SYNTHESIS AND SOME BIOLOGICAL PROPERTIES OF ANALOGUE OF ANGIOTENSIN WITH MODIFIED PROLINE STRUCTURE*

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The [1-asparagine]angiotensin analogue was synthesized by fragment condensation employing the azide method. Proline in the position 7 in this analogue and phenylalanine in the position 8 were substituted with 2-[1-(2-oxo-3-aminopyrrolidinyl)]-3-phenylpropanoic acid. The analogue does not exhibit any agonistic nor antagonistic activity in myotropic, histamin releasing and pressoric tests.

Tissue hormone angiotensin**, a linear octapeptide, is one of the most studied low-molecular vasopressor and hypertensive compounds. Several reviews[2,3] have been published on the significance of individual positions for the hormone activity.

The amino acid in the position 8 is considered to be most sensitive to changes. In this case especially correct orientation of the position 8 carboxyl group relative to the phenyl group of the phenylalanine residue may be significant[4]. Most modifications of the side residue phenylalanine result in almost complete loss of internal activity. Only in the case of preservation of the aromatic residue the activity is conserved partially, exchanges for an aliphatic residue yield analogues with antagonistic properties. 8-N-Me-Phe-analogue of angiotensin[5] has its aromatic residue retained, but the aminogroup is substituted. Its oxytocic activity on rat uterus in vitro is 100% and rat blood pressor activity in vivo is 80% of those of angiotensin II. The presence of a methyl group in the position 8 α-carbon does not, therefore, result in a significant decrease of agonistic potency.

Substitutions in the position 7, i.e. at proline, can be divided into two types. Compounds with preserved local steric properties of the proline residue (e.g. hydroxy-

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** All the chiral amino acids, mentioned in this work, are of the L-series. The nomenclature and symbols of the amino acids and peptides obey the published recommendations[1]: Spr denotes the shifted proline (see text for explanation).

proline[6] or N-methylalanine[7] analogue) exhibit a marked degree of activity. All the other changes in this position result in an almost complete loss of activity (for survey see e.g. ref.[3], p. 125.)

The analogue prepared by us can be compared with both types of substitution. The position 7 is modified together with the substitution of the amino-group of phenylalanine in position 8, but side residue of phenylalanine is preserved.

The modification which we carried out in position 7 is the so-called proline shift. The ring in the side chain is not closed by bond to the nitrogen atom of the amino acid in the position 7 (as in the case of proline) but to the nitrogen of the amino acid in the position 8 (i.e. to the phenylalanine nitrogen). The ring size is thus the same but the cyclic rigid structure si shifted towards the carboxyl end of the molecule. Such a modification fixing the secondary structure was studied by American authors[8] and applied to analogues of the LHRH (ref.[9]) enkephalin[10] and oxytocin[11].

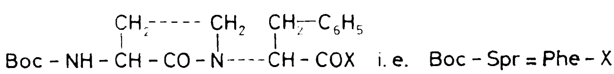
The analogue was prepared by means of fragment condensation utilizing the azide. Protection of side chain function groups was employed only in the case of the guanidine group of arginine. Imidazole ring of histidine and phenolic hydroxyl group of tyrosine were not protected during the synthesis. The carboxy group at the carboxy terminus was protected in the form of a *p*-nitrobenzyl ester. The α-amino groups, of the fragment were protected by means of tert-butoxycarbonyl protecting groups. In the case of the final octapeptide, benzyloxycarbonyl group was used in a manner enabling simultaneous cleavage of all protecting groups (from the amino- and carboxy-terminus as well as from the side chain).

Boc - Met - Phe - OMe

I

Boc - Ile - His - Spr = Phe - OBzl(*p*-NO_2)

III



IIa, X = OH
IIb, X = OH—CH_2—C_6H_4(*p*-NO_2)

Z - Asn - Arg(NO_2) - Val - Tyr - Ile - His - Spr = Phe - OBzl(*p*-NO_2)

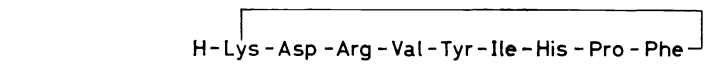
IV

H - Asn - Arg - Val - Tyr - Ile - His - Spr = Phe - OH

V

The key dipeptide 2-(3-tert-butoxycarbonylaminopyrrolidin-2-on-1-yl)-3-phenylpropane acid (*IIa*) was prepared by the treatment of N^{α}-tert-butoxycarbonylmethionylphenylalanine methylester (*I*), which was synthesized from the dicyclohexylammonium salt of tert-butoxycarbonylmethionine and phenylalanine methylester hydrochloride by the carbodiimide method, with methyl iodide to give the sulfonium salt, which in turn reacted with sodium hydride to close the five-membered ring under simultaneous hydrolysis of the ester group. The resulting derivative *IIa* was esterified with *p*-nitrobenzylbromide to the ester *IIb* and after the cleavage of the Boc protecting group with trifluoroacetic acid, it was coupled with tert-butoxycarbonylisoleucyl-histidine hydrazide[12] to yield the protected tetrapeptide *III*. Similarly, this tetrapeptide was coupled with trifluoroacetate of N^{α}-benzyloxycarbonylasparaginyl-N^{G}-nitroarginyl-valyl-tyrosine hydrazide[13] after cleavage of the Boc protecting group, to yield the protected octapeptide *IV*. Finally, all protecting groups were cleaved by hydrogenolysis and the free octapeptide *V* was purified on a column of carboxymethylcellulose.

Biological activities of the analogue *V* were tested in vitro in experiments measuring myotropic and histamin-releasing activities and in experiments in vivo measuring the influence on arterial blood pressure of anesthesized rats. Angiotensin amide (AT-NH_2) was employed as a standard for the myotropic and pressor activities and the cyclic angiotensin analogue (cAT) or bradykinin triacetate (BK) served as standard



for the histamin-releasing measurements. In experiments carried out on isolated rat colon ascendens it was ascertained that compound *V* does not exhibit any myotropic activity in the concentration range $10^{-10}-10^{-6}$ mol l^{-1} and does not influence myotropic effects of AT-NH_2. Experiments with isolated rat peritoneal mast cells showed that compound *V* has no histamin-releasing activity within the concentration range of $10^{-8}-10^{-4}$ mol l^{-1} and does not influence the histamin-releasing activity of cAT or BK at the concentration of the 10^{-5} mol l^{-1}. In experiments carried out on anesthetized rats it was ascertained that the compound in the dose range 0·5 – 500 μg/kg also did not exhibit any influence on the arterial blood pressure nor on the hypertensive effect of AT-NH_2 at the concentration of 0·5 μg/kg. Thus, the results indicate that the angiotensin analogue *V* does not exhibit agonistic nor antagonistic effect in the tests mentioned.

EXPERIMENTAL

Melting points were determined on a Kofler block and are uncorrected. Analytical samples were dried at room temperature and 150 Pa for 24 h. Thin-layer chromatography (TLC) was carried

out on silica gel coated plates (Silufol, Kavalier, Czechoslovakia (S); Merck, FRG (M)) in the following systems: 2-butanol–98% formic acid–water (75 : 13·5 : 11·5, S1), 2-butanol–25% ammonia–water (85 : 7·5 : 7·5, S2), 1-butanol–acetic acid–water (4 : 1 : 1, S3), 1-butanol–ethanol–acetic acid–water (80 : 10 : 5 : 30, S4), chloroform–2-propanol–ethanol–ethylacetate–acetic acid–water (340 : 100 : 20 : 32 : 8 : 1, S5), chloroform–ethanol–ethylacetate–acetic acid–water (340 : 20 : 32 : 8 : 1, S6), 1-butanol–pyridine–acetic acid–water (15 : 10 : 3 : 12, S7), 1-butanol–pyridine–acetic acid–water (4 : 1 : 1 : 2, S8). Paper electrophoresis was performed in a moist chamber in 1M acetic acid (pH 2·4), in 5M acetic acid (pH 1·9) and in pyridine–acetate buffer (pH 5·7) on Whatman 3MM or FN-16 (G.D.R.) paper at 20 V/cm for 60 min. Spots in TLC and electrophoresis were detected with ninhydrin, in UV, according to Pauli, Sakaguchi or by chlorination method. Samples for the amino acid analysis were hydrolyzed with 6M-HCl at 105°C for 20 h and analyzed on a D-500 analyzer (Durrum Corp.). Optical rotations were determined on a Perkin–Elmer instrument type 141 MCA (Norwalk, U.S.A.). High performance liquid chromatography (HPLC) was carried out on an SP-8700 instrument equipped with an SP-8400 detector using an SP-4100 integrator (all from Spectra Physics, Santa Clara, U.S.A.).

N$^{\alpha}$-Tert-butoxycarbonylmethionyl-phenylalanine Methylester (*I*)

The solution of the dicyclohexylammonium salt of tert-butoxycarbonylmethionine (8·95 g; 20 mmol) and phenylalanine methylester hydrochloride (4·3 g; 20 mmol) in dimethylformamide (30 ml) was cooled to −20°C. Dicyclohexylcarbodiimide (4·5 g) was added and the solution was stirred at −20°C for 1 h. The reaction mixture was left overnight in a refrigerator. The dicyclohexylurea was filtered off, filtrates were evaporated to dryness, dissolved in ethylacetate and washed successively with 1M-HCl, 0·5M-$NaHCO_3$, H_2O, evaporated and dried azeotropically (benzene). The crystalline residue was treated with ether, diluted with petrolether, filtered and washed with petrolether. The yield of the crude product was 5·85 g (71%) with a melting point 80°C. Recrystallization from ethylacetate and petrolether yielded 5·3 g (65%) of the product with a melting point in the range 81−83°C, pure according to HPLC (*k* 1·61; methanol–0·05% trifluoroacetic acid 1 : 1). For $C_{20}H_{30}N_2O_5S$ (410·5) calculated: 58·51% C, 7·37% H, 6·82% N; found: 58·75% C, 7·62% H, 6·46% N.

2-(3-Tert-butoxycarbonylaminopyrrolidin-2-on-1-yl)-3-phenylpropanoic Acid (*IIa*)

To the solution of the dipeptide *I* (2·05 g; 5 mmol) in methanol (20 ml) methyliodide (1·5 ml; 25 mmol) was added and the course of the reaction was monitored by HPLC until the starting material disappeared. Methanol was evaporated, the solid was dissolved in dimethylformamide (20 ml) and sodium hydride (0·24 g; 10 mmol) was added to the solution under intensive stirring. Further hydride was added (ca 0·15 g), so that a part of it was not dissolved. The reaction mixture was agitated for 2 h, then acetic acid was added in order to decompose the excess hydride (intensive foaming). The reaction mixture was poured into water (150 ml), acidified with 1M-HCl to pH 2 and the product was shaken into ethylacetate. The ethylacetate extract was shaken successively with a solution of sodium thiosulfate, water, dried over sodium sulfate. filtered and evaporated. Azeotropic drying (benzene) yielded 0·87 g (45%) of foam, pure according to HPLC (*k* 0·86; methanol–0·05% trifluoroacetic acid 1 : 1). $E_{2.4}^{Gly}$ 0·90, $E_{5.7}^{His}$ 0·00 (after cleavage of tert-butyloxycarbonyl group with trifluoroacetic acid).

2-(3-Tert-butoxycarbonylamino-pyrrolidin-2-on-1-yl)-3-phenylpropanoic Acid *p*-Nitrobenzylester (*IIb*)

To a solution of the dipeptide *IIa* (0·85 g; 2·4 mmol) in ethylacetate (10 ml) *p*-nitrobenzylbromide

(0·78 g; 3·6 mmol) and triethylamine (0·58 ml; 3·6 mmol) were added and the reaction mixture was refluxed for 5 h. Triethylamine hydrobromide was filtered off and the filtrate was shaken successively with 1M-HCl, water, 0·5M-$NaHCO_3$, water, dried over sodium sulfate and evaporated. The solid residue was crystallized from ethylacetate and petrolether: 1·0 g (82%) of a product was obtained with m.p. 124—127°C. The yield of the product after double recrystallization from ethylacetate was 0·45 g (37%), m.p. 148—150°C. Product was pure according to HPLC (k 3·14; methanol–0·05% trifluoroacetic acid 70 : 30). R_F (S) 0·89 (S1), 0·78 (S2), 0·82 (S3). For $C_{25}H_{29}$. $.N_3O_7$ (483·5) calculated: 62·10% C, 6·05% H, 8·69% N; found: 62·08% C, 6·03% H, 8·71% N.

N^{α}-Tert-butoxycarbonylisoleucyl-histidyl-[2-(3-amino-pyrrolidin-2-on-1-yl)-3-phenylpropanoic Acid] p-Nitrobenzylester (*III*)

Dipeptide *IIb* (250 mg; 0·5 mmol) was dissolved in trifluoroacetic acid (4 ml), benzene was added after 1 h and the reaction mixture was evaporated. The solid was dissolved in dimethylformamide (2 ml), pH of the solution was adjusted to 7—8 with ethyldiisopropylamine.

Tert-butoxycarbonylisoleucyl-histidine hydrazide[12] (190 mg; 0·5 mmol) was dissolved in dimethylformamide (3 ml), 9M-HCl in dioxane (0·28 ml; 2·5 mmol) was added to the solution. After cooling to —20°C, tert-butylnitrite (68 μl; 0·6 mmol) was added and the reaction mixture was warmed slowly to 0°C (the test for the presence of hydrazide negative). The mixture was cooled again to —40°C, solution of the dipeptide *IIb* prepared as described above was added and the pH was adjusted to 8 with ethyldiisopropylamine. After 1 h stirring at —10°C, the reaction mixture was left overnight in a refrigerator and evaporated. The solid was triturated with ether, water and dried azeotropically (benzen). The product with an R_F (M) 0·75 (S4), 0·56 (S5) and $E^{His}_{1.9}$ 0·50 was used for further reaction.

N^{α}-Benzyloxycarbonylasparaginyl-N^{G}-nitroarginyl-valyl--tyrosyl-isoleucyl-histidyl-[2-(3-aminopyrrolidin-2-on-1-yl)-3-phenylpropanoic Acid] p-Nitrobenzylester (*IV*)

The tetrapeptide-ester *III* was dissolved in trifluoroacetic acid (4 ml), benzene was added to the solution after 1 h. The reaction mixture was evaporated, triturated with ether, filtered and dried overnight in a dessicator over NaOH. Tetrapeptide trifluoracetate (285 mg; 0·37 mmol) was obtained.

To the solution of N^{α}-benzyloxycarbonyl-asparaginyl-N^{G}-nitroarginyl-valyl-tyrosine hydrazide trifluoracetate[13] (0·34 g; 0·4 mmol) in dimethylformamide (4 ml), 9M-HCl in dioxane (0·25 ml) was added. After cooling to —40°C, tert.-butylnitrite (55 μl) was added and the reaction mixture was warmed slowly up to 0°C. The mixture was after 15 min cooled again to —40°C and the tetrapeptide trifluoracetate *III* (prepared as described above) dissolved in dimethylformamide (3 ml) was added and the pH was adjusted to 8 with ethyldiisopropylamine. After 1 h stirring at —10°C, the reaction mixture was left overnight in a refrigerator and evaporated. Saturated solution of NaCl was added to the residue, the precipitate was left in the refrigerator overnight, crushed, filtered and washed with water. The crude product was dissolved in acetic acid (20 ml), filtered and the filtrate was applied onto column Lobar (Size A, 1 × 24 cm) with LiChroprep™-Si60 (40—63 μm). The column was washed with chloroform and the product was eluted with a gradient system (S6) and isopropanol (from 6 : 1 to 7 : 3). The main fraction was rechromatographed (ethanol–chloroform–acetic acid–water 80 : 10 : 5 : 5) on a same type of column. The yield was 145 mg (30%; 0·11 mmol). $[\alpha]_D$ —40·0° (c 0·5; dimethylformamide). R_F (M) 0·60 (S3); 0·00 (S6). $E^{His}_{1.9}$ 0·46.

[5-Isoleucine, 7,8-(2-(3-aminopyrrolidin-2-on-1-yl))-3-phenylpropanoic Acid]angiotensin II (*V*)

Octapeptide *IV* (130 mg; 0·097 mmol) was suspended in a mixture of methanol, acetic acid and water (10 : 1 : 1). Palladium black was added and mixture was saturated with hydrogen for 30 h. The course of the hydrogenolysis was followed by TLC (R_f (M) 0·05 (S8); 0·35 (S7) for free octapeptide) and electrophoresis ($E^{His}_{1.9}$ 0·83; Sakaguchi positive). The palladium black was filtered off and the filtrate was concentrated and diluted with ethanol. Crystalline product was dissolved in water, filtered through a column of carboxymethylcellulose (H^+-cycle), the filtrate was lyophilized and purified by HPLC. The yield of pure product: 28 mg (24%). $[\alpha]_D$ 51·2° (*c* 0·33; 0·5M-acetic acid). R_F and *E* have been mentioned previously. Amino acid analysis: His 0·99, Arg 1·04, Asp 1·10, Val 1·02, Ile 0·92, Tyr 0·98. For $C_{49}H_{70}N_{14}O_{11}$.2 AcOH.3 H_2O (1 205·4) calculated: 52·82% C, 7·02% H, 16·26% N; found: 52·57% C, 7·28% H, 16·01% N.

Pharmacological Methods

Angiotensin amide (Experimental Plant of the Institute of Organic Synthesis, Latvian Academy of Sciences), bradykinin triacetate (Reanal, Hungary) and the cyclic angiotensin analogue (cAT) (ref.[14]) were used as standards for biological tests.

The myotropic activity was determined by van Rossum's method[15] on rats (180—200 g, both sexes). Agonistic activity was determined within the range 10^{-10}—10^{-6} mol l^{-1}. The antagonistic activity was followed by measuring the influence of the analogue *V* on the myotropic effect of AT-NH_2. After the exposure to compound *V* for 3 min, myotropic effect of AT-NH_2 was registered by the sensor TB-6IIT on a polygraph RM-6000 (Nihon Kohden, Japan). Histamin-releasing activity was tested on isolated rat peritoneal mast cells[16] within the concentration range of 10^{-8}—10^{-4} mol l^{-1}. For the determination of the antagonistic activity of the analogue *V*, its influence on the histamin-releasing effect of cAT and BK (concentration 10^{-5} mol . . l^{-1}) was followed. The influence on arterial blood pressure was tested on rats of both sexes (180—200 g) anesthesized with uretan (0·5 ml of 25% solution per 100 g weight, i.p.). The pressure was recorded from arteria carotis by means of transducer Bentley Trantec Physiological Pressure and registered by a double-channel registration device Gemini (Ugo Basile, Italy). Compounds were administered i.v. into vena femoralis in a volume 0·1 ml/200 g at doses 0·5, 5·0, 50, and 500 μg/kg. During observation of the influence of analogue *V* on the hypertensive effect of AT-NH_2, AT-NH_2 (0·5 μg/kg) was administered to rats 1 min before administration of the analogue at doses mentioned above.

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