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Cyclic and Linear Oligocarbamate Ligands for Human Thrombin

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Abstract—Several classes of compounds have been tested as potential inhibitors of the serine protease thrombin, an important regulator of blood coagulation cascades. We describe here the discovery of a new class of thrombin inhibitors based on an unnatural carbamate biopolymer. Oligocarbamate thrombin inhibitors were identified through the screening of diverse cyclic trimer, cyclic tetramer, and linear tetramer libraries using the one bead, one peptide method. Whereas the cyclic trimer oligocarbamate ligands bound thrombin with modest affinity, a cyclic tetramer oligocarbamate inhibited thrombin with an apparent K_i of 31 nM. Linear oligocarbamate tetramers bound thrombin with inhibition constants in the 100-nM range. These nonpeptidic, oligomeric molecules may provide the basis for further drug development and studies of thrombin–ligand interactions.

Introduction

The enzyme thrombin plays a critical role in the process of blood coagulation.[1] Thrombin cleaves fibrinogen to form fibrin, whose polymerization forms a major component of blood clots; it also activates factors V, VIII, and XIII and stimulates the aggregation of platelets. Because of its importance in multiple pathways that lead to thrombosis, thrombin has been thoroughly studied by many groups seeking to design effective inhibitors that can be used as therapeutics. Thrombin is a serine protease consisting of two chains. The smaller A chain (36 residues) is not homologous to other proteases, but the larger B chain (259 residues) has the same active site sequence as the pancreatic proteases, trypsin and chymotrypsin. Like trypsin, thrombin has an Asp residue at the base of the S1 specificity pocket, resulting in a preference for cleavage of Arg-Xxx or Lys-Xxx bonds.[2] Thrombin's specificity, however, is more stringent than trypsin's, with a preference for proteins containing a Pro at the P2 position and an apolar amino acid in the P3 position.[3]

Two distinct classes of active-site directed thrombin inhibitors have been characterized. One group of inhibitors forms a covalent bond between the nucleophilic Ser^{195} in the enzyme and an electrophilic center on the inhibitor. Examples of these inhibitors include C-terminal aldehydes,[4,5] chloromethylketones,[6] phosphonate esters,[7] boronates,[8,9] and α-ketoamides.[10] A second class of inhibitors binds noncovalently to thrombin through hydrogen-bonding, hydrophobic, and electrostatic interactions with the S1-S3 specificity pockets. Examples of high-affinity noncovalent ligands include peptides containing arginine residues,[11] benzamidine groups,[12] and guanidinium moieties.[13] In addition, small molecule inhibitors with phenylamidine groups have been rationally designed based on thrombin crystal structures.[14]

To further explore the structural determinants for thrombin binding as well as identify biologically active inhibitors, we have screened a combinatorial library of oligocarbamates for binding to thrombin. Combinatorial libraries have been successfully applied to the discovery of inhibitors for HIV protease,[15] cathepsin D,[16] and thermolysin.[17] Many of these inhibitors incorporate a transition-state analogue in their design, such as a (hydroxyethyl)amine for aspartyl proteases or phosphonates for zinc proteases. Two libraries have been screened against thrombin that consist of peptides with no unnatural functionality. Screening of a library of peptides on phage identified ligands for thrombin, but the selected peptides did not inhibit enzymatic activity, suggesting that they do not bind in the active site.[18] A second peptide library has been synthesized using the 'one bead, one peptide' method, which was screened specifically for active-site ligands.[19] The selected peptides, however, showed only modest inhibitory activity towards thrombin (Arg-Gly-Arg-Pro-D-Phe,

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$K_i = 5.7\,\mu M$). Here we describe the screening of large, diverse combinatorial libraries of linear and cyclic oligocarbamates against thrombin. This 'unnatural' biopolymer is constructed from the stepwise coupling of *N*-protected aminoethyl nitrophenylcarbonates containing a variety of side chains. A cyclic tetramer was identified (Cyclo(S)-Gly-Hpb^c-Tym^c-arg^c-Val^c-Cys^c-NH_2) with an apparent K_i of 31 nM. This ligand may provide the basis for further improvements in affinity as well as an opportunity to explore the pharmacological properties of this novel class of oligomeric molecules.

Results

Combinatorial oligocarbamate libraries

The oligocarbamate backbone consists of a chiral ethylene backbone linked via carbamate groups, with side chains that contain a variety of functional groups. The efficient synthesis of linear and cyclic oligocarbamate libraries has been previously described,[20] and the frameworks for these compounds are shown in Figure 1.

These libraries were synthesized with a chemically and structurally diverse monomer set, including a large number of guanidinium containing monomers. The 27 monomers used in the synthesis of the library are shown in Figure 2, along with our assigned three letter abbreviations (superscript 'c' indicates a carbamate bond). Cyclic linear oligocarbamates were synthesized by the intramolecular reaction of the side-chain thiol of a C-terminal cysteine with an N-terminal bromoacetamide.[23] The libraries were synthesized for screening in the 'one bead, one peptide' format using a divide and recombine strategy.[24] The cyclic trimer library had 27^3 or 19683 different members, while the cyclic and linear tetramer libraries had 27^4 or 531441 members. Following completion of the synthesis, the libraries were stored at 4 °C and could be used for several assay types and trials.

Screening of molecules attached to solid support for binding to thrombin

Screening of one bead, one peptide libraries is generally accomplished by incubating the beads with the receptor of interest, washing away receptor that does not bind, and identifying the beads that bind the receptor through an enzymatic tag linked to an antibody.[25] In order to find suitable screening conditions, initial assays were performed on the compound BMS-183507, a well-characterized peptide inhibitor ($K_i = 17$ nM),[26] attached to Tentagel resin with an aminocaproic acid-Ser-Arg-Ser-linker. Equal portions of beads covalently modified with BMS-183507 and beads with linker alone were washed and blocked with tris buffer containing 1% BSA and 0.5% Tween-20. Binding of thrombin to beads was detected by incubation with antithrombin antibodies, followed by secondary antibodies conjugated to alkaline phosphatase, with subsequent enzyme-mediated staining of the beads. The binding of thrombin to BMS-183507 on solid support was abolished by coincubation of the soluble inhibitor with thrombin, suggesting that the interaction observed is specific. For each library, several concentrations of thrombin were used to vary the stringency of the screen.

Sequences of oligocarbamates

Positive beads were picked and the oligocarbamates were cleaved and sequenced by matrix assisted laser desorption mass spectrometry (MALDI-MS). The efficiency of the sequencing was similar to previous results (66/69 beads or 96% could be sequenced). The results from the various screens are tabulated in Tables 1–3. For the monomers Pro^c, Asp^c, Glu^c, Ind^c, and Arg^c, both enantiomers were used in the synthesis of the library to increase the structural diversity of the framework. We could not distinguish between the diastereomers for sequences that contain one of these monomers, so the active diastereomer was determined for selected sequences by a deconvolution strategy

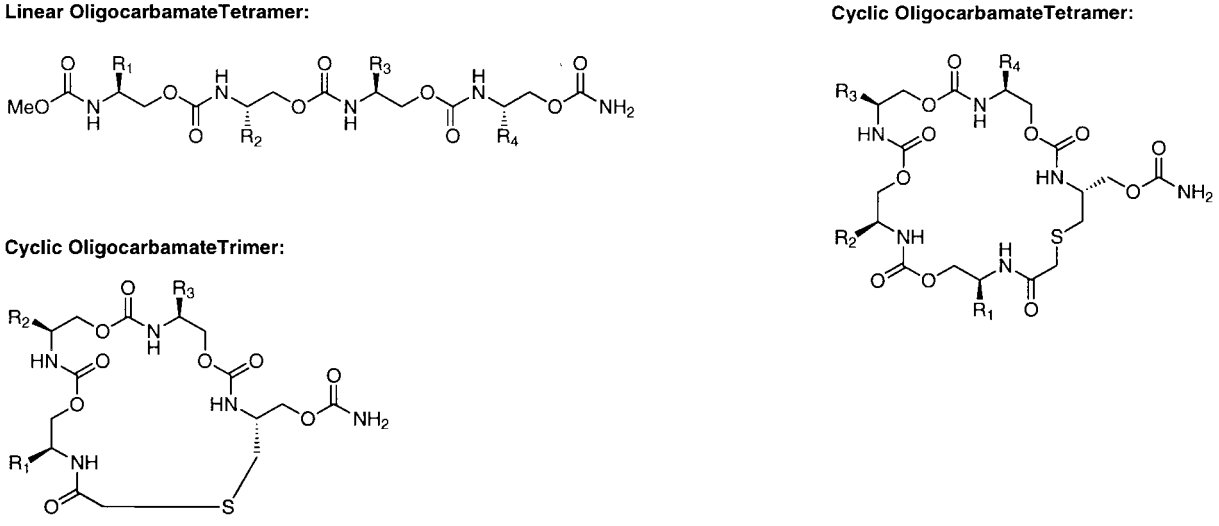


Figure 1. Frameworks for linear tetramer, cyclic trimer, and cyclic tetramer oligocarbamate libraries. The synthesis of these libraries has been previously described.[20] Monomers used in these libraries are shown in Figure 2.

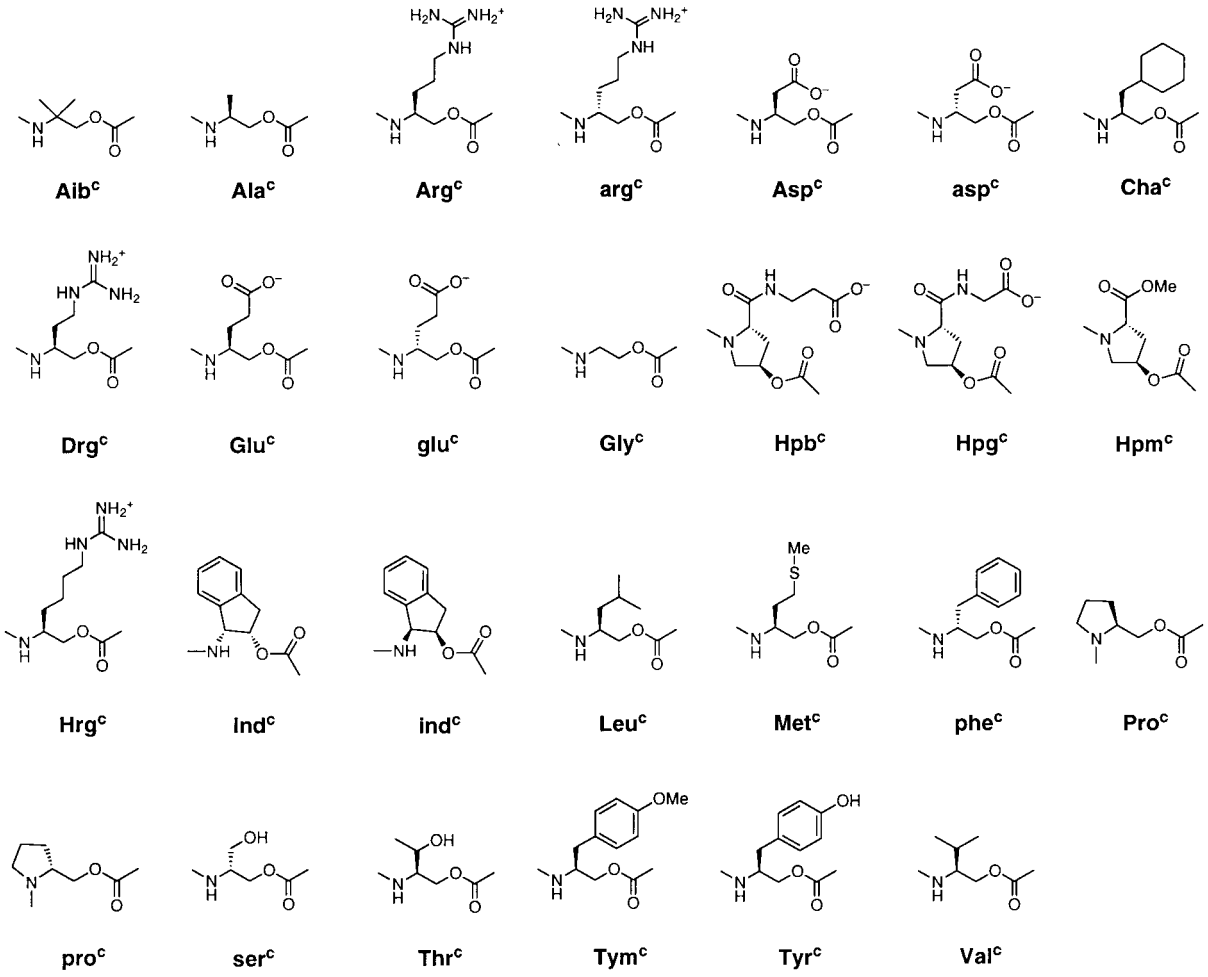


Figure 2. Monomeric units used in the construction of one bead, one peptide oligocarbamate libraries. The subscript 'c' refers to the carbamate linkage. Capital letters refer to *S* stereochemistry at the alpha carbon stereocenter, whereas lower case letters refer to *R* stereochemistry.

previously described.[20] In Tables 1–3 the single active diastereomer is shown for sequences where it was determined, whereas other entries do not show a single sequence.

The sequence arrangement is based on sequence similarity or motifs. For the cyclic tetramer and linear tetramer libraries (Tables 2 and 3), we define a motif as a group of sequences that have at least two identical monomers with the same positional relationship to each other. For example, both Ac^c-Pro^c-Cha^c-Hrg^c-Glu^c-NH_2 and Ac^c-Glu^c-Pro^c-Tym^c-Hrg^c-NH_2 are part of motif 6, Ac^c-(Xxx^c)-Pro^c-Xxx^c-Hrg^c-(Xxx^c)-NH_2, because each sequence has a Pro^c monomer two positions before Hrg^c. For both libraries, however, there were sequences that did not fall into any motif, and are therefore categorized as 'orphan' sequences. For the cyclic trimer library, the definition of a motif is relaxed since there is considerable divergence among the selected sequences (Table 1). Nevertheless, the sequences fall into two basic motifs, one in which the first residue is Hrg^c (with a preference for Pro^c in the second position), and a second in which the first residue is pro^c (with a guanidinium-containing monomer in the third position).

Thrombin inhibitory activity of selected oligocarbamates

The apparent K_is of several oligocarbamates are shown in Tables 4–6. Apparent K_is were determined by conversion of ligand IC_{50}s for thrombin at 10 μM s-2238 substrate in accord with previously described protocols.[4,22] In our hands, however, the K_m for s-2238 under these conditions with thrombin (Sigma) was 1.3 μM compared with the literature value of 2.5 μM.[13] Correspondingly, our determination for the apparent K_i of BMS-183507 was 8.5 nM compared to $K_i = 17$ nM. The values reported here use $K_m = 1.3$ μM.

Apparent K_i determinations were measured for representative ligands of most of the sequence motifs. Table 4 lists the ligands from the cyclic trimer library. All of the ligands from this library bound thrombin with modest affinity; apparent K_i values ranged from 760 nM to 2.9 μM. Carbamates **3** (Cyclo(S)-Gly-Hrg^c-Pro^c-Leu^c-Cys^c-NH_2, $K_i = 1.0$ μM) and **5** (Cyclo(S)-Gly-Hrg^c-Aib^c-Hpg^c-Cys^c-NH_2, $K_i = 2.9$ μM) are representative of motif 1 (Cyclo(S)-Gly-Hrg^c-Xxx^c-Xxx^c-Cys^c-NH_2). Representatives of motif 2 (Cyclo(S)-Gly-pro^c-Xxx^c-Xxx^c-Cys^c-NH_2) include carbamates **1** (Cyclo(S)-Gly-pro^c-

Pro^c-Hrg^c-Cys^c-NH$_2$, $K_i = 760$ nM), **2** (Cyclo(S)-Gly-pro^c-Pro^c-Arg^c-Cys^c-NH$_2$, $K_i = 960$ nM), and **4** (Cyclo(S)-Gly-pro^c-Ala^c-Arg^c-Cys^c-NH$_2$, $K_i = 2.3$ μM). Despite the distinct nature of the sequence motifs and the relative rigidity of these structural frameworks, none of the cyclic trimer oligocarbamates inhibited enzymatic catalysis with high potency.

The range of binding affinities was considerably larger for ligands from the cyclic tetramer library (Table 5). The highest affinity ligands came from motif 3 (Cyclo(S)-Gly-Xxx^c-Tym^c-arg^c-Xxx^c-Cys^c-NH$_2$), which also had the most members. Carbamate **6** (Cyclo(S)-Gly-Hpb^c-Tym^c-arg^c-Val^c-Cys^c-NH$_2$, $K_i = 31$ nM) had the highest affinity of any of the ligands tested; it bound thrombin with approximately fourfold lower affinity than the peptide BMS-183507. The other members of motif 3, however, bound thrombin with considerably lower affinity. Replacement of the Val^c in position 4 of carbamate **6** with either Pro^c or pro^c resulted in a greater than 10-fold loss in activity (carbamate **8**, Cyclo-(S)-Gly-Hpb^c-Tym^c-arg^c-Pro^c-Cys^c-NH$_2$, $K_i = 440$ nM; carbamate **9**, Cyclo(S)-Gly-Hpb^c-Tym^c-arg^c-pro^c-Cys^c-NH$_2$, $K_i = 440$ nM). Similarly, carbamate **7** was also of lower affinity (Cyclo(S)-Gly-ser^c-Tym^c-arg^c-Ala^c-Cys^c-NH$_2$, $K_i = 390$ nM). Although there is a range of affinities within motif 3, the recognition is dependent on the common core of -Tym^c-arg^c- in the second and third positions, which likely bind in the S1

Table 1. Sequences from oligocarbamate cyclic trimer library

Receptor concentration	Sequence			Frequency
Motif 1: Cyclo(S)-Gly-Hrg^c-Xxxx^c-Xxx^c-Cys^c-NH$_2$				
0.24 U/mL	Hrg^c	Aib^c	Hpg^c	2
0.24 U/mL	Hrg^c	Leu^c	Hpg^c	2
0.24 U/mL	Hrg^c	Pro^c	Leu^c	1
0.017 U/mL	Hrg^c	Pro^c	Leu^c	1
0.24 U/mL	Hrg^c	Pro^c/pro^c	Met^c	1
0.24 U/mL	Hrg^c	Pro^c/pro^c	Tym^c	1
0.24 U/mL	Hrg^c	Pro^c/pro^c	Cha^c	1
Motif 2: Cyclo(S)-Gly-pro^c-Xxx^c-Xxx^c-Cys^c-NH$_2$				
0.24 U/mL	pro^c	Ala^c	Arg^c	1
0.017 U/mL	pro^c	Ala^c	Arg^c	3
0.24 U/mL	pro^c	Pro^c	Arg^c	1
0.017 U/mL	pro^c	Pro^c	Arg^c	2
0.24 U/mL	pro^c	Pro^c	Hrg^c	1
0.017 U/mL	pro^c	Pro^c	Hrg^c	1
0.24 U/mL	Pro^c/pro^c	Pro^c/pro^c	Drg^c	1
0.24 U/mL	Pro^c/pro^c	Thr^c	Drg^c	1
0.24 U/mL	Pro^c/pro^c	Ala^c	Drg^c	1
0.017 U/mL	Pro^c/pro^c	Thr^c	Arg^c/arg^c	1
0.017 U/mL	Pro^c/pro^c	Ala^c	Hrg^c	1
0.017 U/mL	Pro^c/pro^c	Val^c	Drg^c	1

Table 2. Sequence motifs from oligocarbamate cyclic tetramer library

Receptor concentration	Sequence			
Motif 3: Cyclo(S)-Gly-Xxx^c-Tym^c-arg^c-Xxx^c-Cyc^c-NH$_2$				
0.017 U/mL	ser^c	Tym^c	arg^c	Ala^c
0.017 U/mL[†]	Hpb^c	Tym^c	arg^c	Val^c
0.008 U/mL[†,††]	Hpg^c	Tym^c	arg^c	Pro^c
	Hpg^c	Tym^c	arg^c	pro^c
0.017 U/mL	Glu^c/g;u^c	Tym^c	arg^c	Pro^c/pro^c
0.017 U/mL	Hpm^c	Tym^c	Arg^c/arg^c	Val^c
Motif 4: Cyclo(S)-Gly-Xxx^c-ind^c-arg^c-Xxx^c-Cys^c-NH$_2$				
0.008 U/mL	Gly^c	ind^c	arg^c	Thr^c
0.017 U/mL	Arg^c/arg^c	Ind^c/ind^c	Arg^c/arg^c	Glu^c/glu^c
0.017 U/mL	Pro^c/pro^c	Ind^c/ind^c	Arg^c/arg^c	Thr^c
Motif 5: Cyclo(S)-Gly-Xxx^c-Drg^c-Glu^c/glu^c-Xxx^c-Cys^c-NH$_2$				
0.008 U/mL	Arg^c/arg^c	Drg^c	Glu^c/glu^c	Ala^c
0.008 U/mL	Hrg^c	Drg^c	Glu^c/glu^c	Thr^c
'Orphan' sequences: Cyclo(S)-Gly-Xxx^c-Xxx^c-Xxx^c-Xxx^c-Cys^c-NH$_2$				
0.017 U/mL	ser^c	Pro^c	Tym^c	Arg^c
0.017 U/mL	Ind^c/ind^c	Hpb^c	Arg^c/arg^c	Arg^c/arg^c
0.008 U/mL	Pro^c/pro^c	Pro^c/pro^c	Drg^c	Ind^c/ind^c

[†]Sequence occurred twice.
[††]Both diasteremoers found to be active.

Table 3. Sequence motifs from oligocarbamate linear tetramer library

Receptor concentration	Sequence				
Motif 6: Ac^c-(Xxx^c)-Pro^c-Xxx^c-Hrg^c-(Xxx^c)-NH$_2$					
0.17 U/mL		Pro^c	Tyn^c	Hrg^c	Gly^c
0.17 U/mL		Pro^c	Cha^c	Hrg^c	Gly^c
0.17 U/mL[†]		Pro^c	Cha^c	Hrg^c	Glu^c
0.033 U/mL	Glu^c	Pro^c	Tym^c	Hrg^c	
0.17 U/mL	Gly^c	Pro^c/pro^c	Cha^c	Hrg^c	
0.17 U/mL	Thr^c	Pro^c/pro^c	Cha^c	Hrg^c	
0.033 U/mL	Asp^c/asp^c	Pro^c/pro^c	Cha^c	Hrg^c	
Motif 7: Ac^c-Xxx^c-Pro^c-Xxx^c-Arg^c-NH$_2$					
0.17 U/mL	ser^c	Pro^c	Tym^c	Arg^c	
0.033 U/mL	ser^c	Pro^c	Tym^c	Arg^c	
0.17 U/mL	Gly^c	Pro^c	Cha^c	Arg^c	
0.033 U/mL	Ala^c	Pro^c/pro^c	Tym^c	Arg^c	
Motif 8: Ac^c-(Xxx^c)-Ind^c-Cha^c-Xxx^c-(Xxx^c)-NH$_2$					
0.17 U/mL		Ind^c	Cha^c	Hrg^c	Val^c
0.17 U/mL		Ind^c/ind^c	Cha^c	Hrg^c	
0.17 U/mL	Hpm^c	Ind^c/ind^c	Cha^a	Arg^c/arg^c	
Motif 9: Ac^c-(Xxx^c)-Tym^c-arg^c-Xxx^x-(Xxx^c)-NH$_2$					
0.033 U/mL		Tym^c	arg^c	Val^c	Gly^c
0.17 U/mL	Gly^c	Tym^c	Arg^c/arg^c	Thr^c	
'Orphan' sequences: Ac^c-Xxx^c-Xxx^c-Xxx^c-Xxx^c-NH$_2$					
0.033 U/mL	pro^c	Tym^c	Gly^c	arg^c	
0.033 U/mL	Arg^c	Ala^c	Val^c	Hrg^c	
0.17 U/mL	Asp^c/asp^c	Pro^c/pro^c	Drg^c	Pro^c/pro^c	
0.033 U/mL	Leu^c	Tym^c	Drg^c	Glu^c/glu^c	
0.033 U/mL	Gly^c	Ind^c/ind^c	Val^c	Hrg^c	
0.033 U/mL	Hpg^c	Cha^c	Hrg^c	Hpm^c	

[†]Sequence occurred twice.

Table 4. Thrombin inhibition by cyclic trimer oligocarbamates

Carbamate	Sequence	Apparent K_i
	BMS-183507	8.5 ± 0.5 nM
1	Cyclo(S)-Gly-pro^c-Pro^c-Hrg^c-Cys^c-NH$_2$	760 ± 60 nM
2	Cyclo(S)-Gly-pro^c-Pro^c-Arg^c-Cys^c-NH$_2$	960 ± 60 nM
3	Cyclo(S)-Gly-Hrg^c-Pro^c-Leu^c-Cys^c-NH$_2$	1.0 ± 0.1 μM
4	Cyclo(S)-Gly-pro^c-Ala^c-Arg^c-Cys^c-NH$_2$	2.3 ± 0.3 μM
5	Cyclo(S)-Gly-Hrg^c-Aib^c-Hpg^c-Cys^c-NH$_2$	2.9 ± 0.4 μM

Table 5. Thrombin inhibition by cyclic tetramer oligocarbamates

Carbamate	Sequence	Apparent K_i
	BMS-183507	8.5 ± 0.5 nM
6	Cyclo(S)-Gly-Hpb^c-Tym^c-arg^c-Val^c-Cys^c-NH_2	31 ± 2 nM
7	Cyclo(S)-Gly-ser^c-Tym^c-arg^c-Ala^c-Cys^c-NH_2	390 ± 40 nM
8	Cyxlo(S)-Gly-Hpb^c-Tym^c-arg^c-Pro^c-Cys^c-NH_2	470 ± 40 nM
9	Cyclo(S)-Gly-Hpb^c-Tym^c-arg^c-pro^c-Cys^cNH_2	470 ± 40 nM
10	Cyclo(S)-Gly-Hpb^c-Pnf^c-arg^c-Val^c-Cys^c-NH_2	510 ± 20 nM
11	Cyclo(S)-Gly-Hpb^c-Phe^c-arg^c-Val^c-Cys^cNH_2	980 ± 190 nM
12	Cyclo(S)-Gly-Hpg^c-Cha^c-arg^c-Val^c-Cys^c-NH_2	2.6 ± 0.3 μM
13	Cyclo(S)-Gly-ser^c-Pro^c-Tym^c-Arg^c-Cys^c-NH_2	3.5 ± 0.4 μM
14	Cyclo(S)-Gly-Gly^c-ind^c-arg^c-Thr^c-Cys^c-NH_2	> 100 μM

and S3 sites of thrombin and are specific for positively charged and hydrophobic residues, respectively. Structure–activity studies of BMS-183507 suggested that electron-deficient aromatic systems in thrombin ligands improve binding affinity (E. J. Iwanowicz, personal communication). To determine whether a similar trend would be seen in oligocarbamate ligands, we replaced the Tym^c residue in carbamate **6** with three other large hydrophobic groups, Cha^c, Phe^c, and Pnf^c (the *p*-nitrophenylalanine carbamate derivative). The three resulting derivatives, carbamate **10** (Cyclo(S)-Gly-Hpb^c-Pnf^c-arg^c-Val^c-Cys^c-NH_2, K_i = 510 nM), carbamate **11** (Cyclo-(S)-Gly-Hpb^c-Phe^c-arg^c-Val^c-Cys^c-NH_2, K_i = 980 nM), and carbamate **12** (Cyclo(S)-Gly-Hpb^c-Cha^c-arg^c-Val^c-Cys^c-NH_2, K_i = 2.6 μM), had at least 15-fold lower affinity than the parent compound. Two other sequences from the cyclic tetramer were of even lower affinity. Carbamate **13** (Cyclo(S)-Gly-ser^c-Pro^c-Tym^c-Arg^c-Cys^c-NH_2, K_i = 3.5 μM) was an orphan sequence that is similar in form to the linear motif 7 (Ac^c-Xxx^c-Pro^c-Xxx^c-Arg^c-NH_2). Carbamate **14**, (Cyclo(S)-Gly-Gly^c-ind^c-arg^c-Thr^c-Cys^c-NH_2, K_i > 100 μM), a member of motif 4 (Cyclo(S)-Gly-Xxx^c-ind^c-arg^c-Xxx^c-Cys^c-NH_2), did not inhibit thrombin activity. The cyclic tetramer framework, perhaps due to its greater flexibility and broader range of functional group presentation, thus has ligands that are of significantly higher affinity than seen for the cyclic trimer framework.

The linear oligocarbamate ligands showed a similarly wide range of thrombin inhibition. Ligands from motif 6 (Ac^c-(Xxx^c)-Pro^c-Xxx^c-Hrg^c-(Xxx^c)-NH_2), which had the most members, generally showed the highest level of inhibitory activity (Table 6). Within the Ac^c-(Xxx^c)-Pro^c-Xxx^c-Hrg^c-(Xxx^c)-NH_2 framework, five ligands had Cha^c as the residue between -Pro^c- and -Hrg^c-, while two ligands had Tym^c (Table 3). Correspondingly, ligands with Cha^c as the central residue had higher binding affinity: carbamate **15** (Ac^c-Pro^c-Cha^c-Hrg^c-Gly^c-NH_2, K_i = 100 nM) bound almost threefold more tightly than carbamate **18** (Ac^c-Pro^c-Tym^c-Hrg^c-Gly^c-NH_2, K_i = 290 nM). In addition, ligands with Gly^c at the C-terminus bound slightly more tightly than those that did not; carbamate **15** had approximately 1.3-fold higher inhibitory activity than carbamate **16** (Ac^c-Pro^c-Cha^c-Hrg^c-Glu^c-NH_2, K_i = 130 nM). Moreover, the guanidinium-containing residue was generally in the third position, since carbamate **23** (Ac^c-Glu^c-Pro^c-Tym^c-Hrg^c-NH_2, K_i = 3.7 μM) with Hrg^c in position 4 had considerably lower affinity than carbamate **18**. In addition to the selected ligands, we synthesized two other derivatives to test the specificity of the presumed interaction between this class of ligands and the thrombin S3 pocket. Substitution of Cha^c with Phe^c resulted in a ninefold reduction in binding affinity (carbamate **20**, Ac^c-Pro^c-Phe^c-Hrg^c-Glyc-NH_2, K_i = 890 nM), while substitution with Pnf^c had similar effects (carbamate **22**, Ac^c-Pro^c-Pnf^c-Hrg^c-Gly^c-NH_2, K_i = 1.0 μM).

Table 6. Thrombin inhibition by linear tetramer oligocarbamates

Carbamate	Sequence	Apparent K_i
	BMS-183507	8.5 ± 0.5 nM
15	Ac^c-Pro^c-Cha^c-Hrg^c-Gly^c-NH_2	100 ± 10 nM
16	Ac^c-Pro^c-Cha^c-Hrg^c-Glu^c-NH_2	130 ± 3 nM
17	Ac^c-Gly^c-Pro^c-Cha^c-Arg^c-NH_2	230 ± 10 nM
18	Ac^c-Pro^c-Tym^c-Hrg^c-Gly^c-NH_2	290 ± 20 nM
19	Ac^c-Tym^c-arg^c-Val^c-Gly^c-NH_2	590 ± 170 nM
20	Ac^c-Pro^c-Phe^c-Hrg^c-Gly^c-NH_2	890 ± 260 nM
21	Ac^c-ser^c-Pro^c-Tym^c-Arg^c-NH_2	910 ± 100 nM
22	Ac^c-Pro^c-Pnf^c-Hrg^c-Gly^c-NH_2	1.0 ± 0.1 μM
23	Ac^c-Glu^c-Pro^c-Tym^c-Hrg^c-NH_2	3.7 ± 1.2 μM
24	Ac^c-pro^c-Tym^c-Gly^c-arg^c-NH_2	> 100 μM
25	Ac^c-Arg^c-Ala^c-Val^c-Hrg^c-NH_2	> 100 μM

The other motifs in the library represented other binding constructs. Rather than -Cha^c-Hrg^c- as a hydrophobic/guanidinium-containing monomer pair that serves as a recognition element, motif 9 (Ac^c-(Xxx^c)-Tym^c-arg^c-Xxx^c-(Xxx^c)-NH_2) features -Tym^c-arg^c-, which is highly analogous to the cyclic tetramer construct motif 3 (Cyclo(S)-Gly-Xxx^c-Tym^c-arg^c-Xxx^c-Cys^c-NH_2). The linear version, however, was considerably less potent than the cyclic version (carbamate **19**, Ac^c-Tym^c-arg^c-Val^c-Gly^c-NH_2, K_i = 590 nM). A number of the linear sequences, as observed for the cyclic tetramer series, did not fall into any of the defined sequence motifs. Two of these sequences were synthesized and characterized, and neither had thrombin inhibitory activity. These compounds could be binding in a region of the protein that is removed from the active site, or could have been selected due to an artifact in the screening process.

Discussion

We have described the screening of diverse combinatorial libraries of unnatural oligocarbamate biopolymers

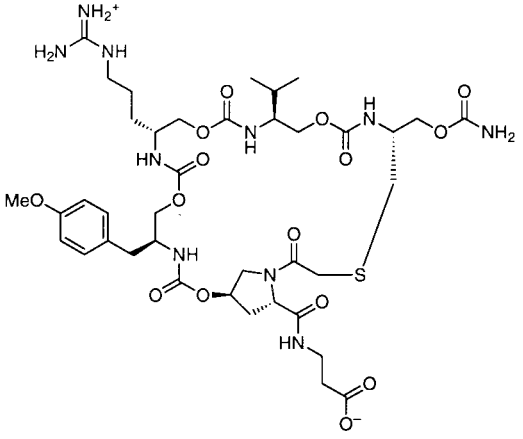
presented in three structural contexts for their ability to bind and inhibit the enzyme thrombin. This screen resulted in the identification of a cyclic tetramer with an inhibition constant of 31 nM. Carbamate-based oligomers of this sort may have a number of potential advantages compared to some of the thrombin inhibitors reported in the literature. These ligands are not mechanism-based protease inhibitors, and consequently do not have electrophilic groups that may lead to reduced stability and enhanced toxicity. The carbamate backbone is non-peptidic, and therefore is resistant to other proteases that could potentially cleave peptidic thrombin inhibitors. Moreover, the screening of combinatorial libraries opens the possibility of discovering previously unexpected modes of inhibition, as demonstrated by the selection of DNA aptamers that inhibit thrombin activity at nanomolar concentrations.[27] In addition, screening of oligocarbamate libraries against a protease is an important test of the utility of unnatural backbones as potential therapeutic compounds, since among natural receptors, proteases are among the most specific receptors for peptides. Several peptide-based thrombin inhibitors are known to make backbone-mediated parallel or anti-parallel β-sheet-type interactions with the protein, an interaction unavailable to the oligocarbamate backbone. Interactions between the protease and an oligocarbamate inhibitor will likely be primarily through side-chain contacts. Finally, in addition to the discovery of thrombin inhibitors and the examination of the oligocarbamate framework's molecular recognition properties, these studies also demonstrate a further application of the one bead, one peptide methodology for library synthesis.

Cyclic tetramer oligocarbamate ligands

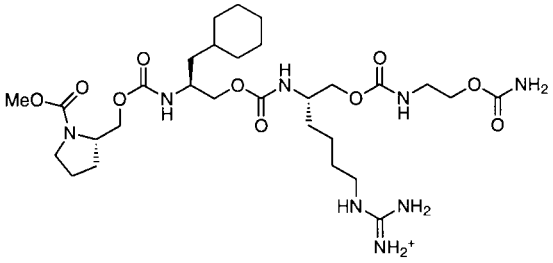
The ligands identified from the cyclic tetramer library show a strong sequence consensus within a motif. In addition, the highest affinity thrombin ligand found among the three libraries is a cyclic tetramer, carbamate **6** (Cyclo(S)-Gly-Hpb^c-Tym^c-arg^c-Val^c-Cys^c-NH_2, $K_i = 31$ nM, Figure 3). In contrast to Arg-containing peptide ligands, which have *S*-stereochemistry at the α-carbon, carbamate **6** binds to the S1 recognition site with the *R*-arg^c monomer. *R*-stereochemistry at the guanidinium-containing residue has been observed previously in the N^{α}-(2-naphthyl-sulphonyl-glycyl)-D-*p*-amidinophenylalanyl-piperidine (NAPAP) thrombin inhibitor (Fig. 3).[12]

NAPAP and carbamate **6** have other general similarities as well. The large hydrophobic naphthyl portion of NAPAP is separated from the benzamidine side chain by 5 atoms along the backbone, while the Tym^c side chain is separated from the arg^c side chain by 6 atoms along the backbone. Using the structure of NAPAP bound to trypsin, Bode et al. modeled NAPAP binding to thrombin.[12] In the modeled structure, the distal phenyl ring of the naphthalene portion of NAPAP was superimposable with the phenyl ring of D-Phe in the D-Phe-Pro-Arg-CH_2Cl structure. This phenyl ring has many interactions with the S3 specificity pocket,

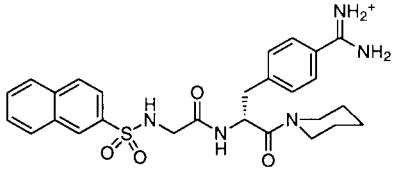
Carbamate **6**: Cyclo (S)
Gly-Hpb^c-Tym^c-arg^c-Val^c-Cys^c-NH_2



Carbamate **15**: Ac^c-Pro^c-Cha^c-Hrg^c-Gly^c-NH_2



N^{α}-(2-naphthyl-sulphonyl-glycyl)-
D-*p*-amidinophenylalanyl-piperidine (NAPAP)



BMS-183507: Guanidino-GABA-Phe-alloThr-Phe-OMe

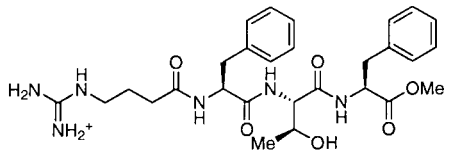


Figure 3. Thombin inhibitors. Structures of carbamates and other thrombin inhibitors referred to in the text.

including Trp215 and Ile174. It is likely that Tym^c in carbamate **6** makes similar contacts with thrombin. In addition, Tym^c is the optimal residue in this position in the carbamate **6** framework, since substitution with Phe^c, Pnf^c, or Cha^c (carbamates **10**–**12**) in this position resulted in a greater than 10-fold loss in affinity. Since there is an almost 100-fold loss in activity compared to the Cha^c derivative, there is most likely an aromatic–aromatic interaction in this pocket. However, the nature of this interaction is unclear without structural information, since the electronic character of the ligands does not correlate simply with binding affinities. For example, the electron-poor carbamate **10** (Cyclo(S)-Gly-Hpb^c-Pnf^c-arg^c-Val^c-Cys^c-NH_2, $K_i = 510$ nM) has a higher affinity than the unsubstituted phenyl-containing carbamate **11** (Cyclo(S)-Gly-Hpb^c-Phe^c-arg^c-Val^c-Cys^c-NH_2, $K_i = 980$ nM), the converse of what is expected based on the high affinity of the electron rich Tym^c residue in carbamate **6**. Steric factors are therefore also likely to play an important part.

The roles of other residues within motif 3 (Cyclo(S)-Gly-Xxx^c-Tym^c-arg^c-Xxx^c-Cys^c-NH_2) are less clear. It is likely that hydroxyproline-type residues are preferred in the first position for conformational reasons, since all three monomers of this type (Hpm^c, Hpg^c, and Hpb^c) were present in selected sequences. Moreover, replacement of a hydroxyproline monomer with ser^c resulted in an order of magnitude loss in activity (carbamate **7**, Cyclo(S)-Gly-ser^c-Tym^c-arg^c-Ala^c-Cys^c-NH_2, $K_i = 390$ nM) compared to carbamate **6**. Position 4 in motif 3 shows a preference for small hydrophobic residues, which may bind in the P2 pocket in analogy to the piperidine moiety of NAPAP. Although hydrophobic, it is likely that substitution with Pro^c or pro^c monomers in this position deleteriously effects the conformation of the cyclic construct, since carbamates **8** and **9** both bind considerably less tightly than carbamate **6** (Cyclo(S)-Gly-Hpb^c-Tym^c-arg^c-Pro^c/pro^c-Cys^c-NH_2, $K_i = 440$ nM). Finally, cyclic oligomers can potentially bind with higher affinity to receptors than their linear counterparts since in the latter case more conformational entropy is lost upon binding to the receptor. Comparison of carbamate **6** with a linear analogue showed this to be the case: carbamate **19** (Ac^c-Tym^c-arg^c-Val^c-Gly^c-NH_2, $K_i = 590$ nM) has the same principal residues as carbamate **6** but binds nearly 20-fold less tightly.

Cyclic trimer oligocarbamate ligands

In contrast to the cyclic tetramer case, ligands from the cyclic trimer oligocarbamate library were grouped into two motifs, one defined by Hrg^c in the first position and a second defined by pro^c in the first position. The ligands from these two motifs were all of modest affinity (760 nM to 2.9 μM), suggesting that the cyclic trimer framework does not have the optimal geometry for effective binding in the active site. Motif 1 (Cyclo(S)-Gly-Hrg^c-Xxx^c-Xxx^c-Cys^c-NH_2) uses the Hrg^c monomer to bind in the thrombin S1 pocket, as distinct from peptides, which generally use Arg. There is a preference for Pro^c in the second position and hydrophobic residues in the third, but no single residue predominates in the third position. The lack of a preference for a particular monomer in the third position suggests that binding is primarily dependent only on the guanidinium monomer in the first position, which may explain the low binding affinity of these residues. A similar situation is seen for the second motif (Cyclo(S)-Gly-pro^c-Xxx^c-Xxx^c-Cys^c-NH_2), although for this group of sequences there is variability in the guanidinium-containing monomers, with Drg^c, Arg^c, and Hrg^c all being found in the third position. In addition, no aromatic or large hydrophobic monomers are found in this motif's sequences, suggesting no strong interaction with the thrombin S3 pocket.

Linear oligocarbamate ligands

Linear oligocarbamate ligands bound thrombin with lower affinity than the cyclic tetramer carbamate **6,** but many had significantly higher affinity than ligands with the cyclic trimer framework. All of the linear carbamates that inhibited thrombin had a large hydrophobic residue (generally Cha^c or Tym^c) followed by a guanidinium-containing residue (generally Hrg^c). The overall binding mode for these ligands is likely to be similar to that described for carbamate **6**, with the guanidinium residue binding in the S1 pocket and the hydrophobic residue binding in the S3 pocket. However, there are distinct differences in both sites. The linear ligands have a preference for Hrg^c compared to Arg^c, suggesting a looser fit compared to carbamate **6** or many of the peptide ligands. In the hydrophobic site, Cha^c is preferred compared with the aromatic monomers Tym^c, Phe^c, and Pnf^c. The difference in binding is probably due to differences in the shape of the monomers: aromatic systems are flat while the cyclohexyl system is in a chair conformation. The roles of the other residues in the linear sequences are less clear. In the first position, there is preference for Pro^c among the high affinity ligands, but the Ind^c monomer was also found in the selected sequences. A similar situation is observed for the fourth position, where ligands that have inhibitory activities within a factor of 2 have the monomers Gly^c and Glu^c.

Use of oligocarbamate libraries for screening multiple receptors

The oligocarbamate libraries screened in this study have been previously screened for binding to the integrin GPIIb/IIIa.[20] GPIIb/IIIa is thought to bind Arg-Gly-Asp sequences in a β-turn configuration; the higher affinity of cyclic peptides compared to linear peptides for GPIIb/IIIa is consistent with this notion, since cyclic peptides may be preorganized in β-turn configurations.[23] The results from the screen of the oligocarbamate libraries were in broad agreement with the peptide results. The two highest affinity ligands (Cyclo(S)-Gly-asp^c-ind^c-Arg^c-Cys^c-NH_2, $IC_{50} = 3.9$ nM; Cyclo(S)-Gly-phe^c-Hrg^c-Hrg^c-asp^c-Cys^c-NH_2, $IC_{50} = 4.9$ nM) were a cyclic trimer and a cyclic tetramer.[20] In contrast, thrombin binds peptide ligands in an extended conformation, forming hydrogen bonds with ligands in a β-sheet-like fashion.[2] However, the highest affinity

oligocarbamate ligand found in these screens was a cyclic tetramer, which bound thrombin with a 19-fold higher affinity than a comparable linear ligand. These results suggest that the advantages in ligand binding affinity conferred by a cyclic structure are not confined to receptors that bind to ligands in an apparently cyclic conformation, as was the case with GPIIb/IIIa. This conclusion is highly dependent on the nature of the cyclic framework, however, since in these studies none of the cyclic trimer ligands bound with high affinity. The unpredictable results from both of these screens, both in terms of optimal framework and particular sequences, underscore the importance of screening diverse combinatorial libraries that have distinct core structures for the discovery of high affinity ligands.

Screening of oligocarbamates on solid support

Many of the applications of the one bead, one peptide method of library synthesis have centered on antibody epitope mapping. More recently, this methodology has been extended to the discovery of protease inhibitors. In one study, low-nanomolar inhibitors of chymotrypsin were identified from a library of 8000 cyclic peptides.[28] All of the selected ligands were found to have high activity. In a second study, an encoded peptide library was screened for binding to thrombin. A two-stage screen was used in which binding was shown to be directed to the active site by a second incubation of beads with thrombin and a competitive inhibitor.[19] Of 15 beads originally selected for binding to thrombin, only 5 were found to contain peptides that bound in the active site. While the screen used by Vágner et al. used biotinylated-thrombin coincubated with streptavidin–alkaline phosphatase, our screen consists of three steps and uses polyclonal antibodies rather than streptavidin to detect thrombin binding. Since the assays were performed with different libraries, they cannot be compared directly; however, there are similarities and differences of note. The three-stage antibody assay also identified sequences that did not bind to the active site. Of the carbamate ligands tested, carbamates **14** (Cyclo(S)-Gly-Gly^c-ind^c-arg^c-Thr^c-Cys^c-NH_2), **24** (Ac^c-pro^c-Tym^c-Gly^c-arg^c-NH_2), and **25** (Ac^c-Arg^c-Ala^c-Val^c-Hrg^c-NH_2) did not inhibit thrombin activity. Carbamate **14** is a member of motif 4 (Cyclo(S)-Gly-Xxx^c-ind^c-arg^c-Xxx^c-Cys^c-NH_2), while carbamates **24** and **25** are orphan linear sequences. Motif 4 has 3 members, and 6 orphan linear tetramer sequences were found, suggesting that 9 of 66 oligocarbamates (14%) found in the screen do not inhibit thrombin activity with s-2238 as a substrate. The screen using biotinylated-thrombin found 10 of 15 beads (67%) had peptide ligands which did not bind in the active site. It is uncertain in either case whether these ligands are binding thrombin in a region distant from the active site or they are false positives from some other source. The differences between the portion of false positives found in the two assay systems could result from the stringency level. Inclusion of streptavidin with biotinylated-thrombin would result in a multivalent interaction in the primary incubation, which would presumably lower the stringency of the screen. While antibodies are also multivalent, use of a three-stage screen leaves the primary incubation as a monovalent interaction. In general, these results suggest that thrombin is a more difficult receptor to work with in the one bead, one peptide format. No such false positives were found in our previous work with the integrin GPIIb/IIIa.

In conclusion, we have described the discovery of a series of oligocarbamate inhibitors of thrombin from diverse combinatorial libraries. These ligands can serve as lead compounds for the development of higher affinity ligands that may be effective as therapeutics. In addition, this work takes advantage of previously synthesized oligocarbamate libraries suggesting that oligocarbamate ligands can be found against a wide range of protein targets, as long as the proteins are compatible with ELISA type formats.

Experimental

Materials

Human thrombin for use in screening oligocarbamate libraries was obtained from Böhringer Mannheim, and human thrombin for use in enzymatic assays was obtained from Sigma. Thrombin substrate S-2238 was from Diapharma (Franklin, OH) and used without further purification. Polyclonal sheep anti-human thrombin was from Accurate Chemical and Scientific Corp. (Westbury, NY), and alkaline phosphatase-conjugated donkey anti-sheep antibody was from Sigma. Materials used for chemistry are as previously listed.[20]

Chemistry

The solid-phase synthesis of linear and cyclic oligocarbamates[20] has been previously described. The oligocarbamate libraries used in this study were previously synthesized and screened for their ability to bind to the integrin receptor GPIIb/IIIa. The detailed characterization of carbamates **6** (Cyclo(S)-Gly-Hpb^c-Tym^c-arg^c-Val^c-Cys^c-NH_2) and **16** (Ac^c-Pro^c-Cha^c-Hrg^c-Gly^c-NH_2) by 2-D ^{1}H NMR has also been previously described.[20]

Assay of oligocarbamate libraries for binding to thrombin

Cyclic and linear oligocarbamate libraries were screened for binding to thrombin using a solid-phase screening procedure similar to that previously described for screening with GPIIb/IIIa. Samples of beads (2 mg for the cyclic trimer library, 5 mg for the cyclic and linear tetramer libraries) were washed (3×0.3 mL×2 min) with blocking buffer (20 mM Tris, 150 mM NaCl, 2 mM $CaCl_2$, 0.02% NaN_3, 0.1% Tween-20, 1% BSA, pH 7.4). Following washing, the beads were blocked with blocking buffer for 1 h. The blocking buffer was then removed by centrifugation of the beads and removal of the supernatant, followed by treatment with varying concentrations of human thrombin (Böhringer-Mannheim) in blocking buffer (see tables for thrombin concentrations) for 45 min. The beads were washed with

blocking buffer (4×0.3 mL×1 min) and incubated with sheep anti-thrombin antibody (5×10^{-4} mg/mL in blocking buffer) for 1 h. The washing procedure was repeated and the beads were treated with alkaline phosphatase conjugated donkey anti-sheep antibody for 1 h, followed by final washes. Positive beads were stained by treatment with bromochloroindolyl phosphate and nitro blue tetrazolium as described.[21] Positive beads were picked and the oligocarbamates were cleaved and sequenced as previously described.[20]

Enzyme inhibition kinetics

The inhibitory activity of oligocarbamates was determined using a procedure similar to that found in previously published protocols.[4] Stock solutions of HPLC purified oligocarbamates were prepared by dissolving freshly lyophilized fractions in 50% DMF in thrombin assay buffer (145 mM NaCl, 5 mM KCl, 1 mg/mL polyethylene glycol-8000, 30 mM *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid, pH 7.4) at concentrations of 20–50 mM. Thrombin assay buffer (220 μL), inhibitor solution (10 μL), and human thrombin (Sigma, 10 μL) were mixed in an ELISA plate for 3 min. The reaction was initiated by addition of 60 μL of substrate s-2238 (50 μM), and monitored by reading the change in optical density at 405 nm on a Spectramax 250 kinetic plate reader (Molecular Devices). The thrombin concentration was set to give a total OD change of approximately 0.030 over 0.5 min with linearity over this time period. The K_m for s-2238 under these conditions with human thrombin from Sigma was 1.3 μM. The IC_{50} for oligocarbamate inhibitors was determined graphically from a plot of OD versus log[inhibitor] fit to a sigmoidal curve. The IC_{50} was related to the apparent K_i using the equation $K_i = IC_{50}/[1+(S/K_m)]$ as described.[22]

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