I. AN INVESTIGATION OF CHYMOTRYPSIN A$_\gamma$ INHIBITION
WITH PEPTIDE ALDEHYDES AND RELATED ANALOGS

II. INHIBITION OF ELASTASE BY TETRAPEPTIDE
CHLOROMETHYL KETONES

A THESIS

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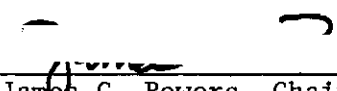
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Ronald Jay Whitley

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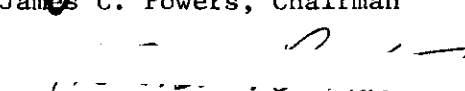School of Chemistry

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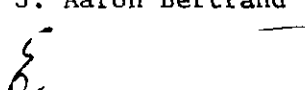INHIBITION OF ELASTASE BY TETRAPEPTIDE
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SUMMARY

PART I. AN INVESTIGATION OF CHYMOTRYPSIN A_γ INHIBITION
WITH PEPTIDE ALDEHYDES AND RELATED ANALOGS

Three peptide aldehydes and related analogs were synthesized
and tested for their ability to generate models of the tetrahedral
transition state for chymotrypsin-catalyzed substrate hydrolysis.
Competitive inhibition experiments with chymotrypsin A_γ show that
N-benzyloxycarbonylphenylalaninal (Z-Phe-H) was the most effective
inhibitor, binding about 400-fold more tightly than comparable amide
substrates. Two naphthalene aldehydes were also found to effectively
inhibit A_γ, but significant spectral perturbations were not observed
when either chromophoric aldehyde was complexed with the enzyme. X-ray
crystallographic studies, however, suggest that Z-Phe-H and the dipep-
tide aldehyde Ac-Ala-Phe-H inhibit chymotrypsin A_γ crystals without
altering unit cell dimensions. Semicarbazone derivatives of our pep-
tide aldehydes were seen to be slightly less reactive than the corre-
sponding aldehydes; peptide alcohols were unreactive. On the basis of
these kinetic results, together with data from other investigators, we
suggest that our aldehydes reversibly bind to chymotrypsin as covalent
tetrahedral hemiacetals, and that these enzyme-aldehyde complexes are
stereochemically analogous to the tetrahedral transition state for the
hydrolysis reaction.

PART II.   INHIBITION OF ELASTASE BY
TETRAPEPTIDE CHLOROMETHYL KETONES

Tetrapeptide chloromethyl ketones containing valine, isoleucine and threonine as the P_1 residues were synthesized as active-site directed irreversible inhibitors of elastase. Based on relative $k_{obsd}/[I]$ values, the P_1-valine and isoleucine chloromethyl ketones were observed to be reactive and specific inhibitors for both porcine pancreatic elastase and human leukocyte elastase. The analogous threonine chloromethyl ketone was relatively unreactive. Trypsin and chymotrypsin which are closely related to elastase were not significantly inhibited by our tetrapeptide chloromethyl ketones. With porcine pancreatic elastase, the valine and isoleucine chloromethyl ketone inhibitors were as effective or slightly more effective than our standard P_1-alanine tetra-peptide inhibitor. With human leukocyte elastase, on the other hand, the valine and isoleucine inhibitors were markedly more effective than the alanine ketone (the best one being Ac-Ala-Ala-Pro-Val-CH_2Cl). These results imply that the two elastolytic enzymes are different and dis-tinct. Human leukocyte elastase is now implicated in the pathology of pulmonary emphysema and related inflammatory diseases. Eventually chloromethyl ketone inhibitors may become valuable in treating these diseases.

PART I

AN INVESTIGATION OF CHYMOTRYPSIN A_γ INHIBITION

WITH PEPTIDE ALDEHYDES AND RELATED ANALOGS

CHAPTER I

INTRODUCTION

Among those enzymes which catalyze hydrolysis reactions, proteases, or protein-cleaving enzymes, are certainly the most well-known and intensively studied. These enzymes provide a gentle, and in some cases, specific method of affecting hydrolysis of peptide bonds of polypeptide substrates. Moreover, proteolytic enzymes are efficient catalysts. They increase the hydrolysis rate by a factor of about 10^5, thereby reducing the time taken for digestive processes involving these proteins from 50 years to a few hours.

Proteases are widely distributed in nature. In humans and other animals proteases are involved physiologically in such diverse biological functions as food digestion, blood coagulation, ovum fertilization and hormone production. In plants they catalyze the turnover of protein; in bacteria they presumably function as digestive enzymes. Because most of these proteases can be readily isolated and purified in large quantities, they are suitable for systematic study. Proteases for the most part are relatively simple, stable, low molecular weight enzymes, and the reactions they catalyze are generally not difficult to measure experimentally. Recently, they have attracted consideration attention in biomedicine due to their implication in certain diseases. Proteases are even becoming utilized commercially in the detergent and food processing industries.

Chymotrypsin (mw 25000) is a very well-characterized protease, and

most studies of this enzyme have been based on bovine pancreatic chymo-
trypsin A (EC 3.4.21.1). Originating in the acinous cells of the pancreas,
chymotrypsin A is produced in the form of a catalytically inactive precur-
sor called chymotrypsinogen. Activation of this zymogen is achieved by
specific proteolytic hydrolysis of certain peptide bonds (Wilcox, 1970;
Northrop et al., 1948). Biologically, active chymotrypsin serves as a
catalyst in the hydrolysis of food proteins in the mammalian small intes-
tine. Preferential cleavage of amide bonds occurs adjacent to the carboxyl
function of aromatic amino acid residues such as L-Phe, L-Tyr and L-Trp
(Cunningham, 1965; Nemath and Schwert, 1950). Proteolysis also occurs
adjacent to other large hydrophobic residues, as, for example, leucine and
methionine, but usually at a slower rate (Brenner et al., 1960; Roveny
et al., 1957). Although its natural substrates are polypeptides, chymo-
trypsin will hydrolyze specific substrates such as amides and esters of
aromatic amino acids as well as a broad variety of other hydrophobic
synthetic substrates and substrate analogs (Cunningham, 1965). As is
the case with most enzymes, chymotrypsin activity varies with pH and is
optimal between 7.5 and 9.0.

Four forms of chymotrypsin (α, γ, δ, π) result from proteolytic
activation of chymotrypsinogen, but the α-form is the one that has been
studied the most extensively. No structural differences have been found
between the α and γ forms even though the two enzymes crystallize in
different crystal habits and vary only slightly in their association
behavior and reactivity in solution (Segal et al., 1971a). α-chymotrypsin
contains 241 amino acid residues and 3 peptide chains (Meedom, 1959); its
amino acid sequence has been determined by Hartley (1964) and Meloun et al.

(1966). X-ray crystallographic investigations have revealed the three-dimensional structures of tosyl-chymotrypsin and native chymotrypsin A at high resolution (Blow, et al., 1969; Blow, 1971; Birktoft and Blow, 1972). Further investigations have studied the three-dimensional structures of chymotrypsin A bound with a variety of substrates and inhibitors (Steitz et al., 1969; Henderson, 1970; Segal et al., 1971a,b). Based on crystallographic investigations of α-chymotrypsin, Blow and his coworkers (1969) have postulated a catalytic mechanism in which the linear arrangement of residues Ser-195, His-57 and Asp-102 functioned in such a way as to allow charge polarization at the active site. In this theory the electrons on the buried aspartate are relayed along hydrogen bonds to the serine hydroxyl group helping to make it strongly nucleophilic and thus explaining its reactivity. Powerful support for this theory has recently been received from nmr studies in solution (Robillard and Shulman, 1972, 1974a,b).

A comprehensive mechanism for chymotrypsin action (eq. 1), whose various features have been extensively studied, is schematically depicted in Figure 1. ES_1 is the noncovalent

$$E + S \rightleftharpoons ES_1 \rightleftharpoons ES_{Tet} \xrightarrow{\overset{P_1}{\uparrow}} ES' \overset{H_2O}{\rightleftharpoons} ES'_{Tet} \longrightarrow E + P_2 \qquad (1)$$
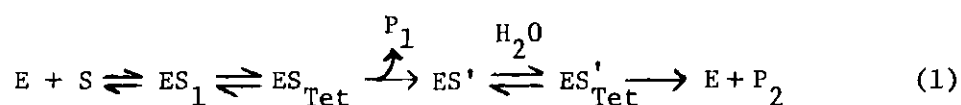
Michaelis complex, and ES_{Tet} is a tetrahedral intermediate which precedes formation of the covalent acyl-enzyme, ES'. P_1 represents the initially released amine of an amide substrate, and ES'_{tet} is a second tetrahedral intermediate preceding the regeneration of free enzyme and P_2, the
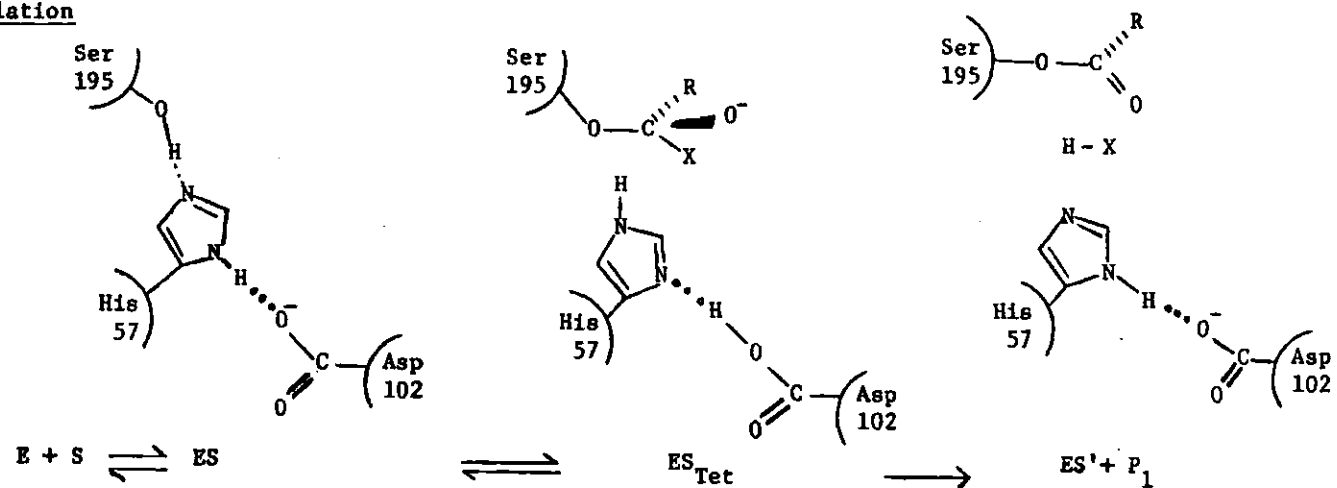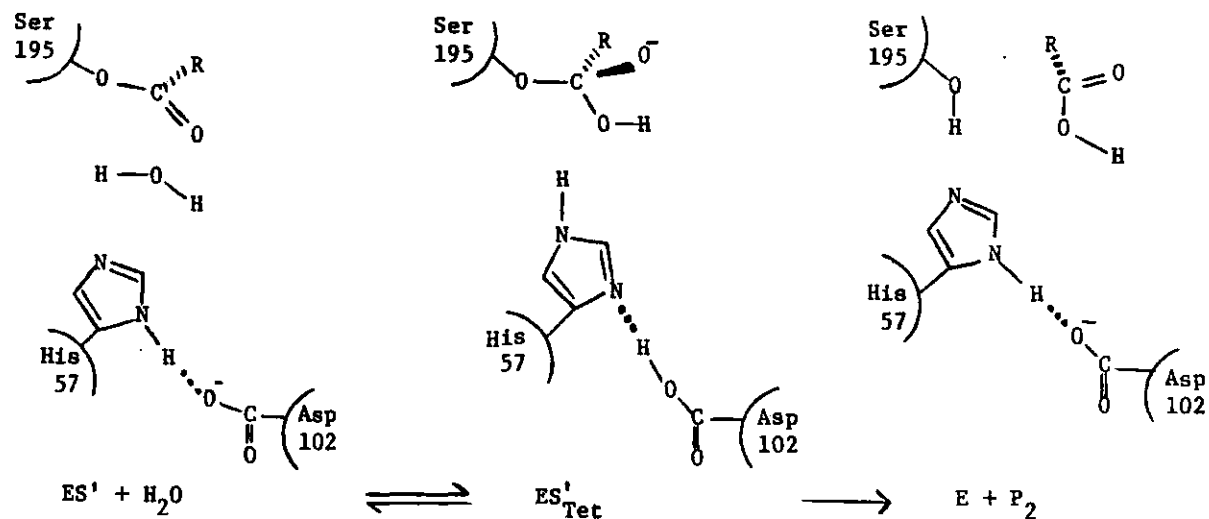
Figure 1. A Possible Mechanism of Action for Chymotrypsin. Note: From Zeffren and Hall, 1973.

carboxylic acid moiety. The inclusion of the two tetrahedral intermedi-
ates in this catalytic mechanism has been justified on several grounds
(Zeffren and Hall, 1973; Hess, 1971; Jencks, 1969). Direct kinetic evi-
dence for tetrahedral intermediate formation, however, has been supplied
by Hess et al., (1970). By employing temperature jump and stopped-flow
kinetic techniques, the direct observation of two distinct chymotrypsin-
substrate complexes prior to the formation of the acyl enzyme and release
of P_1 were made possible. These intermediates were thought to be the
Michaelis complex and a tetrahedral complex. Recently, crystallographic
observations of tetrahedral adducts have been made in the bovine trypsin-
basic pancreatic trypsin inhibitor complex (Ruhlman et al., 1973), in the
porcine trypsin-soybean trypsin inhibitor complex (Sweet et al., 1974),
and between aromatic boronic acids and subtilisin (Matthews et al., 1975).
In the case where the homologous chymotrypsin protease, trypsin, was
complexed with the large pancreatic trypsin inhibitor (Figure 2), the
O_γ atom of trypsin's Ser-195 was found to be covalently linked to the
carbonyl carbon of Lys-15 of the inhibitor. The negatively charged oxy-
gen at the tetrahedral carbon, moreover, was hydrogen bonded to the amide
nitrogens of Gly-193 and Ser-195 of trypsin.

A great deal of information about binding specificity has been
obtained from crystallographic investigations of chymotrypsin complexed
with small molecules. For example, calculated electron density maps
showed quite clearly that chymotrypsin's primary specificity site was a
hydrophobic depression or pocket about 12 x 6.5 x 4 Å in size on the
enzyme surface and into which the aromatic side chain of substrates or
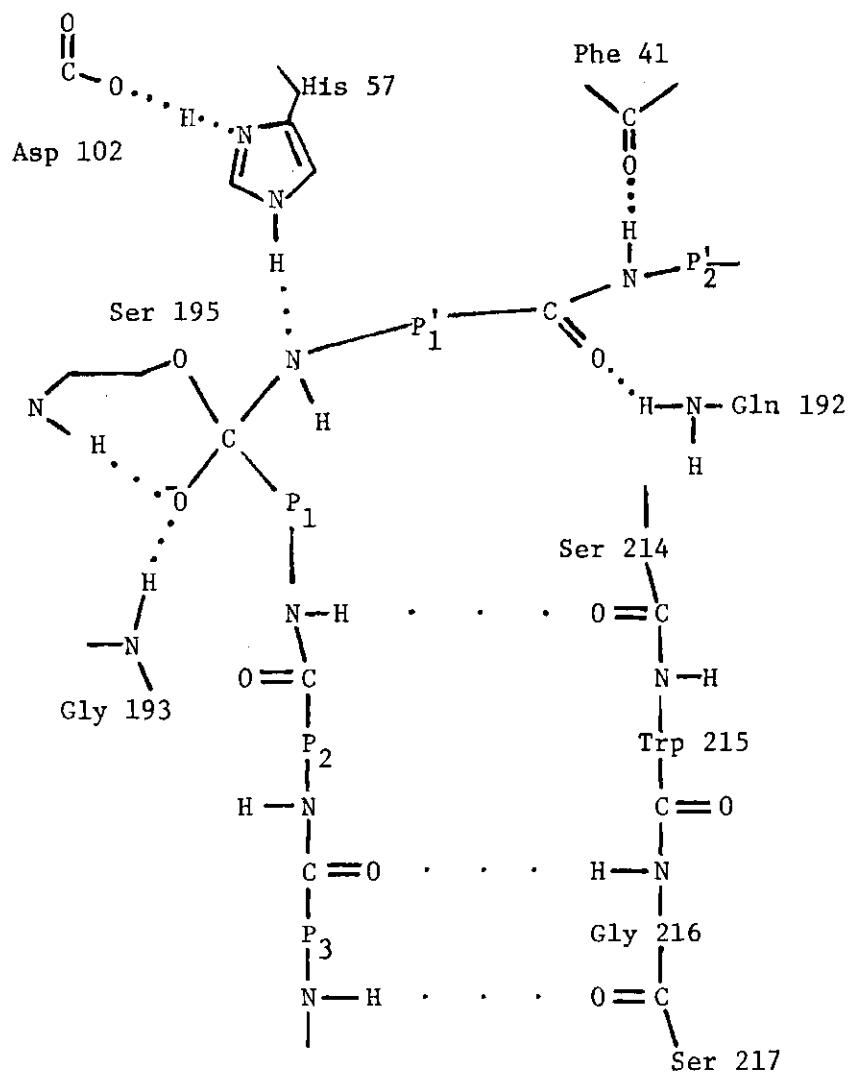
Figure 2.   An Illustration of the Binding Interactions Between
            Trypsin and the Pancreatic Trypsin Inhibitor.
            Note: From Lilljas and Rossmann, 1974.

inhibitors could fit snugly (Steitz et al., 1969). Even though these and other binding interactions have been studied, little detailed information has been revealed concerning the structural and conformational changes that take place during chymotrypsin catalysis. This situation provides the incentive for the development of a structural model of the enzyme-substrate complex preceding acyl enzyme formation. As previously implied, it is generally supposed that this complex may be a highly activated tetrahedral intermediate which resembles a transition state for the hydrolysis reaction.

This thesis, Part I, reports our approach toward studying this important tetrahedral enzyme-substrate complex. The substrate analogs chosen for study are peptide aldehydes and related compounds. Their synthesis as well as their kinetics, ultraviolet, and crystallographic behavior are described. In addition, some explanations and implications of the data are considered.

CHAPTER II

EXPERIMENTAL

Materials and Methods

Chymotrypsin A (lot 53C-8130) and subtilisin BPN' (lot 102C-0650) were purchased from Sigma Chemical Co. and used without further purification in the kinetic studies; their substrate, N-acetyl-L-tyrosine ethyl ester (Ac-Tyr-OEt), was synthesized in our laboratory and had mp 80-81°. Chymotrypsinogen A (lot 111C-8170) and acetyl-trypsin (lot 90C-2200) were also obtained from Sigma Chemical Co. and used without further purification. Diaflo ultrafilters used in enzyme concentration were purchased from Amicon Corp. Carboxymethyl cellulose, CM-52 microgranular, was purchased from Whatman Biochemicals Ltd. Sephadex G-25 fine was obtained from Pharmacia Fine Chemicals.

N-Benzyloxycarbonyl-L-phenylalanine chloromethyl ketone (Z-Phe-CH$_2$Cl) was prepared from Z-PheCHN$_2$ by the method of Shaw (1967). AgF and N,N-carbonyldiimidazole were purchased from PCR. N-methylaniline was obtained from Eastman Organic Chemicals and redistilled before use (B.P. 39°/300μ). 2-naphthaldehyde was purchased from Aldrich Chemical Co., Inc.; 1-naphthaldehyde was obtained from Eastman Organic Chemicals. Optically active amino acids were obtained from commercial sources. All other reagents and solvents were analytical grade.

Mass spectra were taken on a Perkin-Elmer Hitachi RMU-7L instrument, and nuclear magnetic resonance (nmr) spectra were taken on Varian A-60 or

T-60 instruments. Enzyme inhibition kinetics were performed on a
Radiometer automated pH-stat system (model TTT 11). Ultraviolet absorp-
tion studies were performed on a Beckman Model-25 spectrophotometer.
X-ray precession photographs were taken on a Buerger Precession Camera
(Model 3000) supplied by Supper Co. Integrated intensities were measured
from diffraction photographs by means of an automated Syntex microdensi-
tometer.

Dihydro-4-Benzyl-2H-1,3-Oxazine-2,5(3H)-Dione

N-benzyloxycarbonyl-L-phenylalanine diazoketone (1.5 gm, 4.6 mmol),
Prepared from Z-Phe-OH and diazomethane using a mixed anhydride method
(Penke et al, 1970), was dissolved in anisole (50 ml) and then added to a
10% ethereal solution of liquid anhydrous HF (3.0 gm, 15 mmol) in a poly-
ethylene bottle at 0°C. Stirring was continued at room temperature until
the reaction was complete as indicated by thin-layer chromatography.
Work-up of the mixture involved evaporation of the solvent in vacuo,
extraction of the residue into ethyl acetate, washing with citric acid and
$NaHCO_3$ solutions, drying over anhydrous magnesium sulfate and evaporation
to a yellow oil. The residue was then dissolved in chloroform and chroma-
tographed on Merck silica gel G 0.063 - 0.2 mm. The product was isolated
after elution with 2% methanol in chloroform. Recrystallization from
ethyl acetate-cyclohexane gave 0.48 gm (51%) of a white solid, mp 135-136°,
R_f .28 ($CHCl_3$ - CH_3OH, 9:1).

The nmr spectrum ($CDCl_3$) showed peaks at δ 7.30 (5H, s, Ph),
5.81(1H, b, NH), 4.53(2H, d, $PhCH_2O$), 4.11(1H, m, NHCHCO), 3.05(2H,
complex, $CH(CH_2)Ph$). The mass spectrum showed major peaks at m/e

$205(M^+, 45.8\%)$, $161(M - CO_2^+, 16.7\%)$, $118(NH = C(CH_2OH)+, 83.7\%)$ and

$91(C_7H_7+,$ base peak).

<u>Anal</u>. Calcd for $C_{11}H_{11}NO_3$: C, 64.39; H, 5.36; N, 6.81.

Found: C, 64.49; H, 5.22; N, 6.61.

<u>N-Benzyloxycarbonyl-L-Phenylalanine Hydroxymethylketone (Z-Phe-CH$_2$OH)</u>

N-Benzyloxycarbonyl-L-phenylalanine iodomethylketone

was prepared by iodination of Z-Phe-CH$_2$Cl with sodium iodide in acetone.

To a solution of Z-Phe-CH$_2$I (5.0 gm, 11.8 mmol) in acetonitrile (100 ml)

was added a solution of AgF (4 gm, 32 mmol) in water (5 ml). After stir-

ring for 30 minutes at 50°, the mixture was filtered and extracted

with ethyl acetate (2 x 50 ml). The organic extracts were dried over

magnesium sulfate and concentrated <u>in</u> <u>vacuo</u> to an oil. A solution of

this oil in chloroform was then chromatographed on silica gel (60 gm).

The crystalline product was isolated after elution with methanol-chloro-

form (1:100). Recrystallization from ethyl acetate-cyclohexane gave

1.8 gm (48%) of a white solid, mp 88-89°, R_f .41 (CHCl$_3$-CH$_3$OH, 1:24),

ir(CHCl$_3$), 1717 cm^{-1}(C=O). The nmr spectrum (CDCl$_3$) showed peaks at

$\delta 7.36(10H, m, Ph)$, $7.05(1H, m, N\underline{H})$, $5.10(2H, s, PhC\underline{H}_2O)$, $5.28(1H, b, C\underline{H}_2OH)$,

$4.60(1H, m, NHC\underline{H}CO)$, $4.18(2H, s, \underline{CH}_2OH)$, $3.06(2H, d, PhC\underline{H}_2CH)$. The nmr

spectrum (DMSO) showed the hydroxyl proton peak at 5.35(1H, t, CH$_2$O$\underline{H}$,

which disappeared upon adding D$_2$O). A singlet instead of a triplet

appeared in the double resonance spectrum (DMSO) of this compound, indi-

cating that the spin-spin coupling between the methylene and hydroxyl

hydrogens had been destroyed when the methylene hydrogens were irradiated.

The mass spectrum showed major peaks at m/e $313(M^+, .16\%)$,

282($PhCH_2OCONHCH(CH_2Ph)CO^+$, 1.1%), 254($PhCH_2OCONHCH(CH_2Ph)^+$, base peak),

210($PhCH_2NH = CHCH_2Ph^+$, 73.7%), 91($C_7H_7^+$, 78.9%).

<u>Anal</u>. Calcd for $C_{18}H_{19}NO_4$: C, 68.98; H, 6.11; N, 4.47.

Found: C, 68.87; H, 6.14; N, 4.42.

<u>N-Benzyloxycarbonyl-L-Phenylalaninal (Z-Phe-H)</u> was prepared using

methods developed by Japanese workers (Saeki <u>et al</u>., 1973; Shimizu <u>et al</u>.,

1972; Ito <u>et al</u>., 1972). N-benzyloxycarbonyl-L-phenylalanine (4.5gm,

15 mmol) was dissolved in dry tetrahydrofuran and cooled to 10° in a

three-necked flask equipped with drying tube, thermometer, rubber sep-

tum and magnetic stirrer. N,N-carbonyldiimidazole (2.7 gm, 16.8 mmol) was

added and the resulting pale yellow solution stirred for 15 minutes.

After cooling the reaction mixture to -15° in ethylene glycol/dry ice,

.788 <u>M</u> lithium aluminum hydride in tetrahydrofuran (28.5 ml, 22.5mmol)

was slowly added dropwise. Stirring was continued an additional 45 min-

utes at -15°. Excess $LiAlH_4$ was then destroyed by slowly adding dropwise

30 ml of ice cold 1 <u>M</u> NH_4Cl. The reaction mixture was filtered and the

precipitates washed with boiling ethyl acetate (3 x 50 ml) to remove any

occluded organic product. After evaporation of the tetrahydrofuran, the

residue was suspended in ethyl acetate. The combined ethyl acetate

extracts were dried over anhydrous magnesium sulfate and evaporated to

give a crude pale yellow oil.

This oil was dissolved in chloroform and chromatographed on

Merck silica gel G 0.063 - 0.2 mm. The product which eluted with chlor-

oform-methanol (65:1) was recrystallized from ethyl acetate-cyclohexane

to give a white crystalline solid (1.3 gm, 30%), mp 78-79°,

R_f .73($CHCl_3$-CH_3OH, 19:1), ir($CHCl_3$), 1720 cm (C = 0). The nmr spectrum

($CDCl_3$) showed peaks at δ9.75(1H, s, CHO), 7.47(10H, m, Ph), 5.17(2H,

s, Ph$\underline{CH}_2$O), 4.52(1H, m, NH$\underline{CH}$CO), 3.14(2H, d, CH$\underline{CH}_2$Ph).

The mass spectrum showed major peaks at m/e 283(M^+, .13%),

254(($M-CHO)^+$, 22%), 210(PhCH$_2$CHCHCH$_2$Ph$^+$, 14%), 164 (PhCH$_2$OCONHCH$_2^+$, 5.3%)

91($C_7H_7^+$, base peak).

Further elution with chloroform-methanol (24:1) gave a second

crystalline product, N-benzyloxycarbonyl-L-phenylalaninol. Recrystalli-

zation from ethyl acetate-cyclohexane gave 0.8 gm(19%) of a white solid,

mp 94-94.5°, R_f .51($CHCl_3$-CH_3OH, 19:1).

The nmr spectrum ($CDCl_3$) showed peaks at δ7.30(10H, d, Ph), 5.18

1H, b, NH), 5.07(2H, s, Ph$\underline{CH}_2$O), 3.89(1H, m, NH$\underline{CH}$), 3.61(2H, m, CH$\underline{CH}_2$OH),

2.84(2H, d, CH$\underline{CH}_2$Ph), 2.31(1H, s, CH$_2\underline{OH}$, which disappeared upon adding

$D_2$0).

The mass spectrum showed major peaks at m/e 285(M^+, .15%),

254(($M-CH_2OH)^+$, 1.4%), 210(PhCH$_2$NHCHCH$_2$Ph$^+$, 1.4%), 194(($M-C_7H_7)^+$, 11.6%),

150(PhCH$_2$CH(NH)CH$_2$OH$^+$, 4.8%), 91($C_7H_7^+$, base peak).

Anal. Calcd for $C_{17}H_{19}NO_3$: C, 71.58; H, 6.67; N, 4.91.
Found: C, 71.60; H, 6.74; N, 4.89

N-Benzyloxycarbonyl-L-Phenylalaninal Semicarbazone

To the crude oil of N-benzyloxycarbonylphenylalaninal was imme-

diately added a solution of semicarbazide hydrochloride (4.5 g) and

sodium acetate (7.8 gm) in water (50 ml). The mixture was shaken vigor-

ously, warmed on a steam bath for 15 minutes, treated with 95% ethanol

until turbidity just disappeared, and then placed in the refrigerator

overnight. The reaction mixture was diluted with a small amount of water to separate an oil. The solvent was decanted and the residual oil dissolved in ethanol and filtered. The filtrate was cooled in an ice bath and water gradually added until turbidity. The mixture was allowed to stand for several days in the refrigerator. A crystalline product was isolated and recrystallized from ethyl acetate-cyclohexane-methanol to give 1.8 gm (43%) of a white solid, mp 130-131°, R_f 0.74 ($CHCl_3$-CH_3OH, 4:1).

The nmr spectrum ($CDCl_3$) showed peaks at δ 9.66(1H, s, CH=N$\underline{NH}$), 7.36 (10H, d, Ph), 7.07(1H, d, CH$\underline{CH}$NNH), 6.18(1H, b, CON$\underline{H}$CH), 5.68(2H, b, CON$\underline{H}_2$), 5.04(2H, s, Ph$\underline{CH}_2$O), 4.56(1H, m, NH$\underline{CH}$CH$_2$Ph), 2.95(2H, d, CH$\underline{CH}_2$Ph).

The mass spectrum showed major peaks at m/e 340 (M^+, .6%), 282($PhCH_2OCONHCH(CH_2Ph)CH=NH^+$, 4.8%), 189 ($PhCH_2CHCNNHCONH_2^+$, 11.4%), 150($PhCH_2OCONH^+$, 14%), 108($PhCH_2OH^+$, 33%), 91($C_7H_7^+$, base peak).

Anal. Calcd for $C_{18}H_{20}N_4O_3$: C, 63.53; H, 5.88; N, 16.47 Found: C, 63.58; H, 5.96; N, 16.42.

Formyl-L-Phenylalanine (CHO-Phe-OH)

Formyl-L-phenylalanine was prepared by the peptide coupling method of du Vigneaud et al. (1932). To a mixture of L-phenylalanine (49.5 gm, 0.30 mole) in refluxing 97% formic acid (160 ml) was added dropwise acetic anhydride (29.1 ml, 0.28 mmol). The reaction mixture was stirred for 5 minutes and then quenched with 90 ml of ice water. Evaporation of the mixture in vacuo gave a crystalline product which was recrystallized from hot water to give 51 gm (88%) of a white solid, mp 171-172°. Lit: mp 168° (Fisher and Schoeller, 1907).

Formyl-L-Phenylalaninal (CHO-Phe-H)

To a solution of formyl-L-phenylalanine (3.9 gm, 20 mmol) in
anhydrous tetrahydrofuran (150 ml) was added N,N-carbonyldiimidazole
(3.6 g, 22.2 mmol). The colorless solution was stirred at 15° for 10
minutes and then cooled to -15°. 1.15 $\underline{M}$ lithium aluminum hydride in
tetrahydrofuran (26 ml, 30 mmol) was subsequently added dropwise over a
15 minute period; excess reagent was destroyed by addition of ice cold
1 $\underline{M}$ NH_4Cl (50 ml). After evaporation of the tetrahydrofuran under N_2,
the residue was suspended in ethyl acetate. The precipitates were then
quickly filtered and washed with solvent to remove occluded organic pro-
ducts. The ethyl acetate extracts were combined, dried over anhydrous
magnesium sulfate, and evaporated to a colorless oil. This crude oil
rapidly decomposes and yellows unless stored immediately under nitrogen
in the freezer.

Isolation of pure aldehyde involved several purification steps.
The crude oil was dissolved in chloroform and chromatographed on Merck
silica gel G, 0.063-0.2 mm. Elution with chloroform-methanol (19:1)
gave aldehyde-containing fractions which were then pooled and evaporated.
Further separation of the aldehyde from traces of imidazole was
effected by means of liquid chromatography using a 6' x 3/4" silica Woelm
column and a chloroform-methanol solvent system (19:1). Aldehyde frac-
tions were pooled, evaporated to a small volume (1 or 2 ml) and trans-
ferred to a Hickman still. After the complete removal of organic sol-
vents, the pale yellow oil was distilled (160-190°, 400μ) to give a vis-
cous, colorless oil which crystallized on cooling. Recrystallization from

ethyl acetate-cyclohexane gave a white solid (.4 gm, 12%), mp 65-70°,

R_f .47 ($CHCl_3$-CH_3OH, 9.1), ir (neat) 1728 cm^{-1}(C=O).

The nmr ($CDCl_3$) showed peaks at δ9.56(1H, s, C$\underline{H}$O), 8.23(1H, s,

$\underline{H}$CONH), 7.28(5H, s, Ph), 7.19(1H, b, CON$\underline{H}$), 4.75(1H, q, NHC$\underline{H}$),

3.14(2H, d, $\underline{CH}_2$Ph).

The mass spectrum showed major peaks at m/e 177(M^+, 2.9%),

159((M- $H_2O)^+$, 7.0%), 148((M- CHO$)^+$, 19.3%), 132($PhCH_2CHCHO^+$, 24.0%),

120($PhCH_2CHNH_2{}^+$, 32.1%), 91($C_7H_7{}^+$, base peak). The mass of the m/e

177 peak (M^+) was exactly measured at 177.0781 (177.0789 calculated for

$C_{10}H_{11}NO_2$).

Formyl-L-Phenylalaninal Semicarbazone

To the crude oil of formylphenylalaninal was immediately added a

solution of semicarbazide hydrochloride (4 gm) and sodium acetate (6 gm)

in water (40 ml) and shaken vigorously. The mixture was treated with

95% ethanol until turbidity disappeared and then allowed to stand at 0-5°

overnight. The semicarbazone derivative which separated from the solu-

tion was recrystallized from water, affording white needles (2.8 gm, 60%)

mp 178-179°, R_f .46 ($CHCl_3$-CH_3OH, 4:1).

The nmr (DMSO-d6) showed peaks at δ9.96(1H, s, NN$\underline{H}$), 8.18(1H, d,

CHCHNN$\underline{H}$), 7.92(1H, s, $\underline{H}$CO), 7.10(5H, s, Ph), 6.97(1H, b, CON$\underline{H}$CH), 6.19(2H,

s, CON$\underline{H}_2$), 4.60(1H, m, NHC$\underline{H}$CO), 2.80(2H, d, $\underline{CH}_2$Ph)

The mass spectrum showed major peaks at m/e 234(M^+, 5.3%),

189((M-HCONH$_2)^+$, 5.7%), 174 $PhCH_2CHCHNNHCO^+$, 6.1%), 143(M-$C_7H_7)^+$, base

peak), 100($HCONHCHCHNNH_2{}^+$, 77.2%), 91($C_7H_7{}^+$, 95.6%).

Anal. Calcd for $C_{11}H_{14}N_4O_2$: C, 56.41; H, 5.98; N, 23.93.
Found: C, 56.51; H, 6.12; N, 23.79.

N-Acetyl-L-Alanine (Ac-Ala-OH)

The acetylation of L-alanine was accomplished by adding dropwise

acetic anhydride (40.0 ml,0.47 m) to L-alanine (51.1 gm,0.57m) in

refluxing glacial acetic acid (300 ml). After stirring for an addi-

tional 20 minutes, the reaction mixture was cooled to room temperature

by the addition of water (100 ml) and then evaporated to a yellow oil.

The oil was washed with hot benzene (250 ml), the benzene was removed,

and the resulting oil treated with ethyl acetate (375 ml). The product

which crystallized immediately was isolated and recrystallized from hot

ethyl acetate to yield a white solid (50.5 gm, 67%) mp 126-127°,

$[\alpha]_D^{23}$ = -65.7°(C3.8, H_2O); Birnbaum et al. (1952) report mp 125°,

$[\alpha]_D^{25}$ = -66.2°(C2.0, H_2O).

N-Benzyloxycarbonyl-L-Phenylalanine N-Methylanilide (Z-Phe-N(Me)Ph).

The N-methylanilide of N-benzyloxycarbonyl-L-phenylalanine was

prepared from Z-Phe-OH and N-methylaniline using a mixed anhydride

coupling method. One equivalent of isobutyl chloroformate (2.6 ml)

was added to a solution of 1 equivalent of Z-Phe-OH (6.0 gm, 20 mmol)

and 1 equivalent of N-methylmorpholine (2.2 ml) dissolved in anhydrous

tetrahydrofuran at -15°. This mixture was stirred for approximately

10 minutes, 1 equivalent of N-methylaniline (2.1 ml) was added,

and the mixture stirred for 1 hour as the solution was allowed to

warm to room temperature. The hydrochloride salts were filtered, water

was added, and then the tetrahydrofuran was evaporated. The water solu-

tion was extracted at least three times with ethyl acetate. The com-

bined ethyl acetate extracts were washed with 0.5 M citric acid and 1.0

M NaHCO$_3$ and then dried over anhydrous magnesium sulfate. Evaporation

of the ethyl acetate resulted in a colorless oil. This oil was dis-
solved in chloroform and chromatographed on Merck silica gel G 0.063 -
0.2 mm. The product eluted with 2% methanol in chloroform to give a
colorless oil (6.8 gm, 87%), R_f .65 ($CHCl_3$-CH_3OH, 19:1).

The nmr spectrum ($CDCl_3$) showed peaks at δ7.01 (15H, m, Ph),
6.56 (1H, b, N̲H̲), 5.01(2H, s, PhC̲H̲$_2$O), 4.60(1H, m, NHC̲H̲), 3.13(3H, s,
NC̲H̲$_3$), 2.88(2H, b, C̲H̲$_2$Ph).

L-Phenylalanine N-Methylanilide Hydrobromide (HBr·PheN(Me)Ph).

Z-Phe-N(Me)Ph (4.0 gm, 10.3 mmol) was deblocked by dropwise
addition of a saturated solution of HBr in acetic acid. The reaction
mixture was stirred for approximately 30 minutes. This procedure was
repeated again until visible reaction ceased. Anhydrous ether (200 ml)
was then slowly added to precipitate a yellow-orange oil. After vigor-
ously shaking the reaction vessel, the ether was decanted from the
residual oil. The oil was then washed with several portions of ether.
The product crystallized but was too hygroscopic to isolate. It was
immediately dissolved in dimethylformamide (10 ml) and used in further
coupling reactions.

Acetyl-L-Alanyl-L-Phenylalanine N-Methylanilide (Ac-Ala-Phe-N(Me)Ph)

Acetyl-L-alanine (prepared by the acetylation of L-alanine with
1 equiv of acetic anhydride in hot glacial acetic acid) was activated
using the mixed-anhydride procedure. Acetyl-L-alanine (1.62 gm, 12.2
mmol) was dissolved in anhydrous tetrahydrofuran and cooled to -15°.
N-methylmorpholine (1 equiv.) was added followed by 1 equiv. of

isobutyl chloroformate (1.6 ml). Deblocked Z-Phe-N(Me)Ph (4 gm, 12.2

mm, in 10 ml DMF) was added followed by 1 equiv. N-methylmorpholine

(1.35 ml). The reaction mixture was allowed to warm to room temperature

and stirring was continued for 1 hour. The hydrochloride salts were

then filtered, water was added, the organic solvent evaporated, and the

aqueous solution extracted with ethyl acetate. After aqueous citric

acid and bicarbonate washes, the solvent was evaporated and gave a color-

less oil. Purification was effected by chromatography on 0.063-0.2 mm

silica gel G by Merck. The product which eluted with 4% methanol in

chloroform was recrystallized from ethyl acetate-cyclohexane giving white

needles (2.1 gm, 47%), mp 143.5-144, R_f .52 (CHCl$_3$-MeOH, 19:1).

The nmr spectrum (CDCl$_3$) showed peaks at δ 7.77(1H, d, $\underline{NH}$), 7.08

(10H, m, Ph), 6.90(1H, b, $\underline{NH}$), 4.73(2H, m, NH$\underline{CH}$CO), 3.24(3H, s, N$\underline{CH}_3$),

2.87(2H, m, $\underline{CH}_2$Ph), 1.86(3H, s, $\underline{CH}_3$CO), 1.34(3H, d, CH($\underline{CH}_3$)CO).

The mass spectrum showed major peaks at m/e 367(M^+, 2.7%), 281

(PhN(Me)COCH(CH$_2$Ph)NHCO+, 1.6%), 276((M-C$_7$H$_7^+$, 1.0%), 261((M-NMePh)+,

19.5%), 237(PhCHCHCON(Me)Ph+, 15.9%), 233(M-CON(Me)Ph, 2.7%), 146(PhCHCH

(NH)CO+, 20.0%), 120(PhCH$_2$CHNH$_2^+$, base peak), 114(CH$_3$CONCHCH(CH$_3$)CO+,

10.5%), 107(Ph(Me)NH+, 68.4%), 91(C$_7$H$_7$+, 8.6%).

Anal. Calcd for C$_{21}$H$_{25}$N$_3$O$_3$: C, 68.67; H, 6.81; N, 11.44.
Found: C, 68.57; H, 6.77; N, 11.38.

N-Acetyl-L-Phenylalaninal (Ac-ala-Phe-H)

Acetyl-L-alanyl-L-phenylalaninal was prepared from the N-methylanil-

ide derivative of N-acetylalanylphenylalanine. Ac-Ala-Phe-N(Me)Ph

(4.0 gm, 10.8 mmol) was dissolved in anhydrous tetrahydrofuran and cooled

to -15°. 1.15 $\underline{M}$ lithium aluminum hydride in tetrahydrofuran (19 ml,

21.6mmol) was slowly added dropwise. Stirring was continued an addi-

tional 75 minutes at -15°. Excess LiAlH$_4$ was destroyed by slowly add-

ing dropwise cold 1 $\underline{M}$ NH$_4$Cl (15 ml). The precipitates were then

filtered, the tetrahydrofuran evaporated and the residue suspended in

ethyl acetate. After extracting the occluded solids with boiling ethyl

acetate, the organic fractions were combined, dried over anhydrous mag-

nesium and evaporated to a crude pale yellow oil.

This oil was dissolved in chloroform and chromatographed on 100-

200 mesh Florisil. Elution with 3% methanol in chloroform gave the

desired product as a colorless glass (1.2 gm, 42%), R$_f$ .41 (CHCl$_3$-CH$_3$OH,

9:1), ir (CHCl$_3$) 1730 cm^{-1}(C=O).

The nmr spectrum (CDCl$_3$ showed peaks at δ9.38(.5H, s, C$\underline{H}$O),

7.10(5H, s, Ph), 6.63(2H, b, N$\underline{H}$), 4.43(2H, m, NHC$\underline{H}$), 3.33(.5H, s,

$\underline{C}$ H (OH)$_2$), 3.03(2H, d, C$\underline{H}_2$Ph), 2.70(1H, b, CH(O$\underline{H}$)$_2$, which disappeared

upon adding D$_2$O), 1.87(3H, s, C$\underline{H}_3$CO), 1.27(3H, m, CH(C$\underline{H}_3$)CO)

The mass spectrum showed major peaks at m/e 262(M+, 2.3%),

245((M-OH)+, 29.7%), 234((M-CO)+, 10.3%), 201((245-CH$_3$CO)+, 20.0%),

120(PhCH$_2$CHNH$_2$+, base peak), 114(CH$_3$CONHCH(CH$_3$)CO+, 36.0%), 91(C$_7$H$_7$+,

88.6%). The mass of the m/e 244 peak (M-H$_2$O) was exactly measured at

244.1224 (244.1212 calculated for C$_{14}$H$_{16}$N$_2$O$_2$).

In addition to the desired aldehyde, two additional products from

the Florisil chromatography were isolated and crystallized.

N^1-(acetylalanyl)-N^2-phenyl-N^2-methyl-1-benzyl-1,2-ethylenediamine

(Ac-Ala-NHCH(CH$_2$Ph)CH$_2$N(Me)Ph) was eluted with 2% methanol in chloroform

and was recrystallized from ethyl acetate-cyclohexane to yield a white

solid (.5 gm, 13%), mp 181-181.5°, R$_f$ .60 (CHCl$_3$-CH$_3$OH, 19:1).

The nmr spectrum (CDCl$_3$) showed peaks at δ 7.00(10H, m, Ph),

6.36(1H, b, NH), 4.36(2H, m, NHCHCO), 3.45(2H, t, CHCH$_2$NMePh), 2.99(3H,

s, NCH$_3$), 2.88(2H, d, CH$_2$Ph), 1.89(3H, s, CH$_3$CO), 1.13(3H, d, CH(CH$_3$)CO).

The mass spectrum showed major peaks at m/e 353(M+, 3.3%),

267(PhN(Me)CH$_2$CH(CH$_2$Ph)NHCO+, .7%), 223(PhCH$_2$CHCHN(Me)Ph+, 14.0%),

208(PhCH$_2$CHCHNPh+, .6%), 132(PhN(Me)CCH$_2$+, 2.7%), 120(PhN(Me)CH$_2$+,

base peak), 91(C$_7$H$_7$+, 5.9%).

Anal. Calcd for C$_{21}$H$_{27}$N$_3$O$_2$: C, 71.39 H, 7.65; N,11.90.
Found: C, 71.41; H, 7.70; N, 11.88.

Further elution with chloroform-methanol (19:1) gave a second

crystalline product, N-acetylalanylphenylalaninol (Ac-Ala-NHCH(CH$_2$Ph)

CH$_2$OH). Recrystallization from ethyl acetate-cyclohexane gave .8 gm,

(28%) of a white solid, mp 136-138°, R$_f$ .35 (CHCl$_3$-CH$_3$OH, 19:1),

The nmr spectrum (CDCl$_3$) showed peaks at δ 7.28 (5H, s, Ph),

7.11(1H, b, NH), 6.62(1H, b, NH), 4.45(2H, m, NHCHCO), 3.64(2H, d,

CH$_2$OH), 3.56(1H, b, OH, which disappeared upon adding D$_2$O), 2.92(2H, d,

CH$_2$Ph), 1.98(3H, s, CH$_3$CO), 1.37(3 H, d, CH(CH$_3$)CO).

The mass spectrum showed major peaks at m/e 265(M+1, 2.2%),

264(M+, 1.5%), 246(M-H$_2$O, .38%), 234 ((265-CH$_2$OH)+, 9.8%), 173((M-C$_7$H$_7$)+,

155((M-H$_2$O-C$_7$H$_7$)+, 10.5%), 134(PhCH$_2$CH$_2$CHO+, 15.8%), 130(CH$_3$CONHCH(CH$_3$)

C(OH)=NH$_2$+, 65.4%), 120(PhCH$_2$CHNH$_2$+, base peak), 114(CH$_3$CONHCH(CH$_3$)CO+,

82.0%), 91(C$_7$H$_7$+, 40.6%, 86(CH$_3$CONHCH(CH$_3$(+, 54.1%).

Anal. Calcd for C$_{14}$H$_{20}$N$_2$O$_3$: C, 63.64; H, 7.58; N, 10.60.
Found: C, 63.65; H, 7.63; N, 10.52.

N-Acetyl-L-Alanyl-L-Phenylalaninal Semicarbazone

N-acetylalanylphenylalaninal semicarbazone was prepared by adding

a solution of semicarbazide hydrochloride (.9 gm) and sodium acetate

(1.4 gm) in water (9 ml) to N-acetylalanylphenylalaninal (.9 gm). The

reaction mixture was vigorously shaken, warmed on a steam bath for 10

minutes, diluted with 95% ethanol (9 ml), shaken again and finally

allowed to stand at 0-5°. After several days, the reaction mixture was

evaporated to an oil _in vacuo_, dissolved in 10% methanol in chloroform,

and chromatographed on Merck silica gel 0.063 - 0.2 mm. The semi-

carbazone derivative was eluted with chloroform-methanol (3:1) and was

recrystallized from methanol-acetone to give a white solid (.6 gm, 55%),

mp 206-207°, R_f .48 (CHCl$_3$-CH$_3$OH, 4:1), R_f .48(CHCl$_3$-CH$_3$OH, 4:1).

The nmr spectrum (DMSO-d6) showed peaks at δ9.95(1H, s,NNH̲), 8.17(1H,

b, CH̲CHNNH), 7.98(1H,b,CONH̲CH), 7.28(5H,s,ph), 7.20(1H,b,CONH̲CH), 6.32(2H,

s, CONH̲$_2$), 4.50(2H, m, NHCH̲CO), 2.90(2H, d, PhCH̲$_2$), 1.87(3H, s, CH̲$_3$CO),

1.07(3H, d, CH(CH̲$_3$)CO).

The mass spectrum showed major peaks at m/e 319 (M+, .26%),

261(CH$_3$CONHCH(CH$_3$)CONHCH(CH$_2$Ph)C=NH+, .88%), 234(CH$_3$CONHCH(CH$_3$)CONHCH$_2$

(CH$_2$Ph)+, 6.4%), 228((M-C$_7$H$_7$)+, 10.8%), 185(CH$_3$CONHCH(CH$_3$)CONHC=CH-N=NH$_2$+,

6.5%), 148(NH$_2$CH(CH$_2$Ph)CO+, 4.2%), 130(CH$_3$CONHCH(CH$_3$)C(OH)=NH+, 18.3%),

120(PhCH$_2$CHNH$_2$+, 39.4%), 91(C$_7$H$_7$+, base peak).

Anal. Calcd for C$_{15}$H$_{21}$N$_5$O$_3$: C, 56.43; H, 6.58; N, 21.94.

Found: C, 56.33; H, 6.67; N, 21.87.

3-(Benzyloxycarbonylamino)-4-Phenyl-1,2-Butanediol

3-Benzyloxycarbonylamino-4-phenyl-1,2-butanediol was prepared by

the sodium borohydride reduction of N-benzyloxycarbonylphenylalanine

hydroxymethylketone. To a stirred solution of Z-Phe-CH$_2$OH (50 mg) in

isopropanol (6 ml) was added solid NaBH$_4$ (1.6 mg). 90 minutes later,

water (6 ml) and sodium chloride (.5 gm) were added. The mixture was

then extracted with chloroform (2 times), dried over anhydrous mag-

nesium sulfate, and evaporated to give a white solid, mp 110-111°.

The nmr spectrum (CDCl$_3$) showed peaks at δ 7.24(10H, d, Ph),

5.03(2H, s, PhCH$_2$O), 3.85(1H, b, NHCH(CH$_2$Ph)), 3.55(3H, m, CH(OH)CH$_2$(OH)),

2.84(4H, b, CH(CH$_2$Ph); and two OH, which were exchanged upon adding D$_2$O).

The mass spectrum showed major peaks at m/e 315(M+, 13%),

284(PhCH$_2$OCONHCH(CH$_2$Ph)CH(OH)+, .27%), 254(PhCH$_2$OCONHCH(CH$_2$Ph)+, 1.7%),

224 ((M-C$_7$H$_7$)+, 6.4%), 210(PhCH$_2$NHCHCH$_2$Ph+, 3.0%), 164(PhCH$_2$NHCHCH$_2$Ph+,

3.4%), 116(CH$_2$(OH)CH(OH)CH=NCO+, 70.9%) 108(PhCH$_2$OH+, 43.6%),

91(C$_7$H$_7$+, base peak).

Oxidation of Diol

N-Benzyloxycarbonylphenylalaninal (Z-Phe-H) was prepared by the

periodic acid oxidation of 3-benzyloxycarbonylamino-4-phenyl-1,2-butane-

diol. The diol (.16mm) was dissolved in anhydrous tetrahydrofuran and

then reacted with H$_5$IO$_6$ (68 mg). After stirring 1 hour at room tempera-

ture, the reaction mixture was filtered to remove precipitated iodic

acid. The tetrahydrofuran was evaporated and the residual oil extracted

with ethyl acetate. The ethyl acetate solution was washed with water,

1 M bicarbonate, water, saturated KCl solution, dried over anhydrous

magnesium sulfate, and evaporated to a colorless oil. The pure product

was identical (nmr, TLC) with an authentic sample of Z-Phe-H.

β-(2-Naphthyl)acrolein(2-$C_{10}H_7$CH=CH-CHO) was prepared from 2-naphthalde-

hyde and acetaldehyde by a procedure similar to that reported by

Israelashvili et al. (1951). Upon workup, the crude aldehyde was

obtained as a black oil. Purification was effected by chromatography

on 0.05 - 0.2 mm silica gel G. The product was eluted with 10% ethyl

acetate in pentane and was crystallized from ethyl acetate-cyclohexane

as yellow needles, mp 125-126° (Jutz (1958) reports mp 125°); R_f .18(ethyl

acetate-cyclohexane, 1:19); γ_{max}(CHCl$_3$), 1678 cm^{-1}(C = 0); λ_{max}(EtOH)

274(ε35000) and 313(ε30000).

The nmr spectrum showed peaks at δ 9.70(1H, d, CHO), 7.2-8.1(8H,

complex, $C_{10}H_7$CH) and 6.72(1H, d of d, J = 8 Hz, CH=$\underline{CH}$-CHO). The mass

spectrum showed major peaks at m/e 182(M^+, base peak), 165((M-OH)$^+$, 7.5%),

153((M-CHO$^+$, 80.5%), 141((M-CHCHO)$^+$, 18.2%), and 128((M-C=CHCHO)$^+$, 66.7%).

β-(1-Naphthyl)acrolein(1-$C_{10}H_7$CH=CH-CHO) was prepared from 1-naphthalde-

hyde and acetaldehyde by a procedure similar to that reported by

Israelashvili et al. (1951). Upon workup, the crude aldehyde was

obtained as a yellow oil (B.P. 190°/400μ). Purification was effected

by chromatography on 0.05-0.2 mm silica gel G. The product eluted with

5% ethylacetate in pentane and was crystallized from 5% ethyl acetate

in cyclohexane as colorless needles, mp48.5-49° (Jutz (1958) reports

mp 47-48°); R_f .14(ethyl acetate-cyclohexane, 1:19);γ_{max}(neat), 1677 cm^{-1}

(C=0); λ_{max}(EtOH) 254(ε21500) and 340(ε12500).

The nmr spectrum showed peaks at δ 9.73(1H, d, $\underline{CH}$O), 7.2-8.3(8H,

complex. $C_{10}H_7$CH), 6.67(1H, d of d, J = 8 Hz, CH=$\underline{CH}$-CHO). The mass spectrum

showed major peaks at m/e 182(M^+, 71.4%) 165((M-OH)$^+$, 10.8%), 153((M-CHO)$^+$,

base peak), and 128((M-C=CHCHO)+, 25.8%).

N-Acetyl-L-Phenylalanine Methyl Ketone (Ac-Phe-CH$_3$)

The diazoketone Ac-Phe-CHN$_2$ was prepared from acetyl-L-phenylala-
nine and diazomethane using a mixed-anhydride procedure. Ac-Phe-OH
(1.0 gm, 5 mmol) was dissolved in tetrahydrofuran and cooled to -10° in
a three-necked flask equipped with stirrer, drying tube and thermometer.
N-methylmorpholine (.55 ml, 5 mmol) was added, followed by isobutylchloro-
formate (.65 ml, 5 mmol); mixed anhydride formation was practically
instantaneous. After ca 10 minutes, the N-methylmorpholine salts were
filtered and diazomethane (10 mmol) added. The reaction mixture was
allowed to stand overnight at 4°C. Excess 51% hydriodic acid was added
at 5°C. Stirring was continued until nitrogen evolution diminished.
After workup of the reaction mixture, the resulting crude oil was chromato-
graphed on silica gel. The product was eluted with 3% methanol in chloro-
form and recrystallized from ethyl acetate-cyclohexane to give white
needles, mp 120-120.5, R$_f$ .47(chloroform-methanol, 9:1). The nmr spec-
trum (CDCl$_3$) showed peaks at δ 7.23(5H, s, Ph), 7.12(1H, b, NH),
4.85(1H, m, NHCHCO), 3.09(2H, d, PhCH$_2$CH), 2.13(3H, s, CHCOCH$_3$),
1.94(3H, s, CH$_3$CONH).

The mass spectrum showed major peaks at m/e 205(M$^+$, 3%), 162(Ac-
NHCH(CH$_2$Ph)+, 50%), 120(NH$_2$CH(CH$_2$Ph)+, base peak), 91(C$_7$H$_7$+, 28%),
43(CH$_3$CO+, 38%).

Anal. Calcd for C$_{12}$H$_{15}$NO$_2$: C, 70.24; H, 7.32; N, 6.83.
Found: C, 70.34; H, 7.40; N, 6.77.

Reaction of Chymotrypsin with Inhibitors

The competitive inhibition of chymotrypsin A_γ was carried out
under pseudo-first-order conditions at 25.0° ± .5 and pH 7.80 ± .10.
Stock inhibitor solutions were prepared by dissolving a weighed amount
of inhibitor in 1,2-dimethoxyethane (DME) which had been redistilled
over CaH_2 and stored over 4Å molecular sieves. Inhibitor solutions at
appropriate concentrations were prepared by serial dilution of the stock
solutions with DME. A stock solution of chymotrypsin, prepared in
1.0 mM HCl, was stored at 4° and had a concentration of 13mM or 3.1 mg/ml
as determined by uv absorbance at 282 nm. N-acetyl-L-tyrosine ethyl ester
(Ac-Tyr-OEt) solutions were prepared by serial dilution of a 5 mM
Ac-Tyr-OEt solution containing $0.1\underline{F}$ $CaCl_2$ and 5% (v/v)DME. The inhibi-
tion reaction was begun by adding 100 µℓ of an inhibitor solution to
10.0 ml of an Ac-Tyr-OEt solution, adjusting the pH to 7.8, and then
adding a 100 µℓ aliquot of an enzyme solution which had been prepared by
diluting 0.2 ml of the stock chymotrypsin solution with distilled water.
The final enzyme concentration was 0.023 µ M. The rate of hydrolysis of
Ac-Tyr-OEt by chymotrypsin was measured potentiometrically using a pH-
stat. All assays were carried out in a thermostated vessel under a
nitrogen atmosphere. The titrant used was 0.0470 $\underline{F}$ NaOH (0.1085 $\underline{F}$ in
some later runs). Three substrate concentrations (1.0, 2.0 and 3.0 mM
Ac-Tyr-OEt) were used for each inhibitor concentration with each point
being replicated at least two times. Initial velocities were obtained
from the pH-stat recorder tracings by the method of Henderson (1971).

For each inhibitor a value of the dissociation constant, K_I, was

calculated from an Eadie-Hofstee form of the Michaelis-Menton equation

(eq. 2)

$$\frac{v}{s} = \frac{V_{max}}{(1 + \frac{I}{K_I})(K_m)} - \frac{v}{(1 + \frac{I}{K_I})(K_m)}$$ (2)

using a least-squares computer program. Correlation coefficients of

better than 0.96 were obtained throughout.

Reaction of Subtilisin with Inhibitors

The competitive inhibition of Subtilisin BPN' was carried out

under conditions virtually identical with those employed in the inhibi-

tion reactions with chymotrypsin. Solutions of inhibitor and substrates

were made up to the correct concentrations as previously described for

chymotrypsin. A stock subtilisin solution was freshly prepared each

day by dissolving 1.9 mg of enzyme in 1.0 ml of a 0.1 $\underline{M}$ phosphate buffer

at pH 7.0. The enzyme concentration was determined by uv absorbance at

280 nm. The inhibition reaction was initiated by adding 100 $\mu\ell$ of an

inhibitor solution to 10.0 ml of an Ac-Tyr-OEt solution, adjusting the

pH to 7.8, and then adding a 250 $\mu\ell$ aliquot of an enzyme solution which

had been prepared by diluting 0.5 ml of the stock subtilisin solution

with distilled water. The inhibition kinetics were assayed and analyzed

as previously described for chymotrypsin.

Preparation of Crystals of Chymotrypsin A$_\gamma$ for X-ray Analysis.

Crystallization leading to a limited number of single crystals of

uniform size was carried out as follows. Lyophilized chymotrypsin A$_\gamma$

(120 mg) was dissolved in 1 mM HCl to a concentration of 60 mg/ml. The

concentrated enzyme solution (2 ml) was filtered through a 0.9 x 25 cm

column of G-25 fine Sephadex which had been thoroughly prewashed with

distilled water. An effluent fraction of 4.0 ml containing most of the

protein (as indicated by trichloroacetic acid precipitation) was col-

lected in a dust free container, and the pH adjusted to 5.6. Aliquots

(2 ml) of the solution were mixed in small dust free crystal dishes

with equal volumes of a salt solution, 4.0 $\underline{M}$ $(NH_4)_2SO_4$-0.02 $\underline{M}$ cacodylate

buffer (pH 5.6). Care was taken not to nucleate the solution during mix-

ing. The final enzyme concentration was $\underline{ca}$. 15 mg/ml.

The dishes were seeded by touching the liquid surface with a glass

fiber that had been dipped into the mother liquor of a stock preparation

of tetragonal A_γ crystals. Each dish was then sealed and stored at 20°.

Well-formed tetragonal bipyramids (the characteristic crystalline habit

of bovine γ-chymotrypsin), appeared within a week. When these crystals

had reached the size of 0.3 - 0.5 mm in the longest dimension (about two

months), they were used in the x-ray studies.

Preparation of Tetragonal A_γ Crystals from Chymotrypsinogen

Seed crystals of chymotrypsin A_γ were grown from enzyme freshly

derived from zymogen by rapid activation (Wilcox, 1970). Chymotrypsinogen

(1.1g) was treated with acetyl-trypsin (40 mg) at 5° and pH 7.5 for 1

hr. The mixture of enzyme species was then separated on CM-cellulose at

5° using a .075 - .225 $\underline{M}$ phosphate linear gradient (pH 6.2). The major

fraction consisting of A_δ was collected, the pH adjusted to 4.0, and the

protein concentrated in an Amicon Diaflo apparatus, using UM 20 membranes.

During this stage of the preparation, the A_δ species was largely converted

to A_γ. The concentrated enzyme solution was then filtered through G-25

fine Sephadex. After adjusting the pH to 5.6, aliquots of the solution

were mixed with equal volumes of the saturated ammonium sulfate-cacodylate

buffer salt solution previously described. Each dish was then sealed

and stored at 20°. Small seed crystals appeared several months later.

Preparation of Inhibited Crystals for X-Ray Analysis

Chymotrypsin A_γ crystals, grown from a half-saturated ammonium

sulfate (pH 5.6) salt solution, were allowed to soak in 2.4 $\underline{M}$ phosphate

(pH 5.6) for 1 day to remove $(NH_4)_2SO_4$. Reaction of the crystals with

each of the two inhibitors, Ac-Ala-Phe-H and Z-Phe-H, was carried out by

the following procedure. Some of the aldehyde was dissolved in acetoni-

trile. This solution was then mixed with the 2.4 $\underline{M}$ phosphate (pH 5.6)

salt solution to give a 1-2% acetonitrile mixture; γ-crystals were seen

to be soluble in salt solutions containing 3-4% acetonitrile. Some of

the inhibitor precipitated from the mixture, indicating saturation. A

slurry was maintained by frequent stirring and was exchanged slowly with

the solution which bathed the enzyme crystals. The reaction was allowed

to proceed at 20° with occasional mixing and additions of fresh inhibitor

solution. After approximately 2 weeks soaking, the crystals were then

used for x-ray analysis since the inhibited crystals began to signifi-

cantly crack after 3-4 weeks soaking.

X-ray Data Collection

Inhibited γ-chymotrypsin crystals of suitable size, typically

0.5 mm x 0.3 mm, were carefully positioned in acid-washed capillary tubes

made of Lindmann glass. The c-axis (longest dimension) was oriented

approximately parallel to the capillary axis. Excess liquid was removed

using slivers of filter paper, but the base of a crystal was left in a

slight puddle. After sealing, the capillary tube was glued to a stand-

ard size metal pin, and the pin was then mounted on a standard goniometer

head.

Crystals were aligned and centered on a Buerger precession camera

fitted with a Polaroid XR-7 film cassett. Intensity data was collected

by the precession method using Illford Industrial G X-ray film. Precession

photographs at 8 and 16 hrs were obtained for the hOl level of each

derivative crystal using filtered CuK_α radiation (λ = 1.53 Å). The

crystal-to-film distance was 100 mm, and the precession angle was 16°45'.

A screen of 10 mm radius and a spacing of 33.3 mm were also used. All zero-

level photographs, taken at spindle settings found during alignment and

centering, possessed mirror-mirror symmetry. Integrated intensities were

measured from these precession films using an automated microdensitometer.

Control photographs of uninhibited crystals were supplied by Mr. D.

Carroll.

Ultraviolet Absorption Studies

Ultraviolet absorption spectra were measured with matched, two-com-

partment cuvets (path length, 0.44 cm per compartment) and a Beckman

Model 25 recording spectrophotometer. Aldehyde solutions were freshly

prepared by dissolving weighed amounts of β-(2-naphthyl)acrolein and

β-(1-naphthyl) acrolein in 1,2-dimethoxyethane (DME) and diluting with

pH 7.8 buffer (.1 $\underline{M}$ phosphate) resulting in a 10% DME solution. A

stock solution of chymotrypsin A_γ was prepared in 1 mM HCl and had a

concentration of 0.11 mM by uv absorbance. Spectra were recorded at
ambient temperature from 410 nm to 230 nm with aldehyde (compartment 1)
and enzyme (compartment 2) in the sample beam $\underline{vs}$ .1 $\underline{M}$ phosphate buffer,
pH 7.8, in the reference beam. Within 5 minutes of mixing the aldehyde
and enzyme solutions contained in the sample cuvette, spectra were again
recorded over the same wavelength range. Before mixing, concentrations
were as follows: $2-C_{10}H_7CH=CH-CHO$, 30 or 60 μM; $1-C_{10}H_7CH=CH-CHO$, 125
or 250 μM; γ-chymotrypsin, 3 or 110 μM.

In separate experiments, difference spectra were measured with
aldehyde (compartment 1) and chymotrypsin (compartment 2) in the sample
beam $\underline{vs}$ aldehyde and chymotrypsin (compartment 1) and pH 7.8 phosphate
buffer (compartment 2) in the reference beam. In order to minimize
error, the same pipet was used to prepare the sample and reference mix-
tures. Chymotrypsin was added to the cuvets within 15 minutes of
recording the spectrum from a stock solution (0.11 mM). Final concen-
trations were: $2-C_{10}H_7CH=CH-CHO$, 15 or 30 μM; $1-C_{10}H_7CH=CH-CHO$, 75 or
125 μM; γ-chymotrypsin, 55 μM. Control runs, in which buffer was sub-
stituted for the enzyme or the inhibitor, were also performed. All
spectra were recorded from 450 nm to 240 nm at 0.1 absorbance units
full scale.

CHAPTER III

RESULTS AND DISCUSSION

Efforts were directed toward the development and study of a structural model of the tetrahedral enzyme-substrate complex preceding acyl enzyme formation. Specific goals were: a) the synthesis of a small inhibitor molecule resembling a natural substrate that would complex with either chymotrypsin or subtilisin such that the enzyme active site serine had added to the carbonyl group of the inhibitor. This derivative would be analogous to the tetrahedral intermediate observed in the enzymatic hydrolysis of the peptide bond by serine proteases; b) a kinetic and crystallographic investigation of this derivative.

Synthesis of the Inhibitors

Peptide fluoromethyl ketones were initially chosen to serve as our substrate analogs. Because peptide fluoromethyl ketones are structurally similar to normal chymotrypsin substrates, they were considered to be ideal analogs.

$$\underset{\underset{R'}{|}}{RCONHCHCONHR''} \qquad \underset{\underset{R'}{|}}{RCONHCHCOCH_2F}$$
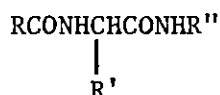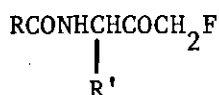
normal substrate   peptide fluoromethyl ketone

For example, the fluorine atom was not expected to participate in

nucleophilic displacement reactions. Hence, fluoromethyl ketones would

probably not react with the enzyme's active site histidine residue.

The fluoromethyl ketone functional group ($COCH_2F$), moreover, is about

the same size and shape as a normal peptide amide bond and, due to the

electronegative fluorine atom, might be expected to have almost the

same polarity as a peptide bond. Thus, a complex of a peptide fluoro-

methyl ketone with chymotrypsin might exist in a form in which the active

site serine (Ser-195) had added across the carbonyl group. This non-

productive adduct would be expected to be an analog of the tetrahedral

intermediate found to intervene in chymotrypsin acylation and deacylation

and as such, would be a good candidate for x-ray crystallography.

Although many different synthetic schemes and chemical systems were tried,

attempts to prepare these peptide fluoromethyl ketones were not success-

ful. For example, the reaction of a peptide diazoketone with anhydrous

HF did not yield the expected fluoroketone even though analogous methods

had been used to prepare chloromethyl ketones (Shaw, 1967). Instead a

cyclization reaction occurred (eq. 3). The products

$$
\begin{array}{ccc}
 & & O \\
 & & \parallel \\
 & & O \\
 & & \parallel \\
C_6H_5CH_2OCNHCHCOCHN_2 & \xrightarrow{\text{HF}} & \\
\quad | & & \\
CH_2C_6H_5 & & \\
\end{array}
\qquad (3)
$$
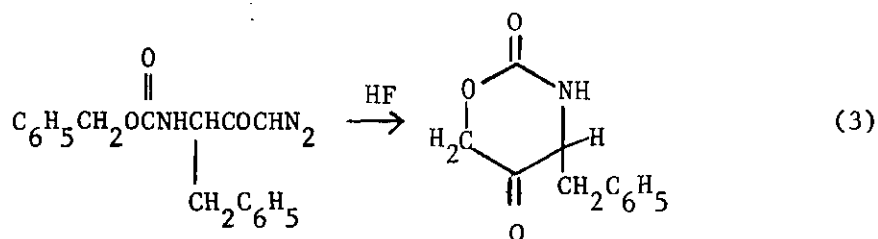
of the reactions of peptide iodomethyl ketones with AgF, on the other

hand, were found to be the corresponding alcohols (eq. 4)

$$\underset{\overset{|}{CH_2C_6H_5}}{RCONHCHCOCH_2I} \quad \xrightarrow[\text{aqueous}]{\text{AgF}} \quad \underset{\overset{|}{CH_2C_6H_5}}{RCONHCHCOCH_2OH} \qquad (4)$$
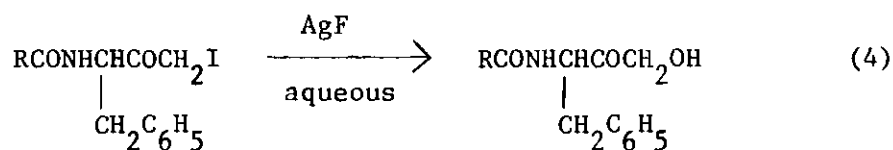
and not the expected primary alkyl halides (Tannhauser et al., 1956).

Failing to realize our goals with peptide fluoromethyl ketones, efforts were then directed toward developing aldehyde analogs of peptide substrates. Being about the same size and shape as a peptide bond, the aldehyde functional group was considered to be ideal. Moreover, aldehydes are known to be very susceptible to carbonyl addition reactions and are frequently unstable in aqueous or alcohol solution, forming hydrates or hemiacetals. Hence, the hemicetal (I), which is probably formed when a serine protease interacts with an aldehyde analog, should resemble the highly activated tetrahedral intermediate suggested in normal substrate hydrolysis of peptide bonds. Recent studies

$$\underset{\overset{\displaystyle O}{\|}}{R-C-H} \qquad \overset{\displaystyle Enzyme}{\underset{\displaystyle OH}{\overset{|}{\underset{|}{CH_2}}}} \qquad\qquad \underset{\overset{\displaystyle OH}{|}}{R-C-H} \qquad \overset{\displaystyle Enzyme}{\underset{\displaystyle O}{\overset{|}{\underset{|}{CH_2}}}}$$

Enzyme | CH₂ | OH — R – C – H ‖ O

Enzyme | CH₂ | O — R – C – H | OH

I
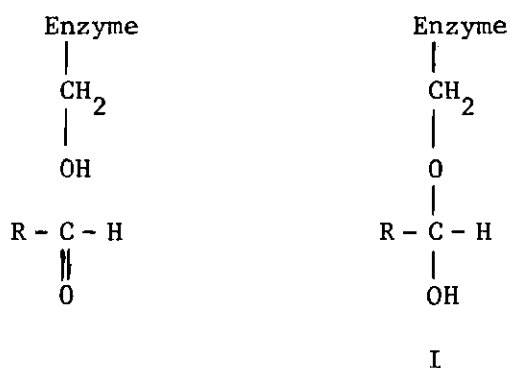
have shown that aldehyde analogs of specific substrates are potent competitive inhibitors of trypsin, chymotrypsin, elastase and papain, binding many times more tightly than substrates. For example, two peptide aldehydes analogous to amide substrates of elastase have been synthesized by Thompson (1973) and shown to bind to the enzyme up to

5000-fold more tightly than the substrates. Aldehydes, structurally

related to the acyl portion of papain substrates, have also been syn-

thesized and found to be extremely potent competitive inhibitors

(Westerik and Wolfenden, 1972). Interestingly, a novel class of

naturally occurring peptide aldehydes, leupeptins, have been shown to

effectively inhibit various proteases such as trypsin and papain

(Aoyagi et al,1969a,b; Maeda, 1971), and their structures have been

confirmed by synthesis (Shimuzu et al., 1972; Kawamura et al., 1969,

Kondo et al., 1969). Furthermore, various synthetic analogs of these

leupeptins have demonstrated marked inhibition of chymotrypsin (Ito,

et al., 1972).

The synthesis of peptides containing aldehyde functional groups

represents a rather new experimental endeavor in synthetic peptide

chemistry. Guided by the studies of several Japanese workers (Ito, et al,

1972; Saeki et al., 1973, Shimuzu et al., 1972), the synthesis of three

P_1-phenylalanine peptide aldehydes was undertaken. The dipeptide

aldehyde, Ac-Ala-Phe-H (II), was to be prepared from Z-Phe-H (III) for use in

$$\begin{array}{lll}
\text{R-NHCHC-H} & R = \text{Ac-Ala-} & \text{(II)} \\
\quad\quad| & & \\
\quad\text{CH}_2 & \text{Z-} & \text{(III)} \\
& \text{CHO-} & \text{(IV)}
\end{array}$$
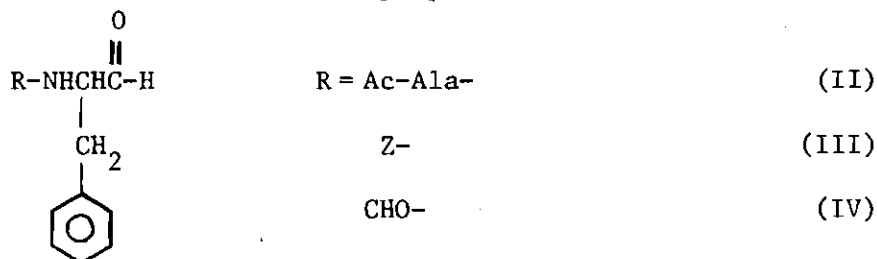
crystallography studies with chymotrypsin A_γ. CHO-Phe-H (IV), on the other

hand, was designed for use with α-chymotrypsin since this enzyme crystal

lattice was not expected to accommodate larger peptides (Birktoft et al.,

1970). Two of these inhibitors, CHO-Phe-H and Z-Phe-H were successfully

synthesized by reducing the imidazolides of CHO-Phe-OH and Z-Phe-OH with

$LiAlH_4$ in anhydrous tetrahydrofuran (eq. 5). Reduction of carboxylic acid

$$R-\underset{\underset{CH_2C_6H_5}{|}}{NH-CH}-\underset{\underset{}{||}}{\overset{O}{C}}-OH \quad \xrightarrow{\qquad \left(\begin{smallmatrix} N \\ N \end{smallmatrix}\right)_2 CO \qquad} \quad R-\underset{\underset{CH_2C_6H_5}{|}}{NH-CH}-\underset{\underset{}{||}}{\overset{O}{C}}-N{\overset{N}{\underset{}{\diagdown}}} \quad \xrightarrow[2)NH_4Cl]{1)LiAlH_4} \quad R-\underset{\underset{CH_2C_6H_5}{|}}{NH-CH}-\underset{\underset{}{||}}{\overset{O}{C}}-H$$

$$(5)$$
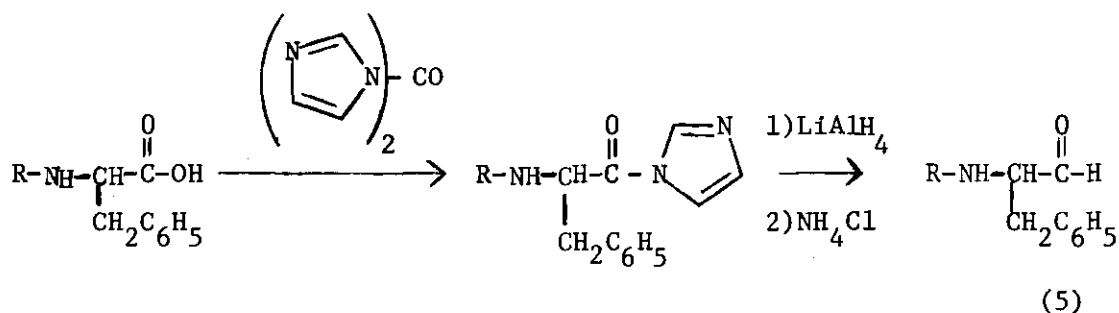
imidazolides with $LiAlH_4$ was originally reported by Staab and Braunling

(1962) but first applied to peptide imidazole derivatives by Shimizu and

coworkers in 1972. Little racemization was reported to have occurred

during these reductions.

Crystalline Z-Phe-H was obtained readily after chromatographic

purification. Conversion of the aldehyde to the corresponding semicar-

bazone confirmed our synthesis. An alternative synthesis of Z-Phe-H was

effected by sodium borohydride reduction of the hydroxymethyl ketone,

$Z-Phe-CH_2OH$, followed by periodic acid oxidation of the resulting 1,2-diol

(eq. 6, $R = Z - NH-CH(CH_2C_6H_5)-$).

$$\underset{R-\overset{\overset{O}{||}}{C}-CH_2OH}{} \quad \xrightarrow{NaBH_4} \quad \underset{R-\overset{\overset{OH}{|}}{CH}-\overset{\overset{OH}{|}}{CH_2}}{} \quad \xrightarrow{H_5IO_6} \quad \underset{R-\overset{\overset{O}{||}}{C}-H}{} \qquad (6)$$
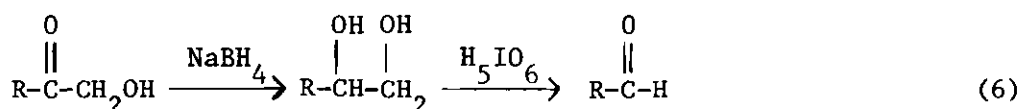
Purification of formylphenylalaninal (CHO-Phe-H) presented

formidable difficulties. This compound was eventually obtained as a

colorless oil by high pressure liquid chromatography. Distillation of

this oil provided an air-sensitive, but crystalline, white solid. The

aldehyde thus obtained was characterized as a crystalline semicarbazone.

Several approaches were considered in our attempts to prepare the

N-acylated dipeptide aldehyde, Ac-Ala-Phe-H. Because the aldehyde func-

tional group has a tendency to react with amino groups, the desired

compound must either be synthesized after the rest of the peptide has been constructed or should be protected (e.g. by means of a semicarbazone) during coupling reactions. The former approach was adopted after failing to remove the semicarbazone blocking group. Direct reduction of the corresponding imidazole derivative of Ac-Ala-Phe-OH was unsatisfactory because of the poor solubility of the starting amino acid in anhydrous tetrahydrofuran or diethyl ether. The synthesis of aldehydes by LiAlH$_4$ reduction of amides derived from N-methylaniline has been reported to give good results (Weygand et al., 1954). Consequently, synthetic efforts were directed toward the preparation of Ac-Ala-Phe-N(Me)Ph. Attempts to couple the dipeptide acid, Ac-Ala-Phe-OH, with N-methylaniline using the dehydrating agent dicyclohexyl carbodiimide (DCCI) resulted in the isolation of a racemized amide product. Varying amounts of racemization are often observed in DCCI coupling reactions, and, in those cases where certain N-acylated peptides are involved, can be identified by the nmr method of Weinstein and Prichard (1972). To circumvent this problem, a mixed-anhydride procedure was employed to prepare the optically active amide. The required sequence of reactions is shown in Figure 3. N-acetyl-L-alanyl-L-phenylalaninal was obtained as a colorless glass after reducing the N-methylanilide with LiAlH$_4$. The corresponding peptide alcohol and amine were also isolated from the reaction mixture. A crystalline semicarbazone derivative of the aldehyde was synthesized and characterized.

Although peptide aldehydes were specifically designed for use in crystallographic studies with chymotrypsin A, it seemed reasonable to suppose that aldehydes in general might be used to provide information

Ac-Ala-OH   Z-Phe-OH

$\downarrow$ iBuOCOCl $\downarrow$ iBuOCOCl

  MeNC$_4$H$_8$O   MeNC$_4$H$_8$O

Ac-Ala-OCOO$_i$Bu   Z-Phe-OCOOiBu

                                                   $\downarrow$ C$_6$H$_5$NH(CH$_3$)

                                                   Z-Phe-N(CH$_3$)C$_6$H$_5$

                                                   $\downarrow$ HBr/HOAc

  MeNC$_4$H$_8$O   HBr·Phe-N(CH$_3$)C$_6$H$_5$

                  $\downarrow$

            Ac-Ala-Phe-N(CH$_3$)C$_6$H$_5$

            $\downarrow$ 1) LiAlH$_4$

                 2) NH$_4$Cl

            Ac-Ala-Phe-H

Peptide Aldehyde
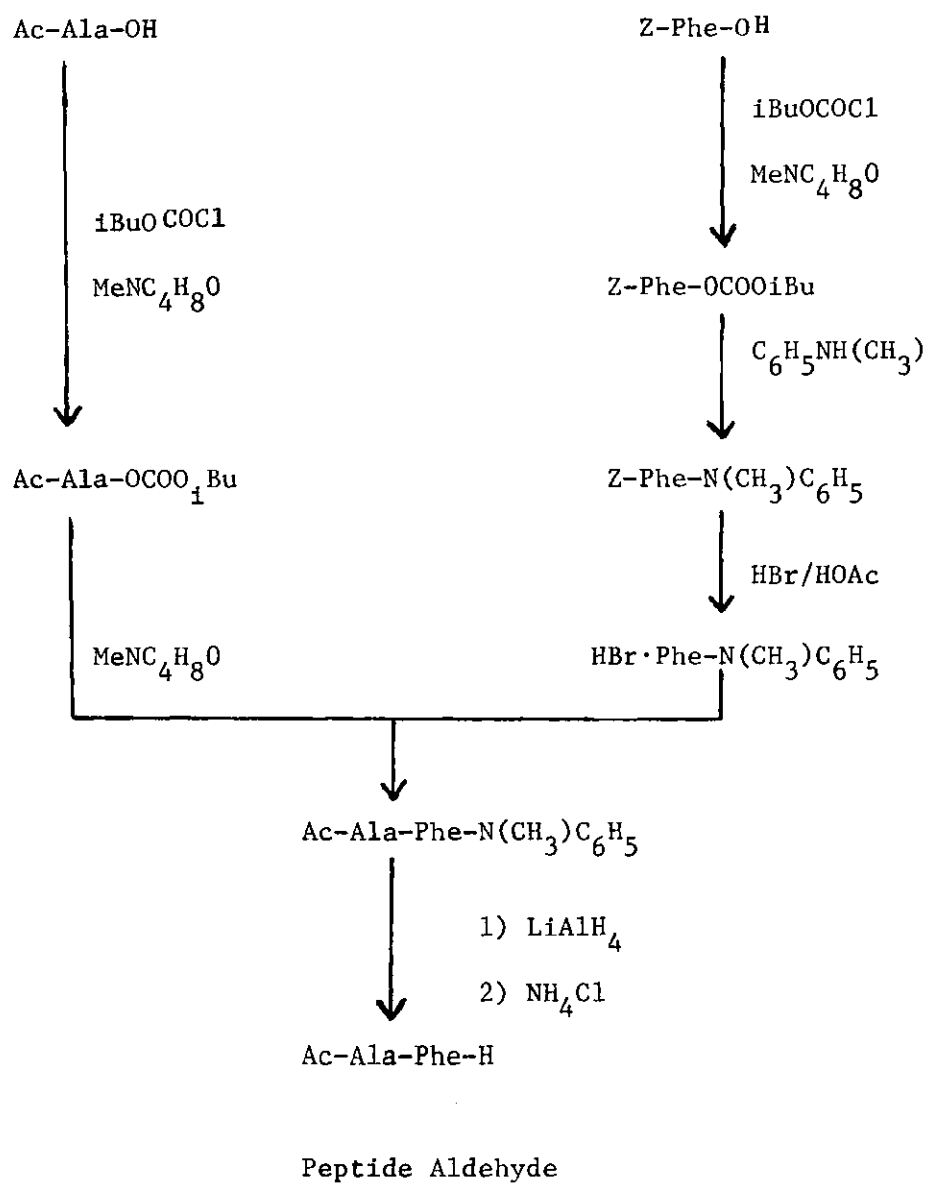
Figure 3. Reaction Scheme for a Synthetic Preparation of
           Ac-Ala-Phe-H.

about the structure of the enzyme complex in solution. Consequently,

we prepared two intensely chromophoric aldehyde inhibitors,

β-(1-naphthyl)acrolein   β-(2-naphthyl)acrolein

V   VI
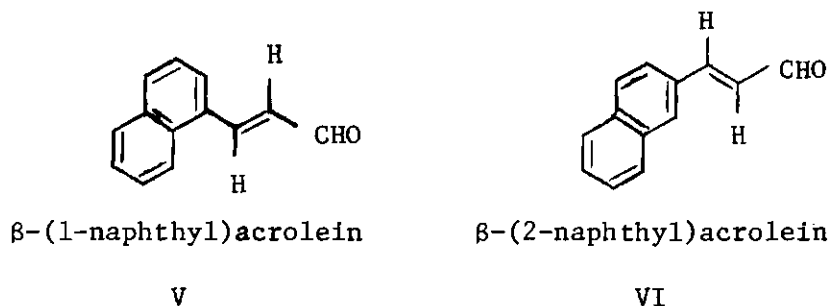
V and VI, by slight modifications of published procedures (Israelashvili

et al., 1951). Both naphthalene aldehydes were crystallized and character-

ized for further use.

Kinetics of Inhibition

The most appropriate parameter used to compare the relative bind-

ing strengths of the various competitive inhibitors is the dissociation

constant, K_I. For competitive inhibition (eq. 7) the rate of reaction of an

$$E \cdot I \xrightleftharpoons[-I]{+I} E \xrightleftharpoons{+S} E \cdot S \longrightarrow E + P \qquad (7)$$
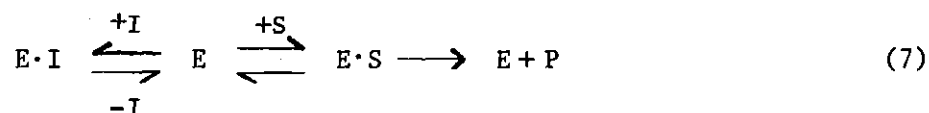
enzyme and its substrate in the presence of an inhibitor is described

by eq. 8 where K_I is the

$$v = \frac{V_{max} S}{K_m (1 + \frac{I}{K_I}) + S} \qquad (8)$$

dissociation constant of the enzyme-inhibitor complex (eq. 9), K_m is

the Michaelis constant and V_{max} is the maximum initial velocity.

$$K_I = \frac{[E][I]}{[E \cdot I]} \tag{9}$$

In this study the chymotrypsin or subtilisin catalyzed hydrolysis of Ac-Tyr-OEt is followed both in the absence and presence of inhibitors by means of an automatic pH-stat titrator, which adds base to titrate the acid formed as a product in order to maintain the pH constant at 7.8 (eq. 10).

$$CH_3CONHCHCOC_2H_5 + H_2O \xrightarrow[\text{pH 7.8}]{\text{enzyme}} CH_3CONHCHC\ O^- + HOC_2H_5 + H^+ \tag{10}$$

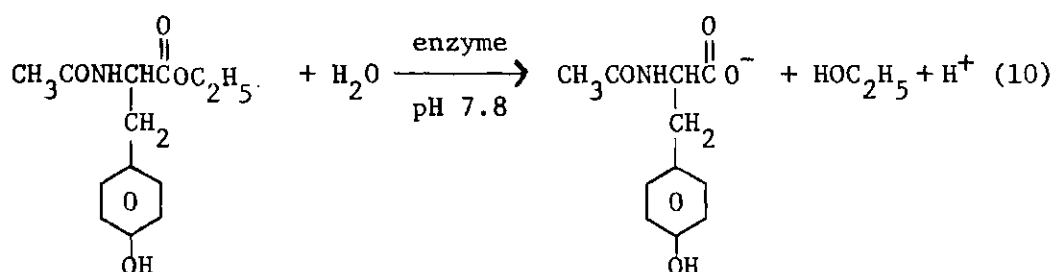A strip chart recording was made of the volume of standard base added as a function of time, and this of course gave a direct measure of the rate of the reaction in question (Wilcox, 1970). These rates were then used to construct Eadie-Hofstee (v/s vs v) plots where v is the initial velocity of the Ac-Tyr-OEt hydrolysis and s is the substrate concentration. Of the various transformations of the Michaelis-Menten equation, this transformation has been shown to give more reliable results than the standard Lineweaver-Burke plot (Dowd and Riggs, 1965). The inhibition constants, K_I, were determined by calculation from the y-intercepts of these plots according to the relationship given in eq. 11. For comparison purposes, accurate dissociation

$$[\text{y-intercept}]_I = [\text{y-intercept}]_{\text{no I}} \left[\frac{1}{1 + \frac{[I]}{K_I}} \right] \tag{11}$$

constants were also conveniently calculated from Dixon (1/v $\underline{vs}$ I) plots

(Dixon, 1953) and found to give the same results. Typical data is shown

is Figure 4. Table I presents the observed dissociation constants for

chymotrypsin A$_\gamma$ binding of several aldehydes, alcohols and semicarbazones.

Effectiveness of Chymotrypsin Inhibitors

The experimental results indicate that our strategy was generally

successful for designing aldehydes which would be excellent competitive

inhibitors at pH 7.8 and 25.0°. The best inhibitor was the peptide alde-

hyde N-benzyloxycarbonyl-L-phenylalaninal (Z-Phe-H, III). Its observed

dissociation constant, 7.8 x 10^{-5} $\underline{M}$, is indicative of a very strong

enzyme-aldehyde complex, binding $\underline{ca}$. 400-fold more tightly than the

specific amide substrate Ac-Phe-NH$_2$ (VII) and CHO-Phe-NH(Et) (VIII).

The magnitude of this dissociation

$$CH_3 \overset{O}{\overset{\|}{C}} NH \underset{\underset{CH_2C_6H_5}{|}}{CH} \overset{O}{\overset{\|}{C}} NH_2$$

$$\underset{\underset{CH_2C_6H_5}{|}}{HCNH} \overset{O}{\overset{\|}{C}} \overset{O}{\overset{\|}{C}} NH(CH_2CH_3)$$
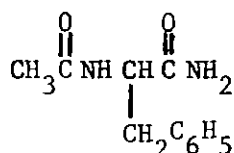
VII   VIII

K_S = 31 mM(Himoe $\underline{et}$ $\underline{al}$., 1967) K_S = 20 mM(Fersht and Requena, 1971)
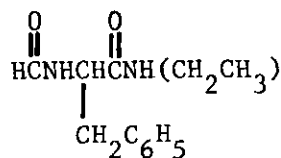
constant compares favorably with that found by Ito $\underline{et}$ $\underline{al}$. (1972) for

Ac-Leu-Leu-Phe-H (K_I = 5.2 x 10^{-5} $\underline{M}$).

The dipeptide aldehyde, Ac-Ala-Phe-H (II), was found to inhibit

chymotrypsin with a K_I value of 6.7 x 10^{-4} $\underline{M}$. In contrast, a structurally

similar inhibitor, Ac-Ala-NHCH$_2$CH$_2$C$_6$H$_5$(K_I = 8.8mM) (Powers $\underline{et}$ $\underline{al}$., 1974),

lacking the reactive aldehyde functional group, was observed to bind 13

less effectively than II. Our dipeptide aldehyde, however, were not
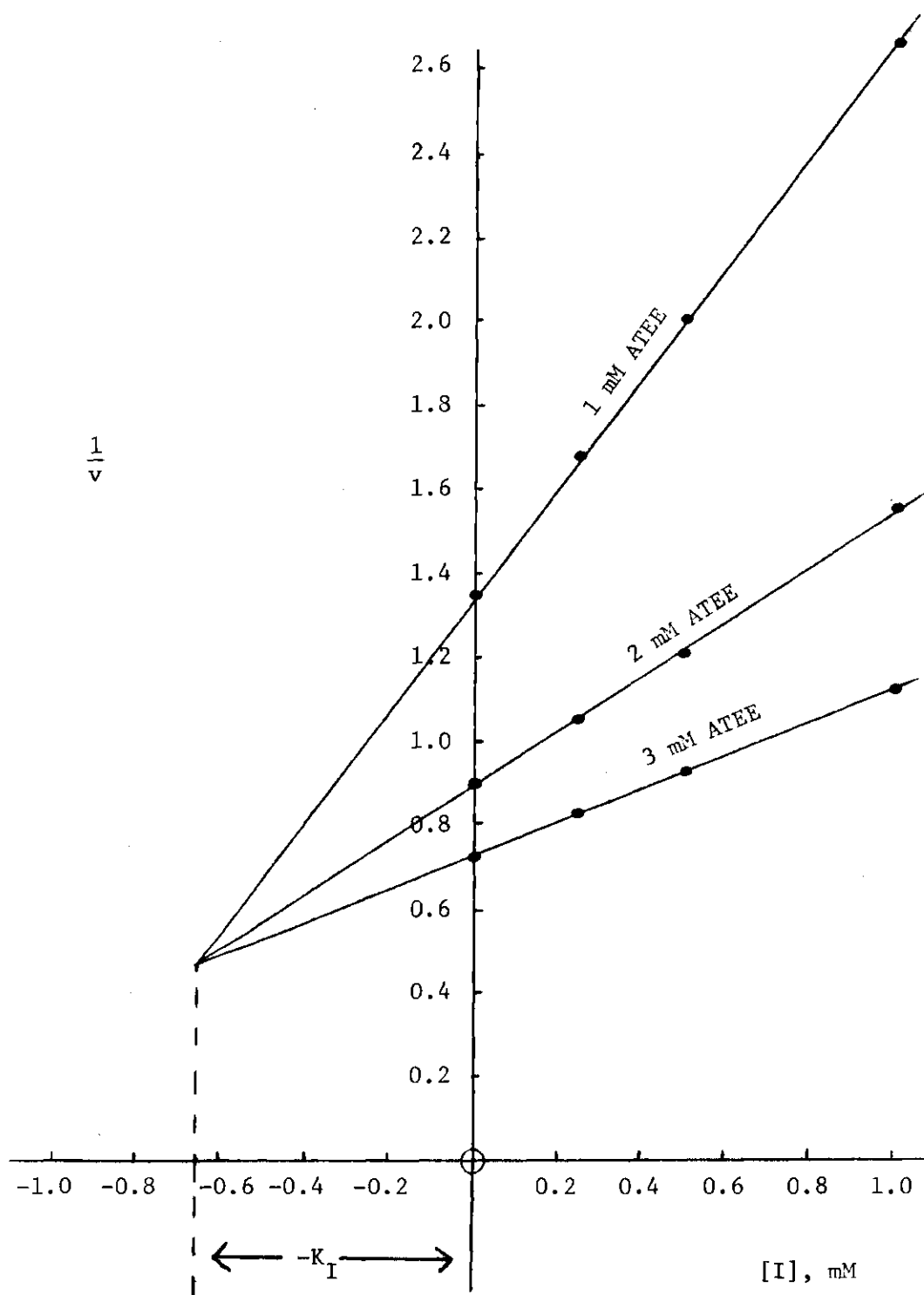
Figure 4. Graphical Determination of the Dissociation Constant,
           K_I, for Ac-Ala-Phe-H by the Method of Dixon.
           Note: ATEE is the substrate, acetyltyrosine ethyl ester.

Table 1. Observed Dissociation Constants for Chymotrypsin A_γ-Binding of Aldehydes, Alcohols and Semicarbazones.[a]

Inhibitor	K_I, mM (Std. deviation)	[I] range, mM
Z-Phe-H	0.078 (.003)	.03 - .05
semicarbazone	0.68 (.02)	.125 - .50
Z-phenylalaninol	> 5	.10 - .50
Ac-Ala-Phe-H	0.67 (.03)	.25 - 1.0
semicarbazone	4.5 (0.3)	1.0 - 3.5
Ac-Ala-Phenylalaninol	> 10	.10 - 1.0
CHO-Phe-H	ca. 8	1.0 - 5.0
semicarbazone	> 50	1.0 - 5.0
$2\text{-}C_{10}H_7CH{=}CH\text{-}CHO$	0.20 (.06)	.05 - .075
$1\text{-}C_{10}H_7CH{=}CH\text{-}CHO$	0.56 (.08)	.10 - .25
$C_6H_5CH_2CH_2CHO$	0.38 (.04)	.25 - 1.0
C_6H_5CHO	2.65 (.11)	.5 - 2.0
None	1.95 (.16)[b,c]	

[a] 5% (v/v) 1,2-dimethoxyethane, 0.10 F $CaCl_2$, pH 7.80, 25.0°

[b] K_m for the hydrolysis of N-Acetyl-L-Tyrosine Ethyl Ester (Ac-Tyr-OEt)

[c] V_{max} = 371 ± 26 μmoles substrate hydrolyzed/min/mg of enzyme.

as effective as the papain and elastase specific substrate aldehyde

analogs which had binding constants of 10^{-7} ~ 10^{-8} $\underline{M}$.

Formyl-L-phenylalaninal (IV) was observed to bind relatively poorly

to γ-chymotrypsin. Surprisingly, the K_I value found for the binding of

CHO-Phe-H at pH 7.8 is only 2-4 times better than the binding constants found

for the amides VII or VIII. On the other hand, our data indicate that both

chromophoric aldehydes, V and VI, were effective, competitive inhibitors

of chymotrypsin, binding 50 to 150 times more tightly than VII.

Table I also includes apparent binding constants for a number of

compounds related to our peptide aldehydes. These compounds, which

include peptide semicarbazones and alcohols, were generally observed to

be less tightly bound than the corresponding aldehydes. For example, the

semicarbazone derivatives of Z-Phe-H and Ac-Ala-Phe-H were found to inhibit

chymotrypsin approximately 7 to 9 times less effectively than their alde-

hyde counterparts. The analogous peptide alcohols, on the other hand,

were not observed to inhibit chymotrypsin within the concentration limits

of our experiments.

Two additional aldehydes were reacted with chymotrypsin. Hydro-

cinnaldehyde (IX), whose

$$C_6H_5CH_2CH_2CHO \qquad\qquad C_6H_5CHO$$

$$\text{IX} \qquad\qquad\qquad\qquad \text{X}$$
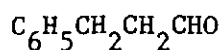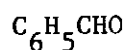
$$K_I = 3.8 \times 10^{-4} M \qquad\qquad K_I = 2.7 \times 10^{-3} M$$

structure resembles hydrocinnamate ester and amide substrates, was

observed to effectively inhibit chymotrypsin at pH 7.8 and 250°. This behavior is consistent with previous binding studies of this aldehyde with chymotrypsin (Rawn and Lienhard, 1974; Schultz and Cheerva, 1975). In contrast, benzaldehyde (X), lacking the ethylene bridge contained in hydrocinnamaldehyde, was a poorer chymotrypsin inhibitor, suggesting to us that X, although capable of binding in the enzyme's hydrophobic specificity pocket, may not react with the nucleop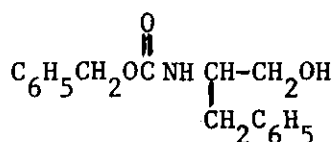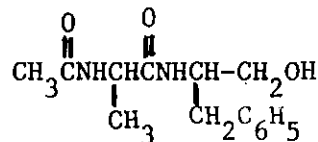hilic serine hydroxyl group. Hydrocinnamaldehyde, on the other hand, is 7 times more tightly bound to the enzyme. This observation is consistent with the additional formation of a reversible covalent bond between hydrocinnamaldehyde and Ser-195.

It is informative at this point to question whether the free aldehydes or their hydrates are the inhibitory species. Several rapidly equilibrating enzyme-inhibitor complexes might conceivably account for the strong binding of our aldehydes. For example, it is possible that a stable noncovalent complex between the enzyme and the free aldehyde could occur. This situation, however, is considered unlikely. Since the aldehyde functional group is about the same size and shape as a peptide bond, it is expected that the carbonyl group of an aldehyde should bind closely to the strongly nucleophilic hydroxyl group of Ser-195. Because aldehydes are quite susceptible to carbonyl addition reactions, this conjunction would be an unstable situation leading rapidly to hemiacetal formation formation. On the other hand, a noncovalent complex of chymotrypsin with an aldehyde hydrate is certainly conceivable. Such a complex would avoid placing a reactive carbonyl group

in the enzyme's active site. However, N-benzyloxycarbonylphenyl-alaninol (XI) and N-acetyl-L-alanyl-L-phenylalaninol (XII), which might be considered reasonable analogs of the

$$C_6H_5CH_2O\overset{\overset{\displaystyle O}{\|}}{C} NH\ \overset{\displaystyle CH-CH_2OH}{\underset{\displaystyle CH_2C_6H_5}{|}}$$

$$CH_3\overset{\overset{\displaystyle O}{\|}}{C}NH\overset{\displaystyle CH}{\underset{\displaystyle CH_3}{|}}\overset{\overset{\displaystyle O}{\|}}{C}NH\overset{\displaystyle CH-CH_2OH}{\underset{\displaystyle CH_2C_6H_5}{|}}$$

(XI)   (XII)

hydrated aldehydes, are extremely poor inhibitors. Furthermore, if noncovalent binding of an aldehyde hydrate to chymotrypsin is indeed the structure of the complex, it is difficult to see why aldehyde hydrates, which are gem-diols, should bind so much better than the analogous alcohols. Hydrogen bonds between the enzyme and a second hydroxyl group, should they exist, would not be likely to greatly favor the binding of a diol over the corresponding alcohol. Therefore, it is probable that the free aldehyde (rather than the gem-diol) is the inhibitory species, and the covalent tetrahedral hemiacetal (I) best represents the structure of the aldehyde-enzyme complex.

To the extent that aldehyde hydrates are present in solution in equilibrium with the free aldehydes, apparent K_I values for the aldehydes in Table I are presumably higher than the true values. Thompson (1973) has shown that
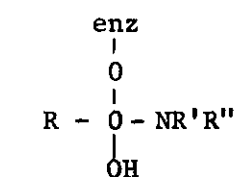
$$K_I(obsd) = K_I(1 + K_{Hy})$$

where K_{Hy} is the formation constant for an aldehyde hydrate. Since to a first approximation K_{Hy} will be independent of remote structural
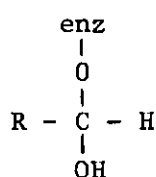
features of an aldehyde, this formation constant may equal that found

for the hydration of acetaldehyde in water, i.e., K_{Hy} = 1.4 (Bell,

1966). Thus, the true K_I value may be only 2.4 times smaller than K_I

experimentally observed.

Transition-State Analogs of Chymotrypsin

The results described above would suggest that chymotrypsin-

aldehyde complexes might be considered transition state analogs

for the enzymatic hydrolysis reaction. It has been forcibly argued that

an enzyme will complex most tightly an activated form of its substrate

and that such a complex would resemble the transition state for the

reaction being catalyzed (Wolfenden, 1972). It is likely, therefore,

that a particular substrate analog might also bind very tightly if its

structure in the bound complex featured relevant elements of the cor-

responding substrate in its transition state complex. Because such an

analog is predicted to be a powerful inhibitor, the dissociation con-

stant, K_I, may be used to estimate the degree to which this inhibitor-

enzyme complex resembles the true transition state. A good transition

state analog would be expected to have a K_I significantly lower than that

of a comparable Michaelis complex. By this criterion, our aldehyde-

chymotrypsin complexes are possible transition state analogs since

they have dissociation constants up to 400 times lower than observed K_s

values for comparable peptide amide substrates. As previously argued,

the hemiacetal (I) is the most reasonable structure for the aldehyde-

chymotrypsin complex. Therefore, a plausible candidate for the transition

state for chymotrypsin-catalyzed peptide bond hydrolysis might be a

```
        enz                      enz
         |                        |
         O                        O
         |                        |
R  -  O - NR'R"          R  -  C - H
         |                        |
        OH                       OH

    tetrahedral
    intermediate              hemiacetal

        XIII                      I
```

tetrahedral adduct not very different from the proposed intermediate (XIII).

Recently, alkylboronic acids, which readily form tetrahedral addition complexes, have been proposed as transition state analogs for α-chymotrypsin (Koehler and Lienhard, 1971; Rawn and Lienhard, 1974). Their very low dissociation constants were the first indication that they were bound at the enzyme active site as tetrahedral analogs of the transition state. This proposal has now been supported by X-ray crystallographic (Matthews et al., 1975) and laser Raman spectroscopic studies (Hess and Seybert, 1975).

Effectiveness of Inhibitors with Subtilisin

In addition to chymotrypsin, a number of other well-known proteases possess a reactive serine residue important for catalytic activity. These serine proteases bear a surprising amount of structural and chemical similarities including crystal structure, active site geometry, extended binding site, amino acid sequence, catalytic mechanism, and biological function. Investigations have shown, however, that differences among the serine proteases arise principally from structural variations in substrate specificity. For example, the binding pocket in

subtilisin is more flexible and larger in size than the corresponding

binding pocket in chymotrypsin (Kraut, 1971). Consequently, subtilisin

manifests a broader substrate and inhibitor specificity at P_1 (e.g. phe,

leu, and lys). It seemed reasonable to suppose, therefore, that our

aldehydes might be excellent competitive inhibitors of subtilisin. Of

the three aldehydes tested (Z-Phe-H, Ac-Ala-Phe-H, and $2-C_{10}H_7CH=CH-CHO$),

only Ac-Ala-Phe-H was found to significantly inhibit subtilisin BPN'.

With an observed dissociation constant of 1.1×10^{-3} M, this aldehyde

binds over 40 times more tightly than a dipeptide amide substrate such

as Z-Ala-Leu-NH$_2$ ($K_S = 4.6 \times 10^{-2}$ M) (Morihara et al., 1970). A

detailed inhibition study with the other two aldehydes was difficult

because of limited solubility. Negligibly small inhibition was observed

in both cases.

Ultraviolet Absorption Studies

Having demonstrated that aldehydes were effective inhibitors of

chymotrypsin, we rationalized that certain chromophoric analogs would

strongly complex with the enzyme and be spectrally sensitive to chemical

and physical effects of their environment. Consequently, we designed

two reactive and intense ultraviolet-absorbing aldehydes which incorpora-

ted the structural features of extended naphthalene π-resonance systems.

These aldehydes were expected to complex with chymotrypsin so that the

aldehyde functional groups were positioned near Ser-195 and the aromatic

nucleus buried in the hydrophobic binding pocket (Figure 5). It was

anticipated that the intense $\pi \longrightarrow \pi^*$ transitions of these extended reso-

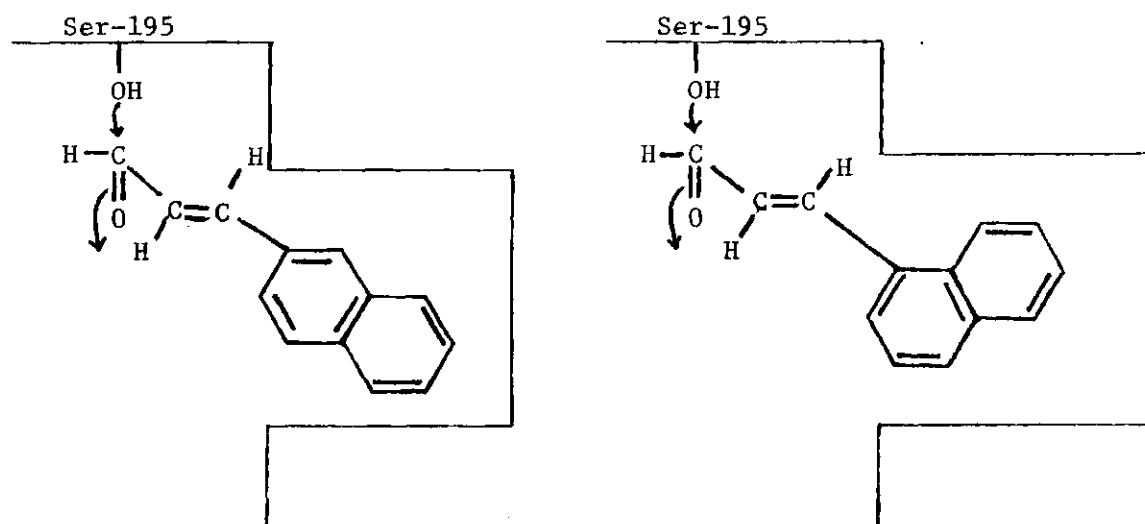nance systems would occur at longer wavelengths and outside the absorption

Figure 5. Schematic Representation of Possible Binding
Interactions Between Two Chromophoric
Aldehydes and the Chymotrypsin Active Site.

regions of the constituent tyrosine and tryptophan residues of the

enzyme. It was hoped, moreover, that the interaction of these aldehydes

with chymotrypsin would result in a derivative that would be suffi-

ciently stable to permit detection of any enzyme-hemiacetal intermediates.

As previously described, both aldehydes did favorably inhibit chymotryp-

sin (Table I) and were observed to display absorption maxima outside the

absorption regions of the enzyme. Unfortunately, significant changes in

these absorption maxima were not observed when chymotrypsin was reacted

with the inhibitors. New absorbances which might have been attributable

to hemiacetal formation were not seen either. Small changes accompanying

the binding of our aldehydes to the enzyme were difficult to precisely

define under the conditions employed in our experiments. Perhaps compari-

sons between a totally inactive, but not denatured, enzyme might more

easily establish the significance of any observed spectral changes.

Although the binding of normal phenylalanine derivatives to the active

site of chymotrypsin generally cause only small spectral changes to occur

(Benmouyal and Trowbridge, 1966; Himoe et al., 1967), our results were

disappointing since reports of unusual spectral perturbations have

appeared in the literature when aroyl acylating agents were reacted with

chymotrypsin ($\Delta\lambda$ as large as 30 nm have been noted) (Charney and Bernhard,

1967).

X-ray Analysis of Inhibited Chymotrypsin A$_\gamma$

In both α and γ chymotrypsins, considerable effort has been

expended in the search for crystals of enzyme-substrate or enzyme-

inhibitor complexes which would be amenable to analysis using difference

Fourier techniques. Chloromethyl ketone-A$_\gamma$ complexes, for example, have

been used in recent studies of the extended binding site of chymotrypsin

(Segal, et al., 1971a,b). The advantages of using irreversible inhibi-

tors such as chloromethyl ketones are two-fold: it makes possible the

achievement of high occupancy at the binding site in the crystal, and

it also permits straightforward characterization of the enzyme. In the

present investigation, however, reversible aldehyde inhibitors were

chosen for study. Although kinetic experiments showed that our aldehydes

were excellent competitive inhibitors of chymotrypsin in solution, ana-

lytical methods were not available for testing the effectiveness of our

compounds as inhibitors of the enzyme in the crystalline state. Conse-

quently, efforts were directed toward the collection and interpretation

of diffraction date from zero-level precession photographs in order to

characterize the inhibited enzymes.

In Table II, the cell parameters of inhibited and native chymo-

trypsin crystals are presented. Inspection of the data will show that

inhibition caused no changes in unit cell dimensions greater than 0.5%.

Provided that A_γ is truly inhibited, these results suggest that the

native and derivative crystals are closely isomorphous. Nevertheless,

obvious intensity differences were not noted when native and derivative

photographs were visually compared. To circumvent this problem, statis-

tical comparisons of zero-level reflections (measured for Z-Phe-H

inhibited and uninhibited A_γ crystals) were attempted. The basis for

comparisons were the fractional differences between derivative (I_D) and

scaled native (I_N) reflection intensities, $(I_D - RI_N)/I_D$. The scaling factor,

$R = \Sigma I_D / \Sigma I_N$, was required because the derivative reflections had been

Table 2. Unit Cell Dimensions of Native and
Inhibited Crystals of Chymotrypsin A_γ
Space Group, $P4_22_12$)

Inhibitor	a = b (Å)	c(Å)
None[a]	69.3	97.4
Z-Phe-H	69.0	98.0
Ac-Ala-Phe-H	69.6	96.8

[a]Native crystals in 2.4 $\underline{M}$ phosphate, pH 5.6.

measured using longer exposure times. Fractional differences were calcu-
lated for each reflection and quantitatively compared with the standard
derivation, σ, calculated for the entire set of 572 reflections. Results
indicate that 9 differences were above 3σ, 22 were above 2σ, and 99 were
above 1σ ($\sigma = 0.47$). These facts imply that the derivative and native
crystals are probably distinct. Alternatively, the slightly misaligned
zero-level native photograph may have elevated the calculated standard
deviation. Because there were doubts that the direct difference Fourier
technique would be effective, further crystallographic studies were not
undertaken.

CHAPTER IV

CONCLUSIONS

In conclusion, we have succeeded in synthesizing three peptide

aldehydes and related analogs, and have investigated their ability to

generate suitable models of the tetrahedral transition state for chymo-

trypsin-catalyzed substrate hydrolysis. Kinetic studies show that

the most effective competitive inhibitor of the group, N-benzyloxy-

carbonylphenylalaninal (Z-Phe-H, $K_I = 7.8 \times 10^{-5}$ M), binds to chymo-

trypsin about 400-fold more tightly than comparable amide substrates.

Two other peptide aldehydes, acetylalanylphenylalaninal (Ac-Ala-Phe-H,

$K_I = 6.7 \times 10^{-4}$ $\underline{M}$) and formylphenylalaninal (CHO-Phe-H, $K_I = 8 \times 10^{-3}$ $\underline{M}$)

are observed to be less reactive. Only the dipeptide aldehyde,

Ac-Ala-Phe-H, is seen to inhibit the homologous serine protease, subtili-

sin BPN'. In addition to our peptide aldehydes, two intensely chromo-

phoric analogs were also synthesized and are seen to be excellent com-

petitive inhibitors of chymotrypsin. Nevertheless, significant

spectral perturbations are not observed when either aldehyde complex is

formed. Results of our x-ray crystallographic studies, however, sug-

gest that both Z-Phe-H and Ac-Ala-Phe-H inhibit crystals of chymotrypsin

A_γ without causing significant changes in unit cell dimensions. Other

kinetic data indicate that semicarbazone derivatives of our peptide

aldehydes compete just 7 - 9 times less effectively in chymotrypsin

inhibition experiments than the corresponding aldehydes, but that

peptide alcohols are quite unreactive.

It is now generally accepted that the transition state for substrate hydrolysis by serine proteases closely resembles a tetrahedral intermediate in which the catalytic serine hydroxyl group forms a covalent adduct with the carbonyl carbon of the labile bond. On the basis of the kinetic results presented here, together with data from other investigators, we suggest that our aldehydes reversibly bind to chymotrypsin as covalent tetrahedral hemiacetals, and that these enzyme-aldehyde complexes are stereochemically analogous to the tetrahedral transition state for the hydrolysis reaction. Direct evidence for the formation of covalent hemiacetals, however, was not obtained in our experiments.

CHAPTER V

RECOMMENDATIONS

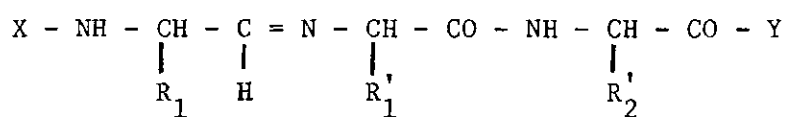A worthwhile extension of the work reported herein might be a kinetic and crystallographic investigation of the S' binding subsites of chymotrypsin and related proteases using peptide aldehyde imines. S' binding subsites may be defined as those subsites on the enzyme surface which interact with extended peptide substrates on the nitrogen side of the scissionable peptide bond (Figure 6). S binding subsites on the other hand, interact with residues on the carbonyl side of the peptide bond which is cleaved. Imines may be synthesized by coupling a peptide aldehyde with an appropriate amine. Competitive inhibition studies as well as difference Fourier studies could be utilized to elucidate the number and character of the S' binding subsites. Such studies would complement those which have already yielded considerable information on the S binding subsites of serine proteases.

$$\downarrow$$

$$X \dashv NH - CH - CO \dashv NH - CH - CO \dashv NH - CH - CO \vdash Y$$
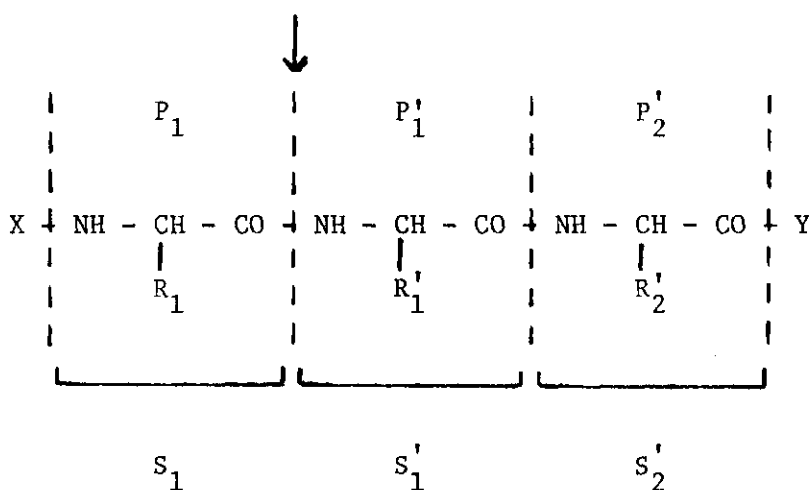
Normal Peptide Substrate

$$X - NH - CH - C = N - CH - CO - NH - CH - CO - Y$$

Imine of a Peptide Aldehyde

Note: Amino acid residues, P_1, P_1', and P_2', are located at subsites S_1, S_1', and S_2', respectively. Normal substrate cleavage occurs between residues P_1 and P_1'. This scheme was originally proposed by Schecter and Berger (1967).

Figure 6. Scheme for Depicting the Positioning of a Polypeptide Substrate and an Imine of a Peptide Aldehyde in the Binding Site of a Protease.

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PART II

INHIBITION OF ELASTASE BY

TETRAPEPTIDE CHLOROMETHYL KETONES

CHAPTER I

INTRODUCTION

Pulmonary emphysema is believed to result from the uninhibited proteolysis of elastic lung tissue by elastase and related neutral proteases derived from leukocytes and macrophages (Mittman, 1972). Normally, the lungs are protected from proteolytic digestion during infection or inflammation by the protease inhibitor α_1-antitrypsin. Individuals with a deficiency of α_1-antitrypsin, however, are biased toward chronic pulmonary lung diseases. The development of appropriate synthetic inhibitors, sufficiently reactive and specific, and capable of replacing α_1-antitrypsin, might therefore prove to be valuable reagents for the treatment of emphysema, arteritis, arthritis and related diseases. In addition, these elastase inhibitors would be important and relevant reagents for the study of the biological function of elastolytic enzymes. This thesis, Part II, reports the synthesis and reactivity of several tetrapeptide chloromethyl ketones which are both reactive and selective elastase inhibitors.

Background

Elastase, like chymotrypsin and trypsin, is a serine protease, and most characterization studies have been carried out on the readily available porcine pancreatic enzyme (EC. 3.4.21.11). Its inactive precursor, proelastase, is secreted by the pancreas and converted to

active enzyme just before use by another enzyme, trypsin. Elastase

has a molecular weight of approximately 25,900, consists of a single

polypeptide chain 240 amino acids in length, and contains 4 disulfide

bonds (Hartley and Shotton, 1971). The active-site residues essential

for catalytic activity have been identified to be Ser-188 and His-45.

Optimal activity occurs at pH 8.5. The detailed molecular structures of

tosyl elastase and native elastase have been determined from 3.5 Å

electron density maps and correlate well with solution kinetic proper-

ties (Shotton and Watson, 1970; Shotton, et al., 1971). A charge-relay

mechanism of elastase catalysis has been proposed, identical with that

for chymotrypsin (Hess, 1971; Hartley and Shotton, 1971). Elastase

differs from other serine proteases chiefly in substrate specificity:

substrates with uncharged nonaromatic side chains, e.g. alanine and

valine, are preferred because the entrance to the specificity pocket

is blocked by residues Val-216 and Thr-226. Both are glycyl residues in

chymotrypsin. Like the other serine proteases, however, elastase is an

endopeptidase and shows esterase activity toward synthetic substrates

such as Ac-Ala-Ala-Ala-OCH$_3$. Unlike them, the reactivity of elastase is

very dependent on the peptide length of substrates or inhibitors.

A number of proteolytic enzymes are known to occur in human

polymorphonuclear (PMN) leukocyte granule fractions, including an elas-

tase which is active at physiological pH(7-8). This human leukocyte

elastase has recently been purified by affinity chromatography and par-

tially characterized (Janoff and Scherer, 1968; Janoff, 1973; Folds

et al., 1972; Ohlsson and Olsson, 1973). It is known to degrade human

lung elastin, arterial walls and basement membrane, digest proteins

of bacteria cell walls in vitro, induce cellular surface changes

associated with loss of growth control, and undergo inhibition by

α_1-antitrypsin and peptide chloromethyl ketones (Janoff et al., 1972;

Janoff and Blondin, 1973, 1974; Mosser et al., 1973; Janoff, 1972b).

Experimental evidence has suggested, furthermore, that human leukocyte

elastase may be involved in pathological processes associated with

elastic tissue damage in acute arthritis, pulmonary emphysema, and

with the tumor-producing action of co-carcinogenic substances (Janoff,

1972a,b; Galdston et al., 1973; Mittman, 1972). Like the more widely

studied porcine pancreatic elastase, human leukocyte elastase is a

serine protease and exhibits esterase activity toward synthetic sub-

strates such as Boc-Ala-ONp and Ac-Ala-Ala-Ala-OCH$_3$.

Although a variety of synthetic inhibitors are known to react

with elastase, certain peptide chloromethyl ketones have received much

recent attention (Powers and Tuhy, 1972, 1973; Tuhy and Powers, 1975;

Thompson and Blout, 1973a; Thomson and Deniss, 1973). Chloromethyl

ketones form a broad class of inhibitors and have been immensely useful

for studying proteolytic enzymes (Shaw, 1970). If properly designed to

resemble natural substrates, chloromethyl ketones can be made fairly

specific for one serine protease or a group of serine proteases with

similar specificity. The reason for the specificity of chloromethyl

ketones is apparent upon consideration of the mechanism of action of

serine proteases.

As revealed by X-ray crystallographic investigations, the cata-

lytic centers of the serine proteases chymotrypsin, subtilisin, trypsin

and elastase are essentially identical in composition and conformation,
all containing a charge-relay system composed of an activated serine,
a histidine, and an aspartic acid residue (Hess, 1971; Blow et al.,
1969). Furthermore, each enzyme contains a binding pocket adjacent to
the catalytic residues whose size and chemistry determine the primary
substrate specificity. When a substrate properly binds to the enzyme,
the carbonyl group of the scissile peptide bond is oriented in such a
way that attack by the active-site serine residue can occur, thus lead-
ing to the formation of an acyl enzyme intermediate which rapidly turns
over (Figure 1). A similarly bound chloromethyl ketone, however, would
place the chloroketone moiety adjacent to the imidazole ring of the
active-site histidine residue. Irreversible alkylation via formation
of a covalent bond between histidine and inhibitor would then occur.
The binding modes of peptide chloromethyl ketones with chymotrypsin A_γ
(Segal et al., 1971a, b) and subtilisin-BPN' (Robertus et al., 1972;
Kraut et al., 1971, 1975; Poulas et al., 1975) have recently been inves-
tigated by X-ray crystallography. These enzyme-inhibitor interactions
are illustrated in Figures 2 and 3. The phenyl group of chloromethyl
ketones was found to be located in the hydrophobic binding pocket, and
the peptide chain of an extended inhibitor and a section of the backbone
of the enzyme formed a β-sheet structure. The solution inhibition
kinetics of these two proteolytic enzymes have also been studied
(Kurachi et al., 1973; J. C. Powers and J. T. Tippett, unpublished
observations). In general, the greater the enzyme-inhibitor interaction,
at the primary as well as the extended binding site, the better the
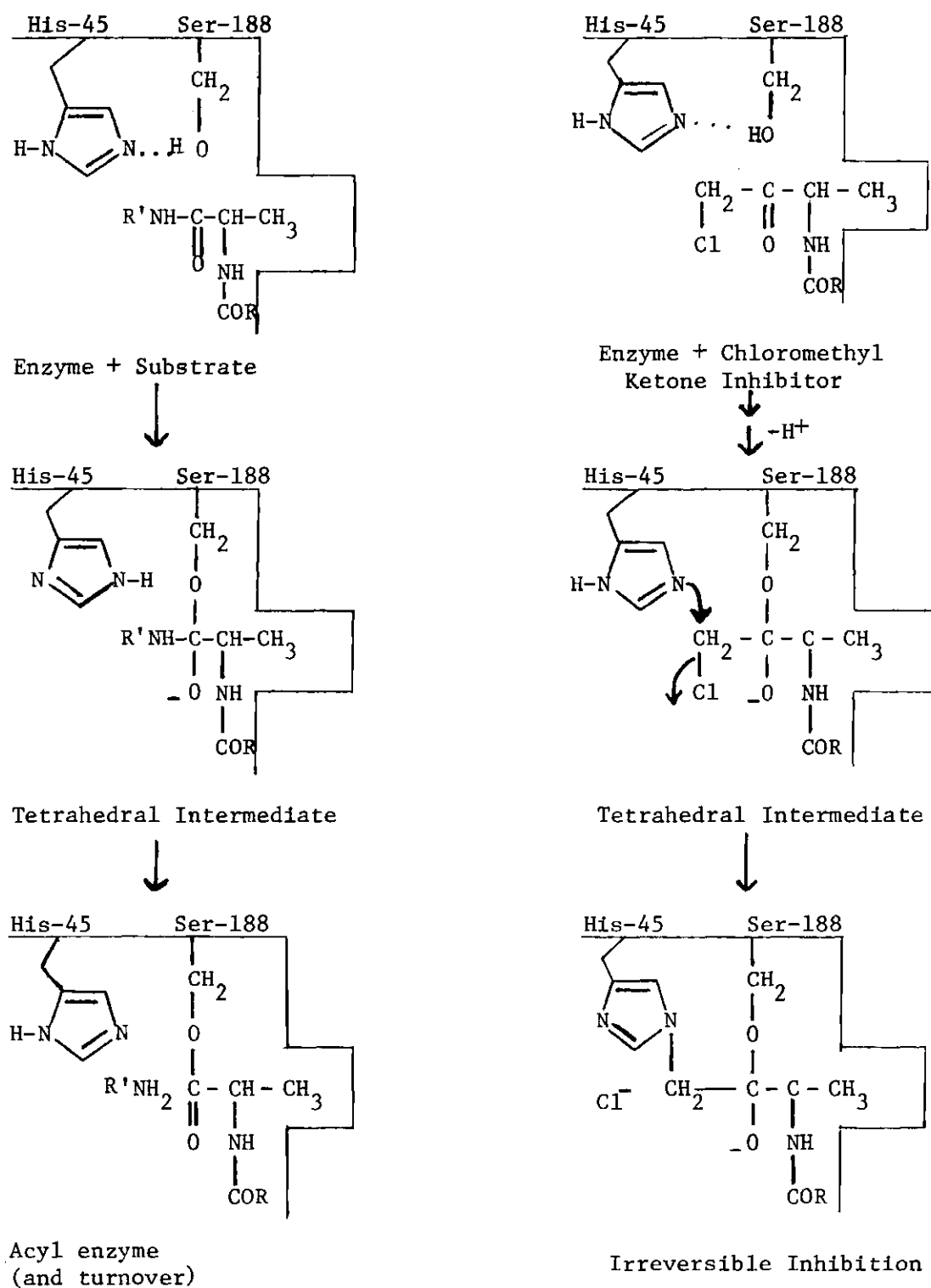inhibition.

Figure 1. A Schematic Representation of Pancreatic Elastase
Reactions with Substrates and Peptide Chloromethyl
Ketone Inhibitors.
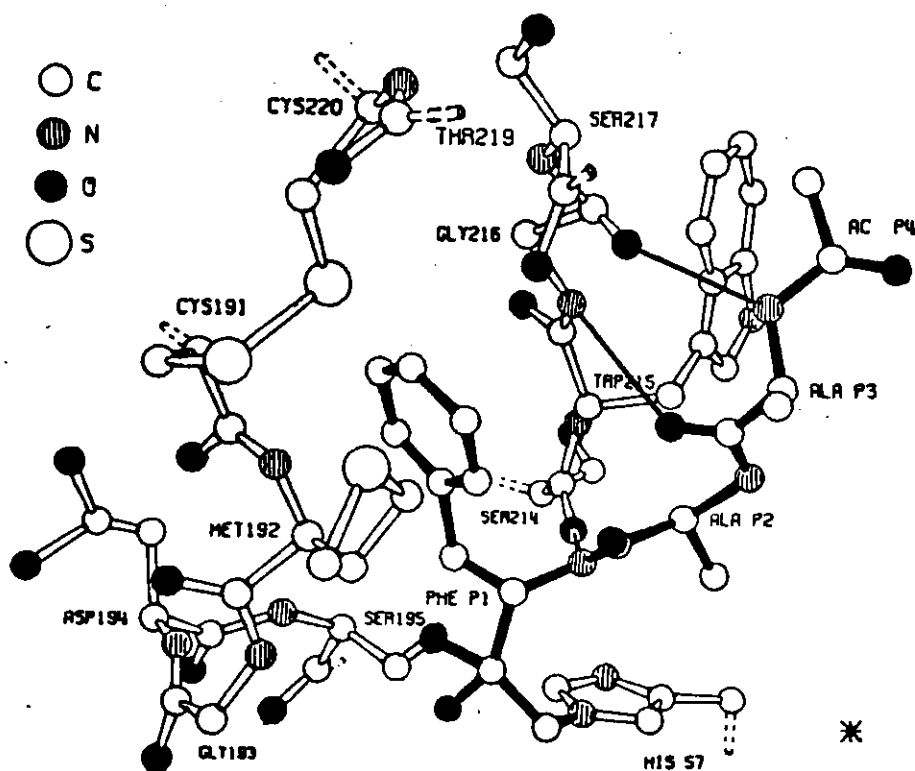
Note: Only pertinent portions of the protease molecule are shown.
This drawing was deduced from X-ray crystallographic
studies (Segal et al., 1971).

Figure 2. Illustration Showing the Proposed Binding
of Ac-Ala-Phe-CH$_2$Cl to Chymotrypsin A$_\gamma$.
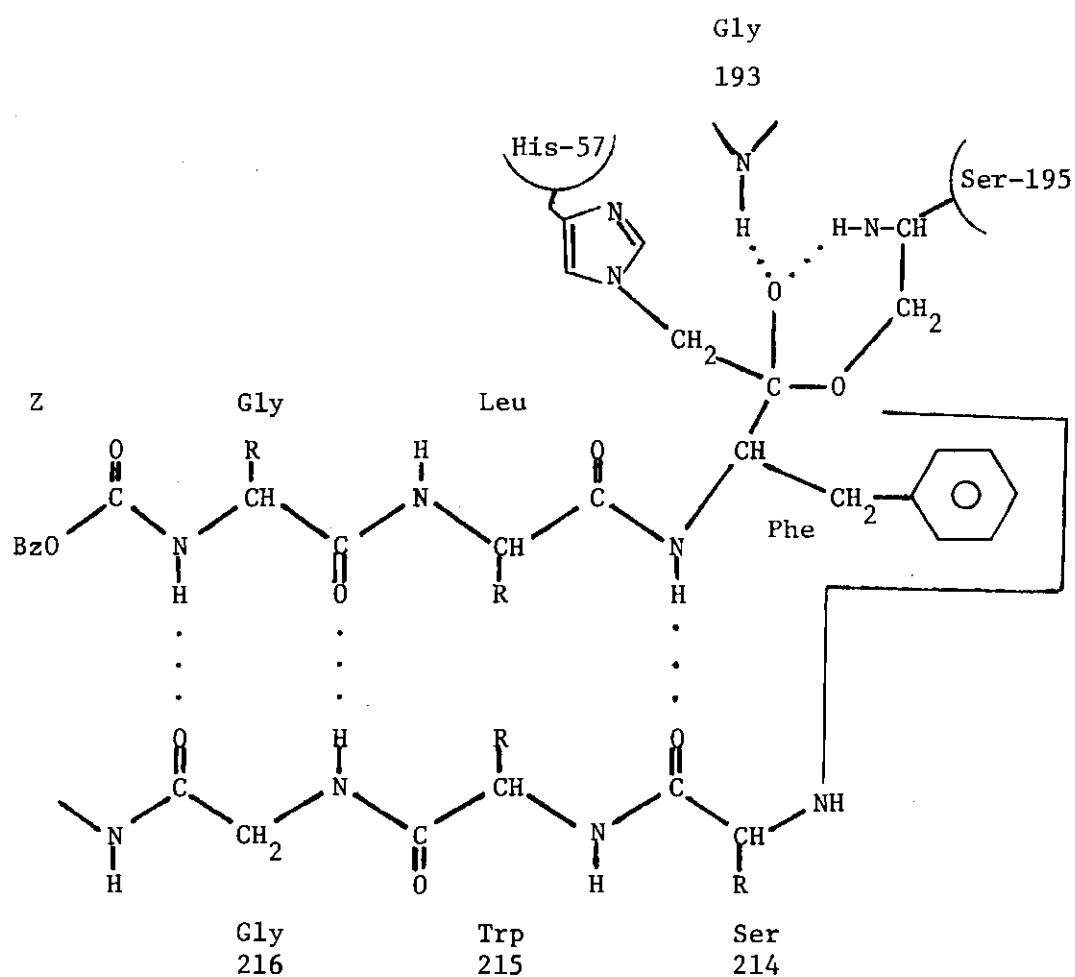
Note: Chymotrypsin (below) and the inhibitor Z-Ala-Leu-Phe-CH$_2$Cl (above) form an anti-parallel β-pleated sheet structure involving three hydrogen bonds.

Figure 3. Diagram of Chymotrypsin A Showing the Extended Binding Site Region.

Recently, a series of P_1-alanine peptide chloromethyl ketones have been demonstrated to be fairly reactive and specific inhibitors for porcine pancreatic elastase and human leukocyte elastase (Powers and Tuhy, 1972, 1973; Tuhy and Powers, 1975). These studies defined two important structural features as being necessary for developing an optimally reactive and selective elastase inhibitor. First, the compounds should contain a terminal alanine chloromethyl ketone moiety because elastase has demonstrated a primary specificity for alanine residues (Narayanan and Anwar, 1969; Atlas et al., 1970; Geneste and Bender, 1969; Kaplan et al., 1970; Atlas and Berger, 1972). Second, the inhibitors should contain an extended peptide chain since the rate of substrate hydrolysis is known to be strongly dependent on chain length (Atlas et al., 1970; Atlas and Berger, 1972; Thompson and Blout, 1970, 1973b). This very important feature was shown to enhance inhibitor reactivity, presumably through an increased interaction with the extended binding site of elastase.

Rationale for Development of Elastase Inhibitors

A recent computer study has examined the bonds split by porcine pancreatic elastase in a sampling of polypeptide substrates (Whitley, et al., 1975). Significant correlations were made concerning the amino acids adjacent to the cleaved peptide bonds. Elastase had been thought to exhibit distinctive substrate or inhibitor specificity for alanine residues at P_1 subsites. Surprisingly, the probabilities of peptide bond cleavages after valine (42.9%) and isoleucine (41.7%) residues

were higher than that for alanine (37.1%). In addition, a 29.2% cleav-age probability was noted for P_1-threonine residues. These results suggested that chloromethyl ketones which incorporated the structural features of an extended peptide chain and an isoleucine, valine or threonine at the P_1 position should be synthesized and compared with the known tetrapeptide elastase inhibitor, Ac-Ala-Ala-Pro-Ala-CH_2Cl.

CHAPTER II

EXPERIMENTAL

Materials and Methods

Porcine pancreatic elastase (lot 55B405) was obtained from Whatman

Biochemicals Ltd.and used without further purification; its substrate,

Boc-Ala-ON$_p$, was purchased from Sigma Chemical Co. Human leukocyte

elastase was gratuitously provided by Dr. Aaron Janoff at S.U.N.Y. at

Stony Brook; it was 95% pure as determined by a gel densitometry scan.

Chymotrypsin A$_\alpha$ (lot CDIOBK) was obtained from Worthington Biochemical

Co., and its substrate, Ac-Tyr-OEt, was synthesized in our laboratory

and had mp 80-81°. Trypsin (lot 102C-1920) and its substrate, Bz-Arg-

OEt, were obtained from Sigma Chemical Co. The tripeptide ester,

Ac-Ala-Ala-Pro-OBz, and the tetrapeptide chloromethyl ketone,

Ac-Ala-Ala-Pro-Ala-CH$_2$Cl, were synthesized by Dr. R. Boone. N-t-Butyl-

oxycarbonyl-L-valine (Boc-Val-OH) was prepared from valine and t-Butyl-

azidoformate using the pH-stat method (Schnabel, 1967), mp 73-75°(lit:

72). Boc-L-isoleucine ·1/2 H$_2$0 and Boc-L-threonine were purchased from

Bachem, Inc. and dried under vacuum overnight. All other reagents and

solvents were analytical grade. Thin-layer chromotography was performed

using Merck silica gel G plates. Elastase inhibition kinetics were

performed on a Beckman Model 25 spectrophotometer. Chymotrypsin and

trypsin inhibition kinetics were performed on a Radiometer automated

pH-stat (model TTTII). Mass spectra were taken on a Hitachi Perkin-

Elmer RMU-7C instrument using a 70 electron volt source, and nuclear magnetic resonance (nmr) spectra were taken on a Varian T-60 instrument. The peak positions in the nmr spectra are reported in ppm relative to tetramethylsilane. The following abbreviations are used: s, singlet; d, doublet; t, triplet; m, multiplet; b, broad.

L-Valine Chloromethyl Ketone Hydrochloride (HCl·Val-CH$_2$Cl)

The diazo ketone Boc-Val-CHN$_2$ was prepared from Boc-Val-OH(10.0 gm, 45 mmol) and diazomethane using a mixed anhydride method (Penke et al., 1970). Anhydrous HCl was bubbled through a solution of Boc-Val-CHN$_2$ in ether/tetrahydrofuran at 5°C for 15 minutes as the solution turned from yellow to colorless. The product was isolated, recrystallized from THF/Et$_2$O and dried in vacuo to give 4.5 gram (55%) of an extremely hygroscopic white solid.

N-Acetyl-L-Alanyl-L-Prolyl-L-Valine Chloromethyl Ketone

Ac-Ala-Ala-Pro-Val-CH$_2$Cl was prepared from deblocked Ac-Ala-Ala-Pro-OBz and HCl·Val-CH$_2$Cl using a mixed anhydride coupling method (Anderson et al., 1967). Ac-Ala-Ala-Pro-OH (1.5 g, 5 mmol) was dissolved in 50 ml anhydrous tetrahydrofuran and stirred at -15°C while N-methylmorpholine (.55 ml, 5mmol) and isobutyl chloroformate (.65 ml, 5 mmol) were added. After 10 minutes, the N-methylmorpholine salts were filtered and HCl.Val-CH$_2$Cl (.93 g, 5 mmol in 5 ml DMF) and N-methylmorpholine (.55 ml, 5 mmol) were added. Stirring was continued for 4 hours as the mixture warmed to 25°C. Then the solvent was removed in vacuo and the resulting yellow oil was dissolved in methanol and chromatographed on Merck silica gel G 0.063-0.2 mm. The product was

isolated after elution with 8% methanol in chloroform. Recrystalliza-

tion from methanol gave 1.05 g (49%) of a white solid, mp 195-196° (d),

R_f 0.41 (CHCl$_3$-CH$_3$OH, 9:1). The nmr spectrum (CDCl$_3$) showed peaks at

8.23 (1H, d, NH), 7 70 (1H,d,NH), 6.43 (1H, d, NH), 4.77 (4H, m, NHCH

(R)CO), 4.23 (2H, s, COCH$_2$Cl), 3.70 (2H, b, N-CH$_2$-(CH$_2$)$_2$-CH),

2.10 (5H, m, CH(CH$_3$)$_2$ and N-CH$_2$-(CH$_2$)$_2$CH), 2.03 (3H, s, CH$_3$CO),

1.27 (6H, m, CH(CH$_3$)$_2$), and 0.87 (6H, m, CH(CH$_3$)CO).

The mass spectrum showed major peaks at m/e 430 (M+), 395(M-Cl),

353(Ac-Ala-Ala-Pro → NHCH(CH(CH$_3$)$_2$)+), 282(Ac-Ala-Ala-Pro+),

254(Ac-Ala-Ala-N-(CH$_2$)$_3$CH+), 238((Ala-Ala-Pro+)-H), 185(Ac-Ala-Ala+),

167((Ala-Pro+)-H), and 114(Ac-Ala+).

Anal. Calcd for C$_{19}$H$_{31}$N$_4$O$_5$Cl: C, 52.96; H, 7.20; N, 13.00.

Found: C, 52.67; H, 7.30; N, 12.96

N-Acetyl-L-Alanyl-Alanyl-L-Prolyl-L-Isoleucine Chloromethyl Ketone

Ac-Ala-Ala-Pro-Ile-CH$_2$Cl was prepared similarly to Ac-Ala-Ala-Pro-

Val-CH$_2$Cl from Ac-Ala-Ala-Pro-Ala-OH and HCl Ile-CH$_2$Cl (prepared analo-

gously to HCl·Val-CH$_2$Cl). The product was isolated from the residue

by chromatography on a silica gel column from which it was eluted with

7% methanol in chloroform. Recrystallization from methanol gave 1.21 g

(55%) of a white solid, mp 207-208° (d), R_f 0.49(CHCl$_3$-CH$_3$OH, 9:1).

The nmr spectrum (CDCl$_3$) showed peaks at δ8.27 (1H, d, NH),

7.77 (1H, d, NH), 6.57 (1H, d, NH), 4.77 (4H, m, NHCH(R)CO),

4.27 (2H, s, COCH$_2$Cl), 3.70 (2H, b, N-CH$_2$(CH$_2$)$_2$-CH),

2.13 (5H, m, CH(CH(CH$_3$)CH$_2$CH$_3$)CO and N-CH$_2$-(CH$_2$)$_2$-CH), 1.99 (3H,s,CH$_3$CO),

1.27 (8H, m, CH(CH$_3$)CO and CH(CH(CH$_3$)CH$_2$CH$_3$)CO), and 0.90(6H,m,CH(CH(CH$_3$)

CH$_2$CH$_3$)CO).

The mass spectrum showed major peaks at m/e 444 (M+),

367(Ac-Ala-Ala-Pro-NH(CH(CH$_3$)CH$_2$CH$_3$)+), 282(Ac-Ala-Ala-Pro+),

254(Ac-Ala-Ala-N-(CH$_2$)$_3$CH+), 185(Ac-Ala-Ala+), 167((Ala-Pro+)-H),

and 114(Ac-Ala+).

<u>Anal</u>. Calcd for C$_{20}$H$_{33}$N$_4$O$_5$Cl: C, 53.99; H, 7.42; N, 12.60.

Found: C, 53.90; H, 7.54; N, 12.61.

N-Acetyl-L-Alanyl-L-Alanyl-L-Prolyl-L-Threonine Chloromethyl Ketone

Ac-Ala-Ala-Pro-Thr-CH$_2$Cl was synthesized from Ac-Ala-Ala-Pro-OH

and HCl·Thr-CH$_2$Cl.   L-Threonine chloromethyl ketone hydrochloride was

prepared similarly to HCl·Val-CH$_2$Cl; in this case the product was

isolated as an oil and immediately dissolved in 5 ml dimethylformamide

(DMF) for use in a subsequent coupling reaction. Ac-Ala-Ala-Pro-OH

(.75 g, 2.5mmol) was dissolved in 50 ml dry tetrahydrofuran and stirred

at -15°C while N-methylmorpholine (.28 ml, 2.5mmol) and isobutyl chloro-

formate (.33 ml, 2.5mmol) were added. After 10 minutes, the solution

was filtered and HCl·Thr-CH$_2$Cl (.43 g, 2.5mmol) and N-methylmorpholine

(.28 ml, 2.5 mmol) were added. After stirring 4 hours at 25°C, the

solvent was removed <u>in vacuo</u> and the resulting oil chromatographed on

a silica gel column. The product was isolated after elution with 9%

methanol in chloroform. Recrystallization from ethyl acetate-methanol

(100:1) gave .21 g (19%) of a white solid, mp 200-201° (d), R$_f$ 0.28

(CHCl$_3$-CH$_3$OH, 9:1). The nmr spectrum (CDCl$_3$) showed peaks at

δ8.07(1H, d, <u>NH</u>),7.67(1H, d, <u>NH</u>), 6.37(1H, d, <u>NH</u>), 4.70(4H, m, <u>CH</u>(R)CO),

4.30(2H, s, CO<u>CH</u>$_2$Cl), 3.70(3H, s, N-<u>CH</u>$_2$(CH$_2$)$_2$-CH and CH(<u>OH</u>)CH$_3$),

3.10(1H, d, <u>CH</u>(OH)CH$_3$), 2.13(4H, m, N-CH$_2$-(<u>CH</u>$_2$)$_2$-CH)), 1.97(3H, s, <u>CH</u>$_3$CO)

and 1.20(9H, m, CH(<u>CH</u>$_3$)).

The mass spectrum showed major peaks at m/e 414(M-H$_2$O), 397(M-Cl),

378(M-H$_2$O-Cl), 311(Ac-Ala-Ala-Pro-NHCH$_2$+), 282(Ac-Ala-Ala-Pro+),

254(Ac-Ala-Ala-N-(CH$_2$)$_3$CH+), 185(Ac-Ala-Ala+), 168(Ala-Pro+), and

114(Ac-Ala+); no M+ peak was observed.

<u>Anal</u>. Calcd for C$_{18}$H$_{29}$N$_4$O$_6$Cl: C,   49.94; H, 6.71; N, 12.95
Found: C, 49.91; H, 6.76; N, 12.89.

Reaction of Porcine Pancreatic Elastase with Inhibitors

Inhibition of porcine pancreatic elastase with the series of

tetrapeptide chloromethyl ketones was carried out under pseudo-first-

order conditions in solutions which contained at least a ten-fold excess

of inhibitor over enzyme. Residual enzymatic activity was measured

periodically using the Boc-Ala-ONp spectrophotometric assay (Visser and

Blout, 1972). A stock solution of elastase prepared in 1 mM HCl was

stored at 4°C and had a concentration of 9.4 µM or 0.24 mg/ml by uv

absorbance. Inhibitor solutions (.10 mM or 10.0 mM) were prepared by

dissolving a weighed amount of inhibitor in methanol and diluting with

pH 5.0 buffer (.1 M acetate) resulting in a 10% methanol solution.

The inhibition reaction was started by mixing 0.5 ml of the inhibi-

tor solution with 0.5 ml of the enzyme solution at 30.0°C and proceeding

through at least two half lives. Initial concentrations (total volume

of 1.0 ml) were as follows: inhibitor, 0.05 or 5.0 mM; elastase, 4.8 µM;

methanol, 5% (v/v). The reaction was monitored by removing 6-8 aliquots

(100 µl each) from the inhibition solution at periodic time intervals

and rapidly mixing with 2.0 ml of a 0.2 mM Boc-Ala-ONp substrate solu-

tion in a sample cuvette. The substrate solution was buffered at pH 6.5

(0.5 M phosphate) and contained 2% (v/v) CH$_3$CN. The release of

p-nitrophenol was followed at 347.5 nm for 1.0-1.5 minutes on the uv

spectrophotometer. The active elastase concentration did not decrease

more than 2% in 1 hour as indicated by control runs which lacked only

inhibitor.

For each inhibition reaction the kinetic parameters k_{obsd} and

$k_{2nd} = k_{obsd}/[I]$ were calculated from eq. 1

$$v = k_{obsd}[E] = k_{2nd}[I][E] \qquad (1)$$

using a least squares computer program. Correlation coefficients of

better than 0.995 were maintained throughout for the linear plots.

Reaction of Human Leukocyte Elastase with Inhibitors

Inhibition experiments with leukocyte elastase were carried out

under conditions which minimized the enzyme consumed yet facilitated

kinetic measurements. In all inhibition reactions, the final concentra-

tion of inhibitor was at least 5-fold greater than the enzyme concentra-

tion. Inhibitor solutions were buffered at pH 6.5 (.1 $\underline{M}$ phosphate) and

contained 10% (v/v) methanol. Elastase solutions ($\underline{ca}$ 0.50 mg/ml) were

prepared by dissolving a weighed amount of lyophilized enzyme in

.1 M phosphate buffer (pH 6.5). The inhibition reaction was started by

mixing 0.025 ml of the inhibitor solution with 0.030 ml of the enzyme

at 30°C, and proceeding through at least two half-lives. Initial concen-

trations (total volume of 0.055 ml) were: elastase, 10 μM; tetrapeptide

inhibitor, 0.05, 0.20, 0.40 or 10.0 mM; NaCl, 0.06 M; methanol, 5% (v/v).

Enzyme assays were performed by removing 10 μl aliquots at periodic time

intervals and rapidly mixing with 0.25 ml of a 0.2 mM Boc-Ala-ON$_p$ sub-

strate solution in a microcuvette. Kinetic analysis were performed as

before.

Reaction of Chymotrypsin and Trypsin with Inhibitors

Inhibition experiments with chymotrypsin and trypsin were carried out under conditions virtually identical with those employed in the inhibition reactions with human leukocyte elastase. All inhibition reactions were carried out at 30.0° and pH 6.5 Stock solutions of enzymes and inhibitors were prepared at appropriate concentrations and used quickly. Enzyme concentrations were determined by ultraviolet absorbance. A typical inhibition reaction was started by mixing 0.25 ml of the inhibitor solution, 0.25 ml of methanol, 0.5 ml of the enzyme solution, and 9 ml of .1 F citrate buffer (pH 6.5) and proceeding for 0-4 hours. Initial concentrations were as follows: enzyme, 10 μM; inhibitor, 0.5 mM – 10.0 mM; and methanol, 5% (v/v).

At various time intervals, an aliquot (100 μl) was removed from the inhibition mixture and assayed for residual enzyme activity using a Radiometer automated pH-stat to measure the initial hydrolysis rate of substrate at pH 7.8. Chymotrypsin was assayed using .01 F N-acetyl-L-tyrosine ethyl ester (Ac-Tyr-OEt) as the substrate and 0.5420 F NaOH as the titrant. The substrate solution contained 0.1 F CaCl$_2$ and 5% (v/v) methanol. Initial velocities were obtained from the pH-stat recorder tracings of base consumption with respect to time using the method of Henderson (1971). Kinetic analyses were performed as previously described for elastase. Correlation coefficients of better than 0.991 were obtained throughout.
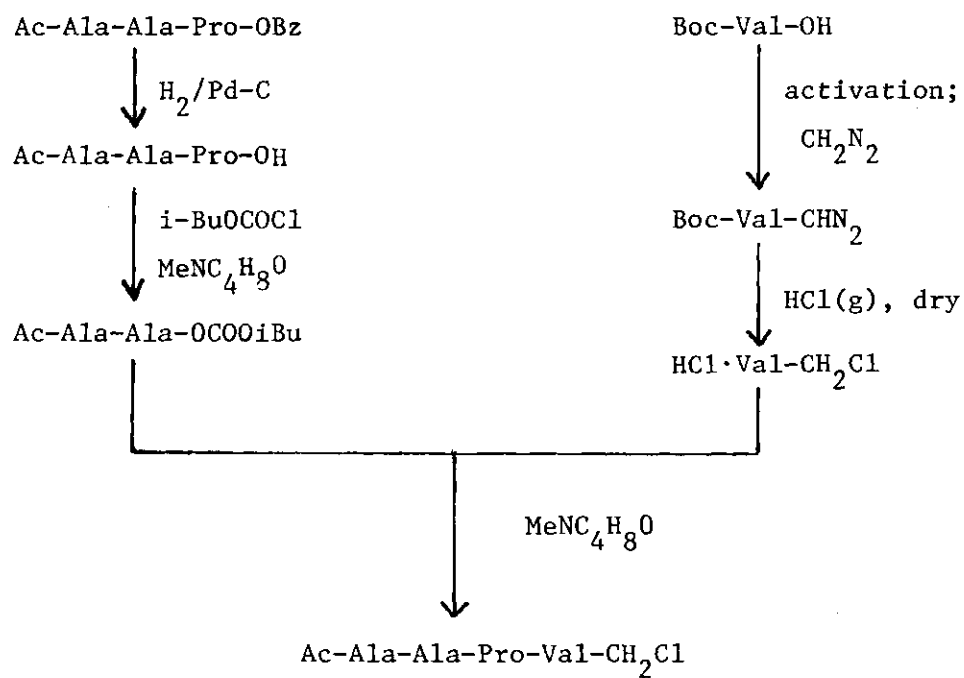
CHAPTER III

RESULTS AND DISCUSSION

Three peptide chloromethyl ketones were synthesized and tested as inhibitors of porcine pancreatic elastase and human leukocyte elastase. By regularly varying the P_1 residues of the inhibitors, the enzyme's primary specificity was studied in an effort to design an optimum elastase inhibitor having a maximum velocity.

Synthesis of the Inhibitors

A series of three tetrapeptide chloromethyl ketones were prepared using a mixed anhydride method. The final steps used in preparing a typical inhibitor, Ac-Ala-Ala-Pro-Val-CH$_2$Cl, are shown in Figure 4. The exemplary structure of this inhibitor as well as a description of the notation adopted for describing its alignment in the binding site of elastase are presented in Figure 5.

All of the peptide chloromethyl ketones were relatively easy to prepare and handle. Minor difficulties, however, were encountered with the unstable L-threonine chloromethyl ketone hydrochloride intermediate. This compound could not be crystallized but was immediately used in a subsequent coupling reaction. Although the desired coupling reaction was apparently achieved, the fact that the isolation of the product occurred in only 19% yield is presumably explicable on the basis of complicating side reactions which involve the free hydroxyl group.

Ac-Ala-Ala-Pro-OBz   Boc-Val-OH

↓ H_2/Pd-C   ↓ activation; CH_2N_2

Ac-Ala-Ala-Pro-OH   Boc-Val-CHN$_2$

↓ i-BuOCOCl, MeNC$_4$H$_8$O   ↓ HCl(g), dry

Ac-Ala-Ala-OCOOiBu   HCl·Val-CH$_2$Cl

MeNC$_4$H$_8$O

↓

Ac-Ala-Ala-Pro-Val-CH$_2$Cl

Tetrapeptide Chloromethyl Ketone

Figure 4.   Final Synthetic Steps Used in the Preparation of
Ac-Ala-Ala-Pro-Val-CH$_2$Cl.

Ac   Ala   Ala   Pro   Val $CH_2 - Cl$
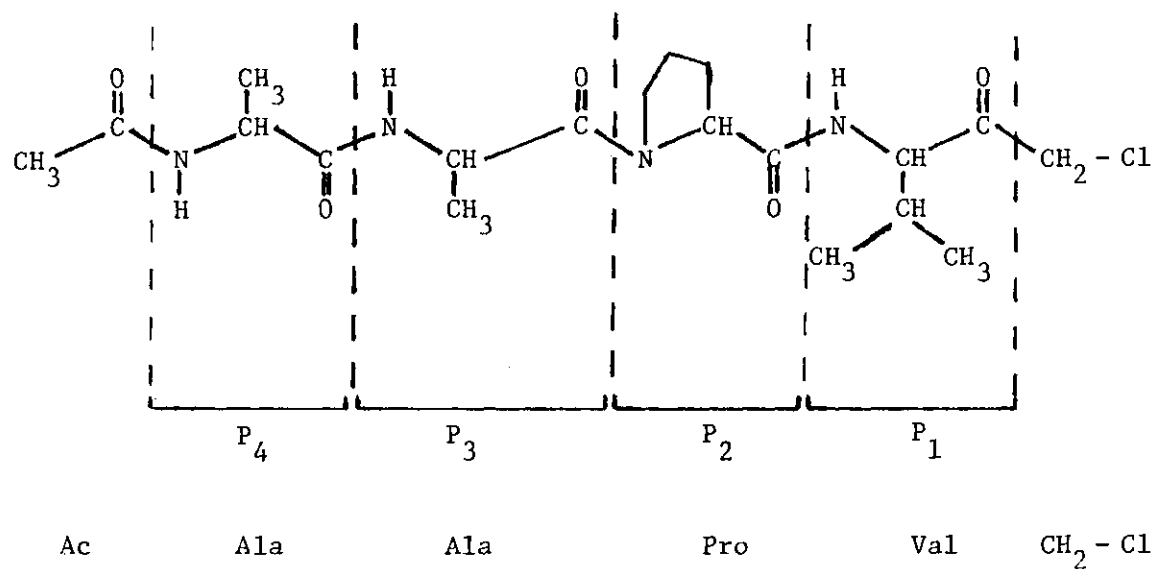
Note: The residues P_1 - P_4 are designated according to the notation of Schechter and Berger (1967).

Figure 5. Structure of the Typical Peptide Chloromethyl Ketone Ac-Ala-Ala-Pro-Val-CH_2Cl.

Tetrapeptide chloromethyl ketones were found to be stable in the solid state but to undergo slow hydrolysis in aqueous solution.

Kinetics of Inhibition

The irreversible reaction of an active-site-directed inhibitor with an enzyme may be represented by eq. 2 and 3

$$E + I \xrightleftharpoons{K_I} E \cdot I \xrightarrow{k_3} E - I \qquad (2)$$
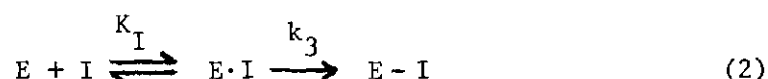
$$K_I = \frac{[E][I]}{[E \cdot I]} \qquad (3)$$

where $E \cdot I$ represents a noncovalently bound complex of the enzyme with the inhibitor, $E-I$ is the final product with the inhibitor irreversibly bound to the enzyme <u>via</u> a covalent linkage, K_I is the dissociation constant of the $E \cdot I$ complex, and k_3 is the limiting rate of inactivation. If the inhibitor concentration is sufficiently great, then the decrease in the total enzyme concentration $(E + E \cdot I)$ in the inhibition reaction follows pseudo-first-order kinetics at any fixed value of $[I]$. In this case, the observed first-order rate constant can be represented by eq. (4)

$$k_{obsd} = \frac{k_3[I]}{K_I + [I]} \qquad (4)$$

(Kitz and Wilson, 1962, Kurachi <u>et</u> <u>al.</u>, 1973).

If the chosen inhibitor concentration is approximately equal to $K_I(I \doteqdot K_I)$, then the ratio $k_{obsd}/[I]$ will vary over a range of inhibitor concentrations and both K_I and k_3 can be evaluated. If, on the other hand, the value of K_I should be much greater than the chosen inhibitor

concentration $(K_I > I)$, then eq. (4) reduces to eq. (5)

$$\frac{k_{obsd}}{[I]} = \frac{k_3}{K_I} \tag{5}$$

and the inhibition parameter, k_3/K_I, comparable to a second-order rate

constant (K_{2nd}), can be determined.

The reactivity of a series of related inhibitors can be compared

on the basis of their relative $k_{obsd}/[I]$ values. The differences in

these $k_{obsd}/[I]$ values will thus reflect both the effect of structural

changes upon binding of inhibitor to enzyme as well as the rate of reac-

tion within the E·I complex. $K_{obsd}/[I]$ values, however, are subject to

distortion from nonlinear concentration effects when $[I] \gtrsim K_I$.

Elastase Inhibition Studies

The series of peptide chloromethyl ketones generally acted as

highly reactive irreversible inhibitors of porcine pancreatic and

human leukocyte elastase. Kinetic results for the porcine pancreatic

enzyme are presented in Table 1 and for the human leukocyte enzyme in

Table 2. Relative values of $k_{obsd}/[I]$ were calculated and are the basis

for reactivity comparisons. Reaction conditions were selected in order

to obtain feasible reaction rates. Both the inhibitor concentration

and pH were decreased to practicable levels so that rates could be

accurately measured. Although optimal elastase activity occurs at pH

8.5, rapid inhibition occurred even at pH 5.0. Good pseudo-first-order

kinetics were observed in all cases. The specific activity of the

enzyme, expressed as $\Delta abs./min./\mu g$ enzyme, was .032 in the 2.1 ml

Table 1. Inhibition of Porcine Elastase with Tetrapeptide Chloromethyl Ketones.[a]

Inhibitor P_4 P_3 P_2 P_1	$[I] \times 10^4$, M	$10^4\ k_{obsd}$,[b] sec^{-1} (Std. dev.)	Half-life, min	$k_{obsd}/[I]$ $M^{-1}\ sec^{-1}$ (rel. values)
Ac-Ala-Ala-Pro-Ile-CH$_2$Cl	0.5	24 (.96)	4.8	48 (1.2)
Ac-Ala-Ala-Pro-Val-CH$_2$Cl	0.5	18 (.38)	6.7	35 (0.9)
Ac-Ala-Ala-Pro-Ala-CH$_2$Cl	0.5	20 (.77)	5.8	40 (1.0)
Ac-Ala-Ala-Pro-Thr-CH$_2$Cl	50.0	4.1 (.11)	27.9	.08 (.002)

[a]Elastase concentration 5 μM, at pH 5.0, 30°C, 5% (v/v) methanol.

[b]Average of three runs.

Table 2. Inhibition of Human Leukocyte Elastase with Tetrapeptide Chloromethyl Ketones.[a]

Inhibitor P_4 P_3 P_2 P_1	$[I]$ x 10^4, M	10^3 k_{obsd}, sec^{-1} (std. dev.)	Half-life, min	$k_{obsd}/[I]$ M^{-1} sec^{-1} (rel. values)
Ac-Ala-Ala-Pro-Val-CH$_2$Cl	0.5	7.64 (.04)[b]	1.6	160 (49)
Ac-Ala-Ala-Pro-Ile-CH$_2$Cl	0.5	6.38 (.04)[c]	1.9	133 (40)
Ac-Ala-Ala-Pro-Ala-CH$_2$Cl	2.0	.68 (.04)	17.0	3.4
	4.0	1.33 (.03)	8.7	3.3 (1.0)
Ac-Ala-Ala-Pro-Thr-CH$_2$Cl	100.0	1.54 (.03)	8.2	0.15 (.05)

[a]Elastase concentration 10 μM, at pH 6.5, 30°C, 5% (v/v) methanol.

[b]Average of three runs.

[c]Average of two runs.

porcine pancreatic elastase assay and .020 in the .26 ml human leukocyte

elastase assay. Because the peptide chloromethyl ketones hydrolyzed

slowly in buffered aqueous solution, the inhibition solutions were

always freshly prepared. Just enough methanol (5%, v/v) was added to

solubilize the inhibitors at 30°C. In all experiments, the reaction was

allowed to proceed through at least two half-lives. No direct evi-

dence was produced to indicate that peptide chloromethyl ketones

actually reacted with the active site histidine residue of elastase

(His-45). By analogy with related serine proteases, it is almost cer-

tain that histidine alkylation does take place. That peptide chloromethyl

ketone elastase inhibitors do indeed react with a histidine residue has
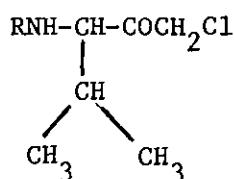
been verified by Thompson and Blout (1973a).

Effectiveness of Porcine Pancreatic Elastase Inhibitors

The overall rate of an active-site-directed irreversible inhibitor

depends both on the amount of $E \cdot I$ complex present at equilibrium as well

as the rate-limiting reaction of the bound inhibitor with the enzyme

to form the inactivated enzyme $(E - I)$. Covalent bond formation would

certainly be influenced by the steric arrangement of the inhibitor

with enzyme in the $E \cdot I$ complex and by electronic effects. On the other

hand, the dissociation constant, K_I, of the $E \cdot I$ complex is determined

primarily by the increase in entropy upon binding the inhibitor to the

enzyme. This increase is due presumably to the disruption of water

structure. Obviously, a structural change in the inhibitor could

affect both the extent of binding to the enzyme, measured by K_I, or the

stereoelectronic relationship between the inhibitor and enzyme in the E·I complex, measured by k_3. That the affinity of an inhibitor for an enzyme is not necessarily related to the rapidity of inactivation has been shown by Shaw and Glover (1970). Due to the generally low inhibitor concentrations used, these two effects were not separately evaluated in the present study. Instead, a comparison of the effectiveness of the tetrapeptide chloromethyl ketone inhibitors was based on their relative $k_{obsd}/[I]$ values, thus reflecting both the influence of K_I and k_3.

An examination of the data in Table 1 will show that both the valine and isoleucine chloromethyl ketones were as effective or slightly more effective than the standard inhibitor, Ac-Ala-Ala-Pro-Ala-CH_2Cl. The best inhibitors of the series, Ac-Ala-Ala-Pro-Ile-CH_2Cl, has a second-order rate constant $(k_{obsd}/[I])$ equal to 48 M^{-1} sec^{-1} at 30.0°C and pH 5.0. These limited results suggest that the primary specificity pocket of porcine pancreatic elastase is large enough to accommodate Val, Ile and Ala as P_1 inhibitors without fostering steric repulsion and perhaps even provides small hydrophobic interactions.

The peptide chloromethyl ketone, Ac-Ala-Ala-Pro-Thr-CH_2Cl, was discovered to be relatively unreactive toward porcine pancreatic elastase under the inhibition conditions employed here. Enzyme inactivation was observed to be over 400 times slower for this threonine derivative than for the isosteric valine inhibitor.

RNH-CH-COCH$_2$Cl
      |
      CH
     / \
  CH$_3$ CH$_3$

valine chloromethyl ketone

RNH-CH-COCH$_2$Cl
      |
      CH
     / \
  CH$_3$ OH
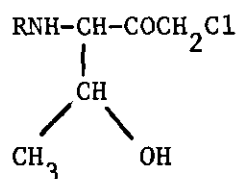
threonine chloromethyl ketone

This decrease in reactivity was quite surprising and might be interpre-
ted in terms of intermolecular hydrogen-bonding between the hydroxyl
group of the threonine chloromethyl ketone and some residue in the
catalytic site of elastase. Such an enzyme-inhibitor interaction could
force a rotation about the C$_\alpha$ bond of the threonine inhibitor. The
result would be a decreased rate of inactivating alkylation within the
bound complex due to the altered conformation of the inhibitor. On the
other hand, intramolecular inhibitor hydrogen-binding (between the β-OH
of threonine and the C = O of the adjacent proline)
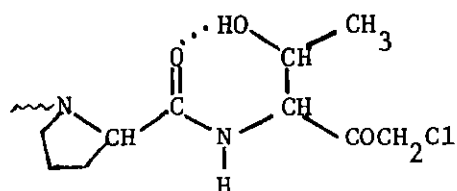
might occur. Irreversible alkylation _via_ formation of a covalent bond
between His-45 and this chloromethylketone would be severely diminished.
It is unlikely, however, that the failure of the threonine chloromethyl
ketone to effectively inhibit elastase is a result of steric repulsion
in the S$_1$ subsite of the enzyme. Both valine and isoleucine deriva-
tives also have medium-sized alkyl side chains but are seen to react
quite rapidly with elastase.

Effectiveness of Human Leukocyte Elastase Inhibitors

A comparison of the peptide chloromethyl ketone inhibitors, based on their relative $k_{obsd}/[I]$ values, leads to several interesting relationships between the structure of inhibitors and reactivity toward human leukocyte elastase. Two tetrapeptides, Ac-Ala-Ala-Pro-Val-CH$_2$Cl and Ac-Ala-Ala-Pro-Ile-CH$_2$Cl were observed to be 40-50 times more reactive than the standard inhibitor, Ac-Ala-Ala-Pro-Ala-CH$_2$Cl (Table II). This results suggests that the side chains of valine and isoleucine, extending about 2-4 Å from C$_\alpha$, are better able to make van der Waals contact within the hydrophobic S$_1$ binding pocket of human elastase. Alanine, on the other hand, has a side chain too small (1 - 2 Å from C$_\alpha$) to interact significantly with the enzyme. That hydrophobic bonding is an important driving force for noncovalent intermolecular interactions in aqueous solution has been discussed by Jencks (1969).

The threonine peptide chloromethyl ketone was again observed to be relatively unreactive. An explanation for this behavior with human leukocyte elastase is similar to that outlined for the porcine pancreatic enzyme. The relative reactivity of various P$_1$ inhibitors with human elastase can be represented by Val, Ile >> Ala > Thr.

Comparisons of the Two Kinds of Elastase

At this point it is of interest to examine certain inhibition characteristics of human leukocyte elastase as compared to the more thoroughly studied porcine pancreatic elastase. The nature of such differences may provide some insight into their separate biological functions. For example, leukocyte elastase generally reacts slightly less rapidly

with the group of peptide chloromethyl ketones than does pancreatic

elastase, as indicated by the somewhat smaller values of $k_{obsd}/[I]$

for inhibition (Table 5). This behavior is most likely due to the

lesser specific activity of the leukocyte enzyme. More significantly,

however, the P_1 valine or isoleucine chloromethyl ketones were dra-

matically more reactive than the standard alanine tetrapeptide in the

case of leukocyte elastase. This substantial difference in the rates

of inhibition definitely establishes that the human leukocyte enzyme

is distinct from the porcine pancreatic enzyme.

Inhibition Studies with Trypsin and Chymotrypsin

To be useful in physiological experiments, our chloromethyl

ketones should selectively as well as effectively inactivate elastolytic

enzymes in preference to such related serine proteases as chymotrypsin

and trypsin. Although these proteolytic enzymes are homologous with

elastase, they show structural differences at their primary binding

sites to accommodate different kinds of substrate P_1 residues. Each

enzyme possesses a pronounced pocket adjacent to the catalytic resi-

dues having special dimensions and steric and electrostatic properties.

For example, chymotrypsin contains a hydrophobic, slot-shaped pocket and

can easily accommodate residues with aromatic side chains, e.g. Phe, Tyr

and Trp, as well as other large residues such as leucine. Trypsin's

binding pocket, on the hand hand, contains an anionic carboxylate group.

Consequently, trypsin is specific for amino acid residues with posi-

tively-charged side chains such as lysine or arginine. Because the

binding pocket of porcine pancreatic elastase is partially crowded with

the alkyl group of Val-216, this enzyme is specific for residues with relatively small alkyl side chains.

Table 3 presents the kinetic results for the attempted inactivation of trypsin with the four tetrapeptide chloromethyl ketones. These compounds were completely unreactive toward trypsin even after 4 hours under our standard inhibition conditions. Consequently, no kinetic constants could be determined. Control experiments without inhibitor indicated, however, that the enzyme remained almost fully active during this time period.

The results for the inhibition experiments with chymotrypsin are shown in Table 4. $K_{obsd}/[I]$ values appeared to increase as the P_1 alanine, valine and isoleucine side chain residues were made larger. Not surprisingly, these inhibitors were seen to inactivate chymotrypsin only 1 to 6 times less effectively than Boc-Gly-Leu-Phe-CH$_2$Cl, a chloromethyl ketone reported to be an excellent inhibitor for α-chymotrypsin (Kurachi et al., 1973). As previously mentioned, chymotrypsin is known to show a specificity for P_1 residues possessing aromatic or large hydrophobic alkyl side chains. Since the reaction conditions employed in these inhibition studies with chymotrypsin were virtually identical with those used for the human leukocyte elastase, a direct comparison of results is possible (refer to Table 5). In general, those tetrapeptide inhibitors with Ile, Val or Ala P_1 residues were observed to inactivate chymotrypsin slower than either the human leukocyte or porcine pancreatic elastase enzymes. For example, Ac-Ala-Ala-Pro-Val-CH$_2$Cl was found to inhibit chymotrypsin with

Table 3. Inhibition of Trypsin with Tetrapeptide Chloromethyl Ketones.[a]

Inhibitor P_4 P_3 P_2 P_1	[I] x 10^4, M	Time (hr)	Activity[b] (%)
Ac-Ala-Ala-Pro-Ile-CH_2Cl	0.5	4	88
Ac-Ala-Ala-Pro-Val-CH_2Cl	0.5	4	90
Ac-Ala-Ala-Pro-Ala-CH_2Cl	2.0	4	87
Ac-Ala-Ala-Pro-Thr-CH_2Cl	100.0	4	89
None[c]		4	89

[a]Trypsin concentration 10 μM, at pH 6.5, 30.0°, 5% (v/v) methanol

[b]Enzyme activity after t (hr) measured as percent of initial activity at t = 0.

[c]Control experiment.

Table 4. Inhibition of α-Chymotrypsin with Tetrapeptide Chloromethyl Ketones.[a]

Inhibitor P_4 P_3 P_2 P_1	$[I] \times 10^4$, M	$10^4\ k_{obsd}$, sec^{-1} (std. dev.)	Half-life, min	$k_{obsd}/[I]$ $M^{-1}\ sec^{-1}$ (rel. values)
Ac-Ala-Ala-Pro-Ile-CH_2Cl	0.5	3.20 (.03)	36.1	6.4 (5.8)
Ac-Ala-Ala-Pro-Val-CH_2Cl	0.5	.98 (.03)	117.8	2.0 (1.8)
Ac-Ala-Ala-Pro-Ala-CH_2Cl	2.0	2.27 (.06)	52.5	1.1 (1.0)
Ac-Ala-Ala-Pro-Thr-CH_2Cl	100.0	5.36 (.12)	21.5	.05 (.05)
Boc-Gly-Leu-Phe-CH_2Cl				6.6^{b} (6.0)

[a]Chymotrypsin concentration 10 μM, at pH 6.5, 30.0°, 5% (v/v) methanol.

[b]Value adjusted to pH 6.5 from measurement at pH 5.0 (Kurachi et al., 1973) assuming $K_{obsd}/[I]$ at pH 5.0 would be ca 20% of $k_{obsd}/[I]$ at pH 6.5.

Table 5. Inhibition of Chymotrypsin and Elastase with Tetrapeptide Chloromethyl Ketones.[a]

Inhibitor	Chymotrypsin		Leukocyte Elastase		Porcine Elastase	
P_4 P_3 P_2 P_1	$k_{obsd}/[I]$ $(M^{-1} sec^{-1})$	$k_{obsd}/[I]$ $(rel)^c$	$k_{obsd}/[I]$ $(M^{-1} sec^{-1})$	$k_{obsd}/[I]$ $(rel)^c$	$k_{obsd}/[I]$ $(M^{-1} sec^{-1})$	$k_{obsd}/[I]$ $(rel)^c$
Ac–Ala–Ala–Pro–Val–CH_2Cl	2.0	1.8	160	150	175	160
Ac–Ala–Ala–Pro–Ile–CH_2Cl	6.4	5.8	133	120	240	220
Ac–Ala–Ala–Pro–Ala–CH_2Cl	1.1	1.0	3.4	3.1	200	180
Ac–Ala–Ala–Pro–Thr–CH_2Cl	.05	.04	.15	.04	.4	.002

[a] Elastase concentration 5-10 µM, inhibitor concentration 0.05 - 10.0 mM, 5% (v/v) methanol, at 30.0° and pH 6.5.

[b] These values were calculated for pH 6.5 assuming that $k_{obsd}/[I]$ values measured at pH 5.0 would be ca 20% of $k_{obsd}/[I]$ values at pH 6.5 (Powers and Tuhy, 1973).

[c] These three sets of results are on an identical scale.

$k_{obsd}/[I] = 2.0 \text{ M}^{-1}\text{sec}^{-1}$ and half-life = 118 minutes. However, this inhibition rate was only about 1% of that observed with the leukocyte elastase. Even the isoleucine chloromethyl ketone, with its somewhat extended alkyl side chain, inhibited chymotrypsin at a rate less than 5% of that observed with the human leukocyte elastase under identical conditions.

Significance of Results

Peptide chloromethyl ketones have proved useful for studies of human leukocyte elastase and its biological activity. For example, Dr. Aaron Janoff has shown that chloromethyl ketones inhibit the digestion of elastin-rich human lung tissue and rat aortic tissue by a human granulocyte elastase in vitro (Janoff, 1972a). A number of further studies using chloroketone inhibitors are currently in progress.

Our results suggest that peptide chloromethyl ketones, if properly designed, can be made fairly specific for human leukocyte elastase. Such specificity is necessary if chloromethyl ketone inhibitors are to be utilized safely in the treatment of emphysema and related diseases. Side reaction interference with other critical biological functions is often a very real problem when drug-type agents are medically evaluated.

CHAPTER IV

CONCLUSIONS

In conclusion, the kinetic results indicate that P_1-valine, isoleucine and alanine tetrapeptide chloromethyl ketones are reactive and specific inhibitors for both porcine pancreatic elastase and human leukocyte elastase. The analogous threonine chloromethyl ketone was relatively unreactive, presumably due to hydrogen-bonding induced conformational changes. These tetrapeptide inhibitors were also shown to be fairly specific for elastolytic enzymes in preference to trypsin or chymotrypsin. With porcine pancreatic elastase, both the valine and isoleucine chloromethyl ketone inhibitors were as effective or slightly more effective than our standard alanine inhibitor. With human leukocyte elastase, however, the valine and isoleucine inhibitors were markedly more effective than the alanine inhibitor (the best one being Ac-Ala-Ala-Pro-Val-CH_2Cl). This result implies that these two elastolytic enzymes are different and distinct.

Human leukocyte elastase has recently been implicated in the pathology of emphysema and related inflammatory diseases. Eventually chloromethyl ketone inhibitors may become valuable in the treatment of these important diseases.

CHAPTER V

RECOMMENDATIONS

The anomalous kinetic behavior of Ac-Ala-Ala-Pro-Thr-CH$_2$Cl with
elastase reported herein might form the basis for further chemical
inquiry. A profitable extension of our work would be to mask the
hydroxyl group of the threonine amino acid residue by 0-methylation
and investigate the reactivity of this new chloromethyl ketone with
elastase. This derivative, approximately isosteric with the very reac-
tive isoleucine inhibitor, should be easily accommodated within the
primary binding pocket of elastase. A comparison with the relatively
ineffective parent inhibitor, therefore, would be meaningful. A rapid
rate of inactivation would affirm previous interpretations of the
ineffective kinetic behavior of the parent threonine-P$_1$ inhibitor. A
slow rate of inactivation by the masked chloromethyl ketone, however,
would not eliminate the possibility that the oxygen of either threonine
inhibitor was hydrogen-bonded to some enzyme hydrogen-donor.

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VITA

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