THE 1- AND 2-NAPHTHYLALANINE ANALOGS OF OXYTOCIN AND VASOPRESSIN*

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Solid phase technique on *p*-methylbenzhydrylamine resin was used for the synthesis of four analogs of oxytocin and four analogs of vasopressin with the non-coded amino acids L- or D- and 1- or 2-naphthylalanine and D-homoarginine. [L-1-Nal^2]oxytocin, [D-1-Nal^2]oxytocin, [L-2-Nal^2]oxytocin, [D-2-Nal^2]oxytocin, [L-1-Nal^2, D-Har^8]vasopressin, [D-1-Nal^2, D-Har^8]vasopressin, [L-2-Nal^2, D-Har^8]vasopressin and [D-2-Nal^2, D-Har^8]vasopressin were synthesized. All eight analogs were found to be uterotonic inhibitors in vitro and in vivo. Analogs with 2-naphthylalanine are stronger inhibitors, particularly in the vasopressin series than the analogs with 1-naphthylalanine. Analogs with 1-naphthylalanine have no activity in the pressor test, analogs with 2-naphthylalanine are weak pressor inhibitors.

Both 1- and 2-naphthylalanines**, despite of their synthesis as early as in the beginning of this century[2], appeared in peptide analogs not until the last two decades. They started to be introduced into peptides as substitutes of tryptophan due to their similarity (1-naphthylalanine is an isoster of tryptophan in which NH group in the indole ring is substituted by vinyl group). LH-RH (substitution of tryptophan in position 3, refs[3,4]) belongs among the first biologically active peptides which were modified in this way. As substitutes for phenylalanine they were first used in deltorphin C (ref.[5]). In the case of neurohypophyseal hormones both naphthylalanines of D configuration were used for the synthesis of analogs of arginine vasopressin ([D-1-Nal^2] and [D-2-Nal^2]arginine-vasopressin, ref.[6]).

In this paper we describe the use of naphthylalanines for the replacement of tyrosine in position 2 in the oxytocin and vasopressin molecule.

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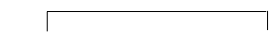
**All the chiral amino acids unless otherwise stated are of the L-series. The nomenclature and symbols of the amino acids and peptides obey the published recommendations[1]: 1-Nal denotes the 1-naphthylalanine (2-amino-3-(1-naphthyl)propionic acid), 2-Nal the 2-naphthylalanine (2-amino-3-(2-naphthyl)-propionic acid) and Har the homoarginine.

Recently we have described the inhibitory properties of oxytocin analogs (refs[7,8]) and vasopressin analogs (refs[9–14]) having substituted phenylalanines in position 2. In an effort to estimate the effect of substantial more bulky substituent in the side chain of amino acid in position 2, two possibilities come into consideration. Either bulky substituent on the benzene ring of phenylalanine (e.g. *p*-phenyl, *tert*-butyl, pentamethyl), or more bulky aromatic ring than benzene as the side chain of the amino acid. The 1-naphthylalanine and 2-naphthylalanine were thus natural candidates.

The synthesis and biological properties of four analogs of oxytocin (*I–IV*) and four analogs of vasopressin (*V–VIII*) are described in this paper having L- or D-1-naphthylalanine or L- or D-2-naphthylalanine in position 2 and (in the case of vasopressin analogs) D-homoarginine in position 8. Some preliminary results were presented recently[15].



	X	Y	Z
I	L-1-Nal	Ile	Leu
II	D-1-Nal	Ile	Leu
III	L-2-Nal	Ile	Leu
IV	D-2-Nal	Ile	Leu
V	L-1-Nal	Phe	D-Har
VI	D-1-Nal	Phe	D-Har
VII	L-2-Nal	Phe	D-Har
VIII	D-2-Nal	Phe	D-Har

EXPERIMENTAL

General Methods

Thin-layer chromatography (TLC) was carried out on silica gel coated plates (Silufol, Kavalier, 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). 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 Whatmann 3MM paper at 20 V/cm for 60 min. Spots after 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 and analyzed on Amino Acid Analyzer T 339 (Mikrotechna Praha, 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.) at 20 °C. Fast atom bombardment mass spectra were obtained on a ZAB-EQ spectrometer (VG Analytical, Manchester, U.K.) at 8 kV with xenon as the bombarding gas. High performance liquid chromatography (HPLC) was carried out on SP-8800 instrument

equipped with SP-8450 detector and SP-4290 integrator (all from Spectra Physics, Santa Clara, U.S.A.). Preparative HPLC was carried out using Vydac 218TP-510 (5 μm, 250 × 10 mm) column. Purity of the products was determined on the Vydac 218TP54 column. Before use, all amino acid derivatives were subjected to the ninhydrin test[16]. Chirality of the naphthylalanine in the pure peptide was determined in hydrolysates either by digestion, using L-amino acid oxidase[17,18] (digestion time 100 h) or on chiral plates[19].

Solid Phase Peptide Synthesis

Incorporation of each amino acid residue into the growing peptide chain on a resin consisted of the following steps: *1*. Cleavage of the Fmoc group by 20% piperidine in dimethylformamide; *2*. washing with dimethylformamide; *3*. addition of 3 equivalents of the Fmoc-protected amino acid in dimethylformamide followed by HOBt (3 equivalents) and by *N*,*N*′-diisopropylcarbodiimide (DIC) (3 equivalents); *4*. washing with dimethylformamide. The synthesis was monitored using the bromophenol blue method[20]. For the side chain protection we have used the nitro group (Har) and the *p*-methylbenzyl group (Cys). Side chain protecting group removal was simultaneous with the cleavage of the peptide from the resin using liquid hydrogen fluoride.

N^{α}-Fluorenyloxycarbonyl-N^{G}-nitro-D-homoarginine

The N^{G}-nitro-D-homoarginine[9] (0.70 g, 3 mmol) was dissolved in acetonitrile (5 ml), water (5 ml) and ethyldiisopropylamine (0.51 ml, 3 mmol). Fmoc-ONSu (1.2 g, 3.6 mmol) was added to this mixture and stirring was continued for 1 h at room temperature. After evaporating of acetonitrile the residue was acidified with 1 M HCl and the product was extracted into ethyl acetate. Extract was washed by water, dried with sodium sulfate, filtered, evaporated and the residue was triturated with ether. The product was filtered and washed with ether. Yield 0.78 g (58%), m.p. 75–77 °C, $[\alpha]_D$ –2.2°, $[\alpha]_{578}$ +20.2° (*c* 0.75, methanol). HPLC: *k* 0.43, (methanol–0.05% trifluoroacetic acid 7 : 3). For $C_{22}H_{25}N_5O_6$ (455.5) calculated: 58.02% C, 5.53% H, 15.38% N; found: 57.88% C, 5.60% H, 15.17% N. FAB MS (*m/z*): 456 ($M + H^+$), 478 ($M + Na^+$).

N^{α}-Fluorenyloxycarbonyl-DL-1-naphthylalanine

The DL-1-naphthylalanine (0.43 g, 2 mmol) was suspended in the mixture of water–acetonitrile (1 : 1, 6 ml) and Fmoc-ONSu (0.8 g, 2.4 mmol) and ethyldiisopropylamine (0.34 ml, 2 mmol) was added. After stirring for 3 h at room temperature acetonitrile was evaporated, the residue was acidified with 1 M HCl and the product was extracted into ethyl acetate. Extract was dried with sodium sulfate, filtered and evaporated. The residue was crystallized from ethyl acetate. The crystals were filtered and washed with light petroleum. Yield 0.74 g (85%), m.p. 295–295.5 °C. HPLC: *k* 4.45, (methanol–0.05% trifluoroacetic acid 7 : 3). For $C_{28}H_{23}NO_4$ (437.2) calculated: 76.87% C, 5.3% H, 3.2% N; found: 76.61% C, 5.32% H, 3.19% N. FAB MS (*m/z*): 438.2 ($M + H^+$).

Fmoc-Oxytocin Nonapeptide Resins

p-Methylbenzhydrylamine resin (Peptides International; 0.92 mmol NH_2/g, 1.0 g) was suspended in dichloromethane and after washing with 5% diisopropylethylamine in dichloromethane and with dimethylformamide Fmoc-Gly-OH (0.82 g, 2.8 mmol) was coupled in the presence of *N*-hydroxybenzotriazole (0.38 g, 2.8 mmol) and diisopropylcarbodiimide (2.8 mmol, 2.8 ml of 1 M solution in DMFA) in dimethylformamide. Coupling was interrupted after 2 h and the resin substitution (0.56 mmol/g) was determined by measuring the optical density (301 nm) of the liberated *N*-(9-fluorenyl-

methyl)piperidine[21,22]. The polymer was then acetylated (5 ml acetanhydride, 2 ml triethylamine in 50 ml dichloromethane). The free amino groups disappeared during 2 h (according to the ninhydrin test). Then the amino acids were coupled using cycle described above. Protected amino acids were used in the following order: Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Cys(pMeBzl)-OH, Fmoc-Asn-OH, Fmoc-Gln-OH, Fmoc-Ile-OH. After this step the resin was divided into three parts (2 : 1 : 1). The parts were then coupled according to the general scheme with either Fmoc-DL-1-Nal-OH (two portions) or Fmoc-L-2-Nal-OH (one portion) or Fmoc-D-2-Nal-OH (one portion) and finally with Fmoc-Cys(pMeBzl)-OH.

Fmoc-Vasopressin Nonapeptide Resins

The synthesis of Fmoc-Gly-resin was the same as described above. Protected derivatives were used in the following order: Fmoc-Har(NO_2)-OH, Fmoc-Pro-OH, Fmoc-Cys(pMeBzl)-OH, Fmoc-Asn-OH, Fmoc-Gln-OH, Fmoc-Phe-OH. After this step the resin was divided into three parts (2 : 1 : 1). The parts were then coupled according to the general scheme with either Fmoc-DL-1-Nal-OH (two portions) or Fmoc-L-2-Nal-OH (one portion) or Fmoc-D-2-Nal-OH (one portion) and finally with Fmoc-Cys(pMeBzl)-OH.

Cleavage of the Peptides from the Resins

After removal of the Fmoc-protecting group, the nonapeptide resin was treated with liquid hydrogen fluoride (5 ml, 60 min, 0 °C) in the presence of anisole (0.5 ml). After the evaporation of hydrogen fluoride, the nonapeptide together with the resin was triturated with ethyl acetate, filtered, and washed with ethyl acetate. The free peptide was extracted successively with acetic acid, 50% acetic acid, and water, and then lyophilized.

Oxidation and Purification of Analogs *I–VIII*

The linear peptide was dissolved in water (150 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 maintained 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 (8 ml), which was washed with 0.25% acetic acid and the product eluted with 50% acetic acid (30 ml). After freeze-drying, the crude product was purified by HPLC in the gradient running from 40% to 60% methanol in 0.05% trifluoroacetic acid for 60 min (analogs *I* and *II*) or from 40% to 60% methanol in 0.05% trifluoroacetic acid for 40 min (analogs *III*, *IV*, *VII* and *VIII*) or from 30% to 65% methanol in 0.05% trifluoroacetic acid for 70 min (analogs *V* and *VI*) and the eluates were lyophilized.

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[23] in Munsick[24] solution. Inhibitory activity was characterized by the pA_2 value[25]. Pressor activity was tested on pithed rat according to refs[26,27].

RESULTS AND DISCUSSION

Eight analogs of oxytocin and vasopressin were synthesized. The values of k, R_F, electrophoretic mobility and FAB MS are given in Table I and the results of amino acid analyses in Table II. The syntheses of analogs *I*, *II*, *V* and *VI* were performed using

racemic amino acids (refs[14,28,29]), in our case DL-1-naphthylalanine and the peptides containing the appropriate diastereoisomers were separated by reversed phase chroma-

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

Compound	k	R_F		$E^{Gly}_{2.4}/E^{His}_{5.7}$	FAB MS[a] m/z
		S1	S4		
I	1.41[b]	0.26	0.69	0.62/0.19	1 041.6
II	3.53[b]	0.22	0.69	0.62/0.16	1 041.7
III	2.44[c]	0.25	0.69	0.60/0.21	1 041.5
IV	5.82[c]	0.21	0.70	0.59/0.13	1 041.3
V	4.56[d]	0.02	0.45	1.07/0.67	1 132.5
VI	5.88[d]	0.02	0.44	1.10/0.65	1 132.5
VII	0.74[c]	0.02	0.50	1.10/0.70	1 132.2
VIII	1.53[c]	0.02	0.49	1.09/0.65	1 132.2

[a] $M + H^+$. [b] Methanol–0.05% trifluoroacetic acid (5.5 : 4.5). [c] Methanol–0.05% trifluoroacetic acid (5 : 5). [d] Methanol–0.05% trifluoroacetic acid (4 : 5).

TABLE II
Amino acid analysis of analogs *I–VIII*

Amino acid	*I*	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*
Asp	1.02	1.00	0.96	1.03	1.05	1.04	1.01	0.96
Glu	1.01	1.01	0.97	1.00	1.00	1.00	0.97	1.00
Pro	0.99	1.01	1.07	1.02	1.00	0.95	0.96	0.92
Gly	1.04	1.09	0.97	1.01	1.07	1.07	1.03	1.05
Cys	1.70	1.69	1.22	1.28	1.13	1.30	1.60	1.65
Ile	0.97	0.92	1.06	1.00	–	–	–	–
Leu	1.02	0.99	0.97	1.01	–	–	–	–
Phe	–	–	–	–	0.86	0.90	0.99	1.15
Har	–	–	–	–	0.94	0.93	1.07	1.08
1-Nal	0.93	0.87	–	–	0.78	0.88	–	–
2-Nal	–	–	0.93	0.91	–	–	1.03	1.07

tography at the end of the preparation. Formation of both diastereoisomers was accomplished using only 1.1 equivalents of protected racemic amino acid (higher amount of the protected racemic amino acid resulted in preferential formation of the L-diastereoisomer) with a good yield. The value of *k* in HPLC on reverse phase was in both cases lower for the L-diastereoisomer, which is consistent with the previous findings[14,28,29].

TABLE III
Biological activities (rat) of oxytocin and vasopressin analogs (I.U./mg or pA_2) with modifications in position 2

Compound		Activity			Reference
		uterotonic		pressor	
		in vitro	in vivo		
OXT		450	–	5	30
[D-Tyr^2]OXT		6.6(inhibition)	–	0.01	31
[Trp^2]OXT		0.24	–	~0.1	32,33
		0.08	–	0.08	34
[D-Trp^2]OXT		pA_2 = 6.9	–	0	32,33
[1-Nal^2]OXT	(*I*)	pA_2 = 7.4	pA_2 = 6.6	0	[a]
[D-1-Nal^2]OXT	(*II*)	pA_2 = 8.0	pA_2 = 6.7	0	[a]
[2-Nal^2]OXT	(*III*)	pA_2 = 8.0	pA_2 = 6.6	pA_2 = 6.7	[a]
[D-2-Nal^2]OXT	(*IV*)	pA_2 = 8.3	pA_2 = 7.4	pA_2 = 6.2	[a]
AVP		17		412	30
[D-Arg^8]VP		0.4		4.1	35
[D-Har^8]VP		0.9		0.83	9,36
[D-Tyr^2]AVP		1.53		194	37
		0.01		39.5	38
[1-Nal^2,D-Har^8]VP	(*V*)	pA_2 = 6.6	0	0	[a]
[D-1-Nal^2,D-Har^8]VP	(*VI*)	pA_2 = 6.6	0	0	[a]
[D-1-Nal^2]AVP		antagonist	–	potent antagonist	6
[2-Nal^2,D-Har^8]VP	(*VII*)	pA_2 = 8.0	pA_2 = 6.4	pA_2 = 6.5	[a]
[D-2-Nal^2,D-Har^8]VP	(*VIII*)	pA_2 = 8.1	pA_2 = 7.5	pA_2 = 7.4	[a]
[D-2-Nal^2]AVP		pA_2 = 8.0	–	pA_2 = 8.45	6

[a] This paper; 0 means inactive up to the dose 2 . 10^{-2} mg/rat.

Biological activities of the analogs are given in Table III. All analogs were found to be uterotonic inhibitors in vitro and in vivo. Analogs with 2-naphthylalanine are stronger inhibitors, particularly in vasopressin series than analogs with 1-naphthylalanine. The pA_2 values in vitro rank them with the strongest uterotonic inhibitors. Particularly surprising is the high antagonistic activity (uterus in vitro) of analogs *III* and *VII*. These analogs having L-2-naphthylalanine in position 2 are the strongest uterotonic inhibitors having L-amino acid in this position in both the oxytocin and vasopressin series. In comparison to the isosteric amino acid tryptophan, the analog having L-1-naphthylalanine does not posses any agonistic activity contrary to L-tryptophan analog, the D-1-naphthylalanine has one order of magnitude higher inhibitory potency than the D-tryptophan analog. The in vivo anti oxytocic activity is substantially lower, however still high especially in the case of D-2-analogs in both the oxytocin and [D-Har^8]vasopressin.

Analogs with 1-naphthylalanine have no activity in the pressor test, analogs with 2-naphthylalanine are weak pressor inhibitors.

In comparison to analogs having the same amino acid in position 2, we can say that the D-Har substitution in position 8 is expressed in higher selectivity, i.e. the inhibitory potency in the pressor test is reduced.

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