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Position Three in Vasopressin Antagonist Tolerates Conformationally Restricted and Aromatic Amino Acid Substitutions: A Striking Contrast with Vasopressin Agonists

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Abstract: We report the solid-phase synthesis and some pharmacological properties of 12 position three modified analogues (peptides 1–12) of the potent non-selective antagonist of the antidiuretic (V_2-receptor), vasopressor (V_{1a}-receptor) responses to arginine vasopressin (AVP) and of the uterine contracting (OT-receptor) responses to oxytocin (OT), [1(-β mercapto-β,β-pentamethylenepropionic acid)-2-*O*-ethyl-D-tyrosine 4-valine] arginine vasopressin [d$(CH_2)_5$D-Tyr(Et)^{2}VAVP] (A) and two analogues of (**B**) (peptides 13,14), the 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid^3 (Tic^3) analogue of (**A**). Peptides 1–12 have the following substituents at position three in (**A**): (1) Pro; (2) Oic; (3) Atc; (4) D-Atc; (5) Aic; (6) D-Phe; (7) Ile; (8) Leu; (9) Tyr; (10) Trp; (11) Hphe; (12) [HO]Tic; Peptide (13) is the Tyr-NH_2^9 analogue of (**B**): Peptide (14) is the D-Cys^6 analogue of (**B**). All 14 new peptides were evaluated for agonistic and antagonistic activities in *in vivo* V_2 and V_{1a} assays and in *in vitro* (no $Mg^{2+})_n$ oxytocic assays. With the exception of the D-Phe^3 peptide (No. 6), which exhibits very weak V_2 agonism ($\sim$0.0017 U/mg), none of the remaining 13 peptides exhibit any agonistic activities in these assays. In striking contrast to their deleterious effects on agonistic activities in AVP, the Pro^3, Oic^3, Tyr^3, Trp^3 and Hphe^3 substitutions in (**A**) are very well tolerated, leading to excellent retention of V_2, V_{1a} and OT antagonistic potencies. All are more potent as V_2 antagonists than the Ile^3 and Leu^3 analogues of (**A**). The Tyr-NH_2^9 and D-Cys^6 substitutions in (**B**) are also well tolerated. The anti-V_2 pA_2 values of peptides 1–5 and 7–14 are as follows (1) 7.77 ± 0.03; (2) 7.41 ± 0.05; (3) 6.86 ± 0.02; (4) 5.66 ± 0.09; (5) $\sim$5.2; (7) 7.25 ± 0.08; (8) 6.82 ± 0.06; (9) 7.58 ± 0.05; (10) 7.61 ± 0.08; (11) 7.59 ± 0.07; (12) 7.20 ± 0.05; (13) 7.57 ± 0.1; (14) 7.52 ± 0.06. All analogues antagonize the vasopressor responses to AVP, with anti-V_{1a} pA_2 values ranging from 5.62 to 7.64, and the *in vitro* responses to OT, with anti-OT pA_2 values ranging from 5.79 to 7.94. With an anti-V_2 potency of 7.77 ± 0.03, the Pro^3 analogue of (**A**) is surprisingly equipotent with (**A**), (anti-V_2 pA_2=7.81 ± 0.07). These findings clearly indicate that position three in AVP V_2/V_{1a} antagonists, in contrast to position three in AVP agonists, is much more amenable to structural modification than had heretofore been anticipated. Furthermore, the surprising retention of V_2 antagonism exhibited by the Pro^3, Oic^3, Tyr^3, Trp^3 and Hphe^3 analogues of (**A**), together with the excellent retention of V_2 antagonism by the Tyr-NH_2^9 and D-Cys^6 analogues of (**B**) are promising new leads to the design of potent and possibly orally active V_2 antagonists for use as pharmacological tools and/or as radioiodinatable ligands and for development as potential therapeutic agents for the treatment of the hyponatremia caused by the syndrome of the inappropriate secretion of the antidiuretic hormone (SIADH).

Keywords: vasopressin; antagonist; Tic; Oic; Atc; Aic; receptor

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Abbreviations: AVP, arginine vasopressin; LVP, lysine vasopressin; AVT, arginine vasotocin; OT, oxytocin; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; Oic, 2S, 3aS, 7aS-octahydroindole-2-carboxylic acid; Atc, 2-aminotetraline-2-carboxylic acid; Aic, 2-aminoindane-2-carboxylic acid; Hphe, homophenylalanine; D-Tyr(Me), *O*-methyl-D-tyrosine; D-Tyr(Et), *O*-ethyl-D-tyrosine; d$(CH_2)_5$[D-Tyr(Et)^2]VAVP, [1-(β-mercapto-β, β-pentamethylenepropionic acid), 2-O-ethyl-D-tyrosine, 4-valine] arginine vasopressin (**A**); d$(CH_2)_5$[D-Tyr-(Et)^2,Tic^3]VAVP (**B**), Tic^3

analogue of (**A**); d(CH_2)$_5$[D-Tyr(Et)^2, Pro^3]VAVP, Pro^3 analogue of (**A**); (CH_2)$_5$[D-Tyr(Et)^2,Oic^3]VAVP, Oic^3 analogue of (**A**); d(CH_2)$_5$[D-Tyr(Et)^2, Atc^3]VAVP, Atc^3 analogue of (**A**); d(CH_2)$_5$[D-Tyr(Et)^2,D-Atc^3]VAVP, D-Atc^3 analogue of (**A**); d(CH_2)$_5$[D-Tyr(Et)^2,Aic^3]VAVP, Aic^3 analogue of (**A**);d(CH_2)$_5$[D-Tyr(Et)^2, D-Phe^3]VAVP, D-Phe^3 analogue of (**A**); d(CH_2)$_5$[D-Tyr(Et)^2,Ile^3]VAVP, Ile^3 analogue of (**A**); d(CH_2)$_5$[D-Tyr(Et)^2,Leu^3]VAVP, Leu^3 analogue of (**A**); d(CH_2)$_5$[D-Tyr(Et)^2,Tyr^3]VAVP, Tyr^3 analogue of (**A**); d(CH_2)$_5$[D-Tyr(Et)^2,Trp^3]VAVP, Trp^3 analogue of (**A**); d(CH_2)$_5$[D-Tyr(Et)^2,Hphe^3]VAVP, Hphe^3 analogue of (**A**); d(CH_2)$_5$[D-Tyr(Et)^2,(HO)Tic^3]VAVP, [HO]Tic^3 analogue of (**A**); d(CH_2)$_5$[D-Tyr(Et)^2,Tic^3,Tyr-NH_2^9]VAVP, Tyr-NH_2^9 analogue of (**B**); d(CH_2)$_5$[D-Tyr(Et)^2,Tic^3,D-Cys^6]VAVP, D-Cys^6 analogue of (**B**); d(CH_2)$_5$[D-Tyr(Et)^2, D-Cys^6]VAVP(**C**), D-Cys^6 analogue of (**A**); d(CH_2)$_5$[D-tyr(Me)^2]VAVP(**D**), D-Tyr(Me)^2 analogue of (**A**); d(CH_2)$_5$[D-Phe^2]VAVP (**E**), D-Phe^2 analogue of (**A**); d(CH_2)$_5$[D-Tyr(Me)^2,Ile^3]VAVP (**F**), Ile^3 analogue of (**D**); d(CH_2)$_5$[D-Phe^2,Ile^3]VAVP (**G**), Ile^3 analogue of (**E**); DCC, dicyclohexylcarbodiimide; ONp, *p*-nitrophenylester; Et_3N, triethylamine; Et_2O, diethyl ether; ACE, angiotensin converting enzyme; MBA, α-methylbenzylamine; DIEA, *N*-nitrophenylester; ,*N*-nitrophenylester; -diisopropylethylamine; PTC, phenyl isothiocyanate; V_2, antidiuretic; V_{1a}, vasopressor; SIADH, syndrome of the inappropriate secretion of antidiuretic hormone.

Antagonists of arginine vasopressin (AVP) and oxytocin (OT), besides being of potential therapeutic value, have found widespread use as (a) powerful pharmacological tools in studies on the physiological, pathophysiological and putative behavioural roles of AVP and OT, and (b) in select instances either directly or as specific or non-specific radioligands for the localization and characterization of the receptors which mediate the vascular (V_{1a} receptor), pituitary (V_{1b} receptor), renal (V_2 receptor) and uterine (OT receptor) responses to these two peptides [1–10].

Since our original discovery of V_2/V_{1a} antagonists [11], we and others have uncovered many surprising differences between AVP agonists and AVP V_2/V_{1a} antagonists in the structural requirements for (a) binding to and activating receptors in the case of agonists and (b) simply binding to receptors in the case of antagonists [12–15]. Perhaps the most notable difference is the discovery that the characteristic ring structure of AVP and of the earlier V_2/V_{1a} antagonists [11] is not a requirement for binding to V_2 or V_{1a} receptors [15]. Thus acyclic peptides can exhibit potent V_2 and/or V_{1a} antagonism [15,16] and can be converted to high-affinity mono- or bifunctional radioactive and/or photoactivatable ligands for V_{1a} receptors [17–20]. In this regard, it may also be noted that the recently discovered non-peptide V_{1a} and V_2 antagonists bear no structural relationship to AVP [21–23]. We now report surprising and striking differences between AVP agonists and AVP antagonists with regard to their structural requirements at position three.

The phenylalanine residue at position three in arginine vasopressin (AVP) [24] has long been believed to be important for V_2 and V_{1a} agonistic activities. With the exceptions of the Ile^3/Phe^3 interchange in the naturally occurring potent V_2,V_{1a}, OT agonist arginine vasotocin (AVT) [25] and a thienylalanine^3 (Thi)^3/Phe^3 interchange in lysine vasopressin (LVP) [26], other reported modifications at position three in AVP and in LVP have led to drastic reductions in the characteristic antidiuretic and vasopressor activities of these agonists [9, 27–32]. Thus, the Trp^3, Tyr^3 and Ser^3 analogues of LVP exhibit drastic losses of V_2 and V_{1a} agonistic activities [9, 27, 28]. Replacement of the Phe^3 residue in the highly potent antidiuretic agonist 1-deamino-LVP by *p*-aminophenylalanine also resulted in a drastic loss of V_2 agonism [29]. More recently, we and others have shown that replacement of the Phe^3 residue in AVP or in LVP by the conformationally restricted amino acid 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic) led to drastic losses of V_2 and V_{1a} agonistic activities [30, 31]. Based on these findings with AVP and LVP agonists, it has also been assumed that position three in AVP antagonists is highly intolerant of change and that a Phe^3 residue is essential for optimal V_2 antagonism. Thus, all of the potent AVP V_2 antagonists reported to date have a Phe residue at position three [2, 4–20].

Consequently, we were highly surprised by our recent finding that a Tic^3/Phe^3 interchange in the AVP V_2/V_{1a} antagonist, [1(β-mercapto-β,β-pentamethylene propionic acid)2-*O*-ethyl-D-tyrosine, 4-valine] arginine vasopressin (d(CH_2)$_5$[D-Tyr(Et)^2]-VAVP) (**A**) [32] (**A**, Table 1) to give d(CH_2)$_5$ [D-Tyr(Et)^2,Tic^3] VAVP [31] (**B**, Table 1) led to full retention of anti-V_2 potency and to enhanced anti-V_2/anti-V_{1a} selectivity [31]. Since, however, Tic [33] can be viewed as a conformationally restricted analogue of Phe [34] and thus might act simply by fixing the phenyl ring at position three in (**B**) into the correct alignment required for optimal binding to the V_2 receptor [31], we wondered if these findings were unique to Tic or whether, in fact, they indicated that position three in (**A**) might possibly be amenable to substitutions with other conformationally constrained amino acids and/or by other aromatic and aliphatic amino acids. In this regard, it may be recalled that an Ile^3/Phe^3 interchange in two potent V_2/V_{1a} antagonists had led to significant losses of both V_2 and V_{1a} antagonism [35]. Thus the Ile^3 analogues of d(CH_2)$_5$[D-Tyr(Me)^2] VAVP (**D**) [32] and d(CH_2)$_5$ [D-Phe^2]VAVP (**E**) [36] retain only about 20%

Table 1 Pharmacological Properties of Analogues of AVP V_2/V_{1a}/OT Antagonist $d(CH_2)_5$[D-Tyr(Et)^2]VAVP (**A**) with Phe^3 Replaced by Some Conformationally Restricted Amino Acids and by D-Phe

No.	Peptide	Anti-antidiuretic (Anti-V_2)		Antivasopressor (anti-V_{1a})		Antioxytocic (*in vitro*)	ED ratio[d]
		ED[a]	pA_2[b]	ED[a]	pA_2[b]	pA_2[c] (no Mg^{2+})	
A	$d(CH_2)_5$[D-Tyr(Et)^2]VAVP[e]	1.10 ± 0.15	7.81 ± 0.07	0.45 ± 0.11	8.22 ± 0.12	8.32 ± 0.10	0.4
B	$d(CH_2)_5$[D-Tyr(Et)^2,Tic^3]VAVP[f]	1.38 ± 0.15	7.69 ± 0.05	7.53 ± 0.58	6.95 ± 0.03	7.54 ± 0.05	5.5
1	$d(CH_2)_5$[D-Tyr(Et)^2,Pro^3]VAVP[g]	1.14 ± 0.08	7.77 ± 0.03 long-acting	2.17 ± 0.40	7.49 ± 0.05	7.94 ± 0.05 very long-acting	1.9
2	$d(CH_2)_5$[D-Tyr(Et)^2,Oic^3]VAVP[g]	2.60 ± 0.27	7.41 ± 0.05	2.09 ± 0.13	7.51 ± 0.03	7.58 ± 0.14 very long-'acting	0.8
3	$d(CH_2)_5$[D-Tyr(Et)^2,Atc^3]VAVP[g,h]	9.15 ± 0.54	6.86 ± 0.02	2.35 ± 0.22	7.45 ± 0.04	7.44 ± 0.04	0.3
4	$d(CH_2)_5$[D-Tyr(Et)^2,D-Atc^3]VAVP[g,h]	145 ± 26	5.66 ± 0.09	161 ± 9	5.62 ± 0.03	~ 6.2	1.1
5	$d(CH_2)_5$[D-Tyr(Et)^2,Aic^3]VAVP[g]	~ 400	~ 5.2	27.1 ± 4.8	6.39 ± 0.09	5.79 ± 0.05	0.07
6	$d(CH_2)_5$[D-Tyr(Et)^2,D-Phe^3]VAVP[g]		Agonist ~ 0.0017 u/mg	62 ± 0.11	6.06 ± 0.08	6.84 ± 0.09	

[a]The effective dose (ED) is defined as the dose (in nanomoles/kilogram) of antagonist that reduced the response to 2*x* units of agonist to the response with *x* units of agonist administered in the absence of antagonist.
[b]Estimated *in vivo* pA_2 values represent the negative logarithm of the effective dose divided by the estimated volume of distribution (67 ml/kg).
[c]The *in vitro* pA_2 value represents the negative logarithm to the base 10 of the average molar concentration of antagonist which reduced the response to 2*x* units of agonist to the response with *x* units of agonist.
[d]ED ratio = antivasopressor ED/anti-antidiuretic ED.
[e]Data from Manning *et al.* [32].
[f]Data from Manning *et al.* [31].
[g]This publication.
[h]Preliminary data reported in Manning *et al.* [2] and Klis *et al.* [60].
Oic = octahydroindole-2-carboxylic acid; Atc = 2-aminotetralin-2-carboxylic acid; Aic = 2-aminoindan-2-carboxylic acid.

and 2% respectively of the V_2 antagonism of their respective parent Phe^3 analogues (**D**) and (**E**) [35]. These later findings [35] seemed to point to a clearly unique role for Tic^3 in leading to full retention of V_2 antagonism in (**B**) [31]. Nonetheless, until other position three modified analogues of (**A**) had been synthesized and examined, we could not be sure about this.

We were also intrigued by the V_2 antagonist design possibilities offered by our discovery that a Tic^3/Phe^3 interchange in (**A**) is well tolerated, with retention of V_2 antagonism and with enhanced anti-V_2/anti-V_{1a} selectivity exhibited by the resulting peptide (**B**) [31]. We wondered whether (**B**) would be a useful lead in the design of potential radioiodinatable ligands for V_2 and V_{1a} receptors and whether it might also serve as a template for the design of orally active V_2 antagonists. To address all of these questions, we report the synthesis and pharmacological properties of the three series of peptides (I, II and III) in Tables 1–3. These tables contain analogues of (**A**) modified at position three. Table 3 contains analogues of (**B**) modified at positions six and nine. These peptides were designed according to the following rationale.

Series I: Peptides 1–6 (Table 1). These are analogues of $d(CH_2)_5$[D-Tyr(Et)^2]VAVP (**A**) [32] which have the Phe^3 residue replaced by a series of conformationally restricted amino acids and by D-Phe. The conformationally restricted amino acids selected for this study are: Pro; 2S, 3aS, 7aS-octahydroindole-2-carboxylic acid (Oic); L- and D-aminotetraline-2-carboxylic acid (L- and D-Atc) and 2-aminoindane 2-carboxylic acid (Aic). These are logical choices for further exploring our finding that a Tic^3/Phe^3 interchange in (**A**) leads to excellent retention of V_2 antagonist by the resulting peptide (**B**) [31]. Proline substitutions have long been utilized to good effect in studies aimed at measuring the effects of conformational constraints in peptides [14, 37, 38]. Furthermore, besides being utilized as a conformationally constrained surrogate for Phe, Tic has also been employed successfully as a conformationally constrained surrogate for Pro in a potent AVP V_{1a} antagonist [31] and in the design of potent bradykinin antagonists [39, 40]. However, in

Table 2 Pharmacological Properties of Analogues of AVP V_2/V_{1a}/OT Antagonist $d(CH_2)_5$[D-Tyr(Et)^2]VAVP (**A**) with Phe^3 Replaced by Aliphatic and Aromatic Amino Acids

No.	Peptide	Anti-antidiuretic (Anti-V_2)		Antivasopressor (anti-V_{1a})		Antioxytocic (*in vitro*)	ED ratio[d]
		ED[a]	pA_2[b]	ED[a]	pA_2[b]	pA_2[c](no Mg^{2+})	
A	$d(CH_2)_5$[D-Tyr(Et)^2]VAVP[e]	1.10 ± 0.15	7.81 ± 0.07	0.45 ± 0.11	8.22 ± 0.12	8.32 ± 0.10	0.4
7	$d(CH_2)_5$[D-Tyr(Et)^2,Ile^3]VAVP[f,g]	4.1 ± 0.7	7.25 ± 0.08	2.5 ± 0.2	7.44 ± 0.03	7.76 ± 0.11	0.6
8	$d(CH_2)_5$[D-Tyr(Et)^2,Leu^3]VAVP[g]	10.0 ± 1.5	6.82 ± 0.06	12.2 ± 0.9	6.74 ± 0.03	7.70 ± 0.04	1.2
9	$d(CH_2)_5$[D-Tyr(Et)^2,Tyr^3]VAVP[g,h]	1.75 ± 0.17	7.58 ± 0.05	2.39 ± 0.32	7.45 ± 0.05	7.53 ± 0.04	1.4
10	$d(CH_2)_5$[D-Tyr(Et)^2,Trp^3]VAVP[g,h]	1.63 ± 0.28	7.61 ± 0.08	1.54 ± 0.04	7.64 ± 0.01	7.63 ± 0.04	0.9
11	$d(CH_2)_5$[D-Tyr(Et)^2,Hphe^3]VAVP[g]	1.73 ± 0.27	7.59 ± 0.07	2.56 ± 0.24	7.42 ± 0.04	7.58 ± 0.05	1.5

[a–e]See corresponding footnotes to Table 1.
[f]Anti-OT activities: *in vitro* with Mg^{2+} (0.5 mM) pA_2 8.22 ± 0.05; *in vivo* ED $= 4.7 \pm 0.9$, $pA_2 = 7.18 \pm 0.08$.
[g]This publication.
[h]Preliminary data reported in Manning *et al.* [2] and Klis *et al.* [60]

Table 3 Pharmacological Properties of Analogues of AVP V_2/V_{1a}/OT Antagonist $d(CH_2)_5$[D-Tyr(Et)^2,Tic^3]VAVP (**B**) Modified at Positions 3, 6 and 9 and Related Peptides (**A**) and (**C**)

No.	Peptide	Anti-antidiuretic (Anti-V_2)		Antivasopressor anti-V_{1a}		Antioxytocic (*in vitro*)	ED ratio[d]
		ED[a]	pA_2[b]	ED[a]	pA_2[b]	pA_2[c](no Mg^{2+})	
A	$d(CH_2)_5$[D-Tyr(Et)^2]VAVP[e]	1.10 ± 0.15	7.81 ± 0.07	0.45 ± 0.11	8.22 ± 0.12	8.32 ± 0.10	0.4
B	$d(CH_2)_5$[D-Tyr(Et)^2,Tic^3]VAVP[f]	1.38 ± 0.15	7.69 ± 0.05	7.53 ± 0.58	6.95 ± 0.03	7.54 ± 0.05	5.5
12	$d(CH_2)_5$[D-Tyr(Et)^2,(HO)Tic^3]VAVP[g,h]	4.20 ± 0.45	7.20 ± 0.05	3.55 ± 0.42	7.27 ± 0.05	7.67 ± 0.05	0.8
13	$d(CH_2)_5$[D-Tyr(Et)^2,Tic^3,Tyr-$NH_2{}^9$]VAVP[g,h]	1.79 ± 0.37	7.57 ± 0.10	19.8 ± 2.1	6.53 ± 0.04	7.61 ± 0.03	11
C	$d(CH_2)_5$[D-Tyr(Et)^2,D-Cys^6]VAVP[i]	3.3 ± 0.7	7.33 ± 0.07	0.60 ± 0.05	8.06 ± 0.05	7.43 ± 0.07	0.18
14	$d(CH_2)_5$[D-Tyr(Et)^2,Tic^3,D-Cys^6]VAVP[g,h]	2.05 ± 0.30	7.52 ± 0.06	26.0 ± 0.5	6.41 ± 0.08	7.39 ± 0.07	13

[a–f]See corresponding footnotes to Table 1.
[g]This publication.
[h]Preliminary data reported in Manning *et al.* [2] and Klis *et al.* [60].
[I]Data from Manning *et al.* [55].

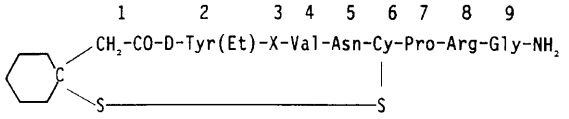
the studies on bradykinin antagonists [39, 40] a D-Tic/L-Pro interchange was superior to a L-Tic/L-Pro interchange in enhancing potency [40]. These studies raised the intriguing, if somewhat remote, possibility that a Pro^3/Tic^3 interchange might be tolerated in (**B**) with retention of V_2 antagonism.

The conformationally restricted amino acids Oic, Atc and Aic have been shown to be valuable tools in the design of enzyme inhibitors and of antagonists of some peptide hormones [39–54]. To date, Oic, Atc and Aic have not been employed in the design of AVP or OT antagonists. We report the first use of these three amino acids in the study on V_2 antagonist design presented here. First synthesized in 1981 [41], Oic has been utilized in the design of angiotensin converting enzyme (ACE) inhibitors and in the design of potent antagonists of bradykinin [39, 40, 42–44]. Atc, first synthesized as ±Atc in 1980 [45], has been utilized in its racemic form as a conformationally restricted analogue of Phe in a variety of studies on selective drug design and in the opioid peptide field [46–49]. The resolution of ±Atc by chiral TLC and the absolute configuration of the enantiomers, characterized by a combination of enzymatic digestion and by NMR spectroscopy, has been reported by Hruby and colleagues [47]. Here, we report the chemical resolution of Boc±Atc and confirm the optical configurations previously obtained [47]. We thus were able to utilize for the first time both Boc-L-Atc and Boc-D-Atc as clearly defined

enantiomers in this study. First synthesized in 1948 [50], Aic has been used as a conformationally restricted analogue of Phe in the design of enzyme inhibitors [51, 52] and in the design of angiotensin II agonists and antagonists [49, 53, 54].

Substitutions with D-amino acid have been very useful in AVP V_2-antagonist design [32, 36]. To date, there have been no reports on the effects of D-amino acid substitutions at position three in AVP antagonists. We thus take this timely opportunity to report our unpublished findings on the profound effects of a D-Phe^3/L-Phe^3 interchange in the AVP V_2/V_{1a} antagonist (**A**) (Peptide 6).

Series II: Peptides 7–11 Table 2. These are analogues of d$(CH_2)_5$[D-$Tyr(Et)^2$]VAVP (**A**) [32] which have the Phe^3 residue replaced by the aliphatic amino acids Ile and Leu and by the aromatic amino acids Tyr, Trp and homophenylalanine (Hphe). From our previous studies [35] noted above, we had reason to expect that an Ile^3/Phe^3 interchange in (**A**) might be tolerated, albeit with reduced antagonistic potencies. However, based on their effects in LVP [9, 27, 28] also noted above, we have very little reason to expect that the replacement of Phe^3 in the AVP antagonist (**A**) by Tyr and by Trp or by Hphe would be tolerated. Nonetheless, a broad study required their inclusion. The new peptides designed according to the above rationale together with the [HO]-Tic^3 analogue (peptide 12) of Series III are position three analogues of d$(CH_2)_5$[D-$Tyr(Et)^2$]VAVP (**A**) [32] which has the following structure:



(**A**), (**B**) and peptides 1–12 have the following substituents at position three (X): (**A**) Phe; (**B**) Tic; (1) Pro; (2) Oic; (3) L-Atc; (4) D-Atc; (5) Aic; (6) D-Phe; (7) Ile; (8) Leu; (9) Tyr; (10) Trp; (11) Hphe; (12) [HO]Tic. The structures of these position three (X^3) substituents are shown in Figure 1.

Series III: Peptides 12–14 (Table 3): These are analogues of d$(CH_2)_5$[D-$Tyr(Et)^2$,Tic^3]VAVP (**B**), [31] with modifications at positions three ([HO] Tic), six (D-Cys) and nine (Tyr-NH_2). The [HO] Tic^3 and Tyr-$NH_2{}^9$ analogues were designed as potential radioiodinatable ligands for V_2 and V_{1a} receptors. We and others have previously reported the usefulness of a

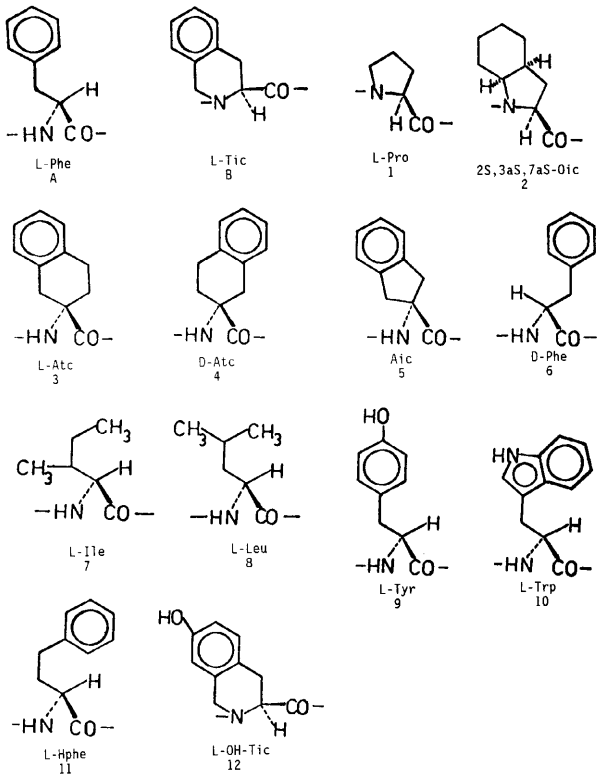
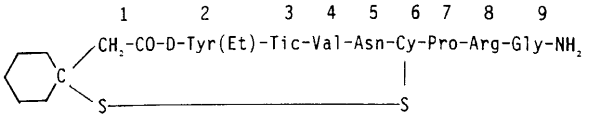


Figure 1

Tyr-$NH_2{}^9$/Gly-$NH_2{}^9$ interchange for the design of receptor-specific cyclic and linear radioiodinatable ligands [1, 2, 17, 19]. However, we had not previously employed a phenolic substituent at position three and were intrigued by the possibility that since Tic^3 was very well tolerated in (**A**) to give (**B**), its hydroxy analogue ([HO]Tic) might also be well tolerated at position three and that the resulting peptide (12) might thus be a potentially useful radioiodinatable ligand. The D-Cys^6 analogue was designed to further investigate the potential of a D-Cys^6/L-Cys^6 interchange in the design of orally bioavailable V_2/V_{1a} antagonists [55].

d$(CH)_5$[D-$Tyr(Et)^2$,Tic^3]VAVP (**B**) [31] has the following structure:



Peptides 13 and 14 (Table 3) contain the following modification of (**B**): (13) Tyr-$NH_2{}^9$; (14) D-Cys^6.

Based on the above rationale, we now report the synthesis by the solid-phase method [56–59] of the following 14 new peptides:

1 $d(CH_2)_5$[D-Tyr(Et)^2, Pro^3]VAVP
2 $d(CH_2)_5$[D-Tyr(Et)^2,Oic^3]VAVP
3 $d(CH_2)_5$[D-Tyr(Et)^2,Atc^3]VAVP
4 $d(CH_2)_5$[D-Tyr(Et)^2,D-Atc^3]VAVP
5 $d(CH_2)_5$[D-Tyr(Et)^2,Aic^3]VAVP
6 $d(CH_2)_5$[D-Tyr(Et)^2,D-Phe^3]VAVP
7 $d(CH_2)_5$[D-Tyr(Et)^{2}Ile^3]VAVP
8 $d(CH_2)_5$[D-Tyr(Et)^2,Leu^3]VAVP
9 $d(CH_2)_5$[D-Tyr(Et)^2,Tyr^3]VAVP
10 $d(CH_2)_5$[D-Tyr(Et)^2,Trp^3]VAVP
11 $d(CH_2)_5$[D-Tyr(Et)^2,Hphe^3]VAVP
12 $d(CH_2)_5$[D-Tyr(Et)^2,[HO]Tic^{3}VAVP
13 $d(CH_2)_5$[D-Tyr(Et)^2,Tic^3,Tyr-$NH_2{}^9$]VAVP
14 $d(CH_2)_5$[D-Tyr(Et)^2,Tic^3,D-Cys^6]VAVP

Preliminary reports on peptides 3, 4, 9, 10 and 12–14 have been presented [2, 60].

Starting from Boc-Gly-resin, or Boc-Tyr(Bzl)-resin, we synthesized the protected precursors I–XIV (Table 4) of the free peptides 1–14 (Table 5), entirely by the Merrifield solid-phase method [56–59]. HCl (1 M)/AcOH was used in all the deprotection steps. Neutralizations were carried out with 10% Et_3N/CH_2Cl_2. Coupling reactions were mediated primarily by DCC/HOBt [61] in CH_2Cl_2/DMF except for Boc-Asn which was incorporated as its *p*-nitrophenyl ester [62] in DMF. Cleavage from the acylpeptide resin was by ammonolysis in methanol [59, 63], with the normal DMF extraction [59] or a modified MeOH extraction procedure [31] (necessitated by the unusual solubility in DMF/H_2O of the Tic^3 protected peptides [31] and of the (HO)Tic^3 protected peptides XII–XIV) to give the protected peptide amides I–XIV (Table 4). Na in NH_3 [64] was used to deblock each protected precursor as previously described [31, 32, 35] and the resulting disulphydryl compounds were oxidatively cyclized with $K_3[Fe(CN)_6]$ using the normal procedure [65] or a 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 [31, 32, 35]. 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 1–14 (Table 5) was checked by thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), amino acid analysis and electron spray mass spectrometry (ESMS).

Peptides were assayed for agonistic activity or antagonistic activity in the rat antidiuretic assay, rat vasopressor assay and *in vitro* rat oxytocic assay. For agonists, the four-point assay design [68] was used, and for antagonists, Schild's pA_2 method [69] was employed. The pA_2 is the negative logarithm of the effective molar concentration of the antagonist that will reduce the response to 2*x* units of the agonist to equal the response to 1*x* unit of the agonist in the absence of antagonist. In practice, this concentration is estimated by finding concentrations above and below the effective concentration and interpolating on a logarithmic scale. In the rat *in vivo* assays, the effective dose (ED) of antagonist is divided by an arbitrarily assumed volume of distribution of 67 ml/kg to allow estimation of its molar concentration for the pA_2 [70]. Synthetic arginine-vasopressin and oxytocin which had been standardized in vasopressor and oxytocic units against the USP Posterior Pituitary Reference Standard were used as working standards in all bioassays. Antidiuretic assays were on water-loaded rats under ethanol anesthaesia as described by Sawyer [71]. Vasopressor assays were performed on urethane-anaesthetized and phenoxybenzamine-treated rats as described by Dekanski [72]. Oxytocic assays were performed on isolated uteri from diethylstilbestrol-primed rats in a Mg^{2+}-free Van Dyke-Hasting's solution [73]. When standard errors are presented in the tables, the means reflect results from at least four independent assay groups.

The antiantidiuretic (anti-V_2), antivasopressor (anti-V_{1a}) and antioxytocic (*in vitro*, no Mg^{2+}) properties of the three series of peptides (I–III), together with those of $d(CH_2)_5$[D-Tyr(Et)^2]VAVP (**A**), $d(CH_2)_5$[D-Tyr(Et)^2, Tic^3]VAVP (**B**) and (**C**), the related D-Cys^6 analogue of (**A**), are presented in Tables 1–3. It should be noted that apart from the D-Phe^3 analogue of (**A**) (peptide 6, Table 1) which exhibits very weak V_2 agonism, none of the remaining 13 new peptides exhibits detectable antidiuretic, vasopressor or oxytocic agonism.

Effects of Conformationally Restricted Amino Acids at Position Three in $d(CH_2)_5$[D-Tyr(Et)^2]VAVP (A) Table 1

V_2-Antagonism. Table 1 contains data on the effects of replacing the Phe^3 residue in (**A**) with the five conformationally restricted amino acids Pro,

Table 4 Physicochemical Properties of the Protected Peptides I–XIV[a]

No.	Peptide	Yield (%)[b]	m.p. (°C)	$[\alpha]_D^{25}$ ($c = 1$) DMF	TLC[c] *a*	*b*	*c*	*d*
I	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Pro-Val-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	92.6	90–92	−33.6	0.49,	0.53,	0.72,	0.96
II	d$(CH_2)_5$(Bzl)-DTyr(Et)-Oic-Val-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	83.7	123–24	−30.2	0.61,	0.66,	0.82,	0.99
III	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Atc-Val-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	60.4	151–53	+10.2	0.60,	0.77,	0.80	
IV	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-D-Atc-Val-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	73.7	161–64	−11.0	0.55,	0.74,	0.87	
V	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Aic-Val-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	52.0	145–47	+5.2	0.67,	0.61,	0.73	
VI	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-D-Phe-Val-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	82.3	201–03	−11.0		0.85,	0.76,	0.88
VII	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Ile-Val-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	60.4	238–4	−17.0	0.85,	0.94,	0.70	
VIII	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Leu-Val-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	98.0	209.6–11.9	−19.1		0.48,	0.71,	0.73
IX	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Tyr(Bzl)-Val-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	95.0	201–03	−19.2	0.77,	0.69,	0.75	
X	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Trp-Val-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	97.0	182–84	−23.0	0.56,	0.60,	0.60	
XI	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Hphe-Val-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	86.1	209–12	−19.8	0.68,	0.64,	0.72	
XII	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-(HO)Tic-Val-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	86.8	151–53	−24.9	0.57,	0.61,	0.73	
XIII	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Tic-Val-Asn-Cys(Bzl)-Pro-Arg(Tos)-Tyr(Bzl)-NH_2	33.1	146–48	−30.6		0.69,	0.75	
XIV	d$(CH_2)_5$(Bzl)-D-Tyr(Et)-Tic-Val-Asn-D-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	75.0	137–41	−14.0		0.56,	0.79,	0.94

[a]The protected peptides I–XIV are the immediate protected precursors for the free peptides 1–14 given in Tables 1–3 and 5.
[b]Yields were calculated on the basis of amino acid content of the resin.
[c]Solvent systems are described in the Experimental part.

Table 5 Physicochemical Properties of Free Peptides 1–14

No.	Peptide	Yield[a,b] (%)	$\alpha]^{25}_{D}$ ($c = 0.1$, 1N) AcOH	TLC, R_F[c]			HPLC t_R[d] (min)	Formula	MW	$[M+H]^{1+}$
				a	b	c				
1	d$(CH_2)_5$[D-Tyr(Et)^2,Pro^3]VAVP	25.5	−82	0.20	0.24	0.43	32.9	$C_{49}H_{75}O_{11}N_{13}S_2$	1086.4	1087
2	d$(CH_2)_5$[D-Tyr(Et)^2,Oic^3]VAVP	27.6	−76	0.23	0.28	0.46	39.7	$C_{53}H_{80}O_{11}N_{13}S_2$	1139.4	1140
3	d$(CH_2)_5$[D-Tyr(Et)^2,Atc^3]VAVP	37.5	−33	0.18	0.21	0.50	40.3	$C_{55}H_{79}O_{11}N_{13}S_2$	1162.4	1163.5
4	d$(CH_2)_5$[D-Tyr(Et)^2,D-Atc^3]VAVP	50.0	−67	0.18	0.21	0.50	41.0	$C_{55}H_{79}O_{11}N_{13}S_2$	1162.4	1163.5
5	d$(CH_2)_5$[D-Tyr(Et)^2,Aic^3]VAVP	27.0	−25	0.43	0.40	0.54	35.8	$C_{54}H_{77}O_{11}N_{13}S_2$	1148.5	1148.5
6	d$(CH_2)_5$[D-Tyr(Et)^2,D-Phe^3]VAVP	24.5	−29	0.24		0.61	45.6	$C_{51}H_{71}O_{11}N_{13}S_2$	1135.4	1136.3
7	d$(CH_2)_5$[D-Tyr(Et)^2,Ile^3]VAVP	26.0	−115	0.30	0.38	0.60	44.4	$C_{50}H_{79}O_{11}N_{13}S_2$	1102.4	1102.3
8	d$(CH_2)_5$[D-Tyr(Et)^2,Leu^3]VAVP	51.8	−151.6	0.22	0.18	0.37	39.9	$C_{50}H_{79}O_{11}N_{13}S_2$	1102.4	1103.5
9	d$(CH_2)_5$[D-Tyr(Et)^2,Tyr^3]VAVP	62.4	−169	0.25	0.25	0.40	35.6	$C_{53}H_{77}O_{12}N_{13}S_2$	1152.4	1153
10	d$(CH_2)_5$[D-Tyr(Et)^2,Trp^3]VAVP	29.5	−136	0.30	0.22	0.50	38.3	$C_{55}H_{78}O_{11}N_{14}S_2$	1173.3	1175.5
11	d$(CH_2)_5$[D-Tyr(Et)^2,Hphe^3]VAVP	26.1	−108	0.22	0.29	0.45	44.6	$C_{54}H_{79}O_{11}N_{13}S_2$	1150.5	1151.5
12	d$(CH_2)_5$[D-Tyr(Et)^2,(HO)Tic^3]VAVP	22.9	−40	0.21	0.45	0.56	35.0	$C_{54}H_{77}O_{12}N_{13}S_2$	1164.4	1165.5
13	d$(CH_2)_5$[D-Tyr(Et)^2,Tic^3,Tyr-$NH_2{}^9$]VAVP	20.4	−10	0.36	0.25	0.54	41.6	$C_{53}H_{83}O_{12}N_{13}S_2$	1254.5	1255
14	d$(CH_2)_5$[D-Tyr(Et)^2,Tic^3,D-Cys^6]VAVP	15.0	−20	0.26	0.34	0.34	51.3	$C_{54}H_{77}O_{11}N_{13}S_2$	1148	1149.5

[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]All peptides gave the expected amino acid analysis ratios after hydrolysis ±5%.
[c]Solvent systems and condition are given in the Experimental part.
[d]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.

Oic, Atc, D-Atc and Aic and by D-Phe. These findings show that a Pro^3/Phe^3 interchange is surprisingly tolerated as well as a Tic^3/Phe^3 interchange in (**A**). With an anti-V_2 $pA_2 = 7.77$, the Pro^3 analogue of A (peptide 1) is equipotent as a V_2 antagonist with both the Phe^3 parent (**A**) (anti-V_2 $pA_2 = 7.81$) and the Tic^3 analogue (**B**) (anti-V_2 $pA_2 = 7.69$). It may be noted also that the Pro^3 analogue exhibits protracted V_2 antagonism. These findings are in striking contrast to the effects on agonistic activities of a Pro^3/Phe^3 interchange in AVP. We have recently found that [Pro^3] AVP is devoid of agonistic activities [unpublished]. The effectiveness of a Pro^3/Phe^3 interchange in (**A**) in leading to retention and prolongation of V_2 antagonism is a promising new lead in V_2 antagonist design. With an anti-V_2 $pA_2 = 7.41$, the Oic^3 analogue (peptide 2) retains about 40% of the V_2 antagonism of (**A**). The Atc^3/Phe^3 interchange in (**A**) led to a significant reduction in anti-V_2 potency. Thus, with an anti-V_2 $pA_2 = 6.86$, the resulting Atc^3 analogue (peptide 3) retains only about 10% of the V_2 antagonism of (**A**). Replacement of the Phe^3 residue in (**A**) by D-Atc and by Aic brought about drastic losses of V_2 antagonism. With anti-V_2 pA_2 values of 5.66 and ~ 5.2, respectively, the resulting D-Atc^3 and Aic^3 analogues (peptides 4 and 5) retain less than 1% of the V_2 antagonism of (**A**). That the D-Atc^3 analogue exhibits any V_2 antagonism is rather surprising in view of the fact that a D-Phe^3/L-Phe^3 interchange in (**A**) to give peptide 6 brought about the total abolishment of V_2 antagonism. The D-Phe^3 analogue (peptide 6) exhibits no detectable V_2 antagonism and is in fact a very weak V_2 agonist. This clearly points to the importance of the L-configuration at position three for V_2 antagonism. The relative anti-V_2 potencies of the L-Atc^3/D-Atc^3 analogues of (**A**) (peptides 3 and 4), with the L-diastereoisomer being almost 16 times more potent than its D-diastereoisomer, is further evidence for a critical requirement for the L-configuration at position three in (**A**) for binding to V_2 receptors.

The data in Table 1, while supporting the importance of aromaticity at position three in contributing to potent V_2 antagonism, clearly shows that aromaticity *per se* is not an absolute requirement for an effective position three substituent . Thus, the non-aromatic Pro^3 residue is tolerated as well as the aromatic Phe^3 and Tic^3 residues in (**A**). Of the remaining new analogues, the non-aromatic Oic^3 residue is tolerated much better than the aromatic Atc^3 and Aic^3 residues in (**A**). These findings also show that subtle structural differences at position three can exert strikingly different effects on V_2 antagonism. Thus, Atc and Aic are very closely related, yet when substituted at position three in (**A**) they exert profoundly different effects on V_2 antagonism. The Atc^3 analogue is over 40 times more potent than the Aic^3 analogues as a V_2 antagonist.

V_{1a} Antagonism. All of the position three analogues in Table 1 exhibit V_{1a} antagonism. Their anti-V_{1a} pA_2 values range from 5.62 to 7.51. All, however, are less potent than (**A**) (anti-V_{1a} $pA_2 = 8.22$). The D-Atc^3 and D-Phe^3 substitutions led to drastic losses of V_{1a} antagonism. With anti-V_{1a} pA_2 values of 5.62 and 6.05 respectively, these two peptides (4 and 6) have retained less than 1% of the V_{1a} antagonism of (**A**). With an anti-V_{1a} $pA_2 = 6.39$, the Aic^3 analogue (peptide 5) retains about 2% of the V_{1a} antagonism of (**A**). Whereas, the D-Atc^3 analogue (peptide 4) displays equally low anti-V_2 and anti-V_{1a} potencies, the Atc^3 analogue (peptide 3) is three times more potent as a V_{1a} than as a V_2 antagonist. With an anti-V_{1a} $pA_2 = 7.51$, the Oic^3 analogue (peptide 2) retains about 22% of the V_{1a} antagonism of (**A**). It exhibits virtually identical anti-V_2 and anti-V_{1a} potencies. The Pro^3 analogue (peptide 1), with an anti-V_{1a} $pA_2 = 7.49$ has retained about 20% of the V_{1a} antagonism of (**A**). It is about twice as potent as a V_2 antagonist than as a V_{1a} antagonist. Its anti-V_2/anti-V_{1a} selectivity is thus enhanced about 5-fold relative to (**A**). Both the Tic^3 and Pro^3 substituents effected enhancements in anti-V_2/anti-V_{1a} selectivity. By contrast, the Atc^3 and Aic^3 substitutions resulted in peptides which are more potent as V_{1a} antagonists than as V_2 antagonists. Of the conformationally restricted position three substituents examined to date, Tic^3 and Pro^3 are clearly the most effective in leading to retention of V_2 antagonism and to enhanced anti-V_2/anti-V_{1a} selectivity. These findings represent promising new clues for design of potent, selective and possibly orally active, peptide-based AVP V_2 antagonists.

Effects on (in vitro) OT Antagonism. Peptide (**A**) is a highly potent *in vitro* OT antagonist ($pA_2 = 8.32$). Replacement of the Phe^3 residue in (**A**) by Aic^3, D-Atc^3 and D-Phe^3 led to drastic losses *in vitro* OT antagonism. By contrast, the Pro^3, Oic^3 and Atc^3 analogues (peptides 1–3) with anti-OT (*in vitro*) pA_2 of 7.94, 7.58 and 7.44, although less potent than (**A**), exhibit substantial OT antagonist potencies (*in vitro*). The (*in vitro*) OT antagonism of the Pro^3 and Oic^3 peptides is also protracted. The finding that some conformationally restricted amino acids at

position three in the AVP V_2/V_{1a}/OT antagonist (**A**) are tolerated, with retention of (*in vitro*) OT antagonism as well as AVP V_2 and V_{1a} antagonism may prove to be of value in the design, not only of V_2 and V_{1a} antagonists, but also of OT antagonists.

Effects of Aliphatic and Aromatic Amino Acids of Position Three in d(CH$_2$)$_5$ [D-Tyr(Et)^2]VAVP (A) Table 2

Table 2 lists five analogues of (**A**) with the Phe^3 residue replaced by two aliphatic amino acids, Ile and Leu, and by three aromatic amino acids, Tyr, Trp and Hphe. All five peptides in this series exhibit V_2, V_{1a} and OT (*in vitro*) antagonism. They exhibit anti-V_2 pA_2 values ranging from 6.82 to 7.61; anti-V_{1a} pA_2 values ranging from 6.74 to 7.64; and anti-OT pA_2 values ranging from 7.53 to 7.76. The Tyr^3, Trp^3 and Hphe^3 aromatic substitutions are surprisingly much more effective than the aliphatic Ile^3 and Leu^3 substituents in leading to retention of V_2 antagonism. With anti-V_2 pA_2 values of 7.58, 7.61 and 7.59 respectively, the Tyr^3, Trp^3 and Hphe^3 analogues (peptides 9–11) exhibit excellent retention of V_2 antagonism and are almost equipotent with (**A**). It will be recalled that Tyr^3 and Trp^3 substitutions in lysine vasopressin (LVP) resulted in drastic losses of V_2 and V_{1a} agonism, clearly showing that these two position three modifications are deleterious for the manifestation of agonistic activities [9, 27, 28]. We have recently shown that Pro^3/Phe^3, Tyr^3/Phe^3 and Trp^3/Phe^3 interchanges in AVP also lead to drastic losses of V_2 and V_{1a} agonistic activities (unpublished). The fact that all four modifications, Pro^3, Tyr^3, Trp^3 and Hphe^3, in the potent V_2/V_{1a}/OT antagonist d(CH$_2$)$_5$[D-Tyr(Et)^2]VAVP (**A**) lead to excellent retention of antagonistic potencies is yet another clear illustration of the profound structural differences between AVP antagonists and AVP agonists required for binding to and activating V_2, V_{1a} and OT receptors [13–16].

As noted above, the aliphatic amino acids, Ile and Leu, at position three in (**A**) were much less effective in leading to retention of V_2 antagonism. Thus, the Ile^3 analogue of (**A**) (peptide 7) retains only about 20% of the V_2 antagonism of (**A**), whereas the Leu^3 analogue of (**A**) (peptide 8) retains only about 10% of the V_2 antagonism of (**A**). We had shown previously that an Ile^3/Phe^3 interchange in other potent V_2/V_{1a} antagonists, d(CH$_2$)$_5$[D-Tyr(Me)^2]VAVP (**D**) (anti-V_2 $pA_2 = 7.77$ [32] and d(CH$_2$)$_5$ [D-Phe^2] VAVP (**E**) (anti-V_2 $pA_2 = 8.06$) [36], could be tolerated, but with significantly diminished V_2 antagonism [35]. Thus, the respective Ile^3 analogues, d(CH$_2$)$_5$ [D-Tyr(Me)^2, Ile^3]VAVP (**F**) and d(CH$_2$)$_5$[D-Phe^2,Ile^3]VAVP (**G**), exhibit drastically reduced anti-V_2 pA_2 values of 6.88 and 6.21 respectively [35]. With an anti-V_2 $pA_2 = 7.25$, the Ile^3 analogue of (**A**) (peptide 7), which differs from (**F**) only by a D-Tyr(Et)/D-Tyr(Me) interchange at position two, is surprisingly much more potent than (**F**) or (**G**). The retention of V_2 antagonism by these three Ile^3-containing peptides is not, however, unexpected. Thus, in contrast to the deleterious effects on V_2 agonism of Phe^3, Tyr^3 and Hphe^3 substitutions in LVP and in AVP, Ile^3 substitutions in AVP and LVP agonists are very well tolerated, with excellent retention of V_2 agonistic activities [9, 27, 28]. All five aliphatic and aromatic position three substituents appear to be equally effective in leading to excellent retention of *in vitro* OT antagonism. With the exception of the Leu^3 analogue, which has an anti-V_{1a} $pA_2 = 6.74$, there appears to be virtually no difference in the effectiveness of the aliphatic Ile^3 and the aromatic Tyr^3, Trp^3 and Hphe^3 substituents in leading to equipotent retentions of $\sim$20% of the V_{1a} antagonism of (**A**). The surprising finding that the Phe^3 residue in the AVP V_2/V_{1a}/OT antagonist (**A**) can be replaced by other aromatic amino acids with excellent retention of V_2 antagonism opens up a new avenue of exploration in V_2 antagonist design.

d(CH$_2$)$_5$[D-Tyr(Et)^{2}Tic^3]VAVP (B) as a lead for the design of Radioiodinateable Ligands and Orally Active V_2 Antagonists (Table 3)

An [HO]Tic^3/Tic^3 interchange in (**B**) resulted in a decrease of V_2 antagonism and an increase in V_{1a} antagonism. With an anti-V_2 $pA_2 = 7.20$, peptide 12 cannot be considered a very promising candidate for radioiodination. By contrast, a Tyr-NH$_2^9$/Gly-NH$_2^9$ interchange in (**B**) led to selective retention of V_2 antagonisms in the resulting peptide 13. With an anti-V_2 $pA_2 = 7.57$ and an anti-V_2/anti-V_{1a} selectivity of 11, peptide 13 could be a promising new radioiodinatable ligand, as indeed could the equipotent Tyr^3 analogue of (**A**) (peptide 9, Table 2). A D-Cys^6/L-Cys^6 interchange in (**B**) has been very well tolerated with selective retention of V_2 antagonism and a significant loss of V_{1a} antagonism. With an anti-V_2 $pA_2 = 7.52$, the D-Cys^6 analogue of (**B**) (peptide 14) retains almost 50% of the V_2 antagonism of (**B**). It retains less than 30% of the V_{1a} antagonism of (**B**) and is thus over twice as selective as (**B**). With an ED ratio of 13, its anti-V_2/anti-V_{1a} selectivity is in fact enhanced 30-fold relative to (**A**) (ED-ratio = 0.4). A D-Cys^6/L-Cys^6 interchange in (**B**)

is significantly more effective than in (**A**). The latter leads to a retention of only about 30% of the V_2 antagonism of (**A**) by the resulting peptide (**C**). Looked at another way, a Tic^3/Phe^3 interchange in (**C**) to give peptide 14 has resulted in a 50% increase in anti-V_2 potency in peptide 14. These findings illustrate that Tic^3 substituted V_2 antagonists can be modified at other positions with excellent retention of V_2 antagonism. Furthermore, the finding that the combination of Tic^3 and D-Cys^6 substitutions in (**A**) is very well tolerated offers the hope that these modifications, together with other appropriate modifications, may be of value in the design of orally active V_2 antagonists.

General Considerations

We have reported the synthesis and some pharmacological properties of 12 analogues of the potent AVP V_2/V_{1a} antagonist $d(CH_2)_5$[D-$Tyr(Et)^2$]VAVP (**A**) [32] modified at position three and two analogues of the equipotent more selective V_2/V_{1a} antagonist, $d(CH_2)_5$[D-$Tyr(Et)^2$,Tic^3]VAVP (**B**) [31] modified at positions six and nine, for a total of 14 analogues. Our findings show that the Pro^3, Oic^3, Tyr^3, Trp^3 and $Hphe^3$ substituents are surprisingly well tolerated with excellent retention of V_2,V_{1a} and OT (*in vitro*) antagonism. With an anti-V_2 $pA_2 = 7.77$, the Pro^3 analogue of (**A**) is, in fact, equipotent with (**A**) (anti-V_2 $pA_2 = 7.81$). We have also shown that a D-Cys^6/L-Cys^6 interchange in (**B**) is tolerated very well, better in fact than a D-Cys^6/L-Cys^6 interchange in (**A**). A Tyr-$NH_2{}^9$/Gly-$NH_2{}^9$ interchange in (**B**) is also well tolerated. Thus, the Tyr-$NH_2{}^9$ analogue of **B** (peptide 13 in Table 3) and the Tyr^3 analogue of (A) (Peptide 9, Table 2) could be promising new radioiodinatable ligands for V_2 receptors. These findings on the position three modifications of (**A**) are in striking contrast to the effects of these substituents in AVP and provide new evidence that AVP agonists and antagonists differ profoundly in the manner in which they interact with V_2,V_{1a} and OT receptors. In agonistic analogues of AVP, the structural requirements at position three for binding to and activating V_2,V_{1a} and OT receptors are very rigid, especially with regard to the lack of tolerance for conformationally restricted and aromatic amino acids other than Phe. Consequently, it has long been assumed that the structural requirements at position three in AVP V_2/V_{1a} antagonists would likewise be highly precise. The findings reported here clearly repudiate this assumption. Thus, position three in the AVP antagonist (**A**), in contrast to position three in AVP, tolerates replacement of the Phe^3 residue by conformationally restricted amino acids and by other aromatic acids. Examination of the CD, NMR and X-ray structures of (**A**), (**B**) and the Pro^3 analogue of (**A**), combined with molecular modelling, should provide important insights to the differences in the conformations of AVP agonists and of AVP antagonists involved in binding to V_2 receptors. Our findings that (**A**) and (**B**) can be modified at positions 3, 6 and 9 with excellent retention of V_2 antagonism, provide significant new clues to the design of potent, orally active V_2 antagonists for use as pharmacological tools and radioiodinatable ligands and for development as potential therapeutic agents for the treatment of the hyponatremia caused by SIADH [2, 3, 74].

EXPERIMENTAL PART

The [β-(benzylthio)-β,β-cyclopentamethylenepropionic acid [75], 2-aminoindan-2-carboxylic acid (Aic) [49, 54], Boc-Aic [53], D,L-2-aminotetralin-2-caboxylic acid (Atc) [45, 49] and Boc-D,L-Atc [48] were synthesized in this laboratory using routine procedures. For both Aic and D,L-Atc the spirohydantoin version of the Strecker synthesis [51, 52] was applied. The racemic Boc-Atc was resolved by its diastereoisomeric α-methylbenzylamine (MBA) salts as described below. The configurations of Boc-L-Atc and Boc-D-Atc were determined following deprotection by comparison of the R_F and $R_{F(L)}/R_{F(D)}$ values of the deblocked amino acids on chiral TLC (Macherey-Nagel, Chiralplate, solvent system: MeCN:MeOH:-H_2O (4 : 1 : 1) with those described in the literature [47]. The Boc-(2S,3aS,7aS)-Oic [39, 40] and other amino acid derivatives were purchased from Bachem California, Inc. Thin-layer chromatography was run on precoated silica gel plates (60F-254, E. Merck) with the following solvent systems: (a) 1-butanol : AcOH : H_2O (4 : 1 : 5, upper phase); (b) 1-butanol : AcOH : H_2O (4 : 1 : 1); (c) 1-butanol : AcOH : H_2O : pyridine (15 : 3 : 3 : 10); (d) chloroform : -methanol (7 : 3). Loads of 10–15 μg were applied and chromatograms were developed at a minimal length of 10 cm. The chlorine gas procedure for the KI-starch reagent was used for detection [58]. Optical rotations were measured with a Rudolph Autopol III polarimeter. Analytical HPLC was performed on a Waters 810 instrument under the following conditions: 90 : 10 to 30 : 70 0.05% aqueous TFA : 0.05% TFA in MeCN, linear gradient over 60 min at

1.0 ml/min ($\lambda = 210$ nm), on a Microsorb C_{18} column (Rainin Instrument Co., Inc.). All peptides were at least 95% pure. Amino acid analyses (AAA) and electrospray mass spectrometry (ESMS) were done by the University of Michigan Protein and Carbohydrate Structure Facility. Amino acid analyses were performed using an ABI automated derivatizer/analyser (Model 420H). Automated hydrolysis was carried out with 6N HCL at 200°C for 75 min. The free amino acids were derivatized with phenyl isothiocyanate in the presence of DIEA and the PTC-amino acid derivatives were separated using an ABI model 130A separation system. Data analysis was performed on an ABI Model 610A system. Electron spray mass spectra were obtained on a Vestec 201 single quadropole mass spectrometer using AcOH : H_2O : MeCN (4 : 46 : 50) as a solvent. Amino acid analyses and ESMS spectra of the free peptides were in agreement with the composition of each peptide.

2-Tert-butyloxycarbonyl-D-aminotetralin-2-carboxylic acid (Boc-D-Atc)

12 g (41.2 mmol) of ±Boc-Atc was dissolved in 420 ml warm EtOH. 4.99 g (41.2 mmol) of (R)-(+)α-methylbenzylamine (MBA) was added and after four days the crystalline product was filtered: yield 8.1 g crude Boc-D-Atc.MBA, m.p. 209–210.6°C. Recrystallization from 300 ml EtOH gave 5.8 g Boc-D-Atc.MBA, m.p. 211.8–213.2°C. The combined mother liquors after concentration to 100 ml gave an additional 0.5 g of the product (this mother liquor was also used for the isolation of Boc-L-Atc as described below). Total yield: 6.3 g (74.1%) Boc-D-Atc.MBA. The MBA salt was decomposed by treatment with ethyl acetate/0.1 M HCl. The organic layer was washed with brine, water, dried over $MgSO_4$ and evaporated *in vacuo*. Crystallization from EtOH gave 3.95 g Boc-D-Atc (65.8%, calculated from the starting ± Boc-Atc); m.p. 164–165°C, $[\alpha]_D^{25} = -8.2$ ($c = 2$, DMF); $[\alpha]_D^{25} = -31.8$ ($c = 2$, MeOH).

2-Tert-butyloxycarbonyl-L-aminotetralin-2-carboxylic acid (Boc-L-Atc)

The ethanolic solution of Boc-L-Atc.MBA (see above) was evaporated *in vacuo* and the residue converted to the free carboxylic acid with ethyl acetate/0.1 M HCl: yield 6.6 g (22.7 mmol) crude Boc-L-Atc. This product after treatment with 2.7 g (22.7 mmol) of S-(−)-α-MBA in 150 ml warm EtOH gave 5.3 g crude Boc-L-Atc. MBA, m.p. 210.5–212.8°C (with sublimation). Recrystallization from 220 ml EtOH gave 4.9 g (57.6%) Boc-L-Atc.MBA, m.p. 211°C (with sublimation). The Boc-L-Atc.MBA was transformed to the free acid as described above. Yield 2.89 g (48.2%) Boc-L-Atc, m.p. 165–166°C (crystallization from EtOH. $[\alpha]_D^{25} = +7.8$ ($c = 2$, DMF); $[\alpha]_D^{25} = +31.7$ ($c = 2$, MeOH).

Characterization of L and D-Atc

The steric identity of Boc-L-Atc and Boc-D-Atc was determined by removal of the Boc protecting group (treatment with HCl (1 M)/AcOH; precipitation and washing with Et_2O), followed by chiral TLC on a Macherey-Nagel Chiralplate in solvent system MeCN : MeOH : H_2O (4 : 1 : 1). The R_F values $R_{F(L)} = 0.56$; $R_{F(D)} = 0.49$ and $R_{F(D)}$ ratio (1.14) are in agreement with those described in the literature [47]. HCl · L-Atc, m.p. 230–232°C (with sublimation) $[\alpha]_D^{25} = +19.2$ ($c = 1$, MeOH); HCl · D-Atc, m.p. 234–236°C $[\alpha]_D^{25} = -19.2$ ($c = 1$, MeOH).

Solid-phase Synthesis Procedure

The procedure of solid-phase synthesis conformed to that published [31, 32, 56–59]. Chloromethylated resin (Bachem 1% cross-linked S-DVB, 200–400 mesh, 0.7–1.00 mmol/g) was esterified with either Boc-Gly or Boc-Tyr(Bzl) to an incorporation of approximately 0.5 mmol/g by the caesium salt method [76]. For the synthesis of protected peptidyl resins, eight cycles of deprotection, neutralization and coupling were carried out by the DCC/HOBt [61] or the active ester [62] procedure. Ammonolysis in MeOH [59, 63] was used to split the protected peptides from the resin. The protected precursors obtained by ammonolysis were purified either with warm MeOH(EtOH) [31] (peptides XII–XIV) or with hot DMF [59] (peptides I–XI) followed by reprecipitations with MeOH(EtOH)/Et_2O or EtOH/H_2O for the methanol-soluble peptides and with DMF/MeOH/Et_2O for those peptides extracted with DMF, until adjudged pure by TLC. The protected peptides (I–XIV, Table 4) were deblocked with sodium in liquid ammonia [64]. The resulting disulphydryl compounds were oxidatively cyclized with K_3 [Fe(CN)$_6$] using the normal [65] or a modified reverse procedure [66]. The free peptides were purified by a two-step gel filtration procedure [67] on Sephadex G-15 (eluent 50% AcOH) and Sephadex LH-20 (eluent 2M AcOH). For some peptides an additional purification by gel filtration on Sephadex G-15 and/or LH-20 was used. The physicochemical

properties of the free peptides 1–14 are given in Table 5.

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

Boc-Gly-resin (1.0 g, 0.5 mmol) was subjected to eight cycles of deprotection, neutralization and coupling with Boc-Arg(Tos), Boc-Pro, Boc-Cys (Bzl), Boc-Asn-ONp, Boc-Val, Boc-Pro, Boc-D-Tyr(Et) and β-(benzylthio)-β,β-pentamethylenepropionic acid respectively. The resulting protected peptidyl resin was cleaved by ammonolysis. The protected peptide was extracted with hot DMF (30 ml) and the product precipitated by addition of hot water (*ca*. 300 ml). After cooling, the product was collected, dried *in vacuo* over P_2O_5, reprecipitated from methanol (30 ml) and ether (*ca*. 200 ml). Collection and drying *in vacuo* over P_2O_5 gave the required acyloctapeptide amide (I, Table 4). The same procedure was used for the synthesis and purification of the protected acylpeptide amides II–XI (Table 4). Warm MeOH or EtOH was utilized for the extraction of the remaining peptide amides to give, following purification by repeated precipitations from MeOH(EtOH)/Et_2O or reprecipitation from EtOH/H_2O, the protected acylpeptide amides XII, XIII and XIV respectively (Table 4).

[1-(β-Mercapto-β,β-pentamethylenepropionic acid}, 2-*O*-Ethyl-D-Tyrosine, 3-Pro, 4-Valine] Arginine Vasopression (1, Tables 1 and 5)

A solution of protected acyloctapeptide amide (I, Table 4) (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 colour persisted in the solution for *ca*. 30 s [31, 32, 64]. NH_4Cl was added to discharge the colour. The ammonia was evaporated. Reoxidation of the deblocked disulphydryl peptide was carried out by a modified-reverse procedure [66] as follows. The residue was dissolved in 25 ml 50% AcOH and the solution was diluted with 75 ml H_2O. The peptide solution was added dropwise with stirring over a period of 15–30 min to an 800 ml aqueous solution which contained 20 ml of a 0.01 M solution of potassium ferricyanide. Meanwhile, the pH was adjusted to approximately 7.0 with concentrated ammonium hydroxide. The yellow solution was stirred for an additional 20 min. The same procedure was utilized for the reoxidation of the free peptides 2, 3, 5, 8, 9, 11, 12 (Tables 1–3 and 5). The remaining free peptides 4, 6, 7, 10, 14 (Tables 1–3 and 5) were reoxidized by the normal method [65], i.e. by the addition of a solution of potassium ferricyanide (0.01 M, 20 ml) to a diluted solution of the peptide at pH 7 as previously described [31, 32]. Following oxidation, the free peptide 1 was isolated and purified as follows: after acidification with AcOH to pH 4.5 and stirring for 20 min with anion-exchange resin (Bio-Rad, AG 3 × 4, Cl form, 5 g damp weight), the suspension was slowly filtered and washed with 0.2 M AcOH (3 × 30 ml), the combined filtrate and wasings 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 [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 (100 × 1.5 cm) eluting with aqueous acetic acid (2 and 0.2 M respectively with a flow rate of 4 ml/h. The peptide was eluted in a single peak (absorbance at 254 nm). Lyophilization of pertinent fractions gave the desired vasopressin analogue (1, Tables 1 and 5). For peptides 2, 3 and 6–14, the two-step purification procedure [67] on Sephadex G-15 and Sephadex LH-20 with eluents 50% AcOH and 2M AcOH respectively was used. Peptide 4 was purified in four steps as follows: Sephadex G-15, 50% AcOH; Sephadex LH-20, 2 M AcOH; Sephadex G-15, 2 M AcOH and Sephadex LH-20, 0.2 M AcOH. With the modification for the reoxidation method described above, this procedure was utilized to give the remaining free peptides in Table 5.

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