

- I. INHIBITION OF ACID PROTEASES BY PEPTIDE
DIAZOMETHYL KETONES
- II. INVESTIGATION OF SERINE PROTEASE ACTIVITY
WITH ACYL CARBAZATES

A THESIS

Presented to

the Faculty of the Division of Graduate Studies

by

Bernard Franklin Gupton


In Partial Fulfillment
of the Requirements for the Degree
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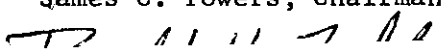
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TABLE OF CONTENTS

	Page
ACKNOWLEDGMENTS.	ii
LIST OF TABLES	v
LIST OF ILLUSTRATIONS.	vi
SUMMARY.	vii

PART I

INHIBITION OF ACID PROTEASES BY PEPTIDE DIAZOMETHYL KETONES

Chapter

I. INTRODUCTION.	2
II. METHODS	12
III. RESULTS	19
IV. DISCUSSION.	28
V. CONCLUSION.	31
VI. RECOMMENDATIONS	32
APPENDIX - ACID PROTEASE ASSAY PROCEDURE	33
BIBLIOGRAPHY	34

PART II

INVESTIGATION OF SERINE PROTEASE ACTIVITY WITH ACYL CARBAZATES

Chapter

I. INTRODUCTION.	38
II. METHODS	46

TABLE OF CONTENTS (Concluded)

PART II (Continued)

Chapter	Page
III. RESULTS	50
IV. DISCUSSION.	60
V. CONCLUSIONS	66
VI. RECOMMENDATIONS	67
APPENDIX - CHYMOTRYPSIN ASSAY PROCEDURE.	68
BIBLIOGRAPHY	69

LIST OF TABLES

PART I

INHIBITION OF ACID PROTEASES BY PEPTIDE DIAZOMETHYL KETONES

Table		Page
1.	Inhibition of Pepsin with Peptide Diazomethyl Ketones	21
2.	Inhibition of <u>Rhizopus chinensis</u> Protease with Diazomethyl Ketones	23
3.	Concentration-Dependent Inhibition of Pepsin with Peptide Diazomethyl Ketones.	26

PART II

INVESTIGATION OF SERINE PROTEASE ACTIVITY WITH ACYL CARBAZATES

Table		Page
1.	Reaction of Acyl Carbazates with Chymotrypsin A _α	51
2.	Reaction of Acyl Carbazates with Subtilisin BPN'.	53
3.	Reaction of Acyl Carbazates with Subtilisin Carlsburg	55

LIST OF ILLUSTRATIONS

PART I

INHIBITION OF ACID PROTEASES BY PEPTIDE DIAZOMETHYL KETONES

Figure		Page
1.	Enzyme-catalyzed Reactions Involving Covalent Acyl Intermediate	5
2.	Enzyme-catalyzed Reactions Involving Amino Intermediate.	7
3.	Alternative Pathways for Enzyme-catalyzed Reactions	9
4.	Structures of Peptide Carbazates.	11
5.	The Synthetic Preparation of Tos-PheCHN ₂ and Ips-PheCHN ₂	18

PART II

INVESTIGATION OF SERINE PROTEASE ACTIVITY WITH ACYL CARBAZATES

Figure		Page
1.	A Diagram of the Role of the Charge Transfer System for Acylation and Deacylation of Chymotrypsin.	40
2.	Three Dimensional Drawing of the Active Site of Monocarbamyl Chymotrypsin A _α	42
3.	Structures of Peptide Carbazates.	43

SUMMARY

PART I

Two peptide diazomethyl ketones, Tos-PheCHN₂ and its heavy atom analog Ips-PheCHN₂, were prepared for use as inhibitors of pepsin and Rhizopus chinensis acid protease in kinetic studies and future crystallographic investigations. Both compounds were shown to be extremely good active-site-directed irreversible inhibitors of the two enzymes. The relative $k_{\text{obsd}}/[I]$ values were found to be very similar indicating that the replacement of the iodine atom for the methyl substituent had little effect upon the inhibitory activity of the compounds. However, in concentration-dependent studies the relative k_3/k_1 values were unobtainable due to the velocity of the inhibition reaction.

PART II

A series of five acyl carbazates was tested for reactivity towards chymotrypsin A, subtilisin BPN', and subtilisin Carlsburg. The five compounds Ac-Bzc-ONp, Ac-Ala-Bzc-ONp, Ac-Ala-Mec-ONp, Ac-Ala-Ala-Mec-ONp, and Ac-Ala-Ala-Pro-Mec-ONp (Bzc = NH-N(CH₂C₆H₅)CO; Mec = NH-N(CH₃)CO) were shown to react with the three enzymes at substantially different rates. Chymotrypsin was rapidly inactivated by the two benzyl carbazates and reacted with Ac-Ala-Ala-Pro-Mec-ONp somewhat more slowly. The two subtilisins reacted with all five compounds and were rapidly inactivated by the two benzyl carbazates. However, the three methyl carbazates had

very fast deacylation rates. This rapid turnover may be a result of a loose fit of the methyl group in the binding pocket of the subtilisins since these enzymes are known to have a broader specificity for large hydrophobic side chains. In all cases where a rate could be measured, subtilisin Carlsburg was more reactive towards the carbazates than subtilisin BPN'. The burst of p-nitrophenol obtained upon reaction of chymotrypsin or the subtilisins was always essentially stoichiometric.

I. INHIBITION OF ACID PROTEASES BY PEPTIDE
DIAZOMETHYL KETONES

CHAPTER I

INTRODUCTION

Enzymes which catalyze protein hydrolysis, referred to as proteolytic enzymes, are among the most widely studied enzymes in the field of biochemistry to date. These enzymes are involved in such physiological processes as food digestion, zymogen activation, ovum fertilization, blood coagulation, and tissue inflammation. The utility of proteolytic enzymes ranges from their use in industrial processes involved with the degradation of protein raw materials to pharmaceutical studies involved with the investigation of diseases which facilitate tissue destruction.

Acid proteases are a particular type of proteolytic enzyme which are characterized by possessing an acidic pH optimum. Some of the types of enzymes which belong to this class are the gastric proteases of pepsin as well as the microbial acid proteases such as penicillopepsin, Rhizopus chinensis acid protease and the milk clotting enzyme, chymosin. A considerable amount of information has been obtained about these enzymes with respect to amino acid sequences, primary substrate specificities and overall reaction mechanism. Although much of the overall catalytic mechanism is still in question, it is thought that these enzymes appear to possess similar catalytic centers, since they are inhibited selectively by active-site-directed reagents such as diazoacetyl-L-phenylalanine ethylester and p-toluenesulfonyl-L-phenylalanyl diazomethane (Delpierre and Fruton, 1966) or diazoacetyl-DL-norleucine methyl ester (Rajagopalan, et al., 1966).

It was found that, in the case of gastric pepsin (Bayliss, et al., 1969) and penicillopepsin (Sodek and Hofmann, 1970), the site of attack by such diazo compounds is the β -carboxyl group of an aspartic acid residue in homologous sequences (pepsin: Ile-Val-Asp-Thr-Gly-Thr-Ser; penicillopepsin: Ile-Ala-Asp-Thr-Gly-Thr-Leu). Upon completion of the amino acid sequence of porcine pepsin, the aspartyl residue sensitive to the diazo compounds was identified as Asp-215 (Tang, et al., 1973). It was also observed that substrate-like epoxides selectively inhibited pepsin (Tang, 1972). However, it was shown that the aspartyl group which was modified was Asp-32 (Hartsuck and Tang, 1973).

Another function of acid proteases is their ability to catalyze transpeptidation reactions under certain conditions. This property has aided in the understanding of the catalytic mechanism for enzymatic hydrolysis. A number of mechanisms have been proposed for the action of porcine pepsin, with many having the central feature of a covalent amino intermediate (Clement, 1973). The evidence for the amino intermediate comes from transpeptidation reactions catalyzed by pepsin involving the transfer of the C-terminal amino acid from a small substrate. Neuman, et al. (1959) and Fruton, et al. (1961) showed that these transpeptidation reactions required a covalently bound intermediate. Furthermore, studies indicated that in hydrolytic reactions there is a nonrandom release of products in an order that is consistent with the postulate of an amino enzyme (Kitson and Knowles, 1971). It has been pointed out, however, that the same observations would be expected for an acyl intermediate in these pepsin catalyzed reactions. This proposal is supported by the observation that pepsin catalyzes the exchange of O^{18} between a substrate

and water (Sharon, et al., 1962). It was shown that an O^{18} exchange catalyzed by pepsin also occurs in the absence of substrate (Shkarenkova, et al., 1968). This observation consequently led to the proposal of a mechanism which accounted for the O^{18} exchange without involving a covalent acyl intermediate. More recently Takahashi and Hofmann (1975) showed that transpeptidation reactions involving N-terminal amino acids with penicillopepsin exhibited specific binding requirements for catalysis. The major products obtained when Phe-Tyr-Thr-Pro-Lys-Ala and Met-Leu-Gly were used as substrates were Phe-Phe and Met-Met, respectively. When Met-Leu-Gly was employed as the substrate, the tetrapeptide Met-Met-Leu-Gly was observed as a probable intermediate. The dipeptides Leu-Phe, Phe-Leu, Leu-Leu, and Phe-Phe were all observed as products when Leu-Tyr-Leu and Phe-Tyr-Thr-Pro-Lys-Ala were co-incubated with the enzyme. These results suggested that two amino acids with large hydrophobic side chains are required in order for transpeptidation to occur. The addition of nucleophilic compounds other than peptides (hydroxyl amine, dinitrophenylhydrazine and aliphatic alcohols) to the reaction mixture did not result in attack on an enzyme acyl intermediate.

On the basis of these observations several mechanisms have been proposed most of which involve an acyl intermediate (Bender and Kezdy, 1965), amino intermediate (Knowles, 1970), or both (Takahashi and Hofmann, 1975). The major features of the mechanism involving the acyl intermediate are shown in Figure 1. The initial binding to form the enzyme substrate complex is believed to occur through the hydrophobic side chains in the S_1 and S_1' subsites (Berger and Schechter, 1970). The acyl intermediate is subsequently formed resulting from nucleophilic attack on the

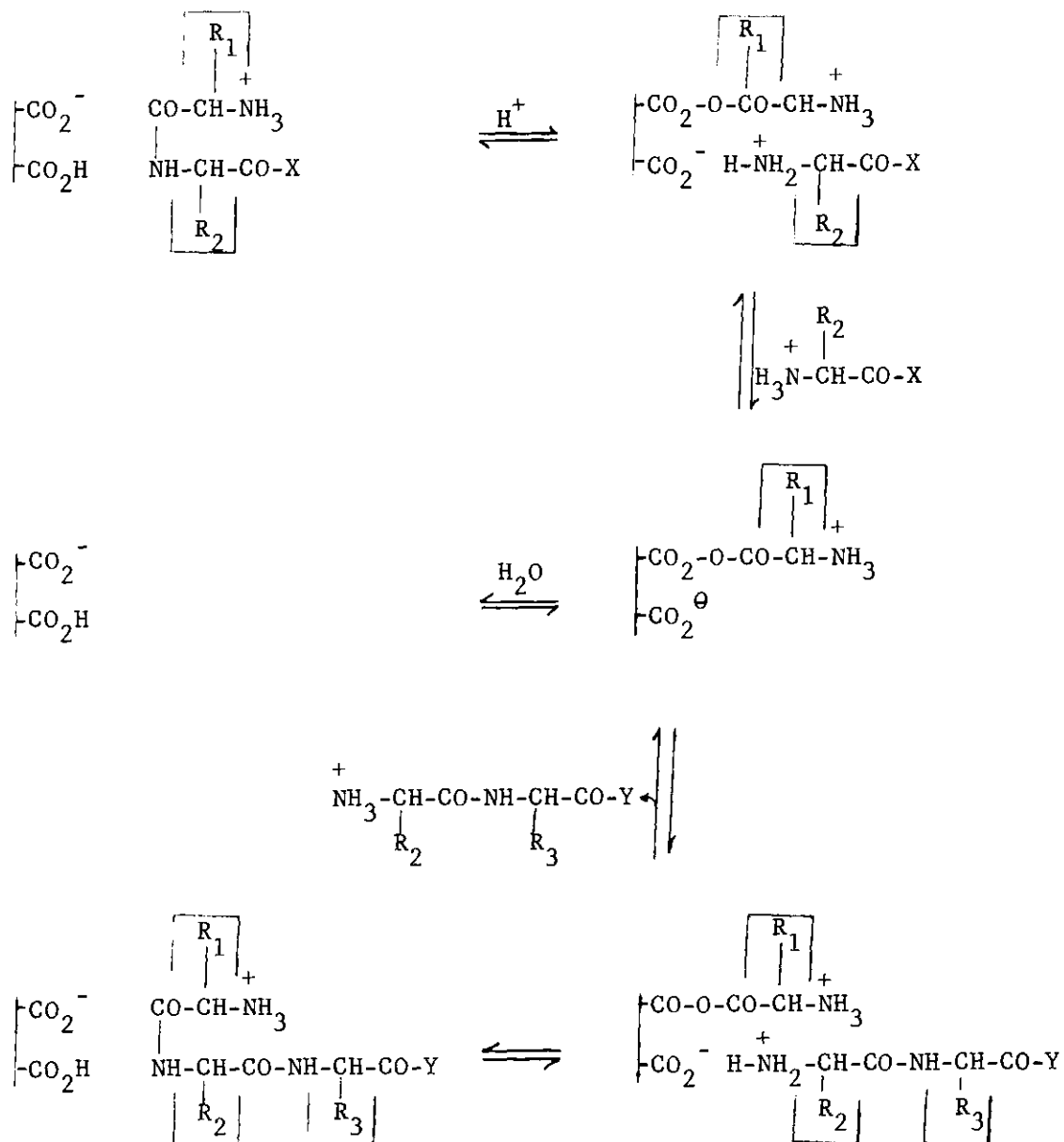


Figure 1. Enzyme-catalyzed Reactions Involving Covalent Acyl Intermediate (Extended peptide chains are represented by x and y.)

carbonyl carbon by one of the active site aspartic acid residues and leaving the amino moiety of the peptide to form an ion pair with the other active site aspartic acid. The acyl intermediate is believed to be stabilized by the hydrophobic environment and the ion pair which is formed. At this point the product at the S_1' subsite may be released allowing for the binding of a new molecule of substrate at the S_1' and S_2' subsites and reacting with the free carboxylate group on the enzyme (Wang, et al., 1974). The partial withdrawal of the proton on the amino group by the active site aspartic acid may aid in the nucleophilic attack on the anhydride to yield the transpeptidation product and the regenerated active site.

The proposed mechanism for catalysis involving an amino intermediate is summarized in Figure 2 (Knowles, 1970). The initial binding of the substrate is subsequently followed by nucleophilic attack of one aspartic acid residue on the carbonyl carbon (II). This step is aided by the second aspartic acid residue acting as a proton donor. This leads to a rearrangement to yield the amino enzyme intermediate. The release of the carboxylate group may lead to either hydrolysis of the amino enzyme intermediate or the binding of an acyl amino acid acceptor specifically which would result in transpeptidation and the synthesis of a new peptide.

It has recently been postulated (Takahashi and Hofmann, 1975) that not all pepsin- and penicillopepsin-catalyzed reactions proceed via the same mechanism. It was further suggested that the course of the reaction would be determined by the nature of the substrate and thus would dictate the type of intermediate formed. The proposed pathways are sum-

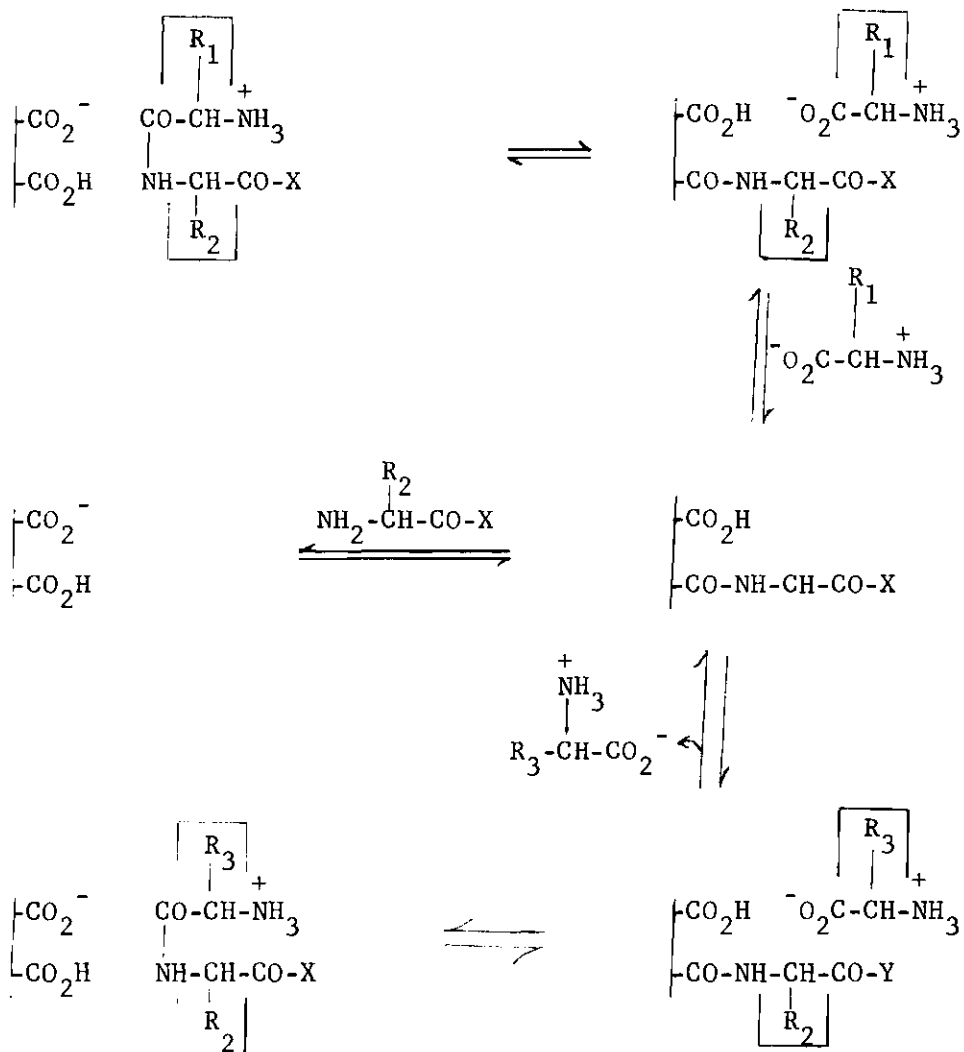


Figure 2. Enzyme-catalyzed Reactions Involving Covalent Amino Intermediate (Extended peptide chains are represented by x and y.)

marized in Figure 3. The first step again would involve the formation of an enzyme-substrate complex. The complex may then follow one of three pathways. One of the possible routes would lead to the formation of an acyl intermediate which would then react with an acceptor or undergo hydrolysis. A second possible route would be that the substrate would undergo hydrolysis without the formation of a covalent intermediate. This possibility has been suggested previously by Silver and Stodard (1972). The third possible route of catalysis would involve the formation of an amino intermediate from the enzyme-substrate complex. The amino intermediate may then undergo hydrolysis or react with an acceptor to yield the transpeptidation product.

It is apparent from the above discussion that several questions remain to be resolved with respect to the catalytic mechanism for transpeptidation reactions and hydrolysis of peptide substrates with acid proteases. In hopes of obtaining a better understanding of the mode of action for acid proteases, an investigation into the irreversible inhibition of a homologous enzyme, Rhizopus chinensis acid protease (Hofmann, et al., 1972) was undertaken with the purpose of obtaining "heavy atom" derivatives of the enzyme for future crystallographic study. Since it had been shown previously that peptide analogs of diazo ketones irreversibly inhibited the Rhizopus acid protease selectively (Tsuru, et al., 1973) it appeared that this class of compounds would be good candidates for our studies. Inhibition studies of pepsin employing Tos-PheCHN₂ showed this particular inhibition to be extremely potent as well as selective (Del-pierre and Fruton, 1965). It appeared that the isomorphous replacement of the methyl substituent on the tosyl group with an iodine atom would

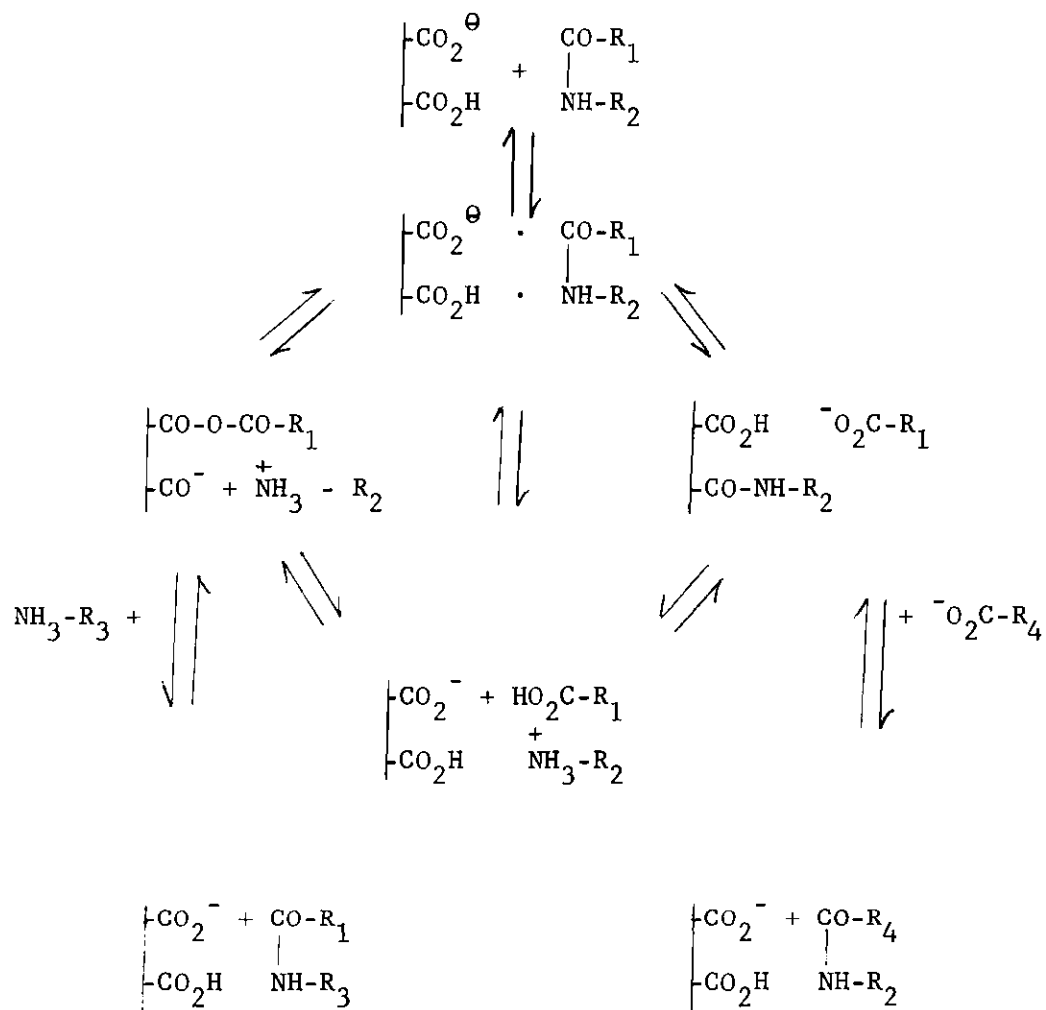


Figure 3. Alternative Pathways for Enzyme-catalyzed Reactions

serve as a suitable "heavy atom" derivative of the inhibitor (Figure 4). This thesis, Part I, is a report on the synthesis and kinetic investigation of two peptide diazomethyl ketones as possible specific irreversible inhibitors of Rhizopus chinensis acid proteinase.

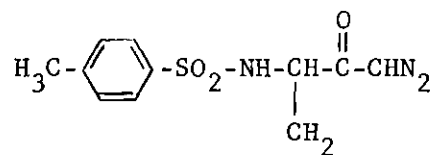
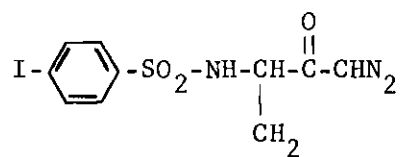
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Figure 4. Structures of Peptide Carbazates

(1 represents N-Tosyl-L-phenylalanyl diazomethyl ketone (Tos-Phe-CHN₂) while 2 represents N-p-(Iodobenzene sulfonyl)-phenylalanyl diazomethyl ketone.)

CHAPTER II

METHODS

Reagents and Materials

Procine pepsin (twice crystallized) was obtained from Worthington Biochemical Corporation (lot PM 3AU). The enzyme was further purified by dissolving 25 mg in 15 ml of 6.25 mM acetate buffer (pH 5.4) and dialyzing the solution overnight at three degrees against one liter of buffer (Delpierre and Fruton, 1966). Rhizopus chinensis protease (three times crystallized) was obtained from Seikagaku Kogyo Company (lot 8U06) and was purified as described for pepsin. The hemoglobin substrate was obtained from Worthington Biochemical Corporation. All reagents and solvents used were of analytical grade. Mass spectra were taken on a Hitachi Perkin-Elmer RMU-7L instrument. Nuclear magnetic resonance (nmr) spectra were taken on a Varian T-60 instrument. Solvents used for nmr spectra were deuteriochloroform (CDCl_3) and deuterodimethylsulfoxide (DMSO-d_6). Infra-red (ir) spectra were taken on a Perkin-Elmer 457 instrument. All ir spectra were taken in either chloroform (CHCl_3) or in a nujol mull. Inhibition experiments were carried out with the aid of a Beckman Model-25 spectrophotometer. Thin layer chromatography (tlc) was performed using Merck silica gel G plates. Column chromatography was carried out using Fisher brand florisil.

N-Tosyl-L-phenylalanine (Tos-Phe-OH)

N-tosyl-L-phenylalanine was prepared by the method of Fischer and

Lipshitz (1915) with modifications. To a solution containing 10.5 g (63.0 mmoles) of L-phenylalanine in 100 ml of 1.0 N NaOH was added 12.0 g (63.0 mmoles) of tosyl chloride in 200 ml of diethyl ether. The mixture was allowed to stir at room temperature for four hours followed by acidification to pH 2. The ether layer was removed in vacuo. The resulting emulsion was extracted with ethyl acetate and evaporated down to the oil. The oil was crystallized from methanol-water (1:8) to give 11.59 g (57.9%) of a white solid, mp 166-168° (Fischer and Lipshitz (1915) report 165°). The mass spectrum had major peaks at m/e 171 (Tos-NH₂⁺), 155 (Tos⁺), 91 (C₇H₇⁺), and 64 (SO₂). The nmr (DMSO-d₆) had major peaks at δ 8.28 (d,1H), 7.48 (m,9H), 3.98 (m,1H), 2.98 (d,2H), and 2.48 (s,3H). The infrared spectrum (nujol) had bands at 1710 cm⁻¹ (carbonyl). Anal. calcd for C₁₆H₁₇NO₄S: C, 56.89; H, 4.77. Found: C, 56.60; H, 4.65.

N-p-Iodobenzenesulfonyl-L-phenylalanine (Ips-Phe-OH)

N-p-iodobenzenesulfonyl-L-phenylalanine was prepared by a method similar to Tos-Phe-OH. The product was recrystallized from methanol-water (1:8) to give 2.29 g (53.5%) of a white solid, mp 164-165°. The mass spectrum had major peaks at m/e 283 (Ips-NH₂⁺), 267 (Ips⁺), 91 (C₇H₇⁺), and 64 (SO₂). The nmr (DMSO-d₆) had peaks at δ 8.28 (d,1H), 7.48 (m,9H), 3.9 (m,1H), and 2.98 (d,2H). The infrared spectrum (nujol) had a band at 1710 cm⁻¹ (carbonyl). Anal. calcd for C₁₅H₁₄INO₄S: C, 41.76; H, 3.25. Found: C, 41.83; H, 3.34.

N-Tosyl-L-phenylalanyl chloride (Tos-Phe-Cl)

N-tosyl-L-phenylalanyl chloride was prepared by the method of Popenoe and Vigneaud (1954) with modifications. To a solution containing 1.0 g (2.31 mmol) of the previously prepared Tos-Phe-OH in anhydrous ether

(15 ml) was added 0.85 g of PCl_5 . This was followed by the addition of 3.0 ml of anhydrous tetrahydrofuran. The mixture was allowed to stir overnight at room temperature. The acid chloride was crystallized from tetrahydrofuran-pet ether (1:5). The crystalline product was washed with 30 ml of cold water and recrystallized from tetrahydrofuran-pet ether (1:5) to give 0.81 g (78.6%) of a white solid, mp $130-131^\circ$ (Popenoe and Vigneaud (1954) report mp $128-129^\circ$), R_f 0.76 (CHCl_3 - CH_3OH , 9:1). The mass spectrum had major peaks at m/e 171 (Tos-NH_2^+), 155 (Tos^+), 91 (C_7H_7^+), 64 (SO_2), and 36 (HCl). The nmr spectrum (DMSO-d_6) had peaks at δ 8.2 (d,1H), 7.4 (m,9H), 3.9 (m,1H), 2.9 (d,2H), and 2.4 (s,3H). The infrared spectrum (nujol) had a band at 1810 cm^{-1} (carbonyl). Anal. calcd for $\text{C}_{16}\text{H}_{16}\text{ClNO}_3\text{S}$: C, 56.89; H, 4.77; Cl, 10.50. Found: C, 56.88; H, 4.78; Cl, 0.56.

N-p-Iodobenzenesulfonyl-phenylalanyl chloride (Ips-Phe-Cl)

N-p-iodobenzenesulfonyl-phenylalanyl chloride was prepared by a method similar to Tos-Phe-Cl. The product was recrystallized from tetrahydrofuran-pet ether (1:5) to give 0.80 g (76.2%) of a white solid, mp $176-177^\circ$, R_f 0.75 (CHCl_3 - CH_3OH , 9:1). The mass spectrum had major peaks at m/e 283 (Ips-NH_2^+), 267 (Ips^+), 91 (C_7H_7^+), 64 (SO_2), and 36 (HCl). The nmr (DMSO-d_6) had major peaks at δ 8.2 (d,1H), 7.4 (m,9H), 3.9 (m,1H), and 2.9 (d,2H). The infrared spectrum (nujol) had a band at 1810 cm^{-1} (carbonyl). Anal. calcd for $\text{C}_{15}\text{H}_{14}\text{ClINO}_3\text{S}$: C, 38.60; H, 3.02; Cl, 7.60. Found: C, 38.99; H, 2.95; Cl, 7.83.

N-Tosyl-L-phenylalanyl diazomethyl ketone (Tos-PheCHN₂)

N-tosyl-L-phenylalanyl diazomethyl ketone was prepared by the method of Delpierre and Fruton (1966) with modifications. To a solution

containing diazomethane (ca. 25 mmol) in 20 ml of anhydrous ether was added 1.1 g (3.25 mmol) of Tos-Phe-Cl at 0°. After the mixture had been kept at room temperature (in the dark) for 16 hours, the solvent was removed in vacuo, and the residue was recrystallized from ethyl acetate-cyclohexane (1:3). A second recrystallization yielded 0.37 g (33.0%) of a pale yellow solid, mp 110-112° (Schoellman and Shaw (1963) report 94-97° while Delpierre and Fruton (1966) report 112-114°), R_f 0.68 (CHCl₃-CH₃OH, 9:1). The mass spectrum had major peaks at m/e 185 (Tos-NH-CH₂⁺), 155 (Tos⁺), 91 (C₇H₇⁺), and 64 (SO₂). The nmr (CDCl₃) had peaks at δ 7.4 (m,9H), 5.3 (s,1H), 5.2 (d,1H), 3.9 (m,1H), 2.9 (d,2H), and 2.4 (s,3H). The infrared spectrum (nujol) had bands at 2100 cm⁻¹ (diazo group) and 1625 cm⁻¹ (carbonyl). Anal. calcd for C₁₇H₁₇N₃O₃S: C, 59.46; H, 4.99; N, 12.24. Found: C, 59.68; H, 4.98; N, 11.83.

N-p-Idobenzenesulfonyl-L-phenylalanyl diazomethyl ketone (Ips-PheCHN₂)

N-p-idobenzenesulfonyl-L-phenylalanyl diazomethyl ketone was prepared by the method of Schoellmann and Shaw (1954) with modifications. To a solution containing diazomethane (ca. 5 mmole) in 20 ml of anhydrous ether was added 0.8 g (1.78 mmole) of Ips-Phe-Cl. The mixture was allowed to stir at ice bath temperature for 1.5 hours followed by the removal of the solvent in vacuo. Work up of the crude mixture involved taking up the residue with 15 ml of ether and washing with NaHCO₃ solution, drying over anhydrous MgSO₄, and evaporation of the ether solvent. The product was crystallized from ethyl acetate-cyclohexane (3:1) to give a yellow solid (39.0%) mp 104-105°, R_f 0.68 (CHCl₃-CH₃OH, 9:1). The mass spectrum had major peaks at m/e 297 (Ips-NH-CH₂⁺), 267 (Ips⁺), 91 (C₇H₇⁺), and 64 (SO₂). The nmr (CDCl₃) had peaks at δ 7.4 (m,9H), 5.3 (s,1H), 5.2 (d,1H),

3.9 (m,1H), and 2.9 (d,2H). The ir spectrum (CHCl_3) had bands at 2100 cm^{-1} (diazo group) and 1625 cm^{-1} (carbonyl). Anal. calcd for $\text{C}_{16}\text{H}_{14}\text{IN}_3\text{O}_3\text{S}$: C, 42.21; H, 3.10; N, 9.23. Found: C, 42.35; H, 2.98; N, 8.76.

Reaction of Pepsin with Inhibitors

Inhibition of pepsin with the peptide diazomethyl ketones was carried out with at least a tenfold excess of inhibitor over enzyme and at a cupric acetate concentration of 0.01 M. Stock solutions of inhibitor in ethanol were prepared at a concentration of 3.0 mM and used within four days. Aliquots of the stock solutions were diluted to 0.3-0.6 mM with ethanol. A stock solution of pepsin was prepared in a 6.25 mM buffer (pH 5.4) and stored at 4° and had a concentration of 0.11 mg/ml by uv absorbance. The inhibition reactions were carried out at 23° and were started by mixing 2.0 ml of the stock enzyme solution with 250 μl of the inhibitor (0.3-0.6 mM). The final concentrations were as follows: inhibitor 0.03 mM or a graded series (concentration-dependent studies); enzyme, 2.5 μM ; cupric acetate, 0.001 M cupric acetate; ethanol 10% (v/v). In the concentration dependent studies, the inhibitor concentration varied over a range of 0.3 to 0.6 mM. Six or seven aliquots (100 μl) were removed from the inhibition mixture at given time intervals and measured for residual pepsin activity by means of the hemoglobin assay. Control experiments which were identical with the inhibition runs but excluded inhibitor indicated that less than 2% decrease in enzymatic activity occurred over a three hour period. For each inhibition reaction the kinetic parameters k_{obsd} and $k_{2\text{nd}} = k_{\text{obsd}}/[\text{I}]$ were obtained from the equation

$$V = k_{\text{obsd}}[\text{E}] = k_{2\text{nd}}[\text{I}][\text{E}]$$

by the use of a least-squares computer program. Correlation coefficients of 0.992 or better were obtained.

Reaction of *Rhizopus chinensis* with Inhibitors

Inhibition experiments of *Rhizopus chinensis* protease with the peptide diazomethyl ketones were carried out under essentially the same conditions as those used for the inhibition of pepsin. Stock solutions of enzyme, inhibitor, and cupric acetate were prepared as previously described. The final concentrations were as follows: enzyme, 2.2 μM (by uv); inhibitor, 0.03 mM; cupric acetate, 0.001 M; and ethanol, 10% (v/v). Measurement of the residual enzymatic activity was again performed by the hemoglobin method of Anson (see Appendix). It was necessary to run blanks on the inhibitor solutions as well as the control due to the fact that the inhibitor absorbed in the region where enzymatic activity was being monitored. Again correlation coefficients of better than 0.992 were obtained.

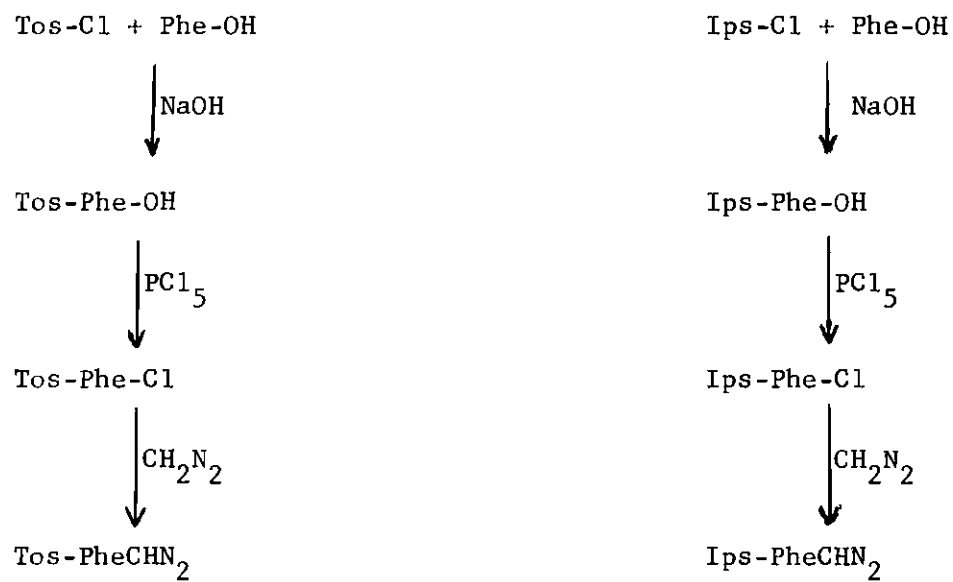


Figure 5. The Synthetic Preparation of Tos-PheCHN₂ and Ips-PheCHN₂

CHAPTER III

RESULTS

Synthesis of the Inhibitors

The two peptide diazomethyl ketones were prepared by the coupling of alanine with the appropriate sulfonic acid followed by reaction with PCl_5 and subsequent reactions with diazomethane (Scheme 1). Attempts were also made at the preparation of the peptide diazomethyl ketones by the use of a mixed anhydride procedure (Penke, et al., 1970); however, a crystalline product was unobtainable. All new compounds were characterized by mass spectra, tlc, combustion analysis, nmr, and ir. The ir spectra proved to be especially useful in the isolation of the peptide diazomethyl ketones since the inhibitors displayed a characteristic diazo band (2100 cm^{-1}).

It was observed that prolonged exposure to air and light resulted in a relative decrease in the intensity of the diazo band indicating the probability of decomposition. Despite this fact, the synthesis and isolation of the peptide diazomethyl ketones was relatively easy with the exception of minor difficulties in the recrystallization of the product. The compounds all exist as crystalline solid materials, moderately soluble in ethanol, but only slightly soluble in water. Due to the aromatic side chains, they are also soluble in benzene. The compounds are believed to decompose around their melting point by the observation of the evolution of nitrogen upon changing phases.

Pepsin Inhibition Studies

The two peptide diazomethyl ketones were found to be excellent irreversible inhibitors of pepsin. Good pseudo-first-order kinetics, i.e. where $[I]/[E] \cong 10$, were observed for all inhibition reactions. The specific activity of pepsin, expressed as Δ absorbance/10 min/mg in the initial assay, was 1.5×10^3 . The inhibitor solutions were prepared fresh since the peptide diazomethyl ketones appeared to be somewhat unstable in the solid state. Also inhibitor solutions contained 10% (v/v) ethanol to aid in the solvation of the compounds. The reaction conditions were selected in order to obtain optimal rates which could be followed for the hemoglobin assay. This was accomplished by adjusting the inhibitor concentration as low as practicable so that rates could be measured easily and accurately. In all experiments the reaction was allowed to proceed through ca. two half-lives.

Table 1 shows the results for the fixed-concentration experiments in which pepsin was inhibited by the two peptide diazomethyl ketones. It was observed that both compounds were very potent irreversible inhibitors. The values of $k_{\text{obsd}}/[I]$ for the two peptide diazomethyl ketones were relatively comparable in value indicating that the substitution of the iodine atom for the methyl substituent had little effect on the inhibitory activity of these compounds. The second-order rate constant $k_{\text{obsd}}/[I]$ may be utilized for purposes of approximate reactivity comparisons between the two inhibitors.

Table 1. Inhibition of Pepsin with Peptide Diazomethyl Ketones^a

Inhibitor	[I] x 10 ⁵ (M)	10 ³ k _{obsd} (sec ⁻¹)	Half-life (min)	k _{obsd} /[I] (M ⁻¹ sec ⁻¹)
Tos-PheCHN ₂	3.0	1.49	7.73	50
Ips-PheCHN ₂	3.0	1.39	8.31	46

^aPepsin concentration 2.5 μM, cupric acetate concentration 5.0 mM acetate buffer (pH 5.4), 10% ethanol, 23°.

Rhizopus chinensis Inhibition Studies

The inhibition of Rhizopus chinensis acid protease by the peptide diazomethyl ketones was performed under similar conditions to those used for the pepsin inhibition studies. Inhibition solutions contained 10% (v/v) ethanol to aid in dissolving of the inhibitor in the inhibition mixture. Both inhibition reactions were carried out to at least two half-lives with both half-lives being less than seven minutes.

Table 2 gives the results for the fixed concentration rate studies of the Rhizopus chinensis acid protease with the two peptide diazomethyl ketones. The compounds were shown to be very effective inhibitors of the enzyme when a tenfold excess of inhibitor to enzyme was employed. The values obtained for $k_{\text{obsd}}/[I]$ with Tos-PheCHN₂ were approximately the same as those obtained for the inhibition of pepsin with Tos-PheCHN₂ and Ips-PheCHN₂ while the $k_{\text{obsd}}/[I]$ for the inhibition of Rhizopus chinensis acid protease with Ips-PheCHN₂ was found to have a somewhat greater value.

Kinetics of Inhibition

In certain cases, the kinetics of inhibition with irreversible inhibitors indicates the existence of a reversible complex between enzyme and inhibitor prior to the formation of a covalent bond. This sequence may be expressed by Eq. 1 while the dissociation constant for the reversible binding step is shown as Eq. 2. In this sequence E·I represents the noncovalently-bound enzyme-inhibition complex while the irreversibly inhibited enzyme is represented by E-I. The limiting rate of inactivation is given as k_3 . Pseudo-first-order kinetics are obtained in the inhibition reaction when the initial enzyme concentration is much greater than

Table 2. Inhibition of Rhizophus chinensis Protease with Diazo-
methyl Ketones^a

Inhibitor	[I] x 10 ⁵ (M)	10 ³ k _{obsd} (sec ⁻¹)	Half-life (min)	k _{obsd} /[I] (M ⁻¹ sec ⁻¹)
Tos-PheCHN ₂	3.0	1.6	7.0	55
Ips-PheCHN ₂	3.0	2.1	5.4	71

^aRhizophus chinensis protease concentration 2.2 μM, cupric acetate concentration 0.001 M, 5.0 mM acetate buffer (pH 5.4), 10% ethanol, 23°.

the initial inhibitor concentration. Under these conditions k_{obsd} , the observed first-order rate constant, is given by Eq. 3 and Eq. 4 (Kitz and Wilson, 1962; Kurachi, et al., 1973).



$$K_I = [E][I]/[E \cdot I] \quad (2)$$

$$k_{\text{obsd}} = \frac{k_3[I]}{K_I + [I]} \quad (3)$$

$$\frac{1}{k_{\text{obsd}}} = \frac{K_I}{k_3} \cdot \frac{1}{[I]} + \frac{1}{k_3} \quad (4)$$

The parameter most often used to evaluate inhibitors on a relative basis is the inhibition constant k_3/K_I which is analogous to the catalytic constant k_{cat}/K_M and is comparable to the second-order rate constant ($k_{2\text{nd}}$). When the inhibitor concentration is on the order of K_I then $k_{\text{obsd}}/[I]$ will vary over a range of inhibitor concentrations and the values of k_3 and K_I can be obtained from a double-reciprocal plot of $1/k_{\text{obsd}}$ vs. $1/[I]$. If, however, the inhibitor concentrations used are appreciably smaller than the value of K_I , then Eq. 4 reduces to Eq. 5 and the value of $k_{\text{obsd}}/[I]$ will remain constant over the inhibitor concentration measured. Under these conditions the value of k_3/K_I may be obtained while the values of k_3 and K_I may not.

$$\frac{k_{\text{obsd}}}{[I]} = \frac{k_3}{K_I} \quad (5)$$

When the value of the inhibition constant k_3/K_I has not been obtained, it is possible to compare the reactivity of the two inhibitors on the basis of their $k_{\text{obsd}}/[I]$ values. This is shown in Tables 1 and 2 for the fixed concentration experiments. Thus, the magnitude of $k_{\text{obsd}}/[I]$ reflects the effects of substituent changes upon the binding of the inhibitors to the enzyme as well as the turnover rate. At this point a comparison of these values on this basis should be made with reservation since the $k_{\text{obsd}}/[I]$ values may vary under conditions where the initial inhibition concentrations are on the order of the inhibitor K_I values. Once it is shown that the inhibitor concentrations are not on the order of the K_I values, a more rigorous comparison of these values may be employed.

Concentration-Dependent Studies

The results of the concentration-dependent studies are shown in Table 3 for the inhibition of pepsin with the two peptide diazomethyl ketones in a graded series of inhibitor concentrations. A set of five inhibition runs was performed for both peptide diazomethyl ketones. The inhibition reactions were carried out over a tenfold range, with the highest inhibitor concentrations being limited by the velocity of the reaction. In both cases, the values of $k_{\text{obsd}}/[I]$ remained relatively constant over the inhibitor concentrations tested, indicating that the inhibitor concentrations were not large enough to be within the region of the K_I value. Pseudo-first-order kinetics were maintained over all inhibition runs through two half-lives by maintaining $[I]/[E] > 10$.

The effectiveness of the two peptide diazomethyl ketones as

Table 3. Concentration-Dependent Inhibition of Pepsin
with Peptide Diazomethyl Ketones^a

Inhibitor	[I] x 10 ⁵ (M)	10 ⁴ k _{obsd} (sec ⁻¹)	k _{obsd} /[I] (M ⁻¹ sec ⁻¹)
Tos-PheCHN ₂	3.0	1.50	49.8
	3.8	2.02	54.0
	4.5	2.54	56.5
	5.25	2.70	51.4
	6.0	3.60	51.9
Ips-PheCHN ₂	3.0	1.39	46.3
	3.25	1.50	46.2
	3.8	1.84	48.2
	4.5	2.09	46.4
	6.0	2.64	44.0

^aPepsin concentration 2.5 μM, cupric acetate concentration 0.001 M, 5.0 mM acetate buffer (pH 5.4), 10% ethanol, 23°.

inhibitors may now be evaluated on the basis of their relative $k_{\text{obsd}}/[\text{I}]$ which is approximately equal to k_3/K_I (Eq. 5), since it has been shown that $k_{\text{obsd}}/[\text{I}]$ does not vary appreciably over increasing inhibitor concentrations. The individual values of the parameters k_3 and K_I , however, could not be obtained for the reasons previously stated.

CHAPTER IV

DISCUSSION

Two peptide diazomethyl ketones were prepared and tested as possible inhibitors of both porcine pepsin and Rhizopus chinensis acid protease. In both cases the two compounds proved to be extremely potent inhibitors with excellent correlation of results.

Preparation of Acid Protease Inhibitors

The synthesis of the two peptide diazomethyl ketones as previously stated proceeded with relatively few complications. The critical portion of the synthesis proved to be the last step in the sequence in which the peptide chloroformate was allowed to react with diazomethane. Once the peptide diazoketone had been generated, the possibility existed for the addition of HCl across the diazo group which would yield an undesired peptide chloroketone. The preparation of Tos-PheCHN₂ has been reported by Schoellman and Shaw (1963) as well as by Delpierre and Fruton (1966). The melting point of the compound reported by Schoellman and Shaw was 94-97° while that for the compound reported by Delpierre and Fruton was 112-114°. It was observed in the preparation of Tos-PheCHN₂ as well as in Ips-PheCHN₂ that the further purification of both compounds yielded a compound of a higher melting point. In the preparation of Tos-PheCHN₂ the further purification of the compound yielded a compound which had a melting point approximately the same as that reported by Delpierre and Fruton.

Effectiveness of Peptide Diazoketones as Inhibitors

The results of the kinetic studies of the inhibition of pepsin and Rhizopus chinensis acid protease with the two peptide diazomethyl ketones were excellent acid protease inhibitors. It has been shown previously in the case of Tos-PheCHN₂ that the inhibitor reacts stoichiometrically with pepsin at an active site aspartate residue (Delpierre and Fruton, 1966).

Upon consideration of the mode of inhibition, one may envision a reaction pathway expressed by Eq. 6. The first step is represented as the initial binding of the inhibitor to the enzyme.



The produce of this equilibrium step E·I represents the noncovalently-bound enzyme-inhibitor complex, where K_I is the dissociation constant of the E·I complex. This is subsequently followed by the inactivation step, where E - I is the covalently bound irreversibly inhibited enzyme product. The rate constant of this rate limiting step is given by k₃. This type of reaction scheme has been proposed for other proteolytic enzymes (Bender and Kezdy, 1964).

An alternative explanation for these results is that the enzyme and inhibitor are undergoing a simple bimolecular reaction in which no prior binding of inhibitor to enzyme is involved. This possibility may not be discounted since it has been shown that certain acid protease catalyzed reactions require the presence of extended binding site interactions. In particular, inhibition studies employing peptide aldehydes as competitive inhibitors of pepsin showed that the peptide analog

Z-Phe-H was not a competitive inhibitor of pepsin while Z-Phe-Phe-H was an excellent competitive inhibitor of pepsin (Kishi and Nakamigo, 1974).

CHAPTER V

CONCLUSIONS

In conclusion, the kinetic results of the inhibition studies of the two peptide diazomethyl ketones with porcine pepsin and Rhizopus chinensis acid protease showed the two compounds to be excellent irreversible inhibitors of both enzymes. However, concentration-dependent studies were unsuccessful in yielding the individual parameters k_3 and K_I . As a result, there exists some question as to the nature of the inhibition reaction, id est, whether the reaction is proceeding in such a way that the inhibitor is bound to the enzyme prior to the formation of a covalent linkage between the enzyme and the compound or is merely undergoing a simple bimolecular reaction between the enzyme and the inhibitor in which no prior binding is involved.

Although these studies have provided some insight into the inhibition of the two acid proteases with the two peptide diazoketones, the major goal of this project will not be realized until the crystal structure of the "heavy atom" derivative of the Rhizopus chinensis acid protease is completed.

CHAPTER VI

RECOMMENDATIONS

A useful extension of these studies would be the determination of the subsite specificities for these acid proteases since few reasonably good synthetic substrates of these enzymes are known. This may be accomplished by employing a series of acyl carbazates as potential acid protease inhibitors. These compounds can be readily prepared by a coupling reaction of the appropriate peptide hydrazide with the proper isocyanate compound. These studies may be aided by the future x-ray crystallographic investigation.

APPENDIX

ACID PROTEASE ASSAY PROCEDURE

The decreasing activity of the two acid proteases during inhibition runs was measured by the hemoglobin assay method of Anson (1938). The substrate stock solution consisted of 2.5% denatured hemoglobin in 0.06 M HCl. The stock solution was kept refrigerated and was good for up to one week. The residual enzymatic activity was determined by adding 100 μ l of an aliquot containing 0.7 μ g from the inhibition mixture to 1.0 ml of the substrate stock solution. The mixture was allowed to stir for exactly ten minutes at which point the reaction was quenched by the addition of 2.0 ml of a 5.0% trichloroacetic acid solution. The suspension was filtered through Whatman No. 50 hardened filter paper and the absorbance of the soluble peptides was measured at 280 nm against distilled water. A blank was also taken by adding the 5.0% trichloroacetic acid to the substrate stock solution prior to the addition of the 100 μ l aliquot from the inhibition mixture. The value obtained from the blank was subtracted from the values of the test runs and the figures thus obtained represent the absorbance of the hydrolysis product.

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II. INVESTIGATION OF SERINE PROTEASE ACTIVITY
WITH ACYL CARBAZATES

CHAPTER I

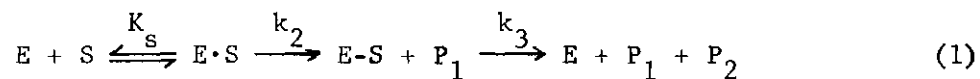
INTRODUCTION

For a discussion of the function and importance of the class of enzymes known as proteolytic enzymes, please refer to the Introduction of this thesis, Part I. These enzymes are known to cleave peptide bonds at certain points along a given peptide chain.

Of the several groups of proteolytic enzymes, a group of enzymes referred to as "serine proteases" has undergone a most intensive investigation. These enzymes are characterized by the presence of a reactive serine residue at the enzyme's active site. Chymotrypsin, subtilisin, trypsin, elastase, thrombin, and plasmin are among the enzymes which belong to this family. Studies directed at the determination of the action of these enzymes have yielded a wealth of information about their physical as well as chemical properties including a detailed three dimensional crystal structure for four of the enzymes, primary substrate specificities, complete amino acid sequences for several species as well as a theory for the catalytic action of this group of enzymes. Although the active sites of serine proteases are essentially identical, the major differences between serine proteases lie in their individual substrate specificities for certain types of amino acids.

Much of the characterization studies dealing with these enzymes was performed with the use of small peptides and peptide esters and amides. On the basis of these studies a mechanism was proposed by which

all of these serine proteases are believed to operate (Bender and Kezdy, 1964). This mechanism is shown in Eq. 1.



The initial binding of a substrate molecule to the enzyme which forms a reversible enzyme-substrate complex $E \cdot S$, is followed by the cleavage of an appropriate peptide bond by an active site serine residue to form an acyl-enzyme intermediate. The formation of this acyl-enzyme intermediate is usually found to be the rate-limiting step with amides. A water molecule subsequently attacks the intermediate resulting in deacylation and regeneration of the free enzyme. Such methods as amino-acid analysis, x-ray crystallographic investigation as well as various kinetic studies have shown an extensive amount of homology among these enzymes, especially in the active site region. This has been shown by the fact that subtilisin, chymotrypsin, trypsin, and elastase all contain the same catalytic groups, serine, histidine, and aspartic acid at the active site in a charge relay system with essentially the same spatial arrangement. A charge relay system between these three catalytic residues is believed to be responsible for the unusually high nucleophilicity of the active-site serine residue. Figure 1 depicts the overall mechanism for the catalytic activity of serine proteases.

The major differences observed among serine proteases lies in the variations in the substrate specificity of each enzyme for certain types of amino acids in the portion of the substrate undergoing cleavage. Each

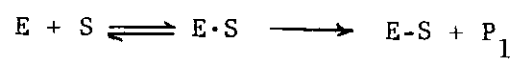
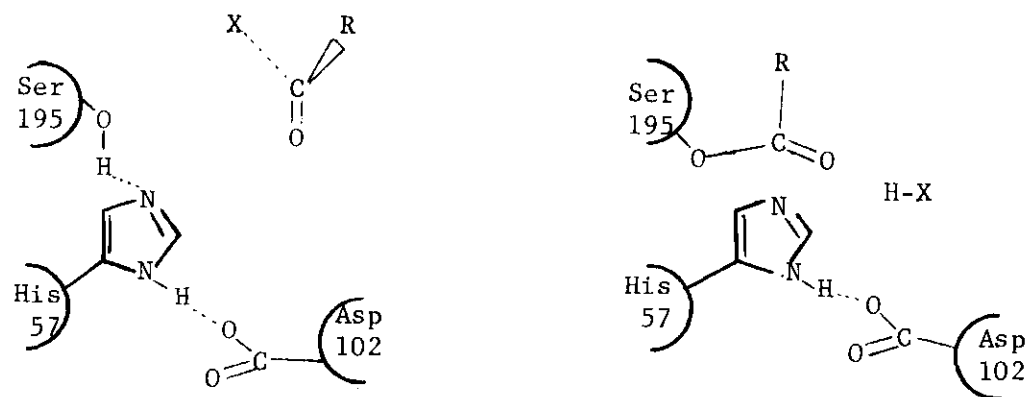
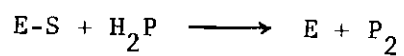
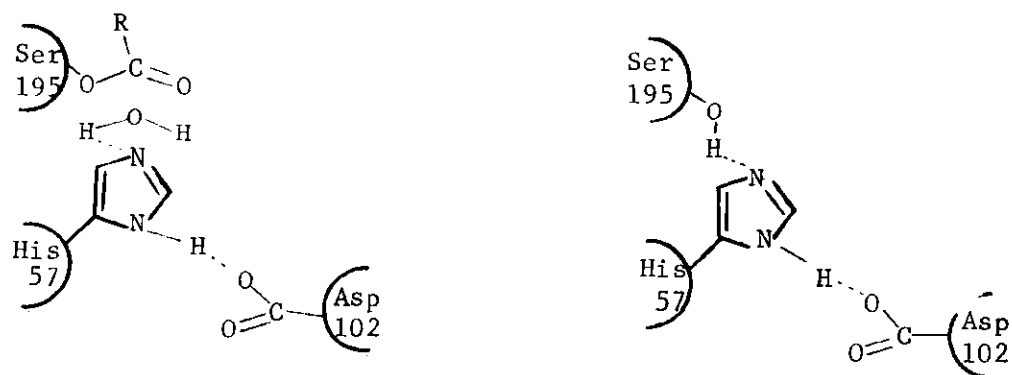
AcylationDeacylation

Figure 1. A Diagram of the Role of the Charge Transfer System for Acylation and Deacylation of Chymotrypsin

serine protease contains a "pocket" adjacent to the catalytic residues which has a specific size, shape, and polarity. The binding pocket or "tosyl hole" (Steitz, et al., 1969) at the enzyme S_1 's subsite (nomenclature of Schechter and Berger, 1967) dictates its primary substrate specificity. This has been shown by the use of x-ray structural studies of bound enzyme derivatives (see Figure 2). From these studies it has been shown that for chymotrypsin, the binding pocket is hydrophobic and narrow in shape which allows it to accommodate a phenyl group and thus gives it a primary substrate specificity for phenylalanine, tyrosine, and tryptophane residues. In the case of trypsin, however, the presence of an anionic carboxylate group near the rear of the pocket results in a specificity towards positively charged side chains such as arginine or lysine. In elastase, the binding pocket is found to be partially filled by the alkyl side chain of Val-216 which decreases the overall size of the pocket. As a result, the specificity of elastase is for residues with small side chains, such as alanine, serine, and valine. For subtilisin, the binding pocket is found to be somewhat larger and more flexible. Because of this fact, this enzyme has a much broader specificity for residues with aromatic or large branched aliphatic side chains such as phenylalanine and leucine. Although it has been shown that the primary subsite specificity is very important in binding interactions, the enzyme's overall specificity is determined by the total interaction with a substrate which includes extended binding site interactions.

The most widely studied of the serine proteases is chymotrypsin. This enzyme has been extensively characterized by x-ray crystallographic investigation as well as numerous kinetic studies. The essential cata-

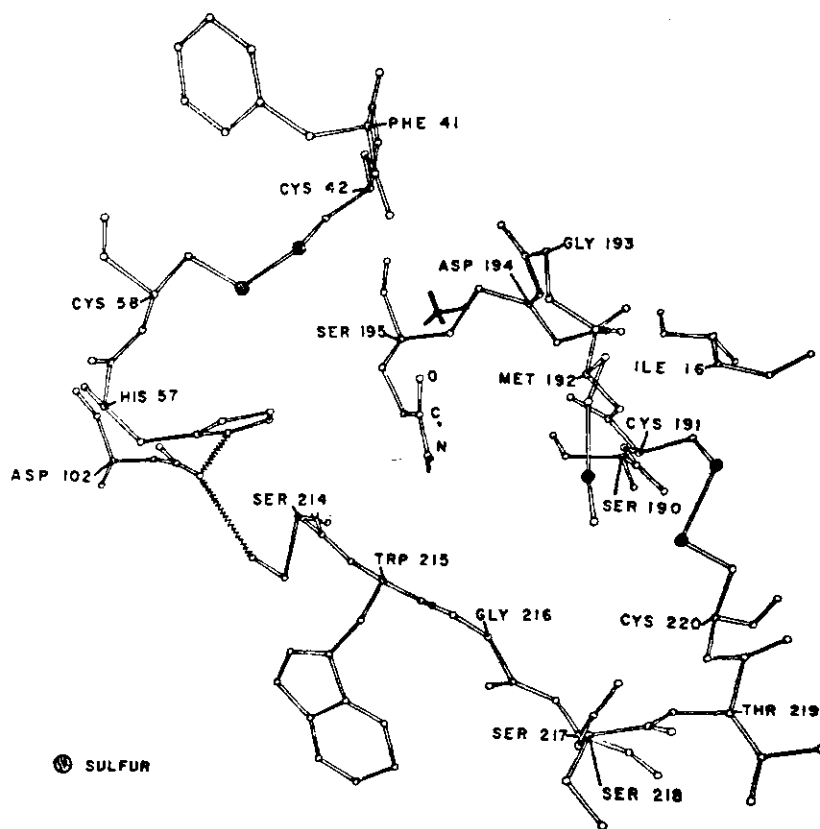


Figure 2. Three Dimensional Drawing of the Active Site of Monocarbamyl Chymotrypsin A α (Robillard, J. D., Powers, J. C., and Wilcox, P. E. (1972), Biochem., 11, 349)

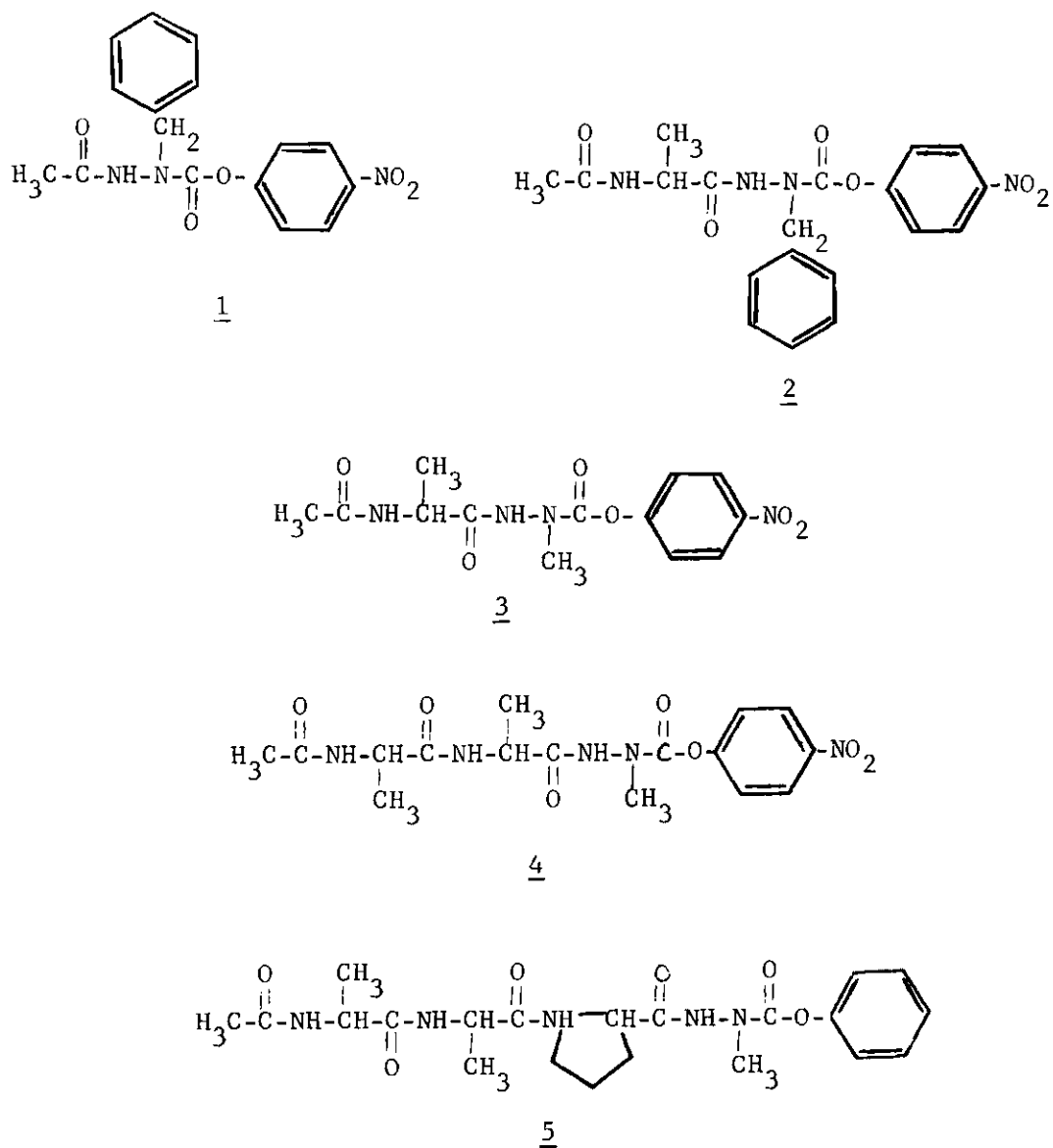


Figure 3. Structures of Peptide Carbazates

(Structures 1 and 2 represent the two benzyl carbazates Ac-Bzc-ONp and Ac-Ala-Bzc-ONp while structures 3, 4, and 5 represent Ac-Ala-Mec-ONp, Ac-Ala-Ala-Mec-ONp, and Ac-Ala-Ala-Pro-Mec-ONp, respectively.)

lytic residues within the charge relay system are Ser-195, His-157, and Asp-102. The three dimensional structure of tosyl-chymotrypsin as well as the native enzyme was determined by x-ray studies which gave the spatial arrangement of the catalytic groups (Blow, et al., 1969; Blow, 1971; Birktoft and Blow, 1972). On the basis of the structural studies as well as kinetic investigations (Henderson, et al., 1971; Hess, 1971) the overall mechanism for chymotrypsin activity has become well established. As previously stated, chymotrypsin exhibits a substrate specificity for aromatic residues, with a phenyl side chain fitting tightly into the enzyme's binding pocket.

Likewise, the subtilisins have undergone an intensive investigation. The present nomenclature for these enzymes (DeLong and Smith, 1968) is as follows: the enzyme isolated by Ottesen and Spector (1960) is called subtilisin Carlsburg and the enzyme isolated by Hagihara (1954) is referred to as subtilisin BPN'. Subtilisin BPN', which is the more widely studied enzyme, has been the subject of x-ray crystallographic investigation (Wright, et al., 1969; Kraut, et al., 1972) as well as detailed kinetic analysis (Moriyama and Oka, 1970). All of these studies indicate that these enzymes employ the same general mechanism as chymotrypsin. The major property which distinguishes these enzymes from chymotrypsin is their broader primary subsite specificity for aromatic side chains.

Acyl carbazates have been shown to react with serine proteases (Kurtz and Neimann, 1961; Elmore and Smyth, 1968) when the appropriate substituents are added. These compounds are analogs of amino acids in which the α -methine group has been replaced by a nitrogen atom (Figure 3). This adjacent nitrogen atom gives a special stability to the acyl enzyme

against deacylation (Robillard, et al., 1972). The reaction of the enzyme with the substrate follows the general reaction scheme shown in Eq. 1 (Kezdy and Kaiser, 1970). The use of p-nitrophenol as P₁ offers several advantages. p-Nitrophenol is an excellent leaving group and its esters have high k_2/k_3 values with chymotrypsin (Hartley and Kilby, 1954). These esters are relatively stable in aqueous solutions at neutral pH. More importantly, the release of p-nitrophenol as a product serves as a useful means of monitoring the reaction between the acyl carbazate and the enzyme since p-nitrophenol has a high absorbance in the visible region of the spectrum ($E = 18,300$) (Kezdy and Bender, 1962).

More recently, Powers and Carroll (1975) prepared three acyl carbazates: Ac-Ala-Bzc-ONp, Ac-Ala-Mec-ONp, and Z-Ala-Ala-Pro-Mec-ONp. The reactions of these compounds and Elmore's reagent (Ac-Bzc-ONp) with chymotrypsin, subtilisin BPN', and elastase were performed. Chymotrypsin was inactivated by both benzyl carbazates and slowly reacted with Z-Ala-Ala-Pro-Mec-ONp while subtilisin BPN' was acylated by all four compounds but underwent a rapid deacylation with the two methyl carbazates. This thesis, Part II, is a report on the detailed investigation of the reaction of a series of five acyl carbazates (see Figure 3) with chymotrypsin A_Q, subtilisin BPN', and subtilisin Carlsburg. These studies were aimed at obtaining a better understanding of the requirements for reactivity of carbazates with these serine proteases and in particular obtaining an explanation for the unusual reactivity of the subtilisins towards the methyl carbazates with respect to the acylation and deacylation rates.

CHAPTER II

METHODS

Materials and Methods

Chymotrypsin A_α (lot CD11BK) was obtained from Worthington Biochemical Company and used without further purification. The chymotrypsin substrate, Ac-Tyr-OEt was prepared by Dr. Peter Tuhy. Subtilisin BPN' (lot P-5255) and subtilisin Carlsburg (lot P-5380) were obtained from Sigma Chemical Company and used without further purification. The benzyl carbazate Ac-Bzc-ONp was obtained from Nutritional Biochemicals Corporation. The methyl carbazate Ac-Ala-Ala-Pro-Mec-ONp was prepared by Dr. Ronald Boone. The benzyl hydrazide Ac-Ala-NH-NH-Bz and the methyl carbazate Ac-Ala-Mec-ONp were prepared by Mr. David Carroll. The methyl carbazate Ac-Ala-Ala-Mec-ONp was prepared by Dr. Peter Tuhy. Thin layer chromatography was carried out with Merck silica gel G plates. Column chromatography was performed with silica gel 60 adsorbent. Mass spectra were taken on a Hitachi Perkin-Elmer RMU-71 instrument and nuclear magnetic resonance (nmr) spectra were taken on a Varian T-60 instrument. Deuteriochloroform (CDCl₃) was the solvent used with all nmr spectra. Infrared (ir) spectra were taken on a Perkin-Elmer 457 instrument. The ir spectra were taken in nujol mulls. Assays were performed on a Beckman Model-25 spectrophotometer and a Radiometer automated pH-stat Model TTT11.

N-Acetyl-L-alanyl-2-benzylcarbazate Acid-p-nitrophenyl Ester

Ac-Ala-Bzc-ONp was prepared by the method of Powers and Carroll

(1975) with modifications. To a 100 ml round bottom flask was added 1.16 g (5.02 mMoles) of Ac-Ala-NH-NH-Bz in 40 ml of pyridine. After five minutes of stirring at 0°, 1.08 g (5.31 mMoles) of p-nitrophenylchloroformate in 5.0 ml of anhydrous tetrahydrofuran was added dropwise to the reaction mixture over a 30 minute period. The reaction mixture was allowed to stir for one hour at 0°, and one additional hour at room temperature. The solvent was then removed in vacuo and the resulting yellow oil was chromatographed on a 30X2 cm silica gel column. The product was recrystallized from acetone to give 0.86 g (43.3%) of a white solid, mp 156-158°, R_f 0.36 (CHCl₃-CH₃OH, 9:1). The mass spectrum had major peaks at m/e 261 (M-HO-Ph-NO₂), 139 (HO-Ph-NO₂⁺), 114 (Ac-Ala), and 91 (C₇H₇⁺). The nmr spectrum (CDCl₃) had peaks at δ 10.0 (s,1H), 8.4 and 7.3 (q,4H), 7.4 (s,5H), 6.2 (d,1H), 4.8 (s,2H), 4.5 (t,1H), 1.9 (s,3H), and 1.3 (d,3H). The ir spectrum showed bands at 1735 cm⁻¹ (carbonyl of ester) and 1670 cm⁻¹ (carbonyl of amide). Anal. calcd for C₁₉H₂₀N₄O₆: C, 57.00; H, 5.00; N, 14.00. Found: C, 56.94; H, 4.97; N, 14.21.

Reaction of Enzymes with Acyl Carbazates

The reaction of the three serine proteases with the four acyl carbazates was carried out with ca. a fiftyfold excess of substrate over enzyme. Stock solutions of substrate in acetonitrile were prepared at a concentration of 5.0 mM and used within four days of preparation. Stock solutions of chymotrypsin A _{α} , subtilisin BPN¹, and subtilisin Carlsburg were made up in 1 mM HCl, stored at 4°, and had a concentration of ca. 100 μ M (exact concentration determined by uv absorbance). Four buffer solutions were prepared for pH dependence studies: pH 7.0 (0.10 M phos-

phate), pH 6.0 (0.10 M citrate), pH 5.0 (0.10 M citrate), and pH 4.0 (0.10 M acetate). All reactions were carried out at 25° and were initiated by mixing 100 μ l of the substrate with 2.0 ml of the appropriate buffer in a cuvet. An identical sample was prepared as a reference and a baseline recorded spectrophotometrically at 345 nm. This was followed by the addition of 100 μ l of 1 mM HCl to the reference cuvet and 100 μ l of the enzyme stock solution to the sample cuvet and the reaction rate observed. The initial concentrations were as follows: enzyme, ca. 5 μ M; substrate, 250 μ M; acetonitrile, 5% (v/v); in a total volume of 2.2 ml.

Determination of Deacylation Rates

The measurement of "slower" deacylation rates was performed by the method of Wilcox (1970) employing Ac-Tyr-OEt as the substrate for chymotrypsin A $_{\alpha}$. A stock solution of Ac-Ala-Bzc-ONp in acetonitrile was prepared at a concentration of 5.0 mM and used immediately. A stock solution of chymotrypsin A $_{\alpha}$ was made up in 1 mM HCl and had a concentration of 100 μ M (exact concentration determined by uv absorbance). The reaction was initiated by the addition of 100 μ l of the substrate stock solution to 2.0 ml of the 0.10 M citrate buffer (pH 5.0). This was followed by the addition of 100 μ l of the enzyme stock solution to the reaction mixture. The mixture was allowed to incubate for five minutes. A 200 μ l portion of the reaction mixture was withdrawn and added to 2.0 ml of the appropriate buffer solution. At given time intervals, 100 μ l fractions were withdrawn and assayed employing the substrate Ac-Tyr-OEt and a pH-stat (see Appendix for further description of assay procedure). The initial concentrations were as follows: enzyme, ca. 5×10^{-6} ; substrate, 23 mM;

acetonitrile 0.5% (v/v); in a total volume of 2.2 ml. The determination of deacylation rates was aided by the use of a least-squares computer program. Correlation coefficients of better than 0.994 were obtained.

CHAPTER III

RESULTS

Reaction of Chymotrypsin with Acyl Carbazates

The series of peptide carbazates reacted with chymotrypsin at various rates depending upon the nature of the substituents. Results were very reproducible under conditions where $[S] \gg [E]$. Assay solutions were freshly prepared since the peptide carbazates underwent a slow hydrolysis upon standing in buffered aqueous solutions. Also inhibitor solutions contained 5% (v/v) acetonitrile to increase the solubility of the compounds in the assay solutions. The reaction conditions were selected in order to obtain reaction rates which could be measured easily and accurately.

Table 1 shows the results for the reaction between chymotrypsin and each of the five peptide carbazates. The enzyme was acylated by the two benzyl carbazates very rapidly over a pH range of 4 to 7 although the pH optimum for chymotrypsin is ca. 8. In contrast, the methyl carbazate Ac-Ala-Mec-ONp did not react with the enzyme at all over this pH range while the methyl carbazate Ac-Ala-Ala-Mec-ONp acylated the enzyme at a substantially slower rate than the two benzyl carbazates. The peptide carbazate Ac-Ala-Ala-Pro-Mec-ONp acylated chymotrypsin rather slowly at lower pH values but acylated the enzyme rapidly at higher pH values. In all cases where chymotrypsin reacted with an acyl carbazate, the decylation rates for these reactions could not be monitored on the time scale

Table 1. Reaction of Acyl Carbazates with Chymotrypsin A_α^a

Substrate	pH	[E] x 10 ⁶	Burst x 10 ² ^b	Acylation x 10 ² ^c	k _{ac}	k _{cat}
		(M)	(A)	(A)	(min ⁻¹)	(min ⁻¹)
Ac-Ala-Mec-ONp	4.0	4.4	2.8	0	0	---
	5.0	4.4	2.7	0	0	---
	6.0	4.4	2.7	0	0	---
	7.0	4.4	2.8	0	0	---
Ac-Ala-Ala-Mec-ONp	4.0	5.0	2.9	0	0	---
	5.0	5.4	3.3	0	1.7 x 10 ⁻¹	---
	6.0	5.0	3.2	3.0	1.4	< 1.1 x 10 ⁻²
	7.0	5.4	3.5	3.1	1.9	< 1.1 x 10 ⁻²
Ac-Ala-Ala-Pro-Mec-ONp	4.0	5.0	2.5	2.2	1.2 x 10 ⁻¹	< 1.1 x 10 ⁻²
	5.0	5.0	2.5	2.3	3.8	< 1.1 x 10 ⁻²
	6.0	5.0	2.5	2.3	> 7.0	< 1.1 x 10 ⁻²
	7.0	5.0	2.6	2.3	> 7.0	< 1.1 x 10 ⁻²
Ac-Bzc-ONp	4.0	4.4	2.3	2.3	> 7.0	< 1.1 x 10 ⁻²
	5.0	4.4	2.4	2.3	> 7.0	< 1.1 x 10 ⁻²
	6.0	4.4	2.3	2.2	> 7.0	< 1.1 x 10 ⁻²
	7.0	4.4	2.4	2.2	> 7.0	< 1.1 x 10 ⁻²
Ac-Ala-Bzc-ONp	4.0	5.4	3.2	2.8	> 7.0	< 1.1 x 10 ⁻²
	5.0	5.4	3.2	2.7	> 7.0	< 1.1 x 10 ⁻²
	6.0	5.4	3.1	2.7	> 7.0	< 1.1 x 10 ⁻²
	7.0	5.4	3.0	2.9	> 7.0	< 1.1 x 10 ⁻²

^a Acyl carbazate concentration 2.3 x 10⁻⁴ M, 5% (v/v) acetonitrile, at 25°.

^b Theoretical burst of p-nitrophenol calculated from enzyme concentration and acyl carbazate extinction coefficient.

^c Observed initial release of p-nitrophenol due to acylation.

of the assay procedure being employed.

The lower limit value of $1.1 \times 10^{-2} \text{ min}^{-1}$ for the deacylation rates was determined by preparing a series of lines of known slopes and superimposing each line on a previously recorded baseline. The line with the smallest slope value which had a perceptible deviation from the baseline was used to calculate the lower limit value. The upper limit value of 7.0 min^{-1} for the acylation rates was determined from Eq. 2 where k_{ac} is the acylation rate and $t_{0.9}$ is the time required to acylate 90% of the enzyme.

$$k_{ac} = \frac{\ln 10}{t_{0.9}} \quad (2)$$

Since a minimum of twenty seconds was required to mix the assay solution and allow for instrument equilibration, this number was used to calculate the upper limit value.

Reaction of Subtilisins with Acyl Carbazates

All of the five peptide carbazates reacted rapidly with both subtilisin BPN' and subtilisin Carlsburg. Identical assay conditions were employed for these studies in order to allow for a ready comparison of results between chymotrypsin and these enzymes. Since identical reaction conditions were used, the same limiting rate values may be utilized for these studies.

Table 2 shows the results for the studies in which subtilisin BPN' was reacted with each of the five peptide carbazates. The enzyme was acylated rapidly by all five compounds over the pH range of 4 to 7. In most cases the acylation rates exceeded the upper limit value and could not be measured.

Table 2. Reaction of Acyl Carbazates with Subtilisin BPN^a

Substrate	pH	[E] x 10 ⁶ (M)	Burst x 10 ² ^b (A)	Acylation x 10 ² ^c (A)	k _{ac} ⁻¹ (min ⁻¹)	k _{cat} ⁻¹ (min ⁻¹)
Ac-Ala-Mec-ONp	4.0	4.4	2.8	2.7	7.1 x 10 ⁻²	< 1.1 x 10 ⁻²
	5.0	3.6	2.2	1.9	> 7.0	9.5 x 10 ⁻²
	6.0	3.6	2.3	2.0	> 7.0	6.6 x 10 ⁻¹
	7.0	4.4	2.9	2.7	> 7.0	4.8
Ac-Ala-Ala-Mec-ONp	4.0	4.1	2.4	2.2	> 7.0	1.4 x 10 ⁻²
	5.0	4.1	2.5	2.4	> 7.0	3.3 x 10 ⁻¹
	6.0	4.1	2.6	2.4	> 7.0	5.3
	7.0	4.1	2.7	2.4	> 7.0	1.1 x 10 ¹
Ac-Ala-Ala-Pro-Mec-ONp	4.0	3.4	1.9	1.6	> 7.0	1.1
	5.0	3.5	1.9	1.7	> 7.0	2.1
	6.0	3.5	1.9	1.6	> 7.0	1.0 x 10 ¹
	7.0	3.5	1.9	1.6	> 7.0	1.5 x 10 ¹
Ac-Bzc-ONp	4.0	4.1	2.1	---	1.7 x 10 ⁻²	---
	5.0	4.1	2.1	2.1	9.3 x 10 ⁻²	< 1.1 x 10 ⁻²
	6.0	4.4	2.2	2.1	1.1	< 1.1 x 10 ⁻²
	7.0	4.4	2.2	2.1	> 7.0	< 1.1 x 10 ⁻²
Ac-Ala-Bzc-ONp	4.0	4.1	2.3	2.0	> 7.0	< 1.1 x 10 ⁻²
	5.0	4.1	2.3	2.2	> 7.0	1.7 x 10 ⁻²
	6.0	4.1	2.3	2.0	> 7.0	6.4 x 10 ⁻²
	7.0	4.1	2.2	2.0	> 7.0	1.1 x 10 ⁻¹

^aAcyl carbazate concentration 2.3 x 10⁻⁴ M, 5% (v/v) acetonitrile, at 25°.

^bTheoretical burst of p-nitrophenol calculated from enzyme concentration and acyl carbazate extinction coefficient.

^cObserved initial release of p-nitrophenol due to acylation.

The deacylation rates for the reactions involving the two benzyl carbazates were rather slow. This is particularly true for Ac-Bzc-ONp which had slower deacylation rates than Ac-Ala-Bzc-ONp. In contrast, the reactions involving the enzyme and each of the three methyl carbazates had very fast deacylation rates. Of these three methyl carbazates, Ac-Ala-Ala-Pro-Mec-ONp had the most rapid deacylation rate when it reacted with subtilisin BPN',

Table 3 shows the results for the experiments in which each of the five acyl carbazates was reacted with subtilisin Carlsburg. All five compounds acylated this enzyme at a rate which was too rapid to follow on the time scale of this assay procedure. The two benzyl carbazates were again noted to have rather slow deacylation rates and the methyl carbazate Ac-Bzc-ONp was shown to have a slower deacylation rate than Ac-Ala-Bzc-ONp. Likewise, the three methyl carbazates were again shown to have very rapid deacylation rates and of the three methyl carbazates, Ac-Ala-Ala-Pro-Mec-ONp had the fastest rates. A comparison of deacylation rates for the reaction between a given acyl carbazate and each of the two subtilisins shows subtilisin Carlsburg to consistently have more rapid deacylation rates than subtilisin BPN'.

Determination of Deacylation Rates

The peptide carbazate Ac-Ala-Bzc-ONp was reacted with chymotrypsin and the rate of deacylation was measured by employing the substrate Ac-Tyr-OEt to monitor the residual enzymatic activity. Although the reaction mixture was diluted by a factor of ten, the relative ratios of carbazate to enzyme were essentially identical to those used in previous experiments

Table 3. Reaction of Acyl Carbazates with Subtilisin Carlsburg^a

Substrate	pH	[E] x 10 ⁶ (M)	Burst x 10 ² ^b (A)	Acylation x 10 ² ^c (A)	k _{ac-1} (min ⁻¹)	k _{cat-1} (min ⁻¹)
Ac-Ala-Mec-ONp	4.0	3.3	2.0	1.7	> 7.0	1.4 x 10 ⁻¹
	5.0	3.3	2.0	1.6	> 7.0	5.5 x 10 ⁻¹
	6.0	3.3	2.0	1.8	> 7.0	4.3
	7.0	3.3	2.0	1.8	> 7.0	1.6 x 10 ¹
Ac-Ala-Ala-Mec-ONp	4.0	4.0	2.4	2.1	> 7.0	8.3 x 10 ⁻¹
	5.0	4.0	2.4	2.2	> 7.0	2.2
	6.0	4.0	2.5	2.2	> 7.0	1.3 x 10 ¹
	7.0	4.0	2.6	2.2	> 7.0	2.6 x 10 ¹
Ac-Ala-Ala-Pro-Mec-ONp	4.0	3.8	1.9	1.7	> 7.0	1.5
	5.0	3.8	1.9	1.7	> 7.0	4.5
	6.0	3.8	1.9	1.6	> 7.0	2.2 x 10 ¹
	7.0	3.8	2.0	1.6	> 7.0	3.6 x 10 ¹
Ac-Bzc-ONp	4.0	3.3	1.7	1.5	> 7.0	< 1.1 x 10 ⁻²
	5.0	3.3	1.7	1.5	> 7.0	2.3 x 10 ⁻²
	6.0	3.3	1.6	1.5	> 7.0	3.1 x 10 ⁻²
	7.0	3.3	1.7	1.5	> 7.0	9.2 x 10 ⁻²
Ac-Ala-Bzc-ONp	4.0	3.5	2.0	1.6	> 7.0	4.1 x 10 ⁻²
	5.0	3.5	2.0	1.8	> 7.0	6.5 x 10 ⁻²
	6.0	3.5	2.0	1.8	> 7.0	3.5 x 10 ⁻¹
	7.0	3.5	1.9	1.8	> 7.0	7.2 x 10 ⁻¹

^a Acyl carbazate concentration 2.3 x 10⁻⁴ M, 5% (v/v) acetonitrile, at 25°.

^b Theoretical burst of p-nitrophenol calculated from enzyme concentration and acyl carbazate extinction coefficient.

^c Observed initial release of p-nitrophenol due to acylation.

with chymotrypsin and subtilisin. By maintaining this ratio, a comparison of rates with previous results can be more readily made.

Table 4 shows the results of the experiments in which the deacylation rates were measured for the reaction between chymotrypsin and Ac-Ala-Bzc-ONp. All of the deacylation rates determined for this reaction were substantially slower than any turnover rate observed from the spectrophotometric assay. The experiments in which the deacylation rates were determined over the pH range from 5 to 7 showed that at pH 5 the deacylation rate was slowest. However, as the pH was increased up to 7, the deacylation rates were substantially more rapid. An attempt was made to also obtain a deacylation rate at pH 4; however, the reaction proceeded too slowly to give reproducible values.

Determination of Extinction Coefficients and Background

Hydrolysis Rates for Acyl Carbazates

The molar extinction coefficients and background hydrolysis rates were measured for all five peptide carbazates. In all studies 5% (v/v) acetonitrile was present in order to simulate conditions used for the spectrophotometric assay. The experimental conditions were selected in order to obtain values which could be measured easily and accurately.

Table 5 shows the results for the experiments in which the rates of background hydrolysis and the molar extinction coefficients were determined with all five peptide carbazates. Extinction coefficients were measured for each compound at pH 4, 5, 6, and 7 and showed good reproducibility. All peptide carbazates had relatively slow background hydrolysis rates over a pH range of 4 to 7 showing that the molar ratio of enzyme to

Table 4. Determination of Deacylation Rates for Reaction of Chymotrypsin A_α with Ac-Ala-Bzc-ONp^a

Substrate	pH	Half-life (min ⁻¹)	k _{cat} (min ⁻¹)
Ac-Ala-Bzc-ONp	5	1.95 x 10 ³	3.6 x 10 ⁻⁴
	6	2.48 x 10 ²	2.8 x 10 ⁻³
	7	1.34 x 10 ²	5.2 x 10 ⁻³

^aChymotrypsin A_α concentration 3.7 x 10⁻⁹ M, acyl carbazate concentration 2.3 x 10⁻⁷ M, 5% (v/v) acetonitrile, at 25°.

Table 5. Molar Extinction Coefficients and Background Hydrolysis Rates for Acyl Carbazates

Compound	pH	$\Sigma_{345} \times 10^{-3}^a$	$k_{bk} \times 10^3^b$ (min^{-1})
Ac-Ala-Mec-ONp	4	5.92	0.9
	5	6.19	0.9
	6	5.97	1.2
	7	6.02	1.9
Ac-Ala-Ala-Mec-ONp	4	5.88	1.1
	5	6.12	1.4
	6	6.32	1.9
	7	6.58	3.2
Ac-Ala-Ala-Pro-Mec-ONp	4	4.89	1.0
	5	4.93	1.0
	6	4.98	1.1
	7	5.16	2.2
Ac-Bzc-ONp	4	5.22	1.0
	5	5.11	1.1
	6	4.92	1.7
	7	5.11	2.2
Ac-Ala-Bzc-ONp	4	5.59	1.0
	5	5.59	1.2
	6	5.51	1.7
	7	5.34	2.9

^aAcyl carbazate concentration 2.3×10^{-4} M, KOH concentration 1.8×10^{-4} M, 5% (v/v) acetonitrile in 2.2 ml.

^bAcyl carbazate concentration 7.0×10^{-4} M, 5% (v/v) acetonitrile in 2.2 ml.

substrate remains relatively unchanged over the time span required for the spectrophotometric assay.

CHAPTER IV

DISCUSSION

A selected variety of peptide carbazates was tested as possible inhibitors of chymotrypsin, subtilisin BPN', and subtilisin Carlsburg with relatively good correlation of results. The effects of substituent replacement upon the inhibitory ability against these three enzymes is the major finding of this work and the basis for future chemical investigation.

Effectiveness of Serine Protease Inhibitors

The experimental results of these studies demonstrate that several of the peptide carbazates are highly reactive inhibitors of the three serine proteases. In certain cases the inhibition reactions proceeded rapidly to form a relatively stable acyl intermediate even at low pH values. The k_{cat} values obtained from these studies serve as a means of evaluating the effectiveness of these peptide carbazates as inhibitors of these enzymes. Peptide carbazates which had k_{cat} values which were less than $1.1 \times 10^{-2} \text{ min}^{-1}$ were considered to be good inhibitors of the three enzymes while compounds with k_{cat} values greater than $1.1 \times 10^{-2} \text{ min}^{-1}$ were considered to be poorer serine protease inhibitors. The best inhibitors of these enzymes were the two benzyl carbazates Ac-Bzc-ONp and Ac-Ala-Bzc-ONp. Both of these compounds acylated the enzyme rapidly over a pH range of 4 to 7 while maintaining very slow deacylation rates. No direct evidence was obtained to indicate that these peptide carbazates

were reacting with the active site serine residue. However, it is relatively certain that alkylation of the reactive serine group does occur on the basis of previous studies with analagous systems. It has been shown that p-nitrophenyl cyanate acylated chymotrypsin A_α at Ser-195 on the basis of the crystallographic investigation of the carbamylated-enzyme derivative (Robillard, et al., 1972). Furthermore, the benzyl carbazates have been shown to be very specific for chymotrypsin and the subtilisins since they were found to be virtually inactive towards the closely related enzymes elastase and trypsin (Powers and Carroll, 1975). This specificity makes these compounds advantageous towards physiological experiments which require the selective inhibition of chymotrypsin and subtilisin in preference to related enzymes.

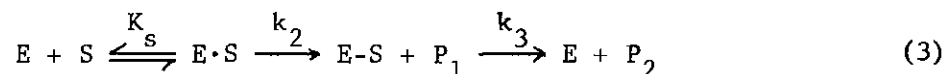
pH Dependence of Inhibitor Activity

The experimental results of these studies showed that where relative reaction rates were measurable, there was a very distinct effect upon the rates of reaction as a result of changes in pH. It was further shown that in all measurable cases, these reaction rates increased with increasing pH. Since the pH optimum for chymotrypsin and the subtilisins with synthetic ester substrates is between 7 and 8 (Cunningham and Brown, 1956; Glazer, 1966) these results would be anticipated if the peptide carbazates were reacting with the active site serine residue.

Stoichiometry of Inhibition

In all cases where acylation was observable, the reactions between the serine proteases and the peptide carbazates were always stoichiometric. Since the peptide carbazates possess this property, these compounds should

be considered as possible active site titrants. The overall reaction scheme for serine proteases (Bender and Kezdy, 1964) is shown in Eq. 3 where the reaction of the enzyme with the substrate leads to the formation of an acyl enzyme intermediate.



Since optimal enzyme titrations should make use of a stoichiometric reaction between the enzyme and the specific substrate, this system offers an excellent opportunity to quantitate the transformation of E to E-S. However, a stoichiometric transformation is only possible under conditions where $k_3 \ll k_2$ (Kezdy and Kaiser, 1970). Furthermore, this transformation can only occur quantitatively under conditions where substrate concentrations are high enough to transform all of the enzyme into the enzyme substrate complex and maintain the complex upon formation. Since these peptide carbazates exhibit very slow deacylation rates with these enzymes under pseudo-first-order reaction conditions, these compounds should be considered as likely active site titrants of subtilisin and chymotrypsin.

Stability of the Acyl Enzyme

The acyl enzyme formed from the reaction of chymotrypsin, subtilisin BPN', or subtilisin Carlsburg with either of the two benzyl carbazates exhibited an exceptional stability which is reflected in its very slow rate of deacylation. In contrast, the three methyl carbazates which reacted with the subtilisins had substantially more rapid deacylation

rates implying that the acyl enzyme formed from these reactions is less stable.

One contributing factor to the stability of the acyl enzyme is the electronic contribution of the adjacent nitrogen atom. The presence of the unshared pair of electrons on the α -nitrogen atom would lead to a reduction in the electrophilicity of the adjacent carbonyl group which would cause the acyl enzyme to be more resistant to hydrolysis. Since a similar situation would be expected to prevail in the base catalyzed hydrolysis of these peptide carbazates, experiments were conducted to quantitate the overall contribution of this effect (Kurtz and Neiman, 1960). A fivefold difference in the rates of base catalyzed hydrolysis of Ac-Ala-ONp and Ac-Mec-ONp was observed. Although these studies indicate that the α -nitrogen atom does contribute to the stability of the acyl enzyme by reducing the electrophilicity of the adjacent carbonyl group, it does not appear reasonable that this effect can completely account for the loss of substrate activity.

A second factor which may contribute to the stabilization of the acyl enzyme would be the stereochemical requirements for hydrolysis. From a stereochemical standpoint, it would be expected that the highest rate of deacylation would occur when the p orbital of the carbonyl carbon is in a position for maximum overlap with the orbitals of the incoming water molecule. Furthermore, it has been proposed that one of the primary functions of the binding sites on these serine proteases is to bring the p orbitals of the acyl carbon into the proper orientation for maximum overlap with the orbitals of the attacking water (Robillard, et al., 1972). It has been shown that indoleacryloyl and carbonyl derivatives of chymo-

trypsin (Henderson, 1970; Robillard, et al., 1972) are also exceptionally resistant to deacylation which is attributed to the orientation of the p orbitals of the carbonyl moiety. The effect of extending the p orbital interactions of the carbonyl group for the acylated enzyme such as in the case of the indoleacryloyl and carbamyl chymotrypsin derivatives could result in an orientation of the carbonyl group which is less favorable for overlap with the orbitals of the attacking water molecule and resulting in a slower rate of deacylation. It has also been shown in crystallographic studies of chloromethyl ketone-inhibited subtilisin BPN' (Kraut, et al., unpublished results) that the carbonyl oxygen of the inhibitor portion of the derivative is held in place by hydrogen bonds from the side chain NH₂ of Asn-155 and the backbone NH of the active site Ser-221. The hydrogen bonding of these residues with the carbonyl oxygen of an acylated enzyme would be expected to increase the nucleophilic susceptibility of the carbonyl carbon making the acyl enzyme less resistant to hydrolysis. The improper orientation of the carbonyl group which could minimize these hydrogen bonding interactions would have the effect of decreasing the electrophilicity of the carbonyl carbon making it even less susceptible to nucleophilic attack by a water molecule.

These concepts may be employed to explain the large difference in the deacylation rates between the methyl and the benzyl carbazates with the subtilisins. Since it has been previously proposed that one of the functions of the binding site of these enzymes is to bring the p orbital of the carbonyl group into the proper orientation for deacylation, the proper side chain substituent would be required for this process by maximizing these binding interactions. Likewise, acylated enzymes which

possess unusual resistance to deacylation due to improper orientation of the carbonyl moiety would be expected to maintain this level of stability only as long as the p orbital of the carbonyl group does not assume an orientation which is favorable for hydrolysis. Thus the stabilization of the carbonyl group in an orientation which is unfavorable for deacylation would result in a more stable acylated enzyme. This may be accomplished by taking advantage of the binding site interactions. Since it is known that the subtilisins possess a broad primary subsite specificity for long hydrophobic side chains, it would be expected that the two benzyl carbazates would bind tightly to the enzyme resulting in the stabilization of the acylated enzyme by fixing the carbonyl in an improper orientation due to the presence of the α -nitrogen atom. In contrast, the methyl carbazates would be expected to fit in the primary binding site more loosely which may allow the carbonyl group to assume a conformation which would be more susceptible to nucleophilic attack by a water molecule resulting in a more rapid rate of deacylation. It is apparent, however, that a better understanding of this wobbling effect can be obtained only by studying the effects of varying the side chain length on these peptide carbazates.

CHAPTER V

CONCLUSIONS

A series of five acyl carbazates was tested for reactivity towards chymotrypsin A_α, subtilisin BPN', and subtilisin Carlsburg. The results of these studies indicate that the rate of reaction of these compounds with the enzymes was very dependent upon the nature of the carbazate substituents. Chymotrypsin was rapidly inactivated by the two benzyl carbazates Ac-Bzc-ONp and Ac-Ala-Bzc-ONp, while the three methyl carbazates Ac-Ala-Mec-ONp, Ac-Ala-Ala-Mec-ONp, and Ac-Ala-Ala-Pro-Mec-ONp reacted much slower (if at all). The two subtilisins reacted with all five compounds; however, a comparison of deacylation rates showed a marked difference between the methyl and the benzyl carbazates. The turnover rates of the methyl carbazates were very rapid compared to the significantly slower rates observed for the benzyl carbazates. These observations may be a result of a loose fit of the methyl group in the binding pocket of the subtilisins since these enzymes are known to have a specificity for long hydrophobic side chains. A comparison of the relative reaction rates of the two subtilisins with the five carbazates indicates that subtilisin Carlsburg is consistently more reactive towards these substrates than subtilisin BPN'. The initial burst of p-nitrophenol obtained upon reaction of chymotrypsin or the subtilisins with the carbazates was always observed to be stoichiometric indicating that these enzymes may be considered as possible active-site titrants.

CHAPTER VI

RECOMMENDATIONS

As stated above, a worthwhile experiment would be to prepare an appropriate isobutyl carbazate and measure the reaction rates of this compound with the two subtilisins. Since the reaction rates have been determined for Ac-Ala-Bzc-ONp and Ac-Ala-Mec-ONp, it would be best to prepare Ac-Ala-Ibc-ONp which would offer a ready comparison of the three rates. The isobutyl side chain should offer a better fit into the binding pocket than the corresponding methyl carbazate but should fit somewhat looser than the benzyl group. If the wobble hypothesis is correct, one would expect to see a rate of hydrolysis which is intermediate between that observed for the methyl and the benzyl carbazates. If this trend is observed, it may be appropriate to prepare a valine and an isoleucine analog of these compounds which would give a more complete picture of this effect.

APPENDIX

CHYMOTRYPSIN ASSAY PROCEDURE

The regeneration of enzymatic activity during the deacylation studies was measured according to the general assay method of Cunningham and Brown (1956). The substrate solution consisted of 0.01 M Ac-Tyr-OEt and 0.10 M CaCl_2 in 0.05 M phosphate (pH 7.0) and contained 5% acetonitrile. The substrate solutions were kept refrigerated and were used within three days of preparation. After the acylated enzyme solution had been prepared, several 100 μl aliquots were removed over a given time span and added to 10.0 ml of the substrate solution. The residual enzymatic activity was monitored on the pH-stat at pH 7.8 with 0.10 N NaOH as the titrate. The rates of deacylation were determined from the pH stat tracings obtained over the course of the enzyme reactivation.

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