Cyclic RGD Peptide Analogues as Antiplatelet Antithrombotics

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Stimulation of platelets activates GPIIbIIIa, the heterodimeric integrin receptor, to bind fibrinogen (Fg), which results in platelet aggregation. GPIIbIIIa/Fg binding inhibitors are potentially suitable for acute use during and after thrombolytic therapy as antithrombotic agents. Incorporation of the tripeptide sequence Arg-Gly-Asp (RGD), a common structural element of many integrin ligands, into cyclic peptides produced a series of peptides of the general structure BrAc-(AA1)-RGD-Cys-OH, which were prepared by solid-phase peptide synthesis. Cyclization was accomplished by reaction of the N-terminal bromoacetyl group with the cysteine sulfhydryl at pH 8 at high dilution, resulting in thioether-bridged cyclic peptides [*cyclo-S*-Ac-(AA1)-RGD-Cys-OH]. Use of α-substituted bromoacetyl groups gave rise to an analogous series of acetyl-substituted thioether-bridged cyclic peptides. Oxidation of the thioethers produced separable diastereomeric sulfoxide-bridged cyclic peptides. After thorough evaluation in a GPIIbIIIa ELISA assay and a platelet aggregation assay, G-4120 (**70A**; AA1 = D-Tyr; sulfoxide bridge) was selected for further investigation as an antithrombotic agent. G-4120 was equipotent in the platelet aggregation assay to kistrin, a highly potent inhibitor of fibrinogen-mediated platelet aggregation isolated from snake venom (IC_{50} = 0.15 μM).

The heterodimeric glycoprotein receptor GPIIbIIIa, present on platelets, has been implicated in critical platelet functions including aggregation and adhesion. Fibrinogen (Fg) binding to GPIIbIIIa is activation dependent and is a final common pathway of aggregation shared by a variety of stimuli, such as ADP, thrombin, or epinephrine.[1] Under certain pathologic conditions, platelet aggregation can result in thrombus formation and ultimately stenosis of the vessel.[2]

Recently, GPIIbIIIa-specific antibodies that inhibit fibronigen binding to the receptor have been shown to be potent inhibitors of platelet aggregation.[3] Furthermore, in vivo studies have shown that antibody-mediated inhibition of fibrinogen binding prevents thrombosis and also enhances the rate of thrombolysis with agents such as tissue plasminogen activator (t-PA) and prevents reocclusion.[4] In some cases, inhibition of GPIIbIIIa alone without exogenous thrombolytic agent led to reperfusion.[5] Although one GPIIbIIIa antibody is under clinical investigation,[6] the general utility of this antibody in antiplatelet therapy may be limited due to potential immunogenicity.[7] Furthermore, the inhibition of platelet aggregation by this antibody is only slowly reversible (48–72 h).[6] Due to the risk of hemorrhage during treatment, a short-acting, rapidly reversible inhibitor would be a more desirable alternative in the context of thrombolytic therapy.[8]

GPIIbIIIa, like several other members of the integrin superfamily of adhesion receptors, contains a binding site for the tripeptide sequence Arg-Gly-Asp (RGD), a common structural element of many integrin ligands. In addition to the Fg molecule, the RGD triad occurs in vitronectin, fibronectin, von Willebrand factor, osteopontin, thrombospondin, and the collagens.[9] Thus, the RGD tripeptide unit is a ubiquitous recognition sequence, the conformation of which in the individual ligands may be critical for recognition specificity.[10] Inhibition of ligand binding to GPIIbIIIa, as well as other integrins, can be accomplished using small peptides containing the RGD sequence.[11-14]

Prior to the initiation of this project, the isolation of the 71-residue protein trigramin from the venom of the viper *Trimeresurus gramineus* was reported.[15] Trigramin is a cysteine-rich protein which contains the RGD sequence.

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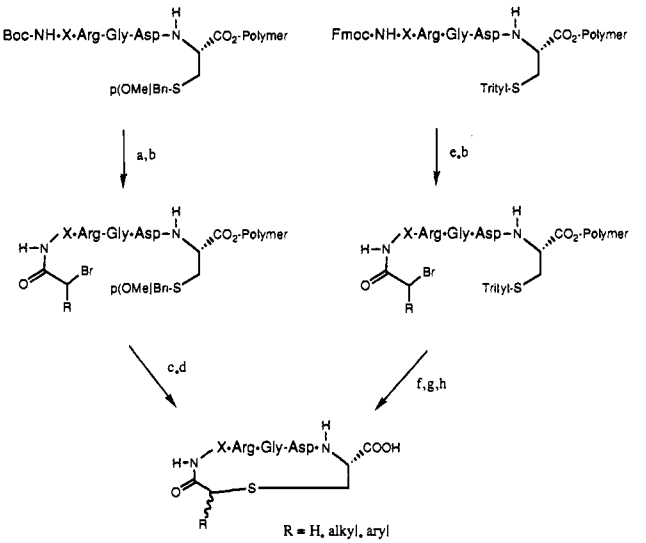
Subsequently, many more RGD-containing viper venom proteins have been isolated and characterized, including echistatin[16] and kistrin.[17] These proteins were the most potent antiaggregatory agents known prior to this work, with IC_{50} values 500–2000 times lower than those of small synthetic RGD peptides.[18] Kistrin has been demonstrated to potentiate the thrombolytic action of t-PA in an animal model of coronary arterial thrombosis.[19]

To complement the investigation of kistrin as an antiplatelet antithrombotic, we initiated a drug design program aimed at small peptide or peptidomimetic GPIIbIIIa/Fg binding inhibitors having a pharmacological profile suitable for acute use during thrombolytic therapy. Studies have shown that peptides with identical sequences can assume unrelated conformations in different proteins[20] and that constraining the conformation of a peptide by cyclization can impart enhanced activity on the peptide.[10,21] We thus set out to increase the rather weak IIbIIIa antagonist activity of linear RGD-containing peptides by incorporating the RGD sequence in cycic peptides.

Chemical Synthesis

Synthesis of peptides was performed by solid-phase peptide synthesis (SPPS),[22] employing either Boc or Fmoc protocols. Boc groups were cleaved with 50% TFA in DCM containing 5% ethanedithiol and 5% anisole. After neutralization with *N,N'*-diisopropylethylamine, couplings of Boc amino acids were accomplished with (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP), 1-hydroxybenzotriazole (HOBt), and catalytic 4-(*N,N*-dimethylamino)pyridine (DMAP) in *N,N*-dimethylacetamide (DMA). For couplings of Asn or Gln, excess HOBt was employed to minimize dehydration of the side-chain amides. Fmoc groups were cleaved by a solution of 20% piperidine in DMA. After the resin was rinsed free of excess piperidine, the Fmoc amino acids were coupled using BOP, HOBt, and catalytic DMAP as with Boc amino acids. Couplings of both Boc and Fmoc amino acids were monitored by a Kaiser ninhydrin test.[23] Peptides were cleaved from the solid support by hydrogen fluoride (Boc peptides)[24] or a solution of TFA containing

Scheme I. Synthesis of Cyclic (Substituted) Acetyl-X-Arg-Gly-Asp-Cys[a]



[a] Reagents: (a) TFA/CH_2CL_2; (b) RCH(Br)COOH/DIPC; (c) HF; (d) 1 mg/mL, pH = 7–8; (e) piperidine/*N,N*-dimethylacetamide; (f) 2% TFA/2% Et_3SiH/CH_2Cl_2; (g) 5% *N*-methylmorpholine/*N,N*-dimethylacetamide; (h) 50% TFA/CH_2Cl_2.

Table I. Inhibition of GPIIbIIIa/Fibrinogen Binding and Platelet Aggregation by Linear RGD Peptides

X-Arg-Gly-Asp-Y

peptide	X	Y	ELISA IC_{50}, nM[a]	platelet aggregation IC_{50}, μM[b]
1	H_2N	Phe-OH	31	43
2	H_2N	Tyr-OH	70	94
3	H_2N	Leu-OH	105	>2 mM
4	H_2N-Gly	Ser-OH	133	160
5	H_2N-Gly	Asp-OH	231	>500
6	H_2N-Gly	Tyr-OH	240	170
7	H_2N-Gly	Leu-OH	500	170
8	H_2N-Gly	Phe-OH	83	20
9	H_2N-Gly	Val-OH	30	75
10	Ac-Gly	Val-OH	12	77
11	Ac-Gly	Val-NH_2	434	>500
kistrin			2.0	0.15

[a] Inhibition of GPIIbIIIa/fibrinogen binding; $n \geq 3$. Confidence limits ±25–30%. [b] Inhibition of platelet aggregation in human platelet-rich plasma induced by ADP; $n \geq 3$. Confidence limits ±25%.

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2% phenol, 2% thiocresol, and 2% ethanedithiol (Fmoc peptides). Purification by reverse-phase high-performance liquid chromatography (RP-HPLC) yielded peptides which showed a single peak by analytical RP-HPLC. Peptides were >95% pure. All peptides were characterized by fast atom bombardment (FAB) mass spectrometry and amino acid analysis.

Synthesis of the thioether-cyclized peptides was carried out by SPPS, with bromoacetylation (bromoacetic acid/*N,N'*-diisopropylcarbodiimide, DIPC) of the amino terminus, as shown in Scheme I. Cleavage of the peptides from the solid support gave crude N-bromoacetylated peptides, which were dissolved in water (ca. 1 mg/mL) and were cyclized by adjusting the pH to 7–8 with ammonium hydroxide. After 6–24 h, the aqueous solutions were acidified to pH 3 and lyophilized; the resultant crude cyclic peptides were purified by RP-HPLC.

An alternative procedure was developed which allowed for cyclization of the bromoacetylated peptides on the solid support. *N*-(Bromoacetyl)-(AA1)-Arg(PMC)-Gly-Asp(*t*-Bu)-Cys(Trt)-resin peptides were prepared employing standard Fmoc protocols.[25] The cysteine trityl group was selectively removed by use of a solution of 2% trifluoroacetic acid/2% triethylsilane in dichloromethane. The peptides cyclized upon standing for several hours in *N,N*-dimethylacetamide in the presence of *N*-methylmorpholine; cleavage of the cyclic peptides from the resin and purification were carried out in the usual fashion.

Results

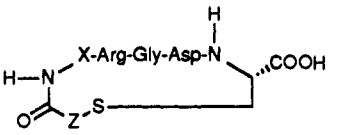
Numerous reports have described limited numbers of linear RGD-containing peptides and their inhibitory effect on the GPIIbIIIa/Fg interaction,[11-14] as well as other integrins with their respective ligands (e.g., vitronectin–vitronectin receptor[10]). To provide a baseline structure–activity correlation, a comprehensive study of approximately 200 linear peptides containing the RGD sequence as antagonists of GPIIbIIIa/Fg binding was undertaken. The results, which are similar to those reported by other groups,[26] are summarized by representative peptides (Table I) and suggest some of the features necessary for optimal inhibitory activity in an RGD-containing peptide. Two conclusions can be drawn from this study: for optimal activity the carboxy terminus of an RGD peptide should be adjacent to the aspartic acid residue and have a lipophilic side chain, and the amino terminus may be blocked. The pentapeptide Ac-Gly-Arg-Gly-Asp-Val-OH (10) is typical of such a GPIIbIIIa antagonist.

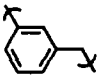
For the investigation of cyclic RGD peptides which incorporate the features of optimized linear peptides, we sought to cyclize a pentapeptide (AA1)-Arg-Gly-Asp-(AA5)-OH, while preserving a free carboxy terminus. This necessitates a bond formation from a side-chain functional group of (AA5) to either the amino terminus or a side-chain functional group of (AA1). Such cyclizations are achievable in a variety of ways.[27] A recent report of the facile cyclization of peptides via a thioether bond prompted the investigation of such cyclic peptides containing RGD.[28]

The systematic variation of thioether-bridged peptides began with the synthesis of *N*-(bromoacetyl)-Gly-Arg-Gly-Asp-Cys-OH by SPPS. Cyclization occurred at high dilution at pH 8, yielding *cyclo-S*-Ac-Gly-Arg-Gly-Asp-Cys-OH (12) as the prototype cyclic peptide. This cyclic peptide was evaluated in a GPIIbIIIa/Fg ELISA assay and a platelet aggregation assay. The ELISA assay, which measures the inhibition of binding of soluble GPIIbIIIa to immobilized fibrinogen, is highly sensitive and reproducible and allows for the rapid evaluation of large numbers of potential inhibitors.[17] The platelet aggregation assay measures the inhibition of ADP-induced aggregation of human platelets in platelet-rich plasma by test peptides. Kistrin was used as a positive control in both assays. The cyclic RGD peptide 12 displayed greatly improved activities, relative to the linear RGD peptide Ac-Gly-Arg-Gly-Asp-Val-OH, in both the GPIIbIIIa ELISA assay (IC_{50} = 4.2 vs 30 nM) and the platelet aggregation assay (IC_{50} = 5.0 vs 70 μM; Table II). Compound 12 was approximately 25-fold less potent than kistrin in the inhibition of platelet aggregation. Compound 12 contains 18 atoms in its backbone, equivalent to a cyclic hexapeptide. Increasing the ring size to 19 atoms by substituting β-alanine for the N-terminal glycine (13), or by bridging via a 3-bromopropionyl group (14), resulted in analogues with diminished activity compared to 12. Deletion of the N-terminal glycine [*cyclo-S*-Ac-Arg-Gly-Asp-Cys-OH (15)] also led to lower activity. Peptide 16, an 18-membered ring cyclized from the amino terminus of Arg to the cysteine sulfhydryl via a *m*-(chloromethyl)benzoyl moiety is also less potent than 12.

Having established the basic structural requirement of the cyclic peptide backbone, attention turned to introducing substituents on the bridging acetyl group through

Table II. Inhibition of GPIIbIIIa/Fibrinogen Binding and Platelet Aggregation



peptide	X	Z	ELISA IC_{50}, nM[a]	platelet aggregation IC_{50}, μM[a]
12	Gly	$-CH_2-$	4.2[b]	5.0[b]
13	β-Ala	$-CH_2-$	12	34.3
14	Gly	$-CH_2CH_2-$	75	64.3
15		$-CH_2-$	132	>2 mM
16			19	30.8

[a] See Table I. [b] $n > 50$. Confidence limits ±5%.

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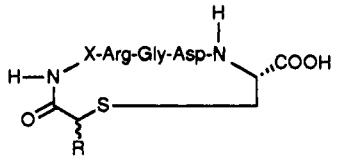
Table III. Inhibition of GPIIbIIIa/Fibrinogen Binding and Platelet Aggregation



peptide	R[a]	ELISA IC_{50}, nM[b]	platelet aggregation (ratio to 12)[c]	peptide	R[a]	ELISA IC_{50}, nM[b]	platelet aggregation (ratio to 12)[c]
17B	1-naphthyl	1.4	0.02	**17A**	1-naphthyl	5.3	0.78
18B	phenyl	1.5	0.17	**18A**	phenyl	15	neg[d]
19B	*p*-biphenylyl	3.5	0.05	**19A**	*p*-biphenylyl	5.0	1.48
20B	*o*-(trifluoromethyl)phenyl	2.7	0.15	**20A**	*o*-(trifluoromethyl)phenyl	23	nt[e]
21B	2-naphthyl	2.8	0.14	**21A**	2-naphthyl	11.5	4.0
22B	*p*-(trifluoromethyl)phenyl	4	0.90	**22A**	*p*-(trifluoromethyl)phenyl	180	nt[e]
23B	*n*-propyl	5.4	1.5	**23A**	*n*-propyl	21	nt[e]
24B	*m*-(trifluoromethyl)phenyl	5.7	nt[e]	**24A**	*m*-(trifluoromethyl)phenyl	35	nt[e]
25B	ethyl	6	4.85	**25A**	ethyl	20	nt[e]
26	(*S*)-(−)-benzyl[f]	14	1.9	**27**	(*R*)-(+)-benzyl[g]	29	7.0

[a] Absolute stereochemistry unknown. [b] See Table I [c] Ratio of IC_{50} of test peptide to IC_{50} of 12 in platelet aggregation assay (see Table I). [d] IC_{50} > 20 μM. [e] Not tested. [f] From (*R*)-(+)-α-bromo-3-phenylpropionic acid (see text). [g] From (*S*)-(−)-α-bromo-3-phenylpropionic acid (see text).

the use of substituted α-bromoacetic acids. As shown in Scheme I, acylation of the amino terminus with racemic bromoacetic acids (e.g., α-bromophenylacetic acid), followed by cyclization, gave rise to pairs of diastereomeric cyclic peptides, which were separated and tested independently in the GPIIbIIIa ELISA assay and in the platelet aggregation assay. Platelet aggregation inhibitory potency is expressed as a ratio of the IC_{50} values of the substituted acetyl-bridged peptides to the IC_{50} of the parent unsubstituted peptide (**12**). All IC_{50} values and ratios have been adjusted to reflect quantitative amino acid analyses. The data in Table III reveal that aromatic substituents on the acetyl group confer enhanced activity relative to **12** in one of the diastereomers, while the other diastereomer generally possesses reduced activity. Smaller substituents such as ethyl or propyl reduce the activity of the cyclic peptides in both diastereomers. The most potent of the aromatic acetyl-substituted peptides is one of the diastereomers of *cyclo*-*S*-(1-naphthylacetyl)-Gly-Arg-Gly-Asp-Cys-OH (**17B**), with an IC_{50} in the ELISA assay of 1.4 nM, equipotent to kistrin. This peptide is 50 times as potent as **12** in inhibiting platelet aggregation. The absolute stereochemistry of the naphthyl-bearing carbon is unknown. Attempts to identify and selectively synthesize one of the diastereomers of **18** (R = phenyl) through the use of (*S*)-α-bromophenylacetic acid [from (*S*)-phenylglycine and nitrosyl bromide][29] produced a mixture of diastereomeric peptides **18A** and **18B**, presumably due to racemization of the chiral bromo acid via phenylbromoketene formation during activation of the bromo acid. Such a ketene would still serve as an acylating agent, giving rise to racemic bromo(phenyl)acetyl peptides, followed by alkylation of the cysteine sulfhydryl group. Attempts to identify the stereochemistry of these arylacetyl-bridged cyclic peptides are still being pursued. Employing chiral 2-bromo-3-phenylpropionic acids derived from D- and L-phenylalanine resulted in enantiomerically pure cyclic peptides of known stereochemistry possessing chiral benzyl substituents on the bridging acetyl group (i.e., **26** and **27**). The more active of these peptides, **26**, possesses the (*S*)-configuration at the carbon bearing the benzyl substituent. While this cyclic peptide displays activity significantly less than those bearing aromatic substituents directly on the acetyl carbon, the relative order of activity in the ELISA and platelet aggregation assays may allow us to infer similar stereochemistry for the more active diastereomers of the arylacetyl-bridged cyclic peptides.

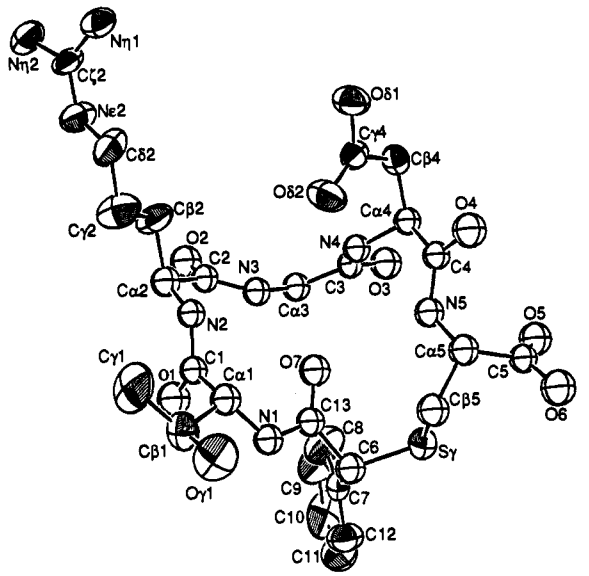
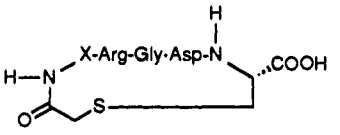


Figure 1. Absolute stereochemistry of **34B** as determined by X-ray crystallography.

Replacement of the N-terminal Gly of **12** with a variety of D- and L-amino acids (Table IV) reveals that most D-amino acids in that position enhance activity relative to glycine in both the ELISA and platelet aggregation assays; hydrophobic D-amino acids (e.g., D-Tyr, D-Val) impart the greatest increases in antagonist activity. All substitutions for the N-terminal glycine with L-amino acids resulted in diminished activity relative to glycine. These results mirror those from the diastereomeric arylacetyl-bridged peptides (Table III) and very likely reflect a conformational effect on the backbone of the cyclic peptides. Combining D-threonine at the N-terminus with a phenylacetyl group at the bridging position gave diastereomeric compounds **34A** and **34B**, which were separated chromatographically and tested in the ELISA and platelet aggregation assays. Crystals were obtained for the less

(29) Shimohigashi, Y.; Waki, M.; Izumiya, N. Stereospecific Synthesis of D-Isothreonine from L-Threonine. *Bull. Chem. Soc. Jpn.* **1979**, *52*, 949–950.

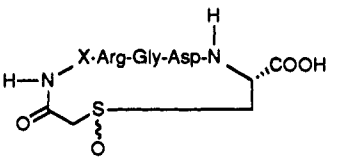
Table IV. Inhibition of GPIIbIIIa/Fibrinogen Binding and Platelet Aggregation



peptide	X[a]	ELISA IC_{50}, nM[b]	platelet aggregation (ratio to 12)[c]	peptide	X[a]	ELISA IC_{50} nM[b]	platelet aggregation (ratio to 12)[c]
12	Gly	4.2	1.0				
28	pro	1.0	0.43	29	Pro	11.4	1.96
30	thr	1.3	0.24	31	Thr	11.6	2.92
32	tyr	1.5	0.15	33	Tyr	20	3.45
34A	thr[d]	1.7	0.06	34B	thr[d]	7.0	3.26
35	val	2.0	0.08	36	Val	17	8.74
37	ala	2.5	0.59	38	Ala	9.5	4.00
39	asn	2.7	0.31	40	Asn	35	nt[e]
41	his	3.0	0.54	42	His	6.8	1.08
43	leu	3.0	0.20	44	Leu	38	nt[e]
45A	t-leu[f]	3.6	nt[e]	45B	t-Leu[f]	22	nt[e]
46	met	4.0	1.06	47	Met	28	nt[e]
48	ser	4.0	0.48	49	Ser	22	nt[e]
50	trp	4.0	0.46	51	Trp	29	4.78
52	gln	43.0	0.68	53	Gln	110	nt[e]
54	orn	5.6	0.25	55	Orn	31	nt[e]
56	ile	6.0	0.27	57	Ile	18	6.08
58	phe	7.0	0.22	59	Phe	35	18.46
60	lys	7.5	0.60	61	Lys	20	nt[e]
62	glu	15	4.6	63	Glu	260	nt[e]
64	asp	25	nt[e]	65	Asp	500	nt[e]

[a] All lower-case letters indicate D-amino acids. [b] See Table I. [c] See Table III. [d] Phenylacetyl-bridged. [e] Not tested. [f] From racemic t-Leu; absolute stereochemistry inferred.

Table V. Inhibition of GPIIbIIIa/Fibrinogen Binding and Platelet Aggregation



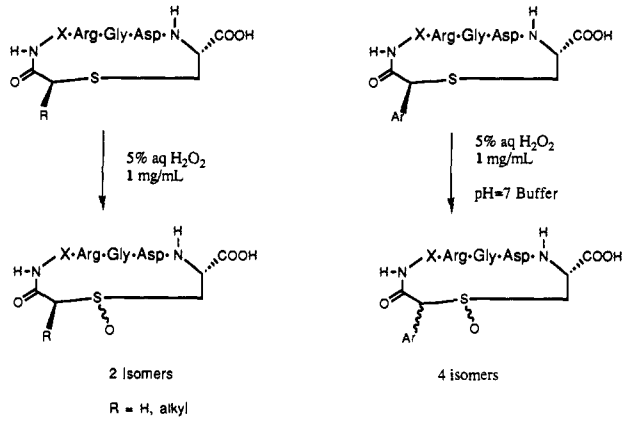
peptide	X[a]	ELISA IC_{50}, nM[b]	platelet aggregation (ratio to 12)[c]	peptide	X[a]	ELISA IC_{50}, nM[b]	platelet aggregation (ratio to 12)[c]
66A	Gly	1.4	0.37	66B	Gly	25	nt[d]
66C[e]	Gly	18	7.7				
67A	asn	6.0	0.46	67B	asn	5.3	1.73
68A	ala	1.6	0.10	68B	ala	4.7	0.61
69A	Tyr	7.3	0.12	69B	Tyr	40.0	3.0
70A	tyr	1.5[f]	0.03[f]	70B	tyr	4.8	0.45
70C[e]	tyr	2.1	0.21				
71A	thr	1.8	0.10	71B	thr	10	nt[d]
73A	glu	4.7	0.90	73B	glu	68.0	17.0
74A	asp	6.0	2.14	74B	asp	nt	66.0
75A	lys	2.6	0.09	75B	lys	14	2.80
76A	val	2.9	0.04	76B	val	4.5	0.21
77A	leu	3.9	0.10	77B	leu	25	nt[d]
78A	Phe	5.6	0.44	78B	Phe	33	nt[e]
79A	phe	6.6	0.70	79B	phe	16	3.83
80A	ser	8	0.49	80B	ser	50	nt[d]

[a] All lower-case letters indicate D-amino acids. [b] See Table I. [c] See Table III. [d] Not tested. [e] Sulfone [f] IC_{50} = 0.15 μM (n > 50; confidence limits ± 5%).

potent of these isomers, **34B**, which were suitable for analysis by X-ray diffraction. Analysis of the X-ray data resulted in the assignment of the (*R*)-configuration to the carbon bearing the phenyl substituent (C-6; Figure 1); hence, the corresponding carbon atom in **34A**, the more active diastereomer, possesses the (*S*)-configuration. This is consistent with the absolute configuration of **26** and lends support to the assignment of the (*S*)-configuration to the more potent diastereomers of the arylacetyl-bridged peptides.

The sulfoxide analogues of the cyclic thioether peptides were prepared by dissolving the sulfide-containing peptides in 5% hydrogen peroxide solution at ca. 1 mg/mL. This procedure yielded two diastereomeric sulfoxides, which were chromatographically separable (Scheme II). Little or no sulfone was observed, and the few sulfones isolated showed activity equal to or less than the parent sulfides. The diastereomeric sulfoxides were evaluated in the ELISA and platelet aggregation assays, and the results are shown in Table V. Within each pair of diastereomeric sulfoxides, one of the diastereomers demonstrated enhanced activity relative to the parent sulfide, while the other sulfoxide generally showed diminished activity. In all cases, the more active isomer of the diastereomeric pairs of sulfoxides eluted earlier from a reverse-phase (C-18) column. As in the case of the cyclic sulfides, the sulfoxide-bridged cyclic

Scheme II. Synthesis of Cyclic (Substituted) Acetyl-X-Arg-Gly-Asp-Cys Sulfoxides



peptides containing hydrophobic residues at the amino-terminal position (e.g., Tyr, Pro, Val) were the most potent inhibitors of platelet aggregation. Compound **70A** was more than 10-fold more active than **66A**, which bears a glycine in the amino-terminal position, and equipotent to kistrin in both ELISA and platelet aggregation assays. The corresponding sulfoxides from the L-Tyr containing cyclic peptides **69A** and **69B** were significantly less active than the D-Tyr-containing cyclics.

The sulfoxides were shown to be chemically stable and did not epimerize under the acidic conditions of reverse-phase HPLC (pH ca. 1) or under the somewhat alkaline conditions of ion-exchange chromatography (pH ca. 8–9). However, oxidation of optically pure arylacetyl-bridged cyclic thioether peptides (e.g., **18B**) at pH 7 yielded four diastereomeric products, which were shown to be the two sulfoxides each of *racemized* arylacetyl peptides. This racemization of the chiral acetyl methine position was likely due to the increased acidity of the methine proton, which being adjacent to an amide carbonyl, a sulfoxide, and an aryl group is sufficiently labile to undergo epimerization in a pH 7 buffer. This was demonstrated by the following reverse-phase HPLC experiments (Figure 2). Cyclization of *N*-(α-bromophenylacetyl)-Gly-Arg-Gly-Asp-Cys-OH produced the separable diastereomers **18A** and **18B**. These thioether-bridged cyclic peptides were independently oxidized at pH 3 to two pairs of separable diastereomeric sulfoxides, **18A.1**, **18A.2** and **18B.1**, **18B.2**. All four of these diastereomers had distinct HPLC retention times. Treatment of **18B.1** with pH 7 buffer resulted in the formation of a mixture of two diastereomers corresponding to **18B.1** and **18A.2**. Similarly, exposure of **18B.2** to pH 7 buffer gave a mixture of **18B.2** and **18A.1**. Finally, oxidation of **18B** at pH 7 gave rise to a mixture of all four diastereomers, each HPLC peak of which could be enriched by coinjection with any of the isolated diastereomers. Due to the inability to control the chiral integrity of the arylacetyl sulfoxides at physiological pH, this subclass of compounds was not investigated further.

Examination of the ELISA and platelet aggregation data for all of the peptides in Tables I–V reveals a generally good correlation between the order of the IC_{50} values in the GPIIbIIIa ELISA assay and the order in the platelet aggregation assay. The large discrepancies in the IC_{50} values between the two assays reflect the many differences in the assay parameters. The increased sensitivity of the purified ligand/receptor assay is likely due, in part, to the reduction in receptor concentration and absence of plasma proteins, including fibrinogen. The ELISA assay generally serves as a good predictor of inhibitory activity in the platelet aggregation assay at IC_{50} levels above ca. 10 nM.

Table VI. Comparison of Carboxy-Terminal Acids vs Amides of RGD Peptides

peptide	structure	ELISA IC_{50}, nM[a]	platelet aggregation IC_{50}, μM[a]
10	Ac-GRGDV-OH	12	77
11	Ac-GRGDV-NH_2	434	>500
12	*cyclo-S*-Ac-GRGDC-OH	4.2[b]	5.0[b]
81	*cyclo-S*-Ac-GRGDC-NH_2	12	150
32	*cyclo-S*-Ac-yRGDC-OH	1.5	0.8
82	*cyclo-S*-Ac-yRGDC-NH_2	2.5	0.7
70A	*cyclo-S*-Ac-yRGDC-OH (sulfoxide)	1.5[b]	0.15[b]
83	*cyclo-S*-Ac-yRGDC-NH_2 (sulfoxide)	4.5	0.2

[a] See Table I. [b] $n > 50$; confidence limits ±5%.

However, the correlation between the two assays decreases for peptides with IC_{50} values less than 10 nM in the ELISA assay. Similar discrepancies between GPIIbIIIa ELISA results and platelet aggregation results have been reported by others.[30] Therefore, peptides with $IC_{50} < 10$ nM in the ELISA assay were evaluated in the platelet aggregation assay as well.

Having developed a number of highly potent inhibitors of platelet aggregation, the initial premise in this study that a free carboxy terminus on the residue following Asp is necessary for activity in an RGD-containing peptide was reexamined. This premise was based on the observation that when the modestly potent linear peptide Ac-GRGDV-OH (**10**) was tested as its carboxy-terminal amide (Ac-GRGDV-NH_2, **11**), an almost complete abatement of its inhibitory activity resulted. As seen in Table VI, the carboxy-terminal amide version of **12** (**81**) similarly displayed a 30-fold decrease in activity in the platelet aggregation assay relative to the C-terminal acid. However, when **32** was prepared with a carboxy-terminal amide (**82**), its ability to inhibit platelet aggregation remained unaffected. Similarly, the carboxy-terminal amide of **70A** (**83**) is essentially equipotent to the parent free acid, with a ratio of IC_{50} values of 1.3. It is possible that for the linear peptides such as Ac-GRGDV, and relatively nonrigid cyclic peptides such as **12**, the electronic repulsion between the carboxylate anions from Asp and the C-terminal Val or Cys is necessary to impart some conformational restriction to the peptide backbone. The maintaining of activity in the amide versions of the more potent and more rigid cyclic peptides, such as **70A**, suggests that electronic repulsion has an almost negligible effect on the peptide conformation. Thus, the possibility, for example, of a fortuitous electrostatic interaction of a free C-terminus of these small RGD peptides with some counterion on the receptor is unlikely.

Due to its high degree of inhibitory activity in the ELISA and platelet aggregation assays, as well as its ease of preparation, **70A** (G-4120) has been selected for further evaluation in animal models. The results from that study will be reported elsewhere.

Experimental Section

Peptides were synthesized by employing standard solid-phase protocols, using either Fmoc or Boc chemistry. Boc and Fmoc amino acids, Boc amino acid polystyrene resins (Merrifield resins),

(30) Klein, S. I.; Molino, B. F.; Chu, V.; Ruggeri, Z.; Czekaj, M.; Gardner, C. J.; Newman, J.; Barrett, J. A. Novel RGD Analogs as Antithrombotic Agents. In *Peptides 1990: Proceedings of the Twenty-First European Peptide Symposium*; Giralt, E., Andreu, D., Eds.; ESCOM Science Publishers B. V.: Leiden, 1991; pp 374–375.

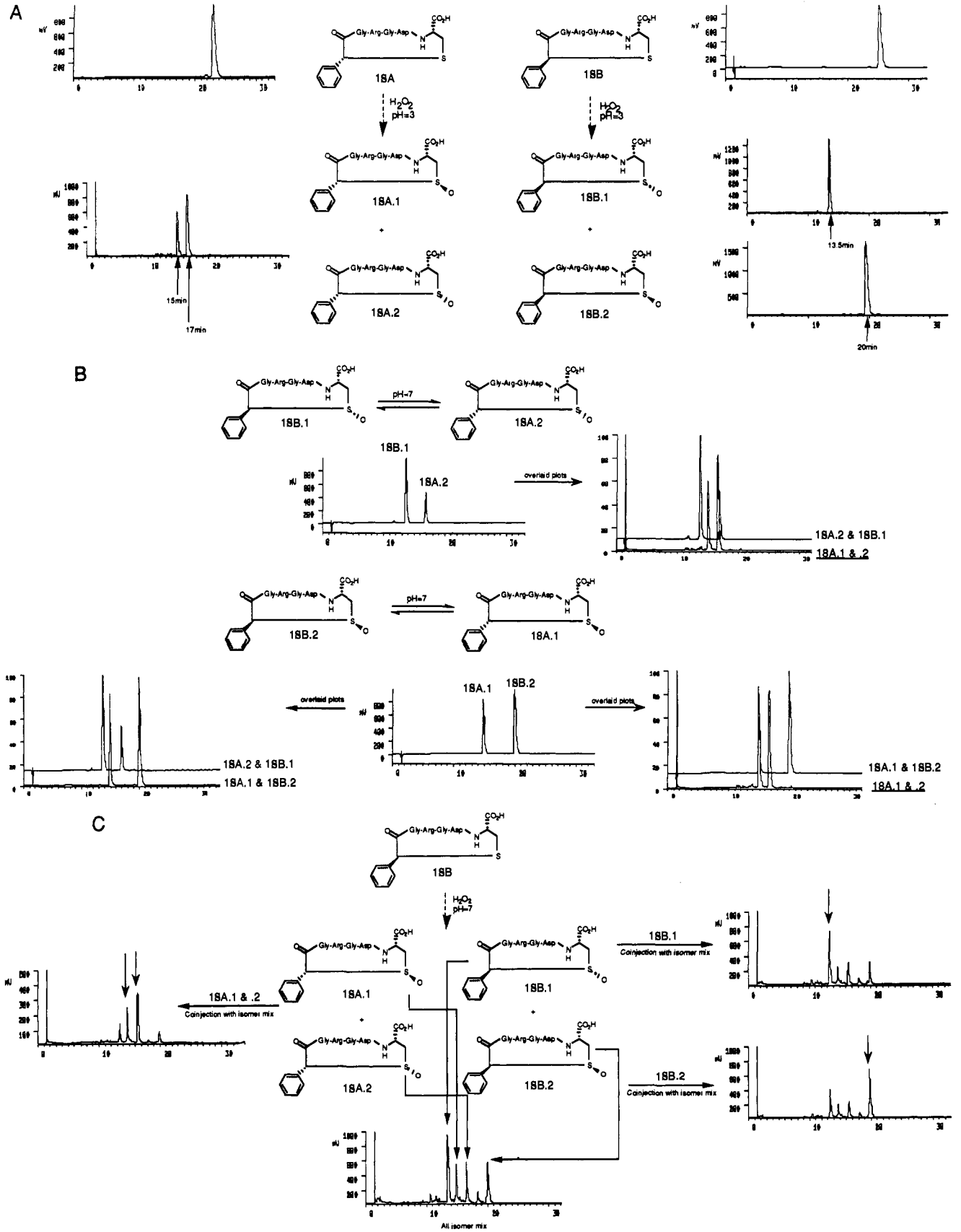


Figure 2. Racemization of phenylacetyl–sulfoxide-bridged cyclic peptides. (A) Oxidation of diastereomeric phenylacetyl–thioether-bridged cyclic peptides at pH 3 produces two sets of separable sulfoxides. (B) Dissolution of a single diastereomer (**1SB.1** or **17B.2**) at pH 7 (buffer) gives rise to a mix of two diastereomers corresponding to racemization of the phenyl group. (C) Oxidation of a single diastereomeric phenylacetyl–thioether-bridged cyclic peptide at pH 7 produces all four possible diastereomeric phenylacetyl–sulfoxide-bridged cyclic peptides. Absolute and relative configurations are unknown; the stereochemistry as shown is for illustrative purposes.

and Fmoc amino acid PepSyn K (polyacrylamide-kieselguhr) resins were purchased from Peninsula Laboratories and Bachem. Peptides were synthesized using either Boc or Fmoc protocols. Boc peptides were cleaved from the resin with HF, and Fmoc peptides were cleaved with trifluoroacetic acid as described below. All peptides were characterized by FAB (fast atom bombardment) mass spectroscopy and amino acid analysis.

Boc Peptide Synthesis. Starting with 1–2 g of Boc amino acid Merrifield resin (substitution at 0.2–0.6 mM amino acid/g of resin), the synthesis was carried out in a sequential manner employing a Biosearch 9500 automated peptide synthesizer using standard double-coupling protocols. The amino termini were bromoacetylated using bromoacetic acid and *N,N'*-diisopropylcarbodiimide (DIPC). Side-chain protection was arg(tosyl), asp(cyclohexyl), cys(4-methylbenzyl), glu(benzyl), his(benzyloxymethyl), lys(2-chlorobenzyloxycarbonyl), orn(benzyloxycarbonyl), ser(benzyl), thr(benzyl), tyr(2-bromobenzyloxycarbonyl).

Fmoc Peptide Synthesis. Starting with 0.5–1 g of Fmoc amino acid PepSyn K resin (substitution at 0.2–0.6 mM amino acid/g of resin), the synthesis was carried out in a sequential manner employing a Milligen 9050 automated peptide synthesizer using standard coupling protocols. Side-chain protection was arg(2,2,5,7,8-pentamethylchroman-6-sulfonyl), cys(trityl), asp, glu, ser, thr, tyr (all *tert*-butyl), his(Boc), lys(Boc).

HF Cleavage. The resin-bound peptide was suspended in a mixture of 4 mL of 1:1 (v/v) anisole/ethyl methyl sulfide per gram of resin in an HF apparatus (Peninsula Laboratories). The system was cooled using liquid nitrogen and evacuated using a mechanical vacuum pump; HF was then condensed (20–30 mL/g of resin) into the reaction vessel. The reaction mixture was stirred for 90 min at 0 °C and evaporated in vacuo by employing a calcium hydroxide trap. The residue was triturated with ether, filtered, and triturated three more times with ether to remove the anisole and ethyl methyl sulfide. The resin was sequentially triturated with 10% aqueous acetic acid (3 × 50 mL), glacial acetic acid (10 mL), and water (100 mL); lyophilization of the aqueous extracts yielded the crude peptide as a white, fluffy powder.

Trifluoroacetic Acid Cleavage. The resin-bound peptide was treated with 10–15 mL of a mixture of TFA and triethylsilane (98:2) per gram of resin for 1 h at ambient temperature. The reaction mixture was evaporated in vacuo and triturated with ether as above. Trituration with aqueous acetic acid and lyophilization as above yielded crude peptide.

Cyclization of Peptides. (A) Solution Cyclization. Crude *N*-bromoacetyl peptides were dissolved in water (ca. 1 mg/mL), the pH was adjusted to 8 with concentrated ammonium hydroxide, and the solution was stirred at ambient temperature for 24 h. The pH was then adjusted to 2–3 with acetic acid, and the solution was frozen, lyophilized, and purified as described below.

(B) Cyclization on Resin. *N*-Bromoacetyl peptides containing a C-terminal *S*-tritylcysteine residue bound to the resin were treated with a solution of 2% TFA and 2% triethylsilane in methylene chloride for 10 min. This treatment was repeated until an aliquot of the resin-bound peptide no longer gave a yellow solution when treated with neat TFA (usually three to four treatments). The resin was washed several times with methylene chloride and then agitated overnight in a solution of 5% *N*-methylmorpholine in *N,N*-dimethylacetamide. Peptide cleavage using trifluoroacetic acid was performed as described above.

Purification of Peptides. The crude peptides were purified by RP-HPLC using a 4.6 mm × 250 mm column containing 10-μm, 300-Å pore-size C-18 packing. Elution from the column was with an acetonitrile/0.1% aqueous trifluoroacetic acid gradient from 0% to 40% acetonitrile linearly over 80 min. All peptides were ≥95% pure.

2-Bromo-2-phenylacetic acid, 2-bromobutyric acid, and 2-bromovaleric acid were purchased from Aldrich.

(*S*)-2-Bromo-3-phenylpropionic acid was prepared from (*S*)-phenylalanine by the method of Izumiya.[29] Thus, 15.16 g (92 mmol) of (*S*)-phenylalanine was dissolved in 100 mL of 1.25 M H_2SO_4 containing 35 g of KBr at 0 °C. Sodium nitrite (9.5 g, 92 mmol) was added portionwise over 15 min, with evolution of an orange gas. After complete addition, the solution was stirred for 1 h at ambient temperature. The aqueous solution was extracted with ethyl acetate (2 × 100 mL), and the combined organic extracts were dried (Na_2SO_4) and evaporated to yield 16.5 g (72 mmol) of a pale yellow oil: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.2–7.4 (m, 5 H, aromatic H), 4.45 (dd, 1 H, J = 7.1 Hz, J' = 7.1 Hz, C*H*BrCO$_2$H), 3.49 (dd, 1 H, J = 8.5 Hz, J' = 14.2 Hz, benzylic H), 3.26 (dd, 1 H, J = 7.1 Hz, J' = 14.2 Hz, benzylic H); HRMS calcd for $C_9H_9O_2Br$ 227.9786, found 227.9799; $[\alpha]^{23}_D$ = −8.26° (*c* 21.3, $CHCl_3$).

(*R*)-2-Bromo-3-phenylpropionic acid was prepared as above from 10.32 g (62.5 mmol) of (*R*)-phenylalanine, yielding 12.7 g (55.5 mmol) of a pale yellow oil: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.2–7.4 (m, 5 H, aromatic H), 4.45 (dd, 1 H, J = 7.1 Hz, J' = 7.1 Hz, C*H*BrCO$_2$H), 3.49 (dd, 1 H, J = 8.5 Hz, J' = 14.2 Hz, benzylic H), 3.26 (dd, 1 H, J = 7.1 Hz, J' = 14.2 Hz, benzylic H); HRMS calcd for $C_9H_9O_2Br$ 227.9786, found 227.9786; $[\alpha]^{23}_D$ = +7.64° (*c* 21.3, $CHCl_3$).

2-Bromo-2-arylacetic Acids. General Procedure.[31] Commercially available arylacetic acids were converted to their acid chlorides by employing oxalyl chloride and catalytic *N,N*-dimethylformamide. After evaporation of the solvent, the crude acid chlorides were subjected to reflux in carbon tetrachloride containing 120 mol % *N*-bromosuccinimide and 3–5 drops of 33% HBr/acetic acid for 3 h. Filtration of the insoluble succinimide after cooling to room temperature, followed by vigorous stirring of the filtrate for ca. 12 h with water and extraction with ethyl acetate, yielded the crude bromo acids contaminated with 10–20% of unbrominated starting acid.

2-Bromo-2-(2-naphthyl)acetic acid was prepared as described above and isolated as a yellow solid: mp 161–162 °C (benzene/hexane); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.2–8.8 (br s, 1 H, carboxylate H), 7.4–8.0 (m, 7 H, aromatic H), 5.56 (d, 1 H, J = 2.5 Hz, benzylic H); HRMS calcd for $C_{12}H_9O_2Br$ 263.9786, found 263.9792.

2-Bromo-2-(1-naphthyl)acetic acid was prepared as described above and isolated as a yellow solid: mp 73–74 °C (benzene/hexane); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.4–8.0 (m, 7 H, aromatic H), 6.2 (s, 1 H, benzylic H); HRMS calcd for $C_{12}H_9O_2Br$ 263.9786, found 263.9791.

2-Bromo-2-[*o*-(trifluoromethyl)phenyl]acetic acid was prepared as described above and isolated as a yellow oil: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.0 (d, 1 H, J = 9.7 Hz, aromatic H), 7.4–7.7 (m, 3 H, aromatic H), 5.8 (s, 1 H, benzylic H); HRMS calcd for $C_9H_6O_2BrF_3$ 281.9503, found 281.9507.

2-Bromo-2-[*m*-(trifluoromethyl)phenyl]acetic acid was prepared as described above and isolated as a yellow oil: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.5–7.9 (m, 4 H, aromatic H), 5.4 (s, 1 H, benzylic H); HRMS calcd for $C_9H_6O_2BrF_3$ 281.9503, found 281.9507.

2-Bromo-2-[*p*-(trifluoromethyl)phenyl]acetic acid was prepared as described above and isolated as a yellow oil: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.7 (dd, 4 H, J = 8.5 Hz, J' = 25 Hz, aromatic H), 5.4 (s, 1 H, benzylic H); HRMS calcd for $C_9H_6O_2BrF_3$ 281.9503, found 281.9504.

2-Bromo-2-(*p*-biphenyl)acetic acid was prepared as described above and isolated as a yellow solid: mp 129–130 °C (benzene/hexane); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.6–8.9 (br s, 1 H, carboxylate H), 7.3–7.7 (m, 9 H, aromatic H), 5.45 (d, J = 6.4 Hz, benzylic H); HRMS calcd for $C_{14}O_{11}O_2Br$ 289.9943, found 289.9943.

X-ray Crystal Structure of 34B. Crystals of formula $C_{27}H_{40}N_8O_{10}S\cdot 6H_2O$ with formula weight 668.73 g/mol were grown from acidified phosphate buffer in space group $P4_3$ with cell dimensions a = 16.084 (2) Å and c = 14.271 (1) Å. With four molecules per unit cell, the calculated density is 1.203 g/cm^3. The density was not measured. A total of 3601 unique reflections were measured by the $\theta/2\theta$ technique using Cu Kα radiation (λ = 1.54184 Å) on an Enraf-Nonius CAD4 diffractometer, of which 786 were considered unobserved. The structure was solved by direct methods and refined[32] to final unweighted and weighted

(31) Gleason, J. G.; Harpp, D. N. α-Bromination of Acid Halides. *Tetrahedron Lett.* **1970**, *39*, 3431.

(32) Frenz, B. A. The Enraf-Nonius CAD 4 SDP—A Real-time System for Concurrent X-ray Data Collection and Crystal Structure Determination. In *Computing in Crystallography*; Schenk, H., Olthof-Hazelkamp, R.; vanKonigsveld, H., Bassi, G. C., Eds.; Delft University Press: Delft, Holland, 1978; pp 64–71.

R-factors of 8.1% and 10.0%, respectively. The alternate space group $P4_1$ was discounted on the basis of the known stereochemistry in the molecule. The final difference map had a maximum peak height of 0.51 $e^-/Å^3$, 0.7 Å from atom O2. The largest negative feature was -0.32 $e^-/Å^3$, 0.6 Å from a poorly ordered water oxygen.

Materials. The following materials were used. Purified fibrinogen (Kabi), dissolved in 20 mM Tris and 120 mM NaCl buffer (pH 7.5). PBS/Tween: 0.137 M NaCl, 0.003 M KCl, 0.008 M Na_2HPO_4, 0.001 M KH_2PO_4, pH 7.4, 0.05% Tween 20 (Sigma). TNCNT/BSA: 20 mM Tris, pH 7.5, 120 mM NaCl, 0.2% NaN_3, 2 mM $CaCl_2$, 0.05% Tween 20, 0.5% BSA (Calbiochem). GPIIbIIIa receptor, purified from human platelets, stored at −70 °C, reconstituted in TNCNT with BSA. ELISA buffer: PBS, 0.5% BSA, 0.05% Tween 20, 0.01% Thimerosal. GAM-HRP: horseradish peroxidase conjugate of goat anti-mouse IgG (Tago), dissolved in ELISA buffer. OPD: o-phenylenediamine hydrochloride, 10-mg tablets (Sigma). Hydrogen peroxide, 30% solution (Sigma). Phosphate/citrate buffer: 16 mM citric acid, 50 mM Na_2HPO_4, pH 5.0.

Fibrinogen/GPIIbIIIa Solid-Phase ELISA. Samples were tested for inhibition of purified human platelet GPIIbIIIa binding to human fibrinogen immobilized onto microtiter plates. Fibrinogen (100 μL, 10 μg/mL) was coated onto 96-well, Nunc Maxisorp plates. After being blocked with BSA (200 μL, 5 mg/mL), samples (50 μL) were added at appropriate dilutions, followed by addition of GPIIbIIIa (50 μL, 40 μg/mL). After a 1-h incubation at room temperature, plates were washed and antibody to GPIIbIIIa (AP3, from P. Newman, Blood Center of Southeast Wisconsin) was added (100 μL, 1 μg/mL in ELISA buffer). After an additional 1-h incubation, plates were washed, GAM-Fc-HRP was added (100 μL, 1:15,000 dilution in ELISA buffer), and the solution was incubated for 1 h and washed. OPD/H_2O_2 (0.67 mg/mL, 0.0003%) in 50 mM Na_2HPO_4/citric acid (pH 5, 100 μL) buffer was added and the peroxidase reaction stopped by addition of H_2SO_4 (50 μL, 1 M). Absorbance at 492–405 nm was determined. The IC_{50} values were estimated by a nonlinear four-parameter curve fit analysis of the data. The coefficient of variation for the IC_{50} values for this assay was 25–30%.

In Vitro Platelet Aggregation in Human PRP. Blood was drawn on 3.8% sodium citrate (9:1) and spun at 180*g* at 22 °C for 12 min, and PRP was removed. The remaining fraction was spun at 1000*g* at 22 °C for 25 min for PPP. The platelet count of PRP was adjusted to 3×10^8/mL with PPP. PRP was incubated with the test sample at 37 °C for 5 min in the aggregometer, followed by addition of an appropriate concentration of aggregating agonist. Percent inhibition was expressed as 100 × the ratio of the change in transmittance in the presence of the test sample to the maximum change in transmittance in the absence of test sample, at 5 min after addition of the agonist. The reproducibility of the assay values of ca. 25% was determined for nine experiments. ADP concentration was 17.2 μM final concentration. The test sample was used at 12–14 concentrations, generally over a 200-fold range. The IC_{50} values were estimated by a nonlinear four-parameter curve fit analysis of the inhibition results.

Supplementary Material Available: One table giving RP-HPLC retention times, FAB mass spectral data, and amino acid analyses (7 pages). Ordering information is given on any current masthead page.

Ester and Amide Derivatives of E64c as Inhibitors of Platelet Calpains

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Ester and amide derivatives of E64c, (+)-(2*S*,3*S*)-3-[[(*S*)-3-methyl-1-[(3-methylbutyl)carbamoyl]butyl]carbamoyl]-2-oxiranecarboxylic acid, an inhibitor of calpains, were synthesized and tested for ability to inhibit calpain in lysed cells, ability to enter intact cells, and ability to inhibit calpain in intact cells. The esters were from halogen-substituted alcohols and alcohols with increasing size. There were no appreciable differences in the inhibitory potency of any of the halogen-substituted esters from ethyl to trifluoroethyl, indicating that ease of hydrolysis of this class of ester is not important for activity. The only ester with impaired activity was the largest, Z-leucyl-norleucyl, which was about 5% as effective as the ethyl ester, E64d. Amides of amino acid esters also had impaired activity. To explore the possibility of targeting E64c derivatives to specific cells, esters and amides of E64c with 5-hydroxytryptamine were tested on the rationale that the active 5-hydroxytryptamine uptake mechanism of platelets might selectively concentrate the drug in platelets. Both the ester and amide inhibited calpain in lysed cells, but only the ester inhibited in intact cells. The 5-hydroxytryptamine ester showed no advantage over the ethyl ester in entering platelets.

Calpains are Ca^{2+}-activated, intracellular cysteine proteases.[1-5] While their specific cleavage of certain protein substrates has been demonstrated in cell-free systems, there is less known about their physiological function. Cell-specific inhibitors of calpains would be valuable for studies of physiological function.

E64c, (+)-(2*S*,3*S*)-3-[[(*S*)-3-methyl-1-[(3-methylbutyl)carbamoyl]butyl]carbamoyl]-2-oxiranecarboxylic acid, is a calpain inhibitor,[6] and its ethyl ester, E64d, is able to cross plasma membranes and inhibit calpains within cells.[7,8] (For a review of calpain inhibitors, see refs 9–12.) We have explored the possibility of synthesizing cell-specific E64c analogues by esterification to an agent that is specifically transported into a certain cell. This required initial studies of the effect of variation of the ester group

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