Solution-Phase Synthesis of α-Rat Atrial Natriuretic Peptide (α-rANP)[1)]

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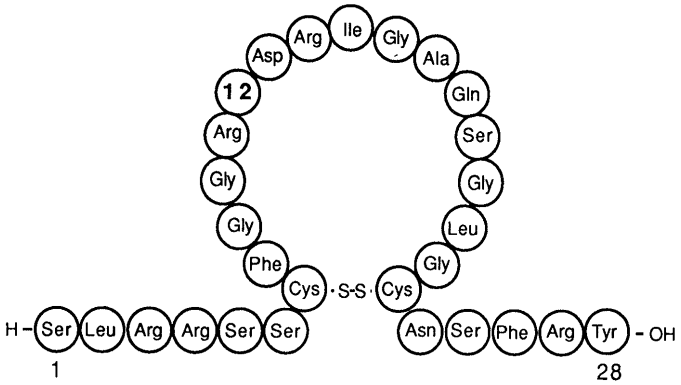
α-Rat atrial natriuretic peptide (α-rANP) was synthesized by assembling five peptide fragments in solution, followed by HF–dimethylselenide–*m*-cresol deprotection and subsequent air-oxidation. Synthetic α-rANP exhibited more potent diuretic and natriuretic activity in rats than synthetic α-hANP.

Keywords solution-phase peptide synthesis; α-rat atrial natriuretic peptide; HF–dimethylselenide–*m*-cresol deprotection; 2,4,6-trimethylbenzylcysteine; 2,2,2-trichloro-*tert*-butoxycarbonylhydrazine; diuretic activity; natriuretic activity

From rat atrial extracts, Flynn *et al.*[2)] have isolated a peptide consisting of 28 amino acids which shows a single amino acid replacement of methionine by isoleucine at position 12 when compared with α-human atrial natriuretic peptide (α-hANP) (Fig. 1). In addition to this rat ANP (α-rANP), several other peptides, both amino- and/or carboxy-terminally deleted forms and amino-terminally elongated forms of α-rANP, have been identified from the same source by several groups.[3-7)] It was considered that the structure of α-rANP, consisting of 28 amino acids, was essential for full potency of diuresis, natriuresis and smooth muscle relaxation.[6)]

Following our solution-phase synthesis of α-hANP,[8)] we undertook the solution-phase synthesis of α-rANP(1-28) to examine the effect of a single amino acid replacement at position 12 on the biological activity. A part of this work has been preliminarily reported.[9-11)] Several other research groups have also reported solid-phase[12)] or solution-phase syntheses of this peptide.[13)]



position	human	rat
12	Met	Ile

Fig. 1. Structure of α-ANPs

In the present synthesis, we employed two new synthetic improvements, *i.e.*, i) use of the Cys(Tmb) derivative and ii) use of the HF–dimethylselenide (Me_2Se) deprotection method. Cys(Tmb) is less susceptible to possible air-oxidation than the usual Cys(MBzl) derivative and the HF–Me_2Se method can reduce the sulfoxide derivative during the deprotection reaction. These improvements were successfully applied to our previous synthesis of α-hANP. Thus, amino acid derivatives bearing protecting groups removable by HF were employed, *i.e.*, Asp(OBzl), Arg(Mts),[14)] and Cys(Tmb)[9,11,15)] mentioned above. Six peptide fragments were selected as building blocks to construct the entire peptide backbone of α-rANP (Fig. 2). Five of the six fragments, *i.e.*, fragments [1]—[3], [5], and [6], are the same as those employed for our previous synthesis of α-hANP. Fragment [4], which covers the area of species variation in human and rat α-ANPs, is an intermediate peptide derivative used in the preparation of fragment [3].

In the assembly of the six peptide fragments, fragment [4] was coupled to fragment [3] before peptide chain elongation, considering the poor reactivity of this fragment, in which isoleucine was located at the carboxy-terminal position. The condensation reactions for chain elongation *via* the azide[16)] then proceeded smoothly without particular difficulty. Completion of each coupling reaction was checked by means of the ninhydrin test, and the amount of acyl component was increased from 1.5 to 3 eq as the chain elongation progressed. Protected products were purified by simple precipitation from DMF–DMSO with MeOH. Throughout this synthesis, Phe was used as a diagnostic

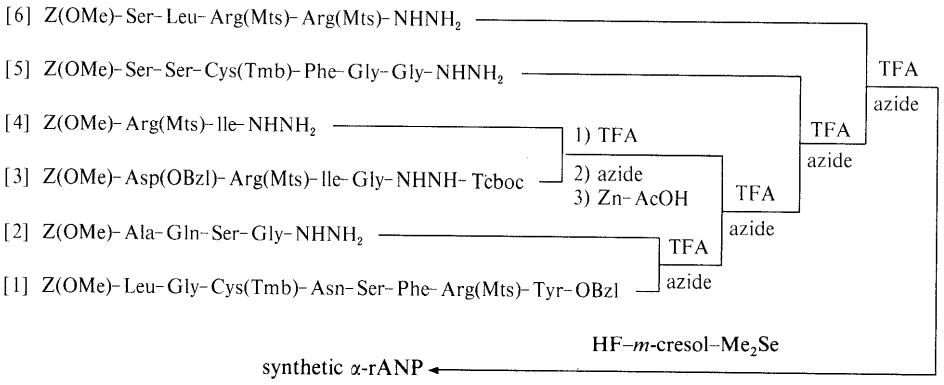


Fig. 2. Synthetic Route to α-rANP

TABLE I. Amino Acid Ratios in 6 N HCl Hydrolysates of Synthetic α-rANP and Its Intermediates

	Protected peptide			Synthetic α-rANP[a]
	11—28	5—28	1—28	
Asp	1.92	1.91	1.86	2.02 (2)
Ser	1.84	3.41	4.15	4.54 (5)
Glu	1.17	1.14	1.10	1.06 (1)
Gly	3.12	5.72	5.26	5.15 (5)
Ala	1.06	1.05	1.02	1.02 (1)
Cys	N.D.	1.12	1.08	0.89 (1)
Ile	1.70	1.44	1.77	1.92 (2)
Leu	1.05	0.74	1.77	1.98 (2)
Tyr	0.94	0.79	0.80	0.95 (1)
Phe	1.00	2.00	2.00	2.00 (2)
Arg	2.91	3.11	4.95	4.92 (5)
Recovery (%)	79	90	78	74

a) Numbers in parentheses are theoretical values. N.D. = not determined.

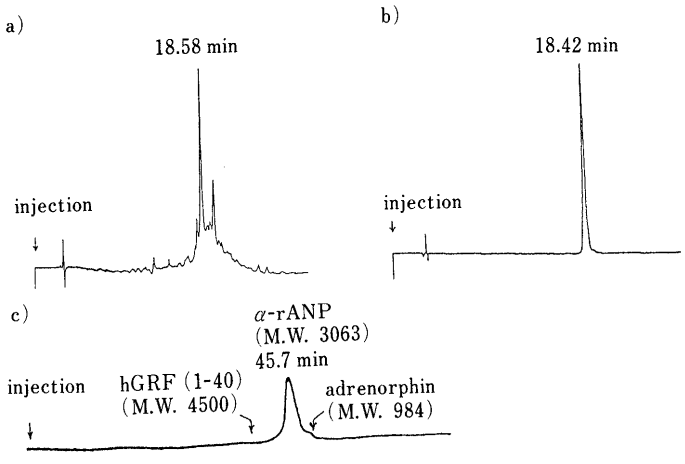


Fig. 3. HPLC Profile of Synthetic α-rANP
a) Gel-filtered sample. b) Purified sample. c) Gel-permiation HPLC.

amino acid in amino acid analyses. Each intermediate was subjected to acid hydrolysis and the recovery of Phe was compared with those of newly added amino acids in order to ascertain satisfactory incorporation, after each condensation (Table I). The homogeneity of every intermediate was further ascertained by thin-layer chromatography (TLC) and elemental analysis.

In the final step, deprotection with HF in the presence of Me_2Se–*m*-cresol and subsequent air-oxidation were carried out in essentially the same manner as described for α-hANP synthesis.[8] The crude product obtained after air-oxidation was purified by gel-filtration on Sephadex G-25, followed by preparative reverse-phase high performance liquid chromatography (HPLC) on an ODS column using gradient elution with acetonitrile in 0.3% aqueous TFA. The purified peptide thus obtained exhibited a single peak on analytical HPLC (Fig. 3b) and a single spot on TLC in different solvent systems. Its acid hydrolysate gave the amino acid ratios predicted by theory. In addition, synthetic α-rANP was proved to be a monomer by using gel-permeation HPLC (Fig. 3c).

Our synthetic α-rANP exhibited about ten times higher natriuretic and diuretic potency than synthetic α-hANP in rats.[10] Relatively low activity of α-hANP might be reasonable, since the *in vivo* assay was carried out in rats. Similar results were also reported by others.[12,13] Details of the biological activities of synthetic α-rANP and α-hANP will be published elsewhere.

Experimental

General experimental procedures employed in this investigation were essentially the same as these used in our α-hANP synthesis.[8]

TLC of products obtained in this series was performed on silica gel (Kieselgel 60 F_{254}, Merck). *Rf* values refer to the following solvent systems (v/v): Rf_1 $CHCl_3$–MeOH–H_2O (8:3:1, lower phase), Rf_2 *n*-BuOH–AcOH–pyridine–H_2O (4:1:1:2), Rf_3 *n*-BuOH–AcOH–pyridine–H_2O (30:20:6:24).

Analytical HPLC was conducted with a Hitachi 655A. Preparative HPLC and gel-permeation HPLC were conducted with Shimadzu LC-4A and LC-5A instruments, respectively.

Z(OMe)–Arg(Mts)–Ile–Asp(OBzl)–Arg(Mts)–Ile–Gly–NHNH–Tcboc Z(OMe)–Asp(OBzl)–Arg(Mts)–Ile–Gly–NHNH–Tcboc[8] (2.03 g, 1.82 mmol) was treated with TFA–anisole (4 ml–0.2 ml) in an ice-bath for 60 min as usual, then dry ether was added. The N^{α}-deprotected peptide, precipitated by ether, was dried over KOH pellets *in vacuo* for 3 h, then dissolved in DMF (5 ml) containing Et_3N (0.25 ml, 1.82 mmol). The azide [prepared from 1.77 g (2.73 mmol) of Z(OMe)–Arg(Mts)–Ile–$NHNH_2$[8]] in DMF (5 ml) and Et_3N (0.36 ml, 2.18 mmol) were added to the above ice-chilled solution and the mixture was stirred at 5 °C overnight. The ninhydrin negative solution was diluted with H_2O (50 ml) and the resulting powder was purified by precipitation from DMF with 2-propanol; yield 1.70 g (60%), mp 160—162 °C, $[\alpha]_D^{20}$ −5.4° (*c* = 0.6, DMSO), Rf_2 0.75. *Anal.* Calcd for $C_{69}H_{97}Cl_3N_{14}O_{17}S_2$: C, 52.95; H, 6.25; N, 12.53. Found: C, 52.68; H, 6.14; N, 12.44.

Z(OMe)–Arg(Mts)–Ile–Asp(OBzl)–Arg(Mts)–Ile–Gly–$NHNH_2$, Z-(OMe)–(α-rANP 11—16)–$NHNH_2$ The above protected hexapeptide (1.70 g, 1.09 mmol) in a mixture of DMF–AcOH (10 ml–2.5 ml) was treated with Zn powder (1.43 g) at room temperature overnight. After filtration of the solution, the filtrate was concentrated and 2% EDTA (30 ml) was added. The resulting powder was washed with 2% EDTA, 5% $NaHCO_3$, and H_2O. The powder was purified by precipitation twice from DMF with 2-propanol; yield 0.88 g (60%), mp 176—178 °C, $[\alpha]_D^{20}$ −11.3° (*c* = 0.7, DMF), Rf_1 0.57. Amino acid ratios in 6 N HCl hydrolysate: Asp 0.93, Gly 1.00, Ile 1.90, Arg 2.07 (recovery of Gly 86%). *Anal.* Calcd for $C_{64}H_{92}N_{14}O_{13}S \cdot H_2O$: C, 55.71; H, 6.87; N, 14.21. Found: C, 55.55; H, 6.67; N, 14.15.

Z(OMe)–Arg(Mts)–Ile–Asp(OBzl)–Arg(Mts)–Ile–Gly–Ala–Gln–Ser–Gly–Leu–Gly–Cys(Tmb)–Asn–Ser–Phe–Arg(Mts)–Tyr–OBzl, Z(OMe)–(α-rANP 11—28)–OBzl Z(OMe)–(α-ANP 17—28)–OBzl[8] (0.5 g, 0.27 mmol) was treated with TFA–anisole (2 ml–0.2 ml) and the N^{α}-deprotected dodecapeptide obtained as described above was dissolved in DMSO–DMF (1:1, 5 ml) containing Et_3N (37 μl, 0.27 mmol). The azide [prepared from 0.55 g (0.40 mmol) of Z(OMe)–(α-rANP 11—16)–$NHNH_2$] in DMF (5 ml) and Et_3N (67 μl, 0.48 mmol) were added to the above ice-chilled solution and the mixture was stirred at 5 °C overnight. The ninhydrin-negative solution was diluted with H_2O (50 ml) and the resulting powder was purified by precipitation from DMSO–DMF (1:1) with MeOH; yield 0.63 g (72%), mp 233—235 °C, $[\alpha]_D^{20}$ −16.0° (*c* = 0.5, DMSO), Rf_2 0.83. *Anal.* Calcd for $C_{144}H_{199}N_{29}O_{35}S_4 \cdot 2H_2O$: C, 56.35; H, 6.69; N, 13.14. Found: C, 56.15; H, 6.52; N, 13.25.

Z(OMe)–Ser–Ser–Cys(Tmb)–Phe–Gly–Gly–Arg(Mts)–Ile–Asp(OBzl)–Arg(Mts)–Ile–Gly–Ala–Gln–Ser–Gly–Leu–Gly–Cys(Tmb)–Asn–Ser–Phe–Arg(Mts)–Tyr–OBzl, Z(OMe)–(α-rANP 5—28)–OBzl The above protected octadecapeptide ester (0.63 g, 0.21 mmol) was treated with TFA–anisole (3 ml–0.2 ml) and the N^{α}-deprotected peptide ester isolated as stated above was dissolved in DMSO–DMF (1:1, 10 ml) containing Et_3N (29 μl, 0.21 mmol). The azide [prepared from 0.27 g (0.31 mmol) of Z(OMe)–(α-ANP 5—10)–$NHNH_2$[8]] in DMF (3 ml) and Et_3N (52 μl, 0.37 mmol) were added to the above ice-chilled solution and the mixture was stirred at 5 °C overnight. The ninhydrin-negative solution was diluted with H_2O (50 ml) and the resulting powder was purified by precipitation from DMSO–DMF (1:1) with MeOH; yield 0.73 g (95%), mp 226 °C (dec.), $[\alpha]_D^{20}$ −20.0° (*c* = 0.5, DMSO), Rf_2 0.76. *Anal.* Calcd for $C_{177}H_{241}N_{35}O_{43}S_5$: C, 56.25; H, 6.64; N, 12.97. Found: C, 56.29; H, 6.67; N, 12.97.

Z(OMe)–Ser–Leu–Arg(Mts)–Arg(Mts)–Ser–Ser–Cys(Tmb)–Phe–Gly–Gly–Arg(Mts)–Ile–Asp(OBzl)–Arg(Mts)–Ile–Gly–Ala–Gln–Ser–Gly–Leu–Gly–Cys(Tmb)–Asn–Ser–Phe–Arg(Mts)–Tyr–OBzl, Protected α-rANP The above protected tetracosapeptide ester (733 mg, 0.20 mmol) was

treated with TFA–anisole (3 ml–0.3 ml) and the N^{α}-deprotected peptide ester isolated as stated above was dissolved in DMSO–DMF (1 : 1, 10 ml) containing Et_3N (28 μl, 0.20 mmol). The azide [prepared from 637 mg (0.60 mmol) of Z(OMe)–(α-ANP 1—4)–$NHNH_2$[8]] in DMF (3 ml) and Et_3N (100 μl, 0.72 mmol) were added to the above ice-chilled solution and the mixture was stirred at 5 °C overnight. H_2O (50 ml) was added and the resulting powder was purified by precipitation from DMSO–DMF (1 : 1) with MeOH; yield 803 mg (88%), mp 249 °C (dec.), $[\alpha]_D^{20}$ −16.0° (c=0.5, DMSO), Rf_2 0.71. *Anal*. Calcd for $C_{216}H_{301}N_{45}O_{52}S_7 \cdot 4H_2O$: C, 55.71; H, 6.69; N, 13.54. Found: C, 55.54; H, 6.66; N, 13.68.

Synthetic α-rANP The protected α-rANP (200 mg) was treated with HF (10 ml) in the presence of Me_2Se (160 μl, 50 eq) and *m*-cresol (229 μl, 50 eq) in an ice-bath for 60 min. After evaporation of HF, dry ether was added and the precipitate was dried over KOH pellets *in vacuo* for 30 min. The resulting deprotected peptide was dissolved in H_2O (2 ml). After being adjusted to pH 8 with 5% NH_4OH, the solution was incubated with 2-mercaptoethanol (300 μl, 100 eq) under an argon atmosphere at 37 °C overnight. The above solution was applied to a column of Sephadex G-25 (1.6 × 140 cm), which was eluted with 0.2 N AcOH. The ultraviolet (UV) absorption at 280 nm was determined in each fraction (5.6 ml). The fractions corresponding to the front main peak (tube Nos. 32—39) were combined and diluted with H_2O (500 ml). The pH of the solution was adjusted to 7.5 with 28% NH_3. The solution was kept standing at room temperature for 120 h, during which time the Ellman[17] test value (absorption at 412 nm) dropped from 0.041 to 0.009. The entire solution was lyophilized and the residue was dissolved in 0.2 N AcOH (*ca.* 3 ml); this solution was applied to a column of Sephadex G-25 (1.6 × 140 cm) using 0.2 N AcOH as an eluant. Individual fractions (5.6 ml each) were collected and their absorption at 280 nm was determined. The fractions corresponding to the main peak (tube Nos. 31—39) were combined and the solvent was removed by lyophilization to give a fluffy powder; yield 58 mg (43%). The above G-25-purified sample (3 mg) was dissolved in 0.3% aqueous TFA containing 20% acetonitrile, and applied to a TSK-gel ODS-120T (21.5 × 300 mm) column, which was eluted with a linear gradient of acetonitrile (20—40%) in 0.3% aqueous TFA for 100 min at 25 °C (flow rate, 5.0 ml/min). The eluate corresponding to the main peak were collected. The remainder of the sample (24 mg) was similarly purified and the combined eluates were evaporated *in vacuo*. The residue was treated with Amberlite IRA-400 (acetate form, *ca.* 1 g) and lyophilized to give a white fluffy powder; yield 5.6 mg (21%) (overall yield calculated from the protected α-rANP, 9.1%). The purified peptide exhibited a single peak on a Chemcosorb 5-ODS-H (4.6 × 150 mm) column eluted with a linear gradient of acetonitrile (10—60% in 30 min) in 0.1% aqueous TFA (flow rate: 0.7 ml/min) as shown in Fig. 3b. Synthetic α-rANP (M.W. 3063) was eluted at 45.7 min on a gel-permeation HPLC column (TSK-gel G2000SW, 7.5 × 600 mm), on which hGRF (M.W. 4500) and adrenorphin (M.W. 984) were used as molecular weight standards (Fig. 3c).[8] $[\alpha]_D^{20}$ −75.1° (c=0.1, 0.2 N AcOH), Rf_2 0.39, Rf_3 0.70. Amino acid ratios in 6 N HCl hydrolysates of synthetic α-rANP are shown in Table I.

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References and Notes

1) Amino acid and peptide derivatives mentioned in this investigation are of the L-configuration. The following abbreviations are used: Z(OMe) = 4-methoxybenzyloxycarbonyl, Bzl = benzyl, Mts = mesitylene-2-sulfonyl, Tmb = 2,4,6-trimethylbenzyl, MBzl = 4-methoxybenzyl, Np = *p*-nitrophenyl, TFA = trifluoroacetic acid, DMF = *N,N*-dimethylformamide, DMSO = dimethyl sulfoxide, THF = tetrahydrofuran, NMM = *N*-methylmorpholine, Tcboc = 2,2,2-trichloro-*tert*-butoxycarbonyl, Me_2Se = dimethylselenide, EDTA = ethylenediaminetetraacetic acid.

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