Highly Selective Tripeptide Thrombin Inhibitors†

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Tripeptide aldehydes such as Boc-D-Phe-Pro-Arg-H (**5l**) exhibit potent direct inhibition of thrombin. This distinction offers important insight for the design of more potent and selective serine protease inhibitors which may be useful pharmacological tools and hold promise for development of clinically useful agents. The structure–activity relationships (SAR) on a series of anticoagulant peptides with high selectivity for the enzyme thrombin are discussed. The SAR is centered on a series of di- and tripeptide arginine aldehydes based on the structure of **5l**. The structural and conformational role of the amino acid residue in position 1 was investigated by substitution with conformationally restricted aromatic amino acids, aromatic acids, and a dipeptide isostere containing the $\psi[CH_2N]$ amide bond replacement. Many of these peptides demonstrate potent antithrombotic activity along with selectivity toward thrombin, determined by comparison of in vitro inhibitory effects on trypsin, plasmin, factor Xa, and tissue plasminogen activator. Compound **5f**, D-1-Tiq-Pro-Arg-H·sulfate is highly active and the most selective tripeptide aldehyde inhibitor of thrombin reported to date.

The process of blood coagulation is triggered by a complex proteolytic cascade leading to the formation of fibrin. Thrombin proteolytically removes activation peptides from the Aα-chain of fibrinogen and thereby initiates insoluble and soluble fibrin formation.[1] Current parenteral pharmacological control of thrombosis is based on inhibition of thrombin through the use of heparin.[2] Heparin acts indirectly on thrombin by accelerating the inhibitory effect of endogenous antithrombin III (the main physiological inhibitor of thrombin).[3] Because antithrombin III levels can vary in plasma and because surface-bound thrombin seems resistant to this indirect mechanism, heparin can be an ineffective treatment. Tripeptide aldehydes such as Boc-D-Phe-Pro-Arg-H (**5l**)[4] exhibit potent direct inhibition of thrombin.[5] This class of reagents was investigated with the goal of developing a novel anticoagulant with high selectivity for the enzyme. In this paper a series of di- and tripeptide arginine aldehydes are described which exhibit improved selectivity for inhibition of thrombin versus a number of other serine proteases of the fibrinolytic system.

Chemistry

The target peptides were synthesized by standard solution-phase peptide synthesis using the general procedures outlined in Scheme I. The unnatural amino acids incorporated into the dipeptides **3f**, **3h**, and **3t** were prepared using catalytic hydrogenation and a standard literature procedure for incorporation of Boc protection into the amino acids (cf. ref 16). The dipeptides were prepared in two steps from the 2,4,5-trichlorophenyl active ester of the Boc-amino acids and L-proline. In the example in Scheme I, crystallization of the diastereomeric dipeptide of **3f** gave the compound with the desired stereochemistry. The properties of the intermediate dipeptides are described in Table I. The final tripeptides were prepared by a convergent synthetic procedure including a mixed anhydride coupling of protected dipeptide units with arginine, protected as the lactam. The protected tripeptide lactams (**4f**) were reduced by $LiAlH_4$ to give the tripeptide aldehydes. Finally, the protecting groups were removed by catalytic hydrogenation and subsequent heating of the amorphous solid in presence of 5 N H_2SO_4. The excess acid in the deblocking was neutralized with Bio Rad AG-1X8 resin (hydroxide form), and the solution was freeze-dried. The free peptides were then purified by reversed-phase HPLC (RP-HPLC).

Enzyme Assays

All enzymes and substrates used were obtained from commercial sources and used without additional purification. Amidolytic assay for the synthetic inhibitors were carried out at 25 °C in 0.1 M Tris-HCl buffer (pH 7.4) using a known amount of enzyme and substrate. The enzyme and substrate concentrations for each assay are in parentheses following the respective reagents. Experiments were conducted in 96-well polystyrene plates, and rates of reactions were determined from hydrolysis rates by measuring the release of *p*-nitroaniline (using a Thermomax plate reader, Molecular Devices, San Francisco, CA) from Bz-Phe-Val-Arg-pNA (0.15 mg/mL or 0.125 mg/mL, respectively, with bovine thrombin at 0.77 U/mL and trypsin at 93 ng/mL), Bz-Ile-Glu-Gly-Arg-pNA (0.135 mg/mL with bovine factor Xa at 73.8 ng/mL), H-DVal-Leu-Lys-pNA (0.278 mg/mL with human plasmin at 270 ng/mL) or H-D-Ile-Pro-Arg-pNA (0.472 mg/mL with human recombinant tissue plasminogen activator at 81.6 ng/mL) using absorbance at 405 nm. Varying concentration of peptides were used to construct concentration–response curves for the IC_{50} values. The IC_{50}'s were then determined, after a 5-min preincubation period, as the concentration of peptide that results in 50% inhibition of enzyme activity relative to a blank control. No time-

† Symbols and abbreviations are in accordance with the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (*Eur. J. Biochem.* 1984, *138*, 9). All amino acids are in the L configuration unless otherwise noted. Other abbreviations used are as follows: Bz, benzoyl; 2,4,5-TCP, 2,4,5-trichlorophenol; Boc, *tert*-butyloxycarbonyl; TFA, trifluoroacetic acid; DCC, dicyclohexylcarbodiimide; DCHA, dicyclohexylamine; Chg, cyclohexylglycine; 1-Tiq, 1-carboxy-1,2,3,4-tetrahyroisoquinoline; MePhg, N^{α}-methylphenylglycine; Phg, phenylglycine; 3-Tiq, 3-carboxy-1,2,3,4-tetrahyroisoquinoline; Nag, naphthylglycine; Thg, 3-thienylglycine; 2-Tqu, 2-carboxy-1,2,3,4-tetrahydroquinoline; 2-Ind, 2-carboxyindoline; Phg(F), 4-fluorophenylglycine; Phg(OH), 4-hydroxyphenylglycine.

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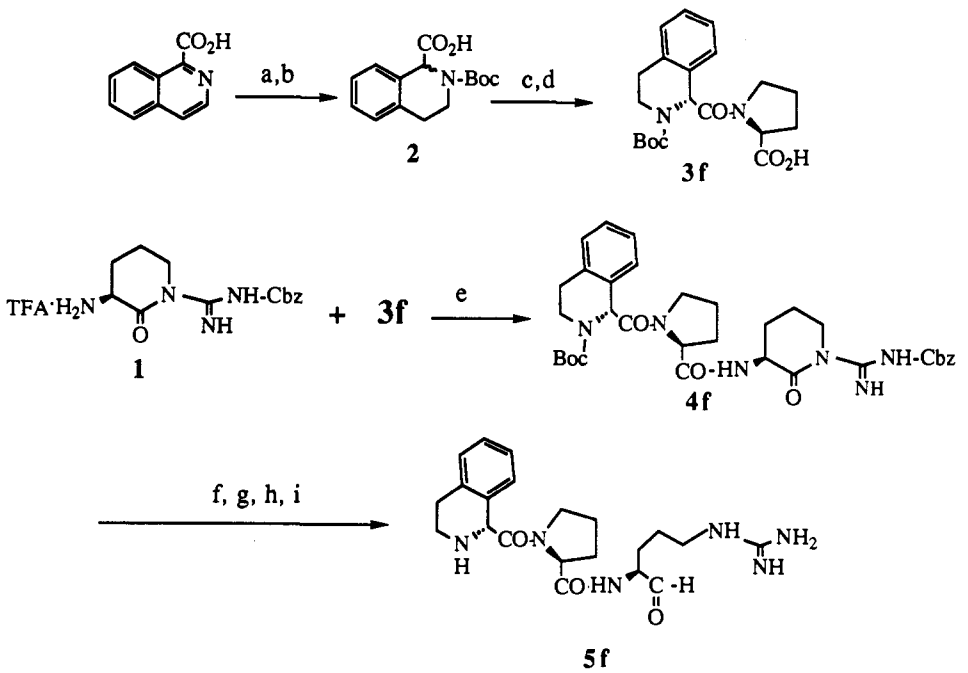
Scheme I[a]



[a] (a) H_2/5% Pt–C/60 psi; (b) Boc_2O/aq NaOH; (c) DCC/EtOAc/2,4,5-TCP; (d) pyridine/L-proline/Et_3N/crystallization; (e) $ClCO_2CH_2CH(CH_3)_2$/DMF/NMM/−20 °C; (f) $LiAlH_4$/THF/−20 °C; (g) H_2/5% Pd–C/THF/H_2O; (h) 5 N H_2SO_4/50 °C; (i) RP-HPLC.

Table I. Physicochemical Properties of the Dipeptides AA-Pro-OH

no.	AA	method[a]	formula	anal.[b]	FAB-MS[c]	$[\alpha]^{25}D$,[d] deg	TLC[e] R_f
3a	Boc-D-Chg	B	$C_{19}H_{32}N_2O_5$	NA[f]	NA[f]	NA[f]	0.54 (B)
3b	Boc-D-Phg		$C_{18}H_{24}N_2O_5$	C,H,N	349	−130.5	0.28 (A)
3c	Cbz-D-MePhg		$C_{23}H_{26}N_2O_5$	C,H,N	397	+1.6	0.33 (C)
3d	Boc-D-Phg		$C_{18}H_{24}N_2O_5$	C,H,N	349	−130.5	0.28 (A)
3e	Boc-D-2-Nag	A	$C_{22}H_{26}N_2O_5$	C,H,N	399	−185.9	0.34 (A)
3f	Boc-D-1-Tiq	B	$C_{19}H_{26}N_2O_5$	C,H,N	375	+43.1	0.44 (A)
3g	Boc-D-1-Nag	A	$C_{22}H_{26}N_2O_5$	C,H,N	399	+98.1	0.36 (A)
3h	Cbz-D-3-Tiq	B	$C_{23}H_{24}N_2O_5$	C,H,N	409	−21.5	0.40 (A)
3i	Boc-DL-Phg(3-CF_3)	A	$C_{19}H_{23}F_3N_2O_5$	NA[f]	NA[f]	NA[f]	0.40, 0.32 (A)
3j	Boc-DL-Phg(3,4-Cl)	A	$C_{18}H_{22}Cl_2N_2O_5$	C,H,N	418	−30.6	0.34 (A)
3k	Boc-DL-Phg(F)	A	$C_{18}H_{23}FN_2O_5$	H,N,F	367	−22.9	0.41 (A)
3l	Boc-D-Phe		$C_{19}H_{26}N_2O_5$	C,H,N	363	−55.7	0.39 (A)
3m	Boc-1-Nag	A	$C_{22}H_{26}N_2O_5$	C,H,N	399	+96.3	0.32 (A)
3n	Boc-D-Thg		$C_{16}H_{22}N_2O_5S$	C,H,N	355	−55.5	0.39 (A)
3o	Boc-DL-Phg(F)	A	$C_{18}H_{23}FN_2O_5$	H,N,F	367	−22.9	0.41 (A)
3p	Boc-D-Phg(OH)		$C_{18}H_{24}N_2O_6$	C,H,N	365	−187.2	0.22 (A)
3q	(*R*)-(+)-MeCH(Ph)CO		$C_{14}H_{17}NO_3$	C,H,N	248	−159.6	0.33 (A)
3r	(*R*)-(+)-EtCH(Ph)CO		$C_{15}H_{19}NO_3$	C,H,N	262	−153.9	0.34 (a)
3s	(*R*)-(+)-MeOCH(Ph)CO		$C_{14}H_{17}NO_4$	C,H,N	264	−65.6	0.30 (A)
3t	Boc-DL-2-Tqu		$C_{20}H_{26}N_2O_5$	C,H,N	375	−48.9	0.6 (A)
3u	Boc-DL-2-Ind		$C_{19}H_{24}N_2O_5$	C,H,N	360	−142.9	0.33 (A)
3v	(*S*)-(−)-MeCH(Ph)CO		$C_{14}H_{17}NO_3$	C,H,N	248	+23.3	0.38 (A)
3w	Boc-D-Pheψ[CH_2N]		$C_{19}H_{28}N_2O_4$	C,H,N	349	−18.9	0.38 (B)

[a] Method used for the synthesis of the AA component, all other dipeptides were prepared from commercially available acids or amino acids. [b] Compounds gave satisfactory analyses (±0.4%). [c] *M/Z* (NH^+). [d] *c* = 0.5, MeOH. [e] Solvent System used in parentheses. [f] NA = not available.

dependent inhibition with this class of inhibitors was observed. A more detailed description of the data analysis will be reported elsewhere.

Results and Discussion

In an effort to obtain improved enzyme inhibitory potency and selectivity over known tripeptide aldehydes, the evaluation of the structural and conformational role of the amino acid residue in position one of Boc-D-Phe-Pro-Arg-H (**5l**) was undertaken. A series of analogs was prepared and evaluated for their ability to inhibit various serine proteases (Table II). In order for these compounds to be therapeutically useful it is important that they do not inhibit the fibrinolytic processes through inhibition of the enzymes plasmin and tissue plasminogen activator (t-PA).[6] A measure of the predicted therapeutic usefulness of these inhibitors might be obtained by examination of the plasmin to thrombin or the t-PA to thrombin IC_{50} ratios, where higher values denote greater selectivity (Table III).

As a first approach, the effect of increasing the conformational flexibility of the peptide was examined by modification of the rigid amide bond between residues one and two. This was accomplished by replacement of this amide bond between the phenylalanine and proline by the dipeptide isostere Boc-D-Pheψ[CH_2N]Pro. This type of modification has led to peptides which exhibited unexpected biological activity.[7] However, in the present

Table II. In Vitro Enzyme Inhibitory Activity[a] of R-Pro-Arg-H

no.	R	thrombin	tgrypsin	plasmin	factor Xa	t-PA[b]
5a	Boc-D-Chg	0.012	0.013	0.081	0.081	2.3
5b	Boc-D-Phg	0.016	0.0098	0.098	0.26	8.7
5c	D-MePhg	0.018	0.018	0.49	2.2	42
5d	D-Phg	0.018	0.019	0.91	1.6	39
5e	Boc-D-2-Nag	0.018	0.0090	0.055	0.050	12
5f	D-1-Tiq	0.019	0.023	1.5	0.64	430
5g	Boc-D-1-Nag	0.023	0.014	0.62	1.4	20
5h	D-3-Tiq	0.025	0.019	0.37	4.1	90
5i	Boc-DL-Phg(3-CF_3)	0.027	0.019	0.13	1.4	15
5j	Boc-DL-Phg(3,4-Cl)	0.028	0.015	0.18	0.069	31
5k	Boc-D-Phg(F)	0.030	0.019	0.085	1.6	18
5l	Boc-D-Phe	0.045	0.014	0.19	1.6	0.95
5m	Boc-1-Nag	0.055	0.029	0.18	0.21	NA[c]
5n	Boc-D-Thg	0.073	0.013	0.15	0.19	13
5o	Boc-Phg(F)	0.085	0.024	0.77	6.7	91
5p	Boc-D-Phg(OH)	0.089	0.017	0.094	0.20	16
5q	(*R*)-(+)-MeCH(Ph)CO	0.094	0.18	18	31	>10
5r	(*R*)-(+)-EtCH(Ph)CO	0.13	0.35	21	33	>100
5s	(*R*)-(+)-MeOCH(Ph)CO	0.25	NA[c]	15	27	860
5t	DL-2-Tqu	3.1	0.57	15	12	660
5u	DL-2-Ind	3.6	0.26	18	20	420
5v	(*S*)-(−)-MeCH(Ph)CO	5.1	0.89	41	81	NA[c]
5w	Boc-D-Pheψ[CH_2N]	78	64	400	190	NA[c]

[a] IC_{50} (μM). Tissue plasminogen activator. [c] Not available.

Table III. In Vitro Enzyme Selectivity[a] R-Pro-Arg-H

no.	R	plasmin/ thrombin	t-PA[b]/ thrombin
5a	Boc-D-Chg	7	190
5b	Boc-D-Phg	6	540
5c	D-MePhg	30	2300
5d	D-Phg	50	2200
5e	Boc-D-2-Nag	3	670
5f	D-1-Tiq	80	23000
5g	Boc-D-1-Nag	30	870
5h	D-3-Tiq	15	3600
5i	Boc-DL-Phg(3-CF_3)	5	560
5j	Boc-DL-Phg(3,4-Cl)	6	1100
5k	Boc-D-Phg(F)	4	600
5l	Boc-D-Phe	4	20
5m	Boc-1-Nag	3	NA[c]
5n	Boc-D-Thg	2	180
5o	Boc-Phg(F)	9	1100
5p	Boc-D-Phg(OH)	1	180
5q	(*R*)-(+)-MeCH(Ph)CO	190	>100
5r	(*R*)-(+)-EtCH(Ph)CO	160	>800
5s	(*R*)-(+)-MeOCH(Ph)CO	60	3400
5t	DL-2-Tqu	5	210
5u	DL-2-Ind	5	120
5v	(*S*)-(−)-MeCH(Ph)CO	8	NA[c]
5w	Boc-D-Pheψ[CH_2N]	5	NA[c]

[a] Selectivity, defined as the ratio of IC_{50}'s. [b] Tissue plasminogen activator. Not available.

example, an analog (**5w**) was prepared which exhibited little or no ability to inhibit any serine protease. This finding could be the result of the introduction of a charged moiety (amine) where one did not previously exist, changes in the overall conformational space occupied by the new analog, or a combination of effects.

A second approach explored decreases in conformational flexibility of the peptide aldehydes. Conformational restriction can be achieved by introduction of sterically demanding amino acids. One such amino acid is phenylglycine. Peptides containing phenylglycine have fewer rotational degrees of freedom than those which contain phenylalanine since the aromatic ring is spatially closer to the peptide backbone. The substitution of the phenylalanine residue in **5l** with a phenylglycine residue gave **5b** which exhibited a 3-fold increase in potency with respect to its ability to inhibit the enzyme thrombin. This change was unexpected since the same modification in a similar series of thrombin inhibitors had been reported to produce a 10-fold decrease in potency.[8]

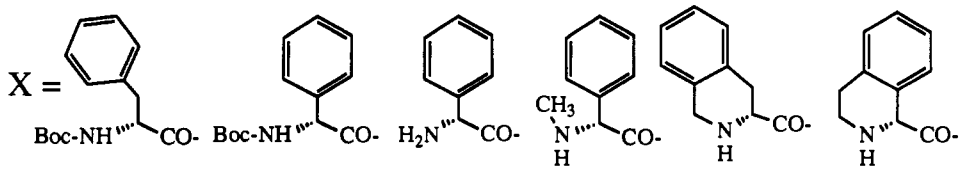
Substitutions on the aromatic ring of phenylglycine with the highly electronegative fluoro (**5k**) or trifluoromethyl (**5i**) moieties did not significantly alter potency or selectivity. Isolation of the L,L,L diasteroeisomer of **5k** (during the final RP-HPLC purification step) gave compound **5o** which exhibited a slight increase in selectivity (2×) along with the expected lower potency (5×). Incorporation of the hydroxy group on the aromatic ring of phenylglycine gave compound **5p** with decreased potency (6×) and selectivity (3×).

Replacement of the phenylglycine in **5b** with a different sterically demanding aromatic amino glycine residue (thienylglycine) resulted in analog **5n** which exhibited a loss in potency (4×) and selectivity (3×). In another analog the overall lipophilicity of peptide **5b** was increased by saturation of the aromatic ring to give the cyclohexylglycine analog **5a**. This analog was the most potent of all analogs tested in its ability to inhibit thrombin; however, it also exhibited a 3-fold loss in t-PA to thrombin selectivity.

Addition of steric bulk to an amino acid residue in a peptide can have the effect of decreasing conformational flexibility. The replacement of the phenylglycine residue in **5b** with either the D or L isomer of 1-naphthylglycine gave compounds **5q** and **5m**. Analog **5q** exhibited a 5-fold increase in the plasmin to thrombin selectivity without any significant loss in potency. The substitution of phenylglycine with either 2-naphthylglycine or **Phg**(3,4 Cl), the latter being an amino acid which is isosteric with 2-naphthylglycine, gave analogs (**5e** and **5j**) with slight decreases in antithrombotic potency and a slight improvement in the t-PA to thrombin ratio. However, the plasmin to thrombin ratios remained unchanged.

Removal of the Boc group in the phenylglycine analog **5b** gave an analog (**5d**) with equal potency and with a 4-fold increase in the selectivity toward thrombin versus t-PA. The addition of a methyl group to the amine of phenylglycine resulted in compound **5c** with no change in potency and selectivity. A similar finding was reported by Bajusz et al.[9-11] on a series of phenylalanine modifications. Replacement of the amino group of phenylglycine

X-Pro-Arg-H



Boc-D-Phe	Boc-D-Phg	D-Phg	D-MePhg	D-3-Tiq	D-1-Tiq
20	540	2200	2300	3600	23,000

Figure 1. The t-PA to thrombin selectivities of several conformationally constrained phenylalanine and phenylglycine analogs.

in **5d** with a methyl (**5q**), ethyl (**5r**), or methoxy (**5s**) group results in compounds with 5-, 7-, and 13-fold decreases in antithrombotic potency, respectively. However, these analogs exhibited dramatic decreases in their ability to inhibit other serine proteases such as plasmin and t-PA, resulting in very large plasmin to thrombin and t-PA to thrombin IC_{50} ratios.

One can introduce additional conformational restrictions by the use of physically constrained (bicyclic) aromatic amino acids. These amino acids possess bicyclic ring structures which incorporate the amino group of the amino acid. Thus, these analogs lack the rotational freedom of the phenylalanine or phenylglycine ring. Two of the constrained bicyclic amino acids incorporated into the 1 position gave compounds **5t** and **5u**. The nitrogen of these compounds are tied back directly to the aromatic ring (aniline amines) and exhibited little ability to inhibit any serine protease. Replacement of phenylglycine with the constrained amino acids D-3-carboxy-1,2,3,4-tetrahydroisoquinoline and D-1-carboxy-1,2,3,4-tetrahydroisoquinoline resulted in analogs (**5h** and **5f**) which exhibited no significant losses in potency. However, analog **5f** exhibited the highest degree of t-PA to thrombin selectivity (23 000) reported to date (Figure 1).

Conclusion

This work has demonstrated the critical importance of the amino terminal aromatic residue of compound **5l** toward the potent and selective inhibition of thrombin. The limited SAR of the phenylglycine aromatic residue resulted in moderate changes in potency and selectivity. In general, simple modifications of the amine in the amino terminus can have an adverse effect on potency and a beneficial affect on selectivity. Compounds **5c**, **5d**, **5f**, **5h**, **5r**, and **5s**, which possess phenylglycine or constrained aromatic amino acids, are clearly more selective than compound **5l** which possesses a phenylalanine residue. The findings from this series of thrombin inhibitors provide useful clues for the design of more potent and selective serine protease inhibitors. Furthermore, some of these compounds could be useful pharmacological tools for studies on the importance of the physiological role of thrombin and hold promise for development of clinically useful agents.

It is important to point out that this class of compounds also inhibit trypsin with approximately the same potency as compared to thrombin.[12] There is no compelling evidence that this fact imposes any limitations upon these compounds as potential anticoagulant agents. A point that should be noted is that plasma contains a number of nonspecific protease inhibitors (including α_1-antitrypsin, α_2-macroglobulin, inter-α-antitrypsin, and antithrombin-III) that could potentially inhibit trypsin.[13,14]

Experimental Section

Chemistry. The unnatural amino acids used in this study were obtained from commercial sources or prepared according to methods A or B and used without detailed characterization. The TFA·Arg(Cbz) lactam (**1**) in Scheme I was prepared according to a literature procedure.[5b] Reactions were monitored, and the homogeneity of the products was checked by TLC on Kieselgel-60 F_{254} plates (Merck, Darmstadt, BRG) with the following eluents (all v/v): (A) $CHCl_3$/MeOH/HOAc (135:15:1); (B) (18:6:1); (C) EtOAc/hexane (3:2). Analytical RP-HPLC was performed on an Pharmacia FPLC liquid chromatography instrument (LCC-500) with UV–visible detector at 214 nm, utilizing a Vydac C_{18} 5-μm particle size, 0.46- × 15-cm column, with eluent system 0.1% TFA (pH 2.0)/CH_3CN under gradient condition at a flow rate of 0.5 mL/min. Molecular weights of peptides were determined by fast-atom bombardment (FAB) mass spectra on an VG Analytical Zab 2 SE mass spectrometer. Amino acid analyses were performed on a Beckman System 6300 High-Performance Amino Acid Analyzer equipped with a 3-mm × 20-cm column of cation exchange resin (Na^+ form). Elemental analysis, indicated by symbols of the elements, refer to data within ±0.4% of the theoretical values. All reagents used were obtained from commercial sources and used without additional purification.

Preparation of Amino Acids (General Procedure, Method A): Boc-DL-Phg(3,4-Cl) (2j). Prepared by a modified method of Kukolja et al.[15] Isobutyl nitrite (29.8 mL, 0.25 mol) was added dropwise to a stirred (50 °C) solution of 3,4-dichlorophenylacetonitrile (37.2 g, 0.2 mol) in water (130 mL), MeOH (130 mL), and KOH (44.8 g, 0.8 mol). The resulting mixture was stirred at 50 °C for 2 h and heated to 90 °C under reflux for 24 h (the bulk of MeOH was allowed to boil off for 3 h). After the mixture cooled to room temperature, water (100 mL) and $CHCl_3$ (200 mL) was added. The aqueous phase was separated and extracted two times with $CHCl_3$. The aqueous layer was acidified to pH 2.0 with 5 N HCl and extracted with EtOAc (300 mL). The organic solution was dried ($MgSO_4$) and concentrated in vacuo to yield 46 g (98%) of the solid oximino acid. The solid (45.5 g, 0.194 mol) was dissolved in EtOH (200 mL) containing concentrated HCl (33 mL, 0.39 mol) and was hydrogenated in the presence of 5% Pd/C catalyst (3.0 g) at ambient temperature and pressure. After the reaction was completed (6 h), the catalyst was removed by filtration. The filtrate was concentrated to 100 mL, water (200 mL) was added, and the pH was adjusted to 6.0 with 2 N NaOH. The resultant precipitate was filtered and dried to give 24.3 g (57%) of DL-Phg(3,4-Cl). The amino acid (24 g, 0.11 mol) was dissolved in 2 N NaOH (110 mL, 0.22 mol) and *tert*-butyl alcohol (100 mL), and di-*tert*-butyl dicarbonate (28.5 g, 0.13 mol) was added to the reaction mixture. After 24 h at room temperature the bulk of the *tert*-butyl alcohol was evaporated, and the resulting aqueous solution was extracted once with diethyl ether. The aqueous layer was separated and acidified with 2 N HCl to pH 2.0 and extracted with ethyl acetate. The organic solution was dried ($MgSO_4$), filtered, and concentrated in vacuo to afford pure **2j** (17 g, 48%): MS (FAB) *m/e* 321 (MH^+). Anal. ($C_{13}H_{15}Cl_2NO_4$) C, H, N.

Preparation of Amino Acids (General Procedure, Method B): Boc-DL-1-Tiq·DCHA (2f). Prepared by the method of Shuman et al.[16] A solution of 1-isoquinolinecarboxylic acid (12.5 g, 0.072 mol) in glacial acetic acid (185 mL) was reacted with hydrogen over platinum oxide (2 g) at 60 psi in a Parr shaker

apparatus at room temperature for 24 h. The reaction mixture was filtered through a Celite pad, and the filtrate was concentrated in vacuo. The solid was triturated with water, filtered, and dried to give the amino acid (8 g, 63%). The solid (7.08 g, 0.040 mol) was dissolved in 2 N NaOH (40 mL, 0.080 mol) and *tert*-butyl alcohol (40 mL), and di-*tert*-butyl dicarbonate (10.5 g, 0.048 mol) was added to the reaction mixture. After 24 h at room temperature the bulk of the *tert*-butyl alcohol was evaporated, and the resulting aqueous solution was extracted once with diethyl ether. The aqueous layer was separated and acidified with 2 N HCl to pH 2.0 and extracted with ethyl acetate. The organic solution was dried ($MgSO_4$), filtered, and concentrated in vacuo. The oil was dissolved in diethyl ether and dicyclohexylamine (7.9 mL, 0.040 mol) was added to the solution. After 4 h of standing at 4 °C, the precipitate was filtered, washed with diethyl ether, and dried in vacuo to afford pure **2f** (15.7 g, 86%): MS (FAB) *m/e* 459 (MH^+). Anal. ($C_{27}H_{42}N_2O_4$) C, H, N.

Preparation of Peptides (General Procedure): Boc-D-1-Tiq-Pro (3f). Compound **2f** (73.4 g, 0.160 mol) was suspended in EtOAc (200 mL), washed with 1.5 N citric acid, water, dried ($MgSO_4$), and filtered. The EtOAc was removed in vacuo. The oil was dissolved in EtOAc and cooled to 0 °C, and 2,4,5-trichlorophenol (31.6 g, 0.160 mol) was added followed by DCC (33 g, 0.160 mol). The reaction was stirred for 1 h at 0 °C, warmed to room temperature, and stirred (1.5 h). The reaction was cooled (0 °C), the resultant precipitate was removed by filtration, and the mother liquor was concentrated in vacuo. The resultant oil was dissolved in pyridine (100 mL), and proline (18.42 g, 0.160 mol) and triethylamine (22.3 mL, 0.160 mol) were added to the reaction mixture. After the mixture stirred for 24 h at room temperature, the pyridine was removed in vacuo. The residue was dissolved in EtOAc/water and the pH adjusted to 9.5 with 2 N NaOH. The aqueous layer was separated, EtOAc was added, and the solution was acidified with 2 N HCl to pH 2.0. The organic layer was separated, dried ($MgSO_4$), filtered, and concentrated in vacuo. The oil was dissolved in CH_2Cl_2/EtOAc (1:1), and after standing at 4 °C for 4 h, the precipitate was filtered and washed with cold EtOAc. The solid was recrystallized once from CH_2Cl_2/EtOAc (1:1). The solid was then dried in vacuo to afford pure **3f**[17] (19.6 g, 33%). The physiochemical properties of the dipeptides are listed in Table I.

Boc-D-1-Tiq-Pro-Arg(Z) Lactam (4f). In flask 1, **3f** (17.8 g, 0.0475 mol) was dissolved in DMF (100 mL) and cooled to −15 °C, and *N*-methylmorpholine (5.3 mL, 0.0523 mol) was added followed by isobutyl chloroformate (6.2 mL, 0.0475 mol). The reaction mixture was stirred at −15 °C for 2 min. In flask 2, TFA·Arg(Z) lactam[4] (19.2 g, 0.0475 mol) was dissolved in DMF (40 mL) and cooled to 0 °C, and *N*-methylmorpholine (5.3 mL, 0.0523 mol) was added to the solution and stirred at 0 °C for 2 min. The contents of flask 2 was added to flask 1, and the reaction mixture was stirred for 4 h (−15 °C) followed by 24 h at room temperature. A solution of 5% $NaHCO_3$ (5 mL) was added, and the mixture was concentrated in vacuo. The residue was dissolved in EtOAc (175 mL) and water (150 mL). The organic layer was separated and washed sequentially with 5% $NaHCO_3$ (150 mL), water (150 mL), 0.01 N HCl (150 mL), and water (150 mL). The EtOAc layer was dried ($MgSO_4$), filtered, and concentrated in vacuo to give an amorphous solid **4f** (24.3 g, 79%): TLC R_f (A) 0.71; MS (FAB) *m/e* 647 (MH^+); $[\alpha]^{25}_D$ −32.8° (*c*, 0.5, $CHCl_3$).

D-1-Tiq-Pro-Arg-H·H_2SO_4 (5f). To a stirred, cooled (−60 °C) solution of **4f** (23.4 g, 0.0362 mol) under a N_2 atmosphere in dry THF (300 mL) was added lithium aluminum hydride 1 M in THF (37 mL, 0.037 mol). The reaction stirred for 30 min at −60 °C. A solution of 20 mL of THF and 20 mL of 0.5 N H_2SO_4 was added dropwise to the reaction. The reaction was diluted with EtOAc (400 mL) and water (400 mL). The EtOAc layer was separated and washed with water (2 × 150 mL), dried ($MgSO_4$), and filtered. The organic solvent was removed in vacuo to give an amorphous solid (21 g, 89%). The solid (18.1 g, 0.0279 mol), dissolved in THF (200 mL), water (80 mL), and 1 N H_2SO_4 (28 mL), was hydrogenated in the presence of 5% Pd/C catalyst (3.0 g) at ambient temperature and pressure. After the reaction was completed, the catalyst was removed by filtration. The filtrate was concentrated to 100 mL in vacuo and *n*-BuOH (200 mL) was added. The organic layer was separated, and the aqueous layer was extracted three times with *n*-BuOH (100 mL). The organic layers were combined and concentrated in vacuo. The residue was triturated with Et_2O/isopropyl ether (1:1), and the solid was filtered and dried to give 11.1 g of crude peptide. The peptide (10.8 g, 0.0192 mol) was dissolved in water (20 mL) and 10 N H_2SO_4 (20 mL), and the solution was heated at 50 °C (25 min) and cooled to room temperature, and the pH was adjusted to 4.0 with Bio Rad AG1-X8 resin (hydroxide form). The resin was removed by filtration and the filtrate lyophilized to give 8.44 g of crude **5f**. The crude **5f** (4.2-g portions) was dissolved in 0.01% H_2SO_4 (pH 2.5) and applied to a 5- × 50-cm column (Vydac C_{18} resin). A step gradient of increasing concentrations of CH_3CN (2% to 10%) was used to elute the peptide from the column. Fractions were collected and pooled on the basis of analytical reverse-phase HPLC profile. The combined fractions were adjusted to pH 4.0 using AG1-X8 resin (Bio Rad analytical anion exchange resin 50–100 mesh in hydroxide form). The solution was filtered, and the filtrate was lyophilized to give pure **5f** (4.8 g, 57%): MS (FAB) *m/e* 415 (MH^+); $[\alpha]^{25}_D$ −76° (*c* 0.5, 0.01 N H_2SO_4); amino acid analysis 1-Tiq 1.0, Pro 0.92. Anal. ($C_{21}H_{30}N_6O_3 \cdot H_2SO_4 \cdot 2.5H_2O$) C, H, N.

Boc-D-Chg-Pro-Arg-H·2HOAc (5a). Prepared from **3a** in a similar manner to that for **5f** except the hydrogenation was carried out in THF/water (1:1) containing glacial HOAc (2 mL). After removal of catalyst and isolation of the crude peptide by lyophilization the peptide was purified by dissolving in 0.1 M NH_4OAc and applied to a 5- × 25-cm column (Vydac C_{18} resin). A step gradient of increasing concentrations of CH_3CN (10% to 40%) was used to elute the peptide from the column. Fractions collected and pooled on the basis of analytical reverse-phase HPLC profile. The combined fractions were lyophilized to give pure **5a** (32%) as a lyophilized white solid: $[\alpha]^{25}_D$ −48° (*c* 0.5, 1 N HOAc); MS (FAB) *m/e* 495 (MH^+); amino acid analysis Pro 1.0, Chg 0.79. Anal. ($C_{24}H_{42}N_6O_4 \cdot 2C_2H_4O_2 \cdot H_2O$) C, H; N: calcd, 13.29; found, 14.24.

Boc-D-Phg-Pro-Arg-H·0.5H_2SO_4 (5b). Prepared from **3b** in a similar manner to that for **5f** as a lyophilized white solid (31%): MS (FAB) *m/e* 489 (MH^+); $[\alpha]^{25}_D$ −126° (*c* 0.5, $CHCl_3$); amino acid analysis Phg 0.92, Pro 1.0. Anal. ($C_{24}H_{36}N_6O_5 \cdot 0.5H_2SO_4$) C, H, N, S.

D-MePhg-Pro-Arg-H·H_2SO_4 (5c). Prepared from **3c** in a similar manner to that for **5f** as a lyophilized white solid (41%): $[\alpha]^{25}_D$ −151° (*c* 0.5, 0.01 N H_2SO_4); MS (FAB) *m/e* 403 (MH^+); amino acid analysis MePhg not calculated, Pro 1.0, Phg 0.002. Anal. ($C_{20}H_{30}N_6O_3 \cdot H_2SO_4$) C, H, N, S.

D-Phg-Pro-Arg-H·3HOAc (5d). Prepared from **3d** in a similar manner to that for **5a** as a lyophilized white solid (27%): MS (FAB) *m/e* 389 (MH^+); amino acid analysis Phg 0.90, Pro 1.0.

Boc-D-2-Nag-Pro-Arg-H·2HOAc (5e). Prepared from **3e** in a similar manner to that for **5a** as a lyophilized white solid (51%): MS (FAB) *m/e* 539 (MH^+). Anal. ($C_{28}H_{38}N_6O_5 \cdot 2C_2H_4O_2$) C, H, N.

Boc-D-1-Nag-Pro-Arg-H·2HOAc (5g). Prepared from **3g** in a similar manner to that for **5a** as a lyophilized white solid (22%): MS (FAB) *m/e* 539 (MH^+); $[\alpha]^{25}_D$ +18.9° (*c* 0.5, 50% HOAc). Anal. ($C_{28}H_{38}N_6O_5 \cdot 2C_2H_4O_2 \cdot 0.5H_2O$) C, H, N.

D-3-Tiq-Pro-Arg-H·H_2SO_4 (5h). Prepared from **3h** in a similar manner to that for **5f** as a lyophilized white solid (72%): MS (FAB) *m/e* 415 (MH^+); $[\alpha]^{25}_D$ −15.2° (*c* 0.5, 0.01 N H_2SO_4); amino acid analysis 3-D-Tiq 1.0, Pro 0.93. Anal. ($C_{21}H_{30}N_6O_3 \cdot H_2SO_4$) C, H, N, S.

Boc-DL-Phg(3-CF_3)-Pro-Arg-H·2HOAc (5i). Prepared from **3i** in a similar manner to that for **5a** as a lyophilized white solid (13%): MS (FAB) *m/e* 557 (MH^+). Anal. ($C_{25}H_{35}N_6O_5F_3 \cdot 2C_2H_4O_2 \cdot H_2O$) C, H, N.

Boc-DL-Phg(3,4-Cl)-Pro-Arg-H·HCl (5j). Prepared from **3j** in a similar manner to that for **5f** as a lyophilized white solid (64%): MS (FAB) *m/e* 557 (M^+); $[\alpha]^{25}_D$ −65° (*c* 0.5, 0.1 N HCl). Anal. ($C_{24}H_{34}N_6O_5Cl_2 \cdot 1.5H_2O$) C, H, N.

Boc-D-Phg(F)-Pro-Arg-H·HOAc (5k). Prepared from **3k** in a similar manner to that for **5a**. The two diastereomeric peptide aldehydes were separated during the RP-HPLC step. Aldehyde **5k** eluted second (separation factor α 1.02) and was obtained as a lyophilized white solid (3%): MS (FAB) *m/e* 507 (MH^+). Anal. ($C_{26}H_{39}N_6O_7F \cdot C_2H_4O_2 \cdot 2H_2O$) C, H, N.

Boc-D-Phe-Pro-Arg-H·2HOAc (5l). Prepared from **3l** in a similar manner to that for **5a** as a lyophilized white solid (42%):

MS (FAB) *m/e* 503 (MH^+); $[\alpha]^{25}_D$ -49.9° (*c* 1.0, $CHCl_3$); amino acid analysis Pro 1.0, Phe 1.0. Anal. ($C_{25}H_{38}N_6O_5 \cdot 2C_2H_4O_2$) C, H, N.

Boc-l-Nag-Pro-Arg-H·2HOAc (5m). Prepared from **3m** in a similar manner to that for **5a** as a lyophilized white solid (82%): $[\alpha]^{25}_D$ -137.5° (*c* 0.5, 50% HOAc); MS (FAB) *m/e* 539 (MH^+). Anal. ($C_{28}H_{38}N_6O_5 \cdot 2C_2H_4O_2$) C, H, N.

Boc-D-Thg-Pro-Arg-H·2HOAc (5n). Prepared from **3n** in a similar manner to that for **5a** as a lyophilized white solid (19%): MS (FAB) *m/e* 495 (MH^+); $[\alpha]^{25}_D$ -83.3° (*c* 0.5, 1 M HOAc). Anal. ($C_{22}H_{34}N_6O_5S \cdot 2C_2H_4O_2 \cdot H_2O$) C, H; N: calcd 13.28; found, 14.47.

Boc-Phg(F)-Pro-Arg-H·2HOAc (5o). Prepared from **3o** in a similar manner to that for **5a**. The two diastereomeric peptide aldehydes were separated during the RP-HPLC step. Aldehyde **5o** eluted first (separation factor α 0.97) and was obtained as a lyophilized white solid (9%): MS (FAB) *m/e* 507 (MH^+).

Boc-D-Phg(OH)-Pro-Arg-H·2HOAc (5p). Prepared from **3p** in a similar manner to that for **5a** as a lyophilized white solid (69%): MS (FAB) *m/e* 505 (MH^+); amino acid analysis Pro 1.0, Phg(OH) 0.98. Anal. ($C_{24}H_{36}N_6O_6 \cdot C_2H_4O_2 \cdot 3H_2O$) C, N; H: calcd, 7.49; found, 6.83.

(*R*)-(+)-MeCH(Ph)CO-Pro-Arg-H·2HOAc (5q). Prepared from **3q** in a similar manner to that for **5a** as a lyophilized white solid (6%): MS (FAB) *m/e* 388 (MH^+). Anal. ($C_{20}H_{29}N_5O_3 \cdot 2C_2H_4O_2 \cdot 0.5H_2O$) C, H, N.

(*R*)-(+)-EtCH(Ph)CO-Pro-Arg-H·2HOAc (5r). Prepared from **3r** in a similar manner to that for **5a** as a lyophilized white solid (30%): MS (FAB) *m/e* 402 (MH^+); $[\alpha]^{25}_D$ -134.5° (*c* 0.5, 1 M HOAc). Anal. ($C_{21}H_{31}N_5O_3 \cdot 2C_2H_4O_2 \cdot 0.25H_2O$) C, H; N: calcd, 13.31; found, 14.16.

(*R*)-(+)-MeOCH(Ph)CO-Pro-Arg-H·2HOAc (5s). Prepared from **3s** in a similar manner to that for **5a** as a lyophilized white solid (39%): MS (FAB) *m/e* 404 (MH^+); $[\alpha]^{25}_D$ -82.9° (*c* 0.5, 1 M HOAc). Anal. ($C_{20}H_{29}N_5O_4 \cdot 2C_2H_4O_2 \cdot 2H_2O$) C, H; N: calcd, 12.52; found, 13.09.

DL-2-Tqu-Pro-Arg-H·H_2SO_4 (5t). Prepared from **3t** in a similar manner to that for **5f** as a lyophilized white solid (48%): MS (FAB) *m/e* 415 (MH^+). Anal. ($C_{21}H_{30}N_6O_3 \cdot H_2SO_4 \cdot H_2O$) C, H, N.

DL-2-Ind-Pro-Arg-H·H_2SO_4 (5u). Prepared from **3u** in a similar manner to that for **5f** as a lyophilized white solid (79%): MS (FAB) *m/e* 401 (MH^+); $[\alpha]^{25}_D$ -49.2° (*c* 0.5, H_2O). Anal. ($C_{20}H_{28}N_6O_3 \cdot H_2SO_4 \cdot 2.5H_2O$) C, H; N: calcd, 15.46; found, 14.91.

(*S*)-(-)-MeCH(Ph)CO-Pro-Arg-H·2HOAc (5v). Prepared from **3v** in a similar manner to that for **5a** as a lyophilized white solid (6%): MS (FAB) *m/e* 388 (MH^+). Anal. ($C_{20}H_{29}N_5O_3 \cdot 2C_2H_4O_2 \cdot 0.5H_2O$) C, H, N.

Boc-D-Pheψ[CH_2N]Pro-Arg-H·2HOAc (5w). Prepared from **3w** in a similar manner to that for **5a** as a lyophilized white solid (36%): MS (FAB) *m/e* 489 (MH^+); $[\alpha]^{25}_D$ +5.1° (*c* 0.5, 1 M HOAc). Anal. ($C_{25}H_{40}N_6O_4 \cdot 2C_2H_4O_2$) C, H, N.

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