and "dummy" atoms defining vectors perpendicular to the tetrazole rings during the matching procedure.

The atomic coordinates corresponding to the figures presented in this work are available upon request to the authors.

Biological Methods. Angiotensin II Receptor Binding. ^{125}I-labeled AII (2200 Ci/mmol) was obtained from Anawa (Wangen, Switzerland). Unlabeled AII was purchased from Bachem (Bubendorf, Switzerland). Bovine serum albumin (BSA) was from Fluka (Buchs, Switzerland). The peptidase inhibitors were from Novabiochem (Läufelfingen, Switzerland). The culture media was purchased from Amimed AG (Muttenz, Switzerland).

Membrane Preparation. The original primary culture of rat aorta smooth muscle cells (SMC) was obtained from Dr. Pfeilschifter (Ciba-Geigy, Basel, Switzerland). The cells were further grown on Dulbecco's Minimum Essential Medium (MEM) containing 4.5 g/L glucose and supplemented with 4 mM L-glutamine, 15% fetal calf serum, and penicillin-streptomycin (200 IU-200 μg/mL). The cells were obtained after 5-20 passages, up to which no changes in AII-binding characteristics or AII-induced IP_3 formation were noted. At confluence, the cells were washed twice with phosphate-buffered saline (PBS) and harvested with a rubber policeman. The pellet was resuspended as described above. The membrane preparations were kept in liquid nitrogen or in aliquots at -80 °C until used; no apparent loss of AII-binding activity was seen under these conditions.

Binding Assay. The experiments were performed with an automatic pipetting and filtration device (Filter-Prep 101, Ismatec, Zurich, Switzerland). Briefly, 20-60 μg of protein was incubated at 25 or 30 °C for 60 min with radioactive tracer (150 pM) and varying concentrations of unlabeled competitors in the buffer used for resuspension of the pellet. The reaction was terminated by the addition of 2 mL of ice-cold buffer. Bound and free radioactivity were separated by immediate filtration through Whatman GF/F filters, which were washed three times with 2 mL of cold PBS. The radioactivity trapped on the filter was measured in a counter (Pharmacia-LKB, Uppsala, Sweden) at 80% efficiency. Nonspecific binding was determined in the presence of 1 μM unlabeled AII.

Data Analysis. The binding data were analyzed by using the four-parameter logistic dose-response method of De Lean et al.[17] for the IC_{50} estimation.

Inhibition of Angiotensin II Induced Vasoconstriction in Rabbit Aortic Rings. Methods. Isolated Rabbit Thoracic Aorta Rings. Rabbits (2-2.5 kg, Chincilla, male) were stunned by a blow to the neck and the descending thoracic aorta quickly removed. From each aorta, rings 2-3 mm wide were prepared and mounted between two parallel hooks under an initial resting tension of 2 g. Thereafter rings were immersed in a 20-mL tissue bath containing a modified Krebs-Henseleit solution of the following composition (mM): 118 NaCl, 4.7 KCl, 2.52 $CaCl_2$, 24.8 $NaHCO_3$, 1.2 Mg_2SO_4, 1.2 KH_2PO_4, 10 glucose, at 37 °C, and gassed with 95% O_2 and 5% CO_2. Each preparation was allowed to equilibrate for at least 1 h. Isometric responses were measured with a force transducer (K30, Hugo Sachs Electronics Freiburg, FRG) coupled to a tissue bath data acquisition system (Buxco Electronics, Inc., Sharon, CT). At 20-min interval, rings were challenged with 10 nM angiotensin II (Hypertensin-CIBA). Two control values to each agonist were obtained. Thereafter rings were incubated with graded concentrations of the test compound for 5 min prior to each agonist challenge. Three to four concentrations were tested for each preparation. Controls rings were incubatued with the vehicle (DMSO). Data were analyzed with the Buxco system and a software package (Branch Technology, Dexter, MI). Responses were expressed as a percentage of the initial control values. The concentration producing a 50% inhibition of the initial value is given as IC_{50}.

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Development of a Small RGD Peptide Fibrinogen Receptor Antagonist with Potent Antiaggregatory Activity in Vitro

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The development of potent antithrombotic agents from the fibrinogen platelet receptor binding sequences Fg-α 572-575 -Arg-Gly-Asp-Ser- and Fg-γ 400-411 -HHLGGAKQAGDV, believed to be a cryptic RGD-type sequence, is described. The tetrapeptide Ac-RGDS-NH_2 itself is capable of inhibiting platelet aggregation in vitro at high concentrations, IC_{50} 91.3 ± 0.1 μM [in vitro antiaggregatory activity employing dog platelet rich plasma (PRP)/ADP], due to low platelet fibrinogen receptor affinity, K_i 2.9 ± 1.9 μM (purified, reconstituted human platelet GPIIb/IIIa), relative to fibrinogen, K_i 38.0 ± 6.0 nM. The peptide is also unstable to plasma, suffering total loss of in vitro activity upon incubation in PRP for 3 h ($T^{1/2}$ 90 min). Only modest improvements in potency were achieved with linear analogues of Ac-RGDS-NH_2, while dramatic results were achieved with cyclic analogues, culminating in the cyclic disulfide Ac-cyclo-*S*,*S*-[Cys-(N^{α}-Me)Arg-Gly-Asp-Pen]-NH_2 (SK&F 106760) with improved plasma stability (100% activity after 3 h), affinity (K_i 58 ± 20 nM purified human receptor), and potency (IC_{50} 0.36 ± 0.4 μM dog PRP/ADP). The affinity of this peptide is 2 orders of magnitude greater than that of Ac-RGDS-NH_2. The affinity of the analogue is also comparable to fibrinogen. This peptide constitutes a first potent small peptide entry into the class of novel antithrombotic agents called fibrinogen receptor antagonists.

Introduction

The most critical step in platelet aggregation is the cross-linking of activated platelets by the multifunctional plasma protein fibrinogen.[1] A logical approach toward inhibition of aggregation and hence thrombus formation would be through inhibition of the binding of fibrinogen to its platelet receptor, the glycoprotein complex of GPIIb and GPIIIa.[1,2] The first fibrinogen receptor antagonists

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were monoclonal antibodies, e.g., PAC-1,[3] 10E5,[4] and AP-2,[5] which bind to GPIIb/IIIa with high affinity and inhibit platelet aggregation in vitro stimulated by a variety of agents. The monoclonal antibodies or $F(ab')_2$ fragments have been shown, furthermore, to inhibit thrombus formation in a canine model of coronary thrombus formation,[6] to inhibit thrombus formation in baboons that have received arterial grafts,[5] and to enhance the thrombolytic efficacy of tPA by reducing the time to reperfusion and by inhibiting reocclusion.[7] The monoclonal antibodies demonstrated that fibrinogen receptor antagonists represent a highly effiacious class of antithrombotic agents. In vivo studies of the $F(ab')_2$ fragment in dogs, baboons, and humans[8] have revealed problems of thrombocytopenia and immunogenicity that will hamper chronic administration of this particular embodiment of the fibrinogen receptor antagonist. Presumably a small peptide fibrinogen receptor antagonist would be less prone to these problems.

Segments of fibrinogen sequence that inhibit platelet fibrinogen receptor binding were identified from the Fg-α chain, residues 572–575 -Arg-Gly-Asp-Ser-,[9] and from the C-terminus of the Fg-γ chain, residues 400–411 - HHLGGAKQAGDV.[10] Fg-γ 400–411 was implicated ultimately as a cryptic RGD-type sequence through the facts that each peptide inhibits the binding of the other peptide to the platelet fibrinogen receptor,[11] and in a structure–activity study of Fg-γ 400–411 the single most important modification was the replacement of Ala-408 with Arg.[12] (Lamprey fibrinogen, furthermore, contains an Fg-γ C-terminal -QQQSKGNS*RGD*N sequence.[13]) The tetrapeptide RGDS inhibits platelet aggregation in vitro at high concentrations, IC_{50} 60–80 μM [employing washed platelets stimulated with ADP],[9] due to low platelet fibrinogen receptor affinity (K_i 15.6 μM).[14] In the canine coronary thrombus model, thrombus inhibition was previously demonstrated by *intracoronary infusion* of >100 μM Ac-RGDS-NH_2 at 0.1 mL/min.[15] This antithrombotic activity was not seen at lower concentrations or infusion rates, due to low potency. Ac-RGDS-NH_2 is relatively unstable in plasma, as demonstrated by a loss of antiaggregatory in plasma after 3 h ($T^{1/2}$ 90 min).[15] This paper describes the development of a highly potent fibrinogen receptor antagonist derived by modifications of Ac-RGDS-NH_2.[16]

Table I. Linear Analogues of Ac-Arg-Gly-Asp-Ser-NH_2

no.		antiaggregatory[a] dog PRP/ADP IC_{50} (μM)	% inhibtn[b] at 3 h
1	Ac-Fg-γ 400–411 Ac-HHLGGAKQAGDV-OH	>200	
2	Ac-Arg-Gly-Asp-Ser-NH_2	91.3 ● 0.1 67.2 ± 18.1[c]	0
3	Ac-*Arg-Arg*-Gly-Asp-Phe-NH_2	113 ± 14	5.9
4	Ac-D-*Arg*-Gly-Asp-Ser-NH_2	>200[a]	
5	Ac-*Lys*-Gly-Asp-Ser-NH_2	>200	
6	Ac-*Cit*-Gly-Asp-Ser-NH_2	>200	
7	Ac-*His*-Gly-Asp-Ser-NH_2	>200	
8	Ac-*hArg*-Gly-Asp-Ser-NH_2	68.2 ● 27.8	33.3
9	Ac-Arg-D-*Ala*-Asp-Ser-NH_2[d]	>200	
10	Ac-Arg-*Pro*-Asp-Ser-NH_2	>200	
11	Ac-Arg-*Sar*-Asp-Ser-NH_2	>200[c]	
12	Ac-Arg-Gly-D-*Asp*-Ser-NH_2	>200 83 ± 24.1[c]	
13	Ac-Arg-Gly-*Asn*-Ser-NH_2	>200	
14	Ac-Arg-Gly-*Glu*-Ser-NH_2	>200	
15	Ac-Arg-Gly-D-*Glu*-Ser-NH_2	97 ● 19.8[c]	
16	Ac-Arg-Gly-*β-Asp*-Ser-NH_2	>200	
17	Ac-*Lys*-Gly-*Glu*-Ser-NH_2	>200	
18	Ac-Arg-Gly-Asp-D-*Ser*-NH_2	138 ± 4	33.3
19	Ac-Arg-Gly-Asp-*Val*-NH_2	55.5 ● 14	92
20	Ac-Arg-Gly-Asp-*Tyr*-NH_2	102 ± 14	98
21	Ac-Arg-Gly-Asp-(2)*NaI*-NH_2	40.5 ● 4.7	100
22	Ac-Arg-Gly-Asp-$\_$-NH_2	138 ± 16	54
23	Ac-Arg-Gly-Asp-*NHCH$_2$CH$_2$Ph*	75.9 ● 17.4	100
24	Ac-Arg-Gly-Asp-Ser-*NHEt*	>200	

[a] Inhibition of platelet aggregation in canine platelet-rich plasma induced by ADP. [b] Percent inhibition of platelet aggregation upon addition of ADP 3 h after incubation of 200 μM peptide in PRP. [c] Aggregation assay employing washed dog platelets stimulated with thrombin. [d] H-Arg-L-Ala-Asp-Ser-OH was reported previously to be considerably less active (IC_{50} 3 mM compared to 30 μM for H-Arg-Gly-Asp-Ser-OH).[19]

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Results

The primary bioassay in this investigation employed the determination of platelet aggregation in an aggregometer. Washed platelets were employed initially for some analogues (as noted in the tables), but for most analogues platelet-rich plasma (PRP) was used. As seen in Table I, analogue potencies are generally higher when washed platelets are employed. This is to be expected, since the RGD-containing plasma proteins fibrinogen, fibronectin, and von Willebrand factor have been removed. We chose PRP since it more closely resembles the in vivo condition and constitutes a more stringent test of analog efficacy. After addition of peptide analogues, aggregation was initiated with ADP. A virtue of this assay system is that it allowed for ready evaluation of the loss of activity over time by retarding the addition of ADP for a chosen period (3 h).

Previous investigations into the development of small peptide fibrinogen receptor antagonists have worked with either RGDS Fg-α 572–575[9,17-24] or HHLGGAKQAGDV Fg-γ 400–411.[11,25] In our hands the γ chain peptide analogue 1 was at least 2 times less active than the α chain peptide analogue 2, Table I. We subsequently found that the receptor affinity of 1 was over 3 times lower than that of 2, Table III. Due to greater potency and smaller structure, most of our work has focused upon the α chain tetrapeptide. In this study we chose to work with analogues bearing an N-terminal acetyl group and a C-terminal carboxamide group since a cationic N-terminal ammonium group and an anionic C-terminal carboxylate group are not present in the intact Fg-α 572–575.

Table II. Cyclic Analogues of Ac-Arg-Gly-Asp-Ser-NH_2

no.		antiaggregatory[a] dog PRP/ADP IC_{50} (μM)	% inhibitn[b] at 3 h
25	Ac-*Cys*-Arg-Gly-Asp-Ser-*Cys*-NH_2	32.7 ± 11	100
26	Ac-Cys-Gly-Arg-Gly-Asp-Cys-NH_2	52.0 ± 17.8	100
27	Ac-Cys-Gly-Arg-Gly-Asp-Pen-NH_2	11.4 ± 2.0	100
28	Ac-Cys-Arg-Gly-Asp-_-Cys-NH_2	16.2 ± 5.9	55[c]
29	Ac-Cys-Arg-Gly-Asp-*Pen*-NH_2	4.12 ± 0.6	100
30	Ac-Pen-Arg-Gly-Asp-Cys-NH_2	7.97 ± 1.47	100
31	Ac-Cys-D-*Arg*-Gly-Asp-Pen-NH_2	4.1 ± 1.1	100
32	Ac-Cys-*hArg*-Gly-Asp-Pen-NH_2	3.85 ± 0.75	56[c]
33	Ac-Cys-*Lys*-Gly-Asp-Pen-NH_2	55.3 ± 5.9	27[c]
34	Ac-Cys-(Et^{trans}_2)*Arg*-Gly-Asp-Pen-NH_2	82 ± 5	5[c]
35	Ac-Cys-(*N-Me*)*Arg*-Gly-Asp-Pen-NH_2 SK&F 106760	0.355 ± 0.035	100[c]
36	Ac-Pen-(*N-Me*)Arg-Gly-Asp-Pen-NH_2	0.37 ± 0.13	100[c]
37	Ac-Cys-Arg-*Ala*-Asp-Pen-NH_2	~200	
38	Ac-Cys-Arg-*Sar*-Asp-Pen-NH_2	73.4 ± 8.18	57
39	Ac-Cys-Arg-Gly-D-*Asp*-Pen-NH_2	>200	
40	Ac-Cys-(*N*-Me)Arg-Gly-*Asn*-Pen-NH_2	>200	
41	Ac-KCAGD(Pen)-OH	>200	
42	Ac-HHLGGAKCRGD(Pen)-NH_2	9.6 ± 2.0	100[c]

[a] Inhibition of platelet aggregation in canine platelet-rich plasma induced by ADP. [b] Percent inhibition of platelet aggregation upon addition of ADP 3 h after incubation of 200 μM peptide in PRP. [c] Percent inhibition of platelet aggregation after addition of ADP 3 h after incubation of IC_{50} dose of peptide in PRP.

Linear Analogues of Ac-Arg-Gly-Asp-Ser-NH_2: Sequence Modifications. We prepared a number of analogues of the linear tetrapeptide Ac-Arg-Gly-Asp-Ser-NH_2 (2), none of which were substantially superior to 2, Table I. From the activities of these analogues, we conclude that the arginine side chain cannot be modified without at least a 2-fold decrease in potency (as demonstrated by analogues 4–7), the glycine residue cannot be replaced with amino acids bearing groups off either the α carbon or α nitrogen (as demonstrated by analogues 9–11), and the aspartic acid side chain cannot be modified without a decrease in potency (as demonstrated by analogues 12–17). Other groups[17-24] have come to similar conclusions.

We have observed increases in potency through replacement of the serine side chain with more lipophilic groups. This has been observed by other groups as well. An exception between our study and others[21,23] is the effect of Tyr substitution, which in our hands gave an analogue (20) with potency only comparable to the Ser analogue 2.

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We found that the position-4 side chain could be deleted to give the tripeptide **22** or the position-4 α-carboxyl group could be deleted to give the "tripeptide" **23**, with little loss of potency. The homoarginine analogue **8** and the analogues bearing lipophilic substitutions for serine (**19–21**) suffered a lower decrease in activity at 3 h than did **2**.

Among the linear analogues in Table I, the maximal gain in potency was only 2-fold with analogue **21**.

Cyclic Analogues of Ac-Arg-Gly-Asp-Ser-NH_2 and Other Conformational Constraints. In contrast to the relatively modest results from the studies with linear analogues, Table I, dramatic results were achieved in structure–activity studies of cyclic disulfide analogues, Table II. Simple enclosure of RGDS in a cystine-containing disulfide ring analogue (**25**) resulted in a 3-fold increase in activity. An alternate six-membered disulfide cyclic structure is analogue **26**, which is somewhat less potent than **25**. In this type of structure the C-terminal Cys corresponds to Ser in Ac-RGDS-NH_2, and the substitution of the more lipophilic Pen gave analogue **27** with enhanced potency. Deletion of the serine in analogue **25**, however, gave the pentapeptide analogue **28**, which was twice as potent as **25**, three times more potent than **26**, and over five times more potent than Ac-RGDS-NH_2 (**2**). Since the C-terminal Cys in **28** corresponds to the serine residue in Ac-RGDS-NH_2, it followed that further gain in potency might be accomplished with the more lipophilic Pen. Accordingly, the Pen-5 analogue **29** was four times more potent than the Cys-5 analogue **28** and over 20 times more potent than Ac-RGDS-NH_2 (**2**). Pen-1 substitution in analogue **30** increased potency 2-fold over Cys-1 **28**.

Due to the enhanced potency of analogue **29**, a number of modifications were incorporated into **29** to explore further gains in potency. Unlike the linear tetrapeptide series, replacement of Arg with either D-Arg in analogue **31** or hArg in analogue **32** had little effect upon potency. Like the linear tetrapeptide series, however, several types of modifications resulted in losses of potency by at least 1 order of magnitude: modification of the cationic group, to either Lys in analogue **33** or (Et^{guan}_2)Arg in analogue **34**; addition of a methyl group to either the α carbon or α nitrogen of Gly in the Ala analogue **37** and Sar analogue **38**; isomerization of L-Asp to the D-Asp analogue **39**; or replacement of the Asp carboxylate with an Asn carboxamide in **40**.

Only one modification to the -RGD- sequence in **29** improved activity. The addition of a methyl group to the Arg α amino group resulted in a 10-fold increase in potency in analogue **35** (SK&F 106760). Analogue **35** is over 250 times more potent than Ac-RGDS-NH_2 (**2**).

Analogue **35** displayed comparable potency in antiaggregatory assays employing either canine PRP and ADP (IC_{50} 0.36 ± 0.04 μM) or human PRP and ADP (0.23 ± 0.06 μM) and against other types of activating agents such as collagen and the thromboxane mimic U-46619 (0.26 ± 0.02 μM dog PRP/collagen, 0.49 ± 0.09 μM dog PRP/U-46619).

Since Pen-1 substitution in **30** gave an analogue with enhanced potency relative to **28**, the Pen-1/Pen-5 analogue of **35** was prepared, but this analogue (**36**) displayed identical potency.

In summary, the employment of three design strategies resulted in dramatic increases in the potency of Ac-RGDS-NH_2: (1) incorporation of the -RGD- sequence into a cyclic pentapeptide disulfide analogue; (2) addition of a methyl group to the α amino group of Arg; and (3) replacement of Cys-5 (corresponding to Ser in Ac-RGDS-NH_2) with the more lipophilic Pen.

If Fg-γ 400–411 is a cryptic version of Fg-α 572–575, then it should be possible to alter the -Cys-Arg-Gly-Asp-Pen- structure of analogue **29** to mimick the structure of Fg-γ 400–411. In other words, replacement of Arg with Ala, the C-terminal carboxamide with a free carboxyl group, and addition of a Lys to the N-terminus gave analogue **41**. This analogue proved to be inactive, however. Addition of the N-terminal residues of Fg-γ 400–411 to the analogue **29** gave analogue **42**, which was almost equipotent with analogue **29**. The addition of these residues did not enhance the potency of **29**. Thus, the modifications that enhance the activity of Fg-α 572–575 did not enhance the activity of Fg-γ 400–411.

Reductions in Loss of Activity in Plasma. If ADP is added to plasma 3 h after incubation of 200 μM Ac-RGDS-NH_2 in plasma, inhibition of platelet aggregation is not observed. The half-life for this loss of antiaggregatory activity for Ac-RGDS-NH_2 is 90 min. A number of analogues of Ac-RGDS-NH_2 did not suffer as severe an activity loss. In fact the observation of complete inhibition of aggregation at 200 μM for 3 h became sufficiently commonplace, especially for the potent cyclic analogues, that the 3-h test was performed subsequently at the IC_{50} dose [noted by footnote *c* in Table II]. Modifications that reduced this loss of activity include those that enhance the lipophilicity of the C-terminal serine amide, incorporation into a cyclic disulfide structure, and modifications to arginine that do not reduce activity.

Correlation of Increase in Antiaggregatory Activity with Increases in Affinity. Recent deployment of a receptor binding assay employing purified human GPIIb/IIIa reconstituted into liposomes has allowed for a partial examination of the correlation of antiaggregatory activities with receptor affinities. As seen in Table III, receptor affinity of Ac-RGDS-NH_2 (**2**) was approximately 100 times lower than that of fibrinogen **43**. The three modifications that resulted in the potent analogue **35**, i.e., incorporation of RGDS into a cyclic pentapeptide disulfide, replacement of Ser with a more lipophilic amino acid, and addition of a methyl group to the Arg α amino group increased receptor affinity 2 orders of magnitude in analogue **35** compared to Ac-RGDS-NH_2 (**2**).

Discussion

Linear Analogues of Ac-RGDS-NH_2: Sequence Modifications. It is important to underscore the number of modifications that failed to enhance the potency of Ac-RGDS-NH_2 significantly as a fibrinogen receptor antagonist. As others have found, most modifications to -Arg-Gly-Asp- fail to even maintain potency. A number of plasma and extracellular matrix glyproteins contain -RGD- sequences that are critical for cellular receptor binding, including fibronectin,[26,27] von Willebrand factor,[28,29] vitronectin,[30] and thrombospondin.[31] In all cases

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Table III. Increases in Affinity and Antiaggregatory Activity

no.		M_r	antiaggregatory[a] activity dog PRP/ADP IC_{50} (μM)	binding inhibition: human GPIIb/IIIa[b] K_i (μM)	binding inhibition: human platelets[c] K_i (μM)
43	fibrinogen	450 000		0.043	0.707 ± 0.308
1	Ac-HHLGGAKQAGDV-OH	1231	>200	14.5 (n = 1)	
2	Ac-Arg-Gly-Asp-Ser-NH_2	474	91.3 ± 0.1	4.2 ± 3.0	62 ± 30
19	Ac-Arg-Gly-Asp-Val-NH_2	486	55.5 ± 14	<10	
25	Ac-Cys-Arg-Gly-Asp-Ser-Cys-NH_2	678	32.7 ± 11	5.3 ± 3.0	
28	Ac-Cys-Arg-Gly-Asp-Cys-NH_2	591	16.2 ± 6	0.78 ± 0.01	
29	Ac-Cys-Arg-Gly-Asp-Pen-NH_2	619	4.12 ± 0.6		
31	Ac-Cys-D-Arg-Gly-Asp-Pen-NH_2	619	4.1 ± 1.1	0.72 ± 0.04	
35	Ac-Cys-(N-Me)Arg-Gly-Asp-Pen-NH_2	633	0.36 ± 0.04	0.058 ± 0.020[d]	0.062 ± 0.010
	SK&F 106760				
44	trigramin[e]	7500	0.13		0.021
45	echistatin[f]	5414	0.03[g]		0.008
46	MAb 10E5[h]	~150 000	<0.05[i]		0.016 (K_d)[j]

[a] Inhibition of platelet aggregation in canine platelet-rich plasma induced by ADP. [b] Purified GPIIb/IIIa isolated from human platelets, reconstituted in liposomes. [c] Gel-filtered human platelets. [d] The K_i was reported previously as 2.7 ± 0.8 nM.[16] [e] Reference 40. [f] Reference 42. [g] Inhibition of ADP-stimulated aggregation of washed platelets. [h] Reference 4. [i] 100% inhibition at ~50 nM.[h] [j] Dissociation constant of ^{125}I-10E5.[h]

RGD-containing peptide fragments of these proteins inhibit cellular receptor binding. All attempts to date to modify the -RGD- sequence in this inhibitory peptides have failed as well.

Potency increases of less than an order of magnitude have been attained through replacement of Ser with more lipophilic groups. In essence, our structure–activity relationship study (SAR) of linear analogues defined a minimum sequence for antiaggregatory activity: -RGD-. Others have shown that the Ac-Arg and Ser-NH_2 amide groups can be removed and the Arg-Gly amide replace with an ethylene group to give analogues that retain micromolar potency.[23]

Analogues of Ac-RGDS-NH_2 Bearing Cyclic and Other Modifications That Reduce Flexibility. The linear analogues were important in defining the minimum sequence for antiaggregatory activity. Through the utilization of modifications that reduce flexibility, however, analogues with considerably enhanced potency have been developed. All three of the design approaches that resulted in increased affinity toward the platelet fibrinogen receptor *can* reduce flexibility: **(1) Incorporation into a Cyclic Pentapeptide Disulfide.** It is well known that the enclosure of a peptide into a cyclic structure dramatically reduces the degrees of flexibility in a linear peptide.[32] **(2) *N*-Methylarginine.** Marshall and Bosshard[33] as well as Manavalan and Momany[34] have shown that an *N*-α-methyl amino group in a peptide restricts the flexibility of a peptide. **(3) Pen Substitution in Cyclic Disulfide Peptides.** The *gem*-dimethyl groups in penicillamine can further constrain cyclic disulfide peptides.[35] Thus, it is feasible that the conformation of RGD in Fg-α 572–575 imposed by the secondary structure of fibrinogen would be lost upon removal from the protein and that these modifications have forced the -RGD- in analogue **35** into the conformation that GPIIb/IIIa recognizes in fibrinogen. Alternatively, the constraints upon flexibility in analogue **35** may have imposed a unique conformation upon -RGD- that promotes a superior interaction with GPIIb/IIIa which compensates for the lack of other receptor interactions available to fibrinogen. Given that the affinity of analogue **35** is comparable to native fibrinogen, we cannot distinguish between the two hypotheses, although the latter hypothesis seems more plausible. A conformational analysis of the analogue **35** is being performed and will be reported in another paper. The data presented in this paper is not a complete analysis of conformational constraints in analogue **29**. Since the discovery of analogue **35**, we have been exploring modifications to the Ac-Cys-, -(NMe)Arg-, and Pen-NH_2 groups, which will be detailed in subsequent papers.

The fact that alteration of the cyclic RGD structure of analogue **29** into a cyclic Fg-γ structure gave an analogue (**41**) that was inactive, and the fact that simple appendage of the Fg-γ residues onto analogue **29** gave an analogue (**42**) with no enhancement of activity suggests that the Fg-γ 400–411 peptide may adopt a conformation that is different from that which may be adopted by analogue **29**. This theory would be supported by the identification of constrained high affinity Fg-γ analogues with structures that are distinctly different from analogue **29**. It is quite plausible that translocation of the cationic binding element two residues up-sequence from its position in -RGD- to the Lys-406 in Fg-γ would require different conformations to achieve high affinity interaction with the same receptor.

The fact that modifications that enhanced the potency of Fg-α 572–575 failed to enhance the potency of Fg-γ 400–411, however, may imply that the latter is not simply

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a cryptic version of the former but may interact with a different but proximal binding site on GPIIb/IIIa and thereby display a structure–activity relationship that differs from Fg-α 572–575. Bennet et al.[3] showed that the inhibitory effects of both Fg-α 572–575 and Fg-γ 402–411 on the binding of fibrinogen to ADP-stimulated platelets was additive and that the binding of the monoclonal antibody PAC-1 was more readily inhibited by the Fg-α peptide than the Fg-γ peptide while the monoclonal antibody A2A9 was not inhibited by the Fg-α peptide but partially inhibited by the Fg-γ peptide. These authors concluded from their studies that the binding sites of these peptides are spatially distinct. Santoro and Lawing,[36] furthermore, found that radiolabeled photoactivatable aryl azide derivatives of Fg-α 572–575 specifically labeled both GPIIb and GPIIIa while the analogous derivative of the Fg-γ peptide 400–411 specifically labeled only GPIIb. Thus, GPIIb/IIIa may undergo a divalent interaction with fibrinogen at two potential binding sites, only one of which may need to be blocked to prevent effective fibrinogen binding.

The influence of conformation on RGD peptide affinity and selectivity was also postulated by Pierschbacher and Ruoslahti.[28,37] They have shown that RGD selectivity toward the vitronectin receptor can be enhanced through incorporation of RGD into the cyclic disulfide peptide cyclo-*S,S*-[H-Gly-Pen-Gly-Arg-Gly-Asp-Ser-Cys-Ala-OH] (**43**).[38] Incorporation of RGD into the cyclic pentapeptide structure of SK&F 106760 has had an important influence on affinity. The role of the pentapeptide structure in SK&F 106760 on selectivity is under current investigation, but a test of "apparent" selectivity may be derived from comparison of the IC_{50} for platelet aggregation with the IC_{50} for inhibition of L8 smooth muscle cell adhesion to vitronectin (the L8 cell expresses $\alpha_v\beta_3$).[39] Accordingly, comparison of the ratio of activities (antiaggregatory IC_{50}/L8 cell antiadhesive IC_{50}) for Ac-RGDS-NH_2 (**2**) (91.3/12.3 μM), **43** (10.1/12.3 μM), and SK&F 106760 (**35**) (0.355/67.2 μM) suggests that SK&F 106760 may be selective for the platelet receptor (Jeff Stadel, unpublished results).

Reductions in Loss of Activity in Plasma. In this study a number of modifications to Ac-RGDS-NH_2 were shown to reduce the loss of activity in plasma suffered by Ac-RGDS-NH_2: those that enhance the lipophilicity of the C-terminal serine amide, incorporation into a cyclic disulfide structure, and modifications to arginine that do not reduce activity. In fact, those modifications that enhanced potency through constraint of conformation also reduced loss of activity with time. It is assumed that the loss of activity is due to enzymatic degradation (which is mainly mediated by aminopeptidases and carboxypeptidases), although loss of active peptide through protein binding could also be involved. The fact that the kinds of modifications which reduced loss of activity also tend to reduce either aminopeptidase or carboxypeptidase degradation reinforcing this as the major mechanism for loss of activity in plasma. More rigorous pharmacokinetic studies of SK&F 106760, which will be reported elsewhere, support this hypothesis as well.

Correlation of Increases in Antiaggregatory Activity with Increases in Affinity. The often dramatic differences between affinity and in vitro potency can at least partially be explained by the presence of micromolar concentrations of fibrinogen and the RGD-containing proteins fibronectin, von Willebrand factor, and thrombospondin found in plasma and released by activated platelets.[1] It follows then that the potency of an analogue in various assays would rank in order as follows: (1) K_i(GPIIb/IIIa) > (2) K_i(platelets) > (3) IC_{50}(washed platelets) > (4) IC_{50}(PRP) [where (1) is the inhibition of ^{125}I-fibrinogen binding to isolated purified GPIIb/IIIa, (2) is the inhibition of ^{125}I-fibrinogen binding to activated washed platelets, (3) is the inhibition of the aggregation of washed platelets, and (4) is the inhibition of the aggregation of platelet-rich plasma]. In fact, Ac-Arg-Gly-Asp-Ser-NH_2 was measured in all four assays with the following rank order: (1) 4.2 μM, (2) 62 μM, (3) 67.2 μM, and (4) 91.3 μM. Note also that the potencies for SK&F 106760 analogue **35** are (1) 58 nM, (2) 62 nM, (3) 115 nM, and (4) 355 nM.

Variations in Analogue Relative Potencies with Aggregation Assay. Throughout most of our work we have employed the same assay conditions to avoid ambiguities that may arise from differences in assay conditions. As noted above, plasma contains a multiplicity of ligands for GPIIb/IIIa. The relative affinities of these ligands are different as are their relative abundances in plasma and in platelet α granules.[1] The number of receptor sites varies with agonist type and agonist concentration as well.[40] Thus, antagonist efficacy can be expected to vary with type of platelet preparation (whole blood, PRP, or washed platelets) and type of agonist employed (thrombin, collagen, ADP, epinephrine, etc.). For example, the potency of the D-Asp linear analogue **12** is over two times lower than the parent L-Asp analogue **2** in dog PRP stimulated with ADP, whereas the potency of **12** and **2** are essentially the same in dog washed platelets stimulated with thrombin, Table I. In our washed-platelet assay the Sar analogue **11** was three times less active than the parent Gly analogue **2**, whereas H-Arg-Sar-Asp-Val-OH was recently shown to be equipotent with H-Arg-Gly-Asp-Val-OH in whole blood stimulated with thrombin.[41] In our dog PRP/ADP assay the Sar analogue **38** is 18 times less active than the parent cyclic disulfide **29**. Inhibitory efficacy of RGDS, furthermore, varies between human, rabbit, and rat making comparisons of analogue efficacies across species difficult.[42] It is entirely possible that groups that employ different assay conditions will develop analogues with different efficacies in each other's assays. At least with regard to SK&F 106760, potency is relatively constant under a variety of different assay conditions: IC_{50} (μM) in dog PRP stimulated with ADP, 0.355 ± 0.04, collagen, 0.26 ± 0.02; U-4619 0.49 ± 0.09; in human PRP stimulated with ADP 0.23 ± 0.06; and in dog whole blood stimulated with ADP

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0.30 ± 0.06. The question of which assay should be employed to give an analogue with the greatest antithrombotic efficacy under human pathologic conditions is a matter of speculation. It would be interesting to evaluate the in vivo antithrombotic efficacies of potent analogues developed in different labs under different assay conditions.

Regional Approach to Fibrinogen Receptor Antagonists Versus the Local Approach. Ostensibly, two different approaches could be taken in the development of fibrinogen receptor antagonists, a "regional approach" in which the potency of -RGDS- would be enhanced by appendage of ancillary binding groups or a "local" approach in which simply the interaction between -RGDS- and its receptor would be enhanced. An example of the regional approach is the generation of monoclonal antibodies to GPIIb/IIIa, which presumably inhibit binding through a number of interactions with the receptor.[3,43]

Another example of the regional approach includes the discovery of the family of snake venom peptides, including trigramin,[44,45] echistatin,[46,47] and bitistatin,[48] all of which contain the -RGD- sequence. It is believed that the cyclic disulfide structures of the snake venom peptides bring other binding elements into the proximity of the -RGD- sequence.[46] In support of this hypothesis is the fact that the affinities of trigramin and echistatin in washed platelets are greater than SK&F 106760 (Table III). Rank ordering by potency, SK&F 106760 < trigramin < echistatin, parallels rank ordering by affinity, suggesting that the potency of the macromolecular snake venom inhibitors is due to high affinity receptor interactions and not other factors.

The local approach toward enhancement of potency has been successful in this study. The enhancements in potency have been rationalized mostly in terms of optimizing conformation. It is also possible, however, that the number of interactions between SK&F 106760 and GPIIb/IIIa could be greater than those between Ac-RGDS-NH_2 and GPIIb/IIIa. In this case the local approach may have resulted in the development of receptor interactions for SK&F 106760 that are not available to the native -RGDS- peptide sequence.

The fact that SK&F 106760 displays an affinity for GPIIb/IIIa that is comparable to fibrinogen is fascinating in light of the vast difference in molecular weights (634 vs 450 000). It is remarkable that such a small inhibitor can so effectively block the interaction of such a large molecule with its receptor. Although many small potent inhibitors have been developed toward protein/protease interactions, the development of SK&F 106760 is one of the few examples[49] of small potent antagonists toward protein/protein interactions.

In other papers it will be shown that SK&F 106760 is a potent, novel, and versatile antithrombotic agent in vivo.

Experimental Section

In Vitro Inhibition of ^{125}I-Fibrinogen Binding to Stimulated Platelets. The procedure of Bennett et al.[50] was followed.

In Vitro Inhibition of ^{125}I-Fibrinogen Binding to Purified, Reconstituted, Human Platelet Fibrinogen Receptor. (a) Purification of GPIIb/IIIa (Modified from Knudsen et al.).[51] Ten units of outdated human platelets were diluted 1:1 in 20 mM Tris-HCl pH 7.3, 140 mM NaCl, 1 mM EDTA and centrifuged at 2000*g* for 15 min. The pelleted platelets were resuspended and washed 3× in the same buffer to remove residual erythrocytes. The packed platelets were resuspended in 5-fold excess extraction buffer (20 mM Tris-HCl pH 7.4, 140 mM NaCl, 2 mM $CaCl_2$, 1 mM PMSF, 3% octylglucoside) and gently stirred for 2 h at 4 °C. This solution was then centrifuged at 100 000*g* for 1 h. The supernatant was applied to a 5-mL column of lentil lectin Sepharose 4B (E. Y. Labs) preequilibrated with 20 mM Tris-HCl pH 7.4, 100 mM NaCl, 2 mM $CaCl_2$ and 1% octylglucoside. The column was washed with 50 mL of cold equilibration buffer and eluted with 10% dextrose in equilibration buffer.

(b) Incorporation of GPIIb/IIIa in Phospholipid Vesicles (Modified from Parise and Phillips).[52] A mixture of 70% phosphatidylserine and 30% phosphatidylcholine (Avanti) was dried to the walls of a glass tube under a stream of nitrogen. Peak protein fractions from the lentil lectin column were pooled and diluted to a final concentration of 0.5 mg protein/mL and a protein/phospholipid concentration of 1:3, w/w. The phospholipids were resuspended and sonicated in a batch sonicator for 5 min. This mixture was dialyzed overnight using 12 000–14 000 molecular weight cutoff dialysis tubing against 2 × 1000-fold excess of 50 mM Tris-HCl, 100 mM NaCl, 2 mM $CaCl_2$. The phospholipid vesicles were centrifuged at 12 000*g* for 15 min and resuspended in the dialysis buffer at a final protein concentration of 1 mg/mL. The vesicles were stored at −70 °C until needed.

(c) Iodination of Human Fibrinogen. Human fibrinogen (2.5 mg) (Calbiochem) in 50 mM Tris-HCl pH 7.4, 100 mM NaCl were incubated at room temperature with 200 μg of Iodogen (Pierce) and 2 mCi of $Na^{125}I$. After 10 min the mixture was desalted over a NAP-10 column (Pharmacia) into 10 mM citrate pH 7.4 and 100 mM NaCl. Peak fraction were pooled and stored at −70 °C until needed.

(d) Fibrinogen Binding Assay (Modified from Parise and Phillips).[52] Peptides were solubilized in 1 M acetic acid at a final concentration of 5 mM. Further dilutions were made in 50 mM Tris-HCl pH 7.3, 100 mM NaCl, 3 mM $CaCl_2$ and 0.5% BSA. GPIIb/IIIa containing vesicles (10 μg protein) were incubated with ^{125}I-fibrinogen (36 nM) and various concentrations of the peptides in a final volume of 200 μL for 1 h at 23 °C. Nonspecific binding was determined by using a 40-fold excess of unlabeled fibrinogen. The ^{125}I-fibrinogen bound to the vesicles was separated from the unbound ^{125}I-fibrinogen by vacuum filtration over 0.2-μm polycarbonate filters (Nucleopore) presoaked in 0.5% BSA for

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1 h. The filters were washed twice with 2 mL of ice-cold buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 3 mM $CaCl_2$). The filters were dried and counted in a Packard γ-counter. Radioligand binding data was analyzed by a nonlinear least-squares curve-fitting program.

In Vitro Inhibition of Aggregation of Washed Platelets. The procedure of Gartner et al.[9] was followed.

In Vitro Inhibition of Aggregation of Canine Platelet-Rich Plasma. Arterial and venous blood was collected from pentobarbitol-anesthetized and conscious dogs, respectively, and anticoagulated by the addition of 0.1 vol of 3.8% sodium citrate. Platelet-rich plasma (PRP) was prepared by low speed centrifugation (180 g) for 10 min at room temperature. The PRP was removed and the remaining blood was centrifuged at 1000g for 5 min to obtain platelet-poor plasma (PPP). The PRP was diluted with PPP to give a final platelet count of 300 000/μL; 300 μL of PRP was added to a glass cuvette and stirred at 1200 rpm at 37 °C. Vehicle (0.9% NaCl) or various concentrations of the peptides was added in a volume of 5 μL and incubated for 3 min before platelet aggregation was induced by ADP (10 μM). Platelet aggregation was determined by the change in light transmission through the PRP on a Chono-Log Model 400VS Lumi-Aggregometer. The ability of the peptides to inhibit platelet aggregation was measured and the IC_{50} was determined as the concentration of peptide required to produce 50% inhibition of the response to ADP in the presence of the vehicle.

Peptide Synthesis. (a) Solid-Phase Synthesis. Protected peptide–resin intermediates were synthesized by the solid-phase method on 4-methylbenzhydrylamine resin [synthesis of analogue **41** was initiated through addition of Boc-Pen(pMeBzl) cesium salt to chloromethyl resin], using the automated Beckman synthesizer on either 0.5 or 1.0 mmol scale. N-terminal *tert*-butyloxycarbonyl protection was employed for all amino acids employed as the following side-chain-protected derivatives: Arg(Tos), D-Arg(Tos), (NMe)Arg(Tos) (procedure for preparation, below), Et_2^{guan}Arg (without side-chain protection),[53] Asp(β-cHex), D-Asp(β-Bzl), Asp(α-Bzl), Cys(pMeBzl), D-Cys(pMeBzl), Glu(Bzl), D-Glu(Bzl), His(Tos), Lys(Cbz), Pen(pMeBzl), D-Pen(pMeBzl), Ser(O-Bzl), D-Ser(OBzl), and Tyr(2-Cl-Cbz). 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). After coupling of the last amino acid, the peptides were acetylated by using a mixture of acetic anhydride and diisopropylethylamine. The peptides were cleaved from resin with deprotection of side-chain protecting groups using anhydrous HF (30 mL for 1.0 mmol scale) in the presence of anisole (3.0 mL for 1.0 mmol scale) at 0 °C for 60 min. Cyclic disulfide peptides were prepared from linear precursors either containing free N-terminal and C-terminal thiols in which case disulfide ring closure was effected with $K_3Fe(CN)_6$ or containing an N-terminal or C-terminal (Cys(SEt) in which case cyclization occurred by the disulfide exchange reaction after removal of the (pMe)Bzl group from the protected Cys or Pen.

(b) Workup of HF Treatment of Linear Peptides. The residues from HF cleavage were washed with anhydrous ether (50 mL for 1.0 mmol scale), extracted with glacial acetic acid (50 mL for 1.0 mmol scale), and lyophilized to a dry powder.

(c) Workup of HF Treatment of Cyclic Disulfide Peptides. The residues from HF cleavage were washed with anhydrous ether (50 mL for 1.0 mmol scale), and crude peptides were extracted with 50% acetic acid (20 mL for 1.0 mmol scale) and diluted to 2 L with deionized water. The pH of aqueous solutions were adjusted to pH 7.5 with concentrated ammonium hydroxide, and either nitrogen or argon was bubbled through the solution to sweep out liberated ethyl mercaptan into a bubbler containing Ellman's reagent. The cyclization process takes place within 24–48 h as determined by HPLC. Solutions were then passed through a reversed-phase C-18 silica column previously equilibrated with water. Crude peptides were then eluted with 15% of acetonitrile in 0.1% aqueous TFA solution. As an example, 530 mg (78% crude yield) of N^{α}-Ac-cyclo-*S,S*-[Cys-Arg-Gly-Asp-Ser-Cys]-NH_2 was obtained by this method.

Peptide Purification. Peptides were purified by one or more of the following methods: Sephadex-G-15 gel filtration using 0.2 M acetic acid; flash medium pressure reversed-phase C-18 silica using an appropriate mixture of acetonitrile in 0.1% aqueous TFA solution as eluent; counter current distribution (CCD) using B:A:W, 4:1:5 (200 transfers).

Peptide Analysis. Peptide structures were verified through FABMS (VG Zab mass spectrometer using fast atom bombardment) and amino acid analysis (72 h, acid hydrolysate, 6 N HCl, 100 °C, performed on a Dionex Autoion 100 analyzer). Peptide purity was determined by analytical TLC (Brinkman 0.25 mm silica gel on 8 × 20 mm TLC plates, visualizing spots with ninhydrin or iodine vapor) in at least one solvent mixture, Table IV, and by analytical HPLC (Beckman dual pump HPLC system employing Beckman 4.6 × 250 mm analytical columns packed with 5-μm spherical 80 Ang. ODS-silica beads at a flow rate of 1.5 mL/min, analyzing by UV at 220 nm) under the conditions listed in Table IV. Analytical data for all purified peptides are contained in Table IV.

Preparation of Ac-hArg-Gly-Asp-Ser-NH_2 (8). Guanidination of the Lys analogue **5** (100 mg, 0.197 mmol) proceeded via the preparation of the hArg analogue **32**, below, with recovery of product from the reaction mixture by Sephadex G-10 gel filtration, eluting with 0.1 M AcOH. Lyophilization of fractions containing product gave a white powder (48 mg, 44%).

Preparation of N^{α}-Ac-Arg-Gly-Asp-NHCH$_2$CH$_2$C$_6$H$_5$ (23). (a) Preparation of Boc-Asp(Bzl)-NH(CH$_2$)$_2$C$_6$H$_5$ (47). To a solution of Boc-Asp(Bzl) (5.0 g, 15.5 mmol) and *N*-methylmorpholine (15.5 mmol) in dry THF (50 mL) was added isobutyl chloroformate (15.5 mmol) at −15 °C under argon. After a few minutes of stirring, a solution of phenethylamine (2.0 mL, 15.5 mmol) in dry THF (15 mL) was added. After 15 min, the mixture was allowed to warm to room temperature. After 2 h the mixture was filtered and rotary evaporated to an oil. An ethyl acetate solution of the oil (100 mL) was washed successively with 1 M HCl (2 × 50 mL), water (50 mL), 10% Na_2CO_3 (2 × 50 mL), and brine (50 mL), dried over Na_2SO_4, and rotary evaporated to given an amorphous white solid (5.59 g, 85%): >95% pure by TLC ($CHCl_3$/MeOH/AcOH, 95:4:1) R_f 0.51; NMR ($CDCl_3$) δ 7.38 (s, 5 H), 7.28 (m, 5 H), 6.53 (m, 1 H), 5.60 (d, 1 H), 5.13 (s, 2 H), 4.48 (m, 1 H), 3.48 (dd, 2 H), 2.75 (m, 4 H), 1.41 (s, 9 H).

(b) Preparation of Boc-Gly-Asp(*O*-Bzl)-NH(CH$_2$)$_2$C$_6$H$_5$ (48). A solution of **47** (4.18 g, 12.8 mmol) in anhydrous TFA (10 mL/g) was allowed to stir for 30 min and rotary evaporated to an oil. A solution of the oil in try THF was employed in a mixed anhydride coupling with Boc-Gly (2.24 g, 12.8 mmol) following the procedure for **47** appropriately scaled. Following acid/base workup crude product was purified on a flash silica column, eluting with 3:1 EtOAc/hexane. Fractions containing pure product were rotary evaporated to give a light yellow glassy material (3.35 g, 54%): >95% pure by TLC (EtOAc) R_f 0.47; NMR ($CDCl_3$) δ 7.41 (s, 5 H), 7.28 (m, 5 H), 6.91 (m, 1 H), 5.18 (m, 3 H), 4.84 (m, 1 H), 3.73 (d, 2 H), 3.52 (dd, 2 H), 2.80 (m, 4 H), 1.50 (s, 9 H).

(c) Preparation of Boc-Arg(Tos)-Gly-Asp(*O*-Bzl)-NH(CH$_2$)$_2$C$_6$H$_5$ (49). As in **b**, a portion of **48** (2.61 g, 6.8 mmol) was treated with TFA, and the resulting TFA salt was employed in a mixed anhydride coupling with Boc-Arg(Tos) (3.42 g, 6.8 mmol) following the procedure for **48** appropriately scaled. Following acid/base workup, crude product was purified on a flash silica column, eluting with 50% EtOAc/iPrOH. Fractions containing pure product were rotary evaporated to give a white glassy material (3.41 g, 63%): >95% pure by TLC ($CHCl_3$/MeOH/AcOH, 90:9:1) R_f 0.41; NMR ($CDCl_3$) δ 7.81 (m, 2 H), 7.38 (dd, 4 H), 7.35 (s, 5 H), 7.25 (m, 5 H), 6.56 (m, 2 H), 5.78 (d, 1 H), 5.10 (s, 2 H), 4.82 (m, 1 H), 4.28–3.79 (m, 4 H), 3.30 (m, 4 H), 2.73 (m, 4 H), 2.32 (s, 3 H), 1.8–1.0 (m, 14 H).

(d) Preparation of Ac-Arg(Tos)-Gly-Asp(*O*-Bzl)-NH(CH$_2$)$_2$C$_6$H$_5$ (50). As in **b**, a portion of **49** (4.2 mmol) was treated with TFA to give an oil. A solution of the oil in DMF (30 mL) and acetic anhydride (2.0 mL, 21.2 mmol) was added EtN(iPr)$_2$ (0.73 mL, 4.2 mmol). After 30 min the mixture was rotary evaporated to an oil. The crude product was purified on a flash silica column, eluting with 50% EtOAc/iPrOH. Fractions containing pure product were rotary evaporated to give a white glass

(53) Nestor, J. L.; Tahilramani, R.; Ho, T. L.; McRae, G. I.; Vikery, B. H. Potent, Long-Acting Luteinizing Hormone-Releasing Hormone Antagonists Containing New Synthetic Amino Acids: *N,N*-Dialkyl-D-homoarginines. *J. Med. Chem.* 1988, *31*, 65–72.

Table IV. Peptide Analytical Data

TLC		HPLC			FABMS	amino acid analysis
sys[a]	R_f	CH_3CN	K'	% purity[c]	$(M + H)^+$	molar ratios
				Ac-Fg-γ 400–411, Ac-HHLGGAKQAGDV-OH (1)		
1	0.22	12i[b]	1.2	97	1231.7	His (1.34), Leu (0.95), Gly (3.03), Ala (2.00), Lys (0.87), Glu (1.10), Asp (1.11), Val (0.94)
				N^{α}-Ac-N^{α}-Arg-Gly-Asp-Ser-NH_2 (2)		
1	0.2	0–50g	5.4	>97	475.0	Asp (0.99), Ser (0.24), Gly (1.03), Arg (1.00)
2	0.49					
				N^{α}-Ac-Arg-Arg-Gly-Asp-Phe-NH_2 (3)		
1	0.4	10i	2.78	98	691.0	Asp (1.00), Gly (1.00), Phe (0.91), Arg (1.89)
2	0.4					
				Ac-D-Arg-Gly-Asp-Ser-NH_2 (4)		
1	0.2			95[d]	475	Asp (1.04), Gly (0.89), Ser (0.83), Arg (1.00)
				Ac-Lys-Gly-Asp-Ser-NH_2 (5)		
1	0.32	2i	1.0	95	447.2	Lys (0.95), Gly (0.95), Asp (1.00), Ser (0.98)
3	0.03					
				Ac-Cit-Gly-Asp-Ser-NH_2 (6)		
1	0.42	2i	1.7	95	476.1	Asp (1.00), Ser (1.00), Gly (0.98), Cit (0.60)
3	0.05					
				Ac-His-Gly-Asp-Ser-NH_2 (7)		
1	1.4	1i	1.4	93	456.2	His (1.04), Gly (0.90), Asp (1.00), Ser (0.98)
3	0.44					
				Ac-hArg-Gly-Asp-Ser-NH_2 (8)		
1	0.33	0–50g	2.1	95	489.2	Asp (1.00), Ser (0.97), Gly (0.99), hArg (0.88)
3	0.04					
				Ac-Arg-Pro-Asp-Ser-NH_2 (10)		
1	0.4			95[d]	515.3	Asp (1.01), Ser (0.86), Pro (0.99), Arg (1.00)
2	0.41					
				Ac-Arg-Sar-Asp-Ser-NH_2 (11)		
1	0.44			95[d]	489.2	Asp (1.00), Ser (0.95), Arg (1.00)
2	0.44					
				Ac-Arg-Gly-D-Asp-Ser-NH_2 (12)		
1	0.29	0–50g	5.2	94	475.4	Asp (1.00), Ser (1.00), Gly (0.98), Arg (1.07)
				Ac-Arg-Gly-Asn-Ser-NH_2 (13)		
1	0.19			95[d]	474.5	Asp (1.00), Ser (0.79), Gly (1.19), Arg (0.98)
2	0.31					
				Ac-Arg-Gly-Glu-Ser-NH_2 (14)		
1	0.27	0–50g	6.7	97	489.3	Arg (1.01), Gly (1.04), Glu (1.00), Ser (1.09)
				Ac-Arg-Gly-D-Glu-Ser-NH_2 (15)		
1	0.41	0–50g	4.6	>97	488.9	
				Ac-Arg-Gly-β-Asp-Ser-NH_2 (16)		
1	0.9	0–50g	0.6	>97	475.2	Asp (1.00), Ser (0.99), Gly (0.98), Arg (1.01)
3	0.03					
				Ac-Lys-Gly-Glu-Ser-NH_2 (17)		
1	0.27	1i	1.7	95	461.1	Ser (1.00), Glu (1.00), Gly (0.93), Lys (0.84)
3	0.04					
				N^{α}-Ac-Arg-Gly-Asp-D-Ser-NH_2 (18)		
1	0.3	0–50g	3.4	94	475.3	Asp (1.00), Ser (0.99), Gly (0.96), Arg (1.01)
				N^{α}-Ac-Arg-Gly-Asp-Val-NH_2 (19)		
1	0.43	5i	1.3	>98	487.3	Asp (1.00), Gly (0.99), Val (1.06), Arg (0.96)
3	0.11					
				N^{α}-Ac-Arg-Gly-Asp-Tyr-NH_2 (20)		
1	0.41			95[d]	551.0	Asp (1.00), Gly (1.02), Tyr (1.00), Arg (0.90)
2	0.4					
				N^{α}-Ac-Arg-Gly-Asp-(2)Nal-NH_2 (21)		
1	0.60	18	1.2	95	585.3	Asp (1.00), Gly (0.99), Arg and Nal present but coelute
3	0.23					
				N^{α}-Ac-Arg-Gly-Asp-_-NH_2 (22)		
1	0.2			95[d]	388.0	Asp (1.00), Gly (1.13), Arg (0.93)
2	0.4					
				N^{α}-Ac-Arg-Gly-Asp-$NHCH_2CH_2Ph$ (23)		
1	0.58	12i	2.3	97	492.1	Asp (0.51), Gly (0.99), Arg (1.00)
3	0.17					

Table IV (Continued)

TLC		HPLC			FABMS	amino acid analysis
sys[a]	R_f	CH_3CN	K'	% purity[c]	$(M + H)^+$	molar ratios
				Ac-Arg-Gly-Asp-Ser-NHEt (24)		
1	0.38	0–50g	3.5	95	503.0	Asp (1.08), Gly (1.00), Ser (1.02), Arg (1.07)
3	0.08					
				N^{α}-Ac-cyclo-*S,S*-[Cys-Arg-Gly-Asp-Ser-Cys]-NH_2 (25)		
1	0.32	0–50g	6.4	>98	678.5	Asp (1.00), Ser (0.98), Gly (0.97), Arg (0.90), Cys/Pen (+)[e]
3	0.06					
				N^{α}-Ac-cyclo-*S,S*-[Cys-Gly-Arg-Gly-Asp-Cys]-NH_2 (26)		
1	0.36	5i	1.9	95	649.1	Asp (1.00), Gly (1.90), Cys/Pen (+), Arg (1.01)
3	0.14					
				N^{α}-Ac-cyclo-*S,S*-[Cys-Gly-Arg-Gly-Asp-Pen]-NH_2 (27)		
1	0.45	7i	2.6	92	677.1	Asp (1.00), Gly (1.96), Cys/Pen (+), Arg (1.01)
3	0.13					
				N^{α}-Ac-cyclo-*S,S*-[Cys-Arg-Gly-Asp-_-Cys]-NH_2 (28)		
1	0.52	3i	2.7	94	592.2	Asp(1.00), Gly (1.00), Arg (0.90), Cys (+)
3	0.57					
				N^{α}-Ac-cyclo-*S,S*-[Cys-Arg-Gly-Asp-Pen]-NH_2 (29)		
1	0.37	6i	2.2	95	620.2	Asp (1.00), Gly (1.01), Arg (0.67), Cys/Pen (+)
3	0.097					
				N^{α}-Ac-cyclo-*S,S*-[Pen-Arg-Gly-Asp-Cys]-NH_2 (30)		
1	0.61	3i	8.4	95	620.2	Asp (1.00), Gly (1.07), Arg (0.85), Cys (+)
2	0.69					
				N^{α}-Ac-cyclo-*S,S*-[Cys-D-Arg-Gly-Asp-Pen]-NH_2 (31)		
1	0.31	6i	5.2	95	620.4	Asp (1.01), Gly (1.03), Arg (1.00), Cys/Pen (+)
3	0.54					
				N^{α}-Ac-cyclo-*S,S*-[Cys-hArg-Gly-Asp-Pen]-NH_2 (32)		
1	0.44	4i	2.0	98	634.2	Asp (1.00), Gly (1.16), Cys/Pen (+)
3	0.19					
				N^{α}-Ac-cyclo-*S,S*-[Cys-Lys-Gly-Asp-Pen]-NH_2 (33)		
1	0.38	3i	3.5	97	592.3	Asp (1.00), Gly (1.13), Lys(1.09), Cys/Pen (+)
				N^{α}-Ac-cyclo-*S,S*-[Cys-(Et^{guan}_2)Arg-Gly-Asp-Pen]-NH_2 (34)		
1	0.45	9i	3.2	95	676.4	Asp (1.00), Gly (1.12), Cys/Pen (+)
2	0.64					
				N^{α}-Ac-cyclo-*S,S*-[Cys-(NMe)Arg-Gly-Asp-Pen]-NH_2 (35)		
1	0.62	5i	2.39	95	634.7	Asp (1.00), Gly (0.98), Cys/Pen (+)
2	0.39					
				N^{α}-Ac-cyclo-*S,S*-[Pen-(NMe)Arg-Gly-Asp-Pen]-NH_2 (36)		
1	0.60	10i	4.4	95	662.3	Asp (1.00), Gly (1.03), Cys/Pen (+)
3	0.56					
				N^{α}-Ac-cyclo-*S,S*-[Cys-Arg-Ala-Asp-Pen-NH_2] (37)		
1	0.53	4i	4.3	95	634.3	Asp (1.00), Ala (0.97), Arg (1.09), Cys/Pen (+)
2	0.58					
				N^{α}-Ac-cyclo-*S,S*-[Cys-Arg-Sar-Asp-Pen]-NH_2 (38)		
1	0.57	7i	3.07	95	634.2	Asp (1.00), Arg (1.03), Cys/Pen (+)
2	0.61					
				N^{α}-Ac-cyclo-*S,S*-[Cys-Arg-Gly-D-Asp-Pen]-NH_2 (39)		
1	0.40	8i	1.9	95	620.2	Asp (1.00), Gly (0.96), Arg (0.96), Cys/Pen (+)
2	0.29					
				N^{α}-Ac-cyclo-*S,S*-[Cys-(NMe)Arg-Gly-Asn-Pen]-NH_2 (40)		
1	0.54	7i	3.9	95	633.4	Asx (1.00), Gly (1.03), Cys/Pen (+)
2	0.56					
				N^{α}-Ac-cyclo-*S,S*-[KCAGD(Pen)]-OH (41)		
1	0.32	4i	6.11	95	664.7	Asp (1.05), Gly (1.02), Ala (1.00), Cys/Pen (+), Lys (0.89)
2	0.43					
				N^{α}-Ac-cyclo-*S,S*-[HHLGGAKCRGD(Pen)]-NH_2 (42)		
1	0.03	8i	3.5	95	1320.5	His (1.74), Leu (0.98); Gly (2.78), Ala (0.68), Lys (1.02), Cys/Pen (+), Arg (1.00), Asp (0.59)

[a] TLC systems: 1 = nBuOH:AcOH:H_2O:EtOAc, 1:1:1:1, 2 = nBuOH:AcOH:H_2O:pyridine, 15:5:10:10; 3 = nBuOH:H_2O:iPrOH:$CHCl_3$, 6.5:2:1.5:0.3. [b] Analytical HPLC run either in an isocratic mode (i) or gradient mode (g) with the percentage CH_3CN employed in mixture with 0.1% TFA. [c] Purity determined by HPLC determination. [d] Purity estimated by TLC determination. [e] Qualitative determination of the presence of Cys and Pen indicated by "+" due to difficult quantitation.

(2.94 g, 95%): >95% pure by TLC ($CHCl_3$/AcOH/MeOH, 90:9:1) R_f 0.23. NMR (CD_3OD) indicated the absence of Boc and the presence of an acetyl group (δ 1.95), proportional to the tosyl methyl group (δ 2.32). The material was used without further

characterization.

(e) Preparation of 23. Anhydrous HF (25 mL) was distilled under vacuum into a mixture of **50** (2.5 g, 3.4 mmol) and anisole (2.5 mL) chilled on a dry ice/acetone bath. After being stirred at 0 °C for 30 min, the mixture was evaporated to an oil. A solution of the oil in 1 M acetic acid (150 mL) was washed with anhydrous Et_2O (3 × 100 mL) and lyophilized to an amorphous solid (1.52 g). The crude product was purified on reversed-phase flash C-18 silica, eluting with 40% MeOH in 0.1% TFA. Fractions containing pure product were reduced in volume by rotary evaporation and lyophilized to give a white powder (326 mg, 97% purity).

Preparation of Ac-Arg-Gly-Asp-Ser-NHEt (24). (a) Preparation of Boc-Ser(Bzl)-NHEt (51). To a solution of Boc-Ser(Bzl) (6.0 g, 20.3 mmol) and *N*-methylmorpholine (2.3 mL, 20.9 mmol) in dry THF (50 mL) was added isobutyl chloroformate (2.7 mL, 20.8 mmol) at −15 °C under argon. After a few minutes of stirring, $EtNH_2$ was bubbled through the mixture for several minutes. After 30 min, the mixture was allowed to warm to room temperature, filtered, and rotary evaporated to an oil. An ethyl acetate solution of the oil (100 mL) was washed successively with 1 M HCl (2 × 50 mL), water (50 mL), 10% Na_2CO_3 (2 × 50 mL), and brine (50 mL), dried over Na_2SO_4, and rotary evaporated to give an amorphous white solid (6.0 g, 92%); >95% pure by TLC ($CHCl_3$/MeOH/AcOH, 90:9:1) R_f 0.57; NMR ($CDCl_3$) δ 7.33 (s, 5 H), 6.54 (d, 1 H), 5.5 (d, 1 H), 4.55 (s, 2 H), 4.29 (m, 1 H), 3.82 + 3.63 (m, 2 H), 3.31 (m, 2 H), 1.43 (s, 9 H), 1.11 (t, 3 H).

(b) Preparation of Boc-Asp(Bzl)-Ser(Bzl)-NHEt (52). A solution of **51** (6.0 g, 18.6 mmol) in anhydrous TFA (10 mL/g) was allowed to stir for 30 min and rotary evaporated to an oil. A solution of the oil in dry THF was employed in a mixed anhydride coupling with Boc-Asp(Bzl) (5.82 g, 18 mmol), following the procedure for **51** appropriately scaled. Acid/base workup gave a glassy material (8.81 g, 93%): >95% pure by TLC ($CHCl_3$/MeOH/AcOH, 90:9:1) R_f 0.50; NMR ($CDCl_3$) δ 7.34 (s, 10 H), 7.24 (d, 1 H), 6.78 (dd, 1 H), 5.62 (d, 1 H), 5.14 (s, 2 H), 4.54 (m, 4 H), 4.02 + 3.58 (m, 2 H), 3.31 (m, 2 H), 2.93 (d, 2 H), 1.48 (s, 9 H), 1.11 (t, 3 H).

(c) Preparation of Boc-Asp-Ser(Bzl)-NHEt (53). To a solution of **52** (4.0 g, 7.6 mmol) in EtOAc (100 mL) and MeOH (25 mL) was added 5% Pd/$BaSO_4$ (2.0 g). The mixture was shaken on a Parr shaker under 45 psi hydrogen for 30 min. The mixture was degassed, filtered, and rotary evaporated to a white glass (3.18 g, 96%): >90% pure by TLC ($CHCl_3$/MeOH/AcOH, 90:9:1) R_f 0.06; NMR ($CDCl_3$) δ 10.32 (s, 1 H), 7.55 (d, 1 H), 7.40 (s, 5 H), 7.17 (s, 1 H), 6.08 (d, 1 H), 4.60 (m, 4 H), 4.02 + 3.63 (m, 2 H), 3.48 (m, 2 H), 2.94 (d, 2 H), 1.48 (s, 9 H), 1.08 (t, 3 H).

(d) Preparation of Ac-Arg-Gly-Asp-Ser-NHEt (24). The dipeptide **53** (131 g, 3 mmol) was coupled to hydroxymethyl resin (1% cross-linked, 1 g, 1 mmol) via DCC (620 mg, 3 mmol) and 4-pyrolidinopyridine (0.15 g, 1 mmol) swelled in excess CH_2Cl_2. Ac-Arg(Tos)-Gly-Asp(hydroxymethyl resin)-Ser(Bzl)-NHEt was prepared by stepwise couplings of the protected amino acids, as described above. HF treatment and workup proceeded as above with purification by flash chromatography on reversed-phase C18 silica, eluting with 5% CH_3CN/0.1% TFA, concentrating fractions containing product by rotary evaporation, and lyophilization to a white powder (173 mg in >95% purity).

Preparation of Ac-cyclo-*S*,*S*-[Cys-hArg-Gly-Asp-Pen]-NH_2 (32). The Lys analogue **33** (100 mg, 0.17 mmol) was dissolved in a solution of *O*-methylisourea hydrogen sulfate (290 mg, 1.7 mmol) in 2.5 M NaOH, pH 11. After 12 h the mixture was passed through an Amberlite XAD-2 column and eluted with 50% CH_3CN/0.1% TFA. Fractions containing crude product were concentrated by rotary evaporation and lyophilized to a white powder. The crude product was purified on reversed-phase flash C-18 silica, eluting with 10% CH_3CN in 0.1% TFA. Fractions containing pure product were reduced in volume by rotary evaporation and lyophilized to give a white powder (40 mg, 37%).

Preparation of Boc-(*N*-Me)Arg(Tos)-OH (57).[54] (a) Preparation of H-(*N*-Bzl)Arg(Tos)-OH (54). To a solution of H-Arg(Tos)-OH (14.75 g, 44.9 mmol) in MeOH (65 mL) were added $NaBH_3CN$ (1.98 g, 31.4 mmol) followed by benzaldehyde (5.25 g, 49.5 mmol). The mixture was stirred at room temperature under argon for 18 h. A white precipitate was filtered from solution and dried in vacuo to a white powder (9.55 g, 50.89%, mp 160–167 °C) which was carried on without further purification.

(b) Preparation of H-(*N*-Bzl,*N*-Me)Arg(Tos)-OH (55). A mixture of **54** (8.35 g, 2.0 mmol) in formic acid (95–97%, 2.3 mL, 60 mmol) and formaldehyde (37–40%, 1.95 mL, 24 mmol) was heated on a steam bath for 10 min. The mixture was allowed to cool and was rotary evaporated to a gum, which was carried on without further purification: FABMS M + H^+ 433; ^1H NMR ($CDCl_3$/d_6-DMSO) δ 7.9 (d, 2 H), 7.5 (d, 2 H), 7.1 (s, 5 H), 4.8 (m, 1 H), 4.1 (s, 2 H), 3.3 (m, 2 H), 2.6 (s, 3 H), 2.4 (s, 3 H), 1.9 (m, 4 H).

(c) Preparation of H-(*N*-Me)Arg(Tos)-OH (56). A mixture of 5% Pd/C (ca. 150 mg) and a solution of the gum **55** (2 mmol) in glacial AcOH (50 mL), water (10 mL), and 3 N HCl (5 mL) was shaken under a hydrogen atmosphere (40 psi) in a Parr apparatus for 6 h. The solution was degassed, filtered, and rotary evaporated to an oil. An aqueous solution (20 mL) of the oil was neutralized to pH 7 with concentrated NH_4OH. A precipitate was filtered from solution and recrystallized twice from hot H_2O and dried in vacuo to a white powder (2.5 g; 37%; mp 210–216 °C dec: FABMS M + H^+ 343; ^1H NMR (D_2O, DCl) δ 7.9 (d, 2 H0, 7.5 (d, 2 H), 4.0 (t, 1 H), 3.35 (t, 2 H), 2.8 (s, 3 H), 2.4 (s, 3 H), 1.75 (m, 4 H); $[\alpha]^{25}_D$ (0.1 MeOH) +5.0. Anal. ($C_{14}H_{22}N_4O_4S \cdot H_2O$) C, H, N.

(d) Preparation of Boc-(*N*-Me)Arg(Tos)-OH (57). To a solution of **56** (2.5 g, 7.3 mmol) in aqueous NaOH (0.3 g in 1 mL) and t-BuOH (1.5 mL) was added di-*tert*-butyl dicarbonate 1.75 g, 8.03 mmol) slowly with stirring. After the solution temperature rose from 23 to 28 °C, more t-BuOH (1.15 mL) was added, and the reaction mixture was allowed to stir overnight. Following addition of water (10 mL), the mixture was washed with hexane (3 × 10 mL). The aqueous solution was acidified with $KHSO_4$ (1.0 g) to pH 2.2 with stirring on an ice bath. A white solid was filtered and recrystallized from EtOAc/hexane to give a white powder (1.37 g; 43%; mp 80–84 °C dec: FABMS M + H^+ 443; ^1H NMR ($CDCl_3$/d_6-DMSO) δ 7.75 (d, 2 H), 7.25 (d, 2 H), 6.6 (m, 2 H), 4.0 (m, 1 H), 3.2 (m, 2 H), 2.75 (s, 3 H), 2.4 (s, 3 H), 1.5 (s, 9 H). Anal. ($C_{19}H_{30}N_4O_6S \cdot 0.5H_2O$) C, H, N.

^1H NMR Spectrum of Nᵅ-Ac-cyclo-*S*,*S*-[Cys-(NMe)Arg-Gly-Asp-Pen]-NH_2 (35). Proton chemical shifts of **40** in a 5:3 mixture of d_6-DMSO/d_8-sulfolane were obtained with a JEOL GX500 spectrometer operating at 500 MHz at 303 K. Coupling constants were obtained from the P.E. COSY.

residue	HN	Hα	Hβ	Hγ	Hδ	HNε	J(HN/Hα)
			(Ac-Me at 1.88 ppm)				
Cys	8.30	4.89	3.05/ 2.96				
(Nᵅ-Me)	2.99	4.99	1.81/	1.42/	3.13	7.57	
Arg	(Nᵅ-Me)		1.64	1.39			
Gly	7.30	4.15/ 3.39					7.3 Hz/ 3.6 Hz
Asp	8.13	4.61	2.71/ 2.54				
Pen	7.17	4.38	1.38				
			(C-terminal amide NH_2: 7.07, 7.50)				

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Registry No. 1, 135284-56-9; 2, 122207-62-9; 3, 126053-53-0; 4, 135284-57-0; 5, 135284-58-1; 6, 135284-59-2; 7, 135284-60-5; 8, 126053-55-2; 9, 135284-61-6; 10, 135284-62-7; 11, 135284-63-8; 12, 135284-64-9; 13, 135284-65-0; 14, 122207-63-0; 15, 135284-66-1; 16, 135284-67-2; 17, 135284-68-3; 18, 126053-50-7; 19, 126053-51-8; 20, 123491-66-7; 21, 126053-52-9; 22, 126053-49-4; 23, 126070-88-0; 24, 126070-89-1; 25, 126053-61-0; 26, 135284-69-4; 27, 135284-70-7; 28, 126053-78-9; 29, 126053-68-7; 30, 126053-76-7; 31, 126053-73-4;

(54) Ali, F. E.; Huffman, W. F.; Marshall, G. R.; Moore, M. L. U.S. Patent 4,687,758, August 18, 1987.

32, 126053-70-1; 33, 126053-69-8; 34, 126053-79-0; 35, 126053-71-2; 36, 126053-75-6; 37, 135284-71-8; 38, 135284-72-9; 39, 135284-73-0; 40, 135284-74-1; 41, 135284-75-2; 42, 135284-76-3; 47, 108279-08-9; 48, 126054-20-4; 49, 126054-21-5; 50, 126054-22-6; 51, 126054-00-0; 52, 126054-01-1; 53, 126054-02-2; 54, 74071-58-2; 55, 74071-59-3; 56, 74071-60-6; 57, 108695-16-5; BOC-Asp(Bzl)-OH, 7536-58-5; $PhCH_2CH_2NH_2$, 64-04-0; BOC-Gly-OH, 4530-20-5; BOC-Arg(Tos)-OH, 13836-37-8; BOC-Ser(Bzl)-OH, 23680-31-1; $EtNH_2$, 75-04-7; $MeOC(NH_2)=NH\cdot H_2SO_4$, 52328-05-9; H-Arg(Tos)-OH, 4353-32-6; PhCHO, 100-52-7.

Conformational Restriction of the Phenylalanine Residue in a Cyclic Opioid Peptide Analogue: Effects on Receptor Selectivity and Stereospecificity†

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In an effort to determine the effect of side chain conformational restriction on opioid receptor selectivity, the cyclic phenylalanine analogues 2-aminoindan-2-carboxylic acid (Aic), 2-aminotetralin-2-carboxylic acid (Atc), and tetrahydroisoquinoline-3-carboxylic acid (Tic) were substituted for Phe in the potent cyclic opioid peptide analogue H-Tyr-D-Orn-Phe-Glu-NH_2, which lacks significant opioid receptor selectivity. Compounds were tested in μ- and δ-opioid receptor representative binding assays and bioassays in vitro. The analogue H-Tyr-D-Orn-Aic-Glu-NH_2 was found to be a potent agonist with high preference of μ receptors over δ receptors. Opening of the five-membered ring of Aic in the latter peptide, as achieved through substitution of C^{α}-methylphenylalanine or o-methylphenylalanine, resulted in only slightly selective compounds, indicating that the high μ selectivity of the Aic analogue is exclusively the consequence of the imposed side chain conformational restriction. Both diastereoisomers of H-Tyr-D-Orn-(D,L)-Atc-Glu-NH_2 were highly μ-selective and, in contrast to the weak affinity observed with the D-Phe^3 analogue as compared to the L-Phe^3 analogue, both had similar potency. Thus, stereospecificity was lost as a consequence of side chain conformational restriction. Further structure–activity data obtained with analogues containing L- or D-homophenylalanine (Hfe) or 3-(1′-naphthyl)alanine (Nap) in place of Phe^3 and consideration of geometric interrelationships between Nap and the L and D isomers of Atc, Hfe, and Phe led to the proposal that the D-Phe^3 and the D-Atc^3 analogue may have different modes of binding to the receptor. The very low potency observed with H-Tyr-D-Orn-N^{α}MePhe-Glu-NH_2 (N^{α}MePhe = N^{α}-methylphenylalanine) and H-Tyr-D-Orn-Tic-Glu-NH_2 indicated that N^{α}-alkylation at the 3-position is detrimental to activity.

Most of the small linear opioid peptides isolated to date are known to be structurally flexible molecules whose conformations are strongly dependent on the environment (for a review, see ref 2). This structural flexibility not only prevents meaningful conformational studies aimed at elucidating the bioactive (receptor-bound) conformation(s) but also may be one of the major reasons for the lack of significant selectivity of most of these peptides toward one or the other of the different opioid receptor types (μ, δ, κ). In efforts to obtain better conformational integrity and to improve receptor selectivity, various conformationally restricted opioid peptide analogues have been developed. In particular, peptide cyclizations via side chains led to cyclic opioid peptide analogues displaying quite high μ-receptor selectivity (e.g. H-Tyr-cyclo[-D-A_2bu-Gly-Phe-Leu-][3] or H-Tyr-D-Orn-Phe-Asp-NH_2[4]) or greatly improved δ-receptor selectivity (e.g. H-Tyr-D-Pen-Gly-Phe-D(or L)-Pen-OH[5] or H-Tyr-D-Cys-Phe-D-Pen-OH).[6] Molecular mechanics studies recently performed with the cyclic analogues H-Tyr-D-Orn-Phe-Asp-NH_2 and H-Tyr-D-Pen-Gly-Phe-D-Pen-OH revealed that the exocyclic Tyr^1 residue and the Phe^3 (Phe^4) side chain in these compounds still enjoy considerable orientational freedom.[7,8] Since the latter moieties are crucial for opioid activity, it is obvious that they also need to be conformationally restricted in order to obtain more definitive insight into the distinct bioactive conformations of these cyclic opioid peptide analogues at the μ and δ receptor.

In the present paper we describe a series of analogues

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(1) Symbols and abbreviations are in accordance with recommendations of the IUPAC–IUB Joint Commission on Biochemical Nomenclature: Nomenclature and Symbolism for Amino Acids and Peptides. *Biochem. J.* 1984, *219*, 345–373. The following other abbreviations were used: A_2bu, α,γ-diaminobutyric acid; Aic, 2-aminoindan-2-carboxylic acid; Atc, 2-aminotetralin-2-carboxylic acid; Boc, tert-butoxycarbonyl; BOP, benzotriazol-1-yl-oxytris(dimethylamino)phosphonium hexafluorophosphate; C^{α}MePhe, C^{α}-methylphenylalanine; DAGO, H-Tyr-D-Ala-Gly-N^{α}MePhe-Gly-ol; DCC, dicyclohexylcarbodiimide; DIEA, diisopropylethylamine; DSLET, H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH; FAB, fast atom bombardment; Fmoc, (fluoren-9-ylmethoxy)carbonyl; GPI, guinea pig ileum; Hfe, homophenylalanine; MVD, mouse vas deferens; Nap, 3-(1′-naphthyl)alanine; N^{α}MePhe, N^{α}-methylphenylalanine; oMePhe, o-methylphenylalanine; Pen, penicillamine; TFA, trifluoroacetic acid; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; U69,593, (5α,7α,8β)-(−)-*N*-methyl-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]benzeneacetamide.

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