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Synthesis and Conformational Characterisation of Hexameric β-Peptide Foldamers by Using Double POAC Spin Labelling and cw-EPR**

Karen Wright,*[a] Michel Wakselman,[a] Jean-Paul Mazaleyrat,[a] Lorenzo Franco,[b] Antonio Toffoletti,[b] Fernando Formaggio,[b] and Claudio Toniolo*[b]

Abstract: A selected set of terminally protected β-hexapeptides, each containing two nitroxide-based (3*R*,4*R*)-4-amino-1-oxyl-2,2,5,5-tetramethylpyrrolidine-3-carboxylic acid (POAC) residues combined with four (1*S*,2*S*)-2-aminocyclopentane-1-carboxylic acid (ACPC) residues, was synthesised by using solution methods and was fully characterised. The two POAC residues are separated in the sequences by different numbers of intervening ACPC residues. The conformational features of the doubly spin-labelled β-hexapeptides were examined in chloroform by FTIR absorption and continuous-wave electron paramagnetic resonance spectroscopic techniques. In particular, the biradical exchange coupling (*J*) between two POAC residues within each peptide indicates unambiguously that the secondary structure overwhelmingly adopted is the 12-helix. Taken together, these results support the view that POAC is an excellent β-amino acid for exploring this type of helical conformation in doubly labelled β-peptides.

Keywords: conformation analysis · EPR spectroscopy · IR spectroscopy · peptides

Introduction

Peptides are folded in many different types of helical conformations. Not unexpectedly, β-peptides, with their higher number of backbone single bonds per each amino acid unit, are known to adopt a variety of helices much larger than that authenticated for α-peptides.[1,2] Therefore, it is evident that the efforts of structural biochemists would be facilitated by the possibility of relying on a large array of physicochemical techniques.

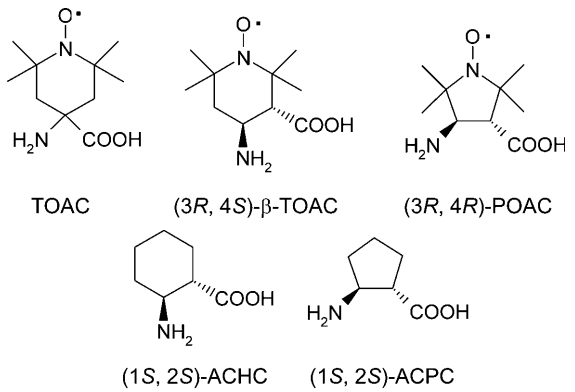
In particular, to solve the numerous 3D structural issues that arise in the study of model α-peptides, the use of double-labelled electron paramagnetic resonance (EPR) spectroscopy was explored.[3–7] In this method, nitroxide spin labels are covalently linked to two α-amino acid side chains in the peptide. These labels may interact over distances that are usually larger than those sampled by NOE interactions in NMR spectroscopy. Thus, double-labelled EPR provides a unique and complementary view of the local peptide structure. More specifically, the amino acid 4-amino-1-oxyl-2,2,6,6-tetramethylpiperidine-4-carboxylic acid (TOAC;[4,8–10] Scheme 1), with strong helix-promoting characteristics and a rigidly positioned nitroxide group in the six-membered ring piperidine structure, was extensively exploited to this end.[4–7]

We have explored the extension of this approach to β-peptide foldamers.[11] A model β-hexapeptide, containing two (3*R*,4*S*)-β-TOAC residues[12] combined with four (1*S*,2*S*)-2-aminocyclohexane carboxylic acid (ACHC) residues (Scheme 1), was synthesised and the preferred conformation (i.e., 3_{14}-helix)[1,2] was assessed in particular by continuous-wave (cw) EPR spectroscopic analysis.[12] Herein, we have expanded this area by utilising the enantiopure five-membered ring (3*R*,4*R*)-4-amino-1-oxyl-2,2,5,5-tetramethylpyrrolidine-3-carboxylic acid (POAC).[13–16] This β-amino acid has a backbone chemical structure identical to that of (1*S*,2*S*)-2-aminocyclopentane carboxylic acid (ACPC), the homo-oligo(β-peptide)s of which[17] are folded in the 2.6_{12}-(also termed 12-) helical conformation in the crystal state and in secondary-structure supporting solvents. According to authoritative review reports,[1,2] the two distinct, experi-

[a] Dr. K. Wright, Dr. M. Wakselman, Dr. J.-P. Mazaleyrat
ILV, UMR CNRS 8180, University of Versailles
78035 Versailles (France)
Fax: (+33) 01-3925-4452
E-mail: wright@chimie.uvsq.fr

[b] Dr. L. Franco, Prof. A. Toffoletti, Prof. F. Formaggio, Prof. C. Toniolo
Department of Chemistry, University of Padova
35131 Padova (Italy)
Fax: (+39) 049-827-5239
E-mail: claudio.toniolo@unipd.it

[**] POAC = (3*R*,4*R*)-4-amino-1-oxyl-2,2,5,5-tetramethylpyrrolidine-3-carboxylic acid; cw-EPR = continuous-wave electron paramagnetic resonance.



Scheme 1. Chemical structures of the nitroxide spin labelled α- or β-amino acids TOAC, β-TOAC, and (3*R*,4*R*)-4-amino-1-oxyl-2,2,5,5-tetramethylpyrrolidine-3-carboxylic acid (POAC) and the β-amino acids (1*S*,2*S*)-2-aminocyclohexane carboxylic acid (ACHC) and (1*S*,2*S*)-2-aminocyclopentane-1-carboxylic acid (ACPC).

mentally observed, types of helical structures promoted in β-peptides by the six- and five-membered, cyclic ACHC and ACPC residues should be attributed to differences in the degree of flexibility of the ring torsion angle -NH-C-C-CO-. Terminally protected β-peptide hexamers were chosen for this study because they are sufficiently long to form stable 12-helices.[1,2] The amino acid sequences of the four β-hexapeptides **1**–**4** investigated are shown in Scheme 2. The two guest nitroxide-containing probes were inserted at the (*i*, *i*+1), (*i*, *i*+2), (*i*, *i*+3), and (*i*, *i*+4) relative positions, respectively. Peptide synthesis was carried out in solution by using a segment-condensation approach. Initial evidence of the overall conformation of the β-hexapeptides was achieved by FTIR absorption spectroscopic analysis. A preliminary report of a limited part of this work has already been published.[18]

Results and Discussion

Peptide synthesis: The five-membered ring (3*R*,4*R*)-POAC and (1*S*,2*S*)-ACPC β-amino acid residues (Scheme 1) were incorporated into four doubly spin labelled β-hexapeptides, in which the POAC residues were placed at positions *i*, *i*+*n* (*n* = 1–4) by using solution-phase synthesis (Scheme 2).

The (3*R*,4*R*)-POAC derivatives used were prepared as previously described.[16] The synthetic strategy employed was N→C chain elongation of peptide segments protected with an *N*-Boc group by using C-activation with 2-(7-aza-1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU).[19,20] As the nitroxide group of POAC is sensitive to acidic conditions,[2,6–8,16] N-deprotection of the Boc group was avoided and the base-labile fluorenyl-9-methyloxycarbonyl (Fmoc) group was used for temporary N-protection of the segments.

The synthesis and characterisation of hexapeptide **3** and its synthetic intermediates have already been reported.[16] For hexapeptide **1**, we started from the protected derivative Fmoc-(3*R*,4*R*)-POAC-O-(*aR*)-Bin (Bin = binaphthol),[16] which was N-deprotected in CH_3CN/Et_2NH and coupled with Boc-(1*S*,2*S*)-ACPC-(3*R*,4*R*)-POAC-OH[16] with HATU in the presence of *N*,*N*-diisopropyl-*N*-ethylamine (DIEA) to afford tripeptide Boc-(1*S*,2*S*)-ACPC-(3*R*,4*R*)-POAC-(3*R*,4*R*)-POAC-O-(*aR*)-Bin in 62% yield (Scheme 3). This tripeptide ester was saponified with sodium hydroxide in $MeOH/H_2O/THF$ at 50 °C, and the resulting crude product Boc-(1*S*,2*S*)-ACPC-(3*R*,4*R*)-POAC-(3*R*,4*R*)-POAC-OH was coupled with HCl·H-[(1*S*,2*S*)-ACPC]$_3$-OMe (obtained by *N*-deprotection in HCl/dioxane of Boc-[(1*S*,2*S*)-ACPC]$_3$-OMe) to afford hexapeptide **1**.

The hexapeptide **2** was also synthesised by coupling two tripeptide segments. Fmoc-(3*R*,4*R*)-POAC-OH[14,16] was

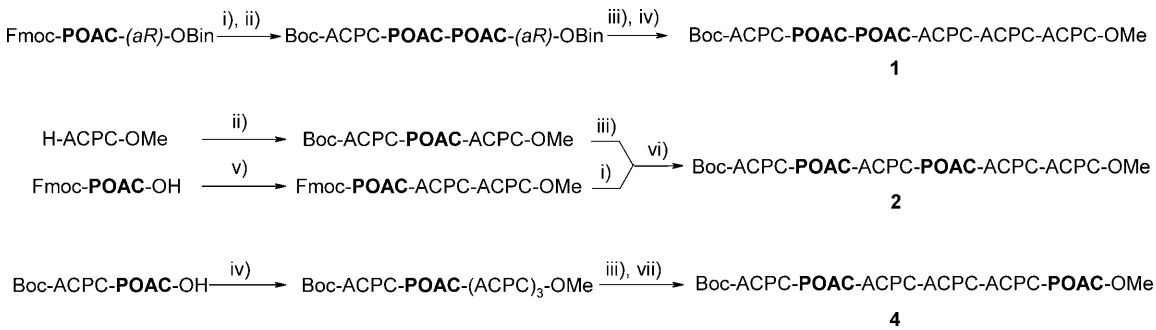
Boc-(1*S*,2*S*)-ACPC-(3*R*,4*R*)-**POAC**-(3*R*,4*R*)-**POAC**-(1*S*,2*S*)-ACPC-(1*S*,2*S*)-ACPC-(1*S*,2*S*)-ACPC-OMe **1**

Boc-(1*S*,2*S*)-ACPC-(3*R*,4*R*)-**POAC**-(1*S*,2*S*)-ACPC-(3*R*,4*R*)-**POAC**-(1*S*,2*S*)-ACPC-(1*S*,2*S*)-ACPC-OMe **2**

Boc-(1*S*,2*S*)-ACPC-(3*R*,4*R*)-**POAC**-(1*S*,2*S*)-ACPC-(1*S*,2*S*)-ACPC-(3*R*,4*R*)-**POAC**-(1*S*,2*S*)-ACPC-OMe **3**

Boc-(1*S*,2*S*)-ACPC-(3*R*,4*R*)-**POAC**-(1*S*,2*S*)-ACPC-(1*S*,2*S*)-ACPC-(1*S*,2*S*)-ACPC-(3*R*,4*R*)-**POAC**-OMe **4**

Scheme 2. POAC/ACPC β-peptides discussed herein. Boc = *tert*-butyloxycarbonyl.



Scheme 3. Synthetic path for the preparation of the hexapeptides **1**, **2**, and **4**. i) CH_3CN/Et_2NH (4:1), RT; ii) Boc-(1*S*,2*S*)-ACPC-(3*R*,4*R*)-POAC-OH, HATU, DIEA, THF, RT; iii) NaOH, $MeOH/H_2O/THF$ (1:1:1), 50 °C; iv) HCl·H-[(1*S*,2*S*)-ACPC]$_3$-OMe, HATU, DIEA, THF, RT; v) HCl·H-[(1*S*,2*S*)-ACPC]$_2$-OMe, HATU, DIEA, THF, RT; vi) HATU, DIEA, THF, RT; vii) H-(3*R*,4*R*)-POAC-OMe, HATU, DIEA, THF, RT.

coupled with HCl·H-[(1*S*,2*S*)-ACPC]$_2$-OMe[16] to afford Fmoc-(3*R*,4*R*)-POAC-(1*S*,2*S*)-ACPC-(1*S*,2*S*)-ACPC-OMe in 62% yield, which was N-deprotected. Boc-(1*S*,2*S*)-ACPC-(3*R*,4*R*)-POAC-OH and HCl·H-(1*S*,2*S*)-ACPC-OMe[16] were coupled to form tripeptide Boc-(1*S*,2*S*)-ACPC-(3*R*,4*R*)-POAC-(1*S*,2*S*)-ACPC-OMe. Saponification and coupling of the two segments gave hexapeptide **2** in 47% yield.

Finally, hexapeptide **4** was formed from pentapeptide Boc-(1*S*,2*S*)-ACPC-(3*R*,4*R*)-POAC-[(1*S*,2*S*)-ACPC]$_3$-OH (from the coupling of Boc-(1*S*,2*S*)-ACPC-(3*R*,4*R*)-POAC-OH and HCl·H-[(1*S*,2*S*)-ACPC]$_3$-OMe followed by saponification), and H-(3*R*,4*R*)-POAC-OMe[16] in 60% yield. Detailed characterisations of all newly synthesised peptides are described in the Experimental Section.

Conformational analysis: We performed a preliminary conformational analysis of hexapeptides **1**–**4** in solution by using FTIR absorption spectroscopy (neither NMR[21] nor CD[22] spectroscopic techniques could be advantageously exploited because the peptides are characterised by paramagnetic and chromophoric nitroxide free radicals).

In the conformationally informative N–H stretching (amide A) region, peptides **1**–**4** generally exhibit two bands in $CDCl_3$, one weaker and located at $\tilde{\nu}=3442$–$3444\ cm^{-1}$ (associated with "free", solvated N–H vibrations)[23–27] and the other, remarkably more intense and broader, in the $\tilde{\nu}=3268$–$3241\ cm^{-1}$ range (associated with hydrogen-bonded N–H vibrations; for a typical spectrum (i.e., peptide **3**), see Figure 1). Moreover, the results of our concentration-dependence (i.e., 1.0–0.1 mM) study (not shown) clearly indicate that there is no evidence for significant self-association through intermolecular hydrogen bonding; that is, all the NH groups are either solvated by $CDCl_3$ or, for the most part, intramolecularly hydrogen bonded. These findings are typical of medium-length peptides largely folded in well-developed helical secondary structures.

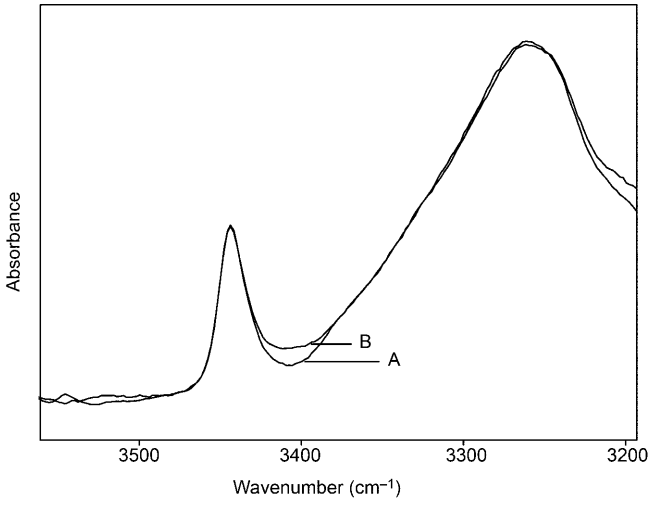


Figure 1. FTIR absorption spectra (N–H stretching region) of hexapeptide **3** at concentrations A) 1.0 and B) 0.1 mM in $CDCl_3$. Cells with path lengths of 1.0 and 10 mm, respectively, were used.

There are only a few studies that discuss the IR amide I ($\tilde{\nu}=1700$–$1600\ cm^{-1}$) region of β-peptides. Muñoz-Guerra and co-workers[28] reported this band at $\tilde{\nu}=1654$–$1656\ cm^{-1}$ for films of three different poly(β-peptide)s with α-alkyl side chains. Recently, DeGrado and co-workers[29] showed that the amide I′ spectrum of a helical β-peptide in D_2O is quite complex because it is characterised by three bands centred at $\tilde{\nu}=1612$, 1624, and $1650\ cm^{-1}$. Finally, our ACHC/β-TOAC model hexapeptide mentioned above[12] shows a main band at $\tilde{\nu}=1654\ cm^{-1}$ accompanied by weak shoulders at $\tilde{\nu}=1624$ and $1682\ cm^{-1}$ in $CDCl_3$. All of these β-peptides are folded in the 14-helix structure. The ACPC/POAC hexapeptides **1**–**4** studied in this work exhibit three bands at $\tilde{\nu}=1686$–1688 (weak), 1654–1666 (strong), and 1622–$1638\ cm^{-1}$ (weak) in $CDCl_3$ (spectra not shown). At this point, by considering the quite different environments utilised for the IR spectroscopic investigations of the 14-helical peptides and the absence of reported reference spectra for 12-helical peptides, any assignment of hexapeptides **1**–**4** to a specific helix conformation on the basis of their IR absorption spectra would only be rather hazardous.

The determination of the spin–spin distance in doubly nitroxide-labelled molecular systems can be obtained by using a number of EPR techniques;[30–34] most of them rely on the measurement of the dipolar interaction between the two electron spins. Unfortunately, for distances in the range expected for our peptides (i.e., 8–13 Å; see below), the measurement of the dipolar interaction in frozen solution is hampered by the almost coincident values of electron–electron dipolar exchange and hyperfine interactions. This phenomenon gives rise to complex cw-EPR spectra in frozen solution, from which extraction of the dipolar interaction alone by means of deconvolution or spectral fitting methods is rather unreliable. On the other hand, all anisotropic interactions are averaged out in fluid solutions, thus leaving only the exchange interaction to influence the EPR spectrum. Although the exact dependence on the distance of the exchange interaction between two electron spins is not easily obtained, it is however true that in a series of rigid, doubly labelled molecules the exchange interaction depends solely on the distance between the two labels, provided that the solvent and temperature are the same. For this reason, in our case, liquid-phase EPR spectra offered the best possibility of determining the relative intramolecular distances between the two nitroxide probes, although in a qualitative way.

The cw-EPR spectra of peptides **1**–**4** in $CHCl_3$ at 295 K are reported in Figure 2 along with the reference amino acid derivative Fmoc-POAC-OH at the same temperature. The spectrum of the amino acid derivative is composed of three lines due to the hyperfine interaction between the unpaired electron of the N–O group and the ^{14}N nuclear spin. The three lines are separated by the hyperfine coupling constant a_N (1.49 mT). Small satellite lines are also present, which are due to the hyperfine interaction with the ^{13}C nuclear spins at different positions in the molecule ($a_{^{13}C}=0.495$ and 0.890 mT in 4 and 2 equivalent positions, respectively).

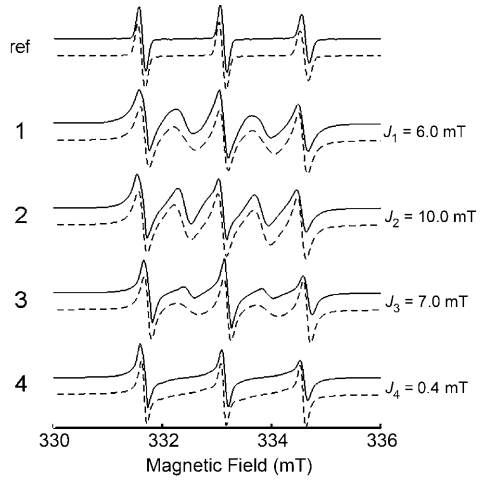


Figure 2. Experimental (full line) and calculated (dashed line) cw-EPR spectra of Fmoc-POAC-OH (reference) and the bis-nitroxide labelled POAC/ACPC hexapeptides **1**–**4** in $CHCl_3$ at 295 K. Peptide concentration: 1.0 mM. ref = reference.

In the case of the doubly spin-labelled peptides **1**–**4**, the EPR spectra are more complex (solid traces in Figure 2) and show additional lines that arise from the intramolecular spin–spin exchange interaction, *J*, between the unpaired electrons of the two nitroxide radicals. The well-resolved lines indicate that the intermolecular *J* interaction is negligible. The spectra have been fitted (dashed traces in Figure 2) to calculate the EPR line positions by using equations reported by Luckhurst[35] with $a_N = 1.46$ mT and $J_1 = 6.0$, $J_2 = 10.0$, $J_3 = 7.0$ and $J_4 = 0.4$ mT for peptides **1**–**4**, respectively. The strength of exchange interaction *J* depends on both the number of bonds between the unpaired electrons and their mutual distance. If the nitroxide groups are separated by many single bonds, the through-bond contribution to the value of *J* becomes negligible relative to the through-space contribution. The latter depends on the conformation of the biradical molecules and is responsible for the observed order of exchange constants: $J_2 > J_3 \cong J_1 \gg J_4$

An inspection of molecular models (Figure 3), based on the structure revealed by X-ray diffraction studies of the ACPC β-homo-hexapeptide folded in the 12-helix conformation,[17] shows that the midpoints of the two nitroxide bonds are separated by about 8.5, 8.0, 8.5, and 13.5 Å in peptides **1**–**4**, respectively. This rank order of distances is in excellent agreement with the best-fit *J* values mentioned above and reported in Figure 2. From these results, we conclude that the ACPC/POAC β-hexapeptides do indeed adopt a 12-helix conformation at room temperature. As for the thermal helix stability of our β-peptides, we recorded the cw-EPR spectra from 270 to 320 K. We did not observe any significant variation in the spectral parameters, apart from a slight decrease in the width of the three narrow lines present in all of the spectra. This property and the smoothness of the thermal change, both similar to those reported in previous studies on other β-peptides,[2] indicate a very limited, non-cooperative destructuration of the 12-helix of our compounds under these experimental conditions.

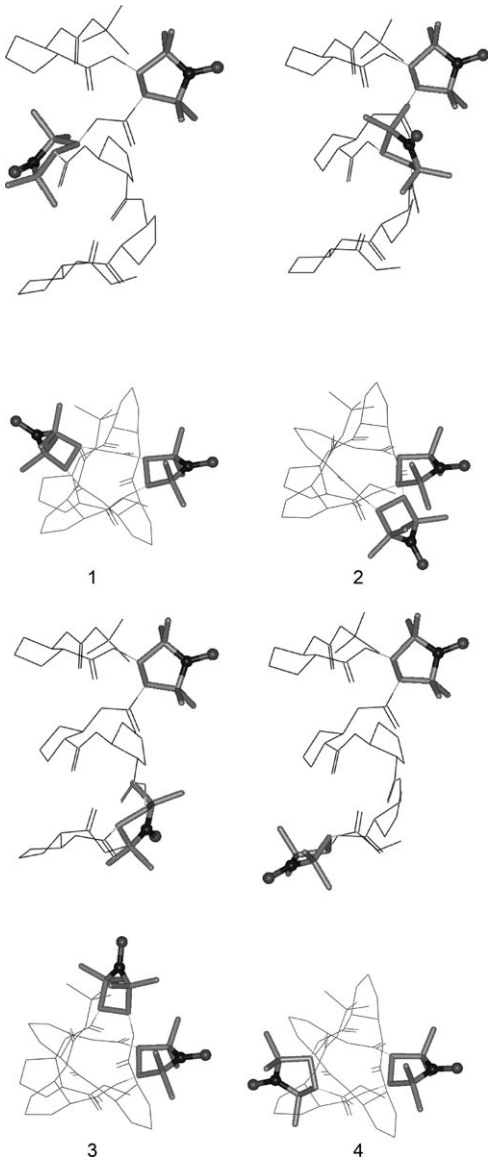


Figure 3. Computer-drawn molecular models for the bis-nitroxide-labelled POAC/ACPC hexapeptides **1**–**4** in the 12-helix conformation (top: view perpendicular to the helix axis; bottom: view parallel to the helix axis).

Conclusion

We have prepared a series of host ACPC β-oligopeptides, each incorporating two guest POAC β-amino acids with a paramagnetic, stable nitroxide moiety in their cyclic structure at appropriate relative positions. The synthesis was carried out by employing solution methods that used a C-activation procedure with HATU.

Our preliminary FTIR absorption spectroscopic analysis provided general information on the global peptide conformation in $CDCl_3$, namely, that the β-hexamers largely adopt

an intramolecularly hydrogen-bonded, nonaggregated helix. Under the same experimental conditions, the additional results presented herein, in particular those results that refer to the exchange interaction *J*, demonstrate that cw-EPR measurements of the doubly POAC-labelled β-peptide foldamers is an extremely useful approach to probe in detail the nature of the helical structure formed. Specifically, it is reassuring that the bulkier, tetramethylated pyrrolidin-*N*-oxide moiety of the POAC residue could be easily accommodated into the 12-helix typically generated by the structurally related cyclopentyl ACPC β-amino acid.

Experimental Section

Synthesis and characterisation of peptides: Melting points were determined with a temperature increase of 3 °C min^{-1} and are uncorrected. Mass spectrometry (MS) analyses (electrospray ionisation (ESI) mode) were performed by V. Steinmetz (University of Versailles, France) on a Hewlett–Packard (Palo Alto, CA) HP5989MS spectrometer. High-resolution MS analyses (ESI mode) were performed by Dr. G. Evano (University of Versailles, France) on a Waters (Milford, MA) Xevo QToF mass spectrometer. Optical rotations were measured with an accuracy of 0.3% in a 1 dm thermostatted cell. Analytical TLC analysis, preparative TLC, and column chromatography were performed on silica gel F 254 (Merck, Darmstadt, Germany), silica gel G-25 (1 mm; Merck), and Kieselgel gel 60 (0.040–0.063 mm; Merck), respectively. The syntheses and characterisations of the compounds HCl·H-(1*S*,2*S*)-ACPC-OMe, Boc-[(1*S*,2*S*)-ACPC]$_2$-OMe, Fmoc-(3*R*,4*R*)-POAC-OH, H-(3*R*,4*R*)-POAC-OMe, Fmoc-(3*R*,4*R*)-POAC-O-(*aR*)-Bin, Boc-(1*S*,2*S*)-ACPC-(3*R*,4*R*)-POAC-OH, and **3** have already been reported.[16]

Boc-(1*S*,2*S*)-ACPC-(3*R*,4*R*)-POAC-(3*R*,4*R*)-POAC-O-(*aR*)-Bin: Fmoc-(3*R*,4*R*)-POAC-O-(*aR*)-Bin (21 mg, 0.03 mmol) was dissolved in CH_3CN (2 mL) and Et_2NH (0.5 mL) was added. The mixture was stirred at room temperature for 3 h and evaporated in vacuo. Boc-(1*S*,2*S*)-ACPC-(3*R*,4*R*)-POAC-OH (25 mg, 0.06 mmol) and THF (1 mL) were added to the resulting crude product containing H-(3*R*,4*R*)-POAC-O-(*aR*)-Bin (not isolated). The solution was cooled on an ice bath and HATU (25 mg, 0.066 mmol) and DIEA (0.01 mL) were added. The mixture was stirred at room temperature for 42 h and concentrated under reduced pressure. The residue was taken up in CH_2Cl_2 and the organic phase was washed successively with HCl (0.5 M), H_2O, and saturated $NaHCO_3$ solution; dried over $MgSO_4$; filtered; and evaporated in vacuo. The residue was purified by preparative TLC on silica gel with CH_2Cl_2/MeOH (92.5:7.5) as the eluant to give the tripeptide (16 mg, 62%) as a solid. R_f = 0.71 (CH_2Cl_2/MeOH 90:10); m.p. 133–135 °C; $[\alpha]_{589}^{25}$ = +92 (c = 0.10 in CH_2Cl_2); MS: m/z (%): 886.5 $[M+Na]^+$; HRMS: m/z calcd for $C_{49}H_{61}N_5O_9Na$: 886.4367; found: 886.4327.

Boc-[(1*S*,2*S*)-ACPC]$_3$-OMe: Sodium hydroxide (60 mg) was added to a solution of Boc-[(1*S*,2*S*)-ACPC]$_2$-OMe (70 mg, 0.2 mmol) in THF (5 mL), MeOH (2 mL), and H_2O (1 mL). The mixture was heated on a water bath at 55 °C for 2 h and concentrated under reduced pressure, diluted with H_2O (3 mL), and neutralised by careful addition of HCl (0.5 M). The solution was extracted with CH_2Cl_2 (3×). The combined extracts in CH_2Cl_2 were dried over $MgSO_4$, filtered, and evaporated in vacuo to give crude Boc-[(1*S*,2*S*)-ACPC]$_2$-OH as a solid. HATU (80 mg, 0.21 mmol) and DIEA (0.05 mL) were added to an ice-cold solution of this residue and HCl·H-(1*S*,2*S*)-ACPC-OMe (43 mg, 0.24 mmol) in THF (4 mL). The mixture was stirred at room temperature for 18 h and evaporated in vacuo. The residue was taken up in CH_2Cl_2 and the organic phase was washed successively with HCl (0.5 M), H_2O, and saturated $NaHCO_3$ solution; dried over $MgSO_4$; filtered; and evaporated in vacuo. The crude product was purified by column chromatography on silica gel with CH_2Cl_2/MeOH (95:5) as the eluant to give the tripeptide as a solid (85 mg, 91%). R_f = 0.28 (CH_2Cl_2/MeOH 95:5); m.p. 207–208 °C; $[\alpha]_{589}^{25}$ = +55 (c = 0.26 in CH_2Cl_2); MS: m/z (%): 488.3 $[M+Na]^+$; HRMS: m/z calcd for $C_{24}H_{39}N_3O_6Na$: 488.2737; found: 488.2746.

Boc-(1*S*,2*S*)-ACPC-(3*R*,4*R*)-POAC-(3*R*,4*R*)-POAC-[(1*S*,2*S*)-ACPC]$_3$-OMe (1): 1) Tripeptide Boc-[(1*S*,2*S*)-ACPC]$_3$-OMe (9 mg, 0.02 mmol) was dissolved in CH_2Cl_2 (1.5 mL), the solution was cooled on an ice bath, and HCl in dioxane (0.3 mL, 4 M) was added. The solution was magnetically stirred at 0 °C for 30 min and at room temperature for 1 h, then evaporated in vacuo at 30 °C. The residue was repeatedly triturated in CH_2Cl_2 and the suspension evaporated in vacuo to give crude HCl·H-[(1*S*,2*S*)-ACPC]$_3$-OMe.

2) Sodium hydroxide (20 mg) was added to a solution of Boc-(1*S*,2*S*)-ACPC-(3*R*,4*R*)-POAC-(3*R*,4*R*)-POAC-O-(*aR*)-Bin (12 mg, 0.014 mmol) in THF (1 mL), MeOH (0.4 mL), and H_2O (0.2 mL). The mixture was heated for 3 h at 50 °C, diluted with H_2O, and concentrated under reduced pressure. The resulting aqueous solution was washed twice with diethyl ether and neutralised by careful addition of HCl (0.5 M). The solution was extracted three times with CH_2Cl_2. The combined extracts in CH_2Cl_2 were dried over $MgSO_4$, filtered, and evaporated in vacuo to give crude Boc-(1*S*,2*S*)-ACPC-[(3*R*,4*R*)-POAC]$_2$-OH. HATU (5 mg, 0.013 mmol) and DIEA (0.01 mL) were added to an ice-cold solution of this residue and HCl·H-[(1*S*,2*S*)-ACPC]$_3$-OMe (6 mg, 0.015 mmol) in THF (1 mL). The mixture was stirred at room temperature for 72 h and evaporated in vacuo. The residue was taken up in CH_2Cl_2 and the organic phase was washed successively with HCl (0.5 M), H_2O, and saturated $NaHCO_3$ solution; dried over $MgSO_4$; filtered; and evaporated in vacuo. The crude product was purified by preparative TLC on silica gel with CH_2Cl_2/MeOH (92.5:7.5) as the eluant to give hexapeptide **1** as a solid (6 mg, 30% for two steps). R_f = 0.48 (CH_2Cl_2/MeOH 90:10); m.p. 211–213 °C; $[\alpha]_{589}^{25}$ = +83 (c = 0.21 in CH_2Cl_2); MS: m/z (%): 965.9 $[M+Na]^+$; HRMS: m/z calcd for $C_{48}H_{78}N_8O_{11}Na$: 965.5688; found: 965.5719.

Boc-(1*S*,2*S*)-ACPC-(3*R*,4*R*)-POAC-(1*S*,2*S*)-ACPC-OMe: HATU (50 mg, 0.13 mmol) and DIEA (0.02 mL) were added to an ice-cold solution of Boc-(1*S*,2*S*)-ACPC-(3*R*,4*R*)-POAC-OH (50 mg, 0.12 mmol) and HCl·H-(1*S*,2*S*)-ACPC-OMe (27 mg, 0.15 mmol) in THF (3 mL). The mixture was stirred at room temperature for 24 h and evaporated in vacuo. The residue was taken up in CH_2Cl_2 and the organic phase was washed successively with HCl (0.5 M), H_2O, and saturated $NaHCO_3$ solution; dried over $MgSO_4$; filtered; and evaporated in vacuo. The crude product was purified by preparative TLC on silica gel with CH_2Cl_2/MeOH (95:5) as the eluant to give the tripeptide as a solid (46 mg, 68%). R_f = 0.46 (CH_2Cl_2/MeOH 95:5); m.p. 184–186 °C; $[\alpha]_{589}^{25}$ = +103 (c = 0.20 in CH_2Cl_2); MS: m/z (%): 560.4 $[M+Na]^+$; HRMS: m/z calcd for $C_{27}H_{45}N_4O_7Na$: 560.3186; found: 560.3187.

Fmoc-(3*R*,4*R*)-POAC-[(1*S*,2*S*)-ACPC]$_2$-OMe: The dipeptide Boc-[(1*S*,2*S*)-ACPC]$_2$-OMe (36 mg, 0.10 mmol) was dissolved in CH_2Cl_2 (2 mL), the solution was cooled on an ice bath, and HCl in dioxane (0.4 mL, 4 M) was added. The solution was magnetically stirred at room temperature for 3 h and evaporated in vacuo at 30 °C. The residue was repeatedly triturated in CH_2Cl_2 and the suspension evaporated in vacuo. Fmoc-(3*R*,4*R*)-POAC-OH (36 mg, 0.085 mmol), HATU (34 mg, 0.09 mmol), and THF (2 mL) were added successively to the resulting crude product HCl·H-[(1*S*,2*S*)-ACPC]$_2$-OMe. The resulting suspension was cooled to 0 °C and DIEA (0.02 mL) was added. The reaction mixture was magnetically stirred from 0 °C to room temperature for 24 h and evaporated in vacuo. The residue was taken up in CH_2Cl_2 and washed successively with HCl (0.5 M), H_2O, and saturated $NaHCO_3$ solution; dried over $MgSO_4$; filtered; and evaporated in vacuo. The crude product was purified by chromatography on a preparative TLC plate of silica gel with CH_2Cl_2/MeOH (95:5) as the eluant to afford the tripeptide as a solid (35 mg, 62%). R_f = 0.31 (CH_2Cl_2/MeOH 95:5); m.p. 104–106 °C; $[\alpha]_{589}^{25}$ = +71 (c = 0.21 in CH_2Cl_2); MS: m/z (%): 682.4 $[M+Na]^+$; HRMS: m/z calcd for $C_{37}H_{47}N_4O_7Na$: 682.3342; found: 682.3360.

Boc-(1*S*,2*S*)-ACPC-(3*R*,4*R*)-POAC-(1*S*,2*S*)-ACPC-(3*R*,4*R*)-POAC-[(1*S*,2*S*)-ACPC]$_2$-OMe (2): 1) Sodium hydroxide (20 mg) was added to a solution of Boc-(1*S*,2*S*)-ACPC-(3*R*,4*R*)-POAC-(1*S*,2*S*)-ACPC-OMe (34 mg, 0.063 mmol) in THF (2 mL), MeOH (1 mL), and H_2O (0.5 mL). The mixture was heated on a 50 °C bath for 3 h, diluted with H_2O, and concentrated under reduced pressure. The resulting aqueous solution was

neutralised by careful addition of HCl (0.5 M). The solution was extracted with CH_2Cl_2 (3×). The combined extracts in CH_2Cl_2 were dried over $MgSO_4$, filtered, and evaporated in vacuo to give the crude product Boc-(1*S*,2*S*)-ACPC-(3*R*,4*R*)-POAC-(1*S*,2*S*)-ACPC-OH.

2) Et_2NH (0.6 mL) was added to Fmoc-(3*R*,4*R*)-POAC-[(1*S*,2*S*)-ACPC]$_2$-OMe (33 mg, 0.05 mmol) dissolved in CH_3CN (2.4 mL). The mixture was stirred at room temperature for 3 h and evaporated in vacuo. The crude product Boc-(1*S*,2*S*)-ACPC-(3*R*,4*R*)-POAC-(1*S*,2*S*)-ACPC-OH (26 mg, 0.05 mmol) in THF (2 mL) was added to the resulting crude product containing H-(3*R*,4*R*)-POAC-[(1*S*,2*S*)-ACPC]$_2$-OMe (not isolated). The solution was cooled on an ice bath and HATU (20 mg, 0.052 mmol) and DIEA (0.01 mL) were added. The mixture was stirred at room temperature for 48 h and concentrated under reduced pressure. The residue was taken up in CH_2Cl_2 and the organic phase was washed successively with HCl (0.5 M), H_2O, and saturated $NaHCO_3$ solution; dried over $MgSO_4$; filtered; and evaporated in vacuo. The residue was purified by preparative TLC on silica gel with CH_2Cl_2/MeOH (95:5) as the eluant to give hexapeptide **2** as a solid (22 mg, 47%). R_f = 0.18 (CH_2Cl_2/MeOH 95:5); m.p. 213–215 °C; $[\alpha]_{589}^{25}$ = +142 (c = 0.21 in CH_2Cl_2); MS: m/z (%): 965.6 $[M+Na]^+$; HRMS: m/z calcd for $C_{48}H_{78}N_8O_{11}Na$: 965.5688; found: 965.5719.

Boc-(1*S*,2*S*)-ACPC-(3*R*,4*R*)-POAC-[(1*S*,2*S*)-ACPC]$_3$-OMe: Tripeptide Boc-[(1*S*,2*S*)-ACPC]$_3$-OMe (37 mg, 0.08 mmol) was dissolved in CH_2Cl_2 (2 mL), the solution was cooled on an ice bath, and HCl in dioxane (0.5 mL, 4 M) was added. The solution was magnetically stirred at 0 °C for 30 min and at room temperature for 1 h, then evaporated in vacuo at 30 °C. The residue was repeatedly triturated in CH_2Cl_2 and the suspension evaporated in vacuo to give crude HCl·H-[(1*S*,2*S*)-ACPC]$_3$-OMe. A solution of Boc-(1*S*,2*S*)-ACPC-(3*R*,4*R*)-POAC-OH (42 mg, 0.102 mmol) in THF (3 mL) was added. The resulting solution was cooled on an ice bath and HATU (43 mg, 0.112 mmol) and DIEA (0.03 mL) were added. The mixture was stirred at room temperature for 24 h and concentrated under reduced pressure. The residue was taken up in CH_2Cl_2 and the organic phase was washed successively with HCl (0.5 M), H_2O, and saturated $NaHCO_3$ solution; dried over $MgSO_4$; filtered; and evaporated in vacuo. The residue was purified by preparative TLC on silica gel with CH_2Cl_2/MeOH (92.5:7.5) as the eluant to give the pentapeptide as a solid (32 mg, 55%). R_f = 0.46 (CH_2Cl_2/MeOH 92.5:7.5); m.p. 93–95 °C; $[\alpha]_{589}^{25}$ = +94 (c = 0.65 in CH_2Cl_2); MS: m/z (%): 782.5 $[M+Na]^+$; HRMS: m/z calcd for $C_{39}H_{63}N_6O_9Na$: 782.4554; found: 782.4586.

Boc-(1*S*,2*S*)-ACPC-(3*R*,4*R*)-POAC-[(1*S*,2*S*)-ACPC]$_3$-(3*R*,4*R*)-POAC-OMe (4): Sodium hydroxide (10 mg) was added to a solution of Boc-(1*S*,2*S*)-ACPC-(3*R*,4*R*)-POAC-[(1*S*,2*S*)-ACPC]$_3$-OMe (23 mg, 0.03 mmol) in THF (1.5 mL), MeOH (0.7 mL), and H_2O (0.3 mL). The mixture was heated for 6 h at 50 °C, diluted with H_2O, and concentrated under reduced pressure. The resulting aqueous solution was washed with CH_2Cl_2 and neutralised by careful addition of HCl (0.5 M). The solution was extracted with CH_2Cl_2 (3×). The combined extracts in CH_2Cl_2 were dried over $MgSO_4$, filtered, and evaporated in vacuo to give crude Boc-(1*S*,2*S*)-ACPC-(3*R*,4*R*)-POAC-[(1*S*,2*S*)-ACPC]$_3$-OH. HATU (12.5 mg, 0.033 mmol) and DIEA (0.01 mL) were added to an ice-cold solution of this residue and H-(3*R*,4*R*)-POAC-OMe (7 mg, 0.033 mmol) in THF (2 mL). The mixture was stirred at room temperature for 72 h and evaporated in vacuo. The residue was taken up in CH_2Cl_2 and the organic phase was washed successively with HCl (0.5 M), H_2O, and saturated $NaHCO_3$ solution; dried over $MgSO_4$; filtered; and evaporated in vacuo. The crude product was purified by preparative TLC on silica gel with CH_2Cl_2/MeOH (95:5) as the eluant to give hexapeptide **4** as a solid (17 mg, 60%). R_f = 0.21 (CH_2Cl_2/MeOH 95:5); m.p. 216–218 °C; $[\alpha]_{589}^{25}$ = +76 (c = 0.21 in CH_2Cl_2); MS: m/z (%): 965.7 $[M+Na]^+$; HRMS: m/z calcd for $C_{48}H_{78}N_8O_{11}Na$: 965.5688; found: 965.5719.

IR spectroscopy: The FTIR absorption spectra were recorded on a Perkin–Elmer (Norwalk, CT) 1720 X FTIR spectrophotometer, nitrogen-flushed, equipped with a sample-shuttle device, at a nominal resolution of 2 cm^{-1} with an average of 100 scans. Cells with path lengths of 0.1, 1.0, and 10 mm (with CaF_2 windows) were used. Spectrograde $CDCl_3$ (99.8% D) was purchased from Aldrich (St. Louis, MO). Solvent (baseline) spectra were recorded under the same conditions.

EPR spectroscopy: Solutions of hexapeptides **1**–**4** in chloroform (1 mM) were placed in EPR quartz tubes (i.d. = 2 mm), which were connected to a vacuum line. After gaseous oxygen was removed from the solutions by repeated freeze/pump/thaw cycles, the tubes were sealed. The EPR spectra were recorded on a Bruker (Karlsruhe, Germany) ER 200D X-band spectrometer (≈9.4 GHz). Sample temperature was regulated by a nitrogen-flow cryostat controlled by a Bruker BVT 2000 unit. EPR spectra were acquired with a field modulation amplitude of 0.05 mT and microwave power of 1 mW.

[1] R. P. Cheng, S. H. Gellman, W. F. DeGrado, *Chem. Rev.* **2001**, *101*, 3219–3232.
[2] D. Seebach, A. K. Beck, D. J. Bierbaum, *Chem. Biodiversity* **2004**, *1*, 1111–1239.
[3] S. M. Miick, G. V. Martinez, W. R. Fiori, A. P. Todd, G. L. Millhauser, *Nature* **1992**, *359*, 653–655.
[4] C. Toniolo, E. Valente, F. Formaggio, M. Crisma, G. Pilloni, C. Corvaja, A. Toffoletti, G. V. Martinez, P. Hanson, G. L. Millhauser, C. George, J. L. Flippen-Anderson, *J. Pept. Sci.* **1995**, *1*, 45–47.
[5] P. Hanson, G. V. Martinez, G. L. Millhauser, F. Formaggio, M. Crisma, C. Toniolo, C. Vita, *J. Am. Chem. Soc.* **1996**, *118*, 271–272.
[6] P. Hanson, G. L. Millhauser, F. Formaggio, M. Crisma, C. Toniolo, *J. Am. Chem. Soc.* **1996**, *118*, 7618–7625.
[7] P. Hanson, D. J. Anderson, G. V. Martinez, G. L. Millhauser, F. Formaggio, M. Crisma, C. Toniolo, C. Vita, *Mol. Phys.* **1998**, *95*, 957–966.
[8] R. Marchetto, S. Schreier, C. R. Nakaie, *J. Am. Chem. Soc.* **1993**, *115*, 11042–11043.
[9] C. Toniolo, M. Crisma, F. Formaggio, *Biopolymers* **1998**, *47*, 153–158.
[10] L. Martin, A. Ivancich, C. Vita, F. Formaggio, C. Toniolo, *J. Pept. Res.* **2001**, *58*, 424–432.
[11] S. H. Gellman, *Acc. Chem. Res.* **1998**, *31*, 173–180.
[12] K. Wright, M. Sarciaux, A. De Castries, M. Wakselman, J.-P. Mazaleyrat, A. Toffoletti, C. Corvaja, M. Crisma, C. Peggion, F. Formaggio, C. Toniolo, *Eur. J. Org. Chem.* **2007**, 3133–3144.
[13] A. Rassat, P. Rey, *Bull. Soc. Chim. Fr.* **1967**, *3*, 815–817.
[14] M. Tominaga, S. R. Barbosa, E. F. Poletti, J. Zukerman-Schpector, R. Marchetto, S. Schreier, A. C. M. Paiva, C. R. Nakaie, *Chem. Pharm. Bull.* **2001**, *49*, 1027–1029.
[15] K. Wright, F. Formaggio, C. Toniolo, R. Török, A. Péter, M. Wakselman, J.-P. Mazaleyrat, *Tetrahedron Lett.* **2003**, *44*, 4183–4186.
[16] K. Wright, L. Dutot, M. Wakselman, J.-P. Mazaleyrat, M. Crisma, F. Formaggio, C. Toniolo, *Tetrahedron* **2008**, *64*, 4416–4426.
[17] D. H. Appella, L. A. Christianson, D. A. Klein, M. R. Richards, D. R. Powell, S. H. Gellman, *J. Am. Chem. Soc.* **1999**, *121*, 7574–7581.
[18] C. Toniolo, F. Formaggio, A. Toffoletti, L. Franco, J.-P. Mazaleyrat, M. Wakselman, K. Wright, in *Peptides 2008* (Ed.: H. Lankinen), Finnish Peptide Society, Helsinki, **2008**, pp. 56–57.
[19] L. A. Carpino, *J. Am. Chem. Soc.* **1993**, *115*, 4397–4398.
[20] L. A. Carpino, H. Imazumi, A. El-Faham, F. J. Ferrer, C. Zhang, Y. Lee, B. M. Foxman, P. Henklein, C. Hanay, C. Mügge, H. Wenschuh, J. Klose, M. Beyermann, M. Bienert, *Angew. Chem.* **2002**, *114*, 457–461; *Angew. Chem. Int. Ed.* **2002**, *41*, 441–445.
[21] G. M. Clore, J. Iwahara, *Chem. Rev.* **2009**, *109*, 4108–4139.
[22] T. T. T. Bui, F. Formaggio, M. Crisma, V. Monaco, C. Toniolo, R. Hussain, G. Siligardi, *J. Chem. Soc. Perkin Trans. 2* **2000**, 1043–1046.
[23] S. Mizushima, T. Shimanouchi, M. Tsuboi, R. Souda, *J. Am. Chem. Soc.* **1952**, *74*, 270–271.
[24] M. T. Cung, M. Marraud, J. Néel, *Ann. Chim.* **1972**, 183–209.
[25] E. S. Pysh, C. Toniolo, *J. Am. Chem. Soc.* **1977**, *99*, 6211–6219.
[26] S. C. Yasui, T. A. Keiderling, F. Formaggio, G. M. Bonora, C. Toniolo, *J. Am. Chem. Soc.* **1986**, *108*, 4988–4993.
[27] G. P. Dado, S. H. Gellman, *J. Am. Chem. Soc.* **1994**, *116*, 1054–1062.
[28] F. López-Carrasquero, C. Aleman, S. Muñoz-Guerra, *Biopolymers* **1995**, *36*, 263–271.

[29] G. Montalvo, M. M. Waegele, S. Shandler, F. Gai, W. F. DeGrado, *J. Am. Chem. Soc.* **2010**, *132*, 5616–5618.
[30] S. S. Eaton, G. E. Eaton in *Biological Magnetic Resonance, Vol. 19* (Eds.: L. J. Berliner, S. S. Eaton, G. E. Eaton), Kluwer, New York, **2001**, pp. 1–27.
[31] L. V. Kulik, S. A. Dzuba, I. A. Grigoryev, Y. D. Tsvetkov, *Chem. Phys. Lett.* **2001**, *343*, 315–324.
[32] H.-J. Steinhoff, *Biol. Chem.* **2004**, *385*, 913–920.
[33] R. Garzelli, V. K. Khlestkin, N. H. Williams, V. Chechik, *Tetrahedron Lett.* **2008**, *49*, 5887–5889.
[34] S. Milikisyants, E. J. J. Groenen, M. Huber, *J. Magn. Reson.* **2008**, *192*, 275–279.
[35] G. R. Luckhurst in *Spin Labelling Theory and Applications* (Ed.: L. J. Berliner), Academic Press, New York, **1976**, pp. 133–181.

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