Development of a Potent Thrombin Receptor Ligand†

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The N-terminal thrombin receptor peptide H-Ser-Phe-Leu-Leu-Arg-Asn-Pro-Asn-Asp-Lys-Tyr-Glu-Pro-Phe-OH (**1**) fully activates the thrombin receptor with an EC_{50} of 10 μM. Structural features in the tetradecapeptide which are responsible for receptor activation have been elucidated. Agonist potency has been enhanced 1000-fold with the design of the shortened peptide H-Ala-Phe(*p*-F)-Arg-Cha-HArg-Tyr-NH_2 (**56**). This analog exhibits an EC_{50} of 0.01 μM and is the most potent agonist for receptor activation reported to date. The monoiodinated derivative H-Ala-Phe(*p*-F)-Arg-Cha-HArg-Tyr(3-I)-NH_2 (**59**) exhibits an EC_{50} of 0.03 μM, a level sufficient for development of a radioligand.

Introduction

Thrombin plays a pivotal role in blood coagulation. It cleaves fibrinogen to form fibrin and activates factors V, VIII, and XIII and protein C, processes which are essential for the control of thrombosis and hemostasis. Thrombin is the most potent stimulator of platelet aggregation,[1] is chemotactic for monocytes,[2] is mitogenic for lymphocytes and mesenchymal cells including vascular smooth muscle cells,[3] and has effects on vascular endothelium.[4] According to a novel mechanism of receptor activation proposed by Coughlin et al.,[5,6] thrombin cleaves the extracellular domain of the receptor to create a new N-terminus, which can then act as a tethered ligand to activate the receptor. A 14-amino acid peptide, H-Ser-Phe-Leu-Leu-Arg-Asn-Pro-Asn-Asp-Lys-Tyr-Glu-Pro-Phe-OH, derived from the new N-terminus of the receptor is also able to fully activate human platelets and cause aggregation in the absence of thrombin with an EC_{50} of 10 μM. Carboxy-truncated analogs of this 14-residue peptide have also been found to have full receptor-activating potencies.[7–14] In order to develop a high-affinity ligand, structural modifications for agonist potency enhancement were carried out. Structure–activity relationship (SAR) studies resulted in the discovery of potent thrombin receptor-activating peptides. Potent peptides suitable for radiolabeling were also developed.

Experimental Section

Peptide Synthesis. Chemical syntheses were carried out by the solid-phase method using an Applied Biosystems (ABI) 430 A synthesizer on either a 0.5- or 1.0-mmol scale. N^{α}-*tert*-Butyloxycarbonyl protection was employed for all amino acids in the solid-phase synthesis and the following side-chain-protected derivatives: Arg(Tos), HArg(NO_2), N-Me-Arg(Tos), Ser(Bzl), Tyr(Br-Z), Lys(Cl-Z), Glu(Chx), Asp(Chx), His(Bom), and Tyr(3-I)(O-Bzl). Boc-Phe(3,4-Cl_2) or Boc-D-Phe(3,4-Cl_2) was synthesized by standard procedures.[15] Peptides with a C-terminal amide were synthesized employing 4-methylbenzhydrylamine poly(styrene) resin (1% cross-linked S-DVB, 200–400 mesh, 0.77 mol/g; ABI), and peptides with a C-terminal carboxylate were synthesized using Pam resins (ABI). Each amino acid was coupled sequentially to the peptide chain using symmetrical anhydride or HOBt active ester couplings by standard ABI-supplied protocol. For couplings onto an *N*-methyl-amino acid, a single DCC/HOBt coupling was followed by another coupling using (benzotriazolyloxy)bis(pyrrolidino)carbonium hexafluorophosphate. The completion of coupling in each step was monitored by the Kaiser ninhydrin test. After the last amino acid was coupled, the growing peptide on the resin was acylated by using either a mixture of the corresponding acid anhydride and DIEA or the symetrical anhydride of acetic acid. The peptide was cleaved from the resin with concomitant removal of all protecting groups by treatment with distilled HF at 0 °C for 1 h in the presence of ca. 10% anisole or cresol. The HF was evaporated, in vacuo, and the residue was triturated with ether. The crude peptide was removed from the resin by dissolution in aqueous acetic acid, filtration, and lyophilization of the filtrate.

Purification and Characterization. Purification was achieved by preparative HPLC on a Delta-Pak C^{18} column, 300 Å, pore size 15 μm, with 0.1% trifluoroacetic acid–aqueous acetonitrile solvent systems using various linear gradients. Fractions containing product of 99% purity as assayed by HPLC were combined to give samples for bioassay. Peptides were characterized by amino acid analysis, NMR, and FABMS where the molecular ion peak clusters at $(M + H)^+$ and $(M - H)^-$ in the negative ion spectra were observed for each peptide. The purity was further checked by HPLC analysis on a Vydac C_{18} column with 0.1% aqueous TFA–acetonitrile, 0.1 M ammonium acetate–acetonitrile, and 0.1% aqueous ammonium hydrocarbonate–acetonitrile solvent systems using various linear gradients. The flow rate was 1.5 mL/min, and the absorbance was recorded at 210 nm. All compounds showed 99% purity in the three HPLC systems, except anlog **60** which was only assayed in the 0.1% aqueous TFA–acetonitrile system.

Amino acid analyses were carried out on a Beckman 6300 CL amino acid analyzer after hydrolysis in constant boiling HCl for 24 h using standard procedures. The unnatural amino acids were quantified. Analytical data are shown in Table 7.

Platelet Aggregation Assay. Platelet rich plasma (PRP) or washed platelets were incubated with 0.2 mg/mL human fibrinogen for 2 min at 37 °C. Thrombin or a thrombin receptor-derived peptide was added to the sample in a Chronolog aggregometer, and aggregation was monitored as a change in light transmittance. When thrombin was used as an agonist, the PRP was preincubated for 2 min with 2 mM Gly-Pro-Arg-Pro peptide to prevent fibrin polymerization. The final amount of light transmittance (extent of aggregation) or the rate of change of light transmittance (rate of aggregation) was calculated. Biological potency of analogs was measured as the peptide effect on the extent or rate of human blood platelet aggregation.

† Abbreviations: PheGly, phenyl-Gly; APhe, *p*-amino-Phe; Tyr(Me), *O*-methyl-Tyr; Phe(*p*-F), 4-fluoro-Phe; Amf, *p*-(aminomethyl)-Phe; Cha, cyclohexyl-Ala; Tyr(3,5-I_2), 3,5-diiodo-Tyr; HArg, homo-Arg; Tyr(3-I), 3-iodo-Tyr; HPhe, homo-Phe; Phe(3,4-Cl_2), 3,5-dichloro-Phe; 2-Nal, 3-(2′-naphthyl)-Ala.

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Table 1. Effect of Chain Length of Ser-Phe-Leu-Leu-Arg-Asn-Pro-Asn-Asp-Lys-Tyr-Glu-Pro-Phe-OH on Activation of Human Thrombin Receptor

no.	structure	no. of amino acids	platelet aggregation[a] EC_{50} (μM)
1	Ser-Phe-Leu-Leu-Arg-Asn-Pro-Asn-Asp-Lys-Tyr-Glu-Pro-Phe-OH	14	10.3 ± 2.0
2	Ser-Phe-Leu-Leu-Arg-Asn-Pro-Asn-Asp-Lys--OH	10	8.0 ± 0.5
3	Ser-Phe-Leu-Leu-Arg-Asn-Pro-Asn-Asp-Lys-NH_2	10	2.0 ± 0.8
4	Phe-Leu-Leu-Arg-Asn-Pro-Asn-Asp-Lys-NH_2	9	>800
5	Ser-Phe-Leu-Leu-Arg-Asn-Pro-Asn-Asp-NH_2	9	3.3 ± 1.2
6	Ac-Ser-Phe-Leu-Leu-Arg-Asn-Pro-Asn-Asp-NH_2		>800
7	Ser-Phe-Leu-Leu-Arg-Asn-Pro-Asn-NH_2	8	24.5 ± 0.5
8	Ser-Phe-Leu-Leu-Arg-Asn-Pro-NH_2	7	0.5 ± 0.1
9	Ser-Phe-Leu-Leu-Arg-Asn-NH_2	6	0.2 ± 0.0
10	Ser-Phe-Leu-Leu-Arg-NH_2	5	1.0 ± 0.5
11	Ser-Phe-Leu-Leu-NH_2	4	185 ± 1.2
12	Ser-Phe-NH_2	2	>800

[a] EC_{50} for stimulation of aggregation of human platelets.

Table 2. Alanine, *N*-Methyl-, and D-Amino Acid Scans

no.	structure						platelet aggregation EC_{50} (μM)
10	Ser	Phe	Leu	Leu	Arg	-NH_2	1.0 ± 0.5
13	DSer						800 ± 0
14		DPhe[a]					>800
15			DLeu[b]				>800
16				DLeu			500 ± 10
17					DArg		109.3 ± 13.3
18	Ala	Phe	Leu	Leu	Arg	-NH_2	0.8 ± 0.1
19		Ala					>800
20			Ala				1.3 ± 0.5
21				Ala			15 ± 1.2
22					Ala		70 ± 21
23	N-Me-Ala						600 ± 60
24		N-Me-Phe					>800
25			N-Me-Leu				350 ± 10
26				N-Me-Leu			260 ± 10
27					N-Me-Arg		800

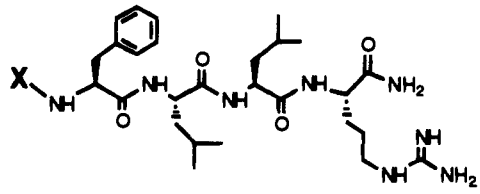
[a] Ala^1 in analogs **26** and **27**.

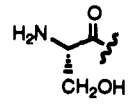
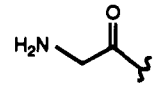
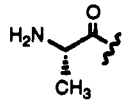
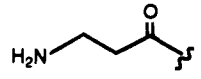
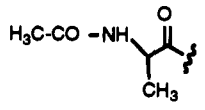
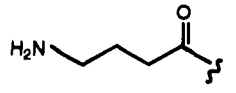
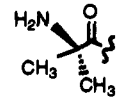
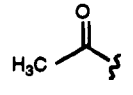
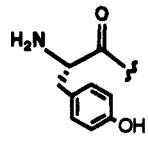
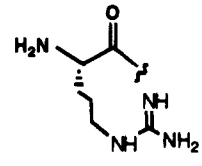
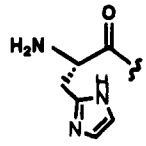
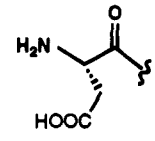
Results and Discussion

The activity of the previously described,[1] 14-residue peptide H-Ser-Phe-Leu-Leu-Arg-Asn-Pro-Asn-Asp-Lys-Tyr-Glu-Pro-Phe-OH (**1**) was confirmed as 10 μM. Toward the goal of improving the potency of this natural ligand, our initial studies focused on elucidation of the effect of chain length on agonist activity. The results of activation of the human thrombin receptor as measured by platelet aggregation using truncated analogs of the tetradecapeptide **1** are shown in Table 1. Shortening chain length from the C-terminus to the pentapeptide **10** retained potency or showed increased potency with the exception of the octapeptide **7** which showed decreased potency. The pentapeptide analog H-Ser-Phe-Leu-Leu-Arg-NH_2 (**10**) displayed a 10-fold enhancement in potency when compared with the 14-residue prototype peptide **1**. The hexapeptide **9** displayed optimal potency. Shortening to the tetrapeptide **11** dramatically decreases potency, and dipeptide **12** was completely inactive. In contrast to successful C-terminal shortning, N-terminal modifications, such as deletion or N-acetylation, resulted in totally inactive compounds (**4**, **6**). When the C-terminal carboxyl group of **2** was replaced with an amide group (**3**), agonist potency increased 4-fold. This suggests that a neutral group at the C-terminus is preferable. Further studies therefore focused on C-terminal amides. Our initial studies had suggested that pentapeptide **10** might contain the minimum structural requirements for effective binding to the thrombin receptor, so we undertook an SAR investigation based on analog **10** used as a lead for further structural modification and optimization. It was only later that the hexapeptide **9** was found to be optimal, so that it is only the final group of compounds that incorporate the sixth amino acid.

The systematic replacement of each amino acid of a bioactive peptide with alanine gives an indication of the importance of the side-chain functional group at the replacement residue. The systematic replacement of each residue with N-methylated or D-residues can give important information about the receptor-bound conformation when an active analog is obtained. The results of Ala, *N*-methyl, and D-residue scans of analog **10** are shown in Table 2. Ser^1 and Leu^3 can be replaced by Ala (**18**, **20**) with complete retention of potency. The hydrophobic side chain of Phe^2, however, is critical for receptor activation, as indicated by the inactive analog **19**. Some degree of binding is contributed by the side chains of Leu^4 and Arg^5, as indicated by the loss of activity seen in analogs **21** and **22**. N-Methylation of peptide bonds is compatable with activity when the amide N-H is not involved in hydrogen bonding and when the active conformation is not disrupted by this bulky group. The N-methylated analogs **23**–**27** displayed a detrimental effect on potency, suggesting that the unmethylated nitrogens in the peptide backbone are required to retain potency. D-Amino acid substitution can also provide important conformational information if an active analog is obtained. Substitution with D-amino acids at position 1, 2, or 3 (**13**–**15**) results in analogs which show no activity at 800 μM. D-Residues

Table 3. Effect of Modifications in Position 1 on Agonist Potencies



No.	X	Plat. Aggr. EC_{50} (μM)	No.	X	Plat. Aggr. EC_{50} (μM)
10		1.0±0.5	31		1.2±0.1
18		0.8±0.1	32		35.0±0.0
23		600±60	33		730±10.0
28		415±125	34		200±30
29		400±85	35		>1200
30		31±10	36		>800
			37	—	>800

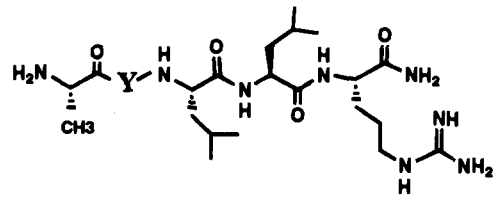
in position 4 or 5 (**16** or **17**) also reduce potency significantly. The L-stereochemistry is, therefore, essential for receptor recognition.

In the pentapeptide series, side-chain modifications of position 1 were made, and the bioassay results are shown in Table 3. A neutral, short, or no side chain in position 1 is preferred for agonist activity, since removal of the hydroxyl group of Ser^1, as in the Ala analog **18**, does not alter the potency. Even deletion of this side chain as in Gly^1 (**31**) also retained full potency. Replacement of Ser^1 with the hydrophilic, charged residues Asp (**36**) and Arg (**35**), or the aromatic residues Tyr (**29**) and His (**30**), causes a severe loss of potency.

An investigation of the N-terminus revealed the considerable contribution of the amino group to agonist potency. As shown in Table 3, removal of this functionality (analog **34**) from Gly reduces potency by about 200-fold. Extension of peptide backbone by the insertion of one (**32**) or two (**33**) CH_2 groups results in a severe loss of potency, possibly because these important amino groups are displayed relative to other receptor-binding elements.

Since alanine in position 1 appeared to be slightly more active, analog **18** became a basis structure for further structure–activity investigation of the adjacent amino acid residue (Phe). The replacement of Phe^2 with Ala causes a total loss in activity.[6,7] As illustrated in Table 4, there appears to be strong preference for a particular placement of the phenyl ring at this position, since replacement with HPhe (**39**) or PhGly (**38**) results in no activity at 800 μM. Derivatives with electron-withdrawing, aromatic ring substituents as is seen in analogs Phe(*p*-Cl) (**47**), Phe(3,4-Cl_2) (**46**), or Phe(*p*-F) (**48**) (EC_{50}s 0.7–0.2 μM) appear to be more potent than the unsubstituted one (**18**); however, analogs with electron-donating substituents on the aromatic ring as in analogs Tyr(Me) (**45**), APhe (**43**), or Amf (**44**) (EC_{50}s 21–800 μM) are much less potent. The most effective side chain at this position is the *p*-fluorophenyl (**48**) which shows a 4-fold potency enhancement over phenyl. Analogs having a saturated ring (**40**) or a naphthyl (**41**) at residue 2 show reduced potency. Incorporation of the strongly electron deficient aromatic pentafluorophenyl group (**42**) causes a decrease from 0.8 to 600 μM (EC_{50}).

Table 4. Effect of Modifications in Position 2 on Agonist Potencies



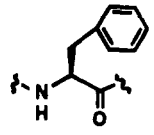
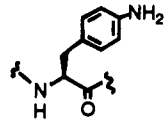
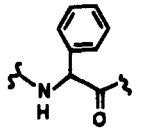
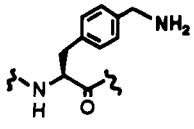
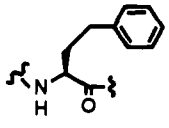
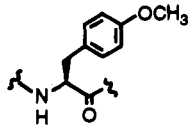
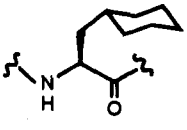
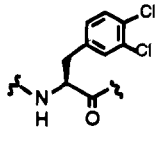
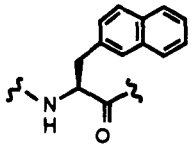
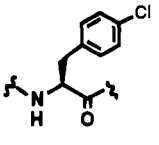
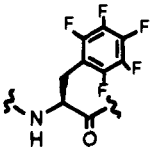
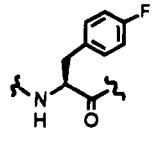
No.	Y	Plat. Aggr. EC_{50} (μM)	No.	Y	Plat. Aggr. EC_{50} (μM)
18		0.8±0.1	**43**		235±80
38		>800	**44**		>800
39		>800	**45**		21.4±4.0
40		28.0±3.0	**46**		0.5±0.1
41		19.0±0.1	**47**		0.7±0.3
42		600±50	**48**		0.2±0.0

Table 5. Effect of Modifications in Positions 3–5 on Agonist Potencies

no.	structure	platelet aggregation EC_{50} (μM)
18	Ala Phe Leu Leu Arg -NH_2	0.8 ± 0.1
48	Ala Phe(*p*-F) Leu Leu Arg -NH_2	0.2 ± 0.05
51	Ala Phe Leu Cha Arg -NH_2	0.4 ± 0.14
52	Ala Phe(*p*-F) Leu Cha Arg -NH_2	0.14 ± 0.025
53	Arg	0.13 ± 0.06
54	Arg	40 ± 4.0
55	HArg	0.12 ± 0.03
56	Arg Arg -NHEt -NH_2	0.16 ± 0.02
57	Arg HArg -NH_2	0.27 ± 0.11
58	Arg Harg Tyr -NH_2	0.01 ± 0.005

In positions 3–5, combinations of individual position modifications have been carried out using Ala-Phe-Leu-Leu-Arg-NH_2 (**18**) as base structure. Leu[4] can be replaced with the more hydrophobic cyclohexylalanine (Cha) with a 2-fold increase in potency in either the phenyl (**49**) or the *p*-fluorophenyl (**50**) series. The most potent analog, Ala-Phe(*p*-F)-Leu-Cha-Arg-NH_2 (**50**), has an EC_{50} of 0.14 μM. An arginine at position 3 (**51**) retains activity and gives added solubility. HArg[5] appeared in the bioassy to give a small increase in potency (**53**). In contrast, a change to Arg at position 4 (**52**) reduces potency dramatically. Moreover, replacement of the C-terminal amide with an ethylamide does not alter the potency (**54**).

Table 6. Activity of Potential Radioligand Peptides

no.	structure	platelet aggregation EC_{50} (μM)
57	Ala-Phe(3,4-Cl_2)-Leu-Cha-Arg-NH_2	0.3 ± 0.1
58	Ala-Phe(3,4-Cl_2)-Tyr(3,5-I_2)-Cha-Arg-NH_2	1.5 ± 0.5
56	Ala-Phe(*p*-F)-Arg-Cha-HArg-Tyr-NH_2	0.01 ± 0.005
59	Ala-Phe(*p*-F)-Arg-Cha-HArg-Tyr(3-I)-NH_2	0.03 ± 0.01
60	Ala-Phe(*p*-F)-Arg-Cha-HArg-Tyr(3,5-I_2)-NH_2	0.15 ± 0.07

Table 7. Chromatographic Data and Amino Acid Analyses on Thrombin Agonist Peptides

no.	HPLC[a] t_R (min)	purity (%)	FABMS	amino acid analyses									
				Ser	Phe	Leu	Arg	Ala	Pro	Lys	Asp	Tyr	Glu
1	21.30	99	1740	0.93	2.00	2.05	1.00		2.01	0.99	2.98	1.01	1.02
2	16.82	99	1203	0.98	1.00	2.04	0.94		1.02	1.00	2.97		
3	14.93	99	1202	0.93	1.02	2.08	0.98		1.01	1.01	2.98		
4	14.19	99	1115		1.00	2.04	0.98		1.02	1.01	2.95		
5	15.53	99	1076	0.91	1.00	2.03	0.98		1.02		3.06		
6	18.20	99	1116	0.97	1.00	2.06	0.95		0.98		3.01		
7	15.49	99	959	0.93	0.90	2.03	0.97		1.02		2.16		
8	15.90	99	846	0.92	0.98	2.01	0.99		0.99		1.00		
9	15.36	99	749	1.00	1.00	2.03	0.97				1.00		
10	15.85	99	634	0.98	0.99	2.05	0.97						
11	17.56	99	478	1.00	0.98	2.02							
12	5.11	99	252	1.00	1.00								
13	16.20	99	634	0.93	1.00	2.00	1.02						
14	20.12	99	618		0.99	2.04	0.97	1.00					
15	17.11	99	618		1.00	2.03	0.98	1.02					
16	17.42	99	634	1.00	0.99	2.01	0.99						
17	16.20	99	634	1.02	1.00	2.03	0.96						
18	15.88	99	618		0.99	2.01	1.01	0.99					
19	10.77	99	558	0.99		1.99	1.01	1.00					
20	12.77	99	592	0.99	1.00	1.01	1.01	1.00					
21	12.41	99	592	1.00	0.99	1.01	1.02	0.99					
22	15.90	99	549	1.00	1.00	2.03		0.97					
23	16.63	99	632		0.99	1.99	0.97		N-Me-Ala 1.04				
24	17.44	99	632			2.04	1.04	1.03	N-Me-Phe 0.89				
25	16.48	99	632		0.92	1.07	1.00	1.01					
26	16.43	99	632		0.98	1.00	0.98	1.04					
27	18.20	99	632		0.98	2.01		1.00	N-Me-Arg 0.94				
28	16.10	99	632		0.99	2.03	1.00		α-Me-Ala 0.97				
				Ala	Phe	Leu	Arg	others					
29	17.89	99	710		0.98	2.00	1.02	Tyr 0.99					
30	15.10	99	684		0.98	2.01	1.00	His 1.01					
31	16.35	99	604		0.98	2.00	0.99	Gly 1.03					
32	13.40	99	618		0.99	2.05	1.00	β-Ala 0.96					
33	16.26	99	632		0.97	2.06	0.97	γ-Abu 1.03					
34	19.98	99	586		0.99	2.02	0.98						
35	14.54	99	703		0.99	2.00	2.00						
36	16.24	99	662		0.98	2.01	0.99	Asp 1.01					
37	14.70	99	547		0.99	2.02	0.98						
38	20.97	99	604	0.99		2.02	0.99	PheGly 0.94					
39	17.62	99	632	0.99		1.98	0.99	HPhe 1.04					
40	18.28	99	624	0.99		2.02	1.00	Cha 0.98					
41	16.43	99	667	0.91		2.00	1.05	2Nal(+)					
42	18.37	99	708	1.01		2.02	0.95	F_5Phe 1.02					
43	11.61	99	633	1.02		1.97	1.01	p-NH_2Phe 0.95					
44	11.90	99	647	1.00		2.05	1.02	Amf 0.92					
45	22.31	99	648	0.98		1.98	0.98	Tyr(Me) 1.06					
46	20.13	99	686	1.03		2.01	1.00	Phe(3,4-Cl_2) 0.96					
47	18.27	99	652	1.00		2.00	0.99	Phe(p-Cl) 1.00					
48	16.59	99	636	1.02		1.99	1.01	Phe(p-F) 0.98					
49	17.38	99	658	1.04	0.97	0.98	1.02	Cha 0.98					
50	20.36	99	676	1.01		0.97	1.03	Phe(p-F) 1.02, Cha 0.97					
51	17.36	99	719	0.97			2.06	Phe(p-F) 0.99, Cha 0.97					
52	12.43	99	679	0.99		0.96	2.04	Phe(p-F) 1.01					
53	19.90	99	690	0.99		0.99		Phe(p-F) 1.02, Cha 0.97, HArg 1.06					
54	19.16	99	747	1.03			1.96	Phe(p-F) 1.04, Cha 0.96					
55	17.53	99	733	0.98			0.96	Phe(p-F) 1.01, Cha 0.97, HArg 1.08					
56	18.95	99	897	1.03			1.01	Phe(p-F) 0.93, Cha 0.98, Tyr 1.01, HArg 1.02					
57	23.25	99	726	1.04		1.05	1.01	Phe(3,4-Cl_2) 0.96, Cha 0.94					
58	24.02	99	1028	1.03			1.01	Phe(3,4-Cl_2) 1.02, Cha 0.97, Tyr(3,5-I_2) 1.05					
59	21.13	99	1022	1.07			1.08	Phe(p-F) 1.03, Cha 0.90, HArg 1.00, Tyr(3-I) 0.92					
60	22.47	99	1148	0.99			0.95	Phe(p-F) 1.02, Cha 0.98, Tyr(3,5-I_2) 0.98, HArg 1.08					

[a] HPLC on a column of Vydac C_{18} (3.9 cm × 30 cm). Gradient: 95% A–50% A, 30 min. A: 0.1% TFA. B: 0.1% TFA in CH_3CN. N-Me-Leu does not give a ninhydrin reaction. Gradient: 80–45% A, 30 min.

When it was discovered that the C-terminally extended hexapeptide **9** is more potent than the pentapeptide **10**, we decided to use this additional side chain as a site for possible radiolabeling. The introduction of tyrosine in position 6 of the modified pentapeptide **55** further enhances potency and offers a site for radiolabeling with iodine. This modification resulted in Ala-Phe(*p*-F)-Arg-Cha-HArg-Tyr-NH_2 (**56**) with an EC_{50} of 0.01 μM, a 27-fold potency enhancement over the corresponding pentapeptide **55**. Analog **56** is the most potent agonist at the thrombin receptor reported to date. The replacement of Tyr with Tyr(3-I) resulted in Ala-Phe(*p*-F)-Arg-Cha-HArg-Tyr(3-I)-NH_2 (**59**) (Table 6), which shows an EC_{50} of 0.03 μM. This level of potency is useful as a receptor radioligand for a binding assay (details of this assay will be published elsewhere). In

contrast, replacement of Leu in position 3 of analog **57** with Tyr(3,5-I_2) to give analog **58** resulted in a 5-fold loss of potency. When Tyr(3,5-I_2) is placed in position 6 (**60**), a significant further loss of potency also is seen. These latter two analogs have not been deemed suitable for development of a binding assay.

Summary

The important binding features of hexapeptide agonists of the thrombin receptor have been elucidated. A small neutral side chain is preferred in position 1. Small, electron-withdrawing groups on aromatic rings in position 2 increase potency. Neutral or basic side chains are preferred in position 3, and aliphatic side chains are preferred in position 4. A cationic binding element is necessary in position 5. A basic side chain in position 3, when combined with Tyr in position 6, is beneficial for potency and solubility. L-Stereochemistry at all centers is necessary for agonist activity.

Through the use of this systematic approach to the development of peptide SARs, the agonist potency of thrombin receptor peptides has been enhanced about 1000-fold. The introduction of unnatural amino acid side chains has provided potential tool compounds for the development of a receptor radioligand and establishment of a binding assay.

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