CHAPTER III

RESULTS AND DISCUSSION

3.1 Peptide synthesis

In this research, a series of dipeptides and tripeptides were synthesized. The chemical reactions involved in the following steps.

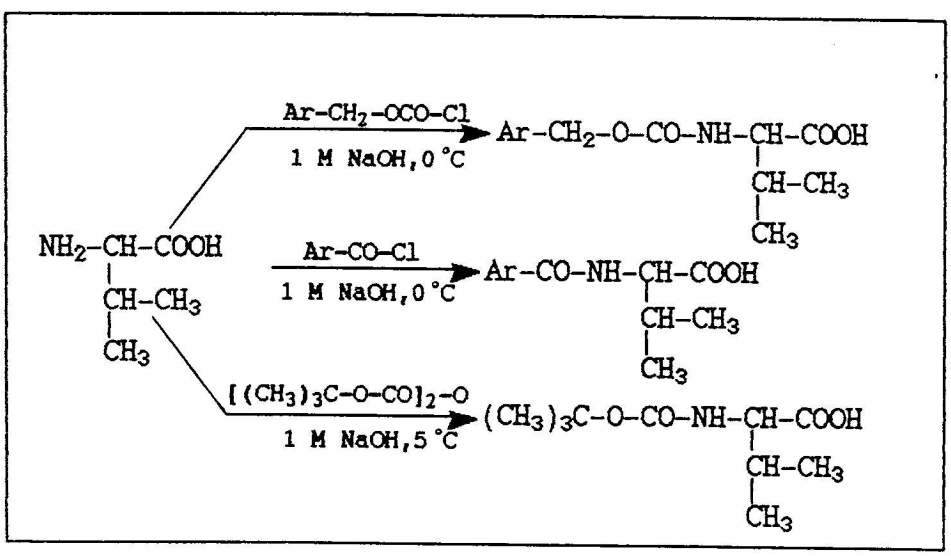
3.1.1 Protection of the N-terminus step

In order to prevent any undesired reaction occurred at the reactive N-terminus of the starting amino acids, they must be protected by a blocking agent. The N-terminus of amino acids were acylated with one of the following blocking groups; the benzoyl (BZ-), benzyloxy carbonyl (Z-) or t-butyloxy carbonyl (BOC) groups. The method of Schwartz et.al.[63] was used for the preparation of benzoyl valine (BZ-V) and benzyloxycarbonylvaline (Z-V). The procedure involved the addition of either benzoylchloride or benzylchloroformate accordingly into the unprotected amino acid, valine, at 0 °C in the presence of 1 M NaOH (Scheme3.1).

It was found that the carbobenzoxy derivative of valine (Z-V) crystallized after a few weeks of storage in the refrigerator. In the case of BZ-V and Z-V, the reaction must

be carefully handled by not using too excessive base, otherwise the protecting agents would be hydrolyzed. The mole ratio of the protecting agents and sodium hydroxide was approximately 1:1 at the beginning of the reaction and a little excess amount of sodium hydroxide was added in the reaction. It was stirred very vigorously at 0 °C since benzoylchloride or benzylchloroformate was in different phase with valine in sodium hydroxide. In addition, the N-terminus protected valine can be easily hydrolyzed in an acidic condition, and thus the acidity of the reaction solution must be carefully checked.

In the case of N-tertiary-butyloxycarbonyl-valine (BOC-V), it was prepared in dioxane at 5 °C by the method of Moroder et.al.[64](Scheme 3.1). Precautions of careful handling in running this reaction similar to what mentioned above must also be strictly followed.

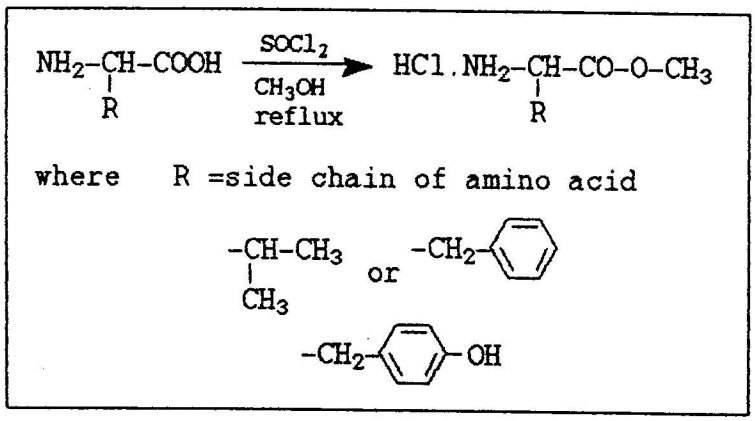


Scheme 3.1 Protection of the N-terminus, Valine.

3.1.2 Protection of the C-terminus step

Prior to the application of any further reaction with an amino acid, the C-terminus must also be protected. In this case a method of synthesizing methyl esters was carried out by refluxing the amino acid in dry methanol with a little excess of thionylchloride (Scheme 3.2).

The synthesized amino acid methyl ester hydrochlorides were hygroscopic so it should be washed with anhydrous ether and kept in a desiccator.

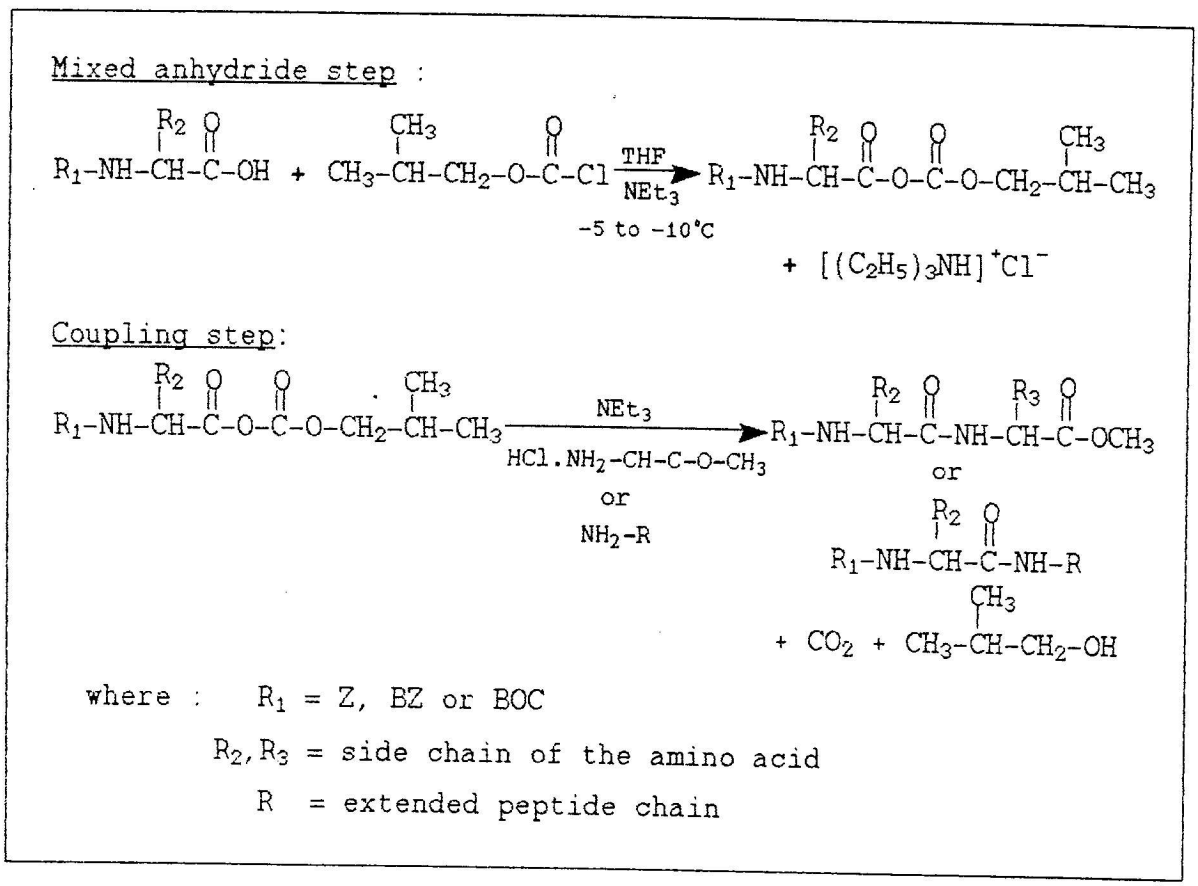


Scheme 3.2 Protection of the C-terminus.

3.1.3 Amide formation step

To build an amide linkage of the peptides, the mixed anhydride method described by Anderson and Zimmerman[13] was applied by adding into the N-protected valine, isobutyl chloroformate as a peptide-forming reagent in the presence of triethylamine as the HCl-acceptor in dry THF at -5 to -10 °C. Then the anhydrides were attacked by the primary amine of the

C-terminus protected amino acid or alkyl amine as a nucleophile in the presence of triethylamine. After the coupling of amino acid via the mixed anhydride was carried out in about 10 hours, the products were formed as shown in the Scheme 3.3.

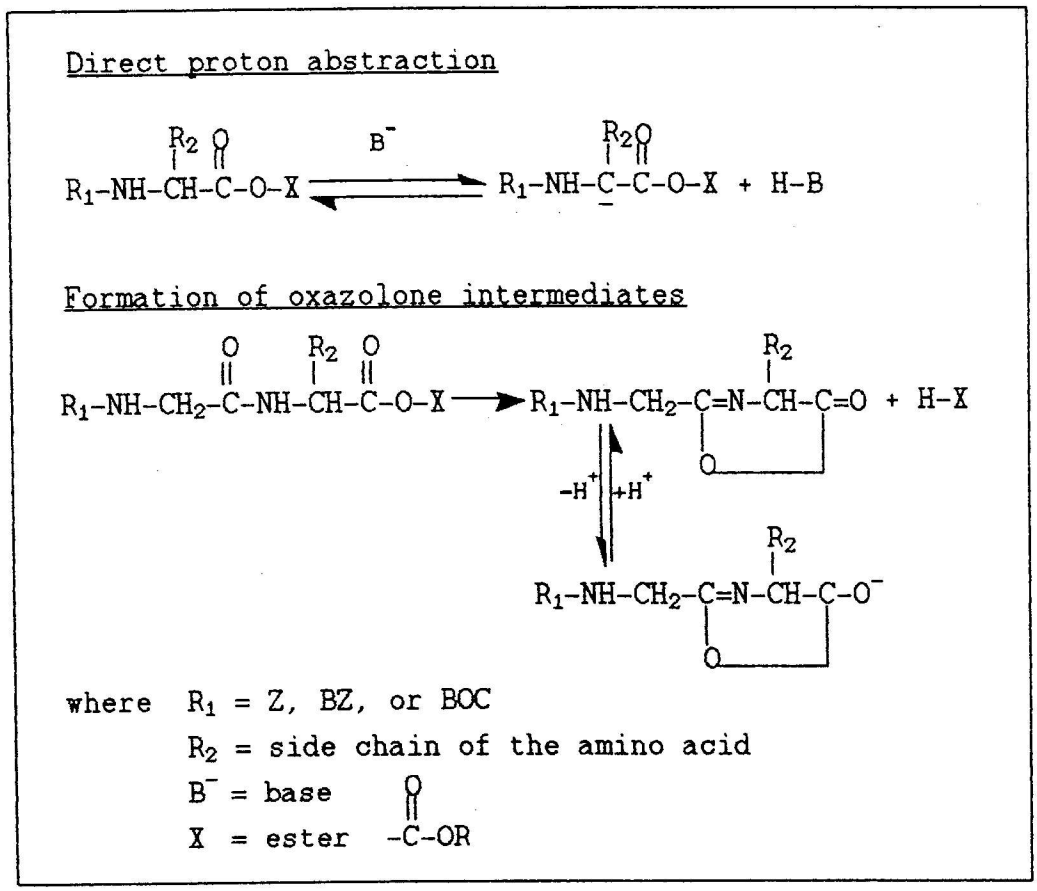


Scheme 3.3 The coupling of amino acid via the mixed anhydride method.

Although the mixed anhydride method has some advantages on activation time, the yield and the purity of products, but the limitation was the racemization which occurred when acylpeptides were used as the coupling components. The evaluation of the synthetic peptide as inhibitors would be

rather difficult for the enantiomers because of the enzyme stereospecific preference. In order to minimize the degree of racemization occuring during the reaction, the optimization conditions of the reactions were carefully chosen. An optimum activation time of 10-15 minutes was allowed before the addition of the N-protected valine. The reaction temperature was carried out between -5 to -10 °C. In this research isobutylchloroformate was chosen to be used in the mixed anhydride step since it was stable upon storage and gave the best yield of the products. The ethylchloroformate could also be used, but it took much longer time in the formation of the anhydride and the yield of the products was also lower than those of using isobutylchloroformate.

Regarding the solvent effect, it was reported that ethyl acetate and tetrahydrofuran gave the best yield without racemic forms. In addition, triethylamine being used as the HCl acceptor was also an important factor for conducting towards racemization, if it was used in excessive amount. Nevertheless, triethylamine was one of the most suitable bases for such sterically controlled racemization. Bodanszky and Ondetti[65] summarized about the racemization mechanism as shown in Scheme 3.4



Scheme 3.4 Racemization mechanism.

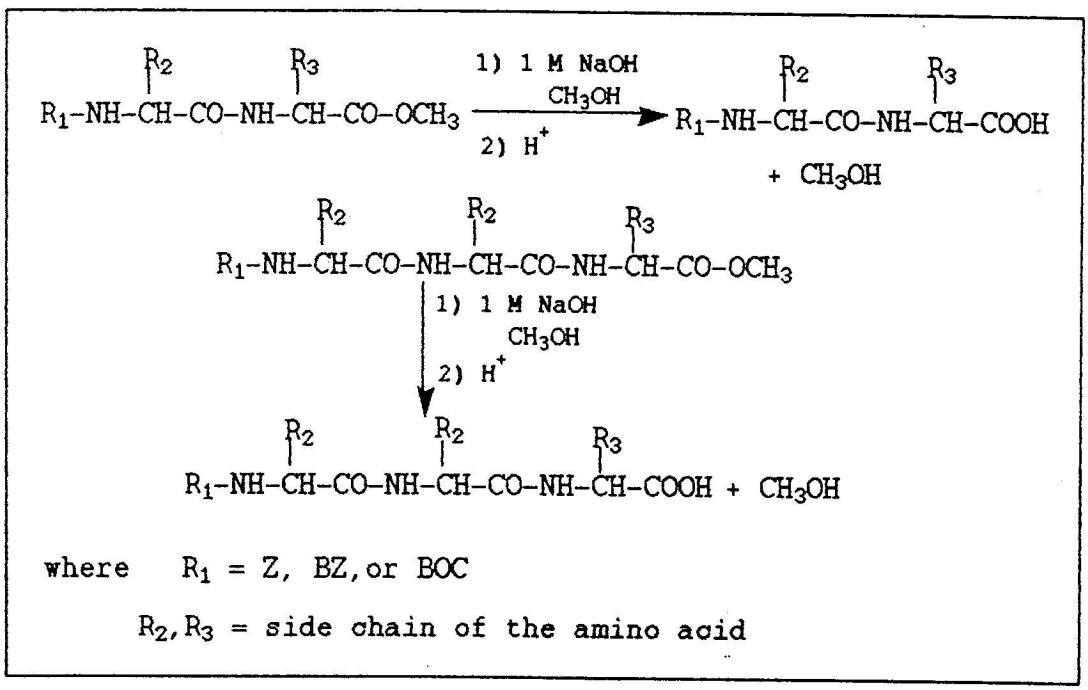
Generally speaking, it is difficult to identify whether the synthetic compounds are exactly in the L-form configuration only or not. Dobashi, A.et.al. studied on the self-induced nonequivalence in the association of D- and L-amino acid derivatives[66] and reported that NMR signal at a high frequency could be used to assign the configuration of the amino acid derivatives. However, attempts in this investigation were made only to synthesize the peptides in order to reduce the degree of racemization by following the optimum conditions as mentioned above.

Another important criteria for the formation of the mixed anhydride was to use the dry solvent. The presence of

water inhibited the formation of the anhydride. In the coupling step, alkyl amines were soluble in tetrahydrofuran while the amino acid methyl esters were soluble in a mixture of water and tetrahydrofuran. So it was unavoidable to have some water in the coupling reaction between the amino acid methyl ester and its anhydride. In contrast to the coupling reaction of alkyl amines in which only dry tetrahydrofuran was used as the solvent.

3.1.4 Removal of the protecting group of C-terminus methyl ester step

Removal of a methoxy group from the C-terminal part of a peptide after the completion of the coupling step, can usually be done by a mild alkaline hydrolysis in methanol, preferably at or below room temperature, and if possible, without an excess of alkali. Such precautions are necessary to avoid undesired side reactions along the already synthesized peptide chain. Frequently even a mild alkaline treatment is undesirable, as it may lead to the hydrolysis of some sensitive amide bonds.[67] In order to carry out the addition of another amino acid or an alkyl amine residue the method of deesterification to obtain the free carboxylic acid should be achieved (Scheme 3.5). The results of deesterification were confirmed by IR spectrum showing a 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 3.6 ppm.



Scheme 3.5 The deesterification of a di- or tripeptide fragment.

3.1.5 Other problems encountered in peptide synthesis

During the course of synthesizing peptide fragments and C-terminal alkyl peptide, it was found that several intermediate and final compounds were difficult to be crystallized. For the intermediates, they were identified with IR and ^1H NMR. If any one of them was confirmed to be the desired compound, it would be used in the following step without further purification. Some of the final compounds obtained as an oil or gel, which composed of more than 2 compounds as shown by TLC, needed to be crystallized. Some of them were crystallized as needle forms in their oil bases, but

they could not be separated from the oil. After the solvent was evaporated under reduced pressure, some final compounds were obtained in pale yellow powder solids. These powder solids were washed with an appropriate solvent such as ether, petroleum ether, methanol and they were recrystallized, or purified by column chromatography. Unfortunately, attempts were made unsuccessfully to purify the following compounds by recrystalization, column chromatography and even by preparative HPLC techniques: Z-V-V-P-C_{12}, BZ-V-V-T-C_{10}, BZ-V-V-T-C_{12}, BZ-V-V-P-C_{10}, BZ-V-V-P-C_{12}, BOC-V-P-C_{10}, and BOC-V-P-C_{12}. Therefore the results of these compounds were not fully accomplished.

In addition, there were other problems related to the possibility of racemization during the peptide synthesis. Generally speaking, it was very difficult to prove their actual configurations and in fact there was no attempt in this research to verify the stereochemistry aspect of the synthetic compounds.

3.2 Structural elucidation of the synthetic peptides

The purity of all of the synthetic peptides were confirmed by TLC, showing a regular round spot without tailing when exposed to both I_2 vapor and UV radiation, They were further proved by elemental analysis (Table 3.1) and HPLC technique.

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

IR spectra of all synthetic peptide showed the characteristic absorption bands for symmetric and asymmetric N-H stretching bands of secondary amine at 3280-3340 cm^{-1},and amide I-II bands at 1500-1720 cm^{-1}. The presence of phenyl groups was revealed by the appearance of weak asymmetric bands at 3000-3100 cm^{-1} and IR spectra of the compounds III-IV and VII-VIII showed C-H out of plane bending of monosubstituted benzene at 720-750 and 690-700 cm^{-1}. In addition to the compounds I-II, V-VI, IX-X, and XII-XIII showed the C-H out of plane bending of para-substituted benzene at 820-840 cm^{-1}. For the presence of CH_2 's chain was revealed by the appearance of strong bands at 2840-2980 cm^{-1}.

^1H NMR of all synthetic peptide showed multiplet signal for methyl protons adjacent to methylene chains $[(CH_2)_n\text{-}CH_3]$ and adjacent to methine proton of valine $[CH(CH_3)_2]$ at δ

0.68-0.90 ppm.[67] ^{1}H NMR of all peptides showed signal of methylene protons adjacent to amide proton [NH-C**H**$_2$-(CH$_2$)$_n$] and adjacent to benzene ring of phenylalanine or tyrosine [C**H**$_2$-Ar] at δ 2.49-3.73 ppm. In addition, the signals for methine proton adjacent to dimethyl group of valine [C**H**(CH$_3$)$_2$] appeared as multiplet at δ 1.92-2.16 ppm and methine proton adjacent to methylene group of phenylalanine or tyrosine [-C**H**-CH$_2$-Ar] together with methine proton adjacent to amide proton with valine [HN-C**H**-CH(CH$_3$)$_2$] appeared at δ 3.73-4.85 ppm. ^{1}H NMR spectra of compounds V-XI showed sharp singlet signals for methylene protons adjacent to oxygen [Ar-C**H**$_2$-O] at δ 5.00-5.10 ppm. ^{1}H NMR spectra of compounds XII-XIII showed sharp singlet signals for trimethyl protons of tertiary butyl group [C(C**H**$_3$)$_3$] at δ 1.35, 1.45 ppm. ^{1}H NMR spectra of compounds I-II, V-VI, IX-X, and XII-XIII showed doublet of doublet signals for aromatic protons of tyrosine that exhibited the existence of para-substituted benzene ring [-CH$_2$-⟨⊙⟩-OH]. For compounds VII-VIII and XI, their spectra showed only sharp singlet signals for both aromatic protons of phenylalanine and carbobenzoxy group [-CH$_2$-**Ar**,**Ar**-CH$_2$-O], but compounds III-IV showed sharp singlet signals for aromatic protons of phenylalanine and multiplet signals for aromatic protons of benzoyl group [-CH$_2$-**Ar**,**Ar**-C=O]. In the case of compounds I-II showed multiplet signals for aromatic protons of benzoyl group and compound V-VI, IX-X showed singlet signal for aromatic protons of carbobenzoxy group, too. ^{1}H NMR spectra of compounds I-II, V-VI, IX-X, and XII-XIII showed -OH signal for tyrosine varied

the position depending on the solvent such as compound XII in $CDCl_3$ showed -OH signal at δ 6.94 ppm while in compounds I-II, V-VI, and IX-X showed -OH signals at δ more than 7.00 ppm. ^1H NMR spectra of all peptides showed broad signals for amide protons which were affected by overlapping with adjacent protons. It was found that the chemical shift was shifted to downfield when DMSO was used instead of $CDCl_3$. This was essentially due to the hydrogen bonding between such protons and the solvent. ^1H NMR of all compounds were shown in Table 3.2-3.7.

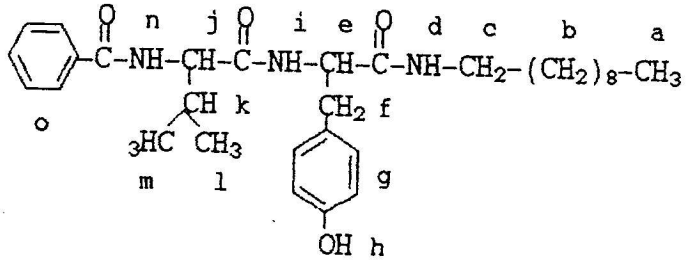
^{13}C NMR spectra of all synthetic compounds showed the signals of methyl carbon adjacent to methylene chain [$(CH_2)_n$-CH_3] and dimethyl carbon of valine [$CH(CH_3)_2$] together with methine carbon adjacent to dimethyl of valine [$CH(CH_3)_2$] appeared at δ 13.6-19.1 ppm. Methylene carbon chains showed many signals at δ 22.2-39.0 ppm. The signals of methylene carbon of phynylalanine or tyrosine [CH-CH_2-Ar] appeared at δ 38.9-39.4 ppm, but methylene carbon of carbobenzoxy group [Ar-CH_2-O] showed its signals at δ 65.0-67.5 ppm. ^{13}C NMR spectra also showed methine carbon adjacent to amide proton with valine [HN-CH-$CH(CH_3)_2$] signal and methine carbon adjacent to methylene carbon of phenylalanine and tyrosine [CH-CH_2-Ar] signal appeared at δ 59.9-71.1 ppm. ^{13}C NMR spectra showed many peaks of aromatic carbon atoms at δ 113.9-136.9 ppm. For the carbonyl carbon atoms, their spectra showed signals at δ 155.2-171.9 ppm. ^{13}C NMR of all the compounds which were shown in Table 3.8-3.13.

Table 3.1 The elemental analysis of synthetic peptides(a)

No.	Compound	Elemental Analysis					
		C(%)		H(%)		N(%)	
		calc'd	found	calc'd	found	calc'd	found
I	BZ-V-T-NH-C_{10}	71.10	71.05	8.66	8.68	8.02	8.05
II	BZ-V-T-NH-C_{12}	71.83	71.54	8.95	8.87	7.62	7.56
III	BZ-V-P-NH-C_{10}	73.34	73.12	8.93	9.02	8.28	8.20
IV	BZ-V-P-NH-C_{12}	73.98	73.80	9.22	9.20	7.84	7.72
V	Z-V-T-NH-C_{10}	69.41	69.34	8.56	8.74	7.59	7.67
VI	Z-V-T-NH-C_{12}	70.19	69.40	8.84	8.42	7.22	7.01
VII	Z-V-P-NH-C_{10}	71.47	71.01	8.81	8.64	7.81	7.63
VIII	Z-V-P-NH-C_{12}	72.18	72.13	9.09	9.12	7.43	7.14
IX	Z-V-V-T-NH-C_{10}	68.07	68.85	8.65	8.95	8.58	8.66
X	Z-V-V-T-NH-C_{12}	68.79	68.64	8.88	8.74	8.23	8.43
XI	Z-V-V-P-NH-C_{10}	69.78	69.82	8.86	8.90	8.80	8.72
XII	BOC-V-T-NH-C_{10}	67.02	67.19	9.50	9.73	8.09	8.20
XIII	BOC-V-T-NH-C_{12}	67.97	67.41	9.75	9.34	7.67	7.46

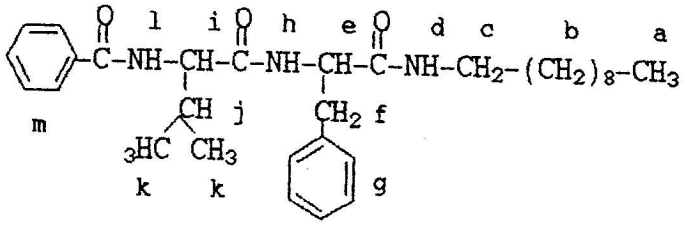
(a) obtained the results from La Trobe University, Australia and Mahidol university, Thailand.

Table 3.2 Assignment of the ^1H NMR of compound I and II ($CDCl_3$+DMSO as solvent)



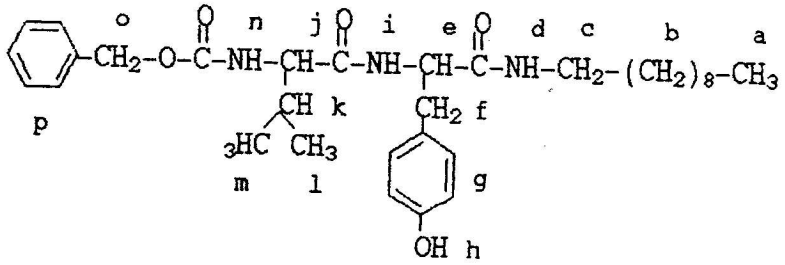
Chemical shift (ppm)		Multiplicity	Assignments
I	II		
0.83	0.86	triplet	a
0.60, 0.83	0.65, 0.86	doublet	l, m
1.21	1.18	singlet	b
2.04	1.95	multiplet	k
2.49	2.51	quartet	c
2.70	2.67	doublet	f
4.21	4.03	triplet	j
4.40	4.40	quartet	e
6.56, 6.96	6.56, 6.97	doublet of doublet	g
7.47	7.39	multiplet	o
7.70	7.48	triplet, broad	h
7.86	7.82	doublet	n
8.20	8.05	triplet	d
9.01	8.62	doublet	i

Table 3.3 Assignment of the ^1H NMR of compound III and IV ($CDCl_3$ as solvent)



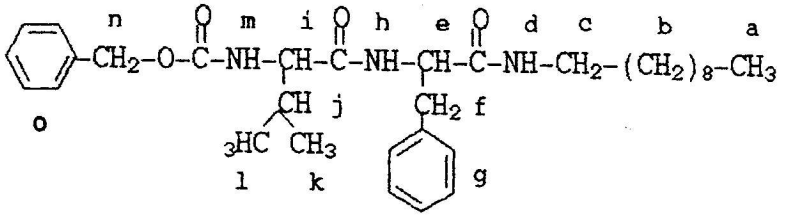
Chemical shift (ppm)		Multiplicity	Assignments
III	IV		
0.84	0.89	multiplet	a,k
1.24	1.23	singlet	b
2.05	1.92	multiplet	j
3.04	3.02	multiplet	c
3.67	3.73	doublet	f
4.01	3.95	triplet	i
4.26	4.30	quartet	e
5.52	5.60	broad	l
5.94	6.14	broad	d
6.78	6.92	broad	h
7.19	7.17	multiplet	g
7.41,7.78	7.36,7.75	multiplet	m

Table 3.4 Assignment of the ^1H NMR of compound V and VI ($CDCl_3$+DMSO as solvent)



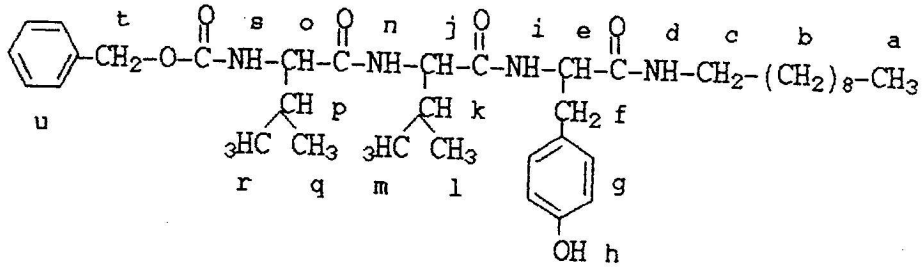
Chemical shift (ppm)		Multiplicity	Assignments
V	VI		
0.82	0.84	triplet	a
0.79, 0.82	0.75, 0.84	doublet of doublet	m, l
1.18	1.20	singlet	b
1.95	1.79	multiplet	k
2.49	2.50	quartet	c
2.88	2.70	doublet	f
3.97	3.77	multiplet	j
4.53	4.40	multiplet	e
5.00	5.00	singlet	o
6.60, 6.89	6.58, 6.90	doublet of doublet	g
7.26	7.28	singlet	p
6.54	7.43	triplet	h
7.66	7.64	doublet	n
7.72	7.94	triplet	d
8.81	8.79	singlet, broad	i

Table 3.5 Assignment of the ^1H NMR of compound VII and VIII ($CDCl_3$+DMSO, $CDCl_3$ as solvent respectively)



Chemical shift (ppm)		Multiplicity	Assignments
VII	VIII		
0.78	0.87	multiplet	a,k,l
1.18	1.25	singlet	b
1.91	2.08	multiplet	j
2.88	3.07	multiplet	c,f
3.96	4.07	triplet	i
4.58	4.66	quartet	e
5.00	5.07	singlet	n
6.32	5.57	doublet	m
7.78	6.15	triplet,broad	d
7.90	7.09	doublet	h
7.11	7.21	multiplet	g
7.24	7.32	singlet	o

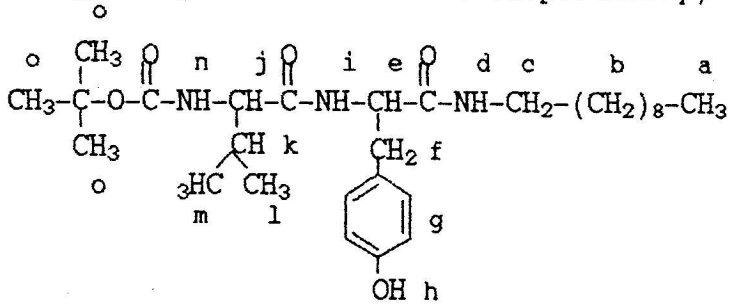
Table 3.6 Assignment of the ^1H NMR of compound IX, X and XI ($CDCl_3$, $CDCl_3$+DMSO and $CDCl_3$ as solvent respectively)



Chemical shift (ppm)			Multiplicity	Assignments
IX	X	XI		
0.90	0.68, 0.82	0.87	multiplet	a, r, g, m, l
1.24	1.20	1.24	singlet	b
2.12	1.93	2.10	broad	p, k
3.08	2.83	3.10	quartet	c
3.70	3.56	3.70	doublet	f
4.10	3.98	4.00	triplet	e
4.50	4.23, 4.42	4.22, 4.85	triplet	o, j
5.07	5.00	5.10	singlet	t
5.56	8.15	5.32	doublet	s
6.05	9.05	-	broad	h
6.47	7.77	6.25	triplet	d
6.65, 6.93	6.59, 6.91	-	doublet of doublet	g
6.78	8.15	6.44	doublet	n
7.09	7.59	7.08	broad	i
7.28	7.29	7.25	singlet	u
-	-	7.33	singlet	g

Table 3.7 Assignment of the ^1H NMR of compound XII and XIII

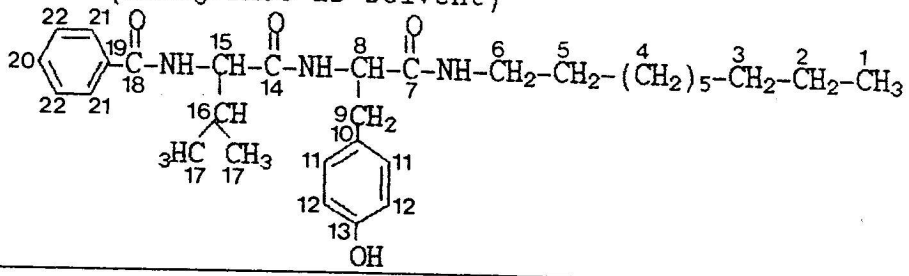
($CDCl_3$, $CDCl_3$+DMSO as solvent respectively)



Chemical shift (ppm)		Multiplicity	Assignments
XII	XIII		
0.87	0.81	multiplet	a,m,l
1.24	1.18	singlet	b
1.45	1.35	singlet	o
2.16	1.90	multiplet	k
2.93	2.82	doublet	f
3.17	3.00	quartet	c
3.88	3.75	triplet	j
4.57	4.45	quartet	e
4.91	5.92	doublet	n
6.14	7.15	broad	d
6.56	7.41	doublet	i
6.76,7.06	6.56,6.92	doublet of doublet	g
6.94	8.71	broad	h

Table 3.8 Assignment of the ^{13}C NMR of compound I and II

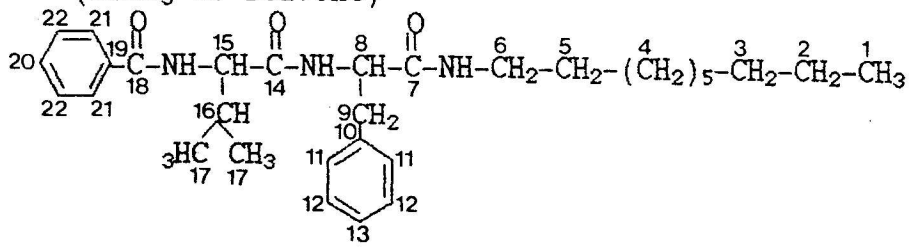
($CDCl_3$+DMSO as solvent)



Chemical shift (ppm)		Assignments
I	II	
13.6	13.8	C1
18.5	18.9	C17
18.7	19.0	C16
22.0,26.5,28.7 29.2,29.7,31.2	22.2,26.6,28.9, 29.2,29.5,31.4	C2-C5
36.0	36.4	C6
38.4	39.0	C9
55.2	54.7	C15
60.2	60.6	C8
114.1	114.8	C13
127.5	127.3	C10
127.9	127.8	C11,C22
129.2	129.8	C12,C21
130.5	130.8	C20
134.5	134.0	C19
167.5	167.1	C18
170.2	170.7	C14
171.5	171.2	C7

Table 3.9 Assignment of the ^{13}C NMR of compound III and IV

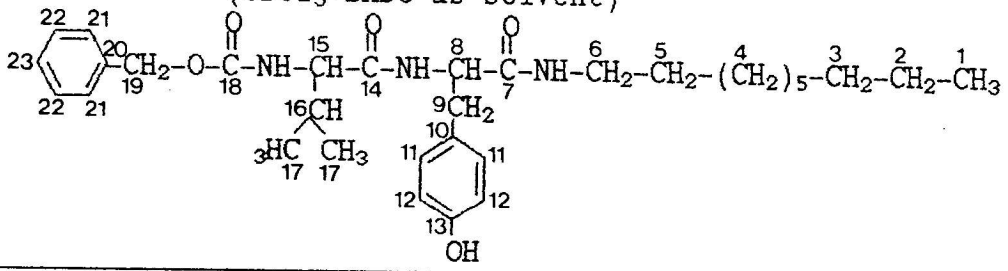
($CDCl_3$ as solvent)



Chemical shift (ppm)		Assignments
III	IV	
14.1	14.1	C1
19.0, 19.1	19.0	C16, C17
22.6, 26.7, 27.9 29.5, 29.6, 31.8	22.6, 26.7, 27.7 29.3, 29.6, 31.8	C2-C5
39.0	36.4	C6
39.3	39.3	C9
56.4	56.3	C15
71.1	71.1	C8
126.5	126.7	C10
127.9	127.2	C11
128.0	128.3	C12
128.4	128.4	C22
129.2	129.2	C20, C13
131.4	131.2	C21
136.8	136.8	C19
156.2	156.0	C18
167.0	166.8	C14
169.8	170.5	C7

Table 3.10 Assignment of the ^{13}C NMR of compound V and VI

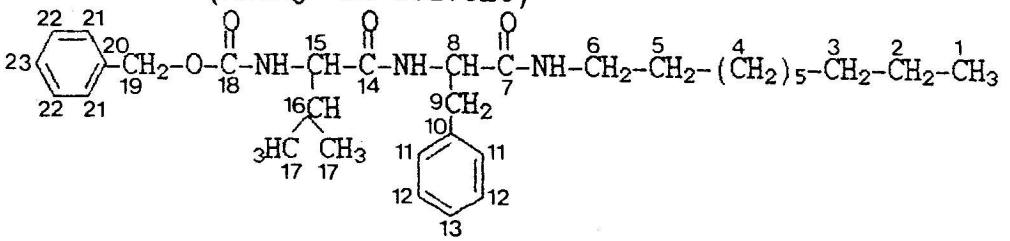
($CDCl_3$+DMSO as solvent)



Chemical shift (ppm)		Assignments
V	VI	
14.1	13.8	C1
17.9	18.9	C17
19.1	19.0	C16
22.6,26.7,27.8 29.4,31.4	22.2,26.5,28.6 29.2,31.4	C2-C5
37.1	36.9	C6
39.0	38.9	C9
53.1	54.4	C15
59.8	60.7	C8
67.1	65.7	C19
115.5	114.8	C13
126.5	127.3	C10
127.9	127.6	C11,C22
128.3	128.0	C12,C21
130.2	129.8	C23
136.4	136.5	C20
155.8	155.7	C18
156.8	156.2	C14
171.9	171.6	C7

Table 3.11 Assignment of the ^{13}C NMR of compound VII, VIII

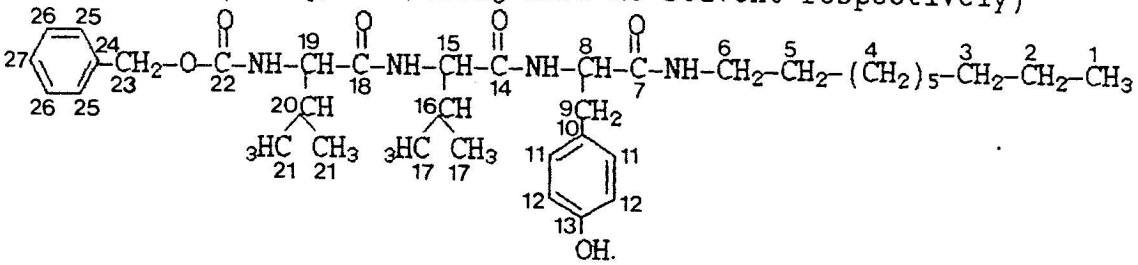
($CDCl_3$ as solvent)



Chemical shift (ppm)		Assignments
VII	VIII	
14.1	14.1	C1
18.0, 18.3	18.3	C17
19.1	19.0	C16
22.6, 26.9, 29.3 29.7, 31.9	22.6, 26.9, 29.3 29.6, 31.8	C2-C5
38.6	38.5	C6
39.4	39.3	C9
54.5	54.6	C15
60.2	60.2	C8
66.5	66.5	C19
126.4	126.4	C10
127.7	127.8	C11, C22
128.1	128.2	C12, C21
129.3	129.3	C13, C23
136.9	136.7	C20
156.2	156.2	C18
170.9	170.6	C14
171.8	171.5	C7

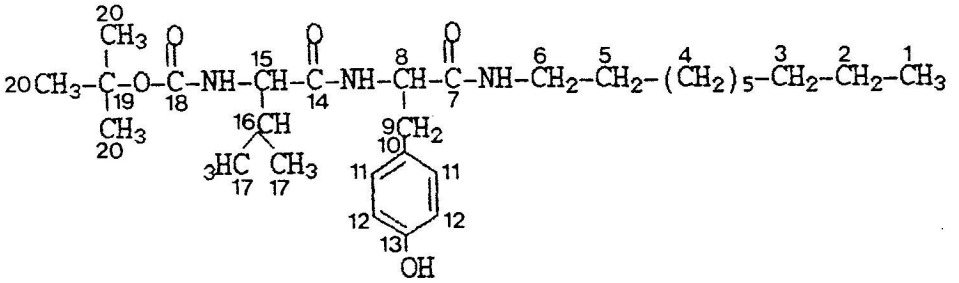
Table 3.12 Assignment of the ^{13}C NMR of compound IX, X, XI

($CDCl_3$, DMSO, $CDCl_3$+DMSO as solvent respectively)



Chemical shift (ppm)			Assignments
IX	X	XI	
17.7	15.6	16.4	C1
18.1	16.5	16.6	C17, C21
18.9, 19.1	17.6	18.3, 18.5	C16, C20
26.5, 27.2 29.4, 29.8	26.8, 29.1 29.3	26.0, 27.1 28.4, 28.6	C2-C5
31.1	35.1	30.2	C6
51.8	50.3	51.5	C9
56.8	53.1, 56.8	56.4	C15, C19
60.2	59.9	60.4	C8
66.7	65.0	67.5	C23
120.5	113.9	116.1	C13
126.4	126.4	126.8	C10
127.8	126.5	127.4, 127.6	C11, C25
128.2	126.6	127.9	C12, C26
130.2	127.1	128.2	C27
136.3	128.9	136.5	C24
156.0	155.2	154.4	C22
170.9	170.4, 170.9	170.1	C14, C18
171.9	171.8	171.7	C7

Table 3.13 Assignment of the ^{13}C NMR of compound XII, XIII ($CDCl_3$+DMSO as solvent)



Chemical shift (ppm)		Assignments
XII	XIII	
13.8	13.8	C1
17.7	17.6	C17
18.9	18.9	C16
22.2,26.5,28.0 28.9,29.1,31.4	22.2,26.5,28.0 28.9,29.1,31.4	C2-C5
30.5	30.4	C20
37.2	37.1	C6
38.9	39.1	C9
53.9	54.2	C15
59.9	60.1	C8
114.6	114.9	C13
127.1	127.2	C10
129.8	129.8	C11,C12
155.7	155.7	C18
170.4	170.4	C14
170.9	171.0	C7

3.3 Enzyme kinetic results

3.3.1 Enzyme kinetic assay conditions

During the study of colouring products, It was found that serine proteases can hydrolyze amide substrate via acyl-enzyme mechanism. The nitroanilide substrates were cleaved to yield the yellow product free *p*-nitro aniline in which its concentration will be determined at 400 nm. It is necessary to determine the optimum conditions for the enzyme kinetics in order to obtain a suitable initial velocity of the reaction and the optimum enzyme activity under the physiological conditions. Factors affecting initial velocity are substrate concentration, enzyme concentration, pH and temperature.

In principle, rate of enzyme reaction is independent of the substrate concentration, so the reaction shows zero-order kinetic. The suitable enzyme concentrations that were chosen for determining the percentage of inhibitions were 100 µL and 200 µL for trypsin and chymotrypsin,respectively, as shown in Fig.II.1-II.13. N-benzoyl-DL-arginine-*p*-nitroanilide, a substrate for trypsin and Suc-Ala-Ala-Pro-Phe-pNA, a substrate for chymotrypsin were studied as shown in Fig. II.14-27. The concentration of both substrates for determining the percentage of inhibitions was 200 µL. The enzyme kinetic experiments were assayed at the physiological temperature of

37 °C and physiological pH 7.5 which gave an optimum activity for each enzyme.(42)

3.3.2 Structure activity relationships of synthetic peptides

The results of enzyme inhibitions of those synthetic peptides (compound I-XIII) under this investigation are shown in Table 3.14. It was interesting to point out the highest concentration as well as the highest inhibition against the trypsin and chymotrypsin. For the minimum effective concentration (MEC) of all synthetic peptides, it was found mostly to be equal to or less than 0.1 μM against trypsin and chymotrypsin under the chosen conditions (Table 2.7). In comparison of the chain length at P_0, it was found that both trypsin and chymotrypsin showed nearly equal in the preference for C-terminal alkyl chain length because of their little diferences in percentages of inhibition between 10 and 12 carbon units. The influence of the N-terminal protecting groups was shown by the comparisons between compounds III and VII and between compounds IV and VIII. It was found that the benzyloxycarbonyl(Z) protecting groups at P_3 gave percentages of inhibition against both enzymes higher than that of benzoyl (BZ) protecting group. In addition, the same result was confirmed by the comparisons between compounds I and V, and between compounds II and VI against trypsin, and chymotrypsin. Regarding the effects of amino acid sequences on the enzyme

inhibitions, chymotrypsin had a trend of preferring phenylalanine to tyrosine which was shown by comparing between compounds V and VII,VI and VIII,II and IV,IX and XI. On the contrary, trypsin has a trend of preferring tyrosine to phenylalanine. Addition of valine into the amino acid sequences, as shown by comparing between compounds V and IX,VI and X, gave the increasing degree of inhibition against trypsin but when comparing between compounds VII and XI,V and IX, as well as VI and X little changes on the decrease in the degree of inhibition against chymotrypsin were found. Evidently, after the studies of all the cases, it was clearly indicated that not only the type of amino acids presented in the peptide sequence but the position at which the amino acid was resided along the length of the inhibitors was also an important factor to determine the the structure activity relationships (SAR).

Most of all the synthetic compounds showed a higher degree of inhibition against chymotrypsin than that against trypsin. This might indicate that structures of the synthetic compounds were possibly similar to the structure of the chymotrypsin's substrate. As a matter of fact chymotrypsin is an acyl enzyme which prefers to cleave amide bond adjacent to an aromatic amino acid. In addition, chymotrypsin has a preference for hydrophobic region. Nevertheless, the degrees of inhibitions against both enzymes were found to be rather high values.

The experiments on the inhibitions of those synthetic compounds against elastase were unsucessfully done. Since the elastase had been stored in the refrigerator for a long time before it was used for testing in the experiments. As the result it showed very low enzyme activities in all cases. However, the synthetic compounds were not suitable to be used for antiarthritis and antiemphysema in any way because they showed high degree of inhibitions against such normal useful protease as chymotrypsin and trypsin. A good candidate for an enzyme inhibitor in this kind of research should show a little or no inhibition against any useful or essential enzymes in human body but a highly specific and effective inhibition for the harmful enzymes which cause the diseases such as elastase and cathepsin G.

According to the results of earlier studies[68,69] on the type of inhibition it was shown that the peptides were competitive inhibitiors against the tested enzymes.

Table 3.14 Inhibition by N-protected amino acid derivatives.

No.	Inhibitor P_4 P_3 P_2 P_1 P_0	conc.(μM)	% Inhibition	
			trypsin	chymotrypsin
I	BZ-V-T-NH-$C_{10}H_{21}$	10	41.76	73.27
II	BZ-V-T-NH-$C_{12}H_{25}$	10	53.85	53.08
III	BZ-V-P-NH-$C_{10}H_{21}$	10	38.02	66.73
IV	BZ-V-P-NH-$C_{12}H_{25}$	10	36.84	55.64
V	Z-V-T-NH-$C_{10}H_{21}$	10	54.95	62.50
VI	Z-V-T-NH-$C_{12}H_{25}$	10	51.65	59.04
VII	Z-V-P-NH-$C_{10}H_{21}$	10	48.79	67.69
VIII	Z-V-P-NH-$C_{12}H_{25}$	10	44.62	77.31
IX	Z- V-V-T-NH-$C_{10}H_{21}$	10	60.22	61.15
X	Z- V-V-T-NH-$C_{12}H_{25}$	10	58.46	57.88
XI	Z- V-V-P-NH-$C_{10}H_{21}$	10	46.15	56.35
XII	BOC-V-T-NH-$C_{10}H_{21}$	10	52.31	71.95
XIII	BOC-V-T-NH-$C_{12}H_{25}$	10	51.43	43.65