THE DEAMINO-CARBA ANALOGS OF VASOTOCIN. SYNTHESIS AND SOME BIOLOGICAL PROPERTIES*

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Solid phase methodology on benzhydrylamine resin with linkage agent and Fmoc technique was used for the synthesis of two analogs of deamino carba-1 and carba-6 oxytocin with phenylalanine in position 2 and with ornithine in position 8. The following analogs were prepared: [Phe^2,Orn^8]deamino-carba-1-oxytocin (*I*) and [Phe^2,Orn^8]deamino-carba-6-oxytocin (*II*). Moreover, analogs with δ-amino group of ornithine protected by benzyloxycarbonyl group and its corresponding sulfoxides were isolated and tested. Both deprotected analogs were found to have high uterotonic activity in vivo, and moderate pressor and antidiuretic agonistic activity. Surprisingly, in uterotonic test they are only partial agonists. The analogs with protected ornithine have preserved high uterotonic in vivo activity in contrary to the pressor activity that was strongly decreased.

Although the number of pharmaceutical companies working in the area of cardiovascular research is big, there is still room for improvement in the design of selectively acting pressor agonists. Analogs of neurohypophyseal hormones are not the most potent compounds influencing the blood pressure, but they offer several advantages which make them still interesting from the point of practical application in management of blood pressure disturbances. It is for example their relative stability in the organism which makes the design of long acting agonists possible (for a review see ref.[1]). Several analogs of vasotocin were introduced into the clinical practice (see ref.[2]). We were interested to see if it is possible to increase pressor activity of selectively acting analogs of vasotocin by carba modification of disulfide bridge.

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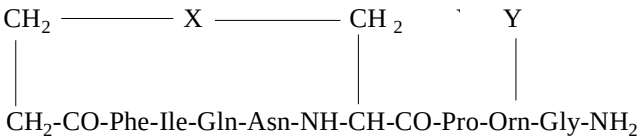
All synthesized analogs* are of deamino series (amino group on amino acid moiety in position 1 is replaced by hydrogen atom), tyrosine in position 2 is replaced by phenylalanine and leucine in position 8 by a basic amino acid, ornithine. Disulfide bridge is modified by so called carba substitution. In the first analog sulfur atom in position one is replaced by methylene group (carba 1 analog), in the second analog position six is modified (carba 6 analog). Moreover the analogs with δ-amino group of ornithine protected by benzyloxycarbonyl group (the sulfides and its corresponding sulfoxides) were isolated and in the case of the carba 6 modification also tested.

The following analogs were prepared:



	X	Y
I	CH_2-S	H
Ia	CH_2-S	Z
Ib	CH_2-SO	Z
II	S-CH_2	H
IIa	S-CH_2	Z
IIb	SO-CH_2	Z

As the key intermediates for the synthesis of carba analogues were used pentafluorophenyl ester of N^{α}-fluorenylmethoxycarbonyl-*S*-(3-*tert*-butyloxycarbonylpropyl)cysteine and pentafluorophenyl ester of N^{α}-fluorenylmethoxycarbonyl-*S*-(2-*tert*-butyloxycarbonylethyl)homocysteine[3].

The syntheses of analogs were performed by solid phase technique on benzhydrylamine resin with a linker. The 4-(α-Fmoc-amino-2′,4′-dimethoxybenzyl)phenoxyacetic acid linkage[4] was connected via glycine on benzhydrylamine resin and following synthesis was performed by current procedure. As the α-amino group protection we have

* 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 (Eur. J. Biochem. *138*, 9 (1984)).

used fluorenylmethyloxycarbonyl group. For the side chain protection of ornithine we have used benzyloxycarbonyl group. Protected amino acids were coupled by *N*,*N*′- diisopropylcarbodiimide (DIC) and *N*-hydroxybenzotriazole (HOBt) or pentafluorophenyl active ester method in *N*-methyl-2-pyrrolidone. Acid labile side chain protecting groups (Bu^t) were removed simultaneously with the cleavage of the peptide from the resin by trifluoroacetic acid. Under these conditions partial cleavage of Z-protecting group from the side chain of ornithine occurred. Therefore the mixture was purified by continuous free-flow electrophoresis before cyclization. To prevent acetylation of free amino group during cyclization[5], hydrochloric acid was added to lyophilisate to remove the traces of acetic acid. The cyclization was effected by diphenylphosphorylazide in dimethylformamide with dipotassium hydrogen phosphate[6] and cyclic protected analogs were isolated and purified by HPLC. Two products, sulfide and sulfoxide of protected analogs always were isolated. The stabile side chain protecting group (Z on ornithine) was cleaved by the liquid hydrogen fluoride with anisole. Cyclic free analogs were purified again by HPLC.

Biological activities of the analogs are given in the Table I, together with the activities of earlier synthesized analogs. The following conclusion can be drawn from the results obtained: (i) carba substitution increased activity in the pressor test; (ii) carba substitution, however, increased also antidiuretic activity. As a consequence the specificity achieved by combination of substitution of position 2 and 8 was lost; (iii) no difference in activity was found between carba-1 and carba-6 substitution; (iv) all tested analogs had very similar uterotonic activity in vivo – independent of the substitution in position 8 (basic group or lipophilic protected amino group), and the character of the substitution of sulfur in the bridge (carba-1, carba-6, or sulfoxide of carba-6); (v) analogs with protected ornithine side chain are more potent uterotonic agonists in vitro but at the same time they are weaker antagonists then their deprotected counterparts.

The influence of carba substitution on the biological activity of an analogue is still not completely clear and surprising results can be obtained. Carba-1 substitution in deamino-oxytocin leads to a substantial increase of the pressor (and also antidiuretic) activity. Carba-6 substitution improved dramatically antidiuretic activity, but actually decreased pressor activity. Similar trend is observable in in vitro and in vivo uterotonic activity of carba analogs of deamino-oxytocin and also [Phe^2]deamino-oxytocin. However, multiply substituted analogs shows activities completely independent on the substitution of sulfur in the disulfide bridge.

EXPERIMENTAL

General Methods

Thin-layer chromatography (TLC) was carried out on silica gel coated plates (Silufol, Kavalier, The Czech Republic) in the following systems: 2-butanol–98% formic acid–water (10 : 3 : 8) (S1); 2-butanol–25% ammonia–water (85 : 7.5 : 7.5) (S2). Paper electrophoresis was performed in a moist chamber

TABLE I
Biological activities (rat) of some neurohypophyseal hormone analogs (I.U./mg)

Compound	Uterotonic		Pressor	Antidiuretic	Ref.
	in vitro	in situ			
OXT	450		5	5	7
dOXT	750		1.4	19	7
dC^{1}OXT	1 800	1 200	17.5	24.1	7
dC^{6}OXT	930	2 790	1.5	115	7
[Orn^8]VP	10		360	88	8
[Orn^8]dVP	15.5		355	202	9
[Orn^8]dC^{6}VP	27		172	650	10
[Phe^2,Orn^8]VP	~0.5		153	16	11
[Phe^2,Orn^8]dVP	<1		45	13	9
[Phe^2]OXT	32		0.4		12
	pA_2 = 6.78				13
[Phe^2]dOXT	~25		~0.04	~0.35	14
[Phe^2]dC^{1}OXT	233	95		0.6	15
[Phe^2]dC^{6}OXT	70	450	0.9	1.5	16
[Phe^2,Orn^8]OXT	~1		120	0.55	11
[Phe^2],Orn^8]dOXT	<2		100	~4	9
[Phe^2,Orn^8]dC^{1}OXT (*I*)	~6.0	86.1	217	[c]	[a]
	pA_2 = 7.7				
[Phe^2,Orn^8]dC^{6}OXT (*II*)	~1.0	81.3	225	[c]	[a]
	pA_2 = 7.6				
[Phe^2,Orn(Z)^8]dC^{6}OXT (*IIa*)	~31.7[b]	85.5	1.7		[a]
	pA_2 = 6.5				
[Phe^2,Orn(Z)^8]dC^{6}OXT-sulfoxid (*IIb*)	~15.0[b]	81.4	1.8		[a]
	pA_2 = 6.8				

[a] This paper. [b] Maximal contractions do not reach the maximal contractions evoked by oxytocin; [c] Activity in nonanaesthetized rat was comparable to the activity of lysine-vasopressin.

in 1 M 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 plates and electrophoresis were detected with ninhydrin or by a chlorination method. Samples for amino acid analysis were hydrolyzed with 6 M HCl at 105 °C for 20 h 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, U.K.) with xenon at 8 kV as the bombarding gas. Continuous free-flow electrophoresis was carried out in an apparatus described previously[17,18] (conditions: 0.5 M acetic acid, 4 °C, 2.6 kV across the electrodes). High performance liquid chromatography was carried out on an SP-8800 instrument equipped with an SP-8450 detector and SP-4290 integrator (all from Spectra Physics, Santa Clara, U.S.A.). HPLC purities of products were determined on the Vydac 218TP54 column. Preparative liquid chromatography was carried out on above described equipment using Vydac 218TP510 (5 μm, 250 × 10 mm) column.

Solid-Phase Peptide Synthesis

A cycle for incorporation of each amino acid residue into the growing peptide chain consisted of the following: 1. cleaving the Fmoc group by 20% piperidine in dimethylformamide; 2. washing with dimethylformamide; 3. washing with dichloromethane; 4. washing with dimethylformamide; 5a. adding the Fmoc-protected amino acid pentafluorophenyl ester in *N*-methyl-2-pyrrolidone; 5b. adding the Fmoc-protected amino acid derivative in *N*-methyl-2-pyrrolidone followed by HOBt and by DIC; 6. washing with dimethylformamide. The synthesis was monitored by bromophenol blue method[19].

Tripeptide Resin

Benzhydrylamine resin (1.20 g, 0.6 mmol) was suspended in dichloromethane and after washing with 5% diisopropylethylamine in dichloromethane and with dimethylformamide was coupled with 4 molar excess of Boc-Gly-OH in the presence of *N*-hydroxybenzotriazole and dicyclohexylcarbodiimide in dimethylformamide. Coupling was finished after 3 h, the resin was washed consequently by dimethylformamide and dichloromethane and the Boc group was cleaved by 50% trifluoroacetic acid in dichloromethane containing 5% anisole. After washing with dichloromethane, isopropanol, dichloromethane, neutralization by 5% diisopropylethylamine in dichloromethane and washing with dichloromethane, dimethylformamide and *N*-methyl-2-pyrrolidone, glycine-resin was coupled with 4-(α-Fmoc-amino-2′,4′-dimethoxybenzyl)phenoxyacetic acid linkage. The following procedure was performed according to general scheme given above (starting from the point 1.). Fmoc-amino acids were coupled to the resin by the DIC/HOBt procedure. All reagents were used in 3 molar excess and coupling was monitored by bromophenol blue method[19]. Protected derivatives were used in the following order: Fmoc-Gly-OH, Fmoc-Orn(Z)-OH (ref.[3]) and Fmoc-Pro-OH. Following this last coupling cycle, the resin was divided into two parts.

[2-Phenylalanine, 8-N^{δ}-benzyloxycarbonylornithine]deamino-1-carba-oxytocin (*Ia*) and Sulfoxide (*Ib*)

Linear octapeptide. Tripeptide resin (0.85 g, 0.3 mmol) was coupled according to general scheme with Fmoc-Cys($C_3H_6COOBu^t$)-OPfp (ref.[3]), Fmoc-Asn-OH, Fmoc-Gln-OH, Fmoc-Ile-OH and Fmoc-Phe-OH. After deprotection of Fmoc group octapeptide resin (0.98 g) was treated with 95% trifluoroacetic acid (20 ml, 60 min) in the presence of anisole (2%) and water (3%). The resin was filtered off, washed with trifluoroacetic acid, ethanol, water and filtrate was diluted with water and after addition of benzene evaporated. The residue was triturated with ethyl acetate. Standing in the

refrigerator overnight afforded a fine precipitate which was centrifuged and dried in desiccator. Yield 244 mg of crude product which, in addition to required product, contained also about 30% of product with free δ-amino group of ornithine. $E_{2.4}^{Gly}$ 0.68 and 1.18, $E_{5.7}^{His}$ 0.15 and 0.45. The crude product (224 mg) was purified by continuous free-flow electrophoresis and lyophilization of the corresponding fractions afforded 75 mg of the product, pure according to electrophoresis: $E_{2.4}^{Gly}$ 0.68, $E_{5.7}^{His}$ 0.15, and 21 mg by-product ($E_{2.4}^{Gly}$ 1.18, $E_{5.7}^{His}$ 0.45).

Cyclization. To the solution of the octapeptide (75 mg) in dimethylformamide (20 ml), cooled to 0 °C, K_2HPO_4 (85 mg) and diphenylphosphoryl azide (DPPA, 45 μl) were added. Cyclization was followed by HPLC. After 5 h, more DPPA (50 μl) was added. After 24 h suspension was centrifuged, dimethylformamide was evaporated, residue was treated with ethyl acetate, left in refrigerator overnight, the product was centrifuged and dried in desiccator. Cyclic crude product (mixture of sulfide, sulfoxide and a by-product from cyclization) was purified by HPLC on Vydac 218TP510 column with 50% methanol in 0.05% trifluoroacetic acid (10 min), continuing by gradient running from 50% to 100% of methanol (25 min). Lyophilization of the corresponding fractions afforded 1.4 mg of the sulfoxide (*k* 3.45, methanol–0.05% trifluoroacetic acid 1 : 1, Vydac. FAB MS (*m*/*z*): 1 109 ($M + H^+$)) and 6.7 mg of the sulfide (*k* 3.86, methanol–0.05% trifluoracetic acid 1 : 1, Vydac. FAB MS (*m*/*z*): 1 093 ($M + H^+$)).

[2-Phenylalanine, 8-ornithine]deamino-1-carba-oxytocin (*I*)

The cyclic peptide *Ia* (6.5 mg) was treated with liquid hydrogen fluoride (5 ml, 10 min, 20 °C) in the presence of anisole (0.5 ml). Deprotected peptide was dissolved in a mixture of ethyl acetate and water after evaporation of hydrogen fluoride, ethyl acetate layer was separated and aqueous solution containing free peptide was lyophilized. The product was purified by HPLC on Vydac 218TP510 column in a gradient running from 30% to 50% methanol in 0.05% trifluoroacetic acid in 40 min. Lyophilisation of the corresponding fractions afforded 2.1 mg of the product pure according to HPLC (*k* 3.80, methanol–0.05% trifluoroacetic acid 3 : 7, Vydac). Amino acid analysis: Asp 1.06, Glu 1.07, Pro 0.98, Gly 1.11, Cys($C_3H_6CO_2H$) 0.76, Ile 1.00, Phe 0.96, Orn 1.06. FAB MS (*m*/*z*): 959.5 ($M + H^+$).

[2-Phenylalanine, 8-N^{δ}-benzyloxycarbonylornithine]deamino-6-carba-oxytocin (*IIa*) and Sulfoxide (*IIb*)

Linear octapeptide. Tripeptide resin (0.85 g, 0.3 mmol) was coupled according to general scheme with Fmoc-Hcy($C_2H_4COOBu^t$)-OPfp (ref.[3]), Fmoc-Asn-OH, Fmoc-Gln-OH, Fmoc-Ile-OH and Fmoc-Phe-OH. After deprotection of Fmoc group octapeptide resin (0.98 g) was treated with 95% trifluoroacetic acid (20 ml, 90 min) in the presence of anisole (2%) and water (3%). The resin was filtered off, washed with trifluoroacetic acid, ethanol, water and the filtrate was diluted with water and after addition of benzene evaporated. The residue was triturated with ethyl acetate. Standing in the refrigerator overnight afforded a fine precipitate which was centrifuged and dried in desiccator. Yield 305 mg of a crude product which, in addition to the required product, contained about 30% of a product with free δ-amino group of ornithine. $E_{2.4}^{Gly}$ 0.67 and 1.18, $E_{5.7}^{His}$ 0.15 and 0.07 (sulfide and sulfoxide), and 0.40. The crude product (260 mg) was purified by continuous free-flow electrophoresis and lyophilization of the corresponding fractions afforded 130.5 mg of the product, which, according to electrophoresis did not contain free amino group on side chain of ornithine ($E_{2.4}^{Gly}$ 0.67, $E_{5.7}^{His}$ 0.15 and 0.07 (sulfide and sulfoxide forms were not separated)), and 34.6 mg of a by-product ($E_{2.4}^{Gly}$ 1.18, $E_{5.7}^{His}$ 0.40).

Cyclization. To the solution of the octapeptide (78 mg) in dimethylformamide (20 ml), cooled to 0 °C, K_2HPO_4 (85 mg) and diphenylphosphoryl azide (45 μl) were added. Cyclization was followed by HPLC. After 5 h, another DPPA (50 μl) was added. After 24 h dipotassium hydrogen phosphate

was centrifuged, dimethylformamide was evaporated, residue was treated with ethyl acetate, left in refrigerator, product centrifuged and dried in desiccator. Cyclic crude product (mixture of sulfide, sulfoxide and by-product from cyclization) was purified by HPLC on Vydac 218TP510 column by gradient running from 0% to 30% of methanol in 0.05% trifluoroacetic acid (3 min) continuing by gradient running from 30% to 70% of methanol (80 min). Lyophilization of the corresponding fractions afforded 17.0 mg of the sulfoxide and 16.6 mg of the sulfide.

Sulfoxide IIb: *k* 2.86 (methanol–0.05% trifluoroacetic acid 1 : 1, Vydac). Amino acid analysis: Asp 1.02, Glu 1.02, Pro 0.95, Gly 1.00, Hcy($C_2H_4CO_2H$) 0.58, Ile 1.00, Phe 0.99, Orn 1.00. For $C_{51}H_{72}N_{12}O_{14}S$. TFA . 3 H_2O (1 277.3) calculated: 49.84% C, 6.23% H, 13.15% N; found: 49.44% C, 5.73% H, 13.33% N. FAB MS (*m*/*z*): 1 109 ($M + H^+$).

Sulfide IIa: *k* 3.92 (methanol–0.05% trifluoroacetic acid 1 : 1, Vydac). Amino acid analysis: Asp 1.07, Glu 1.07, Pro 1.00, Gly 1.07, Hcy($C_2H_4CO_2H$) 0.81, Ile 0.85, Phe 0.96, Orn 1.08 For $C_{51}H_{72}N_{12}O_{13}S$. 0.5 TFA . 3 H_2O (1 204.3) calculated: 51.86% C, 6.57% H, 13.96% N; found: 51.52% C, 5.91% H, 14.47% N. FAB MS (*m*/*z*): 1 093 ($M + H^+$) and 1 115 ($M + Na^+$).

[2-Phenylalanine, 8-ornithine]deamino-6-carba-oxytocin (*II*)

The cyclic peptide, sulfide (8.5 mg) was treated with liquid hydrogen fluoride (5 ml) for 10 min at 20 °C in the presence of anisole (0.5 ml). Deprotected peptide was dissolved in 10% acetic acid and lyophilized. The crude product (according to HPLC containing anisole, electrophoretically pure) was purified by HPLC on Vydac 218TP510 column in slow gradient running from 30% to 60% methanol in 0.05% trifluoroacetic acid in 60 min. Lyophilization of the corresponding fractions afforded 3.1 mg of the product pure according to HPLC (*k* 1.36, methanol–0.05% trifluoroacetic acid 4 : 6; *k* 6.50, methanol–0.05% trifluoroacetic acid 3 : 7, Vydac). Amino acid analysis: Asp 1.07, Glu 1.07, Pro 0.99, Gly 1.05, Hcy($C_2H_4CO_2H$) 0.82, Ile 0.99, Phe 0.95, Orn 1.07. FAB MS (*m*/*z*): 959 ($M + H^+$).

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[20] in Munsick[21] solution. For the determination of the in vivo activity[22] oestrogenized rats in ethanol anaesthesia were used. Inhibitory activity is characterized by pA_2 value (refs[23 – 25]). Pressor activity was tested on pithed rat preparation according to refs[26,27]. Antidiuretic potency on nonanaesthetized rat was followed according to the method in ref.[28].

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