J. Am. Chem. Soc. **1994**, *116*, 3220–3230

Template-Constrained Cyclic Peptides: Design of High-Affinity Ligands for GPIIb/IIIa

Sharon Jackson,[†] William DeGrado,[*,†] Anil Dwivedi,[†] Anju Parthasarathy,[†] Anne Higley,[†] James Krywko,[†] Arlene Rockwell,[†] Jay Markwalder,[†] Greg Wells,[†] Ruth Wexler,[†] Shaker Mousa,[†] and Richard Harlow[‡]

Contribution from The DuPont Merck Pharmaceutical Company, Experimental Station, P.O. Box 80328, Wilmington, Delaware 19880-0328, and Central Research & Development Department, E. I. du Pont de Nemours and Company, P.O. Box 80228, Wilmington, Delaware 19880-0228

Received September 20, 1993[●]

Abstract: Although peptides adopt a large ensemble of conformations in aqueous solution, they are generally believed to bind to a receptor in a unique conformation. Thus, there is considerable interest in devising methods to restrict the conformational freedom of peptides. One such approach involves tying the amino and carboxy terminal ends of the peptide onto a semirigid template that will lock the intervening peptide backbone into a single conformer or a family of related conformers. This general strategy has been tested using the tripeptide sequence Arg-Gly-Asp (RGD), which binds with low affinity to the platelet glycoprotein IIb/IIIa (GPIIb/IIIa or $\alpha_{IIb}\beta_3$). Mimics of RGD are of interest as antithrombotics because of their ability to inhibit the aggregation of platelets. Prior to this study, other workers (Samanen et al. *J. Med. Chem.* **1991**, *34*, 3114–3125) prepared a disulfide-containing cyclic pentapeptide that bound to GPIIb/IIIa with an affinity of approximately 0.1 μM. NMR analysis of the solution conformation of this peptide suggested that replacing the disulfide-containing portion of the cycle with the amino acid *m*-(aminomethyl)benzoic acid would lead to a more rigid structure. Indeed, introduction of this template into a cyclic RGD-containing peptide resulted in compounds with high affinity for the receptor. Further, systematic inclusion of additional conformational constraints in the form of N^α- and C^α-alkyl groups led to a peptide with an affinity of approximately 100 pM for binding to the receptor. This peptide also showed good activity in the platelet aggregation assay at oral doses as low as 0.1 mg/kg.

Introduction

Peptides have long been recognized as an amazingly diverse class of biologically active agents with numerous potential therapeutic applications. However, their development into pharmaceutically acceptable drugs has been hindered by their limited stability and absence of oral activity.[1] These problems arise in large part from their conformational flexibility; in aqueous solution most peptides lack fixed three-dimensional structures and adopt an ensemble of conformational states. Thus, there is a current need for general approaches to increasing the structural rigidity of peptides, which in turn should lead to enhanced specificity, affinity, and oral activity.

One approach to this problem is to design nonpeptidic compounds that mimic the structural properties of peptides by virtue of having the necessary functional groups oriented appropriately for interaction with a receptor. This method has had some noteworthy successes; mimics of several peptide hormones have been discovered from natural sources or through random screening of synthetic compounds,[2] and in recent studies nonpeptidic mimics for Arg-Gly-Asp,[3] angiotensin,[4] and somatostatin[5] have been described. Nevertheless, there are few well-documented general strategies for the conversion of a peptide to a nonpeptidic mimic, and the design of mimics of peptides whose active portions span more than about three residues may be challenging indeed. For instance, mimetics of the active tetrapeptide sequence of somatostatin bind with low affinity when compared to the native peptide.[5]

Another approach has been to design compounds that more closely resemble peptides, but have added functional groups to increase their rigidity and decrease their proteolytic susceptibility (for reviews see ref 6); examples of successful modification of this class include C^α- or N^α-alkylation, replacement of the peptide bond with olefinic or other linkages, and the introduction of dehydroamino acids. Cyclization is also a highly successful strategy used by both nature and synthetic chemists to restrict the conformation of peptides.[6,7] The initial[8] and subsequent[9] work on somatostatin has shown how cyclization of a peptide can give rise to impressive gains in affinity, receptor subtype specificity, and restriction of conformational mobility.

[†] The DuPont Merck Pharmaceutical Co.

[‡] E. I. du Pont de Nemours and Co.

[●] Abstract published in *Advance ACS Abstracts*, February 15, 1994.

(1) Plattner, J. J.; Norbeck, D. W. In *Drug Discovery Technologies*; Clark, D. R., Moos, W. H., Eds.; Ellis Horwood Limited: Chichester, England, 1990; pp 92–138. Humphrey, M. J.; Ringrose, P. S. *Drug Metab. Rev.* **1986**, *17*, 283.

(2) Hirschmann, R. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 1278–1301.

(3) (a) Alig., L.; Edenhofer, A.; Hadváry, P.; Hürzeler, M.; Knopp, D.; Müller, M.; Steiner, B.; Trzeciak, A.; Weller, T. *J. Med. Chem.* **1992**, *35*, 4393–4407. (b) Hartman, G. D.; Egbertson, M. S.; Halczenko, W.; Laswell, W. L.; Duggan, M. E.; Smith, R. L.; Naylor, A. M.; Manno, P. D.; Lynch, R. J.; Zhang, G.; Chang, C. T.-C.; Gould, R. J. *J. Med. Chem.* **1992**, *35*, 4640–4642. (c) Hirschmann, R.; Sprengeler, P. A.; Kawasaki, T.; Leahy, J. W.; Shakespeare, W. C.; Smith, A. B., III. *J. Am. Chem. Soc.* **1992**, *114*, 9699–9701. (d) Ku, T. W.; Ali, F. E.; Barton, L. S.; Bean, J. W.; Bondinell, W. E.; Burgess, J. L.; Callahan, J. F.; Calvo, R. R.; Chen, L.; Eggleston, D. S.; Gleason, J. G.; Huffman, W. F.; Hwang, S. M.; Jakas, D. R.; Karash, C. B.; Keenan, R. M.; Kopple, K. D.; Miller, W. H.; Newlander, K. A.; Nichols, A.; Parker, M. F.; Peishoff, C. E.; Samanen, J. M.; Uzinskas, I.; Venslavsky, J. W. *J. Am. Chem. Soc.* **1993**, *115*, 8861–8862.

(4) Duncia, J. V.; Chiu, A. T.; Carini, D. J.; Gregory, G. B.; Johnson, A. L.; Price, W. A.; Wells, G. J.; Wong, P. C.; Calabrese, J. C.; Timmermans, P. B. M. W. M. *J. Med. Chem.* **1990**, *33*, 1312–1329.

(5) Hirschmann, R.; Nicolaou, K. C.; Pietranico, S.; Salvino, J.; Leahy, E. M.; Sprengeler, P. A.; Furst, G.; Smith, A. B., III; Strader, C. D.; Cascieri, M. A.; Candelore, M. R.; Donaldson, C.; Vale, W.; Maechler, L. *J. Am. Chem. Soc.* **1992**, *114*, 9217–9218.

(6) (a) Milner-White, E. J. *Trends Pharm. Sci.* **1989**, *10*, 70–74. (b) Hruby, V. J. *Trends Pharm. Sci.* **1985**, *6*, 259–262. (b) DeGrado, W. F. *Adv. Protein Chem.* **1988**, *39*, 51–124. (c) Marshall, G. R. *Tetrahedron* **1993**, *49*, 3547–3558.

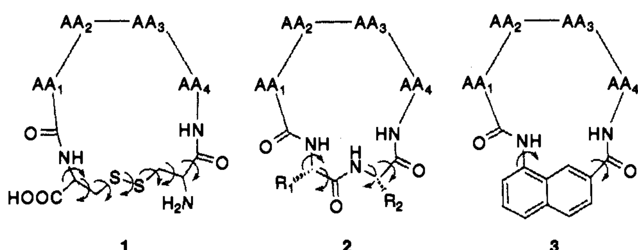
(7) (a) Schwyzer, R.; Ludescher, U. *Helv. Chim. Acta* **1969**, *52*, 2033–2040. Kessler, H. *Angew. Chem., Int. Ed. Engl.* **1982**, *21*, 512–523. (b) Gilon, C.; Halle, D.; Chorev, M.; Selinger, Z.; Byk, G. Biopolymers **1991**, *31*, 745–750. (c) Toniolo, C. *Int. J. Pept. Protein Res.* **1990**, *35*, 287–300.

(8) Veber, D. F.; Freidinger, R. M.; Perlow, D. S.; Palveda, W. F., Jr.; Holly, F. W.; Strachan, R. G.; Nutt, R. F.; Arison, B. H.; Hommick, C.; Randall, W. C.; Glitzer, M. S.; Saperstein, R.; Hirschmann, R. *Nature* **1981**, *292*, 55–58.

(9) (a) Schiller, P. W. *Peptides* **1984**, *6*, 219–268. (b) Rose, G. D.; Gierasch, L. M.; Smith, J. A. *Adv. Protein Chem.* **1985**, *37*, 1–109.

0002-7863/94/1516-3220$04.50/0 © 1994 American Chemical Society

A logical extension of current studies on cyclic peptides would be to incorporate a rigid nonpeptide linker into the macrocycle. If the template was chosen appropriately, it might serve to restrict the intervening peptide sequence to a conformation complementary to that of the receptor. Further, addition of functional groups to the linker might help fine-tune the receptor specificity or other pharmacological properties of the peptide. The relative advantage of incorporating a rigid nonpeptide template into the macrocycle can be appreciated by considering a tetrapeptide cyclized by either a Cys–Cys disulfide linker (**1**), a dipeptide linker (**2**), or a naphthalene linker[10] (**3**): a disulfide linker introduces seven



rotatable bonds, and the dipeptide linker introduces four rotatable bonds. In contrast, the naphthalene linker introduces only two rotatable bonds, which should confer the greatest degree of conformational definition to the intervening tetrapeptide sequence.

While the approach of template-constrained cyclic peptides has been described,[11] and although a number of potential linkers exist,[10,11] it appears that the full utility of this approach has yet to be demonstrated. Therefore, we have studied cyclic peptides containing Arg-Gly-Asp (RGD), a tripeptide sequence known to bind to the glycoprotein IIb/IIIa (GPIIb/IIIa or $\alpha_{IIb}\beta_3$).[12] GPIIb/IIIa is a membrane protein that mediates the aggregation of platelets. In response to stimulation by a variety of agonists including thromboxane, ADP, thrombin, or epinephrine, this protein undergoes a conformational change[13] allowing it to bind to fibrinogen. Multiple GPIIb/IIIa molecules appear to bind to a single molecule of fibrinogen, which leads to cross-linking of the platelets and formation of a platelet-rich clot. Thus, inhibitors of this process have potential as antithrombotic agents,[14] and a number of cyclic, RGD-containing peptides with affinities for GPIIb/IIIa in the micromolar to nanomolar range have been described.[15-17]

We felt that inhibitors of GPIIb/IIIa might provide an attractive model system for evaluating template-constrained cyclic peptides because of the relatively simple nature of the Arg-Gly-Asp recognition sequence. The limited length of this sequence would simplify both the synthetic strategies as well as the conformational analysis of the products. Also, there is a large and growing body of data on the interaction of RGD-containing peptides and peptide mimetics with GPIIb/IIIa, allowing a convenient comparison of this strategy with other approaches. This manuscript describes how the incorporation of a simple template into cyclic RGD-containing peptides has led to orally active compounds with extremely high affinity and specificity for GPIIb/IIIa.

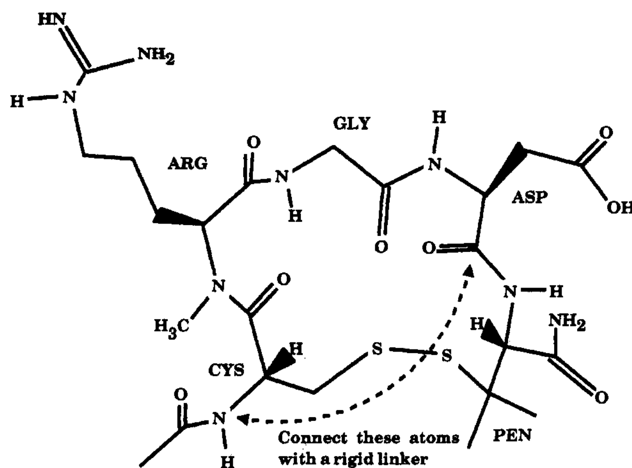


Figure 1. Preferred solution structure of SK&F 106760, as determined by proton NMR.

Results

Design of a Linker. The design of a linker to insert into a cyclic RGD-containing peptide began with an NMR investigation of a cyclic disulfide-containing peptide, SK&F 106760 acetyl-Cys-N^α-methyl-Arg-Gly-Asp-Pen-$CONH_2$ (Pen = penicillamine or $C^\beta,C^{\beta'}$-dimethyl cysteine) previously described by Samanen et al.[15] In our assays, this peptide is capable of inhibiting ADP-induced platelet aggregation with a midpoint (IC_{50}) of 5 μM, and its dissociation constant for binding to GPIIb/IIIa is approximately 0.1 μM. NMR of this peptide dissolved in H_2O indicated that the backbone of the structure adopted a predominant conformation or group of related conformers (Figure 1) in which the RGD tripeptide was highly extended, although the spectra also showed evidence for some conformational averaging. Indeed, Kopple and co-workers have recently published an extensive NMR and crystallographic study on this and related peptides,[18] providing strong evidence for a conformation similar to that described in Figure 1, as well as other conformations.

We next attempted to design a linker that would constrain the tripeptide to adopt the same conformation as in the predominant solution conformation of SK&F 106760. The following criteria were considered in designing the linker: (i) it should have a small molecular weight and be chemically stable; (ii) it should be relatively simple to prepare derivatives of the linker; (iii) the linker should be rigid, but should maintain some flexibility until the precise stereochemical requirements for receptor binding were fully understood; (iv) it should be readily prepared from inexpensive starting materials in a few synthetic steps; (v) the linker should have favorable solubility characteristics; (vi) it should be chemically inert and devoid of known toxicological properties.

Visual inspection of a physical, space-filling (CPK) model of the NMR structure of SK&F 106760 suggested that Mutter's tetralin-containing amino acid (**4**) would be a good candidate as a conformationally constrained linker to span from the amino

(10) Ernest, I.; Kalvoda, J.; Rihs, G.; Mutter, M. *Tetrahedron Lett.* **1990**, *31*, 4011–4014.

(11) (a) Boger, D. L.; Myers, J. B., Jr. *J. Org. Chem.* **1991**, *56*, 5385–5390. (b) Díaz, H.; Espina, J. R.; Kelly, J. W. *J. Am. Chem. Soc.* **1992**, *114*, 8316–8318. (c) Hann, M. M.; Carter, B.; Kitchin, J.; Ward, P.; Pipe, A.; Broomhead, J.; Hornby, E.; Forster, M.; Perry, C. In *Molecular Recognition: Chemical and Biochemical Problems II*; Roberts, S. M., Ed.; The Royal Society of Chemistry: Cambridge, 1992. (c) Wilson, S. R.; Tam, W. K.; Di Grandi, M. J.; Cui, W. *Tetrahedron* **1993**, *49*, 3655–3663. Sarabu, R.; Lovey, K.; Madison, V. S.; Fry, D. C.; Greeley, D. N.; Cook, C. M.; Olson, G. L. *Tetrahedron* **1993**, *49*, 3629–3640. Ripka, W. C.; De Lucca, G. V.; Bach, A. C.; Pottorf, R. S.; Blaney, J. M. *Tetrahedron* **1993**, *49*, 3609–3628.

(12) Gartner, T. K.; Bennett, J. S. *J. Biol. Chem.* **1985**, *260*, 11891–11894.

(13) (a) Plow, E. F.; Ginsberg, M. H. *Prog. Hemostasis Thromb.* **1989**, *9*, 117–156. (b) Phillips, D. R.; Charo, I. F.; Scarborough, R. M. *Cell* **1991**, *65*, 359–362.

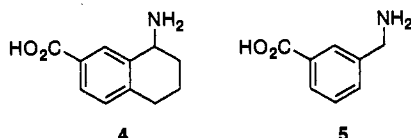
(14) Coller, B. S.; Folts, J. D.; Scudder, L. E.; Smith, S. R. *Blood* **1986**, *68*, 783–786.

(15) Samanen, J.; Ali, F.; Romoff, T.; Calvo, R.; Sorenson, E.; Vasko, J.; Storer, B.; Berry, D.; Bennett, D.; Strohsacker, M.; Powers, D.; Stadel, J.; Nichols, A. *J. Med. Chem.* **1991**, *34*, 3114–3125.

(16) (a) Barker, P. L.; Bullens, S.; Bunting, S.; Burdick, D. J.; Chan, K. S.; Deisher, T.; Eigenbrot, C.; Gadek, T. R.; Gantzos, R.; Lipari, M. T.; Muir, C. D.; Napier, M. A.; Pitti, R. M.; Padua, A.; Quan, C.; Stanley, M.; Struble, M.; Tom, J. Y. K.; Burnier, J. P. *J. Med. Chem.* **1992**, *35*, 2040–2048. (b) McDowell, R. S.; Gadek, T. R. *J. Am. Chem. Soc.* **1992**, *114*, 9245–9253.

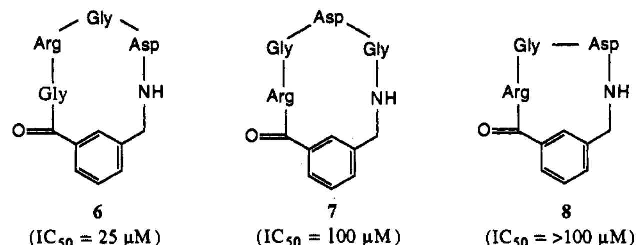
(17) (a) Bogusky, M. J.; Naylor, A. M.; Pitzenberger, S. M.; Nutt, R. F.; Brady, S. F.; Colton, C. D.; Sisko, J. T.; Anderson, P. S.; Veber, D. F. *Int. J. Pept. Protein Res.* **1992**, *39*, 63–76. (b) Scarborough, R. M.; Naughton, M. A.; Teng, W.; Rose, J. W.; Phillips, D. R.; Nannizzi, L.; Arfsten, A.; Campbell, A. M.; Charo, I. F. *J. Biol. Chem.* **1993**, *268*, 1066–1073.

(18) Kopple, K. D.; Baures, P. W.; Bean, J. W.; D'Ambrosio, C. A.; Hughes, J. L.; Peishoff, C. E.; Eggleston, D. S. *J. Am. Chem. Soc.* **1992**, *114*, 9615–9623.



nitrogen of the Cys residue to the carbonyl carbon of Asp. Although this residue was originally prepared as a β-turn mimic, our modeling indicates that it instead prefers a more extended conformation. Thus, if it were incorporated into a cyclic RGD-containing peptide it might help to stabilize the extended conformation of the RGD tripeptide. However, the preparation of this amino acid in racemic form is accomplished via a multistep synthesis, and it might also be overly rigid for the purposes of an initial linker. Therefore, we simplified its structure to **5**, *m*-(aminomethyl)benzoic acid (Mamb), which has also been described in the peptide-mimetic literature.[19] The Mamb moiety was incorporated into an initial cyclic peptide, *cyclo*(Gly-Arg-Gly-Asp-Mamb) (**6**), in which the Cys-Pen disulfide of SK&F 106760 has been replaced by Gly-Mamb. In **6**, Gly formally replaces the Cys residue of SK&F 106760; Gly was chosen to provide a moderate degree of flexibility in the initial design.

Compound **6**, *cyclo*(Gly-Arg-Gly-Asp-Mamb), was moderately active in the platelet aggregation assay, showing an IC_{50} = 25 μM (the concentration required to achieve 50% inhibition of the aggregation of platelets in response to the agonist, ADP). To assess the correctness of the guiding model, two other control peptides *cyclo*(Arg-Gly-Asp-Gly-Mamb) and *cyclo*(Arg-Gly-Asp-Mamb) (**7** and **8**), which were predicted to be less active were also prepared. As expected, **6** was found to be most active in the platelet aggregation assay.

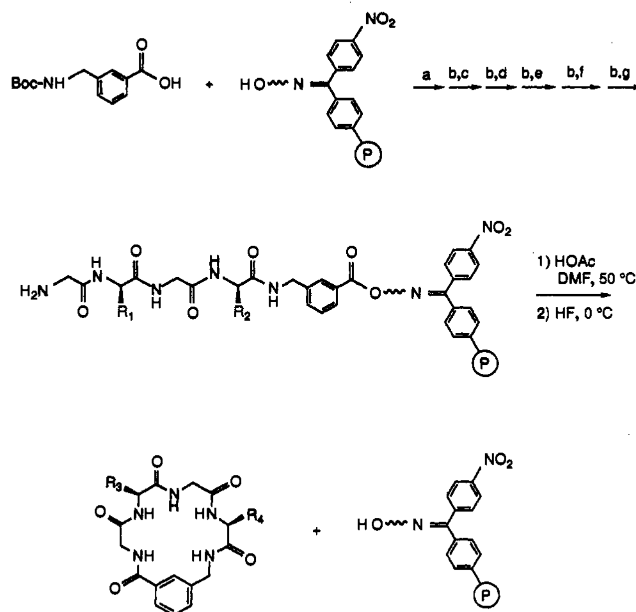


Synthesis of Cyclic Peptides Using the *p*-Nitrobenzophenone Oxime Resin. *cyclo*(Gly-Arg-Gly-Asp-Mamb) (**6**) and the other peptides described in this manuscript were prepared on a polymeric oxime support.[20,21] Peptides are cleaved from this support under mild conditions by nucleophilic attack with agents such as hydrazine, amino acids, or peptide esters. The oxime ester is sufficiently stable toward nucleophilic cleavage to allow solid-phase synthesis under carefully controlled conditions, but can be cleaved by amines in the presence of a single equivalent of acetic acid as a catalyst. Peptides can also be cleaved from the resin in an intramolecular aminolysis reaction, leading to a cyclic peptide.[20,22] We have found this method to be very rapid and convenient for the synthesis of the cyclic RGD-containing peptides.

Compound **6** was prepared using the approach outlined in Scheme 1, Briefly, Boc-*m*-(aminomethyl)benzoic acid[19] was esterified to the oxime polymer using 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) as a coupling agent, and the remaining amino acids were sequentially coupled using HBTU. After removal of the last protecting group, the peptide was cyclized by treatment with diisopropylethylamine (DIEA) to neutralize the trifluoroacetate salt, followed by acetic acid in DMF at 50 °C, The crude product thus obtained showed a single major peak on HPLC and was deprotected with anhydrous

(19) Stewart, F. H. C. *Aust. J. Chem.* **1983**, *36*, 2511–2516.
(20) DeGrado, W. F.; Kaiser, E. T. *J. Org. Chem.* **1980**, *45*, 1295–1300.
(21) DeGrado, W. F.; Kaiser, E. T. *J. Org. Chem.* **1982**, *47*, 3258–3261.
(22) Ösapay, G.; Profit, A.; Taylor, J. W. *Tetrahedron Lett.* **1990**, *31*, 6121–6124.

Scheme 1. Solid-Phase Synthesis of *cyclo*(Gly-Arg-Gly-Asp-Mamb) (**6**), P = Polystyrene, R_1 = $(CH_2)_3NHC(=NH)NHTos$, R_2 = $CH_2C(=O)OcHex$, R_3 = $(CH_2)_3NHC(=NH)NH_2$, R_4 = CH_2COOH, (a) HBTU/NMM, (b) 25% TFA in CH_2Cl, (c) Boc-Asp(OcHex), HBTU, NMM, (d) Boc-Gly, HBTU, NMM, (e) Boc-Arg(Tos), HBTU, NMM, (f) Boc-Gly, HBTU, NMM, (g) 10% DIEA in CH_2Cl_2



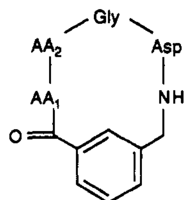
HF. The product was then purified in 11% overall yield (based on the esterification of the first amino acid on the resin) by reverse-phase HPLC.

Subsequent peptides in this series were prepared using this solid-phase method. The first amino acid was attached to the resin using dicyclohexylcarbodiimide (DCC) with (dimethylamino)pyridine added as a catalyst, which gave better results than HBTU. In cases where multigram to kilogram quantities were desired, some of the peptides were resynthesized using a closely related solution-phase method employing the 4-nitrobenzophenone oxime ester of Mamb, as described in the Experimental Section,

Improving the Affinity for GPIIb/IIIa. We next asked whether the addition of auxiliary groups to **6** would lead to a compound with enhanced affinity for the receptor. One way that this might easily be accomplished would be to change the first Gly to an L- or D-amino acid. (The following numbering will be used to refer to amino acids in this series of peptides: *cyclo*(Gly_1-Arg_2-Gly_3-Asp_4-$Mamb_5$).) This substitution would be expected to have two important consequences: side-chain-containing amino acids are far more conformationally constrained than Gly,[23] and hence the resulting peptides might be more potent if the active conformation was enforced. Secondly, the added functional groups might interact favorably with groups on GPIIb/IIIa proximal to the RGD-binding site and thereby increase the specificity and affinity of the peptide for the receptor. Thus, D-Ala or L-Ala was substituted for Gly at the first position of the peptide: D-Ala led to an increase in potency (IC_{50} = 8 μM), while L-Ala led to a decrease in potency. The steric requirements for the side chain projecting from this position were then investigated through the synthesis of a series of analogues, some of which are indicated in Figure 2. An ethyl group led to the greatest inhibition of platelet aggregation, and groups larger than isopropyl interfered with binding.

Introduction of N^{α}- and C^{α}-Methyl Groups as Conformational Constraints. Substituting methyl groups for hydrogen atoms in

(23) Zimmerman, S. S.; Pottle, M. S.; Nèmethy, G.; Scheraga, H. A. *Macromolecules* **1977**, *10*, 1–9.



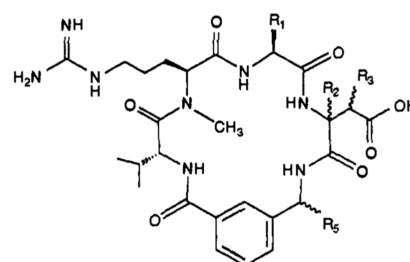
	AA1	AA2	IC50 (μM) Aggregation	IC50 (μM) Fibrinogen Binding
9	D-Ala	Arg	8	0.09
10	L-Ala	Arg	100	nd
11	D-Abu	Arg	7	nd
12	D-Ser	Arg	12	nd
13	D-Val	Arg	8	nd
14	D-Leu	Arg	15	nd
15	D-Phe	Arg	10	10
16	D-Ala	NMeArg	0.08	0.006
17	D-Abu	NMeArg	0.02	0.006
18	D-Val	NMeArg	0.02	0.006
19	D-Pro	NMeArg	0.02	0.006
20	L-Pro	NMeArg	60	nd
21	D-Leu	NMeArg	0.20	0.55

Figure 2. IC_{50} values for inhibition of platelet aggregation and fibrinogen binding for a number of peptides containing various substituents at positions 1 and 2.

the backbones of peptides dramatically decreases their available conformational space.[6a,b] In particular, N^{α}-methylation of an amino acid restricts a residue's Φ and Ψ angles as well as the Φ and Ψ angles of the residue preceding it in sequence,[24] although it also adds the potential complication of cis peptide bond formation. This modification also restricts the number of low-energy side chain rotomers of the residue to which it is attached. N^{α}-methylation of the Arg residue in a series of disulfide-containing RGD peptides[15] has led to highly potent compounds, and we were pleased to find that this substitution also led to a dramatic increase in the activity of our series of peptides (Figure 2).

Figure 2 lists the IC_{50} values for inhibition of platelet aggregation for a number of peptides containing *N*-MeArg (N^{α}-methylarginine) at position 2 together with various substitutions at position 1. As observed for the peptides with Arg at position 2, the compounds with D-amino acids at position 1 were most potent, and the activity was optimal with D-Abu (D-2-aminobutyric acid), D-Val, or D-Pro. The most potent inhibitors (**17–19**) had IC_{50} values of 20 nM, which represents the lowest value that could be obtained under our experimental conditions for this assay. Therefore, the actual receptor-binding affinities of some of the more active compounds were first estimated with an ELISA competition assay, and subsequently the dissociation constants for the most high affinity analogues were determined by a competition assay employing tritium-labeled **17** and purified, immobilized human GPIIb/IIIa.[25] Direct binding studies with **17** indicate a dissociation constant of approximately 100 pM for binding to GPIIb/IIIa. Also, the dissociation constant for **18** was determined to be 160 pM on the basis of its ability to compete with **17** for binding to GPIIb/IIIa. The structure and stereochemistry of compound **17** was also confirmed by X-ray crystallography (supplementary material), which showed the *N*-methyl amide bond to be in the trans geometry.

We next evaluated the effect of additional N^{α}-alkyl groups on the activities of **17** and **18**. Replacement of D-Abu with D-Pro leads to compound **19**, in which the N^{α} group is alkylated by virtue of being in a pyrrolidine ring. This peptide is equipotent with compound **17** in the receptor-binding assay. Also, N-methylation of the Asp at position 4 or the Mamb at position 5 gave compounds **23** (*cyclo*(D-Val-*N*-MeArg-Gly-*N*-MeAsp-Mamb)) and **24** (*cyclo*(D-Val-*N*-MeArg-Gly-Asp-*N*-Mamb)), both of which had reduced antiaggregatory potencies (IC_{50} = 6.0 and 0.6 μM, respectively). We found it difficult to prepare cyclic structures in which the Arg at position 2 and the Gly at position 3 were simultaneously alkylated, but an analogue (**22**) in which position 2 was Arg and position 3 was sarcosine *cyclo*(D-Ala-Arg-*N*-MeGly-Asp-Mamb) had an IC_{50} greater than 100 μM.



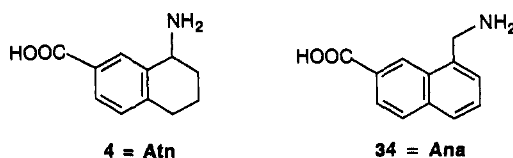
	R1	R2	R3	R5	IC50 (μM) Aggregation	IC50 (μM) Fibrinogen Binding
25	CH3	H	H	H	10	10
26	H	CH3(S)	H	H	15	10
27	H	CH3(R)	H	H	inactive	nd
28	H	H	CH3 (S)	H	0.10	nd
29	H	H	CH3 (R)	H	90	1
30	H	H	H	CH3 (?)	0.08	0.0052
31	H	H	H	CH3 (?)	0.05	0.005
32	H	H	H	Ph(R)	0.08	0.0025
33	H	H	H	Ph(S)	2.0	0.1

Figure 3. The effect of the addition of methyl and phenyl groups on the activity of compound **18**.

The effect of C^{α}-methylation has also been investigated in this series of peptides (Figure 3). Although synthetic difficulties were encountered in the synthesis of analogues with dialkyl substituents at positions 1 or 2, the remaining positions have been examined. C^{α}-methylation at position 3 or 4 gave rise to compounds with reduced potency, but methylation at either of the prochiral centers of the Mamb led to compounds that were nearly as active as the parent compound, **18**. Similarly, addition of a phenyl to the methylene of Mamb led to two diastereomers, only one of which was very active.

In an effort to define the orientation of the carboxylate side chain, C^{β}-methyl derivatives of L-Asp[26] have been incorporated into the cyclic peptide, **18**. Both isomers were prepared, only one of which was highly potent (Figure 3). The chirality at the C^{β} carbon of the isomers was determined on the basis of coupling constants and nuclear Overhauser effects in the NMR spectra of the peptides. The additional constraint of the β-methyl group has allowed the determination of the orientation of the Asp side chain when bound to GPIIb/IIIa (unpublished results).

Finally, we replaced the Mamb linker with the bicyclic linkers[10] 8-amino-5,6,7,8-tetrahydro-2-naphthoic acid (**4**, Atn) or 8-(aminomethyl)-2-naphthoic acid (**34**, Ana) in order to determine



whether the additional rigidity of the bicyclic ring systems would influence the pharmacological properties of the peptides. Introduction of racemic tetralin (**4**) into a cyclic peptide gave two isomeric peptides of sequence *cyclo*(D-Val-*N*-MeArg-Gly-Asp-

(24) Manavalan, P.; Momany, F. A. *Macromolecules* **1980**, *19*, 1943–1973.

(25) (a) Mousa, S. A.; Bozarth, J. M.; Forsythe, M. S.; Lorelli, W.; Thoolen, M. J.; Ramachandran, N.; Jackson, S.; DeGrado, W. F.; Reilly, T. M. *Cardiology* **1993**, *83*, 374–382. (b) Mousa, S. Manuscript in preparation.

(26) Wolf, J.-P.; Rapaport, H. *J. Org. Chem.* **1989**, *54*, 3164.

Atn) (**35** and **36**), with IC_{50} values in the platelet-binding assay of 100 and 20 nM, respectively. The peptide **37**, cyclo(D-Val-*N*-MeArg-Gly-Asp-Ana), showed an IC_{50} of 2 μM. Thus, these modifications failed to substantially increase the activity of the compounds beyond the level observed for the initially designed Mamb linker.

Role of the Macrocycle in Determining Activity. A linear version (CH_3CO-D-Abu-*N*-MeArg-Gly-Asp-Mamb-$CONH_2$, **38**) of the most active compound (**17**) was prepared to determine the role of the macrocycle in modulating the potency and conformational properties of the peptides. As expected, it was considerably less potent (IC_{50} = 9 μM) than the corresponding cyclic analogue. Thus, the cyclic structure plays an important role in maintaining the peptide in a conformation that is appropriate for interaction with the receptor.

Oral Activity. The activity of the most potent compound (**17**) was determined after oral administration to unanesthetized canines. At various times after administering the compound, blood was removed, and the ability of the platelets to aggregate in response to 10^{-4} M ADP was measured. When administered at a dose of 0.1 mg/kg, the drug elicited 75 ± 15% inhibition of platelet aggregation after 15 min and 45 ± 10% inhibition after 6 h. At higher doses (1 mg/kg) 100% inhibition was observed over this entire time period.

CD Studies. To determine how the constraints included in the design of compounds **17** and **18** affected their conformational properties, we measured the CD spectra of these, and other, cyclic peptides.

The CD spectra of **17** and **18** in aqueous solution (Figure 4a) suggest that they adopt rigid structures containing a β turn. Although CD is a qualitative tool when applied to peptide and protein structures, the comparison of the spectra of **17** and **18** with the spectra of some of the less active compounds is illuminating. The spectra of **17** and **18** (Figure 4a) have a maximum near 195 nm and a minimum at 208 nm, typical of type II′ turns.[27] The spectra also have minimum at 235 nm, which should be compared with the value of 220–227 nm more typically observed for type II′ turns. It is likely that this shift is due to other unresolved bands arising from coupling between the aromatic and amide chromophores. In contrast, the spectrum of *cyclo*(Gly-Arg-Gly-Asp-Mamb) (**6**), which lacks both a D-amino acid at position 1 as well as an *N*-methyl group at position 2, shows much less intense bands. This finding suggests that this compound adopts a number of different conformers whose CD signals tend to cancel one another.

Examination of the CD spectra of all the peptides described herein indicates that a CD spectrum similar to that for **17** or **18** is a necessary (but not sufficient) condition for high biological activity. Thus, this spectrum appears to be a signature of a conformer of the ring system that positions the side chains appropriately for a very favorable interaction with the receptor. The spectrum of **13**, *cyclo*(D-Val-Arg-Gly-Asp-Mamb) (Figure 4b), which lacks the *N*-methyl group at position 2, appears to be a linear combination of the spectra of **17** and that of a disordered polypeptide.[28] Other compounds which contain backbone modifications that increase the rigidity of the backbone, but are deleterious to activity such as the *N*-methyl at position 4 in *cyclo*-(D-Val-*N*-MeArg-Gly-*N*-MeAsp-Mamb) (**23**), often showed spectra distinct from those of **17** and **18** (Figure 4c).

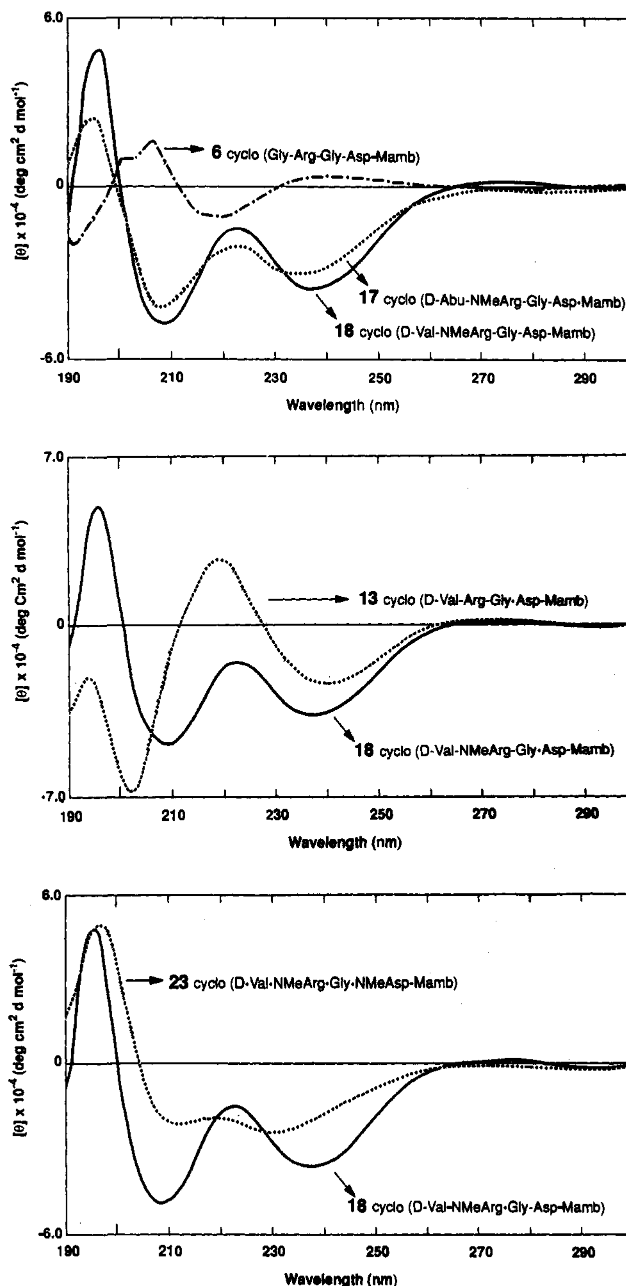


Figure 4. CD spectra of cyclic peptides.

Conclusions

This paper describes the design of template-constrained cyclic peptides as an approach to provide peptides with highly favorable physical and pharmacological properties. This approach is a logical outgrowth of recent studies in which turn-inducing dipeptides[29,30] or anthranilic acid[31] has been incorporated into cyclic peptides to restrict their conformational space. The advantages of TCCP as compared with more traditional medicinal chemistry approaches can be appreciated by comparing the receptor-binding affinities of **17** with SK&F 106760, which was the starting point of our design. Compound **17** binds to GPIIb/IIIa with approximately 3 orders of magnitude greater affinity than SK&F 106760. It is also highly specific for GPIIb/IIIa and has at least 10^3 lower affinity for the closely related RGD-binding vitronectin receptor. A third interesting property is its extreme metabolic stability: it is indefinitely stable in whole blood, rat liver homogenates, and rat intestinal homogenates and shows encouraging oral activity. All of these properties can be understood in terms of the successful rigidification of the RGD

(27) Bush, C. A.; Sarkar, S. K.; Kopple, K. D. *Biochemistry* **1978**, *17*, 4951–4954.
(28) Percel, A.; Hollósi, M.; Sándor, P.; Fasman, G. D. *Int. J. Pept. Protein Res.* **1993**, *41*, 223–236.
(29) Müller, G.; Gurrath, M.; Kessler, H.; Timpl, R. *Angew. Chem., Int. Ed. Engl.* **1992**, *31*, 326–328.
(30) Bean, J. W.; Kopple, K. D.; Pieshoff, C. E. *J. Am. Chem. Soc.* **1992**, *114*, 5328–5334.
(31) Convert, O.; Mazaleyrat, J.-P.; Wakselman, M.; Morize, I.; Reboud-Revaux, M. *Biopolymers* **1990**, *30*, 583–591.

backbone through the conformational restrictions included in its design. It is likely that the proteolytic stability of the compound is a result of it being unable to assume a conformation complementary to that of a protease active site. The specificity of **17** for GPIIb/IIIa appears to be a result of subtle differences in the conformations recognized by various RGD-binding integrins. Thus, by locking the compound into a very restricted set of conformers, it has been possible to obtain a very high-affinity and specific agent.

The success that we and others[15-18] have encountered in the design of conformationally constrained RGD-containing peptides suggests a general approach to the design of template-constrained cyclic peptides. The first step is to define a minimal sequence capable of binding to a receptor. This can be accomplished by preparing analogues of a naturally-occurring peptide or by the more recent approach of screening random peptide libraries.[32] The next step is to insert a semirigid linker into a cyclic form of the sequence of interest, In the present work, biophysical studies of a disulfide-cross-linked form of the active sequence helped define the linker, but in other situations, much less might be known about the bioactive conformation of the peptide. In these cases it would be more appropriate to screen a family of related linkers for one that provides a highly active compound when incorporated into the peptide macrocycle. A number of aromatic compounds[10,11] bearing both amino and carboxyl functionalities are known and could be readily employed for this purpose. The final step in the optimization process involves systematic addition of functional groups to the cyclic peptide to further restrict the conformational freedom as well as to improve the fit with the receptor. Cyclic peptides resulting from this optimization process may prove to be useful as pharmaceutically active agents and also can serve as points of departure for the development of nonpeptidic compounds. Indeed, recent work along these lines has shown that it is possible to prepare high-affinity nonpeptidic mimics of the RGD sequence.[3]

Experimental Section

General Methods. All chemicals and solvents (reagent grade) were used as supplied without further purification. Mass spectra were obtained using a VG Zab-E double-focusing mass spectrometer using a Xenon FAB gun as the ion source or on a Finnigan MAT 8230. NMR spectra were recorded on a 300-MHz General Electric QE-300, Varian 300, Varian 400 spectrometer, or Bruker 600 MHz. Melting points were determined using a Thomas Hoover or Electrothermal 9200 melting-point apparatus and are uncorrected. HPLC analyses were performed on either a Hewlett-Packard 1090, Waters Delta Prep 3000, Rainin, or DuPont 8800 system. Thin-layer chromatography was performed on Silica Gel 60 $^{F}254$ TLC plates (layer thickness 0.2 mm). TLC visualization was accomplished using UV light, iodine, and/or ninhydrin spray. Solvent systems for eluting TLC plates were chloroform/methanol 95:5 = CM; chloroform/acetic acid 95:5 = CA; and chloroform/methanol/acetic acid 85:10:5 = CMA.

Materials. Amino acids and organic reagents were purchased from Bachem Bioscience Inc. (Philadelphia, PA), Advanced ChemTech (Louisville, KY), Sigma, or Aldrich. Boc-D-Abu was purchased as the dicyclohexylammonium salt from Peptides International (Louisville, KY). Manual solid-phase peptide synthesis was performed in 25-mL polypropylene tubes purchased from BioRad Inc. Oxime resin (substitution level = 0.7–0.96 mmol/g) was prepared according to published procedures.[20,21] SK&F 106760 was prepared by a modification of methods described in the literature.

(32) (a) Lam, K. S.; Salmon, S. E.; Hersh, E. M.; Hruby, V. J.; Kazmierski, W. M.; Knapp, R. J. *Nature* **1991**, *354*, 82–84. (b) Cwirla, S. E.; Peters, E. A.; Barrett, R. W.; Dower, W. J. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 6378–6382. (c) Devlin, J. J.; Panganiban, L. C.; Devlin, P. E. *Science* **1990**, *249*, 404–406. Scott, J. K.; Smith, G. P. *Science* **1990**, *249*, 386–390. (d) Houghton, R. A.; Pinilla, C.; Blondelle, S. E.; Appel, J. R.; Dooley, C. T.; Cuervo, J. H. *Nature* **1991**, *354*, 84–86. (e) O'Neil, K. T.; Hoess, R. H.; Jackson, S. A.; Ramachandran, N. S.; Mousa, S. A.; DeGrado, W. F. *Proteins* **1992**, *14*, 509–515.

(33) Sarin, V. K.; Kent, S. B. H.; Tam, J. P.; Merrifield, R. B. *Anal. Biochem.* **1981**, *117*, 147–157.

(34) Gisin, B. F. *Anal. Chim. Acta* **1972**, *58*, 248–249.

***m*-(Aminomethyl)benzoic Acid (Hydrochloride Salt) (39).** 3-Cyanobenzoic acid (10.0 g, 68 mmol) was dissolved in ethanol (EtOH, 200 mL) by heating in a 35–50 °C water bath. Concentrated hydrochloric acid (HCl, 6.12 mL, 73 mmol) was added, and the solution was transferred to a 500-mL nitrogen-flushed round-bottom flask containing palladium on carbon catalyst (1.05 g, 10% Pd/C). The suspension was stirred under an atmosphere of hydrogen for 38 h, filtered through a sintered glass funnel, and washed thoroughly with H_2O. The EtOH was removed under reduced pressure, and the remaining aqueous layer, which contained a white solid, was diluted to 250 mL with additional H_2O. Ethyl ether (Et_2O, 250 mL) was added, and the suspension was transferred to a separatory funnel. Upon vigorous shaking, all solids dissolved, and the aqueous layer was then washed with Et_2O (2×), evaporated under reduced pressure to a volume of 150 mL, and lyophilized to give the title compound (**39**) (8.10 g, 64%) as a beige solid: ^{1}H NMR (D_2O) δ 8.06 (d, 2H), 7.72 (d, 1H), 7.60 (t, 1H), 4.27 (s, 2H).

(*tert*-Butyloxycarbonyl)-*m*-(aminomethyl)benzoic Acid (Boc-Mamb) (40). *m*-(Aminomethyl)benzoic acid·HCl (**39**) (3.0 g, 16.0 mmol) was dissolved in H_2O (60 mL). To this was added a solution of Boc-ON (4.33 g, 17.6 mmol) in acetone (60 mL) followed by triethylamine (5.56 mL, 39.9 mmol). The solution turned yellow, and the pH was adjusted to 9 (wet pH paper) by adding additional triethylamine (1.0 mL, 7.2 mmol). The solution was stirred overnight at room temperature, at which time the acetone was removed under reduced pressure and the remaining aqueous layer was washed with Et_2O (3×). The aqueous layer was then acidified to pH 2 with 2 N HCl and extracted with ethyl acetate (EtOAc, 3×). The combined organic layers were washed with H_2O (3×), dried over anhydrous magnesium sulfate ($MgSO_4$), and evaporated to dryness under reduced pressure. The material was recrystallized from EtOAc/hexane to give two crops of the title compound (**40**) (2.58 g, 64%) as an off-white solid: mp 123–125 °C; ^{1}H NMR ($CDCl_3$) δ 8.02 (d, 2H), 7.55 (d, 1H), 7.45 (t, 1H), 4.95 (br s, 1H), 4.38 (br s, 2H), 1.47 (s, 9H).

N^{α}-(*tert*-Butyloxycarbonyl)-N^{α}-methyl-*m*-(aminomethyl)benzoic Acid (Boc-*N*-MeMamb) (41). Boc-*m*-(aminomethyl)benzoic acid (**40**) (0.87 g, 3.47 mmol), silver(I) oxide (3.22 g, 13.9 mmol), and methyl iodide (1.73 mL, 27.8 mmol) were placed in *N,N*-dimethylformamide (DMF, 12 mL) in a 25-mL round-bottom flask fitted with a reflux condenser. The suspension was heated at 45 °C for 26 h, after which additional methyl iodide (1.73 mL, 27.8 mmol) was added and heating was continued for 16 h. The resulting mixture was filtered and washed with DMF, and chloroform ($CHCl_3$, 120 mL) was then added to the filtrate with rapid stirring. The precipitate which formed was filtered and washed with $CHCl_3$. The filtrate was washed with 5% aqueous potassium cyanide (2×) and H_2O (4×) and dried over anhydrous $MgSO_4$, and the solvent was removed under reduced pressure. To the resulting oil was added EtOH (30 mL) followed by 1 N aqueous sodium hydroxide (6 mL). The mixture was stirred at room temperature for 24 h and then at reflux for 1.5 h. The EtOH was removed under reduced pressure, and water (30 mL) was added. The aqueous solution was cooled and acidified to pH 3 with concentrated HCl. The aqueous layer was extracted with EtOAc (3×), and the combined organic layers were dried over anhydrous $MgSO_4$ and evaporated under reduced pressure. The material obtained was recrystallized from EtOAc/petroleum ether to afford a white waxy solid (**41**) (0.48 g, 53%): ^{1}H NMR (DMSO-d_6) δ 7.81 (m, 2H), 7.43 (m, 2H), 4.39 (s, 2H), 3.26 (s, 3H), 1.28 (br d, 9H).

3-[1′-[(*tert*-Butyloxycarbonyl)amino]ethyl]benzoic Acid (Boc-MeMamb) (42). 3-Acetylbenzoic acid (0.5 g, 3 mmol), hydroxylamine hydrochloride (0.70 g, 10 mmol), and pyridine (0.70 mL, 9 mmol) were refluxed in EtOH (10 mL) for 2 h. The reaction mixture was then concentrated, and the residue was triturated with water, filtered, and dried. The oxime was isolated as a white solid (0.51 g, 94.4% yield): ^{1}H NMR (CD_3OD) δ 7.45–8.30 (m, 4H), 2.30 (s, 3H); MS (CH_4-CI) [M + H – O] = 164.

A solution of the oxime (0.51 g, 3 mmol) in EtOH, containing 10% Pd/C (1.5 g) and concentrated HCl (0.25 mL, 3 mmol), was hydrogenated (30 psi) in a Parr hydrogenator for 5 h. The catalyst was filtered and the filtrate concentrated. The residue was triturated with Et_2O, and the amine hydrochloride was isolated as a white solid (0.48 g, 85.7% yield): ^{1}H NMR (CD_3OD) δ 7.6–8.15 (m, 4H), 4.55 (q, 1H), 1.70 (s, 3H); MS $[M + H]^+$ = 166.

The amine hydrochloride (0.40 g, 2 mmol) was dissolved in water (15 mL), and a solution of Boc-ON (0.52 g, 2.1 mmol) in acetone (15 mL) was added followed by the addition of triethylamine (0.8 mL, 6 mmol). The reaction was allowed to proceed for 20 h, after which the mixture was concentrated and then partitioned between EtOAc and water. The aqueous layer was acidified to pH 2 using 10% HCl. The product was

extracted in EtOAc, which, after the usual workup and recrystallization from EtOAc/hexane, gave the title compound (**42**) as a white solid (0.30 g, 57% yield): mp 116–118 °C; ^{1}H NMR ($CDCl_3$) δ 7.35–8.2 (m, 4H), 4.6 (bs, 1.5H), 1.50 (d, 3H), 1.40 (s, 9H); MS (NH_3-CI) [M + NH_4] = 283.

3-[1'-[(*tert*-Butyloxycarbonyl)amino]benzyl]benzoic Acid (Boc-Ph-Mamb) (43). A solution of 3-benzoylbenzoic acid (2.00 g, 9 mmol), hydroxylamine hydrochloride (2.00 g, 29 mmol), and pyridine (2.00 mL, 25 mmol) in EtOH was refluxed for 12 h. After the usual extractive workup, a white solid was obtained (2.41 g). The product still contained traces of pyridine, but was used in the next step without further purification.

The crude product (2.00 g, ~8 mmol) was dissolved in EtOH (200 mL). Pd/C (10%, 2.00 g) and concentrated HCl (1.3 mL, 16 mmol) were added. The reaction mixture was hydrogenated (30 psi) for 1 h. The catalyst was filtered, and the reaction mixture was concentrated. Upon trituration of the residue with Et_2O and drying under vacuum, the amine hydrochloride was obtained as a white solid (2.12 g, 97% yield): ^{1}H NMR (CD_3OD) δ 7.4–8.15 (m, 10H), 5.75 (s, 1H); MS (CH_4-CI) [M + H – OH] = 211.

The amine hydrochloride (1.00 g, 4 mmol) was converted to its Boc derivative by a procedure similar to the above examples. The recrystallized (from EtOH/hexane) title compound (0.6 g, 48% yield) (**43**) was obtained as a white solid: mp 190–192 °C; ^{1}H NMR (CD_3OD) δ 7.2–8.0 (m, 10H), 5.90 (2s, 1H, 2 isomers), 1.40 (s, 9H); MS (NH_3-CI) [M + NH_4 – C_4H_8].

8-Amino-5,6,7,8-tetrahydro-2-naphthoic Acid Hydrochloride (44). The title compound was prepared from 4-phenylbutyric acid according to the procedures previously reported in the literature[10] to give the racemic mixture of 8-amino-5,6,7,8-tetrahydro-2-naphthoic acid hydrochloride (**44**) in 27% overall yield (0.225 g, 0.001 mol) as a white solid: mp 289–291 °C; ^{1}H NMR (DMSO-d_6) δ 8.55 (bs, 3H), 8.2–8.1 (m, 1H), 7.85–7.8 (m, 1H), 7.35–7.25 (m, 1H), 4.5 (m, 1H), 2.9–2.8 (m, 2H), 2.1–1.9 (m, 3H), 1.85–1.7 (m, 1H); IR (KBr) 3411, 3032, 2950, 1720, 1630, 1610, 1439, 1310, 1253 cm^{-1}.

***N*-(*tert*-Butyloxycarbonyl)-8-(aminomethyl)-2-naphthoic Acid (45).** This compound was prepared by a variation of the literature procedure.[10]

Methyl 8-Cyano-5,6-dihydro-2-naphthoate (46). Using a procedure from Gregory and Johnson,[35] a solution of 1-tetralon-7-carboxylic acid methyl ester[10] (3.50 g, 0.017 mol), (trimethylsilylcyanide (1.98 g, 0.02 mol), and zinc iodide (0.10 g) in benzene (20 mL) was stirred at ambient temperature over 15 h. Then added, sequentially and dropwise, were pyridine (20 mL) and phosphorus oxychloride (4.0 mL, 6.55 g, 0.0425 mol). The reaction mixture was stirred at reflux over 1 h and then evaporated to dryness under reduced pressure. The residue was taken up in $CHCl_3$, backwashed with H_2O, dried over anhydrous $MgSO_4$, and evaporated to dryness under reduced pressure to give **46** (1.70 g, 0.008 mol) in 47% yield as a yellow solid: mp 73–75 °C; ^{1}H NMR ($CDCl_3$) δ 8.0–7.9 (m, 1H), 7.3–7.2 (m, 1H), 6.95 (t, 1H, *J* = 4.8 Hz), 3.95 (s, 3H), 2.9 (t, 2H, *J* = 8.3 Hz), 2.6–2.4 (m, 3H); IR (KBr) 2952, 2224, 1722, 1436, 1264 cm^{-1}.

Methyl 8-(Aminomethyl)-2-naphthoate (47). The methyl ester of 8-cyano-5,6-dihydro-2-naphthoate (**46**) (1.0 g, 0.0047 mol) was transformed according to the procedures of Earnest et al.[10] to give **47** (0.76 g, 0.0035 mol) in 75% yield as an oil: ^{1}H NMR (DMSO-d_6) δ 8.75 (s, 1H), 8.5 (bs, 2H), 8.2–8.05 (m, 3H), 7.75–7.70 (m, 2H), 4.6 (s, 2H), 3.95 (m, 3H); IR (KBr) 2952, 2622, 1718, 1520, 1282 cm^{-1}.

8-(Aminomethyl)-2-naphthoic Acid (48). To a solution of methyl 8-(aminomethyl)-2-naphthoate (**47**) (0.75 g, 0.0035 mol) in dry tetrahydrofuran (50 mL), cooled to 0 °C, was added a solution of lithium hydroxide (0.5 M, 5.83 mL). All was stirred at ambient temperature over 20 h. Another aliquot of lithium hydroxide was added, and stirring was continued for an additional 20 h. The solid was collected, and the filtrate was evaporated to dryness under reduced pressure. The solids were triturated with Et_2O to give **48** (0.67 g, 0.0033 mol) in 95% yield as a white solid: mp 223–225 °C; ^{1}H NMR (DMSO-d_6) δ 8.6 (s, 1H), 8.1–7.9 (m, 1H), 7.8–7.7 (m, 4H), 7.55–7.5 (m, 1H), 7.45–7.35 (m, 2H), 4.2 (s, 2H); IR 3354, 2870, 2670, 2198, 1632, 1604, 1570, 1384 cm^{-1}.

***N*-(*tert*-Butyloxycarbonyl)-8-(aminomethyl)-2-naphthoic Acid (45).** To a solution of 8-(aminomethyl)-2-naphthoic acid (**48**) (0.50 g, 0.00025 mol) and triethylamine (0.038 mL, 0.028 g, 0.000275 mol) in aqueous tetrahydrofuran (50%, 5 mL) was added, portionwise as a solid, 2-(*tert*-butyloxycarbonyloxyimino)-2-phenylacetonitrile (0.068 g, 0.000275 mol). All was stirred at ambient temperature over 5 h. The solution was concentrated to half volume and extracted with Et_2O. The aqueous layer was then acidified to a pH of 1.0 using HCl (1 N) and then extracted with EtOAc. The combined organic layers were dried over anhydrous $MgSO_4$ and evaporated to dryness under reduced pressure to give the title compound (**45**) (0.050 g, 0.00017 mol) in 68% yield as a white solid: mp 190–191 °C; ^{1}H NMR (DMSO-d_6) δ 13.1 (bs, 1H), 8.8 (s, 1H), 8.0 (q, 2H, *J* = 7.9 Hz), 7.9 (d, 1H, *J* = 8.1 Hz), 7.6 (t, 1H, *J* = 7.5 Hz), 7.65–7.55 (m, 2H), 4.6 (d, 2H, *J* = 5.5 Hz), 1.4 (s, 9H); IR 3386, 2934, 1694, 1534, 1298 cm^{-1}.

(2*S*,3*R*)- and (2*S*,3*S*)-N^α-(*tert*-Butyloxycarbonyl)-3-methyl-L-aspartic Acid β-Cyclohexyl Ester (49) was synthesized as a mixture of diastereomers by the procedures outlined below.

(2*S*,3*R*)- and (2*S*,3*S*)-N^α-(9-Phenylfluorenyl)-3-methyl-L-aspartic Acid α-*tert*-Butyl β-Methyl Diester (50) was prepared by the method of Rapoport.[26]

(2*S*,3*R*)- and (2*S*,3*S*)-N^α-(9-Phenylfluorenyl)-3-methyl-L-aspartic Acid α-*tert*-Butyl Ester (51). To a stirred solution of 2.00 g (4.37 mmol) of (2*S*,3*R*)- and (2*S*,3*S*)-N^α-(9-phenylfluorenyl)-3-methyl-L-aspartic acid α-*tert*-butyl β-methyl diester (**50**) in 20 mL of dioxane was added 8 mL of 25% aqueous NaOH. The mixture was stirred for 4 h at 40 °C, cooled, and poured into 1 N aqueous HCl. The mixture was extracted with EtOAc, and the organic extract was washed with brine, dried ($MgSO_4$), and concentrated under reduced pressure to afford 1.59 g (82%) of **51** as a colorless oil: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.17–7.72 (m, 13H, aromatic), 2.93, 2.81 (d, 1H, *J* = 4.2, 8.4 Hz, Hα), 2.38–2.56 (m, 1H, Hβ), 1.19, 1.13 (s, 9H, $C(CH_3)_3$), 1.06, 0.99 (d, 3H, CH_3); MS (NH_3-DCI) 444 ($(M + H)^+$, 100%). HRMS calcd for $C_{28}H_{30}NO_4$ $[M + H]^+$, 444.2175; found, 444.2169.

(2*S*,3*R*)- and (2*S*,3*S*)-N^α-(*tert*-Butyloxycarbonyl)-3-methyl-L-aspartic Acid β-Cyclohexyl Ester (49). To a stirred solution of 1.58 g (3.56 mmol) of (2*S*,3*R*)- and (2*S*,3*S*)-N^α-(9-phenylfluorenyl)-3-methyl-L-aspartic acid α-*tert*-butyl ester (**51**) and cyclohexanol (0.7 g, 7.0 mmol) in 16 mL of CH_2Cl_2 was added dicyclohexylcarbodiimide (1.24 g, 6.0 mmol), followed by (*N*,*N*-dimethylamino)pyridine (50 mg, catalytic). The solution was stirred for 16 h, filtered, and concentrated to give the crude (2*S*,3*R*)- and (2*S*,3*S*)-N^α-(9-phenylfluorenyl)-3-methyl-L-aspartic acid α-*tert*-butyl β-cyclohexyl diester. This material was redissolved in 10 mL of CH_2Cl_2 and treated with 7 mL of trifluoroacetic acid. The solution was stirred for 5 h at ambient temperature and poured into Et_2O. The mixture was washed with H_2O (3×), and the combined aqueous phases were concentrated under reduced pressure to afford a syrup. The crude product was dissolved in *tert*-butyl alcohol (30 mL) and treated with triethylamine (2.1 mL, 15 mmol) and di-*tert*-butyl dicarbonate (2.18 g, 10 mmol). The solution was stirred for 16 h at 40 °C and concentrated under reduced pressure. The residue was redissolved in 1:1 Et_2O/EtOAc and washed with dilute aqueous K_2CO_3 (5×). The combined aqueous washings were acidified to pH 1. The resulting colloidal suspension was extracted with $CHCl_3$ (2×), and the combined organic extracts were dried ($MgSO_4$) and concentrated under reduced pressure. The crude product was chromatographed on silica gel to afford 0.650 g (56%) of **49** as a gel: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.53, 5.30 (d, 1H, *J* = 9, 10 Hz, NH), 4.49–4.85 (m, 2H, Hα, CHO), 2.95–3.09 (m, 1H, Hβ), 1.25 (s, 9H, $C(CH_3)_3$), 1.20–1.87 (m, 13H, CH_3, cyclohexyl); MS (NH_3-DCI): 347 ($(M + NH_4)^+$, 100%), 330 ($(M + H)^+$, 8%), 291 ($(M + NH_4 - C_4H_8)^+$, 18%). HRMS calcd for $C_{16}H_{28}NO_6$ $[M + H]^+$, 330.1917; found, 330.1914.

N^α-(*tert*-Butyloxycarbonyl)-N^α-methyl-L-aspartic Acid β-Cyclohexyl Ester (52). To a stirred, cooled (0 °C) solution of N^α-BocAsp(OcHex)-OH (1.00 g, 3.15 mmol) in 5 mL of THF was added 240 mg (8.0 mmol) of an 80% dispersion of sodium hydride in mineral oil. The solution was stirred for 5 min at 0 °C, and CH_3I (1.87 mL, 30 mmol) was introduced. The mixture was stirred for 16 h, warming to ambient temperature. The reaction was quenched with 0.1 N HCl and extracted with ether. The organic extract was washed with brine, dried ($MgSO_4$), and concentrated under reduced pressure to give an oil. The crude product was dissolved in 25 mL of THF at 3 mL of MeOH. To this solution was added 25 mL (10 mmol) of 0.4 N aqueous LiOH. The solution was stirred at ambient temperature for 3 h and poured into water. The mixture was washed with dilute aqueous K_2CO_3 (4×), and the combined aqueous washings were acidified to pH 1. This mixture was extracted with $CHCl_3$ (3×), and the combined organic extracts were dried ($MgSO_4$) and concentrated under reduced pressure to afford 0.734 g (72%) of **52** as an oily solid: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 4.53–4.84 (m, 1H, CHO), 2.76–3.19 (m, 2H, Hβ), 2.97, 2.91 (s, 3H, CH_3), 1.46 (s, 9H, $C(CH_3)_3$), 1.22–1.93 (m, 10H, cyclohexyl); MS (NH_3-CI/DDIP) 330 ($(M + H)^+$, 53%), 291 ($(M + NH_4 - C_4H_8)^+$, 82%), 274 ($(M + H - C_4H_8)^+$, 70%), 230 ((M + H

(35) Gregory, G. B.; Johnson, A. L.; Ripka, W. C. *J. Org. Chem.* **1990**, *55*, 1479–1483.

$- C_4H_8 - CO_2)^+$, 100%). HRMS calcd for $C_{16}H_{28}NO_6$ $[M + H]^+$, 330.1917; found, 330.1914.

N^{α}-(*tert*-Butyloxycarbonyl)-C^{α}-methylaspartic Acid β-Cyclohexyl Ester (53) was synthesized in racemic form by the following reactions.

C^{α}-Methyl-D,L-aspartic Acid α-Benzyl β-Methyl Diester (54). To a stirred, cooled (−78 °C) solution of L-alanine benzyl ester benzophenone imine (1.00 g, 2.91 mmol) in 10 mL of THF was added 6.40 mL (3.20 mmol) of a solution of potassium bis(trimethylsilyl)amide in toluene over 3 min. The solution was stirred for 5 min at −78 °C, and methyl α-bromoacetate (0.47 mL, 5.0 mmol) was introduced. The solution was stirred for 30 min at −78 °C and removed from the cold bath. The solution was stirred an additional 5 min, whereupon it was quenched by the addition of 1 mL of anhydrous MeOH. The reaction was treated with 20 mL of 1 N aqueous HCl, and the two-phase system was vigorously stirred for 1.5 h at ambient temperature. The mixture was shaken with 30 mL of 2:1 Et_2O/EtOAc, and the phases were separated. The organic phase was washed twice with 0.1 N HCl, and the combined aqueous phases were made strongly basic with saturated aqueous K_2CO_3. This mixture was extracted with $CHCl_3$ (2×), and the combined organic extracts were dried ($MgSO_4$) and concentrated under reduced pressure to give 0.610 g (83%) of **54** as an oil: ^1H NMR ($CDCl_3$, 300 MHz) δ 7.30–7.41 (m, 5H, aromatic), 5.18 (ABq, 2H, J_{AB} = 12.5 Hz, $\Delta\nu$ = 20.0 Hz, OCH_2), 3.60 (s, 3H, OCH_3), 2.79 (ABq, 2H, J_{AB} = 16.8 Hz, $\Delta\nu$ = 118 Hz, Hβ), 2.38 (br s, 2H, NH_2), 1.36 (s, 3H, CH_3).

N^{α}-(*tert*-Butyloxycarbonyl)-C^{α}-methyl-D,L-aspartic Acid α-Benzyl β-Methyl Diester (55). To a stirred solution of C^{α}-methyl-D,L-aspartic acid α-benzyl β-methyl diester (**54**) (4.26 g, 16.9 mmol) and triethylamine (2.78 mL, 20 mmol) in 25 mL of *t*-BuOH was added di-*tert*-butyl dicarbonate (4.37 g, 20 mmol). After stirring for 1 h at ambient temperature TLC indicated very little consumption of starting material had occurred. The solution was warmed to 80 °C for 2 h and then concentrated under reduced pressure. The residue was redissolved in 100 mL of 1:1 Et_2O/EtOAc and washed twice with 0.5 N HCl. The combined organic washings were back-extracted with Et_2O, and the combined organic phases were washed sequentially with dilute aqueous K_2CO_3 and brine. The solution was dried ($MgSO_4$) and concentrated under reduced pressure to give 5.81 g (98%) of **55** as an oil: ^1H NMR ($CDCl_3$, 300 MHz) δ 7.32–7.38 (m, 5H, aromatic), 5.65–5.72 (br s, 1H, NH), 5.19 (s, 2H, OCH_2), 3.60 (s, 3H, OCH_3), 3.17 (ABq, 2H, J_{AB} = 15.8 Hz, $\Delta\nu$ = 81 Hz, Hβ), 1.53 (s, 3H, CH_3), 1.41 (s, 9H, $C(CH_3)_3$); MS (NH_3-CI/DDIP) 369 ($(M + NH_4)^+$, 21%), 352 ($(M + H)^+$, 44%), 313 ($(M + NH_4 - C_4H_8)^+$, 100%), 296 ($(M + H - C_4H_8)^+$, 48%), 252 ($(M + H - C_4H_8 - CO_2)^+$, 41%).

N^{α}-(*tert*-Butyloxycarbonyl)-C^{α}-methyl-D,L-aspartic Acid α-Benzyl Ester (56). To a stirred solution of N^{α}-(*tert*-butyloxycarbonyl)-C^{α}-methyl-D,L-aspartic acid α-benzyl β-methyl diester (**55**) (1.00 g, 2.85 mmol) in 8 mL of THF and 16 mL of dioxane was added 4 mL of 25% aqueous NaOH. The mixture was vigorously stirred for 2.5 h, and then it was poured into 50 mL of 1:1 Et_2O/EtOAc. The mixture was washed once with water and once with 0.1 N NaOH. The combined aqueous washings were acidified to pH 1 with concentrated aqueous HCl, and the resulting colloidal suspension was extracted with $CHCl_3$ (2×). The organic extracts were dried ($MgSO_4$) and concentrated under reduced pressure to give 450 mg (47%) of **56** as an oil: ^1H NMR ($CDCl_3$, 300 MHz) δ 7.31–7.39 (m, 5H, aromatic), 5.67 (br s, 1H, NH), 5.19 (s, 2H, OCH_2), 3.20 (ABq, 2H, J_{AB} = 14 Hz, $\Delta\nu$ = 85 Hz, Hβ), 1.45 (s, 3H, CH_3), 1.41 (s, 9H, $C(CH_3)_3$); MS (NH_3-CI/DDIP) 355 ($(M + NH_4)^+$ + 22%), 338 ($(M + H)^+$, 32%), 299 ($(M + NH_4 - C_4H_8)^+$, 94%), 281 ($(M + H - C_4H_8 - H_2O)^+$, 100%), 238 ($(M + H - C_4H_8 - CO_2)^+$, 36%).

N^{α}-*tert*-Butyloxycarbonyl)-C^{α}-methyl-D,L-aspartic Acid α-Benzyl β-Cyclohexyl Diester (57). To a stirred solution of N^{α}-(*tert*-butyloxycarbonyl)-C^{α}-methyl-D,L-aspartic acid α-benzyl ester (**56**) (0.880 g, 2.61 mmol), cyclohexanol (0.31 mL, 2.9 mmol), and DMAP (50 mg, catalytic) in 15 mL of CH_2Cl_2 was added DCC (0.60 g, 2.9 mmol). The solution was stirred for 1.5 h with the formation of a white precipitate. The suspension was filtered, and the filter cake was washed with CH_2Cl_2. The solution was concentrated under reduced pressure and chromatographed on silica gel. The product was eluted with 3:1 hexanes/EtOAc to afford, after concentration, 1.01 g (93%) of **57** as an oil: ^1H NMR ($CDCl_3$, 300 MHz) δ 7.28–7.37 (m, 5H, aromatic), 5.73–5.77 (m, 1H, NH), 5.18 (s, 2H, OCH_2), 4.69–4.77 (m, 1H, OCH), 3.08 (ABq, 2H, J_{AB} = 15.4 Hz, $\Delta\nu$ = 90 Hz, Hβ), 1.25–1.83 (m, 10H, cyclohexyl), 1.45 (s, 3H, CH_3), 1.41 (s, 9H, $C(CH_3)_3$); MS (NH_3-CI/DDIP) 420 ($(M + H)^+$, 13%), 381 ($(M + NH_4 - C_4H_8)^+$, 8%), 364 ($(M + H - C_4H_8)^+$, 29%), 320 ($(M + H - C_4H_8 - CO_2)^+$, 100%). HRMS calcd for $C_{23}H_{34}NO_6$ $(M + H)^+$, 420. 2386; found, 420.2386.

N^{α}-(*tert*-Butyloxycarbonyl)-C^{α}-methyl-D,L-aspartic Acid β-Cyclohexyl Ester (53). A suspension of N^{α}-(*tert*-butyloxycarbonyl)-C^{α}-methyl-D,L-aspartic acid α-benzyl β-cyclohexyl diester (**57**) (0.990 g, 2.36 mmol), HOAc (0.2 mL), and 250 mg of 10% Pd/C in 30 mL of EtOH was hydrogenated at 45 psi for 4 h. The suspension was filtered and concentrated under reduced pressure. The residue was redissolved in 50 mL of 1:1 Et_2O/EtOAc and washed with dilute aqueous K_2CO_3 (4×). The combined aqueous washings were acidified to pH 1 with concentrated aqueous HCl and extracted with $CHCl_3$ (2×). The combined organic extracts were dried ($MgSO_4$) and evaporated under reduced pressure to give 0.650 g (84%) of **53** as an oil: ^1H NMR ($CDCl_3$, 300 MHz) δ 5.81 (br s, 1H, NH), 4.73–4.83 (m, 1H, OCH), 3.06 (ABq, 2H, J_{AB} = 15.7 Hz, $\Delta\nu$ = 56 Hz, Hβ), 1.12–1.89 (m, 10H, cyclohexyl), 1.64 (s, 3H, CH_3), 1.44 (s, 9H, $C(CH_3)_3$); MS (NH_3 CI/DDIP) 347 ($(M + NH_4)^+$, 1%), 330 ($(M + H)^+$, 7%), 291 ($(M + NH_4 - C_4H_8)^+$, 4%), 273 ($(M + NH_4 - C_4H_8 - H_2O)^+$, 100%), 230 ($(M + H - C_4H_8 - CO_2)^+$, 11%). Anal. Calcd for $C_{16}H_{27}NO_6$: C, 58.34; H, 8.26; N, 4.25. Found: C, 58.66; H, 8.29; N, 4.08.

General Procedure for Solid-Phase Synthesis. (*tert*-Butyloxycarbonyl)-*m*-(aminomethyl)benzoic acid (Boc-Mamb) is coupled to oxime resin using 1 equiv of the *m*-(aminomethyl)benzoic acid (with respect to the substitution level of the resin), 1 equiv of 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) or 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU), and 3 equiv of 4-methylmorpholine (NMM). Alternatively, Boc-Mamb (1 equiv) may be coupled to the oxime resin using 1 equiv each of dicyclohexylcarbodiimide (DCC) and 4-(dimethylamino)pyridine (DMAP) in methylene chloride (CH_2Cl_2) or a CH_2Cl_2/DMF mixture. The reactions are generally allowed to proceed overnight or longer, although shorter reaction times (ca. 2–5 h) also give satisfactory results. The substitution level is then determined using either the picric acid test[33] or the quantitative ninhydrin assay.[34] Unreacted oxime groups are blocked using 0.5 M trimethylacetylchloride/0.5 M diisopropylethylamine (DIEA) or 0.5 M pyridine in DMF for 2 h. Deprotection of the Boc protecting group is accomplished using 25% trifluoroacetic acid (TFA) in CH_2Cl_2 for 30 min. The remaining amino acids or amino acid derivatives are coupled using between a 2- and 10-fold excess (based on the loading of the first amino acid or amino acid derivative) of the appropriate amino acid or amino acid derivatives and HBTU in approximately 8 mL of DMF per gram of resin. The resin is then neutralized in situ using 3 equiv of 4-methylmorpholine (NMM) (based on the amount of amino acid used); coupling times of 1–2 h generally provide complete couplings, although overnight or longer reaction times are also used when convenient. These longer reaction times are necessary when coupling to peptides with an N-terminal *N*-methyl amino acid. The completeness of coupling is monitored by qualitative ninhydrin assay, or picric acid assay in cases where the amino acid was coupled to a secondary amine. Amino acids are recoupled if necessary on the basis of these results.

After the linear peptide has been assembled, the N-terminal Boc group is removed by treatment with 25% TFA in CH_2Cl_2 for 30 min. The resin is then neutralized by rapidly washing with 10% DIEA in CH_2Cl_2. Cyclization with concomitant cleavage of the peptide is accomplished by suspending the resin in approximately 10 mL/g of DMF, adding 1 equiv of acetic acid (HOAc) (based on the loading of the first amino acid), and stirring at 50–60 °C for 60–72 h. Following filtration through a sintered glass funnel the DMF filtrate is evaporated, redissolved in HOAc or 1:1 acetonitrile/H_2O, and lyophilized to obtain protected, cyclized material. Alternatively, the material may be dissolved in methanol and precipitated with ether to obtain the protected, cyclized material. Protecting groups are then removed using anhydrous hydrogen fluoride (HF) containing *m*-cresol or anisole (1 mL per gram of peptide cleaved) as scavenger at 0 °C for 20–60 min to remove side chain protecting groups. In some cases, extending the reaction time beyond 20 min gave rise to ring-opened byproducts. The crude product may be purified by reverse-phase HPLC using a 2.5-cm preparative Vydac C18 column with a linear acetonitrile gradient containing 0.1% TFA to produce pure cyclized material in overall yields ranging from 2% to 72%.

Solid-Phase Synthesis of *cyclo*(D-Val-*N*-MeArg-Gly-Asp-Mamb) (18). To a 25-mL polypropylene tube fitted with a frit (BioRad, Econo-Pac disposable chromatography columns) was added Boc-Mamb (0.126 g, 0.5 mmol) and 6 mL of DMF. To this were added 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, 0.194 g, 0.5 mmol), oxime resin (0.52 g, substitution level = 0.96 mmol/g), and NMM (0.165 mL, 1.50 mmol). The suspension was mixed at room temperature for 24 h. The resin was then washed thoroughly (10–12-mL volumes) with DMF (3×), methanol (MeOH, 1×), CH_2Cl_2 (3×), MeOH

Table 1. Yields and Mass Spectral Data on Selected Peptides (nd = Yield Not Determined; Asterisk = Stereochemistry Not Determined)

	compound	yield (%)	FAB-MS $[M + H]^+$ observed	FAB-MS $[M + H]^+$ expected
6	*cyclo*(Gly-Arg-Gly-Asp-Mamb)	11.1	519.26	519.23
7	*cyclo*(Arg-Gly-Asp-Gly-Mamb)	17.8	519.30	519.23
8	*cyclo*(Arg-Gly-Asp-Mamb)	4.8	462.33	462.21
9	*cyclo*(D-Ala-Arg-Gly-Asp-Mamb)	13.8	533.26	533.25
10	*cyclo*(Ala-Arg-Gly-Asp-Mamb)	10.5	533.25	533.25
11	*cyclo*(D-Abu-Arg-Gly-Asp-Mamb)	72.2	547.21	547.26
12	*cyclo*(D-Ser-Arg-Gly-Asp-Mamb)	26.7	549.31	549.24
13	*cyclo*(D-Val-Arg-Gly-Asp-Mamb)	58.2	561.22	561.28
14	*cyclo*(D-Leu-Arg-Gly-Asp-Mamb)	14.8	575.45	575.29
15	*cyclo*(D-Phe-Arg-Gly-Asp-Mamb)	29.3	609.25	609.28
16	*cyclo*(D-Ala-*N*-MeArg-Gly-Asp-Mamb)	nd	547.23	547.26
17	*cyclo*(D-Abu-*N*-MeArg-Gly-Asp-Mamb)	12.4	561.46	561.28
18	*cyclo*(D-Val-*N*-MeArg-Gly-Asp-Mamb)	9.4	575.45	575.29
19	*cyclo*(D-Pro-*N*-MeArg-Gly-Asp-Mamb)	3.8	573.35	573.28
20	*cyclo*(Pro-*N*-MeArg-Gly-Asp-Mamb)	2.0	573.46	573.28
21	*cyclo*(D-Leu-*N*-MeArg-Gly-Asp-Mamb)	16.5	589.48	589.31
22	*cyclo*(D-Ala-Arg-*N*-MeGly-Asp-Mamb)	5.96	547.41	547.26
23	*cyclo*(D-Val-*N*-MeArg-Gly-*N*-MeAsp-Mamb)	10.9	589.42	589.31
24	*cyclo*(D-Val-*N*-MeArg-Gly-Asp-*N*-MeMamb)	29.9	589.33	589.31
25	*cyclo*(D-Val-*N*-MeArg-Ala-Asp-Mamb)	10.6	589.31	589.31
26	*cyclo*(D-Val-*N*-MeArg-Gly-(*)-C^{α}-MeAsp-Mamb)	4.9	589.29	589.31
27	*cyclo*(D-Val-*N*-MeArg-Gly-(*)-C^{α}-MeAsp-Mamb)	5.4	589.27	589.31
28	*cyclo*(D-Val-*N*-MeArg-Gly-(*S*)-C^{β}-MeAsp-Mamb)	9.6	589.43	589.31
29	*cyclo*(D-Val-*N*-MeArg-Gly-(*R*)-C^{β}-MeAsp-Mamb)	10.5	589.45	589.31
35	*cyclo*(D-Val-*N*-MeArg-Gly-Asp-Atn) (isomer 1)	12.5	615.34	615.24
36	*cyclo*(D-Val-*N*-MeArg-Gly-Asp-Atn) (isomer 2)	18.6	615.35	615.24
37	*cyclo*(D-Val-*N*-MeArg-Gly-Asp-Ana)	7.7	625.32	625.38

(2×), and CH_2Cl_2 (3×). The substitution level was determined to be 0.389 mmol/g by quantitative ninhydrin assay. Unreacted oxime groups were blocked by treatment with 0.5 M trimethylacetyl chloride/0.5 M DIEA in DMF for 2 h.

The following steps were then performed: (1) The resin was washed with DMF (3×), MeOH (1×), CH_2Cl_2 (3×), MeOH (2×), and CH_2Cl_2 (3×). (2) The *t*-Boc group was deprotected using 25% TFA in CH_2Cl_2 for 30 min. (3) The resin was washed with CH_2Cl_2 (3×), MeOH (1×), CH_2Cl_2 (2×), MeOH (3×), and DMF (3×). (4) Boc-Asp(OcHex) (0.613 g, 1.94 mmol), HBTU (0.753 g, 1.99 mmol), 8 mL of DMF, and NMM (0.642 mL, 5.84 mmol) were added to the resin, and the reaction was allowed to proceed for 2.5 h. (5) The coupling reaction was found to be complete as assessed by the qualitative ninhydrin assay. Steps 1–5 were repeated until the desired sequence had been attained. The coupling of Boc-D-Val to NMeArg was monitored by the picric acid test.

After the linear peptide was assembled, the N-terminal *t*-Boc group was removed by treatment with 25% TFA in CH_2Cl_2 (30 min). The resin was washed thoroughly with CH_2Cl_2 (3×), MeOH (2×), and CH_2Cl_2 (3×) and then neutralized by washing twice with 10% DIEA (2 × 1 min). The resin was washed thoroughly with CH_2Cl_2 (3×) and MeOH (3×) and then dried. Half of the resin (0.101 mmol) was cyclized by treating with 6 mL of DMF containing HOAc (5.8 mL, 0.101 mmol) and heating at 50 °C for 72 h. The resin was then filtered through a sintered glass funnel and washed thoroughly with DMF. The DMF filtrate was evaporated to an oil, redissolved in 1:1 acetonitrile/H_2O, and lyophilized to give the protected cyclic peptide (49 mg, 60%). The peptide (42 mg) was treated with anhydrous HF at 0 °C, in the presence of *m*-cresol as scavenger, for 30 min to remove side chain protecting groups. The crude material was precipitated with Et_2O, redissolved in 10% HOAc, and lyophilized to generate the title compound (23 mg, 70%; calculated as the acetate salt). Purification was accomplished using reverse-phase HPLC with a preparative Vydac C18 column (2.5 cm) and a 0.23%/min gradient of 7–18% acetonitrile containing 0.1% TFA to give the TFA salt of the title compound (25% recovery; overall yield 9.4%); FAB-MS $[M + H]^+$ = 575.45.

Solid-Phase Synthesis of CH_3CO-D-Abu-*N*-MeArg-Gly-Asp-Mamb-$CONH_2$ (38). Boc-Mamb was attached to 4-methylbenzylhydrylamine resin (Bachem, 1.28 g, substitution 0.8 mmol/g) by first neutralizing the resin with 25% DIEA/DMF and then mixing overnight with Boc-Mamb (0.756 g, 3.0 mmol), HBTU (1.14 g, 3.0 mmol), and NMM (0.66 mL, 6.0 mmol). The resin was washed thoroughly with DMF (3×), MeOH (1×), CH_2Cl_2 (3×), MeOH (2×), and CH_2Cl_2 (3×). Qualitative ninhydrin assay showed coupling to be complete. The subsequent deprotection, coupling, and monitoring procedures were the same as those for **18**, with the exception that a neutralization wash with 25% DIEA/DMF is needed just prior to the coupling step.

After the desired pentapeptide was assembled, the resin-bound peptide was deprotected, neutralized, and acetylated (mixing 1 h with 0.94 mL of acetic anhydride and 0.8 mL of pyridine in 10 mL of DMF). After final washing and drying under vacuum, anhydrous HF treatment of the resin (1.5 mL of anisole, 10 mL of HF, 1 h at 0 °C) similar to that in **18** produced free linear peptide. Recovery and HPLC purification were the same as in **18**, giving the TFA salt of the title compound (52% recovery from HPLC purification; overall yield 24%); FAB-MS $[M + H]^+$ = 620.3 (expected 620.3).

Synthesis of Compounds 6–17, 19–29. The remaining compounds **6–29** were synthesized using methods similar to those described above in the general method and in the more specific synthesis of **18**. Yields and analytical data are summarized in Table 1.

Solution-Phase Peptide Synthesis. Boc-*N*-MeArg(Tos)-Gly-OBzl (58). Boc-*N*-MeArg(Tos) (25 mmol, 11.07 g, Bachem), 30 mmol of Gly-OBzl tosylate (10.10 g), 25 mmol of HBTU, and 75 mmol of DIEA were dissolved in 25 mL of CH_2Cl_2. The reaction was allowed to proceed 1 h, and the solvent was evaporated under reduced pressure at 50 °C to a syrup, which was dissolved in 400 mL of EtOAc. This solution was extracted with (150 mL each) 5% citric acid (2×), H_2O (1×), saturated sodium bicarbonate ($NaHCO_3$, 2×), and saturated NaCl (1×). The organic layer was dried over $MgSO_4$ and the solvent evaporated under reduced pressure. The resulting oil was triturated with petroleum ether and dried under high vacuum for a minimum of 1 h: yield 14.7 g (99.5%); TLC $R_{f(CM)}$ = 0.18 $R_{f(CA)}$ = 0.10; NMR is consistent with structure; FABMS $[M + H^+]$ = 590.43 (expected 590.26).

***N*-MeArg(Tos)-Gly-OBzl (59).** Boc-*N*-MeArg(Tos)-Gly-OBzl 14.5 g (24.5 mmol) was dissolved in 30 mL of TFA and allowed to react for 5 min, and the solvent was evaporated at room temperature at 1 mm of pressure. The resulting syrup was dissolved in 400 mL of ice cold EtOAc and extracted with 100 mL of ice cold saturated $NaHCO_3$, the aqueous phase was extracted with EtOAc (2 × 200 mL), and the combined organic phases were extracted once with 25 mL of saturated NaCl. The solvent was evaporated under reduced pressure giving a viscous oil that was triturated with 300 mL of Et_2O. The resulting solid was filtered and washed with Et_2O, giving a hygroscopic compound that was dried in a vacuum desiccator: yield 10.33 g (86.2%); TLC $R_{f(CM)}$ = 0.03; $R_{f(CMA)}$ = 0.20; NMR is consistent with structure; FABMS $[M + H]^+$ = 490.21 (expected 490.20).

Boc-D-Val-*N*-MeArg(Tos)-Gly-OBzl (60). *N*-MeArg(Tos)-Gly-OBzl (9.80 mmol, 4.80 g), 9.82 mmol of Boc-D-Val (2.13 g, Bachem), and 10.0 mmol of HBTU (3.79 g) were dissolved in 10 mL of CH_2Cl_2. The flask

was placed on an ice bath, and 20 mmol of DIEA (3.48 mL) was added. The reaction was allowed to proceed at 0 °C for 15 min and for 2 days at room temperature. The reaction mixture was diluted with 400 mL of EtOAc, extracted with (200 mL each) 2 × 5% citric acid and 1 × saturated NaCl, dried over $MgSO_4$, and evaporated under reduced pressure. The resulting oil was triturated with 50 mL and then 30 mL of Et_2O for 30 min with efficient mixing: yield 4.58 g (69%); TLC $R_{f(CM)}$ = 0.27 (also contains a spot near the origin, which is an aromatic impurity that is removed during trituration of the product in the next step); NMR is consistent with structure; FABMS $[M + H]^+$ = 689.59 (expected 689.43).

Boc-D-Val-*N*-MeArg(Tos)-Gly (61). Boc-D-Val-*N*-MeArg(Tos)-Gly-OBzl (4.50 g, 4.44 mmol) dissolved in 80 mL of MeOH was purged with N_2 for 10 min. Pd/C (1.30 g) catalyst (10%, Fluka lot #273890) was then added, and then H_2 was passed directly over the surface of the reaction. TLC showed the reaction to be complete within approximately 0.5 h. After 1 h the catalyst was removed by filtering through a bed of Celite, and the solvent was removed at 40 °C under reduced pressure. The resulting solid was triturated well with 50 mL of refluxing Et_2O, filtered, and washed with petroleum ether: yield 3.05 g (78%); TLC $R_{f(CM)}$ = 0.33; $R_{f(CMA)}$ = 0.37; NMR is consistent with structure; FABMS $[M + H]^+$ = 599.45 (expected 599,29).

4-Nitrobenzophenone Oxime (Ox) (62). 4-Nitrobenzophenone (50 g, 220 mmol, Aldrich) and 30.6 g of hydroxylamine hydrochloride (Aldrich, 440 mmol) were heated at reflux in 0.5 L of MeOH/pyridine (9:1) for 1 h. The reaction mixture was evaporated under reduced pressure, dissolved in 500 mL of Et_2O, extracted with 200 mL each of 5% citric acid (2×) and saturated NaCl (1×), dried over $MgSO_4$, evaporated under reduced pressure, and triturated with Et_2O, giving 44.35 g (83%) of the oxime as a mixture of the cis and trans isomers: TLC $R_{f(CM)}$ = 0.50; $R_{f(CMA)}$ = 0.82; NMR is consistent with structure; FABMS $[M + H]^+$ = 242.07 (expected 242.07).

Boc-Mamb-Ox (63). Boc-Mamb (22 mmol, 5.522 g), 20 mmol of nitrobenzophenone oxime (4.84 g), and 20 mmol of DMAP were dissolved in 40 mL of CH_2Cl_2. The flask was placed on an ice bath, and 21 mmol of DCC was added. The reaction was allowed to proceed on ice for 30 min and at room temperature overnight. The dicyclohexylurea formed was filtered and washed with 40 mL of CH_2Cl_2. The filtrate was evaporated under reduced pressure at room temperature to a syrup and dissolved in 400 mL of EtOAc. This solution was extracted with (150 mL each) 5% citric acid (2×), H_2O (1×), saturated $NaHCO_3$ (2×), and saturated NaCl (1×). The organic layer was dried over $MgSO_4$ and the solvent evaporated under reduced pressure. The resulting oil was triturated with petroleum ether and dried under high vacuum: yield 7.51 g (79%); TLC $R_{f(CM)}$ = 0.41; $R_{f(CMA)}$ = 0.66; NMR is consistent with structure; FABMS $[M + H]^+$ = 476.30 (expected 476.18).

TFA·Mamb-Ox (64). Boc-Mamb-Ox (7.4 g, 15.5 mmol) was dissolved in 30 mL of CH_2Cl_2 plus 10 mL of TFA (25% TFA). The reaction was allowed to proceed at room temperature for 1 h, and the solvent was evaporated under reduced pressure at room temperature for 10 min and then at 40 °C for 15 min. The resulting syrup was triturated with Et_2O (200 mL) at -5 °C. The resulting crystals were filtered after 1 h and washed well with Et_2O: yield 7.22 g (95%); $R_{f(CMA)}$ = 0.25; NMR is consistent with structure; FABMS $[M + H]^+$ = 376.22 (expected 376.12).

Boc-Asp(OcHex)-Mamb-Ox (65). Boc-Asp(OcHex) (20 mmol, 6.308 g, Bachem) and 44 mmol of DIEA (7.66 mL), were dissolved in 20 mL of DMF. HBTU (20 mmol, 7.58 g) was added and the reaction allowed to proceed for 2 min with vigorous stirring. TFA·Mamb-Ox (7.13 g, 15 mmol) was added and the reaction allowed to proceed overnight at room temperature. The solvent was removed under reduced pressure, giving an oil, which was dissolved in 500 mL of EtOAc, and this solution was extracted with (150 mL each) 5% citric acid (2×), H_2O (1×), saturated $NaHCO_3$ (2×), and saturated NaCl (1×). The organic layer was dried over $MgSO_4$ and the solvent evaporated under reduced pressure. The resulting oil was triturated with petroleum ether and dried under high vacuum: yield 9.76 g (97%); TLC $R_{f(CM)}$ = 0.55; NMR is consistent with structure; FABMS $[M + H]^+$ = 673.45 (expected 673.23).

TFA·Asp(OcHex)-Mamb-Ox (66). Boc-Asp(OcHex)-Mamb-Ox (15 mmol) was dissolved in 50 mL of 35% TFA in CH_2Cl_2 and allowed to react 90 min. The solvent was evaporated under reduced pressure at room temperature for 10 min and then at 40 °C for 15 min. To remove traces of TFA, 25 mL of DMF was added and the solvent evaporated at 50 °C. The resulting syrup was triturated with Et_2O (200 mL) and then dried under high vacuum, giving a waxy solid: yield 9.61 g (93%); $R_{f(CMA)}$ = 0.45; NMR is consistent with structure; FABMS $[M + H]^+$ = 573.56 (expected 573.23).

Boc-D-Val-*N*-MeArg(Tos)-Gly-Asp(OcHex)-Mamb-Ox (67). TFA·Asp(OcHex)-Mamb-Ox, Boc-D-Val-*N*-MeArg(Tos)-Gly, and HBTU (10 mmol each) plus 30 mmol of DIEA were dissolved in 20 mL of DMF. After 4 h, the solvent was removed under reduced pressure and the residue taken up in 600 mL of EtOAc, which was extracted with 300 mL each of 5% citric acid, H_2O, and saturated NaCl. The organic layer was dried over $MgSO_4$, evaporated under reduced pressure, triturated with Et_2O, and dried in vacuo: yield 9.90 g (86%); $R_{f(CM)}$ = 0.10; NMR is consistent with structure; FABMS $[M + H]^+$ = 1153.22 (expected 1153.47).

TFA·D-Val-*N*-MeArg(Tos)-Gly-Asp(OcHex)-Mamb-Ox (68). This compound was prepared from Boc-D-Val-*N*-MeArg(Tos)-Gly-Asp-(OcHex)-Mamb-Ox (9.8 g, 8.5 mmol) by treatment with TFA/CH_2Cl_2 (1:1) for 45 min. The solvent was evaporated and the product triturated with Et_2O: yield 9.73 g (98%); $R_{f(CM)}$ = 0.10; NMR is consistent with structure; FABMS $[M + H]^+$ = 1053.22 (expected 1053.4).

***cyclo*(D-Val-*N*-MeArg(Tos)-Gly-Asp(OcHex)-Mamb) (69).** TFA·D-Val-*N*-MeArg(Tos)-Gly-Asp(OcHex)-Mamb-Ox (1.80 g, 1.54 mmol) and 2 mmol each of DIEA and acetic acid were dissolved in 200 mL of DMF. The mixture was heated to 50 °C for 2 days and then evaporated under reduced pressure. The syrup was dissolved in 400 mL of EtOAc/*n*-butanol (1:1) and extracted with 200 mL each of 5% citric acid (3×) and saturated NaCl (1×). The organic layer was dried over $MgSO_4$, evaporated under reduced pressure, and triturated twice with 200 mL of Et_2O: yield 1.07 g (86%); $R_{f(CM)}$ = 0.10; NMR is consistent with structure; FABMS $[M + H]^+$ = 811.25 (expected 811.38).

***cyclo*(D-Val-*N*-MeArg-Gly-Asp-Mamb) (18).** *cyclo*(D-Val-*N*-MeArg-(Tos)-Gly-Asp(OcHex)-Mamb) (0.50 g) was treated with 5 mL of HF at 0 °C, in the presence of 0.5 mL of anisole for 30 min. The HF was removed under reduced pressure and the crude peptide triturated with Et_2O, EtOAc, and Et_2O. The resulting solid was dissolved in 10% HOAc and lyophilized: yield 0.321 g (82% calculated as the acetate salt). The product was purified with a recovery of approximately 40% using HPLC as described for the material synthesized by the solid-phase procedure.

***cyclo*(D-Abu-*N*-MeArg-Gly-Asp-Mamb) (17).** This compound was prepared by the solution-phase route described for **18** and was purified by crystallization: crude peptide (5 g) was dissolved in water (50 mL), and the solution was filtered. The pH was adjusted to neutrality using concentrated ammonium hydroxide, and 150 mL of acetone was then added. The mixture was stirred on an ice bath for 1 h, giving 2.25 g of product (**17**).

Compounds 30–33, 35–36. These peptides were synthesized using the appropriate amino acids and the solution-phase route described for the synthesis of **18**. The products were purified by reverse-phase HPLC.

NMR Structural Studies of SK&F 106760. NMR spectra were collected on an AM600 (Bruker) spectrometer at pH 5.0 at 15 °C. ROESY spectra were collected with mixing times of 150 and 75 ms, and TOCSY spectra (for assignment of spin systems) were collected with a 66-ms spin lock. Spectral widths of 6250 Hz were recorded with 2048 complex points in ω_2 and 512 complex points in ω_1. Assignments were easily made based on the TOCSY spectrum. Data were processed in FELIX (Hare Research), and significant cross peaks in the spectrum were classified as weak, medium, or strong and were fed into molecular dynamics calculations.

An initial structure conforming to the NOE constraints was built in INSIGHTII (BIOSYM Technologies, Inc.) and minimized using DISCOVER. Dynamics were then run for 18 ps starting at 30 K and stepping up in 50-deg increments up to 400 K. A predominant structure minimized from the dynamics trajectories is given in Figure 1.

Crystallography. Compound **17** was recrystallized from a water solution. A rather large crystal, 0.30 × 0.25 × 0.50 mm, was placed in a glass capillary and mounted on a Syntex R3 diffractometer equipped with Mo radiation (λ = 0.71069 Å). The crystal was placed in a cold stream of nitrogen, which kept it cooled to -67 °C. The orthorhombic unit cell parameters were refined from the Bragg angles of 48 computer-centered reflections: a = 8.758(2) Å, b = 18.073(4) Å, c = 22.656(6) Å, and V = 3597 Å^3. The space group was determined to be $P2_12_12_1$ (no. 19) on the basis of the systematic extinctions. With Z = 4 and fw = 722.75 (assuming 9 mol of water per 1 mol of peptide), the density and the linear absorption coefficient of the crystal were calculated to be 1.334 g/cm^3 and 1.04 cm^{-1}, respectively. The intensities of 11 574 reflections, comprising two full octants of data (4° < 2θ < 55.0°) and one partial octant (4° < 2θ < 30°), were collected using 1.2° ω-scans. Standard reflections showed a decrease of 3% over the course of the data collection, and a correction was applied. There was a 2.3% change in the intensity of an azimuthal scan; no absorption correction was applied. R_{merge} for the redundant data was 0.032.

The structure was solved by direct methods and refined by full-matrix least-squares based on F. The refinement of 482 parameters (all peptide nonhydrogen atoms were refined with anisotropic thermal parameters; all water molecules and the hydrogen atoms bonded to the peptide nitrogen atoms, with isotropic thermal parameters; all hydrogen atoms bound to carbons were idealized) using 2360 unique reflections with $I > 2\sigma(I)$ converged at $R = 0.058$ and $R_w = 0.048$. The scattering factors were taken from the *International Tables for X-ray Crystallography*, Vol IV. Attempts to model the water structure in a chemically meaningful fashion were totally frustrated by disorder; the largest peaks were placed into the refinement with variable occupancies. The final difference map had numerous small peaks in the water region; the largest peak had a height of 0.40 e/Å^3 and was located near O3W. The disorder of the water molecules has limited the amount of observable data and, therefore, the accuracy of the structure. Attempts to lower the temperature of the crystal even more were frustrated by a phase transition to a twinned monoclinic cell.

CD Spectroscopy. CD spectra were recorded on a Jasco-600 spectropolarimeter. Peptides were dissolved in deionized water at concentrations of approximately 250 μg/mL. A cylindrical quartz cuvette of 0.05-cm pathlength was used, and the spectra were recorded at room temperature. Eight scans of background spectra (water blanks) and peptide spectra were acquired, and the background was subtracted from the peptide spectra.

Platelet Aggregation Assay. Venous blood was obtained from the arm of a healthy human donor who was drug-free and aspirin-free for at least two weeks prior to blood collection. Blood was collected into 10-mL citrated vacutainer tubes. The blood was centrifuged for 15 min at 150 × g at room temperature, and platelet-rich plasma (PRP) was removed. The remaining blood was centrifuged for 15 minutes at 1500*g* at room temperature, and platelet-poor plasma (PPP) was removed. Samples were assayed on an aggregometer (PAP-4 platelet aggregation profiler), using PPP as the blank (100% transmittance). PRP (200 μL) was added to each micro test tube, and transmittance was set to 0%. Various agonists (20 μL) (ADP, collagen, arachidonate, epinephrine, thrombin) were added to each tube, and the aggregation profiles were plotted (percent transmittance versus time). The results were expressed as percent inhibition of agonist-induced platelet aggregation. For the IC_{50} evaluation, the test compounds were added at various concentrations prior to the activation of the platelets. For some samples, ADP alone was used as the agonist, with comparable results to that of the mixture of agonists described above. The fibrinogen-binding assay was conducted as described elsewhere.[25]

Acknowledgment. We thank Joe Lazaar, Bob Carney, and Mike Haas for mass spectral data, and Rob Houghton for expert technical assistance.

Supplementary Material Available: Tables of atomic coordinates, anisotropic thermal parameters, and interatomic distances and angles (13 pages); table of observed and calculated structure factor amplitudes (6 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.