2-O-METHYL AND 2-O-ETHYL-TYROSINE DERIVATIVES OF [8-ARGININE]VASOPRESSIN ANALOGS: HIGHLY SPECIFIC ANTIDIURETIC AGONISTS

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Dedicated to the memory of Dr Karel Bláha.

The solid phase synthesis of seven 2-O-methyl- and 2-O-ethyl-tyrosine substituted analogs of [8-arginine]vasopressin (AVP) with enhanced antidiuretic agonistic specificity is reported. These peptides are: [2-O-ethyltyrosine, 8-arginine]vasopressin (*I*) [2-O-methyltyrosine, 8-D-arginine]vasopressin (*II*), [2-O-ethyltyrosine, 8-D-arginine]vasopressin (*III*), [2-O-methyltyrosine, 4-valine, 8-arginine]vasopressin (*IV*), [2-O-ethyltyrosine, 4-valine, 8-arginine]vasopressin (*V*), [2-O-methyltyrosine, 4-valine, 8-D-arginine]vasopressin (*VI*), [2-O-ethyltyrosine, 4-valine, 8-D-arginine]vasopressin (*VII*). All analogs were tested for antidiuretic, antivasopressor and antioxytocic activities. Although all these new analogs are antidiuretic agonists they are antagonists of vasopressor responses to AVP, and of responses by the rat uterus to oxytocin. Thus, all seven new Tyr(Me) and Tyr(Et) containing analogs exhibit high antidiuretic specificity and have infinite antidiuretic/pressor (A/P) and antidiuretic oxytocic (A/O) activity ratios. Some of these analogs e.g. Tyr(Me)DAVP, Tyr(Me)VAVP and Tyr(Me)VDAVP, which possess high antidiuretic activity with no pressor or oxytocic agonism, could be useful new pharmacological tools for characterizing receptors mediating specific responses to the neurohypophyseal hormones. They could also be potentially useful in the treatment of diabetes insipidus.

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[8-Arginine]vasopressin (AVP)* has an antidiuretic/pressor (A/P) activity ratio of approximately 1. Because of the potent vasoconstrictor properties of AVP, large doses may cause cardiovascular and gastrointestinal complications. AVP also has substantial oxytocic activity which could complicate its therapeutic use in certain circumstances. Thus, potent and specific antidiuretic agonists have long been sought as potentially useful drugs for the treatment of diabetes insipidus. Intensive structure–activity relationship studies have shown that AVP can be modified in a variety of ways to give analogs which exhibit enhancement of antidiuretic specificity relative to that of AVP (refs[2,3]).

Among the single modifications that enhance anidiuretic specificity, the relative effects of O-methyl and O-ethyl substituents on the tyrosine residue in position two in neurohypophyseal hormone analogs is not clear. It is of interest to note that while some analogs of [8-lysine]vasopressin (LVP)[4,5] and oxytocin (OXT)[6,7], which contain an O-alkyltyrosine instead of tyrosine at position two, have been reported, only one such analog of [8-arginine]vasopressin, namely [Tyr(Me)^2]AVP has been reported to date[8]. [Tyr(Me)^2]AVP retained the antidiuretic activity of AVP but showed greatly reduced pressor activity, i.e. less than 1/30th that of AVP. This lack of effect of the Tyr(Me) substitution in AVP on antidiuretic activity was in striking contrast to its effects in LVP (ref.[4]) and in OXT (refs[6,7]), where it was shown to bring about drastic reductions in antidiuretic activity. More recently, the substitution of Tyr(Et) in LVP was reported to lead to an even further reduction in antidiuretic activity[5].

The aims of the present study were to determine the effects of Tyr(Me) and Tyr(Et) substituents in AVP and some analogs of AVP which have enhanced antidiuretic specificity, namely: [8-D-arginine]vasopressin, (DAVP); [4-valine,8--arginine]vasopressin, (VAVP) and [4-valine, 8-D-arginine]vasopressin, (VDAVP). We thus report the synthesis and some pharmacological properties of the following seven new Tyr(Me) and Tyr(Et) analogs of AVP, DAVP, VAVP and VDAVP:

I, [2-O-ethyltyrosine, 8-arginine]vasopressin (Tyr(Et)AVP);
II, [2-O-methyltyrosine, 8-D-arginine]vasopressin (Tyr(Me)DAVP);
III, [2-O-ethyltyrosine, 8-D-arginine]vasopressin (Tyr(Et)DAVP);
IV, [2-O-methyltyrosine, 4-valine, 8-arginine]vasopressin (Tyr(Me)VAVP);
V, [2-O-ethyltyrosine, 4-valine, 8-arginine]vasopressin (Tyr(Et)VAVP);

* Symbols and abreviations are in accordance with the recommendations of Biochemical Nomenclature[1]. All amino acids are in the L-configuration unless otherwise noted. Other abbreviations used are: Tyr(Me), O-methyltyrosine; Tyr(Et), O-ethyltyrosine; OXT, oxytocin; AVP, [8-arginine]vasopressin; LVP, [8-lysine]vasopressin; DAVP, [8-D-arginine]vasopressin; VAVP, [4-valine, 8-arginine]vasopressin; VDAVP, [4-valine, 8-D-arginine]vasopressin; DMF, dimethylformamide; DCCI, dicyclohexylcarbodiimide; HOBt, N-hydroxybenzotriazole.

VI, [2-O-methyltyrosine, 4-valine, 8-D-arginine]vasopressin (Tyr(Me)VDAVP);
VII, [2-O-ethyltyrosine, 4-valine, 8-D-arginine]vasopressin (Tyr(Et)VDAVP).

Cys-Tyr(X)-Phe-Y-Asn-Cys-Pro-Z-Gly-NH_2

I, X = Et, Y = Gln, Z = Arg;
II, X = Me, Y = Gln, Z = D-Arg;
III, X = Et, Y = Gln, Z = D-Arg;
IV, X = Me, Y = Val, Z = Arg;
V, X = Et, Y = Val, Z = Arg;
VI, X = Me, Y = Val, Z = D-Arg;
VII, X = Et, Y = Val, Z = D-Arg.

The protected peptide precursors required for the synthesis of the vasopressin analogs were prepared by the Merrifield method of solid-phase synthesis[9,10] using previously described modifications[11-14]. Coupling reactions were mediated either by the active ester method[15,16] or by the DCCI/HOBt method[17,18]. The protected nonapeptide amides were obtained by ammonolytic cleavage[14,19] from the respective protected nonapeptide resins. Sodium in liquid ammonia was used to deblock each protected precursor as previously described[11-14,20,21] and the resulting disulfhydryl compounds were oxidatively cyclized with $K_3[Fe(CN)_6]$ (ref.[22]). The free peptides were desalted and purified by gel filtration[23] on Sephadex G-15 as previously described[24].

RESULTS AND DISCUSSION

The antidiuretic and antivasopressor properties of the seven new Tyr(Me) and Tyr(Et) containing analogs of AVP, DAVP, VAVP, VDAVP together with those for Tyr(Me)AVP and each parent analog are given in Table I. The antioxytocic potencies (in vitro) of these analogs together with those for Tyr(Me)AVP are given in Table II.

Effects of Tyr(Me) *and* Tyr(Et) *Substitution on Antidiuretic Activities*

The substitution of Tyr(Me) for Tyr in DAVP did not reduce antidiuretic activity. This is consistent with the result obtained for AVP. In the two remaining peptides, VAVP and VDAVP, the same substitution led to apparent reductions in antidiuretic potencies of about 40 and 50%.

Replacement of Tyr by Tyr(Et) resulted in substantial or dramatic reductions in antidiuretic activity in all 4 pairs studied. Also, in all cases, the Tyr(Et) containing peptide was less potent than the corresponding Tyr(Me) containing peptide. Thus, the Tyr(Et) substitution is much more deleterious to

antidiuretic activity than the Tyr(Me) substitution. However, the degree of loss of activity with the Tyr(Et) substitution depends very much on which peptide the substitution is made in, and is as follows: 53% for AVP, 84% for VDAVP, 93% for DAVP and 96% for VAVP.

Effects of Tyr(Me) *and* Tyr(Et) *Substitutions on Vasopressor Activity*

Tyr(Me)AVP has greatly reduced pressor activity i.e. less than 1/30th that of AVP. All of the seven new Tyr(Me) and Tyr(Et) containing analogs exhibit no vasopressor agonism and are, in fact, antagonists of the vasopressor response to AVP.

TABLE I
Effects of Tyr(Me)/Tyr(Et) substitution on the antidiuretic and vasopressor properties of [8-arginine]vasopressin (AVP) and related 4-valine, and 8-D-arginine agonistic analogs

No.	Compound	Antidiuretic activity units/mg	Antivasopressor activity		A/P ratio
			effective dose[a] nmol/kg	pA_2[b]	
	AVP	323 ± 16	Agonist (370 units/mg)	–	0.9
	Tyr(Me)AVP	386 ± 36	Agonist (9.7 units/mg)	–	40
I	Tyr(Et)AVP	152 ± 13	5.8 ± 1.0	7.17 ± 0.07	infinite
	DAVP	257 ± 35	Agonist (1.1 units/mg)	–	238
II	Tyr(Me)DAVP	309 ± 13	16 ± 3	6.74 ± 0.10	infinite
III	Tyr(Et)DAVP	17.1 ± 0.9	3.2 ± 0.4	7.31 ± 0.07	infinite
	VAVP	738 ± 65	Agonist (32 units/mg)	–	23
IV	Tyr(Me)VAVP	443 ± 33	19 ± 3	6.63 ± 0.07	infinite
V	Tyr(Et)VAVP	29 ± 2	5.3 ± 0.5	7.10 ± 0.04	infinite
	VDAVP	653 ± 51	Agonist (0.037 units/mg)	–	17.649
VI	Tyr(Me)VDAVP	350[c]	19 ± 2	6.63 ± 0.04	infinite
VII	Tyr(Et)VDAVP	106 ± 11	24 ± 4	6.54 ± 0.04	infinite

[a] The effective dose is defined as the dose (in nanomoles per kilogram) that reduces the response seen with $2x$ units of agonist to equal the response seen with x units of agonist administered before antagonist; [b] estimated in vivo pA_2 values represent the negative logarithms of the "effective dose" divided by the estimated volume of distribution (67 ml/kg); [c] there was a significant difference between the slopes of the log dose-response regressions for this analog and the standard and this value represents a rough approximation only.

The Tyr(Et) substituted analogs are equipotent or more potent than their Tyr(Me) counterparts as vasopressor antagonists. With an antivasopressor pA_2 of 7.31, Tyr(Et)DAVP appears to be most potent V_1 antagonist of this series.

Effects of Tyr(Me) *and* Tyr(Et) *Substitutions on Antidiuretic/Pressor (A/P) Selectivity*

Alkylation of the tyrosine residue in position 2 in AVP, DAVP, VAVP and VDAVP resulted in (i) retention of appreciable antidiuretic agonistic activity and (ii) abolishment of vasopressor agonism in virtually all cases, with the concomitant conversion of the parent agonists into moderately potent antagonists. Thus, all new Tyr(Me) and Tyr(Et) containing analogs presented here exhibit high antidiuretic/pressor selectivity and have, in fact, infinite A/P ratios.

Effects of Tyr(Me) *and* Tyr(Et) *Substitutions on Oxytocin Antagonism*

The parent compounds AVP, DAVP and VDAVP exhibit oxytocic agonism. The Tyr(Me) and Tyr(Et) substitutions convert these agonists into antagonists of the in vitro oxytocic responses to oxytocin in the absence and in the presence of magnesium, with pA_2 values ranging from 7.28 up to 7.96 (without magnesium). The effect of alkylation in this series of AVP analogs is much more pronounced than for oxytocin[6,7] and [8-lysine]vasopressin[4,5] analogs: Tyr(Me)

TABLE II
Antioxytocic potencies of Tyr(Me), Tyr(Et) substituted analogs

No.	Compound	Antioxytocic (in vitro) pA_2[a]	
		no Mg^{2+}	0.5 mM-Mg^{2+}
	Tyr(Me)AVP	7.44 ± 0.12	6.34 ± 0.19
I	Tyr(Et)AVP	7.28 ± 0.04	6.96 ± 0.09
II	Tyr(Me)DAVP	7.69 ± 0.06	6.99 ± 0.05
III	Tyr(Et)DAVP	7.71 ± 0.05	6.76 ± 0.05
IV	Tyr(Me)VAVP	7.96 ± 0.04	7.42 ± 0.08
V	Tyr(Et)VAVP	7.44 ± 0.04	7.38 ± 0.06
VI	Tyr(Me)VDAVP	7.46 ± 0.11	7.50 ± 0.30
VII	Tyr(Et)VDAVP	7.49 ± 0.08	7.09 ± 0.08

[a] pA_2 is the negative logarithm of the molar concentration of antagonist that reduces the response to $2x$ units of agonist to equal the response to $1x$ units in the absence of antagonist.

and Tyr(Et) oxytocins display a very low uterotonic activity and under certain conditions inhibit the uterine contracting effects of oxytocin. Tyr(Me) and Tyr(Et) [8-lysine]vasopressins show weak inhibitory effects on the uterotonic activity of oxytocin.

Thus, the new Tyr(Me) and Tyr(Et) containing analogs presented here are more potent or as potent as some of the early oxytocin antagonists, [1-deaminopenicillamine]oxytocin[32] (pA_2 7.14) or [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid]oxytocin[33] (pA_2 7.43).

CONCLUSIONS

We have reported the synthesis and some properties of seven new Tyr(Me) and Tyr(Et) substituted analogs of AVP, DAVP, VAVP, and VDAVP. While the Tyr(Me) substitution did not reduce antidiuretic activity, the Tyr(Et) substitution resulted in substantial losses of antidiuretic activity depending on the peptide in which the substitution is made. All of new analogs are antagonists of the vasopressor response to AVP, and of the uterine response to oxytocin.

Thus, all of the new Tyr(Me) and Tyr(Et) containing analogs reported here exhibit high antidiuretic specificity with infinite antidiuretic/pressor (A/P) and antidiuretic/oxytocic ratios. Some of these analogs e.g. Tyr(Me)DAVP, Tyr(Me)VAVP and Tyr(Me)VDAVP, which possess high antidiuretic activity with no pressor or oxytocic agonism, could be very useful new pharmacological tools for characterizing the receptor types mediating responses to the neurohypophyseal hormones. They could also be potentially useful for the treatment of diabetes insipidus.

EXPERIMENTAL

The protected peptide intermediates *VIII*—*XII* (Table III) were synthesized by the solid-phase method[9,10] by previously described procedures[11—14]. Chloromethylated resin (Bio-Rad Bio-Beads SX1) was esterified with Boc-Gly to a load of 0.43 mmol g according to Gisin[34]. Amino acid derivatives, including Boc-Tyr(Me) and Boc-Tyr(Et), were supplied by Bachem. Triethylamine (TEA) and N-methylmorpholine (NMM) were distilled from ninhydrin. Dimethylformamide was distilled under reduced pressure. Methanol was dried with magnesium methoxide and distilled. Other solvents and reagents were of analytical grade. Thin-layer chromatography (TLC) was on silica gel (0.25 mm, Brinkmann Silplate). The following solvent systems were used: (A) 1-butanol acetic acid water (4:1:5, v v, upper phase); (B) chloroform methanol (7:3, v v); (C) 1-butanol acetic acid water pyridine (15:3:3:10, v v). Loads of 10—50 μg were applied and chromatograms were a minimum length of 10 cm. Iodine vapor was used for detection. For amino acid analysis[35], peptides (approximately 0.5 mg) were hydrolyzed with constant-boiling hydrochloric acid (500 μl) containing phenol (10 μl) in evacuated and sealed ampules for 18 h at 120 °C. The analyses were performed on a Model 121 M Beckman automatic amino acid analyzer. Molar ratios

Czechoslovak Chem. Commun. (Vol. 53) (1988)

TABLE III

Physicochemical properties of the protected peptides: Z-Cys(Bzl)-X^2-Phe-Y^4-Asn-Cys(Bzl)-Pro-Z^8-Gly-NH_2

No.	Structure			Formula M. w.	Yield, % M. p., °C	$[\alpha]_D^{25}$ (*c* 1, DMF)	R_F			Calculated	Found	
	X^2	Y^4	Z^8				A	B	C	%C	%H	%N
VIII	Tyr(Et)	Gln	Arg(Tos)	$C_{77}H_{95}N_{15}O_{16}S_3$ (1582.9)	76.0 224–226	−40.5	0.60	0.51	0.63	58.43	6.05	13.27
										58.47	6.24	13.27
IX	Tyr(Me)	Gln	D-Arg(Tos)	$C_{76}H_{93}N_{15}O_{16}S_3$ (1568.9)	89.7 205–207	−31.2	0.45	0.48	0.48	58.18	5.98	13.45
										57.92	6.11	13.26
X	Tyr(Et)	Gln	D-Arg(Tos)	$C_{77}H_{95}N_{15}O_{16}S_3$ (1582.9)	83.4 233–234	−23.4	0.78	0.46	0.80	58.41	6.05	13.28
										58.10	6.01	13.11
XI	Tyr(Me)	Val	Arg(Tos)	$C_{76}H_{94}N_{14}O_{15}S_3$ (1539.9)	90.6 243–244	−39.1	0.61	0.75	0.65	59.28	6.15	12.73
										59.16	6.30	12.70
XII	Tyr(Et)	Val	Arg(Tos)	$C_{77}H_{96}N_{14}O_{15}S_3$ (1553.9)	72.2 243–245	−38.9	0.56	0.84	0.58	59.52	6.31	12.62
										59.25	6.40	12.45
XIII	Tyr(Me)	Val	D-Arg(Tos)	$C_{76}H_{94}N_{14}O_{15}S_3$ (1539.9)	80.0 231–232	−21.7	0.66	0.79	0.85	59.28	6.15	12.73
										59.06	6.32	12.79
XIV	Tyr(Et)	Val	D-Arg(Tos)	$C_{77}H_{96}N_{14}O_{15}S_3$ (1553.9)	96.5 228–229	−19.8	0.56	0.76	0.61	59.52	6.31	12.62
										58.37	6.33	12.27

were referred to Gly = 1.00. The cysteine content of the free peptides was estimated on the cysteic acid (Cya) to Gly ratio from analyses following performic acid oxidation[36]. All peptides (protected and free) gave the expected amino acid ratios (± 3%). Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN. Optical rotations were measured with a Rudolph polarimeter, Autopol III. Melting points were determined on Thomas–Hoover apparatus and are uncorrected.

Z-Cys(Bzl)-Tyr(Et)-Phe-Gln-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2 (*VIII*)

Boc-Gly-resin (2 g, 0.86 mmol) was converted to protected acyl nonapeptidyl resin in eight cycles of solid-phase peptide synthesis using as the carboxy component Boc-Arg(Tos), Boc-Pro, Boc-Cys(Bzl), Boc-Asn-ONp, Boc-Gln-ONp, Boc-Phe, Boc-Tyr(Et), and finally Z-Cys(Bzl), respectively. The protected acylnonapeptide was cleaved by ammonolysis[14]. The crude product was extracted with hot DMF, and after removal of the resin, precipitated by the addition of hot water. The precipitate was collected, dried in vacuo, and reprecipitated from DMF–ethanol–ethyl ether to give the required protected peptide amide (1.02 g, 75%) based on the Gly content of the starting resin). The physicochemical properties of this and the remaining protected peptides *IX*–*XIV*, which were prepared in essentially the same manner, are given in Table III.

[2-(O-Ethyl)tyrosine, 8-arginine]vasopressin (Tyr(Et)AVP, *I*)

A solution of the protected acyl nonapeptide amide (*VIII*, 158 mg, 0.1 mmol) in sodium-dried and redistilled ammonia (500 ml) was treated at the boiling point and with stirring with sodium from

TABLE IV
Physicochemical properties of analogs *I*–*VII*

No.	X	Y	Z	Yield %	$[\alpha]_D^{25}$ (*c* 0.3; 1M-AcOH)	R_F A / C	Amino acid analysis: Arg / Phe	Asp / Pro	Cys / Tyr	Glu / Val	Gly / NH_3	Cys : Gly
I	Et	Gln	Arg	68.4	−40.3	0.11	1.00	1.00	1.18	0.99	1.00	2.03 : 1.00
						0.23	0.98	1.02	0.97	–	3.07	
II	Me	Gln	D-Arg	84.3	−23.3	0.22	1.03	1.02	1.11	1.02	1.00	2.04 : 1.00
						0.11	0.99	1.03	0.96	–	3.01	
III	Et	Gln	D-Arg	43.5	−24.0	0.19	0.98	1.00	1.20	1.01	1.00	2.03 : 1.00
						0.17	0.97	1.02	0.92	–	2.12	
IV	Me	Val	Arg	74.1	−25.7	0.14	1.02	1.01	1.41	–	1.00	2.08 : 1.00
						0.27	0.99	1.05	0.96	1.01	1.98	
V	Et	Val	Arg	70.9	−24.1	0.14	0.99	1.00	1.00	–	1.00	2.01 : 1.00
						0.45	1.02	1.01	0.99	1.01	1.94	
VI	Me	Val	D-Arg	91.0	−12.0	0.14	1.01	0.97	0.91	–	1.00	2.02 : 1.00
						0.31	1.05	0.96	0.95	0.96	2.32	
VII	Et	Val	D-Arg	76.0	−14.9	0.16	1.01	0.99	2.03	–	1.00	2.09 : 1.00
						0.32	0.98	1.04	0.97	1.04	1.98	

a stick of the metal contained in a small-bore glass tube untill a light-blue color persisted in the solution for 30 s. Dry acetic acid (0.4 ml) was added to discharge the color. The ammonia was evaporated, and nitrogen was passed through the flask. After 5 min, the residue was dissolved in degassed aqueous acetic acid (20%, 50 ml) and quickly poured into ice-cold water (approximately 1 500 ml). The pH was adjusted to approximately 7.0 with concentrated ammonium hydroxide. Following the neutralization, an excess of a solution of potassium ferricyanide (0.01 mol l^{-1}, 18 ml) was added gradually with stirring. The yellow solution was stirred for an additional 20 min and for 10 min with anion-exchange resin (Bio-Rad AG 3, Cl forms, 10 g damp weight). The suspension was slowly filtered through a bed of resin (10 g damp weight). The bed was washed with water (100 ml), and the combined filtrate and washings were lyophilized. The resulting powder was desalted on a Sephadex G-15 column (100 × 2.7 cm) eluting with aqueous acetic acid (50%) with a flow rate of 5 ml h. The eluate was monitored for absorbance at 254 nm and fractioned. The fractions comprising the major peak were checked by TLC (A), pooled, and lyophilized, and the residue was further subjected to gel filtration on a Sephadex G-15 column (100 × 1.5 cm) eluting with aqueous acetic acid (0.2 mol l^{-1})[24] with a flow rate of 4 ml/h. The peptide was eluted in a single peak (absorbance 280 nm). Lyophilization of the pertinent fractions gave the vasopressin analogue *I* as a white powder (84 mg, 76%). The physicochemical properties of this and the remaining free peptides *II* – *VII*, which were prepared in essentially the same manner, are given in Table IV.

Pharmacological Methods

Analogs were assayed for antidiuretic activities by intravenous injection into ethanol-anesthetized and water-loaded rats[25] and for vasopressor activities by intravenous injection into phenoxybenzamine-treated rats under urethane anesthesia[26]. The USP posterior pituitary reference standard was used in all assays. Antagonistic activities were estimated with the same bioassay preparations. The "effective dose" (ED) of an antagonist was estimated as the dose, in nmoles/kg, that reduces the response to 2*x* units of agonist to equal the response to 1*x* units given in the absence of antagonist. In vivo pA_2's were calculated as the negative logarithms of the ED's divided by an arbitrarily chosen volume of distribution (67 ml/kg)[27]. Oxytocic agonism and antagonism were estimated by assays on uteri isolated from estrogen-treated female rats and suspended in media containing no Mg^{++} or 0.5 nM-Mg^{++} (refs[28,29]). Agonistic activities were estimated by the method of Holton[30] and antagonistic pA_2 values were determined as described by Schild[31].

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