THE ANALOGS OF [D-Har^8]VASOPRESSIN WITH DI- AND TRISUBSTITUTED PHENYLALANINE IN POSITION 2; SYNTHESIS AND SOME BIOLOGICAL PROPERTIES*

Miroslava ŽERTOVÁ, Zdenko PROCHÁZKA, Jiřina SLANINOVÁ, Tomislav BARTH, Pavel MAJER and Michal LEBL

Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, 166 10 Prague 6, The Czech Republic

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Dedicated to Professor Otto Wichterle on the occasion of his 80th birthday.

Four analogs of vasopressin with non-coded amino acids D-homoarginine (in position 8) and 2,6-di- or 2,4,6-trisubstituted L- or D-phenylalanine (in position 2) were synthesized using the solid phase method on *p*-methylbenzhydrylamine resin. All the analogs were found to be uterotonic inhibitors, the most potent one in vitro and in vivo being [D-Phe(2,4,6-triMe)^2,D-Har^8]vasopressin with pA_2 values equal to 8.1 and 7.5, respectively. All of them had negligible antidiuretic activity and were weak pressor inhibitors.

Recently we described the inhibitory qualities of vasopressin analogs modified by homoarginine in position 8 and *p*-substituted phenylalanine in position 2 (ref.[1]). The considerably high antagonistic potency of these analogs prompted us to combine the abovementioned modifications with deamination in position 1 (ref.[2]), and to investigate the influence of other benzene ring substitutions of phenylalanine. Consequently, we have successively prepared analogs with (i) ortho-alkyl substituted (ref.[3]), (ii) alkoxy substituted (ref.[4]) and (iii) multiply substituted phenyl ring (this paper). Four analogs of vasopressin** (*I – IV*) described in this paper having D-homoarginine in position 8 and 2,6-dimethyl or 2,4,6-trimethyl substituted phenylalanine in position 2 were synthesized similarly to the ortho-substituted vasopressin analogs[3].

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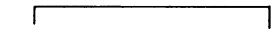
**All the chiral amino acids unless otherwise stated are of the L-series. The nomenclature and symbols of the amino acids and peptides obey the published recommendations[5]: Har denotes the homoarginine, Phe(2,6-diMe) the 2,6-dimethylphenylalanine and Phe(2,4,6-triMe) the 2,4,6-trimethylphenylalanine.

N^{α}-*tert*-Butoxycarbonyl-N^{G}-nitrohomoarginine (ref.[1]) was used in the synthesis. D,L-2,4,6-Trimethylphenylalanine was prepared via the modified acetamidomalonane method[6] starting from 2,4,6-trimethylbenzyl chloride. D,L-2,4-Dimethylphenylalanine was prepared as described earlier[7].



Cys-X-Phe-Gln-Asn-Cys-Pro-D-Har-Gly-NH_2

I, X = L-Phe(2,6-diMe)
II, X = D-Phe(2,6-diMe)
III, X = L-Phe(2,4,6-triMe)
IV, X = D-Phe(2,4,6-triMe)

The syntheses of all four analogs were performed by the solid phase technique on *p*-methylbenzhydrylamine resin. A *tert*-butoxycarbonyl group was used for the α-amino group protection. For the side chain protection either a nitro group (D-Har) or a 4-methylbenzyl group (Cys) was used. The protected amino acids were then coupled by *N,N'*-dicyclohexylcarbodiimide (DCC) and *N*-hydroxybenzotriazole (HOBt) in dimethylformamide. Side chain protecting group removal was simultaneous with the cleavage of the peptide from the resin using liquid hydrogen fluoride. Sulfhydryl group oxidation was performed by potassium ferricyanide. Because of easy separability of diastereoisomeric peptides by means of RP HPLC (refs[1 - 4,8 - 10]), the syntheses were thus performed using racemic amino acids D,L-2,6-dimethylphenylalanine or D,L-2,4,6-trimethylphenylalanine. Peptides containing the appropriate diastereoisomers were separated at the end of the preparation. Formation of both diastereoisomers was accomplished using only 1.1 equivalents of protected racemic amino acid (*tert*-butoxycarbonyl-D,L-2,4-dimethylphenylalanine or *tert*-butoxycarbonyl-D,L-2,4,6-trimethylphenylalanine, see ref.[1]). Chirality of the amino acid in the pure peptide was determined in hydrolysates either by digestion, using L-amino acid oxidase[11,12] (digestion time 100 h), or on chiral plates[13]. In HPLC on reverse phase the *k* value in both cases was found to be lower for the L-diastereoisomer, which is consistent with previous findings (refs[1 - 4,8,9]).

Electrophoresis in pyridine–acetate buffer (pH 5.7) could also have been used to separate the diastereoisomeric mixtures, as in previous experiments[3,4]. Electrophoretic mobility in 1 M acetic acid was, however, identical for both diastereoisomers. Analogs containing L-amino acid in position 2 are significantly more basic than those containing D-amino acid. This means that the configuration of the amino acid in position 2 influences the p*K* of the N-terminal amino group. The influence of the amino acid configuration on the p*K* of its amino group has been described[14,15] for oxytocin analogs

having L- or D-cysteine in position 1; however the transfer of this effect over one amino acid residue was quite surprising.

The biological activities of the analogs are given in Table I. Substitution in position 2 led to a substantial decrease in antidiuretic activity. All analogs with a polysubstituted phenylalanine in position 2 had very low inhibitory activity in the pressor test. As in the previous cases[1-4], all the analogs were found to be antagonists of oxytocin-evoked uterine contractions. Earlier publications have reported that the combination of D-amino acids in both positions, i.e. position 2 and 8, results in more potent inhibitors. The same holds for analogs prepared in this study. The low inhibitory potency of analogs with dimethylphenylalanine was surprising. On the other hand, trimethyl substituted analog *IV* is the most potent in vivo uterotonic inhibitor ($pA_2 = 7.5$) in the group of amino and deamino analogs of [D-Har[8]]vasopressin and one of the most potent inhibitors described up to now (see ref.[18]).

When viewing hitherto prepared analogs of [D-Har[8]]vasopressin containing variously substituted benzene rings of D- or L-phenylalanine in position 2, it is possible to find several interesting connections between the type of the substituent and the magnitude of the uterotonic inhibition (see Table II).

TABLE I
Biological activities (rat) of [D-Har[8]]vasopressin analogs (I.U./mg or pA_2) with modifications in position 2

Compound	Activity				Ref.
	uterotonic		pressor	antidiuretic	
	in vitro	in vivo			
AVP	17		412	465	16
[D-Har[8]]VP	–		0.83	83	17
	0.9		–	1%[a]	1
I	$pA_2 = 5.7$	–	$pA_2 = 5.9$	0[a]	[b]
II	$pA_2 = 6.4$	$pA_2 = 6.5$	$pA_2 = 6.1$	~0.1%[a]	[b]
III	$pA_2 = 6.1$	0	$pA_2 = 5.8$	0[a]	[b]
IV	$pA_2 = 8.1$	$pA_2 = 7.5$	$pA_2 = 6.1$	~0.1%[a]	[b]

[a] Antidiuretic activity was determined on conscious rats using dDAVP as standard; it is expressed in per cent of dDAVP activity, 0 means less than 10^{-4}%; no anti-antidiuretic activity was observed. [b] This paper.

In the case of the analogs of the D-series, the order of their antioxytocin inhibitory potency is as follows:

$$o\text{-Et} > p\text{-Et} = 2,4,6\text{-triMe} > p\text{-OMe} > o\text{-Me} = p\text{-Me} >> p\text{-OEt} >> 2,6\text{-diMe}.$$

The strongest inhibitor is that with an ethyl substituent in the ortho-position closely followed by that with the same substituent in the para-position and that with a group of corresponding size, i.e. *p*-methoxy group. There is no difference in the inhibitory potencies of *o*- and *p*-methyl substituted analogs. The *p*-ethoxy substituted analog has substantially lower potency and the dimethyl substituted analog with methyl groups in ortho- and ortho'-positions the lowest. An interesting finding is the fact that the presence of the third methyl in the para-position places the relevant analog among the strongest inhibitors.

In the in vivo uterotonic test, the difference in potency between the strongest and weakest antagonists is smaller than in the case of the in vitro test. However, the three

TABLE II
Antioxytocic activities (pA_2, rat) of [D-Har^8]vasopressin analogs with modifications in position 2

Modification	Uterotonic activity		Ref.
	in vitro	in vivo	
D-Phe(*o*-Et)	8.4	6.9	3
D-Phe(*p*-Et)	8.1	6.6[a]	1
D-Phe(2,4,6-triMe)	8.1	7.5	[b]
D-Tyr(Me)	7.9	6.9	4
D-Phe(*o*-Me)	7.8	7.3	3
D-Phe(*p*-Me)	7.8	–	1
D-Tyr(Et)	7.3	6.3	4
D-Phe(2,6-diMe)	6.4	6.7	[b]
L-Tyr(Me)	7.7	6.7[a]	4
L-Phe(*p*-Et)	7.4	–	1
L-Tyr(Et)	6.9	7.0	4
L-Phe(*p*-Me)	6.8	6.1[a]	1
L-Phe(*o*-Et)	6.8	6.1	3
L-Phe(*o*-Me)	6.4	6.7	3
L-Phe(2,4,6-triMe)	6.1	0	[b]
L-Phe(2,6-diMe)	5.7	–	[b]

[a] Tested for this paper. [b] This paper.

most potent analogs are more or less the same in both the in vitro and in vivo tests. The strongest antagonist ranks among the strongest in vivo antioxytocin inhibitors ever synthesized, as mentioned above.

As far as the L-series is concerned, where the analogs show one order of magnitude lower inhibitory potency in the in vitro test than the D-stereoisomers, we can see a slightly different potency order:

$$p\text{-OMe} > p\text{-Et} >> p\text{-OEt} = p\text{-Me} = o\text{-Et} > o\text{-Me} > \text{2,4,6-triMe} > \text{2,6-diMe}.$$

The strongest inhibitors are those with para-substituents, followed by those with ortho-substituents. In addition, the third methyl group in the para-position has a positive effect – the trimethyl substituted analog has a higher inhibitory potency than the dimethyl substituted one.

If we compare the differences in inhibitory potency among the diastereoisomeric pairs (see Table III), we get the following order:

TABLE III
Difference of uterotonic inhibitory activity in vitro as a consequence of the configuration change of amino acid in position 2

Modification	Inhibitory potency, pA_2	Logarithmic difference in the potency of the D- and L- stereoisomer
L-Phe(2,4,6-triMe)	6.1	
D-Phe(2,4,6-triMe)	8.1	2.0
L-Phe(*o*-Et)	6.8	
D-Phe(*o*-Et)	8.4	1.6
L-Phe(*o*-Me)	6.4	
D-Phe(*o*-Me)	7.8	1.4
L-Phe(*p*-Me)	6.8	
D-Phe(*p*-Me)	7.8	1.0
L-Phe(*p*-Et)	7.4	
D-Phe(*p*-Et)	8.1	0.7
L-Phe(2,6-diMe)	5.7	
D-Phe(2,6-diMe)	6.4	0.7
L-Tyr(Et)	6.9	
D-Tyr(Et)	7.3	0.4
L-Tyr(Me)	7.7	
D-Tyr(Me)	7.9	0.2

2,4,6-tri-Me >> *o*-Et > *o*-Me >> *p*-Me > *p*-Et > 2,6-diMe >> *p*-OEt > *p*-OMe.

The difference in activities is greatest in the case of 2,4,6-trimethyl substituted phenylalanine. The inhibitory potency of the D-analog is 100 times higher than that of the L-analog. The least difference (about 2-fold) occurs in the cases of 2,6-dimethyl substituted phenylalanine and the alkoxy type substitutions (ethoxy and methoxy).

Even if a large number of data had been accumulated it would still be difficult to estimate what quality of the substituent (its bulkiness, its effect on electron distribution in the benzene ring, or its effect on the basicity of the amino group in position 1) is crucial for the antagonistic potency.

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), 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, Germany) in the following system: acetonitrile–water–methanol (4 : 1 : 1) (CII). Paper electrophoresis was performed in a moist chamber 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 in the TLC and electrophoresis were developed with ninhydrin or by the chlorination method. Samples for amino acid analysis were hydrolyzed with 6 M HCl at 105 °C for 20 h or with a mixture of propionic acid–hydrochloric acid (1 : 1) at 160 °C for 15 min, and analyzed on an Amino Acid Analyzer T 339 (Mikrotechna Praha, The Czech Republic) or D-500 Analyzer (Durrum, 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, Manchester, U.K.) 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-8450 detector and SP-4290 integrator (all from Spectra Physics, Santa Clara, U.S.A.). Preparative HPLC was carried out using column Vydac 218TP510 (5 μ, 250 × 10 mm). Purity of the products was determined on the column of Separon SIX C-18 (S) or Vydac 218TP54 (V). Before use, all amino acid derivatives were subjected to the ninhydrin test[19].

Solid phase peptide synthesis: Incorporation of each amino acid residue into the growing peptide chain consisted of the following cycle: 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% *N*-ethyldiisopropylamine in dichloromethane, 2 and 5 min; 4. washing with dichloromethane and dimethylformamide; 5. adding the Boc-protected amino acid derivative in dimethylformamide 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 using the bromophenol blue method[20].

D,L-2,4,6-Trimethylphenylalanine

Sodium (2.3 g, 100 mmol) was dissolved in boiling absolute ethanol (150 ml), and after 20 min diethyl acetamidomalonate (21.7 g, 100 mmol) and 2,4,6-trimethylbenzyl chloride (18.6 g, 117 mmol) were added. The reaction mixture was refluxed for 12 h, the precipitate was filtered hot and the filtrate was evaporated. Petroleum ether was added to the residue. After overnight refrigeration, the

crystalline product, i.e. diethyl 2,4,6-trimethylbenzylacetamidomalonate, was filtered and washed with petroleum ether. Yield: 30.5 g (81%), m.p. 86 – 88 °C. The literature gives m.p. 81 – 82 °C (ref.[6]).

This compound (10.5 g, 30 mmol) was refluxed for 4 h in 25% HCl (120 ml), cooled in a refrigerator, and filtered and the product (D,L-2,4,6-trimethylphenylalanine hydrochloride) was suspended in water. The pH was adjusted using aqueous ammonia to 7.0. Overnight refrigeration produced crystals which were washed with water. Yield: 5.2 g (83%), m.p. 237 – 238 °C. R_F (CH) 0.52 (L-) and 0.39 (D-). The literature gives m.p. 242 – 244 °C (ref.[6]).

tert-Butoxycarbonyl-D,L-2,4,6-trimethylphenylalanine

A solution of D,L-2,4,6-trimethylphenylalanine (4.14 g, 20 mmol) in a mixture of dioxane (36 ml), water (18 ml) and 1 M NaOH (18 ml) was treated with Boc-anhydride (4.8 g, 22 mmol) under stirring at pH 9 – 9.5 (adjusted by addition of 1 M NaOH) for 3 h. The reaction mixture was extracted with ether. The aqueous layer was cooled to 0 °C, acidified by 20% citric acid, and the product was extracted using ethyl acetate. The extracts were subsequently washed with water, 0.5 M H_2SO_4, water and then dried with sodium sulfate. Filtration and evaporation yielded 5.5 g (90%) of pure crystalline product, m.p. 162 – 165 °C. For $C_{17}H_{25}NO_4$ (307.3) calculated: 66.43% C, 8.20% H, 4.56% N; found: 66.45% C, 8.16% H, 4.49% N. FAB MS (*m/z*): 308 ($M + H^+$).

Nonapeptide Resin

p-Methylbenzhydrylamine resin (Peptides International, 0.79 meq/g, 8 g) was suspended in dichloromethane. After washing with 5% *N*-ethyldiisopropylamine 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. The coupling reaction was interrupted after 2 h, the resin was subsequently washed consequently by dimethylformamide (3 times) and dichloromethane (3 times) and the resin substitution was determined by amino acid analysis (0.55 mmol/g). The polymer was then acetylated (5 ml acetanhydride, 2 ml triethylamine in 50 ml dichloromethane). The following procedure was performed according to the general scheme given in the beginning of the Experimental (starting from point 1.). Boc-amino acids were coupled to the resin by the DCCI/HOBt procedure. All reagents were used in 3 molar excess. The protected derivatives were used in the following order: Boc-D-Har(NO_2)-OH (ref.[1]), Boc-Pro-OH, Boc-Cys-(4-MeBzl)-OH (ref.[21]), Boc-Asn-OH, Boc-Gln-OH and Boc-Phe-OH. Amount of the heptapeptide resin: 14.5 g. Amino acid analysis on the 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 into two parts, 1.0 g (0.3 mmol) each. The parts were then coupled according to the general scheme with either Boc-D,L-Phe(2,6-diMe)-OH (ref.[7]) or Boc-D,L-Phe(2,4,6-triMe)-OH and with Boc-Cys(4-MeBzl)-OH (ref.[21]).

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

After removal of the 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). After the evaporation of hydrogen fluoride, the nonapeptide together with the resin was triturated with ether, filtered, and washed with ethyl acetate. The free peptide was successively extracted with acetic acid, 50% acetic acid, and water, and then lyophilized. The substance was then dissolved in water (900 ml) and the pH of the solution was adjusted with 0.1 M NaOH to 7.0. Potassium ferricyanide (0.01 M solution) was added to this solution until a stable yellow color persisted. During the oxidation (20 min), the pH was main-

tained at 7.2 by adding 0.1 M NaOH and then adjusted with acetic acid to 4.5. The solution was then put on a column of Amberlite CG-50I (50 ml), which was washed with 0.25% acetic acid and the product eluted with 50% acetic acid (150 ml). After freeze-drying, the crude product was purified by HPLC on a Vydac 218TP510 column 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 IV. Elemental analyses for analogs *I* – *IV* are given in Table V.

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[22] in Munsick[23] solution. For the determination of in vivo activity, estrogenized rats in ethanol anaesthesia were used[24]. Inhibitory activity was characterized by the pA_2 value (ref.[25]). Pressor activity was tested on pithed rat according to refs[26,27]. Antidiuretic potency on unanesthetized rats was followed as in Burn et al.[28]. [Mpa^1,-D-Arg^8]vasopressin was used as the standard in the antidiuretic test.

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

? ? ?	*I*	*II*	*III*	*IV*
Yield, %	17.6	18.2	16.8	12.9
k^a	1.05	2.47	1.06	2.25
R_F (S1)	0.04	0.04	0.04	0.04
(S4)	0.46	0.49	0.48	0.50
$E_{2.4}^{Gly}$	1.20	1.20	1.12	1.12
$E_{5.7}^{His}$	0.67	0.60	0.69	0.59
$[\alpha]_D$ (c 0.1; 1M AcOH)	+28.0	−33.7	+13.9	−25.8
FAB MS (m/z)^b	1 110.4	1 110.4	1 124.5	1 124.5
		Amino acid analysis		
Asp	1.03	1.02	1.00	1.00
Glu	1.08	1.09	1.07	1.05
Pro	1.26	1.20	1.07	1.08
Gly	1.03	1.06	1.02	1.01
Cys	1.47	1.96	1.46	1.53
Phe	0.99	1.06	0.82	0.84
Phe(diMe)	0.82	0.87	–	–
Phe(triMe)	–	–	0.78	0.80
Har	1.07	0.91	0.71	0.82

^{a} Methanol–0.05% trifluoroacetic acid (4.5 : 5.5), Vydac. ^{b} For ($M + H^+$).

TABLE V
Elemental analyses of analogs *I – IV*

Compound	Formula	M.w.	Calculated/Found		
			% C	% H	% N
I	$C_{49}H_{70}N_{15}O_{11}S_2$. 3.5 TFA . 3 H_2O	1 562.5	43.04	5.13	13.44
			42.74	4.80	13.97
II	$C_{49}H_{70}N_{15}O_{11}S_2$. 3.5 TFA . 5 H_2O	1 598.5	42.08	5.26	13.14
			41.54	4.69	13.47
III	$C_{50}H_{72}N_{15}O_{11}S_2$. 4 TFA . 2.5 H_2O	1 624.5	42.88	5.03	12.93
			42.39	4.69	13.48
IV	$C_{50}H_{72}N_{15}O_{11}S_2$. 4 TFA . 2.5 H_2O	1 624.4	42.88	5.03	12.93
			42.78	4.79	13.21

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