SYNTHESIS AND BIOLOGICAL PROPERTIES OF VASOPRESSIN ANALOGUES CONTAINING 1,2,3,4-TETRAHYDROISOQUINOLINE--3-CARBOXYLIC ACID*

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Solid phase synthesis methodology on a benzhydrylamine resin was used for the synthesis of three analogues of vasopressin with the non-coded amino acid, 1,2,3,4-tetrahydroisoquinoline-3--carboxylic acid (Tic), in the position 2 ([Tic^2, Lys^8]VP (*I*)) and in the position 3 ([Tic^3, Lys^8]VP (*II*)). The analogue containing only one Tic in place of both aromatic residues was also isolated (des-Tyr^2-[Tic^3, Lys^8]VP (*III*)). The biological activities of all analogues were negligible.

Molecules of neurohypophyseal hormones have been shown to be very flexible in solution. A conformation of oxytocin, necessary for the interaction with the uterotonic receptor, was suggested on the basis of extensive physico-chemical studies. Similar attempts were also made in the vasopressin area (for the review see e.g.[1]). Detailed mapping of the appropriate receptors requires modification of the hormone molecule limiting its conformational freedom. One of conformational constraints which may limit the number of available spatial structures is alkylation of the backbone amide group. Introduction of 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid*** (ref.[2]) into the peptide chain in the place of an aromatic amino acid combines backbone alkylation with side-chain fixation. In addition to local rigidization of the peptide backbone in the immediate neighborhood of the above mentioned amino--acid, increased global rigidity of the whole molecule may be expected due to transannular effects. Conformationally restricted analogues of hormones allow easier

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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[3]: Tic denotes the 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid moiety.

determination of solution conformation which might be (in the case of potent agonists or inhibitors) more similar to that which is present during interaction with the receptor. In the most favourable case, selectively acting analogs might be obtained and conformational and structural features which are important for binding and transduction can be more precisely determined. Additionally, increased enzymatic stability may be expected.

Three analogues containing Tic were synthesized. In the first one, L-Tic replaced tyrosine in position 2 ([Tic^2, Lys^8]VP (*I*)), and in the second one, phenylalanine in position 3 ([Tic^3, Lys^8]VP (*II*)). The third analogue is the octapeptide which was isolated as a side product in an unsuccessful synthesis of an analogue containing two residues of L-Tic (des-Tyr^2-[Tic^3, Lys^8]VP (*III*)).

H-Cys-X-Y-Gln-Asn-Cys-Pro-Lys-Gly-NH_2

I, X = Tic, Y = Phe
II, X = Tyr, Y = Tic
III, X = —, Y = Tic

Syntheses of all three analogues were performed by solid phase technique on a benzhydrylamine resin. For α-amino group protection we have used the tert.butyloxycarbonyl (Boc) group. For side chain protection we have used: 2-bromobenzyloxycarbonyl (Lys), benzyl (Tyr), 4-methylbenzyl (Cys). Protected amino acids were coupled by N,N′-dicyclohexylcarbodiimide (DCC) and N-hydroxybenzotriazole (HOBt) in dimethylformamide. In the case of L-Tic coupling we expected problems due to the sterically hindered secondary amino group. Condensation of the protected Tic with the free amino group proceeded smoothly, but problems arose in the subsequent coupling steps. Removal of Boc-protection required repeated exposure to trifluoroacetic acid or the use of a higher concentration of this acid. Since the secondary amino group of Tic does not give a positive ninhydrin test, we employed the chloranil test[4]. We also tested the possibility of determining the free secondary amino group by its acylation using a large excess of an amino acid not contained in the sequence of vasopressin and, simultaneously, with a high probability of easy coupling. We used alanine and the remaining free secondary amino group was determined by amino acid analysis. Acylation of the Tic amino group proceeded extremely slowly, and complete coupling was achieved only after an addition of dimethylaminopyridine. We have not succeeded in acylation of Tic by a second Tic residue, and the attempt to synthesize [Tic^2, Tic^3]VP led only to the isolation of the analogue *III* as the main product.

Side chain protecting groups were cleaved simultaneously with the cleavage of the peptide from the resin by liquid hydrogen fluoride. Sulfhydryl groups oxidation was performed with potassium ferricyanide and the analogues were purified by HPLC and gel filtration.

Screening of biological activities of analogues *I*–*III* (Table I) have shown that all the compounds exhibit negligible agonistic activities in tests typical for neurohypophyseal hormones. Antagonistic activities were also not found. It is well known that both positions 2 and 3 are important for the biological activity of vasopressin analogues. However, such complete elimination of the activity can be compared only with the results of testing of [Phe^2, Tyr^3, Lys^8]VP (which was, however, questioned recently by the synthesis of [Phe^2, Phe(NH$_2$)^3, Arg^8]VP having significant (102 I.U./mg) antidiuretic activity[5]).

The large decrease in potency of compounds *I* and *II* is most probably due to the conformational changes of the molecule which are related to the conformational constraints imposed by a Tic residue in position 2 and/or 3 of vasopressin.

EXPERIMENTAL

General methods: Thin-layer chromatography (TLC) was carried out on silica gel coated plates (Silufol, Kavalier, Czechoslovakia) in the following systems: 2-butanol–98% formic acid–water (10 : 3 : 8) (S1), 1-butanol–acetic acid–water–pyridine (15 : 3 : 6 : 10) (S4), 1-butanol–acetic acid–water (50 : 15 : 40) (S13). Paper electrophoresis was performed in a moist chamber in 1M acetic acid (pH 2·4) and in pyridine–acetate buffer (pH 5·7) on Whatman 3MM paper, at 20 V/cm for 60 min. Spots on TLC and electrophoresis were developed with ninhydrin or by the chlorination method. Samples for amino acid analysis were hydrolyzed with 6M-HCl at 105°C for 20 h or with a mixture propionic acid–hydrochloric acid (1 : 1) at 160°C for 15 min and analyzed on an Amino acid analyzer T 339 (Mikrotechna, Praha, Czechoslovakia) or D-500 analyzer (Durrum Corp., U.S.A.). Optical rotations were determined on a Perkin–Elmer instrument type 141 MCA (Norwalk, U.S.A.). Fast atom bombardment mass spectra were obtained on a ZAB-EQ spectrometer (VG Analytical Ltd., Manchester) with xenon at 8 kV as the bombarding gas. ^{1}H NMR spectra were measured on a FT NMR spectrometer Varian XL-200 in hexadeuterodimethylsulfoxide with tetramethylsilane as an internal reference. High per-

TABLE I

Biological activities (rat) of vasopressin analogues (I.U./mg)

Compound	Uterotonic (in vitro)	Galactogogic (in situ)	Pressor	Antidiuretic	Ref.
[Lys^8]VP	5	65[a]	285	260	5
[Phe^2, Lys^8]VP	<0·1	2·5–3·0[b]	55–79	20–29	5
[Phe^2, Tyr^3, Lys^8]VP	<0·01		0·14	0·013	6
I	<0·0045	<0·02	<0·01	0·005	
II	<0·0045	<0·01	<0·01	0·046	
III	0·006[c]	<0·01	<0·01	—	

[a] Ref.[7]; [b] rabbit; [c] in vivo <0·01.

formance liquid chromatography (HPLC) was carried out on an SP-8700 instrument equipped with an SP-8400 detector and SP-4100 integrator (all from Spectra Physics, Santa Clara, U.S.A.). Preparative liquid chromatography was carried out on a Modulprep preparative liquid chromatograph (Jobin Yvon, Longjumeau Cédex, France). As the final purification step, Biogel P-2 column chromatography in 1M acetic acid was employed (1 × 100 cm). Chelaton III was added to the sample in order to remove the residues of heavy metals, if present. Before use, all amino-acid derivatives were subjected to ninhydrin test[8].

Solid-phase peptide synthesis: A cycle for incorporation each amino acid residue into the growing peptide chain consisted of the following: *1*) washing with dichloromethane (3 × 20 ml, 1 min/wash); *2*) cleaving the Boc group by adding 20 ml of 45% trifluoracetic acid in dichloromethane containing 5% anisole, one treatment for 5 min, the second one for 30 min; *3*) washing with dichloromethane (3 × 20 ml, 1 min/wash); *4*) washing with isopropanol (3 × 20 ml, 1 min/wash); *5*) washing with dichloromethane (3 × 20 ml, 1 min/wash); *6*) neutralizing by addition 5% diisopropylethylamine in dichloromethane (2 × 20 ml, 2 min/wash); *7*) washing with dichloromethane (4 × 20 ml, 1 min/wash); *8*) washing with dimethylformamide (3 × 20 ml, 1 min/wash); *9*) addition of the Boc-protected amino acid derivative in 20 ml of dimethylformamide followed by HOBt, followed by DCCI and stirring for 0·5—16 h; *10*) washing with dimethylformamide (3 × 20 ml, 1 min/wash); *11*) washing with dichloromethane (3 × 20 ml, 1 min/wash). Between steps *9* and *10*, several milligrams of the resin were removed and used for ninhydrin test[8] to determine the progress of the coupling. In the case of the coupling onto the imino group of proline or Tic the chloranil test[4] was used.

Nᵅ-Tert.butyloxycarbonylglycyl-resin

Benzhydrylamine resin (UCB, 0·6 mmol/g, 2·5 g) was suspended in dichloromethane, washed with dimethylformamide and coupled with 3 molar excess of Nᵅ-tert.butyloxycarbonylglycine, N-hydroxybenzotriazole and dicyclohexylcarbodiimide in dimethylformamide. Coupling was interrupted after 2 h, the resin was washed consequently with dimethylformamide (3 × 30 ml) and dichloromethane (3 × 30 ml) and the substitution of 0·56 mmol/g was determined by quantitative amino acid analysis. Free amino groups were acetylated with a mixture of 5 ml acetanhydride and 2 ml triethylamine in 50 ml dichloromethane for 3 h.

Hexapeptide-resin (Peptide-resin A)

The synthesis was performed according to the general scheme given above. Boc-amino acids were coupled to the resin by the DCCI/HOBt procedure. All reagents were used in 3 molar excess and the coupling was monitored by ninhydrin or chloranil test. Protected derivatives were used in the following order: Boc-Lys(2-Br-Z)-OH (ref.[9]), Boc-Pro-OH, Boc-Cys(4-Me-Bzl)-OH (ref.[10]) Boc-Asn-OH and Boc-Gln-OH. Finally, the Boc-group was cleaved from the hexapeptide-resin, the resin was washed and dried.

[2-(1,2,3,4-Tetrahydroisoquinoline-3-carboxylic acid), 8-lysine]vasopressin (*I*)

Peptide-resin A (1·3 g, 0·5 mmol) was acylated according to the general scheme with Boc-Phe-OH, Boc-Tic-OH (ref.[11]) and Boc-Cys(4-Me-Bzl)-OH (ref.[10]). The deprotection of Boc-octapeptide-resin was achieved by the treatment with 45% TFA and 10% anisole in dichloromethane (5 min and 1 h), followed by 60 min treatment in 95% TFA with 5% of anisole. Deprotection was checked by acetylation[12] and chloranil test[4]. Condensation of the cysteine derivative was performed for 3 h and after this period it was repeated with the addition of dimethylamino-

pyridine. Finally, the Boc-group was cleaved from the nonapeptide-resin, the resin was washed and dried (1·35 g), and treated with liquid hydrogen fluoride (10 ml, 60 min, 0°C) in the presence of thioanisole (1 ml). The unprotected nonapeptide, together with the resin, was triturated with ethyl acetate after evaporation of the hydrogen fluoride, filtered off, washed with ethyl acetate and the free peptide was extracted successively by acetic acid, 50% acetic acid, water and lyophilized. The lyophilizate (0·48 g) was dissolved in water (500 ml) and the pH of the solution was adjusted with 0·1M-NaOH to 7·0. Potassium ferricyanide (180 mg in 60 ml of water) was added to this solution in a period of 10 min. During the oxidation (20 min), pH was maintained at 7·0 by addition of 0·1M-NaOH and after oxidation adjusted with acetic acid to 4·5. The solution was put onto a column of Amberlite CG-50I, the column was washed with 0·25% acetic acid and the product eluted with 50% acetic acid. After freeze-drying, the product (370 mg) was purified by HPLC (Separon SIX C-18, 7 μm, 25 × 0·8 cm). Elution with methanol–0·05% trifluoroacetic acid mixture (3 : 7) and lyophilization of the corresponding fractions afforded 105 mg (15%, based on the glycine-resin) of the product, pure according to HPLC (k' 3·96; methanol–0·05% trifluoroacetic acid 4 : 6). Gel filtration of a part (50 mg) of this product on Biogel P2 column (1 × 100 cm) in 1M acetic acid afforded two peaks of the same amino acid composition. The first, probably the dimer, was eluted at 72 ml (5 mg), the second at 76 ml (38 mg). R_F: 0·00 (S1), 0·29 (S4), 0·34 (S13). $E_{2.4}^{Gly}$ 1·08, $E_{5.7}^{His}$ 0·68. $[\alpha]_D$ −5·3° (c 0·32; 1M acetic acid). Amino acid analysis: Asp 1·00, Glu 1·02, Pro 1·07, Gly 1·02, Cys 1·42, Phe 0·94, Tic 0·95, Lys 1·02. MS: 1 054 ($M + H^+$), 1 076 ($M + Na^+$). According to elemental analysis the lyophilizate contains 87·8% of peptide bis-trifluoroacetate (for $C_{47}H_{65}N_{13}O_{11}S_2.2$ TFA (1 280·3) calculated: 14·22% N; found: 12·48% N).

Heptapeptide-resin (Peptide-resin B)

Peptide-resin A (2·6 g, 1·0 mmol) was coupled with Boc-Tic-OH (ref.[11]) (2·5 h). The deprotection of Boc-heptapeptide-resin was performed by the treatment with 45% TFA and 10% anisole in dichloromethane (5, 30 and 60 min). Deprotection was checked by acetylation test[12] and chloranil test[4]. A small sample of the resin was quantitatively acylated by Boc-Ala-OH and tested by amino acid analysis. Only 75% incorporation of alanine was found and therefore deprotection was continued for another 90 min in 95% TFA with 5% of anisole. Yield: 3·0 g.

[3-(1,2,3,4-Tetrahydroisoquinoline-3-carboxylic acid), 8-lysine]vasopressin (*II*)

Peptide-resin B (1·5 g, 0·5 mmol) was coupled according to the general scheme with Boc-Tyr-OH, and Boc-Cys(4-Me-Bzl)-OH (ref.[10]). Condensation of tyrosine derivative was performed for 4 h and after this period it was repeated with the addition of dimethylaminopyridine. Finally the Boc-group was cleaved from the nonapeptide-resin, the resin was washed and dried (1·50 g) and was treated with liquid hydrogen fluoride (10 ml, 60 min, 0°C) in the presence of thioanisole (1 ml). Oxidation of the unprotected nonapeptide was performed analogously to the above described procedure. The product after desalting (370 mg) was purified by HPLC (Separon SI-C-18, 10 μm, 35 × 2 cm). Elution with a methanol–0·05% trifluoroacetic acid mixture (step gradient 15 : 85, 30 : 70 and 50 : 50) and lyophilization of the corresponding fractions afforded 72 mg (10%) of the product, pure according to HPLC (k' 1·80; methanol–0·05% trifluoroacetic acid 40 : 60). Gel filtration on Biogel P2 (1 × 100 cm) in 1M acetic acid afforded only one peak (52 mg). R_F: 0·00 (S1), 0·28 (S4), 0·32 (S13). $E_{2.4}^{Gly}$ 1·06, $E_{5.7}^{His}$ 0·65. $[\alpha]_D$ −5·5° (c 0·28; 1M acetic acid). Amino acid analysis: Asp 1·00, Glu 1·00, Pro 1·06, Gly 1·02, Cys 1·35, Tyr 0·80, Tic 1·03, Lys 0·96. MS: 1 069 ($M + H^+$), 1 091 ($M + Na^+$). According to elemental analysis the lyophilizate contains 81·5% of peptide bis trifluoroacetate (for $C_{47}H_{65}N_{13}O_{12}S_2.2$ TFA (1 296·3) calculated: 14·05% N; found: 11·45% N).

Des-2-tyrosine-[3-(1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid), 8-lysine]vasopressin (*III*)

Analogue *III* was isolated as the main product from the mixture resulting from the attempt to synthesize [2,3-(1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid), 8-lysine]vasopressin. The synthesis performed as described above afforded after freeze-drying 350 mg of crude peptide. One part (150 mg) was purified by HPLC (Separon SIX C-18, 7 μm, 25 × 0·8 cm). Elution with a methanol–0·05% trifluoroacetic acid mixture (35 : 65) and lyophilization of the fractions with k' 4·52 afforded 12·0 mg of the product, pure according to HPLC (k' 4·29; methanol–0·05% trifluoroacetic acid 40 : 60). R_F: 0·00 (S1), 0·11 (S4), 0·11 (S13). $E_{2,4}^{Gly}$ 0·81, $E_{5,7}^{His}$ 0·64. $[\alpha]_D$ −47·4° (*c* 0·1; 1M acetic acid). Amino acid analysis: Asp 1·00, Glu 1·00, Pro 1·07, Gly 1·04, Cys 1·25, Tic 1·13, Lys 0·93. ^{1}H NMR spectroscopy demonstrated the presence of only one Tic residue. According to elemental analysis the lyophilizate contains 72·2% of peptide bis trifluoroacetate (for $C_{38}H_{56}N_{12}O_{10}S_2.2$ TFA (1 133·1) calculated: 14·83% N; found: 10·71% N).

Pharmacological Methods

All pharmacological tests were performed using Wistar rats weighing 200—300 g. The uterotonic potency in vitro was evaluated using the Holton procedure[13,14] in Munsick[14] solution, and in vivo, according to ref.[15]. The galactogogic potency in vivo was established according to refs[16,17]. Pressor activity was tested on pithed rat preparation according to refs[18,19]. The activity on unanaesthetized rat was evaluated by modified Burn's method[20,21]. Male rats (160 to 200 g) after overnight fasting with free access to water were applied the water load (4 ml/100 g) per os and at the same time, s.c. injection of the tested compound or standard preparation was administered. Urine was collected for 5 h and its volume was read every 15 min. The time in minutes ($T_{1/2}$) in which one half of the water load was excreted was taken as a measure for the delay of water diuresis. LVP was used as the standard at dose 1·0 mU/100 g in 0·2 ml. Analogues *I* and *II* were applied at doses from 200 to 18 000 ng/100 g, analogue *III* at doses up to 10 000 ng/100 g. Control rats were injected with physiological solution.

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REFERENCES

1. Hruby V. J., Lebl M. in: *CRC Handbook of Neurohypophyseal Hormone Analogs* (K. Jošt, M. Lebl and F. Brtník, Eds), Vol. I., Part 1, p. 105. CRC Press, Boca Raton 1987.
2. Pictet A., Spengler T.: Chem. Ber. *44*, 2030 (1911).
3. IUPAC-IUB Joint Commission on Biochemical Nomenclature: Eur. J. Biochem. *138*, 9 (1984).
4. Christensen T.: Acta Chem. Scand. *33*, 763 (1979).
5. Lebl M., Jošt K., Brtník F. in: *CRC Handbook of Neurohypophyseal Hormone Analogs* (K. Jošt, M. Lebl and F. Brtník, Eds), Vol. II, Part 2, p. 127. CRC Press, Boca Raton 1987.
6. Boissonnas R. A., Guttmann S.: Helv. Chim. Acta *43*, 190 (1960).
7. Procházka Z., Ancans J. E., Slaninová J., Machová A., Barth T., Škopková J., Budějšinský M., Pavlíková F., Lebl M.: Collect. Czech. Chem. Commun. *53*, 2604 (1988).

8. Kaiser E., Colescott R. L., Bossinger C. D., Cook P. I.: Anal. Biochem. *34*, 595 (1970).
9. Yamashiro D., Li C. H.: J. Am. Chem. Soc. *95*, 1310 (1973).
10. Cosand W. L., Merrifield R. B.: Proc. Natl. Acad. Sci. U.S.A. *74*, 2771 (1977).
11. Hruby V. J., Cody W. L., Castrucci A. M. D. L., Hadley M. E.: Collect. Czech. Chem. Commun. *53*, 2549 (1988).
12. Krchňák V., Malý J., Flegel M.: Int. J. Pept. Protein Res. *31*, 239 (1988).
13. Holton P.: Br. J. Pharmacol. *3*, 328 (1984).
14. Munsick R. A.: Endocrinology *66*, 451 (1960).
15. Pliška V.: Eur. J. Pharmacol. *5*, 253 (1969).
16. Bisset G. W., Clark B. J., Haldar J., Harris M. C., Lewis G. P., Rocha e Silva M.: Br. J. Pharmacol. Chemother. *31*, 537 (1967).
17. Barth T., Jošt K., Rychlík I.: Endocr. Exp. *9*, 35 (1975).
18. Shipley R. E., Tilden J. H.: Proc. Soc. Exp. Biol. *64*, 453 (1947).
19. Krejčí I., Kupková B., Vávra I.: Br. J. Pharmacol. Chemother. *30*, 497 (1967).
20. Burn J. H., Finney D. J., Goodwin L. G. in: *Biological Standardization*, 2nd ed., p. 187. Oxford University Press, London 1950.
21. Vávra I., Machová A., Krejčí I.: J. Pharmacol. Exp. Ther. *188*, 241 (1974).

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