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Analogues of Arginine Vasopressin (AVP) Modified in the N-Terminal Part of the Molecule with N-Benzylglycine*

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The synthesis and some pharmacological properties of five new analogues of arginine vasopressin (AVP) substituted with N-benzylglycine are described. All new peptides were tested for pressor and uterotonic activity. The results obtained imply that the structural change studied is in general incompatible with interaction of the analogues with V_{1A} and OT receptors, however, in combination with suitable additional changes, may be of value in the design of new antagonists of these receptors.

Key words: arginine vasopressin (AVP), N-benzylglycine, V_{1A} antagonists

The characterization of arginine vasopressin (AVP) in the early 1950s stimulated not only the fields of synthetic peptide chemistry and peptide endocrinology, but also the structure-activity relationship studies of this hormone, and particularly the search for analogues with more specific activity [1]. In the beginning of this research, modifications of the vasopressin molecule yielded a variety of peptides with higher and selective antidiuretic or vasoconstriction activities [2]. Later, the successful synthesis of potent antagonists of V_{1A} receptors greatly stimulated research on the role of vasopressin in cardiovascular regulation [3,4]. As the next step, Manning and coworkers synthesized first antagonists of the antidiuretic activity [5]. Investigations continued in many laboratories resulted in progress which allowed to improve the potency and selectivity of designed analogues and we are beginning to obtain a fairly good picture of structural factors, which afford the required type of biological activ-

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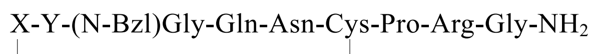
*Abbreviations: The three-letter symbols of amino acid are in accordance with the 1983 Recommendation of the IUPAC-IUB Joint Commission on Biochemical Nomenclature [*Eur. J. Biochem.*, **138**, 9–37 (1984)]. Other abbreviations used are: AVP, arginine vasopressin; Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; Cpa, 1-mercaptocyclohexaneacetic acid; dAVP, deamino arginine vasopressin; DCM, dichloromethane; DIEA, diisopropylethylamine; DMF, dimethylformamide; HATU, O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOAt, 1-hydroxy-7-azabenzotriazole; HOBt, 1-hydroxybenzotriazole; MBHA, *p*-methylbenzhydrylamine; Mob, 4-methoxybenzyl; Mpa, 3-mercaptopropionic acid; NMP, 1-methyl-2-pyrrolidone; OT, oxytocin; TBTU, 2-1H(benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate.

ity. However, the preparation of compounds that are very active, truly selective and useful as drugs, is still a challenge.

It is believed that L-phenylalanine residue in position 3 of AVP is mainly involved in recognition of the hormone and its binding to the receptors [6]. So far, most of the analogues modified in this position still contained an aromatic amino acid residue [2,7]. Continuing this direction we synthesized and pharmacologically evaluated analogues having L-1- or L-2-naphthylalanine in position 3 [8]. One of the peptides, [L-2-Nal^3,D-Arg^8]VP, was among the most potent antagonists of the pressor response to AVP. This analogue designed without any modification in position 1, which was previously thought to be essential for substantial pressor antagonism, is the first potent V_{1A} antagonist devoid of antiuterotonic activity. Recently, we also described the synthesis and some pharmacological properties of AVP analogues containing ethylene-bridged dipeptide Phe-Phe in positions 2 and 3. One of these, [Cpa^1,(Phe-Phe)^{2,3},Val^4]AVP, is a very potent V_{1A} antagonist, which does not interact with either V_2 or oxytocic receptors [9].

As a continuation of our efforts to elucidate the role of position 3 in AVP and its analogues, we designed and synthesized three new peptides modified in this position with N-benzylglycine [(N-Bzl)Gly]. An additional stimulus to our studies came from our earlier results describing that AVP substituted in positions 2 and 3 with N-methyl-L-phenylalanine was converted into a potent antagonist [9]. The modification proposed in the present study may be considered, both as an introduction of peptoid unit, analogue of phenylalanine, in which the aromatic side chain is moved from C^α to nitrogen, *i.e.* nearer to the aromatic chain of tyrosine, or as N-alkylation of the peptide bond different from methylation. These observations and knowledge that even minor changes in the structure of N-terminal part of AVP analogues are important for their activity [8] prompted us to synthesize [(N-Bzl-Gly)^3]AVP and two peptides, in which we combined the above modification with Mpa (3-mercaptopropionic acid) or Cpa (β-mercapto-β,β-cyclopentamethylene propionic acid, 1-mercaptocyclohexane-acetic acid) substitutions. We also decided to check how substitution of both Tyr^2 and Phe^3 in AVP and [Cpa^1]AVP molecules will influence the activity of these peptides. The synhesized analogues have the following general structures:

X-Y-(N-Bzl)Gly-Gln-Asn-Cys-Pro-Arg-Gly-NH_2



where: X = Cys, Y = Tyr **I**; X = Mpa, Y = Tyr **II**; X = Cpa, Y = Tyr **III**;
X = Cys, Y = (N-Bzl)Gly **IV**; X = Cpa, Y = (N-Bzl)Gly **V**.

EXPERIMENTAL

General: All solvents were purified by conventional methods. Evaporations were carried out under reduced pressure. Melting points were determined on a capillary melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded at 250 MHz on a Bruker Avance model DPX 250. Splitting patterns are abbreviated as follows: (s) singlet, (d) doublet, (t) triplet, (q) quartet, (m) multiplet. The optical rota-

tions were measured in a 1 dcm cell (1 ml) on a Horiba high speed automatic polarimeter at 589 nm (NaD line). Thin layer chromatography (TLC) was carried out on silica plates (Merck), and 250 nm silica gel GF protected uniplates (Analtech), the spots being visualized by iodine, ninhydrin or chlorine followed by starch/KJ. The A, butan-1-ol-acetic/acid/water (4:1:5, v/v, upper phase) solvent system 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, 10 × 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 from 20 to 80% of (*2*) for 25 min 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. HPLC for N-benzylglycine derivatives was performed on an LDC Analytical instrument using a Vydac C_{18} (4.6 × 250 mm) column, flow rate 1.0 ml/min., detection at 220 nm, and solvents: (A) 0.05% trifluoroacetic acid in water and (B) 0.038% trifluoroacetic acid in acetonitrile/H_2O 90:10 in a gradient application. Mass spectra were determined on the APO Electron Model MI 12001E and Finningan Mat 95 spectrometers with FAB ionization technique.

(N-Bzl)Gly-OH (2). To a stirred solution of glycine (15 g, 0.2 mol) in 100 ml 2N aqueous NaOH, benzaldehyde (21.1 g, 0.2 mol) was added and stirring continued for 1.5 h. (a homogeneous mixture was formed). The solid $NaBH_4$ (2.28 g, 0.06 mol) was then added in portions at a temperature below 15°C. The reaction mixture had been stirred for 0.5 h and the second part of benzaldehyde (10.6 g, 0.1 mol) and $NaBH_4$ (1.14 g, 0.03 mol) was added. After an additional 1.5 h of stirring, it was extracted by ether (2 × 100 ml) and neutralized by 2N aqueous HCl. The crystalline precipitate of N,N-dibenzylglycine was removed by filtration and the filtrate was evaporated to the form of wet paste. Water (20 ml) was then added and crystals of N-benzyl-glycine were filtered, washed with water (2 × 10 ml) and dried. Yield 12 g (37%); m.p. 187–190°C (m.p. 196–198°C [10]); HPLC purity 95%, t_R = 12.5 min (gradient 20–60% B in 25 min). ^1H NMR (D_2O) δ: 3.61 (s, 2 H, -CH_2-Gly), 4.26 (s, 2H, -$CH_2C_6H_5$), 7.49 (s, 5H, -C_6H_5).

Boc-(N-Bzl)Gly-OH (3). To a stirred solution of N-benzylglycine **2** (5 g, 0.03 mol) in 70 ml of the mixture dioxane:water (1:1, v/v), Boc-anhydride (7.85 g, 0.036 mol) was added, followed by triethylamine (3.13 g, 0.031 mol). After 2.5 h of stirring, the solvents were removed under reduced pressure and the oily residue was extracted with 5% aqueous $NaHCO_3$ (3 × 30 ml). The water layer was washed with ethyl acetate (2 × 30 ml), acidified with solid $KHSO_4$ and extracted with ethyl acetate (3 × 50 ml). The organic layer was dried over anhydrous $MgSO_4$ and evaporated to dryness, giving 5.85 g (73%) of white crystalline product. M.p. 102–105°C; HPLC purity 98%, t_R = 10.32 min, (gradient 20–60% B in 25 min). ^1H NMR ($CDCl_3$) δ: 1.46 and 1.48 (s, 9H, Boc, two rotamers), 3.83 and 3.96 (s, 2H, CH_2-Gly, rotamers), 4.52 and 4.55 (s, 2H, CH_2-C_6H_5, rotamers), 7.24–7.37 (m, 5H, -C_6H_5).

(N-Bzl)Gly-OBzl×TsOH (4). N-Benzylglycine **2** (5 g, 0.3 mol) and *p*-toluenesulfonic acid (monohydrate, 5.7 g, 0.03 mol) were added to a mixture of freshly distilled benzyl alcohol (15 ml) and benzene (25 ml). The mixture was refluxed, while the water formed in the reaction was trapped in a Dean-Stark receiver. When no more water appeared in the distillate, solvents were removed under reduced pressure and the crude residue was crystallized from ethanol:water yielding 6.3 g (82%) of white crystalline product. M.p. 144–146°C; HPLC purity 98%, t_R = 12.53 min (gradient 20–60% B in 25 min). Without any further characterization this product has been used for coupling.

Boc-(N-Bzl)Gly-(N-Bzl)Gly-OBzl (5). To the stirred suspension of Boc-(N-Bzl)Gly-OH **3** (2.12 g, 0.008 mol), HOBt (1.08 g, 0.008 mol) and TBTU (2.56 g, 0.008 mol) in DCM (8 ml), DIPEA (2.74 ml, 0.016 mol) was added. After 20 min of stirring, the crystalline N-Bzl-Gly-OBzl x TsOH **4** (3.28 g, 0.008 mol) and DIPEA (1.37 ml, 0.008 mol) was added. The reaction mixture has been left with stirring for 70 h, diluted with ethyl acetate (50 ml) and washed with 5% aqueous $KHSO_4$ (2 × 25 ml), 5% aqueous $NaHCO_3$ (2 × 25 ml) and water (2 × 25 ml). The organic layer was dried over anhydrous $MgSO_4$ and evaporated to dryness yielding 3.4 g of crude oily product which was purified on "flash" chromatography in the solvent system DCM:MeOH = 98:2. Yield 1.5 g (37%) of pure homogeneous product as a white solid; HPLC purity 98%, t_R = 22.4 min (gradient 40–90% B in 25 min); FAB-MS $[M+H]^+$ 503 calculated for $C_{30}H_{34}N_2O_5$ 502.

Boc-(N-Bzl)Gly-(N-Bzl)Gly-OH (6). Boc-(N-Bzl)Gly-(N-Bzl)Gly-OBzl **5** (502 mg, 1 mmol) in methanol (5 ml) was hydrogenated in the presence of 10% Pd/C at a pressure of 4–5 Kg/cm^2 on a Parr apparatus for 1.5 h (monitored by TLC). After evaporation of filtered solution, it yielded 450 mg (99%) of homogeneous product as a white foam. HPLC purity 98%, t_R = 13.1 min (gradient 40–90% B in 25 min); FAB-MS $[M+H]^+$ 413, $[M+Na]^+$ 435 calculated for $C_{23}H_{28}N_2O_5$ 412. This product without any further characterization was used in the solid phase synthesis.

S-p-methoxybenzyl-1-mercaptocyclohexaneacetic acid (Cpa(Mob)) [11] and S-p-methoxybenzyl-3-mercaptopropionic acid (Mpa(Mob)) [12] were synthesized using procedures described in the literature. All amino acid derivatives were purchased from Bachem AG.

Peptide synthesis: All peptides were synthesized manually by solid phase method, *i.e.* by the stepwise coupling of Boc-amino acids to the growing peptide chain on a methoxybenzhydryl 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 (only peptide I *in situ* neutralization [13]), (iii) couplings in DCM or DCM/DMF (1:1, v/v) carried out of DIEA. The couplings of Boc-(N-Bzl)Gly, Boc-(N-Bzl)Gly^{2,3}, Boc-Tyr(2Br-Z), Boc-Cys(Mob), Cpa(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 [14] or chloranil test [15]. Re-coupling was performed when the test was positive. After completion of the synthesis, the protected nonapeptidyl or acyloctapeptidyl resins 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 [16]. After the removal of HF and anisole *in vacuo*, the mixture was washed with anhydrous diethyl ether, then with acetic acid and the solution diluted with methanol. The resulting dithiols were oxidatively cyclized with 0.1 M I_2 in methanol using the normal procedure. 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. After freeze-drying, the fractions comprising the major peak were purified by HPLC. Preparative HPLC of analogues were carried out in a gradient running from 10 to 50% (*2*) for 120 min. 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–V are presented in Table 1.

Table 1. Physicochemical properties of peptides I–V.

No.	Peptide	HPLC t_r	Formula	M^+ *calculated*	$[M + H^+]$
I	[(N-Bzl)Gly^3]AVP	7.35	$C_{46}H_{66}N_{15}O_{12}S_2$	1083.5	1085
II	[Mpa^1,(N-Bzl)Gly^3]AVP	10.63	$C_{46}H_{64}N_{14}O_{12}S_2$	1068.5	1069.6
III	[Cpa^1,(N-Bzl)Gly^3]AVP	13.63	$C_{51}H_{72}N_{14}O_{12}S_2$	1136.5	1137.7
IV	[(N-Bzl)Gly^{2,3}]AVP	11.58	$C_{46}H_{65}N_{15}O_{11}S_2$	1067.5	1068.9
V	[Cpa^1,(N-Bzl)Gly^{2,3}]AVP	17.19	$C_{51}H_{72}N_{14}O_{11}S_2$	1120.5	1121.9

Biological evaluation: The activities of the analogues were determinated in the rat uterotonic test *in vitro* in the absence of magnesium ions [17,18] and in the rat pressor test using phenoxybenzamine-treated male rats [19]. Wistar rats were used in all experiments. Synthetic oxytocin and vasopressin were used as standards in the uterotonic and pressor tests, respectively. A detailed description can be found in [20]; standard activity values for vasopressin and oxytocin were taken from [7]. Values of activities found are given in Table 2.

Table 2. Pharmacological properties of new analogues of AVP (IU/mg or pA_2).

Compound		Activity			
		Uterotonic *in vitro*, no Mg^{2+} (IU/mg)	Antiuterotonic (pA_2)	Pressor (IU/mg)	Antipressor (pA_2)
AVP[a]		17	–	412	–
[(N-Bzl)Gly[3]]AVP	(I)	0	–	0	–
dAVP[a]		27–63	–	346–370	–
[Mpa[1],(N-Bzl)Gly[3]]AVP	(II)	0	–	0	–
[Cpa[1]]AVP[a]		–	8.15	0	8.35
[Cpa[1],(N-Bzl))Gly[3]]AVP	(III)	0	–	0	–
[(N-Bzl)Gly[2,3]]AVP	(IV)	0	–	0	–
[Cpa[1],(N-Bzl)Gly[2,3]]AVP	(V)	–	6.5	–	6.5

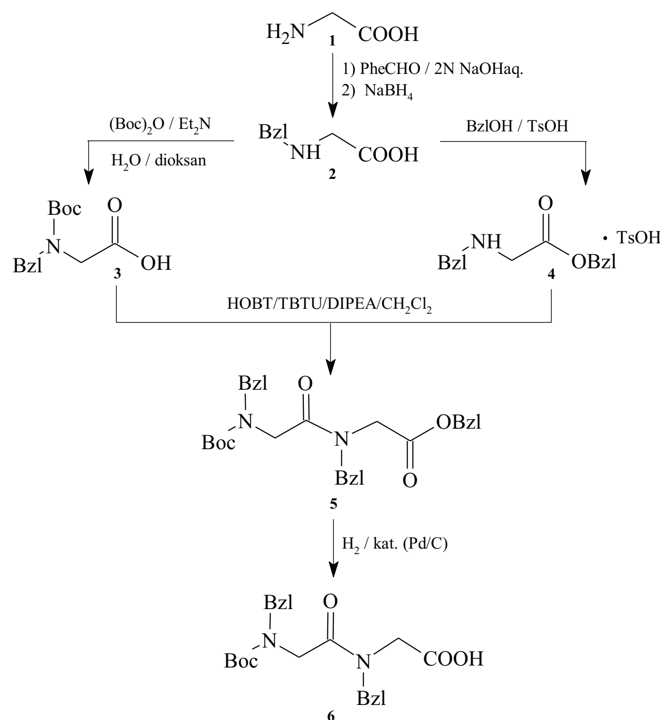
0 means inactive up to concentration 2×10^{-5} M (uterotonic test) or up to the dose of 0.4 mg/kg of experimental animal (pressor test); preliminary test of peptide V for antidiuretic or antiantidiuretic activity on conscious rats showed no effect. The pA_2 represent the negative logarithm to the base of 10 of the average molar concentration of the antagonist that will reduce the appropriate biological response to 2× units of agonist to the level of x units of it.
[a]Data from [7].

RESULTS AND DISCUSSION

As a part of our systematic study on the role of L-phenylalanine in an AVP molecule, we investigated the influence of its replacement in this hormone or its analogues with N-benzylglycine (peptides I–III). Moreover, our previous observation, that substitution of positions 2 and 3 in AVP with N-methyl-L-phenylalanine resulted in a potent V_{1A} blocker, prompted us to synthesize two additional analogues having two N-benzylglycine residues in the peptide chain in positions 2 and 3 (peptides IV and V). As we mentioned in the introduction, the structural change performed may be considered both as an introduction of peptoid analogue of phenylalanine, in which side chain was transferred from C^{α} to N, or as N-alkylation of the peptide bond different from methylation. To prepare N-benzylglycine we applied previously reported strategy of reductive alkylation [10,21]. First, glycine was converted into its N-benzyl analogue **2** using benzaldehyde and sodium borohydride as a reducing agent (Scheme 1). The N-benzylglycine **2** was obtained with moderate yield (37%) using this procedure and was accompanied by a significant amount of N,N-dibenzylglycine as a byproduct. To protect the amino terminus of **2,** the Boc group was introduced using *tert*-butyl pyrocarbonate as a reagent at the standard reaction conditions [22]. The synthesis of N-benzylglycine benzyl ester *p*-toluenosulfonate **4** has been achieved using esterification procedure catalyzed by *p*-toluenosulfonic acid [23].

Compound **3** has been coupled with **4** to the fully protected dipeptide Boc-(N-Bzl)Gly-(N-Bzl)Gly-OBzl **5** using TBTU as a condensing agent [24]. A low yield of the desired dipeptide **5** has been observed and was probably due to the high

Scheme 1



steric hindrance of both the amino and carboxy components. Removal of the benzyl ester group from **5** by hydrogenolysis in methanol over 10% Pd on carbon yielded the dipeptide **6** with the unprotected carboxylic group.

Synthesis of our peptides was based on Boc chemistry and started with MBHA resin. For the side-chain protection we used the 4-methoxybenzyl group (Cpa, Mpa, Cys). The protected amino acids were coupled using TBTU and HOBt in dimethylformamide. In the case of peptides IV and V, we used dipeptide Boc-(N-Bzl)Gly-(N-Bzl)Gly to avoid possible difficulties in the coupling step. Moreover, couplings of Boc-(N-Bzl)Gly or its dipeptide as well as the reactions of N-terminal residues (Cpa, Mpa, Cys) were mediated by HATU/HOAt/DIEA. Nevertheless, the presence of the units mentioned above in the sequence, caused great difficulties. The peptides were obtained in relatively low yields and with a considerable amount of byproducts. This was especially so in the case of peptide I and we repeated this synthesis applying, in the last two couplings, the procedure with *in situ* neutralization [13]. Namely, after the last washing of trifluoroacetate salt of peptidylresin, Boc-derivative and HATU were added, followed by 3 equivalents of DIEA. On completion of the syntheses, the protected peptidyl or acylpeptidyl resins were treated with liquid hydrogen fluoride (HF in the presence of anizole at –70°C) and oxidized

with I_2 in methanol. The crude products were purified on Sephadex G-15 and preparative HPLC. We noticed a significant improvement of the yield and purity of crude peptide I obtained using the procedure with *in situ* neutralization, which makes this protocol worth recommending for the synthesis when bulky acylating agents are used.

Some pharmacological properties of compounds I–V are summarized in Table 2. As can be seen in the case of substitution at position 2 or 2 and 3 the natural hormone, the modification results in the loss of pressor or uterotonic potencies (peptides I and IV): the analogues have neither agonistic nor antagonistic activity in the two tests. This would imply that they do not interact at all with V_{1A} and OT receptors. In the case of deaminovasopressin – an analogue of AVP with high and prolonged activity – this change (peptide II) has the same effect as for I and IV. Also, if we perform N-benzylglycine substitution in the potent vasopressor antagonist, $[Cpa^1]$AVP, (peptide III), the analogue loses all its inhibitory potency. This disappointing observation is very interesting as the presence of 1-mercaptocyclohexaneacetic acid residue (Cpa) in position 1 usually results in high antipressor activity [2,7]. This means that the loss of the proton donating NH group able to form hydrogen bonding, and at last but not least probable shift of the *cis*/*trans* equilibrium of the peptide bond between residues 2 and 3 for higher *cis* conformer content, changes the conformation of the molecule to such a degree that it is not able to interact with the receptor.

As regards peptide V, it is rather surprising that additional modification of inactive analogues III and IV (*i.e.* either additional replacement of Tyr^2 for (N-Bzl)Gly in III or substitution of Cys^1 for Cpa^1 in IV) resulted in a weak antagonist at V_{1A} and oxytocic receptors. The results presented imply that the structural change studied is in general incompatible with the interaction with V_{1A} and OT receptors, however, in combination with suitable additional changes in the structure of an analogue may be of value in the design of new V_{1A} and/or for oxytocin antagonists.

Acknowledgments

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