AMINO ACIDS AND PEPTIDES. CVI.*

CYCLIC PART OF VASOPRESSIN AND OXYTOCIN MOLECULE; SYNTHESIS AND BIOLOGICAL PROPERTIES

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The vasopressin cycle (*I*) and the oxytocin cycle (*II*) were prepared by condensation of glutaminyl-asparaginyl-S-benzylcysteine amide (*III*) with tosyl-S-benzylcysteinyl-tyrosyl-phenylalanine azide or by stepwise extension of *III* by isoleucine, tyrosine, and S-benzylcysteine, respectively, followed by reduction and oxidative cyclization of the obtained protected linear hexapeptide amides *VI* and *IX*, and by purification of the cyclization products. Both products showed hormonal activities approximately by two orders lower than the two naturally-occurring hormones.

The molecule of the vasopressins (and of oxytocin) consists of two different parts: of the heterodetic cyclic moiety containing cystine, tyrosine, phenylalanine (isoleucine), glutamine, and asparagine, and of the linear tripeptide side chain, comprising proline, arginine or lysine (leucine), respectively, and glycine. The disulfide cycle represents the most characteristic structural feature of these hormones and is of utmost importance for their biologic activity. Any interference with its size results mostly in a drastic decrease of the hormonal activities. The presence of the cycle, however, is not absolutely necessary. Linear vasopressin analogs[1-3] show distinct activities. Neither agonistic nor antagonistic effects were observed with oxytocin- and vasopressin-(7—9)-tripeptide amides.** Both parts of the molecule contain one of the key sites (positions in which both types of hormones differ). So far, interest has been focused predominantly on the molecule of oxytocin and vasopressins as a whole, though it is obvious that it is the disulfide cycle alone which represents an extremely interesting object for physico-chemical, biochemical, and pharmacological studies. Of the four cyclic peptides which come first in question, *i.e.* the vasopressin cycle, the oxytocin cycle, and their deamino analogs, only the oxytocin peptide has been prepared and partially studied so far[7].***

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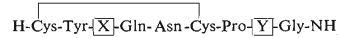
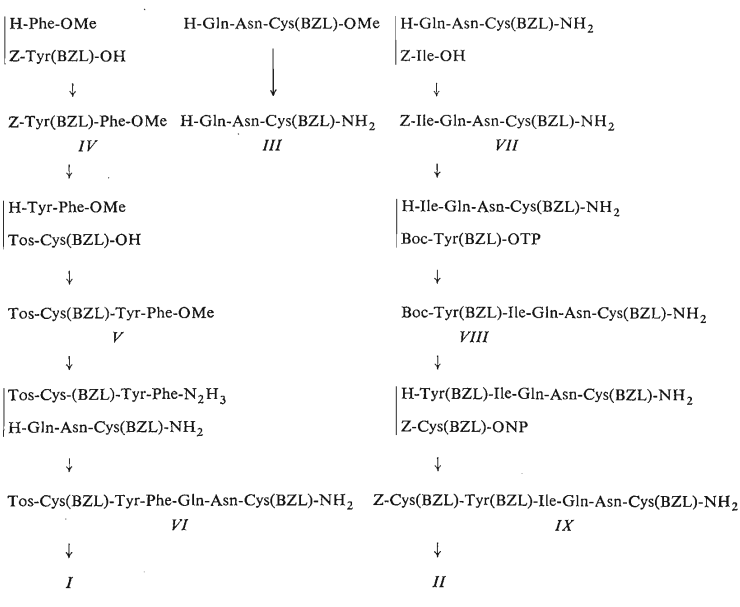
** The abbreviations used are those described in papers[4-6]. All amino acids, with the exception of glycine, are of L-configuration.

*** Vasopressin-(1-6)-, and oxytocin-(1-6)-hexapeptide amides have also been prepared by Chipens and Papsuevič (Izv. AN Latv. SSR, Ser. Chim. *1969*, 751), Kaurov and coworkers. (Ž. Obšč. Chim. *40*, 904 (1970)), and quite recently, by Hruby and coworkers (J. Am. Chem. Soc. *93*, 5593 (1971)). For the vasopressin cycle a uterotonic activity of 0·01 I.U./mg and pressor activity of 0·03 I.U./mg has been reported in the first communication. No activity data are given in the second paper.



Arginine vasopressin X = Phe, Y = Arg
Lysine vasopressin X = Phe, Y = Lys
Oxytocin X = Ile, Y = Leu

The synthesis was based on the scheme employed for the preparation of oxytocin. Tosyliso-leucyl-glutaminyl-asparagine was condensed with S-benzylcysteine amide. The resulting tetra-peptide amide derivative was reduced by sodium in liquid ammonia, rebenzylated, purified by countercurrent distribution, and condensed with benzyloxycarbonyl-S-benzylcysteinyl-tyrosine. The protecting groups were removed by sodium in liquid ammonia, the disulfide cycle was closed by oxidation (aeration), and the cyclopeptide was purified by countercurrent distribution. When tested on isolated rat uterus, it showed an activity corresponding to 3·3 I.U./mg, on the mammary gland of lactating female rabbit an activity of 1·1 I.U./mg, it lacked, however, depressor and pressor effects.



SCHEME 1

To complement the existing data and some of our preceding results[8-12] we decided to synthesize the vasopressin and oxytocin ring. The key intermediate in both syntheses was the tripeptide ester common to positions 4 through 6 of both vasopressin and oxytocin molecule[13]. We converted it into the amide by the action of ammonia in methanol (Scheme I). When preparing the protected vasopressin fragment, we coupled the amide with tosyl-S-benzylcysteinyl-tyrosyl-phenylalanine azide. (The azide was obtained from tosyl-S-benzylcysteinyl-tyrosyl-phenylalanine methyl ester[14] (*V*) *via* the hydrazide.) Ester *V* was prepared by condensation (mixed anhydride method) of N,O-dibenzyloxycarbonyltyrosine with phenylalanine methyl ester, decarbobenzoxylation of the reaction product by hydrogen bromide in glacial acetic acid, and acylation of the arising dipeptide ester by tosyl-S-benzylcysteine chloride. The protected linear oxytocin peptide was prepared from *III* by stepwise synthesis from the carboxyl end. Product *III* was condensed with benzyloxycarbonylisoleucine (mixed anhydride method), the reaction product was decarbobenzoxylated (by hydrogen bromide in glacial acetic acid) and coupled with N-tert-butyloxycarbonyl-O-benzyltyrosine 2,4,5-trichlorophenyl ester[15]. The tert-butyloxycarbonyl group was split off by trifluoroacetic acid and the free pentapeptide amide was acylated by benzyloxycarbonyl-S-benzylcysteine *p*-nitrophenyl ester. Hexapeptide amides *VI* and *IX* were then freed of the protecting groups (sodium in liquid ammonia[16]), the disulfide ring was closed by oxidation (potassium ferricyanide[17]), the obtained cyclic peptides were desalted on a column of Amberlite IRC-50 (XE-64) and purified by continuous flow electrophoresis[8,18]. The obtained peptides, the vasopressin ring (*I*) and the oxytocin ring (*II*), behaved on thin-layer chromatography and paper electrophoresis as uniform compounds. The results of elemental analysis and amino acid analysis[19] afforded satisfactory results which were in agreement with the expected composition of both products.

Both cyclic peptides were assayed for antidiuretic, pressor, uterotonic, and galactogogue effects using the synthetic, purified vasopressin and the National Standard for Oxytocin and Vasopressin Compounds as references.* The effect of vasopressin fragment *I* on diuresis was examined by two procedures[20,21]. In the first case its activity was 0·49 I.U./mg (average),** in the second case 0·39 I.U./mg (doses 20 to 100 ng) and 1·05 I.U./mg (doses 1–10 ng). When assayed for the pressor effect (despinalized rat[22]) *I* was inactive in doses up to 30 μg. The uterotonic activity[23] was 0·26 I.U./mg (medium free of Mg^{2+}-ions) and 0·082 I.U./mg (in the presence of Mg^{2+}-ions). When tested on an isolated strip of mammary gland[24], *I* showed an activity of approximately 0·3 I.U./mg. The antidiuretic activity of oxytocin frag-

* The biological assays were carried out by Dr I. Krejčí and Dr A. Machová, Research Institute for Pharmacy and Biochemistry, Prague, Doc. MUDr V. Holeček, IIIrd Clinic of Internal Medicine, Charles University, Prague, and by Dr T. Barth, Department of Biochemistry of this Institute.

** All data were processed statistically.

ment *II* tested on rat in ethanol narcosis[20] corresponded approximately to 1·3 I.U./mg. Similarly to *I* and in agreement with previous data[7], *II* did not affect the blood pressure of rat in doses lower than 50 μg. When tested for uterotonic activity *in situ*[25], *II* was inactive in doses below 25 μg while its activity on isolated rat uterus[23] was 2·7 I.U./mg (in the absence of Mg^{2+}-ions) and 0·75 I.U./mg (in the presence of Mg^{2+}-ions). The galactogogue activity determined as described above was 5·9 I.U./mg (in the absence of Mg^{2+}-ions) and 2·7 I.U./mg (in the presence of Mg^{2+}-ions). These results will be discussed in detail elsewhere.

The oxytocin ring shows a distinct uterotonic and galactogogue effect and residual antidiuretic activity. The vasopressin ring has a marked influence on diuresis and possesses also a certain uterotonic and milk-ejecting properties. In these respects the two fragments resemble the vasopressins and oxytocin, which retain – in addition to their typical effects – the residual activities of their counterparts. In other respects they differ. Neither *I* nor *II* show effect on the blood pressure of despinalized rat. While the cyclic fragment of the molecule is entirely sufficient for the manifestation of the first three activities, the tripeptide side chain is essential for the pressor effect. To a similar result have led the results of experiments with the shortening[26,27] of the tripeptide chain, changes in configuration[8,9,11,12], and changes[10] involving position 9, not, however, changes in the side chain of the basic amino acid[28,29].

EXPERIMENTAL

The melting points were determined on a Kofler block and are not corrected. The optical activity was measured in an objective polarimeter and in Zeiss–Winkel 0·01 circular polarimeter. Zeiss (Jena) VSU 2-P Spectrophotometer was employed for colorimetry in the ultraviolet region. Polarographic measurements were carried out on LP 7 Polarograph equipped with a linear recorder (Laboratorni přistroje, Prague) in combination with Kalousek vessel with isolated saturated calomel electrode. Preparative electrophoreses were performed in the apparatus of Hannig[18]. The purity of intermediary and final products was checked by chromatography in thin layer on aluminum sheets (Silufol and Silufol UV 254, Kavalier) using the systems n-butanol–acetic acid–water (4 : 3 : 2 and 4 : 1 : 5) and by paper electrophoresis (in 5% acetic acid and pyridine acetate buffer at pH 5·7). Unless stated otherwise, the samples for analysis were dried 8 h at 60°C and 10^{-4} Torr over phosphorus pentoxide.

Glutaminyl-asparaginyl-S-benzylcysteine Amide (*III*)

Glutaminyl-asparaginyl-S-benzylcysteine methyl ester[13] (10·0 g, 21·5 mmol) was allowed to stand at room temperature 3 days with 200 ml of approximately 20% ammonia solution in methanol (pressure bottle). The solid product was filtered off at reduced pressure. Yield 7·5 g (77%), m.p. 192–194°C, after recrystallization from aqueous methanol m.p. 205–207°C; $[\alpha]_D^{20}$ −48·7° ± ± 0·5° (*c* 1·08, water). For $C_{19}H_{28}N_6O_5S$ (452·5) calculated: 50·43% C, 6·24% H, 18·58% N, 7·09% S; found: 50·68% C, 6·36% H, 18·80% N, 7·07% S.

N,O-Dibenzyloxycarbonyltyrosyl-phenylalanine Methyl Ester (*IV*)

To a solution of 4·5 g (10 mmol) of N,O-dibenzyloxycarbonyltyrosine[30] in 30 ml of chloroform were added at −10°C 1·4 ml (10 mmol) of N-ethylpiperidine, 0·7 ml (10 mmol) of pyridine, and after 15 min, 1·15 ml (10 mmol) of pivalic acid chloride. The reaction mixture was set aside for 5 min at −5° and then a solution of 1·8 g (10 mmol) of phenylalanine methyl ester in 5 ml

of chloroform was added. After 60 min standing at room temperature the chloroform layer was evaporated and the neutral product was isolated. After recrystallization from aqueous acetic acid and drying, 4·8 g (79%) of a product melting at 186—188°C was obtained. $[\alpha]_D^{20}$ −24·4 ± 0·5° (*c* 1·5, chloroform). For $C_{35}H_{34}N_2O_8$ (610·7) calculated: 68·87% C, 5·61% H, 4·58% N; found: 68·88% C, 5·64% H, 4·50% N. A product of identical properties was obtained by using ethyl chloroformate in a yield by approximately 10% lower.

Tosyl-S-benzylcysteinyl-tyrosyl-phenylalanine Methyl Ester (*V*)

Protected dipeptide ester *IV* (2·75 g, 4·5 mmol), was decarbobenzoxylated by a solution of hydrogen bromide in glacial acetic acid. The hydrobromide was precipitated from the reaction mixture by ether, filtered off at reduced pressure, and dried *in vacuo* over phosphorus pentoxide (yield 1·5 g). The product was dissolved in 10 ml of water and 10 ml of ethyl acetate and 0·5 g of sodium carbonate in 18 ml of water were added to the solution. The mixture was cooled to 0°C and a solution of 1·3 g (3·5 mmol) of tosyl-S-benzylcysteine chloride in 7 ml of ethyl acetate was added with vigorous stirring. The neutral product was isolated from the organic phase. Yield 1·65 g (71%), m.p. 168—171°C. After recrystallization from aqueous ethanol, 1·2 g (52%) was obtained of a product of m.p. 173—175°C which was used in subsequent work. A part of the sample was again recrystallized from aqueous ethanol. M.p. 180°C, $[\alpha]_D^{20}$ −23·1° ± 0·5° (*c* 2·4, 95% acetic acid). Recorded[14] m.p. 180—181°C.

Tosyl-S-benzylcysteinyl-tyrosyl-phenylalanyl-glutaminyl-asparaginyl-S-benzylcysteine Amide (*VI*)

Hexapeptide amide *VI* was prepared by the method used for the preparation of vasopressin analogs[13]. From 3·2 g (4·6 mmol) of tosyl-S-benzylcysteinyl-tyrosyl-phenylalanine hydrazide (prepared by hydrazinolysis[13] of *V*), 2·1 g (4·6 mmol) of *III*, 0·32 g of sodium nitrite (in 1 ml of water) in 55 ml of dimethylformamide, 5·0 g (97%) of a product of m.p. 215—230°C was obtained. After recrystallization from aqueous acetic acid, 3·8 g (74%) was obtained of hexapeptide amide *VI*, m.p. 253—255°C, $[\alpha]_D^{20}$ − 41·3° ± 0·5° (*c* 2·05, 95% acetic acid). For $C_{54}H_{63}N_9O_{11}S_3$ (1 110·4) calculated: 58·41% C, 5·72% H, 11·35% N, 8·66% S; found: 58·71% C, 5·81% H, 11·49% N, 8·58% S. Amino-acid composition[19]: Asp 1·03, Glu 1·05, Tyr 0·94, Phe 0·99.

Benzyloxycarbonylisoleucyl-glutaminyl-asparaginyl-S-benzylcysteine Amide (*VII*)

Benzyloxycarbonylisoleucine (3·0 g, 10·6 mmol) was coupled with *III* (4·8 g, 10·6 mmol) using ethyl chloroformate (1·1 ml, 10·6 mmol) and N-ethylpiperidine (1·50 ml, 10·6 mmol) in 60 ml of dimethylformamide. The yield of the neutral product was 6·1 g (81%). After recrystallization from aqueous acetic acid, 5·5 g (72%) was obtained of a product of m.p. 274—277°C which was used in subsequent work. After one additional crystallization the m.p. increased to 280—283°C, $[\alpha]_D^{20}$ −31·9° (*c* 0·5, dimethylformamide). For $C_{33}H_{45}N_7O_8S$ (699·8) calculated: 56·63% C, 6·49% H, 14·01% N, 4·58% S; found: 56·88% C, 6·58% H, 14·06% N, 4·65% S.

N-tert-Butyloxycarbonyl-O-benzyltyrosyl-isoleucyl-glutaminyl-asparaginyl-S-benzylcysteine Amide (*VIII*)

Protected tetrapeptide amide *VII* (2·5 g) was decarbobenzoxylated by a solution of hydrogen bromide in glacial acetic acid. The hydrobromide was precipitated from the reaction mixture by ether, filtered off at reduced pressure, dried *in vacuo* over phosphorus pentoxide and dissolved in water (40 ml). The pH of the solution was adjusted to 8·5—9 by the addition of ammonium

hydroxide. The reaction mixture was set aside for 2 h at 0°C and the isoleucyl-glutaminyl-asparaginyl-S-benzylcysteine amide which had separated was filtered off at reduced pressure. The amide was washed on the filter with water and dried. Yield 1·2 g (58%). The product (1·0 g, 1·7 mmol) was dissolved in 15 ml of dimethylformamide, N-tert-butyloxycarbonyl-O-benzyltyrosine 2,4,5-trichlorophenyl ester[15] (0·95 g, 1·7 mmol) was added to the solution, and the reaction mixture was set aside for 16 h at room temperature. The yield of the neutral product, which was used without any additional purification in the subsequent reactions, was 1·2 g (74%), m.p. 249—253°C. The sample for analysis was repeatedly crystallized from dimethylformamide–water, m.p. 254 to 256°C, $[\alpha]_D^{20}$ −27·8° (*c* 0·25, dimethylformamide). For $C_{46}H_{62}N_8O_{10}S$ (919·0) calculated: 60·11% C, 6·81% H, 12·19% N, 3·49% S; found: 60·35% C, 6·70% H, 12·12% N, 3·42% S.

Benzyloxycarbonyl-S-benzylcysteinyl-O-benzyltyrosyl-isoleucyl-glutaminyl-asparaginyl-S-benzylcysteine Amide (*IX*)

Product *VIII* (2·2 g) was allowed to stand 60 min with 10 ml of trifluoroacetic acid. The solution was evaporated *in vacuo* and the dry residue was recrystallized from ethanol–water. Yield 1·94 g (87%). The trifluoroacetate was dissolved in 35 ml of dimethylformamide and 0·29 ml (2·1 mmol) of triethylamine and 0·90 g (2·1 mmol) of benzyloxycarbonyl-S-benzylcysteine *p*-nitrophenyl ester[31] were added to the solution. The reaction mixture was allowed to stand 16 h at room temperature and the neutral product was isolated. Yield 1·8 g (81%), m.p. 264—267°C. The sample for analysis was purified by crystallization from aqueous acetic acid. M.p. 275—277°C, $[\alpha]_D^{20}$ −41·5° (*c* 0·2, dimethylformamide). For $C_{59}H_{71}N_9O_{11}S_2$ (1146) calculated: 61·82% C, 6·26% H, 10·99% N, 5·59% S; found: 61·98% C, 6·19% H, 10·76% N, 5·6% S.

Vasopressin-(1—6)-hexapeptide Amide *I*

The reduction, oxidation, desalting, and purification were carried out as described elsewhere[8,13]. From 750 mg of protected hexapeptide amide *VI*, 96 mg of the lyophilisate was obtained. After electrophoretic purification, 21 mg of cyclopeptide *I* was obtained. For $C_{33}H_{43}N_9O_9S_2$ (773·3) calculated: 16·29% N; found: 16·01% corresponding to a 98·3% content of the peptide in the lyophilisate. $[\alpha]_D^{20}$ −17·6° (*c* 0·25, 1M-CH_3CO_2H). The results of elemental analysis after 8 h drying of the product at 120°C/10^{-4} Torr over phosphorus pentoxide corresponded to the sesquihydrate. For $C_{33}H_{43}N_9O_9S_2.1{\cdot}5\ H_2O$ (800·9) calculated: 49·55% C, 5·79% H, 15·76% N; found: 49·45% C, 6·08% H, 16·01% N. Amino-acid composition[19]: Cys 1·85, Tyr 0·95, Phe 1·00, Glu 1·04, Asp 1·00.

Oxytocin-(1—6)-hexapeptide Amide *II*

The preparation of *II* was carried out as in the preceding experiment. Product *IX* (540 mg) afforded 167 mg of unpurified and 85 mg of purified peptide. For $C_{30}H_{45}N_9O_9S_2$ (740·0) calculated: 17·04% N; found: 13·34% N, corresponding to 78·3% content of peptide in the lyophilisate. $[\alpha]_D^{20}$ −16·9° (*c* 0·13, 1M-CH_3CO_2H). Repeated analyses of samples dried 10—16 h at 120°C/10^{-4} Torr over phosphorus pentoxide gave values corresponding to hemiacetate monohydrate. For $C_{30}H_{45}N_9O_9S_2.0{\cdot}5\ CH_3CO_2H.H_2O$ (788·0) calculated: 47·25% C, 6·28% H, 15·99% N; found: 47·06% C, 6·61% H, 15·78% N. Amino-acid composition[19]: Cys 1·72, Tyr 0·99, Ile 0·98, Glu 1·07, Asp 1·06.

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