Synthesis and Biological Property of α-Human Atrial Natriuretic Peptide Analogs with a Constrained or Stereochemically Modified Cyclic Moiety[1)]

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Conformationally restricted analogs of α-human atrial natriuretic peptide (α-hANP) containing L- or D-penicillamine, or D-cysteine in place of cysteine residues at positions 7 and 23 were synthesized by the liquid phase procedure. Their biological properties in the assays of receptor binding and cyclic guanosine monophosphate (cGMP) accumulation employing rat vascular smooth muscle cells (VSMC), vasorelaxant activity using rat isolated aorta were evaluated. We found that the constrained and/or stereochemically altered ring moiety generally did not influence the receptor binding activity, however, cGMP accumulation and vasorelaxant activities were quite sensitive to conformational perturbation. Furthermore, a lack of correlation between cGMP accumulation activity and vasorelaxant activity was observed. Dissociation between these activities was typical in the case of [^{D}Pen^{7,23}]-α-hANP(7—28), which showed quite weak vasorelaxant activity in spite of its full cGMP accumulation and receptor binding potencies. This result suggests that cGMP accumulation alone is not sufficient to promote ANP-induced vasorelaxation, and that the other second messenger(s) may mediate this activity.

Keywords α-human atrial natriuretic peptide analog; liquid phase synthesis; biological activity; cyclic GMP; receptor binding; vasorelaxation; vascular smooth muscle cells (VSMC)

Atrial natriuretic peptide (ANP) comprises a family of peptides with potent natriuretic/diuretic and peripheral vasodilatory activities which are present in the storage granules of atrial muscle. This hormone has been recognized as playing an important role in pressure/volume homeostasis. Several low-molecular weight (M.W.) (around 3000) peptides with different chain lengths such as atriopeptins (I, II, and III)[2)] and α-rat atrial natriuretic peptide (α-rANP)[3)] have been isolated from rat atria, however, the major circulating form in the rat appeared to be a 28-residue peptide.[4,5)] Alpha-human atrial natriuretic peptide (α-hANP)[6)] was isolated by Kangawa and Matsuo, and this type has also proved to be the major circulating form in human.[7)] Figure 1 shows the primary structure of α-hANP. This hormone is a 28-amino acid peptide having a 53-membered cyclic structure created by two half cystine^{7,23} flanking a hexapeptide head and a pentapeptide tail.

Following the structure elucidation of rat or human ANP, extensive studies on the relationship between structure and activity have been carried out in an attempt to obtain a more active and/or selective agonist or to clarify the molecular mechanisms of ANP actions. Using fragment analogs, it has become clear that the N-terminal exocyclic hexapeptide (H–Ser–Leu–Arg–Arg–Ser–Ser–) is unessential for diuresis/natriuresis or hypotensive activity.[8)] In contrast, the C-terminal exocyclic portion (–Asn–Ser–Phe–Arg–Tyr–OH) is critical for the expression of several ANP actions.[8a,c)] The importance of the cyclic structure was demonstrated by several groups,[9)] *e.g.*, a linear analog such as [Cys(Acm)^{7,23}]-α-hANP, which is unable to cyclize during assay, showed only 1/40 the diuretic activity of α-hANP.[9b)] This result implies that the cyclic form *via* two half cystine plays the critical role for the induction of the ANP activities. In addition the importance of the sulfur atoms in Cys residues is apparent by comparing the following results: Replacement of the disulfide bond in the molecule by a carba-type unit, which has been successfully applied to the eel-calcitonin analog with prolonged activity,[10)] resulted in reduced potencies.[11)] However, the analog substituted by mercaptopropionic acid (Mpr), having the same ring size, showed remarkably enhanced activities *in vivo*.[12)] These results strongly suggest that the disulfide acts not only to hold the ANP molecule in adequate conformation but to interact directly with the receptors discriminating between the receptor subtypes.

Based on these results, we were interested in the disulfide moiety which was supposed to be important as a trigger of ANP actions and we synthesized a series of analogs in which conformational changes were restricted. Our approaches were as follows: i) to reduce the flexibility in the ring moiety by introducing the Pen residue in which two methyl groups are contained in place of hydrogen atoms on the β-carbon in the cysteine residue, ii) to provide a stereochemically altered ring structure by introducing ^{D}Cys or ^{D}Pen residue. These approaches have been successfully applied to oxytocin or vassopressin analogs giving antagonistic property on the molecules.[13)] As mentioned before, a fully active fragment analog, α-hANP(7—28) [abbreviated as NP], was chosen for the parent compound. Using these analogs, we examined the effects of constrained or stereochemically modified cystine moiety on the ANP actions and discussed the relationships between receptor binding, cyclic guanosine monophosphate (cGMP) accumulation and vasorelaxant activities.

Peptide Synthesis All the peptide analogs were synthesized by the liquid phase procedure applying the maximum protection method combined with HF deprotection.[14)] The following protected amino acids were used for the synthesis:

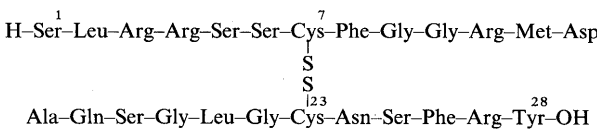

Fig. 1. Primary Structure of α-hANP

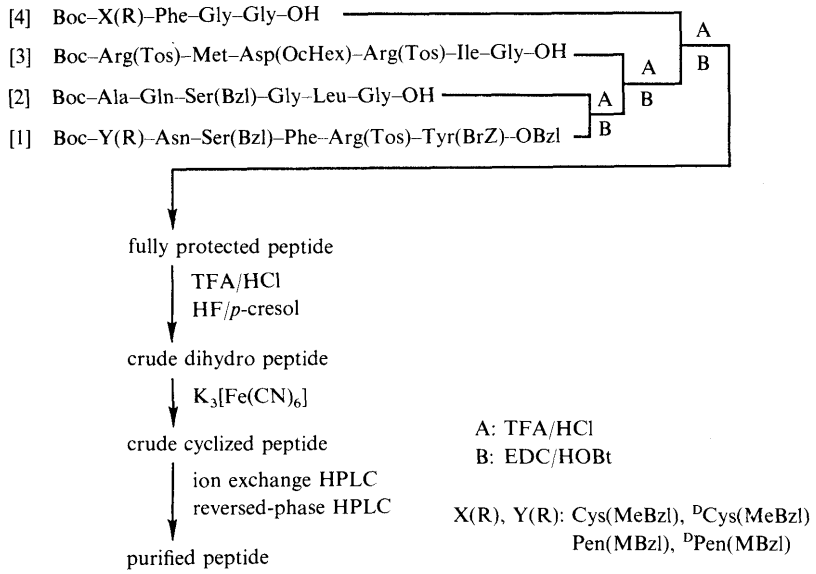

Fig. 2. Synthetic Scheme for [X^7,Y^{23}]-α-hANP(7—28)

TABLE I. Yields and Physical Constants of [X^7,Y^{23}]-α-hANP(7—28)

Compd. No.	Position 7	Position 23	Yield[a] (%)	$[\alpha]_D^{30}$ (c, 1% AcOH) (deg)		TLC[b] Rf_6	TLC[b] Rf_7	HPLC[c] (min)	Purity (%)	$[M+H]^+$ Found	$[M+H]^+$ Calcd
1	D-Cys	Cys	34	−38.1	(0.2)	0.46	0.53	16.09	99.8	2393.1	2391.1
2	Pen	Cys	27	−39.4	(0.5)	0.49	0.57	15.94	99.8	2421.1	2421.1
3	D-Pen	Cys	29	−37.4	(0.5)	0.49	0.57	16.99	99.5	2421.1	2421.1
4	Cys	D-Cys	33	−27.0	(0.6)	0.49	0.53	15.68	99.3	2393.1	2393.1
5	D-Cys	D-Cys	38	−20.6	(0.6)	0.47	0.51	16.14	98.3	2393.1	2393.1
6	Pen	D-Cys	28	−28.7	(0.5)	0.48	0.51	16.03	98.3	2421.1	2421.1
7	D-Pen	D-Cys	26	−17.2	(0.6)	0.49	0.51	17.10	98.9	2421.1	2421.1
8	Cys	Pen	17	−23.3	(0.4)	0.47	0.54	15.81	97.5	2421.1	2421.1
9	D-Cys	Pen	18	−34.9	(0.4)	0.46	0.53	16.48	98.7	2421.1	2421.1
10	Pen	Pen	13	−27.7	(0.4)	0.49	0.53	16.49	99.0	2449.1	2449.1
11	D-Pen	Pen	18	−27.8	(0.3)	0.47	0.54	17.57	98.3	2449.1	2449.1
12	Cys	D-Pen	18	−31.9	(0.4)	0.48	0.51	15.97	98.7	2421.1	2421.1
13	D-Cys	D-Pen	15	−28.7	(0.6)	0.49	0.53	16.44	97.8	2421.1	2421.1
14	Pen	D-Pen	11	−28.2	(0.4)	0.47	0.51	16.88	99.3	2449.1	2449.1
15	D-Pen	D-Pen	9	−34.4	(0.3)	0.47	0.51	18.13	97.0	2449.1	2449.1

a) Yields were calculated from the protected peptide and were not optimized. Amino acid analyses gave the acceptable ratios (data not shown). *b*) *n*-BuOH–AcOH–Pyr–H_2O: $Rf_6 = 4:1:1:2$, $Rf_7 = 30:6:20:24$. *c*) A: 0.1% TFA, B: 50% CH_3CN in 0.1% TFA, flow rate: 1 ml/min, gradient from B = 30 to B = 100 for 30 min. (α-hANP: 14.67 min).

Arg(Tos), Asp(OcHex), L-, D-Cys(MeBzl), L-, D-Pen(MBzl), Ser(Bzl), and Tyr(BrZ). As shown in Fig. 2, the peptide was divided into four fragments containing the Gly residue at the C-terminal end. These fragments were synthesized in a stepwise manner using Boc for the α-amino protecting group and Pac ester for the α-carboxyl protecting group except for fragment [1] with Bzl ester.

Preparation of the fragments: Starting from H–Tyr-(BrZ)–OBzl, fragment [1] was prepared by stepwise elongation using the EDC/HOBt method. Fragment [2] was prepared as follows: Since direct aminoacylation of H–Gly–OPac usually causes serious side reactions due to the formation of a cyclic schiff base, we first prepared Boc–Leu–Gly–OEt by DCC for the preparation of Boc–Leu–Gly–OPac. After saponification of this ethyl ester, the resulting Boc–Leu–Gly–OH was reacted with phenacyl bromide to provide Boc–Leu–Gly–OPac. Boc–Ser(Bzl)–Gly–OH · DCHA, which was prepared from the corresponding ethyl ester by saponification, was coupled to the TFA/HCl treated sample of Boc–Leu–Gly–OPac to provide Boc–Ser(Bzl)–Gly–Leu–Gly–OPac. From this, Boc–Gln–OH, and Boc–Ala–OH were assembled by EDC/HOBt in a stepwise manner, and finally fragment [2] was obtained by Zn/AcOH treatment. Fragment [3] was prepared, starting from Boc–Ile–Gly–OPac in a similar manner, by stepwise coupling using EDC/HOBt followed by Zn/AcOH treatment. Fragment [4] was prepared as follows: Boc–Gly–OH was coupled to H–Gly–OBzl by DCC to give Boc–Gly–Gly–OBzl which was hydorogenated and then converted to the corresponding phenacyl ester, giving Boc–Gly–Gly–OPac. Stepwise couplings were followed, and finally fragment [4] was obtained by Zn/AcOH treatment.

Fragment coupling: Starting from the TFA/HCl treated sample of fragment [1], 1.0—1.03 eq of fragments [2], [3], and [4] were successively assembled using EDC/HOBt. Low solubility of the generating intermediates in DMF

was overcome by use of additional DMSO and NMP. Each coupling was completed within 6 h and the minimum usage of acyl component made the purification simple. After each coupling, precipitation with boiling MeOH of the product actually gave a pure intermediate.

Deprotection: Each of the fully protected peptides (150—300 mg) was treated with HF/*p*-cresol (8.5/1.5 ml) at −2 °C for 1 h. The deprotected peptide thus obtained was converted to the corresponding acetate by passing it through a column of Bio Rad AG1-X2, and the eluate was concentrated by evaporation.

Cyclization: Oxidative cyclization by use of $K_3[Fe(CN)_6]$ was performed applying the same procedure as reported by Rivier *et al.*[15] This procedure was achieved by reversing du Vigneaud's procedure[16] which had been employed for oxytocin synthesis. Its advantage is supposed to minimize disulfide bond scrambling between the correctly cyclized peptide and dihydropeptide by keeping the content of dihydropeptide as low as possible. Cyclization in this series was carried out as follows: Dihydropeptide solution in H_2O (10 mM) was added dropwise to a well-stirred 8 M urea solution containing 40% excess of $K_3[Fe(CN)_6]$ over 30 min to keep the final peptide concentration below 1 mM. However, this condition resulted in poor yields when Pen or D-Pen was positioned at 23, and especially [^{D}Pen^{7,23}]-NP was obtained in a low yield, presumably due to the steric hindrance of Pen.

Purification: The solution of cyclized peptide was passed through a columun of Bio Rad AG3-X4A to remove ferro-, and ferri ions. The ionic strength of the eluate was lowered, if necessary, to around 3 mS/cm with H_2O. After carboxymethyl (CM) column chromatography, reversed-phase high performance liquid chromatography (RP-HPLC) was carried out for the final purification. Physical constants and analytical data of the final compounds were summarized in Table I. The structures of the purified peptides were confirmed by amino acid analysis and fast atom bombardment mass spectrometry (FAB-MS). The purities of the compounds were checked using analytical RP-HPLC and thin layer chromatography (TLC).

TABLE II. Relative Biological Activities of [X^7,Y^{23}]-α-hANP(7—28)

Compd. No.	Position 7	Position 23	VSMC: Binding activity	VSMC: cGMP accumulation	Rat aorta: Vasorelaxation
α-hANP(7—28)			100[a]	100[b]	100[c]
1	D-Cys	Cys	76	12	42
2	Pen	Cys	82	22	16
3	D-Pen	Cys	65	35	31
4	Cys	D-Cys	121	17	9
5	D-Cys	D-Cys	115	8	6
6	Pen	D-Cys	130	23	3
7	D-Pen	D-Cys	130	8	3
8	Cys	Pen	72	43	10
9	D-Cys	Pen	87	15	37
10	Pen	Pen	66	28	11
11	D-Pen	Pen	82	32	6
12	Cys	D-Pen	129	23	2
13	D-Cys	D-Pen	41	8	2
14	Pen	D-Pen	115	3	2
15	D-Pen	D-Pen	92	84	4

Values are mean of 2 to 3 experiments. *a*) $IC_{50}=3.4\pm1.8\times10^{-9}$ M ($n=3$), *b*) $ED_{50}=10.5\pm3.6\times10^{-7}$ M ($n=3$), *c*) $ED_{50}=2.8\pm0.8\times10^{-8}$ M ($n=3$). Mean ±S.E.

Results and Discussion

We examined the biological properties of the analogs in the assay of receptor binding and cGMP accumulation in vascular smooth muscle cells (VSMC), and vasorelaxation using rat aorta. Results of the assays are shown in Table II. The compounds were classified into four groups based on the amino acid residue at position 23; **1** through **3** (Cys^{23}), **4** through **7** (^{D}Cys^{23}), **8** through **11** (Pen^{23}) and **12** through **15** (^{D}Pen^{23}).

Regarding the receptor binding activity, each analog showed relatively high binding activity and the differences among the analogs were not great. The activity ranged from 41% for [^{D}Cys^{7},^{D}Pen^{23}]-NP at minimum to 130% for [Pen^{7},^{D}Cys^{23}]-NP and [^{D}Pen^{7}, Cys^{23}]-NP at maximum. However, apparent tendencies of the respective substituent-effects could not be pointed out. Spear *et al.* reported that the conformationally constrained analogs having two disulfide bonds or the smaller ring size in the molecule were

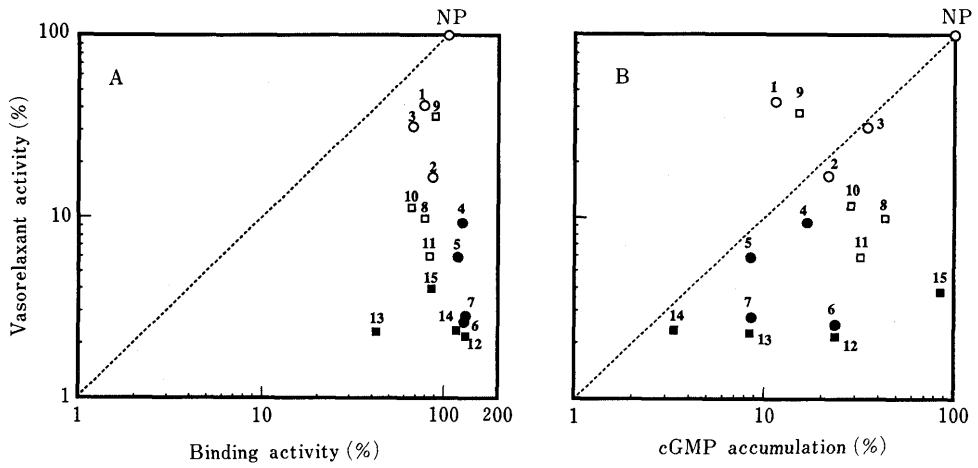

Fig. 3. Comparison of ANP Activities

A: Comparison between vasorelaxant activity and binding activity of the analogs. B: Comparison between vasorelaxant activity and cGMP accumulation activity of the analogs. Each number above the mark corresponds to the compound number shown in Table I or Table II. The analogs are divided into four groups based on the 23th residue as follows: [○], [X^7, Cys^{23}]-NP; [●], [X^7, ^{D}Cys^{23}]-NP; [□], [X^7, Pen^{23}]-NP; [■], [X^7, ^{D}Pen^{23}]-NP.

potent or even more potent binders in spite of their drastic difference in the cystine-location from the native molecule.[17] In our previous study, the Cys-modified linear analog such as [Cys(Me)^{7,23}]-α-hANP also exhibited full binding potency.[18] It is therefore concluded that the conformational requirement attributed to the cyclic structure is not stringent and that critical factors for binding might also exist in other functional groups of the constituent amino acids in the ANP molecule.

Regarding the vasorelaxant activity, all the analogs were weaker vasorelaxants as compared with NP. Attenuation of this activity was pronounced when the D-residue was positioned at 23, while the configuration at position 7 did not so strongly affect this activity. As shown in Fig. 3A, the analogs can be grouped into three classes with respect to their vasorelaxant activities. The analogs having Cys^{23} are relatively potent vasorelaxants (16—42%). When Pen was substitued for Cys^{23}, this activity slightly decreased (6—37%). A drastic decrease was observed when the D-residues, ^{D}Cys or ^{D}Pen, were positioned at 23 (2—9%). This result suggests that the configuration or flexibility of Cys^{23} is one of the major determinants of the active conformation of the ANP molecule.

In contrast, cGMP accumulation activities broadly ranged from 3% for [Pen^{7},^{D}Pen^{23}]-NP to 84% for [^{D}Pen^{7,23}]-NP (Fig. 3B). All the analogs exhibited lower activity compared with NP. Recent studies on ANP receptors have revealed that ANP-binding sites are classified into two categories based on whether or not they are coupled to the stimulation of particulate guanylate cyclase.[19] In the smooth muscle cells, it was reported that the major population (75—98%) of ANP-binding sites (C-receptor), which is assumed to be responsible for clearance or storage of ANP, is uncoupled to guanylate cyclase, and the rest (B-receptor) is coupled to the cyclase to elicit vasorelaxation.[20] Thus, the binding activity in the VSMC assay system mainly reflects the non-coupled receptor occupancy, while coupled receptor occupancy is expressed by the value of cGMP accumulation. Considering these, most of the analogs in this series are the C-receptor specific ligands rather than the B receptor ligands because each analog has high binding potency compared with the respective cGMP accumulation activity, suggesting that the conformational requirement for the B-receptor binding is stringent, while the C-receptor recognizes a variety of conformers. In the case of oxytocin having a smaller ring moiety (20-membered), a series of [Pen^{1}]-oxytocin analogs are known to bind its "biological receptor" and inhibit several oxytocin actions.[13] When the ring size of α-hANP is compared to that of oxytocin, contribution of the Pen residue in the ANP molecule to the restriction-effect on the cyclic structure is apparently less than that of oxytocin, however, it still remains possible that the conformationally constrained analogs, including this series, are substantially bound to both receptor subunits and the subsequent mobility of the ligand/B-receptor complex is suppressed. It should be noted that [^{D}Pen^{7,23}]-NP (**15**), the conformation of which is supposedly so different from that of NP, has full binding and cGMP accumulation activities.

Vasorelaxation induced by ANP is generally recognized to be mediated by cGMP.[21] Using fragment analogs, a high correlation between cGMP accumulation and vasorelaxation has been observed. In our case, however, a good relationship between cGMP accumulation and vasorelaxant activity was not observed (Fig. 3B). Except for **1** and **9**, all the analogs exhibited lower vasorelaxant activities in spite of their higher cGMP levels. Dissociation between these activities was especially typical in the case of [^{D}Pen^{7,23}]-NP (**15**) which exhibited less than 4% vasorelaxant potency despite its full cGMP accumulation potency (84%). Although the comparison between cGMP accumulation in VSMC and vasorelaxation in rat aorta should be carefully discussed, because both assay results are obtained from basically different assay systems, cGMP induced by this analog does not seem to be utilized as a second messenger for vasorelaxation. This result indicates that cGMP accumulation, at least in our VSMC assay system, is only a secondary event which occurs after the ANP-receptor occupancy or that the cGMP may mediate other biological responses which are not identified. Similar results were reported by Budzik *et al.* using the synthetic analog, [Lys^{8}]-α-rANP(5—28) with a different design, in VSMC assay.[22]

In conclusion, the introduction of bulky residue and/or D-residue into the ANP molecule in place of Cys^{7,23} did not affect the receptor binding activity, however, vasorelaxant activity was significantly influenced indicating that the configuration and flexibility in Cys^{23} are involved in modulating the ligand-receptor complex for subsequent vasorelaxation. A lack of correlation between cGMP accumulation activity and vasorelaxant activity was observed, especially in the case of [^{D}Pen^{7,23}]-NP, suggesting the possible existence of another second messenger(s) which mediates vasorelaxation.

Experimental

Melting points were determined with a Yanagimoto micro-melting point apparatus and are uncorrected. Optical rotations were measured with a Jasco DIP-360 polarimeter in a half dm microcell. *Rf* values on TLC of silica gel (Merck 5714) were observed in the following systems: Rf_1; $CHCl_3$–MeOH–AcOH (95 : 5 : 3), Rf_2; $CHCl_3$–MeOH–H_2O (8 : 3 : 1, lower phase), Rf_3; $CHCl_3$–TFE (6 : 1), Rf_4; $CHCl_3$–MeOH–AcOH (85 : 15 : 5), Rf_5; $CHCl_3$–TFE–AcOH (9 : 1 : 1), Rf_6; *n*-BuOH–AcOH–Pyr–H_2O (4 : 1 : 1 : 2), Rf_7; *n*-BuOH–AcOH–Pyr–H_2O (30 : 20 : 6 : 24). Spots were revealed by ultraviolet (UV) absorption and ninhydrin. Amino acid analysis was performed on a Hitachi 835 analyzer after 24 or 72 h hydrolysis in 6 N HCl containing 0.1% phenol in an evacuated and sealed ampoule. Analytical HPLC was performed on a Shimadzu LC-6A system equipped with a YMC-A-302 (5 μm) C-18 column.

N$^{\alpha}$-Deprotection The Boc-group used for N$^{\alpha}$-protection was removed by treating the peptide with TFA (3—10 ml/g) in the presence of 1.2 eq of HCl in dioxane for 10 min at −10 °C and then for 50 min at room temperature. The solution was concentrated to an oil by evaporation at room temperature and the acids remaining were removed as a benzene azeotrop by means of repeated evaporation. The residue was treated with dry ether to give a solid which was collected by filtration and was followed by washing with ether until the washings were no longer strongly acidic (pH 5—6). After drying *in vacuo* over NaOH pellets for 1 h, the N$^{\alpha}$-deprotected peptide hydrochloride was provided for the coupling.

Coupling Procedure All the couplings were performed using the EDC/HOBt method. DMF was used as the solvent unless otherwise mentioned. A solution of Boc-protected acyl component (1.0—1.05 eq), amine component (1 eq), and HOBt (1.05—1.10 eq) in DMF was cooled to −20 °C. EDC (1.05—1.10 eq) was added dropwise to the solution, and the mixture was stirred for 1 h at −15 °C and for 2—6 h at room temperature. Work-up procedures were made as follows: A) The reaction mixture was diluted with a 5 to 7-fold volume of AcOEt or $CHCl_3$. The extract was washed with 5% $NaHCO_3$ (twice), 1 N HCl (twice) and H_2O–NaCl (three times). After drying over Na_2SO_4, the extract was

concentrated to dryness, then crystallized or precipitated with appropriate solvents. B) The reaction mixture was poured into stirred ice-H_2O to produce a solid. After drying, the crude sample was precipitated with appropriate solvents.

Boc-Arg(Tos)-Tyr(BrZ)-OBzl (16) This compound was obtained by coupling Boc-Arg(Tos)-OH (84.5 g, 168 mmol) with H-Tyr(BrZ)-OBzl·TosOH (100.0 g, 152 mmol) in DMF (300 ml) using EDC (32.0 ml, 175 mmol) and HOBt (23.6 g, 175 mmol). After 4 h of stirring, the mixture was diluted with AcOEt and the extract was washed according to procedure A. The solvent was removed by evaporation, the residue was washed with ether/pet. ether by decantation, and triturated with hexane; yield 128.3 g (94%), mp 69—70 °C, $[\alpha]_D^{30}$ −9.6° (c=0.7, MeOH), Rf_1 0.42, Rf_2 0.76. *Anal.* Calcd for $C_{42}H_{48}BrN_5O_{10}S$: C, 56.37; H, 5.41; N, 7.83. Found: C, 56.15; H, 5.48; N, 7.73.

Boc-Phe-Arg(Tos)-Tyr(BrZ)-OBzl (17) Treatment of **16** (127.3 g, 142 mmol) with TFA (340 ml) and 4 N HCl/DXN (42.5 ml, 170 mmol) provided N$^\alpha$-deprotected peptide hydrochloride, which was dissolved in DMF (250 ml) together with HOBt (20.3 g, 150 mmol) and Boc-Phe-OH (37.7 g, 142 mmol). EDC (27.5 ml, 150 mmol) was added to this solution under cooling to −20 °C. After 4 h of stirring, the mixture was taken up in AcOEt and the extract was worked up according to procedure A. The solvent was removed by evaporation to an oil which was washed with ether/pet.ether by decantation, and triturated with hexane twice; yield 139.2 g (94%), mp 82—84 °C, $[\alpha]_D^{30}$ −11.3° (c=0.7, MeOH), Rf_1 0.40, Rf_2 0.78. *Anal.* Calcd for $C_{51}H_{57}BrN_6O_{11}S$: C, 58.78; H, 5.51; N, 8.07. Found: C, 58.63; H, 5.60: N, 7.91.

Boc-Ser(Bzl)-Phe-Arg(Tos)-Tyr(BrZ)-OBzl (18) Compound **17** (138.7 g, 133 mmol) was treated with TFA (300 ml) and 4 N HCl/DXN (39.9 ml, 160 mmol). N$^\alpha$-Deprotected peptide isolated as described above was reacted with Boc-Ser(Bzl)-OH (39.3 g, 133 mmol) in DMF (240 ml) using EDC (25.6 ml, 140 mmol) and HOBt (18.9 g, 140 mmol). After 3 h of stirring, the mixture was diluted with AcOEt and the extract was worked up according to procedure A. Concentration of the extract gave an oil which was precipitated with ether twice; yield 149.6 g (92%), mp 89 °C, $[\alpha]_D^{30}$ −12.2° (c=0.5, MeOH), Rf_1 0.38, Rf_2 0.82. *Anal.* Calcd for $C_{61}H_{68}BrN_7O_{13}S$: C, 60.09; H, 5.62; N, 8.04. Found: C, 59.80; H, 5.63; N, 7.88.

Boc-Asn-Ser(Bzl)-Phe-Arg(Tos)-Tyr(BrZ)-OBzl (19) The N$^\alpha$-deprotected sample of **18** [prepared by treating **18** (148.9 g, 122 mmol) with TFA (350 ml) and 4 N HCl/DXN (36.6 ml, 147 mmol)] was dissolved in DMF (350 ml) together with HOBt (17.7 g, 131 mmol) and Boc-Asn-OH (28.8 g, 124 mol). EDC (24.0 ml, 131 mol) was added to this solution. After 6 h of stirring, according to procedure B the mixture was poured into stirred ice-H_2O to produce a solid which was washed with H_2O and dried *in vacuo* over P_2O_5. The crude material thus obtained was precipitated from $CHCl_3$/MeOH and ether twice; yield 149.7 g (92%), mp 142—143 °C, $[\alpha]_D^{30}$ −22.4° (c=1.0, DMF), Rf_1 0.33, Rf_2 0.72. *Anal.* Calcd for $C_{65}H_{74}BrN_{15}O_9S$: C, 58.55; H, 5.59; N, 9.46. Found: C, 58.27; H, 5.54; N, 9.47.

Boc-Y(R)-Asn-Ser(Bzl)-Phe-Arg(Tos)-Tyr(BrZ)-OBzl (20a) Y(R)= Cys(MeBzl). The N$^\alpha$-deprotected sample of **19** [preparered by treating **19** (145.0 g, 109 mmol) with TFA (300 ml) and 4 N HCl/DXN (32.7 ml, 131 mmol)] was reacted with Boc-Cys(MeBzl)-OH (35.5 g, 109 mmol) in DMF (250 ml) using EDC (22.0 ml, 120 mmol) and HOBt (16.2 g, 120 mmol). Stirring was continued for 6 h and the mixture was worked up according to procedure B. The resulting solid was precipitated from $CHCl_3$/MeOH with ether twice: yield 156.6 g (93%), mp 249 °C, $[\alpha]_D^{30}$ −17.0° (c=0.5, DMF), Rf_2 0.48, Rf_4 0.71. *Anal.* Calcd for $C_{76}H_{87}BrN_{10}O_{16}S$: C, 59.25; H, 5.69; N, 9.09. Found: C, 59.10; H, 5.69; N, 8.91. Amino acid ratio in 6 N HCl hydrolysate: Asp 1.01 (1), Ser 0.85 (1), Cys N.D. (1), Tyr 0.97 (1), Phe 1, Arg 1.01 (1), recovery of Phe 95%.

In a similar manner, **20b**: Y(R) = ^{D}Cys(MeBzl), **20c**: Y(R) = Pen(MBzl), and **20d**: Y(R) = ^{D}Pen(MBzl) were prepared.

Boc-Ser(Bzl)-Gly-Leu-Gly-OPac (21) Treatment of Boc-Leu-Gly-O-Pac (80.0 g, 197 mmol) with TFA (200 ml) and 4 N HCl/DXN (59.0 ml, 236 mmol) provided the hydrochloride, which was dissolved in DMF (300 ml) together with Boc-Ser(Bzl)-Gly-OH·DCHA (110.5 g, 207 mmol) and HOBt (29.3 g, 217 mmol). To this mixture was added EDC·HCl (41.5 g, 217 mmol) in several portions at −20 °C. After 6 h of stirring, DCHA·HCl salt was removed by filtration, and the filtrate was diluted with AcOEt. The extract was washed and dried as usual and concentrated by evaporation. Precipitation of the residue with hexane gave a solid which was recrystallized from AcOEt and ether; yield 120.0 g (95%), mp 64—67 °C, $[\alpha]_D^{30}$ −19.5° (c=0.4, MeOH), Rf_1 0.40, Rf_2 0.44. *Anal.* Calcd for $C_{33}H_{44}N_4O_9 \cdot 1/2H_2O$: C, 61.00; H, 6.98; N, 8.62. Found: C, 61.08; H, 7.02; N, 8.47.

Boc-Gln-Ser(Bzl)-Gly-Leu-Gly-OPac (22) The N$^\alpha$-deprotected sample of **21** [prepared by treating **21** (119.0 g, 186 mmol) with TFA (220 ml) and 4 N HCl/DXN (55.7 ml, 223 mmol)] was coupled with Boc-Gln-OH (48.0 g, 195 mmol) in DMF (220 ml) using EDC (37.4 ml, 204 mmol) and HOBt (27.6 g, 204 mmol). After 4 h of stirring, a gelatinous product was triturated with H_2O. The soid was precipitated from $CHCl_3$/MeOH with ether twice; yield 126.7 g (89%), mp 208—210 °C, $[\alpha]_D^{30}$ −11.8° (c=0.6, DMSO), Rf_2 0.66, Rf_4 0.77. *Anal.* Calcd for $C_{38}H_{52}N_6O_{11}$: C, 59.36; H, 6.82; N, 10.93. Found: C, 59.25; H, 6.83; N, 10.83.

Boc-Ala-Gln-Ser(Bzl)-Gly-Leu-Gly-OPac (23) The N$^\alpha$-deprotected sample of **22** [prepared by treatment of **22** (7.7 g, 10 mmol) with TFA (30 ml) and 4 N HCl/DXN (3.0 ml, 12 mmol)] was coupled with Boc-Ala-OH (2.0 g, 10.5 mmol) in DMF (30 ml) using EDC (2.0 ml, 11 mmol) and HOBt (1.5 g, 11 mmol). After 4 h of stirring, the mixture was worked up according to procedure B to give a solid which was precipitated from $CHCl_3$/MeOH with ether twice; yield 7.8 g (93%). mp 217 °C, $[\alpha]_D^{30}$ −13.4° (c=0.6, DMSO), Rf_2 0.65, Rf_4 0.76. *Anal.* Calcd for $C_{41}H_{57}N_7O_{12}$: C, 58.63; H, 6.84; H, 11.67. Found: C, 58.33; H, 6.80; N, 11.55.

Boc-Ala-Gln-Ser(Bzl)-Gly-Leu-Gly-OH (24) To a solution of **23** (5.8 g, 6.9 mmol) in AcOH (70 ml) zinc powder (9.0 g, 20 eq) was added. The mixture was stirred for 1 h at 40 °C. Zinc was removed by filtration and the filtrate was concentrated to dryness by evaporation. The residue was triturated with H_2O to provide a solid which was precipitated from DMF with ether; yield 4.3 g (87%), mp 202 °C (dec.), Rf_2 0.13, Rf_4 0.22. $[\alpha]_D^{30}$ −12.3° (c=0.5, DMSO), *Anal.* Calcd for $C_{33}H_{51}N_7O_{11} \cdot H_2O$: C, 53.57; H, 7.22; N, 13.25. Found: C, 53.52; H, 7.05; N, 12.99. Amino acid ratio in 6 N HCl hydrolysate: Ser 0.88 (1), Glu 0.93 (1), Gly 1.87 (2), Ala 0.98 (1), Leu 1, recovery of Leu 98%.

Boc-Arg(Tos)-Ile-Gly-OPac (25) Boc-Ile-Gly-OPac (8.1 g, 20 mmol) was treated with TFA (50 ml) and 4 N HCl/DXN (6.0 ml, 24 mmol) as usual and the N$^\alpha$-deprotected peptide was dissolved in DMF (80 ml) together with HOBt (3.0 g, 22 mmol) and Boc-Arg(Tos)-OH (10.6 g, 21 mmol). EDC (4.0 ml, 22 mmol) was added and the solution was stirred for 6 h. The mixture was diluted with AcOEt and the extract was worked up by procedure A. The resulting oil was cystallized with hexane. Recrystallization was carried out from $CHCl_3$ with ether; yield 12.7 g (89%), mp 93—97 °C, $[\alpha]_D^{30}$ −25.7° (c=0.6, MeOH), Rf_2 0.69, Rf_3 0.31. *Amal.* Calcd for $C_{34}H_{48}N_6O_9S \cdot 1/2H_2O$: C, 56.25; H, 6.80; N, 11.58. Found: C, 56.11; H, 6.74; N, 11.52.

Boc-Asp(OcHex)-Arg(Tos)-Ile-Gly-OPac (26) The N$^\alpha$-deprotected sample of **25** [prepared by treating **25** (9.0 g, 12.6 mmol) with TFA (30 ml) and HCl/DXN (3.8 ml, 15.1 mmol)] was reacted with Boc-Asp(OcHex)-OH (4.0 g, 12.6 mmol) in DMF (50 ml) using EDC (2.5 ml, 13.9 mmol) and HOBt (1.9 g, 13.9 mmol). After 6 h of stirring, the mixture was taken up in $CHCl_3$. The extract was worked up by procedure A to an oil which was precipitated from $CHCl_3$/MeOH with ether twice; yield 8.3 g (72%), mp 173—174 °C, $[\alpha]_D^{30}$ −23.2° (c=0.6, MeOH), Rf_2 0.75, Rf_3 0.33. *Anal.* Calcd for $C_{44}H_{63}N_7O_{12}S$: C, 57.81; H, 6.95; N, 10.74. Found; C, 57.51; H, 7.04; N, 10.75.

Boc-Met-Asp(OcHex)-Arg(Tos)-Ile-Gly-OPac (27) The N$^\alpha$-deprotected sample of **26** [prepared by treating **26** (5.0 g, 5.5 mmol) with TFA (30 ml) and 4 N HCl/DXN (1.7 ml, 6.6 mmol)] was reacted with Boc-Met-OH (1.4 g, 5.8 mmol) in DMF (40 ml) using EDC (1.1 ml, 6.0 mmol) and HOBt (816 mg, 6.0 mmol). After 6 h of stirring, the mixture was worked up by procedure B to a solid which was precipitated from $CHCl_3$/MeOH with ether twice; yield 5.5 g (96%), mp 144 °C, $[\alpha]_D^{30}$ −23.6° (c=0.7, MeOH), Rf_2 0.76, Rf_3 0.29. *Anal.* Calcd for $C_{49}H_{72}N_8O_{13}S_2$: C, 56.30; H, 6.94; N, 10.72. Found: C, 56.02; H, 6.96; N, 10.54.

Boc-Arg(Tos)-Met-Asp(OcHex)-Arg(Tos)-Ile-Gly-OPac (28) The N$^\alpha$-deprotected sample of **27** [prepared by treating **27** (3.3 g, 3.2 mmol) with TFA (20 ml) and 4 N HCl/DXN (950 μl, 3.8 mmol)] was reacted with Boc-Arg(Tos)-OH (1.7 g, 3.3 mmol) in DMF (25 ml) using EDC (637 μl, 3.5 mmol) and HOBt (470 mg, 3.5 mmol). After 6 h of stirring, the mixture was worked up by procedure B to a solid which was precipitated from MeOH with ether; yield 4.1 g (96%), mp 132—135 °C, $[\alpha]_D^{30}$ −23.1° (c=0.7, MeOH), Rf_2 0.74, Rf_4 0.76. *Anal.* Calcd for $C_{62}H_{90}N_{12}O_{16}S_3 \cdot H_2O$: C, 54.21; H, 6.75; N, 12.24. Found: C, 54.26; H, 6.75; N, 12.17.

Boc-Arg(Tos)-Met-Asp(OcHex)-Arg(Tos)-Ile-Gly-OH (29) To a solution of **28** (2.5 g, 184 μmol) in AcOH (30 ml) zinc powder (2.4 g, 20-eq) was added. the mixture was stirred for 1 h at 40 °C under N_2. Zinc was removed by filtration, the filtrate was concentrated to dryness then treated with H_2O to give a solid. Crystallization was performed with MeOH and ether, yield 2.2 g (97%), mp 139—145 °C, $[\alpha]_D^{30}$ −27.2° (c=0.4, MeOH),

Rf_2 0.43, Rf_4 0.55. *Anal.* Calcd for $C_{54}H_{84}N_{12}O_{15}S_3$: C, 52.41; H, 6.84; N, 13.58. Found: C, 52.20; H, 6.89; N, 13.42. Amino acid ratio in 6 N HCl hydrolysate: Asp 1.01 (1), Gly 1, Met 0.96 (1), Ile 0.97 (1), Arg 1.92 (2), recovery of Gly 99%.

Boc-Phe-Gly-Gly-OPac (30) Boc-Gly-Gly-OPac (11.0 g, 31.4 mmol) was treated with TFA (30 ml) and 4 N HCl/DXN (9.4 ml, 37.7 mmol) and the N^{α}-deprotected peptide was isolated as usual. Five grams of this peptide hydrochloride, HOBt (2.6 g, 19.2 mmol) and Boc-Phe-OH (4.9 g, 18.3 mmol) were dissolved in DMF (40 ml). EDC (3.5 ml, 19.2 mmol) was added and the solution was stirred for 4 h. The mixture was taken up in AcOEt, the extract was worked up by procedure A to an oil which was precipitated with ether. Crystallization was performed from $CHCl_3$ and ether; yield 7.9 g (91%), mp 141—143 °C, $[\alpha]_D^{30}$ +11.8° (c=0.6, MeOH), Rf_1 0.29, Rf_2 0.74. *Anal.* Calcd for $C_{26}H_{31}N_3O_7 \cdot 1/2H_2O$: C, 61.65; H, 6.37; N, 8.30. Found; C, 61.87; H, 6.11; N, 8.31.

Boc-X(R)-Phe-Gly-Gly-OPac (31a) X(R)=Cys(MeBzl). The N^{α}-deprotected sample of **30** [prepared by treating **30** (1.7 g, 3.5 mmol) with TFA (5 ml) and 4 N HCl/DXN (950 μl, 3.8 mmol)] was reacted with Boc-Cys(MeBzl)-OH (1.2 g, 3.6 mmol) in DMF (7 ml) using EDC (695 μl, 3.8 mmol) and HOBt (515 mg, 3.8 mmol). After 6 h of stirring, the mixture was diluted with $CHCl_3$ and the extract was worked up by procedure A to an oil which was precipitated with ether, and then crystallized from $CHCl_3$/MeOH with ether, yield 2.1 g (85%), mp 157—159 °C, $[\alpha]_D^{30}$ −6.1° (c=0.8, MeOH), Rf_1 0.34, Rf_3 0.54. *Anal.* Calcd for $C_{37}H_{44}N_4O_8S$: C, 63.05; H, 6.29; N, 7.95. Found: C, 62.82; H, 6.29; N, 7.92.

The sample of **31b**: X(R)=^{D}Cys(MeBzl), **31c**: X(R)=Pen(MBzl), and **31d**: X(R)=^{D}Pen(MBzl), were prepared in a similar manner as described above.

Boc-X(R)-Phe-Gly-Gly-OH (32a) X(R)=Cys(MeBzl). To a solution of **31a** (2.0 g, 2.8 mmol) in AcOH (15 ml) zinc powder (3.7 g, 20 eq) was added. After 1 h of stirring under N_2 atmosphere at 40 °C, zinc was removed by filtration, and the filtrate was concentrated by evaporation to dryness. The residue was taken up in AcOEt and the extract was worked up by procedure A to an oil which was precipitated with ether, yield 1.6 g (93%), mp 80—83 °C, $[\alpha]_D^{30}$ −19.8° (c=0.7, MeOH), Rf_2 0.30, Rf_4 0.55. *Anal.* Calcd for $C_{29}H_{38}N_4O_7S$: C, 59.37; H. 6.53; N, 9.55. Found: C, 59.15; H, 6.47; N, 9.55. Amino acid ratio in 6 N HCl hydrolysate: Gly 2, Cys N.D. (1), Phe 1.02 (1), recovery of Gly 99%.

The samples of **32b**: X(R)=^{D}Cys(MeBzl), **32c**: X(R)=Pen(MBzl), and **32d**: X(R)=^{D}Pen(MBzl), were prepared in a similar manner as described above.

Boc-Ala-Gln-Ser(Bzl)-Gly-Leu-Gly-Y(R)-Asn-Ser(Bzl)-Phe-Arg-(Tos)-Tyr(BrZ)-OBzl (33a) Y(R)=Cys(MeBzl). The N^{α}-deprotected sample of **20a** [prepared by treating **20a** (4.6 g, 3.0 mmol) with TFA (30 ml) and 4 N HCl/DXN (896 μl, 3.6 mmol)] was dissolved in DMF (15 ml). A pre-cooled mixture of **24** (2.3 g, 3.1 mmol) and HOBt (445 mg, 3.3 mmol) in DMSO (5 ml) was added to the above solution, and was followed by EDC (602 μl, 3.3 mmol). After stirring for 6 h, H_2O was added to the mixture to give a solid which was washed with H_2O and MeOH. After drying, the product was precipitated with boiling MeOH, and was followed by washing with ether; yield 5.9 g (93%), mp 244—252 °C (dec.), $[\alpha]_D^{30}$ −17.3° (c=1.7, DMSO), Rf_3 0.32, Rf_5 0.61. *Anal.* Calcd for $C_{104}H_{128}N_{17}BrO_{24}S_2 \cdot H_2O$: C, 57.77; H, 6.06; N, 11.01. Found: C, 57.83; H, 6.24; N, 11.27. Amino acid ratio in 6 N HCl hydrolysate: Asp 0.99 (1), Ser 1.70 (2), Glu 0.99 (1), Gly 2.00 (2), Ala 1.00 (1), Cys N.D. (1), Leu 1.00 (1), Tyr 0.94 (1), Phe 1, Arg 0.94 (1), recovery of Phe 97%.

Other samples, **33b**: Y(R)=^{D}Cys(MeBzl), **33c**: Y(R)=Pen(MBzl), and **33d**: Y(R)=^{D}Pen(MBzl), were prepared in a similar manner as described above.

Boc-Arg(Tos)-Met-Asp(OcHex)-Arg(Tos)-Ile-Gly-Ala-Gln-Ser(Bzl)-Gly-Leu-Gly-Y(R)-Asn-Ser(Bzl)-Phe-Arg(Tos)-Tyr(BrZ)-OBzl (34a) Y(R)=Cys(MeBzl). The N^{α}-deprotected sample of **30a** [prepared by treating **30a** (1.2 g, 564 μmol) with TFA (10 ml) and 4 N HCl/DXN (141 μl, 677 μmol)] was reacted with **29** (733 mg, 592 μmol) in DMSO-NMP (5—3 ml) using EDC (113 μl, 620 μmol) and HOBt (84 mg, 620 μmol). The mixture was stirred for 6 h and precipitated with ice-H_2O. The solid was washed with H_2O and MeOH. After drying it was treated with boiling MeOH, and was followed by washing with ether; yield 1.8 g (98%), mp 245—248 °C (dec.), $[\alpha]_D^{30}$ −26.0° (c=0.5, DMSO), Rf_5 origin. *Anal.* Calcd for $C_{153}H_{202}BrN_{29}O_{36}S_5 \cdot 2H_2O$: C, 55.69; H, 6.29, N, 12.31. Found: C, 55.93; H, 6.26; N, 12.04. Amino acid ratio in 6 N HCl hydrolysate: Asp 2.01 (2), Ser 1.71 (2), Glu 1.03 (1), Gly 3.01 (3), Ala 0.99 (1), Cys N.D. (1), Met 0.92 (1), Ile 0.93 (1), Leu 1, Tyr 0.91 (1), Phe 0.98 (1), Arg 3.00 (3), recovery of Leu 96%.

Other samples, **34b**: Y(R)=^{D}Cys(MeBzl), **34c**: Y(R)=Pen(MBzl), and **34d**: Y(R)=^{D}Pen(MBzl), were prepared in a similar manner as described above.

Boc-X(R)-Phe-Gly-Gly-Arg(Tos)-Met-Asp(OcHex)-Arg(Tos)-Ile-Gly-Ala-Gln-Ser(Bzl)-Gly-Leu-Gly-Y(R)-Asn-Ser(Bzl)-Phe-Arg(Tos)-Tyr-(BrZ)-OBzl (35a) X(R)=^{D}Cys(MeBzl), Y(R)=Cys(MeBzl). The N^{α}-deprotected sample of **34a** [prepared by treating **34a** (202 mg, 62 μmol) with TFA (3 ml) and 4 N HCl/DXN (19 μl, 74 μmol)] was reacted with **32b** (40 mg, 68 μmol) in DMSO-NMP (1—5 ml) using EDC (12.4 μl, 68 μmol) and HOBt (9.2 mg, 68 μmol). After 2 h of stirring, the mixture became a gelatinous solid which was allowed to stand overnight. The mixture was worked up by procedure B to a solid which was treated with boiling MeOH, and was followed by washing with ether, yield 215 mg (93%), mp >260 °C, Rf_5 origin. *Anal.* Calcd for $C_{187}H_{241}BrN_{34}O_{42}S_6 \cdot 4H_2O$: C, 56.41; H, 6.30; N, 11.96. Found: C, 56.18; H, 6.15; N, 11.97. Amino acid ratio in 6 N HCl hydrolysate: Asp 1.94 (2), Ser 1.78 (2), Glu 0.98 (1), Gly 4.93 (5), Ala 1.07 (1), Cys N.D. (2), Met 0.95 (1), Ile 0.94 (1), Leu 1, Tyr 0.91 (1), Phe 1.97 (2), Arg 2.65 (3), recovery of Leu 94%.

Other fully protected peptides, **35b** [X(R)=Pen(MBzl), Y(R)=Cys-(MeBzl)], **35c** [X(R)=^{D}Pen(MBzl), Y(R)=Cys(MeBzl)], **35d** [X(R)=Cys(MeBzl), Y(R)=^{D}Cys(MeBzl)], **35e**[X(R), Y(R)=^{D}Cys(MeBzl)], **35f** [X(R)=Pen(MBzl), Y(R)=^{D}Cys(MeBzl)], **35g** [X(R)=^{D}Pen(MBzl), Y(R)=^{D}Cys(MeBzl)], **35h** [X(R)=Cys(MeBzl), Y(R)=Pen(MBzl)], **35i** [X(R)=^{D}Cys(MeBzl), Y(R)=Pen(MBzl)], **35j** [X(R), Y(R)=Pen (MBzl)], **35k** [X(R)=^{D}Pen(MBzl), Y(R)=Pen(MBzl)], **35l** [X(R)=Cys(MeBzl), Y(R)=^{D}Pen(MBzl)], **35m** [X(R)=^{D}Cys(MeBzl), Y(R)=^{D}Pen(MBzl)], **35n** [X(R)=Pen(MBzl), Y(R)=^{D}Pen(MBzl)], and **35o** [X(R), Y(R)=^{D}Pen-(MBzl)] were prepared in a similar manner as described above.

H-X-Phe-Gly-Gly-Arg-Met-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Y-Asn-Ser-Phe-Arg-Tyr-OH (1) X=^{D}Cys, Y=Cys. The N^{α}-deprotected sample of **35a** [prepared by treating **35a** (200 mg, 53 μmol) with TFA (1 ml) and 4 N HCl/DXN (14 μl, 64 μmol)] was treated with HF (8.5 ml) in the presence of *p*-cresol (1.5 ml, 250 eq) at −2 °C for 1 h. Exess HF was removed by evaporation and the residue was precipitated with ether to a solid. The deprotected peptide was dissolved in 10% AcOH (10 ml) and the solution was passed through a column of Bio Rad AG1-X2 (20 ml, acetate) previously equilibrated with 10% AcOH. The flow through fraction (25 ml) was concentrated by evaporation, and the residue was dissolved in H_2O (5.3 ml). This solution (10 mM) was added dropwise to the well-stirred $K_3[Fe(CN)_6]$ solution (24.4 mg in 48 ml of 8 M urea/H_2O) over 30 min. In the course of the reaction, the pH of the mixture was kept at 7—7.5 with 10% NH_4OH. After complete cyclization, the pH was brought to 5 with AcOH and the solution was passed through a column of Bio Rad AG3-X4A (5 ml, chloride) previously equilibrated with 1% AcOH to remove ferri- and ferrocyanide anions. The flow through fraction (60 ml), the ionic strength of which was lowered to 3 mS/cm with H_2O (10 ml), was applied onto a CM packed column (Toyopearl CM-2SW, 2×15 cm). The peptide was eluted with a linear salt (NH_4OAc) gradient from 0 to 0.3 M (pH 7.5) at a flow rate of 7 ml/min over 1 h. Each fraction (3.5 ml) was checked by UV absorption at 280 nm. The major peak fractions were pooled (6 frcs., more than 95% purity each) and applied onto a column of C-18 (YMC-Pack D-ODS-5, 2×25 cm). The peptide was eluted with a gradient of CH_3CN (24% to 36% in 0.1% TFA for 1 h at a flow rate of 10 ml/min). The desired fractions (4 frcs, more than 98% purity each) were combined and lyophilized to give a title compound; yield 43.1 mg (34% based on the fully protected peptide).

Other final peptides, **2** through to **15**, were prepared essentially in the same procedure. The yields and analytical data of the compounds (**1** through **15**) were shown in Table I.

Receptor Binding Assay in VSMC VSMC were derived from explants of rat thoracic aorta. Cells were cultured in Dulbeco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (Flow) at 37 °C in a 95% air/5% CO_2 atmosphere, and were used for the assay between the 4th to 12th passages. Iodination of α-hANP was carried out by the lactoperoxidase method,[7] and the specific activity of [^{125}I] α-hANP was 22.2 MBq/mg. For the binding assay, the cells were cultured in 24-well multiplate (Corning) to confluent (*ca.* 4×10^5 cell/well), and incubated with 5×10^{-10} M [^{125}I] α-hANP (*ca.* 3×10^5 cpm) and various doses of the analogs ranging from 5×10^{-11} M to 5×10^{-7} M in 0.25 ml of the binding buffer (DMEM containing 0.1% bovine serum albumin and 20 mM HEPES, pH 7.4) for 45 min at 25 °C. After incubation, the cells were washed extensively with the same buffer and the bound [^{125}I] α-hANP were solubilized with 0.5 M NaOH. The radioactivity was measured by γ-spectrophotometer (ARC-500, Aloka). Non-specific binding was defined by incubating parallel culture wells with [^{125}I] α-hANP in the presence of 5×10^{-7} M unlabelled α-hANP and was routinely less than 10% of the

total binding. ID_{50} values of the analogs were obtained after creating displacement curves.

cGMP Quantification in VSMC VSMC were cultured in 24-well multiplates as described above, and confluent cells were incubated with the analogs in 0.25 ml of the binding buffer containing 0.5 mM 1-methyl-3-isobutylxanthine for 15 min at 37 °C. The incubation was terminated by aspirating the assay medium and the addition of 0.5 ml of 6% perchloric acid. The cells were disrupted by sonication and cellular cGMP was quantified using a cGMP radioimmunoassay kit (Yamasa Shoyu). Activity curves were generated for the ED_{50} calculation.

Vasorelaxation in Isolated Rat Aorta Aortic strips were prepared from the thoracic aortas of Sprague-Dawley rats and mounted in a 7 ml chamber containing Krebs–Henseleit solution (118 mM NaCl, 4.76 mM KCl, 2.54 mM $CaCl_2$, 2.44 mM $MgCl_2$, 1.19 mM $NaHCO_3$ and 5.55 mM glucose) aerated with 95% O_2/5% CO_2 at 37 °C. After 1 h equilibration, the aortic strips were contracted with 2×10^{-7} M norepinephrine, and once the contractile response was stabilized, they were exposed to the analogs cummulatively. Dose-response curves were obtained for the calculation of ED_{50} values.

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References and Notes

1) Presented in part at the 26th Symposium on Peptide Chemistry, Tokyo, October 1988, Y. Kitajima, Y. Minamitake, M. Furuya, M. Takehisa, T. Narikawa, and S. Tanaka, "Peptide Chemistry 1988," ed. M. Ueki, Protein Res. Foundation, Osaka, 1989, p. 195. Unless otherwise mentioned, amino acids, peptides and their derivatives described in this investigation are of the L-configuration. The following abbreviations were used: Boc = *tert*-butyloxycarbonyl, Bzl = benzyl, Tos = tosyl, cHex = cyclohexyl, MeBzl = 4-methylbenzyl, MBzl = 4-methoxybenzyl, Pac = phenacyl, BrZ = 2-bromobenzyloxycarbonyl, DCC = dicyclohexylcarbodiimide, EDC = 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide, TFA = trifluoroacetic acid, DCHA = dicyclohexylamine, HOBt = *N*-hydroxybenzotriazole, DMF = dimethylformamide, NMP = *N*-methylpyrrolidone, DMSO = dimethylsulfoxide, DXN = dioxane, TFE = trifluoroethanol, NP = α-hANP (7—28), Pen = penicillamine.
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