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Analogs of arginine vasopressin modified in the *N*-terminal part of the molecule with a conformationally constrained *cis*-peptide bond motif

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Abstract: The present work is part of our studies aimed at clarifying the influence of steric constraints in the *N*-terminal part of arginine vasopressin (AVP) and its analogs on the pharmacological activity of the resulting peptides. We describe the synthesis of eight new analogs of AVP or [3-mercaptopropionic acid (Mpa)^1]AVP (dAVP) substituted at positions 2 and 3 or 3 and 4 with two diastereomers of 4-aminopyroglutamic acid. The steric constraints provided by this modification turned out, however, so strong that all the peptides were inactive in all of the bioassays (pressor, antidiuretic and uterotonic tests). Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: arginine vasopressin (AVP); 4-aminopyroglutamic acid; conformational constraints

INTRODUCTION

Arginine vasopressin (AVP), a neurohypophyseal hormone, is a nonapeptide with a disulfide bridge between Cys residues at positions 1 and 6. This results in a peptide constituted of a six-amino acid cyclic part and a *C*-terminal amidated tripeptide tail. AVP is an important regulator of the fluid and electrolyte homeostasis in mammals through its antidiuretic action in the kidney. Furthermore, it participates in the regulation of blood pressure via its potent vasoconstrictor effect [1,2]. These different activities are mediated by binding to four different types of receptors. The V_2 vasopressin receptors are coupled to adenylyl cyclase and are responsible for the antidiuretic effect of AVP. The V_{1a} and V_{1b} (V_3) receptors are both coupled to phospholipase C. The former is involved in blood pressure control and in all other known functions of AVP, except for the stimulation of corticotropin secretion by the adenohypophysis, which is mediated via V_{1b} (V_3) receptor subtype. The oxytocin (OT) receptors are also coupled to phospholipase C and are responsible for the galactogogic and uterotonic effects [3].

Many vasopressin agonists and antagonists have been used as pharmacological and therapeutic tools in animal and human physiology and pathophysiology [4,5]. However, the design and structure–activity studies of analogs that are truly selective for AVP receptors still remain an area of great interest.

Biologically active peptides exhibit multiple conformations in solution. Therefore, the synthesis of conformationally restricted analogs is a valuable approach for determining structure–activity relationships. Restrictions can be imposed, e.g., by formation of cyclic structures within the peptide framework either through disulfide and lactam bridges or by substitution of chosen amino acid residues or building blocks with sterically restricted fragments that limit conformational freedom, forcing the peptide backbone and/or side chains to adopt specific orientations.

In our laboratory, we have shown that such an approach could result in analogs with very interesting pharmacological properties [6,7]. In 1997, we described the synthesis and some pharmacological properties of analogs having L-1-naphthylalanine (L-1-Nal) or L-2-naphthylalanine (L-2-Nal) at position 3 [8]. One of the new peptides [L-2-Nal^3, D-Arg^8] VP, was among the most potent and selective antagonists of V_{1a} receptors reported to date. Moreover, it was the first V_{1a} antagonist devoid of antiuterotonic activity, and its high antipressor potency arises without modification of position 1 previously thought to be essential for substantial pressor antagonism [8]. Two other peptides

Abbreviations: The symbols of the amino acids and peptides are in accordance with the 1983 Recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature and 'Abbreviations and symbols in peptide science: a revised guide and commentary' published in *J. Pept. Sci.* 2006; **12**: 1–12. Other abbreviations: APy, *cis*-(2*S*,4*S*)-4-aminopyroglutamic acid; Apy, *trans*-(2*R*,4*S*)-4-aminopyroglutamic acid; FC, flash chromatography; LHDMS, lithium bis (trimethylsilylamide); Mpa, 3-mercaptopropionic acid; NMP, 1-methyl-2-pyrrolidone; PyBOP, benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium-hexafluorophosphate; TIS, triisopropylsilane.

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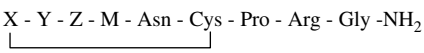
[Mpa^1, L-1-Nal^3, D-Arg^8] VP and [Mpa^1, L-2-Nal^3, D-Arg^8] VP, were highly potent V_2 agonists. The latter peptide was exceptionally selective. Our results suggested that position 3 in AVP and its analogs is important not only for binding and recognition, as previously thought, but also for pressor, antidiuretic, and uterotonic activities [8].

Recently, continuing our efforts in this direction, we replaced the residues at positions 2 or 3 of AVP and some of its analogs with 1-aminocyclohexane-1-carboxylic acid (Acc) and 1-aminocyclopentane-1-carboxylic acid (Apc) [9,10]. Our results have shown that both the Acc^3 and Apc^3 modifications were deleterious to interaction with V_1, V_2 and OT receptors, except for [Mpa^1, Acc^3, Val^4, D-Arg^8] VP, which is a potent and selective antidiuretic agonist. In the case of the Acc^2 and Apc^2 substitution, the situation was different, as it selectively modified interaction with the aforementioned receptors.

Bearing all this in mind, we have designed, synthesized and determined some pharmacological properties of four new analogs of AVP with two amino acid residues substituted at positions 2 and 3 or 3 and 4 with one amino acid residue, either a (2*S*, 4*S*) or (2*R*, 4*S*) diastereomer of 4-aminopyroglutamic acid (see Figure 1 for their structures), our novel *cis*-peptide bond motif. Computer modeling data suggest that this modification, e.g. short-range cyclization, should stand for β-turn type VI mimetics [11]. Introduction of such motifs into bioactive peptides might be used to explore the impact of β-turn geometry on their potency and selectivity. Moreover, this modification, apart from reducing the flexibility, also changed the character of a fragment of the molecule from aromatic to aliphatic. In the next four peptides, we combined the above modification with a deamino modification (Mpa) at position 1. The structures of our analogs are as follows:



where:

X = Cys,	Y-Z = **APy**,	M = Gln	**I**
X = Mpa,	Y-Z = **APy**,	M = Gln	**II**
X = Cys,	Y = Tyr,	Z-M = **APy**	**III**
X = Mpa,	Y = Tyr,	Z-M = **APy**	**IV**
X = Cys,	Y-Z = **Apy**,	M = Gln	**V**
X = Mpa,	Y-Z = **Apy**,	M = Gln	**VI**
X = Cys,	Y = Tyr,	Z-M = **Apy**	**VII**
X = Mpa,	Y = Tyr,	Z-M = **Apy**	**VIII**

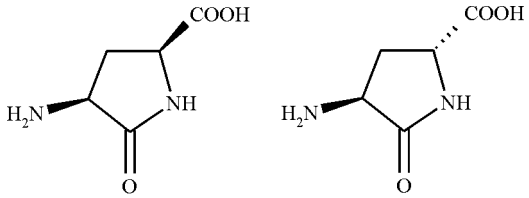


Figure 1 Structure of *cis*-(2*S*,4*S*)-4-aminopyroglutamic acid (**APy**) and *trans*-(2*R*,4*S*)-4-aminopyroglutamic acid (**Apy**).

MATERIALS AND METHODS

General

Thin-layer chromatography (TLC) was carried out on silica plates (Merck), and spots were visualized with iodine or ninhydrin. The solvent system used was butan-1-ol/acetic acid/water/ethyl acetate (1 : 1 : 1 : 1, v/v). High-performance liquid chromatography (HPLC) was carried out on a Waters (analytical and preparative) chromatograph equipped with a UV detector ($\lambda = 226$ nm). The purity of the peptides was determined on a Vydac C_{18} column (5 μm, 4.6 × 250 mm). The following solvent systems were used: (A) 0.1% aqueous TFA, (B) acetonitrile/0.1% aqueous TFA (80 : 20 v/v). A linear gradient from 1% to 40% of solution (B) for 20 min was applied at a flow rate of 1 ml/min. Preparative HPLC was carried out using a Kromasil C_8 column (5 μm, 25 × 250 mm) in a gradient running from 10 to 50% of (B) for 120 min at a flow rate of 10 ml/min ($\lambda = 226$ nm). The mass spectra of the peptides were recorded on a MALDI TOF mass spectrometer.

Mpa (Trt) was obtained as described for Cys (Trt) [12] using Mpa instead of L-cysteine hydrochloride.

All amino acid derivatives were purchased from NovaBiochem, except Fmoc-APy and Fmoc-Apy, which were synthesized according to the literature [13–15].

Peptide Synthesis

All the peptides were obtained manually by solid-phase peptide synthesis using Fmoc chemistry on polystyrene resin (Fmoc-Gly TentaGel S RAM, Rapp Polymere, 0.23 mmol/g) on a 150 μmol scale.

Mixtures of protected amino acid/TBTU/HOBt/NMM (1 : 1 : 1 : 2) in DMF or protected amino acid/PyBOP/HOBt/NMM (1 : 1 : 1 : 2) in DMF or in a mixture of DMF/NMP (1 : 1 v/v) containing 1% Triton were used for coupling. The completeness of each coupling reaction during synthesis was monitored by the Kaiser test [16] or chloranil test [17]. Recoupling was performed when the test was positive.

The Fmoc deprotection was accomplished using a 20% solution of piperidine in DMF. A solution of TFA/H_2O/TIS/PhOH (92.5 : 2.5 : 2.5 : 2.5) was used for the cleavage of peptides from the TentaGel resin (3 h). Solutions of the cleaved peptides were filtered off and evaporated *in vacuo* to *ca.* 1 ml. Then, the peptides were precipitated with diethyl ether to afford the crude products.

The resulting dithiols were oxidatively cyclized with a 0.1 M I_2 in methanol using the standard procedure [18]. The solvents were evaporated under reduced pressure and the residue was dissolved in water and lyophilized. The crude

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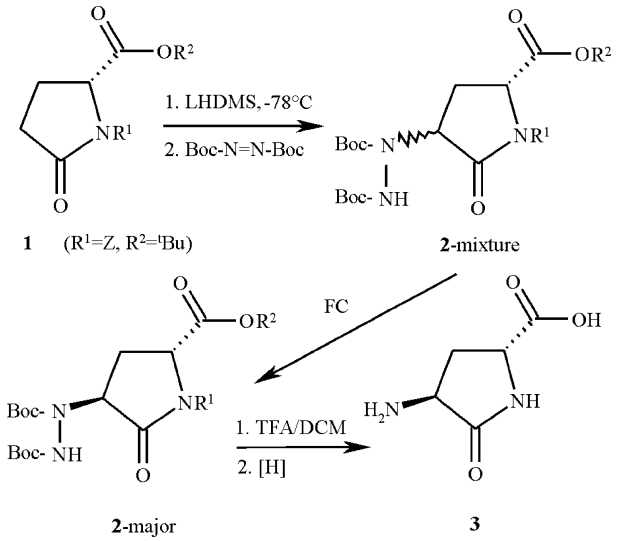
products were desalted on a Sephadex G-15 column, and eluted with aqueous acetic acid (30%) at a flow rate of 3 ml/h. After freeze-drying, the fractions comprising the major peak were purified by RP-HPLC. The peptides were eluted as single peaks. The purity and identity of each peptide were determined by HPLC and MALDI TOF mass spectroscopy (molecular ion). Physicochemical properties of the eight new analogs (**I–VIII**) are presented in Table 1.

Biological Evaluation

Wistar rats were used in all experiments. Female rats were estrogenized 48 h before the experiment. The uterotonic test was carried out *in vitro* in the absence or in the presence of 1 mM of magnesium ions [19–21]. The vasopressor test was performed using phenoxybenzamine-treated male rats [22]. Synthetic OT was used as a standard in uterotonic tests, and synthetic AVP was used in the pressor test. Dose–response (single administration) or cumulative dose–response (measurements without washing steps between the administration of enhanced doses) curves were constructed. Tests to assess the antidiuretic or diuretic properties were conducted on conscious male rats in two variations of the modified Burn test [23,24] as recently described in detail in Ref. 9 (for details concerning all tests, see Ref. 25).

RESULTS AND DISCUSSION

For the preparation of *trans*-(2*R*,4*S*)-4-aminopyroglutamic acid **3**, the previously reported procedure of electrophilic amination of the protected (*R*)-pyroglutamic acid [13] was used (Scheme 1). Racemization-free method [26] for enol generation from **1** with LHDMS in THF at −78 °C and subsequent reaction with di-*tert*-butyl azadicarboxylate afforded the expected intermediate **2** as a diastereomeric mixture. The diastereoisomers were separated chromatographically (FC) in ethyl acetate–hexane giving both pure compounds as oils, which crystallized after prolonged standing. The major isomer *trans* was deprotected, followed by reduction of the hydrazine moiety to amine.



Scheme 1 Preparation of *trans*-(2*R*,4*S*)-4-aminopyroglutamic acid **3**.

Protection of this freshly generated amino group by means of FmocOSu yielded useful SPPS Fmoc-Apy.

Diastereomeric *cis*-(2*S*,4*S*)-4-aminopyroglutamic acid was synthesized according to the procedure based on Michael-type addition of the *in situ* generated *Z*-ΔAla-OMe to enolates prepared from diethyl acylaminomalonates (Scheme 2) followed by base-catalyzed hydrolysis and decarboxylation in boiling dioxane [14,15]. The crude mixture of four stereoisomers of *Z*-4-aminopyroglutamic acid was separated through fractional crystallization into *Z-cis* and *Z-trans* racemates. The *Z-cis* racemate was converted into a mixture of diasteomeric salts with the aid of cinchonidine, which was then separated into enantiomers, again through fractional crystallization. After hydrogenolytic removal of the *Z* group and protection of the amino group with FmocOSu, we obtained optically pure derivative, Fmoc-APy [15].

Both motifs as Fmoc derivatives were incorporated into the peptide chains using standard SPPS methodology.

Table 1 Physicochemical properties of peptides **I–VIII**

Peptide		Formula	HPLC (T_R)	Molecular ion	
				Calculated	$[M + H^+]$
[APy^{2,3}] AVP	I	$C_{33}H_{53}N_{15}O_{11}S_2$	12.1	899.9	900.3
[Mpa^1, APy^{2,3}] AVP	II	$C_{33}H_{52}N_{14}O_{11}S_2$	13.5	884.9	885.2
[APy^{3,4}] AVP	III	$C_{37}H_{54}N_{14}O_{11}S_2$	12.7	934.9	935.1
[Mpa^1, APy^{3,4}] AVP	IV	$C_{37}H_{53}N_{13}O_{11}S_2$	14.3	919.9	920.5
[Apy^{2,3}] AVP	V	$C_{33}H_{53}N_{15}O_{11}S_2$	11.9	899.9	900.3
[Mpa^1, Apy^{2,3}] AVP	VI	$C_{33}H_{52}N_{14}O_{11}S_2$	13.2	884.9	885.2
[Apy^{3,4}] AVP	VII	$C_{37}H_{54}N_{14}O_{11}S_2$	12.5	934.9	935.3
[Mpa^1, Apy^{3,4}] AVP	VIII	$C_{37}H_{53}N_{13}O_{11}S_2$	13.9	919.9	920.2

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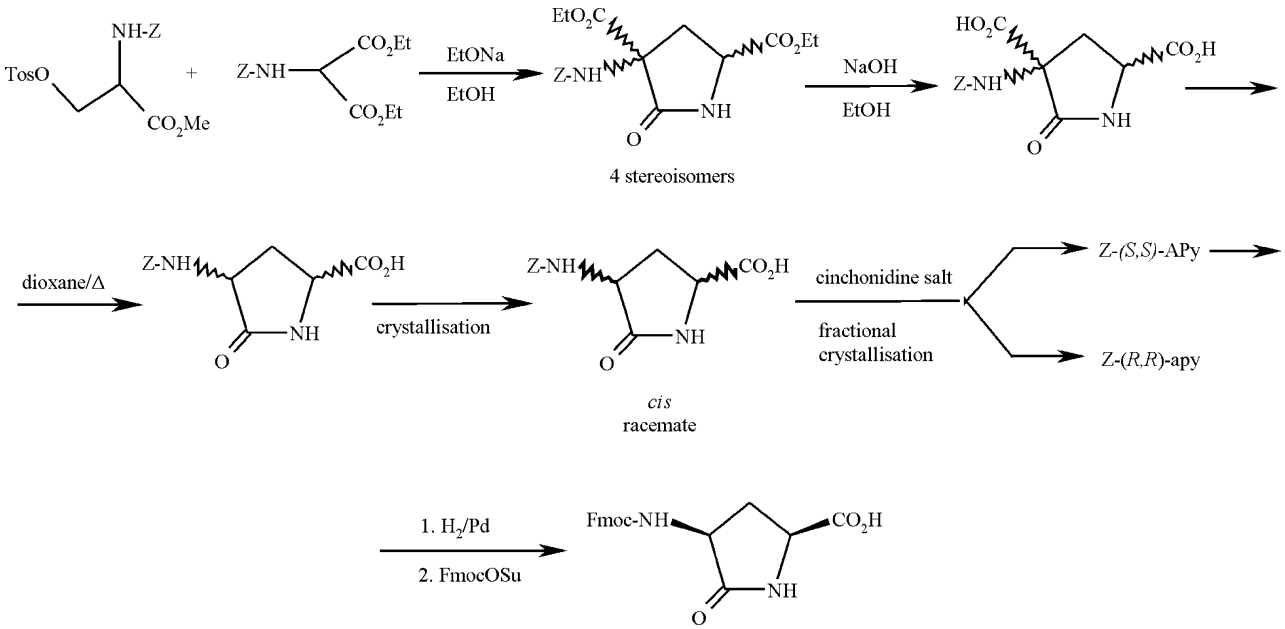

Scheme 2 Synthesis of Fmoc-APy.

The eight new analogs of AVP (**I–VIII**) were synthesized manually using Fmoc chemistry on polystyrene resin (Fmoc-Gly TentaGel S RAM, Rapp Polymere, 0.23 mmol/g) on a 150 μmol scale. The coupling was mediated by either the TBTU/HOBt or PyBOP/HOBt methods. For peptides **II**, **IV**, **VI** and **VIII**, Mpa (Trt) was used in the final coupling step. The crude products were desalted on a Sephadex G-15 and purified by RP-HPLC. The purity and identity of each peptide were determined by HPLC and MALDI TOF mass spectroscopy (molecular ion).

The activities of the analogs were determined in the *in vitro* rat uterotonic test in the absence or presence of 1 mM magnesium ions, in the rat pressor test, and in the antidiuretic assay on conscious rats as described in the experimental section. None of the eight analogs showed any activity in these assays.

The present work is a continuation of our studies aimed at clarifying the impact of steric restrictions in the *N*-terminal part of the AVP molecule on pharmacological properties. Previously, we reported that a bulky naphthyl moiety at position 2 or 3 of AVP and some of its analogs influenced significantly the interaction with V_{1a}, V_2 and oxytocic receptors as expressed by modulated pharmacological activities [8,27]. Some of the peptides displayed very interesting pharmacological properties. In other studies, we imposed steric restrictions by replacement of amino acid residues at positions 2 and 3 with an *N*,*N*′-ethylene-bridged dipeptide, Phe-Phe. Again, this resulted in highly potent and selective V_{1a} antagonists [6,7].

Moreover, interest in understanding the role of the *cis*-amide bond for peptide bioactivity has led to the synthesis of several surrogates, which are able to lock the peptide bond in the *cis*-geometry. Burney *et al.* have designed a mimetic of the *cis*-peptide bond on the basis of extension of the pyroglutamic residue at C^{γ} [11]. Their computer modeling data suggests that this should stand for β-turn mimetics and can serve as hydrogen-bond acceptor as well as donor, having an amide bond unchanged. The incorporation of such cyclic structure elements into short flexible peptides reduces the number of existing conformers and can be used to explore the impact of β-turn geometry for their selectivity and potency. It is even more obvious when one realizes that each motif replaces in the peptide backbone two subsequent amino acid residues. However, it should also be emphasized that our modifications did not change the size of the 20-membered ring believed to be essential for pharmacological activity of AVP analogs.

On the basis of all these findings, we decided to synthesize four new analogs of AVP substituted at positions 2 and 3 or 3 and 4 with two diastereomers of 4-aminopyroglutamic acid and four peptides in which we combined the above modification with the placement of Mpa at position 1. Unfortunately, all the new analogs were inactive in all assays performed.

CONCLUSIONS

In summary, our results, although not impressive in terms of the biological activities of the reported peptides, appear to offer new information about the structure–activity relationships useful for designing AVP analogs.

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