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Selectivity Enhancement Induced by Substitution of Non-natural Analogues of Arginine and Lysine in Arginine-Based Thrombin Inhibitors

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Abstract—Seven non-natural analogues of arginine and lysine have been substituted in an established arginine-based thrombin inhibitor. Four of the new compounds exhibited significant thrombin inhibition (K_i's 0.53–3.95 μM) and were subsequently tested for selectivity against trypsin. The two best compounds gave selectivity ratios of 962 and 525 (trypsin/thrombin), improving upon the parent compound. © 2001 Elsevier Science Ltd. All rights reserved.

The serine protease thrombin plays a multifunctional role in the regulation of thrombosis and hemostasis and can participate in the evolution of thrombotic disorders.[1] Consequently, the development of potent, selective, and orally bioavailable inhibitors of thrombin is of significant clinical interest; various peptide-based compounds containing arginine (Arg) or lysine (Lys) residues have entered clinical trials.[2] Failures in these trials were partially attributable to lack of oral bioavailability and selectivity for thrombin.[3]

Research in our laboratory focuses on the design, synthesis, and exploitation of non-natural analogues of Arg and Lys.[4–7] These compounds can effect increased receptor binding affinities and enhance partitioning through the blood–brain barrier[8] when incorporated into pharmaceutically relevant peptides such as neurotensin (8–13)[9–11] and bradykinin.[12] Since the analogues are more lipophilic[6] than their natural counterparts, enhanced oral bioavailability might be anticipated[13] when substituted into peptide-based thrombin inhibitors.

The analogues are also larger[6] than Arg and Lys, hence changes in selectivity also might be anticipated when substituted into peptides. This would be of great utility if desired receptor binding is maintained or enhanced. The objective of this study was to evaluate whether improvements in binding and selectivity could be induced by substitution of our analogues in the simple Arg-based thrombin inhibitor **1** (Fig. 1) previously described by Kikumoto et al.[14] and St. Laurent et al.[15] While these compounds are not of current interest as therapeutic candidates, their modification and study constitutes an initial evaluation of the potential of our analogues for substitution in the more chemically complex peptide-based thrombin inhibitors that fell out of clinical trials.

Synthesis

Guanylation of Nα-Dansyl-L-Orn-Pip with the requisite thiopseudourea hydriodide salt provided Arg derivatives **2**–**5** using methods previously developed in our laboratory (Scheme 1).[5,6]

Synthesis of the Lys analogues **6** and **7** required an asymmetric approach (Scheme 2).[4,6] First, 7-(*R*,*S*)-aminooctanoic acid was protected with benzyl chloroformate (Z-Cl) in the usual manner. Next, the oxazolidinone chiral auxiliary[16] was added in order to direct azide addition in an enantiospecific fashion. Following removal of auxiliary, the acid was activated with IBCF and amidated with piperidine. The azide was reduced with $SnCl_2$ and the resulting amine protected with 5-dimethylamino-1-naphthalene-sulfonyl chloride (DNS-Cl).

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Finally, the Z group was removed via catalytic hydrogenation to produce compounds **6** and **7**. Enantiomeric purity at the α-carbon (>95%) was assessed utilizing RP-HPLC. However, efforts to separate the diastereomers were unsuccessful and these analogues were used as a mixture.

Preparation of the ethylene linked (N^{δ}–N^{ω}) Arg analogue **8** (Scheme 3) began with amidation of (2*S*)-azido-5-bromovaleric acid[7] with piperidine as described above. The resulting product was reacted with ethylenediamine and CNBr to produce the cyclic guanidyl functionality. The azide was reduced with $SnCl_2$ subsequent to side-chain protection with Z-Cl. Finally, the α-amine was protected with dansyl-Cl and the Z group removed to produce **8**. All final products were subjected to RP-HPLC utilizing a Kontrosorb C-18 semiprep column and purified to >95%.

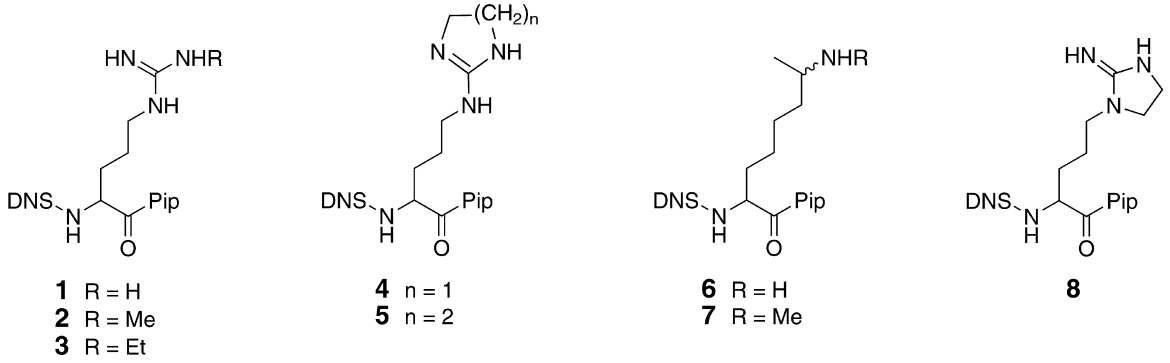
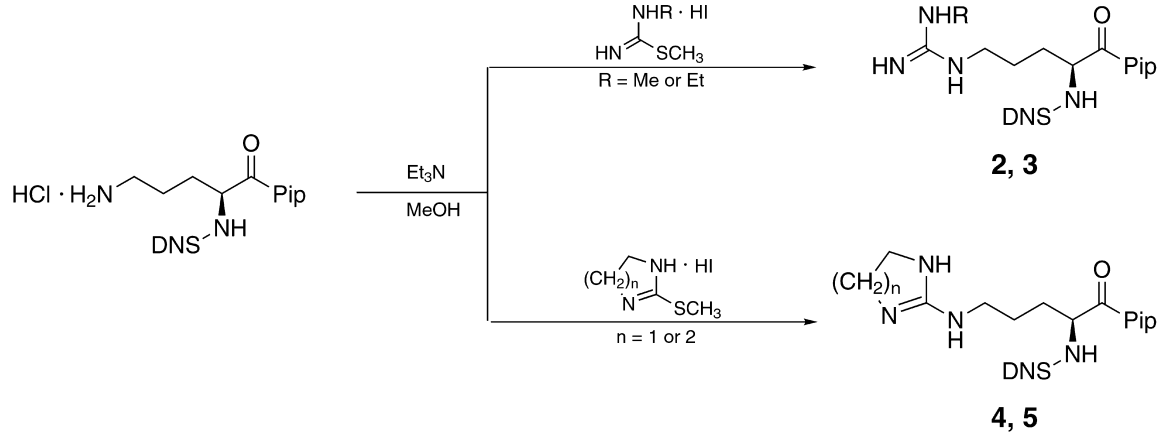
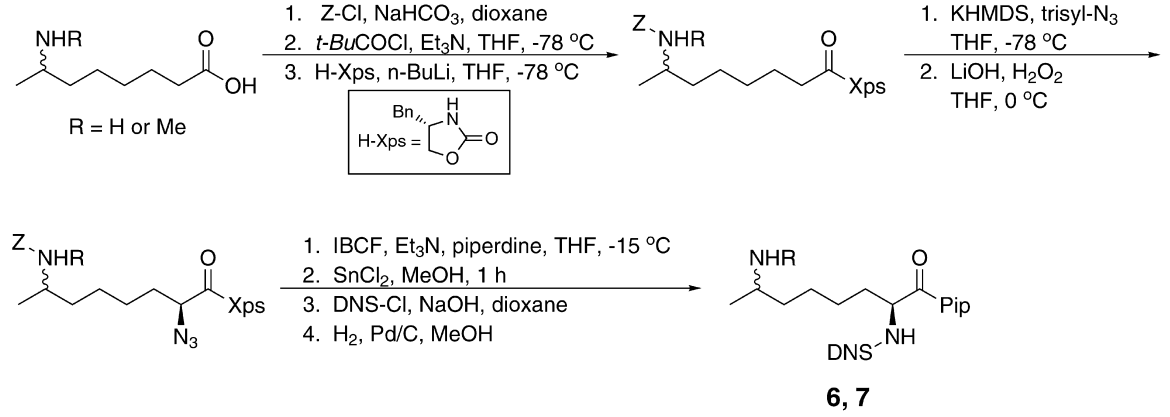


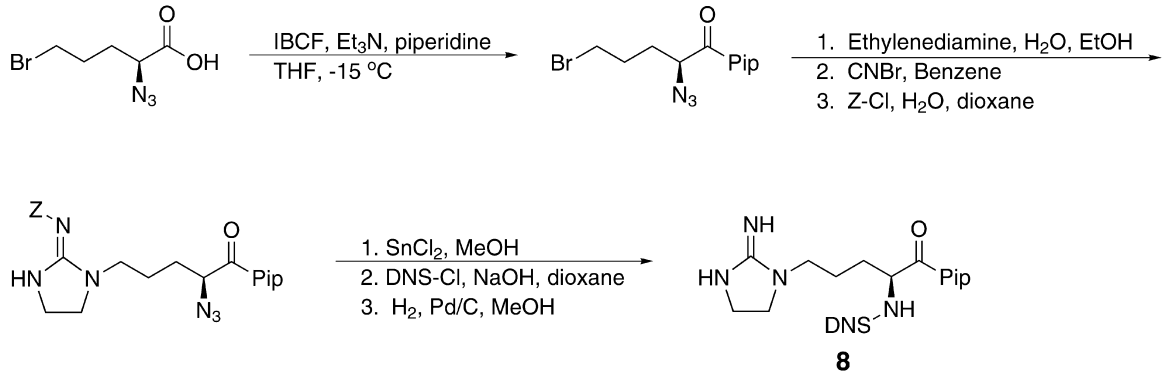
Figure 1. Arg-based thrombin inhibitor **1** and non-natural Arg (**2**–**5** and **8**) and Lys (**6** and **7**) derivatives prepared for this study (DNS-Cl = 5-dimethylamino-1-naphthalenesulfonyl chloride; Pip = piperidine).



Scheme 1. Synthesis of Arg derivatives **2**–**5** (DNS-Cl = 5-dimethylamino-1-naphthalenesulfonyl chloride; Pip = piperidine).



Scheme 2. Synthesis of Lys derivatives **6** and **7** (Z = benzyloxycarbonyl, IBCF = isobutylchloroformate, DNS-Cl = 5-dimethylamino-1-naphthalenesulfonyl chloride; Pip = piperidine).



Scheme 3. Synthesis of ethylene linked (N^{δ}–N^{ω}) Arg analogue **8** (DNS-Cl = 5-dimethylamino-1-naphthalenesulfonyl chloride; Pip = piperidine).

Characterization and Analysis

Thrombin and trypsin binding affinities, selectivities, and physiochemical properties of compounds **1**–**8** are summarized in Table 1. Compounds **2**–**4** and **8** exhibited significant thrombin inhibition although less than the parent compound (**1**). With the exception of compound **8**, increasing the amount of alkylation on the guanidinium side chain of the Arg analogues correlated to an increasing K_i, with **5** having a K_i at least 2 orders of magnitude greater than **1**. We hypothesize that the ethylene (N^{δ}–N^{ω}) linkage reduced the entropy of the side chain and enabled it to exist in a favored binding conformation, thus leading to a K_i of 530±40.0 nm. Compound **4** is of particular interest because the terminal ethylene linkage prevents the guanidinium side chain from interacting with Asp-189 of thrombin in the traditional fashion. This data suggests that the guanidinium portion of **4** binds to the active site of thrombin in a manner different from **1**–**3** and **8**.[17,18] In contrast, **5** failed to inhibit thrombin. This indicates that nonplanar structures are poorly tolerated by the enzyme. Likewise, the nonplanar Lys analogues (**6** and **7**) exhibited limited inhibition and were not evaluated further.

Compounds **1**–**4** and **8** were tested for selectivity against trypsin (chosen as the benchmark serine protease); compounds **2** and **8** exhibited significant increases in selectivity for thrombin. We hypothesize that thrombin's ability to accept larger lipophilic side chains in the guanidinium binding (S1) position than trypsin[19–22] accounted for the increased specificity. Therefore, one would predict compounds with a greater extent of alkylation would exhibit greater selectivity compared to **2** and **8**. However, compounds **3** and **4** are less selective. This finding reinforces our hypothesis that the guanidinium of compound **4** binds to thrombin in a manner different from that previously described. The expected relative binding exhibited by **3** and **4** to trypsin likely is a result of the entropy cost in fixing the ethyl group of **3** in the binding conformation of **4**.

Table 1. Binding affinity, selectivity and analytical data for compounds **1**–**8**

Compound	Thrombin K_i (nM)[a]	Trypsin K_i (μM)[b]	Selectivity ratio (Try/Thr)	Retention time (min)[c]	M_r (calcd:found)[d]
1	83.0±24.8	2.34±0.15	28.2	20.9±0.02	475.6:475.3
2	958±184	922±55.6	962	21.8±0.02	489.7:489.3
3	1890±65.6	337±27.7	178	23.1±0.01	503.7:503.3
4	3950±101	116±6.11	29.4	21.9±0.01	501.7:501.3
5	>10,000	—	—	—	515.7:515.3
6	>10,000	—	—	—	475.7:475.3
7	>10,000	—	—	—	489.7:489.3
8	530±40.0	278±3.61	525	22.3±0.01	501.7:501.3

[a]Thrombin binding assays were carried out in 1 mL cuvettes utilizing a Perkin–Elmer Lambda 4A UV/vis spectrophotometer. Inhibitor concentration was determined using an extinction coefficient of 4.3×10^6 cm^2/mol at $\lambda = 369$ nm.[23] Human α-thrombin (0.09 units) was incubated with inhibitor (0.1–4.0 μM) in buffer (1 mg/mL PEG 8000, 5 mM KCl, 145 mM NaCl, 30 mM HEPES, pH 7.4). Chromogenic substrate (D-Phe-Pip-Arg-pNA, 10–40 μM) was then added to a total volume of 1 mL and the change in optical density was monitored at 405 nm for 3 min. K_i was determined graphically according to the method of Dixon (plots of reciprocal enzyme velocity (1/v) versus inhibitor concentration at various enzyme substrate concentrations).

[b]Trypsin binding assays were carried out in 1 mL cuvettes utilizing a Perkin–Elmer Lambda 4A UV/vis spectrophotometer. Inhibitor concentration was determined using an extinction coefficient of 4.3×10^6 cm^2/mol at $\lambda = 369$ nm.[23] Bovine trypsin (43 units) was incubated with inhibitor (1.0–100.0 μM) in buffer (0.1 M Tris, 20 mM $CaCl_2$, pH 8.2). Chromogenic substrate (Bz-Arg-pNA, 46.0–184 μM) was then added to a total volume of 1 mL and the change in optical density was monitored at 405 nm for 3–5 min. K_i was determined graphically according to the method of Dixon (plots of reciprocal enzyme velocity (1/v) versus inhibitor concentration at various enzyme substrate concentrations).

[c]RP-HPLC: The mobile phase consisted of 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent B). Analytical HPLC was performed on a Waters HPLC system in combination with a Kontrasorb, C-18 column at a flow rate of 4 mL/min. A linear gradient from 15 to 30% B over 15 min was used and the effluent detected by UV absorbance at 369 nm. All peptides were of greater than 95% purity.

[d]An electrospray mass spectrum for each analogue was obtained using a Finnigan LCQ instrument. The mass spectrum of the $[M+H]^+$ ion for each analogue gave correct sequence data.

The addition of alkyl groups to the guanidinium side chain of Arg resulted in compounds that are more lipophilic than the parent compound as indicated by comparison of RP-HPLC retention times (Table 1). Compounds **3**, **4**, and **8**, which incorporate an ethyl(ene) group, are more lipophilic than **2**, which has a methyl group on the side chain. Furthermore, the ethyl group on **3** has more degrees of freedom and appears able to interact more favorably with the hydrophobic environment of the column's carbon packing when compared to the ring systems of **4** and **8**. Although losses in overall thrombin activity were noted, we hypothesize that the enhanced lipophilicities of these analogues, which may promote passive diffusion across the gastrointestinal tract, could lead to compounds with greater oral bioavailablity.

Summary

We have synthesized a series of thrombin inhibitors based upon non-natural analogues of Arg and Lys. This study represents the third system (with neurotensin and bradykinin) in which we have explored the utilization of these novel compounds. Previously, enhanced receptor binding and blood–brain barrier access were demonstrated; in this study, selectivity enhancements (with some potency losses) were observed. These results provide the impetus for further investigation of our analogues in alternate scaffolds with the objective of developing clinically suitable thrombin inhibitors.

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