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Stapling of a 3₁₀-Helix with Click Chemistry

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Abstract

Short peptides are important as lead compounds and molecular probes in drug discovery and chemical biology, but their well-known drawbacks, such as high conformational flexibility, protease lability, poor bioavailability and short half-lives *in vivo*, have prevented their potential from being fully realized. Side chain-to-side chain cyclization, e.g. by ring-closing olefin metathesis, known as stapling, is one approach to increase the biological activity of short peptides which has shown promise when applied to 3_{10} - and α -helical peptides. However, atomic resolution structural information on the effect of side chain-to-side chain cyclization in 3_{10} -helical peptides is scarce, and suggests that there is significant potential for improvement of existing methodologies. Here, we report a novel stapling methodology for 3_{10} -helical peptides using the copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction in a model Aib rich peptide and examine the structural effect of side chain-to-side chain cyclization by NMR, X-ray diffraction, linear IR and femtosecond 2D IR spectroscopy. Our data show that the resulting cyclic peptide represents a more ideal 3_{10} -helix than its acyclic precursor and other stapled 3_{10} -helical peptides reported to date. Side chain-to-side chain stapling by CuAAC should prove useful when applied to 3_{10} -helical peptides and protein segments of interest in biomedicine.

Introduction

The concept of introducing conformational constraints in peptides which stabilize their biologically active secondary structure has attracted a lot of interest as a way to improve the pharmacological properties of peptides. In particular, this concept has been successfully applied to α -helical peptides and protein segments. Examples include peptides with intramolecular H-bond surrogates¹ and so-called stapled peptides, the latter deriving helix stabilization from side chain-to-side chain hydrophobic interactions,² salt bridges,³ disulfide bridges,⁴ lactams⁵ and metathesis derived hydrocarbon bridges.^{6–8} Significantly, hydrocarbon stapling of α -helical peptides has afforded several compounds which could have clinical potential, e.g. against cancer.^{9–11} The structural and pharmacological effects of ring-closing metathesis in peptides have recently been reviewed.¹² Recently, hydrocarbon stapling has also been successfully applied to 3_{14} -helical β -peptides,¹³ extending its range of applicability beyond α -peptides.

The 3_{10} -helix, which is defined by intramolecular $i \rightarrow i+3$ H-bonds, is an important structural element in proteins, peptide antibiotics known as peptaibols,¹⁴ and many biological recognition processes, as well as a postulated intermediate structure in protein folding.¹⁵

Over the last decade the predominant water channel in the mammalian brain, aquaporin-4 (AQP4), has emerged as an important target for treatment of brain edema after stroke or trauma.^{16–19} As part of an ongoing project to design selective inhibitors of AQP4 we have been interested in side chain-to-side chain bridges that allow some stabilization of the 3_{10} -helical conformation of the Pro138-Gly144 segment of human AQP4,²⁰ which has been postulated to mediate adhesive interactions between two AQP4 tetramers.^{21–23}

Examples of $i \rightarrow i+3$ and $i \rightarrow i+4$ side chain-to-side chain crosslinking in 3_{10} -helical peptides by Glu-Lys lactam formation,²⁴ ferrocenedicarboxylic acid Lys diamides,²⁵ photoinduced 1,3-dipolar cycloaddition,²⁶ metathesis derived hydrocarbon bridges,^{20,27,28} and a *p*-phenylenediacetic acid bridge²⁹ between two α,α -disubstituted 4-aminopiperidine-4-carboxylic acid (Api) residues have been reported. However, only two studies^{28,29} have provided atomic resolution detail of the effect of cyclization on helix regularity, i.e. on backbone dihedral angles and H-bond lengths.

In the first X-ray crystallographic study²⁸ of the effect of side chain-to-side chain cyclization in a 3₁₀-helical peptide it was observed that the backbone is distorted by an $i \rightarrow i+3$ metathesis derived olefinic bridge, resulting in the breakage of one intramolecular H-bond, thus disrupting the 3₁₀-helix. The *p*-phenylenediacetic acid bridge on the other hand appears to afford a highly regular Api/Aib based 3₁₀-helix.²⁹ However, α,α -disubstituted amino acids like Aib and *N*-acylated Api are generally hydrophobic and tend to distort the dihedral angles of neighbouring monosubstituted, proteinogenic residues away from ideality.^{24,28,30} In the context of a helical peptide primarily consisting of proteinogenic amino acids monosubstituted residues are expected to be better tolerated. Hence, new methodology for crosslinking of monosubstituted residues, which does not significantly distort the regularity of the 3₁₀-helix, is highly desirable. If, at the same time, the crosslinking results in a more hydrophilic bridge, thus increasing the aqueous solubility of the stapled peptide, such a methodology could potentially have broad utility. In particular, the resulting stapled peptides should be useful for the study and modulation of biologically important recognition processes involving 3₁₀-helical peptides and protein segments.

There has been an explosion of interest in click chemistry³¹ in recent years, exemplified by the highly popular copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction.³²⁻³⁵ This reaction has been successfully applied to $i \rightarrow i+4$ side chain-to-side chain cyclization in an α -helical peptide^{36,37} and $i \rightarrow i+3$ cyclization in peptoids (peptides composed of *N*-substituted glycines).³⁸ The high functional group tolerance of the CuAAC reaction, the very large dipole moment ($\sim 5D$)³⁹ and the relatively high resistance to metabolic degradation^{40,41} of the 1,2,3-triazole moiety make 3₁₀-helical peptides with a side chain-to-side chain triazole bridge highly interesting objects of study.

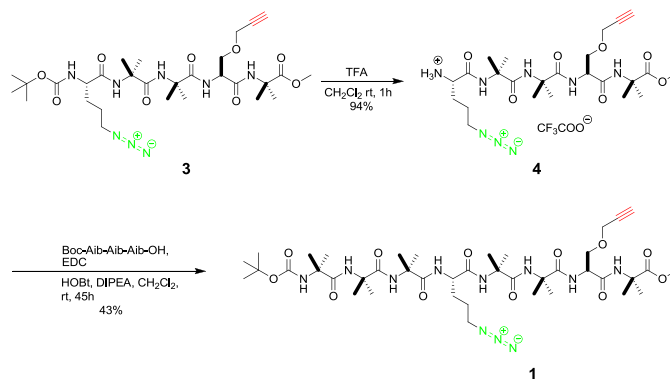
In this paper we report the installation of an $i \rightarrow i+3$ constraint by side chain-to-side chain CuAAC between two monosubstituted residues in the context of a 3₁₀-helical Aib rich peptide and examine in detail the effect of cyclization on helix regularity by X-ray crystallography, NMR, linear IR and 2D IR spectroscopy. To allow a direct comparison with the results for the $i \rightarrow i+3$ hydrocarbon bridge, two octapeptides **1** (Scheme 1) and **2** (Scheme 2) with the reactive/crosslinked residues in the same Aib rich context as the olefinic peptides of Boal *et al*²⁸ were chosen as synthetic targets.

To the best of our knowledge, this study represents the first X-ray structural investigation of a (α - or 3_{10} -) helical peptide after stapling by CuAAC or with a triazole derived conformational constraint and the first 2D IR structural investigation of a 3_{10} -helical peptide with a conformational constraint installed. Given the widespread interest in the CuAAC reaction, it is important to note that this study also affords what appears to be the first crystal structure of a bifunctional azide-alkyne compound.

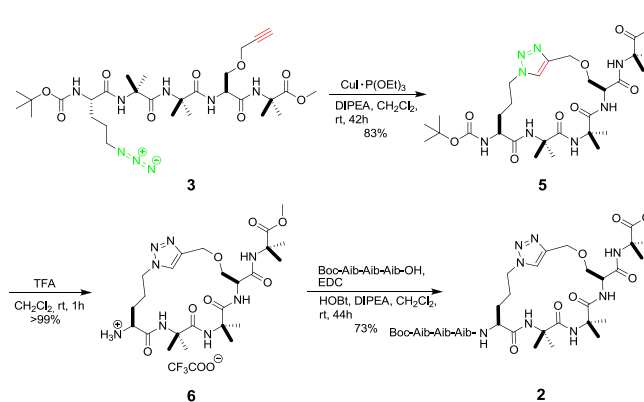
Results and Discussion

Peptide Synthesis

The building block N^α -Boc- δ -azido-L-norvaline⁴² was synthesised in 63% yield from N^α -Boc-L-ornithine using a recently developed shelf-stable and crystalline diazo transfer reagent, imidazole-1-sulfonyl azide hydrochloride.⁴³ N -Boc- O -propynyl-L-serine⁴⁴ was synthesised in 77% yield by a variation of Sugano's method for synthesis of N -Boc- O -benzyl-L-serine.⁴⁵ The octapeptide **1** was assembled by a segment condensation strategy using standard solution phase peptide coupling chemistry employing EDC/HOBt in DMF or CH_2Cl_2 (Supporting Information). The final steps involved deprotection of pentapeptide **3** with TFA/ CH_2Cl_2 (1:1) and coupling of the resulting trifluoroacetate **4** with Boc-Aib-Aib-Aib-OH²⁸ to afford the octapeptide **1** in 43% yield (Scheme 1).

Scheme 1. Final steps of the synthesis of the acyclic peptide **1**.

A recent investigation of Aib oligopeptides by 2D IR spectroscopy revealed that the onset of 3_{10} -helical structure appears to occur already at the pentapeptide level in CDCl_3 .⁴⁶ This, together with our previous success in cyclizing an olefinic pentapeptide by ring closing metathesis²⁰ in CH_2Cl_2 suggested that cyclization by CuAAC might be possible at the pentapeptide level in CH_2Cl_2 . At high dilution (~ 0.15 mM) **3** was cyclized to **5** in 83% yield (Scheme 2) in the presence of 0.31 eq. of the organic-soluble copper(I) complex $\text{CuI}\cdot\text{P}(\text{OEt})_3$, which was synthesised according to a literature procedure.^{47,48} Dimerization and cyclodimerization are competing processes and have resulted in relatively low yields of cyclic monomer in several instances of intramolecular CuAACs, even at high dilution.^{35,49,50} The relatively high yield in this case suggests a high degree of substrate preorganization in CH_2Cl_2 . Deprotection of **5** with TFA/ CH_2Cl_2 (1:1), yielding the trifluoroacetate **6**, followed by segment condensation with Boc-Aib-Aib-Aib-OH afforded octapeptide **2** in 73% yield over two steps (Scheme 2). This synthesis strategy for the synthesis of **2** has the advantage over direct cyclization of **1** that it allows the most challenging synthetic step, namely the macrocyclization, to be carried out at the earliest possible stage. Later structural studies (below) demonstrated that **1** is predominantly 3_{10} -helical in CH_2Cl_2 solution, suggesting that cyclization would have been successful also at the octapeptide level. However, the yield obtained on cyclization of **3** was considered satisfactory and rendered the alternative strategy superfluous and untested in this particular study.

Scheme 2. Final steps of the synthesis of the cyclic peptide **2**.**X-ray crystallography**

Peptide **1** ($\text{C}_{41}\text{H}_{69}\text{N}_{11}\text{O}_{12}$, $M_w = 908.05$) (Scheme 1) crystallized as colourless, plate shaped crystals in space group $P2_12_12_1$, with unit cell parameters $a = 16.239(12)$, $b = 18.236(14)$, $c = 18.655(14)$, $\alpha = 90.00^\circ$, $\beta = 90.00^\circ$, $\gamma = 90.00^\circ$ (orthorhombic crystal system) and $Z = 4$. The X-ray structure was refined to a final R -factor of 0.068 for data obtained for a very small crystal ($0.100 \text{ mm} \times 0.010 \text{ mm} \times 0.009 \text{ mm}$). The peptide forms a fully developed right handed 3_{10} -helix with all possible intramolecular $i \rightarrow i+3$ H-bonds present, including between the *tert*-butoxycarbonyl (Boc) group and the amide NH of Aib3 (Figure 1).

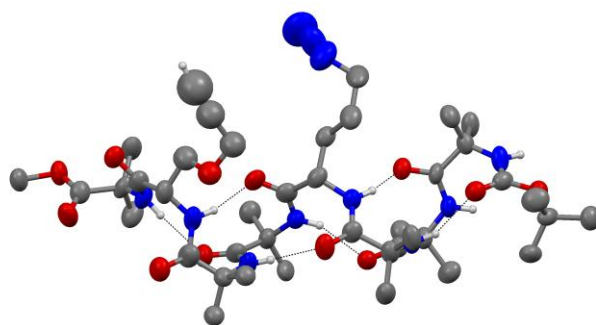


Figure 1. Thermal ellipsoid plot of X-ray crystal structure of acyclic peptide **1** at the 50% probability level with intramolecular H-bonds indicated. Apolar hydrogens have been omitted for clarity. The major backbone conformation at Aib6 is shown (see Supporting Information for details).

With the exceptions of residues 4, 6, 7 and 8 the conformations of all remaining residues fall into the 3_{10} -helical region of (ϕ, ψ) -space, with the mean absolute deviations from the ideal (i.e. average observed in peptides) 3_{10} -helical angles of $(-57^\circ, -30^\circ)^{51}$ being 3.68° and 4.48° , respectively (Figure 2).

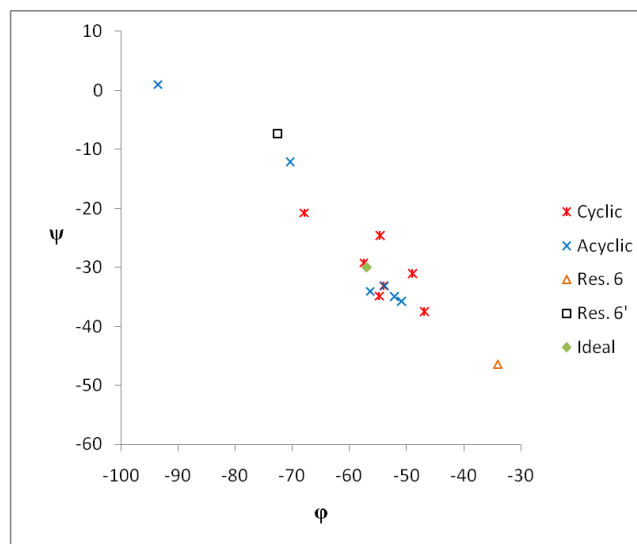


Figure 2. Partial Ramachandran plot for residues 1-7 of **1** and **2**. Both conformations of Aib6 in the crystal structure of **1** are indicated (Res. 6: major conformation; Res. 6': minor conformation). See Supporting Information for details and complete Ramachandran plots.

However, the dihedral angles of the two chiral, monosubstituted residues 4 and 7 deviate significantly from the ideal 3_{10} -helical angles, with $[(|\Delta\phi|, |\Delta\psi|) = (13.38^\circ, 17.90^\circ)$ and $(36.55^\circ, 30.96^\circ)]$ respectively. Similarly large deviations from ideality, albeit slightly smaller for residue 4, were observed for the acyclic olefinic peptide of Boal *et al.*²⁸ Here, residue 7, for which $(\phi, \psi)_7 = (-93.55^\circ, 0.96^\circ)$, forms part of a type-I β -turn^{52,53} together with residue 6 in its minor conformation $(-72.56^\circ, -7.39^\circ)$. In contrast to the acyclic olefinic analog, where residue 8 is in the α_R conformation,²⁸ the C-terminal residue in **1** adopts a left-handed polyproline II (P_{II}) conformation with $(\phi, \psi) = (-57.83^\circ, 159.12^\circ)$. This fits well, however, into the empirical pattern found in a recent survey of non-helical conformations of Aib residues in peptides. In a database of 143 crystal structures of Aib-containing helices with > 3 residues

with a C-terminal Aib 86.5% adopted the opposite helix sense than the rest of the molecule and 20.3% of these fell within the P_{III} region.⁵⁴

The intramolecular H-bond lengths ($d_{C=O...HN}$) vary between 2.115 Å (Aib1→Aib4) and 2.341 Å (Aib3→Aib6), with mean 2.193 Å and standard deviation 0.088 Å. These values are very similar to the ones found for the acyclic olefinic analog (2.210 Å and 0.115 Å).²⁸ For both of these acyclic peptides the same pattern of H-bond length variations is observed. For both acyclic peptides, the longest H-bonds are between pairs of Aib residues on the N-terminal (3 and 6) and on the C-terminal (5 and 8) sides of the monosubstituted residues respectively.

The carbonyl group of Aib7 forms an intermolecular H-bond to the carbamate NH of the Boc group, but there is no intermolecular peptide-peptide H-bond to the carbonyl of the methyl ester as is often seen in structures of Aib rich peptides.⁵⁴

The cyclic peptide **2** (C₄₁H₆₉N₁₁O₁₂, M_w = 908.05) (Scheme 2) crystallized as colourless, plate shaped crystals in space group C2, with unit cell parameters **a** = 36.417(12), **b** = 13.382(5), **c** = 11.873(4), **α** = 90.00°, **β** = 102.360(4)°, **γ** = 90.00° (monoclinic crystal system) and *Z* = 4. The X-ray structure was refined to a final *R*-factor of 0.039, which is unusually low for a molecule of this size.

Like the acyclic peptide **1** the cyclic octapeptide **2** forms a fully developed right handed 3₁₀-helix with all possible *i* → *i*+3 intramolecular H-bonds present (Figure 3).

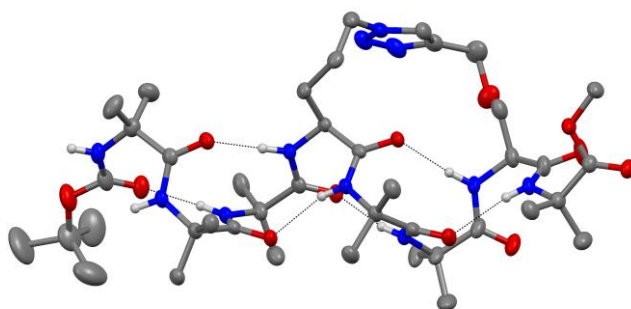


Figure 3. Thermal ellipsoid plot of X-ray crystal structure of cyclic peptide **2** at the 50% probability level with intramolecular H-bonds indicated. Apolar hydrogens have been omitted for clarity.

Whereas significant deviations from an ideal 3_{10} -helix with respect to individual dihedral angles were observed in the crystal structure of peptide **1**, the structure of peptide **2** represents a strikingly ideal 3_{10} -helix from residue 1-7. The average (ϕ, ψ)-angles are $(-54.96^\circ, -30.17^\circ)$, deviating a mere 2.04° and 0.17° from ideality, making peptide **2** the most perfect crosslinked 3_{10} -helix to date (Table 1).

Compound	ϕ	σ_ϕ	ψ	σ_ψ
Ideal 3_{10} -helix	-57	-	-30	-
1 (major)	-58.75	18.68	-27.93	16.34
1 (minor)	-64.22	15.61	-22.38	15.63
2	-54.96	6.79	-30.17	5.87
Acyclic olefinic ²⁸	-62.32	15.23	-24.29	10.27
Cyclic olefinic ²⁸	-68.35	24.04	-16.94	24.50
Cyclic hydrogen ²⁸	-59.99	14.94	-27.52	15.59
Acyclic Api ²⁹	-54.20	4.35	-28.97	8.07
Cyclic Api (mol.1) ²⁹	-55.52	2.61	-26.77	6.11
Cyclic Api (mol.2) ²⁹	-54.94	3.83	-27.73	7.20

Table 1. Mean (ϕ, ψ)-angles with standard deviations for the crosslinked 3_{10} -helical peptides for which crystallographic data have been reported. The dihedral angles for residue 8 have been omitted for all peptides.

Importantly, the triazole bridge appears to strongly enforce a 3_{10} -helical conformation for residues 4, 6 and 7, effectively removing these as outliers in the Ramachandran plot (Figure 2). The deviations from ideality for these residues in peptide **2** are $(|\Delta\phi|, |\Delta\psi|) = (2.21^\circ, 4.88^\circ); (0.51^\circ, 0.78^\circ)$ and $(10.93^\circ, 9.25^\circ)$, respectively, dramatically improved relative to **1**. This is opposite to the trend observed in the structures of the cyclic olefinic peptide and its hydrogenated analog, where cyclization appeared to cause larger or unchanged deviations, with $(\phi, \psi)_4 = (-96.57^\circ, 19.84^\circ)$ and $(-66.64^\circ, -22.33^\circ)$ and $(\phi, \psi)_7 = (-108.91^\circ, 13.69^\circ)$ and $(-90.06^\circ, 3.05^\circ)$, respectively.²⁸ The sizes of the macrocycles and the rigid elements (triazole and carbon-carbon double bond) are important parameters which could be partially responsible for the different effects of the two staples on the backbone conformation. The macrocycle in **2** is slightly larger (19 atoms along the shortest path) than in the corresponding olefinic peptide (18

atoms). In addition, the rigid element in the CuAAC-derived bridge is also slightly larger than a carbon-carbon double bond.

The residues of the *p*-phenylene diacetic acid crosslinked Api/Aib peptide generally have close to ideal dihedral angles from residue 1 through to 7, but residue 4 (next to the first Api residue) has a slightly distorted ψ -angle (-22.69°) and the deviation from the ideal ψ -angle for residue 6 (-14.26° , $|\Delta\psi| = 15.74^\circ$) is larger than any ϕ/ψ -deviation for **2**.²⁹

The C-terminal residue adopts an α_L conformation, in other words the opposite helix sense as the rest of the molecule. This is statistically the most common conformation for a C-terminal Aib in Aib rich helices with > 3 residues and is often due to head-to-tail intermolecular interactions with the Boc group in capped peptides or with solvent.⁵⁴ Interestingly, in the structure of **2** the dihedral angles of (46.80° , 49.93°) allow two peptide molecules to contact each other in a tail-to-tail fashion forming two bifurcated non-classical C-H...O=C H-bonds between the triazole hydrogen and the carbonyl groups of residues 7 and 8 (Figure 4). This underlines a potential added advantage of a triazole in a helix stabilizing bridge, namely its ability to make useful contacts to peptides/proteins. The triazole is approximately coplanar with the bifurcated H-bond. Interestingly, all the Api peptides,²⁹ which also are highly 3_{10} -helical at residue 7, have C-terminal residues with very similar conformation to peptide **2**.

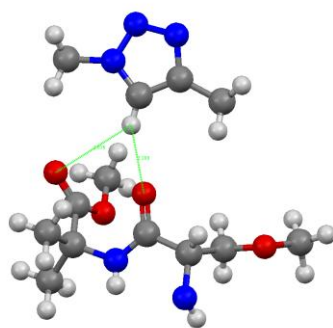


Figure 4. The triazole proton of one molecule forms a bifurcated non-classical H-bond with residues 7 and 8 in another molecule. Only one of the two bifurcated H-bonds between two peptide molecules is shown. The remainder of the molecules has been omitted for clarity.

The overall similarity to an ideal 3_{10} -helix is also reflected in significantly shorter intramolecular H-bonds compared to **1** and the hydrocarbon stapled analogs (Table 2). The data for **1** refer to the structure with the alkyne side chain in its major orientation and the backbone in its major conformation (see Supporting Information for details).

Compound	O...H	σ	O...H*	σ^*
1	2.193	0.088	2.164	0.056
2	2.118	0.075	2.102	0.071
2 (scaled)	2.087	0.084	2.058	0.051
Acyclic olefinic ²⁸	2.210	0.115		
Cyclic olefinic ²⁸	2.384	0.760	2.075	0.357
Cyclic hydrogen ²⁸	2.335	0.195	2.257	0.034

Table 2. Mean O...H distances with standard deviations for the intramolecular H-bonds in the crystal structures of **1** and **2**. Positional parameters for amide H atoms were refined for **2** only. To facilitate comparison with **1**, scaled values are included for **2** after normalization of all N-H bonds to 0.880 Å (i.e. H atoms are moved along the covalent bond vectors so as to make the N-H distances equal to 0.880 Å, the fixed N-H distance used in the refinement of **1**). *The longest H-bond, i.e. between residue 4 and 7 for **2** and between 3 and 6 for **1** and the hydrocarbon stapled peptides, has been omitted.

As expected the longest H-bond observed in the structure of **2** is between residues 4 and 7 (2.232 Å, $\Delta_{2-1} = +0.103$ Å), whose conformations change the most as they are pulled in towards more ideal 3_{10} -helical dihedral angles. However, all the remaining 5 intramolecular H-bonds are shorter in **2** than in **1**. The largest improvements are seen for Boc→Aib3 ($\Delta_{2-1} = -0.172$ Å), Aib3→Aib6 ($\Delta_{2-1} = -0.193$ Å) and Aib5→Aib8 ($\Delta_{2-1} = -0.184$ Å). Interestingly, the Aib3→Aib6 H-bond is the one stretched the most in the cyclic hydrocarbon stapled peptides relative to their acyclic precursor ($\Delta_{\text{cyclic-acyclic}} = +0.386$ Å and $\Delta_{\text{cyclic-acyclic}} = +1.585$ Å), and is in fact broken in the cyclic olefinic peptide ($d_{\text{O...H}} = 3.927$ Å).²⁸

NMR spectroscopy

While the X-ray crystallographic studies provided atomic resolution detail of the conformations of **1** and **2** in the solid state these may not be very representative of their solution phase structures. A careful investigation of the solution phase structures of **1** and **2** in the polar aprotic solvent CD₂Cl₂, including a detailed comparison with the corresponding crystal structures, was therefore undertaken using various NMR spectroscopic techniques.

The ¹H-NMR spectra of **1** and **2** were found to be concentration dependent (Figure 5). In particular, the NH(Aib₁) (Δδ on the order of +0.4 ppm) and NH(Aib₂) (Δδ on the order of +0.2 ppm) resonances display remarkable downfield shifts when moving from a relatively dilute solution (~10 mM) to a more concentrated solution (> 50 mM). The chemical shifts of the remaining protons, with the notable exception of one of the methyl groups, are much less concentration dependent than δ_{NH(Aib1)} and δ_{NH(Aib2)}. Remarkably, the concentration dependence of the ¹H-NMR spectra, the selectivity of the effect on δ_{NH(Aib1)} and δ_{NH(Aib2)} and the magnitude and sign of the chemical shift changes may all be explained by invoking a (concentration-independent) predominantly 3₁₀-helical backbone structure for **1** and **2** in CD₂Cl₂. It should be noted that NH(Aib₁) and NH(Aib₂) are the only amide NHs which do not participate in *intramolecular* H-bonding if the peptides adopt a 3₁₀-helical structure (Figures 1 and 3) and the only NHs which thus may engage in *intermolecular* H-bonding without compromising the overall 3₁₀-helical structure of the peptides. We believe that the large and selective downfield shifts of δ_{NH(Aib1)} and δ_{NH(Aib2)} could be explained by hypothesizing that NH(Aib₁) and NH(Aib₂) form intermolecular H-bonds in concentrated CD₂Cl₂ solution, while the other NHs remain intramolecularly H-bonded. This would imply the presence of a monomer-dimer equilibrium, which could explain the concentration dependence of the ¹H-NMR spectra.

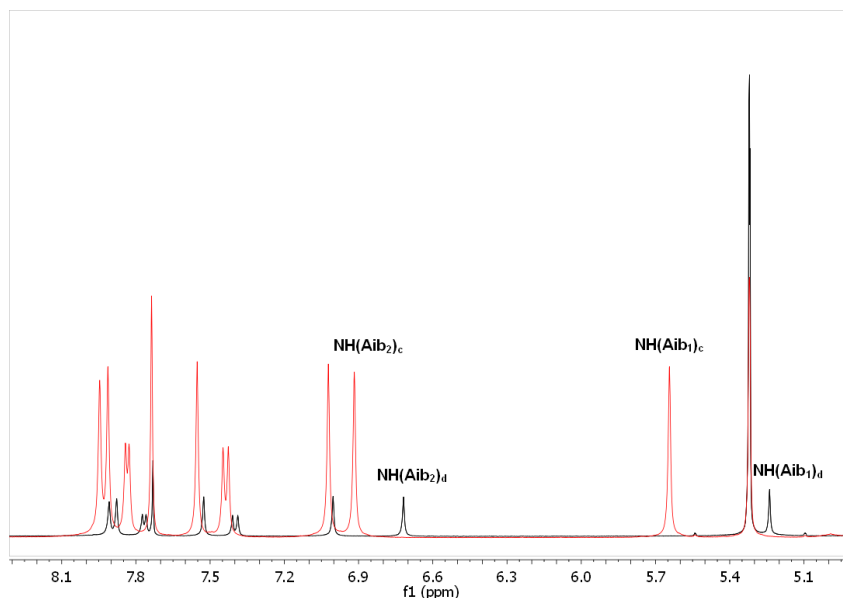


Figure 5. Overlay of the NH region of the ^1H -NMR spectra of **2** in dilute (~ 10 mM, black) and concentrated solution (>50 mM, red). The NH(Aib₁) and NH(Aib₂) resonances are significantly downfield shifted in the more concentrated solution.

The concentration dependence of the ^1H -NMR spectra prompted the question of whether the structures in dilute solutions or in concentrated solutions are more similar to the crystal structures. The fact that NH(Aib₁) and NH(Aib₂) are not H-bonded to other peptide molecules in the crystal structures suggests that the structures in dilute solution are likely to be most similar to the solid state structures. It should be added that this concentration regime is also of greater biomedical relevance. The cyclic peptide **2** does form a dimer in the crystal, but this does not involve H-bonding to NH(Aib₁) or NH(Aib₂) (Figure 4).

At any rate, caution is required when comparing solution phase structures in CD_2Cl_2 with the crystal structures, given the fact that the crystals were grown by slow evaporation of EtOAc (**1**) and (effectively) *i*PrOH solutions (**2**) rather than CD_2Cl_2 solutions.

To extract more detailed structural information 2D ROESY spectra were recorded in CD_2Cl_2 solution (Figure 6 and Supporting Information). The 2D ROESY spectrum of **1** clearly demonstrated the presence of all possible $\text{NH}(i) \rightarrow \text{NH}(i+1)$ ROEs and the only possible $\text{C}^\alpha\text{H}(i) \rightarrow \text{NH}(i+2)$ ROE

(Supporting Information), which are indicative of 3_{10} - or α -helical peptides.⁵⁵⁻⁵⁷ However, the presence of the long range $C^{\alpha}H(i) \rightarrow NH(i+3)$ ROE also typical of 3_{10} -helical peptides could not be established with certainty as the $C^{\alpha}H(\text{azidonorVal})$ peak is overlapping with the $C^{\alpha}H(\text{propSer})CH_2O$ peak, which has an ROE to $NH(\text{propSer})$ (Supporting Information).

Gratifyingly, ROEs characteristic of a 3_{10} -helical structure was also found in the 2D ROESY spectrum of **2**, including all possible short range $NH(i) \rightarrow NH(i+1)$, medium range $C^{\alpha}H(i) \rightarrow NH(i+2)$ and long range $C^{\alpha}H(i) \rightarrow NH(i+3)$ ROEs (Figure 6).

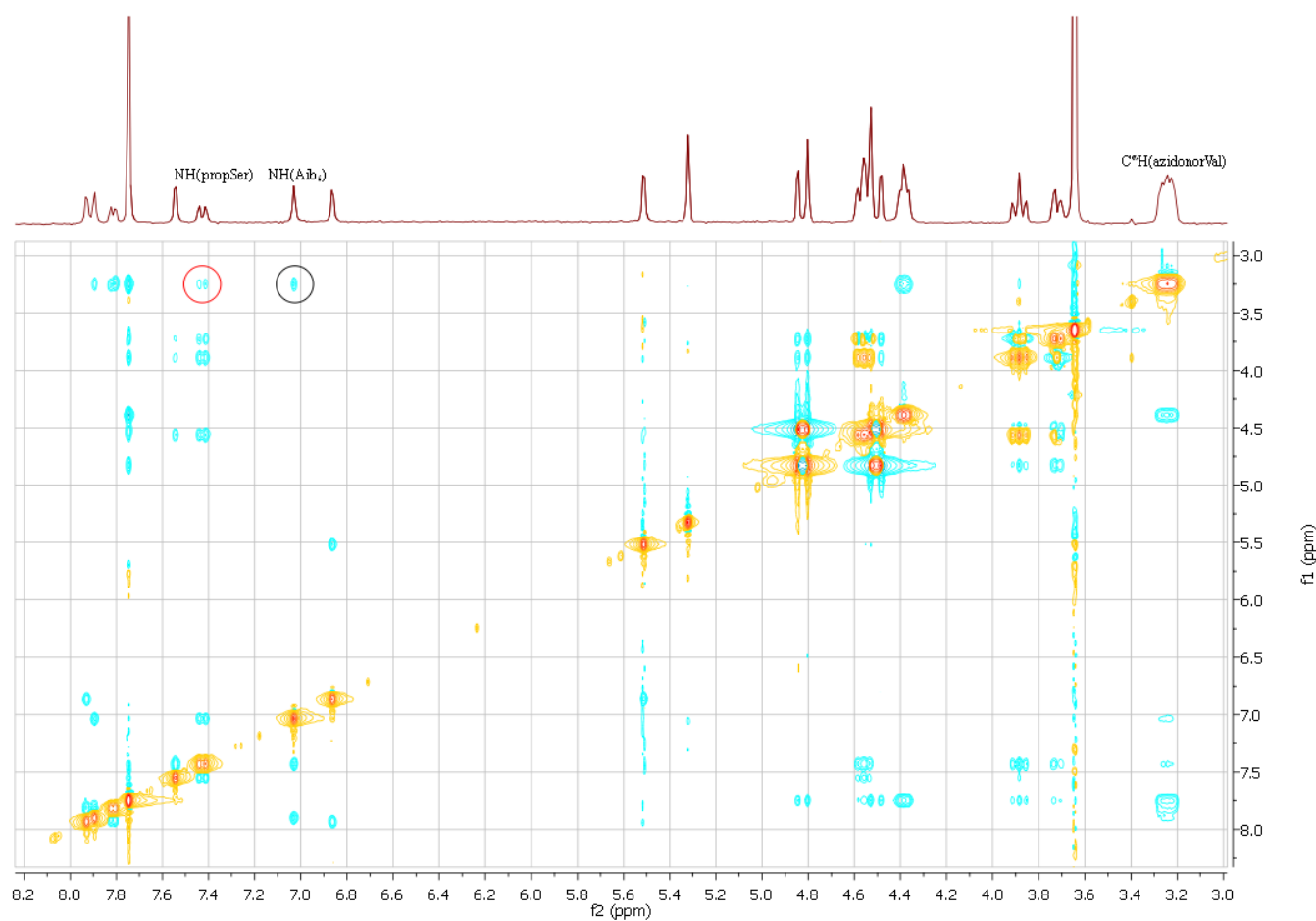


Figure 6. Partial ROESY spectrum of **2** displaying the only possible medium range $C^{\alpha}H(i) \rightarrow NH(i+2)$ (black circle) and long-range $C^{\alpha}H(i) \rightarrow NH(i+3)$ ROE (red circle).

A commonly used upper distance constraint for a weak ROE is on the order of 5.0 Å.⁵⁸ In order to determine more precisely the extent to which the solution structures of **1** and **2** mimic their corresponding crystal structures all proton pairs closer than 5.0 Å in the crystal structures, which should

give rise to an ROE if the crystal structure is preserved in solution, were identified. In the case of **1** the distance measurements were performed on the structure with the alkyne side chain and backbone at Aib6 in their major conformations. A careful analysis of experimental 2D ROESY spectra recorded in dilute CD₂Cl₂ solution for the presence or absence of these ROEs was undertaken. The results are summarized in Figure 7.

	NH1	NH2	NH3	NH4	NH5	NH6	NH7	NH8	C ^α H4	C ^α H7	C ^β H ₂ 4	C ^β H ₂ 7	C ^δ H ₂ 4	C ^δ H ₂ 7	CCH
NH1															
NH2															
NH3															
NH4															
NH5															
NH6															
NH7															
NH8															
C ^α H4															
C ^α H7															
C ^β H ₂ 4															
C ^β H ₂ 7															
C ^δ H ₂ 4															
C ^δ H ₂ 7															
CCH															

Figure 7. ROEs observed in the 2D ROESY spectra of **1** and **2** in dilute CD₂Cl₂ solution and comparison of the solution structures of **1** and **2** in CD₂Cl₂ with their respective crystal structures. Colour code: Green signifies an ROE which should be observed if the crystal structure is preserved in solution and is *found* experimentally. Yellow signifies an ROE which should be observed if the crystal structure is preserved in solution, but cannot unambiguously be identified experimentally because of overlap with other ROEs, artifact peaks (e.g. TOCSY peaks) or diagonal peaks under the given experimental conditions. Red signifies an ROE which should be observed if the crystal structure is preserved in solution, but is *not found* in the experimental spectra or could not be distinguished from background noise. White fields correspond to distances greater than 5.0 Å in the crystal structure. Blue signifies the presence of a correlation in the experimental spectrum which was not predicted by the crystal structure. For each correlation the left hand columns refer to peptide **1** and the right hand

columns to peptide 2. Note that CCH is the alkyne proton in 1 and the triazole proton in 2. All methyl groups and the Boc group have been omitted.

For 1 the general correlation between the predictions based on the crystal structure and the experimental solution phase spectrum is quite good, with 18 (45%) predicted ROEs present and 11 (27.5%) absent. However, the presence/absence of an additional 11 (27.5%) ROEs could not unambiguously be decided due to overlap with other peaks. Importantly, the correlation appears to be even better for the cyclic peptide 2, with 27 (63%) predicted ROEs present in the experimental spectrum, only 3 (7%) undecidable and 13 (30%) absent. For both compounds it should be added that a large fraction of the correlations that were not found experimentally corresponds to distances between 4.0-5.0 Å in the crystal structure and are expected to be weak and potentially difficult to distinguish from background noise in dilute solutions.

An examination of the individual *backbone-to-backbone* ROEs reveals the presence of all possible $\text{NH}(i) \rightarrow \text{NH}(i+1)$ ROEs, but none of the predicted $\text{NH}(i) \rightarrow \text{NH}(i+2)$ ROEs. However, the latter finding should not be considered as strong evidence against a 3_{10} -helical structure, as the $\text{NH}(i) \rightarrow \text{NH}(i+2)$ distance is approximately 4.5 Å in a 3_{10} -helix, giving rise to a weak ROE at best, but can be much shorter in non-helical peptides, as can be appreciated from a molecular model. When it comes to the predicted $\text{C}^{\alpha}\text{H} \rightarrow \text{NH}$ type ROEs the accordance between theory and experiment is very good for both peptides.

Some of the *side chain-to-backbone* ROEs, i.e. side chain-to-NH and side chain-to- $\text{C}^{\alpha}\text{H}$, are quite informative, as their presence/absence appear to be particularly sensitive to small changes in backbone structure away from the 3_{10} -helical crystal structures. This is especially the case for the $\text{C}^{\delta}\text{H}_2(i) \rightarrow \text{NH}(i)$ interactions in the acyclic peptide 1, and to a lesser degree the $\text{C}^{\beta}\text{H}_2(i) \rightarrow \text{NH}(i)$ interactions. In the 3_{10} -helical crystal structure the torsional angle $\text{C}^{\beta}-\text{C}^{\alpha}-\text{N}-\text{H}$ is small and positions the NH proton and the side chain on the same side of the backbone with close to minimal distance separation. The distance is 4.20 Å in the crystal structure, making this a quite sensitive test for local helical structure. The absence of an

observable $C^{\delta}H_2(4) \rightarrow NH(4)$ correlation for **1** could indicate that the peptide backbone enjoys substantial conformational freedom, at least locally. The $C^{\delta}H_2(4) \rightarrow NH(4)$ ROE is present in the 2D ROESY spectrum of **2**, for which the corresponding crystal structure distance is 4.87 Å. This is an important difference between the two compounds. The $C^{\delta}H_2(7) \rightarrow NH(7)$ correlation (3.56 Å in the crystal structure) is, however, present in **1**. The shorter distance compared with $C^{\delta}H_2(4) \rightarrow NH(4)$ (4.20 Å) suggests that it is less sensitive to backbone changes away from the crystal structure than the $C^{\delta}H_2(4) \rightarrow NH(4)$ ROE. In contrast to **1**, the $C^{\delta}H_2(7) \rightarrow NH(7)$ is absent from the 2D ROESY spectrum of the cyclic peptide **2**, but this does not necessarily imply a large deviation from the crystal structure, as the crystal structure distance is 4.69 Å, placing it close to the detection limit already in a perfect 3_{10} -helix.

There are no observable differences between **1** and **2** regarding the presence/absence of $C^{\beta}H_2 \rightarrow NH$ type ROEs. The presence of $C^{\beta}H_2(i) \rightarrow NH(i)$ interactions in both peptides should be noted in support of a predominantly 3_{10} -helical backbone structure. However, larger backbone conformational changes are required to completely abolish these than is the case for the $C^{\delta}H_2(i) \rightarrow NH(i)$ ROEs discussed earlier. The absence of predicted $NH(i) \rightarrow C^{\beta}H_2(i+1)$ and presence of $C^{\beta}H_2(i) \rightarrow NH(i+1)$ correlations in both peptides are less informative.

The absence of $C^{\alpha}H(4) \rightarrow C^{\beta}H_2(7)$ interactions in the cyclic peptide **2** is a bit surprising (distance 4.21 Å in the crystal). For the acyclic peptide **1** the presence or absence of this ROE cannot be decided.

Side chain-to-side chain correlations may also be informative, especially for the acyclic peptide, for which the side chains of residues 4 and 7 are likely to be too far apart to give rise to observable ROEs in many non-helical conformations. Most of these are absent in both peptides, including the expected $C^{\beta\gamma}H_2(4) \rightarrow C^{\beta}H_2(7)$ and $C^{\delta}H_2(4) \rightarrow C^{\beta}H_2(7)$ ROEs for **2** and the $C^{\beta\gamma}H_2(4) \rightarrow C^{\delta}H_2(7)$ ROE for **1**.

Finally, a very weak ROE which was not predicted on the basis of the X-ray structure was observed between $C^{\delta}H_2(4)$ and $C^{\delta}H_2(7)$ for **1**. In general, the absence of many extra peaks not predicted by the crystal structure provides further evidence that the solution structure closely mimics the crystal structure. However, the presence of the unexpected $C^{\delta}H_2(4) \rightarrow C^{\delta}H_2(7)$ ROE may nevertheless be taken as

evidence in favour of a 3_{10} -helical structure, because it means that the side chains of residues 4 and 7 are on the same side of the molecule, which is what one would expect for a 3_{10} -helical peptide.

As a final note, the 2D ROESY spectra display ROEs characteristic of 3_{10} -helices independently of concentration. Hence, the possible presence of a monomer-dimer equilibrium is not expected to constitute a major complication for the structural analysis of **1** and **2** in solution.

The signal dispersion of the methyl resonances is significantly greater for **2** than for **1** (Figure 8). This indicates a smaller degree of conformational mobility/averaging on the NMR timescale in the former case and thus provides evidence for a more stable 3_{10} -helix.

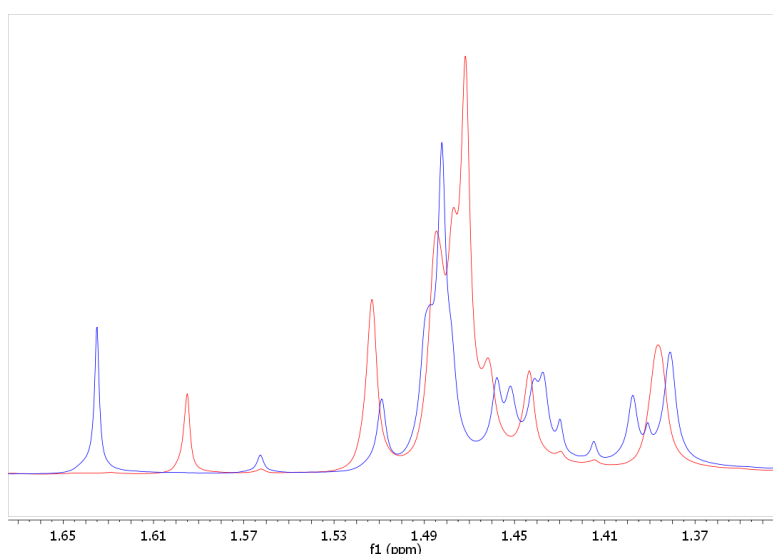


Figure 8. Methyl resonances in the ^1H -NMR spectra of **1** (red) and **2** (blue) ($c \sim 10$ mM). The greater dispersion of the resonances for the cyclic peptide may be taken as evidence for a more structured peptide in solution (less conformational averaging).

The chemical shifts of the NH resonances of the cyclic peptide **2** are generally very similar to those of **1** (Table 3), and demonstrate that the H-bond lengths and angles of the two peptides most likely are relatively similar in CD_2Cl_2 solution. The only exceptions are NH(Aib₆) and NH(propSer), which are shifted 0.29 ppm and 0.15 ppm upfield for **2**. The 1D and 2D NMR data taken together demonstrate that $i \rightarrow i+3$ side chain-to-side chain macrocyclization by CuAAC results in a close-to-ideal and conformationally stable 3_{10} -helical peptide.

Residue	δ_{acyclic}	δ_{cyclic}	$\Delta\delta$
Aib1	5.21	5.24	+0.03
Aib2	6.69	6.72	+0.03
Aib3	7.92	7.91	-0.01
azidonorVal	7.80	7.77	-0.03
Aib5	7.84	7.88	+0.04
Aib6	7.29	7.00	-0.29
propSer	7.55	7.40	-0.15
Aib8	7.56	7.53	-0.03

Table 3. Chemical shifts of NH resonances in dilute (5 mM, 296 K) CD₂Cl₂ solution.

Measurement and simulation of 2D IR spectra

Conventional linear IR response of the amide-I mode is widely used to obtain structural information of polypeptides.⁵⁹ Going beyond 1D, 2D IR spectroscopy measures nonlinear response of the mode, which has higher sensitivity to the underlying biomolecular structure.^{60–62} In this study we measured FT IR and 2D IR spectra of **1** and **2** in CH₂Cl₂ to obtain insights into the backbone conformation of these peptides in a polar aprotic solvent. Also, the spectral profiles were simulated based on crystal structures established by the X-ray diffraction analysis. Details of our 2D IR spectrometer, data collection, and calculation protocol have been described previously.^{46,63,64} The specifics relevant to the data discussed below are given in the Supporting Information.

The top panels in Figure 9a and b show the measured FT IR spectra (black solid) of **1** and **2**, respectively, in CH₂Cl₂ (~ 6 mM, a thickness of 180 μ m). The spectra were normalized by the peak absorbance of the methyl ester C=O band at 1738 cm⁻¹ after subtracting the solvent spectrum. We assign the small band at 1701 cm⁻¹ to the urethane C=O of the N-terminus Boc group and the broad band at 1662 cm⁻¹ to the amide-I modes. The line shapes of the amide-I bands of the two peptides are slightly different, more rounded at the peak for **2** but pointy for **1**. The full-width-half-maxima are 27.7 and 30.8 cm⁻¹, respectively. It is not straightforward, however, to infer from the linear spectra whether **1** and **2** in CH₂Cl₂ maintain the same ₃₁₀-helical conformation as observed in the crystal states, or if their structures have changed to other conformations, such as α -helix and P_{III}.

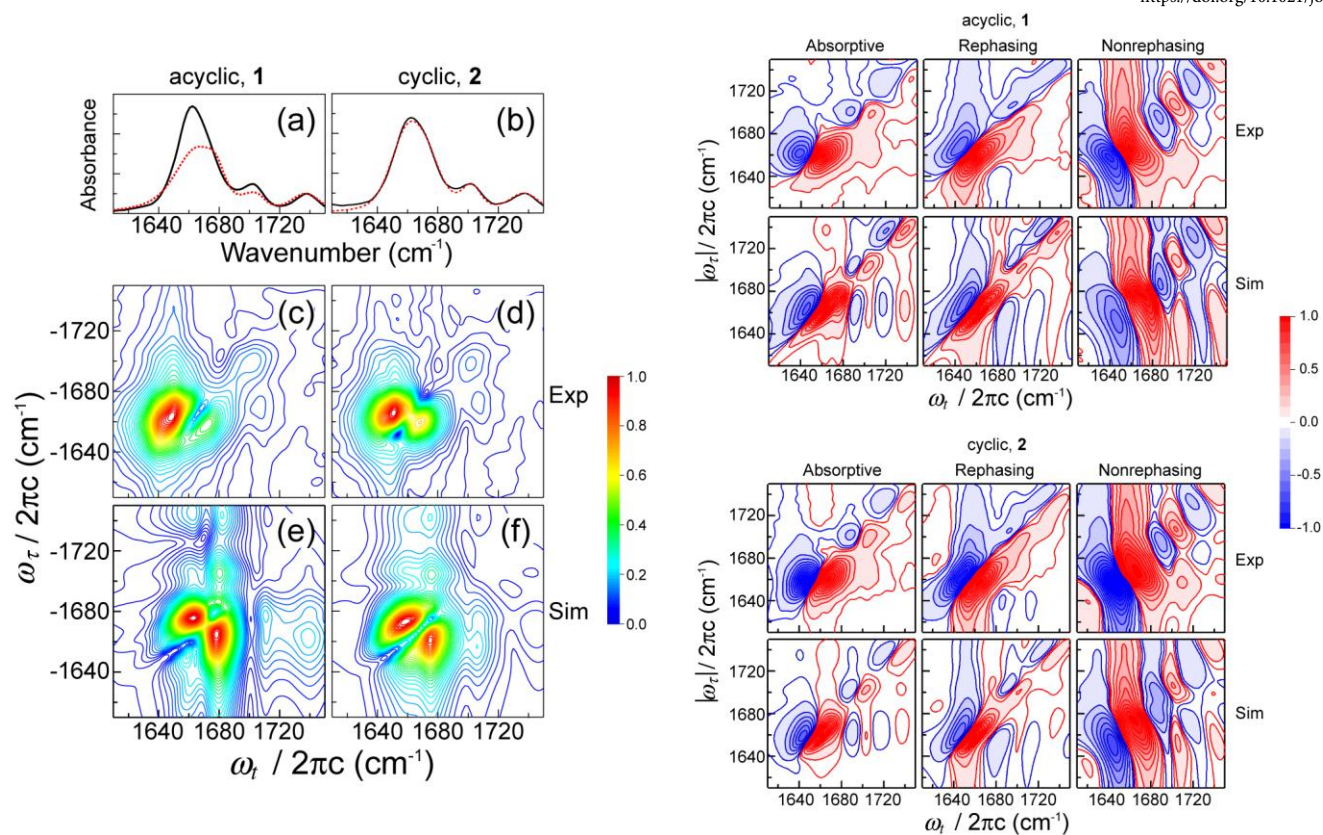


Figure 9. (a, b) Measured (black solid) and simulated (red dashed) linear IR spectra for **1** and **2**. (c, d) 2D IR cross-peak patterns in CH_2Cl_2 observed under the double-crossed polarization configuration. (e, f) Simulated cross-peak patterns based on the peptide backbone conformations of **1** and **2** in the crystal state. Shown on the right are the measured and simulated absorptive and the real parts of the rephasing and nonrephasing 2D IR spectra under the perpendicular polarization configuration.

The absorptive and the real parts of rephasing and nonrephasing 2D IR spectra of **1** and **2** measured under the $\langle Y, Y, Z, Z \rangle$ polarization configuration are shown on the right in Figure 9. The three positive peaks along the diagonal line of the absorptive spectrum correspond to the 0-1 transitions, whereas the three negative peaks are the 1-2 transitions, which are anharmonically shifted from the 0-1 in the ω_t direction. For the rephasing and nonrephasing spectra, the nodal lines between the 0-1 and 1-2 transitions are parallel and perpendicular to the diagonal, respectively. These 2D IR spectra look almost indistinguishable between **1** and **2**.

The amide-I 2D IR cross-peak pattern obtained under the double-crossed polarization can much more sensitively distinguish subtle structural differences, for example, between 3_{10} - and α -helices.^{63,64} In general, the experimental and simulated 2D profile exhibits a doublet pattern for the former and a multiple-peak pattern for the latter.^{64,65} Figure 9c and d presents the absolute magnitude cross-peak patterns of **1** and **2**, respectively. A doublet clearly shows up in the amide-I region. Weak cross-peaks between the amide-I and the urethane C=O modes are also observed. The doublet pattern of **1** is similar to that of **2** but some subtle differences can also be noticed. For **2**, the line shape and relative intensity of the lower diagonal peak to the upper peak (0.68) are very close to those observed for other 3_{10} -helical peptides with Aib and (α Me)Val residues.^{63,64} For **1**, the two peaks in the doublet are more elongated along the diagonal, merged into each other at a lower frequency, and their intensity ratio is 0.45. This result suggests that **1** and **2** are both 3_{10} -helical in the solution but their structures are slightly different. It is conceivable that **1** may be more disordered than **2** because it lacks the side chain-to-side chain CuAAC constraint. Our previous theoretical study shows that the rephasing cross-peak pattern differs from a doublet with the appearance of extra features as the peptide conformation increasingly deviates from an ideal 3_{10} -helix.⁶⁵ The absence of extra features suggests that the structural difference between **1** and **2** is small in CH₂Cl₂ despite the quite different dihedral angles of the residues 4, 6, and 7 observed in the crystal state.

To further address these points, we performed model calculations to examine how similar or different the 2D IR spectral patterns would be if the crystal structures are preserved in solution. Figure 9e and f presents the simulated 2D IR cross-peak pattern using an ensemble of peptide structures with the backbone dihedral angles in Gaussian distributions centered at the crystal structures of **1** and **2**, respectively. The simulated 2D IR pattern of **2** shows a clear amide-I doublet along with some cross-peaks between the amide-I and the capping C=Os. The linear IR spectrum (Figure 9b, red dashed) and 2D absorptive and the real parts of the rephasing and nonrephasing spectra (Figure 9, bottom panels on the right) were also calculated using the same parameters, and the agreement with the experimental spectrum is quite good. This suggests that the solution structure of **2** is close to that of an ideal 3_{10} -

helix. In contrast, the crystal structure of **1** gave rise to quite different linear and 2D IR spectra from those of **2**. The calculated linear spectrum of the major backbone conformer consists of two overlapping, broad amide-I bands with the lower frequency band having a stronger peak intensity. The linear spectrum of the minor backbone conformer also exhibits two bands with an opposite trend in the peak intensity. The population weighted spectrum (Figure 9a) shows hints of overlapping features. The cross-peak pattern in Figure 9e exhibits a more spread doublet with additional shoulders than that characteristic of an ideal 3_{10} -helix. Note that the simulated cross-peak pattern is still different from the ideal α -helix conformation $[(\phi, \psi) = (-63^\circ, -42^\circ)]$ we obtained previously.^{63,64} Also, the calculated 2D absorptive spectrum of **1** is much more elongated along the diagonal than that of **2**. The experimental and simulation results indicate that the different backbone conformations of **1** and **2** in the crystal state are no longer preserved in CH_2Cl_2 .

The results from our linear and 2D IR experiments and simulations are consistent with the findings from 1D and 2D NMR measurements. Both peptides remain 3_{10} -helical in solution. Comparing to **2**, the solution structure of the acyclic peptide **1** is more flexible and/or disordered. It also exhibits larger deviations from the crystal structure. The difference in their behavior can be attributed to the effect of the side chain-to-side chain constraint on peptide conformation.

Conclusion

In summary, the feasibility of side chain-to-side chain crosslinking by CuAAC in a 3_{10} -helical Aib rich peptide has been demonstrated. An attractive feature of the cyclic product **2** is its significantly higher aqueous solubility ($> 1 \text{ mM}$) compared to **1**. 2D IR and 2D ROESY experiments confirmed that the cyclic peptide **2** retained a 3_{10} -helical structure in the polar aprotic solvent CD_2Cl_2 . The first X-ray crystallographic investigation of a helical peptide with a triazole derived crosslink has revealed that **2** is the most perfect crosslinked 3_{10} -helical peptide so far studied in the crystal state, with mean (ϕ, ψ) -angles deviating less than 2° from ideality.

The closeness to ideality of the conformational angles in the solid state strongly suggests that the CuAAC side chain-to-side chain crosslinking methodology may have significant utility applied to peptides and peptidomimetics of interest in chemical biology and biomedicine, in particular to synthetic analogs of the Pro138-Gly144 segment of human AQP4.²⁰

Experimental Section

Full experimental details for the crystallization of **1** and **2**, acquisition of NMR, FT IR and 2D IR data, model calculations of linear and 2D IR spectra and syntheses of intermediates may be found in the available Supporting Information. The crystal structures of **1** and **2** have been deposited at the Cambridge Crystallographic Data Centre (accession codes CCDC 770131 and CCDC 770132).

N-*tert*-butoxycarbonyl α,α -dimethylglycyl α,α -dimethylglycyl α,α -dimethylglycyl δ -azido-L-norvalyl α,α -dimethylglycyl α,α -dimethylglycyl *O*-propynyl-L-seryl α,α -dimethylglycyl methyl ester **1** δ -azido-L-norvalyl α,α -dimethylglycyl α,α -dimethylglycyl *O*-propynyl-L-seryl α,α -dimethylglycyl methyl ester trifluoroacetate **4** (0.551 g, 0.827 mmol) and *N*-*tert*-butoxycarbonyl α,α -dimethylglycyl α,α -dimethylglycyl α,α -dimethylglycine²⁵ (0.309 g, 0.827 mmol) (see Supporting Information for details on preparation) were suspended in CH₂Cl₂ (5 mL) and a solution of *N,N*-diisopropylethylamine (0.107 g, 0.828 mmol) in CH₂Cl₂ (7 mL) added. HOBt hydrate (0.127 g, 0.829 mmol) and then EDC hydrochloride (0.174 g, 0.908 mmol) were added together with more CH₂Cl₂ (5 mL) at room temperature. The reaction mixture was stirred for 45 h at room temperature before being diluted with CH₂Cl₂ (65 mL). The solution was washed with 5% (w/w) citric acid monohydrate solution (3 $\times$ 35 mL), 7.5% (w/w) K₂CO₃ solution (3 $\times$ 35 mL) and saturated brine (35 mL). The solution was dried with anhydrous MgSO₄ and the solvent evaporated affording a white solid (0.595 g). The solid (0.573 g) was purified by flash column chromatography (eluent: CH₂Cl₂/acetone (3:1)) affording the title compound as a white solid (0.323 g, 43%); ¹H NMR (400 MHz, CD₂Cl₂, ~10 mM) δ 7.92 (s, 1H, NH(Aib₃)), 7.84 (s, 1H, NH(Aib₅)), 7.80 (d, *J* = 5.1 Hz, 1H, NH(azidonorVal)), 7.56 (s, 1H, NH(Aib₈)), 7.55 (d, *J* = 8.0 Hz, 1H, NH(propSer)), 7.29 (s, 1H, NH(Aib₆)), 6.69 (s, 1H, NH(Aib₂)), 5.21 (s, 1H,

NH(Aib₁)), 4.44 (td, J = 8.3, 3.5 Hz, 1H, C ^{α} H(propSer)), 4.23 (dd, J = 15.6, 2.4 Hz, 1H, CHHCCH), 4.18 (dd, J = 16.0, 2.4 Hz, 1H, CHHCCH), 3.97-3.80 (m, 3H, C ^{α} H(azidonorVal)/CH₂O), 3.65 (s, 3H, OCH₃), 3.33 (t, J = 6.8 Hz, 2H, CH₂N₃), 2.48 (t, J = 2.3 Hz, 1H, CCH), 2.02-1.62 (m, 4H, CH₂CH₂CH₂N₃), 1.61-1.36 (m, 45H, CH₃/(CH₃)₃); ¹³C NMR (50 MHz, CD₂Cl₂) δ 177.4, 176.2, 175.9, 175.6, 175.5, 175.4, 174.0, 170.1, 156.3, 81.8, 80.6, 74.7, 70.0, 58.8, 57.4, 57.4, 57.0, 57.0, 56.4, 54.9, 52.5, 51.7, 28.6, 28.6, 28.6, 28.5, 27.9, 27.9, 27.7, 27.4, 27.3, 26.5, 25.5, 25.2, 23.7, 23.5, 23.4, 23.3, 23.1; HRMS (m/z): [M+Na]⁺ calcd. for C₄₁H₆₉N₁₁O₁₂Na, 930.5024; found, 930.5017; Anal. Calcd. for C₄₁H₆₉N₁₁O₁₂: C, 54.23; H, 7.66; N, 16.97. Found: C, 54.2; H, 7.6; N, 16.6.

Methyl 2-methyl-2-((5S,14S)-8,8,11,11-tetramethyl-5-(2,2,6,6,9,9,12,12-octamethyl-4,7,10-trioxo-3-oxa-5,8,11-triazatridecanamido)-6,9,12-trioxo-16-oxa-1,7,10,13,19,20-hexaazabicyclo[16.2.1]henicosa-18(21),19-dienecarboxamido)propanoate 2 (5S,14S)-14-(1-methoxy-2-methyl-1-oxopropan-2-ylcarbamoyl)-8,8,11,11-tetramethyl-6,9,12-trioxo-16-oxa-1,7,10,13,19,20-hexaazabicyclo[16.2.1]henicosa-18(21),19-dien-5-aminium 2,2,2-trifluoroacetate **6** (0.332 g, 0.498 mmol) and *N*-tert-butoxycarbonyl α,α -dimethylglycyl α,α -dimethylglycyl α,α -dimethylglycine²⁵ (0.186 g, 0.498 mmol) (see Supporting Information for details on preparation) were suspended in CH₂Cl₂ (3 mL) and a solution of *N,N*-diisopropylethylamine (0.065 g, 0.50 mmol) in CH₂Cl₂ (4 mL) added. HOBt hydrate (0.076 g, 0.50 mmol) and then EDC hydrochloride (0.105 g, 0.548 mmol) were added at room temperature together with additional CH₂Cl₂ (3 mL). The reaction mixture was stirred for 44 h at room temperature before being diluted with CH₂Cl₂ (40 mL). The solution was washed with 5% (w/w) citric acid monohydrate solution (3 $\times$ 20 mL), 7.5% (w/w) K₂CO₃ solution (3 $\times$ 20 mL) and saturated brine (20 mL). The solution was dried with anhydrous MgSO₄ and the solvent evaporated affording the title compound as a white solid (0.331 g, 73%); ¹H NMR (400 MHz, CD₂Cl₂, ~10 mM) δ 7.91 (s, 1H, NH(Aib₃)), 7.88 (s, 1H, NH(Aib₅)), 7.77 (d, J = 6.0 Hz, 1H, NH(azidonorVal)), 7.73 (s, 1H, C₂HN₃), 7.53 (s, 1H, NH(Aib₈)), 7.40 (d, J = 8.4 Hz, 1H, NH(propSer)), 7.00 (s, 1H, NH(Aib₆)), 6.72 (s, 1H, NH(Aib₂)), 5.24 (s, 1H, NH(Aib₁)), 4.82 (d, J = 13.0 Hz, 1H, OCHHC₂HN₃), 4.55 (td, J = 8.4, 2.8 Hz, 1H, C ^{α} H(propSer)), 4.51 (d, J = 13.2 Hz, 1H, OCHHC₂HN₃), 4.38 (t, J = 5.7 Hz, 2H, CH₂N₃C₂H), 3.88

(t, $J = 8.5$ Hz, 1H, $C^{\alpha}HCHHO$), 3.72 (dd, $J = 9.0, 2.9$ Hz, 1H, $C^{\alpha}HCHHO$), 3.65 (s, 3H, OCH_3), 3.23 (ddd, $J = 11.7, 5.9, 3.8$ Hz, 1H, $C^{\alpha}H(\text{azidonorVal})$), 2.31–2.17 (m, 1H, CH_2CHHCH_2), 2.07–1.93 (m, 2H, $C^{\alpha}HCHHCH_2CH_2/CH_2CHHCH_2$), 1.79–1.68 (m, 1H, $C^{\alpha}HCHHCH_2CH_2$), 1.64 (s, 3H, CH_3), 1.58–1.35 (m, 42H, $CH_3/(CH_3)_3$); ^{13}C NMR (75 MHz, CD_2Cl_2) δ 177.4, 175.9, 175.9, 175.6, 175.5, 175.5, 173.7, 170.0, 156.4, 145.3, 125.1, 81.8, 70.0, 65.3, 57.5, 57.5, 57.4, 57.3, 57.1, 56.4, 55.2, 54.5, 52.5, 49.7, 28.6, 28.3, 27.9, 27.9, 27.3, 27.2, 27.0, 25.4, 25.3, 24.8, 23.7, 23.4, 23.3, 23.2, 23.0; HRMS (m/z): $[M + H]^+$ calcd for $C_{41}H_{70}N_{11}O_{12}$, 908.5205; found, 908.5194; Anal. Calcd for $C_{41}H_{69}N_{11}O_{12}$: C, 54.23; H, 7.66; N, 16.97. Found: C, 54.0; H, 7.6; N, 16.5.

Acknowledgments

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Supporting Information Available: Synthesis schemes, full experimental details for syntheses, compound characterization data, crystallization conditions, crystallographic data in CIF format, experimental details for acquisition of X-ray, NMR, linear IR and 2D IR data, model calculations of linear and 2D IR spectra, Ramachandran plots and partial 2D ROESY spectra. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Table of Contents Graphic

