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The use of boronic acids in the study of hydrolytic enzymes

de Soyza, Tushini Viveka, Ph.D.

City University of New York, 1990

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THE USE OF BORONIC ACIDS IN THE STUDY OF
HYDROLYTIC ENZYMES

by

Tushini V. de Soyza

A dissertation submitted to the Graduate Faculty
in Biochemistry in partial fulfillment of the
requirement for the degree of Doctor of
Philosophy, The City University of New York.

1990

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This manuscript has been read and accepted for the Graduate
Faculty in Biochemistry in satisfaction of the dissertation
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ABSTRACT**THE USE OF BORONIC ACIDS IN THE STUDY OF
HYDROLYTIC ENZYMES**

by

Tushini V. de Soyza

Adviser: Professor Manfred Philipp

**Part I: Substrate specificity and boronic acid inhibition
of E. coli RTEM-2 beta-lactamase**

In this study, kinetic data for E. coli RTEM-2 beta-lactamase were obtained in order to understand the mechanism of action of this enzyme.

The substrate specificity of this Class A beta-lactamase for the two cephalosporins PADAC and nitrocefin was studied. This study revealed the presence of essential groups on the free enzyme which have pKs of 5.5 and 7.5. These pKs are tentatively assigned to the active site groups Glu-166 and Lys-73. pK_2 was shifted by substrate binding, while pK_1 was unchanged.

Inhibition of E. coli RTEM-2 beta-lactamase by boronic acids was rapid, occurring within a minute of mixing. Of the tested boronic acids, the arylboronic acids with bulky hydrophobic substituents were the most potent inhibitors. Of the other substituted phenylboronic acids, those with electron-withdrawing substituents were better inhibitors. The pH-dependence of inhibition for this group

of compounds showed the ionization of a basic group with a pK of about 5.3. pK_2 reflected the ionization of the substituted phenylboronic acid.

**Part II: A comparison of the inhibition of the serine
protease thrombin and the cysteine protease papain
by a peptide boronic acid**

Inhibition of bovine serum thrombin by 1-(Z-D-Phenylalanyl-L-Prolineamido)-1-dihydroxyborono-4-methoxybutane was competitive. Inhibition occurred soon after mixing, to an observation time of about a minute. The pH-dependence of inhibition was bell-shaped and was governed by a pK_1 of 7.05, which is attributed to the ionization of the active site histidine. The pK_2 of 8.75 reflects the ionization of the boronic acid group. The $K_i(\text{Lim})$ was 6.9 nM.

Inhibition of bovine serum thrombin by Z-D-Phenylalanyl-L-Prolyl-L-Arginine was also competitive. The pH profile of inhibition was sigmoidal with a pK of 8.0. $K_i(\text{Lim})$ was 1.5 μM . Inhibition was rapid.

Papain, a cysteine protease was also inhibited by the above peptide boronic acid. Inhibition occurred soon after mixing. However, inhibition was noncompetitive at pH 7. The pH profile of inhibition was bell-shaped and was governed by a pK_1 of 4.4 and a pK_2 of 7.4. $K_i(\text{Lim})$ was 12.5 μM .

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FOR MY PARENTS

List of Abbreviations

Ac = acetyl

AIE = 5-amidoisophthalic acid dimethyl ester

Am = Amidino

AMC = aminomethylcoumarin

beta-BPA = beta-bromopenicillanic acid

t-Boc = tertiary-butyloxycarbonyl

Bz = benzoyl

Bzl = benzyl

BSA = bovine serum albumin

CBZ or Z = benzyloxycarbonyl

DMF = dimethylformamide

DMSO = dimethylsulfoxide

Dpm = diphenylmethyl

EDTA = ethylenediaminetetraacetate

I = ionic strength

Lim = pH-independent limiting value (see Appendix 1)

Nas = N-arylsulfonyl

pNP = para-nitrophenylacetate

Chromobora I =

dimethylaminonaphthylazomethoxyphenylsulphamidophenyl-3-
boronic acid

Chromobora II = 3-(dimethylaminophenylazophenyl-
thioureido)-phenylboronic acid

Fluorobora II = 3-(darpsylamido)-phenylboronic acid

Fluorobora P = 3-(pyrenesulfamino)-phenylboronic acid

nitrocefin = 3-(2,4-dinitrostyryl) (6R,7R)-7-(2-thienylacetamido)-ceph-3-em-4-carboxylic acid

PADAC = 7-(thienyl-2-acetamido)-3-[[2-[[4-(dimethylamino)phenyl]azo]pyridino]-methyl]cephem-4-carboxylic acid

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1. Substrate Specificity and Boronic Acid Inhibition of E. coli RTEM-2 beta-lactamase

INTRODUCTION

Bacterial resistance to penicillins is due to the enzyme, beta-lactamase. Beta-lactamase is both membrane-bound and excreted by penicillin-resistant bacteria (1). It hydrolyzes beta-lactam rings. Beta-lactamase activity was first recorded in 1940 (1).

Of the three classes of beta-lactamases, the Class A and C enzymes have a serine group at the active site (2). Class B enzymes are Zn or Mg-requiring metallo-enzymes (2).

The TEM-2 beta-lactamase, which is a class A enzyme, is a constitutive enzyme of some resistant strains of Escherichia coli. This is indistinguishable by substrate profile, sensitivity to beta-lactam inhibitors, molecular weight, and reaction with anti-serum, to the TEM-1 enzyme. They differ in their isoelectric points (3). Of the 186 amino acids present, there is only a single amino acid difference, that of lysine replacing glutamine at residue 14 in the TEM-2 beta-lactamase (4,5).

The bactericidal effect of penicillin and related beta-lactam antibiotics is due to their irreversible inactivation of D-alanine carboxypeptidase, which is the enzyme responsible for the transpeptidation step of bacterial cell wall biosynthesis (6). Penicillin acts as a

structural analog of the dipeptide terminus of peptidoglycan strands (7). The substrate forms an acyl-enzyme intermediate with the trans-peptidase. This is followed by elimination of D-Alanine. An inactive penicilloyl-enzyme is formed when penicillin, acting as a structural analog of the substrate, binds to the enzyme. An intact beta-lactam bond, a D-asymmetric center and the presence of a free carboxyl group are the specific structural features that are required of an antibiotic in order for it to bind to a carboxypeptidase from Bacillus cereus. This binding activity is abolished by mild heat or an ionic detergent (6). This indicates that this is not simply a chemical acylation of an active nucleophile, but the result of specific interactions between penicillin and the native enzyme. The covalent bond which is formed between penicillin and the enzyme is stable to boiling with SDS. A nearly stoichiometric complex is recovered after denaturation (6).

The covalent bond between the enzyme and penicillin is believed to be formed via the beta-lactam carbonyl bond (6). The hydroxylaminolysis of the bound penicillin is catalyzed by the carboxypeptidase, resulting in restoration of enzymatic activity, accompanied by the release of penicilloyl hydroxamate (8).

The serine proteases of the chymotrypsin and subtilisin families have similar, although evolutionarily independent catalytic mechanisms (9,10). Both have, as

active site functional groups, a serine, an adjacent histidine and an aspartic acid which interacts with the histidine. Two NH groups form hydrogen bonds to the anionic carbonyl oxygen atom adjacent to the scissile bond in the transition state (9). When crystal structures were compared, lysine-73 of the class A beta-lactamases was found to be aligned with the active site histidine of the serine proteases (11). No analogous aspartic acid residue was found at the equivalent position in beta-lactamase (11).

Photooxidation inactivates the E. coli RTEM enzyme, with accompanying destruction of histidine (12). This may indicate the involvement of histidine-112 in the mechanism, since this is the only conserved histidine residue, with the only exception being the enzyme from Staphylococcus aureus, where it is replaced by tyrosine (13). A completely inactivated enzyme is obtained, with the RTEM-2 enzyme, in the presence of N-bromosuccinimide, N-chlorosuccinimide and ethoxyformic anhydride, when two of the four available tryptophan residues are modified (14). B. cereus beta-lactamase I as well as the E. coli RTEM-2 beta-lactamase can be irreversibly modified by methanesulfonylfluoride (MSF) and diisopropylfluorophosphate (DFP) (15). The enzyme was protected from inactivation by these agents in the presence of a poor substrate, carbenicillin. This indicated active site modification at a serine residue (15).

Most studies on the inhibition of beta-lactamases

have concentrated on irreversible inactivators. In one of these the inactivation of Bacillus cereus beta-lactamase I by 6-beta-bromopenicillanic acid (beta-BPA) was found to be accompanied by covalent modification of the enzyme (16). 6-beta-bromopenicillanic acid is a probable suicide substrate of this enzyme (16). The inactivation reaction was competitively inhibited by cephalosporin C, a competitive inhibitor, as well as by covalently bound clavulanic acid (17). This suggests that the inhibitor reacts with the enzyme active site. It was proposed that the initial reaction is as a normal substrate and the rate-determining step of inactivation is acylation of the enzyme. This is followed by a rapid irreversible inactivation reaction rather than normal hydrolysis of the acyl-enzyme.

The pH-rate profile for inactivation of the enzyme gave apparent pK_a values of 5.25 and 8.6. These are likely to be due to ionization of groups on the free enzyme. These agree with the pK values obtained for ampicillin hydrolysis, which were 5.4 and 8.6 (18). This finding lends support to the hypothesis that the kinetics of beta-BPA inactivation of this enzyme simulate those of the interaction of the enzyme with a poor substrate (17).

A similar study (19) found inactivation of B. cereus beta-lactamase by beta-BPA to be a second-order process and inactivation was slower in the presence of substrate. It has been suggested that in the inactivation of the thiol RTEM-I beta-lactamase by beta-BPA, the inhibitor is

covalently bound to the cysteine residue (20). A study using ^3H -labelled beta-BPA found that in B. cereus beta-lactamase, the site labelled is serine-44 (21).

The first examples of potentially useful beta-lactamase inactivators were isolated from natural sources (22). One of these which is in clinical use is clavulanic acid. The initial interaction of clavulanic acid with the E. coli beta-lactamase is as a substrate which undergoes normal hydrolysis. Second, a transient intermediate is then formed, and the free enzyme is slowly regenerated. The third interaction is the irreversible inactivation of the enzyme (23). More than 100 molecules of clavulanate are required to inactivate one enzyme molecule. About one-third of the enzyme activity is restored on treatment of the inactivated enzyme with hydroxylamine. The rate of recovery of enzyme activity was consistent with the hydroxylaminolysis of an O-acyltyrosine (23).

Although irreversible inactivators are useful in practical applications, more mechanistic insight may be obtained with reversible inhibitors, especially if they are transition state analog inhibitors. Boric and boronic acids have been found to be reversible, competitive inhibitors of B. cereus beta-lactamase I (24) and the Class C beta-lactamase from Pseudomonas aeruginosa (25). In the latter study some of the compounds which contained potentially reactive functional groups, such as the aldehyde group, did not behave as irreversible inhibitors, as would be

expected if the aldehyde group reacted with an amino group in the enzyme to form a Schiff base. Similarly, m-iodoacetamidobenzeneboronic acid, a potential alkylating agent did not behave as an irreversible inhibitor. Within each of the series of arylsubstituted boronic acids tested, inhibitory ability fell in the order meta > para > ortho, where the differences between the meta- and para- compounds were barely significant. The ortho-compound would be expected to bind weakly due to an unfavourable steric interaction. It was not established in that study if a covalent bond forms between the active-site serine and the boronic acid, although it is thought that structure-specific boronic acids inhibit beta-lactamases in a similar manner to the serine proteases, that is by virtue of being covalently-binding transition state analogs.

Of the other reversible competitive inhibitors of beta-lactamases, the products of the enzymic reaction, penicilloic acids and their decarboxylation products, penilloic acids fall into the category of inhibitors which lack the beta-lactam ring. These were some of the first such inhibitors to be discovered (24).

EXPERIMENTAL

Materials

Water used in the experiments was deionized by passing it through an ion-exchange resin (Barnstead). Glassware was cleaned with soap and water and a cleaning solution of chromic acid where necessary, rinsed with deionized water and dried in an oven at 60°C.

Buffers were prepared from reagent grade chemicals. Initial 0.1 and 1 molar hydrochloric acid and sodium hydroxide solutions were prepared by adding a weighed amount into a volumetric flask followed by addition of water. Initial 0.5 molar potassium dihydrogen phosphate, 1 molar acetic acid, 1 molar ammonia, and 1 molar sodium bicarbonate solutions were prepared similarly. Buffer solutions of known ionic strength were prepared by mixing these solutions and diluting them according to the proportions given in The Biochemists' Handbook (26). These were sodium dihydrogen phosphate-disodium hydrogen phosphate within the pH range 5.8 to 7.8; acetic acid-sodium acetate within the pH range 3.8 to 5.8; 5:5'-diethylbarbituric acid-sodium 5:5-diethylbarbiturate within the pH range 8.0 to 8.8 and sodium bicarbonate-sodium carbonate within the pH range 9.2 to 10. Buffers were filtered through a Millipore filter (pore size: 0.45 μ m) at regular intervals.

The pH meter was calibrated with standard buffers of pH 4, 7 and 10 (Fisher Scientific Co.).

Acetonitrile was from Aldrich Chemical Co. Dimethylsulfoxide and dimethylformamide were from Fisher Scientific. These solvents were used without further purification.

The E. coli RTEM-2 beta-lactamase was a gift from Dr. K. Bush.

The lactamase substrate benzylpenicillin (Penicillin G) hydrochloride was purchased from Sigma. Stock solutions of this were made by dissolving in pH 7.00 buffer of 0.1M ionic strength (3 mg/ml). 3-(2,4-dinitrostyryl)(6R,7R)-7-(2-thienylacetamido)-ceph-3-em-4-carboxylic acid (nitrocefin) was purchased from BBL Microbiology. Stock solutions of this were in dimethylsulfoxide (0.3-3 mg/ml) with dilutions into buffer. Pyridinium-2-azo-p-dimethylaniline cephalosporin (PADAC) was purchased from Calbiochem and stock solutions were made in pH 7.00 buffer to a level of 3 mg/4 ml.

Of the compounds which were tested as inhibitors, 2,4-dichlorophenylboronic acid was purchased from Alfa Chem. Co. Stock solutions were in 95% ethanol (40 mg/ml).

3,5-Bis(trifluoromethyl)phenylboronic acid was purchased from Lancaster Synthesis. Stock solutions were made in 95% ethanol (30 mg/ml).

4-Bromophenylboronic acid, 3-nitrophenylboronic acid, 3-dansylamidophenylboronic acid, 3-aminophenylboronic

acid, ortho-phthalaldehydic acid and 4-carboxybenzaldehyde were purchased from Aldrich. 3-Carboxyphenylboronic acid was purchased from Calbiochem. Isophthalaldehydic acid was purchased from Pfalz and Bauer.

Other than for the carboxy-substituted compounds which were dissolved in acetone (~3 mg/ml) stock solutions of all the other compounds were in 95% ethanol (10-40 mg/ml).

3-Succinylamidophenylboronic acid, N-acetyl-D-boro-Valine, and N-acetyl-D-boro-Leucine were synthesized by Dr. D. Matteson and co-workers. Stock solutions were made in acetonitrile (2-4 mg/ml).

Dimethylaminonaphthylazomethoxyphenylsulfamidophenyl-3-boronic acid (Chromobora I), 3-(dimethylaminophenylazophenyl-thioureido)-phenylboronic acid (Chromobora II), 3-(darpsylamido)-phenylboronic acid (Fluorobora II), and 3-(pyrenesulfamino)-phenylboronic acid (Fluorobora P) were purchased from Polysciences Inc.

Stock solutions of Chromobora I, Fluorobora II and Fluorobora P were made in 95% ethanol (1 mg/ml). Stock solutions of Chromobora II were in dimethyl formamide (1 mg/ml).

Methods

Kinetic data were initially obtained on a GCA/McPherson Spectrophotometer (Model EU-707-SA). This

instrument was attached to a temperature control system. Enzyme assays were performed at 25 or 30°C.

Kinetic studies were also done on a Vmax kinetic microplate reader (Molecular Devices), at known ambient temperatures. Microplates were purchased from PerfeCTOR Scientific. Aspirations, mixing, and dilutions were performed by a Biomek 1000 automated laboratory workstation.

Benzylpenicillin hydrolysis was observed at 232 nm. At this wavelength, absorbance decreases as product is formed. (Δ extinction coefficient = $940 \text{ M}^{-1}\text{cm}^{-1}$).

Stock solutions of E. coli RTEM-2 beta-lactamase were made in pH 7 buffer, I=.1 M. (usually 10 microliters/ml). Further dilutions were also in buffer. The enzyme was kept frozen. Aliquots of 100-200 microliters were thawed and kept at 0-5°C., and used within a month.

Buffers of ionic strength 0.1 M and 1 M NaCl were used to prepare the reaction buffers which were 0.5M in ionic strength and had 1 mM EDTA. These were filtered (Millipore filtration unit) daily, when absorbance measurements were taken in the U.V. region. Cuvettes were washed in chromate wash solution and then thoroughly rinsed with deionized water between kinetic runs. These were either air-dried or dried in an oven at 60°C. Quartz cuvettes were used in the U.V. region. All cuvettes used were of one cm path length and were of 1 ml or 3 ml capacity.

For the most part, continuously adjustable digital microliter pipettes from Rainin Instrument Co. were used in this study.

The substrate and the inhibitor being studied were added in aliquots of 50 - 200 microliters to the buffer (one or three ml) in a cuvette. The solution was mixed by inversion and placed in the cuvette holder of the spectrophotometer. A baseline absorbance reading would then be taken over a one to two minute time interval. The enzyme, in aliquots of 100 - 200 microliters would then be added, the solution again mixed by inversion and the change in absorbance due to product formation was observed as a function of time. The delay in taking the first absorbance reading after adding the enzyme was less than a minute.

The McPherson spectrophotometer was typically used with the following settings: span of 0.1 absorbance units, signal averaging over 100 milliseconds with a chart speed of 30 seconds per division.

The dissociation constant for 3-carboxyphenylboronic acid was calculated in the presence of 10 mM EDTA in the assay mix. This value was compared to the value obtained with no EDTA in the assay mix. This study was done in order to see if there was significant complexation of the boronic acid with EDTA. EDTA was introduced in order to inactivate any beta-lactamase II which would have interfered with the kinetics.

The inhibitory abilities of the boronic acid analogs

of N-acylamino acids and all hydrophobic arylboronic acids other than 3-(dimethylaminophenylazophenyl-thioureido)-phenylboronic acid were tested with benzylpenicillin as the substrate. The reaction was followed to completion and pseudo first order kinetic plots were obtained. The pseudo-first-order rate constants were derived from time-course absorption data. Typically, for each inhibitor, at least three different inhibitor concentrations were used. Kinetic data for one compound were obtained within a short period of time, usually within twelve hours. The same enzyme stock solution was used at all inhibitor concentrations. Fresh enzyme stock solutions were made daily.

10^{-4} M boric acid-containing buffers were used in the assays which used the boronic acid analogs of N-acyl amino acids. This was done in order to remove the pinane-diol blocking group from the boronic acid moiety, at concentrations of boronic acid higher than 10^{-6} M. (This group comes off spontaneously in solution, at lower concentrations.)

All other boronic acids as well as the aldehydes were tested with nitrocefin as the substrate. Stock solutions of nitrocefin were made up in DMSO (3 mg/ml). The reaction progress was measured at 486 nm (delta extinction coefficient $16,000 \text{ M}^{-1}\text{cm}^{-1}$). Initial rates with, and without the inhibitor were obtained, at a substrate concentration which was well below K_m (11 μM).

The molar enzyme concentration of the stock solution

was determined using the literature value of k_{cat}/K_m for benzylpenicillin hydrolysis.

In order to see if the enzyme was being adsorbed on to the glass walls of the cuvette, pseudo first order rate constants were obtained at different enzyme concentrations, with benzylpenicillin as the substrate (20 μM). These were plotted as a function of enzyme concentration.

The substrate specificity of E. coli RTE-2 beta-lactamase to nitrocefin and PADAC was studied on the Vmax kinetic microplate reader. The components of the assay mixture were essentially as before except that the ionic strength of the assay mixture was 0.35 M. The wavelengths used were 490 and 450 nm respectively. (Δ extinction coefficient for PADAC at 450 nm = $12,400 \text{ M}^{-1}\text{cm}^{-1}$). pH profiles were constructed using a program written on a Lotus-123 spreadsheet (see Appendix 1). These were constructed to give theoretical curves of one ionization. The pH profiles of $1/K_m$ for nitrocefin hydrolysis were constructed by combining the spreadsheets of k_{cat} and k_{cat}/K_m .

The pH profiles for the inhibition of E. coli RTE-2 beta-lactamase by arylboronic acids were also obtained.

A study was done to see if there was appreciable adsorption of enzyme on to the surface of the microplate reader well walls. Initial rates were obtained over a row of wells (8), at enzyme concentrations arrived at by serial dilution.

RESULTS

The substrate specificity study for the two cephalosporins 3-(2,4-dinitrostyryl)-(6R,7R)-7-(2-thienylacetamido)-ceph-3-em-4-carboxylic acid (nitrocef) and pyridinium-2-azo-p-dimethylaniline cephalosporin (PADAC) show that pH dependency of k_{cat}/K_m is governed by a pK_1 which is 5.5 (Figures 2, 5 & 9).

pK_2 for nitrocef hydrolysis is 6.96 in 8% DMSO and 7.30 in 0.4% DMSO. pK_2 for PADAC hydrolysis is 7.72 in the absence of organic solvent.

The limiting value for k_{cat}/K_m for nitrocef shows a two-fold increase at 0.4% DMSO compared to 8% DMSO in the assay mix. The value at 0.4% DMSO is $4.64 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ (Figure 5). The limiting value for k_{cat}/K_m for PADAC hydrolysis is $6.95 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ (Figure 9) in the absence of organic solvent.

At pH values below 4.5, the observed initial rate of the reaction for both substrates was low at the highest substrate concentrations used, which were about $2 \times K_m$. These values were excluded when constructing Lineweaver-Burk plots.

This was more apparent with PADAC, where deviant kinetics were observed when using high substrate concentrations. It was therefore decided to use substrate concentrations that were well below K_m for constructing Lineweaver-Burk plots. As a consequence, the individual pH-

dependencies of k_{cat} and K_m could not be determined.

pK_1 of k_{cat}/K_m for PADAC hydrolysis is 5.48 and pK_2 is 7.72. $k_{\text{cat}}/K_m(\text{Lim})$ is $6.95 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (Figure 9).

pK_1 in the pH-dependence of k_{cat} for nitrocefin hydrolysis at both DMSO concentrations is 5.5. pK_2 at 8% DMSO is 7.71 and at 0.4% DMSO is 8.22. $k_{\text{cat}}(\text{Lim})$ at 8% DMSO is 2180 s^{-1} and at 0.4% DMSO is 2656 s^{-1} (Figures 3 & 6).

The pH profiles of $1/K_m$ were constructed so that $\text{pK}_E = \text{pK}_2$ of k_{cat}/K_m and $\text{pK}_{ES} = \text{pK}_2$ of k_{cat} (Figures 4 & 7)

Table I

Substrate	pK_1	pK_2	$k_{\text{cat}}/K_m(\text{Lim})$ $\times 10^{-6} \text{ M}^{-1} \text{ s}^{-1}$	Conditions
Nitrocefin	5.44	6.96	23.1	8% DMSO
Nitrocefin	5.50	7.30	46.4	0.4% DMSO
PADAC	5.48	7.72	6.95	0% DMSO

Table II

Substrate	pK_1	pK_2	$k_{\text{cat}}(\text{Lim})$	Conditions
Nitrocefin	5.49	7.71	2180 s^{-1}	8% DMSO
Nitrocefin	5.46	8.22	2656 s^{-1}	0.4% DMSO

Inhibition by Chromobora I, which is dimethylaminonaphthylazomethoxyphenylsulfamidophenyl-3-boronic acid, by Chromobora II, which is 3-(dimethylaminophenylazophenyl-thioureido)-phenylboronic acid, by Fluorobora II, which is 3-(darpsylamido)-phenylboronic acid, and by Fluorobora P, which is 3-(pyrenesulfamino)-phenylboronic acid is rapid and dissociation constants are in the low micromolar range at pH 7 (Figures 12, 13 & 14). Of these, the inhibition by dimethylaminonaphthylazomethoxyphenylsulfamidophenyl-3-boronic acid (Chromobora I) is best with a K_i of 0.5 μM (Figure 14).

Inhibition of notrocefin hydrolysis by Chromobora II is competitive (Figure 11). Inhibition by these phenylboronic acids with bulky hydrophobic substituents at the 3- position occurred soon after mixing (Figure 15).

Table III

Inhibitor	Substrate	K_i μM
Chromobora I	Benzylpenicillin	0.5
Chromobora II	Nitrocefin	3.4
Fluorobora II	Benzylpenicillin	2.9
Fluorobora P	Benzylpenicillin	1.75

Inhibition by N-acetyl-D-boro-leucine and N-acetyl-D-boro-Valine was rapid with slightly better inhibition seen with N-acetyl-D-boro-Leucine which has a K_i of 1.5×10^{-5} M. N-acetyl-D-boro-Valine has a K_i of 4.7×10^{-5} M (Figure 16). Inhibition by these amino acid analogs is better when compared with the inhibition by the substituted arylboronic acids (Table XIV).

There was no inhibition by the aldehydes ortho-phthalaldehydic acid, isophthalaldehydic acid and 4-carboxybenzaldehyde at a concentration of 3 mM in the assay mix.

The substituted arylboronic acids all show bell shaped $1/K_i$ vs pH relationships (Figures 18 to 23).

The Brønsted plot of $\log 1/K_i(\text{Lim})$ vs the observed $\text{p}K_2$ of the 3- substituted phenylboronic acids gives a slope of -1.2 (Figure 24).

Figure 25 shows the relationship between $1/K_i(\text{Lim})$ and the observed $\text{p}K_1$ of the substituted phenylboronic acids.

Table IV

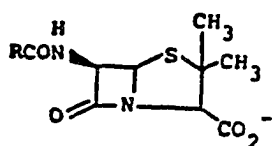
Inhibitor substituent	pK_1	pK_2	K_i (Lim) $\times 10^5 M$	Conditions	pK_{lit}^*
<u>p</u> -Br	4.84	8.16	4.63	0.4% DMSO	8.66
<u>m</u> -NO ₂	5.50	7.50	6.41	0.4% DMSO	7.23
-H	5.32	8.68	84.7	0.4% DMSO	8.83
2,4-Cl ₂	5.46	7.74	44.8	0.4% DMSO	
<u>m</u> -NH ₂	5.51	8.88	200	0.4% DMSO	
<u>m</u> -CO ₂ H	6.33	7.66	2.63	3.0% DMSO	

*Reference 27

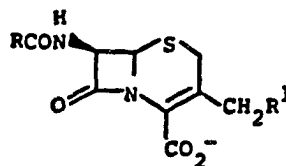
There is no significant difference in the binding constant of m-carboxyphenylboronic acid with 10 mM EDTA in the assay mixture (Figure 17). Therefore all assay buffers contained 1 mM EDTA. The error in K_i values introduced by 1 mM EDTA is estimated to be about 1.5% (the K_i with 1 mM EDTA would be 1.5% higher than the K_i with no EDTA).

It can be seen that neither the glass cuvettes nor the microtiter plates (Perfector Scientific) used in this study adsorbed the enzyme to any significant degree. (Figures 26 & 27). If such adsorbance had occurred, it would have adversely affected the kinetic data.

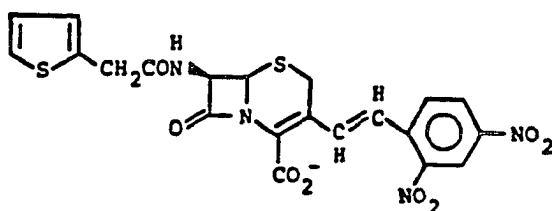
Figure 1. Substrates of *E. coli* RTEM-2 beta-lactamase



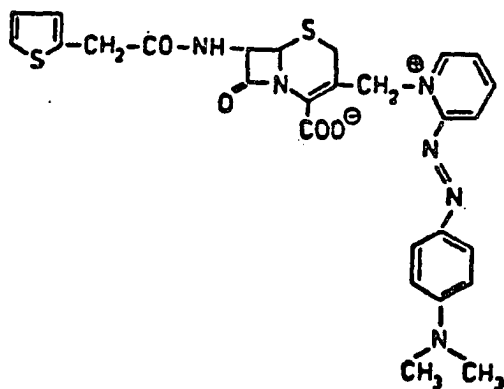
Penicillins



Cephalosporins



3-(2,4-dinitrostyryl)(6R,7R)-7-(2-thienylacetamido)-ceph-3-em-4-carboxylic acid (nitrocefin)



7-(thienyl-2-acetamido)-3-[[2-[[4-(dimethylamino)phenyl]azo]pyridino]-methyl]cephem-4-carboxylic acid (PADAC)

TABLE V

pH-dependent values of nitrocefin hydrolysis by E. coliRTEM-2 beta-lactamase

DMSO in the assay mix. = 8% v/v. I = 0.35 M

pH	$k_{\text{cat}}(\text{sec}^{-1})$	$K_{\text{m}}(\mu\text{M})$	$k_{\text{cat}}/K_{\text{m}} (\times 10^{-6} \text{ M}^{-1}\text{s}^{-1})$
4.50	360	122	2.95
4.90	520	148	3.51
5.30	507	71.6	7.08
5.70	1210	79.2	15.3
6.32	2010	125	16.1
6.85	1626	100	16.3
7.05	2139	195	11.0
7.40	1264	171	7.37
7.70	1010	121	8.35
8.10	1036	841	1.23
8.60	259	576	0.450
9.10	98.6	623	0.158

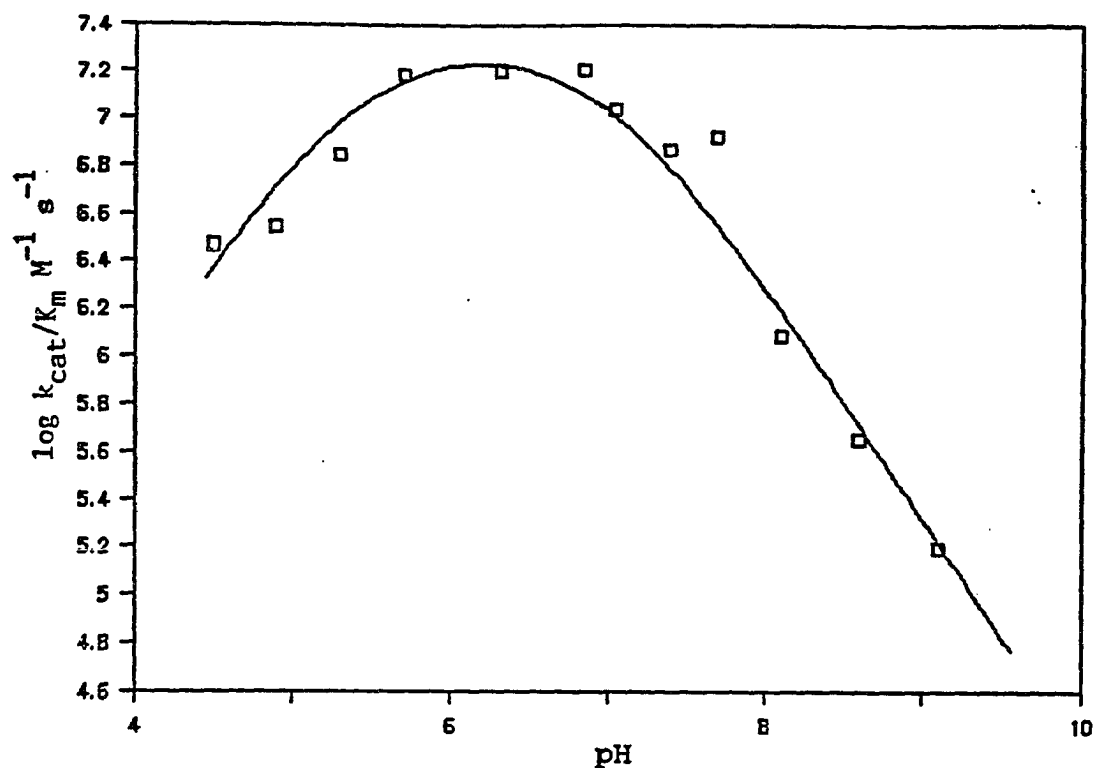


Figure 2. pH-dependence of nitrocefin hydrolysis by E. coli RTEM-2 beta-lactamase
DMSO in the assay mix. = 8% v/v. I = 0.35 M
 $\text{pK}_1 = 5.44 \pm 0.22$ $\text{pK}_2 = 6.96 \pm 0.22$
 $k_{\text{cat}}/K_m(\text{Lim}) = (2.31 \pm 0.353) \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$
Data of Table V

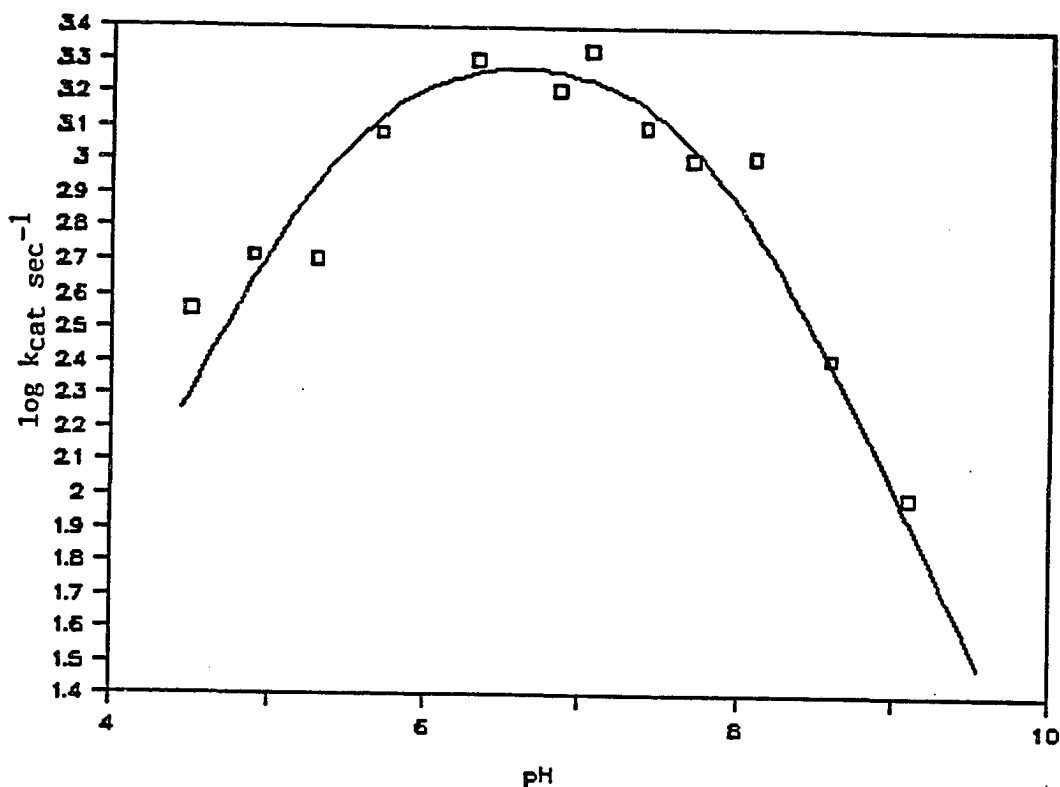


Figure 3. pH-dependence of k_{cat} for nitrocefin hydrolysis by *E. coli* RTEM-2 β -lactamase
 DMSO in the assay mix. = 8% v/v. $I = 0.35$ M
 $pK_1 = 5.49 \pm 0.37$ $pK_2 = 7.71 \pm 0.37$
 $k_{cat}(Lim) = 2180 \pm 206$ s⁻¹
 Data of Table V

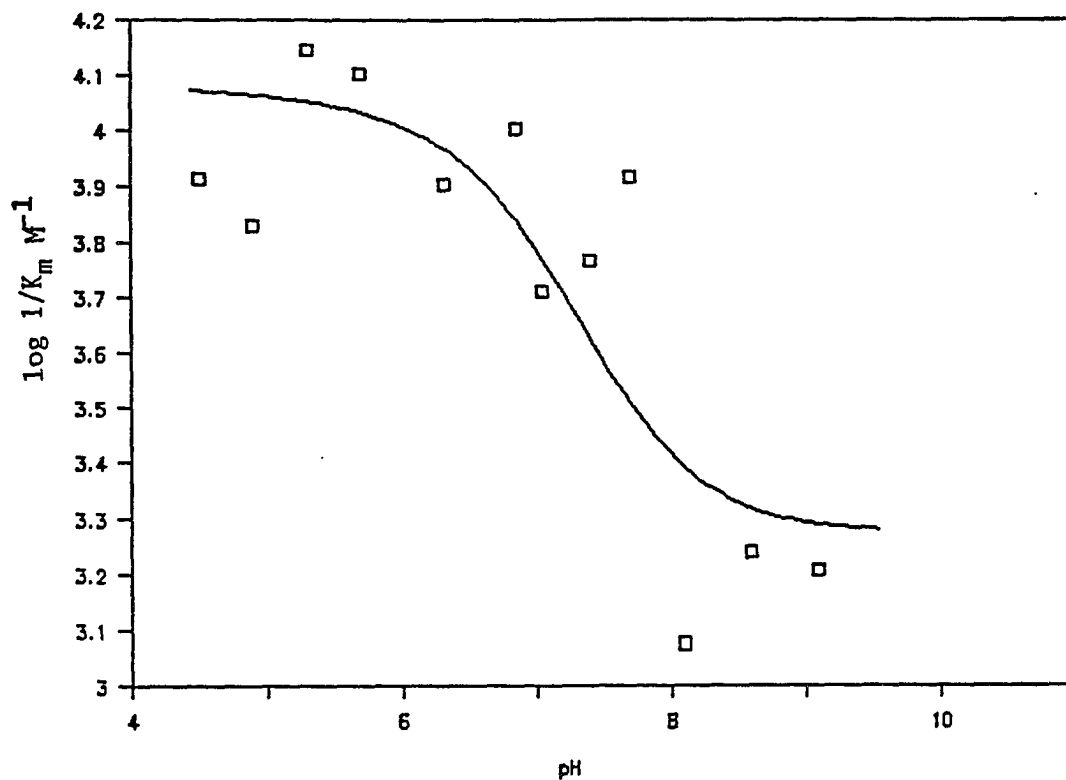


Figure 4. pH-dependence of $1/K_m$ for nitrocefin hydrolysis by *E. coli* RTEM-2 beta-lactamase
 DMSO in the assay mix. = 8% v/v. I = 0.35 M
 The curve parameters are :
 $pK_E = 6.96 \pm 0.22$ $pK_{ES} = 7.71 \pm 0.37$
 The limiting values for K_m are $94.4 \pm 24 \mu\text{M}$ and $233 \pm 24 \mu\text{M}$

Table VI

pH-dependent values of nitrocefin hydrolysis by E. coli

RTEM-2 beta-lactamase

DMSO in the assay mix. = 0.4% v/v. I = 0.35 M

pH	$k_{\text{cat}} (\text{sec}^{-1})$	$K_{\text{m}} (\mu\text{M})$	$k_{\text{cat}}/K_{\text{m}} (\times 10^{-6} \text{ M}^{-1} \text{ s}^{-1})$
4.94	653	54.6	12.0
5.30	1515	98.5	15.4
5.67	1763	54.9	32.1
6.25	2145	58.7	36.5
6.85	2248	68.2	33.0
6.55	2513	72.4	34.7
7.15	2582	74.7	34.6
7.87	2118	247	8.57
8.10	1432	193	7.21
8.60	688	415	1.66
9.00	252	356	0.708
9.15	353	378	0.934
9.20	124	276	0.449
9.50	68.9	203	0.339

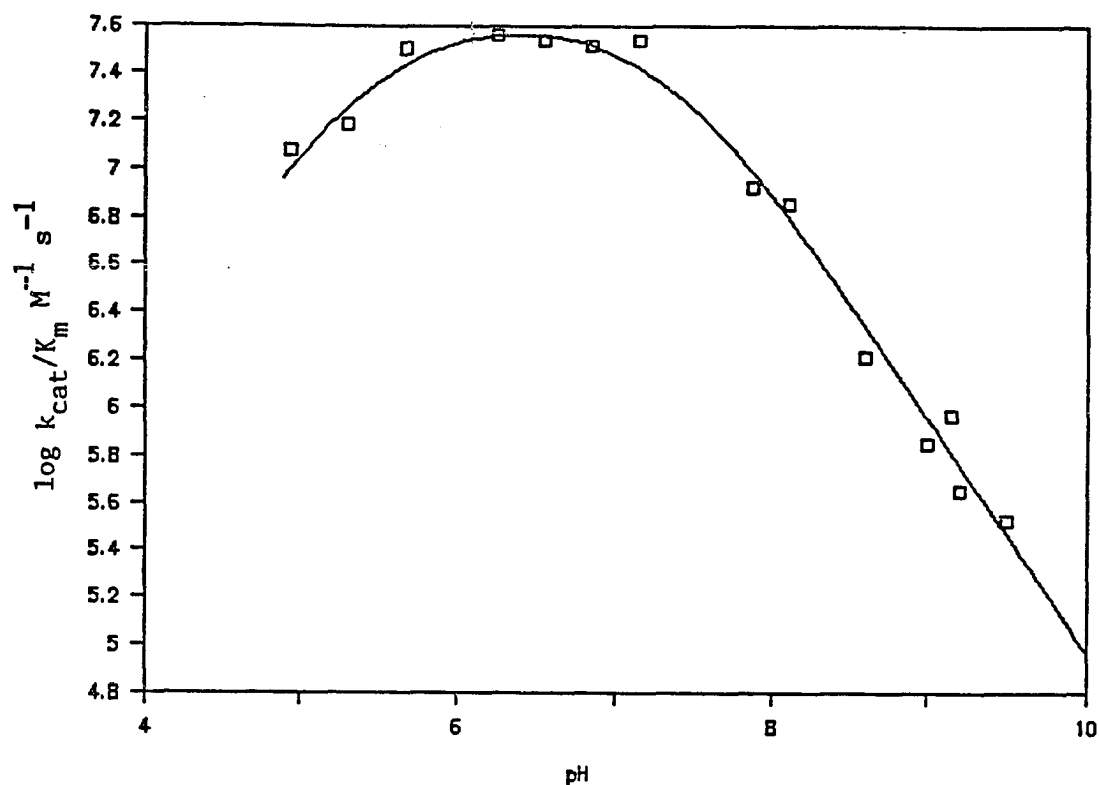


Figure 5. pH-dependence of nitrocefin hydrolysis by
E. coli RTEM-2 beta-lactamase
 DMSO in the assay mix. = 0.4% v/v. I = 0.35 M
 $\text{pK}_1 = 5.50 \pm 0.16$ $\text{pK}_2 = 7.30 \pm 0.16$
 $k_{\text{cat}}/K_m(\text{Lim}) = (4.64 \pm 0.28) \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$
 Data of Table VI

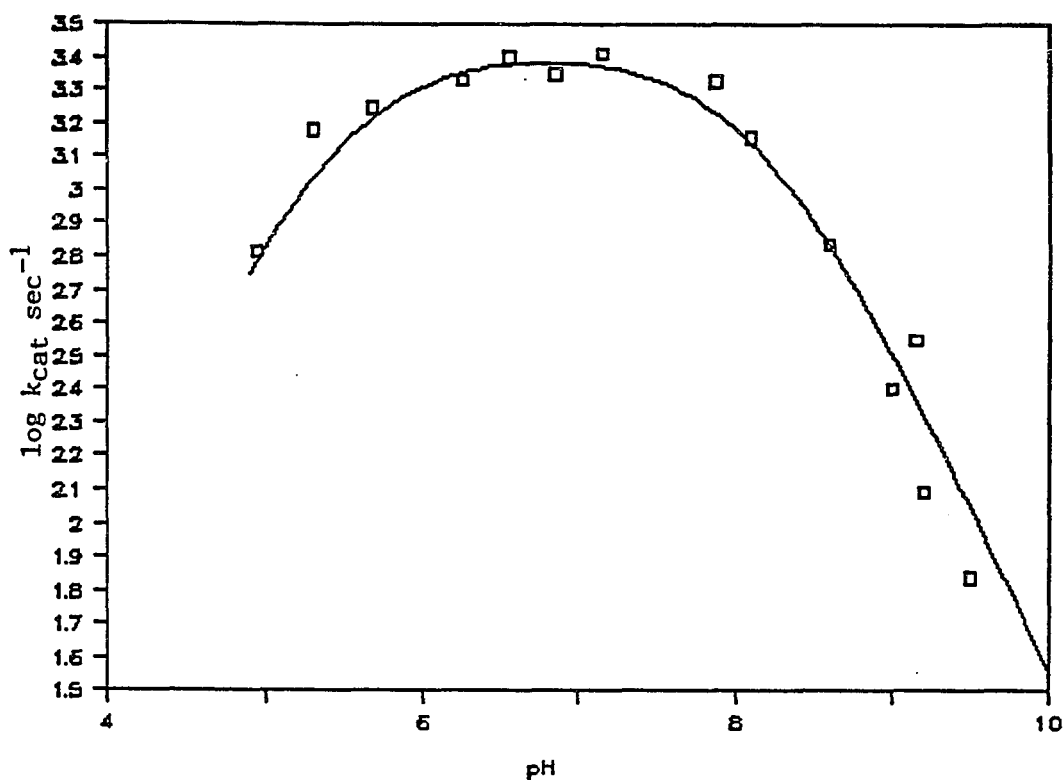


Figure 6. pH-dependence of k_{cat} for nitrocefin hydrolysis by *E. coli* RTEM-2 β -lactamase
 DMSO in the assay mix. = 0.4% v/v. $I = 0.35$ M
 $pK_1 = 5.46 \pm 0.28$ $pK_2 = 8.22 \pm 0.28$
 $k_{cat}(Lim) = 2656 \pm 157$ s⁻¹
 Data of Table VI

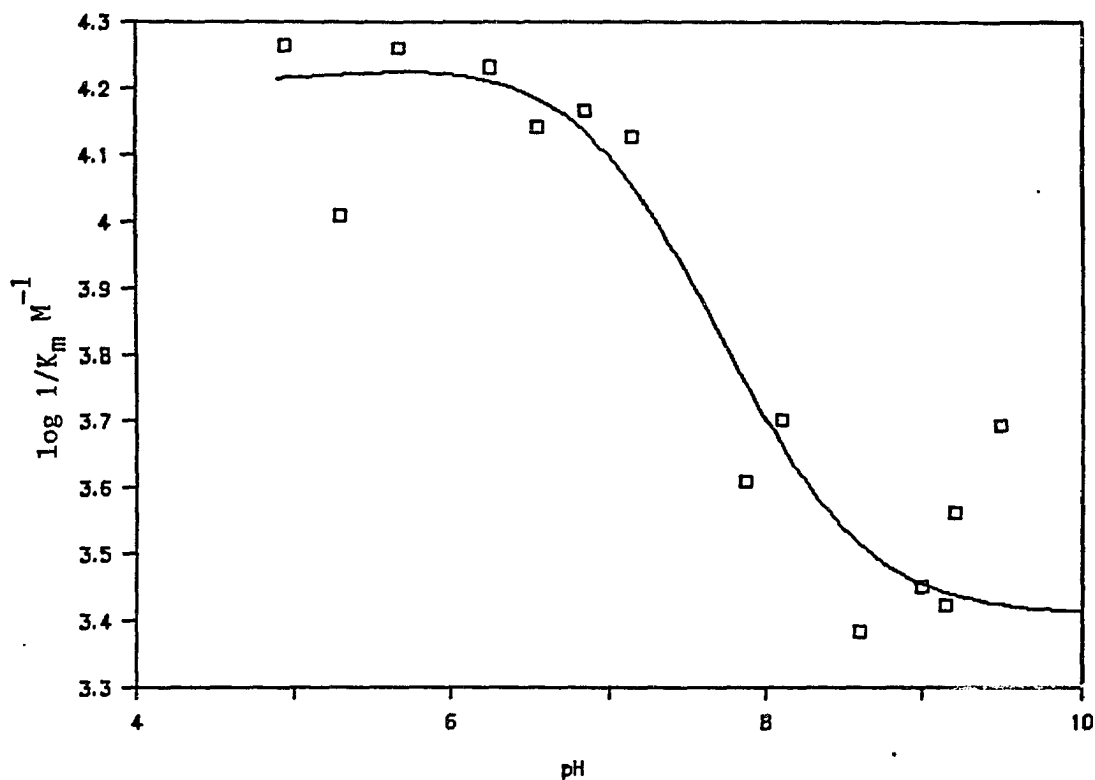


Figure 7. pH-dependence of $1/K_m$ for nitrocefin hydrolysis by E. coli RTEM-2 beta-lactamase
 DMSO in the assay mix. = 0.4% v/v. I = 0.35 M
 The curve parameters are:
 $pK_E = 7.30 \pm 0.16$ $pK_{ES} = 8.22 \pm 0.28$
 The limiting values for K_m are $57.2 \pm 6.85 \mu\text{M}$ and $172 \pm 6.85 \mu\text{M}$

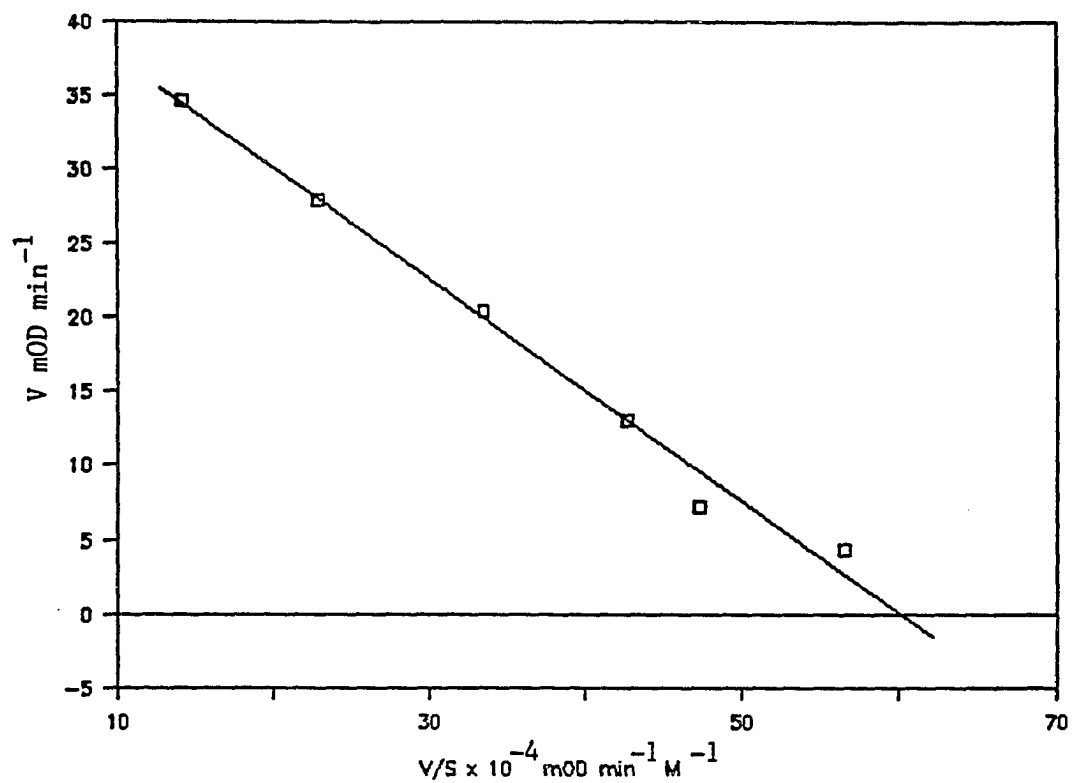


Figure 8. Nitrocefin hydrolysis by E. coli RTEM-2
beta-lactamase at pH 7.15
DMSO in the assay mix. = 0.4% v/v. I = 0.35 M
[enzyme] = 3.0×10^{-10} M

Table VII

PADAC hydrolysis by E. coli RTEM-2 beta-lactamase

I = 0.35 M

pH	$k_{\text{cat}}/K_m (\times 10^{-6} \text{ M}^{-1}\text{sec}^{-1})$
4.61	1.89
5.10	2.10
5.34	2.87
6.30	5.30
6.58	6.40
7.02	5.50
7.28	7.70
7.52	6.50
8.33	1.25
8.69	0.506
8.72	0.610
8.90	0.342
9.28	0.230

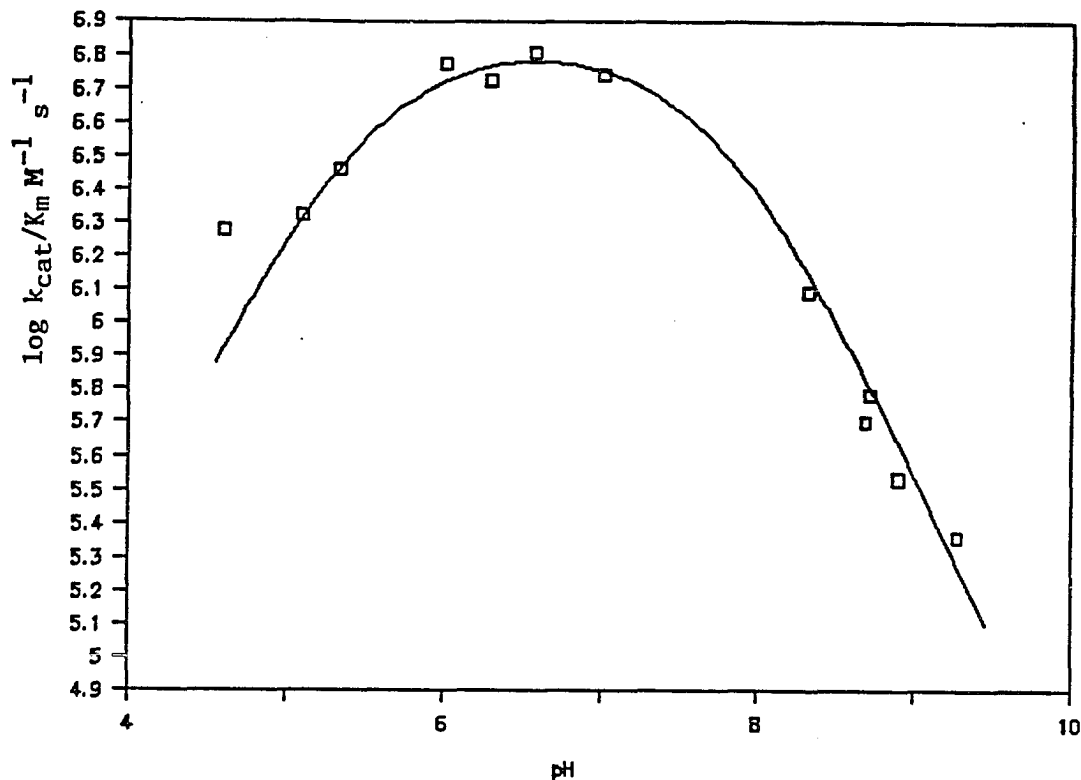


Figure 9. pH-dependence of PADAC hydrolysis by E. coli RTEM-2 beta-lactamase in the absence of organic solvent. $I = 0.35 \text{ M}$
 $pK_1 = 5.48 \pm 0.20$ $pK_2 = 7.72 \pm 0.20$
 $k_{cat}/K_m(\text{Lim}) = (6.95 \pm 0.34) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$
 Data of Table VII

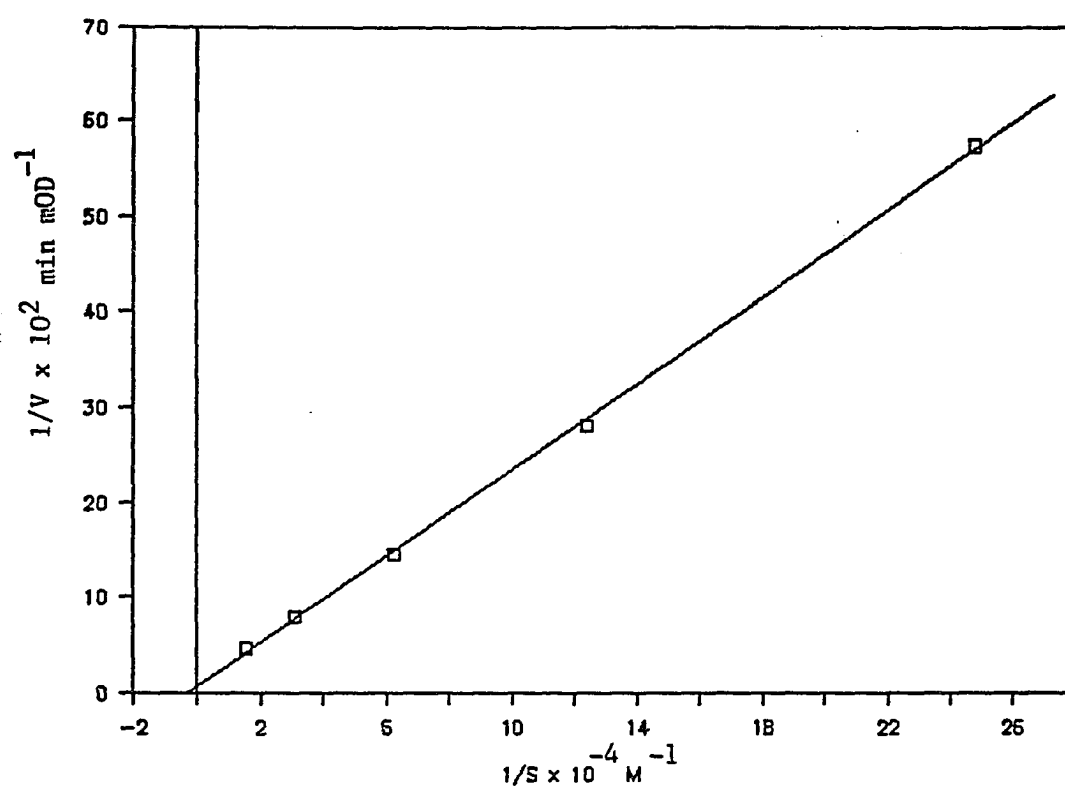


Figure 10. PADAC hydrolysis by *E. coli* RTEM-2 beta-lactamase at pH 7.02. $I = 0.35 \text{ M}$
 $[\text{enzyme}] = 2.2 \times 10^{-10} \text{ M}$

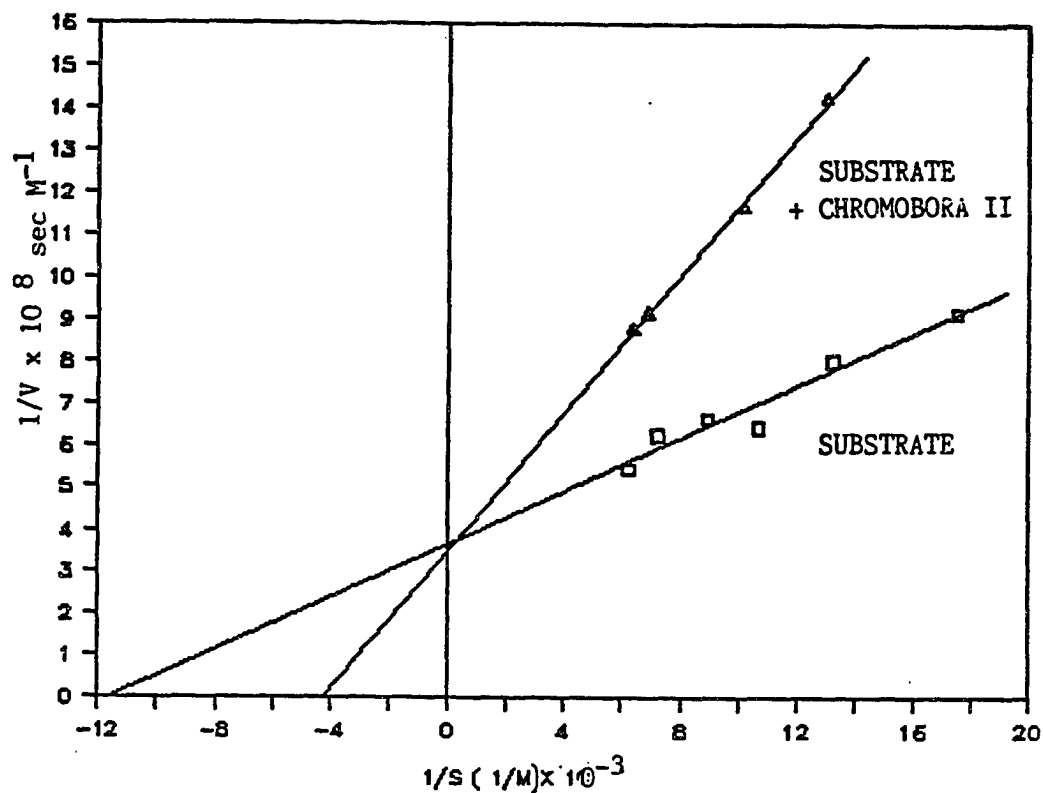


Figure 11. Nitrocefin hydrolysis by *E. coli* RTEM-2 beta-lactamase in the presence and in the absence of Chromobora II at pH 7 and 30°C. $I = 0.5 \text{ M}$
 $[\text{Chromobora II}] = 5.2 \times 10^{-6} \text{ M}$
 $[\text{enzyme}] = 8.0 \times 10^{-12} \text{ M}$
 $K_m = 8.6 \times 10^{-5} \text{ M}$ $k_{cat} = 1712 \text{ s}^{-1}$
 $K_i(\text{Chromobora II}) = 3.4 \times 10^{-6} \text{ M}$

Table VIII

Nitrocefin hydrolysis by E. coli RTEM-2 beta-lactamase in the presence of the inhibitor 3-(dimethylaminophenylazophenyl-thioureido)-phenylboronic acid (Chromobora II), at pH 7 and 30°C.

DMSO in the assay mix. = 3 % v/v. I = 0.5 M

[substrate] = 2.2 μ M

[enzyme] = 3.9×10^{-10} M

[I] (μ M)	k_1 (sec^{-1})
0	1.10×10^{-2}
1.62	7.35×10^{-3}
3.11	5.75×10^{-3}

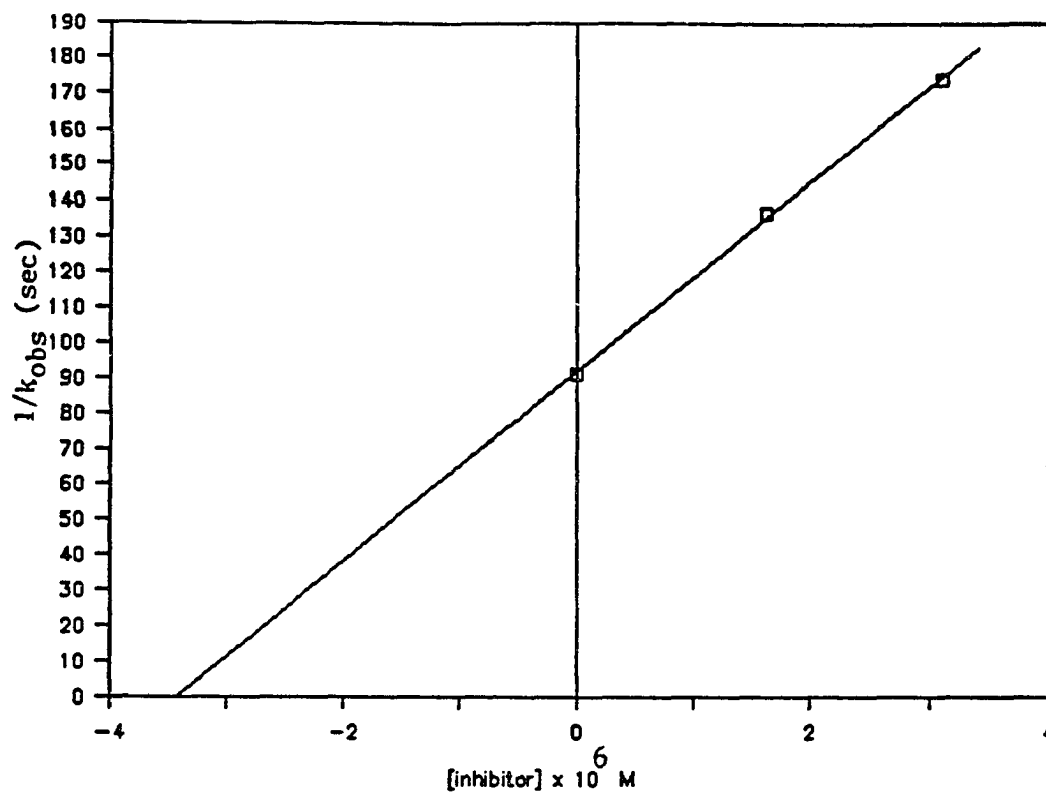


Figure 12. Chromobora II inhibition of E. coli RTM-2
beta-lactamase at pH 7 and 30°C. I = 0.5 M
 $K_i = 3.4 \times 10^{-6}$ M
 Data of Table VIII

Table IX

Benzympenicillin hydrolysis by E. coli RTEM-2 beta-
lactamase in the presence of the inhibitor 3-
(darpsylamido)-phenylboronic acid (Fluorobora II), at pH 7
and 30⁰C. I = 0.5 M
[substrate] = 40 μ M
[enzyme] = 3.9×10^{-10} M

[I] (μ M)	k_1 (sec ⁻¹)
1.33	4.4×10^{-2}
1.95	4.2×10^{-2}
2.25	3.9×10^{-2}
2.54	3.3×10^{-2}
3.11	3.1×10^{-2}
3.65	2.9×10^{-2}

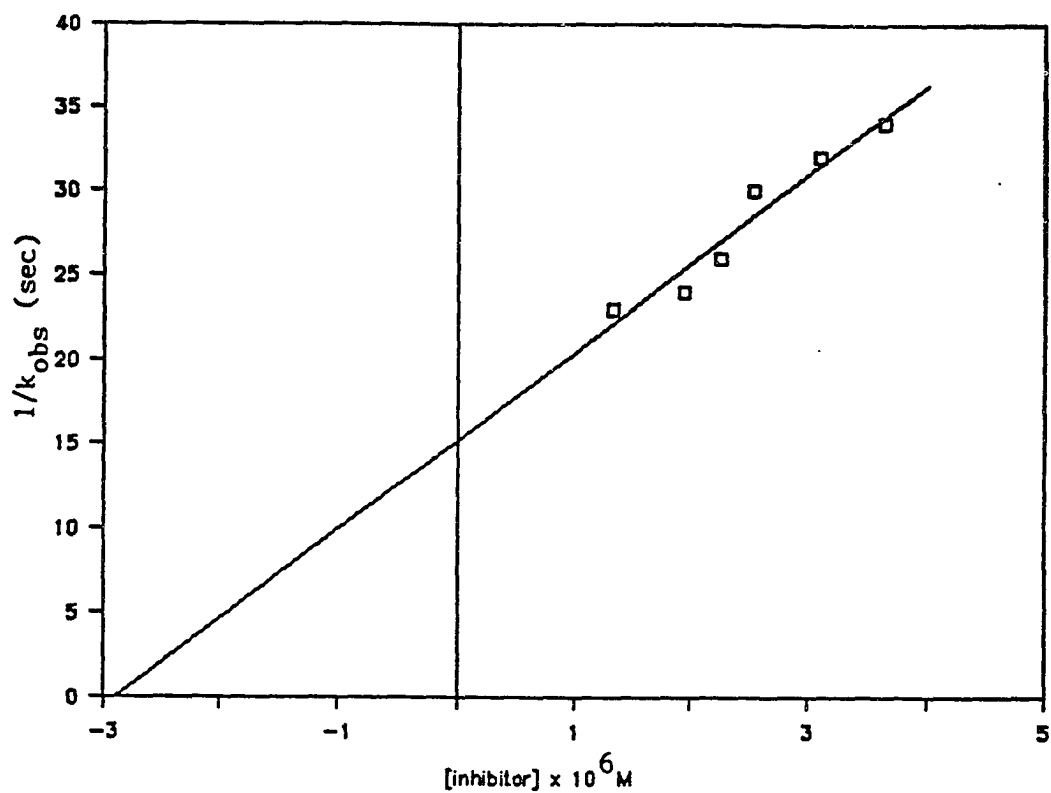


Figure 13. Fluorobora II inhibition of *E. coli* RTM-2
beta-lactamase at pH 7 and 30°C. I = 0.5 M
 $K_i = 2.9 \times 10^{-6}$ M
 Data of Table IX

Table X

Benzympenicillin hydrolysis of E. coli RTEM-2 beta-
lactamase in the presence of the inhibitor
dimethylaminonaphthylazomethoxyphenylsulphamidophenyl-3-
boronic acid (Chromobora I), at pH 7 and 30⁰C. I = 0.5 M
[substrate] = 41.6 μ M
[enzyme] = 6.8×10^{-10} M

[I] (μ M)	k_1 (sec ⁻¹)
0	1.60×10^{-2}
1.77	3.85×10^{-3}
2.21	2.90×10^{-3}
2.65	2.58×10^{-3}

Table XI

Benzympenicillin hydrolysis by E. coli RTEM-2 beta-lactamase in the presence of the inhibitor 3-(pyrenesulfamino)-phenylboronic acid (Fluorobora P), at pH 7 and 30⁰C.

I = 0.5 M

[substrate] = 41.6 μ M

[enzyme] = 6.8×10^{-10} M

[I] (μ M)	k_1 (sec ⁻¹)
0	1.6×10^{-2}
0.50	1.25×10^{-2}
1.01	1.0×10^{-2}
2.00	7.5×10^{-3}

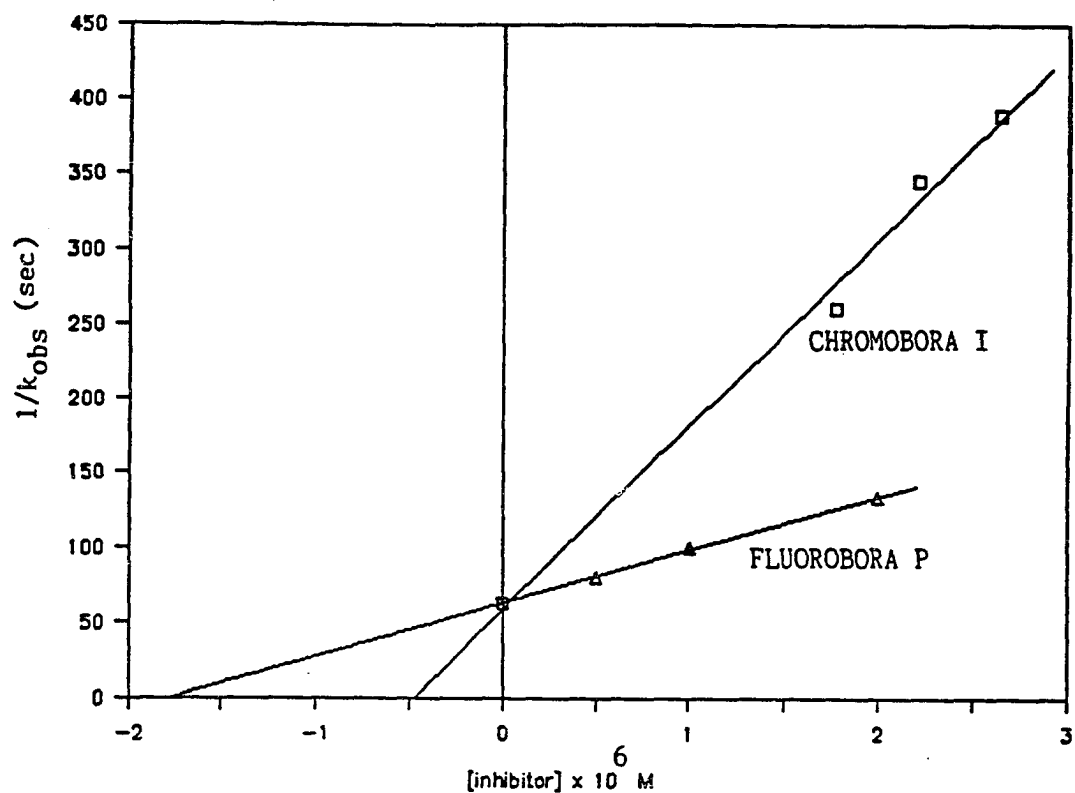


Figure 14. Inhibition of *E. coli* RTM-2 beta-lactamase by Chromobora I and Fluorobora P at pH 7 and 30°C.
 K_i (Chromobora I) = 0.5×10^{-6} M
 K_i (Fluorobora P) = 1.75×10^{-6} M
 Data of Tables X and XI

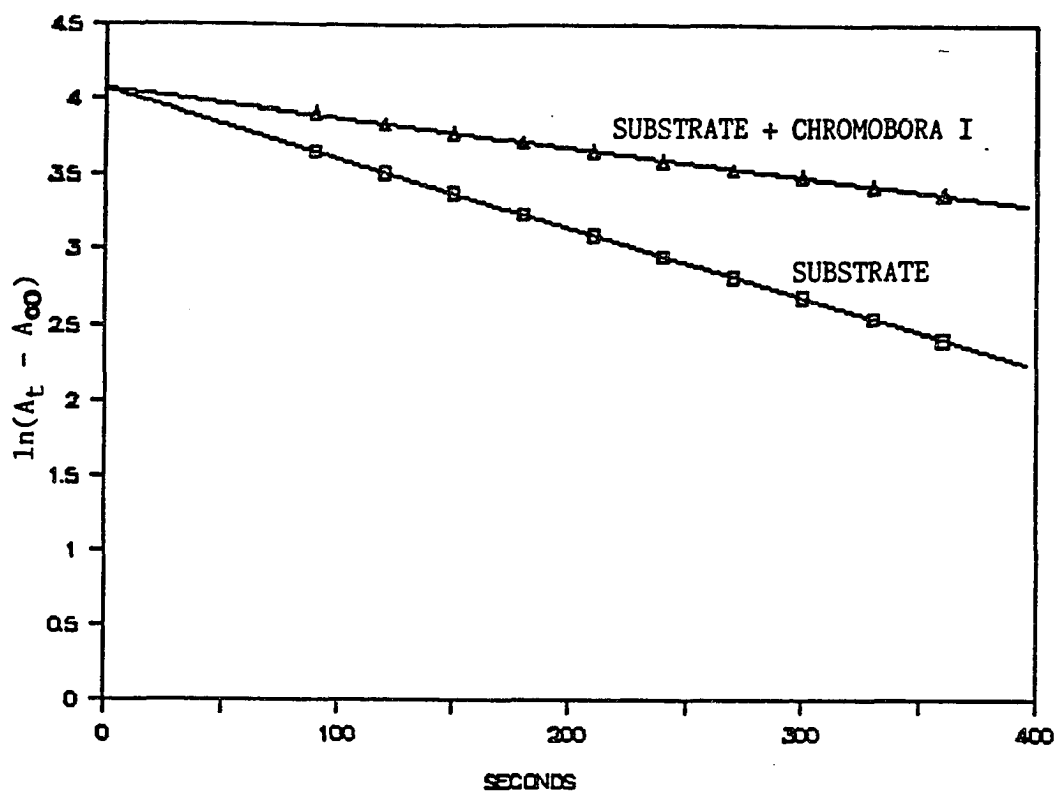


Figure 15. Inhibition of *E. coli* RTEM-2 beta-lactamase by Chromobora I at pH 7 and 30°C. I = 0.5 M
The plot shows the pseudo-first-order hydrolysis of 41.6 μ M benzylpenicillin.
[Chromobora I] = 1.77 μ M

Table XII

Benzylpenicillin hydrolysis by E. coli RTEM-2 beta-lactamase in the presence of the inhibitor, N-acetyl-D-boro-Leucine at pH 7 and 28⁰C. I = 0.5 M

[substrate] = 40 μ M

[enzyme] = 11.2×10^{-10} M

[I] (μ M)	k_1 (sec ⁻¹)
0	4.54×10^{-2}
44	1.17×10^{-2}
61	9.22×10^{-3}

Table XIII

Benzylpenicillin hydrolysis by E. coli RTEM-2 beta-lactamase in the presence of the inhibitor, N-acetyl-D-boro-Valine at pH 7 and 30⁰C. I = 0.5 M

[substrate] = 40 μ M

[enzyme] = 3.40×10^{-10} M

[I] (μ M)	k_1 (sec ⁻¹)
0	1.40×10^{-2}
85.0	5.05×10^{-3}
99.8	4.45×10^{-3}

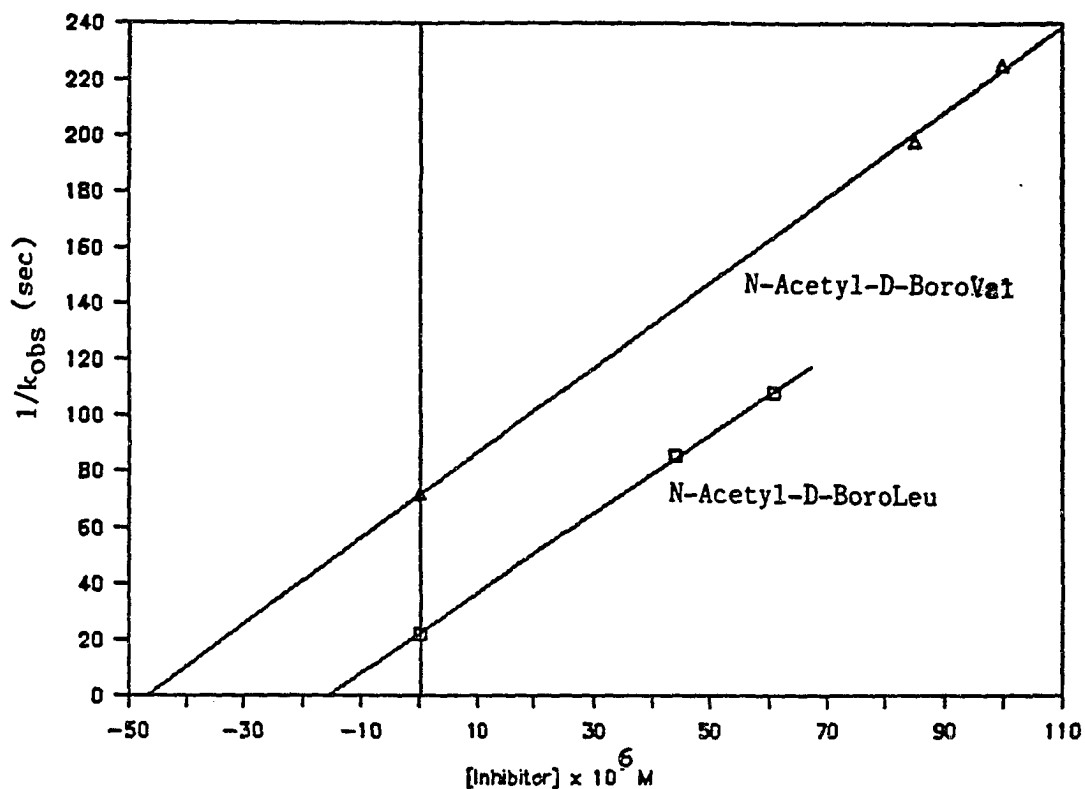


Figure 16. Inhibition of *E. coli* RTEM-2 beta-lactamase by N-acetyl-D-boro-Leucine and N-acetyl-D-boro-Valine at pH 7 and 28°C. I = 0.5 M
 K_i (leucine analog) = 15 μ M
 K_i (valine analog) = 47 μ M
 Data of Tables XII and XIII

TABLE XIV

Inhibition constants for boronic acids at pH 7 and 30°C.

DMSO in the assay mix. = 3 % v/v. I = 0.5 M

[substrate] = 11 μ M

[enzyme] = 8.7×10^{-11} M

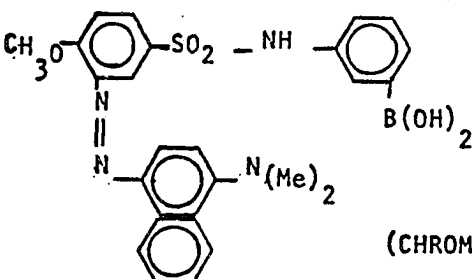
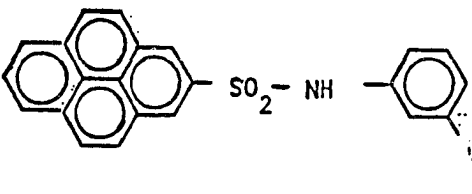
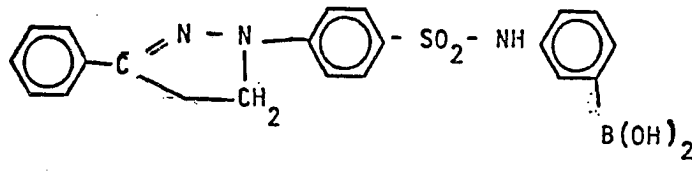
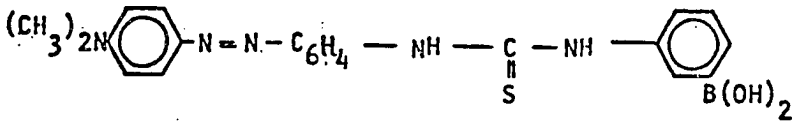
Compound	K_i (μ M)
N-Acetyl-D-boro-Leucine*	16
N-Acetyl-D-boro-Valine*	47
3-Nitrophenylboronic acid	73
3-Carboxyphenylboronic acid	150
3,5(trifluoromethyl)-phenylboronic acid	220
3-Dansylamidophenylboronic acid	228
2,4-Dichlorophenylboronic acid	702
Phenylboronic acid	1000
3-Aminophenylboronic acid	3500
3-Succinylamidophenylboronic acid	1051

*Carboxylic acid group replaced by boronate-pinanediol complex

The substrate used was nitrocefin

+

Table XV

COMPOUND	K_I ($\times 10^6$ M)
 (CHROMOBORA I)	0.5
 (FLOROBORA P)	1.75
 (FLOROBORA II)	3.0
 (CHROMOBORA II)	3.4

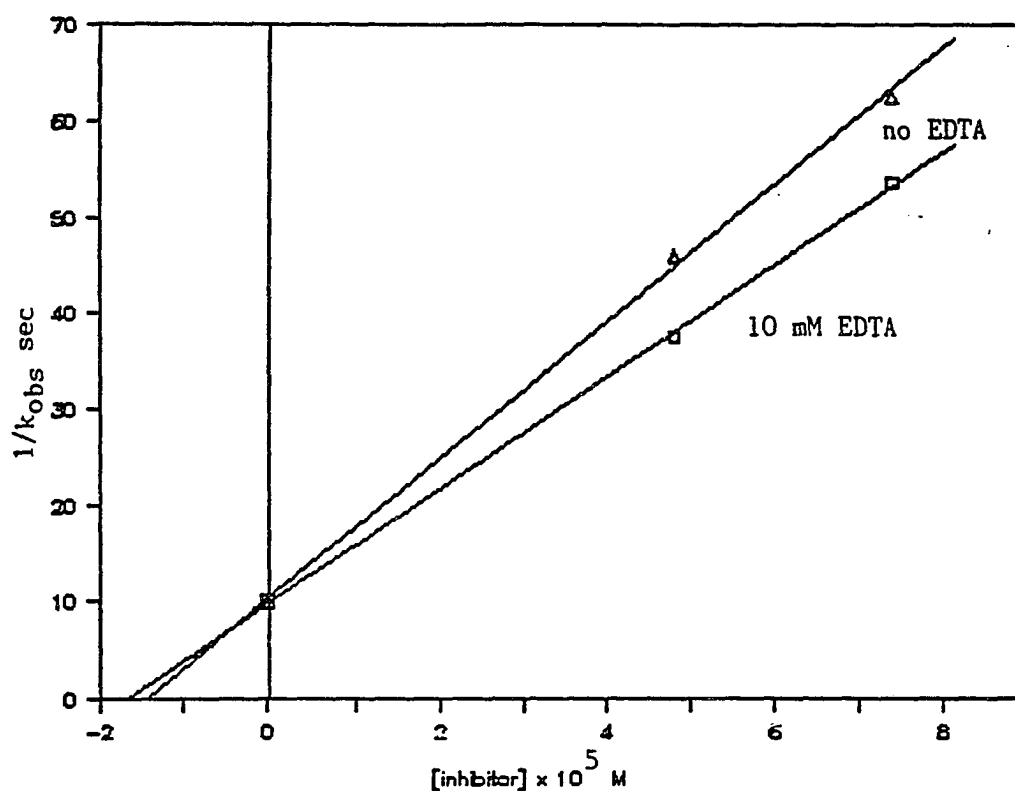


Figure 17. Inhibition of *E. coli* TEM-2 β -lactamase by *m*-carboxyphenylboronic acid with no EDTA and with 10 mM EDTA in the assay mixture at pH 7 and 30°C. $I = 0.5 \text{ M}$
 K_i (no EDTA) = $1.45 \times 10^{-4} \text{ M}$
 K_i (10 mM EDTA) = $1.67 \times 10^{-4} \text{ M}$
 $[\text{nitrocefin}] = 10 \text{ }\mu\text{M}$

Table XVI

m-Carboxyphenylboronic acid inhibition of E. coli RTE^M-2
beta-lactamase

DMSO in the assay mix. = 3 % v/v. I = 0.35 M

[nitrocefin] = 7.2 - 51.9 μ M

pH	K_i ($\times 10^5$ M)
4.55	30.2
5.00	20.6
5.42	19.6
6.10	10.9
6.52	7.26
7.00	5.16
7.20	5.30
7.30	5.94
7.64	7.54
7.80	10.9
8.30	26.1
8.55	35.6
9.20	301

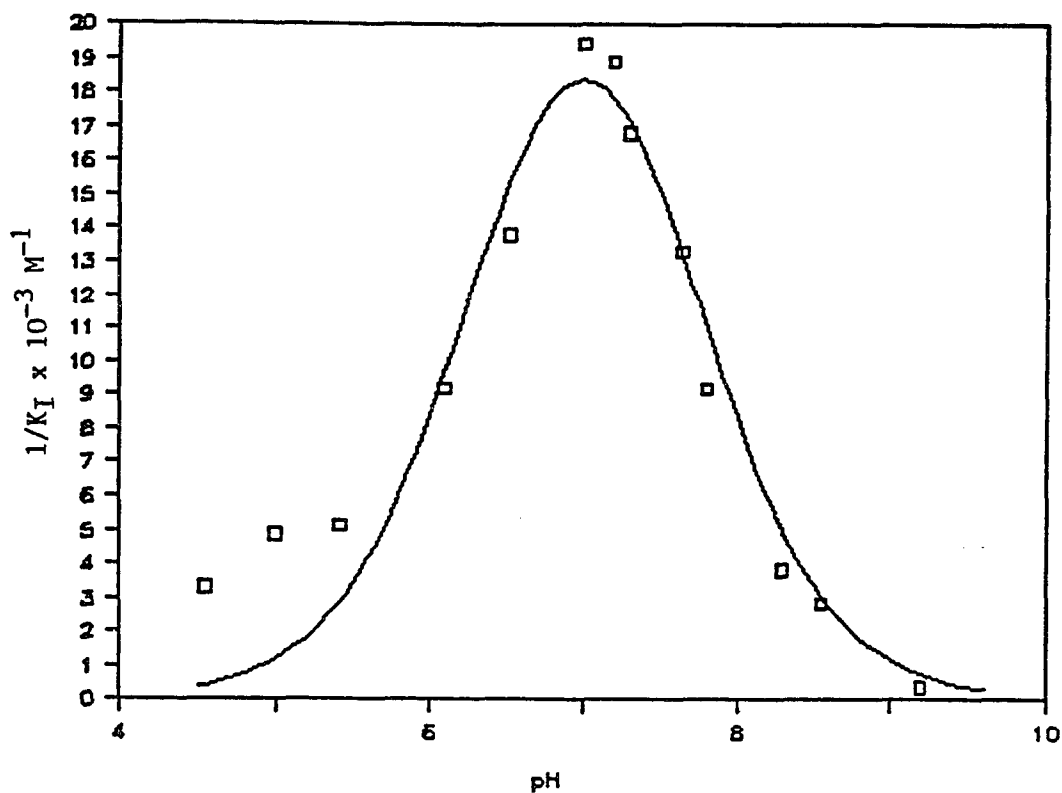


Figure 18. m-Carboxyphenylboronic acid inhibition of
E. coli RTEM-2 beta-lactamase
DMSO in the assay mix. = 3% v/v. I = 0.35 M
 $pK_1 = 6.33 \pm 0.37$ $pK_2 = 7.67 \pm 0.37$
 $K_i(\text{Lim}) = (2.63 \pm 0.755) \times 10^{-5} \text{ M}$
Data of Table XVI

Table XVII

m-Nitrophenylboronic acid inhibition of E. coli RTEM-2

beta-lactamase

DMSO in the assay mix. = 0.4 % v/v. I = 0.35 M

[nitrocefin] = 14.4 - 115 μ M

pH	K_i ($\times 10^5$ M)
4.95	17.5
5.40	12.7
6.10	10.0
6.58	7.68
6.95	6.84
7.30	9.98
7.75	14.0
8.18	61.2
8.30	61.2

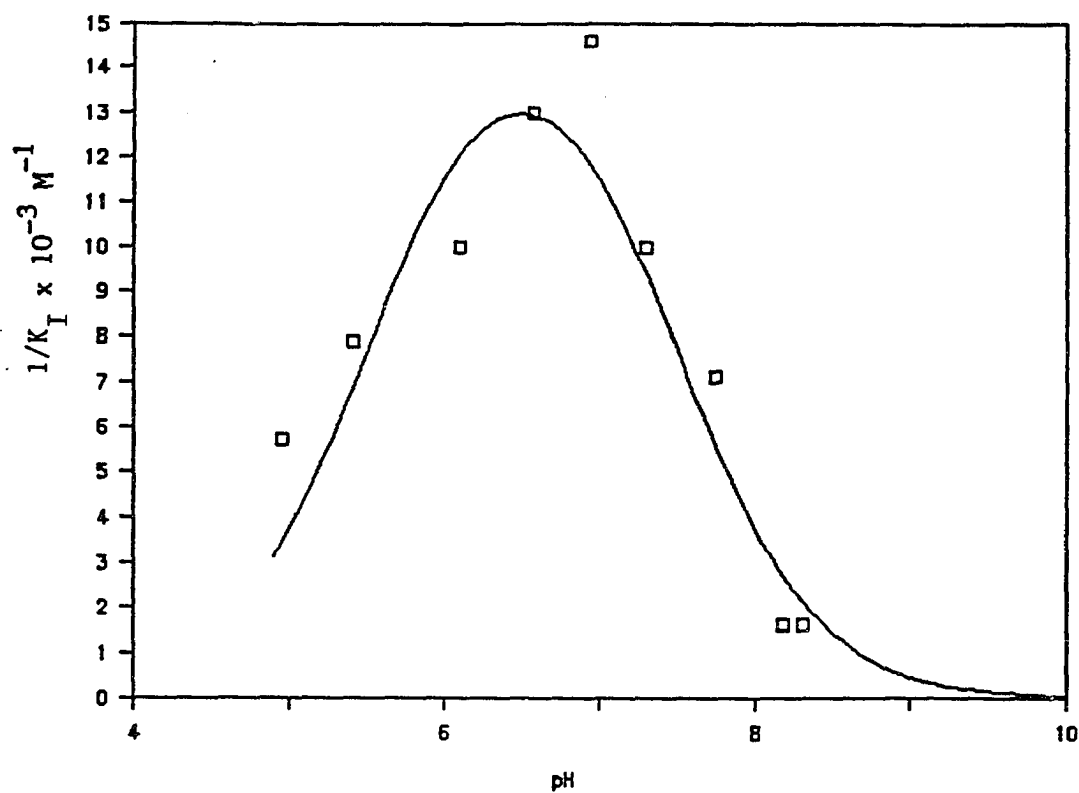


Figure 19. m-Nitrophenylboronic acid inhibition of
E. coli RTEM-2 beta-lactamase
DMSO in the assay mix. = 0.4% v/v. I = 0.35 M
 $\text{pK}_1 = 5.50 \pm 0.39$ $\text{pK}_2 = 7.50 \pm 0.39$
 $K_i(\text{Lim}) = (6.41 \pm 0.804) \times 10^{-5} \text{ M}$
Data of Table XVII

Table XVIII

p-Bromophenylboronic acid inhibition of E. coli RTEM-2
beta-lactamase

DMSO in the assay mix. = 0.4 % v/v. I = 0.35 M

[nitrocefin] = 7.3 - 58.4 μ M

pH	K_i ($\times 10^6$ M)
4.50	109
4.99	90.3
5.20	60.6
5.40	56.6
6.20	47.8
6.30	48.0
7.00	51.7
7.30	53.7
7.55	62.7
8.20	80.6
8.80	269

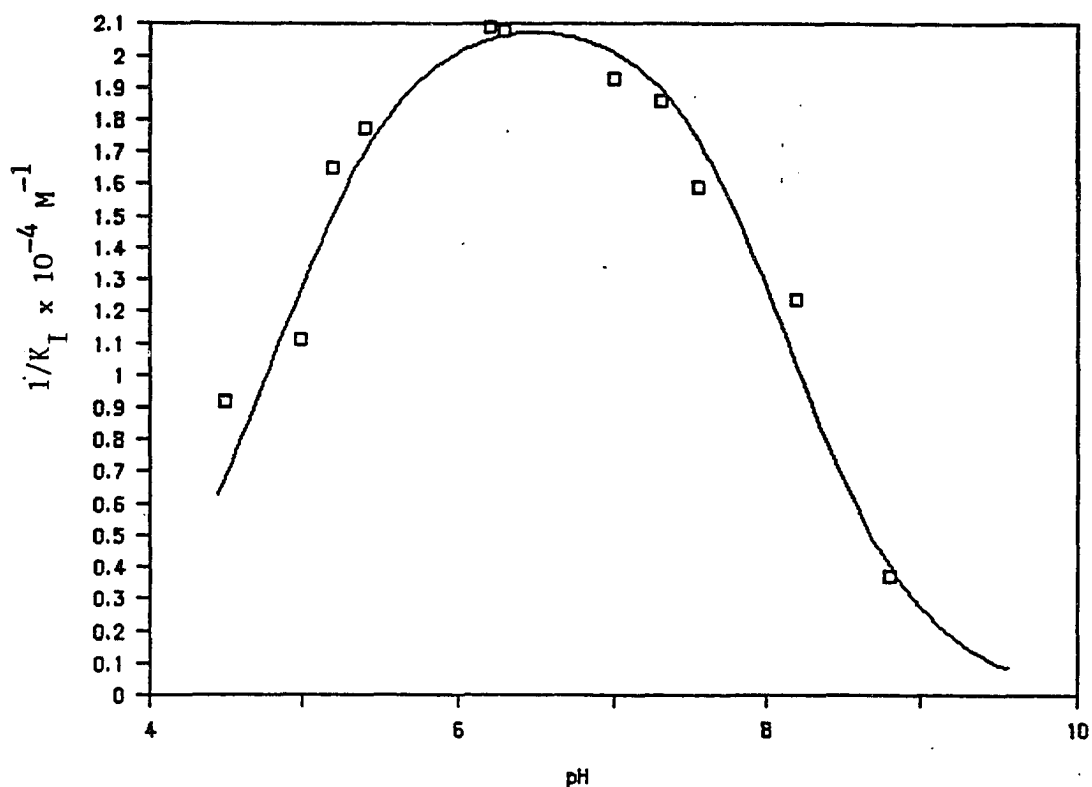


Figure 20. p-Bromophenylboronic acid inhibition of
E. coli RTEM-2 beta-lactamase
DMSO in the assay mix. = 0.4% v/v. I = 0.35 M
 $pK_1 = 4.84 \pm 0.17$ $pK_2 = 8.16 \pm 0.17$
 $K_i(\text{Lim}) = (4.63 \pm 0.0731) \times 10^{-5} \text{ M}$
Data of Table XVIII

Table XIX

Phenylboronic acid inhibition of E. coli RTEM-2 beta-lactamase

DMSO in the assay mix. = 0.4 % v/v. I = 0.35 M

[nitrocefin] = 7.3 - 58.4 μ M

pH	K_i ($\times 10^3$ M)
4.51	5.47
4.96	2.49
5.17	1.67
5.27	1.67
5.96	1.14
6.32	1.00
6.89	0.900
7.38	0.766
8.18	0.950
8.62	1.52
8.80	2.78

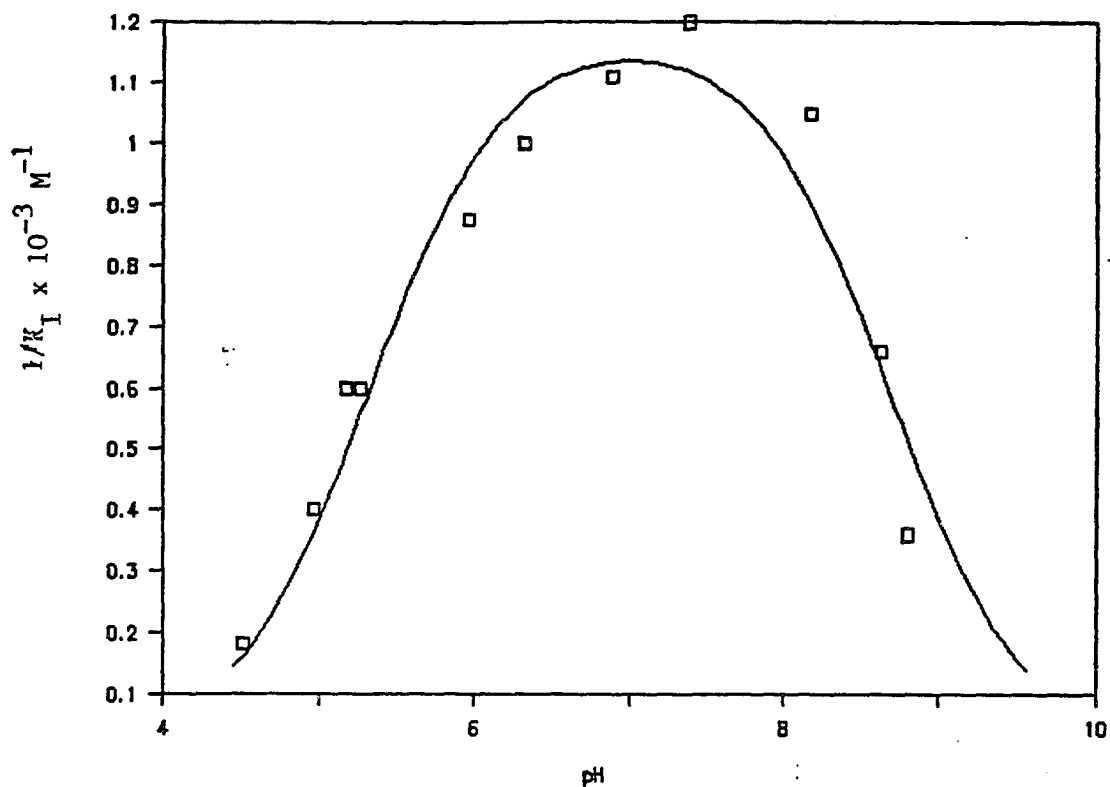


Figure 21. Phenylboronic acid inhibition of
E. coli RTEM-2 beta-lactamase
 DMSO in the assay mix. = 0.4% v/v. I = 0.35 M
 $\text{pK}_1 = 5.32 \pm 0.35$ $\text{pK}_2 = 8.68 \pm 0.35$
 $K_i(\text{Lim}) = (8.47 \pm 0.220) \times 10^{-4} \text{ M}$
 Data of Table XIX

Table XX

2,4-Dichlorophenylboronic acid inhibition of E. coli RTEM-2
beta-lactamase

DMSO in the assay mix. = 0.4 % v/v. I = 0.35 M

[nitrocefin] = 7.3 - 58.4 μ M

pH	K_i ($\times 10^4$ M)
4.68	15.8
5.18	12.8
5.36	9.87
5.45	9.87
6.18	6.17
6.32	5.45
7.12	4.89
7.84	9.22
8.35	19.7
8.90	126

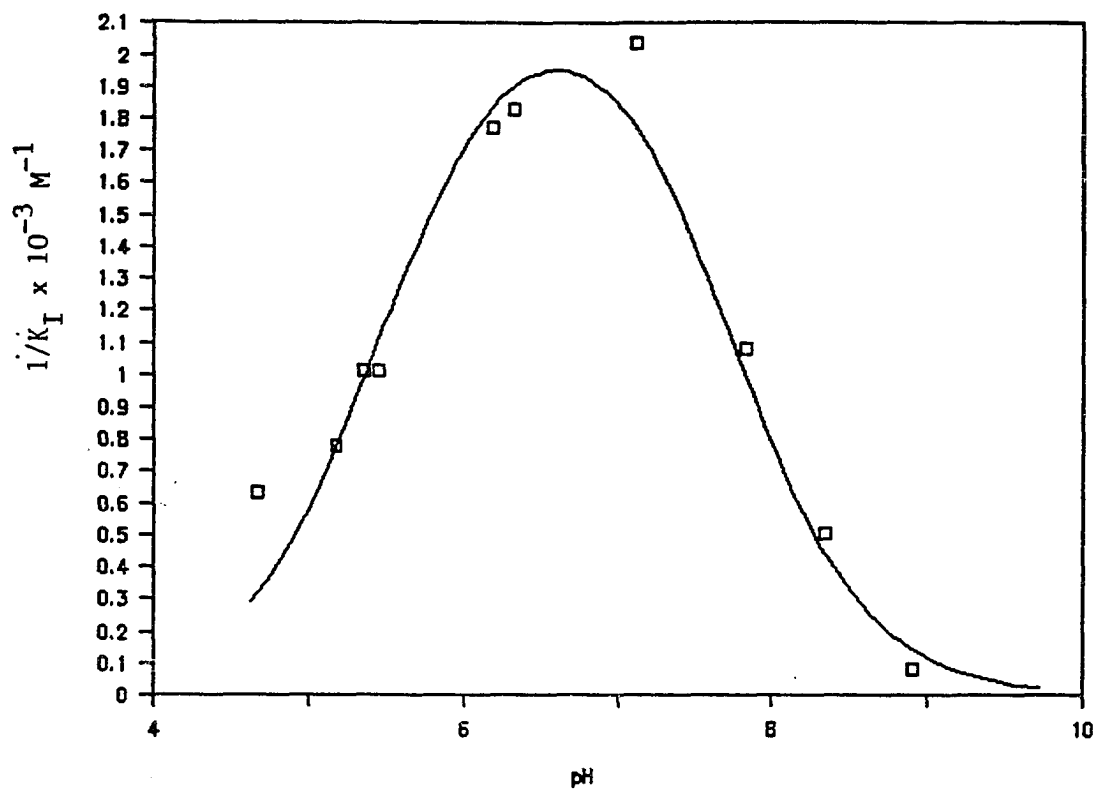


Figure 22. 2,4-Dichlorophenylboronic acid inhibition of E. coli REM-2 beta-lactamase
 DMSO in the assay mix. = 0.4% v/v. I = 0.35 M
 $pK_1 = 5.46 \pm 0.25$ $pK_2 = 7.74 \pm 0.25$
 $K_i(\text{Lim}) = (4.48 \pm 0.331) \times 10^{-4} \text{ M}$
 Data of Table XX

Table XXI

m-Aminophenylboronic acid inhibition of E. coli RTEM-2
beta-lactamase

DMSO in the assay mix. = 0.4% v/v. I = 0.35 M

[nitrocefin] = 7.2 - 51.9 μ M

pH	K_i ($\times 10^3$ M)
4.90	5.99
5.41	4.76
6.10	2.62
6.42	2.11
7.05	2.09
7.80	2.08
8.18	2.28
8.60	3.08
8.80	4.69
9.21	6.29

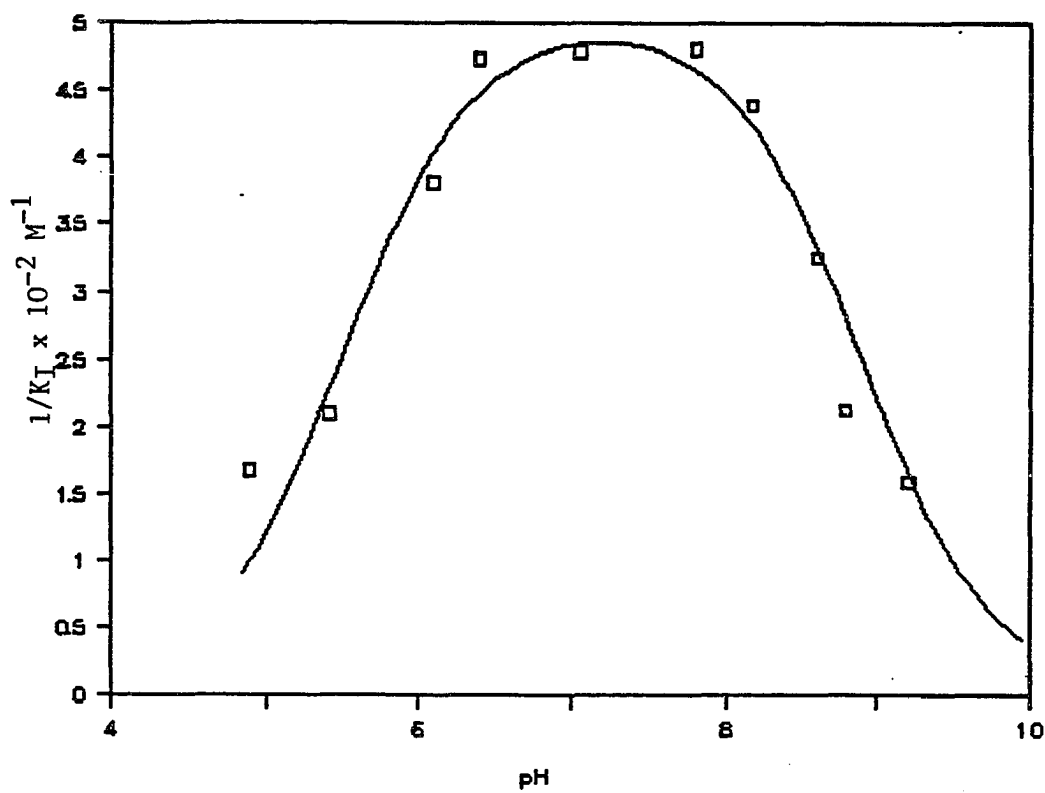


Figure 23. m-Aminophenylboronic acid inhibition of
E. coli RTEM-2 beta-lactamase
DMSO in the assay mix. = 0.4% v/v. I = 0.35 M
 $pK_1 = 5.51 \pm 0.20$ $pK_2 = 8.88 \pm 0.20$
 $K_i(\text{Lim}) = (2.0 \pm 0.0379) \text{ mM}$
Data of Table XXI

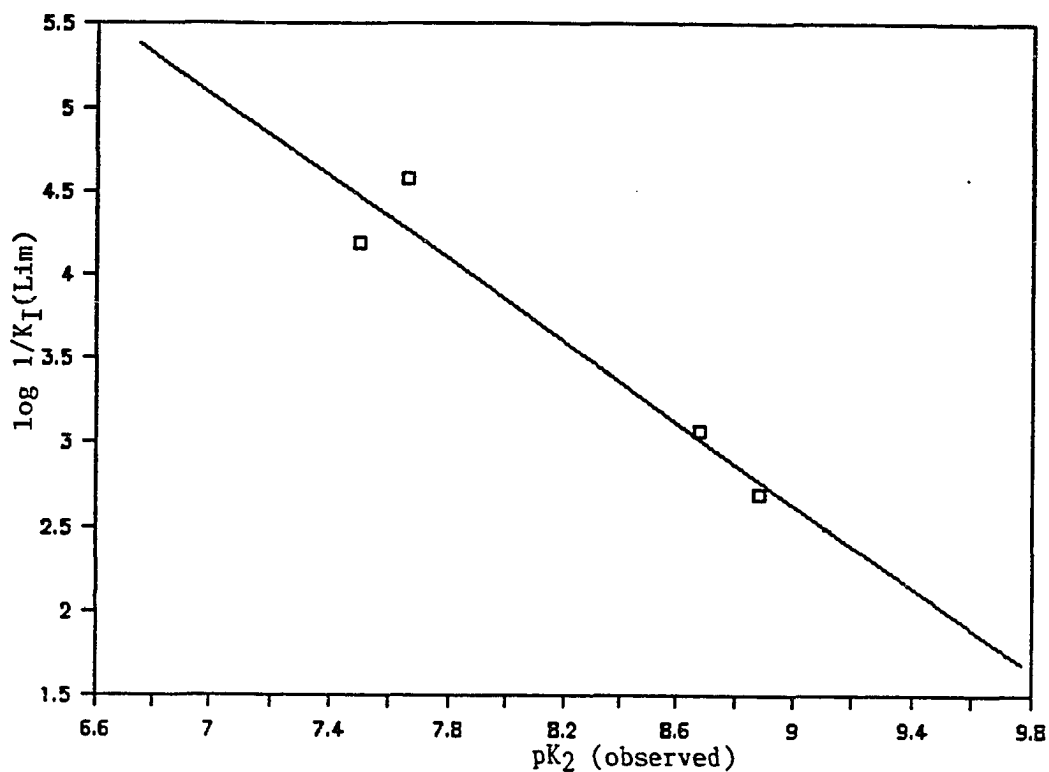


Figure 24. Brønsted plot for meta-substituted phenylboronic acid inhibition of E. coli RTEM-2 beta-lactamase
 The plot has a slope of -1.2 ± 0.25
 Data of Figures 18, 19, 21 & 23

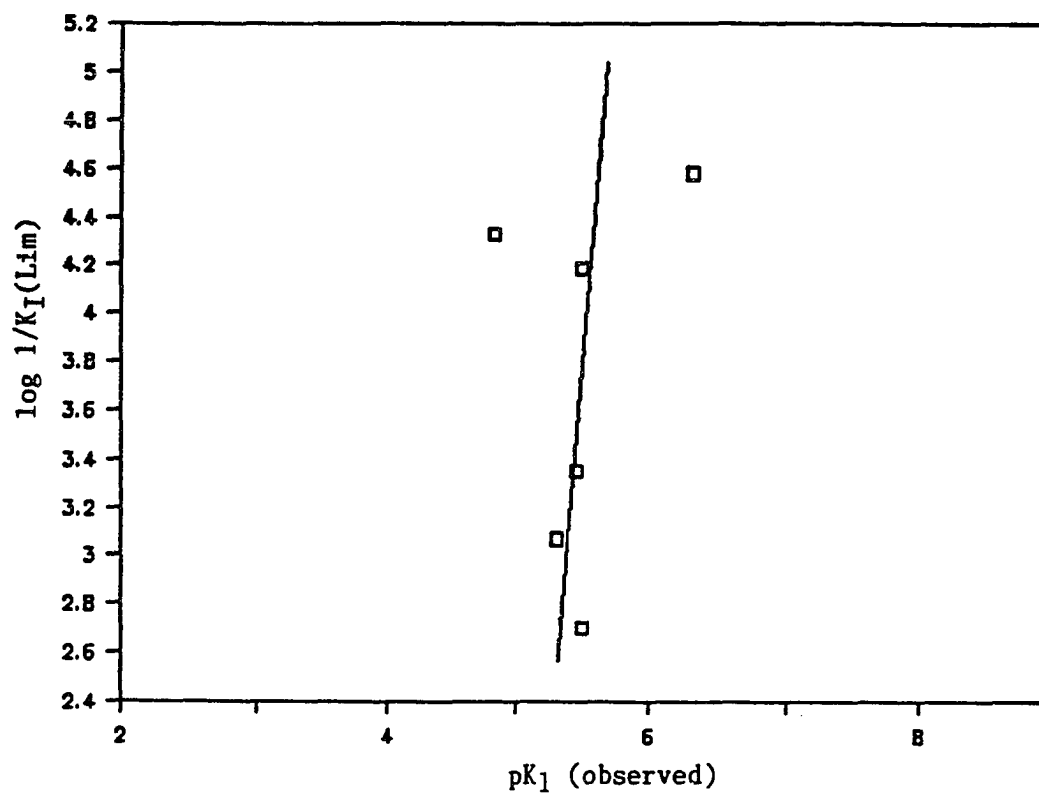


Figure 25. Dependence of $K_i^{-1}(\text{Lim})$ on the pK_1 of the substituted phenylboronic acid in the inhibition of *E. coli* RTEM-2 beta-lactamase
Data of Figures 18 - 23

Table XXII

Benzympenicillin hydrolysis by E. coli RTEM-2 beta-lactamase, at pH 7 and 30°C. I = 0.5 M

The enzyme assay was done in a quartz cuvette.

[substrate] = 20 μ M

[E] ($\times 10^{10}$ M)	k_1 (sec ⁻¹)
0.660	8.98×10^{-3}
1.00	1.20×10^{-2}
1.33	1.70×10^{-2}
1.66	2.30×10^{-2}
2.00	3.00×10^{-2}
2.33	3.80×10^{-2}

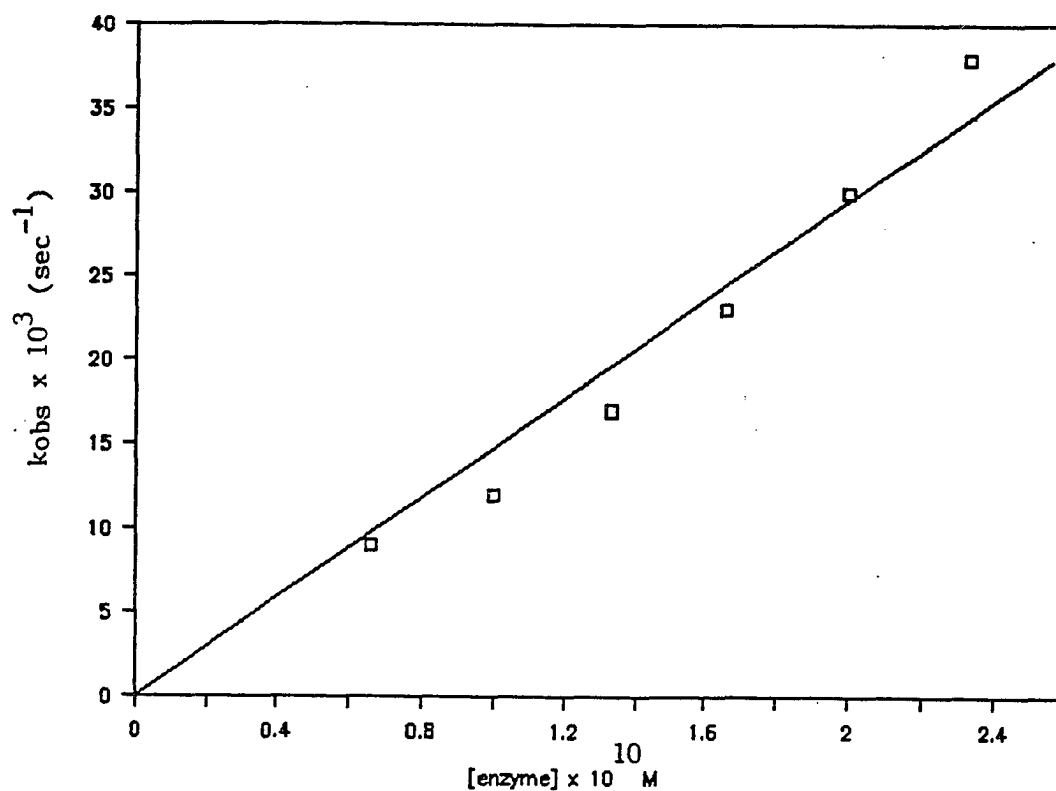


Figure 26. Benzylpenicillin hydrolysis by E. coli RTEM-2
beta-lactamase at pH 7 and 30°C. I = 0.5 M
 Data of Table XXII

Table XXIII

Nitrocefin hydrolysis by E. coli RTEM-2 beta-lactamase at pH 7. The enzyme assay was done in a 96 well PerfeCTOR microtiter plate.

Absorbance readings were taken on a Vmax kinetic microplate reader.

DMSO in the assay mix. = 0.4 % v/v. I = 0.35 M

[substrate] = 49.8 μ M

[Enzyme] $\times 10^{10}$ M	Rate mOD min ⁻¹
14.8	114.8
7.40	75.00
3.70	43.00
1.85	21.72
0.925	11.28
0.462	5.170
0.231	2.464
0.115	1.125

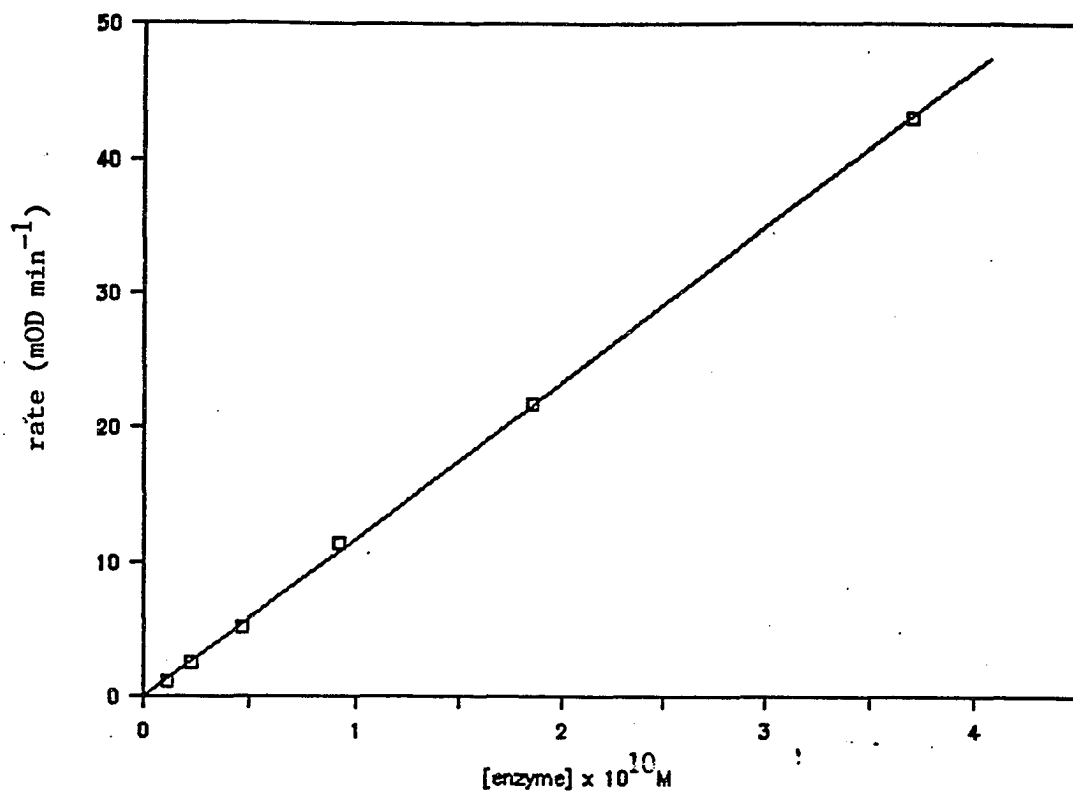


Figure 27. Nitrocefin hydrolysis by E. coli RTEM-2
beta-lactamase at pH 7 and 25°C
DMSO in the assay mix. = 0.4% v/v. I = 0.35 M
Data of Table XXIII

DISCUSSION AND CONCLUSIONS

Mechanistic information gained by the substrate hydrolyses studies:

In an enzymatic reaction, the pH-dependence of k_{cat}/K_m follows the ionizations in the free enzyme and the free substrate (28):



A pK_1 of 5.5 is seen in the pH profile of k_{cat}/K_m for both nitrocefin and PADAC. The pK values for a pH profile of k_{cat}/K_m are in principle attributable to groups on the free enzyme and the free substrate. When there is rate-determining binding (diffusion-controlled encounter of the enzyme and the substrate) k_{cat}/K_m may reach values as high as $3 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ (28). Briggs-Haldane kinetics applies in these instances (28). When $k_2 > k_{-1}$, $k_{\text{cat}}/K_m = k_1$, which is the rate constant for the association of enzyme with substrate. This has been found to be on the order of $10^8 \text{ M}^{-1} \text{ s}^{-1}$ (28).

In benzylpenicillin hydrolysis by E. coli RTEM-2 beta-lactamase, k_{cat}/K_m , which is $10^8 \text{ M}^{-1} \text{ s}^{-1}$ (29), is close to the expected diffusion-controlled association rate. In the case where association of substrate is rate-determining the apparent pK values in the pH profile of k_{cat}/K_m will be modified by the diffusion-controlled limiting value of k_{cat}/K_m (28).

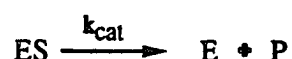
A pK_1 of 5.4 for ampicillin hydrolysis (18) and nitrocefin hydrolysis (30) by beta-lactamase I from Bacillus cereus and for cephalosporin C and benzylpenicillin hydrolysis by the Class C enzyme from Pseudomonas aeruginosa (31) has been observed. In the first case, chemical modification studies using Woodward's reagent K and a carbodi-imide indicated that this group could be a carboxylate acting as a base.

From the crystal structure studies on the Class A enzyme from Staphylococcus aureus (32) Glu-166 is positioned at the active site, with Lys-73 and Lys-234 in close proximity. These are conserved residues in the E. coli RTEM-2 beta-lactamase. pK_1 could therefore be tentatively assigned to the ionization of Glu-166. The substrate nitrocefin is expected to have a pK between 1.6 and 3 due to the ionization of the carboxylate group (30).

pK_2 of k_{cat}/K_m , which is about 7.5, could tentatively be assigned to Lys-73. Lys-73 makes a salt bridge interaction with Glu-166 (32).

In the pH profiles of k_{cat} for nitrocefin hydrolysis, pK_1 is the same value as the pK_1 of k_{cat}/K_m . There is a shift of about one pH unit in the value of pK_2 in k_{cat} from the observed value of pK_2 of k_{cat}/K_m .

The ionizations of the enzyme-substrate complex will be reflected in the pH-dependence of k_{cat} (28).

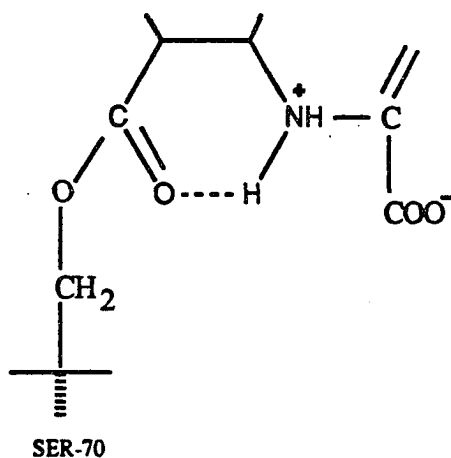


It is possible that nonproductive binding will affect the pH-dependence of k_{cat} but not of k_{cat}/K_m (33). The pK values of groups essential to catalysis and binding may be expected to be different in the enzyme-substrate complex than in the free enzyme, since substrate binding can change the environment at the active site.

The possibility that pK_2 is due to the ionization of a group that is involved in only the deacylation step has to be considered since such an ionization will be reflected in the pH profile of k_{cat} and not of k_{cat}/K_m .

The transition state prior to deacylation may be expected to be stabilized by hydrogen bonding between the cephaloridine ring nitrogen and the carbonyl group of the scissile bond. The pK of this group which can be compared to that of proline, is 10.6. This structure is supported by results (34) showing that thionocephalosporin in which the beta-lactam carbonyl oxygen atom is replaced with a sulphur atom is a much poorer substrate for the E. coli RTEM-2 beta-lactamase when compared to the analogous oxo-beta-lactam.

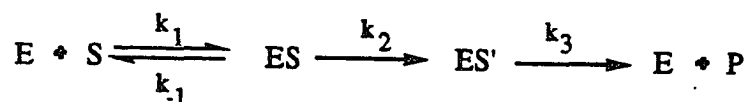
Figure 28



If pK_2 in the pH profiles of k_{cat}/K_m and k_{cat} reflects the ionization of Lys-73, this group may be involved in the protonation of the beta-lactam ring nitrogen in acylation. In deacylation it could act as a proton sink in the protonation of the serine-acyl complex by Glu-166. The shift in pK_2 of k_{cat} could reflect the change in the pK of Lys-73 due to the presence of the bound substrate (Figures 29 and 30). This shift could result from a protonic equilibrium between the cephaloridine ring N atom and the ϵ -amino group of Lys-73.

This shift in pK_2 is reflected in the pH profile of $1/K_m$, where pK_E and pK_{ES} correspond to pK_2 of k_{cat}/K_m and pK_2 of k_{cat} respectively.

These data support a mechanism where $k_2 > k_3$. The presence of slow and suicide substrates (35, 36) is also supportive of the rate limiting step being deacylation for some substrate analogs.



When $k_2 \gg k_3$, the general relationship $K_m = k_3/(k_2 + k_3) * K_S$ becomes $K_m = k_3/k_2 * K_S$ where $K_S = (k_{-1} + k_2)/k_1$. Since the experimental pH profile of $1/K_m$ shows only the pK values that are seen in k_{cat}/K_m and k_{cat} , K_S is likely to be independent of pH within the pH range 5.0 to 9.5.

When the pH profiles of k_{cat}/K_m for the two substrates nitrocefin and PADAC are compared, pK_1 is the

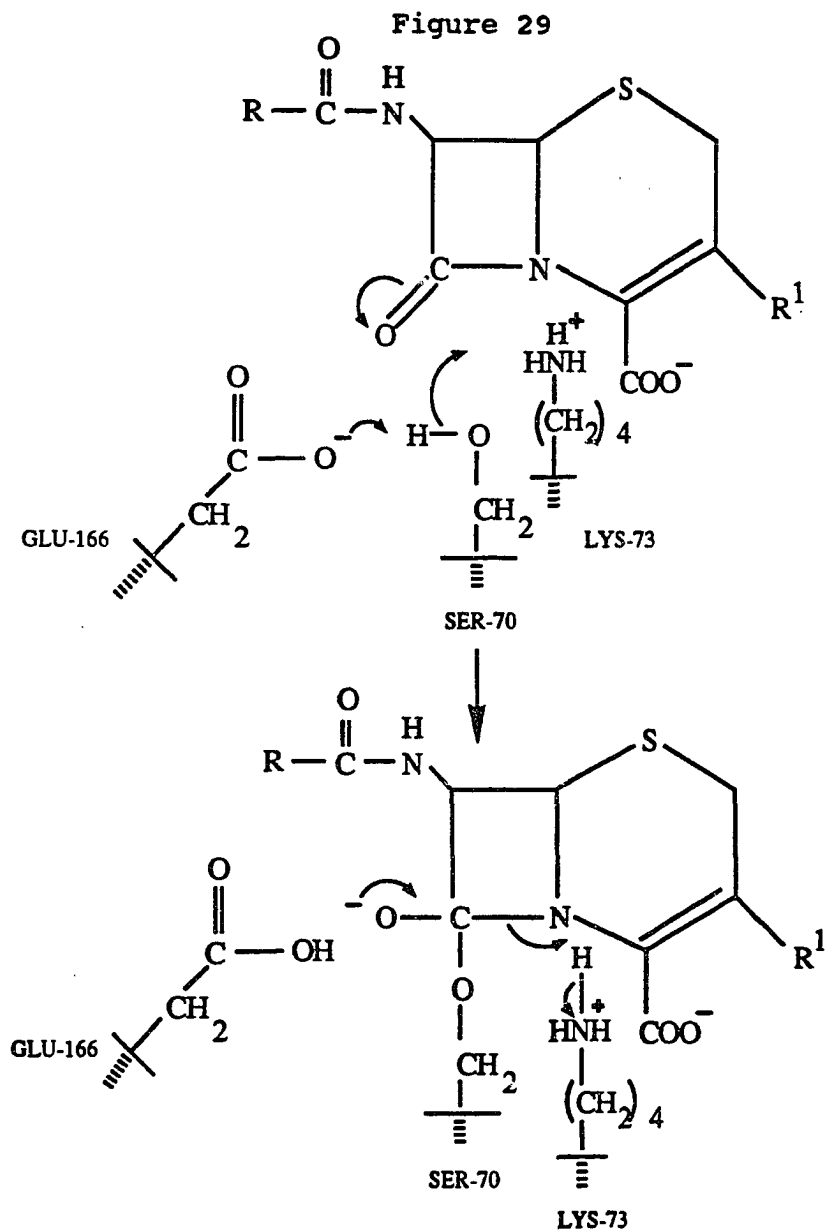
same whereas pK_2 of PADAC is 7.72, which is a shift of 0.5 pH units from that of the value for nitrocefin at 0.4% DMSO. The side-chain leaving group of PADAC, N,N-dimethylaniline-4-azo-2'-pyridinium can be expected to have a pK of about 3.5 due to the dimethylaniline group (pK of p-dimethylaminoazobenzene = 3.5) (37) and a pK of about -2.5 due to the azo group (pK of azobenzene = -2.48) (38). The pyridinium ring nitrogen atom which is alkylated cannot show a pK. Therefore the pK of 7.72 is clearly not due to the ionization of the substrate.

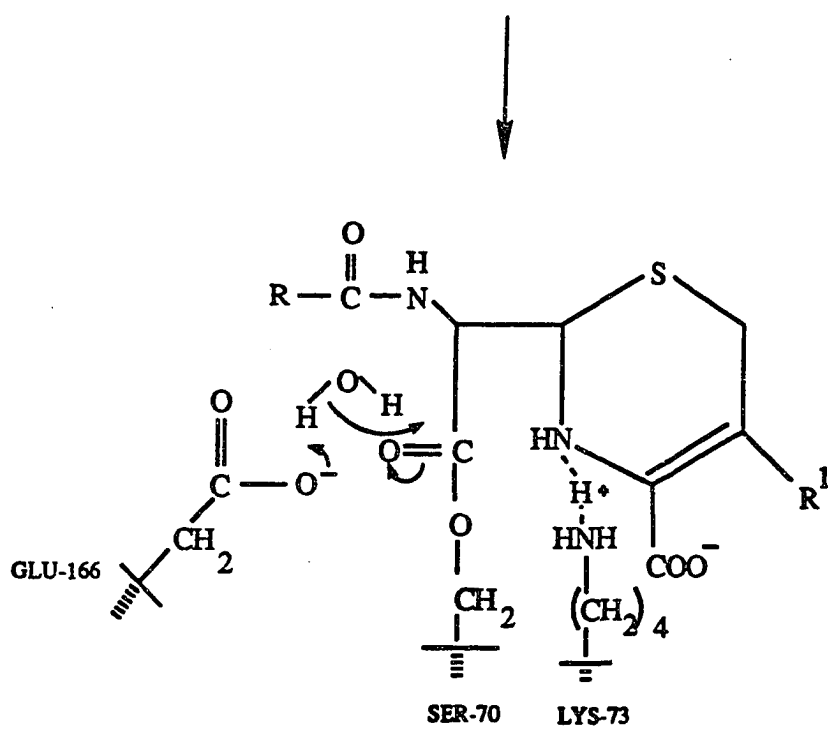
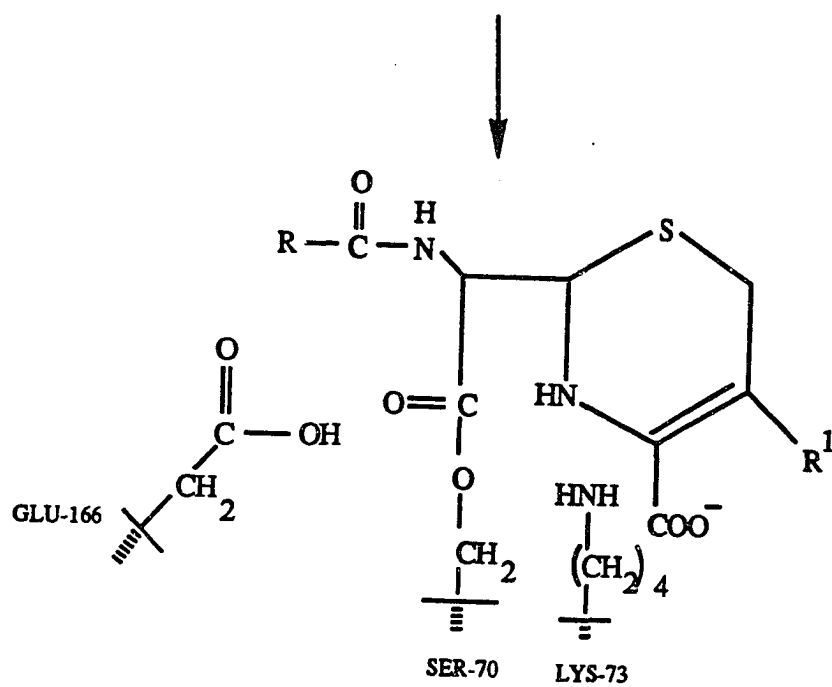
$k_{cat}/K_m(\text{Lim})$ for nitrocefin hydrolysis is $4.64 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ at 0.4% DMSO. PADAC is a poorer substrate with a $k_{cat}/K_m(\text{Lim})$ which is $6.95 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$. In the case of nitrocefin, $k_{cat}/K_m(\text{Lim})$ is close to the limiting value seen with rate-determining binding which is about $10^8 \text{ M}^{-1} \text{ s}^{-1}$. However if this was true for nitrocefin, when compared to the much slower substrate PADAC, a shift in pK_1 would have been expected. The shift in pK_2 is in the opposite direction to what would have been seen if rate-determining binding had occurred (28). It is therefore clear that although nitrocefin is a good substrate, diffusion-controlled association does not take place.

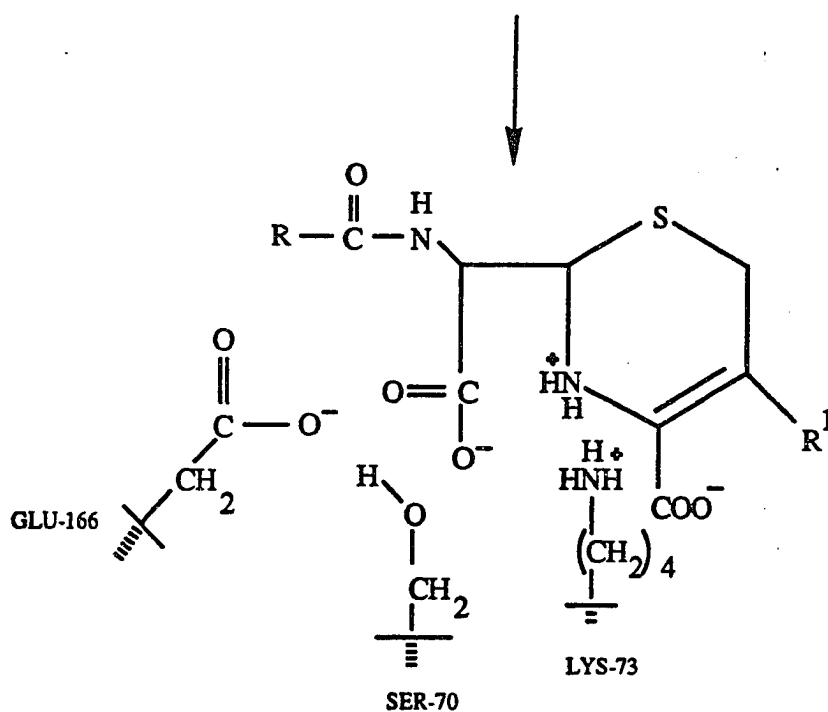
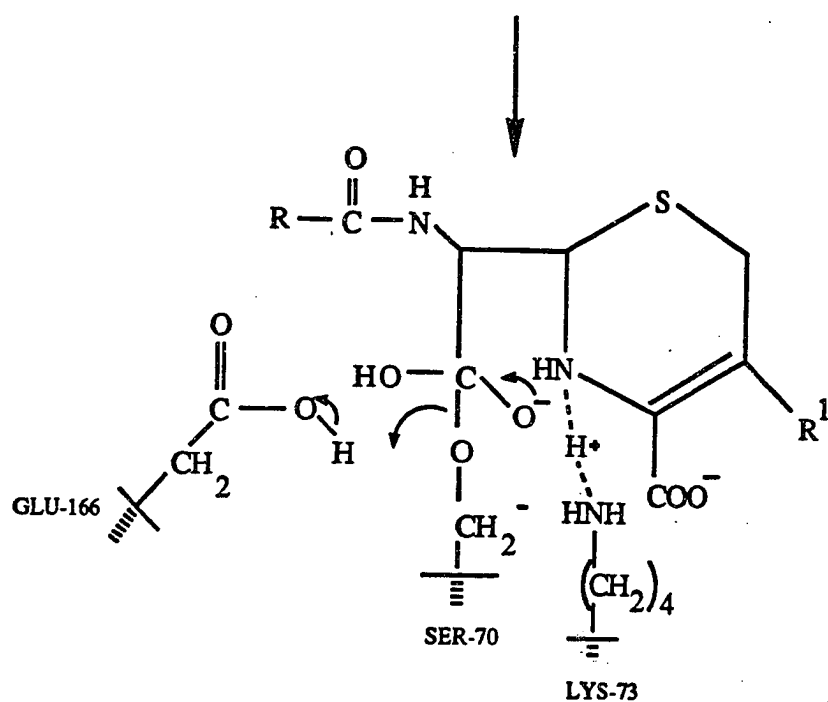
The limiting value of k_{cat}/K_m is affected by the DMSO concentration in the assay mix. The effect is seen in both K_m and k_{cat} . The effect of DMSO on the interaction of proflavine with alpha-chymotrypsin has been studied in some detail (39). In that study the effect of DMSO on K_m and K_p

(dissociation constant for the alpha-chymotrypsin-proflavine complex) was accounted for by a combination of competitive inhibition and dielectric effects by DMSO on substrate and inhibitor binding.

A mechanism of action for substrate hydrolysis by E. coli RTEM-2 beta-lactamase can be proposed based on this study:







In this mechanism, the low pK for Lys-73 (pK of the ϵ -amino group of free lysine = 10.5) could be due to the influence of the adjacent Lys-234 which is expected to interact with the exocyclic carboxylate of substrates (32). This is consistent with what is observed, where the pK of Lys-73 goes up by about one pH unit in the presence of the bound substrate.

Three parameters, inductive, field and resonance effects can influence the behaviour of amino acids (40). The α -amino group of cysteine has pK of 8.9 when the sulfhydryl group is protonated and a pK of 10.4 when the sulfhydryl group is non-protonated (40). It is likely that a similar effect by Lys-234 on the ionization of Lys-73 occurs, thereby lowering the pK of the ϵ -amino group.

The pK of groups at the active site are also dependent on the hydrophobicity or polarity at the active site. The pK of the α -amino group of glycine at 0% dioxane at 25⁰ C is 9.78, whereas at 70% dioxane the pK is 7.42 (28). Similarly, the pK of the α -carboxyl group of glycine at 0% dioxane is 2.35, and at 70% dioxane the pK is 3.96 (28). Thus, the lowered pK of Lys-73 could be also due to the presumed hydrophobicity at the active site of this enzyme. Chymotrypsin is presumed to have a similar hydrophobic active site (41).

Lys-73 could also act as a cationic group which promotes general base (Glu-166) mediated attack by Ser-70 on the substrate.

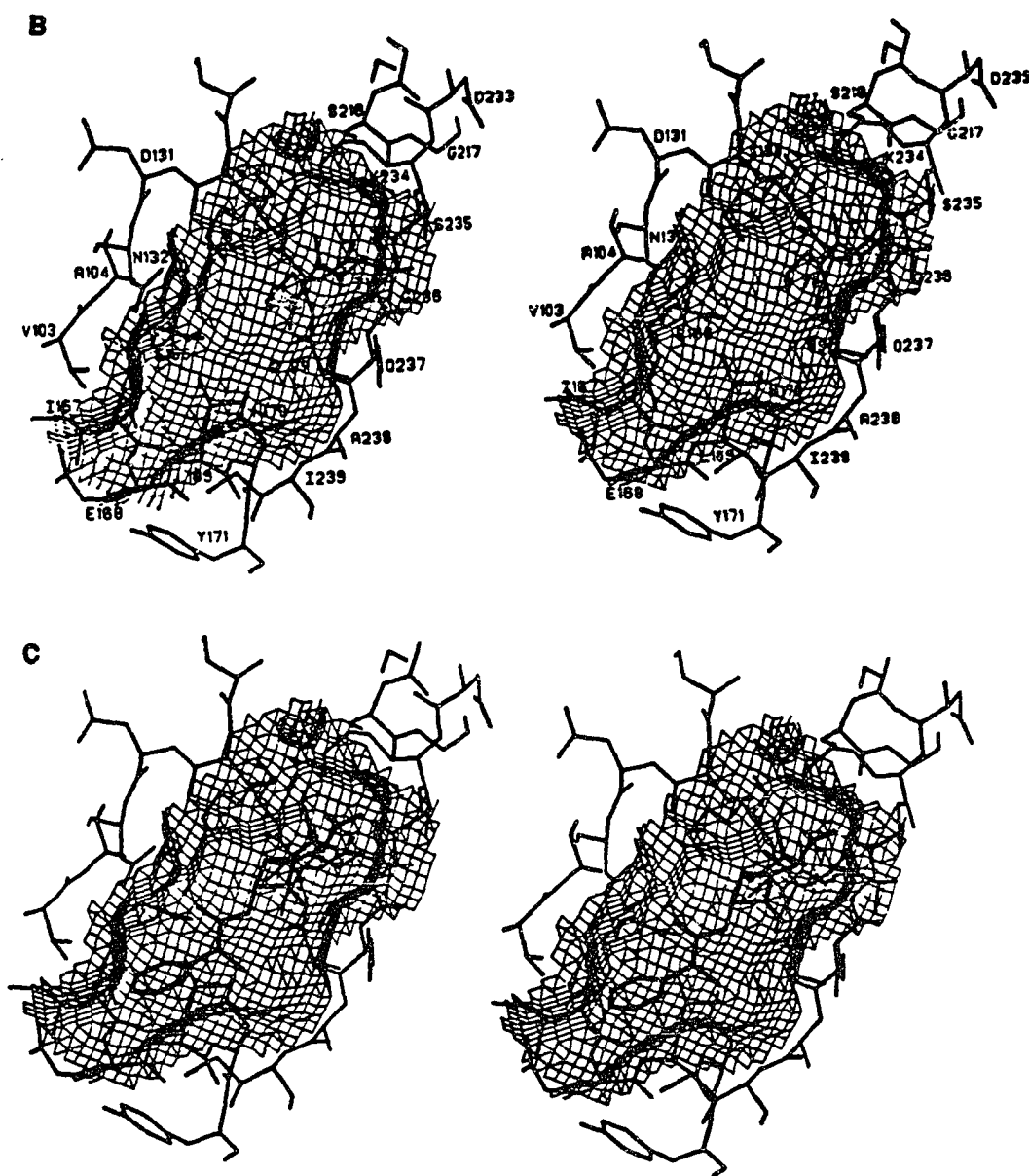


Figure 30. Active site of beta-lactamase from Staphylococcus aureus PC1 at 2.5 Å resolution (from C. Herzberg and J. Moult, (1987) Science 236, 694)

B. Stereoscopic view of the residues that make up the active site depression.

C. As B, with the proposed position of a bound ampicillin molecule

Boronic acid inhibition of E. coli RTEM-2 beta-lactamase:

Inhibition is best with the arylboronic acids with bulky hydrophobic substituents at the meta position. There is a 1000-fold increase in affinity compared to phenylboronic acid. Since the dissociation constants for these compounds are similar, this indicates considerable flexibility in the binding region on the enzyme as well as indicating that there is a hydrophobic binding region. In the crystal structure of the S. aureus enzyme, Val-103 and Ile-239 form a hydrophobic gully at the active site. This is thought to accomodate the hydrophobic end groups on the 7-alpha-substituents of the beta-lactams (32).

The amino acid analogs N-acetyl-D-boro-Leucine and N-acetyl-D-boro-Valine are structurally analogous to the exocyclic acylamido group on penicillins.

The pH profiles of inhibition of E. coli RTEM-2 beta-lactamase by the phenylboronic acids reflect the pK_1 which was seen in the substrate pH profiles. The two exceptions are m-carboxyphenylboronic acid and p-bromophenylboronic acid. In these cases the deviation from a pK_1 of 5.5 could be due to the presence of alternate binding modes.

The pK_2 in the inhibition pH profiles can in each case be assigned to the ionization of the boronic acid group. These values are in close agreement with the literature pK values where available. Since the theoretical pH profiles are drawn to reflect single ionizations, the pK_2 of substrate hydrolysis is not seen in these pH

profiles.

The mechanism for boronic acid complexation at the active site could thus be:

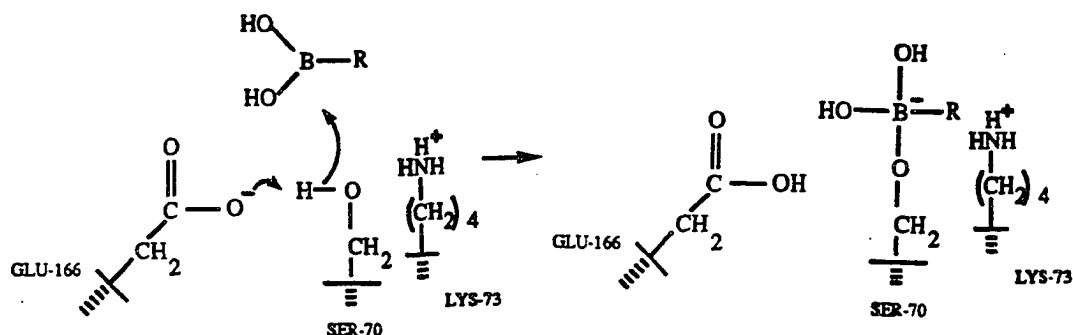


Figure 31

The dependence of $K_i(\text{Lim})$ on the pK_2 of the pH profile of inhibition for the meta substituted phenylboronic acids (Figure 23) is consistent with inhibition being governed by electronic effects which contribute to stabilization of the boronate complex at the active site, for this group of compounds. As such, better inhibition is seen with the phenylboronic acids with electron-withdrawing substituents. *p*-Bromophenylboronic acid deviates from this relationship due probably to better binding due to hydrophobic interactions. 2,4-Dichlorophenylboronic acid is a poor inhibitor which also deviates from the above relationship. This is attributable to the substituent in the ortho position which makes it sterically unfavourable for binding.

m-Carboxyphenylboronic acid may have been expected to bind better due to the presence of the carboxylic acid group. This can be expected to make the same interaction in binding that the exocyclic carboxylate on the substrate makes. It is postulated that the carboxylate interacts with the side chain ammonium group of the conserved Lys-234 (32). However enhanced binding by m-carboxyphenylboronic acid is not observed (Figure 23). This may indicate that the additional binding energy from this interaction is not a significant contribution towards binding of substrate or inhibitor.

The arylboronic acids with hydrophobic substituents (Figures 11, 12, and 13) are far superior in their binding ability compared to the more simply substituted arylboronic acids where binding is determined by electronic effects (Figures 17 to 23). The difference is in the range of two orders of magnitude. This indicates that hydrophobic interactions are significant in binding and play a greater role in inhibition than substituent effects attributable to resonance and induction.

II. A Comparison of the Inhibition of the Serine Protease Thrombin and the Cysteine Protease Papain by a Peptide Boronic Acid

INTRODUCTION

Thrombin is generated in blood serum from its zymogen, prothrombin. It is the central bioregulatory enzyme in hemostasis (1). Prothrombin contains two homologous 'kringle' structures in the non-catalytic subunit that are removed on activation (2). Factor X_a hydrolyzes two peptide bonds on prothrombin to form active thrombin (3).

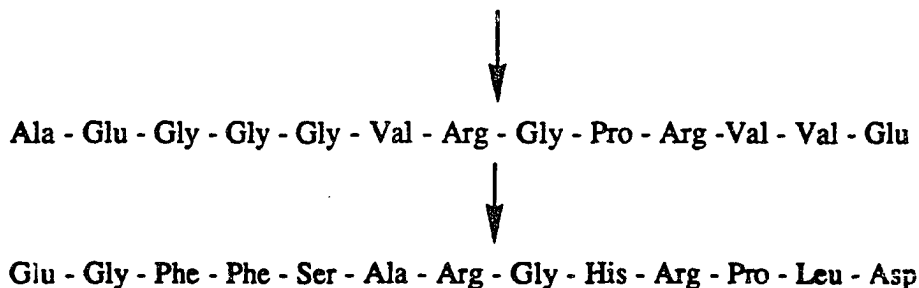
Thrombin is a proteolytic enzyme in the majority of its interactions. It also has nonenzymic hormone-like activities involving receptor-mediated processes (4). In the clotting of whole blood, although > 80% of the zymogen is activated, less than 10% of the potential enzyme concentration is ever achieved. This is thought to be due to inclusion of thrombin within fibrin clots (5).

Thrombin has no net charge at neutral pH (6). It adheres to apolar and negatively charged surfaces (7). Thus the enzyme is readily separated on cationic exchange resins (6). Human alpha-thrombin has an 'exosite' which is independent of the catalytic site. This exosite is involved in fibrinogen recognition (1).

A small protein, hirudin (M.W. ~ 7000) which has a

hydrophobic disulfide knot followed by a noncleavable Pro-Lys-Pro sequence and a very negatively charged tail, is a potent ($K_i \sim 60$ pM) noncompetitive inhibitor of human alpha-thrombin. It competitively inhibits gamma-thrombin 200-fold less efficiently (8). Cleavage of either the disulfide bridges or the tail destroys its inhibitory ability (9). Hirudin binding appears to involve the apolar binding site, regions neighbouring the catalytic site and the anionic binding exosite (10). Hirudin binding blocks various hormonal activities of thrombin (4).

One of the physiological roles attributed to thrombin is its proteolytic action on fibrinogen to release fibrinopeptides A and B. This proteolysis occurs at particular Arg-Gly bonds of the A-alpha and B-beta chains of fibrinogen (11).



The site of thrombin action on fibrinogen, which is also one of the polymerization sites, is in the central nodule of the linear trinodular structure of fibrinogen (12).

The generation of a fibrin clot, which is initiated by thrombin, proceeds by means of three reversible steps

(13): thrombin induced conversion of fibrinogen to fibrin monomer, formation of intermediate polymers from fibrin monomer, and the incorporation of the intermediate polymers into the fibrin gel network.

Although the crystal structure of thrombin has not yet been determined, a preliminary 3-dimensional structure has been suggested (14).

The peptide-chloromethylketone D-Phe-Pro-ArgCH₂Cl is an effective inhibitor of thrombin with a k_1/K_i ratio of $2.8 \times 10^8 \text{ M}^{-1} \text{ min}^{-1}$ (15). (K_i (inhibitor constant) = $2.5 \times 10^{-8} \text{ M}$); k_1 (first-order rate constant = 6.9 min^{-1})). Substitution of a phenylalanine residue for a proline residue at the P₂ position of the inhibitor results in a decrease in k_1/K_i of four orders of magnitude (15).

The rate of formation of the covalent enzyme-inhibitor complex for the P₂-Proline analog is only 2.5 times faster than that of the P₂-Phenylalanine analog. The difference in potency is therefore attributed almost exclusively to a pronounced decrease in affinity with which thrombin binds the latter inhibitor compared to the former inhibitor, before covalent bond formation (15).

Only a small difference in overall inhibitor effectiveness was observed in inhibitors with common P₁ and P₂ residues but varying in the P₃ position. The presence of a bulky, unnatural ϵ -benzoyl-D-lysine residue in P₃ was tolerated well by thrombin with only a four-fold decrease in inhibitor efficacy (15). This may imply the existence of

a very hydrophobic region in the region of the fibrinopeptide-binding site. Proflavin which is very rigid and hydrophobic is bound by thrombin with a dissociation constant of 30 μ M (16). Thrombin which has been inactivated with D-Phe-Pro-ArgCH₂Cl binds proflavin with a marked decrease in affinity ($K_d \sim 1.2$ mM). This suggests overlapping binding regions for the two reagents (16).

Substrate-dependent inhibition of thrombin by peptide aldehydes has been observed. Inhibition by D-Phe-Pro-Arg-H with the substrate Z-Phe-Val-Arg-pNA is competitive, whereas with Z-D-Phe-Pro-Arg-pNA as a substrate, noncompetitive inhibition was seen. Other examples of this variation in the mode of inhibition with substrate were presented (17).

The importance of P₂-Proline for thrombin inhibition was also demonstrated in reference 18. A D-isomer at P₃ position was shown to be particularly advantageous in thrombin and trypsin inhibition. This was most noticeable with a P₂-Proline residue. The strong substrate-dependence of the inhibitory effect has not been adequately explained.

Computer-assisted comparisons made on the X-ray coordinates of homologous atoms in the serine protease derivatives tosyl chymotrypsin A-alpha, tosyl elastase and diisopropylphosphoryl trypsin have shown that sequence homology results in close conformational similarity. Inferences were drawn, on this basis, about the three-dimensional structure of thrombin, for which atomic

coordinates have not yet been determined experimentally. An explanation was sought for the structural origins of the unique substrate specificity of thrombin (14).

Thrombin specifically cleaves four Arg-Gly bonds in fibrinogen, whereas trypsin cleaves peptide linkages in proteins where the acyl residue is arginine or lysine. Both trypsin and thrombin have an aspartic acid residue at position 189, thus their specificity for substrates with positively charged side chains. The occurrence of some 35 amino acid insertions in the main or B chain of thrombin relative to trypsin was recorded (14).

On the basis of the model provided, all of these insertions occur at or near the surface of the molecule, away from the active site and binding pocket. In addition it has been found that 90% of the terminal sialic acid and substantial amounts of the other carbohydrates can be removed from thrombin by glycosidase treatment, without effect on the fibrinogen clotting activity (18).

Thrombin possesses a second polypeptide chain, the A chain, connected to the main or B chain by a disulfide linkage at cysteine 122 (19). An analogous A chain is not found in trypsin. In bovine thrombin, the A chain consists of 49 residues, while in the human enzyme the A chain is 13 residues shorter at the amino terminus. In both enzymes, the C-terminal portion of the A chain extends for 27 residues beyond the disulfide linkage and terminates in an arginine residue.

Chymotrypsin A-alpha also possesses an A chain which consists of only 15 residues, attached through a cysteine at the N-terminus. On the basis of the assumed homology in tertiary structure between thrombin and chymotrypsin, it has been suggested that in thrombin, the C-terminal portion of the A chain could mask the active site or the substrate binding region (14).

In the suggested mechanism for the specificity of thrombin, the active site region of thrombin, which is postulated to be very similar in structure to that of trypsin, is normally masked by the A chain, possibly with the side chain of the C-terminal arginine occupying the substrate binding pocket. Thus the substrate regions of fibrinogen should have a higher affinity for binding with the active site region of thrombin than the masking chain. This will enable selective proteolysis to take place. The existence of multiple contacts between thrombin and fibrinogen in an extended binding region was proposed (14).

In a study where four fibrinogen-like heptapeptides were synthesized and studied as possible substrates for thrombin, the 'native' heptapeptide Gly-Val-Arg-Gly-Pro-Arg-Leu-NH₂ was a poor substrate for thrombin compared to fibrinogen (20). The primary sequence of the peptide itself, is therefore not sufficient to explain the specificity of thrombin for the arg-gly bond in fibrinogen, and the efficiency with which thrombin cleaves this bond selectively ($k_{\text{cat}}/K_m = 2 \times 10^5 \text{ M}^{-1} \text{ sec}^{-1}$).

Proline in site P_2' was preferred over glycine in this subsite. However, the reverse was true for trypsin. Arginine was preferred at P_3' over glycine, and also although less noticeably so, over alanine. Trypsin again showed the opposite effect preferring glycine over arginine in site P_3' . LeuNH_2 was preferred over LeuOH in subsite P_4' . In the A-alpha chain of human fibrinogen this site is occupied by valine (21).

Some of the overall conclusions reached by these investigators were that both thrombin and trypsin contain at least three subsites corresponding to the carboxyl side of the hydrolyzed linkage of the substrate, which are involved in substrate binding. Arginine is preferred over ornithine in binding to subsite S_1 of both enzymes (21).

Both enzymes prefer nonpolar residues at subsite S_2 . The two enzymes deviate in subsite S_3 . The values of k_{cat}/K_m for thrombin hydrolysis decreases as the amino acid corresponding to this subsite is increased in size. This is consistent with the hypothesis that the active site of thrombin involves a narrower cleft than that of trypsin. Trypsin shows a strong preference for a large nonpolar residue in this subsite. Thrombin prefers alanine over glycine at S_1' , but further replacement by leucine reduces the rate of hydrolysis to the same value as for glycine. This is not seen with trypsin, where the rate of hydrolysis is largest with leucine at this position. Nine subsites in total, five to the left of the Arg-Gly bond, and four to

the right were considered (21).

The values of k_{cat}/K_m with the best synthetic substrates were still several orders of magnitude below that for fibrinogen.

In a subsequent study (22), a 51-residue N-terminal cyanogen bromide fragment from the A-alpha chain of human fibrinogen was found to have similar kinetic constants to that of fibrinogen. It was speculated that similar kinetic constants will be obtained with the 27-residue N-terminal portion of CNBr-A-alpha since residues 28, 36, 45, and 49 participate in disulfide bonds in fibrinogen, and were carboxymethylated cysteines in the CNBr-A-alpha fragment which was prepared. For this reason, the region between residues 28 and 51 was not expected to interact with thrombin (22).

Based on kinetic data, the presently accepted model contains a hairpin-like structure on fibrinogen around the Arg-Gly bond that is cleaved (23). This model places Phe-8, which was found to be kinetically important, near this Arg-Gly bond. Phe-8 is presumed to make hydrophobic interactions with thrombin in the enzyme-substrate complex. This structure is thought to be stabilized by intramolecular salt links (23).

Irreversible inhibitors of thrombin are known, of which hirudin is perhaps the most potent. Few reversible inhibitors of thrombin are known.

Peptide boronic acids are effective reversible

inhibitors of other serine proteases. Some peptide boronic acids exhibit time-dependent inhibition. The peptide boronic acids corresponded to the better substrates for chymotrypsin, pancreatic elastase and leukocyte elastase. The most effective boronate inhibitor of chymotrypsin was MeO-Suc-Ala-Ala-Pro-boro-Phe-OH, with a K_i (initial) of 3.4 nM and a K_i (final) of 0.16 nM. MeO-Suc-Ala-Ala-Pro-boro-Val-OH was the most effective inhibitor of leukocyte elastase. The boro-Ala and boro-Phe peptides were 100 and 500 fold less effective with elastase. This demonstrated the importance of the residue in the P_1 site in inhibition (24).

The $t_{1/2}$ for slow-binding inhibition was about 15 min. Some of the structural requirements for boronic acids to exhibit slow-binding inhibition of chymotrypsin were characterized. Binding in the primary specificity site (P_1) alone, as is the case for the inhibition of chymotrypsin by Ac-boro-Phe-OH does not give slow-binding inhibition (25). For peptide boronic acids, the specificity requirement of the enzyme in the P_1 site must be met for progressive inhibition since the boro-Val-OH and boro-Ala-OH analogs of the peptide behaved as simple competitive inhibitors. The stereochemistry of the P_1 boroamino acid is also important since MeO-Suc-Ala-Ala-Pro-D-boro-Phe-OH behaves as a simple competitive inhibitor with a K_i which is about 2 orders of magnitude higher than the corresponding all-L isomer (24).

The present study shows that thrombin, like other serine proteases, is reversibly inhibited by a sequence-specific peptide boronic acid.

In order to study the general specificity of boronic acids to different types of proteases, especially cysteine proteases, it was decided to see if the peptide boronic acid inhibited papain.

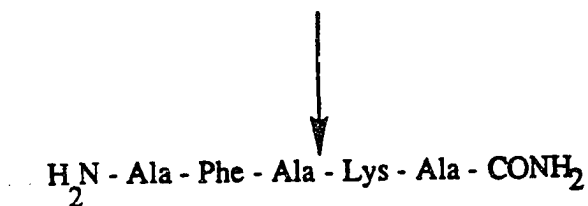
Papain

Papain, a cysteine protease, is one of the major protein constituents of the latex and the fruit of Carica papaya (26).

Papain is inactivated by iodoacetamide, with conversion of cysteine-25 to its alkylated derivative (27). One of the three disulfide bridges of papain (Cys-56-Cys-95) is reduced selectively with 0.32 M 2-mercaptoethanol in 8M urea at pH 8.2 (28). The derivative is crystallised, on the addition of HgCl_2 , after removing the denaturing and reducing agents (29). This derivative contains two bound atoms of Hg per molecule of protein, one of which is bound to the active site sulfhydryl group (Cys-25), the other cross-links Cys-56 to Cys-95, forming a S-Hg-S bridge.

Although there is no known natural substrate for papain, the enzyme cleaves a wide variety of peptide bonds (30). The active site of papain extends over 2.5 nm. 7 amino acid residues in this active site are thought to be

important in substrate binding. These are located on both sides of the catalytic site, four on one side and three on the other. S_2 specifically interacts with a phenylalanine side chain (31). Thus the presence of phenylalanine in P_2 enhances the susceptibility of a peptide to hydrolysis by papain.



Due to the influence of the positioning of the phenylalanine residue on the point of cleavage, there is no cleavage of the above peptide at the lysine residue (31). Subsite S_1 shows preference for lysine over alanine. Peptides containing the sequences Ala-Ala-Phe-Ala or Ala-Ala-Phe-Lys have been found to be strong inhibitors. Val and Leu also interact strongly with subsite S_2 (31).

Due to its broad specificity, papain is not frequently used for the purpose of sequencing proteins and very long polypeptides. However, it is used in the hydrolysis of peptides of moderate size, particularly when they do not contain bonds which are susceptible to hydrolysis by trypsin and chymotrypsin (32).

Aldehydes which are structurally related to the acyl portion of substrates are extremely potent competitive

inhibitors of papain (33,34). The thiohemiacetal formed at the active site with Cys-25 resembles the tetrahedral intermediates in substrate hydrolysis. Although the accumulation of such an intermediate is not favoured for thermodynamic reasons (35), it is generally accepted that papain hydrolyses proceed via a tetrahedral intermediate which is similar to that seen with the serine proteases. Acylation occurs at the active site cysteine (Cys-25), while in the serine proteases acylation is at the active site serine.

Similar to the case with the serine proteases, the involvement of a histidine group (His-159) is implicated in the mechanism, possibly in deacylation (36). The crystal structure of papain itself (37) and that of papain complexed to a thiol protease inhibitor (38) places His-159 at the active site of papain. In the latter study it was shown that N¹ of His-159 is involved in hydrogen bond formation with the inhibitor.

Further evidence for a histidine residue at the active site was supplied in a study where papain was irreversibly inhibited by 1,3-dibromoacetone which is a possible cross-linking agent. The active site thiol and the N¹ of an adjacent imidazole group were alkylated by this reagent indicating that these groups are within 5 Å of each other in the tertiary structure (39).

The inhibition of cysteine proteases by boronic acids has not previously been reported in the literature. The

pBR322 RTEM beta-lactamase was mutated at the active site to replace Ser-70 with Cys-70 (40). Although the wild-type enzyme was competitively inhibited by boric acid ($K_i = 1$ mM), the same was not seen with the thiol beta-lactamase. A small degree of noncompetitive inhibition was observed at a boric acid concentration of 10 mM. Phenylboronic acid was a poor inhibitor of thiolsubtilisin ($K_i > .1$ M). This shows poorer binding by three orders of magnitude compared to phenylboronic acid inhibition of subtilisin. Inhibition was independent of time (41). In that study when activated papain was incubated with 10^{-2} M n-butylboronic acid, phenylboronic acid, m-nitrophenylboronic acid and phenylarsonate, using benzoyl-L-Arginine ethyl ester as the substrate there was no detectable inhibition at pH 6.7 for any of the reagents, and the K_i s were estimated to be in the order of 10^{-2} to 10^{-3} M.

Various aldehydes were moderately good inhibitors of thiolsubtilisin with K_i values ranging from 10^{-3} - 10^{-5} M. Of these Ac-(Leu)₂-Phe-CHO was the best with a K_i of 5×10^{-5} M (41).

Spectrophotometric data utilising 4-dimethylaminocinnamaldehyde show that aldehyde binding to thiolsubtilisin does not involve the formation of a thiohemiacetal (41).

The active site Cys-25 to Ser-25 mutation was accomplished in papain by chemical means. The resulting enzyme showed no enzymic activity towards good substrates

for the native enzyme, such as Z-L-Glycine-p-nitrophenyl ester or Z-L-Lysine-p-nitrophenyl ester (42). As a result no inhibitory studies could be carried out.

In the present study, papain is noncompetitively inhibited by 1-(Z-D-Phenylalanyl-L-Prolineamido)-1-dihydroxyborono-4-methoxybutane in the micromolar range, with pH-dependent binding.

EXPERIMENTAL

Materials

Bovine plasma thrombin was purchased from Sigma. When reconstituted with one ml water, the resulting solution contained 0.15 M NaCl and 0.05 M sodium citrate at pH 6.5. 120 or 240 NIH units of thrombin were contained in a vial. Thrombin concentration was calculated on the basis of 1860 NIH units/mg protein. Dilutions were in deionized water.

The thrombin substrate, N-t-Boc-L-Valyl-L-Prolyl-L-Arginine-7AMC.HCl was purchased from Sigma. Stock solutions were made in DMF (2 mg/ml). 10-fold diluted solutions were in DMF. 50-fold and further dilutions were in pH 7 buffer, ionic strength 0.1M.

The thrombin substrate, H-D-Phenylalanyl-L-Pipecolyl-L-Arginine-p-nitroanilide dihydrochloride (S-2238) was a gift from KabiVitrum. Stock solutions were made in water (3 mg/10 ml).

Peptide inhibitors, Z-D-Phenylalanyl-L-Prolyl-L-Arginine and 1-(benzyloxycarbonyl-D-Phenylalanyl-L-Prolineamido)-1-dihydroxyborono-4-methoxybutane were synthesized by Dr. G. Claesen. The latter compound was obtained as the pinanediol ester of the boronic acid. (This group spontaneously dissociates in solution at concentrations below 10^{-6} M, and the free boronic acid is exposed.) Stock solutions of the former were made in pH 7

buffer (2 mg/ml). Dilutions were also in buffer. Stock solutions of the latter were in methanol (1.7 mg/ml). Dilutions were also in methanol.

Fluorescence measurements were taken on a Hitachi Perkin-Elmer fluorescence spectrophotometer (MPF-3A). This had a temperature-controlled sample compartment.

Absorbance measurements were made on a GCA/McPherson spectrophotometer (Model EU-707-SA) with an attached temperature control system.

Buffers of ionic strength 0.1M were used in the enzyme assays. These were prepared according to the formulae given in The Biochemists Handbook (26). In addition to the buffers which were used in Part I of this thesis tris(hydroxymethyl)aminomethanehydrochloride-tris(hydroxymethyl)aminomethane was used in the pH range 8.0 to 8.8.

Bovine serum albumin was purchased from Sigma. Stock solutions were made in water (1.5 mg/5 ml). The assay mixture contained 0.01 mg BSA/ml.

Studies with papain

Water used in the experiments, organic solvents and the buffers used were treated as in the previous sections. Formic acid-sodium formate buffer was used in the pH range 3.2 to 3.6.

Mercuripapain was purchased from Worthington

Biochemical Corp. The vial contained 8.8 mg protein/ml. Dilutions were in buffers of 0.1 M ionic strength.

N-CBZ-L-Lysine-p-nitrophenyl ester.HCl was purchased from Sigma. Stock solutions were made in acetonitrile (10 mg/3.5 ml). Dilutions were also in acetonitrile.

N-CBZ-L-Lysine-thiobenzyl ester.HCl was purchased from Sigma. Stock solutions were made in acetonitrile (10 mg/3.2 ml). Dilutions were also in acetonitrile.

2-Mercaptoethanol was purchased from Sigma. Dilutions were in buffer.

1-(Z-D-Phe-L-Prolineamido)-1-dihydroxyborono-4-methoxybutane was synthesized by Dr. Metternich. Stock solutions were in methanol. Dilutions were also in methanol.

Methods

Inhibition of bovine thrombin by Z-D-Phe-L-Pro-L-Arginine and 1-(Z-D-Phe-L-Prolineamido)-1-dihydroxyborono-4-methoxybutane, when studied with the substrate N-t-Boc-L-Val-L-Pro-L-Arg-7AMC.HCl was observed on a Hitachi Perkin-Elmer fluorescence spectrophotometer. Fluorescence due to product formation was observed as a function of time at an emission wavelength of 440 nm (excitation at 350 nm). Excitation and emission slits were set at 6 nm and the sample sensitivity was 10. Assays were done at 24-26°C.

The dissociation constant (K_m) for the substrate was determined at each pH. This was done by constructing Lineweaver-Burk plots, using initial rates at different substrate concentrations, at each pH.

Enzyme assays were done by sequential addition of 3 ml buffer, 50-300 microliters substrate, 100 microliters BSA, 50-300 microliters buffer and 50-200 microliters enzyme.

Since there was substrate fluorescence at the wavelengths used, a baseline reading was taken before adding the enzyme. Typically, product formation was observed over a 2-4 minute time interval.

The pH of the assay mixture was read immediately after the assay, on a Radiometer PHM61 Laboratory pH meter.

The inhibitor dissociation constant (K_i), was determined at each pH by comparing the initial rates of the inhibited and uninhibited reactions.

The reaction progress curve, when using the substrate, H-D-Phe-L-Pip-L-Arg-p-nitroanilide.2HCl, was observed on the McPherson spectrophotometer at 405 nm (delta extinction coefficient = $9920 \text{ M}^{-1} \text{ cm}^{-1}$).

Studies with papain

A GCA/McPherson spectrophotometer (model EU-707-SA) with an attached temperature control system was used in the kinetic studies. Substrate hydrolysis with N-CBZ-L-Lysine-

p-nitrophenyl ester was observed at 400 nm at pH 7 and above (delta extinction coefficient $6667 \text{ M}^{-1}\text{cm}^{-1}$ at pH 7). (the p-nitrophenolate ion which is released on substrate hydrolysis has maximal absorbance at this wavelength). Absorbance was read at 340 nm (delta extinction coefficient $6360 \text{ M}^{-1}\text{cm}^{-1}$) for assays carried out at pH values below 7.

Adjustments were made for spontaneous hydrolysis which was apparent at near neutral pH s.

N-CBZ-L-Lysine thiobenzyl ester.HCl was the substrate used at the more basic pHs since there was no spontaneous hydrolysis. Substrate hydrolysis was observed at 240 nm (delta extinction coefficient $3000 \text{ M}^{-1}\text{cm}^{-1}$).

Buffers were deaerated by aspiration before use in order to prevent oxidation of the active site cysteine (Cys-25). They were 0.5 M in NaCl, 1 mM in EDTA and 10^{-4}M in boric acid. Mercaptoethanol was introduced to the assay mixture at a concentration of 60 mM.

Quartz cuvettes of one cm path length were used throughout this study. Assays were done by the sequential addition of 0.9 or 1 ml buffer, 5-20 μl substrate from an appropriately diluted solution, 50-200 μl of methanol or inhibitor, 5 μl mercaptoethanol and 50-200 μl of appropriately diluted murcuripapain. A baseline absorbance reading was taken before adding the enzyme.

Reactions were run to completion and the pseudo-first order rate constant determined at each pH for the inhibited and the uninhibited runs. The K_i at each pH was obtained.

RESULTS

Dissociation constants for the thrombin substrate, N-t-Boc-L-Valyl-L-Prolyl-L-Arginine-7AMC are essentially pH-independent between pH 5 and 10 (Table I, Figure 2).

Inhibition of thrombin by 1-(Z-D-Phe-L-prolineamido)-1-dihydroxyborono-4-methoxybutane is competitive at pH 7.1 in the presence of the substrate N-t-Boc-L-Val-L-Pro-L-Arg-7AMC (Figure 4).

Inhibition is pH-dependent (Table II). Binding is maximal at pH 7.75, which is close to the physiological pH.

The pH profile of inhibition is bell-shaped and shows the ionization of essential groups which have pKs of 7.05 and 8.75 (Figure 3).

The pseudo-first-order hydrolysis of the substrate N-t-Boc-L-Val-L-Pro-L-Arg-7AMC in the presence of the inhibitor 1-(Z-D-Phe-L-Prolineamido)-1-dihydroxyborono-4-methoxybutane at pH 7.75 shows the time-dependence of substrate hydrolysis (Figure 5). It is clear that inhibition is immediate upon mixing of the solution. The time lag between mixing and recording the first data point was less than a minute.

Inhibition by Z-D-Phe-L-Pro-L-Arginine is also competitive at pH 7.1 with the substrate H-D-Phe-L-Pip-L-Arg-p-nitroanilide (Figure 7).

Inhibition is pH-dependent with maximal binding occurring at acid pH's (Table III). The pH profile of

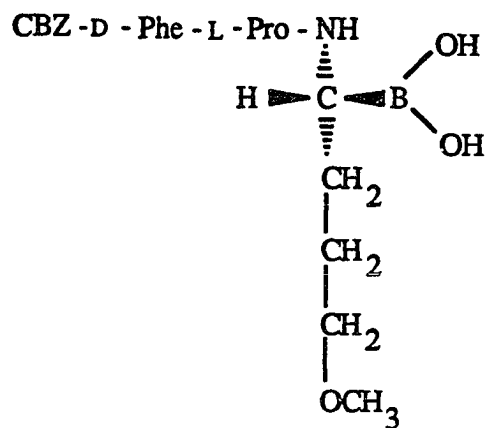
inhibition is sigmoidal and shows the ionization of an essential group of pH 8.0 (Figure 6).

Here too, time course data for inhibition show that inhibition is rapid (Figure 8).

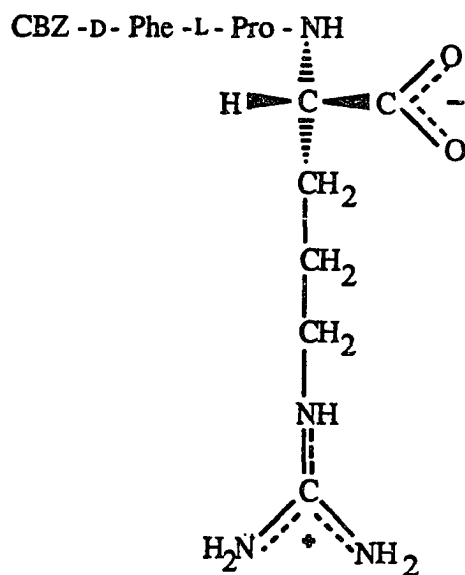
Inhibition of papain by 1-(Z-D-Phe-L-Prolineamido)-1-dihydroxyborono-4-methoxybutane was noncompetitive at pH 6.87 in the presence of the substrate N-CBZ-L-Lys-p-nitrophenyl ester (Figure 10). Inhibition was pH-dependent with maximal binding occurring at slightly acidic pH (Table IV). The pH profile of inhibition is bell-shaped and shows the ionization of essential groups which have pKs of 4.38 and 7.47 (Figure 9). Inhibition was rapid and occurred soon after mixing of the solution (Figure 11).

In this study the dissociation (via hydrolysis) of the pinanediol blocking group from the boronic acid was observed to take place with a half-life of about 1 minute. Absorbance at 240 nm decreases on dissociation, with a delta extinction coefficient of $\sim 2460 \text{ M}^{-1} \text{ cm}^{-1}$ with 10^{-4} M borate in the solution.

Figure 1. Thrombin Inhibitors



1-(Z-D-Phenylalanyl-L-Prolineamido)-1-dihydroxyborono-4-methoxybutane



Z -D-Phenylalanyl-L-Prolyl-L-Arginine

Table I

Dissociation constants for the substrate N-t-Boc-L-Val-L-Pro-L-Arg-7AMC with bovine serum thrombin at 25⁰ C.

Buffers were of ionic strength 0.1 M. [BSA] = 0.01 mg/ml

pH	K _m (μM)
5.10	1.7
6.10	3.3
7.13	2.5
7.75	2.5
8.95	1.9
9.20	1.3
9.50	1.2
10.05	6.2

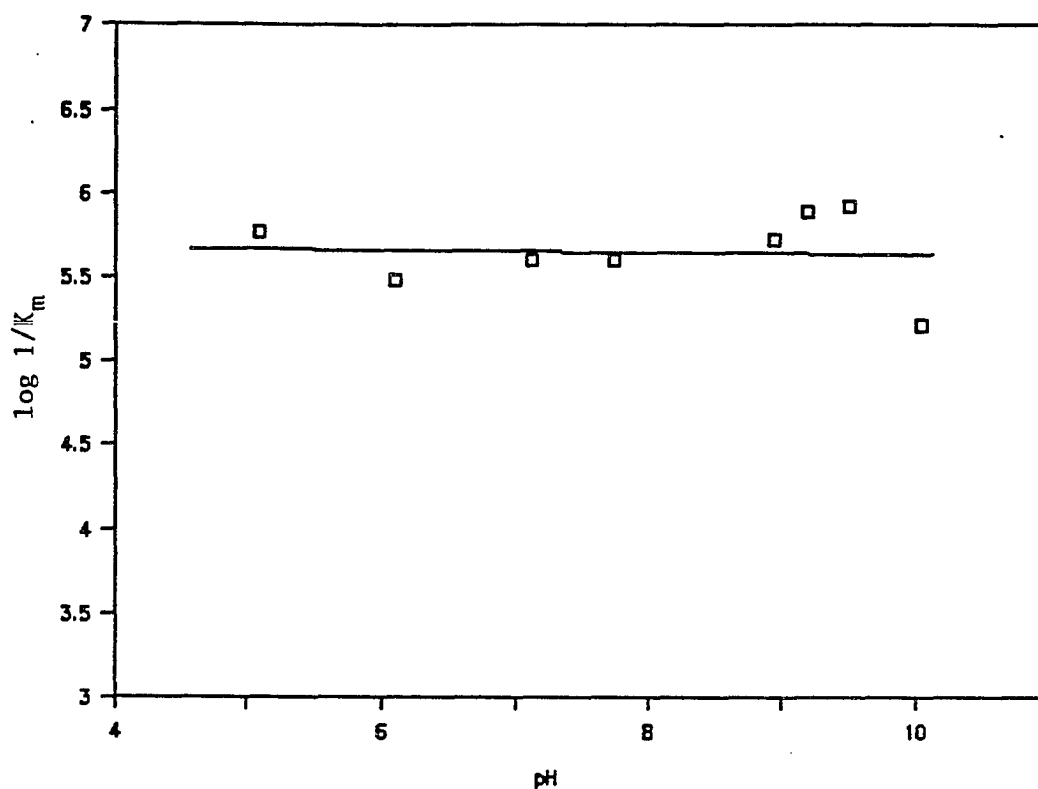


Figure 2. pH-dependence of $1/K_m$ for the thrombin substrate N-t-Boc-L-Val-L-Pro-L-Arg-7AMC
 $t = 25^{\circ}\text{C}$ $I = 0.1 \text{ M}$ $[\text{BSA}] = 0.01 \text{ mg/ml}$
Data of Table I

Table II

pH-dependent dissociation constants for the inhibitor 1-(2-D-Phe-L-Prolineamido)-1-dihydroxyborono-4-methoxybutane, with bovine serum thrombin, in the presence of the substrate, N-t-Boc-L-Val-L-Pro-L-Arg-7AMC at 26⁰ C. Buffers were of ionic strength 0.1 M. [BSA] = 0.01 mg/ml.

pH	K _i (x 10 ⁸ M)
5.10	38
6.10	7.5
7.13	1.5
7.75	0.86
8.95	1.5
9.20	3.9
9.50	4.7
10.05	89

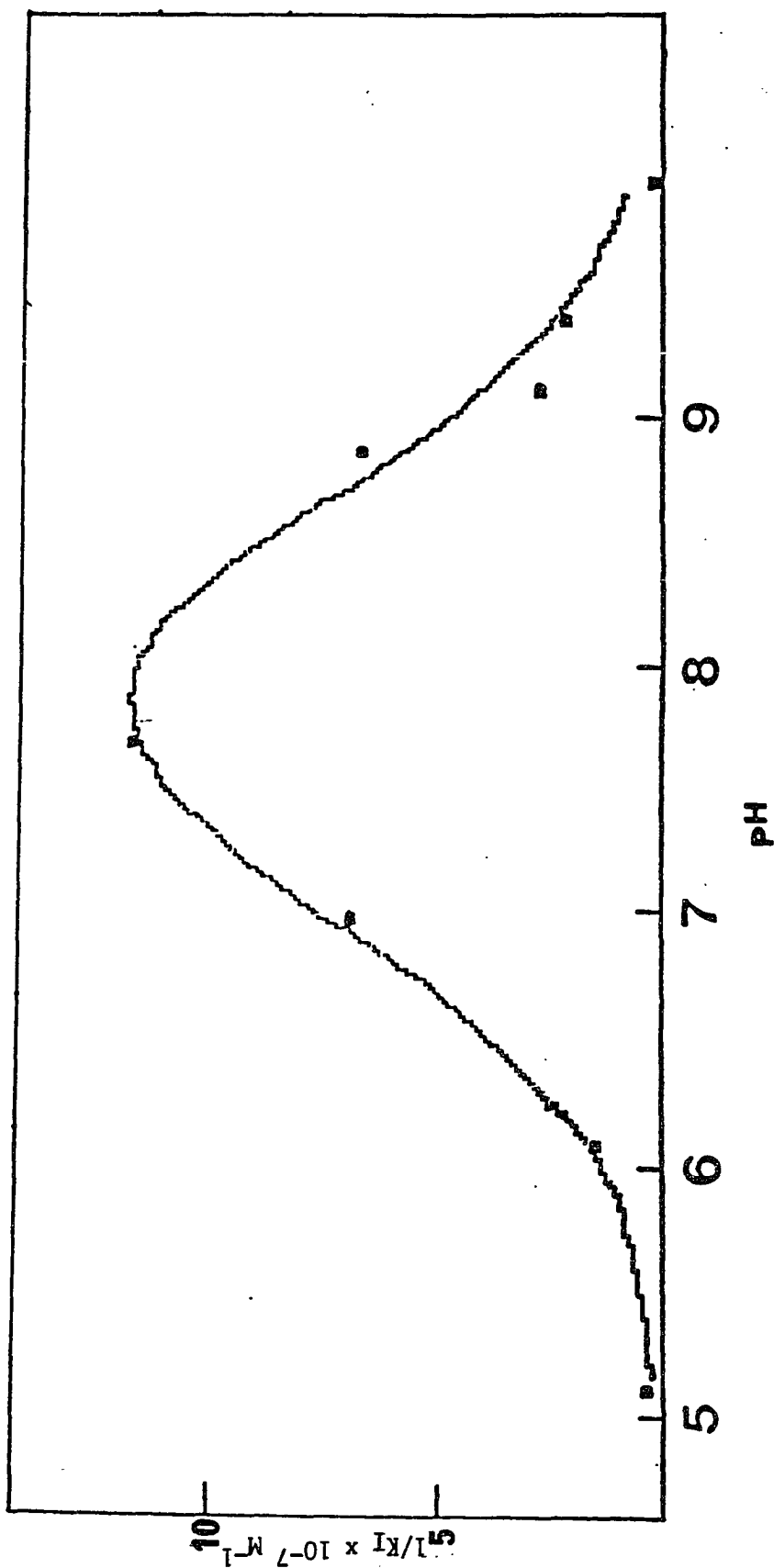


Figure 3. pH-dependence of the 1-(2-D-Phe-L-Prolineamido)-1-dihydroxyborono-4-methoxybutane inhibition of bovine serum thrombin at 25°C. $I = 0.1 \text{ M}$. $[BSA] = 0.01 \text{ mg/ml}$
 $pK_1 = 7.05$ $pK_2 = 8.75$ $K_1(\text{Lim}) = 6.9 \times 10^{-9} \text{ M}$
 Data of Table II

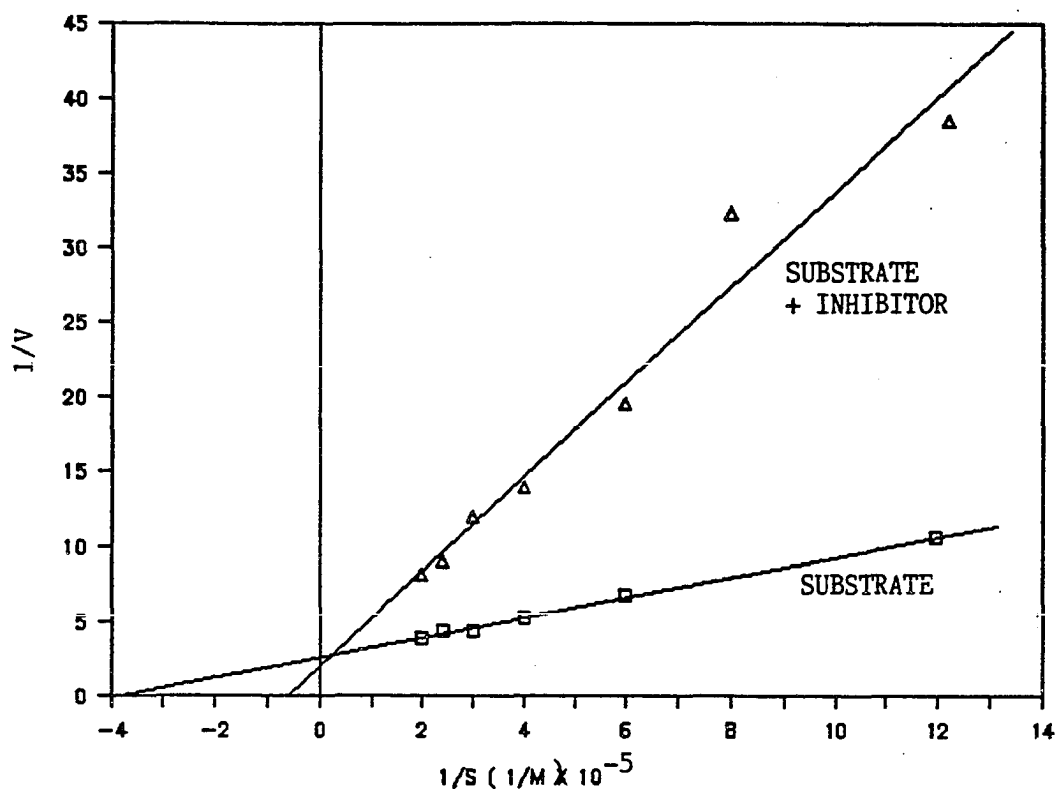


Figure 4. 1-(Z-D-Phe-L-Prolineamido)-1-dihydroxyborono-4-methoxybutane inhibition of bovine serum thrombin at pH 7.1 and 26°C. I = 0.1 M phosphate buffer [BSA] = 0.01 mg/ml. [thrombin] = 8×10^{-10} M The substrate used was N-t-Boc-L-Val-L-Pro-L-Arg-7AMC

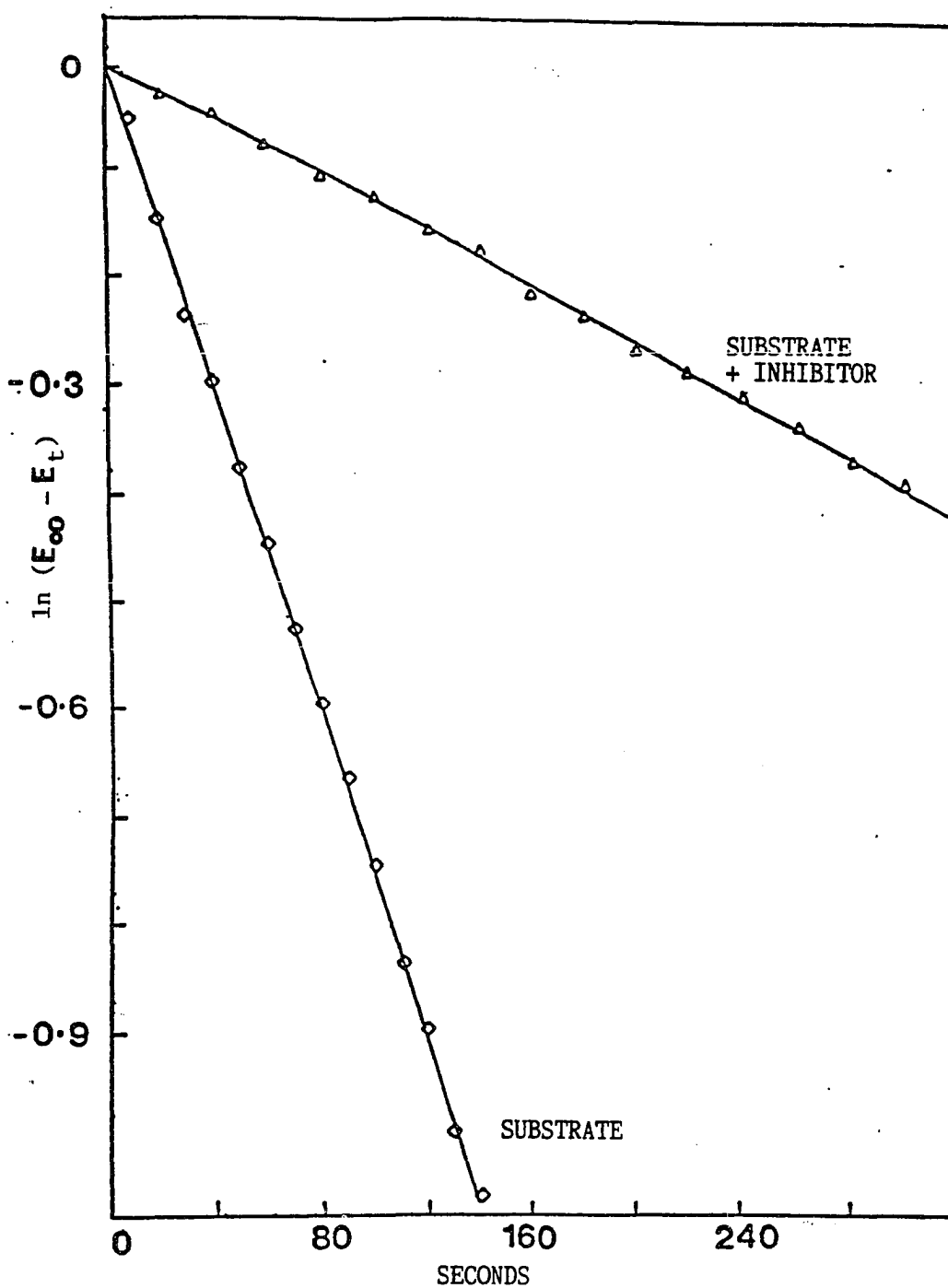


Figure 5. Inhibition of thrombin by 1-(Z-D-Phe-L-Prolineamido)-1-dihydroxyborono-4-methoxybutane. The plot shows the pseudo-first-order hydrolysis of 0.28 μM N-t-Boc-L-Val-L-Pro-L-Arg-7AMC in pH 7.75 phosphate buffer I = 0.1 M at 25°C. [inhibitor] = 37 nM [thrombin] = 2.0 nM [BSA] = 0.01 mg/ml

Table III

pH-dependent dissociation constants for the inhibitor Z-D-Phe-L-Pro-L-Arginine, with bovine serum thrombin, in the presence of the substrate, N-t-Boc-L-Val-L-Pro-L-Arg-7AMC at 30° C.

Buffers were of ionic strength 0.1 M. [BSA] = 0.01 mg/ml

pH	K_i ($\times 10^6 M$)
5.42	1.59
6.00	1.60
7.00	2.00
7.62	2.53
7.75	4.00
7.99	3.11
8.28	3.74
8.52	4.38
8.74	9.30
9.00	19.0
9.20	23.8
9.40	40.0

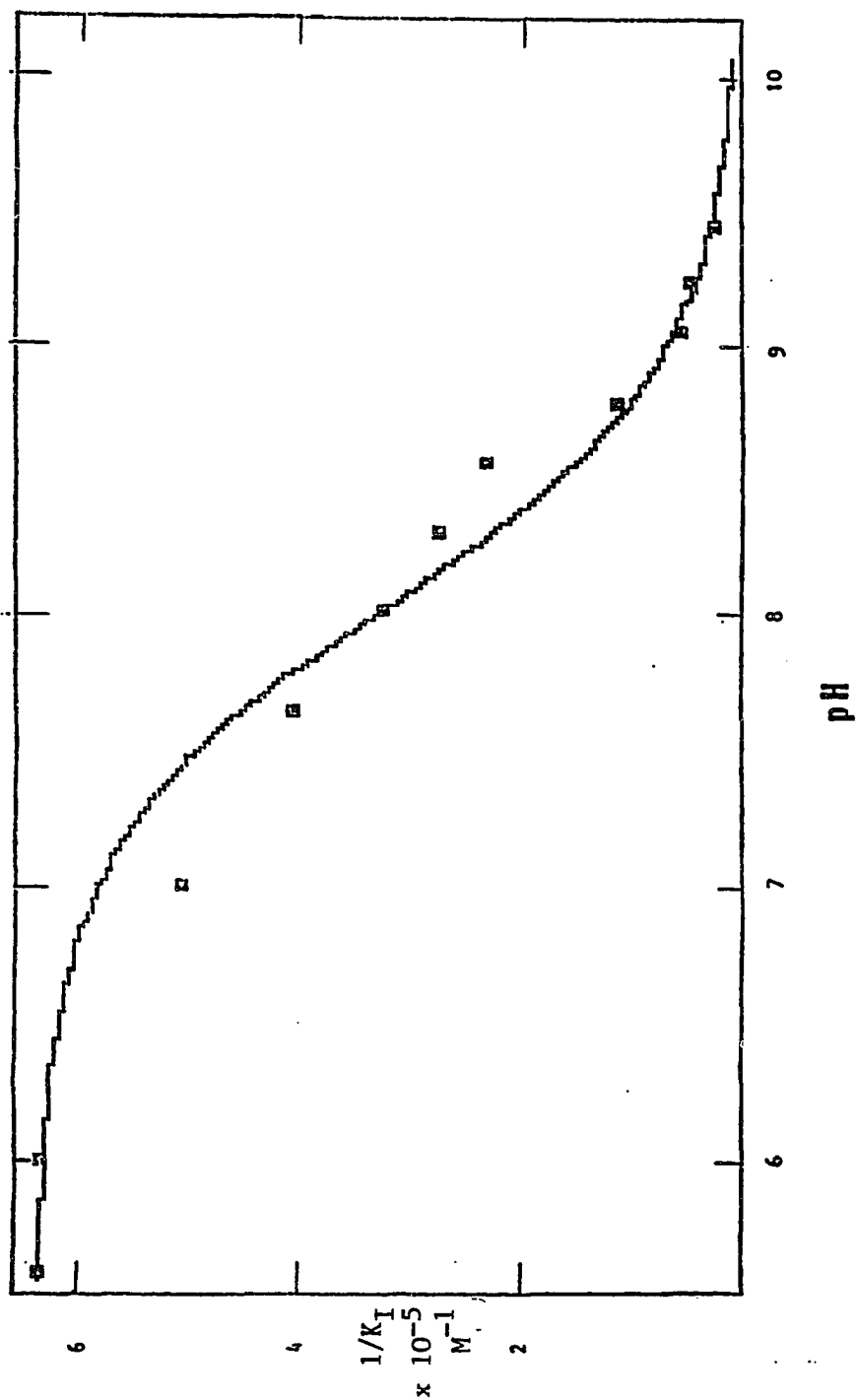


Figure 6. pH-dependence of the Z-D-Phe-L-Pro-L-Arginine inhibition of bovine serum thrombin at 25°C. $I = 0.1 \text{ M}$. $[BSA] = 0.01 \text{ mg/ml}$
 $pK = 8.0$ $K_i(\text{Lim}) = 1.5 \times 10^{-6} \text{ M}$
 Data of Table III

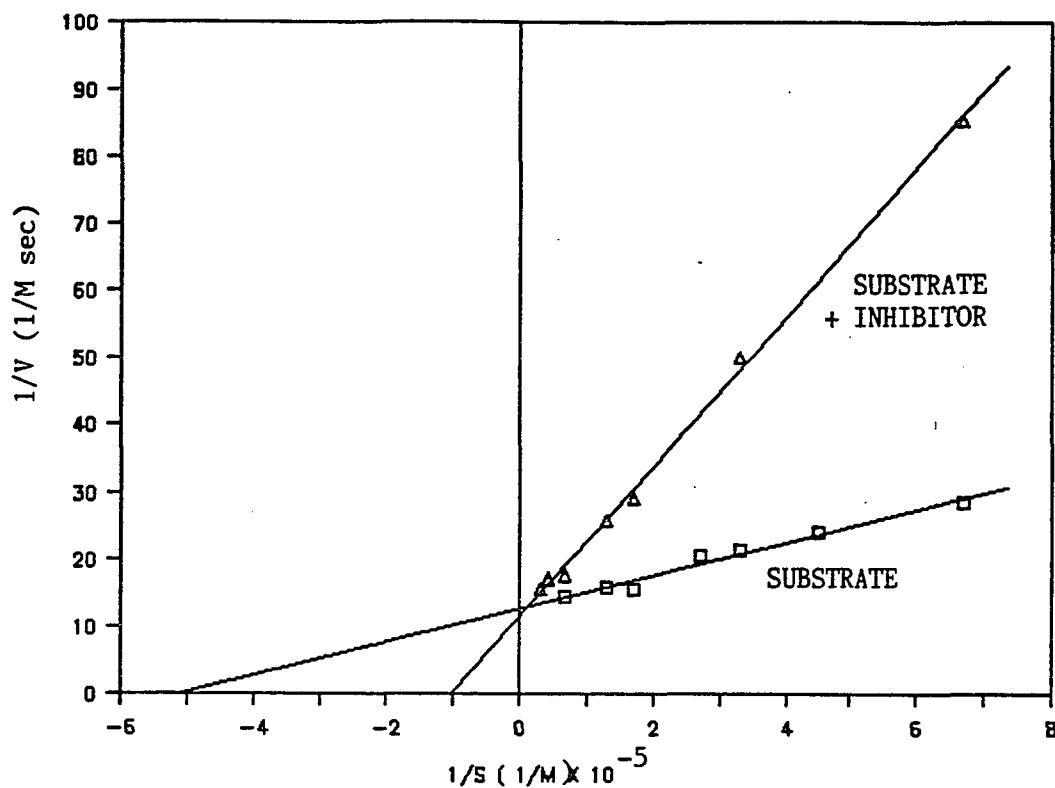


Figure 7. Z-D-Phe-L-Pro-L-Arginine inhibition of bovine serum thrombin at pH 7.12 and 30°C
 I = 0.1 M phosphate buffer [BSA] = 0.01 mg/ml
 [thrombin] = 3.7 nM
 The substrate used was D-Phe-L-Pip-L-Arg-p-nitroanilide

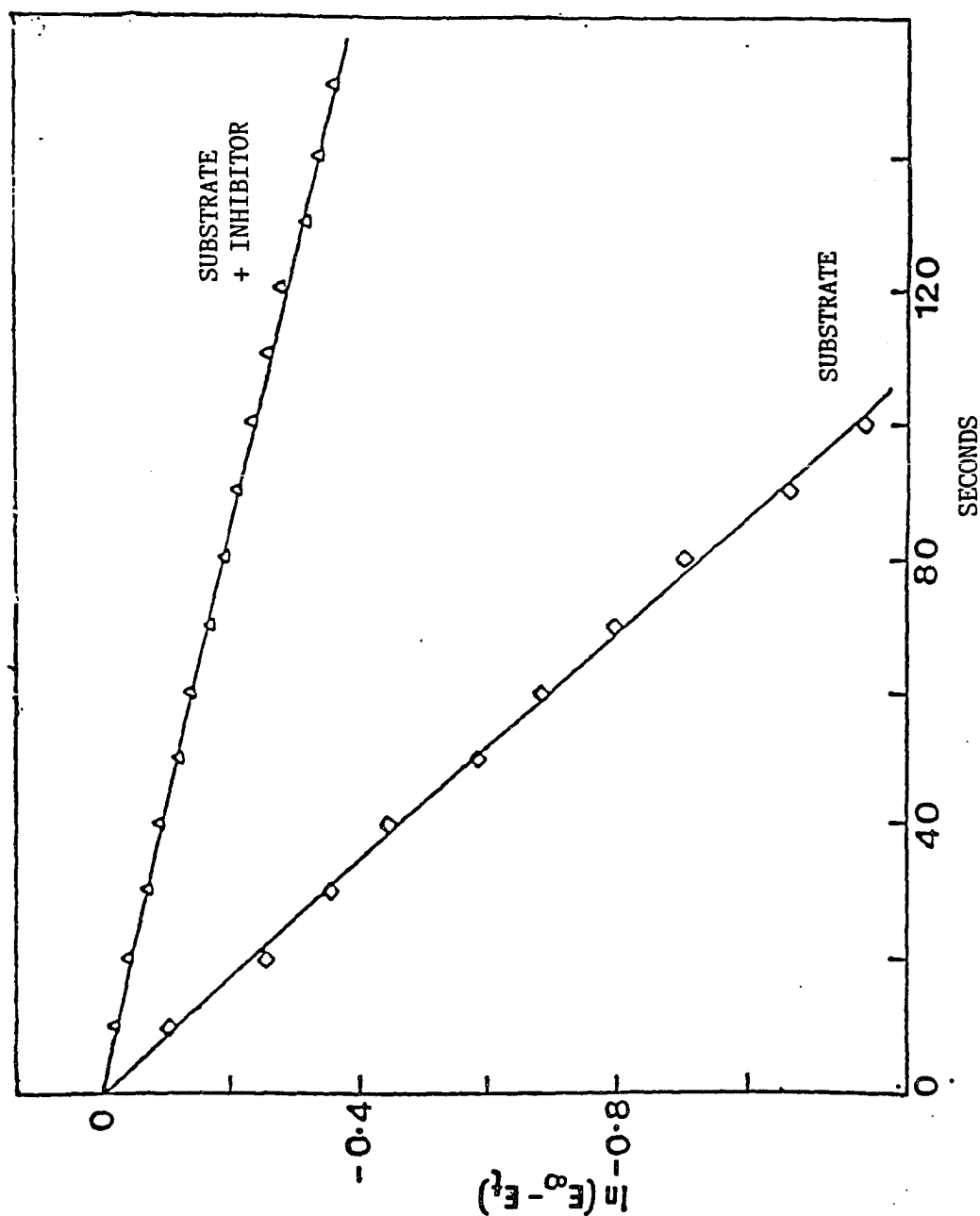


Figure 8. Inhibition of thrombin by Z-D-Phe-L-Pro-L-Arginine. The plot shows the pseudo-first-order hydrolysis of $0.3 \mu\text{M}$ N-t-Boc-L-Val-L-Pro-L-Arg-7AMC in pH 7.75 phosphate buffer at 25°C . $I = 0.1 \text{ M}$.
 $[\text{BSA}] = 0.01 \text{ mg/ml}$ $[\text{inhibitor}] = 20 \mu\text{M}$
 $[\text{thrombin}] = 1.6 \text{ nM}$

Table IV

Dissociation constants for the inhibitor 1-(Z-D-Phe-L-Prolineamido)-1-dihydroxyborono-4-methoxybutane with papain at 25⁰ C.

The assay mixtures contained 1 mM EDTA, 0.1 mM boric acid, and 60 mM 2-mercaptoethanol. Buffers were of ionic strength 0.1 M.

pH	$K_i \times 10^6$ M
3.90	49.3
4.47	24.5
5.00	15.3
5.86	13.9
6.60	10.6
6.87	14.8
*7.65	43.4
*7.80	72.7

The substrate used was N-CBZ-L-Lys-p-nitrophenyl ester

*The substrate used was N-CBZ-L-Lys-thiobenzyl ester

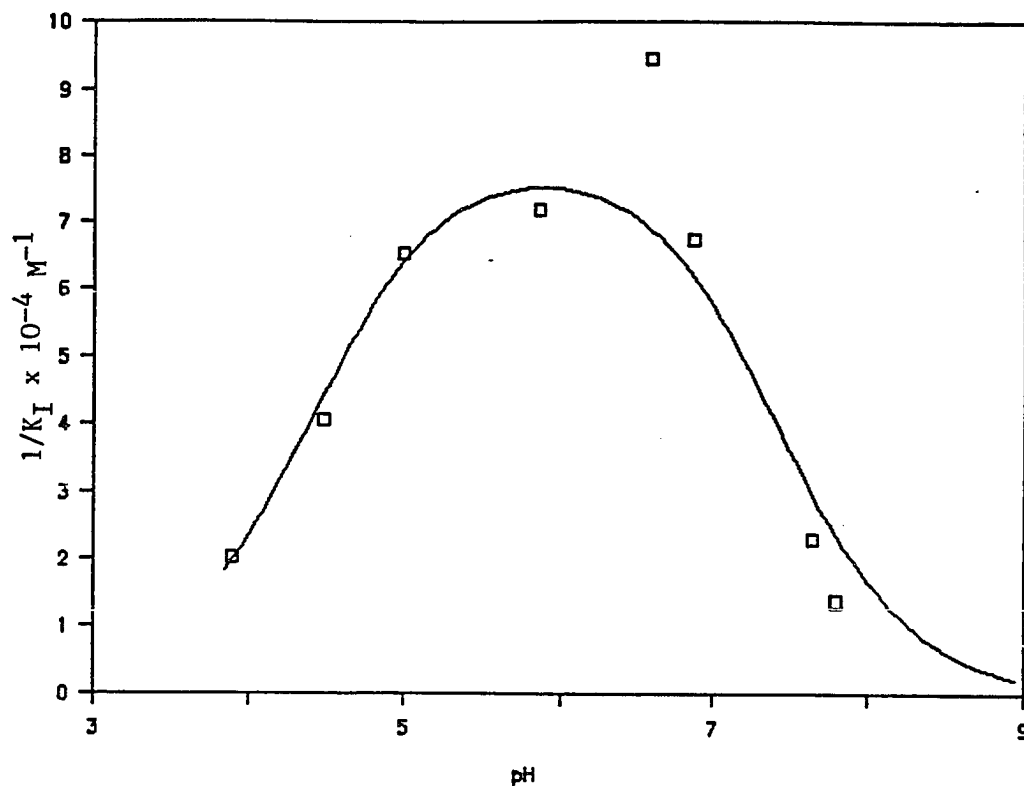


Figure 9. pH-dependence of the 1-(Z-D-Phe-L-Prolineamido)-1-dihydroxybgrono-4-methoxybutane inhibition of papain at 25°C.

$$pK_1 = 4.38 \pm 0.29 \quad pK_2 = 7.42 \pm 0.29$$

$$K_i(\text{Lim}) = 12.5 \pm 0.00873 \mu\text{M}$$

Data of Table IV

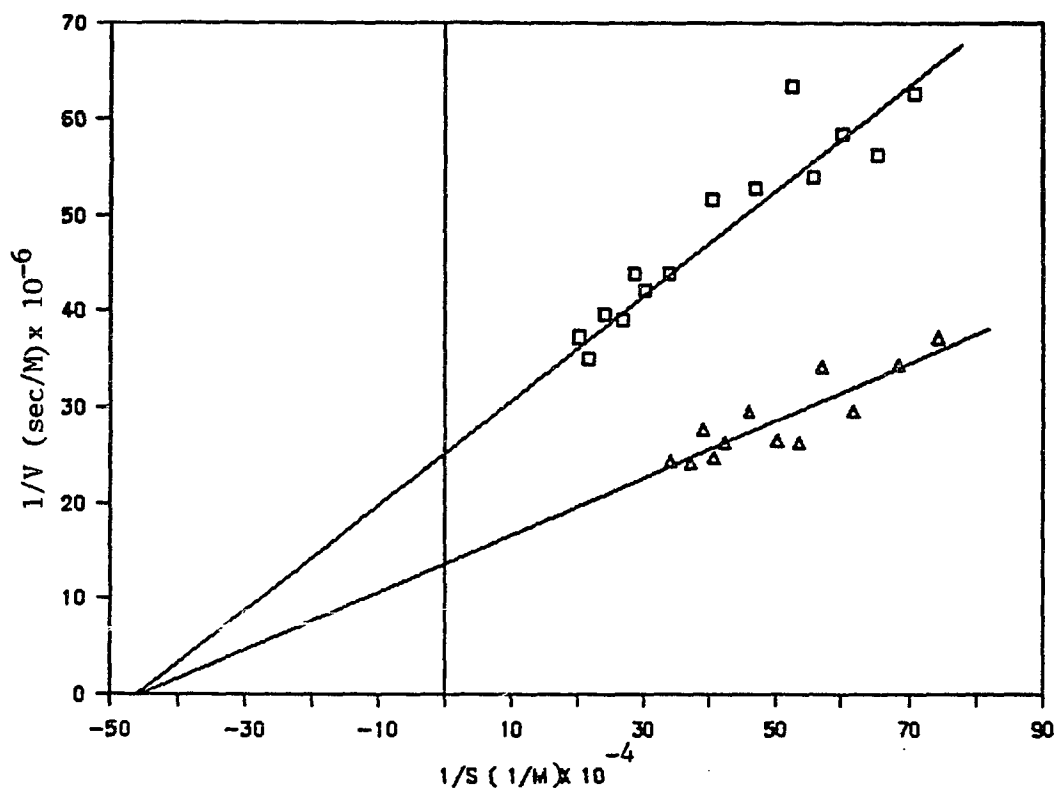


Figure 10. 1-(Z-D-Phe-L-Prolineamido)-1-dihydroxyborono-4-methoxybutane inhibition of papain at pH 6.87 and 25°C. I = 0.1 M phosphate buffer
 [NaCl] = 0.5 M [EDTA] = 0.1 mM
 [boric acid] = 10^{-4} M [2-mercaptoethanol] = 60 mM
 The substrate used was N-CBZ-L-Lys-p-nitrophenyl ester
 $K_m = 2.2 \times 10^{-6}$ M $V_{\max}(\text{uninh}) = 7.34 \times 10^{-8}$ M s $^{-1}$
 $V_{\max}(\text{inh}) = 3.71 \times 10^{-8}$ M s $^{-1}$

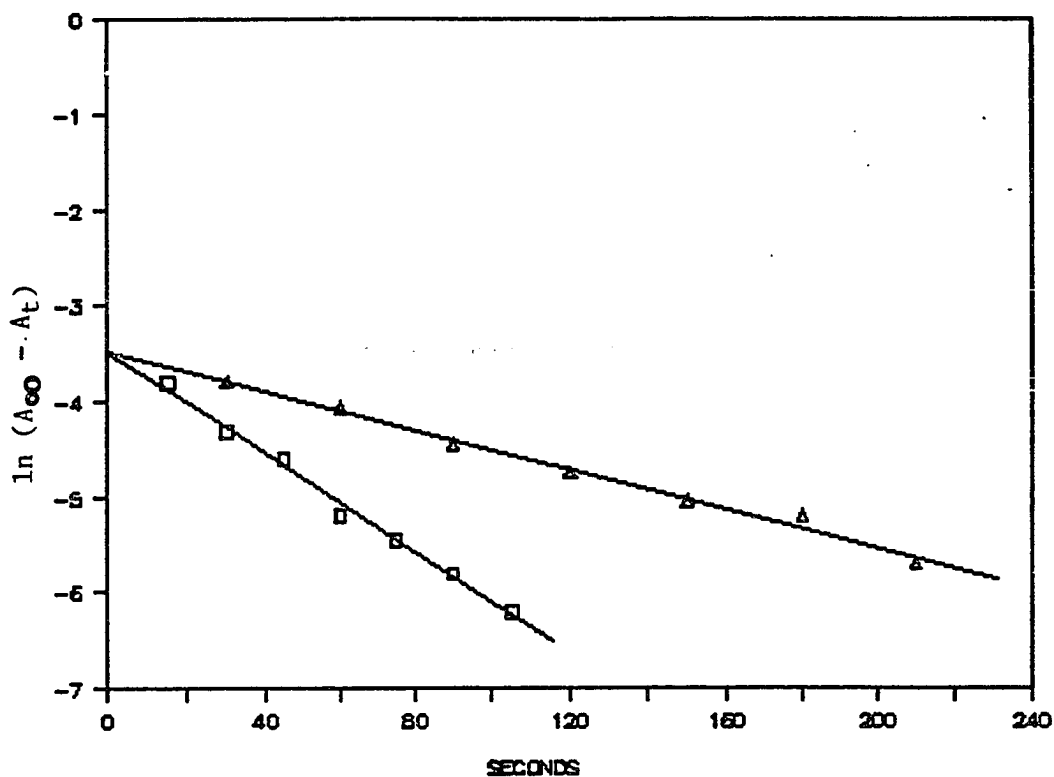


Figure 11. Inhibition of papain by 1-(Z-D-Phe-L-Prolineamido)-1-dihydroxyborono-4-methoxybutane. The plot shows the pseudo-first-order hydrolysis of 5.2 μM N-CBZ-L-Lys-p-nitrophenyl ester in pH 5.8 acetate buffer I = 0.1 M at 25°C. [inhibitor] = 16.8 μM [papain] = 3 nM

DISCUSSION AND CONCLUSIONS

The mechanism of the association of boronic acids with serine proteases has been extensively studied (43-48). The currently accepted model, which is consistent with crystallographic data (48) suggests that the boronic acid initially binds noncovalently as the trigonal acid. This is followed by esterification to the active site serine.

The X-ray study, which was done on Subtilisin BPN' (Novo) at a resolution of 2.5 Å, showed that with both phenylboronic acid and 2-phenylethaneboronic acid the boron atom forms a covalent tetrahedral adduct with the oxygen of the catalytic site Ser-221. One hydroxyl group of the boronic acid is at a distance of 2.8 Å from the N of the catalytic histidine residue. This is the expected distance for a hydrogen bond between the two. The second inhibitor hydroxyl group is situated in the oxyanion hole and forms hydrogen bonds to the side chain amide of Asn-155 and to the backbone amido group of Ser-221. The lengths of these hydrogen bonds are 2.5 and 3.0 Å.

The tetrahedral complex which is formed by the inhibitor is analogous in structure to the tetrahedral intermediate which is formed about the carbonyl carbon of susceptible peptide bonds (49). The negatively charged carbonyl oxygen atom is stabilized by two hydrogen bonds from donors on the enzyme. Simultaneous positioning of the leaving group to accept a proton from His-64 occurs.

These models are represented in Figure 12:

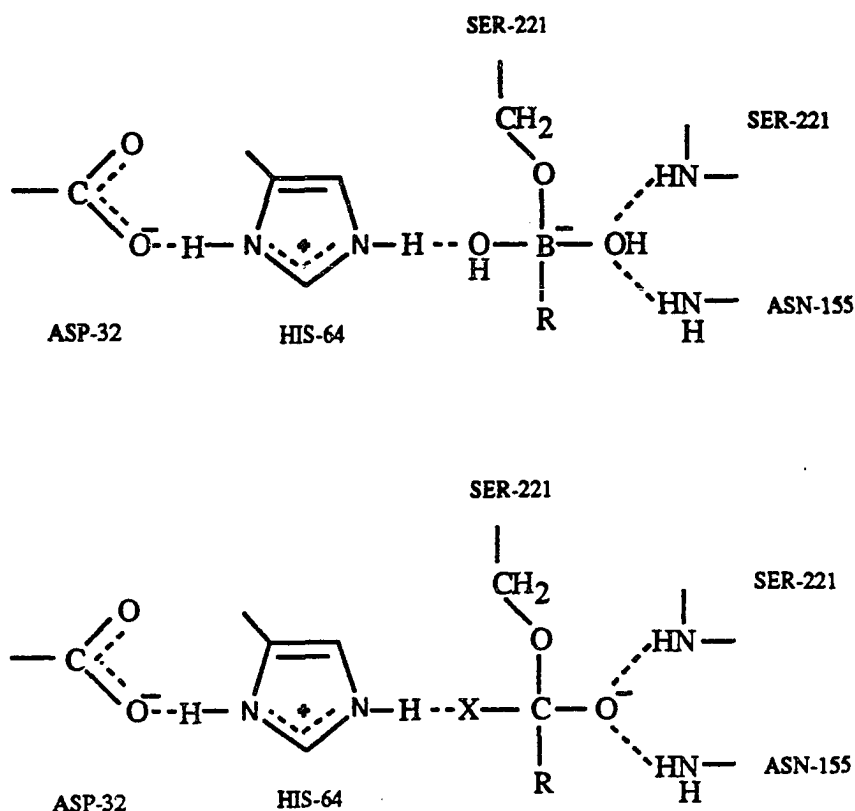


Figure 12

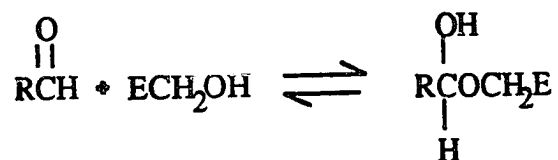
In contrast, results from ^{15}N NMR spectroscopic studies indicate that some boronic acid inhibitors may exhibit formation of an adduct between the active site histidyl nitrogen atom and the boron atom of the inhibitor (50). A recent crystallographic study on the inhibition of porcine pancreatic elastase by a peptide boronic acid shows a direct contact between the boron atom of the inhibitor and the ϵ -N of the active site histidine as well as covalent bonding to the γ -O of the active site serine (51).

In the inhibition of chymotrypsin and subtilisin BPN'

by borate ion, phenylboronic acid, and 2-phenylethylboronic acid, the bound inhibitors at pH 9.5 were found to shift the resonance position of the His-Asp hydrogen bonded proton indicating that negatively charged tetrahedral adducts of the boronic acids are formed at the active site (52). A later study found no evidence for a His-Asp hydrogen bond in native subtilisins. However it was present in thiolsubtilisin and in the phenylboronic acid derivatives of serine enzymes (53).

Evidence for a shift in the pK of the active site histidine when bound to an anionic inhibitor was found in the inactivation of trypsin, chymotrypsin and subtilisin by diisopropylfluorophosphate (DIFP). While the pK in the diisopropyl phosphoryl (DIP) derivative was near 7.4, the apparent pK in the monoisopropyl phosphoryl (MIP) derivative was between 9.7 and 11.4. Both values are for the ionization of the active site imidazolium ion. The elevation in the pK of the imidazolium in the presence of MIP was thought to be due to the interaction of the imidazolium with the monoanionic phosphodiester (54).

Inhibition of serine and cysteine proteases by aldehydes is well known (55-58). A hemiacetal or hemimercaptal is formed with the active site serine or cysteine residue:



This structure is supported by NMR data (59).

Although of similar structure to the boronic acid-enzyme complex, the hemiacetals are neutral. The negatively charged boronate complex better resembles the tetrahedral anionic transition state that is likely to be formed during substrate hydrolysis. The much poorer binding exhibited by the aldehydes compared to the boronic acids is probably due to this significant difference.

In this study, the inhibition of thrombin by a peptide boronic acid was investigated. The peptide boronic acid chosen for this purpose, 1-(2-D-Phe-L-Prolineamido)-1-dihydroxyborono-4-methoxybutane has the sequence specificity that is required by thrombin. Although this is different from the sequence at the point of cleavage on the natural substrate fibrinogen, previous studies have shown that this is an effective sequence for thrombin inhibitors and synthetic thrombin substrates (19,60).

The aldehyde inhibitors D-Phe-L-Pro-L-Arg-H and Bz-D-Phe-L-Pro-L-Arg-H had dissociation constants of 75 and 90 nM respectively, with fibrinogen as the substrate (19). Inhibition was noncompetitive.

The peptide boronic acid, which has a K_i (lim) of 7 nM, shows 10-fold better inhibition when compared to these analogous peptide aldehydes. The value of 7 nM is close to the dissociation constant for N-arylsulfonyl-Gly-4-amidino-Phe-Piperidine which is 6 nM (61).

Table V

Thrombin Dissociation Constants Towards Small-Molecule
Ligands

Ligand	K_{diss} nM	Reference
1-(Z-D-Phe-L-Prolineamido)- 1-dihydroxyborono-4- methoxybutane	7	this thesis
<u>b</u> -Nas-Gly-(<u>p</u> -Am) Phe -Piperidine	6	61
H-D-Phe-Pro-Arg-H	75	19
Bz-D-Phe-Pro-Arg-H	90	19
4-Amidinophenylpyruvate	1000	62
Z-D-Phe-Pro-Arg	1500	this thesis
Boc-Val-Pro-Arg-7AMC	3500	this thesis
H-D-Phe-Pip-Arg-pNA	6000	63
H-D-Phe-Pro-Arg-AIE	54,000	64
4-Aminobenzamidine	80,000	65

Inhibition by 1-(Z-D-Phe-L-Prolineamido)-1-dihydroxyborono-4-methoxybutane is competitive, which indicates that binding is at the active site. It is seen that the K_m value of N-t-Boc-L-Val-L-Pro-L-Arg-7AMC is

essentially pH-independent within the range that was studied (Table I). The pK of 7.05 in the pH profile of inhibition is presumably due to the ionization of the active site histidine group (His-92 in human alpha-thrombin).

Like other serine proteases, thrombin possesses a catalytic triad consisting of aspartate, histidine and serine residues (66). In the suggested mechanism for boronic acid inhibition of serine proteases, a deprotonated imidazole is required for inhibitor binding (Figure 12). This is consistent with the observed pH-dependent binding of 1-(Z-D-Phe-L-Prolineamido)-1-dihydroxyborono-4-methoxybutane, where binding is better as the pH is increased, with maximal binding occurring at around pH 7.75.

The second pK of 8.75 seen in Figure 2 is attributable to the ionization of the boronic acid group (this thesis, 44). Inhibition of chymotrypsin by a specific aldehyde showed that inhibition was essentially pH independent. In that study, binding of N-benzoyl-L-phenylalaninal to alpha-chymotrypsin was observed and it was found that in the pH dependency of K_i , there was only a 4-fold increase between pH 8.0 and pH 3.0 (67).

Based on these data, a possible mechanism for the peptide boronic acid inhibition of thrombin can be proposed:

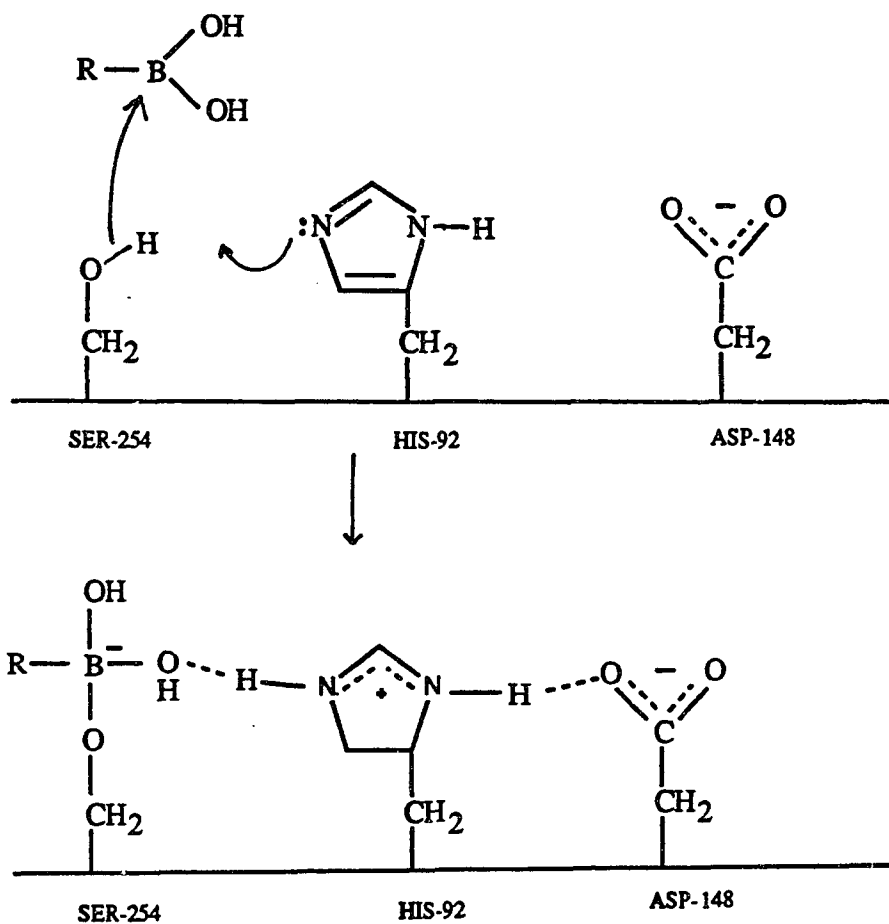


Figure 13

The preference for an arginine at the P₁ site by thrombin is met with the inhibitor Z-D-Phe-L-Pro-L-Arginine which inhibits thrombin competitively with maximal binding occurring at acid pH (Figure 6). This is an expected result since the negatively charged carboxylate group on the inhibitor will be stabilized by hydrogen bonding to the acid form of the enzyme.

Sigmoidal pH profiles of inhibition were obtained in the inhibition of chymotrypsin by tripeptide carboxylic acids, where binding was better at lower pH values. The pK reflected in the pH profiles of inhibition was that of the active site histidine (68). Acetylamino acids bind with the carboxyl group near to His-57 and Ser-195 of the catalytic site of chymotrypsin (69).

Binding of N-acetyl-D-tryptophanamide to chymotrypsin was independent of pH over the pH range 2.28 to 9.65, while binding of N-acetyl-D-Tryptophan was pH-dependent with better binding in the acidic range (70). The pH-dependency was interpreted in terms of an ionizing group on the enzyme having an apparent pK of 7.3. The negative charge of the active site Asp-102 would repel the carboxyl group of the acetylamino acid. This negative charge is neutralized by the protonated His-57. Therefore inhibitor binding is governed by the pK of the active site His-57.

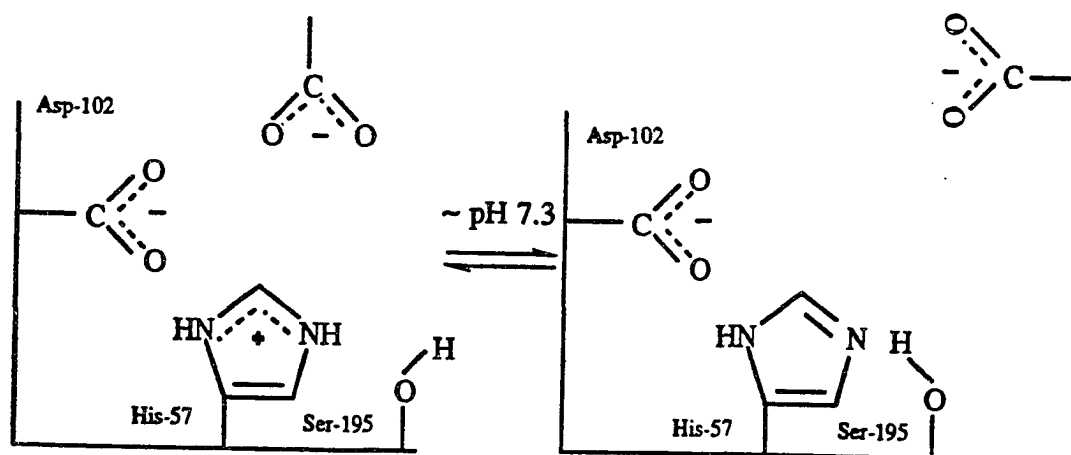


Figure 14

The pK of 8.0 which is reflected in the pH profile of inhibition of thrombin by the peptide carboxylate (Figure 5) is probably due to the ionization of the active site histidine. The pK differs from that seen in the inhibition by the peptide boronic acid. Such a perturbation in the pK at the active site is not uncommon especially with an inhibitor such as this, which has a charged residue. It is possible that the charged arginine residue on the inhibitor affects the ionization of the histidine so that a deviation is observed in the pK from that of the free enzyme.

For chymotrypsin (68, 69, 70) the pK for binding of peptide carboxylates is identical to pK_1 of k_{cat}/K_m . This is not the case for thrombin, and it is not clear why thrombin behaves differently.

Inhibition of Papain by 1-(Z-D-Phe-L-Prolineamido)-1-dihydroxyborono-4-methoxybutane

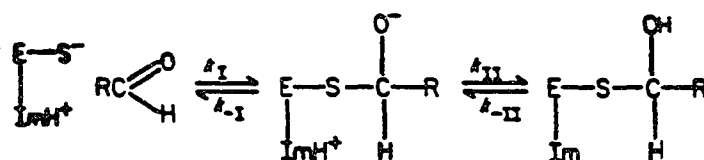
The peptide boronic acid was an effective inhibitor of papain. This is despite the fact that papain is specific towards substrates with L-amino acid residues (71). Papain is known to have a preference for phenylalanine in the second or further residue from the C-terminal end of a substrate (71). Cleavage occurs effectively with lysine or arginine at the P_1 site.

Analogous aldehydes probably inhibit papain by the formation of thiohemiacetals. Bz-glycinal binds over 10^5 -

fold more tightly than the analogous alcohol which is Bz-glycinol (K_i for Bz-glycinal = 0.025 mM; K_i for Bz-glycinol > 1000 mM). This is indicative of thiohemiacetal formation in inhibition. The analogous free carboxylate and amide are not effective inhibitors compared to the aldehyde (33).

In the pH profile of inhibition of papain by N-acetyl-L-Phenylalanylglycinal, the observed pK_1 was 4.3 and pK_2 was 8.6 (34). pK_2 was assigned to the ionization of the Cys-25 sulfhydryl group perturbed by its proximity to His-159. In that study, the rate of dissociation of the covalent thiohemiacetal adduct was found to be independent of pH between pH 3.5 and 9.0. The histidine in the complex appeared to be fully unprotonated in this pH range. It was proposed that the decomposition of the neutral thiohemiacetal would be facilitated by His-159 acting as a base (34).

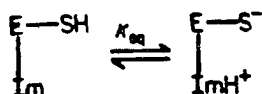
Thiohemiacetal formation in papain is believed to proceed in the following manner (34):



The pH profile of inhibition of papain by 1-(Z-D-Phenylalanyl-L-Prolineamido)-1-dihydroxyborono-4-methoxybutane shows a dependence on two ionizable groups of pK 4.38 and 7.42. The former has, in previous studies, been

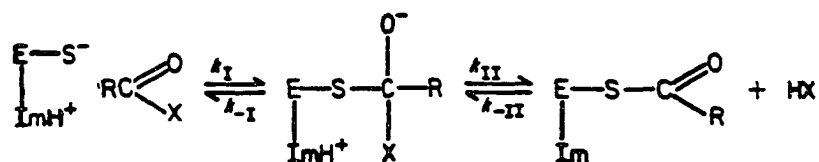
assigned to an essential carboxylate group (Asp-158) which must be ionized for maximal activity (36,72). However, a recent study (73) has shown that the active site His-159 has an abnormally low pK of about 4 due to its proximity to the active site Cys-25. The pK of His-159 can be expected to be influenced by the chemical state of Cys-25. The pK of the thiol group changes from 3.3 to 7.6 on deprotonation of His-159. Similarly, the pK of His-159 shifts from 4.3 to 8.5 when Cys-25 is deprotonated.

The cysteine-histidine catalytic pair may exist as a mixture of two tautomeric forms (73):

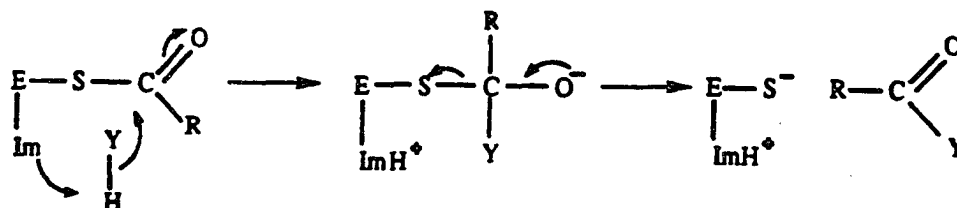


Since the ion-pair form is predominantly present between pH 5 and 8, it has been suggested that this is the catalytically active form of papain (74). Since the proton that originated on Cys-25 is already located on His-159, general base catalysis is not thought to occur in the acylation of papain. This is supported by the observed lack of a significant solvent deuterium isotope effect on k_2/K_S for substrate hydrolysis (75).

The following scheme was proposed for the acylation of papain by substrates (76):



Deacylation would follow:



With different substrates, pK_2 in k_{cat}/K_m has been observed to vary between 8.0 and 8.5. This was true for substrates where acylation was rate determining ($k_2 < k_3$), such as the amides and for substrates where deacylation was rate determining ($k_3 < k_2$), such as the esters (36,72,77). This has been generally assigned to the ionization of the Cys-25 sulfhydryl group.

For the substrates benzoyl-L-Citrulline methyl ester (78), benzoyl-L-Arginine ethyl ester (79), and benzoyl-L-Glycine ethyl ester (80) K_m is essentially independent of pH. K_m for the substrates N-acetyl-DL-Tryptophan-p-nitrophenyl ester (81) and CBZ-L-Glycine-p-nitrophenyl ester (82) are independent of pH from pH 4.0 to 8.0. For the amide substrate N-benzoyl-L-arginineamide where k_2 (acylation) is rate determining, K_m was independent of pH above pH 4.5.

In the relationship $K_m = k_3/(k_2 + k_3) * K_S$, (page 67), when acylation is rate-limiting $K_m = K_S$. Hence, when K_m is pH-independent K_S is likely to be pH-independent.

k_3 (deacylation) was dependent on a single ionizable group of pK 3.9 with activity on the alkaline side of the pK (79).

For the substrate used in this study, CBZ-L-Lysine-p-nitrophenyl ester, $k_2 \gg 20 * k_3$ (81). Therefore the general relationship $K_m = k_2/(k_2 + k_3) * K_S$ becomes $K_m = (k_3/k_2) * K_S$. For this substrate also K_S is believed to be pH-independent above pH 5 (81).

In the pH profile of inhibition of papain by 1-(Z-D-Phenylalanyl-L-Prolineamido)-1-dihydroxyborono-4-methoxybutane with the substrates CBZ-L-Lysine-p-nitrophenyl ester and CBZ-L-Lysine-thiobenzyl ester, pK_1 is 4.38. This is likely to be the same group which gave a pK_1 of 4.30 in substrate hydrolysis and which gave a pK_1 of 4.3 in the inhibition pH profile of a peptide aldehyde (34). This can be assigned to the active site Cys-25 - His-159 ion-pair.

The pK_2 of 7.42 reflected in the inhibition pH profile of the peptide boronic acid could be assigned to the ionization of a histidine. In the alkylation of papain by L(-)-alpha-iodopropionic acid, the pH profile of inhibition shows that inhibition is dependent on an unprotonated group having a pK of 4.0 and a protonated group with a pK of 7.8 (83). The assignment of the latter

pK was to an essential histidine group.

pK₂ of the pH profile of inhibition of thrombin by the peptide boronic acid is attributed to the ionization of the boronic acid since this is the expected pK of the boronic acid moiety as evidenced by this and other studies (Part I of this thesis, 44).

However, in the case of papain, it is not possible to assign unambiguously the pK₂ that is reflected in the pH profile of inhibition by the peptide boronic acid to a group on the enzyme. (This is clearly not due to the ionization of the peptide boronic acid. The peptide boronic acid is expected to have a pK of 8.75 based on the results obtained in the thrombin inhibition study.)

The noncompetitive nature of the inhibition of papain by the peptide boronic acid compounds the difficulty in assigning this pK to a group on the enzyme. However, on this issue, several studies have shown that for some inhibitors, although inhibition is noncompetitive, binding is at the active site.

In the case of subtilisin Novo, Bz-Arginine noncompetitively inhibits the hydrolysis of Ac-Ala-Ala-Ala-OMe and Ac-Ala-Ala-Phe-OMe even though the X-ray structure shows that binding is at the active site (84). Peptide ester hydrolysis by subtilisin Carlsberg is inhibited competitively by Bz-Arginine (85). This has been explained in terms of two different productive substrate binding sites. On subtilisin Novo, Bz-Arginine occupies only the B

site, whereas in subtilisin Carlsberg it is presumed to occupy the A site, with the benzoyl group in the S_1 crevice (nomenclature and reference 86).

The inhibition of subtilisin Novo and subtilisin Carlsberg by Z-Ala-Ala-Phe and Z-Ala-Ala-Tyr was competitive with the substrates Ac-Ala-Ala-Ala-OMe and Ac-Tyr-OEt. The same peptides inhibited N-trans-cinnamoylimidazole hydrolysis by the subtilisins noncompetitively while mixed inhibition was seen with the inhibitors Z-Ala-Ala-Cys(Bzl) and Z-Ala-Ala-Cys(Dpm) (68). This indicates that there is considerable variation in the mode of inhibition depending on the substrate that is used. Therefore it is possible that in papain, although inhibition by the peptide boronic acid is noncompetitive, that binding is at the active site, especially since pK_1 of inhibition seems to reflect the ionization of the active site Cys-25 - His-159 ion-pair.

It is a significant result that papain, a cysteine protease, is potently inhibited by the peptide boronic acid with possible complexation at the active site. In a previous study papain was not inhibited by phenylboronic acid, n-butylboronic acid and m-nitrophenylboronic acid (41). It is also significant that thiol RTEM beta-lactamase is not inhibited by boric acid (40). Phenylboronic acid inhibits thiolsubtilisin with a $K_i > 0.1$ M which shows 10^3 -fold poorer binding compared to inhibition of subtilisin where K_i is 2.29×10^{-4} M (41).

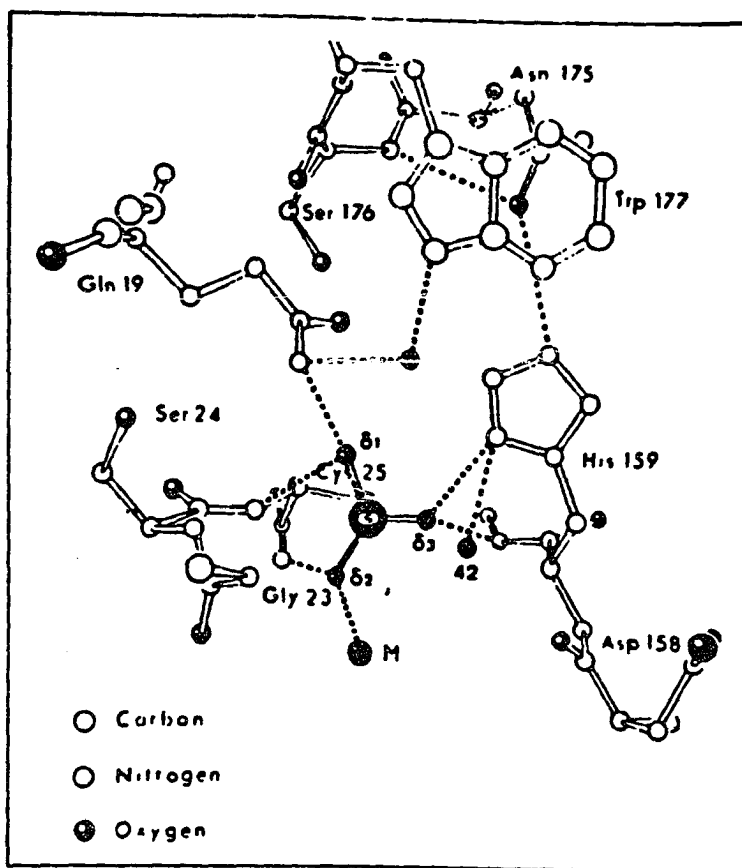


Figure 15. Schematic picture of the papain active site (from I. G. Kamphuis, K. H. Kalk, M. B. Swarte and J. Drenth, (1984) *J. Mol. Biol.* 179, 233)

Hydrogen bonds are shown as broken lines. M is a methanol and 42 a water molecule. A second water molecule is between Gln-19 and Trp-177.

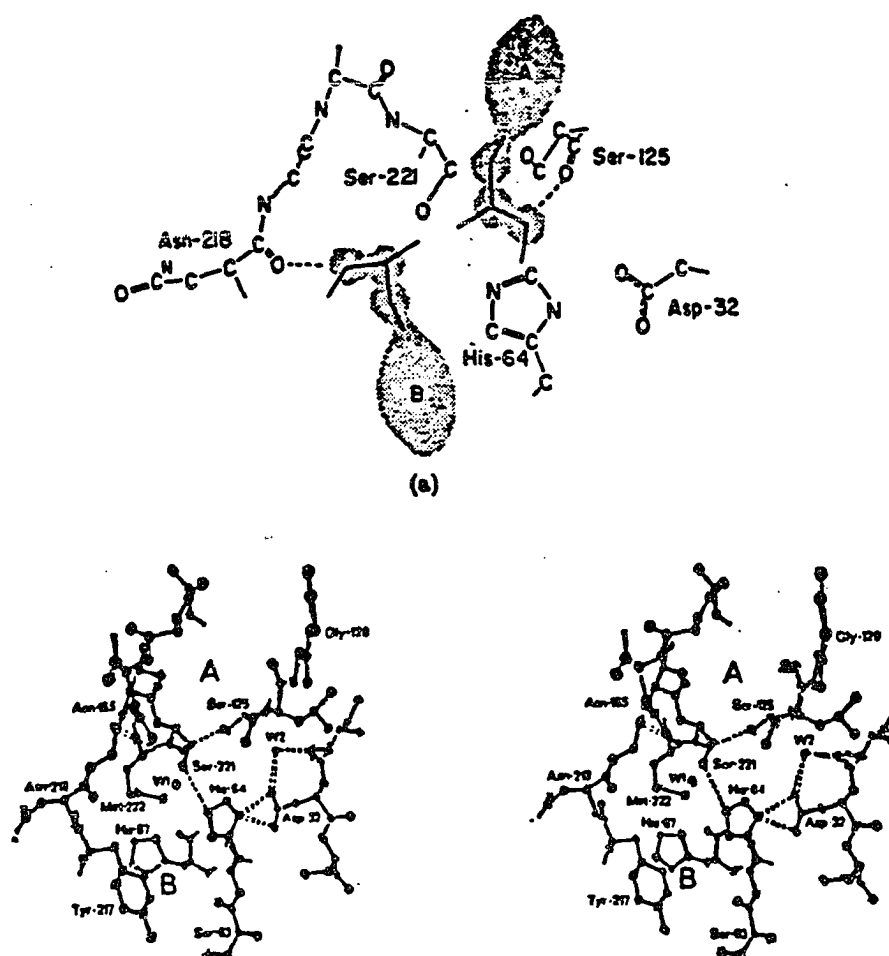


Figure 16. The geometrical relationship between the catalytic residues of subtilisin and the substrate binding sites A and B is presented schematically in (a) and in a detailed stereoscopic drawing in (b). W1 and W2 are water molecules. Dashed lines are hydrogen bonds (from C. S. Wright (1972) J. Mol. Biol. 67, 151).

It is likely that peptide boronic acids which better resemble the specificity requirement of papain will be much better inhibitors. These peptide boronic acids would ideally have a boro-Arg or a boro-Lys at the P_1 site and a phenylalanine at the P_2 site.

Some of the overall conclusions from this study can be summarized as follows:

Thrombin is competitively inhibited by 1-(Z-D-Phe-L-Prolineamido)-1-dihydroxyborono-4-methoxybutane with the substrate N-t-Boc-L-Val-L-Pro-L-Arg-7AMC. The ionization of the active site histidine and that of the boronic acid group are implicated in the mechanism of inhibition, as deduced from the pH-dependence of inhibition. This indicates either the possible complexation of the boronic acid group with the active site histidine or covalent modification of the active site serine by the boronic acid. The K_i (Lim) of 7 nM for thrombin inhibition makes this one of the most potent synthetic inhibitors of this enzyme.

Thrombin is competitively inhibited by Z-D-Phe-L-Pro-L-Arginine with the substrate D-Phe-L-Pip-L-Arg-pNA. The pH profile of inhibition with the substrate N-t-Boc-L-Val-L-Pro-L-Arg-7AMC is sigmoidal with better binding in the acid region. The pK of 8.0 reflected in the pH profile of inhibition was attributed to the active site histidine perturbed by its proximity to the charged inhibitor.

When compared with the inhibition by 1-(Z-D-Phe-L-Prolineamido)-1-dihydroxyborono-4-methoxybutane which has identical residues in the P_3 and P_2 positions and differs only in the residue at the P_1 position, inhibition is poorer by about 2 orders of magnitude ($K_i(\text{Lim}) = 1.5 \mu\text{M}$). The charged side-chain of P_1 -Arginine provides additional binding energy in inhibition (by interacting with Asp-248). This is absent in the peptide boronic acid where this position is occupied by 4-methoxybutaneboronic acid.

The better binding by the peptide boronic acid indicates the effectiveness of the boronic acid group in the inhibition of thrombin.

In contrast to thrombin, papain is noncompetitively inhibited by the same peptide boronic acid. The ionization of the active site Cys-25 - His-159 ion-pair is implicated in the mechanism of inhibition reflected as pK_1 in the pH profile of inhibition. It is not possible to assign unambiguously the pK_2 reflected in the pH profile of inhibition. The peptide boronic acid inhibition of papain is poorer by about 3 orders of magnitude ($K_i(\text{Lim}) = 12.5 \mu\text{M}$) compared to thrombin inhibition.

Future studies using specific peptide boronic acids as inhibitors of serine and cysteine proteases may lead to the discovery of compounds which are of importance in therapeutic and enzyme mechanistic studies.

APPENDIX 1

The following equation was used to construct single ionization pH profiles on Lotus-123 (The spreadsheet is also attached)

$$k_{\text{obs}} = \frac{k_{\text{Lim}}}{1 + \frac{K_2}{K_1} \frac{1}{[\text{H}^+]}} \quad \text{----- (1)}$$

$$\frac{1}{k_{\text{obs}}} = \frac{1 + \frac{K_2}{K_1} \frac{1}{[\text{H}^+]}}{k_{\text{Lim}}}$$

$$\text{Since } \text{pH}_{\text{max}} = 1/2(\text{p}K_1 + \text{p}K_2)$$

$$[\text{H}^+]_{\text{max}}^2 = K_1 * K_2$$

Substituting for K_2 in equation (1)

$$\frac{1}{k_{\text{obs}}} = \frac{1}{k_{\text{Lim}}} + \frac{[\text{H}^+] + \frac{[\text{H}^+]_{\text{max}}^2}{[\text{H}^+]}}{k_{\text{Lim}} * K_1}$$

This allows for a linear Lineweaver-Burk type plot to be constructed. When $\frac{1}{(k_{\text{obs}} * [\text{H}^+] + [\text{H}^+]_{\text{max}}^2)}$ is on the y axis and $\frac{1}{([\text{H}^+] + [\text{H}^+]_{\text{max}}^2)}$ is on the x axis

the slope = $1/k_{\text{Lim}}$; the intercept = $1/(k_{\text{Lim}} * K_1)$

This substitution and the resulting linear plot was first made use of by Dr. H. C. Keifer.

For enzyme inhibition,

$$K_i = K_i(\text{Lim}) + \frac{[\text{H}^+] + \frac{[\text{H}^+]_{\text{max}}^2}{[\text{H}^+]}}{K_1/K_i(\text{Lim})} \quad \text{-- (2)}$$

	A	B	C	D
	1/(Hx + V) vs. 1/Hx Plot for 2 pK's		pH Input	Vel. Input
1				
2				
3	1. Enter your Data in Columns C & D.		4.940	6.5300E+02
4	Copy the data to F & G.		5.300	1.5150E+03
5	2. Set the pH-optimum in B6.		5.670	1.7630E+03
6	pH-Optimum =	6.800	6.250	2.1450E+03
7			6.850	2.2480E+03
8	3. Run a Regression on Columns G & H		6.550	2.5130E+03
9	G = Y Axis, H = X Axis		7.150	2.5820E+03
10	4. Make sure that the theoretical pH		7.870	2.1180E+03
11	profile exists in Columns E and F		8.100	1.4320E+03
12	And goes to	+B36	8.600	6.8800E+02
13			9.000	2.5200E+02
14	5. Graph a Linear X-Y Plot Using:		9.150	3.5300E+02
15	A VARIABLE = COLUMN G3..G25		9.200	1.2400E+02
16	X-VARIABLE = COLUMN F3...F		9.500	6.8900E+01
17	B-VARIABLE = COLUMN E3...E			
18	To E AND F	+B36		
19	(Or Use Named Graph)			
20	6. READ NUMERICAL DATA BELOW			
21	pK1 =	(-@LN(B22))/@B\$53		
22	K1 =	+@C\$49/@D\$43		
23				
24	pK2 =	(2*@B\$6)-@B\$21		
25	K2 =	10^(-B24)		
26				
27	Klim =	1/@C\$49		
28				
29	Delta pH =	0.050		
30				
31	Theoretical pH Values			
32	Start at pH =	@MIN(F3..F25)-0.05		
33	Stop at pH =	@MAX(F3..F25)+0.05		
34				
35	Set Theoretical Points From			
36	E and F 26 to	(B33-B32)/B29+26		
37	Copy From E & F 27			
38				
39	Caution: Do Not Erase			
40	E 26-27 and F 26-27			
41				
42	Regression Output:			
43	Constant			1.0954E+02
44	Std Err of Y Est			6.0144E+01
45	R Squared			9.8159E-01
46	No. of Observations			1.4000E+01
47	Degrees of Freedom			1.2000E+01
48				
49	X Coefficient(s)		3.7657E-04	
50	Std Err of Coef.		1.4889E-05	
51				
52				
53	Ln 10 =	2.3025850930		
54				
55				
56				
57				
58				
59				
60				

Y_Parameters for Line on
Lineweaver-Burk type plot are at S26, S27

X_Parameters for Line on
Lineweaver-Burk type plot are at I26, I27

	E Theor. Vel.	F pH	G Observed Rate	H 1/(V * Hx)	I 1 / Hx
1					
2					
3		4.940	6.5300E+02	1/(G3*J3)	1/J3
4		5.300	1.5150E+03	1/(G4*J4)	1/J4
5		5.670	1.7630E+03	2.6386E+02	4.6518E+05
6		6.250	2.1450E+03	7.6803E+02	1.6474E+06
7		6.850	2.2480E+03	1.3941E+03	3.1340E+06
8		6.550	2.5130E+03	1.0727E+03	2.6957E+06
9		7.150	2.5820E+03	9.0998E+02	2.3496E+06
10		7.870	2.1180E+03	2.5173E+02	5.3317E+05
11		8.100	1.4320E+03	2.2028E+02	3.1544E+05
12		8.600	6.8800E+02	1.4531E+02	9.9975E+04
13		9.000	2.5200E+02	1.5797E+02	3.9809E+04
14		9.150	3.5300E+02	7.9839E+01	2.8183E+04
15		9.200	1.2400E+02	2.0257E+02	2.5118E+04
16		9.500	6.8900E+01	1.8272E+02	1.2589E+04
17					1.0000E+00
18					1.0000E+00
19					1.0000E+00
20					1.0000E+00
21					1.0000E+00
22					1.0000E+00
23					1.0000E+00
24					1.0000E+00
25					1.0000E+00
26					0.0000E+00
27					0.0000E+00
28		6.6766E+02	+F27+\$B\$29		(@MAX(\$I\$3..\$I\$25))
29		7.2679E+02	+F28+\$B\$29	Ymin =	@MIN(G3..G25)
30		7.8908E+02			
31		8.5433E+02			
32		9.2229E+02			
33		9.9266E+02			
34		1.0651E+03			
35		1.1391E+03			
36		1.2143E+03			
37		1.2902E+03			
38		1.3663E+03			
39		1.4421E+03			
40		1.5170E+03			
41		1.5905E+03			
42		1.6623E+03			
43		1.7319E+03			
44		1.7988E+03			
45		1.8629E+03			
46		1.9237E+03			
47		1.9812E+03			
48		2.0352E+03			
49		2.0856E+03			
50		2.1322E+03			
51		2.1752E+03			
52		2.2146E+03			
53		2.2503E+03			
54		2.2825E+03			
55		2.3113E+03			
56		2.3367E+03			
57		2.3589E+03			
58		2.3780E+03			
59		2.3941E+03			
60		2.4071E+03			

	J	K	L	M	S
1	[H]+[Hm]^2/[H]	Log Vtheor	Log Vobs		
2	-----				
3	(10^(-F3))+(((10^(-B\$6))^2)/(10^(-F3)))				
4	-(10^(-F4))+(((10^(-B\$6))^2)/(10^(-F4)))				
5	2.1497E-06		(@LN(G5))/B\$53		
6	6.0701E-07		(@LN(G6))/B\$53		
7	3.1908E-07		3.3518E+00		
8	3.7096E-07		3.4002E+00		
9	4.2561E-07		3.4120E+00		
10	1.8756E-06		3.3259E+00		
11	3.1702E-06		3.1559E+00		
12	1.0003E-05		2.8376E+00		
13	2.5120E-05		2.4014E+00		
14	3.5482E-05		2.5478E+00		
15	3.9811E-05		2.0934E+00		
16	7.9433E-05		1.8382E+00		
17	1.0000E+00				
18	1.0000E+00				
19	1.0000E+00				
20	1.0000E+00				
21	1.0000E+00				
22	1.0000E+00				
23	1.0000E+00				
24	1.0000E+00				
25	1.0000E+00				
26		2.7476E+00		+D43	
27		2.7866E+00		(C49*I27)+D43	
28		2.8246E+00			
29		2.8614E+00			
30		2.8971E+00			
31		2.9316E+00			
32		2.9649E+00			
33		2.9968E+00			
34		3.0374E+00			
35		3.0566E+00			
36		3.0843E+00			
37		3.1107E+00			
38		3.1356E+00			
39		3.1590E+00			
40		3.1810E+00			

APPENDIX 2

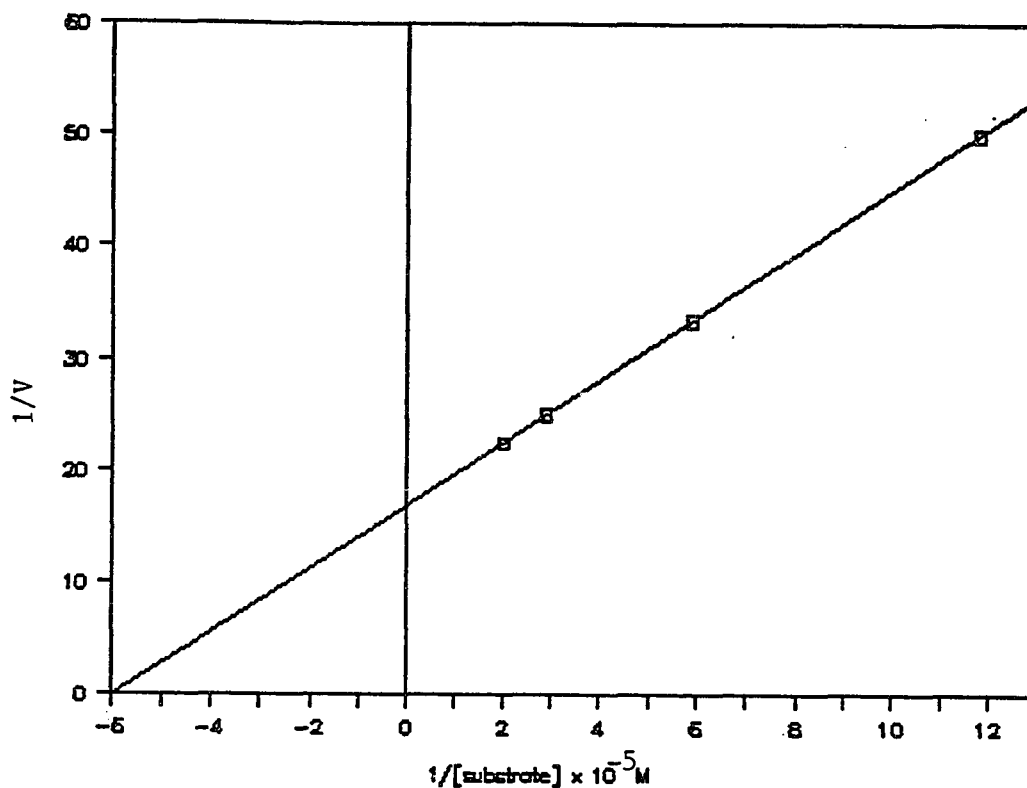


Figure A. Lineweaver-Burk plot for N-t-Boc-L-Val-L-Pro-L-Arg-7AMC hydrolysis by bovine serum thrombin at pH 5.1 and 25°C.

$K_m = 1.7 \mu\text{M}$

$[\text{thrombin}] = 7.9 \text{ nM}$

Conditions of the assay are given in the "Methods" section

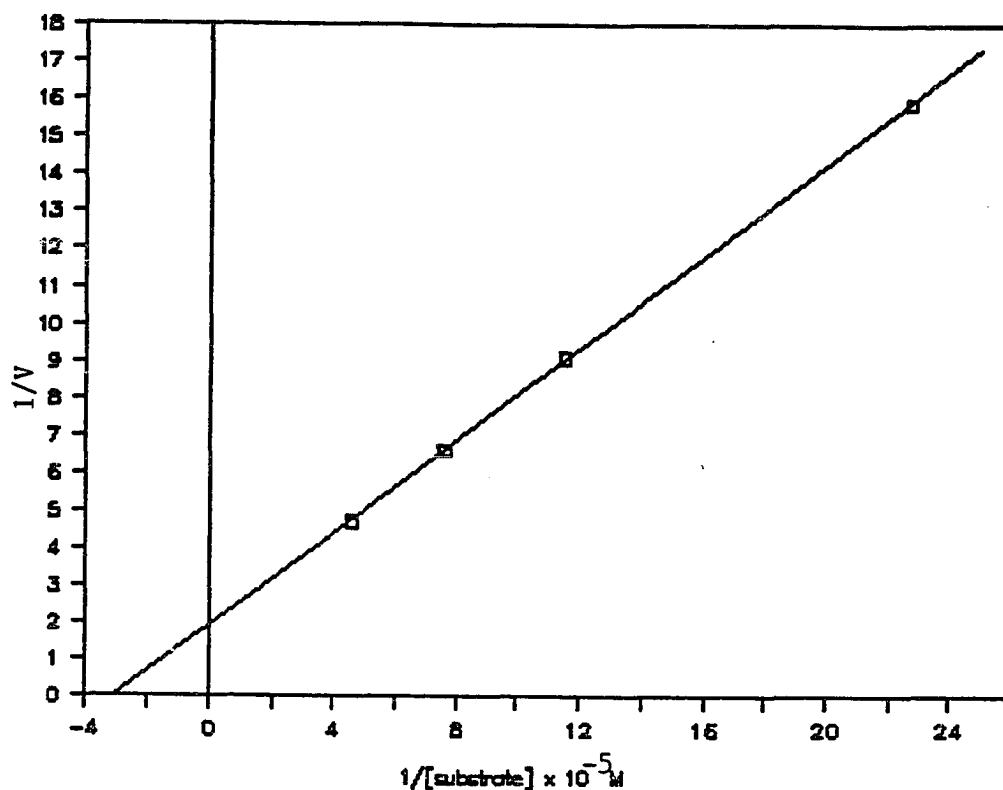


Figure B. Lineweaver-Burk plot of N-t-Boc-L-Val-L-Pro-L-Arg-7AMC hydrolysis by bovine serum thrombin at pH 6.1 and 25°C.
 $K_m = 3.3 \mu\text{M}$
 $[\text{thrombin}] = 2.2 \text{ nM}$
 Conditions of the assay are given in the "Methods" section

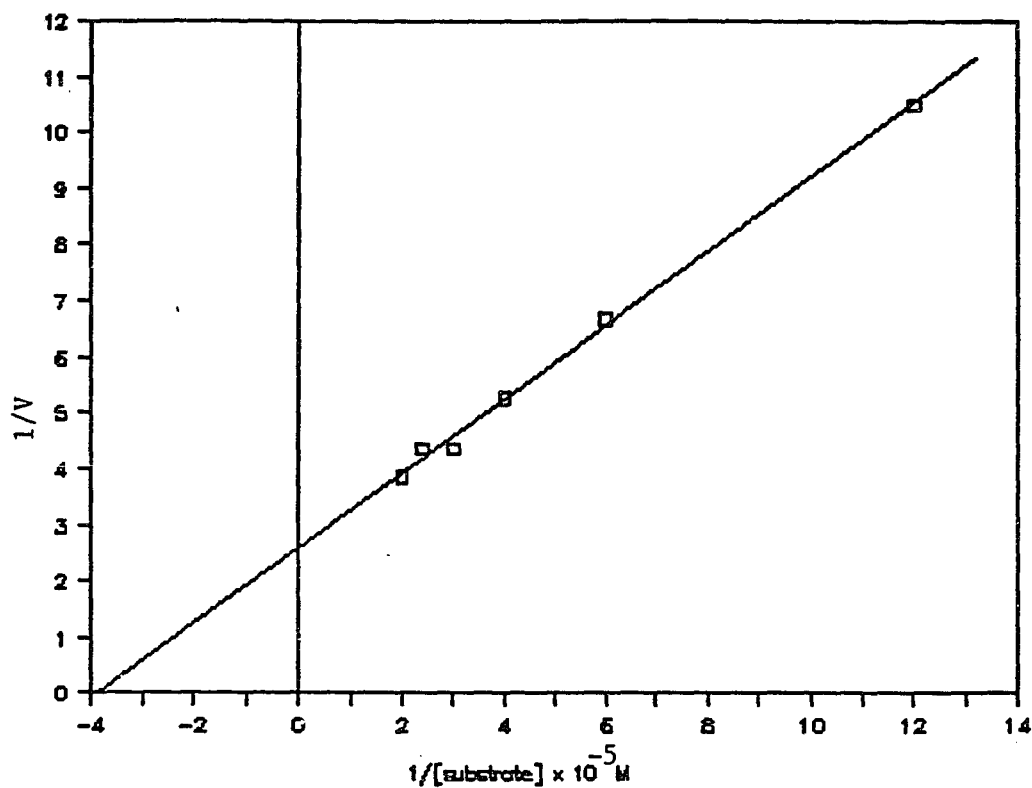


Figure C. Lineweaver-Burk plot of N-t-Boc-L-Val-L-Pro-L-Arg-7AMC hydrolysis by bovine serum thrombin at pH 7.05 and 25°C.

$$K_m = 2.5 \mu M$$

$$[thrombin] = 8.1 \times 10^{-10} M$$

Conditions of the assay are given in the "Methods" section

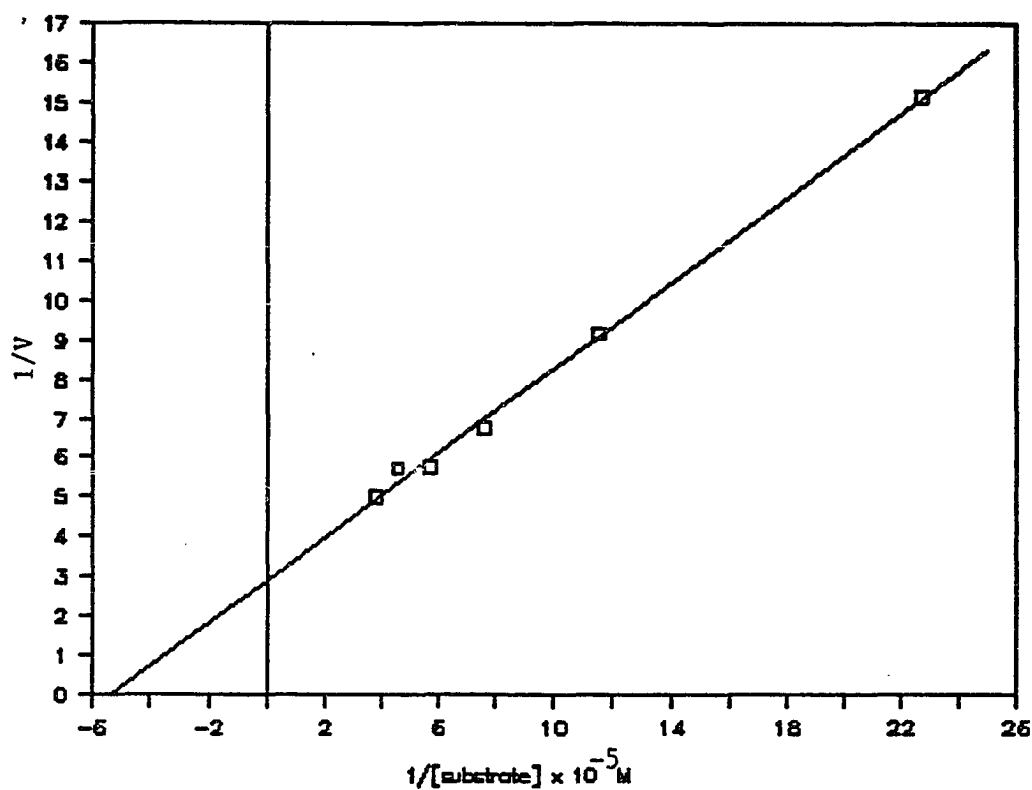


Figure D. Lineweaver-Burk plot of N-t-Boc-L-Val-L-Pro-L-Arg 7AMC hydrolysis by bovine serum thrombin at pH 8.95 and 25°C.

$$K_m = 1.9 \mu\text{M}$$

$$[\text{thrombin}] = 2.2 \times 10^{-10} \text{ M}$$

Conditions of the assay are given in the "Methods" section

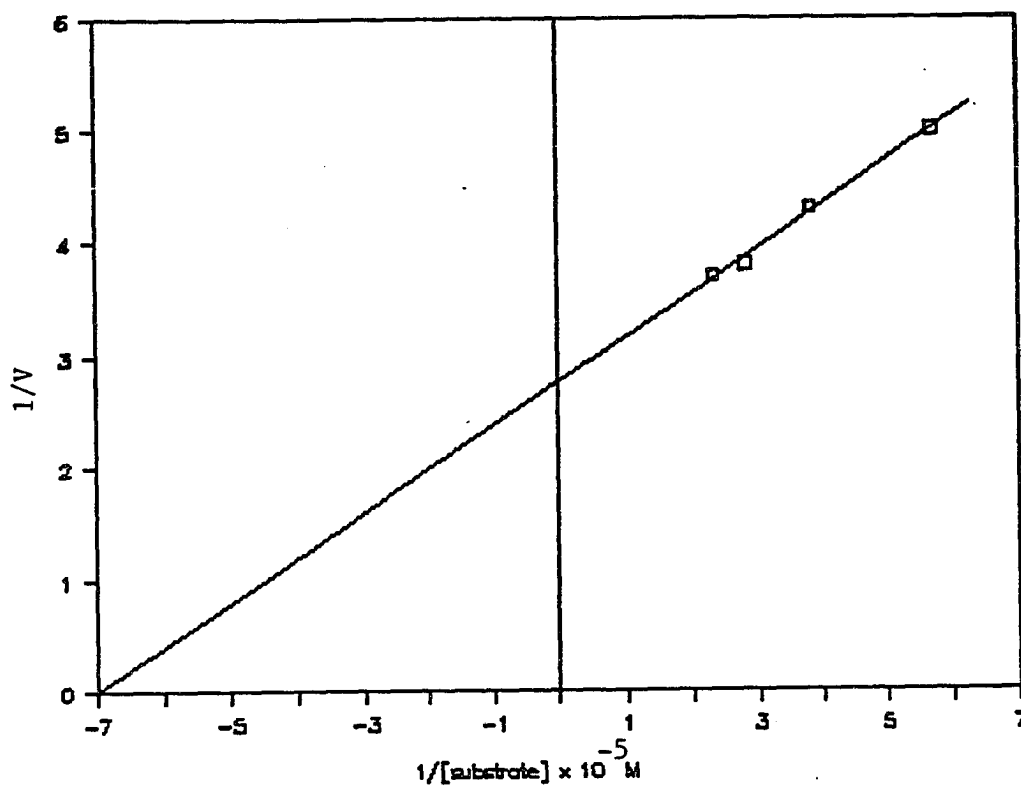


Figure E. Lineweaver-Burk plot of N-t-Boc-L-Val-L-Pro-L-Arg-7AMC hydrolysis by bovine serum thrombin at pH 9.2 and 25°C.

$$K_m = 1.3 \mu\text{M}$$

$$[\text{thrombin}] = 2.2 \times 10^{-10} \text{ M}$$

Conditions of the assay are given in the "Methods" section

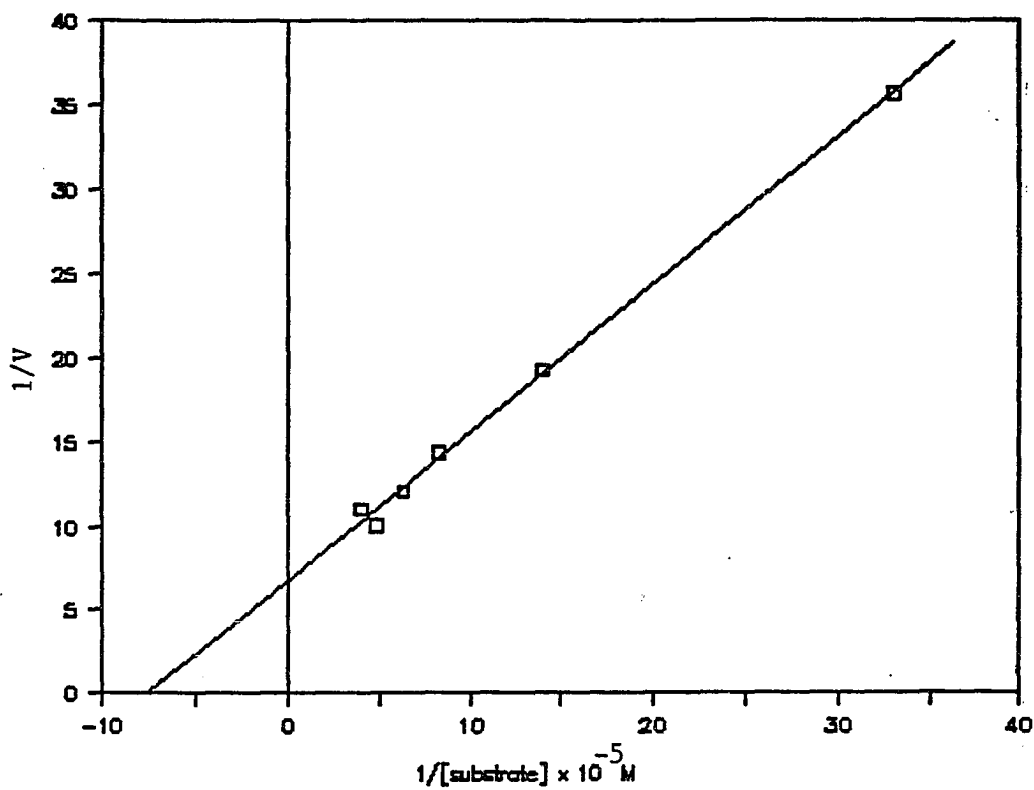


Figure F. Lineweaver-Burk plot of N-t-Boc-L-Val-L-Pro-L-Arg-7AMC hydrolysis by bovine serum thrombin at pH 9.55 and 25°C.

$$K_m = 1.25 \mu\text{M}$$

$$[\text{thrombin}] = 4.5 \times 10^{-10} \text{ M}$$

Conditions of the assay are given in the "Methods" section

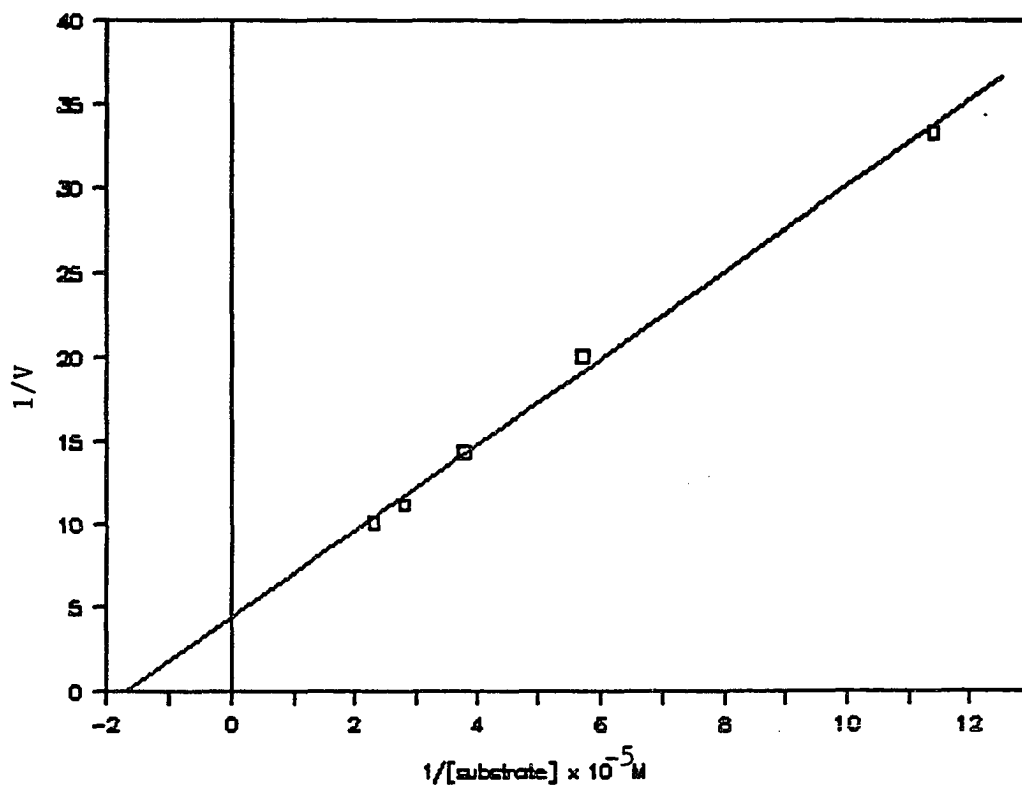


Figure G. Lineweaver-Burk plot of N-t-Boc-L-Val-L-Pro-L-Arg-7AMC hydrolysis by bovine serum thrombin at pH 10 and 25°C.

$$K_m = 6.2 \mu M$$

$$[thrombin] = 5.5 \times 10^{-10} M$$

Conditions of the assay are given in the "Methods" section

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