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β^2-Amino Acids in the Design of Conformationally Homogeneous *cyclo*-Peptide Scaffolds

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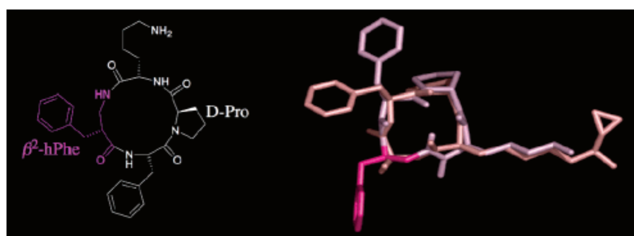

Herein, we report studies on the influence of chiral β^2-amino acids in the design of conformationally homogeneous cyclic tetrapeptide scaffolds. The cyclic α-tetrapeptide *cyclo*(-Phe-D-Pro-Lys-Phe-) (**1**) and its four mixed analogues, having one of the α-Phe replaced by either an (*S*)- or an (*R*)-β^2hPhe residue (i.e., *cyclo*(-(*R*)-β^2hPhe-D-Pro-Lys-Phe) (**2a**), *cyclo*(-(*S*)-β^2hPhe-D-Pro-Lys-Phe-) (**2b**), *cyclo*(-Phe-D-Pro-Lys-(*R*)-β^2hPhe-) (**3a**), and *cyclo*(-Phe-D-Pro-Lys-(*R*)-β^2hPhe-) (**3b**)), were all synthesized through solid-phase procedures followed by solution-phase cyclization. Initially, all five *cyclo*-peptides were analyzed by ^{1}H NMR spectroscopic studies in different solvents and at variable temperatures. Subsequently, a detailed 2D NMR spectroscopic analysis of three of the mixed peptides in water was performed, and the information thus extracted was used as restraints in a computational study on the peptides' conformational preference. An X-ray crystallographic study on the side chain-protected (Boc) **2a** revealed the solid-state structure of this peptide. The results presented herein, together with previous literature data on β^3-amino acid residues, conclusively demonstrate the potential of β-amino acids in the design of conformationally homogeneous cyclic peptides that are homologous to peptides with known applications in biomedicinal chemistry and as molecular receptors.

Introduction

Natural products comprising cyclic peptides are widespread in nature and often display unique biological effects (e.g., cytotoxic, immunosuppressive, and antibacterial activities).[1] Cyclization is also a common methodology for reducing the conformational freedom of linear peptides,[2] as the high flexibility that is characteristic of smaller linear peptides often is undesirable for biological applications.[3] Cyclization also enhances bioavailability and metabolic stability of a peptide.

Cyclic tetrapeptides, such as chlamydocin (Figure 1a), trapoxin B, and HC toxin, are an especially interesting class of natural products, which have been shown to modify histone deacetylase function and thereby affect cell growth and morphology.[4] Cyclic tetrapeptides are also sufficiently small to be considered "druglike" and have been used in the search for novel

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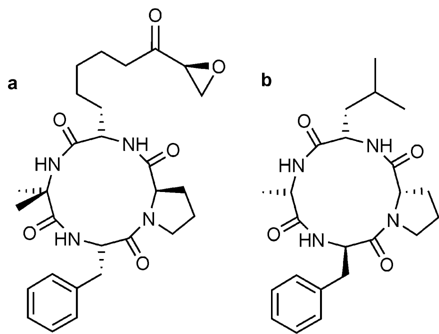

FIGURE 1. Examples of cyclic tetrapeptides. (a) Natural product chlamydocin, which inhibits mammalian histone acetylases. (b) Synthetic peptide designed and synthesized to be used as a stereoselective molecular receptor.

antibacterial[5] and cytotoxic[6] agents. They have also been used as stereoselective hosts in supramolecular applications (Figure 1b)[7] and could probably find future applications in asymmetric catalysis.[8] Unfortunately, the use of cyclic tetrapeptides in these fields is limited by short supply of the few known naturally occurring derivatives and by the difficulties of synthesizing them.[9] In all applications listed above, reduced conformational freedom of the peptide backbone is desirable. Although cyclization reduces the conformational space available to a peptide, cyclic peptides as short as tetramers most often still exist as multiple conformers in polar solvents such as water or DMSO.[10] Thus, all areas where cyclic tetrapeptides have been successfully applied in the past would benefit from new ways of increasing the structural stability and simultaneously enhance the synthetic accessibility and increase the structural diversity of compounds related to these important peptides.

Cyclic peptides of necessity contain a turn in the sequence. D-Amino acids and proline residues have been widely used for incorporating designed turns into peptides.[1] The insertion of a D-amino acid strongly influences the position of this turn and does in most cases control the overall conformation of the peptide.[11] Likewise, proline residues strongly affect the conformation of a peptide chain by restraining the conformational space and inducing different types of turns.[12] Further, proline has the ability to change the conformation of the peptide chain by undergoing *cis*-/*trans*-isomerization.

β-Amino acids (i.e., amino acids containing an extra methylene group in the backbone) occur naturally in peptidic structures[13] and have been used in peptide design to obtain mixed peptides that retained their biological activities,[14] while showing enhanced proteolytic stability.[15] In addition to di-, tri-, and tetra-substituted analogues, proteinogenic β-amino acids with the side chain either at the α-carbon (β^2-amino acids) or at the β-carbon (β^3-amino acids) may be synthesized. Oligomers of β-amino acids (i.e. β-peptides)[16] have also received tremendous interest as foldamers[17] with promising biological activities (e.g., antibacterial,[18] somatostatin mimicking,[19] and disruption of protein−protein interactions).[20] Further, they are completely resistant to proteolytic[21] and metabolic[22] degradation.

Theoretically, each additional methylene group incorporated into the backbone of a peptide introduces extra degrees of torsional freedom due to rotation around the $C\alpha-C\beta$ bond.[23] However, similar to D-amino acids and proline, β-amino acids are proven to be turn-inducing residues with potential to enhance structural stability of short peptides.[24] Sewald et al.[25] have reported studies concerning the incorporation of a β^3-amino acid residue into cyclic tetra- and pentapeptides otherwise only

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containing α-amino acids. They found an enhanced stability of the overall secondary structure, more specifically that the incorporated unnatural β-amino acid residue preferably occupied the central sequence position of a modified γ-turn conformation. Similar studies were published by Fairlie et al.,[26] where they investigated how β^3-amino acids influence the stability of cyclic tetrapeptides by incorporating a β^3-hPhe (β^3-(*S*)-homophenylalanine) residue into an α-peptide sequence, which contained another conformationally biasing residue (i.e., D-proline). They too observed an enhanced conformational stability of the resulting peptide, but only when the β^3-hPhe residue was positioned opposite and not adjacent to the D-proline residue.

In line with our interest on the synthesis and conformational analysis of cyclic peptides consisting merely of β-amino acids[27] and the recent interest in designing turn mimetics for disruption of protein−protein interactions,[28] we became interested in finding out whether a β^2-amino acid residue would impose similar restriction on the conformational space of cyclic peptides otherwise composed only of α-amino acids. Theoretical studies by Wu and Wang have shown that β-substitution (β^3-amino acid) is more efficient than α-substitution (β^2-amino acid) in reducing the flexibility of a β-peptide backbone.[29] However, the unique structures found for β^2-amino acid containing peptides[24b,30] suggest that β^2-amino acids could also have a stabilizing effect on cyclic α-peptides. Herein, we wish to report our findings on the influence of both enantiomers of a β^2-amino acid on the conformational preference of a cyclic tetrapeptide.

Results

Design and Synthesis. To study the effect of β^2-amino acids and to be able to compare the result to the influence of β^3-amino acids, as studied by Sewald et al.[25] and Fairlie et al.,[26] we chose a sequence closely related to that used by Fairlie et al. Hence, a cyclic tetrapeptide consisting merely of α-amino acids, that is, *cyclo*(-Phe-D-Pro-Lys-Phe-) (**1**), was synthesized for comparison. Although this peptide does not contain the nonproteinogenic amino acid (2*S*,9*S*)-2-amino-8-oxo-9,10-epoxydecanoic acid (Aoe), known to be necessary for the biological activity of chlamydocin,[31] this sequence still has close structural resemblance to such entities, suggesting that the structures identified in this study could serve as a scaffold for further developments of biologically active substances and molecular receptors.

The positional effect of the β^2-amino acid incorporation was of high interest, whereupon we synthesized mixed cyclic tetramers with the β^2hPhe residue either in position $i - 1$ (**2a**,**b**) or $i + 2$ (**3a**,**b**) with respect to the D-proline. Additionally, the effect of the chirality of the side chain of the β^2-amino acid was also of significant interest, and therefore (*R*)-β^2hPhe was inserted in **2a** and **3a** and (*S*)-β^2hPhe in **2b** and **3b**, respectively (Figure 2).

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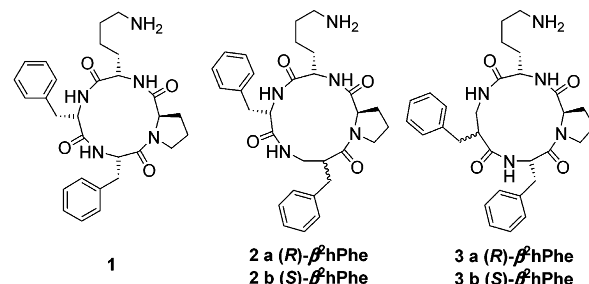

FIGURE 2. Cyclic tetrapeptide **1** and mixed cyclic α/β^2-tetrapeptides **2a**,**b** and **3a**,**b**.

In contrast to the readily available (both synthetically and commercially) β^3-amino acids, the synthesis of enantiomerically pure β^2-amino acids is far more tedious and still requires laborious multistep procedures despite numerous developments in this field.[32]

The protected β^2-amino acids, Fmoc-(*R*)-β^2hPhe-OH and Fmoc-(*S*)-β^2hPhe-OH, were synthesized enantiomerically pure utilizing a procedure developed by Seebach and co-workers,[30,33] with the exception of using the Evans type of chiral oxazolidinone auxiliary.[34]

The general strategy for the syntheses of the cyclic tetrapeptide **1** and the mixed cyclic α/β^2-peptides **2a/b** and **3a/b** is outlined in Scheme 1 and included first solid-phase synthesis of the linear peptide, followed by cleavage of the precursors from the resin and final cyclization in solution. The linear peptides were all synthesized by standard Fmoc solid-phase peptide synthesis protocols on 2-chlorotrityl chloride resin (**4**, Barlos resin).[35] Cleavage of Fmoc-deprotected **5** from the solid support was performed under the typical mild conditions for this resin (i.e., acetic acid) and gave the side chain Boc-protected linear tetrapeptide **6**. For cyclization, the linear precursor **6** was dissolved in dry DMF followed by addition of DIEA. This mixture was then added slowly over 20 h via a syringe pump to a flask containing a vigorously stirred solution of PyBOP and DIEA in dry DMF to get a final peptide concentration of about 1 mM. The products were obtained after preparative reversed-phase HPLC, and the yields for the cyclization step were in a narrow range of 36−40% for all peptides (Table 1).

Characterization of the mixed cyclic peptides by reversed-phase HPLC on an analytical C18 column revealed interesting results. The retention time of **2b** is only about half as long as that for the other cyclopeptides (Table 1). This behavior represents a remarkable change in overall hydrophobicity under these conditions, suggesting a different conformation of **2b** caused by the reversed stereochemistry at the β^2hPhe-OH residue compared to that of its diastereomer **2a**.

Solid-State Structure through X-ray Diffraction. Before deprotection of the Boc-protected lysine side chain, we obtained crystals of **2a** suitable for X-ray diffraction (Figure 3).

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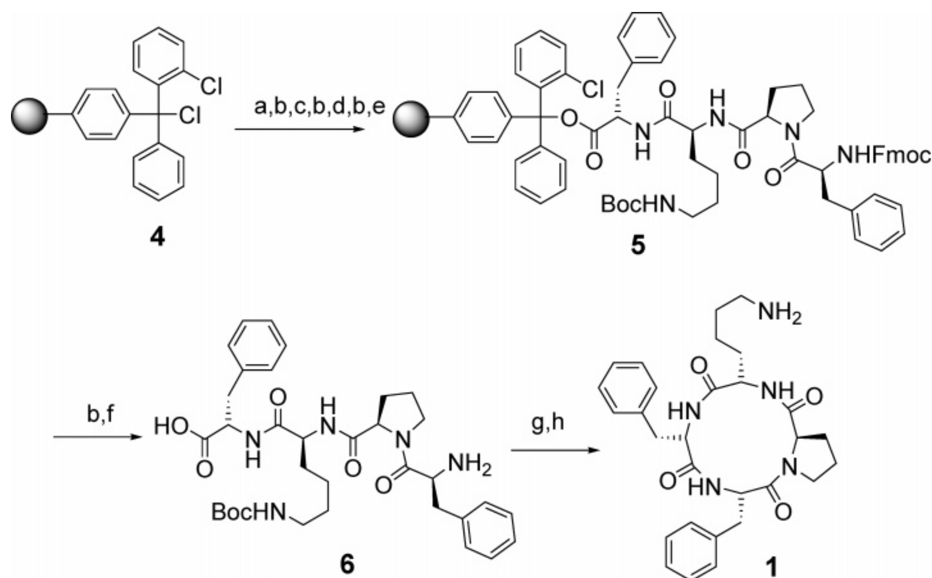
SCHEME 1. General Synthetic Route for the Cyclic Tetrapeptides, Exemplified by 1[a]



[a] Reagents and conditions: (a) Fmoc-Phe-OH, DIEA, DCM, room temperature, 1 h; (b) 2% DBU/2% piperidine in DMF, 5 × 5 min; (c) Fmoc-Lys(Boc)-OH, HBTU, HOBt, DIEA, DMF, room temperature, 3 h; (d) Fmoc-D-Pro-OH, HBTU, HOBt, DIEA, DMF, room temperature, 3 h; (e) Fmoc-Phe-OH, HBTU, HOBt, DIEA, DMF, room temperature, 3 h; (f) DCM/CH_3COOH/TFE, (3:1:1), room temperature, 2 h; (g) PyBOP, DIEA, DMF, room temperature, 24 h; (h) TFA.

TABLE 1. Isolated Chemical Yields and Retention Times during Analytical RP-HPLC of Purified Mixed Cyclic α/β-Tetrapeptides

peptide	t_r (min)	yield (%)
2a	19.7	37
3a	22.8	40
2b	10.9	36
3b	23.0	36

All *trans*-amide bonds were observed in the solid-state structure of Boc-protected **2a**. While the $i + 1$ amide nitrogen (relative to the D-Pro residue) formed a hydrogen bond intermolecularly, the two amide nitrogens at $i + 2$ and $i - 1$ positions formed intramolecular hydrogen bonds to carbonyl oxygen atoms of the $i + 1$ residue and of D-Pro, respectively. These two intramolecular H-bonds, with distances of 2.94(1) Å and 3.17(1) Å between the heavy atoms, respectively, contribute to the rigid conformation of this cyclic peptide. Despite much effort, no crystals of sufficient quality for X-ray diffraction measurements were obtained for any of the other peptides in protected or deprotected form.

Solution-State Structures through NMR Spectroscopy and Calculations. Before investigating the conformational preference of the peptides by NMR spectroscopy and Monte Carlo conformational search, we made some brief predictions of the expected conformations from how the different amino acid residues are positioned relative to each other. Structure **2a** (*cyclo*(-(*R*)-β^2hPhe-D-Pro-Lys-Phe-)) and **2b** (*cyclo*(-(*S*)-β^2hPhe-D-Pro-Lys-Phe-)) have the turn-inducing residues D-Pro and β^2hPhe at i and $i - 1$ positions, respectively, assuming a stabilizing effect in the D-Pro-β^2hPhe sequence. The cyclic peptides **3a** (*cyclo*(-Phe-D-Pro-Lys-(*R*)-β^2hPhe-)) and **3b** (cyclo(-Phe-D-Pro-Lys-(*S*)-β^2hPhe-)), however, have the two turn-promoting residues separated from each other in an i and $i + 2$ relation. This could lead to stabilization of the conformer at two positions independent of each other, similar to the observations made by Fairlie and co-workers.[26] Further, the chirality of the β^2-amino acid was expected to influence the conformational stability, which rationalized the synthesis of β^2hPhe residues with either the side chain of the same relative configuration as natural α-amino acids ((*R*)-β^2hPhe) or with the opposite chirality ((*S*)-β^2hPhe).[36]

^{1}H NMR spectra of the five cyclic tetrapeptides **1** and **2**−**3a**/**b** were first recorded in D_2O/H_2O (1:9). The amide region of cyclic α-tetramer **1** gives the expected indication of several conformers in slow interconversion (Figure 4a). Further, the coupling constants are smaller than what would be expected for an ordered conformation.

The amide regions of the spectra arising from the four mixed tetrapeptides all give the indication that replacement of one α-amino acid residue with a β-amino acid analogue gives rise to peptides with enhanced structural stability.

Comparing the spectra given for diastereomers **2a** and **2b**, the only significant difference observed is a drastic change in shift of the α-Phe amide proton signal (Figure 4b,c, respectively). In comparison between the amide regions of the ^{1}H

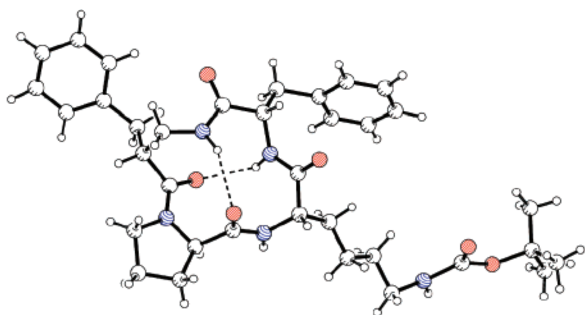


FIGURE 3. Perspective drawing of the crystal structure of Boc-protected **2a**. Two intramolecular hydrogen bonds are shown in dashed lines.

(36) It should be noted that, as the backbone of the amino acids are prolonged with an extra methylene group, the nomenclature of *R* and *S* according to the CIP rules can change even though the absolute configuration remains unaltered.

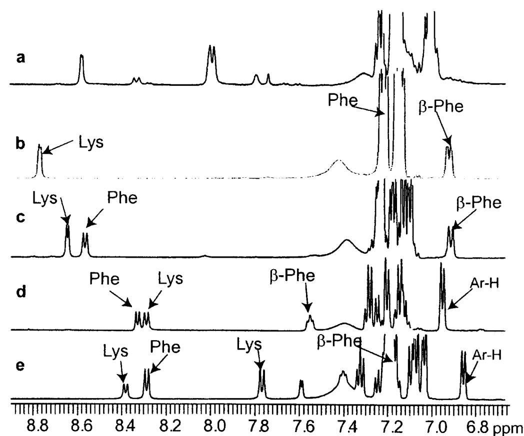


FIGURE 4. ^{1}H NMR (500 MHz) spectra of **1** (a), **2a** (b), **2b** (c), **3a** (d), and **3b** (e) in D_2O/H_2O (1:9). Expansions of the amide region laid on top of each other for comparison. The pH was measured to be 4.7 (**1**), 4.3 (**2a**, **2b**, and **3a**), and 3.3 (**3b**).

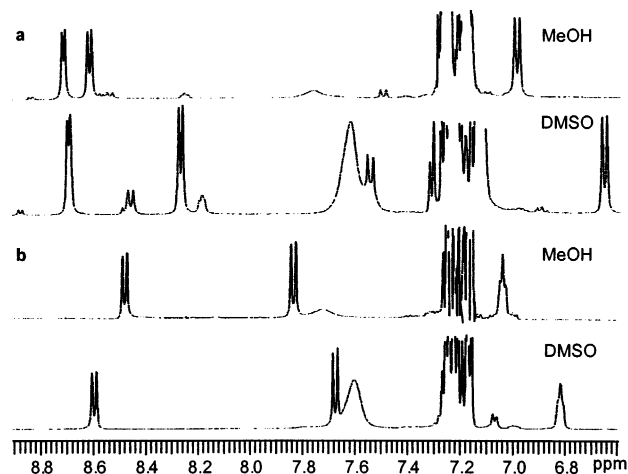


FIGURE 5. Solvent contribution to the conformational stability evaluated by ^{1}H NMR (500 MHz) spectroscopy in CD_3OD/CH_3OH (1:9) and DMSO-d_6. Two conformations of **2b** (a) appear in DMSO-d_6 but not in methanol or water. The existence of multiple conformers seen in aqueous solution of cyclic tetramer **3b** (b) (see Figure 4e) is, however, reduced to one in both CD_3OD/CH_3OH (1:9) and DMSO-d_6.

NMR spectra arising from the diastereomers **3a** and **3b**, there is no dramatic change in shifts due to the change of chirality; however, the high degree of conformational stability seen in the spectra of **3a** has been reduced in the spectra of **3b** (Figure 4d,e). One observation arising from this is the existence of two separate amide proton signals arising from the Lys residue (δ = 8.38 and 7.77 ppm, with $^3J_{NHCH\alpha}$ = 6.9 Hz in both cases).

Cyclic tetramers **2a** and **2b** give rise to sharp amide proton signals and large (>8 Hz) $^3J_{NHCH\alpha}$ for the α-Phe and β^2hPhe residues, whereas **3a** shows large $^3J_{NHCH\alpha}$ (7.8 and 8.5 Hz, respectively) for the α-Phe and Lys residues and a well-defined triplet signal from β^2hPhe. The large coupling constants observed strongly support that **2a**, **2b**, and **3a** all exist as one predominant conformer in aqueous solution. Interestingly, the coupling constant of Lys is small ($^3J_{NHCH\alpha} \approx 5-6$ Hz) in **2a** and **2b**, but in cyclic tetrapeptide **3a** and **3b** large NH−CHα coupling constants of >8 Hz are observed.

The solvent contribution to the conformational stability was investigated by running additional ^{1}H NMR spectra of the five compounds in CD_3OD/CH_3OH (1:9) and DMSO-d_6. This analysis revealed interesting details about compounds **2b** and **3b**. When the spectrum of **2b** was recorded in CD_3OD/CH_3OH, the conformational stability seen in aqueous solution was left unchanged. However, when DMSO-d_6 was used as a solvent the cyclic peptide adopted higher flexibility and gave rise to a double set of amide proton signals (Figure 5a). Despite the existence of more than one conformer in DMSO, these appear as greatly ordered ones according to the observations of large $^3J_{NHCH}$ arising for both conformers. In the case of **3b**, the multiple numbers of conformers seen in aqueous solution are reduced to one stable conformer in both DMSO-d_6 and methanol (Figure 5b). The mixed cyclic tetramers **2a** and **3a** do not give rise to any significant conformational change upon change of solvent.

The amide proton temperature coefficients[37] were measured for all peptides in DMSO-d_6 (Table 2). The measured values suggest that two amide protons are involved in intramolecular hydrogen bonding in peptide **2a**, while peptides **2b** and **3b** only have one amide proton involved in intramolecular hydrogen bonding in each peptide. The value of the temperature coefficient for the β^2hPhe amide proton in **3a** in DMSO suggests that this proton is between solvent-exposed and hydrogen-bonded states. From the variable temperature measurements, it could also be noted that only the major conformation of peptide **2b** in DMSO-d_6 was observed at temperatures above 85 °C.

TABLE 2. Amide Proton Temperature Coefficients $\Delta\delta/\Delta T$ (ppb/K) of the Cyclic β^2hPhe Containing Peptides 2a, 2b, 3a, and 3b Measured in DMSO-d_6 over the Temperature Range 25−85 °C

peptide	$\Delta\delta/\Delta T$ Lys	Phe	β^2hPhe
2a	6.6	1.1	1.2
2b	4.7	5.1	1.2
3a	5.3	5.2	3.8
3b	4.9	2.1	5.3

The peptides giving rise to only one major conformer in D_2O/H_2O (1:9) (**2a**, **2b**, and **3a**) were next evaluated by 2D NMR experiments (ROESY, TOCSY, PE-COSY, and gHMBC). To exclude the possibility of analyzing incorrect peaks arising from TOCSY side effects in the ROESY spectra, NOESY was additionally run for structures **2a** and **3a**. From the 2D NOE experiments we could establish that all secondary amide bonds had the *trans*-configuration according to the lack of NOEs between $CH\alpha_i$ and $CH\alpha_{i+1}$. Concerning the D-Pro residue, ROESY cross-peaks between the δ-protons of the D-Pro and a backbone proton on the adjacent Phe residue ($CH\beta_{Pro-S}$ in the case of β^2hPhe and CHα in the case of Phe) were observed for the cyclic tetrapeptides **2a** and **3a**, indicating a *trans*-amide bond in these peptides. The 2D spectra of **2b**, however, lacked the presence of such NOEs, thus suggesting the presence of a *cis*-amide bond. The expected NOEs arising between $CH\alpha_i$ and $CH\alpha_{i+1}$, characteristic for such a *cis*-amide bond, were difficult to observe in the ROESY spectrum of **2b** due to overlap; however, the *cis*-amide bond could be concluded to be present due to the dramatic change in chemical shift of CHα of the D-Pro residue as compared to that of **2a** and **3a** (cf. 3.70 ppm (**2b**) to 4.38 and 4.52 ppm from **2a** and **3a**, respectively).

As a complement to the experimental conformational studies, a theoretical conformational search was performed on the mixed

(37) (a) Kopple, K. D.; Ohnishi, M.; Go, A. *J. Am. Chem. Soc.* **1969**, *91*, 4264. (b) Ohnishi, M.; Urry, D. W. *Biochem. Biophys. Res. Commun.* **1969**, *36*, 194. (c) Kessler, H. *Angew. Chem., Int. Ed. Engl.* **1982**, *21*, 512.

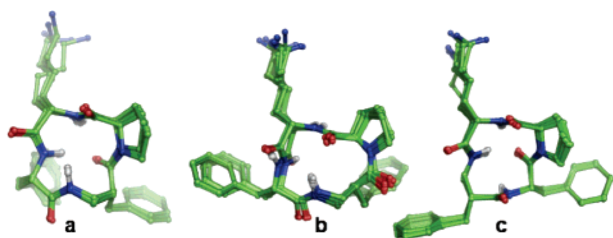


FIGURE 6. Results from the computational conformational search made by Monte Carlo search shown as bundles of six (2 × 6 for **2b**) low-energy structures from **2a** (a), **2b** (b), and **3a** (c).

cyclic peptides evaluated by the 2D NMR studies obtained in D_2O/H_2O (1:9). NOEs derived from ROESY or NOESY cross-peaks of the mixed cyclic tetrapeptides were introduced using the upper bond distance limits: strong 2.5 ± 1 Å, medium 3.0 ± 1 Å, and weak 4.0 ± 1 Å in a conformational sampling (see Experimental Section for details). Further, the three secondary amide bonds were all fixed to 180° ± 20°. For compounds **2a** and **3a**, the tertiary amide bond was locked into a trans-position by 180° ± 20°, whereas in structure **2b** it was fixed to a *cis*-amide bond by 0° ± 20°.

For **2a**, 16 NOEs were extracted and used in the conformational search. Only one family of conformers was found, and the results of the computational studies are seen as a bundle of six low-energy structures in Figure 6a. This theoretical structure extracted from NMR studies in solution is in almost perfect agreement with the structure given for the Boc-protected derivative by X-ray crystallography (Figure 3). More specifically, the energetically most favored conformer in both the solid and solution states of a highly polar solvent, such as water, is the one characterized by two intramolecular hydrogen bonds between residues in an i to $i + 2$ arrangement.

A conformational study of the cyclic peptide possessing opposite chirality of β^2hPhe residue (**2b**) was performed using 20 extracted NOEs in a way similar to that described above, but here two sets of conformers were found. These are represented by six low-energy structures each and are shown in Figure 6b. Comparing the results from this cyclic peptide with those extracted from the diastereomer **2a**, the presence of a *cis*-amide bond in the backbone prevents the formation of one of the stabilizing intramolecular hydrogen bonds, thus leaving one stabilizing i to $i + 2$ hydrogen bond. As a consequence, the amide bond positioned opposite to the *cis*-amide bond possesses an enhanced flexibility, which causes the amide bond to fluctuate in orientation at this position. It is reasonable to assume that the rate of this rotation is solvent-dependent and that the two conformations observed for this peptide in DMSO-d_6 (Figure 5a) represent these two conformations in slow exchange on the NMR time scale.

Regarding **3a**, 16 NOEs were used in the computational calculation, giving rise to only one conformational family, represented in Figure 6c as a bundle of six conformers. The position and chirality of the β^2hPhe residue in this peptide allow the formation of one intramolecular i to $i + 2$ hydrogen bond that involves both the stabilizing D-Pro residue and the opposing β^2hPhe residue, thus again leading to a highly conformationally homogeneous peptide. The overall shape of this cyclic peptide is similar to that of **2a** with the exception of the orientation of the amide bond between the two phenylalanine residues, which is naturally dependent on the position of the β^2-amino acid.

Discussion

It should be noted that the NMR restrained calculations were performed in a water continuum and did not involve any hydrogen-bonding restraints. Still, the resulting structures show excellent agreement with the predictions made from the DMSO temperature coefficients concerning the presence and location of hydrogen bonding. Thus, the structure of **2a** in Figure 6 features two hydrogen bonds, involving the amide protons on the β^2hPhe residue and the Phe residue, just as predicted from the data listed in Table 2. Likewise, the amide proton of the β^2hPhe residue in peptide **2b** was predicted to be involved in hydrogen bonding and found to be so in the calculation. The solution-state structures calculated for peptide **3a** are also seen to involve a hydrogen bond involving the β^2hPhe residue; again, the temperature coefficient in DMSO suggested this, even if the value here was not as small as that observed for the other peptides. The solution structure of peptide **3b** in water was not calculated due to the presence of two conformers. Still, as the temperature coefficient suggested involvement of the lysine residue in hydrogen bonding, it may be envisaged that the opposite chirality of the β^2hPhe residue in **3b**, as compared to that of **3a**, forces the molecule into a conformation where the β^2hPhe amide proton is no longer involved in hydrogen bonding, but instead allows the α-Phe residue to engage in hydrogen bonding to the Lys and/or the D-Pro residue. Overall, these findings consistently support the initial assumptions made at the beginning; namely, that a β^2-amino acid is capable of stabilizing cyclic tetrapeptides into one main conformation even in aqueous solution. The stabilizing effect may be obtained either by placing the turn-inducing residues D-Pro and β^2hPhe at i and $i - 1$ position or by having the two turn-promoting residues separated from each other in an i and $i + 2$ relationship.

Interestingly, all cyclic peptides with a β^2-amino acid investigated here were shown to incorporate modified β-turns. This is in contrast to the studies reported by Sewald et al.,[25] where γ-turns were observed in a larger cyclic mixed peptide incorporating β^3-amino acids, and in the studies by Fairlie et al.,[26] where both γ- and β-turns were observed depending on the chirality of one of the α-amino acids in the cyclic tetrapeptide sequence. This observation suggests that a β^2-amino acid has a larger propensity to induce β-turns in cyclic mixed α,β-peptides than a β^3-amino acid residue has and thus may provide a useful complement to β^3-amino acids in the design of conformationally homogeneous cyclic peptides with predictable structural characteristics.

As stated in the Introduction, these findings are expected to find practical use in the area of medicinal chemistry, molecular recognition, and catalysis. To illustrate the similarity of the cyclic mixed tetrapeptides to those of naturally occurring bioactive cyclic tetrapeptides (e.g., HC toxin and chlamydocin), we superpositioned the X-ray structure of chlamydocin[38] on the solution-state structures of the two mixed cyclic peptides **2a** and **3a** that were found to have all *trans*-amide bonds. As seen in Figure 7, the Cα-carbons of these structures overlay well, showing that a β2-amino acid in both the $i - 1$ and $i + 2$ position, relative to D-proline, places all the side chains of the mixed α/β-peptide in a spatial orientation similar to that of a natural all-α-peptide.

Furthermore, Fairlie et al. demonstrated that the 13-membered ring of their mixed α/β-cyclic tetrapeptides was a potential

(38) Flippen, J. L.; Karle, I. L. *Biopolymers* **1976**, *15*, 1081.

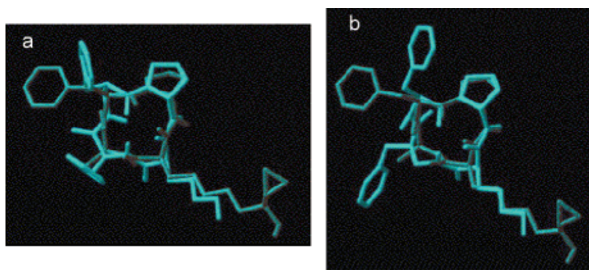


FIGURE 7. Superposition of the X-ray structure of chlamydocin (brown) and the solution state structure of cyclic tetrapeptides **2a** (a) and **3a** (b) (blue). RMSD: 0.57 Å (a) and 0.43 Å (b) for the four Cα-carbons.

peptidomimetic scaffold that had a good match between the side-chain positions and a range of structurally diverse nonpeptidic templates found in natural products.[26] Thus, mixed cyclic α/*β*-peptides may be regarded as promising rigid backbones that offer several sites for tailoring the molecule's properties and function at will.

Conclusions

Investigations by ^{1}H NMR showed enhanced conformational homogenity of cyclic tetrapeptides due to the incorporation of a β^2hPhe amino acid residue. By analyzing spectra recorded in different solvents, it could be concluded that peptides **2a** and **3a**, which both contain (*R*)-β^2hPhe (i.e., corresponding to the "natural" L-configuration of the α-amino acids), possessed an even higher degree of conformational rigidity as compared to the mixed peptides **2b** and **3b** containing (*S*)-β^2hPhe, independent of where in the sequence the *β*-amino acid is placed.

Fairlie et al. concluded in their study that incorporation of a β^3hPhe residue into cyclic tetrapeptides enhanced the conformational homogenity when the β^3-residue was positioned $i + 2$ relative to the other biasing element (i.e., D-Pro).[26] In our case, this increased conformational stability was observed independently of the position of the β^2hPhe residue. On the other hand, the side-chain chirality of the β^2-residue was shown to be relevant. The stabilizing effect of a *β*-amino acid in the $i + 2$ position relative to D-Pro, as noted by Fairlie et al. for β^3-amino acids, is also noted in our study, provided that the chirality of β^2-amino acid matches that of the flanking α-amino acids (i.e., all "L"-configuration, **3a**). However, a β^2-amino acid of the opposite chirality at this position (i.e., "D"-amino acids in both i and $i + 2$ positions) is conformationally destabilizing as seen for **3b**. Perhaps more surprising is the finding that a β^2-amino acid next to the D-Pro element is capable of enhancing the conformational stability. Incorporation of a β^2-amino acid, in the $i - 1$ position as compared to the D-Pro residue, of the same relative configuration as a natural α-amino acid enhances the conformational homogenity (cf. **1** and **2a**), while the incorporation of a "D"-configured β^2-amino acid residue in this position is capable of inducing a *cis*-amide bond (**2b**).

Taken together, this study demonstrates that both single β^3- and β^2-amino acid residues may be used to enhance the conformational homogenity of small cyclic peptides in aqueous solution. This is a particularly important finding in the case of cyclic tetrapeptides, due to their great practical utility as biologically active substances, as molecular hosts in supramolecular recognition, and possibly in the future development of novel peptides with catalytic function. The finding that both β^2- and β^3-amino acids provide enhanced conformational stability of cyclic peptides offers increased variability for designed placement of functional groups (in space) and proclaims further elaboration of entities such as conformational restraints in future peptidomimetic designs.

Experimental Section

General Method for the Synthesis of Tetrapeptides 1−3 via Solution-Phase Cyclization. 1. Loading of the Resin. Freshly activated 2-chlorotrityl chloride resin (1.49 mmol/g, 1 equiv) was put into a manual peptide synthesis vessel and was swollen in dry DCM.[39] A solution of 1 equiv of Fmoc-Phe-OH and 2.5 equiv of DIEA in dry DCM was added, and argon was bubbled through the reaction mixture for 45 min. The resin was washed with DMF (2 × 5 mL), a mixture of DCM/MeOH/DIEA = 80:15:5 was added, and the suspension was stirred for 10 min. This step was repeated once. The resin was further washed with DMF, and subsequent Fmoc deprotection was executed with 2% DBU/2% piperidine in DMF (5 × 5 min). The resin was washed with DMF, shrunk with MeOH (5 × 2 min), and finally dried under high vacuum for 10 h. Determination of the substitution level of a small sample of the resin based on Fmoc-UV spectrophotometry resulted in a typical loading yield of 60% (0.89 mmol/g).

2. Amino Acid Coupling. These steps were performed on the Quest 210 synthesizer, and for Fmoc deprotection the resin was stirred with 2% DBU/2% piperidine in DMF for 5 × 5 min. After washing the resin with DMF (5 × 2 min), we added the solution of the activated amino acid derivative (3 equiv α- or β^2-amino acid, 2.9 equiv of HBTU, 3 equiv of HOBt, and 6 equiv of DIEA in DMF). The resin suspension was stirred at room temperature for 3 h, and the TNBS test[35] showed completion of the reaction. The resin was washed with DMF (5 × 2 min) and DCM (5 × 2 min) and was shrunk with MeOH (5 × 2 min).

3. Cleavage of the Linear Tetrapeptides from the Resin. The cleavage mixture (DCM/TFE/AcOH = 3:1:1) was added to the dry resin, and the suspension was stirred at room temperature for 2 h. The resin was filtered off and washed two times with cleavage mixture for 2 min. After adding *n*-hexane (ca. 15 times excess) to the combined filtrates, we evaporated the mixture under reduced pressure. Water was added, and the crude product was lyophilized.

4. Cyclization in Solution. The linear tetrapeptide (1 equiv) was added to a solution of DIEA (5 equiv) in approximately 15 mL of dry DMF. This mixture was added slowly over 20 h via syringe pump to a flask containing a well stirred solution of PyBOP (3 equiv) and DIEA (5 equiv) in dry DMF (final peptide concentration: 1 mmol/L). The reaction mixture was stirred at room temperature for a further 4 h. DMF was evaporated, and the residue was purified by RP-HPLC. The cyclic peptides **1**, **2a**, and **2b** were cyclized between the α-Phe and *β*-Phe residues, whereas mixed cyclic peptides **3a** and **3b** were cyclized between α-Phe and the D-Pro.

***cyclo*(-Phe-D-Pro-Lys-Phe-) (1).** The purification was done in two steps. First step: 30−75% B over 29 min, t_r = 18.7 min. Evaporation of the combined fractions resulted in Boc cleavage, and the subsequent second purification step (10−60% B over 29 min, t_r = 19.2 min) led after final lyophilization to the target peptide **1** in 36% yield. Analytical RP-HPLC (20−40% B over 27 min): t_r = 15.2 min. MALDI-TOF MS: calcd. for $C_{29}H_{37}N_5O_4$: 519.285; found: 520.182 ([M + H]$^+$), 542.133 ([M + Na]$^+$), 558.095 ([M + K]$^+$).

(39) DCM: dichloromethane; DIEA: *N*,*N*-diisopropylethylamine; DMAP: 4-(dimethylamino)pyridine; HBTU: 2-(1*H*-benzotriazole-1-yl-)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt: 1-hydroxy-1*H*-benzotriazole; MeOH: methanol; NMM: *N*-methylmorpholine; PyBOP: benzotriazole-1-yl-oxytris(pyrrolidono)phosphonium hexafluorophosphate; TFE: trifluoroethanol; TNBS: 2,4,6-trinitrobenzenesulfonic acid.

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Supporting Information Available: Experimental procedure for **2a**,**b** and **3a**,**b**, NMR details, computational details, copies of ^{1}H NMR spectra of **1**−**3** in H_2O/D_2O (9:1), CH_3OH/CD_3OD (9:1), and DMSO and copies of 2D NMR spectra (TNTOCSY, ROESY (**2b**), NOESY (**2a** and **3a**)) in H_2O/D_2O (9:1), and ELSD spectra of all compounds. CIF file containing the X-ray structure data of Boc-protected **2a**. This information is available free of charge via the Internet at http://pubs.acs.org.

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