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THROMBIN RECEPTOR (PAR-1) ANTAGONISTS. HETEROCYCLE-BASED PEPTIDOMIMETICS OF THE SFLLR AGONIST MOTIF

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Abstract: The thrombin receptor (PAR-1) is activated by α-thrombin to stimulate various cell types, including platelets, through the tethered-ligand sequence SFLLRN. A series of azole-based carboxamides, designed after SFLLR, were synthesized and evaluated in vitro. The compounds inhibited platelet aggregation induced by SFLLRN-NH_2 or α-thrombin, and blocked the binding of [^{3}H]-S-(*p*-F-Phe)-Har-L-Har-KY-NH_2 to a CHRF membrane preparation of PAR-1. Oxazole **30** bound to PAR-1 with an IC_{50} of 1.6 μM, and gave IC_{50} values of 25 μM and 6.6 μM against α-thrombin- and SFLLRN-NH_2-induced platelet aggregation, respectively.

Thrombin is a key trypsin-like serine protease in hemostasis that has roles in both coagulation and thrombosis. Its various cellular actions, such as platelet aggregation, lymphocyte mitosis, monocyte chemotaxis, and endothelial cell proliferation,[1-5] are mediated by specific receptors on the cell surface. The first example of such a receptor was the human thrombin receptor (protease-activated receptor-1; PAR-1), which Coughlin and coworkers cloned, expressed, and identified as a member of the vast G-protein coupled receptor (GPCR) superfamily.[6] Thrombin activates PAR-1 by proteolytic cleavage of the extracellular domain at the Arg-41/Ser-42 peptide bond to reveal a truncated N-terminus containing the activation sequence SFLLRN, which serves as a "tethered ligand." Recently, other protease-activated receptors with close homology to the thrombin receptor, PAR-2,[7,8] PAR-3,[9a] and PAR-4,[9b,c] have been cloned as well.

Structure-activity relationships of SFLLR-based agonists have received considerable attention in platelet activation studies.[4,5,10-12] The minimum structural requirements for agonist peptides are, generally, a small uncapped N-terminal residue at position 1, an aromatic residue at position 2 (with agonist potency enhanced ca. 4-fold by *p*-F-Phe substitution[13]), and a basic or aromatic residue at position 5. A large hydrophobic amino acid at position 4 is important as well, but widely varied substitution is tolerated at position 3.

Some SFLLR analogues have proven to be antagonists of PAR-1 activation in terms of blocking platelet aggregation.[12-14] In fact, peptide analogues with a cinnamoyl-(*p*-F-Phe) at positions 1/2 and a *p*-guanidino-Phe at position 3 are potent inhibitors of platelet aggregation induced by SFLLRNP-NH_2, although no results were reported relative to inhibition of aggregation induced by α-thrombin.[14,15] For example, *E*-cinnamoyl-(*p*-F-Phe)-(*p*-guanidino-Phe)-LRR-NH_2 exhibited an IC_{50} value of 0.021 μM in platelet aggregation induced by SFLLRNP-NH_2 and an IC_{50} value of 0.0075 μM in binding to PAR-1 (vs. [^{3}H]-SFFLRR-NH_2).[14,15] Thus, there is a need for new thrombin receptor antagonists that work against both agonist peptides and α-thrombin, the endogenous activator.

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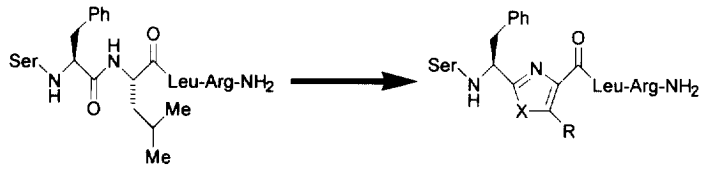
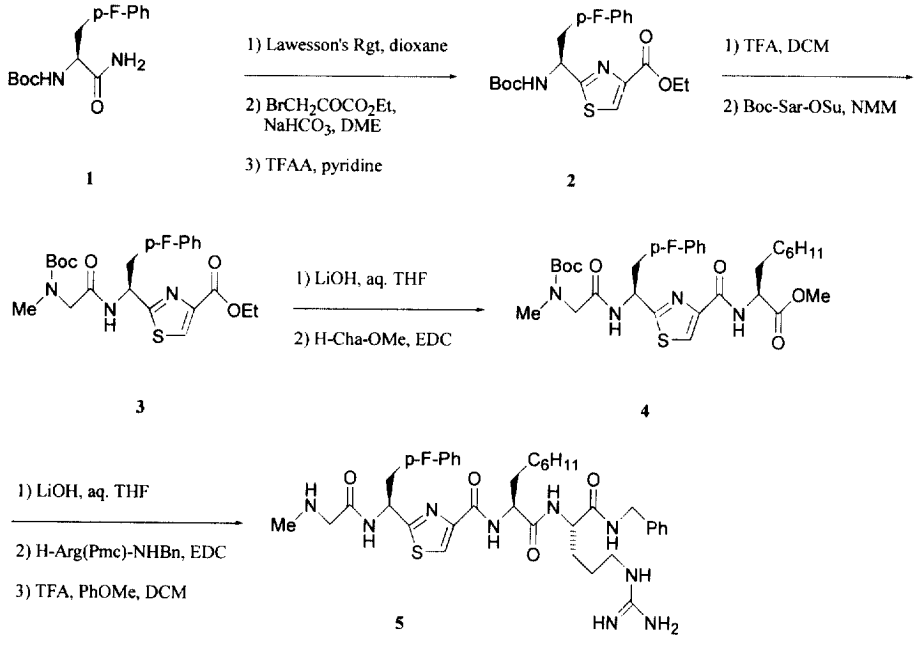


(1)

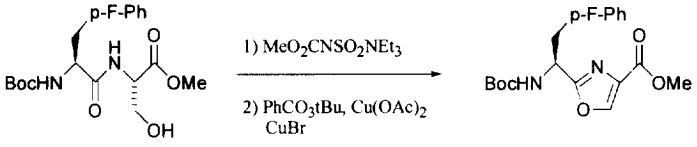
Given the flexibility for substitution at position 3 in SFLLR analogues, we decided to investigate a heterocyclic replacement at positions 2/3, employing a central constraint in the peptide backbone in the form of an azole entity (Eq 1, X = S or O),[16] with a *p*-F-Phe substituent to represent the best side chain at position 2 of SFLLR. This dipeptide mimetic would alter the preferred conformations of the backbone at the 2/3 position, as well as influence the adjacent residues, especially in the context of ligand-receptor interaction. Significantly, this change has led to a series of novel azole-based thrombin receptor (PAR-1) antagonists, which inhibit platelet aggregation induced not only by SFLLRN-NH_2, but also by α-thrombin.



(2)

Analogue Synthesis. The azole-based dipeptide mimetics (e.g., **2**) were synthesized in solution and then carried forward by using standard peptide synthesis techniques (solid or liquid phase) to furnish the azole targets. For the thiazole compounds, the first step was conversion of a Boc-Phe-NH_2 to a thioamide by using Lawesson's reagent. Hantzsch cyclization of the thioamide with bromopyruvate readily yielded key intermediate **2** (Eq 2).[17] The Boc esters represented by **2** were deprotected at the N-terminus with trifluoroacetic acid (TFA) and acylated with *N*-oxysuccinimide-activated Boc-amino acids to give pseudotripeptides (**3**). Homologation of the C-terminus was initiated by ester hydrolysis with LiOH followed by sequential 3-ethyl-1-(3-dimethylaminopropyl)carbodiimide (EDC)-mediated coupling with a hydrophobic α-amino ester, ester

hydrolysis, and coupling again with a basic residue. The stereochemical integrity at the α-position was maintained during saponification of the Cha-type residue with <1% isomerization. Typically, the basic residue was capped as a benzylamide for superior activity (e.g., Arg-NHBn). The arginine side chain was protected with the Pmc (2,2,5,7,8-pentamethylchroman-6-sulfonyl) group, which is conveniently labile to TFA.



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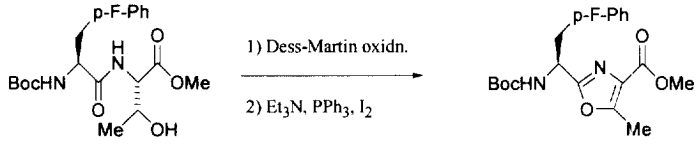
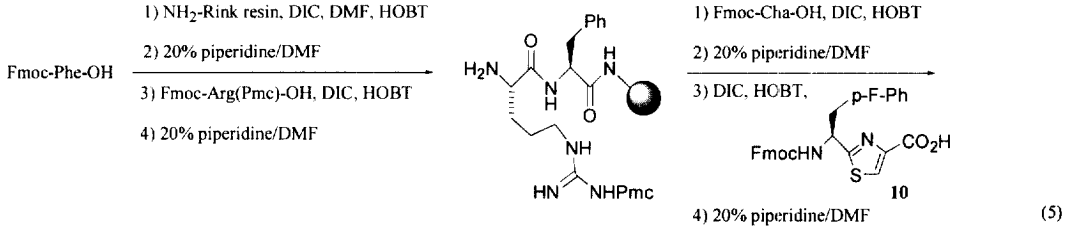


(4)

8 9

The oxazole targets were synthesized by using similar solution-phase methodology. A serine-derived oxazole dipeptide mimetic was prepared by assembling Boc-(*p*-F-Phe)-Ser-OMe (EDC·HCl in CH_2Cl_2; **6**) and cyclizing it with Burgess' reagent to an oxazoline (Eq 3),[18] which was oxidized to an oxazole with *tert*-butylperoxybenzoate/copper (II);[19] intermediate **7** was then used as shown in Eq 2 to prepare target compounds. 5-Methyloxazole **9** was prepared from **8** by reversing the two-step sequence (Eq 4). First, **8** was converted to



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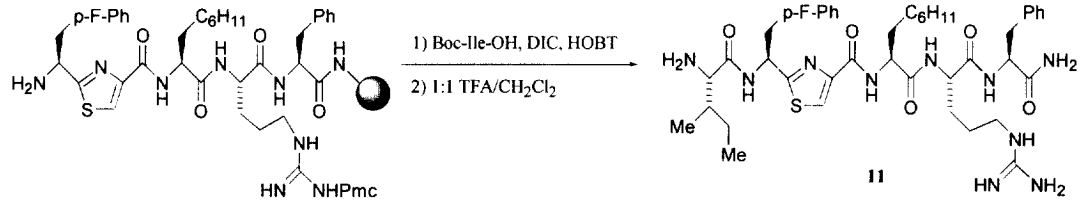
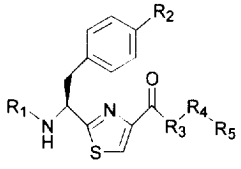


a methyl ketone by Dess-Martin oxidation, then cyclized with I_2/Ph_3P to afford **9**.[20] Thiazole intermediate **2** turned out to be the most synthetically accessible vis-à-vis oxazoles **7** and **9**; a high-yielding cyclization and a straightforward purification contributed to a useful process for the synthesis of the final thiazole targets.

Fmoc-protected thiazole **10** was introduced at an intermediate stage of our solid-phase synthesis of C-terminal phenylalanine amides (Eq 5). The C-terminal Phe was anchored to a Rink resin for parallel synthesis of arrays of targets. Iterative Fmoc removal and diisopropylcarbodiimide (DIC)/1-hydroxybenzotriazole (HOBT) coupling, then TFA deprotection/resin cleavage, afforded reasonably pure products (>90%) in ca. 50 mg quantities. Given the calculated initial resin loading of 1.0 mmol/g, isolated yields were ca. 40-60%.

Table 1. Platelet aggregation and PAR-1 binding IC_{50} values (μM) for thiazoles

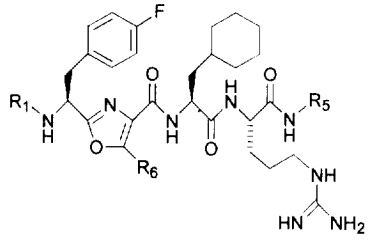


No.[a]	R_1[b]	R_2	R_3[c]	R_4	R_5	IC_{50}, Human GFP[d] Thrombin	IC_{50}, Human GFP[d] TRAP-6	IC_{50}, PAR-1 Binding[e]
5	Sar	F	Cha	Arg	NHBn	27 ± 4.4	11 ± 1.7	10 ± 3.5
11	Ile	F	Cha	Arg	Phe-NH_2	32 ± 6.7	4.0 ± 1.3	3.9 ± 0.4
12	Sar	F	Cha	Arg	Phe-NH_2	41 ± 8.3	14 ± 2.4	5.7 ± 1.8
13	Val	F	Cha	Arg	Phe-NH_2	22 ± 7.8	4.6 ± 3.5	2.7 ± 0.5
14	Val	OMe	Cha	Arg	Phe-NH_2	49 ± 13	9.5 ± 1.4	8.2 ± 0.8
15	H	F	Cha	Arg	Phe-NH_2	28 ± 8.0	16 ± 4.2	8.0
16	Sar	F	Nip	Arg	Phe-NH_2	>100	>100	>100
17	Sar	F	Arg	Arg	Phe-NH_2	>100	>100	>100
18	Sar	F	D-Cha	Arg	Phe-NH_2	96 ± 1.2	42 ± 3.6	94 ± 4.8
19	Ile	F	N-Bn-Gly	Arg	Phe-NH_2	>100	80 ± 17	>100
20	Sar	F	Cha	Har	Phe-NH_2	59 ± 7.3	7.7 ± 1.8	5.0
21	Sar	F	Cha	Arg	Cha-NH_2	46 ± 6.1	6.8 ± 1.7	2.7 ± 0.5
22	Sar	F	Cha	Arg	hPhe-NH_2	45	5.5	4.1 ± 0.9
23	Sar	F	Cha	Arg	$NH(CH_2)_2Ph$	27 ± 6.8	10 ± 3.6	5.6 ± 1.8
24	Sar	F	Lys	Arg	$NH(CH_2)_2Ph$	>100	>100	>100
25	Sar	F	Phe	Arg	NHBn	46 ± 8.8	13 ± 2.8	24 ± 4.8
26	Sar	F	Cha	Arg	$NHCH_2CH(Me)Ph$	40 ± 3.5	16 ± 4.1	24 ± 6.

a. See ref 21. b. Sar = sarcosine. c. Cha = cyclohexylalanine; Nip = nipecotic acid. d. α-Thrombin- or SFLLRN-NH_2-induced gel-filtered platelet aggregation (at least n = 2; n = 1 for values without error limits).[22] A level of 80-100% aggregation was achieved at a single concentration per platelet preparation. For SFLLRN-NH_2 (EC_{50} = 0.30 ± 0.15 μM), the concentration was 0.5-1.0 μM; for thrombin (EC_{50} = 73 ± 12 pM), the concentration was 0.05-0.5 nM. e. Inhibition of [^{3}H]-S-(*p*-F-Phe)-Har-L-Har-KY-NH_2 binding to a thrombin receptor membrane preparation (n = 2; n = 1 for values without error limits).[23]

Biological Results. The thiazole (Table 1) and oxazole (Table 2) derivatives[21] exemplify 120 target compounds that were synthesized and tested for inhibition of thrombin- and SFLLRN-NH_2-induced platelet aggregation,[22] as well as competitive binding of [^{3}H]-S-(*p*-F-Phe)-Har-L-Har-KY-NH_2 (Har = homoarginine) to a membrane preparation of PAR-1.[23] Since little difference in potency was observed between the two heterocycles, thiazoles in excess of oxazoles were prepared because of their relative ease of synthesis. In the oxazole series, H at R_6 was preferred over Me for both binding and inhibition of SFLLR-induced aggregation (**30** vs. **31**). At the N-terminal R_1 position of either series, a small uncapped residue (i.e., Sar or Gly) was preferred for inhibition of binding (**30**, IC_{50} = 1.6 μM). Fluoro substitution at R_2 was important for activity as H (**34**) or OMe (**14**) substitution afforded only weak antagonists (Table 1). At the R_3 site, a large hydrophobic residue of L-configuration (e.g., L-Cha) appeared necessary, and a guanidine-bearing residue of L-configuration at the R_4 residue was crucial for activity (Table 1). Interestingly, each of these aforementioned amino acid criteria are reflective of the SAR for SFLLR-based agonists. Since an aromatic residue at R_5 had provided good agonist activity,[12] a Phe or aralkyl group was incorporated in these series with success (Tables 1 and 2); an aliphatic residue was also acceptable (**21**). The role of these agents in PAR-2, PAR-3, and PAR-4 recognition is yet to be determined.

Table 2. Platelet aggregation and PAR-1 binding IC_{50} values (μM) for oxazoles



No.[a]	R_1[b]	R_6	R_5	IC_{50}, Human GFP[c] Thrombin	TRAP-6	IC_{50}, PAR-1 Binding[c]
27	Gly	H	H	19 ± 0.6	13 ± 4.2	6.2 ± 0.5
28	Gly	H	CH_2Ph	17 ± 4.2	10 ± 1.7	5.4 ± 0.2
29	β-Ala	H	CH_2Ph	>100	36 ± 15	6.7 ± 1.3
30	Sar	H	CH_2Ph	25 ± 2.5	6.6 ± 1.5	1.6 ± 0.5
31	Sar	Me	CH_2Ph	23 ± 3.0	18 ± 2.5	5.0 ± 2.0
32	Dpr	Me	CH_2Ph	24 ± 3.1	25 ± 6.9	53 ± 18
33	Ac	Me	CH_2Ph	18 ± 2.7	19 ± 4.9	30 ± 3.0
34[d]	Sar	Me	CH_2Ph	45 ± 4.6	36 ± 7.3	60

a. See ref 21. b. Dpr = 2,3-diaminopropionic acid; Sar = sarcosine. c. See Table 1. d. Des-fluoro-Phe(oxazole).

In conclusion, Sar-oxazole **30** exhibited superior thrombin receptor affinity (IC_{50} = 1.6 μM), while Ile-thiazole **11** exhibited the best inhibition of platelet aggregation induced by TRAP-6 (IC_{50} = 4.0 μM). Since thrombin is the endogenous agonist for PAR-1, a prerequisite of pharmacological efficacy for a PAR-1 antagonist would be inhibition of thrombin-induced platelet aggregation. To this end, several analogues (**13**,

27, **28**, **30-33**) had thrombin-mediated IC_{50} values in the vicinity of 20 μM, while exhibiting similar inhibitory potency against TRAP-6. Representative compounds from our azole series were inactive in a chromogenic assay for thrombin inhibition at 100 μM, thereby excluding a direct enzyme-based mechanism of inhibition. Furthermore, antagonist **30** displayed 10-fold weaker platelet aggregation inhibition with arachidonic acid as the agonist (IC_{50} = 63 μM) than with TRAP-6. Thus, the PAR-1 affinity and inhibition of SFLLRN-NH_2-induced platelet aggregation are consistent with a thrombin receptor mechanism for the biological activity.

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21. All target compounds were homogenous by HPLC with satisfactory ^{1}H NMR and FABMS data. C-Terminal secondary amides exhibited satisfactory combustion analysis as well. For example, oxazole **30** was isolated as a white powder: mp 127-130°C; $[\alpha]^{25}_D$ -21.8° (*c* 0.28, MeOH). FABMS m/*z* 720 (MH^+); ^{1}H NMR (DMSO-d_6, 400 MHz) δ 0.9 (m, 2 H), 1.1 (m, 4 H), 1.2 (m, 2 H), 1.4-1.8 (m, 12 H), 2.38 (s, 3 H), 3.1 (m, 2 H), 3.3 (m, 2 H), 3.6 (m, 2 H), 4.3 (m, 4 H), 4.6 (m, 1 H), 5.3 (m, 1 H), 7.1 (m, 2 H), 7.2 (m, 7 H), 7.5 (m, 1 H), 7.92 (d, J = 6 Hz, 1 H), 8.3 (m, 4 H), 8.60 (s, 1 H), 9.1 (m, 1 H). Analysis calculated for $C_{37}H_{50}FN_9O_5$•2 TFA: C, 51.95; H, 5.53; N, 13.30. Found: C, 51.55; H, 5.78; N, 13.05.
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