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Article

Sculpting Secondary Structure of a Cyclic Peptide: Conformational Analysis of a Cyclic Hexapeptide Containing a Combination of L-Leu, D-Leu, and Aib Residues

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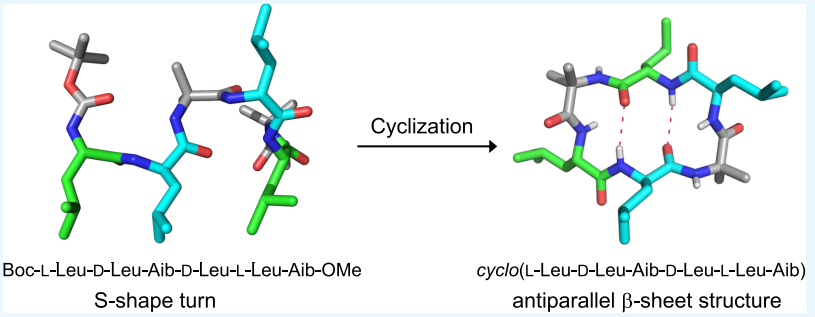

ABSTRACT: We have previously reported that *cyclo*(L-Leu-D-Leu-Aib-L-Leu-D-Leu-Aib) (**2**), a cyclic hexapeptide consisting of heterochiral L-Leu and D-Leu (L-Leu-D-Leu) residues with achiral 2-aminoisobutyric acid (Aib) residues, forms a figure-8 conformation. In this study, we newly designed *cyclo*(L-Leu-D-Leu-Aib-D-Leu-L-Leu-Aib)+ (**4**), an epimer of **2**, and examined the conformational differences between **2** and **4** by X-ray crystallographic analysis. Peptide **4** formed a planar cyclic conformation with an antiparallel *β*-sheet hydrogen-bonding pattern. This investigation demonstrates the potential to manipulate the molecular conformation of cyclic peptides by simply arranging the L- and D-amino acids and emphasizes that diverse conformations can be obtained by using cyclic peptides. Harnessing cyclic peptides as platforms for distinct molecular structures is a promising approach to expanding the chemical space for various applications.

INTRODUCTION

The rational design of peptides holds significant importance across diverse fields, such as organic chemistry, nanotechnology, and medicinal chemistry.[1−7] There often arises a necessity to fold these peptides into precise configurations. Therefore, a spectrum of strategies that modulate peptide conformations has been explored. These strategies typically encompass the incorporation of nonproteinogenic amino acids.[8−13] Such peptides containing nonproteinogenic amino acids adopt a clear and compact conformation commonly called a "foldamer."[14−17] In particular, drug discovery research has been energized by efforts employing these foldamers, and among them, those forming cyclic or helical structures are considered ideal candidates for advancing novel pharmaceutical agents.[18−21] Foldamers that simulate ring and helical structures are being used in the drug discovery field as inhibitors of protein−protein interactions and DDS carriers. In particular, small- to medium-sized cyclic peptides have been highlighted for the diverse attributes that arise from their distinct structures. These features include improved steric and chemical stability, increased affinity and specificity in binding to target proteins, and enhanced cell membrane permeability.[22−26] In peptide cyclization research, a common strategy involves amalgamating an L-amino acid and a D-amino acid (L-AA−D-AA) as templates.[27−29] A precedent study conducted by our group showcased the capacity of a cyclic hexapeptide *cyclo*(L-Leu-D-Leu-Aib-L-Leu-D-Leu-Aib) (**2**), which incorporates the achiral 2-aminoisobutyric acid (Aib)[30−33] into the L-Leu-D-Leu segment, to adopt a unique figure-8 configuration within its crystalline structure (Figure 1).[34] The distinct structure adopted by **2** is primarily driven by the robust bending-inducing propensity of the two Aib residues within its sequence. Additionally, a pivotal factor contributing to the emergence of the twisted configuration was the strategic incorporation of a rippled sheet motif, cleverly crafted from opposing L-Leu and D-Leu residues. Based on this previous result, in this study, we designed *cyclo*(L-Leu-D-Leu-Aib-D-Leu-L-Leu-Aib) (**4**), which is an epimer of peptide **2** (Figure 1). We

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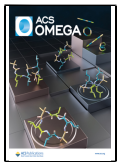


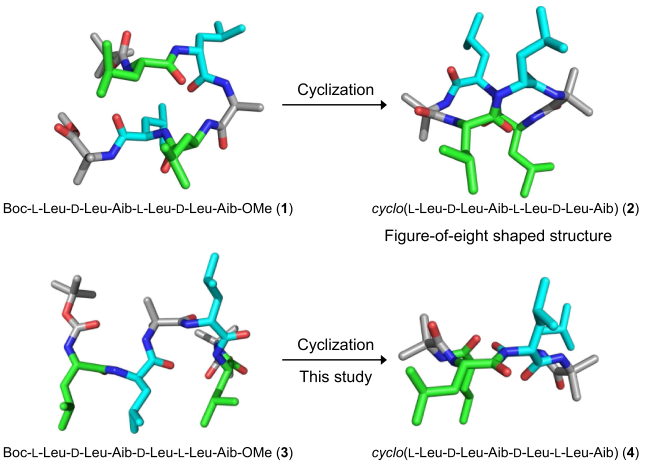


Figure 1. Chemical structures of linear hexapeptides **1** and **3** and cyclic hexapeptides **2** and **4**, with amino acids L-Leu (green), D-Leu (cyan), and Aib (gray) color-coded.

examined the conformational differences between the two structures by analyzing their respective crystal arrangements.

RESULTS AND DISCUSSION

The cyclic hexapeptide **4** was synthesized starting from the linear hexapeptide **3**[35] (Scheme 1). After deprotecting the *N*- and *C*-terminal protecting groups, intramolecular cyclization of these free terminals using EDC/HOBt gave the desired peptide **4** in 52% yield.

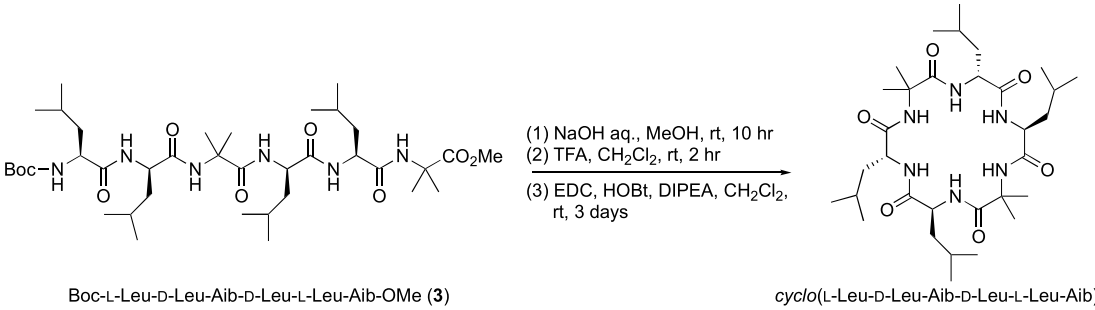
Cyclic hexapeptide **4** was dissolved in MeOH/H_2O (30/1). After the gradual evaporation of a mixture of MeOH and H_2O at room temperature, well-defined crystals suitable for X-ray crystallographic analysis were obtained. The structure of **4** was determined using the direct method with SHELXS-2014[36] and subsequently refined with SHELXL-2016/4.[37] Anisotropic thermal parameters were assigned to all non-hydrogen atoms, whereas some hydrogen atoms underwent isotropic refinement. The remaining hydrogen atoms were positioned based on calculated coordinates. The pertinent torsion angles for the backbone and side chains and the intramolecular and intermolecular hydrogen-bond parameters are presented in Tables 1 and 2.[38] The structure of **4** was solved in spacer group $P2_1/n$, and in the asymmetric unit, two crystallographically independent molecules **a** and **b** coexisted alongside methanol and water molecules (Figure 2a,2b). Molecules **a** and **b** exhibited nearly identical conformations, with minor variations observed in their side-chain conformations (Figure 2c,2d). Notably, the structure of **4** contrasts that of **2**, which adopts a twisted figure-8 configuration.[34] In their structures, β-turn

Table 1. Selected Torsion Angles (ω, ϕ, Ψ, and χ) [deg] for Peptide 4

residue	torsion angle ϕ	Ψ	ω	χ
		Molecule **a**		
L-Leu(1)	−120.47	21.28	179.48	−55.19
D-Leu(2)	122.93	165.47	164.84	64.21
Aib(3)	58.70	23.62	172.16	
D-Leu(4)	120.47	−21.28	−179.48	55.19
L-Leu(5)	−122.93	−165.47	−164.84	−64.21
Aib(6)	−58.70	−23.62	−172.16	
		Molecule **b**		
L-Leu(1)	−118.38	14.76	−178.75	−53.32
D-Leu(2)	123.53	168.01	165.93	65.60
Aib(3)	60.35	20.50	172.27	
D-Leu(4)	118.38	−14.76	178.75	53.32
L-Leu(5)	−123.53	−168.01	−165.93	−65.60
Aib(6)	−60.35	−20.50	−172.27	

structures were observed at the Aib(3) and Aib(6) residues, and antiparallel β-sheet structures were observed between L-Leu(1)-D-Leu(2) and D-Leu(4)-D-Leu(5) residues. Examination of the hydrogen-bonding interactions of the amide protons revealed the establishment of antiparallel β-sheet-type hydrogen bonds between the D-Leu and L-Leu residues. Thus, in both molecules **a** and **b**, two intramolecular hydrogen bonds were observed between H−N(5a) and C(2a)═O(2a) [N(5a)···O(2a) = 3.175 Å; ∠N−H···O = 165.01°] and H−N(2a) and C(5a)═O(5a) [N(2a)···O(5a) = 3.175 Å; ∠N−H···O = 165.01°], and between H−N(5b) and C(2b)═O(2b) [N(5b)···O(2b) = 3.152 Å; ∠N−H···O = 166.96°] and H−N(2b) and C(5b)═O(5b) [N(2b)···O(5b) = 3.152 Å; ∠N−H···O = 166.96°], respectively. Furthermore, interactions between other amide protons and solvent molecules (methanol and water) led to the formation of interparticle hydrogen bonds, ultimately contributing to an A-B-type packing arrangement (Figure 3). Examination of the amino acid side chains in **2** and **4** revealed distinct differences. The conformational disparity between cyclic peptides **2** and **4** may be attributed to the presence or absence of steric repulsion among these optically active amino acid residues that are oriented toward each other. In the case of cyclic peptide **2**, L-Leu(1) and L-Leu(4), as well as D-Leu(2) and D-Leu(5), share the same orientation, resulting in the formation of a "figure-eight" conformation. This conformation may have served to prevent these side chains from colliding with each other. Conversely, in cyclic peptide **4**, L-Leu(1) and D-Leu(4), as well as D-Leu(2) and L-Leu(5), assume opposing

Scheme 1. Synthesis of Cyclic Hexapeptide 4



Boc-L-Leu-D-Leu-Aib-D-Leu-L-Leu-Aib-OMe (**3**)

cyclo(L-Leu-D-Leu-Aib-D-Leu-L-Leu-Aib) (**4**)

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Table 2. Intra- and Intermolecular H-bond Parameters for Peptide 4[a]

donor D−H	acceptor A	distance D···A	angle [deg] D−H···A	symmetry operations
		Molecule **a**		
$O_{M'}$−H[b]	O_{1a}	2.692	170.51	x, y, z
$O_{W'}$−H[c]	O_{2a}	2.812	165.05	$-x, -y, -z$
N_{5a}−H	O_{2a}	3.175	165.01	$1 - x, -y, -z$
$O_{W''}$−H	O_{3a}	2.769	169.04	x, y, z
$O_{M'}$−H	O_{4a}	2.692	170.51	x, y, z
$O_{W'}$−H	O_{5a}	2.769	169.04	$-x, -y, -z$
N_{2a}−H	O_{5a}	3.175	165.01	$1 - x, -y, -z$
$O_{W''}$−H	O_{6a}	2.769	169.04	x, y, z
N_{4a}−H	$O_{W'}$	2.841	150.72	$1 + x, y, z$
N_{3a}−H	$O_{M''}$	2.978	139.72	$1/2 - x, -1/2 + y, 1/2 - z$
N_{1a}−H	$O_{W'}$	2.841	150.72	$1 + x, y, z$
N_{6a}−H	$O_{M''}$	2.978	139.72	$1/2 - x, -1/2 + y, 1/2 - z$
		Molecule **b**		
$O_{M''}$−H	O_{1b}	2.708	175.03	x, y, z
$O_{W''}$−H	O_{2b}	2.805	162.36	$-x, 1 - y, -z$
N_{5b}−H	O_{2b}	3.152	166.96	$-x, 1 - y, -z$
$O_{W'}$−H	O_{3b}	2.795	164.32	x, y, z
$O_{M''}$−H	O_{4b}	2.708	175.03	x, y, z
$O_{W''}$−H	O_{5b}	2.805	162.36	$-x, 1 - y, -z$
N_{2a}−H	O_{5b}	3.152	166.96	$-x, 1 - y, -z$
$O_{W'}$−H	O_{6b}	2.795	164.32	x, y, z
N_{4b}−H	$O_{W''}$	2.836	150.34	x, y, z
N_{3b}−H	$O_{M'}$	2.937	145.83	$1/2 - x, 1/2 + y, 1/2 - z$
N_{1b}−H	$O_{W''}$	2.836	150.34	x, y, z
N_{6b}−H	$O_{M'}$	2.937	145.83	$1/2 - x, 1/2 + y, 1/2 - z$

[a]The numbering of the amino acid residues begins at the *N*-terminus of the peptide. [b]O_M: MeOH molecule. [c]O_W: H_2O molecule.

orientations, thus eliminating steric repulsion between them. The result would have formed a conformation similar to that of conventional cyclic peptides composed of LD-peptides. This result indicates that cyclic peptide structures with tailored amino acid configurations can serve as basic templates that fill chemical spaces in the field of drug discovery.

Finally, MD simulations of peptide **4** were executed using the Molecular Operating Environment (MOE) program. The minimum energy conformation closely resembled those observed in the crystal, albeit with specific differences present in the side chains of each amino acid (Figure 4).

CONCLUSIONS

In this study, we newly designed the cyclic peptide *cyclo*(L-Leu-D-Leu-Aib-D-Leu-L-Leu-Aib) (**4**), an epimer of *cyclo*(L-Leu-D-Leu-Aib-L-Leu-D-Leu-Aib) (**2**), and analyzed the differences in conformation between the two structures in their crystalline states. As a result, peptide **4** adopted a flat cyclic structure with an antiparallel β-sheet hydrogen-bonding pattern. The insights gained from this comparative study demonstrate the potential to control the overall molecular conformation by manipulating the spatial arrangement of amino acids within cyclic peptide sequences. This highlights the versatility of cyclic peptides as a platform for designing molecules with unique conformations, thereby opening the possibility of creating molecules with various steric structures and properties. Overall, our research underscores the promising prospect that cyclic peptides serve as highly adaptable templates for designing molecules with distinct conformations. These peptides may expand the chemical space and provide an approach for finding valuable applications in various fields, including drug development, biomolecular control, and chemical biology research.

EXPERIMENTAL SECTION

Synthesis of Peptide 4. A solution of linear hexapeptide **3** (566 mg, 0.75 mmol) dissolved in 10 mL of MeOH was stirred at room temperature for 10 h after the addition of 1.5 mL (1.5 mmol) of 1 M NaOH aqueous solution. Subsequently, the solution was neutralized with 1 N aqueous HCl, and MeOH was then evaporated. The resulting aqueous solution was

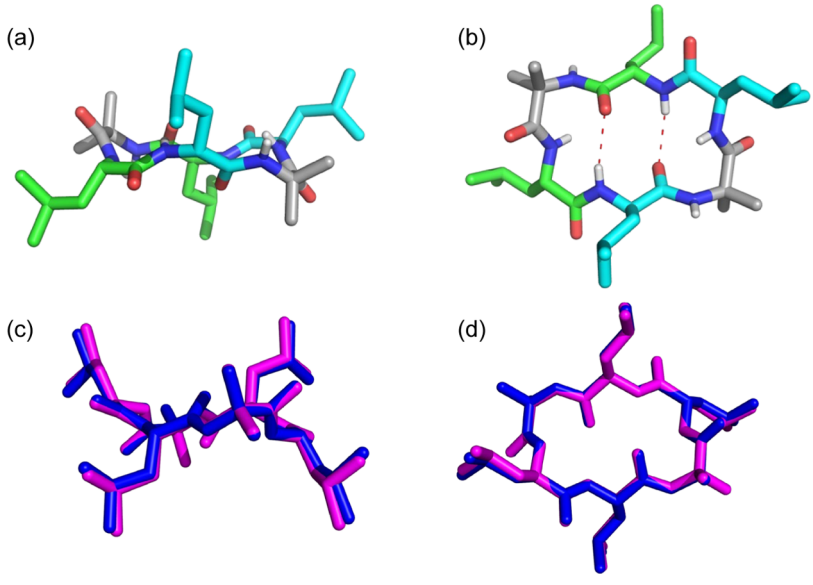

Figure 2. Structure of molecule **a** from (a) side and (b) overhead views. Hydrogen bonds are represented by red dashed lines. Amino acids L-Leu (green), D-Leu (cyan), and Aib (gray) are shown. The superimposed structures of molecules **a** (blue) and **b** (magenta) from (c) side and (d) overhead views. H atoms in parts (c) and (d) are not shown.

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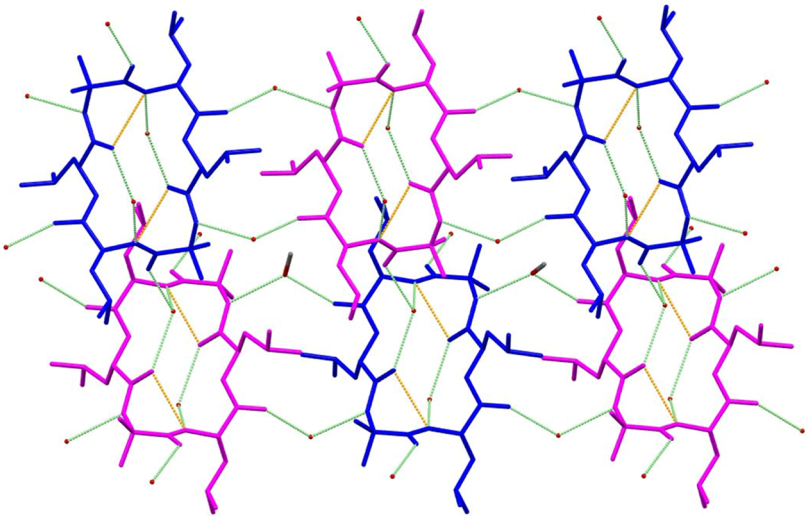

Figure 3. Packing of molecules **a** (blue) and **b** (magenta) in the crystalline state. Intramolecular (orange) and intermolecular (light green) hydrogen bonds are indicated as red dashed lines.

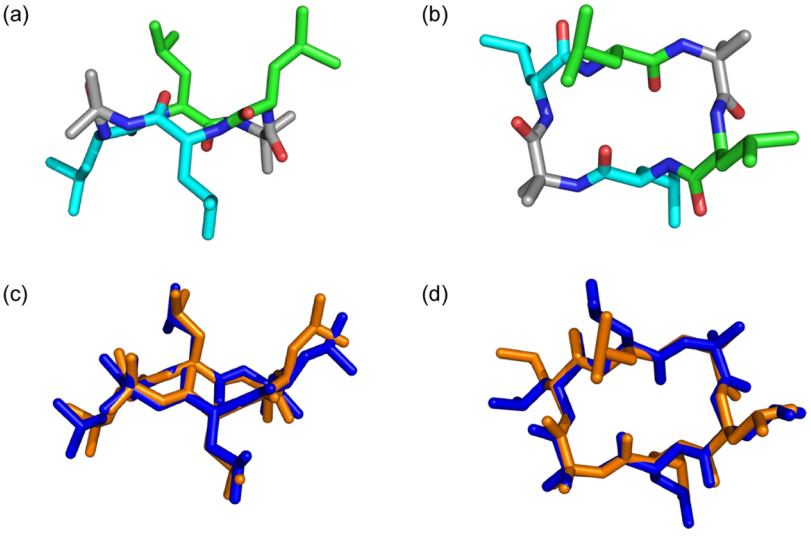

Figure 4. (a, b) Minimum energy conformation of peptide **4**, and (c, d) the superimposed structure of calculated (orange) and X-ray (blue) structures.

extracted with AcOEt and subsequently dried by using Na_2SO_4. After removing the solvent, hexapeptide-carboxylic acid (555 mg, 99%) was obtained as a solid. The solid was used for the subsequent reaction without further purification. To a solution of the acid in CH_2Cl_2 (5 mL) at 0 °C was added trifluoroacetic acid (1 mL). The mixture was stirred at room temperature for 2 h. After solvent removal, a crude N- and C-terminal free hexapeptide was obtained and utilized without additional purification. A mixture of EDC (173 mg, 0.9 mmol), HOBt (122 mg, 0.9 mmol), DIPEA (314 μL, 1.8 mmol), and the above N- and C-terminal free hexapeptide in CH_2Cl_2 (100 mL) was stirred at room temperature for 3 days. The resulting solution was sequentially washed with 3% aqueous HCl, 5% aqueous $NaHCO_3$, and brine. Afterward, the mixture was dried over Na_2SO_4. The solvent was removed, and the remaining residue was subjected to column chromatography on silica gel (*n*-hexane/AcOEt = 1:1). This purification step yielded cyclic hexapeptide **4** (242 mg, 52%).^{1}H NMR (600 MHz, $CDCl_3$) δ 8.72−7.29 (br, 4H), 6.56−5.92 (brs, 2H), 4.95−4.22 (brs, 2H), 4.22−3.51 (brs, 2H), 2.02−1.72 (br, 6H), 1.69−1.47 (m, 12H), 1.41 (s, 6H), 0.97 (d and t overlapped, J = 5.4 Hz, 12H), 0.93 (d, J = 6.6 Hz, 6H), 0.90 (d, J = 6.6 Hz). ^{13}C NMR (151 MHz, $CDCl_3$) δ: 174.8 (2C), 174.5 (2C), 173.9 (2C), 56.2 (4C), 40.3 (4C), 37.1 (2C), 25.1 (4C), 24.9 (4C), 23.4 (2C), 22.4 (4C), 21.6 (2C). HRMS (ESI): m/z calcd. for $C_{32}H_{59}N_6O_6$ $[M + H]^+$ 623.4491, found 623.4505.

Molecular Dynamics Simulations of Peptides. The MD simulations were performed by using MOE 2022.02 under the Amber10:EHT force field. The initial structure of peptide **4** was chosen based on its X-ray structure. Water molecules were added around the peptide, and the system was neutralized by adding 0.1 M NaCl salt. The simulation's temperature was regulated by NAMD. The initial energy minimization for each simulation was carried out by using the standard protocol in MOE 2022.02. A time step of 2 fs was consistently used in all simulations. The system was saved every 0.5 ps during the simulations. The simulation proceeded through several steps. An initial 10 ps time evolution occurred at 0 K for minimization. Subsequently, a 100 ps time evolution occurred, during which the temperature increased from 10 to 300 K

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(heating), followed by an equilibration step lasting 100 ps, with tethers ranging from 0.5 to 100 Å. Finally, a 100,000 ps (100 ns) production step was performed at a constant temperature of 300 K. Following the completion of MD simulations for all systems, the results were analyzed using MOE 2022.02.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c06397.

Crystal and experimental data for peptide **4** (Table S1); stereoview (ORTEP) of the asymmetric cell (Figure S1); stereoview (ORTEP) of the molecule A (Figure S2.); stereoview (ORTEP) of the molecule B (Figure S3); packing diagram (Figure S4); torsion angles (Table S2); and geometry of hydrogen bonds (Table S3) (PDF)

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Author Contributions

T.I., H.Y., T.K., and M.D. conducted the experiments and analyzed the results, while T.K. and Y.D. were responsible for the research design and paper writing. All authors contributed to result discussions and manuscript feedback.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

Aib, 2-aminoisobutyric acid; Leu, leucine; DDS, drug delivery system; AA, amino acids; Boc, *tert*-butoxycarbonyl; TFA, trifluoroacetic acid; EDC, 1-ethyl-(3-(dimethylamino)propyl)-carbodiimide; HOBt, 1-hydroxybenzotriazole; MeOH, methanol; MD, molecular dynamics; AcOEt, ethyl acetate; DIPEA, *N*,*N*-diisopropylethylamine; NMR, nuclear magnetic resonance; HRMS, high resolution mass spectrometry; ESI, electrospray ionization; EHT, extended Hückel theory; RMSD, root-mean-square deviation; MM, molecular mechanics; NAMD, Nanoscale Molecular Dynamics program

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(38) Crystal data for **4**: $C_{32}H_{58}N_6O_6{\cdot}2(CH_3OH){\cdot}2(H_2O)$; M_r = 722.96; monoclinic; $P2_1/n$, a = 12.930 Å, b = 15.229 Å, c = 21.348 Å; α = 90°, β = 103.354°, γ = 90°; V = 4089.91 Å^3; Z = 4; D_x = 1.174 g/cm^3; μ = 0.702 mm^{-1}; No. of observations ($I > 2\sigma(I)$) = 7707; No. of parameters = 479; R_1 = 0.0373, and R_w = 0.0990. CCDC 2288074 for **4** contains the supplementary crystallographic data for this paper. These data can be obtained free of charge at https://www.ccdc.cam.ac.uk/.

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