Journal of Peptide Science
J. Peptide Sci. **11**: 91–96 (2005)
Published online 15 September 2004 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.600

JOURNAL OF PEPTIDE SCIENCE

Analogues of arginine vasopressin modified in position 2 and 3 with conformationally constrained dipeptide fragments

ELŻBIETA ŁEMPICKA,[a] IZABELA DERDOWSKA,[a] WIOLETA KOWALCZYK,[a] OLGA DAWIDOWSKA,[a]* ADAM PRAHL,[a] MARCIN JANECKI,[b] TOMASZ JASIŃSKI,[c] HENRYK I. TRZECIAK[c] and BERNARD LAMMEK[a]

[a] Department of Chemistry, University of Gdańsk, Sobieskiego 18, 80-952 Gdańsk, Poland
[b] Department of Palliative Medicine and Palliative Care, Silesian Medical University, Medyków 14, 40-752 Katowice, Poland
[c] Department of Pharmacology, Silesian Medical University, Jagiellońska 4, 41-200 Sosnowiec Poland

Received 17 April 2004; *Revised 7 May 2004*; *Accepted 19 May 2004*

Abstract: This study describes the synthesis and some pharmacological properties of ten new analogues of arginine vasopressin (AVP) containing a conformationally constrained dipeptide fragment in the *N*-terminal part of their molecules. Amino acid residues in positions 2 and 3 of AVP and some of its agonistic analogues were replaced with ┌Phe┐Phe and D┌Phe┐D-Phe, dipeptides having a $-CH_2-CH_2-$ link bridging two nitrogens. All the new peptides were tested for vasopressor and antidiuretic activities. Four peptides with pA_2 values ranging from 5.96 to 7.21 turned out to be weak or moderately potent V_{1a} antagonists. The results supplied new information about the structure — activity relationship of AVP analogues. As some of these were unexpected, they point to the need for caution when extrapolating previously known effects of modifications to analogues having conformationally constrained fragments in their molecules.

Keywords: analogues of arginine vasopressin (AVP); antivasopressor activity; conformational constraints

INTRODUCTION

The neurohypophysis is the posterior lobe of the pituitary. It secretes oxytocin (OT) and arginine vasopressin (AVP), two cyclic nonapeptides of similar structure that are primarily synthesized in the hypothalamus. Vasopressin, also known as antidiuretic hormone, stimulates the kidneys to retain water and increases blood pressure. Vasopressin exerts its action through at least three types of cell surface receptor (V_{1a}, V_{1b} and V_2). V_{1a} receptors have been shown to be present in many tissues, including the brain [1]. V_{1b} receptors mediate the ACTH-releasing effects of AVP from the anterior pituitary [2]. Both receptor subtypes function via the phosphoinositide pathway. V_2 receptors, present in the renal tubule and collecting duct, modulate the antidiuretic response to AVP, they are linked to adenylate cyclase activity [2].

Many vasopressin agonists and antagonists have been used as pharmacological and therapeutic tools in animal and human physiology and pathophysiology [3, 4]. However, the design and structure — activity studies of analogues that are truly selective for AVP receptors is still a field of great interest.

Biologically active peptides exhibit multiple conformations in solution. Thus, the synthesis of conformationally restricted analogues is a valuable approach for determining structure — activity relationships. Restrictions can be imposed by the formation of cyclic structures within the peptide framework by disulphide and lactam bridges, or by substitution of chosen amino acid residues with sterically restricted fragments that limit conformational freedom, forcing the peptide backbone and/or side chains to adopt specific orientations. Another approach for achieving certain stabilization of the structure is the preparation of various types of pseudopeptides through short-range cyclization. One such constraint consists of the $-CH_2-CH_2-$ link bridging two consecutive peptide nitrogens leading to the formation of a piperazinone ring that incorporates the relevant $-N-C-C'-N'$ peptide segment as its inherent part [5]. Several constrained enkephalinamides and antagonists of B_2 receptors of bradykinin have been prepared using the above-mentioned approach [6–8].

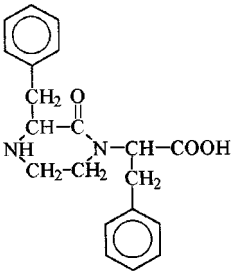
In 1998 this unit was used to replace the amino acid residues occupying positions 2 and 3 of AVP, and some of its agonistic and antagonistic analogues [9]. Two peptides were highly potent V_{1a} antagonists. One of them, namely [Cpa[1], ┌Phe┐

Abbreviations: Symbols used for the amino acids and peptides are in accordance with the 1983 Recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature and 'A Revised Guide to Abbreviations in Peptide Science' published in *J. Pept. Sci.* 2003; **9**: 1–8. Other abbreviations: AVP, arginine vasopressin; Cpa, 1-mercaptocyclohexaneacetic acid; Mpa, 3-mercaptopropionic acid.

*Correspondence to: Olga Dawidowska, Department of Chemistry, University of Gdańsk, Sobieskiego 18, 80-952 Gdańsk, Poland;
e-mail: olad@chem.univ.gda.pl

Phe^{2,3},Val^{4}]AVP, which appears not to interact with either V_2 and oxytocic receptors, was exceptionally selective. In this context, it is interesting to note that the modification discussed does not change dramatically the chemical character of the designed analogues.

These interesting results prompted us to continue our efforts to gain an insight into the role of the shape of the *N*-terminal part of AVP analogues for their pharmacological activity. It was decided to replace the amino acid residues in positions 2 and 3 of AVP and some of its agonistic analogues with ⌜Phe⌝Phe and D⌜Phe⌝D-Phe. The structures of the dipeptide unit and analogues **I–X** are as follows:



⌜Phe⌝Phe or D⌜Phe⌝D-Phe

X-⌜Phe⌝Phe-Y-Asn-Cys-Pro-Z-Gly-NH_2

where:	X = Cys	Y = Val	Z = Arg	**(I)**
	X = Mpa	Y = Val	Z = Arg	**(II)**
	X = Cys	Y = Gln	Z = D-Arg	**(III)**
	X = Mpa	Y = Gln	Z = D-Arg	**(IV)**
	X = Cys	Y = Val	Z = D-Arg	**(V)**
	X = Mpa	Y = Val	Z = D-Arg	**(VI)**

X-D-⌜Phe⌝D-Phe-Y-Asn-Cys-Pro-Z-Gly-NH_2

where:	X = Cys	Y = Gln	Z = Arg	**(VII)**
	X = Mpa	Y = Gln	Z = Arg	**(VIII)**
	X = Cys	Y = Val	Z = Arg	**(IX)**
	X = Mpa	Y = Val	Z = Arg	**(X)**

EXPERIMENTAL

General

All solvents were purified by conventional methods. Evaporations were carried out under reduced pressure. Thin layer chromatography (TLC) was carried out on silica plates (Merck), the spots being visualized by iodine, ninhydrin or chlorine followed by starch/KJ. The solvent system, butan-1-ol/acetic acid/water (4 : 1 : 5, v/v, upper phase) was used. High-performance liquid chromatography (HPLC) for peptides was carried out on a Gold System Beckman (analytical) and Waters chromatograph (preparative) equipped with a UV detector. The purity of the peptides was determined on a Vydac C_{18} column (5 μm, 4.6 × 250 mm) with precolumn Ultrasphere ODS (5 μm, 4.6 × 45 mm). The following solvent systems were used: (1) 0.1% aqueous trifluoroacetic acid (TFA), (2) acetonitrile — 0.1% TFA (80 : 20 v/v); a linear gradient of 20% to 80% of (2) for 25 min (from 0 to 100% of (2) for 40 min for peptide **VII**) at a flow rate of 1 ml/min (λ = 226 nm). Preparative HPLC was carried out using a Chromasil C_8 column (5 μm, 25 × 250 mm), flow rate 10 ml/min. FAB/MS of peptides were recorded on a TRIO-3 mass spectrometer at 7 keV with argon as the bombarding gas.

All amino acid derivatives were purchased from Bachem AG. S-p-methoxybenzyl-3-mercaptopropionic acid (Mpa(Mob)) was synthesized using procedures described in the literature [10].

N-t-Butyloxycarbonyl-[(2R,3′R)-3-phenyl-2-(1′-piperazine-3′-benzyl-2′on)] propionic acid and its (2S, 3′S) enantiomer were synthesized as described [9, 11].

Peptide Synthesis

All peptides were synthesized manually by the solid phase method, i.e. by the stepwise coupling of Boc-amino acids to the growing peptide chain on a 4-methylbenzhydrylamine resin (MBHA resin, Senn Chemicals AG, 1% DVB, 200–400 mesh, 0.67 mmol/g). Fully protected peptide resins were synthesized according to standard procedures involving: (i) deprotection steps using 33% TFA in the presence of anisole (1%), 5 and 25 min; (ii) neutralization with 10% TEA/DCM, 3 and 7 min; (iii) couplings in DCM/DMF (1 : 1, v/v) carried out using DCC and HOBT. The couplings of Boc-Cys(Mob) and Mpa(Mob) were mediated by HATU and HOAt in the presence of DIEA, in a mixture of DMF, NMP and DCM (1 : 1 : 1 v/v) containing 1% triton. The completeness of each coupling reaction was monitored by the Kaiser [12] or chloranil test [13]. Re-coupling was performed when the test was positive. After completion of the synthesis, the protected nonapeptidyl resins (2 mmol) were treated with 10 ml of liquid hydrogen fluoride (HF) containing 0.5 ml of anisole at −70 °C and stirred for 60 min at 0 °C [14]. After the removal of HF and anisole *in vacuo*, the mixture was washed with anhydrous diethyl ether, then with acetic acid and the solution was diluted with methanol. The resulting dithiols were oxidatively cyclized with 0.1 M I_2 in methanol using the normal procedure [9]. The solvents were evaporated under reduced pressure and the resulting materials dissolved in water and lyophilized. The crude products were desalted on a Sephadex G-15 column, eluted with aqueous acetic acid (50%) at a flow rate of 3.0 ml/h. The eluates were fractionated, and the fractions containing the major peak were pooled and lyophilized. The residue was then subjected to gel filtration on a Sephadex LH-20 column eluted with 30% aqueous acetic acid at a flow rate of 2 ml/h. The peptides were eluted as single peaks. The purity and identity of each peptide was determined by HPLC and FAB mass spectrometry (molecular ion). The values of the molecular ions were as expected. The physicochemical properties of peptides **I–X** are presented in Table 1.

Bioassay Methods

The vasopressor and antidiuretic activity of AVP analogues were tested on male Wistar rats, 250–270 g, bred at the Central Experimental Animal Farm of the Silesian Medical University.

Table 1 Physicochemical Properties of Peptides **I–X**

Peptide		R_F	HPLC t_r	Formula	M^+ calculated	$[M + H^+]$
[Phe-Phe^{2,3},Val^4]AVP	**I**	0.21	17.2[a]	$C_{48}H_{68}N_{14}O_{10}S_2$	1064.5	1065.5
[Mpa^1, Phe-Phe^{2,3},Val^4]AVP	**II**	0.20	16.8	$C_{48}H_{67}N_{13}O_{10}S_2$	1049.5	1050.3
[Phe-Phe^{2,3},D-Arg^8]VP	**III**	0.21	10.8	$C_{48}H_{67}N_{15}O_{11}S_2$	1093.5	1094.5
[Mpa^1, Phe-Phe^{2,3},D-Arg^8]VP	**IV**	0.16	18.4[a]	$C_{48}H_{66}N_{14}O_{11}S_2$	1078.5	1079.5
[Phe-Phe^{2,3},Val^4,D-Arg^8]VP	**V**	0.22	13.4	$C_{48}H_{68}N_{14}O_{10}S_2$	1064.5	1065.8
[Mpa^1 Phe-Phe^{2,3},Val^4,D-Arg^8]VP	**VI**	0.23	20.6	$C_{48}H_{67}N_{13}O_{10}S_2$	1049.5	1050.6
[D Phe-D-Phe^{2,3}]AVP	**VII**	0.10	18.2	$C_{48}H_{67}N_{15}O_{11}S_2$	1093.5	1094.5
[Mpa^1,D Phe-D-Phe^{2,3}]AVP	**VIII**	0.13	13.6	$C_{48}H_{66}N_{14}O_{11}S_2$	1078.5	1079.1
[D Phe-D-Phe^{2,3},Val^4]VP	**IX**	0.18	13.5	$C_{48}H_{68}N_{14}O_{10}S_2$	1064.5	1065.3
[Mpa^1, D Phe-D-Phe^{2,3},Val^4]VP	**X**	0.25	16.2	$C_{48}H_{67}N_{13}O_{10}S_2$	1049.5	1050.5

[a] Vydac C_{18} column (5 μm, 10 × 250 mm), flow rate 2 cm^3/min.

The use of animals was reviewed and approved by the Local Ethics Committee for Animals Experiments in Katowice (Poland).

Vasopressor Activity

Vasopressor assays of AVP analogues were performed *in vivo* according to the method of Dekanski [15] as described by Stürmer [16]. The rats were given 1.75 g/kg urethane intraperitoneally (i.p.). Blood pressure stabilization was evoked by phenoxybenzamine (SmithKline Beecham, USA) in two or three repeated doses, each amounting to 1 mg/kg intravenously (i.v.). Peptides were injected into the cannulated jugular vein in a volume of 0.1 ml (saline) and the cannula was rinsed with 0.2 ml saline. Analogues were injected at 30–40 min intervals. Systolic blood pressure measurements were taken by a pressure transducer (type PL3JD, Gould-Statham, USA) fixed into the carotid artery.

Antidiuretic Activity

The antidiuretic activity of the peptides was estimated in rats *in vivo* according to Stürmer's modification [16] of Sawyer's method [17]. Anaesthesia, however, was induced by injecting 150 mg/kg of thiobutabarbital (Inactin, RBI, USA) i.p. [18,19]. Catheters were inserted into the jugular vein for peptide infusion. After laparotomy, a polyethylene (PE) cannula was introduced into the urinary bladder and ligated. The urine flow was collected direct from the urinary bladder until urine flow rate stabilization (0.8–1.2 ml/10 min).

From the beginning of the experiment the animals were connected by PE catheter to a syringe infusion pump (Medipan, type 612, Poland). Water balance was kept constant for each rat by rehydrating with a hypoosmotic water solution (180 mOsm/kg) containing 0.24% NaCl and 1.8% glucose, adding arbitrary excess (1–2 ml/h) to compensate for extrarenal water loss.

Peptides were injected into the jugular vein as a bolus in a total volume of ~0.3 ml (including wash) after achieving urine flow stabilization in three consecutive 10 min periods at 40 min intervals. Anaesthesia was maintained throughout the experiment.

Agonistic activity was expressed in international units per milligram (IU/mg) of peptide by the comparison of regression lines for AVP and related peptides [20]. The antidiuretic response was defined as $V_p/V_o \times 100$, where V_o is the urine volume collected in the 10 min period preceding the injection of AVP or analogue; V_p is the volume of urine collected during the 10 min starting period immediately after injection.

The antagonistic activity of AVP analogues was measured by the method of Schild [19] and described as the effective dose (ED in nmol/kg) and pA_2 value.

Each peptide was administered in two doses: high, which reduced the response to 2× units of agonist to less than the response to 1× unit of agonist; and low, which did not fully reduce the response obtained by 2× units of agonist. pA_2 values estimated *in vivo* represent the negative logarithms (to base 10) of effective doses divided by an assumed volume of distribution (V_d) in rats (67 ml/kg) according to the formula:

$$pA_2 = -\log ED/V_d$$

Antivasopressor activity was estimated after injection of AVP analogues (10^{-4}–10 IU/kg i.v.) 1 min before AVP administration (1×10^{-2} IU/kg i.v.).

AVP was used as a standard agonist in all assays. Its pressor and antidiuretic activities were accepted as 369 IU/mg and 323 IU/mg, respectively [21].

Urinary Electrolytes Analysis

During the antidiuretic experiments the urine was collected in consecutive 10 min periods before and after peptide

administration. The urinary sodium, potassium, calcium and chloride concentrations of all the samples were determined using a Synchrom EL-ISE Electrolite System (model 4410, Beckman, Instruments Inc., USA). The electrolyte excretion (mEq/10 min) was expressed as the percent after vs before the peptide administration.

Statistical significance of differences of baseline values between experimental groups was determined by unpaired two-tailed *t*-test. A $p < 0.05$ value was considered significant.

RESULTS AND DISCUSSION

The ten new analogues of AVP (**I**–**X**) were synthesized using standard solid-phase Boc methodology. For peptides **II, IV, VI, VIII** and **X** Mpa(Mob) was used in the final coupling step. On completion of the syntheses, the peptides or acylpeptides were split from the resin with simultaneous deprotection by treatment with liquid HF in the presence of anisole at 0 °C and oxidized with I_2 in methanol. The crude peptides were purified on Sephadex G-15 and LH-20. Some of the pharmacological properties of the new analogues **I**–**X** and those of AVP, [Mpa^1]AVP and previously obtained in our laboratory, [Mpa^1, [Phe-Phe]^{2,3}]AVP are presented in Table 2. None of the new analogues exhibited pressor or *anti-antidiuretic* activity.

With regard to the antidiuretic properties of the new analogues, it is clear that the modifications resulted in an outstanding decrease or removal of interaction of peptides with V_2 receptors, as compounds **I**–**VI** showed only negligible antidiuretic potency, whereas peptides **VII**–**X** were not active.

None of the analogues of AVP tested changed the urinary excretion of Na^+, K^+, Ca^{2+} and Cl^- except for peptide **VII**, which significantly decreased sodium excretion ($p < 0.05$) (data not shown).

Passing on to the antivasopressor activity, analogues **II**–**IV** and **VI** with pA_2 values ranging from 5.96 to 7.21 turned out to be weak or moderately potent blockers of V_{1a} receptors, while the remaining new compounds were inactive.

Previously, it was reported that the introduction of the [Phe-Phe] fragment into the AVP molecule resulted in a loss of all the activities checked, although the same change when applied to [Mpa^1]AVP gave a weak pressor antagonist. However, spectacular results were obtained by placing this unit in positions 2 and 3 of two V_{1a}

Table 2 Pharmacological Data on Vasopressin Analogues[a]

Peptide		Vasopressor (IU/mg)	Antivasopressor (pA_2)	Antidiuretic (IU/mg)
AVP[b]		369 ± 6	—	323 ± 16
[Mpa^1]AVP[b]		346	—	1745 ± 385
[Mpa^1, [Phe-Phe]^{2,3}]AVP[c]		—	5.8 ± 0.2 (6)	0.105 ± 0.07 (7)
[[Phe-Phe]^{2,3}]AVP[c]		—	ND	0.085 ± 0.027 (8)
[Cpa^1, [Phe-Phe]^{2,3}]AVP[c]		—	8.9 ± 0.03 (9)	7.6 ± 2.9 (8)
[[Phe-Phe]^{2,3},Val^4]AVP	**I**	ND (4)	ND (4)	<0.001 (8)
[Mpa^1, [Phe-Phe]^{2,3},Val^4]AVP	**II**	ND (4)	7.21 ± 0.78 (8)	<0.001 (8)
[[Phe-Phe]^{2,3},D-Arg^8]VP	**III**	ND (4)	6.23 ± 0.23 (8)	<0.001 (8)
[Mpa^1, [Phe-Phe]^{2,3},D-Arg^8]VP	**IV**	ND (6)	6.67 ± 0.29 (8)	<0.001 (8)
[[Phe-Phe]^{2,3},Val^4,D-Arg^8]VP	**V**	ND (5)	ND (6)	<0.001 (8)
[Mpa^1, [Phe-Phe]^{2,3},Val^4,D-Arg^8]VP	**VI**	ND (4)	5.96 ± 0.15 (8)	<0.001 (8)
[D [Phe-D-Phe]^{2,3}]AVP	**VII**	ND (4)	ND (4)	ND (5)
[Mpa^1, D [Phe-D-Phe]^{2,3}]AVP	**VIII**	ND (4)	ND (7)	ND (6)
[D [Phe-D-Phe]^{2,3},Val^4]VP	**IX**	ND (4)	ND (4)	ND (8)
[Mpa^1, D [Phe-D-Phe]^{2,3},Val^4]VP	**X**	ND (4)	ND (7)	ND (7)

[a] Results are expressed as mean ± SEM. Values in parentheses indicate the numbers of rats tested with each compound.
[b] Values taken from [10].
[c] Values taken from [9].
ND, not detectable to a dose of 10 IU/kg.

antagonists, namely [Cpa^1]AVP and [Cpa^1,Val^4]AVP, as a significant increase of anti-V_1 potency and selectivity was observed. These observations prompted the further exploration of the impact of the shape of the *N*-terminus of vasopressin analogues on their pharmacological properties. The results of the present work are in good conformity with our previous findings concerning the influence of ⌜Phe⌝Phe on the activity of the analogues, as in this study the substitution at position 2 and 3 also resulted in the case of four peptides with antipressor properties.

The results showed that a combination of ⌜Phe⌝Phe^{2,3} modification together with substitution at position 4 with Val in the AVP molecule, resulted in the practically inactive peptide **I**. However, passing on to peptide **II**, which may be treated as a derivative of **I**, it was observed that deamination resulted in moderate antipressor potency. It is an interesting finding, as such a measure is known to enhance antidiuretic activity [22]. On the other hand, peptide **II** may be considered a derivative of [Mpa^1, ⌜Phe⌝Phe^{2,3}]AVP, a weak V_{1a}-blocker previously obtained by us. From this point of view, the substitution at position 4 of this peptide with valine, resulted in more than a 10-fold enhancement of antipressor potency. This was an unexpected effect, as Val^4 in AVP and its analogues in many cases improves antidiuretic potency and/or selectivity [22]. It is also worth pointing out that antagonist **II** was designed without 1-mercaptocyclohexaneacetic acid substitution in position 1, which was long thought to be essential for the substantial pressor antagonism of cyclic analogues of AVP [3, 22]. With regard to compound **III**, it was designed by inversion of the configuration of Arg^8 in [⌜Phe⌝Phe^{2,3}]AVP, previously obtained in our laboratory [9]. It converted this inactive peptide into a weak V_{1a}-blocker. Again, it is a surprising result as the effect of D-Arg substitution in the analogues described in the literature resulted in a decrease of the pressor activity making the analogues very selective [3].

Going one step further it was decided to synthesize analogue **IV**, by combining both the above-mentioned modifications with the deamination of position 1. This measure gave an approximately 4-fold enhancement of anti-V_{1a} activity.

Finally, the lack of activity of the next peptide **V**, designed by modification of positions 2 and 3 with ⌜Phe⌝ Phe unit, combined with Val^4 substitution and conversion of configuration of Arg^8, and the very low antipressor potency of **VI**, which contained all four modifications used in this study, points to the need for caution when extrapolating previously known effects to analogues that have conformationally constrained fragments in their molecules. One can only assume that analogue — receptor interaction, that occurred with numerous peptides, may be disturbed owing to the more rigid structure of the *N*-terminal part of the molecule.

As far as the peptides designed by D⌜Phe⌝D-Phe^{2,3} substitution are concerned, this modification alone, or in combination with other changes, resulted in the complete removal of all activities determined.

In summary, these results supplied new information about the structure–activity relationships in the design of V_{1a} antagonists.

Acknowledgements

Partial funding for this work was provided by the Polish State Committee for Scientific Research in the form of grant no. 6 PO5F 010 21.

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