Conformationally Constrained Peptides and Semipeptides Derived from RGD as Potent Inhibitors of the Platelet Fibrinogen Receptor and Platelet Aggregation

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Structure–activity studies have been pursued on cyclo-*S*,*S*-[Ac-Cys-(N^{α}-Me)Arg-Gly-Asp-Pen]-NH_2, **2** (SK&F 106760), a potent inhibitor of platelet aggregation, in an effort to improve potency and affinity for the GPIIb/IIIa receptor. Modifications on the N- and C-termini of **2** produced a series of peptides which indicate that the C-terminal carboxylate group may be a secondary receptor-binding element. Further modification by replacing the disulfide tether N^{α}-acetylcysteine/penicillamineamide with the novel, inexpensive, achiral, constrained, and more lipophilic tether 2-mercaptobenzoyl/2-mercaptoaniline (Mba/Man) afforded the semipeptide cyclo-*S*,*S*-[Mba-(N^{α}-Me)Arg-Gly-Asp-Man], **18** (SK&F 107260), which exhibited significant enhancement in both affinity and potency. To further investigate the effect of the phenyl ring at the C-terminus, peptides bearing the novel (2*R*,3*S*)- and (2*R*,3*R*)-β-phenylcysteines were synthesized, which culminated in the cyclo-*S*,*S*-[Ac-Cys-(N^{α}-Me)Arg-Gly-Asp-(2*R*,3*S*)-β-phenylCys]-OH peptide, **22**, which displayed substantial affinity and potency. We describe, herein, the development of both **18** and **22** and the additional structural modifications within the constrained cyclic disulfide ring to probe the stereochemical and steric requirements for receptor interaction.

Introduction

Binding of the multimeric adhesive protein fibrinogen (Fg) to activated GPIIb/IIIa platelet plasma membrane receptors is believed to be an important step for platelet aggregation.[1-4] Platelet activation (stimulated by a variety of agents such as ADP, thrombin, and epinephrine) and aggregation are involved in thrombus formation and other pathophysiological processes in atherosclerosis and related vasoocclusive disorders.[5,6] Inhibition of Fg binding to its receptor (GPIIb/IIIa) blocks platelet aggregation and thrombus formation.[7,8] GPIIb/IIIa-specific antibodies have been shown to inhibit platelet aggregation and thrombus formation both in vitro and in vivo in various species.[9-12] One GPIIb/IIIa monoclonal antibody (7E3-F(ab″$_2$) is under clinical investigation which demonstrates the utility of such antibodies as antithrombotic agents.[13a,b] Similarly, a small potent Fg-receptor antagonist peptide may also have utility for antithrombotic therapy.[14a,b]

As with many members of the integrin superfamily of adhesion receptors, GPIIb/IIIa recognizes proteins and peptides bearing the tripeptide sequence Arg-Gly-Asp (RGD). The RGD sequence occurs twice in the α-chain of Fg (95–97 and 572–574) and once in other natural integrin ligands such as vitronectin, fibronectin, von Willebrand factor, osteopontin, thrombospondin, and collagen.[15] Inhibition of natural ligand binding to integrins can be accomplished by RGD-containing small proteins or peptides.[16] The disintegrin family of RGD-containing proteins derived from snake venom such as trigramin, echistatin, and kistrin are potent inhibitors of platelet aggregation.[17-21] However, small linear peptides containing the RGD sequence are typically much less potent inhibitors.[22-24] We generated potent fibrinogen antagonists from small linear peptides, e.g., Ac-RGDS-NH_2, **1**, by (1) enclosing -RGD- into cyclic structures bearing the disulfide tether Ac-Cys-S-S-Cys-NH_2,[25] (2) replacing the C-terminal cysteine with the more lipophilic β-branched penicillamine (Pen), and (3) adding a methyl group to the α-amine of Arg. These modifications gave rise to cyclo-*S*,*S*-[Ac-Cys-(N^{α}-Me)Arg-Gly-Asp-Pen]-NH_2, **2** (SK&F 106760), a highly potent Fg-receptor antagonist both in vitro and in vivo.[26-30] Since our initial report of **2**, other cyclic RGD-containing peptides have been reported as potent Fg-receptor antagonists.[31,32]

The search for potent fibrinogen-receptor antagonists was continued using the general strategies that produced **2**, e.g., conformational constraints introduced via cyclization and increased lipophilicity at the C-terminus. This has led to a highly potent cyclic peptide, cyclo-*S*,*S*-[Mba-(N^{α}-Me)Arg-Gly-Asp-Man], **18** (SK&F 107260).[33] To probe the effect of the phenyl rings at the C-terminus, cyclic pentapeptides bearing the novel β-phenylcysteines were prepared which culminated in cyclo-*S*,*S*-[Ac-Cys-(N^{α}-Me)Arg-Gly-Asp-(2*R*,3*S*)-β-phenylCys]-OH, **22**, the most active peptide in the Ac-Cys/Pen series. Further studies were undertaken to evaluate the effect of N^{α}-alkyation on arginine and other residues as well as to probe for stereochemical requirements within the cyclic disulfide structure. This paper describes the synthesis and an extensive SAR study of cyclic disulfide peptides leading to **18**, **22**, and other related peptides.

Results and Discussion

The primary bioassay employed was inhibition of ADP-induced platelet aggregation in canine platelet-rich plasma (PRP).[29] Fg-receptor affinity was assessed by inhibition of [^{125}I]Fg and [^{3}H]**18** binding to purified human GPIIb/IIIa receptor reconstituted in liposomes.[34-36] The [^{3}H]**18** radioligand has proven superior to radioiodinated fibrin-

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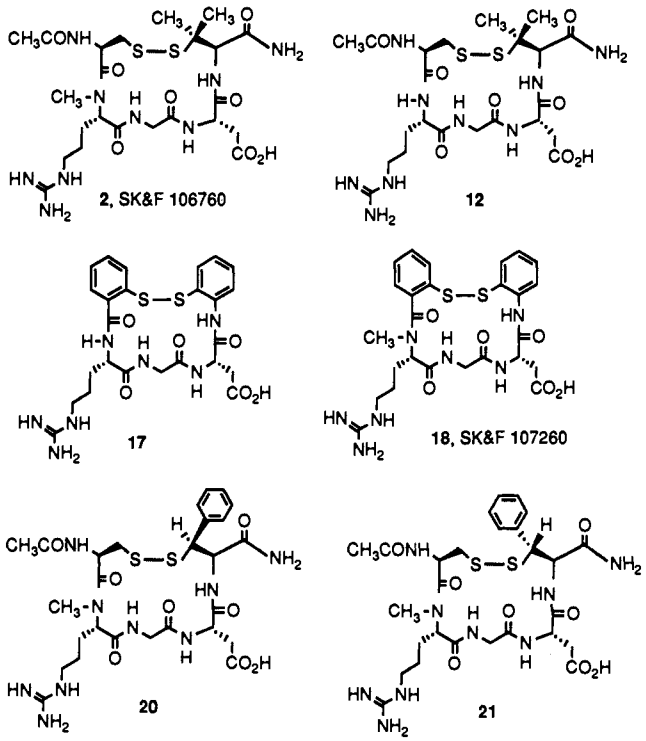
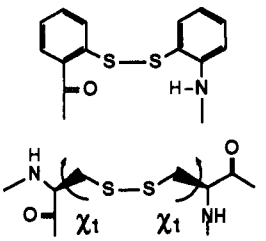


Figure 1. Chemical structures of lead compounds.

ogen due to enhanced stability relative to [^{125}I]Fg. The discussion will be focusing on the antiaggregatory potency because although the affinity data tend to demonstrate that potency of compounds in the platelet antiaggregation assay is related to Fg-receptor interaction, the correlation is not always maintained. All IC_{50} and K_i values have been adjusted for the peptide content derived from quantitative amino acid analyses. The structures of key analogues are shown in Figure 1.

Analogues of cyclo-*S,S*-[Ac-Cys-(N^{α}-Me)Arg-Gly-Asp-Pen]-NH_2: Amino and Carboxy Terminal Modifications. The RGD analogues were initially prepared as *N*-acetyl carboxamides since both -RGD- sequences in the fibrinogen α-chain lack an N-terminal ammonium or C-terminal carboxylate. To test the roles of N- and C-termini within the cyclic disulfide peptides, analogues shown in Table1 were prepared. Substituting the N^{α}-acetyl of **2** with more steric and lipophilic acyl substituents resulted in peptides **3–5** with comparable potency to **2**. In fact, the des-acyl analogue **6** and the des-acylamino analogue **7** displayed activities comparable to **2**. These results suggest that modification at the amino terminus of the cyclic pentapeptide disulfide, by substitution with more lipophilic acyl groups or even deletion of the acyl or acylamino groups, does not contribute significantly to Fg-receptor antagonist potency. It has been reported that the C-terminal free carboxylate may contribute to better bioactivity than the C-terminal amide.[37] This was observed for the free C-terminal carboxylate analogue **8**, which displayed better potency than **2**; however, the amino carboxylate analogue **9** displayed comparable potency to **6**. These results suggest that although a free carboxylate at the carboxy terminus may increase antiaggregatory activity, the N-terminal may influence the overall potency of the peptide. The C-terminal carboxylate could enhance affinity and potency through a favorable receptor interaction, which may reflect features of enhanced hydrogen bonding or electrostatic interaction.

Development of cyclo-*S,S*-[Mba-(N^{α}-Me)Arg-Gly-Asp-Man], 18, cyclo-*S,S*-[Ac-Cys-(N^{α}-Me)Arg-Gly-Asp-(2*R*,3*S*)-β-phenylCys]-OH, 22, and Other Structurally Related Potent Peptides. (a) Conformation Constraint and Lipophilic Replacement of the Disulfide Tether. The next portion of the cyclic pentapeptide structure to be modified was the disulfide tether. We sought replacements for the Cys/Cys dipeptide that would be more lipophilic and would place a greater degree of conformational constraint upon the macrocyclic ring. As can be seen in Table 2, substitution of the lipophilic, β-branched Pen for either the N- or C-terminal Cys in **10** afforded analogues **11** and **12** that displayed enhanced potency. The introduction of Pen at both the N- and C-termini afforded little advantage over the C-terminal substitution alone (**13** vs **2**). We continued our search to replace the Cys-Pen or Pen/Pen tethers with ones that would remove the chiral centers in both Cys and Pen residues. The 2-mercaptoaryl compounds, 2-mercaptobenzoic acid (Mba) and 2-mercaptoaniline (Man), may be capable of functioning as replacements for Cys and Pen as they contain the sulfhydryl group for cyclic disulfide ring formation, constrain the χ_1 torsion angle to 0°, are more lipophilic than Cys or Pen, and are achiral.



Single substitution of Mba for the N^{α}-Ac-Cys in **10** resulted in **14** which displayed potency comparable to the N^{α}-Ac-Cys analogue **10**. Likewise, substitution of Man for the C-terminal cysteinamide in **10** resulted in **15** which also displayed comparable potency to **10**. Thus, the Mba and Man substitutions for Cys and/or Pen displayed no loss of the antiaggregatory activity. Surprisingly, replacing both N^{α}-Ac-Cys/Cys-NH_2 in **10** with Mba/Man afforded analogue **17** which demonstrated a significant enhancement in potency relative to **10**, **11**, or **12**. It is also 30-fold more potent than either singly-substituted analogue **14** or **15**. In fact, **17** displayed potency and affinity similar to our lead peptide **2**. Thus, substitution of both Mba and Man had a greater effect than either substitution alone.

(b) Effect of (N^{α}-Me)Arg in 17. We have reported previously on the significant increase in potency and affinity of both linear and cyclic RGD peptides which resulted from replacement of the arginine residue with the N^{α}-methylarginine.[26–29] This trend has been maintained in the series containing Mba/Man as well. The (N^{α}-Me)Arg analogue **16** displays considerably greater potency and receptor affinity compared to analogue **15**. As seen in Table 2, N^{α}-methylarginine substitution for the arginine in the Mba/Man analogue **17** gave **18**, SK&F 107260, which displays high in vitro potency (IC_{50} = 0.09 μM) and affinity (K_i = 2 nM). The effect of this substitution on affinity is particularly noteworthy since **18** exhibits affinity for GPIIb/IIIa which is 20 times better than the natural ligand fibrinogen (K_i = 43 nM). Cyclic semipeptide **18** has also been evaluated in the Folt's model of coronary thrombosis, demonstrating enhanced efficacy over **2**: at 0.3 mg/kg bolus in doses, **2** (n = 6) inhibited

Table 1. N- and C-Termini Modification of cyclo-*S,S*-[Ac-C(N^{α}-Me)RGD-Pen]-NH_2

no.	compound	plat agg IC_{50} (μM)[a]	binding K_i (μM)[b]
1	Ac-RGD-NH_2	91.3 ± 0.1	4.2
2	cyclo-*S,S*-[Ac-C(N^{α}-Me)RGD-Pen]-NH_2[c] (SK&F 106760)	0.36 ± 0.04	0.058 ± 0.02 0.102 ± 0.018[d]
3	cyclo-*S,S*-[Bz-C(N^{α}-Me)RGD-Pen]-NH_2[e]	0.20 ± 0.07	0.0137
4	cyclo-*S,S*-[Bu-C(N^{α}-Me)RGD-Pen]-NH_2[f]	0.25 ± 0.06	0.026
5	cyclo-*S,S*-[Pac-C(N^{α}-Me)RGD-Pen]-NH_2[g]	0.28 ± 0.07	0.035
6	cyclo-*S,S*-[C(N^{α}-Me)RGD-Pen]-NH_2	0.20 ± 0.04	0.0093 ± 0.005
7	cyclo-*S,S*-[Mpr-(N^{α}-Me)RGD-Pen]-NH_2[h]	0.34 ± 0.11	0.0243
8	cyclo-*S,S*-[Ac-C(N^{α}-Me)RGD-Pen]-OH	0.08 ± 0.02	0.0085
9	cyclo-*S,S*-[C(N^{α}-Me)RGD-Pen]-OH	0.12 ± 0.01	0.0048

[a] Inhibition of platelet aggregation in canine platelet-rich plasma induced by ADP as measured by light transmittance in a Chrono-Log aggregometer. [b] Inhibition of [^{125}I]fibrinogen binding to purified GPIIb/IIIa isolated from human platelets and reconstituted in liposomes; no statistical limits indicate only one determination; also, see ref 56. [c] Pen = penicillamine. [d] Inhibition of [^{3}H]SK&F 107260 binding to purified GPIIb/IIIa isolated from human platelets and reconstituted in liposomes; numbers represent the mean of quadruplicate determinations of a single experiment. [e] Bz = benzoyl. [f] Bu = valaryl. [g] Pac = phenacyl. [h] Mpr = mercaptopropionyl.

Table 2. Development of SK&F 107260 and Other Structurally Related Potent Peptides

no.	compound	plat agg IC_{50} (μM)[a]	binding K_i (μM)[b]
2	cyclo-*S,S*-[Ac-C(N^{α}-Me)RGD-Pen]-NH_2	0.36 ± 0.04	0.058 ± 0.02
10	cyclo-*S,S*-[Ac-CRGDC]-NH_2	16.2 ± 5.8	0.78 ± 0.01
11	cyclo-*S,S*-[Ac-Pen-RGDC]-NH_2	7.97 ± 1.47	0.188
12	cyclo-*S,S*-[Ac-CRGD-Pen]-NH_2[c]	4.12 ± 0.6	NA[d]
13	cyclo-*S,S*-[Ac-Pen-(N^{α}-Me)RGD-Pen]-NH_2	0.37 ± 0.13	0.0025
14	cyclo-*S,S*-[Mba-RGDC]-NH_2[e]	8.08 ± 2.7	NA
15	cyclo-*S,S*-[Ac-CRGD-Man][f]	9.71 ± 1.05	0.28
16	cyclo-*S,S*-[Ac-C(N^{α}-Me)RGD-Man]	0.17 ± 0.04	0.026 ± 0.058
17	cyclo-*S,S*-[Mba-RGD-Man]	0.29 ± 0.09	0.027 ± 0.009
18	cyclo-*S,S*-[Mba-(N^{α}-Me)RGD-Man] (SK&F 107260)	0.09 ± 0.02	0.0021 ± 0.006 0.0026 ± 0.0006[g]
19	cyclo-*S,S*-[Mba-(N^{α}-Me)RGD-Mea][h]	0.236 ± 0.051	0.0095
20	cyclo-*S,S*-[Ac-C(N^{α}-Me)RGD-(2*R*,3*S*)-β-PhC]-NH_2[i]	0.05 ± 0.02	0.045 ± 0.025
21	cyclo-*S,S*-[Ac-C(N^{α}-Me)RGD-(2*R*,3*R*)-β-PhC]-NH_2	0.098 ± 0.01	0.125
22	cyclo-*S,S*-[Ac-C(N^{α}-Me)RGD-(2*R*,3*S*)-β-PhC]-OH	0.04 ± 0.01	0.0021

[a] As in footnote *a* (Table 1). [b] As in footnote *b* (Table 1). [c] Reference 28. [d] Not available. [e] Mba = 2-mercaptobenzoic acid. [f] Man = 2-mercaptoaniline. [g] As in footnote *d* (Table 1). [h] Mea = 2-mercaptoethylamine. [i] β-PhC = 3-phenylcysteine = 3-mercaptophenylalanine.

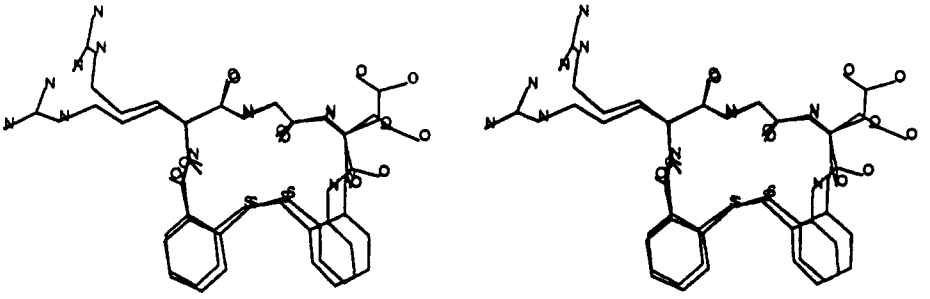


Figure 2. Stereoplot of compound 18, as determined from X-ray crystal structure and ^{1}H NMR.

thrombus formation for 28 ± 2 min while 18 (n = 3) inhibited thrombus formation for 105 ± 21 min.[30,38]

(c) Effect of the Phenyl Rings in the Mba/Man Tether. To probe the role of the conformational constraint imposed by the Man aryl ring tether, analogue 19 was prepared with the unsubstituted 2-mercaptoethylamine (Mea) in place of Man in 18. As can be seen in Table 2, 19 displayed potency and affinity lower than 18. These results suggest that the contribution of the Man phenyl ring (either as an additional receptor interaction or through its constraint on conformation) is favoring the Fg-receptor interaction. The effect of the aromatic ring in Mba on receptor affinity remains an open question since the analogous Mpr/Man analogue was not prepared as a test of the role of Mba in 18. The Mpr/Pen analogue 7 was found to be equipotent with Ac-Cys/Pen 2, however, suggesting that the constraint imposed by Mba may be unnecessary. As discussed below, however, Mba and Man were critical in terms of imposing a high degree of conformational constraint on the macrocyclic ring, giving rise to a highly defined solution conformation.

The low-temperature ^{1}H-NMR spectrum of semipeptide 18 in CD_3OH displays, in a 4:1 ratio, two sets of signals attributable to two distinct conformations which could be determined using an NOE-constrained-distance geometry procedure.[39] Surprisingly, these two conformations are quite similar, differing primarily in the orientation of the plane of the Asp-anilide amide group. In both conformations, the arginine resides in a turn followed by an extended glycine followed by a turn about aspartic acid, Figure 2. The nitrate salt of 18 gave crystals suitable for X-ray diffraction analysis, yielding a single conformation identical to that of the minor conformation determined in solution.[39] By contrast, the low-temperature ^{1}H-NMR spectrum of the Cys/Pen peptide 2 broadened but could not be resolved into distinct sets of signals, indicating conformational averaging.[39a] Utilization of the NOEs from the ^{1}H-NMR spectrum in a similar constrained-distance

Table 3. N^{α}-Substituted Amino Acid Modifications

no.	compound	plat agg IC_{50} (μM)[a]	binding K_i (μM)[b]
2	cyclo-*S,S*-[Ac-C(N^{α}-Me)RGD-Pen]-NH_2	0.36 ± 0.04	0.058 ± 0.02
12	cyclo-*S,S*-[Ac-CRGD-Pen]-NH_2	4.12 ± 0.6	NA
23	cyclo-*S,S*-[Ac-CR(N^{α}-Me)GD-Pen]-NH_2	73.4 ± 8.2	1.25[c]
24	cyclo-*S,S*-[Ac-CRG(N^{α}-Me)D-Pen]-NH_2	136.7 ± 21.9	25.3[c]
25	cyclo-*S,S*-[Ac-C(N^{α}-Et)RGD-Pen]-NH_2	0.9 ± 0.1	0.065
26	cyclo-*S,S*-[Ac-C(N^{α}-Bzl)RGD-Pen]-NH_2	7.74 ± 1.78	>100
27	cyclo-*S,S*-[Ac-C(N^{α}-Me)R(N^{α}-Me)GD-Pen]-NH_2	39.5 ± 6.0	15.1[c]
28	cyclo-*S,S*-[Ac-C(N^{α}-Me)RG(N^{α}-Me)D-Pen]-NH_2	23.4 ± 8.7	7.04[c]

[a] As in footnote *a* (Table 1). [b] As in footnote *b* (Table 1). [c] As in footnote *d* (Table 1).

geometry study yielded, nonetheless, a conformation similar to that of the major component determined in solution for **18**, again with the characteristic turn–extended–turn conformation about RGD. Subsequent work has revealed other cyclic RGD analogues that give alternate solution conformations about RGD but display lower affinity for GPIIb/IIIa.[40] The ^{1}H-NMR spectrum of the relatively unconstrained cyclo-*S,S*-[Ac-Cys-Arg-Gly-Asp-Cys]-OH furthermore could only be rationalized as a mixture of two rather different conformations.[41] Thus, the Mba-Man analogue **18** has been quite valuable in providing a highly defined solution conformation that has been explored subsequently as a pharmacophore model of RGD interaction with GPIIb/IIIa.[42,43]

(d) Effect of the C-Terminal β-Phenylcysteine. We and others[43] have found that the serine residue in a linear RGDS peptide could be replaced with the more lipophilic phenylalanine with enhancement of activity. This earlier finding, along with our work with Pen and Man replacements at the C-terminus, suggested exploration of phenyl-substituted cysteines in place of the Pen residue in **2**, so as to increase the bulk of the side chain. The protected syn- and anti-β-phenylcysteines were synthesized via a stereoselective route[45] and incorporated into the two diastereomers **20** and **21** utilizing solid-phase peptide synthesis. The 2*R*,3*S* diastereoisomer **20** and the 2*R*,3*R* diastereomer **21** displayed a better potency than **2** and similar activity to the potent Mba/Man semipeptide **18**. The fact that both isomers are comparable to **18** and more active than **2** suggests the importance of the phenyl ring to Fg-receptor antagonist activity, as was also seen with comparable linear analogues derived from **18**.[46] The phenyl ring may contribute favorably to activity as a receptor-binding element through a hydrophobic interaction. The fact that both diastereomers are of comparable activity suggests a flexibility in this position, allowing either diastereomer to adopt a high-affinity conformation. The free C-terminal carboxylate analogue **22** displayed comparable potency and enhanced receptor affinity relative to the C-terminal amide analogue **20**. The fact that no apparent enhancement of potency was displayed by **22** over **20**, in contrast to 8 vs 2, suggests that the β-phenylcysteine in **20** and **22** may force a different receptor interaction, which lacks a favorable interaction of the carboxylate group. Peptide **22** is the most active peptide analogue in our cyclic pentapeptide series.

The data in Table 2 reveal that: (a) replacement of Mba-Man for the N^{α}-Ac-Cys/Pen-NH_2 or N^{α}-Ac-Pen/Pen-NH_2 in the cyclic pentapeptide **2** or **13** confers enhanced potency and Fg-receptor affinity; (b) replacement of the carboxy terminal Pen with (2*R*,3*S*)-β-phenylcysteine, as in **22**, enhanced receptor affinity and Fg-antagonist potency; and (c) favorable contribution of the N^{α}-methyl substitution on arginine within the -RGD- sequence for Fg-receptor antagonist potency has been demonstrated once again in the pentapeptide Mba-Man analogues.

Effect of Modification of N^{α}-Substitution on Arginine and Other Amino Acids. In addition to arginine, the effect of α-amine methylation upon antiaggregatory potency has been examined for Gly and Asp within the framework of the cyclic peptide **12**. As can be seen in Table 3, the corresponding (N^{α}-Me)Gly and (N^{α}-Me)Asp peptides **23** and **24** lost an order of magnitude in activity as compared with **12**. Similar modification on peptide **2** gave analogues **27** and **28** with even greater losses of potency as compared with **2**.

Since the probable solution conformations of **2** have been determined by ^{1}H NMR via spectroscopic and molecular modeling,[39] the loss of potency obtained from the N^{α}-methyl substitution in analogues **23**, **27**, **24**, and **28** may be considered in terms of potential perturbations to this conformation. Three of the four conformations determined for Asp in **2** (ϕ,ψ = −63°,142°, 53°,74°, and −93°,146°) coincide with the low-energy conformations determined for Ac-(N^{α}-Me)Ala-NHMe by Manavalan and Momany.[47] These data suggest that the (N^{α}-Me)Asp peptide **28** could adapt the same conformations as **2**, and it would appear that the loss of activity with the (N^{α}-Me)Asp peptide is not due to a perturbation of receptor-bound conformation. It can be argued, in a similar fashion, that the loss of activity with the (N^{α}-Me)Gly peptide is not due to perturbation of receptor-bound conformation. The extended glycine in **2**, predicted by our model, is also consistent with the effect of N^{α}-methylation on the aspartic acid residue. Thus, loss of activity upon methylation of either Gly or Asp in **2** appears to be due either to a negative steric receptor interaction or to loss of a favorable receptor interaction.

The enhanced activity from N^{α}-methylation of arginine could be due to a favorable receptor interaction. The favorable interaction could arise from a number of factors: (a) promotion of a cis-amide bond, (b) increasing the electronegativity of the amide oxygen through a donating effect by the N^{α}-methyl group, or (c) providing a positive lipophilic interaction with the receptor. The conformations determined in solution for both **2** and **18** did not contain a cis-amide involving (N^{α}-Me)Arg.[39] We attempted to address the latter two possibilities with peptides **25** and **26** with N^{α}-ethyl- and N^{α}-benzylarginine replacements for arginine in **12**. A modest loss in potency of the N^{α}-ethyl analogue **25** and a larger loss of potency in **26** were observed as compared with **2**. However, similar receptor affinity and weaker potency to peptide **2** were displayed by the N^{α}-ethyl analogue **25**, while diminished potency and apparent loss of affinity were displayed by the N^{α}-benzyl analogue **26**. Thus, there is a certain degree of steric bulk in the N^{α}-substituent that is tolerated by the receptor. Unfortunately, the small difference between

Table 4. Reverse Chirality Effect of the Chiral Residues

no.	compound	plat agg IC_{50} (μM)[a]	binding K_i (μM)[b]
2	cyclo-*S,S*-[Ac-C(N^{α}-Me)RGD-Pen]-NH_2	0.36 ± 0.04	0.058 ± 0.02
12	cyclo-*S,S*-[Ac-CRGD-Pen]-NH_2	4.12 ± 0.6	NA
29	cyclo-*S,S*-[Ac-CrGD-Pen]-NH_2	4.12 ± 1.1	0.72 ± 0.04
30	cyclo-*S,S*-[Ac-CRGd-Pen]-NH_2[c]	>200	>1000
31	cyclo-*S,S*-[Ac-CRGD-pen]-NH_2	20.3 ± 2.2	3.0
32	cyclo-*S,S*-[Ac-cRGD-Pen]-NH_2	98.7 ± 1.9	2.65[d]
33	cyclo-*S,S*-[Ac-c(N^{α}-Me)RGD-Pen]-NH_2	1.9 ± 0.35	0.11[d]
34	cyclo-*S,S*-[Ac-C(N^{α}-me)rGD-Pen]-NH_2	1.79 ± 0.23	0.082 ± 0.0
18	cyclo-*S,S*-[Mba-(N^{α}-Me)RGD-Man]	0.09 ± 0.02	0.0024
35	cyclo-*S,S*-[Mba-(N^{α}-me)rGD-Man]	0.6 ± 0.01	0.020 ± 0.008
36	cyclo-*S,S*-[Mba-(N^{α}-Me)RGd-Man]	25.97 ± 7.03	8.75[d]

[a] As in footnote *a* (Table 1). [b] As in footnote *b* (Table 1). [c] Lower case letter = single letter D-amino acid. [d] As in footnote *d* (Table 1).

Table 5. N^{α}-Substitution on Gly-2 in cyclo-*S,S*-[Ac-CGRGD-Pen]-NH_2

no.	compound	plat agg IC_{50} (μM)[a]	binding K_i (μM)[b]
2	cyclo-*S,S*-[Ac-C(N^{α}-Me)RGD-Pen]-NH_2	0.36 ± 0.04	0.058 ± 0.02
12	cyclo-*S,S*-[Ac-CRGD-Pen]-NH_2	4.12 ± 0.6	NA
37	cyclo-*S,S*-[Ac-CGRGD-Pen]-NH_2	11.4 ± 2.0	0.61 ± 0.13
38	cyclo-*S,S*-[Ac-C(N^{α}-Me)GRGD-Pen]-NH_2	1.2 ± 0.71	0.077 ± 0.0
39	cyclo-*S,S*-[Ac-CG(N^{α}-Me)RGD-Pen]-NH_2	0.36 ± 0.05	0.135[c]
40	cyclo-*S,S*-[Ac-C(N^{α}-Me)G(N^{α}-Me)RGD-Pen]-NH_2	0.15 ± 0.03	0.02 ± 0.01
41	cyclo-*S,S*-[Ac-C(N^{α}-Bzl)G(N^{α}-Me)RGD-Pen]-NH_2	0.29 ± 0.06	0.43 ± 0.059
42	cyclo-*S,S*-[Ac-CP(N^{α}-Me)RGD-Pen]-NH_2	24.4 ± 9.81	>10
17	cyclo-*S,S*-[Mba-RGD-Man]	0.29 ± 0.09	0.027
18	cyclo-*S,S*-[Mba-(N^{α}-Me)RGD-Man]	0.09 ± 0.02	0.0024
43	cyclo-*S,S*-[Mba-(N^{α}-Me0GRGD-Man]	3.46 ± 1	0.143 ± 0.072
44	cyclo-*S,S*-[Mba-(N^{α}-Me)G(N^{α}-Me)RGD-Man]	0.102 ± 0.043	0.0245 ± 0.006
45	cyclo-*S,S*-[Mba-P(N^{α}-Me)RGD-Man]	1.01 ± 0.44	0.82[c]

[a] As in footnote *a* (Table 1). [b] As in footnote *b* (Table 1). [c] As in footnote *d* (Table 1).

the N^{α}-Me and N^{α}-Et analogues precludes any discussion about the role of the substituent, except that the receptor appears to somewhat tolerate the ethyl group.

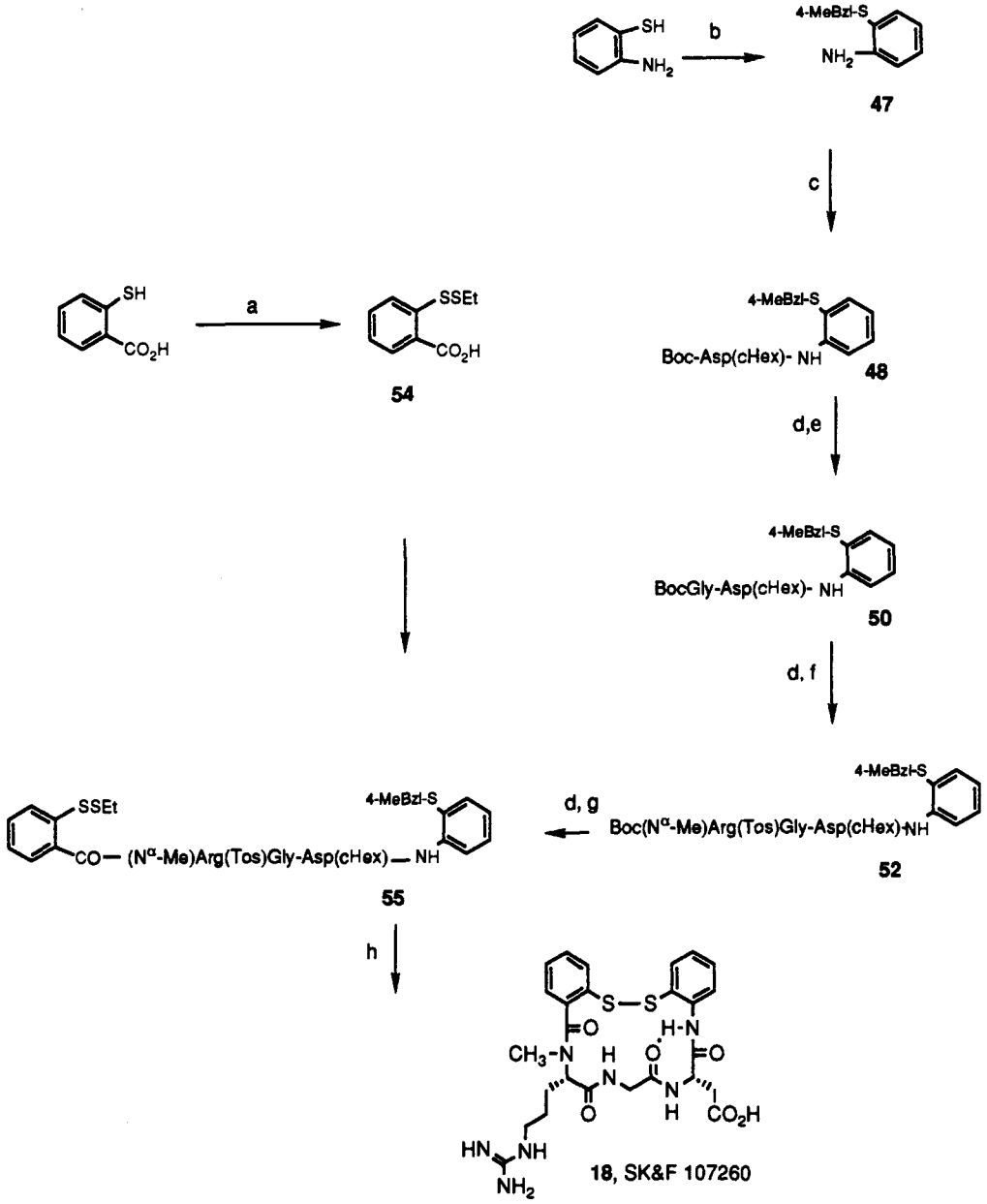
Effect of Reversing Amino Acid Chirality in the Cyclic Pentapeptides. During the course of the structure–activity studies to delineate the requirements for the Fg-receptor activity, we prepared the series of peptides in Table 4, with single D-amino acid replacements. Replacement of D-Arg for L-Arg in **12** gave **29** with retention of potency. Replacement of D-N^{α}-methylarginine for N^{α}-methylarginine in the Cys/Pen peptide **2** gave analogue **34** with apparent similar affinity but loss in potency. Similar replacement of D-N^{α}-methylarginine for N^{α}-methylarginine in the Mba/Man semipeptide **18** gave analogue **35** with loss of affinity and potency.

Substantial losses of potency and affinity were obtained upon inversion of Asp chirality in the Cys/Pen peptide **30** relative to **12** and Mba/Man **36** relative to **18**. Less dramatic losses of potency and receptor affinity (compared to **12**) were observed upon inversion of chirality of the N-terminal and C-terminal amino acid residues in the Cys/Pen analogues **31** and **32**, comparable to the losses observed in the D-(N^{α}-Me)Arg analogue **34**. Thus, the receptor is extremely sensitive to inversion of chirality at aspartic acid but more tolerant toward alterations of the orientations of the cation-bearing side chain in arginine and the side chains in the amino and carboxy terminal amino acids. These results are consistent with the N^{α}-Me modifications described above and with other previously reported modifications in cyclic disulfide pentapeptide RGD analogues.[29]

Effect of N^{α}-Alkylation on the Intercalated Amino Acid between Cys and Arg. In our inital search for optimal cyclic disulfide peptides, the hexapeptide **37** displayed reasonable activity compared to the pentapeptide **12**, Table 5. We have examined the effect of N-alkylation on the intercalated amino acid residue in the cyclic hexapeptide disulfide **37**. The N^{α}-methylglycine analogue **38** displayed an increase in potency compared with **12** and a dramatic enhancement in potency compared with the des-methyl analogue **37**. However, N-methylation of the arginine residue in **37** gave **39** with enhanced potency over **37** or **38** and similar potency to **2**. Relative to **2**, the N^{α}-methyl group in **38** is in the same position with regard to the N-terminal Cys but is not located on the Arg α-amine. Nonetheless, the doubly-N^{α}-methylated analogue **40** was superior to either mono-N^{α}-methylated analogue **38** or **39**. These results demonstrate that, despite the increase in the ring size to a hexapeptide, an enhancement in potency was obtained by N^{α}-methylation of arginine. In the cyclic hexapeptide disulfide series, the location of the N^{α}-methyl group is dependent upon the specific amino acid and not upon its position in the sequence. Replacing N^{α}-benzylglycine for N^{α}-methylglycine in **40** gave analogue **41** with no drastic reduction of potency; however, replacing proline for N^{α}-methylglycine, as in **42**, produced a dramatic loss of potency. The conformational restriction caused by the proline residue is apparently not favorable for a high-affinity receptor interaction. There is at least one other report of a drastic loss in Fg-receptor antagonist activity in a cyclic RGD peptide where a proline residue proceeded the arginine.[37]

A similar intercalation of N^{α}-alkyl amino acids was carried out on the cyclic Mba-RGD-Man, **17**, as represented in analogues **43–45**. Although the intercalation of N^{α}-methylglycine between Cys and Arg in the Cys-Pen analogues **12** and **2** gave analogues with enhanced potency, the same intercalation between Mba and Arg in the Mba-Man analogue **17** gave analogue **43** with more than a 10-fold drop in potency. Intercalation of (N^{α}-Me)Gly in **18** gave **44** with no loss of activity but with a trend of decreasing receptor affinity. Like the cyclic peptide **2**, the intercalation of Pro between Mba and N^{α}-methylarginine in **18** produced **45** which has reduced potency.

Scheme 1. Synthesis of cyclo-*S*,*S*-(Mba-(N^{α}-Me)Arg-Gly-Asp-Man] 18 and Analogues[a]



[a] Reagents: (a) $EtSH/SO_2Cl_2$, toluene (36%); (b) *p*-MeBzl-Br/Et_3N, EtOH (52%); (c) Boc-Asp(c-Hex)-OH, *i*-BuO_2CCl, NMM (88%); (d) TFA/CH_2Cl_2; (e) Boc-Gly-OH, EDC, HOBt/DMF (82%); (f) Boc-(N^{α}-Me)Arg(Tos), EDC, HOBt/DMF (99%); (g) compound **54**, EDC, DMAP, DIEA (80%); (h) anhydrous HF, Et_2O/50% AcOH (95%).

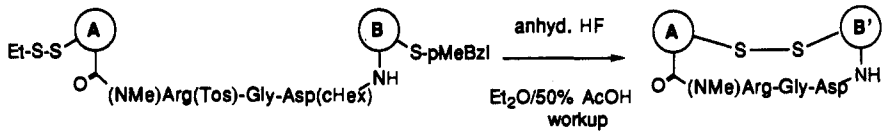
In conclusion, highly potent fibrinogen-receptor antagonists have been designed from cyclo-*S*,*S*-[Ac-Cys-(N_{α}-Me)Arg-Gly-Asp-Pen]-NH_2 by: (1) replacement of the N^{α}-Ac-Cys/Pen-NH_2 tether with a novel mimetic Mba/Man, (2) substitution of a free carboxyl group at the carboxy terminus for the carboxyamide, (3) intercalation of a simple N^{α}-methylglycine between the Cys and Arg residues, and (4) replacement of one of the hydrogens in the carboxy terminal Cys with the novel (2*R*,3*S*)- and (2*R*,3*R*)-β-phenylcysteines. This investigation has resulted in constrained cyclic peptides, the conformations of which have been utilized to design potent nonpeptide antagonists, which will be detailed elsewhere.[43]

Chemical Synthesis

Synthesis of peptides was performed by either solid-phase peptide synthesis (SPPS) or standard solution-phase synthesis employing availabe Boc amino acids (Experimental Section). The syntheses of novel protected N^{α}-alkylarginines are described in the Experimental Section. Chiral syntheses of the protected (2*R*,3*R*)- and (2*R*,3*S*)-β-phenylcysteines have been described elsewhere.[45] Analogues of peptide **2** were synthesized with standard solid-phase procedures as described previously.[29] Semipeptide **18** bearing the novel Mba-Man diaryldisulfide tether and its related analogues were synthesized by standard solution-phase methods utilizing the readily available Mba and Man and the appropriately protected (N^{α}-Me)arginine and -aspartic acid as shown in Scheme 1. In this work, Mba was protected as Mba(SEt) and Man as Man(4-methylBzl), although other thiol protection schemes should be feasible.

Rapid Disulfide Ring Closure. Disulfide ring closure via disulfide-exchange reaction usually is performed at alkaline pH and high dilution of the linear peptide precursors.[48] With semipeptide **18** and its analogues,

Table 6. Protecting Group Removal and Disulfide Bridge Formation



				percent disulfide product (%)				
				after HF[a]	after dilution and neutralization[b]			
no.	A	B	B'	0 h	40 min	2 h	24 h	complete, @ X h
2	Ac-Cys	Pen-NH-*p*-MeBHA resin	Pen-NH_2	22	67		77	95, 72
46	Ac-Cys	Cys-NH-*p*-MeBHA resin	Cys-NH_2	48	48[c]			95, 96
				54	57[c]	60	66[c]	95, 60
14	Mba	Cys-NH-*p*-MeBHA resin	Cys-NH_2	40	70		94	
				69	83[c]	88[c]	91[c]	
18[d]	Mba	Man (solution)	Man	95				
19	Mba	Mea (solution)	Mea	95				

[a] Percent disulfide (HPLC of crude product) in aqueous AcOH solution after Et_2O/50% AcOH workup. [b] Percent disulfide (HPLC of crude product) in aqueous solution after dilution of aqueous workup solution to 0.5 mM and pH to 7.6 with concentrated NH_4OH. [c] Rotary evaporation of these samples gave solids that contained ~95% disulfide. [d] All Man peptides to date give ~95% disulfide upon HF reaction and workup, regardless of N-terminal thiol group A or sequence.

however, cyclization occurred spontaneously upon completion of HF deprotection and ether/aqueous acetic acid workup, Table 6. Other Cys/Pen peptides were only partially cyclized at that point. If these peptides were allowed to stand in a dilute, neutral solution, however, cyclization typically went to completion after 24 h, Table 6. Upon further analysis of the phenomenon, we found that the aqueous solutions, obtained after HF reaction and Et_2O/aqueous AcOH workup of various Cys/Pen peptides, could be concentrated by rotary evaporation to promote removal of ethanethiol and, thereby, drive the cyclization into completion, Table 6 (entries denoted by footnote *c*). None of the products were seriously contaminated with multimeric product. The C-terminal Man appears to have a greater influence upon accelerated closure of the disulfide ring, Table 6, than the N-terminal Mba. This accelerative effect does not appear to be due to an enhanced reactivity of the arylalkyldisulfide in Mba(SEt) over the dialkyldisulfide in Cys(SEt), since the Mba-Cys peptide was not completely cyclized after HF. The accelerative effect, however, may be due to the lack of an α-carboxylate or -carboxamide, since an analogue bearing a C-terminal Mea was completely cyclized after workup of the HF reaction. This latter fact disfavors consideration of the difference in pK_a for aromatic vs aliphatic thiols as a possible influence on cyclization in acidic solution.

In other respects, the diaryldisulfide appears to behave like other peptidic disulfides. For example, the aryl mercaptan gives a red color with DTNB, and the diaryldisulfide is readily reduced with DTT.[48]

Experimental Section

Peptide Synthesis. Peptides in Tables 1–5 were synthesized employing either the solid-phase method for peptide 2 analogues or the standard solution-phase synthesis for semipeptide 18 analogues, with the exception of peptides 14–16 and 19 which were synthesized by the solid-phase method.[49] Peptides with a C-terminal amide were synthesized employing 4-methylbenzhydrylamine polystyrene resin (1% cross-linked S-DVB, 200–400 mesh, ~1.0 mequiv/g), and peptides with a C-terminal carboxylate were synthesized using the chloromethyl Merrifield resin (1% cross-linked S-DVB, ~1.0 mequiv/g). The C-terminal amino acid was incorporated to the resin via its cesium salt.[50] The protected peptide–resin intermediates were synthesized using an automated Beckman 990-MP synthesizer on either a 0.5- or 1.0-mmol scale. N-terminal *tert*-(butyloxy)carbonyl protection was employed for all amino acids in the solid-phase synthesis with the following side-chain protected derivatives: L- and D-Arg(Tos), L- and D-N^{α}-methylArg(Tos),[51] N^{α}-ethylArg(Tos), N^{α}-BzlArg(Tos) (procedure for preparation follows), Asp(β-cHex), D-Asp(β-Bzl), Cys(4-MeBzl) or Cys(SEt), D-Cys(4-MeBzl), (2*R*,3*S*)- and (2*R*,3*R*)-β-phenylcysteines(4-MeBzl),[45] Pen and D-Pen(4-MeBzl), Mba(SEt), and Man(4-MeBzl) (procedure for preparation follows). Each amino acid was coupled sequentially to the peptide chain grown from the C-terminal amino acid using *N*,*N*-dicyclohexylcarbodiimide/1-hydroxybenzotriazole (DCC/HOBt) using a standard double-coupling protocol. For a difficult coupling for the amino acids following an N^{α}-alkyl amino acid, a single DCC/HOBt coupling followed by another coupling using benzotriazolyloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP reagent) was performed.[52] A complete coupling in each step was monitored by a Kaiser ninhydrin test.[53] Boc group was cleaved with 50% trifluoroacetic acid (TFA) in methylene chloride, and the generated amino terminus was neutralized with treatment of 7% diisopropylethylamine (DIEA) in methylene chloride. 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 diisopropylethylamine or the symmetrical anhydride of the corresponding acid. The peptides were cleaved from the resin with deprotection of the side-chain protecting groups using anhydrous HF in the presence of 10% anisole as a carbonium-ion scavenger at 0 °C for 60 min. Cyclic disulfide peptide analogues of 2 were prepared from linear precursors using either $K_3Fe(CN)_6$ for those containing free N-terminal and C-terminal thiols or the disulfide-exchange reaction for those containing N-terminal or C-terminal Cys(SEt) at one end and a free thiol at the other end.[29] For analogues related to the cyclic peptide 18, a spontaneous cyclization was observed upon completion of the HF reaction and ether wash. We elaborated on this observation by subjecting the crude residue obtained after HF and ether wash to vacuum for a brief period of time to remove the ethanethiol and drive the cyclization into completion. The peptides were purified by one of the following methods: Sephadex G-15 gel filtration using 0.2 M acetic acid, flash medium-pressure reverse-phase C-18 silica using an appropriate mixture of acetonitrile in 0.1% aqueous TFA solution as eluent, or preparative reverse-phase HPLC using C-18 column with acetonitrile–0.1% aqueous TFA solution to >95% purity. The peptides were analyzed for structure confirmation using FABMS (VG Zab high-resolution mass spectrometer with fast atom bombardment technique), where the molecular ion peak clusters at $(M + H)^+$ and $(M - H)^-$ in the negative ion spectra were observed for each peptide, and amino acid analysis (24 h, 6 N HCl hydrolysis at 100 °C, performed on a Dionex Autoion 100 analyzer). Peptide purity was determined by: (a) analytical TLC (precoated silica gel, 0.25 mm, 5 × 20 cm; E. Merck), developed for a minimum length of 10 cm in at least one solvent system, Table 7, and visualized by spraying with 10% clorox solution followed by 1% KI–starch solution and (b) HPLC analysis (Beckman dual pump system using either ODS silica 5-μm Altex or Vydac 218 TP, 4.6- × 250-mm analytical column)

Table 7. Peptide Analytical Data

no.	compound	TLC sys[a]	TLC R_f	HPLC CH_3CN[b]	HPLC K'	FABMS $(M+H)^+$	amino acid analyses, molar ratio[c]
2	cyclo-*S,S*-[Ac-C(N^{α}-Me)RGD-Pen]-NH_2	1	0.62	5i	2.39	634.7	Asp (1.00), Gly (0.98), (N^{α}-Me)Arg (+), Cys/Pen (+)
		2	0.56	5–50g	4.16		
3	cyclo-*S,S*-[Bzl-C(N^{α}-Me)RGD-Pen]-NH_2	1	0.67	10–50g	7.3	696.3	Asp (1.00), Gly (0.97), (N^{α}-Me)Arg (+), Cys/Pen (+)
		2	0.61				
4	cyclo-*S,S*-[Bu-C(N^{α}-Me)RGD-Pen]-NH_2	1	0.63	10–50g	3.99	662.3	Asp (1.00), Gly (0.97), (N^{α}-Me)Arg (+), Cys/Pen (+)
		2	0.60				
5	cyclo-*S,S*-[Pac-C(N^{α}-Me)RGD-Pen]-NH_2	1	0.7	10–50g	5.99	710.2	Asp (1.00), Gly (1.01), (N^{α}-Me)Arg (+), Cys/Pen (+)
		2	0.66				
6	cyclo-*S,S*-[C(N^{α}-Me)RGD-Pen]-NH_2	1	0.43	10–50g	1.4	592.3	Asp (1.00), Gly (1.02), (N^{α}-Me)Arg (+), Cys/Pen (+)
		2	0.49				
7	cyclo-*S,S*-[Mpr-C(N^{α}-Me)RGD-Pen]-NH_2	1	0.58	10i	6.11	577.2	Asp (1.00), Gly (1.15), (N^{α}-Me)Arg (+), Mpr/Pen (+)
		2	0.5	0–50g	10.93		
8	cyclo-*S,S*-[Ac-C(N^{α}-Me)RGD-Pen]-OH	1	0.54	10–5g	4.27	635.3	Asp (1.00), Gly (1.02), (N^{α}-Me)Arg (+), Cys/Pen (+)
		2	0.53				
9	cyclo-*S,S*-[C(N^{α}-Me)RGD-Pen]-OH	1	0.39	10–50g	3.52	593.1	Asp (1.00), Gly (1.05), (N^{α}-Me)Arg (+), Cys/Pen (+)
		2	0.43				
10	cyclo-*S,S*-[Ac-CRGDC]-NH_2	1	0.52	3i	2.7	592.2	Asp (1.00), Gly (1.00), Arg (0.90), Cys (+)
		2	0.57	1–50g	5.1		
11	cyclo-*S,S*-[Ac-Pen-RGDC]-NH_2	1	0.61	3i	8.4	620.2	Asp (1.00), Gly (1.07), Arg (0.85), Cys/Pen (+)
		2	0.69	1–50g	5.8		
12	cyclo-*S,S*-[Ac-CRGD-Pen]-NH_2	1	0.37	5i[d]	2.2	620.2	Asp (1.00), Gly (1.01), Arg (0.67), Cys/Pen (+)
		3	0.097	0–50g[e]	3.7		
13	cyclo-*S,S*-[Ac-Pen-(N^{α}-Me)RGD-Pen]-NH_2	1	0.6	10i	4.4	662.3	Asp (1.00), Gly (1.03), (N^{α}-Me)Arg (+), Pen/Pen (+)
		2	0.56	1–50g	6.8		
14	cyclo-*S,S*-[Mba-RGDC]-NH_2	1	0.48	12i[d]	2.2	583	Asp (0.94), Gly (1.00), Arg (0.42), Cys (+)
		3	0.22	0–30g[f]	4.5		
15	cyclo-*S,S*-[Ac-CRGD-Man]	1	0.57	10i[d]	4.9	587.2	Asp (1.00), Gly (1.03), Arg (0.94), Cys (+)
		3	0.23	0–50g[e]	4.5		
16	cyclo-*S,S*-[Ac-C(N^{α}-Me)RGD-Man]			5–50g[d,g]	4.6	611.2	Asp (1.00), Gly (1.06), (N^{α}-Me)Arg (+), Cys (+)
17	cyclo-*S,S*-[Mba-RGD-Man]	1	0.68	20i	3.6	588	Asp (0.81), Gly (1.28), Arg (1.00)
		2	0.70	10–50g	6.0		
18	cyclo-*S,S*-[Mba-(N^{α}-Me)RGD-Man]	1	0.75	21i	3.0	602.1	Asp (0.93), Gly (1.00), (N^{α}-Me)Arg (+)
		2	0.77	10–50g	3.4		
19	cyclo-*S,S*-[Mba-(N^{α}-Me)RGD-Mea]			5–50g[d,g]	6.6	554.2	Asp (1.00), Gly (0.89), (N^{α}-Me)Arg (+)
20	cyclo-*S,S*-[Ac-C(N^{α}-Me)RGD-(2*R*,3*S*)-β-PhC]-NH_2	1	0.56	15i	5.7	682.2	Asp (0.97), Gly (1.00), (N^{α}-Me)Arg (+), Cys/β-PhCys (+)
		2	0.61	0–50g	13.1		
21	cyclo-*S,S*-[Ac-C(N^{α}-Me)RGD-(2*R*,3*R*)-β-PhC]-NH_2	1	0.7	10i	8.51	682	Asp (1.00), Gly (0.96), (N^{α}-Me)Arg (+), Cys/β-PhCys (+)
		2	0.67	1–50g	8.35		
22	cyclo-*S,S*-[Ac-C(N^{α}-Me)RGD-(2*R*,3*S*)-β-PhC]-OH	1	0.53	14i	6.3	683.1	Asp (1.00), Gly (1.05), (N^{α}-Me)Arg (+), Cys/β-PhCys (+)
		2	0.5	1–50g	9.3		
23	cyclo-*S,S*-[Ac-CR(N^{α}-Me)GD-Pen]-NH_2	1	0.57	7i	3.07	634.2	Asp (1.00), (N^{α}-Me)Gly (+), Arg (1.03), Cys/Pen (+)
		2	0.61	0–50g	9.3		
24	cyclo-*S,S*-[Ac-CRG(N^{α}-Me)D-Pen]-NH_2	1	0.47	4i	7.49	634.2	(N^{α}-Me)Asp (+), Gly (1.00), Arg (1.00), Cys/Pen (+)
		2	0.32	1–50g	6.63		
25	cyclo-*S,S*-[Ac-C(N^{α}-Et)RGD-Pen]-NH_2	1	0.47	7i	6.14	648.8	Asp (1.00), Gly (1.08), (N^{α}-Et)Arg (+), Cys/Pen (+)
		2	0.43	1–50g	14.08		
26	cyclo-*S,S*-[Ac-C(N^{α}-Bzl)RGD-Pen]-NH_2	1	0.74	15i	8.4	710.5	Asp (1.07), Gly (1.00), (N^{α}-Bzl)Arg (+), Cys/Pen (+)
		2	0.73	1–50g	3.9		
27	cyclo-*S,S*-[Ac-C(N^{α}-Me)R(N^{α}-Me)GD-Pen]-NH_2	1	0.62	7i	7.44	648.2	Asp (1.00), (N^{α}-Me)Gly, (N^{α}-Me)Arg (+), Cys/Pen (+)
		2	0.6	0–50g	10.27		
28	cyclo-*S,S*-[Ac-C(N^{α}-Me)RG(N^{α}-Me)D-Pen]-NH_2	1	0.41	10i[d]	1.0	648.4	Gly (1.00), Gly (1.05), (N^{α}-Me)Asp (+), (N^{α}-Me)Arg (+), Cys/Pen (+)
29	cyclo-*S,S*-[Ac-CrGD-Pen]-NH_2	1	0.31	6i	5.2	620.4	Asp (1.00), Gly (1.03), Arg (1.01), (N^{α}-Me)Arg (+), Cys/Pen (+)
		2	0.54	5–50g	3.9		
30	cyclo-*S,S*-[Ac-CRGd-Pen]-NH_2	1	0.4	8i	1.9	620.2	Asp (1.00), Gly (0.96), Arg (0.96), Cys/Pen (+)
		2	0.29	5–50g	3.5		
31	cyclo-*S,S*-[Ac-CRGD-Pen]-NH_2	1	0.42	8i[d]	1.0	620.2	Asp (1.00), Gly (1.03), Arg (0.88), Cys/Pen (+)
		2	0.11	0–50g[e]	2.8		
32	cyclo-*S,S*-[Ac-cRGD-Pen]-NH_2	1	0.40	6i	2.05	620.2	Asp (1.00), Gly (1.03), Arg (1.20), Cys/Pen (+)
		2	0.46	1–50g	4.42		
33	cyclo-*S,S*-[Ac-c(N^{α}-Me)RGD-Pen]-NH_2	1	0.79	10i	3.8	634.2	Asp (1.00), Gly (0.99), (N^{α}-Me)Arg (+), Cys/Pen (+)
		2	0.83	1–50g	6.3		
34	cyclo-*S,S*-[Ac-C(N^{α}-me)rGD-Pen]-NH_2	1	0.61	6i	5.8	634.3	Asp (1.01), Gly (1.00), (N^{α}-Me)Arg (+), Cys/Pen (+)
		2	0.54	1–50g	6.4		
35	cyclo-*S,S*-[Mba-(N^{α}-me)rGD-Man]	1	0.74	21i	6.1	602	Asp (1.00), Gly (1.21), (N^{α}-Me)Arg (+)
		2	0.68	10–50g	6.7		
36	cyclo-*S,S*-[Mba-(N^{α}-Me)RGd-Man]	1	0.76	25i	2.2	602.2	Asp (1.00), Gly (1.051), (N^{α}-Me)Arg (+)
		2	0.9	10–60g	6.7		
37	cyclo-*S,S*-[Ac-CGRGD-Pen]-NH_2	1	0.45	7i[d]	2.6	677.1	Asp (1.00), Gly (1.96), Arg (1.01), Cys/Pen (+)
		3	0.13	0–50g[e]	0.9		
38	cyclo-*S,S*-[Ac-C(N^{α}-Me)GRGD-Pen]-NH_2	1	0.53	7i	4.2	691.2	Asp (1.00), Gly (1.14), (N^{α}-Me)Gly (+), Arg (1.04), Cys/Pen (+)
		2	0.67	1–20g	6.5		
39	cyclo-*S,S*-[Ac-CG(N^{α}-Me)RGD-Pen]-NH_2	1	0.41	9i	3.8	691.4	Asp (1.00), Gly (1.95), (N^{α}-Me)Arg (+), Cys/Pen (+)
		2	0.46	1–50g	6.67		
40	cyclo-*S,S*-[Ac-C(N^{α}-Me)G(N^{α}-Me)RGD-Pen]-NH_2	1	0.42	6i	5.66	721.3	Asp (1.00), Gly (1.00), Ser (0.96), (N^{α}-Me)Arg (+), Cys/Pen (+)
		2	0.41	0–50g	3.19		

Table 7. (Continued)

no.	compound	TLC sys[a]	TLC R_f	HPLC CH_3CN[b]	HPLC K'	FABMS $(M+H)^+$	amino acid analyses, molar ratio[c]
41	cyclo-*S,S*-[Ac-C(N^α-Bzl)G(N^α-Me)RGD-Pen]-NH_2	1	0.61	17i	12.8	781.1	Asp (1.00), Gly (1.00), (N^α-Bzl)Gly, (N^α-Me)Arg (+), Cys/Pen (+)
		2	0.64	0–50g	10.1		
42	cyclo-*S,S*-[Ac-CP(N^α-Me)RGD-Pen]-NH_2	1	0.33	17i	6.58	731.4	Asp (1.00), Pro (0.98), Gly (1.03), (N^α-Me)Arg (+), Cys/Pen (+)
		2	0.5	1–50g	8.42		
43	cyclo-*S,S*-[Mba-(N^α-Me)GRGD-Man]	1	0.73	20i	6.9	659.1	Asp (1.00), Gly (1.03), Arg (1.11), (N^α-Me)Gly (+)
		2	0.67	10–50g	6.6		
44	cyclo-*S,S*-[Mba-(N^α-Me)G(N^α-Me)RGD-Man]	1	0.68	20i	8.6	673.2	Asp (1.04), Gly (1.00), (N^α-Me)Gly (+), (N^α-Me)Arg (+)
		2	0.74	10–50g	11.84		
45	cyclo-*S,S*-[Mba-P(N^α-Me)RGD-Man]	1	0.6	22i	5.57	699.2	Asp (1.00), Pro (0.84), Gly (1.10), (N^α-Me)Arg (+)
		2	0.57	1–50g	10.13		

[a] TLC system: 1 = *n*-BuOH:AcOH:H_2O:EtOAc, 1:1:1:1; 2 = *n*-BuOH:AcOH:H_2O:pyridine, 15:5:10:10; 3 = *n*-BuOH:H_2O:*i*-PrOH:$CHCl_3$, 6.5:2:5:0.3. [b] Analytical HPLC run using Altex Ultrasphere ODS, 4.5 mm × 25 cm, or as noted in footnote *d*; detection at 220 nm either in an isocratic mode (i) or a gradient mode for 20 min (g), or as noted in footnote *e*, with the percentage CH_3CN employed in the mixture with 0.1% TFA. [c] Quantitative determination of the presence of (N^α-Me)Arg, Cys, and Pen indicated by (+) due to difficult quantitation. [d] Employed Vydac 218 TP, ODS, 4.6 mm × 25 cm. [e] 15-min gradient run. [f] 10-min gradient run. [g] 30-min gradient run.

with 1.5 mL/min flow rate with a UV detection at 220 nm under the conditions listed in Table 7. Analytical data for all the purified peptides are listed in Table 7.

Preparation of cyclo-*S,S*-[2-mercaptobenzoyl-N^α-methylarginyl-glycyl-aspartyl-2-mercaptophenylamide] (cyclo-*S,S*-[Mba-(N^α-Me)Arg-Gly-Asp-Man]) (18). (a) Preparation of 2-(4-Methylbenzyl)thioaniline (Man-4-MeBzl) (47). To a solution of 2-thioaniline (5.0 mL, 42 mmol) in ethanol (50 mL) was added triethylamine (5.9 mL, 423 mmol) under argon. α-Bromo-*p*-xylene (7.78 g, 42 mmol) in ethanol (50 mL) was then added dropwise. The reaction mixture was stirred for 1 h, concentrated in vacuo to a small volume, diluted with anhydrous ether, and filtered to remove triethylamine hydrobromide. The filtrate was concentrated to dryness to give a yellow oil (5.0 g, 52%). Flash column chromatography (silica gel, 20% ethyl acetate/hexane) yielded **47** as a yellow oil (4.26 g): DCIMS $(M+H)^+$ 230; ^1H NMR ($CDCl_3$, 90 MHz) δ 2.35 (s, 3 H), 3.85 (s, 2 H), 4.25 (br s, 2 H), 6.7 (m, 2 H), 7.2 (m, 6 H).

(b) Preparation of Boc-Asp(*O*-cHex)-Man(4-MeBzl) (48). To a cold solution of Boc-Asp(*O*-cHex) (31.5 g, 100 mmol) in THF (500 mL) and *N*-methylmorpholine (13.1 g, 120 mmol) was added isobutyl chloroformate (15.6 mL, 1.2 mmol) dropwise. The reaction mixture was stirred for a few minutes, and a solution of **47** (22.0 g, 96 mmol) in THF (500 mL) was added. The reaction mixture was allowed to warm to room temperature and stirred for 18 h. Upon completion of the reaction, the reaction mixture was filtered and the filtrate was concentrated to dryness. The residue was dissolved in ethyl acetate (500 mL) and washed successively with 5% aqueous citric acid (3 × 150 mL), water (1 × 400 mL), 10% aqueous $NaHCO_3$ (1 × 400 mL), water (1 × 400 mL), and saturated salt solution (1 × 300 mL). The solution was dried (anhydrous K_2CO_3), filtered, and concentrated to yield **48** (53 g): TLC (EtOAc/hexane, 3:7) R_f 0.72; FABMS $(M+H)^+$ 527; ^1H NMR ($CDCl_3$, 90 MHz) δ 1.2–1.9 (m, s, 20 H), 2.3 (s, 3 H), 2.8 (d, 2 H), 3.9 (s, 2 H), 4.4–4.9 (m, 2 H), 5.5 (m, 1 H), 6.8–7.4 (m, 6 H), 8.2 (d, 1 H), 9.2 (s, 1 H).

(c) Preparation of Asp(*O*-cHex)-Man(4-MeBzl) (49). A solution of **48** (52 g) in methylene chloride (CH_2Cl_2) was treated with 50% TFA/CH_2Cl_2 (400 mL) for 45 min at room temperature. The solvent was evaporated and chased several times with CH_2Cl_2 to eliminate traces of TFA. The product precipitated as its TFA salt upon addition of ether. The solid was collected and air-dried to yield a white solid of **49** (46.7 g, 88%): FABMS $(M+H)^+$ 427.4.

(d) Preparation of Boc-Gly-Asp(*O*-cHex)-Man(4-MeBzl) (50). To a cold solution of **49** (46.7 g, 86.4 mmol) in DMF (100 mL) was added DIEA (15 mL, 86.1 mmol). *N*-Hydroxybenzotriazole (HOBt) (14.0 g, 104 mmol) was added followed by Boc-Gly (16.6 g, 94.8 mmol). The reaction mixture was stirred in the cold for a few minutes, and *N*-ethyl-*N'*-[(dimethylamino)propyl]-carbodiimide (EDC) (18.2 g, 94.9 mmol) was added. The reaction mixture was allowed to warm to room temperature and stirred for 18 h. The reaction mixture was concentrated to a small volume and poured into 1.5 L of aqueous 10% K_2CO_3. The precipitated product was collected by filtration and washed with water to neutral pH to give **50** (50.6 g): TLC (EtOAc/hexane, 3:7) R_f 0.37; FABMS $(M+H)^+$ 584.6; ^1H NMR ($CDCl_3$, 90 MHz) δ 1.1–1.9 (m, s, 19 H), 2.3 (s, 3 H), 2.8 (m, 3 H), 3.9 (m, 4 H), 4.7–5.2 (m, 3 H), 6.9–7.5 (m, 7 H), 8.3 (d, 1 H), 9.2 (s, 1 H).

(e) Preparation of Gly-Asp(*O*-cHex)-Man(4-MeBzl) (51). A solution of **50** (11.7 g, 20 mmol) in CH_2Cl_2 was treated with 50% TFA/CH_2Cl_2 (80 mL) as described in (c) to give 12.4 g of **51**.

(f) Preparation of Boc-(N^α-Me)Arg(Tos)-Gly-Asp(*O*-cHex)-Man(4-MeBzl) (52). To an ice-cold solution of **51** (12.4 g, 20 mmol) in DMF (20 mL) was added DIEA (3.6 mL, 20 mmol). To this solution was added HOBt (3.4 g, 20 mmol) followed by Boc-(N^α-Me)Arg(Tos) (10.1 g, 22 mmol). The reaction mixture was stirred for several min, and EDC (4.4 g, 22 mmol) was added portionwise. The reaction mixture was allowed to warm to room temperature and stirred for 18 h. The reaction mixture was concentrated to a small volume and poured into 10% aqueous K_2CO_3. The resulting solid was collected by filtration and washed with water to neutral pH to provide **52** (20.4 g): TLC (CH_2Cl_2/MeOH, 9:1); FABMS $(M+H)^+$ 908.1; ^1H NMR ($CDCl_3$, 90 MHz) δ 1.2–2.0 (m, s, 20 H), 2.3 (2s, 7 H), 2.7–3.3 (m, 10 H), 3.9 (m, 4 H), 4.5–5.1 (m, 3 H), 6.4–6.6 (br s, 2 H), 6.8–8.2 (m, 14 H), 9.1 (s, 1 H).

(g) Preparation of (N^α-Me)Arg(Tos)-Gly-Asp(*O*-cHex)-Man(4-MeBzl) (53). A solution of **52** (17.7 g, 19.5 mmol) in CH_2Cl_2 was treated with 50% TFA/CH_2Cl_2 (80 mL) as described in (c) to give the TFA salt of **53**.

(h) Preparation of 2-*S*-(Ethylthio)mercaptobenzoic Acid (Mba(SEt)) (54). To argon-purged hexane (50 mL) were added ethanethiol (3.7 mL, 50 mmol) and sulfuryl chloride (4.0 mL, 50 mmol), and the solution was stirred for 30 min. Toluene (100 mL) was added followed immediately by 2-mercaptobenzoic acid (17.71 g, 50 mmol). The reaction mixture was stirred at room temperature for 3 h, and the solid product precipitated out. The solid was filtered, dried, and chromatographed (flash silica gel column, ethyl acetate) to yield **54** as a tan solid (3.8 g, 36%): DCIMS $(M+H)^+$ 215; ^1H NMR ($CDCl_3$, 90 MHz) δ 1.15 (t, 3 H), 2.5 (q, 2 H), 7.1 (t, 1 H), 7.4 (t, 1 H), 8.1 (d, 2 H), 11.0 (s, 1 H).

(i) Preparation of Mba(SEt)-(N^α-Me)Arg(Tos)-Gly-Asp-(*O*-cHex)-Man(4-MeBzl) (55). To a cold solution of **53** (20 mmol) in DMF (20 mL) was added DIEA (3.5 mL, 22 mmol) dropwise. Mba(SEt) (**54**) (4.6 g, 22 mmol) and EDC (4.2 g, 22 mmol) were added successively followed by 4-*N,N*-(dimethylamino)pyridine (DMAP) (2.9 g, 24 mmol). The reaction mixture was allowed to warm to room temperature, and stirring was continued for another 24 h. Another portion of **54** (4.6 g, 22 mmol), DMAP (2.9 g, 24 mmol), and EDC (4.2 g, 22 mmol) was added, and stirring was continued for another 24 h to obtain complete reaction. The reaction mixture was washed successively with water, 5% aqueous citric acid, water, and saturated salt solution. The organic extracts was dried (anhydrous Na_2SO_4), filtered, and concentrated to a solid residue (19.1 g). Chromatography (flash silica gel column, 10% MeOH/CH_2Cl_2) gave **55** (9.0 g): FABMS $(M+H)^+$ 1004.4; ^1H NMR ($CDCl_3$, 90 MHz) δ 1.1–2.0 (m, 19 H), 2.2 (s, 3 H), 2.3 (s, 3 H), 2.7 (m, 8 H), 3.8–3.9 (m, 4 H), 4.8 (m, 4 H), 6.35 (br s, 3 H), 6.9–8.2 (m, 17 H), 8.1 (s, 1 H).

(j) Preparation of cyclo-*S,S*-[Mba-(N^{α}-Me)Arg-Gly-Asp-Man] (18). The protected linear peptide **55** (8.5 g, 8.5 mmol) was treated with anhydrous HF (90 mL) and anisole (8.5 mL) at 0 °C for 1 h. The HF was removed at 0 °C under vacuum, and the residue was washed with ether to yield a tan solid (5.0 g). The solid was subjected to vacuum for about 1 h to achieve complete cyclization. About 2.5 g of the crude product was purified by flash chromatography (medium-pressure ODS reverse-phase column, eluted with 21% acetonitrile/water–0.1% TFA). Fractions which contained > 90% pure peptides were combined and lyopholized. Further purification using Sephadex G-15 gel filtration (0.2 M acetic acid) provided 18 (1.0 g) with >98% purity. The analytical data for 18 are listed in Table 7.

Preparation of Boc-(N^{α}-Bzl)Arg(Tos). (a) Preparation of (N^{α}-Bzl)Arg(Tos) (56). To a solution of N^{G}-tosylarginine (25 g, 76.1 mmol) and benzaldehyde (8.0 mL, 76.1 mmol) in methanol (30 mL) was added $NaBH_3CN$ (1.6 g, 25.5 mmol) at room temperature, and the reaction mixture was stirred under argon for 90 min. The pH of the reaction mixture was maintained at 6.5–7.0 by adding the appropriate amount of 1 N HCl. An additional amount of benzaldehyde (8.0 mL) and $NaBH_3CN$ (1.6 g) was added, and stirring was continued for 18 h. N^{α}-Bzl-N^{G}-tosylarginine (**56**) precipitated and was collected by filtration. The solid was triturated with hot methanol to give 10.5 g of **56**: mp 160–167 °C dec; FABMS $(M + H)^+$ 419; ^1H NMR ($CDCl_3$, 90 MHz) δ 1.9 (m, 4 H), 2.4 (s, 3 H), 3.3 (m, 2 H), 4.1 (s, 2 H), 4.8 (m, 4 H), 7.1 (s, 5 H), 7.5 (d, 2 H), 7.9 (d, 2 H).

(b) Preparation of Boc-(N^{α}-Bzl)Arg(Tos) (57). To a solution of **56** (6.6 g, 15.8 mmol) in DMF (80 mL) and DIEA (2.7 mL, 15.8 mmol) was added in a dropwise manner di-*tert*-butyl dicarbonate ($(Boc)_2O$) (1.3 mL, 15.8 mmol). The reaction mixture was stirred at room temperature for 2 h; another portion of $(Boc)_2O$ (1.3 mL, 15.8 mmol) was added, and stirring was continued for 18 h. The reaction mixture was concentrated, and the residue was dissolved in 1 N NaOH and extracted twice with *n*-hexane. The basic aqueous solution was acidified with $KHSO_4$ to pH 2.3. The formed precipitate was filtered and air-dried to yield 7.2 g of **57**; TLC ($CH_2Cl_2/CH_3OH/AcOH$, 8.5:1:0.5) R_f 0.8; FABMS $(M + H)^+$ 519; ^1H NMR ($CDCl_3$, 90 MHz) δ 1.5 (s, 9 H), 1.9 (m, 4 H), 2.4 (s, 3 H), 3.3 (m, 2 H), 4.1 (s, 2 H), 4.8 (m, 3 H), 7.1 (s, 5 H), 7.5 (d, 2 H), 7.9 (d, 2 H).

Preparation of Boc-(N^{α}-Me)Arg(Tos). (a) Preparation of (N^{α}-Bzl)(N^{α}-Me)Arg(Tos) (58).[54] To a finely powdered **56** (8.35 g, 2.0 mmol) were added formic acid (95–97%) (2.3 mL, 60 mmol) and formaldehyde (37–40%) (1.95 mL, 24 mmol). The reaction mixture was heated on a steam bath for 45 min and then concentrated in vacuo to a gummy material of **58**: FABMS $(M + H)^+$ 433; ^1H NMR ($CDCl_3$, 90 MHz) δ 1.9 (m, 4 H), 2.4 (s, 3 H), 2.6 (s, 3 H), 3.3 (m, 2 H), 4.1 (s, 2 H), 4.8 (m, 4 H), 7.1 (s, 5 H), 7.5 (d, 2 H), 7.9 (d, 2 H).

(b) Preparation of (N^{α}-Me)Arg(Tos) (59). To a solution of **58** (2.0 mmol) in glacial acetic acid (50 mL) was added a mixture of water (10 mL) and 3 N HCl (5 mL) and then a catalytic amount of 5% palladium on carbon. The mixture was hydrogenated on a Parr shaker for about 6 h for complete uptake of hydrogen. The catalyst was removed by filtration, and the filtrate was concentrated to an oil. The oil residue was dissolved in water and neutralized to pH 7 with a dilute solution of ammonium hydroxide where **59** precipitated as a white solid. It was recrystallized from hot water twice to give 2.5 g of pure **59**. The structure was confirmed with: mp 210–216 °C dec; FABMS $(M + H)^+$ 343; ^1H NMR (D_2O/DCL, 90 MHz) δ 1.75 (m, 4 H), 2.4 (s, 3 H), 2.8 (s, 3 H), 3.3 (t, 2 H), 4.0 (t, 1 H), 7.5 (d, 2 H), 7.9 (d, 2 H). Anal. Calcd for $C_{14}H_{22}N_4O_4S \cdot H_2O$: C, 46.65; H, 6.71; N, 15.55. Found: C, 46.64; H, 6.70; N, 15.02.

(c) Preparation of Boc-(N^{α}-Me)Arg(Tos) (60). To a solution of **59** (2.5 g, 7.3 mmol) in sodium hydroxide solution (0.3 g in 1 mL of water) and *tert*-butyl alcohol (1.5 mL) was added $(Boc)_2O$ (1.75 g, 8.03 mmol) slowly with good stirring. After a short induction period, the temperature rose from 23 to 28 °C. The reaction was brought to completion after the addition of another portion of *tert*-butyl alcohol (1.15 mL), and the mixture was stirred for 18 h. Water (10 mL) was then added to the reaction mixture, and it was extracted with *n*-hexane (3 × 10 mL). The aqueous solution was acidified, while cooling with $KHSO_4$ (1.0 g), to pH 2.2. The formed white precipitate was filtered and recrystallized from ethyl acetate–hexane mixture to give **60** (1.37 g). The structure was confirmed with: $[\alpha]^{25}_D$ (0.1 MeOH) −18.5°; TLC (*n*-BuOH/AcOH/H_2O, 8:2:2) R_f 0.83; mp 80–84 °C dec; FABMS $(M + H)^+$ 443; ^1H NMR ($CDCl_3$, 90 MHz) δ 1.5 (s, 9 H), 2.4 (s, 3 H), 2.75 (s, 3 H), 3.2 (m, 2 H), 4.0 (m, 1 H), 6.6 (br, 3 H), 7.25 (d, 2 H), 7.75 (d, 2 H). Anal. Calcd for $C_{19}H_{30}N_4O_6S \cdot 0.5H_2O$: C, 50.54; H, 6.92; N, 12.41. Found: C, 50.59; H, 6.81; N, 12.26.

Preparation of Boc-(N^{α}-Et)Arg(Tos) (61). To a solution of N^{G}-tosylarginine (6.6 g, 20 mmol) in methanol (120 mL) and glacial acetic acid (3.0 mL) was added acetaldehyde (1.1 g, 22 mmol). The reaction mixture was stirred at room temperature for 15 min, and then, sodium cyanoborohydride (1.4 g, 22 mmol) was added. After stirring for 1 h, an additional portion of acetaldehyde (0.5 g) and $NaBH_3CN$ (0.2 g) was added, and stirring was continued for an additional 15 min. The reaction mixture was concentrated to dryness to give (N^{α}-Et)Arg(Tos) (6.4 g, 83%). To a solution of (N^{α}-Et)Arg(Tos) (3.28 g, 10 mmol) in *n*-BuOH (25 mL) and sodium hydroxide (25 mL of 1 N solution) was added $(Boc)_2O$ (2.19 g, 10 mmol), and stirred at room temperature for 18 h. The pH of the reaction mixture was adjusted to 5.5 with 6 N HCl, and it was extracted with EtOAc. It was purified on a flash silica gel column (CH_2Cl_2/MeOH/AcOH, 9:10:0.3) to give pure **60** (1.3 g): TLC (CH_2Cl_2/MeOH, 9:1); mp 116–118 °C; FABMS $(M + H)^+$ 457.2; ^1H NMR ($CDCl_3$, 90 MHz) δ 0.9–1.7 (m, 7 H), 1.3 (s, 9 H), 2.3 (s, 3 H), 3.3 (br m, 4 H), 4.2 (m, 1 H), 6.0 (br, 1 H), 6.5 (br, 3 H), 7.25 (d, 2 H), 7.75 (d, 2 H). Anal. Calcd for $C_{20}H_{32}N_4O_6S$: C, 52.61; H, 7.06; N, 12.27. Found: C, 52.67; H, 7.31; N, 11.61.

In Vitro Inhibition of Platelet Aggregation of Canine Platelet-Rich Plasma (PRP).[29] Platelet aggregation was measured optically on a Chrono-Log Lume aggregometer Model 400VS. The test peptide was dissolved in 1M acetic acid and diluted with water to give a stock solution of 12 mM peptide concentration in 0.2 M acetic acid. The stock peptide solution (5 μL) was added to the citrated canine PRP (300 μL) and ADP (5 μL) in a glass cuvette to give a solution with final concentrations of 200 μM peptide and 10 μM ADP, pH 7.7. The ability of a peptide (5-min preincubation) to inhibit platelet aggregation was determined, and the IC_{50} was measured as the concentration of the peptide required to produce 50% inhibition of the control response to the agonist.

In Vitro Inhibition of [^{125}I]Fibrinogen Binding to Purified, Reconstituted, Human Platelet Fibrinogen Receptor. The detailed experimental procedure for the Fg-binding assay, purification of GPIIb/IIIa receptor and its incorporation in phospholipid vesicles was discussed in a previous publiction.[29] A similar procedure was employed to assay all the peptides in Tables 1–5.

[^{3}H]SK&F 107260 Competition Binding Assay.[34] Binding assay was performed in a 96-well filtration plate assembly (Millipore Corporation, Bedford, MA) using 0.22-μm hydrophilic durapore membranes. The wells were precoated with 0.2 mL of 10 μg/mL polylysine (Sigma, St. Louis, MO) at room temperature for 1 h to block nonspecific binding. Various concentrations of the competing ligands (0.001–100 μM, diluted in a buffer containing 50 mM Tris–HCl, pH 7.4, 100 mM NaCl, and 2 mM $CaCl_2$ (buffer B)) were applied to the wells followed by the addition of 0.0045 μM [^{3}H]SK&F 107260 (85.6 Ci/mmol). The liposome-incorporated, lectin-column-purified GPIIb/IIIa (0.5 μg) was added to initiate the reaction. The samples were incubated at room temperature for 1 h. GPIIb/IIIa-bound [^{3}H]-SK&F 107260 was separated from the unbound by filtration using a Millipore filtration manifold followed by washing with ice-cold buffer B (4 × 0.2 mL). Bound radioactivity remaining on the filters was counted in 1.5 mL of Ready Solve (Beckman, Fullerton, CA) in a Beckman liquid scintillation counter Model LS6800 with 40% efficiency. The IC_{50} (concentration of the antagonist to inhibit 50% binding of [^{3}H]SK&F 107260) was determined by a nonlinear, least-squares curve-fitting routine, which was modified from the LUNDON-2 program. The K_i (dissociation constant of the antagonist) was calculated according to the Cheng and Prusoff equation: $K_i = IC_{50}/(1 + LK_d)$, where L and K_d are the concentration (0.0045 μM) and the dissociation constant (0.0045 μM) of [^{3}H]SK&F 107260, respectively.[55] The data

presented were from a single experiment. All data points were the mean ± SEM of quadruplicate determinations.

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