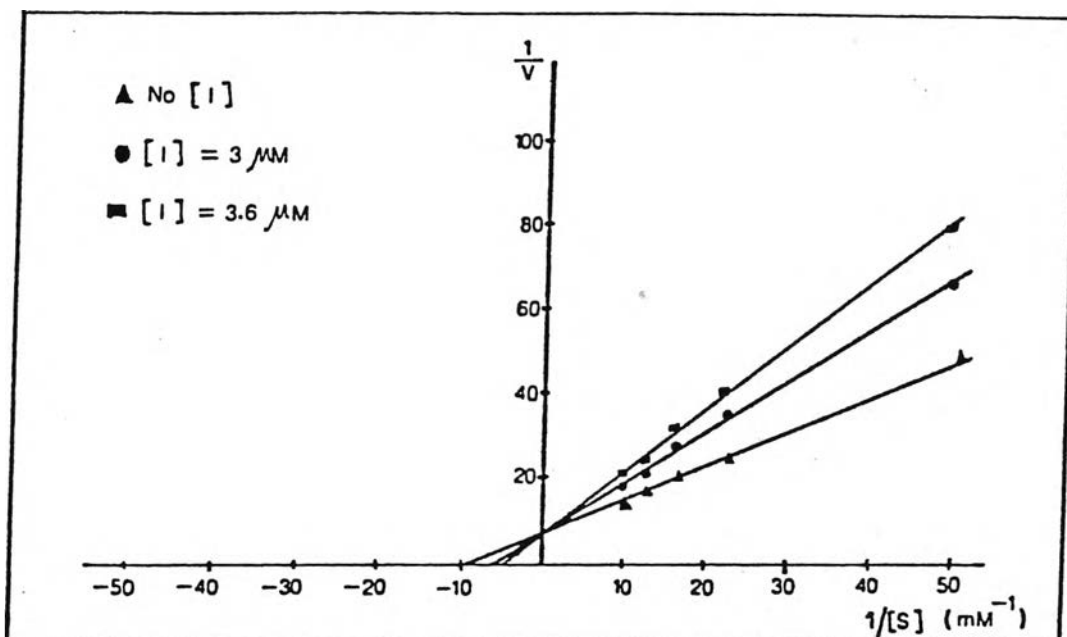


Fig.3.5 Lineweaver-Burk double-reciprocal plots of kinetic data of compound V by chymotrypsin.

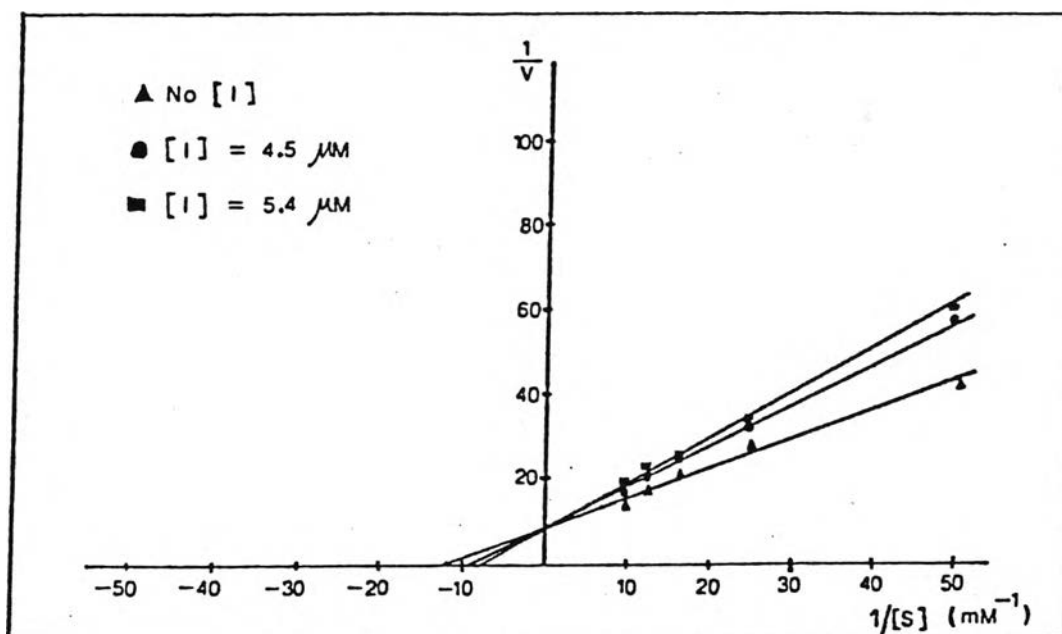


Fig.3.6 Lineweaver-Burk double-reciprocal plots of kinetic data of compound VI by chymotrypsin.

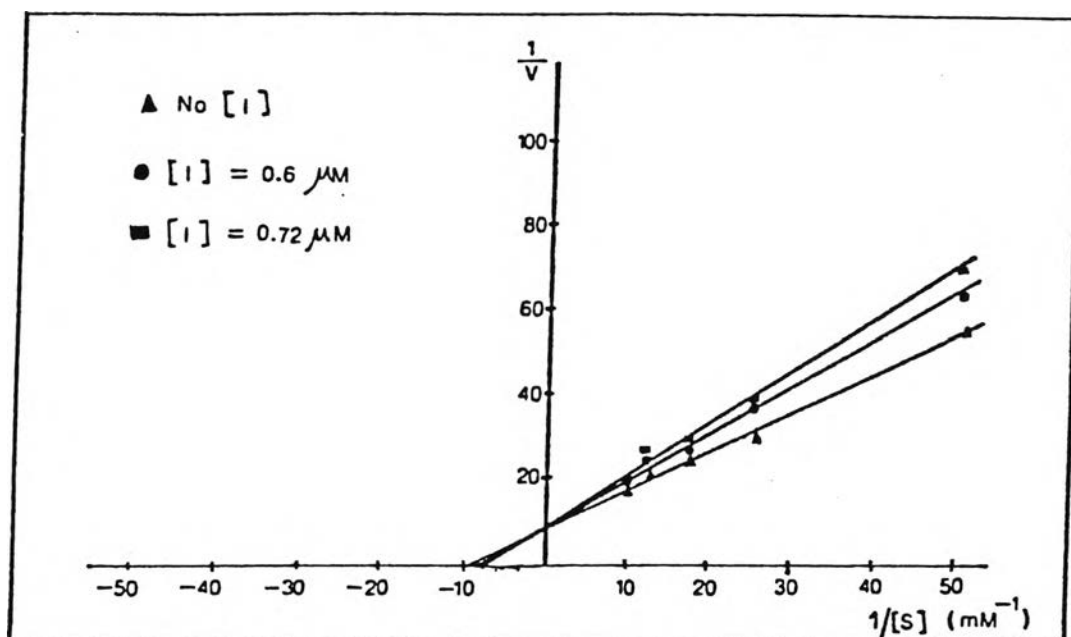


Fig.3.7 Lineweaver-Burk double-reciprocal plots of kinetic data of compound VII by chymotrypsin.

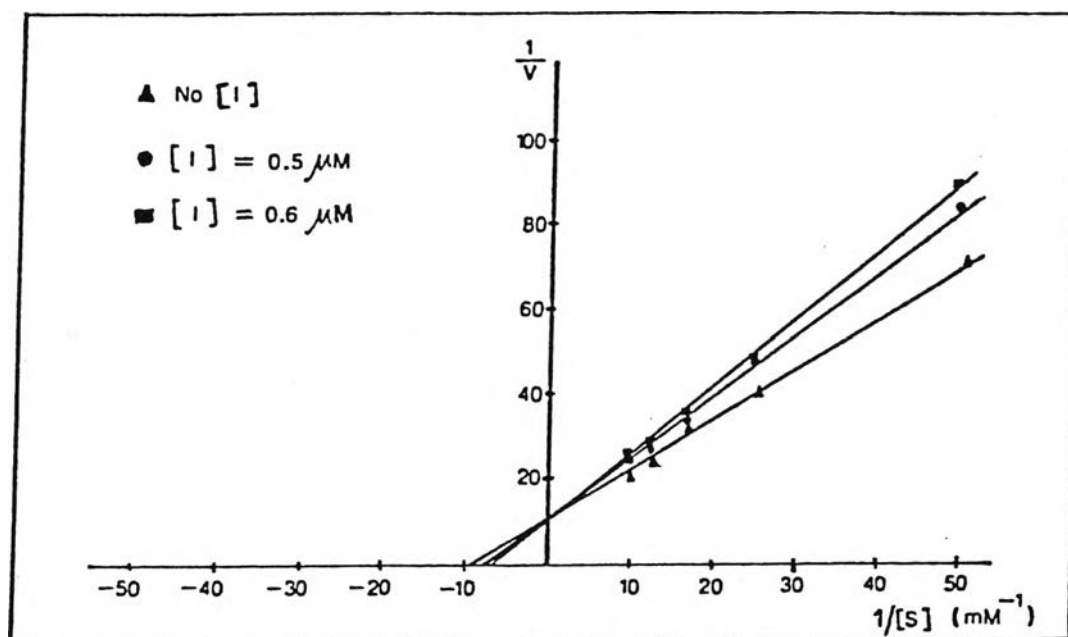


Fig.3.8 Lineweaver-Burk double-reciprocal plots of kinetic data of compound VIII by chymotrypsin.

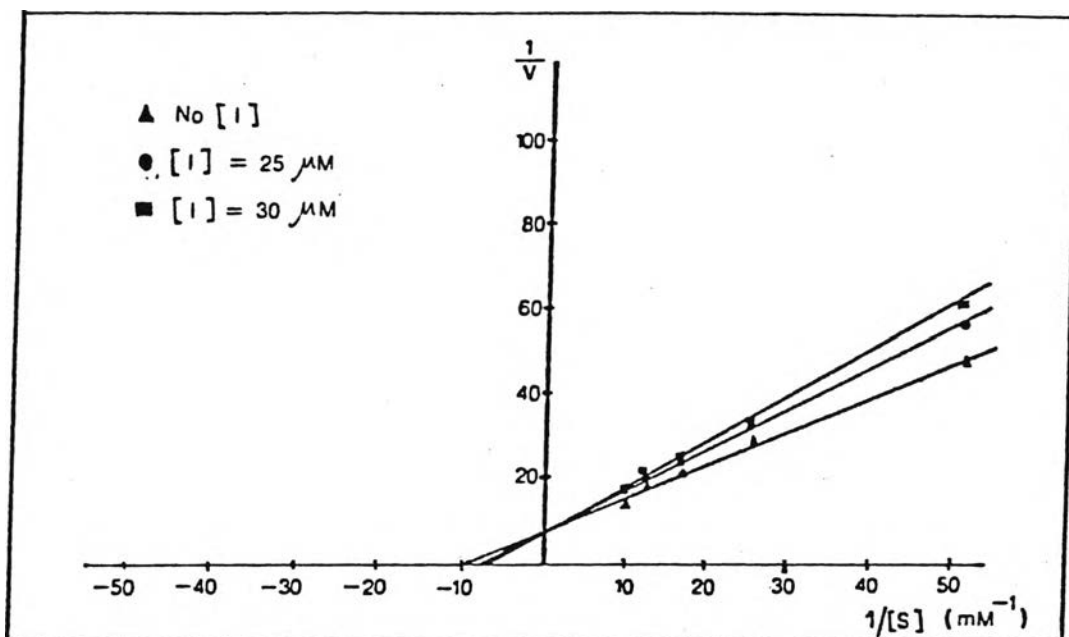


Fig.3.9 Lineweaver-Burk double-reciprocal plots of kinetic data of compound IX by chymotrypsin.

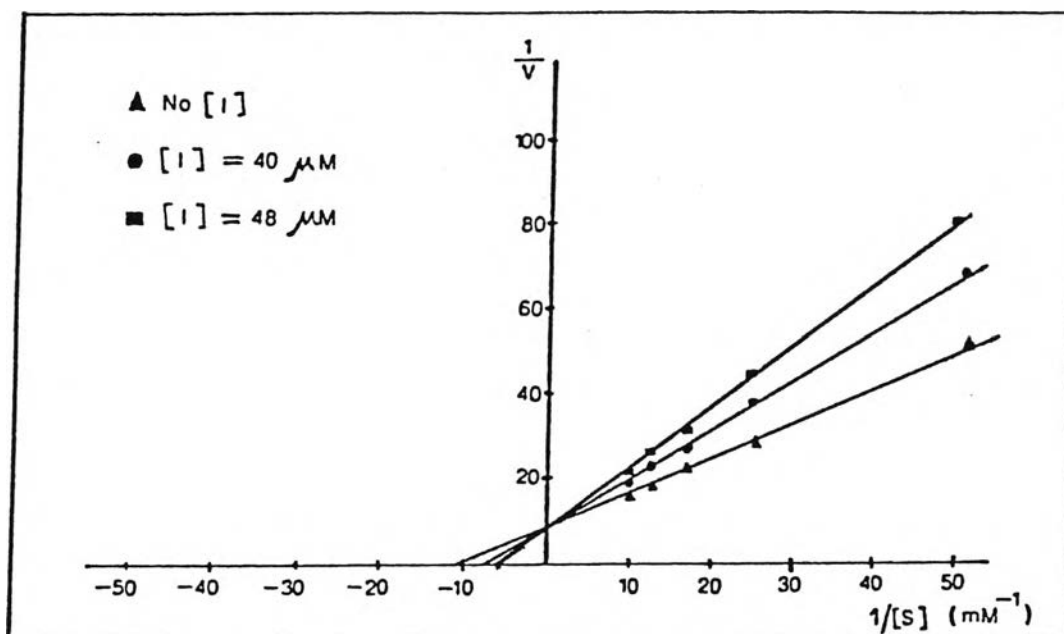


Fig.3.10 Lineweaver-Burk double-reciprocal plots of kinetic data of compound X by chymotrypsin.



### 3.3.4 Determination of The Value of Kinetic Parameters

The value of  $K_m$  was determined by using a plot of  $1/V$  against  $1/[S]$  in a Lineweaver-Burk plot. Whereas the slope of each of these lines ( $K_m/V_{max}$ ) 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 concentration of inhibitors being used, gave the  $K_I$  by extrapolation back to  $[I]$  abscissa. A computer programme was used to calculate the values of  $K_m$ ,  $K_i$  and  $K_I$  by a least square method.

In this experiment, initial velocities were determined by varying the substrate concentrations ( 0.02 - 0.1 mM ) for individual enzyme. Owing to the low inhibition for each inhibitor, the value of  $K_m$  was only determined for trypsin. The results of Lineweaver-Burk data without inhibitor for determining  $K_m$  of trypsin was presented in Table 3.2 and the data was plotted as shown in Fig. 3.11. For chymotrypsin, the value of  $K_m$  was determined by using the average of  $K_m$  calculated from Fig. 3.1 to Fig. 3.10 without inhibitor. In this experiment, the value of  $K_i$  and  $K_I$  were also determined for chymotrypsin by using the results in Tables II.1 to Table II.10 in Appendix II.

Table 3.2 Lineweaver-Burk data without inhibitor for determining  $K_m$  of trypsin.

[S] mM	1/[S]	V	1/V	Regression analysis
0.10	10.00	0.0470	21.28	slope ( $K_m/V_{max}$ ) = 1.7997 $x = -1.8475$ $y = 3.3249$ correlation coefficient = 0.9998
0.08	12.50	0.0376	26.60	
0.06	16.67	0.0306	32.68	
0.04	25.00	0.0208	48.08	
0.02	50.00	0.0107	93.46	

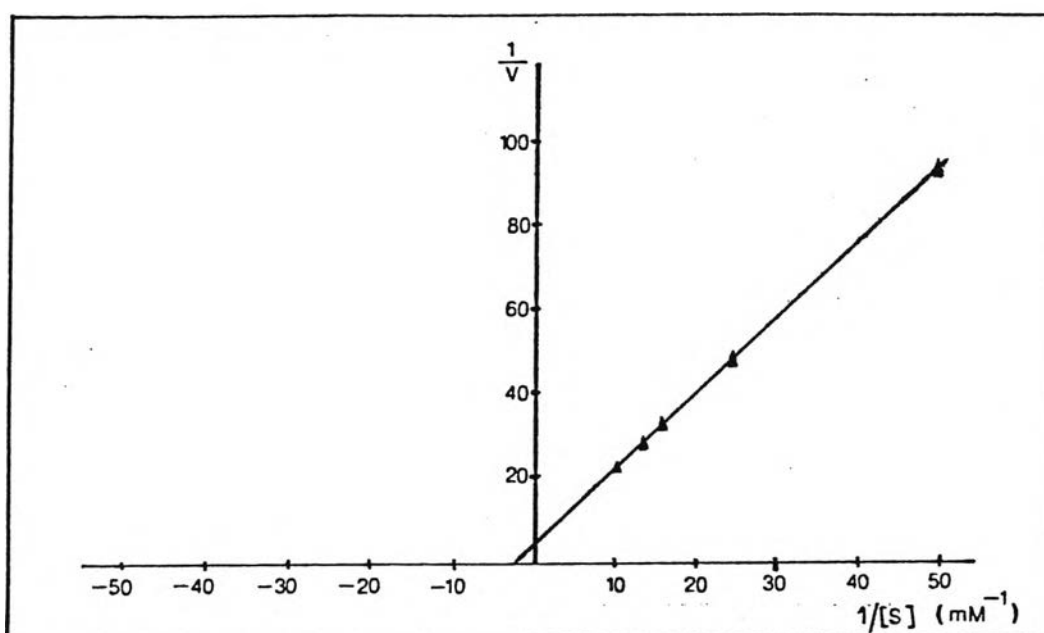


Fig.3.11 Lineweaver-Burk double-reciprocal plot of kinetic data without inhibitor by trypsin.