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Folded Structures in Protonated Reduced Dipeptides

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Abstract:Reduced dipeptides with the general formula RCO-Xaa-rXbb-$N^+HR'R''$ (rXbb, reduced analogue of residue Xbb: NH-$C^{\alpha}HR^1$-$C_r H_2$) are shown to adopt a folded conformation in solution and in the solid state. The protonated reduced amide bond is an active proton donor capable of interacting with a peptide carbonyl to give a strong hydrogen bond topologically equivalent to the $i+2$ or $i+3 \rightarrow i$ interaction. The resulting conformation is similar to the γ- or β-turn structure found in peptides and proteins.

Keywords: conformational analysis; crystal structure; folded structures; pseudopeptides; reduced peptides

Because of its resistance against enzymic degradation, the reduced amide bond (CH_2—NH) has been largely used for the design of peptidase inhibitors [1–6] and introduced in bioactive peptide analogues [6–14]. Despite their common use, the reduced peptides have been the subject of only a limited number of conformational NMR [11, 12, 15–18] and theoretical analyses [19, 20]. The structures of some reduced protease-inhibitor complexes have been solved by X-ray diffraction, but the resolution accuracy is rather low [2, 3, 5]. The crystal structures of N^{α}-Z and N^{α}-Boc-Proψ[CH_2—NH]Leu-Gly-NH_2, derived from the oxytocin C-terminal tripeptide and containing a reduced Pro-Leu amide bond, have been reported [21].

In previous investigations we have studied two reduced analogues of the Piv-Pro-Gly-NHR' model dipeptide, containing a C-terminal reduced amide bond [22]. The reduced amide (amine) group, may be protonated at the physiological pH, and we have shown that the protonated reduced amide link is a strong proton-donating group capable of interacting with the acyl carbonyl. The resulting folded structure, resembling the βII-turn in peptides, has been solved by X-ray diffraction when the protonated reduced peptide is associated with the BPh_4^- anion [22, 23].

The present study reports the conformational analysis of similar reduced dipeptides, derived from the Pro-Gly, Pro-Ala, Gly-Ala and Ala-Pro sequences, and having the general formula RCO-Xaa-rXbb-$N^+HR'R''$ (Table 1), where rXbb denotes the reduced amino acid residue NH-$C^{\alpha}HR_t$-C_rH_2. In order that the intramolecular interactions could prevail over the ammonium–anion interactions, the protonated reduced dipeptides have been associated with the weakly polar BPh_4^- or PF_6^- anion [24, 25], and examined by FT-IR and ^{1}H-NMR in organic solution. The crystal structure of Piv-Pro-rAla-N^+HMe_2, BPh_4^- has been solved by X-ray diffraction.

Abbreviations: b, broad; COSY, correlated spectroscopy; d, doublet; DCC, *N,N'*-dicyclohexylcarbodiimide; DCM, dichloromethand; DMA, dimethylacetamide; DMAP, 4-dimethylamino-pyridine; DMSO, dimethylsulphoxide; FT-IR, Fourier-transform infrared spectroscopy; *J*, coupling constant, m, multiplet; NMM, *N*-methylmorpholine; ONp, 4-nitrophenyl; Ph, phenyl; Piv, pivaloyl; rXaa or rXbb, reduced amino acid residue; s, singlet; TOCSY, totally correlated spectroscopy; Xaa or Xbb, amino acid residue; Z, benzyloxycarbonyl.

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Table 1 The Protonated Reduced Dipeptides Investigated with Their Given Codes[a]

Protonated reduced dipeptide	Code	Anion
Piv-Pro-rAla-N^+HMe_2	**PrA**	BPh_4^-
Piv-Pro-rGly-N^+HMe_2[b]	**PrG**	BPh_4^- or PF_6^-
Piv-Pro-rGly-N^+H_2Et[b]	**PrG'**	BPh_4^-
Boc-Gly-rAla-N^+HMe_2	**GrA**	BPh_4^-
Piv-Gly-rGly-N^+H_2Et[c]	**GrG'**	BPh_4^-
Boc-Ala-rPro-N^+H_2Me	**ArP**	BPh_4^-
Piv-rPro-N^+H_2iPr	**rP**	PF_6^-

[a]The letter r preceding the three- or one-letter code denotes the reduced anlogue (NH-$C^\alpha HR_r$-CH_2) of the peptide residue. The rAla and rPro reduced residues have the same absolute *S*-configuration as the cognate L-Ala and L-Pro residues.
[b]Reference [22].
[c]Reference [23].

Crystal structures

The bond lengths and bond angles of the protonated reduced amide group C^α–C_r–N^+–C in **PrA** and **GrG'** are indicated in Figure 1. Due to the absence of electronic conjugation, the C_r–N^+ bond is significantly longer, and both C^α–C_r–N^+ and C_r–N^+–C angles significantly smaller than their homologues in peptides [37]. However, the $C^\alpha \cdots C$ distance of about 3.8 Å between the transoid carbons is unchanged with reference to peptides. The N^+–H site in **PrA** is strongly intramolecularly hydrogen bonded to the Piv-carbonyl, with a short $N^+ \cdots O$ distance of 2.82 Å, in a similar way to the $i+3 \rightarrow i$ interaction typical of the β-turn structure in peptides [38]. The same interaction is also present in the crystal structures of **PrG**, **PrG'** and **GrG'** [22, 23].

All the crystallized protonated reduced dipeptides adopt a folded conformation which, according to the Pro–ψ_1 angle (Table 2), resembles the βII-turn in peptides [38]. Actually, these reduced dipeptides assume two conformations, noted βII^+ and βII^- (Figure 2), differing in the rotational states of the N–C^α and C^α–C_rH_2 bonds ('ϕ_2', 'ψ_2' $\approx 50°,50°$ or $90°,-60°$). Conformation βII^+ places the (rAla)$C^\beta H_3$ group of **PrA** in a pseudo-equatorial orientation with respect to the 10-membered ring (Fig. 3(a)). Both conformations βII^+ and βII^- are present in the crystal structure of **PrG** [22].

There is no intermolecular hydrogen bond in the crystal structure of **PrA**. The (rAla)N-H group is in contact with the BPh_4^- anion as illustrated in Figure 3 with three short $N \cdots C$ distances of 3.38–3.46 Å. In Figure 3 we also note the stacking of the Pro pyrrolidine cycle with one BPh_4^- aromatic ring.

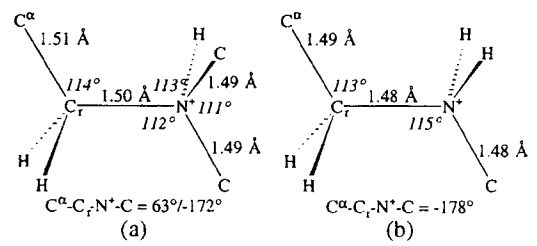


Figure 1 Dimensions of the protonated reduced amide link in **PrA** (a) and **GrG'** (b) [23].

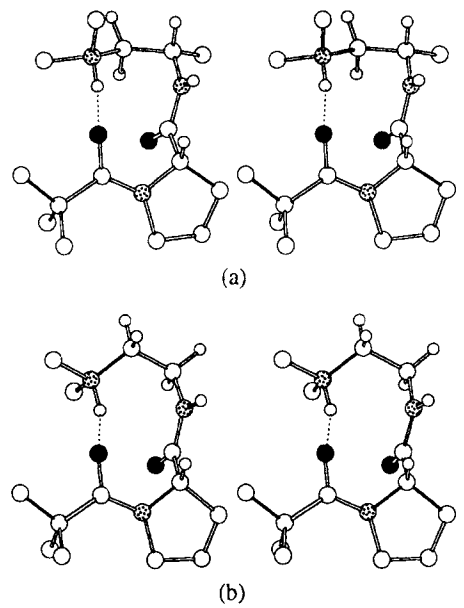


Figure 2 Stereoviews of the folded crystal molecular conformation of **PrA** (a, βII^+-type) and **PrG** (b, βII^--type [22]) stabilized by an N^+—H···O=C interaction closing a 10-membered cycle.

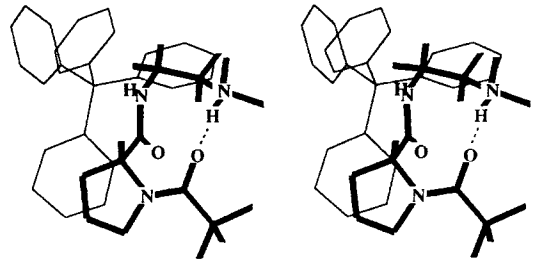


Figure 3 Stereoviews showing the relative disposition of the BPh_4^- anion (thin line) and the protonated reduced dipeptide **PrA** (heavy line) in the crystal.

Table 2 Backbone Torsional Angles (degrees) in the Molecular Structures of Crystallized Protonated Reduced Dipeptides

	PrA[a]	**PrG**[b]		**PrG'**		**GrG'**[c]
Conformation[d]	βII^+	βII^+	βII^-	βII^-	βII^-	βII^-
ω_0	175.6(5)	178	178	−178	180	−176
Pro/Gly						
ϕ_1	−52.4(8)	−53	−53	−58	−48	−53
ψ_1	140.5(5)	136	136	145	141	140
ω_1	−178.2(6)	−171	−171	−173	−177	−167
rAla/rGly[e]						
'ϕ_2'	52.4(8)	41	97	95	99	92
'ψ_2'	41.5(8)	53	−63	−61	−63	−63
'ω_2'	−171.7(6)	−170	167	−176	−177	−178
	62.2(8)	60	−72			

[a]Present work.
[b]Two independent molecules per asymmetric unit [22].
[c]Reference [23].
[d]This folded conformation is denoted with reference to the cognate β-turn type, indicating in superscript the sign of angle 'ψ_2'.
[e]The torsional angles in the reduced rAla/rGly residue are defined as follows: 'ϕ' = C-N-C^α-C_r, 'ψ' = N-C^α-C_r-N^+; 'ω' = C^α-C_r-N^+-C

Conformations in solution

The conformational preferences of such small protonated reduced dipeptides in organic solution is essentially governed by the formation of intramolecular hydrogen bonds, which can be revealed by the shift to lower frequencies of both the N—H and C=O stretching IR absorptions.

PrA and PrG Reduced Peptides

PrG and **PrA**, associated with the BPh_4^- anion, exhibit in DCM very similar IR data (Table 3): (1) the low (Piv)C=O absorption near 1585 cm^{-1}, superimposed on the weak and sharp BPh_4^- aromatic contribution at 1580 cm^{-1}, denotes the participation of this vibrator in a strong hydrogen bond; (2) the (Pro)C=O absorption at 1687 cm^{-1} is typical of a free vibrator; (3) the absence of any absorption at about 3100 cm^{-1}, expected for the N^+—H···BPh_4^- ionic pair, and the presence of a broad and multicomponent absorption band centered at 2755 cm^{-1} are in favour of a N^+—H to (Piv)C=O hydrogen bond; (4) the (Gly/Ala)N—H gives rise to a two or three-component absorption, with one or two minor peaks above 3400 cm^{-1} assigned to the free N—H in different environments [39], and a major peak at about 3360 cm^{-1} due to a weakly perturbed N—H bond. The substitution of PF_6^- for BPh_4^- with **PrG** has no influence on the C=O absorptions, but results in a single sharp peak at 3414 cm^{-1}, indicating that the above absorption at 3360 cm^{-1}, which is not an intrinsic contribution of the BPh_4^- anion, is due to weak N—H···BPh_4^- interaction. Thus the N—H bond is mostly oriented outside of the folded molecule in order to interact with the anion, as in the crystal structure of **PrA** (Figure 3), and the two weak contributions for the free N—H bond in **PrA** probably denote the existence of two other minor conformers with the N—H shielded from the BPh_4^- anion.

Substitution of MeCN for DCM has very little influence on the C=O stretching frequencies (Table 3), indicating that the N^+—H···O=C hydrogen bond is retained in this weakly aprotic solvent. However, the broad (rGly/rAla)N—H absorption near 3355 cm^{-1}, typical of a solvated N—H site, denotes the dissociation of the amide···anion interaction. In DMSO, disruption of the N^+—H···O=C hydrogen bond is illustrated by the low N^+—H frequency, typical of a solvated site, and the high (Piv)C=O frequency assigned to a free vibrator.

The variation with solvent polarity of the proton resonances for the **PrA** $C^\alpha H$-C_rH_2-N^+HMe_2 fragment (Table 4) illustrates the contact of the reduced peptide with the BPh_4^- anion in $CDCl_3$. Moreover, the occurrence of one small and one high vicinal coupling constant (Table 4) is in favour of a highly preferred *gauche* conformation for both $C^\alpha H$-C_rH_2 (only the value 'ψ_2' ~ 60° is compatible with the N^+—H···O=C interaction) and C_rH_2-N^+H ('ω_2' ~ ±60°) fragments in $CDCl_3$ and MeCN-d_3 [34, 36]. The small NH-$C^\alpha H$ coupling constant (J_{MY}) in $CDCl_3$ is compatible with the folded structure

Table 3 N—H, N^+—H and C=O Stretching Frequencies (cm^{-1}) for the Protonated Reduced Dipeptides Associated with the BPh_4^- or PF_6^- Anion in Various Solvents[a]

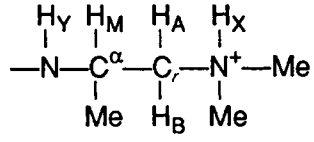
Compound, solvent	Gly/Ala N—H	rAla/rGly N—H	N^+—H	Piv/Boc C=O	Pro/Gly/Ala C=O
PrA, BPh_4^-		3431[w]			
DCM		3416[w]; 3360[s]	*2700*[c]	*1583*	1687
MeCN		3354[b]	*2700*[c]	*1582*	1685
DMSO		3260[b]	2500[bc]	1617	1679
PrG, BPh_4^-					
DCM		3427[w]; *3359*[s]	*2700*[c]	*1585*	1688
MeCN		3359[b]	*2700*[c]	*1583*	1688
DMSO		3265[b]	2500[bc]	1617	1682
PrG, PF_6^-					
DCM		3414[s]	2700[c]	*1584*	1687
GrA, BPh_4^-					
DCM	3448[m]; *3406*[m]	*3365*[s]	3100[w]; *2700*[c]	1721[w]; *1689*[m]	1678
MeCN	3360[b]	3360[b]	*2700*[c]	1717[m]; *1694*[w]	1681
ArP, BPh_4^-					
DCM	3435[s]		*3000*[c]	1710; *1626*	
MeCN	3427[w]; 3384[b]		*3000*[c]	1711	1648[w]; *1628*[m]
DMSO	3250[b]		2500[bc]	1702	1650[s]
rP, PF_6^-					
DCM			3245[w]; *3000*[c]	1602[m]; *1584*[m]	

[a]Strong (s), medium (m) and weak (w) absorptions. The frequencies in roman and italics denote free and intramolecularly hydrogen bonded vibrators, respectively.
[b]Absorption of a solvated vibrator.
[c]Average frequency for a multicomponent absorption band.

observed in the crystal, with a cisoid disposition ('ϕ' ~ 60°) of the NH-C$^\alpha$H fragment (Figure 2) [33]. The increase of J_{MY} in the ionic pair-breaking solvent MeCN-d_3 (Table 4) denotes the appearance of some NH-C$^\alpha$H transoid conformer. In DMSO-d_6, the magnetic equivalence of the C_rH_2 protons supports a flexible C$^\alpha$H-C_rH_2 fragment, and therefore an open structure of **PrA** in this strong solvating medium.

The folded structures of **PrA** and **PrG** have been investigated by SYBYL molecular dynamics simulation with substitution of an acetyl for the pivaloyl group. The simulation was started from the extended structure for 200 ps at 400 K, generating 200,000 conformers which cover the whole conformational space accessible to these small molecules. After the first 20 ps for stabilization of the temperature at 400 K, one of every 20 conformers set was retained for 0.02 ps intervals, and among the 9000 conformers generated, we have considered those having a short $(N^+)H\cdots O(Ac)$ distance (< 2.5 Å), typical of a N^+—H···O=C hydrogen bond, and a molecular energy of less than 10 kcal above the

Table 4 NMR Data for the NH—C$^\alpha$H—C_rH_2—NH^+ System in **PrA**



	Chemical shifts (p.p.m.)					Coupling constants (Hz)					
Solvent	δ_A	δ_B	δ_M	δ_X	δ_Y	J_{AM}	J_{BM}	J_{AB}	J_{AX}	J_{BX}	J_{MY}
$CDCl_3$	3.21	2.76	3.70	8.05	5.32	11.8	3.5	12.5	1.5	9.5	6.5
MeCN-d_3	3.22	3.04	4.10	8.04	6.72	11.4	3.9	12.9	1.5	9.3	7.3
DMSO-d_6	3.09		4.10	8.82	7.93	6.7		–	[a]	[a]	8.2

[a]Not visible.

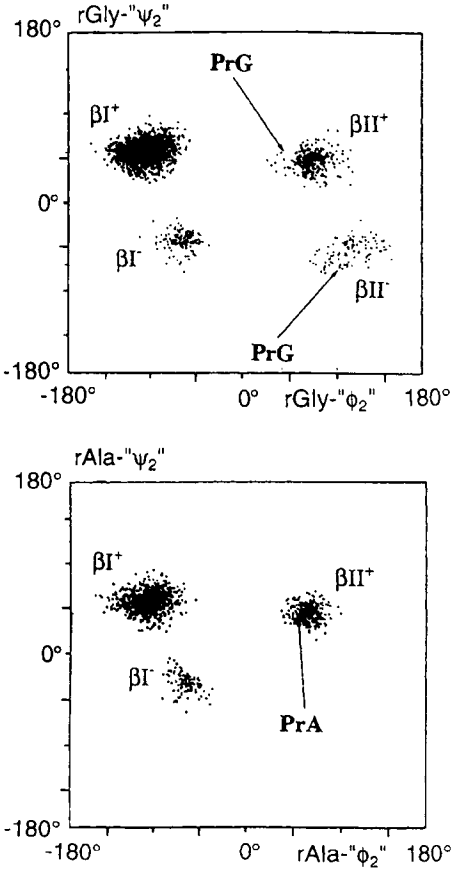


Figure 4 rGly/rAla-'ϕ_2', 'ψ_2' Ramachandran map showing the different conformers folded by an N^+—H···O=C hydrogen bond, and generated by SYBYL molecular dynamics for Ac-Pro-rGly-N^+HMe_2 (upper) and Ac-Pro-rAla-N^+HMe_2 (lower). The conformations of the rGly and rAla residues in the crystal structures of **PrG** and **PrA** are indicated for comparison.

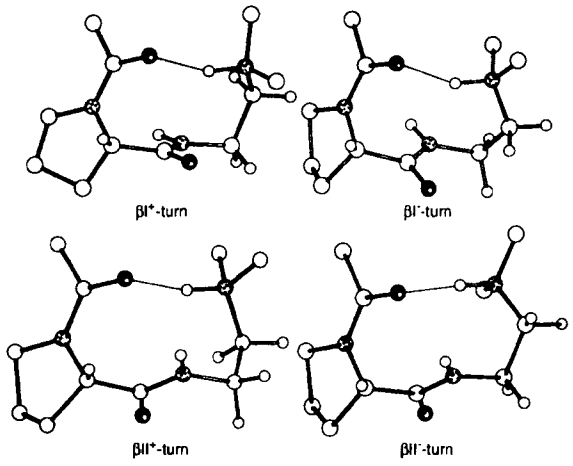


Figure 5 Four β-like folded conformers of Ac-Pro-rGly-N^+HMe_2 presenting an N^+—H···O=C hydrogen bond of the $i+3 \rightarrow i$ type (SYBYL minimization).

absolute minimum. The Ramachandran maps in Figure 4 show the existence of four folded conformers for **PrG**, and only three for **PrA**, differing in the sign of the rGly/rAla 'ψ_2' angle, and in the orientation of the Pro-rXaa amide plane. In Table 5 are listed the main conformational data for the energy minimized folded conformers (Figure 5), which are denoted with reference to the cognate β-turn with the sign of the 'ψ_2' angle indicated in superscript. The sterical hindrances due to the (rAla)$C^{\beta}H_3$ side chain with the Pro oxygen and one of the N^+Me_2 methyl groups is responsible for the absence of the βII^- folded structure for **PrA**.

For both **PrG** and **PrA**, the βI^+-turn is the most frequent folded form according to SYBYL simulation

Table 5 Conformational Data According to Molecular Simulation for the Folded Structures of Two Reduced Peptides with the Pro-rGly and Pro-rAla Sequences

Compound	Pro ϕ	Pro ψ	rGly/rAla 'ϕ'	rGly/rAla 'ψ'	H···O (Å)	Energy (kcal)	[a]
Ac-Pro-rGly-N^+HMe_2							
βI^+	−64	−23	−104	53	1.79	6.2	1250
βI^-	−66	−20	−66	−40	1.78	6.7	138
βII^+	−65	106	75	45	1.75	8.2	230
βII^-	−66	97	128	−49	1.76	8.5	102
Ac-Pro-rAla-N^+HMe_2							
βI^+	−49	−35	−101	57	1.73	5.6	788
βI^-	−65	−30	−59	−40	1.79	8.2	133
βII^+	−50	131	60	42	1.77	7.4	255

[a]Number of folded conformers in each family among the 9000 conformers retained after molecular dynamics, and presenting a short H···O distance (< 2.5 Å) (see Figure 4).

(Figure 4) whereas only βII^+ (**PrA** and **PrG**) and βII^- (**PrG**, **PrG'** and **GrG'**) are observed in the solid state (Table 2). The simulation was carried out disregarding the peptide···anion interaction which requires the outer orientation of the amide N—H as in βII^- and βII^+. Under these conditions, the inner orientation of the N—H bond as in βI^+ (Figure 5) becomes favoured, exactly as in the ion pair dissociating solvent MeCN, where the increase of the NH-$C^{\alpha}H$ coupling constant for **PrA** (Table 4) probably reflects the transition from a cisoid (βII^+) to a transoid (βI^+) arrangement of the rAla NH-$C^{\alpha}H$ system.

GrA Reduced Dipeptide

The Gly-Ala sequence is not frequently found in β-turns [38], and only one-third of the Piv-Gly-Ala-NHMe molecules is β-folded in $CHCl_3$ [40]. The IR spectrum of **GrA** in DCM exhibits three C=O and three N—H contributions (Table 3). The weak N^+—H contribution at 3100 cm^{-1}, compensated by a broad and multicomponent absorption band about 2700 cm^{-1}, and the double absorption of (Boc)C=O at 1721 and 1689 cm^{-1}, denote a conformational equilibrium between an open conformer (35% occurrence) and a folded conformer (65% occurrence) presenting a N^+—H···O=C(Boc) hydrogen bond. All the other frequencies are typical of free vibrators, except the absorption at 3365 cm^{-1} due to the (rAla)N—H···$BPhPro_4^-$ interaction, also responsible for the shielded (Gly)$C^{\alpha}H_2$ (3.33 p.p.m.) and (rAla)$C^{\alpha}H$ proton (3.71 p.p.m.) resonances in $CDCl_3$, compared with 3.64 and 4.27 p.p.m., respectively, in MeCN-d_3. In MeCN, the relative peak intensities of the (Boc)C=O absorptions (65/35 in favour of the free contribution) are inverted with reference to the $CHCl_3$.

rP and ArP Reduced Peptides

The Xaa-Pro peptide sequences are not frequently β-folded, except in βVI-turns where the Xaa-Pro amide bond adopts a *cis* disposition [3, 41, 42]. Both **rP** (Piv)C=O and N^+—H vibrators exhibit two double absorptions in DCM (Table 3), denoting the occurrence of an intramolecular N^+—H···O=C interaction, closing a seven-membered cycle, in half of the molecules. Under the same conditions, the cognate Piv-Pro-NHiPr molecule exhibits only a very small percentage of the N—H···O=C interaction of the same $i+2 \rightarrow i$ type [43]. The high (Boc)C=O absorption at 1710 cm^{-1} for **ArP** in DCM, typical of a free vibrator, excludes the possibility of a β-like folded

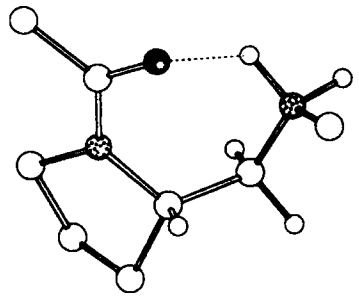


Figure 6 γ-like folded conformation of Ac-rPro-N^+H_2Me presenting an N^+—H···O=C hydrogen bond of the $i+2 \rightarrow i$ type (SYBYL minimization).

structure (Table 3). On the other hand, the low (Ala)C=O and N^+—H stretching frequencies demonstrate that these sites are hydrogen-bonded in DCM (Table 3). Contrary to the cognate peptide Piv-Ala-Pro-NHMe [44], no *cis* Ala-rPro amide bond is detected for **ArP** in $CDCl_3$. It follows that both **rP** and **ArP** adopt in DCM the same γ-like folded structure stabilized by a N^+—H···O=C hydrogen bond closing a seven-membered cycle [38], which imposes the *trans* disposition on the rPro-preceding amide bond (Figure 6).

This structure, similar to that adopted by the rPro residue in the crystallized neutral reduced tripeptide Boc-rPro-Leu-Gly-NH_2 [21], is essentially retained in MeCN where a small contribution of the free (Ala)C=O carbonyl appears at 1648 cm^{-1}, and where 30% of the Ala-rPro amide bonds adopt the *cis* conformation. The γ-like folded structure disappears completely by solvation in DMSO (Table 3).

GENERAL CONSIDERATIONS

In order to investigate the possible influence of a reduced amide bond in a peptide chain, we have considered reduced analogues of peptides known to adopt preferentially the γ- or β-turn structure, where the reduced amide bond has been introduced at the C-terminal position. Owing to the pK_a value of the reduced amide (amine) group, the reduced analogues have been studied in their protonated form associated with a bulky and weakly polar anion in order to minimize the peptide···anion interactions.

Although the protonated reduced amide group $C^{\alpha}C_r$–N^+–C^{α} seems to prefer the transoid conformation, the absence of electronic conjugation cannot exclude the possibility of a *gauche* disposition. Nevertheless, the transoid form exhibits overall dimensions quite similar to those of the peptide

unit. On these bases, it appears to mimic the amide transient state during enzymatic cleavage more closely than the phosphinic (PO_2^-—CH_2) [45], sulphinamide (SO—NH) [46] or sulphonamide (SO_2—NH) [47] group.

The protonated reduced amide group is a very active proton donor engaged in short contacts with the peptide carbonyls. Hydrogen bonds of the $i+2 \rightarrow i$ and $i+3 \rightarrow i$ types, giving rise to γ- and β-like folded structures, respectively, have been shown in the present work. The former is especially favoured with the reduced rPro residue, and it appears to be more stable against solvation than the homologous γ-turn in peptides. The latter is also more stable than the β-turn for the cognate peptide sequence, but assumes different conformations differing both in the orientation of the central amide bond and the g^+ or g^- disposition of the N-C^α-C_r-N^+ system. The type of the β-like folded structure, i.e. the orientation of the middle amide bond, not only depends on the sequence, but also on eventual intermolecular interactions such as the peptide $\cdots$anion interaction in the present case.

It is highly probable that the preferential interactions of the ammonium group would depend on the environment, and particularly on the presence and the nature of an anionic counterpart. We have shown that a negative charge distributed on a large surface by electronic conjugation is not tightly bound to the ammonium group. On the contrary, we can envisage that a negative charge located on a limited site, creating a strong local electrical field, would allow stronger ionic interactions than in the present case.

EXPERIMENTAL PART

Synthesis

The reduced dipeptides we have prepared are listed in Table 1 with their abbreviated code derived from the one-letter code. The pivaloyl group has been used to prevent *cis/trans* isomerization of the tertiary Pro-preceding amide bond [26] and the N^+HMe_2 terminus, having a single N^+–H bond, to give easily interpretable IR and NMR data. The BPh_4^- anion has been introduced by anion exchange between the reduced peptide hydrochloride salt and $Na^+BPh_4^-$ (Fluka) in water where the reduced peptide BPh_4^- salt precipitates. The PF_6^- salt has been obtained by lyophilizing a water solution of the neutral reduced peptide and hexafluorophosphoric acid diethyloxide (Aldrich). The neutral reduced peptides are stable, but often obtained as an oily, more or less carbonated product. The PF_6^- and BPh_4^- reduced peptide salts are more stable in the solid state than in solution where the rapid evolution with time of their IR and NMR data denotes a chemical instability preventing long-term NMR experiments.

The syntheses of **PrG**, **PrG'** and **GrG'** have been already reported [22, 23]. **GrA** has been prepared from Boc-Gly-ONp and the racemic NH_2-CHMe-CH_2-NMe_2 diamine (Fluka) abbreviated as H-DL-rAla-NMe_2. Coupling of Piv-Pro-OH and H-DL-rAla-NMe_2, using the mixed anhydride with isobutyl chloroformate, gave a mixture of the two Piv-L-Pro-L-rAla-NMe_2 and Piv-L-Pro-D-rAla-NMe_2 diastereoisomers. The former (**PrA**) eluted first by flash-chromatography with ethanol on silica gel, and its stereochemistry was validated by X-ray crystallography (see below).

Boc-Gly-rAla-NMe$_2$. Racemic oil, $R_F = 0.12$ (EtOH). ^{1}H-NMR ($CDCl_3$): 1.18 (d, rAla-$C^\beta\underline{H}_3$, $J = 6.5$ Hz); 1.45 (s, Boc-$(C\underline{H}_3)_3$); 2.21 (s, $N^+(C\underline{H}_3)_2$); 2.35 (A) and 2.18 (B) (ABX, rAla-$C_r\underline{H}_2$, $J_{AB} = 12.4$ Hz, $J_{AX} = 9.4$ Hz, $J_{BX} = 5.5$ Hz); 3.77 (d, Gly-$C^\alpha\underline{H}_2$, $J = 5.7$ Hz); 3.97 (m, rAla-$C^\alpha\underline{H}$); 5.32 (d, rAla-N$\underline{H}$); 6.38 (t, Gly-N$\underline{H}$). IR (film): 1710 cm^{-1} (Boc-*CO*); 1645 cm^{-1} (Gly-*CO*).

Boc-Gly-rAla-N$^+$HMe$_2$, BPh$_4^-$ (GrA). Racemic, ^{1}H-NMR (MeCN-d_3): 1.18 (d, rAla-$C^\beta\underline{H}_3$, $J = 6.8$ Hz); 1.42 (s, Boc-$(C\underline{H}_3)_3$); 2.85 (s, $N^+(C\underline{H}_3)_2$); 3.07 (d, rAla-$C_r\underline{H}_2$, $J = 6.7$ Hz); 3.64 (d, Gly-$C^\alpha\underline{H}_2$, $J = 5.7$ Hz); 4.27 (m, rAla-$C^\alpha\underline{H}$); 5.72 (b, Gly-N$\underline{H}$); 6.80–7.32 (m, $B(C_6\underline{H}_5)_4$ + rAla-N*H* + N^+H). IR (MeCN): 1681 cm^{-1} (Ala-*CO*); 1717 cm^{-1} (Boc-*CO*); 2700 cm^{-1} (N^+H); 3360 cm^{-1} (Gly-*NH* + rAla-*NH*).

Piv-Pro-rAla-NMe$_2$. Melting point m.p. = 82°C, $R_F = 0.23$ (EtOH) and 0.36 (MeOH/DCM 40/60 v/v). ^{1}H-NMR ($CDCl_3$): 1.14 (d, rAla-$C^\beta\underline{H}_3$, $J = 6.5$ Hz); 1.27 (s, Boc-$(C\underline{H}_3)_3$; 1.80–2.26 (m, Pro-$C^\beta\underline{H}_2 + C^\gamma\underline{H}_2$); 2.22 (s, $N^+(C\underline{H}_3)_2$); 2.36 (A) and 2.16 (B) (ABX, rAla-$C_r\underline{H}_2$, $J_{AB} = 12.2$ Hz, $J_{AX} = 5.9$ Hz, $J_{BX} = 8.9$ Hz); 3.70 (m, Pro-$C^\delta\underline{H}_2$); 3.91 (m, rAla-C^α*H*); 4.58 (m, Pro-$C^\alpha\underline{H}$); 6.69 (d, rAla-N$\underline{H}$, $J = 4.4$ Hz). IR (KBr): 1625 cm^{-1} (Piv-*CO*); 1665 cm^{-1} (Pro-*CO*).

Piv-Pro-rAla-N$^+$HMe$_2$,BPh$_4^-$ (PrA). $[\alpha]_D^{20} = -10.8$ ($c = 1$, MeOH), ^{1}H-NMR (MeCN-d_3): 1.21 (d, rAla-$C^\beta\underline{H}_3$, $J = 7.1$ Hz); 1.25 (s, Boc-$(C\underline{H}_3)_3$); 1.70–2.32

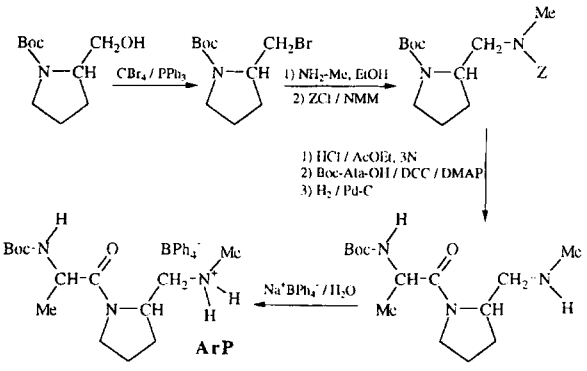


Figure 7 Synthesis of **ArP** by the nucleophilic substitution procedure.

(m, Pro-C$^{\beta}\underline{H}_2$ + C$^{\gamma}\underline{H}_2$); 2.87 (s, N$^+$(C$\underline{H}_3$)$_2$); 3.22 (A), 3.04 (B) (ABX, rAla-C$_r\underline{H}_2$, J_{AB} = 12.9 Hz, J_{AX} = 3.8 Hz, J_{BX} = 11.8 Hz); 3.75 (m, Pro-C$^{\delta}\underline{H}_2$); 4.00–4.35 (m, Pro-C$^{\alpha}\underline{H}$ + rAla-C$^{\alpha}\underline{H}$); 6.72 (d, rAla-N$\underline{H}$, J = 7.3 Hz); 6.75–7.20 (m, B(C$_6\underline{H}_5$)$_4$); 8.04 (b, N$^+\underline{H}$). IR (MeCN): 1617 cm^{-1} (Piv-*CO*); 1679 cm^{-1} (Pro-*CO*); 2700 cm^{-1} (*N*$^+\underline{H}$); 3354 cm^{-1} (rAla-*N*$\underline{H}$).

rP was prepared by reductive animation of the racemic Boc-prolinal [27], and **ArP** by nucleophilic substitution (Figure 7) of Boc-rPro-Br, obtained by bromination of Boc-prolinol (Merck) [28]. In both cases, the temporary Z-protection of the reduced amide function was required to prevent further undesirable acylation.

Piv-rPro-NHiPr. Racemic oil. R_F = 0.20 (iPrOH/DCM 10/90 v/v). ^{1}H-NMR (CDCl$_3$): 1.32 (m, Piv-(C$\underline{H}_3$)$_3$ + iPr-(C$\underline{H}_3$)$_2$); 1.78 (m, rPro-C$^{\beta}\underline{H}$); 1.92–2.09 (m, Pro-C$^{\gamma}\underline{H}_2$); 2.22 (m, rPro-C$^{\beta}\underline{H}$); 3.15 (A) and 2.93 (B) (ABX, rPro-C$_r\underline{H}_2$, J_{AB} = 12.4 Hz, J_{AX} = 3.5 Hz, J_{BX} = 6.8 Hz); 3.18 (m, iPr-C$\underline{H}$); 3.60 and 3.87 (m, rPro-C$^{\delta}\underline{H}_2$); 4.36 (m, rPro-C$^{\alpha}\underline{H}$); 7.66 (d, iPr-N$\underline{H}$)). IR (film): 1625 cm^{-1} (Piv-*CO*).

Piv-rPro-N$^+$H$_2$iPr, PF$_6^-$ (rP). Racemic. ^{1}H-NMR (CDCl$_3$/DMSO-d$_6$ 90/10): 0.94 (s, Piv-(C$\underline{H}_3$)$_3$); 0.98 and 1.00 (2d, iPr-(C$\underline{H}_3$)$_2$, J = 6.4 Hz); 1.25–1.92(m, rPro-C$^{\beta}\underline{H}_2$ + C$^{\gamma}\underline{H}_2$); 2.70 (m, rPro-C$_r\underline{H}_2$); 3.26 and 3.51 (m, rPro-C$^{\delta}\underline{H}_2$); 3.72 (m, iPro-C$\underline{H}$); 3.85 (m, rPro-C$^{\alpha}\underline{H}$); 8.46 and 8.76 (b, N$^+\underline{H}_2$). IR (KBr): 1595 cm^{-1} (Piv-*CO*); 2800 cm^{-1} (N$^+$*H*$_2$).

Boc-Ala-rPro-NHMe. Oil. R_F = 0.32 (iPrOH/DCM 7/93 v/v). ^{1}H-NMR (CDCl$_3$): 1.35 (d, Ala-C$^{\beta}\underline{H}_3$, J = 7.0 Hz); 1.41 (s, Boc-(C$\underline{H}_3$)$_3$); 1.60–2.40 (m, rPro-C$^{\beta}\underline{H}_2$ + rPro-C$^{\gamma}\underline{H}_2$ + Me-N$\underline{H}$); 2.73 (s, N-C$\underline{H}_3$); 2.92–3.21 (m, rPro-C$_r\underline{H}_2$); 3.55 and 3.81 (m, rPro-C$^{\delta}\underline{H}_2$); 4.46 (m, rPro-C$^{\alpha}\underline{H}$) + Ala-C$^{\alpha}\underline{H}$); 5.56 (d, Ala-N$\underline{H}$), J = 7.9 Hz. IR (film): 1710 cm^{-1} (Boc-*CO*); 1645 cm^{-1} (Ala-*CO*).

Boc-Ala-rPro-N$^+$H$_2$Me, BPh$_4^-$ (ArP). $[\alpha]_D^{20}$ = − 32.6 (c = 1, MeOH), ^{1}H-NMR (MeCN-d$_3$): *trans* (t)/*cis* (c) 70/30; 1.24 (c) and 1.26 (t) (d, Ala-C$^{\beta}\underline{H}_3$, J = 6.8 Hz); 1.38 (t) and 1.44 (c) (s, Boc-(C$\underline{H}_3$)$_3$); 1.60–2.38 (m, rPro-C$^{\beta}\underline{H}_2$ + C$^{\gamma}\underline{H}_2$); 2.61 (c) and 2.63 (t) (s, N$^+$-C$\underline{H}_3$); 3.04 (m, rPro-C$_r\underline{H}_2$); 3.47 and 3.75 (m, rPro-C$^{\delta}\underline{H}_2$); 4.13 (m, rPro-C$^{\alpha}\underline{H}$); 4.37 (m, Ala-C$^{\alpha}\underline{H}$); 5.58 (t) and 5.81 (c) (b, Ala-N$\underline{H}$); 6.71–7.80 (m, B(C$_6\underline{H}_5$)$_4$ + N$^+\underline{H}_2$).

X-ray Diffraction

Single crystals of **PrA** were grown by cooling an AcOEt solution (orthorhombic, $P2_12_12_1$, a = 11.322 (1) Å, b = 14.222(2) Å, c = 22.002(3) Å, Z = 4, calculated density 1.15 g/cm^3). The X-ray diffraction data were collected on an Enraf Nonius CAD-4 four-circle diffractometer in the $\omega/2\theta$-scan mode. Reflections numbering 3788 in total, of which 2212 were observed with $I > \sigma(I)$, were measured at room temperature in the 1–70° θ range using Cu–Kα radiation (λ = 1.54178 Å) monochromatized by a graphite crystal. During data collection, two standard reflections were measured every 2 h to check the stability of the crystal. Intensities were corrected for Lorentz and polarization effects but no absorption correction was applied. The crystal structure was solved by direct methods using SHELXS 90 [29]. The E-maps revealed the whole molecule except the hydrogen atoms, and the structure was refined through the least-squares procedure with the complete matrix of normal equations [30]. Heavy atoms were affected by anisotropic thermal factors, and hydrogen atoms were located on E-map differences and affected by an isotropic thermal factor of 4 Å^2. The residual R factors are R = 0.056 and R_w = 0.057 [$w = 0.056/[\sigma^2(F) + 0.00084\ F^2]$, with largest difference peak and hole of 0.19 and − 0.16 e/Å^3, respectively. The NH hydrogen atoms were placed 1.03 Å from N in the direction obtained by refinement [31].

FT-IR and ^{1}H-NMR Spectroscopy

IR spectra were run in the Fourier transform mode on a Bruker IFS-25 apparatus using a cell path of 0.5 mm in order to investigate the N—H (3200–3500 cm^{-1}), C=O (1580–1720 cm^{-1}) and N$^+$—H (2500–3300 cm^{-1}) stretching frequencies in DCM, MeCN and DMSO. The peptide concentration was

0.005 M, and further dilution confirmed the absence of any molecular aggregation. The N—H and C=O stretching frequencies were assigned on the basis of previous studies on peptides [32] and ammonium groups [24, 25]. The free amide N—H is expected to give a sharp absorption at 3400–3450 cm^{-1}, and the free (Piv)C=O at 1620–1625 cm^{-1} in DCM. When engaged in an intramolecular hydrogen bond, their absorption is shifted to lower frequencies by 100–200 cm^{-1} and 10–25 cm^{-1}, respectively. The N^+H and N^+H_2 stretching frequencies greatly depend both on the solvent and on the associated anion. In a weakly polar solvent such as DCM, the N^+H bond gives rise to a sharp peak at about 3250 or 3100 cm^{-1} when associated with the PF_6^- or BPh_4^- anion, respectively, which turns into a multicomponent and broad absorption shifted down to 2500–3000 cm^{-1} upon formation of an $N^+—H\cdots O=C$ hydrogen bond in DMA [25]. The same is true for the N^+H_2 cation which absorbs at 3245 or 3140 cm^{-1} in DCM when associated with the PF_6^- or BPh_4^- anion, respectively. The N^+H_2 cation also presents a very weak absorption at 1596 cm^{-1} in DCM. Both the PF_6^- or BPh_4^- anions have no visible absorption in the 2200–2800 and 3150–3600 cm^{-1} regions, but BPh_4^- exhibits a weak and sharp aromatic contribution at 1580 cm^{-1}.

^{1}H-NMR spectra were run on a Bruker AC-200P apparatus with Me_4Si as internal reference, and spin systems have been solved by COSY and TOCSY experiments. The vicinal coupling constants J in the NH-C^αH-C_rH_2-N^+H moiety have been exploited in terms of torsional angles from the Karplus correlations adapted to the NH-C^αH [33] and C^αH-$C^\beta H_2$ fragments [34], provided the electronegativity correction J (corrected) = 1.06 J (observed) is applied [35], and to the CH_2-N^+H fragment [36].

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