

Synthesis, Antidiuretic and Pressor Activities of [Arginine⁴]Arginine-Vasopressin and Two Related Analogues

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Using well-established solid-phase techniques, three new analogues of arginine-vasopressin (AVP) were synthesized. In these the glutamine residue in position 4 was replaced with an additional arginine. The new analogues were: [Arginine⁴]arginine-vasopressin ([Arg⁴]AVP), [2-thiopropionic acid¹,arginine⁴]arginine-vasopressin (d[Arg⁴]AVP) and [1-thiocyclohexaneacetic acid¹,arginine⁴]arginine-vasopressin (d(CH₂)₅[Arg⁴]AVP). [Arg⁴]AVP showed about the same antidiuretic activity as AVP but had only about 40 % of its pressor activity. Unexpectedly, deamination caused a drop in the antidiuretic activity to about 50 %. d(CH₂)₅[Arg⁴]AVP had practically negligible antidiuretic and low pressor effects.

After the syntheses of oxytocin and vasopressin were accomplished by du Vigneaud and co-workers,^{1,2} much effort has been made to study the structure-activity relationships of both hormones. During the past 30 years about 600 analogues have been synthesized and pharmacologically evaluated. These studies have aimed at determining how some structural changes of oxytocin (OT) and arginine-vasopressin (AVP) affect their characteristic spectra of activities. Thus, the design of analogues possessing specific biological properties has been greatly facilitated. However, one approach to the design of more active and selective agonists or antagonists, respectively, is based on combining into a single molecule those structural modifications which in the first case enhance a given activity and in the second case enhance one activity selectively and relatively to another.³

It is well known that some molecular fragments of both OT and AVP are essential for their biological activities. Thus, in the design of analogues, these positions must not be violated. This is not the case for the glutamine residue in position 4.⁴ Glutamine occupies this position in the mammalian hormones, but both serine and asparagine have been identified in this position in oxytocin derivatives found in some fishes.⁵ In fact, replacement of the glutamine residue with a wide variety of amino acids has resulted in compounds with very interesting properties.⁶⁻⁹ However, the effect of substituting in position 4 the pituitary hormones with basic amino acid residues is not well explored. Only two analogues of OT were synthesized in which the glutamine residue was replaced with ornithine¹⁰ and lysine.¹¹ In the first case, [Orn⁴]-oxytocin possesses about 10 % of oxytocic activity, about 30 % of

Table 1. Physico-chemical data for protected octa- and nonapeptide intermediates.

Substance ^a	<i>R_F</i> in system			$[\alpha]_D^{20}$ (c 1.3, DMF)	M.p./°C
	A	B	D		
Boc-Tyr(Bzl)-Phe-Arg(Tos)- -Asn-Cys(Bzl)-Pro-Arg(Tos)- -Gly-NH ₂ (1)	0.32	0.80	0.69	-36.4°	78-82
Z-Cys(Bzl)-Tyr(Bzl)-Phe-Arg(Tos)- -Asn-Cys(Bzl)-Pro-Arg(Tos)- -Gly-NH ₂ (2)	0.41	0.80	0.86	-25.7°	172-173
β -Mpa(Bzl)-Tyr(Bzl)-Phe-Arg(Tos)- -Asn-Cys(Bzl)-Pro-Arg(Tos)- -Gly-NH ₂ (3)	0.39	0.82	0.82	-26.0°	164-166
d(CH ₂) ₅ (Bzl)-Tyr(Bzl)-Phe-Arg(Tos)- -Asn-Cys(Bzl)-Pro-Arg(Tos)- -Gly-NH ₂ (4)	0.47	0.81	0.85	-30.6°	185-190

^a The symbols β -Mpa and d(CH₂)₅ are used to indicate the 2-thiopropionic acid and 1-thiocyclohexaneacetic acid, respectively. All four protected peptides gave correct C, H and N analyses ($\pm 0.4\%$).

milk-ejecting activity but only very low pressor and antidiuretic activities. These findings prompted us to synthesize and evaluate the biological properties of AVP-analogues, in which the glutamine residue was replaced with an additional arginine. Here are described the synthesis and chemical as well as some biological properties of three new peptides, [Arg^{4,8}]vasopressin, its deamino compound and [1-thiocyclohexaneacetic acid¹, Arg^{4,8}]-vasopressin.

RESULTS AND DISCUSSION

Chemistry. The three peptides in Table 1 were synthesized by stepwise coupling of Boc-amino acids to the growing peptide chain on a Merrifield resin. The couplings were mediated by dicyclohexylcarbodiimide or by the *p*-nitrophenyl ester for the asparaginyl residue. The protected peptide was cleaved from the resin by ammonolysis,¹² deblocked by sodium in liquid ammonia^{1,13} and the resulting dithiols were oxidatively cyclised with K₃[Fe(CN)₆].¹⁴ The crude peptides were desalted and then further purified by gel filtration

Table 2. Yield of nonapeptides.

Protected nonapeptide	Crude protected nonapeptide	Peptide after deprotection, oxidation and 1st column	Peptide after 2nd column	Peptide after 3rd column
2	207 mg	80 mg	52 mg	41 mg
3	193 mg	68 mg	50 mg	42 mg
4	195 mg	100 mg	72 mg	-

Table 3. Physico-chemical characteristics of [Arg⁴]AVP's.

Analogue	R_F in system				$[\alpha]_D^{20}$ (°) (c 0.9 1 M HOAc)	Amino acid analyses
	A	B	C	E		
[Arg ⁴]AVP	0.07	—	0.20	0.05	-26.1	Tyr 0.99; Phe 1.05; Arg 2.13; Asp 0.97; $\frac{1}{2}$ Cys 0.99; Pro 0.99; Gly 1.07; NH ₃ 1.99.
d[Arg ⁴]AVP	0.15	0.20	—	0.11	-5.8	Tyr 1.00; Phe 1.04; Arg 2.07; Asp 0.98; $\frac{1}{2}$ Cys 0.99; Pro 0.92; Gly 1.01; NH ₃ 2.02.
d(CH ₂) ₅ [Arg ⁴]AVP	0.18	0.27	—	0.12	-39.9	Tyr 0.99; Phe 1.05; Arg 2.09; Asp 0.97; $\frac{1}{2}$ Cys 0.99; Pro 0.93; Gly 1.02; NH ₃ 2.12.

on Sephadex G-15.¹⁵ As analogues 2 and 3 were not sufficiently pure after this two-step purification, they were subjected to gel filtration on a Sephadex LH-20 column.

Table 2 summarizes the details of the syntheses of the peptides, characterized in Table 3 with respect to the yields obtained after the different chromatographic purifications. The resin had a glycine content of 0.62 meq/g resin. The first amino acid was coupled to the extent of 70 % as determined by amino acid analysis. The yield of the protected octapeptide resin was 0.6 mmol/g resin which was 97 % of the theoretical value. This result shows that during the deprotection steps there was only a small or no loss of peptide. The data from the amino acid analysis of all analogues revealed the expected ratios of the amino acids.

During the chromatographic purifications of the peptides, fractions were selected in such a manner that the purity was emphasized rather than the yield.

Pharmacology. The three new vasopressins were assayed for their antidiuretic and pressor effects and the results are summarized in Table 4. [Arg⁴]AVP has an antidiuretic activity very close to AVP but is not superactive like [Val⁴]- and [Abu⁴]AVP.¹⁶ On the other hand, [Arg⁴]AVP has a decreased vasopressor activity compared with AVP, but this activity is still high in comparison with those of the [Val⁴]- and [Abu⁴]-analogues mentioned, making it only moderately interesting with regard to its A/P selectivity.

With respect to the pharmacological properties of d[Arg⁴]AVP, the deamination resulted in a decreased instead of an increased antidiuretic activity.¹⁷ The pressor activity

Table 4. Pharmacological properties of analogues (IU/mg).

Peptide	Antidiuretic activity ^a	Pressor activity ^a	A/P	P/A
[Arg ⁴]AVP	430	168	2.6	0.4
d[Arg ⁴]AVP	214	128	1.7	0.6
d(CH ₂) ₅ [Arg ⁴]AVP	1.5	28	0.05	19

^a Relative to international standard.

remained approximately constant. Again the corresponding, deaminated [Val⁴]- and [Abu⁴]-analogues show much higher A/P ratios.¹⁶

The third new compound, d(CH₂)₅[Arg⁴]AVP, had a practically negligible effect in the antidiuretic assay. Its pressor activity, however, was considerably higher than expected, making this compound a pronounced pressor agonist rather than a potential pressor antagonist.

EXPERIMENTAL

N,N-Dimethylformamide (DMF) was distilled under reduced pressure; triethylamine (NEt₃) was distilled from ninhydrin. Other solvents and reagents were of analytical grade. Thin-layer chromatography was carried out on silica plates (Merck), and the spots were visualized by ninhydrin or iodine. The following solvent systems were used: A, 1-butanol-acetic acid-water (4:1:5, v/v, upper phase); B, 1-butanol-acetic acid-water-pyridine (15:3:3:10, v/v); C, 1-butanol-acetic acid-water-pyridine (21:15:2:12, v/v); D, chloroform-methanol (7:3, v/v); E, ethanol-0.1 M acetic acid-0.1 M pyridine (4:1:1, v/v). Solutions containing 10–50 μg of the sample were applied to the plates and the chromatograms were developed for a minimum of 10 cm. The melting points are uncorrected. For amino acid analysis the peptides (~0.5 mg) were hydrolysed with constant-boiling hydrochloric acid (400 μl), containing phenol (20 μl), in evacuated, sealed ampoules for 18 h at 110 °C. The analyses were performed using a one-column system (Durrum). The elemental analyses, determined on a Carlo Erba Model 1106 analyzer, indicated by the element symbols, were within ±0.4 % of the theoretical values. The optical rotations were measured with a Hilger-Watts polarimeter with an accuracy of 0.01°. The analytical samples were dried over P₂O₅ *in vacuo* for 24 h. *p*-Nitrophenyl 1-[(phenylmethyl)thio]-cyclohexaneacetate¹⁸ was synthesized using the method described by Yim and Huffman.¹⁹

Synthesis of the peptides. The peptides were synthesized by the solid-phase method.^{12,20} Chloromethylated resin (Bio-Rad, Bio-Beads Sx1) was esterified²¹ with Boc-Gly to a load of 0.62 mmol/g. Boc-Gly-resin was subjected to seven cycles of deprotection, neutralization and coupling using solid-phase methodology to give Boc-Tyr(Bzl)-Phe-Arg(Tos)-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-resin. In general, the Boc group was used for the protection of the α-amino group and it was removed by treatment with 1.2 N HCl/HOAc. Coupling was effected by dicyclohexylcarbodiimide (DCC) or, in the case of asparagine, by the *p*-nitrophenyl esters. The completeness of each coupling reaction was monitored by the Kaiser test.²² The protected octapeptidyl resin, 1.61 g (0.51 mmol) was ammonolysed¹² and the product was extracted with warm DMF. The product was precipitated from DMF-ethanol-ethyl ether to give 0.750 g (89 %), based on the initial glycine content of the resin. Amino acid analysis: Tyr 0.98; Phe 1.01; Asp 1.03; Cys(Bzl) 0.98; Pro 1.02; Arg 2.05; Gly 1.00; NH₃ 1.96. This material was dissolved in TFA and stirred at RT for 40 min. Cold ether was added and the precipitated material was filtered and washed several times with ether. The product was dried *in vacuo* over sodium hydroxide pellets. This material was divided into three portions, dissolved in DMF and a minute excess of *N*-methylmorpholine was added to give pH 7–8 to moist pH paper. *p*-Nitrophenyl 1-[(phenylmethyl)thio]-cyclohexaneacetate, Boc-Cys(Bzl)-ONp or *p*-nitrophenyl 2-(phenylmethyl)thiopropionate were used in the final coupling steps to obtain the three protected nonapeptide analogues II–IV in Table 1. The protected peptides were treated with sodium in liquid ammonia^{1,13} and the resulting dithiol compounds were subjected to oxidative cyclisation with K₃Fe(CN)₆.¹⁴

Purification of the crude peptides. After lyophilisation the resulting materials were desalted on a Sephadex G-15 column (110×2.5 cm), eluted with 50 % aq. acetic acid at a flow rate of 4–5 ml/h.¹⁵ The fractions corresponding to the major peak were pooled, diluted with water and lyophilised. The residue was subjected to further gel filtration on a Sephadex G-15 column (110×1.5 cm), eluted with aq. acetic acid (0.2 M) at a flow rate of 4 ml/h.¹⁵ The peptides eluted as single peaks, monitored at 280 nm. Lyophilisation of the pertinent

fractions yielded the vasopressin analogues as white powders. Analogues d[Arg⁴]AVP and [Arg⁴]AVP were not pure enough after this two-step purification and were therefore further purified by chromatography on Sephadex LH-20 (135×1.8 cm), again eluted with aq. acetic acid (0.2 M). The fractions containing pure peptides were lyophilised. The data on the synthesis and purification steps are summarized in Table 2. The data in Table 3 summarize the properties of the resulting three arginine-vasopressin analogues.

Bioassays. Pressor assays were performed on rats treated with dibenzamine as described by Dekanski.²³ Antidiuretic activity was assessed in hydrated anaesthetized rats using intravenous injections.²⁴ The change in urine conductivity was taken as the measure of the effect. Arginine-vasopressin (AVP) was used as the standard. The biological results are described in Table 4.

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