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An Investigation of Position 3 in Arginine Vasopressin with Aliphatic, Aromatic, Conformationally-Restricted, Polar and Charged Amino Acids[1]

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Abstract: We report the solid-phase synthesis and some pharmacological properties of 23 new analogs of arginine vasopressin (AVP) which have the Phe^3 residue replaced by a broad variety of amino acids. Peptides 1–9 have at position 3: (1) the mixed aromatic/aliphatic amino acid thienylalanine (Thi) and the aliphatic amino acids; (2) cyclohexylalanine (Cha); (3) norleucine (Nle); (4) Leu; (5) norvaline (Nva); (6) Val; (7) alpha-aminobutyric acid (Abu); (8) Ala; (9) Gly. Peptides 10–23 have at position 3: the aromatic amino acids, (10) homophenylalanine (Hphe); (11) Tyr; (12) Trp; (13) 2-naphthylalanine (2-Nal); the conformationally-restricted amino acids (14) Pro; (15) 2-aminotetraline-2-carboxylic acid (Atc); the polar amino acids (16) Ser; (17) Thr; (18) Gln; and the charged amino acids (19) Asp; (20) Glu; (21) Arg; (22) Lys; (23) Orn. All 23 new peptides were evaluated for agonistic and, where appropriate, antagonistic activities in *in vivo* antidiuretic (V_2-receptor) and vasopressor (V_{1a}-receptor) assays and in *in vitro* (no Mg^{2+}) oxytocic assays. The corresponding potencies (units/mg) in these assays for AVP are: 323 ± 16; 369 ± 6 and 13.9 ± 0.5. Peptides 1–9 exhibit the following potencies (units/mg) in these three assays: (1) 379 ± 14; 360 ± 9; 36.2 ± 1.9; (2) 294 ± 21; 73.4 ± 2.7; 0.33 ± 0.02; (3) 249 ± 28; 84.6 ± 4.3; 4.72 ± 0.16; (4) 229 ± 19; 21.4 ± 0.6; 2.1 ± 0.2; (5) 134 ± 5; 31.2 ± 0.9; 28.4 ± 0.2; (6) 114 ± 9; 45.3 ± 2.3; 11.3 ± 1.6; (7) 86.7 ± 2.5; 4.29 ± 0.13; 0.45 ± 0.03; (8) 15.5 ± 1.5; 0.16 ± 0.01; ~ 0.02; (9) 3.76 ± 0.03; < 0.02; *in vitro* oxytocic agonism was not detected. These data show that the aliphatic amino acids Cha, Nle, Leu, Nva and Val are well-tolerated at position 3 in AVP with retention of surprisingly high levels of antidiuretic activity. Peptides 2–9 exhibit significant gains in both antidiuretic/vasopressor (*A*/*P*) and antidiuretic/oxytocic (*A*/*O*) selectivities relative to AVP. [Thi^3]AVP appears to be a more potent antidiuretic and oxytocic agonist than AVP and is equipotent with AVP as a vasopressor agonist. The antidiuretic potencies of peptides 10–23 exhibit drastic losses relative to AVP. They range from a low of 0.018 ± 0.001 units/mg for the Lys^3 analog (peptide 22) to a high of 24.6 ± 4.6 units/mg for the $Hphe^3$ analog (peptide 10). Their vasopressor potencies are also drastically reduced. These range from a low of < 0.002 units/mg for peptide 22 to a high of 8.99 ± 0.44 units/mg for the Atc^3 analog (peptide 15). Peptides 10–23 exhibit negligible or undetectable *in vitro* oxytocic agonism. The findings on peptides 10–23 show that position 3 in AVP is highly intolerant of changes with aromatic, conformationally-restricted, polar and charged amino acids. Furthermore, these findings are in striking contrast to our recent discovery that position 3 in the potent V_2/V_{1a}/OT antagonist $d(CH_2)_5$D-Tyr(Et)^{2}VAVP tolerates a broad latitude of structural change at position 3 with many of the same amino acids, to give excellent retention of antagonistic potencies. The data on peptides 1–4 offer promising clues to the design of more potent and selective AVP V_2 agonists. Copyright © 1999 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: agonist; aliphatic; aromatic; conformationally-restricted; polar; charged amino acids

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[1] Symbols and abbreviations are in accordance with the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (*Eur. J. Biochem. 180*, A9–A11, 1989).

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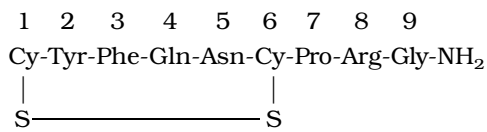
Table 1 Some Pharmacological Activities of AVP Analogs Modified at Position 3 with Thienylalanine and Aliphatic Amino Acids

No.	Peptide	Antidiuretic (*A*) (units/mg)	Vasopressor (*P*) (units/mg)	Oxytocic (*O*) (no Mg^{2+}) (units/mg)	*A*/*P*	*A*/*O*
	AVP[a]	323 ± 16	369 ± 6	13.9 ± 0.5	0.9	23.2
	AVT ([Ile^3]AVP)[a]	231 ± 30	160 ± 4	127 ± 9	1.4	1.8
	LVP (Lys^{8}VP)[a,b]	284 ± 39	270 ± 15	10.1 ± 0.3	1.05	28.1
	LVT ([Ile^3]LVP)[b]	24 ± 3	130 ± 13	78 ± 10	0.2	0.3
A	[Thi^3]LVP[c]	332 ± 32	243 ± 5	19.0 ± 0.5	1.4	17.5
1	[Thi^3]AVP[d,e]	379 ± 14	360 ± 9	36.2 ± 1.9	1.05	10.5
2	[Cha^3]AVP[d]	294 ± 21	73.4 ± 2.7	0.33 ± 0.02	4	890
3	[Nle^3]AVP[d,e]	249 ± 28	84.6 ± 4.3	4.72 ± 0.16	2.9	52.8
4	[Leu^3]AVP[d,e]	229 ± 19[f]	21.4 ± 0.6	2.1 ± 0.2	10.7	109
5	[Nva^3]AVP[d,e]	134 ± 5	31.2 ± 0.9	28.4 ± 0.2	4.3	4.7
6	[Val^3]AVP[d,e]	114 ± 9[f]	45.3 ± 2.3	11.3 ± 1.6	2.5	10.1
7	[Abu]AVP[d,e]	86.7 ± 2.5	4.29 ± 0.13	0.45 ± 0.03	20.2	192
8	[Ala^3]AVP[d]	15.5 ± 1.5	0.16 ± 0.01	~0.02	97	~775
9	[Gly^3]AVP[d]	3.76 ± 0.03	<0.02	ND	>188	

ND, non-detectable up to 100 μg tested.
[a] Data form Manning *et al.* [20].
[b] Data from Berde *et al.* [3].
[c] Data from Smith *et al.* [25].
[d] This publication.
[e] Preliminary data reported in Manning *et al.* [34].
[f] Preliminary data reported in Sawyer *et al.* [31].

Since the original synthesis of arginine vasopressin (AVP) by du Vigneaud and colleagues [1], this cyclic octapeptide and its naturally occurring Lys^8 analog (LVP) [2] have been the subject of intensive investigations aimed at the design of potent and selective agonists and antagonists of the receptors which mediate the characteristic biological responses to these peptides [3–7] and to the closely related peptide, oxytocin (OT) [8]. The four most well-characterized responses and the receptors which mediate them are: antidiuretic (V_2-receptor), vasopressor (V_{1a}-receptor), ACTH-releasing (V_{1b}-receptor) and oxytocic (uterine-receptor) [3,9–11]. Human V_2, V_{1a}, V_{1b} and OT receptors have recently been cloned as well as V_{1a} receptors from rats and V_2 and OT receptors from pigs [12–18]. AVP has the following structure:



While, over the years, hundreds of analogs of AVP with modifications at virtually all positions 1–9 have been synthesized and pharmacologically evaluated [2–7], position 3 has been largely ignored. Accordingly, the syntheses of only a handful of position-3 analogs of AVP and LVP have been reported to date [2–7]. The most notable position-3-modified analog of AVP is the naturally occurring Ile^3 analog, arginine vasotocin (AVT) [3,19]. AVT exhibits potent antidiuretic, vasopressor and oxytocic activities [3,19,20] (Table 1). While it is somewhat less potent than AVP as an antidiuretic and a vasopressor agonist, it is substantially more potent than AVP as an

Abbreviations: Abu, alpha-aminobutyric acid; Atc, 2-aminotetraline-2-carboxylic acid; AVP, arginine vasopressin; AVT, arginine vasotocin; Boc, *tert*-butyloxycarbonyl; Bu^t, *tert*-butyl; Cha, cyclohexylalanine; DCC, dicyclohexylcarbodiimide; DIPEA, *N*,*N*-diisopropylethylamine; Et_2O, diethyl ether; Fm, 9-fluorenylmethyl; HOBt, *N*-hydroxybenzotriazole; Hphe, homophenylalanine; LVP, lysine vasopressin; LVT, lysine vasotocin; MeCN, acetonitrile; 2-Nal, 2-naphthylalanine; Nle, norleucine; Nva, norvaline; ONp, *p*-nitrophenylester; OT, oxytocin; Pmc, 2,2,5,7,8-pentamethyl-chroman-6-sulfonyl; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; Thi, thienylalanine; V_2, antidiuretic; V_{1a}, vasopressor.

Table 2 Pharmacological Activities of AVP Analogs Modified at Position 3 with Aromatic, Conformationally-Restricted, Polar and Charged Amino Acids

No.	Peptide	Antidiuretic (*A*) (units/mg)	Vasopressor (*P*) (units/mg)	Oxytocic (*O*) (no Mg^{2+}) (units/mg)	*A*/*P*	*A*/*O*
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	LVT (Ile^3]LVP)[a,b]	24 ± 3	130 ± 13	78 ± 10	0.2	0.3
10	[Hphe^3]AVP[c]	24.6 ± 4.6	1.88 ± 0.11	~0.01	13.1	~2460
B	[Tyr^3]LVP[b]	0.18 ± 0.08	1.6 ± 0.2	~0.01	0.1	~18
11	[Tyr^3]AVP[c,e]	20.3 ± 2.2	4.93 ± 0.20	0.026 ± 0.003	4.1	781
C	[Trp^3]LVP[b]	–	~0.07	<0.01		
12	[Trp^3]AVP[c,e]	1.60 ± 0.18	0.31 ± 0.01	~0.01	5.2	~160
13	[2-Nal^3]AVP[c,d,e,g]	1.53 ± 0.25	3.60 ± 0.21	~0.31	0.4	~4.9
D	[Tic^3]AVP[f]	0.91 ± 0.08	0.25 ± 0.03	~0.022	3.6	~41.4
14	[Pro^3]AVP[c,e]	0.46 ± 0.03	<0.003	<0.005	>153	>92
15	[Atc^3]AVP[c,e]	12.7 ± 1.2	8.99 ± 0.44	0.11 ± 0.004	1.4	115
E	[Ser^3]LVP[b]	~0.02	<0.01	<0.01	>2.0	>2.0
16	[Ser^3]AVP[c,e]	3.23 ± 0.39	0.38 ± 0.01	0.02 ± 0.003	8.5	161
17	[Thr^3]AVP[c]	6.38 ± 0.19	0.086 ± 0.004	0.083 ± 0.008	74.2	76.9
18	[Gln^3]AVP[c]	2.78 ± 0.63	<0.05	ND	>556	
19	[Asp^3]AVP[c]	~0.05	<0.005	ND	>10	
20	[Glu^3]AVP[c]	~0.04	<0.003	ND	>13.3	
21	[Arg^3]AVP[c]	0.019 ± 0.003	<0.003	<0.005	>6.3	>3.8
22	[Lys^3]AVP[c]	0.018 ± 0.001	<0.002	ND	>9.0	
23	[Orn^3]AVP[c]	0.08 ± 0.01	<0.003	ND	>26.7	

ND, not detectable up to 100 μg tested.
[a,b] See corresponding footnotes to Table 1.
[c] This publication.
[d] Reported in reference [33] as a weak antidiuretic agonist with vasopressor activity = 56 ± 12.7 units/mg.
[e] Preliminary data reported in Manning *et al.* [34].
[f] Data from Manning *et al.* [28].
[g] Also exhibits weak *in vitro* antagonism: $pA_2 \sim 6.1$.

oxytocic agonist (Table 1). By contrast, lysine vasotocin (LVT) [21] the Ile^3 analog of LVP, while possessing potent vasopressor and oxytocic agonism, exhibits only about 10% of the antidiuretic agonism of LVP [3,21] (Table 1).

Most of the early studies of position 3 focused on modifications of LVP [3,21–24]. The Ser^3, Tyr^3 and Trp^3 analogs of LVP exhibit drastic reductions in antidiuretic and vasopressor potencies relative to LVP [3,21–24] (Table 2). Thus, based on these position-3 analogs of LVP and, notwithstanding the pharmacological profile of AVT [3,19,20] and a later report that the thienylalanine^3 analog of LVP, [Thi^3]LVP [25], exhibits surprisingly high antidiuretic and vasopressor potencies, position 3 in AVP has long been considered intolerant of changes [3–5,21–24,26]. Consequently, this position has been virtually ignored in structure/activity studies of AVP. Recent reports of position-3 analogs of LVP and AVP indeed support the conclusion that position 3 in these molecules is highly intolerant of structural modifications [27,28]. Thus, replacement of the Phe^3 residue in LVP and in AVP by the conformationally-restricted amino acid 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic) to give [Tic^3]LVP [27] and [Tic^3]AVP [28], respectively, led in each instance to drastic losses of agonistic activities. In contrast to these findings, we recently reported the surprising finding that the Phe^3 residue in the potent non-selective AVP V_2/V_{1a} antagonist [1-(β-mercapto-β,β-pentamethylenepropionoic acid), 2-*O*-ethyl-D-tyrosine, 4-valine] arginine vasopressin (d$(CH_2)_5$[D-Tyr(Et)^2]VAVP) [29] could be replaced by a variety of amino acids (aromatic, aliphatic and conformationally-restricted) with moderate to full retention of V_2 antagonism [30].

These findings on position-3-modified AVP antagonists prompted us to reinvestigate the role of position 3 in AVP agonists. We decided to explore the effects of replacement of the Phe[3] residue in AVP with a broad variety of aliphatic, aromatic, conformationally-restricted, polar, acidic and basic amino acids. We had indeed previously synthesized two analogs of AVT, [3-valine] vasotocin and [3-leucine] vasotocin, specifically for use as research tools in a study aimed at identifying the vasotocin-like activity in the head ganglia of gastropod molluscs [31] but had not reported their synthesis or detailed pharmacological properties. Both peptides can also be viewed as 3-substituted analogs of vasopressin, i.e. [Leu[3]]AVP and [Val[3]]AVP and thus, could be part of this structure/activity study of position 3 in AVP. We selected the following additional amino acids as replacements for the Phe[3] residue in AVP: Gly, Ala, Abu, Nva, Nle, Cha, Thi, Hphe, Tyr, Trp, Pro, Atc, Ser, Thr, Gln, Asp, Glu, Arg, Lys, Orn. During the course of these studies, Lammek and colleagues reported that the 2-naphthylalanine[3] (2-Nal[3]) analog of AVP exhibits potent antidiuretic agonism, in the order of 5 million units/mg [32]. These findings were very much at variance with the emerging data from the above series of position-3-modified analogs of AVP. We thus decided to resynthesize [2-Nal[3]]AVP and to include our findings in this study. In the meantime, Lammek and collaborators reported that their earlier finding of extraordinary high antidiuretic activity for this peptide was in error [33]. We now report the synthesis and some pharmacological properties of the following 23 position-3-modified analogs of AVP.

1. [Thi[3]]AVP (Thi = thienylalanine)
2. [Cha[3]]AVP (Cha = cyclohexylalanine)
3. [Nle[3]]AVP (Nle = norleucine)
4. [Leu[3]]AVP
5. [Nva[3]]AVP (Nva = norvaline)
6. [Val[3]]AVP
7. [Abu[3]]AVP
8. [Ala[3]]AVP
9. [Gly[3]]AVP
10. [Hphe[3]]AVP (Hphe = homophenylalanine)
11. [Tyr[3]]AVP
12. [Trp[3]]AVP
13. [2-Nal[3]]AVP (2-Nal = 2-naphthylalanine)
14. [Pro[3]]AVP
15. [Atc[3]]AVP (Atc = 2-aminotetraline-2-carboxylic acid)
16. [Ser[3]]AVP
17. [Thr[3]]AVP
18. [Gln[3]]AVP
19. [Asp[3]]AVP
20. [Glu[3]]AVP
21. [Arg[3]]AVP
22. [Lys[3]]AVP
23. [Orn[3]]AVP

Preliminary data on the antidiuretic activities of peptides 4 and 6 were reported by Sawyer *et al.* [31]. A preliminary report on 12 of these peptides (nos. 1, 3–7, 11–16) has also been presented ([34]).

Peptide Synthesis

The synthesis of the free peptides 1–23 (Table 4) was carried out utilizing two solid-phase methods [35–42]. Due to the incompatibility of the thienylalanine and naphthylalanine residues with the Na/NH_3 procedure [25,43], it was necessary to use the Rink amide resin [35] and the Fmoc strategy [36,37] for the synthesis of peptides 1 and 13. The deprotections were carried out with 20% piperidine/DMF [36]. Coupling reactions were performed primarily by DCC/HOBt in DMF [44] except for Fmoc-Asn and Fmoc-Gln which were incorporated as their *p*-nitrophenyl esters [37,45] in DMF. The simultaneous cleavage of the peptides from the resin and their deprotection was mediated by Reagent K [37]. For peptides 2–12 and 14–23 the Merrifield solid-phase method [38,39] with the modifications previously described [20,40–42] was used. Starting from Boc-Gly-resin the protected precursors II–XII, XIV–XXIII (Table 3) were synthesized. HCl (1 M)/AcOH was used in all the deprotection steps except those involving Boc-Gln in which TFA was employed [20,41,42]. Neutralizations were carried out with 10% Et_3N/CH_2Cl_2. Coupling reactions were mediated mainly by DCC/HOBt [44] in CH_2Cl_2/DMF except for Boc-Asn and Boc-Gln which were incorporated as their *p*-nitrophenyl esters [46] in DMF. Cleavage from the acylpeptide resin was by ammonolysis in methanol with DMF extraction [41,47] to give the protected peptide amides II–XII, XIV–XXIII (Table 3). Na in liquid NH_3 [1,2,48] was used to deblock each protected precursor as previously described [20,41,42]. The resulting disulfydryl compounds from both the Reagent K treatment and the Na/Liquid NH_3 treatment were oxidatively cyclized with $K_3[Fe(CN)_6]$ using the normal procedure [49] or a modified reverse procedure [50]. The free peptides were desalted and purified by gel filtration on Sephadex G-15 and Sephadex LH-20 mainly in a two-step procedure [51] using 50% AcOH and 2 M AcOH as eluents, respectively, as previously de-

Table 3 Physicochemical Properties of the Protected Peptides II–XII and XIV–XXIII

No.	Peptide	Yield (%)	M.p. (°C)	$[\alpha]_D^{25}$ ($c = 1$) DMF	TLC			
					a	b	c	d
II	Z-Cys(Bzl)-Tyr(Bzl)-Cha-Gln-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	92.3	208–209	−32.9	0.54	0.49	0.73	0.81
III	Z-Cys(Bzl)-Tyr(Bzl)-Nle-Gln-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	88.8	231–232	−36.3	0.46	0.61	0.74	0.77
IV	Z-Cys(Bzl)-Tyr(Bzl)-Leu-Gln-Asn-Cys(Bzl)-Pro-Arg(Tos)-GIy-NH_2	76.5	208–211	−23.7 ($c = 1.2$)	0.37		0.85	0.48
V	Z-Cys(Bzl)-Tyr(Bzl)-Nva-Gln-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	86.7	235–236	−36.0	0.51	0.44	0.74	0.82
VI	Z-Cys(Bzl)-Tyr(Bzl)-Val-Gln-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	80.0	231–232	−34.8 ($c = 0.5$)	0.30		0.82	0.41
VII	Z-Cys(Bzl)-Tyr(Bzl)-Abu-Gln-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	86.6	239–240	−35.2	0.51	0.46	0.74	0.79
VIII	Z-Cys(Bzl)-Tyr(Bzl)-Ala-Gln-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	86.6	233–234	−34.6	0.53	0.54	0.71	0.75
IX	Z-Cys(Bzl)-Tyr(Bzl)-Gly-Gln-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	89.7	194–195	−37.7	0.52	0.41	0.68	0.66
X	Z-Cys(Bzl)-Tyr(Bzl)-Hphe-Gln-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	89.1	223–224	−34.3	0.50	0.51	0.70	
XI	Z-Cys(Bzl)-Tyr(Bzl)-Tyr(Bzl)-Gln-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	88.8	230–231	−31.7	0.53	0.54	0.75	0.89
XII	Z-Cys(Bzl)-Tyr(Bzl)-Trp-Gln-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	56.1	199–200	−28.5	0.52	0.54	0.72	0.67
XIV	Z-Cys(Bzl)-Tyr(Bzl)-Pro-Gln-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	90.2	124–125	−38.9	0.47	0.46	0.71	0.80
XV	Z-Cys(Bzl)-Tyr(Bzl)-Atc-Gln-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	82.3	98–99	−25.0	0.52	0.55	0.71	0.77
XVI	Z-Cys(Bzl)-Tyr(Bzl)-Ser(Bzl)-Gln-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	88.7	225–226	−30.1	0.57	0.45	0.69	0.80
XVII	Z-Cys(Bzl)-Tyr(Bzl)-Thr(Bzl)-Gln-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	90.3	227–228	−24.7	0.56	0.50	0.68	0.92
XVIII	Z-Cys(Bzl)-Tyr(Bzl)-Gln-Gln-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	87.7	241–242	−34.8	0.43	0.39	0.61	0.28
XIX	Z-Cys(Bzl)-Tyr(Bzl)-Asp-Gln-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	58.4	212–214	−37.2	0.43	0.51	0.49	
XX	Z-Cys(Bzl)-Tyr(Bzl)-Glu-Gln-Asn-Cys(Bzl)-pro-Arg(Tos)-Gly-NH_2	77.1	218–219	−34.8	0.40		0.58	0.08
XXI	Z-Cys(Bzl)-Tyr(Bzl)-Arg(Tos)-Gln-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	86.3	170–171	−29.4	0.47	0.42	0.69	0.98
XXII	Z-Cys(Bzl)-Tyr(Bzl)-Lys(Z)-Gln-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	87.4	210–211	−32.1	0.55	0.58	0.72	0.89
XXIII	Z-Cys(Bzl)-Tyr(Bzl)-Orn(Z)-Gln-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	86.8	205–206	−35.5	0.47	0.49	0.72	0.83

Table 4 Physicochemical Properties of Free Peptides 1–23

No.	Peptide	Yield (%)	$[\alpha]_D^{25}$ ($c = 0.1$, 1 N AcOH)	TLC (R_f)			HPLC (t_R) (min)	Formula	MW	$[M+H]^{1+}$
				a	b	c				
1	[Thi^3]AVP*	9.5	−32.0	0.13	0.04	0.26	34.5	$C_{44}H_{63}O_{12}N_{15}S_3$	1090.3	1090.7
2	[Cha^3]AVP	39.4	−24.0	0.14	0.02	0.16	23.4	$C_{46}H_{71}O_{12}N_{15}S_2$	1090.3	1091.5
3	[Nle^3]AVP	38.3	−29.0	0.19	0.02	0.21	18.2	$C_{43}H_{67}O_{12}N_{15}S_2$	1050.2	1051.0
4	[Leu^3]AVP	44.1	−10.0 ($c = 0.4$)	—	—	0.16		$C_{43}H_{67}O_{12}N_{15}S_2$	1052.2	1050.8
5	[Nva^3]AVP	37.5	−17.0	0.09	0.01	0.19	16.6	$C_{42}H_{65}O_{12}N_{15}S_2$	1036.2	1037.0
6	[Val^3]AVP	47.3	−17.7 ($c = 0.3$)	0.05	0.02	0.16	15.9	$C_{42}H_{65}O_{12}N_{15}S_2$	1036.2	1037.0
7	[Abu^3]AVP	38.7	−45.0	0.06	0.02	0.17	13.5	$C_{41}H_{63}O_{12}N_{15}S_2$	1022.2	1023.0
8	[Ala^3]AVP	55.0	−43.0	0.06	0.02	0.13	10.9	$C_{40}H_{61}O_{12}N_{15}S_2$	1008.2	1009.0
9	[Gly^3]AVP	29.1	−54.0	0.06	0.01	0.08	9.9	$C_{39}H_{59}O_{12}N_{15}S_2$	994.1	994.5
10	[Hphe^3]AVP	46.8	−33.0	0.18	0.05	0.10	20.5	$C_{37}H_{57}O_{12}N_{13}S_2$	1098.3	1099.6
11	[Tyr^3]AVP	58.9	−36.0	0.13	0.01	0.14	14.6	$C_{46}H_{65}O_{13}N_{15}S_2$	1100.3	1001.5
12	[Trp^3]AVP	38.6	−16.0	0.11	0.02	0.16	19.0	$C_{48}H_{65}O_{12}N_{16}S_2$	1123.3	1124.5
13	[2-Nal^3]AVP*	24.6	−30.0	0.18	0.04	0.19	25.3	$C_{50}H_{67}O_{12}N_{15}S_2$	1134.2	1134.9
14	[Pro^3]Avp	31.3	−34.0	0.07	0.02	0.18	36.6	$C_{42}H_{63}O_{12}N_{15}S_2$	1034.2	1034.9
15	[Atc^3]AVP	28.8	−42.0	0.18	0.04	0.21	20.2	$C_{48}H_{67}O_{12}N_{15}S_2$	1110.3	1111.5
16	[Ser^3]AVP	52.9	−36.0	0.06	0.04	0.11	10.69	$C_{40}H_{61}O_{13}N_{15}S_2$	1024.2	1025.0
17	[Thr^3]AVP	64.5	−65.0	0.06	0.02	0.13	10.9	$C_{41}H_{63}O_{13}N_{15}S_2$	1038.2	1039.0
18	[Gln^3]AVP	52.4	−30.0	0.04	0.01	0.09	8.9	$C_{42}H_{65}O_{13}N_{16}S_2$	1065.2	1065.6
19	[Asp^3]AVP	37.0	−69.0	0.05	0.02	0.06	7.8	$C_{41}H_{61}O_{14}N_{15}S_2$	1052.1	1052.8
20	[Glu^3]AVP	48.0	−40.0	0.04	0.13	0.09	8.4	$C_{42}H_{63}O_{14}N_{15}S_2$	1066.1	1066.4
21	[Arg^3]AVP	39.8	−28.0	0.02	0.01	0.04	8.4	$C_{43}H_{68}O_{12}N_{18}S_2$	1093.2	1094.0
22	[Lys^3]AVP	36.0	−49.0	0.02	0.01	0.01	9.0	$C_{43}H_{68}O_{12}N_{16}S_2$	1065.3	1066.5
23	[Orn^3]AVP	25.8	−46.0	0.03	0.01	0.02	11.2	$C_{42}H_{66}O_{12}N_{16}S_2$	1051.2	1052.0

* Peptides 1 and 13 were prepared using Fmoc strategy for SPPS and Rink amide resin [35–37]. All other peptides were synthesized using Merrifield solid-phase method [38–42].

scribed [20,28–30,41,42]. When necessary, an additional purification on Sephadex G-15 and/or Sephadex LH-20 with 0.2 M AcOH as eluent was carried out. The purity of the free peptides 1–23 (Table 4) was checked by thin-layer chromatography (TLC), high performance liquid chromatography (HPLC) and electron spray mass spectrometry (ESMS).

BIOASSAYS

Peptides were assayed for agonistic activity in the rat antidiuretic assay, rat vasopressor assay and *in vitro* rat oxytocic assay using the 4-point assay design [52]. Synthetic arginine vasopressin and oxytocin which had been standardized in vasopressor and oxytocic units against the USP Posterior Pituitary Reference Standard were used as working standards in all bioassays. Antidiuretic assays were on water-loaded rats under ethanol anesthesia as described in Sawyer [53]. Vasopressor assays were performed on urethane-anesthetized and phenoxybenzamine-treated rats as described by Dekanski [54]. Oxytocic assays were performed on isolated uteri from diethylstilbestrol-primed rats in a Mg^{2+}-free van Dyke–Hastings's solution [55]. When standard errors are presented in the tables, the means reflect results from at least four independent assay groups.

The antidiuretic, vasopressor and *in vitro* oxytocic (no Mg^{2+}) agonistic activities of peptides 1–9, which have the Phe^3 residue in AVP replaced by thienylalanine (Thi), and the aliphatic amino acids, cyclohexylalanine (Cha) Nle, Leu, Nva, Val, Abu, Ala and Gly are given in Table 1, together with the corresponding data for AVP, AVT, LVP and LVT. The corresponding data for peptides 10–23, which have the Phe^3 residue in AVP replaced by the aromatic amino acids homophenylalanine (Hphe), Tyr, Trp, 2-naphthylalanine (2-Nal), the conformationally-restricted amino acids Pro, 2-aminotetraline-2-carboxylic acid (Atc), the polar amino acids, Ser, Thr, Gln and the charged amino acids Asp, Glu, Arg, Lys, Orn, together with previously published data

for the related Ser^3, Trp^3 and Tyr^3 analogs of LVP and for [Tic^3]AVP are presented in Table 2. With the exception of the [2-Nal^3]AVP peptide, which is a weak *in vitro* OT antagonist, none of the peptides in Tables 1 and 2 exhibit any evident V_{1a}, V_2 or OT antagonistic potencies. Examination of the agonistic activities of the position-3-modified AVP peptides in both Tables reveal striking differences in the effects of thienylalanine and aliphatic amino acids (Table 1) and those of aromatic, conformationally-restricted, polar and charged amino acids (Table 2). Thus, it is clear from the data in Table 1 that position 3 in AVP tolerates a broad latitude of structural modification with thienylalanine and aliphatic amino acids, leading to excellent retention of antidiuretic agonism and to enhancement of antidiuretic/vasopressor (*A*/*P*) and antidiuretic/oxytocic (*A*/*O*) selectivities. By contrast, virtually all of the position-3-modified AVP peptides in Table 2 exhibit striking losses of antidiuretic, vasopressor and oxytocic activities relative to AVP. The findings on the peptides reported in Table 1 offer promising new clues to the design of novel, potent and selective AVP antidiuretic agonists.

Effects of Thienylalanine and Aliphatic Amino Acid Substitutions at Position 3 in AVP (Table 1)

On Antidiuretic Activity. Replacement of the Phe^3 residue in AVP by the mixed aliphatic/aromatic amino acid, thienylalanine, and by a variety of aliphatic amino acids is very well tolerated. In fact, with antidiuretic potency of 379 ± 14 units/mg, [Thi^3]AVP (peptide 1) appears to be more potent than AVP (antidiuretic activity = 323 ± 16 units/mg) and [Thi^3]LVP (antidiuretic activity = 332 ± 32 units/mg). Also striking are the high antidiuretic potencies of the Cha^3, Nle^3, and Leu^3 analogs of AVP (peptides 2–4). With antidiuretic potencies of 294 ± 28, 249 ± 28 and 229 ± 19 units/mg, respectively, these three peptides are equipotent with AVT (231 ± 30 units/mg). The Nva^3, Val^3, and Abu^3 analogs of AVP (peptides 5–7) exhibit antidiuretic potencies of 134 ± 5, 114 ± 9 and 86.7 ± 2.5 units/mg, respectively. The Ala^3 and Gly^3 analogs (peptides 8 and 9) exhibit drastic reductions relative to AVP. They exhibit antidiuretic potencies of 15.5 ± 1.5 and 3.76 ± 0.03 units/mg, respectively. Thus, the data on peptides 2–9 appear to show a good correlation between the size of the aliphatic substituent at position 3 and the antidiuretic potency of the resultant position 3 analog.

On Vasopressor Activity. With the exception of [Thi^3]AVP (peptide 1), all of the position-3 analogs of AVP in Table 1 exhibit striking losses of vasopressor potencies relative to AVP. Thus, AVP possesses vasopressor potency of 369 ± 6 units/mg. [Thi^3]AVP possesses vasopressor potency of 360 ± 4 units/mg and is thus, equipotent with AVP. The vasopressor potencies of peptides 2–9 range from a low of < 0.02 units/mg for the Gly^3 analog (peptide 9) to 84.6 units/mg for the Nle^3 analog (peptide 4). With vasopressor potencies, respectively, of 84.6 ± 4.3, 21.4 ± 0.6, and 73.4 ± 2.7 units/mg, the Nle^3, Leu^3 and Cha^3 analogs are significantly less potent than AVT (vasopressor activity = 160 ± 4 units/mg).

On Antidiuretic/Vasopressor (A/P) Selectivity. With the exception of [Thi^3]AVP (peptide 1) all of the position-3 analogs of AVP exhibit gains in *A*/*P* selectivity relative to AVP (*A*/*P* = ~ 0.9). Thus, the Val^3, Nle^3, Cha^3 and Nva^3 analogs are, respectively, 2.5, 2.9, 4 and 4.3 times more potent as antidiuretic agonists than as vasopressor agonists. With *A*/*P* ratios of 10.7 and 20.2 respectively, the Leu^3 and Abu^3 analogs exhibit even greater gains in *A*/*P* selectivity. The most striking gains in *A*/*P* selectivity are exhibited by the relatively weak antidiuretic agonists [Ala^3]AVP and [Gly^3]AVP which possess, respectively, *A*/*P* ratios of 97 and > 188. These findings offer promising clues to the design of more potent and selective antidiuretic agonists with properties similar to those of the potent and selective antidiuretic/vasopressor agonist dDAVP [56] and the potent and selective antidiuretic agonist/vasopressor antagonist dVDAVP [57].

On Oxytocic Activity. Peptides 1–9 (Table 1) exhibit a broad spectrum of OT agonism. Two peptides are more potent than AVP (OT activity = 13.9 ± 0.5 units/mg); six peptides are less potent than AVP, and one peptide is virtually equipotent with AVP. With oxytocic activities of 36.2 ± 1.9 and 28.4 ± 0.2 units/mg, respectively, both [Thi^3]AVP (peptide 1) and [Nva^3]AVP (peptide 5) are significantly more potent than AVP as oxytocic agonists. The [Gly^3]AVP analog (peptide 9) possesses undetectable oxytocic activity. With oxytocic activities of 0.02, 0.33, 0.45, 2.1 and 4.72 units/mg, respectively, the Ala^3, Cha^3, Abu^3, Leu^3 and Nle^3 analogs are much less potent than AVP as oxytocic agonists. With oxytocic activity of 11.3 units/mg, [Val^3]AVP retains much of the OT activity of AVP. Remarkably, AVT ([Ile^3]AVP), with oxytocic potency of 127 ± 9 units/mg, is strikingly more potent than all of the peptides 1–9. In this regard, comparison of the oxytocic potencies of AVT, the Leu^3

analog (peptide 4) and the Nle^3 analog (peptide 3) shows clearly the profound effects of subtle structural modifications at position 3 in AVP on OT agonist activity. With oxytocic potencies of 127, 4.72 and 2.1 units/mg, respectively, for the Ile^3, Nle^3 and Leu^3 analogs of AVP, the superiority of the Ile residue at position 3 for binding to and activating OT receptors is clearly illustrated.

On Antidiuretic/Oxytocic (A/O) Selectivity. AVP possesses 323 units/mg of antidiuretic activity and only 13.9 units/mg of *in vitro* (no Mg^{2+}) oxytocic activity. It thus exhibits an *A/O* selectivity of 23.2. AVT possesses 231 units/mg of antidiuretic activity and 127 units/mg of *in vitro* (no Mg^{2+}) oxytocic activity. It thus exhibits a greatly diminished *A/O* ratio of 1.8 relative to AVP. With *A/O* ratios, respectively, of 10.5, 10.1 and 4.7, three of the nine new peptides in Table 1, [Thi^3]AVP (peptide 1), [Nva^3]AVP (peptide 5) and [Val^3]AVP (peptide 6) also exhibit diminished *A/O* ratios relative to AVP. However, the remaining six peptides exhibit modest to striking gains in *A/O* selectivity relative to AVP. In this regard, with antidiuretic potency of 294 units/mg and *in vitro* (no Mg^{2+}) potency of 0.33 units/mg to give an *A/O* ratio = 890, [Cha^3]AVP (peptide 2) is one of the most selective antidiuretic/oxytocic agonists reported to date.

Effects of Aromatic, Conformationally-Restricted, Polar and Charged Amino Acids at Position 3 in AVP (Table 2)

All of the peptides in Table 2 exhibit drastic reductions in antidiuretic, vasopressor and oxytocic potencies relative to AVP. Thus, with antidiuretic potencies ranging from 0.018 to 20.3 units/mg relative to 323 units/mg for AVP, vasopressor potencies ranging from 0.002 to 8.99 units/mg relative to 369 units/mg for AVP and oxytocic potencies ranging from undetectable for peptides 18–23 to 0.31 units/mg for peptide 13 relative to 13.9 units/mg for AVP, peptides 10–23 (Table 2) confirm that position 3 in AVP, is intolerant to modifications with aromatic, conformationally-restricted, polar and charged amino acids. It is of interest to note that with antidiuretic activities of 20.3 ± 2.2 and 3.23 ± 0.39 units/mg, respectively, the Tyr^3 and Ser^3 analogs of AVP (peptides 11 and 16) are, however, 110 and 160 times more potent as V_2 agonists than the previously reported [3,23,24] Tyr^3 and Ser^3 analogs of LVP. These findings are consistent with the striking differences in antidiuretic potencies previously observed for AVT and LVT (Table 1). Thus, it is clear that LVP is much less tolerant of structural change at position 3 than AVP. These findings also illustrate the profound effects on antidiuretic potency of a Lys/Arg interchange at position 8 with substituents other than Phe at position 3.

The relatively high antidiuretic activity (12.7 units/mg) exhibited by [Atc^3]AVP (peptide 15) relative to the Tic^3 and Pro^3 analogs which exhibit about 1 unit and 0.5 unit of antidiuretic activity, respectively, is somewhat surprising. The relatively high antidiuretic potencies of the $Hphe^3$ (peptide 10) and Tyr^3 (peptide 11) analogs which exhibit antidiuretic activities of 24.6 ± 4.6 and 20.3 ± 2.2 units/mg, respectively, compared with those of the Trp^3 and 2-Nal^3 analogs (peptides 12 and 13) which exhibit antidiuretic potencies of 1.60 ± 0.18 and 1.53 ± 0.25 units/mg, respectively, are also quite intriguing. The *A/P* selectivities of peptides 10–23 range from a low of 0.43 for [2-Nal^3]AVP to a high of 556 for [Gln^3]AVP (*A/P* for AVP = 0.9). The *A/O* selectivities of peptides 10–23 range from a low of 4.9 for [2-Nal^3]AVP to a high of ~ 2460 for [$Hphe^3$]AVP (*A/O* for AVP = 23.2). Thus, it is clear from the data in Table 2 that while the replacement of the Phe^3 residue in AVP by aromatic, conformationally-restricted, polar and charged amino acids leads to substantial reductions in antidiuretic potencies, in virtually all cases the reductions in vasopressor and oxytocic potencies are even more drastic. Accordingly, the majority of the resultant peptides exhibit significant gains in both *A/P* and *A/O* selectivities relative to AVP.

Contrasting Effects of Some Position-3 Modifications in AVP Agonists and in an AVP V_2/V_{1a}/OT Antagonist

As noted in the introduction, we recently reported that the Phe^3 residue at position 3 in the potent AVP V_2/V_{1a}/OT antagonist $d(CH_2)_5$[D-$Tyr(Et^2)$, Val^4]AVP [29] can be replaced by a variety of aromatic, conformationally-restricted and aliphatic amino acids with retention of moderate to full V_2 antagonism [30]. It is particularly noteworthy, for example, that the Tic^3 and Pro^3 analogs are virtually equipotent with the parent peptide as V_2 antagonists. By striking contrast, replacement of the Phe^3 residue in AVP by Tic and by Pro led to drastic reductions in the antidiuretic agonistic activities of the resultant analogs. Thus, [Tic^3]AVP and [Pro^3]AVP (peptides D and 6, Table 2) exhibit less than 0.2% of the antidiuretic potency of AVP. The contrasting effects of these and other position-3 modifications in AVP agonists (Tables 1 and 2) and in AVP antagonists [30] present clear evidence that

the structural requirements for agonistic binding to and activation of AVP V_2 receptors are far more stringent than those required for the binding of AVP antagonists to V_2 receptors.

More recently, we reported that replacement of the Phe^3 residue in $d(CH_2)_5$[D-Tyr(Et)^2]VAVP by Arg^3 or Lys^3 resulted in peptides which exhibit selective vasodepressor properties and are devoid of V_2, V_{1a} and OT antagonistic properties [58,59]. The replacement of the Phe^3 residue in AVP by Arg^3, and Lys^3 to give peptides 21 and 22 (Table 2) led to drastic losses of antidiuretic, vasopressor and oxytocic agonistic activities but did not result in peptides with vasodepressor properties. Thus, the studies reported here for the effects of modifications at position 3 in AVP and those previously reported for the effects of many of the same modifications at position 3 in the potent non-selective V_2/V_{1a}/OT antagonist $d(CH_2)_5$[D-Tyr(Et)^2]VAVP [30,58,59], clearly illustrate the profound differences between the effects of the same modifications at position 3 in AVP agonists and in AVP antagonists.

General Considerations

We have reported here the synthesis of 23 analogs of AVP with modifications at position 3. Their pharmacological properties are presented in Tables 1 and 2. We have found that position 3 in AVP is highly intolerant to modifications with aromatic, conformationally-restricted, polar and basic amino acids (Table 2). The drastic losses of antidiuretic agonism exhibited by the AVP analogs which have aromatic and conformationally-restricted amino acids at position 3 are in striking contrast to our recent report that position 3 in the potent V_2/V_{1a}/OT antagonist $d(CH_2)_5$[D-Tyr(Et)^2, Val^4]AVP can be modified with aromatic and conformationally-restricted amino acids with retention of moderate to full V_2 receptor antagonism [30] and offer further evidence for differences in binding to V_2 receptors of AVP agonists and AVP antagonists. We also report that unlike position 3 in LVP (cf. LVP and LVT, Table 2), this position in AVP is highly tolerant to structural modifications with a series of aliphatic amino acids. With antidiuretic potencies of 294 ± 21, 249 ± 28, and 229 ± 19 units/mg, respectively, the Cha^3, Nle^3 and Leu^3 analogs (peptides 2–4, Table 1) are, in fact, equipotent with AVT (antidiuretic activity $= 231 \pm 30$ units/mg) as V_2 agonists. However, with reduced vasopressor and OT potencies, these three peptides and the remaining analogs with aliphatic amino acids at position 3 (peptides 5–9, Table 1) all exhibit significant increases in antidiuretic/vasopressor and antidiuretic/oxytocic selectivities relative to AVP. [Thi^3]AVP (peptide 1, Table 1), like [Thi^3]LVP [25] (A, Table 1), is a highly potent antidiuretic/vasopressor agonist. With antidiuretic activity of 379 ± 14 units/mg, [Thi^3]AVP appears to be a more potent antidiuretic agonist than AVP (antidiuretic activity = 323 ± 16 units/mg). With vasopressor activity of 360 ± 9 units/mg, [Thi^3]AVP is an equipotent vasopressor agonist to AVP (vasopressor activity = 369 ± 6 units/mg). The findings on a number of the peptides in Table 1 provide useful clues for the design of potent and selective AVP V_2 agonists and also show that further study of position 3 in other more potent and selective AVP agonists, such as dDAVP [56] and dVDAVP [57], is well warranted.

EXPERIMENTAL PART

The Merrifield resin and the Rink amide resin were purchased from Bachem California, Inc. Boc-Atc was synthesized in this laboratory as previously described [30]. All other amino acid derivatives were purchased from Bachem California, Inc., except Fmoc-2-Nal which was provided by PepTech Corp., Cambridge, MA. Thin-layer chromatography (TLC) was run on precoated silica gel plates (60F-254, E. Merck) with the following solvent systems: (a) 1-butanol:AcOH:H_2O (4:1:5, upper phase); (b) 1-butanol:AcOH:H_2O (4:1:1); (c) 1-butanol:AcOH:H_2O:pyridine (15:3:3:10); (d) chloroform:methanol (7:3). Loads of 10–15 μg were applied and chromatograms were developed at a minimal length of 10 cm. The chlorine gas procedure for the KI-starch reagent was used for detection [40]. Optical rotations were measured with a Rudolph Autopol III polarimeter. Analytical HPLC was performed on a Waters 810 instrument under the following conditions: 90:10 to 30:70 0.05% aqueous TFA:0.05% TFA in MeCN, linear gradient over 60 min at 1.0 mL/min ($\lambda = 210$ nm), on a Microsorb C_{18} column (Rainin Instrument Co., Inc.). All peptides were at least 95% pure. Electron spray mass spectra (ESMS) were done by the University of Michigan Protein and Carbohydrate Structure Facility on a Vestec 201 single quadropole mass spectrometer using AcOH:H_2O:MeCN (4:46:50) as a solvent. ESMS spectra of the free peptides were in agreement with the composition of each peptide.

Solid-Phase Synthesis Procedures

Peptides 1–23 (Tables 3 and 4) were synthesized using the Fmoc approach [36,37] or the standard Merrifield method [38,39]. For peptides 1 and 13 (Table 4) 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxymethyl-polystyrene resin (Rink amide resin) [35] and the Fmoc strategy were used [36,37]. Protection of the side chain functional groups of the amino acids was as follows: Bu^t for Tyr, Trt for Cys and Pmc for Arg. The deprotections were carried out with 20% piperidine/DMF [36,37] and the coupling reactions were mediated primarily by DCC/HOBt [44] except for the Fmoc-Asn and Fmoc-Gln which were incorporated as their *p*-nitrophenyl esters [37,45] in DMF. For the synthesis of protected peptidyl resins, nine cycles of deprotection and coupling were performed. Cleavage of the peptides from the resin and removal of their protecting groups was carried out simultaneously by Reagent K (16.5 mL TFA, 1 g phenol, 1 mL H_2O, 1 mL thioanisol and 0.5 mL 1,2-ethanedithiol) [37].

For peptides 2–12 and 14–23 the Merrifield solid-phase method [38,39] with the modifications previously described [20,40–42] was used. Chloromethylated resin (1% cross-linked S-DVB, 200–400 mesh, 0.7–1.00 mmol/g) was esterified with Boc-Gly to an incorporation of approximately 0.5 mmol/g by the caesium salt method [60]. For the synthesis of protected peptidyl resins, eight cycles of deprotection, neutralization and coupling were carried out by the DCC/HOBt [44] or the active ester [46] procedure. Ammonolysis in MeOH [41,47] was used to split the protected peptides from the resin. For peptides XIX and XX (Table 3) the Asp^3 and Glu^3 residues were incorporated using Boc-Asp(Fm) and Boc-Glu(Fm) [61,62], respectively, by the DCC/HOBt [44] procedure. For the solid-phase synthesis of peptides XIX and XX, the final two deprotections were performed with HCl (1 M)/AcOH as described above, but the subsequent neutralizations were carried out with 10% DIPEA/CH_2Cl_2 [63]. The ammonolytical cleavage of peptides XIX and XX was accompanied with removal of the side chain Fm protecting groups. The protected precursors obtained by ammonolysis were extracted with hot DMF and isolated by precipitation with hot water as previously described [20,28,29,41,42,57]. They were purified by reprecipitations with DMF/MeOH/Et_2O until adjudged pure by TLC [41] to give the required protected peptides II–XII, XIV–XXIII (Table 3). Deblocking was carried out with sodium in liquid ammonia [1,2,48] as previously described [20,28,29,41,42,57]. The resulting disulfydryl compounds from both the Reagent K treatment and the Na/liquid NH_3 treatment were oxidatively cyclized with $K_3[Fe(CN)_6]$ using the normal [49] or a modified reverse procedure [50]. The free peptides were purified by a two-step gel filtration procedure [51] on Sephadex G-15 (eluent 50% AcOH) and LH-20 (eluent 2 M AcOH). For some peptides an additional purification by gel filtration on Sephadex G-15 and/or LH-20 was used. The physicochemical data for the free peptides 1–23 are given in Table 4.

(Thi^3)Arginine Vasopressin (1, Table 4)

4 - (2′,4′ - Dimethoxyphenyl - Fmoc - aminomethyl)-phenoxymethyl-polystyrene resin (Rink amide resin) 0.48 g, (0.48 mmol/g) was subjected to nine cycles of deprotection and coupling with Fmoc-Gly, Fmoc-Arg(Pmc), Fmoc-Pro, Fmoc-Cys(Trt), Fmoc-Asn-ONp, Fmoc-Gln-ONp, Fmoc-Thi, Fmoc-Tyr(Bu^t) and Boc-Cys(Trt). The resulting protected peptidyl resin (0.98 g) was cleaved and simultaneously deprotected by treatment with Reagent K [37] as follows. The peptidyl resin was stirred with 10 mL Reagent K for 4 h at room temperature, filtered and washed twice with 5 mL TFA. The combined filtrate and washings were precipitated with ether, the collected product was washed with ether and dried *in vacuo* over P_2O_5. The resulting disulfydryl peptide (0.150 g) was reoxidized by the normal procedure [49] as follows. The residue was dissolved in 600 mL 0.2 M AcOH and the pH was adjusted to approximately 7 with concentrated ammonium hydroxide. Following neutralization, an excess of a solution of potassium ferricyanide (0.01 M, 17.5 mL) was added gradually with stirring and the yellow solution was stirred for an additional 20 min. Following oxidation, the free peptide 1 was isolated and purified as follows: after acidification with AcOH to pH 4.5 and stirring for 20 min with an anion exchange resin (Bio-Rad, AG 3×4, Cl^- form, 5 g damp weight), the suspension was slowly filtered and washed with 0.2 M AcOH (3×30 mL), the combined filtrate and washings were lyophilized. The resulting powder was desalted on a Sephadex G-15 column (110×2.7 cm) eluting with aqueous acetic acid (50%) with a flow rate of 5 mL/h [51]. The eluate was fractionated and monitored for absorbance at 254 nm. The fractions making up the major peak were checked by TLC, pooled and lyophilized. The residue was further subjected to two consecutive gel filtrations on Sephadex LH-20 (100×1.5 cm) eluting with aqueous acetic acid (2 and 0.2 M), respectively, with a flow rate of 4

mL/min. The peptide was eluted in a single peak (absorbance at 254 nm). Lyophilization of the pertinent fractions gave the desired vasopressin analog 1 (Table 4). The synthesis, cleavage, reoxidation and purification of peptide 13 were carried out in essentially the same manner. With minor modifications the same procedure was utilized for the reoxidation and purification of the free peptides 3 and 5 (Table 4).

Z-Cys(Bzl)-Tyr(Bzl)-Cha-Gln-Asn-Cys(Bzl)-Pro-Arg-(Tos)-Gly-NH_2 (II, Table 3)

Boc-Gly-resin (0.68 g, 0.35 mmol) was subjected to eight cycles of deprotection, neutralization and coupling with Boc-Arg(Tos), Boc-Pro, Boc-Cys(Bzl), Boc-Asn-ONp, Boc-Gln-ONp, Boc-Cha, Boc-Tyr(Bzl) and Z-Cys(Bzl), respectively. The resulting protected peptidyl resin was cleaved by ammonolysis as previously described [20,28,29,41,42,57]. The protected peptide was extracted with hot DMF (30 mL) and the product precipitated by addition of hot water (ca. 300 mL). After cooling, the product was collected, dried *in vacuo* over P_2O_5, reprecipitated from methanol (30 mL) and ether (ca. 200 mL). Collection and drying *in vacuo* over P_2O_5 gave the required nonapeptide amide (II, Table 3). The same procedure was used for the synthesis and purification of the protected nonapeptide amides III–XII, XIV–XXIII (Table 3). The physicochemical properties of the protected peptides II–XII, XIV–XXIII are given in Table 3.

(Cha^3)Arginine Vasopressin (2, Table 4)

The Na/liquid NH_3 procedure was used for the deprotection of all protected nonapeptide amides II–XII, XIV–XXIII as described here for peptide II. A solution of protected nonapeptide amide (II, Table 3) (120 mg) in sodium-dried ammonia (ca. 400 mL) was treated at the boiling point with stirring with sodium from a stick of metal contained in a small-bore glass tube until a light blue color persisted in the solution for ca. 30 s [20,28,29,41,42,47]. NH_4Cl was added to discharge the color. Reoxidation of the deblocked disulfydryl peptide 2 was performed by the modified reverse procedure [50] as follows. The resulting disulfydryl peptide residue was dissolved in 25 mL 50% AcOH and the solution was diluted with 57 mL H_2O. The peptide solution was added dropwise with stirring over a period of 15–30 min to an 800 mL aqueous solution which contained 20 mL of a 0.01 M solution of potassium ferricyanide. Meanwhile, the pH was adjusted to approximately 7.0 with concentrated ammonium hydroxide. Removal of the excess potassium ferricyanide, using the anion exchange resin Bio-Rad AG 3×4, Cl^- form, desalting and purification procedures by gel filtration, using Sephadex G-15 and Sephadex LH-20 were as described above for peptide 1. With minor modifications the same procedure was used to oxidize the free peptides 2, 4, 6–12 and 14–23 (Table 4).

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