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Analogues of AVP modified in the *N*-terminal part of the molecule with Pip isomers: TFA-catalysed peptide bond hydrolysis‡

Dariusz Sobolewski,[a]* Adam Prahl,[a] Anna Kwiatkowska,[a] Jiřina Slaninová[b] and Bernard Lammek[a]

Using SPPS techniques, six new analogues of AVP and some of its agonists were synthesised. The peptides were designed by substitution of Phe at position 3 of AVP, [Mpa[1]] AVP (dAVP) and [Mpa[1],Val[4],D-Arg[8]]VP (dVDAVP) with L- or D-Pip, a non-coded α-imino acid, also called homoproline. Surprisingly enough, both the analogues of AVP and [Mpa[1]]AVP with identical substituents at position 2 turned out to be highly sensitive to TFA used for deprotection and cleavage of the synthesised peptides from the resin and it was the reason why we were not able to obtain these four peptides. The mechanisms of their fragmentation were proposed in this study. Unfortunately, all the new analogues were inactive in bioassays for the pressor, antidiuretic and uterotonic *in vitro* activities in the rat.

Keywords: AVP; Pip; amide bond hydrolysis; biological activity

In mammals, AVP is a cyclic nonapeptide mainly synthesised and released into the circulation by the magnocellular neurons of the supraoptic and paraventricular hypothalamic nuclei, with axons projecting to the pituitary [1]. Physiological effects of AVP are mediated by at least three distinct VP receptor subtypes: V_{1a}, known to mediate the contractile action of AVP on vascular smooth muscles and stimulate glycogenolysis in the liver; V_{1b} (V_3), involved in the release of ACTH from pituitary; and V_2, receptors mediating antidiuretic action in the kidney [2]. AVP also interacts with OT receptors which are responsible for the galactobolic and uterotonic effects [3].

Conformationally constrained analogues of bioactive peptides have triggered a growing interest in studies of their SARs. Steric restrictions can be imposed, e.g. by formation of cyclic structures within the peptide backbone or reduction of the peptide flexibility by introducing amino acids with a limited conformational freedom. One of the most straightforward approaches for the peptide modification is to introduce changes into the side chains of single amino acids. At this level, a multitude of possibilities for the synthesis of non-proteinogenic amino acids has already been offered, and useful preparative routes for the asymmetric synthesis of many derivatives have been developed. This strategy enabled incorporation of amino acids with side chains that do not or uncommonly occur naturally in peptides or proteins, with the aim to introduce special functional groups, either to restrict conformational flexibility of a peptide or to enhance its metabolic stability. Furthermore, D-configured amino acids, N^{α}-alkylated amino acids or C^{α}-dialkylated amino acids could be employed.

Recently, we replaced the residues at position 2 or 3 of AVP and some of its agonistic and antagonistic analogues with either 1-aminocyclohexane-1-carboxylic acid (Acc) or 1-aminocyclopentane-1-carboxylic acid (Apc) [4–6]. Acc and Apc were chosen to reduce the flexibility of the peptides by implanting a sterically constrained residue, thus forcing the peptide backbone and side chains to adopt specific orientations. The Acc and Apc substitution in position 2 selectively altered biological activity. Some of the analogues turned out to be highly potent V_2 agonists with different pressor and uterotonic potencies. Our results have shown that both the Acc[3] and Apc[3] modifications were detrimental to interaction with V_{1a}, 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.

These findings prompted us to continue our studies by introduction of new sterically restricted residues into this part of the molecule with the aim to find out how the modification would affect biological potency of the analogues. We have designed ten new peptides substituted at position 2 or 3 with Pip isomers, a non-coded α-imino acid, also known as Pip or homoproline (Figure 1). Pip has been found in several important natural products [7–9] as well as it has been extensively used as a proline substitute in numerous syntheses of peptidomimetics [10–12]. Eight of the

* *Correspondence to: Dariusz Sobolewski, Faculty of Chemistry, University of Gdańsk, Sobieskiego 18, 80-952 Gdańsk, Poland. E-mail: dareks@chem.univ.gda.pl*

a *Faculty of Chemistry, University of Gdańsk, Sobieskiego 18, 80-952 Gdańsk, Poland*

b *Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Flemingovo sq. 2, 16610 Prague, Czech Republic*

Abbreviations used: *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: AVP, arginine vasopressin; Pip, pipecolic acid; Mpa, 3-Merceptopropionic acid; NMP, 1-methyl-2-pyrrolidone; TIS, triisopropylsilane.*

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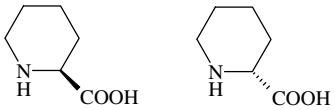

Figure 1. Structure of Pip isomers.

peptides were designed by substitution of Tyr^2 or Phe^3 of the AVP and [Mpa^1]AVP with L- or D-Pip. In the case of a potent V_2 agonist, [Mpa^1,Val^4,D-Arg^8]VP, two new analogues have been prepared by substituting position 3 with L- or D-Pip. It should be noticed that the modification, apart from reducing the flexibility, also changed the character of the molecule from aromatic to aliphatic. We designed the following analogues: [L-Pip^2]AVP (**I**), [Mpa^1,L-Pip^2]AVP (**II**), [D-Pip^2]AVP (**III**), [Mpa^1,D-Pip^2]AVP (**IV**), [L-Pip^3]AVP (**V**), [Mpa^1,L-Pip^3]AVP (**VI**), [D-Pip^3]AVP (**VII**), [Mpa^1,D-Pip^3]AVP (**VIII**), [Mpa^1,L-Pip^3,Val^4,D-Arg^8]VP (**IX**), [Mpa^1,D-Pip^3,Val^4,D-Arg^8]VP (**X**).

The synthesis of ten new peptides (**I**–**X**) was carried out 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 HATU/HOAt methods. For peptides **II, IV, VI, VIII, IX** and **X**, Mpa(Trt) was used in the final coupling step. Cleavage from the resin and side-chain deprotection was carried out by acidolysis with TFA using water, triisopropylsilane (TIS) and phenol as scavengers [13]. Surprisingly enough, a MALDI TOF mass spectrometry of the crude analogues **I**–**IV** did not reveal expected molecular ions. Accordingly, a conclusion has been drawn that this was due to a high sensitivity to TFA used for deprotection and cleavage of the synthesised peptides from the resin. The fragmentation products are shown in Table 1.

These results indicated that the degradation of peptides **I**–**IV** was due to an unexpected TFA-promoted amide bond cleavage under the standard SPPS conditions. Despite previous knowledge that protonation could be an initial step involved in non-enzymatic hydrolysis of amides, peptides and proteins in aqueous solution [14], this backbone integrity remains still highly unusual at room temperature and generally requires additional agents to be carried out [15–17]. Contrary to these reports, fragmentation of peptides containing either *N*-alkylated or Pip residues under mild conditions has already been reported [18–20].

For analogues **II** and **IV** that could be regarded as *N*-acylated oktapeptides, we have suggested a mechanism of cleavage via the formation of an intramolecular tetrahedral intermediate (Scheme 1) [18–20]. This oxazolone-like intermediate is obtained from a species protonated at the Pip residue by nucleophilic attack of an adjacent carbonyl group of the Mpa^1 residue. Apparently, the important factor here is the charge density on the carbonyl oxygen. The inductive effect (I) of the alkyl side-chain of the residue adjacent to the carbonyl group enhances the carbonyl nucleophilicity. Once the tetrahedral intermediate has been formed, the lone-pair electrons on the nitrogen of Phe^3 are no longer in conjugation with the carbonyl π-bond of the Pip residue. As an amine-like structure, the Phe^3 nitrogen becomes a proton acceptor. Thus, the peptide fragment 3–9 is ejected, and the system collapses to an oxazolinium ion intermediate that immediately reacts with traces of water to form the final product (fragment 1–2).

The proposed degradation mechanism of analogues **II** and **IV** is similar to that for the acid-catalysed hydrolysis of peptides containing either *N*-alkylated residues as claimed by Urban *et al*. [18] and Creighton *et al*. [19] or Pip residues as proposed by Rubini *et al*. [20]. Moreover, these authors postulate that peptides in which the above-mentioned modifications are preceded by one additional amino acid show different behaviour depending on the *N*-terminal acylation. Peptides with acylated *N*-terminus are readily hydrolysed, whereas those with free (protonated) *N*-terminus are fairly stable. The results obtained by TFA treatment of analogues **I**–**IV** only partially confirmed these findings, whereas with analogues **I** and **III** they completely ruled out the mechanism of cleavage via the oxazolone-like intermediate. A comparison of the acidolysis products of peptides with the Mpa^1 (**II** and **IV**) and Cys^1 (**I** and **III**) residues reveals the importance of the influence of the *N*-terminal amino group on the mechanism of hydrolysis. Protonation of the free *N*-terminal amino group of peptides **I** and **III** results in both decreased electron density (–I effect) on the carbonyl oxygen of the Cys^1 residue and in the formation of a hydrogen bond between the *N*-terminus and that of oxygen. Consequently, the acid-promoted hydrolysis of the amides can serve as a model for cleavage of the peptide bond in analogues **I** and **III** (Scheme 2) [21–23].

A first step of this mechanism involves specific protonation of the carbonyl oxygen of the Cys^1 residue to activate it towards attack by a water molecule. That attack is assisted by a second water molecule to directly afford a tetrahedral intermediate, the gem-diol derivative. In the next step, a nitrogen atom is protonated and the amide bond is being broken. That process is assisted by simultaneous proton removal from one hydroxyl group by a water molecule to afford final hydrolysis products.

In contrast to AVP analogues modified at position 2 with L- or D-Pip (**I**–**IV**), peptides containing the same substituents at position 3 (**V**–**X**) were fairly resistant to TFA-catalysed peptide bond hydrolysis. In that case, no trace of backbone's degradation has been noticed. The physicochemical properties of compounds **V**–**X** are presented in Table 2. The purity of each peptide was determined by HPLC and their molecular ions were as expected. The activities of these analogues 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 six analogues showed any activity in these assays.

Table 1. TFA-catalysed hydrolysis of peptides **I**–**IV** during TFA cleavage from the resin

Analogue		Molecular ion *calculated*	*found*	Products of cleavage
[L-Pip^2]AVP	**I**	1032.2	931.2	H-Cys + L-Pip-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2
[Mpa^1,L-Pip^2]AVP	**II**	1017.2	820.2	Mpa-L-Pip + Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2
[D-Pip^2]AVP	**III**	1032.2	931.2	H-Cys + D-Pip-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2
[Mpa^1,D-Pip^2]AVP	**IV**	1017.2	820.3	Mpa-D-Pip + Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2

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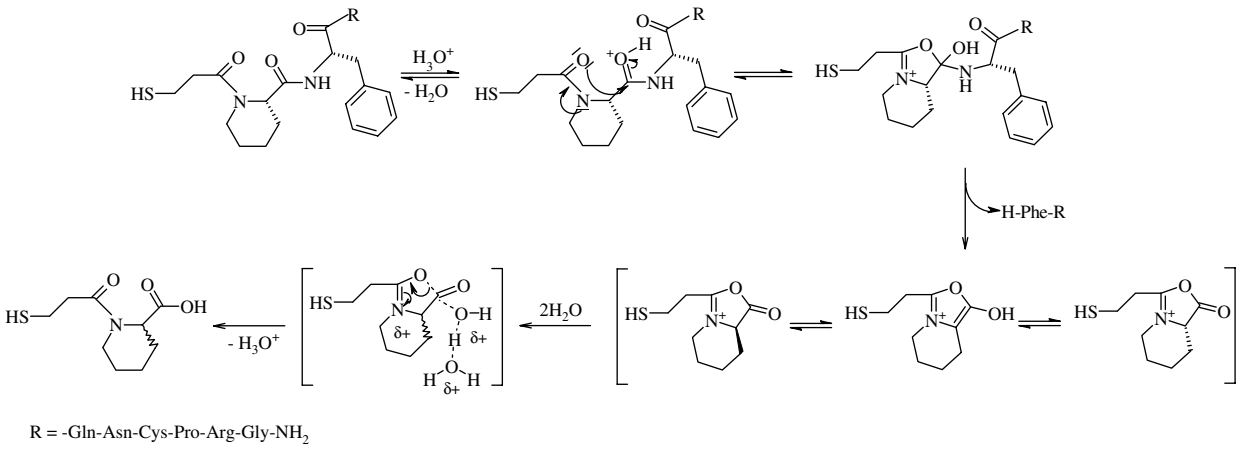
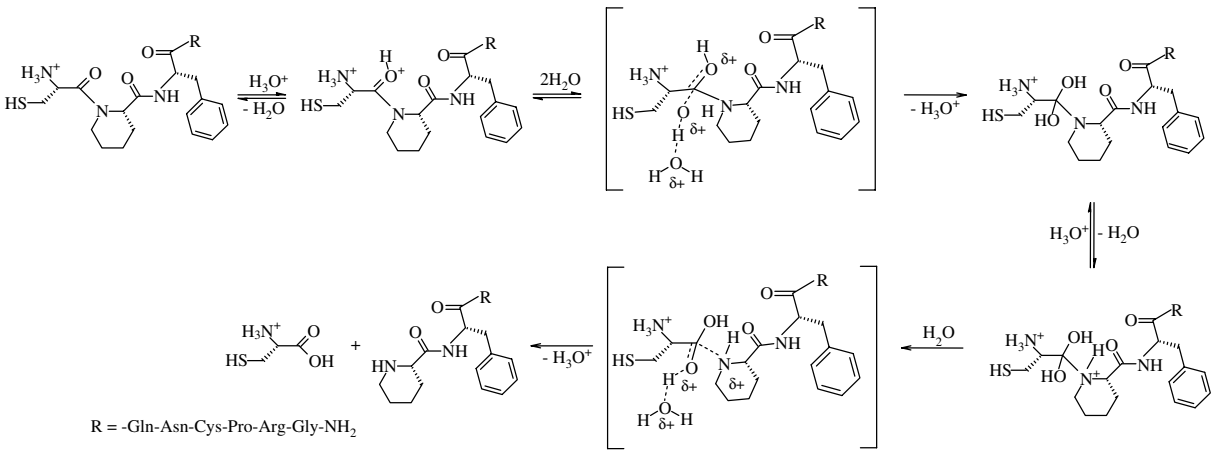


Scheme 1. Proposed mechanism of the acid-promoted hydrolysis of analogue **II** (similar to that of analogue **IV**).



Scheme 2. Proposed mechanism of the acid-promoted hydrolysis of analogue **I** (similar to that of analogue **III**).

The present work is part of our studies aimed at clarifying the influence of steric constraints in the *N*-terminal part of AVP analogues on the pharmacological properties of the resulting peptides. Previously, we have reported that some analogues modified at position 2 or 3 with unusual amino acid residues displayed interesting biological activities [4–6,24,25].

Unfortunately, in this case, incorporation of L- or D-Pip residue into the short flexible peptide molecules resulted in compounds with either a high sensitivity to acid-promoted hydrolysis (**I**–**IV**) or inactive ones (**V**–**X**). However, we do believe that even due to these surprising and disappointing in terms of bioassay results, it was worthwhile to learn how our modification affected pharmacological properties of AVP and its selected agonists. Our results suggest that the Pip isomers probably fix the molecule in a conformation unfavourable for binding to the receptors and for activity. The results imply that our modification is incompatible with the binding sites of the V_{1a}, V_2 and oxytocic receptors.

Our results, while disappointing in terms of biological activities of the reported analogues, offer important information on SARs

Table 2. Physicochemical properties of peptides **V**–**X**

Peptide		Formula	HPLC t_R (min)	Molecular ion[a]	
				Calculated	$[M+H]^+$
[L-Pip^3]AVP	**V**	$C_{43}H_{65}N_{15}O_{12}S_2$	11.7	1048.2	1048.4
[Mpa^1,L-Pip^3]AVP	**VI**	$C_{43}H_{64}N_{14}O_{12}S_2$	13.3	1033.2	1033.7
[D-Pip^3]AVP	**VII**	$C_{43}H_{65}N_{15}O_{12}S_2$	11.9	1048.2	1048.6
[Mpa^1,D-Pip^3]AVP	**VIII**	$C_{43}H_{64}N_{14}O_{12}S_2$	13.6	1033.2	1033.7
[Mpa^1,L-Pip^3,Val^4,D-Arg^8]VP	**IX**	$C_{43}H_{65}N_{13}O_{11}S_2$	9.6	1004.2	1004.9
[Mpa^1,D-Pip^3,Val^4,D-Arg^8]VP	**X**	$C_{43}H_{65}N_{13}O_{11}S_2$	9.8	1004.2	1005.1

[a] The mass spectra of the peptides were recorded on a MALDI TOF mass spectrometer.

and stability of the L- or D-Pip-containing peptides to standard TFA cleavage conditions.

Experimental Procedures

General

TLC was carried out on silica plates (Merck), and spots were visualised with iodine or ninhydrin. The solvent system used was butan-1-ol/acetic acid/water/ethyl acetate (1 : 1 : 1 : 1, v/v). 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 to analogues **V**–**VIII** and linear gradient from 10 to 60% of solution [B] for 20 min was applied for analogues **IX** and **X** at a flow rate of 1 ml/min. Preparative HPLC was carried out using a Waters C_{18} column (15 µm, 7.8 × 250 mm) in a gradient running from 10 to 50% of [B] for 120 min at a flow rate of 2.5 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) [26] using 3-Mpa instead of L-cysteine hydrochloride.

All the amino acid derivatives were purchased from NovaBiochem, except for Fmoc-L-Pip and Fmoc-D-Pip, which were provided by NeoMPS SA.

Peptide Synthesis and Purification

The synthesis of all the new AVP analogues (**I**–**X**) was carried out 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/HATU/HOAt/NMM (1 : 1 : 1 : 2) in DMF or in a mixture of DMF/1-methyl-2-pyrrolidone (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 [27] or chloranil test [28]. 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 crude products (about 150 µmol of each), which were dissolved in acetic acid (250 ml) and the solutions were diluted with methanol (1500 ml).

The resulting dithiols were oxidatively cyclised with a 0.1 M I_2 in methanol using the standard procedure [29]. The solvents were evaporated under reduced pressure and the residue was dissolved in water and lyophilised. With peptides **V**–**X**, the crude 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. Peptides **V**–**X** were eluted as single peaks. Their purity and identity were determined by HPLC and MALDI TOF mass spectroscopy (molecular ion).

Biological Evaluation

Wistar rats were used in all experiments. Female rats were estrogenised 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 [30–32]. The vasopressor test was performed using phenoxybenzamine-treated male rats [33]. Synthetic OT was used as a standard in uterotonic tests, and synthetic AVP was used in the pressor test. Their biological potency in each test was checked using standard preparations of neurohypophyseal hormones. 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 [34,35] as recently described in detail in Ref. 5 (for details concerning all tests, see Ref. 36).

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