SEQUENCE MODIFICATION IN NEUROHYPOPHYSEAL HORMONES *

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Lysine vasopressin with tyrosine and phenylalanine interchanged was resynthesized in an attempt to explain recent results of biological testing of analogs modified in position 3. However, negligible activities found by Boissonnas and Guttmann in 1960 were only confirmed. The second analog, [Leu^7,Pro^8]dCOT-1, proved the positive influence of deamino and carba modifications on the biological activity of neurohypophyseal hormones. This analog was found to be very selective in its oxytocin-like activity.

There are no doubts on the importance of the amino acid sequence in biologically active compounds for their activity. However, all the positions are not equally important. Most results concerning sequence alterations in oxytocin and vasopressin confirmed this presumption, see Table I. However, some time ago, synthesis[1] of an analogue [Phe^2, $Phe(NH_2)^3$]AVP was published, biological activities of which were surprisingly high[2] taking into consideration that phenylalanine substituted in the *p*-position by a hydrophilic amino group may be considered analogous to tyrosine and comparing its activities to [Phe^2, Tyr^3]LVP. One of the explanations of the differences was based on the assumption that there were some problems connected with the synthesis and testing of analogs of this series in 1960. Similar explanation was proven to be correct[7] in the case of another modification originally believed as deteriorating the activity of vasopressin – D-alanine in position 9. However, resynthesis of [Phe^2, Tyr^3, Lys^8]VP and its new biological evaluation completely confirmed the results reported by Boissonnas[4] (see Table I). It means that the high activity of the aminosubstituted analogue may be explained by different characters of the substituents – strong acidity of the OH-group and low basicity of the NH_2--group.

Another sequence interchange which was of interest for us was that in positions 7 and 8 of oxytocin. It was shown by Bláha et al.[6] that such modification conserves

* Part CCXXVII in the series Amino Acids and Peptides; Part CCXXVI: Collect. Czech. Chem. Commun. 604, (1992).

** All the chiral amino acids, mentioned in this work, are of the L-series, if not specified otherwise. The nomenclature and symbols of the amino acids and peptides obey the published recommendations (Eur. J. Biochem. *138*, 9 (1984)).

the biological activity considerably. As a consequence of the same type of interchange in vasopressin, the uterotonic activity in vitro is preserved and all other activities are strongly decreased. We were interested to find out if this interchange would have the same effect on the activity of a highly active analog of oxytocin. Deamination of cysteine in position 1 and mono carba replacement of the disulfide bridge are known to be activity enhancing modifications (for the discussion of structure activity relationship see e.g. ref.[8]).

We have synthesized [Leu^7,Pro^8]dCOT-1 using solid phase synthesis and cyclization in solution. Table I shows that we have obtained an analog exhibiting relatively high in vitro and in vivo uterotonic and galactogogic potency. At the same time the decrease of the uterotonic activity was of the same order of magnitude in the case of analogs of oxytocin and dCOT-1. On the other hand, the decrease of the galactogogic potency was lower with the dCOT-1 analog. Due to the very low pressor activity, the [Leu^7,Pro^8]dCOT-1 analog is a rather oxytocin-like selective agonist.

EXPERIMENTAL

General methods and instrumentation were the same as described previously[7], including the protocol of the solid phase synthesis. Progress of the coupling was followed by the bromophenol blue "BB" method[9].

TABLE I

Biological activities (rat, I.U./mg) of oxytocin, vasopressin and its analogs with two interchanged amino acids

Compound	Uterotonic	Galactogogic	Pressor	Antidiuretic	Ref.
LVP	5	63	285	260	
[Phe^2, Tyr^3]LVP	<0·01		0·14	0·013	4
[Arg^7, Pro^8]VP	20	1·6	<1	3·2	6
[Phe^2, Phe(NH$_2$)^3]AVP			8·9	102	2
[Phe^2, Tyr^3]LVP	0 (0^a)		0·12	0·01	^{b}
OXT	450	450	4	4	
[Ile^2, Tyr^3]OXT	0				3
[Phe^2, Tyr^3]OXT	<0·01		<0·01	%0·001	4
[Asn^4, Glu^5]OXT	0·4		0·01		5
[Leu^7, Pro^8]OXT	27	45			6
dCOT-1	1 898 (1 251^a)	562	17·5	21	8
[Leu^7, Pro^8]dCOT-1	53·2 (118·9^a)	243	<0·04	<0·2	^{b}

[a] In vivo; [b] this paper.

[Phe^2, Tyr^3, Lys^8]VP

p-Methylbenzhydrylamine resin (Peptides International, 0·9 mmol/g, 0·4 g, 0·36 mmol) was suspended in dichloromethane and after washing with 5% diisopropylethylamine in dichloromethane and with dimethylformamide it was coupled with a 3 molar excess of Boc-Gly-OH in the presence of N-hydroxybenzotriazole and diisopropylcarbodiimide in dichloromethane. The following amino acids were coupled to the resin according to general scheme described earlier[7]: Boc-Lys(ClZ)-OH, Boc-Pro-OH, Boc-Cys(pMeBzl)-OH, Boc-Asn-OH, Boc-Gln-OH, Boc-Tyr(BrZ)-OH, Boc-Phe-OH, Boc-Cys(pMeBzl)-OH. Boc-amino acids were coupled to the resin using the DIC/HOBt procedure. All reagents were used in 3 molar excess. Yield for benzhydrylamine resin: 0·83 g. The nonapeptide-resin (0·8 g) was treated with liquid hydrogen fluoride (10 ml, 60 min, 0°C) in the presence of thioanisole (0·5 ml) and *m*-cresol (0·5 ml). The unprotected nonapeptide, together with the resin, was triturated with ether, and after the evaporation of hydrogen fluoride, filtered off, washed with ethyl acetate and then the free peptide was extracted successively with 30% acetic acid, water and lyophilized (0·33 g). The lyophilizate was dissolved in water (400 ml) and pH of the solution was adjusted with 5% NH_4OH to 8·5. Potassium ferricyanide (0·01M solution) was added until stable yellow colour persisted. After stirring for additional 30 min, pH was adjusted to 4·5 with acetic acid. To the solution, Amberlite IR 45 (Cl^- form, 25 ml) was added and stirring continued for 90 min. Filtered solution was freeze-dried (0·35 g), dissolved in 1M acetic acid and applied onto the column of Sephadex G-25 (3 × 60 cm). Part of the main fraction (5 mg) was purified by HPLC on the Vydac 218TP510 column using a gradient running from 0% to 50% methanol in 0·05% trifluoroacetic acid in 50 min. Lyophilization of the corresponding fraction afforded 1·1 mg of the product pure according to HPLC (*k* 3·67, methanol–0·05% trifluoroacetic acid 1 : 4). Electrophoretic mobility of this product was identical to that of lysine-vasopressin ($E_{2.7}^{Gly}$ 1·08, $E_{5.7}^{His}$ 0·54. Amino acid analysis: Asp 0·99, Glu 1·01, Pro 1·02, Gly 1·00, Cys 1·61, Tyr 0·94, Phe 0·99, Lys 1·00. FAB MS (*m*/*z*): 1 057 ($M + H^+$).

[Leu^7, Pro^8]deamino-carba-1-oxytocin

p-Methylbenzhydrylamine resin (Peptides International, 0·79 mmol/g, 0·5 g, 0·39 mmol) was acylated by Boc-Gly-OH in the same manner as above. Similarly, Boc-Pro-OH and Boc-Leu-OH were coupled to it. After the third amino acid was coupled, the synthetic protocol was changed to the Fmoc strategy. A cycle for incorporation of an amino acid residue into the growing peptide chain considered of the following steps: 1. cleaving the Fmoc group by washing with 20% piperidine in dimethylformamide for 15 min; *2.* washing with dimethylformamide (4 × 2 min); *3.* coupling with 3 equivalents of the Fmoc-protected amino acid derivative, N-hydroxybenzotriazole and diisopropylcarbodiimide in dimethylformamide; *4.* washing with dimethylformamide (3 × 2 min). The following protected derivatives were used: Fmoc-Cys($C_3H_6CO_2Bu^t$)-OH, Fmoc-Asn-OH, Fmoc-Gln-OH, Fmoc-Ile-OH and Boc-Tyr-OH. The resin was treated with a mixture of trifluoroacetic acid, dichloromethane and anisole (45 : 45 : 10; twice 15 min). The resin was washed with dichloromethane, methanol, dichloromethane, 5% diisopropylethylamine in dichloromethane and dichloromethane. The nonapeptide-resin (0·64 g) was treated with liquid hydrogen fluoride (10 ml, 0°C, 1 h) and anisole (1 ml). After usual processing, the lyophilizate was subjected to gel filtration (Sephadex G-25, 80 × 2 cm). Yield of the linear peptide: 266 mg.

To the solution of the heptapeptide (200 mg) in dimethylformamide (25 ml), cooled to 0°C, K_2HPO_4 (150 mg) and diphenylphosphoryl azide (70 μl) were added. Cyclization was followed by HPLC. After 24 h, the starting compound was not detected. Reaction mixture was filtered,

evaporated, and the residue was precipitated from the dimethylformamide solution with ether. Part of the product (25 mg) was purified by HPLC on a Vydac 218TP510 column using a slow gradient running from 20% to 45% methanol in 0·05% trifluoroacetic acid in 75 min. Lyophilization of the appropriate fraction afforded 6·3 mg of the product pure according to HPLC (*k* 5·13, 23% of methanol in 0·05% trifluoroacetic acid). Amino acid analysis: Asp 0·96, Glu 1·02, Pro 0·95, Gly 1·00, Cys(C_3H_6COOH) 0·89, Leu 1·02, Ile 0·99, Tyr 0·94. For $C_{46}H_{75}N_{11}O_{16}S$ 0·5 AcOH.2·5 H_2O (1 049) calculated: 51·50% C, 7·11% H, 14·62% N; found: 51·54% C, 7·16% H, 14·66% N. FAB MS (*m/z*): 975 ($M + H^+$).

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