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MACROCYCLIC HEXAPEPTIDE ANALOGUES OF THE THROMBIN RECEPTOR (PAR-1) ACTIVATION MOTIF SFLLRN

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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. Macrocyclic peptide analogues of SFLLRN were synthesized and evaluated in vitro. In general, the compounds were much less potent in inducing platelet aggregation relative to SFLLRN-NH_2 and did not act as antagonists of α-thrombin. Derivative **3c** was the most potent macrocycle in activating PAR-1, with an EC_{50} of 24 μM.

α-Thrombin, a trypsin-like serine protease centrally involved in hemostasis, also promotes diverse cellular responses such as platelet aggregation, lymphocyte mitosis, monocyte chemotaxis, and endothelial cell proliferation.[1] These actions are mediated by specific receptors on the cell surface, which eluded identification for many years. However, in 1991 Coughlin and coworkers cloned and expressed the first example of a thrombin receptor (protease-activated receptor 1; PAR-1) and identified it as a member of the vast G-protein coupled receptor (GPCR) superfamily.[2] Thrombin proteolytically cleaves the lengthy N-terminal extracellular domain of PAR-1 at the Arg-41/Ser-42 peptide bond to reveal a new N-terminus containing the sequence SFLLRN, which apparently serves as a "tethered ligand." Significantly, synthetic SFLLRN-NH_2 (TRAP-6), and peptide analogues thereof, are capable of activating PAR-1 in a manner similar to thrombin, albeit at higher concentrations (50-1000 times greater).[2] Other protease-activated receptors with close homology to the original thrombin receptor, namely PAR-2,[3,4] PAR-3,[5] and PAR-4,[6] have recently been cloned as well, and these possess their own agonist peptide motifs.

SFLLRN-based agonist peptides have received considerable structure-activity attention in terms of platelet activation studies.[1d,e,7-9] The minimum structural requirements for the PAR-1 peptides (thrombin receptor-activating peptides; TRAPs) are, generally, at least an N-terminal pentapeptide with a free amino group at position (P) 1, a small side chain at P1, an aromatic residue at P2 (with agonist potency enhanced ca. four-fold by *p*-F-Phe substitution[10]), and a basic or aromatic residue at P5. A large hydrophobic amino acid at P4 is important as well, but widely varied substitution is tolerated at P3. We[11] and others[12] have also been interested in the structural properties of the peptide backbone, relative to function, to gain an understanding of possible bioactive conformations. Cyclic peptides are expected to adopt folded conformations, such as β- or γ-turns, which are fixed by intramolecular hydrogen bonding. Hence, we sought to investigate macrocyclic

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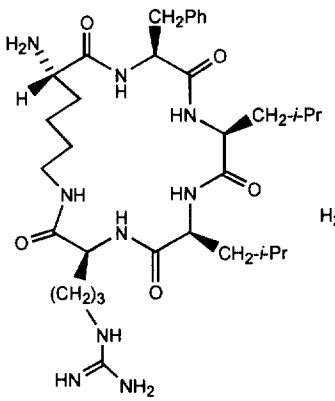
analogues of SFLLRN and ascertain the effect of conformational constraint on PAR-1 agonist activity in the form of platelet activation, as well as possible PAR-1 antagonist activity. During the course of our work, Matsoukas et al.[12b] reported three macrocyclic analogues of SFLLR with a cyclization linkage between the P1 side chain and the C-terminus and found that **1** has nearly the same potency as SFLLR-NH_2 in inducing contractions of gastric smooth muscle. We now report our studies of a variety of macrocyclic hexapeptide analogues of SFLLRN with a cyclization linkage between the P1/P6 (**2**) or the P3/P6 (**3** and **4**) side chains.



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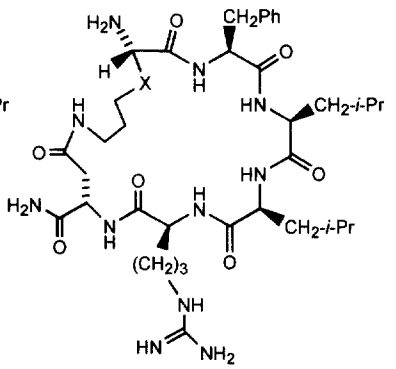
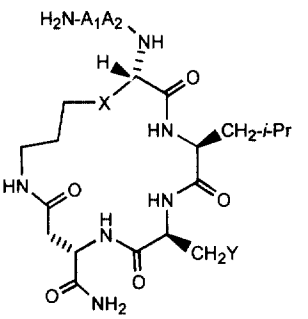


2a X = CH_2
2b X = $(CH_2)_4NHCO$



3a A_1A_2 = Ala-Phe, X = CH_2, Y = $(CH_2)_2NHC(=NH)NH_2$
3b A_1A_2 = Gly-4FPhe, X = CH_2, Y = $(CH_2)_2NHC(=NH)NH_2$
3c A_1A_2 = Gly-Phe, X = CH_2, Y = Ph
4 A_1A_2 = Gly-Phe, X = $(CH_2)_4NHCO$, Y = $(CH_2)_2NHC(=NH)NH_2$

Synthesis. Cyclic peptides **2a**, **2b**, **3a-3c**, and **4** were synthesized by using a classical solution-phase Fmoc protocol[13] exemplified in Scheme 1. Fmoc removal from **5** was accomplished with 20% Et_2NH in acetonitrile and the resultant amine was coupled with protected arginine, as shown, to give a quantitative yield of dipeptide **6**. The dipeptide was elaborated by using the same deprotection/coupling procedure to give hexapeptide **7**. The Boc and *t*-Bu ester groups were removed with 50% CF_3CO_2H (TFA) in CH_2Cl_2 and, after evaporation in vacuo, the amino acid was cyclized by using BOP-Cl/DMAP[14] to afford protected cyclic peptide **8**. Deprotection of the acid-labile groups was performed with HF/anisole at 0 °C for 3.5 h and product **2a** was purified by reverse-phase HPLC. Compounds prepared analogously are shown in Table 1.

To more rapidly obtain compounds **3** in which A_1 and A_2 are widely varied, we resorted to solid-phase synthesis of a mini-library. This entailed preparation of the dipeptide A_1A_2 on a trityl resin, coupling with the appropriate cyclic tetrapeptide, prepared as above, and cleavage from the solid support with TFA (Scheme 2). Thus, trityl chloride resin (Advanced Chemtech) was reacted with the allyl ester of β-alanine and *i*-Pr_2NEt in DMF[15] to give the amino acid-functionalized resin, **9**. Palladium-catalyzed deprotection of the allyl ester[16] and coupling with phenylalanine allyl ester gave **10**, which was deprotected to afford dipeptide **11**. Diisopropylcarbodimide (DIC) coupling of **11** with cyclic tetrapeptide **12**, prepared according to the route in Scheme 1, gave the resin-bound target, which was cleaved from the support with 50% TFA in

CH_2Cl_2. Concentration in vacuo and trituration with ether provided **3p**. Similarly, **3c-3z** were prepared, with arginine being protected by a 2,4,6-trimethylbenzenesulfonyl (Mts) group that was readily removed with triflic acid/TFA/anisole during cleavage from the resin.

Scheme 1

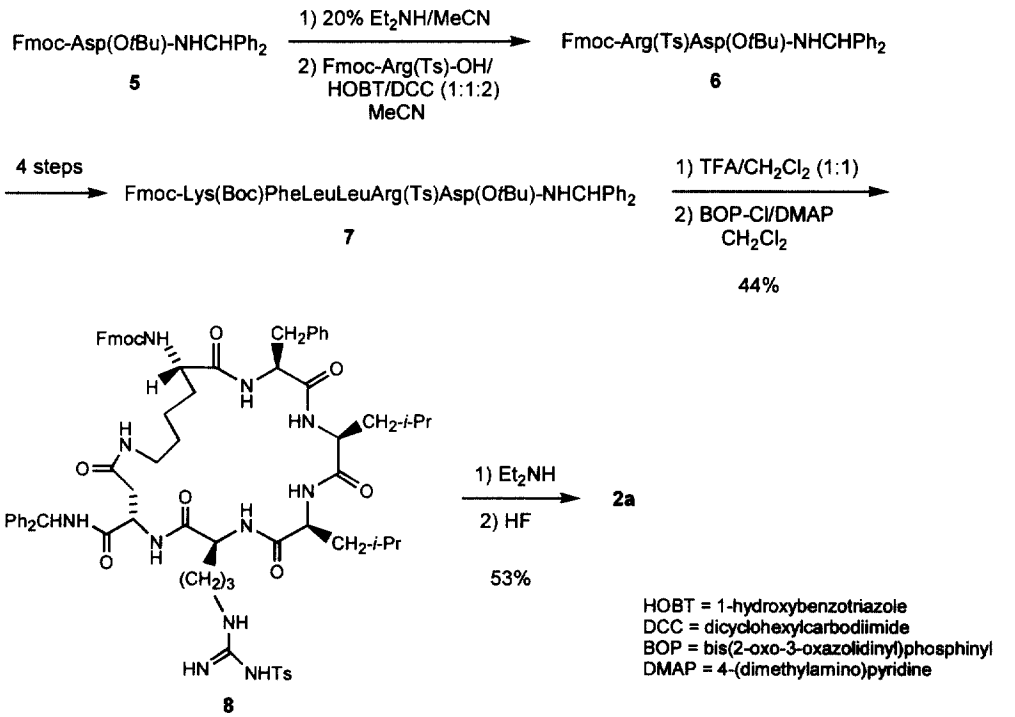
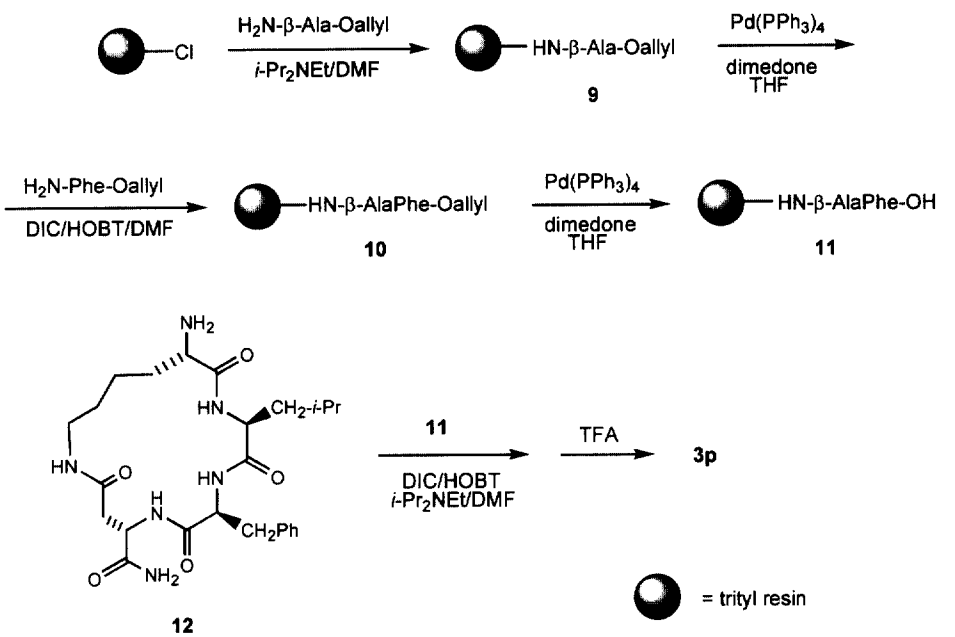


Table 1. Chemical Properties[a]

cmpd	formula	mp, °C[b]	$[\alpha]_D^{25}$ (*c*, MeOH)
2a	$C_{37}H_{61}N_{11}O_7$•2.5 CF_3CO_2H•2.5 H_2O	182-190	-6.9° (0.65)
2b	$C_{41}H_{68}N_{12}O_8$•2.5 CF_3CO_2H•4.0 H_2O	142-150	-30.5° (0.73)
3a	$C_{34}H_{55}N_{11}O_7$•2.5 CF_3CO_2H•3.0 H_2O	225-235	+2.6° (0.51)
3b	$C_{33}H_{52}FN_{11}O_7$•2.2 CF_3CO_2H•4.2 H_2O	>250	+2.3° (0.82)
3c	$C_{36}H_{50}N_8O_7$•3.5 CF_3CO_2H•3.5 H_2O	>250	
3o	$C_{34}H_{55}N_{11}O_7$•3.5 CF_3CO_2H•2.0 H_2O	230-240	-6.7° (0.73)
4	$C_{37}H_{60}N_{12}O_8$•3.0 CF_3CO_2H•2.0 H_2O	225-230	-5.8° (0.67)

a. These compounds were purified by reverse-phase HPLC by using 0.16% TFA in MeCN/0.20% TFA in water and lyophilized to white solids; they were characterized by mass spectrometry and high-field proton NMR. Microanalytical data (C, H, N) were within the accepted range (±0.4%); % water was determined by Karl-Fisher analysis. b. Mp values are corrected to a set of standards.

Scheme 2



Biological Results. The cyclic peptides were screened for agonist and antagonist activity by using human gel-filtered platelet aggregation.[11,17] For inhibition of platelet aggregation α-thrombin was used as the agonist (Table 2); some results were also obtained with TRAP-6 (50 μM) as the agonist (**2b**, 6%; **3a**, 6%;, **3b**, 9%; **3c**, 26%; **4**, 49%). The P1/P6 cyclic peptides **2a** and **2b** showed very little agonist activity, which might be attributed to the larger ring size of 23 or 28 atoms in comparison to **1** with 19 atoms. Although **2b** showed modest antagonist activity with an IC_{50} of ca. 50 μM, whereas **2a** was devoid of antagonist activity, this is probably not solely related to PAR-1 since **2b** inhibited thrombin to a modest degree (26% inhibition at 50 μM). The P3/P6 cyclic tetrapeptides **3a** and **3b**, with a smaller 17-membered ring, exhibited improved, albeit modest, agonist activity (EC_{50} of ca. 60 μM). Given the agonist activity of **3a** and **3b**, we conducted a study with variations at the A_1/A_2 positions of **3**. Changing A_1 and A_2 while retaining the arginine, as in **3d-3o**, led to a loss of agonist activity. However, when Arg was replaced with Phe, as in **3c**, agonist activity was increased; also, **3v-3x** had reasonable agonist activity. Insertion of a GABA unit into the ring of inactive **3g** gave 22-membered macrocycle **4**, which exhibited modest agonist (EC_{50} = 60 μM) and antagonist (44% inhibition at 50 μM) activity. This activity for **3g** and **4** may be attributed to increased flexibility of the larger ring, which can orient the critical side chains into the proper spatial arrangement for binding to the receptor.

In summary, 17-membered P3/P6 macrocycles **3a** and **3b** showed modest agonist activity (EC_{50} = 60 μM), albeit ca. 200 times less than SFLLRN-NH_2. Through structural variations of this macrocyclic tetrapeptide, we found that replacement of Arg with Phe (viz. **3c**) can afford reasonable agonist activity (EC_{50} = 24 μM). Increasing the ring size of inactive **3g** by insertion of a GABA unit, between the Lys and Asp side chains, gave **4**, which exhibited improved, albeit modest, agonist activity.

Table 2. Biological Data[a]

cmpd	A_1A_2	Y	agonist activ.[b] EC_{50} (% aggr.)	antagonist activ.[c] % inh. vs thrombin
2a	- -	- -	(6)	3
2b	- -	- -	(5)	52
3a	Ala-Phe	$(CH_2)_2NHC(=NH)NH_2$	66	2
3b	Gly-4FPhe	$(CH_2)_2NHC(=NH)NH_2$	60	14
3c	Gly-Phe	Ph	24	23
3d	β-Ala-Phe	$(CH_2)_2NHC(=NH)NH_2$	(7)	3
3e	4-Abu-Phe	$(CH_2)_2NHC(=NH)NH_2$	(6)	6
3f	Ser-Phe	$(CH_2)_2NHC(=NH)NH_2$	(16)	13
3g	Gly-Phe	$(CH_2)_2NHC(=NH)NH_2$	(15)	9
3h	β-Ala-3FPhe	$(CH_2)_2NHC(=NH)NH_2$	(7)	3
3i	4-Abu-3FPhe	$(CH_2)_2NHC(=NH)NH_2$	(6)	2
3j	Ser-3FPhe	$(CH_2)_2NHC(=NH)NH_2$	(12)	5
3k	Gly-3FPhe	$(CH_2)_2NHC(=NH)NH_2$	(6)	10
3l	β-Ala-hPhe	$(CH_2)_2NHC(=NH)NH_2$	(7)	1
3m	4-Abu-hPhe	$(CH_2)_2NHC(=NH)NH_2$	(8)	2
3n	Ser-hPhe	$(CH_2)_2NHC(=NH)NH_2$	(16)	5
3o	Gly-hPhe	$(CH_2)_2NHC(=NH)NH_2$	(5)	2
3p	β-Ala-Phe	Ph	NA	11
3q	4-Abu-Phe	Ph	NA	14
3r	Ser-Phe	Ph	NA	12
3s	β-Ala-3FPhe	Ph	NA	24
3t	4-Abu-3FPhe	Ph	NA	27
3u	Ser-3FPhe	Ph	NA	23
3v	Gly-3FPhe	Ph	63	11
3w	β-Ala-hPhe	Ph	43	16
3x	4-Abu-hPhe	Ph	37	15
3y	Ser-hPhe	Ph	NA	2
3z	Gly-hPhe	Ph	NA	1
4	Gly-Phe	$(CH_2)_2NHC(=NH)NH_2$	30	44
std[d]			0.80	0

a. Abbreviations: 4-Abu = 4-aminobutyryl; hPhe = homophenylalanine. b. Activation of human platelet aggregation (gel-filtered platelets), expressed as an EC_{50} value in μM or as percent aggregation induced by the test compound at 50 μM (in parentheses), performed as reported in ref 17. NA indicates no activity at 100 μM. c. Inhibition of human platelet aggregation (gel-filtered platelets) induced by human α-thrombin, expressed as percent inhibition at 50 μM (ref 17). d. Reference standard, SFLLRN-NH_2 (TRAP-6).

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