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Conformationally restricted analogs of the direct thrombin inhibitor FM 19

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ABSTRACT

The serine protease thrombin plays several key roles in the clotting cascade within the hemostatic system, such as in fibrin formation and platelet activation. Thus, development of an inhibitor that binds to the enzyme's active site (a direct thrombin inhibitor) offers an approach for the treatment of thrombus-associated diseases. Previous structure–activity relationship studies originally based on the bradykinin breakdown product Arg-Pro-Pro-Gly-Phe (**RPPGF**) led to the development of lead compound **FM 19** (D-Arg-Oic-Pro-D-Ala-Phe(*p*-Me)-NH_2). The recently determined X-ray structure of **FM 19** in the active site of thrombin has revealed sites of modification to potentially improve inhibition. In this study, we report the synthesis and biological characterization of nine peptides that replace only the D-Arg residue of the **FM 19** sequence, investigating ways to add conformational restriction, modification of the basic moiety at the end of the side chain, and removal of the charge from the N-terminus. Two of these peptides, **6** and **7** (IC_{50} values of 0.51 and 0.45 μM, respectively), show similar potency to the best compounds in the **FM 19** series reported thus far.

1. Introduction

The serine protease thrombin plays a central role in the hemostatic system and coagulation.[1] One main function of thrombin is the conversion of soluble fibrinogen into insoluble fibrin, the protein that cross-links with platelets to form a thrombus.[2] In addition, thrombin is an effective platelet activator, via the protease-activated receptors (PARs) 1 and 4 present on the platelet cell surface.[3] The PARs, which are G protein-coupled receptors, can send downstream signals resulting in platelet activation and aggregation.[3] Thus, thrombin is an attractive target for modulation of the hemostatic system and the prevention of thrombus formation.

Due to its attractiveness as a target for anticoagulation therapy, research focused on thrombin inhibition has been pursued by several groups for at least the last 25 years.[4,5] The 65 amino acid coagulation protein originally isolated from leeches, hirudin, was one of the early examples of the therapeutic potential of thrombin inhibition. Hirudin is a well-studied, bivalent direct thrombin inhibitor (DTI), which binds to both the enzyme's active site as well as an auxiliary site, exosite I, with a sub-picomolar K_i.[6,7] Since this discovery, many compounds have been developed as thrombin inhibitors. Some work as bivalent inhibitors, similar to hirudin,[4,6,7] others work through allosteric inhibition by binding to another auxillary site, exosite II,[8,9] and still others work as univalent DTIs, and bind only to the enzyme's active site.[4–6]

Dabigatran, marketed as the prodrug dabigatran etexilate,[10] has recently been approved by the FDA as the first orally available univalent DTI. When compared to warfarin and related compounds, the only orally available agents indicated for anticoagulation treatment,[11] dabigatran was shown to be at least as effective at preventing stroke in patients with atrial fibrillation, while also showing a similar or decreased risk of major hemmorage.[10] In addition, since dabigatran shows fewer interactions with food and other drugs and does not require frequent monitoring and dose adjustment, dabigatran finally offers an alternative to warfarin treatment[10] and shows that anticoagulation can be achieved by orally administered direct thrombin inhibitors.

Our development of a novel direct thrombin inhibitor was initially based upon the endogenous bradykinin breakdown product Arg-Pro-Pro-Gly-Phe (**RPPGF**) (Fig. 1), which was shown to inhibit thrombin substrate cleavage.[12] Structure–activity relationship (SAR) studies led to the development of the lead compound **FM 19**, with the sequence D-Arg-Oic-Pro-D-Ala-Phe(*p*-Me)-NH_2,[13,14] where Oic is (2*S*,3*aS*,7*aS*)-octahydroindole-2-carboxylic acid (Fig. 1). **FM 19** has been shown to be effective at prolonging thrombin clotting times and delaying carotid artery thrombosis following oral administration in mice and thus represents a promising alternative lead toward the development of DTIs. Although the potency of **FM 19** is rather low, the X-ray structure of **FM 19** in the active

Abbreviations: DTI, direct thrombin inhibitor; HOBt, hydroxybenzotriazole; HBTU, *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uroniumhexafluorophosphate; DIEA, *N*,*N*-diisopropylethylamine; Oic, (2*S*,3*aS*,7*aS*)-octahydroindole-2-carboxylic acid; Phe(*p*-Me), *p*-methyl phenylalanine; NMP, *N*-methyl-2-pyrrolidone; TFA, trifluoroacetic acid; THF, tetrahydrofuran; PAR, protease-activated receptor; SAR, structure–activity relationships; Boc, *tert*-butoxycarbonyl; Fmoc, fluorenylmethyloxycarbonyl.

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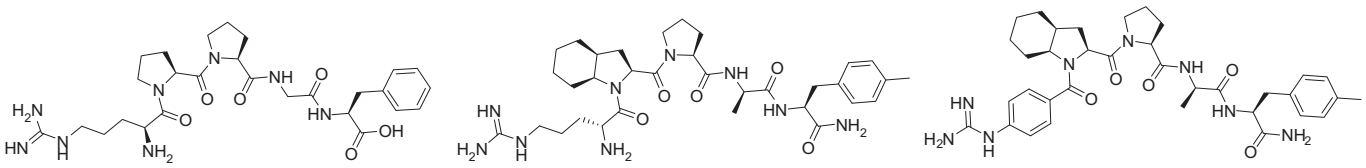


Figure 1. Structures of pentapeptides **RPPGF** (left), **FM 19** (middle) and compound **1** (right).

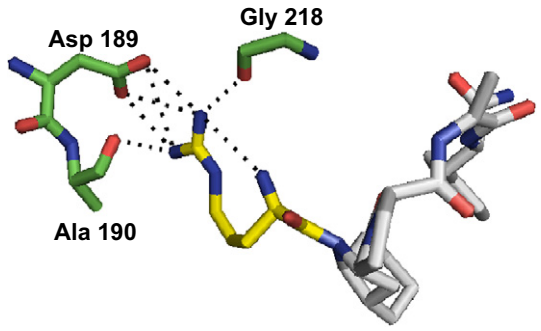


Figure 2. Structure of **FM 19** in the active site of thrombin (PDB 3BV9, 1.8 Å). The D-Arg residue is highlighted in yellow, with key interacting residues of thrombin shown in green.

site of thrombin[15] (Fig. 2), has guided the SAR for improved inhibitors. Previously, we have reported replacements to the D-Arg residue of the **FM 19** sequence, and two of these peptides show a further increase in potency over **FM 19**. One of these pentapeptides, compound **1** (Fig. 1), also adds conformational restriction to this side chain.[16] In this study, we investigate additional replacements of this crucial residue, to better understand the structural features important for thrombin inhibition.

2. Results and discussion

Nine new replacements for the D-Arg residue in the **FM 19** sequence were synthesized and are reported here. These replacements were guided by insights gained from the X-ray structure of **FM 19** in the active site of thrombin[15] (Fig. 2), and are summarized in Figure 3. From the crystal structure, we can first see that

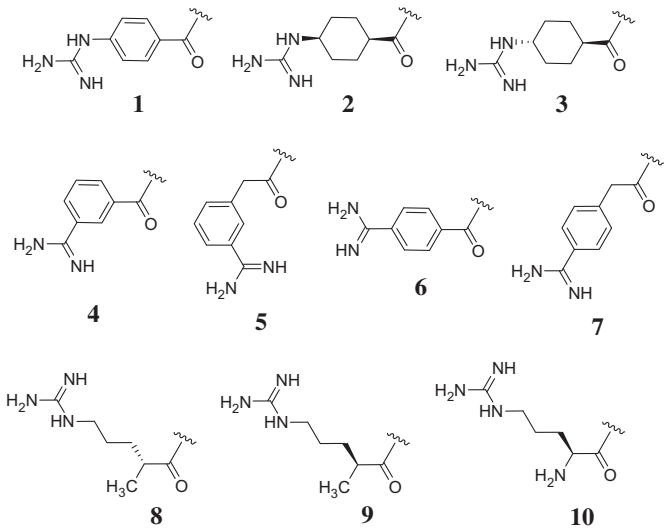


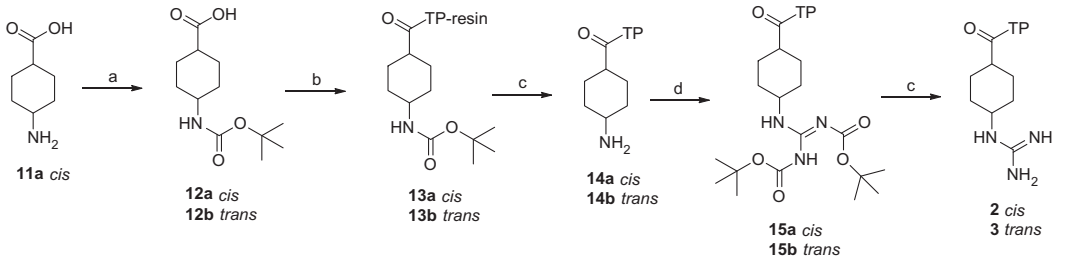
Figure 3. Structures of D-Arg replacements (**X**) in **FM 19** sequence (**X**-Oic-Pro-D-Ala-Phe(*p*-Me)-NH_2) for compounds **1**–**10**.

the two positively charged groups of **FM 19**, the N-terminal amine and guanidino group on the side chain of D-Arg, are in close proximity, creating an unfavorable electrostatic interaction. This can best be alleviated by removal of the N-terminal amine, since the guanidino group makes key interactions with active site thrombin residues Asp 189, Ala 190 and Gly 218, but the N-terminal amine does not participate directly in binding to thrombin.[15] Thus, all replacements except **10** lack the N-terminal amine. Additionally, the D-Arg side chain of **FM 19** assumes a high energy, eclipsed conformation when bound to thrombin. To reduce this energetic penalty, we have designed modifications to stabilize this eclipsed side chain orientation, such as in compounds **1**,[16] **2** and **3**. We also replaced the guanidino with another basic group, an amidino moiety, to explore another way to split the positive charge between more than one atom at the end of the side chain. This splitting of the positive charge at the end of the amino acid side chain allows for improved potency over a simple point charge, due to the side chain's ability to better interact with the catalytic Asp 189.[17] An amidino moiety has been shown to be effectively incorporated in the non-peptide direct thrombin inhibitor argatroban.[17] Here we used this functional group while also adding conformational restriction, as seen in compounds **4**–**7**. Finally, we investigated replacing the N-terminal amine with another group of comparable size, a methyl group (compound **8**), while still maintaining the same stereochemical conformation, to see how removal of only the positive charge impacts potency. For comparison, we have also made the methyl and amino containing replacements with the opposite stereochemistry (compounds **9** and **10**, respectively). Analytical data for all compounds can be found in Table 2.

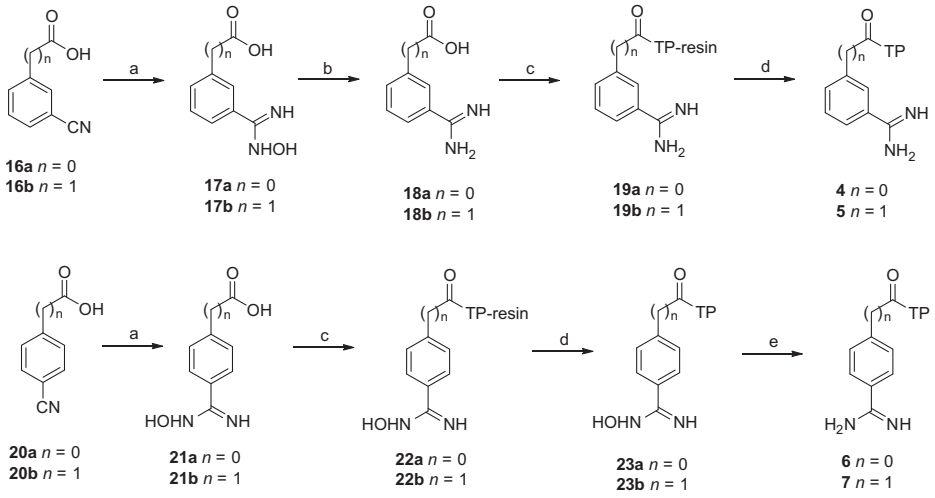
2.1. Chemistry

First, two saturated cyclic analogs of **1** were synthesized using cyclohexanoic acids, with the guanidino group and acid moieties in either the *cis* or *trans*-configurations, compounds **2** and **3**, respectively (Scheme 1). The starting material for the *cis* analog was purchased as **11a**, and the amino group was protected with a *tert*-butoxycarbonyl (Boc) group as **12a**. The starting material for the *trans* analog was purchased with the amine already protected, as **12b**. These acids could then be coupled to the Oic-Pro-D-Ala-Phe(*p*-Me) tetrapeptide on the solid phase resin to yield pentapeptides **13a** and **13b**, followed by cleavage from the resin and simultaneous deprotection of the cyclohexanoic acid residue amino group, resulting in **14a** and **14b**. The amine was converted to a di-Boc protected guanidino group[18] producing **15a** and **15b**, which not only installed the desired functional group into the final peptide, but also allowed for easier purification from the amine-containing peptides. The Boc protecting groups were then removed, giving pentapeptides **2** and **3**, ready for testing.

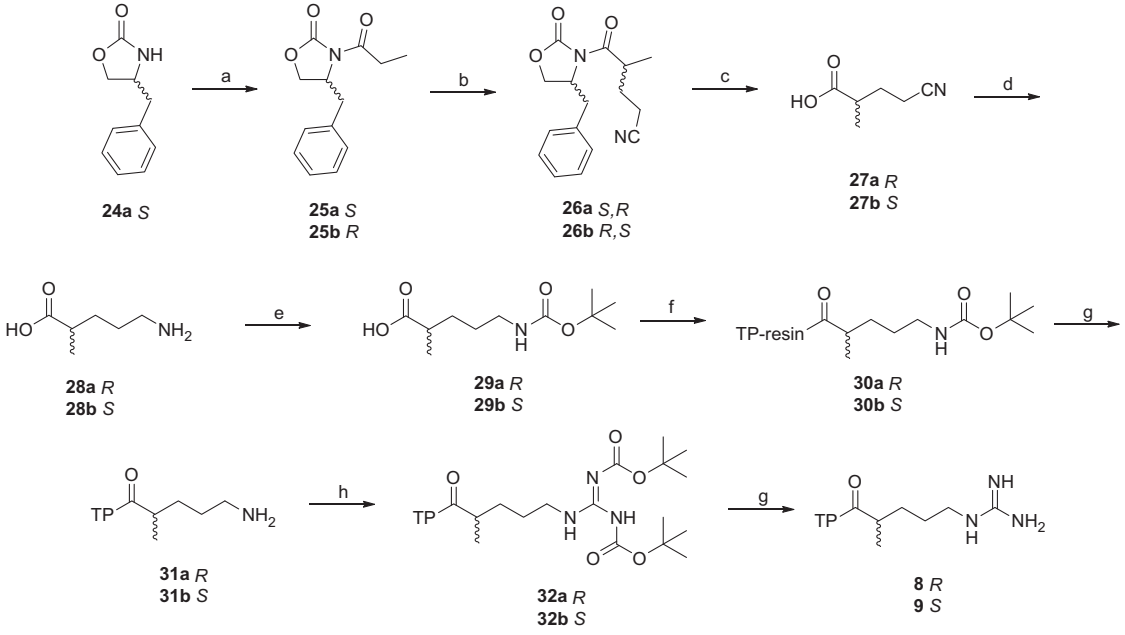
Next, we synthesized four replacements containing an unsaturated ring as in **1**, but replacing the guanidino group with an amidino group. This was done with both benzoic acid (**4** and **6**) and phenylacetic acid (**5** and **7**) scaffolds, substituted in the *meta* (**4** and **5**) and *para* (**6** and **7**) positions. The synthesis of all four replacements began with the commercially available corresponding cyano acids **16a**, **16b**, **20a** and **20b** (Scheme 2). The nitrile was first converted to the hydroxyamidino using hydroxylamine



Scheme 1. Reagents and conditions: (a) di-*tert*-butyldicarbonate, NaOH, 1,4-dioxane–H_2O 2:1, 0 °C to room temperature, 3 h. (b) HOBt, HBTU, DIEA, Oic-Pro-D-Ala-Phe(*p*-Me)-NH_2 tetrapeptide resin (designated TP-resin), NMP, room temperature, 1.5 h. (c) 95:5 TFA–H_2O, room temperature, 2 h. (d) *N*,*N*′-di-Boc-*N*′-trifylguanidine, triethylamine, 1,4-dioxane, room temperature, 24 h.[13]



Scheme 2. Reagents and conditions: (a) hydroxylamine hydrochloride, KOH, ethanol, reflux, 16–18.5 h.[14] (b) H_2, 10% Pd/C, H_2O, 50 °C, 18–20 h. (c) HOBt, HBTU, DIEA, Oic-Pro-D-Ala-Phe(*p*-Me)-NH_2 tetrapeptide resin (designated TP-resin), NMP, room temperature, 1–4 h. (d) 95:5 TFA–H_2O, room temperature, 2 h. (e) acetic anhydride, H_2, 10% Pd/C, glacial acetic acid, room temperature, 21.5–23.5 h.[14]



Scheme 3. Reagents and conditions: (a) propionyl chloride, *n*-butyllithium, THF, −78 °C to room temperature, 14 h.[16] (b) acrylonitrile, $TiCl_3$(OiPr), DIEA, dichloromethane, 0 °C, 4 h.[16] (c) LiOH, THF–H_2O 2:1, 0 °C to room temperature, 1–15 h.[16] (d) H_2, PtO_2, H_2O–methanol 3:7, room temperature, 14–15 h.[16] (e) di-*tert*-butyldicarbonate, NaOH, 1,4-dioxane–H_2O 2:1, 0 °C to room temperature, 4–4.5 h. (f) HOBt, HBTU, DIEA, Oic-Pro-D-Ala-Phe(*p*-Me)-NH_2 tetrapeptide resin (designated TP-resin), NMP, room temperature, 4.5–13 h. (g) 95:5 TFA–H_2O, room temperature, 2 h. (h) *N*,*N*′-di-Boc-*N*′-trifylguanidine, triethylamine, 1,4-dioxane, room temperature, 15 h–6 days.[13]

hydrochloride and base[19] (compounds **17a**, **17b**, **21a** and **21b**). The *meta*-substituted amidino compounds **18a** and **18b** were synthesized via hydrogenation and collected as their hydrochloride salts. Compounds **18a** and **18b** were then coupled to the Oic-Pro-D-Ala-Phe(*p*-Me) tetrapeptide on the resin to yield **19a** and **19b**, followed by cleavage from the resin to give the final desired pentapeptides **4** and **5**.

The synthesis of the *para*-substituted replacements followed a slightly less direct route. Conversion of 4-hydroxyamidinobenzoic acid (**21a**) to 4-amidinobenzoic acid was achieved through the same procedure as described above for the *meta*-substituted replacements, however the resulting compound was only soluble in strong acids, and therefore not suitable for continued peptide synthesis. As a result, **21a** and **21b** were first coupled to the Oic-Pro-D-Ala-Phe(*p*-Me) tetrapeptide on the resin to yield **22a** and **22b**, followed by cleavage from the resin to give pentapeptides **23a** and **23b**. The hydroxyamidino moiety in **23a** and **23b** was then hydrogenated to the desired amidino through the use of acetic anhydride as an acylating agent in acetic acid,[19] to give desired pentapeptides **6** and **7**.

Two compounds, **8** and **9**, were synthesized that replaced the N-terminal amine of **FM 19** with a methyl group (Scheme 3). The replacement in **8** has the *R*-configuration, which corresponds to the same stereochemistry as the D-Arg residue in **FM 19**, while the replacement in **9** has the opposite, *S* stereochemistry. Syntheses of enantiopure replacements were achieved through the use of Evans oxazolidinone chiral auxiliaries.[20] The commercially available oxazolidinone **24a** was functionalized using propionyl chloride and *n*-butyllithium,[21] generating **25a**. The other enantiomer of this compound, **25b**, was commercially available and purchased. Compounds **25a** and **25b** were then functionalized with acrylonitrile,[21] to yield **26a** and **26b**, with the desired stereochemistry. The chiral auxiliary was then hydrolyzed[21] to give the cyanoacids **27a** and **27b**. Compounds **27a** and **27b** were then hydrogenated using PtO_2,[21] resulting in amino acids **28a** and **28b**. The amino group on **28a** or **28b** was protected with a Boc group to give **29a** and **29b**, and the protected amino acids were then coupled to the Oic-Pro-D-Ala-Phe(*p*-Me) tetrapeptide on the resin to yield **30a** and **30b**. Compounds **30a** and **30b** were then cleaved from the resin while simultaneously removing the Boc protecting group, to give amino-containing pentapeptides **31a** and **31b**. The amine was converted to a di-Boc protected guanidino group[18] producing **32a** and **32b**, again, to simultaneously install the desired guanidino group into the final peptide, as well as allow for easier purification from the amine-containing peptides. The Boc protecting groups were then removed, giving pentapeptides **8** and **9**, ready for testing.

2.2. Inhibition of α-thrombin

Peptides **RPPGF** and **2**–**10** were tested for their ability to inhibit human α-thrombin according to the general assay procedure. The results of these assays are summarized in Table 1, along with results for **1**[16] and **FM 19**.[16] A representative inhibition profile for compound **7** is shown in Figure 4. As seen in Figure 4, both **FM 19** and compound **7** were able to completely inhibit α-thrombin. This was also observed for all compounds reported here.

When comparing the guanidino-containing saturated ring replacements **2** and **3**, we can see that the *trans*-substitution (**3**) is more potent than the *cis*-substitution (**2**). Assuming the cyclohexanoic acid residue in **3** adopts the expected, energetically favored chair conformation with the *trans para* substituents arranged equatorially, the residue 1 side chain of **3** adopts a

Table 1
Inhibitory activities of **RPPGF**, **FM 19** and compounds **1**–**10** against human α-thrombin

Compound	D-Arg replacement (**X**) in **FM 19** sequence (**X**-Oic-Pro-D-Ala-Phe(*p*-Me)-NH_2)	IC_{50} (μM)
RPPGF	—	601 ± 21
FM 19	D-Arg	4.4 ± 1.3[16]
1	*p*-Guanidinobenzoic acid	0.57 ± 0.12[16]
2	*cis*-4-Guanidino-1-cyclohexanecarboxylic acid	19.5 ± 5.4
3	*trans*-4-Guanidino-1-cyclohexanecarboxylic acid	2.5 ± 0.5
4	3-Amidinobenzoic acid	1.03 ± 0.25
5	3-Amidinophenylacetic acid	26.2 ± 2.3
6	4-Amidinobenzoic acid	0.51 ± 0.19
7	4-Amidinophenylacetic acid	0.45 ± 0.08
8	(*R*)-5-Guanidino-2-methylpentanoic acid	98 ± 27
9	(*S*)-5-Guanidino-2-methylpentanoic acid	9.8 ± 1.0
10	L-Arg	73 ± 36

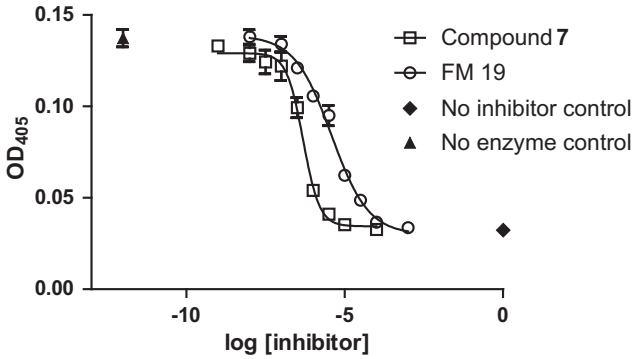


Figure 4. Representative inhibition profile of **7** and **FM 19**.

Table 2
Analytical data for **RPPGF**, **FM 19** and compounds **1**–**10**

Compound	D-Arg replacement (**X**) in **FM 19** sequence (**X**-Oic-Pro-D-Ala-Phe(*p*-Me)-NH_2)	Expected [M+H]	Found [M+H]	Analytical HPLC retention time (min)
RPPGF	—	573.3	573.1	17.4
FM 19	D-Arg	654.4[16]	654.4[16]	26.5[16]
1	*p*-Guanidinobenzoic acid	659.4[16]	659.3[16]	31.6[16]
2	*cis*-4-Guanidino-1-cyclohexanecarboxylic acid	665.4	665.4	34.2
3	*trans*-4-Guanidino-1-cyclohexanecarboxylic acid	665.4	665.4	32.1
4	3-Amidinobenzoic acid	644.4	644.5	31.4
5	3-Amidinophenylacetic acid	658.4	658.5	33.4
6	4-Amidinobenzoic acid	644.4	644.5	30.5
7	4-Amidinophenylacetic acid	658.4	658.5	33.2
8	(*R*)-5-Guanidino-2-methylpentanoic acid	653.4	653.2	31.2
9	(*S*)-5-Guanidino-2-methylpentanoic acid	653.4	653.2	35.1
10	L-Arg	654.4	654.2	26.7

quasi-planar shape similar to that of **1**. In a similar chair conformation of the saturated ring of **2**, such a quasi-planar orientation is not possible since one of the *trans*-substituents will be equatorial, the other axial, thus making key interactions more difficult to achieve. Since the three-dimensional shape of **3** more closely resembles **1**, it would be expected that **3** is more potent than **2**. However, **3** is still approximately four times less potent than **1**, and thus it appears as if the rigidity and planarity of the unsaturated ring is preferred.

Three of the four amidino-containing peptides from Table 1 are more potent than lead compound **FM 19**. Positioning of the amidino substitution relative to the rest of the peptide appears to be the key consideration. In **4** and **6** the connection of the aromatic ring to remainder of the molecule is via a single rotatable bond, which minimizes the positional variation of the amidino group and maintains it in an orientation suitable for interaction with thrombin. In **7** two rotatable bonds link the aromatic moiety to the peptide, but conformational space accessible to the amidino remains limited since rotation about the aryl–alkyl bond does not affect its position. By contrast rotation about either the aryl–alkyl or alkyl–carbonyl bond in **5** significantly alters the position of the aminido group relative to the rest of the molecule, leading to a reduced population of the binding orientation and hence reduced potency.

The methyl-substituted peptides **8** and **9** produce an interesting result. Compound **8** has the same stereochemistry as the N-terminal residue in **FM 19**, while **9** has the opposite stereochemistry. The only difference between **FM 19** and **8** is the elimination of the charge due to replacement of the amino group by a methyl group, since the sterics and stereochemistry of this part of the molecule are maintained. Previous studies have suggested that the *R*-configuration around this stereocenter is preferred (D-Arg is favored over L-Arg),[13] and thus we would expect **8** to be more potent than **9**, but in fact **9** shows an order of magnitude improvement in potency over **8**. Compound **8** has the methyl group in the same position as the amine of **FM 19**, which is more solvent-exposed, while **9** positions the methyl group in a more hydrophobic environment, pointed further into the active site and directed more toward the aliphatic side chain of Oic. Thus, it would be expected that there would be more of a penalty for the methyl group to be solvent-exposed as in **8**, suggesting a reason for the increase in potency seen in **9**.

Finally, due to the unexpected results of **8** and **9**, compound **10** was synthesized to verify that D-Arg as the N-terminal residue is favored over L-Arg. This has been confirmed, as we see that **10** is approximately 16-fold less potent than **FM 19**. Although the D-Arg in **FM 19** places the N-terminal amine in close proximity to the guanidino group creating an unfavorable interaction, this amine can make a water-mediated hydrogen bond to the backbone carbonyl of Gly 216 in thrombin's active site.[15] The amine of **10** is instead pointed in a different direction, deeper into the active site and more toward the side chain of the Oic residue. In this orientation, the amine of **10** is unable to participate in hydrogen bonding to the enzyme and is in an unfavorable hydrophobic environment, hence its observed lower potency. These results are consistent with findings from other research groups who have also utilized a D-Arg residue for thrombin inhibition.[22,23]

3. Conclusions

This study reports nine new replacements of the D-Arg residue in the **FM 19** sequence. Four of these compounds (**3**, **4**, **6** and **7**) are more potent than lead compound **FM 19**, while two (**6** and **7**) show equal, if not slightly improved, potency when compared to the best previously reported compounds in this series.[16] Like **1**, **6** and **7** owe their improved potency to the incorporation of conformational restriction in the side chain to overcome the entropic penalty of the flexible D-Arg side chain of **FM 19**. We have shown that replacing the N-terminal residue with a saturated ring substituted at the 4 position with a guanidine group can yield a potent compound, and this guanidino group is preferred in the *trans*-orientation (**3**), over the *cis*-orientation (**2**). This is consistent with the previously synthesized compound **1**, as the guanidino group in **3** more closely resembles the positioning of this same group in **1**. Also, the guanidino group can be replaced with an amidino moiety on an aromatic ring, and *para*-substitution (**6** and **7**) is preferred over *meta*-substitution (**4** and **5**). Furthermore, although the N-terminal amine in **FM 19** makes an unfavorable electrostatic interaction with the guanidino group on the side chain, this amine can form a water-mediated hydrogen bond with thrombin's active site.[15] Replacement of this amine with a methyl group (**8**) greatly reduces potency, unless the stereochemistry around this chiral center is changed to make the methyl group less solvent-exposed (**9**). Additionally, burying an amine in the less-solvent exposed area of the active site also results in decreased inhibition (**10**). Finally, previous work demonstrates that delocalization of the positive charge on the side chain of the N-terminal residue in this peptide sequence is important for potency,[16] as the splitting of this charge has been shown to improve potency of direct thrombin inhibitors by increasing interactions with catalytic Asp 189.[17] The current work demonstrates that this delocalization can be successfully achieved by the use of either a guanidino or amidino moiety.

4. Experimental section

4.1. Chemistry general procedures

All reagents were purchased from commercial sources and used without further purification. Purity of synthesized compounds was determined on a Waters alliance 2690 Analytical HPLC (Waters Corporation, Milford, MA, USA) and a Vydac Protein and Peptide C_{18} reverse phase column, using a linear gradient of 0% Solvent B (0.1% trifluoroacetic acid (TFA) in acetonitrile) in Solvent A (0.1% TFA in water) to 70% Solvent B in Solvent A in 70 min, and UV absorbance at 230 nm. ESI-MS was performed on either a Finnigan LCQ mass spectrometer (ThermoFinnigan, San Jose, CA, USA) in positive mode or an Agilent Technologies LC/MS system using a 1200 Series LC and 6130 Quadrupole LC/MS (Agilent Technologies, Santa Clara, CA, USA) in positive mode with 10–100 μL injection volume and a linear gradient of 0% Solvent D (0.02% TFA and 0.1% acetic acid in acetonitrile) in Solvent C (0.02% TFA and 0.1% acetic acid in water) to 60% Solvent D in Solvent C in 15 min. NMR data were obtained on either a 300 or 500 MHz Bruker spectrometer. All column chromatography was performed using a Flash+ System (Biotage Corporation, Sweden) and pre-packed silica Flash 40+M cartridges (40 × 150 mm) and Flash 40+ samplets (40–63 μm particle distribution).

4.2. General procedure for solid phase peptide synthesis

Peptides were synthesized using standard fluorenylmethyloxycarbonyl (Fmoc) chemistry, on a CS Bio CS336X Peptide Synthesizer (CS Bio Company, Menlo Park, CA, USA), using previously described protocols.[24] Fmoc-Phe-Wang resin (NovaBiochem, EMD Chemicals, Gibbstown, NJ, USA) was used to produce peptide **RPPGF** as a C-terminal acid; Rink amide resin (Advanced Chem Tech, Louisville, KY, USA) was used to produce peptides **2**–**10** as C-terminal amides. A solution of 20% piperidine in *N*-methyl-2-pyrrolidone (NMP) was used to remove the Fmoc protecting group

before beginning synthesis, and again to remove the Fmoc protecting group after each coupling cycle. Coupling was performed using three to four-fold excess of amino acid and a solution of 0.4 M 1-hydroxybenzotriazole (HOBt; Advanced Chem Tech, Louisville, KY, USA) and *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluorophosphate (HBTU; Advanced Chem Tech, Louisville, KY, USA) in dimethylformamide, in the presence of *N*,*N*-diisopropylethylamine (DIEA). After the synthesis was complete, the resin was washed with NMP followed by a wash with dichloromethane, and dried.

4.3. General peptide purification procedure

Crude peptides were purified using a Waters semipreparative HPLC (Waters Corporation, Milford, MA, USA) with a Vydac or Waters Protein and Peptide C_{18} column, using a linear gradient of 0–10% Solvent B in Solvent A to 50–60% Solvent B in Solvent A, at a rate of 1% per minute.

4.3.1. Synthesis of RPPGF dihydrotrifluoroacetate

RPPGF was synthesized according to the general procedure for solid phase peptide synthesis, using Fmoc-Phe-Wang resin (520 mg, 0.39 mmol/g substitution). Once synthesis was complete, the dried resin (627 mg) was stirred for 2 h at room temperature with 95:5 TFA–H_2O (10 mL). After filtration, the solution was concentrated in vacuo, followed by precipitation with cold diethyl ether. After refrigeration overnight, **RPPGF** dihydrotrifluoroacetate was collected (off-white solid, 120 mg) and purified by HPLC to obtain **RPPGF** dihydrotrifluoroacetate (white solid, 98 mg, 60% yield) which was >95% pure by HPLC.

4.3.2. Synthesis of *cis*-4-guanidino-1-cyclohexanecarboxylic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH_2 hydrotrifluoroacetate (2)

Compound **15a** (97 mg) was stirred for 2 h at room temperature with 95:5 TFA–H_2O (10 mL). The solution was concentrated in vacuo, followed by precipitation with cold diethyl ether. After refrigeration overnight, **2** hydrotrifluoroacetate was collected (off-white solid, 56 mg) and purified by HPLC to obtain **2** hydrotrifluoroacetate (white solid, 42 mg, 39% yield) which was >95% pure by HPLC.

4.3.3. Synthesis of *trans*-4-guanidino-1-cyclohexanecarboxylic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH_2 hydrotrifluoroacetate (3)

Compound **15b** was stirred for 2 h at room temperature with 95:5 TFA–H_2O (10 mL). The solution was concentrated in vacuo, followed by precipitation with cold diethyl ether. After refrigeration overnight, **3** hydrotrifluoroacetate was collected (off-white solid, 42 mg) and purified by HPLC to obtain **3** hydrotrifluoroacetate (white solid, 13 mg, 13% yield) which was 94% pure by HPLC.

4.3.4. Synthesis of 3-amidinobenzoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH_2 hydrotrifluoroacetate (4)

First, compound **18a** (42 mg, 0.2 mmol) was dissolved in a solution of 0.4 M HOBt/HBTU in NMP (3 mL) and then a 1 M DIEA in NMP solution was added (2 mL). This solution was transferred to a vial containing Oic-Pro-D-Ala-Phe(*p*-Me)-resin (321 mg, 0.65 mmol/g substitution; synthesized according to the general solid phase peptide synthesis procedure) in NMP (2 mL). The solution was diluted with NMP (5 mL) and placed on a shaker at room temperature for 1 h. The resin was collected, washed with dichloromethane and dried, to yield the resin-bound pentapeptide **19a**. Compound **19a** then was stirred for 2 h at room temperature with 95:5 TFA–H_2O (10 mL). After filtration, the solution was concentrated in vacuo, followed by precipitation with cold diethyl ether. After refrigeration overnight, **4** hydrotrifluoroacetate was collected (off-white solid, 94 mg) and purified by HPLC to obtain **4** hydrotrifluoroacetate (white solid, 3.7 mg, 2.3% yield) which was >95% pure by HPLC.

4.3.5. Synthesis of 3-amidinophenylacetic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH_2 hydrotrifluoroacetate (5)

First, compound **18b** (53 mg, 0.25 mmol) was dissolved in a solution of 0.4 M HOBt/HBTU in NMP (3 mL) and then a 1 M DIEA in NMP solution was added (2 mL). This solution was transferred to a vial containing Oic-Pro-D-Ala-Phe(*p*-Me)-resin (377 mg, 0.65 mmol/g substitution; synthesized according to the general solid phase peptide synthesis procedure) in NMP (2 mL). The solution was diluted with NMP (5 mL) and placed on a shaker at room temperature for 1 h. The resin was collected, washed with dichloromethane and dried, to yield the resin-bound pentapeptide **19b** (373 mg). Compound **19b** (304 mg) was stirred for 2 h at room temperature with 95:5 TFA–H_2O (10 mL). After filtration, the solution was concentrated in vacuo, followed by precipitation with cold diethyl ether. After refrigeration overnight, **5** hydrotrifluoroacetate was collected (off-white solid, 86 mg) and purified by HPLC to obtain **5** hydrotrifluoroacetate (white solid, 4.2 mg, 2.6% yield) which was >95% pure by HPLC.

4.3.6. Synthesis of 4-amidinobenzoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH_2 hydrotrifluoroacetate (6)

The synthesis of **6** was completed using a modification of a previously reported procedure.[19] In a reaction vessel for a Parr shaker, compound **23a** (15 mg, 0.02 mmol) and acetic anhydride (5 μL, 0.05 mmol) were dissolved in glacial acetic acid (40 mL) and degassed. A catalytic amount of 10% Pd/C was added, and the solution was placed on the shaker under 50 psi H_2 at room temperature for 23.5 h. The solution was filtered through celite and the filtrate was concentrated in vacuo to give **6** as a hydroacetate salt. The oil was purified by HPLC to obtain **6** hydrotrifluoroacetate (white solid, 7.6 mg, 3.4% yield) which was >95% pure by HPLC.

4.3.7. Synthesis of 4-amidinophenylacetic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH_2 hydrotrifluoroacetate (7)

The synthesis of **7** was completed using a modification of a previously reported procedure.[19] In a reaction vessel for a Parr shaker, compound **23b** (8.2 mg, 0.01 mmol) and acetic anhydride (5 μL, 0.05 mmol) were dissolved in glacial acetic acid (40 mL) and degassed. A catalytic amount of 10% Pd/C was added, and the solution was placed on the shaker under 50 psi H_2 at room temperature for 21.5 h. The solution was filtered through celite and the filtrate was concentrated in vacuo to give **7** as a hydroacetate salt. The oil was purified by HPLC to obtain **7** hydrotrifluoroacetate (white solid, 2.1 mg, 0.9% yield) which was >95% pure by HPLC.

4.3.8. Synthesis of (*R*)-5-guanidino-2-methylpentanoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH_2 hydrotrifluoroacetate (8)

Compound **32a** was stirred for 2 h at room temperature with 95:5 TFA–H_2O (10 mL). After filtration, the solution was concentrated in vacuo, followed by precipitation with cold diethyl ether. After refrigeration overnight, **8** hydrotrifluoroacetate salt was collected (off-white solid, 40 mg) and purified by HPLC to obtain **8** hydrotrifluoroacetate (white solid, 35 mg, 57% yield) which was >95% pure by HPLC.

4.3.9. Synthesis of (*S*)-5-guanidino-2-methylpentanoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH_2 hydrotrifluoroacetate (9)

Compound **32b** was stirred for 2 h at room temperature with 95:5 TFA–H_2O (10 mL). After filtration, the solution was concentrated in vacuo, to yield an oil containing **9** (39 mg). The oil was purified by HPLC to obtain **9** hydrotrifluoroacetate (white solid, 22 mg, 57% yield) which was >95% pure by HPLC.

4.3.10. Synthesis of L-Arg-Oic-Pro-D-Ala-Phe(*p*-Me)-NH_2 dihydrotrifluoroacetate (10)

Compound **10** was synthesized according to the general solid phase peptide synthesis procedure, using Rink amide resin (340 mg, 0.59 mmol/g substitution). Once synthesis was complete, the dried resin (468 mg) was stirred for 2 h at room temperature with 95:5 TFA–H_2O (10 mL). After filtration, the solution was concentrated in vacuo, followed by precipitation with cold diethyl ether. After refrigeration overnight, **10** dihydrotrifluoroacetate was collected (off-white solid, 125 mg) and purified by HPLC to obtain **10** dihydrotrifluoroacetate (white solid, 55 mg, 31% yield) which was >95% pure by HPLC.

4.3.11. Synthesis of Boc-*cis*-4-amino-1-cyclohexanecarboxylic acid (12a)

To a stirred solution of the commercially available **11a** (286 mg, 2.0 mmol) in 2:1 1,4-dioxane–H_2O (6 mL) at 0 °C was added 1 M NaOH (3 mL), followed by di-*tert*-butyldicarbonate (480 mg, 2.2 mmol) dissolved in a minimal amount of 1,4-dioxane. The solution was stirred for 3 h and slowly warmed to room temperature, and was then concentrated in vacuo to obtain a white solid. The solid was dissolved in H_2O (4 mL) and ethyl acetate (7 mL) and acidified to pH 1.5 using 1 M HCl, prompting precipitation of **12a**. Compound **12a** was collected by filtration (white solid, 388 mg, 73% yield) and was >95% pure by HPLC. ^{1}H NMR (500 MHz, DMSO): δ 12.07 (s, 1H, br), 6.75 (s, 1H, br), 2.36 (m, 1H), 1.86 (m, 2H), 1.50–1.37 (m, 7H), 1.37 (s, 9H).

4.3.12. Synthesis of *cis*-4-amino-1-cyclohexanecarboxylic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH_2 hydrotrifluoroacetate (14a)

First, compound **13a** was synthesized according to the general procedure for solid phase peptide synthesis, using Rink amide resin (270 mg, 0.75 mmol/g substitution), giving the resin-bound pentapeptide (375 mg). Compound **13a** was then stirred for 2 h at room temperature with 95:5 TFA–H_2O (10 mL). After filtration, the solution was concentrated in vacuo, followed by precipitation with cold diethyl ether. After refrigeration overnight, **14a** hydrotrifluoroacetate was collected (off-white solid, 108 mg, 73% yield) and was >95% pure by HPLC. ESI-MS calculated $[M+H]^+$, 623.4, found, 623.3.

4.3.13. Synthesis of *trans*-4-amino-1-cyclohexanecarboxylic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH_2 hydrotrifluoroacetate (14b)

First, Compound **13b** was synthesized using the commercially available **12b** according to the general procedure for solid phase peptide synthesis, using Rink amide resin (310 mg, 0.70 mmol/g substitution), giving the resin-bound pentapeptide (358 mg). Compound **13b** was then stirred for 2 h at room temperature with 95:5 TFA–H_2O (10 mL). After filtration, the solution was concentrated in vacuo, followed by precipitation with cold diethyl ether. After refrigeration overnight, **14b** hydrotrifluoroacetate was collected (off-white solid, 93 mg, 58% yield) and was 67% pure by HPLC and carried on without further purification or characterization.

4.3.14. Synthesis of *cis*-4-(di-Boc)guanidino-1-cyclohexanecarboxylic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH_2 (15a)

The synthesis of **15a** was completed using a modification of a previously reported procedure.[18] To a stirred solution of *N*,*N*′-di-Boc-*N*′-triflylguanidine (48 mg, 0.12 mmol) and triethylamine (96 μL, 0.69 mmol) in 1,4-dioxane (2 mL) was added a solution of **14a** (101 mg, 0.14 mmol) in 1,4-dioxane (5 mL). The solution stirred at room temperature for 24.5 h, and was then concentrated in vacuo to yield a yellow oil. The oil was dissolved in ethyl acetate (20 mL), washed with 2 M $KHSO_4$ (2 × 5 mL) followed by saturated $NaHCO_3$ (2 × 5 mL), dried over $MgSO_4$ and concentrated in vacuo to give a white solid containing **15a** (97 mg, 60% yield). The solid was 75% pure by HPLC and carried on without further purification or characterization.

4.3.15. Synthesis of *trans*-4-(di-Boc)guanidino-1-cyclohexanecarboxylic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH_2 (15b)

The synthesis of **15b** was completed using a modification of a previously reported procedure.[18] To a stirred solution of *N*,*N*′-di-Boc-*N*′-triflylguanidine (50 mg, 0.13 mmol) and triethylamine (88 μL, 0.63 mmol) in 1,4-dioxane (2 mL) was added a solution of **14b** (93 mg, 0.13 mmol) in 1,4-dioxane (13 mL). The solution stirred at room temperature for 24 h, and was then concentrated in vacuo to yield a yellow-orange oil. The oil was dissolved in ethyl acetate (20 mL), washed with 2 M $KHSO_4$ (2 × 5 mL) followed by saturated $NaHCO_3$ (2 × 5 mL), dried over $MgSO_4$ and concentrated in vacuo to give an oily residue containing **15b**. The solid was carried on without further purification or characterization.

4.3.16. Synthesis of 3-hydroxyamidiobenzoic acid[19] (17a)

To a suspension of the commercially available **16a** (1 g, 6.8 mmol) in ethanol (100 mL) and H_2O (5 mL) was added KOH (954 mg, 17 mmol) and hydroxylamine hydrochloride (709 mg, 10.2 mmol), and the solution was held at reflux for 18.5 h. After cooling to room temperature, the solid KCl was filtered away from the solution, and the filtrate was concentrated in vacuo to give a white solid. The solid was purified using the Flash+ system and a mobile phase of 3:2 dicholoromethane–methanol (R_f = 0.33), to yield **17a** (white solid, 566 mg, 46% yield), which was >95% pure by HPLC. ^{1}H NMR (500 MHz, DMSO): δ 9.71 (s, 1H, br), 8.28 (s, 1H), 7.94 (d, J = 7.7 Hz, 1H), 7.83 (d, J = 7.7 Hz, 1H), 7.46 (dd, J_1 = 7.7 Hz, J_2 = 0.5 Hz, 1H), 5.89 (s, 1H)

4.3.17. Synthesis of 3-hydroxyamidinophenylacetic acid[19] (17b)

To a solution of commercially available **16b** (1 g, 6.25 mmol) in ethanol (100 mL) was added KOH (877 mg, 15.6 mmol) and hydroxylamine hydrochloride (651 mg, 9.4 mmol), and the solution was held at reflux for 16 h. After cooling to room temperature, the solid KCl was filtered away from the solution, and the filtrate was concentrated in vacuo to give a white solid. The solid was purified using the Flash+ system and a mobile phase of 3:2 dicholoromethane–methanol (R_f = 0.27), to yield **17b** (white solid, 608 mg, 50% yield). ^{1}H NMR (500 MHz, DMSO): δ 12.49 (s, 1H, br), 9.60 (s, 1H), 7.57 (s, 1H), 7.52 (d, J = 7.6 Hz, 1H), 7.29 (dd, J_1 = 7.6 Hz, J_2 = 0.5 Hz, 1H), 7.25 (d, J = 6.8 Hz, 1 H), 5.77 (s, 2H), 3.54 (s, 2H).

4.3.18. Synthesis of 3-amidiobenzoic acid hydrochloride (18a)

In a reaction vessel for a Parr shaker, compound **17a** (566 mg, 3.1 mmol) was suspended in H_2O (50 mL) and degassed. A catalytic amount of 10% Pd/C was added, and the solution was placed on the shaker under 50 psi H_2 at 50 °C for 20 h. No longer a suspension, the solution was filtered through celite, and 1 M HCl (3.15 mL) was added to the filtrate which was concentrated in vacuo to yield **18a** hydrochloride (white solid, 518 mg, 82% yield). ^{1}H NMR (500 MHz, DMSO): δ 9.61 (s, 2H), 9.44 (s, 2H), 8.25 (s, 1H), 8.20 (d, J = 7.3 Hz, 1H), 7.98 (d, J = 7.2 Hz, 1H), 7.72 (dd, J_1 = 7.5 Hz, J_2 = 0.5 Hz, 1H).

4.3.19. Synthesis of 3-amidinophenylacetic acid hydrochloride (18b)

In a reaction vessel for a Parr shaker, compound **17b** (608 mg, 3.1 mmol) was suspended in H_2O (50 mL) and degassed. A catalytic amount of 10% Pd/C was added, and the solution was placed on the shaker under 50 psi H_2 at 50 °C for 18 h. No longer a suspension, the solution was filtered through celite, and 1 M HCl (3.13 mL) was added to the filtrate which was concentrated in vacuo to yield **18b** hydrochloride (white solid, 604 mg, 90% yield). ^{1}H NMR (500 MHz, DMSO): δ 12.50 (s, 1H, br), 9.40 (s, 2H), 9.20 (s, 2H),

7.72 (s, 1H), 7.71 (d, J = 7.7 Hz, 1H), 7.64 (d, J = 7.7 Hz, 1H), 7.57 (dd, J_1 = 7.9 Hz, J_2 = 0.5 Hz, 1H), 3.63 (s, 2H). ESI-MS calculated $[M+H]^+$, 179.1, found, 179.2.

4.3.20. Synthesis of 4-hydroxyamidiobenzoic acid[19] (21a)

To a suspension of commercially available **20a** (1 g, 6.8 mmol) in ethanol (100 mL) and H_2O (5 mL) was added KOH (954 mg, 17 mmol) and hydroxylamine hydrochloride (709 mg, 10.2 mmol), and the solution was held at reflux for 18.5 h. After cooling to room temperature, the solid KCl was filtered away from the solution, and the filtrate was concentrated in vacuo to give a white solid. The solid was purified using the Flash+ system and a mobile phase of 3:2 dicholoromethane–methanol (R_f = 0.35), to yield **21a** (white solid, 616 mg, 50% yield). ^{1}H NMR (500 MHz, DMSO): δ 9.76 (s, 1H, br), 7.85 (d, J = 8.1 Hz, 2H), 7.63 (d, J = 8.1 Hz, 2H), 5.80 (s, 2H).

4.3.21. Synthesis of 4-hydroxyamidinophenylacetic acid[19] (21b)

To a suspension of commercially available **20b** (882 mg, 5.5 mmol) in ethanol (100 mL) was added KOH (767 mg, 13.7 mmol) and hydroxylamine hydrochloride (570 mg, 8.2 mmol), and the solution was held at reflux for 15 h. After cooling to room temperature, the solid KCl was filtered away from the solution, and the filtrate was concentrated in vacuo to give a white solid. The solid was purified using the Flash+ system and a mobile phase of 3:2 dicholoromethane–methanol (R_f = 0.27), to yield **21b** (white solid, 286 mg, 27% yield). ^{1}H NMR (300 MHz, DMSO): δ 12.50 (s, 1H, br), 9.59 (s, 1H, br), 7.59 (d, J = 7.3 Hz, 2H), 7.24 (d, J = 7.6 Hz, 2H), 5.78 (s, 2H), 3.53 (s, 2H).

4.3.22. Synthesis of 4-hydroxyamidinobenzoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH_2 hydrotrifluoroacetate (23a)

First, compound **21a** (71 mg, 0.38 mmol) was dissolved in a solution of 0.4 M HOBt/HBTU in NMP (2.5 mL) and then a 1 M DIEA in NMP solution was added (1 mL). This solution was transferred to a vial containing Oic-Pro-D-Ala-Phe(*p*-Me)-resin (360 mg, 0.7 mmol/g substitution; synthesized according to the general solid phase peptide synthesis procedure) in NMP (2 mL). The solution was diluted with NMP (5 mL) and placed on a shaker at room temperature for 4 h. The resin was collected, washed with dichloromethane and dried, to yield the resin-bound pentapeptide **22a** (417 mg). Compound **22a** was then stirred for 2 h at room temperature with 95:5 TFA–H_2O (10 mL). After filtration, the solution was concentrated in vacuo, followed by precipitation with cold diethyl ether. After refrigeration overnight, **23a** hydrotrifluoroacetate was collected (off-white solid, 105 mg) and purified by HPLC to obtain **23a** hydrotrifluoroacetate (white solid, 15 mg, 6.2% yield) which was >80% pure by HPLC. ESI-MS calculated $[M+H]^+$, 660.4, found, 660.3.

4.3.23. Synthesis of 4-hydroxyamidinophenylacetic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH_2 hydrotrifluoroacetate (23b)

First, compound **21b** (101 mg, 0.41 mmol) was dissolved in a solution of 0.4 M HOBt/HBTU in NMP (2.5 mL) and then a 1 M DIEA in NMP solution was added (1 mL). This solution was transferred to a vial containing Oic-Pro-D-Ala-Phe(*p*-Me)-resin (371 mg, 0.7 mmol/g substitution; synthesized according to the general solid phase peptide synthesis procedure) in NMP (2 mL). The solution was diluted with NMP (5 mL) and placed on a shaker at room temperature for 4 h. The resin was collected, washed with dichloromethane and dried, to yield the resin-bound pentapeptide **22b** (443 mg). Compound **22b** was then stirred for 2 h at room temperature with 95:5 TFA–H_2O (10 mL). After filtration, the solution was concentrated in vacuo, followed by precipitation with cold diethyl ether. After refrigeration overnight, **23b** hydrotrifluoroacetate was collected (off-white solid, 100 mg) and purified by HPLC to obtain **23b** hydrotrifluoroacetate (white solid, 14 mg, 5.1% yield) which was >70% pure by HPLC. ESI-MS calculated $[M+H]^+$, 674.4, found, 674.5.

4.3.24. Synthesis of (*S*)-4-benzyl-3-propionyloxazolidin-2-one[21] (25a)

Commercially available compound **24a** (800 mg, 4.5 mmol) was dissolved in anhydrous THF (30 mL) in dry glassware under N_2, and cooled to −78 °C in a dry ice/acetone bath. A solution of 1.6 M *n*-butyllithium in hexanes (5.0 mmol, 3.1 mL) was syringed in over 3 min. After mixing for 30 min, propionyl chloride (5.0 mmol, 0.43 mL) was syringed in over 3 min. The solution was allowed to slowly warm to room temperature over 14 h and quenched by the addition of saturated NH_4Cl (10 mL) and H_2O (30 mL). The final aqueous layer was extracted with ethyl acetate (3 × 40 mL) and the combined organic extracts were dried over $MgSO_4$ and concentrated in vacuo to yield **25a** (oil, 1.0 g, 96% yield) which was >95% pure by HPLC. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.33 (dd, J_1 = 7.4 Hz, J_2 = 7.6 Hz, 2H), 7.29 (d, J = 6.9 Hz, 1H), 7.21 (d, J = 7.4 Hz, 2H), 4.67 (m, 1H), 4.18 (m, 2H), 3.31 (dd, J_1 = 13.3 Hz, J_2 = 3.2 Hz, 1H), 2.96 (m, 2H), 2.77 (dd, J_1 = 13.3 Hz, J_2 = 3.7 Hz, 1H), 1.21 (t, J = 7.4 Hz, 3H).

4.3.25. Synthesis of (*R*)-5-((*S*)-4-benzyl-2-oxooxazolidin-3-yl)-4-methyl-5-oxopentanenitrile[21] (26a)

$TiCl_3$(OiPr) was first prepared using dry glassware under N_2. A solution of 1 M $TiCl_4$ in dichloromethane (3.4 mmol, 3.4 mL) was cooled to 0 °C, and $Ti(OiPr)_4$ (1.1 mmol, 0.34 mL) was added dropwise over 5 min. The solution was diluted with anhydrous dichloromethane (3.5 mL) and stirred at 0 °C for 15 min. Meanwhile, compound **25a** (1.0 g, 4.3 mmol) was dissolved in anhydrous dichloromethane (14 mL) in dry glassware under N_2, and DIEA (0.79 mL) was added dropwise over 5 min, with stirring, at room temperature and then cooled to 0 °C. The $TiCl_3$(OiPr) solution was added to the **25a** solution dropwise over 25 min. The $TiCl_3$(OiPr) flask was rinsed with anhydrous dichloromethane (2 mL), and the rinse added to the combined flask. The solution was stirred at 0 °C for 30 min, and then acrylonitrile (0.43 mL, 6.5 mmol) was added dropwise over 10 min, and this solution stirred at 0 °C for 4 h. The reaction was quenched with the addition of saturated NH_4Cl (25 mL) and H_2O (15 mL), and the aqueous layer was extracted with diethyl ether (3 × 40 mL). The organic extracts were combined and washed with saturated $NaHCO_3$ (55 mL) followed by brine (55 mL), dried over $MgSO_4$ and concentrated in vacuo to yield a yellow oil. The oil was purified using the Flash+ system and a mobile phase of 3:7 ethyl acetate–hexanes (R_f = 0.30), to yield **26a** as a very slightly yellow oil (890 mg, 72% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.35 (dd, J_1 = 7.6 Hz, J_2 = 7.1 Hz, 2H), 7.30 (d, J = 7.3 Hz, 1H), 7.21 (d, J = 7.1 Hz, 2H), 4.69 (m, 1H), 4.20 (m, 2H), 3.83 (sextet, J = 6.9 Hz, 1H), 3.31 (dd, J_1 = 13.4 Hz, J_2 = 3.2 Hz, 1H), 2.78 (dd, J_1 = 13.3 Hz, J_2 = 3.6 Hz, 1H), 2.41 (dt, J_1 = 7.7 Hz, J_2 = 1.2 Hz, 2H), 2.19 (m, 1H), 1.81 (m, 1H), 1.24 (d, J = 6.9 Hz, 3H).

4.3.26. Synthesis of (*S*)-5-((*R*)-4-benzyl-2-oxooxazolidin-3-yl)-4-methyl-5-oxopentanenitrile[21] (26b)

$TiCl_3$(OiPr) was first prepared using dry glassware under N_2. A solution of 1 M $TiCl_4$ in dichloromethane (3.6 mmol, 3.6 mL) was cooled to 0 °C, and $Ti(OiPr)_4$ (1.2 mmol, 0.35 mL) was added dropwise over 5 min. The solution was diluted with anhydrous dichloromethane (3.5 mL) and stirred at 0 °C for 15 min. Meanwhile, the commercially available compound **25b** (1.05 g, 4.5 mmol) was dissolved in anhydrous dichloromethane (14 mL) in dry glassware under N_2, and DIEA (0.75 mL) was added dropwise over 5 min, with stirring, at room temperature and then cooled to 0 °C. The $TiCl_3$(OiPr) solution was added to the **25b** solution dropwise over 25 min. The $TiCl_3$(OiPr) flask was rinsed with anhydrous dichloro-

methane (2 mL), and the rinse added to the combined flask. The solution stirred at 0 °C for 30 min, and then acrylonitrile (0.45 mL, 6.8 mmol) was added dropwise over 10 min, and this solution stirred at 0 °C for 4 h. The reaction was quenched with the addition of saturated NH_4Cl (25 mL) and H_2O (15 mL), and the aqueous layer was extracted with diethyl ether (3 × 40 mL). The organic extracts were combined and washed with saturated $NaHCO_3$ (55 mL) followed by brine (55 mL), dried over $MgSO_4$ and concentrated in vacuo to yield a yellow oil. The oil was purified using the Flash+ system and a mobile phase of 3:7 ethyl acetate–hexanes (R_f = 0.27), to yield **26b** as a very slightly yellow oil (919 mg, 71% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.35 (dd, J_1 = 7.5 Hz, J_2 = 7.2 Hz, 2H), 7.30 (d, J = 7.4 Hz, 1H), 7.21 (d, J = 7.2 Hz, 2H), 4.69 (m, 1H), 4.19 (m, 2H), 3.82 (sextet, J = 6.7 Hz, 1H), 3.31 (dd, J_1 = 13.5 Hz, J_2 = 3.3 Hz, 1H), 2.78 (dd, J_1 = 13.4 Hz, J_2 = 3.6 Hz, 1H), 2.41 (dt, J_1 = 8.2 Hz, J_2 = 1.9 Hz, 2H), 2.18 (m, 1H), 1.81 (m, 1H), 1.23 (d, J = 7.1 Hz, 3H).

4.3.27. Synthesis of (*R*)-4-cyano-2-methylbutanoic acid[21] (27a)

To a stirred solution of **26a** (729 mg, 2.55 mmol) in THF (18 mL) at 0 °C were added 1 M LiOH solution (5.95 mL) and H_2O (50 μL). The solution slowly warmed to room temperature over 1 h. The solution was diluted with H_2O (20 mL) and extracted with ethyl acetate (3 × 30 mL). The aqueous layer was acidified to pH 1.5 with 1 M HCl, and extracted with ethyl acetate (3 × 30 mL). These last organic fractions were combined, dried over $MgSO_4$, and concentrated in vacuo to yield **27a** as a colorless oil (191 mg, 51% yield). ^{1}H NMR (500 MHz, DMSO): δ 12.38 (s, 1H, br), 2.51 (m, 2H), 2.41 (sextet, J = 7.1 Hz, 1H), 1.85 (m, 1H), 1.63 (m, 1H), 1.09 (d, J = 7.1 Hz, 3H).

4.3.28. Synthesis of (*S*)-4-cyano-2-methylbutanoic acid[21] (27b)

To a stirred solution of **26b** (919 mg, 3.2 mmol) in THF (21 mL) at 0 °C were added 1 M LiOH solution (6.42 mL) and H_2O (580 μL). The solution slowly warmed to room temperature over 15 h. The solution was diluted with H_2O (20 mL) and extracted with ethyl acetate (3 × 30 mL). The aqueous layer was acidified to pH 1.5 with 1 M HCl, and extracted with ethyl acetate (3 × 30 mL). These last organic fractions were combined, dried over $MgSO_4$, and concentrated in vacuo to yield **27b** as a colorless oil (233 mg, 57% yield). ^{1}H NMR (500 MHz, DMSO): δ 12.35 (s, 1H, br), 2.52 (m, 2H), 2.40 (sextet, J = 7.0 Hz, 1H), 1.89 (m, 1H), 1.62 (m, 1H), 1.09 (d, J = 7.1 Hz, 3H).

4.3.29. Synthesis of (*R*)-5-amino-2-methylpentanoic acid hydrochloride[21] (28a)

In a reaction vessel for the Parr shaker, **27a** (191 mg, 1.5 mmol) was dissolved in 3:7 H_2O–methanol (40 mL) and degassed. A catalytic amount of PtO_2 was added, and the solution was placed on the shaker at room temperature under 50 psi H_2 for 15 h. The solution was filtered through celite, and 1 M HCl (2 mL) was added to the filtrate. The solution was concentrated in vacuo to yield **28a** hydrochloride (oil, 237 mg, 94% yield). ^{1}H NMR (500 MHz, DMSO): δ 7.96 (s, 3H, br), 2.74 (sextet, J = 7.7 Hz, 2H), 2.33 (sextet, J = 6.9 Hz, 1H), 1.55 (m, 3H), 1.39 (m, 1H), 1.05 (d, J = 7.0 Hz, 3H).

4.3.30. Synthesis of (*S*)-5-amino-2-methylpentanoic acid hydrochloride[21] (28b)

In a reaction vessel for the Parr shaker, **27b** (233 mg, 1.8 mmol) was dissolved in 3:7 H_2O–methanol (40 mL) and degassed. A catalytic amount of PtO_2 was added, and the solution was placed on the shaker at room temperature under 50 psi H_2 for 14 h. The solution was filtered through celite, and 1 M HCl (2 mL) was added to the filtrate. The solution was concentrated in vacuo to yield **28b** hydrochloride (oil, 293 mg, 95% yield). ^{1}H NMR (500 MHz, DMSO): δ 7.97 (s, 3H, br), 2.74 (sextet, J = 7.7 Hz, 2H), 2.34 (sextet, J = 6.1 Hz, 1H), 1.56 (m, 3H), 1.39 (m, 1H), 1.05 (d, J = 6.8 Hz, 3H).

4.3.31. Synthesis of Boc-(*R*)-5-amino-2-methylpentanoic acid (29a)

To a stirred solution of **28a** (237 mg, 1.4 mmol) in 2:1 1,4-dioxane–H_2O (9 mL) at 0 °C was added 1 M NaOH (4 mL), followed by di-*tert*-butyldicarbonate (340 mg, 1.6 mmol) dissolved in a minimal amount of 1,4-dioxane. The solution stirred for 4 h and slowly warmed to room temperature, and the solution was concentrated in vacuo to obtain a white solid. The solid was dissolved in H_2O (10 mL) and acidified to pH 2 using 1 M HCl. The aqueous layer was extracted with ethyl acetate (5 × 20 mL), and the combined organic extracts were washed with brine (2 × 40 mL) followed by H_2O (2 × 40 mL), and dried over $MgSO_4$. The organic layer was concentrated in vacuo to yield **29a** (colorless oil, 248 mg, 76% yield). ^{1}H NMR (500 MHz, DMSO): δ 12.06 (s, 1H, br), 6.81 (t, J = 5.4 Hz, 1H), 2.88 (q, J = 6.3 Hz, 2H), 2.29 (sextet, J = 6.7 Hz, 1H), 1.50 (pentet, J = 6.5 Hz, 2H), 1.37 (s, 9H), 1.32 (m, 2H), 1.02 (d, J = 7.0 Hz, 3H).

4.3.32. Synthesis of Boc-(*S*)-5-amino-2-methylpentanoic acid (29b)

To a stirred solution of **28b** (293 mg, 1.7 mmol) in 2:1 1,4-dioxane–H_2O (9 mL) at 0 °C was added 1 M NaOH (4.5 mL), followed by di-*tert*-butyldicarbonate (540 mg, 1.9 mmol) dissolved in a minimal amount of 1,4-dioxane. The solution stirred for 4.5 h and slowly warmed to room temperature, and the solution was concentrated in vacuo to obtain a white solid. The solid was dissolved in H_2O (10 mL) and acidified to pH 2 using 1 M HCl. The aqueous layer was extracted with ethyl acetate (5 × 20 mL), and the combined organic extracts were washed with brine (2 × 40 mL) followed by H_2O (2 × 40 mL), and dried over $MgSO_4$. The organic layer was concentrated in vacuo to yield **29b** (colorless oil, 340 mg, 84% yield). ^{1}H NMR (500 MHz, DMSO): δ 12.06 (s, 1H, br), 6.81 (t, J = 5.3 Hz, 1H), 2.87 (q, J = 6.3 Hz, 2H), 2.29 (sextet, J = 6.8 Hz, 1H), 1.49 (pentet, J = 6.8 Hz, 2H), 1.37 (s, 9H), 1.33 (m, 2H), 1.02 (d, J = 6.9 Hz, 3H).

4.3.33. Synthesis of (*R*)-5-amino-2-methylpentanoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH_2 hydrotrifluoroacetate (31a)

First, compound **29a** (248 mg, 1.07 mmol) was dissolved in a solution of 0.4 M HOBt/HBTU in NMP (2.75 mL) and then a 1 M DIEA in NMP solution was added (1.1 mL). This solution was transferred to a vial containing Oic-Pro-D-Ala-Phe(*p*-Me)-resin (388 mg, 0.7 mmol/g substitution; synthesized according to the general solid phase peptide synthesis procedure) in NMP (3 mL). The solution was diluted with NMP (6 mL) and placed on a shaker at room temperature for 4.5 h. The resin was collected, washed with dichloromethane and dried, to yield the resin-bound pentapeptide **30a** (437 mg). Compound **30a** was then stirred for 2 h at room temperature with 95:5 TFA–H_2O (10 mL). After filtration, the solution was concentrated in vacuo, followed by precipitation with cold diethyl ether. After refrigeration overnight, **31a** hydrotrifluoroacetate was collected (off-white solid, 101 mg) and purified by HPLC to obtain **31a** hydrotrifluoroacetate (white solid, 67 mg, 54% yield) which was >95% pure by HPLC. ESI-MS calculated $[M+H]^+$, 611.4, found, 611.2.

4.3.34. Synthesis of (*S*)-5-amino-2-methylpentanoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH_2 (31b)

First, compound **29b** (97 mg, 0.42 mmol) was dissolved in a solution of 0.4 M HOBt/HBTU in NMP (2.5 mL) and then a 1 M DIEA in NMP solution was added (1 mL). This solution was transferred to a vial containing Oic-Pro-D-Ala-Phe(*p*-Me)-resin (357 mg, 0.59 mmol/g substitution; synthesized according to the general

solid phase peptide synthesis procedure) in NMP (2 mL). The solution was diluted with NMP (6 mL) and placed on a shaker at room temperature for 13 h. The resin was collected, washed with dichloromethane and dried, to yield the resin-bound pentapeptide **30b** (397 mg). Compound **30b** was then stirred for 2 h at room temperature with 95:5 TFA–H_2O (10 mL). After filtration, the solution was concentrated in vacuo, followed by precipitation with cold diethyl ether. After refrigeration overnight, **31b** hydrotrifluoroacetate was collected (off-white solid, 84 mg) and purified by HPLC to obtain **31b** hydrotrifluoroacetate (white solid, 37 mg, 40% yield) which was >95% pure by HPLC. ESI-MS calculated $[M+H]^+$, 611.4, found, 611.2.

4.3.35. Synthesis of (*R*)-5-(di-Boc)guanidino-2-methylpentanoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH_2 (32a)

The synthesis of **32a** was completed using a modification of a previously reported procedure.[18] To a stirred solution of *N*,*N*′-di-Boc-*N*′-triflylguanidine (32 mg, 0.08 mmol) and triethylamine (57 μL, 0.41 mmol) in 1,4-dioxane (2 mL) was added a solution of **31a** (56 mg, 0.08 mmol) in 1,4-dioxane (18 mL). The solution stirred at room temperature for 15 h, and was then concentrated in vacuo to yield a yellow oil. The oil was dissolved in ethyl acetate (20 mL), and washed with 2 M $KHSO_4$ (2 × 10 mL) followed by saturated $NaHCO_3$ (2 × 10 mL), dried over $MgSO_4$ and concentrated in vacuo to give a white solid containing **32a**. The solid was carried on without further purification or characterization.

4.3.36. Synthesis of (*S*)-5-(di-Boc)guanidino-2-methylpentanoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH_2 (32b)

The synthesis of **32b** was completed using a modification of a previously reported procedure.[18] To a stirred solution of *N*,*N*′-di-Boc-*N*′-triflylguanidine (20 mg, 0.05 mmol) and triethylamine (36 μL, 0.26 mmol) in 1,4-dioxane (2 mL) was added a solution of **31b** (37 mg, 0.05 mmol) in 1,4-dioxane (13 mL). The solution stirred at room temperature for 6 days, and was then concentrated in vacuo to yield a yellow oil. The oil was dissolved in ethyl acetate (20 mL), and washed with 2 M $KHSO_4$ (2 × 10 mL) followed by saturated $NaHCO_3$ (2 × 10 mL), dried over $MgSO_4$ and concentrated in vacuo to give a white solid containing **32b**. The solid was carried on without further purification or characterization.

4.4. Inhibition of human α-thrombin assay protocol

Assays to measure the ability of the new synthetic peptides to inhibit human α-thrombin (>95% pure; specific activity = 5070 U/mg, Haematologic Technologies, Essex Junction, VT, USA) cleavage of the chromogenic substrate tetrapeptide Sarcosine-Pro-Arg-*p*-nitroanilide (Bachem, Torrance, CA, USA) were completed as previously described.[16] Before use in each assay, α-thrombin solutions were stored on ice in assay buffer (10 mM tris(hydroxymethyl)aminomethane (Tris), 150 mM NaCl, pH 7.6), with long-term storage in a 50/50 glycerol/water solution at −20 °C.

Inhibition experiments were done in 96-well plates, using nine different concentrations of synthetic peptides, each in triplicate. Controls included no enzyme, no inhibitor and lead compound **FM 19**. Enzyme was preincubated with peptide in assay buffer at 37 °C for 5 min, and the experiment was initiated with the addition of substrate. The final concentration of enzyme in the assay was 2 nM and the final substrate concentration was 200 μM. After 10 min, the OD_{405} (average and standard deviation of the three wells for each concentration) was plotted as a function of the negative log of inhibitor concentration to generate a dose–response curve using GraphPad Prism (version 5, GraphPad Software, Inc., La Jolla, CA, USA). From these data, an IC_{50} value was determined. Each compound was tested in at least three independent experiments, and the average and standard deviation are reported. A sample inhibition profile, producing a single result for compound **7** can be found in Figure 4.

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