ATRIOPEPTINS. III. SYNTHESIS AND BIOLOGICAL ACTIVITY STUDIES OF α-r-ANF, AP-II, AP-III AND des-Ser[5]-Ser[6]-AP-II

Mikhail V. OVCHINNIKOV, Zhanna D. BESPALOVA, Aleksandr S. MOLOKOEDOV, Mikhail I. TITOV, Inna V. REVENKO, Valentin A. VINOGRADOV, Nikolai V. KOROBOV and Sergei V. ZHUKOVSKII

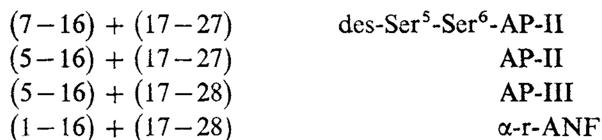
Cardiology Research Center of the U.S.S.R., Academy of Medical Sciences, 121552 Moscow, U.S.S.R.

Received February 26, 1988
Accepted June 14, 1988

Starting from previously synthesized atriopeptin fragments, peptides α-r-ANF, AP-II, AP-III and des-Ser[5]-Ser[6]-AP-II were prepared using complex F. The acetamidomethyl protecting group on cysteine was removed simultaneously with the cyclization. Biological activity of atriopeptin was evaluated.

In our preceding communications[1,2] we have described the synthesis of fragments of atrial natriuretic peptide α-r-ANF, AP-II, AP-III and des-Ser[5]-Ser[6]-AP-II (for abbreviations see ref.[1]). The present paper concerns the synthesis of these compounds and investigation of their relative biological activity.

All the atriopeptins were synthesized by condensation of two large fragments according to the following scheme:

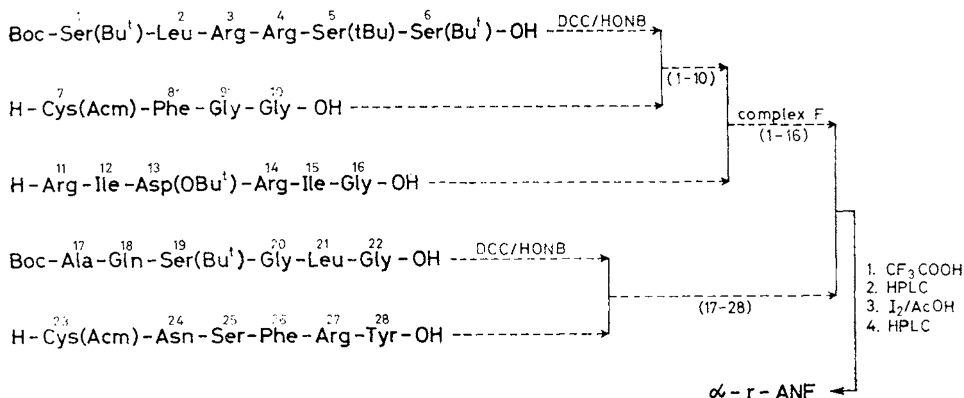


(7 – 16) + (17 – 27) des-Ser[5]-Ser[6]-AP-II
(5 – 16) + (17 – 27) AP-II
(5 – 16) + (17 – 28) AP-III
(1 – 16) + (17 – 28) α-r-ANF

The general synthetic scheme is given in Scheme 1 for α-r-ANF.

Invariably, this last condensation of two large fragments was carried out using complex F. Thus, for example, in the case of α-r-ANF, fragments 1 – 16 and 17 – 28 were condensed after preliminary activation of the N-terminal fragment. Activation of the fragment 1 – 16 was connected with some difficulties. Firstly, the protected hexadecapeptide was sparingly soluble in DMF and, secondly, even after prolonged period of time no activated derivative was formed (according to TLC). These complications were observed with all the N-terminal atriopeptin fragments. We assumed that the formation of activated ester is hindered by ionization of the carboxy group of the peptide and formation of its inner salt with one of the arginine residues.

We added therefore pyridine hydrobromide to the reaction mixture in an amount equivalent to the number or arginine residues in the fragment to be activated. This destroyed the inner salt and, moreover, strongly increased the solubility of the activated compound, leading to a rapid and quantitative formation of pentafluorophenyl



SCHEME 1

ester of the N-terminal fragment which was then condensed with the C-terminal fragment. The obtained protected peptide was treated with trifluoroacetic acid and purified by HPLC (see Fig. 1). Removal of the acetamidomethyl groups with simultaneous cyclization was carried out with iodine in aqueous methanol[3] or in aqueous acetic acid[4], the best results being obtained with 80% aqueous acetic acid. In this solvent the peptide cyclized rapidly and unequivocally (yield about 97%) at a sufficiently high concentration (100 mg of peptide in 70 ml of solution). After purification by HPLC, all the atriopeptins were obtained in about 20% yield, based on the starting N-terminal fragment.

All the atriopeptins synthesized by us showed correct amino acid analyses (see Table I) and were homogeneous according to TLC and HPLC.

It is worth notice that we have found conditions for separation of cyclic and linear peptides (Fig. 1*b*) which allowed monitoring the cyclization. This appeared particularly important in the case of atriopeptins containing a C-terminal tyrosine residue, because prolonged treatment with iodine of solutions of AP-III and α-r-ANF modified the tyrosine residue (as shown by ^1H NMR spectra).

The obtained atriopeptins reduced the arterial pressure and enhanced markedly the excretion of Na^+ ions, whereas diuresis and excretion of K^+ ions were increased only insignificantly (see Fig. 2). The Na^+ excretion increased during the first 10 minutes after administration and then returned to the basal level.

TABLE I
Amino acid analysis data for the obtained atriopeptins (cysteine was not determined)

Compound	Ala	Arg	Leu	Ile	Asp
I	1·14(1)	2·97(3)	0·91(1)	1·85(2)	2·00(2)
II	1·09(1)	3·02(3)	0·95(1)	1·91(2)	2·07(2)
III	1·18(1)	3·11(3)	0·98(1)	1·88(2)	1·91(2)
IV	1·03(1)	4·32(5)	1·99(2)	1·76(2)	1·97(2)

Compound	Ser	Tyr	Phe	Gly	Glu
I	3·20(4)	—	1·95(2)	4·67(5)	1·22(1)
II	3·23(4)	0·78(1)	1·96(2)	5·00(5)	1·07(1)
III	1·86(2)	—	2·04(2)	5·00(5)	1·04(1)
IV	4·27(5)	0·83(1)	1·92(2)	5·00(5)	1·08(1)

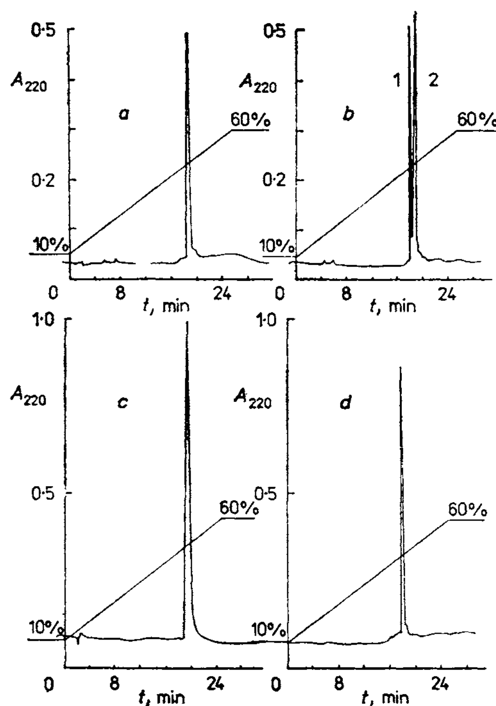


FIG. 1
HPLC curves of atriopeptins; Spherisorb ODS column (250 × 4·6 mm), gradient (25 min) of acetonitrile (10—60%) in 0·05M-KH_2PO_4 (pH 3), flow rate 1 ml/min, detection at 220 nm: *a* AP-III; *b* AP-III before (1) and after (2) cyclization; *c* α-r-ANF; *d* AP-II

The relative natriuretic and hypotensive activity of the studied atriopeptins is depicted in Fig. 3. The most active compound was α-r-ANF; shortening from the N- or C-terminal side decreased the biological activity. The obtained in vivo data show that the relative biological activity decreases in the order α-r-ANF > AP-III > > AP-II > des-Ser[5]-Ser[6]-AP-II.

Atriopeptins induced a concentration-dependent dilatation of rat isolated aorta rings, pre-treated with prostaglandin $F_{2\alpha}$. In the first series of experiments, the activity of AP-II and AP-III was about the same (EC_{50} = 18·8 ± 5·3 and 33·6 ± 10·9 nmol l^{-1}, respectively) and was markedly higher than that of des-Ser[5]-Ser[6]-AP-II (EC_{50} = 243·3 ± 65·4; the difference from AP-II and AP-III is significant at p 0·05). In the second experimental series it has been shown that AP-III and α-r-ANF had the same vasodilatory activity. The obtained data indicate that in the model used, α-r-ANF, AP-II and AP-III exhibit a strong and approximately equal vasodilatation

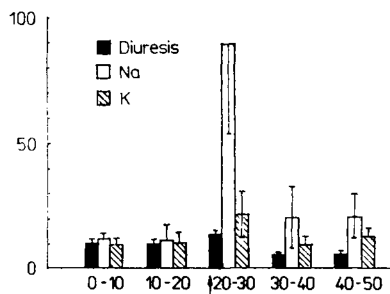


FIG. 2

Effect of atriopeptin II in a dose of 8 μg/kg (intravenously) on diuresis and excretion of electrolytes with urine (x-axis: time intervals in minutes, y-axis: diuresis in 0·1 ml per 10 min and electrolyte excretion in μ equivalents per 10 min). Introduction of AP-II is marked by arrow. The data are given as intervals representing mean values with confidence limits at $p < 0·05$

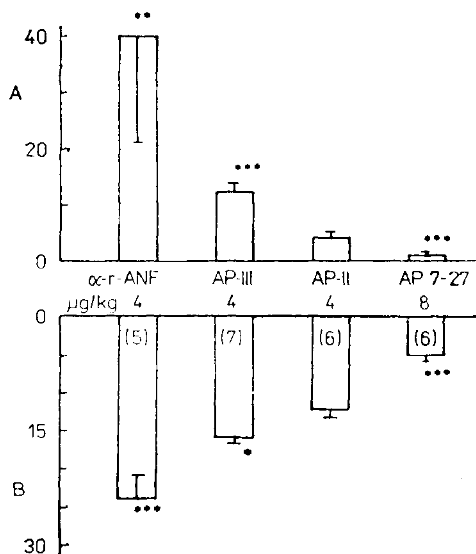


FIG. 3

Relative natriuretic and hypotensive activity of atriopeptins (y-axis: A relative increase of sodium excretion ($M \pm m$), B decrease of systolic pressure in mm Hg ($M \pm m$). The number of experiments is given in parentheses. Significance of difference from the effect of AP-II. *$p < 0·1$, **$p < 0·05$, ***$p <$ $< 0·01$. M denotes mean arithmetic value, m is mean error of the arithmetic value

effect whereas removal of two N-terminal serine residues results in a substantial activity decrease.

Thus, the results show the important role of both the N- and C-terminal part of the peptide in producing various biological activities. At the same time, they represent a basis for the search for new analogues with a given spectrum of pharmacological activity.

Concerning the relative activity of atriopeptins, rather contradictory results are reported. Some of them[5-7] are in accord with our findings. On the other hand, some papers claim that α-r-ANF, AP-III, AP-II and Ap-I (des-Phe^{26}-Arg^{27}-AP-II) are identical in their vasodilatory, hypotensive and natriuretic activity[8,9]. These differences may be connected with different number of observations and different experimental models. According to our opinion, the prospects of use of atriopeptins as drugs for curing arterial hypertension, cardiovascular and renal insufficiency require a further search for highly biologically active and degradation-resistant ANF analogues.

EXPERIMENTAL

The homogeneity of the obtained compounds was checked by TLC on Kieselgel 60 (Merck, F.R.G.) chromatographic plates in the following systems: chloroform–methanol–32% acetic acid (60 : 45 : 20, A); 1-butanol–acetic acid–pyridine–water (10·5 : 6 : 1 : 7·5, B). Spots were detected by spraying with chlorobenzidine. Amino acid analyses were performed on a Labotron Liquimat automatic analyzer (F.R.G.); the peptide samples were hydrolyzed with 6M-HCl containing 2% of phenol, at 110°C for 24 h. The results are given in Table I. HPLC was carried out on an Altex 332 instrument using an Altex/Hitachi 155-40 detector (220 nm). Acetonitrile was purchased from Merck (Lichrosolve), water and trifluoroacetic acid were distilled three times in an all-glass apparatus, potassium hydrogen phosphate was a Sigma product.

H-Ser-Ser-Cys-Phe-Gly-Gly-Arg-Ile-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-OH (AP-II) (*I*)

A) Boc-Ala-Gln-Ser(Bu^t)-Gly-Leu-Gly-Cys(Acm)-Asn-Ser-Phe-Arg-OH (*XI*, ref.[1]; 0·27 g; 0·18 mmol) was heated with trifluoroacetic acid (20 ml) for 1 h. The acid was evaporated and the remaining oil was mixed with ether. The precipitate was filtered, washed with ether, dried, dissolved in water (20 ml) and treated with Dowex 1 (OH^- form). The resin was filtered off, washed on the filter with water, the combined filtrates were concentrated, the dry residue was triturated with ether and the insoluble material was collected on filter, washed with ether and dried under vacuum over phosphorus pentoxide.

B) Pyridine hydrobromide (0·051 g; 0·32 mmol) was added to a suspension of Boc-Ser(Bu^t)-Ser(Bu^t)-Cys(Acm)-Phe-Gly-Gly-Arg-Ile-Asp(OBu^t)-Arg-Ile-Gly-OH (*XXII*, ref.[2]; 0·25 g; 0·16 mmol) in DMF (3 ml), the obtained solution was cooled to 0°C, and complex F (0·24 g; 0·32 mmol) was added with stirring. The reaction mixture was stirred at 0°C for 1 h at 20°C for 6 h. The separated N,N′-dicyclohexylurea was filtered off, the filtrate was concentrated to a small volume and mixed with ethyl acetate–ether (1 : 1; 40 ml). The precipitate was collected, washed on the filter repeatedly with ether, dried and dissolved in DMF (3 ml). To the thus-obtained solution the product prepared under *A*) was added.

The reaction mixture was stirred at 20°C for 1 h, ether (50 ml) was added, the precipitate was collected, washed on filter with ether and dried under vacuum over phosphorus pentoxide. The obtained product was treated with trifluoroacetic acid for 1 h, the reaction mixture was mixed with ether (70 ml), the precipitate was filtered, washed with ether, dried, dissolved in water (20 ml) and purified by HPLC on a 25 × 1·6 cm solumn of Silasorb-C18 (5 μm, Chemapol, Czechoslovakia), using a gradient water→ methanol (both solvents contained 0·02 mol l^{-1} triethylamine acetate, pH 4) in the course of 50 min; flow rate 10 ml/min. After desalting on the same column (gradient from 0·1% aqueous TFA to methanol) and freeze-drying, 0·15 g of the linear product was obtained. The obtained lyophilizate was dissolved in 50% acetic acid (20 ml) and a solution of iodine in acetic acid (2·25 mg/1 ml; 70 ml) was added. The formation of the disulfide bond was monitored by HPLC (see Fig. 1*b*); usually, the reaction was complete within 2 h. Zinc powder (1·8 g) was then added, after 2 min the mixture was filtered, the solid was washed with water on filter and the combined filtrates were concentrated to 30 ml. The concentrate was purified by HPLC on the same column, using a gradient water→ methanol (both solvents containing 0·1% of trifluoroacetic acid) during 30 min. Fractions, corresponding to the principal peak, were combined, the solvents were evaporated and the residue was dissolved in water (30 ml) and freeze-dried, affording 0·10 g (20%) of compound *I*; $[\alpha]_D^{20}$ −44·7° (*c* 1; 10% acetic acid); R_F 0·22 (A), 0·28 (B). For amino acid analysis see Table I.

AP-III (*II*)

The title compound was obtained in an analogous manner as described for compound *I*. Condensation of Boc-Ser(Bu^t)-Ser(Bu^t)-Cys(Acm)-Phe-Gly-Gly-Arg-Ile-Asp(OBu^t)-Arg-Ile-Gly-OH (*XII*, ref.[2]; 0·31 g; 0·2 mmol) with Boc-Ala-Gln-Ser(Bu^t)-Gly-Leu-Gly-Asn-Ser-Phe-Arg-Tyr-OH (*XXIII*, ref.[1]; 0·37 g; 0·22 mmol) gave 0·13 g (26%) of compound *II*; $[\alpha]_D^{20}$ −38·8° (*c* 0·5; 10% acetic acid); R_F 0·24 (A), 0·31 (B).

des-Ser^5-Ser^6-AP-II (*III*)

The title compound was obtained as described for the preparation of compound *I*. Reaction of Boc-Cys(Acm)-Phe-Gly-Gly-Arg-Ile-Asp(OBu^t)-Arg-Ile-Gly-OH (*XI*, ref.[2]; 0·73 g; 0·6 mmol) with Boc-Ala-Gln-Ser(Bu^t)-Gly-Leu-Gly-Cys(Acm)-Asn-Ser-Phe-Arg-OH (*XI*, ref.[1]; 1·05 g; 0·7 mmol) afforded 0·31 g (23%) of compound *III*; $[\alpha]_D^{20}$ −38·0° (*c* 1; 10% acetic acid); R_F 0·29 (A), 0·23 (B).

α-r-ANF (*IV*)

Compound *IV* was synthesized as described for compound *I*. Reaction of Boc-Ser(Bu^t)-Leu-Arg--Arg-Ser(Bu^t)-Ser(Bu^t)-Cys(Acm)-Phe-Gly-Gly-Arg-Ile-Asp(OBu^t)-Arg-Ile-Gly-OH (*XX*, ref.[2]; 0·48 g; 0·18 mmol) with Boc-Ala-Gln-Ser(Bu^t)-Gly-Leu-Gly-Cys(Acm)-Asn-Ser-Phe-Arg-Tyr--OH (*XXIII*, ref.[1]; 0·19 g; 0·18 mmol) furnished 0·10 g (20%) of compound *IV*; $[\alpha]_D^{20}$ −45·4° (*c* 1; 10% acetic acid); R_F 0·25 (B).

Pharmacological Methods

The biological activity of atriopeptins was determined in vitro as well as in vivo. The latter experiments were performed with Wistar male rats, weight 250—350 g. The animals were anesthetized with inactin (120 mg/kg, intraperitoneally). After tracheotomy, polyethylene catheters were introduced into the carotid artery, jugular vein and urinary bladder. The rectal temperature was maintained between 37·5°C and 38°C. The arterial catheter was connected to a pressure transducer

(Statham). The arterial pressure and the heart rate were recorded on a Linearcorder Mark VII (Watanabe) recorder. A solution of 0·3% NaCl and 2% glucose in 100 μl/min was infused into the jugular vein. On the background of constant diuresis, two blank samples were taken and then, after administration of the compound, three 10 minutes' samples of urine were collected. The content of Na^+ and K^+ ions in the urine samples was determined on an atomic emission spectrometer. The tested atriopeptins were dissolved in the infusion solution (100 μl) and applied intravenously during 1 min. Only one dose of the peptide was administered to each animal; no repeated applications of the atriopeptins were made.

The peripheral vasodilatory activity of atriopeptins was estimated on isolated rings cut from the thoracic part of aorta of Wistar rats. Four aorta rings from the same rat were put into thermostated compartments containing Krebs solution (37°C, gas mixture 95% O_2 and 5% CO_2). After equilibration for 2 h at submaximal concentration of prostaglandin $F_{2\alpha}$ (10 or 30 mmol l^{-1}) and a load of 1 g, atriopeptins were applied and cumulative concentration-dependence of the effect was obtained. From these data the dose versus effect plots were constructed and the EC_{50} values estimated (EC_{50} denotes an atriopeptin concentration, producing 50% decrease in tension of prostaglandin $F_{2\alpha}$-pretreated vessels). The peptides were applied in volumes not larger than 25 μl per 10 ml Krebs solution.

The results were evaluated using the paired as well as non-paired *t*-tests.

REFERENCES

1. Ovchinnikov M. V., Bespalova Zh. D., Molokoedov A. S., Revenko I. V., Sepetov N. F., Isakova O. L., Titov M. I.: Collect. Czech. Chem. Commun. *54*, 772 (1989).
2. Ovchinnikov M. V., Bespalova Zh. D., Molokoedov A. S., Revenko I. V., Sepetov N. F. Isakova O. L., Titov M. I.: Collect. Czech. Chem. Commun. *54*, 784 (1989).
3. Veber D. F., Milkowski J. D., Denkewalter R. G., Hirschmann R.: Tetrahedron Lett. *26*, 3057 (1968).
4. Thibaults B., Garcia R., Cantin M., Seidan N. G., Lazure C., Chretien M.: Eur. 0140731 (1985).
5. Thibault G., Garcia R., Carrier F., Seidan N. G., Chretien M., Cantin M.: Biochem. Biophys. Res. Commun. *125*, 938 (1984).
6. Garcia R., Thibaults G., Seidah N. G., Cantin M., Genest J., Chretien M.: Biochem. Biophys. Res. Commun. *126*, 178 (1985).
7. Genest J.: Br. Heart. J. *56*, 302 (1986).
8. Wakitani K., Oshima T., Loewy A. D., Holmberg S. W., Cole B. R., Adams S. P., Fok K. F.: Circ. Res. *56*, 621 (1985).
9. Jonson M. D.: Life Sci. *38*, 225 (1985).

Translated by M. Tichý.