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Design and synthesis of potent, highly selective vasopressin hypotensive agonists

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Abstract: We report here the solid-phase synthesis and vasodepressor potencies of a new lead vasopressin (VP) hypotensive peptide [1(β-mercapto-β,β-pentamethylenepropionic acid)-2-O-ethyl-D-tyrosine, 3-arginine, 4-valine, 7-lysine, 9-ethylenediamine] lysine vasopressin, d(CH_2)$_5$[D-Tyr(Et)^2, Arg^3, Val^4, Lys^7, Eda^9]LVP (**C**) and 21 analogues of **C** with single modifications at positions 9 (**1–13**), 6 (**14**), 2 (**16–20**) and combined modifications at positions 6 and 10 (**15**) and 2 and 10 (**21**). Peptides **1–13** have the following replacements for the Eda residue at position 9 in **C**: (**1**) Gly-NH_2; (**2**) Gly-NH–CH_3; (**3**) Ala-NH_2; (**4**) Ala-NH–CH_3, (**5**) Val-NH_2; (**6**) Cha-NH_2; (**7**) Thr-NH_2; (**8**) Phe-NH_2; (**9**) Tyr-NH_2; (**10**) Orn-NH_2; (**11**) Lys-NH_2; (**12**) D-Lys-NH_2; (**13**) Arg-NH_2. Peptide **14** has the Cys residue at position 6 replaced by Pen. Peptide **15** is the retro-Tyr^{10} analogue of peptide **14**. Peptides **16–20** have the D-Tyr(Et) residue at position 2 in **C** replaced by the following substituents: D-Trp (**16**); D-2-Nal (**17**); D-Tyr(Bu^t)(**18**); D-Tyr(Pr^n) (**19**); D-Tyr(Pr^i) (**20**). Peptide **21** is the retro-Tyr^{10} analogue of peptide **20. C** and peptides **1–21** were evaluated for agonistic and antagonistic activities in *in vivo* vasopressor (V_{1a}-receptor), antidiuretic (V_2-receptor), and in *in vitro* (no Mg^{2+}) oxytocic (OT-receptor) assays in the rat, and, like the original hypotensive peptide, d(CH_2)$_5$[D-Tyr(Et)^2, Arg^3, Val^4]AVP (**A**) (Manning *et al.*, *J. Peptide Science* 1999, 5 : 472–490), 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 (BP) maintained at 100–120 mmHg. The effective dose (ED), in μg/100 g i.v., the dose 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. The EDs measure the vasodepressor potencies of the hypotensive peptides **C** and **1–21** relative to that of **A** (ED = 4.66 μg/100 g) and to each other. The following ED values in μg/100 g were obtained for **C** and for peptides **1–21; C** 0.53; (**1**) 2.41; (**2**) 1.13; (**3**) 1.62; (**4**) 0.80; (**5**) 1.83; (**6**) 1.56; (**7**) 2.12, (**8**) 2.58; (**9**) 1.40; (**10**) 0.88; (**11**) 0.90; (**12**) 0.85; (**13**) 0.68; (**14**) 0.99; (**15**) 1.05; (**16**) 0.66; (**17**) 0.54; (**18**) 0.33; (**19**) 0.18; (**20**) 0.15; (**21**) 0.14. All of the hypotensive peptides reported here are more potent than **A**. Peptides **20** and **21** exhibit a striking 30-fold enhancement in vasodepressor potencies relative to **A**. With a vasodepressor ED = 0.14, peptide **21** is the most potent VP vasodepressor agonist reported to date. Because it contains a retro-Tyr^{10} residue, it is a promising new radioiodinatable ligand for the putative VP vasodilating receptor. Some of these new hypotensive peptides may be of value as research tools for studies on the complex cardiovascular actions of VP and may lead to the development of a new class of antihypertensive agents. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: vasopressin; vasodepressor; hypotensive

Abbreviations: Abbreviations are as in *J. Peptide Science* **9**:1–8 (2003) and references there cited, and as follows. All amino acids are in the L-configuration unless otherwise noted. Other abbreviations used are: AVP, arginine vasopressin; LVP, lysine vasopressin; VP, vasopressin; D-Tyr(Et), *O*-ethyl-D-tyrosine; D-Tyr(Bu^t), *O*-*t*-butyl-D-tyrosine; D-Tyr(Pr^n), *O*-*n*-propyl-D-tyrosine; D-Tyr(Pr^i), *O*-*i*-propyl-D-tyrosine; D-2-Nal, D-2-naphthylalanine; Pen, penicillamine; Eda, ethylenediamine; d(CH_2)$_5$, β-mercapto-β,β-pentamethylenepropionyl; d(CH_2)$_5$(Mob), β-(4-methoxybenzyl)mercapto-β,β-pentamethylenepropionyl; Eda ← Tyr, Eda retro-tyrosine; d(CH_2)$_5$[D-Tyr(Et)^2, Arg^3, Val^4]AVP (**A**), [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, Arg^7, Eda^9]AVP (**B**), the Arg^7, Eda^9 analog of (**A**); d(CH_2)$_5$[D-Tyr(Et)^2, Arg^3, Val^4, Lys^7, Eda^9]LVP (**C**), the Lys^7, Lys^8, Eda^9 analog of (**A**); DMF, dimethylformamide; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; ONp, 4-nitrophenyl ester; Boc, *t*-butyloxycarbonyl; Bzl, benzyl; Tos, tosyl; Z, benzyloxycarbonyl; Mob, 4-methoxybenzyl; Z(2Cl), 2-chlorobenzyloxycarbonyl; AcOH, acetic acid; TFA, trifluoroacetic acid; DIPEA, diisopropylethylamine; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography, ESMS, electro-spray mass spectrometry; AUC, area under the vasodepressor response curve; ED, effective dose. TFMSA, trifluoromethane sulfonic acid; MALDI-TOF, matrix-assisted laser desorption ionization, time-of-flight; DHB, dihyroxybenzoic acid.

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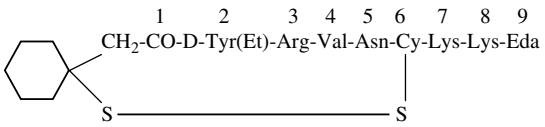
INTRODUCTION

Arginine vasopressin (AVP), long known for its antidiuretic, vasopressor, pituitary ACTH-releasing, and uterine-contracting effects, mediated respectively by the V_2, V_{1a}, V_{1b} (V_3), and the oxytocin (OT) receptors [1], also exhibits vasodilating or hypotensive effects [2,3]. To date, the receptor that mediates the vasodilating effects of AVP has not been characterized [1–3]. Attempts to do so have been hampered by the lack of selective agonists or antagonists for this putative AVP receptor. We recently reported the serendipitous discovery of the first known vasopressin (VP) peptides that exhibit specific hypotensive activity [4–9]. These novel VP hypotensive peptides were discovered during the course of studies on the effects of replacing the Phe^3 residue in the potent nonselective V_2/V_{1a}/OT antagonist, 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 [10] by a variety of amino acids. Replacement of the Phe^3 by an Arg^3 residue in this antagonist had dramatic effects. The resultant peptide, d$(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Val^4]AVP (**A**), exhibited none of the antagonistic properties of its parent – instead it exhibited totally unexpected selective vasodepressor effects in the rat bioassay [4]. Thus was discovered the first selective VP hypotensive agonist [4,5]. Hypotensive potencies are measured as the vasodepressor effective dose (ED) in μg/100 g i.v. [7,9]. The ED is the dose that produces a vasodepressor response of 5 cm^2 AUC (area under the curve) in the 5-min period following injection of the test peptide (see section on Bioassays for how EDs are measured). Peptide **A** exhibits a vasodepressor ED = 4.66 μg/100 g. [7,9]. This is the standard against which all new VP hypotensive peptides analogues are measured. Peptide **A** became the lead compound in extensive structure/activity relationship studies aimed at delineating which structural features of **A** are required for hypotensive activity [6–9]. These studies have shown that the requirements for hypotensive activity are quite rigid [6–9]. However, we also found that **A** could be modified to give peptides with enhanced hypotensive potencies. We found, for example, that the combination of Arg^7 and Eda^9 (where Eda = ethylenediamine) substitutions in **A** led to a 4–5-fold increase in hypotensive potency [7,9]. The resulting peptide, d$(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Val^4, Arg^7, Eda^9]AVP (**B**), exhibits a vasodepressor ED = 1.10 μg/100 g [7,9]. Peptide **B** has been found to be a valuable pharmacological tool in a recent elegant study on the mechanism of the putative hypotensive effects of VP [11]. Using **B** as a new lead, we found that replacement of the Arg^7, Arg^8 dipeptide in **B** by a Lys^7, Lys^8 dipeptide gave a further increase in hypotensive potency. In a preliminary report [12], we found that the resulting peptide, d$(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Val^4, Lys^7, Eda^9]LVP (**C**), exhibits a vasopressor ED = 0.53 μg/100 g [12]. It may be noted also that **C** has been utilized as a pharmacological tool in a recent study on the mechanism of action of its hypotensive effects in rats [13]. d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Eda^9]LVP (**C**) has the following structure:



Peptide **C** was selected as a new lead for the design of the potent hypotensive peptide agonists reported here. The design rationale followed closely that used in our earlier study, in which peptide **A** was the lead compound [7,9]. The present study was guided by the findings from that study. We report here the effects of single modifications of **C** at positions 9, 6 and, 2 and the effects of combined modifications of **C** at positions 6 and 10, and 2 and 10.

Single Modifications of C

Position 9. Gly-NH_2, Gly-NH–CH_3, Ala-NH_2, Ala-NH-CH_3, Cha-NH_2, Val-NH_2, Thr-NH_2, Phe-NH_2, Tyr-NH_2, Orn-NH_2, Lys-NH_2, D-Lys-NH_2, Arg-NH_2. The selection of these position 9 substituents was guided in part by the results of our previous study [7] of position 9 substituents in the first hypotensive VP peptide agonist **A** [5]. In that study, we had utilized a smaller number of position 9 substituents and found that position 9 could be modified with retention, enhancement, and total abolishment of hypotensive activities, depending on the chemical structure of the position 9 substituents [7]. In this study, we expanded the investigation of position 9 in **C** to include the most promising position 9 substituents from our earlier study [7]: Gly-NH_2, Ala-NH_2, Arg-NH_2, Tyr-NH_2, together with the *N*-methylated derivatives of Gly-NH_2 and Ala-NH_2, other aliphatic amino acids, Cha-NH_2 and Val-NH_2, a polar amino acid, Thr-NH_2, another aromatic amino acid, Phe-NH_2, and the basic amino acids, Orn-NH_2 and Lys-NH_2. To evaluate the role of stereochemistry at position 9, the D-Lys-NH_2^9 analogue of **C** was included in this study.

Position 6. Pen^6 (penicillamine 6). Pen^6 substitutions in OT and in LVP resulted respectively in a very weak OT agonist and in a VP antagonist [14,15]. We thus wondered what the effect of a Pen^6 substitution would be in the VP hypotensive agonist **C** – would it be tolerated to retain or enhance hypotensive agonism or would it lead to an antagonist of the VP hypotensive response.

Position 2. D-Trp; D-2-Nal, D-Tyr(Bu^t); D-Tyr(Pr^n), D-Tyr (Pr^i). In our previous study [7] of position 2 modifications of **A**, we reported the effects of D-Tyr(Me)^2 and

L-Tyr(Et)^2 modifications. Both modifications brought about drastic reductions in vasodepressor potencies [7]. We have also found that D-aliphatic amino acids are not tolerated at position 2 [6]. In this study on the effects of position 2 substituents in **C** we selected a series of D-aromatic amino acids which we and others had previously found to be effective position 2 substituents in leading to partial or full retention or to enhancement of antagonistic potencies in VP and OT antagonists [10,16–18]. Thus D-Trp^2 and D-Nal^2 have been utilized in the design of potent OT antagonists [16–18]. We had found that both D-Tyr(Pr^n) and D-Tyr(Pr^i) [19] are reasonably well tolerated as position 2 substituents in a V_2/V_{1a}/OT antagonist [10]. To further evaluate the role of increasing the size of the D-Tyr (alk) substituent at position 2 in **C**, the D-Tyr (Bu^t) substituent was also included. All the five position 2 substituents were thus selected in the hope that one or more might be well tolerated in the potent and selective hypotensive agonist **C**.

Design of Radioiodinatable Hypotensive Ligands Using Combined Modifications of C

Positions 6 and 10. Pen^6, retro-Tyr^{10}; positions 2 and 10: D-Tyr (Pr^i)^2, retro-Tyr [10]. We have previously reported that the retro-Tyr^{10} modification of **B** is very well tolerated [7,9]. In fact, with an ED = 1.05 μg/100 g, the retro-Tyr^{10} analogue of **B** is one of the most potent hypotensive VP agonists reported to date and thus is a promising radioiodinatable ligand for the putative VP vasodilating receptor [7,9]. In this study, with a view to designing more potent radioiodinatable ligands, we selected the Pen^6 and D-Tyr(Pr^i)^2 analogues of **C** for retro-Tyr^{10} modification.

On the basis of the above rationale we now report the synthesis, by the solid-phase method, of d(CH$_2$)$_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Eda^9]LVP (**C**) and the new analogues of **C**, peptides **1–21**, listed below:

C d(CH$_2$)$_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Eda^9]LVP
1 d(CH$_2$)$_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Gly-NH$_2$^{9}]LVP
2 d(CH$_2$)$_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Gly-NH-CH$_3$^{9}]LVP
3 d(CH$_2$)$_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Ala-NH$_2$^{9}]LVP
4 d(CH$_2$)$_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Ala-NH-CH$_3$^{9}]LVP
5 d(CH$_2$)$_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Val-NH$_2$^{9}]LVP
6 d(CH$_2$)$_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Cha-NH$_2$^{9}]LVP
7 d(CH$_2$)$_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Thr-NH$_2$^{9}]LVP
8 d(CH$_2$)$_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Phe-NH$_2$^{9}]LVP
9 d(CH$_2$)$_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Tyr-NH$_2$^{9}]LVP
10 d(CH$_2$)$_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Orn-NH$_2$^{9}]LVP
11 d(CH$_2$)$_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Lys-NH$_2$^{9}]LVP
12 d(CH$_2$)$_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,D-Lys-NH$_2$^{9}]LVP
13 d(CH$_2$)$_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Arg-NH$_2$^{9}]LVP
14 d(CH$_2$)$_5$[D-Tyr(Et)^2,Arg^3,Val^4,Pen^6,Lys^7,Eda^9]LVP
15 d(CH$_2$)$_5$[D-Tyr(Et)^2,Arg^3,Val^4,Pen^6,Lys^7,Eda^9 ← Tyr^{10}]LVP
16 d(CH$_2$)$_5$[D-Trp^2,Arg^3,Val^4,Lys^7,Eda^9]LVP
17 d(CH$_2$)$_5$[D-2-Nal^2,Arg^3,Val^4,Lys^7,Eda^9]LVP
18 d(CH$_2$)$_5$[D-Tyr(Bu^t)^2,Arg^3,Val^4,Lys^7,Eda^9]LVP
19 d(CH$_2$)$_5$[D-Tyr(Pr^n)^2,Arg^3,Val^4,Lys^7,Eda^9]LVP
20 d(CH$_2$)$_5$[D-Tyr(Pr^i)^2,Arg^3,Val^4,Lys^7,Eda^9]LVP
21 d(CH$_2$)$_5$[D-Tyr(Pr^i)^2,Arg^3,Val^4,Lys^7,Eda^9 ← Tyr^{10}]LVP

Preliminary pharmacological properties of **C** and of peptides **1, 9–14**, and **16–20** have been reported respectively in Refs [12] and [20]. We present here for the first time the quantitative vasodepressor potencies of peptide **C** and of peptides **1–21**.

Peptide Synthesis

Peptide **C** and the new analogues of **C**, peptides **1–21**, were synthesized utilizing the solid-phase method as follows: The protected peptide amides **I–XIII** (Table 1) were obtained by a combination of solid-phase [21,22] and solution methods [23]. First, the solid-phase method [21,22] was utilized to prepare intermediate protected acylheptapeptides, followed by DCC/HOBt-mediated '7 + 1' coupling in solution [23] with the appropriate amino acid amide to give the desired protected peptide amide. The protected Eda peptides, **c, XIV**, and **XVI–XX** (Table 1), were synthesized entirely by the solid-phase method [21,22]. The protected retro-modified peptides **XV** and **XXI** (Table 1) were obtained by coupling the protected Eda peptides **XIV** and **XX** with *Z*-Tyr(Bzl) in DMF using the DCC/HOBt procedure [23] as described in Ref. 24. Solid-phase syntheses were carried out by the Merrifield method [21,22] with the modifications previously described [7,10,25–28]. Boc-Lyz(Tos)-resin or Boc-Lys[Z(2Cl)]-resin was converted to the corresponding acylheptapeptidyl resins in seven cycles of deprotection, neutralization, and coupling. A HCl (1 M)/AcOH mixture was used in all the deprotection steps [7,10,25–28]. Neutralizations were carried out with 10% Et_3N/CH_2Cl_2. Coupling reactions were mediated mainly by DCC/HOBt [23] in CH_2Cl_2/DMF except for Boc-Asn which was incorporated as its 4-nitrophenyl ester (ONp) [29] in DMF. The acylpeptide resins were cleaved using the following procedures: acidolysis by HBr/TFA [21,22,30,31] to give the protected acylheptapeptide intermediates required for the preparation of protected acylpeptide amides **I–XIII**, or by aminolysis with Eda in methanol [7,24,32] to give the protected Eda peptides **c, XIV**, and **XVI-XX** (Table 1). All the protected precursors, including the protected peptides **XV** and **XXI**, were purified by the same general method: extraction or dissolving with warm DMF followed by reprecipitations with H_2O or EtOH/Et_2O until adjudged pure by TLC, as previously described [7,10,25–28]. The physicochemical properties of all protected peptides are given in Table 1. Deprotection of all protected peptides was carried out with sodium in liquid

Table 1 Physicochemical properties of protected peptides **c** and **I–XXI**[a]

No	Peptide	Yield (%)[b]	m.p. (^{0}C)	TLC, R_f[c]					
				a	b	c	d	e	f
C	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Lys-[Z(2Cl)]-Lys[Z(2Cl)]-Eda	67.7	214–216	0.86	0.69	0.77	—	0.92	—
I	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Lys-(Tos-Lys(Tos)-Gly-NH_2	86.6	182–184	0.86	0.73	0.73	—	0.37	—
II	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Lys-[Z(2Cl)]-Lys[Z(2Cl)]-Gly-NH-CH_3	81.7	193–195	0.91	0.74	0.78	—	—	0.68
III	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Lys-(Tos)-Lys(Tos)-Ala-NH_2	95.0	200–202	0.87	0.77	0.76	—	—	—
IV	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Lys-[Z(2Cl)]-Lys[Z(2Cl)]-Ala-NH-CH_3	78.4	201–203	0.91	0.81	0.79	—	—	0.72
V	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Lys-[Z(2Cl)]-Lys[Z(2Cl)]-Val-NH_2	79.1	188–190	0.97	0.82	0.87	—	—	0.66
VI	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Lys-(Tos)-Lys(Tos)-Cha-NH_2	74.0	207–209	0.91	0.82	0.77	—	0.54	—
VII	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Lys-(Tos)-Lys(Tos)-Thr(Bzl)-NH_2	84.1	212–214	0.94	0.86	0.91	0.93	—	—
VIII	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Lys-[Z(2Cl)]-Lys[Z(2Cl)]-Phe-NH_2	78.5	210–212	0.97	0.81	0.84	—	—	0.79
IX	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Lys-(Tos)-Lys(Tos)-Tyr-NH_2	86.8	215–217	0.96	0.75	0.77	—	—	0.71
X	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Lys-(Tos)-Lys(Tos)-Orn(Tos)-NH_2	93.5	186–188	0.95	0.84	0.86	0.97	—	—
XI	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Lys-(Tos)-Lys(Tos)-Lys(Z)-NH_2	80.0	192–194	0.97	0.81	0.84	—	—	0.68
XII	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Lys-(Tos)-Lys(Tos)-D-Lys(Z)-NH_2	87.8	219–221	0.94	0.84	0.82	0.96	—	—
XIII	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Lys-(Tos)-Lys(Tos)-Arg(Tos)-NH_2	80.5	216–218	0.96	0.78	0.75	—	—	0.74
XIV	d$(CH_2)_5$(Mob)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Pen(Bzl)-Lys-[Z(2Cl)]-Lys[(Z(2Cl)]-Eda	84.6	212–214	0.77	0.65	0.70	—	—	0.54
XV	d$(CH_2)_5$(Mob)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Pen(Bzl)-Lys-[Z(2Cl)]-Lys[Z(2Cl)]-Eda←Tyr(Bzl)-Z	69.6	222–224	0.88	0.83	0.80	—	—	0.82
XVI	d$(CH_2)_5$(Bzl)-D-Trp-Arg(Tos)-Val-Asn-Cys(Mob)-Lys-[Z(2Cl)]-Lys[Z(2Cl)]-Eda	94.6	191–193	0.73	0.74	0.66	—	—	—
XVII	d$(CH_2)_5$(Bzl)-D-Nal-Arg(Tos)-Val-Asn-Cys(Mob)-Lys-[Z(2Cl)]-Lys[Z(2Cl)]-Eda	80.0	208–210	0.70	0.64	0.69	—	—	0.80
XVIII	d$(CH_2)_5$(Meb)-D-Tyr(Bu^t)-Arg(Tos)-Val-Asn-Cys(Mob)-Lys-[Z(2Cl)]-Lys[Z(2Cl)]-Eda	78.6	198–200	0.64	0.58	0.62	0.78	—	—
XIX	d$(CH_2)_5$(Meb)-D-Tyr(Pr^n)-Arg(Tos)-Val-Asn-Cys(Mob)-Lys-[Z(2Cl)]-Lys[Z(2Cl)]-Eda	94.6	207–209	0.62	0.56	0.67	0.69	—	—
XX	d$(CH_2)_5$(Bzl)-D-Tyr(Pr^i)-Arg(Tos)-Val-Asn-Cys(Mob)-Lys-[Z(2Cl)-Lys[Z(2Cl)]-Eda	91.9	202–203	0.73	0.74	0.66	—	—	—
XXI	d$(CH_2)_5$ (Bzl)-D-Tyr(Pr^i)-Arg(Tos)-Val-Asn-Cys-(Mob)-Lys-[Z(2Cl)]-Lys[Z(2Cl)]-Eda←Tyr(Bzl)-Z	88.0	226–228	—	0.87	0.85	—	0.92	—

[a] The protected peptides **c** and **I–XXI** are the immediate protected precursors for the free peptides **C** and **1–21** given in Tables 3 and 4.
[b] Yields are based on the amino acid content of the resin for protected peptides **c** and **XVI–XX**. For protected peptides **I–XV** and **XXI**, yields were calculated on the basis of the theoretical yields expected from the solution coupling.
[c] Solvent systems are described in the section Experimental.

ammonia [33,34], as previously described [7,10,25–28], except for the 2-naphthylalanine containing peptide **XVII**, which was deprotected by the TFMSA procedure [35,36]. The resulting disulphydryl compounds were oxidatively cyclized with $K_3[Fe(CN)_6]$ [37] using the modified reverse procedure [38]. The free peptides were desalted and purified by gel filtration on Sephadex G-15 and Sephadex LH-20 mainly in a two-step procedure

Table 2 Physicochemical properties of free peptides **C, 1–21**[a]

No	Peptide	Yield(%)[b]	TLC, R_f[c]				HPLC T_R(min)	Formula	MW	MW Found
			a	c	d	F				
C	d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Eda^9]LVP	16.8	0.24	0.03	0.30	0.09	16.0	$C_{51}H_{84}O_{10}N_{15}S_2$	1134.5	1134.5
1	d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Gly-NH$_2^9$]LVP	31.3	0.22	0.03	0.38	0.10	25.9	$C_{51}H_{85}O_{11}N_{15}S_2$	1148.5	1148.9
2	d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Gly-NH-CH$_3^9$]LVP	60.6	0.16	0.02	0.32	0.14	23.7	$C_{52}H_{87}O_{11}N_{15}S_2$	1162.5	1162.5
3	d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Ala-NH$_2^9$]LVP	25.5	0.20	0.04	0.35	0.11	25.6	$C_{51}H_{87}O_{11}N_{15}S_2$	1162.5	1162.7
4	d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Ala-NH-CH$_3^9$]LVP	67.8	0.22	0.02	0.36	0.14	25.5	$C_{53}H_{89}O_{11}N_{15}S_2$	1176.5	1176.6
5	d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Val-NH$_2^9$]LVP	55.0	0.21	0.08	0.38	0.51	26.4	$C_{54}H_{91}O_{11}N_{15}S_2$	1190.6	1190.5
6	d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Cha-NH$_2^9$]LVP	23.3	0.24	0.06	0.47	0.49	31.1	$C_{58}H_{97}O_{11}N_{15}S_2$	1244.6	1244.7
7	d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Thr-NH$_2^9$]LVP	33.4	0.20	0.05	0.43	0.12	24.3	$C_{53}H_{89}O_{12}N_{15}S_2$	1192.5	1192.4
8	d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Phe-NH$_2^9$]LVP	22.4	0.19	0.03	0.36	0.29	29.0	C_{58} $H_{91}O_{11}N_{15}S_2$	1238.6	1238.6
9	d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Tyr-NH$_2^9$]LVP	56.6	0.23	0.15	0.38	0.31	26.3	$C_{58}H_{91}O_{12}N_{15}S_2$	1254.6	1254.6
10	d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Orn-NH$_2^9$]LVP	22.6	0.10	—	0.06	0.02	23.1	$C_{54}H_{92}O_{11}N_{16}S_2$	1205.6	1204.6
11	d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Lys-NH$_2^9$]LVP	61.7	0.18	0.02	0.28	0.10	22.8	$C_{55}H_{94}O_{11}N_{16}S_2$	1219.6	1219.6
12	d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,D-Lys-NH$_2^9$]LVP	21.8	0.10	—	0.05	0.02	19.4	$C_{55}H_{94}O_{11}N_{16}S_2$	1219.6	1218.6
13	d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Arg-NH$_2^9$]LVP	64.2	0.19	—	0.30	0.06	18.6	$C_{55}H_{94}O_{11}N_{18}S_2$	1247.6	1247.6
14	d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4,Pen^6,Lys^7,Eda^9]LVP	12.1	0.10	—	0.26	0.04	16.4	$C_{53}H_{91}O_{10}N_{15}S_2$	1162.5	1162.4
15	d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4,Pen^6,Lys^7,Eda^9 ←Tyr^{10}] LVP	41.8	0.09	0.02	0.30	0.10	16.6	$C_{62}H_{100}O_{12}N_{16}S_2$	1325.7	1325.5
16	d$(CH_2)_5$[D-Trp^2,Arg^3,Val^4,Lys^7,Eda^9]LVP	25.6	0.06	–0.06	0.04		14.7	$C_{51}H_{84}O_9N_{16}S_2$	1129.5	1129.9
17	d$(CH_2)_5$[D-2-Nal^2,Arg^3,Val^4,Lys^7,Eda^9]LVP	23.9	0.09	0.03	0.31	0.09	16.8	$C_{53}H_{85}O_9N_{15}S_2$	1140.5	1140.1
18	d$(CH_2)_5$[D-Tyr(Bu^t)^2,Arg^3,Val^4,Lys^7,Eda^9]LVP	36.0	0.02	0.11	0.22	0.07	17.9	$C_{53}H_{91}O_{10}N_{15}S_2$	1162.5	1162.9
19	d$(CH_2)_5$[D-Tyr(Pr^n)^2,Arg^3,Val^4,Lys^7,Eda^9]LVP	40.1	0.17	0.03	0.28	0.05	18.3	$C_{52}H_{89}O_{10}N_{15}S_2$	1148.5	1148.6
20	d$(CH_2)_5$[D-Tyr(Pr^i)^2,Arg^3,Val^4,Lys^7,Eda^9]LVP	23.1	0.04	—	0.08	0.04	17.1	$C_{52}H_{89}O_{10}N_{15}S_2$	1148.5	1148.9
21	d$(CH_2)_5$[D-Tyr(Pr^i)^2,Arg^3,Val^4,Lys^7,Eda^9 ←Tyr^{10}]LVP	19.1	0.20	—	0.14	0.05	19.1	$C_{61}H_{97}O_{12}N_{16}S_2$	1311.6	1311.6

[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 section Experimental.
[c] All peptides were at least 95% pure. For elution a linear gradient 90 : 10 to 30 : 70 (0.05% aqueous TFA: 0.05 TFA in CH_3CN) over 30 min with flow rate 1.0 ml/min was applied.

[39] using 50% AcOH and 2 M AcOH as eluents, respectively, as previously described [7,10,25–27]. When necessary, 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 **C** and **1–21** (Table 2) was checked by thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), and mass spectrometry (MS).

Bioassays

Peptides **C** and **1–21** were examined in the standard vasopressor, antidiuretic, and oxytocic bioassays for neurohypophysial peptides. Vasopressor assays were performed in urethane-anesthetized male rats, 1.0 g/kg i.p., as described by Dekanski [40]. 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 [41]. The rat was anesthetized (surgical depth) with 12% ethanol, 50 ml/kg p.o. Water diuresis was induced and anesthesia maintained by a constant water load 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 [42] for isotonic contraction recording. In all bioassays, agonistic potencies of the peptides were determined by the four-point (2×2 parallel-line) bioassay design [43]. Antagonistic potencies were measured by the pA_2 method [44]. VP 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 expressed as the mean ± SE.

Determination of Vasodepressor Activity

Vasodepressor activities of the hypotensive VP peptide **C** (Table 3) and peptides **1–21** (Tables 3 and 4) were

Table 3 Vasodepressor potencies of d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Eda^9]LVP (**C**), related analogues **A** and **B**, and analogues of **C** with single modifications at positions 9 (**1–13**) and 6 (**14**) and combined modifications at positions 6 and 10 (**15**)

No	Peptide	Vasodepressor ED, μg/100 g[a,b]
A	d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4]AVP[c]	4.66 ± 0.46
B	d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4,Arg^7,Eda^9]AVP[c]	1.10 ± 0.25
C	d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Eda^9]LVP[d,f]	0.53 ± 0.05
1	d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Gly-NH$_2^9$]LVP[e,f]	2.41 ± 0.24
2	d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Gly-NH-CH$_3^9$]LVP[f]	1.13 ± 0.10
3	d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Ala-NH$_2^9$]LVP[f]	1.62 ± 0.11
4	d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Ala-NH-CH$_3^9$]LVP[f]	0.80 ± 0.09
5	d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Val-NH$_2^9$]LVP[f]	1.83 ± 0.14
6	d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Cha-NH$_2^9$]LVP[f]	1.56 ± 0.11
7	d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Thr-NH$_2^9$]LVP[f]	2.12 ± 0.21
8	d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Phe-NH$_2^9$]LVP[f]	2.58 ± 0.12
9	d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Tyr-NH$_2^9$]LVP[e,f]	1.40 ± 0.14
10	d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Orn-NH$_2^9$]LVP[e,f]	0.88 ± 0.05
11	d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Lys-NH$_2^9$]LVP[e,f]	0.90 ± 0.07
12	d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,D-Lys-NH$_2^9$]LVP[e,f]	0.85 ± 0.048
13	d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Arg-NH$_2^9$]LVP[e,f]	0.68 ± 0.05
14	d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4,Pen^6, Lys^7,Eda^9]LVP[e,f]	0.99 ± 0.04
15	d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4,Pen^6, Lys^7,Eda^9 ← Tyr^{10}]LVP[f]	1.05 ± 0.08

[a] ED, effective dose (in μg 100/g 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 section on Methods for details of vasodepressor assays).
[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] From Chan *et al.* [9].
[d,e] Preliminary data reported are from: [d] Ref. 12; [e] Ref. 20.
[f] This publication.

Table 4 Vasodepressor potencies of d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Eda^9]LVP (**C**) and analogues of **C** with modifications at position 2 (**16–20**) and at positions 2 and 10 (**21**)

No	Peptide	Vasodepressor ED, μg/100 g[a,b]
C	d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Eda^9]LVP[c]	0.53 ± 0.05
16	d$(CH_2)_5$[D-Trp^2,Arg^3,Val^4, Lys^7,Eda^9]LVP[d,e]	0.66 ± 0.05
17	d$(CH_2)_5$[D-2-Nal^2,Arg^3,Val^4, Lys^7,Eda^9]LVP[d,e]	0.54 ± 0.03
18	d$(CH_2)_5$[D-Tyr(Bu^t)^2,Arg^3,Val^4, Lys^7,Eda^9]LVP[d,e]	0.33 ± 0.02
19	d$(CH_2)_5$[D-Tyr(nPr)^2,Arg^3,Val^4, Lys^7,Eda^9]LVP[d,e]	0.18 ± 0.018
20	d$(CH_2)_5$[D-Tyr(iPr)^2,Arg^3,Val^4, Lys^7,Eda^9]LVP[d,e]	0.15 ± 0.01
21	d$(CH_2)_5$[D-Tyr(iPr)^2,Arg^3,Val^4, Lys^7,Eda^9 ←Tyr^{10}]LVP[d,e]	0.14 ± 0.02

[a] See footnote [a] in Table 3 for definition of ED and AUC.
[b] See the corresponding footnote in Table 3.
[c] Preliminary data reported in Ref. 12.
[d] Preliminary data reported in Ref. 20.
[e] This publication.

determined in urethane-anesthetized male rats as previously described [7,9]. BP was monitored via a cannulated carotid artery. The vasodepressor response was measured by the 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 was 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, is the 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 [7,9], 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) was infused at a rate (0.01–0.05 ml/min) 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 vasodepressor potencies of d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3, Val^4,Lys^7,Eda^9]LVP (**C**) and the analogues of **C** with single modifications at positions 9 (peptides **1–13**) and position 6 (peptide **14**), together with the radioiodinatable peptide **15** that contains modifications at position 6 (Pen^6) and 10 (retro-Tyr) are presented in Table 3. The vasodepressor potencies of the position 2 modified analogues of **C** (peptides **16–20**) and of the positions 2 (D-Tyr(Pr^i) and 10 (retro-Tyr) modified radioiodinatable analogue (peptide **21**) of **C** are presented in Table 4. Peptide **C** and all of the new peptides reported here were also examined in the standard rat antidiuretic, vasopressor, and oxytocic bioassays. Like the original VP hypotensive peptide A [5,7], they were found to exhibit negligible or undetectable agonistic activities and no antagonistic activities in these assays. Thus only the vasodepressor potencies of peptides **C** and **1–21** are given in Tables 3 and 4. The data in Table 3 show that all the position 9 analogues of **C** reported here exhibit potent hypotensive agonism. These findings illustrate that position 9 in **C** tolerates a wide variety of substituents without drastic losses of hypotensive potency. With vasodepressor ED values ranging from 0.80 to 2.58 μg/100 g, all are more potent than **A** which has a vasodepressor ED value = 4.66 μg/100 g [7,9]. However, none of the position 9 substituted analogues of **C** are as potent as or more potent than **C** with vasodepressor ED value = 0.53 μg/100 g [12]. With vasodepressor ED values ranging from 0.15–0.66 μg/100 g, all the position 2 analogues of **C** (Table 4) are more potent than all the position 9 analogues of **C** reported in Table 3. In fact, three of the position 2 analogues of **C**, the D-Tyr (Bu^t), D-Tyr (Pr^n)^2, and D-Tyr(Pr^i)^2 analogues (Nos **18**, **19**, and **20**), with vasodepressor EDs = 0.33, 0.18, and 0.15 μg/100 g respectively, are more potent than **C**. One of these, **17**, the D-2-Nal^2 analogue with a vasodepressor ED = 0.54 μg/100 g, is equipotent with **C**. Only one, the D-Trp^2 analogue (**16**) with a vasodepressor ED = 0.66 μg/100 g, is slightly less potent than **C**. With a vasodepressor ED = 0.14 μg/100 g,

the retro-Tyr[10] analogue (**21**) is equipotent with it parent D-Tyr(Pr^i)[2] analogue (**20**). With vasodepressor EDs of 0.15 and 0.14 μg/100 g, both peptides **20** and **21** exhibit 30-fold gains in hypotensive potency over the original hypotensive peptide **A** (vasodepressor ED = 4.66 μg/100 g). These two peptides are clearly the most potent hypotensive VP agonists reported to date.

DISCUSSION

We have previously shown that the hypotensive potency of our original selective hypotensive VP agonist d(CH$_2$)$_5$[D-Tyr(Et)[2],Arg[3],Val[4]]AVP **A** (vasodepressor ED = 4.66 μg/100 g) [7,9] could be enhanced 4–5 fold by replacing the Pro[7] and Gly-NH$_2^9$ residues with Arg[7] and Eda[9] residues [7,9]. The resultant peptide, d(CH$_2$)$_5$[D-Tyr(Et)[2],Arg[3],Val[4],Arg[7],Eda[9]]AVP (**B**), exhibits a vasodepressor ED = 1.10 μg/100 g [7,9]. A further two fold increase in hypotensive potency was effected by replacing the Arg[7], Arg[8] residues in **B** with Lys[7], Lys[8] residues to give d(CH$_2$)$_5$[D-Tyr(Et)[2],Arg[3],Val[4],Lys[7],Eda[9]]LVP (**C**) (vasodepressor ED = 0.53 μg/100 g) [12], used as the new lead in the studies reported here. In this study, this new lead hypotensive agonist **C** was modified with single substitutions at positions 9, 6, and 2. Radioiodinatable ligands were designed by incorporating retro-Tyr[10] modifications at position 9 in peptides **14** and **20** to give respectively peptides **15** and **21**.

Effects of Position 9 Modification of C (Table 3)

Replacement of the Eda[9] residue in **C** by a wide variety of aliphatic, aromatic, polar, and basic amino acids resulted in analogues **1–13**, Table 3, all of which exhibit potent hypotensive agonism. With vasodepressor ED values ranging from a low of 2.58 μg/100 g to a high of 0.68 μg/100 g, all these position 9 analogues of **C** are more potent than the original standard VP hypotensive peptide **A** (vasodepressor ED = 4.66 μg/100 g) [7,9]). With vasodepressor ED values of 0.68, 0.85, 0.90, 0.88 and 0.80 μg/100 g, 5 of these 13 peptides, respectively, the Arg-NH$_2^9$, D-Lys-NH$_2^9$, Lys-NH$_2^9$, Orn-NH$_2^9$, and Ala-NH-CH$_3^{9-}$ derivatives are also more potent than **B** (vasodepressor ED = 1.10 μg/100 g [7,9]). However, none of these position 9 substituted analogues is more potent than **C** (vasodepressor ED = 0.53 μg/100 g). Comparisons of the vasodepressor ED values of peptides **1–13** (Table 3) reveal that the analogues with the basic amino acids Orn-NH$_2^9$, D and L-Lys-NH$_2^9$, and Arg-NH$_2^9$ are the most potent of this series. Of note also is the finding that the L- and D-Lys-NH$_2^9$ analogues are virtually equipotent hypotensive agonists. Thus, there is no specific requirement for an L-amino acid at position 9. Amide *N*-methylation of the *C*-terminal Gly-NH$_2$ (**1**) and Ala-NH$_2$ (**3**) to give the peptides **2** and **4** resulted in both instances in 2-fold enhancements in hypotensive potencies. Comparisons of the vasodepressor ED values of the Phe-NH$_2^9$ analogue (peptide **8**: vasodepressor ED = 2.58 μg/100 g) with the Cha-NH$_2^9$ analogue (peptide **6**: vasodepressor ED = 1.56 μg/100 g) suggest that aliphalicity at position 9 is superior to aromaticity for retention of hypotensive activity. Yet with a vasodepressor ED value = 1.40 μg/100 g, the Tyr-NH$_2^9$ analogue (peptide **9**) is clearly more potent than both peptides **6** and **8**. This supports the view that a combination of aromaticity and polarity at position 9 is of importance in contributing to hypotensive potency. These findings on the effects of position 9 substituents in **C** may provide useful clues for the design of more potent hypotensive agonists.

Effects of Pen[6] Substitution in C

Replacement of the Cys residue at position 6 in **C** by a Pen residue to give peptide **14** resulted in an unexpected retention of hypotensive activity. With a vasodepressor ED = 0.99 μg/100 g, peptide **14** exhibits about 50% of the hypotensive potency of **C**. In light of the fact that previous studies have shown that the Pen[6]/Cys[6] interchange in OT and in LVP resulted respectively in a weak OT agonist and in a V$_{1a}$ antagonist (14,15), the fact that this interchange in the VP hypotensive agonist **C** leads to retention of 50% of hypotensive agonism would appear to indicate that steric hindrance at position 6 in a hypotensive VP agonist is not deleterious to the activation of the putative VP vasodilating receptor.

Effects of Position 2 Substituents in C (Table 4)

Replacement of the D-Tyr(Et)[2] residue in **A** by L-Tyr(Et) and by D-Tyr(Me) had previously been shown to lead to drastic reductions in vasodepressor potencies [7]. Thus, the findings that the D-Tyr(Et)[2] residue in **C** could be replaced with a variety of other position 2 substituents with retention and in some instances clear enhancement of hypotensive potency was a surprise. Furthermore, the finding that the D-Tyr(Pr^n)[2] and D-Tyr(Pr^i)[2] substitutions in **C** led to a marked enhancement of hypotensive potencies relative to the D-Tyr(Et)[2] parent **C** is in marked contrast to their relative effects in the aforementioned V$_2$/V$_{1a}$/OT antagonist, d(CH$_2$)$_5$[D-Tyr(Et)[2],Val[4]] AVP [10]. Replacement of the D-Tyr(Et)[2] residue in this peptide by D-Tyr(Pr^n)[2] and by D-Tyr (Pr^i)[2] resulted in both instances in reductions of rat anti-antidiuretic potencies. Thus, the enhancements of hypotensive agonism exhibited by peptides **19** and **20** relative to **C** was not anticipated. This surprise finding offers promising new clues for the design of more potent hypotensive agonists.

With vasodepressor ED values of 0.33, 0.18, and 0.15 μg/100 g, peptides **18**, **19**, and **20** are

clearly more potent than **C** (vasodepressor ED = 0.53 μg/100 g). With a vasodepressor ED = 0.15 μg/100 g, the D-Tyr(Pr^i)^2 analogue is the most potent hypotensive agonist of this series and is consequently one of the most potent VP hypotensive agonists reported to date. It was thus selected for derivitization with a retro-Tyr^{10} to give Peptide **21**.

Design of Radioiodinatable Hypotensive Agonists (Tables 3 and 4)

We have previously utilized the retro-Tyr^{10} modification of **B** for the design of a highly potent radioiodinatable ligand [7,9]. In this study, we selected peptide **14**, the Pen^6 analogue of **C** (vasodepressor ED = 0.99 μg/100 g), and peptide **20**, the D-Tyr(Pr^i)^2 analogue of **C** (vasodepressor ED = 0.15 μg/100 g), for modification with a retro-Tyr residue. The resulting retro-Tyr^{10} peptides (**15** and **21**) are highly potent hypotensive agonists. With vasodepressor ED values of 1.05 and 0.14 μg/100 g, respectively, peptides **15** (Table 3) and **21** (Table 4) are virtually equipotent with their parent analogues (**14** and **20**). With a vasodepressor ED of 0.14 μg/100 g, peptide **21** is, in fact, the most potent hypotensive peptide reported to date and is thus a very promising new radioiodinatable ligand that could be of value in studies aimed at the location and characterization of the putative VP vasodilating receptor.

CONCLUSION

In this study we have examined the effects of single and multiple modifications of the potent hypotensive VP agonist, d(CH$_2$)$_5$[D-Tyr(Et)^2,Arg^3,Val^4,Lys^7,Eda^7]LVP **C** [12]. Peptide **C** was modified at position 9 by replacing the Eda residue with Gly-NH$_2$ (**1**), Gly-NH-CH$_3$ (**2**), Ala-NH$_2$ (**3**), Ala-NH-Me (**4**), Val-NH$_2$ (**5**), Cha-NH$_2$ (**6**), Thr-NH$_2$ (**7**), Phe-NH$_2$ (**8**), Tyr-NH$_2$ (**9**), Orn-NH$_2$ (**10**), Lys-NH$_2$ (**11**), D-Lys-NH$_2$ (**12**), and Arg-NH$_2$ (**13**). **C** was modified at position 6 by replacing the Cys residue with Pen (**14**). With vasopressor ED values ranging from 2.58–0.68 μg/100 g, all of the position 9 analogues and the Pen^6 analogue of **C** are more potent than the original hypotensive peptide d(CH$_2$)$_5$[D-Tyr (Et)^2,Arg^3,Val^4]AVP (**A**) [7] (vasodepressor ED = 4.66 μg/100 g] [7,9]. None, however, is as potent as **C** (vasodepressor ED = 0.53 μg/100 g) [12]. Nonetheless, this study on the effects of a variety of amino acids and substituted amino acids at position 9 in **C** has uncovered some important clues for the design of more potent VP hypotensive agonists.

This study has also uncovered promising new position 2 substituents in **C**, which enhance hypotensive agonism. We examined the effects of replacing the D-Tyr(Et)^2 residue in **C** with D-Trp (**16**), D-2-Nal (**17**), D-Tyr(Bu^t) (**18**), D-Tyr(Pr^n) (**19**), and D-Tyr(Pr^i) (**20**). Surprisingly, with vasodepressor ED values ranging from 0.66–0.15 μg/100 g, all are clearly more potent than **A**. With vasodepressor ED values of 0.33, 0.18, and 0.15 μg/100, peptides **18–20** are more potent than **C**. The D-Tyr(Pr^i)^2 analogue (**20**) is in fact 30 times more potent than the original hypotensive peptide **A**. With a vasodepressor ED = 0.14 μg/100 g, peptide **21**, the retro-Tyr^{10} analogue of **20**, is a promising new radioiodinatable ligand for the characterization of the putative VP vasodilating receptor. As noted earlier, the hypotensive agonists **B** [7,9] and **C** [12] (Table 3) have been utilized as pharmacological tools to investigate the hypotensive responses to VP [11,13]. The more potent analogues of **C**, peptides **18–21** reported here, are new pharmacological tools for studies on the vasodilating effects of VP in regional vascular beds. They also offer promising clues to the design of more potent hypotensive VP agonists for development as a new class of antihypertensive agents. The findings presented here may also be of value for the design of the first antagonists of the VP hypotensive response.

EXPERIMENTAL

All reagents used were of analytical grade; Boc-Lys(Tos)-resin was prepared in this laboratory by the cesium salt method [45]. Boc-D-Tyr(Et), Boc-D-Tyr(Pr^i), and Boc-D-Tyr(Pr^n) were synthesized as previously described [19]. Gly-NH-CH$_3$ and Ala-NH-CH$_3$ were synthesized by the ONp-ester procedure [46]; Cha-NH$_2$, Thr(Bzl)-NH$_2$, Orn(Tos)-NH$_2$, D-Lys(Z)-NH$_2$, and Arg(Tos)-NH$_2$ were prepared by the mixed anhydride method [47]. Boc-Lys[Z(2Cl)]-resin, Val-NH$_2$, TyrNH$_2$, and Lys(Z)-NH$_2$ were purchased from Chem-Impex International (Wood Dale, IL). The β-S-(4-methoxybenzyl)mercapto-β,β-pentamethylenepropionic acid, Gly-NH$_2$, Ala-NH$_2$, Phe-NH$_2$, and Z-Tyr(Bzl) were purchased from Bachem Bioscience, Inc., (King of Prussia, PA). Fmoc-D-Tyr(Bu^t) was purchased from Bachem, California, Inc. All other amino acid derivatives were purchased from Bachem California, Inc. or Chem-Impex International (Wood Dale, IL). TLC was run on precoated silica gel plates (60F-254, E. Merck) with the following solvent systems: (a) 1-butanol : AcOH : H$_2$O (4 : 1 : 5, upper phase); (b) 1-butanol : AcOH : H$_2$O (4 : 1 : 1); (c) 1-butanol : AcOH :H$_2$O: pyridine (15 : 3 : 3 : 10); (d) 1-butanol : AcOH : H$_2$O (4 : 1 : 2); (e) chloroform : methanol (7 : 3); (f) 1-butanol : AcOH : H$_2$O (2 : 1 : 1). 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 [22]. 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 CH$_3$CN, linear gradient over 30 min at 1.0 ml/min (λ = 210 nm), on a Microsorb C$_{18}$ column (Rainin Instrument Co., Inc.). All peptides were at least 95% pure. Mass spectra (MS) were taken by the University of Oklahoma Health Science Center Molecular Biology Resource Facility on a PE Sciex Q-STAR Quadropole TOF electron spray mass spectrometer using 50 : 50 CH$_3$CN/H$_2$O with 0.5% AcOH as a solvent, or by Tufts Medical School

Core Facility, Medical Department, on a Voyager (Applied Biosystems) matrix-assisted laser desorption ionization, time-of-flight (MALDI-TOF) mass spectrometer using dihydroxybenzoic acid (DHB) as the matrix. Mass spectra of the free peptides were in agreement with the composition of each peptide.

Solid-phase Synthesis Procedures

The protected precursors **I–XIII** (Table 1) of the free peptides **1–13** (Table 2) were synthesized by a combination of the Merrifield solid-phase [21,22] with the modifications previously described [7,10,25–28] and solution synthesis methods. Boc-Lys(Tos)-resin or Boc-Lys[Z(2Cl)]-resin was prepared by esterification of Merrifield resin (chloromethylated resin (1% cross-linked S-DVB, 200–400 mesh, 0.7–1.00 mmol/g) with Boc-Lys(Tos) or Boc-Lys [Z(2Cl)] to an incorporation of approximately 0.5 mmol/g by the cesium salt method [45]. For the synthesis of protected peptidyl resins, seven cycles of deprotection, neutralization, and coupling were carried out starting from Boc-Lys(Tos)-resin or Boc-Lys[Z(2Cl)]-resin. A HCl (1 M)/AcOH mixture [7,10,25–28] was used in all the deprotection steps, except for the Fmoc-protecting group following the incorporation of Fmoc-D-Tyr(Bu^t)[2] (peptide **XVIII**), where 20% piperidine/DMF was used. Neutralizations were carried out with 10% Et_3N/CH_2Cl_2. Coupling reactions were mediated mainly by DCC/HOBt [23] in CH_2Cl_2/DMF, except for Boc-Asn which was incorporated as its ONp [29] in DMF. For peptides **I–XIII** the acylpeptide resins were cleaved by acidolysis with HBr/TFA [21,22,30,31] to give the acylheptapeptide intermediates d(CH_2)$_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Lys(Tos)-Lys(Tos) **D** and d(CH_2)$_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Lys[Z(2Cl)]-Lys[Z(2Cl)] **E**. Protected peptides **I**, **III**, **VI**, **VII**, **IX–XI**, and **XIII** were obtained by coupling the intermediate **D** with Gly-NH_2, Ala-NH_2, Cha-NH_2, Thr(Bzl)-NH_2, Tyr-NH_2, Orn(Tos)-NH_2, Lys(Z)-NH_2, and D-Lys(Z)-NH_2, respectively, by the DCC/HOBt method [23] in DMF. Intermediate **D** also served as the precursor for many other position 9 analogues of **C** not reported here. Using the same approach, protected peptides **II**, **IV**, **V**, **VIII**, and **XII** were synthesized by coupling the protected acylheptapeptide intermediate **E** with Gly-NH-CH_3, Ala-NH-CH_3, Val-NH_2, Phe-NH_2, and Arg(Tos)-NH_2. The protected precursors **c, XIV**, and **XVI–XX** (Table 1) of the free peptides **C, 14**, and **16–20** (Table 2) were prepared entirely by the solid-phase method, followed by aminolysis with Eda in methanol [7,24,32]. The protected retro-modified precursors **XV** and **XXI** (Table 1) of the free peptides **15** and **16** (Table 2) were obtained by coupling the protected Eda peptides **XIV** and **XX** with Z-Tyr(Bzl in DMF using the DCC/HOBt procedure [23] as described in [24]. The protected peptides were purified either by extraction (for peptides **I–IX** and **XVI–XX**) or by dissolving (for peptides **XV** and **XXI**) with warm DMF followed by reprecipitations with H_2O or EtOH/Et_2O until adjudged pure by TLC, as previously described [7,10,25–28]. The physicochemical properties of all protected peptides are given in Table 1. Except for peptide **XVII**, which was deprotected by the TFMSA procedure [35,36], deprotection of all protected peptides was carried out with sodium in liquid ammonia [33,34] as previously described [7,10,25–28]. The resulting disulphydryl compounds were oxidatively cyclized with $K_3[Fe(CN)_6]$ [37] using the modified reverse procedure [38]. The free peptides were desalted and purified by gel filtration on Sephadex G-15 and Sephadex LH-20 mainly in a two-step procedure [39] using 50% AcOH and 2 M AcOH as eluents, respectively, as previously described [7,10,25–27]. When necessary, 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 **C** and **1–21** (Table 2) was checked by TLC, HPLC, and mass spectrometry (MS).

((β-S-(4-Methoxybenzylmercapto)-β,β-pentamethylene-propionyl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Lys(Tos)-Lys(Tos)-Gly-NH_2 (I, Table 1). Boc-Lys(Tos)-resin (17 g, 5.1 mmol) was converted to d(CH_2)$_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Lys(Tos)-Lys(Tos)-resin in seven cycles of deprotection, neutralization, and coupling (mediated by DCC/HOBt or active ester) with Boc-Lys(Tos), Boc-Cys(Bzl), Boc-Asn-ONp, Boc-Val, Boc-Arg(Tos), Boc-D-Tyr(Et), and β-S-(4-methoxybenzyl)mercapto-β,β-pentamethylenepropionic acid, respectively, by the manual method of solid-phase synthesis, as previously described [7,10,25–28]. The resulting protected peptidyl resin (23.15 g, yield 95.2%) was subjected to acidolytic cleavage [21,22,30,31] in 2.5 g aliquots (with nine repetitions) to give the protected acylheptapeptide intermediate d(CH_2)$_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Lys(Tos)-Lys(Tos) **D** as follows: Hydrogen bromide was bubbled through a suspension of 2.5 g (0.6 mmol) d(CH_2)$_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Lys(Tos)-Lys(Tos)resin in TFA (20 ml) and anisole (3 ml) in a glass funnel fitted with a fritted disk, as described in Ref. 22. 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 evaporator. Addition of ether (*ca* 200 ml) to the residual anisole solution gave a precipitate, which after 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 0.89 g (85.8%) of the desired acylheptapeptide **D.** To a cooled (0 °C) solution of peptide **D** (0.35 g, 0.2 mmol) and HOBt (0.034 g, 0.22 mmol) in 1 ml of anhydrous DMF was added 0.11 ml (0.22 mmol) of 2 M solution of DCC in DMF. The reaction mixture was stirred for 1 h, whereupon the dicyclohexylurea (DCU) was removed by filtration. The filtrate was added to a solution of Gly-NH_2 (0.05 g, 0.45 mmol) in 2 ml anhydrous DMF. DIPEA was added to give a pH ~ 7.5. After the mixture was stirred for 18 h at room temperature (TLC monitoring), the peptide was precipitated with 0.5 N HCl, collected, washed with H_2O, and dried overnight *in vacuo* over P_2O_5. The resulting product was dissolved in DMF/methanol (8 : 2) and precipitated with ether to give 0.36 g (86.6%) of the desired protected peptide **I** (Table 1). Utilizing the same approach, protected peptides **III**, **VI**, **VII**, **IX–XI**, and **XIII** (Table 1) were prepared by coupling acylheptapeptide **D** with Ala-NH_2, Cha-NH_2, Thr(Bzl)-NH_2, Tyr-NH_2, Orn(Tos)-NH_2, Lys(Z)-NH_2, and D-Lys-NH_2, respectively. Starting with 2 g (1.5 mmol) of Boc-Lys[Z(2Cl)]-resin, using the manual solid-phase method and Boc-Lys[Z(2Cl)] in place of Boc-Lys(Tos) for position 7, d(CH_2)$_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Lys[Z(2Cl)]-Lys[Z(2Cl)]-resin (4.1 g, yield 98.2%) was synthesized as described above. 3.3 g (1.2 mmol) of this resin was subjected to acidolytic cleavage

(HBr/TFA as described above) to give the protected acylheptapeptide intermediate d(CH_2)$_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Lys[Z(2Cl)]-Lys[Z(2Cl)] **E** (1.9 g, 89.6%). Protected peptides **II**, **IV**, **V**, **VIII**, and **XII** (Table 1) were synthesized by coupling of protected acylheptapeptide intermediate **E** with Gly-NH-CH_3, Ala-NH-CH_3, Val-NH_2, Phe-NH_2, and Arg(Tos)-NH_2 by the DCC/HOBt procedure [23,] as described above.

d(CH$_2$)$_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Lys(Z(2Cl))-Lys(Z(2Cl))-Eda, c (Table 1). Starting from d(CH_2)$_5$(Bzl)-D-Tyr(Et)-Arg(Tos)-Val-Asn-Cys(Bzl)-Lys[Z(2Cl)]-Lys[Z(2Cl)]-resin (2.55 g, 0.98 mmol) prepared as described above, the protected precursor **c** was obtained by aminolysis with ethylenediamine (Eda)/MeOH and DMF extraction [7,24,32] as follows. The protected peptidyl resin was placed in a 250-ml round bottomed flask, 75 ml of anhydrous MeOH was added, the suspension was cooled at *ca* 0 °C, and 30 ml of Eda (99.5+%, redistilled; Aldrich) was added with stirring. After 30 min, the cooling bath was removed and the suspension stirred at room temperature for 2 days. The solvents were removed on a rotary evaporator and the protected precursor **c** (Table 1) was further extracted and purified as described above. The protected Eda peptides **XIV** and **XVI–XX** were prepared by the same procedure starting from the corresponding acylheptapeptide resins.

d(CH$_2$)$_5$(Bzl)-D-Tyr(Pr^i)-Arg(Tos)-Val-Asn-Cys(Mob)-Lys(Z(2Cl))-Lys(Z(2Cl))-Eda←Tyr(Bzl) (XXI, Table 1). The retro-modified peptide **XXI** was synthesized by a DCC/HOBt-mediated coupling [23,24] of the protected Eda peptide **XX** and Z-Tyr(Bzl) as follows: To a cooled (0 °C) solution of Z-Tyr(Bzl) (0.20 g, 0.5 mmol) and HOBt (0.08 g, 0.6 mmol) in 1 ml of anhydrous DMF was added 0.3 ml (0.6 mmol) of 2M solution of DCC in DMF. The reaction mixture was stirred for 1 h, whereupon the dicyclohexylurea (DCU) was removed by filtration. The filtrate was added to a solution of **XX** (0.3 g, 0.16 mmol) in 1 ml anhydrous DMF. DIPEA was added to give a pH ~7.5. After the mixture was stirred for 18 h at room temperature (TLC monitoring), methanol (20 ml) was added followed by ether (250 ml). The precipitated product was collected following overnight storage at 4 °C. Washing with warm methanol gave the required protected peptide **XXI**, 0.32 g, yield 88.0% (Table 1). The protected retro-modified peptide **XV** (Table 1) was prepared by the same procedure utilizing the protected Eda peptide **XIV**.

(β-Mercapto)-β,β-pentamethylenepropionyl-D-Tyr(Et)-Arg-Val-Asn-Cys-Lys-Lys-Eda (d(CH$_2$)$_5$(D-Tyr(Et)^2, Arg^3, Val^4, Lys^7, Eda^9)LVP (C, Table 2). The Na/liq.NH_3 procedure [34] was used for the deprotection of protected peptides **c, I–XVI,** and **XVIII–XXI** as described here for the protected peptide **c**. A solution of the protected precursor **c** (Table 1), 120 mg, in sodium-dried ammonia (*ca* 400 ml) was treated at the boiling point and 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 [7,10,25–28]. NH_4Cl was added to discharge the color and the ammonia evaporated. Reoxidation of the deblocked disulphydryl peptide **C** was performed by the modified reverse procedure [38] as follows: The resulting disulphydryl peptide residue was dissolved in 25 ml, 50% 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 that contained 20 ml of a 0.01 M solution of potassium ferricyanide [37]. Meanwhile, the pH was adjusted to approximately 7.0 with concentrated ammonium hydroxide. Following oxidation, the free peptide **C** 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), and 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 AcOH (50%) with a flow rate of 5 ml/h [39]. 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 AcOH (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 VP analogue **C** (Table 2). With minor modifications, the same procedure was utilized for the deprotection, cyclization, and purification of the remaining protected peptides **I–XVI** and **XVIII–XXI** to give the free peptides **1–16** and **18–21**.

((β-Mercapto)-β,β-pentamethylenepropionyl)-D-2-Nal-Arg-Val-Asn-Cys-Lys-Lys-Eda (d(CH$_2$)$_5$(D-2-Nal^2, Arg^3, Val^4, Lys^7, Eda^9)LVP) (17, Table 2). The presence of a naphthylalanine residue in peptide **XVII** precluded the use of the Na/liq. NH_3 cleavage procedure. We have previously shown that the naphthylalanine ring is partially reduced (the addition of 2H) by this procedure [48,49]. The deprotection of the Eda peptide **XVII** (Table 1) was performed by the standard TFMSA procedure [35,36], as recently described [18,] as follows: The protected peptide **XVII** (150 mg) was placed in a round bottomed flask with a stirring bar, 0.5 ml of thioanisole and 0.25 ml of 1,2-ethanedithiol was added, the mixture was chilled with an ice bath, and 5 ml of TFA was added. After stirring for 5 min at 0°C, 0.5 ml of TFMSA was added slowly dropwise with vigorous stirring. Following additional stirring for 30 min at room temperature, 50 ml of anhydrous ether was added dropwise. The precipitated peptide was filtered, washed with ether, and dried overnight *in vacuo* over P_2O_5 and NaOH. Oxidation of the resulting residue of the deblocked disulphydryl precursor of peptide **17** was performed with $K_3[Fe(CN)_6]$ [37] by the modified reverse procedure [38] followed by purification by gel filtration on Sephadex G-15 and Sephadex LH-20 in a two-step procedure [39] as described above, to give 22.2 mg (23.9%) of the required free peptide **17**. The physicochemical properties of all the free peptides **C** and **1–21** are given in Table 2.

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