

Inhibition of Human α -Thrombin with Small Covalent Inhibitors and r-Hirudin

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Abstract.

Thrombin is a key serine protease involved in the blood cascade. Two small molecular inhibitors, 4-nitrophenyl diethylphosphate (paraoxon) and 4-nitrophenyl 2-propyl methylphosphonate (MPNP), covalently bond to the active-site Ser to form a mimic of the tetrahedral intermediate found in thrombin-catalyzed reactions. The pH dependence of their inhibition of human α -thrombin at $25.0 \pm 0.1^\circ\text{C}$ was determined. The $\text{pK}_{\text{a}1}$ and $\text{pK}_{\text{a}2}$ for paraoxon are 7.8 ± 0.1 and 9.3 ± 0.2 respectively, and for MPNP are 8.0 ± 0.1 and 8.6 ± 0.2 respectively. The pK_{a} s may be attributed to the role of His57 and Leu16 respectively in catalysis. The maximal second order rate constant, k_i/K_i , for the inhibition of human α -thrombin was determined to be $0.47 \pm 0.05 \text{ M}^{-1}\text{s}^{-1}$ with paraoxon and $6.2 \pm 0.1 \text{ M}^{-1}\text{s}^{-1}$ with MPNP.

Hirudin, found naturally in *Hirudo medicinalis*, is a slow and tight binding inhibitor of thrombin, interacting noncovalently over a large area. This study focused on the dependence of this interaction on the presence of Na^+ and Cl^- ions at an ionic strength (μ) of 0.3 M. Kinetic measurements were conducted spectrophotometrically in the presence of the chromophoric substrate, H-D-Phe-Pip-Arg-4-nitroanilide. The dissociation constant and second-order rate constant, when fitted, for the inhibition of human α -thrombin with r-hirudin at $\text{pH} \sim 8.1$ and $25.0 \pm 0.1^\circ\text{C}$ are: $(0.47 \pm 0.15) \text{ pM}$ and $(4.8 \pm 0.1) \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ in NaCl; $(2.8 \pm 0.1) \text{ pM}$ and $(3.49 \pm 0.02) \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ in Na acetate; $(13.8 \pm 0.2) \text{ pM}$ and $(8.2 \pm 0.4) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ in choline Cl; $(12 \pm 3) \text{ pM}$ in choline acetate. The solvent isotope effect, $K_{\text{H}}/K_{\text{D}}$, for K_i is 0.26 ± 0.15 in NaCl; 0.72 ± 0.02 in Na acetate; 2.1 ± 0.5 in choline acetate; and 2.59 ± 0.03 in choline chloride. This suggests that the association of hirudin with thrombin is accompanied with changes in hydrogen bonding as a function of Na^+ and Cl^- ions.

Introduction.

Physiological Role of Thrombin.

Thrombin is a serine protease in the trypsin family which cleaves scissile bonds adjacent to positively charged residues.¹ Its natural substrates contain an arginine in the S1 subsite, a proline in the S2 subsite, and a hydrophobic residue in the S3 subsite. This high specificity is utilized by thrombin, which plays a key physiological role in blood coagulation,² summarized below in Figure 1. A wide range of enzymes are involved in the cascade resulting in the cleaving of prothrombin to activated thrombin, which then plays a heavily coordinated dual role: the cleavage of fibrinogen to fibrin in clot formation, and in clot degradation. Thrombin also plays a role as an activator of further blood coagulation factors.³ As this physiological process has wide implications with respect to human health, thrombin and its inhibitors have been the subject of extended study.

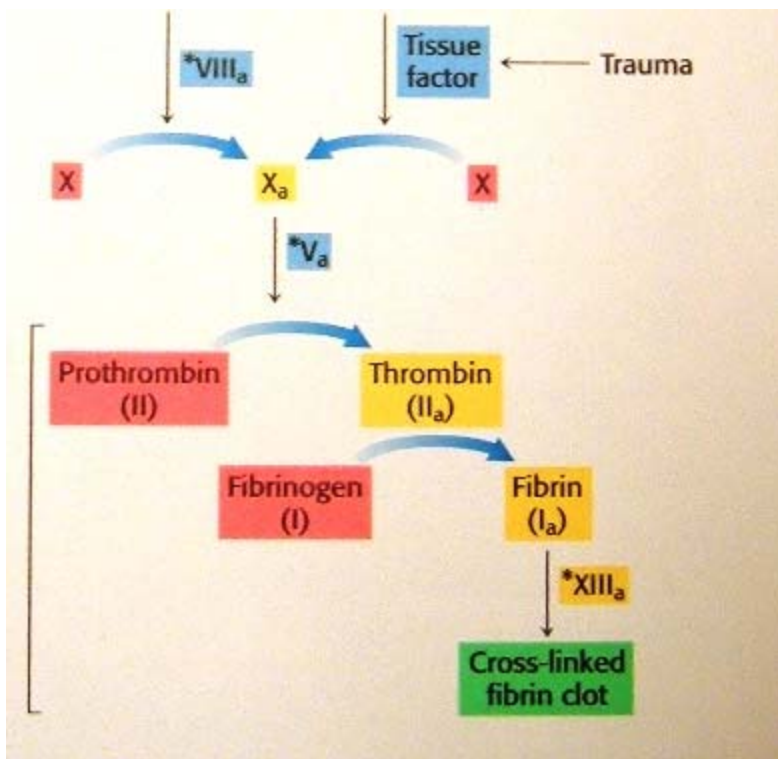


Figure 1: Thrombin in the blood clotting cascade.⁴

Structure of Thrombin.

Thrombin is a large, globular enzyme of roughly spherical structure, with dimensions of 45 x 45 x 50Å.¹ It consists of a thirty-six residue A chain and a 259-residue B chain linked by a disulfide bridge to form a single contiguous structure. The B chain also contains several internal disulfide bridges.² The longer B chain is responsible for catalytic activity and is homologous to the catalytic units on other trypsin-like serine proteases.⁵ Thrombin's active site is located in a narrow cleft between two insertion loops of the enzyme.⁶ Thrombin is prone to autolytic cleavage at Arg77, resulting in β -Thrombin. Similar breaks at other points give rise to other modified, less active forms of thrombin.²

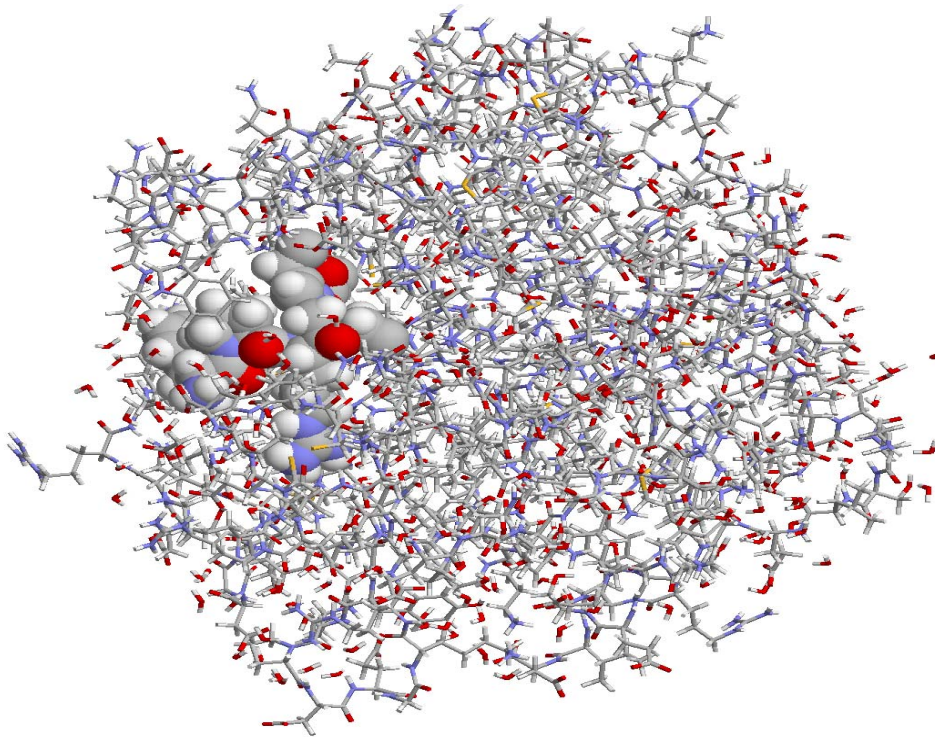


Figure 2: The X-ray structure of human α -thrombin.⁷

Mechanism of Thrombin Catalysis.

The active site of thrombin contains a catalytic triad, consisting of His57, Asp102, and Ser195. These three residues perform the general-base catalysis of a protease, as shown in Figure 3. In the first step, His57 acts as a general base, accepting a proton from Ser195 and so activating it. The Asp102 acts to stabilize His57 in this role. Ser195's γ -O⁻ then attacks the substrate's carboxyl group to form a tetrahedral intermediate, which is stabilized by a group of nearby amides forming the so-called "oxyanion hole". The His57 then donates a proton to the N of the leaving group in the tetrahedral intermediate, causing the release of the amine leaving group and the formation of an acylenzyme intermediate as the tetrahedral intermediate collapses. Again, His57 serves as a general base, activating a water molecule, which attacks the acylenzyme to form a second tetrahedral intermediate. The similar collapse of this second tetrahedral intermediate causes the release of the acid and a return to the original enzyme.^{7,8}

Small Covalent Inhibitors of Thrombin.

Phosphate and phosphonate inhibitors have been widely used in the inhibition of serine hydrolases. The inhibitor attaches covalently to the active site serine and resists nucleophilic attack due to high negative charge density, often compounded by the release of a ligand from the phosphorous atom.⁹ This new covalent adduct resembles the second tetrahedral intermediate formed in thrombin hydrolysis of its natural substrate after the attack by a water molecule on the acylenzyme. Two such small covalent inhibitors are 4-nitrophenyl-diethylphosphate (paraoxon) and 4-nitrophenyl 2-propyl methylphosphonate (MPNP).

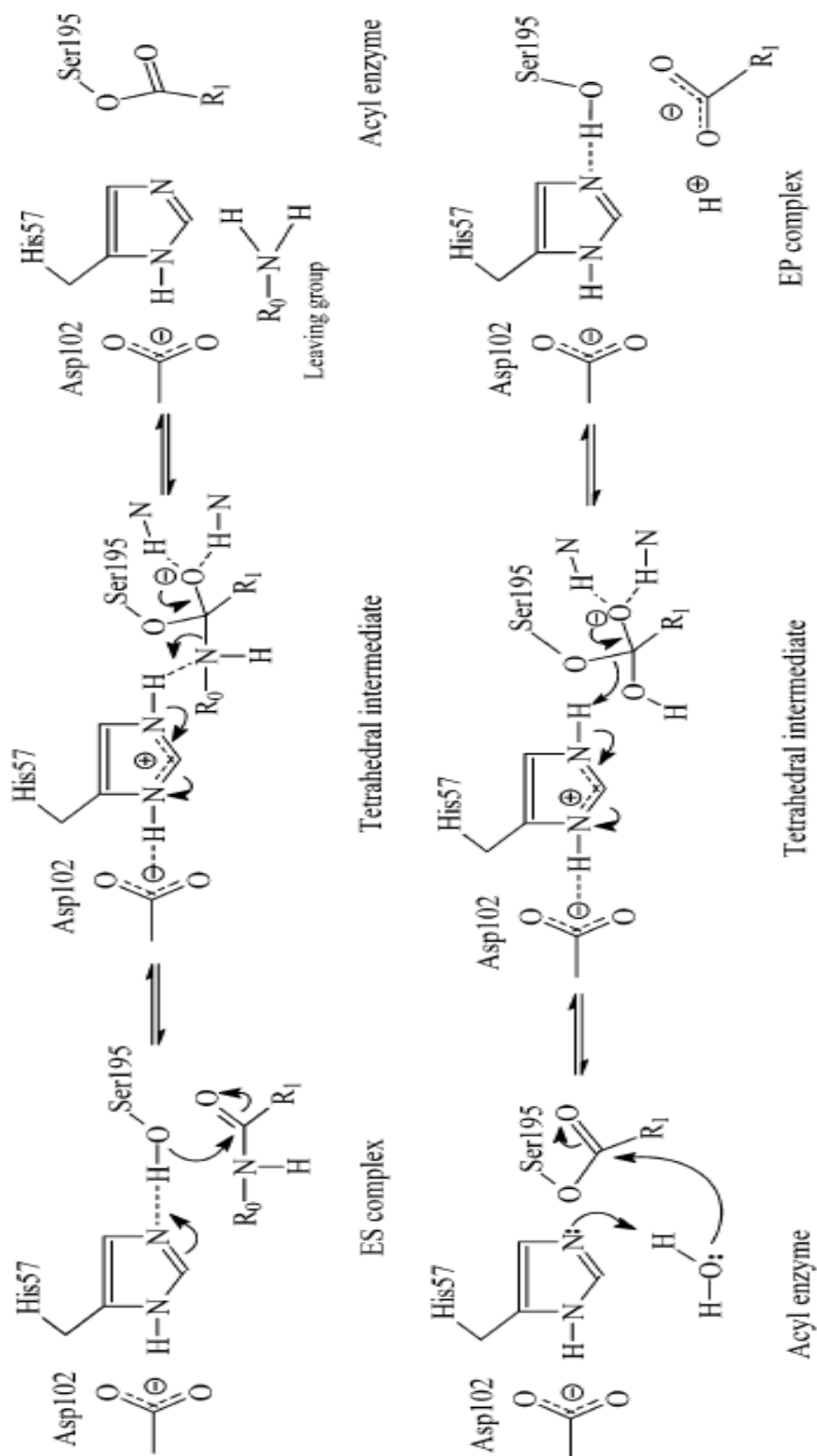


Figure 3: Mechanism of catalysis of thrombin.⁸

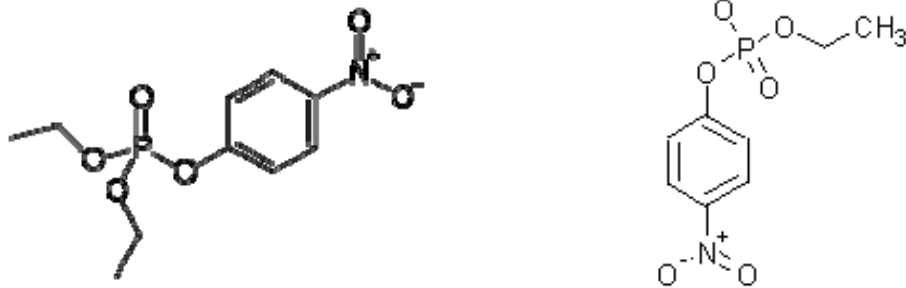


Figure 4: 4-nitrophenyl-diethylphosphate (left) and 4-nitrophenyl 2-propyl methylphosphonate (right).

Hirudin Structure and Inhibition.

Hirudin, derived from *Hirudo medicinalis*, is the most potent known naturally occurring inhibitor of thrombin. It is a single sixty-five residue amino acid chain, with a C-terminal glutamine and an N-terminal isoleucine.² Its primary structure is displayed in Figure 5. Hirudin has two dominant regions: a compact “head” at the N terminus, and a long flexible C-terminal tail. The “head” region contains three disulfide bridges, between cysteine residues at the six and fourteenth, sixteenth and twenty-eighth, and twenty-second and thirty-ninth positions, the latter two of which form the characteristic “double loop”.² The “tail” region runs from fortieth to sixty-fifth residues and, in contrast to the head, is unbound and flexible.

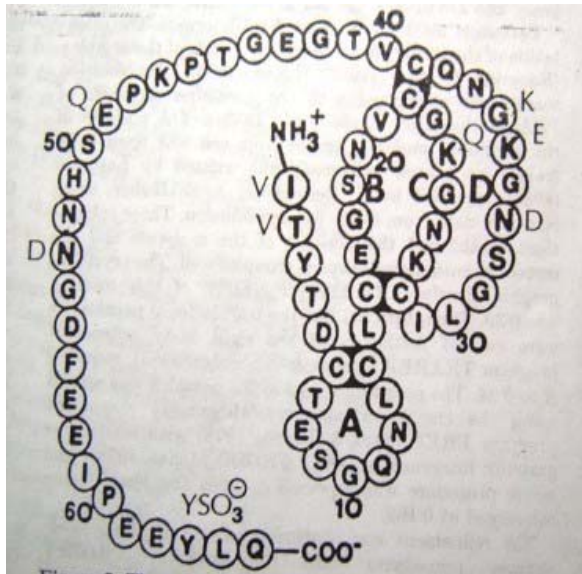


Figure 5: Primary structure of hirudin.¹⁰

Unlike the case with most inhibitors of serine proteases, which make contact strictly in the region of the active site, thrombin and hirudin interact hundreds of times over a wide area of an estimated 1400 Å². It derives its extraordinarily tight binding over a wide range of interresidue interactions both inside and outside the active site.¹⁰ The first forty-eight residues of hirudin, in the N-terminal head, form 103 interactions with thrombin. The C-terminal tail also plays an important role, taking on an unusual extended conformation that also makes several hundred further contacts with the enzyme, all within 4Å.² An illustration of the positioning of hirudin bound to thrombin can be found in Figure 6.



Figure 6: Thrombin-hirudin complex.¹¹

Hirudin is defined as a slow and tight-binding inhibitor, and so “exerts its reversible inhibitory effect on an enzyme-catalyzed reaction at a concentration comparable to that of the enzyme.” The strength of inhibitor-enzyme interactions lead to a delay in the establishment of a steady-state velocity, and allows an identification of a distinct “pre-steady state” phase in a graph of product formation versus time.¹² Additionally the formation of the enzyme-inhibitor complex depends both upon ionic strength and upon the nature of the ions present. Evidence suggests that sodium ions increase the rate of complex formation while the presence of chloride ions has the opposite effect.¹³

The thrombin-hirudin solution structure, as determined by NMR, also shows ~12 H-bonds, some potentially strong, i.e. with a donor-acceptor distance $< 2.6\text{\AA}$.¹⁷ These hydrogen bonds, dubbed short, strong H-bonds or SSHBs, have been on the radar screen of enzymologists as they have potential for great stabilization.¹⁷ These bonds could also be part of the very strong binding witnessed between thrombin and hirudin. NMR evidence of SSHBs is given in Figure 7.

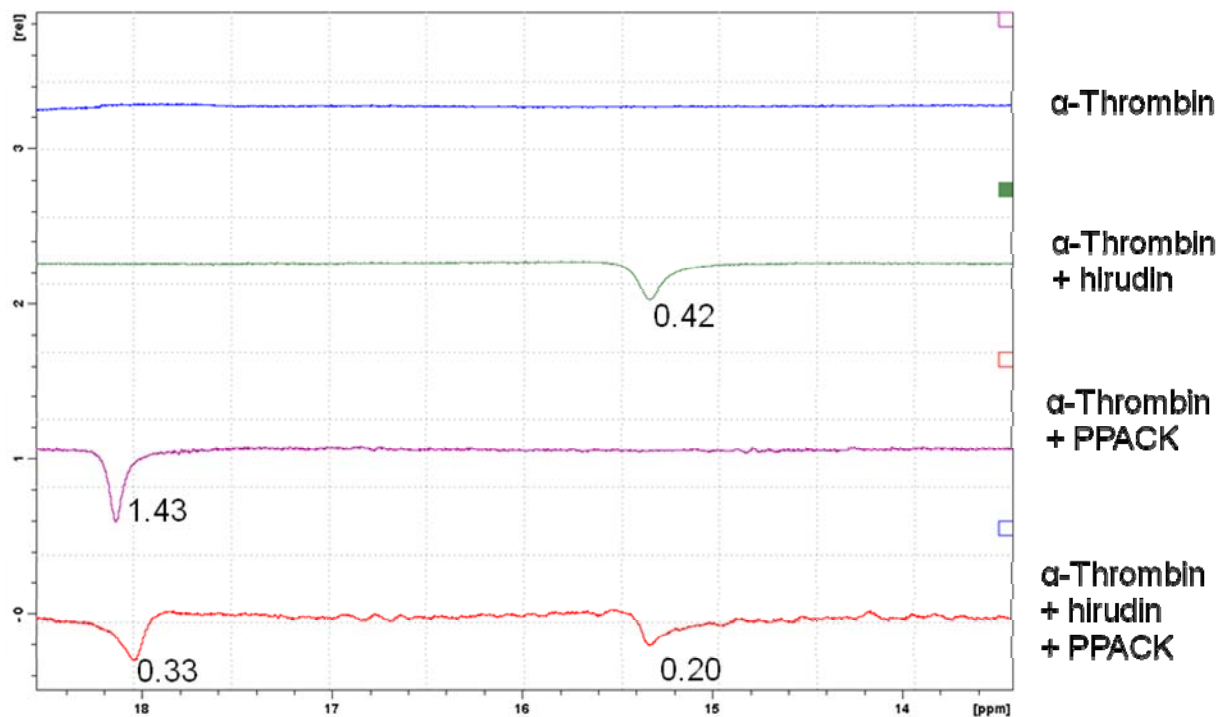


Figure 7: SSHB signals observed in thrombin samples. Values displayed in the spectrum are the relative height of the signals using methyl group at -1.2ppm as internal reference. Intensities of hydrogen bond signals were not calculated because the reference methyl signal intensity cannot be accurately measured.¹⁸

Goals.

The goal of this study is twofold: (1) to determine the pH dependency of the inhibition of thrombin by the small covalent inhibitors paraoxon and MPNP, and (2) to explore the effect of Na^+ and Cl^- ions on the formation of the thrombin-hirudin complex.

Materials and Methods.

Materials.

Anhydrous dimethyl sulfoxide (DMSO), heavy water with 99.9 % deuterium content and anhydrous methanol, were purchased from Aldrich Chemical Co. All buffer salts were reagent grade and were purchased from either Aldrich, Fisher, or Sigma Chemical Co. H-D-Phe-Pip-Arg-4-nitroanilide.HCl (pNA) (S-2238) 99% (TLC) was purchased from Diapharma Group Inc. 4-Nitrophenyl diethylphosphate (paraoxon) was from Aldrich Chemical Co. and 4-nitrophenyl-2-propyl methylphosphonate (MPNP) was synthesized in this lab previously. Human α -thrombin, MM 36,500 d, 3010 NIH u/mg activity in pH 6.5, 0.05 M sodium citrate buffer, 0.2 M NaCl, 0.1% PEG-8000 was purchased from Enzyme Research Laboratories. R-hirudin was purchased from Pentapharm, Lot 40907401/126-05 and Lot 405383/126-05.

Solutions.

Buffer solutions were prepared from the appropriate analytical grade salts using double distilled deionized water. Buffers were prepared by weight from Tris-base and Tris-HCl at 0.02 M, 0.3 M NaCl, 0.1% PEG4000 at pH 8.0. Buffers for pH profile studies were 0.05 M of the respective buffer salt and 0.15 M of NaCl with 0.1% PEG-4000 added. Calculated amounts of HCl or NaOH were used to adjust the buffer pH as necessary. Buffers for pH-dependence studies were made in 0.05 M concentration as follows; phosphate for pH 6.03 - 7.47, citrate for pH 6.52, HEPES for pH 7.49 - 7.79 and barbital for pH 8.07 and 8.54, while 0.02 M Tris was used for pH 8.28. Buffers for the study of hirudin-thrombin interaction were prepared at 0.02 M Tris, ionic strength $\mu = 0.3$ M, 0.1% PEG-4000 at pH \sim 8.1. The salts used were NaCl, Choline Cl, Na Acetate, and Choline Acetate. D₂O buffers were prepared by weight using the same protocol as

their respective H₂O counterparts. All measurements of pH were taken with a Delta electronic pH meter.

Instrumentation.

Spectrophotometric measurements were performed with a Perkin-Elmer Lambda 6 UV-Vis Spectrophotometer interfaced to a PC. Temperature was monitored via a temperature probe and linked digital readout. Temperature control was maintained using a Neslab RTE-4 or a Lauda 20 circulating water bath. All studies were performed at $25.0 \pm 0.1^\circ\text{C}$.

Thrombin Activity Assay.

The concentration of active thrombin enzyme was determined from the initial rates of the hydrolysis of the chromogenic substrate S-2238 (Figure 7). Under conditions of saturation, initial rates are equal to maximal velocities, V_{max} . From these values [E] was calculated given $k_{\text{cat}} = 95 \pm 20 \text{ s}^{-1}$ in pH 8.2, 0.02 M Tris, 0.3M NaCl, 0.1% PEG-4000, buffer at $25.0 \pm 0.1^\circ\text{C}$.⁸ S-2238 is a substrate specific for thrombin used in all of the experimental studies. The substrate stock solution was made by mixing ~10 mg of the S-2238 substrate into 1 mL of DMSO.

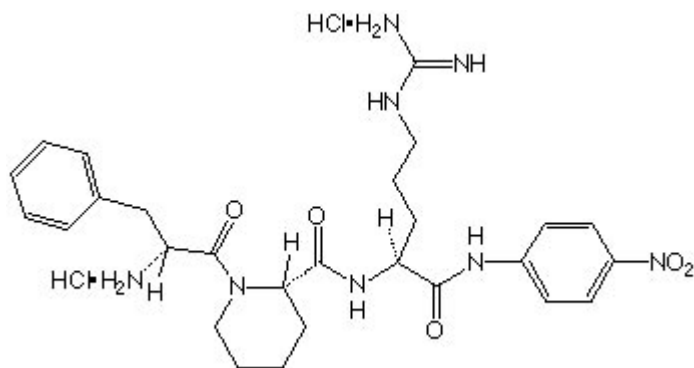


Figure 8: The structure of S-2238.¹⁵

Kinetic Procedure: Inhibition by Paraoxon, MPNP.

In a typical spectrophotometric run, 190 μL of thrombin at the desired pH was drawn and 10 μL of 0.14 M inhibitor solution added. This reaction mixture was incubated under temperature control. At each indicated time interval, 10 μL aliquots were drawn, and the reaction was quenched by dilution into a cuvette containing 980 μL pH 8.00, 0.05M barbital buffer. Ten μL S-2238 was added and the cell inverted several times to initiate the reaction. Kinetic runs were followed by monitoring the absorbance of the released 4-nitroaniline at 400 nm. Pseudo-first-order rate constants for inhibition were calculated from the 5s slopes for declining activities for 4 half-lives. The second-order rate constants were calculated from k_{obs} divided by the concentration of inhibitor.

Kinetic Procedure: Inhibition by Hirudin.

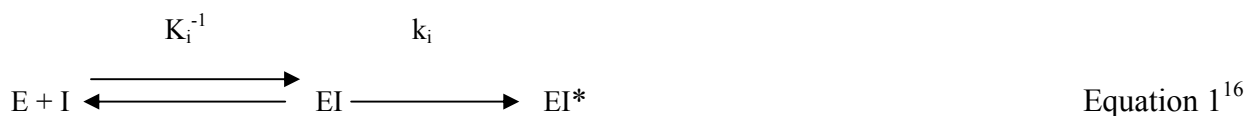
Each individual set of kinetic data was collected in a single contiguous session under strict temperature control. Glass cuvettes were incubated in the cell compartment for ten minutes prior to their use in a run in order to allow the temperature to equilibrate prior to the addition of reagents. During this time the instrument was “zeroed” so that all measured absorbance would be a product of the released 4-nitroaniline, measured at 400 nm. The enzyme and inhibitor, once diluted to the desired concentration, was aliquoted and frozen separately in order to preserve enzyme activity during the course of a given session. The order of addition to the glass cuvette involved the appropriate amount of buffer, 10 μL substrate, 5-15 μL inhibitor, and finally 10 μL enzyme, to a total volume of 1 mL. The lapse in seconds between this addition of enzyme and the commencement of the kinetic run was recorded. Change in absorbance was followed for ~ 1200 seconds.

The concentration of active hirudin in the stock solution was independently determined. 5-10 μL 5.67×10^{-8} M hirudin (this concentration was determined as a result of this process) and 50 μL 2.48×10^{-8} M thrombin were combined in a single glass cuvette such that the thrombin was in significant excess over hirudin, and allowed to incubate until full inhibition was reached. The activity of the remaining (uninhibited) thrombin was assayed by the addition of 10 μL substrate and 965-970 μL buffer. The difference between the remaining activity and that of the original thrombin stock was taken as the concentration of hirudin in the stock solution. These independently determined values were compared with the hirudin activity value, x , calculated during data analysis of hirudin-thrombin inhibition. In most cases the activity value x fell within a range from 0.7-0.9, signifying 70%-90% activity of the calculated value, which was verified by the independent determination.

Data Analysis.

Mechanism Scheme: Inhibition by Paraoxon, MPNP.

The irreversible inhibition of thrombin by covalent inhibitors, such as paraoxon and MPNP, can be modeled as:



Here K_i is an equilibrium constant, $k_{\text{off}}/k_{\text{on}}$. The rate constant of the association of enzyme and inhibitor, by which the enzyme-inhibitor complex (EI) is formed, is k_{on} ; the rate constant of the disassociation of this complex is k_{off} . The first-order rate constant for the formation of the bond resulting in the covalently modified thrombin (EI*) is k_i . *Observed* rate constants were determined by an exponential decay fit of the initial rates of each aliquot from the reaction

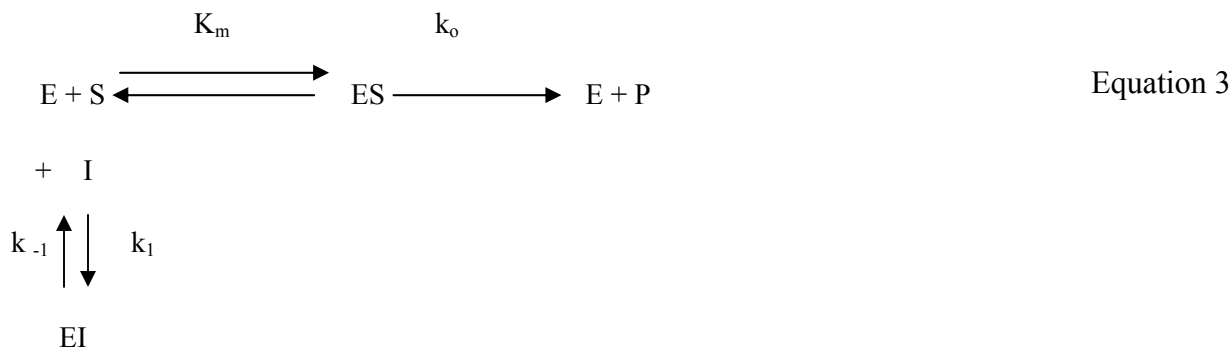
mixture. The pK_a s were determined by a double-bell curve fit of these pseudo-first-order rate constants using the equation:

$$k_{\text{obs}} = L / (1 + 10^{(pK_1 - \text{pH})} + 10^{(\text{pH} - pK_2)}) \quad \text{Equation 2}$$

at NaCl 0.15 M and $25.0 \pm 0.1^\circ\text{C}$. This fitting was performed using GraFit 5.

Mechanism Scheme: Inhibition by Hirudin.

The reaction scheme which describes this inhibition takes into account the reversible inhibition of thrombin in the presence of a substrate and is described by two steps: enzyme-substrate complex formation, and the formation of product and free enzyme.



The formation of product vs. time is described by the equation

$$P = v_s t + (v_s - v_o)(1-d) / (dk) * \log((1 - de^{-kt}) / (1-d)) \quad \text{Equation 4}^{12,13}$$

where v_s is the steady state reaction velocity, v_o is the reaction velocity in the absence of inhibitor, t is time, P is the amount of product formed, and d is a function equivalent to $(K_i + E_t + I_t - ((K_i + E_t + I_t)^2 - 4E_t I_t)^{1/2}) / (K_i + E_t + I_t + ((K_i + E_t + I_t)^2 - 4E_t I_t)^{1/2})$. Here, K_i is the dissociation constant, E_t is the concentration of all enzyme present in solution, I_t is the concentration of all inhibitor present in the solution, and k is a pseudo first-order rate constant, which is equivalent to

$k_1((K_i+E_t+I_t)^2-4E_tI_t)^{1/2}/(1+[S]/K_m)$. Here, k_1 is the forward second-order kinetic constant for inhibition and $[S]$ is the concentration of substrate.

Data Processing: Inhibition by Hirudin.

Data was first processed in Microsoft Excel, including adjustment for the recorded time lag between enzyme addition and the beginning of the actual recording of each kinetic run. Corrections were applied to convert from absorption units to concentration, and subsequently to list the concentrations in μM . The data was then transferred to GraFit 5 where all further data analysis was performed. All fittings were set to “simple robust” with the final values verified using other statistical methods, including explicit fitting. The exact analytic procedure employed was originally pioneered in this laboratory by John Paul Sheehy. First, the steady state velocity was determined via fitting to a simplified version of the progress equation (Equation 4). P was the dependent variable, t the independent variable, and d , v_o , v_s , k , and h were found by means of a non-linear least squares fit. A full set of data curves for this first level of fitting is found in Figure 8 below.

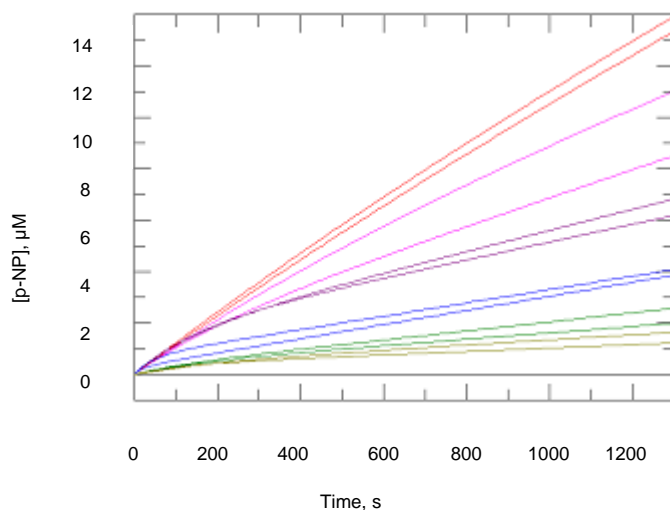


Figure 9: Sample data curves for the inhibition of human α -thrombin by r-hirudin in pH 8.12, 0.02 M Tris, 0.3 M Choline Cl, 0.1% PEG at $25.0\pm 0.1^\circ\text{C}$.

The resulting steady state velocities, v_s , were plotted against the hirudin concentrations of the respective runs, calculated on the assumption that one anti-thrombin unit (ATU) was equal to 8.5 pmol of r-hirudin. The dependence of the steady state velocity on the concentration of inhibitor was modeled using

$$v_s = (v_o / (2E)) \left(\frac{((K_i + Ix - E)^2 + 4K_i E)^{1/2} - K_i + Ix - E}{2} \right) + h \quad \text{Equation 5}$$

Here, v_s and v_o remain previously defined, while E is enzyme concentration, I is inhibitor concentration, x is a factor which represents the ratio of the “true” (active) inhibitor concentration to the concentration calculated by Pentapharm, K_i is the apparent dissociation constant K_i' , and h is an offset. The presence of h is intended to account for residual activity caused by β -thrombin and γ -thrombin, contaminating the α -thrombin sample after autolysis. These alternate forms are less susceptible to inhibition by r-hirudin, resulting in low thrombin activity even at maximal inhibition. To minimize the h factor (<10%), new samples of thrombin were consistently used, retaining the relative purity of α -thrombin. A sample fitting is shown in Figure 9 below. The apparent K_i' here determined could then be corrected for substrate concentration using the formula:

$$K_i \text{ (corrected)} = K_i' \text{ (apparent)} / (1 + ([S]/K_m)) \quad \text{Equation 6}$$

wherein $[S]$ is the substrate concentration and the K_m is for that substrate under the experimental conditions.

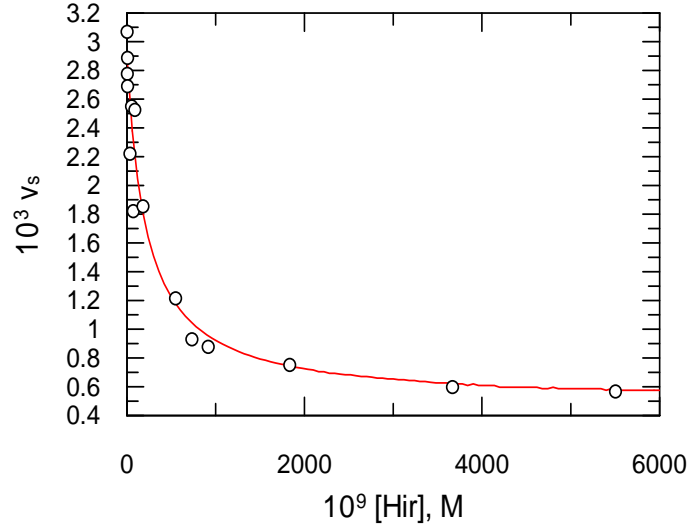


Figure 10: Sample K_i determination curve for the inhibition of human α -thrombin by r-hirudin in pH 8.12, 0.02 M Tris, 0.3 M Choline Cl, 0.1% PEG at $25.0 \pm 0.1^\circ\text{C}$.

The third level of fitting returns to the progress curve, now utilizing the full version of Equation 3, which was made possible by the determination of K_i in the second level of fitting. In this version of the equation, d is fully expanded, but all other variables retain their initial significance. As with the second level of fitting, P and t were the dependent and independent variables respectively. The values for v_o , v_s , and k were left as parameters to be fitted. The “ k ” values derived from this fitting were plotted against the inhibitor concentrations (corrected by x , the calculated hirudin activity value, which maintained a value of 0.7-0.9 for each run), using the formula:

$$k = k_1 K_m \frac{((K_i + E + I)^2 - 4EI)^{1/2}}{(K_m + S)} \quad \text{Equation 7}$$

where the new variable k_1 is the elementary forward second-order rate constant. A sample fit is found in Figure 10. With the corrected values of K_i and k_1 so calculated, k_{-1} was determined using the equation:

$$K_i = k_{-1}/k_1 \quad \text{Equation 8}$$

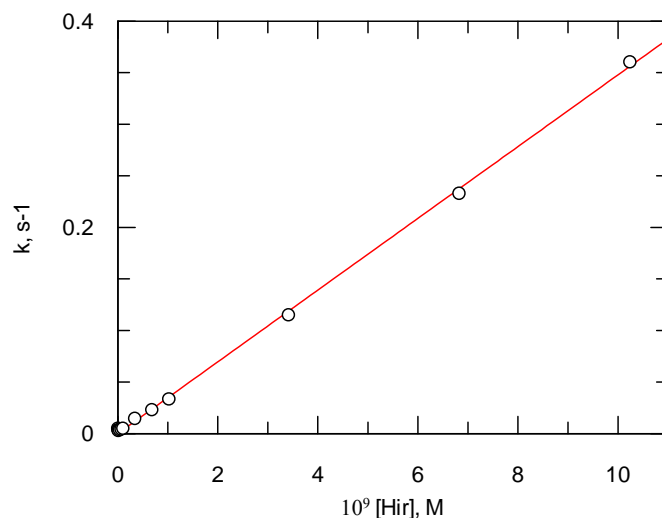


Figure 11: Sample second order rate constant determination for the inhibition of human α -thrombin by r-hirudin in pH 8.12, 0.02 M Tris, 0.3 M Choline Cl, 0.1% PEG at $25.0 \pm 0.1^\circ\text{C}$.

Results and Discussion.

pH dependence of Thrombin Inhibition by Paraoxon, MPNP.

The pH dependence of the inhibition of human α -thrombin by paraoxon and MPNP was bell-shaped, yielding maxima corresponding to a second-order rate constant of $0.47 \pm 0.05 \text{ M}^{-1} \text{ s}^{-1}$ for paraoxon inhibition and $6.2 \pm 0.1 \text{ M}^{-1} \text{ s}^{-1}$ for MPNP inhibition. The two pK_a 's calculated from the data are 7.8 ± 0.1 and 9.3 ± 0.2 for paraoxon inhibition and 8.0 ± 0.1 and 8.6 ± 0.2 for MPNP inhibition. These values are recorded in Table 1, and the trend of the pH profiles in Figure 12.

Table 1: pK_a s and rate constants for the inhibition of thrombin by paraoxon, MPNP at $25.0 \pm 0.1^\circ\text{C}$, 0.15M NaCl.

Inhibitor	pK_{a1}	pK_{a2}	$k_i/K_i, \text{ M}^{-1} \text{ s}^{-1}$ pH 8.1 (max)	$K_i, \text{ M}$ pH 7.47
paraoxon	7.8 ± 0.1	9.3 ± 0.2	0.47 ± 0.05	$> 10^{-5}$
MPNP	8.0 ± 0.1	8.6 ± 0.2	6.2 ± 0.1	$> 10^{-5}$

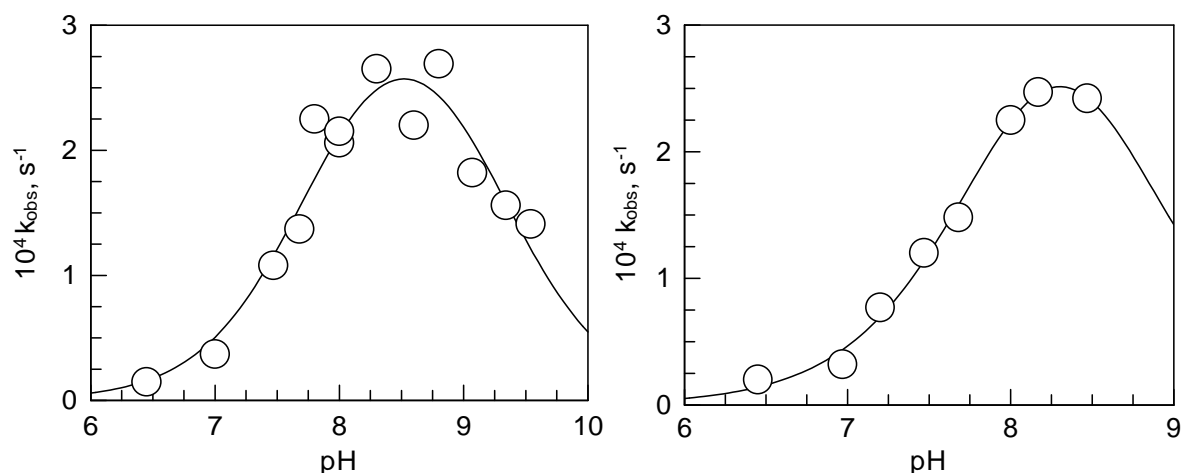


Figure 12: pH dependence of the pseudo-first-order rate constant for the inhibition of thrombin with paraoxon (left) and MPNP (right) at NaCl 0.15 M and $25.0 \pm 0.1^\circ\text{C}$

Effects of Na^+ , Cl^- Ions on Thrombin-Hirudin Complex Formation.

The replacement of sodium with choline caused a rise in K_i for both chlorine and acetate anions: $0.47 \pm 0.15 \text{ pM}$ (NaCl) to $13.8 \pm 0.2 \text{ pM}$ (Choline Cl), and $2.78 \pm 0.04 \text{ pM}$ (Na Acetate) to $12 \pm 3 \text{ pM}$ (Choline Acetate). The effects were mixed when a similar replacement was done for Cl^- . When replaced with acetate in the case of the choline cation, K_i experienced a fall from $13.8 \pm 0.2 \text{ pM}$ (Choline Cl) to $12 \pm 3 \text{ pM}$ (Choline Acetate). However when replaced with acetate in the case of the sodium cation, K_i rose from $0.47 \pm 0.15 \text{ pM}$ to $2.8 \pm 0.1 \text{ pM}$.

Table 2: Disassociation constants and rate constants for hirudin inhibition of α -thrombin, 0.3M indicated salt, 25.0 \pm 0.1 $^{\circ}$ C.

Salt	K_i , pM	k_1 M $^{-1}$ s $^{-1}$	k_{-1} (s $^{-1}$)
NaCl*	0.47 \pm 0.15	(4.8 \pm 0.1) x 10 7	2.3 x 10 $^{-5}$
NaCl (D $_2$ O)*	1.8 \pm 0.9	(6 \pm 2) x 10 7	2 x 10 $^{-4}$
Choline Cl	13.8 \pm 0.2	(8.2 \pm 0.4) x 10 6	1.1 x 10 $^{-4}$
Choline Cl (D $_2$ O)	5.32 \pm 0.01	(2.9 \pm 0.2) x 10 7	1.5 x 10 $^{-4}$
Na Acetate	2.8 \pm 0.1	(3.49 \pm 0.02) x 10 7	10 $^{-3}$
Na Acetate (D $_2$ O)	3.9 \pm 0.1	**	**
Choline Acetate	12 \pm 3	**	**
Choline Acetate (D $_2$ O)	5.7 \pm 0.1	**	**

* These values courtesy of JP Sheehy, 2007-08.

** Fitting has not yet been resolved.

Na $^+$, Cl $^-$ and Solvent Isotope Effect.

The solvent isotope effect is presented as a ratio of dissociation constants, K_i , in H $_2$ O and D $_2$ O, expressed as the dimensionless quantity K_H/K_D . Again, the effect of the presence or absence of the sodium ion is marked, while that of the chloride ion remains mixed.

Table 3: Solvent isotope effects and rate constants for hirudin inhibition of α -thrombin, 0.3M indicated salt, 25.0 \pm 0.1 $^{\circ}$ C.

Salt	K_H/K_D for K_i
NaCl	0.26 \pm 0.15*
Choline Cl	2.59 \pm 0.03
Na Acetate	0.72 \pm 0.02
Choline Acetate	2.1 \pm 0.5

Conclusions.

pH profiles of Paraoxon and IMN.

The rate of inhibition of thrombin by paraoxon and MPNP is too slow to be of great medical use. However the pH dependence for the inhibition of human α -thrombin by paraoxon and MPNP could be elucidated, and proved to be bell-shaped, with maxima at pH values approximating 8.3. The two determined pK_a s corresponded with the classically accepted values for His57 and Ile16 on the thrombin enzyme.

Hirudin-Thrombin K_i Dependency on Na^+ , Cl^- Ions.

The dependency of thrombin-hirudin complex formation on ionic strength, as the result of a decrease in k_1 , is well documented.^{13,14} The role of sodium in this interaction has also been noted, and explored in this study. Replacing sodium in the typical thrombin buffer salt NaCl with choline cation, K_i experienced a significant 30-fold rise. This change was mirrored when the chloride was replaced with the anion acetate, though on a smaller scale, with a four-fold rise in K_i upon substitution of choline for sodium. This suggests that the Na^+ ion specifically plays a significant role in thrombin-hirudin complex formation. There is only mixed evidence, however, that Cl^- plays a lesser, inhibitory, role. These effects did not appear to be mirrored in the calculated values of k_1 , given in Table 2. Of additional note is the small magnitude of all measured values of K_i .

Hirudin-Thrombin SIE Dependency on Na^+ , Cl^- Ions.

A significant difference between the solvent isotope effects in solutions containing the Na^+ ion and those replacing it with choline is evident on the order of magnitude scale. As with the

dissociation constant, the effect of the presence or absence of the Cl^- ion is mixed, causing a threefold increase in the presence of the Na^+ ion and a marginal decrease when sodium is replaced by choline.

References.

- [1] Bode, W.; Turk, D.; Karshikov, A., *Protein Sci.* **1992**, *1*, 426-471
- [2] Rydel, T.; Tulinsky, A.; Wolfram, B.; Huber, R. *J. Mol. Biol* **1991**, *221*, 583-601.
2000.
- [3] Berg, J. M.; Tymoczko, J. L.; Stryer, L., *Biochemistry*. Sixth. 2007. United States, W.H. Freeman and Company
- [4] Berg, J.M.; Tymoczko, J.L.; Stryer, L. *Biochemistry*; 6th ed. W.H. Freeman and Company: New York, NY, 2007; p. 293.
- [5] Berliner, J. L. editor, *Thrombin Structure and Function*, Plenum Press, New York and London
- [6] Huntington, J. A., *J. Thromb. Haemostasis* **2005**, *3*, 1861-1872
- [7] Pineda, A. O.; Carrell, C. J.; Bush, L. A.; Prasad, S.; Caccia, S.; Chen, Z. W.; Mathews, F. S.; Di Cera, E., *J. Biol. Chem.* **2004**, *279*, 318-42.
- [8] Kovach, I. M.; Enyedy, E. J., *J. Am. Chem. Soc.* **2004**, *126*, 6017-6024
- [9] Kovach, I. M. (2004) *J. Phys. Org. Chem.* *17*, 602-614.
- [10] Grutter, M.; Priestle, J.; Rahuel, J.; Grossenbacher, H.; Bode, W.; Hofsteenge, J.; Stone, S. *The EMBO Journal* **1990**, *9*, 2361-2365.
- [11] T.J. Rydel et al., *Science*, **1990**, *249*, 277
- [12] Williams, J.; Morrison, J. *Methods of Enzymology* **1979**, *63*, 437-467.
- [13] Stone, S.R.; Hofsteenge, J. *Biochemistry*, **1986**, *25*, 4622-4628.
- [14] Stone, S. R.; Dennis, S.; Hofsteenge, J. *Biochem.*, **1989**, *28*, 6857-6863.
- [15] S-2238; MSDS No. S820324 [Online]; DiaPharma: Columbus, OH, Sep 2004.
- [16] Kitz, R.; Wilson, I. B. *J. Biol. Chem.* **1962**, *237*, 3245-3249.
- [17] Mildvan, A.S.; Massiah, M.A.; Harris, T.K.; Marks, G.T.; Harrison, D.H.T.; Viragh, C.; Reddy, P.M.; Kovach, I.M., *J. Mol. Struct.*, **2002**, *215*, 163-175.
- [18] Kovach, I.M.; Zhang, D., unpublished results.