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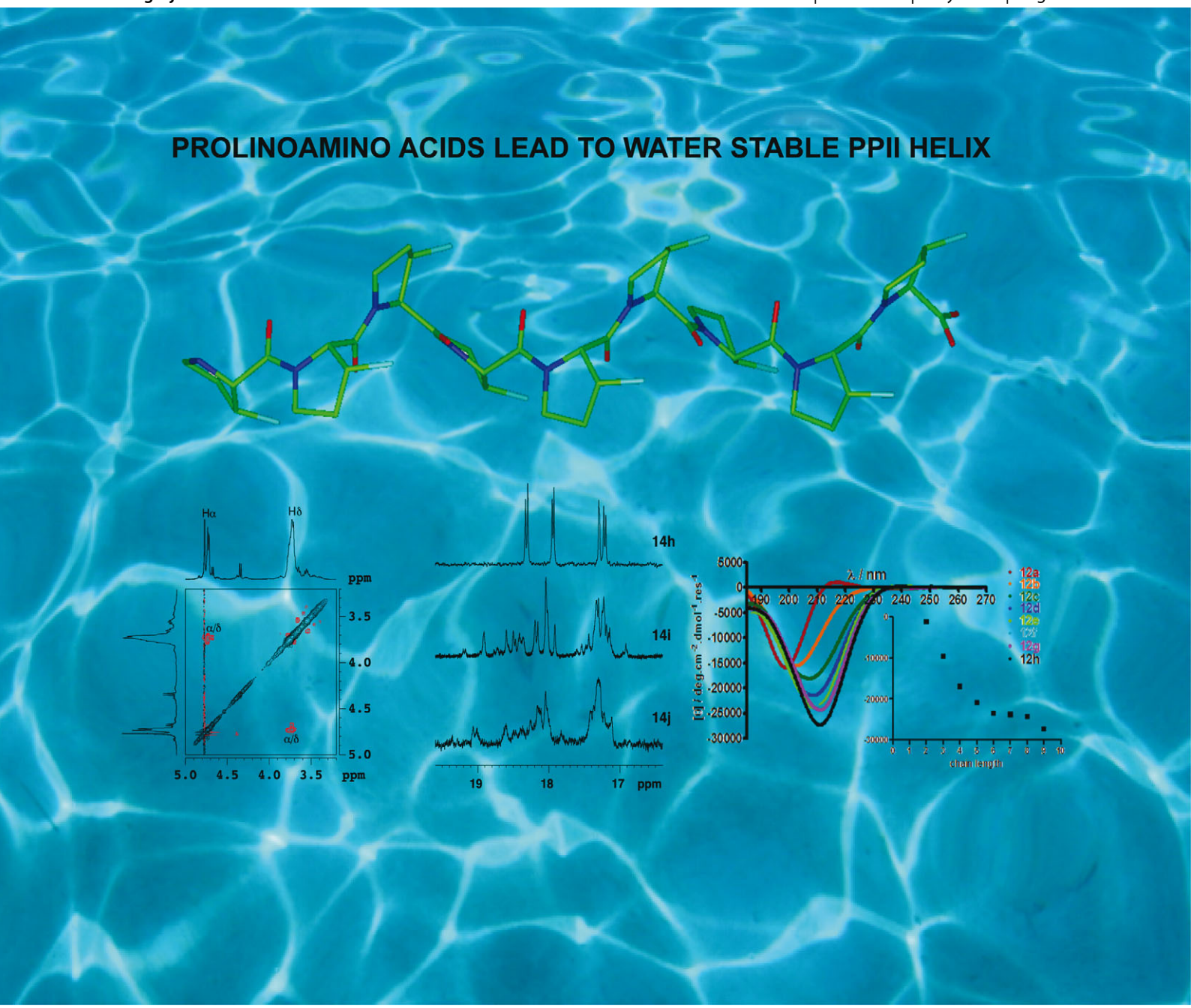

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Philippe Karoyan *et al.*

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Homooligomers of substituted prolines and β-prolines: syntheses and secondary structure investigation†

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Homooligomers of enantiomerically pure (2*S*,3*R*)-3-methyl-proline, (3*R*,4*R*)-4-methyl-β-proline and (3*R*,4*S*)-3,4-dimethyl-β-proline were synthesized and studied using circular dichroism (CD) in water, methanol and propanol and using NMR in water. Changes in the far-UV CD spectrum were observed from dimers to hexamers, but little change was observed from hexamers to octa- or nonamers, both in water and methanol. CD and NMR data allowed us to conclude that oligomers of 3-substituted prolines with more than six residues adopt a characteristic PPII secondary structure both in water and aliphatic alcohols. Oligomers of (3*R*,4*R*)-4-methyl-β-proline bear the same CD signature as non-substituted β-proline oligomers, suggesting that substitution at position 3 is not sufficient to reduce conformational heterogeneity in β-proline oligomers. In the case of 3,4-disubstituted-β-proline oligomers, an atypical signature with an extra negative band at around 225 nm was observed, together with a concentration dependent CD spectrum indicating association properties. Nevertheless, NMR studies of ^{13}C labelled oligomers of 3,4-disubstituted-β-prolines revealed a complex mixture of *cis–trans* conformers even for longer oligomers.

Introduction

Biopolymers display a broad variety of structures that are directly linked to their biological function. Native α peptides made of natural α amino acids are often poor drug candidates owing to bad bioavailability and high susceptibility to protease degradation in biological media. In the last decade, foldamers, *i.e.* short length oligomers with unnatural backbones that adopt well-defined secondary structures, have been developed to mimic biopolymers.[1–4] They are useful tools for understanding the folding propensities and the associated biological functions of biopolymers. Even though foldamers could also represent promising drug candidates since they may overcome the lack of structural and biological stability of natural biopolymers, only a few examples of foldamers that indeed interfere with biological processes by mimicking natural protein have been reported so far.[5,6] This is probably due to two major drawbacks associated with most of the foldamer backbones reported to date. The most significant one is that like α-peptides, stabilisation of their secondary structures almost always relies on specific backbone hydrogen-bonding patterns, which dramatically limit their folding propensity in the aqueous biological medium. The second issue is related to the functionalization of the foldamers backbone with substituents that could participate in molecular recognition, which is often rather tricky.

An interesting strategy to access synthetic backbones capable of folding in water is to design monomers that have the intrinsic propensity to fold without the contribution of hydrogen bond stabilisation. This point has been addressed for example with *N*-alkylglycine oligomers, *i.e.* peptoids, an important class of foldamers that are able to adopt a wide range of secondary structures (PPI,[7,8] PPII helices,[9]...), thanks to the control of the amide bond *cis–trans* isomerism. This is for instance also done by Nature that uses proline to build polyproline helical scaffolds. Among the twenty natural amino acids this cyclic amino acid is unique in its structure since the secondary amine group forms a tertiary amide when involved in a peptidic bond thus preventing hydrogen-bond formation. Although the pyrrolidine ring restricts the flexibility of ϕ and Ψ peptide backbone dihedral angles, the formed tertiary amide bond is more susceptible to *cis–trans* isomerism extending the accessible conformational space. Despite the lack of hydrogen bond, polyproline or proline rich peptide sequences can fold into a helical conformation, *i.e.* the polyproline II (PPII) helix,

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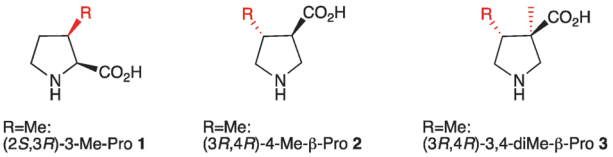


Fig. 1 Substituted proline and β-proline derivatives.

which is often involved in the recognition interface of protein–protein interactions.[10–12] Functionalized PPII helices could be designed by using prolinoamino acids, *i.e.* proline chimeras,[13] combining the pyrrolidine conformational restriction together with amino acid side chain functionalities.

We previously reported an efficient synthetic route to access to β-substituted proline analogues[14–17] (Fig. 1, **1**) by means of the intramolecular carbozincation of zinc enolates derived from *N*-homoallyl α-amino esters. A significant feature of this approach is that it enables the introduction of various functional groups at position 3. In particular, the synthesis of proline chimeras with proteinogenic amino acid side chains has been demonstrated.[13,14,18–22]

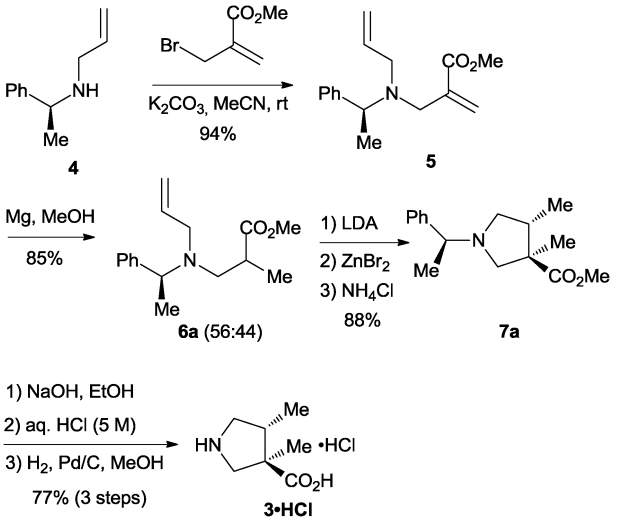
These monomers allow combining the conformational constraints and lack of hydrogen-bond donor of the proline residue in a peptide while keeping the opportunity to decorate them with proteinogenic side chains. We have shown that these residues can be used to build functionalized β-turns that are stable in water,[23] or to introduce a specific functional group in PPII folded peptides that inhibit peptide–protein[24] or protein–protein interaction.[25] Recently the carbocyclization synthetic strategy has been applied to the preparation of β-proline analogues substituted at position 4 (Fig. 1, **2**) and di-substituted at positions 3 and 4 (Fig. 1, **3**).[17,26] We report here a structural study using CD and NMR of the folding abilities of our substituted proline and β-proline derivative oligomers, *i.e.* prolinovalines (**1** R═Me, Fig. 1) and β-prolinovalines (**2** and **3**, R═Me, Fig. 1) in order to evaluate the influence of the substitution of the pyrrolidine ring on the folding of the polymers. This work represents the first step toward the rational design of water-stable functionalized polyproline-like foldamers. The foremost goal of this work is to use these new foldamers to mimic proteins that interact with their partner through an extended domain in order to target interesting biological interactions.

Results and discussion

Synthesis

Amino acid **1**, *i.e.* prolinovaline, was synthesized according to the procedure previously described.[14] By this method the *cis*-(2*S*,3*R*)-prolinovaline **1** was obtained in enantiomerically pure form and at a gram-scale.

The required cyclic β-amino acids **2** and **3** were prepared in diastereo- and enantiomerically pure forms by intramolecular carbocyclization of zinc enolates derived from *N*-allyl β-amino esters (Scheme 1).[26] For gram-scale synthesis, precursor **6a** was best prepared by reduction with magnesium in MeOH of

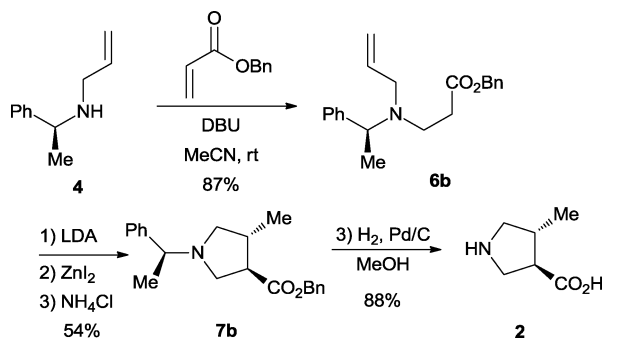


Scheme 1 Synthesis of β-ProMe$_2$ monomer **3**.

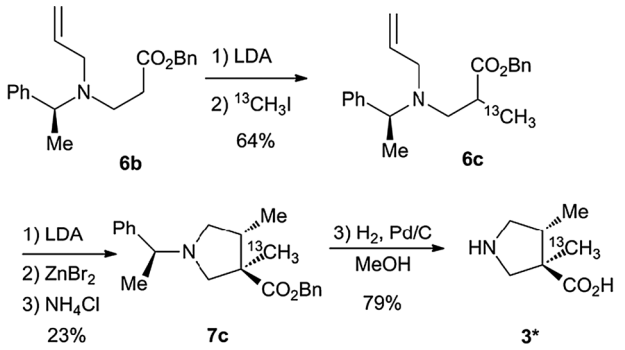
amino-enoate **5**, readily obtained from *N*-allyl-*N*-((*S*)-1-phenylethyl)-amine and methyl 2-bromomethyl-acrylate following our previously reported procedure.[26] Carbocyclization of β-aminoester **6a** by treatment with LDA followed by transmetalation with $ZnBr_2$ yielded pyrrolidine **7a** diastereo- and enantiomerically pure in 88% yield at a gram-scale. Access to enantiopure (3*R*,4*R*)-3,4-dimethyl-β-proline **3** from **7a** was achieved efficiently by a three-step sequence involving saponification of the methyl ester and reductive cleavage of the 1-phenylethyl chiral auxiliary by catalytic hydrogenation over Pd/C in methanol.

By a similar strategy, access to (3*R*,4*R*)-4-methyl-β-proline **2**, *i.e.* β-prolinovaline, devoid of the α methyl substituent proved troublesome as reproducibility problems were met at the hydrogenolysis stage. Thus, we developed an alternate route involving benzyl ester derivatives (Scheme 2). Cyclization precursor **6b** was obtained by reaction of amine **4** with benzyl acrylate in the presence of DBU. Cyclization of **6b** provided methylpyrrolidine **7b** in 54% yield, diastereo- and enantiomerically pure. Deprotection leading to cyclic amino acid **2** was then readily performed in a single step by catalytic hydrogenation over Pd/C.

Finally, we also synthesized a ^{13}C-labelled analogue of compound **3**, *i.e.* **3*** that is ^{13}C-labelled on a specific methyl group (Scheme 3). This compound was prepared in order to help the



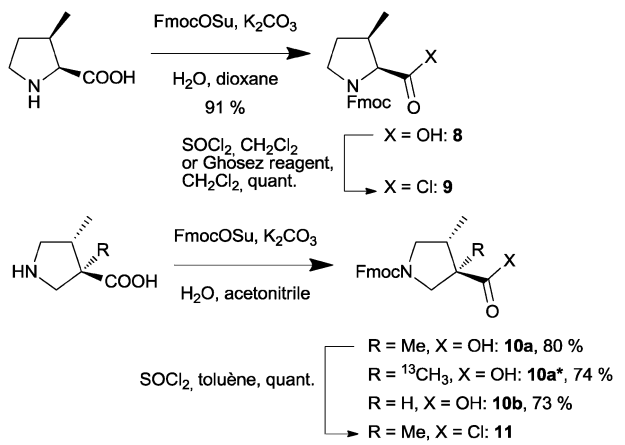
Scheme 2 Synthesis of β-prolinovaline monomer **2**.



Scheme 3 Synthesis of ^{13}C-labelled β-ProMe$_2$ monomer **3***.

oligomer NMR signal interpretation. Indeed, NMR study of proline derived oligomers can be complicated due to *cis–trans* interconversion of tertiary amide bonds giving rise to numerous sets of NMR signals, hampering spectra interpretation. Thus, the preparation of ^{13}C-labelled amino acid **3*** was also achieved *via* the carbocyclization methodology (Scheme 3). The ^{13}C-labelled methyl group was introduced with labelled iodomethane by methylation of the lithium enolate derived from **6b**. Pyrrolidine **7c** was then obtained by carbocyclization of enoate **6c** and the amino acid **3*** was obtained after catalytic hydrogenation over Pd/C.

A final Fmoc protection step of amino groups yielded monomers **8**, **10a**, **10a*** and **10b** suitable for the solid phase synthesis of the oligomers (Scheme 4). For monomers of *N*-Fmoc-(2*S*,3*R*)-prolinovaline **8** and *N*-Fmoc-(3*R*,4*R*)-3,4-dimethyl-β-proline **10a** and **10a***, coupling reactions were achieved after conversion to the corresponding acyl chloride. Indeed, the steric hindrance of these compounds hampered getting satisfactory yields using standard peptide coupling reagents (HBTU, HATU or PyBroP). In the case of 3,4-dimethyl-β-proline **10a** and **10a***, the activation of the carboxylic function through acyl chloride preparation was required only for the coupling of the first amino acid to the resin, HATU giving satisfactory yields in the next steps (Scheme 4). Since charges at both extremities of



Scheme 4 Suitable *N*- and *C*-derivatisation of monomers for oligomers solid phase syntheses.

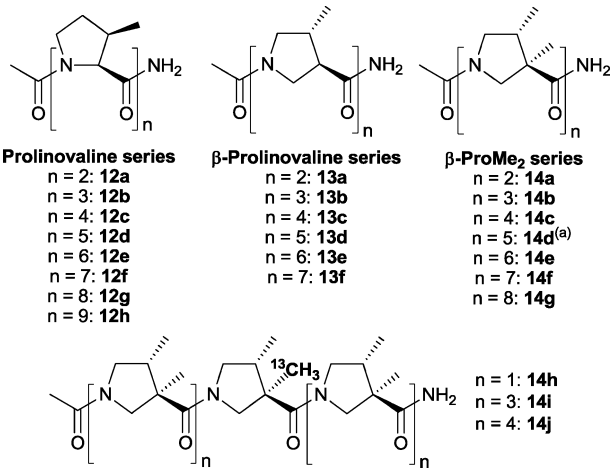


Fig. 2 Oligomers prepared for CD studies. (a) Pentamer **14d** was prepared with the enantiomer of monomer **3** (Fig. 1) and is thus the enantiomer of the compound drawn.

polyproline oligomers can dramatically influence the PPI–PPII conformational equilibrium,[27] the oligomers were acetylated at the *N*-terminus and produced as carboxamides. After cleavage from the resins and purification by RP-HPLC, three series of oligomers (*i.e.* **12**, **13**, **14**, Fig. 2) were obtained, respectively, for prolinovaline derivatives (from dimer to nonamer, **12a** to **12h**), for β-prolinovaline derivatives (from dimer to heptamer, **13a** to **13f**), and for 3,4-dimethyl-β-proline derivatives (from dimer to octamer, **14a** to **14g**).

Three additional compounds in series **14** have been synthesized for NMR studies, namely **14h**, **14i** and **14j** (Fig. 2). They are homooligomers of increasing chain length (respectively trimer, heptamer and nonamer), incorporating one ^{13}C-labelled monomer into the central position. The ^{13}C will be used as a reporter of the *cis–trans* conformational heterogeneity.

Circular dichroism and NMR conformational studies

Circular Dichroism (CD) is a commonly used technique for rapid identification of secondary structures in proteins. It also provides insight into the folding propensities of non-natural oligomers or polyproline oligomers when other techniques fail. For example, PPII conformations are difficult to establish using NMR since the extended conformation prevents the observation of through space correlation (NOEs) between non-adjacent residues. The existence of *cis–trans* interconversion equilibria also often hampers the interpretation of NMR spectra.

Oligomers built from proline adopt extended conformations in solution, *i.e.* PPII conformation in water and PPI conformation in aliphatic alcohols such as MeOH or PrOH. In the case of polyproline conformations, characteristic CD signatures are observed: a weak maximum at 226 nm and a strong minimum at 206 nm in water for PPII conformation, and weak minima at 200 and 232 nm, together with a strong maximum at 215 nm in aliphatic alcohols for PPI conformation.[10] PPII–PPI helix interconversion has been studied using non-natural proline surrogates substituted at various positions.[28–32]

To perform a comparative conformational study in water and aliphatic alcohols, we ensured that the oligomers were well soluble. Interestingly, all oligomers showed a solubility >mM in all solvent systems. The water solubility of these oligomers is promising for construction of foldamers having stable conformation in biological media.

Prolinovaline series 12a to 12h

Since prolinovaline and proline have closely related structures (only an additional methyl at position 3) similar behaviour for the oligomers in solution was expected. Whatever the solvent used, the dimer's CD spectra show a negative band at 200 nm and a positive band at 218 nm. CD spectra of longer oligomers recorded in water (Fig. 3a) show only one strong negative band that shifts from 200 to 210 nm. The positive band at 215 nm disappeared and more surprisingly, no band is observed at around 225 nm where polyprolines usually show a small positive band. This phenomenon has already been reported and is nevertheless consistent with the establishment of a PPII conformation.[33] We used NMR spectroscopy to confirm the presence of major conformers in water. The ^{1}H NMR spectra of oligomers **12a–h** cannot be completely assigned owing to the poor chemical dispersion observed for such homopolymers. However the methyl resonance is a convenient probe to assess *cis–trans* isomerism. Indeed, for each oligomer, major methyl resonances are observed (Fig. 4). Their number corresponds to the number of prolinovaline units, as can be seen in dimer **12a**, trimer **12b** and tetramer **12c** (Fig. 4). This indicates that a major conformer is populated in solution. The analysis of 2D ROESY spectra (Fig. 5) shows strong Hα–Hδ correlations and weak Hα–Hα correlations, demonstrating that the *trans* conformation of peptide bonds predominates.

Upon chain elongation, variation of the normalized intensity of the negative band on CD spectra is not linear and reaches a plateau at six residues (Fig. 3a, inset). This suggests that from six residues the CD spectra do not depict anymore to the sum of the chiral contribution of each monomer but a defined chiral secondary structure in water. For the octamer, upon increasing the temperature from 20 to 90 °C, the shape of the CD signal remains unchanged, only a slight decrease in intensity is observed (see ESI†). This indicates that the secondary structure adopted by the prolinovaline octamer is stable in this temperature range. In MeOH, the negative band also shifts from 200 to 210 nm upon chain elongation (Fig. 3b). As compared to water, an additional positive band is observed at 235 nm for the longer oligomers in MeOH and PrOH. The spectra of heptamer **12f** (Fig. 3c) are superimposable in alcohols and very close to the spectrum obtained in water. As we already reported, the substitution at position 3 of the pyrrolidine ring can be accommodated in PPII conformations,[24,25] thus these data all together suggest that as in water, the PPII conformation is also reached in alcohols.

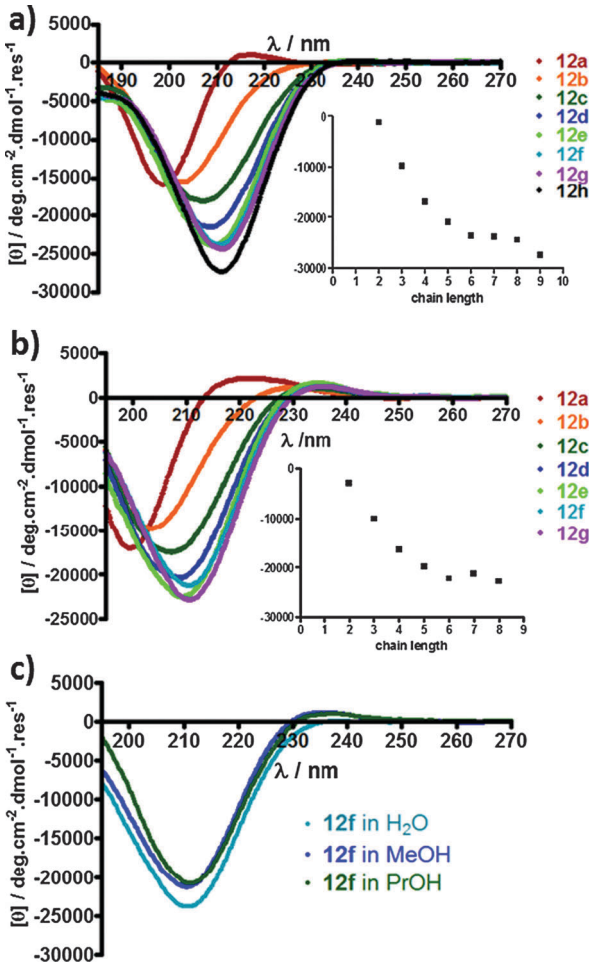


Fig. 3 CD spectra of prolinovaline oligomers **12a–h** recorded at 20 °C using a cell length of 0.1 cm. Concentrations are 400 μM for **12a–c**, 300 μM for **12d**, 200 μM for **12e–g** and 100 μM for **12h** (a) **12a–h** in H_2O, inset: [θ] at 211 nm as a function of chain length, (b) **12a–h** in MeOH, inset: [θ] at 211 nm as a function of chain length. (c) Superimposition of the heptamer (**12f**) CD spectra recorded in water, MeOH and PrOH.

β-Prolinovaline series 13a to 13f

The folding properties of oligomers built from non-substituted β-proline monomers have already been investigated in solution. Although a first study by Yuki *et al.* suggested that poly-β-prolines exist in random coiled conformation,[34] Kim *et al.*[35] described an ordered conformation for the β-proline decamer in 10 mM phosphate buffer (pH 7.0) on the basis of its CD spectrum exhibiting a large positive band at 215 nm and a negative band at 198 nm. Moreover, Gellman and coworkers[36] have shown that the mean residue ellipticity of β-proline oligomers is independent of the chain length from four residues suggesting that a stable secondary structure is established in MeOH. This behaviour is similar to that of α-proline oligomers. Nevertheless, further investigation by NMR on these oligomers showed conformational heterogeneity in solution since a mixture of *cis–trans* amide bond rotamers is observed.[37] So far, conformational stabilisation in β-proline oligomers has only been achieved by restriction of the conformational flexibility of the monomers, using di-substituted derivatives at position 5 of the pyrrolidine ring,[38] or using α,δ ethano-bridge β-proline derivatives, both favouring *cis* rotamers,[39] or α,γ methano-bridge β-proline selecting *trans* rotamers.[40]

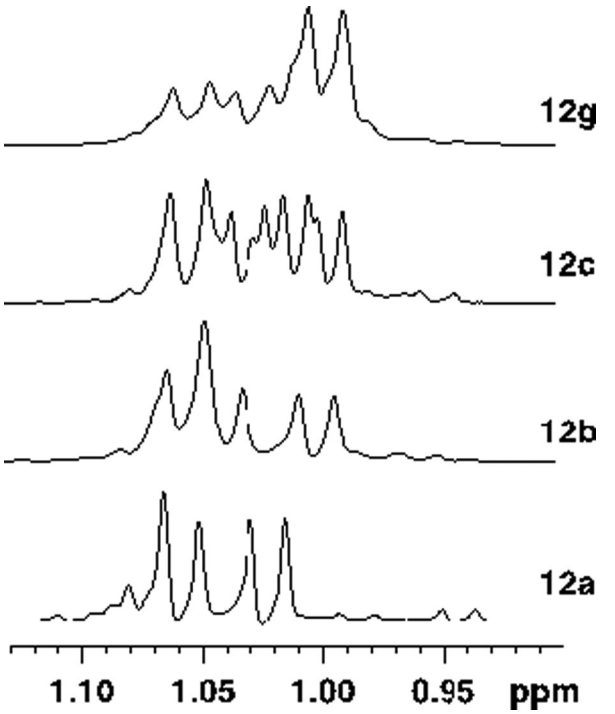


Fig. 4 1D ^{1}H NMR spectra of oligomers **12a–c** and **12g** recorded in D_2O at 25 °C showing the methyl resonances. Methyl signals appear as doublets owing to ^{3}J coupling with the Hβ proton.

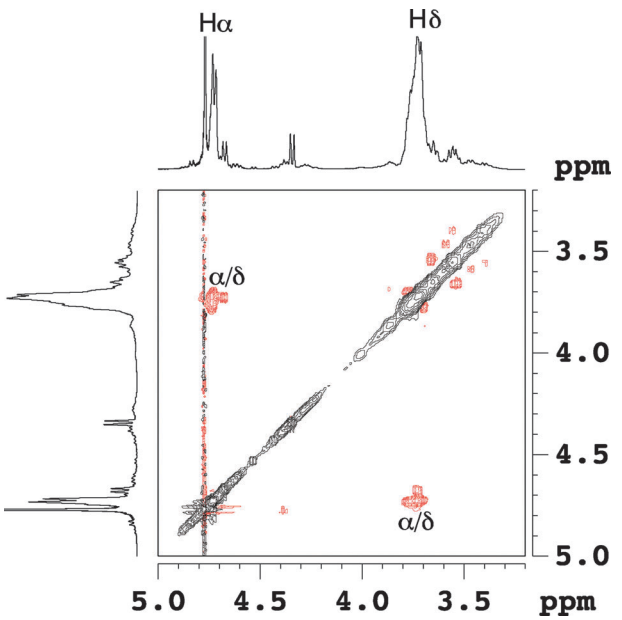


Fig. 5 2D ^{1}H–^{1}H ROESY spectrum of oligomer **12h** recorded in D_2O at 25 °C (300 ms mixing time) showing the strong Hα–Hδ correlations characteristic of *trans* peptide bond conformation.

In an attempt to investigate the influence of the substitution at position 4 of the pyrrolidine ring of β-proline on the secondary structure, CD experiments were performed at 20 °C in water (Fig. 6) and MeOH (see ESI†). β-Prolinovaline oligomers have a CD signal bearing two bands of equal intensity: one negative

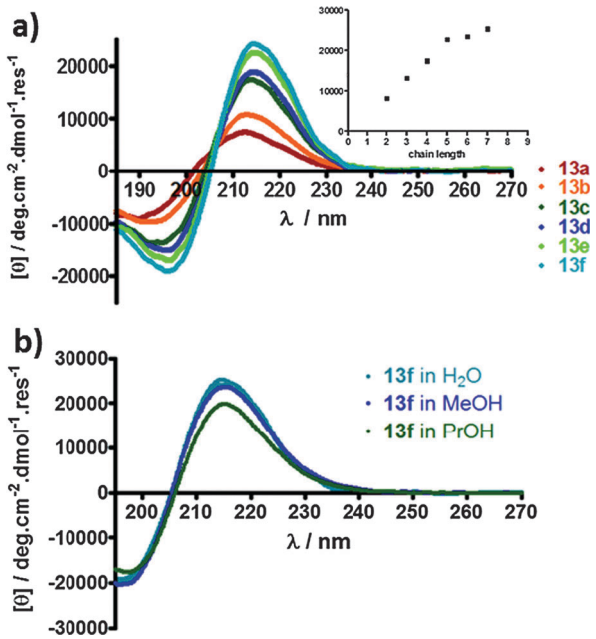


Fig. 6 CD spectra of β-Proval series, *i.e.* oligomers **13a–f** recorded at 20 °C using a 0.1 cm cell length. Concentrations were set at 500 μM for **13a–b**, 200 μM for **13c–d** and 100 μM for **13e–f**. (a) In H_2O, inset: [θ] at 215 nm as a function of chain length. (b) Superimposition of the **13f** heptamer CD spectra recorded in water, MeOH and PrOH.

band at 197 nm and one positive band at 215 nm (Fig. 6). This shape is identical to that observed for unsubstituted β-proline oligomers.[36] The normalised intensity of both bands increases with chain elongation in a non-linear manner, reaching a plateau from 5 residues. CD spectra of the heptamer were recorded at different temperatures and upon heating to 90 °C no conformational transition was observed, evidenced by only slight changes in the spectra (see ESI†). These data would possibly indicate the establishment of a stable secondary structure. However, the same behaviour was observed for non-substituted β-proline oligomers suggesting that these CD spectra likely reflect equilibrium between several secondary structures rather than the presence of a unique secondary structure in solution. The same trend is observed in MeOH and PrOH. The superimposition of the CD spectra recorded in water, MeOH and PrOH suggests that this conformational equilibrium is not influenced by solvation effects (Fig. 6).

Me_2-β-Pro series 14a to 14g

Before investigating the conformations adopted by all oligomers of each series described herein (**12**, **13** and **14**), the CD spectra of the tetramers (*i.e.* **12c**, **13c** and **14c**) were recorded at different concentrations ranging from 25 μM to 1 mM. For compounds **12c** and **13c**, the tetramer signals were superimposable meaning that no association occurred for these oligomers whatever the concentrations were (see ESI†). A different behaviour was evidenced for tetramer **14c**, in both water and MeOH (Fig. 7). Indeed, in this case, a concentration dependent signal was observed. At 25 μM, the CD spectrum exhibited two bands: a minimum at 195 nm

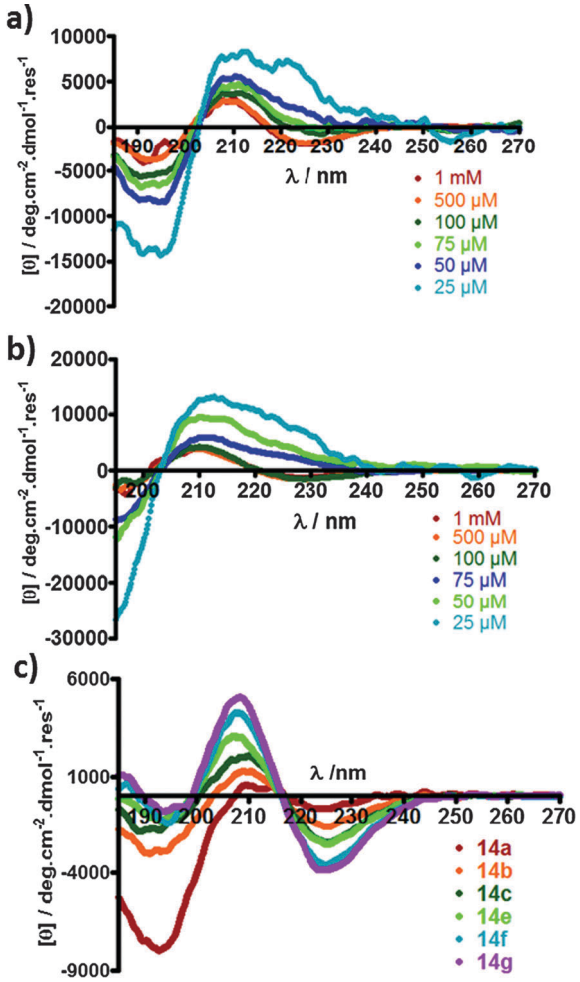


Fig. 7 CD spectra of Me_2-β-Pro series, *i.e.* oligomers **14a–g** recorded at 20 °C using a 0.1 cm cell length. (a) CD spectra of **14c** at different concentrations in H_2O. (b) CD spectra of **14c** at different concentrations in MeOH. (c) CD spectra of **14a–g** in H_2O. Concentrations were set at 400 μM for **14a–c** and 200 μM for **14d–g**.

and a maximum at 210 nm, which was similar to that of β-prolinovaline oligomers. From 25 to 100 μM, the intensities of both bands decreased and a new band at 225 nm appeared in H_2O. Above 100 μM, no change was observed anymore. These results suggest that the tetramer self-assembles above 75 μM in H_2O. In MeOH, the same trend was observed and self-assembling seems to be completed at around 500 μM.

Since self-assembling is often associated with the establishment of a more stable secondary structure, we decided to perform the experiments at 100 μM, the lower concentration at which no more variation is observed on the CD spectrum. Upon elongating the chain, the signals intensity at 210 and 225 nm increases whereas it decreases at 195 nm (Fig. 7c). Variations of the normalised intensity of the bands at 195, 210 and 225 nm show different behaviours: a saturation of the signal seems to be reached at 195 and 225 nm whereas a linear trend is observed at 210 nm (see ESI†). These findings prevented us to conclude regarding the establishment of a unique stable conformation in water.

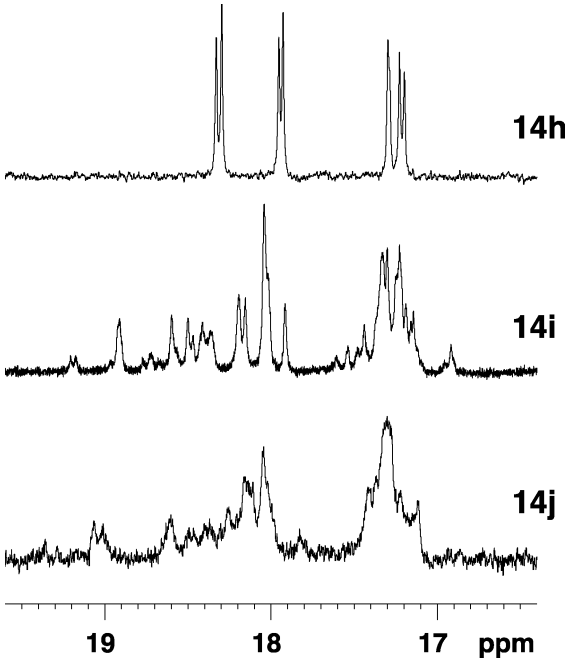


Fig. 8 1D ^{13}C NMR spectra of oligomers **14h–j** in D_2O at 25 °C showing the methyl resonance.

The conformational properties of this series were further analyzed using NMR spectroscopy. The NMR spectra exhibit low chemical shift dispersion and high complexity due to chemical shift heterogeneity. The NMR spectra of dimer **14a** could be unambiguously assigned. Four forms with roughly identical populations are observed, corresponding to *cis–trans* rotamers of both amide bonds in slow exchange on the NMR time scale (ESI†). In order to facilitate the conformational analysis of larger oligomers, we incorporated a ^{13}C-labelled methyl group into a single unit occupying the central position in oligomers. 1D ^{13}C NMR spectra were recorded to probe the *cis–trans* interconversions of amide bonds around the central unit. The 1D ^{13}C NMR spectrum of trimer **14h** (Fig. 8) shows eight signals of comparable intensities that correspond to *cis–trans* isomerism of the three amide bonds. The similar populations prove that there is no conformational preference around the amide bonds in the trimer. An even larger number of peaks is observed on the ^{13}C NMR spectra of heptamer **14i** and nonamer **14j** (Fig. 8). Thus the methyl ^{13}C signal appears to be very sensitive to *cis–trans* isomerism of several amide groups, and not only those directly connecting the labelled unit. The high complexity of ^{13}C NMR spectra clearly demonstrates that there is no stabilisation of an amide bond conformer, even in longer oligomers.

Experimental

Peptide synthesis

Oligomers were synthesized manually on a solid support using an Fmoc-rink amide resin (substitution level = 0.43–0.57 mmol g^{-1} resin, 100–200 mesh). The resin was swollen in DCM for 15 min prior to use. Fmoc removal was performed by using 20% (v:v)

piperidine in 1-methyl-2-pyrrolidone (NMP) once for 1 min and then once for 15 min. The resin was washed five times with NMP.

Fmoc-amino acid 8 couplings. Acyl chloride **9** was formed using 1-chloro-*N*,*N*-2-trimethyl-1-propenylamine (1.1 eq.) in anhydrous DCM (0.2 M) under an Ar atmosphere. The reaction mixture was stirred at room temperature for 1 h 30 min and was directly used for amino acid coupling on the resin: the resin was suspended in anhydrous DCM, *N*,*N*-diisopropylethylamine (4 eq.) then the solution of acyl chloride (2 equivalents) was added. After 1 hour the solution was removed by filtration and the resin was washed with DCM five times then rinsed with NMP 3 times. Reaction completion was checked by the Kaiser or chloranil test for primary or secondary amines, respectively. In the case of a positive test, the same amino acid was condensed again until getting a negative test. Cycles of deprotection-washing–coupling-washing were repeated until the desired sequence was achieved.

Fmoc-amino acid 10a (and 10a*) couplings. Except for the first residue, all couplings were performed using 3 equivalents of Fmoc-amino acid **10a** (or **10a***), 3 equivalents of HATU and HOAt and 6 equivalents of DIEA dissolved in NMP (0.15 M). Each coupling reaction was allowed to proceed for 0.5 h at room temperature. The solution was then removed by filtration and the resin was washed 5 times with NMP. Reaction completion was checked by performing the chloranil test. In the case of a positive test, the coupling was repeated. Cycles of deprotection-washing–coupling-washing were performed until the desired sequence was achieved. The first coupling required prior formation of the acyl chloride **11**. **11** was obtained using $SOCl_2$ (47 eq.) in toluene. The reaction was allowed to proceed at 90 °C for 0.5 hour and the solvents were removed. After Fmoc removal, the resin was rinsed three times with dried DCM and kept under an Ar atmosphere. Anhydrous DCM and dried DIEA were added followed by the solution of the acyl chloride in dried DCM. The resin was shaken at room temperature for 0.5 hour, rinsed five times with DCM. And coupling completion was monitored by the Kaiser test.

Fmoc-amino acid 10b couplings. Stepwise couplings were accomplished with 3 equivalents of Fmoc-amino acid **10b**, 3 equivalents of HBTU and HOBt and 6 equivalents of DIEA in NMP (0.15 M). Each coupling reaction was allowed to proceed for 0.5 h at room temperature. The solution was then removed by filtration and the resin was washed with NMP five times. Reaction completion was checked by the Kaiser or chloranil test for primary or secondary amines, respectively. In the case of a positive test, the same amino acid was condensed again until getting a negative test. Cycles of deprotection-washing–coupling-washing were repeated until the desired sequence was achieved.

Acetylation, cleavage and purification. Final acetylation of all oligomers was performed using 32 equivalents of acetic anhydride in DCM (1.4 M) for 1 hour at room temperature. Cleavage of peptides was accomplished by treatment of the resin with 3 mL of TFA/triisopropylsilane/H_2O (95/2.5/2.5 v:v:v) for 3 h at room temperature. The TFA solution was collected and the beads rinsed 3 times with neat TFA. The combined TFA solutions were evaporated. The peptide was triturated with cold Et_2O, collected by centrifugation, and the pellet was washed three times with cold Et_2O. The pellet was then dried for 3 hours under vacuum, redissolved in deionized water and freeze dried. The peptide dissolved in deionized water containing 0.1% of TFA (v:v) was purified by reversed phase HPLC using a C18 column with an acetonitrile–water gradient both solvents containing 0.1% TFA.

Sample preparation of circular dichroism spectroscopy

Stock solutions of each peptide were prepared in deionised H_2O and were in the millimolar range. The peptide concentration was determined by NMR using sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) as an internal reference compound (see ESI† for more details).

CD solutions of desired concentration in H_2O were further prepared by dilution of stock solutions. To prepare MeOH solutions, the required amount of stock solution was freeze-dried and MeOH was added. MeOH samples were heated at 50 °C for 1 hour and then allowed to stand at room temperature for 24 hours prior to recording CD spectra.

Circular dichroism spectroscopy

CD measurements were performed using a Jasco J 815 spectropolarimeter equipped with a Peltier to control the temperature, using quartz cells with a path length of 0.1 cm, a scan speed of 50 nm min^{-1} and a resolution of 0.2 nm. Spectra were recorded as an average of four scans. For each spectrum, the background was subtracted prior to smoothing and processing. All the measured ellipticities are presented normalised as a function of the concentration of solutions and the number of chromophores (amide bonds).

NMR spectroscopy

NMR experiments were performed on a Bruker Avance III 500 MHz spectrometer equipped with a TCI cryoprobe. NMR spectra were recorded in D_2O at temperatures between 25 °C and 35 °C. ^1H and ^{13}C chemical shifts were referenced to sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS). NMR studies of small oligomers were based on the analysis of 2D homonuclear COSY, TOCSY and NOESY spectra, and 2D ^1H–^{13}C HSQC, HMBC, and HSQC-TOCSY spectra. 2D NOESY experiments were recorded with a mixing time of 1 s.

Conclusions

Homooligomers of enantiomerically pure (2*S*,3*R*)-3-methyl-proline, (3*R*,4*R*)-4-methyl-β-proline and (3*R*,4*S*)-3,4-dimethyl-β-proline were synthesized and studied using circular dichroism (CD) in water and alcohols. In each series, changes in the far-UV CD spectrum were observed from dimers to hexamers, but little change was observed from hexamers to octa- or nonamers, in water, methanol and propanol. We conclude from CD and NMR studies that oligomers of 3-substituted prolines with more than six residues adopt a characteristic PPII secondary structure. CD data on β-prolinovaline oligomers show close similarities

with unsubstituted β-prolines, indicative of *cis–trans* conformational heterogeneity in solution, with no influence of the solvent. In the case of 3,4-disubstituted-β-prolines, the concentration dependent CD spectra reveal association properties. An atypical CD signature was also observed with an extra negative band at around 225 nm. However NMR studies clearly show the absence of favoured amide rotamers even in the longest oligomers. Therefore the conformational restrictions brought by the two methyl substituents are not sufficient to stabilize unique amide bond rotamers of β-prolines. From the structural studies performed here with these proline analogues, we can conclude that only 3-substituted prolines appear to be valuable tools for building short, stable and functionalized PPII helix mimetics.

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