N^{α}-GLYCYL-GLYCYL-GLYCYL-8-ORNITHINE-VASOPRESSIN: A TRYPSIN RESISTANT VASOPRESSIN ANALOG WITH A SPECIFIC PRESSOR EFFECT*

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N^{α}-Glycyl-glycyl-glycyl-8-ornithine-vasopressin (*Ia*) was prepared by fragment condensation in solution. The new analog showed a higher pressor and a lower antidiuretic activity than the corresponding lysine analog *Ib*. Analog *Ia* is not cleaved by trypsin.

Vasopressin analogs** lacking a glycine residue at position 9 have both typical vasopressin activities, the pressor and the antidiuretic activity, markedly decreased. They retain, however, their effects on memory and behavior[2]. We have endeavored to prepare this type of compounds by tryptic cleavage of analogs described earlier[3]. We observed, however, that trypsin does not liberate[4] the glycine amide residue either from [8-ornithine]deamino-6-carba-vasopressin or from [8-ornithine]vasopressin. The replacement of lysine by ornithine at position 8 leads therefore to a certain metabolic stabilization. Moreover, as has been observed earlier[5], this replacement brings about dissociation of the two main activities in favor of the pressor effect. We considered interesting to examine how this replacement would affect a vasopressin analog of the hormonogen type with the α-amino group of cysteine acylated by a peptide chain.

Analog *Ia* was prepared by fragment condensation; the azide method was used and the sulfur atoms of the cysteines were protected by benzyl groups. The N^{δ}-amino group of ornithine was protected by a benzyloxycarbonyl group and the N^{α}-amino groups by 2-nitrobenzenesulfenyl or benzyloxycarbonyl groups. The benzyloxycarbonyl group of tripeptide[6-8] *III* was split off by hydrogen bromide in acetic acid and the product was acylated by the azide prepared from hexapeptide hydrazide[2] *II*. The obtained nonapeptide ester *IVa* was converted into hydrazide *IVb* by treatment with hydrazine and subsequently condensed by the azide method with the tripeptide

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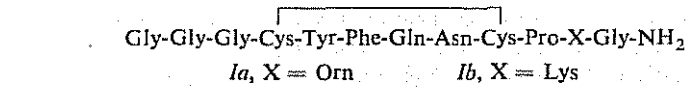
** The nomenclature and symbols comply with the rules proposed elsewhere[1]. The amino acids used were of L-configuration.

amide[3] from which the 2-nitrobenzenesulfenyl group had been removed by hydrogen chloride in ether. All protecting groups of dodecapeptide *VI* were split off by sodium in liquid ammonia and oxidizing cyclization was effected by potassium ferricyanide. Analog *Ia* was purified by free-flow electrophoresis and gel filtration. A product of identical purity can be obtained (with the omission of electrophoretic purification) by repeated gel filtration on Bio-Gel P-4.



Ia, X = Orn *Ib*, X = Lys

Z-Gly-Gly-Gly-Cys(Bzl)-Tyr(Bzl)-Phe-N_2H_3 *II*

Z-Gln-Asn-Cys(Bzl)-OMe *III*

Z-Gly-Gly-Gly-Cys(Bzl)-Tyr(Bzl)-Phe-Gln-Asn-Cys(Bzl)-Y

IVa, Y = OMe *IV*, Y = N_2H_3

Nps-Pro-Orn(Z)-Gly-NH_2 *V*

Z-Gly-Gly-Gly-Cys(Bzl)-Tyr(Bzl)-Phe-Gln-Asn-Cys(Bzl)-Pro-Orn(Z)-Gly-NH_2 *VI*

Compared to analog *Ib*, compound *Ia* prepared in this study has a uterotonic activity *in vitro* lower by one order and is inactive when subjected to the galactogogic test. Its pressor effect was approximately 2·5 times higher than the effect of compound *Ib*; antidiuresis was 3 times lower than the reported value[9]. The trend was therefore the same as that observed earlier with [8-lysine]vasopressin and [8-ornithine]vasopressin[5].

Analog *Ia*, similarly to the two ornithine analogs discussed above, is not cleaved by trypsin. It shows, however, a strongly protracted effect in the two typical vasopressin tests; this effect can obviously be ascribed to its hormonogen character. We cannot, however, regard the persistence index as increased compared to compound *Ib*. Even though the molecule of compound *Ia* is resistant to cleavage by trypsin or trypsin-like enzymes, it may undergo other degradation processes which may lead to a loss of its activity (*cf.*[10]). Tryptic cleavage, at least in the case of this analog, does not represent the main pathway of enzymatic inactivation. The experiments carried out so far do not permit us to decide whether this holds true also for vasopressin and its other analogs or whether other, originally not too important degradation processes will take over if the main inactivation mechanism is not effective.

EXPERIMENTAL

Methods

The samples for elemental analysis were dried 24 h *in vacuo* (1 Torr) at room temperature. The melting points were determined on a Kofler block and are not corrected. Thin-layer chromatography was carried out on silica gel layer sheets (Silufol, Kavalier) 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), and pyridine–1-butanol–acetic acid –water (10 : 15 : 3 : 6) (S4). Electrophoresis was carried out in a wet chamber on Whatman No 3 MM paper, 60 min at a potential gradient of 20 V/cm in the following buffers: 1M acetic acid (pH 2·4) and pyridine–acetic acid (pH 5·7). The compounds were detected by ninhydrin staining or by chlorination. Amino acid analyses were carried out on 20-h hydrolysates (6M-HCl, 105°C, *in vacuo* of 1 Torr) of samples in an automatic amino acid analyzer (Type 6020, Instrument Development Workshops of the Czechoslovak Academy of Sciences). The reaction mixtures were taken to dryness in a rotatory evaporator (bath temperature 30—40°C, oil pump). Free-flow electrophoresis was performed in the apparatus described earlier[11,12]. Gel filtration was carried out on a 100 × 1 cm column of Bio-Gel P-4 (Bio-Rad Laboratories, Richmond, U.S.A.) in 3M acetic acid. Peptide material in the effluent was located in both cases by absorbance measurement at 280 nm. Optical rotation was examined in type 141 MCA Perkin–Elmer polarimeter.

Materials

The characteristics of [8-ornithine]deamino-6-carba-vasopressin were the same as those described earlier[3]. [8-Ornithine]vasopressin was a commercial product of Sandoz (POR 8 Sandoz). Water or acetic acid in elemental analyses were calculated to fit optimally to the values determined and were not estimated analytically. The products are not crystalline solvates or stoichiometric salts of basic peptides with acetic acid in most cases.

N^{α}-Benzyloxycarbonylglycyl-glycyl-glycyl-S-benzylcysteinyl-O-benzyltyrosyl-phenylalanyl--glutaminyl-asparaginyl-S-benzylcysteine Methyl Ester (*IVa*)

A solution of tripeptide ester[6–8] *III* (1·3 g) in acetic acid (3·5 ml) was treated with hydrogen bromide in acetic acid (35%; 3·5 ml). The mixture was heated 10 min at 55°C and diluted with ether. The product was repeatedly triturated with ether, dried, and dissolved in dimethylformamide (7 ml). N-Butyl nitrite (0·34 ml) was added to the solution of protected hexapeptide hydrazide[2] *II* (1·86 g) in a mixture of dimethylformamide (20 ml) and 6M-HCl in tetrahydrofuran (1·7 ml) cooled down to −30°C. After 4 min of stirring at −30°C, the solution of tripeptide hydrobromide was added. The mixture was treated with N-ethylpiperidine until its pH was 8·5—9·0 (wet indicator paper). The mixture was set aside for 4 days at 0°C, then taken to dryness, the residue triturated with 1M-HCl, collected by filtration and washed with 1M-HCl, water, and methanol. The yield of the product precipitated from acetic acid with ether was 2·66 g (98%), m.p. 239—243°C. R_F 0·54 (S1), 0·40 (S2), 0·61 (S3), 0·67 (S4). Electrophoresis was carried out with a sample treated with hydrogen bromide in acetic acid: $E_{5,7}^{His}$ 0·32, $E_{2,4}^{Gly}$ 0·52. The sample for analysis was precipitated from acetic acid with ether, m.p. 239—243°C. $[\alpha]_D^{25}$ −30·1° (*c* 0·2, dimethylformamide). For $C_{69}H_{79}N_{11}O_{15}S_2.C_2H_4O_2.3\ H_2O$ (1480) calculated: 57·59% C, 6·06% H, 10·40% N; found: 57·45% C, 5·87% H, 10·25% N.

N^{α}-Benzyloxycarbonylglycyl-glycyl-glycyl-S-benzylcysteinyl-O-benzyltyrosyl-phenylalanyl--glutaminyl-asparaginyl-S-benzylcysteine Hydrazide (*IVb*)

A solution of compound *IVa* (1·0 g) in dimethylformamide (11 ml) was treated with hydrazine hydrate (2 ml). The mixture was stirred 92 h at room temperature, diluted with water (70 ml) and cooled down to 0°C. The product was collected by filtration and washed with water. The yield was 0·96 g (96%) of a product melting at 235—240°C. The sample for analysis was crystallized from dimethylformamide and water without a change in melting point; $[\alpha]_D^{25}$ —31·8° (*c* 0·2, dimethylformamide). For $C_{68}H_{79}N_{13}O_{14}S_2 . 2 H_2O$ (1 402) calculated: 58·23% C, 5·96% H, 12·98% N; found: 57·97% C, 5·85% H, 13·11% N.

N^{α}-Benzyloxycarbonylglycyl-glycyl-glycyl-S-benzylcysteinyl-O-benzyltyrosyl-phenylalanyl--glutaminyl-asparaginyl-S-benzylcysteinyl-prolyl-N^{δ}-benzyloxycarbonylornithyl-glycine Amide (*VI*)

A solution of protected tripeptide amide[3] *V* (0·40 g) in dimethylformamide (3 ml) was treated with 2M-HCl in ether (0·77 ml). The mixture was set aside for 4 min at room temperature and subsequently the hydrochloride was precipitated with ether, washed with ether, and dissolved in dimethylformamide (3·5 ml). A solution of hydrazide *IVb* (0·47 g) in a mixture of dimethylformamide (10 ml) and 4·5M-HCl in tetrahydrofuran (0·78 ml), cooled down to —30°C, was treated with n-butyl nitrite (60 µl) and after 4 min of stirring with the solution of the tripeptide hydrochloride and N-ethylpiperidine (to pH 8·5, wet indicator paper). The mixture was allowed to stand 70 h at 0°C and the solvents were evaporated. The dry residue was triturated with 1M-HCl, filtered off, and washed with 1M-HCl and water. Precipitation of the material from acetic acid with water afforded 0·51 g (84%) of product of m.p. 175—182°C. A part of the latter (50 mg) was filtered through a 200 × 1 cm column of Sephadex LH-20 (Pharmacia, Uppsala, Sweden) in dimethylformamide. Peptide material was located in the effluent by means of a differential refractometer (manufactured in the workshops of the Institute). In addition to the desired product two peaks were detected: one with a molecular weight lower than compound *VI* and another peak with a higher molecular weight (total quantity 5 mg). The peak containing the desired product was taken to dryness, the dry residue dissolved in dimethylformamide, and the material precipitated with ether. The yield was 37 mg (74%) of a product of m.p. 183—187°C. $[\alpha]_D^{25}$ —34·4° (*c* 0·2, dimethylformamide). Amino acid analysis: Orn 0·94; Asp 1·01; Glu 1·00; Pro 1·00; Gly 3·72; Tyr 0·99; Phe 1·03; Cys(Bzl) 1·74. For $C_{88}H_{104}N_{16}O_{19}S_2.2 H_2O$ (1 808) calculated: 58·46% C, 6·13% H, 12·39% N; found: 58·23% C, 5·92% H, 12·28% N.

N^{α}-Glycyl-glycyl-glycyl-8-ornithine-vasopressin (*Ia*)

Protected peptide *VI* (300 mg) was dissolved in distilled liquid ammonia (30 ml) and reduced by a sodium rod. When the blue color of the solution was stable for 60 s, the mixture was decolorized by ammonium chloride and lyophilized. The lyophilisate was dissolved in 0·1M-HCl (16 ml), the solution was diluted with water (30 ml) and extracted with ethyl acetate and ether. The solution was then diluted with water (250 ml), its pH adjusted to 7·0, and treated 50 min with a solution of potassium ferricyanide (105 mg) in water (30 ml) with stirring. The mixture was stirred 30 min at room temperature, its pH adjusted to 4·1 with acetic acid, and the solution placed onto a column (15 ml) of Amberlite GG-50-I. The column was washed with 0·25% acetic acid (to a negative reaction for chloride ions) and the product was eluted by 50% acetic acid. Lyophilization of the effluent afforded 190 mg of product which was dissolved in 20% acetic acid (4 ml). The solution was subjected to separation by free-flow electrophoresis (2800 V, 2·03 ml/h). Lyophilization of the

peak containing the desired material afforded 108 mg (52%) of a product whose part (50 mg) was submitted to gel filtration. This operation gave 36 mg (37% calculated on protected dodecapeptide) of the following product: $E^{His}_{5.7}$ 0·64, $E^{Gly}_{2.4}$ 1·00; $[\alpha]_D^{25}$ −84·6° (*c* 0·1, water). Amino acid analysis: Orn 1·02; Asp 0·98; Glu 0·98; Pro 1·01; Gly 4·02; Cys 1·88; Tyr 0·97; Phe 1·04. For $C_{51}H_{74}N_{16} \cdot O_{15}S_2 \cdot 3\,C_2H_4O_2 \cdot 4\,H_2O$ (1468) calculated: 46·65% C, 6·45% H, 15·27% N; found: 46·68% C, 6·14% H, 15·02% N.

Another peak (22 mg, $E^{His}_{5.7}$ 0·74 and $E^{Gly}_{2.4}$ 0·72) obtained electrophoretically was also subjected to gel filtration and shown to be dimer of product *Ia* by partial substitution of free amino groups by potassium 4-chloro-3,5-dinitrobenzene sulfonate[13,14]. Amino acid analysis: Orn 0·97, Asp 0·90, Glu 0·94, Pro 1·06, Gly 4·27, Cys 1·50, Tyr 1·03, Phe 1·09.

Protected dodecapeptide *VI* (80 mg) was reduced in a similar manner, the product was freed of organic salts on Amberlite and purified by gel filtration on Bio-Gel P-4 only. Product *Ia* obtained contained approximately 5% of a byproduct; repeated gel filtration on Bio-Gel afforded the pure product in a yield of 13 mg (25%).

Cleavage of Analog *Ia* with Trypsin

A solution of product *Ia* (50 μg) in water (0·7 ml) and Tris-HCl buffer (200 μl; 0·2M, pH 7·8) was mixed with a trypsin solution (0·1 mg; Worthington) in Tris-HCl buffer (0·1 ml) containing 10^{-4}M $CaCl_2$. The mixture was incubated 60 min at 37°C. A blank solution (sample without trypsin) was incubated as a control. The biological activity, determined by the pressor test, was the same as that of the sample incubated in the absence of trypsin. The activity of the trypsin solution was tested with N^{α}-benzoylarginine *p*-nitroanilide as substrate.

Pharmacological Tests

Antidiuretic activity was assayed on anesthetized male rats with 6—8% water load as described elsewhere[15,16]. Pressor activity was determined on despinalized male rats[17]. Galactogogic activity[18] was assayed on lactating rats (5—10 days after the delivery). Uterotonic activity was determined on an isolated strip of rat uterus[19,20] with a Mg^{2+}-free solution.

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