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SELF-ASSEMBLING CYCLIC α,γ-TETRAPEPTIDES

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Abstract –Cyclic α,γ-tetrapeptide self-assembled into dimers by formation of only 4-hydrogen-bonded interactions of amide groups of the two α-amino acids of a cyclic tetrapeptides.

In recent years, considerable effort has been devoted to the synthesis of organic and inorganic nanotubes.[1] Among a number of other nanotube design concepts, self-assembling peptide nanotubes (SPNs)[1-6] have for some time attracted attention because of their structural and functional properties that may be suitable for various applications in biology and materials science.[7] SPNs are formed by stacking cyclic peptides; in particular, the SPNs based on cyclic α,γ-peptides such as **1b** and **1c** (Figure1), in which *cis*-3-aminocyclohexanecarboxylic acid (γ-Ach) alternates with an α-amino acid,[8] hold out considerable promise for the design of nanotubes with novel structural and internal cavity properties. Here we demonstrate that the internal diameter of these self-assembling peptides can be controlled simple by adjusting the ring size of the peptide subunits. In this study we presented the design and synthesis of 4-residue cyclic peptides that associates as dimeric tubelet segments that resembles the features of nanotubes. For those studies we have used selective *N*-methylation of the backbone amide functionalities, showing the dynamic, thermodynamic and structural parameters that govern this process. Additionally, the rigorous control of internal diameter of ensemble has also been shown by preparing self-assembling hexapeptides (m= 1, **1b** and **1c**) and octapeptides (m = 2, **2b** and **2c**). These dimers are by themselves of interest because of the possibility of using them in reversible encapsulation processes of a guest molecule for the purposes of protecting it from the external medium, transporting it between phases, or subjecting it to some kind of reaction in the resulting nanoscale reaction chamber.

The cyclic peptides constituting SPNs are flat because of the chiralities of their amino acids, and they stack together because these chiralities also orient their C=O and N-H groups roughly perpendicular to the plane of the peptide ring, which allows β-sheet-like hydrogen bonding between rings (Figure 1).

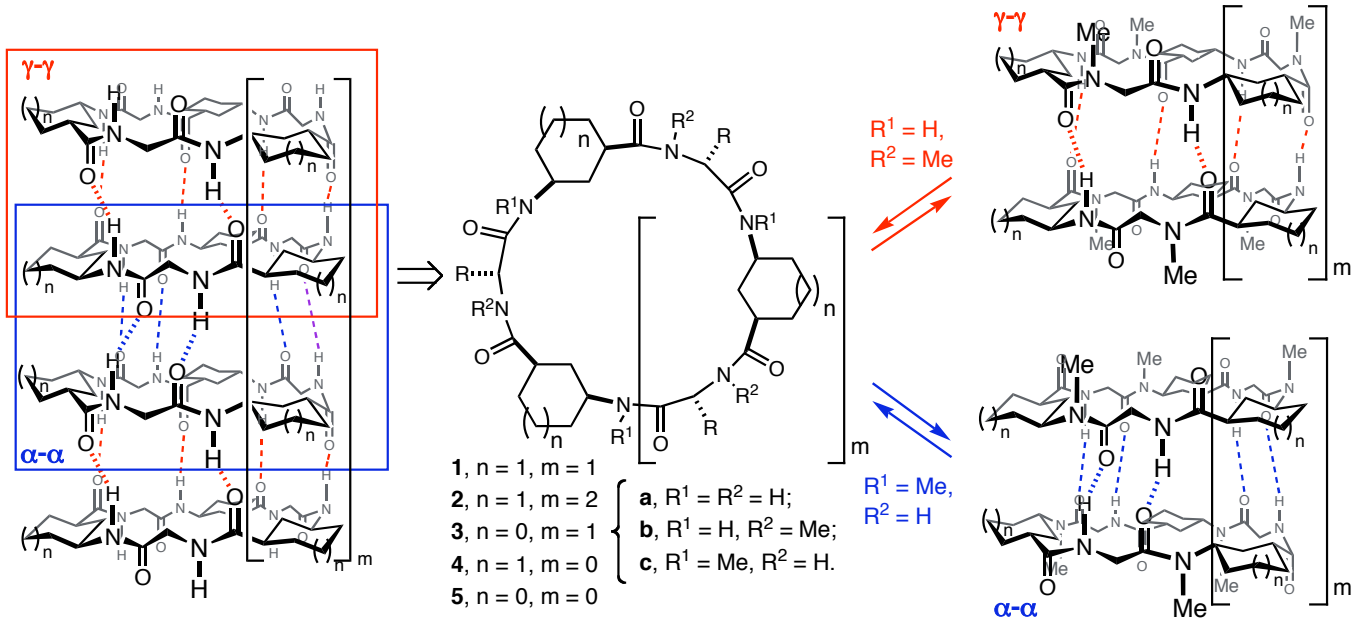
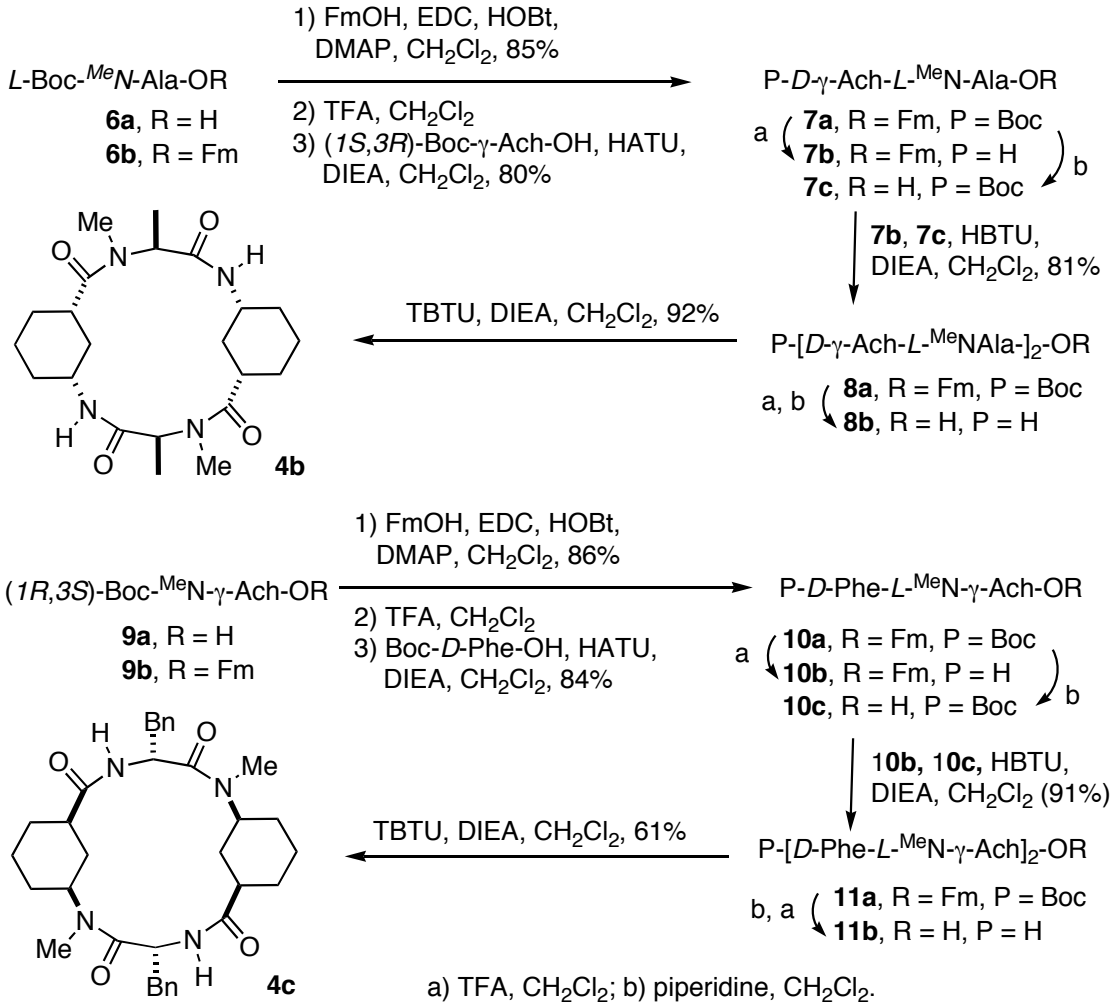


Figure 1. Center: A generic *cyclo*[(*D*-α-Aa-*L*-γ-Aca)$_n$-] peptides (**1-5**). Right: structure of a nanotube self-assembled therefrom by Aca-to-Aca (γ-γ) and Aa-to-Aa (α-α) hydrogen bonding between antiparallel rings. Left: dimers of derivatives with *N*-methylated *D*-α-Aa (upper) or *N*-methylated *L*-γ-Aca (lower) illustrating γ-γ and α-α bonding, respectively. For clarity, amino acid side chains have been omitted in the representations of the nanotube and dimers.

α,γ-CPs, such as **1**-**3**, have their γ-Aca NH and C=O groups on one face (the γ-face) and their α-amino acid (α-Aa) NH and C=O groups on the other (the α-face), and because of the different HN···C=O spacings of the α and γ amino acids these peptide rings can only stack through β-sheet-like hydrogen bond interactions with each other if the orientations of the rings alternate, so that α-faces bond to α-faces and γ-faces to γ-faces.[9] Thus an SPN formed of α,γ-CPs has two alternating types of β-sheet-like sets of hydrogen bonds: one involving exclusively the NH and C=O groups of the γ–amino acid (γ-γ bonding) and the other those of the α-amino acid (α-α bonding).

The structure described above implies that, depending on the reading frame adopted, the α,γ-CP-based SPN can be regarded as composed either of dimeric repeat units that are bound internally by α-α bonding and externally by γ-γ bonding, or of dimeric repeat units that are bound internally by γ-γ bonding and externally by α-α bonding (Figure 1, left). The thermodynamics of the formation of these nanotubes, and other factors affecting the process, can be studied by investigating these two kinds of repeat unit separately, to which end external hydrogen bonding between repeat units can be prevented by methylation of those of the α,γ-CP NH groups that will be outward-facing in the dimer (Figure 1, right).[10] Studies of dimers of this kind have shown that, when the γ-Aca is *cis*-3-aminocyclohexanecarboxylic acid (γ-Ach), α-α bonding is very much more stable than γ-γ bonding for both six-amino acid α,γ-CPs (**1**) and eight-amino acid α,γ-CPs (**2**).[11,12] Intrigued by this, in the work described here we investigated whether the same pattern holds for the smallest possible α,γ-CPs, tetrapeptides of type *cyclo*[(α-Aa-γ-Aca)$_2$-].

To study (γ-γ)-bonding we prepared *cyclo*[(*L*-^{Me}*N*-Ala-*D*-γ-Ach)$_2$-] (**4b**) by solution phase methods as shown in Scheme 1, starting from L-Boc-^{Me}*N*-Ala-OFm (**6b**). Treatment of **6b** with TFA, followed by coupling with *D*-Boc-γ-Ach-OH using HATU, proceeded in high yield to give dipeptide (**7a**) (80%).[13] After treatment of half the synthesized quantity of **7a** with 1:1 TFA/DCM and the other half with 20% piperidine/DCM, the resulting dipeptides (**7b**) and (**7c**), were coupled using HBTU in the presence of DIEA, giving tetrapeptide (**8a**) in 81% yield. Double deprotection of **8a** with piperidine followed by TFA, and cyclization of the resulting peptide (**8b**), afforded the cyclic peptide (**4b**) in 51% yield from **8a**.



Scheme 1. Synthesis of *cyclo*[(*L*-^{Me}*N*-Ala-*D*-γ-Ach)$_2$-] (**4b**) and *cyclo*[(*D*-Phe-*L*-^{Me}N-γ-Ach)$_2$-] (**4c**).

The ^{1}H NMR spectra of **4b** in polar and nonpolar solvents (CCl_4, $CDCl_3$, MeOH, DMSO) are well defined, reflect a high degree of symmetry, and in $CDCl_3$ show a $J_{NH,H\alpha}$ coupling constant of 10.1 Hz that is typical of the all-*trans* backbone conformation required for flatness of the peptide ring. However, neither these spectra nor others run in 9:1 CCl_4/$CDCl_3$ show any signs of intermolecular hydrogen bonding, the N-H resonance of γ-Ach remaining at the same position ($\delta = 5.86$ ppm) [regardless of concentration]. In the FT-IR spectrum,[14] amide I and amide II$_{II}$ bands at respectively 1621 and 1522 cm^{-1} suggest the expected

flatness of the peptide ring, but the position of the amide A band [ν(NH)], 3366 cm^{-1}, is that of an amide proton that is not involved in any hydrogen bond.

To study (α-α)-bonding, we prepared *cyclo*[(*D*-Phe-*L*-^{Me}*N*-γ-Ach)$_2$-] (**4c**) using the same approach as for **4b**. Its ^{1}H NMR spectra in polar and nonpolar solvents once again indicate a flat, all-*trans* configuration; but in this case, unlike that of **4b**, dimerization through intermolecular hydrogen bonds in nonpolar solvents is reflected by the fact that the Phe N-H signal shifts increasingly downfield as concentration is increased, from 6.0 ppm at a concentration of 1 mM to 7.7 ppm at 200 mM. This concentration dependence allowed the dimerization constant K_a in chloroform at 298 K to be determined as 15 M^{-1},[15] and series of experiments carried out at different temperatures in the range 233-313K allowed the corresponding thermodynamic parameters ΔH°_{298} = -30.8 kJ mol^{-1} and ΔS°_{298} = -82.0 J K^{-1} mol^{-1} to be extracted from van't Hoff plots. The negative enthalpy and entropy, together with the observed fall in K_a with increasing solvent polarity, support the idea that the formation of dimers of **4c** is an enthalpy-driven, entropy-opposed[16] process brought about principally by intermolecular hydrogen bonding. Furthermore, the β-sheet-like nature of this bonding is supported by FT-IR spectra recorded in chloroform,[14] which not only show amide I and amide II$_{II}$ bands (at 1627 and 1522 cm^{-1}, respectively), but also amide A bands near 3300 cm^{-1}, while the band at 3411 cm^{-1} may be due to the N-H vibration of the monomer.

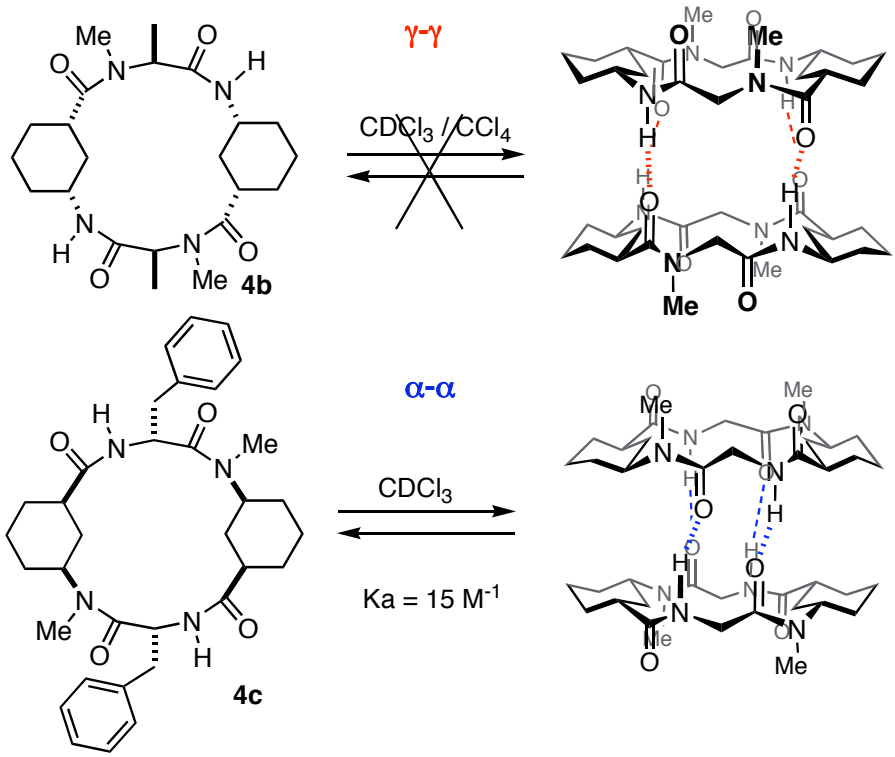


Figure 2. Compound (**4c**) spontaneously forms dimers in nonpolar solvents, compound (**4b**) does not.

Definitive evidence of the existence of dimers of **4c** in the solid state was obtained by X-Ray crystallography. The crystals, formed by vapour-phase equilibration of a tetrachloroethane solution of **4c** with hexane, consist of dimers in which the two CPs are stacked with antiparallel orientation and linked

by a β-sheet-like set of four hydrogen bonds N···O distances of 2.91-3.08 Å (Figure 3). The fact that all the hydrogen-bonding amide groups, with N-H···O angles of 156°-168°, are slightly tilted towards the centre of the dimer (Figure 3) may explain the low enthalpic contribution to the dimerization process. In the peptide rings of dimer of **4c**, all four Phe C(O)-C_α-N angles are very similar (~105°) but slightly smaller of those of hexamers and octamers,[8] with a dimer lumina with approximate Van der Waals diameters of 0.8 Å ($C_{\beta\text{-Ach}}$-$C_{\beta\text{-Ach}}$) and 3.9 Å ($C_{\alpha\text{-Phe}}$-$C_{\alpha\text{-Phe}}$) and the cavity has a Van der Waals volume of approximately 18 $Å^3$.[17]

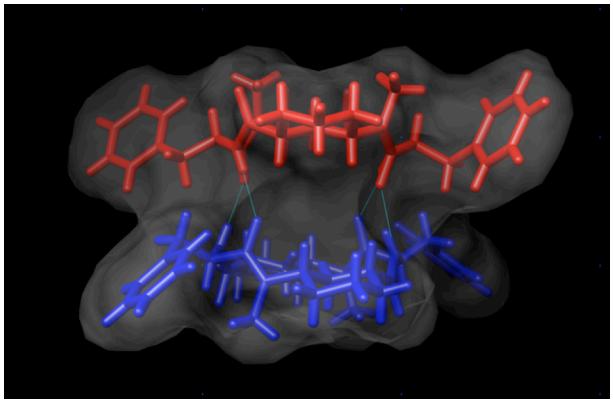
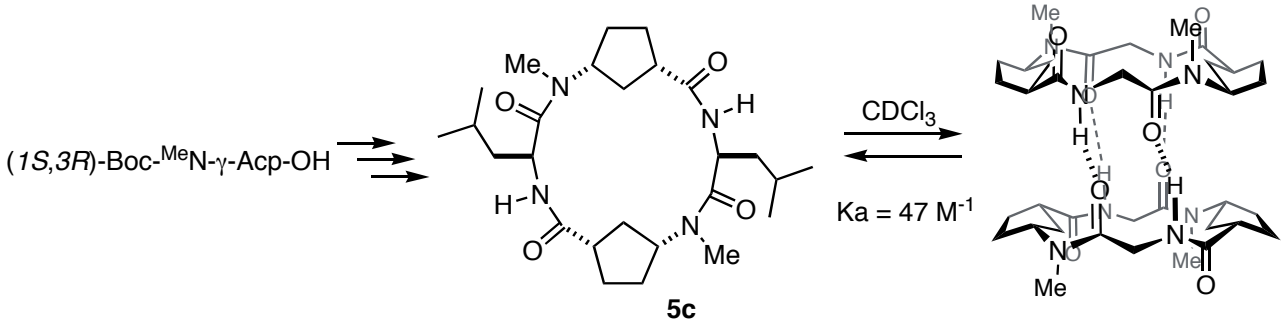


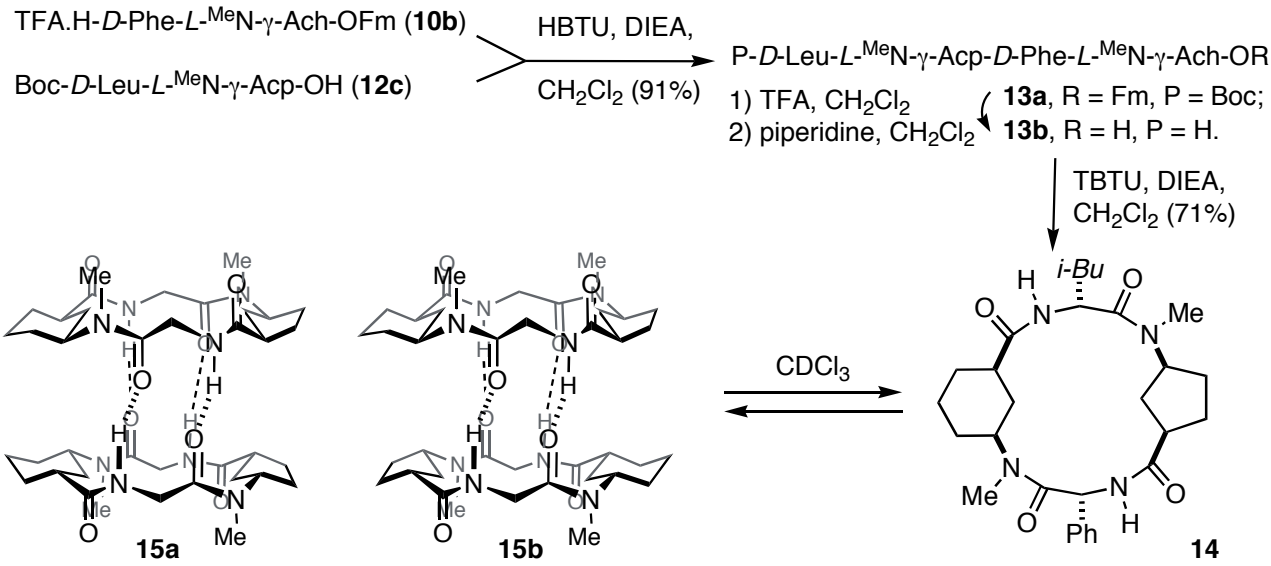
Figure 3. Side view of crystal structure of dimeric **4c**, each monomer is presented in different color (red and blue).

Stable (α–α)-bonded dimers were also formed when *cis*-3-aminocyclopentananecarboxylic acid (γ–Acp) was used as the γ–Aca of the α,γ-CP, instead of γ-Ach.[8d] *Cyclo*[(*L*-Leu-*D*-^{Me}*N*-γ-Acp)$_2$-] (**5c**) was prepared using the same approach as for **4b** and **4c**, but starting from *D*-Boc-^{Me}*N*-γ-Acp-OH (Scheme 2),[8d] and its flat, all-*trans* conformation was indicated by the characteristics of its ^{1}H NMR spectra in polar and nonpolar solvents ($J_{NH,H\gamma}$ = 9.0-9.5 Hz). As in the case of **4c**, the formation of intermolecular hydrogen bonds was indicated by the progressive downfield shift of the α-amino N-H signal from 6.5 to 7.7 ppm as concentration was increased in nonpolar solvents. This concentration dependence shown again an enthalpy-driven, entropy-opposed dimerization process,[16] with an association constant three times larger than for **4c** [K_a(CDCl$_3$) = 47 M^{-1}], and thermodynamic parameters of: ΔH°_{298} = -12.6 kJ mol^{-1} and ΔS°_{298} = -10.4 J K^{-1} mol^{-1}.



Scheme 2. Synthesis of **5c**, which spontaneously dimerizes in nonpolar solvents.

Finally, the Acp-Ach tetrapeptide hybrid (**14**) was prepared to study cyclopentyl/cyclohexyl packing interaction. This peptide lack of *C2* symmetry of the previously prepared peptides (**4c**) and (**5c**), and can, in principle, form two kinds of α-α-bonded dimer (**15a** and **15b**) with stabilities that might differ because of differences in between-monomer interactions (Scheme 3). We were interested to evaluate if packing between cyclopentyl and cyclohexyl rings is more favourable than cyclopentyl-cyclopentyl and cyclohexyl-cyclohexyl packing favoring the selective formation of **15b**, as in previously reported heterodimer formation.[8d] Peptide (**14**) was prepared as before, starting from dipeptides (**10b**) and (**12c**) that were coupled in the presence of HBTU, and the resulting linear tetrapeptide (**13a**) was deprotected with piperidine followed by TFA, and the cyclization of the resulting peptide (**13b**), afforded the cyclic peptide (**14**) in 64% yield from **13a** (Scheme 3). The flat, all-*trans* conformation was indicated by the characteristics of its ^{1}H NMR spectra in polar and nonpolar solvents. In chloroform the formation of intermolecular hydrogen bonds was indicated by the progressive downfield shift of the α-amino N-H signal from 6.0 to 6.7 ppm as concentration was increased from 1.0 mM to 20 mM. At higher concentration the peak is split in two signals that correspond to the two α-amino acids NH (Leu and Phe). This concentration dependence shown a dimerization constant similar to the other two peptides ($K_a(CDCl_3) = 28\ M^{-1}$). Unfortunately this fast exchanging on the NMR time scale of the monomer and the two dimers did not allowed to study the equilibrium between **15a** and **15b** in chloroform even at 210K. Slow dimers equilibration was observed in NMR spectra of **14** (40 mM in 3:1 CH_2Cl_2/CS_2) obtained at 178 K, which showed four NH signals (8.51, 8.33, 7.64 and 7.46) corresponding to both dimers which are in an almost 1:1 ratio, suggesting no preference for packing between cyclopentyl and cyclohexyl rings in these cyclic tetrapeptide dimers.



Scheme 3. Synthesis of peptide (**14**) that dimerizes in nonpolar solvents to give the two diastereomeric assemblies **15a** and **15b**.

In conclusion, NMR, FT-IR spectrum and X-Ray diffraction data have provided conclusive evidence that α,γ-CPs with just four amino acids form dimers held together by β-sheet-like hydrogen bonds between their α faces, while (γ–γ)-bonded dimers seem not to be formed at all.[11,12] The (α-α)-bonded dimers are more stable than the isolated monomers by about 2 kcal mol^{-1}, i.e. by about 0.5 kcal mol^{-1} per hydrogen bond, a value that is similar to those observed in (γ–γ)-bonded dimers of hexa- and octapeptide α,γ-CPs[8] and *L,D*-Cps,[10] is considerably smaller than the values in (α-α)-bonded dimers of hexa- and octapeptide α,γ-CPs (which have not so far been measured because dimerization is virtually complete even at low concentrations of these species).[8] It seems likely that in tetrapeptide α,γ-CP dimers hydrogen bonding is weaker than in the corresponding hexa- and octapetide dimers because of conformational constraints in the small four-amino-acid ring.

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This paper is dedicated to Prof. Barry M. Trost in honor of his 65th birthday.

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