Analogues of Neurohypophyseal Hormones, Oxytocin and Arginine Vasopressin, Conformationally Restricted in the N-Terminal Part of the Molecule

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It is generally accepted that the conformation of the N-terminal part of neurohypophyseal hormones analogues is important for their pharmacological activity. In this work, we decided to investigate how the substitution of positions 2 and 3 with the ethylene-bridged dipeptide -Phe-Phe would alter the pharmacological properties of OT, [Mpa[1]]OT, and [Cpa[1]]OT (OT = oxytocin; Mpa = 3-mercaptopropionic acid; Cpa = 1-mercapto-cyclohexaneacetic acid) and to investigate how a bulky 3,3-diphenyl-L-alanine residue incorporated in position 2 of AVP, [Mpa[1]]AVP, and [Cpa[1]]AVP (AVP = arginine vasopressin) would change the pharmacological profile of the compounds. The next analogues, [Val[4]]AVP, [Mpa[1],Val[4]]AVP, and [Cpa[1],Val[4]]AVP, had *N*-benzyl-L-alanine introduced at position 3. The last peptide was designed by Cys[1] substitution in AVP by its sterically restricted bulky counterpart, α-hydroxymethylcysteine. All the peptides were tested for their in vitro uterotonic, pressor, and antidiuretic activities in the rat. The results of these assays showed that the reduction of conformational freedom of the N-terminal part of the molecule had a significant impact on pharmacological activities.

Introduction

Oxytocin (OT),[a] a physiologically important nonapeptide hormone and neurotransmitter containing a 20-membered ring and an acyclic tripeptide tail, regulates several physiological functions, such as milk ejection, uterine contraction, vascular and cardiac relaxation, etc. Another neurohypophyseal hormone, arginine vasopressin (AVP), in addition to its well-known antidiuretic activity, has also complex cardiovascular actions. The AVP and OT receptor family consists of four subtypes of receptors. V_{1a} receptors modulate the vasopressor actions of AVP,[1] the adrenocorticotropic hormone (ACTH) releasing activity of AVP is evoked by V_{1b} receptors,[2] and OT receptors control milk ejection and uterine smooth muscle contractions. These three receptor subtypes function via the phosphatidylinositol pathway. The last subtype, V_2 receptor, present in the renal tubule and collecting ducts, modulates antidiuretic responses to AVP and is linked to adenylate cyclase activity.[2] The AVP and OT receptor subtypes show a high degree of homology. This, along with the similar structure of both hormones, may be a reason for the overlap of pharmacological profiles of OT and AVP that are seen. However, the affinities of these hormones for receptors are different, as are their biological capacities to transduce signals and elicit a biological response.

In the last few years, there has been an upsurge of interest in both neurohypophyseal hormones, and new physiological and pathophysiological roles for these peptides have been indicated. Most of this work would not have been possible without the aid of OT and AVP antagonists as pharmacological tools.[3,4] However, during the past decade, we noticed that antagonists of OT, which seemed to be less readily investigated than their AVP counterparts, became more and more interesting because of their potential application in the prevention of premature labor and treatment of dysmenorrhea. There is a substantial body of evidence pointing to the significance of OT in both premature and mature parturitions; thus, one alternative to the current therapy for preterm labor is the use of OT receptor blockers.[5] Although in recent years much progress has been made toward improving the selectivity of OT antagonists,[6] the design of compounds that are both potent and selective as OT blockers remains a challenge.

A great deal of evidence points to the importance of the conformation of the N-terminal part of OT and AVP analogues for their pharmacological activity.[3,7−9] This is supported also by our recent description of some pharmacological properties of analogues with bulky L- or D-β-(1-naphthyl)-alanine (Nal) moieties at position 2 of [(Mpa)[1]]AVP (Mpa = 3-mercapto-propionic acid). Both compounds surprisingly exhibited moderate antioxytocic activity in vitro and were very selective.[10] We assumed that the hindering effect caused by the naphthyl ring has a significant impact on conformation of the analogues and thus greatly influences their interactions with receptors. In another study, Prochazka and Slaninová[11] described the synthesis and pharmacological evaluation of analogues with L- or D-Nal at position 2 of OT or AVP. This, together with the results of

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[a] Abbreviations: AVP, arginine vasopressin; Cpa, 1-mercaptocyclohexaneacetic acid; Dap, diaminopropionic acid; DCM, dichloromethane; DIEA, diisopropylethylamine; Dpa, 3,3-diphenyl-L-alanine; DMF, dimethylformamide; HATU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; α-HmC, α-hydroxymethylcysteine; HOAt, 1-hydroxybenzotriazole; HOBt, 1-hydroxy-benzotriazole; MBHA, *p*-methoxybenzhydrylamine; Mob, 4-methoxybenzyl; Mpa, 3-mercaptopropionic acid; Nal, β-(1-naphthyl)-L-alanine; NMP, 1-methyl-2-pyrrolidone; OT, oxytocin, TBTU, 2-1H-(benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid. The symbols of 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.

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our previous study,[12] clearly demonstrates new possibilities opened up by utilizing the bulky Nal residue for modification of both OT or AVP for designing analogues of neurohypophyseal hormones. It has been shown that single substitution with a Nal residue results in selective and quite potent analogues of both hormones.[11,12] Another approach, which also resulted in moderately potent and fairly selective antagonists of OT, was used in Manning's laboratory and consisted of multiple modifications of the OT molecule, e.g., desGly-NH_2, d$(CH_2)_5$[D-Trp[2],Thr[4],Dap[5]]OVT, where Dap is diaminopropionic acid, and d$(CH_2)_5$ stands for 1-mercaptocyclohexaneacetic acid.

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 formation of cyclic structures within the peptide framework by disulfide and lactam bridges, or by substitution of chosen amino acid residues with sterically restricted fragments that limit conformational freedom, forcing peptide backbone and/or side chains to adopt specific orientations. Another approach to achieve certain stabilization of the structure, is the preparation of various types of pseudopeptides through additional, 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, which incorporates the relevant N−C−C′−N′ peptide segment as its inherent part.[13] Using theoretical conformational analysis, the impact of such constraint (*N*,*N*′-ethylene-bridged dipeptide unit) was studied, taking *N*,*N*′-bridged *N*-acetyl-Phe-Phe-*N*′-methylamide as an example. The results indicate equal preferences for assuming either of two enantiomeric twisted chair/boatlike pucker types for the piperazinone ring. The size and/or pucker type of the ring constraint affects little the peptide backbone, which always tends to exist in either of two distinct semi-extended forms. They are equally populated for the piperazinone-constrained peptide. The side-chain benzyl group shows conformational preferences dependent on whether it belongs to the first (in the ring) or to the second (outside the ring) Phe residue. In the first Phe generally two, *v*iz. extended-to-N (EN) and extended-to-O (EO) staggered conformations are preferred over the third folded one (F). The notable exceptions are the piperazinone-constrained peptides where the F form tends to be distinctly favored. The benzyl ring belonging to the second Phe (that outside the rings) typically prefers the EN and never assumes the F conformation.[14]

In summary, all these findings prompted us to continue our studies on the influence of sterically restricted fragments placed in this part of OT or AVP analogues on their pharmacological properties. First, we decided to check how substitution of positions 2 and 3 with an ethylene-bridged dipeptide -Phe-Phe (a $-CH_2-CH_2-$ bridge spanning two subsequent peptide nitrogens, thus forming a piperazinone ring) would alter the pharmacological properties of OT, [Mpa[1]]OT, and [Cpa[1]]OT.

Next, we designed three peptides by substituting position 2 in AVP, [Mpa[1]]AVP, and [Cpa[1]]AVP with bulky 3,3-diphenyl-L-alanine residue. Both modifications restrict the conformational freedom of the resulting peptides, which should influence their pharmacological properties. The third group of analogues was designed by *N*-benzyl-L-alanine substitution at position 3 of [Val[4]]AVP, [Mpa[1],Val[4]]AVP, and [Cpa[1],Val[4]]AVP. The *N*-benzyl-L-alanine may be considered as a structural analogue of phenylalanine, in which the side chain is moved from C^{α} to nitrogen. At the same time, introduction of such a peptoid unit into a peptide chain may also be considered as an alkylation of the peptide bond, which is attractive as a local and subtle mode of conformational constraint. Considerable information exists concerning outlining of the structural perturbations induced by this modification: occurrence of the cis isomer for tertiary amide bonds, steric constraints due to the N-alkyl group, suppression of a proton-donating N−H group capable of hydrogen bonding, and increased basicity of the carbonyl group. Conformational studies indicate that the influence of N-alkylation on a conformation depends to a large extent on the chirality of the residues surrounding the modified peptide bond.[15]

Finally, it seemed worthwhile to modify the Cys[1] in AVP to check how its sterically restricted bulky counterpart, α-hydroxymethylcysteine (α-HmC), will change the pharmacological profile of the hormone.

The analogues have the following structure: [-Phe-Phe[2,3]]-OT (**I**), [Mpa[1], -Phe-Phe[2,3]]OT (**II**), [Cpa[1], -Phe-Phe[2,3]]OT (**III**), [Dpa[2]]AVP (**IV**), [Mpa[1],Dpa[2]]AVP (**V**), [Cpa[1],Dpa[2]]AVP (**VI**), [N-Bzl-Ala[3],Val[4]]AVP (**VII**), [Mpa[1],N-Bzl-Ala[3],Val[4]]AVP (**VIII**), [Cpa[1],N-Bzl-Ala[3],Val[4]]AVP (**IX**), [α-HmC[1]]AVP (**X**).

Results

The 10 new analogues of AVP and OT (**I**−**X**) were synthesized using Boc-chemistry on a methoxybenzhydryl resin (**I**−**IX**) and on a chloromethylated Merrifield resin (**X**). For analogues **II**, **V**, **VIII**, and **III**, **VI**, **IX**, Mpa(Mob) and Cpa(Mob) were used in final step, respectively. As we were not able to couple Boc-N(Bzl)Ala during the synthesis to the growing peptide chain for peptides **VII**−**IX**, Boc-Tyr(Bzl)-N(Bzl)Ala-Val-OH was used in the fifth coupling step. On completion of the syntheses, the protected peptidyl or acylpeptidyl resin (analogues **I**−**IX**) were treated with liquid HF in the presence of anisole at 0 °C (in the case of analogue **X** the protected nonapeptidyl resin was ammonolysed in methanol) and oxidized with I_2 in methanol. The crude products were desalted on a Sephadex G-15 and purified by RP-HPLC. The purity and identity of each peptide were determined by HPLC and FAB mass spectrometry (molecular ion).

Physicochemical properties and pharmacological data of the new peptides **I**−**X**, and those of AVP, OT, [Mpa[1]]AVP, and some related peptides, are presented in Tables 1 and 2. The activities of the analogues were determined in the in vitro rat uterotonic test in the absence of magnesium ions, in the rat pressor test, and in the antidiuretic assay on conscious rats as described in the experimental section. None of these new analogues exhibited antivasopressor activity. A comparison of the antidiuretic activities of the new analogues with those published previously must be done with caution as different testing procedures were used. To compare the effects of compounds with different regression lines of the dose response curve, doses of AVP were compared with such doses of new analogues that gave the same antidiuretic response, i.e., the doses that caused rats to excrete half of the water load ($t_{1/2}$) in 60 and 200 min. For AVP, the activity was arbitrary taken to be 465 IU/mg for both responses. Analogues **IV** and **V** have antidiuretic activity comparable to AVP at the level of $t_{1/2}$ 60 min. However, their dose−response curves are much steeper being similar to those of dDAVP. It means that at a $t_{1/2}$ level of 200 min the activity is much higher than that of AVP (2000 and 10000% of that of AVP, respectively). The remaining analogues (**I**−**III** and **VI**−**IX**) exhibited negligible antidiuretic activity, while compound **X** was a moderately potent agonist.

Peptides **I**−**VI** and **X** exhibited different antioxytocic activities ranging from $pA_2 = 6.40$ to 7.23. Analogues **VII** and **IX** did not show any activity in the uterotonic test, while compound **VIII** showed very low agonistic activity.

Table 1. Physicochemical Properties of Peptides **I**−**X**[a]

analogue		HPLC t_r		formula	$[M + H^+]$		yield [%]	
		a	b		calculated	found	A	B
[Phe-Phe[2,3]]OT	**I**	9.89	11.98	$C_{48}H_{66}N_{12}O_{11}S_2$	1051.2	1052.2	89	59
[Mpa[1], Phe-Phe[2,3]]OT	**II**	13.84	17.72	$C_{48}H_{65}N_{11}O_{11}S_2$	1036.2	1036.1	65	24
[Cpa[1], Phe-Phe[2,3]]OT	**III**	18.37	23.74	$C_{52}H_{76}N_{11}O_{11}S_2$	1104.2	1104.2	83	50
[Dpa[2]]AVP	**IV**	11.36	13.18	$C_{52}H_{69}N_{15}O_{11}S_2$	1143.8	1144.5	86	63
[Mpa[1],Dpa[2]]AVP	**V**	14.00	17.18	$C_{52}H_{68}N_{14}O_{11}S_2$	1128.8	1129.7	79	46
[Cpa[1],Dpa[2]]AVP	**VI**	15.87	19.96	$C_{57}H_{76}N_{14}O_{11}S_2$	1197.4	1197.6	88	59
[N-Bzl-Ala[3],Val[4]]AVP	**VII**	9.70	11.75	$C_{47}H_{68}N_{14}O_{11}S_2$	1069.3	1069.8	71	38
[Mpa[1],*N*-Bzl-Ala[3],Val[4]]AVP	**VIII**	11.63	13.56	$C_{47}H_{67}N_{13}O_{11}S_2$	1054.3	1054.3	85	54
[Cpa[1],*N*-Bzl-Ala[3],Val[4]]AVP	**IX**	13.97	16.99	$C_{52}H_{73}N_{13}O_{11}S_2$	1122.3	1122.2	87	66
[α-HmC[1]]AVP	**X**	7.38	9.82[c]	$C_{47}H_{66}N_{15}O_{13}S_2$	1113.5	1114.7	71	16

[a] Headings: a, Linear gradient from 20 to 80% of [B] for 20 min; b, linear gradient from 20 to 80% of [B] for 30 min; c, linear gradient from 10 to 45% of [B] for 15 min. A, yields were calculated on the base of the glycine content of the starting resin; B, yields are based on the amount of crude peptide.

Table 2. Pharmacological Properties of the New AVP Analogues[a]

analogue		activity		
		uterotonic in vitro no Mg^{2+}	pressor IU/mg or pA_2	antidiuretic IU/mg[c]
AVP[b]		17	412	465
[Mpa[1]]AVP[b]		27−63	346−370	1300−1745
[Cpa[1]]AVP[b]		$pA_2 = 8.15$	$pA_2 = 8.35$	0.033
OT[b]		450	5	5
[Mpa[1]]OT[b]		803	1.44	19
[Cpa[1]]OT[b]		$pA_2 = 7.61$		
[Val[4]]AVP[b]			32	738
[Mpa[1],Val[4]]AVP[b]			51	1150
[Cpa[1],Val[4]]AVP[b]		$pA_2 = 7.34$	$pA_2 = 7.97$	0.32
[Phe-Phe[2,3]]OT	**I**	$pA_2 = 6.82$	0	<0.04
[Mpa[1], Phe-Phe[2,3]]OT	**II**	$pA_2 = 6.50$	0	<0.04
[Cpa[1], Phe-Phe[2,3]]OT	**III**	$pA_2 = 6.72$	0	<0.04
[Dpa[2]]AVP	**IV**	$pA_2 = 7.00$	0	450 (9000)
[Mpa[1],Dpa[2]]AVP	**V**	$pA_2 = 7.23$	0	450 (45000)
[Cpa[1],Dpa[2]]AVP	**VI**	$pA_2 = 6.10$	0	<0.04
[*N*-Bzl-Ala[3],Val[4]]AVP	**VII**	0	0	<0.04
[Mpa[1],*N*-Bzl-Ala[3],Val[4]]AVP	**VIII**	very low agonist	0	<0.04
[Cpa[1],*N*-Bzl-Ala[3],Val[4]]AVP	**IX**	0	0	<0.04
[α-HmC[1]]AVP	**X**	pA_2=6.40	0.18	4.5 (∼22.5)

[a] IU/mg or pA_2. [b] Values taken from ref 7. [c] The activities obtained by comparing doses of AVP and the analogue resulting in an antidiuresis time of $t_{1/2} = 60$ min; in parentheses, the activities obtained by comparing doses of AVP and the analogue resulting in an antidiuresis time of $t_{1/2} = 200$ min.

Discussion

The present work is a continuation of our already 10-year studies aimed at clarifying the impact of steric restrictions and bulky subtituents in the N-terminal part of the AVP and OT molecules on pharmacological properties of the resulting analogues. Previously, we reported that such modifications influenced the interaction of molecules with V_{1a}, V_2, and oxytocic receptors. Our approach to this problem included the ethylene-bridged dipeptide fragment -Phe-Phe, at positions 2 and 3.[16] One peptide from this series was a highly potent and selective blocker of V_1 receptors. It is noteworthy that OT analogues substituted at positions 2 and 3 with the ethylene-bridged dipeptide -D-Phe-D-Phe exhibited either no or very weak activities.[17] We also showed that modification of position 2 of AVP and some of its analogues with 1-aminocyclohexane-1-carboxylic acid or 1-aminocyclopentane-1-carboxylic acid resulted in compounds having differently modified activities (high antidiuretic potency, low and graded pressor activity, and either no activity or low oxytocin antagonizing activity in the uterotonic in vitro test), thus selectively altered interaction with receptors.[18−20] Furthermore, we demonstrated that the analogues of AVP modified at position 2 with bulky L or D β-(1-naphthyl)-alanine were moderately potent and exceptionally selective oxytocin antagonists in vitro.[21] On the other hand, [D-Arg[8]]VP substituted at position 3 with β-(2−naphthyl)-L-alanine turned out to be a potent and selective antagonist of the vasopressor response to AVP.[12]

This paper describes the synthesis and pharmacological evaluation of 10 new analogues with various types of modifications in the N-terminal part of the molecule. First, we substituted positions 2 and 3 of OT, [Mpa[1]]OT, and [Cpa[1]]OT with a conformationally constrained dipeptide unit Phe-Phe, to obtain analogues **I**, **II**, **III**. This constraint consists of the $-CH_2-CH_2-$ link bridging two consecutive peptide chain nitrogens leading to the formation of a piperazinone ring that incorporates the relevant −N−C−C′−N′− peptide segment as its inherent part. This modification converted OT and its agonist [Mpa[1]]OT into rather weak but selective antagonists of oxytocic activity in the in vitro uterus test. With [Cpa[1]]OT, this modification was not beneficial. On the contrary, it significantly reduced the antagonistic potency (see analogue **III**).

Of the next three analogues, **IV**, **V**, and **VI**, peptide **IV** designed by only a single modification of the AVP molecule, i.e., substitution of Tyr[2] with 3,3-diphenyl-L-alanine, has strikingly different pharmacological properties in comparison to the parent hormone. It displays no pressor activity, while its antidiuretic potency is preserved and even prolonged due to the much steeper dose−response curve. Moreover, this compound is a moderately potent blocker of oxytocic uterotonic activity. This combination of pharmacological properties makes our

peptide interesting as a tool in physiological experiments. Deamination of compound **IV** results in peptide **V** having an essentially similar profile of activities. However, both antidiuretic and antiuterotonic potencies are enhanced. It is interesting to notice that additional modification of compound **IV** at position 1 with 1-mercaptocyclohexaneacetic acid (Cpa) (intended to enhance its antagonistic properties), resulted in peptide **VI** with substantially reduced antioxytocic potency and practically devoid of antidiuretic activity. This finding suggests that two bulky groups at positions 1 and 2 together might not be compatible with the binding site of the receptors. Previously, when we combined Cpa[1] modification with substitution of position 2 of sterically restricted amino acids, e.g., 1-aminocyclohexane-1-carboxylic acid or 1-aminocyclopentane-1-carboxylic acid,[18-20] we also observed the reduction of activity. The condition that Cpa[1] (or similar residue) is essential for substantial antagonistic activity[3,4,7] appears not to be valid for analogues that contain either bulky or effectively reducing conformational freedom fragments in the N-terminus.

N-Benzyl-L-alanine, a chiral peptoid unit, may be considered as an analogue of L-phenylalanine, in which the side chain is moved from C^{α} to nitrogen. In other words, the nitrogen of the peptide bond is de facto alkylated by benzyl. This may appear to be a local and subtle conformational change. However, all three analogues with *N*-benzyl-L-alanine in position 3 (**VII**−**IX**) showed no activities in the tests; thus, this modification turned out to be deleterious for the interaction with all V_{1a}, V_2, and oxytocic receptors.

Peptide **X** prepared by substitution of α-hydroxymethylcysteine in position 1 of AVP instead of Cys has an interesting pharmacological profile, although the activities are not too high, being comparable to those of any analogue having the modified amino group. It is a weak antagonist of oxytocin, practically devoid of pressor activity; however, it still has weak antidiuretic properties (about 1% of that of AVP). This modification in combination with single or multiple additional changes in positions 2, 3, and 8 may result in a compound having interesting pharmacological properties.

Summing up, our studies resulted in several analogues with interesting pharmacological properties that may contribute to mapping the receptor binding sites. We believe that even more important is the fact that we could once more demonstrate that the modification of the N-terminal part of the molecule, especially the reduction of conformational freedom, has a dramatic impact on the pharmacological activities, which in turn opens new possibilities for designing new analogues of AVP and OT with desired activities.

Experimental Section

Chemistry. Racemic *S*-benzyl-α-hydroxymethylcysteine [α-HmC(Bzl)] was synthesized by selective *S*-benzyl-cysteine α-hydroxymethylation and was resolved into its enantiomers by fractional crystallization of the diastereomeric salts of their N-benzoyl derivatives with (−)-quinine, using the method previously described.[22] [*R*]- and [*S*]-Boc-α-HmC(Bzl)-OH were prepared in acetonitrile using tetramethylammonium hydrate (TMAH) and Boc_2O according to the published procedure.[23]

The tripeptide building block [Boc-Tyr(Bzl)-N(Bzl)Ala-Val-OH] (**4**) was synthesized separately in solution. Following the routine strategy, the dipeptide [Boc-N(Bzl)-Ala-Val-OBzl (**1**) was deprotected on the N-terminus with HCl solution in ethyl acetate (4 M), and the resulting hydrochloride salt of H-N(Bzl)Ala-Val-OBzl (**2**) was extended by coupling with Boc-Tyr(Bzl)-OH to yield the protected tripeptide Boc-Tyr(Bzl)-N(Bzl)Ala-Val-OBzl (**3**). Removal of the benzyl ester group from **3** by careful hydrogenolysis in methanol over 10% Pd on charcoal afforded the free acid, Boc-Tyr(Bzl)-N(Bzl)Ala-Val-OH (**4**), which was used for the solid-phase peptide synthesis procedure.

Melting points were uncorrected. Optical rotations were measured in a 1-dm cell on a Horiba polarimeter (model SEPA-200) at 589 nm (Na D line). NMR spectra were recorded on a Bruker Avance DPX 250 spectrometer. Analytical HPLC was performed on a Milton Roy instrument with a SpectroMonitor 3100 detector using Vydac C_{18} (250 × 4.6 mm) column, flow rate 1 mL/min, detection at 220 nm, and solvents [A] 0.05% trifluoroacetic acid in water and [B] 0.038% trifluoroacetic acid in acetonitrile/water 90:10 in a gradient application. Thin-layer chromatography (TLC) was carried out on 250 nm silica gel GF precoated uniplates (Merck). The chromatograms were visualized with ninhydrin or with chlorine followed by starch/KI. FAB mass spectra were recorded on an APO Electron model MI 1200 1E mass spectrometer equipped with a FAB ion source.

Boc-N(Bzl)Ala-Val-OBzl (1). To the stirred suspension of Boc-N(Bzl)Ala-OH (1.396 g; 5 mmol), HOBT (0.675 g; 5 mmol) and TBTU (1.605 g; 5 mmol) in DCM (20 mL), DIPEA (0.856 mL; 5 mmol) were added. After 20 min of stirring, the crystalline $TosOHxNH_2$Val-OBzl (1.897 g, 5 mmol) and DIPEA (0.856 mL; 5 mmol) were added. The reaction mixture was left with stirring for 3 days, diluted with ethyl acetate (100 mL), and washed successively with 1 M aqueous $KHSO_4$, 5% aqueous $NaHCO_3$ and water. The organic layer was dried over anhydrous $MgSO_4$ and then evaporated to dryness, yielding 2.17 g of crude oil, which was purified on "flash" chromatography in the solvent system ethyl acetate/hexane = 1:1.5 (A). Yield 2.01 g (85.6%) of pure homogeneous product as a light yellow oil. HPLC purity 100%, t_R = 15.2 min (gradient 70−90%B in 25min), α_D^{20} −42.6 (*c* = 1, MeOH), R_f = 0.6 (A), FAB/MS *m/e* 469 $[M + H]^+$ Calcd for $C_{27}H_{36}O_5N_2$ = 468. ^1H NMR ($CDCl_3$), δ (ppm): 0.84 (dd, 6H, *J* = 7.5; 5 Hz Val γCH_3), 1.24 (d, 3H, *J* = 7.5 Hz Ala βCH_3), 1.44 (s, 9H Boc CH_3), 2.06−2.11 (m, 1H, Val βCH), 4.12 (dd, 1H, *J* = 7.5, 5.0 Hz Val αCH), 4,27 (s, 2H, Ph$\underline{CH_2}$N), 4.33 (s, 2H Ph$\underline{CH_2}$O), 4.71 (1H, d, *J* = 8.2 Hz N$\underline{H}$), 5.08−5.14 (m, 1H, Ala αCH) 7.21−7.35 (m, 10H, $\underline{Ph}CH_2O$ and $\underline{Ph}CH_2N$).

Boc-Tyr(Bzl)-N(Bzl)Ala-Val-OBzl (3). Removal of Boc Group from Dipeptide 1. Dipeptide **1** (1.874 g, 4 mmol) was treated with a 4 M HCl solution in ethyl acetate (15 mL). After 30 min, the solvent was removed at room temperature under reduced pressure. The crude waxy product was treated with ether and filtered off to yield 1.140 g (70%) of **2**, which was used for the next step without further purification.

Coupling with Boc-Tyr(Bzl)-OH. A solution of 1.03 g (2.77 mmol) of Boc-Tyr(Bzl)-OH in 20 mL of dichloromethane was cooled to −15 °C and treated with 0.304 mL (2.77 mmol) of *N*-methylmorpholine (NMM), followed by 0.366 mL (2.77 mmol) of isobutyl chloroformate. The mixture was stirred for 15 min, then 1.12 g (2.77 mmol) of **2** was added, followed by addition of 0.304 mL (2.77 mmol) of NMM. After being stirred 1 h at −10 °C, the mixture was warmed slowly to room temperature and stirred overnight. The solvent was removed under reduced pressure to dryness. The residue was taken up in ethyl acetate (70 mL) and washed with 1 M $KHSO_4$ (2 × 25 mL), 1 M $NaHCO_3$ (2 × 25 mL), water (2 × 25 mL), and brine (1 × 25 mL). After the sample was dried over anhydrous Na_2SO_4, ethyl acetate was evaporated, yielding 1.82 g of crude product, which was purified on "flash" chromatography in the solvent system ethyl acetate/hexane = 1:1.5 (A). The tripeptide **3** was isolated as an amorphous powder. Yield 0.746 g (37%); HPLC purity 99.5%, t_R = 16.85 min (gradient 70−90%B in 25 min); R_f (A) = 0.4; α_D^{20} −28 (*c* = 1, MeOH); FAB/MS *m/e* 722.3 $[M + H]^+$, 744.4 $[M + Na]^+$ Calcd. for $C_{43}H_{51}N_3O_7$ = 721.8; ^1H NMR ($CDCl_3$) δ (ppm): 0.84 (dd, 6H, *J* = 6.75, 5 Hz Val γ-CH_3), 1.30 (d, 3H *J* = 6.75 Hz Ala βCH_3), 1.42 (s, 9H Boc-CH_3), 2.00−2.11 (m, 1H, Val βCH), 2.90−2.97 (m, 2H, Tyr βCH_2), 3.88−4.12 (m, 1H Val αCH), 4.30−4.41 (m, 1H, Tyr αCH), 5.04−5.22 (m, 7H, 6H $\underline{CH_2}$Ph, 1H Ala αCH), 6.05 (d, 1H, *J* = 8.5 Hz Val NH), 6.63 (d, 1$\underline{H}$, *J* = 8.5 Hz Tyr NH), 7.34−7.38 (19H, 4H Tyr Ph, 10H Tyr and Ala $OCH_2\underline{Ph}$, 5H $NCH_2\underline{Ph}$);

Boc-Tyr(Bzl)-N(Bzl)Ala-Val-OH (4). A solution of protected tripeptide **3** (0.516 g, 0.715 mmol) in 5 mL of methanol was hydrogenated (3 h, monitoring by TLC) over 10% Pd on charcoal. The filtered solution was evaporated yielding a homogeneous amorphous powder 0.430 g (95,1%). HPLC purity 98%, $t_R = 6.20$ min. (gradient 70−90% B in 25 min.), α_D^{20} −16,7 ($c = 1$, MeOH); FAB/MS *m/e* 632.1 $[M + H]^+$ Calcd. for $C_{36}H_{46}N_3O_7 = 631.4$; ^1H NMR ($CDCl_3$) δ (ppm): 0.92 (dd, 6H, $J = 6.75$; 5.25 Val γCH_3), 1.22 (d, 3H, $J = 7.00$ Hz, Ala βCH_3), 1.40 (s, 9H, Boc CH_3), 1.96−2.00 (m, 1H Val βCH), 2.87−2.92 (m, 1H, Tyr βCH_2), 3.85−3.87 (m, 1H, Val αCH), 4.36−4.82 (m, 6H, 1H Ala αCH, 1H Tyr αCH, 2H OCH_2Ph, 2H NCH_2Ph), 5.51 (d, 1H, $J = 6.90$ Hz Val NH), 5.77 (d, 1H, $J = 6.75$ Hz Tyr NH), 7.20−7.26 (m, 14H, 4H Tyr Ph, 5H OCH_2Ph and 5H NCH_2Ph). ^{13}C NMR ($CDCl_3$) δ(ppm), 17.74 (Ala β C), 18.68, 18.97 (Val γC), 28.11 (Boc CH_3), 30.64 (Val βC), 52.82 (Tyr β C), 55.62 (Ala αC), 57.63 (Tyr αC), 58.83 (Val αC), 65.80 (CH_2Ph), 80.45 (Boc C=O), 115.6, 126.39, 126.82, 127.96, 130.46, 137.48, (Tyr, OBzl, Ph), 155.10, 155.57, 155.96, (*N*-Bzl Ph), 156.2 (Boc C=O), 170.47, 171.28, 174.32 (Ala, Tyr, Val C=O).

Boc-Phe-Phe was synthesized using a procedure described in the literature.[12]

Mpa(Mob) was obtained as described for Mpa(Bzl)[24] using *p*-methoxybenzyl chloride. Cpa(Mob) was synthesized using a procedure described in the literature.[25]

All amino acid derivatives were purchased from NovaBiochem, except Boc-Dpa, which was provided by Merck.

Peptide Synthesis. Thin-layer chromatography (TLC) was carried out on silica plates (Merck), and spots were visualized with iodine or ninhydrin. The solvent system used was 1-butanol/acetic acid/water/ethyl acetate (1:1:1:1, v/v). High-performance liquid chromatography (HPLC) was carried out on a Waters (analytical and preparative) chromatograph equipped with a UV detector ($\lambda = 226$ nm). The purity of the peptides **I**−**IX** was determined on a Vydac C_{18} column (5 μm, 100A; 25 × 0.46 cm). The following solvent systems were used: [A] 0.1% aqueous trifluoroacetic acid (TFA) and [B] acetonitrile: 0.1% aqueous TFA (80:20 v/v). Linear gradients from 20 to 80% of solution [B] for 20 min and from 20 to 80% of solution [B] for 30 min were applied for peptides at a flow rate of 1 mL/min. Preparative HPLC was carried out using a Kromasil C_8 column (5 μm, 25 × 250 mm) in a gradient running from 10 to 50% of [B] for 120 min at a flow rate of 10 mL/min. The purity and identity of the peptide **X** were determined by HPLC [a Gold System Beckman chromatograph, Vydac C_{18} column (5 μm, 4.6 × 250 mm) with precolumn Ultrasphere ODS (5 μm, 4.6 × 45 mm), solvent systems were used: [A] 0.1% aqueous trifluoroacetic acid (TFA), [B] acetonitrile: 0.1%TFA (80:20 v/v)]. A linear gradient from 10 to 45% of solution [B] for 15 min was applied for peptide at a flow rate of 1 mL/min ($\lambda = 226$ nm). FAB/MS of the peptides were recorded on a MALDI TOF mass spectrometer.

All peptides were obtained by solid-phase peptide synthesis. Analogues **I**−**IX** were synthesized manually using Boc-chemistry on a methoxybenzhydryl resin (MBHA resin, Senn Chemicals AG, 1% DVB, 200−400 mesh, 0.67 mmol/g) on a scale of 150 μmol according to standard procedures, using in situ neutralization.[26] The protected peptide precursor: Boc-α-HmC(Bzl)-Tyr(Bzl)-Phe-Gln-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2 (analogue **X**) was synthesized manually by a solid-phase method, i.e., by the stepwise coupling of Boc-amino acids to the growing peptide chain on a chloromethylated Merrifield resin (Novabiochem, 200−400 mesh, 0.7 mmol/g) on a scale of 150 μmol. Fully protected peptide resin was 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 of Boc-amino acid mediated by TBTU and HOBt in the presence of DIEA in DMF or in a mixture of DMF, NMP, and DCM (1:1:1 v/v) containing 1% Triton, in the case of Boc-(αHm)Cys(Bzl) coupling.[27,28]

The completeness of each coupling reaction during synthesis was monitored by the Kaiser test[29] or chloranil test.[30] Recoupling was performed when the test was positive. With peptides **II**, **III**, **V**, **VI**, **VIII**, and **IX**, Mpa(Mob) or Cpa(Mob) were used in the final coupling step.

After completion of the synthesis, the protected peptidyl or acylpeptidyl resins were treated with 10 mL of liquid hydrogen fluoride (HF) containing 1 mL of anisole at −70 °C and stirred for 60 min at 0 °C.[31] After removal of HF and anisole in vacuo, the mixture was washed successively with anhydrous diethyl ether and acetic acid, and the solution was diluted with methanol.

With analogue **X**, the protected nonapeptidyl resin was ammonolysed in methanol.[27,28] After evaporation of the solvent, the product was extracted into hot DMF, precipitated with boiling water, and left overnight at room temperature. The peptide was collected by filtration, washed with water, and dried in-vacuo over P_2O_5. The product was further purified by dissolving in DMF and reprecipitating with CH_3-OH: Et_2O (3:1). Then a solution of the peptide intermediate (0.15 mmol) in a sodium-dried and redistilled ammonia was treated at boiling point, and with stirring with sodium from a stick of the metal contained in a small-bore glass tube until a light-blue color persisted in the solution for 30 s, NH_4Cl was added to discharge the color. The solution was evaporated, the residue was dissolved in glacial acetic acid (150 mL), and the solution was diluted with methanol (1500 mL).

The resulting dithiols were oxidatively cyclized with 0.1 M I_2 in methanol using the standard procedure. The solvents were evaporated under reduced pressure, and the residue was dissolved in water and lyophilized. The crude products were desalted on a Sephadex G-15 column and eluted with aqueous acetic acid (30%) at a flow rate of 3−4 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 10% aqueous acetic acid at a flow rate of 4.0 mL/h, $\lambda = 254$ nm. The peptide was eluted as a single peak. Peptides **V**, **VII**−**IX** were purified by RP-HPLC. The peptides were eluted as single peaks. The purity and identity of each peptide were determined by HPLC and FAB mass spectrometry (molecular ion).

Biological Evaluation. Wistar rats were used in all experiments. Female rats were estrogenized 48 h before the experiment. The uterotonic test was carried out in vitro in the absence of magnesium ions.[32,33] The vasopressor test was performed using phenoxybenzamine-treated male rats.[34] Synthetic oxytocin was used as a standard in uterotonic tests, and synthetic arginine vasopressin was used in pressor test. Dose−response (single administration) or cumulative dose−response (measurements without washing steps between administration of enhanced doses) curves were constructed. The values reported are averages of three to five separate experiments.

Tests to assess the antidiuretic or diuretic properties were conducted on conscious male rats in two variations of the modified Burn test.[35,36] In the standard manner with hydrated rats, the animals having fasted for 16 h were weighed and then given tap water through a stomach catheter. The water load was 4% of the body weight. Immediately after the water load, the tested substances (or physiological saline as control) were administered subcutaneously at doses of 0.001−100 nmol/kg. The rats were then placed in individual metabolic cages, and their urine was collected over a 5 h period. The time $t_{1/2}$ in which the rats excreted half the water load was determined and then plotted against the dose. For comparison of the compounds, such doses were chosen as to yield $t_{1/2}$ equal to 200 min and the so-called threshold doses yielding $t_{1/2}$ equal to 60 min (equal to the value of $t_{1/2}$ obtained with the physiological solution). On each day of the experiment, 21 rats divided into 5 groups of 4 or 5 animals to which different doses and compounds were administered were used; each dose being tested in two or three independent experiments (different days, different rats). To test for diuretic effects with nonhydrated rats, no water load was given to the fasting animals. For details see ref 37.

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Supporting Information Available: HPLC data for peptides **I**−**IX**. This material is available free of charge via the Internet at http://pubs.acs.org.

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