SYNTHESIS AND SOME PHARMACOLOGICAL PROPERTIES OF [7-GLYCINE, 8-ORNITHINE]VASOPRESSIN AND TWO OF ITS ANALOGUES*

Michal LEBL, Tomislav BARTH, Jana ŠKOPKOVÁ and Karel JOŠT

Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6

Received August 1st, 1979

Protected peptides were prepared by fragment condensation according to the scheme 6 + 3 or 9 + 3, which, after the removal of the protecting groups by sodium in liquid ammonia and oxidative cyclization, afforded [7-glycine,8-ornithine]vasopressin, [7-glycine,8-ornithine]deamino--vasopressin, and N^{ε}-glycyl-glycyl-glycyl[7-glycine,8-ornithine]vasopressin. All the analogues had very low intrinsic vasopressin-like activities; the analogue with hormonogen nature had a depressor effect and inhibited the pressor action of lysine-vasopressin.

A number of studies have been performed concerning oxytocin[1] and vasopressin[2] analogues with hormonogen properties, *i.e.* of compounds from which the active effector is liberated only after they are introduced into the organism and subjected to enzymic cleavage or unspecific (*e.g.* hydrolytic[3]) processes. So far, it has not been easy to establish whether a compound of the hormonogen type has intrinsic activity or not due to the fact that it is practically impossible to prevent the generation of the parent hormone (or its analogue) during pharmacological assays, especially when they are performed under conditions *in vivo*. The synthesis of compounds from which the active effector could not be liberated by the action of enzymes gave ambiguous results[4,5] (it must be stressed that these compounds cannot be considered to be hormonogens in the true sense of the word). The two-phase course of the pressor response to compounds of the hormonogen type led to the assumption that the first phase of the response is caused by the intrinsic activity of the hormonogen; this served as a basis for the theoretical calculation of the course of the pressor response[6].

As yet, two vasopressin analogues with glycine** in position 7 have been prepared, namely [7-glycine,8-lysine]vasopressin[8] and [7-glycine,8-arginine]vasopressin[9]. Both analogues had very low pressor activity (0·15 anf 0·01 I.U./mg). This led to the assumption that other analogues of vasopressin containing glycine in position 7

* Part CLXVI in the series Amino Acids and Peptides; Part CLXV: This Journal *45*, 2855 (1980).

** The amino acids used were of L-configuration. The nomenclature and symbols comply with the rules proposed elsewhere[7]. Mpr denotes the β-mercaptopropionic acid residue.

would have very low vasopressin-like activities, while the oxytocin-like activities would remain relatively unchanged[10,11]. On other hand, it is known[12] that the introduction of ornithine into position 8 increases the selectivity of the pressor effect, even in the case of an analogue of the hormonogen type[13]. In order to investigate the interrelation of the three structural modifications, we synthetized N^{α}-glycyl-glycyl-glycyl-[7-glycine,8-ornithine]vasopressin (*I*), [7-glycine,8-ornithine]-vasopressin (*II*) and [7-glycine,8-ornithine]deamino-vasopressin (*III*) and studied their biological activities.

The common intermediary product for the analogues prepared was tripeptide *IV* obtained by the carbodiimide condensation of *o*-nitrobenzenesulphenylglycine with dipeptide[14] *V*. The amino-protecting group was split off by HCl and the free tripeptide--amide was condensed by the azide method[15] with peptide-hydrazides[13,16,17] *VI*, *VII* and *VIII*. The resulting protected peptides *IX*, *X* and *XI* were purified by gel filtration on a column of Sephadex LH-20 in dimethylformamide. The protecting groups were removed by the action of sodium in liquid ammonia and the disulphide bond was formed by oxidation with potassium ferricyanide. The cyclic peptides were purified by gel filtration on columns of Bio-Gel P–4 in 3M and 1M acetic acid.

The biological activities of the compounds synthetized are given in Table I. The compound of the hormonogen type (*I*) has depressor action, whereas the vasopressin analogues *II* and *III* have a very low pressor effect. The depressor response to compound *I* has a time course typical for compounds of the hormonogen type. It follows that a compound with an even higher depressor effect than that of analogue *I* is gradually generated (*e.g.* the diglycyl or monoglycyl derivative) and it can be seen that the compounds of the hormonogen type have intrinsic activity. It is apparent that the time course of the response cannot be due to the liberation of compound *II* because its action is of opposite nature. Compound *I* inhibits the pressor effect of lysine-vasopressin. This action is comparable to that of N^{α}-acetyl[2-O-methyl-

TABLE I

Biological Activites of the Analogues Prepared (I.U./mg)

Compound	Pressor	Antidiuretic	Galactogogic	Uterotonic
I	[a]	0·032	—	0·006
II	0·78	1·2	7	0·45
III	0·63	0·61	7	1·6

[a] Depressor effect, *cf.* the text.

tyrosine]oxytocin, described elsewhere[18]. Because N^{α}-acetyl[2-O-methyltyrosine]-oxytocin does not have intrinsic depressor activity, it follows that there need not be any direct connection between the depressor action and the inhibition of the pressor effect.

Gly-Gly-Gly-Cys-Tyr-Phe-Gln-Asn-Cys-Gly-Orn-Gly-NH_2

I

Cys-Tyr-Phe-Gln-Asn-Cys-Gly-Orn-Gly-NH_2

II

Mpr-Tyr-Phe-Gln-Asn-Cys-Gly-Orn-Gly-NH_2

III

Nps-Gly-Orn(Z)-Gly-NH_2 Orn(Z)-Gly-NH_2

IV *V*

Z-Gly-Gly-Gly-Cys(Bzl)-Tyr(Bzl)-Phe-Gln-Asn-Cys(Bzl)-$NHNH_2$

VI

Tos-Cys(Bzl)-Tyr-Phe-Gln-Asn-Cys(Bzl)-$NHNH_2$

VII

Mpr(Bzl)-Tyr-Phe-Gln-Asn-Cys(Bzl)-$NHNH_2$

VIII

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

IX

Tos-Cys(Bzl)-Tyr-Phe-Gln-Asn-Cys(Bzl)-Gly-Orn(Z)-Gly-NH_2

X

Mpr(Bzl)-Tyr-Phe-Gln-Asn-Cys(Bzl)-Gly-Orn(Z)-Gly-NH_2

XI

The depressor action of compound *I* was observed only in tests performed on pithed rats; in tests using anaesthetized rats, compound *I* had no effect on the blood pressure. In our experiments, we also simultaneously registered the minute cardiac volume. Its value varied within physiological limits. The difference between the

results of the two pressor assays can be explained by the fact that in the pithed rat the sympathetic section of the autonomous nervous system is almost out of action and one may assume that most regulatory mechanisms do not operate. By contrast, the anaesthetized rat is apparently able to compensate the hypotensive action of compound *I* (*e.g.* by vasoconstriction or by the release of endogeneous vasopressin).

In the case of analogues *II* and *III*, the vasopressin-like activities were decreased to a greater extent than the oxytocin-like activities. The relatively high galactogogic activity is rather surprising. It can be seen that the substitution of the amino acid in position 7 by glycine eliminates the effect of ornithine in position 8 on the specificity of the pressor action (pressor/antidiuretic activity).

However, an analogue with 7-glycine substitution was described[19] ([Mpr^1,Gly^7, D-Arg^8]vasopressin), having a high degree of antidiuretic activity.

EXPERIMENTAL

The samples for elemental analysis were dried for 24 h at 150 Pa at room temperature. The melting points were determined on a Kofler block and were not corrected. Thin-layer chromatography was carried out on silica gel 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), 1-butanol–acetic acid–water (4 : 1 : 1) (S3), pyridine–1-butanol–acetic acid–water (10 : 15 : 3 : 6) (S4). Electrophoresis was performed on paper Whatman 3 MM in a moist chamber for 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). Samples for amino acid analyses were hydrolysed for 20 h (6M-HCl, 105°C, 150 Pa) and analysed by means of an automatic analyser (type 6020, Development Workshops, Czechoslovak Academy of Sciences). Reaction mixtures were evaporated on a rotary evaporator (bath temperature 30—40°C, oil pump). Gel filtration was performed on columns of Sephadex LH-20 (Pharmacia, Uppsala, Sweden) in dimethylformamide or Bio-Gel P—4 (Bio-Rad Laboratories, Richmond, U.S.A.) in 3M or 1M acetic acid. Peptide material was detected by absorption at 280 nm. Optical rotation was measured on a Perkin–Elmer (type 141 MCA) polarimeter.

o-Nitrobenzenesulphenylglycyl-N$^\delta$-benzyloxycarbonylornithyl-glycinamide (*IV*)

To a solution of N$^\alpha$-*o*-nitrobenzenesulphenyl-N$^\delta$-benzyloxycarbonylornithyl-glycinamide (1·3 g) in methanol (50 ml), 2·75M-HCl in ether (2·75 ml) was added, the solution was concentrated in vacuum and the dipeptide hydrochloride *V* was precipitated with ether. After washing with ether and drying, the product was dissolved in dimethylformamide (40 ml), the solution was alkalinized with N-ethylpiperidine (0·31 ml) and, after the addition of *o*-nitrobenzenesulphenylglycine (0·7 g) and 1-hydroxybenzotriazole (305 mg), cooled to —10°C. Then, dicyclohexylcarbodiimide (0·5 g) was added and after 15 h stirring at room temperature, urea was filtered off and dimethylformamide was evaporated. The oily product was triturated with ether, water and 0·05M-H_2SO_4. The crystalline substance was filtered off and washed with 0·5M-$NaHCO_3$, water and ether. The yield was 0·65 g (45%) of product, m.p. 184—187°C. The sample for analysis was crystallized from a mixture of methanol and ethyl acetate; m.p. 187—190°C; $[\alpha]_D$ —26·6° (*c* 0·2, dimethylformamide). For $C_{23}H_{28}N_6O_7S$ (532·6) calculated: 51·87% C, 5·30% H, 15·78% N; found: 51·61%C, 5·31% H, 15·78% N. R_F 0·55 (S1), 0·43 (S2), 0·55 (S3), 0·57 (S4); $E^{His}_{5.7}$ 0·70, $E^{Gly}_{2.4}$ 1·02 (after removing the *o*-nitrobenzenesulphenyl group).

N^{α}-Benzyloxycarbonylglycyl-glycyl-glycyl-S-benzylcysteinyl-O-benzyltyrosyl-phenylalanyl--glutaminyl-asparaginyl-S-benzylcysteinyl-glycyl-N^{δ}-benzyloxycarbonylornithyl-glycinamide (*IX*)

To the solution of tripeptide *IV* (0·65 g) in dimethylformamide (10 ml), 2·75M-HCl in ether (0·5 ml) was added. After 5 min at room temperature, the hydrochloride was precipitated with ether and reprecipitated from methanol by ether. The product was dried and dissolved in dimethylformamide (6 ml). To a solution of nonapeptide hydrazide[13] *VI* (0·4 g) in a mixture of dimethylformamide (10 ml) and 10M-HCl in tetrahydrofuran (0·3 ml), cooled to —30°C, n-butyl nitrite (60 µl) was added. The mixture was stirred for 4 min at —30°C, a pre-cooled solution of tripeptide hydrochloride in dimethylformamide was added and the pH value was adjusted to pH 8·5 (moist pH paper). The mixture was kept for 70 h at 0°C, the solvents were then evaporated and the residue was triturated with 1M-HCl. The crystalline substance was filtered off, washed with 1M-HCl and water, and reprecipitated from acetic acid by water. The yield was 530 mg of product, m.p. 220—225°C. Part of the product (100 mg) was purified by gel filtration on a 2·5 × 100 cm column of Sephadex LH-20 in dimethylformamide; after the evaporation of the fractions containing the required compound, 62 mg of product were obtained with a m.p. of 229—233°C. $[\alpha]_D$ —56·1° (*c* 0·2, dimethylformamide). Amino acid analysis: Orn 1·12, Asp 0·98, Glu 0·98, Gly 4·88, Tyr 0·96, Phe 1·03, Cys(Bzl) 1·34. For $C_{85}H_{100}N_{16}O_{19}S_2.H_2O$ (1732) calculated: 58·94% C, 5·94% H, 12·94% N; found: 58·71% C, 5·91% H, 13·27% N. $E^{His}_{5.7}$ 0·62, $E^{Gly}_{2.4}$ 0·93 (after the removal of benzyloxycarbonyl groups).

N^{α}-*p*-Toluenesulphonyl-S-benzylcysteinyl-tyrosyl-phenylalanyl-glutaminyl-asparaginyl--S-benzylcysteinyl-glycyl-N^{δ}-benzyloxycarbonylornithyl-glycinamide (*X*)

To a solution of hexapeptide hydrazide[16] *VII* (0·56 g) in a mixture of dimethylformamide (10 ml) and 8·5M-HCl in tetrahydrofuran (0·6 ml), cooled to —30°C, n-butyl nitrite (120 µl) was added. After 4 min, a solution of tripeptide hydrochloride (prepared as described earlier from 0·53 g of tripeptide *IV*) in dimethylformamide (4 ml) was added and the pH value was adjusted to 8·5 by N-ethylpiperidine (1·0 ml). The reaction mixture was kept at 0°C for 70 h and then treated as described in the case of compound *IX*; the yield was 660 mg of product, m.p. 230—243°C. A part (100 mg) of the product was subjected to gel filtration under conditions stated in the previous case; the yield was 88 mg of product, m.p. 241—245°C. $[\alpha]_D$ —16·8° (*c* 0·2, dimethylformamide). Amino acid analysis: Orn 1·06, Asp 0·96, Glu 0·93, Gly 2·08, Tyr 0·95, Phe 0·97, Cys(Bzl) 0·90. For $C_{71}H_{85}N_{13}O_{16}S_3$ (1473) calculated: 57·90% C, 5·82% H, 12·36% N; found: 57·64% C, 5·74% H, 12·36% N.

S-Benzyl-β-mercaptopropionyl-tyrosyl-phenylalanyl-glutaminyl-asparaginyl-S-benzylcysteinyl--glycyl-N^{δ}-benzyloxycarbonylornithyl-glycinamide (*XI*)

A solution of hydrazide[17] *VIII* (0·5 g) in a mixture of dimethylformamide (10 ml) and 7M-HCl in tetrahydrofuran (0·71 ml) was cooled to —30°C and mixed with n-butyl nitrite (100 µl). After 4 min, a solution of tripeptide hydrochloride (prepared from 0·53 g of tripeptide *IV*) in dimethylformamide (4 ml) was added and the pH value of the mixture was adjusted to 8·5 by N-ethylpiperidine (0·8 ml). The mixture was stored at 0°C for 70 h and then treated in the above-mentioned way. The yield was 630 mg (92%) of product, m.p. 228—237°C. A part of the product (100 mg) was purified by gel filtration (as described earlier); 71 mg of the compound were obtained, m.p. 242—244°C. $[\alpha]_D$ —31·3° (*c* 0·2, dimethylformamide). Amino acid analysis: Orn 0·97, Asp 1·00, Glu 0·98, Gly 2·02, Cys(Bzl) 0·75, Tyr 1·00, Phe 1·04. For $C_{64}H_{78}N_{12}O_{14}S_2$ (1303) calculated: 58·97% C, 6·03% H, 12·89% N; found: 58·75% C, 6·02% H, 13·02% N.

N^{α}-Glycyl-glycyl-glycyl[7-glycine,8-ornithine]vasopressin (*I*)

Protected peptide *IX* (73 mg of the compound purified by gel filtration) was dissolved in distilled liquid ammonia (20 ml) and reduced by a sodium rod until the blue colour was stable for 60 s. The solution was decolorized by adding ammonium chloride and freeze-dried. The residue was dissolved in 0·1M-HCl (6 ml), the solution was diluted with water (4 ml) and extracted with ethyl acetate and ether. After further dilution with water (130 ml), the pH value of the solution was adjusted to 7·0 by 0·1M-NaOH, a solution of potassium ferricyanide (27 mg) in water (10 ml) was added and the mixture was stirred (while maintaining the above-mentioned pH value) at room temperature for 60 min. Then, the pH value was adjusted to 4·0 by acetic acid, the solution was applied onto a column of Amberlite CG-50-I (7 ml), the column was washed with 0·25% acetic acid and the product was eluted with 50% acetic acid. Freeze-drying yielded 38 mg of the compound which was dissolved in 3M acetic acid (3 ml) and the solution was applied onto a 100 × 1 cm column of Bio-Gel P—4. The material, obtained by freeze-drying the fractions containing the monomer, was dissolved in 1M acetic acid (2 ml) and again subjected to gel filtration on a column of Bio-Gel P—4. After freeze-drying, 11 mg (22%) of material was obtained which, according to the UV spectrum, contained 82% of peptide material. $E_{5.7}^{His}$ 0·57, $E_{2.4}^{Gly}$ 1·03; $[\alpha]_D$ —44·6° (*c* 0·1, 1M acetic acid). Amino acid analysis: Orn 1·07, Asp 0·96, Glu 0·98, Gly 5·05, Cys 1·87, Tyr 1·06, Phe 1·07. For $C_{48}H_{68}N_{16}O_{15}S_2.2\,C_2H_4O_2.2\,H_2O$ (1 389) calculated: 46·67% C, 6·09% H, 16·13% N; found: 46·38% C, 5·90% H, 15·95% N.

[7-Glycine,8-ornithine]vasopressin (*II*)

Protected peptide *X* (100 mg) was reduced and its cyclization was performed by oxidation with potassium ferricyanide (44·7 mg) as described in the case of compound *I*. After two-fold gel filtration on a Bio-Gel P—4 column, 19 mg (28%) of product were obtained; $E_{5.7}^{His}$ 0·64, $E_{2.4}^{Gly}$ 1·11; $[\alpha]_D$ —13·6° (*c* 0·1, 1M acetic acid). Amino acid analysis: Orn 0 94, Asp 0·98, Glu 1·03, Gly 2·01, Cys 1·50, Tyr 1·03, Phe 1·01. For $C_{42}H_{61}N_{13}O_{12}S_2.2\,C_2H_4O_2.2\,H_2O$ (1 100) calculated: 47·62% C, 6·34% H, 15·69% N; found: 47·63% C, 5·99% H, 15·66% N.

[7-Glycine,8-ornithine]deamino-vasopressin (*III*)

Protected peptide *XI* (80 mg) was reduced and subjected to cyclization by means of potassium ferricyanide (44·7 mg) as described in the case of compound *I*. The material was purified in the same way as analogues *I* and *II* and the yield was 11·5 mg (19%) of product with $E_{5.7}^{His}$ 0·31, $E_{2.4}^{Gly}$ 0·61; $[\alpha]_D$ —42·6° (*c* 0·1, 1M acetic acid). Amino acid analysis: Orn 1·04, Asp 0·98, Glu 0·98, Gly 1·93, Cys 0·34, Tyr 1·04, Phe 1·04. For $C_{42}H_{58}N_{12}O_{12}S_2.C_2H_4O_2.2\,H_2O$ (987·1) calculated: 48·32% C, 6·17% H, 14·70% N; found: 48·06% C, 5·86% H, 14·64% N.

Pharmacological Methods

Antidiuretic activity was determined in assays on anaesthetized male rats under 6—8% water load[20,12]. Galactogogic activity was tested on lactating rats (5—10 days after delivery)[22]. Uterotonic activity was measured[23,24] using isolated strips of the rat uterus in the absence of Mg^{2+}. Pressor activity was determined[25] in experiments with pithed or anaesthetized (Pentobarbital) male rates. The minute cardiac volume was measured by the dilution method using Cardio-green dye[26].

We wish to thank Mrs H. Kovářová, Mrs J. Kellerová and Mrs M. Milbauerová for valuable technical assistance, and Mrs H. Farkašová for performing the amino acid analyses. Elemental analyses were performed at the Analytical Laboratory of this Institute (Head Dr J. Horáček).

REFERENCES

1. Beránková-Ksandrová Z., Bisset G. W., Jošt K., Krejčí I., Pliška V., Rudinger J., Rychlík I., Šorm F.: Brit. J. Pharmacol. *26*, 615 (1966).
2. Kynčl J., Řežábek K., Kasafírek E., Pliška V., Rudinger J.: Eur. J. Pharmacol. *28*, 294 (1974).
3. Smyth D. G.: J. Biol. Chem. *242*, 1579 (1967).
4. Kasafírek E., Jošt K., Rudinger J., Šorm F.: This Journal *30*, 2600 (1965).
5. Jošt K., Rudinger J., Šorm F.: This Journal *28*, 2021 (1963).
6. Pliška V.: Il Farmaco, Ed. Sci. *23*, 623 (1968).
7. *Tentative Rules on Biochemical Nomenclature.* Biochemistry *6*, 362 (1967); Biochem. J. *126*, 773 (1972).
8. Kolc J., Zaoral M., Šorm F.: This Journal *32*, 2667 (1967).
9. Papsuevich O. S., Krikis A. J., Chipens G. I.: Zh. Obshch. Khim. *42*, 224 (1972).
10. Bodanszky M., Bath R. J.: J. Chem. Soc., Chem. Commun. *1968*, 766.
11. Lowbridge J., Manning M., Haldar J., Sawyer W. H.: J. Med. Chem. *20*, 120 (1977).
12. Huguenin R. L.: Helv. Chim. Acta *47*, 1934 (1964).
13. Lebl M., Barth T., Jošt K.: This Journal *43*, 1300 (1978).
14. Jošt K., Procházka Z., Cort J. H., Barth T., Škopková J., Prusík Z., Šorm F.: This Journal *39*, 2825 (1974).
15. Honzl J., Rudinger J.: This Journal *26*, 2333 (1961).
16. Studer R. O., duVigneaud V.: J. Amer. Chem. Soc. *82*, 1499 (1960).
17. Zaoral M., Kolc J., Šorm F.: This Journal *32*, 1250 (1967).
18. Krejčí I., Kupková B., Barth T., Jošt K.: Physiol. Bohemoslov. *22*, 315 (1973).
19. Flegel M., Barth T., Krojidlo M., Flegelová Z.: *Peptides* 1978. Proc. 15th Eur. Pept. Symp., Wroclaw 1978, (I. Z. Siemion, G. Kupryszewski, Eds), p. 481. Wroclaw University Press, Wroclaw 1979.
20. Jeffers W. A., Livezey M. M., Austin J. H.: Proc. Soc. Exp. Biol. Med. *50*, 184 (1942).
21. Pliška V., Rychlík I.: Acta Endocrinol. (Copenhagen) *54*, 129 (1967).
22. Bisset G. W., Clark B. J., Haldar J., Harris M., Lewis G. P., Rocha e Silva M.: Brit. J. Pharmacol. Chemother. *31*, 497 (1967).
23. Holton P.: Brit. J. Pharmacol. *3*, 328 (1948).
24. Munsick R. A.: Endocrinology *66*, 451 (1960).
25. Krejčí I., Kupková B., Vávra I.: Brit. J. Pharmacol. Chemother. *30*, 497 (1967).
26. Sapirstein L. A.: Circulation Res. *4*, 689 (1956).

Translated by L. Servitová.