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Synthesis and Structure–Activity Investigation of Novel Vasopressin Hypotensive Peptide Agonists

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Abstract: We report the solid phase synthesis and vasodepressor potencies of the novel hypotensive peptide [1(-β-mercapto-β,β-pentamethylene propionic acid)-2-*O*-ethyl-D-tyrosine, 3-arginine, 4-valine] arginine vasopressin, d$(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Val^4]AVP (**A**), its related Lys^3 (**B**), Tyr-NH_2^9 (**C**), [Lys^3, Tyr-NH_2^9] (**D**) analogs and in a preliminary structure–activity study of positions 2–4 and 7–9, 24 analogs (**1**–**24**) of **A**–**C**. Peptides **1**–**6**, **9**–**14** have the following single substituents at positions 2, 3, 4, 8 and 9 in (**A**): **1**, D-Tyr(Me)^2; **2**, L-Tyr(Et)^2; **3**, Orn^3; **4**, N-Me-Arg^3; **5**, Glu^3; **6**, Arg^4; **9**, D-Arg^8; **10**, Eda^9; **11**, Arg-NH_2^9; **12**, Ala-NH_2^9; **13**, desGly^9; **14**, desGly-NH_2^9. Peptides **15** and **16** are analogs of **B** which possess the following single modifications: **15**, Arg-NH_2^9; **16**, desGly^9. Peptides **7** and **8** are analogs of (**C**) with the following single modification: **7**, Gln^4; **8**, Lys^8. Peptides **17**–**24** are analogs of **A** possessing the following multiple modifications: **17**, [Sar^7, Eda^9]; **18**, [Arg^7, Eda^9]; **19**, [Arg^7, Eda^9 ← Tyr^{10}]; **20**, [Arg^4, Arg-NH_2^9]; **21**, [Ile^4, desGly^9]; **22**, [Arg^4, desGly^9]; **23**, [Arg^7, desGly^9]; **24**, [Arg^7, Lys^8, desGly^9]. All 24 new peptides were evaluated for agonistic and antagonistic activities in *in vivo* antidiuretic (V_2-receptor), vasopressor (V_{1a}-receptor) and in *in*

Abbreviations: Symbols and abbreviations are in accordance with the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (*Eur. J. Biochem.* 1989; **180**: A9–A11). All amino acids are in the L-configuration unless otherwise noted. Other abbreviations used are; AVP, arginine vasopressin; D-Tyr(Et), *O*-ethyl-D-tyrosine; D-Tyr(Me), *O*-methyl-D-tyrosine; L-Tyr(Et), *O*-ethyl-L-tyrosine; MeArg, *N*-methyl-arginine; Eda, ethylendiamine; desGly, desglycine [carboxy terminus is ArgNH_2 or LysNH_2]; desGly(NH_2), desglycineamide [carboxy terminus is ArgOH; Sar, sarcosine; Eda ← Tyr, Eda retro-L-tyrosine; d$(CH_2)_5$[D-Tyr(Et)^2, Val^4]AVP, [1-(β-mercapto-β,β-pentamethylenepropionic acid), 2-*O*-ethyl-D-tyrosine, 4-valine] arginine vasopressin; d$(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Val^4]AVP (**A**), [1-(β-mercapto-β,β-pentamethylene-propionic acid),2-*O*-ethyl-D-tyrosine, 3-arginine, 4-valine]arginine vasopressin; d$(CH_2)_5$[D-Tyr(Et)^2, Lys^3, Val^4]AVP (**B**), Lys^3 analog of **A**; d$(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Val^4, Tyr-NH_2^9]AVP (**C**), Tyr-NH_2^9 analog of **A**; d$(CH_2)_5$[D-Tyr(Et)^2, Lys^3, Val^4, Tyr-NH_2^9]AVP (**D**), Tyr-NH_2^9 analog of **B**; d$(CH_2)_5$[D-Tyr(Me)^2, Arg^3, Val^4]AVP, D-Tyr-(Me)^2 analog of **A**; d$(CH_2)_5$[L-Tyr(Et)^2, Arg^3, Val^4]AVP, L-Tyr(Et)^2, analog of **A**; d$(CH_2)_5$[D-Tyr(Et)^2, Orn^3, Val^4]AVP, Orn^3 analog of **A**; d$(CH_2)_5$[D-Tyr(Et)^2, MeArg^3, Val^4]AVP, *N*-Me-Arg^3 analog of **A**; d$(CH_2)_5$[D-Tyr(Et)^2, Glu^3, Val^4]AVP, Glu^3 analog of **A**; d$(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Arg^4]AVP, Arg^4 analog of **A**; d$(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Gln^4, Tyr-NH_2^9]AVP, Gln^4 analog of **C**; d$(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Val^4, Lys^8, Tyr-NH_2^9]AVP, Lys^8 analog of **C**; d$(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Val^4, D-Arg^8]AVP, D-Arg^8 analog of **A**; d$(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Val^4, Eda^9]AVP, Eda^9 analog of **A**; d$(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Val^4, Arg-NH_2^9]AVP, Arg-NH_2^9 analog of **A**; d$(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Val^4, Ala-NH_2^9]AVP, Ala-NH_2^9 analog of **A**; desGly^9, d$(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Val^4]AVP, desGly^9 analog of **A**; desGly-NH_2^9, d$(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Val^4]AVP, desGly-NH_2^9 analog of **A**; d$(CH_2)_5$[D-Tyr(Et)^2, Lys^3, Val^4, Arg-NH_2^9]AVP, Arg-NH_2^9 analog of **B**; desGly^9, d$(CH_2)_5$[D-Tyr(Et)^2, Lys^3, Val^4]AVP, desGly^9 analog of **B**; d$(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Val^4, Sar^7, Eda^9]AVP, [Sar^7, Eda^9] analog of **A**; d$(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Val^4, Arg^7, Eda^9]AVP, [Arg^7, Eda^9] analog of **A**; d$(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Arg^7, Eda^9 ← Tyr^{10}]AVP, [Arg^7, Eda^9, retro-Tyr^{10}] analog of **A**; d$(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Arg^4, Arg-NH_2^9]AVP, [Arg^4, Arg-NH_2^9] analog of **A**; desGly^9, d$(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Ile^4]AVP, [Ile^4, desGly^9] analog of **A**; desGly^9, d$(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Arg^4]AVP, [Arg^4, desGly^9] analog of **A**; desGly^9, d$(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Val^4, Arg^7]AVP, [Arg^7, desGly^9] analog of **A**; desGly^9, d$(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Val^4, Arg^7, Lys^8]AVP, [Arg^7, Lys^8, desGly^9] analog of **A**; DMF, dimethylformamide; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; ONp, *p*-nitrophenylester; MeCN, acetonitrile; Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; Tos, tosyl; AcOH, acetic acid; TFA, trifluoroacetic acid; DIEA, diisopropylethylamine; V_2, antidiuretic; V_{1a}, vasopressor; AUC, area under the vasodepressor response curve; ND, non-detectable; Z, benzyloxycarbonyl; OFm, 9-fluorenylmethylester; 2-Cl-Z, 2-chlorobenzyloxycarbonyl; TLC, thin-layer chromatography; ESMS, electronspray mass spectrometry; HPLC, high performance liquid chromatography; SPPS, solid phase peptide synthesis; BOP, Castro's Reagent, benzotriazole-1-yl-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate; Phaa, phenylacetic acid; Aaa, 1-adamantaneacetic acid; Abu, α-aminobutyric acid.

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vitro (no Mg^{2+}) oxytocic (OT-receptor) assays and like the parent peptides (**A**–**D**) (Chan *et al. Br. J. Pharmacol.* 1998; **125**: 803–811) were found to exhibit no or negligible activities in these assays. Vasodepressor potencies were determined in anesthetized male rats with baseline mean arterial blood pressure maintained at 110–120 mmHg. The effective dose (ED), in μg 100 g^{-1} i.v., required to produce a vasodepressor response of 5 cm^2, area under the vasodepressor response curve (AUC) during the 5-min period following the injection of the test peptide, was determined. Therefore, the EDs measure the relative vasodepressor potencies of the hypotensive peptides. The following ED values were obtained for **A**–**D** and for peptides **1**–**24**: **A**, 4.66; **B**, 5.75; **C**, 10.56; **D**, 11.60; **1**, ~20; **2**, ~30; **3**, 6.78; **4**, non-detectable (ND); **5**, ND; **6**, ~32; **7**, ND; **8**, 8.67; **9**, ND; **10**, 2.43; **11**, 3.54; **12**, 10.57; **13**, 4.81; **14**, ND; **15**, 4.47; **16**, 9.78; **17**, 5.72; **18**, 1.10; **19**, 1.05; **20**, 10.41; **21**, 9.13; **22**, ~33; **23**, 3.01; **24**, 1.71. **A** is clearly the most potent of the four original hypotensive peptides **A**–**D**. These data provide insights to which modification of **A** enhance, retain or abolish hypotensive potencies. Six of the new hypotensive peptides are significantly more potent than **A**. These are peptides 10, 11, 18, 19, 23 and 24. Peptide 19, a radioiodinatable ligand, is ten times more potent than **C** or **D**. The Gln^4 modification of **C** and the N-Me-Arg^3, Glu^3, D-Arg^8 and desGly-NH_2^9 modifications of **A** abolished hypotensive potency. By contrast, the Eda^9, Arg-NH_2^9, [Sar^7, Eda^9], [Arg^7, $Eda^9 \leftarrow Tyr^{10}$], [$Arg^7$, desGly^9], [$Arg^7$, Lys^8, desGly^9] modifications of **A** all led to enhancements of hypotensive potency. This initial structure–activity exploration provides useful clues to the design of (a) more potent vasodepressor peptides and (b) high affinity radioiodinatable ligands for the putative AVP vasodilating receptor. Some of the peptides here may be of value as pharmacological tools for studies on the complex cardiovascular actions of AVP and may lead to the development of a new class of anti-hypertensive agents. Copyright © 1999 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: vasopressin; vasodepressor; hypotensive

INTRODUCTION

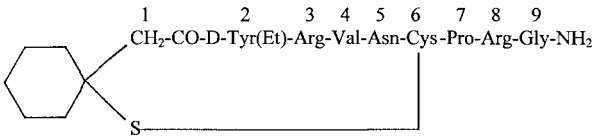
During the course of studies on the role of position three in the potent non-selective cyclic peptide vasopressin (VP), V_2/V_{1a}/OT antagonist, d$(CH_2)_5$[D-Tyr(Et)^2, Val^4]AVP [1], we found that an Arg^3/Phe^3 interchange led to surprising and unexpected findings [2–4]. The resultant peptide, d$(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Val^4]AVP (**A**), was found to possess little or no antidiuretic (V_2-receptor), oxytocic (OT-receptor) and vasopressor (V_{1a}-receptor) agonistic or antagonistic activities but had marked and prolonged vasodepressor activity [2–4]. Replacement of the Phe^3 residue in d$(CH_2)_5$[D-Tyr(Et)^2, Val^4]AVP by Lys^3 to give d$(CH_2)_5$[D-Tyr(Et)^2, Lys^3, Val^4]AVP (**B**) also resulted in a peptide with selective vasodepressor action [2–4]. Peptides **A** and **B** are the first known analogs of VP which exhibit selective vasodepressor action (Table 1). The discovery of these selective hypotensive agonists provides new evidence for the existence of a novel VP receptor responsible for mediating the well known vasodilating action of VP [5,6]. For a review of the known VP and OT receptors see Barberis et al. [7]. In further studies, we have found that the *C*-terminal Gly-NH_2 residue in both **A** and **B** could be replaced by Tyr-NH_2 with retention of hypotensive properties [3,4] (Table 1). The resultant peptides, d$(CH_2)_5$[(D-Tyr(Et)^2, Arg^3, Val^4, Tyr-NH_2^9]AVP (**C**) and d$(CH_2)_5$[D-Tyr(Et)^2, Lys^3, Val^4, Tyr-NH_2^9]AVP (**D**) are potential radioiodinatable ligands for the putative VP vasodilating receptor [3,4].

Besides providing evidence for the existence of the putative vasodilating receptor, the discovery of these four selective hypotensive peptide agonists opened up an entirely new area for the investigation of structure–activity relationships (SAR) in the vasopressin field. In preliminary SAR studies aimed at determining the effects of an Arg^3/Phe^3 interchange in other VP analogs, we found that an Arg^3/Phe^3 replacement in the potent and selective VP V_{1a} antagonist, d$(CH_2)_5$[Tyr(Me)^2]AVP [8] and in the potent and selective VP V_2 antagonist, d$(CH_2)_5$[D-Ile^2, Ile^4, Ala-NH_2^9]AVP [9], while abolishing antagonistic potencies, led in each instance to peptides devoid of hypotensive activities [10]. Likewise, Arg^3/Phe^3 replacements in the two linear V_2/V_{1a} VP antagonists, Aaa-D-Tyr(Et)-Phe-Val-Asn-Abu-Pro-Arg-Arg-NH_2 [11] and Phaa-D-Tyr(Et)-Phe-Val-Asn-Abu-Pro-Arg-Arg-NH_2 [11,12] did not result in hypotensive peptides [10]. We also very recently reported that an Arg^3/Phe^3 interchange in AVP did not result in a hypotensive peptide [13]. We thus decided to focus further structure–activity studies primarily on d$(CH_2)_5$[D-Tyr(Et)^2, Arg^3]VAVP (**A**), since, in preliminary assays, it appeared to be the most potent of the four original hypotensive peptides (**A**–**D**) [2–4]. d$(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Val^4]AVP has the following structure:

Table 1 Pharmacological Properties of Selective Hypotensive VP Peptides **A**–**D** Compared to Those of AVP and V_2/V_{1a}/OT Antagonist, $d(CH_2)_5$[D-Tyr(Et)^2, Val^4]AVP

Peptide	Agonistic activities				Antagonistic activity(pA_2)[a]		
	Antidiuretic (V_2)	Vasopressor (V_{1a})	Oxytocic (OT)	Vasodepressor Ed[b] (μg 100 g^{-1})	Anti-V_2	Anti-V_{1a}	Anti-OT (*in vitro*, no Mg^{2+})
AVP[c]	323 ± 16	396 ± 6	13.9 ± 0.5		–	–	–
$d(CH_2)$[D-Tyr(Et)^2, Val^4]AVP[d]	–	–	–	–	7.81 ± 0.07	8.22 ± 0.12	8.32 ± 0.10
$d(CH_2)$[D-Tyr(Et)^2, Arg^3, Val^4]AVP (**A**)	<0.005	ND	<0.05	4.66[e]	ND[f]	ND	ND
$d(CH_2)$[D-Tyr(Et)^2, Lys^3, Val^4]AVP (**B**)	<0.005	ND	<0.05	5.75[e]	ND	ND	ND
$d(CH_2)$[D-Tyr(Et)^2, Arg^3, Val^4, Tyr-NH_2^9]AVP (**C**)	<0.003	ND	ND	10.56[e]	ND	ND	ND
$d(CH_2)$[D-Tyr(Et)^2, Lys^3, Val^4, Tyr-NH_2^9]AVP (**D**)	<0.003	ND	ND	11.60[e]	ND	ND	ND

[a] For the *in vivo* anti-V_{1a} and anti-V_2 pA_2s, the values are estimates since the molar concentrations are estimated by dividing the i.v. dose (ED) required for the pA_2 by an assumed volume of distribution of 67 ml kg^{-1} [73].
[b] ED, effective dose (in μg 100 g^{-1} i.v.) is the dose that produces a vasodepressor response of 5 cm^2 AUC in the 5-min period following injection of test peptide. AUC, area under the vasodepressor response curve. See 'Methods' section for details of vasodepressor assays.
[c] From Manning *et al.* (1973) [74].
[d] From Manning *et al.* (1982) [1].
[e] This publication. For earlier reports on A–D (see References [2–4,10]).
[f] ND, non-detectable. In anti-V_{1a} assays, up to 20 μg/rat i.v.; higher doses were vasodepressor. In anti-V_2 and anti-OT assays, up to 100 μg no detectable antagonism; estimated $pA_2 \ll 5$.



As noted above, we already knew that **A** could be modified at positions 3 and 9, alone and in combination with Lys^3 and Tyr-NH_2^9, to give peptides **B**, **C** and **D** [3,4]. We were eager to determine how **A** could be further modified at these and at other positions with retention and/or enhancement of hypotensive agonism. The strategy we followed was adapted from that which others had utilized in early SAR studies on the neurohypophysial peptides [14–16] and which we and others utilized with much success in later studies on the design of potent and selective VP V_2/V_{1a} agonists and antagonists. For reviews see [17–26].

In this approach, **A**, and in a very few instances **B** and **C**, would initially be modified at positions 2–4 and 7–9 with a small number of single substitutions to determine which of these positions would tolerate being modified without a serious loss of hypotensive potency. The results of these studies would also provide insights on how **A** and the related molecules **B** and **C** could be modified with retention or enhancement of hypotensive activity. Combinations of the most promising modifications to emerge from these studies would then be combined in **A** in attempts to further enhance hypotensive potency. The structural modifications initially selected were for the most part those which we and others had found to be of most value in SAR and design studies on VP V_{1a} and V_2 agonists and antagonists [17–26]. Following this strategy, molecules **A**, **B** and **C** were modified at positions 2–4 and 7–9 as follows:

Position 2 (in **A**): L-Tyr(Et), D-Tyr(Me). We had previously shown that both modifications are well tolerated in VP antagonists [1,27] and in the case of the L-$Tyr(Et)^2$ substitution, also in VP V_2 agonists [28,29]. These modifications would also provide insights to size and stereochemical requirements at position 2 in **A**.

Position 3 (in **A**): Orn, MeArg, Glu. There have been no reports on the use of these modifications at position 3 in VP agonist or antagonist design. However, we recently found that the Orn^3 and Glu^3 substituents were not tolerated in AVP [13]. These substituents would provide insights to both size and charge at position 3. Although we speculated that an acidic substituent would probably not be tolerated at position 3, we could not be sure about this until the Glu^3 peptide had indeed been synthesized and tested.

Position 4: Gln, Arg, Ile. These modifications at position 4 were carried out in **A**, in **C** and in the $desGly^9$ analog of **A** to provide insight to polarity, charge, size, and lipophilicity at position 4. Gln^4 is present in AVP and is very well tolerated in AVP V_2 and V_{1a} agonists [15,18–21] and in both cyclic and linear VP V_{1a} antagonists [8,9,12,17–20]. It is less well tolerated in VP V_2 antagonists [30,31]. Arg^4 is present in Arg-conopressin-S isolated from *Conus striatus* [32] and in lysine conopressin-G isolated from *Conus geographus* [33]. While synthetic versions of these two peptides were found to exhibit drastic losses of antidiuretic and pressor agonism relative to arginine vasotocin (AVT) [34], Arg^4 was shown to be well tolerated in AVP [35] and in AVT [34]. Arg^4 is also well tolerated in AVP V_{1a} and V_2/V_{1a} antagonists [36]. Although Ile^4 is very well tolerated in VP V_2/V_{1a} antagonists [31] it resulted in a substantial decrease in antidiuretic activity when substituted for Gln^4 in dDAVP [37,38].

Position 7: Sar, Arg. Both of these modifications had been previously shown by others and by us to be suitable substitutes for Pro at position 7 in side chain shortened desPro analogs of VP V_2/V_{1a} and V_{1a} antagonists [39–41]. By contrast, the Arg^7 analog, [$desPro^7$]AVP exhibited drastic losses of agonistic activities [41]. While [Sar^7]AVP exhibited a drastic loss of vasopressor agonism, it retained over 50% of the antidiuretic activity of AVP [42]. We wondered whether one or both of these modifications would be compatible with hypotensive agonist activity. Both substituents were utilized in combination with the ethylenediamine (Eda) modification [43] at position 9 and in the case of the Arg^7 substitution, also with the $Eda^9 \leftarrow Tyr^{10}$ [26] modification.

Position 8: Lys, D-Arg. These modifications were carried out in **C** and in **A**, respectively. They were considered of importance in providing insights to the effects of Lys^8/Arg^8 and of D-Arg^8/L-Arg^8 interchanges in the hypotensive peptides **C** and **A**. The Lys^8 modification was also carried out in combination with the *C*-terminal desGly modification in **A**. It will be recalled that [Lys^8]

VP is a naturally occurring neurohypophysial peptide [15,20] and that the Lys^8 modification is very well tolerated in AVP agonists [15,20] and in AVP/OT antagonists [45]. By contrast, while the D-Arg^8 modification is well tolerated in VP antagonists [27] it results in drastic losses of vasopressor agonism in VP agonists [46–48]. We were thus most curious to determine how the D-Arg^8 modification would affect the hypotensive properties of **A**.

Position 9: Ethylenediamine (Eda), Arg-NH_2, Ala-NH_2, Eda retro-Tyr (Eda←Tyr^{10}), desGly (*C*-terminal = Arg-NH_2^8), desGly-NH_2 (*C*-terminal = Arg^8). We and others have used some or all of these modifications in SAR and design studies on VP and OT agonists and antagonists [9,12,41–44,49–53]. Whereas, in LVP, Eda^9 led to the loss of agonistic activities [43]; it is well tolerated alone and with retro modifications in VP antagonists [44,53]. The Arg-NH_2^9, Ala-NH_2^9, desGly^9 and desGly-NH_2^9 modification are also well tolerated in VP antagonists [9,12,51,52]. By contrast, in AVP, the desGly^9 modification brought about a 50% reduction in antidiuretic activity [51,52] while the desGly-NH_2^9 modification led to a 98% loss of antidiuretic activity [42,51,52]. We wondered whether these position 9 modifications alone and in combination with modifications at other positions in **A** would be tolerated in hypotensive agonists in the same way as in VP antagonists, or whether, as in the case of VP agonists, they would lead to reductions in hypotensive potency.

Based on the above rationale, we now report the synthesis by the solid phase method [54–56] of d$(CH_2)_5$[D-$Tyr(Et)^2$, Arg^3, Val^4]AVP (**A**), its Lys^3 analog (**B**), its Tyr-NH_2^9 analog (**C**), its [Lys^3, Tyr-NH_2^9] analog (**D**) and the new peptides **1**–**24** listed below:

A d$(CH_2)_5$[D-$Tyr(Et)^2$, Arg^3, Val^4]AVP
1 d$(CH_2)_5$[D-$Tyr(Me)^2$, Arg^3, Val^4]AVP
2 d$(CH_2)_5$[L-$Tyr(Et)^2$, Arg^3, Val^4]AVP
B d$(CH_2)_5$[D-$Tyr(Et)^2$, Lys^3, Val^4]AVP
3 d$(CH_2)_5$[D-$Tyr(Et)^2$, Orn^3, Val^4]AVP
4 d$(CH_2)_5$[D-$Tyr(Et)^2$, $MeArg^3$, Val^4]AVP
5 d$(CH_2)_5$[D-$Tyr(Et)^2$, Glu^3, Val^4]AVP
6 d$(CH_2)_5$[D-$Tyr(Et)^2$, Arg^3, Arg^4]AVP
7 d$(CH_2)_5$[D-$Tyr(Et)^2$, Arg^3, Gln^4, Tyr-NH_2^9]AVP
C d$(CH_2)_5$[D-$Tyr(Et)^2$, Arg^3, Val^4, Tyr-NH_2^9]AVP
8 d$(CH_2)_5$[D-$Tyr(Et)^2$, Arg^3, Val^4, Lys^8, Tyr-NH_2^9]AVP
9 d$(CH_2)_5$[D-$Tyr(Et)^2$, Arg^3, Val^4, D-Arg^8]AVP
10 d$(CH_2)_5$[D-$Tyr(Et)^2$, Arg^3, Val^4, Eda^9]AVP
11 d$(CH_2)_5$[D-$Tyr(Et)^2$, Arg^3, Val^4, Arg-NH_2^9]AVP
12 d$(CH_2)_5$[D-$Tyr(Et)^2$, Arg^3, Val^4, Ala-NH_2^9]AVP
13 desGly, d$(CH_2)_5$[D-$Tyr(Et)^2$, Arg^3, Val^4]AVP
14 desGly-NH_2,d$(CH_2)_5$[D-$Tyr(Et)^2$, Arg^3, Val^4]AVP
D d$(CH_2)_5$[D-$Tyr(Et)^2$, Lys^3, Val^4, Tyr-NH_2^9]AVP
15 d$(CH_2)_5$[D-$Tyr(Et)^2$, Lys^3, Val^4, Arg-NH_2^9]AVP
16 desGly, d$(CH_2)_5$[D-$Tyr(Et)^2$, Lys^3, Val^4]AVP
17 d$(CH_2)_5$[D-$Tyr(Et)^2$, Arg^3, Val^4, Sar^7, Eda^9]AVP
18 d$(CH_2)_5$[D-$Tyr(Et)^2$, Arg^3, Val^4, Arg^7, Eda^9]AVP
19 d$(CH_2)_5$[D-$Tyr(Et)^2$, Arg^3, Val^4, Arg^7, Eda^9←Tyr^{10}]AVP
20 d$(CH_2)_5$[D-$Tyr(Et)^2$, Arg^3, Arg^4, Arg-NH_2^9]AVP
21 desGly, d$(CH_2)_5$[D-$Tyr(Et)^2$, Arg^3, Ile^4]AVP
22 desGly, d$(CH_2)_5$[D-$Tyr(Et)^2$, Arg^3, Arg^4]AVP
23 desGly, d$(CH_2)_5$[D-$Tyr(Et)^2$, Arg^3, Val^4, Arg^7]AVP
24 desGly, d$(CH_2)_5$[D-$Tyr(Et)^2$, Arg^3, Val^4, Arg^7, Lys^8]AVP

A detailed pharmacological examination of the properties of **A–D** has been reported in [4]. Preliminary pharmacological properties of peptides **1**–**16**, **17**, **21** have been reported in [10] and/or [57]. We present here for the first time the quantitative vasodepressor potencies of peptides **A**–**D** and of peptides **1**–**24**.

Peptide Synthesis

Starting from Boc-Gly-Resin, Boc-Tyr(Bzl)-Resin, Boc-Arg(Tos)-Resin, Boc-Ala-Resin and Boc-Lys(2-Cl-Z)-Resin, we synthesized all protected precursors **a**–**c**, **I**–**XVIII** and **XX**–**XXIV** (Table 4) of the free peptides **A**–**D**, **1**–**18** and **20**–**24** (Table 5) entirely by the Merrifield solid-phase method [54–56] with the modifications previously described [1,8,27–31,59,60]. The protected retromodified peptide **XIX** (Table 4) was obtained by coupling the protected Eda peptide **XVIII** (Table 4) with Z-Tyr(Bzl) in DMF using the BOP reagent [78,79] as described in [44]. In the SPPS, HCl (1 M)/AcOH was used in all the deprotection steps except those involving Boc-Gln and Boc-Glu(OFm) in which TFA was employed [59]. Neutralizations were carried out with 10% Et_3N/CH_2Cl_2. Coupling reactions were mediated primarily by DCC/HOBt [58] in CH_2Cl_2/DMF except for Boc-Gln and Boc-Asn which were incorporated as their *p*-nitrophenyl esters [61] in DMF. The following cleavage procedures for the acylpeptide resin were used: (a) ammonolysis in methanol with DMF extraction [59,62] to give the protected peptide amides **a**–**d**, **I**–**IX**, **XI**–**XIII**, **XV**, **XVI** and **XX**–**XXIV**; (b) aminolysis with ethylenediamine (Eda) in methanol with DMF extraction [43,53] to give the protected Eda peptides **X**, **XVII** and **XVIII**; (c) acidolysis by HBr/TFA [50,52,55,56] to give the protected peptide **XIV**. Na in NH_3 [63,64] was used to deblock each protected precursor as previously described [1,8,27–31,59,60] and the resulting bis-thiol compounds oxidatively cyclized with $K_3[Fe(CN)_6]$ [65] using the modified reverse procedure [66]. The free peptides were desalted and purified by gel filtration on Sephadex G-15 and Sephadex LH-20 mainly in a two-step procedure [67] using 50% AcOH and 2 M AcOH as eluents, respectively, as previously described [1,8,27–31,60]. 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 **A**–**D** and **1**–**24** (Table 5) was checked by thin-layer chromatography (TLC), high performance liquid chromatography (HPLC) and electron spray mass spectrometry (ESMS).

Bioassays

Standard vasopressor, antidiuretic and oxytocic bioassays for neurohypophysial peptides were performed. Vasopressor assays were performed in urethane-anesthetized male rats, 1.0 g kg^{-1} i.p., as described by Dekanski [68]. The carotid artery and jugular vein were cannulated with PE catheters for blood pressure (BP) recording and for drug administration respectively. Antidiuretic assays were performed in ethanol-anesthetized and water-loaded male rats according to the method described by Sawyer [69]. The rat was anesthetized (surgical depth) with 12% ethanol, 50 ml kg^{-1} p.o. Water diuresis was induced and anesthesia maintained by a constant waterload equal to 8% of body weight with a 2% ethanol–0.05% NaCl solution p.o. The jugular vein was cannulated for drug administration. The urinary bladder was cannulated through an abdominal incision with a PE catheter for urine collection. *In vitro* oxytocic assays were performed on isolated uteri from rats that had been pretreated the previous afternoon with 50 μg diethylstilbestrol in oil per rat injected subcutaneously. The isolated uterine horn was suspended in a Mg^{2+}-free van Dyke-Hasting solution [70] for isotonic contraction recording. In all bioassays, agonistic potencies of the peptides were determined by the four-point (2×2 parallel-line) bioassay design [71]. Antagonistic potencies were measured by the pA method [72]. AVP and OT standardized against the USP Posterior Pituitary Standard for vasopressor and oxytocic activities by the four-point assay design were used as the working standards in the bioassays of all test samples. At least four independent assays ($n = 4$), each in a different animal preparation, were performed for each bioassay. The bioassay value is represented by the mean with SE.

Determination of vasodepressor activity. Vasodepressor activities of the hypotensive VP peptides **A**–**D** (Table 1) and **1**–**24** (Tables 2 and 3) were determined in urethane-anesthetized male rats as previously described [4]. Blood pressure was monitored via a cannulated carotid artery. The vasodepressor response was measured by the area under the vasodepressor response curve (AUC), determined by a polar planimeter, for the 5-min period following the injection of the hypotensive VP peptide. Two predetermined doses of the test peptide

were injected: a low dose that would produce a vasodepressor AUC response of less than 5 cm^2 during the 5-min period following the peptide injection and a high dose that would produce an AUC response of greater than 5 cm^2. The vasodepressor responses of each peptide were determined in six rats. The mean responses to the low and the high doses were computed and the dose–response curve constructed. The dose that would produce an AUC response of 5 cm^2 was interpolated from the dose–response curve. This calculated dose, in μg 100 g^{-1}, is the effective dose (ED) for the 5 cm^2 AUC response and was used to express the vasodepressor potency of the hypotensive peptide. Since the vasodepressor response is baseline BP dependent [4], rats used for the quantitative bioassays of vasodepressor potencies were given an infusion of phenylephrine to elevate and maintain their baseline BP at 110–120 mmHg. Phenylephrine (25 μg ml^{-1}) was infused at a rate (0.01–0.05 ml min^{-1}) to maintain the baseline BP at the required range for the 5-min period before the injection of the test peptide. The infusion was continued for another 5-min period following the peptide injection and then ceased. Upon recovery of the vasodepressor response, phenylephrine infusion was reinstituted for the next peptide injection. Two to three peptide injections could be administered in a stable preparation.

RESULTS

The pharmacological properties, including new data on their quantitative vasodepressor potencies, for peptides **A**–**D** are presented together with those of AVP and the non-selective AVP V_2/V_{1a}/OT antagonist, d(CH_2)$_5$[D-Tyr(Et^2), Val^4]AVP [1] in Table 1. The data in Table 1 clearly show that the peptides **A**–**D**, as previously reported [4], possess undetectable or negligible agonistic or antagonistic activities in the standard neurohypophyseal peptide antidiuretic, vasopressor and oxytocic assays. Peptides **1**–**24** were also found to possess undetectable or negligible activities in these same assays. Thus, only the vasodepressor potencies for peptides **1**–**24** are

Table 2 Vasodepressor Potencies of 16 Analogs of d(CH_2)$_5$[D-Tyr(Et)^2, Arg^3, Val^4]AVP (**A**) and Related Peptides **B** and **C** Possessing Single Modifications at Positions 2, 3, 4, 8 and 9

No.	Peptide	ED (μg 100 g^{-1} i.v. 5 cm^2 AUC 5 min[a,b]
A	d(CH_2)$_5$[D-Tyr(Et)^2, Arg^3, Val^4]AVP[c,d,e,f,g,h]	4.66
1	d(CH_2)$_5$[D-Tyr(Me)^2, Arg^3, Val^4]AVP[f,g,h]	~20
2	d(CH_2)$_5$[L-Tyr(Et)^2, Arg^3, Val^4]AVP[f,g,h]	~30
B	d(CH_2)$_5$[D-Tyr(Et)^2, Lys^3, Val^4]AVP[c,d,e,f,g,h]	5.75
3	d(CH_2)$_5$[D-Tyr(Et)^2, Orn^3, Val^4]AVP[f,g,h]	6.78
4	d(CH_2)$_5$[D-Tyr(Et)^2, MeArg^3, Val^4]AVP[f,g,h]	ND
5	d(CH_2)$_5$[D-Tyr(Et)^2, Glu^3, Val^4]AVP[g,h]	ND
6	d(CH_2)$_5$[D-Tyr(Et)^2, Arg^3, Arg^4]AVP[g,h]	~32
7	d(CH_2)$_5$[D-Tyr(Et)^2, Arg^3, Gln^4, Tyr-NH_2^9]AVP[f,g,h]	ND
C	d(CH_2)$_5$[D-Tyr(Et)^2, Arg^3, Val^4, Tyr-NH_2^9]AVP[d,e,f,g,h]	10.56
8	d(CH_2)$_5$[D-Tyr(Et)^2, Arg^3, Val^4, Lys^8, Tyr-NH_2^9]AVP[g,h]	8.67
9	d(CH_2)$_5$[D-Tyr(Et)^2, Arg^3, Val^4 ,D-Arg^8]AVP[g,h]	ND
10	d(CH_2)$_5$[D-Tyr(Et)^2, Arg^3, Val^4, Eda^9]AVP [g,h]	2.43
11	d(CH_2)$_5$[D-Tyr(Et)^2, Arg^3, Val^4, Arg-NH_2^9]AVP[f,g,h]	3.54
12	d(CH_2)$_5$[D-Tyr(Et)^2, Arg^3, Val^4, Ala-NH_2^9]AVP[f,g,h]	10.57
13	desGly^9, d(CH_2)$_5$[D-Tyr(Et)^2, Arg^3, Val^4]AVP[f,g,h]	4.81
14	desGly-NH_2^9, d(CH_2)$_5$[D-Tyr(Et)^2, Arg^3, Val^4]AVP[f,g,h]	ND
D	d(CH_2)$_5$[D-Tyr(Et)^2, Lys^3, Val^4, Tyr-NH_2^9]AVP[d,e,f,g,h]	11.60
15	d(CH_2)$_5$[D-Tyr(Et)^2, Lys^3, Val^4, Arg-NH_2^9]AVP[g,h]	4.47
16	desGly^9, d(CH_2)$_5$[D-Tyr(Et)^2, Lys^3, Val^4]AVP[h]	9.78

[a] See footnote b in Table 1 for definition of ED and AUC.
[b] All peptides exhibited undetectable or negligible agonistic or antagonistic activities in the standard antidiuretic, vasopressor, and oxytocic (*in vitro*, no Mg^{2+}) assays.
[c–g] Preliminary data reported as follows: [c] Reference [2]; [d] Reference [3]; [e] Reference [4]; [f] Reference [10]; [g] Reference [57].
[h] This publication.

Table 3 Vasodepressor Potencies of Eight Analogs of d$(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Val^4]AVP (**A**) possessing multiple substitutions at Positions 4, 7, 8, 9 and 10

No.	Peptide	ED (μg 100 g^{-1} i.v. 5 cm^2 AUC 5 min[a,b])
A	d$(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Val^4]AVP[c,d,e,f,g,h]	4.66
17	d$(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Val^4, Sar^7, Eda^9]AVP[g,h]	5.72
18	d$(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Val^4, Arg^7, Eda^9]AVP [g,h]	1.10
19	d$(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Val^4, Arg^7, Eda^9←Tyr^{10}]AVP[h]	1.05
20	d$(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Arg^4, Arg-NH_2^9]AVP[h]	10.41
21	desGly^9, d$(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Ile^4]AVP[f,h]	9.13
22	desGly^9, d$(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Arg^4]AVP[h]	~33
23	desGly^9, d$(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Val^4, Arg^7]AVP[h]	3.01
24	desGly^9, d$(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Val^4, Arg^7, Lys^8]AVP[h]	1.71

[a–g] See corresponding footnotes in Table 2.
[h] This publication.

given in Tables 2 and 3. Table 2 contains data on 16 analogs of **A**, **B** and **C** which possess single modifications. Table 2 contains data on eight analogs of **A** which contain multiple substitutions. Examination of the vasodepressor potency data in Table 1 shows that d$(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Val^4]AVP (**A**), with an ED = 4.66, is the most potent of the original four hypotensive peptides **A**–**D**. Examination of the vasodepressor potency data in Table 2 shows that two of the analogs of **A** which possess single modifications (peptides **10** and **11**) exhibit substantially enhanced vasodepressor potencies (EDs = 2.43 and 3.54) relative to **A**. Also noteworthy is the finding that five of the single modifications of **A**, utilized in this study, effected a complete abolishment of vasodepressor activity in the resulting peptides (**4**, **5**, **7**, **9** and **14**). The rest of the peptides in Table 2 exhibit a spectrum of vasodepressor potencies ranging from those with hypotensive potencies similar to **A** (ED = 4.66) (peptides **13** and **15**, which have EDs of 4.81 and 4.47, respectively) to those with potencies significantly lower than **A** (peptides **1**, **2** and **6**; EDs = ~20, ~30 and ~32, respectively). Examination of the vasodepressor potency data in Table 3 shows that four of the peptides which possess multiple substitutions of **A** exhibit significant enhancements of vasodepressor activity relative to **A**. These are peptides **18**, **19**, **23** and **24**, which possess vasodepressor ED = 1.10, 1.05, 3.01 and 1.71, respectively.

Modifications which Retain or Enhance Vasodepressor Activity

Replacement of the *C*-terminal Gly-NH_2 in **A** by Eda and by Arg-NH_2 to give, respectively, peptides **10** and **11** (Table 2), led to enhancements of vasodepressor potency in both cases. Thus, peptides **10** and **11** with EDs of 2.43 and 3.54 are clearly more potent than **A** (ED = 4.66). An Arg-NH_2^9/Gly-NH_2^9 interchange in **B** (ED = 5.75) to give peptide **15** (ED = 4.47) also resulted in an increase in vasodepressor potency. A Lys^8/Arg^8 interchange in **C** (ED = 10.56) to give peptide **8** (ED = 8.67) also led to increased vasodepressor potency. Striking differences were observed for the effects of the desGly^9 modification. This was very well tolerated in **A**, whereas in **B** it led to a significant loss of vasodepressor potency. Thus, with an ED = 4.81 peptide, **13** is almost equipotent with **A** (ED = 4.66), while peptide **16** (Table 2), with an ED = 9.78, is significantly less potent than **B** (ED = 5.75) (Table 2). An Arg^7/Pro^7 interchange in peptide **13** (ED = 4.81) to give peptide **23** (ED = 3.01) led to a significant increase in vasodepressor potency. This potency was further increased by a Lys^8/Arg^8 interchange in peptide **23** to give peptide **24** (ED = 1.71) (Table 3). The combination of the Arg^7 and Eda^9 modification in **A** to give peptide **18** (ED = 1.10) (Table 3) led to a further significant enhancement in vasodepressor potency. Thus, peptide **18** is one of the most potent hypotensive peptides reported to date. Extension of the *C*-terminal of peptide **18** (ED = 1.10) with retro-Tyr to give peptide **19** (ED = 1.05) (Table 3) was well tolerated with full retention of vasodepressor activity. Peptide **19** is about ten times more potent than peptides **C** and **D**, both of which have a *C*-terminal Tyr-NH_2 at position 9. Thus, peptide **19** is potentially a superior radioiodinatable ligand for the location of the putative VP vasodilating receptor.

Modifications of A and C which Abolish Hypotensive Activity

While a relatively small number of the single modifications were well tolerated with enhancements or minimal losses of vasodepressor activity relative to **A**–**D**, five of these modifications were not at all tolerated and resulted in peptides (**4**, **5**, **7**, **9** and **14**, Table 2) totally devoid of vasodepressor activity. These modifications are: MeArg^3, Glu^3, D-Arg^8 and desGly-NH$_2^9$ in **A** and Gln^4 in **C**.

DISCUSSION

As noted in the 'Introduction', most but not all, of the structural modifications utilized in this study have previously been shown to be of value in the design of AVP V_2 and/or V_{1a} agonists and/or V_2/V_{1a} antagonists. Thus, in addition to evaluating the usefulness of these modifications for the design of hypotensive agonists, a comparison of the effects of the structural modifications of hypotensive peptides utilized here with the effects of the same modifications in AVP V_2 and V_{1a} agonists and/or selective and non-selective AVP V_2/V_{1a} antagonists can now be evaluated.

Position 2: D-Tyr(Me), L-Tyr(Et)

With vasodepressor EDs = $\sim$20 and $\sim$30, respectively, peptides **1** and **2** (Table 2) are clearly less potent than **A** (ED = 4.66). These findings, which show that both modifications are rather deleterious, are in sharp contrast to the effects of the D-Tyr(Me)^2 and L-Tyr(Et)^2 modifications in AVP V_2/V_{1a} and in V_{1a} antagonists. Both substituents are well tolerated in AVP antagonists with excellent retention of antagonistic potencies [1,27]. The L-Tyr(Et)^2 modification is also very well tolerated in V_2 agonists [28,29]. It remains to be determined whether other position 2 substituents might be better tolerated than the two examined here.

Position 3: Orn, MeArg, Glu, Lys, Arg

Replacement of the Arg^3 residue in **A** by an Orn^3 residue resulted in a modest decrease in vasodepressor potency. With an ED = 6.78 peptide **3** is somewhat less potent than **A** (ED = 4.66) (Table 2). By contrast, replacement of the Arg^3 residue in **A** by either MeArg (peptide **4**) or by Glu (peptide **5**) (Table 2) led, in each instance, to the abolishment of vasodepressor activity. Of the position three residues examined to date, the Arg^3 substituent appears to be superior to both Lys^3 (ED of **B** = 5.75) and Orn^3 in effecting vasodepressor activity. It will be recalled that we have previously shown 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 a wide variety of aromatic, conformationally restricted and aliphatic amino acids with good to excellent retention of V_2/V_{1a} antagonistic potencies [60]. Also, we have recently reported that position 3 in AVP tolerates a broad spectrum of structural change with retention of antidiuretic agonistic activities [13] and, furthermore, that Lys^3, Arg^3, and Orn^3 substituents in AVP, while abolishing agonistic activity do not lead to peptides possessing vasodepressor activity [13]. Thus, it would now appear that the presence of a basic substituent at position 3 in **A** and in **A**-like peptides appears to be a key structural requirement for vasodepressor activity.

Position 4: Val, Arg, Gln, Ile

Val is very well tolerated at position 4 in **A** and in all of the peptides possessing enhanced vasodepressor potencies (peptides **10**, **11** and **15** (Table 2); peptides **18**, **19**, **23** and **24** (Table 3). Modification by Arg, Ile and Gln at position 4 were not nearly as well tolerated. Thus, an Arg^4/Val^4 interchange in **A** (ED = 4.66) to give peptide **6** (ED = $\sim$32) and in peptide **11** (ED = 3.54) (Table 2) to give peptide **20** (ED = 10.4) (Table 3) led to significant losses of vasodepressor potency. These findings are in contrast to the effects of a similar Arg^4/Val^4 interchange and of an Arg^4/Gln^4 interchange in AVP V_2/V_1 and V_{1a} antagonists [36] and in AVP V_2 agonists [35]. Arg^4 has been shown to be well tolerated in both AVP agonists [35] and antagonists [36]. An Ile^4/Val^4 replacement in peptide **13** (ED = 4.81) to give peptide **21** (ED = 9.13) led to an almost 50% reduction in vasodepressor potency. This is also at variance with our observations for a similar Ile^4/Val^4 interchange in V_2 antagonists. In V_2 antagonists this interchange was very well tolerated and led in a number of instances to an enhancement of V_2 antagonism [31]. The effect of a Gln^4/Val^4 interchange in **C** was most striking. The resulting peptide **7** (Table 2) is totally devoid of hypotensive potency. As noted earlier, Gln is present at position 4 in AVP and is very well tolerated in VP, V_{1a} and V_2 agonists and in V_{1a} and V_{1b} antagonists [18–21].

On the basis of the position 4 substituents examined in this study, Val^4 is the most effective in retaining and/or enhancing vasodepressor activity.

It may be recalled that Val^4 had previously been shown to be the optimal substituent for antidiuretic activity in selective AVP V_2 agonists [37,38,47,48]. To determine the optimal position 4 substituent for vasodepressor activity, a more extensive study of this position needs to be carried out.

Position 7: Pro, Arg, Sar

Proline is well tolerated in the four original hypotensive peptides **A**–**D** (Table 1) and in the three peptides in Table 2 which exhibit enhanced hypotensive potencies relative to **A** – namely, peptides **10**, **11** and **15**. The Sar^7/Pro^7 and Arg^7/Pro^7 interchanges had contrasting effects. Thus, replacement of the Pro^7 residue in peptide **10** (Table 2) (ED = 2.43) by Sar^7 to give peptide **17** (Table 3) (ED = 5.72) led to a significant decrease in vasodepressor potency. By contrast, replacement of the Pro^7 in peptide **10** by an Arg^7 to give peptide **18** (Table 3) (ED = 1.10) led to a greater than twofold enhancement of vasodepressor potency. Comparison of the EDs of peptide **13** (ED = 4.81) (Table 2), and peptide **23** (ED = 3.01) (Table 3) further illustrates the effectiveness of the Arg^7/Pro^7 interchange in enhancing hypotensive potency. These findings, while in sharp contrast to the drastic losses of agonistic activities brought about by an Arg^7/Pro^7 interchange in AVP [41] are very reminiscent of how well this interchange is tolerated in AVP V_2/V_{1a} antagonists [39–41].

Position 8: L-Arg, D-Arg, Lys

As noted earlier, replacement of the L-Arg^8 residue in **A** by its D-Arg^8 enantiomer to give peptide **9** (Table 2) led to a total abolition of vasodepressor activity. Thus, it is clear that the stereochemical requirements of the position 8 residue for interaction with the vasodilating receptor are highly rigid. While this finding is somewhat analogous to the drastic loss of vasopressor agonism observed by a similar D-Arg^8/L-Arg^8 interchange in the potent VP agonists dAVP [46] and d[Val^4] AVP [47,48], it stands in contrast to the effects of this interchange in VP V_2/V_{1a} and in V_{1a} antagonists where it is well tolerated [27]. A Lys^8/Arg^8 interchange in the two peptides studied here was very well tolerated. In fact, in both instances this interchange led to peptides possessing enhanced vasodepressor activity. Thus, a Lys^8/Arg^8 interchange in **C** (ED = 10.56) (Table 2) to give peptide **8**, ED = 8.67 (Table 2) and in peptide **23** (ED = 3.01) (Table 3), to give peptide **24** (ED = 1.71) (Table 3), led in both instances to substantial increases in vasodepressor potencies. Thus, it would appear that the Lys^8 modification may be superior to the Arg^8 modification for binding to and activating vasodilating receptors.

Position 9: Gly-NH_2, desGly, desGly-NH_2, Tyr-NH_2, Ala-NH_2, Arg-NH_2, Eda, Eda←Tyr^{10}

With the sole exception of the desGly-NH_2^9 modification (peptide **14**, Table 2) which resulted in a complete loss of vasodepressor activity, position 9 was found to tolerate all of these substitutents with varying degrees of effectiveness towards retaining or enhancing hypotensive potencies. With an ED = 4.81, the $desGly^9$ peptide (**13**, Table 2) is almost equipotent with **A** (ED = 4.66). Replacement of the *C*-terminal Gly-NH_2 in **A** (ED = 4.66) and **B** (ED = 5.75) by Tyr-NH_2 to give respectively **C** (ED = 10.56) and **D** (ED = 11.60) resulted in both instances in substantial losses of vasodepressor potency. With an ED = 10.57 the Ala-NH_2^9 analog of **A** (peptide **12**, Table 2) also exhibits a substantial loss of vasodepressor potency relative to **A**. Replacement of the *C*-terminal Gly-NH_2 in **A** by Eda to give peptide **10** (ED = 2.43), (Table 2) and by Arg-NH_2 to give peptide **11** (ED = 3.54) (Table 2), brought about significant increases in vasodepressor potencies. The findings that position 9 in AVP hypotensive peptides tolerates some of these structural changes is analogous to how well many of these changes are tolerated in AVP V_2/V_{1a} antagonists [9,12,41–43,49–53]; many, but not all are also tolerated in AVP V_2 agonists [20,21].

The contrasting effects of the $desGly^9$ and desGly-NH_2^9 modifications are particularly intriguing. Thus, as noted above, the $desGly^9$ modification was generally well tolerated, particularly in peptides **23** and **24** (Table 3). Both peptides are significantly more potent than **A**. By contrast, removal of *C*-terminal Gly-NH_2 from **A** to give peptide **14** (Table 2) abolished its vasodepressor activity. Both modifications are well tolerated in AVP antagonists [12,51,52]. The $desGly^9$ modification is also well tolerated in a variety of AVP agonists [51,52]. The effects of the desGly-NH_2^9 modification in AVP agonists was found to be very much structure dependent. Whereas, in AVP it led to an almost total loss of V_2 agonism [51], in dVDAVP it led to a peptide which is almost equipotent with AVP as a V_2 agonist [52]. It may be recalled also that an Eda^9/Gly-NH_2^9 interchange in LVP abolished agonistic activities [43]. However, this same interchange in AVP V_2/V_{1a} antagonists led to full retention of antagonistic potencies [9,53].

CONCLUSION

We have reported here a preliminary SAR investigation on the effects of structural modifications at positions 2–4 and 7–9 of the lead hypotensive peptide, d(CH_2)$_5$[D-Tyr(Et)^2, Arg^3, Val^4]AVP (**A**), its related Lys^3 (**B**), and Tyr-NH_2^9 (**C**) analogs. Based on our findings to date, we have uncovered modifications that are clearly not well tolerated and other modifications which lead to enhanced vasodepressor activity. In between these two extremes are modifications which, although tolerated, lead to significant reductions in vasodepressor activity.

While some caution is called for in assessing the usefulness of many of the structural modifications used in this study, our findings suggest that for the design of more potent hypotensive peptides, none of the five modifications which resulted in inactive peptides, namely Glu^3, MeArg^3, Gln^4, D-Arg^8 and desGly-NH_2^9 would be worth pursuing further. This does not preclude their usefulness for the design of hypotensive peptide antagonists.

These studies have uncovered a number of promising structural modifications of **A** which could lead to the design of (a) more potent hypotensive peptides and (b) more potent radioiodinatable ligands for the putative vasodilating receptor. These are: Arg^7, Lys^8, desGly^9, Eda^9 and Eda^9←Tyr^{10}. With EDs of 1.10, 1.71, 3.01, 2.43, 3.54, peptides **18**, **24**, **23** (Table 3), **10**, **11** (Table 2) are all significantly more potent than **A** (ED = 4.66). These five peptides contain single and/or combined modifications at positions 7–9. Thus, further modifications at these positions are now well warranted. With an ED = 1.05, d(CH_2)$_5$[D-Tyr(Et)^2, Arg^3, Val^4, Arg^7, Eda^9←Tyr^{10}]AVP (peptide **19**, Table 3) is the most potent hypotensive peptides uncovered in this study. It is about ten times more potent than the radioiodinatable peptide **C** (ED = 10.56). It is thus a promising radioiodinatable ligand for the putative VP vasodilating receptor. Furthermore, it offers new clues to the design of more potent radioiodinatable ligands. Some of the peptides reported here may also be of value as pharmacological tools for studies on the complex cardiovascular actions of VP, particularly the vasodilating action of AVP in regional vascular beds, and may lead to the development of a new class of anti-hypertensive agents.

EXPERIMENTAL

The Merrifield resin was purchased from Eastman Chemical Co., Rochester, NY. The Boc-Lys(2-Cl-Z)-resin was purchased from Bachem California, Inc. The Boc-Arg(Tos)-resin was either purchased from Bachem California or prepared by the cesium salt method [75]. The amino acid derivatives were purchased from Bachem California or from Chem-Impex International, USA. The BOP reagent [78,79] was purchased from Richelieu Biotechnologies, Canada. Boc-D-Tyr(Et) [76] and β-(benzylthio)-β,β-pentamethylenepropionic acid [77] were synthesized by previously published procedures. 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 was used for the *KI* – starch reagent was used for detection [56]. 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^{-1} (λ = 210 nm), on a Microsorb C_{18} column (Rainin Instrument Co., Inc.). All peptides were at least 95% pure. 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.

Solid-Phase Synthesis Procedures

The protected precursors **a**–**d**, **I**–**XVIII** and **XX**–**XXIV** (Table 4) of the free peptides **A**–**D**, **1**–**18** and **20**–**24** (Table 5) were synthesized entirely by the Merrifield solid-phase method [54–56] with the modifications previously described [1,8,27–31,59,60]. The protected retro-Tyr-modified peptide **XIX** (Table 4) was prepared by retroaddition of Z-Tyr(Bzl) to the protected Eda peptide XVIII (Table 4) using the BOP reagent [78,79] as described in [44]. Boc-Gly-resin, Boc-Ala-resin, Boc-Tyr(Bzl)-resin and Boc-Arg(Tos)-resin were prepared by esterification of Merrifield resin (chloromethylated polystyrene: 1% divinylbenzene copolymer beads, 200–400 mesh, 0.7 ± 0.10 meq g^{-1}) with either Boc-Gly, Boc-Ala, Boc-Tyr(Bzl), or Boc-Arg(Tos)

Table 4 Physicochemical Properties of the Protected Peptides **a**–**d** and **I**–**XXIV**[a]

No.	Peptide	Yield (%)[b]	m.p. (°C)	$[\alpha]_D^{25}$ ($c = 1$) DMF	TLC, R_f[c]			
					a	*b*	*c*	*d*
a	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	90.2	140–142	−9.4	0.51	0.56	0.72	0.98
b	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Lys(Z)-Val-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	87.2	159–161	−16.2	0.58	0.60	0.75	0.94
c	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Pro-Arg(Tos)-Tyr(Bzl)-NH_2	48.5	162–163	−21.4	0.73	0.71	0.70	0.96
d	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Lys(Z)-Val-Asn-Cys(Bzl)-Pro-Arg(Tos)-Tyr(Bzl)-NH_2	58.6	197–198	−24.8	0.81	0.76	0.78	0.98
I	d$(CH_2)_5$(Bzl)-D-Tyr(Me)-Arg(Tos)-Val-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	77.0	132–134	−10.7	0.74	0.62	0.78	0.83
II	d$(CH_2)_5$(Bzl)-L-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	77.0	151–153	−21.9	0.75	0.64	0.57	0.82
III	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Orn(Z)-Val-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	86.8	195–196	−16.6	0.58	0.63	0.75	0.97
IV	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-MeArg(Tos)-Val-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	57.9	138–140	−27.0	0.74	0.62	0.78	0.07
V	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Glu-Val-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	71.5	214–215	−14.4	0.73	0.62	0.76	0.21
VI	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Arg(Tos)-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	66.0	141–143	−8.7	0.79	0.64	0.76	0.82
VII	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Gln-Asn-Cys(Bzl)-Pro-Arg(Tos)-Tyr(Bzl)-NH_2	70.5	130–132	−24.0	0.68	0.70	0.71	0.99
VIII	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Pro-Lys(Z)-Tyr(Bzl)-NH_2	81.7	200–202	−15.4	0.95	0.80	0.86	0.96
IX	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Pro-D-Arg(Tos)-Gly-NH_2	76.4	170–172	−2.4	0.79	0.66	0.78	0.93
X	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Pro-Arg(Tos)-Eda	98.0	137–139	−12.5	0.51	0.44	0.57	0.07
XI	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Pro-Arg(Tos)-Arg(Tos)-NH_2	71.1	140–142	−3.8	0.79	0.68	0.79	0.92
XII	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Pro-Arg(Tos)-Ala-NH_2	67.3	131–132	−5.1	0.76	0.65	0.80	0.90
XIII	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Pro-Arg(Tos)-NH_2	63.5	134–136	−10.2	0.80	0.61	0.73	0.94
XIV	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Pro-Arg(Tos)-OH	65.4	137–136	−14.4	0.86	0.65	0.73	0.46
XV	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Lys(Z)-Val-Asn-Cys(Bzl)-Pro-Arg(Tos)-Arg(Tos)-NH_2	82.1	148–150	−13.1	0.80	0.60	0.71	0.99
XVI	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Lys(Z)-Val-Asn-Cys(Bzl)-Pro-Arg(Tos)-NH_2	68.2	181–183	−15.2	0.78	0.65	0.69	0.99
XVII	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Sar-Arg(Tos)-Eda	98.0	129–132	−5.8	0.53	0.50	0.57	0.04
XVIII	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Arg(Tos)-Arg(Tos)-Eda	95.9	122–124	−8.2	0.58	0.51	0.65	0.09
XIX	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Arg(Tos)-Arg(Tos)-Eda←Tyr(Bzl)Z	90.7	145–147	−7.7	0.83	0.68	0.82	0.97
XX	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Arg(Tos)-Asn-Cys(Bzl)-Pro-Arg(Tos)-Arg(Tos)-NH_2	79.3	130–132	−4.5	0.81	0.67	0.77	0.90
XXI	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Ile-Asn-Cys(Bzl)-Pro-Arg(Tos)-NH_2	57.9	130–132	−12.0	0.72	0.65	0.73	0.96
XXII	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Arg(Tos)-Asn-Cys(Bzl)-Pro-Arg(Tos)-NH_2	65.7	140–142	−14.7	0.69	0.59	0.68	0.88
XXIII	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Arg(Tos)-Arg(Tos)-NH_2	82.6	137–139	−15.3	0.88	0.72	0.69	0.98
XXIV	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Arg(Tos)-Lys(2-Cl-Z)-NH_2	65.8	176–178	−13.0	0.79	0.80	0.75	0.98

[a] The protected peptides **a**–**d** and **I**–**XXIV** are the immediate protected precursors for the free peptides **A**–**D** and **1**–**24** given in Tables 1–3 and 5.
[b] Yields are based on the amino acid content of the resin except for **XIX**, which was calculated on theoretical yield expected from the solution coupling.
[c] Solvent systems are described in the Experimental.

Table 5 Physicochemical Properties of Free Peptides **A**–**D**, **1**–**24**[a]

No.	Peptide	Yield (%)[b]	$[\alpha]_D^{25}$ ($c = 0.1$, 1 N AcOH)	TLC, R_f[c]			HPLC T_R (min)	Formula	MW	MW (ESMS) found
				a	*b*	*c*				
A	d(CH_2)$_5$[D-Tyr(Et)^2, Arg^3, Val^4]AVP	40.8	−130.0	0.12	0.08	0.28	28.38	$C_{50}H_{80}O_{11}N_{16}S_2$	1145.4	1145.1
B	d(CH_2)$_5$[D-Tyr(Et)^2, Lys^3, Val^4]AVP	31.9	−134.0	0.09	0.08	0.28	29.39	$C_{50}H_{80}O_{11}N_{14}S_2$	1117.4	1117.3
C	d(CH_2)$_5$[D-Tyr(Et)^2, Arg^3, Val^4, Tyr-NH_2^9]AVP	60.2	−108.0	0.24	0.13	0.39	38.66	$C_{57}H_{86}O_{12}N_{16}S_2$	1251.6	1251.0
D	d(CH_2)$_5$[D-Tyr(Et)^2, Lys^3, Val^4, Tyr-NH_2^9]AVP	46.9	−124.0	0.23	0.16	0.38	34.85	$C_{57}H_{86}O_{12}N_{14}S_2$	1223.5	1223.2
1	d(CH_2)$_5$[D-Tyr(Me)^2,Arg^3,Val^4]AVP	18.0	−68.0	0.26	0.07	0.32	30.18	$C_{49}H_{78}O_{11}N_{16}S_2$	1131.4	1130.8
2	d(CH_2)$_5$[L-Tyr(Et)^2, Arg^3, Val^4]AVP	40.0	−103.0	0.28	0.11	0.34	30.35	$C_{50}H_{80}O_{11}N_{16}S_2$	1145.4	1145.4
3	d(CH_2)$_5$[D-Tyr(Et)^2, Orn^3, Val^4]AVP	53.7	−137.0	0.19	0.06	0.22	34.04	$C_{49}H_{78}O_{11}N_{14}S_2$	1103.4	1103.3
4	d(CH_2)$_5$[D-Tyr(Et)^2, MeArg^3, Val^4]AVP	32.4	−79.0	0.21	0.05	0.20	35.43	$C_{51}H_{82}O_{11}N_{16}S_2$	1159.4	1159.0
5	d(CH_2)$_5$[D-Tyr(Et)^2, Glu^3, Val^4]AVP	52.7	−102.0	0.31	0.22	0.37	40.56	$C_{49}H_{75}O_{13}N_{13}S_2$	1118.3	1119.0
6	d(CH_2)$_5$[D-Tyr(Et)^2, Arg^3, Arg^4]AVP	45.0	−86.0	0.22	0.02	0.05	24.90	$C_{51}H_{83}O_{11}N_{19}S_2$	1202.4	1202.6
7	d(CH_2)$_5$[D-Tyr(Et)^2, Arg^3, Tyr-NH_2^9]AVP	52.1	−110.0	0.23	0.07	0.31	31.09	$C_{57}H_{85}O_{13}N_{17}S_2$	1280.6	1280.1
8	d(CH_2)$_5$[D-Tyr(Et)^2, Arg^3, Val^4, Lys^8, Tyr-NH_2^9]AVP	31.0	−106.0	0.32	0.09	0.17	29.88	$C_{57}H_{86}O_{12}N_{14}S_2$	1223.5	1223.0
9	d(CH_2)$_5$[D-Tyr(Et)^2, Arg^3, Val^4, D-Arg^8]AVP	50.0	−124.0	0.23	0.08	0.21	39.18	$C_{50}H_{80}O_{11}N_{16}S_2$	1145.4	1145.1
10	d(CH_2)$_5$[D-Tyr(Et)^2, Arg^3, Val^4, Eda^9]AVP	13.8	−121.0	0.20	0.04	0.17	25.23	$C_{50}H_{81}O_{10}N_{16}S_2$	1130.4	1131.0
11	d(CH_2)$_5$[D-Tyr(Et)^2, Arg^3, Val^4, Arg-NH_2^9]AVP	37.9	−105.0	0.21	0.01	0.12	41.38	$C_{54}H_{89}O_{11}N_{19}S_2$	1244.5	1244.8
12	d(CH_2)$_5$[D-Tyr(Et)^2,Arg^3,Val^4,Ala-NH_2^9]AVP	32.7	−78.0	0.26	0.10	0.10	40.19	$C_{51}H_{82}O_{11}N_{16}S_2$	1159.4	1159.4
13	desGly, d(CH_2)$_5$[D-Tyr(Et)^2, Arg^3, Val^4]AVP	27.5	−135.0	0.28	0.10	0.21	36.38	$C_{48}H_{77}O_{10}N_{15}S_2$	1088.4	1088.8
14	desGly-NH_2, d(CH_2)$_5$[D-Tyr(Et)^2, Arg^3, Val^4]AVP	54.2	−167.0	0.24	0.07	0.18	32.86	$C_{48}H_{76}O_{11}N_{14}S_2$	1089.4	1089.2
15	d(CH_2)$_5$[D-Tyr(Et)^2, Lys^3, Val^4, Arg-NH_2^9]AVP	38.6	−113.0	0.25	0.02	0.17	26.81	$C_{54}H_{89}O_{11}N_{17}S_2$	1216.6	1215.5
16	desGly, d(CH_2)$_5$[D-Tyr(Et)^2, Lys^3,Val^4]AVP	38.0	−106.0	0.27	0.05	0.36	29.41	$C_{48}H_{77}O_{10}N_{13}S_2$	1060.4	1059.4
17	d(CH_2)$_5$[D-Tyr(Et)^2, Arg^3, Val^4, Sar^7, Eda^9]AVP	26.6	−82.0	0.21	0.04	0.16	27.22	$C_{48}H_{79}O_{10}N_{16}S_2$	1104.4	1104.8
18	d(CH_2)$_5$[D-Tyr(Et)^2, Arg^3, Val^4, Arg^7, Eda^9]AVP	28.7	−84.0	0.20	0.02	0.09	28.08	$C_{51}H_{87}O_{10}N_{19}S_2$	1190.5	1190.5
19	d(CH_2)$_5$[D-Tyr(Et)^2, Arg^3, Val^4, Arg^7, Eda^9 ←Tyr^{10}]AVP	27.0	−96.0	0.20	0.02	0.21	29.25	$C_{60}H_{96}O_{12}N_{20}S_2$	1353.7	1354.0
20	d(CH_2)$_5$[D-Tyr(Et)^2, Arg^3,Arg^4,Arg-NH_2^9]AVP	37.4	−75.0	0.01	0.02	0.01	22.38	$C_{55}H_{92}O_{11}N_{22}S_2$	1301.6	1302.6
21	desGly, d(CH_2)$_5$[D-Tyr(Et)^2, Arg^3,Ile^4]AVP	26.0	−154.0	0.31	0.12	0.33	35.08	$C_{49}H_{79}O_{10}N_{15}S_2$	1102.4	1101.9
22	desGly, d(CH_2)$_5$[D-Tyr(Et)^2, Arg^3,Arg^4]AVP	31.7	−133.0	0.16	0.02	0.03	27.13	$C_{49}H_{80}O_{10}N_{18}S_2$	1145.4	1144.9
23	desGly, d(CH_2)$_5$[D-Tyr(Et)^2, Arg^3,Val^4, Arg^7]AVP	26.3	−102.0	0.22	0.02	0.21	30.33	$C_{49}H_{82}O_{10}N_{18}S_2$	1147.5	1146.9
24	desGly, d(CH_2)$_5$[D-Tyr(Et)^2, Arg^3,Val^4, Arg^7, Lys^8]AVP	31.7	−99.0	0.17	0.02	0.16	28.52	$C_{49}H_{82}O_{10}N_{16}S_2$	1119.4	1118.6

[a] Yields are based on the amount of protected peptide used in the reduction–reoxidation step in each case and are uncorrected for acetic acid and water content.
[b] Solvent systems and conditions are given in the Experimental.
[c] All peptides were at least 95% pure. For elution linear gradient 90:10 to 30:70 (0.05% aqueous TFA:0.05% TFA in MeCN) over 60 min with flow rate 1.0 ml/min was applied.

using the cesium salt method [75]. For the synthesis of protected peptidyl resins, seven or eight cycles of deprotection, neutralization and coupling were carried out starting as follows: for protected peptides **a**, **b**, **I**–**VI** and **IX** from Boc-Gly-resin; for protected peptides **c**, **d**, **VII** and **VIII** from Boc-Tyr(Bzl)-resin; for protected peptides **X**, **XI**, **XIII**–**XVIII** and **XX**–**XXIII** from Boc-Arg(Tos)-resin; for protected peptide **XII** from Boc-Ala-resin and for protected peptide **XXIV** from Boc-(2-Cl-Z)-Lys-resin. HCl (1 M)/AcOH was used in all the deprotection steps except these involving Boc-Asn, Boc-Gln and Boc-Glu(OFm) in which TFA was employed [59]. Neutralizations were carried out with 10% Et_3N/CH_2Cl_2. Boc amino acids (except Boc-Asn and Boc-Gln) were coupled by the DCC/HOBt procedure [58] in CH_2Cl_2/DMF (9:1, v/v). Boc-asparagine and Boc-glutamine were coupled as their nitrophenyl esters [61] in DMF. The acylpeptide resin were cleaved using the following procedures: (a) ammonolysis in methanol [59,62] to give the protected peptide amides **a**–**d**, **I**–**IX**, **XI**–**XIII**, **XV**, **XVI** and **XX**–**XXIV**; (b) aminolysis with ethylenediamine (Eda) in methanol [43,53] to give the protected Eda peptides **X**, **XVII** and **XVIII**; (c) acidolysis with HBr/TFA [50, 52,55,56] to give the protected peptide **XIV**. All of the protected precursors including the retroprotected peptide **XIX** were purified by the same general method: extraction with warm DMF followed by reprecipitations with H_2O and EtOH/Et_2O until adjudged pure by TLC, as previously described [1,8,27–31,59,60] to give the required protected peptides **a**–**d**, **I**–**XXIV** (Table 4). The physicochemical properties of all protected peptides (**a**–**d**, **I**–**XXIV** are given in Table 4. Deprotections were carried out with sodium in liquid ammonia [63,64] as previously described [1,8,27–31,59,60]. The resulting bis-thiol compounds were oxidatively cyclized with $K_3[Fe(CN)_6]$ [65] using the modified reverse procedure [66]. The free peptides were desalted and purified by a two-step gel filtration procedure [67] on Sephadex G-15 (eluent 50% AcOH) and LH-20 (eluent 2 M AcOH). For some peptides, an additional purification on Sephadex G-15 or/and Sephadex LH-20 with 0.2 M AcOH as eluent was carried out. The purity of the free peptides **A**–**D**, **1**–**24** (Table 5) was checked by TLC and HPLC. Their structures were confirmed by ESMS. The TLC, HPLC, ESMS data and some other physicochemical properties of the free peptides **A**–**D**, **1**–**24** are presented in Table 5.

((β-Benzylthio)-β,β-pentamethylenepropionyl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2 (a, Table 4)

Boc-Gly-resin (1 g, 0.5 mmol) was converted to protected acyl octapeptidyl resin in eight cycles of deprotection, neutralization and coupling (mediated by DCC/HOBt or active ester) with Boc-Arg(Tos), Boc-Pro, Boc-Cys(Bzl), Boc-Asn-ONp, Boc-Val, Boc-Arg(Tos), Boc-D-Tyr(Et) and β-(benzylthio)-β,β-pentamethylenepropionic acid, respectively by the manual method of solid-phase synthesis as previously described [1,8,27–31,59,60]. The resulting protected peptidyl resin (1.7 g, yield 96.3%) was subjected to ammonolysis with DMF extraction [43,53] as follows. The resin was suspended in anhydrous methanol (100 ml) in a 250-ml round bottomed flask cooled at ca. −70°C and NH_3 (ca. 30 ml) was bubbled through the suspension for a period of approximately 30 min. The tightly stoppered flask was kept at room temperature in the hood for 2 days. The suspension was recooled, the stopper removed and the NH_3 and methanol allowed to evaporate at room temperature. The protected peptide was extracted with warm (ca. 50°C) DMF (ca. 30 ml) and precipitated with warm (ca. 50°C) water (ca. 500 ml). Following overnight storage at 4°C, the product was collected, dried *in vacuo* over P_2O_5 and reprecipitated from warm (ca. 50°C) DMF (ca. 5 ml) with ethyl ether (ca. 300 ml), collected and dried *in vacuo* over P_2O_5 to give the protected acyloctapeptide amide (a) (Table 4). Starting from the appropriate Boc-amino acid resin, this procedure was used for the preparation of all of the remaining protected peptide amides **b**–**d**, **I**–**IX**, **XI**–**XIII**, **XV**, **XVI** and **XX**–**XIV** (Table 4).

((β-Benzylthio)-β,β-pentamethylenepropionyl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Arg(Tos)-Arg(Tos)-Eda (XVIII, Table 4)

Starting from d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Arg(Tos)-Arg(Tos)-resin (2.92 g, 0.74 mmol), prepared as described above for (**a**) with Boc-Arg(Tos) in place of Boc-Pro, the protected Eda-peptide **XVIII** was obtained by aminolysis with Eda/MeOH and DMF extraction [43,53] as follows. The protected peptidyl resin was placed in a 250-ml round bottomed flask, 100 ml of anhydrous methanol was added, the suspension was cooled at ca. 0°C and 30 ml of ethylenediamine (Eda, 99.5 + %, redistilled; Aldrich), was added under stirring. After 30 min the cooling bath was removed and the suspension was stirred at room temperature for 2

days. The solvents were removed on a rotary evaporation and the protected peptide **XVIII** (Table 4) was extracted and purified as described above for the protected peptide **a**. The protected Eda peptides **X** and **XVII** (Table 4) were prepared by the same procedure.

((β-Benzylthio)-β,β-pentamethylenepropionyl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Arg(Tos)-Arg(Tos)-Eda ← Z-Tyr(Bzl) (XIX, Table 4)

The retromodified peptide **XIX** was synthesized by BOP mediated coupling [78,44] of protected peptide **XVIII** and Z-Tyr(Bzl) as follows. Protected peptide **XVIII** (Table 4) 0.46 g (0.25 mmol), Z-Tyr(Bzl) 0.20 g (0.5 mmol) and BOP (Castro's reagent, benzotriazole - 1 - yl - oxy - tris(dimethylamino) - phosphonium-hexafluorophosphate) 0.22 g (0.5 mmol) were dissolved in 3 ml of anhydrous DMF, cooled in an ice bath and DIEA 0.17ml (1 mmol) was added. The reaction mixture was stirred for 30 min at 0°C and for 2.5 h at room temperature and was periodically monitored by TLC. After the reaction was completed (the protected Eda peptide was consumed), 5% $KHCO_3$ (20 ml) was added and the mixture was stirred for 30 min, whereupon an oily product appeared. The reaction mixture was transferred to a separatory funnel; ethyl acetate was added and the organic layer was washed consecutively with H_2O, 1 M HCl, and H_2O, dried over $MgSO_4$ and evaporated on a rotary evaporation. The residue was dissolved in 5 ml DMF, the solution was diluted with 50 ml MeOH and the product was precipitated with 300 ml Et_2O. The residue was collected and dried *in vacuo* over P_2O_5 to give the desired protected retromodified peptide **XIX** (Table 4).

((β-Benzylthio)-β,β-pentamethylenepropionyl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Pro-Arg(Tos)-OH (XIV, Table 4)

The protected peptide **XIV** was split from the resin by acydolytic cleavage [50,52,55,56] as follows. Hydrogen bromide was bubbled through a suspension of 0.9 g (0.3 mmol) of [(β-benzylthio)-β,β-pentamethylenepropionyl]-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Pro-Arg-(Tos)-resin in TFA (20 ml) and anisole (3 ml) in a glass funnel fitted with a fritted disk as described in [56]. After 30 min the filtrate was collected. The resin was resuspended in CH_2Cl_2 (10 ml), TFA (10 ml) and anisole (3 ml). HBr bubbling was resumed for a further 30 min, whereupon the filtrate was collected and the resin was washed with CH_2Cl_2:TFA (1:1, 20 ml × 3). The filtrates and washings were combined and evaporated to dryness on a rotary evaporation. Addition of ether (ca. 200 ml) to the residual anisole solution gave a precipitate, which, following 3 h at 4°C, was collected, washed with ether and dried over P_2O_5. This material was dissolved in warm DMF (ca. 5 ml), reprecipitated with water, collected, and dried *in vacuo* over P_2O_5 to give the desired protected acylheptapeptide **XIV** (Table 4).

(1-(β-Mercapto-β,β-pentamethylenepropionic acid), 2-*O*-ethyl-D-tyrosine, 3-arginine, 4-valine)arginine-vasopressin (d(CH_2)$_5$(D-Tyr(Et)^2, Arg^3, Val^4)AVP) (A, Table 5)

A solution of protected nonapeptide amide (**a**, Table 4) (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 [1,8,27–31,59,60]. NH_4Cl was added to discharge the color and the ammonia evaporated. The resulting bis-thiol peptide residue was dissolved in 25 ml 50% of degassed AcOH and the solution was diluted with 50 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 [66]. Meanwhile, the pH was adjusted to approximately 7.0 with concentrated ammonium hydroxide. The yellow solution was stirred for an additional 20 min. Following cyclization, the free peptide **A** 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 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^{-1} [67]. 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 column (100 × 1.5 cm) eluting with aqueous acetic acid (2 and 0.2 M), respectively, with a flow rate of 4 ml min^{-1}. The peptide was eluted in a single peak (absorbance at 254 nm). Lyophilization of the pertinent fractions gave the desired vasopressin analog **A** (Table 5). With minor

modifications, the same procedure was utilized for the deprotection, cyclization and purification of the remaining protected peptides **b**–**d**, **I**–**XIV** (Table 4) to give the free peptides **B**–**D**, **1**–**24** (Table 5). The desalting and the purification of the free peptides usually required the two-step gel filtration procedure [67] on Sephadex G-15 and Sephadex LH-20 using 50% AcOH and 2 M AcOH as eluents, respectively, as previously described [1,8,27–31,60]. Only when necessary, an additional purification on Sephadex G-15 and/or Sephadex LH-20 with 0.2 M AcOH as eluent was carried out.

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