Characterization of the 3_{10}-Helix in Model Peptides by HRMAS NMR Spectroscopy

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Dedicated to Professor Günther Jung on the occasion of his 65th birthday

Abstract: A tetra- and a hepta-homopeptide from the C^{α}-tetrasubstituted Aib (α-aminoisobutyric acid) residue were covalently linked to the POEPOP resin by the fragment-condensation approach. The conformational preferences of the two model peptides were determined for the first time on a solid support by means of high-resolution magic angle spinning NMR spectroscopy. The results obtained indicate that the Aib homopeptides adopt a regular 3_{10}-helical structure even when they are covalently bound to a polymeric matrix, and thus confirm the remarkable conformational stability of the peptides rich in this amino acid. An ATR-FTIR spectroscopic investigation, performed in parallel, also confirmed that these polymer-bound peptides do indeed adopt a helical conformation. The results of this study open the possibility to exploit the peptide-resin conjugates based on C^{α}-tetrasubstituted α-amino acids as helpful, structurally organized templates in molecular recognition studies or as catalysts in asymmetric synthesis.

Keywords: conformation analysis · 3_{10}-helix · HRMAS NMR spectroscopy · peptides · solid-phase synthesis

Introduction

High-resolution magic angle spinning (HRMAS) NMR spectroscopy is a very promising technique in the field of solid-phase organic chemistry for the characterization of resin-bound compounds.[1] HRMAS NMR has been successfully used for the analysis of multi-step synthesis of small organic molecules,[2] to shed light on the drawbacks of amino acid coupling during solid-phase peptide synthesis,[3] and for the conformational characterization of model and bioactive peptides covalently attached to different resins.[4] The model poly(Ala)$_n$ sequences, for example, have been studied to determine their tendency to self-aggregate when bound to a polystyrene-type resin.[4b] However, the same peptides are able to fold in an α-helix when their loading on the solid support is decreased.[4b] More recently, we have found that the 141–159 peptide sequence from foot-and-mouth disease virus covalently linked to the POEPOP (polyoxyethylene-polyoxypropylene) resin, swollen in an organic solvent, is able to fold into a regular helical structure, which is very close to the secondary structure adopted by the free peptide in solution.[4a, 5] The determination of the conformation adopted by bioactive peptides when bound to a solid support can be strictly related to the use of peptide-resin conjugates for the generation or the purification of specific antibodies. Peptides could also exhibit relevant biological activity when linked to a polymer matrix in which the resin itself plays the role of the carrier, thus replacing the classical carrier proteins.[6]

While the propensity to form α-helices and β-turns has been studied on peptides bound to a solid support,[4] other common peptide secondary structures, the 3_{10}-helix and β-pleated sheet, have not yet been characterized by HRMAS NMR. In particular, the 3_{10}-helix represents the third principal secondary structure in globular proteins after α-helices and β-sheets,[7] and it has been described at atomic resolution both in model peptides and peptaibol antibiotics.[8] A high proportion of C^{α}-tetrasubstituted α-amino acids within a peptide sequence strongly favours the formation of this type of helix. Aib (α-aminoisobutyric acid) can be considered as the prototype and the simplest member of the C^{α}-tetrasubstituted

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residue family.[8a–c, e] It has been demonstrated that Aib-rich peptides tend to adopt helical structures of remarkable stability. In the case of $(Aib)_n$ homo-oligopeptides up to the dodecamer, fully developed and stable 3_{10}-helices are generated either in the crystalline state or in structure-supporting solvents.

In this paper we present an HRMAS NMR conformational analysis of a series of Aib-based peptides covalently linked to the POEPOP resin. We chose the POEPOP resin for its peculiar features to swell in a wide range of solvents and to give HRMAS NMR spectra of the bound molecules very close in resolution to those obtained for the same molecules analyzed free in solution.[2a, 9] Additional information on the preferred conformation adopted by these model compounds was obtained from ATR (ATR, attenuated total reflection) FTIR experiments.

Deuterated DMSO resulted to be the most suitable swelling solvent for the structural characterization of the PEOPOP-bound peptides. The determination of the temperature coefficients of the unambiguously assigned NH proton resonances confirmed the tendency of these peptides to fold into a regular 3_{10}-helix. In this context, Aib-rich peptides, which adopt a conformationally constrained structure with a well-defined spatial geometry, can be exploited as useful templates in molecular recognition studies[10] even if covalently linked to polymeric matrices. The present HRMAS NMR characterization of the structure adopted by solid-supported model peptides containing Aib residues can be viewed as the first step along this direction. Moreover, chiral C^{α}-tetrasubstituted residue-containing peptides covalently linked to a resin and characterized by defined helical motifs could be exploited as catalysts in asymmetric synthesis. Indeed, the only examples reported to date involve short peptides, made up of protein amino acids, that are generally less prone to adopt a well defined secondary structure.[11]

Abstract in French: *Un tetra- et un hepta-homopeptides synthétisés à partir de résidus C^{α}-tetrasubstitués Aib (acide α-aminoisobutyrique), ont été liés de manière covalente à la résine POEPOP par condensation de fragments. Les préférences conformationnelles de ces deux peptides modèles ont ensuite été déterminées pour la première fois sur support solide par spectroscopie RMN haute résolution à rotation à l'angle magique. Les résultats obtenus indiquent que les peptides poly-Aib adoptent une structure régulière en hélice 3_{10} même lorsqu'ils sont liés de manière covalente à une matrice polymérique, confirmant ainsi la remarquable stabilité conformationnelle de ces peptides riches en Aib. Une analyse spectroscopique par ATR-FTIR a été réalisée en parallèle et indique clairement que ces peptides liés au polymère adoptent une conformation en hélice. Les résultats de cette étude permettent d'envisager l'exploitation de conjugués peptide-résine, basés sur des acides aminés C^{α}-tetrasubstitués, comme plate-formes structurellement organisées pour des études de reconnaissance moléculaire ou comme catalyseurs en synthèse asymétrique.*

Abstract in Italian: *Un tetra- e un epta-omopeptide costituiti da residui C^{α}-tetrasostituiti di Aib (acido α-amminoisobutirrico) sono stati legati covalentemente alla resina POEPOP tramite condensazione di frammenti. Le preferenze conformazionali di questi due peptidi modello sono state studiate per la prima volta su un supporto solido mediante NMR ad alta risoluzione a rotazione a l'angolo magico. I risultati ottenuti indicano che gli omopeptidi dell'Aib adottano una struttura elicoidale regolare di tipo 3_{10} anche quando sono legati covalentemente a una matrice polimerica, confermando quindi la notevole stabilità conformazionale dei peptidi ricchi in questo amminoacido. È stata inoltre condotta un'analisi mediante spettroscopia ATR-FTIR. Essa ha confermato che questi peptidi legati a un polimero adottano una conformazione elicoidale. I risultati di questo studio aprono la possibilità di sfruttare i coniugati peptide-resina basati su amminoacidi C^{α}-tetrasostuiti come templati strutturalmente organizzati, utili negli studi di riconoscimento molecolare o come catalizzatori nella sintesi asimmetrica.*

Results and Discussion

Synthesis and characterization: Several methods have been developed for the incorporation of C^{α}-tetrasubstituted α-amino acids on a solid support.[12] In particular, the step-by-step solid-phase synthesis of Aib-rich peptides has allowed to obtain oligomers of a limited length.[13] We decided to explore the possibility to couple model Aib homopeptides of different length to a resin using the fragment condensation strategy. More specifically, two peptides, containing four and seven Aib residues, protected at the N-terminus as *p*BrBz (*p*-bromobenzoyl) and Z (benzyloxycarbonyl) derivatives, respectively, and unprotected at the C-terminus were coupled to the amino groups of POEPOP resin.

Initially, we focused on the synthesis of **1**. *p*BrBz-$(Aib)_4$-OH was activated by treatment with triphosgene and collidine in *N*,*N*-dimethylformamide (DMF) and subsequently added to the resin.[14] The mixture was first shaken at room temperature and then at 50 °C for more than one week. Due to the limited excess of peptide used (see Experimental Section) the reaction was not complete as detected by the positive Kaiser test.[15] The free amino functions on the resin were eventually blocked by acylation with trifluoroacetic anhydride. We reasoned that the introduction of a flexible spacer such as Ahx (6-aminohexanoic acid) between the resin and the Aib sequence could have increased the yield of the coupling step. However, when Z-$(Aib)_7$-OH, activated as described above for *p*BrBz-$(Aib)_4$-OH, was coupled to the Ahx-resin, to give **2**, we did not achieve a better result despite the presence of the spacer. Moreover, in the HRMAS NMR spectra (see below) a number of unidentified signals generated by impurities was observed. Our tentative explanation calls for the 5(4*H*)-oxazolone intermediary role rather than for the absence or the presence of the spacer. Indeed, it is known that peptides based on $C^{\alpha,\alpha}$-disubstituted glycines undergo a rapid, intramolecular conversion to their corresponding 5(4*H*)-oxazolones when C-activated by any procedure.[16] These heterocycles display a modest acylating capability, thus requiring very long reaction times to reach satisfactory coupling yields.[13a, 16] Under these conditions, the use of overactivating reagents, such as triphosgene, may be responsible for the

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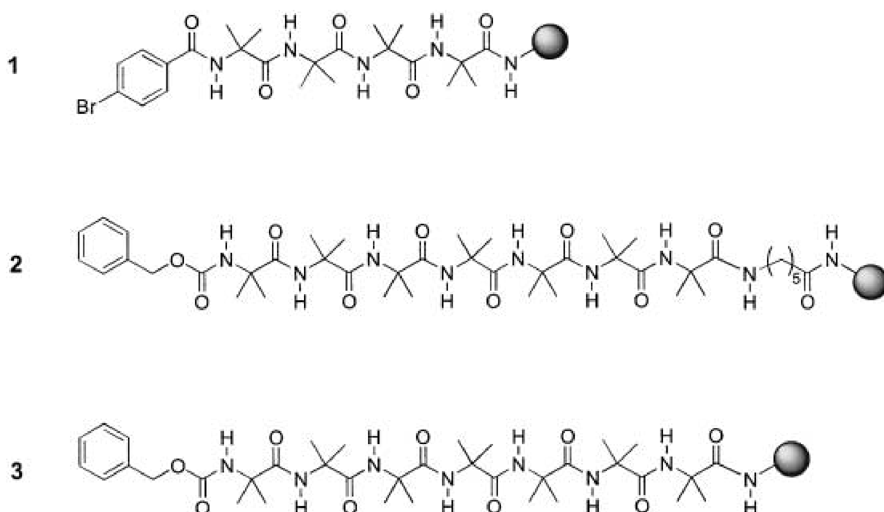
number of side-products observed. We then decided to choose a milder activating reagent. Z-$(Aib)_7$-OH was treated with HATU [*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] in 3% NMM (*N*-methyl morpholine) in DMF[17] and the resulting mixture added to the spacer-free POEPOP resin to give **3**. In this case, complete resin



acylation was achieved after shaking for four days at room temperature without any necessary capping of the resin free NH groups. As expected, the acylating mixture, analyzed by MALDI-TOF mass spectrometry, contained the 5(4*H*)-oxazolone from Z-$(Aib)_7$-OH rather than the peptide/HOAt (7-aza-1-hydroxybenzotriazole) derivative. From the above described experiments we conclude that, due to the slowly reacting 5(4*H*)-oxazolones formation, fragment condensations of peptides made up of C^{α}-tetrasubstituted α-amino acid residues give better results when mild activating reagents are used.

Conformational analysis: The resin bound Aib homopeptides **1**–**3** were initially analyzed by ATR-FTIR. Figure 1 shows the amide N-H stretching (amide A) region. The spectra exhibit an intense band at 3300–3325 cm^{-1} typical of NH groups involved in H bonds,[18] and a broad band between 3400 and 3600 cm^{-1} attributed to a combination of free amide N-H and water O-H stretching. It is worth noting that the spectra of compounds **1** and **2** include also a contribution from the amide groups generated after trifluoroacetic anhydride acylation of the resin free NH groups. Due to the high hygroscopic character of the POEPOP resin, residual water molecules could significantly contribute to the stretching band above 3500 cm^{-1}. As compared to **1**, an increase of the band related to the H-bonded NH groups is displayed by **2** and **3**. In the 1800–1500 cm^{-1} spectral region the amide C=O stretching (amide I) band is observed at 1664, 1660 and 1657 cm^{-1} for **1**–**3**, respectively, while for the same conjugates the amide II band is visible at 1537, 1533 and 1531 cm^{-1}, respectively (Figure 2).[19] In particular, the amide A region is strongly indicative of a peptide conformation stabilized by intramolecular C=O···H-N hydrogen bonds, while the positions of the amide I bands are in accordance with the presence of a helical structure.[18a,b,19a] However, the positions of the amide I bands did not allow us to discriminate between 3_{10}- and α-helices. Indeed, it has been reported that the amide I band for $(Aib)_n$ homo-oligomers in a 3_{10}-helical conformation is located at about 1666 cm^{-1},[19b] while the same band for α-helices is found at slightly lower wavenumbers and, moreover, its position is strongly dependent on the experimental conditions.[19c] Therefore, an unequivocal assignment of the helix type adopted by homopeptides **1**–**3** is not straightforward if based exclusively on the position of the amide I IR absorption.

To determine more precisely the preferred helical conformation of the peptides covalently bound to the resin (constructs **1**–**3**), a series of 1D and 2D HRMAS NMR experiments was performed. Initially, the influence of the deuterated solvents on the resolution of the HRMAS spectra was investigated. Seven different aprotic solvents ranging from low (cyclohexane) to high (DMSO) polarity were used.[20] Figure 3

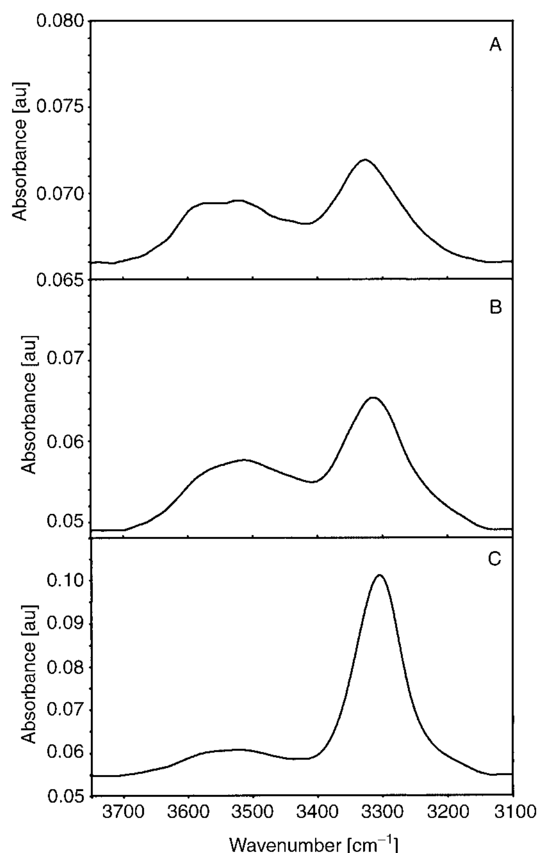


Figure 1. ATR-FTIR spectra in the N-H stretching (amide A) region of the POEPOP-peptides **1** (A), **2** (B) and **3** (C).

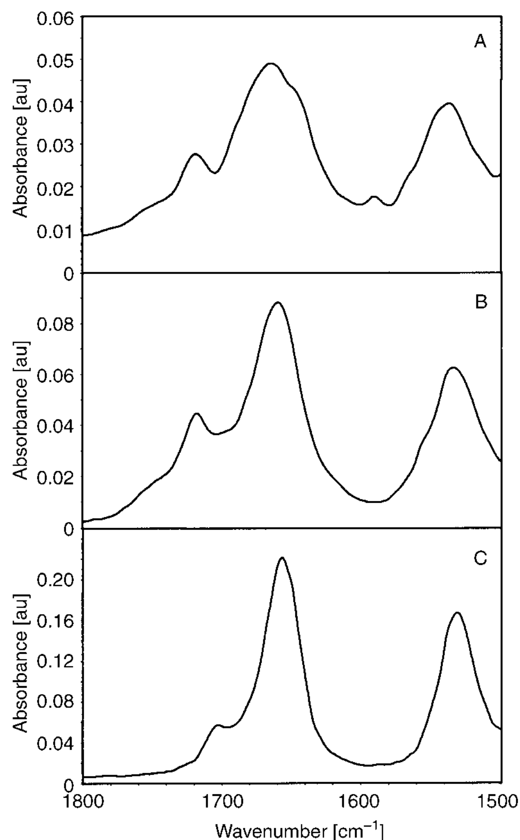


Figure 2. ATR-FTIR spectra in the amide I and II regions of the POEPOP-peptides **1** (A), **2** (B) and **3** (C).

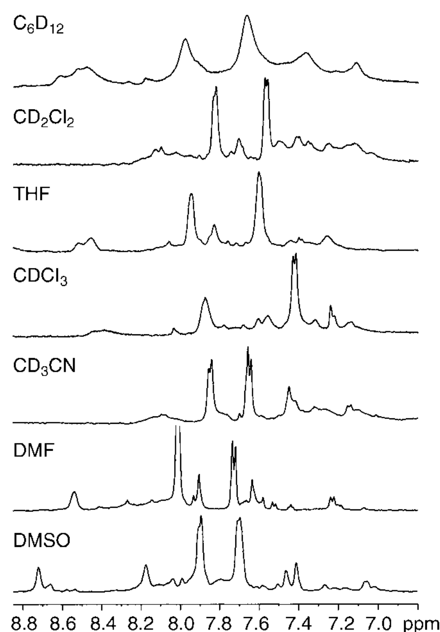


Figure 3. Amide proton region of the HRMAS NMR spectrum of the POEPOP-peptide **1** swollen in different deuterated solvents. The polarity increases from cyclohexane to DMSO.

shows the ^{1}H NMR resonances of peptide **1** in the amide proton region. The best resolved spectra were obtained by swelling the peptide-resin conjugate in DMSO and DMF, while the typical chlorinated solvents used in the solution conformational analysis gave broad and unresolved NH proton signals.

Although the POEPOP resin displays very good swelling properties[9b] in all of the solvents tested, with the exception of cyclohexane, solvents of low polarity such as chloroform afforded HRMAS spectra of poor quality and difficult interpretation. Therefore, for the NMR study of the secondary structure of the POEPOP-bound peptides **1**–**3** we decided to use deuterated DMSO.[21] To unambiguously assign all NH proton signals[22] and to confirm the helical arrangement of the peptides, as suggested by ATR-FTIR spectroscopy, two-dimensional NMR (NOESY) experiments were performed by swelling compounds **1**–**3** in [D$_6$]DMSO. In each case the resonance of the first Aib residue was attributed by means of the NOE correlation between the NH proton of Aib^1 and the protons of the N-terminal protecting group. In compound **1** the Aib^1 NH cross-correlates with the aromatic protons of the *p*BrBz group (Figure 4), while for compounds **2** and **3** a cross-peak between the Aib^1 NH (at 7.83 ppm) and the methylene protons of the Z-group (at 5.10 ppm) was observed in the NOESY spectra. The NH functionality of the POEPOP resin was easily assigned in **1** and **3** thank to the spatial interaction occurring between this amide proton (at 7.06 ppm) and the vicinal methylene protons (at 3.42 ppm) of the polyoxyethylene chain. These interactions, in turn, do confirm that the peptides are covalently bound to the solid support. Compound **2** bears the Ahx spacer between the Aib sequence and the NH function of the resin. In this case dipolar interactions were observed for the resin NH proton (at 7.57 ppm) with both the α-methylene protons of Ahx (at 2.08 ppm) and the polyoxyethylene vicinal methylene protons (at 3.41 ppm). Then, the 2D HRMAS NMR analysis allowed a straightforward assignment of all remaining amide proton resonances. The NOESY spectra showed a series of strong sequential NH($i \rightarrow i+1$) dipolar interactions, the presence of which is considered diagnostic of helical structures[23] (Figures 4 and 5).

However, these interactions alone do not allow to assess if a 3_{10}- or an α-helical conformation is present. Indeed, in peptides and proteins there are two NOE constraints [$d_{\alpha N}$ (i, $i+2$)] and [$d_{\alpha N}$ (i, $i+4$)] believed to be characteristic of the 3_{10}- and the α-helix, respectively.[23] Unfortunately, these interactions do not occur in the case of peptides based exclusively on C$^\alpha$-tetrasubstituted α-amino acids as such residues lack any αCH proton.[22] In the case of the POEPOP-peptide **3**, additional medium-range ($i \rightarrow i+n$, $n > 1$) cross-peaks, albeit weak, were observed in the amide proton region of the NOESY spectrum (Figure 5). In particular, the Aib^2–Aib^4, Aib^5–Aib^7 and Aib^6-resin NH protons correlations of the NH$_i$–NH$_{i+2}$ type were found. These signals helped us also to solve the ambiguities in the sequential assignment of the backbone region from residue 3 to 6.

To investigate the 3D-structure of the POEPOP-bound Aib homopeptides in more detail and, hopefully, to establish whether a 3_{10}- or an α-helical structure is present, a series of monodimensional spectra was recorded by increasing the temperature from 300 to 340 K. On the basis of the high-field shift of the NH resonances, the temperature coefficients of these protons were calculated for the three POEPOP-linked

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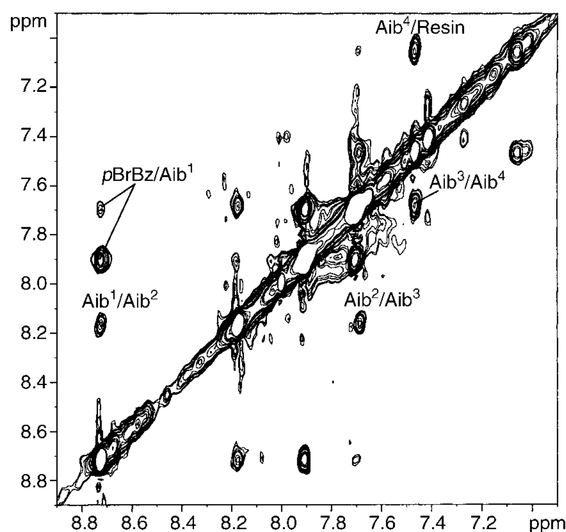


Figure 4. Amide proton region of the HRMAS 2D NOESY ($\tau_m = 300$ ms) spectrum for the POEPOP-peptide **1** swollen in [D6]DMSO.

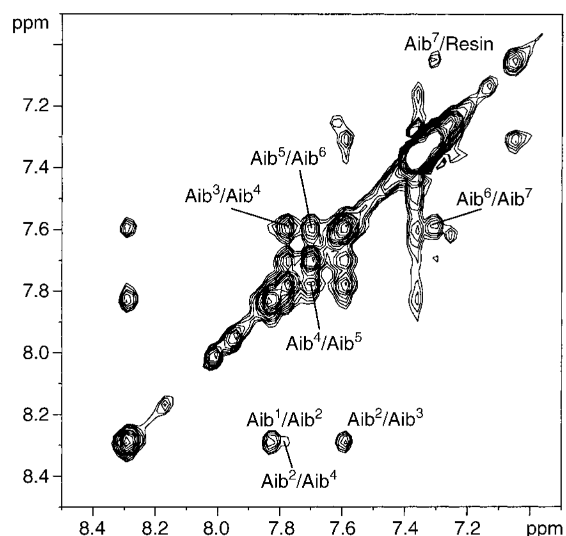
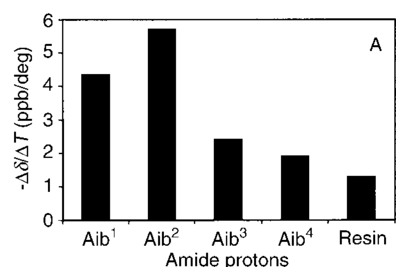
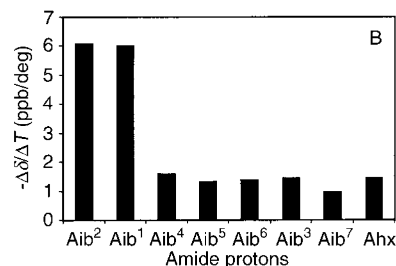


Figure 5. Amide proton region of the HRMAS 2D NOESY ($\tau_m = 500$ ms) spectrum for the POEPOP-peptide **3** swollen in [D6]DMSO.





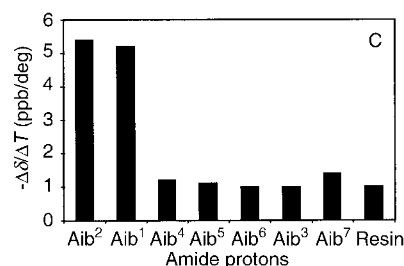


Figure 6. Temperature coefficients of the amide NH protons of the POEPOP-peptides **1** (A), **2** (B) and **3** (C), swollen in [D6]DMSO, measured in the range 300–340 K. The assignment of the residues was performed by NOESY HRMAS experiments (see Figures 4 and 5). The residues are ordered as they appear in the NMR spectrum from high to low chemical shifts, and numbered according to their assignment.

Aibhomopeptides (Figure 6).[21] In order to assess if any influence is exerted by the resin on the conformation of the bound peptides, we also recorded the monodimensional spectra of Z-$(Aib)_7$-O*t*Bu (for synthetic and conformational details on this peptide see ref. [24]) in the same solvent and temperature ranges examined for **1**–**3**. The NH proton temperature coefficients of this terminally protected tetrapeptide are reported in Figure 7. From this study, two classes of NH protons were clearly observed: i) the first class includes two amide protons, namely the Aib1 and Aib2 NH protons, particularly sensitive to the increase of temperature; ii) the second class involves all other amide protons, only marginally perturbed by heating.

These HRMAS NMR findings (Figure 6) strongly support the conclusion that POEPOP-supported Aib homo-oligomers fold into a 3_{10}-helix rather than into a α-helix. As a matter of fact, the latter structural motif would have required the first three backbone NH protons not being involved in the intramolecular hydrogen-bonding stabilization of the helix, while the first two only are expected not to be hydrogen bonded in a 3_{10}-helix.[8a–c, 8e, 24] Moreover, the HRMAS NMR NH proton temperature coefficients of the resin-bound homopeptide **3** are remarkably similar to those displayed by Z-$(Aib)_7$-O*t*Bu (Figure 7), that is known to adopt a 3_{10}-helical structure.[24] From the latter comparison it can be concluded

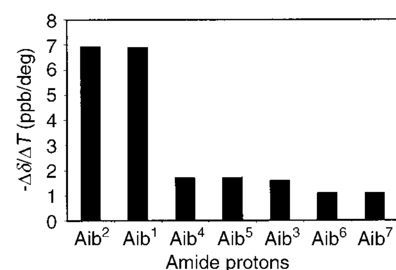


Figure 7. Temperature coefficients of the amide NH protons of Z-$(Aib)_7$-O*t*Bu in [D6]DMSO, measured in the range 300–340 K. The assignment of the residues was performed by a ROESY experiment. The residues are ordered as they appear in the NMR spectrum from high to low chemical shifts, and numbered according to their assignment.

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that the 3_{10}-helical conformation of the -(Aib)$_7$- stretch is entirely preserved when the peptide is bound to the resin.

Conclusion

We successfully linked a series of Aib-based homopeptides of different length to the POEPOP resin by the fragment condensation approach. The conformational analysis of the peptide-resin conjugates was performed for the first time by HRMAS NMR and ATR-FTIR spectroscopy. The two complementary techniques allowed us to demonstrate the high propensity of the model Aib-peptides to fold into a regular 3_{10}-helical structure even when they are covalently bound to an insoluble polymer. We believe that this is an important finding in view of the possibility to exploit solid-supported, conformationally constrained peptides containing C^{α}-tetrasubstituted α-amino acids as useful scaffolds in molecular recognition studies. Moreover, the insertion of chiral residues as hosts within a guest Aib homopeptide sequence or the synthesis of resin-bound homopeptides based on chiral C^{α}-tetrasubstituted α-amino acids can be exploited in asymmetric catalysis. We are currently directing our research along these lines.

Experimental Section

General: All reagents and solvents were obtained from commercial suppliers and used without further purification. Dichloromethane was distilled prior to use. POEPOP-NH_2 resin (substitution, 0.58 mmol g^{-1}) was prepared as described by Furrer et al.[4a]

Abbreviations: Symbols and abbreviations for amino acids and peptides are in accord with the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (*J. Biol. Chem.* **1972**, *247*, 977).

Synthesis of 1: 2,4,6-Collidine (24 µL, 181 µmol) was added to a solution of *p*BrBz-Aib$_4$-OH[25] (35 mg, 65 µmol) and triphosgene (6.4 mg, 21.5 µmol) in dry CH_2Cl_2 (2 mL). After 1 min this solution was added to the POEPOP-NH_2 resin (74 mg, 43 µmol) and the mixture was shaken 3 d at room temperature. The solvent was changed to DMF and the mixture heated to 50 °C for additional 4 d. After the usual work-up the resin was treated for 20 min with a trifluoracetic anhydride/pyridine/CH_2Cl_2 (1:1:1) solution (1.5 mL). The mixture was filtered and the resin extensively washed and dried in vacuo.

Synthesis of 2: A solution of Fmoc-Ahx-OH (59 mg, 165 µmol), benzotriazole-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate (74 mg, 165 µmol) and 1-hydroxybenzotriazole (26 mg, 165 µmol) in DMF (1.5 mL) was added to the POEPOP-NH_2 resin (58 mg, 33 µmol), followed by diisopropylethylamine (87 µL, 500 µmol). The mixture was shaken for 30 min. The coupling was repeated twice. Removal of the Fmoc group was accomplished by treating the resin twice for 15 min with a solution of 25 % piperidine in DMF. The acylating mixture, prepared by dissolving Z-Aib$_7$-OH[24] (50 mg, 67 µmol) and triphosgene (6.6 mg, 22 µmol) in dry CH_2Cl_2 (2 mL) followed by 2,4,6-collidine (25 µL, 185 µmol), was added to the POEPOP-Ahx-NH_2 resin. After the reaction mixture was shaken for 6 d at room temperature the solvent was exchanged for DMF and the mixture heated at 70 °C for additional 4 d. The resin was washed and treated for 20 min with a trifluoracetic anhydride/pyridine/CH_2Cl_2 (1:1:1) solution (1.5 mL). The mixture was filtered and the resin washed and dried in vacuo.

Synthesis of 3: Z-Aib$_7$-OH[24] (84.5 mg, 113 µmol) and HATU (43 mg, 113 µmol) were dissolved in a 3 % NMM solution in DMF (1.5 mL). After 20 min, this solution was added to the POEPOP-NH_2 resin (65 mg, 38 µmol) and the mixture shaken for 4 d at room temperature. Then, the reaction mixture was filtered and the resin washed and dried in vacuo.

ATR-FTIR spectroscopy: The ATR spectral data were obtained in the 4000 – 650 cm^{-1} range with four scans at 4 cm^{-1} resolution on a Spectrum One Perkin-Elmer spectrophotometer. Before data collection, the samples were pressed on a diamond crystal upon being dried on a high-vacuum pump.

NMR spectroscopy: HRMAS 1D NMR spectra were recorded on a Bruker DSX 500 and/or on a MSL 300 spectrometer equipped with 4 mm ^{1}H/X (X tuned to ^{2}H) MAS probe. The samples were swollen in different deuterated solvents (Figure 2) into a 4 mm HRMAS rotor. The solvents were added to about 5 mg of peptide-resin conjugate directly inside the rotor. The samples were spun at 6 or 8 kHz. The 1D spectra for the determination of temperature coefficients of the NH protons were acquired in the range 300 – 340 K with an increment of 5 K. The temperature was calibrated using a mixture of 80 % ethylene glycol/[D$_6$]DMSO. HRMAS 2D NMR spectra were recorded on a Bruker DSX 500 MHz spectrometer equipped with 4 mm ^{1}H/X (X tuned to ^{2}H) MAS probe and operating at 500.03 MHz for ^{1}H. The samples were spun at 8 kHz. The spectra were acquired at a temperature of 300 K and referenced to the peak of the solvent. Through-space dipolar connectivities were obtained from NOESY spectra using mixing times of 300 or 500 ms. The spectra were recorded in the pure phase mode using a states-time proportional phase increment method, with 2048 points in t_2 and 512 increments in t_1. A spectral width of 7575.76 or 5252.10 Hz was used for the proton signals. A presaturation pulse sequence was applied for the suppression of the POEPOP methylene proton signals. 1D and 2D NMR experiments in solution were acquired on a Bruker Avance DMX 600 spectrometer. Z-(Aib)$_7$-O*t*Bu was dissolved in [D$_6$]DMSO at 1 mM concentration. 1D NMR spectra for the determination of the temperature coefficients of the NH protons were recorded in the range 300 – 340 K with an increment of 10 K. ROESY spectrum was acquired at 300 K.

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