Enhanced Selectivity of Oxytocin Antagonists Containing Sarcosine in Position 7[†]

Imre Pavo,[§] Jirina Slaninova,[‡] Uwe Klein, and Falk Fahrenholz[*]

Max-Planck-Institut für Biophysik, Kennedyallee 70, W-60596 Frankfurt, Federal Republic of Germany, and Institute of Organic Chemistry and Biochemistry, Czech Academy of Sciences, 16610 Praha 6, Czech Republic

Neurohypophyseal hormone analogues containing sarcosine (Sar) in position 7 were prepared to design more potent and selective oxytocin antagonists. The three analogues (1–3) of [Sar[7]]arginine-vasopressin ([Sar[7]]AVP) and six analogues (4–9) of [Sar[7]]arginine-vasotocin ([Sar[7]]AVT) had a reduced affinity for antidiuretic V_2 receptors. The [Sar[7]]AVP derivatives (1–3) were potent antiuterotonic (in vitro pA_2 = 7.5–8.4, in vivo 6.6–7.1) and antipressor (pA_2 = 7.2–8.0) agents. The [Sar[7]]AVT analogues (4–9) were more potent and selective uterotonic antagonists (in vitro pA_2 = 7.9–8.6, in vivo 7.1–7.5); their antipressor potencies were reduced (pA_2 = 6.4–7.7). The change of the antagonistic potencies was paralleled by a change in the receptor affinities. Among other antiuterotonic analogues, [Mca[1], D-Phe[2], Sar[7]]AVT (4, Mca = β-mercapto-β,β-cyclopentamethyl-enepropionic acid) and [Mca[1], D-Tyr(OEt)[2],Sar[7]]AVT (6) were synthesized, two highly potent antiuterotonic compounds (in vitro pA_2 = 8.3, in vivo 7.4 and 7.5, respectively) with reduced antipressor activity (pA_2 = 6.4) and reduced binding affinity to V_2 receptors (K_d = 421 and 35 nM, respectively) and no anti-antidiuretic effect. Another potent antiuterotonic analogue, [Mca[1], D-Trp[2],-Sar[7]]AVT (9, in vitro pA_2 = 7.9, in vivo 7.5) has virtually no binding capability to V_2 receptors ($K_d \sim$ 0.3 mM). These analogues should lead to the design of even more potent and selective oxytocin antagonists.

Introduction

In recent years, analogues of the neurohypophyseal hormones arginine–vasopressin (AVP) and oxytocin (OT) with antagonistic properties (antipressor [V₁], anti-antidiuretic [V₂], and antiuterotonic [OT] antagonists) have been developed as potential pharmacological tools in the treatment of several diseases.[1–3] Oxytocin antagonists with antiuterotonic potency can be useful in the therapy of preterm labor and primary dysmenorrhoea.[3,4] For a potential drug, high selectivity is required for the exclusion of side effects.[5] Neurohypophyseal hormone antagonists have recently been synthesized with high V₂/V₁ and V₁/V₂ potency ratios, but only a few antagonists are known with an acceptable OT/V₁, i.e. antiuterotonic/antipressor (aUT/aP), potency ratio. Even in these cases, the antipressor potency of the analogues is only up to 10-fold lower than the antiuterotonic potency.[6–10]

Oxytocin antagonists with sarcosine (Sar) instead of Pro in position 7 exhibit enhanced uterotonic activity and selectivity: the introduction of Sar[7] into oxytocin (OT), deamino-OT ([Mpa[1]]OT), and [Thr[4]]OT yielded analogues with high oxytocic activity, while their vasopressin-like (V₁ and V₂) activities were suppressed.[11,12] [Sar[7]]AVP

had also a lower antidiuretic activity than AVP.[11] In the case of V₁ receptor and OT receptor antagonists, Sar[7]/Pro[7] substitution to give [Dep[1],Sar[7]]AVP, [Mca[1],Sar[7]]-AVP, and [Mca[1],D-Phe[2],Sar[7]]AVP resulted in severely depressed antidiuretic activities, with a slightly weakened pressor and a slightly weakened or maintained uterotonic antagonism.[13,14] This change may be connected with the altered conformational mobility of the C-terminal part of the peptides containing Sar in positin 7.[15] Thus, as concerns antidiuresis, it is likely that further oxytocin and/or pressor antagonists containing Sar[7] probably will have only a weak V₂ effect. If further modifications of these analogues were to result in a higher aUT/aP selectivity, the design of highly specific OT antagonists could be achieved.

We decided to prepare selective OT antagonists containing Sar[7] substitution with a view to attaing an enhanced aUT/aP selectivity. In designing the new analogues, we took into consideration that (a) the most potent pressor inhibitors are AVP analogues, i.e. containing Phe[3] and Arg[8], and (b) the most potent uterotonic inhibitors are oxytocin analogues containing Leu or a basic amino acid in position 8, i.e. containing Ile in position 3 and Leu/Arg/Orn in position 8.[16] We synthesized six analogues with the general structure [X[1],Y[2],Ile[3],Sar[7],Arg[8]]VT ([Sar[7]]-arginine-vasotocin analogues) and three analogues [X[1],Y[2],-Phe[3],Sar[7],Arg[8]]VP ([Sar[7]]AVP analogues). These compounds were tested for binding affinity to the three main types of neurohypophyseal hormone receptors (V₁, V₂, and OT) because preliminary reports suggested a good correlation between receptor binding affinity and antagonistic property.[13,17,18] They were also tested in the rat oxytocic bioassay in vitro in the presence or absence of magnesium salt and in the rat uterotonic assay in vivo. Their antivasopressor potencies were assessed via their ability to inhibit the pressor response to exogenous AVP[19] in the

Table 1. Physicochemical Characteristics of Oxytocin Antagonists[a]

no.	analogue	formula	M + H[+b]	% yield[c]	HPLC, k' [d]	TLC, R_f[e] A	TLC, R_f[e] B
1	[Mca[1], D-Tyr(OEt)[2],Sar[7]]AVP	$C_{51}H_{74}N_{14}O_{12}S_2$	1139.2	19	8.11	0, 39	0, 65
2	[Mca[1],D-Tyr[2],Sar[7]]AVP	$C_{49}H_{70}N_{14}O_{12}S_2$	1111.3	27	7.71	0, 34	0, 61
3	[Mca[1],D-Ile[2],Sar[7]]AVP	$C_{46}H_{72}N_{14}O_{11}S_2$	1062.7	21	7.02	0, 31	0, 56
4	[Mpa[1],D-Phe[2],Sar[7]]AVT	$C_{41}H_{64}N_{14}O_{11}S_2$	993.4	30	5.14	0, 21	0, 45
5	[Mca[1],D-Phe[2],Sar[7]]AVT	$C_{48}H_{72}N_{14}O_{11}S_2$	1061.3	24	6.91	0, 31	0, 57
6	[Mpa[1], D-Tyr(OEt)[2],Sar[7]]AVT	$C_{43}H_{68}N_{14}O_{12}S_2$	1037.2	23	6.37	0, 23	0, 47
7	[Mca[1],D-Tyr(OEt)[2],Sar[7]]AVT	$C_{48}H_{76}N_{14}O_{12}S_2$	1105.5	20	7.90	0, 37	0, 56
8	[Mca[1],Tyr(OMe)[2],Sar[7]]AVT	$C_{47}H_{74}N_{14}O_{12}S_2$	1091.9	22	6.84	0, 60	0, 76
9	[Mca[1],D-Trp[2],Sar[7]]AVT	$C_{48}H_{73}N_{15}O_{11}S_2$	1100.4	14	7.99	0, 38	0, 67

[a] For analytical methods, see the Experimental Section. [b] Data obtained by FAB-MS. [c] These yields are based on the milliequivalents of starting Boc-amino acid-resin. [d] All compounds were at least 95–98% pure as shown by the HPLC pattern. Detection was at 220 nm. [e] The composition of solvents A and B is given in the Experimental Section.

Table 2. Amino Acid Analyses Ratios of Oxytocin Antagonists[a]

no.	analogue	amino acid analyses ratios Tyr	Phe	Ile	Glu	Asp	Cys	Arg	Gly
1	[Mca[1],D-Tyr(OEt)[2],Sar[7]]AVP	0.67	0.97		0.94	1.01	0.43	1.04	1.00
2	[Mca[1],D-Tyr[2],Sar[7]]AVP	0.87	0.98		0.97	0.99	0.45	0.96	1.00
3	[Mca[1],D-Ile[2],Sar[7]]AVP		0.94	1.03	0.89	0.96	0.40	1.01	1.00
4	[Mpa[1],D-Phe[2],Sar[7]]AVT		0.87	0.91	0.98	0.96	0.47	0.99	1.00
5	[Mca[1],D-Phe[2],Sar[7]]AVT		0.90	0.93	0.90	0.97	0.43	1.07	1.00
6	[Mpa[1],D-Tyr(OEt)[2],Sar[7]]AVT	0.63		0.87	0.92	0.81	0.38	0.94	1.00
7	[Mca[1],D-Tyr(OEt)[2],Sar[7]]AVT	0.69		0.90	0.99	0.92	0.43	1.03	1.00
8	[Mca[1],Tyr(OMe)[2],Sar[7]]AVT	0.66		0.94	0.97	1.03	0.31	0.97	1.00
9	[Mca[1],D-Trp[2],Sar[7]]AVT			1.03	1.07	0.96	0.43	0.91	1.00

[a] Applied Biosystem automated amino acid analyzer was used. Peptides were hydrolyzed with HCl in the gas phase. Norleucin was used as internal standard. Molar ratios were referred to Gly (=1.00).

pitched rat preparation. Three of the highly potent antagonists were also tested for anti-antidiuretic activity.[20]

Results and Discussion

Peptides were prepared by the solid-phase method of peptide synthesis on Merrifield resin[21] with Boc chemistry, utilizing a method described earlier.[14] The protected peptide intermediates were cleaved from the resin by ammonolysis. Protecting groups were removed by reduction with sodium in ammonia. Unprotected peptides were cyclized in water with $K_3Fe(CN)_6$, purified by gel chromatography on a Sephadex G-15 column. The purity and structure of the peptides were assessed by TLC, analytical RP-HPLC, amino acid analysis, and FAB-MS. Analytical data are presented in Table 1 and the results of the amino acid analysis in Table 2.

The analogues were examined in the rat in vitro and in vivo uterotonic tests and in the rat in vivo pressor assay.[19] The parameters (dissociation constant, K_d) of binding of the peptides to the V_1 receptor in rat liver plasma membranes,[17] to the V_2 receptor in bovine kidney inner medulla membranes,[14] and to the OT receptor in guinea pig myometrium membranes[14] were determined in competition experiments with [^{3}H]AVP (V_1 and V_2 receptors) and [^{3}H]OT (OT receptors).[14] All biological activities are listed in Table 3, together with the parameters of some other relevant peptides: [Mca[1]]AVP,[22] [Mca[1],Sar[7]]AVP,[13] [Mca[1],D-Phe[2]]AVP,[23] [Mca[1],D-Phe[2],Sar[7]]AVP,[14] [Mca[1],-Tyr(OMe)[2]]AVP,[22] [Mca[1],Tyr(OMe)[2],Orn[8]]VT,[24] and [Mca[1],D-Trp[2]]AVT.[25]

All newly synthesized analogues had a markedly reduced affinity for V_2 receptors as compared with AVP. The apparent dissociation constants (K_d) were 20 to 1.5 × 10^5 times higher than that of AVP. The affinity of [Sar[7]]-AVT analogues (4–9) for V_2 receptor were somewhat lower than those of the [Sar[7]]AVP compounds (1–3). This is in accord with the fact that V_2 antagonists are derived from

AVP containing Phe in position 3 and Arg in position 8.[1] As far as the antidiuretic activity is concerned, three compounds (4, 6, and 7) were tested for anti-antidiuretic potency in the arrangement according to Lammek et al.[20] However, no enhancement of the urine flow was seen in the dose range 1–80 μg/kg. The activity at the V_2 receptor was determined by the same method which has been used for the structurally related reference compounds [Mca[1],D-Phe[2]]AVP[23] (pA_2 = 7.21) and [Mca[1],D-Phe[2],Sar[7]]AVP[14] (0.08 IU/mg). No anti-antidiuretic activity was observed for the above three compounds. It is reasonable to suppose that their agonistic activity does not exceed that of [Mca[1],D-Phe[2],Sar[7]]AVP. Thus, our analogues with low V_2 receptor affinity can have only a very weak biological effect on V_2 receptors.

For the [Sar[7]]AVP ([X[1],Y[2],Phe[3],Sar[7],Arg[8]]VP) analogues (1–3), there was no significant differences between antiuterotonic and antipressor activities. Well-established substitutions of AVP, such as Mca in position 1[26] and D-Tyr or D-Tyr(OEt) in position 2[22] combined with Sar[7], resulted in potent OT and V_1 antagonists without an improved selectivity towards oxytocin activity: aUT/aP ratio of 2.5 and 1, respectively, for analogue 1 and 2. The affinities of these two peptides for V_1 receptors were higher than that of AVP, whereas for OT receptors they were 3–23-fold lower than that of OT. The substitution with D-Ile[2] as in analogue 3, originally known from V_2 antagonists but also used in potent uterotonic antagonists,[27,28] displayed moderately reduced OT and V_1 antagonistic activities, too (aUT/aP ratio 2.0).

As assumed for the change of Phe[3] to Ile[3] (synthesis of [Sar[7]]AVT antagonists ([X[1],Y[2],Phe[3],Sar[7],Arg[8]]VP, 4–9), the antiuterotonic activity was maintained and the antipressor activity was somewhat decreased, the aUT/aP ratio being between 2.5 and 80. Correspondingly, the affinity for OT receptors was relatively higher and that for V_1 receptors lower. Most compounds have an OT/V_1

Table 3. Binding Affinities and Biological Activities of the New Oxytocin Antagonists (1–9) and Some Other Peptides[a]

no.	peptide	affinity to the receptors: apparent dissociation constants (K_d) (nM)			antiuterotonic activity (pA_2 values)			antipressor activity in vivo (pA_2 values)	selectivity aUT/aP ratio[b]
		OT	V_2	V_1	in vitro		in vivo		
					no Mg^{2+}	1 mM Mg^{2+}			
	AVP		1.5 ± 0.1	0.65 ± 0.04					
	oxytocin	6.7 ± 0.5							
	[Mca1]AVP[c]				8.15			8.35	0.6
	[Mca1,Sar7]AVP[d]		240 ± 30	0.59 ± 0.11	6.95 ± 0.13			7.93 ± 0.03	0.1
	[Mca1,D-Phe2]AVP[e]				8.59			8.35	2.0
	[Mca1,D-Phe2,Sar7]AVP[f]	7 ± 1	98 ± 14	0.96 ± 0.14	8.1		7.6	7.8	2.0 (0.6)
1	[Mca1,D-Tyr(OEt)2,Sar7]AVP	21 ± 6	29 ± 6	0.32 ± 0.04	8.4 ± 0.2		7.1 ± 0.1[i]	8.0 ± 0.2	2.5 (0.1)
2	[Mca1,D-Tyr2,Sar7]AVP	160 ± 40	230 ± 50	0.22 ± 0.02	8.0 ± 0.1		7.0 ± 0.1[i]	8.0 ± 0.1	1.0 (0.1)
3	[Mca1,D-Ile2,Sar7]AVP	265 ± 89	182 ± 52	8.28 ± 1	7.5 ± 0.2	7.0 ± 0.1	6.6 ± 0.1	7.2 ± 0.2	2.0 (0.25)
4	[Mpa1,D-Phe2,Sar7]AVT	8 ± 2	421 ± 120	50.1 ± 6	8.3 ± 0.2	8.0 ± 0.1	7.4 ± 0.2	6.4 ± 0.2	80 (10)
5	[Mca1,D-Phe2,Sar7]AVT	8 ± 2	311 ± 88	8.28 ± 1	8.0 ± 0.1	8.0 ± 0.2	7.4 ± 0.1	7.0 ± 0.1	10 (2.5)
6	[Mpa1,D-Tyr(OEt)2,Sar7]AVT	27 ± 7	35 ± 10	13.1 ± 1.6	8.3 ± 0.1	8.1 ± 0.1	7.5 ± 0.2	6.4 ± 0.2	80 (12.6)
7	[Mca1,D-Tyr(OEt)2,Sar7]AVT	15 ± 4	130 ± 36	0.62 ± 0.08	7.9 ± 0.1	7.9 ± 0.2	7.1 ± 0.2[i]	7.5 ± 0.2	2.5 (0.5)
	[Mca1,Tyr(OMe)2]AVP[c]				8.13			8.62	0.3
	[Mca1,Tyr(OMe)2,Orn8]VT[g]				8.52			7.96	3.6
8	[Mca1,Tyr(OMe)2,Sar7]AVT	18 ± 6	811 ± 233	2.0 ± 0.8	8.6 ± 0.1	8.2 ± 0.1	7.5 ± 0.1[i]	7.7 ± 0.1	8.0 (1.0)
	Mca1,D-Trp2]AVT[h]					7.77			
9	[Mca1,D-Trp2,Sar7]AVT	6 ± 2	3 × 10⁵	3.8 ± 0.6	7.9 ± 0.3	7.7 ± 0.1	7.5 ± 0.1[i]	7.5 ± 0.2	2.5 (1.0)

[a] Preliminary results presented in part at the European Peptide Symposium, in Interlaken, Switzerland, September 1992, and in the proceedings of the symposium.[34] Results from rat uterotonic and pressor assays, mean ± SE. [b] Reciprocal values of the ratio of effective doses in the uterotonic in vitro (mol/L) and pressor tests (mol/kg). In parentheses the reciprocal values of that in uterotonic in vivo (mol/kg) and pressor test (mol/kg). [c] From Kruszynski et al.[22] [d] From Gazis et al.[13] [e] From Manning et al.[23] [f] From Kasprzykowski et al.[14] [f] From Bankowski et al.[24] [h] From Flouret et al.[25] [i] The inhibition of the response to oxytocin was stronger 10–20 min after administration that 1 min after administration.

receptor affinity ratio below 0.2 if there is no selectivity of the uterotonic effect. For our AVT analogues which were found selective for a uterotonic effect, there was both a higher affinity OT/V_1 ratio (0.5–6) and a selectivity ratio of in vivo activities aUT/aP of 2.5–12.6. For example [Mca1,D-Phe2,Sar7]AVT (5) had a better aUT/aP ratio than [Mca1,D-Phe2,Sar7]AVP[14]: 10 and 2.0, respectively. The OT/V_1 receptor affinity ratio is also enhanced: 1.04 and 0.14, respectively. Another good example of the enhanced selectivity of [Sar7]AVT analogues over AVP analogues is [Mca1,Tyr(OMe)2,Sar7]AVT (8) in comparison with [Mca1,Tyr(OMe)2]AVP.[22] Analogue 8 had a rat uterus in vitro test pA_2 value of 8.6, while that for its counterpart was 8.13; the aUT/aP ratio was 8 and 0.3, respectively. In comparison with the [Sar7]AVP analogues, the V_2 receptor affinities of the [Sar7]AVT analogues were further decreased. Analogues 4, 6, 8, and 9 are highly potent antiuterotonic agents both in vitro and in vivo.

The time course of the inhibitory effects of compounds 1, 2, and 7–9 are different from those for other competitive inhibitors. Whereas the inhibitory effect for most analogues is highest 1 min after inhibitor administration, and after 10–20 min (depending on the dose) the response to oxytocin is returning (or has already returned) to its uninhibited value, the inhibitory effects of compounds 1, 2, and 7–9 are higher 10–20 min than 1 min after inhibitor application. However, no calculations for determination of the half-life of the response were performed.

Compounds 4 and 6 are relative selective antiuterotonic agents: their aUT/aP ratio is 80. For these peptides the OT/V_1 receptor affinity ratios are among the highest: 6.25 and 0.49, respectively. [Mca1,D-Trp2,Sar7]AVP (9) has a high antiuterotonic activity and only mild selectivity between OT and V_1 effects (an aUT/aP ratio of 2.5), but it underwent practically no binding to V_2 receptors (K_d as high as 0.3 mM). This observation correlates well with the data of Melin et al.[9] that the introduction of D-Trp2 instead of D-Tyr(OEt)2 in some oxytocin antagonists reduced their antidiuretic effect. Recent results of Flouret

et al. show that [Mca1,D-Trp2]AVT has a high potency as an oxytocin antagonist and a very low potency in the antidiuretic assay.[25,29]

In our discussion, as usual in the literature, the OT/V_1 antagonistic potency (aUT/aP) ratios refer to the antiuterotonic and antipressor potency ratios from a rat uterus in vitro test (in the absence of Mg^{2+}) and pressor test in vivo. This is mainly because the uterotonic in vivo data are usually not available. However, this comparison may be misleading because the in vivo tests reflect more complex metabolic processes. Comparison of the new Sar analogues for which uterotonic in vivo data are available shows that two of them may be taken as truly OT-selective, i.e. analogues 4 and 6 show in vivo aUT/aP ratios of 10 and 12.6.

For neurohypophyseal hormone analogues, and especially in the case of antagonists, the receptor binding assay seems to be a good screening method for the evaluation of biological activity. Our data show, in agreement with the observations of Atke et al.,[18] that potent oxytocin antagonists have a lower affinity for oxytocin receptors than oxytocin: the apparent dissociation constants are several times higher for the synthetic compounds. Potent antipressor analogues, on the other hand, have a higher affinity for V_1 receptors than AVP. We found a close correlation between the antipressor potency and the V_1 receptor binding affinity of our peptides (Figure 1); the correlation coefficient was 0.995. The correlation was somewhat weaker for the oxytocin effect (correlation coefficient 0.922). This discrepancy can probably be explained by the species difference and the heterogeneity of the uterine neurohypophyseal hormone receptors.

Conclusions

We have shown that Sar in position 7 in uterotonic and pressor antagonists consequently reduced the affinity for antidiuretic V_2 receptors. The synthesis of [Sar7]arginine-vasotocin analogues permitted maintenance of the uterotonic antagonism and reduction of the pressor antagonism. These changes were paralleled by the changes in the
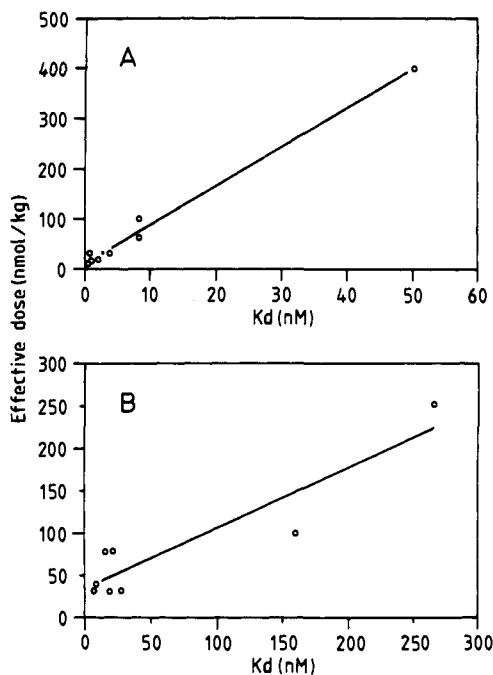
Figure 1. Correlation between receptor affinities (apparent dissociation constants, K_d) and antagonistic potencies (effective doses) of oxytocin antagonists containing sarcosine in position 7: (A) correlation between receptor affinities for rat liver V_1 receptors and antagonistic potencies in rat pressor test; (B) correlation between receptor affinities for guinea pig myometrial oxytocin receptors and antagonistic potencies in rat uterotonic in vivo test.

receptor affinities. Among other antiuterotonic analogues, we synthesized [Mpa^1,D-Phe^2,Sar^7]AVT (4) and [Mpa^1,D-Tyr(OEt)^2,Sar^7]AVT (6), two highly potent antiuterotonic compounds with reduced antipressor activity, reduced binding capability to V_2 receptors and practically no anti-antidiuretic potency. Another potent antiuterotonic analogue, [Mca^1,D-Trp^2,Sar^7]AVT (9), shows practically no binding to V_2 receptors. These analogues can serve as a basis for the design of more potent and selective oxytocin antagonists.

Experimental Section

The analogues were synthesized by solid-phase syntheses described earlier.[14] Chloromethylated resin (Bio-Rad) was esterified[30] by Boc-Gly to an incorporation of 0.81 mmol/g. DCC coupling method was used for the following amino acid derivatives: Boc-Arg(Tos), Boc-Sar, Boc-Cys(Bzl), Boc-Phe, Boc-Ile, Boc-D-Tyr(OEt), Boc-D-Tyr(MeOBzl), Boc-D-Phe, Boc-Tyr-(OMe),Boc-D-Trp, Mpa(Bzl) (Bachem,Bubendorf,Switzerland) and Mca(Bzl) (Novabiochem). Asn and Gln were coupled as active esters: Boc-Asn-ONp and Boc-Gln-ONp (Bachem, Bubendorf, Switzerland). Boc groups were removed in 50% TFA in dichloromethane; in the case of D-Trp, 2% dimethyl sulfoxide was added. The resins containing the protected nonapeptides were ammonolyzed in methanol. After 48 h the solvents were evaporated, and the protected peptide amides were extracted into hot DMF, precipitated with boiling water and left to stand at 4 °C for 2 days. The products were collected by filtration, washed with water, dried, and reprecipitated from EtOH: yield 34-46%. The protected nonapeptides (approximately 200 mg) were dissolved in 300 mL of ammonia. At the boiling point sodium was added until a light blue color persisted. After 20 s, acetic acid was added and the solvent was evaporated. The residues were dissolved in water, the pH was adjusted to 7-7.5, and the solutions were treated with 0.05 M K$_3$[Fe(CN)$_6$]. After the addition of anion-exchange resin and filtration, the crude unprotected and cyclized peptides were lyophilized and chromatographed once or twice on a Sephadex G-15 column. The

major peaks were pooled and lyophilized. The peptides were analyzed by TLC, HPLC, FAB mass spectrometry, and amino acid analysis. Analytical TLC was carried out on 250 μm thick, 5- × 20-cm silica gel plates (60 F-254, E. Merck), and the spots were visualized by ninhydrin or iodine. The following TLC systems were used: A, 1-butanol–acetic acid–water (4:1:1, v/v/v); B, 1-butanol–acetic acid–water (8:5:4, v/v/v). For HPLC characterization (Knauer system) a reversed-phase column (Lichrosorb RP C-18, 4 × 250 mm, Knauer) was used. For elution a 45-mL linear gradient of acetonitrile from 27% to 72% in 0.09% TFA–water solution (v/v/v) with a flow rate of 1.5 mL/min was applied. Detection was at 220 nm. The yields of the syntheses, based on the milliequivalents of starting Boc-amino acid-resins, were 14–30%.

The K_d values of the antagonists were obtained from competitive binding experiments. Plasma membranes from rat liver containing V_1 receptors (prepared by separation based on a two-phase polymer system[31]) and of bovine kidney inner medulla containing V_2 receptors (prepared by differential centrifugation[32]) were incubated with 10 nM [^{3}H]AVP (17.4 Ci/mmol, Amersham) and varying concentrations of the nonlabeled peptides (1-9). Myometrial plasma membranes of late pregnant guinea pig (prepared by the method of Fuchs et al.[33]) were incubated with 10 nM[^{3}H]OT (80 Ci/mmol, NEN) and varying concentrations of the nonlabeled peptides (1-9). The binding parameters were determined by using a weighted, nonlinear least-squares fit to logistic curves.[17]

The biological activities of the analogues were determined in the rat uterus in an in vitro test in Mg^{2+}-free medium or medium containing 1 mM Mg^{2+}.[19] The results are expressed as pA_2 (negative decadic logarithm of the inhibitor concentration [mol/L] halving the effect of oxytocin). An uterotonic in vivo test, using ethanol-anaesthetized rats, was also performed.[19] The results are expressed as the negative decadic logarithm of the effective concentration (mol/kg) reducing the effect of a 10 mIU dose of oxytocin to the level of 5 mIU of oxytocin applied 1 min after administration of the inhibitor. Antipressor activity was measured in the pressor test on pitched rats. Results are expressed as the negative decadic logarithm of the effective concentration (mol/kg) reducing the effect of a 1 × 10^{-5}-mg dose of AVP to the level of 5 × 10^{-5}-mg AVP.[19] The anti-antidiuretic potency of the analogues 4, 6, and 7 was evaluated by their ability to inhibit the antidiuretic effect of endogenous AVP.[20]

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