

Supplementary Information

Each side chain of cyclosporin A is not essential for high passive permeability across lipid bilayers

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Materials and methods

Abbreviation

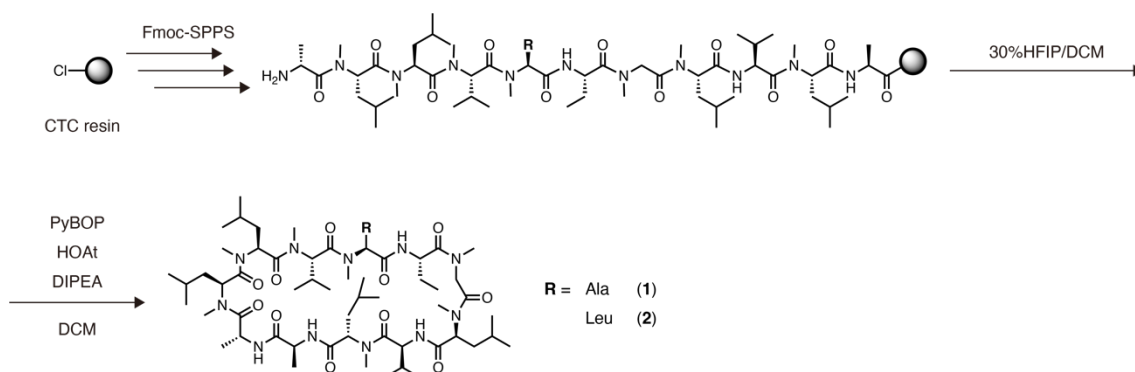
ACN, acetonitrile; COMU, ethyl 2-cyano-2-((dimethyliminio)(morpholino)methoxyimino)acetate hexafluorophosphate; CsA, cyclosporin A; CTC resin, 2-chlorotriyl chloride resin; DCM, dichloromethane; DIPEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; Fmoc, 9-fluorenylmethoxycarbonyl; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; HOAt, 3*H*-1,2,3-triazolo[4,5-*b*]pyridin-3-ol; Horizon-LBA, horizontal lipid bilayer permeability assay; HPLC, high performance liquid chromatography; HPTS, 8-hydroxypyrene-1,3,6-trisulfonic acid; LC/MS, liquid chromatography/mass spectrometry; MeOH, methanol; PBS, phosphate-buffered saline; PyBOP, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; TFA, trifluoroacetic acid; UPLC, ultra performance liquid chromatography.

General

Chemicals and solvents used in this study were purchased from commercial suppliers and used without further purification. CsA was purchased from Tokyo Chemical Industry Co., Ltd. Preparative HPLC was performed on a Prominence HPLC system (Shimadzu) with a 5C₁₈-AR-II column (Nacalai tesque, 10 mm I.D.×150 mm, 34350-41). All the HPLC was performed using two solvents (solvent A: H₂O containing 0.1% TFA; solvent B: acetonitrile containing 0.1% TFA). UPLC for purity check of peptides was performed on LC-2040C 3D Plus (Shimadzu) with Shim-pack Velox C₁₈ 1.8 mm Column (Shimadzu, 2.1 I.D. × 50 mm). LC-MS was performed on an ACQUITY UPLC H-Class/SQD2 (Waters) using ACQUITY UPLC[®] BEH C₁₈ 1.7 mm Column (Waters, 2.1 I.D. × 50 mm). In Horizon-LBA, the current was measured with a CEZ2400 amplifier (Nihon Kohden). The observation of lipid bilayers was performed with an IX-71 microscope (Olympus). Fluorescence was measured using a plate reader, Infinite M200 PRO (TECAN). NMR spectra were recorded using ECS-400 (JEOL).

Synthesis

Peptide 1 and 2

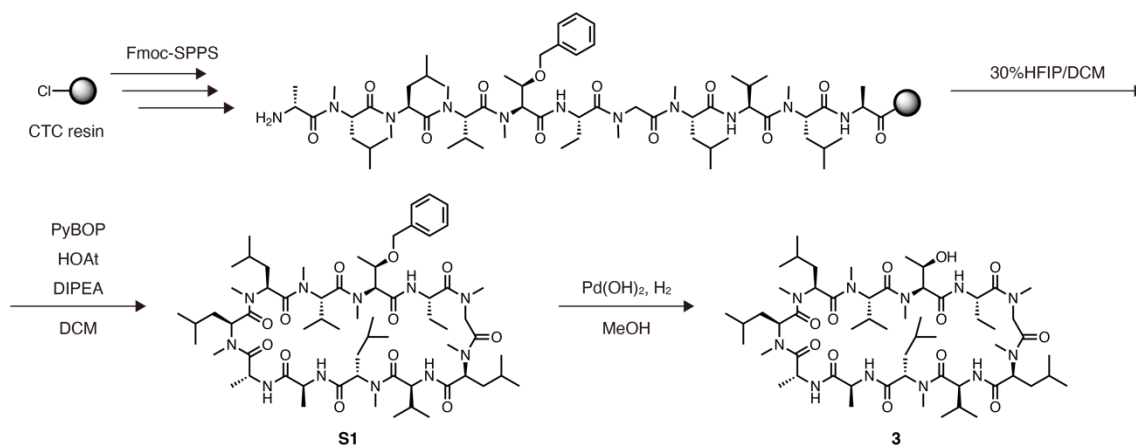


Scheme S1. The synthesis of **1** and **2**.

Peptide **1** and **2** were synthesized on CTC resin (1.32 mmol/g). Resin (300 mg, 0.40 mmol) was first swelled in DCM in a 12 mL fritted syringe with continuous shaking. DIPEA (269 μ L, 1.58 mmol, 4 equiv.) and Fmoc-L-Ala-OH (247 mg, 0.79 mmol, 2 equiv.) were dissolved in 4 mL anhydrous DCM, and the solution was applied to the resin. The resin was incubated for 2 h at room temperature with continuous shaking. After the reaction, the resin was washed with DCM, DCM/methanol/DIPEA = 17/2/1, and DCM three times each. The loading of the beads was quantified according to a previous report.¹ The loading percentage was determined as 78%. For each sequence, 77 mg of the resin (62 μ mol) was applied to further peptide synthesis. Fmoc deprotection was performed by incubating the resin with 20% piperidine in DMF twice (3 min and 12 min). After the reaction, the resin was washed with DMF three times. A coupling reaction of amino acids was performed using Fmoc-protected amino acid (4 equiv.), COMU (4 equiv.) and DIPEA (8 equiv.) in DMF (0.2 M for Fmoc-protected amino acid and COMU, and 0.4 M for DIPEA) for 1–2 h. In the coupling reaction of 8th L-MeVal, triple coupling at room temperature and, subsequently, a single coupling at 70 °C were conducted. In the reaction of 9th MeLeu and 10th MeLeu, a single coupling at 70 °C was conducted. In the coupling reaction of 11th D-Ala, double coupling at 70 °C was conducted. In the coupling reactions of the other amino acids, a single coupling at room temperature was conducted. After the coupling reactions, the resin was washed with DMF three times. The coupling and deprotection were repeated until the 11th residue. The synthesized peptides were cleaved from the resin by incubating the resin with 30% HFIP in DCM for 30 min three times. The resin was washed with DCM and MeOH. The filtrate was collected in a recovery flask. After all the filtrates were combined, the solution was evaporated under reduced pressure. To the residue, PyBOP (65 mg, 124 μ mol, 2 equiv.), HOAt (17 mg, 124 μ mol, 2 equiv.), DIPEA (105 μ L, 0.62 mmol, 10 equiv.) and DCM (31 mL) were added, and the reaction mixture was shaken for 2.5 h at room temperature. The solvent was removed under reduced pressure,

and the residue was purified by a reversed phase column on HPLC to give **1** (23 mg, 21 μ mol, 34%) and **2** (21 mg, 18 μ mol, 30%). The products were identified by LC-ESI-MS (Table S2).

Peptide 3



Scheme S2. The synthesis of **3**.

S1 was synthesized following the same synthetic procedure as **1**. To a recovery flask, **S1** (3.4 mg, 2.8 μ mol), palladium hydroxide on activated carbon (Pd 20%) (3.8 mg), and 6 mL MeOH were added.² The flask was charged with H₂ with a balloon, and the mixture was stirred for 8 h at room temperature. The reaction mixture was filtered through celite. The solvent was removed under reduced pressure to give **3** (7.8 mg, 6.9 μ mol, 11% from the 1st residue on the resin). The product was identified by LC-ESI-MS (Table S2).

Peptide 4–10

Peptides 4–10 (Table S1) were synthesized following the same synthetic procedure as 1. The yields were 10% (4), 17% (5), 12% (6), 11% (7), 24% (8), 14% (9), and 15% (10). The products were identified by LC-ESI-MS (Table S2).

Table S1. The sequence of peptide 4–10

Name	Residue No.										
	1	2	3	4	5	6	7	8	9	10	11
4	MeAla	Ala	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal
5	MeAla	Abu	Sar	MeAla	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal
6	MeAla	Abu	Sar	MeLeu	Ala	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal
7	MeAla	Abu	Sar	MeLeu	Val	MeAla	Ala	D-Ala	MeLeu	MeLeu	MeVal
8	MeAla	Abu	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeAla	MeLeu	MeVal
9	MeAla	Abu	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeAla	MeVal
10	MeAla	Abu	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeAla

Assay

General scheme of permeability measurements by Horizon-LBA

The lipid bilayer permeability of the peptides was measured by Horizon-LBA (horizontal lipid bilayer permeability assay) following the same scheme as our previous report.³ 5 μL of 10 mg/mL DOPC in decane was used as the lipid solution. The lower chamber was used as the donor side, and the upper chamber was used as the acceptor side in the permeability measurements. 1 mL of analytes in 1% DMSO/PBS and 180 μL of 1% DMSO/PBS were used as the donor and acceptor solutions, respectively.

A permeability coefficient P_{app} was calculated using the following equation:

$$P_{app} = \frac{Q}{A \times t \times C_0}$$

Where:

Q (mol) = the amount of analytes in the acceptor well measured in LC/MS

t (s) = the incubation time

C_0 (mol cm^{-3}) = the initial concentration of the analyte in the lower (donor) chamber

A (cm^2) = the hole area. A was calculated based on a picture taken at $t = 1$ min

Permeability measurement of CsA, 1, 2, and 3 by Horizon-LBA

The permeability of CsA, **1**, **2**, and **3** across the lipid bilayer was measured by Horizon-LBA. 6 μM CsA, 15 μM **1**, 10 μM **2**, 15 μM **3**, 10 μM Propranolol, and 10 μM HPTS in 1% DMSO/PBS was used as the donor solution. After the system was incubated for 120 min at room temperature, the acceptor solution was collected. The analytes in the acceptor well were quantified by LC/MS. The experiment was conducted in triplicate.

Permeability measurement of peptides 4–10 by Horizon-LBA

The permeability of **4–10** across the lipid bilayer was measured by Horizon-LBA. The permeability of each peptide was measured in three separate experiments using **1** as a reference compound (**1&4–6**, **1&7**, **1&8–10**). 10 μM peptides, 10 μM propranolol, and 50 μM HPTS in 1% DMSO/PBS was used as the donor solution. After the system was incubated for 1.5 h at room temperature, the acceptor solution was collected. The analytes in the acceptor well were quantified by LC/MS. The experiments were conducted in triplicate.

Measurement of amide temperature coefficient (ATC)

Chemical shifts of NH of the four amides in CsA and its derivatives **1–10** were measured at 25, 28, 31, 34, and 37 $^{\circ}\text{C}$ in chloroform-*d*. Based on the ^1H NMR spectra or COSY, $\Delta\delta/\Delta T$ ppb K^{-1} was calculated.⁴

Supporting figures & Tables

Table S2. Calculated and observed mass in LC-ESI-MS of cyclic peptides

Peptide	Formula	Calculated		Observed
		Exact MS	[M+H] ⁺	
1	C ₅₆ H ₁₀₁ N ₁₁ O ₁₁	1103.8	1104.8	1105.2
2	C ₅₉ H ₁₀₇ N ₁₁ O ₁₁	1145.8	1146.8	1147.4
3	C ₅₇ H ₁₀₃ N ₁₁ O ₁₂	1133.8	1134.8	1134.3
4	C ₅₅ H ₉₉ N ₁₁ O ₁₁	1089.8	1090.8	1091.1
5	C ₅₃ H ₉₅ N ₁₁ O ₁₁	1061.7	1062.7	1062.9
6	C ₅₄ H ₉₇ N ₁₁ O ₁₁	1075.7	1076.7	1076.9
7	C ₅₃ H ₉₅ N ₁₁ O ₁₁	1061.7	1062.7	1062.9
8	C ₅₃ H ₉₅ N ₁₁ O ₁₁	1061.7	1062.7	1063.0
9	C ₅₃ H ₉₅ N ₁₁ O ₁₁	1061.7	1062.7	1062.5
10	C ₅₄ H ₉₇ N ₁₁ O ₁₁	1075.7	1076.7	1076.7

Table S3. Calculated lipophilicity AlogP of cyclic peptides. AlogP was calculated by RDKit.⁵

Peptide	AlogP
CsA	3.27
1	2.33
2	3.35
3	1.69
4	1.94
5	1.3
6	1.69
7	1.3
8	1.3
9	1.3
10	1.69

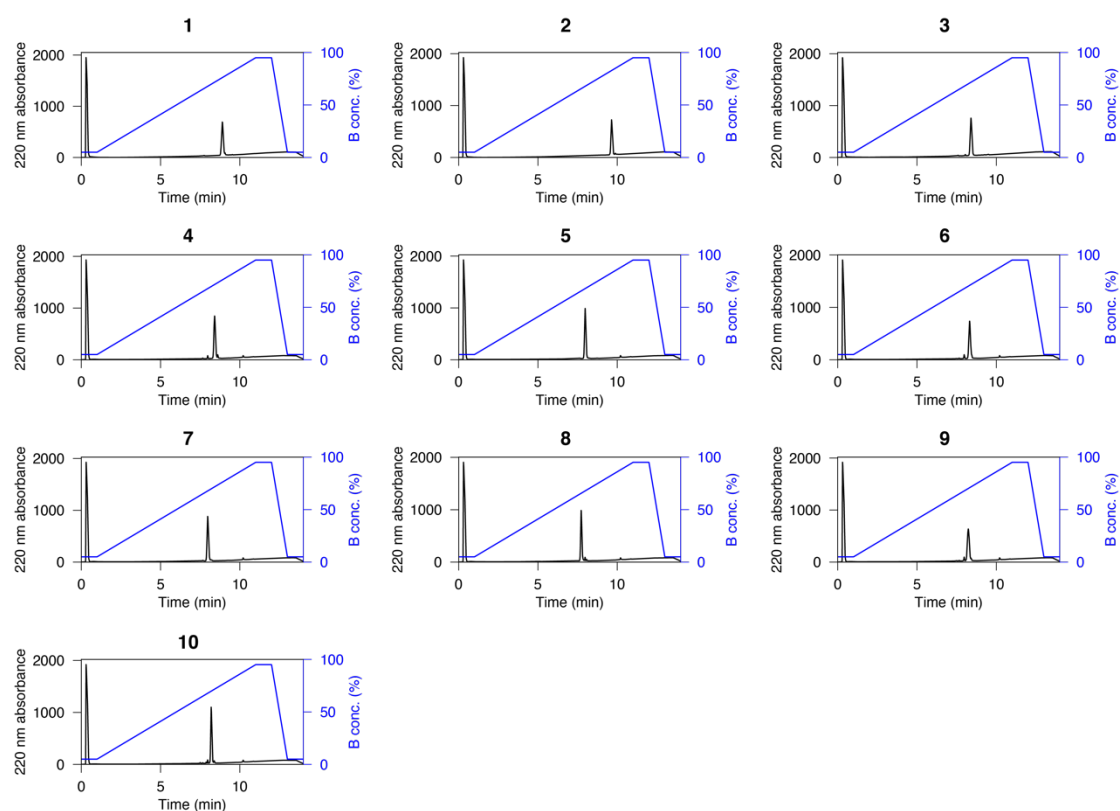


Fig. S1 UV chromatogram of all the peptides analyzed in this study. All the peptides after purification were analyzed using UPLC. The injected peptide solutions were prepared from DMSO stock solutions. The purity of each peptide was checked by running UPLC at a flow rate of 0.4 mL/min using mobile phase A (0.1% TFA in water) and B (0.1% TFA in acetonitrile) over 14 min gradient. The column temperature was set to 60 °C.

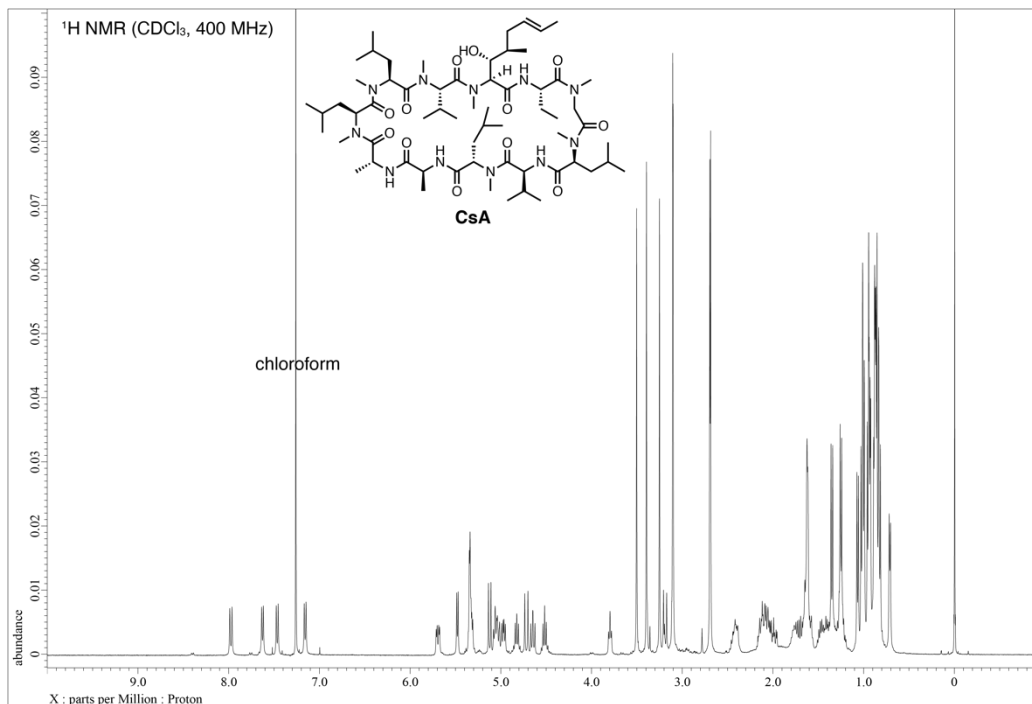


Fig. S2 ¹H NMR spectrum of CsA. Spectrum was recorded in CDCl₃ at 25 °C.

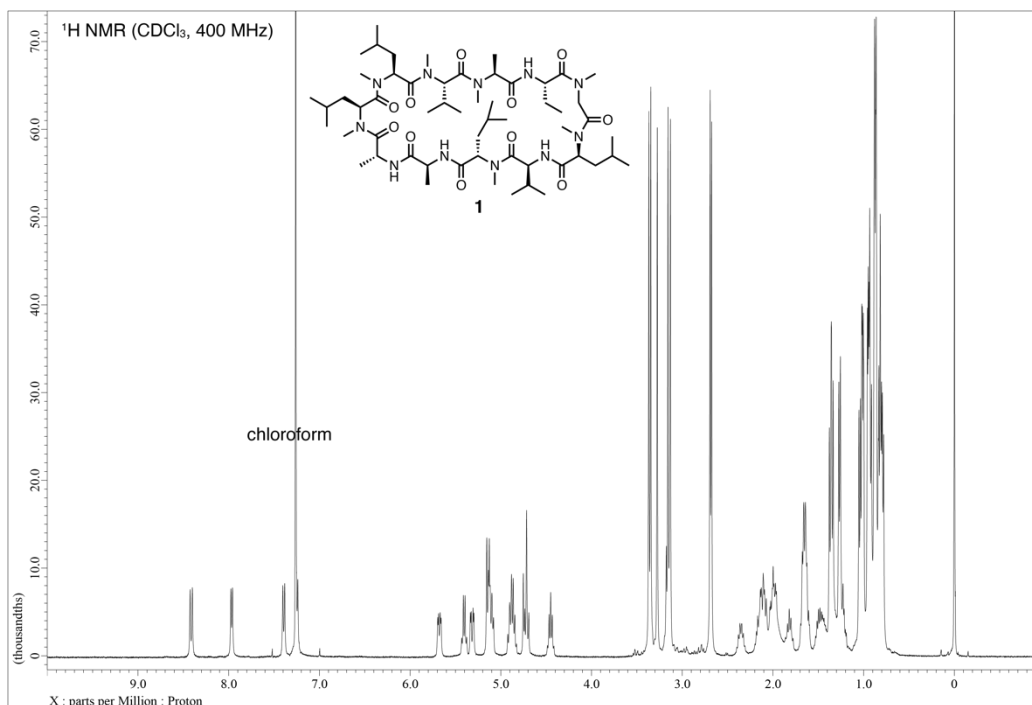


Fig. S3 ¹H NMR spectrum of 1. Spectrum was recorded in CDCl₃ at 25 °C.

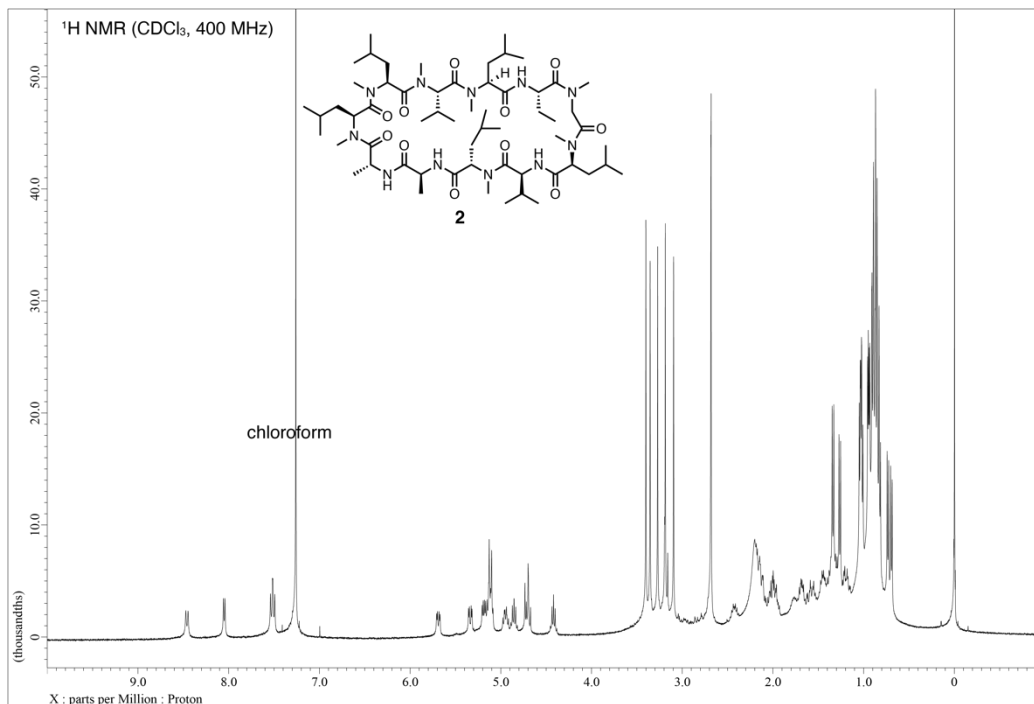


Fig. S4 ¹H NMR spectrum of **2**. Spectrum was recorded in CDCl₃ at 25 °C.

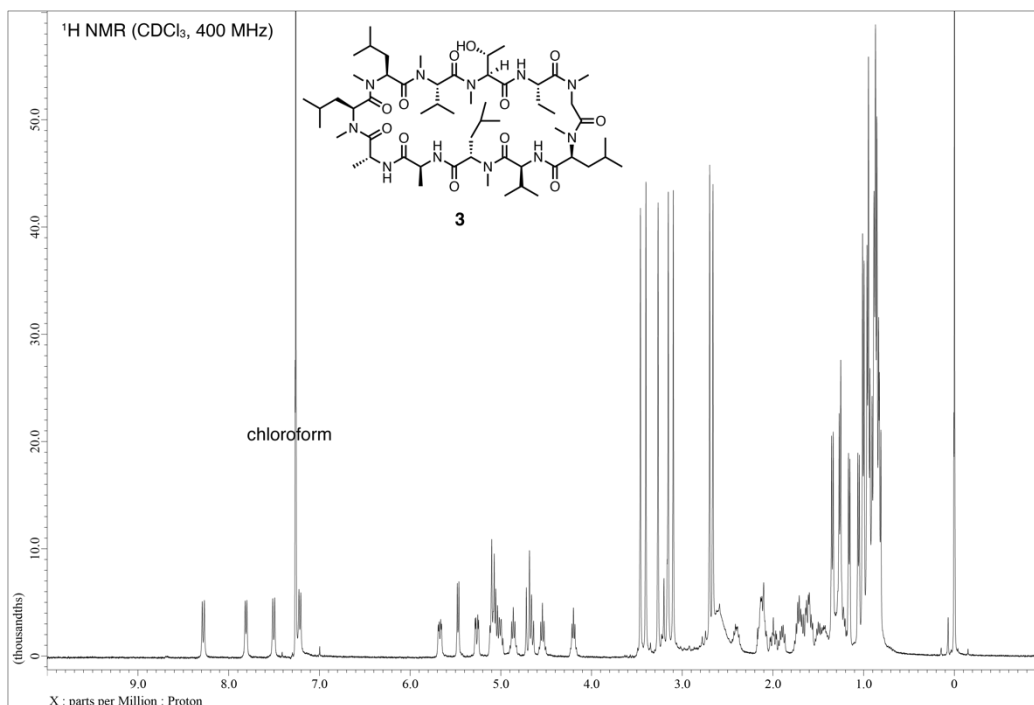


Fig. S5 ¹H NMR spectrum of **3**. Spectrum was recorded in CDCl₃ at 25 °C.

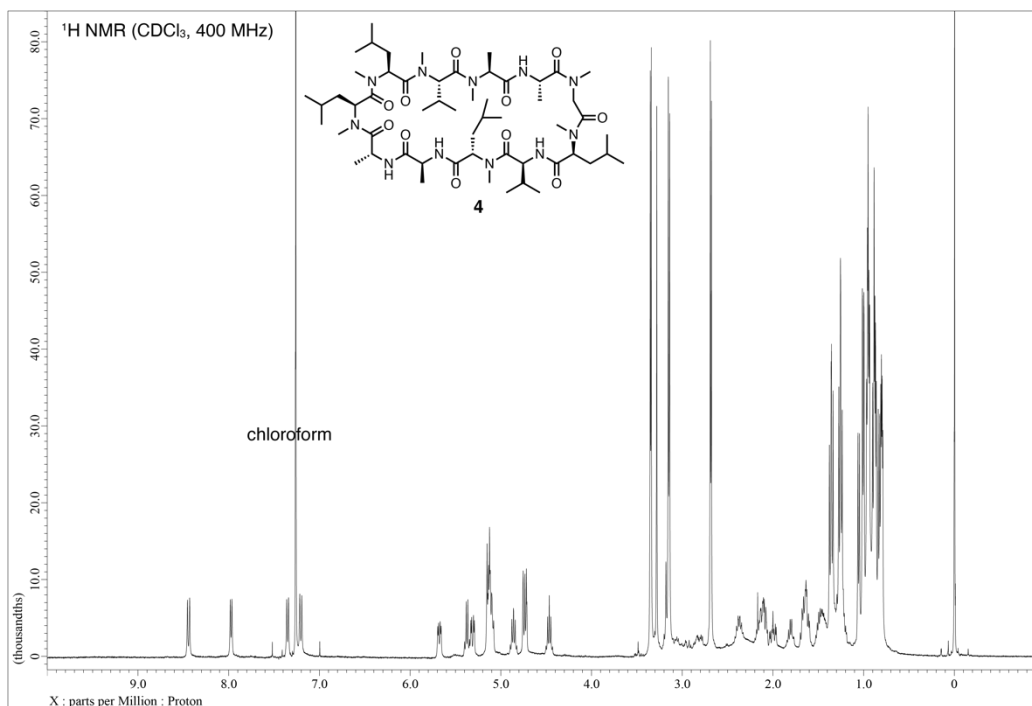


Fig. S6 ¹H NMR spectrum of **4**. Spectrum was recorded in CDCl₃ at 25 °C.

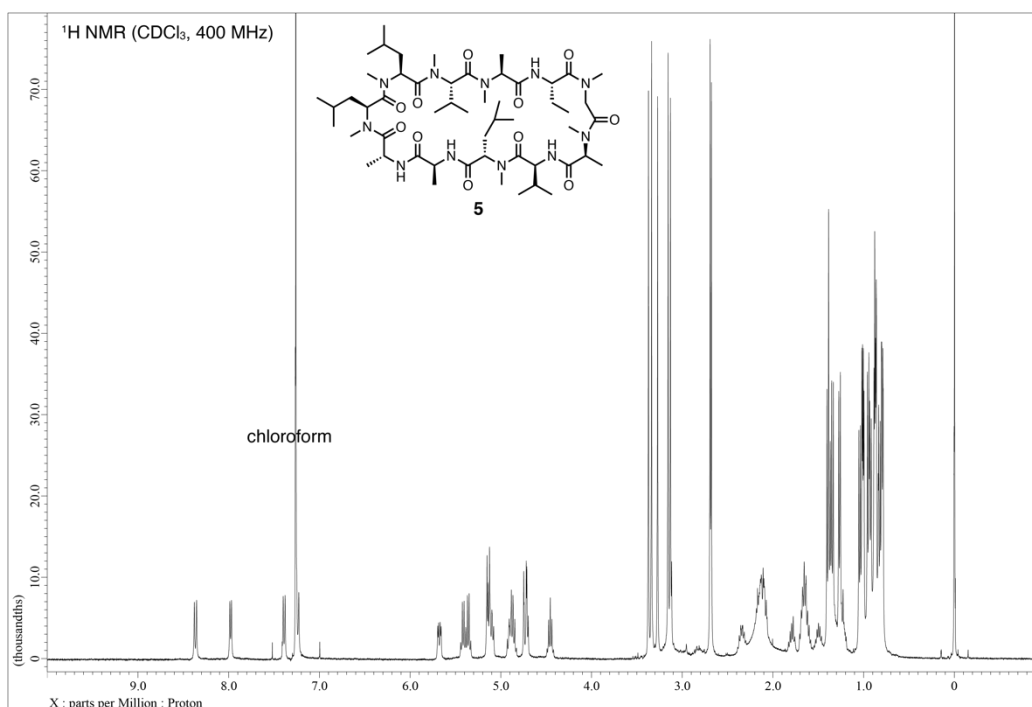


Fig. S7 ¹H NMR spectrum of **5**. Spectrum was recorded in CDCl₃ at 25 °C.

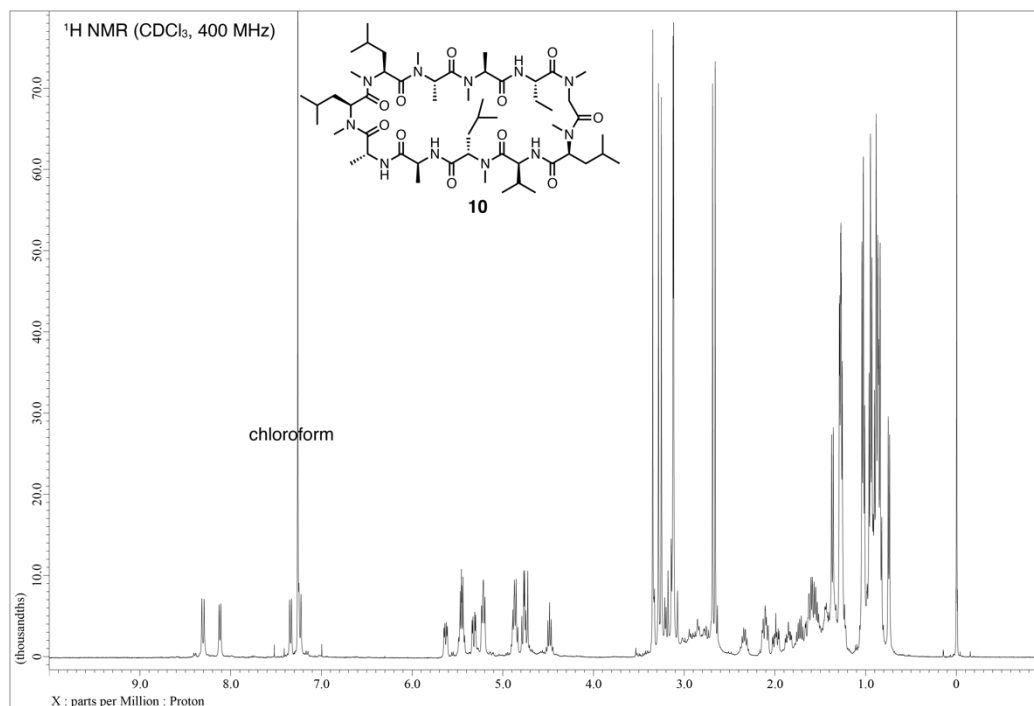


Fig. S12. ^1H NMR spectrum of **10**. Spectrum was recorded in CDCl_3 at 25 $^\circ\text{C}$.

Reference

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