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Syntheses and Biological Activities of Selenium Analogs of α-Rat Atrial Natriuretic Peptide[1)]

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α-Rat atrial natriuretic peptide (7—28) (rANP (7—28)) and a series of its analogs in which half cystine residue(s) were substituted with half selenocystine residue(s) were synthesized by using the Fmoc-based solid-phase method followed by cyclization by means of dimethylsulfoxide (DMSO)–trifluoroacetic acid (TFA) oxidation. These analogs possess comparable activities in both receptor binding and cGMP accumulation in rat vascular smooth muscle cells to those of rAMP (7—28).

Keywords atrial natriuretic peptide; selenocystine; Fmoc-based solid-phase peptide synthesis; dimethylsulfoxide; cyclic GMP

α-Atrial natriuretic peptide (α-ANP) is a member of the natriuretic peptide family, which plays an important role in body fluid homeostasis and blood pressure control through its effect on natriuresis/diuresis, vasorelaxation, and inhibition of aldosterone section.[2)] α-ANP is a 28-residue peptide containing an intramolecular disulfide bridge that forms a 53-membered ring structure conserved in all known members of the family. Intensive structure–activity relationship (SAR) studies have elucidated the importance of this disulfide-bonded ring structure for biological activity. The linear analog of human ANP (hANP) shows much lower activity in both cGMP production in vascular smooth muscle cells and vasorelaxation.[3)] *In vivo* data for ring-size-altered analogs of rANP (7—28), lacking the N-terminal amino group and containing 2-mercaptoacetic acid, 3-mercaptopropionic acid or 4-mercaptobutyric acid in place of the Cys^7 residue, also indicate the significance of this cyclic structure for biological activity.[4)] However, the synthetic analog containing an ethylene unit instead of the disulfide bond exhibited much lower activity in the rat aorta and natriuresis assay in spite of possessing a 53-membered ring structure.[5)] In addition, Minamitake *et al.* reported the synthesis of a series of α-hANP analogs in which Cys residue(s) were replaced with D-Cys, L-Pen, and/or D-Pen residue(s), and found that the vasorelaxant activity was significantly influenced by the side-chain configuration and flexibility of the Cys^{23} residue.[6)] We are interested in the structural specificity of the disulfide bridge in ANP for biological activity, and therefore planned the synthesis and evaluation of ANP analogs in which Cys residues are replaced with Sec residues.

This SAR strategy involving the replacement of Cys residue(s) with Sec residue(s) has already been applied to oxytocin[7)] and somatostatin.[8)] However, the synthetic routes using the *Se*-Bzl group, followed by cleavage with sodium in liquid ammonia, were unsatisfactory because of side reactions. Recently, we developed a synthetic method for incorporating selenocystine into peptides which is compatible with standard Fmoc-based solid-phase synthesis protocols.[9)]

In this paper, we wish to report the synthesis and *in vitro* activities of three selenium analogs of rANP (7—28).

Results and Discussion

We synthesized rANP (7—28) possessing full agonistic activities[10)] and its three selenium analogs, [Sec^7]-rANP (7—28), [Sec^{23}]-rANP (7—28), and [$Sec^{7,23}$]-rANP (7—28) (Fig. 1). The assembly of peptide chains was accomplished manually by Fmoc-based solid-phase techniques[11)] on Wang-type polystyrene supports[12)] using successive DIC–HOBt coupling and N^{α}-deprotection with 20% (v/v) piperidine/DMF. Fmoc-Cys(MBzl)-OH and Fmoc-Sec-

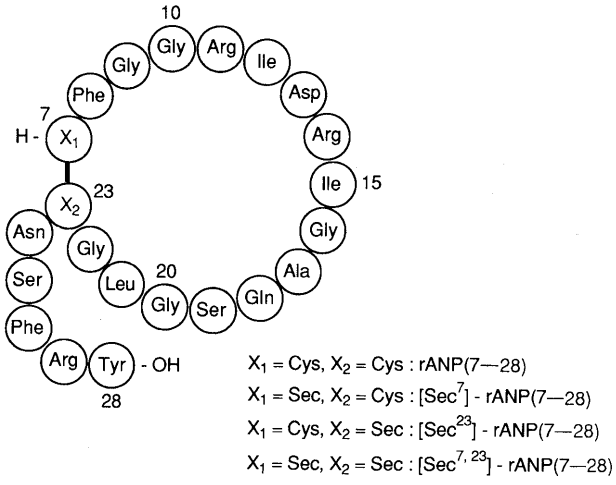

Fig. 1. Structures of rANP (7—28) and Its Selenium Analogs

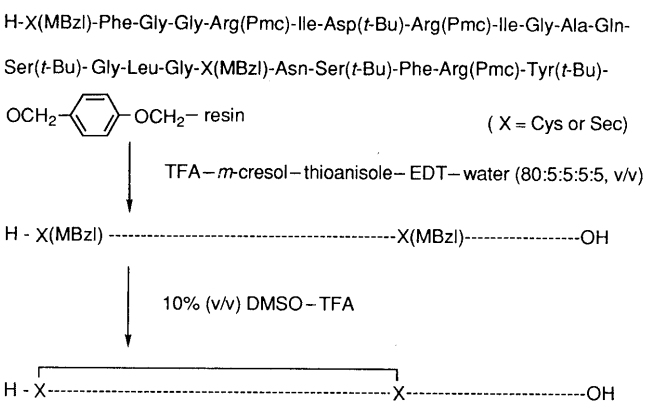

Chart 1. Synthetic Schemes for rANP (7—28) and Its Selenium Analogs

t-Bu = *tert*-butyl.

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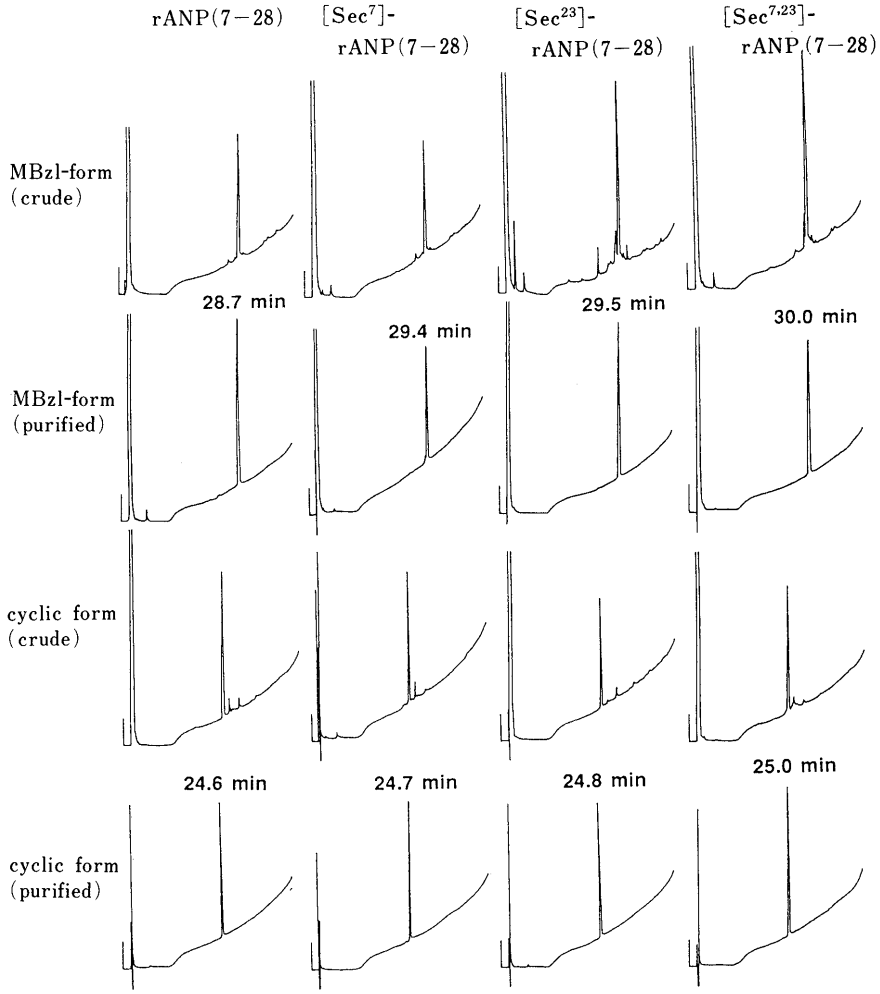

Fig. 2. HPLC Profiles of rANP (7—28), Its Selenium Analogs, and Their Synthetic Intermediates

Column, Cosmosil C18-AR (0.46 × 15 cm); elution, linear gradient of MeCN (10—50% in 30 min) in 0.1% aqueous TFA; flow rate, 1 ml/min; detection: 220 nm

(MBzl)-OH were used as precursors of the Cys and the Sec residues, respectively. For the protection of the guanidino group of Arg residues, the highly acid-labile Pmc group[13] was employed. Other side-chain functional groups were protected with *tert*-butyl-type protective groups.

The scheme for deprotection/cleavage and cyclization is illustrated in the chart. Each peptidyl resin was first treated with TFA–*m*-cresol–thioanisole–EDT–water (80 : 5 : 5 : 5 : 5, v/v)[9] for the detachment of the peptide from the solid support with simultaneous deprotection of side-chain protective groups. During this treatment, the *S*- and *Se*-MBzl groups remained intact. The resulting semi-pure MBzl-peptides were further purified by HPLC prior to cyclization studies.

Cyclization of peptides was accomplished by oxidation of the purified MBzl-peptides with 10% (v/v) DMSO–TFA.[14] Using these reagents, both *S*- and *Se*-MBzl groups were cleaved with concomitant simultaneous formation of S–S, Se–Se, and S–Se bonds. The time required for completion of cyclization varied with the peptides; 60 min for rANP (7—28), 120 min for [Sec^{23}]-rANP (7—28), 15 min for both [Sec^{7}]- and [Sec^{7,23}]-rANP (7—28). Under these reaction conditions (1 mg peptide/ml reagent), no significant side products, such as polymeric products, were observed on analysis by HPLC. The HPLC purification of crude products yielded cyclic peptides in homogeneous form. HPLC profiles and physicochemical characteristics of all rANP analogs and their synthetic intermediates are summarized in Fig. 2 and Tables I—V.

These results demonstrate the synthetic usefulness of this route. Significantly, this direct one step oxidative cyclization procedure circumvents the problem of facile degradation of

Table I. Amino Acid Ratios in 6 N HCl Hydrolysates of *S*- and *Se*-Protected rANP(7—28) Analogs[a,b]

	[Cys(MBzl)^{7,23}]-rANP(7—28)	[Sec(MBzl)^{7}, Cys(MBzl)^{23}]-rANP(7—28)	[Cys(MBzl)^{7}, Sec(MBzl)^{23}]-rANP(7—28)	[Sec(MBzl)^{7,23}]-rANP(7—28)
Asx	2.03 (2)	1.76 (2)	2.07 (2)	1.75 (2)
Ser	1.67 (2)	1.59 (2)	1.58 (2)	1.58 (2)
Glx	1.03 (1)	0.94 (1)	1.04 (1)	0.90 (1)
Gly	5.03 (5)	4.58 (5)	5.18 (5)	4.63 (5)
Ala[c]	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)
Ile	1.94 (2)	1.64 (2)	1.96 (2)	1.61 (2)
Leu	1.03 (1)	0.89 (1)	1.04 (1)	0.86 (1)
Tyr	0.99 (1)	0.87 (1)	1.04 (1)	0.84 (1)
Phe	2.09 (2)	1.83 (2)	2.00 (2)	1.73 (2)
Arg	2.92 (3)	2.61 (3)	3.00 (3)	2.65 (3)
Cys	N.D. (2)	N.D. (1)	N.D. (1)	
Sec		N.D. (1)	N.D. (1)	N.D. (2)

a) Values in parentheses are theoretical. *b*) N.D., not determined. *c*) Diagnostic amino acid.

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TABLE II. FAB-Mass Spectral Data for *S*- and *Se*-Protected rANP (7—28) Analogs

	Formula	FAB-mass *m/z*	
		Found $(M+H)^+$	Calcd $(M+H)^+$
[Cys(MBzl)^{7,23}]-rANP(7—28)	$C_{117}H_{173}N_{33}O_{32}S_2$	2618.6	2619.0
[Sec(MBzl)^7,Cys(MBzl)^{23}]-rANP(7—28)	$C_{117}H_{173}N_{33}O_{32}SSe$	2665.3	2665.9
[Cys(MBzl)^7,Sec(MBzl)^{23}]-rANP(7—28)	$C_{117}H_{173}N_{33}O_{32}SSe$	2665.5	2665.9
[Sec(MBzl)^{7,23}]-rANP(7—28)	$C_{117}H_{173}N_{33}O_{32}Se_2$	2712.1	2712.8

TABLE III. Amino Acid Ratios in 6 N HCl Hydrolysates of rANP(7—28) and Its Selenium Analogs[a,b]

	rANP(7—28)	[Sec^7]-rANP(7—28)	[Sec^{23}]-rANP(7—28)	[Sec^{7,23}]-rANP(7—28)
Asx	2.00 (2)	2.00 (2)	1.98 (2)	1.94 (2)
Ser	1.78 (2)	1.84 (2)	1.74 (2)	1.73 (2)
Glx	1.02 (1)	1.02 (1)	1.02 (1)	1.01 (1)
Gly	5.00 (5)	4.97 (5)	4.93 (5)	4.89 (5)
Ala[c]	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)
Ile	1.92 (2)	1.87 (2)	1.91 (2)	1.86 (2)
Leu	1.00 (1)	1.00 (1)	0.99 (1)	0.97 (1)
Tyr	1.00 (1)	1.00 (1)	0.98 (1)	0.92 (1)
Phe	1.97 (2)	2.02 (2)	1.98 (2)	1.87 (2)
Arg	2.88 (3)	2.88 (3)	2.86 (3)	2.78 (3)
$(Cys)_2$	N.D. (1)			
$(Sec)_2$				N.D. (1)
Cys-Sec		N.D. (1)	N.D. (1)	

a) Values in parentheses are theoretical. *b*) N.D., not determined. *c*) Diagnostic amino acid.

TABLE IV. Amino Acid Ratios in LAP Digests of rANP(7—28) and Its Selenium Analogs[a,b]

	rANP(7—28)	[Sec^7]-rANP(7—28)	[Sec^{23}]-rANP(7—28)	[Sec^{7,23}]-rANP(7—28)
Asp	1.03 (1)	0.97 (1)	1.00 (1)	1.03 (1)
Asn	N.D. (1)	N.D. (1)	N.D. (1)	N.D. (1)
Ser	2.07 (2)	2.03 (2)	2.03 (2)	2.03 (2)
Gln	N.D. (1)	N.D. (1)	N.D. (1)	N.D. (1)
Gly	5.12 (5)	4.80 (5)	4.87 (5)	5.18 (5)
Ala[c]	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)
Ile	1.97 (2)	1.89 (2)	1.85 (2)	1.91 (2)
Leu	1.03 (1)	0.97 (1)	1.01 (1)	1.02 (1)
Tyr	1.01 (1)	0.92 (1)	0.94 (1)	1.00 (1)
Phe	2.05 (2)	2.04 (2)	1.98 (2)	2.06 (2)
Arg	3.08 (3)	2.83 (3)	2.83 (3)	3.00 (3)
$(Cys)_2$	1.03 (1)			
$(Sec)_2$				0.95 (1)
Cys-Sec		N.D. (1)	N.D. (1)	

a) Values in parentheses are theoretical. *b*) N.D., not determined. *c*) Diagnostic amino acid.

free selenol groups by traces of molecular oxygen. Thus, the 10% (v/v) DMSO–TFA oxidation system was shown to be useful for direct formation of the S–Se bond between MBzl-protected Cys and Sec residues, as well as for disulfide and diselenide bond formation.

Next, the newly synthesized peptides were evaluated for receptor binding potencies and for cGMP accumulation in cultured rat vascular smooth muscle cells. In these assay systems, the binding potency is assumed to represent the clearance receptor (C-receptor) occupancy, and cGMP accumulation is thought to reflect the action on the biological receptor (B-receptor). The receptor binding potencies were estimated by measuring the concentration of the peptides inhibiting specific binding of [^{125}I]-α-hANP to the cells, and quantitation of cGMP in the cells was performed by radio immunoassay.[6] Results are shown in Fig. 3. Values of IC_{50} for receptor binding and EC_{50} for cGMP accumulation were derived from corresponding dose–response curves (Table VI). The three selenium analogs showed potencies nearly equal to those of rANP (7—28) in both receptor binding and cGMP accumulation. These results suggest that the substitution (Sec for Cys) did not cause significant conformational change around the disulfide bridge, and indicate that the Sec residue in rANP analogs plays a role similar to that of the Cys residue in exerting biological activity. This is in agreement with the results of SAR using selenium analogs of oxytocin[7] and somatostatin.[8] Although the substitution of an ethylene bridge for the S–S bonds of oxytocin[15] and eel calcitonin[16] does not significantly affect their biological activities, an analog of α-hANP (7—28) with the same substitution possesses much lower spasmolytic activity in rat aorta and much lower natriuretic activity in rat than those of the native compound.[5] Based on the above data, we suggest the disulfide bridge of ANP may interact directly with its functional receptor. It is tempting to speculate that the receptor may possess a "soft pocket" which interacts with

TABLE V. FAB-Mass Spectral Data for rANP(7—28) and Its Selenium Analogs

	Formula	FAB-mass *m/z*	
		Found $(M+H)^+$	Calcd $(M+H)^+$
rANP(7—28)	$C_{101}H_{155}N_{33}O_{30}S_2$	2376.6	2376.7
[Sec^7]-rANP(7—28)	$C_{101}H_{155}N_{33}O_{30}SSe$	2424.3	2423.6
[Sec^{23}]-rANP(7—28)	$C_{101}H_{155}N_{33}O_{30}SSe$	2424.8	2423.6
[Sec^{7,23}]-rANP(7—28)	$C_{101}H_{155}N_{33}O_{30}Se_2$	2470.9	2470.5

TABLE VI. Activities of rANP(7—28) Analogs for Receptor Binding and cGMP Accumulation in Cultured Rat Vascular Smooth Muscle Cells

	Receptor binding IC_{50} ($\times 10^{-9}$ M)[a]	cGMP accumulation EC_{50} ($\times 10^{-7}$ M)[b]
rANP(7—28)	2.3	1.4
[Sec^7]-rANP(7—28)	4.9	1.3
[Sec^{23}]-rANP(7—28)	3.7	1.8
[Sec^{7,23}]-rANP(7—28)	2.8	1.0

Values are the means of two experiments. *a*) The concentration of the peptide inhibiting specific binding of [^{125}I]-α-hANP by 50% (IC_{50}) was derived from an analysis of plots of the percentage of specific binding *vs.* the log concentration of the peptide. *b*) The concentration of the peptide required for half-maximum accumulation of cGMP (EC_{50}) was derived from an analysis of plots of cGMP contents *vs.* log concentration of the peptide.

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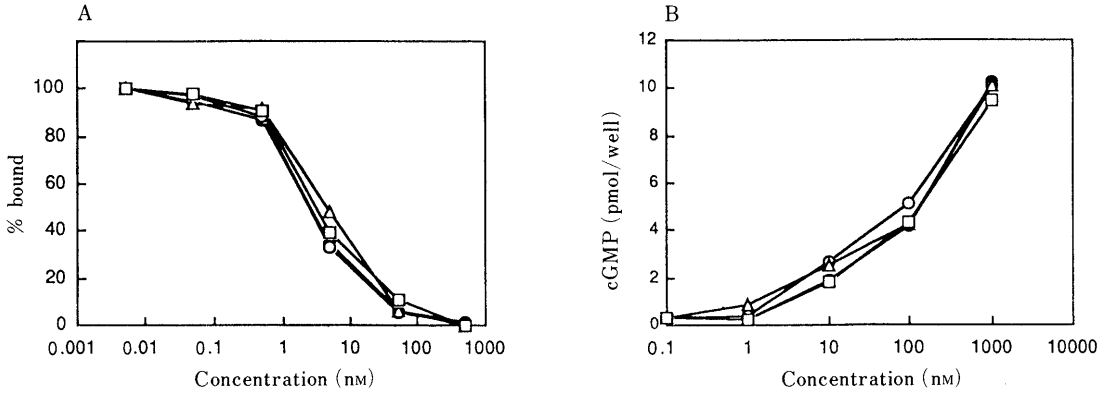

Fig. 3. Activities of rANP (7—28) Analogs for Receptor Binding (A) and cGMP Accumulation (B) in Rat Cultured Vascular Smooth Muscle Cells
●, rANP (7—28); △, [Sec^{7}]-rANP (7—28); □, [Sec^{23}]-rANP (7—28); ○, [Sec^{7,23}]-rANP (7—28). Values are the means of two experiments.

the soft sulfur or selenium atom of ANP or its selenium analogs.

Experimental

General HPLC was performed on a Waters single pump system equipped with a 600 Multisolvent delivery system and a Lambda-Max model 481 spectrometer. The solvents for HPLC were water and MeCN, both containing 0.1% (v/v) TFA, and both were degassed before use. For analytical HPLC, a Cosmosil C18-AR column (5 μm particle size: 0.46 × 15 cm) was used at a flow rate of 1 ml/min. Preparative HPLC was performed on a Cosmosil C18-AR column (5 μm particle size: 2.0 × 25 cm) at a flow rate of 8 ml/min or a YMC Pack D-ODS-5 column (5 μm particle size: 2.0 × 25 cm) at a flow rate of 10 ml/min. FAB-mass spectra were recorded on a VG Analytical ZAB-SE instrument. Acid hydrolysis of peptides was performed with 6 N HCl containing 0.1% (v/v) phenol at 110 °C for 20 h. For LAP digestion of peptides, samples (*ca.* 60 nmol) were incubated with LAP (1 unit, Sigma) in 50 mM Tris·HCl buffer (pH 7) at 37 °C overnight. Amino acid analysis was performed with a Hitachi 835 amino acid analyzer.

Solid-Phase Peptide-Chain Construction Protected resin-bound peptides corresponding to the amino acid sequence of rANP (7—28) and its selenium analogs were constructed manually by Fmoc-based solid-phase methods. For the side chain protection of Fmoc-amino acids, the following protecting groups were used; *tert*-Bu group for Tyr, Ser, and Asp, Pmc group for Arg, and MBzl group for Cys and Sec. Each synthesis was started on a 0.1 mmol scale on Fmoc-Tyr(*tert*-Bu)-*p*-benzyloxybenzyl alcohol resin (0.42 mmol/g). Removal of the N$^{\alpha}$-Fmoc group was performed with 20% (v/v) piperidine/DMF (10 min) followed by washing with DMF. Each coupling reaction was carried out at room temperature for 2 h in DMF using Fmoc amino acid (2.5 eq) with DIC and HOBt (2.5 eq each). Every coupling reaction was continued until the resin became negative to the Kaiser test.[17]

Deprotection and Cleavage of the Peptides For the deprotection of all side-chain protecting groups except for *S*- and *Se*-MBzl groups and simultaneous detachment of the peptide from the resin, each dried peptidyl resin for rANP (7—28), its analogs: [Sec^{7,23}]-rANP (7—28) or [Sec^{23}]-rANP (7—28) (250 mg) was treated with TFA–*m*-cresol–thioanisole–EDT–water (80 : 5 : 5 : 5 : 5, v/v, 12.5 ml) at room temperature for 2 h. The resin for [Sec^{7}]-rANP (7—28) (170 mg) was treated with the same reagents (8.5 ml) under the same conditions. The resin was removed by filtration and the TFA was evaporated. The ice-chilled ether was added to the residue, and the resulting precipitate was collected by centrifugation, washed with ether, and dissolved in 50% (v/v) aqueous acetic acid (5 ml) containing DTT (50 eq). The main product was purified by preparative HPLC and the solvent was removed by lyophilization to give a white powder; yield [Cys(MBzl)^{7,23}]-rANP (7—28): 67.4 mg (60%), [Sec(MBzl)^{7}, Cys(MBzl)^{23}]-rANP (7—28): 37.1 mg (48%), [Cys(MBzl)^{7}, Sec(MBzl)^{23}]-rANP (7—28): 56.3 mg (49%), [Sec(MBzl)^{7,23}]-rANP (7—28): 58.8 mg (51% based on the C-terminal amino acid). Each product was identified by amino acid analysis after acid hydrolysis and by FAB-mass spectrometry. The results are shown in and Tables I and II.

Cyclization of the Peptides Each purified MBzl-peptide (20 mg) was treated with 10% (v/v) DMSO–TFA (20 ml) at room temperature for the optimized reaction time: rANP (7—28) (60 min), [Sec^{7,23}]-rANP (7—28) (15 min), [Sec^{23}]-rANP (7—28) (120 min), [Sec^{7}]-rANP (7—28) (15 min), then ice-chilled ether was added. The resulting precipitate was collected by centrifugation, washed with ether, and dissolved in 50% (v/v) aqueous acetic acid (1.0 ml). The main product was purified by HPLC and lyophilized to give the desired product as a white power; yield rANP (7—28): 7.5 mg (41%), [Sec^{7}]-rANP (7—28): 5.8 mg (32%), [Sec^{23}]-rANP (7—28): 6.2 mg (34%), [Sec^{7,23}]-rANP (7—28); 7.3 mg (40%). Each product was identified by amino acid analysis after acid hydrolysis, LAP digestion and FAB-mass spectrometry. The results are shown in Tables III—V.

Receptor Binding Assay in Cultured Rat Vascular Smooth Muscle Cells[6] Vascular smooth muscle cells were derived from explants of rat thoracic aorta. The cells were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco) at 37 °C in a 95% air/5% CO_2 atmosphere and were used for experiments between the 6th and 9th passages. The cells were cultured in 24-well multiplates (Corning) to confluence (*ca.* 4×10^{5} cells/well), and were incubated with 5×10^{-10} M [^{125}I]-α-hANP and unlabeled sample peptide at a concentration ranging from 5×10^{-11} to 5×10^{-7} M in 0.25 ml of DMEM, pH 7.4, containing 20 mM HEPES and 0.1% BSA for 45 min at 37 °C. After incubation, the cells were washed extensively with the same medium, and the bound [^{125}I]-α-hANP was solubilized with 0.5 M NaOH. Radioactivity was measured by using a γ-spectrometer (ARC-500, Aloka). Non-specific binding was defined by incubating the cells with [^{125}I]-α-hANP in the presence of 5×10^{-7} M unlabeled α-hANP.

cGMP Determination in Cultured Rat Vascular Smooth Muscle Cells[6] Rat vascular smooth muscle cells were cultured in 24-well multiplates as described above, and confluent cells were incubated with the peptide at a concentration ranging from 10^{-9} to 10^{-6} M in 0.25 ml of DMEM, pH 7.4, containing 20 mM HEPES, 0.1% BSA and 0.5 mM 1-methyl-3-isobutylxanthine for 15 min at 37 °C. The incubation was terminated by aspirating the assay medium and adding 0.5 ml of 6% perchloric acid. The cells were disrupted by sonication and cellular cGMP concentration was determined using a cGMP radio immunoassay kit (Yamasa Shoyu).

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References and Notes

1) All amino acids used here are of the L-configuration unless otherwise mentioned. The following abbreviations are used: Pen = penicillamine, Sec = selenocysteine, $(Sec)_2$ = selenocystine, Fmoc = 9-fluorenylmethoxycarbonyl, MBzl = 4-methoxybenzyl, Pmc = 2,2,5,7,8-pentamethylchroman-6-sulfonyl, DMF = *N*,*N*-dimethylformamide, MeCN = acetonitrile, TFA = trifluoroacetic acid, DIC = *N*,*N*′-diisopropylcarbodiimide, HOBt = *N*-hydroxybenzotriazole, EDT = 1,2-ethanedithiol, DTT = dithiothreitol, LAP = leucine aminopeptidase (porcine kidney microsomal), DMEM = Dulbecco's modified Eagle's medium, BSA = bovine serum albumin, DMSO = dimethylsulfoxide.
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