Crystal Structure of a Synthetic Cyclodecapeptide for Template-Assembled Synthetic Protein Design

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The structural prototype of a new generation of regioselectively addressable functionalized templates (RAFTs) for use in protein de novo design has been synthesized and crystallized. The structure of the aromatically substituted cyclodecapeptide was determined by X-ray diffraction; it consists of an antiparallel β sheet spanned by heterochirally induced type II′ β turns, similar to that observed in gramicidin S. The three-dimensional structure of the artificial template was also examined by an NMR spectroscopic analysis in solution and shown to be compatible with a β-sheet plane suitable for accommodating secondary functional peptide fragments for the synthesis of template-assembled synthetic proteins (TASPs).

KEYWORDS:

conformation analysis · cyclopeptides · peptides · protein design · structure elucidation

Introduction

Topological templates are synthetic scaffolds that direct functional groups or structural elements in well-defined spatial arrangements.[1] If their conformational properties are compatible with stereochemical requirements, they ideally mimic structural and functional features of peptide ligands and protein surfaces, such as discontinuous epitopes, binding and catalytic sites. Synthetic constructs find widespread application in protein de novo design based on the template-assembled synthetic protein (TASP) approach and in peptide mimicry for drug design.[2] Examples of molecular scaffolds used in this connection include porphyrins,[3] steroids,[4] calixarenes,[5] glycosides,[6] and a variety of cyclic peptides and peptidomimetics,[7] referable to a general concept of conformational constraint analogues.[8]

Studies in the Lausanne laboratory have focused on the development of regioselectively addressable functionalized templates (RAFTs; for a recent example involving VCAM-1 side chain presentation see ref. [9]). These topological templates are cyclic peptides incorporating two prolylglycine sequences as β-turn[10] inducers to constrain their backbone conformation into an antiparallel β sheet. An orthogonal protection scheme for the template amino acid side chains, that is, lysines, cysteines, or glutamic acids, through the assembly of suitably protected building blocks allows regioselective functionalization above or below the plane of the β sheet (Figure 1 a).[11] RAFT molecules are easily accessible through a combination of solid-phase (modular assembly of the linear sequence) and solution (cyclization) techniques. Their versatility and potential in protein de novo design and peptide mimicry have been demonstrated.[12]

Cyclodecapeptides based on two homochiral L-Pro-Gly type II β-turn hairpins so far investigated showed significant backbone flexibility.[13] Therefore, we designed a new generation of RAFT, aimed at effectively restricting the template conformation to a double β-II′ hairpin similar to that found for gramicidin S in the crystal state.[14] The key feature of this design is the incorporation of two copies of the D-Pro-Gly sequence, which, as a type II′ β-turn promoter, is expected to be a better nucleator of the β-hairpin conformation.[15] So far, crystal structures of cyclic peptides of comparable size with the potential to serve as a structural basis for a functionalizable scaffold include—besides the paradigmatic case of gramicidin S[14]—cyclodecapeptides of the antamanide[16] and cycloleonuripeptide D[17] class. Those compounds feature contiguous repeats of more than one homochiral proline residues and can therefore not be envisaged as first-order mimetics of antiparallel β sheets. In light of the

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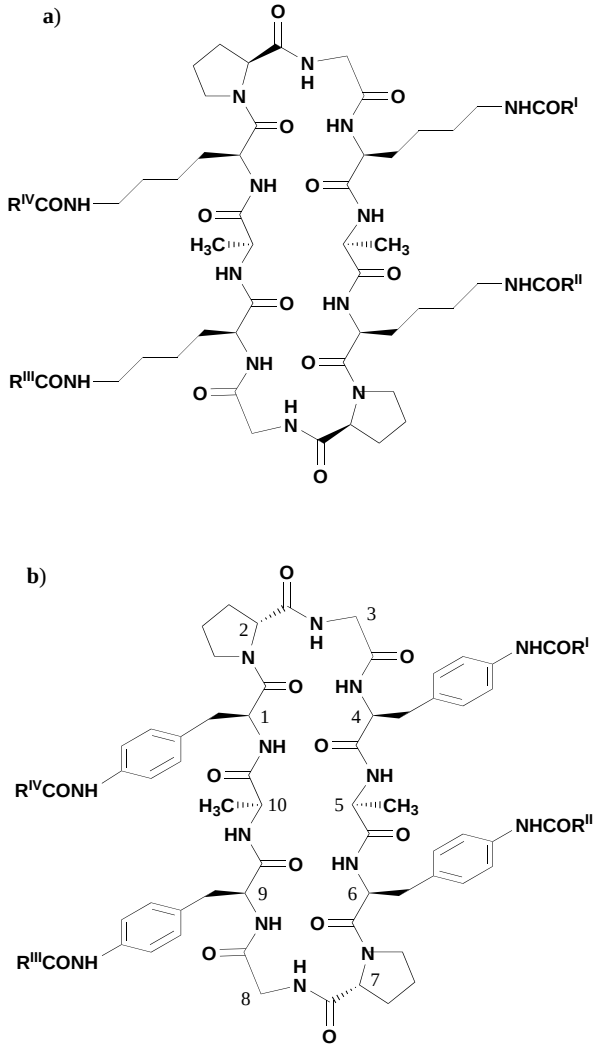

Figure 1. *Regioselectively addressable functional templates (RAFTs). a) Cyclic decapeptide of type* c*[Lys-Ala-Lys-Pro-Gly]$_2$. b) New generation of RAFT molecules of type* c*[aza-Tyr-Ala-aza-Tyr-Ala-*D*-Pro-Gly]$_2$. R^{I} – R^{IV} represent functional groups or peptide blocks for potential use in protein design and mimicry.*

present evidence, a homochiral cyclodecapeptide with the side chain pattern of gramicidin S adopting an antiparallel hairpin conformation spanned by type II and type I hairpin turns in the crystal state[18] will have to be considered as potentially flexible in solution. A number of smaller cyclopeptides characterized in detail[19] seem to be too small to accommodate the propensity of functional side chains required for TASP de novo design.

As structural prototype of this new RAFT generation we chose *cyclo*(Ala-*p*NO_2Phe-D-Pro-Gly-*p*NO_2Phe)$_2$ (**1**; Figure 1 b) for the following reasons: The *para*-nitrophenylalanine (*p*NO_2Phe or *p*NF) residues occupy the sequence positions corresponding to the potential attachment sites of peptide chains for the design of TASP molecules. A further working hypothesis behind the choice of *p*NF lies in its reduced side chain conformational flexibility compared to the commonly used Lys or other aliphatic proteinogenic amino acids with a terminal functional group. This property, in turn, was expected to enhance the chance of obtaining a crystalline compound stabilized by interresidual aromatic stacking interactions, amenable to X-ray diffraction analysis. To confirm the correctness of this structure – property-oriented approach, we herein report the conformational characterization of this new RAFT prototype in the crystal state and provide supporting two-dimensional (2D) ^{1}H NMR spectroscopic evidence for its conformation in solution.

Results

Crystal-state conformation

The molecular structure of **1**·H_2O as determined by X-ray diffraction is shown in Figure 2. Relevant torsion angles[20] are

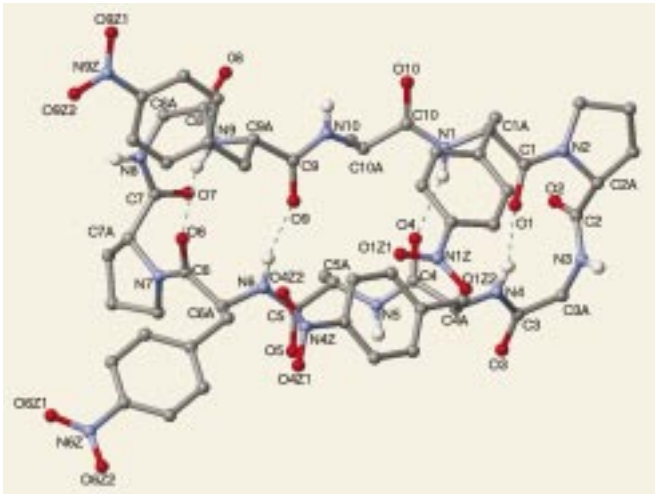

Figure 2. *X-ray crystal structure of* cyclo(L-p*NF-*D*-Pro-Gly-*L*-p*NF-*L*-Ala)$_2$ (**1**) as viewed perpendicularly to the β-sheet plane. Atom numbering is indicated for the nitro groups of* p*NF residues and all backbone atoms. Intramolecular hydrogen bonds are shown as dashed lines.*

listed in Table 1 and the intra- and intermolecular hydrogen bond parameters in Table 2. All peptide bonds are in the *trans* configuration. The largest deviation ($|\Delta\vartheta| = 10.3(5)^\circ$) from *trans* planarity is observed at the *p*NF^6-D-Pro^7 peptide bond. The cyclodecapeptide backbone is folded into an antiparallel β-sheet conformation terminating at both ends each with a type II′ β turn having the D-Pro^2-Gly^3 and D-Pro^7-Gly^8 residues at the corner positions. The antiparallel conformation is stabilized by four alternating N–H···O=C intramolecular H bonds (Table 2). The N4···O1 and N6···O9 H bonds are significantly stronger[21] than their alternating counterparts N1···O4 and N9···O6, respectively. The values of the backbone torsion angles for residues 1 – 5 are similar to those of the corresponding residues 6 – 10, the largest difference being observed between *p*NF^1 and *p*NF^6 residues (Table 1). As a result of the pleated nature of the β sheet (Figure 3) the Ala^5 and Ala^{10} side chains protrude from one side of the average plane of the cyclopeptide ring, while the side chains of all four *p*NF residues point out from the opposite side.

The distances between the C^β carbon atoms of the *p*NF residues flanking the β turns are 5.511(6) Å and 4.683(6) Å for

Table 1. *Relevant torsion angles in the X-ray crystal structure of* cyclo(L-p*NF*-D-*Pro*-*Gly*-L-p*NF*-L-*Ala*)$_2$ *monohydrate.*

Torsion angle [°]	L-*p*NF[1]	D-Pro[2]	Gly[3]	L-*p*NF[4]	L-Ala[5]	L-*p*NF[6]	D-Pro[7]	Gly[8]	L-*p*NF[9]	L-Ala[10]
ϕ	−167.2(5)	51.3(7)	−96.4(7)	−106.1(6)	−142.4(5)	−141.6(6)	55.7(7)	−84.1(9)	−91.3(7)	−150.0(6)
ψ	145.8(5)	−126.1(5)	12.4(8)	135.2(5)	136.7(6)	96.5(6)	−128.8(6)	0.2(10)	114.6(6)	141.0(5)
ω	−177.5(5)	176.5(5)	−175.3(5)	172.0(5)	173.4(5)	−169.7(5)	177.5(6)	176.2(6)	−171.5(6)	179.4(5)
χ^1	46.1(6)			172.4(4)		−167.6(5)			−67.1(6)	
$\chi^{2,1}$	−98.6(5)			−89.8(5)		−56.6(7)			−64.8(6)	
$\chi^{2,2}$	82.0(5)			92.6(5)		123.4(5)			113.2(5)	

Table 2. *Intra- and intermolecular H-bond parameters for* cyclo(L-p*NF*-D-*Pro*-*Gly*-L-p*NF*-L-*Ala*)$_2$ *monohydrate.*

Donor D-H	Acceptor A	Distance D···A [Å]	Distance H···A [Å]	Angle D-H···A [°]	Symmetry equivalence of A
Intramolecular					
N1-H01	O4	3.006(6)	2.19	160	*x*, *y*, *z*
N4-H04	O1	2.864(6)	2.04	161	*x*, *y*, *z*
N6-H06	O9	2.821(6)	2.00	159	*x*, *y*, *z*
N9-H09	O6	2.994(7)	2.19	156	*x*, *y*, *z*
Intermolecular					
N3-H03	O1W	3.090(12)	2.30	153	*x*, *y*, *z*
N5-H05	O10	2.999(6)	2.17	161	$^1/_2+x$, $^1/_2-y$, $1-z$
N8-H08	O6Z2	3.060(10)	2.25	157	$-^1/_2+x$, $^3/_2-y$, $1-z$
N10-H010	O3	3.056(6)	2.24	160	$-^1/_2+x$, $^1/_2-y$, $1-z$
O1W-H1W	O8	2.725(11)	1.90	175	$^1/_2+x$, $^1/_2-y$, $1-z$
O1W-H2W	O1Z1	3.144(16)	2.29	171	$2-x$, $-^1/_2+y$, $^1/_2-z$

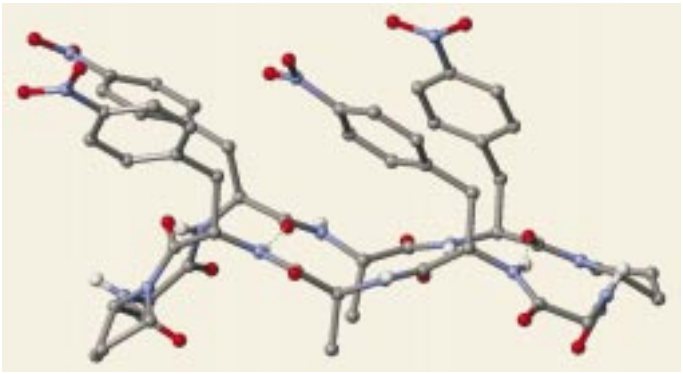

Figure 3. *X-ray crystal structure of* cyclo(L-p*NF*-D-*Pro*-*Gly*-L-p*NF*-L-*Ala*)$_2$ (**1**) *as viewed nearly parallel to the* β*-sheet plane.*

residues 1 – 4 and 9 – 6, respectively. A larger separation is observed between the pairs of the C^β carbon atoms longitudinally within the same strand (7.563(6) Å and 6.995(6) Å for residues 1 – 9 and 4 – 6, respectively). The orientations adopted by the side chains of the *p*NF residues are described by the χ^1, $\chi^{2,1}$, and $\chi^{2,2}$ torsion angles (Table 1). The χ^1 values found for *p*NF[6] and *p*NF[9] match the most probable rotamers (*trans* and *gauche*$^-$, respectively) associated with their backbone conformation, while the *gauche*$^+$ and *trans* side chain dispositions of *p*NF[1] and *p*NF[4], respectively, correspond to allowed rotamers, but not to the most probable ones, which would be again *trans* and *gauche*$^-$, respectively.[22] It has to be noted, however, that the side chain dispositions observed in the crystal structure must also meet packing requirements. In this respect, it is worth recalling that the *para*-nitro groups of both *p*NF[1] and *p*NF[6] residues are involved in intermolecular H bonds (Table 2). Compared to the C^β carbon atoms, a wider dispersion is observed among the distances between the N^ζ nitrogen atoms of the *p*NF side chains, which represent the potential attachment sites of peptide chains for the construction of TASP molecules. The distances are 4.505(8) Å for residues 1 – 4, 10.740(8) Å for residues 9 – 6, 11.136(7) Å for residues 1 – 9, and 7.044(9) Å for residues 4 – 6, respectively. This deviation from a more regular geometry is the result of the different orientations adopted by the *p*NF side chains as described above. In the crystal conformation, the N^ζ nitrogen atoms of residues 1 and 4 would seem to be too close to allow the build-up of two α helices on them. For comparison, the average distance between the α-helix axes in a leucine zipper coiled coil is 9.3 Å.[23] However, in a model built on the basis of the crystal structure, by changing only χ^1 torsion angles of *p*NF[1] and *p*NF[4] residues to match the statistically most probable *trans* and *gauche*$^-$ dispositions experimentally found for *p*NF[6] and *p*NF[9], the distance between N^ζ atoms of residues 1 and 4 becomes 11.5 Å, while those between the pair of N^ζ atoms of residues 1 – 9 and 4 – 6 amount to 16.4 and 15.3 Å, respectively. Model-building studies suggest that a four-helix bundle TASP molecule can be designed on this template without forcing the α helices to diverge, provided that interhelical side chain – side chain interactions have been properly engineered.

The antiparallel β-sheet conformation found in this cyclodecapeptide is remarkably similar to published (D-Pro-Gly)-induced single β-hairpin structures.[15b,f] However, these latter structures show increasing conformational variability with increasing distance from the nucleating type II′ β turn. Therefore, our cyclic double β-hairpin template seems to be better suited as a scaffold in that it allows a stricter conformational control.

The pyrrolidine rings of the D-Pro[2] and D-Pro[7] residues adopt conformations close to the *twist* ($^3T_4$) and the *envelope* (E_4) dispositions, respectively, described by the following puckering parameters:[24] $q_2 = 0.375(8)$ Å and $\phi_2 = 95.6(9)^\circ$ for D-Pro[2]; $q_2 = 0.365(8)$ Å and $\phi_2 = 106(1)^\circ$ for D-Pro[7].

The packing mode of the cyclodecapeptide monohydrate is governed by a complex network of intermolecular H bonds, satisfying all potential H-bond donors not already intramolecularly engaged (Table 2). Chains of symmetry-related molecules are observed parallel to the *a* direction through the formation of N5-H···O10 ($^1/_2+x$, $^1/_2-y$, $1-z$) and N10-H···O3 ($-^1/_2+x$, $^1/_2-y$, $1-z$) H bonds. This arrangement is further stabilized by a water-mediated interaction, involving an N3-H···O1W H bond within the same asymmetric unit, and the donation of an H bond

from the water molecule to the O8 carbonyl oxygen atom of a ($^1/_2+x$, $^1/_2-y$, $1-z$) symmetry-related peptide molecule. An additional H bond connects the N8-H group with one of the *para*-nitro oxygen atoms of the *p*NF[6] residue of a ($-^1/_2+x$, $^3/_2-y$, 1-*z*) symmetry-related peptide molecule. Finally, the water molecule acts as an H-bond donor to one of the *para*-nitro oxygen atoms of the *p*NF[1] residue of a ($2-x$, $-^1/_2+y$, $^1/_2-z$) symmetry-related peptide molecule, thus stabilizing chains of molecules parallel to the *b* direction.

Conformation in solution

To complement the crystallographic results, template **1** has been investigated by ^{1}H NMR spectroscopy in solution. Its 400-MHz spectrum in [D_6]DMSO is well resolved, allowing sequence-specific assignments by using a combination of TOCSY, DQF-COSY, ROESY, and NOESY experiments.[25] At variance from the crystal-state structure, the template is expected to adopt an averaged C_2-symmetry in solution when observed on the NMR time scale, which leads to the degeneracy of the NMR spectrum with only one set of signals corresponding to the sequence *p*NF[1,6]-D-Pro[2,7]-Gly[3,8]-*p*NF[4,9]-Ala[5,10]. Relevant NMR spectroscopic parameters for **1** are listed in Table 3. Large values of vicinal coupling constants $^3J_{NHC\alpha H} \geq 8.2$ Hz and low-field positions

Table 3. *Relevant ^{1}H NMR spectroscopic parameters for **1** in [D_6]DMSO.*[a]

	NH	α	β	γ	δ	$^3J_{NHC\alpha H}$	$\Delta\delta/\Delta T$
*p*NF[1,6]	8.57	4.92	3.21 2.96	7.36	8.02	8.2	−2.6
D-Pro[2,7]	–	4.39	2.00 1.83	1.83	3.55 3.45	–	–
Gly[3,8]	8.64	3.57 3.43	–	–	–	6.3	−5.7
*p*NF[4,9]	7.65	4.87	3.03 2.91	7.57	8.15	9.9	−1.7
Ala[5,10]	8.92	4.92	1.24	–	–	8.5	−5.7

[a] Chemical shifts δ (in ppm) and coupling constants $^3J_{NHC\alpha H}$ (in Hz) were measured at 303 K. Temperature coefficients of the amide signal chemical shift ($\Delta\delta/\Delta$T in ppb K^{-1}) were determined in the range of 300 – 330 K.

$\Delta\delta_{\alpha H} \geq 4.8$ for residues *p*NF[1,6], *p*NF[4,9], and Ala[5,10] strongly support β-strand conformations for the tripeptide sequences *p*NF[4]-Ala[5]-*p*NF[6] and *p*NF[9]-Ala[10]-*p*NF[1]. NOE data and temperature coefficients corroborate distinctive evidence[26] for a β-hairpin structure of template **1** in solution. Specifically, the presence of the D-Pro-Gly type II′ β turns is confirmed by the NOE connectivity NH_i-NH_{i+1} between Gly[3,8] and *p*NF[4,9], the strong NOE connectivity αH_i-NH_{i+1} between D-Pro[2,7] and Gly[3,8], and by the small temperature dependence of NH-*p*NF[4,9] and NH-*p*NF[1,6] signals suggesting that these amide protons are involved in hydrogen bonding. The NOE connectivity αH_i-δH_{i+1} between *p*NF[1,6] and D-Pro[2,7] is characteristic of the *trans* conformer for the amide bond between these residues. Interestingly, no *cis* – *trans* equilibrium could be detected for **1** on the NMR time scale at 303 K, suggesting a good overall isomer stability. Though in principle degenerate, the key long-range NOE connectivity NH-NH between *p*NF[1,6] and *p*NF[4,9] (Figure 4) is compatible with an antiparallel β-sheet structure of **1**. Interestingly, the relative strength of hydrogen bonds across the strands as measured by NMR experiments is in agreement with the X-ray crystallographic structure; however, due to symmetrization in solution all four H bonds have to be regarded as equivalent within the limits of experimental error. The N-H signals with uniformly high temperature coefficients can consistently be assigned to the non-H-bonded residues Gly[3,8] and Ala[5,10]. Some long-range NOE connectivities involving side chains are observed in the ROESY experiment. Most notably, NOE connectivities αH_i-γH_{i-1} between D-Pro[2,7] and *p*NF[1,6], αH_i-γH_{i+2} between D-Pro[2,7] and *p*NF[4,9], and γH-δH between *p*NF[1,6] and *p*NF[4,9] support the view that the aromatic side chains point in the same direction on one side of the average plane of the β sheet. Overall, this complementary study shows that the extended double β-II′-hairpin structure of template prototype **1**, as observed in the crystal state, is entirely compatible with 2D NMR data in solution.

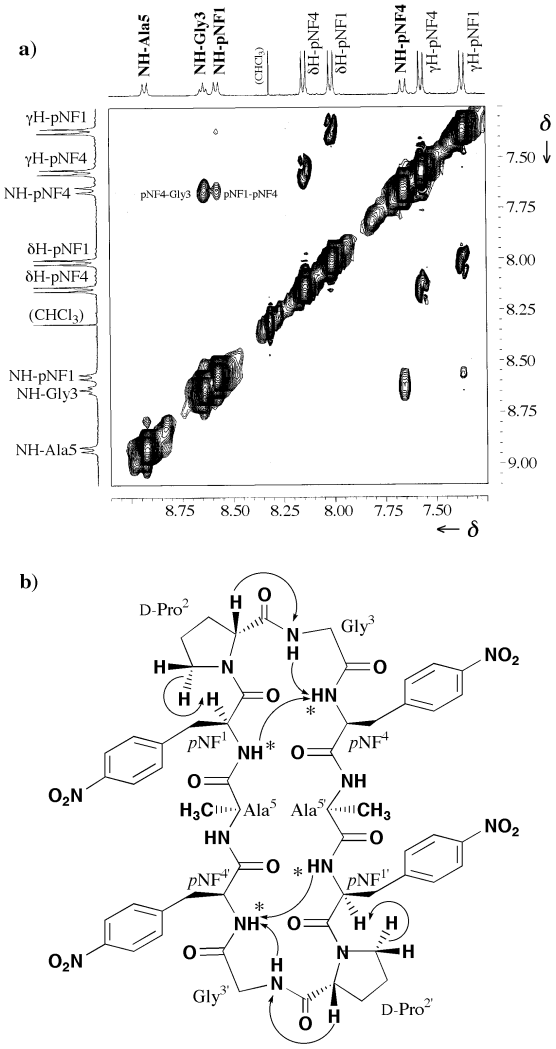

Figure 4. *a) Expanded N-H region of the 2D ^{1}H NMR NOESY spectrum of* cyclo-(L-pNF-D-Pro-Gly-L-pNF-L-Ala)$_2$ (**1**) *(conditions: [D_6]DMSO, 400 MHz, 303 K, τ_m = 150 ms). b) Principal NOE patterns and temperature shift rates showing compatibility with the antiparallel β-sheet conformation.*

In summary, the present data unequivocally prove the postulated three-dimensional structure of a de novo designed cyclic peptide template in the solid state and in solution, that is, an antiparallel β-sheet backbone conformation connected by two type II′ β-turn hairpins. In combination with recent techniques for functionalization and regioselective attachment of peptide blocks, this novel generation of topological templates represents a powerful tool in protein design and mimicry.

Experimental Section

Peptide synthesis: The linear sequence of the peptide was assembled on Fmoc-Ala-SASRIN (Fmoc = 9-fluorenylmethoxycarbonyl) according to Fmoc/*t*Bu protocols and then released from the solid support by treatment with TFA/dichloromethane (5:95). Cyclization was performed in DMF under high dilution conditions (10^{-3} M), using benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) as coupling reagent and diisopropylethylamine (DIEA) as base. The crude was purified by flash chromatography ($CHCl_3$/MeOH, 9:1) to obtain **1** in 57% overall yield. The final product was characterized by electrospray ionization mass spectrometry (ESI-MS). M.p. 210–215 °C; ESI-MS: *m/z*: calcd 1219.4 $[M+H]^+$, found 1219.6.

Crystal structure analysis: Single crystals of *cyclo*(L-*p*NF-D-Pro-Gly-L-*p*NF-L-Ala)$_2$ monohydrate were grown from methanol/water by slow evaporation (diffusion gradient method). Data collection was performed on a Bruker HI STAR area detector coupled to a Bruker M18X-HF rotating anode (Cu target). The crystal–detector distance was 7.00 cm; empirical formula $C_{56}H_{62}N_{14}O_{18}\cdot H_2O$; crystal dimensions $0.15\times0.10\times0.05$ mm, orthorhombic, space group $P2_12_12_1$, $a=18.986(5)$, $b=20.195(5)$, $c=16.665(4)$ Å, $V=6390(3)$ Å^3, $Z=4$, $\rho_{calcd}=1.286$ g cm^{-3}; $2\theta_{max}=101°$, $Cu_{K\alpha}$ radiation ($\lambda=1.54178$ Å), area detector ϑ-scan mode, $T=293(2)$ K. A total of 22 362 reflections were collected, of which 3457 were unique ($R_{int}=0.0792$). Intensities were corrected for Lorentz and polarization effects, no absorption correction was made ($\mu=0.831$ mm^{-1}). The structure was solved by direct methods with the SHELXS 97 program.[27] Refinement was carried out by full-matrix-block least squares on F^2 using all data, with all non-hydrogen atoms anisotropic, by application of the SHELXL 97 program,[28] allowing the positional parameters and the anisotropic displacement parameters of the non-hydrogen atoms to refine at alternate cycles. All phenyl rings were constrained to the idealized geometry. Restraints were applied to most of the 1–2 and 1–3 interatomic distances, as well as to all anisotropic displacement parameters of the non-hydrogen atoms. The data/restraints/parameters ratio was 3457:6001:755. H atoms were calculated at idealized positions, and refined as riding, with U_{iso} set equal to 1.2 (or 1.5 for the methyl groups and the water molecule) times the U_{eq} value of the parent atom. Final *R* indices: $R_1=0.0565$ [on $F\geq4\sigma(F)$] and $wR_2=0.1599$ (on F^2, all data). Residual electron density: $+0.447$ and -0.174 e Å^{-3}. Crystallographic data (excluding structure factors) for the structures reported in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication no. CCDC-147541. Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: (+44) 1223-336-033; e-mail: deposit@ccdc.cam.ac.uk).

NMR spectroscopic analysis: The sample for NMR analysis was prepared by dissolving *cyclo*(L-*p*NF-D-Pro-Gly-L-*p*NF-L-Ala)$_2$ (**1**) in [D$_6$]DMSO (5 mg in 0.5 mL). Data were collected on a Bruker 400DRX spectrometer at 400 MHz ($\tau_m=150$ ms). Chemical shifts were calibrated with reference to the residual DMSO signal (^{1}H, $\delta=2.49$; ^{13}C, $\delta=39.5$). 2D NMR experiments were typically acquired by using $2\,K\times512$ matrices over a 4000-Hz sweep width in both dimensions. Quadrature detection in the indirect dimension was achieved by using the TPPI procedure.[29] Scalar connectivities were recovered from 2D double-quantum filtration (DQF) COSY experiments.[30] Dipolar connectivities were obtained either by applying the conventional NOESY sequence[31] or the ROESY sequence[32] with optimized mixing times from 150 to 200 ms. A randomization of the mixing length (±5%) was introduced in the NOESY experiments in order to minimize coherence transfer. The spin lock mixing interval of the ROESY sequence was applied by coherent CW irradiation at $\gamma B_2/2\pi=2$ kHz. The standard sinebell squared routine was employed for apodization with a shift range of 60–90° and zero filling in both dimensions before 2D transformations were applied to end up with square matrices of $2\,K\times2\,K$ real-point data. Complete proton signals assignments were made by using (DQF) COSY experiments, HMQC was used to assign unambiguously the carbon signals. Coupling constants *J* were directly measured from the high-resolution 1D ^{1}H NMR spectra (±0.1 Hz).

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