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A case study of 2,2-dimethylthiazolidine as locked *cis* proline amide bond: synthesis, NMR and molecular modeling studies of a δ-conotoxin EVIA peptide analog†

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The δ-conotoxin EVIA from the *Conus ermineus* venom, a recently characterized toxin, exhibits *cis*–*trans* isomerism of the Leu[12]-Pro[13] bond associated with the triggering of its biological activity. In this paper we use the pseudoproline concept to target the presumed bioactive *cis* conformation. We report the design and the synthesis of loop 2 analogs from residue 8 to 18 containing either the *cis*-inducing Cys($\Psi^{Me,Me}$Pro)[13] unit **1** or the natural proline residue **2**. NMR studies in water and molecular modeling allowed us to identify the amide bond "locked" in a *cis* conformation for **1** as in the suggested bioactive form of the natural toxin.

Introduction

The prevalence of the proline residue in biological processes such as protein folding[1,2] and protein recognition[3,4] has led to the development of several mimics of proline intended to constrain the peptide backbone in reverse turn motifs or to alter the imide *cis*–*trans* ratio.[5] Indeed, unlike other amino acids which predominantly adopt the *trans* form, the imidic Xaa-Pro peptide bond readily exists in the *cis* as well as in the *trans* form.[6] A statistical analysis of the PDB[7] has shown that in a set of 571 protein structures, 5.2% had a *cis* Xaa-Pro bond and 0.03% a *cis* Xaa-nonPro bond. In several studies the *cis* form was suggested to play a crucial role in biological recognition processes.[8–10] In the case of the HIV-1_{IIIB} V3 peptide fragment, this isomerism has been extensively investigated by NMR. Compared to the *trans* form predominant in solution in the free state, J. Anglister and co-workers[11] have presented NMR evidence of a *cis* proline bond conformation in complex with the Fv fragment of an anti-gp120 antibody (0.5β antibody). The 18-residue HIV-1_{IIIB} V3 peptide presents a central QRGPGR loop and the affinity of 0.5β antibody for this V3 peptide was found to be comparable to its affinity for the intact glycoprotein. This study gives one of the first experimental evidences that a *cis* proline bond can be a prerequisite for the interaction with a receptor. It shows also that due to the isomerization observed between the two forms, it is often difficult to characterize the bioactive conformation.

Thus, the development of proline mimetics able to constrain the peptide bond exclusively to the *cis* conformation is of considerable utility. In addition to facilitate the investigation of the *cis*–*trans* isomerization, it would provide an invaluable tool for targeting *cis*-amide bonds in biologically relevant recognition processes.

There are many examples of proline analogs that have been developed in this way to stabilize the *cis* conformation.[5,12–17] Especially, the substituted thiazolidines or oxazolidines turned out to be useful and efficient tools.[18,19] In fact, within the heterocyclic system, the conformation preference is essentially driven by the number and steric requirements of the substituents at the position C2 which destabilize one of the two conformers. In particular the incorporation of the 2,2-dimethylated thiazolidine derivative, Cys($\Psi^{Me,Me}$Pro), in model peptides can induce up to 100% *cis* conformation in the preceding peptide bond.[19,20] In addition it has also been used successfully in biological peptides such as the HIV-1_{MN} V3 loop of gp120[21] and opioïd peptides.[22] These works have clearly shown by NMR studies that the *cis* conformation is highly predominant in solution and therefore have illustrated the efficiency of the dimethylated thiazolidine to lock a peptide bond in a *cis* conformation.

In this paper, we have targeted the proposed bioactive *cis* conformation of the loop 2 of δ-conotoxin EVIA by preparing a Cys($\Psi^{Me,Me}$Pro)-containing peptide analog. Previous NMR investigations by J.-M. Lancelin *et al.*[23] have shown that the δ-conotoxin EVIA was characterized by a 1/1 *cis*/*trans* isomerism of a Leu[12]-Pro[13] peptide bond in slow exchange on the NMR time scale. This isomerism occurs within a specific long disordered loop 2 including residues 11 to 19. The loop contributes to an important hydrophobic patch on the surface of the toxin suggesting that this *cis*/*trans* isomerism might be important for the toxin–receptor interaction. This toxin from the *Conus ermineus* venom, a fish-hunting sea snail, is the first toxin found to inhibit sodium channel inactivation in neuronal membranes from amphibians and mammals without affecting rat skeletal muscle and human cardiac muscle sodium channel subtypes.[24] Therefore, the Cys($\Psi^{Me,Me}$Pro) analog represents an interesting and useful tool for understanding and studying the specific properties of δ-conotoxin EVIA.

The targeted analog **1** Ac-GFASL[12]C($\Psi^{Me,Me}$pro)[13]ILKNG-NH_2 presents a 2,2-dimethylated thiazolidine instead of the proline involved in the Leu-Pro-Ile peptide bonds (Fig. 1). Unlike the major previous studies in which the thiazolidine analog was included in di- or tripeptides, in cyclic or Gly-Cys($\Psi^{Me,Me}$Pro)-Gly linear sequence, we investigated here its potential in a hydrophobic environment.

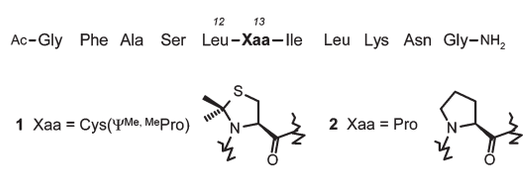

Fig. 1 Sequences of the targeted peptides **1** and **2**.

Herein we report the synthesis and the structural studies of **1** and its cognate peptide **2** by high field NMR and molecular modeling. The peptide **2** Ac-GFASL[12]P[13]ILKNG-NH_2 was indeed also prepared to determine qualitative structural changes produced by the replacement of the proline[13] by the pseudoproline. Studies have been done in water, close to physiological conditions and to our knowledge it is the first NMR structural study of thiazolidine containing peptide in this solvent.

Results and discussion

Synthesis

The most convenient and successful way to introduce a pseudoproline residue in a peptide sequence is to incorporate it as a preformed

† Electronic supplementary information (ESI) available: A list of $^3J_{NH\text{-}H\alpha}$ coupling constants and restraints used for molecular modeling. See http://www.rsc.org/suppdata/ob/b4/b408325c/

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dipeptide building block[25,26] during the solid phase peptide elongation. Using this procedure, several peptides including a pseudoproline (oxazolidine or thiazolidine) residue were prepared in good yields.[21,22] Therefore, we have chosen the standard protocol to introduce the dimethylthiazolidine in our targeted sequence **1** Ac-GFASL^{12}C($\Psi^{Me,Me}$Pro)^{13}ILKNG-NH$_2$. The formation of the carboxy fluoride of the Fmoc-protected pseudoproline-preceding amino acid followed by its coupling to the unprotected pseudoproline in solution is one of the most convenient ways to prepare the dipeptide building blocks. Fmoc amino acid fluorides were reported to be extremely well suited for hindered amino acids.[27] During this work, the (diethylamino)sulfur trifluoride DAST was used as fluorinating agent. This strategy allows access to the needed building block FmocLeu-Cys($\Psi^{Me,Me}$Pro) in very good yield (75% after precipitation) in large scale from the activated FmocLeu fluoride[28] and the 2,2-dimethyl-L-thiazolidine-4-carboxylic acid hydrochloride[29] (Fig. 2).

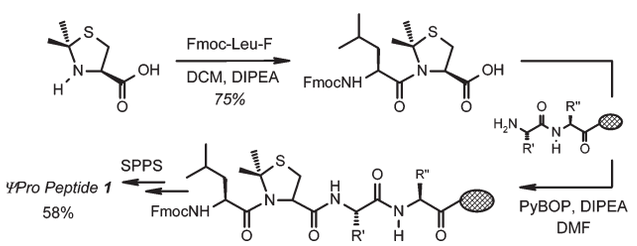

Fig. 2 Supported synthesis of peptide **1** using the dipeptide building block strategy.

The Sieber amid resin® was chosen to prepare the peptides **1** and **2** according to standard procedure of SPPS (solid support peptide synthesis) using Fmoc (9-fluorenylmethoxycarbonyl)/*t*Bu (*tert*-butyl) strategy. The linker of the Sieber amid resin allowed the cleavage of the peptides from the resin under very low acidic conditions (1% trifluoroacetic acid, TFA) yielding an amide at the C-terminal end. These mild conditions are necessary to prevent the opening of the dimethylthiazolidine moiety. Previous works have indeed reported loss of the thiazolidine integrity with more concentrated TFA solution.[21,26] Consequently very acid-labile protecting groups were used throughout the syntheses, trityl for serine and methoxytrityl for lysine. Preceding the cleavage from the resin with a deprotection cocktail of 1% TFA and 5% TIS (triisopropylsilane) as scavenger, the linear peptides were submitted to *N*-terminal acetylation. Under these conditions, the opening of thiazolidine ring has been prevented as judged by HPLC analysis and mass spectroscopy and the peptides **1** and **2** were yielded in 58% and 48%.

Resonance assignments

Assignments of all proton resonances of peptides **1** and **2** were carried out using TOCSY and NOESY experiments. Deviations of Hα chemical shifts from random coil values are not very large (≤0.1 ppm) indicating that both peptides are rather unstructured in solution.[30] Stereospecific assignment of the Hβ protons of the Cys($\Psi^{Me,Me}$Pro) and proline residue has been done on the basis of $J_{H\alpha\text{-}H\beta}$ coupling constants and NOE. The pro-*R* Hβ showed a coupling constant $^3J_{H\alpha\text{-}H\beta}$ greater than the $^3J_{H\alpha\text{-}H\beta}$ coupling constant involving the pro-*S* Hβ proton.[19] Thus, this allowed us to identify unambiguous NOE for the major region Leu^{12}-Cys($\Psi^{Me,Me}$Pro)^{13}-Ile^{14} or Leu^{12}-Pro^{13}-Ile^{14}. Many NOE cross-peaks permitted us to characterize the Leu^{12}-Cys($\Psi^{Me,Me}$Pro)^{13} amide bond in a *cis* conformation for peptide **1** (Fig. 3): significant NOE are indeed observed between Cys($\Psi^{Me,Me}$Pro)^{13}-Hα/Leu^{12}-Hα, Ile^{14}-NH/Leu^{12}-Hα, a medium NOE is observed between Ile^{14}-Hα/Leu^{12}-Hα and no NOE is seen between Leu^{12}-Hα/ Cys($\Psi^{Me,Me}$Pro)^{13}-Hδ, which is only compatible with Cys($\Psi^{Me,Me}$Pro)^{13}*cis* conformation.

In contrast, peptide **2** showed (Fig. 3) strong NOE between Leu^{12}-Hα/Pro^{13}-Hδ and no NOE between Pro^{13}-Hα/Leu^{12}-Hα, which is characteristic of a *trans* amide Leu^{12}-Pro^{13} bond.

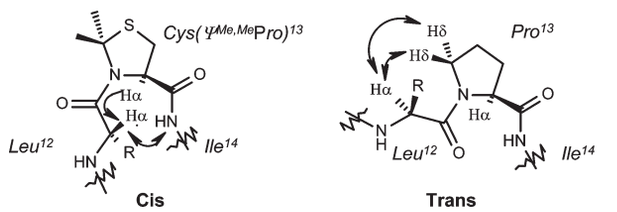

Fig. 3 Characteristic NOEs observed between Leu^{12}, Cys ($\Psi^{Me,Me}$Pro)^{13} and Ile^{14} residues in the *cis* and *trans* forms.

All these data suggest the presence of a turn centred on the Leu^{12} and Cys($\Psi^{Me,Me}$Pro)^{13} or Pro^{13} residues. In the two peptides **1** and **2**, no exchange peaks that could indicate the presence of a *trans* or *cis* amide bond respectively were observed in NMR spectra.

Structure calculations for peptide 1

Our main interest here was to characterize in detail the *cis* conformation. Therefore restrained molecular dynamics have been only performed on the Cys($\Psi^{Me,Me}$Pro)^{13} peptide **1** presenting a *cis* form as identified above by NMR.

NOE data and coupling constants were used to drive a set of 63 distances and 20 dihedral angle restraints. These restraints were then used to generate a set of 50 structures by a simulated annealing protocol as described in the Experimental section. Starting from completely random structures, all the calculated structures fitted the experimental data quite well. No more than 1 to 4 violations greater than 0.2 Å are observed per structure. Most of structures have the R_X and R_C factors issued from the IRMA calculation[31] around 0.6 and 0.05 which are reasonably low values and confirm the consistency of the structures with the experimental data. Analysis of the ϕ and ψ angles showed that all the residues are in the energetically favourable region of the Ramachandran plot. The Leu^{12}/Cys($\Psi^{Me,Me}$Pro)^{13} ω angle has a mean value of 6.4° characteristic of a *cis* amide bond.[32,33] For further details, 10 structures with the least restraints violations (no more than 1 violation greater than 0.2 Å and no violations greater than 5 degrees) and the lowest energy were selected and analysed with the MolMol[34] and Procheck programs.[35]

Superimposition of these 10 structures over all residues gave a poor rmsd of 2.75 Å for the backbone atoms and 3.20 Å for heavy atoms. Better results were obtained by superimposition of only residues 10 to 16, giving an averaged rmsd to the mean structure of 0.56 Å for the backbone atoms only. This can be likely explained by a lack of NOE cross peak for *N*- and *C*-termini because of end fraying commonly observed in short peptides. No particular secondary structure is detected but this is not very surprising as the sequence corresponds to the disordered loop 2 of conotoxin EVIA.[23] However, the Ser^{11}-Leu^{12}-Cys($\Psi^{Me,Me}$Pro)^{13}-Ile^{14} region is very well defined and the 10 structures can be superimposed with a rmsd of 0.22 Å for the backbone atoms and 0.69 Å for the heavy atoms (Fig. 4A).

A detailed analysis of the best representative structure described below shows that this region has the characteristics of a non hydrogen bonded VIb-β-turn (Fig. 4B). This is the common β-turn observed when a proline with a *cis* amide bond is located at position $i + 2$ in a turn.[36–39]

In particular, the distance between the Ser^{11} C=O and the Ile^{14} NH is around 4,6 Å so that no hydrogen bond can be formed. This is one of the characteristics observed in a VIb-β-turn: no intramolecular hydrogen bond is formed between the *i* carbonyl oxygen and the $i + 3$ amide hydrogen whereas it exists in the VIa-β-turn.[36,39] The backbone ϕ and ψ angles measured for Leu^{12} (residue $i + 1$) and Cys($\Psi^{Me,Me}$Pro)^{13} (residue $i + 2$) are around −75°/130° and −80°/142° respectively. They are close to the mean values found in the literature for a VIb-β-turn involving proline[37,40] the largest "deviation" observed being only for ϕ value of residue $i + 1$ (−75° instead of −120° commonly measured for ϕ). However, this difference to the mean value is not significant since it has been showed that the ϕ value of residue $i + 1$ can go from −60° to −170° when its χ^1 angle changed from −180° to 60°.[6]

It is noteworthy that this VIb-β-turn has also been observed by J.-M. Lancelin *et al.*[23] in the native disordered loop 2 of conotoxin

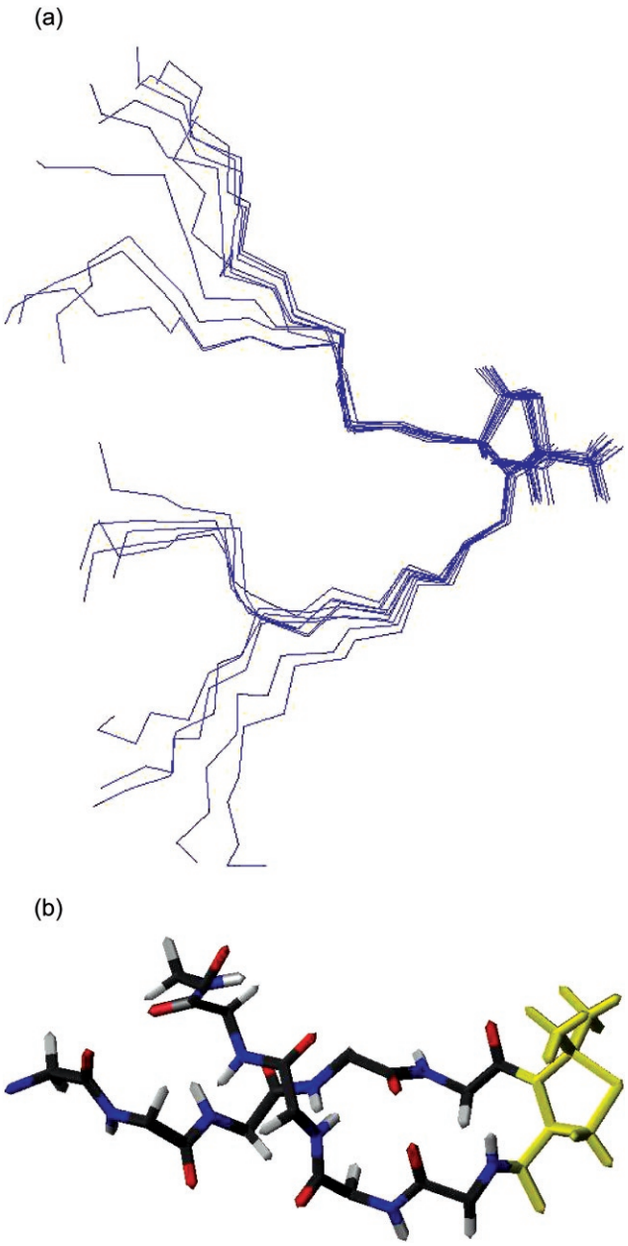

Fig. 4 (A) The 10 best NOE-restrained structures of peptide **1** showing main chain heavy atom and the whole Cys($\Psi^{Me,Me}$Pro)^{13} residue. The structures are overlaid in the Ser^{11}-Leu^{12}-Cys($\Psi^{Me,Me}$Pro)^{13}-Ile^{14} region, showing a very well defined type VIb β-turn. (B) The best representative structure of peptide **1**. The Cys($\Psi^{Me,Me}$Pro)^{13} residue is highlighted in yellow.

EVIA (pdb code 1G1Z). An overlay of the native turn with our calculated turn structure (Ser^{11}-Leu^{12}-Cys($\Psi^{Me,Me}$Pro)^{13}-Ile^{14}) shows similar residue conformations with an rmsd between the two structures of 0.89 Å over backbone atoms.

If we focus now only on the Cys($\Psi^{Me,Me}$Pro)^{13} residue, its ring is very well defined. It adopts a β-exo puckering according to significant values of $\chi^4 = -5.2°$, $\xi 1 = -93°$ and $\xi 2 = 30°$.[41] This preferential "DOWN" envelope conformation has already been observed in previous studies where Pro or Cys($\Psi^{Me,Me}$Pro) are involved in a *cis* amide bond.[6,19,42,43] It is also consistent with the $^3J_{H\alpha\text{-}H\beta}$ coupling constants previously measured.

Finally, the stabilization of the structure might be reinforced by a hydrogen bond possibly formed between Leu^{15} NH and Ile^{14} CO.

Conclusion

In the present work, we have prepared efficiently a Cys($\Psi^{Me,Me}$Pro) containing peptide analog of the loop 2 of δ-conotoxin EVIA. It was studied by NMR and molecular modeling in water. To our knowledge, it is the first time that water was used as solvent for structural investigations of a pseudoproline containing peptide. Our results show that the 2,2-dimethylthiazolidine can be advantageously used to exclusively constrain to a *cis* conformation the Leu^{12}-Cys($\Psi^{Me,Me}$Pro)^{13} bond of the loop 2, a conformation which is proposed to play a crucial role in recognition process of the toxin with its receptor.

On one hand our study is a new illustration of the powerful tool that represents the dimethylthiazolidine as a mimic of the *cis* proline bond. On the other hand this work opens the way to structure–activity relationship studies. Indeed the VIb-β turn centered on Leu^{12} and Cys($\Psi^{Me,Me}$Pro)^{13} which has been characterized in detail by our structural analysis is similar to the one observed in the native δ-conotoxin EVIA. This represents the first step for a better understanding and study of the specific properties of δ-conotoxin EVIA on sodium channels in neuronal membranes.

Experimental

Peptide synthesis and characterization

All protected amino acids were purchased from Calbiochem-Novabiochem or Advanced ChemTech; reagents and solvents were purchased from Fluka, Aldrich, SdS or Carlo-Erba and used without purification. Reverse phase high pressure liquid chromatography (RP-HPLC) was performed on Waters equipment using C_{18} columns (Nucleosil 300 Å). The analytical column (250 × 4.6 mm) was operated at 1 mL min^{-1} and the preparative column (250 × 21 mm) at 22 mL min^{-1} with UV monitoring at 214 nm using an A–B gradient (buffer A: 0.09% TFA in water; buffer B: 0.09% TFA in 90% acetonitrile). Mass spectra were obtained by electron spray ionization (ESI-MS) on a VG Platform II or by chemical ionization (CI-MS) on a Thermofinnigan Polaris Q in the positive mode.

The dipeptide Fmoc-Leu^{12}-Cys($\Psi^{Me,Me}$Pro)^{13} was prepared under argon using Fmoc-LeuF (252 mg, 0.71 mmol)[28] with a small excess of 2,2-dimethyl-L-thiazolidine-4-carboxylic acid hydrochloride (171 mg, 0.86 mmol)[29] and 2 equivalents of diisopropylethylamine DIPEA (0.25 mL, 1.42 mmol) in dry CH_2Cl_2 (10 mL). After one hour at room temperature (CH_2Cl_2/MeOH/AcOH, 90/10/0.1 for TLC analysis), the mixture was washed with an aqueous solution of citric acid 10% (2 × 10 mL), dried on Na_2SO_4 and evaporated. The crude product was purified by RP-HPLC (5–50% B, 30 min, C_{18}) to give 265 mg (75%) of Fmoc-Leu-Cys($\Psi^{Me,Me}$Pro). $C_{27}H_{32}N_2O_5S$ (496.1). t_R = 22.5 min (5–50% B, 30 min, C_{18}, 214 and 299 nm). CI-MS (NH_3, isobutane, *m*/*z*): 497.4 $[M + H]^+$. δ_H (300 MHz, DMSO-d_6): 7.9–7.3 (m, 9H, H_{ar}, N*H*), 4.8 (dd, 1H, C*H*$\alpha_{\Psi Pro}$), 4.3–4.1 (m, 4H, C*H*α_{Leu}, C*H*$_{Fmoc}$, CH_2O_{Fmoc}), 3.5 (dd, 1H, C$H_2\beta_{\Psi Pro}$), 3.3 (d, 1H, C$H_2\beta_{\Psi Pro}$), 2.0–1.3 (m, 9H, 2C$H_{3\Psi Pro}$, C*H*$_{Leu}$, CH_{2Leu}), 0.9 (d, 6H, 2CH_{3Leu}).

Peptides **1** and **2** were built up by a standard solid phase procedure using *N*-9-fluorenylmethyloxycarbonyl (Fmoc) protected amino acids. 125 mg of Sieber amide resin substituted at *ca.* 0.52 mmole g^{-1} was used to afford carboxyl terminus primary amide. Each coupling step was performed in dimethylformamide DMF (5 mL) during 40 min using 2 equivalents of the Fmoc-protected amino acid, 2 equivalents of PyBOP and 4 equivalents of DIPEA. The same reaction time was applied in the synthesis of peptide **1** for the coupling of the Fmoc-Leu-Cys($\Psi^{Me,Me}$Pro). For the introduction of the asparagine residue, 2 equivalents of the pre-activated Fmoc-Asn-Opfp were used in presence of DIPEA. After each coupling step, the completeness of the reaction was controlled by a TNBS test. Deprotection of Fmoc-protected amine groups was achieved during 10 min using a 20% of piperidine in DMF solution (5 mL). After the last Fmoc deprotection, peptides were N-acetylated on the resin using a solution of acetic acid, PyBOP and DIPEA during 20 minutes in CH_2Cl_2. Finally, peptides were deprotected and cleaved from the resin by a solution of TFA/TIS/CH_2Cl_2 1/5/94 (5 mL). The cleaving solutions were evaporated and the salt-containing products were precipitated and washed with diethyl ether. The crude peptides were then purified by RP-HPLC (5–100% B, 30 min, C_{18}) to yield 50 mg (58%) and 40 mg (48%) of peptides **1** and **2**, respectively. The peptides were greater than 98% pure as judged by analytical RP-HPLC.

Peptide **1**. $C_{55}H_{90}N_{14}O_{14}S$ (1203.48). t_R = 20.3 min (5–100% B, 30 min, C_{18}, 214). ESI-MS (*m*/*z*): 1204.93 $[M + H]^+$, 602.25 $[M + 2H]^{2+}$.

Peptide **2**. $C_{54}H_{88}N_{14}O_{14}$ (1157.39). t_R = 19.2 min (5–100% B, 30 min, C_{18}, 214). ESI-MS (*m*/*z*): 579.85 $[M + 2H]^{2+}$, 386.9 $[M + 3H]^{3+}$.

Sample preparation

1 mg of **1** and **2** were dissolved in either 90%/10% H_2O/D_2O or 100% D_2O. The pH was adjusted to an uncorrected value of 3 using DCl. The final concentration of the samples was around 1.3 mM. No symptom of self aggregation was detected such as a concentration dependence of chemical shifts.

NMR spectroscopy

NMR experiments were carried out using a BRUKER Avance 500 MHz spectrometer. One and 2D ^{1}H NMR spectra were calibrated using 3-(trimethylsilyl)propionate-2,2,3,3-d_4 (TSP-d_4).

Spin system identification and sequential assignment were achieved by z-filtered TOCSY and NOESY experiments recorded at 283 K. The TOCSY spectra were recorded with a spin-lock time of 80 ms using a MLEV17 pulse train of 10 kHz. Three mixing times of 250, 110 and 80 ms were used for NOESY.

All 2D NMR experiments were recorded with 512 ($t1$) × 2048 ($t2$) complex data points. 128 scans were acquired over a spectra width of 5000 Hz in both dimensions.

Water resonance was suppressed either by a standard presaturation or by the sculpting gradient scheme using selective pulses of 3–4 ms.[44]

Data processing and analysis was performed using FELIX2000 program (MSI, San Diego, CA) on a Sunray workstation. Prior to Fourier transform, a sine-squared window function shifted by $\pi/4 - \pi/2$ was applied with a zero-filling in F1 dimension to 1k points.

NOE intensities and experimental restraints

NOE intensities were measured from NOESY spectra using the integration routine in the FELIX program. The calibration for the NOE intensities was achieved using the internuclear cross peak intensity of Hα protons of the glycine residue (distance 1.78 Å). Methylene protons without stereospecific assignments and unresolved diastereotopic methyl protons were treated as pseudoatoms and the correction factors were added to the upper and lower distances.

Several $^3J_{NH\text{-}H\alpha}$ coupling constants were measured around 7.5 Hz on the z-filtered TOCSY indicating that some residues populate predominately the β region of ϕ,Ψ space.[45] Thus ϕ and Ψ dihedral angles restraints were set to $-180° < \phi < -30°$ and $90° < \Psi < 180°$ with a force constant of 30 kcal mol^{-1} rad^{-2} for residues 9, 10, 11, 16 and 17. Structure calculations without these dihedral restraints were also carried out. This did not produce any structural modifications, and only a slight increase of the rmsd values was observed for the backbone atoms.

ω angles were constrained to $160° < \omega < -160°$ for residues 8–12 and 13–17 and to $-10° < \omega < 10°$ for residue 13 to maintain respectively *trans* and *cis* amide bond conformation, all with a force constant of 200 kcal mol^{-1} rad^{-2}.

A total of 44 intra-residue distances and 19 inter-residue distances were applied with different force constants: 100 kcal mol^{-1} Å^{-2} for significant restraints involving protons of Leu^{12}, Cys($\Psi^{Me,Me}$Pro)^{13} and Ile^{14} residues and 50 kcal mol^{-1} Å^{-2} for the others. Characteristic restraints involving protons located in the turn (Fig. 3) were in the range of 3–3.6 Å for Ile^{14}-NH/Leu^{12}-Hα, 2.4–3.1 Å for Cys($\Psi^{Me,Me}$Pro)^{13}-Hα/Leu^{12}-Hα and 3.6–4.1 Å for Ile^{14}-Hα/Leu^{12}-Hα.

Molecular modeling

Calculations were performed on a workstation using InsightII 2000 and Discover 2.98 (Biosym/Molecular Simulation). The energy of the system was calculated with the CVFF force-field.[46] A distance-dependent dielectric constant $\varepsilon = 4r$ was used to account for solvent effect. Newton's equation of motion was integrated using the Verlet algorithm with an integration time step of 0.5 fs.[47]

A starting structure was built using the Biopolymer module. A total of 50 structures were then generated starting from randomized coordinates. Experimental restraints (NOE and dihedral) and covalent interactions were gradually scaled up to each of these structures over 15 ps of dynamics calculations at 900 K. Then, MD was continued over 10 ps to scale up non-bonded interactions. The structures generated were slowly cooled down to 300 K by steps of 100 K in 18 ps, while non-bonded interactions were still raised up. Molecules were equilibrated at 300 K during 5 ps with full non-bonded interactions. At the end of these calculations, all 50 stored structures were energy minimized starting with steepest descent followed by conjugated gradients until the derivative of the energy fell below 0.001 kcal mol^{-1} Å^{-1} level.

The structure with the lowest energy was chosen and the iterative relaxation matrix approach (IRMA)[31] was used to generate more accurate distance restraints. Back calculated 2D NOE spectra were generated for comparison with experimental 2D NOE spectra (calculation of R_c and R_x factors).

The refined distances were introduced in the molecular dynamics calculations and the protocol was repeated until good convergence between back-calculated and experimental NOESY spectra was achieved.

10 structures with the least restraint violations and the lowest energy were then selected and analysed with MolMol[34] and Procheck programs.[35] The coordinates of the ensemble of these 10 structures have been deposited in the Protein Data Bank (pdb code: 1SBU).

Acknowledgements

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