

**Synthesis and Structure-Activity Relationships of Novel Direct Thrombin Inhibitors  
based on Pentapeptide FM 19**

by

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This work is dedicated to my family,  
both the one I was born into,  
and the one I made for myself.

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## Table of Contents

<b>Dedication</b>	ii
<b>Acknowledgements</b>	iii
<b>List of Figures</b>	viii
<b>List of Tables</b>	ix
<b>List of Schemes</b>	x
<b>List of Abbreviations</b>	xi
<b>Abstract</b>	xiv
<b>Chapter</b>	
<b>1. Introduction</b>	1
ACS and Coagulation	1
Thrombin	4
Protease Activated Receptors	6
Clinical Use of Anticoagulants	8
Bradykinin, <b>RPPGF</b> , <b>TH 146</b> and <b>FM 19</b>	12
Chapter 1 References	16
<b>2. D-Arg replacements in the FM 19 sequence</b>	20
Chapter 2 References	39
<b>3. Phe(<i>p</i>-Me) replacements in the FM 19 sequence</b>	40
Chapter 3 References	53
<b>4. Pro-D-Ala replacements in the FM 19 sequence and C-terminal substituted amides</b>	54
Pro-D-Ala replacements	54
C-terminal substituted amides	61
Chapter 4 References	69

<b>5. Synthesis and testing of an ‘optimized’ peptide</b>	70
Chapter 5 References	74
<b>6. Conclusions and future directions</b>	75
Conclusions	75
Future Directions	78
Chapter 6 References	82
<b>7. Experimental methods</b>	83
Chemistry general procedures	83
General procedure for SPPS	83
General peptide purification procedure	84
General human $\alpha$ -thrombin inhibition assay protocol	84
Synthesis of peptides <b>1-56</b>	85
Synthesis of intermediates <b>57-119</b>	108
Chapter 7 References	137
 <b>Appendix</b>	 138

## List of Figures

### Figure

1. Coagulation Cascade	3
2. Thrombin bound to PPACK	5
3. Activation of PAR1	7
4. PAR1 G protein partners	8
5. Structures of <b>RPPGF</b> , <b>TH 146</b> , and <b>FM 19</b>	13
6. Crystal Structure of <b>FM 19</b> in the active site of thrombin	14
7. D-Arg residue in the active site of thrombin	21
8. Structures of D-Arg replacements in compounds <b>1-5</b>	22
9. Structures of D-Arg replacements in compounds <b>6-8</b>	25
10. Structures of D-Arg replacements in compounds <b>9-16</b>	29
11. Structures of D-Arg replacements in compounds <b>17-20</b>	35
12. Phe( <i>p</i> -Me) residue in the active site of thrombin	41
13. Structures of Phe( <i>p</i> -Me) replacements in compounds <b>21-26</b>	42
14. Structures of Phe( <i>p</i> -Me) replacements in compounds <b>27-34</b>	44
15. Structures of Phe( <i>p</i> -Me) replacements in compounds <b>35-37</b>	51
16. Pro-D-Ala residues in the active site of thrombin	55
17. Structures of peptides <b>38</b> and <b>39</b>	55
18. Structures of Pro-D-Ala replacements in compounds <b>40-43</b>	57
19. Structures of Pro-D-Ala replacements in compounds <b>44-48</b>	58
20. Structure of CGPM superimposed on <b>FM 19</b>	60
21. Phe( <i>p</i> -Me) residue in the active site of thrombin	62
22. Structures of C-terminal modifications in compounds <b>49-53</b>	63
23. Structures of C-terminal modifications in compounds <b>54-55</b>	66
24. Structure of compound <b>56</b>	71



## List of Tables

### Table

1.	D-Arg replacements to remove the N-terminal amine and alter side chain length	21
2.	D-Arg replacements to add conformational restriction	25
3.	D-Arg replacements to modify the guanidino group	29
4.	D-Arg replacements to investigate $\alpha$ -carbon stereochemistry	35
5.	Phe( <i>p</i> -Me) replacements to investigate alkyl versus aryl substitution and alter side chain length	41
6.	Phe( <i>p</i> -Me) replacements to investigate lipophilicity and <i>para</i> substitution	43
7.	Phe( <i>p</i> -Me) replacements to investigate $\alpha$ -carbon stereochemistry	50
8.	Pro-D-Ala replacements with shortened sequences	55
9.	Pro-D-Ala replacements to investigate ring size and stereochemistry	56
10.	Pro-D-Ala replacements using different scaffolds and peptidomimetics	58
11.	C-terminal modifications using alkyl amides	62
12.	C-terminal modifications using aryl amides	65
13.	Combination of best Modifications	71
A1.	Summary of assay and physiochemical data for <b>RPPGF</b> , <b>TH 146</b> , <b>FM 19</b> , and compounds <b>1-56</b>	139

## List of Schemes

### Scheme

1. Synthesis of compounds <b>1-5</b>	23
2. Synthesis of compound <b>6</b>	25
3. Synthesis of compounds <b>7 and 8</b>	27
4. Synthesis of compounds <b>9-11</b>	30
5. Synthesis of compound <b>12</b>	31
6. Synthesis of compounds <b>13 and 14</b>	33
7. Synthesis of compounds <b>15 and 16</b>	34
8. Synthesis of compounds <b>17, 18 and 20</b>	36
9. Synthesis of intermediate <b>96</b>	45
10. Synthesis of compound <b>31</b>	46
11. Synthesis of compound <b>32</b>	47
12. Synthesis of compound <b>33</b>	49
13. Synthesis of compounds <b>47 and 48</b>	60
14. Synthesis of compound <b>51</b>	63
15. Synthesis of compounds <b>52 and 53</b>	64
16. Synthesis of compound <b>54</b>	66
17. Synthesis of compound <b>55</b>	67
18. Synthesis of compound <b>56</b>	72

## List of Abbreviations

ACE	angiotensin-Converting Enzyme
ACS	acute Coronary Syndrome
ADP	adenosine diphosphate
Aic	2-aminoindane-2-carboxylic acid
Ala	alanine
APC	activated protein C
Arg	arginine
Azt	azetidine-2-carboxylic acid
Bip	biphenylalanine
Bn	benzyl
Boc	<i>tert</i> -butoxycarbonyl
BZA	3-amino-1-carboxymethyl-2,3,4,5-tetrahydro-1H-(1)-benzazapine- 2-one
cAMP	cyclic adenosine monophosphate
Cha	cyclohexylalanine
CGPM	4-amino-6-phenylmethyl-1,2,3,4-tetrahydroquinoline
CPL	3-amino-1-carboxymethylcaprolactam
DCM	dichloromethane
DIEA	<i>N,N</i> -diisopropylethylamine
DMF	dimethylformamide
DTI	direct thrombin inhibitor
ESI-MS	electrospray ionization mass spectrometry
Et	ethyl
Fmoc	9-fluorenylmethyloxycarbonyl
Fmoc-Cl	9-fluorenylmethyloxycarbonyl chloride
Gly	glycine

GPCR	G protein coupled receptor
HATU	2-(7-aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HBTU	<i>O</i> -benzotriazole- <i>N,N,N',N'</i> -tetramethyl-uronium-hexafluorophosphate
HK	high molecular-weight kininogen
HOAt	1-hydroxy-7-azabenzotriazole
HOBt	<i>N</i> -hydroxybenzotriazole
HPLC	high performance liquid chromatography
Idg	indanylglycine
<i>i</i> Pr	<i>iso</i> -propyl
LC/MS	liquid chromatography/mass spectrometry
Me	methyl
MLC	myosin light chain
NMP	<i>N</i> -methylpyrrolidinone
NMR	nuclear magnetic resonance spectroscopy
Oic	(2 <i>S</i> ,3 <i>aS</i> ,7 <i>aS</i> )-octahydroindole-2-carboxylic acid
PAR	protease activated receptor
Pbf	2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl
PDB	protein data bank
Ph	phenyl
Phe	phenylalanine
Phe(F) <sub>5</sub>	pentafluorophenylalanine
PK	prekallikrein
PPACK	thrombin inhibitor with sequence D-Phe-Pro-Arg-CHCl <sub>2</sub>
Pr	propyl
Pro	proline
SAR	structure-activity relationship
SPPS	solid phase peptide synthesis
Sty	styrylalanine
TF	tissue factor

TFA	trifluoroacetic acid
TFPI	tissue factor pathway inhibitor
THF	tetrahydrofuran
Tyr(Me)	<i>O</i> -methyl-L-tyrosine
Tyr(Bzl)	<i>O</i> -benzyl-L-tyrosine

## Abstract

The serine protease thrombin is the main clotting enzyme in the hemostatic system, in addition to being an effective platelet activator. Modulation of the actions of thrombin can be used as a mechanism to treat myocardial infarctions and other acute coronary syndromes. The bradykinin breakdown product, pentapeptide **RPPGF**, was the starting point to investigate new inhibitors that bind to the enzyme's active site. Subsequent SAR studies led to the development of a lead compound, **FM 19**, with the sequence D-Arg-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub>. More recently, the x-ray structure of **FM 19** in the active site of thrombin has been determined, providing insight into modifications to improve binding. The alterations described here include replacements to the D-Arg residue to maintain a crucial interaction with the catalytic Asp189, but eliminate an unfavorable electrostatic interaction between two positively charged groups, the N-terminal amine and guanidino group on the side chain; simultaneous replacement of the Pro-D-Ala residues which do not make any direct contacts with the enzyme, serving instead as scaffolding; modification of the Phe(*p*-Me) residue to enhance van der Waals interactions with surrounding Trp 215, Leu 99 and Ile 174 residues of thrombin; and modification of the C-terminus of the peptide to add alkyl and aryl substituents to the amide nitrogen. Alterations to two portions of the molecule investigated led to more potent compounds than **FM 19** (replacements to the D-Arg residue and the addition of substituents on the C-terminus). Incorporating the best modifications of both types led to the synthesis of compound **56**, with the sequence 5-guanidinopentanoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NHC<sub>6</sub>H<sub>11</sub>, which has an IC<sub>50</sub> of 530 nM, and shows an over 8-fold increase in potency over **FM 19**. Compound **56** is a promising new lead in the development of a new direct thrombin inhibitor.

## Chapter 1

### Introduction

#### ACS and Coagulation

Animals have evolved the ability to heal themselves after an injury in several ways. One such mechanism is the ability to form a blood clot (or thrombus) after injury involving a blood vessel has occurred. While this ability is extremely important to prevent more severe injuries and even death, it sometimes leads to the formation of unnecessary or pathological clots which can lead to acute coronary syndrome (ACS), stroke, or pulmonary embolism. ACS is a broad term that can refer to several different disease states of the heart, which occur when the heart does not get enough blood. One such condition that falls into this category is unstable angina, which is pain associated with ischemia, or lack of blood supply to the heart, and occurs when the body is at rest.<sup>1-3</sup> Myocardial infarction, more commonly referred to as a heart attack, is another related condition. Myocardial infarction occurs when blood supply to the heart is interrupted, resulting in death of a portion of the heart tissue.<sup>1</sup> Both of these conditions are most commonly caused by the formation of a thrombus that blocks a coronary artery, either partially (in the case of unstable angina) or entirely (as in myocardial infarction).<sup>2,4</sup> The thrombus is often formed after rupture of an atherosclerotic plaque, exposing the blood vessel to the lipids, cholesterol and foam cells that were once inside the plaque, as well as exposing tissue factor (TF)-expressing cells.<sup>2</sup>

As seen in Figure 1, thrombin plays a central role in the blood coagulation cascade, which is a series of proteins, many of which are serine proteases, being converted from their normal, circulating, inactive states (designated as roman numerals) into their active forms (designated as their corresponding roman numeral followed by the letter 'a'). The TF that is exposed following the rupture of an atherosclerotic plaque or

due to tissue injury initiates the extrinsic pathway (also known as the tissue factor pathway) of coagulation, which is the primary coagulation initiation pathway.<sup>5</sup> TF is primarily expressed on the surfaces of cells which lie underneath the endothelium of the blood vessels, and so this pathway can and does take place somewhat outside of the normal, healthy vasculature.<sup>6</sup> The TF that is exposed interacts with circulating factor VII to form a complex which can first causes activation of factor VII to generate factor VIIa, and then activates factor X (producing factor Xa), as well as small amounts of factor IXa from factor IX. The factor Xa, along with cofactor factor Va join in the common pathway to form the prothrombinase complex, which then cleaves factor II (prothrombin) into trace amounts of thrombin (factor IIa), in what is often referred to as the initiation phase of coagulation.<sup>2,7</sup> In what is classically referred to as the intrinsic pathway, amplification of the amount of available thrombin can be achieved. The factor IXa previously produced can interact with factor VIIa, assembling in a complex known as tenase, activating more factor X into factor Xa, resulting in propagation. Once enough thrombin has been locally generated, it can then cleave soluble fibrinogen into insoluble fibrin, creating the protein portion of the clot.<sup>2,7</sup>

These pathways (extrinsic and intrinsic) are thought to operate in parallel, and their activation is highly dependent upon the cell type present.<sup>8</sup> TF is an integral membrane protein usually expressed on cells outside of the walls of the blood vessels, and not usually exposed to circulating coagulation factors unless vessel injury occurs. However, since smaller coagulation factors can diffuse outside of the normal blood circulation, it is possible that the TF/factor VIIa complex forms before tissue injury is present, but is not physiologically relevant due to the low concentrations of this complex outside of blood vessels.<sup>8</sup> The intrinsic pathway needs a negatively charged surface for initiation, which is usually the surface of a platelet inside of the blood vessel. The platelet acts to keep necessary coagulation factors nearby, resulting in rapid generation of large amounts of thrombin to assist in thrombus formation.<sup>8</sup>



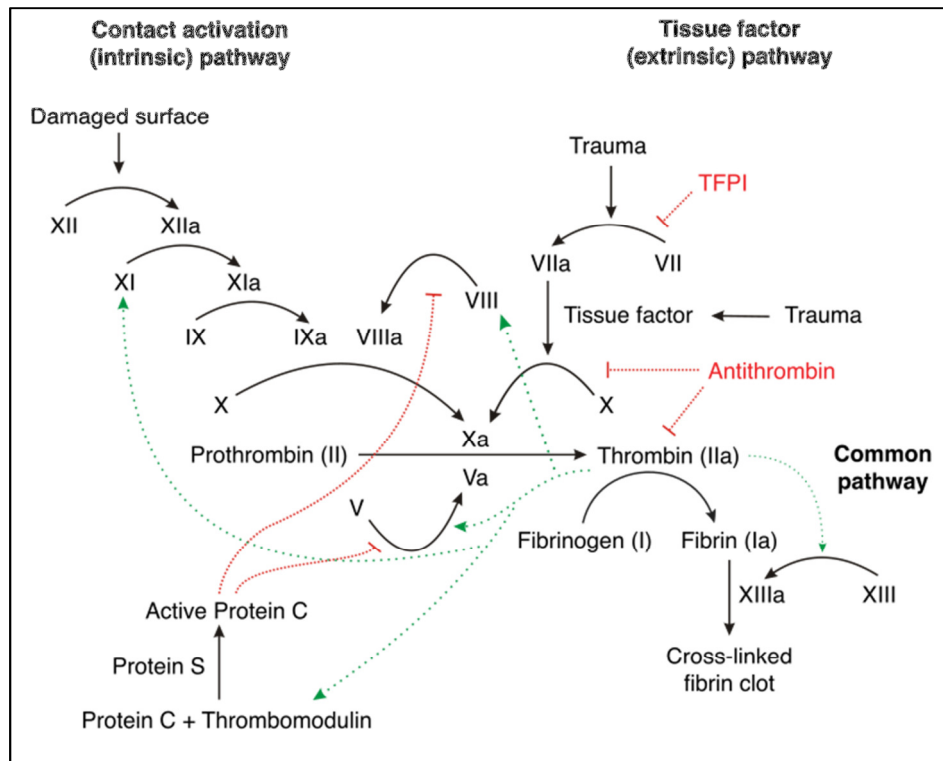


Figure 1: Coagulation Cascade<sup>9</sup>

Once the thrombus has formed and bleeding has stopped, the body needs to be able to control unnecessary coagulation, and so regulatory mechanisms exist, which are shown in red in Figure 1.<sup>10,11</sup> The first of these is the tissue factor pathway inhibitor (TFPI). TFPI is a 43 kDa inhibitor of Factor Xa.<sup>12</sup> Once TFPI binds to factor Xa, this complex then binds to the factor VIIa/TF complex, rendering the factor VIIa/TF complex inactive, and stopping the initiation of the blood coagulation cascade.<sup>10-12</sup> The second pathway is the protein C pathway. Thrombin can bind to the thrombomodulin receptor on the surface of endothelial cells, causing thrombin to undergo a conformational change that potently activates protein C, an endogenous plasma protein, forming activated protein C (APC). APC then degrades factor Va and factor VIIa, resulting in inhibition of coagulation.<sup>10,11</sup> The third pathway involves the serpin antithrombin. Serpins (Serine Protease Inhibitors) are endogenous proteins, most of which function to inhibit proteases. Antithrombin can inactivate the serine proteases factor Xa and thrombin, by preferentially binding to the free forms of these serine proteases, rather than binding to these proteases while they are in complexes with other coagulation factors.<sup>10,11,13</sup>

## Thrombin

Human  $\alpha$ -thrombin is a trypsin-like serine protease which consists of 295 amino acids in two chains, the shorter A chain (36 amino acids) and the longer B chain (259 amino acids) (Figure 2).<sup>14</sup> There are four disulfide bonds, marked in Figure 2 as orange with numbers 1-4. The disulfide bond between Cys 1 and Cys 122 (marked as disulfide bond 1) holds the two chains together, with the A chain shown in green, behind the B chain, which is shown in blue. Figure 2 also shows the active site containing the catalytic triad, contained within the B chain, of H57, D102 and S195 (residues marked with \*) and bound to the inhibitor PPACK (shown in yellow, sequence D-Phe-Pro-Arg-CH<sub>2</sub>Cl).<sup>14</sup> Thrombin prefers to cleave substrates next to an Arg residue, and often cleaves either an Arg-Ser or Arg-Gly bond, as the bottom of the catalytic pocket contains D189 which coordinates well with the side chain of an arginine residue.<sup>14,15</sup> In addition, there are two auxiliary sites that have been identified and named exosite I and II, which help with substrate specificity. Exosite I contains a fibrinogen recognition region, which helps to confer specificity for fibrinogen, while exosite II recognizes glycosaminoglycans.<sup>16</sup> Furthermore, there is a sodium-ion binding site, which also aids in recognition and alters the kinetics of thrombin substrate cleavage.<sup>14</sup> Finally thrombin contains two insertion loops, which are longer than the loops found in the related enzymes trypsin and chymotrypsin. These are the 60-loop, composed of mainly hydrophobic residues, and the  $\gamma$ -loop, which is made of more hydrophilic groups.<sup>17</sup> The 60-loop is more structurally rigid, and can make interactions with substrates on the N-terminal side of the site of cleavage. The  $\gamma$ -loop on the other hand, is more flexible and can interact with substrates on the C-terminal side of the site of cleavage.<sup>17,18</sup> Together, both the 60-loop and the  $\gamma$ -loop help to confer thrombin substrate specificity through sterics as well as by allowing only substrates with complementary surfaces to access to the active site.<sup>19</sup>

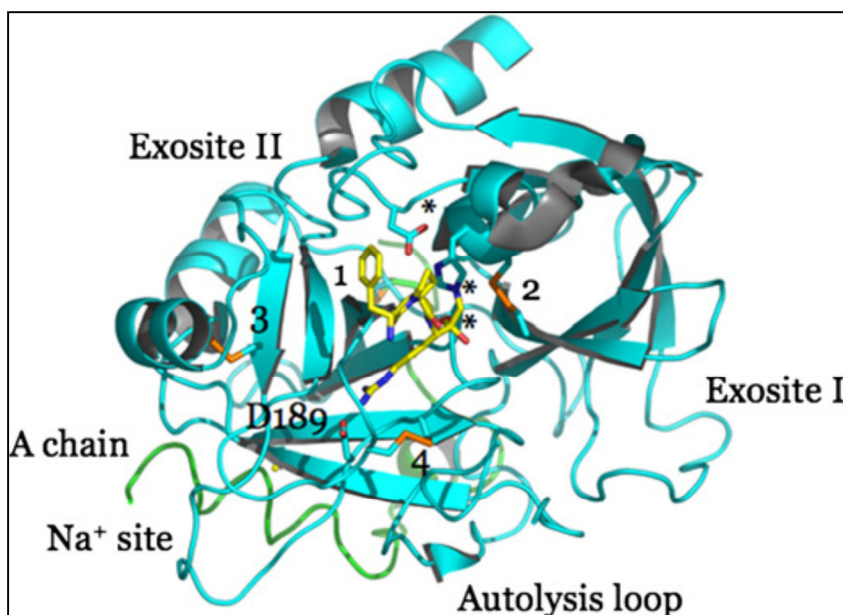


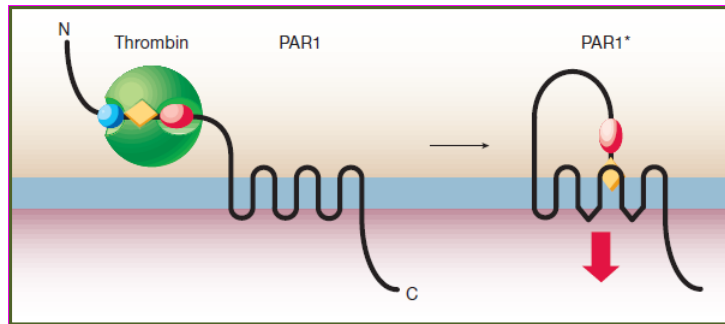
Figure 2: Thrombin Bound to PPACK<sup>14</sup>

As alluded to before, thrombin has both procoagulant and anticoagulant functions. Thrombin is responsible for the cleavage of fibrinogen into fibrin, which is incorporated into a forming thrombus. Thrombin can also activate several other coagulation factors such as V, VII and XI to aid in the amplification of thrombin generation.<sup>2,5-7</sup> However, when enough thrombin has been generated, there is a negative feedback loop which allows for the inactivation of the coagulation cascade. When thrombin binds to the cell surface receptor thrombomodulin on intact endothelial cells through exosite I, circulating protein C can also bind to this complex and become activated. The activated protein C, along with its protein S cofactor, can then degrade and inactivate factors Va and VIIIa.<sup>10,11</sup> Finally Na<sup>+</sup> binding in the allosteric sodium-ion binding site plays a role in determining thrombin's preference for having procoagulant or anticoagulant functions. When Na<sup>+</sup> is bound to thrombin, thrombin is in its so-called fast form, and is able to more efficiently cleave fibrinogen and activate factors V, VIII and XI. However, Na<sup>+</sup> is not required for optimal activation of protein C, and so the non-sodium ion bound, or slow form, of thrombin can efficiently play its anticoagulant role. Thus, it appears that the presence of Na<sup>+</sup> in the blood contributes to coagulation by enhancing the rate of thrombin's procoagulant actions.<sup>14</sup>

Finally, there are different forms of human thrombin –  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\epsilon$  and  $\zeta$ . The most physiologically relevant form is  $\alpha$ -thrombin, which has been described above. The  $\beta$ ,  $\gamma$ ,  $\epsilon$  and  $\zeta$  forms of thrombin are shorter sequences than  $\alpha$ -thrombin, which are a result of either autolysis or by digestion with another serine protease resulting in cleavage of a portion of the B chain.<sup>20,21</sup> Each of these shortened sequences results from a single cleavage of  $\alpha$ -thrombin, except  $\gamma$ -thrombin, which has two cleavages, and it has been suggested that  $\gamma$ -thrombin is the product of further cleavage of  $\beta$ -thrombin.<sup>22,23</sup> Clotting activity is greatest in  $\alpha$ -thrombin, but is seen in a reduced capacity in both  $\epsilon$ - and  $\zeta$ -thrombin. Fibrinogen cleavage is almost eliminated in both the  $\beta$  and  $\gamma$  forms.<sup>22,24,25</sup> However,  $\beta$ - and  $\gamma$ -thrombin still possess the ability to cleave small peptide substrates, suggesting that the active site remains intact in these thrombin forms.<sup>26</sup>

### **Protease Activated Receptors**

One additional procoagulant role that thrombin plays is that of platelet activator. This action of thrombin is produced through the protease activated receptors (PARs), which are a small family of G protein-coupled receptors (GPCRs).<sup>27-29</sup> There are four known members of this family, PAR1-4, which are activated upon cleavage by a serine protease, as shown in Figure 3. Human platelets express PAR1 and PAR4, which are both activated by thrombin.<sup>27-29</sup> PAR1 and PAR4 each carry their own cryptic ligand. Thrombin binds to a portion of the N-terminus of the receptor and cleaves a fragment, creating a *neo*-N-terminus that can then bind intramolecularly, sending downstream signals to activate platelet aggregation.<sup>27-29</sup> Once the receptor is activated, the signal needs to somehow be turned off. Most GPCRs (except the PARs) are activated after an extracellular ligand binds, and the signal propagated can be shut down through phosphorylation of the receptor and arrestin binding, leading to internalization and recycling of the receptor to the cell surface.<sup>30</sup> Since the PARs carry their own ligand, their deactivation mechanism is a little more complex. Instead, the signal is terminated by either internalization of the receptor and subsequent degradation, or by further cleavage of this tethered ligand. PAR1 is also thought to be internalized at a much faster rate than PAR4.<sup>30</sup> In addition, it appears as if PAR1 is activated through cleavage by  $\alpha$ -thrombin, whereas  $\beta$ - and  $\gamma$ -thrombin selectively activate PAR4.<sup>23</sup>



**Figure 3: Activation of PAR1<sup>27</sup>**

Although both PAR1 and PAR4 are activated by thrombin and lead to platelet aggregation, they produce complementary, rather than redundant, signals.<sup>31</sup> PAR1, the prototype of the PAR family, is activated at lower thrombin concentrations and produces a signal that is rapidly silenced. A portion of the PAR1 receptor binds to exosite I in addition to the active site of thrombin, allowing for a tighter and more specific interaction, and becomes quickly desensitized due to phosphorylation. PAR4 requires higher thrombin concentrations due to the fact that there is no portion of the receptor that binds to exosite I, leading to an interaction that is not as extensive, and also produces a longer lasting signal. This may mean that PAR4 can function as a complement to PAR1, or could be activated by other proteases besides thrombin.<sup>14,27-30</sup> In addition, the Na<sup>+</sup> bound form of thrombin increases the rate of PAR activation, which is consistent with Na<sup>+</sup> being an important cofactor for thrombin's procoagulant functions.<sup>14</sup>

Since PAR1 and PAR4 are both GPCRs, they can couple to G proteins within a platelet. PAR1 and PAR4 both couple to the G<sub>12/13</sub> and G<sub>q</sub> families of G proteins, and PAR1 couples to the G<sub>i</sub> family as well.<sup>27,32</sup> The G protein partners are shown in Figure 4. When either PAR1 or PAR4 couples to the G<sub>α,12/13</sub> subunit, this causes the platelet to change its shape drastically with spike-like projections, and release platelet-dense granules, which are secretory organelles that contain a variety of molecules such as ADP and the vasodilator histamine, as well as calcium ions.<sup>32</sup> Additionally, G<sub>12/13</sub> can regulate myosin light chain (MLC) phosphorylation through the Rho/Rho kinase pathway, which results in the platelet changing its shape to produce spike-like projections.<sup>33</sup> When PAR1 couples to G<sub>α,i</sub>, adenylyl cyclase is inhibited. This causes a decrease in cAMP, which itself can lead to platelet inhibition. However, although the signal from PAR1 itself is

short-lived, the ADP secreted can activate another G<sub>i</sub>-coupled receptor, the P2Y<sub>12</sub> receptor, which leads to additional adenylyl cyclase inhibition.<sup>32,34</sup> When PAR1 or PAR4 couples to G<sub>α,q</sub>, there is an increase in the intracellular calcium concentration, which activates the GP IIb/IIIa fibrinogen receptor which is also present on the platelet surface.<sup>32</sup> When the GP IIb/IIIa receptor is activated, a recognition sequence on the outside of the cell is unmasked, allowing this receptor to bind ligands such as fibrin. Once a ligand binds, this causes the cross-link between platelets and fibrin, forming a thrombus.<sup>2,35</sup> With PAR1, this increase in intracellular calcium is quick and short-acting, and so PAR4 is responsible for the majority of the increase in intracellular calcium.<sup>32</sup> It is clear that upon activation, PARs induce changes within platelets which are favorable for the promotion of aggregation and thrombus formation. Thus, like thrombin, the PARs also appear to be an attractive target for anticoagulant drugs.

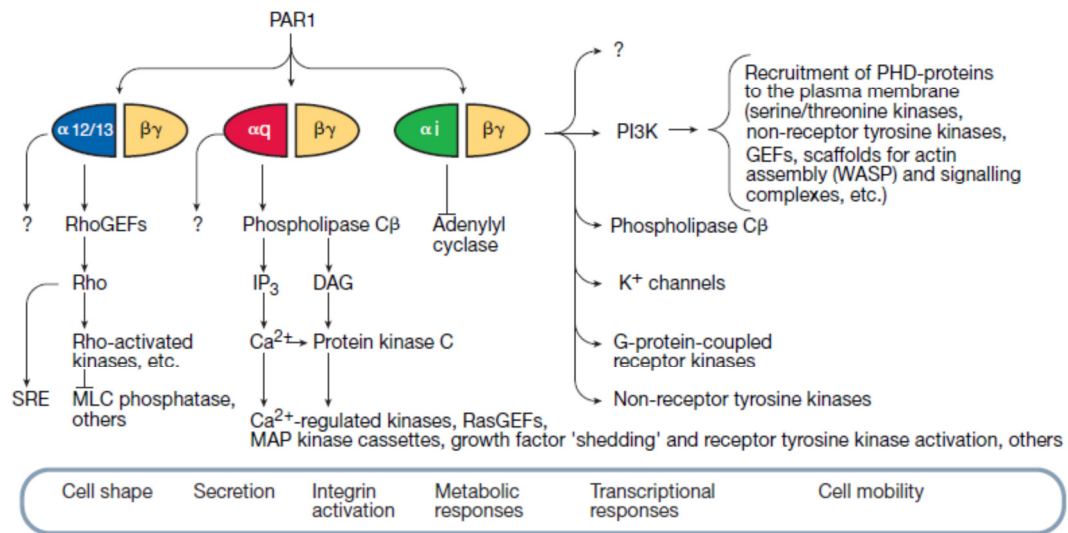


Figure 4: PAR1 G protein partners<sup>27</sup>

### Clinical Use of Anticoagulants

Since myocardial ischemia and other thromboembolic diseases are among the leading causes of death worldwide, antithrombotic and anticoagulant drugs are widely used.<sup>2,11</sup> One such drug is heparin, a very highly sulfated glycosaminoglycan composed of repeating units of sulfated hexuronic acid (D- or L-) and D-glucosamine, which has the

highest negative charge density of any biomolecule known.<sup>2,10,11</sup> Unfractionated heparin, along with smaller versions of heparin called low-molecular weight heparins, work by activating antithrombin, which in turn inhibits factor Xa and thrombin by making the interaction between the antithrombin and the serine protease stronger.<sup>2,11</sup> Heparins have been widely used for many years, and is the treatment of choice during surgery to prevent blood clots. However, there are several issues associated with its use.<sup>2,11</sup> Heparin is derived naturally from slaughtered animals (mostly pig intestines in the U.S.), and it varies in composition of glycosaminoglycans, as well as potency and pharmacokinetic properties between patients.<sup>2,11</sup> Although less of a problem with fractionated versions, all heparins show at least some plasma protein binding, resulting in less heparin available to bind to antithrombin. There are also serious side effect risks, such as heparin-induced osteoporosis and heparin-induced thrombocytopenia.<sup>2</sup> Finally, heparin must be administered subcutaneously or intravenously, and has a narrow therapeutic window and variable dose-response relationship, which requires frequent monitoring of patients.<sup>2</sup>

The most commonly prescribed anticoagulant drug is warfarin, a synthetic derivative of coumarin.<sup>2,10</sup> Warfarin was introduced into clinical practice as an anticoagulant in 1954, and acts indirectly.<sup>10,36,37</sup> Warfarin is a vitamin K antagonist, and works by blocking the conversion of vitamin K epoxide to vitamin K. Vitamin K is needed to convert factors II, VII, IX and X into their active, pro-coagulant form, and it is also required to convert protein C into its active, anti-coagulant form.<sup>38</sup> Warfarin is widely used and administered, but there are still many problems with its use.<sup>11,38</sup> Warfarin is very susceptible to adverse interactions with other drugs and diet, and the dose-response varies greatly between patients. As a result, treatment with warfarin has a narrow therapeutic window and requires frequent monitoring in the form of regular blood tests.<sup>38</sup> In addition, since warfarin's effects on coagulation are indirect, effects are not seen until approximately one day after administration. Warfarin's effects last several days after the last administration, and the only antidote available to stop the antithrombotic effects is vitamin K itself.<sup>11</sup> Thus, warfarin is not an ideal anticoagulant.

Due to the large role that platelets play in thrombus formation, inhibition of platelet activation and aggregation has been another area of interest to produce new anticoagulants. Low-dose aspirin therapy has long been known to reduce the risk of

cardiovascular events such as myocardial infarction or stroke. Aspirin works to inhibit cyclooxygenase which inhibits the formation of thromboxane A<sub>2</sub> in platelets, which then leads to inhibition of platelet aggregation.<sup>39</sup> Another area is inhibition of the ADP receptor on platelet cell surface, the P2Y<sub>12</sub> receptor. This receptor is associated with the amplification of platelet aggregation, and is irreversibly inhibited by thienopyridines such as clopidogrel (marketed as Plavix by Bristol-Meyers Squibb). A study has shown that clopidogrel was actually more effective than aspirin in preventing death by vascular events, but carries the risk of neutropenia, as well as an increased risk of bleeding.<sup>39</sup> Yet another method involving platelets is inhibition of the PARs, which would result in decreased platelet activation.<sup>40</sup> Vorapaxar, a PAR1 antagonist made by Merck was recently shown in a phase III clinical trial to reduce the risk of death due to cardiovascular events when compared to standard therapy, but carries an increased risk of moderate to severe bleeding events.<sup>41</sup> Thus, Vorapaxar or another related compound could become a potential new anticoagulant in the coming years.

In addition to the anticoagulants discussed above, there are also several agents which interact directly with serine proteases in the coagulation cascade. One such agent, the orally available factor Xa inhibitor rivaroxaban and marketed as Xarelto by Janssen Pharmaceuticals Inc., was approved for use in the U.S. in 2011 for the prevention of stroke in patients with non-valvular atrial fibrillation.<sup>42,43</sup> Factor Xa is responsible for thrombin generation, which leads to thrombus formation, and so inhibition of this enzyme produces an anticoagulant effect. Additionally, it is believed that inhibition of factor Xa may result in a lower risk of a major bleeding event, specifically intracranial hemorrhage.<sup>42,43</sup> In addition to rivaroxaban, there are other factor Xa inhibitors currently in clinical trials. The first of these compounds is apixaban, which was approved in Europe in May 2012, and expected to be reviewed for approval in the U.S. in March 2013.<sup>43</sup> One clear advantage of apixaban is the low renal clearance (25%), indicating that this compound (manufactured by Pfizer and Bristol-Meyers Squibb) would be an alternative anticoagulant for patients with decreased kidney function.<sup>43</sup> Finally, another factor Xa inhibitor in phase III clinical trials in the U.S. is edoxaban, marketed by Daiichi Sankyo, which was approved in Japan in 2011.<sup>43,44</sup> Like the other factor Xa inhibitors discussed above, edoxaban has the potential to interact with CYP3A4 inhibitors, which



could potentially lead to adverse interactions.<sup>42-44</sup> However, inhibition of factor Xa has been shown to effectively produce an anticoagulant effect, and continues to be an area of further investigation.

Currently on the market, there are also direct thrombin inhibitors (DTIs), which interact with thrombin's active site to inhibit its actions. One of these is hirudin, a sulfated, 65 amino acid peptide originally isolated from leeches.<sup>11</sup> Hirudin is classified as a bivalent DTI, since it occupies two sites on thrombin for inhibition: the active site as well as exosite I. Hirudin is administered parenterally and is the most potent thrombin inhibitor known thus far with a sub-picomolar  $K_i$ .<sup>11</sup> However, it has a narrow therapeutic window, carries a bleeding risk, and many patients develop antibodies, resulting in an allergic reaction. A smaller, synthetic version is bivalirudin, also a divalent DTI, administered parenterally.<sup>11</sup> Bivalirudin, when compared to hirudin, has a wider therapeutic window, reduced immune response, and a lower bleeding risk, but is also approximately 800 times less potent than hirudin, and much more expensive than unfractionated heparin, and therefore not as widely used.<sup>11</sup>

The first univalent DTI, which interacts with only the active site of thrombin, was argatroban. Argatroban is a synthetic small molecule that must be administered intravenously, and shows significant plasma protein binding and rapid elimination.<sup>11</sup> Argatroban was the first non-covalent, reversible, small molecule univalent DTI.<sup>11</sup> Argatroban proves that a univalent DTI is acceptable option for an anticoagulant, but also leaves room for improvement.

Dabigatran etexilate, a dual prodrug of dabigatran, is the first orally available DTI approved for use in the U.S. It is marketed by Boehringer Ingelheim as Pradaxa, and was approved in October 2010 for prevention of stroke in patients with atrial fibrillation. Dabigatran etexilate has a promising profile, as it is at least as effective in the prevention of stroke when compared to warfarin treatment, with similar or decreased risk of a major bleeding event.<sup>45</sup> Additionally, since dabigatran shows fewer interactions with both food and other drugs, it does not require the frequent monitoring necessary with warfarin treatment.<sup>45</sup> Therefore, there is finally an orally available anticoagulant alternative to warfarin, and this proves that orally available anticoagulation treatment can be achieved through direct thrombin inhibition.

## **Bradykinin, RPPGF, TH 146 and FM 19**

Our search for an orally available direct thrombin inhibitor first began in the Schmaier lab, with the discovery of the pentapeptide **RPPGF** (Figure 5).<sup>46</sup> **RPPGF** is produced from a circulating high molecular-weight kininogen (HK) complexed with prekallikrein (PK).<sup>47</sup> When this HK•PK complex binds to a complex comprised of urokinase plasminogen activator receptor, cytokeratin 1 and gC1qR on the endothelial cell surface, it causes rapid activation of PK into kallikrein. The active kallikrein then cleaves HK to produce kinin-free kininogen and bradykinin.<sup>47</sup> Bradykinin, a nonapeptide with the sequence RPPGFSPFR<sup>48</sup> can be further broken down by angiotensin-converting enzyme (ACE) to produce the stable pentapeptide **RPPGF**.<sup>46</sup>

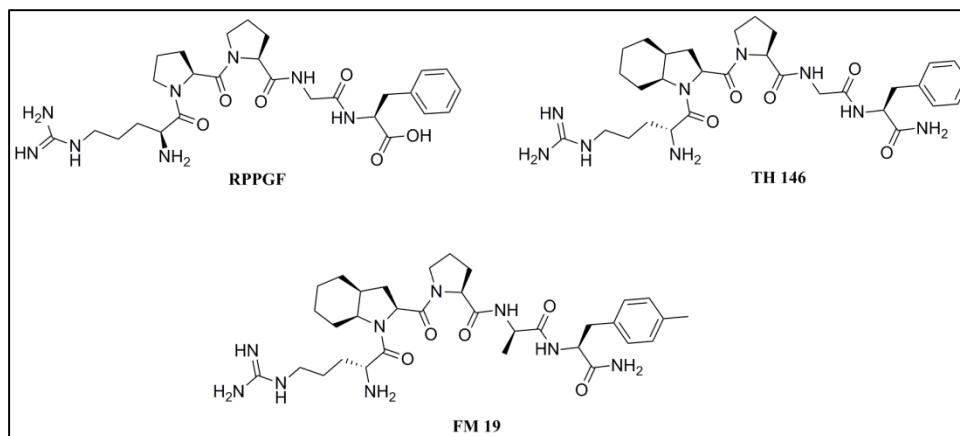
Inhibition of ACE is known to be beneficial for the heart.<sup>49</sup> First, ACE is responsible for generation of angiotensin II, which is implicated in cardiovascular diseases such as atherosclerosis and hypertension when it activates the angiotensin II receptors.<sup>50</sup> Secondly, inhibition of ACE results in higher accumulations of bradykinin, resulting in increased interaction with the corresponding bradykinin receptors, B<sub>1</sub>R and B<sub>2</sub>R.<sup>49</sup> B<sub>1</sub>R, expressed primarily under inflammatory responses due to tissue injury<sup>49</sup>, is activated by a bradykinin metabolite (Lys-DABK, with the sequence KRPPGFSPF)<sup>51</sup>, and is involved in both inflammatory processes and acute and chronic pain sensations.<sup>52</sup> Meanwhile, B<sub>2</sub>R is constitutively expressed, and once activated by bradykinin is responsible for the antihypertensive effect of vasodilation seen with ACE inhibitor administration.<sup>49</sup>

**RPPGF** has been shown to inhibit blood coagulation by two distinct mechanisms.<sup>46</sup> First, **RPPGF** binds directly to the thrombin cleavage site on PAR1. This prevents thrombin from binding and cleaving the N-terminus, and not allowing the tethered ligand required for PAR1 activation to be unmasked. Thus, **RPPGF** works to inhibit platelet activation by binding directly to the PAR1 receptor present on the platelet cell surface.<sup>46</sup> Secondly, **RPPGF** has been shown to bind directly to the active site of thrombin with a K<sub>i</sub> of 1.75 mM.<sup>46</sup> This not only inhibits cleavage of the PAR receptor, but also inhibits the other actions of thrombin, such as propagation of the coagulation cascade and fibrinogen cleavage. **RPPGF** is an unusual direct thrombin inhibitor because it binds in what is termed a retro-binding fashion.<sup>46</sup> This means it binds in the

opposite direction (in terms of linear sequence) when compared to natural thrombin substrates. Thus, **RPPGF** serves as a novel lead for the development of a direct thrombin inhibitor.

The first set of SAR studies using **RPPGF** as a lead compound resulted in the development of a new lead compound, **TH 146** (Figure 5), which was reported by Schmaier and coworkers.<sup>53</sup> **TH 146** was reported to be dual functioning, acting as both a direct thrombin inhibitor as well as binding directly to PAR1, like the precursor **RPPGF**. Pentapeptide **TH 146** has three key structural modifications from **RPPGF**. First, the N-terminal Arg residue was modified from the naturally occurring L configuration to the D configuration.<sup>53</sup> Secondly, the Pro residue in the second position was replaced with an Oic residue ((2*S*,3*aS*,7*aS*)-octahydroindole-2-carboxylic acid). Finally, the C-terminus of the peptide was modified from an acid to an amide.<sup>53</sup> These last two modifications (modification to Oic and creation of a C-terminal amide) have been documented in the literature to improve potency of bradykinin antagonists.<sup>54,55</sup> These combined modifications resulted in a  $K_i$  for thrombin inhibition of 97  $\mu\text{M}$ , which represents an 18-fold increase in potency over **RPPGF**.<sup>53</sup>

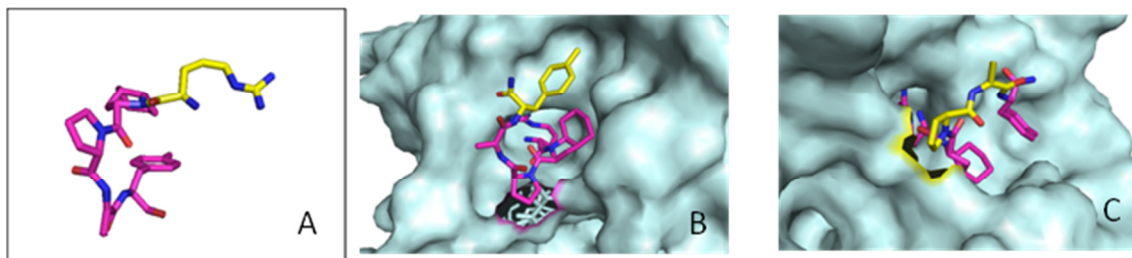
SAR studies using **TH 146** as a lead compound were then completed in the Mosberg lab, and culminated in pentapeptide **FM 19** (Figure 5).<sup>56</sup> **FM 19** made two changes to the C-terminal end of the peptide. First, a methyl group was added in the *para* position of the phenyl ring in the C-terminal Phe residue. Secondly, the Gly residue in the fourth position was modified to a D-Ala residue.<sup>56</sup> Together, these modifications improved thrombin inhibition potency to give a  $K_i$  of 4.4  $\mu\text{M}$ <sup>57</sup>, which is a 22-fold increase over **TH 146** and an almost 400-fold increase in potency seen over **RPPGF**. In addition, **FM 19** has shown promise as an orally active antithrombotic agent.<sup>57</sup> **FM 19** was stable in rat intestinal juices, and after oral administration to rats in drinking water, the *in vivo* time to thrombosis was increased.<sup>57</sup> **FM 19** has been reported to only act as an anticoagulant in one way, by direct thrombin inhibition, and is not believed to have a direct interaction with the thrombin cleavage site on PAR1.<sup>57</sup>



**Figure 5: Structures of RPPGF, TH 146 and FM 19**

Finally, an x-ray structure of **FM 19** in the active site of thrombin has been reported and has given valuable insights to design a new direct thrombin inhibitor.<sup>57</sup> First, the side chain of the D-Arg residue adopts a more eclipsed and strained conformation instead of the lower-energy extended conformation (Figure 6A). Modification of the side chain length or addition of conformational restriction may help to alleviate this higher-energy conformation. In addition, this arrangement places the positively charged N-terminal amine in close proximity with the positively charged guanidino group on the side chain, which would be expected to produce an adverse electrostatic interaction. Removal of the N-terminal amine, modification of the guanidino functionality, or modification of  $\alpha$ -carbon stereochemistry could all help to address this issue of strain. Secondly, the Phe(*p*-Me) makes primarily van der Waals interactions within the active site (Figure 6B). Modification of this residue would determine preference thrombin has for alkyl versus aryl residues, as well as tolerance for lipophilicity and alteration of  $\alpha$ -carbon stereochemistry. In addition, continued investigation of different *para* substituents would continue to build upon the previous studies, which found that longitudinal substituents (as in the case with *para* substitution) are preferred over side chains that are wider.<sup>56</sup> Also, the Pro and D-Ala residues do not directly interact with the active site of the enzyme, but instead act as more of a scaffold to allow the other three residues of **FM 19** to adopt the proper conformations for binding (Figure 6C). Several modifications could be made to this middle portion of the sequence which could still maintain the overall positioning of the peptide, but could also

potentially improve oral bioavailability and make the overall structure less peptide-like, which could result in longer half-life and less metabolism *in vivo*. Finally, modification of the C-terminus of the peptide has the potential to make the compound a more likely candidate for oral administration. The crystal structure gives less obvious clues concerning modifications here, but the addition of either an alkyl or aryl group, via substitution of the nitrogen atom on the C-terminal amide, could potentially add additional van der Waals interactions around the outside of the thrombin binding pocket. Modifications here would add lipophilicity and could potentially increase intestinal absorption.



**Figure 6: Crystal Structure of FM 19 in the active site of thrombin (PDB 3BV9, 1.8 Å resolution).**

**A: D-Arg residue highlighted in yellow with the rest of the peptide in magenta.**

**B: Phe(*p*-Me) residue highlighted in yellow with the rest of the peptide in magenta.**

**C: Pro-D-Ala residues highlighted in yellow with the rest of the peptide in magenta**

This thesis presents the work done to modify four different areas of the **FM 19** sequence independently. Chapter 2 focuses on modifications to the D-Arg residue at the N-terminus of the **FM 19** sequence to improve potency. Chapter 3 describes modifications to the Phe(*p*-Me) residue on the C-terminus, again in an attempt to improve potency over the lead compound **FM 19**. Chapter 4 is divided into two parts, both with the intention to not only improve potency, but also to potentially improve oral bioavailability. Chapter 4 looks at modifications to the Pro-D-Ala residues simultaneously in the first section, and then describes modifications of the primary amide on the C-terminus of **FM 19** in the second section.

## Chapter 1 References

- (1) Goodman & Gilman's *The Pharmacological Basis of Therapeutics*; 10 ed.; McGraw-Hill Medical Publishing Division: New York, NY, 2001.
- (2) Rauch, U.; Osende, J. I.; Fuster, V.; Badimon, J. J.; Zahi Fayad, P.; James H. Chesebro, M. *Annals of Internal Medicine* **2001**, *134*, 224.
- (3) Grech, E. D.; Ramsdale, D. R. *British Medical Journal* **2003**, *326*, 1259.
- (4) Paoli, G.; Merlini, P.; Ardissino, D. *Current Pharmaceutical Design* **2005**, *11*, 3919.
- (5) Dellinger, R. P. *Clinical Infectious Diseases* **2003**, *36*, 1259.
- (6) Hoffman, M. *Journal of Thrombosis and Thrombolysis* **2003**, *16*, 17.
- (7) Gustafsson, D.; Bylund, R.; Antonsson, T.; Nilsson, I.; Jan-Erik Nyström; Eriksson, U.; Bredberg, U.; Teger-Nilsson, A.-C. *Nature Reviews Drug Discovery* **2004**, *3*, 649.
- (8) Hoffman, M.; Monroe, D. M. *Current Hematology Reports* **2005**, *4*, 391.
- (9) D, J.; Wikipedia: 2012, p Coagulation Cascade.
- (10) Hirsh, J. *Thrombosis Research* **2003**, *109*, S1.
- (11) Alban, S. *Current Pharmaceutical Design* **2008**, *14*, 1152.
- (12) Mast, A. E.; Broze, G. J. *Blood* **1996**, *87*, 1845.
- (13) Dahlback, B. *The Lancet* **2000**, *355*, 1627.
- (14) Di Cera, E. *Molecular Aspects of Medicine* **2008**, *29*, 203.
- (15) Ishii, K.; Gerszten, R.; Zheng, Y. W.; Welsh, J. B.; Turck, C. W.; Coughlin, S. R. *The Journal of Biological Chemistry* **1995**, *270*, 16435.
- (16) Huntington, J. A.; Baglin, T. P. *TRENDS in Pharmacological Sciences* **2003**, *24*, 589.
- (17) Huntington, J. A. *Journal of Thrombosis and Haemostasis* **2005**, *3*, 1861.
- (18) Lane, D. A.; Philippou, H.; Huntington, J. A. *Blood* **2005**, *106*, 2605.
- (19) Huntington, J. A. *Biochimica et Biophysica Acta* **2012**, *1824*, 246.

- (20) Bauer, R. S.; Chang, T.-L.; Berliner, L. J. *The Journal of Biological Chemistry* **1980**, *255*, 5900.
- (21) Fenton, J. W.; Fasco, M. J.; Stackrow, A. B.; Aronson, D. L.; Young, A. M.; Finlayson, J. S. *The Journal of Biological Chemistry* **1977**, *252*, 3587.
- (22) Berliner, L. J. *Thrombin Structure and Function*; Plenum Press: New York, 1992.
- (23) Soslau, G.; Goldenberg, S. J.; Class, R.; Jameson, B. *Platelets* **2004**, *15*, 155.
- (24) Brezniak, D. V.; Brower, M. S.; Witting, J. I.; Walz, D. A.; Fenton, J. W. *Biochemistry* **1990**, *29*, 3536.
- (25) Brower, M. S.; Walz, D. A.; Garry, K. E.; Fenton, J. W. *Blood* **1987**, *69*, 813.
- (26) Chang, J.-Y. *Biochemical Journal* **1986**, *240*, 797.
- (27) Coughlin, S. R. *Nature* **2000**, *407*, 258.
- (28) Chung, A. W. Y.; Jurasz, P.; Hollenberg, M. D.; Radomski, M. W. *British Journal of Pharmacology* **2002**, *135*, 1123.
- (29) Cleary, D. B.; Trumbo, T. A.; Maurer, M. C. *Archives of Biochemistry and Biophysics* **2002**, *403*, 179.
- (30) Traynelis, S. F.; Trejo, J. *Current Opinion in Hematology* **2007**, *14*, 23.
- (31) Leger, A. J.; Jacques, S. L.; Badar, J.; Kaneider, N. C.; Derian, C. K.; Andrade-Gordon, P.; Covic, L.; Kuliopulos, A. *Circulation* **2006**, *113*, 1244.
- (32) Leger, A. J.; Covic, L.; Kuliopulos, A. *Circulation* **2006**, *114*, 1070.
- (33) Klages, B.; Brandt, U.; Simon, M. I.; Schultz, G.; Offermanns, S. *The Journal of Cell Biology* **1999**, *144*, 745.
- (34) Brass, S. *Nature* **2001**, *413*, 26.
- (35) Varga-Szabo, D.; Pleines, I.; Nieswandt, B. *Arteriosclerosis, Thrombosis, and Vascular Biology* **2008**, *28*, 403.
- (36) Gandhi, N. S.; Mancera, R. L. *Chemical Biology and Drug Design* **2008**, *72*, 455.

- (37) Bjork, I.; Lindahl, U. *Molecular and Cellular Biochemistry* **1982**, *48*, 161.
- (38) Ansell, J.; Hirsh, J.; Poller, L.; Bussey, H.; Jacobson, A.; Hylek, E. *Chest* **2004**, *126*, 204S.
- (39) Bhatt, D. L.; Topol, E. J. *Nature Reviews Drug Discovery* **2003**, *2*, 15.
- (40) Hamilton, J. R. *Blood Reviews* **2009**, *23*, 61.
- (41) Morrow, D. A.; Braunwald, E.; Bonaca, M. P.; Ameriso, S. F.; Dalby, A. J.; Fish, M. P.; Fox, K. A. A.; Lipka, L. J.; Liu, X.; Nicolau, J. C.; Ophuis, A. J. O.; Paolasso, E.; Scirica, B. M.; Spinar, J.; Theroux, P.; Wiviott, S. D.; Strony, J.; Murphy, S. A. *New England Journal of Medicine* **2012**, *366*, 1404.
- (42) Ageno, W.; Gallus, A. S.; Wittowsky, A.; Crowther, M.; Hylek, E.; Palareti, G. *Chest* **2012**, *141*, e44S.
- (43) Al-Khatib, S. M.; Alexander, J. H.; Lopes, R. D.; Mahaffey, K. W.; Patel, M. R.; Granger, C. B. *Current Cardiology Reports* **2012**, *14*, 70.
- (44) Camm, A.; Bounameaux, H. *Drugs* **2011**, *71*, 1503.
- (45) Eisert, W. G.; Huel, N.; Stangier, J.; Wienen, W.; Clemens, A.; Ryn, J. v. *Arteriosclerosis, Thrombosis, and Vascular Biology* **2010**, *30*, 1885.
- (46) Hasan, A. A. K.; Warnock, M.; Nieman, M.; Srikanth, S.; Mahdi, F.; Krishnan, R.; Tulinsky, A.; Schmaier, A. H. *American Journal of Physiology - Heart and Circulatory Physiology* **2003**, *285*, H183.
- (47) Schmaier, A. H. *The Journal of Clinical Investigation* **2002**, *109*, 1007.
- (48) Hasan, A. A. K.; Amenta, S.; Schmaier, A. H. *Circulation* **1996**, *94*, 517.
- (49) Manolis, A. J.; Marketou, M. E.; Gavras, I.; Gavras, H. *Hypertension Research* **2010**, *33*, 772.
- (50) Higuchi, S.; Ohtsu, H.; Suzuki, H.; Shirai, H.; Frank, G. D.; Eguchi, S. *Clinical Science* **2007**, *112*, 417.
- (51) McLean, P. G.; Ahluwalia, A.; Perretti, M. *The Journal of Experimental Medicine* **2000**, *192*, 367.
- (52) Calixto, J. B.; Medeiros, R.; Fernandes, E. S.; Ferreira, J.; Cabrini, D. A.; Campos, M. M. *British Journal of Pharmacology* **2004**, *143*, 803.

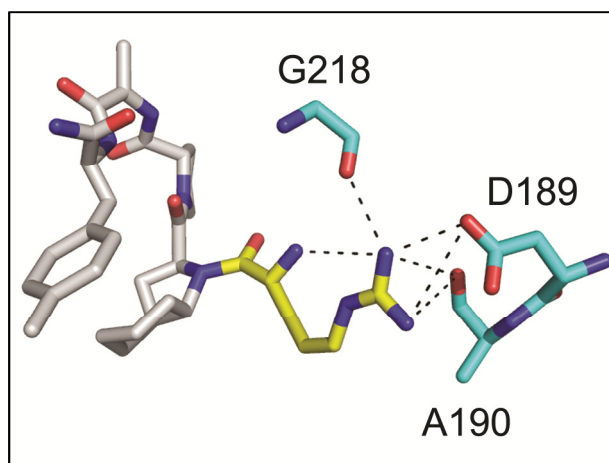


- (53) Nieman, M. T.; Warnock, M.; Hasan, A. A. K.; Mahdi, F.; Lucchesi, B. R.; Brown, N. J.; Murphey, L. J.; Schmaier, A. H. *The Journal of Pharmacology and Experimental Therapeutics* **2004**, *311*, 492.
- (54) Hasan, A. A. K.; Warnock, M.; Srikanth, S.; Schmaier, A. H. *Thrombosis Research* **2001**, *104*, 451.
- (55) Stewart, J. M. *Peptides* **2004**, *25*, 527.
- (56) Burke, F. M.; Warnock, M.; Schmaier, A. H.; Mosberg, H. I. *Chemical Biology and Drug Design* **2006**, *68*, 235.
- (57) Nieman, M. T.; Burke, F.; Warnock, M.; Zhou, Y.; Sweigart, J.; Chen, A.; Ricketts, D.; Lucchesi, B. R.; Chen, Z.; Cera, E. D.; Hilfinger, J.; Kim, J. S.; Mosberg, H. I.; Schmaier, A. H. *Journal of Thrombosis and Haemostasis* **2008**, *6*, 837.

## Chapter 2

### D-Arg Replacements in the FM 19 sequence

Previous work on the development of direct thrombin inhibitors, beginning with the endogenous pentapeptide **RPPGF** led to the development of lead compound **FM 19**, with the sequence D-Arg-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub>.<sup>1,2</sup> The x-ray structure of **FM 19** in the active site of thrombin has provided insight into sites of modification of the structure, to potentially improve potency.<sup>3</sup> The first of these modifications focuses on replacements to the D-Arg residue on the N-terminus of the peptide (Figure 7). First, the side chain of the D-Arg residue adopts a strained, somewhat eclipsed conformation. This conformation places the N-terminal amine of the peptide in close proximity to the guanidino group on the D-Arg side chain. Since both groups are positively charged at physiological pH, we expect these groups to have an adverse electrostatic repulsion. The guanidino group of the D-Arg side chain makes interactions with several residues within the thrombin active site, including the side chain of the catalytic D189, and backbone carbonyls of G218 and A190. In contrast, the N-terminal amine of the peptide does not directly interact with the active site residues, and so elimination of this amine has potential to improve potency by removing this adverse electrostatic interaction with the guanidino group. In addition, the eclipsed conformation that is observed for the D-Arg side chain is itself a higher energy conformation than a fully extended conformation would be. Therefore, we explored alteration of the length of this side chain to try to alleviate this strain, in addition to adding conformational restriction to lock in the eclipsed conformation. We also replaced this guanidino group with other positively charged groups, to see how this would affect potency. Finally, we explored altering the stereochemistry of the  $\alpha$ -carbon to see what effect this would produce.

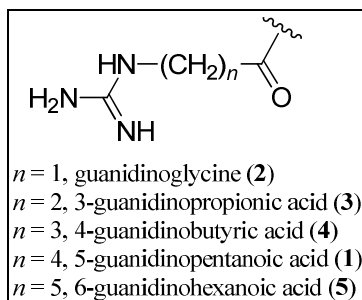


**Figure 7: D-Arg residue in the active site of thrombin. The D-Arg residue is highlighted in yellow with the rest of FM 19 shown in grey, and key residues of thrombin in cyan.<sup>4</sup> PDB: 3BV9, 1.8 Å**

First, we looked at removing the N-terminal amine from **FM 19** and altering the side chain length with compounds **1-5** found in Table 1. The structures of these replacements are shown in Figure 8 and the synthesis of these peptides is shown in Scheme 1.

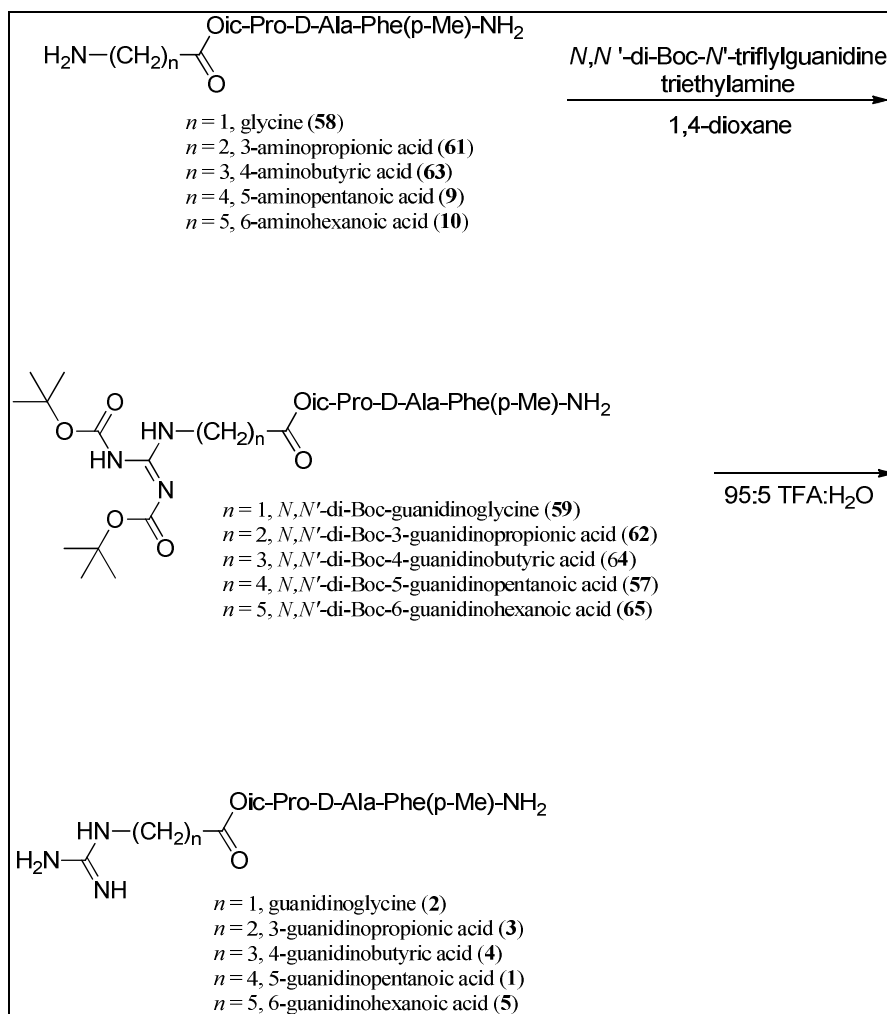
**Table 1: D-Arg replacements to remove the N-terminal amine and alter side chain length**

Compound	D-Arg replacement (X) in the FM 19 Sequence (X-Oic-Pro-D-Ala-Phe( <i>p</i> -Me)-NH <sub>2</sub> )	IC <sub>50</sub> (μM)
<b>FM 19</b>	D-Arg	4.4 ± 1.3 <sup>4</sup>
<b>1</b>	5-guanidinopentanoic acid	0.66 ± 0.20 <sup>4</sup>
<b>2</b>	Guanidinoglycine	36.8 ± 9.4 <sup>4</sup>
<b>3</b>	3-guanidinopropionic acid	6.5 ± 0.6 <sup>4</sup>
<b>4</b>	4-guanidinobutyric acid	8.4 ± 2.0 <sup>4</sup>
<b>5</b>	6-guanidinohexanoic acid	25.6 ± 6.8 <sup>4</sup>



**Figure 8: Structures of D-Arg replacements in compounds 1-5**

Compounds **1-5** (Scheme **1**) were first synthesized using solid phase peptide synthesis as pentapeptides with an amino group in the place of the desired guanidino group. Each pentapeptide was then treated with *N,N'*-di-Boc-*N'*-triflylguanidine<sup>5</sup> to install the desired guanidino functionality (which was protected with 2 Boc groups) from the existing primary amine. The protection of this guanidino group allowed for easier purification of the products from any unreacted amino-containing peptides. The resulting compounds were then treated with TFA to remove the protecting groups, to give final compounds **1-5**.



**Scheme 1: Synthesis of compounds 1-5**

Compound **1** removes the N-terminal amine of the peptide, without making any other alterations to the sequence (the side chain length is identical to **FM 19**). From Table **1**, we can see that simply removing this amine results in an over six-fold improvement in potency. Since both **1** and **FM 19** have the same linker length between the guanidino group on the side chain and the backbone carbonyl, the interactions described in Figure **7** can be maintained, but the adverse electrostatic interaction between the amino group and guanidino group in **FM 19** has been eliminated. Since removal of this amine improved potency as expected, the majority of the rest of the compounds synthesized in this chapter do not contain this amino group.

Compounds **2-5** in Table **1** explore both alteration of side chain length (with compounds **2**, **3**, and **4** having shorter side chains, and **5** having a longer side chain than

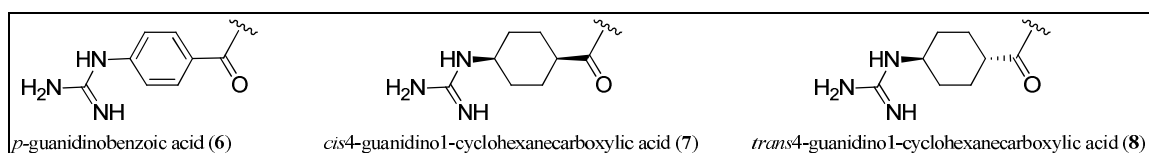
**FM 19**) in addition to removal of the N-terminal amine. Compound **2** (with a guanidinoglycine residue in the first position) appears to have too short of a linker between the guanidino group on the side chain and the backbone carbonyl connecting this residue to the remainder of the peptide, whereas **5** (6-guanidinohexanoic acid substitution) appears to have too long of a side chain, as these compounds are the least potent compounds seen in Table 1. The intermediate analogs, **3** (3-guanidinopropionic acid substitution) and **4** (4-guanidinobutyric acid), show similar potency to **FM 19**. Compound **3** has a shorter side chain than **4**, and this residue is less flexible, and would be expected to have a smaller loss of entropy upon binding to thrombin. However, it is likely that the linker between the guanidino in **3** and the backbone carbonyl is still too short, and therefore does not allow the most ideal interaction between the guanidino group and key active site residues of thrombin. Finally, **4** has a slightly longer and more flexible side chain compared to **3**, and we would expect that **4** might be able to interact with the active site residues of thrombin in an advantageous way without adopting the high-energy eclipsed conformation seen with **FM 19** in Figure 7. Unfortunately, **4** is almost 2-fold less potent than **FM 19**, suggesting that the guanidino group in **4** is not optimally positioned within the active site of thrombin. Thus, although **1** is expected to adopt the same high-energy eclipsed conformation on its side chain as is seen with **FM 19**, it appears to have the optimal linker length of six atoms between the positive charge on the guanidino group of the side chain and the backbone carbonyl to allow for the most favorable interaction with thrombin.

Next, we wanted to look at adding conformational restriction to the side chain of this first residue, in an attempt to lock in the eclipsed conformation seen with **FM 19** in Figure 7, while also removing the N-terminal amine. Table 2 shows the results from these three compounds, **6-8**, with the structures of the replacements shown in Figure 9 and the synthesis displayed in Schemes 2 and 3.

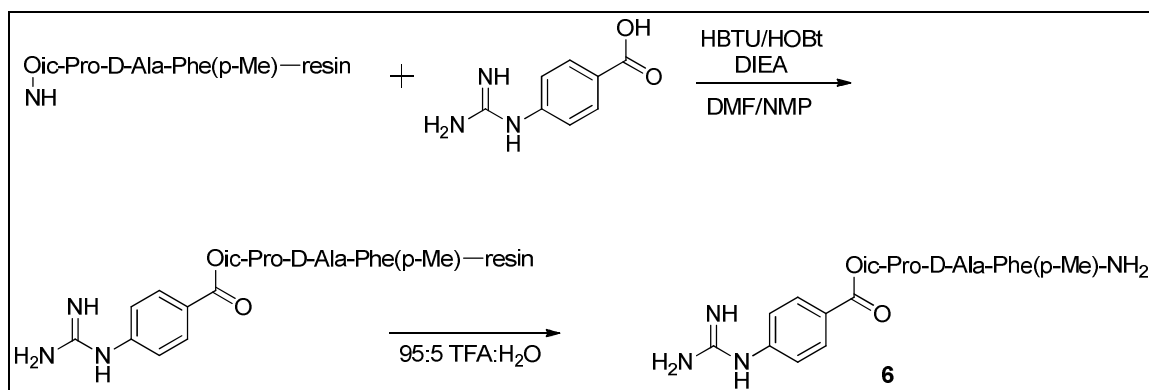
**Table 2: D-Arg replacements to add conformational restriction**

Compound	D-Arg replacement (X) in the FM 19 Sequence (X-Oic-Pro-D-Ala-Phe( <i>p</i> -Me)-NH <sub>2</sub> )	IC <sub>50</sub> (μM)
FM 19	D-Arg	4.4 ± 1.3 <sup>4</sup>
<b>6</b>	<i>p</i> -guanidinobenzoic acid	0.57 ± 0.12 <sup>4</sup>
<b>7</b>	<i>cis</i> -4-guanidino-1-cyclohexanecarboxylic acid	19.5 ± 5.4 <sup>6</sup>
<b>8</b> <sup>†</sup>	<i>trans</i> -4-guanidino-1-cyclohexanecarboxylic acid	2.5 ± 0.5 <sup>6</sup>

<sup>†</sup>Compound was synthesized by Vanessa R. (Porter) Barrus.

**Figure 9: Structures of D-Arg replacements in compounds 6-8**

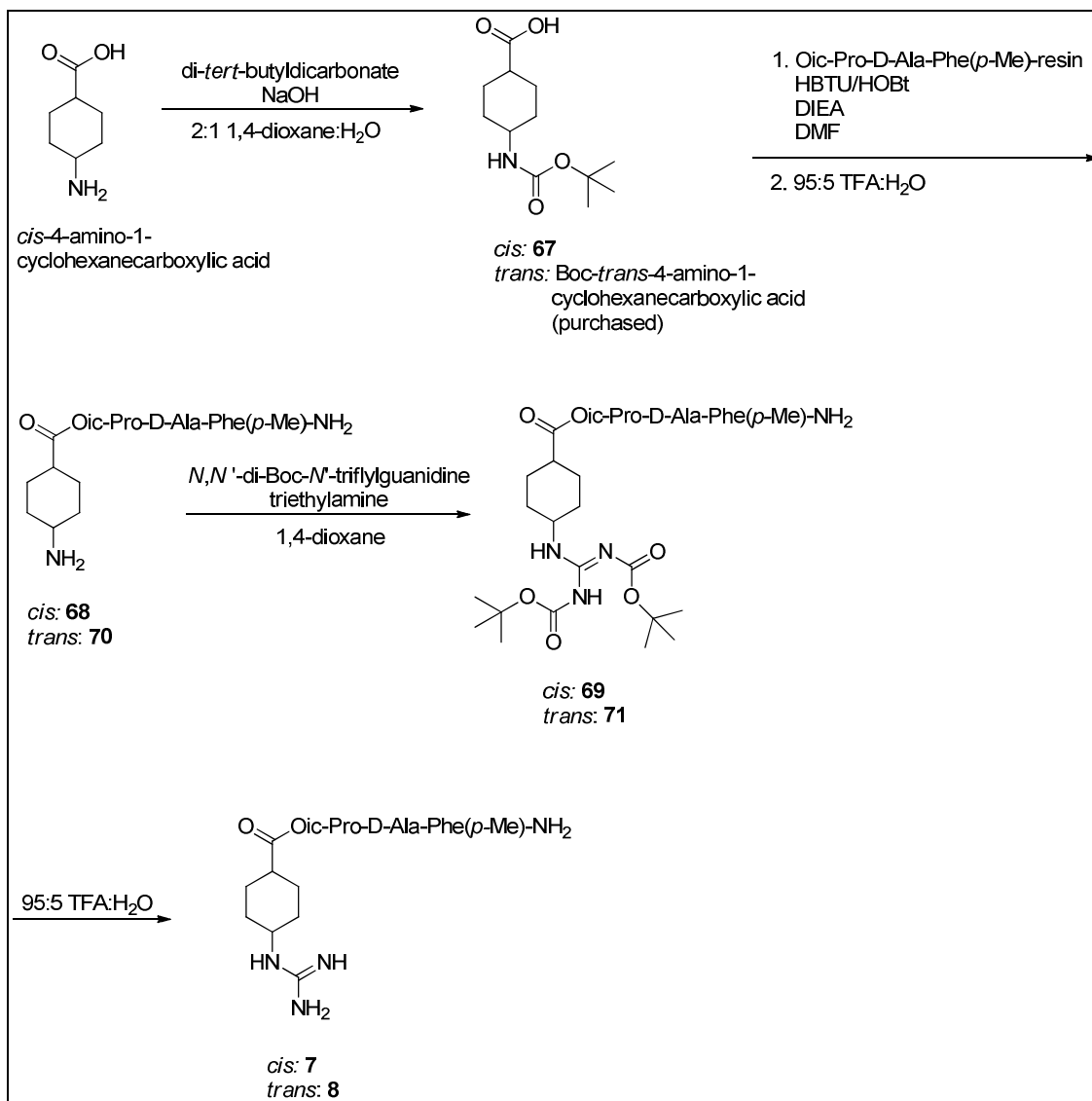
To make compound **6** (Scheme 2), the tetrapeptide Oic-Pro-D-Ala-Phe(*p*-Me) was first synthesized using solid phase peptide synthesis. While still attached to the resin, *p*-guanidinobenzoic acid was coupled to make the desired pentapeptide, followed by treatment with TFA which cleaved the peptide from the resin, to generate the final pentapeptide **6**.

**Scheme 2: Synthesis of Compound 6**

Compounds **7** and **8** (Scheme 3) were synthesized in a similar manner to compounds **1-5**. First, *cis*-4-amino-1-cyclohexanecarboxylic acid was protected on the amine with a Boc group, while the *trans* analog was purchased as the commercially

available Boc-*trans*-4-amino-1-cyclohexanecarboxylic acid. These protected amino acids were then coupled to the resin-bound tetrapeptide Oic-Pro-D-Ala-Phe(*p*-Me), followed by treatment with TFA to give amino-containing pentapeptides **68** (*cis*) and **70** (*trans*). These pentapeptides were then treated with *N,N'*-di-Boc-*N'*-triflylguanidine<sup>5</sup> to install the desired guanidino functionality (which was protected with 2 Boc groups) from the existing primary amine. Again, the protection of this guanidino group allowed for easier purification of the products from any unreacted amino-containing peptides. The resulting compounds were then treated with TFA to remove the protecting groups, to give final compounds **7** (*cis*) and **8** (*trans*).





Scheme 3: Synthesis of Compounds 7 and 8

Compounds **6**, **7** and **8** all replace the first residue of **FM 19** by adding conformational restriction to the side chain while also removing the N-terminal amine, and maintaining the optimal linker length between the guanidino group and the backbone carbonyl. Compound **6** uses an unsaturated benzoic acid ring, while **7** and **8** use saturated cyclohexanoic acid rings, in either the *cis* (compound **7**) or *trans* (compound **8**) conformation. From Table 2, we can see that compound **6** (with a *p*-guanidinobenzoic acid substitution) is over 7-fold more potent than **FM 19**. The side chain of **6** locks in the

eclipsed conformation seen in the D-Arg side chain of **FM 19** in Figure 7, and presumably places the guanidino group in approximately the same position. However, it might be expected that **6** be more potent than **1** (Table 1), but in fact these two compounds are equipotent. Perhaps this could be explained based on the exact configuration of the *p*-guanidinobenzoic acid residue. The linker portion of the side chain of **FM 19** is not completely planar, while the corresponding portion of **6** is, which could result in the guanidino group of **6** pointing in a slightly less than optimal position within the active site. Compound **1**, on the other hand, has a more flexible side chain which would allow more flexibility of the guanidino group, and allow it to orient itself in an ideal position to obtain the most advantageous interaction possible.

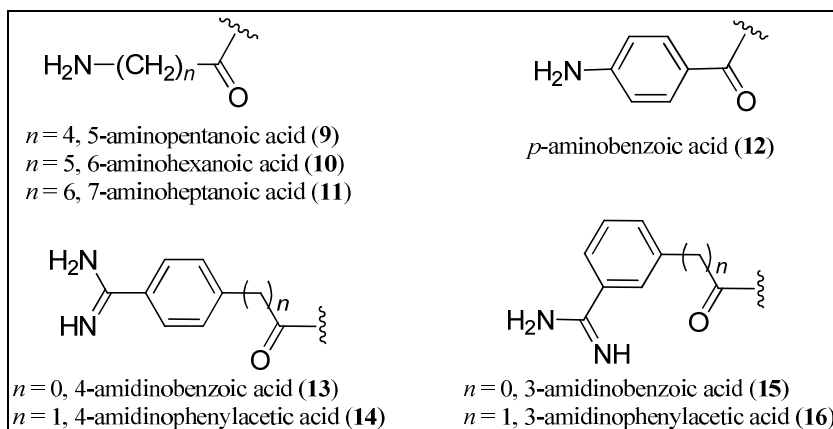
Compounds **7** and **8** use a saturated ring on the side chain of the first residue, with **7** adopting a *cis* conformation and **8** being *trans*. Table 2 shows that **8** is slightly more potent than **FM 19**, and almost eight times as potent as **7**. If we assume that both **7** and **8** adopt the expected and more energetically favorable chair conformations on their side chains, this would put the *trans*-4-guanidino-1-cyclohexanecarboxylic acid residue of **8** in a quasi-planar configuration, which is similar to the configuration of **6** and would presumably orient the guanidino group in approximately the same position. Compound **7** would not be able to adopt the quasi-planar configuration of **8** since one substituent of *cis* compound **7** would be in the equatorial position and the other would be axial, making the key interactions of the molecule much more difficult to achieve. Although **8** is slightly more potent than **FM 19**, it is still less potent than **6**, suggesting that when adding conformational restriction, the planarity and rigidity of an aromatic ring is preferred over a saturated ring.

We have determined that removal of the N-terminal amine of the peptide improves potency, the optimal linker length between the positively charged guanidino nitrogens on the side chain and backbone carbonyl of the rest of the peptide is six atoms, and an aromatic ring is preferred when adding conformational restriction. Now we will look at modification of the guanidino group, with either an amino group (compounds **9-12**) or an amidino group (compounds **13-16**). The aryl amidine functionality seen in compounds **13-16** has been used in other direct thrombin inhibitors, such as argatroban,

as a replacement for the arginine residue.<sup>7</sup> All of these eight compounds lack the N-terminal amine, while some (**12-16**) also add conformational restriction. Table **3** shows the inhibition results for these compounds, and the structures of the replacements are shown in Figure **10**. Synthesis of these compounds is shown in Schemes **4-7**.

**Table 3: D-Arg replacements to modify the guanidino group**

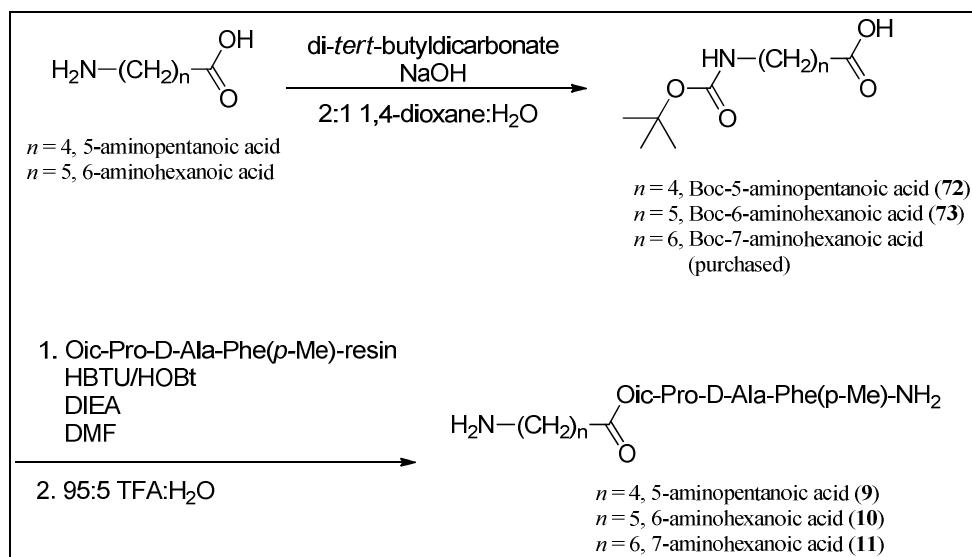
Compound	D-Arg replacement ( <b>X</b> ) in the <b>FM 19</b> Sequence ( <b>X</b> -Oic-Pro-D-Ala-Phe( <i>p</i> -Me)-NH <sub>2</sub> )	IC <sub>50</sub> (μM)
<b>FM 19</b>	D-Arg	4.4 ± 1.3 <sup>4</sup>
<b>9</b>	5-aminopentanoic acid	241 ± 41 <sup>4</sup>
<b>3</b>	3-guanidinopropionic acid	6.5 ± 0.6 <sup>4</sup>
<b>10</b>	6-aminohexanoic acid	130 ± 31 <sup>4</sup>
<b>4</b>	4-guanidinobutyric acid	8.4 ± 2.0 <sup>4</sup>
<b>11</b>	7-aminoheptanoic acid	498 ± 14 <sup>4</sup>
<b>1</b>	5-guanidinopentanoic acid	0.66 ± 0.20 <sup>4</sup>
<b>12</b>	<i>p</i> -aminobenzoic acid	225 ± 102
<b>6</b>	<i>p</i> -guanidinobenzoic acid	0.57 ± 0.12 <sup>4</sup>
<b>13</b>	4-amidinobenzoic acid	0.51 ± 0.19 <sup>6</sup>
<b>14</b>	4-amidinophenylacetic acid	0.45 ± 0.08 <sup>6</sup>
<b>15</b>	3-amidinobenzoic acid	1.03 ± 0.25 <sup>6</sup>
<b>16</b>	3-amidinophenylacetic acid	26.2 ± 2.3 <sup>6</sup>



**Figure 10: Structures of D-Arg replacements in compounds 9-16**

Compounds **9-11** (Scheme **4**) were synthesized by first protecting the primary amine on the D-Arg replacement with a Boc group, to make Boc-5-aminopentanoic acid (for **9**) and Boc-6-aminohexanoic acid (for **10**). The commercially available Boc-7-

aminoheptanoic acid was used in the synthesis of **11**. These protected amino acids were then coupled to the previously synthesized Oic-Pro-D-Ala-Phe(*p*-Me) tetrapeptide on the resin, and the resulting pentapeptide attached to the resin was treated with TFA to remove the protecting group as well as cleave the peptide from the resin, giving final peptides **9-11**.

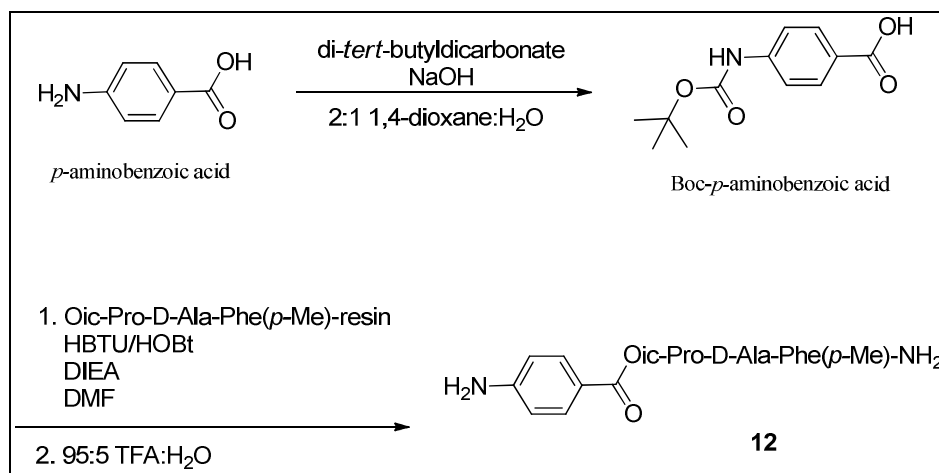


**Scheme 4: Synthesis of compounds 9-11**

Compounds **9-11** contain an amino group on the side chain. When comparing the potencies of these compounds with their guanidino-containing counterparts, it is important to maintain the linker length between the positively charged group at the end of the side chain and the backbone carbonyl of the rest of the peptide. Thus, **9** places the positive charge from the amino group in approximately the same position within thrombin's active site that **3** places the positive charge from its guanidino group, and so on. First, when comparing the shortest of these pairs of compounds, **9** (5-aminopentanoic acid substitution) from Table **3** and **3** (3-guanidinopropionic acid substitution), it appears that the guanidino-containing peptide **3** is more than 30 times more potent than **9**. This pattern continues with **10** (6-aminohexanoic acid) and **4** (4-guanidinobutyric acid), but with less of a difference in potency, as **4** is only approximately 15-fold more potent than **10**. Finally, we see the largest gap with **11** (7-aminoheptanoic acid) and **1** (5-guanidinopentanoic acid), with **1** being more than 750 times more potent than **11**.

Therefore, it is clear that a guanidino group on the side chain here is favored over an amino group in approximately the same position within the active site, which is not a surprising result. The active site of thrombin contains the catalytic residue D189, and there is a delocalization of the negative charge between both oxygen atoms on this side chain. It has been suggested that in order to optimally interact with this residue, a positive charge delocalized on more than one nitrogen atom on the inhibitor side chain is preferred over a point charge,<sup>7</sup> and the results from compounds **9-11** support this theory.

Compound **12** (Scheme 5) was synthesized in the same manner as compounds **9-11**. First, the amine of *p*-aminobenzoic acid was protected with a Boc group, then the amino acid was coupled to the previously synthesized Oic-Pro-D-Ala-Phe(*p*-Me) tetrapeptide on the resin, giving the resin-bound pentapeptide. This was then treated with TFA to remove the Boc group as well as cleave the peptide from the resin, resulting in pentapeptide **12**.

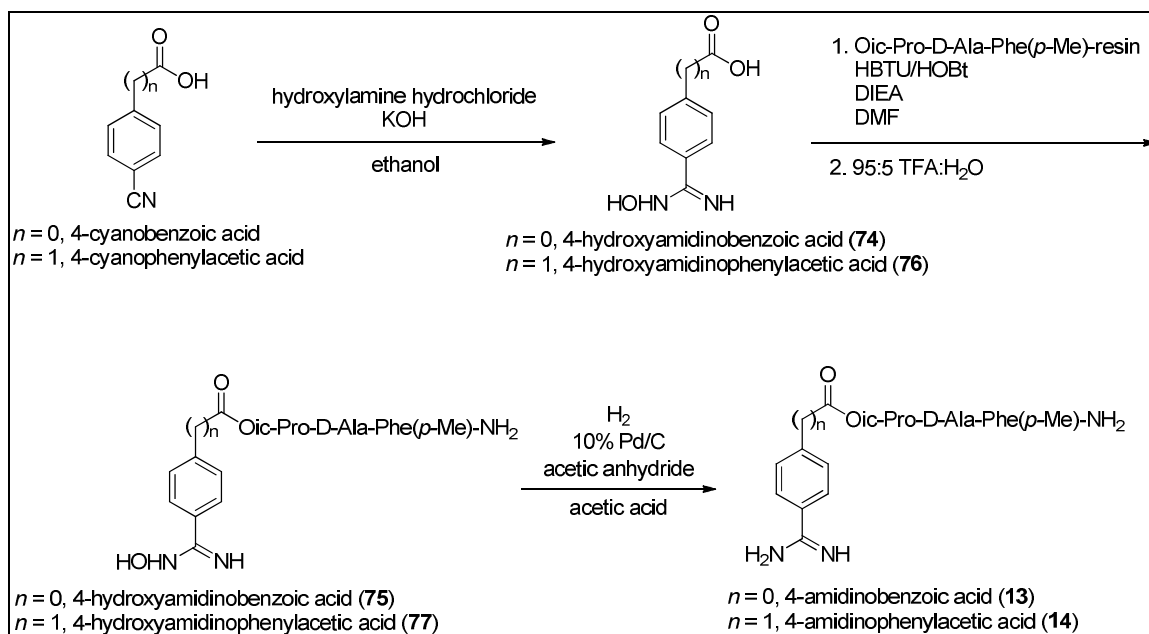


**Scheme 5: Synthesis of compound 12**

Compound **12** contains *p*-aminobenzoic acid in place of the D-Arg residue of **FM 19**, and is somewhat similar in structure to **6**. From Table **3** we can see that **12** is over 50-fold less potent than **FM 19**, and almost 400 times less potent than **6**. We can assume that since **12** contains an amino group, it is less likely to be potent due to the fact that the side chain contains a point charge, which cannot be delocalized over more than one

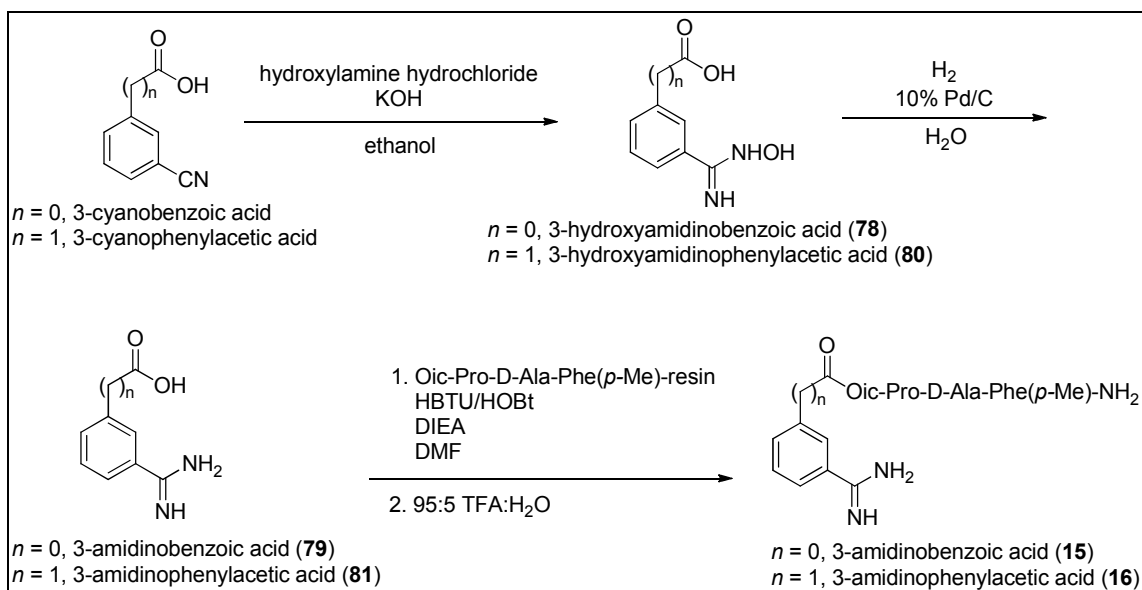
nitrogen. Also, the linker length between the amino in **12** is shorter than what is seen in **6**. Interestingly, we can see from Table 3 that **12** shows the same inhibition as **9** (5-aminopentanoic acid substitution), and both compounds contain amino groups on the end of a side chain with four carbon atoms separating the amino group from the carbonyl carbon on the backbone of the peptide. The only difference between these compounds is the addition of conformational restriction of an aromatic ring in **12**, where **9** has only a straight chain alkyl linker.

Compounds **13-16** (Scheme 6) were synthesized to install an amidino group on the side chain of the amino acid replacement in this first position. Compounds **13** and **14** used conformational restriction in the form of an unsaturated ring, both substituted in the *para* position with an amidino group, with either a benzoic acid (**13**) or phenylacetic acid (**14**) scaffold. First, the corresponding cyano acid was treated with hydroxylamine hydrochloride and KOH to convert the cyano group to the hydroxyamidino group<sup>8</sup> seen in **74** and **76**. These compounds were coupled to the previously synthesized Oic-Pro-D-Ala-Phe(*p*-Me) tetrapeptide on the resin and then treated with TFA to generate the pentapeptide with the hydroxyamidino functionality still in the place of the desired amidino group of the product. The hydroxyamidino group was then reduced using H<sub>2</sub>, 10% Pd/C and acetic anhydride (as an acylating agent)<sup>8</sup> to give the desired amidino-containing pentapeptides **13** and **14**.



**Scheme 6: Synthesis of compounds 13 and 14**

Compounds **15** and **16** (Scheme 7) also replace the D-Arg residue of the **FM 19** sequence with an unsaturated ring containing an amidino group, but are substituted in the *meta* position of the ring, with either a benzoic acid (**15**) or phenylacetic acid (**16**) scaffold. The synthesis of **15** and **16** is similar to the synthesis of **13** and **14**, and again starts with the conversion of the corresponding cyano acid to the hydroxyamidino, using the same procedure with hydroxylamine hydrochloride and KOH.<sup>8</sup> Now, the synthesis of **15** and **16** follows a slightly more direct route. The hydroxyamidino functionality was reduced to an amidino using just H<sub>2</sub> and 10% Pd/C as a catalyst, to make amidino acids **79** and **81**. These amidino acids were then coupled directly to the previously prepared Oic-Pro-D-Ala-Phe(*p*-Me)-resin, generating the resin-bound pentapeptides. After treatment with TFA, the synthesis of the final pentapeptides **15** and **16** was complete.



**Scheme 7: Synthesis of compounds 15 and 16**

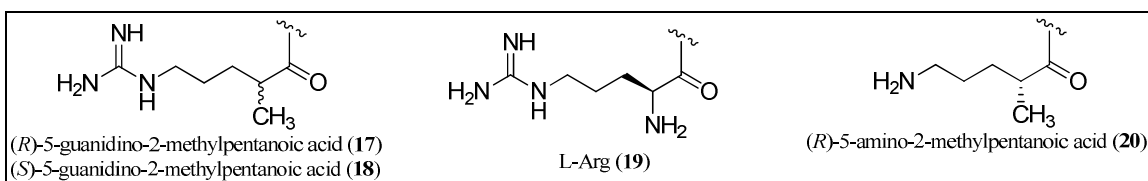
From Table 3, we can see that three of the four amidino-containing peptides are more potent than **FM 19**, with the exception of the 3-amidino phenylacetic substituted peptide, compound **16**. It appears as if positioning of the amidino functionality relative to the remainder of the peptide is the most important factor when determining potency of these compounds. The peptides containing a benzoic acid scaffold, **13** (4-amidino benzoic acid) and **15** (3-amidino benzoic acid) have the amidino group on the side chain connected to the remainder of the peptide through a single rotatable bond, allowing for less variability of the positioning of this crucial moiety. Thus, the amidino group is more firmly held in a favorable binding orientation in both of these cases. On the other hand, the side chains of **14** (4-amidino phenyl acetic acid) and **16** (3-amidino phenylacetic acid) contain two rotatable bonds: an alkyl-aryl bond as well as an alkyl-carbonyl bond. In the case of **14**, rotation around the alkyl-aryl bond does not affect the positioning of the amidino group within thrombin's active site, but rotation of this corresponding bond in **16** causes the amidino group to drastically alter its orientation. This rotation most likely leads to a reduced population of the preferred binding orientation of **16**, resulting in the loss of inhibitor potency observed.



Finally, we would like to replace the N-terminal amino group with a group of similar size that would not carry a positive charge, and so compound **17** was synthesized with a methyl group in place of the amine. We also made compound **18** which inverted the stereochemistry of this N-terminal residue, but also contained the amine to methyl replacement. The results for these compounds are shown in Table 4, the structures of the replacements are displayed in Figure 11, and the synthesis of compounds **17** and **18** is shown in Scheme 8.

**Table 4: D-Arg replacements to investigate  $\alpha$ -carbon stereochemistry**

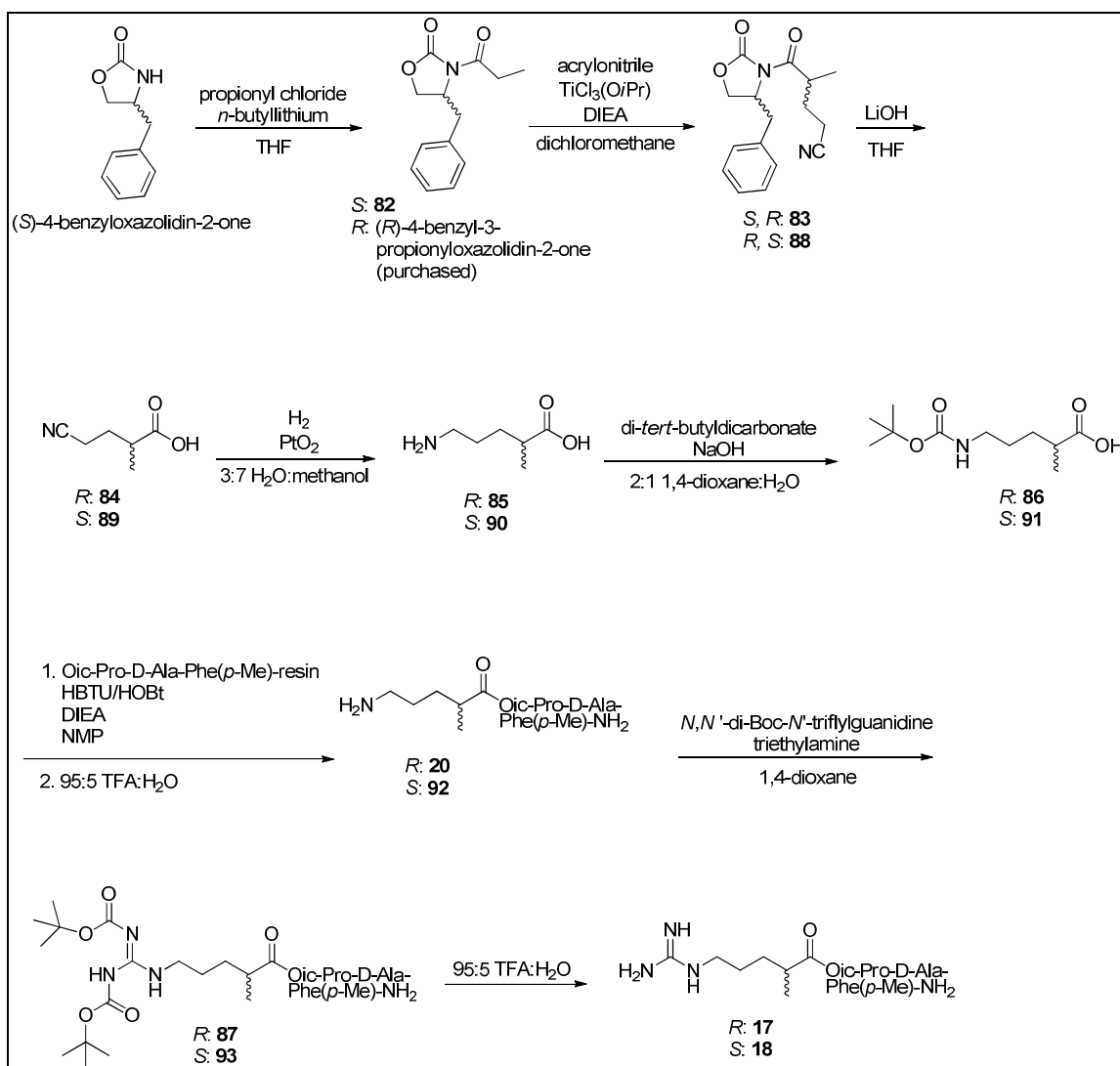
Compound	D-Arg replacement (X) in the FM 19 Sequence (X-Oic-Pro-D-Ala-Phe( <i>p</i> -Me)-NH <sub>2</sub> )	IC <sub>50</sub> ( $\mu$ M)
<b>FM 19</b>	D-Arg	4.4 $\pm$ 1.3 <sup>4</sup>
<b>17</b>	( <i>R</i> )-5-guanidino-2-methylpentanoic acid	98 $\pm$ 27 <sup>6</sup>
<b>18</b>	( <i>S</i> )-5-guanidino-2-methylpentanoic acid	9.8 $\pm$ 1.0 <sup>6</sup>
<b>19</b>	L-Arg	73 $\pm$ 36 <sup>6</sup>
<b>20</b>	( <i>R</i> )-5-amino-2-methylpentanoic acid	2000 $\pm$ 86



**Figure 11: Structures of D-Arg replacements in compounds 17-20**

The syntheses of **17** and **18** (Scheme 8) were designed to produce a single enantiomer of the D-Arg replacement, using Evans oxazolidinone chiral auxiliaries,<sup>9</sup> resulting in a single diastereomer peptide. To begin the synthesis of **17**, the commercially available (*S*)-4-benzyloxazolidin-2-one was functionalized with propionyl chloride<sup>10</sup> to produce intermediate **82**. The other enantiomer of this compound, the commercially available (*R*)-4-benzyl-3-propionyloxazolidin-2-one, was purchased and used as the starting point for the synthesis of **18**. These compounds were then treated with acrylonitrile<sup>10</sup> to yield **83** and **88**, with the desired stereochemistry. Treatment with LiOH removed the auxiliaries,<sup>10</sup> and reduction using H<sub>2</sub> and PtO<sub>2</sub> converted the cyano group to a primary amine,<sup>10</sup> resulting in amino acids **85** and **90**. The amine was Boc protected,

and then compounds **86** and **91** could be coupled to the previously prepared Oic-Pro-D-Ala-Phe(*p*-Me)-resin, generating the resin-bound pentapeptides. After treatment with TFA, pentapeptides **20** and **92** were generated, with an amino group on the side chain. To install the guanidino functionality in place of the amino moiety, these pentapeptides were treated with *N,N'*-di-Boc-*N'*-triflylguanidine,<sup>5</sup> which installed a guanidino group (protected with 2 Boc groups) in place of the amino. Like previous compounds, the protection of this guanidino group allowed for easier purification of the products from any unreacted amino-containing peptides. The resulting compounds were then treated with TFA to remove the protecting groups, resulting in peptides **17** and **18**.



Scheme 8: Synthesis of compounds **17**, **18** and **20**<sup>6,10</sup>

Compound **17** (Table 4) was the first compound in this group to be synthesized and tested, and gave a somewhat surprising result. Since the adverse electrostatic interaction between the two positively charged moieties was removed in this structure, we expected **17** to be more potent, but instead it shows an over 20-fold decrease in potency when compared to **FM 19**. Therefore, compound **18** was synthesized, to invert stereochemistry around this residue, but still maintain the methyl group in place of the N-terminal amine. Again, somewhat surprisingly, compound **18** showed an order of magnitude improvement over **17**, and was only about 2-fold less potent than **FM 19**. We then synthesized compound **19** (Table 4), which included an L-Arg residue on the N-terminus, to verify that D-Arg (or *R* stereochemistry) is preferred. This was in fact what was determined, since L-Arg is approximately 16-fold less potent than **FM 19**.

Further investigation of the crystal structure offered an explanation to the unexpected results of compounds **17** and **18**. Compound **17** places a methyl group in the same position of the amine of **FM 19**, which is a more solvent-exposed location within the active site. Conversely, **18** positions its methyl group in a more hydrophobic environment, deeper in the active site and directed more toward the aliphatic side chain of the Oic residue. We would expect there to be more of a penalty for the methyl group to be more solvent exposed (as in the case of **17**), and so this suggests a reason for the order of magnitude improvement seen with **18** over **17**. Furthermore, although the N-terminal amine of **FM 19** is in close proximity to the guanidino group on the side chain, resulting in an unfavorable electrostatic interaction, this amino group can also form a water-mediated hydrogen bond to backbone carbonyl of G216 within thrombin's active site.<sup>3</sup> Since the amino group on the L-Arg residue of **19** would orient the amine in a different direction and place it deeper into the active site, this favorable hydrogen bonding interaction cannot take place. In addition, placing this positively charged group in a hydrophobic environment would also lead to the loss in potency observed for **19**.

Finally, compound **20** from Table 4 is over two orders of magnitude less potent than **FM 19**. Based on what has been discovered previously, this result is to be expected. First, the side chain of **20** contains a point charge in the form of an amine, which is much less favored than a moiety which would be able to delocalize the charge over more than one atom. Secondly, the side chain is too short, and the positively charged group may not

be able to reach deep enough into thrombin's active site to achieve the most favorable interactions. Finally, the N-terminal amine is replaced with a methyl group, and due to the *R* configuration around the  $\alpha$ -carbon, this methyl group is positioned into a solvent-exposed area, contributing to the decrease in potency observed.

To conclude, we have made replacements to the D-Arg residue of **FM 19**, resulting in several compounds which show superior thrombin inhibition compared the lead compound **FM 19**, and five of these compounds (**1**, **6**, **13**, **14** and **15**) show approximately equal potency to each other. Each of the more potent compounds removed the N-terminal amine, contained a moiety on the end of the side chain which allowed for delocalization of a positive charge (versus a single point charge), and most added conformational restriction of the side chain in the form of an unsaturated six-membered ring.

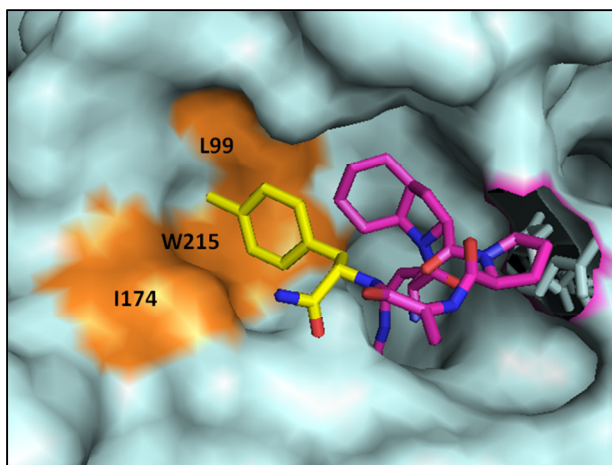
## Chapter 2 References

- (1) Nieman, M. T.; Warnock, M.; Hasan, A. A. K.; Mahdi, F.; Lucchesi, B. R.; Brown, N. J.; Murphey, L. J.; Schmaier, A. H. *The Journal of Pharmacology and Experimental Therapeutics* **2004**, *311*, 492.
- (2) Burke, F. M.; Warnock, M.; Schmaier, A. H.; Mosberg, H. I. *Chemical Biology and Drug Design* **2006**, *68*, 235.
- (3) Nieman, M. T.; Burke, F.; Warnock, M.; Zhou, Y.; Sweigart, J.; Chen, A.; Ricketts, D.; Lucchesi, B. R.; Chen, Z.; Cera, E. D.; Hilfinger, J.; Kim, J. S.; Mosberg, H. I.; Schmaier, A. H. *Journal of Thrombosis and Haemostasis* **2008**, *6*, 837.
- (4) Girnys, E. A.; Sobczyk-Kojiro, K.; Mosberg, H. I. *Chemical Biology and Drug Design* **2010**, *75*, 35.
- (5) Feichtinger, K.; Zapf, C.; Sings, H. L.; Goodman, M. *Journal of Organic Chemistry* **1998**, *63*, 3804.
- (6) Girnys, E. A.; Porter, V. R.; Mosberg, H. I. *Bioorganic and Medicinal Chemistry* **2011**, *19*, 7425.
- (7) Masic, L. P. *Current Medicinal Chemistry* **2006**, *13*, 3627.
- (8) Judkins, B. D.; Allen, D. G.; Cook, T. A.; Evans, B.; Sardharwala, T. E. *Synthetic Communications* **1996**, *26*, 4351.
- (9) Evans, D. A.; Bilodeau, M. T.; Somers, T. C.; Clardy, J.; Cherry, D.; Kato, Y. *Journal of Organic Chemistry* **1991**, *56*, 5750.
- (10) Attwood, M. R.; Conway, E. A.; Dunsdon, R. M.; Greening, J. R.; Handa, B. K.; Jones, P. S.; Jordan, S. C.; Keech, E.; Wilson, F. X. *Bioorganic and Medicinal Chemistry Letters* **1997**, *7*, 429.

## Chapter 3

### Phe(*p*-Me) Replacements in the FM 19 sequence

Previous studies, initially based on the endogenous pentapeptide **RPPGF**, have led to the development of lead compound **FM 19** (sequence D-Arg-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub>), which shows superior direct thrombin inhibition when compared to the earlier compounds in the series.<sup>1,2</sup> An x-ray structure of **FM 19** in thrombin's active site<sup>3</sup> has been used to guide structure-based design of additional analogs to potentially improve thrombin inhibition. Chapter 2 discussed modifications made to the D-Arg residue on the N-terminus of the peptide, and this chapter focuses on modifications made to the C-terminal residue of **FM 19**, with replacements to the Phe(*p*-Me) moiety. From Figure 12, we can see that the interactions the Phe(*p*-Me) residue makes with thrombin are not as extensive as the contacts the D-Arg residue makes with the enzyme, and instead the Phe(*p*-Me) residue engages in mostly van der Waals interactions with active site residues I174, W215 and L99. Thus, the interactions on the C-terminus of this peptide are much less specific than for the N-terminus.



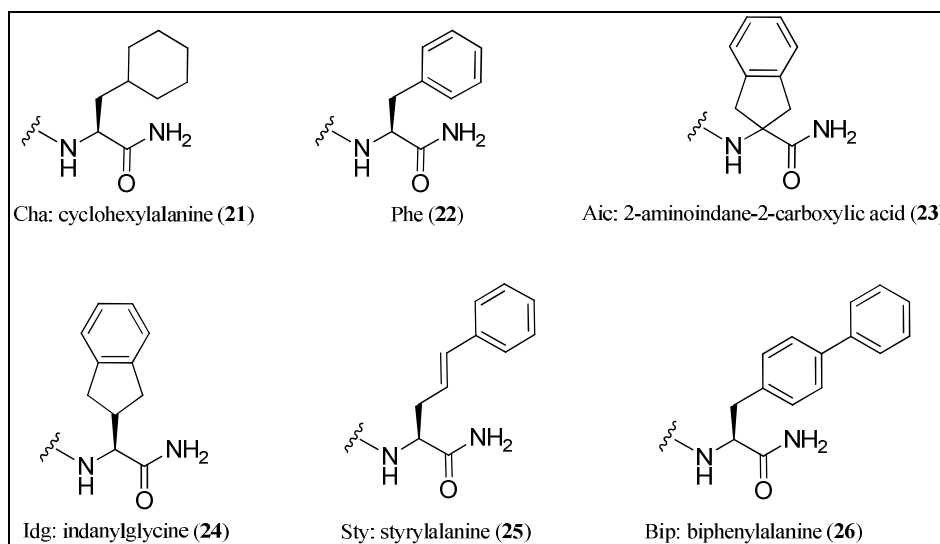
**Figure 12:** Phe(*p*-Me) residue in the active site of thrombin. The Phe(*p*-Me) is highlighted in yellow with the rest of FM 19 shown in magenta, key residues of thrombin in orange, and the rest of thrombin in cyan. PDB: 3BV9, 1.8 Å

First, we decided to investigate thrombin's preference for aryl or alkyl substituents in this position, and also to determine how long of a side chain was tolerated. The results from these compounds, **21-26**, are shown in Table 5, and the structures of these replacements are shown in Figure 13.

**Table 5: Phe(*p*-Me) replacements to investigate alkyl versus aryl substitution and alter side chain length**

Compound	Phe( <i>p</i> -Me) replacement (Y) in the <b>FM 19</b> Sequence (D-Arg-Oic-Pro-D-Ala-Y-NH <sub>2</sub> )	IC <sub>50</sub> (μM)
<b>FM 19</b>	Phe( <i>p</i> -Me)	4.4 ± 1.3 <sup>‡</sup>
<b>21</b>	Cha	154 ± 40
<b>22</b> <sup>‡</sup>	Phe	49.9 ± 1.6
<b>23</b>	Aic	345 ± 15
<b>24</b>	Idg	70.5 ± 15.4
<b>25</b>	Sty	55.5 ± 3.0
<b>26</b>	Bip	12.2 ± 0.9

<sup>‡</sup>Compound synthesized by Athena Flecha.



**Figure 13: Structures of Phe(*p*-Me) replacements in compounds 21-26**

Compounds **21** and **22** both contain a single ring on the side chain, with **22** having a Phe replacement, and **21** consisting of a similar, but saturated cyclohexane ring. Both compounds have the same side chain length, with a single methylene between the terminal ring and the  $\alpha$ -carbon. From Table 5 we can see that **22** is approximately 3 times more potent than **21**. The fact that the aromatic ring is preferred over the saturated ring is not a surprising result, since the portion of the enzyme that this residue will bind to is often referred to as the aryl binding site<sup>5</sup>, and it is assumed that the aromatic ring of the side chain of **22** can interact with the active site W215 in a favorable edge-on arrangement.

Since an aromatic side chain is preferred, compounds **23** – **26** contain an aromatic ring at the end of their side chains, and investigate alteration of side chain length. Compound **23** has the shortest side chain length, with a 2-aminoindane-2-carboxylic acid residue at the C-terminal position. This residue is also rather constrained, since the  $\alpha$ -carbon forms a portion of the side chain's 5-membered ring. Although the aromatic ring is a similar distance from the remainder of the peptide when compared to the unsaturated ring of **22** (with the Phe substitution), it shows a large decrease in potency. This is most likely due to the added constraint on the side chain of **23**, which orients the phenyl ring in a slightly different direction. Compound **24** has a slightly longer side chain, with the addition of an extra carbon from **23** with an indanylglycine residue, moving the 5-



membered ring slightly further from the backbone of the peptide. Again, **24** is less potent than **22**, which is probably due to the orientation of the phenyl ring, from the added constraints on the side chain. However, **24** is more potent than **23**. Compound **25** moves the terminal aromatic ring even further from the rest of the peptide, with a 3 carbon linker between it and the backbone of the peptide, with the addition of a double bond. Compound **25** shows similar potency to **22**, even though the side chain is longer. Finally, **26** has the longest side chain length, as it adds another phenyl ring in the *para* position when compared to the structure of **22**. Compound **26** is the most potent of this series of compounds, showing approximately 4-fold improvement over **22**, but is still about 3-fold less potent than **FM 19**. Thus, as the side chain of the C-terminal residue gets longer, potency increases, which could be a result of increased van der Waals interactions. However, the *para* methyl group of **FM 19** still appears to be optimal for this residue.

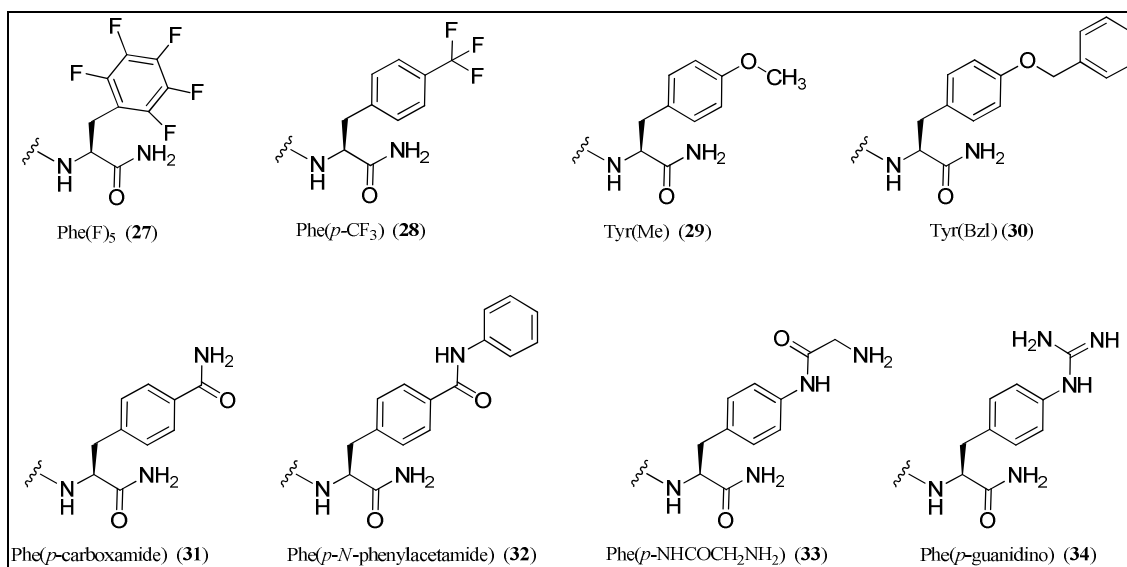
Next, we looked at adding increased lipophilicity with fluoro-substituted compounds, as well as additional *para* substituents. The results for these compounds, **27-32**, are shown in Table 6, and the structures of the replacement residues are shown in Figure 14.

**Table 6: Phe(*p*-Me) replacements to investigate lipophilicity and *para* substitution**

Compound	Phe( <i>p</i> -Me) replacement (Y) in the <b>FM 19</b> Sequence (D-Arg-Oic-Pro-D-Ala-Y-NH <sub>2</sub> )	IC <sub>50</sub> (μM)
<b>FM 19</b>	Phe( <i>p</i> -Me)	4.4 ± 1.3 <sup>‡</sup>
<b>27</b>	Phe(F) <sub>5</sub>	567 ± 181
<b>22</b> <sup>‡</sup>	Phe	49.9 ± 1.6
<b>28</b>	Phe( <i>p</i> -CF <sub>3</sub> )	12.7 ± 4.7
<b>29</b>	Tyr(Me)	28.7 ± 1.6
<b>30</b>	Tyr(Bzl)	66.6 ± 3.7
<b>31</b>	Phe( <i>p</i> -carboxamide)	5.8 ± 1.3
<b>32</b>	Phe( <i>p</i> -N-phenylacetamide)	392 ± 39
<b>33</b>	Phe( <i>p</i> -NHCOCH <sub>2</sub> NH <sub>2</sub> )	2426*
<b>34</b>	Phe( <i>p</i> -guanidino)	174 ± 9
<b>FM 32</b>	Phe( <i>p</i> -NH <sub>2</sub> )	172*

<sup>‡</sup>Compound synthesized by Athena Flecha.

\*This is the average for *n* = 2.

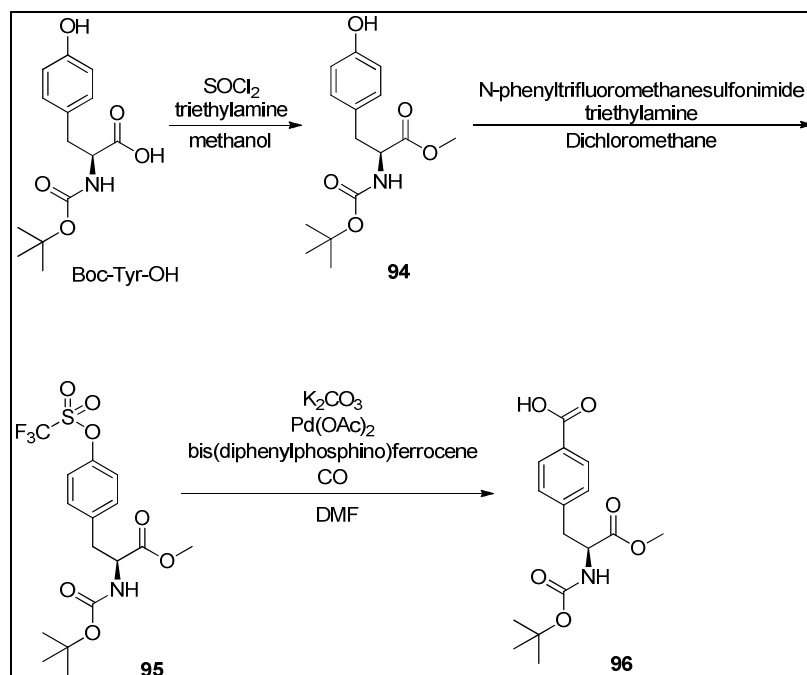


**Figure 14: Structures of Phe(p-Me) replacements in compounds 27-34**

First, we look at two compounds which increase lipophilicity with the addition of fluoro substituents on the phenyl ring of the side chain with compounds **27** (pentafluoro phenylalanine) and **28** (*para*-trifluoromethyl moiety). Compound **27** shows an order of magnitude decrease in potency when compared to **22**, with an unsubstituted Phe residue. Compound **28** also is approximately 3-fold less potent when compared to **FM 19**, with a methyl group in the *para* position. Thus, it appears as if simply adding lipophilicity does not lead to an increase in potency.

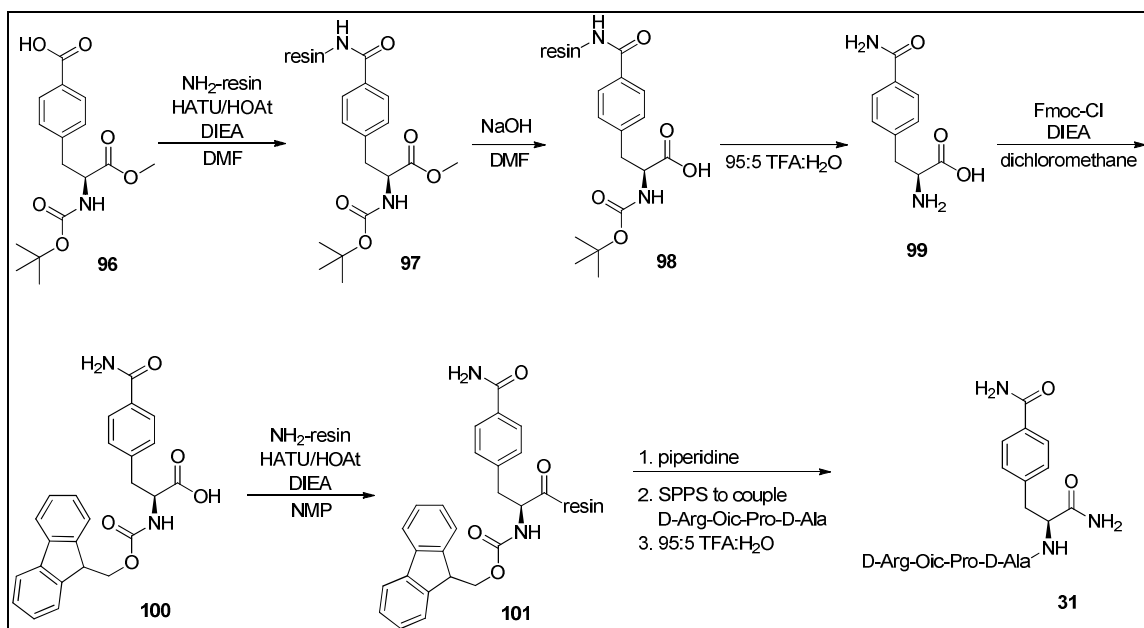
Next, we examined some additional *para* substituents. The first of these compounds uses an ether linkage to add either a methyl group (**29**) or benzyl group (**30**). Compound **29** is a better inhibitor than **30**, although **29** is still over 6-fold less potent than **FM 19**. Perhaps the methyl group of **29** is oriented in a less than optimal position, due to the ether linkage and geometry around the oxygen atom. Compound **30** is much less potent, and has the longest side chain length of any compounds in the series. Although we previously found that increasing side chain length improves potency, it is possible that this side chain has become too long. Or we may see a similar effect to what is seen in **29**, where the addition of the oxygen from the ether linker changes the orientation of the terminal benzyl group so that it is unable to position itself to achieve the optimal interactions with the hydrophobic residues within the aryl binding site.

The next two compounds contain an amide in the *para* position, with **31** containing an unsubstituted amide, and **32** adding a phenyl ring off of the amide nitrogen. Both compounds were synthesized using **96** (Boc-Phe(*p*-COOH)-OMe) as an intermediate, whose synthesis is shown in Scheme 9.



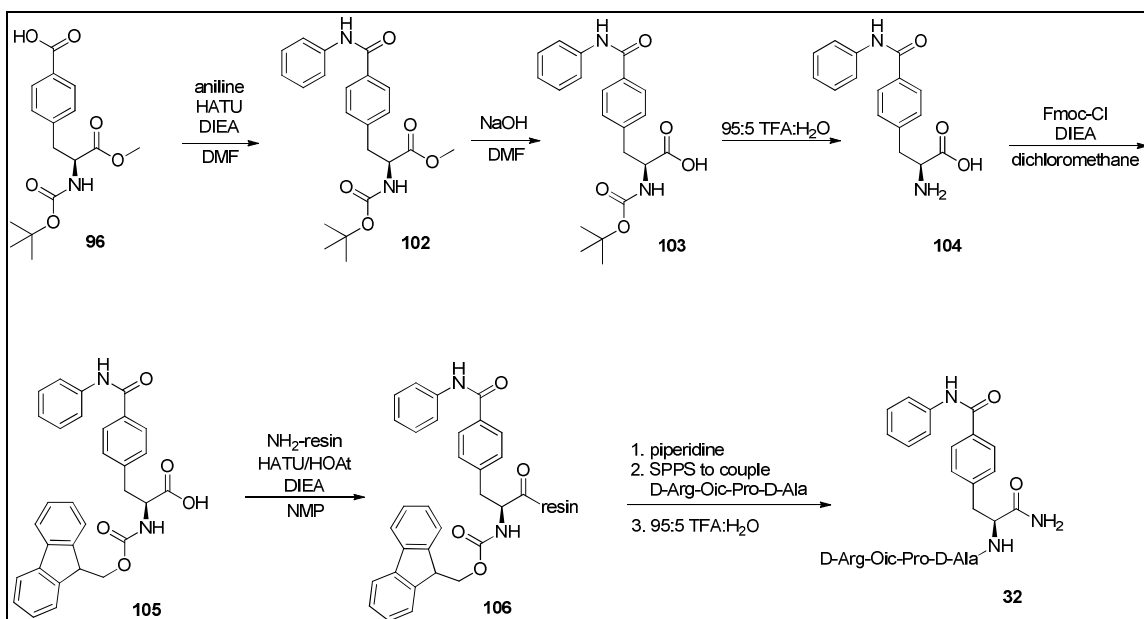
Scheme 9: Synthesis of intermediate **96**

Compound **96** was synthesized by first protecting the acid of the commercially available Boc-Tyr-OH as a methyl ester using thionyl chloride in methanol, to yield **94**. This compound was then treated with *N*-phenyltrifluoromethanesulfonylimide to convert the *para* hydroxyl to a triflate moiety.<sup>6</sup> This could then be converted to the desired *para* carboxylic acid group through a Pd cross coupling reaction<sup>6</sup>, resulting in the desired intermediate **96**.



**Scheme 10: Synthesis of compound 31**

Synthesis of compound **31** (Scheme **10**) began by treating **96** with Rink resin and HATU to convert the *para* carboxylic acid into the desired amide, still attached to the resin (**97**). Next, the methyl ester was removed using NaOH, generating **98** still attached to the resin. The use of the solid support facilitated easier purification of the products from any unreacted starting materials. Next, the Boc group was removed and the amino acid was cleaved from the resin simultaneously using TFA, producing **99**. The amino acid **99** had the amino group protected with an Fmoc group using Fmoc-Cl, giving **100**, which was then coupled to Rink resin on the C-terminus using HATU, to give the Fmoc-protected amino acid **101** attached to the solid support. This was then treated with piperidine to reveal the amino functionality, and standard peptide synthesis was used to complete the remainder of the peptide. After treatment with TFA, the synthesis of the desired final pentapeptide **31** was complete.



Scheme 11: Synthesis of compound 32

Compound **32** (Scheme 11) was synthesized in a similar manner to **31**.

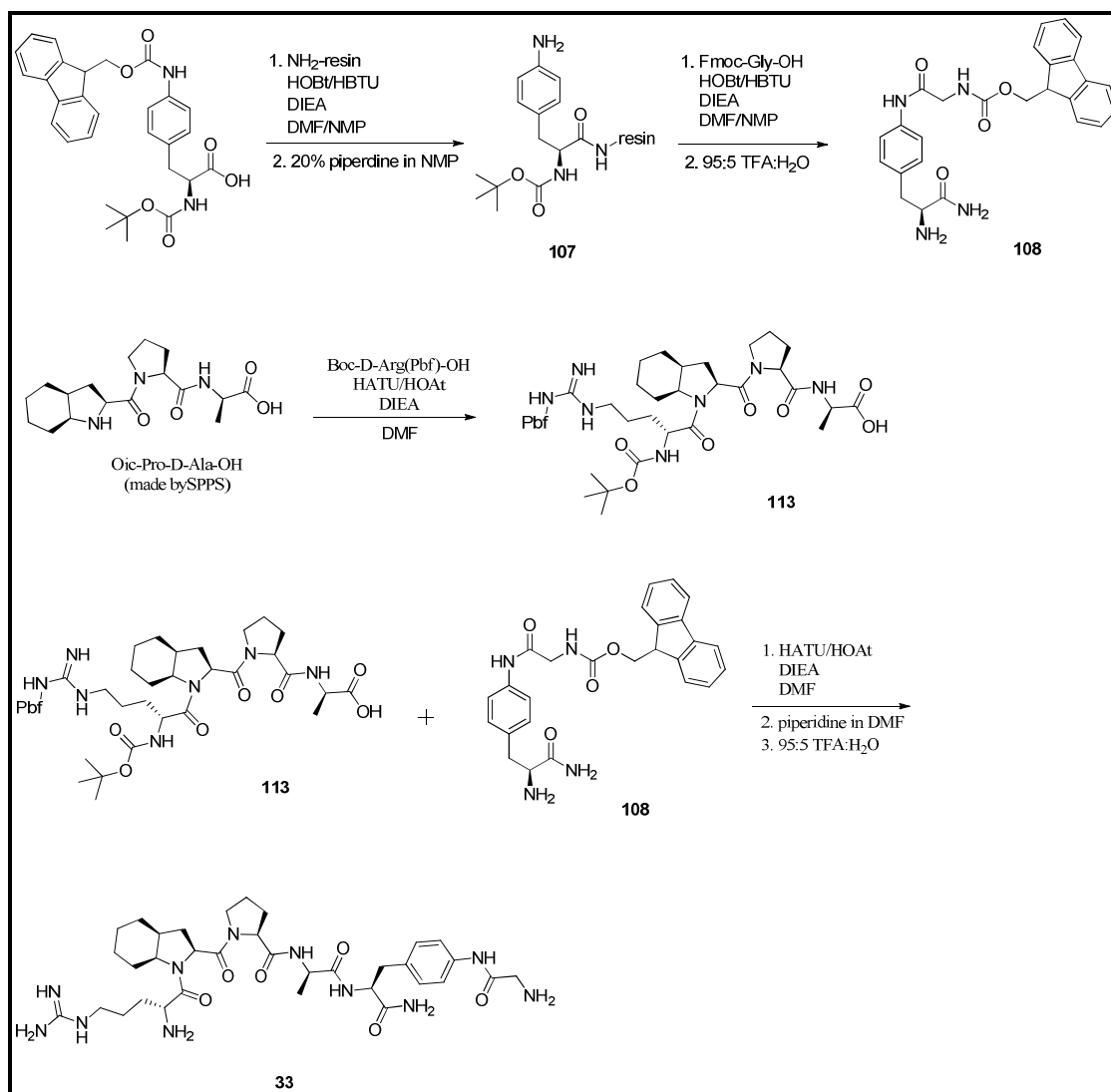
Intermediate **96** was first treated with aniline and HATU to install the phenyl-substituted amide in the *para* position on the phenyl ring, resulting in **102**. The methyl ester on the C-terminus was removed using NaOH to yield **103**, and then the Boc group was removed from the amino group using TFA to give **104**. The amine was then protected using Fmoc-Cl, generating the Fmoc-protected amino acid **105**. This amino acid was then coupled to Rink resin using HATU, generating the resin-bound amino acid **106**. This was treated with piperidine to remove the Fmoc group, and then standard peptide synthesis was used to complete the remainder of the peptide. Final pentapeptide **32** was complete after treatment with TFA.

From Table 6 we can see that the unsubstituted amide in **31** shows similar potency to lead compound **FM 19**, which might seem to be a surprising result at first. However, buried within this aryl binding site between the W215 and L99, is the backbone carbonyl of S214. This carbonyl has been suggested to be available as a hydrogen-bond acceptor,<sup>5</sup> and perhaps this sort of interaction is taking place with **31**, which would explain the result seen. However, the phenyl substituted amide of **32** shows significantly lower potency than either **31** or **FM 19**. This compound is more like **30** (Tyr(Bzl)) in structure, but is also approximately 6-fold less potent than the *para* substituted benzyl ether. Thus, the

large size of this side chain overall most likely contributes to the decrease in inhibitor potency seen, and the large phenyl substituent also makes the hydrogen bonding with S214 less likely.

Finally, we examine two compounds that add a positive charge in the *para* position of this residue, with compounds **33** (Phe(*p*-NHCOCH<sub>2</sub>NH<sub>2</sub>)) and **34** (Phe(*p*-guanidino)). These compounds were made to see if a cation- $\pi$  interaction could be achieved between the side chain of this residue and W215 within the binding pocket, or if additional interactions could be made with the backbone carbonyl of S214. The synthesis of **33** is shown in Scheme **12**, while **34** was made from commercially available materials using SPPS methods.

Compound **33** was synthesized in two pieces. First, the commercially available Boc-Phe(*p*-NHFmoc)-OH was coupled to Rink resin, and the Fmoc protecting group was then removed with piperidine to give resin-loaded intermediate **107** with a free amino group in the *para* position on Phe. Next, commercially available Fmoc-Gly-OH was coupled to resin-bound amino acid **107**, followed by treatment with TFA to remove the Boc protecting group, as well as cleave the amino acid from the resin, producing **108**. Separately, the tripeptide Oic-Pro-D-Ala-OH was synthesized using SPPS, and the commercially available Boc-D-Arg(Pbf)-OH was coupled to this, resulting in tetrapeptide **113**. Finally, tetrapeptide **113** was coupled to amino acid **108**, giving a fully protected pentapeptide. This resulting compound was treated first with piperidine to remove the Fmoc protecting group, followed by treatment with TFA to remove the Boc and Pbf protecting groups, resulting in **33**, ready to test.



Scheme 12: Synthesis of Compound 33

The yield of **33** was very low, and so only enough compound was generated to test twice (see Table 6). And due to the very low potency of this compound, at over 2.2 mM, it was unnecessary to generate more. It is clear that the positively charged amino group of **33** is not tolerated in the aryl binding site created by I174, W215 and L99. Although there remains a theoretical cation- $\pi$  interaction between this amine and W215, and the potential for a hydrogen bonding interaction with the backbone carbonyl of S214, the remainder of the hydrophobic residues here do not allow for a positive charge to be present. In addition, the results of a previous compound, **FM 32** (sequence D-Arg-Oic-Pro-D-Ala-Phe(*p*-NH<sub>2</sub>)-NH<sub>2</sub>, Table 6), appears to be in line with these results. **FM 32**,

with an IC<sub>50</sub> of 172 μM, contains a *para* amino group, which would not be expected to be positively charged at physiological pH, but would still be able to function as a hydrogen bond donor. This compound is much more potent than **33**, but still less potent than **31** (with a *p*-carboxamide substituent). Perhaps the longer *para* substituent of **31** allows this compound to more effectively form hydrogen bonding interactions with the buried backbone carbonyl of S214.

Compound **34** is almost 40-fold less potent than **FM 19**. Although the guanidino group is capable of being a hydrogen bond donor, and could presumably form a hydrogen bond with S214, the guanidino group itself is a rather large, very polar group with a delocalized positive charge, which may not fit well into the remainder of the binding pocket. Also, since the terminal end of this residue resembles the end of the D-Arg residue (and even more closely resembles the D-Arg replacement in compound **6** from Chapter 2 with a *p*-guanidinobenzoic acid residue), it is possible that **34** could be trying to bind in the opposite, and non-retro-binding fashion, with the Phe(*p*-guanidino) residue attempting to bind where the D-Arg residue is supposed to bind, resulting in the loss of key binding interactions from the remainder of the peptide, including the van der Waals interactions needed in the aryl binding site. Therefore, from these data, it appears as if an uncharged, hydrogen bond donor is accepted in the aryl binding site, but a positive charge is not.

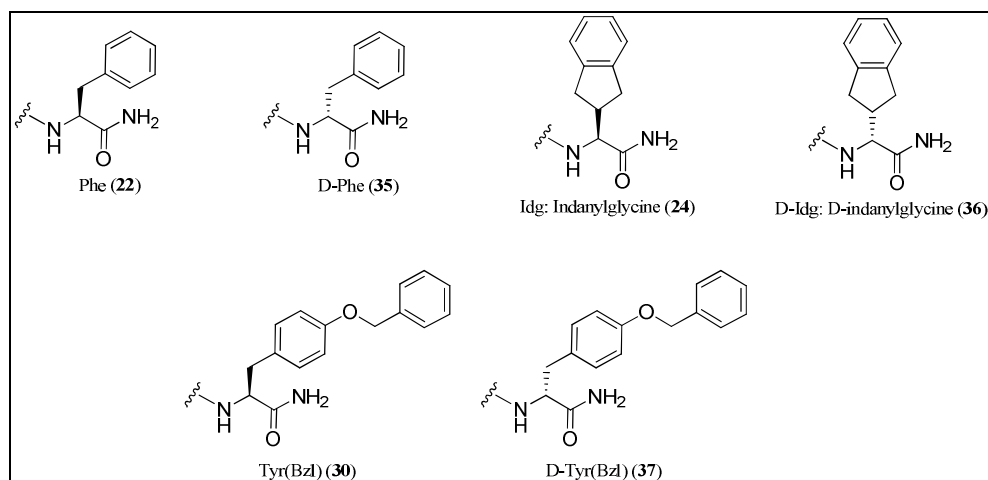
Finally, we made compounds **35-37** which investigate alteration of the stereochemistry around the α-carbon of the C-terminal residue. The results from these compounds are shown in Table 7, with the structures of the Phe(*p*-Me) replacements shown in Figure 15.

**Table 7: Phe(*p*-Me) replacements to investigate α-carbon stereochemistry**

Compound	Phe( <i>p</i> -Me) replacement (Y) in the <b>FM 19</b> Sequence (D-Arg-Oic-Pro-D-Ala-Y-NH <sub>2</sub> )	IC <sub>50</sub> (μM)
<b>FM 19</b>	Phe( <i>p</i> -Me)	4.4 ± 1.3 <sup>‡</sup>
<b>22</b> <sup>‡</sup>	Phe	49.9 ± 1.6
<b>35</b>	D-Phe	697 ± 8
<b>24</b>	Idg	70.5 ± 15.4
<b>36</b>	D-Idg	250 ± 60
<b>30</b>	Tyr(Bzl)	66.6 ± 3.7
<b>37</b>	D-Tyr(Bzl)	233 ± 34

<sup>‡</sup>Compound synthesized by Athena Flecha.





**Figure 15: Structures of Phe(*p*-Me) replacements in compounds 35-37**

Table 7 shows three pairs of compounds which differ only by the stereochemistry of the C-terminal residue. Compounds **22** and **35** have a Phe residue (L and D, respectively), compounds **24** and **36** contain the indanylglycine residue (L and D, respectively), and compounds **30** and **37** contain the Tyr(Bzl) moiety, which has a *para* benzyl ether substituent on the Phe ring (again, L and D, respectively). In each case, the L stereochemistry shows greater potency than the D. The largest difference is seen with the Phe compounds (**22** and **35**), with an almost 14-fold difference. The other two pairs show about 3.5-fold difference in potency, suggesting that maybe when the side chain of is slightly longer, the C-terminal residue has an easier time generating some favorable van der Waals contacts with this portion of the enzyme. It is also possible that this portion of the molecule simply does not provide a lot of favorable interactions with the enzyme.

To conclude, we have discovered that aryl substituents are preferred over alkyl substituents in the fifth position of the **FM 19** sequence, and when the aromatic ring is pushed further into the aryl binding site, thrombin inhibition is improved when compared to a constrained ring close to the remainder of the backbone of the peptide. Adding lipophilicity does not improve inhibition, but adding a small, uncharged, hydrogen bond donor is tolerated. Finally, L-amino acids are preferred over D-amino acids. Although 17 compounds were synthesized which replaced the Phe(*p*-Me) residue in the **FM 19**

sequence, none were more potent than the lead compound itself. Therefore, Phe(*p*-Me) in the fifth position is the optimal residue found in the series thus far.

### Chapter 3 References

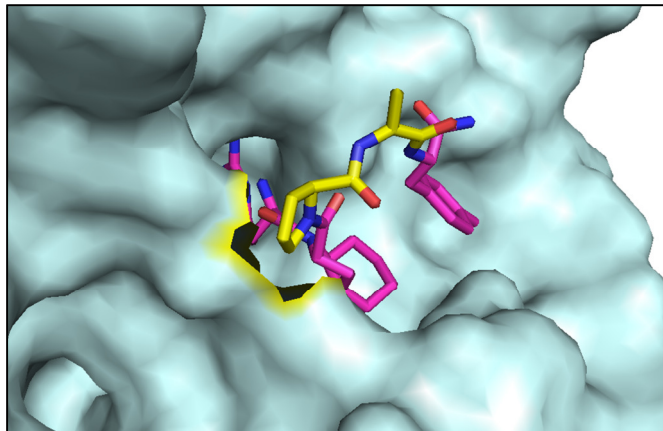
- (1) Nieman, M. T.; Warnock, M.; Hasan, A. A. K.; Mahdi, F.; Lucchesi, B. R.; Brown, N. J.; Murphey, L. J.; Schmaier, A. H. *The Journal of Pharmacology and Experimental Therapeutics* **2004**, *311*, 492.
- (2) Burke, F. M.; Warnock, M.; Schmaier, A. H.; Mosberg, H. I. *Chemical Biology and Drug Design* **2006**, *68*, 235.
- (3) Nieman, M. T.; Burke, F.; Warnock, M.; Zhou, Y.; Sweigart, J.; Chen, A.; Ricketts, D.; Lucchesi, B. R.; Chen, Z.; Cera, E. D.; Hilfinger, J.; Kim, J. S.; Mosberg, H. I.; Schmaier, A. H. *Journal of Thrombosis and Haemostasis* **2008**, *6*, 837.
- (4) Girnys, E. A.; Sobczyk-Kojiro, K.; Mosberg, H. I. *Chemical Biology and Drug Design* **2010**, *75*, 35.
- (5) Bode, W.; Turk, D.; Sturzebecher, J. *European Journal of Biochemistry* **1990**, *193*, 175.
- (6) Berezowska, I.; Chung, N. N.; Lemieux, C.; Wilkes, B. C.; Schiller, P. W. *Journal of Medicinal Chemistry* **2009**, *52*, 6941.

## Chapter 4

### Pro-D-Ala Replacements in the FM 19 sequence and C-terminal substituted amides

#### Pro-D-Ala replacements

Lead compound **FM 19** (sequence D-Arg-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub>) was developed as a univalent direct thrombin inhibitor after SAR studies were performed on the endogenous peptide **RPPGF**.<sup>1,2</sup> Further modifications have been explored, with chapter 2 focusing on replacements to the D-Arg residue, and chapter 3 discussing modifications to the Phe(*p*-Me) residue of **FM 19**. Instead of again looking at modifications of a single residue, this chapter displays alterations made in a slightly different manner, but still guided by examination of the crystal structure of **FM 19** in the active site of thrombin.<sup>3</sup> The first part of this chapter discusses alterations which replace both the Pro and D-Ala residues simultaneously, while the second part investigates modifications on the C-terminus of the peptide. First, the crystal structure shows that the middle Pro-D-Ala residues do not make any direct contacts with the enzyme, but instead stick out of the active site and act more as a scaffold to hold the other residues in their proper positions for binding (Figure 16). We made peptides with shortened sequences which eliminate or reduce this middle portion of the peptide, made the ring size of the Pro residue smaller, as well as peptides which alter the stereochemistry of these residues. Finally, several peptides were synthesized which use turn mimics or peptidomimetics in place of these residues.

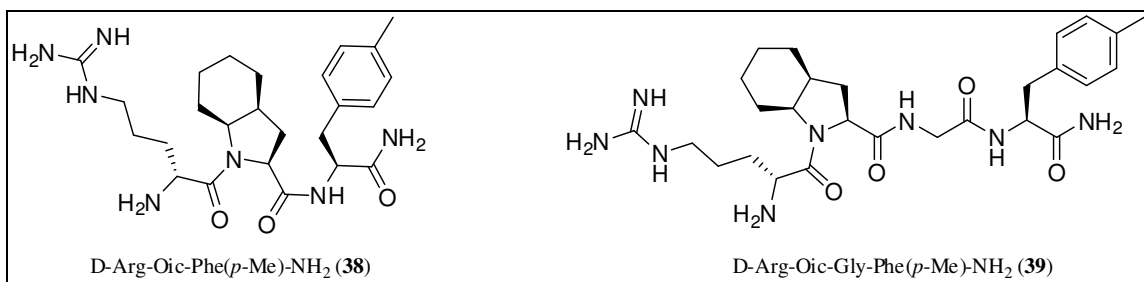


**Figure 16: Pro-D-Ala residues in the active site of thrombin. The Pro-D-Ala residues are shown in yellow with the rest of FM 19 shown in magenta, and thrombin in cyan. PDB: 3BV9, 1.8 Å**

First, we made two peptides (**38** and **39**) with shorter sequences compared to **FM 19**, to try to eliminate this scaffold portion of the peptide. The results are shown in Table **8** and the structures of the peptides are shown in Figure **17**.

**Table 8: Pro-D-Ala replacements with shortened sequences**

Compound	Sequence	IC <sub>50</sub> (μM)
<b>FM 19</b>	D-Arg-Oic-Pro-D-Ala-Phe( <i>p</i> -Me)-NH <sub>2</sub>	4.4 ± 1.3 <sup>4</sup>
<b>38</b>	D-Arg-Oic-Phe( <i>p</i> -Me)-NH <sub>2</sub>	1178 ± 212
<b>39</b>	D-Arg-Oic-Gly-Phe( <i>p</i> -Me)-NH <sub>2</sub>	1021 ± 152



**Figure 17: Structures of peptides 38 and 39**

The shortened sequences were synthesized in an attempt to remove the need for the scaffold created by the Pro-D-Ala residues in the middle of **FM 19**. Removal of this portion of the peptide would result in smaller compounds with decreased molecular

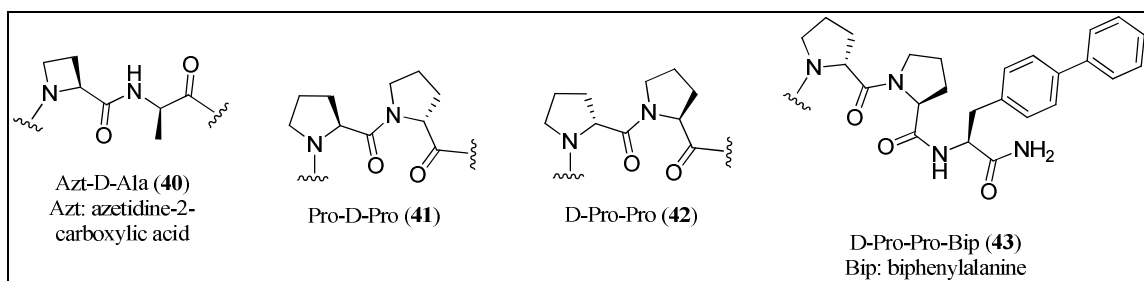
weight, which would likely improve the pharmacokinetic properties of the molecule. Modeling studies performed by Dr. Pogozheva of proposed peptide sequences suggested that it could be possible to shorten the overall sequence. With only the D-Arg residue constrained, the remainder of the C-terminus of the proposed peptide sequence rotated out of the binding pocket, and the Oic residue moved towards the aryl binding site, in an attempt to make the same van der Waals interactions with I174, W215 and L99 of thrombin that the Phe(*p*-Me) residue normally makes. Thus, it appeared as if the middle Pro-D-Ala residues might not be necessary. These shorter peptides, compounds **38** and **39**, are both significantly less potent than **FM 19**, each showing at least a 200-fold decrease in inhibition. These data suggest that both of the sequences of **38** and **39** are too short. Unfortunately, it appears as if both the D-Arg and Phe(*p*-Me) residues are unable to make key interactions when less than three amino acids separate the two terminal residues of the peptides in this series.

Next, we made one compound which decreases the size of the proline ring (**40**), and made three compounds (**41-43**) which investigate the stereochemistry of these middle residues. Results for these compounds are displayed in Table 9, and the structures of the replacements are shown in Figure 18.

**Table 9: Pro-D-Ala replacements to investigate ring size and stereochemistry**

Compound	Pro-D-Ala replacement ( <b>Z</b> ) in <b>FM 19</b> Sequence (D-Arg-Oic- <b>Z</b> -Phe( <i>p</i> -Me)-NH <sub>2</sub> )	IC <sub>50</sub> (μM)
<b>FM 19</b>	Pro-D-Ala	4.4 ± 1.3 <sup>4</sup>
<b>40</b>	Azt-D-Ala	13.3 ± 0.8
<b>41</b>	Pro-D-Pro	6.70 ± 0.43
<b>42</b>	D-Pro-Pro	13.6 ± 0.4
<b>43*</b>	D-Pro-Pro-Bip-NH <sub>2</sub>	677 ± 36

\*Compound **43** also replaces the Phe(*p*-Me) residue



**Figure 18: Structures of Pro-D-Ala replacements in compounds 40-43**

Compound **40** replaces only the Pro residue, with a smaller, 4-membered azetidine ring, and does not change the D-Ala residue. From Table **9**, we can see that this modification with the addition of a little more restriction is fairly well tolerated, with only approximately a 3-fold decrease in potency. However, the Pro residue in this position is slightly more preferred. Next, we made two compounds which use Pro in both the 3 and 4 positions, but alternate stereochemistry. Compound **41** uses the same stereochemistry as **FM 19** at each position, with a single modification of the D-Ala to a D-Pro, while compound **42** replaces the Pro-D-Ala moiety with a D-Pro-Pro dipeptide sequence. Compound **41** shows approximately the same potency as **FM 19**, which is not entirely surprising. Since the side chains of these residues do not interact with thrombin, several different amino acids in this position have been shown to be well tolerated during previous SAR studies.<sup>5</sup> Perhaps the slight decrease in potency can be attributed to the added rigidity of the Pro residue. Compound **42** was synthesized in an attempt to rotate the C-terminal Phe(*p*-Me) residue slightly, to try to make a stacked  $\pi$ - $\pi$  interaction between the Phe(*p*-Me) and W215 in the aryl binding site. Unfortunately, it appears as if this was not achieved, due to the 3-fold decrease in potency when compared to **FM 19**, and 2-fold decrease when compared to **41**. After further investigation of the crystal structure, it appears as if altering the stereochemistry of these two residues as in **42** could potentially pull the Phe(*p*-Me) residue slightly out of the binding pocket, and may remove some of the van der Waals interactions once made within the aryl binding site. Therefore, compound **43** was synthesized, which uses the D-Pro-Pro middle residues of **42**, but also changes the Phe(*p*-Me) residue to biphenylalanine (Bip), which has a longer side chain. Unfortunately, this additional modification did not help, and **43** is significantly less potent than **42**. From these data, it seems as if optimal thrombin

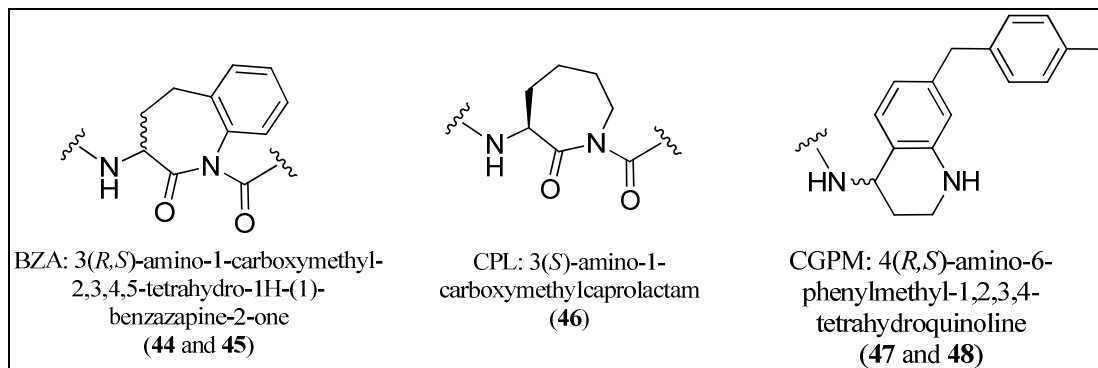
inhibition is achieved through an L amino acid in position 3 and a D amino acid in position 4, as is seen in **FM 19**.

The last replacements to this middle portion of the peptide were compounds **44-48**, which each replace both the Pro and D-Ala residues with some sort of different scaffold structure. Only three different scaffolds were used to synthesize these five compounds, since the BZA scaffold used for **44** and **45** was purchased as a racemic mixture, and the CGPM scaffold for **47** and **48** was previously synthesized by Yafei Jin (Vahlteich Medicinal Chemistry Core) as a mixture of enantiomers. Also, **47** and **48** did not use Phe(*p*-Me) in the sequence, as the scaffold contained a portion which substituted for the entire C-terminus of the peptide. The results for these five compounds is shown in Table 10, and the structures of the replacements is shown in Figure 19.

**Table 10: Pro-D-Ala replacements using different scaffolds and peptidomimetics**

Compound	Pro-D-Ala replacement ( <b>Z</b> ) in <b>FM 19</b> Sequence (D-Arg-Oic- <b>Z</b> -Phe( <i>p</i> -Me)-NH <sub>2</sub> )	IC <sub>50</sub> (μM)
<b>FM 19</b>	Pro-D-Ala	4.4 ± 1.3 <sup>4</sup>
<b>44</b>	BZA	32 ± 13
<b>45</b>	BZA	484 ± 22
<b>46</b>	CPL	389 ± 210
<b>47*</b>	CGPM	379 ± 44
<b>48*</b>	CGPM	371 ± 60

\*Compounds **47** and **48** also replace the Phe(*p*-Me) residue

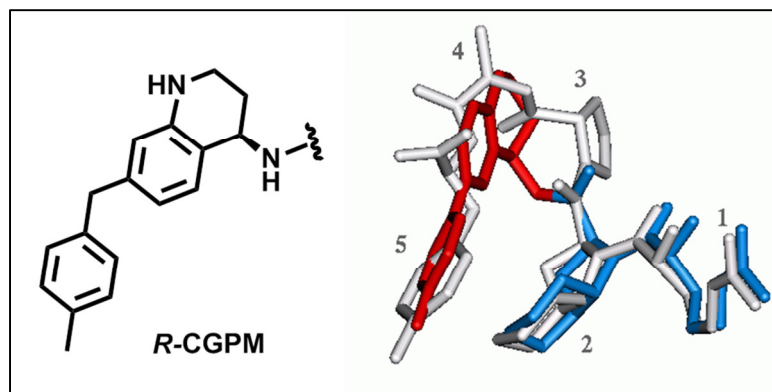


**Figure 19: Structures of Pro-D-Ala replacements in compounds 44-48**

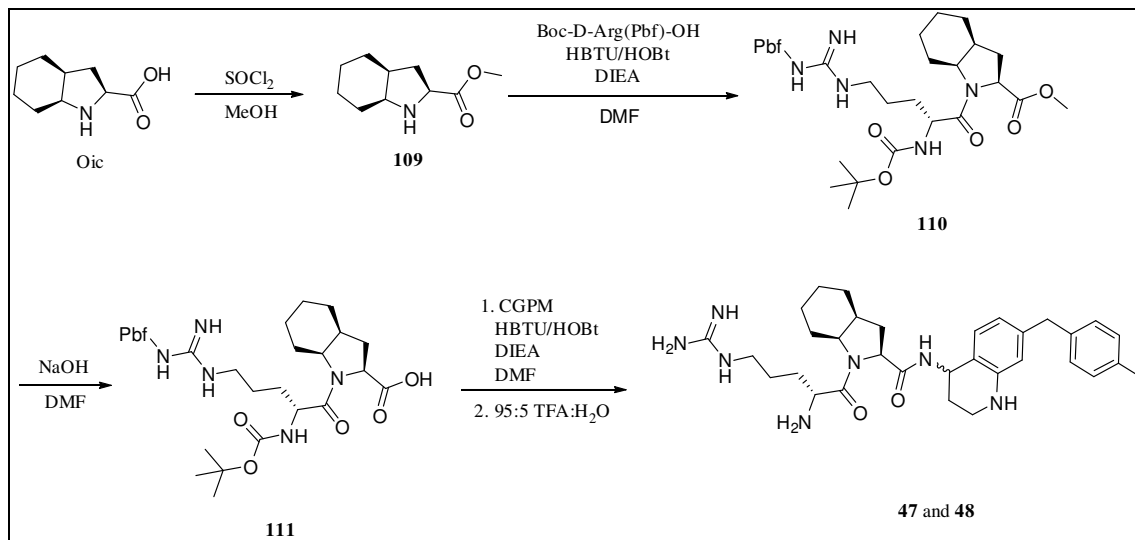


First, we will look at compounds **44** and **45**, which each contain a BZA substitution for the Pro-D-Ala residues. The compounds were synthesized together using SPPS and commercially available amino acids including Fmoc-BZA-OH, and the final peptides were separated during HPLC purification. Compound **44** is assigned as the earlier eluting peak, whereas **45** is the later eluting peak. We can see from Table **10** that **44** is over an order of magnitude more potent than its diastereomer **45**, but **44** still shows a 7-fold decrease inhibition when compared to **FM 19**. Modeling studies have suggested that the *S* enantiomer of BZA would be more potent than the *R* enantiomer, and so it is proposed that **44** contains *S*-BZA, although it is not known for certain. Compound **46** contains a CPL residue, which is similar in structure to BZA, but does not include the fused 6-membered aromatic ring. Compound **46** was also synthesized using enantiomerically pure *S*-CPL, and so we are certain of the stereochemistry in this case. Unfortunately, **46** is 88 times less potent than **FM 19**, and so it appears as if the addition of the fused 6-membered ring on BZA may add some rigidity to the overall structure, resulting in an increase in potency of the final peptide. Due to the fact that this portion of the peptide is solvent-exposed, it is highly unlikely that this additional aromatic ring would be able to make any additional van der Waals interactions. However, none of the scaffolding structures used in **44-46** appear to adequately mimic the Pro-D-Ala dipeptide sequence seen in **FM 19**.

Finally, the last two replacements to the Pro-D-Ala also replace the Phe(*p*-Me) residue by using the peptidomimetic scaffold CGPM. Modeling studies have suggested that the *R* enantiomer of this scaffold could mimic the structure of these three residues in **FM 19** (Figure **20**). Compounds **47** and **48** were synthesized together and the two diastereomers were separated during HPLC purification. The synthesis of these compounds is shown in Scheme **13**.



**Figure 20: Structure of CGPM superimposed on FM 19. CGPM is shown in red with the remainder of the peptide in blue, and FM 19 is shown in grey. Modeling figure provided by Dr. Irina Pogozheva.**



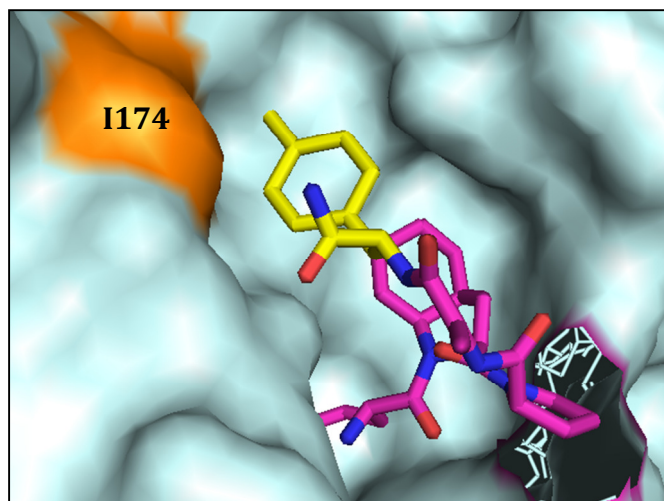
**Scheme 13: Synthesis of compounds 47 and 48**

First, the commercially available Oic had its carboxylic acid functionality protected as a methyl ester using thionyl chloride giving **109**. This was then coupled to commercially available Boc-D-Arg(Pbf)-OH using HBTU, to give protected dipeptide **110**. The methyl ester was then removed, giving **111**. This dipeptide was then coupled to peptidomimetic CGPM, and then the resulting compound was treated with TFA to give final peptides **47** and **48**. Compound **47** was assigned as the early eluting peak during HPLC purification, where the late eluting peak was designated as **48**.

From Table **10**, we can see that both diastereomers **47** and **48** show similar inhibition, with each being approximately 85 times less potent than **FM 19**. Thus, it appears as if neither stereoisomer can adequately mimic the Pro-D-Ala-Phe(*p*-Me) residues of the lead peptide. We would expect one compound to be better than the other, since chapter 3 confirmed that L amino acids in position 5 are preferred over D amino acids. However, the terminal, methyl substituted saturated ring of these two compounds is attached to the remainder of the peptide through a rotatable methylene bond, and so the rotation here may allow each diastereomer to make some favorable interactions, although it is clear from the assay data that neither diastereomer can make an optimal interaction with the aryl binding site. Again, as was shown with compounds **44-46** above, it is clear that the CGPM scaffold is not a suitable modification for the Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub> sequence of **FM 19**. Unfortunately, no explored replacements of the middle Pro-D-Ala residues produce a more potent compound than lead compound **FM 19**.

### **C-terminal substituted amides**

The last set of modifications made to the **FM 19** structure involve modification of the C-terminal amide of the peptide, by the addition of different alkyl and aryl groups. Previous SAR studies have shown that addition of alkyl groups here have the potential to increase potency,<sup>5</sup> and this observation is supported by examining the crystal structure. The nitrogen of the C-terminal amide points out of the aryl binding site, but is still in somewhat close proximity to I174, which makes up a portion of this binding site. The addition of hydrophobic substituents here may add to additional van der Waals interactions, leading to an increase in potency.



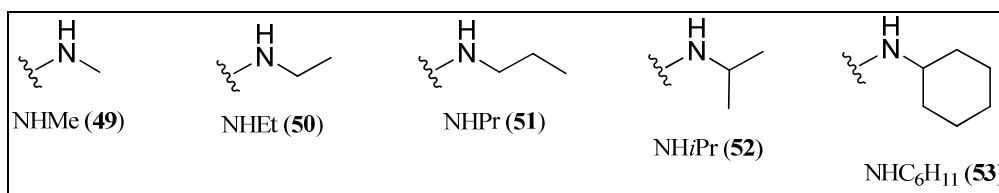
**Figure 21:** Phe(*p*-Me) residue and C-terminus in the active site of thrombin. The Phe(*p*-Me) residue is shown in yellow with the rest of FM 19 shown in magenta, I174 of thrombin shown in orange and the rest of thrombin in cyan. PDB: 3BV9, 1.8 Å.

First, we will look at some alkyl substitutions on the C-terminal amide with compounds **49-53**. The results for these compounds is shown in Table **11** and the structures of these modifications are shown in Figure **22**. In addition, for ease of synthesis, each of the peptides in the following two tables contains a Phe residue on the C-terminus, instead of Phe(*p*-Me). Therefore, each peptide will be compared to **22**, which contains a Phe residue with an unsubstituted amide on the C-terminus.

**Table 11: C-terminal modifications using alkyl amides**

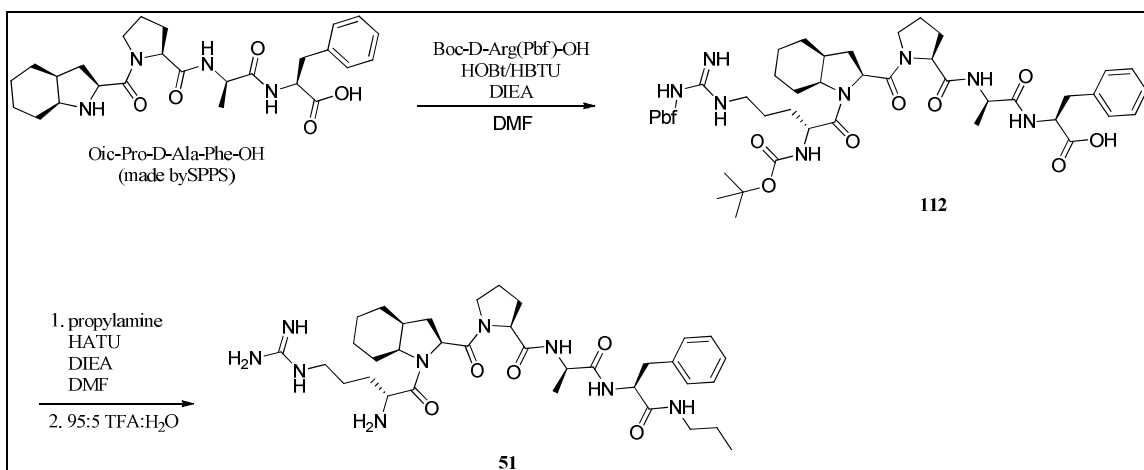
Compound	C-terminal modification (W) of <b>22</b> sequence (D-Arg-Oic-Pro-D-Ala-Phe-W)	IC <sub>50</sub> (μM)
<b>22</b> <sup>‡</sup>	NH <sub>2</sub>	49.9 ± 1.6
<b>49</b>	NHMe	30.8 ± 3.5
<b>50</b>	NHEt	25.3 ± 1.7
<b>51</b>	NHPr	17.5 ± 5.1
<b>52</b>	NHiPr	31.6 ± 4.3
<b>53</b>	NHC <sub>6</sub> H <sub>11</sub>	16.0 ± 1.6

<sup>‡</sup>Compound synthesized by Athena Flecha.



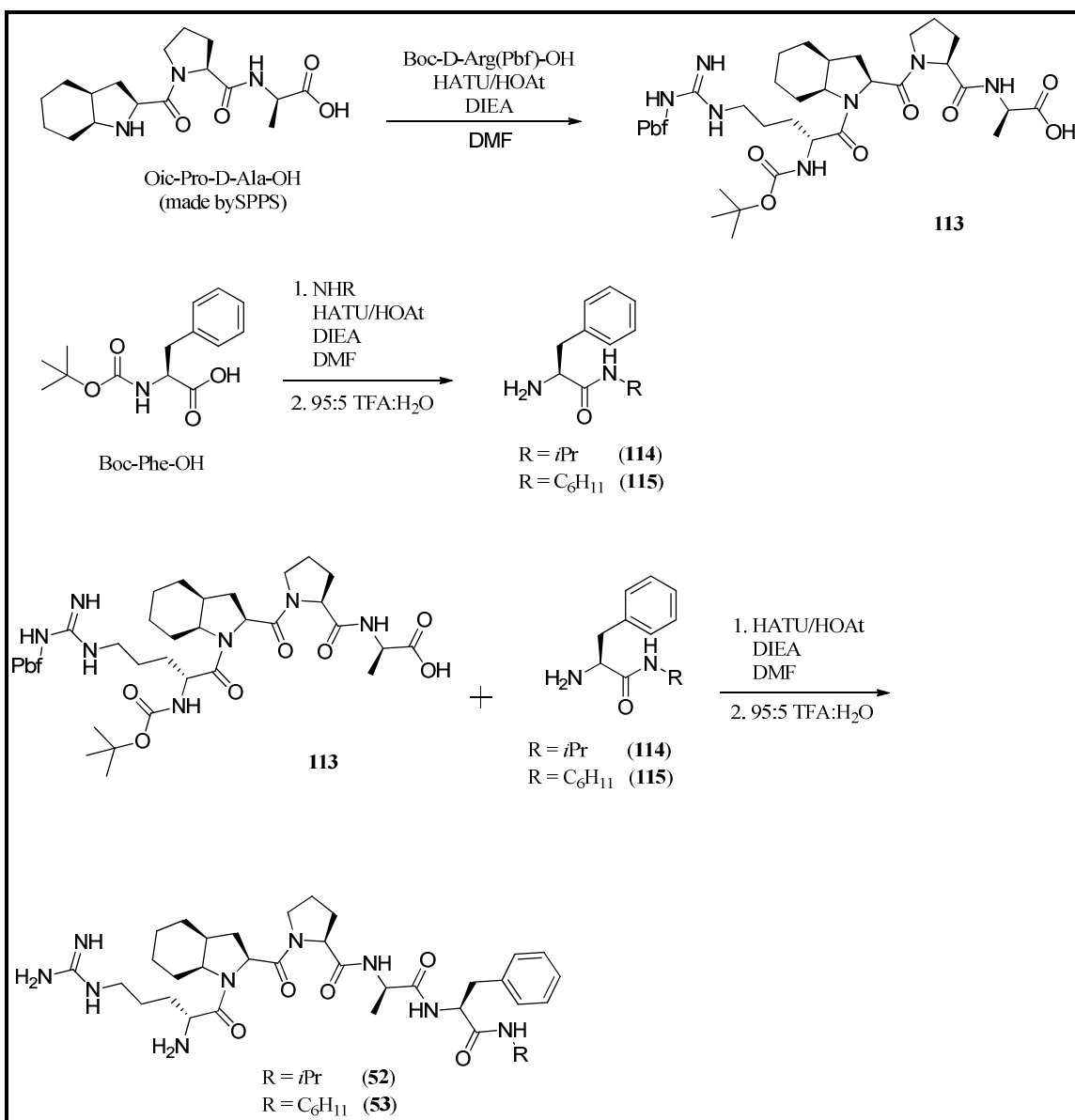
**Figure 22: Structures of C-terminal modifications in compounds 49-53**

The methyl substituted (**49**) and ethyl substituted (**50**) peptides were synthesized using commercially available resins which produced the desired substituted amide upon cleavage. The synthesis of compound **51** is shown in Scheme **14**, and the synthesis of **52** and **53** is shown in Scheme **15**.



**Scheme 14: Synthesis of compound 51**

Compound **51** was synthesized by first generating the tetrapeptide Oic-Pro-D-Ala-Phe-OH using SPPS. This tetrapeptide was then coupled to the commercially available Boc-D-Arg(Pbf)-OH, generating the protected pentapeptide **112**. The C-terminal acid was then functionalized using propylamine and HATU, and the resulting compound was treated with TFA to generate the unprotected pentapeptide **51**.



Scheme 15: Synthesis of compounds **52** and **53**

Compounds **52** and **53** were synthesized in two pieces. First, both peptides begin with synthesis of the tripeptide Oic-Pro-D-Ala-OH using SPPS, which could then be coupled to the commercially available Boc-D-Arg(Pbf)-OH, generating tetrapeptide **113**. Next, the C-terminal amino acid (Phe) was functionalized with the desired amide. Commercially available Boc-Phe-OH was treated with the desired amine (isopropyl amine for the synthesis of **52** and cyclohexylamine for **53**) to give the protected Phe residues. These compounds were then treated with TFA to remove the Boc protecting

group, giving **114** (Phe-NH*i*Pr) or **115** (Phe-NHC<sub>6</sub>H<sub>11</sub>). Finally, the two pieces could be coupled together to give protected pentapeptides, which were then treated with TFA to give final compounds **52** and **53**, which were ready for testing.

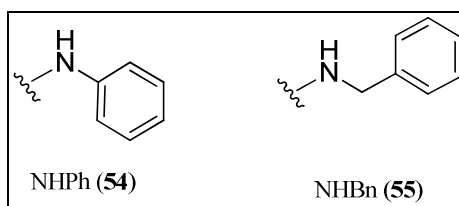
Table **11** shows that each of the C-terminal alkyl amides is more potent than the parent, unsubstituted amide in compound **22**. In addition, as the alkyl chain gets longer, the compound becomes more potent. The methyl amide (**49**) is slightly less potent than the ethyl amide (**50**), and the propyl amide (**51**) is more potent than the ethyl amide. Also, it does not appear as if adding bulk without elongating the alkyl substituent will improve inhibition. This is seen with compound **52** (*i*Pr amide), as this compound is less potent than the longer propyl amide (**51**) and **52** shows comparable potency to **50**. Finally, compound **53** has the longest and the bulkiest substitution here, with a cyclohexyl group. This compound is at least as potent as the propyl amide, suggesting that either the increase in potency observed with elongation of the alkyl chain may reach the upper limit at four carbons, or the addition of a little more steric bulk could negate the improved effects of a longer alkyl chain. Therefore, an even longer, straight alkyl chain could perhaps improve inhibition further.

In addition to the alkyl amides in Table **11**, we also made two compounds, **54** and **55**, which include an aryl amide on the C-terminus. Similar to the alkyl amides, these compounds contain Phe as the C-terminal amino acid. The results for these compounds are shown in Table **12** and the structures of the modifications are shown in Figure **23**.

**Table 12: C-terminal modifications using aryl amides**

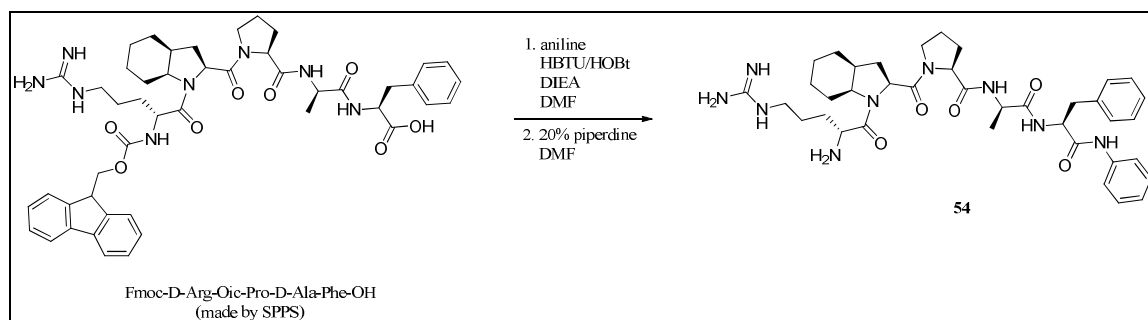
Compound	C-terminal modification ( <b>W</b> ) of <b>22</b> sequence (D-Arg-Oic-Pro-D-Ala-Phe- <b>W</b> )	IC <sub>50</sub> (μM)
<b>22</b> <sup>‡</sup>	NH <sub>2</sub>	49.9 ± 1.6
<b>54</b>	NHPh	12.9 ± 2.2
<b>55</b>	NHBn	36.8 ± 4.7

<sup>‡</sup>Compound synthesized by Athena Flecha.



**Figure 23: Structures of C-terminal modifications in compounds 54 and 55**

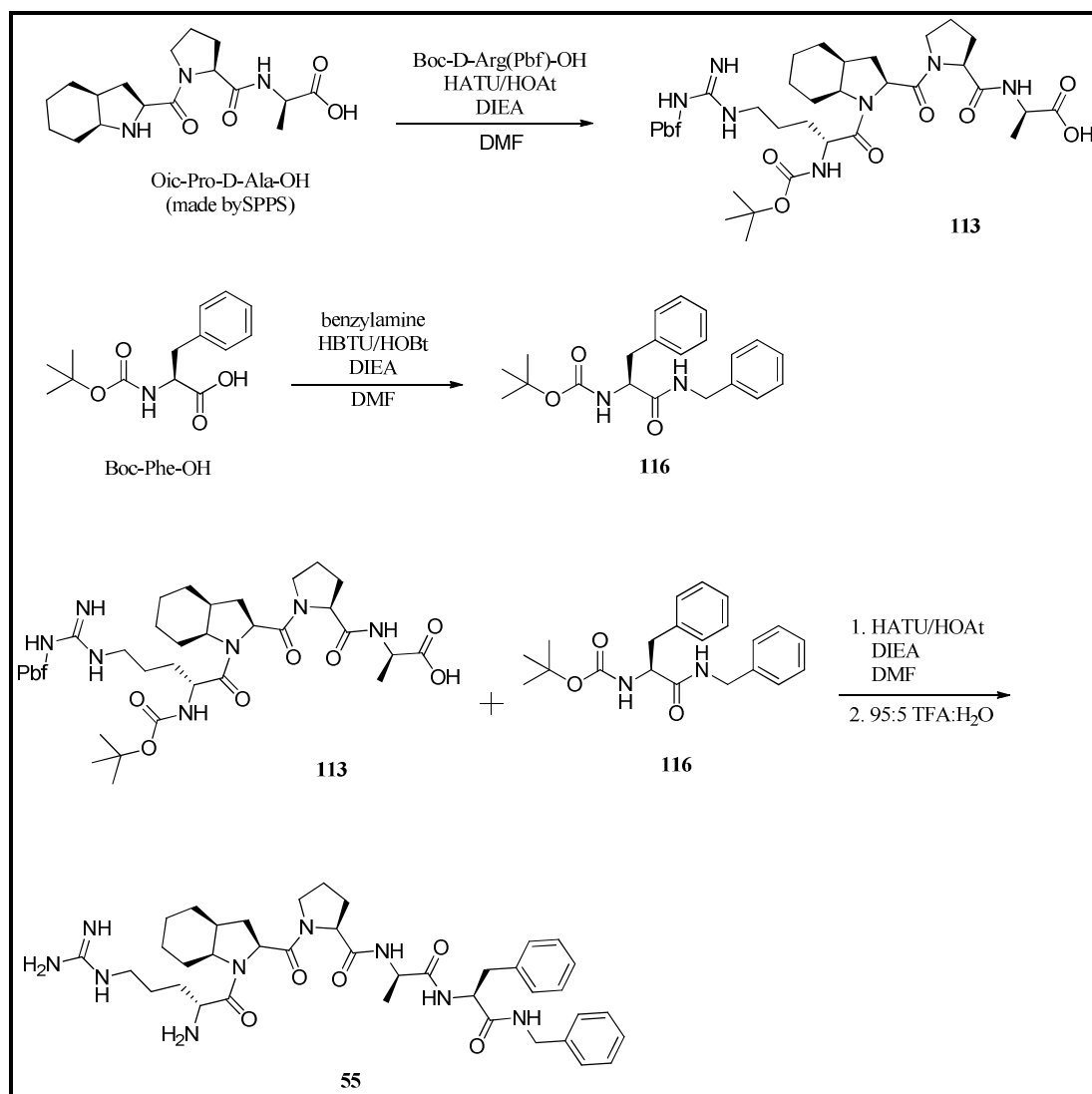
The synthesis of compound **54** is the most straightforward of any of the alkyl or aryl amides, and is shown in Scheme 16. The synthesis of compound **55** was completed in the same manner as compounds **52** and **53**, and is shown in Scheme 17.



**Scheme 16: Synthesis of compound 54**

Compound **54** was generated by first synthesizing the protected pentapeptide Fmoc-D-Arg-Oic-Pro-D-Ala-Phe-OH using SPPS. Next, aniline was coupled to this pentapeptide to produce the desired C-terminal phenyl amide. After this, the Fmoc protecting group was removed using piperidine, giving final pentapeptide **54**.





**Scheme 17: Synthesis of compound 55**

Compound **55** was synthesized in two pieces. First, the tripeptide Oic-Pro-D-Ala-OH was synthesized using SPPS, and this was then coupled to the commercially available Boc-D-Arg(Pbf)-OH, generating tetrapeptide **113**. Next, the C-terminal amino acid (Phe) was functionalized with the desired amide. Commercially available Boc-Phe-OH was treated with benzylamine to give the protected Phe residue. This compound was then treated with TFA to remove the Boc protecting group, giving **116** (Phe-NHBn). Finally, the two pieces (**113** and **116**) could be coupled together to give the protected pentapeptide, which was then treated with TFA to give final compound **55**, ready for testing.

From Table **12** we can see that again, both aryl amides are more potent than the unsubstituted amide of compound **22**. Compound **54** (phenyl amide) has the same length of four carbons from the nitrogen as seen in compound **53** from Table **11**, and compound **55** (benzyl amide) has one extra carbon. We can see that compound **54** is almost four times more potent than **22**, whereas **55** shows only a slight increase in inhibition, if any at all. Therefore, it appears that either four carbons is the ideal length of a chain here for inhibition, or that the added steric bulk of the aromatic ring is more unfavorable than a straight chain substituent.

To conclude, this chapter has focused on alterations to two different portions of the **FM 19** structure; first replacing the Pro-D-Ala residues in the middle of the sequence, and secondly, making C-terminal substituted amides. The Pro-D-Ala replacements looked at shortening the overall peptide sequence, modification of ring size of the Pro residue in the third position, as well as inversion of stereochemistry of these residues. In addition, this middle portion of the sequence was replaced with different scaffold structures. Unfortunately, no compounds were produced which showed superior thrombin inhibition when compared to **FM 19**. However, we also made substituted C-terminal amides, using different alkyl and aryl substituents, and each of these showed an increase in potency when compared to the corresponding unsubstituted amide. Both alkyl and aryl groups were tolerated, and inhibition improved as the alkyl chain got longer, although it appears as if adding extra steric bulk may not be ideal.

## Chapter 4 References

- (1) Nieman, M. T.; Warnock, M.; Hasan, A. A. K.; Mahdi, F.; Lucchesi, B. R.; Brown, N. J.; Murphey, L. J.; Schmaier, A. H. *The Journal of Pharmacology and Experimental Therapeutics* **2004**, *311*, 492.
- (2) Burke, F. M.; Warnock, M.; Schmaier, A. H.; Mosberg, H. I. *Chemical Biology and Drug Design* **2006**, *68*, 235.
- (3) Nieman, M. T.; Burke, F.; Warnock, M.; Zhou, Y.; Sweigart, J.; Chen, A.; Ricketts, D.; Lucchesi, B. R.; Chen, Z.; Cera, E. D.; Hilfinger, J.; Kim, J. S.; Mosberg, H. I.; Schmaier, A. H. *Journal of Thrombosis and Haemostasis* **2008**, *6*, 837.
- (4) Girnys, E. A.; Sobczyk-Kojiro, K.; Mosberg, H. I. *Chemical Biology and Drug Design* **2010**, *75*, 35.
- (5) Burke, F. M., University of Michigan, 2007.

## Chapter 5

### Synthesis and testing of an ‘optimized’ peptide

SAR studies to develop a direct thrombin inhibitor, beginning with endogenous pentapeptide **RPPGF**, led to the development of lead compound **FM 19** (D-Arg-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub>).<sup>1,2</sup> The crystal structure of **FM 19** in the active site of thrombin was determined,<sup>3</sup> and was used to guide the development of additional analogs. Chapter 2 described replacements to the D-Arg residue on the N-terminus of the peptide, resulting in several compounds with superior thrombin inhibition compared to **FM 19**. Chapter 3 explored replacements to the C-terminal Phe(*p*-Me) residue, but we were unable to synthesize any more potent inhibitors when compared to the lead compound. Chapter 4 explored two different types of modifications: replacements to the Pro-D-Ala residues simultaneously, and substitution of the C-terminal amide. While no replacements to the middle Pro-D-Ala residues resulted in compounds with improved potency, all compounds which added a substituent on the C-terminal amide showed an increase in inhibition when compared to the corresponding unsubstituted amide. Since we have found two different types of replacements which increase potency individually, we wanted to combine them in a single peptide.

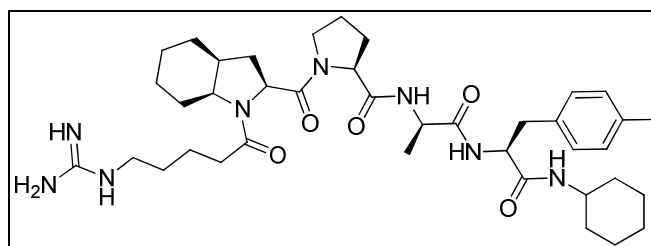
There are several D-Arg Replacements which can be used to synthesize the ‘optimized’ peptide, each which shows an improvement in potency when compared to **FM 19**, and are all within experimental error of each other. However, for the ease of synthesis, we chose the 5-guanidinopentanoic acid replacement of compound **1** which shows approximately a six-fold improvement in potency over **FM 19** (Table 13). The synthesis of this replacement was by far the highest yielding of all of the most potent D-Arg replacements made, which would allow us to generate the ‘optimized’ peptide in the most efficient manner. Additionally, there are three substituted amides on the C-terminus

which show the largest improvement in potency when compared to **FM 19**, and like the D-Arg replacements, are all within experimental error of each other. We chose to substitute the C-terminal amide with a cyclohexane ring, and the addition of this group led to a three-fold increase in inhibition (compound **53**, Table **13**) when compared to the corresponding unsubstituted amide (compound **22**, Table **13**). This addition was chosen partially because of the high yield and cost-effective synthesis of the desired peptide, which made it more favorable than the propyl substituted amide. The phenyl substituted amide was not chosen, as this carries a potential metabolic liability of hydroxylation on the aromatic ring, making this substitution less appealing. The resulting compound is **56**, with the sequence 5-guanidinopentanoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NHC<sub>6</sub>H<sub>11</sub>. The assay results are shown in Table **13**, the structure of the peptide is shown in Figure **24**, and the synthesis is displayed in Scheme **18**.

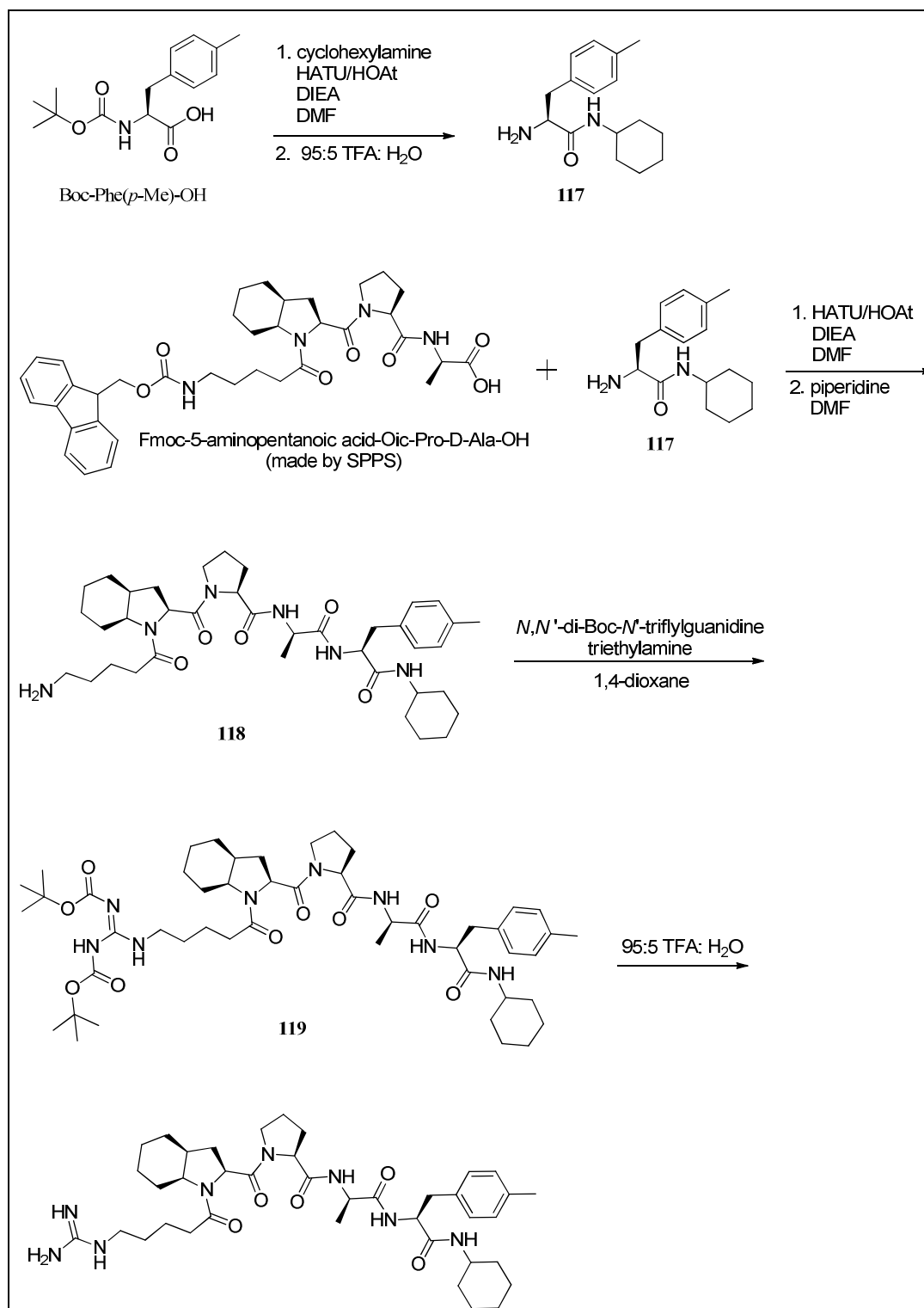
**Table 13: Combination of best Modifications**

Compound	Sequence	IC <sub>50</sub> (μM)
<b>FM 19</b>	D-Arg-Oic-Pro-D-Ala-Phe( <i>p</i> -Me)-NH <sub>2</sub>	4.4 ± 1.3 <sup>4</sup>
<b>1</b>	5-guanidinopentanoic acid-Oic-Pro-D-Ala-Phe( <i>p</i> -Me)-NH <sub>2</sub>	0.66 ± 0.20 <sup>4</sup>
<b>22</b> <sup>‡</sup>	D-Arg-Oic-Pro-D-Ala-Phe-NH <sub>2</sub>	49.9 ± 1.6
<b>53</b>	D-Arg-Oic-Pro-D-Ala-Phe-NHC <sub>6</sub> H <sub>11</sub>	16.0 ± 1.6
<b>56</b>	5-guanidinopentanoic acid-Oic-Pro-D-Ala-Phe( <i>p</i> -Me)-NHC <sub>6</sub> H <sub>11</sub>	0.53 ± 0.04

<sup>‡</sup>Compound synthesized by Athena Flecha.



**Figure 24: Structure of compound 56**



Scheme 18: Synthesis of Compound 56

Compound **56** was synthesized in two pieces. First, the C-terminal Phe(*p*-Me) residue was functionalized with the desired cyclohexyl amide. Commercially available Boc-Phe(*p*-Me)-OH was treated with cyclohexylamine to give the protected Phe(*p*-Me) residue. This compound was then treated with TFA to remove the Boc protecting group, giving **117** (Phe(*p*-Me)-NHC<sub>6</sub>H<sub>11</sub>). Next, the tetrapeptide Fmoc-5-aminopentanoic acid-Oic-Pro-D-Ala-OH was synthesized by SPPS methods, and coupled to **117** to give the protected pentapeptide, which was treated with piperidine to remove the Fmoc protecting group and yield **118**. Compound **118** was then treated with *N,N'*-di-Boc-*N'*-triflylguanidine<sup>5</sup> to install the desired guanidino functionality (which was protected with 2 Boc groups) from the existing primary amine, giving **119**. The protection of this guanidino group allowed for easier purification of the product from any unreacted amino-containing peptide. The resulting compound was then treated with TFA to remove the protecting groups, to give final compound **56**.

From Table **13**, we can see that compound **56** is approximately 8-fold more potent than lead compound **FM 19**. Unfortunately, it does not appear that the combination of the two different replacements in this peptide are additive. Compound **56** might be slightly more potent than the corresponding unsubstituted amide **1**, but the two results are within experimental error. However, compound **56** still represents an improvement in thrombin inhibitor potency over lead compound **FM 19**.

## Chapter 5 References

- (1) Nieman, M. T.; Warnock, M.; Hasan, A. A. K.; Mahdi, F.; Lucchesi, B. R.; Brown, N. J.; Murphey, L. J.; Schmaier, A. H. *The Journal of Pharmacology and Experimental Therapeutics* **2004**, *311*, 492.
- (2) Burke, F. M.; Warnock, M.; Schmaier, A. H.; Mosberg, H. I. *Chemical Biology and Drug Design* **2006**, *68*, 235.
- (3) Nieman, M. T.; Burke, F.; Warnock, M.; Zhou, Y.; Sweigart, J.; Chen, A.; Ricketts, D.; Lucchesi, B. R.; Chen, Z.; Cera, E. D.; Hilfinger, J.; Kim, J. S.; Mosberg, H. I.; Schmaier, A. H. *Journal of Thrombosis and Haemostasis* **2008**, *6*, 837.
- (4) Girnys, E. A.; Sobczyk-Kojiro, K.; Mosberg, H. I. *Chemical Biology and Drug Design* **2010**, *75*, 35.
- (5) Feichtinger, K.; Zapf, C.; Sings, H. L.; Goodman, M. *Journal of Organic Chemistry* **1998**, *63*, 3804.



## Chapter 6

### Conclusions and future directions

#### Conclusions

We have been able to synthesize several compounds with improved thrombin inhibition when compared to lead compound **FM 19** (D-Arg-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub>), using a structure-based approach guided by the crystal structure of **FM 19** in the active site of thrombin.<sup>1</sup> We have made peptides which alter four different areas of the **FM 19** sequence individually; the D-Arg residue, the Pro-D-Ala residues simultaneously, the Phe(*p*-Me) residue, and substitution of the C-terminal amide. First, chapter 2 explored alterations to the D-Arg residue. We found that eliminating the N-terminal amine and hence, eliminating the adverse electrostatic interaction with the guanidino group on the side chain seen in the crystal structure<sup>1</sup> resulted in the expected increase in potency. Additionally, we found that adding conformational restriction can also improve potency, and an aromatic ring appears to be the most favorable. Also, modification of the guanidino group to an amidino group is tolerated, since both groups allow for delocalization of the positive charge at the end of the side chain. Several compounds in this series show increased thrombin inhibition when compared to **FM 19**, and four of these show submicromolar potencies.

Next, chapter 3 described modifications made to the Phe(*p*-Me) residue. First, the preference for aryl substituents here was confirmed in this aryl binding site. In addition, other properties of this residue were explored, such as elongation of the side chain, hydrogen-bonding substituents, and stereochemistry. Unfortunately, no replacements of this Phe(*p*-Me) residue resulted in increased potency.

The first part of chapter 4 investigated replacements to the middle, Pro-D-Ala residues of the peptide sequence simultaneously. Since these residues do not make any

direct interactions with the enzyme and instead are exposed to solvent, we explored several different scaffold and peptidomimetic replacements here, and also inverted the stereochemistry. Again, there were no compounds synthesized in this series which improved thrombin inhibition when compared to **FM 19**. The second part of chapter 4 added substituents onto the C-terminal amide, using both alkyl (straight chain as well as cyclic) and aryl moieties, and every one of the peptides synthesized were more potent than the corresponding unsubstituted amide. As the length of the chain increased, so did the potency of the compound. The addition of steric bulk, such as with a cyclic structure or branched alkane, might influence the inhibitory effect in a negative way, although the results are inconclusive. However, these substituted amides are expected to pick up additional van der Waals interactions, likely leading to the increase in potency observed.

The results from chapters 2-4 culminated in compound **56**, with the sequence 5-guanidinopentanoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NHC<sub>6</sub>H<sub>11</sub>, from chapter 5. This peptide combined favorable modifications to both the D-Arg residue and the substituted C-terminus. Compound **56** shows an over 8-fold improvement in potency over **FM 19**, but only a modest (if any) improvement over the corresponding unsubstituted amide (compound **1**). Although the improvement in potency is not extremely large, compound **56** still represents a promising lead peptide for further direct thrombin inhibitors, especially for the potential to be more orally available than **FM 19**, due to the removal of the positive charge at the N-terminus, and the overall increase in lipophilicity.

Although we were able to synthesize several peptides with improved inhibition when compared to lead compound **FM 19**, it should be noted that the modeling studies performed throughout were not always able to suggest more potent modifications. There are a few reasons why this may have occurred. First, many of the studies were performed by using molecular replacement. A portion of the **FM 19** structure was modified with the proposed replacement (often a single amino acid replacement, but occasionally more than one residue was changed at a time), using the crystal structure of **FM 19** in the active site of thrombin (PDB 3BV9, 1.8 Å resolution). Once the new peptide was in place, energy minimizations were performed on the ligand, while not allowing thrombin itself to undergo any movements or energy minimizations. While this is a simple way to quickly investigate many new structures, the active site of thrombin can and does move in

response to different inhibitors binding, and so maintenance of a static enzyme active site will not always accurately portray what will occur when the ligand interacts with thrombin. For example, the crystal structure of **FM 19** in the active site of thrombin shows that the enzyme has moved to accommodate the inhibitor. The side chain of W60d of thrombin was shown to have  $> 4\text{\AA}$  shift when compared to a previously reported crystal structure of thrombin bound to the inhibitor PPACK (D-Phe-Pro-Arg-CH<sub>2</sub>Cl, PDB 1SFQ, 1.9 Å resolution).<sup>1,2</sup> In addition, the Pro residue of **FM 19** causes additional movement of nearby residues, allowing E192 to form a hydrogen bond with K149e (a rarely seen interaction in thrombin crystal structures), which is thought to contribute to the stability of this complex.<sup>1</sup>

Another potential reason for why the modeling studies were not always predictive occurred when Glide (Schrödinger, LLC) was used for molecular docking studies of new peptides. When these studies were performed, a portion of the new peptide was constrained, and the remainder of the peptide was allowed to move and minimize energy (while again maintaining a static enzyme). During these studies, it was difficult to determine how much of the ligand to constrain. If too much of the structure was held in place, then very few iterations occurred, resulting in a peptide with some unfavorable interactions that was very similar overall when compared to **FM 19**. However, when a smaller fragment of the peptide was constrained, the modeling studies took many days (or longer) to complete, and often gave results that were not repeatable. Part of this is probably due to the fact that overall, these peptides contain many rotatable bonds, and Glide is more predictive for compounds which have fewer rotatable bonds. For instance, when only the guanidino group on the side chain of the D-Arg residue was held in position, the C-terminus of the peptide was fully able to move, and actually moved out of the aryl binding pocket entirely. The software tried to place the Oic residue in this binding site, moving the remaining three residues into solvent and making no interactions with the enzyme at all. This led to the synthesis of the shortened peptide sequences of **38** and **39** (Table 8, chapter 4), which showed a very large decrease in potency. Therefore, although the docking with Glide had hinted that maybe a shorter peptide sequence could make all necessary interactions, this was shown experimentally to not be the case.

Finally, another major shortcoming of the modeling studies has nothing to do with the models themselves. The active site of thrombin has a very shallow binding site, with the majority of the inhibitors binding near the surface of the enzyme. The side chain of the D-Arg residue goes deepest into the enzyme, but the sites where Phe(*p*-Me) and Oic bind are fairly solvent exposed. Therefore, any software program will have trouble modeling a ligand on the surface of an enzyme. To conclude, although the modeling studies were not always predictive of experimental results, the models were able to suggest compounds with improved potency, especially when looking at replacements to the D-Arg residue, such as compound **1** (5-guanidinopentanoic acid replacement, Table **1**, chapter 2), which is one of the most potent compounds in the series.

### **Future Directions**

We have now developed several compounds which show superior thrombin inhibition when compared to lead compound **FM 19**, and so the majority of the future directions for this project focus on further *in vitro* and *in vivo* testing of the most potent inhibitors. Before these additional tests are done, however, it would be worthwhile to explore additional substituted amides on the C-terminus of the peptide sequence. Chapter 4 showed that adding alkyl or aryl substituents here have the potential to lead to an increase in potency over the corresponding unsubstituted amide. However, it also appears as if a longer alkyl chain might be preferred over larger substituents which add steric bulk. Therefore, it would be interesting to see what effect a longer straight alkyl chain would have, beginning with a butyl substituent and moving on to longer chains until the optimal length is determined.

After these additional analogs are synthesized and tested in the inhibition of  $\alpha$ -thrombin assay utilized previously, there are a number of additional *in vitro* assays which can determine if the newly synthesized peptides are able to inhibit thrombin's normal functions, and have potential for clinical relevance as new antithrombotics. The first of these assays would be inhibition of platelet aggregation using previously established methods.<sup>3,4</sup> Using platelet-rich plasma from healthy human donors and  $\gamma$ -thrombin to initiate platelet aggregation, minimal concentration of inhibitor required to achieve 100% inhibition of platelet aggregation could be determined.

Another assay which could be utilized is the inhibition of calcium mobilization. Since  $\alpha$ -thrombin activation of both PAR1 and PAR4 results in calcium mobilization (after downstream signaling associated with G-protein partners), this assay would determine if a compound is able to inhibit this mobilization, which translates to a decrease in stimulation of these receptors, using a previously established method.<sup>4</sup> The minimal concentration of inhibitor required to display a 50% decrease in  $\text{Ca}^{2+}$  mobilization following  $\alpha$ -thrombin stimulation can be determined using human fibroblasts and the fluorescent dye Fura-2, which detects the cytoplasmic concentration of calcium ions. While this assay will determine if the PARs are stimulated through their downstream signals, it can also be a method to determine if a compound might be dual-functioning. The earlier peptides in this series, **RPPGF** and **TH 146** (chapter 1), bind both to the active site of thrombin in addition to binding to the thrombin cleavage site on PAR1, resulting in these compounds to have a dual function in inhibition of thrombus formation.<sup>3,4</sup> However, **FM 19** acts as only a direct thrombin inhibitor, and does not bind to PAR1.<sup>1</sup> If several new compounds (not just the most potent) were tested in this calcium mobilization assay, the results could be correlated with the results from the inhibition of  $\alpha$ -thrombin assays found in chapters 2-5. If a compound shows a greater inhibition in the calcium mobilization assay than what would be predicted from the inhibition of  $\alpha$ -thrombin assay, it is possible that this compound could be dual-functioning as well. This result could prove to be an interesting result and would help to determine which structural features are important for PAR binding independently from thrombin inhibition.

The final *in vitro* functional assay would determine if the newly synthesized peptides are able to inhibit blood clotting by measuring the activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin clotting time (TCT) according to previously described protocols.<sup>5</sup> Each of these three methods determines the length of time required for a thrombus to form in a human plasma sample, although each assay measures a different aspect of clot formation. First, the APTT assay gives information on the intrinsic and common pathways of coagulation through initiation with a contact activator (traditionally kaolin, but more modern activators include micronized silica).<sup>6</sup> The PT assay gives information on the extrinsic and common pathways of

coagulation through initiation with thromboplastin in the presence of tissue factor.<sup>6</sup> Finally, the the TCT assay (also referred to as simply thrombin time), gives information on thrombin cleavage of fibrinogen, through initiation with thrombin.<sup>6</sup>

After confirming that the new inhibitors are able to inhibit thrombin's functions *in vitro*, it will be important to test these compounds for specificity. Thrombin is a serine protease, and there are many other serine proteases both within the coagulation cascade as well as elsewhere in the human body. Assays to determine specificity should include coagulation factors VIIa, IXa, Xa, XIa, and XIIa, as well as the related serine protease trypsin.

Compounds showing promise in the above *in vitro* assays could then be tested *in vivo* by determining if the time to thrombosis can be increased after administration of the new inhibitor. Initially, the compound would be delivered intraperitoneally, and the time to thrombosis measured. If the time to thrombosis increases after the addition of inhibitor, the minimal concentration of compound needed to increase time to thrombosis would be determined. Next, the compound could be administered in drinking water, and again, the minimal concentration to increase the time to thrombosis would be determined. These values can be measured using the Rose Bengal assay or the ferric chloride model.<sup>4,7</sup> These two assays use different stimulation to initiate thrombosis, but both measure occlusions in the right carotid artery. The Rose Bengal assay uses laser stimulation whereas the ferric chloride assay uses FeCl<sub>3</sub>. FeCl<sub>3</sub> causes much greater thrombosis stimulation, resulting in a shorter time to occlusion normally,<sup>4,7</sup> and therefore, any increase in the time to thrombosis in the ferric chloride model suggests a strong anticoagulant effect. Lead compound **FM 19** was able to increase the time to thrombosis using the Rose Bengal assay following oral administration in drinking water (5 mg/mL) to 62 minutes from 23 minutes in untreated animals,<sup>1</sup> and so it is not unreasonable to believe that any newer inhibitors might also be orally available.

Finally, we could determine the metabolic and pharmacokinetic properties of the new compounds. To start, pooled liver microsomes can be used to investigate metabolism. **FM 19** did not show any significant metabolism after 120 minutes, and this was attributed to the structure not having any peptide bonds between two naturally occurring amino acids.<sup>1</sup> All compounds with superior thrombin inhibition compared to

**FM 19** made in this series also lack any natural peptide bonds, and so it is likely that the new inhibitors might also be metabolically stable. Furthermore, **FM 19** was not metabolized *in vivo* in Sprague-Dawley rats,<sup>1</sup> and so *in vivo* metabolic studies should be performed on new compounds as well. Additionally, it would be worthwhile to determine some pharmacokinetic properties of any promising new inhibitors. **FM 19** had a half-life of 32.5 minutes in beagle dogs and 27 minutes in Sprague-Dawley rats, and a clearance of 8.2 mL/min/kg in beagles and 10.9 mL/min/kg in rats. Finally, after administration of 3 mg **FM 19** by duodenal lavage, the bioavailability was found to be 12.7%, which is a very promising result. It would be interesting to determine these parameters for new inhibitors, and see how they compare to the results of **FM 19**. If any new inhibitors produce results to the previously described *in vitro* and *in vivo* assays which are superior or comparable to the results obtained for **FM 19**, these compounds with improved potency represent a promising new lead for an orally available direct thrombin inhibitor.

## Chapter 6 References

- (1) Nieman, M. T.; Burke, F.; Warnock, M.; Zhou, Y.; Sweigart, J.; Chen, A.; Ricketts, D.; Lucchesi, B. R.; Chen, Z.; Cera, E. D.; Hilfinger, J.; Kim, J. S.; Mosberg, H. I.; Schmaier, A. H. *Journal of Thrombosis and Haemostasis* **2008**, *6*, 837.
- (2) Pineda, A. O.; Carrell, C. J.; Bush, L. A.; Prasad, S.; Caccia, S.; Chen, Z.-W.; Mathews, F. S.; Di Cera, E. *Journal of Biological Chemistry* **2004**, *279*, 31842.
- (3) Hasan, A. A. K.; Warnock, M.; Nieman, M.; Srikanth, S.; Mahdi, F.; Krishnan, R.; Tulinsky, A.; Schmaier, A. H. *American Journal of Physiology - Heart and Circulatory Physiology* **2003**, *285*, H183.
- (4) Nieman, M. T.; Warnock, M.; Hasan, A. A. K.; Mahdi, F.; Lucchesi, B. R.; Brown, N. J.; Murphey, L. J.; Schmaier, A. H. *The Journal of Pharmacology and Experimental Therapeutics* **2004**, *311*, 492.
- (5) Hasan, A. A. K.; Warnock, M.; Srikanth, S.; Schmaier, A. H. *Thrombosis Research* **2001**, *104*, 451.
- (6) Perry, D. J.; Todd, T. <http://www.practical-haemostasis.com/index.html>
- (7) Shariat-Madar, Z.; Mahdi, F.; Warnock, M.; Homeister, J.; Srikanth, S.; Krijanovski, Y.; Murphey, L. J.; Jaffa, A. A.; Schmaier, A. H. *Blood* **2006**, *108*, 192.



## Chapter 7

### Experimental Methods

#### 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 C18 reverse phase column, using a linear gradient of 0% Solvent B (0.1% 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  $\mu$ L 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 Bruker spectrometer (300 or 500 MHz) or a Varian spectrometer (400 MHz). All column chromatography was performed using a Flash+ System (Biotage Corporation, Sweden) and pre-packed silica Flash 40+M cartridges (40  $\times$  150 mm) and Flash 40+ samplets (40–63  $\mu$ m particle distribution).

#### General procedure for SPPS

Peptides were synthesized using standard Fmoc chemistry, on a CS Bio CS336X Peptide Synthesizer (CS Bio Company, Menlo Park, CA, USA), using previously described protocols.<sup>1</sup> Rink amide resin (Advanced Chem

Tech, Louisville, KY, USA) was used to produce all peptides with C-terminal amides (**1-44**); see individual protocols for resin used to make peptides **47-53**. A solution of 20% piperidine in 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 HOBt/HBTU (Advanced Chem Tech, Louisville, KY, USA) in DMF, in the presence of DIEA. After the synthesis was complete, the resin was washed with NMP followed by a wash with dichloromethane, and dried.

### **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 C18 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.

### **General human $\alpha$ -thrombin inhibition assay protocol**

Assays were performed to measure the ability of the new synthetic peptides to inhibit human  $\alpha$ -thrombin's (Haematologic Technologies, Essex Junction, VT, USA) cleavage of the chromogenic substrate tetrapeptide Sarcosine-Pro-Arg-*p*-nitroanilide (Bachem, Torrance, CA, USA). 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 (10 mM Tris (tris(hydroxymethyl)aminomethane), 150 mM NaCl, pH 7.6) at 37°C for 5 minutes, and the experiment was started with the addition of substrate. The final concentration of enzyme in the assay was 2 nM and the final substrate concentration was 200  $\mu$ M. After 10 minutes, the OD<sub>405</sub> was plotted as a function of the negative log of inhibitor concentration (average and standard deviation of the three wells for each concentration) to generate a dose-response curve using GraphPad Prism (version 5, GraphPad Software, Inc., La Jolla, CA, USA). From these data, an IC<sub>50</sub> value was determined. Each compound was tested in at least three independent experiments, and the average and standard deviation are reported.

**Synthesis of 5-guanidinopentanoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub> hydrotrifluoroacetate (1)**

NB 2: 134-136 and 139

Compound **57** was dissolved in a cooled solution of 95:5 TFA:H<sub>2</sub>O (10 mL) and stirred at room temperature for 1 hour. The solution was concentrated *in vacuo* followed by precipitation with cold diethyl ether. After refrigeration overnight, **1** hydrotrifluoroacetate was collected as an off-white solid (97 mg) and purified by HPLC using the general peptide purification procedure to obtain **1** hydrotrifluoroacetate as a white solid (55 mg, 40.6% yield for 2 steps) which was >99% pure by HPLC.

**Synthesis of Guanidinoglycine-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub> hydrotrifluoroacetate (2)**

NB 2: 273-274, 281

Compound **59** was dissolved in a cooled solution of 95:5 TFA:H<sub>2</sub>O (10 mL) and stirred at room temperature for 4 hours. The solution was concentrated *in vacuo* followed by precipitation with cold diethyl ether. After refrigeration overnight, **2** hydrotrifluoroacetate was collected as an off-white solid (51.9 mg) and purified by HPLC using the general peptide purification procedure to obtain **2** hydrotrifluoroacetate as a white solid (45.7 mg, 64.3% yield for 2 steps) which was >99% pure by HPLC.

**Synthesis of 3-guanidinopropionic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub> hydrotrifluoroacetate (3)**

NB 2: 297, 300-301

Compound **62** was dissolved in a cooled solution of 95:5 TFA:H<sub>2</sub>O (10 mL) and stirred at room temperature for 3.25 hours. The solution was concentrated *in vacuo* followed by precipitation with cold diethyl ether. After refrigeration overnight, **3** hydrotrifluoroacetate was collected as an oily purple solid (64.5 mg) and purified by HPLC using the general peptide purification procedure to obtain **3** hydrotrifluoroacetate as a white solid (32.2 mg, 31.7% yield for 2 steps) which was >97% pure by HPLC.

**Synthesis of 4-guanidinobutyric acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub>  
hydrotrifluoroacetate (4)**

NB 2: 276, 282

Compound **64** was dissolved in a cooled solution of 95:5 TFA:H<sub>2</sub>O (10 mL) and stirred at room temperature for 3.25 hours. The solution was concentrated *in vacuo* followed by precipitation with cold diethyl ether. After refrigeration overnight, **4** hydrotrifluoroacetate was collected as an off-white solid (50.7 mg) and purified by HPLC using the general peptide purification procedure to obtain **4** hydrotrifluoroacetate as a white solid (37.7 mg, 42.5% yield for 2 steps) which was >97% pure by HPLC.

**Synthesis of 6-guaidinohexanoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub>  
hydrotrifluoroacetate (5)**

NB 2: 305, 308-309

Compound **65** was dissolved in a cooled solution of 95:5 TFA:H<sub>2</sub>O (10 mL) and stirred at room temperature for 4 hours. The solution was concentrated *in vacuo* followed by precipitation with cold diethyl ether. After refrigeration overnight, **5** hydrotrifluoroacetate was collected as an off-white solid (30.4 mg) and purified by HPLC using the general peptide purification procedure to obtain **4** hydrotrifluoroacetate as a white solid (8.6 mg, 12.5% yield for 2 steps) which was >97% pure by HPLC.

**Synthesis of *p*-guanidinobenzoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub>  
hydrotrifluoroacetate (6)**

NB 2: 172-174, 182-183

First, Oic-Pro-D-Ala-Phe(*p*-Me)-resin was synthesized according to the general solid phase peptide synthesis procedure, using Rink amide resin (270 mg, 0.75 mmol/g substitution) and commercially available amino acids. After synthesis was complete, the resin was washed with NMP followed by dichloromethane, dried and transferred to a scintillation vial (271.6 mg). Compound **66** (70.4 mg, 0.19 mmol) was dissolved in a pre-made solution of 0.4 M HOBt/HBTU in DMF (6 mL, 2.4 mmol), NMP (5 mL) and DIEA (3 mL, 17.2 mmol). The solution of **66** was added to the tetrapeptide resin and agitated at room temperature for 21 hours. The resin was washed with NMP followed by

dichloromethane and dried (326.6 mg). A cooled solution of 95:5 TFA:H<sub>2</sub>O (10 mL) was added and the mixture stirred at room temperature for 4 hours. The solution was filtered away from the resin, concentrated *in vacuo* followed by precipitation with cold diethyl ether. After refrigeration overnight, **6** hydrotrifluoroacetate was collected as an off-white solid (66.0 mg) and purified by HPLC using the general peptide purification procedure to obtain **6** hydrotrifluoroacetate as a white solid (5.1 mg, 3.3% yield) which was >97% pure by HPLC.

**Synthesis of *cis*-4-guanidino-1-cyclohexanecarboxylic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub> hydrotrifluoroacetate<sup>2</sup> (7)**

NB 2: 177-179, 186-187

Compound **69** was dissolved in a cooled solution of 95:5 TFA:H<sub>2</sub>O (10 mL) and stirred at room temperature for 2 hours. The solution was concentrated *in vacuo* followed by precipitation with cold diethyl ether. After refrigeration overnight, **7** hydrotrifluoroacetate was collected as an off-white solid (56.2 mg) and purified by HPLC using the general peptide purification procedure to obtain **7** hydrotrifluoroacetate as a white solid (42.2 mg, 41.% yield for 2 steps) which was >97% pure by HPLC.

**Synthesis of *trans*-4-guanidino-1-cyclohexanecarboxylic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub> hydrotrifluoroacetate<sup>2</sup> (8)**

Compound **71** was dissolved in a cooled solution of 95:5 TFA:H<sub>2</sub>O (10 mL) and stirred at room temperature for 2 hours. The solution was concentrated *in vacuo* followed by precipitation with cold diethyl ether. After refrigeration overnight, **8** hydrotrifluoroacetate was collected as an off-white solid (42 mg) and purified by HPLC using the general peptide purification procedure to obtain **8** hydrotrifluoroacetate as a white solid (13 mg, 13.% yield for 2 steps) which was >97% pure by HPLC. This compound was synthesized by Vanessa R. (Porter) Barrus.

**Synthesis of 5-aminopentanoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub>  
hydrotrifluoroacetate (9)**

NB 2: 295, 302-303, 312

Compound **9** was synthesized according to the general solid phase peptide synthesis procedure, using Rink amide resin (320 mg, 0.7 mmol/g substitution) and commercially available Fmoc-protected amino acids, in addition to **72**. Once synthesis was complete, the dried resin (409 mg) was stirred for 3.5 hours at room temperature with 95:5 TFA:H<sub>2</sub>O (10 mL). After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **9** hydrotrifluoroacetate was collected (108 mg) and purified by HPLC using the general peptide purification procedure to obtain **9** hydrotrifluoroacetate (31.8 mg, 20.0% yield) which was >97% pure by HPLC.

**Synthesis of 6-aminohexanoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub>  
hydrotrifluoroacetate (10)**

NB 2: 294, 298-299

Compound **10** was synthesized according to the general solid phase peptide synthesis procedure, using Rink amide resin (350 mg, 0.7 mmol/g substitution) and commercially available Fmoc-protected amino acids, in addition to **73**. Once synthesis was complete, the dried resin (451 mg) was stirred for 3 hours at room temperature with 95:5 TFA:H<sub>2</sub>O (10 mL). After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **10** hydrotrifluoroacetate was collected (97.6 mg) and purified by HPLC using the general peptide purification procedure to obtain **10** hydrotrifluoroacetate (50.3 mg, 28.3% yield) which was >97% pure by HPLC.

**Synthesis of 7-aminoheptanoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub>  
hydrotrifluoroacetate (11)**

NB 2: 260-261, 268-269

Compound **11** was synthesized according to the general solid phase peptide synthesis procedure, using Rink amide resin (310 mg, 0.7 mmol/g substitution) and

commercially available Fmoc-protected amino acids, in addition to the commercially available Boc-7-aminohexanoic acid. Once synthesis was complete, the dried resin (427 mg) was stirred for 3.5 hours at room temperature with 95:5 TFA:H<sub>2</sub>O (10 mL). After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **11** hydrotrifluoroacetate was collected (121.5 mg) and purified by HPLC using the general peptide purification procedure to obtain **11** hydrotrifluoroacetate (83.9 mg, 52.3% yield) which was >97% pure by HPLC.

**Synthesis of *p*-aminobenzoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub> hydrotrifluoroacetate (**12**)**

NB 2: 270-271, 279-280

Compound **12** was synthesized according to the general solid phase peptide synthesis procedure, using Rink amide resin (320 mg, 0.7 mmol/g substitution) and commercially available Fmoc-protected amino acids, in addition to Boc-*p*-aminobenzoic acid made previously in the lab by Dr. Sobczyk-Kojiro. Once synthesis was complete, the dried resin (445 mg) was stirred for 4 hours at room temperature with 95:5 TFA:H<sub>2</sub>O (10 mL). After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **12** hydrotrifluoroacetate was collected (107.2 mg) and purified by HPLC using the general peptide purification procedure to obtain **12** hydrotrifluoroacetate as a white solid (53.8 mg, 32.9% yield) which was >97% pure by HPLC.

**Synthesis of 4-amidinobenzoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub> hydrotrifluoroacetate<sup>2</sup> (**13**)**

The synthesis of **13** was completed using a modification of a previously reported procedure.<sup>3</sup> In a reaction vessel for a Parr shaker, compound **75** (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<sub>2</sub> at room temperature for 23.5 h. The solution was filtered through celite and the filtrate was

concentrated *in vacuo* to give **13** as a hydroacetate salt. The oil was purified by HPLC using the general peptide purification procedure to obtain **13** hydrotrifluoroacetate as a white solid (7.6 mg, 3.4% yield) which was >97% pure by HPLC.

#### **Synthesis of 4-amidinophenylacetic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub> hydrotrifluoroacetate<sup>2</sup> (**14**)**

The synthesis of **14** was completed using a modification of a previously reported procedure.<sup>3</sup> In a reaction vessel for a Parr shaker, compound **77** (8.2 mg, 0.01 mmol) and acetic anhydride (5  $\mu$ L, 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<sub>2</sub> at room temperature for 21.5 h. The solution was filtered through celite and the filtrate was concentrated *in vacuo* to give **14** as a hydroacetate salt. The oil was purified by HPLC to obtain **14** hydrotrifluoroacetate as a white solid (2.1 mg, 0.9% yield) which was >97% pure by HPLC.

#### **Synthesis of 3-amidinobenzoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub> hydrotrifluoroacetate<sup>2</sup> (**15**)**

First, compound **79** (42 mg, 0.2 mmol) was dissolved in a solution of 0.4 M HOBt/HBTU in DMF (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 3-amidinobenzoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-resin. The pentapeptide resin was then stirred for 2 h at room temperature with 95:5 TFA:H<sub>2</sub>O (10 mL). After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **15** hydrotrifluoroacetate was collected as an off-white solid, 94 mg) and purified by HPLC using the general peptide purification procedure to obtain **15** hydrotrifluoroacetate as a white solid (3.7 mg, 2.3% yield) which was >97% pure by HPLC.



**Synthesis of 3-amidinophenylacetic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub> hydrotrifluoroacetate<sup>2</sup> (16)**

First, compound **81** (53 mg, 0.25 mmol) was dissolved in a solution of 0.4 M HOBt/HBTU in DMF (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 3-amidinophenylacetic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-resin (373 mg). The pentapeptide resin (304 mg) was then stirred for 2 h at room temperature with 95:5 TFA:H<sub>2</sub>O (10 mL). After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **16** hydrotrifluoroacetate was collected as an off-white solid (86 mg) and purified by HPLC using the general peptide purification procedure to obtain **16** hydrotrifluoroacetate as a white solid (4.2 mg, 2.6% yield) which was >97% pure by HPLC.

**Synthesis of (*R*)-5-guanidino-2-methylpentanoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub> hydrotrifluoroacetate<sup>2,4</sup> (17)**

Compound **87** was stirred for 2 h at room temperature with 95:5 TFA:H<sub>2</sub>O (10 mL). After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **17** hydrotrifluoroacetate salt was collected as an off-white solid (40 mg) and purified by HPLC to obtain **17** hydrotrifluoroacetate as a white solid (35 mg, 57% yield) which was >97% pure by HPLC.

**Synthesis of (*S*)-5-guanidino-2-methylpentanoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub> hydrotrifluoroacetate<sup>2,4</sup> (18)**

Compound **93** was stirred for 2 h at room temperature with 95:5 TFA:H<sub>2</sub>O (10 mL). After filtration, the solution was concentrated *in vacuo*, to yield an oil containing **18**

(39 mg). The oil was purified by HPLC to obtain **18** hydrotrifluoroacetate as a white solid (22 mg, 57% yield) which was >97% pure by HPLC.

#### **Synthesis of Arg-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub> hydrotrifluoroacetate<sup>2</sup> (19)**

Compound **19** was synthesized according to the general solid phase peptide synthesis procedure, using Rink amide resin (340 mg, 0.59 mmol/g substitution) and commercially available Fmoc-protected amino acids. Once synthesis was complete, the dried resin (468 mg) was stirred for 2 hours at room temperature with 95:5 TFA:H<sub>2</sub>O (10 mL). After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **19** hydrotrifluoroacetate was collected as an off-white solid (125 mg) and purified by HPLC using the general peptide purification procedure to obtain **19** hydrotrifluoroacetate (55 mg, 31% yield) which was >97% pure by HPLC.

#### **Synthesis of (*R*)-5-amino-2-methylpentanoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub> hydrotrifluoroacetate<sup>2,4</sup> (20)**

First, compound **86** (248 mg, 1.07 mmol) was dissolved in a solution of 0.4 M HOBt/HBTU in DMF (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 Boc-(*R*)-5-amino-2-methylpentanoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-resin (437 mg). The pentapeptide resin was then stirred for 2 h at room temperature with 95:5 TFA:H<sub>2</sub>O (10 mL). After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **20** hydrotrifluoroacetate was collected as an off-white solid (101 mg) and purified by HPLC using the general peptide purification procedure to obtain **20** hydrotrifluoroacetate as a white solid (67 mg, 54% yield) which was >97% pure by HPLC.

### **Synthesis of D-Arg-Oic-Pro-D-Ala-Cha-NH<sub>2</sub> dihydrotrifluoroacetate (21)**

NB 4: 174-175, 177-179, 181-186

Compound **21** was synthesized according to the general solid phase peptide synthesis procedure, using Rink amide resin (344 mg, 0.59 mmol/g substitution) and commercially available Fmoc-protected amino acids. Once synthesis was complete, the dried resin (474 mg) was stirred for 4 hours at room temperature with a solution of 0.75 g phenol, 0.25 mL triisopropylsilane, 0.5 mL thioanisole, 0.5 mL H<sub>2</sub>O and 10 mL TFA. After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **21** dihydrotrifluoroacetate was collected (138 mg) and a portion was purified by HPLC using the general peptide purification procedure to obtain **21** dihydrotrifluoroacetate (44.9 mg) which was >97% pure by HPLC.

### **Synthesis of D-Arg-Oic-Pro-D-Ala-Phe-NH<sub>2</sub> dihydrotrifluoroacetate (22)**

AF NB: 9-12, BG NB 5: 118

Compound **22** was synthesized according to the general solid phase peptide synthesis procedure, using Rink amide resin (339 mg, 0.59 mmol/g substitution) and commercially available Fmoc-protected amino acids. Once synthesis was complete, the dried resin (527 mg) was stirred for 2 hours at room temperature with 95:5 TFA:H<sub>2</sub>O (10 mL). After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **22** dihydrotrifluoroacetate was collected and a portion was purified by HPLC using the general peptide purification procedure to obtain **22** dihydrotrifluoroacetate (26.1 mg) which was >97% pure by HPLC. (Compound made by Athena Flecha.)

### **Synthesis of D-Arg-Oic-Pro-D-Ala-Aic-NH<sub>2</sub> dihydrotrifluoroacetate (23)**

NB 4: 193-195, 212-215, 221-223

Compound **23** was synthesized according to the general solid phase peptide synthesis procedure, using Rink amide resin (347 mg, 0.59 mmol/g substitution) and commercially available Fmoc-protected amino acids. Once synthesis was complete, the dried resin (494 mg) was stirred for 5 hours at room temperature with a solution of 0.75 g

phenol, 0.25 mL triisopropylsilane, 0.5 mL thioanisole, 0.5 mL H<sub>2</sub>O and 10 mL TFA. After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **23** dihydrotrifluoroacetate was collected (132 mg) and a portion was purified by HPLC using the general peptide purification procedure to obtain **23** dihydrotrifluoroacetate (42.2 mg) which was >97% pure by HPLC.

#### **Synthesis of D-Arg-Oic-Pro-D-Ala-Idg-NH<sub>2</sub> dihydrotrifluoroacetate (24)**

NB 4: 176, 177-179, 181-186

Compound **24** was synthesized according to the general solid phase peptide synthesis procedure, using Rink amide resin (340 mg, 0.59 mmol/g substitution) and commercially available Fmoc-protected amino acids. Once synthesis was complete, the dried resin (465 mg) was stirred for 4 hours at room temperature with a solution of 0.75 g phenol, 0.25 mL triisopropylsilane, 0.5 mL thioanisole, 0.5 mL H<sub>2</sub>O and 10 mL TFA. After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **24** dihydrotrifluoroacetate was collected (145 mg) and a portion was purified by HPLC using the general peptide purification procedure to obtain **24** dihydrotrifluoroacetate (34.6 mg) which was >97% pure by HPLC.

#### **Synthesis of D-Arg-Oic-Pro-D-Ala-Sty-NH<sub>2</sub> dihydrotrifluoroacetate (25)**

NB 4: 174-175, 177-179, 181-186

Compound **25** was synthesized according to the general solid phase peptide synthesis procedure, using Rink amide resin (345 mg, 0.59 mmol/g substitution) and commercially available Fmoc-protected amino acids. Once synthesis was complete, the dried resin (454 mg) was stirred for 4 hours at room temperature with a solution of 0.75 g phenol, 0.25 mL triisopropylsilane, 0.5 mL thioanisole, 0.5 mL H<sub>2</sub>O and 10 mL TFA. After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **25** dihydrotrifluoroacetate was collected (152 mg) and a portion was purified by HPLC using the general peptide

purification procedure to obtain **25** dihydrotrifluoroacetate (32 mg) which was >97% pure by HPLC.

#### **Synthesis of D-Arg-Oic-Pro-D-Ala-Bip-NH<sub>2</sub> dihydrotrifluoroacetate (26)**

NB 4: 176, 177-179, 181-186

Compound **26** was synthesized according to the general solid phase peptide synthesis procedure, using Rink amide resin (344 mg, 0.59 mmol/g substitution) and commercially available Fmoc-protected amino acids. Once synthesis was complete, the dried resin (488 mg) was stirred for 4 hours at room temperature with a solution of 0.75 g phenol, 0.25 mL triisopropylsilane, 0.5 mL thioanisole, 0.5 mL H<sub>2</sub>O and 10 mL TFA. After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **26** dihydrotrifluoroacetate was collected (155 mg) and a portion was purified by HPLC using the general peptide purification procedure to obtain **26** dihydrotrifluoroacetate (41 mg) which was >97% pure by HPLC.

#### **Synthesis of D-Arg-Oic-Pro-D-Ala-Phe(F)<sub>5</sub>-NH<sub>2</sub> dihydrotrifluoroacetate (27)**

NB 4: 270-273, 278-280

Compound **27** was synthesized according to the general solid phase peptide synthesis procedure, using Rink amide resin (350 mg, 0.59 mmol/g substitution) and commercially available Fmoc-protected amino acids. Once synthesis was complete, the dried resin (524 mg) was stirred for 2 hours at room temperature with 95:5 TFA:H<sub>2</sub>O (10 mL). After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **27** dihydrotrifluoroacetate was collected (146 mg) and a portion was purified by HPLC using the general peptide purification procedure to obtain **27** dihydrotrifluoroacetate (37 mg) which was >97% pure by HPLC.

### **Synthesis of D-Arg-Oic-Pro-D-Ala-Phe(*p*-CF<sub>3</sub>)-NH<sub>2</sub> dihydrotrifluoroacetate (28)**

NB 4: 270-273, 278-280

Compound **28** was synthesized according to the general solid phase peptide synthesis procedure, using Rink amide resin (350 mg, 0.59 mmol/g substitution) and commercially available Fmoc-protected amino acids. Once synthesis was complete, the dried resin (488 mg) was stirred for 2 hours at room temperature with 95:5 TFA:H<sub>2</sub>O (10 mL). After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **28** dihydrotrifluoroacetate was collected (132 mg) and a portion was purified by HPLC using the general peptide purification procedure to obtain **28** dihydrotrifluoroacetate (45 mg) which was >97% pure by HPLC.

### **Synthesis of D-Arg-Oic-Pro-D-Ala-Tyr(Me)-NH<sub>2</sub> dihydrotrifluoroacetate (29)**

NB 6: 59-60, 64

Compound **29** was synthesized according to the general solid phase peptide synthesis procedure, using Rink amide resin (386 mg, 0.6 mmol/g substitution) and commercially available Fmoc-protected amino acids. Once synthesis was complete, the dried resin (571 mg) was stirred for 2 hours at room temperature with 95:5 TFA:H<sub>2</sub>O (10 mL). After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **29** dihydrotrifluoroacetate was collected (154 mg) and a portion was purified by HPLC using the general peptide purification procedure to obtain **29** dihydrotrifluoroacetate (84 mg) which was >97% pure by HPLC.

### **Synthesis of D-Arg-Oic-Pro-D-Ala-Tyr(Bzl)-NH<sub>2</sub> dihydrotrifluoroacetate and D-Arg-Oic-Pro-D-Ala-D-Tyr(Bzl)-NH<sub>2</sub> dihydrotrifluoroacetate (30 and 37)**

NB 6: 61-62, 65-66, 79

Compounds **30** and **37** were synthesized according to the general solid phase peptide synthesis procedure, using Rink amide resin (367 mg, 0.6 mmol/g substitution) and commercially available Fmoc-protected amino acids. The Fmoc-Tyr(Bzl)-OH had racimized into both D and L enantiomers. Once synthesis was complete, the dried resin

(545 mg) was stirred for 2 hours at room temperature with 95:5 TFA:H<sub>2</sub>O (10 mL). After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **30** dihydrotrifluoroacetate and **37** dihydrotrifluoroacetate were collected (142 mg total of both compounds) and purified by HPLC using the general peptide purification procedure. The early eluting peak was assigned as **37**, due to its lower prevalence and comparison of HPLC retention times to other D amino acids in this position. Compounds **30** dihydrotrifluoroacetate (43 mg) and **37** dihydrotrifluoroacetate (28 mg) were each >97% pure by HPLC.

### **Synthesis of D-Arg-Oic-Pro-D-Ala-Phe(*p*-carboxamide)-NH<sub>2</sub> dihydrotrifluoroacetate (31)**

NB 6: 108-109, 113, 119

Compound **31** was synthesized according to the general solid phase peptide synthesis procedure, using **101** (approximately 0.2 mmol) and commercially available Fmoc-protected amino acids. Once synthesis was complete, the dried resin (415 mg) was stirred for 4 hours at room temperature with 95:5 TFA:H<sub>2</sub>O (10 mL). After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **31** dihydrotrifluoroacetate was collected (56.8 mg) and purified by HPLC using the general peptide purification procedure to obtain **31** dihydrotrifluoroacetate (4.5 mg, 2.1% yield for 2 steps) which was >97% pure by HPLC.

### **Synthesis of D-Arg-Oic-Pro-D-Ala-Phe(*p*-*N*-phenylacetamide)-NH<sub>2</sub> dihydrotrifluoroacetate (32)**

NB 6: 33-34, 35, 37-38

Compound **32** was synthesized according to the general solid phase peptide synthesis procedure, using **106** (approximately 0.2 mmol) and commercially available Fmoc-protected amino acids. Once synthesis was complete, the dried resin (452 mg) was stirred for 2.25 hours at room temperature with 95:5 TFA:H<sub>2</sub>O (10 mL). After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **32** dihydrotrifluoroacetate was collected as an off-white solid (93 mg) and purified by HPLC using the general peptide purification procedure to

obtain **32** hydrotrifluoroacetate (7.7 mg, 3.6% yield for 2 steps) which was >97% pure by HPLC.

**Synthesis of D-Arg-Oic-Pro-D-Ala-Phe(*p*-NHCOCH<sub>2</sub>NH<sub>2</sub>)-NH<sub>2</sub> trihydrotrifluoroacetate (**33**)**

NB 6: 210-211, 212-213, 214, 229

To a stirred solution of **113** (17 mg, 0.02 mmol), HATU (39 mg, 0.09 mmol) and HOAt (13 mg, 0.09 mmol) in DMF (5 mL) was added DIEA (32  $\mu$ L, 0.18 mmol) followed by **108** (11 mg, 0.02 mmol), and the solution stirred at room temperature for 4.5 days. The solution was concentrated *in vacuo* to give a yellow solid, which was dissolved in ethyl acetate (25 mL) and washed with 0.5 M HCl (3  $\times$  20 mL), saturated NaHCO<sub>3</sub> (3  $\times$  20 mL) and brine (25 mL). After drying over MgSO<sub>4</sub>, the solution was concentrated *in vacuo* to yield a colorless residue. This residue was dissolved in piperidine (15 mL) and DMF (2 mL), and the solution stirred at room temperature for 2 hours before concentrating *in vacuo* to give a yellow residue. This residue was then dissolved in 95:5 TFA:H<sub>2</sub>O, stirred at room temperature for 2.25 hours, concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After two hours of refrigeration, **33** trihydrotrifluoroacetate was collected (26 mg) and purified by HPLC using the general peptide purification procedure to obtain **33** trihydrotrifluoroacetate (2 mg, 11% yield) which was >97% pure by HPLC.

**Synthesis of D-Arg-Oic-Pro-D-Ala-Phe(*p*-guanidino)-NH<sub>2</sub> dihydrotrifluoroacetate (**34**)**

NB 4: 98-99, 104-105

Compound **34** was synthesized according to the general solid phase peptide synthesis procedure, using Rink amide resin (368 mg, 0.6 mmol/g substitution) and commercially available Fmoc-protected amino acids. Once synthesis was complete, the dried resin (581 mg) was stirred for 2 hours at room temperature with 95:5 TFA:H<sub>2</sub>O (10 mL). After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **34** hydrotrifluoroacetate was collected (166 mg) and a portion was purified by HPLC using the general peptide



purification procedure to obtain **34** hydrotrifluoroacetate (35 mg) which was >97% pure by HPLC.

#### **Synthesis of D-Arg-Oic-Pro-D-Ala-D-Phe-NH<sub>2</sub> dihydrotrifluoroacetate (35)**

NB 5: 159-160, 164-165

Compound **35** was synthesized according to the general solid phase peptide synthesis procedure, using Rink amide resin (401 mg, 0.59 mmol/g substitution) and commercially available Fmoc-protected amino acids. Once synthesis was complete, the dried resin (539 mg) was stirred for 2 hours at room temperature with 95:5 TFA:H<sub>2</sub>O (10 mL). After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **35** hydrotrifluoroacetate was collected (158 mg) and a portion was purified by HPLC using the general peptide purification procedure to obtain **35** hydrotrifluoroacetate (57 mg) which was >97% pure by HPLC.

#### **Synthesis of D-Arg-Oic-Pro-D-Ala-D-Idg-NH<sub>2</sub> dihydrotrifluoroacetate (36)**

NB 4: 209-210, 212-215, 221-223

Compound **36** was synthesized according to the general solid phase peptide synthesis procedure, using Rink amide resin (248 mg, 0.59 mmol/g substitution) and commercially available Fmoc-protected amino acids. Once synthesis was complete, the dried resin (504 mg) was stirred for 2 hours at room temperature with 95:5 TFA:H<sub>2</sub>O (10 mL). After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **36** hydrotrifluoroacetate was collected (154 mg) and purified by HPLC using the general peptide purification procedure to obtain **36** hydrotrifluoroacetate (70.3 mg, 53.7% yield) which was >97% pure by HPLC.

#### **Synthesis of D-Arg-Oic-Phe(*p*-Me)-NH<sub>2</sub> dihydrotrifluoroacetate (38)**

NB 4: 155-156, 163-164, 165-167

Compound **38** was synthesized according to the general solid phase peptide synthesis procedure, using Rink amide resin (380 mg, 0.59 mmol/g substitution) and

commercially available Fmoc-protected amino acids. Once synthesis was complete, the dried resin (477 mg) was stirred for 4 hours at room temperature with a solution of 0.75 g phenol, 0.25 mL triisopropylsilane, 0.5 mL thioanisole, 0.5 mL H<sub>2</sub>O and 10 mL TFA. After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **38** hydrotrifluoroacetate was collected (118 mg) and a portion was purified by HPLC using the general peptide purification procedure to obtain **38** hydrotrifluoroacetate (45 mg) which was >97% pure by HPLC.

#### **Synthesis of D-Arg-Oic-Gly-Phe(*p*-Me)-NH<sub>2</sub> dihydrotrifluoroacetate (39)**

NB 4: 155-156, 163-164, 165-167

Compound **39** was synthesized according to the general solid phase peptide synthesis procedure, using Rink amide resin (331 mg, 0.59 mmol/g substitution) and commercially available Fmoc-protected amino acids. Once synthesis was complete, the dried resin (421 mg) was stirred for 4 hours at room temperature with a solution of 0.75 g phenol, 0.25 mL triisopropylsilane, 0.5 mL thioanisole, 0.5 mL H<sub>2</sub>O and 10 mL TFA. After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **39** hydrotrifluoroacetate was collected (109 mg) and a portion was purified by HPLC using the general peptide purification procedure to obtain **39** hydrotrifluoroacetate (56 mg) which was >97% pure by HPLC.

#### **Synthesis of D-Arg-Oic-Azt-D-Ala-Phe(*p*-Me)-NH<sub>2</sub> dihydrotrifluoroacetate (40)**

NB 81-82, 87-88, 102, 107

Compound **40** was synthesized according to the general solid phase peptide synthesis procedure, using Rink amide resin (368 mg, 0.59 mmol/g substitution) and commercially available Fmoc-protected amino acids. Once synthesis was complete, the dried resin (545 mg) was stirred for 2.25 hours at room temperature with 95:5 TFA:H<sub>2</sub>O (10 mL). After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **40** hydrotrifluoroacetate was collected (149 mg) and a portion was purified by HPLC using the general peptide purification procedure to obtain **40** hydrotrifluoroacetate (38 mg) which was >97% pure by HPLC.

### **Synthesis of D-Arg-Oic-Pro-D-Pro-Phe(*p*-Me)-NH<sub>2</sub> dihydrotrifluoroacetate (41)**

NB 4: 207-208, 212-215, 221-223

Compound **41** was synthesized according to the general solid phase peptide synthesis procedure, using Rink amide resin (345 mg, 0.59 mmol/g substitution) and commercially available Fmoc-protected amino acids. Once synthesis was complete, the dried resin (440 mg) was stirred for 2 hours at room temperature with 95:5 TFA:H<sub>2</sub>O (10 mL). After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **41** hydrotrifluoroacetate was collected (158 mg) and a portion was purified by HPLC using the general peptide purification procedure to obtain **41** hydrotrifluoroacetate (51 mg) which was >97% pure by HPLC.

### **Synthesis of D-Arg-Oic-D-Pro-Pro-Phe(*p*-Me)-NH<sub>2</sub> dihydrotrifluoroacetate (42)**

NB 4: 199-201, 212-215, 221-223

Compound **42** was synthesized according to the general solid phase peptide synthesis procedure, using Rink amide resin (344 mg, 0.59 mmol/g substitution) and commercially available Fmoc-protected amino acids. Once synthesis was complete, the dried resin (504 mg) was stirred for 4 hours at room temperature with 95:5 TFA:H<sub>2</sub>O (10 mL). After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **42** hydrotrifluoroacetate was collected (166 mg) and a portion was purified by HPLC using the general peptide purification procedure to obtain **42** hydrotrifluoroacetate (31 mg) which was >97% pure by HPLC.

### **Synthesis of D-Arg-Oic-D-Pro-Pro-Bip-NH<sub>2</sub> dihydrotrifluoroacetate (43)**

NB 4: 230-232, 236-237

Compound **43** was synthesized according to the general solid phase peptide synthesis procedure, using Rink amide resin (365 mg, 0.59 mmol/g substitution) and commercially available Fmoc-protected amino acids. Once synthesis was complete, the dried resin (550 mg) was stirred for 2 hours at room temperature with 95:5 TFA:H<sub>2</sub>O (10 mL). After filtration, the solution was concentrated *in vacuo*, followed by precipitation

with cold diethyl ether. After refrigeration overnight, **43** hydrotrifluoroacetate was collected (142 mg) and a portion was purified by HPLC using the general peptide purification procedure to obtain **43** hydrotrifluoroacetate (54 mg) which was >97% pure by HPLC.

#### **Synthesis of D-Arg-Oic-BZA-Phe(*p*-Me)-NH<sub>2</sub> dihydrotrifluoroacetate (**44** and **45**)**

NB 4: 68-70, 75-76, 80-82

Compounds **44** and **45** were synthesized according to the general solid phase peptide synthesis procedure, using Rink amide resin (347 mg, 0.59 mmol/g substitution) and commercially available Fmoc-protected amino acids. Note that Fmoc-BZA-OH was purchased as a racemic mixture, giving two peptides (**44** and **45**) as final products. Once synthesis was complete, the dried resin (402.4 mg) was stirred for 4.5 hours at room temperature with a solution of 0.75 g phenol, 0.25 mL triisopropylsilane, 0.5 mL thioanisole and 0.5 mL H<sub>2</sub>O in 10 mL TFA. After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **44** dihydrotrifluoroacetate and **45** dihydrotrifluoroacetate were collected (132 mg of both compounds) and purified by HPLC using the general peptide purification procedure. The early eluting peak was assigned as compound **44** and the later eluting peak was assigned as compound **45**. Compounds **44** dihydrotrifluoroacetate (40 mg) and **45** dihydrotrifluoroacetate (30 mg) were both >97% pure by HPLC.

#### **Synthesis of D-Arg-Oic-CPL-Phe(*p*-Me)-NH<sub>2</sub> dihydrotrifluoroacetate (**46**)**

NB 2: 212-214, 219-221, 226, 229

Compound **46** was synthesized according to the general solid phase peptide synthesis procedure, using Rink amide resin (286 mg, 0.7 mmol/g substitution) and commercially available Fmoc-protected amino acids. Once synthesis was complete, the dried resin (227.3 mg) was stirred for 3 hours at room temperature with a solution of 0.75 g phenol, 0.25 mL triisopropylsilane, 0.5 mL thioanisole and 0.5 mL H<sub>2</sub>O in 10 mL TFA. After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **46** dihydrotrifluoroacetate was collected (59 mg) and purified by HPLC using the general peptide purification procedure

to obtain **46** dihydrotrifluoroacetate (22 mg, 12.2% yield) which was >97% pure by HPLC.

### Synthesis of D-Arg-Oic-CGPM dihydrotrifluoroacetate (**47** and **48**)

NB 5: 209-210, 211, 218-219

The orange solid containing **111** (139 mg, 0.2 mmol) was dissolved in a pre-made solution of 0.4 M HOBt/HBTU in DMF (0.6 mL, 0.24 mmol), and DIEA (0.21 mL, 1.2 mmol) and DMF (2 mL) was added. To this solution was added a solution of CGPM (4-amino-6-phenylmethyl-1,2,3,4-tetrahydroquinoline, mixture of *R* and *S* enantiomers, synthesized by Yafei Jin in the Vahlteich Medicinal Chemistry Core, 48 mg, 0.19 mmol) in DMF (3 mL). The solution stirred at room temperature for 3 hours, and was concentrated *in vacuo* to give a dark brown material. This material was dissolved in ethyl acetate (50 mL) and washed with 1 M HCl (3 × 25 mL), saturated NaHCO<sub>3</sub> (3 × 25 mL) and brine (1 × 25 mL), dried over MgSO<sub>4</sub> and concentrated *in vacuo* to give an orange residue (121 mg). To this residue was added 95:5 TFA:H<sub>2</sub>O (10 mL), and the solution stirred at room temperature for 2 hours. The solution was concentrated *in vacuo*, and compounds **47** dihydrotrifluoroacetate and **48** dihydrotrifluoroacetate were precipitated with cold diethyl ether. After refrigeration overnight, **47** dihydrotrifluoroacetate and **48** dihydrotrifluoroacetate were collected (122 mg combined mass) and purified by HPLC using the general peptide purification procedure to obtain **47** dihydrotrifluoroacetate (12 mg) and **48** dihydrotrifluoroacetate (33 mg), which were each >97% pure by HPLC. Compound **47** dihydrotrifluoroacetate was assigned as the peak to elude earlier on HPLC, while **48** dihydrotrifluoroacetate was assigned as the later eluting peak, since the exact stereochemistry of either compound is unknown.

### Synthesis of D-Arg-Oic-Pro-D-Ala-Phe-NHMe dihydrotrifluoroacetate (**49**)

NB 5: 275-278, 290, 291

Compound **49** was synthesized using resin to produce a C-terminal methyl amide ({3[(Methyl-Fmoc-amino)-methyl]-indol-1-yl}-acetyl AM resin, 305 mg, 0.67 mmol/g substitution) and commercially available Fmoc-protected amino acids. First, the resin was swelled for 5 minutes using 3 mL NMP in a vial. To this vial was added a solution

of 20% piperidine in DMF (10 mL). After shaking at room temperature for 1 hour, the resin was rinsed with NMP and transferred to a new vial. Then, Fmoc-Phe-OH (334 mg, 0.86 mmol) was dissolved in a solution of HATU (154 mg, 0.4 mmol), HOAt (58 mg, 0.4 mmol), and DIEA (0.14 mL, 0.8 mmol) in NMP (12 mL), and this solution was added to the resin vial. After shaking at room temperature for 11 days, the resin was washed with NMP followed by dichloromethane and dried (293 mg resin). A portion of this resin (136 mg) was transferred to a reaction vessel for the CS Bio peptide synthesizer, and the remainder of the peptide was synthesized according to the general peptide synthesis procedure. Once synthesis was complete, the dried resin (166 mg) was stirred for 2.25 hours at room temperature with 95:5 TFA:H<sub>2</sub>O (10 mL). After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **49** dihydrotrifluoroacetate was collected (56 mg) and purified by HPLC using the general peptide purification procedure to obtain **49** dihydrotrifluoroacetate (15 mg, 18% yield) which was >97% pure by HPLC.

#### **Synthesis of D-Arg-Oic-Pro-D-Ala-Phe-NHEt dihydrotrifluoroacetate (50)**

Compound **50** was synthesized using resin to produce a C-terminal ethyl amide ({3[(Ethyl-Fmoc-amino)methyl]-indol-1-yl}-acetyl AM resin, 305 mg, 0.67 mmol/g substitution) and commercially available Fmoc-protected amino acids. First, the resin was swelled for 5 minutes using 3 mL NMP in a vial. To this vial was added a solution of 20% piperidine in DMF (10 mL). After shaking at room temperature for 1 hour, the resin was rinsed with NMP and transferred to a new vial. Then, Fmoc-Phe-OH (334 mg, 0.86 mmol) was dissolved in a solution of HATU (154 mg, 0.4 mmol), HOAt (58 mg, 0.4 mmol), and DIEA (0.14 mL, 0.8 mmol) in NMP (12 mL), and this solution was added to the resin vial. After shaking at room temperature for 11 days, the resin was washed with NMP followed by dichloromethane and dried (293 mg resin). A portion of this resin (136 mg) was transferred to a reaction vessel for the CS Bio peptide synthesizer, and the remainder of the peptide was synthesized according to the general peptide synthesis procedure. Once synthesis was complete, the dried resin (166 mg) was stirred for 2.25 hours at room temperature with 95:5 TFA:H<sub>2</sub>O (10 mL). After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After

refrigeration overnight, **50** dihydrotrifluoroacetate was collected (56 mg) and purified by HPLC using the general peptide purification procedure to obtain **50** dihydrotrifluoroacetate (15 mg) which was >97% pure by HPLC.

#### **Synthesis of D-Arg-Oic-Pro-D-Ala-Phe-NHPr dihydrotrifluoroacetate (51)**

NB 5: 161-163, 168, 171-172

First, **112** (109 mg) was dissolved in DMF (2 mL), and to this solution was added HATU (98 mg, 0.26 mmol) and DIEA (0.30 mL, 1.72 mmol). Commercially available propylamine hydrochloride (19 mg, 0.20 mmol) was dissolved in DMF (2 mL) and transferred to the **113** solution, which stirred at room temperature for 9 days, and was concentrated *in vacuo* to give a yellow oil. The oil was dissolved in ethyl acetate (20 mL), washed with 1 M HCl (3 × 20 mL), saturated NaHCO<sub>3</sub> (3 × 20 mL), and brine (1 × 25 mL), dried over MgSO<sub>4</sub>, and concentrated *in vacuo* to yield a yellow residue. This residue was dissolved in 95:5 TFA:H<sub>2</sub>O (10 mL) and the solution stirred at room temperature for 2 hours. The solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **51** dihydrotrifluoroacetate was collected (60 mg) and purified by HPLC using the general peptide purification procedure to obtain **51** dihydrotrifluoroacetate (3.6 mg, 4.4% yield for 2 steps) which was >97% pure by HPLC.

#### **Synthesis of D-Arg-Oic-Pro-D-Ala-Phe-NHiPr dihydrotrifluoroacetate (52)**

NB 6: 95-97, 103

To a stirred solution of **113** (62 mg, 0.07 mmol), HATU (57 mg, 0.15 mmol) and HOAt (21 mg, 0.15 mmol) in DMF (5 mL) was added DIEA (130 μL, 0.74 mmol) followed by **114** (50 mg, 0.16 mmol), and the solution stirred at room temperature for 19 hours. The solution was concentrated *in vacuo* to give a yellow oil, which was dissolved in ethyl acetate (30 mL) and washed with 1 M HCl (3 × 25 mL), saturated NaHCO<sub>3</sub> (3 × 25 mL) and brine (25 mL). After drying over MgSO<sub>4</sub>, the solution was concentrated *in vacuo* to yield a yellow solid. This solid was dissolved in 95:5 TFA:H<sub>2</sub>O, stirred at room temperature for 2.25 hours, concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **52** dihydrotrifluoroacetate was collected (28

mg) and purified by HPLC using the general peptide purification procedure to obtain **52** dihydrotrifluoroacetate (15 mg, 24% yield for 2 steps) which was >97% pure by HPLC.

#### **Synthesis of D-Arg-Oic-Pro-D-Ala-Phe-NHC<sub>6</sub>H<sub>11</sub> dihydrotrifluoroacetate (53)**

NB 6: 215-216, 221, 222, 228

To a stirred solution of **113** (93 mg, 0.11 mmol), HATU (86 mg, 0.23 mmol) and HOAt (33 mg, 0.24 mmol) in DMF (5 mL) was added DIEA (192  $\mu$ L, 1.10 mmol) followed by **115** (60 mg, 0.17 mmol), and the solution stirred at room temperature for 23 hours. The solution was concentrated *in vacuo* to give a yellow oil, which was dissolved in ethyl acetate (30 mL) and washed with 1 M HCl (3  $\times$  25 mL), saturated NaHCO<sub>3</sub> (3  $\times$  25 mL) and brine (25 mL). After drying over MgSO<sub>4</sub>, the solution was concentrated *in vacuo* to yield a yellow residue. This residue was dissolved in 95:5 TFA:H<sub>2</sub>O, stirred at room temperature for 2 hours, concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **53** dihydrotrifluoroacetate was collected (45 mg) and purified by HPLC using the general peptide purification procedure to obtain **53** dihydrotrifluoroacetate (4.8 mg, 4.6% yield for 2 steps) which was 96% pure by HPLC.

#### **Synthesis of D-Arg-Oic-Pro-D-Ala-Phe-NHPh dihydrotrifluoroacetate (54)**

NB 5: 26-28, 31-32, 33-35, 38-39

First, Fmoc-D-Arg(Pbf)-Oic-Pro-D-Ala-Phe-OH was synthesized according to the general solid phase peptide synthesis procedure, using Fmoc-Phe-Wang resin (310 mg, 0.65 mmol/g substitution) and commercially available Fmoc-protected amino acids. Once synthesis was complete, the dried resin (454 mg) was stirred for 2 hours at room temperature with 95:5 TFA:H<sub>2</sub>O (10 mL). After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, the tetrapeptide Fmoc-D-Arg-Oic-Pro-D-Ala-Phe-OH hydrotrifluoroacetate was collected (163 mg) and was >87% pure by HPLC. To a solution of this tetrapeptide (76 mg, 0.08 mmol) in DMF (5 mL), was added a premade solution of 0.4 M HOBt/HBTU in DMF (0.77 mL, 0.31 mmol) and DIEA (107  $\mu$ L, 0.61 mmol), followed by aniline (28  $\mu$ L, 0.31 mmol). The solution stirred at room temperature for 15 hours, and was then concentrated *in vacuo* to give a yellow oil. The



oil was dissolved in a premade solution of 20% piperidine in DMF (5 mL), and the solution stirred at room temperature for 1 hour. After concentrating *in vacuo*, the resulting yellow oil was dissolved in ethyl acetate (25 mL) and washed with saturated NaHCO<sub>3</sub> (3 × 25 mL). The pH of the combined aqueous fractions was adjusted to 14 using 4 M NaOH, and this was extracted with ethyl acetate (3 × 50 mL). The combined organic fractions were dried over MgSO<sub>4</sub> and concentrated *in vacuo* to give a yellow residue. This residue was purified by HPLC using the general peptide purification procedure to obtain **54** dihydrotrifluoroacetate (18 mg, 9.5% yield) which was >97% pure by HPLC.

#### **Synthesis of D-Arg-Oic-Pro-D-Ala-Phe-NhHBn dihydrotrifluoroacetate (55)**

NB 6: 53-55, 63

To a stirred solution of **113** (62 mg, 0.07 mmol), HATU (59 mg, 0.16 mmol) and HOAt (24 mg, 0.18 mmol) in DMF (5 mL) was added DIEA (130 μL, 0.74 mmol) followed by **116** (55 mg, 0.15 mmol), and the solution stirred at room temperature for 19 hours. The solution was concentrated *in vacuo* to give a dark yellow oil, which was dissolved in ethyl acetate (30 mL) and washed with 1 M HCl (3 × 25 mL), saturated NaHCO<sub>3</sub> (3 × 25 mL) and brine (30 mL). After drying over MgSO<sub>4</sub>, the solution was concentrated *in vacuo* to yield a yellow solid. This solid was dissolved in 95:5 TFA:H<sub>2</sub>O, stirred at room temperature for 2.25 hours, concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **55** dihydrotrifluoroacetate was collected (53 mg) and purified by HPLC using the general peptide purification procedure to obtain **55** dihydrotrifluoroacetate (18 mg) which was >97% pure by HPLC.

#### **Synthesis of 5-guanidinopentanoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NHC<sub>6</sub>H<sub>11</sub> hydrotrifluoroacetate (56)**

NB 6: 289, 294

Compound **119** was dissolved in a solution of 95:5 TFA:H<sub>2</sub>O (10 mL) and the solution stirred at room temperature for 2 hours. The solution was concentrated *in vacuo* and **56** hydrotrifluoroacetate was precipitated with cold diethyl ether. After refrigeration

for 5 hours, **56** hydrotrifluoroacetate was collected, dried (16 mg), and purified according to the general peptide purification procedure to give **56** hydrotrifluoroacetate (4.5 mg, 26% overall yield for all steps) which was >97% pure by HPLC.

### **Synthesis of *N,N'*-di-Boc-5-guanidinopentanoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub> hydrotrifluoroacetate (**57**)**

NB 2: 130-134

The synthesis of **57** was completed using a modification of a previously reported procedure.<sup>5</sup> First, triethylamine (137  $\mu$ L, 0.98 mmol) was added to a stirred solution of *N,N'*-di-Boc-*N'*-triflylguanidine (69 mg, 0.18 mmol) in 1,4-dioxane (2 mL). A solution of **9** (139 mg, 0.20 mmol) in 1,4-dioxane (3 mL) was then added and the resulting mixture was stirred at room temperature for 21 hours and then concentrated *in vacuo* to yield a yellow oil. The oil was dissolved in ethyl acetate (20 mL) and washed with 2 M potassium bisulfate (2  $\times$  5 mL), dried over MgSO<sub>4</sub>, and concentrated *in vacuo* to yield a white solid (133 mg) which contains **56** (71% pure by HPLC). The solid was carried on without further purification or characterization.

### **Synthesis of Gly-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub> hydrotrifluoroacetate (**58**)**

NB 2: 259, 264-265

Compound **58** was synthesized according to the general solid phase peptide synthesis procedure, using Rink amide resin (310 mg, 0.7 mmol/g substitution) and commercially available Fmoc-protected amino acids. Once synthesis was complete, the dried resin (391.9 mg) was stirred for 3.75 hours at room temperature with 95:5 TFA:H<sub>2</sub>O (10 mL). After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **58** hydrotrifluoroacetate was collected (102.7 mg) and purified by HPLC using the general peptide purification procedure to obtain **58** hydrotrifluoroacetate as a white solid (75 mg, 51.7% yield) which was >95% pure by HPLC.

### Synthesis of *N,N'*-di-Boc-Gly-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub> (**59**)

NB 2: 272-273

The synthesis of **59** was completed using a modification of a previously reported procedure.<sup>5</sup> First, triethylamine (79  $\mu$ L, 0.56 mmol) was added to a stirred solution of *N,N'*-di-Boc-*N'*-triflylguanidine (39.5 mg, 0.10 mmol) in 1,4-dioxane (2 mL). A solution of **58** (75 mg, 0.11 mmol) in 1,4-dioxane (5 mL) was then added and the resulting mixture was stirred at room temperature for 18 hours and then concentrated *in vacuo* to yield a yellow oil. The oil was dissolved in ethyl acetate (20 mL) and washed with 2 M potassium bisulfate (2  $\times$  5 mL) followed by saturated sodium bicarbonate (2  $\times$  5 mL), dried over MgSO<sub>4</sub>, and concentrated *in vacuo* to yield a white solid (71.2 mg) which contains **59** (95% pure by HPLC). The solid was carried on without further purification or characterization.

### Synthesis of Boc-3-aminopropionic acid (**60**)

NB 2: 286-287

To a stirred solution of 3-aminopropionic acid (181 mg, 2.0 mmol) in 2:1 1,4-dioxane:H<sub>2</sub>O (6 mL) at 0°C was added 1M 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 4 hours and slowly warmed to room temperature, and was then concentrated *in vacuo* to obtain a white solid. The solid was dissolved in H<sub>2</sub>O (6 mL) and ethyl acetate (15 mL) and acidified to pH 2.5 using 10% citric acid. The acidified aqueous layer was extracted with ethyl acetate (5  $\times$  15 mL), and the pooled organic fractions were washed with saturated NaCl (2  $\times$  40 mL) followed by H<sub>2</sub>O (2  $\times$  40 mL) and dried over MgSO<sub>4</sub>. The organic layer was concentrated *in vacuo* to give a white solid (240 mg, 63.4% yield) and was >95% pure by HPLC. <sup>1</sup>H NMR (300 MHz, DMSO):  $\delta$  12.19 (s, 1H, br), 6.80 (s, 1H, br), 3.11 (t, *J* = 6.93 Hz, 2H), 2.34 (t, *J* = 6.99 Hz, 2H), 1.37 (s, 9H)

**Synthesis of 3-aminopropionic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub>  
hydrotrifluoroacetate (61)**

NB 2: 292

Compound **61** was synthesized according to the general solid phase peptide synthesis procedure, using Rink amide resin (370 mg, 0.7 mmol/g substitution) and commercially available Fmoc-protected amino acids, in addition to **60**. Once synthesis was complete, the dried resin (470 mg) was stirred for 3.5 hours at room temperature with 95:5 TFA:H<sub>2</sub>O (10 mL). After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **61** hydrotrifluoroacetate was collected as an off-white solid (108 mg) and was 60% pure by HPLC. Compound **61** was carried on without further purification. ESI-MS calculated [M + H]<sup>+</sup>, 569.3, found, 569.3.

**Synthesis of *N,N'*-di-Boc-3-aminopropionic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub> (62)**

NB 2: 296-297

The synthesis of **62** was completed using a modification of a previously reported procedure.<sup>5</sup> First, triethylamine (117  $\mu$ L, 0.83 mmol) was added to a stirred solution of *N,N'*-di-Boc-*N'*-triflylguanidine (55.7 mg, 0.14 mmol) in 1,4-dioxane (2 mL). A solution of **61** (107.7 mg, 0.16 mmol) in 1,4-dioxane (5 mL) was then added and the resulting mixture was stirred at room temperature for 22.5 hours and then concentrated *in vacuo* to yield a yellow oil. The oil was dissolved in ethyl acetate (20 mL) and washed with 2 M potassium bisulfate (2  $\times$  6 mL) followed by saturated sodium bicarbonate (2  $\times$  6 mL), dried over MgSO<sub>4</sub>, and concentrated *in vacuo* to yield a colorless residue which contains **62**. The residue was carried on without further purification or characterization.

**Synthesis of 4-aminobutyric acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub>  
hydrotrifluoroacetate (63)**

NB 2: 260-261, 266-267

Compound **63** was synthesized according to the general solid phase peptide synthesis procedure, using Rink amide resin (310 mg, 0.7 mmol/g substitution) and commercially available Fmoc-protected amino acids, in addition to Boc-4-aminobutyric

acid made previously in the lab by Dr. Sobczyk-Kojiro. Once synthesis was complete, the dried resin (397 mg) was stirred for 3.5 hours at room temperature with 95:5 TFA:H<sub>2</sub>O (10 mL). After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **63** hydrotrifluoroacetate was collected (113.8 mg) and purified by HPLC using the general peptide purification procedure to obtain **63** hydrotrifluoroacetate (93.8 mg, 62.0% yield) which was >95% pure by HPLC.

#### **Synthesis of *N,N'*-di-Boc-3-aminopropionic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub> (**64**)**

NB 2: 275-276

The synthesis of **64** was completed using a modification of a previously reported procedure.<sup>5</sup> First, triethylamine (95  $\mu$ L, 0.68 mmol) was added to a stirred solution of *N,N'*-di-Boc-*N'*-triflylguanidine (47.7 mg, 0.12 mmol) in 1,4-dioxane (2 mL). A solution of **63** (93.8 mg, 0.14 mmol) in 1,4-dioxane (5 mL) was then added and the resulting mixture was stirred at room temperature for 19 hours and then concentrated *in vacuo* to yield a yellow oil. The oil was dissolved in ethyl acetate (20 mL) and washed with 2 M potassium bisulfate (2  $\times$  6 mL) followed by saturated sodium bicarbonate (2  $\times$  6 mL), dried over MgSO<sub>4</sub>, and concentrated *in vacuo* to yield a colorless oil which contains **64**. The oil was carried on without further purification or characterization.

#### **Synthesis of *N,N'*-di-Boc-6-guanidinohexanoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub> (**65**)**

NB 2: 304-305

The synthesis of **65** was completed using a modification of a previously reported procedure.<sup>5</sup> First, triethylamine (72  $\mu$ L, 0.51 mmol) was added to a stirred solution of *N,N'*-di-Boc-*N'*-triflylguanidine (35.9 mg, 0.09 mmol) in 1,4-dioxane (2 mL). A solution of **10** (73.9 mg, 0.10 mmol) in 1,4-dioxane (5 mL) was then added and the resulting mixture was stirred at room temperature for 19 hours and then concentrated *in vacuo* to yield a yellow oil. The oil was dissolved in ethyl acetate (20 mL) and washed with 2 M potassium bisulfate (2  $\times$  6 mL) followed by saturated sodium bicarbonate (2  $\times$  6 mL),

dried over MgSO<sub>4</sub>, and concentrated *in vacuo* to yield a colorless oil which contains **65**. The oil was carried on without further purification or characterization.

### Synthesis of *N,N'*-di-Boc-*p*-guanidinobenzoic acid (**66**)

NB 2: 145-152

The synthesis of **66** was completed using a modification of a previously reported procedure.<sup>5</sup> First, triethylamine (560  $\mu$ L, 4.00 mmol) was added to a stirred solution of *N,N'*-di-Boc-*N'*-triflylguanidine (431.6 mg, 1.10 mmol) in 1,4-dioxane (2 mL). A solution of *p*-aminobenzoic acid (164.0 mg, 1.20 mmol) in 1,4-dioxane (4 mL) was then added and the resulting mixture was stirred at room temperature for 8 days and then concentrated *in vacuo* to yield a yellow oil. The oil was dissolved in ethyl acetate (20 mL) and water (30 mL), and acidified to pH 2 with 10% citric acid. The acidified aqueous layer was extracted with ethyl acetate (4  $\times$  15 mL), and the combined organic fractions were washed with H<sub>2</sub>O acidified to pH 1.5 with HCl (3  $\times$  25 mL). The organic solution was concentrated to a volume of 50 mL, and 20 mL saturated sodium bicarbonate solution was added, prompting precipitation of **66** as an off-white solid (252.6 mg, 60.5% yield), which was >95% pure by HPLC. The solid was carried on without further purification or characterization.

### Synthesis of Boc-*cis*-4-amino-1-cyclohexanecarboxylic acid<sup>2</sup> (**67**)

NB 2: 161-163

To a stirred solution of the commercially available *cis*-4-amino-1-cyclohexanecarboxylic acid (286 mg, 2.0 mmol) in 2:1 1,4-dioxane:H<sub>2</sub>O (6 mL) at 0  $^{\circ}$ 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<sub>2</sub>O (4 mL) and ethyl acetate (7 mL) and acidified to pH 1.5 using 1 M HCl, prompting precipitation of **67**. Compound **67** was collected by filtration as a white solid (388 mg, 73% yield) and was >95% pure by HPLC. <sup>1</sup>H NMR (500 MHz, DMSO):  $\delta$  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).

**Synthesis of *cis*-4-amino-1-cyclohexanecarboxylic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub> hydrotrifluoroacetate<sup>2</sup> (68)**

NB 2: 170-171

Compound **68** was synthesized according to the general solid phase peptide synthesis procedure, using Rink amide resin (270 mg, 0.75 mmol/g substitution) and commercially available Fmoc-protected amino acids, in addition to **67**. Once synthesis was complete, the dried resin (375 mg) was stirred for 2 hours at room temperature with 95:5 TFA:H<sub>2</sub>O (10 mL). After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **68** hydrotrifluoroacetate was collected as an off-white solid (100.9 mg, 67.7% yield) which was >95% pure by HPLC. ESI-MS calculated [M+H]<sup>+</sup>, 623.4, found, 623.3.

**Synthesis of *N,N'*-di-Boc-*cis*-4-guanidino-1-cyclohexanecarboxylic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub><sup>2</sup> (69)**

NB 2: 175-176

The synthesis of **69** was completed using a modification of a previously reported procedure.<sup>5</sup> First, triethylamine (96 μL, 0.69 mmol) was added to a stirred solution of *N,N'*-di-Boc-*N'*-triflylguanidine (50.7 mg, 0.13 mmol) in 1,4-dioxane (2 mL). A solution of **68** (100.8 mg, 0.12 mmol) in 1,4-dioxane (5 mL) was then added and the resulting mixture was stirred at room temperature for 24.5 hours and then concentrated *in vacuo* to yield a yellow oil. The oil was dissolved in ethyl acetate (20 mL) and washed with 2 M potassium bisulfate (2 × 5 mL) followed by saturated sodium bicarbonate (2 × 5 mL), dried over MgSO<sub>4</sub>, and concentrated *in vacuo* to yield a white solid which contains **69** (97.4 mg). The solid was 75% pure by HPLC and was carried on without further purification or characterization.

**Synthesis of *trans*-4-amino-1-cyclohexanecarboxylic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub> hydrotrifluoroacetate<sup>2</sup> (70)**

Compound **70** was synthesized according to the general solid phase peptide synthesis procedure, using Rink amide resin (310 mg, 0.70 mmol/g substitution) and commercially available Fmoc-protected amino acids, in addition to commercially

available Boc-*trans*-4-amino-1-cyclohexanecarboxylic acid. Once synthesis was complete, the dried resin (358 mg) was stirred for 2 hours at room temperature with 95:5 TFA:H<sub>2</sub>O (10 mL). After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **70** hydrotrifluoroacetate was collected as an off-white solid (93 mg, 58% yield) which was 67% pure by HPLC, and was carried on without further purification or characterization.

### **Synthesis of *N,N'*-di-Boc-*trans*-4-guanidino-1-cyclohexanecarboxylic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub> hydrotrifluoroacetate<sup>2</sup> (71)**

The synthesis of **71** was completed using a modification of a previously reported procedure.<sup>5</sup> First, triethylamine (88 μL, 0.63 mmol) was added to a stirred solution of *N,N'*-di-Boc-*N'*-triflylguanidine (50 mg, 0.13 mmol) in 1,4-dioxane (2 mL). A solution of **68** (100.8 mg, 0.12 mmol) in 1,4-dioxane (13 mL) was then added and the resulting mixture was stirred at room temperature for 24 hours and then concentrated *in vacuo* to yield a yellow-orange oil. The oil was dissolved in ethyl acetate (20 mL) and washed with 2 M potassium bisulfate (2 × 5 mL) followed by saturated sodium bicarbonate (2 × 5 mL), dried over MgSO<sub>4</sub>, and concentrated *in vacuo* to yield an oily residue which contains **71**. The residue was carried on without further purification or characterization.

### **Synthesis of Boc-5-aminopentanoic acid (72)**

NB 2: 290-291

To a stirred solution of 5-aminopentanoic acid (1.18 g, 10.0 mmol) in 2:1 1,4-dioxane:H<sub>2</sub>O (30 mL) at 0°C was added 1M NaOH (10 mL), followed by di-*tert*-butyldicarbonate (2.41 g, 11.0 mmol) dissolved in a minimal amount of 1,4-dioxane. The solution was stirred for 4 hours and slowly warmed to room temperature, and was then concentrated *in vacuo* to obtain a white solid. The solid was dissolved in H<sub>2</sub>O (20 mL) and ethyl acetate (25 mL) and acidified to pH 2.5 using 1 M HCl. The acidified aqueous layer was extracted with ethyl acetate (5 × 25 mL), and the pooled organic fractions were washed with saturated NaCl (2 × 50 mL) followed by H<sub>2</sub>O (2 × 50 mL) and dried over MgSO<sub>4</sub>. The organic layer was concentrated *in vacuo* to give a white solid (1.74 g, 80.0% yield) and was >95% pure by HPLC. <sup>1</sup>H NMR (500 MHz, DMSO): δ 12.00 (s,



1H, br), 6.81 (s, 1H, br), 2.90 (q,  $J = 6.60$ , 2H), 2.20 (t,  $J = 7.20$  Hz, 2H), 1.47 (pentet,  $J = 7.75$  Hz, 2H), 1.38 (s, 9H), 1.35 (pentet,  $J = 7.75$  Hz, 2H).

### Synthesis of Boc-6-aminohexanoic acid (73)

NB 2: 288-289

To a stirred solution of 6-aminohexanoic acid (1.31 g, 10.0 mmol) in 2:1 1,4-dioxane:H<sub>2</sub>O (30 mL) at 0°C was added 1M NaOH (15 mL), followed by di-*tert*-butyldicarbonate (2.46 g, 11.0 mmol) dissolved in a minimal amount of 1,4-dioxane. The solution was stirred for 4 hours and slowly warmed to room temperature, and was then concentrated *in vacuo* to obtain a white solid. The solid was dissolved in H<sub>2</sub>O (20 mL) and ethyl acetate (25 mL) and acidified to pH 3 using 10% citric acid. The acidified aqueous layer was extracted with ethyl acetate (5 × 25 mL), and the pooled organic fractions were washed with saturated NaCl (2 × 50 mL) followed by H<sub>2</sub>O (2 × 50 mL) and dried over MgSO<sub>4</sub>. The organic layer was concentrated *in vacuo* to give a white solid (2.05 g, 88.6% yield) and was >95% pure by HPLC. <sup>1</sup>H NMR (300 MHz, DMSO): δ 11.98 (s, 1H, br), 6.78 (s, 1H, br), 2.87 (t,  $J = 5.91$  Hz, 2H), 2.18 (t,  $J = 7.32$  Hz, 2H), 1.47 (pentet,  $J = 7.56$  Hz, 2H), 1.37 (s, 9H), 1.32 (pentet,  $J = 6.45$  Hz, 2H), 1.20 (pentet, 2H,  $J = 6.45$  Hz).

### Synthesis of 4-hydroxyamidinobenzoic acid<sup>2,3</sup> (74)

To a suspension of commercially available 4-cyanobenzoic acid (1 g, 6.8 mmol) in ethanol (100 mL) and H<sub>2</sub>O (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 dichloromethane:methanol ( $R_f = 0.35$ ), to yield **74** as a white solid (616 mg, 50% yield). <sup>1</sup>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).

### Synthesis of 4-hydroxyamidinobenzoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub> hydrotrifluoroacetate<sup>2</sup> (75)

First, compound **74** (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 (1 mL) was added. 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 4-hydroxyamidinobenzoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-resin (417 mg). This pentapeptide resin was then stirred for 2 h at room temperature with 95:5 TFA:H<sub>2</sub>O (10 mL). After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **75** hydrotrifluoroacetate was collected as an off-white solid (105 mg) and purified by HPLC using the general peptide purification procedure to obtain **75** hydrotrifluoroacetate as a white solid (15 mg, 6.2% yield) which was >80% pure by HPLC. ESI-MS calculated [M+H]<sup>+</sup>, 660.4, found, 660.3.

### Synthesis of 4-hydroxyamidinophenylacetic acid<sup>2,3</sup> (76)

To a suspension of commercially available 4-cyanophenylacetic acid (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 dichloromethane:methanol ( $R_f = 0.27$ ), to yield **76** as a white solid (286 mg, 27% yield). <sup>1</sup>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).

### Synthesis of 4-hydroxyamidinophenylacetic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub> hydrotrifluoroacetate<sup>2</sup> (**77**)

First, compound **76** (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 (1 mL) was added. 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 4-hydroxyamidinophenylacetic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-resin (443 mg). The pentapeptide resin was then stirred for 2 h at room temperature with 95:5 TFA:H<sub>2</sub>O (10 mL). After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **77** hydrotrifluoroacetate was collected as an off-white solid (100 mg) and purified by HPLC using the general peptide purification procedure to obtain **77** hydrotrifluoroacetate as a white solid (14 mg, 5.1% yield) which was >70% pure by HPLC. ESI- MS calculated [M+H]<sup>+</sup>, 674.4, found, 674.5.

### Synthesis of 3-hydroxyamidinobenzoic acid<sup>2,3</sup> (**78**)

To a suspension of the commercially available 3-cyanobenzoic acid (1 g, 6.8 mmol) in ethanol (100 mL) and H<sub>2</sub>O (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 dichloromethane:methanol (*R<sub>f</sub>* = 0.33), to yield **78** as a white solid (566 mg, 46% yield), which was >95% pure by HPLC. <sup>1</sup>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*<sub>1</sub> = 7.7 Hz, *J*<sub>2</sub> = 0.5 Hz, 1H), 5.89 (s, 1H)

### Synthesis of 3-amidinobenzoic acid hydrochloride<sup>2</sup> (79)

In a reaction vessel for a Parr shaker, compound **78** (566 mg, 3.1 mmol) was suspended in H<sub>2</sub>O (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<sub>2</sub> 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 **79** hydrochloride as a white solid (518 mg, 82% yield). <sup>1</sup>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*<sub>1</sub> = 7.5 Hz, *J*<sub>2</sub> = 0.5 Hz, 1H).

### Synthesis of 3-hydroxyamidinophenylacetic acid<sup>2,3</sup> (80)

To a solution of commercially available 3-cyanophenylacetic acid (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 dichloromethane:methanol (*R*<sub>f</sub> = 0.27), to yield **80** as a white solid (608 mg, 50% yield). <sup>1</sup>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*<sub>1</sub> = 7.6 Hz, *J*<sub>2</sub> = 0.5 Hz, 1H), 7.25 (d, *J* = 6.8 Hz, 1H), 5.77 (s, 2H), 3.54 (s, 2H).

### Synthesis of 3-amidinophenylacetic acid hydrochloride<sup>2</sup> (81)

In a reaction vessel for a Parr shaker, compound 17b (608 mg, 3.1 mmol) was suspended in H<sub>2</sub>O (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<sub>2</sub> 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 **81** hydrochloride as a white solid (604 mg, 90% yield). <sup>1</sup>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*<sub>1</sub> = 7.9 Hz, *J*<sub>2</sub> = 0.5 Hz, 1H), 3.63 (s, 2H). ESI-MS calculated [M+H]<sup>+</sup>, 179.1, found, 179.2.

### Synthesis of (*S*)-4-benzyl-3-propionyloxazolidin-2-one<sup>2,4</sup> (**82**)

Commercially available (*S*)-4-benzoyloxazolidin-2-one (800 mg, 4.5 mmol) was dissolved in anhydrous THF (30 mL) in dry glassware under N<sub>2</sub>, 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<sub>4</sub>Cl (10 mL) and H<sub>2</sub>O (30 mL). The final aqueous layer was extracted with ethyl acetate (3 × 40 mL) and the combined organic extracts were dried over MgSO<sub>4</sub> and concentrated *in vacuo* to yield **82** as an oil (1.0 g, 96% yield) which was >95% pure by HPLC. <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 7.33 (dd, *J*<sub>1</sub> = 7.4 Hz, *J*<sub>2</sub> = 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*<sub>1</sub> = 13.3 Hz, *J*<sub>2</sub> = 3.2 Hz, 1H), 2.96 (m, 2H), 2.77 (dd, *J*<sub>1</sub> = 13.3 Hz, *J*<sub>2</sub> = 3.7 Hz, 1H), 1.21 (t, *J* = 7.4 Hz, 3H).

### Synthesis of (*R*)-5-((*S*)-4-benzyl-2-oxooxazolidin-3-yl)-4-methyl-5-oxopentanenitrile<sup>2,4</sup> (**83**)

TiCl<sub>3</sub>(*OiPr*) was first prepared using dry glassware under N<sub>2</sub>. A solution of 1 M TiCl<sub>4</sub> in dichloromethane (3.4 mmol, 3.4 mL) was cooled to 0 °C, and Ti(*OiPr*)<sub>4</sub> (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 **82** (1.0 g, 4.3 mmol) was dissolved in anhydrous dichloromethane (14 mL) in dry glassware under N<sub>2</sub>, and DIEA (0.79 mL) was added dropwise over 5 min, with stirring, at room temperature and then cooled to 0 °C. The TiCl<sub>3</sub>(*OiPr*) solution was added to the **82** solution dropwise over 25 min. The TiCl<sub>3</sub>(*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<sub>4</sub>Cl (25 mL) and H<sub>2</sub>O (15 mL), and the aqueous layer was extracted with diethyl ether (3 × 40 mL). The organic extracts were combined and washed with saturated NaHCO<sub>3</sub> (55 mL) followed by brine (55 mL), dried over MgSO<sub>4</sub> 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 **83** as a very slightly yellow oil (890 mg, 72% yield).  $^1\text{H NMR}$  (500 MHz,  $\text{CDCl}_3$ ):  $\delta$  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).

#### Synthesis of (*R*)-4-cyano-2-methylbutanoic acid<sup>2,4</sup> (**84**)

To a stirred solution of **83** (729 mg, 2.55 mmol) in THF (18 mL) at 0 °C were added 1 M LiOH solution (5.95 mL) and  $\text{H}_2\text{O}$  (50  $\mu\text{L}$ ). The solution slowly warmed to room temperature over 1 h. The solution was diluted with  $\text{H}_2\text{O}$  (20 mL) and extracted with ethyl acetate (3  $\times$  30 mL). The aqueous layer was acidified to pH 1.5 with 1 M HCl, and extracted with ethyl acetate (3  $\times$  30 mL). These last organic fractions were combined, dried over  $\text{MgSO}_4$ , and concentrated *in vacuo* to yield **84** as a colorless oil (191 mg, 51% yield).  $^1\text{H NMR}$  (500 MHz, DMSO):  $\delta$  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).

#### Synthesis of (*R*)-5-amino-2-methylpentanoic acid hydrochloride<sup>2,4</sup> (**85**)

In a reaction vessel for the Parr shaker, **84** (191 mg, 1.5 mmol) was dissolved in 3:7  $\text{H}_2\text{O}$ :methanol (40 mL) and degassed. A catalytic amount of  $\text{PtO}_2$  was added, and the solution was placed on the shaker at room temperature under 50 psi  $\text{H}_2$  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 **85** hydrochloride as an oil (237 mg, 94% yield).  $^1\text{H NMR}$  (500 MHz, DMSO):  $\delta$  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).

#### Synthesis of Boc-(*R*)-5-amino-2-methylpentanoic acid<sup>2,4</sup> (**86**)

To a stirred solution of **85** (237 mg, 1.4 mmol) in 2:1 1,4-dioxane: $\text{H}_2\text{O}$  (9 mL) at 0°C was added 1M NaOH (4 mL), followed by di-*tert*-butyldicarbonate (340 mg, 1.6 mmol) dissolved in a minimal amount of 1,4-dioxane. The solution was stirred for 4 hours and slowly warmed to room temperature, and was then concentrated *in vacuo* to

obtain a white solid. The solid was dissolved in H<sub>2</sub>O (10 mL) and ethyl acetate (20 mL) and acidified to pH 2 using 1 M HCl. The acidified aqueous layer was extracted with ethyl acetate (5 × 20 mL), and the pooled organic fractions were washed with saturated NaCl (2 × 40 mL) followed by H<sub>2</sub>O (2 × 40 mL) and dried over MgSO<sub>4</sub>. The organic layer was concentrated *in vacuo* to give **86** as a colorless oil (248 mg, 76% yield). <sup>1</sup>H NMR (300 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).

### Synthesis of *N,N'*-di-Boc-(*R*)-5-guanidino-2-methylpentanoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub><sup>2</sup> (**87**)

The synthesis of **87** was completed using a modification of a previously reported procedure.<sup>5</sup> 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 **20** (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<sub>4</sub> (2 × 10 mL) followed by saturated NaHCO<sub>3</sub> (2 × 10 mL), dried over MgSO<sub>4</sub> and concentrated *in vacuo* to give a white solid containing **87**. The solid was carried on without further purification or characterization.

### Synthesis of (*S*)-5-((*R*)-4-benzyl-2-oxooxazolidin-3-yl)-4-methyl-5-oxopentanenitrile<sup>2,4</sup> (**88**)

TiCl<sub>3</sub>(*OiPr*) was first prepared using dry glassware under N<sub>2</sub>. A solution of 1 M TiCl<sub>4</sub> in dichloromethane (3.6 mmol, 3.6 mL) was cooled to 0 °C, and Ti(*OiPr*)<sub>4</sub> (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 (*R*)-4-benzyl-3-propionyloxazolidin-2-one (1.05 g, 4.5 mmol) was dissolved in anhydrous dichloromethane (14 mL) in dry glassware under N<sub>2</sub>, and DIEA (0.75 mL) was added dropwise over 5 min, with stirring, at room temperature and then cooled to 0 °C. The TiCl<sub>3</sub>(*OiPr*) solution was added to the (*R*)-4-benzyl-3-propionyloxazolidin-2-one solution dropwise over 25 min. The TiCl<sub>3</sub>(*OiPr*) flask was

rinsed with anhydrous dichloromethane (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<sub>4</sub>Cl (25 mL) and H<sub>2</sub>O (15 mL), and the aqueous layer was extracted with diethyl ether (3 × 40 mL). The organic extracts were combined and washed with saturated NaHCO<sub>3</sub> (55 mL) followed by brine (55 mL), dried over MgSO<sub>4</sub> 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<sub>f</sub> = 0.27), to yield **88** as a very slightly yellow oil (919 mg, 71% yield). <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 7.35 (dd, *J*<sub>1</sub> = 7.5 Hz, *J*<sub>2</sub> = 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*<sub>1</sub> = 13.5 Hz, *J*<sub>2</sub> = 3.3 Hz, 1H), 2.78 (dd, *J*<sub>1</sub> = 13.4 Hz, *J*<sub>2</sub> = 3.6 Hz, 1H), 2.41 (dt, *J*<sub>1</sub> = 8.2 Hz, *J*<sub>2</sub> = 1.9 Hz, 2H), 2.18 (m, 1H), 1.81 (m, 1H), 1.23 (d, *J* = 7.1 Hz, 3H).

#### Synthesis of (*S*)-4-cyano-2-methylbutanoic acid<sup>2,4</sup> (**89**)

To a stirred solution of **88** (919 mg, 3.2 mmol) in THF (21 mL) at 0 °C were added 1 M LiOH solution (6.42 mL) and H<sub>2</sub>O (580 μL). The solution slowly warmed to room temperature over 15 h. The solution was diluted with H<sub>2</sub>O (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<sub>4</sub>, and concentrated *in vacuo* to yield **89** as a colorless oil (233 mg, 57% yield). <sup>1</sup>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).

#### Synthesis of (*S*)-5-amino-2-methylpentanoic acid hydrochloride<sup>2,4</sup> (**90**)

In a reaction vessel for the Parr shaker, **89** (233 mg, 1.8 mmol) was dissolved in 3:7 H<sub>2</sub>O:methanol (40 mL) and degassed. A catalytic amount of PtO<sub>2</sub> was added, and the solution was placed on the shaker at room temperature under 50 psi H<sub>2</sub> 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 **90** hydrochloride as an oil (293 mg, 95%



yield).  $^1\text{H}$  NMR (500 MHz, DMSO):  $\delta$  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).

### Synthesis of Boc-(*S*)-5-amino-2-methylpentanoic acid<sup>2</sup> (**91**)

To a stirred solution of **90** (293 mg, 1.7 mmol) in 2:1 1,4-dioxane:H<sub>2</sub>O (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<sub>2</sub>O (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<sub>2</sub>O (2 × 40 mL), and dried over MgSO<sub>4</sub>. The organic layer was concentrated *in vacuo* to yield **91** as a colorless oil (340 mg, 84% yield).  $^1\text{H}$  NMR (500 MHz, DMSO):  $\delta$  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).

### Synthesis of (*S*)-5-amino-2-methylpentanoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub> hydrotrifluoroacetate<sup>2</sup> (**92**)

First, compound **91** (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 Boc-(*S*)-5-amino-2-methylpentanoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-resin (397 mg). The pentapeptide resin was then stirred for 2 h at room temperature with 95:5 TFA:H<sub>2</sub>O (10 mL). After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, **92** hydrotrifluoroacetate was collected as an off-white solid (84 mg) and purified by HPLC using the general peptide purification

procedure to obtain **92** hydrotrifluoroacetate as a white solid (37 mg, 40% yield) which was >95% pure by HPLC. ESI-MS calculated  $[M+H]^+$ , 611.4, found, 611.2.

### Synthesis of *N,N'*-di-Boc-(*S*)-5-guanidino-2-methylpentanoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NH<sub>2</sub><sup>2</sup> (**93**)

The synthesis of **93** was completed using a modification of a previously reported procedure.<sup>5</sup> To a stirred solution of *N,N'*-di-Boc-*N'*-triflylguanidine (20 mg, 0.05 mmol) and triethylamine (36  $\mu$ L, 0.26 mmol) in 1,4-dioxane (2 mL) was added a solution of **92** (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<sub>4</sub> (2  $\times$  10 mL) followed by saturated NaHCO<sub>3</sub> (2  $\times$  10 mL), dried over MgSO<sub>4</sub> and concentrated *in vacuo* to give a white solid containing **93**. The solid was carried on without further purification or characterization.

### Synthesis of Boc-Tyr-OMe (**94**)

NB 4: 44-45

To a solution of the commercially available Boc-Tyr-OH (2.36 g, 8.4 mmol) in anhydrous methanol (40 mL) at 0°C under N<sub>2</sub> was added anhydrous triethylamine (3.6 mL, 25.2 mmol), followed by the addition of thionyl chloride (1.3 mL, 16.8 mmol) over 15 minutes. The solution stirred under N<sub>2</sub> for 2.5 hours before warming to room temperature and concentrating *in vacuo* to yield a light yellow solid. The solid was dissolved in ethyl acetate (40 mL) and washed with 10% citric acid (3  $\times$  25 mL), saturated NaHCO<sub>3</sub> (3  $\times$  25 mL) and brine (2  $\times$  25 mL). After drying over MgSO<sub>4</sub>, the solution was concentrated *in vacuo* to yield **94** as a light yellow solid (1.41 g, 57% yield). <sup>1</sup>H NMR (500 MHz, DMSO):  $\delta$  9.21 (s, 1H), 7.22 (d,  $J$  = 7.95 Hz, 1H), 7.00 (d,  $J$  = 8.30, 2H), 6.65 (d,  $J$  = 8.35, 2H), 4.03 (m, 1H), 3.59 (s, 3H), 2.84 (dd,  $J_1$  = 8.60 Hz,  $J_2$  = 5.25 Hz, 1H), 2.72 (dd,  $J_1$  = 9.85 Hz,  $J_2$  = 3.85 Hz, 1H), 1.32 (s, 9H).

### Synthesis of Boc-Tyr(4-OTf)-OMe (95)

NB 4: 62-63

To a solution of **94** (2.1 g, 7.15 mmol) in anhydrous dichloromethane (20 mL) at 0°C in a dry flask under N<sub>2</sub> was added triethylamine (3.0 mL, 21.4 mmol) followed by *N*-phenyltrifluoromethanesulfonimide (2.4 g, 6.8 mmol). The solution slowly warmed to room temperature, and stirred for 43.5 hours. The solution was concentrated *in vacuo* to yield a yellow oil, which was dissolved in ethyl acetate (40 mL) and washed with 10% citric acid (3 × 20 mL), saturated NaHCO<sub>3</sub> (3 × 20 mL) and brine (2 × 20 mL). After drying over MgSO<sub>4</sub>, the organic layer was concentrated *in vacuo* to obtain **95** as a yellow oil (3.6 g). The oil was carried on without further purification. <sup>1</sup>H NMR (500 MHz, DMSO): δ 7.42 (m, 4H), 7.16 (d, *J* = 7.80 Hz, 1H), 4.23 (m, 1H), 3.71 (s, 3H), 3.07 (dd, *J*<sub>1</sub> = 9.00 Hz, *J*<sub>2</sub> = 4.80 Hz, 1H), 2.89 (dd, *J*<sub>1</sub> = 10.80 Hz, *J*<sub>2</sub> = 2.85 Hz, 1H), 1.29 (s, 9H).

### Synthesis of Boc-Phe(*p*-COOH)-OMe (96)

NB 4: 83-85

To a solution of **95** (3.54 g impure yellow oil) in anhydrous DMF (30 mL) was added K<sub>2</sub>CO<sub>3</sub> (5.6 g, 40.5 mmol), bis(diphenylphosphino)ferrocene (DPPF, 967 mg, 1.77 mmol) and palladium II acetate (193 mg, 0.85 mmol). Carbon monoxide was bubbled through the suspension for 30 minutes, and then the solution stirred at 60°C for 13.5 hours. The suspension was filtered through celite and concentrated *in vacuo* to obtain a brown solid, which was dissolved in saturated NaHCO<sub>3</sub> (40 mL) and extracted with ethyl acetate (3 × 70 mL). The aqueous layer was acidified to pH 1 with 1 M HCl, and extracted with ethyl acetate (3 × 50 mL). These last organic extracts were combined and washed with saturated NaCl (50 mL), dried over MgSO<sub>4</sub>, and concentrated *in vacuo* to give **96** as a light orange solid (766 mg, 33.1% yield for 2 steps) which was >90% pure by HPLC. The solid was carried on without further purification. <sup>1</sup>H NMR (500 MHz, DMSO): δ 12.84 (s, 1H, br), 7.85 (d, *J* = 8.00 Hz, 2H), 7.35 (d, *J* = 8.05 Hz, 3H), 4.21 (m, 1H), 3.62 (s, 3H), 3.06 (dd, *J*<sub>1</sub> = 8.70 Hz, *J*<sub>2</sub> = 4.85 Hz, 1H), 2.92 (dd, *J*<sub>1</sub> = 10.30 Hz, *J*<sub>2</sub> = 2.95 Hz, 1H), 1.31 (s, 9H).

### Synthesis of Boc-Phe(*p*-CONH-resin)-OMe (**97**)

NB 6: 90

Rink resin (1.01g, 0.6 mmol/g) was treated with NMP (7 mL) in a vial for 10 min to swell, then piperidine (12 mL) was added and the vial was agitated at room temperature for 3 hours, and the resin then washed with NMP. A solution of **96** (397 mg, 1.20 mmol), HATU (689 mg, 1.80 mmol), HOAt (247 mg, 1.80 mmol) and DIEA (1.05 mL, 6.00 mmol) in DMF (3 mL), which had stirred at room temperature for 2 hours, was then added to the washed resin in a new vial, along with additional DMF (9 mL). The solution was agitated for 6 days at room temperature, and then the resin-bound **97** was collected and washed with NMP. Compound **97** was carried on without further purification or characterization.

### Synthesis of Boc-Phe(*p*-CONH-resin)-OH (**98**)

NB 6:93

To a vial containing **97** (approximately 1 g of loaded resin) was added DMF (12 mL) and 1 M NaOH (3 mL), and was agitated at room temperature for 3 hours. The resin-bound **98** was collected and washed with NMP, followed by DCM, and dried. Compound **98** (1.03 g) was carried on without further characterization or purification.

### Synthesis of Phe-(*p*-carboxamide)-OH trifluoroacetate (**99**)

NB 6:94

To a vial containing **98** (1.03 g) was added a cooled solution of 95:5 TFA:H<sub>2</sub>O (10 mL) and stirred at room temperature for 2 hours. After filtration, the solution was concentrated *in vacuo* followed by precipitation with cold diethyl ether. After refrigeration overnight, **99** hydrotrifluoroacetate was collected as an off-white solid (187 mg) and was 93% pure by HPLC. The solid was carried on without further characterization or purification.

### Synthesis of Fmoc-Phe(*p*-carboxamide)-OH (**100**)

NB 6: 101-102

To a stirred suspension of **99** (146 mg, 0.46 mmol) and DIEA (0.5 mL, 2.3 mmol) in anhydrous dichloromethane (10 mL) in a dry flask under N<sub>2</sub> at 0°C, was added a solution of Fmoc-Cl (241 mg, 0.93 mmol) in dichloromethane (1.3 mL) slowly over 20 minutes. The solution slowly warmed to room temperature with stirring over 2.75 hours, and was then concentrated *in vacuo* to give a dark yellow solid. The solid was dissolved in an aqueous solution of H<sub>2</sub>O (10 mL) and 1 M HCl (4 mL) and extracted with ethyl acetate (3 × 20 mL). The organic fractions were combined, washed with saturated NaCl (25 mL), dried over MgSO<sub>4</sub>, and concentrated *in vacuo* to yield a yellow oil (222 mg) which contained **100**. Compound **100** was carried on without further purification or characterization.

### Synthesis of Fmoc-Phe(*p*-carboxamide)-resin (**101**)

NB 6: 106-107

Rink resin (386 mg, 0.6 mmol/g) was treated with NMP (4 mL) in a vial for 10 min to swell, then a solution of 20% piperidine in NMP (15 mL) was added and the vial was agitated at room temperature for 1 hour and washed with NMP. A solution of **100** (222 mg), HATU (156 mg, 0.40 mmol), HOAt (57 mg, 0.40 mmol) and DIEA (175 μL, 1.00 mmol) in NMP (4 mL), which had stirred at room temperature for 30 minutes, was then added to the washed resin in a new vial, along with additional NMP (14 mL). The solution was agitated for 20 hours at room temperature, and then the resin-bound **101** was collected and washed with NMP. Compound **101** was carried on without further purification or characterization.

### Synthesis of Boc-Phe(*p*-*N*-phenylacetamide)-OMe (**102**)

NB 5: 131-132

To a stirred solution of **96** (974 mg, 3.0 mmol) in DMF (15 mL) was added HATU (1.37 g, 3.6 mmol) and DIEA (4.7 mL, 12.0 mmol), followed by aniline (1.1 mL, 12.0 mmol). The solution stirred at room temperature for 41 hours and was concentrated *in vacuo* to leave a dark orange solid. The solid was dissolved in ethyl acetate (150 mL)

and the organic layer was washed with 10% citric acid (2 × 50 mL), 10% NaHCO<sub>3</sub> (2 × 50 mL), and saturated NaCl (1 × 50 mL). The organic layer was then dried over MgSO<sub>4</sub> and concentrated *in vacuo* until a small amount of solvent remained, prompting precipitation of **102** as an orange solid, which was collected by filtration and dried (879 mg, 74% yield). Compound **102** was >99% pure by HPLC. <sup>1</sup>H NMR (500 MHz, DMSO): δ 10.18 (s, 1H, br), 7.88 (d, *J* = 7.85 Hz, 2H), 7.77 (d, *J* = 8.30 Hz, 2H), 7.39 (d, *J* = 8.15 Hz, 2H), 7.37 (d, *J* = 8.05 Hz, 1H), 7.34 (t, *J* = 7.60 Hz, 2H), 7.10 (dt, *J*<sub>1</sub> = 6.50 Hz, *J*<sub>2</sub> = 0.85 Hz, 1H), 4.23 (m, 1H), 3.63 (s, 3H), 3.09 (dd, *J*<sub>1</sub> = 8.75 Hz, *J*<sub>2</sub> = 5.00 Hz, 1H), 2.93 (dd, *J*<sub>1</sub> = 10.50 Hz, *J*<sub>2</sub> = 3.00 Hz, 1H), 1.33 (s, 9H).

### Synthesis of Boc-Phe(*p*-*N*-phenylacetamide)-OH (**103**)

NB 5: 137-138

To a stirred solution of **102** (879 mg, 2.2 mmol) in DMF (25 mL) was added 2 M NaOH (2.2 mL, 4.4 mmol) over 2 minutes. The solution stirred at room temperature for 1.5 hours and concentrated *in vacuo* to give a yellow solid. The solid was dissolved in H<sub>2</sub>O (30 mL) and acidified to pH 2 with 1 M HCl. The aqueous layer was extracted with ethyl acetate (2 × 50 mL) and the combined organic extracts were washed with brine (50 mL), dried over MgSO<sub>4</sub> and concentrated *in vacuo* to yield an off-white solid which contained **103** (947 mg). The solid was carried on without further purification. <sup>1</sup>H NMR (400 MHz, DMSO): δ 12.70 (s, 1H, br), 7.87 (d, *J* = 8.00 Hz, 2H), 7.77 (d, *J* = 8.00 Hz, 2H), 7.39 (d, *J* = 8.00 Hz, 2H), 7.34 (t, *J* = 8.00 Hz, 2H), 7.20 (d, *J* = 8.00 Hz, 1H), 7.10 (t, *J* = 8.00 Hz, 1H), 4.14 (m, 1H), 3.12 (dd, *J*<sub>1</sub> = 12.00 Hz, *J*<sub>2</sub> = 4.00 Hz, 1H), 2.92 (dd, *J*<sub>1</sub> = 12.00 Hz, *J*<sub>2</sub> = 8.00 Hz, 1H), 1.32 (s, 9H).

### Synthesis of Phe(*p*-*N*-phenylacetamide)-OH trifluoroacetate (**104**)

NB 5: 139

Compound **103** (947 mg) was dissolved in a solution of 95:5 TFA:H<sub>2</sub>O (10 mL) and stirred at room temperature for 3 hours, concentrated *in vacuo* and precipitated with diethyl ether. After refrigeration overnight, **104** was collected as a light orange solid (666 mg, 76% yield for 2 steps) and was >98% pure by HPLC. <sup>1</sup>H NMR (400 MHz, DMSO): δ 10.23 (s, 1H, br), 7.92 (d, *J* = 8.00 Hz, 2H), 7.78 (d, *J* = 8.00 Hz, 2H), 7.42 (d, *J* = 8.00

Hz, 2H), 7.35 (t,  $J = 8.00$  Hz, 2H), 7.10 (t,  $J = 8.00$  Hz, 1H), 3.72 (m, 1H), 3.37 (s, 3H, br), 3.22 (dd,  $J_1 = 8.00$  Hz,  $J_2 = 4.00$  Hz, 1H), 3.01 (dd,  $J_1 = 12.00$  Hz,  $J_2 = 4.00$  Hz, 1H).  $^{13}\text{C}$  NMR (100 MHz, DMSO):  $\delta$  170.01, 165.63, 141.10, 139.59, 133.60, 128.92, 128.16, 127.34, 124.04, 120.81, 54.96, 36.89.

### Synthesis of Fmoc-Phe(*p*-N-phenylacetamide)-OH (**105**)

NB 6: 25-26

To a stirred suspension of **104** (150 mg, 0.38 mmol) and DIEA (0.40 mL, 2.30 mmol) in anhydrous dichloromethane (10 mL) in a dry flask under  $\text{N}_2$  at  $0^\circ\text{C}$ , was added a solution of Fmoc-Cl (198 mg, 0.75 mmol) in dichloromethane (1.8 mL) slowly over 25 minutes. The solution slowly warmed to room temperature with stirring over 2.5 hours, and was then concentrated *in vacuo* to give a yellow solid which contained **105**, and was carried on without further purification or characterization.

### Synthesis of Fmoc-Phe(*p*-N-phenylacetamide)-resin (**106**)

NB 6: 29

Rink resin (358 mg, 0.6 mmol/g) was treated with NMP (4 mL) in a vial for 5 min to swell, then a solution of 20% piperidine in NMP (15 mL) was added and the vial was agitated at room temperature for 1 hour and washed with NMP. A solution of **105**, HATU (153 mg, 0.40 mmol), HOAt (64 mg, 0.40 mmol) and DIEA (175  $\mu\text{L}$ , 1.00 mmol) in NMP (4 mL), which had stirred at room temperature for 45 minutes, was then added to the washed resin in a new vial, along with additional NMP (14 mL). The solution was agitated for 22 hours at room temperature, and then the resin-bound **106** was collected and washed with NMP. Compound **106** was carried on without further purification or characterization.

### Synthesis of Boc-Phe(*p*-NH<sub>2</sub>)-resin (**107**)

NB 6: 180-183

First, Rink amide resin (417 mg, 0.6 mmol/g) was treated with 3 mL NMP, then a deprotection solution of 20% piperidine in NMP (15 mL) was added, and the solution agitated at room temperature for 1 hour, followed by several rinses with NMP.

Separately, the commercially available Boc-Phe(*p*-NH-Fmoc)-OH (251 mg, 0.50 mmol) was dissolved in NMP (12 mL) and a pre-made solution of 0.4 M HOBt/HBTU in DMF (1.88 mL, 0.75 mmol), and DIEA (0.22 mL, 1.25 mmol) was added. This solution was added to the deprotected Rink amide resin, and the solution agitated at room temperature for 19.5 hours. The resulting compound, Boc-Phe(*p*-NH-Fmoc)-resin, was first rinsed with NMP, and then deprotected using a solution of 20% piperidine in NMP (18 mL) and was agitated at room temperature for 1.5 hours, yielding **107**, which was washed with NMP. Compound **107** was carried on without further purification or characterization.

### Synthesis of Phe(*p*-NHCOCH<sub>2</sub>NH-Fmoc)-NH<sub>2</sub> hydrotrifluoroacetate (**108**)

NB 6: 180-183, 189

First, the commercially available Fmoc-Gly-OH (223 mg, 0.75 mmol) was dissolved in NMP (12 mL) and a pre-made solution of 0.4 M HOBt/HBTU in DMF (1.88 mL, 0.75 mmol), and DIEA (0.22 mL, 1.25 mmol) was added. This solution was added to **107**, and the solution agitated at room temperature for 19 hours. After rinsing with NMP followed by dichloromethane, the resulting compound, Boc-Phe(*p*-NHCOCH<sub>2</sub>NH-Fmoc)-resin, was collected and dried (461 mg). To the dried resin was added a cooled cleavage and deprotection solution comprised of 95:5 TFA:H<sub>2</sub>O (10 mL), and the solution stirred at room temperature for 2 hours. After filtration, the solution was concentrated *in vacuo*, and **108** hydrotrifluoroacetate was precipitated with cold diethyl ether. After refrigeration overnight, **108** hydrotrifluoroacetate was collected (27 mg) and purified according to the general peptide purification procedure, to obtain **108** hydrotrifluoroacetate (13 mg, 9.4% yield) as a white solid, which was >97% pure by HPLC. <sup>1</sup>H NMR (400 MHz, DMSO): δ 9.99 (s, 1H), 8.06 (s, 3H, br), 7.90 (d, *J* = 7.48 Hz, 2H), 7.80 (s, 1H), 7.73 (d, *J* = 7.40 Hz, 2H), 7.63 (t, *J* = 5.64 Hz, 1H), 7.55 (s, 1H), 7.54 (d, *J* = 7.52 Hz, 2H), 7.43 (t, *J* = 7.32 Hz, 2H), 7.34 (t, *J* = 7.40 Hz, 2H), 7.18 (d, *J* = 8.12 Hz, 2H), 4.31 (d, *J* = 6.76, 2H), 4.23 (dd, *J*<sub>1</sub> = 7.04 Hz, *J*<sub>2</sub> = 6.44 Hz, 1H), 3.89 (t, *J* = 7.28 Hz, 1H), 3.79 (d, *J* = 5.96 Hz, 2H), 3.03 (dd, *J*<sub>1</sub> = 14.00 Hz, *J*<sub>2</sub> = 8.20 Hz, 1H), 2.92 (dd, *J*<sub>1</sub> = 13.88 Hz, *J*<sub>2</sub> = 6.36 Hz, 1H). <sup>13</sup>C NMR (100 MHz, DMSO): δ 170.15, 168.33, 157.02, 144.27, 141.16, 138.45, 130.21, 130.01, 128.07, 127.51, 125.66, 120.56, 119.60, 66.15, 53.92, 47.07, 44.42, 36.75.



### Synthesis of Oic-OMe hydrochloride (**109**)

NB 5: 173-174

To a solution of the commercially available H-Oic-OH (511 mg, 3.0 mmol) in anhydrous methanol (5 mL) at -5°C under N<sub>2</sub> was added thionyl chloride (0.57 mL, 7.8 mmol) over 10 minutes. The solution stirred under N<sub>2</sub> for 24.5 hours, slowly warming to room temperature, and then concentrated *in vacuo* and dried to yield a slightly orange, off-white solid containing **109**. This solid was carried on without further purification or characterization.

### Synthesis of Boc-D-Arg(Pbf)-Oic-OMe (**110**)

NB 5: 175-176

The commercially available Boc-D-Arg(Pbf)-OH (1.74 g, 3.3 mmol), was dissolved in a pre-made solution of 0.4 M HOBt/HBTU in DMF (15 mL, 6.0 mmol), and DIEA (4.2 mL, 24.0 mmol) was added. To this solution was added a solution of compound **109** in a solution of 15 mL DMF, and the combined solution was stirred at room temperature for 1.5 days. After concentration *in vacuo*, the resulting red/brown material was dissolved in ethyl acetate (50 mL) and washed with 10% citric acid (3 × 25 mL), saturated NaHCO<sub>3</sub> (3 × 25 mL), and brine (1 × 25 mL), dried over MgSO<sub>4</sub>, and concentrated *in vacuo* and dried to give an orange solid containing **110** (2.2 g). The solid was carried on without further purification or characterization.

### Synthesis of Boc-D-Arg(Pbf)-Oic-OH (**111**)

NB 5: 177-178, 181-182

The orange solid containing **110** (2.2 g) was dissolved in DMF (20 mL), and to this solution was added 2 M NaOH (6.0 mL, 12.0 mmol), and the resulting solution was stirred at room temperature for 1 hour. The solution was concentrated *in vacuo*, and the resulting material dissolved in H<sub>2</sub>O (50 mL) and acidified to pH 2 with 1 M HCl. The acidified aqueous layer was extracted with ethyl acetate (2 × 50 mL), and the combined organic extracts were washed with brine (1 × 50 mL), dried over MgSO<sub>4</sub>, and concentrated *in vacuo* to yield an orange solid containing **111** (2.0 g). This solid was carried on without further purification or characterization.

### Synthesis of Boc-D-Arg(Pbf)-Oic-Pro-D-Ala-Phe-OH (112)

NB 5: 140-141, 145-146, 157-158

First, the tetrapeptide Oic-Pro-D-Ala-Phe-OH was synthesized according to the general peptide synthesis procedure, using Fmoc-Phe-Wang resin (652 mg, 0.65 mmol/g substitution) and commercially available Fmoc-protected amino acids. Once the synthesis was complete, the dried resin (646 mg) was stirred for 2 hours at room temperature with 95:5 TFA:H<sub>2</sub>O (10 mL). After filtration, the solution was concentrated *in vacuo*, followed by precipitation with cold diethyl ether. After refrigeration overnight, tetrapeptide Oic-Pro-D-Ala-Phe-OH hydrotrifluoroacetate was collected (115 mg) and purified by HPLC using the general peptide purification procedure to obtain Oic-Pro-D-Ala-Phe-OH hydrotrifluoroacetate (102 mg, 40% yield) which was >97% pure by HPLC. (ESI-MS calculated [M+H]<sup>+</sup>, 485.3, found, 485.2.) Next, commercially available Boc-D-Arg(Pbf)-OH (72 mg, 0.14 mmol) was dissolved in a pre-made solution of 0.4 M HOBt/HBTU (0.33 mL, 0.13 mmol), and DIEA (0.15 mL, 0.86 mmol) was added. This solution was added to a solution of Oic-Pro-D-Ala-Phe-OH hydrotrifluoroacetate (52 mg, 0.09 mmol) in DMF (5 mL). The combined solution stirred at room temperature for 16.5 hours, and was concentrated *in vacuo* to obtain a colorless residue. The residue was dissolved in ethyl acetate (25 mL) and washed with 0.5 M HCl (3 × 20 mL) and brine (1 × 25 mL), dried over MgSO<sub>4</sub>, and concentrated *in vacuo* to give a colorless residue (109 mg), which contains **112**, which was carried on without further purification or characterization.

### Synthesis of Boc-D-Arg(Pbf)-Oic-Pro-D-Ala-OH (113)

NB 5: 293-294; NB 6: 10-11, 46-47

First, Oic-Pro-D-Ala-OH was synthesized according to the general solid phase peptide synthesis procedure, using Fmoc-D-Ala-Wang resin (259 mg, 0.8 mmol/g substitution) and commercially available Fmoc-protected amino acids. Once synthesis was complete, the dried resin (268 mg) was stirred for 2.25 hours at room temperature with 95:5 TFA:H<sub>2</sub>O (10 mL). After filtration, the solution was concentrated *in vacuo* to obtain a light yellow solid. This solid was purified by HPLC using the general peptide purification procedure to obtain Oic-Pro-D-Ala-OH hydrotrifluoroacetate (67 mg, 72%

yield) which was >97% pure by HPLC. (ESI-MS calculated [M+H]<sup>+</sup>, 338.2, found, 338.2.) Next, commercially available Boc-D-Arg(Pbf)-OH (120 mg, 0.23 mmol), HATU (90 mg, 0.24 mmol) and HOAt (33 mg, 0.24 mmol) were dissolved in DMF (8 mL), and DIEA (0.26 mL, 1.50 mmol) was added. This solution was added to a solution of Oic-Pro-D-Ala-OH hydrotrifluoroacetate (67 mg, 0.15 mmol) in DMF (2 mL). The solution stirred at room temperature for 20 hours, and was concentrated *in vacuo* to obtain an orange residue. The residue was dissolved in ethyl acetate (25 mL) and washed with 1 M HCl (3 × 20 mL) and brine (1 × 25 mL), dried over MgSO<sub>4</sub>, and concentrated *in vacuo* to give a yellow residue (170 mg), which contains **113**, which was carried on without further purification or characterization.

#### Synthesis of Phe-NH*i*Pr hydrotrifluoroacetate (**114**)

NB 6: 86-88, 91-92

To a stirred solution of the commercially available Boc-Phe-OH (268 mg, 1.01 mmol), HATU (763 mg, 2.01 mmol) and HOAt (286 mg, 2.10 mmol) in DMF (15 mL) was added DIEA (5.3 mL, 30.4 mmol) followed by isopropyl amine (0.30 mL, 3.66 mmol). The solution stirred at room temperature for 19.5 hours, and was then concentrated *in vacuo* until a dark orange oil remained. The oil was dissolved in ethyl acetate (25 mL) and was washed with 1 M HCl (3 × 20 mL), saturated NaHCO<sub>3</sub> (3 × 20 mL) and brine (1 × 25 mL), dried over MgSO<sub>4</sub>, and concentrated *in vacuo* to give a dark orange residue. To this residue was added 95:5 TFA:H<sub>2</sub>O (10 mL), and the solution stirred at room temperature for 2.25 hours. After concentration *in vacuo*, a portion of this material was purified by HPLC using the general peptide purification procedure to obtain **114** hydrotrifluoroacetate (122 mg) which was >95% pure by HPLC. <sup>1</sup>H NMR (400 MHz, DMSO): δ 8.21 (s, 1H, br), 8.13 (d, *J* = 7.48 Hz, 2H), 7.33 (d, *J* = 8.00 Hz, 2H), 7.28 (d, *J* = 7.00 Hz, 1H), 7.22 (d, *J* = 4.00 Hz, 2H), 3.87 (s, 1H, br), 3.79 (septet, *J* = 6.76 Hz, 1H), 2.98 (dd, *J*<sub>1</sub> = 5.64 Hz, *J*<sub>2</sub> = 2.08 Hz, 2H), 1.04 (d, *J* = 6.56 Hz, 3H), 0.87 (d, *J* = 6.56, 3H). <sup>13</sup>C NMR (100 MHz, DMSO): δ 166.94, 135.33, 129.91, 128.86, 127.52, 53.91, 41.16, 37.56, 22.46, 22.37.

### Synthesis of Phe-NHC<sub>6</sub>H<sub>11</sub> hydrotrifluoroacetate (115)

NB 6: 202-204, 208-209

To a stirred solution of the commercially available Boc-Phe-OH (276 mg, 1.04 mmol) in DMF (15 mL) was added HATU (762 mg, 2.00 mmol) and HOAt (273 mg, 2.00 mmol), followed by DIEA (5.3 mL, 30.00 mmol). To this solution was added cyclohexylamine (0.35 mL, 3.00 mmol), and the solution stirred at room temperature 16.5 hours. After concentration *in vacuo*, the resulting dark orange oil was dissolved in ethyl acetate (25 mL) and washed with 0.5 M HCl (3 × 20 mL), saturated NaHCO<sub>3</sub> (3 × 20 mL) and brine (1 × 25 mL), dried over MgSO<sub>4</sub>, and concentrated *in vacuo* to give a light orange solid. To this solid was added 95:5 TFA:H<sub>2</sub>O (10 mL), and this solution stirred at room temperature for 2.25 hours. After concentration *in vacuo*, this material was purified by HPLC using the general peptide purification procedure to obtain **115** hydrotrifluoroacetate (176 mg, 47% yield) which was 96% pure by HPLC. <sup>1</sup>H NMR (400 MHz, DMSO): δ 8.24 (s, 3H), 8.13 (d, *J* = 8.00 Hz, 1H), 7.32 (t, *J* = 6.80 Hz, 2H), 7.26 (d, *J* = 7.20 Hz, 1H), 7.21 (d, *J* = 6.80 Hz), 3.91 (t, *J* = 6.80 Hz, 1H), 3.50 – 3.46 (m, 1H), 3.03 – 2.93 (m, 2H), 1.71 – 1.63 (m, 2H), 1.56 – 1.49 (m, 3H), 1.26 – 1.07 (m, 4H), 0.94 – 0.87 (m, 1H). <sup>13</sup>C NMR (100 MHz, DMSO): δ 166.96, 135.32, 129.91, 128.85, 127.51, 53.84, 48.04, 37.61, 32.42, 32.34, 25.47, 24.72, 24.62.

### Synthesis of Phe-NHBn hydrotrifluoroacetate (116)

NB 5: 199-201, 205, 220-221

To a stirred solution of the commercially available Boc-Phe-OH (276 mg, 1.04 mmol) in a pre-made solution of 0.4 M HOBt/HBTU in DMF (5.0 mL, 2.00 mmol) was added DIEA (5.3 mL, 30.4 mmol) followed by benzylamine (0.33 mL, 3.02 mmol). The solution stirred at room temperature for 1.5 days, and was then concentrated *in vacuo* until an orange oil remained. The oil was dissolved in ethyl acetate (50 mL) and was washed with 10% citric acid (3 × 25 mL), saturated NaHCO<sub>3</sub> (3 × 25 mL) and brine (1 × 25 mL), dried over MgSO<sub>4</sub>, and concentrated *in vacuo* to give a light orange residue. To this residue was added 95:5 TFA:H<sub>2</sub>O (10 mL), and the solution stirred at room temperature for 2 hours. After concentration *in vacuo*, this material was purified by HPLC using the general peptide purification procedure to obtain **116**

hydrotrifluoroacetate (194 mg, 53% yield) which was >97% pure by HPLC. <sup>1</sup>H NMR (400 MHz, DMSO): δ 8.84 (t, *J* = 5.76, 1H), 8.28 (s, 3H, br), 7.35-7.21 (m, 8H), 7.08 (d, *J* = 6.56, 2H), 4.33 (dd, *J*<sub>1</sub> = 9.08 Hz, *J*<sub>2</sub> = 6.08 Hz, 1H), 4.22 (dd, *J*<sub>1</sub> = 9.76 Hz, *J*<sub>2</sub> = 5.40 Hz, 1H), 4.03 (t, *J* = 7.16 Hz, 1H), 3.04 (dd, *J*<sub>1</sub> = 6.84 Hz, *J*<sub>2</sub> = 2.16 Hz, 1H). <sup>13</sup>C NMR (100 MHz, DMSO): δ 168.17, 138.50, 135.30, 129.88, 128.98, 128.70, 127.79, 127.58, 127.42, 54.05, 42.71, 37.51

### Synthesis of Phe(*p*-Me)-NHC<sub>6</sub>H<sub>11</sub> hydrotrifluoroacetate (117)

NB 6: 258-259, 262-263

To a stirred solution of the commercially available Boc-Phe(*p*-Me)-OH (289 mg, 1.04 mmol) in DMF (10 mL) was added HATU (766 mg, 2.01 mmol) and HOAt (273 mg, 2.00 mmol), followed by DIEA (5.3 mL, 30.00 mmol). To this solution was added cyclohexylamine (575 μL, 5.00 mmol), and the solution stirred at room temperature 18 hours. After concentration *in vacuo*, the resulting dark orange oil was dissolved in ethyl acetate (30 mL) and washed with 5% citric acid (3 × 20 mL), saturated NaHCO<sub>3</sub> (3 × 20 mL) and brine (1 × 25 mL), dried over MgSO<sub>4</sub>, and concentrated *in vacuo* to give an orange solid. To this solid was added 95:5 TFA:H<sub>2</sub>O (10 mL), and this solution stirred at room temperature for 2 hours. After concentration *in vacuo*, this material was purified by HPLC using the general peptide purification procedure to obtain **119**

hydrotrifluoroacetate (147 mg, 38% yield) which was >97% pure by HPLC. <sup>1</sup>H NMR (400 MHz, DMSO): δ 8.19 (s, 3H), 8.16 (d, *J* = 8.00 Hz, 1H), 7.13 (d, *J* = 8.00 Hz, 2H), 7.09 (d, *J* = 8.40 Hz, 2H), 3.87 (t, *J* = 7.20 Hz, 1H), 3.51 (m, 1H), 2.93 (d, *J* = 7.20 Hz, 2H), 2.27 (s, 1H), 1.72-1.64 (m, 2H), 1.59-1.53 (m, 3H), 1.24-0.91 (m, 5H). <sup>13</sup>C NMR (100 MHz, DMSO): δ 167.06, 136.59, 132.22, 129.79, 129.41, 53.92, 48.06, 37.20, 32.43, 32.37, 25.48, 24.71, 24.62, 21.10.

### Synthesis of 5-aminopentanoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NHC<sub>6</sub>H<sub>11</sub> hydrotrifluoroacetate (118)

NB 6: 278-279, 282-283, 284-285

First, the tetrapeptide Fmoc-5-aminopentanoic acid-Oic-Pro-D-Ala-OH was synthesized using standard SPPS methods, using Fmoc-D-Ala-Wang resin (435 mg, 0.48

mmol/g) and commercially available Fmoc-protected amino acids. Once synthesis was complete, the dried resin (681 mg) was stirred for 1.75 hours at room temperature with 95:5 TFA:H<sub>2</sub>O (10 mL). After filtration, the solution was concentrated *in vacuo* to obtain a yellow residue. This residue was purified by HPLC using the general peptide purification procedure to obtain Fmoc-5-aminopentanoic acid-Oic-Pro-D-Ala-OH hydrotrifluoroacetate. Next, to a stirred solution of Fmoc-5-aminopentanoic acid-Oic-Pro-D-Ala-OH, HATU (76 mg, 0.02 mmol), HOAt (35 mg, 0.26 mmol) and DIEA (114  $\mu$ L, 0.65 mmol) in DMF (6 mL) was added **117** (37 mg, 0.01 mmol), and the resulting solution stirred at room temperature for 24 hours. The solution was concentrated *in vacuo* to leave a yellow residue, which was dissolved in ethyl acetate (30 mL) and washed with 10% citric acid (3  $\times$  20 mL), saturated NaHCO<sub>3</sub> (3  $\times$  20 mL), and brine (1  $\times$  25 mL). The final organic layer was dried over MgSO<sub>4</sub> and concentrated *in vacuo* to give an off-white solid (57 mg), which contains Fmoc-5-aminopentanoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NHC<sub>6</sub>H<sub>11</sub>. Finally, to a stirred solution of Fmoc-5-aminopentanoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NHC<sub>6</sub>H<sub>11</sub> in DMF (5 mL) was added piperidine (5 mL), and the solution stirred at room temperature for 35 minutes. The solution was concentrated *in vacuo* to give a white solid, which was purified according the general peptide purification procedure to obtain **118**. ESI-MS calculated [M+H]<sup>+</sup>, 679.5, found, 679.5.

### Synthesis of di-Boc-5-guanidinopentanoic acid-Oic-Pro-D-Ala-Phe(*p*-Me)-NHC<sub>6</sub>H<sub>11</sub> (**119**)

NB 6: 286-287

To a stirred solution of *N,N'*-di-Boc-*N'*-triflylguanidine (31 mg, 0.08 mmol) and triethylamine (100  $\mu$ L, 0.72 mmol) in 1,4-dioxane (7 mL) was added **118** and the solution stirred at room temperature for 19 hours. The solution was concentrated *in vacuo* to give a colorless oil, which was dissolved in ethyl acetate (20 mL) and washed with 10% citric acid (3  $\times$  10 mL), saturated NaHCO<sub>3</sub> (3  $\times$  10 mL) and brine (1  $\times$  10 mL). The final organic layer was dried over MgSO<sub>4</sub> and concentrated *in vacuo* to yield a white solid containing **119**. This solid was carried on without further purification or characterization.

## Chapter 7 References

- (1) Przydzial, M. J.; Pogożheva, I. D.; Bosse, K. E.; Andrews, S. M.; Tharp, T. A.; Traynor, J. R.; Mosberg, H. I. *Peptide Research* **2005**, *65*, 333.
- (2) Girnys, E. A.; Porter, V. R.; Mosberg, H. I. *Bioorganic and Medicinal Chemistry* **2011**, *19*, 7425.
- (3) Judkins, B. D.; Allen, D. G.; Cook, T. A.; Evans, B.; Sardharwala, T. E. *Synthetic Communications* **1996**, *26*, 4351.
- (4) Attwood, M. R.; Conway, E. A.; Dunsdon, R. M.; Greening, J. R.; Handa, B. K.; Jones, P. S.; Jordan, S. C.; Keech, E.; Wilson, F. X. *Bioorganic and Medicinal Chemistry Letters* **1997**, *7*, 429.
- (5) Feichtinger, K.; Sings, H. L.; Baker, T. J.; Matthews, K.; Goodman, M. *Journal of Organic Chemistry* **1998**, *63*, 8432.

## Appendix



Table A1: Summary of assay and physiochemical data for **RPPGF**, **TH 146**, **FM 19**, and compounds **1-56**

Compound	Sequence	50% Inhibition of human $\alpha$ -thrombin (IC <sub>50</sub> , $\mu$ M)	Expected [M + H]	Found [M + H]	RP-HPLC retention time (min)	Notebook Compound Code
<b>RPPGF</b>	Arg-Pro-Pro-Gly-Phe-OH	601 $\pm$ 21	573.3	573.1	17.4	RPPGF
<b>TH 146</b>	D-Arg-Oic-Pro-Gly-Phe -NH <sub>2</sub>	49.5 $\pm$ 5.2	626.4	626.2	21.9	TH 146
<b>FM 19</b>	D-Arg-Oic-Pro-D-Ala-Phe( <i>p</i> -Me)-NH <sub>2</sub>	4.4 $\pm$ 1.3	654.4	654.4	26.5	FM 19
<b>1</b>	5-guanidinopentanoic acid-Oic-Pro-D-Ala-Phe( <i>p</i> -Me)-NH <sub>2</sub>	0.66 $\pm$ 0.20	639.4	639.4	32.6	BG 5
<b>2</b>	Guanidino glycine-Oic-Pro-D-Ala-Phe( <i>p</i> -Me)-NH <sub>2</sub>	36.8 $\pm$ 9.4	597.3	597.3	30.1	BG 10
<b>3</b>	3-guanidinopropionic acid-Oic-Pro-D-Ala-Phe( <i>p</i> -Me)-NH <sub>2</sub>	6.5 $\pm$ 0.6	611.4	611.2	30.1	BG 15
<b>4</b>	4-guanidinobutyric acid-Oic-Pro-D-Ala-Phe( <i>p</i> -Me)-NH <sub>2</sub>	8.4 $\pm$ 2.0	625.4	625.3	31.4	BG 11
<b>5</b>	6-guanidino hexanoic acid-Oic-Pro-D-Ala-Phe( <i>p</i> -Me)-NH <sub>2</sub>	25.6 $\pm$ 6.8	653.4	653.5	33.9	BG 18
<b>6</b>	<i>p</i> -guanidinobenzoic acid-Oic-Pro-D-Ala-Phe( <i>p</i> -Me)-NH <sub>2</sub>	0.66 $\pm$ 0.20	659.4	659.3	31.6	BG 6

Compound	Sequence	50% Inhibition of human $\alpha$ -thrombin (IC <sub>50</sub> , $\mu$ M)	Expected [M + H]	Found [M + H]	RP-HPLC retention time (min)	Notebook Compound Code
<b>7</b>	<i>cis</i> -4-guanidino-1-cyclohexanecarboxylic acid-Oic-Pro-D-Ala-Phe( <i>p</i> -Me)-NH <sub>2</sub>	0.57 $\pm$ 0.12	665.4	665.4	34.2	BG 7
<b>8</b>	<i>trans</i> -4-guanidino-1-cyclohexanecarboxylic acid-Oic-Pro-D-Ala-Phe( <i>p</i> -Me)-NH <sub>2</sub>	2.5 $\pm$ 0.5	665.4	665.4	32.1	VRP 2
<b>9</b>	5-aminopentanoic acid-Oic-Pro-D-Ala-Phe( <i>p</i> -Me)-NH <sub>2</sub>	241 $\pm$ 41	597.4	597.2	30.6	BG 17
<b>10</b>	6-aminohexanoic acid-Oic-Pro-D-Ala-Phe( <i>p</i> -Me)-NH <sub>2</sub>	130 $\pm$ 31	611.4	611.4	31.6	BG 16
<b>11</b>	7-aminohexanoic acid-Oic-Pro-D-Ala-Phe( <i>p</i> -Me)-NH <sub>2</sub>	498 $\pm$ 14	625.4	625.3	33.5	BG 12
<b>12</b>	<i>p</i> -aminobenzoic acid-Oic-Pro-D-Ala-Phe( <i>p</i> -Me)-NH <sub>2</sub>	225 $\pm$ 102	617.3	639.5 (+Na)	32.0	BG 14
<b>13</b>	4-amidinobenzoic acid-Oic-Pro-D-Ala-Phe( <i>p</i> -Me)-NH <sub>2</sub>	0.51 $\pm$ 0.19	644.4	644.5	30.5	BG 22
<b>14</b>	4-amidinophenylacetic acid-Oic-Pro-D-Ala-Phe( <i>p</i> -Me)-NH <sub>2</sub>	0.45 $\pm$ 0.08	658.4	658.5	33.2	BG 24
<b>15</b>	3-amidinobenzoic acid-Oic-Pro-D-Ala-Phe( <i>p</i> -Me)-NH <sub>2</sub>	1.03 $\pm$ 0.25	644.4	644.5	31.4	BG 19

Compound	Sequence	50% Inhibition of human $\alpha$ -thrombin (IC <sub>50</sub> , $\mu$ M)	Expected [M + H]	Found [M + H]	RP-HPLC retention time (min)	Notebook Compound Code
<b>16</b>	3-amidinophenylacetic acid-Oic-Pro-D-Ala-Phe( <i>p</i> -Me)-NH <sub>2</sub>	26.2 $\pm$ 2.3	658.4	658.5	33.4	BG 20
<b>17</b>	( <i>R</i> )-5-guanidino-2-methylpentanoic acid-Oic-Pro-D-Ala-Phe( <i>p</i> -Me)-NH <sub>2</sub>	98 $\pm$ 27	653.4	653.2	31.2	BG 25
<b>18</b>	( <i>S</i> )-5-guanidino-2-methylpentanoic acid-Oic-Pro-D-Ala-Phe( <i>p</i> -Me)-NH <sub>2</sub>	9.8 $\pm$ 1.0	653.4	653.2	35.1	BG 26
<b>19</b>	Arg-Oic-Pro-D-Ala-Phe( <i>p</i> -Me)-NH <sub>2</sub>	73 $\pm$ 36	654.4	654.2	26.7	BG 27
<b>20</b>	( <i>R</i> )-5-amino-2-methylpentanoic acid-Oic-Pro-D-Ala-Phe( <i>p</i> -Me)-NH <sub>2</sub>	2000 $\pm$ 86	611.4	611.2	30.0	BG 25a
<b>21</b>	D-Arg-Oic-Pro-D-Ala-Cha-NH <sub>2</sub>	154 $\pm$ 40	646.4	646.3	27.6	BG 31
<b>22</b>	D-Arg-Oic-Pro-D-Ala-Phe-NH <sub>2</sub>	49.9 $\pm$ 1.6	640.4	640.4	23.8	AF 1
<b>23</b>	D-Arg-Oic-Pro-D-Ala-Aic-NH <sub>2</sub>	345 $\pm$ 15	652.4	652.2	23.3	BG 35
<b>24</b>	D-Arg-Oic-Pro-D-Ala-Idg-NH <sub>2</sub>	70.5 $\pm$ 15.4	666.4	666.2	26.5	BG 33
<b>25</b>	D-Arg-Oic-Pro-D-Ala-Sty-NH <sub>2</sub>	55.5 $\pm$ 3.0	666.4	666.2	27.1	BG 32

Compound	Sequence	50% Inhibition of human $\alpha$ -thrombin (IC <sub>50</sub> , $\mu$ M)	Expected [M + H]	Found [M + H]	RP-HPLC retention time (min)	Notebook Compound Code
26	D-Arg-Oic-Pro-D-Ala-Bip-NH <sub>2</sub>	12.2 $\pm$ 0.9	716.4	716.2	31.8	BG 34
27	D-Arg-Oic-Pro-D-Ala-Phe(F) <sub>5</sub> -NH <sub>2</sub>	567 $\pm$ 181	730.3	730.4	27.8	BG 42
28	D-Arg-Oic-Pro-D-Ala-Phe( <i>p</i> -CF <sub>3</sub> )-NH <sub>2</sub>	12.7 $\pm$ 4.7	708.4	708.4	29.2	BG 41
29	D-Arg-Oic-Pro-D-Ala-Tyr(Me)-NH <sub>2</sub>	28.7 $\pm$ 1.6	670.4	670.3	22.6	BG 59
30	D-Arg-Oic-Pro-D-Ala-Tyr(Bzl)-NH <sub>2</sub>	66.6 $\pm$ 3.7	746.4	746.3	32.3	BG 60L
31	D-Arg-Oic-Pro-D-Ala-Phe( <i>p</i> -carboxamide)-NH <sub>2</sub>	5.8 $\pm$ 1.3	683.4	683.3	16.1	BG 58
32	D-Arg-Oic-Pro-D-Ala-Phe( <i>p</i> - <i>N</i> -phenylacetamide)-NH <sub>2</sub>	392 $\pm$ 39	759.4	759.3	27.3	BG 28
33	D-Arg-Oic-Pro-D-Ala-Phe( <i>p</i> -NHC(O)CH <sub>2</sub> NH <sub>2</sub> )-NH <sub>2</sub>	2426 ( <i>n</i> = 2)	712.4	712.4	15.7	BG 65
34	D-Arg-Oic-Pro-D-Ala-Phe( <i>p</i> -guanidino)-NH <sub>2</sub>	174 $\pm$ 9	697.4	697.4	16.0	BG 61
35	D-Arg-Oic-Pro-D-Ala-D-Phe-NH <sub>2</sub>	697 $\pm$ 8	640.4	640.3	20.6	BG 51
36	D-Arg-Oic-Pro-D-Ala-D-Idg-NH <sub>2</sub>	250 $\pm$ 60	666.4	666.2	23.8	BG 36

Compound	Sequence	50% Inhibition of human $\alpha$ -thrombin (IC <sub>50</sub> , $\mu$ M)	Expected [M + H]	Found [M + H]	RP-HPLC retention time (min)	Notebook Compound Code
37	D-Arg-Oic-Pro-D-Ala-D-Tyr(Bzl)-NH <sub>2</sub>	233 $\pm$ 34	746.4	746.3	27.826	BG 60E
38	D-Arg-Oic-Phe( <i>p</i> -Me)-NH <sub>2</sub>	1178 $\pm$ 212	486.3	486.2	24.9	BG 29
39	D-Arg-Oic-Gly-Phe( <i>p</i> -Me)-NH <sub>2</sub>	1021 $\pm$ 152	543.3	543.2	23.2	BG 30
40	D-Arg-Oic-Azt-D-Ala-Phe( <i>p</i> -Me)-NH <sub>2</sub>	13.3 $\pm$ 0.8	640.4	640.3	24.9	BG 49
41	D-Arg-Oic-Pro-D-Pro-Phe( <i>p</i> -Me)-NH <sub>2</sub>	6.70 $\pm$ 0.43	680.4	680.2	25.5	BG 38
42	D-Arg-Oic-D-Pro-Pro-Phe( <i>p</i> -Me)-NH <sub>2</sub>	13.6 $\pm$ 0.4	680.4	680.2	34.0	BG 37
43	D-Arg-Oic-D-Pro-Pro-Bip-NH <sub>2</sub>	677 $\pm$ 36	742.4	742.4	39.3	BG 40
44	D-Arg-Oic-BZA-Phe( <i>p</i> -Me)-NH <sub>2</sub>	31.7 $\pm$ 12.7	702.4	702.2	29.8	BG 8a
45	D-Arg-Oic-BZA-Phe( <i>p</i> -Me)-NH <sub>2</sub>	484 $\pm$ 22	702.4	702.2	36.4	BG 8b
46	D-Arg-Oic-CPL-Phe( <i>p</i> -Me)-NH <sub>2</sub>	389 $\pm$ 210	654.4	654.4	24.7	BG 9
47	D-Arg-Oic-CGPM	379 $\pm$ 44	560.4	560.3	27.9	BG 39E
48	D-Arg-Oic-CGPM	371 $\pm$ 60	560.4	560.3	32.0	BG 39L
49	D-Arg-Oic-Pro-D-Ala-Phe-NHMe	30.8 $\pm$ 3.5	654.4	654.3	23.9	BG 53
50	D-Arg-Oic-Pro-D-Ala-Phe-NHEt	25.3 $\pm$ 1.7	668.4	668.3	24.6	BG 56

Compound	Sequence	50% Inhibition of human $\alpha$ -thrombin (IC <sub>50</sub> , $\mu$ M)	Expected [M + H]	Found [M + H]	RP-HPLC retention time (min)	Notebook Compound Code
<b>51</b>	D-Arg-Oic-Pro-D-Ala-Phe-NHPr	17.5 $\pm$ 5.1	682.4	682.3	28.2	BG 52
<b>52</b>	D-Arg-Oic-Pro-D-Ala-Phe-NHtPr	31.6 $\pm$ 4.3	722.5	722.5	34.3 (95.81%)	BG 66
<b>53</b>	D-Arg-Oic-Pro-D-Ala-Phe-NHC <sub>6</sub> H <sub>11</sub>	16.0 $\pm$ 1.6	668.4	668.3	24.7	BG 46
<b>54</b>	D-Arg-Oic-Pro-D-Ala-Phe-NHPh	12.9 $\pm$ 2.2	716.4	716.5	32.1	BG 44
<b>55</b>	D-Arg-Oic-Pro-D-Ala-Phe-NHIBn	36.8 $\pm$ 4.7	730.4	730.3	30.8	BG 45
<b>56</b>	5-guanidinopentanoic acid-Oic-Pro-D-Ala-Phe( <i>p</i> -Me)-NHC <sub>6</sub> H <sub>11</sub>	0.53 $\pm$ 0.04	721.5	721.5	41.8	BG 68