(m, 1 H, J = 7 Hz, C*H*Me$_2$), 4.25 (q, 2 H, J = 7.2 Hz, CO_2CH_2), 4.35 (dd, 1 H, J = 7.6, 2.0 Hz, H-4), 4.46 (dd, 1 H, J = 7.6, 7.0 Hz, H-5), 6.95 (d, 1 H, J = 2.0 Hz, H-2); ^{13}C NMR ($CDCl_3$) δ 14.1, 17.2, 32.18, 61.6, 70.1, 86.46, 156.67, 171.08.

(±)-*threo*-*N*-Methyl-β-hydroxyleucine (10). To a stirred solution of oxazoline **8** (1.0 g, 4.6 mmol) in dry Et_2O (4 mL) at 0 °C was added methyl triflate (1.06 g, 6.5 mmol) via a syringe. The reaction mixture was stirred at room temperature for 1 h under N_2 atmosphere and then diluted with ether (15 mL), and H_2O (15 mL) was added. The aqueous layer was separated and diluted with concentrated HCl (3 mL). The mixture was refluxed for 15 h and then concentrated. The oily orange hydrochloride salt **10** was purified by ion-exchange chromatograhy over a basic ion-exchange resin (Dowex-1, hydroxide form) column, eluting with H_2O and 0.2 N acetic acid. Fractions containing the product were pooled and lyophilized to give **10** (0.48 g, 64%) as an off-white powder. Recrystallization from aqueous acetone gave white needles: mp 238–240 °C dec; TLC (solvent C) R_f 0.46; IR (KBr), 1616 cm^{-1}; ^{1}H NMR (D_2O) δ 0.96 (d, 3 H, J = 6.5 Hz, $CHCH_3CH_3$), 0.97 (d, 3 H, J = 6.9 Hz, $CHCH_3CH_3$), 1.83 (m, 1 H, C*H*Me$_2$), 2.74 (s, 3 H, *N*-CH_3), 3.57 (d, 1 H, J = 7.2 Hz, H-2), 3.69 (dd, 1 H, J = 7.2, 5.1 Hz, H-3); ^{13}C NMR (D_2O) δ 18.47, 21.67, 32.83, 35.32, 69.94, 77.63, 174.60. Anal. Calcd for $C_7H_{15}NO_3$: C, 52.15; H, 9.40; N, 8.69. Found: C, 52.0; H, 9.22; N, 8.62.

***N*,*O*-Isopropylidene-*N*-methyl-β-hydroxyleucine (11c).** A suspension of *threo*-*dl*-*N*-methyl-β-hydroxyleucine [(MeLeu(3-OH)] (0.08 g, 0.49 mmol) in dry acetone (50 mL) was refluxed for 24 h (in the presence of N_2 atmosphere) until a clear solution was obtained. The solvent was concentrated to 0.5 mL and was used as such in the next step.

***N*,*O*-Isopropylidene-MeLeu(3-OH)-Abu-Sar-MeLeu-Val-MeLeu-Ala-OBzl (14c).** The above residue of **11c** was diluted with dry THF (5 mL), and *N*-methylmorpholine (0.55 mL, 0.5 mmol) was added. This solution was cooled to 0 °C, and a solution of hexapeptide **16b** (0.28 g, 0.4 mmol) in THF (3 mL) followed by 1-hydroxybenzotriazole (HOBT) (0.15 g, 1 mmol) and DCC (0.13 g, 0.6 mmol) was added. The reaction mixture was stirred at 0 °C for 4 h and 36 h at room temperature. The reaction mixture was cooled, filtered, evaporated to dryness, and worked up as for **16a**. The crude product was purified on a silica gel column using chloroform-methanol (99:1) as eluant, to give pure heptapeptide **14c** as oil: yield 0.28 g (73%); ^{1}H NMR ($CDCl_3$) multiple conformers δ 0.7–1.08 (m, 27 H, CH_3 of Abu, MeLeu, Val, MeLeu (3-OH), 1.2 (s, 6 H, CH_3 isopropylidene), 1.33 (d, 3 H, Ala-CH_3), 1.40–1.50 (m, 10 H, Abu, MeLeu, β-CH_2, MeLeu-γ-CH, Val β-CH), 2.18–3.23 (6 major s inside m, 13 H, *N*-Me and Sar α-CH), 3.34 (t, 1 H, α-CH), 3.5–5.27 (series of m, 8 H, α-CH and benzyl CH_2), 6.49 (d, 1 H, NH), 6.62 (d, 1 H, NH), 7.35 (s, 5 H, ArH), 7.8–8.1 (m, 1 H, NH).

***N*-MeLeu(3-OH)-Abu-Sar-MeLeu-Val-MeLeu-Ala-OBzl.** The protected peptide **14c** (0.18 g, 0.2 mmol) was stirred at room temperature in a solution of MeOH–1 N HCl (2:1) for 15 h under a N_2 atmosphere. The reaction mixture was concentrated and water (4 mL) added. The aqueous layer was basified with $NaHCO_3$ solution, and the oily material separated out was extracted with $CHCl_3$ (2 × 10 mL). The organic layer separated and washed with NaCl solution, dried (Na_2SO_4), and concentrated to give N-deprotected heptapeptide in 85% yield. ^{1}H NMR showed the loss of the isopropylidene group.

Biological Methods. Female, 4–5-week-old BALB/c mice were obtained from Harlan Sprague-Dawley, Indianapolis, IN, and were maintained in our laboratories until used. Murine single-cell thymocyte suspensions were prepared as described previously.[22] Briefly, excised thymus tissue was minced, pressed through wire mesh with a syringe barrel, and centrifuged at 200*g* for 3 min, and the cells were washed 1× with media and distributed into 96 well microtiter plates (10^6 cells/well) in a final volume of 250 μL. Con A was added to the cell suspension at a final concentration of 1 μg/mL. Inhibitors were added in 5 μL of 25% EtOH (controls received 5 μL of 25% EtOH alone). In these experiments, Dulbecco's MEM-Hams F12 (1:1, v/v) media containing 5 ng/L of insulin, 5 ng/L transferrin, and 5 pg/L of selenium was used in place of EHAA media with mouse serum. The level of mitogenic activation was determined at 24 and 48 h by pulsing with [^{3}H]thymidine for 12 h, followed by harvesting and measurement of cellular [^{3}H]-TdR incorporation by scintillation counting as described previously.[22]

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Potent Vasopressin Antagonists Lacking the Proline Residue at Position 7

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As part of a program to design potent antidiuretic vasopressin antagonists and to define the minimum effective pharmacophore requirements for vasopressin (VP) antagonist activity, we studied the importance of the C-terminal tripeptide of a previously reported peptide antagonist of arginine-vasopressin (AVP, **1**). The proline residue at position 7 in AVP is proposed to impart a conformational constraint to the peptide backbone that is essential for V_2-receptor agonist activity. Since the structure–activity relationships for VP agonists and antagonists are different, we investigated the effect of proline on antagonist activity, by synthesizing analogue **3** lacking this residue. This analogue was found to retain a high degree of antidiuretic antagonist activity. Since deletion of the Gly residue at position 9 of the antagonist did not adversely affect VP antagonist potency, several vasopressin antagonist analogues (**4–7** and **9**) that lacked both the Pro and Gly residues were also studied. These, too, were found to block vasopressin V_2-receptor activity. Our results indicate that neither the proline nor glycine residues are essential for antagonism of the V_2 receptor.

Some highly potent and selective antagonists of the antidiuretic and vascular effects of the nonapeptide arginine-vasopressin (AVP) have been described.[1–7] These antagonists were derived by modification of amino acid residues at various positions of AVP. In an effort to determine the miniumum structural requirements for anti-

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Table I. Vasopressin V_2-Receptor Antagonist Activity: Pmp-D-Tyr(Et)-Phe-Val-Asn-Cys-X[a]

no.	X	K_{bind},[b]	K_i,[c] nM	ED_{300},[d] μg/kg
1	Pro-Arg-Gly-NH_2[e]	12	6.4	11
2	Pro-Arg-NH_2	12	3.9	9
3	Arg-Gly-NH_2	13	2.7	23
4	Arg-NH_2	9.1	2.5	58
5	Lys-NH_2	26	5.8	59
6	D-Arg-NH_2	4.4	1.2	28
7	Harg-NH_2	31	13	193
8	Arg-Gly-OH	45	41	88
9	Arg-OH	25	23	150

[a] Abbreviations: Pmp, β-mercapto-β,β-pentamethylenepropionic acid; D-Tyr(Et) is D-tyrosine ethyl ether. All amino acids are in the L configuration unless otherwise noted. [b] K_{bind} is a measure of the affinity of ligand for receptor in the porcine renal medullary membrane. It is derived from the equation for competitive inhibition $K_b = IC_{50}/(1 + L/K_D)$[15] where IC_{50} is the concentration of the ligand for 50% inhibition of [^{3}H]LVP binding, L is the concentration of the ligand, and K_D is the dissociation constant of [^{3}H]LVP. [c] K_i is the inhibition constant measured for inhibition of LVP-stimulated adenylate cyclase of pig kidney medullary membrane and is derived from the equation for competitive inhibition as described.[16] Mean of triplicate determinations. [d] ED_{300} is the dose (μg/kg) required to decrease urine osmolality in rats from the hydropenic levels (~1500 mOsm/kg of H_2O) to 300 mOsm/kg of H_2O. [e] Compound 1 has been described in ref 3 as a potent antagonist in anesthesized rat model; the data presented for 1 in the table is generated in our laboratories for comparison.

diuretic antagonist activity and to design small, potent molecules, we investigated the significance of the C-terminal tripeptide (Pro-Arg-GlyNH$_2$)[8] of a potent antagonist 1 (Table I), originally described by Manning et al.[3] Independent studies in our laboratories[9] as well as those of Manning et al.[10] revealed that the Gly at position 9 in 1 can be deleted with retention of potent antagonist activity (cf. 2). To the contrary, removal of the glycinamide from the carboxy terminus of AVP drastically reduces its potency.[11] In the model proposed by Walter et al.[12] for the biologically active conformation of vasopressin agonists at the renal V_2 receptor, the proline at position 7 is proposed to play a key role in the orientation of the tripeptide tail with respect to the cyclic hexapeptide ring and is proposed to be an important binding element by virtue of its exposure as a corner residue in a β turn. Since we have shown that the pharmacophore requirements at the renal V_2 receptor differ for vasopressin agonists and antagonists,[13] a similar observation for other hormones have been documented,[14] a study was undertaken to determine if the proline residue is critical for antagonist activity. In order to address this question several peptide analogues, i.e., **3–9** (table I) as well as two agonist analogues **10** and **11** (Table II), all of which lack the Pro residue or both the Pro and Gly residues, were prepared.[15] In this paper the synthesis and preliminary in vitro (pig) and in vivo (rat) pharmacological properties of these peptides are reported.

Table II. Vasopressin V_2-Receptor Agonist Activity: X-Tyr-Phe-Val-Asn-Cys-Y[a]

no.	X	Y	K_{bind},[b] nM	K_a,[c] nM
10	Mpr	Arg-Gly-NH_2	11000	partial agonist with relative V_{max} of 17% at 3×10^{-5} M[d]
11	Mpr	Arg-NH_2	15300	partial agonist with relative V_{max} of 8% at 3×10^{-5} M
	Cys	Pro-Arg-Gly-NH_2(AVP)	4.2	0.67
	Cys	Pro-Lys-Gly-NH_2(LVP)	5.2	1.0

[a] Abbreviation: Mpr, β-mercaptopropionic acid. [b] K_{bind} as identified in footnote b, Table I. [c] K_a is the adenylate cyclase activation constant in the porcine renal medullary preparation. [d] Relative V_{max} is the maximum stimulation obtained by the compound divided by the V_{max} reached by LVP, multiplied by 100.

Peptide Synthesis

The protected peptide precursors required for the synthesis of each of the peptides **2–11** with the exception of **7** were prepared entirely by the solid-phase method of peptide synthesis[17,18] on either benzhydrylamine resin for the C-terminal amides or chloromethylated resin for the C-terminal acid. The homoarginine analogue **7** was prepared by converting the ε-amino group of the Lys analogue **5** to a guanidino group by use of aqueous *O*-methylisourea at pH 10.[19] The [(4-methylbenzyl)thio]-β,β-pentamethylenepropionic acid and the Boc-D-Tyr ethyl ether were synthesized according to published procedures.[20,21] Couplings of all the protected dicyclohexylcarbodiimide activated amino acid were facilitated by the addition of 1-hydroxybenzotriazole (HOBT). Cleavage of the peptides from the resin, with simultaneous deprotection of the side-chain protecting groups, was achieved with use of anhydrous HF. The deblocked disulfhydryl compounds were oxidatively cyclized with dilute aqueous $K_3Fe(CN)_6$. The peptides were purified by either counter-current distribution (CCD) or partition column chromatography

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followed by gel filtration on Sephadex G-15 and preparative HPLC on reversed-phase C-18 if necessary. All peptides were homogeneous by HPLC and TLC, and their structures were confirmed by FAB mass spectrometry and amino acid analysis.

Bioassay Methods

Peptides 1–11 were tested in vitro as previously described[22] for vasopressin V_2-receptor binding and antagonist activity on the pig renal medullary membrane preparations. Affinity for the receptors was determined by measuring the binding and is expressed as K_{bind}. The antagonist activity for analogues **1–9** was determined by their ability to inhibit LVP-stimulated adenylate cyclase in the same preparation and is expressed as the inhibition constant K_i. Agonist activity was determined by the ability of the peptides to activate adenylate cyclase and is expressed as K_a. The in vivo evaluation for the antagonists **1–9** was carried out as described[23,24] with use of the hydropenic rat model. Briefly, groups of male rats were deprived of food and water overnight to establish a stable hydropenic state, characterised by elevated plasma vasopressin levels and new maximal urine concentrating ability (urine osmolality ~1500 mOsm/kg of H_2O).[24] On the morning of the study, rats were housed in individual metabolism cages (four rats/cage) and administered vehicle or one of four doses of antagonist. Urine volume and osmolality (Uosm) were determined, and dose–response relationships for dilution of Uosm were developed. Potency was expressed as the effective dose (ED, μg/kg) required to dilute Uosm from hydropenic levels to plasma osmolality levels (300 mOsm/kg of H_2O), the level of Uosm at which the renal free water clearance switches from a net negative to a net positive value (e.g., water diuresis). Potency values presented represent average based upon one to three full dose–response relationships per peptide. One analogue, **2**, has been evaluated in >3 times and its ED_{300} was 8.7 ± 1.2 (mean ± SEM, n = 9).

Results and Discussion

The V_2-receptor affinity and antagonist activities of peptides **1–9** are presented in Table I. The affinity and agonist activity of analogues **10** and **11** are presented in Table II. As indicated in Table I, single deletions of the Gly and the Pro residues from **1** gave analogues **2** and **3**, which displayed potent antagonist activity both in vitro and in vivo. Simultaneous deletion of both the Gly and the Pro residues from the antagonist **1** resulted in analogues **4** and **5**, which have activity comparable to that of **1**. In contrast, deletion of the Pro residue or the Pro and Gly residues from the agonist AVP resulted in analogues **10** and **11** (Table II), which exhibited a greater than 1000-fold loss of affnity for the porcine V_2 receptor relative to AVP and LVP. These results imply that the presence of the Pro residue at position 7 in AVP is clearly important for binding and activation of the porcine V_2 receptor whereas it is not necessary for V_2-receptor antagonistic activity. Peptide **6**, which is the D-antipode of the L-argininamide **4** was prepared in an attempt to enhance enzymatic stability; analogue **6** exhibited antagonist activity similar to that of **4** (see Table I).

The importance of side-chain length in analogue **4** was probed with analogue **7**, which represents insertion of an addional methylene unit. The antagonist activity of **7** appears to be less than the lower homologue **4** (see Table I). This suggests that the length of the side chain of the basic amino acid may be important for interaction with the receptor.

To probe the importance of the C-terminal amide group on antagonist activity, analogues **8** and **9**, lacking the C-terminal amide, were prepared. These molecules, while still retaining significant antagonist activity, appeared to be less potent than their carboxamide counterparts (**3** and **4**).

In conclusion, we have shown that neither the Pro nor the Gly residue in the tripeptide tail of **1** appears essential for potent antidiuretic antagonist activity. Thus an effective pharmacophore for vasopressin V_2-receptor antagonist can be presented by molecules simultaneously lacking both the Pro and Gly residues. In contrast, deletion of Pro in AVP results in substantial loss of agonist activity. Finally, these results continue to support our hypothesis that the pharmacophore requirements for agonists and antagonists are different.

Experimental Section

The protected peptide resin intermediates required for the synthesis of peptides **2–11**, with the exception of **7**, were synthesized by the solid-phase method[17,18] on either benzhydrylamine resin for the C-terminal amides or chloromethylated resin for the C-terminal acid. Benzhydrylamine resin (BHA, 1% cross-linked S-DVB, 200–400 mesh, ~1.0 mequiv/g) was synthesized according to an improved procedure.[25] Chloromethylated resin (1% cross-linked S-DVB, 200–400 mesh, 1.0 mequiv/g) was obtained from Aldrich Chemical Co. Boc-Arg(Tos)-*O*-Bzl-resin (1% cross-linked S-DVB, 0.35 mequiv/g) was obtained from Peninsula Laboratories. Amino acid derivatives were supplied by Peninsula Laboratories or Chemalog Inc. Solvents and reagents were analytical grade, methylene chloride (CH_2Cl_2) was HPLC grade, dimethylformamide (DMF) was filtered from molecular sieves (4 Å) prior to its use, and trifluoroacetic acid (TFA) used for HPLC was redistilled prior to its use. The yield of pure peptides was not optimized. Greater emphasis was given to obtaining high purity, which resulted in decreased yields. When counter-current distribution (CCD, Graig-Post, 240 transfer)[26] or partition column chromatography (Sephadex G-25) were used for purification, 1-butanol–acetic acid–water (B:A:W, 4:1:5, v/v) solvent system was used. Purity and homogeneity of the peptides were routinely checked by high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC). HPLC was performed with 4.5 mm × 25 cm Altex Ultrasphere 5 μ ODS column for analytical work and 10 mm × 25 cm column for preparative work with UV detection at 220 nm. The following solvent system was used for HPLC: acetonitrile–water containing 0.1% TFA (20:80 to 50:50 in gradient or 40:60 in isocratic runs unless otherwise noted). TLC was on precoated silica gel (0.25 mm; 5 × 20 cm, E. Merck) and developed in one of the following solvent systems: A, 1-butanol–acetic acid–water–ethyl acetate (B:A:W:E, 1:1:1:1, v/v); B, 1-butanol–acetic acid-water (B:A:W, 4:1:5, v/v (upper phase)); C, 1–butanol–acetic acid–water–pyridine (B:A:W:P, 15:3:3:10, v/v). Loads of 10–20 μg were applied, and chromatograms were minimum length of 10 cm. Spraying with clorox (10%) and KI–starch (1%) solutions was used for detection. For amino acid analysis, peptides (~1.0 mg) were hydrolyzed for 18 h at 110 °C with concentrated hydrochloric acid–propionic acid (1:1, v/v, 1–2 mL) in evacuated and sealed ampules. The analyses were performed with a Kontron Liquimat III automatic amino acid analyzer. FAB

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Table III. Physicochemical Properties of Pmp-D-Tyr(Et)-Phe-Val-Asn-Cys-X Antagonists (1–9)

no.	X	formula	FAB (M + H)$^+$	amino acid analysis									R_f		
				Asp	Pro	Gly	Cys	Val	Tyr[a]	Phe	Lys	Arg	A[b]	B[c]	C[d]
2	Pro-Arg-NH_2	$C_{51}H_{74}N_{12}O_{10}S_2$	1079	1.09	1.37		0.42	1.00	0.61	1.17		1.00		0.4	
3	Arg-Gly-NH_2	$C_{48}H_{70}N_{12}O_{10}S_2$	1039	1.00		1.00	0.56	0.96	0.70	0.96		0.96	0.64		0.55
4	Arg-NH_2	$C_{46}H_{67}N_{11}O_9S_2$	982	1.06			0.37	1.00	0.65	0.91		1.00	0.60	0.52	
5	Lys-NH_2	$C_{46}H_{67}N_9O_9S_2$	954	1.00			0.43	0.98	0.55	0.98	1.01		0.63	0.36	
6	D-Arg-NH_2	$C_{46}H_{67}N_{11}O_9S_2$	982	1.00			0.40	1.01	0.62	1.09		1.12	0.70	0.49	
7	Harg-NH_2	$C_{47}H_{69}N_{11}O_9S_2$	996	1.00			0.47	0.91	0.18[e]	0.85		1.04	0.65	0.50	
8	Arg-Gly-OH	$C_{48}H_{69}N_{11}O_{11}S_2$	1040	1.00		1.08	0.43	1.03	0.46	1.07		0.98	0.62	0.38	
9	Arg-OH	$C_{46}H_{66}N_{10}O_{10}S_2$	983	1.00			0.27	0.95	0.55	0.99		1.07	0.60	0.27	

[a] This is the ratio of hydrolyzed deethylated Tyr from Tyr(Et), the low values observed here due to the presence of incomplete deethylation of Tyr(Et) which was observed in amino acid analysis. [b] The solvent system A is described in the Experimental Section. [c] The solvent system B is described in the Experimental Section. [d] The solvent system C is described in the Experimental Section. [e] Substantial amount of Tyr(Et) was observed.

Table IV. Physicochemical Properties of Mpr-Tyr-Phe-Val-Asn-Cys-X Agonist (10 and 11)

no.	X	formula	FAB (M + H)$^+$	amino acid analysis							R_f		
				Asp	Gly	Cys	Val	Tyr[a]	Phe	Arg	A	B	C
10	Arg-Gly-NH_2	$C_{41}H_{58}N_{12}O_{10}S_2$	943	0.99	1.00	0.54	1.01	0.72	0.88	0.94	0.60		0.55
11	Arg-NH_2	$C_{39}H_{55}N_{11}O_9S_2$	886	1.00		0.21	1.03	0.60	0.85	0.98	0.58		0.54

[a] See footnote *a*, Table IV.

mass spectrometry was performed by the Analytical, Physical and Structural Chemistry Department of Smith Kline & French Laboratories on the VG ZAB high-resolution spectrometer. FABMS gave a strong molecular ion peak cluster at (m + H)$^+$ of each peptide as well as (M – H)$^-$ in the negative ion spectra. Fragmentations of the molecules and the iminium ions of the form H_2N^+=CHR (where R = an amino acid side chain) were also observed. The protected peptide–resin intermediates prior to coupling of the β-[(4-methylbenzyl)thio]-β,β-pentamethylenepropionic acid (Pmp-4-MeBzl) or β-[(4-methylbenzyl)thio]-propionic acid (Mpr-4-MeBzl) were subjected to Edman degradation for detection of deleted peptides on the automated sequencer (Sequemat mini 15).[27] The physicochemical data for peptides **2–11** are shown in Tables III and IV.

Pmp(4-MeBzl)-D-Tyr(Et)-Phe-Val-Asn-Cys-(4-MeBzl)-Arg(Tos)-Gly-BHA-R (12). Boc-Gly-BHA-R was prepared by the symmetrical anhydride method[28] of $(Boc\text{-}Gly)_2O$ on BHA-resin (1.0 mmol/g) in DMF for 2 h to give a loading of Boc-Gly-BHA-R of 0.95 mmol/g by amino acid analysis. It was subjected to seven cycles of deprotection with 50% TFA–CH_2Cl_2 (v/v), neutralization with 7% *N,N*-diisopropylethylamine (DIEA–CH_2Cl_2, v/v), and coupling with protected amino acids.[17,18] The protected amino acids (3.0 mmol) were activated with dicyclohexylcarbodiimide (DCC, 3.0 mmol), and coupling was facilitated by the addition of *N*-hydroxybenzotriazole (HOBT, 6.0 mmol). β-[(4-Methylbenzyl)thio]-β,β-pentamethylenepropionic acid (Pmp-4-MeBzl) was activated with DCC (3.0 mmol), and coupling was facilitated with 4-(dimethylamino)pyridine (DMAP,3.0 mmol). All coupling steps were carried out in DMF–CH_2Cl_2 solvent mixture for 2–4 h, using either an automated peptide synthesizer (Beckman 990B) or manual shaker. Completion of the coupling reactions was monitored by the ninhydrin test.[29] A general protocol used for each coupling on the resin was as follows: (1) wash with CH_2Cl_2 (three times, 1 min), (2) prewash with TFA–CH_2Cl_2 (1:1, v/v, 1 min), (3) deprotection with TFA–CH_2Cl_2 (1:1, v/v, 20 min), (4) wash with CH_2Cl_2 (six times, 1 min), (5) prewash with DIEA–CH_2Cl_2 (7%, v/v, 1 min), (6) neutralize with DIEA–CH_2Cl_2 (7%, v/v, 8 min), (7) wash with CH_2Cl_2 (four times, 1 min), (8) couple protected amino acid (3 mmol), HOBT (6.0 mmol) in DMF–CH_2Cl mixture, followed by DCC (3.0 mmol) for 2–4 h, (9) wash with DMF two times, 1 min), (10) wash with ethanol–CH_2Cl_2 (1:1, v/v, three times, 1 min), and (11) wash with CH_2Cl_2 (four times, 1 min). The protected peptide–resin intermediate was washed with a series of solvents and dried in vacuo.

Pmp-D-Tyr(Et)-Phe-Val-Asn-Cys-Arg-Gly-NH_2 (3). The protected peptide-resin intermediate **12** (2.0 g) in anisole (2.5 mL) was cleaved and deprotected with anhydrous liquid HF, 25 mL at 0 °C for 1 h, using Teflon Kel-F vacuum line in the hood. The HF was evaporated and the residue was treated with anhydrous ether to eliminate anisole and byproducts. The crude peptide was extracted from the resin with degassed DMF (50 mL) and acetic acid (HOAc, 33%, 50 mL) into 4 L of degassed water. The aqueous diluted disulfhydryl octapeptide was oxidatively cyclized with use of 0.01 M potassium ferricyanide solution at pH 7.2 until color persisted for 30 min. After the completion of the oxidation reaction, the pH of the solution was adjusted to 4.5 with use of glacial HOAc. This solution was passed through a weakly acidic cation-exchange resin (Bio-Rex 70, 50–100 mesh, 2.5 × 12 cm column). The column was eluted with pyridine–acetate buffer (pyridine–HOAc–H_2O, 30:4:66, v/v). The pyridine acetate was removed by distillation in vacuo, and the residue was lyophilized from 10% HOAc to give 420 mg (42.6%) of partially purified peptide. It was purified (250 mg) on CCD followed by preparative reverse-phase isocratic HPLC (50 mg) to give 24 mg of pure peptide.

Pmp-D-Tyr(Et)-Phe-Val-Asn-Cys-Pro-Arg-NH_2 (2). The protected peptide-resin intermediate Pmp-(4-MeBzl)-D-Tyr-(Et)-Phe-Val-Asn-Cys(4-MeBzl)-Pro-Arg(Tos)-BHA-R was synthesized from Boc-Arg(Tos)-BHA-R in seven cycles of deprotection, neutralization, and coupling similar to that for **12**. Boc-Arg(Tos)BHA-R was prepared from Boc-Arg(Tos) (3.0 mmol) on BHA-resin (1.0 mmol), using DCC (3.0 mmol) and HOBT (6.0 mmol). Anhydrous HF cleavage–deprotection, oxidative cyclization, and purification by flash chromatography on a C-18 reverse-phase column using 50% aqueous CH_3CN containing 1% TFA as an eluent gave 350 mg of partially purified peptide. Further purification on CCD, followed by gel filtration on a Sephadex G-15 column using 0.2 M HOAc as an eluent gave 122 mg of **2**.

Pmp-D-Tyr(Et)-Phe-Val-Asn-Cys-Arg-NH_2 (4). The protected peptide-resin intermediate Pmp(4-MeBzl)-D-Tyr(Et)-Phe-Val-Asn-Cys(4-MeBzl)-Arg(Tos)-BHA-R was synthesized from Boc-Arg(Tos)-BHA-R in six cycles of deprotection, neutralization, and coupling similar to that for **12**. Boc-Arg(Tos)-BHA-R was prepared from Boc-Arg(Tos) (1.5 mmol) on BHA-resin (0.5 mmol), using DCC (1.5 mmol) and HOBT (3.0 mmol) to give a loading of 0.70 mmol/g. Anhydrous HF cleavage–deprotection, oxidative cyclization, and purification on Bio-Rex 70

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gave 80 mg (31%) of partially purified peptide. Purification by partition column chromatography followed by preparative reverse-phase isocratic HPLC (42 mg) gave 37 mg of 4.

Pmp-D-Tyr(Et)-Phe-Val-Asn-Cys-Lys-NH_2 (5). The protected peptide-resin intermediate Pmp(4-MeBzl)-D-Tyr(Et)-Phe-Val-Asn-Cys(4-MeBzl)-Lys(Cl-Z)BHA-R was synthesized from Boc-Lys(Cl-Z)-BHA-R in six cycles of deprotection, neutralization, and coupling similar to that for 12. Boc-Lys(Cl-Z)-BHA-R was prepared from Boc-Lys(Cl-Z) (3.0 mmol) on BHA (1.0 mmol), using DCC (3.0 mmol) and HOBT (6.0 mmol) to give a 0.97 mmol/g loading. Anhydrous HF cleavage–deprotection and oxidative cyclization followed by purification on Bio-Rex 70 gave 463 mg (50.3%) of partially purified peptide. Purification by partition column chromatography (65 mg) followed by Bio-Gel polyacrylamide P2 column using HOAc (0.2 M) as an eluent gave 15.0 mg of pure 5.

Pmp-D-Tyr(Et)-Phe-Val-Asn-Cys-D-Arg NH_2 (6). The protected peptide-resin intermediate Pmp(4-MeBzl)-D-Tyr-(Et)-Phe-Val-Asn-Cys(4-MeBzl)-D-Arg(Tos)-BHA-R was synthesized from Boc-D-Arg(Tos)-BHA-R in six cycles of deprotection, neutralization, and coupling similar to that for 12. Boc-D-Arg-(Tos)-BHA-R was prepared from Boc-D-Arg(Tos) (3.0 mmol) on BHA-resin (1.0 mmol), using DCC (3.0 mmol) and HOBT (6.0 mmol) to give a loading of 0.62 mmol/g. Anhydrous HF cleavage–deprotection and oxidative cyclization followed by purification on Bio-Rex 70 gave 279 mg (45%) of partially purified peptide. Further purification through partition column (150 mg), followed by preparative isocratic HPLC (52 mg), gave 41.50 mg of pure 6. Coinjection of 6 with the L-Arg isomer 4 showed a separation of the two diastereomeric peptides.

Pmp-D-Tyr(Et)-Phe-Val-Asn-Cys-Harg-NH_2 (7). To a solution of *O*-methylisourea hydrogen sulfate 294 mg (1.7 mmol) in H_2O (8.0 mL) at pH 10.0 (using 2 N aqueous NaOH) was added an aqueous solution of peptide 5 (26.7 mg, 28 μmol) in water (5 mL). The reaction mixture was stirred at room temperature for 24 h. The pH of the solution was readjusted to 10 (2 N NaOH) every hour for the first 5 h. The progress of the reaction was monitored with reverse-phase HPLC to >90% completion. The reaction mixture pH was adjusted to 4.5 (1% HOAc), concentrated to dryness in vacuo, and purified by preparative isocratic HPL to give 18 mg of 7.

Pmp-D-Tyr(Et)-Phe-Val-Asn-Cys-Arg-GlyOH (8). The protected peptide-resin intermediate Pmp(4-MeBzl)-D-Tyr-(Et)-Phe-Val-Asn-Cys(4-MeBzl)-Arg(Tos)-GlyO-Bzl-R was synthesized from Boc-GlyOBzl-R in seven cycles of deprotection, neutralization, and coupling. Boc-Gly-OBzl-R was prepared from Boc-Gly and chloromethylated resin to an incorporation of 0.58 mmol/g by cesium salt method.[28] Anhydrous HF cleavage–deprotection, and oxidative cyclization followed by flash chromatography on a C-18 reverse-phase column using 50% aqueous CH_3CN containing 1% TFA as an eluent gave 938 mg (86%) of partially purified 8. Further purification by preparative reverse-phase isocratic HPLC (50 mg) using 45:55 CH_3CN–H_2O containing 0.1% TFA gave 40 mg of 8.

Pmp-D-Tyr(Et)-Phe-Val-Asn-Cys-ArgOH (9). The protected peptide-resin intermediate Pmp(4-MeBzl)-D-Tyr(Et)-Phe-Val-Asn-Cys(4-MeBzl)-Arg(Tos)-OBzl-R was synthesized from Boc-Arg(Tos)-OBzl-R (Peninsula, 0.35 mequiv/g) in six cycles of deprotection, neutralization, and coupling. Anhydrous HF cleavage–deprotection and oxidative cyclization followed by flash chromatography as described for 8 gave 245 mg (25%) of partially purified 9. Further purification by preparative isocratic HPLC (22 mg) gave 10 mg of pure 9.

Mpr-Tyr-Phe-Val-Asn-Cys-ArgGly-NH_2 (10). The protected peptide-resin intermediate Mpr(4-MeBzl)-Tyr-Phe-Val-Asn-Cys(4-MeBzl)Arg(Tos)-Gly-BHA-R was synthesized from Boc-Gly-BHA-R in seven cycles of deprotection, neutralization, and coupling similar to 12. Boc-Gly-BHA-R was prepared from Boc-Gly and BHA-resin to an incorporation of 1.0 mmol/g. Anhydrous HF cleavage–deprotection and oxidative cyclization followed by flash chromatography as described for the preparation of 8 gave 703 mg (51%) of partially purified 10. Further purification by CCD, followed by gel filtration on Sephadex G-15 (83 mg) eluted with aqueous HOAc (0.2 M), gave 48 mg of 10.

Mpr-Tyr-Phe-Val-Asn-Cys-Arg-NH_2 (11). The protected peptide-resin intermediate Mpr(4-MeBzl)-Tyr-Phe-Val-Asn-Cys(4-MeBzl)-Arg(Tos)-BHA-R was synthesized from Boc-Arg-(Tos)-BHA-R in six cycles of deprotection, neutralization, and coupling. Boc-Arg(Tos)-BHA-R was prepared from Boc-Arg(Tos) and BHA-resin to an incorporation of 0.45 mmol/g. Anhydrous HF cleavage–deprotection, oxidative cyclization, and purification by flash chromatography on C-18 reverse-phase column using 50% aqueous CH_3CN containing 0.1% TFA gave 12.0 mg of purified material. Further purification by preparative reverse-phase isocratic HPLC using 70:30 aqueous CH_3CN containing 0.1% TFA gave 8.0 mg of pure 11.

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