

**THE ANALOGS OF 8-D-HOMOARGINININ-VASOPRESSIN
WITH *o*-SUBSTITUTED PHENYLALANINE IN POSITION 2:
SYNTHESIS AND SOME BIOLOGICAL PROPERTIES***

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Solid phase methodology on *p*-methylbenzhydrylamine resin was used for the synthesis of four analogs of vasopressin with the non coded amino acids, D-homoarginine (in position 8) and *o*-substituted L- or D-phenylalanine (in position 2). [L-Phe(*o*-Me)²,D-Har⁸]vasopressin (*I*), [D-Phe(*o*-Me)²,D-Har⁸]vasopressin (*II*), [L-Phe(*o*-Et)²,D-Har⁸]vasopressin (*III*) and [D-Phe(*o*-Et)²,D-Har⁸]vasopressin (*IV*) were synthesized. All analogs had very low antidiuretic activity. Analogs *I* and *IV* were low pressor inhibitors. All analogs were found to be the uterotonic inhibitors, the most potent one in vitro being [D-Phe(*o*-Et)²,D-Har⁸]vasopressin with a $pA_2 = 8.4$.

Recently we described the inhibitory activities of vasopressin analogs modified by homoarginine in position 8 and *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 (ref.²) and also to investigate the influence of other benzene ring substitution of phenylalanine. Thus, analogs having (a) *o*-alkylsubstituted (this paper, some preliminary results were presented at the symposium³), (b) multiply (dialkyl- or trialkyl-) substituted (ref.⁴) and (c) alkoxy substituted rings (ref.⁵) were prepared.

Four analogs of vasopressin** (*I–IV*) described in this paper with D-homoarginine in position 8 and *o*-substituted phenylalanine in position 2 were synthesized similarly as the *p*-substituted vasopressin analogs¹. N^α-Tert-butoxycarbonyl-N^G-nitrohomoarginine¹ was found to be a suitable derivative of D-homoarginine for solid phase

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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 recommendations⁶: Har denotes the homoarginine moiety, Phe(*o*-Me) the *o*-methylphenylalanine and Phe(*o*-Et) the *o*-ethylphenylalanine moiety.

synthesis. D,L-*o*-Ethylphenylalanine was prepared via the modified acetamidomalonanane method^{7,8} starting from a mixture of *o*- and *p*-ethylbenzyl chloride⁹. Different

Cys-X-Phe-Gln-Asn-Cys-Pro-D-Har-Gly-NH₂

- I X = L-Phe(*o*-Me)
- II X = D-Phe(*o*-Me)
- III X = L-Phe(*o*-Et)
- IV X = D-Phe(*o*-Et)

reactivities of *o*- and *p*-ethylbenzyl chloride enable us by choice of suitable conditions of synthesis to prepare either practically pure *p*-derivative or a mixture of both *o*- and *p*-derivatives. Using an excess of *o*- and *p*-ethylbenzyl chloride mixture, only one product, (diethyl 4-ethylbenzylacetamidomalonate), is obtained. Using equimolar amount of ethylbenzyl chloride, the mixture of *p*- and *o*-derivatives (diethyl 4-ethylbenzylacetamidomalonate and diethyl 2-ethylbenzylacetamidomalonate) is obtained in approximately the same proportion as that of the starting mixture. Identification of the *o*- and *p*-ethylphenylalanine was performed by NMR spectroscopy. Amino groups of racemic amino acids were protected by the Boc-group.

The syntheses of all four analogs were performed by solid phase technique on the *p*-methylbenzhydrylamine resin. Tert-butoxycarbonyl group was used for the α -amino group protection. For the side chain protection either a nitro group (D-Har) or 4-methylbenzyl (Cys) was used. Protected amino acids were coupled by N,N'-dicyclohexylcarbodiimide (DCC) and N-hydroxybenzotriazole (HOBT) in dimethylformamide. Side chain protecting groups were removed simultaneously with the cleavage of the peptide from the resin using liquid hydrogen fluoride. Sulfhydryl group oxidation was performed by potassium ferricyanide. The analogs were purified by HPLC. We made use of the fact that diastereoisomeric peptides could be easily separated through reverse phase chromatography^{1,2,5,10-12}. As such, the synthesis was performed using racemic amino acids D,L-*o*-methylphenylalanine or D,L-*o*-ethylphenylalanine and peptides containing the appropriate diastereoisomers were separated at the end of the preparation. Identification of the L- or D-amino acid in the pure peptides was performed on hydrolysates either by digestion with L-amino acid oxidase^{13,14} (the digestion was prolonged for 100 h) or on chiral plates¹⁵. Moreover, in both cases the value of *k* in HPLC on reverse phase was always lower for the L-diastereoisomer – consistent with the previous findings^{1,2,5,10,11}. The formation of both diastereoisomers was accomplished with only 1:1 equivalents of protected racemic amino acid (tert-butoxycarbonyl-D,L-*o*-methylphenylalanine or tert-butoxycarbonyl-D,L-*o*-ethylphenylalanine).

Electrophoresis in pyridine-acetate buffer (pH 5.7) could be used for the separation of the diastereoisomeric mixtures too. (However, electrophoretic mobility in 1M acetic acid is identical for both diastereoisomers.) Analog containing L-amino acid

in position 2 are significantly more basic than D-amino acid containing ones. This means that the configuration of the amino acid in position 2 influences p*K* of the N-terminal amino group. Influence of the configuration of an amino acid on the p*K* of its amino group was described^{16,17} for oxytocin analogs with L- or D-cysteine in position 1; however the transfer of this effect over one amino acid residue was quite surprising.

Biological activities of the analogs are given in Table I. Substitution in position 2 led to a substantial decrease in antidiuretic activity. The analogs with *o*-substituted phenylalanine in position 2 had either no activity (*II, III*) or very low inhibitory activity (*I, IV*) in the pressor test. As in the previous cases^{1,2,5}, all analogs were found to be uterotonic inhibitors. The earlier published analogs evidenced that more potent inhibitors result from the combination of D-amino acids in both position 2 and 8. This is also true for analogs prepared in this study. Analogously, as analog containing *p*-ethylphenylalanine of D-configuration was found superior in the *p*-substituted series, the *o*-ethylphenylalanine of D-configuration containing analog (with p*A*₂ = 8.4) is superior in the *o*-substituted series.

TABLE I

Biological activities (rat) of [D-Har⁸]vasopressin analogs (I.U./mg or p*A*₂) with modifications in position 2

Compound	Activity				Ref.
	Uterotonic		Pressor	Anti-diuretic	
	in vitro	in vivo			
AVP	17		412	465	18
[D-Har ⁸]VP	—		0.83	83	19
	0.9		—	1% dDAVP	1
<i>I</i>	p <i>A</i> ₂ = 6.4	p <i>A</i> ₂ = 6.7	p <i>A</i> ₂ < 6.0	< 10 ⁻⁴ % dDAVP	^a
<i>II</i>	p <i>A</i> ₂ = 7.8	p <i>A</i> ₂ = 7.3	0	< 10 ⁻⁴ % dDAVP	^a
<i>III</i>	p <i>A</i> ₂ = 6.8	p <i>A</i> ₂ = 6.1	0	< 10 ⁻⁴ % dDAVP	^a
<i>IV</i>	p <i>A</i> ₂ = 8.4	p <i>A</i> ₂ = 6.9	p <i>A</i> ₂ = 5.6	< 10 ⁻⁴ % dDAVP	^a

^a This paper.

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) or on silica gel RP with Cu^{2+} and chiral reagent coated plates (Chiralplate, Macherey–Nagel, F.R.G.) in the following system: acetonitrile–water–methanol (4 : 1 : 1) (CH). 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 chlorination method. Samples for amino acid analysis were hydrolyzed with 6M HCl at 105°C for 20 h or with 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. High performance liquid chromatography (HPLC) was carried out on an SP-8800 instrument equipped with an SP-8405 detector and SP-4290 integrator (all from Spectra Physics, Santa Clara, USA). 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 above described equipment using column Vydac 218TP510 (5 μm , 250 \times 10 mm). Before use, all amino acid derivatives were subjected to ninhydrin test²⁰.

Solid phase peptide synthesis: A cycle for incorporation each amino acid residue into the growing peptide chain consisted of the following: 1. cleaving the Boc group by 50% trifluoroacetic acid in dichloromethane containing 5% anisole, 5 min and 30 min; 2. washing with dichloromethane, 2-propanol and dichloromethane; 3. neutralizing by 5% diisopropylethylamine in dichloromethane, 2 and 5 min; 4. washing with dichloromethane and dimethylformamide; 5. adding the Boc-protected amino acid derivative in dimethyl formamide followed by HOBT, followed by DCC and stirring for 1–2.5 h; 6. washing with dimethylformamide, dichloromethane, 2-propanol and dichloromethane. The synthesis was monitored by bromophenol blue method²¹.

D,L-o-Ethylphenylalanine

Sodium (1.73 g, 75 mmol) was dissolved in boiling absolute ethanol (110 ml) and after 20 min diethyl acetamidomalonate (16.3 g, 75 mmol) and mixture of *o*- and *p*-ethylbenzyl chloride⁹ (22% *o*- and 78% *p*-derivative according to NMR) (11.6 g, 75 mmol) were added. Reaction mixture was refluxed for 12 h, the precipitate was hot filtered and filtrate was evaporated and to residue petroleum ether was added. After overnight standing in refrigerator was the product, mixture of diethyl 2-ethylbenzylacetamidomalonate (13%) and diethyl 4-ethylbenzylacetamidomalonate (87% according to HPLC), filtered off and washed with petroleum ether. Yield: 15.6 g (62%) of mixture. HPLC: *k* 2.04 (*o*-) and 2.70 (*p*-), methanol–0.05% trifluoroacetic acid 7 : 3 (S). Better solubility of *o*-derivative in ethanol is possible to use for partial prepurification of this mixture. Crude product was suspended in little amount of ethanol, triturated and filtered off. Product on filter (7.5 g) is almost pure *p*-derivative (1.8% *o*-derivative), mother liquor is enriched by *o*-derivative (40% *o*-, 60% *p*-). Mother liquor was evaporated (6.7 g) and pure *o*- and *p*-derivative were separated by preparative HPLC. (SepTech ST/Lab 800C instrument with column Kromasil C18 100 A 7.5 \times 25 cm A/E Macrobore Annular expansion column: product was eluted by gradient running from 0% to 32% of acetonitrile in 120 min continuing by gradient running from 32% to 40% of acetonitrile in 120 min at flow 60 ml/min.) Yield of diethyl 2-ethylbenzylacetamidomalonate (oil): 1.9 g (7.5%).

Diethyl 2-ethylbenzylacetamidomalonate (1.9 g) was refluxed 4.5 h in 25% HCl (25 ml), cooled in refrigerator, filtered off and product (D,L-*o*-ethylphenylalanine hydrochloride) was suspended in water and pH was adjusted by aqueous ammonia to 7.0. Overnight standing in the refrigerator afforded crystals which were washed by water. Yield 0.8 g (72%), m.p. 191–194°C; $R_F(\text{CH})$ 0.56 (L-) and 0.46 (D-). HPLC: k 1.53, methanol–0.05% trifluoroacetic acid 3 : 7 (V). For $\text{C}_{11}\text{H}_{15}\text{NO}_2 \cdot 0.1 \text{H}_2\text{O}$ (193.2) calculated: 68.09% C, 7.22% H, 7.38% N; found: 68.11% C, 7.76% H, 7.29% N.

D,L-*p*-Ethylphenylalanine

Sodium (0.87 g, 37.5 mmol) was dissolved in boiling absolute ethanol (55 ml) and after 20 min diethyl acetamidomalonate (8.15 g, 37.5 mmol) and a mixture of *o*- and *p*-ethylbenzyl chloride⁹ (11.6 g, 75 mmol) were added. Reaction mixture was refluxed for 12 h, the precipitate was hot filtered and filtrate was evaporated and to the residue petroleum ether was added. After overnight standing in refrigerator was the crystalline product, diethyl 4-ethylbenzylacetamidomalonate, filtered off and washed with petroleum ether. Yield 6.36 g (51%), m.p. 89–91°C. HPLC: k 2.70, methanol–0.05% trifluoroacetic acid 7 : 3 (S).

Diethyl 4-ethylbenzylacetamidomalonate (6.33 g) was refluxed 4.5 h in 25% HCl (80 ml), cooled in refrigerator, filtered off and product (D,L-*p*-ethylphenylalanine hydrochloride) was suspended in water and pH was adjusted by aqueous ammonia to 7.0. Overnight standing in the refrigerator afforded crystals which were washed by water. Yield: 3.0 g (82%), m.p. 202–206°C. HPLC: k 2.05, methanol–0.05% trifluoroacetic acid 3 : 7 (V). The literature gives m.p. 185 to 193°C (ref.²²) and 244–247°C (capillary) (ref.²³).

Tert-butoxycarbonyl-D,L-*o*-ethylphenylalanine

Solution of D,L-*o*-ethylphenylalanine (480 mg, 2.5 mmol) in the mixture of dioxane (5 ml), water (2.5 ml) and 1M NaOH (2.5 ml) was treated with Boc-anhydride (0.6 g, 2.75 mmol) with stirring at pH 8–9 (adjusted by addition of 1M NaOH) for 3 h. Reaction mixture was extracted by ether, aqueous layer was cooled to 0°C, acidified by 20% citric acid and product was extracted by ethyl acetate. Extracts were washed by water, 0.5M H_2SO_4 , water and dried by sodium sulfate. Filtration, evaporation and treating with petroleum ether afforded 517 mg (71%) of pure crystalline product, m.p. 122–125°C. HPLC: k 7.24, methanol–0.05% trifluoroacetic acid 3 : 2 (V). For $\text{C}_{16}\text{H}_{23}\text{NO}_4$ (293.3) calculated: 65.51% C, 7.90% H, 4.77% N; found: 65.46% C, 7.77% H, 4.75% N.

Nonapeptide-Resins

p-Methylbenzhydrylamine resin (Peptides International, 0.79 meq/g, 8 g) was suspended in dichloromethane and after washing with 5% diisopropylethylamine in dichloromethane and with dimethylformamide was coupled with 3 molar excess of Boc-Gly-OH in the presence of *N*-hydroxybenzotriazole and dicyclohexylcarbodiimide in dimethylformamide. Coupling was interrupted after 2 h, the resin was washed consequently by dimethylformamide (3×) and dichloromethane (3×) and resin substitution was determined by amino acid analysis (0.55 mmol/g). Unreacted amino groups were acetylated (5 ml acetic anhydride and 2 ml triethylamine in 50 ml dichloromethane 2 h). The following procedure was performed according to general scheme given at the beginning of 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.

Protected derivatives were used in the following order: Boc-D-Har(NO₂)-OH (ref.¹), Boc-Pro-OH, Boc-Cys(4-Me-Bzl)-OH (ref.²⁴), Boc-Asn-OH, Boc-Gln-OH and Boc-Phe-OH. Yield for heptapeptide resin: 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.

Following this step the resin was divided in several parts: for *o*-Me-Phe analogs 1.0 g (0.3 mmol) and was coupled according to general scheme with Boc-D,L-Phe(*o*-Me)-OH (ref.²⁵) or Boc-D,L-Phe(*o*-Et)-OH and with Boc-Cys(4-Me-Bzl)-OH (ref.²⁴).

The Cleavage of the Peptide from the Resin, Oxidation and Purification of Analogs I–IV

After cleavage of Boc-protecting group the nonapeptide-resin was treated with liquid hydrogen fluoride (15 ml, 60 min, 0°C) in the presence of anisole (1 ml). Unprotected nonapeptide, together with the resin, was triturated with ether after evaporation of hydrogen fluoride, filtered off, washed with ethylacetate and then free peptide was extracted successively by acetic acid, 50%

TABLE II
Physico-chemical and analytical data for analogs I–IV

	I	II	III	IV
Yield %	12.3	9.3	12.3	10.2
<i>k</i>	1.41 ^a	2.73 ^a	1.24 ^b	2.88 ^b
<i>R_F</i> (S1)	0.4	0.04	0.04	0.04
(S4)	0.44	0.42	0.44	0.48
<i>E</i> _{2.4} ^{Gly}	1.12	1.07	1.12	1.12
<i>E</i> _{5.7} ^{His}	0.74	0.62	0.69	7.58
[α] _D (c 0.1, 1M AcOH)	+26.8	−20.0	0.0	−19.2
FAB MS (<i>m/z</i>) ^c	1 096.3	1 096.3	1 110.4	1 110.4
Amino acid analysis				
Asp	1.03	1.01	0.96	0.95
Glu	0.99	1.03	1.09	0.99
Pro	0.99	0.94	1.23	1.15
Gly	1.00	1.08	1.00	0.97
Cys	1.69	1.44	1.50	1.40
Phe	0.96	0.89	0.97	0.98
Phe(<i>o</i> -Me)	1.06	1.10	—	—
Phe(<i>o</i> -Et)	—	—	1.02	1.00
Har	1.00	0.94	0.95	0.98

^a Methanol–0.05% trifluoroacetic acid (4 : 6). ^b Methanol–0.05% trifluoroacetic acid (4.5 : 5.5), Vydac. ^c For M+H⁺.

TABLE III
Elemental analyses of analogs I–IV

Compound	Formula	M.W.	Calculated/Found		
			% C	% H	% N
I	C ₄₈ H ₆₈ N ₁₅ O ₁₁ S ₂ .3 TFA.2.5 H ₂ O	1 482.4	43.75	5.17	14.17
			43.46	4.93	14.68
II	C ₄₈ H ₆₈ N ₁₅ O ₁₁ S ₂ .3 TFA.2.5 H ₂ O	1 482.4	43.75	5.17	14.17
			43.60	4.92	14.63
III	C ₄₉ H ₇₀ N ₁₅ O ₁₁ S ₂ .3 TFA.4.5 H ₂ O	1 532.5	43.11	5.38	13.71
			42.91	4.79	13.92
IV	C ₄₉ H ₇₀ N ₁₅ O ₁₁ S ₂ .4 TFA.1.5 H ₂ O	1 592.4	42.99	4.86	13.18
			42.83	4.38	12.73

acetic acid, water and lyophilized. The lyophilizate was dissolved in water (900 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 put on a column of Amberlite CG-50I (50 ml), the column was washed with 0.25% acetic acid and the product elued with 50% acetic acid (150 ml). After freeze-drying, the crude product was purified by HPLC on column Vydac 218TP510 in a slow gradient running from 30% to 50% methanol in 0.05% trifluoroacetic acid in 60 min and lyophilized. Yields, *k*, *R_F*, electrophoretic mobilities, optical rotation values, FAB MS and amino acid analyses are given in Table II. Elemental analyses for analogs I–IV are given in Table III.

Pharmacological Methods

All pharmacological test were performed using Wistar rats weighing 200–300 g. The uterotonic potency *in vitro* was evaluated using the Holton procedure²⁶ in Munsick²⁷ solution. For the determination of the *in vivo* activity²⁸ oestrogenized rats in ethanol anaesthesia were used. Inhibitory activity was characterized by pA₂ value (ref.²⁹). Pressor activity was tested on pithed rat preparation according to refs^{30,31}. Antidiuretic potency on nonanaesthetized rats was followed according to the method in ref.³². As the standard in the antidiuretic test [deamino¹, D-arginin⁸]vasopressin was used.

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