Polysubstituted cyclohexane γ-amino acids induce a double α-/β-turn in short non-natural peptides

David Reza,[a,b] Pau Nolis,[c] Víctor M. Sánchez-Pedregal,[b] Young Kee Kang,[d] Ramón J. Estévez,[a,b] Juan C. Estévez[a,b,*]

[a] Centro Singular de Investigación en Química Biolóxica e Materiais Moleculares (CiQUS), Universidade de Santiago de Compostela, c/ Jenaro de la Fuente s/n, 15782 Santiago de Compostela, Spain

[b] Departamento de Química Orgánica, Universidade de Santiago de Compostela, Avda. das Ciencias s/n, 15782 Santiago de Compostela, Spain

[c] Servei de Resonancia Magnetica Nuclear, Universitat Autonoma de Barcelona, Barcelona, Spain

[d] Department of Chemistry, Chungbuk National University, Cheongju, Chungbuk 28644, Republic of Korea

* Corresponding author: Juan C. Estévez, juancarlos.estevez@usc.es

Abstract: We describe short non-natural peptides that adopt α- and β-turn folds in solution and in the crystal. The peptides are constituted by a core of *trans* and *cis* stereoisomers of polyhydroxylated cyclohexane γ-amino acids, flanked by dimers of *L*-α-alanine, resulting in hybrid hexapeptides with an ααγγαα backbone. DFT calculations and spectroscopic analysis by NMR, CD and FT-IR in solution are consistent with structural changes upon deprotection of certain hydroxyl groups of the central polyhydroxylated γ-amino acids. X-ray diffraction analysis of a crystalline sample revealed a double α-/β-turn that was also identified by NMR spectroscopy in acetonitrile-d_3 solution.

Introduction

The tertiary structure of globular proteins is driven in part by the presence of reverse turns, which are secondary structures of short and non-regular peptide sequences where the polypeptide chain reverses its overall direction, conditioning the arrangement of the other secondary structures (helix, sheets, or random coil parts) present in the protein.[1] These structures not only condition protein folding but, due to their exposure to the outside of the protein, also play an important role in protein stabilization in biological media[2] and may also constitute molecular recognition sites for the protein/protein or protein/nucleic acid interactions.[3,4]

Reverse turns are constituted by peptide sequences in which the CO group of one residue is hydrogen-bonded to the HN group of another residue of the same chain separated by 1 to 5 positions, while keeping their Cα atoms close in space (less than 7 Å). Typically, turns cause a dramatic change in the direction of the polypeptide chain. Turns are usually classified according to the number of residues intervening in the hydrogen bond between the carbonyl group of residue *i* and the HN group of residue *i*+n: *i*+1 (δ-turn), *i*+2 (γ-turn), *i*+3 (β-turn), *i*+4 (α-turn) and *i*+5 (π-turn).[5,6]

The extraordinary importance of these secondary structures in the final tertiary structure of proteins stimulated that they were searched and studied profusely in natural proteins, finding examples of all of them. The β-turns are the most common ones in natural proteins. Also, peptides with this type of secondary structure have important biological properties, including antimicrobial and antiviral activities.[7] In contrast, reverse α-turns are rarely found in natural proteins and, when they appear, they are usually stabilized by smaller turns, especially reverse β-turns. They have been found in certain active sites of enzymes, and especially in cyclopeptides, playing an important role in cell proliferation signaling processes, among others.[8]

In addition to the peptide sequences that induce turns in natural proteins, formed by proteinogenic α-amino acids, similar types of structures have been discovered in recent years in molecules generally made up of non-natural amino acids (β-, γ-, δ- or ε-), either conformationally restricted or not.[9,10] Furthermore, turn-type structures in which the constituent units are not amino acids have also been described.[11–13]

The discovery of these non-natural turn structures has been of enormous importance to mimic natural turn structures in the design of non-natural peptides and proteins.[14] It was recognized the potential of introducing new functionalities in the turn that can improve biocompatibility or selectivity of the structures,[15] or can act as new catalysts.[16] This is one of the reasons why the development of this field continues to be of crucial interest in areas such as biological chemistry and new materials design.

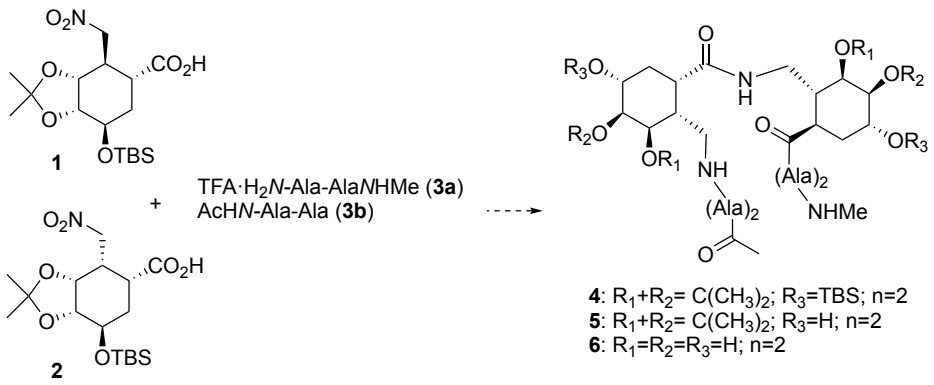
Previous computational studies showed that peptides constituted by a central core of two cyclohexane γ-amino acids with hetero and homo chiralities can generate turn structures in peptide chains.[17] The conformational constraint imposed by the cyclohexane ring on the Cα-Cβ bond has an important effect to favour certain β-turn folds. However, the effect of further substituents on the cyclohexane ring has not yet been explored. One of the reasons is

the lack of efficient methods to synthetize those complex non-natural amino acids. More recently, we described the synthesis of polyhydroxylated cyclohexane γ-amino acids from (–)-shikimic acid as well as a protocol to incorporate them into peptides.[18] Here we present the synthesis and conformational studies of hybrid ααγγαα hexapeptides constituted by a core of *trans* and *cis* stereoisomers of such polyhydroxylated cyclohexane γ-amino acids with the goal of exploring their ability to form peptide turns.

RESULTS

Peptide design

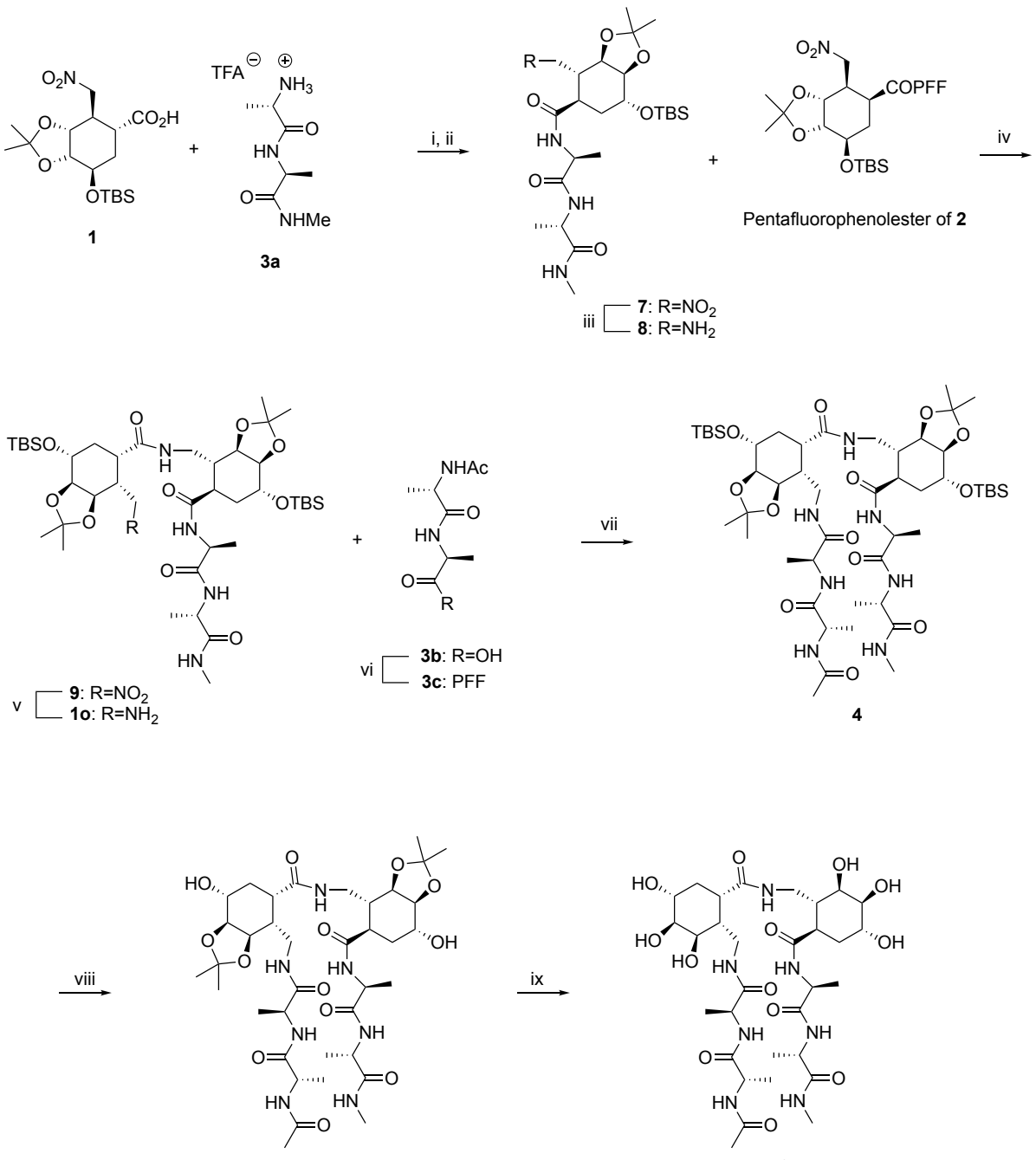
The hexameric hybrid peptide comprises a central core of two trihydroxylated cyclohexane γ-amino acids with Cα-Cβ *trans* and *cis* relative configurations (derived from precursors **1** and **2** respectively) flanked by *L*-α-Alanine di-residues (Scheme 1). This peptide design was chosen because it combines the tendency of γ-amino acid dimeric peptides to fold into reverse turns[17] with the potential to change the peptide turn geometry by modulating the polarity and/or steric hindrance of the hydroxyl groups depending on their degree of protection or deprotection.



Scheme 1

Synthesis

Peptide **4** was synthesized starting from commercial TFA·H2N-Ala-Ala-NH-Me (**3a**), AcHNAla-Ala (**3b**) and nitro acids **1** and **2** previously obtained in our laboratory using (–)-shikimic acid as starting material, following the procedure described previously by us (Scheme 1).[18] Peptides **5** and **6** were obtained by stepwise selective deprotection of the OH groups of peptide **4** (Scheme 2).



Scheme 2. (i) Pentafluorophenol, DIC, Cl_2CH_2, rt, 12 h. (ii) TFA·H_2NAlaAlaNHMe, DIEA, DMF, rt, 2 h (80%, two steps). (iii) H_2 (P=1 atm), Raney-Ni, MeOH, rt, 12 h. (iv) perfluorophenolester of **2** (Ref. XX), DIEA, DMF, rt, 12 h (83 % two steps). (v) H_2 (P= 1atm), Raney-Ni, MeOH, rt, 12 h. (vi) Pentafluorophenol, DIC, Cl_2CH_2, rt, 12 h. (vii) AcNAla-AlaOPFF (3c), DIEA, DMF, rt, 12 h (54 %, two steps). (viii) TBAF, THF, rt, 48 h (68%). (ix) AcOH/THF/H2O, rt, (50 %).

IR spectroscopy

The solution IR spectrum of peptide **4** in $CHCl_3$ shows large red shifts of bands Amide A and Amide I, and a blue shift of the Amide II band, relative to peptide **6**. This is in accordance with the presence of strong intramolecular hydrogen bonds in peptide **4** and the absence of

such hydrogen bonds in peptide **6** (Table 1, Figure 1). Compound **5** was studied in acetonitrile and in water, but the frequency shift of its bands is less conclusive about the presence of hydrogen bonds, as values are intermediate between those of peptides **4** and **6**.

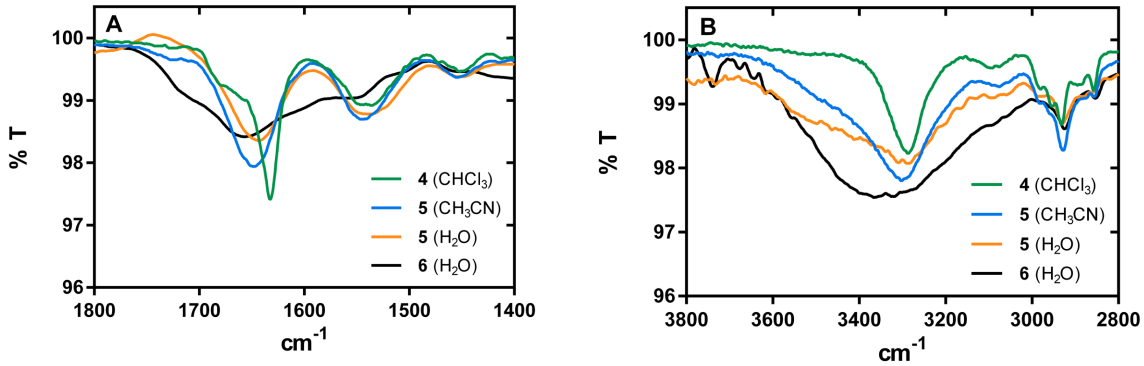


Figure 1. Enlargement of the Amide A (A), Amide I and Amide II (B) regions of the FTIR spectra of peptides **4**, **5** and **6** (solvents are indicated in parentheses).

Table 1. $\bar{\nu}_{max}$ (cm^{-1}) of the characteristic Amide A, Amide I, and Amide II bands of compounds **4**, **5** and **6**.

Compound	Solvent	Amide A	Amide I	Amide II
4	$CHCl_3$	3287	1633	1542
5	CH_3CN	3302	1648	1544
5	H_2O	3288	1643	1538
6	H_2O	3343	1657	1555

Circular Dichroism

The ECD spectra of compounds **4** and **5** in acetonitrile show a positive band at 192 nm and an intense negative band at ≈ 220 nm that is characteristic of peptide secondary structures,[19] while the ECD spectra of compounds **5** and **6** in water show less intense negative and positive bands at 205 nm and 220 nm, respectively, that is the characteristic pattern of peptides lacking a defined secondary structure (Figure 2).

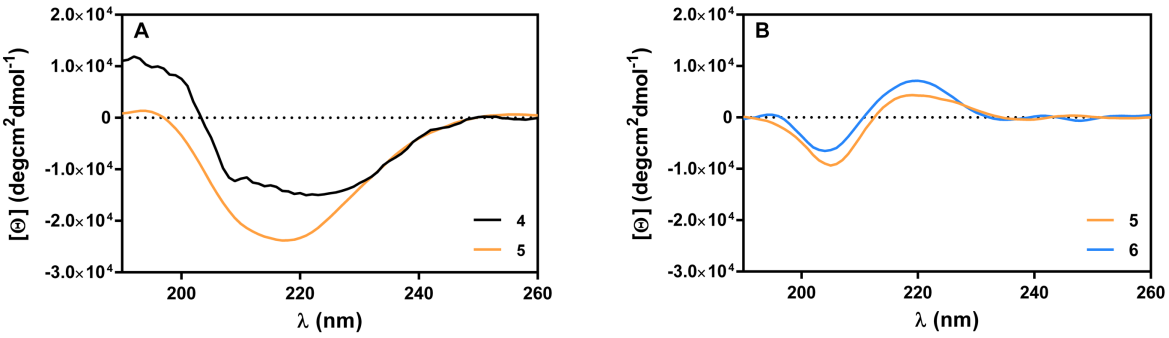


Figure 2. (A) circular dichroism spectra of **4** (black) and **5** (orange) in CH_3CN (c=1 mg/mL, 25 °C). (B) Circular dichroism spectra of **5** (orange) and **6** (blue) in water (c=1 mg/mL, 25 °C).

Structural study by NMR and X-Ray crystallography

Peptide 5

Single crystals of **5** were obtained by slow evaporation of an acetonitrile solution. The unit cell contains two peptide molecules in hairpin conformation with slightly different geometries in an antiparallel relative orientation (Figure 4). Each peptide molecule forms five intramolecular and several intermolecular hydrogen bonds with neighbouring molecules in the crystal. The intramolecular hydrogen bonds define the complex double α-/β-turn due to a bifurcated hydrogen bond of the Ala1 carbonyl oxygen (Figure 6 and Table 2). The hydrogen bond Ala1:O → Ala5:H_N corresponds to an α-turn *i* → *(i+4)* forming a 17-membered ring. The hydrogen bond Ala1:O → Tcy4:H_N establishes a β-turn *i* → *(i+3)* forming a 12-membered ring. The structure is further stabilized by an additional NH···O=C and two OH···O=C hydrogen bonds: Ala5:O → Ala1:H_N , Ccy3:O → Ccy3:HOε , and Ala2:O → Tcy4:HOε. Note that the latter two hydrogen bonds are not possible in peptide **4** as its two ε-hydroxyl groups are protected with TBS.

The cyclohexane rings of the two γ-amino acids are in chair conformations, only slightly distorted. It is of note that the *L*-α-Ala di-residues flanking the core γ-amino acids are not totally extended but adopt a curved shape, which approximates some atoms to establish intramolecular interactions (Figure 5).

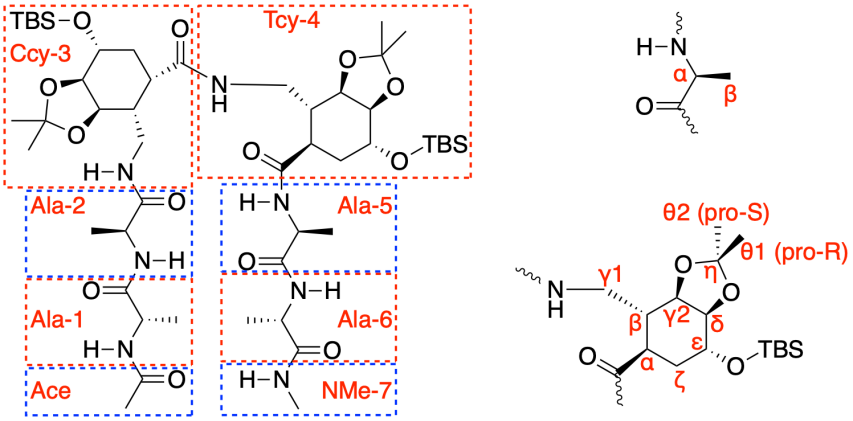


Figure 3. Residue and atom nomenclature used in this manuscript for peptides **4**, **5** and **6**. These peptides differ only in the protecting groups on oxygens γ2, δ and ε.

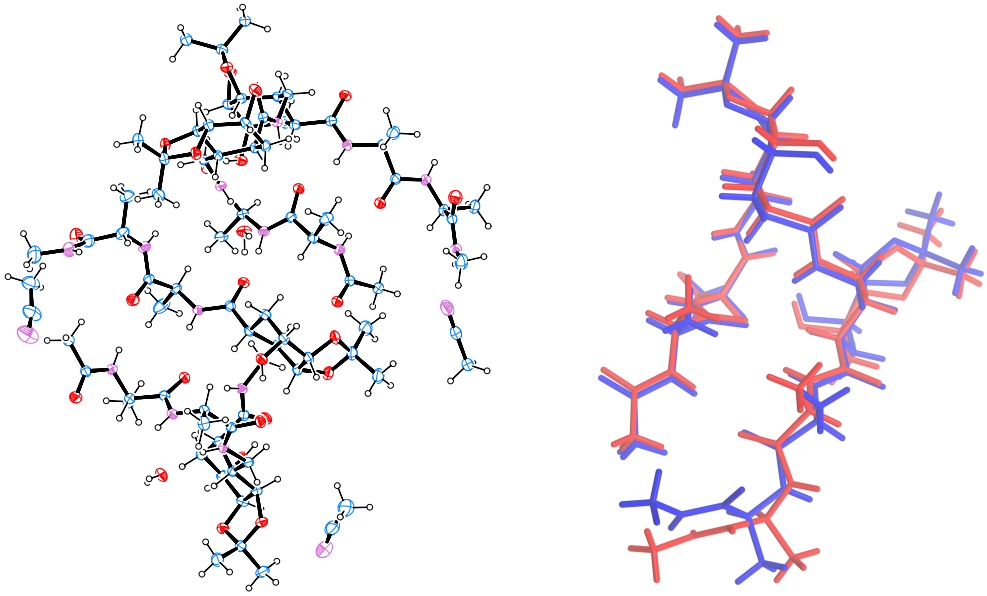


Figure 4. *Left*: ORTEP diagram of the unit cell containing two peptide **5** molecules with slightly different geometries. *Right*: overlay of the two molecules of the unit cell (heavy atom RMSD = 0.610 Å).

Table 2. Hydrogen bond parameters in the X-ray structure of peptide **5** (Å, °).

Donor (D)	Acceptor (A)	D—H (Å)	H...A (Å)	D...A (Å)	∠D—H...A (°)	Hbond type
Ala1:HN	Ala5:O	0.88	2.15	3.010(6)	167	C_{20}
Ccy3:HOε	Ccy3:O	0.84	1.86	2.654(5)	157	
Tcy4:HN	Ala1:O	0.88	2.24	3.107(6)	166	C_{12}
Tcy4:HOε	Ala2:O	0.84	1.91	2.745(5)	173	
Ala5:HN	Ala1:O	0.88	2.12	2.935(6)	153	C_{17}

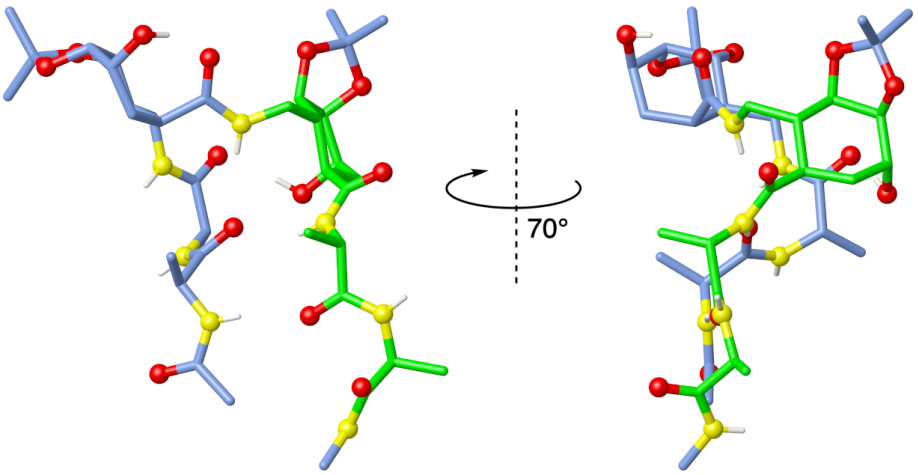


Figure 5. Geometry of peptide **5** in the crystal. Left and right views differ by a rotation of 70°. Colours: C *blue* (residues 1-3) and *green* (residues 4-6), O *red*, N *yellow*, H_N and H_O *white*; the other H atoms are not shown for clarity.

The solution NMR analysis of peptide **5** was done in acetonitrile-d_3 as it was crystallized from this solvent. Its low solubility in chloroform precluded its NMR analysis in $CDCl_3$ solution. NMR spectra of peptide **5** in acetonitrile-d_3 solution were recorded at 500 and 750 MHz. Resonances were assigned from a set of 1D and 2D homonuclear spectra (TOCSY, CLIP-COSY, ROESY and NOESY) spectra. Amide temperature coefficients were determined between 273 and 343 K in 10 K steps at 500 MHz. DOSY spectra recorded at two concentrations (1 and 10 mM, 298 K, 750 MHz) confirmed that peptide **5** is monomeric in solution. The NOESY spectrum (t_{mix} 600 ms) was recorded at a temperature of 308 K and 750 MHz to minimize peak overlap in the amide H_N region. The analysis of the NOE data is fully consistent with the geometry found in the crystal, *i.e.* with the double α-/β-turn conformation (Figure 6).

Two further structures of peptide **5** were generated by DFT calculations. The first one was optimized from the experimental XRD structure and the second one was generated from the cyclohexyl moiety of XRD structure and the stem of the hairpin structure with un-substituted cyclohexane moiety previously published.[17] The natural bond orbital (NBO) analysis indicated that the H-bond between the carbonyl oxygen of Ala2 and the ε-HO group of Tcy4 is decisive to stabilize this double turn structure. Although some dynamics are expected for such a small peptide in solution, the fact that the observed NOEs are consistent with the geometry determined by diffraction analysis supports that it is highly populated in solution. This agrees well with the ECD spectrum recorded in acetonitrile solution (Figure 2), that has the characteristic maximum (*ca.* 192 nm) and minimum (≈ 215 nm) of a peptide turn.

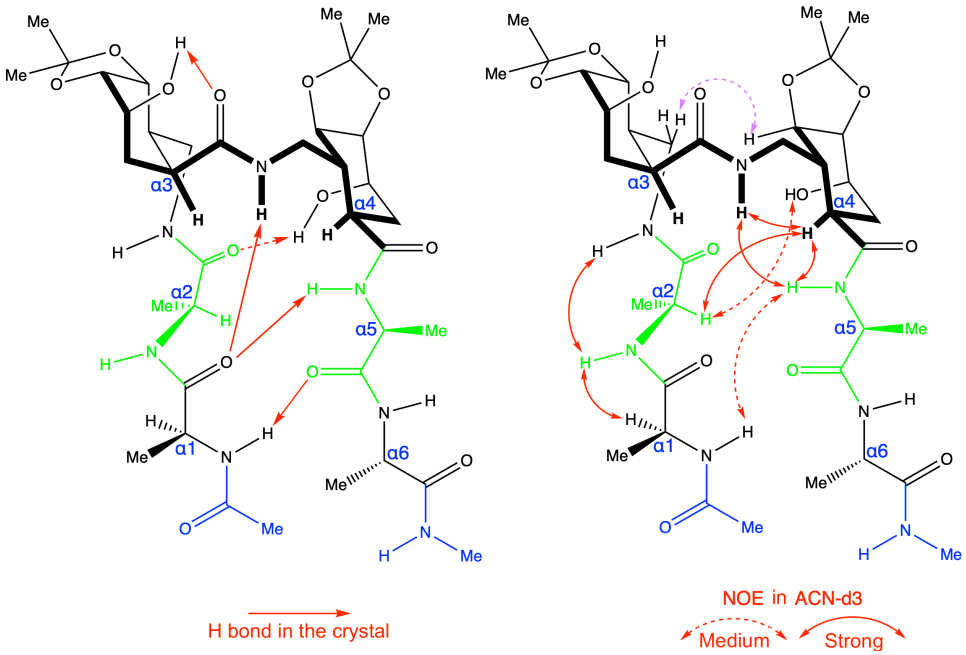


Figure 6. *Left*: summary of the intramolecular hydrogen bonds identified in the crystal structure of peptide **5**. *Right*: summary of the inter-residual NOE contacts of peptide **5** in acetonitrile-d_3 solution (t_{mix} = 600 ms, T = 308 K, 750 MHz). NOE cross-peaks were classified by intensity as *strong* (S), *medium* (M) or *weak* (W) and inter-proton distances were measured from the coordinates of the crystal structure.

Peptide 4

The solution structure of peptide **4** was further studied by NMR in $CDCl_3$ solution in conjunction with DFT calculations. The initial structures of peptide **4** with ε-O-TBS groups were generated from the optimized XRD and hairpin structures of peptide **5**, and were then optimized at the M06-2X/6-31G(d) level of theory. NMR resonances were assigned from a set of 1D (^{1}H and ^{13}C{^{1}H}) and 2D (TOCSY, ROESY, multiplicity edited HSQC, HMBC and band-selective HMBC) spectra. Amide temperature coefficients were determined between 273 and 308 K in 5 K steps at 500 MHz. The NOESY spectrum recorded at 328 K (t_{mix} 400 ms, 600 MHz) showed the NOE contacts Ala2:HN/Ala5:HN and Ala2:HN/Ala6:Hα, that are compatible with a hairpin β-turn (distances = 3.72 and 2.75 Å, respectively) but not with the XRD structure of peptide **5**, in which those distances are 4.91 Å and 7.70 Å, respectively. The NOE contact Tcy4:HN/Ala5:HN is compatible with the α-/β-turn XRD structure (d = 3.03 Å) but also with the hairpin structure of peptide **4** obtained by DFT calculations (d = 3.81 Å). In summary, the DFT calculated hairpin structure of **4** is the one that best fits the NOE data, while the XRD-like DFT calculated structure of **4** does not comply with some NOE peaks.

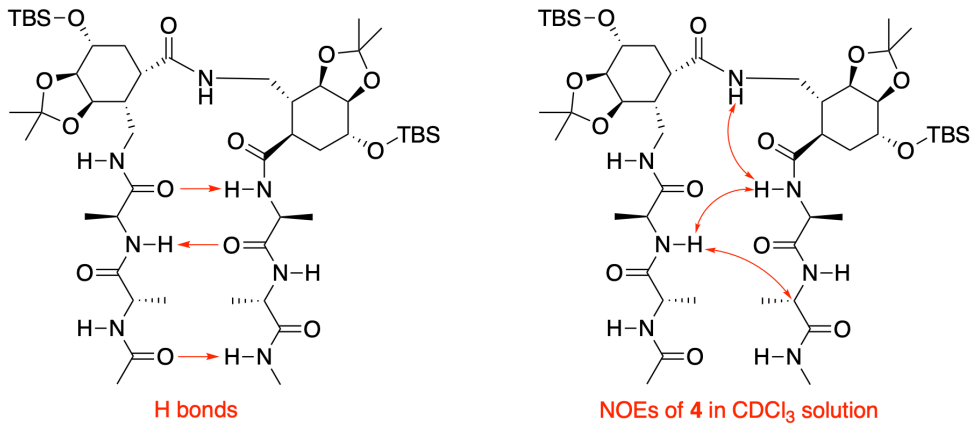


Figure 7. *Left*: hydrogen bonds expected in a β-turn hairpin. *Right*: long-range NOE contacts detected in the NOESY spectrum of peptide **4** in $CDCl_3$ solution (t_{mix} = 400 ms, T = 328 K, 600 MHz).

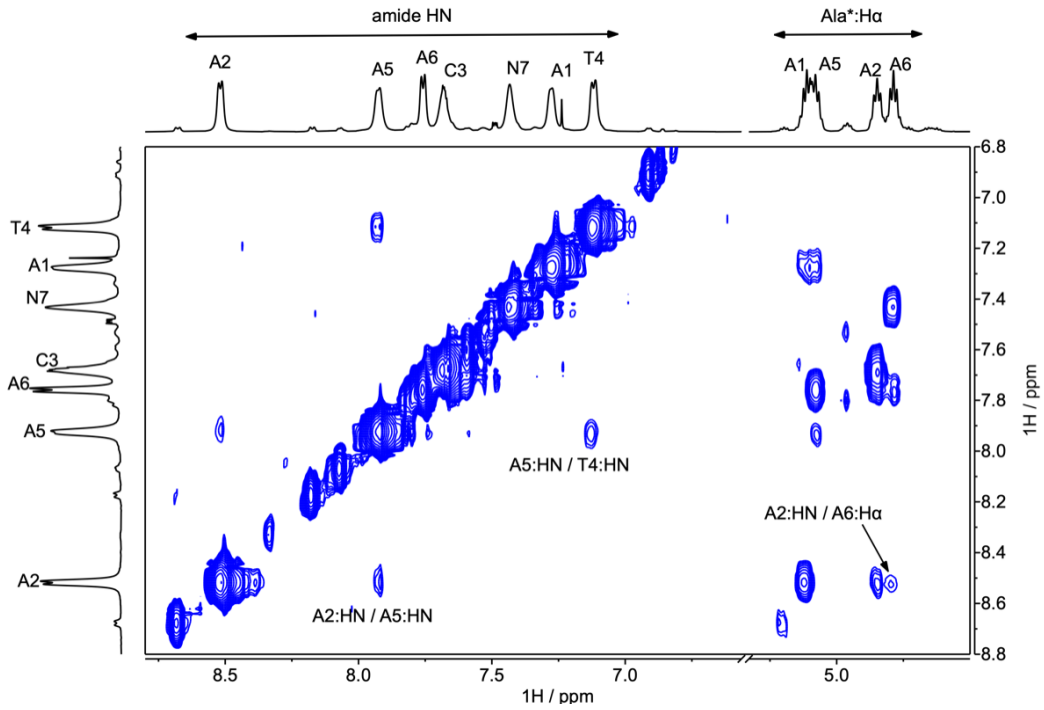


Figure 8. Enlargement of the amide H_N and Hα region of the NOESY of peptide **4** (t_{mix} = 400 ms, $CDCl_3$, 328 K, 600 MHz). Inter-residue NOE contacts Ala2:HN/Ala5:HN, Ala2:HN/Ala6:Hα, and Tcy4:HN/Ala5:HN are labeled.

Peptide 6

The solubility of the totally deprotected peptide **6** in acetonitrile is low, so its NMR spectra were measured in water solution (90% H_2O / 10% D_2O). The ROESY spectrum was recorded with a mixing time of 600 ms (298 K, 600 MHz). Only short-range or sequential NOEs (e.g. Ala2:HN/Ccy3:HN and Ala6:HN/NMe7:HN) were detected. The long-range NOEs

Ala2:HN/Ala5:HN and Ala5:HN/Tcy4:HN that support the β-turn conformation of **4** in $CDCl_3$ solution are not detected in the spectrum of **6** in water solution. This indicates that peptide **6** is mostly unstructured, which is also in agreement with its ECD and IR spectra.

DISCUSSION

Hybrid peptide sequences of non-natural amino acids of the class αγγα (tetramers) and ααγγαα (hexamers) have been shown to form β-turns computationally.[17] Molecular modeling calculations indicate that conformational restriction of the Cα-Cβ bond of γ-amino acids favours the formation of β-turns in tetrameric and hexameric peptides. Those DFT calculations were done with simple cyclohexane rings lacking any other substituents. In our current design, we introduced highly functionalized polyhydroxylated cyclohexane γ-amino acids with the goal of accessing to new turns and/or of modulating the accessible secondary structures by modifying the degree of protection of the OH groups. We have studied three peptides with a common backbone that only differ in the degree of protection of their OH groups. This affects solubility and fold, as anticipated by ECD and IR analysis. With the more detailed structural analysis by NMR and XRD, we demonstrate that the degree of protection affects the type of turn that forms. It is unclear if this is due to the changes in flexibility imposed by the protecting groups, or if it is due to differential interactions of the protected/unprotected OH groups with the solvent. Unfortunately, differences in solubility precluded the comparison of peptides **4**, **5** and **6** in the same solvent.

The crystal structure of peptide **5** (crystallized from acetonitrile solution) presents a remarkable fold that has the features of an α-turn (C17 ring) and a β-turn (C12 ring) due to the bifurcated H-bond between the carbonyl Ala2:O and the amide hydrogens Tcy4:H_N and Ala5:H_N, respectively. There is a further H-bond Ala1:H_N → Ala5:O that contributes to stabilize the hairpin. The NMR NOE data recorded at 308 K in acetonitrile-d_3 solution are fully compatible with the 3D structure in the crystal (Figure 7). The existence of a well-defined fold of peptide **5** was anticipated by its CD spectrum in acetonitrile, that displays an intense maximum at 192 nm and an intense minimum at 220 nm.[5] While the CD spectra of **4** in acetonitrile is similar to that of **5**, suggesting a similar fold, its NOESY spectrum in $CDCl_3$ gives cross-peaks that are not compatible with the XRD structure, like Ala2:HN / Ala5:HN and Ala2:HN / Ala6:Hα, as they correspond to H-H distances of 4.91 and 7.70 Å in the crystal, respectively. In fact, these NOEs are compatible with a β-turn fold, as these H-H distances were calculated as 2.75 and 3.72 Å, respectively, in the hairpin structure optimized by DFT

calculations. Furthermore, NOE cross-peaks Ccy3:HN / Tcy4:HN and Ccy3:HN / Ala5:HN do not appear in the NOESY spectrum of **4**, in agreement with the long H-H distances (6.54 and 5.08 Å, respectively) in the hairpin structure optimized by DFT calculations.

Peptide **6** was obtained by deprotecting all the OH groups of residues 3 and 4, so it has six free hydroxyl groups in total. The increase in polarity relative to peptide **5** made peptide **6** barely soluble in acetonitrile but soluble in the more polar DMSO and water. The CD and IR spectra of **6** in water were indicative of lack of defined structure in solution (Figure 2). NMR spectroscopy in water solution confirmed this, as the only detected inter-residue NOE peaks are sequential, *i.e*. no NOEs between the N-terminal and C-terminal alanine residues were detected.

Conclusion

We have demonstrated experimentally that hybrid peptide sequences of non-natural amino acids of the class ααγγαα are able to form β-turns with properties that anticipate their utility as hairpins inducers in the design of non natural peptides. Furthermore, the structure of **5** as a double α-/β-turn illustrates the important role of amino acid side chain substituents (the OH groups on the cyclohexane rings in this example) to stabilize certain conformations. Exploration of other configurations or protection/unprotection schemes in the amino acid building blocks may give access to novel structures in the field of non natural peptides.

Acknowledgements

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Experimental

Materials and methods

Specific rotations were recorded on a JASCO DIP-370 optical polarimeter. Infrared spectra were recorded on a *MIDAC Prospect FT-IR PerkinElmer Spectrum Two* spectrometer. Nuclear

magnetic resonance spectra were recorded on Varian Mercury 300, Bruker Avance III 500, Bruker Avance III 600 and Bruker NEO 750 spectrometers. Mass spectra were obtained on a Kratos MS 50 TC mass spectrometer. X-ray experiments were obtained with a Bruker Appex II apparatus. Thin layer chromatography (tlc) was performed using Merck GF-254 type 60 silica gel and ethyl EtOAc/hexane mixtures as eluents; the tlc spots were visualized with a Hanessian stain (dipping into a solution of 12.5 g of $(NH_4)_4Mo_7O_{24}\cdot 4H_2O$, 5 g of $Ce(SO_4)_2\cdot 4H_2O$ and 50 mL of H_2SO_4 in 450 mL of H_2O, and warming). Column chromatography was carried out using Merck type 9385 silica gel. Solvents were purified as described in the literature.[20]

Synthesis of (3aR,4R,5R,7R,7aR)-7-((tert-butyldimethylsilyl)oxy)-2,2-dimethyl-N-((S)-1-(((S)-1-(methylamino)-1-oxopropan-2-yl)amino)-1-oxopropan-2-yl)-4-(nitromethyl)hexahydrobenzo[d][1,3]dioxole-5-carboxamide (7)

To a solution of **1** (0.50 g, 1.28 mmol) in dry dichloromethane (30 mL), cooled at 0 ºC diisopropylcarbodiimide (0.30 mL, 1.92 mmol) and pentafluorophenol (0.71 g, 3.84 mmol) were added, and the resulting mixture was stirred for 3 h at 0 °C and then at room temperature for 12 h. The reaction mixture was poured into a sodium bicarbonate aqueous saturated solution (20 mL), and the suspension was extracted with dichloromethane (3 × 15 mL). The joined organic layers were dried with anhydrous sodium sulfate, filtered, and concentrated under reduced pressure. To a solution of the resulting residue in dry DMF (10 mL), DIEA (0.18 mL, 1.039 mmol), and trifluoroacetic salt (**3a**) (0.43 g, 0.77 mmol) were added and the resulting mixture was stirred at room temperature for 12 h. The mixture was then added to 50 mL of brine and extracted with EtOAc (3 × 25 mL). The joined organic layers were washed with water (100 mL), dried with anhydrous sodium sulfate, filtered, and concentrated under reduced pressure. Flash column chromatography of the obtained residue (EtOAc/hexane 1:2) provided (0.46 g, 80% yield) of tripeptide **7**, as an amorphous solid. $\mathbf{[\alpha]_D^{20}}$ –2.46 (*c* 7.5, $CHCl_3$). **IR** ($CHCl_3$) ν 3306, 1734, 1687, 1660, 1531 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$) δ 0.09 (s, 6H, $Si(CH_3)_2$), 0.89 (s, 9H, $SiC(CH_3)_3$), 1.32 – 1.44 (m, 9H, 3xCH_3), 1.50 (s, 3H, CH_3), 1.76 (d, J = 13.8 Hz, 1H, C<u>H</u>-H), 2.03 (d, J = 6.1 Hz, 1H, C<u>H</u>-H), 2.39 (t, J = 10.3 Hz, 1H, CH), 2.60 (t, J = 10.8 Hz, 1H, CH), 2.82 (d, J = 4.5 Hz, 3H, NCH_3), 4.03 (m, 1H, CH-N), 4.18 (dd, *J* = 9.3, 4.9 Hz, 1H, CH-O), 4.26 (m, 1H, CH-N), 4.38 – 4.48 (m, 2H, CH_2-NO_2), 4.52 (dd, *J* = 16.8, 8.6 Hz, 1H, CH-O), 4.68 (dd, J = 12.3, 4.0 Hz, 1H, CH-O), 6.10 (s, 1H, NH), 6.32 (d, J = 7.0 Hz, 1H, NH), 6.65 (d, J = 7.5 Hz, 1H, NH) ppm.

^{13}C{^{1}H} NMR (300 MHz, $CDCl_3$) δ –5.02 ($SiCH_3$), –4.85 ($SiCH_3$), 18.01 (SiC_c), 18.96 ($SiC(\underline{C}H_3)_3$), 19.19 (CH_3), 25.72 ($C_c(\underline{C}H_3)_2$), 26.25 (CH_3), 26.37 (CH_3), 28.16 (NCH_3), 33.39 (CH_2), 38.59 (OCH), 41.07 (OCH), 48.88 (OCH), 67.28 (NCH), 74.07 (NCH), 75.47 (NCH_2), 77.17 (NCH), 109.51 (C_c), 172.00 (C=O), 172.82 (C=O) ppm.

EMAR (ESI+) m/z, calcd. for $C_{24}H_{45}N_4O_8Si$ $[M+H]^+$: 545.2972. Found: 545.3014.

Synthesis of (3aR,4R,5R,7R,7aR)-7-((tert-butyldimethylsilyl)oxy)-4-(((3aR,4R,5S,7R,7aR)-7-((tert-butyldimethylsilyl)oxy)-2,2-dimethyl-4-(nitromethyl)hexahydrobenzo[d][1,3]dioxole-5-carboxamido)methyl)-2,2-dimethyl-N-((S)-1-(((S)-1-(methylamino)-1-oxopropan-2-yl)amino)-1-oxopropan-2-yl)hexahydrobenzo[d][1,3]dioxole-5-carboxamide (9)

To a deoxygenated solution of compound **7** (0.16 g, 0.31 mmol) in methanol (25 mL) Raney-Ni 1% (1.25 ml, 10% wt) was added and the resulting mixture was stirred under a hydrogen atmosphere (P = 1 atm) for 18 h. This reaction was then filtered through a Celite pad, which was washed with methanol and the filtrate was concentrated under reduced pressure. The resulting solid was taken up in dry DMF (3 mL), DIEA (0.065 mL, 0.37 mmol), and then perfluorophenylester of compound **2** (0.19 g, 0.34 mmol) (obtained as previously described)[18] were added and the resulting mixture was stirred at room temperature for 12 h. Then diluted with EtOAc (20 mL) and washed with saturated aqueous solution of ammonium chloride (2 × 25 mL). The organic layer was washed with water (40 mL), dried with anhydrous sodium sulfate and concentrated to dryness under reduced pressure. The residue was purified by flash column chromatography (EtOAc) to give tetrapeptide **9** (0.228 g, 83 % yield), as an amorphous white solid. **$[\alpha]_D^{24}$:** +10.4 (*c* 2.1, $CHCl_3$). **IR** ($CHCl_3$) ν 3301; 1630; 1553; 1530 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$) δ 0.05 (s, 3H, $SiCH_3$), 0.08 (s, 3H, $SiCH_3$), 0.09 (s, 3H, $SiCH_3$), 0.11 (s, 3H, $SiCH_3$), 0.86 (s, 6H, $C(CH_3)_3$), 0.89 (s, 6H, $C(CH_3)_3$), 1.32 (6H, 2xCH_3), 1.38 (6H, 2xCH_3), 1.46 (s, 3H, CH_3), 1.49 (3H, CH_3), 1.56 – 1.81 (m, 2H, CH_2), 1.96 (t, *J* = 11.5 Hz, 3H, CH_2 + CH), 2.37 – 2.53 (m, 1H, COCH), 2.65 (p, J = 7.1 Hz, 1H, COCH), 2.79 (d, J = 4.5 Hz, 4H, NCH_3 + CH), 3.13 – 3.44 (m, 2H, NCH_2), 3.82 – 4.15 (m, 1H, 4xCH), 4.22 (s, 1H, CH), 4.36 (t, J = 7.4 Hz, 1H, CH), 4.50 (q, J = 6.7 Hz, 2H, 2xCH), 4.60 (d, J = 6.9 Hz, 2H, NCH_2), 6.82 (s, 1H, NH), 6.85 (d, *J* = 4.9 Hz, 1H, NH), 6.99 (d, J = 7.6 Hz, 1H ,NH), 7.52 (d, J = 6.8 Hz, 1H, NH) ppm.

^{13}C{^{1}H} NMR (300 MHz, $CDCl_3$) δ -4.9 (CH_3), -4.8 (CH_3), -4.6 (CH_3), -4.5 (CH_3), 18.1 (C), 18.2 (C), 18.8 (CH_3), 19.2 (CH_3), 25.4 (CH_3), 25.9 (6xCH_3), 26.3 (CH_3), 26.4 (CH_3), 27.8 (CH_3), 28.4 (CH_3), 31.4 (CH_2), 32.5 (CH_2), 38.1 (CH), 39.3 (CH), 39.7 (CH), 41.4 (CH), 48.9 (NCH_2), 49.4 (CH), 67.9 (CH), 70.2 (CH), 73.6 (CH), 75.0 (NCH_2), 76.7 (CH), 77,6 (CH), 79.7 (CH), 108.9 (C), 109.2 (C), 172.1 (C=O), 172.8 (C=O), 173.3 (C=O), 174.7 (C=O) ppm.

EMAR (ESI+) m/z, calcd. for $C_{41}H_{76}N_5O_{12}Si_2$ $[M+H]^+$:886.5020; Found: 886.5024.

AcNAla-Ala-OPFF (3c)

To a solution of commercial **3b** (0.1 g, 0.27 mmol) in dry dichloromethane (10 mL), cooled at 0 ºC diisopropylcarbodiimide (0.06 mL, 0.40 mmol) and pentafluorophenol (0.15 g, 0.81 mmol) were added, and the resulting mixture was stirred for 3 h at 0 °C and then at room

temperature for 12 h. The reaction mixture was poured into a sodium bicarbonate aqueous saturated solution (10 mL), and the suspension was extracted with dichloromethane (3 × 10 mL). The joined organic layers were dried with anhydrous sodium sulfate, filtered, and concentrated under reduced pressure to give **3c** (0.12 g, 0.27 mmol) that was used in the next step.

Synthesis of (3aR,4R,5S,7R,7aR)-4-(((S)-2-((S)-2-acetamidopropanamido)propanamido)methyl)-7-((tert-butyldimethylsilyl)oxy)-N-(((3aR,4R,5R,7R,7aR)-7-((tert-butyldimethylsilyl)oxy)-2,2-dimethyl-5-(((S)-1-(((S)-1-(methylamino)-1-oxopropan-2-yl)amino)-1-oxopropan-2-yl)carbamoyl)hexahydrobenzo[d][1,3]dioxol-4-yl)methyl)-2,2-dimethylhexahydrobenzo[d][1,3]dioxole-5-carboxamide (4)

Raney-Ni 1% (0.06 ml, 10% wt) was added to a deoxygenated solution of **9** (0.055 g, 0.062 mmoles) in methanol (0.31 mL). The resulting mixture was stirred under hydrogen atmosphere (P = 1 atm) for 18 h. Then, the mixture was filtered through Celite and washed with methanol. The solvent was evaporated under reduced pressure and then redissolved in dry DMF (2.5 mL). DIEA (0.06 mL, 0.44 mmol) and **3c** (0.12 g, 0.27 mmol) were then added and the mixture was stirred under Argon atmosphere for 12h. The resulting mixture was diluted with EtOAc (50 mL), washed with ammonium chloride (2 × 40 mL) and distilled water (40 mL). The organic layer was dried with anhydrous sodium sulfate and concentrated to dryness under reduced pressure. The residue was purified by flash column chromatography (CH_2Cl_2/MeOH 95:5) to give the hexapeptide **4** (0.1 g, 54 %) as a white solid. **$[\alpha]_D^{20}$:** +6.21 (*c* 1.0, $CHCl_3$). **IR** ($CHCl_3$) ν 3288; 1633; 1547; 1535 cm^{-1}. **EMAR** (ESI+) m/z, calcd. for $C_{49}H_{89}N_7O_{13}Si_2$ $[M+Na]^+$: 1062.5948. Found:1062.5949.

Synthesis of (3aR,4R,5S,7R,7aS)-4-(((S)-2-((S)-2-acetamidopropanamido)propanamido)methyl)-7-hydroxy-N-(((3aR,4R,5R,7R,7aS)-7-hydroxy-2,2-dimethyl-5-(((S)-1-(((S)-1-(methylamino)-1-oxopropan-2-yl)amino)-1-oxopropan-2-yl)carbamoyl)hexahydrobenzo[d][1,3]dioxol-4-yl)methyl)-2,2-dimethylhexahydrobenzo[d][1,3]dioxole-5-carboxamide (5)

TBAF (0.05 mL, 0.05 mmol) was added to a stirred solution of the hexapeptide **4** (0.024 g, 0.023 mmol) in dry THF. The resulting mixture was stirred under argon atmosphere for 48 h. and the solvent was then evaporated under reduced pressure. The residue was purified by flash column chromatography (CH_2Cl_2/MeOH 10:1), to give the hexapeptide **5** (0.013 g, 68 %) as a white solid. **$[\alpha]_D^{20}$:** +7.72 (*c* 1.0, MeCN). **IR** (MeCN) ν 3302; 1647; 1543 cm^{-1}. **EMAR** (ESI+) m/z, calcd. for $C_{37}H_{61}N_7NaO_{13}$ $[M+Na]^+$: 834.4241. Found: 834.4220.

Synthesis of (1S,2R,3R,4S,5R)-2-(((S)-2-((S)-2-acetamidopropanamido) propanamido) methyl)-3,4,5-trihydroxy-N-(((1R,2R,3S,4R,6R)-2,3,4-trihydroxy-6-(((S)-1-(((S)-1-(methylamino)-1-oxopropan-2-yl) amino)-1-oxopropan-2-yl) carbamoyl) cyclohexyl) methyl) cyclohexane-1-carboxamide (6)

Hexapeptide **7** (23 mg, 0.022 mmol) was dissolved in 3 mL of a mixture of TFA/H_2O/Dioxane (1:1:1) and stirred for 24 h. The solvent was evaporated then under reduced pressure and the solid obtained was washed with hexane The residue was purified by HPLC to give hexapeptide **6** (10 mg, 50 %) as a white solid. **$[\alpha]_D^{20}$:** +8.24 (*c* 1.0, H_2O). **IR** (H_2O) ν 3278; 1624; 1541 cm^{-1}. **EMAR** (ESI+) m/z, calcd. for $C_{31}H_{54}N_7O_{13}$ $[M+H]^+$: 732.3776. Found:732.3774.

DFT calculations

The initial hairpin structure of peptide **5** was generated from the cyclohexyl moiety of the XRD structure and the stem of the hairpin structure with the unsubstituted cyclohexane moiety previously described.[17] The initial structures of peptide **4** with TBS groups were generated from the optimized XRD and hairpin structures of peptide **5**. All structures were optimized at the M06-2X/6-31G(d) level of theory, for which single-point energy calculations ($E_{0,dTZ}$) were calculated at the M06-2X/def2-TZVP level of theory and followed by solvation free energy (ΔG_{solv}) calculations at the PCM M06-2X/6-31G(d) level of theory in chloroform and acetonitrile. Each relative conformational energy (ΔE_s) in solution was calculated by the sum of ($\Delta E_{0,dTZ}$) and ($\Delta\Delta G_{solv}$).

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