Supporting Information

Synthesis of cyclic peptide-based [2]rotaxanes via copper-catalyzed azide–alkyne cycloaddition

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EXPERIMENTAL SECTION

Materials.

The chemical reagents were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), Watanabe Chemical Industries Ltd. (Hiroshima, Japan), and Fujifilm Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and used without further purification.

Characterization procedures.

^{1}H nuclear magnetic resonance (NMR) spectra were recorded with a Bruker Avance III 400 MHz spectrometer (Bruker BioSpin, Rheinstetten, Germany) at 25 °C. Chloroform-*d*, dichloromethane (DCM)-d_2, and methanol-d_4 were used as the NMR solvents. Electrospray ionization (ESI) mass spectrometry (MS) analysis was conducted with a Thermo Fisher Scientific Exactive Plus Orbitrap ESI mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra were obtained with an Autoflex-T2 instrument (Bruker Daltonics) in linear/positive mode with α-cyano-4-hydroxy cinnamic acid (α-CHCA) as the matrix. Reversed-phase high-performance liquid chromatography (HPLC) was performed using a Shimadzu LC–6AD liquid chromatography system equipped with an ultraviolet–visible (UV–vis) detector (220 nm, Shimadzu SPD–10AVvp) and an Inertsil WP300 C18 column (250 × 4.6 mm and 250 × 20 mm, GL Science Inc., Tokyo, Japan) or Cosmosil C18-MS packed column (75 × 4.6 mm, Nacalai Tesque, Inc., Kyoto, Japan).

Synthesis of (*R*)-2,2-dimethylthiazolidine-4-carboxylic acid (H-C($\Psi^{Me,Me}$Pro)-OH).

Cysteine hydrochloride (5.0 g, 31.7 mmol) was heated to reflux in dry acetone (300 mL) under argon for 12 h. The reaction mixture was concentrated to a small volume, and the residual slurry

was cooled at 4 °C for 30 min. The resulting crystalline solid was collected by filtration, washed with cold acetone (3 × 20 mL) and dried under reduced pressure to afford H-C($\Psi^{Me,Me}$Pro)-OH hydrochloride as colorless crystals. Yield: 5.5 g, 30.6 mmol (96.5%).

^{1}H NMR (400 MHz, $CDCl_3$, δ in ppm): 4.00 (t, J = 7.5 Hz, 1H), 3.35 (t, J = 8.4 Hz, 1H), 2.97 (t, J = 9.4 Hz, 1H), 1.60 (s, 3H), 1.44 (s, 3H). (Figure S8)

ESI-MS (m/z): [M+Na]+ calcd for $C_7H_{13}NO_2Na$, 166.0884; found, 166.0887.

Synthesis of Fmoc-Gly-C($\Psi^{Me,Me}$Pro)-OH.

$SOCl_2$ (4.6 g, 40.4 mmol) was added to a solution of Fmoc-Gly-OH (3.0 g, 10.1 mmol) in DCM (3 mL). The resulting mixture was stirred for 24 h and then concentrated in vacuo. The residue was dissolved in acetonitrile, and H-C($\Psi^{Me,Me}$Pro)-OH (1.51 g, 8.41 mmol) and *N,N*-diisopropylethylamine (4.39 mL, 25.2 mmol) were added. The resulting mixture was stirred for 12 h at 25 °C under an atmosphere of nitrogen. The reaction mixture was concentrated under reduced pressure to yield a yellow oil. This oil was taken up in ethyl acetate and washed sequentially with 5% aq. citric acid twice and brine, and then the organic phase dried over Na_2SO_4, filtered, and evaporated to dryness. The crude material was used without further purification.

Synthesis of the cyclic peptides.

Cyclo$(PG)_4$ and cyclo[$(PG)_3$PC(StBu)] were synthesized according to a previous study.[1] H-[GC($\Psi^{Me,Me}$Pro)$(GP)_3$]-OH was prepared via Fmoc solid-phase peptide synthesis on a Biotage Initiator+ Alstra fully automated microwave peptide synthesizer (Biotage, Sweden). The syntheses were carried out on H-Pro-Trt(2-Cl)-Resin (Watanabe Chemical Ind. Ltd.) on a 0.25 mol scale in a 10 mL reaction vial. The Fmoc group was deprotected at 25 °C in two stages, the first of which

used piperidine-dimethylformamide (DMF) (1:4) for 3 min, followed by piperidine-DMF (1:4) for 10 min. The linear peptide coupling reactions were performed via a 4 equiv. of amino acids, 4 equiv. of *N*,*N*′-diisopropylcarbodiimide (DIC), and 4 equiv. of ethyl cyano(hydroxyimino)acetate (Oxyma) in DMF for 5 min at 75 °C. After each coupling and deprotection step, washes with DMF (×5) were performed. After the synthesis was complete, the resin was washed with DCM (×4) and dried thoroughly. H-[GC($\Psi^{Me,Me}$Pro)$(GP)_3$]-OH was cleaved from the resin by treatment with 2 mL of 1% trifluoroacetic acid (TFA) in DCM (5 × 5 min). The reaction mixture was filtered to remove the resin, and the filtrate was concentrated under reduced pressure. The peptides were precipitated by adding cold diethyl ether to the residue, and the supernatant was decanted. The crude peptide was used without further purification. H-[GC($\Psi^{Me,Me}$Pro)$(GP)_3$]-OH (1 equiv.), (1-cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate (3 equiv.), and Oxyma (3 equiv.) were dissolved in dry DMF (0.5 mM). Diisopropylethylamine (5 equiv.) was then added at 0 °C, and the reaction mixture was stirred for 72 h at 25 °C under an argon atmosphere. The solvent was removed under reduced pressure, and the residue was washed with diethyl ether and purified by reversed-phase HPLC eluted with a linear gradient of CH_3CN/water containing 0.1% TFA. The synthesis of cyclo[GC($\Psi^{Me,Me}$Pro)$(GP)_3$] was confirmed by MALDI-TOF MS (Fig S7).

Synthesis of 5-hexyn-1-amine.

6-Chloro-1-hexyne (1.00 g, 8.58 mmol) and phthalimide potassium salt (1.91 g, 10.3 mmol) in DMF (20 mL) were stirred at 25 °C for 24 h. Diethyl ether was then added to the reaction mixture, and the organic layer was washed with water and brine, dried over Na_2SO_4, filtered, and evaporated to dryness. The residue was purified by column chromatography (silica gel, eluent: DCM) to obtain

a white solid. Hydrazine monohydrate (1.67 mL, 34.3 mmol) was added dropwise to a solution of this white solid (1.95 g, 8.58 mmol) in tetrahydrofuran (THF; 20 mL) at 25 °C. The reaction mixture was heated to reflux (70 °C) for 3 h and then allowed to cool at 25 °C, after which the precipitate was dissolved in water. The organic phase was separated, and the water phase was extracted twice with diethyl ether. The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and evaporated, affording 5-hexyn-1-amine as a transparent oil. Yield: 415 mg, 4.27 mmol (49.8%).

^{1}H NMR (400 MHz, $CDCl_3$, δ in ppm): 2.71 (m, 2H), 2.22 (td, J = 4.7 Hz, 2.1 Hz, 2H), 2.13 (br, 2H) 1,96 (t, J = 2.6 Hz, 1H), 1.57 (m, 4H). (Figure S9)

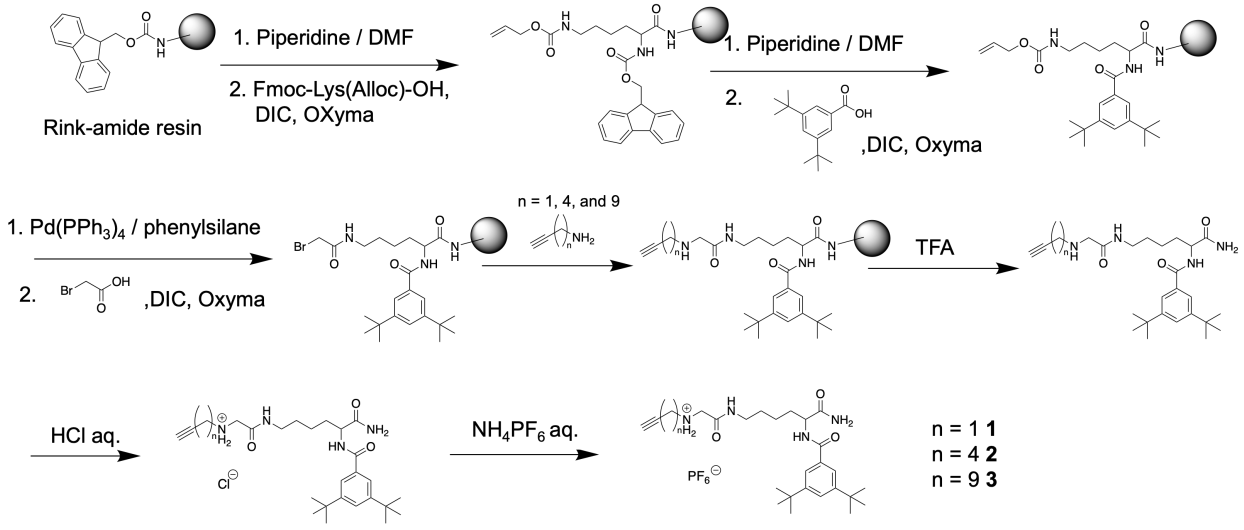
Synthesis of 10-undecane-1-amine.

10-Undecyn-1-ol (2.00 g, 11.9 mmol) and carbon tetrabromide (4.34 g, 13.1 mmol) in anhydrous DCM (10 mL) were stirred with ice bath for 10 min. Triphenylphosphine (3.42 g, 13.1 mmol) was added and the mixture was stirred at 25 °C for 1 h and then concentrated in vacuo. The residue was purified by column chromatography (silica gel, eluent: $CHCl_3$) to obtain 11-Bromo-1-undecyne. 11-Bromo-1-undecyne. (2.75 g, 11.9 mmol) and phthalimide potassium salt (2.64 g, 14.3 mmol) in DMF (30 mL) were stirred at 25 °C for 24 h. Diethyl ether was then added to the reaction mixture, and the organic layer was washed with water and brine, dried over Na_2SO_4, filtered, and evaporated to dryness. The residue was purified by column chromatography (silica gel, eluent: DCM) to obtain a white solid. Hydrazine monohydrate (1.97 mL, 40.5 mmol) was added dropwise to a solution of this white solid (3.00 g, 10.1 mmol) in THF (30 mL) at 25 °C. The reaction mixture was heated to reflux (70 °C) for 3 h and then allowed to cool at 25 °C, after which the precipitate was dissolved in water. The organic phase was separated, and the water phase was

extracted twice with diethyl ether. The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and evaporated, affording 10-undecane-1-amine as a transparent oil. Yield: 690 mg, 4.12 mmol (40.9%).

^{1}H NMR (400 MHz, $CDCl_3$, δ in ppm): 1.21-1.56 (m, 14H), 1.93 (t, J = 2.6 Hz, 1H), 2.16-2.20 (m, 2H) 2.68 (t, J = 7.0 Hz, 2H). (Figure S10)

Synthesis of monocationic ammonium threads 1, 2, and 3.



Scheme S1. Synthesis of monocationic ammonium threads 1, 2, and 3.

A total of 0.25 mol of Fmoc-protected Rink amide resin (100–200 mesh, Watanabe Chemical Ind. Ltd.) was weighed in a 10 mL reaction vial, the Fmoc group was removed with 20% piperidine/DMF (1 × 3 min and 1 × 10 min) at 25 °C, and then the resin was washed with DMF (×4). Then, a solution of Fmoc-Lys(Alloc)-OH (4 equiv.), DIC, and Oxyma in DMF (3 mL) was added. Aminolysis was performed for 5 min at 75 °C on a Biotage Initiator+ Alstra fully automated microwave peptide synthesizer. The Alloc group was removed with a solution of $PhSiH_3$ (20

equiv.) and $Pd(PPh_3)_4$ (0.3 equiv.) in 6 mL of DCM, and the resulting mixture was stirred for 30 min at 25 °C. The peptide resin was subsequently washed with DCM (×4) and DMF (×4). Then, 2.0 M bromoacetic acid, 2.0 M DIC and Oxyma in dry DMF (2.5 mL) were added, and the aminolysis reaction was performed for 5 min at 75 °C on a Biotage Initiator+ Alstra fully automated microwave peptide synthesizer. The resin was subsequently washed with DMF (×4). Then, a solution of amine (1.0 M) in dry DMF (2 mL) was added, and amination was performed for 5 min at 75 °C on a Biotage Initiator+ Alstra fully automated microwave peptide synthesizer. The resin was washed with DMF (×4) and DCM (×4). The desired peptide was cleaved from the resin by treatment with a cleavage cocktail containing TFA (5.94 mL), triisopropylsilane (156 μL), and water (156 μL) at 25 °C for 1.5 h. The reaction mixture was filtered to remove the resin, and the filtrate was concentrated under reduced pressure. The peptides were precipitated by adding cold diethyl ether to the residue, and the supernatant was decanted. The crude peptides were dried and purified via reversed-phase HPLC eluted with a linear gradient of CH_3CN/water containing 0.1% TFA. The obtained compound was dissolved in the least amount of methanol possible, and a large amount of 1 M hydrochloric acid was added. The formed precipitates were collected by filtration and dried in vacuo. Then, saturated aq. ammonium hexafluorophosphate was poured into a solution of the precipitate (dissolved in the least amount of methanol possible) until precipitate formed. The precipitate was collected by filtration, washed with water, and dried in vacuo to afford **1**, **2**, or **3** as a white solid.

1: ^{1}H NMR (400 MHz, MeOH-d_4, δ in ppm): 7.73 (d, J = 1.8 Hz, 2H), 7.65 (t, J = 1.8 Hz, 1H), 4.60-4.56 (m, 1H), 3.89 (d, J = 4.1 Hz, 2H), 3.79 (s, 2H), 3.17 (t, J = 2.5 Hz, 2H), 1.99-1.92 (m, 2H), 1.88-1.79 (m, 2H), 1.61 (quin, J = 7.3 Hz, 2H) 1.52-1.45 (m, 2H), 1.36 (s, 18H). (Figure S11)

ESI-MS (m/z): [M+Na]+ calcd for $C_{26}H_{40}N_4O_3Na$, 479.3001; found, 479.2998.

2: ^{1}H NMR (400 MHz, DCM-d_2, δ in ppm): 8.26 (br, 2H) 7.64 (t, J = 1.6 Hz, 1H), 7.56 (d, J = 1.7 Hz, 2H), 7.20 (d, J = 7.8 Hz, 1H), 6.85 (t, J = 5.9 Hz, 1H), 6.51 (s, 1H), 5.85 (s, 1H), 4.60 (t, J = 5.7 Hz, 1H), 4.10, 4.06, 3.87, 3.83 (s, 2H), 3.49-3.41 (m, 2H), 3.29-3.06 (m, 2H), 2.12 (td, J = 2.5 Hz, J = 6.9 Hz, 2H), 1.92-1.84 (m, 2H), 1.63-1.48 (m, 8H), 1.33 (s, 18H). (Figure S12)

ESI-MS (m/z): [M+Na]+ calcd for $C_{29}H_{46}N_4O_3Na$, 521.3471; found, 521.3468.

3: ^{1}H NMR (400 MHz, MeOH-d_4, δ in ppm): 7.78 (d, J = 1.6 Hz, 2H), 7.65 (s, 1H), 4.60-4.55 (m, 1H), 3.73 (s, 2H), 3.28-3.24 (m, 2H), 2.99 (t, J = 7.9 Hz, 2H), 2.19-2.13 (m, 3H), 2.01-1.78 (m, 2H), 1.72-1.30 (m, 18H), 1.37 (s, 18H). (Figure S13)

ESI-MS (m/z): $[M+Na]^+$ calcd for $C_{34}H_{56}N_4O_3Na$, 591.4245; found, 591.4244

Synthesis of *N*-(3-azidopropyl)-3,5-di-*tert*-butylbenzamide (5).

3-Azido-1-propanamine (200 mg, 0.91 mmol), 3,5-di-*tert*-butylbenzoic acid (191 mg, 1.09 mmol), 1-hydroxybenzotriazole monohydrate (184 mg, 1.37 mmol), and triethylamine (380 μL, 2.73 mmol) were dissolved in chloroform (10 mL). Then, a solution of 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride salt (261 mg, 2.73 mmol) in chloroform (2.0 mL) was added dropwise to the above mixture at 0 °C under nitrogen. The resulting mixture was stirred at 0 °C for 30 min and at 25 °C for 24 h. The mixture was subsequently diluted with chloroform and washed three times with 4 wt% $KHSO_4$ (aq.) and saturated $NaHCO_3$ (aq.). The organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography (silica gel, eluent: chloroform) to obtain a white solid. Yield: 220 mg, 0.583 mmol (57%).

^{1}H NMR (400 MHz, $CDCl_3$, δ in ppm): 7.58 (s, 3H), 6.34 (s, 1H), 3.57 (q, J = 3.1 Hz, 2H), 3.47 (t, J = 6.5 Hz, 2H), 1.94 (quin, J = 6.5 Hz, 2H), 1.34 (s, 18H). (Figure S14)

ESI-MS (m/z): [M+Na]+ calcd for $C_{18}H_{28}N_4ONa$, 339.2167; found, 339.2161.

Spectroscopic microscopy.

Circular dichroism (CD) spectra were recorded at 25 °C using a JASCO J-1500 CD spectrometer (JASCO, Tokyo, Japan). Peptide solutions at a concentration of 1.25×10^{-4} M were placed in an optical cell with a 1 mm path length for analysis.

Molecular dynamics (MD) simulations.

C($\Psi^{Me,Me}$Pro) was constructed in PyMOL, energy minimization and geometry optimization were performed with quantum mechanics with the Hartree–Fock/6-31G* basis set, and the restraint electrostatic potential (RESP) charges were calculated via the Hartree–Fock/6-31G* basis set with Gaussian 16.[2] The Antechamber module in AMBER18[3] was used with the RESP fit of the calculated potentials to generate amber files. The tLeap module in AMBER18 was used to construct the starting structures of cyclo[GC($\Psi^{Me,Me}$Pro)$(GP)_3$] using the AMBER ff14SB force field.[4] The system was subsequently minimized and equilibrated to 298.15 K, first by heating from 0 K to the desired temperature with constant volume for 100 ps, followed by 300 ps of simulation at a constant pressure of 1 bar to achieve the correct density. The resulting equilibrated structures were then independently heated to 44 different temperatures ranging from 298.15 to 798.15 K for 500 ps to generate initial replica exchange molecular dynamics (REMD) replicas. REMD simulations were performed in the NVT ensemble using a Langevin thermostat for temperature coupling with a collision frequency of 1 ps^{-1}. Using these equilibrated replicas, 200 ns of REMD

simulations were performed on each replica, resulting in 8.8 μs of MD simulation data. Exchanges between neighboring replicas were allowed every 2 ps in the NVT ensemble. Analysis of the simulations was carried out with CPPTRAJ analysis simulation software, which includes the amber tools.[5]

Principal component analysis (PCA).

PCA was performed on the MD trajectories of cyclo[GC($\Psi^{Me,Me}$Pro)$(PG)_3$]. The variance–covariance matrix was calculated from the C_α and N atoms and the C atom of the carbonyl coordinates of the MD trajectories between 10 and 200 ns after their superimposition on their average coordinates. The matrix was then diagonalized to obtain the principal component eigenvectors. Each structure in each MD trajectory was projected onto the space defined by the first and second principal component eigenvectors to graphically display the conformational distribution.

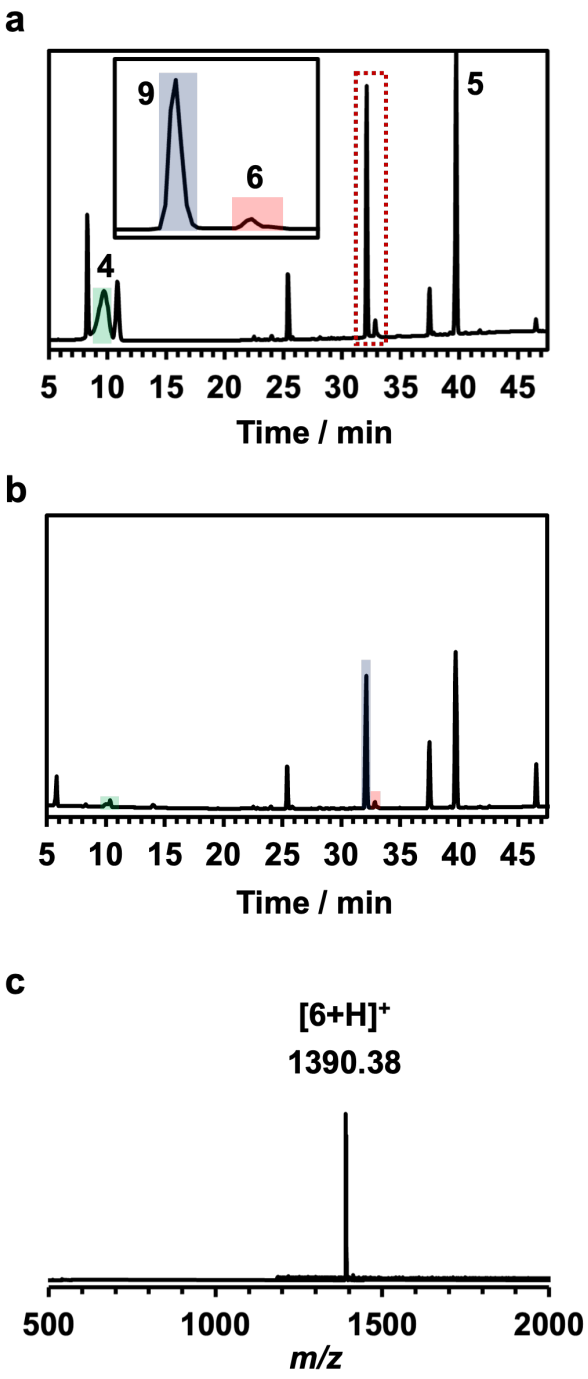


Figure S1. (a, b) HPLC chromatogram of the crude reaction mixture of entry 1 detected at (a) 220 and (b) 280 nm. Red peak: rotaxane; blue peak: free thread; and green peak: free cyclic peptide. (c) MALDI-TOF MS spectrum of the fraction containing the red peak from the chromatogram in (a).

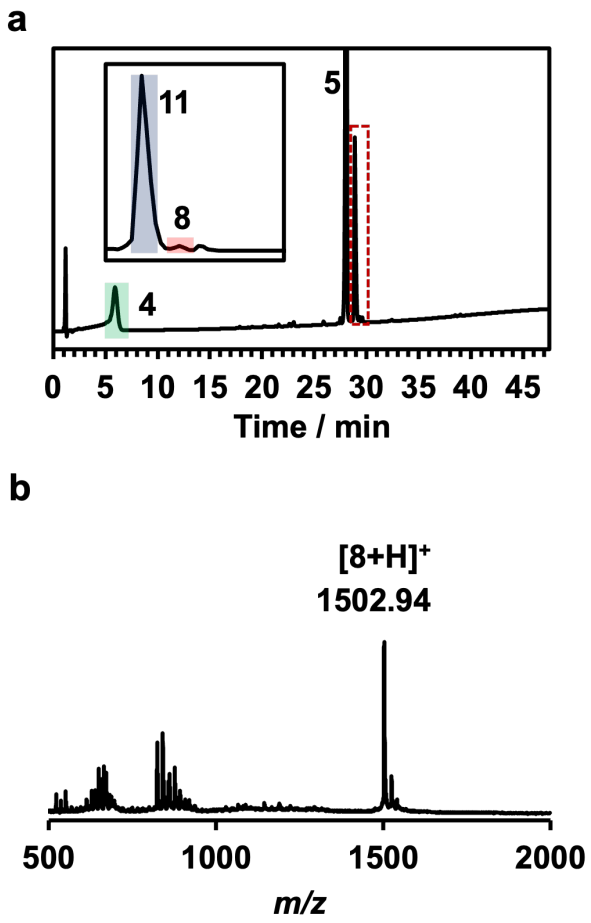


Figure S2. (a) HPLC chromatogram of the crude reaction mixture of entry 3 detected at 220 nm. Cosmosil C18-MS packed column was used. Red peak: rotaxane; blue peak: free thread; and green peak: free cyclic peptide. (b) MALDI-TOF MS spectrum of the fraction containing the red peak from the chromatogram in (a).

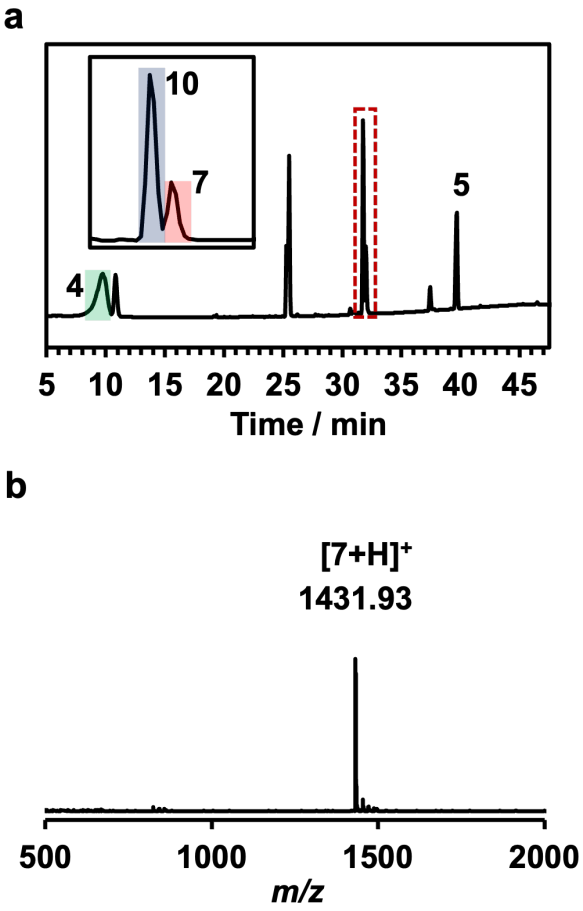


Figure S3. (a) HPLC chromatogram of the crude reaction mixture of entry 4 detected at 220 nm. Red peak: rotaxane; blue peak: free thread; and green peak: free cyclic peptide. (b) MALDI-TOF MS spectrum of the fraction containing the red peak from the chromatogram in (a).

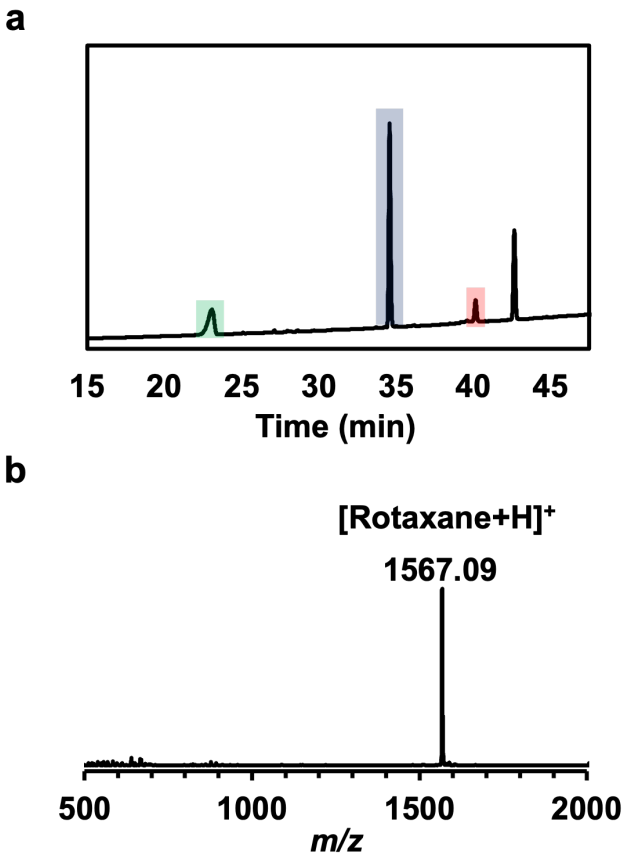


Figure S4. (a) HPLC chromatogram of the crude reaction mixture of entry 5 detected at 220 nm. Red peak: rotaxane; blue peak: free thread; and green peak: free cyclic peptide. (b) MALDI-TOF MS spectrum of the fraction containing the red peak from the chromatogram in (a).

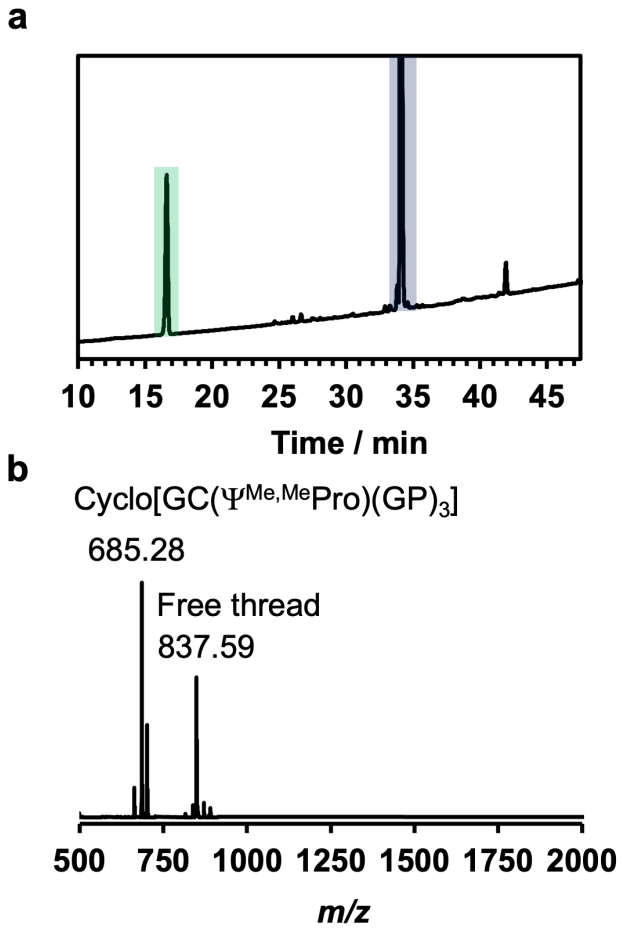


Figure S5. (a) HPLC chromatogram of a crude reaction mixture of entry 6 detected at 220 nm. Blue peak: free thread; and green peak: free cyclic peptide. (b) MALDI-TOF MS spectrum of the crude reaction mixture of entry 4.

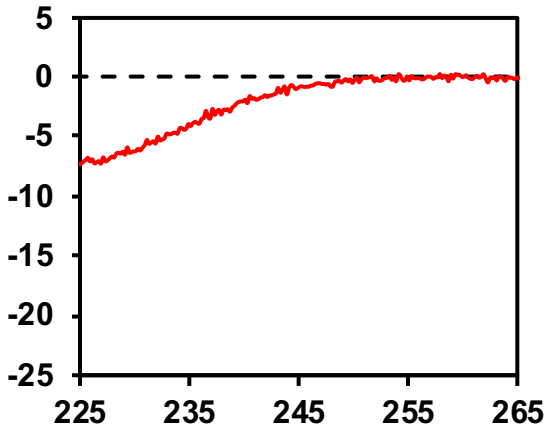


Figure S6. CD spectrum of 0.10 mM cyclo[GC($\Psi^{Me,Me}$Pro)$(GP)_3$] in DCM.

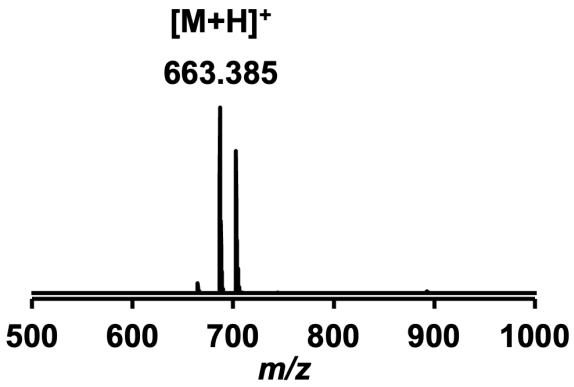


Figure S7. MALDI-TOF MS spectrum of cyclo[GC($\Psi^{Me,Me}$Pro)$(GP)_3$].

NMR spectra of new compounds.

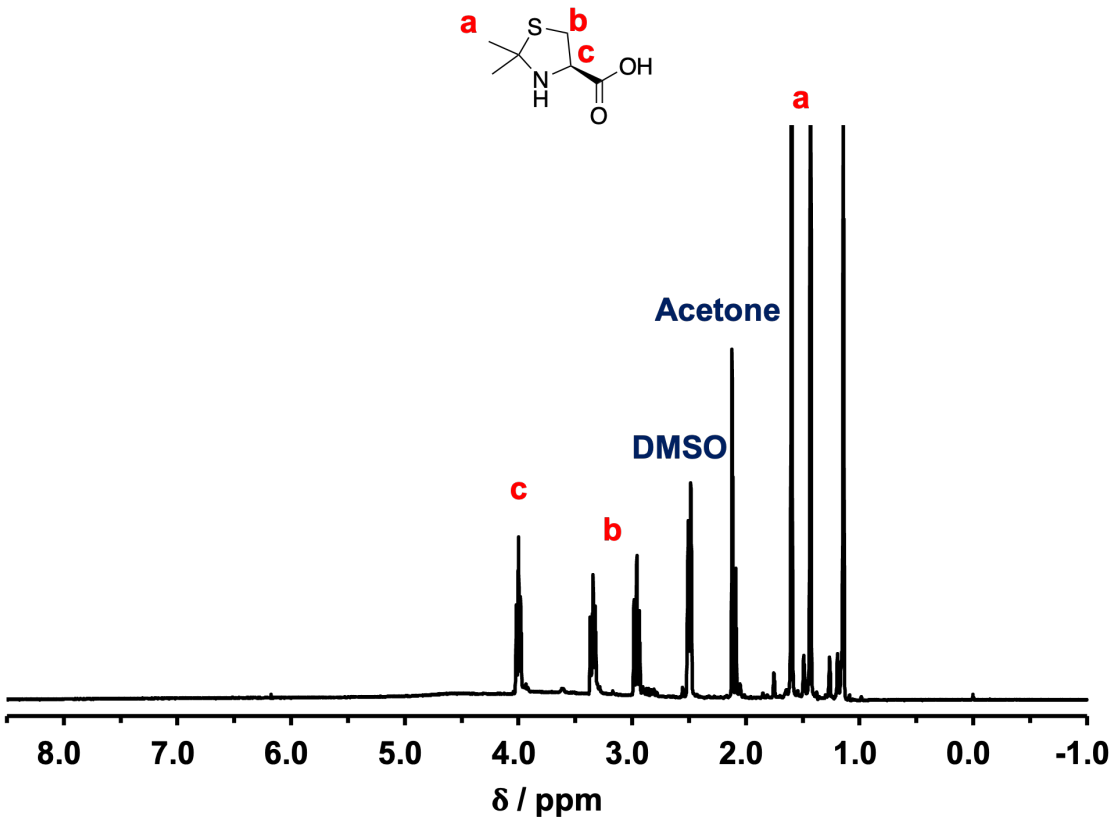


Figure S8. ^{1}H NMR spectrum of (*R*)-2,2-dimethylthiazolidine-4-carboxylic acid.

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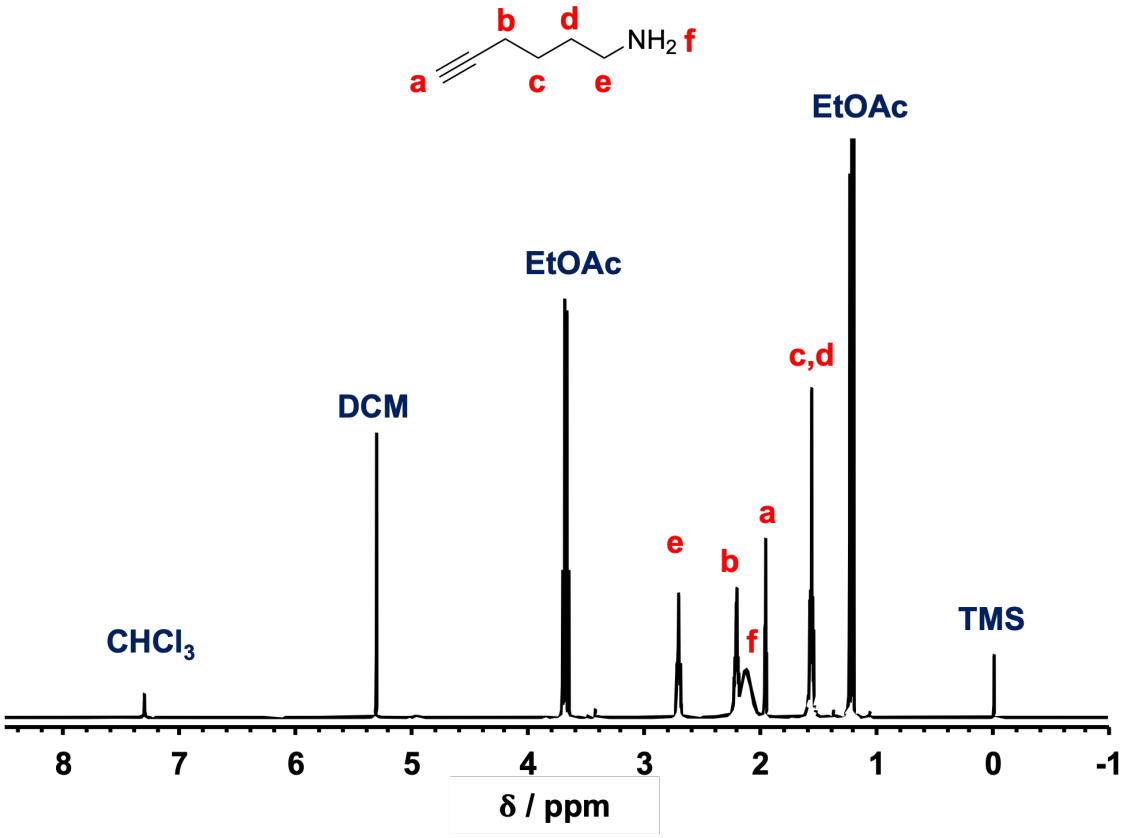

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3 **Figure S9.** ^{1}H NMR spectrum of 5-hexyn-1-amine.

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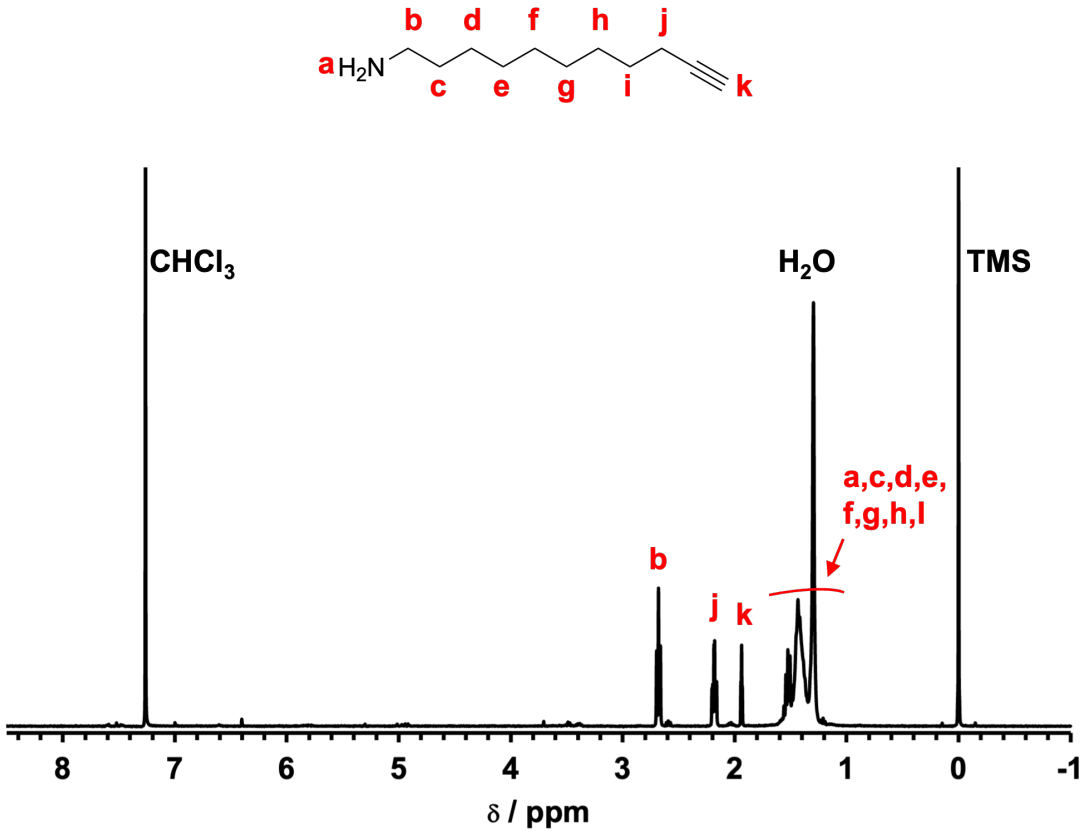

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3 **Figure S10.** ^{1}H NMR spectrum of 10-undecane-1-amine.

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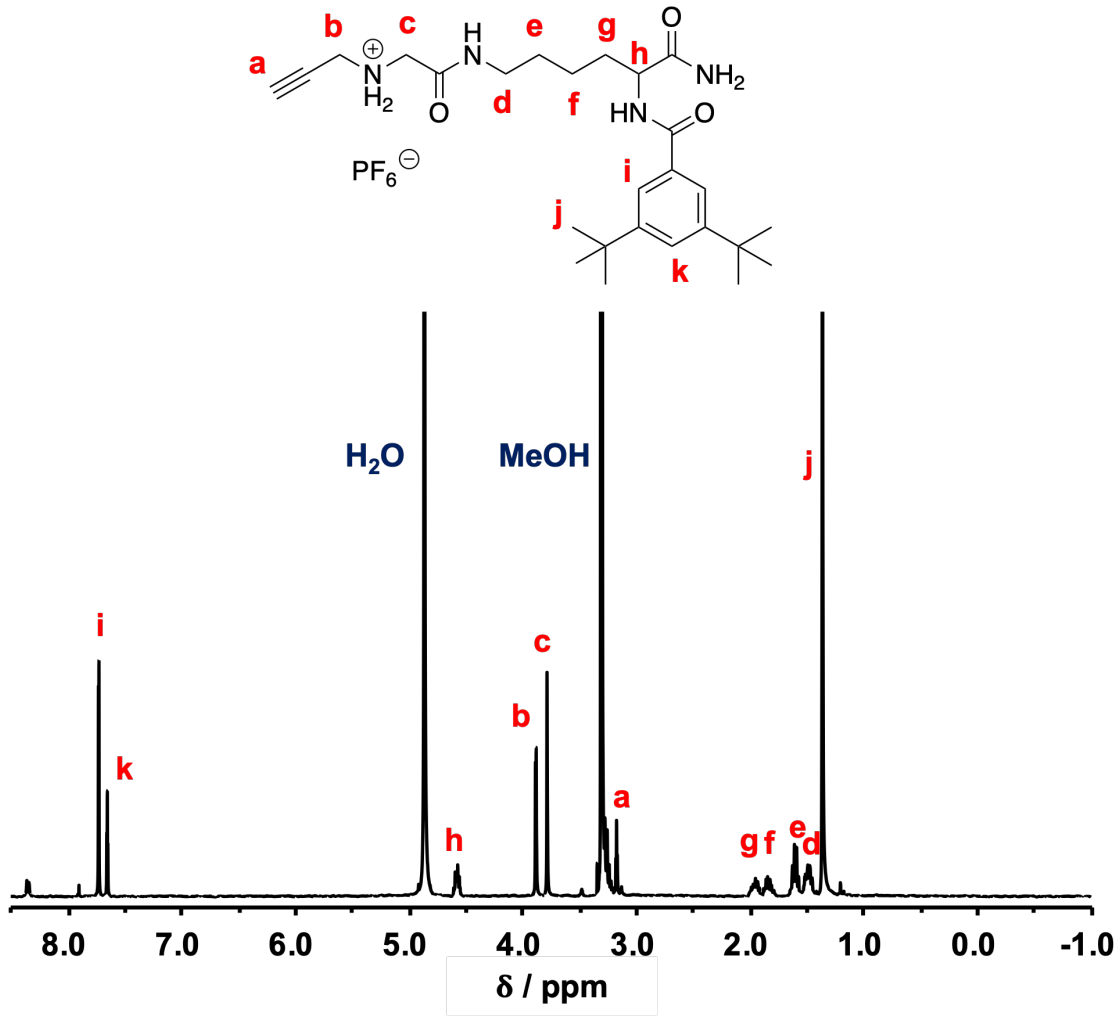

Figure S11. ^{1}H NMR spectrum of monocationic ammonium thread **1**.

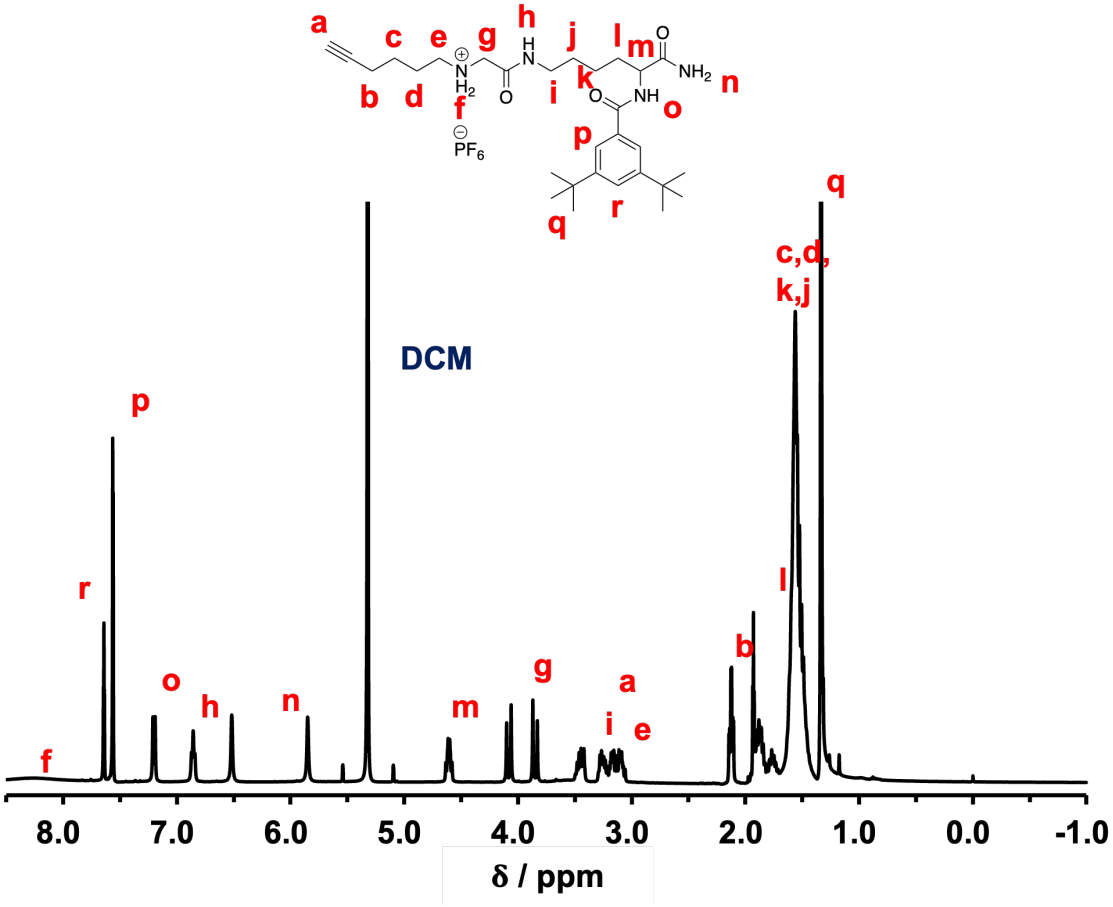

Figure S12. ^{1}H NMR spectrum of monocationic ammonium thread **2**.

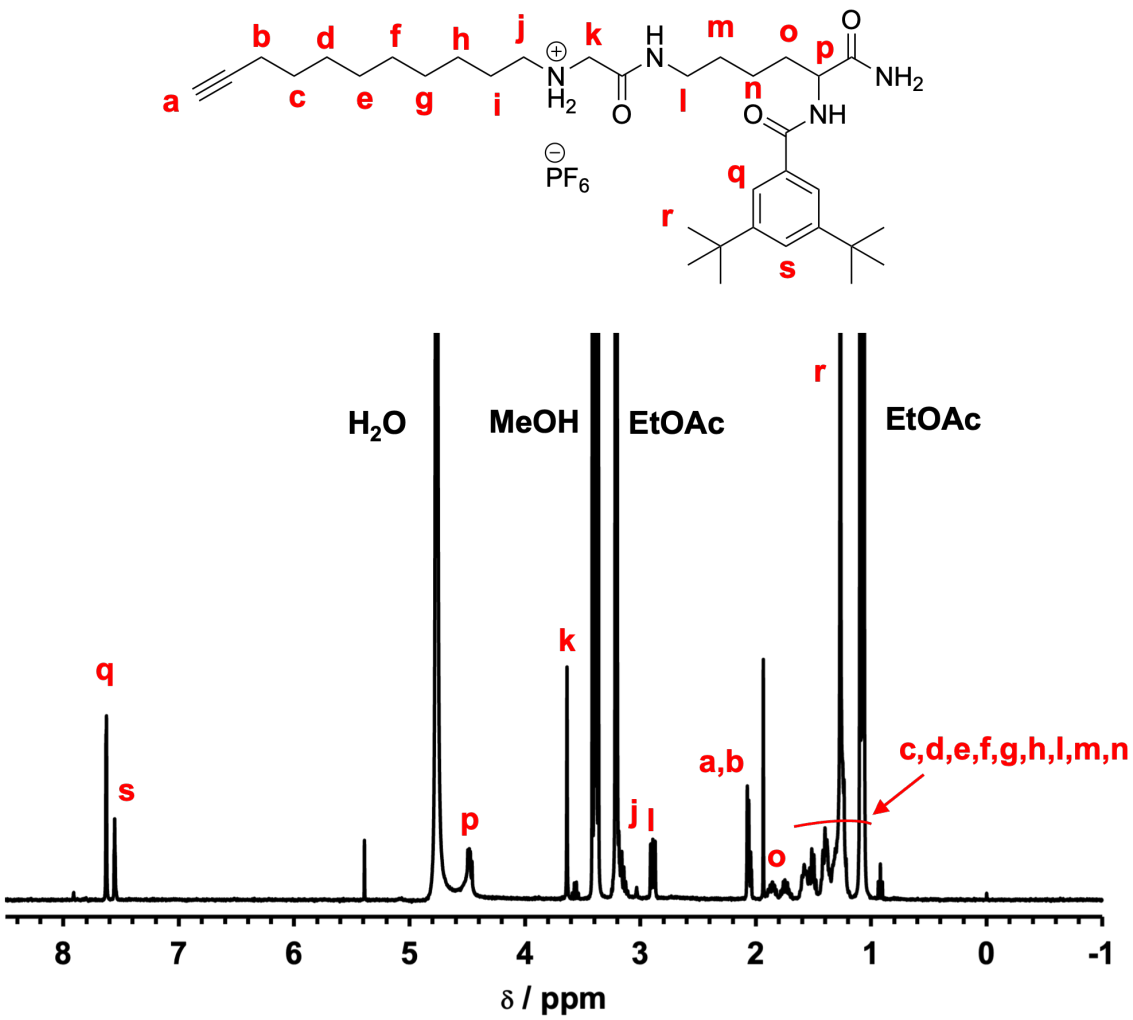

Figure S13. ^{1}H NMR spectrum of monocationic ammonium thread **3**.

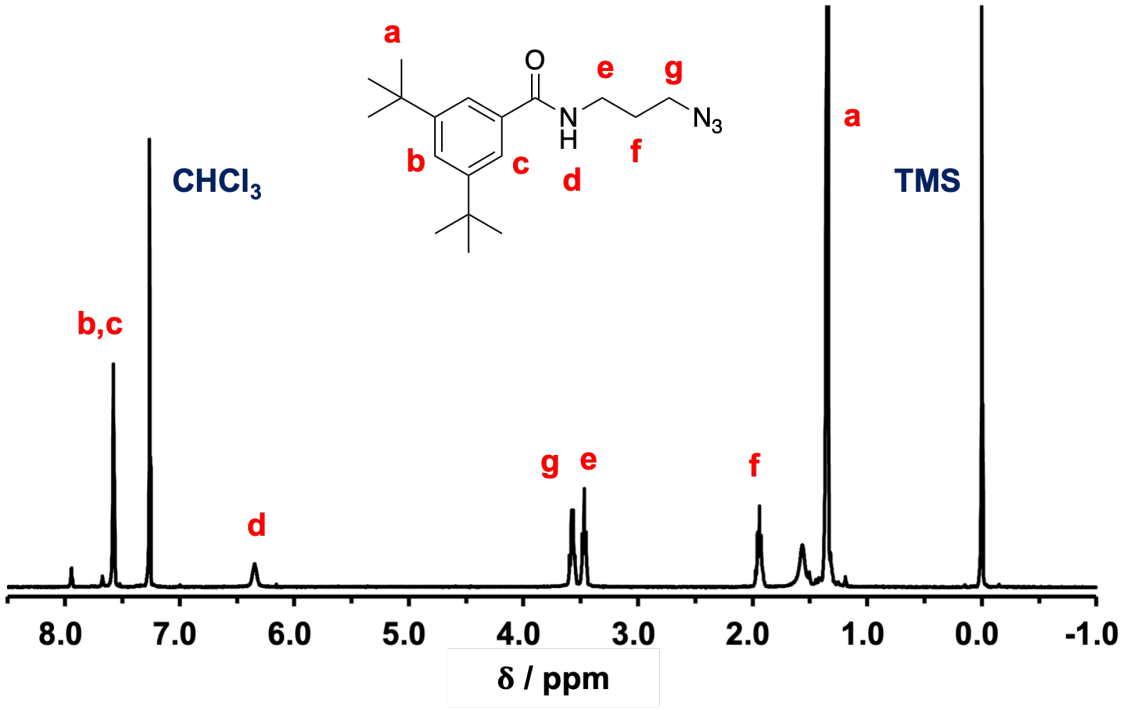

Figure S14. ^{1}H NMR spectrum of *N*-(3-azidopropyl)-3,5-di-*tert*-butylbenzamide.

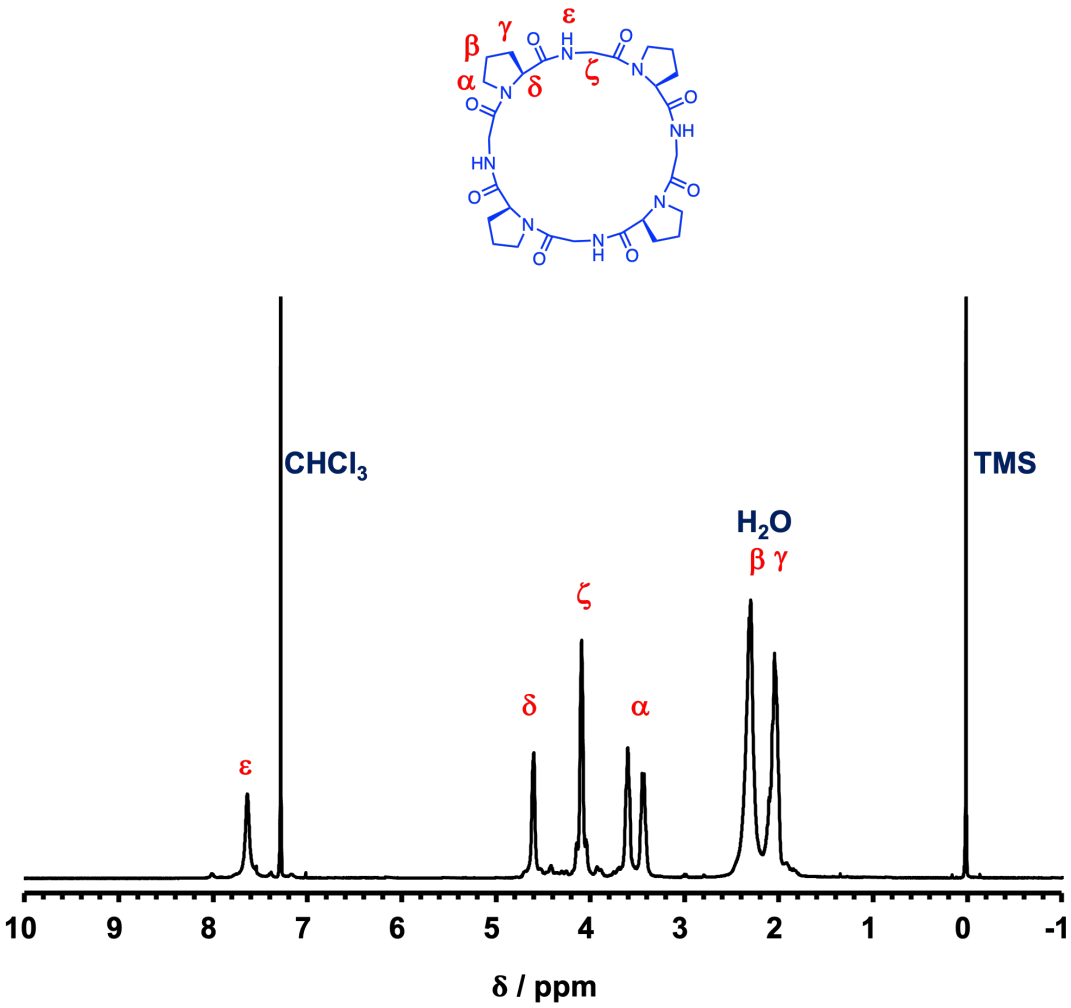

Figure S15. ^{1}H NMR spectrum of cyclo(PG)$_4$.

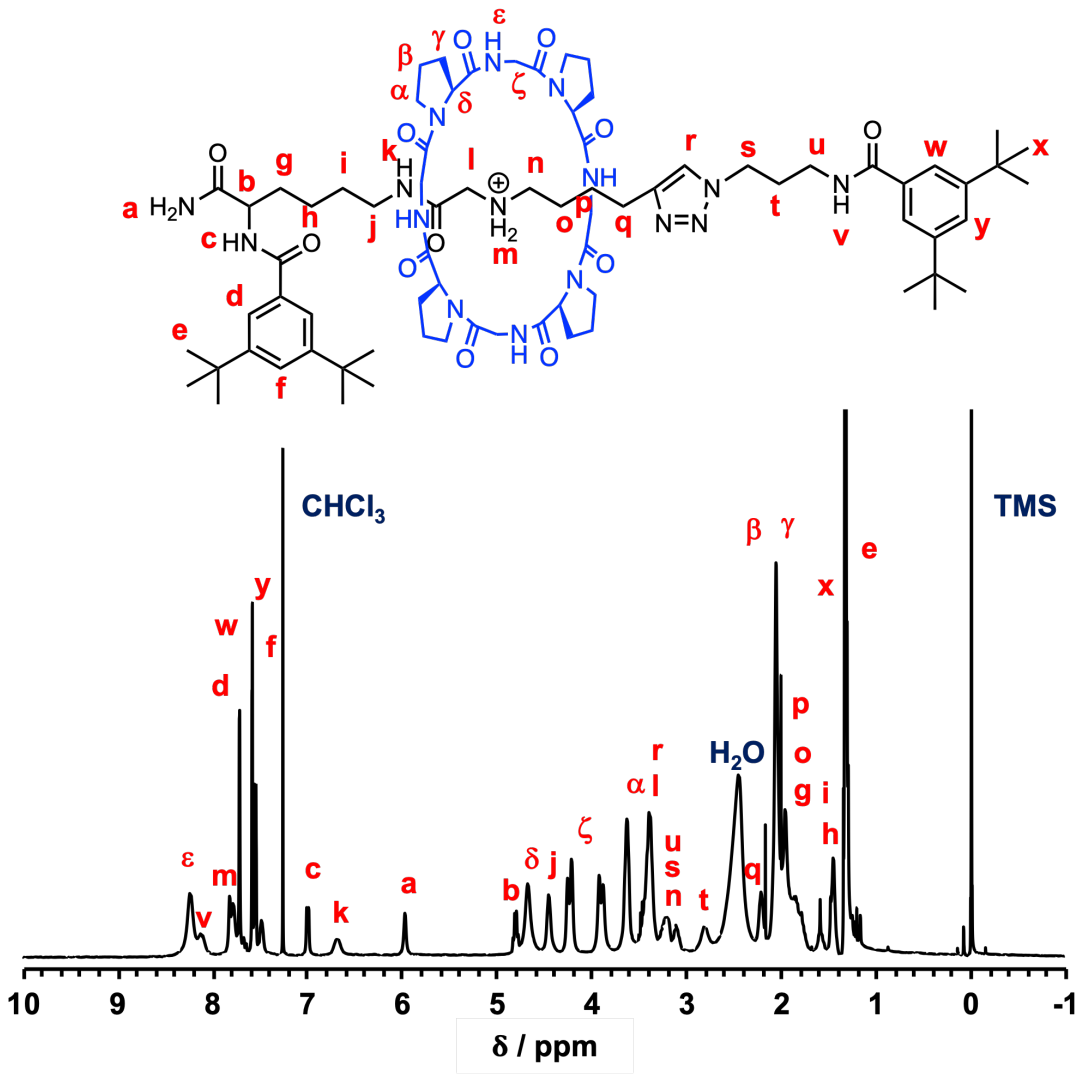

Figure S16. ^{1}H NMR spectrum of rotaxane **7**.

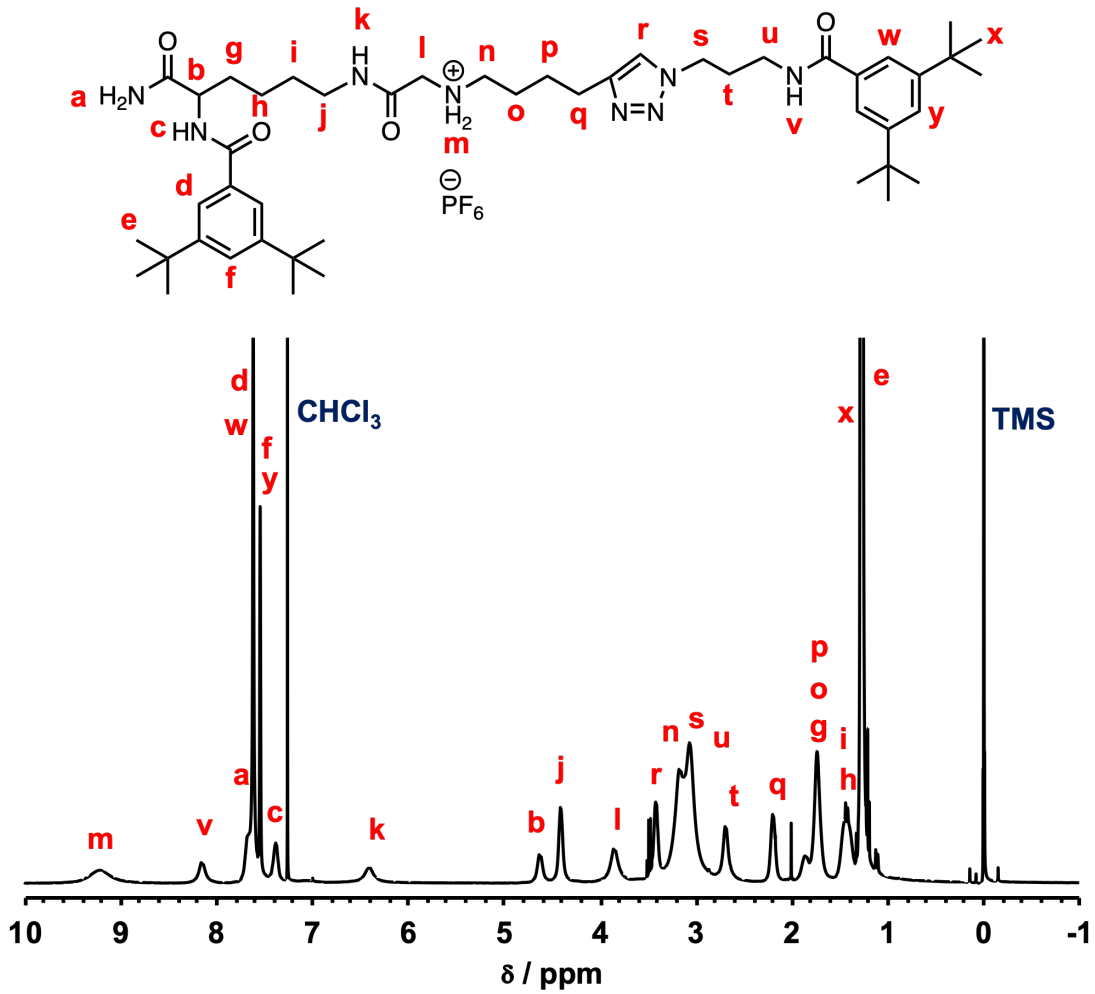

Figure S17. ^{1}H NMR spectrum of free thread **10**.

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