CHAPTER III

RESULTS AND DISCUSSION

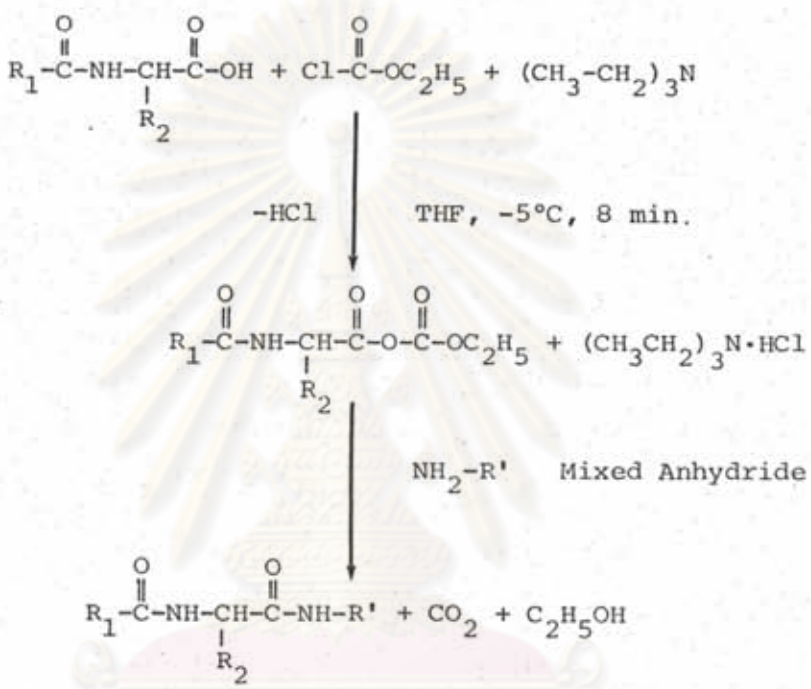
3.1 Peptide Synthesis

The synthetic peptides in this research were synthesised using the coupling reaction of the mixed anhydride method of Anderson and Zimmerman (22) which gave good yields. It was important at the coupling stage that dipeptides of reasonable purity and yield were obtained, so that stepwise procedure of adding one amino acid residue at a time would markedly decrease the overall yield, based on the first two amino acids used. In this research, the method of mixed anhydride was used with the use of ethyl chloroformate to build the amide linkage and triethylamine is the tertiary base was used as the HCl acceptor.

3.1.1 The effect of racemisation

An activation time of 8-15 minutes was allowed before the addition of the carboxyl-protected amino acid. The reaction was carried out at -5 to -10°C. This optimisation condition of the reactions were chosen so as to obtain maximum yield (60%-80%) and high purity, as well as reduction in the degree of racemisation occuring during the reaction. It was important to reduce the degree of racemisation to the D-form, since the proteases are only active against the L-amino acid. The conditions described by Anderson and Zimmerman were intended to accomplish this result by using specific

activation times which were important in the reduction of racemisation. It was also found that using the mixed anhydride method they were able to obtain a ratio of 5% in the DL-form and 75% in the L-form. The mixed anhydride mechanism is proposed as shown in scheme 3.1 which corresponds to the previous report (21).



R_1 = t-BOC or Z

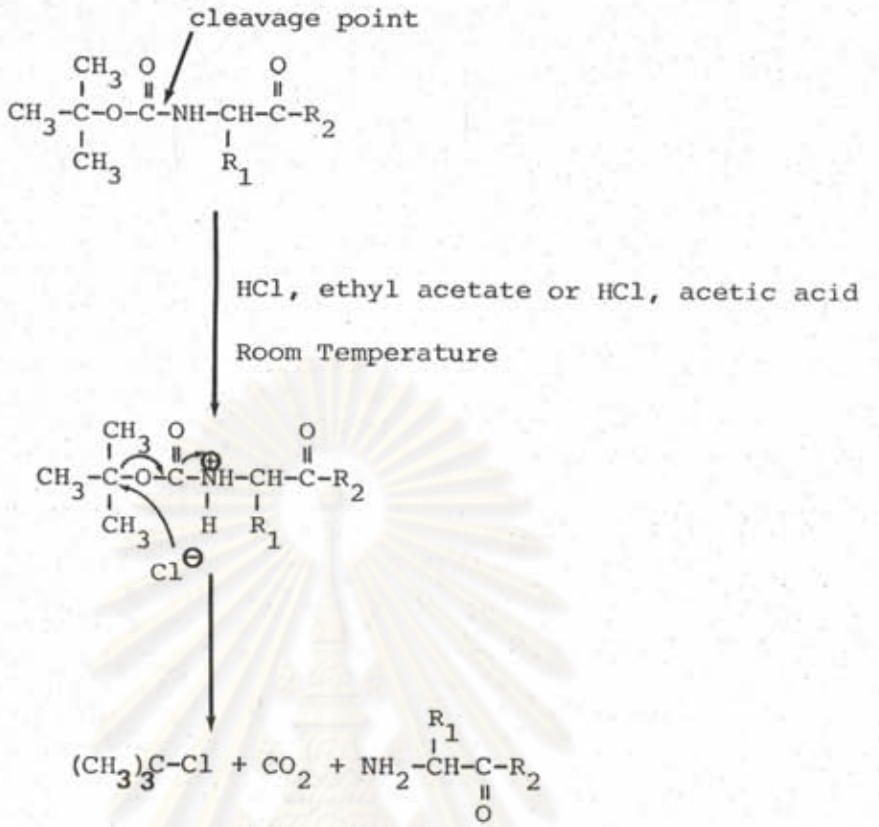
R_2 = amino acid side chain of valine ($CH(CH_3)_2$) or alanine (CH_3)

R' = extended peptide chain

Scheme 3.1 An extended fully protected peptide synthesis

3.1.2 Removal of protecting groups

The synthetic peptides were synthesised in the course of this research, No.9; No.11-15 that is t-butyloxy carbonyl (t-BOC) amino acids which are frequently used as intermediates in peptide synthesis and the reagents normally used for the removal of the protecting group are acidic. The t-BOC group is extremely labile by acidolysis and a saturated solution of HCl in ethyl acetate or a solution of HCl in acetic acid was used as the cleaving agent. After 3 hours of stirring in the acidic medium at room temperature, the t-BOC group was successfully removed. The mechanism for removal of t-BOC amino acids are presented in scheme 3.2



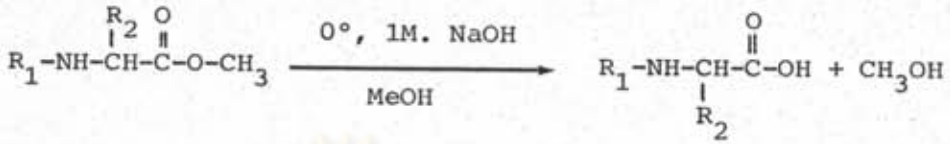
R_1 = amino acid side chain of valine ($CH(CH_3)_2$) or alanine (CH_3)

R_2 = extended peptide chain

Scheme 3.2 Removal of t-BOC protecting group

The conditions for the deprotection of the amino acid depend upon the particular derivative, for instance, a longer reaction time is required for the removal of the t-BOC amino acid. It was found that the best time was 3 hours. The deprotection was detected by the disappearance of the t-butyl proton peak at δ1.44-1.5 in the proton NMR spectrum. Product were obtained in 80% yields and were used in the next step without purification.

Removal of the methyl group from the carboxyl-terminal end of the peptide chain required the use of 1M.NaOH as basic hydrolyte agent.



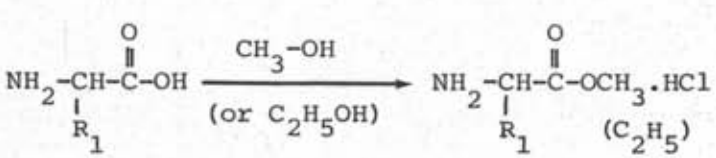
R_1 = t-BOC or Z

R_2 = amino acid side chain of valine or alanine

Scheme 3.3 Removal of methyl protecting group

After one hour stirring in an alkaline solution of methanol at 0°C, the hydrolysis of methyl ester to a free carboxyl group was achieved in 65% yield. This result was confirmed by proton NMR with the loss of the $-OCH_3$ proton peak at δ 3.7.

The carboxyl end was protected by esterification (29). Methyl and ethyl esters were synthesised by bubbling dry hydrochloric acid gas through a suspension of the amino acid in cold dry MeOH (or EtOH). The amino acid esters were produced as their hygroscopic hydrochloride salts and were stored at below 0°C. The proton NMR confirmed the peak of hydrochloride salt of the amino acid esters by the appearance of a peak at δ6.8.



In the first step of the synthesis of compound 15 (valine methyl ester HCl), valine methyl ester was introduced into the peptide by the mixed anhydride method described by Vinogrodova(30). Valine methyl ester was used as its free base in synthesis according to the method of Anderson and Zimmerman(22). In the synthesis requiring the valine methyl ester, water was used as its solvent, according to the method of Vinogrodova(31), as the solubility of this compound in organic solvents was negligible.

3.2 Structural Characterisation of the synthetic peptides

Each of the synthetic peptides was purified by repeated recrystallisation from a mixed solvent of ethyl acetate and hexane. Purity was confirmed by both TLC, which was shown a single regular round spot without tailing when exposed to UV radiation, and correct elemental analysis.

The characterisation of reaction products is always difficults, however, it was successfully accomplished by the assistance of the modern instruments such as elemental analyzer, IR, both ^{1}H NMR and ^{13}C NMR.

IR spectra clearly exhibited the characteristic of an amide by showing all the peaks for amide I-VI band, and secondary amine group. Other assignments for the IR absorption peaks were described in Table 2.2 to Table 2.5. The most significant indication for the presence of a carbonyl group was clearly indicated by both IR spectra at around 1660 cm^{-1} and ^{13}C NMR spectra at around 173.17-175.98 ppm. The ^{13}C NMR revealed the number of methylene groups in the molecule. The number of carbon atoms in the molecule was confirmed by the appearance of the appropriate number of peaks in the ^{13}C NMR spectra. (Fig.2.17 to Fig.2.24 and Table 2.10 to Table 2.13)

Compound 9,11,12 and 13; the most significant indication for the presence of t-butyl protons was clearly indicated by both ^{1}H NMR spectra at 1.44 ppm. and IR spectra was shown the tertiary butyl group, a strong doublet peak at around 1395-1385 cm^{-1} and 1370-1365 cm^{-1}.

Compound 14 and 15; the presence of t-butyl protons were clearly indicated by both ^{1}H NMR spectra at 1.44 ppm. and IR spectra was shown the isopropyl groups give rise to a strong doublet peak, with peaks of almost equal intensity at around 1357-1360 cm^{-1} and 1378-1380 cm^{-1} because of the overlap of the methyl groups involved in this structure.

Compound 16 and 17 ; the most significant indication for the presence of carbonyl group was shown by the ^{13}C NMR. The compound 16 and 17 showed the absorption peak of a carbonyl carbon atom at the range of 175.87-172.85 ppm. In addition there was always a pair of absorption peak in the range of 175.87-172.85 ppm. in the ^{13}C NMR spectra. They were assigned for $-CH_2-\overset{O}{\overset{\|}{C}}-OH$ at the lower field and $-CH_2-\overset{O}{\overset{\|}{C}}-NH$ at the higher field, according to their electronegativity. Other assignments for the ^{13}C NMR as well as for the ^{1}H NMR were set in Table 2.18, 2.19, 2.12 and 2.13 respectively.

3.3 Mechanism of substrate hydrolysis

Peptide and synthetic ester substrates are hydrolysed by the serine proteases by the acyl enzyme mechanism. The mechanism for the trypsin, chymotrypsin-catalysed hydrolysis of ester, which involves acetylation of hydroxyl group of serine 195(32) has been proposed by Hartley and Kilby(33). The mechanism in Fig. 3.1 show that in the trypsin and chymotrypsin-catalysed hydrolysis of BAN, steady state liberation of p-nitrophenol resulting from hydrolysis of the acyl enzyme.

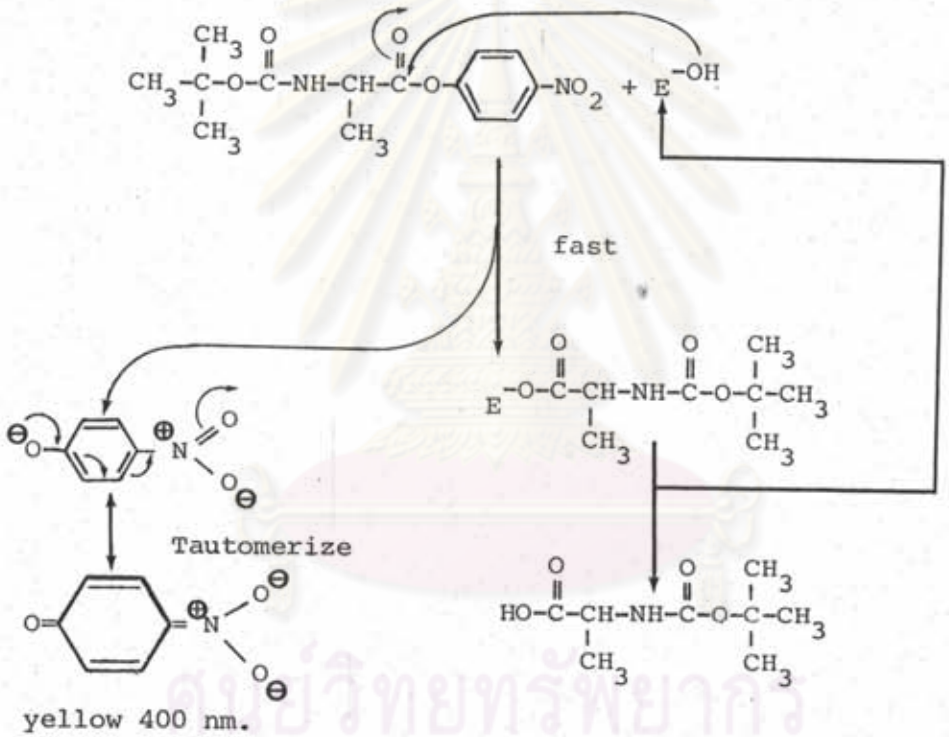


Fig. 3.1 Mechanism of substrate hydrolysis

In this research work was found that BAN, a non-specific substrate was also hydrolysed by trypsin and chymotrypsin. The two-intermediate mechanism (eqn.3.1) has been well established for trypsin and chymotrypsin-catalysed hydrolysis of non-specific ester substrates under optimum conditions.

$$E + S \underset{}{\overset{k_s}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' + P_1 \xrightarrow{k_3} E + P_2 \quad (3.1)$$

where,

ES = reversible enzyme-substrate complex

k_s = the enzyme-substrate dissociation constant

ES'= the acyl-enzyme intermediate

P_1 = the leaving group of substrate

P_2 = the carboxylic acid

k_2,k_3 = rate constant of P_1 and P_2 respectively

The acyl-enzyme intermediate has been characterised as an ester of the substrate carboxyl and the hydroxyl of serine residue in trypsin and chymotrypsin. In addition to the similarities in mechanism of action, trypsin also has several structural features of its active site which are very similar to those of chymotrypsin. The polypeptide chains form at the surface of the enzyme molecule a conformationally fixed grouping of amino acids acts the catalytic site for substrate hydrolysis. Other grouping act as the binding sites for fixation of substrates or competitive inhibitors.

3.4 The optimum condition for the enzyme kinetics

Trypsin and chymotrypsin activity increased with the enzyme concentration, showing a first order relationship as shown in Fig.2.25. For both enzyme, the limiting value of 500 µl of 0.2 mg./ml. trypsin and 0.1 mg./ml. chymotrypsin, there ceased to be a linear relationship between the enzyme added and intial velocity, while the maximum and constant velocities were obtained above 30 µl. of substrate solution. The initial substrate concentration in the manner was shown in Fig.2.26. The first part of the curve represents conditions where the limiting factor is the substrate concentration. The second part of the curve (the plateau) indicates that the reaction velocity no longer increases with continuing increase in the concentration of the substrate, thus the amount of enzyme is the limiting factor. At low concentration of substrate the reaction shows first-order kineties with respect to the substrate, but later becomes zero-order when the reaction velocity becomes independent of the concentration of the substrate.

The dependence of trypsin and chymotrypsin activity on the pH of the reaction was studied and comparison of the results are shown in Fig.2.27. The pH optima of trypsin and chymotrypsin reaction were found to be around 7.9 and 7.5 respectively. The pH-activity curve for the action of chymotrypsin on BAN occurs at a higher pH than trypsin, indeed, the whole curve is shifted toward a more alkaline region. For example, at pH 7.5 where the rate of hydrolysis of chymotrypsin is 0.196 Abs./min. of the maximal rate, the rate of hydrolysis of trypsin is only 0.098 Abs./min. of the maximum.

If any one of the reactants and the products of an enzymatic reaction absorbs any wavelength in the regions of ultraviolet, visible or infrared spectra, then it is possible to monitor the progress of such as enzymatic reaction spectrophotometrically. In studying enzyme reaction spectrophotometrically, it is essential that the temperature of the cell must be controlled with in ± 0.4°C. The optimum temperature of trypsin and chymotrypsin was 37°C as presented in Fig.2.28. Howerer, the curve begins to fall off at high temperature and eventually the enzyme loses all activity as the protein becomes denatured by heat.

3.5 Tetradecanoyl and dodecanoyl peptide esters

In Table 3.1 is given inhibition results on HLE, PPE, trypsin and chymotrypsin for tetradecanoyl and dodecanoyl peptide esters. These inhibitors tested show that the divaline derivatives are by far the best inhibitors of HLE with high specificity. By introduction of an alanine residue at either P_1 or P_2 in place of a valine residue, the specificity of the inhibitor is removed and a reduction in the activity toward HLE and chymotrypsin is also observed. This indicates a preference for two valine residues attached to a tetradecanoyl hydrocarbon chain. By comparing compound 4 and 7, on reduction of a two carbon fragement from the alkyl chain of compound 4, show that the specificity of the inhibitor is removed, and an overall reduction in inhibitory activity towards HLE and chymotrypsin also occurs. This indicates that the length of the inhibitor is important to the inhibitory activity of the compound.

The series of these inhibitors in Table 3.1 show a decrease in the activity for HLE and chymotrypsin when an alanine residue is introduced at P_1 or P_2. This indicates not only that the type of amino acid present in the peptide sequence is important, but also that the position at which the amino acid is situated along the length of the inhibitors is a factor which determines both specificity and activity towards the four enzyme. This points out delicate differences between the active sites of HLE, PPE, trypsin and chymotrypsin. It is also interesting to note none of the tetradecanoyl peptide esters, are as good inhibitors of HLE (23) as the very specific tetradecanoyl divaline inhibitor, and not specific for chymotrypsin. The increase in chain length associated with the introduction of an

extra amino acid, and the effect that an extra alkyl side chain has on the overall structural make-up of the inhibitor, may play important roles.

Table 3.1 Inhibition by tetradecanoyl and dodecanoyl peptide ester

No.	Inhibitor P_4 P_3 P_2 P_1 P_1'	concentration ($X10^{-5}$M.)	% inhibition HLE[a]	PPE[a]	chymo-trypsin[b]	trypsin[b]
3	C_{14}- A - V - OMe	5	33	30	14.18	0
4	C_{14}- V - V - OMe	5	56	0	52.41	0
5	C_{14}- V - A - OEt	5	43	44	16.99	0
7	C_{12}- V - V - OMe	5	50	48	13.20	23.03
6	C_{14}- V - V - A - OEt	5	46	21	10.28	0
10	C_{14}- V - V - V - OMe	5	67	62	22.58	11.02

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

(b) These values represent the mean of four experiments

3.6 N-Protected amino acids and peptides

Table 3.2 lists inhibition results of dodecyl amino acids and peptides protected at the N-terminal end by a carbobenzoxy, t-butoxy carbonyl or succinyl group. Comparison of compounds 1, 14, 17 which differ only in their protecting groups at the P_2 position, shows a switch in specificity from HLE, PPE, trypsin and chymotrypsin. Compound 1 is specific to HLE, PPE and chymotrypsin,compound 14 is specific to HLE, and compound 17 is specific to PPE, trypsin and chymotrypsin. The structural changes at the P_2 position of the inhibitor was recognised by the four enzymes, thus pointing out differences between HLE, PPE and trypsin, chymotrypsin in their perferences to interact with different groups in their active sites.

HLE preferred the t-BOC group at P_2 while PPE preferred the Z-group (23) and trypsin, chymotrypsin preferred the succinyl group. It is interesting to note also, that the t-BOC group closely resembles the alkyl side chain of valine and the Z-group resembles the side chain of phenylalamine and succinyl group resembles the side chain of serine.

By comparision of compound 9 and 14 which differ only at the P_1 position, by having different amino acid residues, further differences between the four enzymes can be seen. The two inhibitors give some information about the P_1 position of the inhibitor by varying either a valine or alanine residue. The results show that HLE preferred a valine residue at P_1 since the inhibitor's activity toward HLE has markedly increased with compound 14 while the activity toward PPE, trypsin and chymotrypsin remained similar. This seems to fit the observation that HLE prefers valine residues to alanine, much more than

does chymotrypsin and PPE, trypsin is uneffected.

Observing compounds 11, 12, 9 and 13 which differ only at P'_1 position by having different the alkyl side chain, further differences in the role in the inhibitors differences in activities can be seen. Percentage of inhibition of trypsin and chymotrypsin would decrease as the effect of an extra alkyl side chain. The results set out in Table 3.2, show that the compounds 9, 11, 12 inhibit HLE, whereas the other three enzymes are uneffected, showing that these compounds are specific to HLE and non-specific to trypsin and chymotrypsin while the activity of compound 14 has remained similar. But for compound 13, the result seems to show no inhibition at a concentration of 0.10 mM. are reported as inactive for four enzymes. This indicates that the carbon chain length of the inhibitor is important to the inhibitory activity if does not consider the inhibitory effect of compound 13. Therefore, the preference for valine amino acids along the length of the inhibitor by HLE is evident while PPE, trypsin and chymotrypsin lack such a specific preference for valine.

Comparing of compounds 16 and 17 clearly show the effect of the P_1 position of the inhibitor, varying in the P_1 position by having either a valine or alanine residue. By introduction of an alanine residue in place of a valine residue, the specificity of the inhibitor is removed. The compounds 16 and 17 inhibit trypsin and chymotrypsin whilst these two compounds have on effect on HLE and PPE.

Table 3.2 gives some indication of the effect of the protecting group in the activity of these compound toward HLE and PPE. Comparing of compounds 8 and 9 clearly show the effect of t-BOC group at

P_2 position. On removal of this protecting group, the inhibitory activity towards HLE and chymotrypsin is reduced while that for PPE has slightly increased and for trypsin has no inhibition at a concentration of 0.05 mM. are reported as inactive.

Table 3.2 Inhibition by N-Protected amino acids and peptides

No.	Inhibitor P_3 P_2 P_1 P_1'	concentration (mM.)	% Inhibition			
			HLE[a]	PPE[a]	chymo trypsin[b]	trypsin[b]
1	Z - V - NH - C_{12}	0.05	37	14	15.99	0
2	Z - V - V - NH - C_{12}	0.05	62	20	22.09	0
8	NH_2 - A - NH - C_{12}	0.05	15	28	0	0
11	t-BOC- A - NH - C_8	0.10	84	10	37.40	20.14
12	t-BOC- A - NH - C_{10}	0.10	96	0	35.34	12.82
9	t-BOC- A - NH - C_{12}	0.05	40	0	3.12	0
13	t-BOC- A - NH - C_{14}	0.10	1	2	2.68	0
14	t-BOC- V - NH - C_{12}	0.05	64	0	7.44	0
15	t-BOC - V - V - NH - C_{12}	0.05	100	0	13.92	5.40
16	Suc- A - NH - C_{12}	0.10	0	4	39.41	17.42
17	Suc- V - NH - C_{12}	0.10	0	3	66.23	50.61

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

(b) These values represent the mean of four experiments

3.7 The type of inhibition

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 result measured for velocity with varying substrate concentration at three different inhibitor concentration on trypsin and chymotrypsin are shown in Table 2.31 to Table 2.64 and graphical schemes were plotted as shown in Fig. 2.29 to Fig. 2.62 respectively. 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 1-17 and the values of inhibitor constants are summarized in Table 3.3 and Table 3.4 on trypsin and chymotrypsin respectively. (The values of K_i and K_I are useful when comparing strengths of binding of inhibitors to the enzyme and to the ES complex respectively. (Appendix II)) The K_m values for BAN by trypsin and chymotrypsin were 22.1 μM. and 18.1 μM. respectively.

The type of inhibition of all the synthetic inhibitors were determined. Compounds 1 and 2 are un-competitive types, compounds 3-8, 10 and 16-17 are non-competitive types, and compounds 9 and 11-15 are competitive types. The studies carried out on proteinase inhibitors by Dorn et. al. (34) also reported, indicated non-competitive inhibition with peptide sequences of up to five amino acids. The inhibitors developed in this research contained an alkyl chain attached to short peptides, it was considering to find that both alkyl chain and peptides were also reported to inhibit in the same mode.

Table 3.3 The type of inhibition and the inhibitor constants of tetradecanoyl and decanoyl peptides ester

No.	Inhibitor	Enzyme	type	Inhibitor constant	
				K_I (μM.)	K_i (μM.)
3	C_{14}- A - V - OMe	trypsin	non-competitive	72.10	109.27
		chymotrypsin		64.73	89.78
4	C_{14}- V - V - OMe	trypsin	non-competitive	84.58	195.05
		chymotrypsin		2.31	11.05
5	C_{14}- V - A - OEt	trypsin	non-competitive	65.18	99.41
		chymotrypsin		62.92	84.78
7	C_{12}- V - V - OMe	trypsin	non-competitive	8.69	14.95
		chymotrypsin		75.91	107.60
6	C_{14}- V - V - A - OEt	trypsin	non-competitive	134.28	185.06
		chymotrypsin		99.59	179.06
10	C_{14}- V - V - V - OMe	trypsin	non-competitive	28.92	28.92
		chymotrypsin		13.28	26.99

Table 3.4 The type of inhibition and the inhibitor constants of N-protected amino acids and peptides

No.	Inhibitor	Enzyme	Type	Inhibitor constant	
				K_I (μM.)	K_i (μM.)
1	Z - V - NH - C_{12}	trypsin chymotrypsin	un-competitive	226.46 198.04	
2	Z - V - V - NH - C_{12}	trypsin chymotrypsin	un-competitive	143.69 95.68	
8	NH_2- A - NH - C_{12}	trypsin chymotrypsin	non-competitive	n.d. (a) n.d. (a)	
11	t-BOC- A - NH - C_8	trypsin chymotrypsin	competitive		71.29 23.76
12	t-BOC- A - NH - C_{10}	trypsin chymotrypsin	competitive		112.85 42.11
9	t-BOC- A - NH - C_{12}	trypsin chymotrypsin	competitive		205.97 123.98
13	t-BOC- A - NH - C_{14}	trypsin chymotrypsin	competitive		345.56 127.82
14	t-BOC- V - NH - C_{12}	trypsin chymotrypsin	competitive		153.26 59.71
15	t-BOC- V - V - NH - C_{12}	trypsin chymotrypsin	competitive		150.59 45.17
16	Suc - A - NH - C_{12}	trypsin chymotrypsin	non-competitive	39.58 25.97	82.34 14.39
17	Suc - V - NH - C_{12}	trypsin chymotrypsin	non-competitive	5.24 7.97	9.65 8.13

(a) n.d. = not determined