STRONG UTEROTONIC INHIBITORS — ANALOGS OF 1-DEAMINO--8-D-HOMOARGININE-VASOPRESSIN WITH *p*-SUBSTITUTED PHENYLALANINE IN POSITION 2*

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Solid phase methodology on benzhydrylamine or *p*-methylbenzhydrylamine resin was used for the synthesis of five analogs of deamino-vasopressin with non-coded amino acids. D-homoarginine, in position 8 and *p*-substituted D- or L-phenylalanine in position 2. Besides the mother analog, [Mpr[1],D-Har[8]]vasopressin (*I*), [Mpr[1],L-Phe(*p*-Me)[2], D-Har[8]]vasopressin (*II*), [Mpr[1],D--Phe(*p*-Me)[2], D-Har[8]]vasopressin (*III*), [Mpr[1],L-Phe(*p*-Et)[2],D-Har[8]]vasopressin (*IV*) and [Mpr[1], D-Phe(*p*-Et)[2], D-Har[8]]vasopressin (*V*) were synthesized. All analogs have very low antidiuretic and pressor activities. Analogs containing *p*-methylphenylalanine of L-configuration and *p*-ethylphenylalanine of both D- and L-configuration are pressor inhibitors. All analogs substituted in position 2 were found to act as the uterotonic inhibitors, the most potent being [Mpr[1],D-Phe-(*p*-Et)[2], D-Har[8]]vasopressin (*V*) with $pA_2 = 8{\cdot}30$.

Inhibitors of oxytocin uterotonic activity are still being sought for by peptide chemists and pharmacologists. Their practical value might be found in several applications, the most prominent being the prevention of premature delivery. For this implication, an analog with very high and selective inhibitory activity is desirable. Its enzymatic stability and therefore potential oral applicability and/or long action would be extremely beneficial. We have described recently inhibitory activities of vasopressin analogs modified by homoarginine in position 8 and by *p*-substituted phenylalanine in position 2 (ref.[1]). The considerably high inhibitory activity of these analogs prompted us to combine the above mentioned modifications with deamination in position 1 expecting the increase of inhibitory activity and higher metabolic stability of the analogs. Preliminary results were presented at the symposium[2].

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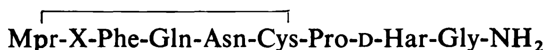
** All the chiral amino acids, mentioned in this work, are of the L-series. The nomenclature and symbols of the amino acids and pepides obey the published recommendations[3]: Har denotes the homoarginine moiety, Mpr the β-mercaptopropionic acid, Phe(*p*Et) the *p*-ethylphenylalanine and Phe(*p*Me) the *p*-methylphenylalanine moiety.

Five analogs of deamino-vasopressin** (*I* – *V*) with D-homoarginine in position 8 and modified phenylalanine in position 2 were synthesized similarly as the vasopressin analogs containing α-amino group[1], with the only exception of using β-mercaptopropionic acid instead of cysteine in position 1.



I, X = Tyr
II, X = L-Phe(*p*Me)
III, X = D-Phe(*p*Me)
IV, X = L-Phe(*p*Et)
V, X = D-Phe(*p*Et)

N^{α}-Tert-butoxycarbonyl-N^{G}-nitrohomoarginine[1] was found to be the suitable derivative of D-homoarginine for solid phase synthesis. *p*-Methylphenylalanine and *p*-ethylphenylalanine residues were applied in the form of Boc derivative[1] as a mixture of enantiomers. β-Mercaptopropionic acid was introduced as an appropriate S-*p*-methylbenzyl or S-benzyl protected derivative. The use of the former derivative was always more successful.

Syntheses of all five analogs were performed by solid phase technique on the benzhydrylamine or *p*-methylbenzhydrylamine resin. As the α-amino group protection we have used tert-butoxycarbonyl group. For the side chain protection we have used: nitrogroup (D-Har), 4-methylbenzyl (Cys, Mpr), 2,6-dichlorobenzyl or benzyloxycarbonyl (Tyr) and benzyl (Mpr). Protected amino acids were coupled by N,N'-dicyclohexylcarbodiimide (DCC) and N-hydroxybenzotriazole (HOBt) in dimethylformamide. Side chain protecting groups were cleaved simultaneously with the cleavage of the peptide from the resin by the liquid hydrogen fluoride. Sulfhydryl groups oxidation was performed by potassium ferricyanide and analogs were purified by HPLC. In syntheses of analogs with *p*-ethylphenylalanine and *p*-methylphenylalanine we made use of the fact that diastereoisomeric peptides can be easily separated by reversed phase chromatography[4–6]. Syntheses of these analogs were performed with racemic amino acids and the appropriate diastereoisomers separated at the end of the synthesis. Identification of L- or D-amino acid was performed by the digestion with L-amino acid oxidase[7,8]. Moreover, in both cases the value of *k* in HPLC on reversed phase was always lower for L-diastereoisomer, what is consistent with previous findings[4,5]. For the formation of both diastereoisomers only 1·1 equivalent of the protected racemic amino acid (tert-butoxycarbonyl-D,L-*p*-methylphenylalanine or tert-butoxycarbonyl-D,L-*p*-ethylphenylalanine[1]) were used.

Biological activities of the analogs are given in Table I. [Mpr^1,D-Har^8]vasopressin (*I*) exhibits approximately 5% of the antidiuretic activity of dDAVP. This result is comparable to the finding of other authors[10,12] who determined the activity of

analog *I* in the test on anaesthetized rat to be 196 IU/mg or 2% of the activity of dDAVP. Analog *I* was also found to be a weak agonist in the uterotonic test in vitro. Substitution in position 2 led to a substantial decrease of the antidiuretic activity. Analogs with substituted position 2 have either no pressor activity (*III*) or very low inhibitory activity (*II*, *IV*, *V*). As well as in the case of α-amino group containing analogs[1], a weak uterotonic agonist *I* was transformed by modification of position 2 to a rather potent uterotonic inhibitor. Similarly as described earlier[13], and also in preceeding paper[1], *p*-ethylphenylalanine of D-configuration was found superior in producing an inhibitor.

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–pyridine–water (15 : 3 : 10 : 6) (S4). 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 in TLC and electrophoresis were detected 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.). Fast atom bombardment mass spectra were obtained

TABLE I

Biological activities (rat) of vasopressin analogs (IU/mg or pA_2 values) with the modification in the position 2

Compound	Uterotonic in vitro	Pressor	Anti-diuretic	Ref.
AVP	17[a]	412[a]	465[a]	—
[D-Har[8]]VP	—	0·83	83	10
	0·9	—	1% dDAVP	1
[Mpr[1],D-Har[8]]VP	—	0·06	196	10
	2	0·5	1 540	11
	2	0·05	2% dDAVP	12
I	0·8	0·28	5% dDAVP	[b]
II	pA_2 = 7·50	pA_2 = 6·2	0·02% dDAVP	[b]
III	pA_2 = 8·20	0	0·02% dDAVP	[b]
IV	pA_2 = 8·00	pA_2 = 6·2	0·02% dDAVP	[b]
V	pA_2 = 8·30	pA_2 = 6·35	0·02% dDAVP	[b]

[a] Values quoted according to ref.[9]; [b] this paper.

on a ZAB-EQ spectrometer (VG Analytical Ltd., Manchester) with xenon at 8 kV as the bombarding gas. HPLC purities of products were determined on the column of Separon SIX C-18 (S) or Vydac 218TP54 (V). Preparative liquid chromatography was carried out on Vydac 218TP510 (5 µm, 250 × 10 mm) column or on column filled with Separon SGX-C18 (10 µm, 250 × 16 mm). Before use, all amino acid derivatives were subjected to ninhydrin test[14].

Solid-Phase Peptide Synthesis

A cycle for incorporation each amino acid residue into the growing peptide chain consisted of the following:

1. cleavage of the Boc group by adding 40 ml of 50% trifluoroacetic acid in dichloromethane containing 5% anisole, one treatment for 5 min, a second one for 30 min; 2. washing with dichloromethane (3 × 40 ml, 1 min/wash); 3. washing with isopropanol (3 × 40 ml, 1 min/wash); 4. wahing with dichloromethane (3 × 40 ml, 1 min/wash); 5. neutralizing by adding 40 ml of 5% diisopropylethylamine in dichloromethane, one treatment for 2 min, a second one for 5 min. 6. washing with dichloromethane (2 × 40 ml, 1 min/wash); 7. washing with dimethylformamide (3 × 40 ml, 1 min/wash); 8a. addition of the Boc-protected amino acid hydroxybenzotriazole ester in dichloromethane and stirring for 30 min; 8b. addition of the Boc-protected amino acid derivative in 40 ml dimethylformamide followed by HOBt, followed by DCC and stirring for 1—2·5 h; 9. washing with dimethylformamide (3 × 40 ml, 1 min/wash); 10. washing with dichloromethane (3 × 40 ml, 1 min/wash); 11. washing with isopropanol (3 × 40 ml, 1 min/wash); 12. washing with dichloromethane (3 × 40 ml, 1 min/wash);

Hydroxybenzotriazole active ester was prepared as follows: 1 equivalent of the Boc-protected amino acid, 1 equivalent of dicyclohexylcarbodiimide (1M solution in dichloromethane) and 1 equivalent of 1-hydroxybenzotriazole (2M solution in dimethylformamide) were stirred 20 to 30 min at room temperature, urea was filtered off and washed by dichloromethane. The synthesis was monitored by ninhydrin test[14] (analog *I*) or bromophenol blue method[15] (analogs *II*—*V*).

Heptapeptide-Resin (Peptide-Resin A)

Benzhydrylamine resin (UCB, 0·56 mmol/g, 4·46 g, 2·5 mmol) was suspended in dichloromethane and after washing with 5% diisopropylethylamine in dichloromethane and with dimethylformamide it was coupled with 3 molar excess of Boc-Gly-OBt. Coupling was finished after 2 h and the resin was washed consequently with dimethylformamide (3 × 40 ml) and dichloromethane (3 × 40 ml). The following procedure was performed according to the general scheme given at the beginning of the experimental part (starting from the point 1.). Boc-amino acids were coupled to the resin by the hydroxybenzotriazole active ester procedure. All reagents were used in 3 molar excess and coupling was monitored by ninhydrin test[14]. Protected derivatives were used in the following order:

Boc-D-Har(NO_2)-OH (ref.[1]), Boc-Pro-OH, Boc-Cys(4-Me-Bzl)-OH (ref.[16]), Boc-Asn-OH, Boc-Gln-OH and Boc-Phe-OH. In the case of homoarginine, cysteine and phenylalanine coupling, the reaction was not complete after 30 min, and therefore nucleophilic acylation catalyst (4-dimethylaminopyridine, 50 mg) was used. Yield: 6·9 g. Amino acid analysis on resin: Asp 0·95, Glu 1·13, Pro 1·01, Gly 0·67, Cys 0·50, Phe 1·10, Har 0·82.

Heptapeptide-Resin (Peptide-Resin B)

p-Methylbenzhydrylamine resin (Peptides International, 0·79 mmol/g, 8·0 g) was suspended in dichloromethane and after washing with 5% diisopropylethylamine in dichloromethane and

with dimethylformamide it was coupled with 3 molar excess of Boc-Gly-OH in the presence of N-hydroxybenzotriazole and dicyclohexylcarbodiimide in dimethylformamide. Coupling was finished after 2 h, the resin was washed with dimethylformamide (3 × 40 ml) and dichloromethane (3 × 40 ml) and resin substitution was determined by amino acid analysis 0·55 mmol/g. Polymer was acetylated (5 ml acetanhydride, 2 ml triethylamine in 50 ml dichloromethane). The free amino groups disappeared during 2 h (according to the ninhydrin test). The following procedure was performed according to the general scheme given at the beginning of the experimental part (starting from the point 1.). Boc-amino acids were coupled to the resin by the DCCI/HOBt procedure. All reagents were used in 3 molar excess and coupling was monitored by bromophenol blue method[15] Protected derivatives were used in the following order: Boc-D-Har. .(NO_2)-OH (ref.[1]), Boc-Pro-OH, Boc-Cys(4-Me-Bzl)-OH (ref.[16]), Boc-Asn-OH, Boc-Gln-OH and Boc-Phe-OH. Yield: 14·5 g. Amino acid analysis on resin: Asp 0·90, Glu 1·03, Pro 1·06, Gly 1·02, Cys 0·70, Phe 1·01, Har 0·98.

[1-Mercaptopropionic Acid,8-D-homoarginine]vasopressin (*I*)

Peptide-resin A (0·91 g, 0·33 mmol) was coupled according to the general scheme with Boc-Tyr(Z)-OH and Mpr(Bzl)-OH. The nonapeptide-resin (1·0 g) was treated with liquid hydrogen fluoride (10 ml, 60 min, 0°C) in the presence of anisole (1·5 ml). HF was blown out by nitrogen at 0°C during 30 min. Unprotected nonapeptide, together with the resin, was triturated with ether, filtered off, washed with ethyl acetate and then the free peptide was extracted with 20% acetic acid (100 ml, 40°C), diluted with water and lyophilized. The lyophilizate was dissolved in water (300 ml) and the pH of the solution was adjusted with NH_4OH to 7·0. Potassium ferricyanide (0·01M solution) was added to this solution until stable yellow colour persisted. During the oxidation (30 min), pH was maintained at 7·0 and then adjusted with acetic acid to 4·5. The solution was applied to a column of Amberlite CG-50I (30 ml), the column was washed with 0·25% acetic acid (150 ml) and the product eluted with 50% acetic acid (90 ml). After freeze-drying, the crude product (94 mg) was purified by HPLC (Knauer) using a slow gradient of methanol in 0·1% trifluoroacetic acid on a column of Separon SGX-C18. Lyophilization of the corresponding fractions afforded 23 mg of the product pure according to HPLC (*k* 7·48, methanol–0·05% trifluoroacetic acid 4 : 6 — Separon). R_F 0·00 (S1), 0·43 (S4). $E_{2.4}^{Gly}$ 0·70, $E_{5.7}^{His}$ 0·39. $[\alpha]_D$ −57·1° (*c* 0·2; 1M acetic acid) ($[\alpha]_D$ −67·4° (*c* 0·5; 1M acetic acid)[10], $[\alpha]_D$ −59·4° (*c* 0·4; 1M acetic acid)[12]). Amino acid analysis after oxidation: Asp 1·01, Glu 0·96, Pro 1·05, Gly 1·00, $CySO_3H$ 1·10, Tyr 0·75, Phe 0·93, Har 0·94. For $C_{47}H_{66}N_{14}O_{12}S_2$. 2 TFA.4 H_2O (1 383·4) calculated: 44·28% C, 5·53% H, 14·17% N; found: 44·12% C, 5·21% H, 13·99% N. FAB MS (*m/z*): 1 084, $(M + H^+)$.

[1-Mercaptopropionic Acid, 2-*p*-Methyl-L-phenylalanine, 8-D-Homoarginine]vasopressin (*II*) and [1-Mercaptopropionic Acid, 2-*p*-Methyl-D-phenylalanine, 8-D-Homoarginine]vasopressin (*III*)

Peptide-resin B (2·0 g, 0·6 mmol) was coupled according to the general scheme with 1·1 equivalents Boc-L,D-Phe(*p*-Me)-OH (ref.[1]) for 18 h and with Mpr(4-Me-Bzl)-OH. After cleavage of the Boc-protecting group, the nonapeptide-resin (2·0 g) was treated with liquid hydrogen fluoride (15 ml, 60 min, 0°C) in the presence of anisole (2·0 ml). Unprotected nonapeptide, together with the resin, was triturated with ether after evaporation of hydrogen fluoride, filtered off, washed with ethyl acetate and then, the free peptide was extracted successively with acetic acid, 50% acetic acid, water, and lyophilized. The lyophilizate (920 mg) was dissolved in water (300 ml) and the pH of the solution was adjusted with 0·1M NaOH to 7·0. Potassium ferricyanide

(0·01M solution) was added to this solution until stable yellow colour persisted. During the oxidation (20 min), pH was maintained at 7·2 by addition of 0·1M NaOH and then adjusted with acetic acid to 4·5. The solution was applied to a column of Amberlite CG-50I (30 ml), the column was washed with 0·25% acetic acid and the product eluted with 50% acetic acid. After freeze--drying, the product (318 mg) was purified by HPLC on a column Vydac 218TP510 using a slow gradient running from 25% to 45% methanol in 0·05% trifluoroacetic acid in 60 min. Lyophilization of the corresponding fractions afforded 73 mg and 68 mg of the products pure according to HPLC.

The first product (73 mg; *k* 1·26, methanol–0·05% trifluoroacetic acid 1 : 1 — Vydac) corresponds to the analog with *p*-methyl-L-phenylalanine (*II*) in position 2. R_F 0·07 (S1), 0·56 (S4). $E_{2.4}^{Gly}$ 0·64; $E_{5.7}^{His}$ 0·30 (detection by chlorination method). $[\alpha]_D$ −52·6° (*c* 0·1; 1M acetic acid). Amino acid analysis: Asp 1·06, lu 1·02, Pro 1·08, Gly 1·08, Cys 0·81, 4-Me-Phe 0·92, Phe 1·02, Har 0·93. For $C_{48}H_{68}N_{14}O_{11}S_2$.2 TFA.2 H_2O (1 345·4) calculated: 46·42% C, 5·54% H, 14·58% N; found: 46·03% C, 5·33% H, 14·99% N. FAB MS (*m*/*z*): 1 082 (M + H^+).

The second product (68 mg; *k* 1·94, methanol–0·05% trifluoroacetic acid 1 : 1 — Vydac) cor. responds to the analog with *p*-methyl-D-phenylalanine (*III*) in position 2. R_F 0·06 (S1), 0·56 (S4) $E_{2.4}^{Gly}$ 0·64; $E_{5.7}^{His}$ 0·30 (detection by chlorination method). $[\alpha]_D$ −60·8° (*c* 0·1; 1M acetic acid)- Amino acid analysis: Asp 1·02, Glu 0·96, Pro 1·03, Gly 1·12, Cys 0·81, 4-Me-Phe 1·07, Phe 0·98, Har 0·82. For $C_{48}H_{68}N_{14}O_{11}S_2$.1·5 TFA.3·5 H_2O (1 315·4) calculated: 46·57% C, 5·86% H, 14·91% N; found: 46·53% C, 5·43% H, 15·16% N. FAB MS (*m*/*z*): 1 082 (M + H^+).

[1-Mercaptopropionic Acid, 2-*p*-Ethyl-L-phenylalanine, 8-D--Homoarginine]vasopressin (*IV*) and [1-Mercaptopropionic Acid, 2-*p*-Ethyl-D-phenylalanine, 8-D-Homoarginine]vasopressin (*V*)

Peptide-resin B (2·9 g, 0·88 mmol) was coupled according to the general scheme with 1·1 equivalents of Boc-L,D-Phe(*p*-Et)-OH (ref.[1]) for 24 h and the coupling was repeated twice with another 0·5 equivalents for 24 and 3 h and with Mpr(4-Me-Bzl)-OH. Treatment of the protected nonapeptide was analogous to that of compound *II* and *III*. Freeze-drying afforded 368 mg of crude product which was purified by HPLC on a column Vydac 218TP510 using a slow gradient running from 30% to 50% of MeOH in 0·05% trifluoroacetic acid in 60 min. Lyophilization of the corresponding fractions after dilution with 1M acetic acid afforded 97 mg and 91 mg of the products pure according to HPLC.

The first product (97 mg; *k* 1·94, methanol–0·05% trifluoroacetic acid 1 : 1 — Vydac) corresponds to the analog with *p*-ethyl-L-phenylalanine (*IV*) in position 2. R_F 0·08 (S1), 0·56 (S4). $E_{2.4}^{Gly}$ 0·64; $E_{5.7}^{His}$ 0·30 (detection by chlorination method). $[\alpha]_D$ −49·0° (*c* 0·1; 1M acetic acid). Amino acid analysis: Asp 1·03, lGu 1·07, Pro 0·98, Gly 1·02, Cys 0·33, 4-Et-Phe 0·78, Phe 0·93, Har 0·96. For $C_{49}H_{70}N_{14}O_{11}S_2$.3 TFA.AcOH.$H_2O$ (1 515·5) calculated: 45·18% C, 5·25% H, 12·94% N; found: 45·23% C, 5·33% H, 12·85% N. FAB MS (*m*/*z*): 1 095 (M^+).

The second product (91 mg; *k* 3·46, methanol–0·05% trifluoroacetic acid 1 : 1) corresponds to the analog with *p*-ethyl-D-phenylalanine (*V*) in position 2. R_F 0·06 (S1), 0·56 (S4). $E_{2.4}^{Gly}$ 0·64; $E_{5.7}^{His}$ 0·30 (detection by chlorination method). $[\alpha]_D$ −69·1° (*c* 0·1; 1M acetic acid). Amino acid analysis: Asp 1·00, Glu 1·08, Pro 1·00, Gly 0·96, Cys 0·40, 4-Et-Phe 0·88, Phe 0·94, Har 1·02. For $C_{49}H_{70}N_{14}O_{11}S_2$.3 TFA.AcOH.2 H_2O (1 533·5) calculated: 44·64% C, 5·32% H, 12·78% N; found: 44·48% C, 5·25% H, 12·78% N. FAB MS (*m*/*z*): 1 095 (M^+).

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[17] in Munsick[18] solution. Inhibitory activity is characterized by pA_2 value (ref.[19]). Pressor activity was tested on pithed rat preparation according to refs[20,21]. Antidiuretic potency on a nonanaesthetized rat was followed according to the method in ref.[22]. As the standard in the antidiuretic test [deamino^1,D-arginine^8]-vasopressin was used.

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