CHAPTER IV

RESULTS AND DISCUSSION

4.1 Peptide synthesis

4.1.1 Amide formation

The synthetic peptides, amino acid derivatives in this research, were synthesized by using the coupling reaction of the mixed anhydride method of Anderson and Zimmerman (76). An important prerequisite is the control of racemization of the L-form to yield the D-form, since the presence of enantiomers would make the evaluation of these inhibitors difficult, as it is likely that the enzymes would have stereospecific preferences.

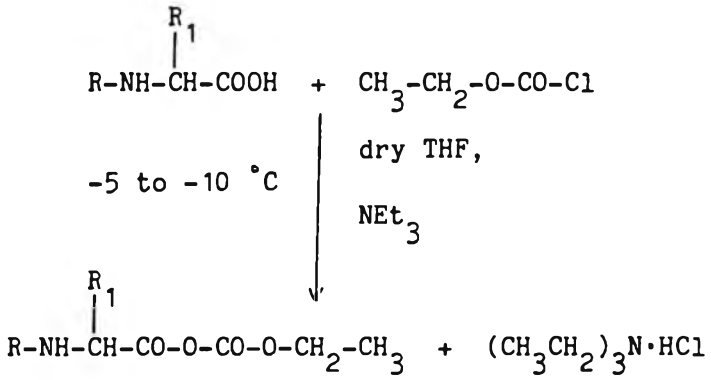
For this reason, the optimization conditions of the reactions were carefully chosen so as to obtain maximum yield and high purity, as well as to reduce in the degree of racemization occuring during the reaction. By using the mixed anhydride method of Anderson et. al., it was found that using the optimum activation time of 10-15 mins before the addition of an N-protected amino acid, and then conducting the reaction between -5 to -10 °C, optimum yields ranging from 60 % to 90 % with a high purity as well as a reduction in the degree of racemization were obtained. Thus these conditions were carefully taken and used in all the experiments for making the synthetic peptides.

Despite the fact that the conditions as mentioned will reduce the degree of racemization, it is still difficult to tell whether all the synthetic compounds are in the L-form only. For this

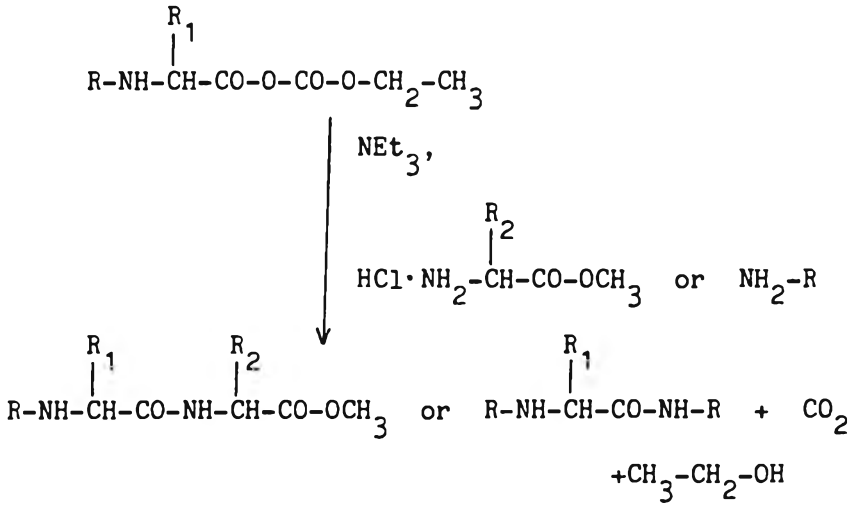
reason, it is necessary to find the selective methods that can identify the structures of these compounds. It was reported that NMR method at high frequency can be used to identify the configuration of some amino acid derivatives (77). However, there is not a single method to be used for finding out the absolute configuration of peptides or proteins. So throughout this research, we only synthesized these peptides under the condition mentioned above and identified the structures by elemental analyzer, IR spectrophotometer, ^{1}H and ^{13}C NMR spectrometer.

The mixed anhydride method involves the addition of ethyl chloroformate to the N-protected amino acid in dry THF at -5 to -10 °C. This gives a result in the "activation" of the carboxyl group. Consequently, the carboxyl group of the mixed anhydride will then be more susceptible to nucleophile attacked by the primary amine of the amino acid or alkyl amine to which it is to be coupled. In addition, triethylamine, the tertiary base was used as the HCl acceptor (Scheme 4.1). Since triethylamine is a strong base, it is necessary to control the quantity of triethylamine used for the experiments. It was found that the unreacted triethylamine was the racemizer (76). It is interesting to note that during the preparation of Bz-Phe-NH-$C_{14}H_{29}$, different products with different melting points, but almost the same IR spectra, were obtained by changing the amounts of triethylamine from 1 equivalence to 2 equivalence . There is much evidence that racemization of activated acylamino acids or acylpeptides can occur by direct proton abstraction or by formation of oxazolone intermediates which are readily racemized (78) (Scheme 4.2).

(1) Formation of the mixed anhydride.



(2) Coupling



where: R = Z or Bz

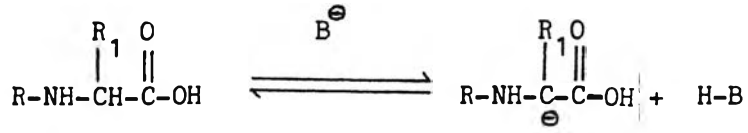
R_1, R_2 = side chain of the amino acid

R = extended peptide chain

Scheme 4.1 The coupling of amino acid via the mixed anhydride method.

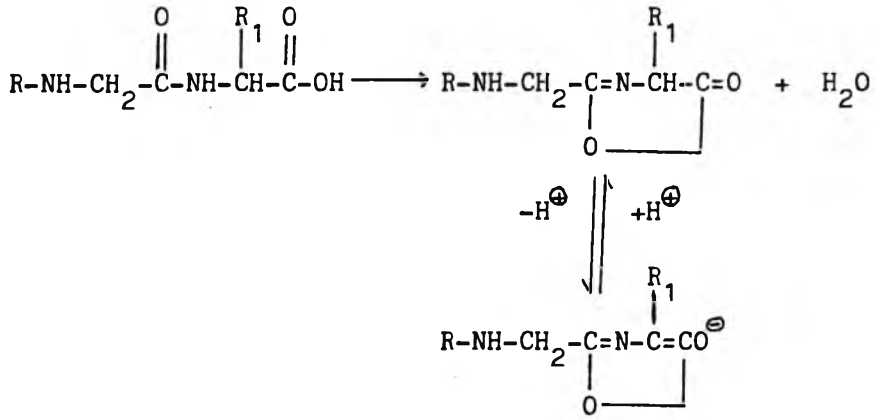
Another important criteria for the formation of the mixed anhydride, is that the solvent must be dry, since the presence of water inhibits the formation of the anhydride. However, the

(1) Direct proton abstraction



or

(2) Formation of oxazolone intermediates



where: R = Z or Bz

R_1 = side chain of the amino acid

$B^{\ominus}$ = base

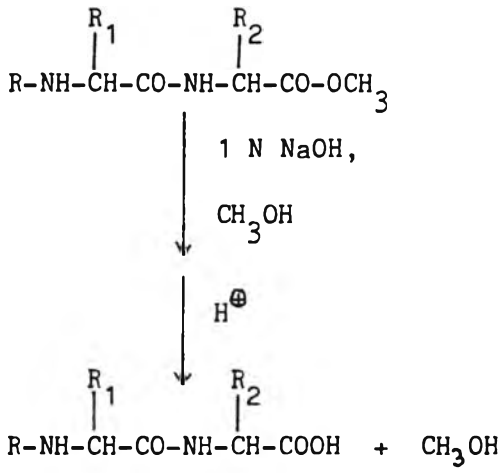
Scheme 4.2 Racemization mechanisms.

coupling of amino acid or alkyl amine residue, requires slightly different conditions. While the alkyl amines are soluble in organic solvents (e.g. THF), the amino acid methyl esters are only soluble in a mixture of water and THF. It was found that even though water was present in the solvent containing the amino acid methyl ester, neither the yield nor the purity of the synthesized peptide fragment was affected which was quite different from the coupling of alkyl amines. It was also found that the addition of amino acid methyl

esters to the mixed anhydride at low temperatures decreased the chances of moisture hydrolysing the anhydride.

4.1.2 Deprotection

As part of the peptide synthesis, it is necessary at certain stages to deprotect the C-terminus of a dipeptide fragment so that the addition of another amino acid or an alkyl amine residue can be carried out. The most common method of deesterification is basic hydrolysis in 1 N NaOH (Scheme 4.3).



where: R = Z or Bz

R_1, R_2 = side chain of the amino acid

Scheme 4.3 The deesterification of a dipeptide fragment.

The hydrolysis of the methyl ester to the free carboxylic acid was achieved in high yields of over 70 %, but in this step the main problem was the purification of the product. Owing to a long period of time (2-3 weeks) taken for recrystallisation of some intermediates in this step, these fractions were used without further purification in the successive synthetic pathway for the synthesis of the desired final compounds. The results of

deesterification were confirmed by IR spectrum showing the broad peak of the -COOH group at 2500-3200 cm^{-1} and by proton NMR with the loss of the $-OCH_3$ signal at δ 3.7 ppm.

4.2 Structural elucidation of the synthetic peptides

Each of the synthetic peptides was purified by fractional recrystallisation from ethyl acetate. Their purity was confirmed by both TLC, showing a single regular round spot without tailing when exposed to UV radiation, and correct elemental analysis within $\pm$ 0.4 % of the calculated values.

The characterisation of reaction product was successfully accomplished by the assistance of the instruments such as elemental analyzer, IR spectrophotometer, ^1H NMR and ^{13}C NMR spectrometer.

IR spectra of all synthetic peptides (compound I-X) exhibited the characteristic absorption bands for symmetric and asymmetric N-H stretching bands of secondary amine at 3290-3360 cm^{-1}, and amide I-II bands at 1520-1660 cm^{-1}. The presence of phenyl groups were revealed by the appearance of weak bands at 3000-3100 cm^{-1}, and IR spectra of the compounds I-VIII showed C-H out of plane bending of monosubstituted benzene at 730-745 and 690-695 cm^{-1}, but of the compounds IX and X exhibited C-H out of plane bending of para-substituted benzene at 830 cm^{-1}. In addition, the presence of CH_2's chains were revealed by the appearance of strong bands at 2800-2950 cm^{-1}.

^1H NMR of 10 compounds showed sharp singlet signal for methylene proton chains at δ 1.24-1.26 ppm and broad triplet signals for methyl protons adjacent to methylene chains. ^1H NMR spectra of

all peptides also showed broad multiplet signals which occurred from overlapping between signals of methylene protons adjacent to amide proton with signals of methylene protons adjacent to benzene ring of phenylalanine or tyrosine. In addition, the signals for methine proton of phenylalanine or tyrosine appeared as quintet which occurred from interaction between methine proton with neighbouring protons, methylene protons and amide proton, at δ 4.54-4.83 ppm.

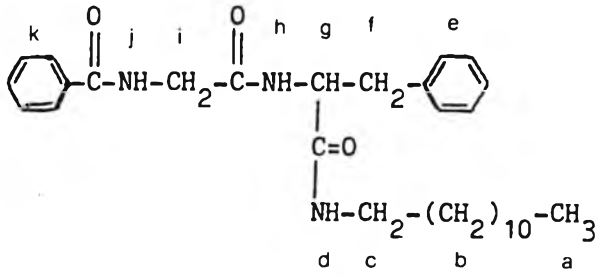
^{1}H NMR spectra of compounds III, IV, V and IX showed multiplet signals for methine proton ($\underline{CH}$-CH_3) of alanine at δ 4.15-4.19 ppm, and doublet signals for methyl protons at δ 1.34 ppm, and sharp singlet signals for methylene protons (Ar-$\underline{CH_2}$-O) at δ 5.07 ppm. For compounds VI, VII, VIII and X, their spectra showed broad doublet signals for methylene protons of glycine which were results appeared of the interaction with neighbouring amide proton at δ 3.84-4.08 ppm.

The aromatic proton signals for all synthetic compounds are different. ^{1}H NMR spectra of compounds I, II, VII and VIII showed sharp singlet signals for aromatic protons of phenylalanine and multiplet signals for aromatic protons of benzoyl group. For compounds III, IV, V and VI, their spectra showed only sharp singlet signals for both aromatic protons of phenylalanine and carbobenzoxy group. However, compound IX showed a singlet signal for aromatic protons of carbobenzoxy group and doublet of doublet signals for aromatic protons of tyrosine which exhibited the existence of para-substituted benzene ring. For compound X, its spectra showed multiplet signals for aromatic protons of benzoyl group and doublet of doublet signals for aromatic protons of tyrosine-like compound IX.

^{1}H NMR signals for compounds IX and X also showed -OH signal for tyrosine at δ 7.33 and 7.61 ppm respectively. ^{1}H NMR signals of all peptides also showed broad signals for amide protons which were affected by overlapping with adjacent protons. In addition, it was found that the chemical shifts of amide protons and hydroxy protons will be shifted to downfield when DMSO was used instead of $CDCl_3$ because of hydrogen bonding between such protons and solvent. ^{1}H NMR of compounds VII and IX were shown in Table 4.1 and 4.2, respectively.

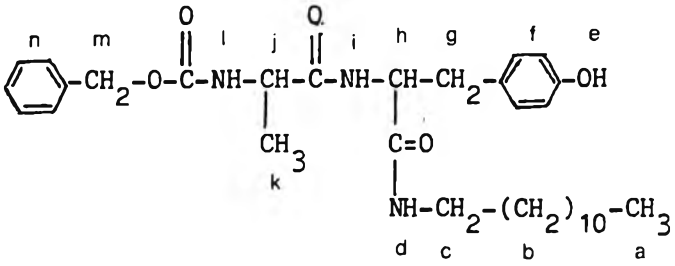
^{13}C NMR signals of these ten compounds showed carbonyl carbon atoms at δ 167.18-172.59 ppm. The number of carbon atoms in the molecule was also confirmed by the appearance of the appropriate number of signals in the ^{13}C NMR spectra. The signals of methyl carbon adjacent to methylene chain and methyl carbon of alanine appeared at δ13.92-14.08 and 17.98-18.52 ppm, respectively. Methylene carbon chains showed many signals at δ 22.37-38.89 ppm. The signals of methylene carbon of phenylalanine or tyrosine appeared at δ 39.38-39.76 ppm, but methylene carbon of carbobenzoxy group showed its signal at δ 67.12-67.23 ppm, and methylene carbon of glycine showed the signal at δ 43.23-44.58 ppm. ^{13}C NMR spectra also showed methine carbon signal of alanine at δ 50.97-51.08 ppm. For compound I-X, ^{13}C NMR spectra showed many peaks of aromatic carbon atom at δ 115.22-155.91 ppm, especially the presence of -OH group in compounds IX and X, aromatic carbon atom attached by -OH group, its signal was shifted and appeared at δ 155.91 ppm. In addition, the signal for the ortho aromatic carbon atom with -OH group appeared at the upfield frequency at δ 115.22 ppm. ^{13}C NMR of compounds IV and X were shown in Tables 4.3 and 4.4 respectively.

Table 4.1 Assignment of the ^1H NMR of compound VII ($CDCl_3$ as solvent).



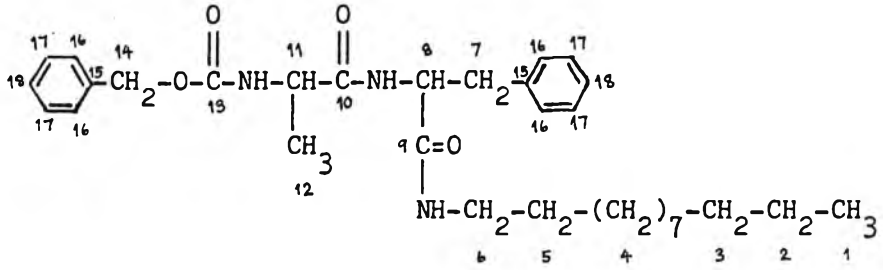
Chemical shift (ppm)	Multiplicity	Assignments
0.88	triplet, broad	a
1.25	singlet	b
3.09	multiplet	c
5.82	broad	d
7.20	singlet	e
3.09	multiplet, overlapped with peak c	f
4.56	quartet	g
6.75	doublet, broad	h
4.09	doublet	i
6.99	broad	j
7.62	multiplet	k
7.26	singlet	$CDCl_3$

Table 4.2 Assignment of the ^1H NMR of compound IX ($CDCl_3$ as solvent).



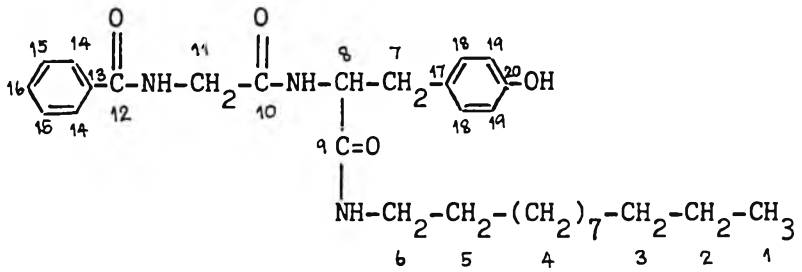
Chemical shift (ppm)	Multiplicity	Assignments
0.87	triplet, broad	a
1.24	singlet	b
3.05	multiplet	c
5.93	singlet, broad	d
7.33	singlet, overlapped with peak n	e
6.87	doublet of doublet	f
3.05	multiplet, overlapped with peak c	g
4.54	quartet	h
-	no observed	i
4.15	multiplet	j
1.34	doublet, overlapped with peak b	k
5.27	broad	l
5.07	singlet, broad	m
7.33	singlet	n
7.26	singlet	$CDCl_3$

Table 4.3 Assignment of the ^{13}C NMR of compound IV ($CDCl_3$ as solvent).



Assignments	Chemical shift (ppm)
C1	14.08
C2-C5	22.64, 26.81, 29.25, 29.52, 29.63, 31.90
C6	38.40
C7	39.65
C8	54.60
C9	172.05
C10	170.21
C11	50.97
C12	18.41
C13	155.96
C14	67.17
C15	136.08
C16	129.26
C17	128.06, 128.28, 128.61
C18	126.98
$CDCl_3$	75.62, 77.03, 78.44

Table 4.4 Assignment of the ^{13}C NMR of compound X ($CDCl_3$ + DMSO-d_6 as solvent).



Assignments	Chemical shift (ppm)
C1	13.97
C2-C5	22.42, 26.76, 29.14, 29.41, 31.69
C6	38.78
C7	39.76
C8	54.66
C9	169.34
C10	167.88
C11	43.66
C12	171.02
C13	133.70
C14	127.58
C15	127.41
C16	131.48
C17	128.28
C18	130.12
C19	115.33
C20	155.91
$CDCl_3$	76.44, 77.90, 79.31
DMSO-d_6	39.43

4.3 Enzyme assay conditions

In this research, spectrophotometric method was used for enzyme assays by measuring of the increase in absorbance of colouring products. BAPNA was used as substrate for trypsin and Suc-Ala-Ala-Pro-Phe-pNA was used as substrate for cathepsin G and chymotrypsin. All of these enzymes are serine proteases which hydrolyze amide substrates by acyl-enzyme mechanism. The nitroanilide substrates are cleaved to yield free 4-nitroaniline, the yellow product, whose concentration was determined at 405 nm (Eq.1 and 2).

$$\text{Bz-DL-Arg-NA} + H_2O \xrightarrow{\text{trypsin}} \text{Bz-DL-Arg} + \text{4-nitroaniline} \quad ....(1)$$

$$\text{Suc-Ala-Ala-Pro-Phe-pNA} + H_2O \xrightarrow{\text{cathepsin G or chymotrypsin}} \text{Suc-Ala-Ala-Pro-Phe} + \text{4-nitroaniline} \quad ...(2)$$

In enzyme kinetic study, many factors affecting initial velocity have to be taken into consideration such as substrate concentration and enzyme concentration. In addition, pH and temperature also affect enzyme activity. For this reason, it is necessary to select and control such conditions to give suitable rate of reaction and optimum activity under the physiological conditions.

The concentration of substrate in enzyme kinetic study was generally prepared in saturation to enzyme because at high substrate concentration the reaction will show zero-order kinetic and the reaction velocity will become independent of the concentration of

the substrate. However, both substrates being used in this experiment were synthetic substrates. It was found that the preparation of saturated substrate concentrations of each enzyme in order to give maximal velocity was difficult. Substrates were not completely dissolved in the solvent. In addition, both synthetic substrates were very high expensive. So each substrate was prepared at 2 mM concentration for this study.

For enzyme concentration, it is well known that initial velocity increases with the enzyme concentration at constant substrate concentration. Due to the problem of the preparation of the saturated substrate concentration for each enzyme, it is necessary to choose the suitable enzyme concentration which shows reasonable activity for each enzyme. The enzyme activity (Abs/min) for this study is in the range of 0.05-0.07.

The solution for enzyme assays was prepared to give pH 7.5. Because this physiological pH gives optimum activity for cathepsin G (79) and chymotrypsin (80) as well as gives 95 % activity for trypsin (81). All experiments were assayed at the physiological temperature, 37 °C.

4.4 Enzyme Inhibition of Synthetic Inhibitors

Table 4.5 exhibits the inhibition results of amino acid derivatives protected at the N-terminal end by a carbobenzoxy (Bz) or benzoyl group (Z). The influence of the amino protecting groups was investigated to determine their effect on enzyme specificity and inhibition. In Comparison compound VI and VII which are different only in their protecting group, it was found that both trypsin and chymotrypsin preferred Bz to Z. And compounds III-VI with Z as their protecting groupg also showed no inhibition for trypsin. In all cases, the results showed that the inhibitions against chymotrypsin of all synthetic inhibitors are higher than these against trypsin. It is in fact that chymotrypsin has a significant preference for hydrophobic region, especially the aromatic amino acid and that all synthetic compounds have aromatic amino acids at P_1.

In order to determine the optimum chain length requirement for the specific inhibition, three series of compounds were synthesized: Bz-Phe-NHR, Z-Ala-Phe-NHR and Bz-Gly-Phe-NHR. Each compound carries an alkyl chain (R) of various length. In all cases, it was found that both trypsin and chymotrypsin showed little differences in percentages of inhibition regarding alkyl chain length. However, chymotrypsin showed its preference to the length of the C-terminal alkyl chain in the following order 14>12>10.

The effects of amino acid sequences, comparing investigated by taking compounds I, II, VII and VIII as inhibitors against trypsin. It was found that the degree of inhibition increased when

they have a glycine moiety at P_2. Compounds IV and IX with the same Z protecting group but different amino acids at P_1, showed that trypsin preferred Tyr to Phe at P_1. On the other hand, compounds VII and X with the same Bz protecting group, showed that trypsin preferred Phe to Tyr at P_1.

For chymotrypsin in comparing the effect of the amino acid sequences between compounds I, II, VII and VIII, it was found that the results were the same as trypsin. Comparing between compounds IV and IX and between compounds VII and X, it showed that chymotrypsin preferred Phe to Tyr at P_1. While compounds IV and VI, showed that this enzyme preferred Ala to Gly at P_2.

In all cases, they showed that all synthetic inhibitors were not good for trypsin, they showed a little inhibition or no inhibition. But for chymotrypsin, compounds I-VIII showed a good inhibitory activity at concentrations less than 50 μM. Compounds IX-X percentage inhibitions are not so high as compounds I-VIII, but they also showed good inhibition for chymotrypsin. It may be possible that the structures of synthetic inhibitors are similar to the structure of its substrate and chymotrypsin is acyl enzyme which prefers to cleave amide bond adjacent to aromatic amino acid.

For cathepsin G, in this research it was found that the activity of this enzyme was very low under the chosen conditions and its activity could not be determined to get the percentage of inhibition with synthetic inhibitors. It may be possible that the physiological temperature, 37 °C, being used in this study is not suitable. Starkey and Barrett found that cathepsin G showed its high activity and adequate stability at 50 °C (9). In addition, Tanaka et. al. (2) showed that cathepsin G is exceptionally

unreactive and they concluded that the low intrinsic kinetic reactivity of cathepsin G was an inherent property of the enzyme and was related to the function of this enzyme. In this research, the reaction time was only 5 mins. in which it might not be enough for cathepsin G to complete its reactions.

Table 4.5 Inhibition by N-protected amino acid derivatives.

No	Inhibitor P_3 P_2 P_1 P_1'	conc. (μM)	% Inhibition	
			chymotrypsin	trypsin
I	Bz-Phe-NH-$C_{12}H_{25}$	50	72.92	7.50
II	Bz-Phe-NH-$C_{14}H_{29}$	50	78.72	10.61
III	Z-Ala-Phe-NH-$C_{10}H_{21}$	50	89.58	0
IV	Z-Ala-Phe-NH-$C_{12}H_{25}$	50	90.38	0
V	Z-Ala-Phe-NH-$C_{14}H_{29}$	50	94.64	0
VI	Z-Gly-Phe-NH-$C_{12}H_{25}$	50	87.93	0
VII	Bz-Gly-Phe-NH-$C_{12}H_{25}$	10	89.39	0
VIII	Bz-Gly-Phe-NH-$C_{14}H_{29}$	5	72.73	-
		10	-	0
IX	Z-Ala-Tyr-NH-$C_{12}H_{25}$	50	28.04	5.55
X	Bz-Gly-Tyr-NH-$C_{12}H_{25}$	50	34.18	7.04

4.5 The type of inhibition and the value of kinetic parameters

The type of inhibition and the kinetic constants were determined from the initial rates of hydrolysis by the Lineweaver-Burk double reciprocal method, based on five separate substrate concentrations. The velocities measured with varying substrate concentration at three different inhibitor concentrations of chymotrypsin were shown in Table II.1 to Table II.10 and their graphical schemes were plotted as shown in Fig. 3.1 to Fig. 3.10. For trypsin, the result with varying substrate concentration without inhibitor was shown in Table 3.2 and its graphical scheme was exhibited in Fig. 3.11. In compliance with the requirements of regression analysis the reciprocal value of the Michaelis constant, $1/K_m$ is presented. Correlation coefficients were greater than 0.99. The type of inhibition of compounds I-X and the values of inhibitor constants are summarized in Table 4.6 on chymotrypsin.

The K_m value of trypsin in BAPNA was 0.54 mM and that of chymotrypsin in Suc-Ala-Ala-Pro-Phe-pNA was 0.10 mM. The type of inhibition of all synthetic inhibitors against chymotrypsin was found to be a competitive one, therefore only the value of K_i not K_I can be determined. The K_i value is useful for comparing the strengths of binding of the inhibitors to the enzymes. In comparision between the percentages inhibition of each inhibitor against chymotrypsin is shown in Table 3.1. In addition, there was an evident to say that the higher the percentage of inhibition is, the less the K_i value is. Because chymotrypsin has been shown to prefer aromatic amino acid residues at P_1 (82). It is possible that

this enzyme will bind to substrate or inhibitor at the same site. So it may be possible that any new synthetic inhibitors having the similar structure as the inhibitors developed in this research may show the same type of inhibition.

Table 4.6 The type of inhibition and the inhibitor constants of N-protected amino acids and peptides for chymotrypsin.

No	Inhibitor	Type	K_i (μM)
I	Bz-Phe-NH-$C_{12}H_{25}$	competitive	13.02
II	Bz-Phe-NH-$C_{14}H_{29}$	competitive	12.19
III	Z-Ala-Phe-NH-$C_{10}H_{21}$	competitive	6.08
IV	Z-Ala-Phe-NH-$C_{12}H_{25}$	competitive	4.87
V	Z-Ala-Phe-NH-$C_{14}H_{29}$	competitive	4.65
VI	Z-Gly-Phe-NH-$C_{12}H_{25}$	competitive	11.16
VII	Bz-Gly-Phe-NH-$C_{12}H_{25}$	competitive	2.67
VIII	Bz-Gly-Phe-NH-$C_{14}H_{29}$	competitive	1.96
IX	Z-Ala-Tyr-NH-$C_{12}H_{25}$	competitive	96.31
X	Bz-Gly-Tyr-NH-$C_{12}H_{25}$	competitive	70.93