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A Type-II β-Turn, Proline-containing, Cyclic Pentapeptide as a Building Block for the Construction of Models of the Cleavage Site of Pro-oxytocin

MONICA DETTIN[a], LUCIA FALCIGNO[b], TIZIANO CAMPANILE[b], CLAUDIA SCARINCI[a], GABRIELLA D'AURIA[b], MARINA CUSIN[a], LIVIO PAOLILLO[b] and CARLO DI BELLO[a,*]

[a] Department of Chemical Process Engineering, University of Padova, 35131 Padova, Italy
[b] Department of Chemistry, University of Naples, 80134 Naples, Italy

Abstract: Previous studies have indicated that proteolytic activation of pro-hormones and pro-proteins occurs most frequently at the level of basic amino acids arranged in doublets and that the dibasic sites are situated in or next to β-turns. Investigations utilizing synthetic peptides reproducing the *N*-terminal processing domain of pro-oxytocin-neurophysin have suggested a close relationship between the secondary structure of the cleavage locus and enzyme recognition, the minimal recognized sequence being the -Pro-Leu-Gly-Gly-Lys-Arg-Ala-Val-Leu- segment of the native precursor. NMR investigations and energy minimization studies have demonstrated that this sequence is organized in two type-II β-turns involving the -Pro-Leu-Gly-Gly- and -Lys-Arg-Ala-Val- sequences. To further strengthen the above reported hypothesis and to study the role of turn subtypes, a new proline containing cyclic substrate of the processing enzyme, in which the *N*-terminal side that comes before the Lys-Arg pair is constrained to adopt a type-II β-turn, has been synthesized. The presence of a type-II β-turn structure in this cyclic peptide model has been demonstrated by a combined NMR, CD and FT-IR absorption investigation. A preliminary study shows that PC1 is able to recognize and process our constrained substrate.

Keywords: cyclic peptide; peptide conformation; peptide synthesis; pro-oxytocin; β-turn

INTRODUCTION

Secretory peptides are generated from larger precursor proteins by proteolytic cleavage at sites consisting of one or more basic amino acids. Analyses of sequences flanking the basic residues indicate that these segments might participate in the recognition of the cleavage loci by providing accessible zones constituted by β-turns [1] or Ω-loops [2]. The presence of a β-turn at the *N*-terminus of the dibasic cleavage site is a recurrent conformational motif in several peptides of different length used as substrates for pro-oxytocin-neurophysin convertases and, consequently, it was concluded that the

Abbreviations: Abbreviations used for amino acids follow the recommendations of the IUPAC-IUB Commission of Biochemical Nomenclature, *Eur. J. Biochem.* 1984; **138:** 9–37. The following additional abbreviations are used: γAbu, γ-aminobutyric acid; δAva, δ-aminovaleric acid; Boc, *tert*-butyloxycarbonyl; CD, circular dichroism; CVFF, consistent valence force field; DCM, dichloromethane; DIEA, diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethylsulphoxide; DQFCOSY, double quantum filtered correlated spectroscopy; EDT, ethanedithiol; FID, free induction decay; Fmoc, 9-fluorenylmethoxycarbonyl; FT-IR, Fourier-transform infrared; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; MALDI, matrix assisted laser desorption ionization; MBHA, methylbenzhydramine; MeCN, acetonitrile; NMM, *N*-methylmorpholine; NMP, *N*-methylpyrrolidone; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhancement spectroscopy; OAl, allyloxy; Pmc, 2,2,5,7,8-pentamethyl-chroman-6-sulfonyl; RMD, restrained molecular dynamics; r.m.s.d., root mean square deviation; ROESY, rotating frame nuclear Overhauser enhancement correlation spectroscopy; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; TMS, tetramethylsilane; TSP, 3-(trimethylsilyl) sodium propionate; TOCSY, total correlation spectroscopy.

* Correspondence to: Department of Chemical Process Engineering, Via Marzolo 9, 35131 Padova, Italy.

β-turn situated on the *N*-terminus of the dibasic cleavage site is an important feature in the processing enzyme recognition [3,4].

Previous studies on synthetic peptides have demonstrated that the minimal recognized sequence is -Pro-Leu-Gly-Gly-Lys-Arg-Ala-Val-Leu-(-Pro^7-Leu^{15}-). In addition, NMR investigations and energy minimization studies have shown that the -Pro^7-Leu^{15}- folded peptide population is organized in two type-II β-turns [5] (-Pro^7-$G1y^{10}$- and -Lys^{11}-Val^{14}- sequences) that locate the two side chains of Lys and Arg on the same side of the molecule [4].

To further strengthen the above reported hypotheses and to study the role of the β-turn subtypes, we have now synthesized a novel substrate for the processing enzyme in which the *N*-terminal side of the Lys-Arg doublet is constrained to adopt a type-II β-turn. The selected model is derived from a Pro-containing homodetic cyclopeptide of sequence -δAva-Gly-Pro-Gly-Gly- for which NMR, CD and FT-IR absorption investigations have evidenced the presence of a type-II β-turn structure involving the sequence -Gly-Pro-Gly-Gly-, not only in organic solvents but also in aqueous solution [6]. The original model peptide proposed by Fasman and coworkers has been modified by addition of the -Lys-Arg-Ala-Val-Leu- sequence to the peptide chain. To this purpose, in our peptide model the Gly^{i+3} residue has been replaced by an Asp residue. The side chain of the Asp residue maintains the cyclic structure whereas the α-carboxylic function is used to extend the main chain. In addition, the original δAva residue has been replaced by a γAbu residue in order to maintain the dimension of the cyclic structure.

The CD, FT-IR absorption and NMR investigations presented in this paper demonstrate that the *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-NH_2 peptide (Figure 1) adopts a $1 \leftarrow 4$ hydrogen-bonded type-II β-turn conformation in solution. Consequently we propose the *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-NH_2 as a type-II β-turn model for the insertion of such a secondary structure into peptide chains. Accordingly, this type-II β-turn model has also been used in the synthesis of a longer peptide, namely *cyclo*(1-5β)γAbu - Gly - Pro - Gly - Asp - Lys - Arg - Ala - Val - Leu-NH_2, whose CD and NMR conformational characterization is also presented here.

MATERIALS AND METHODS

Materials

Fmoc-protected amino acids and resins were obtained from Novabiochem (Langelfingen, Switzerland); 0.45 M HBTU/HOBt solution in DMF from Applied Biosystems (Perkin–Elmer, Norwalk, CT); MeCN, NMP, DCM and TFA from Janssen (Geel, Belgium); piperidine, DIEA, 1,2-ethandithiol, TSP sodium salt, TMS, DMSO-d_6 and thioanisole from Aldrich (Deisenhofen, Germany); TFE-d_3 from Cil (Woburn, MA).

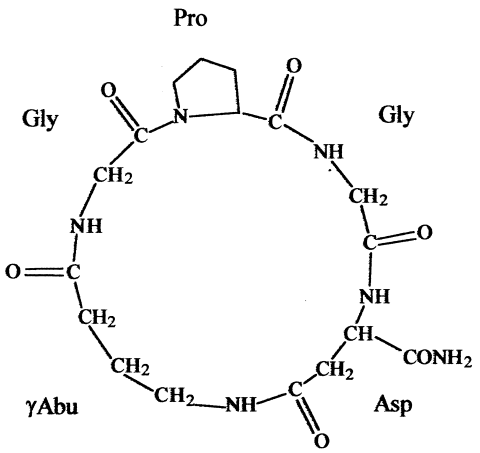

Figure 1 Formula of *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-NH_2.

Synthesis of *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-NH_2

The linear peptide sequence was synthesized by the solid-phase method, using a fully automated peptide synthesizer (Applied Biosystems model 431A). Rink-MBHA resin (0.466 g, 0.47 mmol/g) and HBTU as coupling agent were used. The side-chain protection used for Asp was the O*t*Bu group. After each single coupling step, a resin sample was submitted to a quantitative ninhydrin test; the extent of coupling resulted over 99.6% for each step. The Fmoc group of the last residue was deprotected. Cleavage from the resin and the side-chain deprotection were achieved treating 500 mg of peptide-resin with 0.5 ml of H_2O and 9.5 ml of TFA for 90 min. After concentration, the peptide was precipitated with cold diethyl ether.

Crude H-γAbu-Gly-Pro-Gly-Asp-NH_2 (0.13 mmol) was combined with 1.5 eq. of HATU and 3 eq. of DIEA in DMF at a concentration of 1×10^{-3} M. The solution was stirred for 24 h. The reaction mixture was evaporated to dryness and the residue was dissolved in 18 ml of water. Half solution was directly loaded onto a semi-preparative Delta Pak C_{18} column (15 μm, 100 Å, 7.8×300 mm Waters). The

mobile phase was 0.05% TFA/H_2O and the elution was isocratic at a flow rate of 4 ml/min. A total of 11 mg of purified peptide was obtained. The final analytical chromatogram on a Vydac C_{18} column (5 μm, 100 Å, 4.6 × 250 mm) gave a 90% of purity grade under the following conditions: eluant A, 0.05% TFA in water; eluant B, 0.05% TFA in MeCN; flow rate, 1 ml/min; gradient, 0–15% B over 30 min; detector, 214 nm. The identity of the product was confirmed by MALDI spectrometric analysis (theoretical value: 410 Da; experimental value: 410 Da).

cyclo(1-5β)γAbu-Gly-Pro-Gly-Asp-Lys-Arg-Ala-Val-Leu-NH_2

The first synthetic protocol for *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-Lys-Arg-Ala-Val-Leu-NH_2 was designed to maximize the number of reactions on the peptide still anchored to the solid support (Figure 2(A)). The aim was to ensure easy separation of the impurities from the peptide at the end of each reaction. Unfortunately, this protocol did not give satisfactory results, probably due to the incomplete deprotection of the Asp side chain from the OAl group.

The second protocol, reported in Figure 2(B) and here described in detail, gave the target peptide. The fully protected peptide was synthesized by the solid-phase method, using the above reported automated peptide synthesizer and 0.25 mmol of Sieber-amide-resin (0.25 mmol/g of substitution). Double coupling was made for the first amino acid insertion. The incorporation of the Leu residue (0.09 mmol/g) was evaluated by quantitative amino acid analysis of a 0.81 mg-resin sample after acid hydrolysis (0.5 ml of 6 N HCl at 118°C for 18 h under vacuum). The coupling agent used was HBTU. The side-chain protections employed were Asp(OAl), Lys (Boc) and Arg (Pmc). After each step a resin sample was submitted to a quantitative ninhydrin test to determine the extent of coupling that resulted over 99.1%. A total of 1.476 g of protected peptide-resin was obtained, corresponding to a 23% yield with respect to the initial substitution of the resin. At the end of the solid-phase synthesis a resin sample was hydrolysed at 130°C for 16 h with 0.5 ml of 12 N HCl and 0.5 ml of propionic acid. The amino acid analysis gave the following results: Asp 0.93 (1); Gly 1.79 (2); Arg 1.62 (1); Ala 0.94 (1); Pro 0.65 (1); Val 1.05 (1); Leu 1.08 (1); Lys 0.96 (1). The exceedingly high value of Arg is probably due to the presence of secondary products and is in agreement with the high yield of Arg coupling in comparison with the coupling yields of the previous residues. To deprotect the N^{α}-function from the Fmoc group, 736 mg of fully protected peptide-resin was stirred in 20% piperidine/NMP (v/v) for 20 min. The resin was filtered, washed extensively with NMP and DCM, and dried under vacuum. The peptide-resin was combined with 10 ml of a 1% TFA/DCM solution for 2 min, then the resin was filtered over 2 ml of CH_3OH added with 200 μl of piridine. The solution was concentrated and the product precipitated with a cold solution of 10% acetic acid in water. The operation was repeated several times to obtain a complete precipitation. To deprotect the Asp β-COOH function from the OAl group, the crude and partially protected peptide was treated with 0.85 g of $Pd(PPh_3)_4$ (0.73 mmol) and 2 ml of a HOAc/NMM/$CHCl_3$ (2:1:37) solution. Because the $Pd(PPh_3)_4$ was not completely dissolved, 7 ml of the above reported solution was added and the mixture was stirred for 17 h at 25°C under Ar. Then, 2 ml of DMF was added and the mixture was evaporated under vacuum. The DMF solution was loaded onto an LH-20 column (3.5 × 30 cm) equilibrated with DMF and 100 drop fractions were collected. The product (0.122 g) was detected in fractions 24 ÷ 30. These fractions were dried under vacuum and added with 91 ml of DMF. Cyclization was achieved by adding 1.5 eq. of HATU and 3.0 eq. of DIEA for 8 h. The solution was concentrated to a volume of 2 ml and loaded onto an LH-20 column. The target peptide was collected from fractions 19 to 21. The fractions were dried and treated with 0.75 g of phenol and an EDT/thioanisole/H_2O/TFA (1:2:2:20) solution for 90 min at 25°C. The product was precipitated from the concentrated mixture with cold diethyl ether, filtered, dissolved in water and loaded onto a semi-preparative Delta Pak C_{18} column. The elution was achieved using the following conditions: eluant A, 0.05% TFA in water; eluant B, 0.05% TFA in MeCN; gradient, 10–40% B over 60 min; flow rate, 4 ml/min; detector, 214 nm. The purified product was analysed by HPLC using the following conditions: column, Vydac C_{18}; eluant A, 0.05% TFA in water; eluant B, 0.05% TFA in MeCN; gradient, 13–20% B over 14 min; flow rate, 1 ml/min; detector, 214 nm. Integration of the chromatographic pattern gave 90% purity grade. The MALDI mass spectrometric analysis of the purified product gave the expected result (theoretical value: 979 Da; experimental value: 977 Da). A scheme of synthesis is shown in Figure 2(B).

Enzymatic Digestion

The PC1 preparation used in the assay was kindly provided by N.G. Seidah (Clinical Research Institute

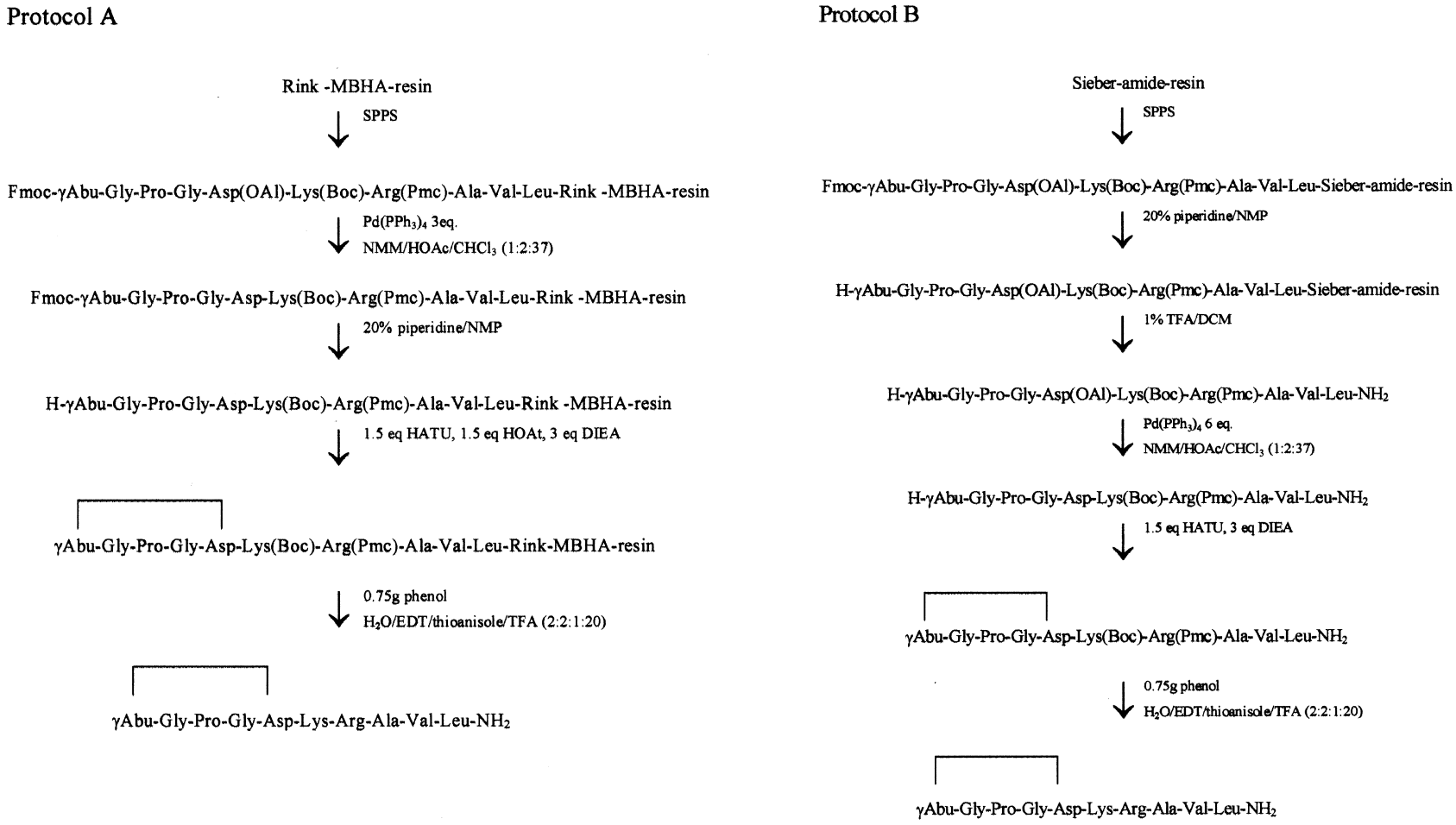

Figure 2 Schemes of synthesis of the peptide *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-Lys-Arg-Ala-Val-Leu-NH_2 using protocols A and B.

of Montreal, Montreal, Quebec, Canada). Incubations with synthetic peptide substrate were conducted for various time intervals with 10 μl of a purified hPC1 fraction representing an average of 2.6 nmol pro-oxytocin-neurophysin (1–20) cleaved $\times$ h^{-1} at 37°C in a total volume of 50 μl. The final incubation mixture contained 15 μl of 2.98 mM peptide solution; 5 μl of 100 mM $CaCl_2$ solution; 50 μl of 100 mM sodium acetate solution at pH 5.5; 10 μl of PC1 and 20 μl of H_2O. Each sample was analysed by HPLC using the following conditions: column, Vydac C_{18}; eluant A, 0.05% TFA in water; eluant B, 0.05% TFA in MeCN; gradient, 5–40% B over 70 min; flow rate, 1 ml/min; detector, 214 nm.

HPLC

Peptide purification and characterization were carried out using a Waters 600E HPLC instrument equipped with a Waters 490E programmable multiwavelength detector and an automatic injector Waters model 717 or a HPLC Waters model 600E associated system equipped with a photodiode array detector Waters model 996 and an automatic injector Waters model 712.

CD

CD spectra were recorded at room temperature using a Jasco model J-710 automatic recording circular dichrograph. Cylindrical fused quartz cells of 0.05 and 0.1 cm path-lengths were used. Spectra are reported in units of mean residue ellipticity (peptide molecular weight/number of amide bonds), $[\Theta]_R$ (deg $\times$ cm^2 $\times$ $dmol^{-1}$). Each weighted quantity of peptide was dissolved in the minimum amount of water to which 10 mM phosphate buffer pH 7 or TFE was added to a final content of 99% (v/v). Peptide concentration, determined by quantitative amino acid analysis, ranged from 4.7×10^{-5} to 4.8×10^{-4} M.

FT-IR Absorption

Each FT-IR absorption spectrum was measured at room temperature using a nitrogen-flushed Jasco model 300E infrared spectrometer at 2 cm^{-1} nominal resolution. A cell with a CaF_2 window and 0.2 mm path-length was used. Peptide concentrations in DMSO were 0.2 mg/ml and 0.4 mg/ml for cyclic and linear peptides, respectively. For the spectra in TFE and D_2O, peptide concentration was $\sim$10 mg/ml. The solutions in TFE were obtained by dissolving each weighed quantity of the peptide in 10 μl of D_2O to which 90 μl of TFE were added. Infrared absorption spectra of each solvent were obtained under identical conditions and subtracted from the spectra of the peptides in the corresponding solvents. Fourier self-deconvolution was used for band narrowing. Area measurements of multi-component bands were achieved using a curve-fitting program purchased from Jasco.

NMR

One-dimensional (1D) and two-dimensional (2D) NMR spectra were recorded on a Varian Unity 400 spectrometer, operating at 400 MHz, located at the 'Centro di Studio di Biocristallografia del C.N.R.', University of Naples 'Federico II'. NMR experiments were run in DMSO and TFE/H_2O at 298 K. Samples used in all ^{1}H-NMR experiments were prepared by dissolving 2–3 mg of solid peptides in 700 μl of DMSO-d_6 (99.98% isotopic purity) and in TFE-d_3/H_2O 80:20 (99% isotopic purity) for a 2–3 mM final concentration. Chemical shifts were referred to TMS in the case of DMSO and to internal TSP in the case of aqueous solutions. 2D-experiments, such as DQFCOSY [7], TOCSY [8], NOESY [9] and ROESY [10] were recorded by the phase-sensitive States-Haberkorn method. The data file generally consisted of 256 points and 2048 (4096 for the DQFCOSY) points in the $\omega 1$ and $\omega 2$ dimensions, respectively. Mixing times (τ_m) used in the TOCSY experiments were typically 70 ms; NOESY experiments were performed with mixing times of 100, 200, 300 and 400 ms. A continuous spin-lock was used for the ROESY spectra ($\tau_m = 100$ and 120 ms). Off-resonance effects, associated to the low-power spin-lock field, have been compensated by means of two 90° hard pulses before and after the spin-lock period [11]. Free induction decays were multiplied in both dimensions with weighting functions, and the data points were zero-filled to 1K in $\omega 1$ prior to Fourier transformations. Temperature coefficients of NH signals were measured from 1D and TOCSY spectra recorded in the temperature range 298–310 K. NOE analysis was achieved by means of NOESY spectra; NOE intensities were evaluated by integration of cross-peaks, making use of the appropriate Varian software, and then converted into inter-proton distances using the $1/r^6$ relationship for rigid molecules [12]. Geminal protons (γAbu^1 $\alpha\alpha'CH_2$ in TFE/H_2O and terminal CONH-CONH′ in DMSO for *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-NH_2; Pro^3 $\beta\beta'CH_2$ in TFE/H_2O and Asp^5 $\beta\beta'CH_2$ in DMSO for

cyclo(1-5β)γAbu-Gly-Pro-Gly-Asp-Lys-Arg-Ala-Val-Leu-NH_2) were chosen as reference with a relative distance of 178 ppm. Vicinal $^3J_{NH\text{-}\alpha CH}$ coupling constants were measured from DQFCOSY spectra. In all NMR experiments performed in TFE/H_2O, the water resonance was suppressed during the relaxation delay by pre-saturation. The decoupler offset was set equal to the transmitter to prevent phase distortions around the water peak due to imperfect cancellation of the dispersive component of the residual water magnetization [13].

Computational Methods

All calculations were performed on a Silicon Graphics Indigo 2 workstation. The Insight/Discover (Biosym Tecnologies, San Diego, CA) program with the consistent valence force field (CVFF) [14–16] was employed for energy minimization and molecular dynamics calculations, including NOE effects as constraints on inter-atomic distances. Minimizations were performed using a conjugate algorithm for the initial stages of refinement and a quasi-Newton–Raphson algorithm for the final ones [17]. The motion equation algorithm was the leapfrog [18]. Computational conditions were chosen to avoid boundary effects [19]. Starting models for the simulation were hand-built using standard parameters for amino acid residues supplied with the Insight software package. By starting from these models an energy minimization procedure was used to generate structures consistent with NOE data. Interproton distances evaluated from NOEs were inserted as restraints with a tolerance of $\pm$10% during the simulations [20]. The RMD simulations were carried out for 50 ps in the equilibration phase and for 160 ps without velocity rescaling since the temperature was kept constant at 300 K. The statistical analyses were performed during the last 50 ps of the simulations.

RESULTS AND DISCUSSION

Peptide Synthesis

The synthesis of peptide *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-NH_2 did not present particular problems. The peptide was grown on a Rink-MBHA resin using O*t*Bu for side-chain protection of the Asp residue. Cleavage of crude peptide from the solid support and concomitant deprotection of the Asp side chain were followed by condensation of the amino terminal group of the main chain with the β-carboxylic function of the Asp residue. The condensation reaction was carried out by adding 1.5 eq. of HATU and 3 eq. of DIEA to a 1×10^{-3} M solution of the peptide in DMF. The reaction was monitored by HPLC. As reported in Figure 2(A), the first synthetic protocol for peptide *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-Lys-Arg-Ala-Val-Leu-NH_2 was designed to maximize the number of reactions on the peptide still anchored to the solid support. This protocol, however, did not give the desired product (data not shown). The limitation of the method concerns the low deprotection yield of the OAl group.

The second protocol (Figure 2(B)) introduces another orthogonality level by growing the peptide chain on an more acid-labile resin (Sieber-amide-resin). HPLC monitoring of the OAl group deprotection and of the condensation between the γ-amino group of γAbu and the β-carboxylic group of Asp has allowed us to optimize the reaction times and to obtain the target peptide.

CD Study

For the sake of comparison CD spectra of the *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-NH_2 peptide are plotted together with those of the corresponding linear peptide in the same solvents.

In aqueous solution (Figure 3(A)) the linear pentapeptide spectrum is characterized by a strong negative band at 195 nm, indicative of a disordered conformation [21]. The formation of an amide bond, between the γAbu amino group and the Asp β-carboxylic group, substantially modifies the spectrum that presents now two positive maxima at 185 and 200 nm and a broad negative band at 225 nm. At wavelength values above 190 nm, our cyclic analogue presents a class-B spectrum indicative of type-II β-turn conformation [22] with band intensity and position identical to the ones shown by the Fasman peptide model *cyclo*(1-5)δAva-Gly-Pro-Gly-Gly. Under 190 nm the spectra of *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-NH_2 and of *cyclo*(1-5)δAva-Gly-Pro-Gly-Gly are different. On the other hand, the replacement of a Gly residue by an Asp residue is expected to decrease the β-turn propensity ($f_{i+3} = 0.152$ for Gly and $f_{i+3} = 0.081$ for Asp) [23].

In TFE the spectrum of the *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-NH_2 peptide (Figure 3(B)) is blue shifted: indeed, the intense positive band is located at 190 nm whereas the negative band is at 220 nm. This CD spectrum resembles a type-C

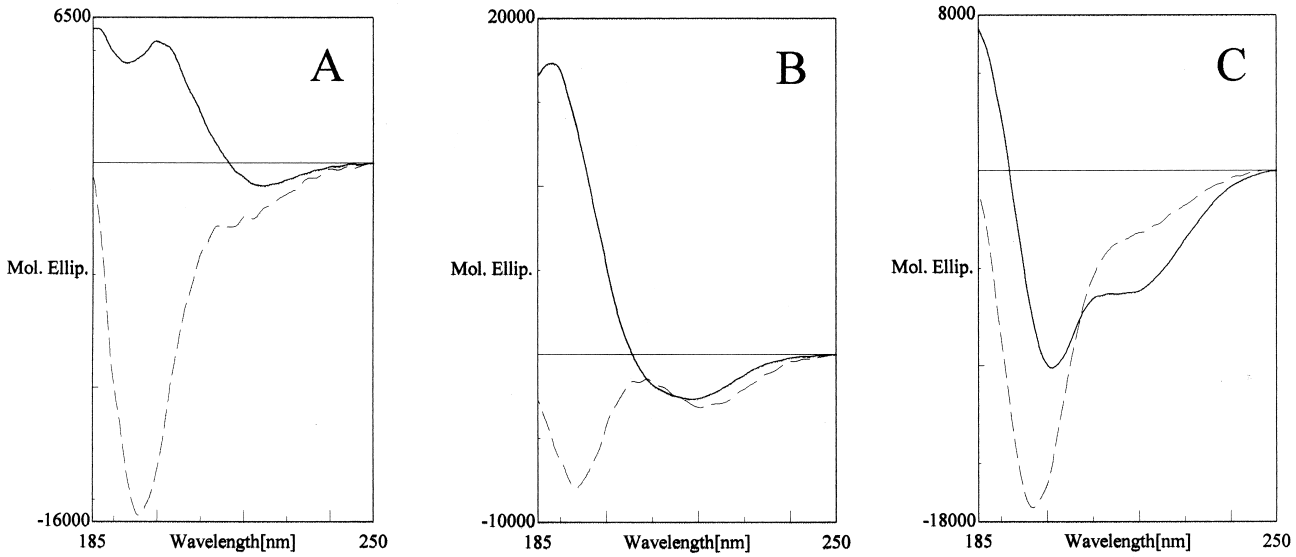

Figure 3 CD spectra of *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-NH_2 (—) and H-γAbu-Gly-Pro-Gly-Asp-NH_2 (– – –) in 10 mM phosphate buffer, pH 7 (A) and in 99% TFE/H_2O (B). CD spectra of *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-Lys-Arg-Ala-Val-Leu-NH_2 in 10 mM phosphate buffer, pH 7 (– – –) and in 99% TFE/H_2O (—) (C).

spectrum according to the definition given by Woody [22]. In the same solvent the linear analogue presents a major negative band at 195 nm attributed to a disordered conformation but also a negative broad band at 220 nm indicative of the existence of some ordered conformations. The addition of the -Lys-Arg-Ala-Val-Leu- sequence to the cyclic peptide substantially modifies the dichroic spectra (Figure 3(C)). More specifically:

- In aqueous solution the spectrum is characterized by a strong negative band at 195 nm. It seems likely that a contribution of disordered conformations, due to the -Lys-Arg-Ala-Val-Leu- sequence, would overlap the positive band at 195 nm attributed to the type-II β-turn conformation of *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp.
- In TFE the spectrum shows two negative bands at 202 and 222 nm and positive values for $\lambda <$ 190 nm. The shape of this CD curve, but not the amplitude, is reminiscent of the one characteristic of α-helix spectra.

FT-IR Absorption Study

The *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-NH_2 peptide and its linear analogue have been studied by the FT-IR absorption technique in different solvents. The solvents used can be divided in the following categories: (a) hydrogen-bond acceptor organic solvent (DMSO); (b) mostly hydrogen-bond donor organic solvent (TFE); (c) aqueous solvent (D_2O).

The rationale for using DMSO stems from the known properties of this organic solvent to partially disrupt the secondary structures of globular proteins and polypeptides, since the NH groups can hydrogen bond to the S→O group, leaving the corresponding amide C=O groups 'naked' or non-hydrogen-bonded and, consequently, characterized by a strong IR band in the range 1660–1670 cm^{-1} [24]. Accordingly, it seemed of interest to determine whether DMSO is able to disrupt the hydrogen bonds in order to pinpoint the presence, strength and stability of intramolecular hydrogen bonds. The spectrum of cyclic pentapeptide (Figure 4) shows two main amide I bands, one at 1670 cm^{-1} with a shoulder at 1660 cm^{-1}, and a second at 1640 cm^{-1}. While the former is clearly in line with 'free' C=O stretching bands [24], the presence of the latter demonstrates that there are intramolecular hydrogen bonds in this cyclic peptide that remain intact and are not affected by DMSO. The intensity ratio of the 1670/1640 cm^{-1} bands is approximately 4:2. Consequently, it seems reasonable to assign the band at 1670–1660 cm^{-1} to four solvent-exposed C=O groups and the band at 1640 cm^{-1} to two hydrogen-bonded C=O groups. However, a contribution of the Gly-Pro tertiary peptide bond is possible.

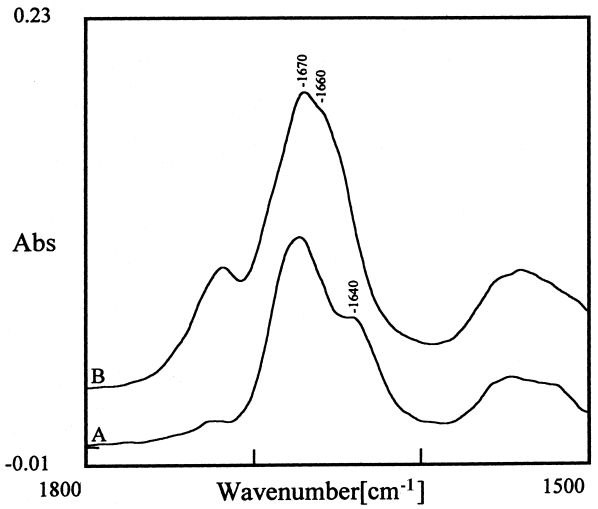

Figure 4 Amide I region of the FT-IR absorption spectra of *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-NH_2 (A) and H-γAbu-Gly-Pro-Gly-Asp-NH_2 (B) in DMSO.

The spectrum of the linear peptide in DMSO (Figure 4) presents a composite band at high frequencies; however, the band at 1640 cm^{-1}, assigned to hydrogen-bonded C=O groups, is absent, as expected [25].

Even in the presence of a hydrogen donor solvent, the spectrum of the cyclic analogue (Figure 5(A)) retains the frequency band at 1647 cm^{-1} which is assigned to intramolecular hydrogen-bonded C=O groups [25], together with two bands at higher frequencies (1661 and 1674 cm^{-1}) due to C=O groups not involved in intramolecular hydrogen bonds.

Excluding an intermolecular H-bonding on the basis of the invariant CD spectra at different concentrations, in the same solvent (data not shown) the band at 1627 cm^{-1} might be related to a double-acceptor carbonyl [26].

For the linear peptide (Figure 5(B)) the region of the amide I modes is characterized by three main bands: 1677, 1656 and 1629 cm^{-1}, while the band at 1640 cm^{-1} is absent. The increased flexibility of the molecule with no conformational constraint is confirmed by the presence of a negative band at 195 nm in the CD spectrum in the same solvent which is assigned to aperiodic structures.

The amide I bands in aqueous solution (D_2O) are considerably broader as compared to those found in organic solvents. The composite band centred at 1650 cm^{-1} is constituted of three components, one at 1670 cm^{-1}, a second at 1650 cm^{-1} and the last at 1640 cm^{-1} (Figure 6). The high-frequency bands can be assigned to solvent-exposed C=O groups, whereas the band at 1640 cm^{-1} can be attributed to C=O groups engaged in intramolecular hydrogen bonds persisting in aqueous solution. The band at 1621 cm^{-1}, which is observed only in D_2O, may represent a population with very strong hydrogen-bonded C=O groups. This band has been assigned by different authors to bifurcated hydrogen-bonded C=O groups [24]. Then the persistence of intramolecular hydrogen bonds, even in the presence of a bifunctional solvent such as water, agrees with the CD spectrum profile that is typical of type-II β-turn structure.

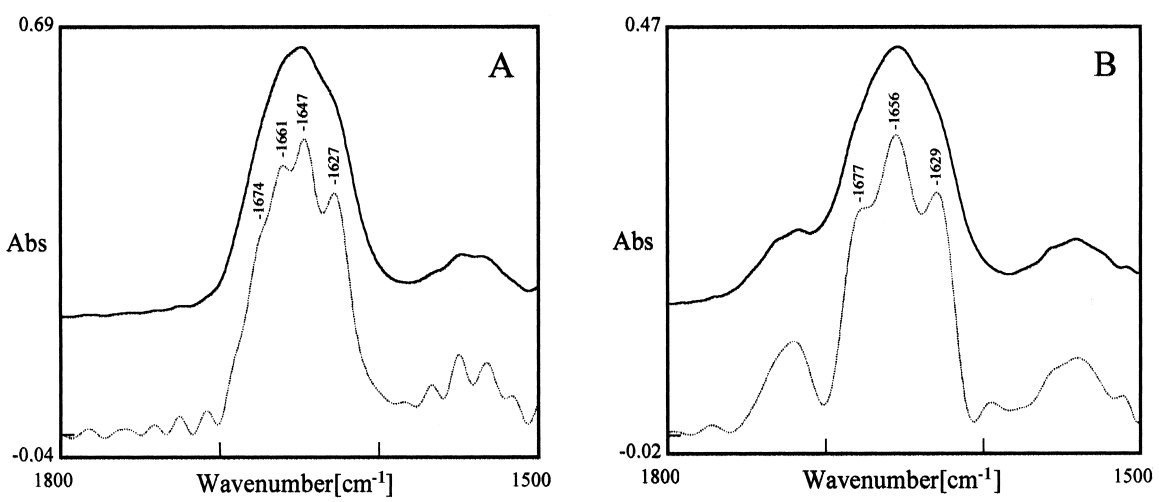

Figure 5 FT-IR absorption spectrum of *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-NH_2 (A) and H-γAbu-Gly-Pro-Gly-Asp-NH_2 (B) in a 90% TFE/D_2O solution in the region of the amide I and II bands. Broken traces are spectra after band narrowing *via* Fourier self-deconvolution.

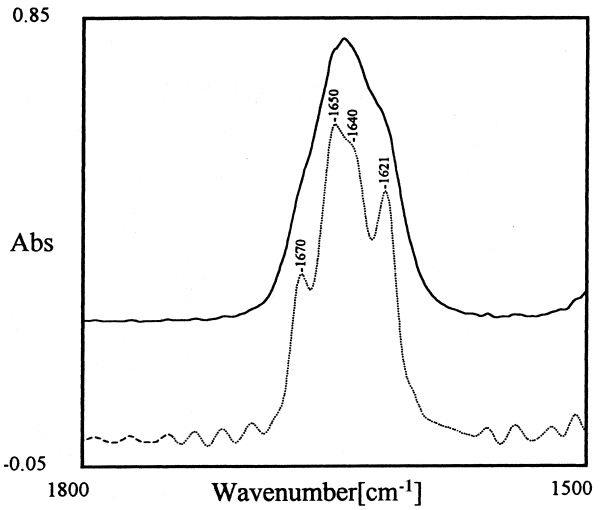

Figure 6 FT-IR absorption spectrum of *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-NH_2 in D_2O solution in the region of the amide I and II bands. Broken traces are spectra after band narrowing *via* Fourier self-deconvolution.

Peptide H-γAbu-Gly-Pro-Gly-Asp-NH_2 has been characterized exclusively in DMSO and TFE because it proved to be impossible to dissolve the peptide in MeCN and D_2O. The FT-IR absorption spectra of linear peptides show a band at frequency higher than 1700 cm^{-1} that can be assigned to the β-carboxylic group of the Asp residue.

NMR Study

NMR studies were performed in TFE/H_2O and in DMSO for both *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-NH_2 and *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-Lys-Arg-Ala-Val-Leu-NH_2 peptides.

The sequential assignment of all amino acid spin systems of *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-NH_2 peptide (Table 1) was obtained by analysing the TOCSY and NOESY spectra. Prochiral assignment of the Asp^5 and γAbu^1 residues was achieved from the vicinal $^3J_{\alpha\beta}$ and $^3J_{\alpha\beta'}$ coupling constants and from the relative intensities of the (α, β), (α, β'), (NH, β), (NH, β') NOE contacts [12]. Both residues show a stereospecific *gauche* conformation ($\chi = 180°$). The prochiral assignment of Pro^3 $\beta\beta'CH_2$ protons was not feasible since they exhibit very close chemical shifts. The Gly^2-Pro^3 peptide bond conformation, too, was not fully established due to the severe overlap of Gly and Pro αCHs with the residual water signal.

The strong NOE contacts Gly^4 NH/Asp^5 NH and Pro^3 αCH/Gly^4 NH indicate the presence of a type-II β-turn structure in the Gly^2-Asp^5 segment. This β-turn could be stabilized by H-bond interaction between the Gly^2 C=O and the Asp^5 NH group, as suggested by the low temperature coefficient of the Asp^5 amide proton (Table 1).

The addition of the -Lys-Arg-Ala-Val-Leu-NH_2 sequence to the short cyclic peptide complicates the NMR analysis. First of all, the 1D spectrum shows the presence of many conformers and, of these, only the most abundant was completely characterized. Moreover, not all resonances are observed (Lys^6 NH, Lys^6 εNH and γAbu^1 NH signals are missing). The sequential assignment of the proton resonances was carried out by analysing the cross-peak patterns in the TOCSY and NOESY spectra. Again, even for *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-Lys-Arg-Ala-Val-Leu-NH_2 it was not possible to establish the Gly^2-Pro^3 peptide bond conformation. In Table 2 proton chemical shifts together with vicinal $^3J_{NH\text{-}\alpha CH}$

Table 1 Chemical Shifts δ (ppm), $^3J_{NH\text{-}\alpha CH}$ Coupling Constants (Hz) and Temperature Coefficients $\Delta\delta/\Delta T$ (ppb/K) of *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-NH_2 in TFE/H_2O

Amino acid	NH	α	β	γ	δ	Others	$^3J_{NH\text{-}\alpha CH}$	$-\Delta\delta/\Delta T$
γAbu^1	7.55	3.06 3.52	1.85^{proR} 1.98^{proS}	2.41			6.6 4.1	6.6
Gly^2	7.82	3.83					5.1	5.7
Pro^3		4.31	1.98 2.36	2.08	3.60 3.80			
Gly^4	8.50	3.85 3.96					5.8 5.0	6.8
Asp^5	7.74	4.70	2.79^{proR} 2.88^{ProS}			CONH 6.78 CONH′ 7.35	7.4	1.1

Table 2 Chemical Shifts δ (ppm), $^3J_{NH\text{-}\alpha CH}$ Coupling Constants (Hz) and Temperature Coefficients $\Delta\delta/\Delta T$ (ppb/K) of *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-Lys-Arg-Ala-Val-Leu-NH_2 in TFE/H_2O

Amino acid	NH	α	β	γ	δ	Others	$^3J_{NH\text{-}\alpha CH}$	$-\Delta\delta/\Delta T$
γAbu[1]	[a]	3.08	1.99	2.53			–	–
Gly[2]	7.64	3.99 4.13					5.5	5.9
Pro[3]		4.41	2.03 2.28	2.06	3.56 3.69		–	–
Gly[4]	8.03	3.93					5.9	6.4
Asp[5]	8.30	4.39	2.74 3.16				6.6	3.5
Lys[6]	[a]	4.79	2.15	1.42	1.68 1.75	$\varepsilon\varepsilon'$ 3.00	–	–
Arg[7]	7.72	4.32	1.88	1.63	3.16	εNH 6.89	6.7	3.6
Ala[8]	7.58	4.30	1.41				5.7	5.7
Val[9]	7.27	4.11	2.14	0.97			7.8	3.9
Leu[10]	7.36	4.38	1.64	1.64	0.93	CONH 6.30 CONH′ 6.90	7.6	5.1

[a] This resonance is not observed.

coupling constants and temperature coefficients of amide protons are reported. The NOE analysis in the Gly[2]-Asp[5] fragment reveals the same strong contacts Gly[4] NH/Asp[5] NH and Pro[3] αCH/Gly[4] NH that were observed for the *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-NH_2 peptide, indicating again the presence of a type-II β-turn. This evidence is confirmed by the chemical shift deviations of the Pro[3] αCH and Gly[4] $\alpha\alpha'CH_2$ protons that exhibit the typical trend of residues in the $i+1$ and $i+2$ positions of a type-II β-turn [27]. In the case of this tailed cyclic peptide, however, strong NOEs between Asp[5] NH and Gly[4] $\alpha\alpha'CH_2$ are also observed, suggesting the presence of chemical equilibria among different types of turns.

In the -Lys-Arg-Ala-Val-Leu-NH_2 fragment no NH_i/NH_{i+1} NOE contacts are observed, but the αCH chemical shift deviations are consistent with a type-I β-turn in the -Lys[6]-Arg-Ala-Val[9]- segment.

The complete sequential assignment of *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-NH_2 was obtained following the standard procedure indicated by Wüthrich [12]. Proton chemical shifts are reported in Table 3 together with $^3J_{NH\text{-}\alpha CH}$ coupling constants and temperature coefficients of amide protons. For Asp[5] $\beta\beta'CH_2$, NMR data indicate a stereospecific *trans* conformation ($\chi = -60°$). In the segment -Gly[2]-Pro[3]-Gly[4]-Asp[5]-, the relative intensities of NOE contacts between Gly[4] NH/Asp[5] NH and Pro[3] αCH/Gly[4] NH resonances point to a type-II β-turn. Furthermore, the low $\Delta\delta/\Delta T$ value of the Asp[5] NH proton (-0.2 ppb/K), together with the αCH and NH proton chemical shift deviations from the random coil values, confirms this hypothesis. Indeed, αCH protons for Pro[3] and Gly[4] residues show negative deviations, a trend characteristic of a type-II β-turn [24]. The Asp[5] amide proton deviation from the random coil value, too, confirms that Asp[5] is involved in the β-turn formation [24].

For the longer *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-Lys-Arg-Ala-Val-Leu-NH_2 peptide, as already observed in TFE/H_2O, many conformers are present even in DMSO. Only the major conformer has been fully characterized. All NMR data are shown in Table 4. Chemical shift sequential assignment was carried out by analysing the cross-peak patterns in the TOCSY, NOESY and ROESY spectra. The prochiral assignment for Pro[3] $\beta\beta'CH_2$, Asp[5] $\beta\beta'CH_2$ and Lys[6] $\beta\beta'CH_2$ protons was established from the $^3J_{\alpha\beta}$ and $^3J_{\alpha\beta'}$ coupling constant values and from the

Table 3 Chemical Shifts δ (ppm), $^3J_{NH\text{-}\alpha CH}$ Coupling Constants (Hz) and Temperature Coefficients $\Delta\delta/\Delta T$ (ppb/K) of *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-NH_2 in DMSO

Amino acid	NH	α	β	γ	δ	Others	$^3J_{NH\text{-}\alpha CH}$	$-\Delta\delta/\Delta T$
γAbu[1]	7.44	3.07	1.65	2.10 2.37			5.8	0.3
Gly[2]	7.94	3.72 4.33					8.0	3.2
Pro[3]		4.18	1.80 2.15	1.90	3.56 3.63		–	–
Gly[4]	8.54	3.64					5.4	4.8
Asp[5]	7.42	4.33	2.50^{proR} 2.65^{proS}			CONH 6.97 CONH′ 7.20	8.6	0.2

(α, β), (α, β'), (NH, β), (NH, β') NOE contacts [11]. For Asp[5] $\beta\beta'CH_2$ and Lys[6] $\beta\beta'CH_2$ protons, the data show a stereospecific *trans* conformation ($\chi = -60°$), while for Pro[3] $\beta\beta'CH_2$ the stereospecific conformation is *gauche* ($\chi = 180°$). Due to extensive overlaps of the NH peaks, temperature coefficients were measured from TOCSY spectra at different temperatures. The observed strong NOE contacts Gly[2] $\alpha\alpha'CH_2$/Pro[3] $\delta\delta'CH_2$ are diagnostic of a *trans* peptide bond between the Gly[2]-Pro[3] residues. In the *cyclo*(1-5β) moiety, the Gly[4] NH/Asp[5] NH and Pro[3] αCH/Gly[4] NH NOEs suggest a type-II β-turn in the Gly[2]-Asp[5] sequence. This conclusion is confirmed by the αCH and NH chemical shift deviations. This

Table 4 Chemical Shifts δ (ppm), $^3J_{NH\text{-}\alpha CH}$ Coupling Constants (Hz) and Temperature Coefficients $\Delta\delta/\Delta T$ (ppb/K) of *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-Lys-Arg-Ala-Val-Leu-NH_2 in DMSO

Amino acid	NH	α	β	γ	δ	Others	$^3J_{NH\text{-}\alpha CH}$	$-\Delta\delta/\Delta T$
γAbu[1]	7.71	2.83	1.77	2.26			–	1.0
Gly[2]	8.05	3.90 3.98					5.7	3.9
Pro[3]		4.29	1.89^{proR} 2.04^{proS}	1.92	3.49 3.57		–	–
Gly[4]	8.23	3.73					5.8	4.2
Asp[5]	8.54	4.48	2.59^{proS} 3.03^{proR}				7.5	3.0
Lys[6]	[a]	4.51	1.80^{proS} 2.05^{proR}	1.24 1.31	1.51	$\varepsilon\varepsilon'$ 2.75 εNH 7.65	–	–
Arg[7]	7.89	4.27	1.66	1.40	3.05	εNH 7.41	7.8	3.9
Ala[8]	8.09	4.29	1.21				7.9	5.0
Val[9]	7.78	4.11	1.98	0.83			7.3	3.6
Leu[10]	7.77	4.25	1.45	1.56	0.86	CONH 6.97 CONH′ 7.27	6.1	4.3

[a] This resonance is not observed.

Table 5 Dihedral Angles (deg) of *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-NH_2 Molecular Model Averaged over the Last 50 ps of RMD Using NOEs in TFE/H_2O

Residue	ϕ	ψ	μ^1	μ^2	χ
γAbu^1	162.3	−34.4	57.2[a]	−84.7[b]	
Gly^2	−148.6	−79.1			
Pro^3	−71.0	102.1			
Gly^4	120.7	−29.3			
Asp^5	−75.6		162.1[c]	123.7[d]	−67.6

[a] N γAbu^1-CA γAbu^1-CB γAbu^1-CG γAbu^1.
[b] CA γAbu^1-CB γAbu^1-CG γAbu^1-CO γAbu^1.
[c] CO Gly^4-N Asp^5-CA Asp^5-CB Asp^5.
[d] CA Asp^5-CB Asp^5-CG Asp^5-N γAbu^1.

structure appears stabilized by a Gly^2 C=O/Asp^5 NH H-bond considering the Asp^5 NH low temperature coefficient (−3.0 ppb/K). In the -Lys-Arg-Ala-Val-Leu- segment the Arg^7 NH/Ala^8 NH and the Ala^8 NH/$Va1^9$ NH NOE contacts suggest a type-I β-turn in the Lys^6-Val^9 sequence. Nevertheless, considering the presence of other strong NOE contacts, such as Lys^6 αCH/Arg^7 NH, Arg^7 αCH/Ala^8 NH, Ala^8 αCH/Val^9 NH, and that temperature coefficients and coupling constants are not typical of turns, it is fair to state that in the fragment -Lys-Arg-Ala-Val-Leu- folded structures are in equilibrium with random conformers.

RMD Calculations Based on NMR Data

Energy minimizations and RMD simulations were carried out using the experimental NMR data collected in TFE/H_2O and in DMSO. All peptide bonds were assumed to be *trans* since no indication of a *cis* peptide bond (αCH_i-αCH_{i+1} NOEs) was found [28]. In the case of *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-NH_2 peptide in TFE/H_2O 33 interproton diatances (25 intraresidual and eight sequential) were inserted as restraints. The minimized structure reveals the presence of a type-II β-turn in the -Gly^2-Pro^3-Gly^4-Asp^5- segment, stabilized by a H-bond between the Gly^2 C=O and the Asp^5 NH groups. This finding is in line with the low temperature coefficient of the Asp^5 amide proton (Table 1). This model was used as a starting point for RMD simulations *in vacuo*. In Figure 7 the average molecular model obtained after RMD *in vacuo* at 300 K by using NOEs in TFE/H_2O is shown. By looking at Table 5, where the ϕ and ψ dihedral angles are reported, a type-II β-turn in the -Gly^2-Pro^3-Gly^4-Asp^5- segment can be inferred, in good agreement with the experimental data. Moreover, the Gly^2 C=O-Asp^5 NH distance analysis shows the presence of an H-bond with a 60% statistical occurrence during the simulation. Finally, the examination of χ dihedral angle values is consistent with the NMR stereospecific assignment.

The *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-Lys-Arg-Ala-Val-Leu-NH_2 model was built starting from the *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-NH_2 minimized structure and then adding the -Lys-Arg-Ala-Val-

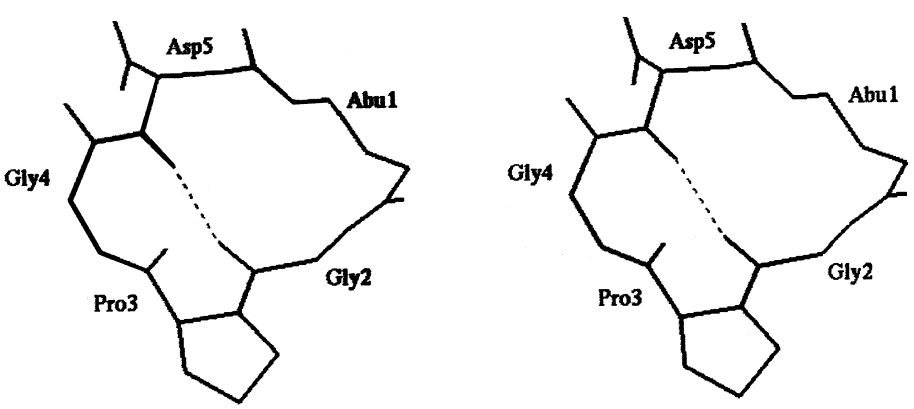

Figure 7 Stereoview of the *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-NH_2 average molecular model after RMD *in vacuo* using NMR data in TFE/H_2O. The intramolecular H-bond is indicated as a dashed line.

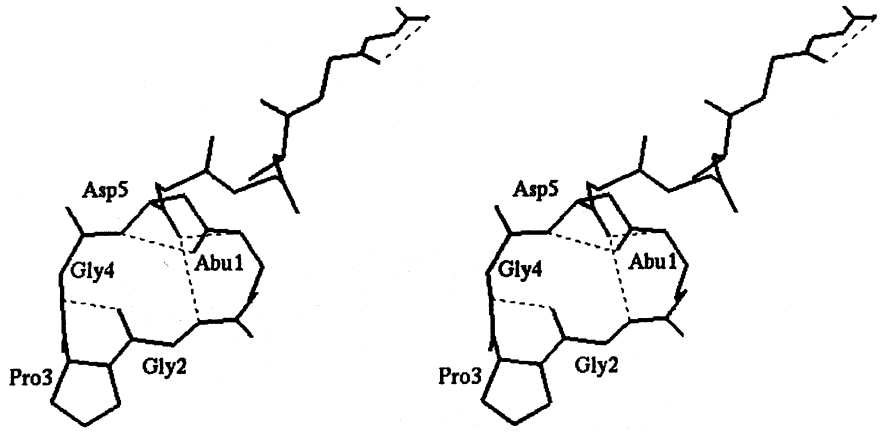

Figure 8 Stereoview of the *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-Lys-Arg-Ala-Val-Leu-NH_2 average molecular model after RMD *in vacuo* using NMR data in TFE/H_2O. The intramolecular H-bonds are indicated as dashed lines.

Leu-NH_2 segment on the Asp terminal C=O group. Lys and Arg side chains were considered charged. This model was minimized using NOE contacts in TFE/H_2O as distance restraints on the interproton distances and with a dielectric constant $\varepsilon = 40$, to avoid non-physical folds of the charged side chains [29]. These NOEs include 38 intraresidue and 15 sequential contacts, giving a total of 53 NOEs. In Figure 8 the average molecular model obtained after RMD ($\varepsilon = 40$) calculations is shown. In the *cyclo*-(1-5β)γAbu-Gly-Pro-Gly-Asp moiety, as indicated by ϕ and ψ angles (Table 6), a type-II β-turn earlier suggested by NMR data in the -Gly^2-Asp^5- segment appears quite distorted. This turn is stabilized by an intramolecular $4 \rightarrow 1$ H-bond between the Asp^5 N–H and Gly^2 C=O groups. Moreover, an intramolecular $3 \rightarrow 1$ H-bond between the Gly^4 N–H and Gly^2 C=O groups, indicating a γ-turn conformation around the Pro^3 residue, is also observed. The average r.m.s.d. is 0.80 Å for the backbone of the *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp moiety of these two peptides in TFE/H_2O. The -Lys-Arg-Ala-Val-Leu-NH_2 segment is in an extended conformation.

In the case of *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-NH_2 peptide in DMSO, 18 interproton distances (12 intraresidue and six sequential) have been used as distance restraints for RMD calculations *in vacuo* at 300 K. The average molecular model obtained after RMD is quite similar to that calculated from NMR

Table 6 Dihedral Angles (deg) of *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-Lys-Arg-Ala-Val-Leu-NH_2 Molecular Model Averaged over the Last 50 ps of RMD Using NOEs in TFE/H_2O

Residue	ϕ	ψ	μ^1	μ^2	χ
γAbu^1	78.6	58.6	68.8[a]	−165.4[b]	
Gly^2	−179.4	−106.5			
Pro^3	−80.0	88.3			
Gly^4	144.7	−84.0			
Asp^5	−87.0	117.4	145.9[c]	104.9[d]	52.5
Lys^6	−97.1	127.5			
Arg^7	−127.5	90.1			
Ala^8	−150.8	94.2			
Val^9	−133.4	160.1			
Leu^{10}	−112.3	140.5			

[a] N γAbu^1-CA γAbu^1-CB γAbu^1-CG γAbu^1.
[b] CA γAbu^1-CB γAbu^1-CG γAbu^1-CO γAbu^1.
[c] CO Gly^4-N Asp^5-CA Asp^5-CB Asp^5.
[d] CA Asp^5-CB Asp^5-CG Asp^5-N γAbu^1.

Table 7 Dihedral Angles (deg) of *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-NH_2 Molecular Model Averaged over the Last 50 ps of RMD Using NOEs in DMSO

Residue	ϕ	ψ	μ^1	μ^2	χ
γAbu^1	−147.9	34.3	84.6[a]	−91.5[b]	
Gly^2	126.7	−85.5			
Pro^3	−72.1	98.5			
Gly^4	116.6	1.69			
Asp^5	−93.5	122.0	147.3[c]	75.3[d]	−93.6

[a] N γAbu^1-CA γAbu^1-CB γAbu^1-CG γAbu^1.
[b] CA γAbu^1-CB γAbu^1-CG γAbu^1-CO γAbu^1.
[c] CO Gly^4-N Asp^5-CA Asp^5-CB Asp^5.
[d] CA Asp^5-CB Asp^5-CG Asp^5-N γAbu^1.

data in TFE/H_2O. The average r.m.s.d. is 0.65 Å for the backbone (including the oxygen atoms).

The -Gly^2-Pro^3-Gly^4-Asp^5- segment shows ϕ and ψ angles (Table 7) typical of a type-II β-turn stabilized by a H-bond between Gly^2 C=O and Asp^5 NH. The presence of a type-II β-turn results in the solvent exposition of the Gly^4 amide proton in both solvents. Moreover, the inward orientation of Gly^2 and Asp^5 NHs is observed. The only structural difference regards the γAbu^1 amide proton orientation that is inward in DMSO and outward in TFE/H_2O, in agreement with the different temperature coefficients measured in both solvents.

In the case of the *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-Lys-Arg-Ala-Val-Leu-NH_2 peptide in DMSO, preliminary calculations were performed by using 61 distance restraints (42 intraresidue and 19 sequential). The -Lys-Arg-Ala-Val-Leu-NH_2 segment is in an extended conformation. Starting from the minimized structure, two additional long-range distance restraints were used (Gly^2 αCH_2/Val^9 $\gamma\gamma' CH_3$ and Asp^5 αCH/Val^9 $\gamma\gamma' CH_3$). In Figure 9 the average molecular model, obtained after *in vacuo* RMD ($\varepsilon = 40$) calculations, is shown. By superimposing the *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-NH_2 average model to the cyclic moiety of the tailed peptide an average r.m.s.d. of 0.95 Å is found for the backbone. As observed in TFE/H_2O, a distorted β-turn in the -Gly^2-Asp^5- segment, including a γ-turn around the Pro^3 residue, is present. The hydrophilic -Lys-Arg-Ala-Val-Leu-NH_2 tail is reversed toward the *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp moiety, in contrast to the structure found in TFE/H_2O.

CONCLUSIONS

In the proteolytic activation of prohormones the presence of a β-turn at the *N*-terminus of the dibasic cleavage site appears to be a crucial feature for molecular recognition, as suggested by previous studies on pro-oxytocin/neurophysin [1,4,30] and pro-somatostatin [31].

To further strengthen this hypothesis we have synthesized a new substrate for the processing enzyme, i.e. the *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-Lys-Arg-Ala-Val-Leu-NH_2 peptide, in which the *N*-terminal side of the Lys-Arg doublet is constrained to adopt a type-II β-turn. The *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp moiety of the new substrate, designed by modifying the Pro-containing peptide *cyclo*(δAva-Gly-Pro-Gly-Gly), that according to Fasman and coworkers adopts a type-II β-turn, is here proposed as a type-II β-turn model for the insertion of such a secondary structure into peptide chains.

Experimental CD, FT-IR absorption and NMR data indicate a $1 \leftarrow 4$ hydrogen-bonded type-II β-turn conformation for the *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-NH_2 peptide in solution. Average molecular models, evaluated by RMD using NOE data collected either in TFE/H_2O or in DMSO, are quite similar with a backbone r.m.s.d. of 0.65 Å. Both models show a type-II β-turn in the segment -Gly^2-Pro^3-Gly^4-Asp^5- stabilized by a H-bond between Gly^2 C=O and Asp^5 NH groups.When the *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp moiety is elongated by adding the -Lys-Arg-Ala-Val-Leu-NH_2 tail, an increased flexibility of the system is observed. Indeed, CD spectra in TFE/H_2O solution show a negative band at 195 nm due to a disordered conformation of the -Lys-Arg-Ala-Val-Leu-NH_2 tail, that cancel out the positive contribution at 195 nm typical of the type-II β-turn of the *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp moiety. This hypothesis seems to be confirmed by the NMR data that show the existence of several conformational families for the *cyclo*(1-5β)γAbu-

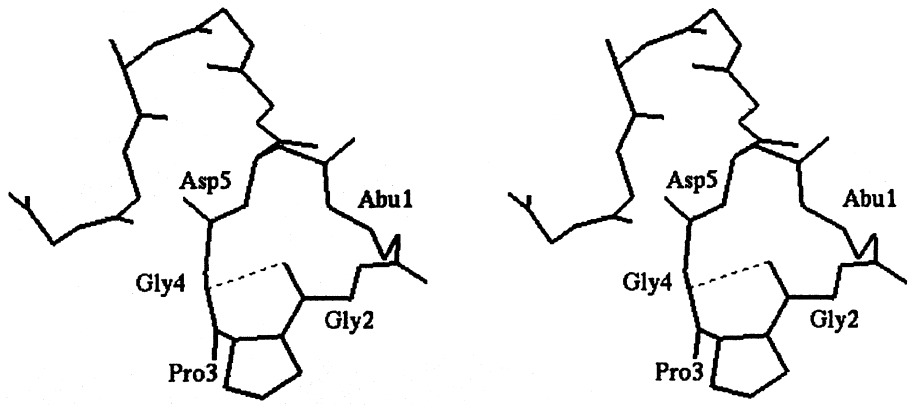

Figure 9 Stereoview of the *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-Lys-Arg-Ala-Val-Leu-NH_2 average molecular model after RMD *in vacuo* using NMR data in DMSO. The intramolecular H-bond is indicated as a dashed line.

Gly-Pro-Gly-Asp-Lys-Arg-Ala-Val-Leu-NH_2 peptide. However, in the most abundant species, a type-II β-turn motif for the *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp moiety is still present. The average molecular models of the *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-Lys-Arg-Ala-Val-Leu-NH_2 peptide, calculated by RMD using NOEs measured either in TFE/H_2O or in DMSO, are well superimposed in the *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp moiety (r.m.s.d. is 0.92 Å for the backbone). By superimposing the *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp backbone of *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-NH_2 and *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp-Lys-Arg-Ala-Val-Leu-NH_2 average molecular models in each solvent, r.m.s. deviations of 0.80 Å in TFE/H_2O and 0.95 Å in DMSO were found, thus confirming that the global fold of the cyclic moiety is preserved in both solvents. Larger differences are found for the tail conformation. Indeed, the -Lys-Arg-Ala-Val-Leu-NH_2 segment is in an extended conformation in the TFE/H_2O average molecular model, whilst it is bent toward the *cyclo*(1-5β)γAbu-Gly-Pro-Gly-Asp moiety in DMSO. These findings suggest that in an aqueous environment the Lys^6-Leu^{10} hydrophilic *N*-terminal sequence prefers an extended conformation, probably stabilized by solvent interactions.

Preliminary investigations show that PC1, a subtilisin/kexin family convertase, already used to study synthetic substrates of pro-oxytocin [32], is able to recognize and process our constrained substrate confirming the importance of a type-II β-turn moiety preceding the basic doublet in determining enzyme recognition.

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