

Supporting Information

**Solid-Phase Total Synthesis and Dual Mechanism of Action of the Channel-Forming 48-mer Peptide
Polytheonamide B**

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60 pages

Table of Contents

Supplementary Figures and Tables	S3
Syntheses of Compounds	S11
General Remarks	S11
Preparation of Amino Acids 37, 41, 43, and 45	S12
Solid-Phase Synthesis of Polytheonamide B and Fluorescent Polytheonamide	S16
Cytotoxicity Assay	S26
Cell Culture	S26
WST-1 Assay	S26
Sulforhodamine B Assay	S27
Plasma Membrane Potential Assay	S29
Plasma Membrane Depolarization Assay Using DiBAC ₄ (3)	S29
Cellular Imaging Analysis	S30
Preparation of MCF-7 Cells	S30
Endocytosis Inhibition Assay	S30
Subcellular Localization Analysis of Fluorescent Polytheonamide with LysoTracker Red	S31
Subcellular Localization Analysis of Fluorescent Polytheonamide with RFP-LAMP1	S31
Localization Analysis of LysoTracker Red	S31
Cathepsin B Activity Assay	S31
Lysosomal pH Measurement with FRD	S32
Mitochondrial Membrane Potential Assay	S32
Apoptosis Assay	S33
PI-annexin V Assay	S33
NMR Spectra	S34
HPLC Charts for Purification of Synthetic Peptides	S48
HPLC Charts for Analysis of Synthetic Peptides	S53
HPLC Charts of Purified Synthetic Peptides	S58
References	S60

Supplementary Figures and Tables

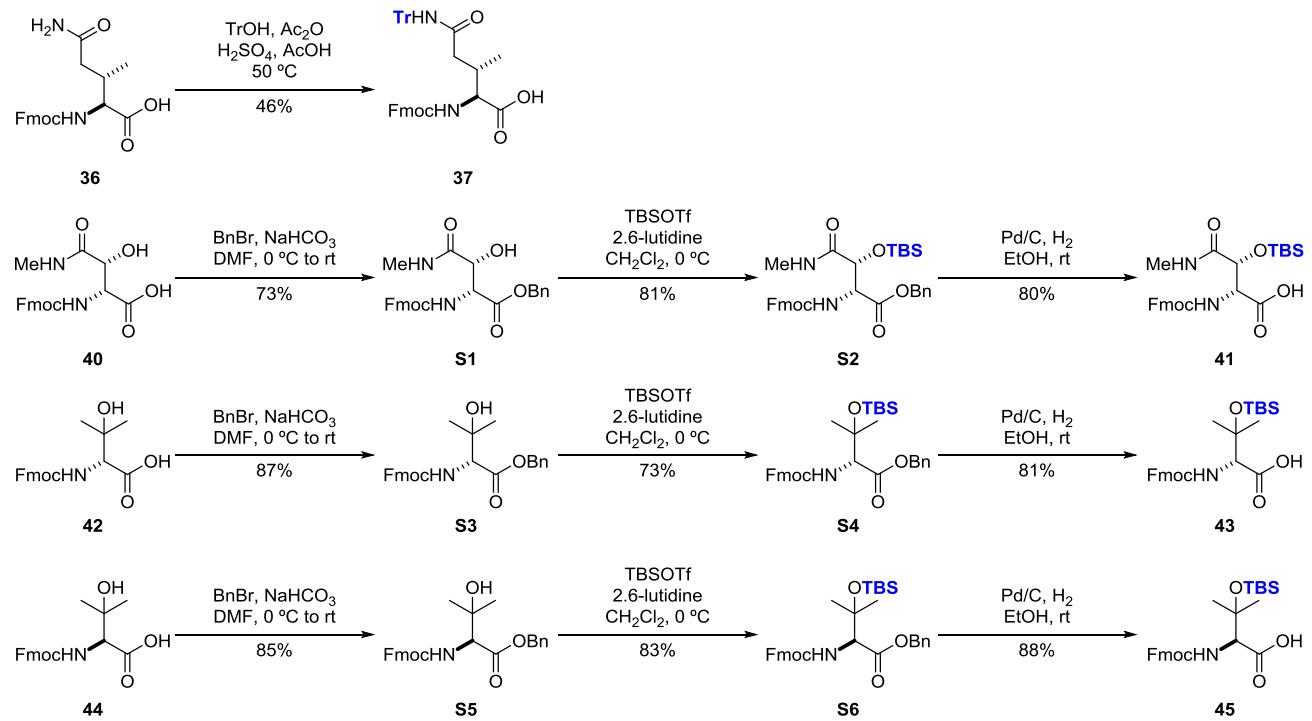
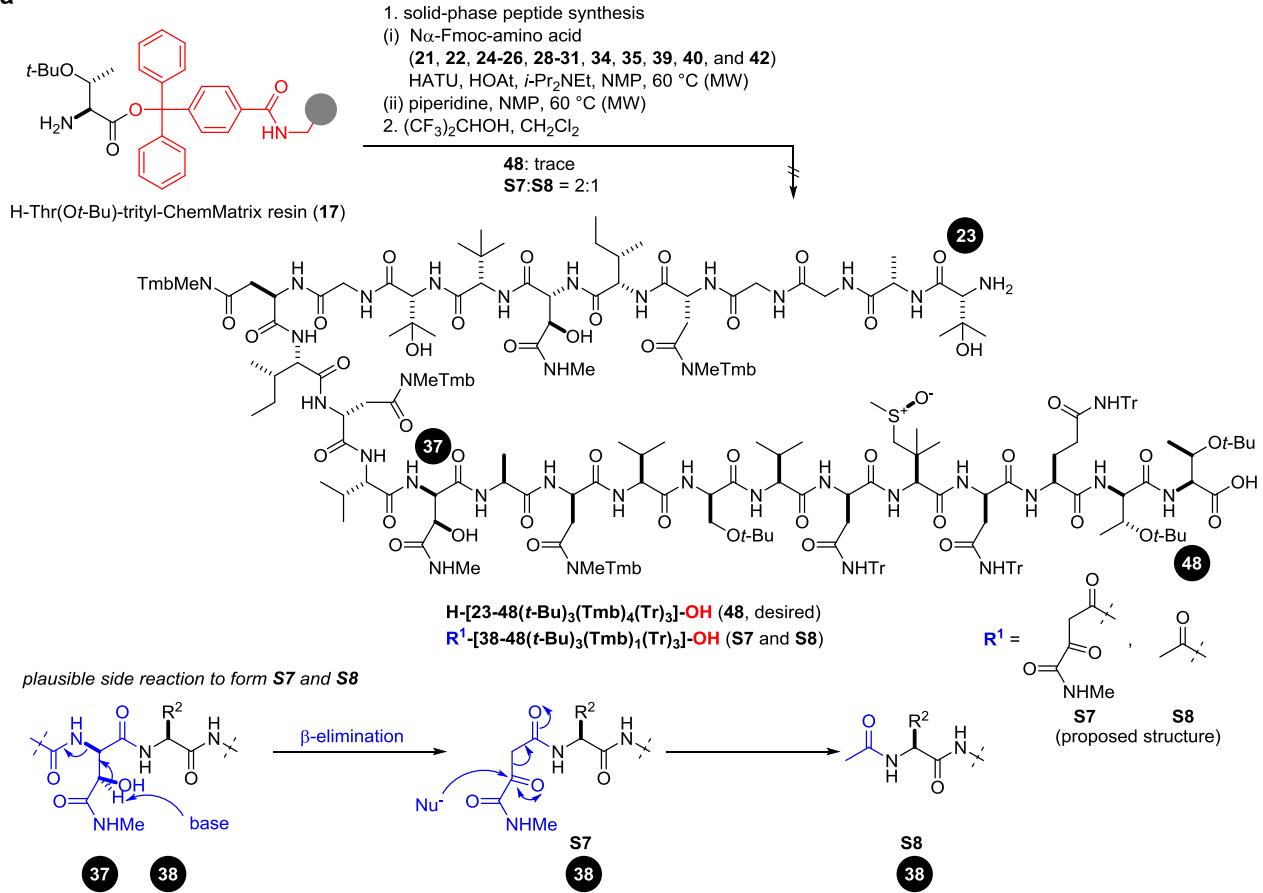
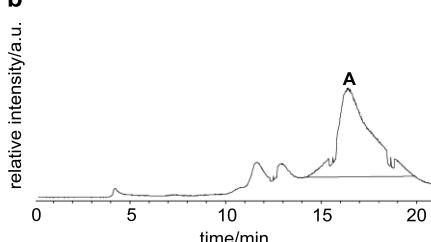


Figure S1. Preparation of amino acid components **37**, **41**, **43**, and **45**.

a**b**

c

relative intensity/a.u.

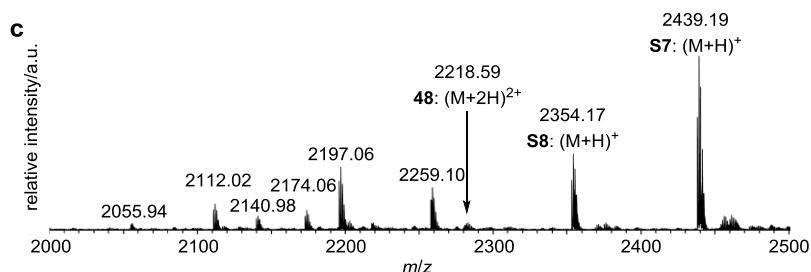


Figure S2. (a) Attempted synthesis of C-terminal fragment **48** and plausible side reaction to form **S7** and **S8**. (b) Total ion chromatogram of the crude material on LC-MS analysis. LC-MS conditions [column: COSMOSIL Protein-R 4.6 × 150 mm, eluent A: *i*-PrOH + 0.05% HCO₂H, eluent B: H₂O + 0.05% HCO₂H, linear gradient A/B = 50/50 to 100/0 over 40 min, flow rate: 0.5 mL/min, temperature: 35 °C, detection: ESI-TOF MS (m/z = 50–3000)]. (c) MS (ESI-TOF) signals of peak A in Figure S2b (m/z = 2000–2500). The trace amount of desired **48** was detected as the divalent ion 2218.59 ($[\text{M}+2\text{H}]^{2+}$). The signals 2439.19 and 2354.17 correspond to the monovalent ions ($[\text{M}+\text{H}]^+$) of fragmented byproducts **S7** and **S8**, respectively. The signals 2259.10, 2197.06, 2174.06, 2140.98, 2112.02, and 2055.94 are presumed to be formed from thermal deprotection of **S7** and **S8** (−Tmb, −Tr, and −Tr and *t*-Bu). a.u. = arbitrary unit.

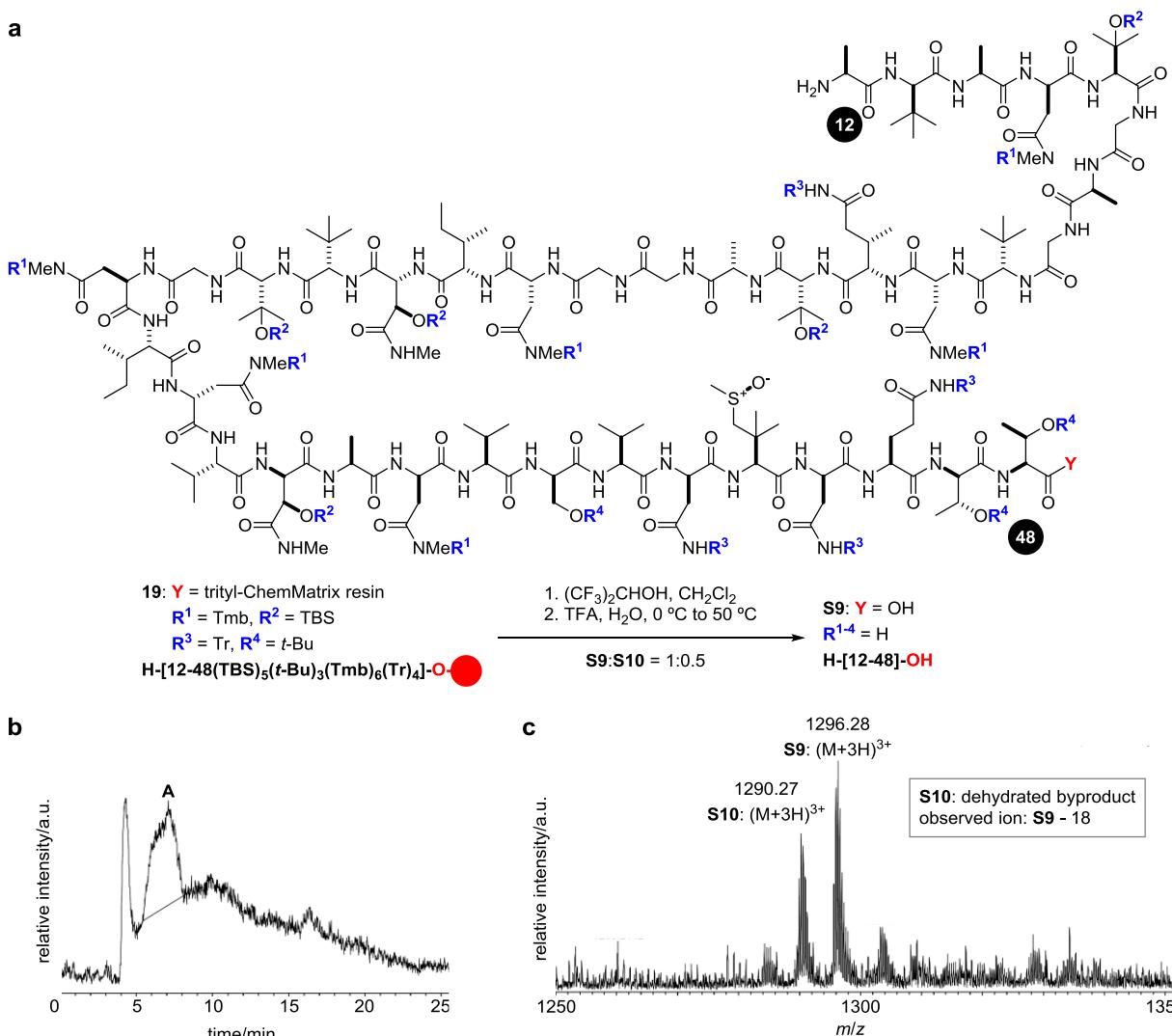


Figure S3. (a) Attempted global deprotection of C-terminal fragment **19**. (b) Total ion chromatogram of the crude material on LC-MS analysis. LC-MS conditions [column: Inertsil WP300 C8 4.6 × 150 mm, eluent A: CH₃CN + 0.05% TFA, eluent B: H₂O + 0.05% TFA, linear gradient A/B = 25/75 to 60/40 over 40 min, flow rate: 0.5 mL/min, temperature: 40 °C, detection: ESI-TOF MS (m/z = 50–3000)]. (c) MS (ESI-TOF) signals of peak A in Figure S3b (m/z = 1250–1350). The signal 1296.28 ($[M+3H]^{3+}$) corresponds to the trivalent ion ($[M+3H]^{3+}$) of **S9**. The signal 1290.27 corresponds to the trivalent ion of dehydrated byproduct **S10** ($[M+3H]^{3+}$).

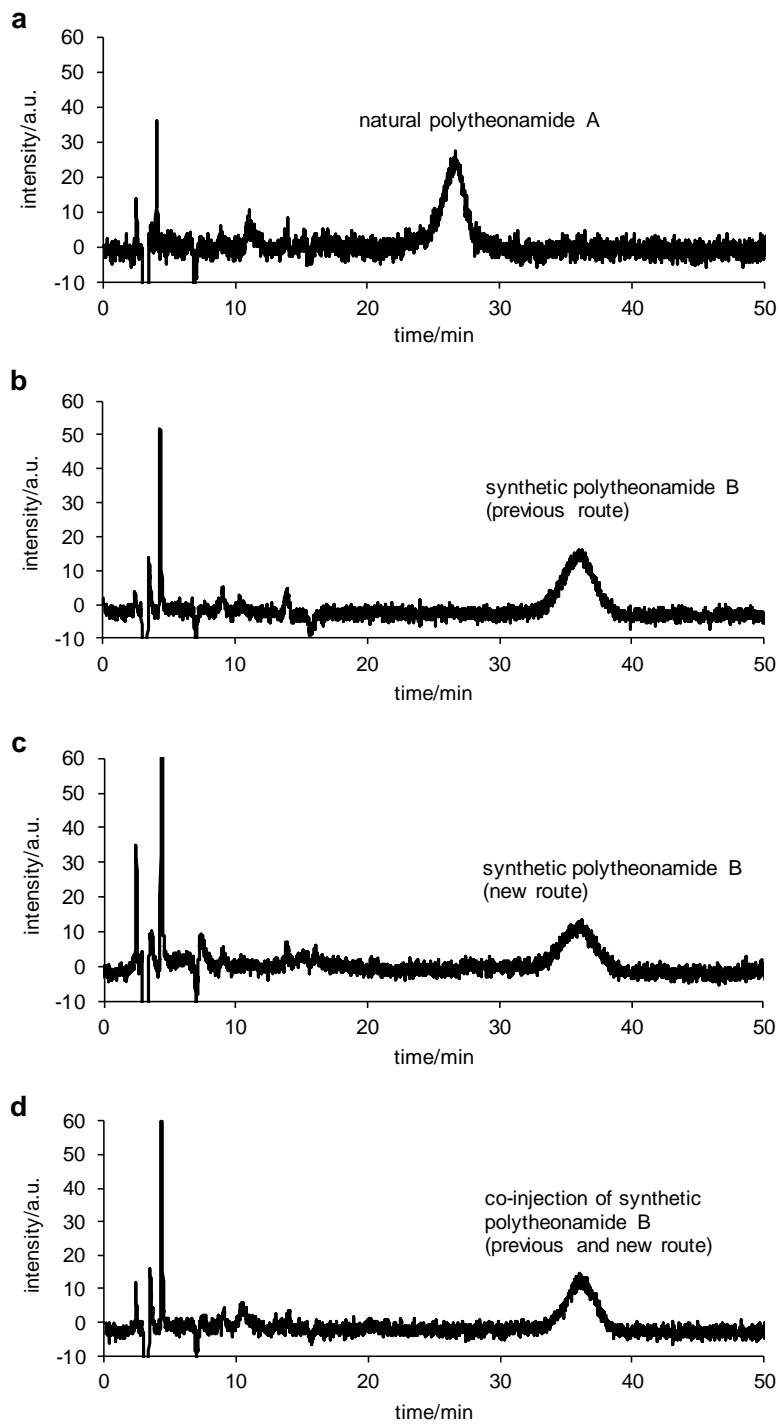


Figure S4. HPLC charts of polytheonamides. HPLC conditions (column: Inertsil C8-3 4.6 × 150 mm, eluent A: *n*-PrOH + 0.05% TFA, eluent B: H₂O + 0.05% TFA, isocratic: A/B = 37/63, flow rate: 0.5 mL/min, detection: UV 200 nm, temperature: 45 °C). (a) HPLC chart of natural polytheonamide A, (b) synthetic polytheonamide B (previous route), (c) synthetic polytheonamide B (new route), and (d) co-injection of synthetic polytheonamide B (previous route) and synthetic polytheonamide B (new route).

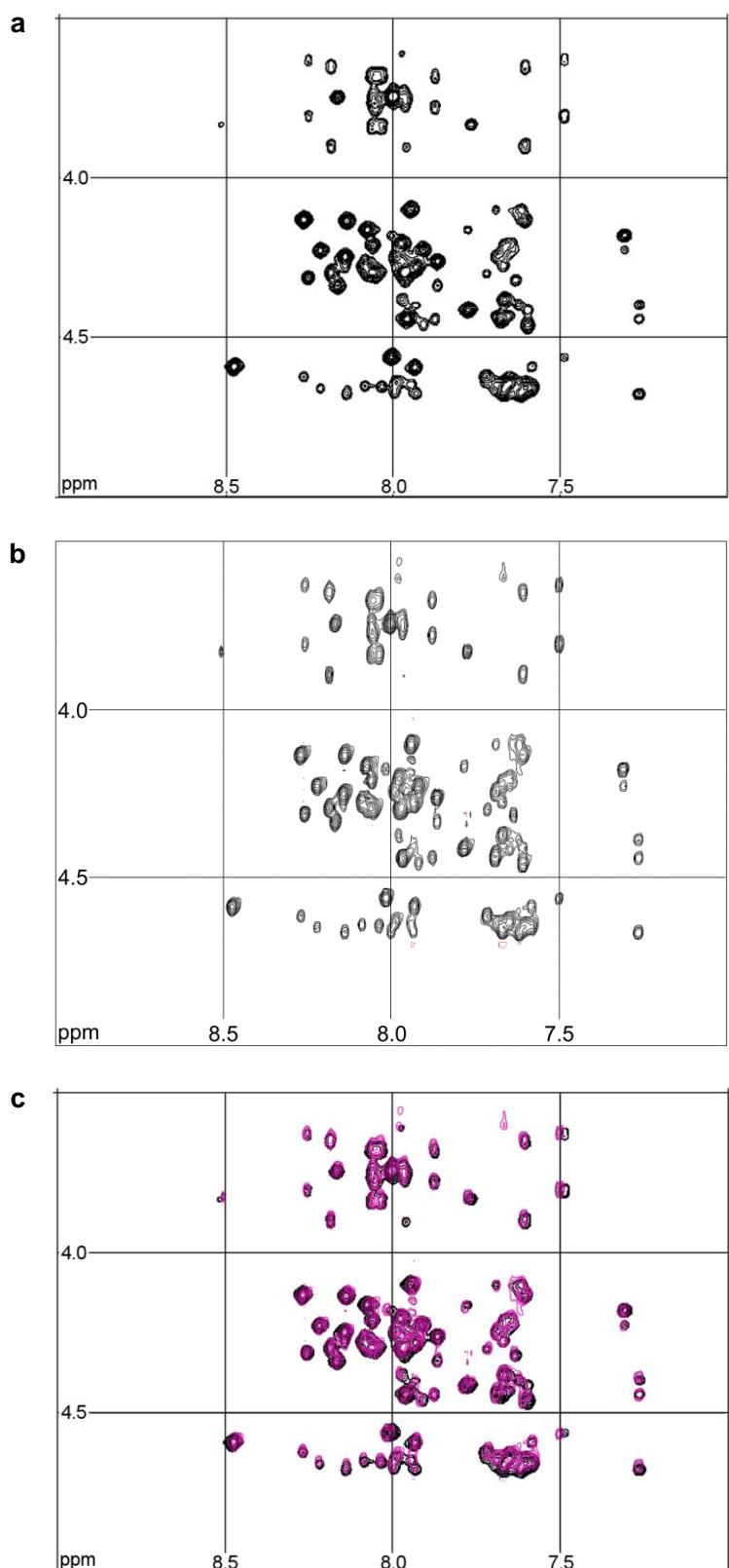


Figure S5. Comparison of fingerprint region of ^1H - ^1H NOESY spectrum (800 MHz) of **1** prepared via previous route (a) and new route (b). The spectrum was recorded in $\text{DMSO}-d_6$ (0.312 mM) at 40 °C on Varian Unity Inova 800 system equipped with a cold probe (a)^{S1} or Bruker Avance III HD 800 MHz equipped with CryoProbe (b). (c) An overlay of the two spectra. The spectra of Figures S5a and S5b were displayed in black and purple, respectively.

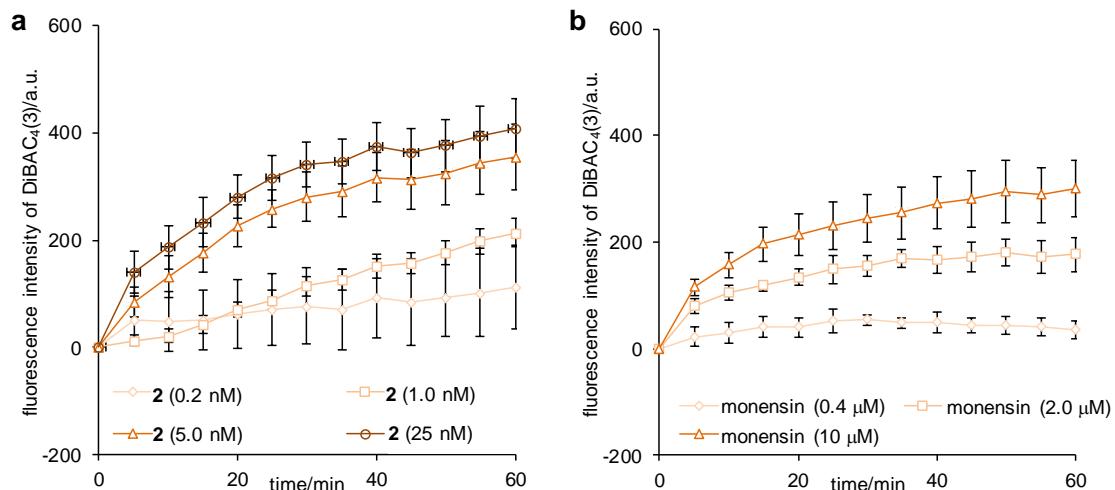


Figure S6. Time-course of the change of fluorescence intensity of DiBAC₄(3) in MCF-7 cells caused by adding **2** (a) or an ionophore monensin as a positive control (b). Peptide **2** or monensin was added at 0 min. Each plot is displayed as mean \pm S.D.

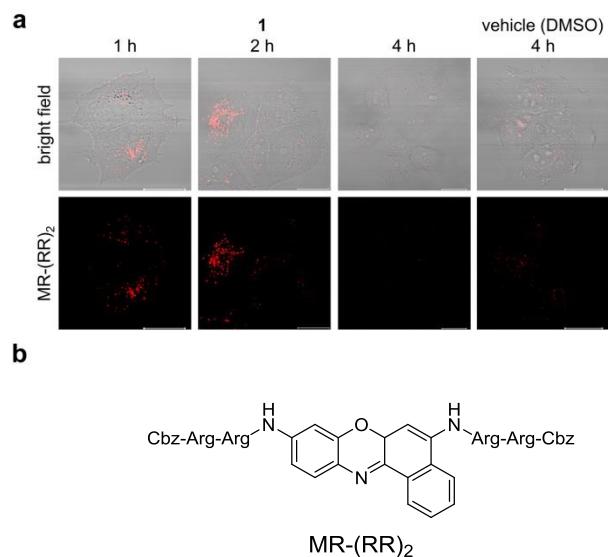


Figure S7. (a) Time-dependent fluorescence change of cresyl violet [also known as Magic Red (MR)] generated from MR-(RR)₂ by cathepsin B. MCF-7 cells were incubated with 5.0 nM **1** or vehicle (DMSO) for 1, 2, and 4 h. Scale bar represents 20 μ m. (b) Structure of cathepsin B substrate, MR-(RR)₂.

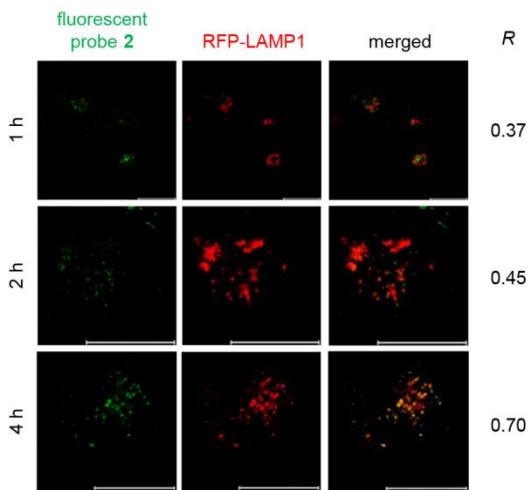
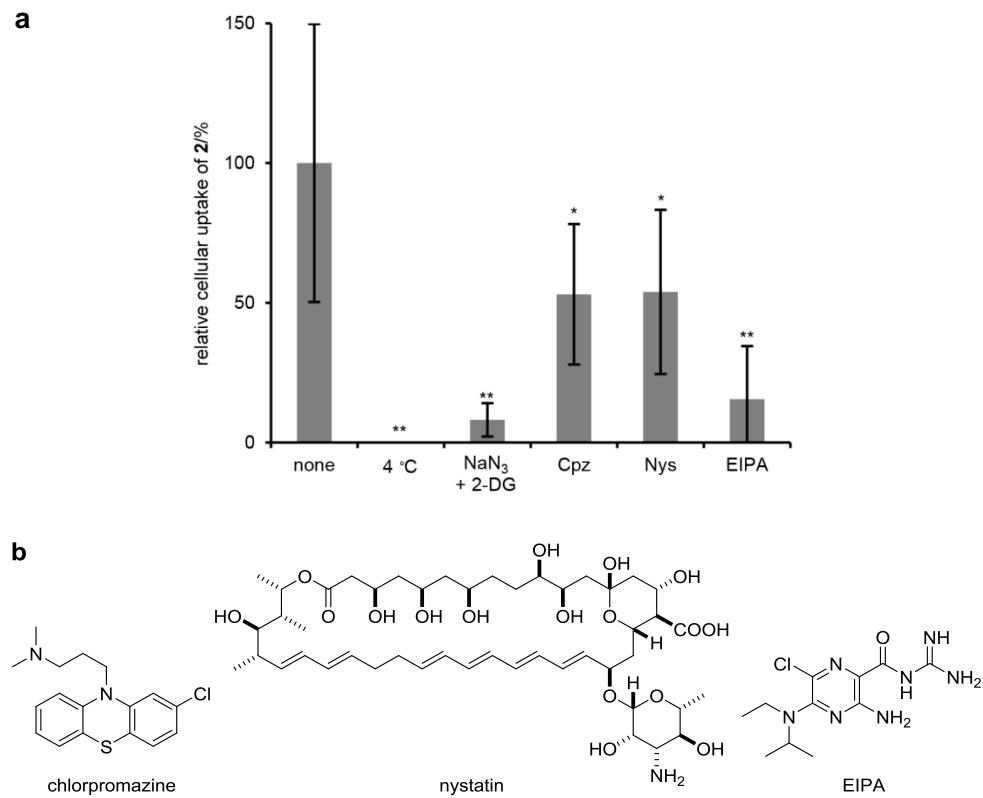


Figure S8. Subcellular localization of **2** (10 nM) in MCF-7 cells after incubation for 1 h, 2 h, and 4 h in the presence of a lysosomal marker RFP-LAMP1. In merged images, yellow signals indicate overlapping of fluorescent probe **2** with RFP-LAMP1. *R* value represents Pearson's correlation coefficient calculated from the green and red fluorescence in each merged image. Scale bar represents 20 μ m.



compounds	concentration (μ M)	function ^{S2}
chlorpromazine (Cpz)	10	inhibitor of clathrin-dependent endocytosis
nystatin (Nys)	50	inhibitor of lipid raft/caveolae pathway
5-(<i>N</i> -ethyl- <i>N</i> -isopropyl)-amiloride (EIPA)	10	selective inhibitor of Na^+/H^+ exchanger and blocker of macropinocytosis

Figure S9. (a) Effects of endocytosis and metabolic inhibitors on the cellular uptake of **2** (10 nM). The cellular uptake value was normalized against the mean value of the control. * $P < 0.05$ and ** $P < 0.01$ determined by *t*-test. (b) Structures, tested concentrations, and functions of endocytosis inhibitors.

Table S1. Comparison of the previous and new routes to **1**

		previous route ^{S1}	new route	
equivalent of peptide fragments				
resin	residue-48	1.0 eq	1.0 eq	
peptide fragment	residues 1–11	0.14 eq	0.92 eq	
	residues 12–25	0.09 eq	-	
	residues 26–32	0.15 eq	-	
overall yields and number of steps and HPLC purifications				
overall yield		4.5%	4.5%	
longest linear steps from residue-48	37steps (92%/step)	76 steps (96%/step)		
total steps		161 steps	169 steps	
preparation of component amino acids	SPPS	58 steps	70 steps	
solid-phase reaction		90 steps	94 steps	
		0	1 step	
		4 steps	2 steps	
		0	1 step	
total		94 steps	98 steps	
solution-phase reaction	thioesterification	3 steps	0	
	fragment coupling	3 steps	0	
	deprotection	3 steps	1 step	
	total	9 steps	1 step	
number of HPLC purifications		9	2	

Syntheses of Compounds

General Remarks

All reactions sensitive to air and/or moisture were carried out under argon (Ar) atmosphere in dry solvents, unless otherwise noted. THF, CH₂Cl₂, DMF, and Et₂O were purified by a Glass Contour solvent dispensing system (Nikko Hansen). *i*-Pr₂NEt was distilled from KOH. All other reagents were used as supplied unless otherwise stated. Analytical thin-layer chromatography (TLC) was performed using E. Merck Silica gel 60 F254 pre-coated plates. Flash column chromatography was performed using 40–50 µm Silica Gel 60 N (Kanto Chemical). Solid-phase peptide synthesis (SPPS) was performed on a microwave-assisted peptide synthesizer MWS-1000A (EYELA) using a sealed reaction vessel, a reaction temperature of which was monitored by an internal temperature probe. Optical rotations were recorded on a P-2200 polarimeter (JASCO). Infrared (IR) spectra were recorded on an FT/IR-4100 spectrometer (JASCO) as a thin film on a KBr, NaCl, or CaF₂. ¹H and ¹³C NMR spectra were recorded on an ECX 500 (500 MHz for ¹H NMR, 125 MHz for ¹³C NMR) spectrometer, an ECA 500 (500 MHz for ¹H NMR, 125 MHz for ¹³C NMR) spectrometer, an ECS 400 (400 MHz for ¹H NMR, 100 MHz for ¹³C NMR) (JEOL), or an Avance III HD 800 MHz equipped with CryoProbe (800 MHz for ¹H NMR) (Bruker). Chemical shifts are denoted in δ (ppm) relative to residual solvent peaks as internal standard (CDCl₃, ¹H δ 7.26, ¹³C δ 77.0; DMSO-*d*₆, ¹H δ 2.50, ¹³C δ 39.5). HRMS and LRMS spectra were recorded on a T100LP (JEOL) or MicrOTOFII (Bruker Daltonics) electrospray ionization time-of-flight (ESI-TOF) mass spectrometer. UV absorbance was measured on a UV-1800 UV-VIS spectrophotometer (Shimadzu). High performance liquid chromatography (HPLC) experiments were performed on an Agilent 1100 HPLC system (Agilent).

Preparation of Amino Acids 37, 41, 43, and 45

Trityl amide 37. To a solution of **36** (400 mg, 1.05 mmol) in AcOH (10.5 mL) and Ac₂O (395 μ L, 4.18 mmol) were added TrOH (1.09 g, 4.18 mmol) and H₂SO₄ (16.8 μ L, 0.32 mmol) at 50 °C. The reaction mixture was stirred at 50 °C for 3 h, and then Ac₂O (198 μ L, 2.09 mmol), TrOH (550 mg, 2.09 mmol), and H₂SO₄ (11.2 μ L, 0.210 mmol) were added. The reaction mixture was stirred at 50 °C for 5 h, and then TrOH (550 mg, 2.09 mmol) and H₂SO₄ (11.2 μ L, 0.210 mmol) were added. After being stirred at 50 °C for 11 h, the reaction mixture was poured into H₂O (30 mL) at 0 °C. The precipitate was filtered and dissolved in AcOEt (50 mL). The solution was washed with H₂O, dried over anhydrous Na₂SO₄, filtered, and concentrated. The residue was recrystallized from hexane/AcOEt to give **37** (302 mg, 0.483 mmol, 46%): white solid; $[\alpha]_D^{23} = -20.8^\circ$ ($c = 1.06$, CHCl₃); IR (film) ν 908, 1036, 1081, 1212, 1327, 1448, 1494, 1714, 2360, 3060, 3316 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 0.98 (3H, d, $J = 6.9$ Hz), 2.42 (1H, dd, $J = 13.2$ Hz), 2.55 (1H, dd, $J = 13.7$, 8.5 Hz), 2.72 (1H, m), 4.24 (1H, t, $J = 7.3$ Hz), 4.41 (3H, m), 5.89 (1H, d, $J = 6.0$ Hz), 7.08 (1H, s), 7.19–7.43 (19H, m), 7.61 (2H, t, $J = 6.0$ Hz), 7.78 (2H, d, $J = 7.3$ Hz); ¹³C NMR (100 MHz, CDCl₃) δ 15.9, 33.3, 40.9, 47.1, 56.5, 67.0, 71.5, 120.0, 125.06, 125.13, 127.1, 127.4, 127.7, 128.2, 128.6, 141.3, 143.5, 143.7, 155.5, 171.1, 173.1; HRMS (ESI-TOF) calcd for C₄₀H₃₅N₂Na₂O₅ [M–H+2Na]⁺ 669.2336, found 669.2344.

Benzyl ester S1. To a solution of **40** (989 mg, 2.57 mmol) in DMF (12.9 mL) were added BnBr (642 μ L, 5.40 mmol) and NaHCO₃ (691 mg, 8.22 mmol) at 0 °C. The reaction mixture was gradually warmed to room temperature and stirred for 37 h, and then H₂O (10 mL) was added. The resultant mixture was extracted with AcOEt (30 mL \times 2). The combined organic layers were washed with H₂O (\times 4), dried over anhydrous Na₂SO₄, filtered, and concentrated. The residue was purified by flash column chromatography on silica gel (20 g, hexane/AcOEt 1/1 to 0/1) to give **S1** (888 mg, 1.87 mmol, 73%): white solid; $[\alpha]_D^{27} = +22.8^\circ$ [$c = 1.05$, CHCl₃/MeOH (9/1)]; IR (film) ν 1003, 1052, 1081, 1106, 1251, 1335, 1411, 1450, 1540, 1658, 1718, 2947, 3064, 3325 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 2.81 (3H, d, $J = 4.6$ Hz), 4.20–4.27 (1H, m), 4.32–4.39 (2H, m), 4.55 (1H, m), 4.80 (1H, dd, $J = 9.2$, 2.9 Hz), 5.23 (2H, s), 5.91 (1H, br), 6.58 (1H, br), 7.27–7.33 (7H, m), 7.40 (2H, t, $J = 7.5$ Hz), 7.56 (2H, m), 7.76 (2H, d, $J = 7.5$ Hz); ¹³C NMR (125 MHz, DMSO-d₆) δ 25.6, 46.5, 57.4, 66.1, 66.3, 71.5, 120.1, 125.3, 127.1, 127.6, 127.7, 128.0, 128.4, 135.8, 140.7, 143.7, 156.2, 170.3, 170.7; HRMS (ESI-TOF) calcd for C₂₇H₂₆N₂NaO₆ [M+Na]⁺ 497.1683, found 497.1676.

TBS ether S2. To a solution of **S1** (876 mg, 1.85 mmol) in CH₂Cl₂ (18.5 mL) were added 2,6-lutidine (688 μ L, 5.91 mmol) and TBSOTf (848 μ L, 3.69 mmol) at 0 °C. The reaction mixture was stirred at 0 °C for 1 h, and then H₂O (10 mL) was added. The resultant mixture was extracted with CH₂Cl₂ (30 mL \times 3). The combined organic layers were dried over anhydrous Na₂SO₄, filtered, concentrated, and azeotroped with toluene (\times 3). The residue was purified by flash column chromatography on silica gel (20 g, hexane/AcOEt 9/1 to 7/3) to give **S2** (883 mg, 1.50 mmol, 81%): colorless oil; $[\alpha]_D^{27} = +5.2^\circ$ ($c = 0.99$, CHCl₃); IR (film) ν 983, 1057, 1109, 1162, 1201, 1254, 1331, 1409, 1450, 1533, 1669, 1727, 2361, 2857, 2951, 3436 cm⁻¹; ¹H

NMR (500 MHz, CDCl₃) Signals derived from rotamers A and B (4:1) were observed, and those of major rotamer A were listed. δ 0.06 (3H, s), 0.07 (3H, s), 0.92 (9H, s), 2.75 (3H, d, *J* = 5.2 Hz), 4.21–4.30 (2H, m), 4.41 (1H, dd, *J* = 10.1, 6.9 Hz), 4.56 (1 H, d, *J* = 2.3 Hz), 4.86 (1H, m), 5.17 (2H, d, *J* = 12.6 Hz), 5.80 (1H, d, *J* = 9.8 Hz), 6.36 (1H, br), 7.28–7.42 (9H, m), 7.59 (2H, t, *J* = 8.0 Hz), 7.76 (2H, d, *J* = 7.5 Hz); ¹³C NMR (125 MHz, CDCl₃) δ –5.4, –5.2, 17.9, 25.6, 25.9, 47.0, 57.2, 67.4, 67.6, 73.2, 120.0, 125.0, 125.2, 127.00, 127.05, 127.7, 128.5, 128.6, 134.9, 141.2, 143.7, 143.9, 155.8, 169.7, 170.7; HRMS (ESI-TOF) calcd for C₃₃H₄₀N₂NaO₆Si [M+Na]⁺ 611.2548, found 611.2551.

Carboxylic acid 41. To a solution of **S2** (883 mg, 1.50 mmol) in EtOH (15 mL) was added 10 wt% Pd/C (79.8 mg, 75 μmol) at room temperature. The flask equipped with a balloon was evacuated and recharged with H₂ (× 3). After being stirred at room temperature for 3 h under H₂ atmosphere, the reaction mixture was filtered through a pad of Celite with AcOEt (50 mL). The filtrate was concentrated. The residue was purified by flash column chromatography on silica gel (15 g, CHCl₃/MeOH 100/0 to 96/4) to give **41** (596 mg, 1.20 mmol, 80%): white solid; [α]_D²⁷ = +2.8° (*c* = 0.98, CHCl₃); IR (film) ν 910, 984, 1057, 1108, 1162, 1254, 1333, 1414, 1440, 1534, 1667, 1723, 2360, 2858, 2952, 3434 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) Signals derived from rotamers A and B (3:1) were observed, and those of major rotamer A were listed. δ 0.10 (3H, s), 0.12 (3H, s), 0.93 (1H, s), 2.81 (3H, d, *J* = 5.2 Hz), 4.20–4.32 (2H, m), 4.38 (1 H, m), 4.64 (1H, d, *J* = 1.8 Hz), 4.84 (1H, dd, *J* = 9.8, 2.3 Hz), 5.72 (1H, d, *J* = 9.2 Hz), 6.58 (1H, d, *J* = 4.6 Hz), 7.29 (2H, t, *J* = 6.9 Hz), 7.37 (2H, m), 7.58 (2H, m), 7.75 (2H, d, *J* = 7.5 Hz); ¹³C NMR (125 MHz, CDCl₃) δ –5.4, –5.1, 18.0, 25.6, 26.1, 47.0, 56.9, 67.4, 73.1, 120.0, 125.0, 125.2, 127.0, 127.7, 141.2, 143.6, 143.8, 156.0, 171.5, 173.0; HRMS (ESI-TOF) calcd for C₂₆H₃₃N₂Na₂O₆Si [M–H+2Na]⁺ 543.1898, found 543.1891.

Benzyl ester S3. To a solution of **42** (1.02 g, 2.87 mmol) in DMF (7.2 mL) were added BnBr (715 μL, 6.02 mmol) and NaHCO₃ (772 mg, 9.18 mmol) at 0 °C. The reaction mixture was gradually warmed to room temperature and stirred for 42 h, and then H₂O (10 mL) was added. The resultant mixture was extracted with AcOEt (30 mL × 2). The combined organic layers were washed with H₂O (× 4), dried over anhydrous Na₂SO₄, filtered, concentrated, and azeotroped with toluene (× 3). The residue was purified by flash column chromatography on silica gel (15 g, hexane/AcOEt 9/1 to 7/3) to give **S3** (1.11 g, 2.49 mmol, 87%): white solid; [α]_D²⁷ = +15.4° (*c* = 1.06, CHCl₃); IR (film) ν 1048, 1211, 1337, 1450, 1510, 1715, 2978, 3431 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 1.23 (3H, s), 1.24 (3H, s), 2.40 (1H, br), 4.22 (1H, t, *J* = 7.4 Hz), 4.31 (1 H, d, *J* = 8.6 Hz), 4.42 (2H, m), 5.22 (2H, d, *J* = 12.1 Hz), 5.65 (1H, d, *J* = 9.2 Hz), 7.28–7.42 (8H, m), 7.59 (2H, d, *J* = 6.9 Hz), 7.76 (2H, d, *J* = 7.5 Hz); ¹³C NMR (125 MHz, CDCl₃) δ 26.3, 26.8, 47.1, 61.6, 67.2, 67.3, 71.9, 119.9, 125.0, 127.0, 127.7, 128.4, 128.56, 128.61, 134.9, 141.2, 143.6, 143.8, 156.3, 171.5; HRMS (ESI-TOF) calcd for C₂₇H₂₇NNaO₅ [M+Na]⁺ 468.1781, found 468.1785.

TBS ether S4. To a solution of **S3** (1.11 g, 2.48 mmol) in CH₂Cl₂ (24.8 mL) were added 2,6-lutidine (925 μL, 7.94 mmol) and TBSOTf (1.14 mL, 4.96 mmol) at 0 °C. The reaction mixture was stirred at 0 °C for 1 h, and then H₂O (10 mL) was added. The resultant mixture was extracted with CH₂Cl₂ (30 mL × 3).

combined organic layers were washed with saturated aqueous NH₄Cl, dried over anhydrous Na₂SO₄, filtered, concentrated, and azeotroped with toluene ($\times 5$). The residue was purified by flash column chromatography on silica gel (20 g, hexane/AcOEt 97/3 to 93/7) to give **S4** (1.01 g, 1.80 mmol, 73%): colorless oil: $[\alpha]_D^{27} = +0.7^\circ$ ($c = 1.18$, CHCl₃); IR (film) ν 1040, 1153, 1208, 1253, 1327, 1451, 1503, 1729, 2856, 2930, 2952, 3445 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 0.04 (3H, s), 0.11 (3H, s), 0.87 (9H, s), 1.30 (3H, s), 1.34 (3H, s), 4.19–4.24 (2H, m), 4.36 (1 H, d, $J = 7.3$ Hz), 5.10–5.23 (2H, d, $J = 12.4$ Hz), 5.62 (1H, d, $J = 9.6$ Hz), 7.28–7.42 (9H, m), 7.59 (2H, d, $J = 7.3$ Hz), 7.76 (2H, d, $J = 7.8$ Hz); ¹³C NMR (125 MHz, CDCl₃) δ –2.4, -2.3, 14.1, 18.0, 22.6, 25.6, 27.6, 28.1, 31.6, 47.1, 63.1, 66.9, 67.0, 74.9, 119.9, 125.1, 127.0, 127.7, 128.3, 128.5, 128.6, 135.3, 141.3, 143.7, 144.0, 156.1, 170.1; HRMS (ESI-TOF) calcd for C₃₃H₄₁NNaO₅Si [M+Na]⁺ 582.2646, found 582.2654.

Carboxylic acid 43. To a solution of **S4** (679 mg, 1.21 mmol) in EtOH (12.1 mL) was added 10 wt% Pd/C (12.9 mg, 12.1 μ mol) at room temperature. The flask equipped with a balloon was evacuated and recharged with H₂ ($\times 3$). After being stirred at room temperature for 4 h under H₂ atmosphere, the reaction mixture was filtered through a pad of Celite with AcOEt (50 mL). The filtrate was concentrated. The residue was purified by flash column chromatography on silica gel (10 g, hexane/AcOEt 70/30 to 50/50) to give **43** (460 mg, 0.979 mmol, 81%): white solid; $[\alpha]_D^{27} = -18.0^\circ$ ($c = 1.01$, CHCl₃); IR (film) ν 908, 1040, 1160, 1211, 1254, 1323, 1450, 1511, 1720, 2857, 2932, 2952, 3443 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 0.18 (6H, s), 0.91 (9H, s), 1.29 (3H, s), 1.44 (3H, s), 4.24 (1H, t, $J = 6.9$ Hz), 4.29 (1H, d, $J = 8.6$ Hz), 4.41 (2 H, d, $J = 6.9$ Hz), 5.53 (1H, d, $J = 9.2$ Hz), 7.31 (2H, t, $J = 7.5$ Hz), 7.39 (2H, m), 7.59 (2H, d, $J = 7.5$ Hz), 7.76 (2H, d, $J = 7.5$ Hz); ¹³C NMR (125 MHz, CDCl₃) δ –2.3, 17.9, 25.6, 27.2, 27.5, 47.1, 62.8, 67.2, 75.6, 120.0, 125.07, 125.12, 127.0, 127.7, 141.3, 143.6, 143.9, 156.3, 174.8; HRMS (ESI-TOF) calcd for C₂₆H₃₅NNaO₅Si [M+Na]⁺ 492.2177, found 492.2186.

Benzyl ester S5. To a solution of **44** (923 mg, 2.60 mmol) in DMF (6.5 mL) were added BnBr (648 μ L, 5.45 mmol) and NaHCO₃ (699 mg, 8.32 mmol) at 0 °C. The reaction mixture was gradually warmed to room temperature and stirred for 45 h, and then H₂O (10 mL) was added. The resultant mixture was extracted with AcOEt (30 mL $\times 2$). The combined organic layers were washed with H₂O ($\times 4$), dried over anhydrous Na₂SO₄, filtered, concentrated, and azeotroped with toluene ($\times 3$). The residue was purified by flash column chromatography on silica gel (15 g, hexane/AcOEt 9/1 to 7/3) to give **S5** (849 mg, 1.91 mmol, 73%): white solid; $[\alpha]_D^{27} = -15.7^\circ$ ($c = 1.36$, CHCl₃); IR (film) ν 910, 1050, 1158, 1208, 1335, 1385, 1452, 1519, 1715, 2250, 2893, 2979, 3424 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 1.23 (3H, s), 1.24 (3H, s), 2.40 (1H, s), 4.22 (1H, t, $J = 6.9$ Hz), 4.31 (1 H, d, $J = 9.7$ Hz), 4.42 (2H, m), 5.22 (2H, d, $J = 12.1$ Hz), 5.65 (1H, d, $J = 9.2$ Hz), 7.27–7.42 (9H, m), 7.59 (2H, d, $J = 7.5$ Hz), 7.76 (2H, d, $J = 7.5$ Hz); ¹³C NMR (125 MHz, CDCl₃) δ 26.3, 26.8, 47.1, 61.5, 67.2, 67.3, 71.9, 120.0, 125.0, 127.0, 127.7, 128.4, 128.6, 134.9, 141.2, 143.6, 143.7, 156.3, 171.4; HRMS (ESI-TOF) calcd for C₂₇H₂₇NNaO₅ [M+Na]⁺ 468.1781, found 468.1804.

TBS ether S6. To a solution of **S5** (771 mg, 1.73 mmol) in CH₂Cl₂ (17.3 mL) were added 2,6-lutidine

(645 μ L, 5.54 mmol) and TBSOTf (795 μ L, 3.46 mmol) at 0 °C. The reaction mixture was stirred at 0 °C for 1 h, and then H₂O (10 mL) was added. The resultant mixture was extracted with CH₂Cl₂ (30 mL \times 3). The combined organic layers were washed with saturated aqueous NH₄Cl, dried over anhydrous Na₂SO₄, filtered, concentrated, and azeotroped with toluene (\times 5). The residue was purified by flash column chromatography on silica gel (15 g, hexane/AcOEt 95/5 to 93/7) to give **S6** (894 mg, 1.60 mmol, 92%): colorless oil; $[\alpha]_D^{27} = -1.3^\circ$ ($c = 1.25$, CHCl₃); IR (film) ν 1041, 1157, 1183, 1209, 1253, 1328, 1451, 1504, 1731, 2856, 2930, 2952, 3445 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 0.04 (3H, s), 0.11 (3H, s), 0.88 (9H, s), 1.30 (3H, s), 1.35 (3H, s), 4.20–4.25 (2H, m), 4.36 (1H, d, $J = 7.3$ Hz), 5.10–5.23 (2H, d, $J = 12.4$ Hz), 5.62 (1H, d, $J = 9.6$ Hz), 7.28–7.42 (9H, m), 7.59 (2H, d, $J = 7.3$ Hz), 7.76 (2H, d, $J = 7.8$ Hz); ¹³C NMR (125 MHz, CDCl₃) δ -2.4, -2.3, 14.1, 18.0, 22.6, 25.6, 27.6, 28.1, 31.6, 47.1, 63.1, 66.9, 67.0, 74.9, 119.9, 125.1, 125.2, 127.0, 127.63, 127.65, 128.3, 128.5, 128.6, 135.2, 141.2, 141.3, 143.7, 144.0, 156.1, 170.1; HRMS (ESI-TOF) calcd for C₃₃H₄₁NNaO₅Si [M+Na]⁺ 582.2646, found 582.2659.

Carboxylic acid 45. To a solution of **S6** (583 mg, 1.04 mmol) in EtOH (10.4 mL) was added 10 wt% Pd/C (11.1 mg, 10.4 μ mol) at room temperature. The flask equipped with a balloon was evacuated and recharged with H₂ (\times 3). After being stirred at room temperature for 4 h under H₂ atmosphere, the reaction mixture was filtered through a pad of Celite with AcOEt (50 mL). The filtrate was concentrated. The residue was purified by flash column chromatography on silica gel (15 g, hexane/AcOEt 70/30 to 50/50) to give **45** (432 mg, 0.920 mmol, 88%): white solid; $[\alpha]_D^{27} = +15.4^\circ$ ($c = 1.04$, CHCl₃); IR (film) ν 908, 1040, 1161, 1210, 1254, 1323, 1450, 1510, 1720, 2856, 2932, 2952, 3443 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 0.19 (6H, s), 0.91 (9H, s), 1.29 (3H, s), 1.44 (3H, s), 4.24 (1H, t, $J = 7.5$ Hz), 4.29 (1H, d, $J = 8.6$ Hz), 4.41 (2H, d, $J = 7.5$ Hz), 5.53 (1H, d, $J = 8.1$ Hz), 7.31 (2H, t, $J = 8.0$ Hz), 7.39 (2H, m), 7.59 (2H, t, $J = 7.4$ Hz), 7.76 (2H, d, $J = 7.5$ Hz); ¹³C NMR (125 MHz, CDCl₃) δ -2.3, 18.0, 25.6, 27.2, 27.5, 47.1, 62.8, 67.2, 75.6, 120.0, 125.07, 125.12, 127.0, 127.7, 141.3, 143.6, 143.9, 156.3, 174.8; HRMS (ESI-TOF) calcd for C₂₆H₃₅NNaO₅Si [M+Na]⁺ 492.2177, found 492.2173.

Solid-Phase Synthesis of Polytheonamide B and Fluorescent Polytheonamide

Determination of loading rate

Fmoc-protected resin was treated with piperidine/NMP (1/3, 1.00 mL) at room temperature for 15 min. The supernatant was collected. The same treatment and collection of the supernatant were repeated ($\times 2$). The resin was washed with NMP (2 mL $\times 4$), and then the supernatants were collected. UV absorption at 301 nm of the combined supernatants was measured. The background absorbance was canceled by subtracting the control absorbance obtained from a solution of piperidine/NMP (1/24). The loading rate (x mmol/g) was determined by the following equation, where a is the weight of Fmoc-protected resin (mg), and b is absorbance at 301 nm.

$$x = (10000 \times b) / (7800 \times a)$$

Procedures for solid-phase peptide synthesis

Peptides **14**, **19** and **47** were prepared on a peptide synthesizer MWS-1000A (EYELA). Standard operation was shown as follows:

Step 1: The solid supported N_{α} -Fmoc peptide was deprotected with piperidine/NMP (1/4, 40 or 60 °C, 200 W; 5 min).

Step 2: The reaction vessel (LibraTube) containing the resin was washed with NMP (2 mL, 30 sec $\times 5$).

Step 3: A N_{α} -Fmoc-amino acid (4.0 eq) was activated by a solution of *O*-(7-aza-1*H*-benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU)/1-hydroxy-7-azabenzotriazole (HOAt) (4.0 eq, 0.45 M) in NMP. To the solution of activated Fmoc-amino acid was added a solution of *i*-Pr₂NEt (8.0 eq, 2.0 M) in NMP. The resultant mixture was transferred to the reaction vessel.

Step 4: The activated N_{α} -Fmoc-amino acid was coupled with the peptide on the resin (40 or 60 °C, 200 W; 20 min) and the reaction vessel containing the resin was washed with NMP (2 mL, 30 sec $\times 5$).

Steps 1–4 were repeated and amino acids were condensed on the solid support. Condensation of Fmoc-L-Ala-OH (**22**), Fmoc-L-Val-OH (**24**), Fmoc-L-Ile-OH (**25**), Fmoc-L-Tle-OH (**26**), Fmoc-D-Tle-OH (**27**), Fmoc-L-βMeIle-OH (**33**), Fmoc-L-β,βMe₂Met(O)-OH (**34**), Fmoc-L-βMeGln(Tr)-OH (**37**), Fmoc-D-βOTBSAsm-OH (**41**), Fmoc-D-βOTBSVal-OH (**43**), and Fmoc-L-βOTBSVal-OH (**45**) were performed by the double coupling method (Steps 3 and 4 were repeated before Step 1).

Quantification of peptides

The quantification of **14** and **47** was carried out using ^1H NMR. HPLC-purified peptide was dissolved in DMSO- d_6 (0.20 mL, 99.5 atom%D). Then, the peptide in DMSO- d_6 solution was transferred into a NMR tube and subjected to ^1H NMR measurement at 40 °C. Peptide concentration was calculated from the ^1H NMR intensity using the signal intensity of residual DMSO- d_5 as the reference. The concentration of residual DMSO- d_5 in DMSO- d_6 was standardized using 0.18 mM Fmoc-L-Val-OH (**24**) in DMSO- d_6 solution.

The quantification of **2** was carried out using HPLC (column: Inertsil Inertsil C8-3 4.6 × 150 mm, eluent A: *n*-PrOH + 0.05% TFA, eluent B: H₂O + 0.05% TFA, linear gradient: A/B = 25/75 to 50/50 over 35 min, flow rate: 0.6 mL/min, detection: UV 500 nm, temperature: 45 °C). HPLC chromatograms were recorded under the UV detection, and the peak areas of the peptides (UV 500 nm) were calculated using HyStar LC integration software (Bruker Daltonics). The quantity of **2** was determined from its peak area using the peak of compound **51** (UV 500 nm) as the reference (column: Inertsil Inertsil C8-3 4.6 × 150 mm, eluent A: *n*-PrOH + 0.05% TFA, eluent B: H₂O + 0.05% TFA, linear gradient: A/B = 15/85 to 35/65 over 30 min, flow rate: 0.6 mL/min, detection: UV 500 nm, temperature: 45 °C).

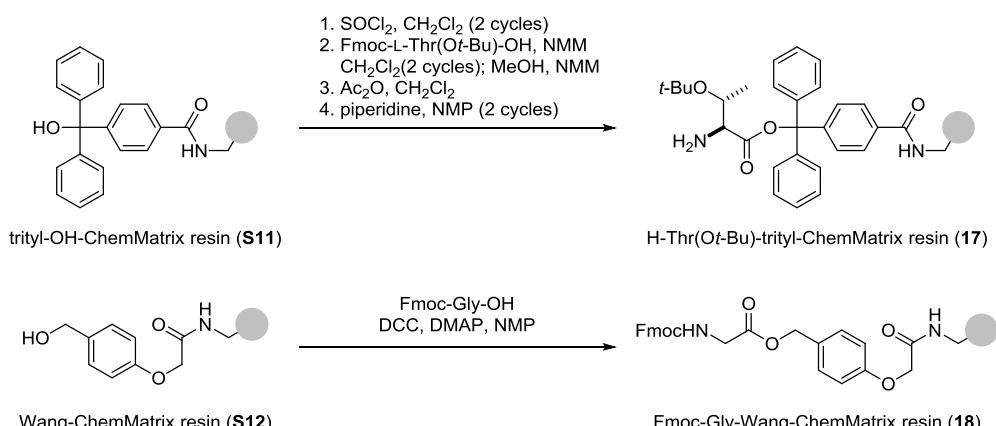


Figure S10. Preparation of **17** and **18**.

Resin 17. To trityl-OH-ChemMatrix resin **S11** (203 mg, 0.40–0.65 mmol/g) in LibraTube was added $\text{SOCl}_2/\text{CH}_2\text{Cl}_2$ (1/49, 2.7 mL). After being stirred at room temperature for 11 h, the reaction mixture was filtered. The same treatment was repeated ($\times 1$).

To the above resin were added a solution of Fmoc-L-Thr(Ot-Bu)-OH (310 mg, 0.780 mmol) in CH_2Cl_2 (1.0 mL) and a solution of *N*-methylmorpholine (NMM, 114 μL , 1.04 mmol) in CH_2Cl_2 (1.0 mL). After being stirred at room temperature for 10 h, the reaction mixture was filtered. The same treatment was repeated ($\times 1$). To the mixture was added NMM/MeOH (1/3, 67 μL). After being stirred at room temperature for 1 h, the resultant mixture was filtered, and washed with CH_2Cl_2 (2.0 mL $\times 3$).

To the above resin was added $\text{Ac}_2\text{O}/\text{CH}_2\text{Cl}_2$ (1/3, 2.0 mL). After being stirred at room temperature for 15 min, the reaction mixture was filtered, washed with CH_2Cl_2 (2.0 mL $\times 5$), DMF (2.0 mL $\times 5$), MeOH (2.0 mL $\times 5$), and Et_2O (2.0 mL $\times 5$), and dried under vacuum to give the dried resin (253 mg). The loading rate was determined to be 0.345 mmol/g by the above method.

The above dried resin (247 mg, 85.2 μ mol) was washed with CH₂Cl₂ (2.0 mL \times 6) and NMP (2.0 mL \times 6). To the resin was added piperidine/NMP (1/4, 2.0 mL). After being stirred at room temperature for 15 min, the reaction mixture was filtered. The same treatment was repeated (\times 1). The resin was washed with NMP (2.0 mL \times 5), CH₂Cl₂ (2.0 mL \times 5), MeOH (2.0 mL \times 5), and Et₂O (2.0 mL \times 5), and dried under vacuum to give resin **17** (228 mg).

Resin 18. To a solution of Fmoc-Gly-OH (240 mg, 807 μ mol) in CH₂Cl₂ (2.5 mL) were added N,N'-dicyclohexylcarbodiimide (DCC, 166 mg, 807 μ mol) and 4-(N,N-dimethylamino)pyridine (DMAP, 2.00 mg, 16.4 μ mol) at room temperature. After being stirred at room temperature for 20 min, the reaction mixture was concentrated to give the crude acid anhydride, which was used in the next reaction without further purification.

Wang-ChemMatrix resin **S12** (265 mg, 161 μ mol) in LibraTube was washed with CH₂Cl₂ (2.0 mL \times 3) and NMP (2.0 mL \times 3). To the resin was added a solution of the above crude acid anhydride in NMP (2.0 mL). After being stirred at room temperature for 60 min, the reaction mixture was filtered. The same treatment was repeated (\times 2). The resin was washed with NMP (2.0 mL \times 3), CH₂Cl₂ (2.0 mL \times 3), MeOH (2.0 mL \times 3), and Et₂O (2.0 mL \times 3), and dried under vacuum to give the preloaded resin (309 mg, 119 μ mol, 74%). The loading rate was determined to be 0.386 mmol/g by the above method.

To the above preloaded resin (304 mg) was added Ac₂O/CH₂Cl₂ (1/3, 4.0 mL) for the capping of remaining hydroxy groups. After being stirred at room temperature for 15 min, the reaction mixture was filtered, washed with NMP (2.0 mL \times 3), CH₂Cl₂ (2.0 mL \times 3), MeOH (2.0 mL \times 3), and Et₂O (2.0 mL \times 3), and dried under vacuum to give resin **18**, which was used in the next reaction.

Fragment 14. Resin **18** (300 mg, 95.3 μ mol) was subjected to 10 cycles (**21**, **22**, **23**, **26**, **27**, and **33**) of microwave-assisted SPPS protocol (steps 1 and 4 were performed at 40 °C) to give the resin-bound peptide **46** (394 mg).

The above resin-bound peptide **46** (189 mg) was washed with CH₂Cl₂ (2 mL \times 3) and NMP (2 mL \times 3). To the resin-bound peptide was added piperidine/NMP (1/4, 2 mL). After being stirred at 40 °C (MW) for 5 min, the reaction mixture was filtered, and washed with NMP (2 mL \times 3), CH₂Cl₂ (2 mL \times 3), and THF (2 mL \times 3). To the resin-bound peptide were added a solution of (benzotriazol-1-yloxy)tritypyrrolidinophosphonium hexafluorophosphate (PyBOP, 95.0 mg, 183 μ mol) in THF (1.0 mL) and a solution of *i*-Pr₂NEt (32.0 μ L, 183 μ mol) and Ncap-OH (**32**, 58.0 mg, 365 μ mol) in THF (1.0 mL). After being stirred at room temperature for 1 h, the reaction mixture was filtered, washed with THF (2 mL \times 3), NMP (2 mL \times 3), and CH₂Cl₂ (2 mL \times 3), and dried under vacuum to give Ncap-[1–11]-Wang-ChemMatrix resin.

To the above resin-bound peptide was added TFA/H₂O (19/1, 2.0 mL). After being stirred at room temperature for 1 h, the reaction mixture was filtered, and concentrated under a stream of Ar to give the crude **14**. The crude **14** was dissolved in *i*-PrOH, and filtered through 0.20 μ m PTFE filter. The filtrate was purified by reversed-phase HPLC (column: Inertsil WP300 C8 7.6 \times 250 mm, eluent A: CH₃CN + 0.05% TFA, eluent B: H₂O + 0.05% TFA, linear gradient: A/B = 50/50 to 100/0 over 40 min, flow rate: 1.5 mL/min,

detection: UV 220 nm, temperature: 40 °C) to give **14** ($t_R = 34\text{--}39$ min, 6.51 mg, 5.59 μmol , 12% over 23 steps): LRMS (ESI-TOF) calcd for $\text{C}_{57}\text{H}_{101}\text{N}_{11}\text{O}_{14} [\text{M}+\text{H}]^+$ 1164.76, found 1164.73.

Fragment 47. Resin **18** (300 mg, 95.3 μmol) was subjected to 10 cycles (**21**, **22**, **23**, **26**, **27**, and **33**) of microwave-assisted SPPS protocol (steps 1 and 4 were performed at 40 °C) to give the resin-bound peptide **46** (394 mg).

To the above resin-bound peptide (81.6 mg) was added TFA/H₂O (19/1, 1.5 mL). After being stirred at room temperature for 1 h, the reaction mixture was filtered, and concentrated under a stream of Ar to give the crude **47**. The crude **47** was dissolved in *i*-PrOH, and filtered through 0.20 μm PTFE filter. The filtrate was purified by reversed-phase HPLC (column: Inertsil WP300 C8 7.6 \times 250 mm, eluent A: CH₃CN + 0.05% TFA, eluent B: H₂O + 0.05% TFA, linear gradient: A/B = 50/50 to 100/0 over 40 min, flow rate: 1.5 mL/min, detection: UV 256 nm, temperature: 40 °C) to give **47** ($t_R = 31\text{--}37$ min, 4.72 mg, 3.79 μmol , 20% over 21 steps): LRMS (ESI-TOF) calcd for $\text{C}_{64}\text{H}_{100}\text{N}_{11}\text{O}_{14} [\text{M}+\text{H}]^+$ 1246.74, found 1246.71.

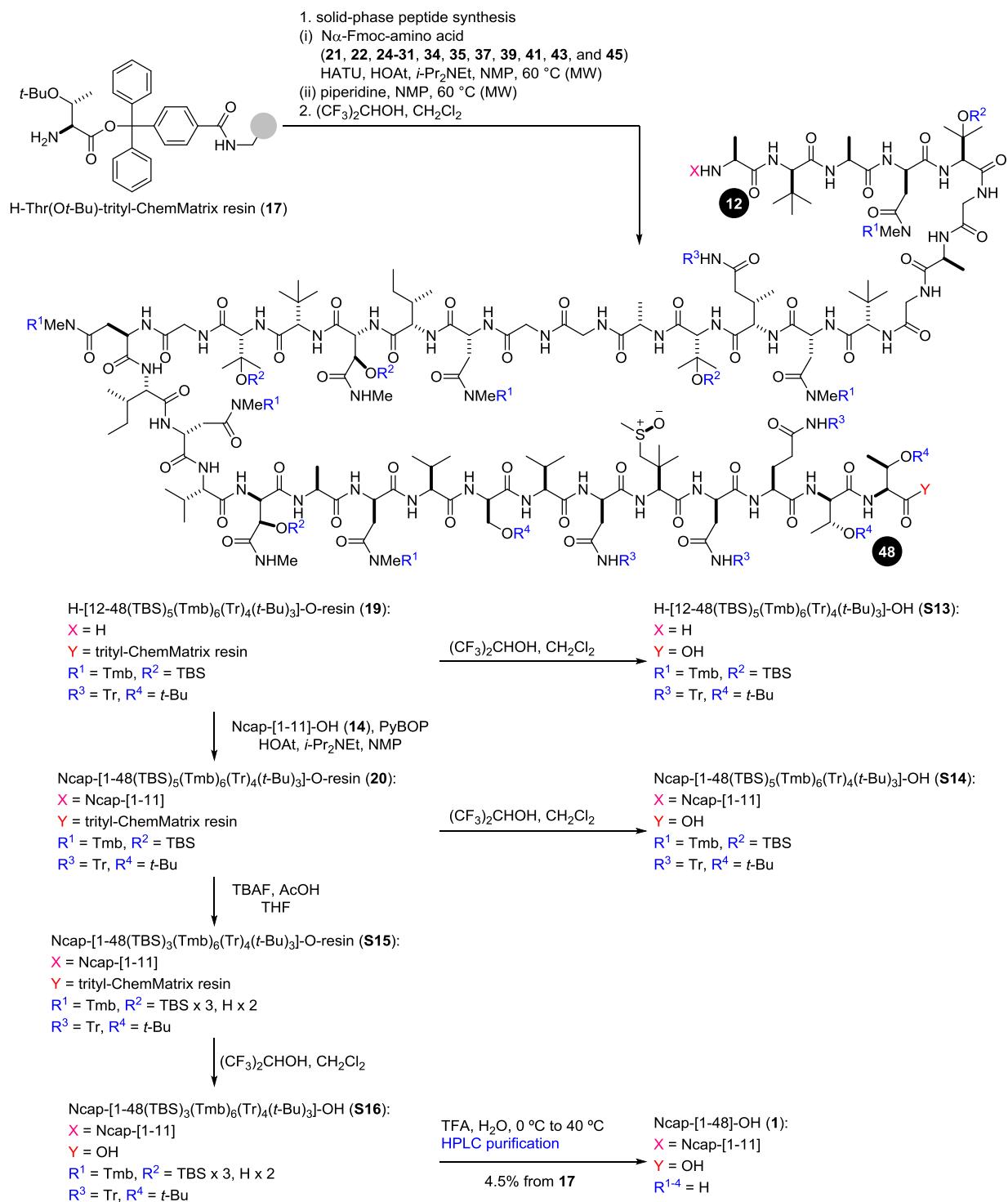


Figure S11. Synthesis of **S13–S16**.

Fragment 19. Resin **17** (55.6 mg, 24.4 μmol) was subjected to 36 cycles (**21**, **22**, **24–26**, **28–31**, **34**, **35**, **39**, **40**, and **42**) of microwave-assisted SPPS protocol (60 °C) to give resin-bound peptide **19** (138 mg). For characterization of the compound, a small amount of resin-bound peptide **19** was separated. To the separated resin-bound peptide **19** was added $(\text{CF}_3)_2\text{CHOH}/\text{CH}_2\text{Cl}_2$ (1/3, 200 μL). After being stirred at room temperature for 1 h, the reaction mixture was concentrated under a stream of Ar to give the crude peptide **S13**. The crude peptide **S13** was dissolved in *i*-PrOH/H₂O (4/1), and filtered with 0.20 μm PTFE filter. The filtrate was analyzed by reversed-phase HPLC (column: COSMOSIL Protein-R 4.6 × 150 mm, eluent A: *i*-PrOH + 0.05% TFA, eluent B: H₂O + 0.05% TFA, linear gradient: A/B = 50/50 to 100/0 over 40 min, flow rate: 0.5 mL/min, detection: UV 220, 254 nm, temperature: 40 °C, $t_{\text{R}} = 25.2\text{--}26.5$ min): LRMS (ESI-TOF) calcd for C₃₄₀H₅₀₃N₄₉O₇₇SSi₅ [M+4H]⁴⁺ 1669.89, found 1669.83.

Polytheonamide B (1). Resin-bound peptide **19** (30.8 mg) in LibraTube was washed with CH₂Cl₂ (2 mL × 3) and NMP (2 mL × 3), and transferred to a glass vial with NMP (300 μL).

To Ncap-[1-11]-OH (**14**, 5.86 mg, 5.03 μmol) were added a solution of PyBOP (2.62 mg, 5.03 μmol) and HOAt (680 μg , 5.03 μmol) in NMP (200 μL), and a solution of *i*-Pr₂NEt (1.76 μL , 10.1 μmol) in NMP (18 μL), and NMP (100 μL). After being stirred at room temperature for 10 min, the reaction mixture was transferred with NMP (100 μL) to the above resin-bound peptide **19** in the glass vial. The reaction mixture was stirred at room temperature for 24 h, and then the solvent was removed with a microsyringe. The resultant resin-bound peptide was washed with NMP (2 mL × 3) and THF (2 mL × 3) to give resin-bound peptide **20**. For characterization of the compound, a small amount of resin-bound peptide **20** was separated. To the separated resin-bound peptide **20** was added $(\text{CF}_3)_2\text{CHOH}/\text{CH}_2\text{Cl}_2$ (1/3, 200 μL). After being stirred at room temperature for 1 h, the reaction mixture was concentrated under a stream of Ar to give the crude peptide **S14**. The crude peptide **S14** was dissolved in *i*-PrOH, and filtered through 0.20 μm PTFE filter. The filtrate was analyzed by reversed-phase HPLC (column: COSMOSIL Protein-R 4.6 × 150 mm, eluent A: *i*-PrOH + 0.05% TFA, eluent B: H₂O + 0.05% TFA, linear gradient: A/B = 50/50 to 100/0 over 40 min, flow rate: 0.5 mL/min, detection: UV 220, 254 nm, temperature: 40 °C, $t_{\text{R}} = 30.7\text{--}34.1$ min): LRMS (ESI-TOF) calcd for C₃₉₇H₆₀₂N₆₀O₉₀SSi₅ [M+4H]⁴⁺ 1956.58, found 1956.59.

To resin-bound peptide **20** in THF (1.0 mL) was added a solution of tetrabutylammonium fluoride (TBAF, 1.0 M in THF, 50.2 μL , 50.2 μmol) and AcOH (2.58 μL , 45.2 μmol) in THF (100 μL). After being stirred at room temperature for 1 h, solvent was removed with a microsyringe. The resultant resin-bound peptide was washed with THF (2 mL × 3) and CH₂Cl₂ (2 mL × 3) to give resin-bound peptide **S15**.

To resin-bound peptide **S15** was added $(\text{CF}_3)_2\text{CHOH}/\text{CH}_2\text{Cl}_2$ (1/3, 1.6 mL). After being stirred at room temperature for 1 h, the reaction mixture was concentrated under a stream of Ar to give the crude peptide **S16**. For characterization of the compound, a small amount of the crude peptide **S16** was dissolved in *i*-PrOH, and filtered through 0.20 μm PTFE filter. The filtrate was analyzed by reversed-phase HPLC (column: COSMOSIL Protein-R 4.6 × 150 mm, eluent A: *i*-PrOH + 0.05% TFA, eluent B: H₂O + 0.05% TFA, linear gradient: A/B = 50/50 to 100/0 over 40 min, flow rate: 0.5 mL/min, detection: UV 220, 254 nm, temperature:

40 °C, $t_R = 29.2\text{--}31.0$ min): LRMS (ESI-TOF) calcd for $C_{385}H_{574}N_{60}O_{90}SSi_3$ [M+4H] $^{4+}$ 1899.53, found 1899.52.

To the above crude peptide **S16** was added TFA/H₂O (19/1, 1.0 mL). After being stirred at 0 °C for 1 h and at 40 °C for 1 h, the reaction mixture was concentrated under a stream of Ar to give the crude peptide. The crude peptide was dissolved in *n*-PrOH/H₂O (1/1), and filtered through 0.20 μm PTFE filter. The filtrate was purified by reversed-phase HPLC (column: Inertsil C8-3 4.6 × 150 mm, eluent A: *n*-PrOH + 0.05% TFA, eluent B: H₂O + 0.05% TFA, linear gradient: A/B = 25/75 to 50/50 over 35 min, flow rate: 0.6 mL/min, detection: UV 220, 254 nm, temperature: 45 °C) to give **1** ($t_R = 30.2\text{--}31.6$ min, 1.15 mg, 228 nmol, 4.5% over 76 steps): LRMS (ESI-TOF) calcd for $C_{219}H_{379}N_{60}O_{72}S$ [M+3H] $^{3+}$ 1678.59, found 1678.52. ¹H NMR, ¹H–¹H DQF-COSY, ¹H–¹H TOCSY, and ¹H–¹H NOESY spectra in DMSO-*d*₆ (0.313 mM) at 40 °C (800 MHz) were shown in Figures S27 and S28, which are identical to those of the previous report.¹ Comparison of fingerprint region of the ¹H–¹H NOESY spectrum is shown in Figure S5.

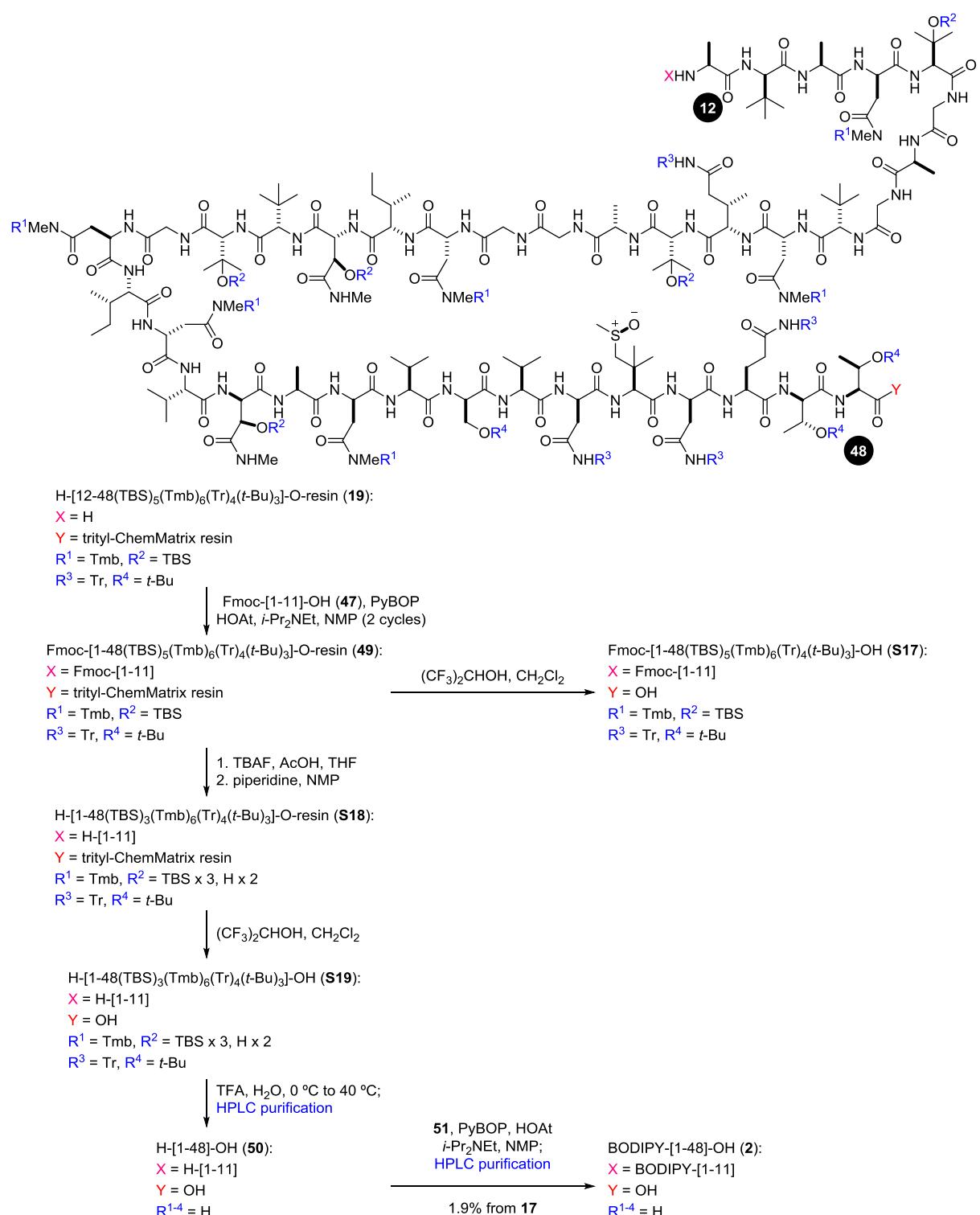


Figure S12. Synthesis of **S17–S19**.

Fluorescent polytheonamide (2). Resin-bound peptide **19** (16.2 mg) in LibraTube was washed with CH₂Cl₂ (2 mL × 3) and NMP (2 mL × 3), transferred to a glass vial with NMP, and then the solvent was removed with a microsyringe.

To Fmoc-[1–11]-OH (**47**, 2.55 mg, 2.05 μmol) were added a solution of PyBOP (1.07 mg, 2.05 μmol) and HOAt (280 μg, 2.05 μmol) in NMP (100 μL) and a solution of *i*-Pr₂NEt (0.714 μL, 4.10 μmol) in NMP (50 μL). After being stirred at room temperature for 10 min, the reaction mixture was transferred with NMP (100 μL) to resin **19** in the glass vial. The reaction mixture was stirred at room temperature for 22 h, and then solvent was removed with a microsyringe. To Fmoc-[1–11]-OH (**47**, 0.472 mg, 0.379 μmol) were added a solution of PyBOP (0.197 mg, 0.379 μmol) and HOAt (51.6 ng, 0.379 μmol) in NMP (100 μL) and a solution of *i*-Pr₂NEt (0.132 μL, 0.758 μmol) in NMP (50 μL). After being stirred at room temperature for 10 min, the mixture was transferred with NMP (100 μL) to the above resin in the glass vial. The reaction mixture was stirred at room temperature for additional 22 h, and then solvent was removed with a microsyringe. The resin was washed with NMP (2 mL × 3) and THF (2 mL × 3) to give resin-bound peptide **49**. For characterization of the compound, a small amount of resin-bound peptide **49** was separated. To the separated resin-bound peptide was added (CF₃)₂CHOH/CH₂Cl₂ (1/3, 200 μL). After being stirred at room temperature for 1 h, the reaction mixture was concentrated under a stream of Ar to give the crude peptide **S17**. The crude peptide **S17** was dissolved in *i*-PrOH, and filtered with 0.20 μm PTFE filter. The filtrate was analyzed by reversed-phase HPLC (column: COSMOSIL Protein-R 4.6 × 150 mm, eluent A: *i*-PrOH + 0.05% TFA, eluent B: H₂O + 0.05% TFA, linear gradient: A/B = 50/50 to 100/0 over 40 min, flow rate: 0.5 mL/min, detection: UV 220, 254 nm, temperature: 40 °C, *t*_R = 32.0–33.3 min): LRMS (ESI-TOF) calcd for C₄₀₄H₆₀₀N₆₀O₉₀SSi₅ [M+4H]⁴⁺ 1977.07, found 1977.07.

To resin-bound peptide **49** in THF (400 μL) was added a solution of TBAF (1.0 M in THF, 26.4 μL, 26.4 μmol) and AcOH (1.36 μL, 23.8 μmol) in THF (136 μL). After being stirred at room temperature for 1 h, solvent was removed with a microsyringe. The resultant resin-bound peptide was washed with THF (2 mL × 3) and NMP (2 mL × 3).

To the above resin-bound peptide was added piperidine/NMP (1/4, 500 μL). After being stirred at room temperature for 30 min, the solvent was removed with a microsyringe. The resin was washed with NMP (2 mL × 3) and CH₂Cl₂ (2 mL × 3) to give resin-bound peptide **S18**.

To resin-bound peptide **S18** was added (CF₃)₂CHOH/CH₂Cl₂ (1/3, 800 μL). After being stirred at room temperature for 1 h, the reaction mixture was concentrated under a stream of Ar to give the crude peptide **S19**. For characterization of the compound, a small amount of the crude peptide **S19** was dissolved in *i*-PrOH, and filtered through 0.20 μm PTFE filter. The filtrate was analyzed by reversed-phase HPLC (column: COSMOSIL Protein-R 4.6 × 150 mm, eluent A: *i*-PrOH + 0.05% TFA, eluent B: H₂O + 0.05% TFA, linear gradient: A/B = 50/50 to 100/0 over 40 min, flow rate: 0.5 mL/min, detection: UV 220, 254 nm, temperature: 40 °C, *t*_R = 28.6–30.4 min): LRMS (ESI-TOF) calcd for C₃₇₇H₅₆₂N₆₀O₈₈SSi₃ [M+4H]⁴⁺ 1864.51, found 1864.46.

To the above crude peptide **S19** was added TFA/H₂O (19/1, 500 μL). After being stirred at 0 °C for 1 h and at 40 °C for 1 h, the reaction mixture was concentrated under a stream of Ar to give the crude peptide.

The crude peptide was dissolved in *n*-PrOH/H₂O (1/1), and filtered through 0.20 μm PTFE filter. The filtrate was purified by reversed-phase HPLC (column: Inertsil C8-3 4.6 × 150 mm, eluent A: *n*-PrOH + 0.05% TFA, eluent B: H₂O + 0.05% TFA, linear gradient: A/B = 20/80 to 37/63 over 25 min, flow rate: 0.6 mL/min, detection: UV 220, 254 nm, temperature: 45 °C) to give **50** (*t_R* = 19.5–20.4 min), which was used in the next reaction without further purification: LRMS (ESI-TOF) calcd for C₂₁₁H₃₆₇N₆₀O₇₀S [M+3H]³⁺ 1631.89, found 1631.83.

To **51** (46.2 μg, 114 nmol) were added a solution of PyBOP (59.3 μg, 114 nmol) and HOAt (15.5 μg, 114 nmol) in NMP (10 μL), and a solution of *i*-Pr₂NEt (39.7 nL, 228 nmol) in NMP (10 μL), and NMP (80 μL). After being stirred at room temperature for 5 min, the mixture was transferred with NMP (100 μL) to one third of the crude peptide **50** in a glass vial. The reaction mixture was stirred at room temperature for 21 h, and then solvent was removed under a stream of Ar to give the crude peptide. The crude peptide was dissolved in *n*-PrOH/H₂O (1/1), and filtered through 0.20 μm PTFE filter. The filtrate was purified by reversed-phase HPLC (column: Inertsil C8-3 4.6 × 150 mm, eluent A: *n*-PrOH + 0.05% TFA, eluent B: H₂O + 0.05% TFA, linear gradient: A/B = 25/75 to 50/50 over 35 min, flow rate: 0.6 mL/min, detection: UV 220, 254 nm, temperature: 45 °C) to give **2** (*t_R* = 23.4–24.4 min, 16.9 nmol, 1.9% over 78 steps): LRMS (ESI-TOF) calcd for C₂₃₁H₃₉₁BF₂N₆₃O₇₂S [M+3H]³⁺ 1760.96, found 1760.93.

Cytotoxicity Assay

Cell Culture

P388 mouse leukemia cells were obtained from Institute of Development Aging and Cancer (Tohoku University). Cells were maintained in RPMI1640 growth medium [RPMI1640 with phenol red (FUJIFILM Wako Pure Chemical), 10v/v% heat-inactivated fetal bovine serum, penicillin G (100 units/mL), and streptomycin (100 µg/mL)] under atmosphere of 5% CO₂ at 37 °C.

MCF-7 human breast cancer cells were obtained from the American Type Culture Collection. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) growth medium [DMEM-low glucose (Sigma), 10v/v% heat-inactivated fetal bovine serum, penicillin G (100 units/mL), and streptomycin (100 µg/mL)] under atmosphere of 5% CO₂ at 37 °C. The growth medium was refreshed every 2 or 3 d to reach 70–90% cell confluence.

WST-1 Assay

Various concentrations of compounds **1** and **2** in DMSO/RPMI1640 growth medium (1/49) were prepared by 5-fold serial dilutions. P388 cells were cultured in the growth medium at 37 °C and harvested at 4 °C by centrifugation at 1000 rpm for 3 min using a MRX-150 centrifugator equipped with a TMA-3 rotor (Tomy). The collected cells were resuspended into the growth medium at 2 × 10⁴ cells/mL. Aliquots of the former medium (100 µL) containing peptides and the latter medium containing P388 cells (100 µL) were mixed in 96-well plates. The final concentration of **1** and **2** ranged from 0.16 pM to 320 nM. The final concentration of P388 cells and DMSO were 1 × 10⁴ cells/mL and 1%, respectively. The cells were incubated at 37 °C under atmosphere of 5% CO₂ for 92 h, and then the number of viable cells was determined by WST-1 assay. A solution of 1-methoxy-PMS (1-methoxy-5-methylphenazinium methylsulfate) /WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt] in growth medium [1-methoxy-PMS 0.70 mg/mL in H₂O:WST-1 3.6 mg/mL in pH 7.4 buffer (20 mM HEPES) = 1/9 v/v, 20 µL] were added to each well and further incubated for 4 h under atmosphere of 5% CO₂ at 37 °C. The absorbance of each well at 415 nm was measured using on Benchmark microplate reader (Bio-Rad). The cytotoxicity of each compound was evaluated as IC₅₀ (nM) by means of three replicates. Sigmoidal curve fittings were performed on Graphpad Prism (Graphpad Software). The cell viability was normalized against untreated cells as 100% and the growth medium as 0%. The obtained IC₅₀ values by three independent assays were 0.086 ± 0.011 nM for **1** and 0.30 ± 0.046 nM for **2**, respectively.

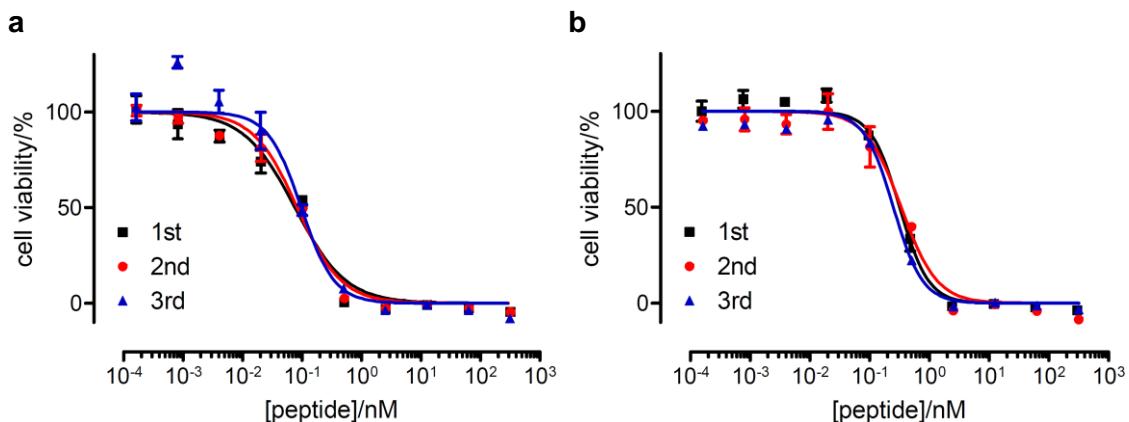


Figure S13. Dose-dependent effects of (a) **1** and (b) **2** on the cell viability (%) of P388 mouse leukemia cells (3 independent experiments). Each plot is displayed as mean \pm S.D.

Sulforhodamine B Assay

Various concentrations of compounds **1** and **2** in DMSO/DMEM growth medium (1/49) were prepared by 5-fold serial dilutions. MCF-7 cells were cultured in the growth medium at 37 °C and harvested by trypsinization at 37 °C for 5–10 min. The collected cells were resuspended into the growth medium at 12.5×10^5 cells/mL. The cell suspension (100 μ L/well) was seeded into black polystyrene flat-bottom 96-well plates (Greiner bio-one). The cells were incubated at 37 °C under atmosphere of 5% CO₂ for 24 h. Aliquots of the former medium (100 μ L) containing compounds were added to each well. The final concentration of **1** and **2** ranged from 0.19 pM to 380 nM. The final concentration of cells and DMSO were 6.25×10^5 cells/mL and 1%, respectively. After being incubated at 37 °C under atmosphere of 5% CO₂ for 48 h, the cells were fixed by ice-cold trichloroacetic acid/H₂O (3/7 w/v%) at 4 °C for 60 min. The fixed cells were washed with water (\times 4) and dried. To each well was added a solution of sulforhodamine B (SRB) in AcOH/H₂O (1/99) (0.57 mg/mL, 100 μ L/well). The fixed cells were stained at room temperature for 30 min in the dark. The cells were washed with AcOH/H₂O (1/99, \times 4) and dried. To the stained cells was added a solution of Tris-base in H₂O (10 mM, 200 μ L/well). The plate was vortexed at room temperature for 10 min. The fluorescence (Ex. 485 nm/Em. 585 nm) of each well was measured on Spectra Max Gemini EM microplate reader (Molecular Devices). The cytotoxicity of each compound was evaluated as IC₅₀ (nM) by means of three replicates. Sigmoidal curve fittings were performed on Graphpad Prism (Graphpad Software). The cell viability was normalized against untreated cells as 100% and the growth medium as 0%. The obtained IC₅₀ values by three independent assays were 5.9 ± 0.33 nM for **1** and 13 ± 0.20 nM for **2**, respectively.

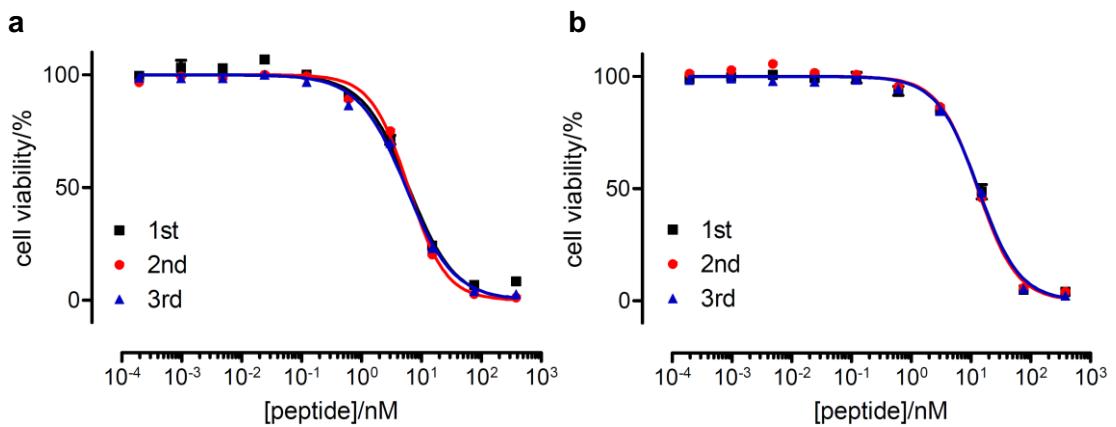


Figure S14. Dose-dependent effects of (a) **1** and (b) **2** on the cell viability (%) of MCF-7 human breast cancer cells (3 independent experiments). Each plot is displayed as mean \pm S.D.

Plasma Membrane Potential Assay

Plasma Membrane Depolarization Assay Using DiBAC₄(3)

Various concentrations of compounds **1** and **2**, and monensin in DMSO were prepared by 5-fold serial dilutions. MCF-7 cells were cultured in the growth medium at 37 °C and harvested by trypsinization at 37 °C for 5–10 min. The collected cells were resuspended into the growth medium at 3.00×10^5 cells/mL. The cell suspension (100 µL/well) was seeded into a black polystyrene flat-clear-bottom 96-well plate (Greiner bio-one). The cells were incubated at 37 °C under atmosphere of 5% CO₂ for 2 d, and then each well was washed (200 µL × 2) and filled (198 µL) with the buffer [20 mM HEPES, 120 mM NaCl, 2 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 5 mM glucose, 1 µM DiBAC₄(3)]. The cells were incubated at 37 °C under atmosphere of 5% CO₂ for 1 h. Aliquots of DMSO solution containing the compounds (2 µL/well) were added to each well. The final concentration of the compounds ranged from 0.2 nM to 25 nM for **1** and **2**, and 0.4 µM to 10 µM for monensin. The fluorescence (Ex. 485 nm/Em. 530 nm) of each well was measured at every 5 min for 60 min with a Spectra Max Gemini EM microplate reader. The background drift from DMSO addition and non-specific interaction between the tested peptides and DiBAC₄(3) was canceled by subtracting the control traces obtained from vehicle control (DMSO without peptides) and peptides with DiBAC₄(3) without cells, respectively. The fluorescence intensities were evaluated by means of four replicates.

Cellular Imaging Analysis

Preparation of MCF-7 Cells

MCF-7 cells were cultured in the growth medium at 37 °C and harvested by trypsinization at 37 °C for 5–10 min. The collected cells were resuspended into the growth medium at 5.0×10^4 cells/mL. The cell suspension (1.0 mL) was seeded to 3.5 cm poly-L-lysine coated glass-bottom dish (Matsunami Glass Ind.).

Endocytosis Inhibition Assay

The cells were preincubated at 37 °C under atmosphere of 5% CO₂ for 1 d, and then the medium was refreshed (1.0 mL). The cells were incubated at 37 °C under atmosphere of 5% CO₂ for 1 d. A solution of the endocytosis or metabolic inhibitor [a solution of NaN₃ and 2-deoxyglucose in PBS (+) (NaN₃: 1.0 M, 10 µL, final concentration = 10 mM; 2-deoxyglucose: 0.5 M, 10 µL, final concentration = 5 mM), a solution of chlorpromazine in DMSO (2.0 mM, 5.0 µL, final concentration = 10 µM), a solution of nystatin in DMSO (10 mM, 5.0 µL, final concentration = 50 µM), or a solution of EIPA in DMSO (2.0 mM, 5.0 µL, final concentration = 10 µM)] was added, and then the cells were incubated for 30 min. A solution of **2** in DMSO (2.0 µM, 5.0 µL, final concentration = 10 nM) was added to the cells, and then the cells were incubated at 37 °C under atmosphere of 5% CO₂ for 50 min. A solution of Hoechst 33342 (Thermo Fisher Scientific) in H₂O (1 mM, 10 µL, final concentration = 10 µM) was added to the cells, and then the cells were incubated for 10 min. The dish was washed (1.0 mL × 3) and filled (1.0 mL) with Hank's Balanced Salt Solution (HBSS containing calcium, magnesium and no phenol red), and subjected to confocal fluorescence microscopic analysis on LSM510-META equipped with a 63× oil-immersion objective lens (Carl Zeiss).

For low temperature experiment (Figure S9a, 4 °C), the cells were preincubated at 4 °C for 30 min. A solution of **2** in DMSO (2.0 µM, 5.0 µL, final concentration = 10 nM) was added, and then the cells were incubated at 4 °C for 50 min. A solution of Hoechst 33342 in H₂O (1 mM, 10 µL, final concentration = 10 µM) was added to the cells, and then the cells were incubated for 10 min. The dish was washed (1.0 mL × 3) and filled (1.0 mL) with HBSS, and subjected to confocal fluorescence microscopic analysis on LSM510-META.

The number of particles and particle size were analyzed by ‘Analyze Particles’ function using ImageJ.^{S3}

Subcellular Localization Analysis of Fluorescent Polytheonamide with LysoTracker Red

The cells were preincubated at 37 °C under atmosphere of 5% CO₂ for 1 d, and then the medium was refreshed (1.0 mL). The cells were incubated at 37 °C under atmosphere of 5% CO₂ for 1 d. A solution of **2** in DMSO (0.2 or 2.0 μM, 5.0 μL, final concentration = 1.0 or 10 nM) was added, and then the cells were incubated at 37 °C under atmosphere of 5% CO₂ for different period (1 h, 2 h, 4 h, or 8 h). A solution of LysoTracker Red DND-99 (Thermo Fisher Scientific) in DMSO (10 μM, 5.0 μL, final concentration = 50 nM) was added to the cells, and then the cells were incubated for 50 min. A solution of Hoechst 33342 in H₂O (1 mM, 10 μL, final concentration = 10 μM) was added to the cells, and then the cells were incubated for 10 min. The dish was washed (1.0 mL × 3) and filled (1.0 mL) with HBSS, and subjected to confocal fluorescence microscopic analysis on SD-OSR (Olympus). Pearson's correlation coefficients (PCC) were calculated by Costes' analysis using ImageJ with JACoP Plug-in^{S4}.

Subcellular Localization Analysis of Fluorescent Polytheonamide with RFP-LAMP1

The cells were preincubated at 37 °C under atmosphere of 5% CO₂ for 1 d, and then the medium was refreshed (1.0 mL). CellLight Lysosomes-RFP, BacMam 2.0 (30 μL, particles per cell = 60, Thermo Fisher Scientific) was added to the cells for expressing RFP-LAMP1. The cells were incubated at 37 °C under atmosphere of 5% CO₂ for 1 d, and then a solution of **2** in DMSO (2.0 μM, 10 μL, final concentration = 20 nM) was added. The cells were incubated at 37 °C under atmosphere of 5% CO₂ for different period (1 h, 2 h, 4 h, or 8 h). The dish was washed (1.0 mL × 3) and filled (1.0 mL) with HBSS, and subjected to confocal fluorescence microscopic analysis on LSM510-META. PCCs were calculated by Costes' analysis using ImageJ with JACoP Plug-in.

Localization Analysis of LysoTracker Red

The cells were preincubated at 37 °C under atmosphere of 5% CO₂ for 1 d, and then the medium was refreshed (1.0 mL). The cells were incubated at 37 °C under atmosphere of 5% CO₂ for 1 d, and then a solution of **1** in DMSO (1.0 μM, 5.0 μL, final concentration = 5.0 nM) or vehicle (DMSO) was added. The cells were incubated at 37 °C under atmosphere of 5% CO₂ for different period (0 h, 1 h, or 3 h). A solution of LysoTracker Red DND-99 in DMSO (10 μM, 5.0 μL, final concentration = 50 nM) was added to the cells, and then the cells were incubated for 50 min. A solution of Hoechst 33342 in H₂O (1 mM, 10 μL, final concentration = 10 μM) were added to the cells, and then the cells were incubated for 10 min. The dish was washed (1.0 mL × 3) and filled (1.0 mL) with HBSS, and subjected to confocal fluorescence microscopic analysis on LSM510-META. The number of particles and particle size were analyzed by 'Analyze Particles' function using ImageJ.

Cathepsin B Activity Assay

The cells were preincubated at 37 °C under atmosphere of 5% CO₂ for 1 d, and then the medium was refreshed (1.0 mL). The cells were incubated at 37 °C under atmosphere of 5% CO₂ for 1 d, and then a solution of **1** in DMSO (1.0 μM, 5.0 μL, final concentration = 5.0 nM) or vehicle (DMSO) was added. The

cells were incubated at 37 °C under atmosphere of 5% CO₂ for different period (0.5 h, 1.5 h, or 3.5 h). A solution of MR-(RR)₂ (5.0 μL #6133, Immunochemistry Technologies) in DMSO/H₂O (1/9, 500 μL) was added to the cells, and then the cells were incubated for 20 min. A solution of Hoechst 33342 in H₂O (1 mM, 10 μL, final concentration = 10 μM) were added to the cells, and then the cells were incubated for 10 min. The dish was washed (1.0 mL × 3) and filled (1.0 mL) with HBSS, and subjected to confocal fluorescence microscopic analysis on LSM510-META. The number of particles and particle size were analyzed by ‘Analyze Particles’ function using ImageJ.

Lysosomal pH Measurement with FRD

The cells were preincubated at 37 °C under atmosphere of 5% CO₂ for 1 d, and then the medium was refreshed (0.5 mL). Fluorescein-tetramethylrhodamin-tagged dextran (FRD, average molecular weight 70 kDa, 0.25 mg, final concentration 0.5 mg/mL, Thermo Fisher Scientific) was added to the cells. The cells were incubated at 37 °C under atmosphere of 5% CO₂ for 15 h, and then a solution of **1** in DMSO (1.0 μM, 5.0 μL, final concentration = 5.0 nM) or vehicle (DMSO) was added. The cells were incubated at 37 °C under atmosphere of 5% CO₂ for different period (1 h, 2 h, 4 h, or 8 h for **1**, 8 h for the vehicle). The dish was washed (1.0 mL × 3) and filled (1.0 mL) with HBSS, and subjected to confocal fluorescence microscopic analysis on ECLIPSE Ti2 (Nikon) with Dragonfly system (Oxford instruments).

For calibration of lysosomal pH, cells were seeded to the independent dish. FRD was loaded to the cell as the same procedure described above, and the dish was washed (1.0 mL × 3) and filled (1.0 mL) with buffer (pH 4.02, 5.03, 6.08, 6.94, or 8.05, 10 mM HEPES, 120 mM KCl, 20 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂). A solution of the additives (1.0 mM nigericin, 0.1 mM monensin, and 10 μM bafilomycin A1) in DMSO (10 μL, final concentration = 10 μM nigericin, 1.0 μM monensin, and 0.1 μM bafilomycin A1) were added to the cells. The cells were incubated for 15 min at room temperature, and subjected to confocal fluorescence microscopic analysis on ECLIPSE Ti2.

Mitochondrial Membrane Potential Assay

The cells were preincubated at 37 °C under atmosphere of 5% CO₂ for 1 d, and then the medium was refreshed (1.0 mL). The cells were incubated at 37 °C under atmosphere of 5% CO₂ for 1 d, and then a solution of **1** in DMSO (1.0 μM, 5.0 μL, final concentration = 5.0 nM) or vehicle (DMSO) was added. The cells were incubated at 37 °C under atmosphere of 5% CO₂ for different period (0.5 h, 1.5 h, 3.5 h, or 7.5 h). A solution of tetramethyl rhodamine methyl ester (TMRM) in DMSO (50 μM, 5.0 μL, final concentration = 250 nM) was added to the cells, and then the cells were incubated for 20 min. A solution of Hoechst 33342 in H₂O (1 mM, 10 μL, final concentration = 10 μM) was added to the cells, and then the cells were incubated for 10 min. The dish was washed (1.0 mL × 3) and filled (1.0 mL) with HBSS, and subjected to confocal fluorescence microscopic analysis on LSM510-META.

Apoptosis Assay

PI-annexin V assay

MCF-7 cells were cultured in the growth medium at 37 °C and harvested by trypsinization at 37 °C for 5–10 min. The collected cells were resuspended into the growth medium (5.0 mL). The cell suspension (1.0 mL) was seeded to 6.0 cm tissue culture dishes (Asahi Glass) with 5.0 mL growth medium. The cells were preincubated at 37 °C under atmosphere of 5% CO₂ for 2 d, and then the medium was refreshed (2.0 mL). The cells were incubated at 37 °C under atmosphere of 5% CO₂ for different period (47, 46, 44, 24, or 0 h), and then a solution of **1** in DMSO (0.5 µM, 20 µL, final concentration = 5.0 nM) or vehicle (DMSO) was added to the cells. The cells were incubated for different period (1, 2, 4, 8, 24, or 48 h). After the incubation, the medium was collected and the cells were harvested by trypsinization at 37 °C for 3 min. The collected cells were resuspended into the fresh growth medium (5.0 mL), mixed with supernatant, and harvested at 4 °C by centrifugation at 1000 rpm for 3 min using a MRX-150 centrifugator equipped with a TMA-3 rotor. The collected cells were resuspended into the binding buffer (0.5 mL: 10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1.8 mM MgCl₂, 1 mM CaCl₂). The cell suspension (0.1 mL) was transferred to a microtube (1.5 mL, BM Equipment), and then a solution of propidium iodide (PI) in H₂O (1.5 µL: PI 100 µg/mL) and FITC-annexin V (3.0 µL 4700-100, Medical & biological laboratories) were added to the cell suspension. The cell suspension was incubated at room temperature for 5 min in the dark, and then the binding buffer (400 µL) was added. The resultant suspension was analyzed by BD FACSaria II (Becton Dickinson).

NMR Spectra

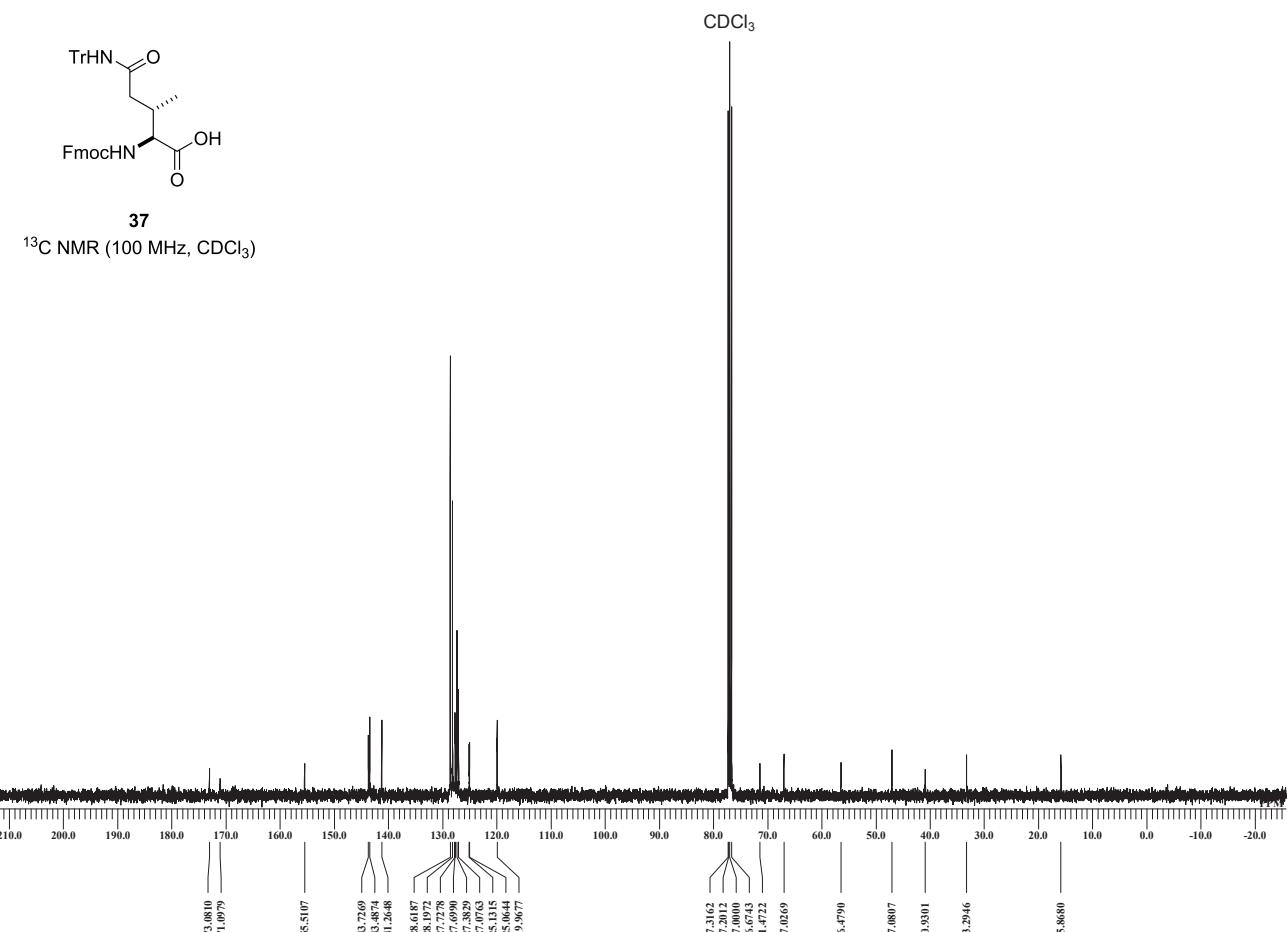
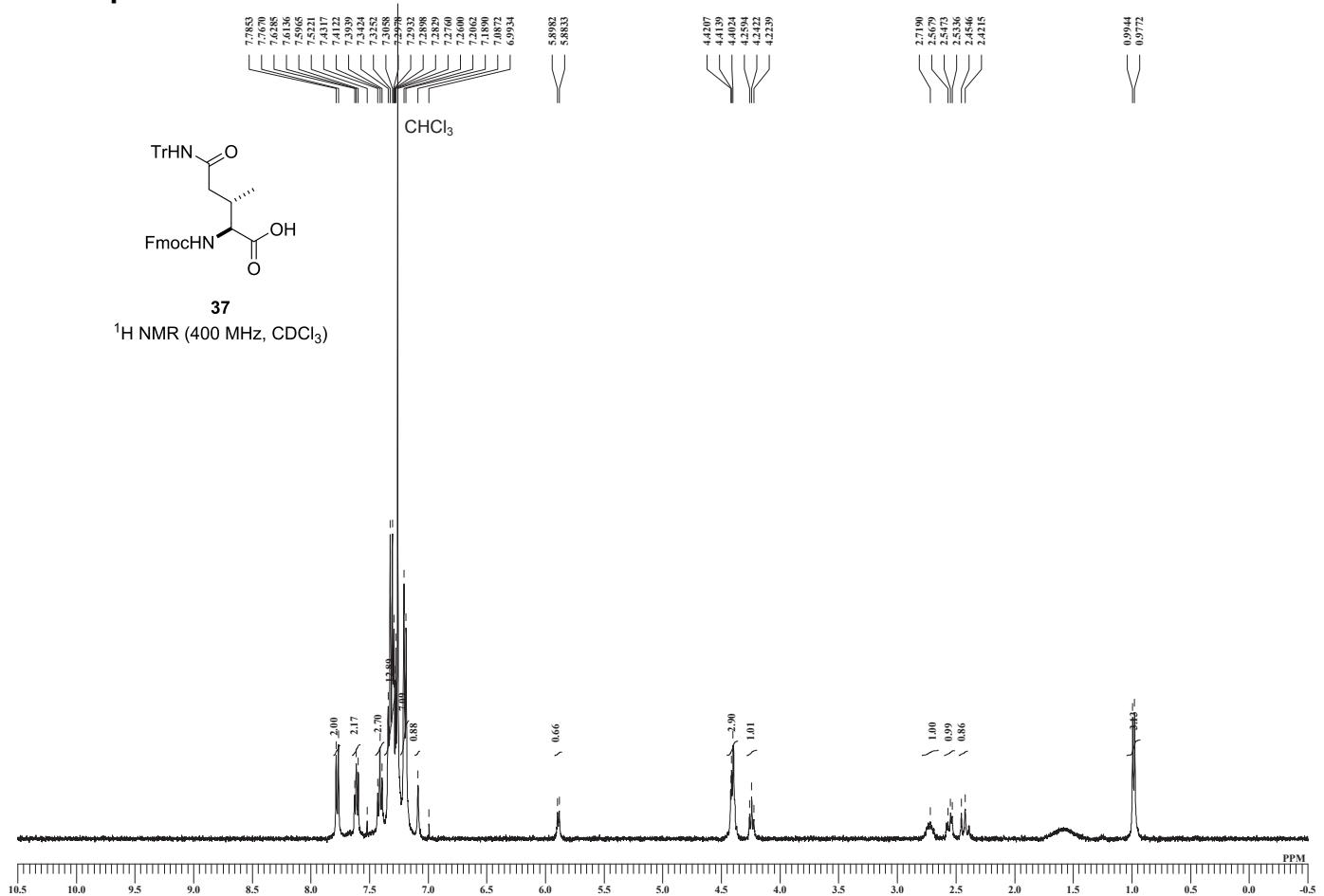


Figure S15. ^1H and ^{13}C NMR spectra of **37**.

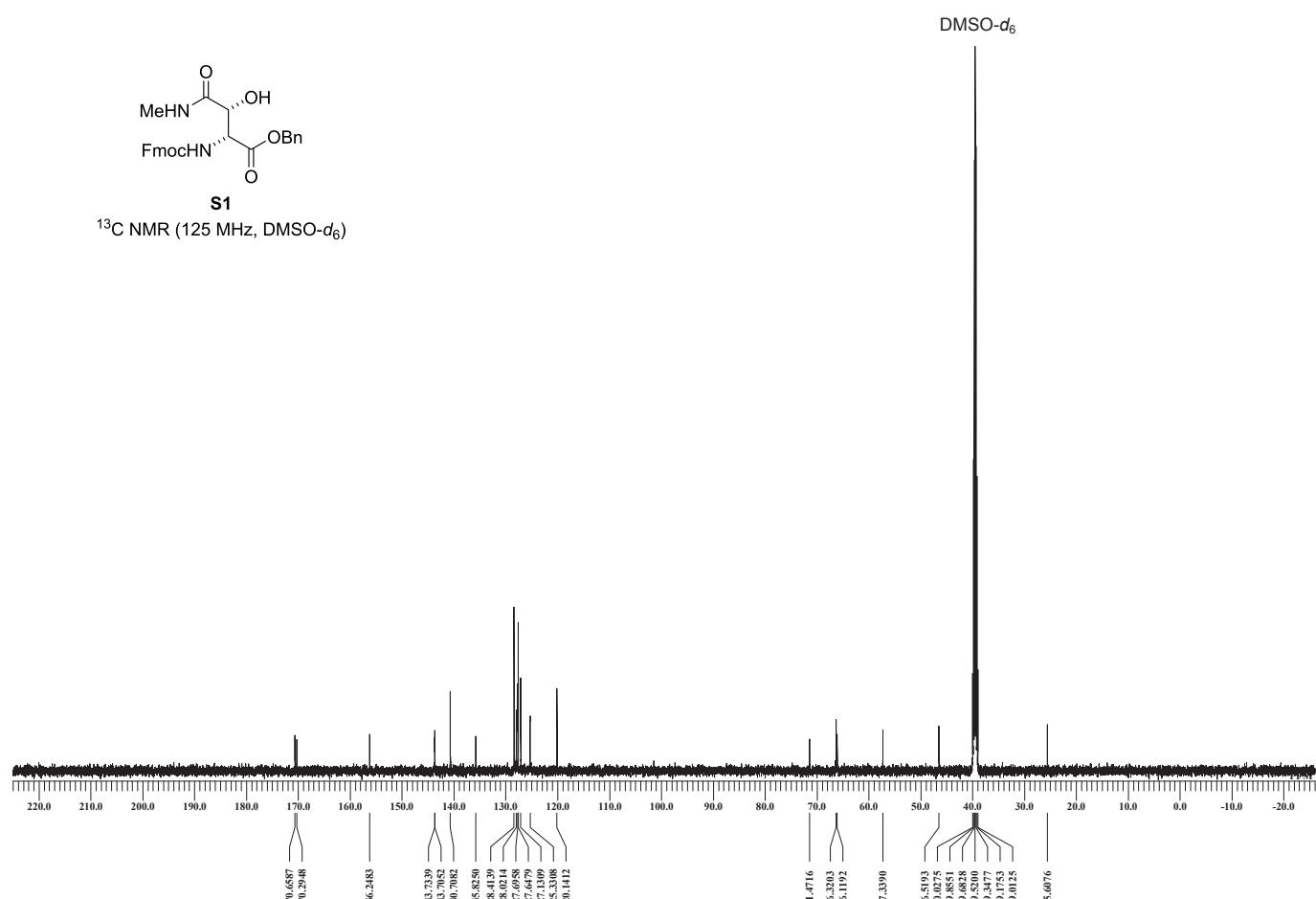
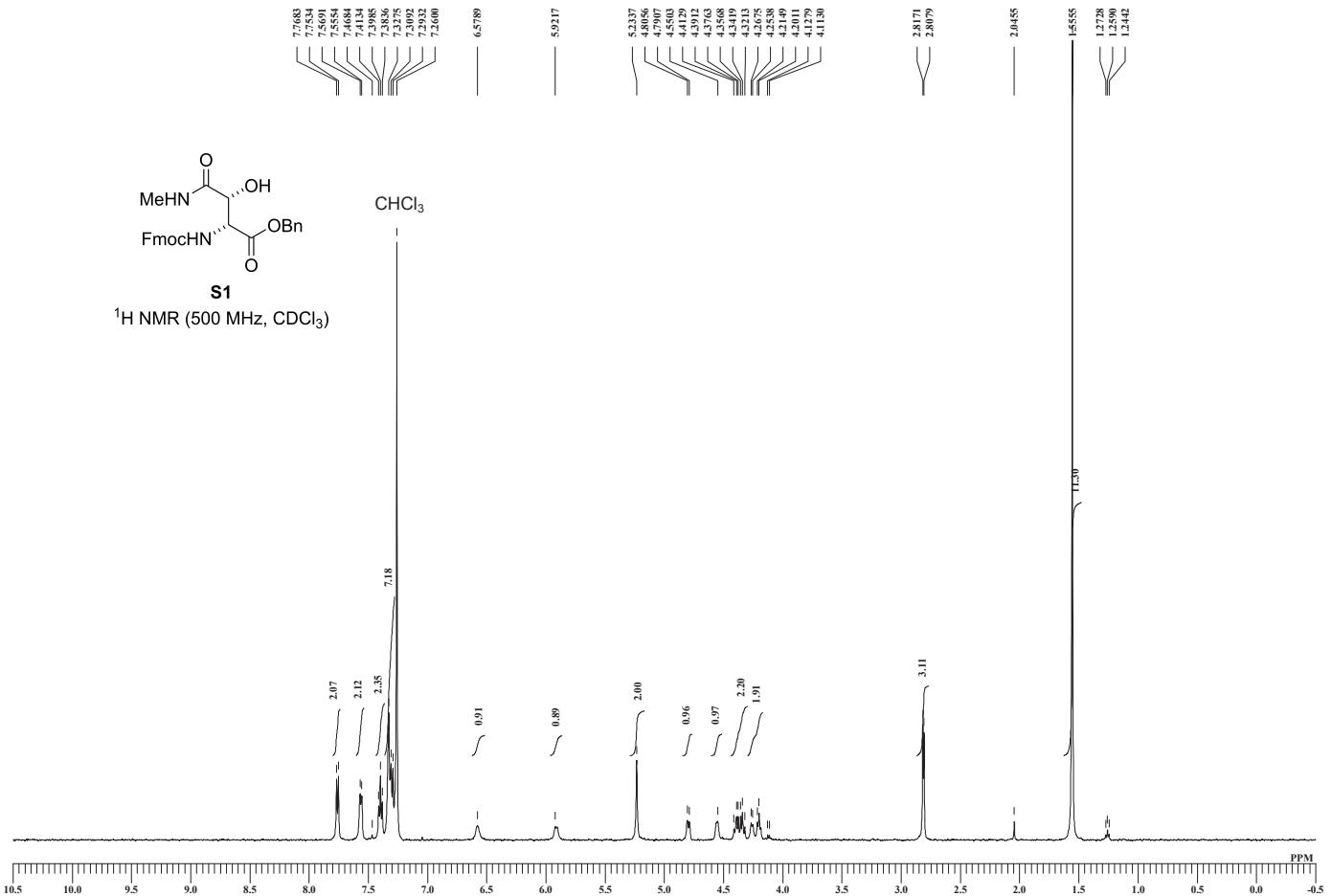
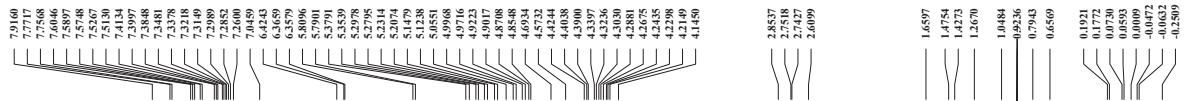
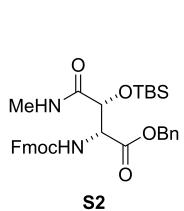
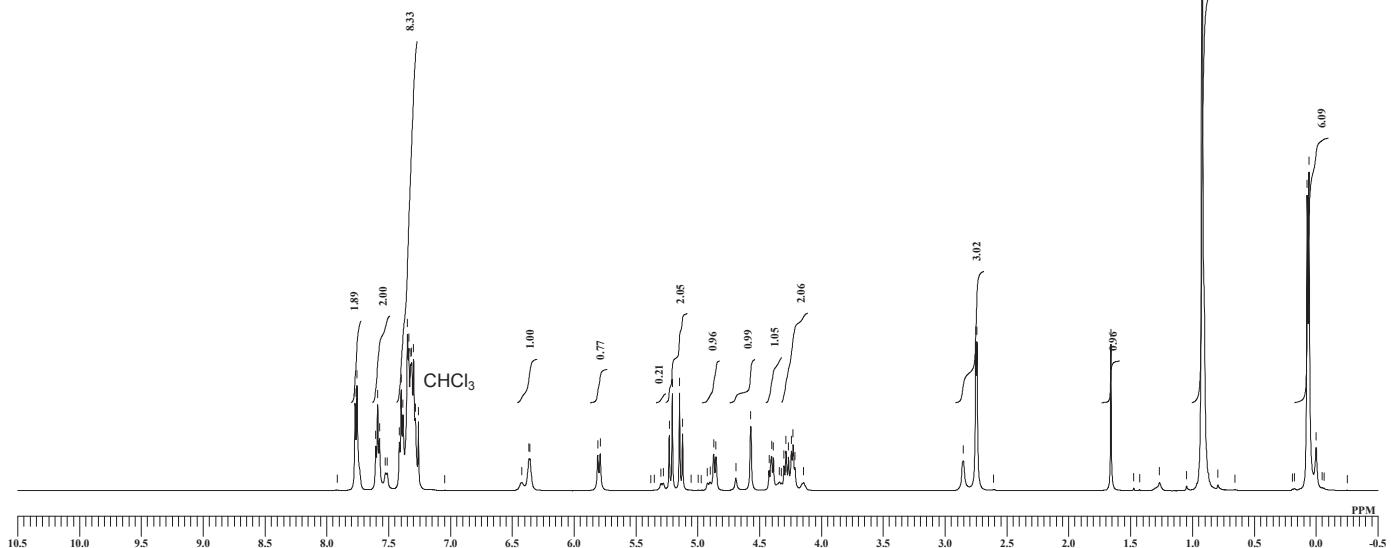


Figure S16. ¹H and ¹³C NMR spectra of S1.



S2
¹H NMR (500 MHz, CDCl₃)



¹³C NMR (125 MHz, CDCl₃)

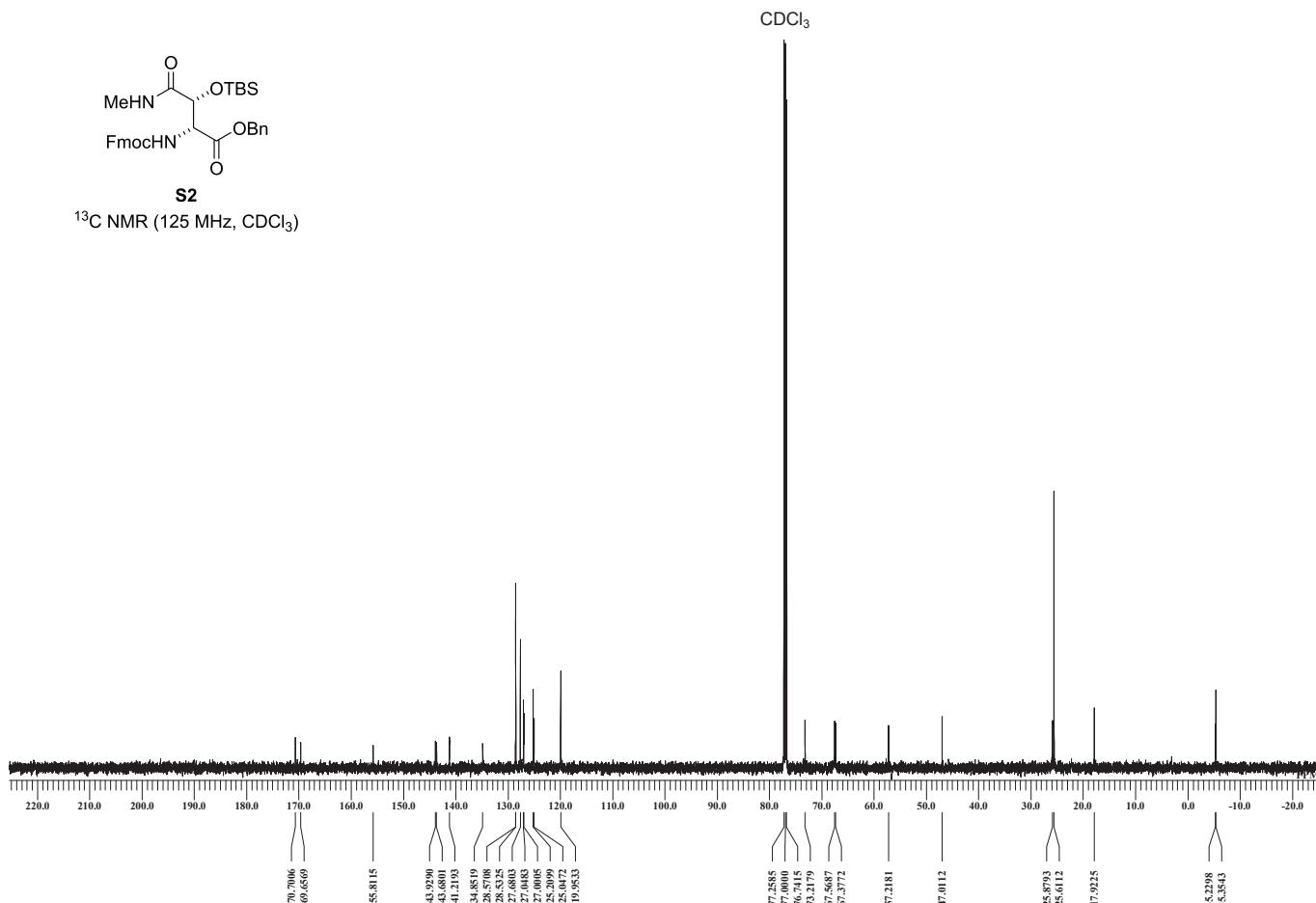


Figure S17. ¹H and ¹³C NMR spectra of S2.

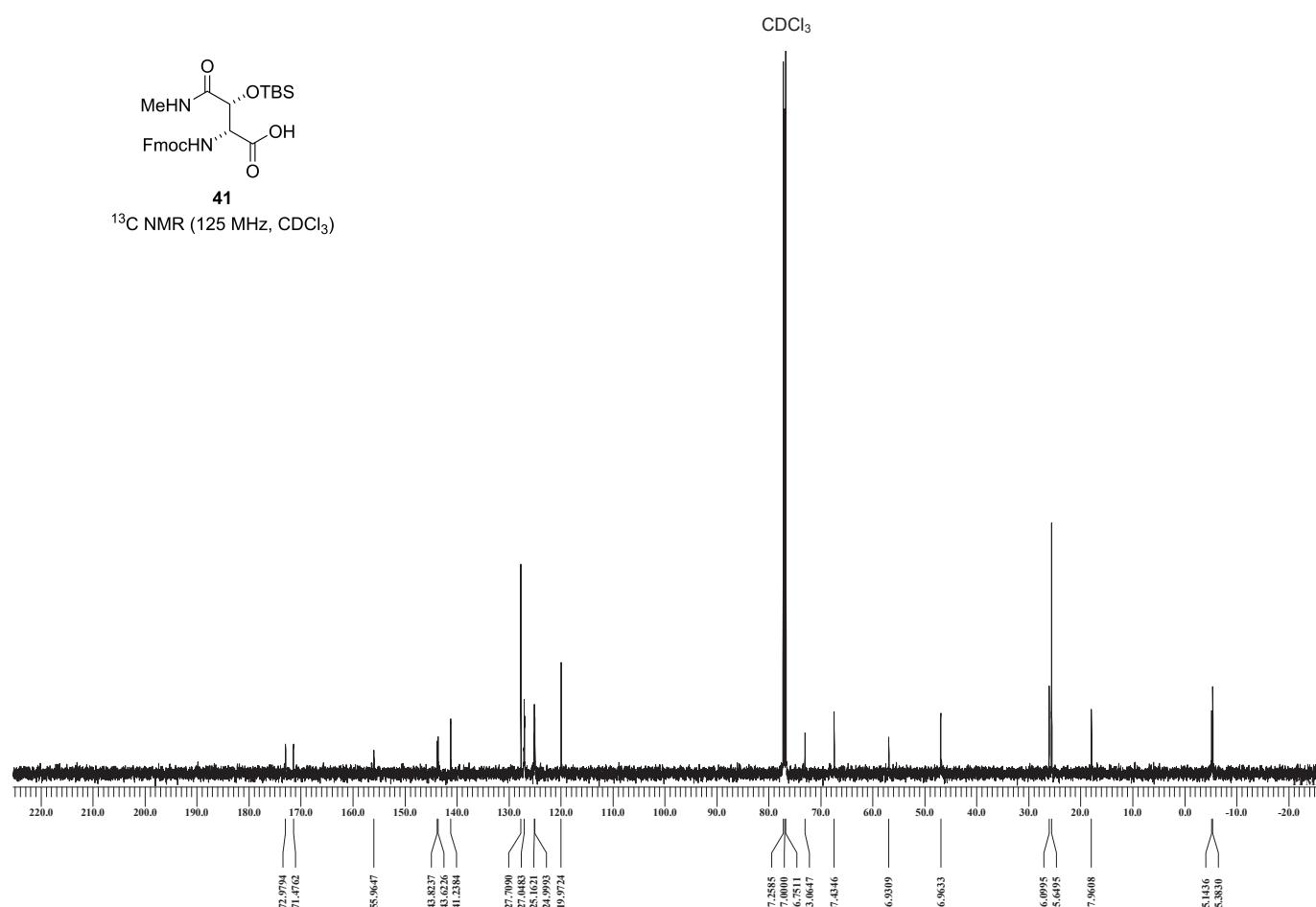
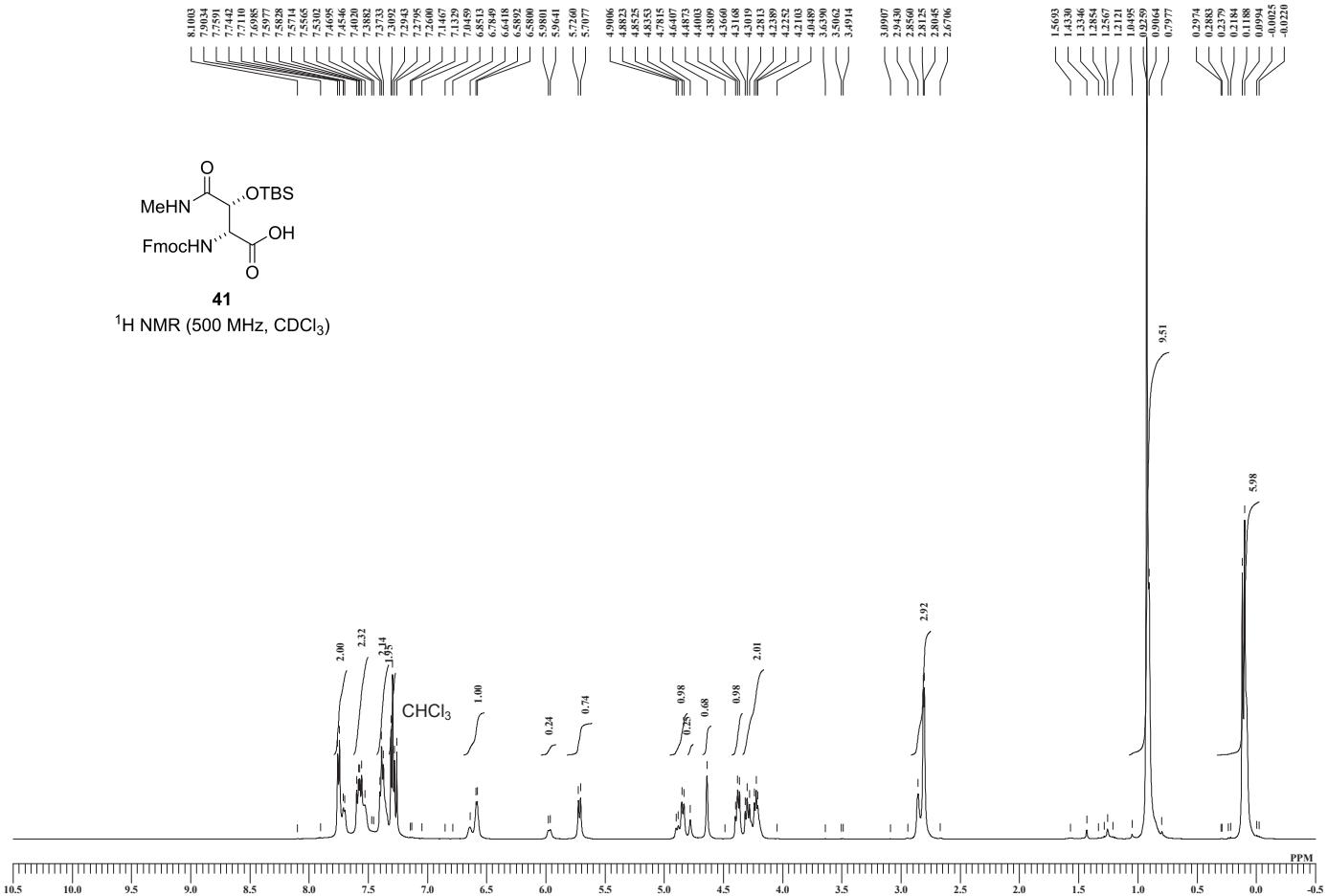


Figure S18. ^1H and ^{13}C NMR spectra of **41**.

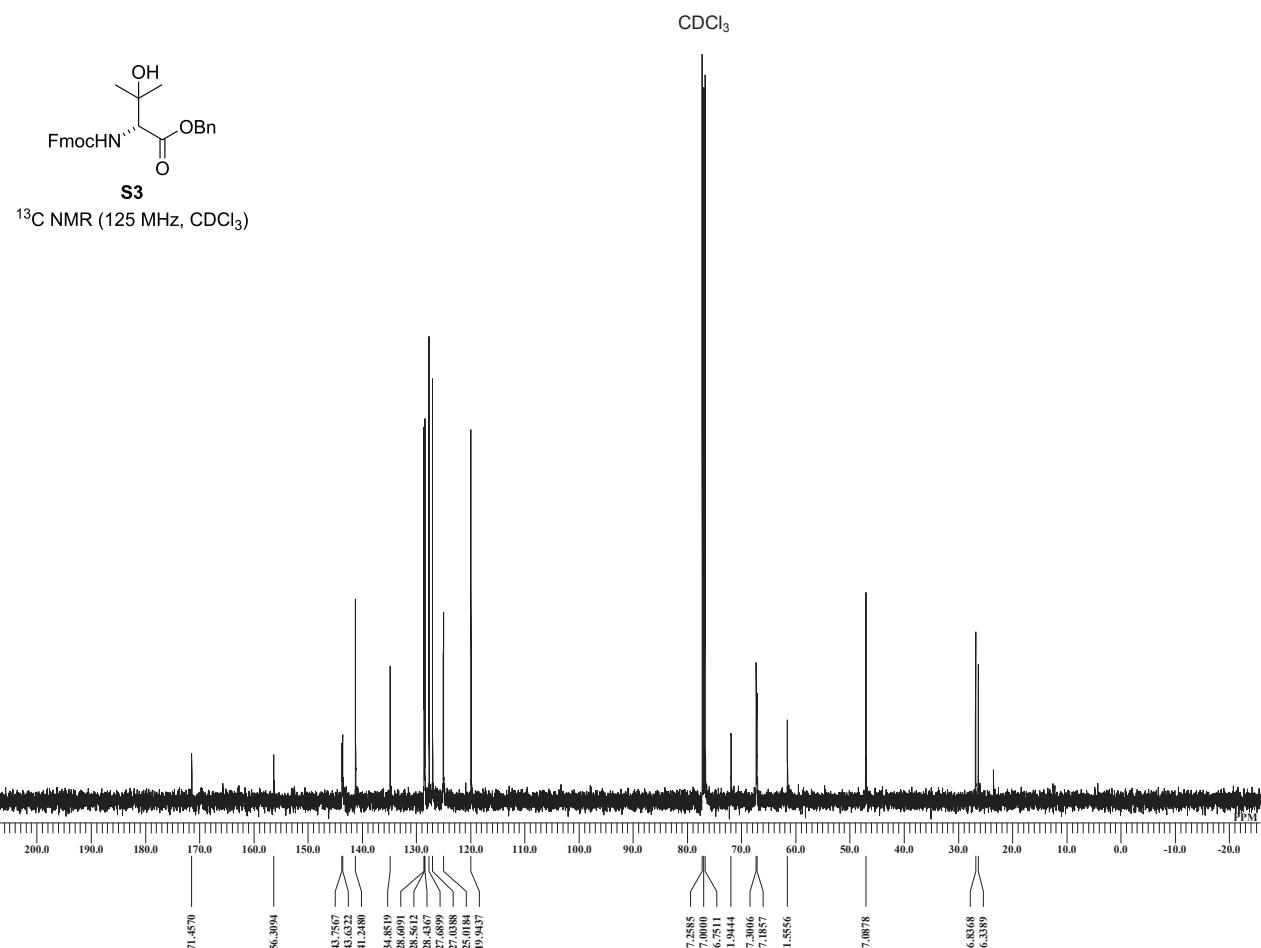
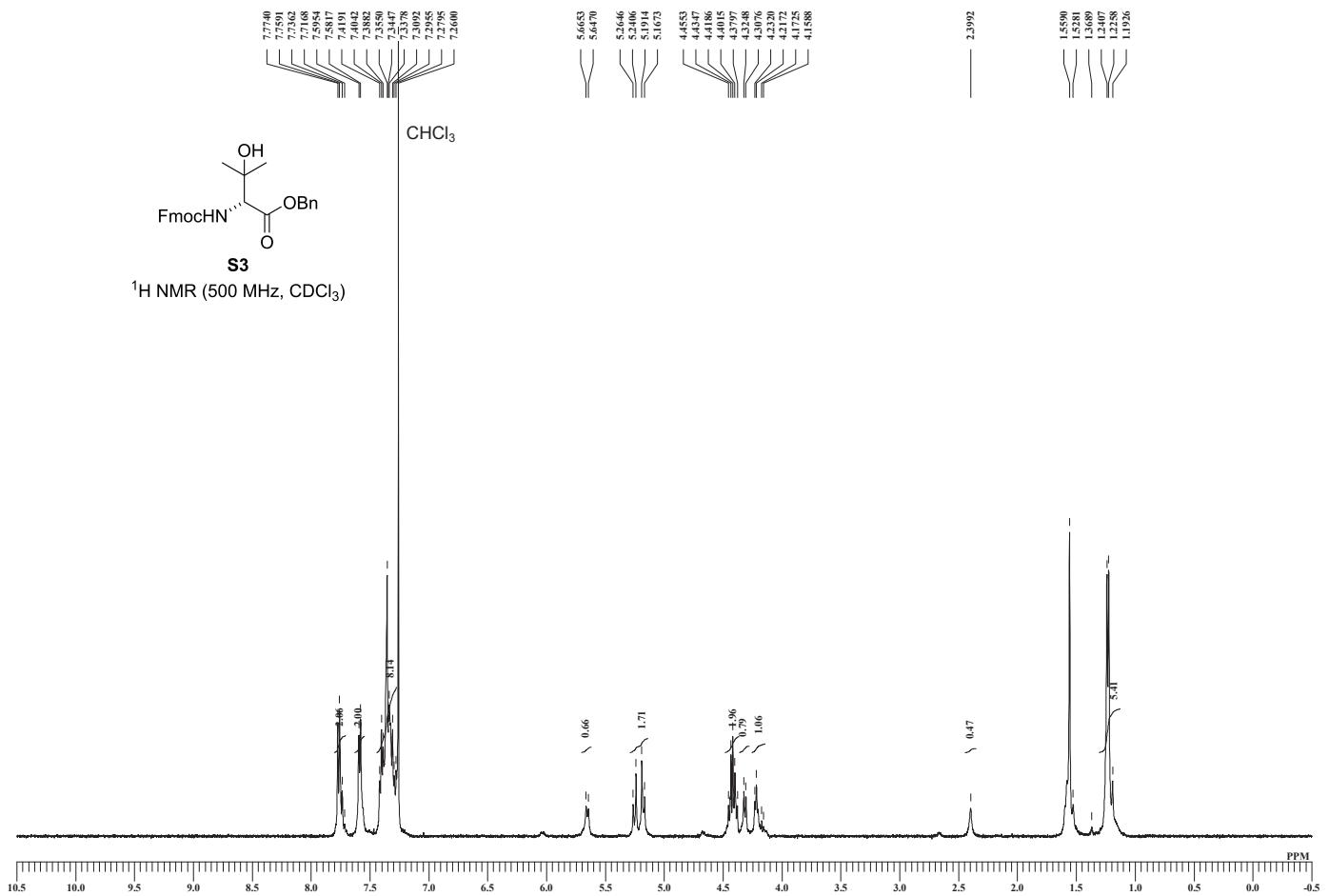


Figure S19. ^1H and ^{13}C NMR spectra of **S3**.

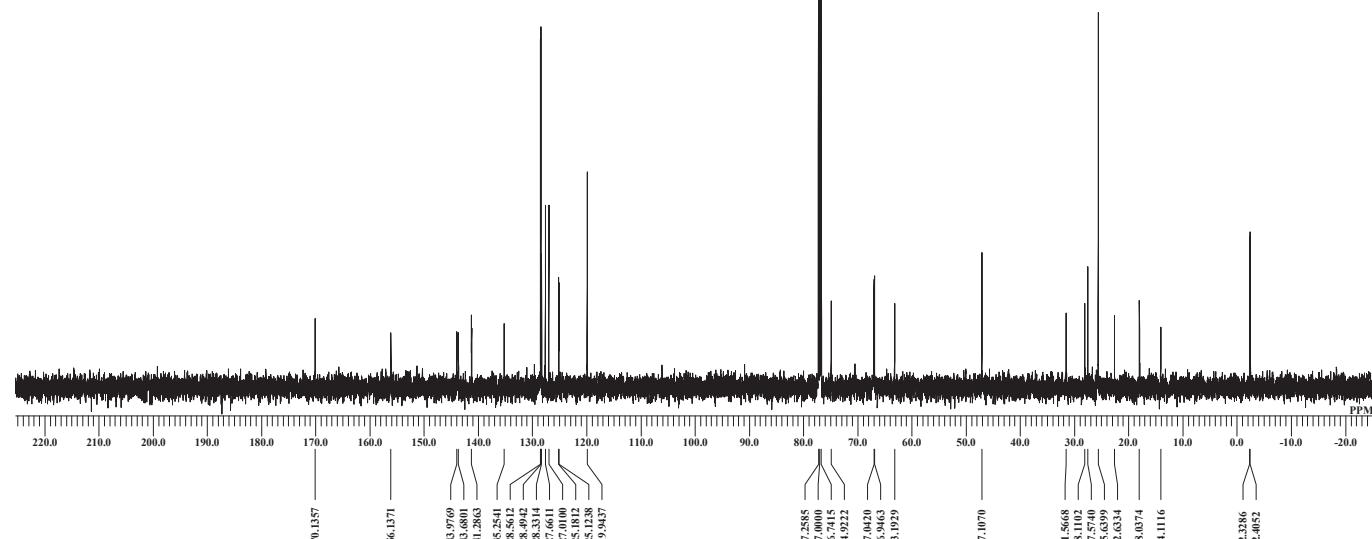
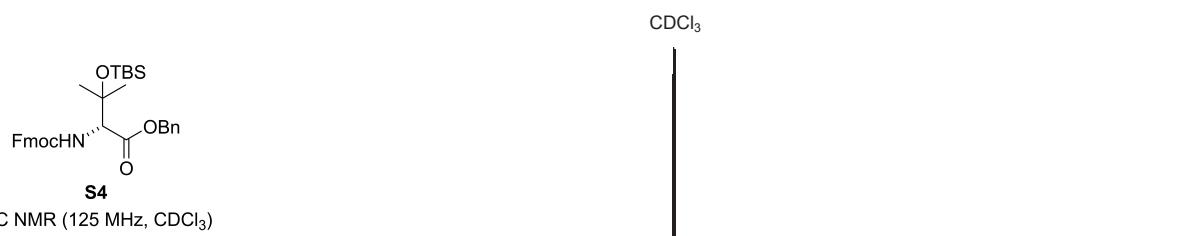
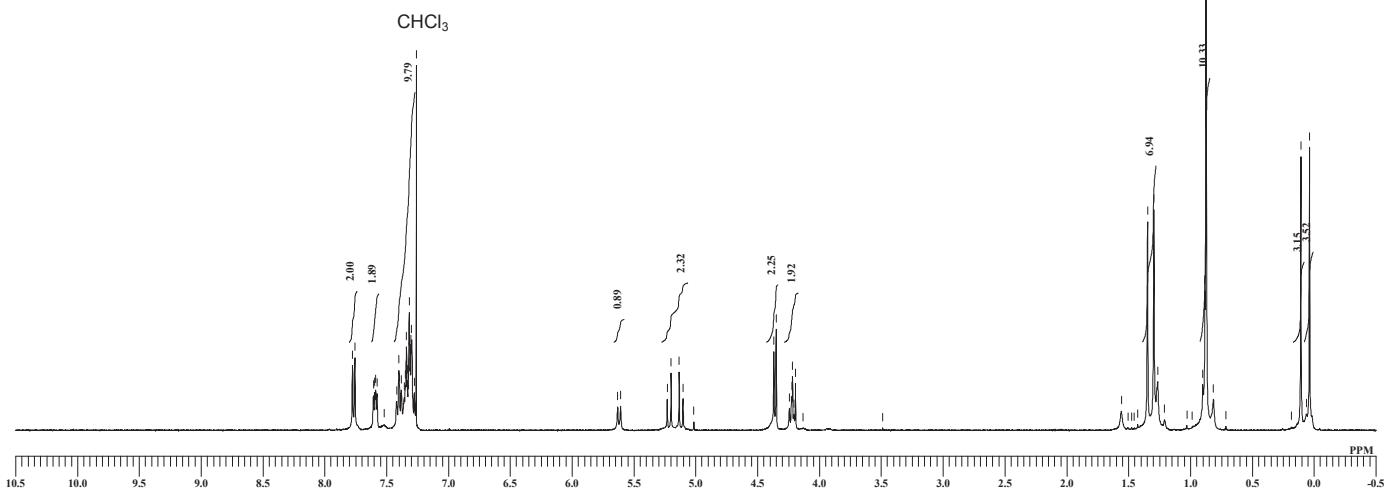
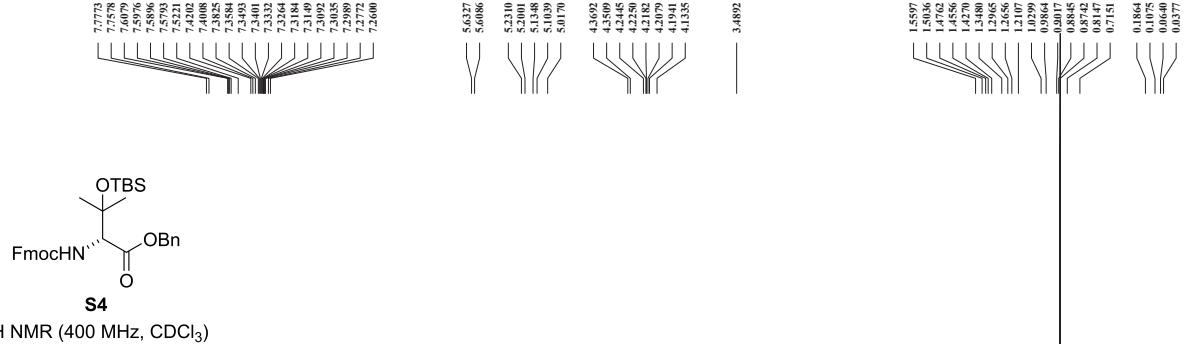


Figure S20. ^1H and ^{13}C NMR spectra of S4.

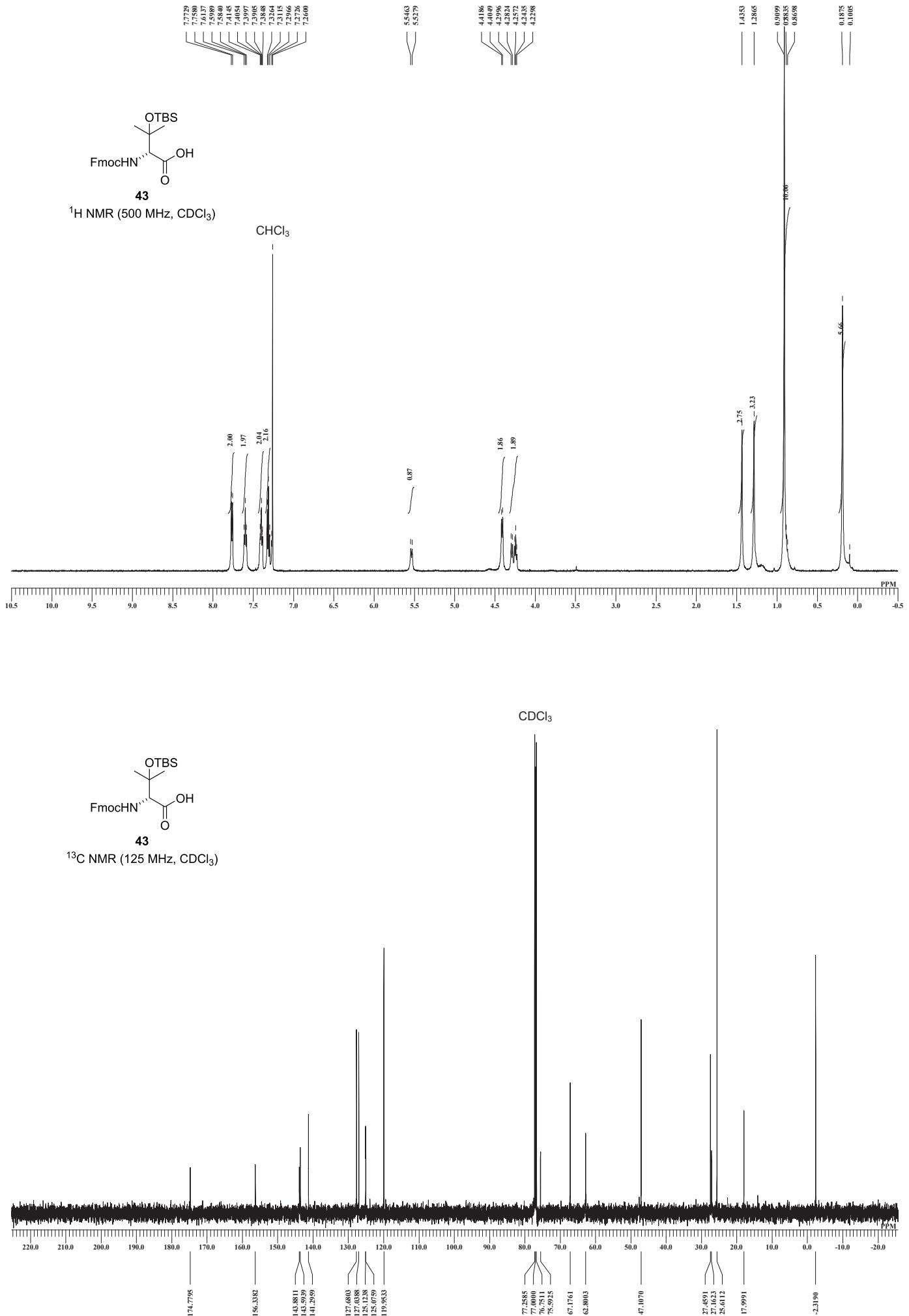


Figure S21. ¹H and ¹³C NMR spectra of **43**.

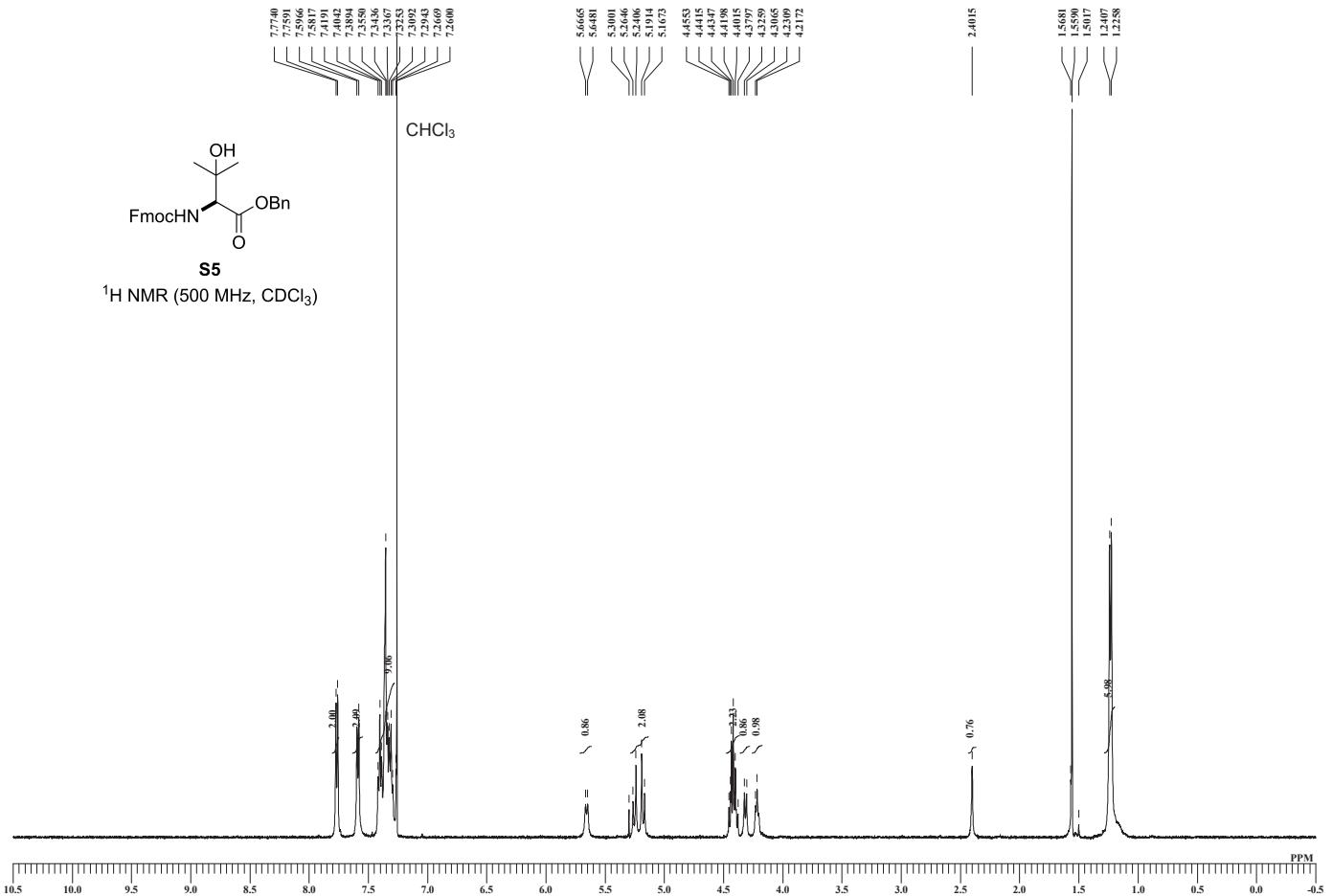
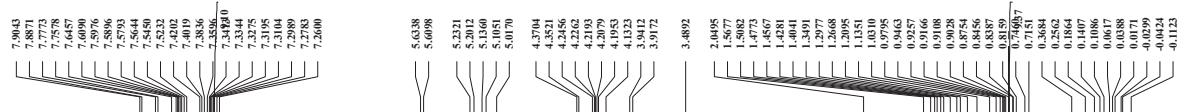
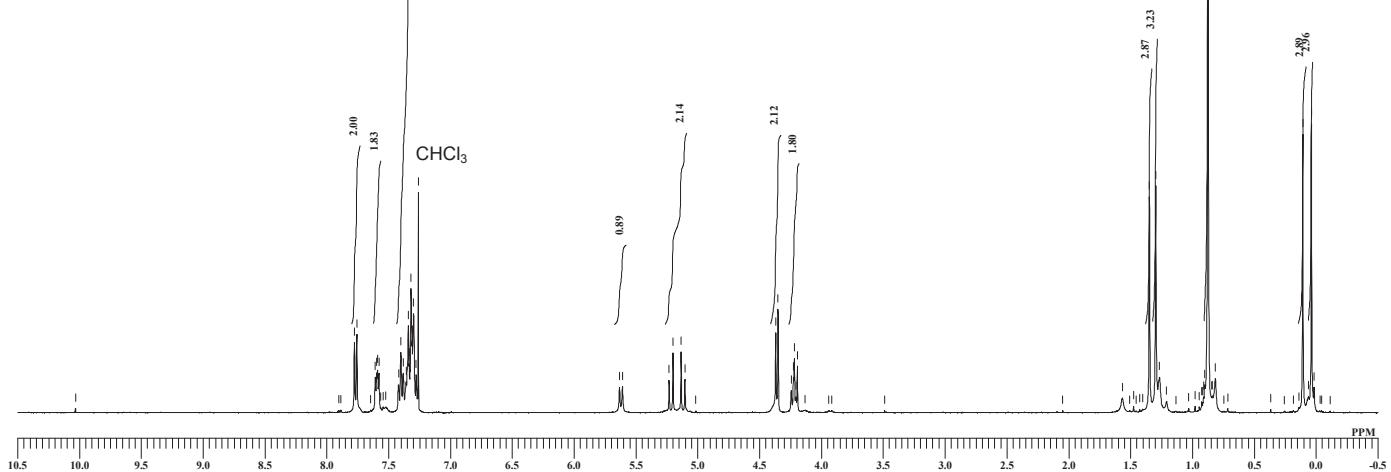


Figure S22. ¹H and ¹³C NMR spectra of S5.



S6
 ^1H NMR (400 MHz, CDCl_3)



S6
 ^{13}C NMR (125 MHz, CDCl_3)

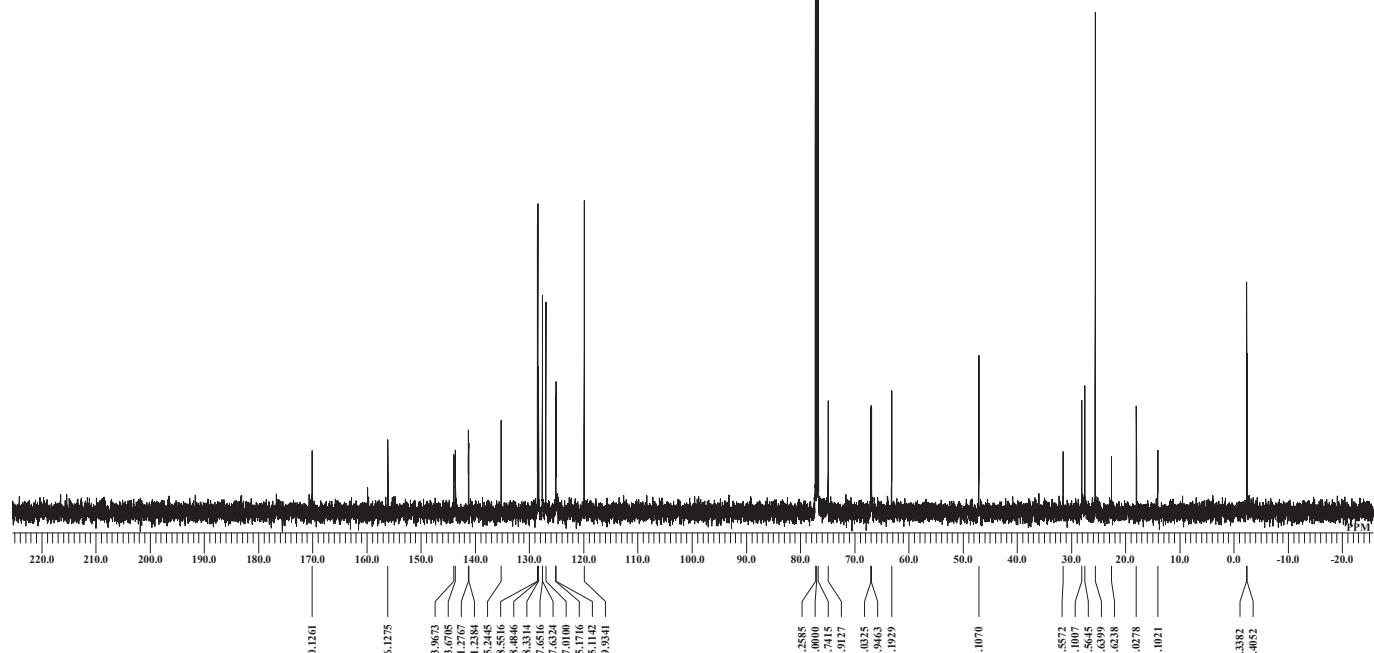


Figure S23. ^1H and ^{13}C NMR spectra of S6.

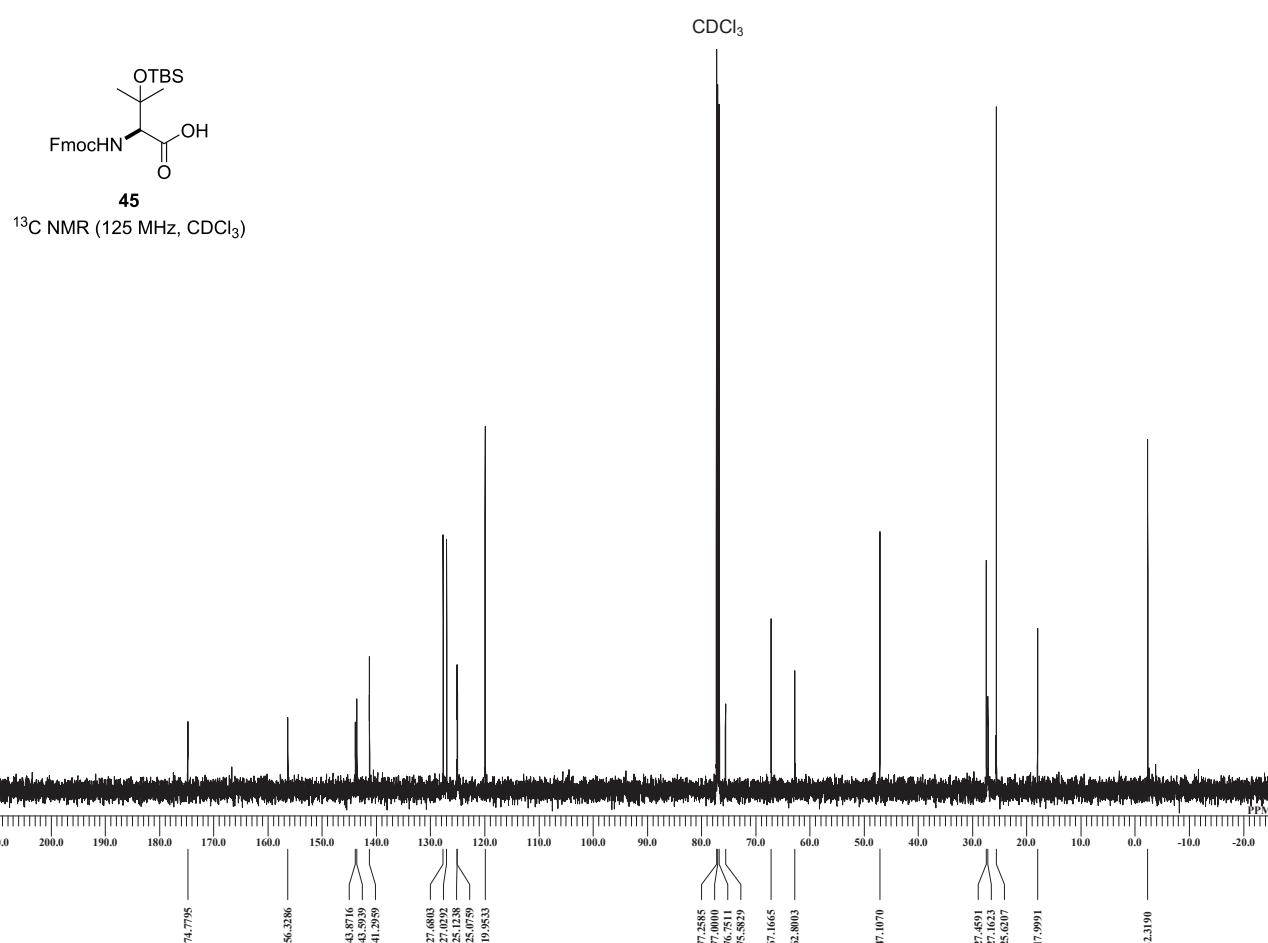
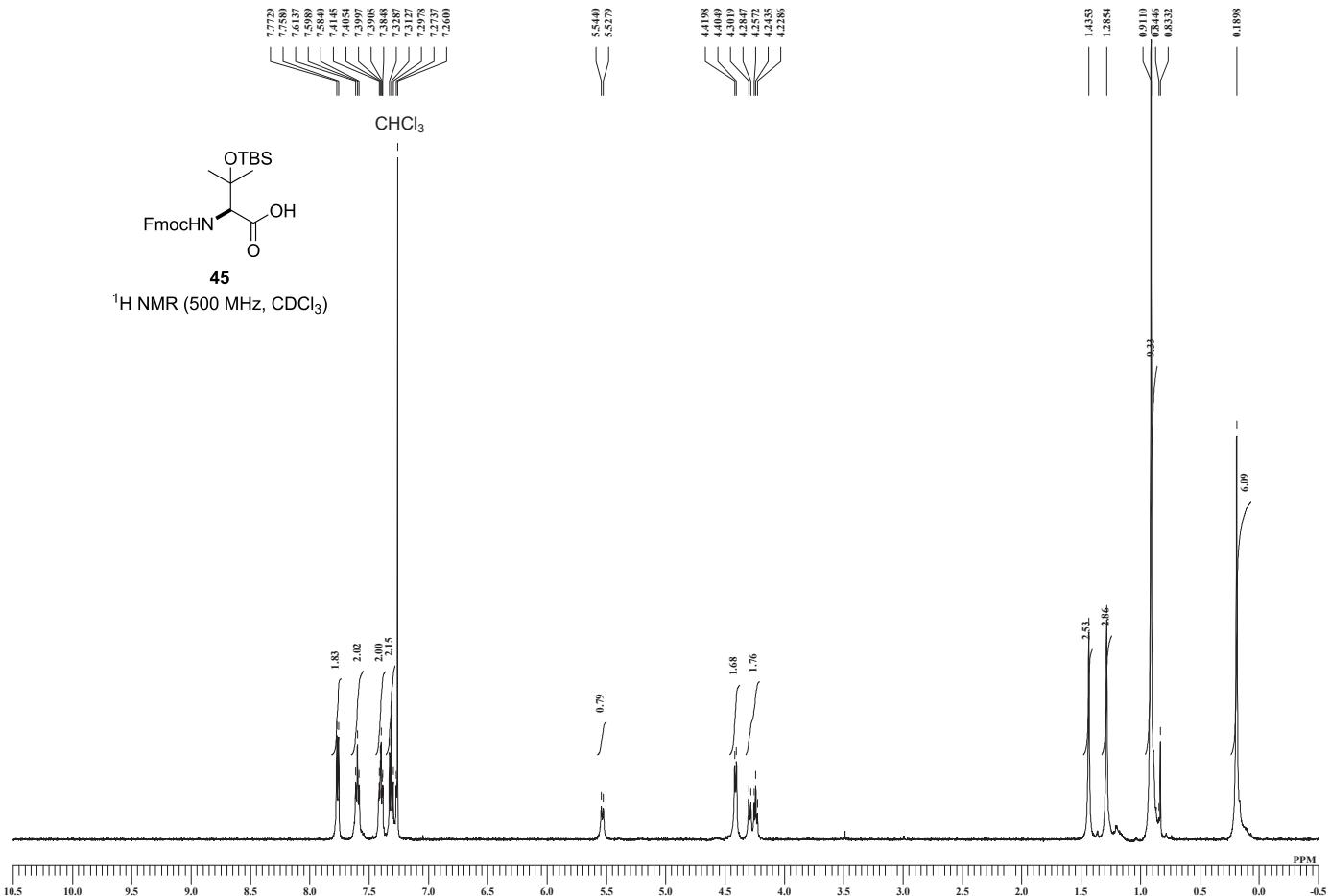


Figure S24. ^1H and ^{13}C NMR spectra of **45**.

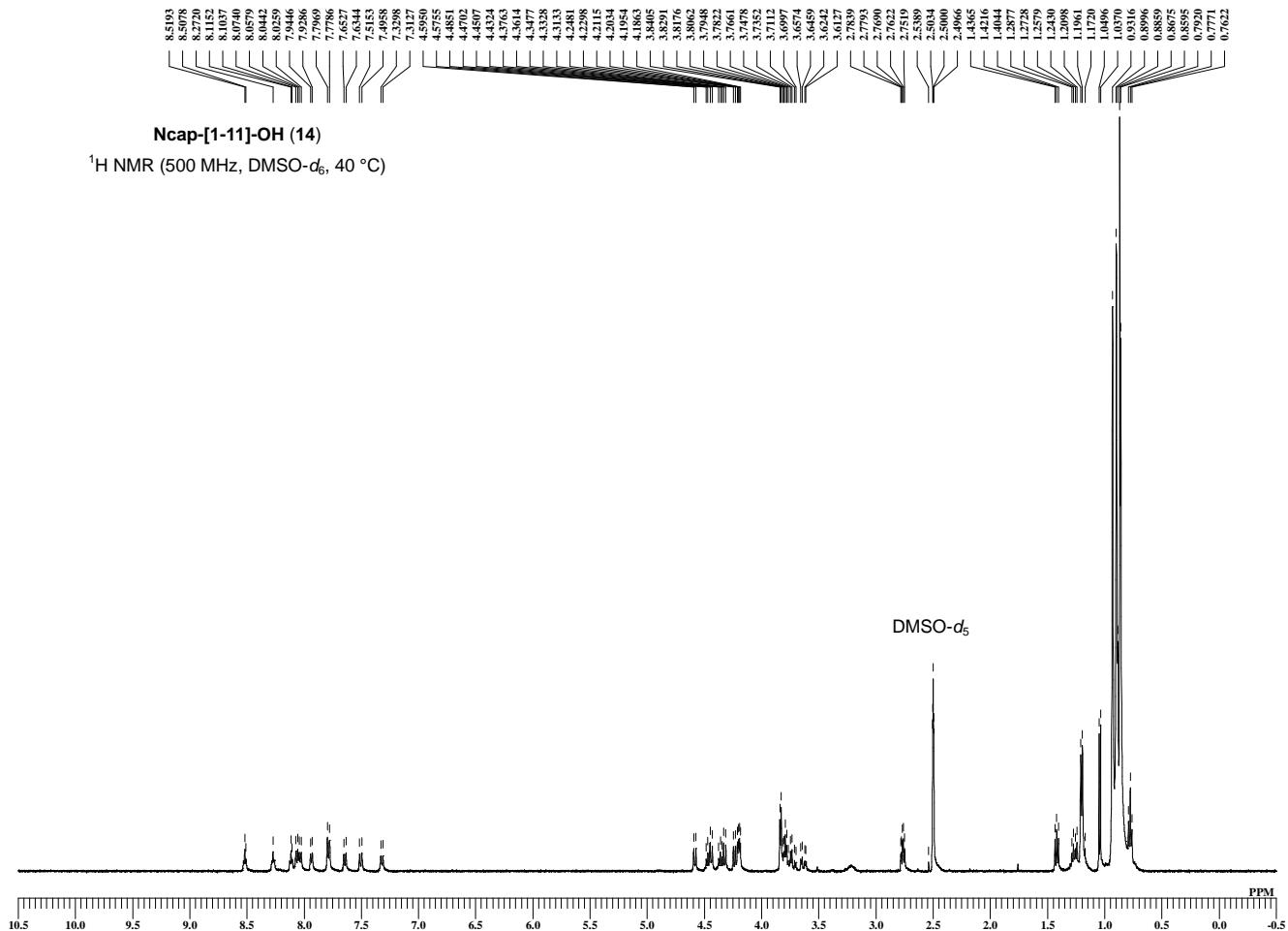


Figure S25. ¹H NMR spectrum of **14**.

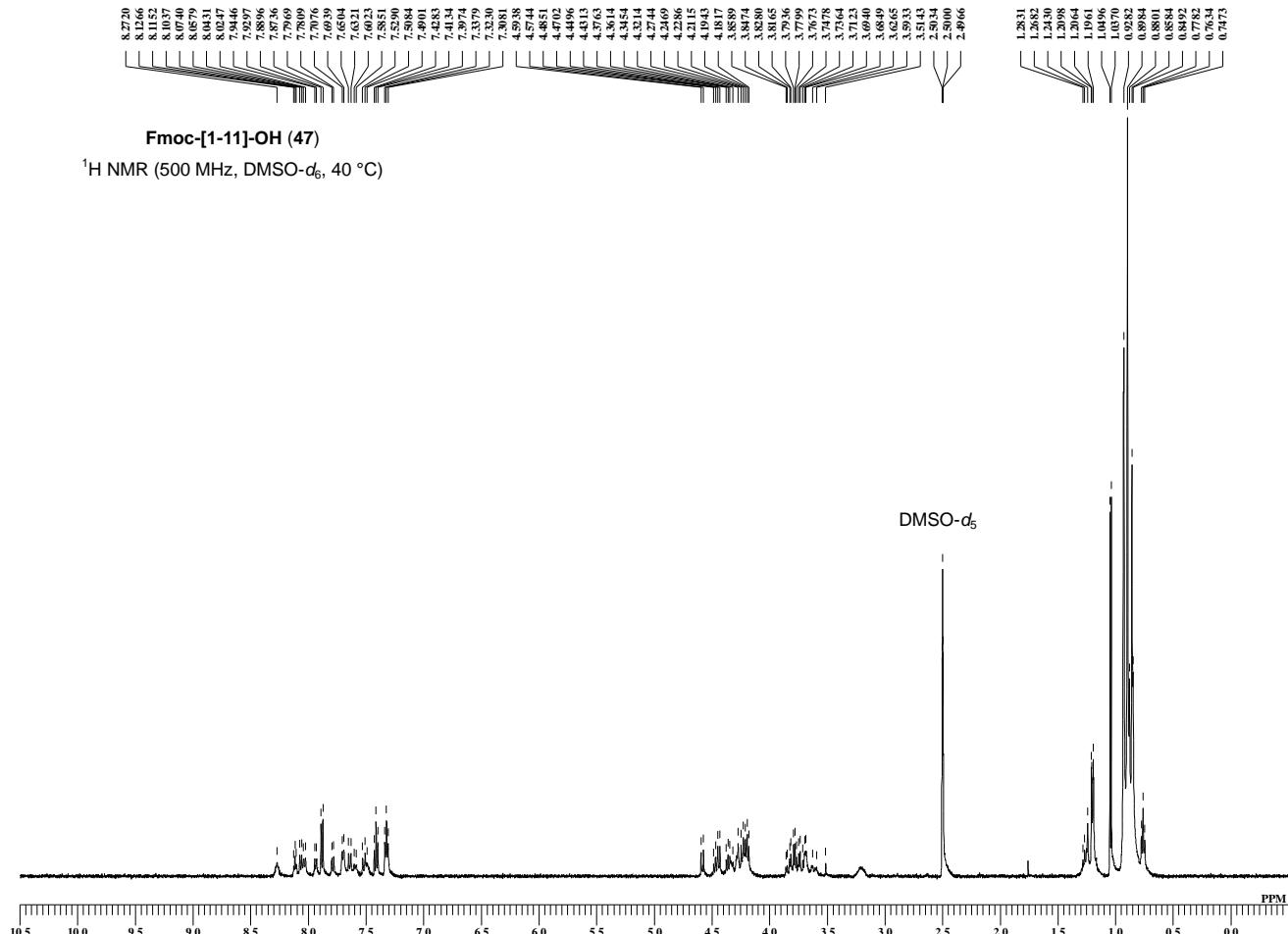
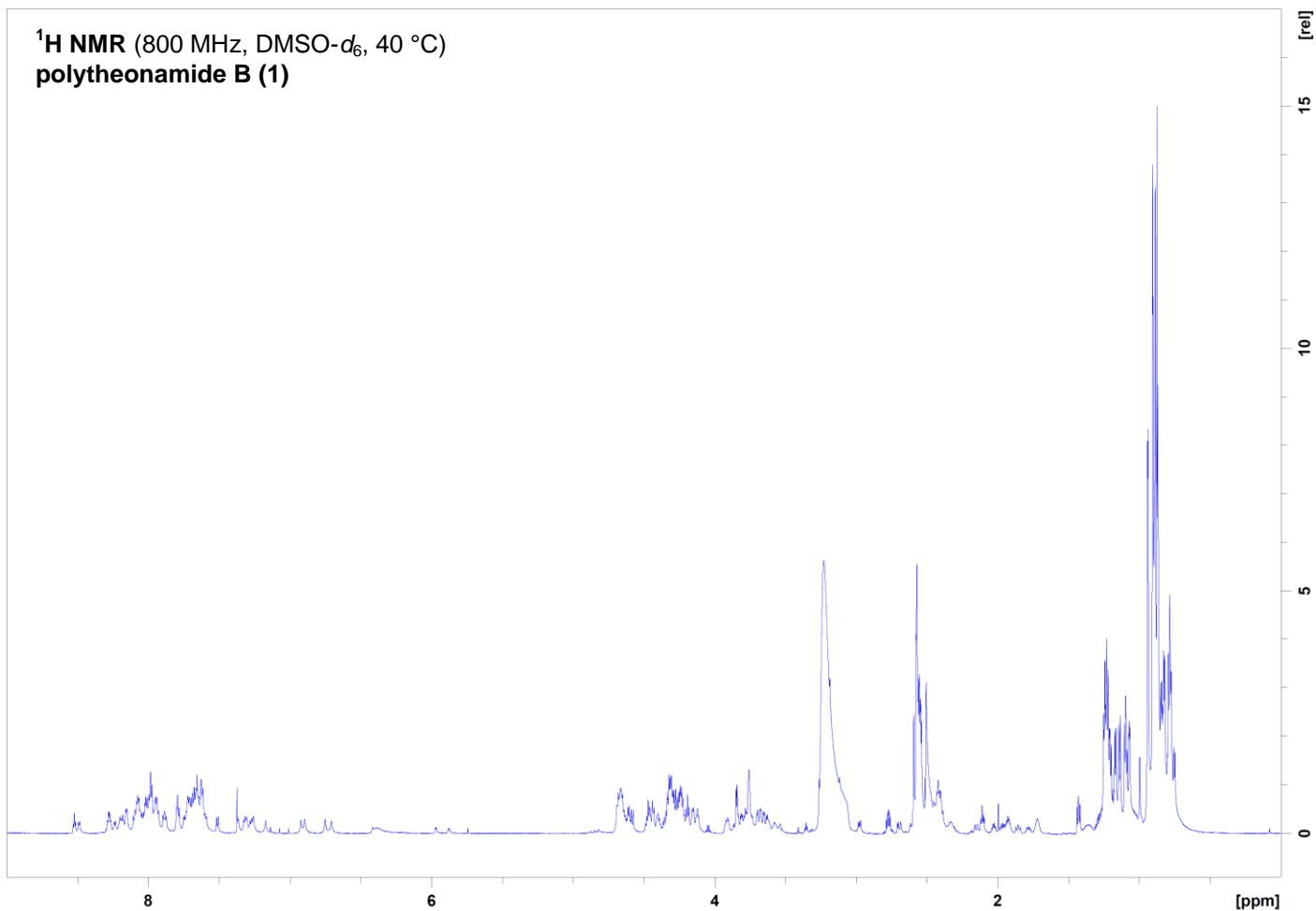


Figure S26. ¹H NMR spectrum of **47**.

^1H NMR (800 MHz, DMSO- d_6 , 40 °C)
polytheonamide B (1)



^1H - ^1H DQF-COSY (800 MHz, DMSO- d_6 , 40 °C)
polytheonamide B (1)

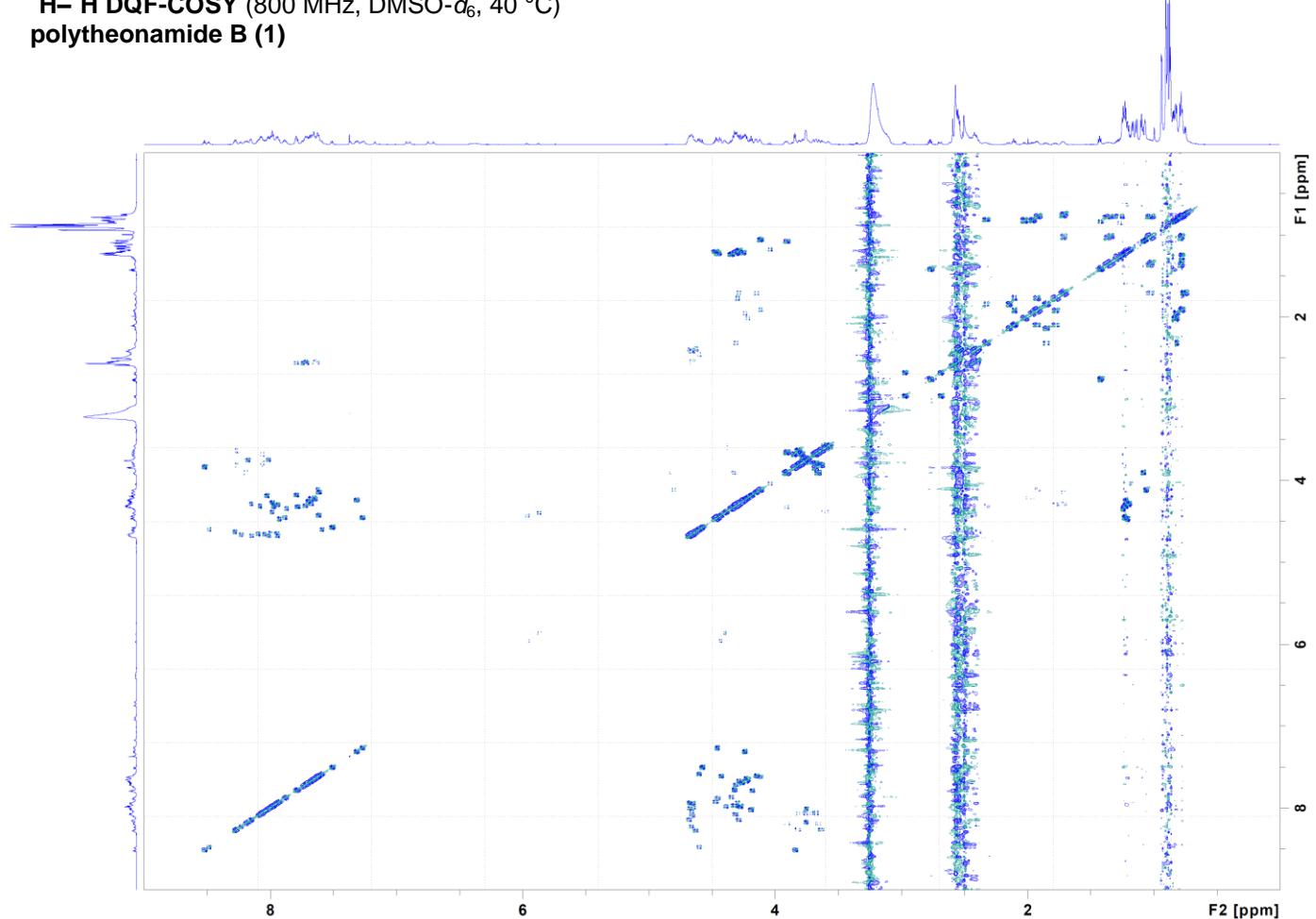
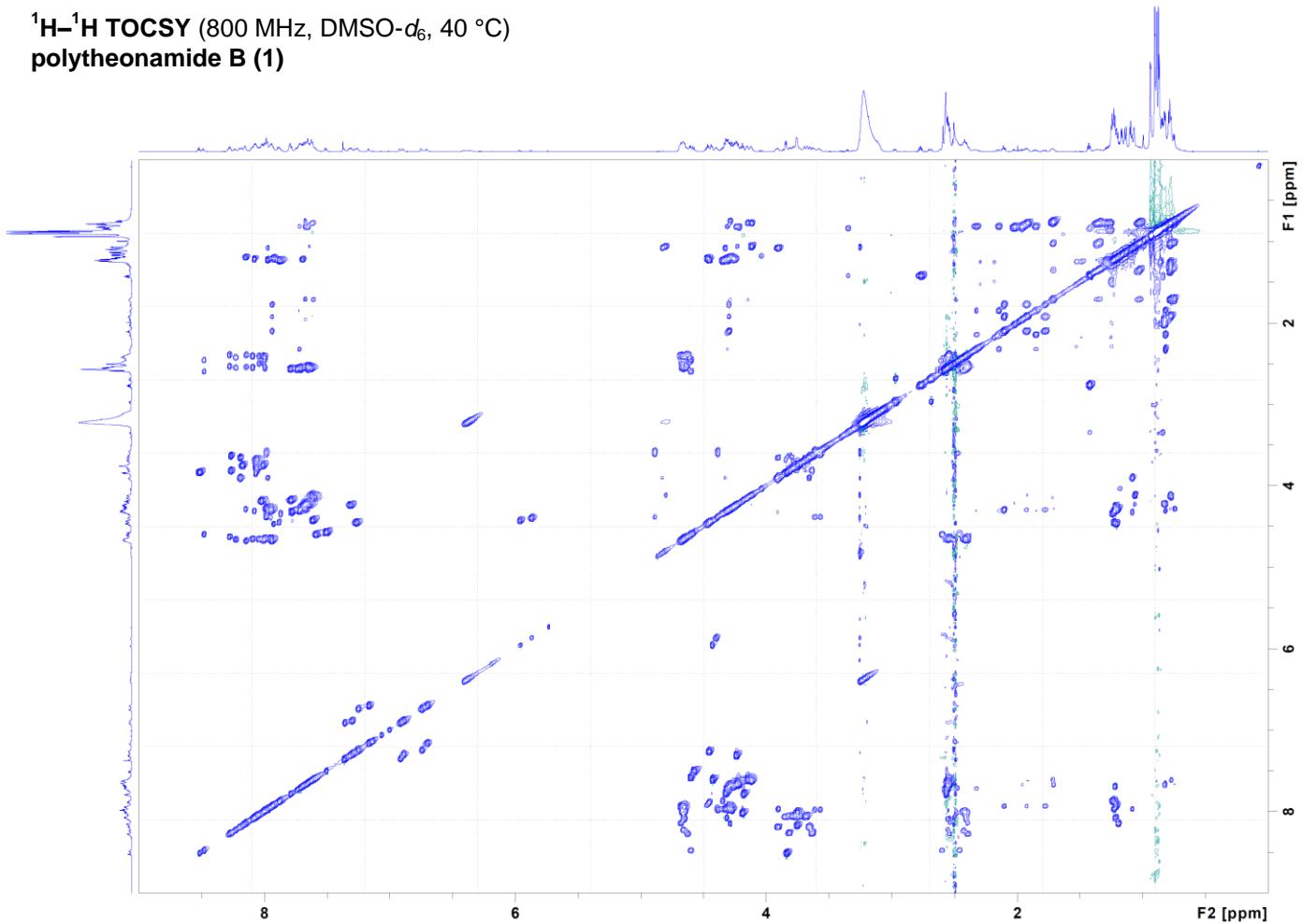


Figure S27. ^1H NMR and ^1H - ^1H DQF-COSY spectra of **1**.

^1H - ^1H TOCSY (800 MHz, DMSO- d_6 , 40 °C)
polytheonamide B (1)



^1H - ^1H NOESY (800 MHz, DMSO- d_6 , 40 °C)
polytheonamide B (1)

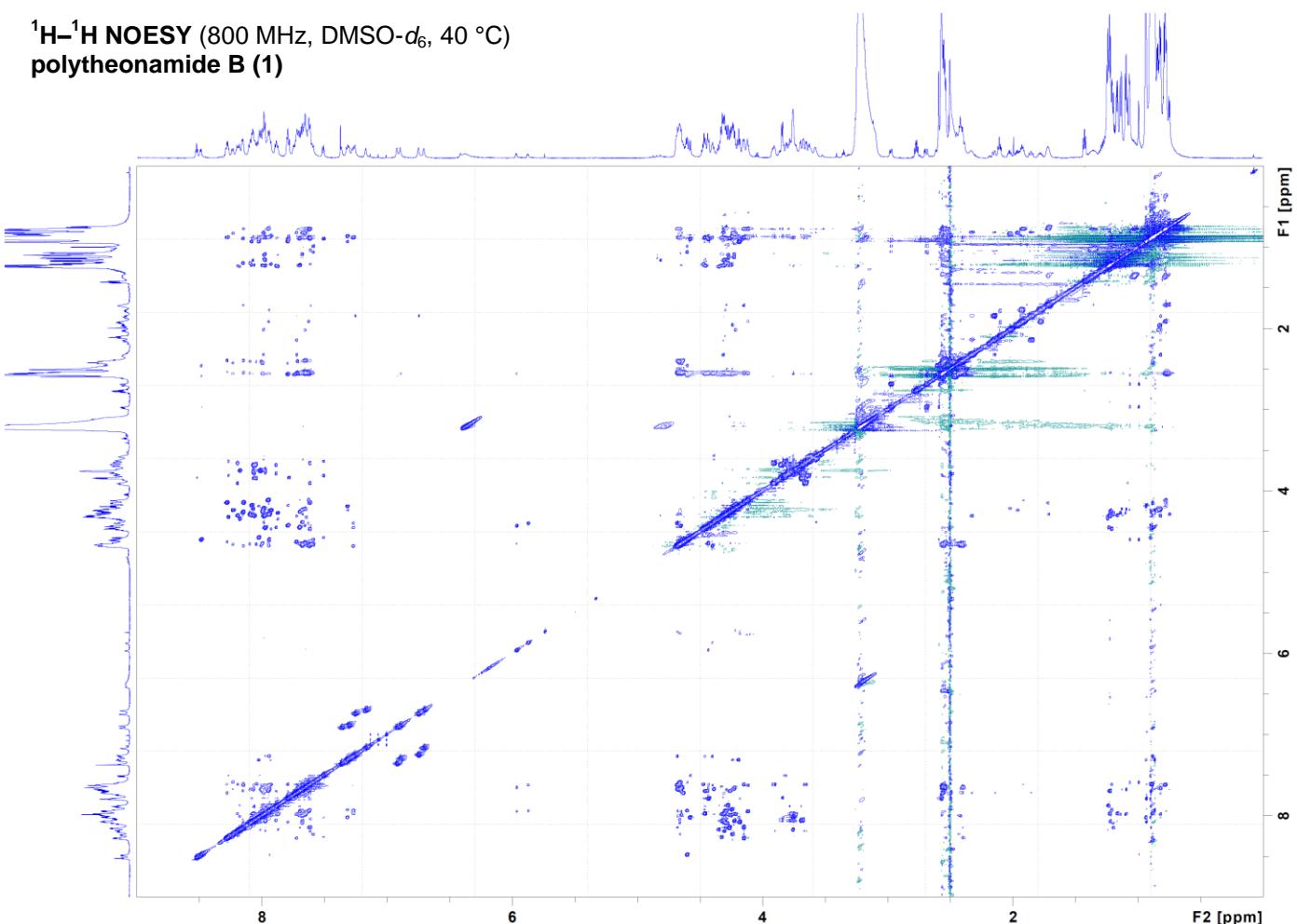
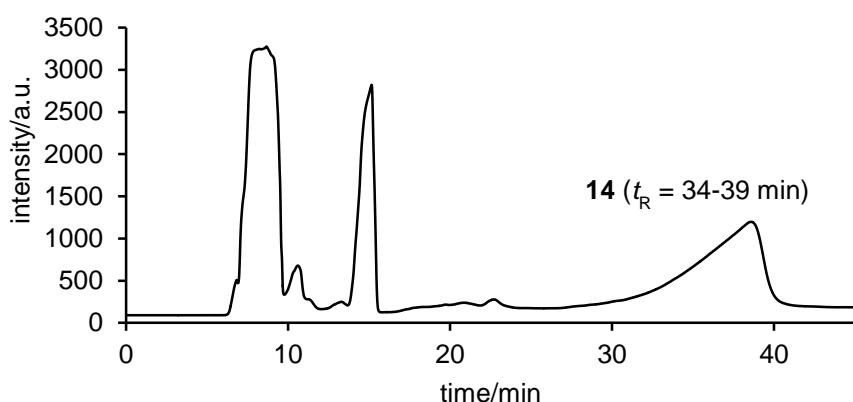


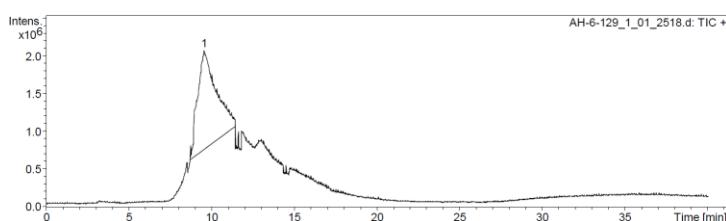
Figure S28. ^1H - ^1H TOCSY and ^1H - ^1H NOESY spectra of **1**.

HPLC Charts for Purification of Synthetic Peptides

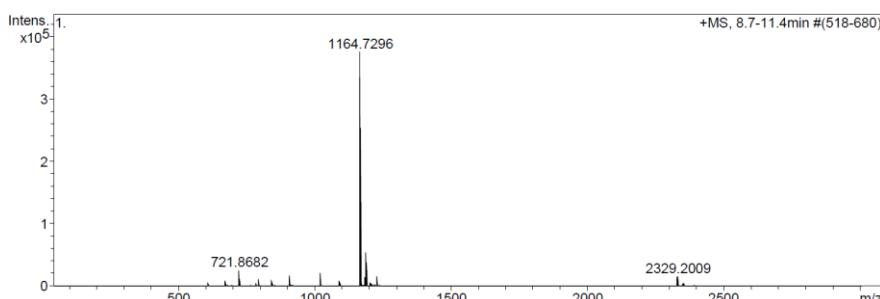
a



b



c



d

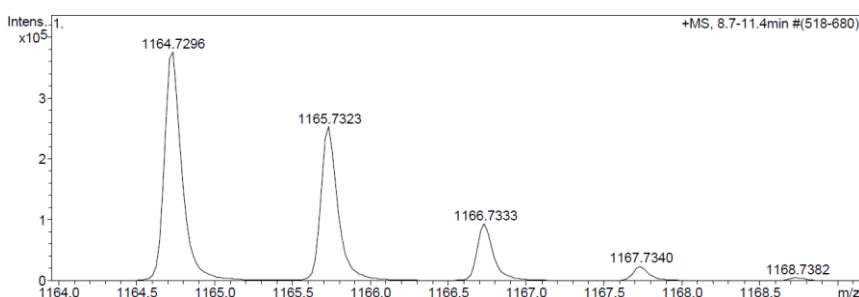


Figure S29. (a) HPLC chart for purification of **14**. Column: Inertsil WP300 C8 7.6 × 250 mm, eluent A: CH₃CN + 0.05% TFA, eluent B: H₂O + 0.05% TFA, linear gradient: A/B = 50/50 to 100/0 over 40 min, flow rate: 1.5 mL/min, detection: UV 220 nm, temperature: 40 °C. (b) Total ion chromatogram of the crude material. Column: Inertsil WP300 C8 4.6 × 150 mm, eluent A: i-PrOH + 0.05% HCO₂H, eluent B: H₂O + 0.05% HCO₂H, linear gradient: A/B = 50/50 to 100/0 over 40 min, flow rate: 0.5 mL/min, detection: ESI-TOF MS, temperature: 35 °C. (c) MS (ESI-TOF) signals of peak 1 in Figure S29b (m/z = 0–3000) and (d) (m/z = 1164–1169).

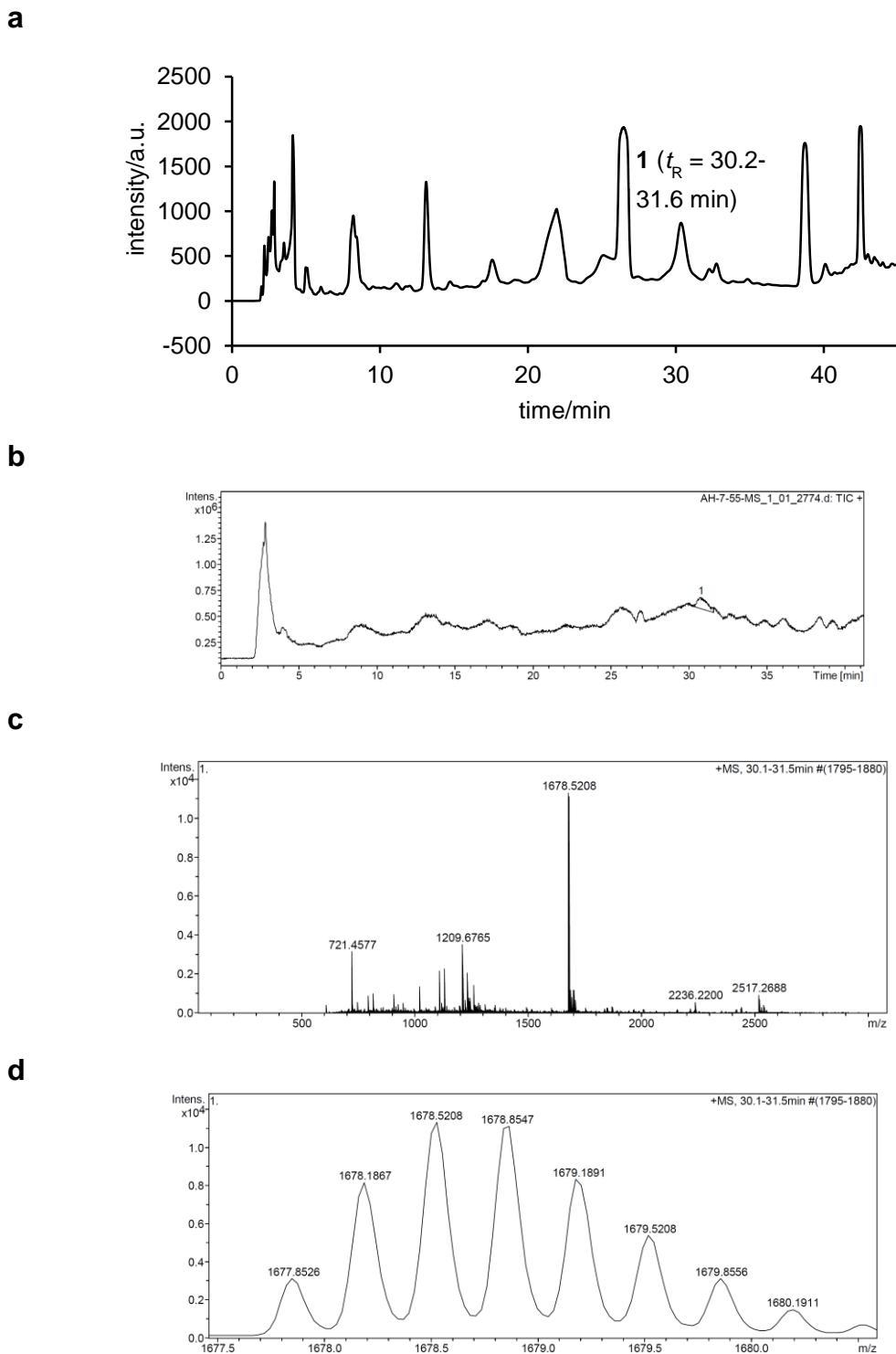


Figure S30. (a) HPLC chart for purification of **1**. Column: Inertsil C8-3 4.6 × 150 mm, eluent A: *n*-PrOH + 0.05% TFA, eluent B: H₂O + 0.05% TFA, linear gradient: A/B = 25/75 to 50/50 over 35 min, flow rate: 0.6 mL/min, detection: UV 220 and 254 nm, temperature: 45 °C. (b) Total ion chromatogram of the crude material. Column: Inertsil C8-3 4.6 × 150 mm, eluent A: *n*-PrOH + 0.05% TFA, eluent B: H₂O + 0.05% TFA, linear gradient: A/B = 25/75 to 50/50 over 35 min, flow rate: 0.6 mL/min, detection: ESI-TOF MS, temperature: 45 °C. (c) MS (ESI-TOF) signals of peak 1 in Figure S30b (*m/z* = 0–3000) and (d) (*m/z* = 1677.5–1680.5).

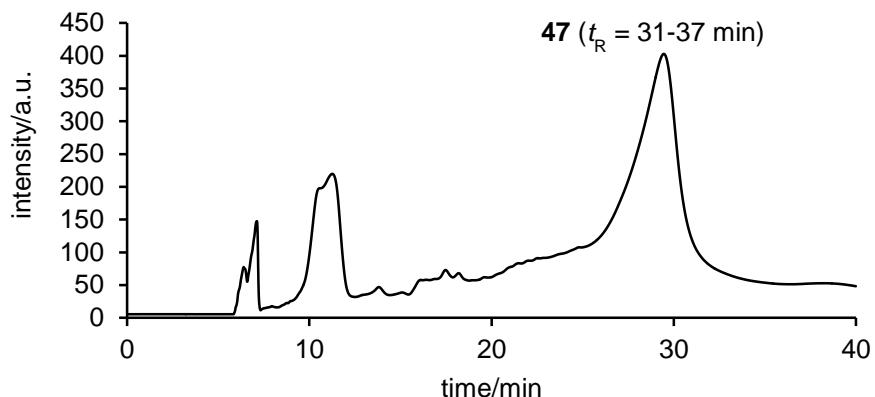
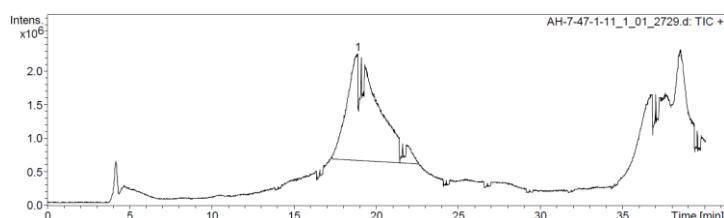
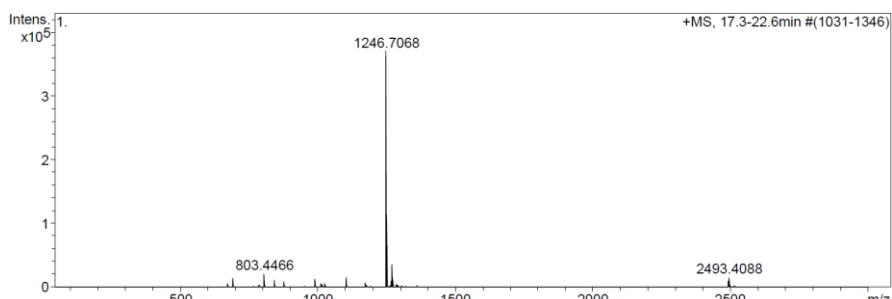
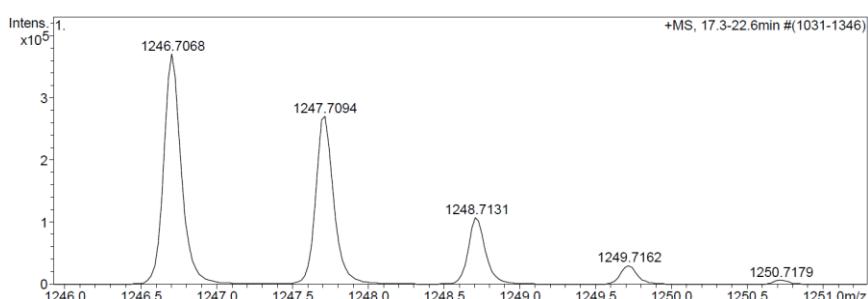
a**b****c****d**

Figure S31. (a) HPLC chart for purification of **47**. Column: Inertsil WP300 C8 7.6 \times 250 mm, eluent A: $\text{CH}_3\text{CN} + 0.05\%$ TFA, eluent B: $\text{H}_2\text{O} + 0.05\%$ TFA, linear gradient: A/B = 50/50 to 100/0 over 40 min, flow rate: 1.5 mL/min, detection: UV 256 nm, temperature: 40 °C. (b) Total ion chromatogram of the crude material. Column: Inertsil WP300 C8 4.6 \times 150 mm, eluent A: $i\text{-PrOH} + 0.05\%$ HCO_2H , eluent B: $\text{H}_2\text{O} + 0.05\%$ HCO_2H , linear gradient: A/B = 40/60 to 80/20 over 40 min, flow rate: 0.5 mL/min, detection: ESI-TOF MS, temperature: 40 °C. (c) MS (ESI-TOF) signals of peak 1 in Figure S31b ($m/z = 0\text{--}3000$) and (d) ($m/z = 1246\text{--}1251$).

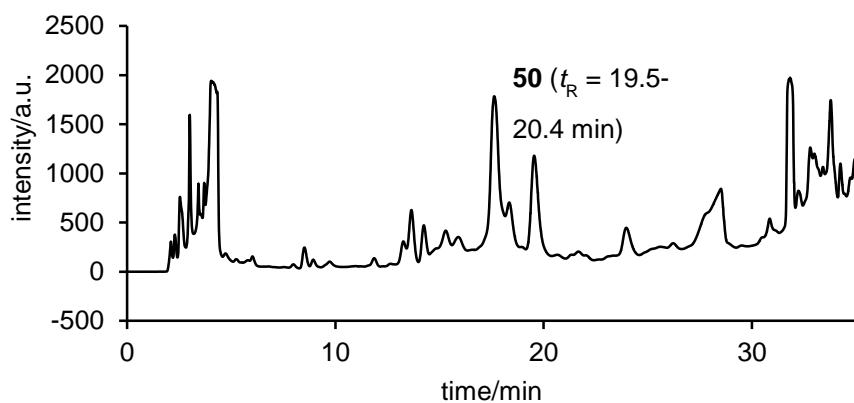
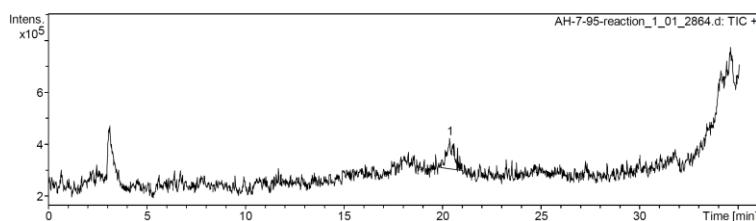
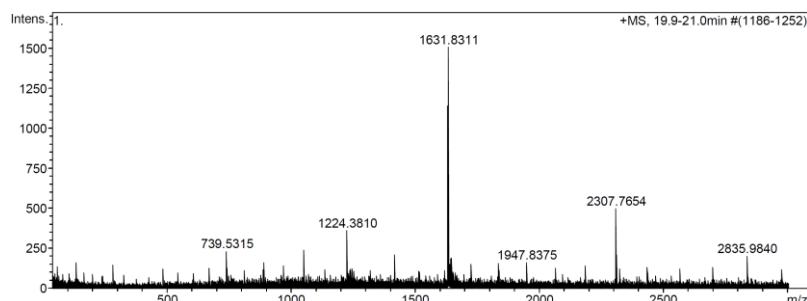
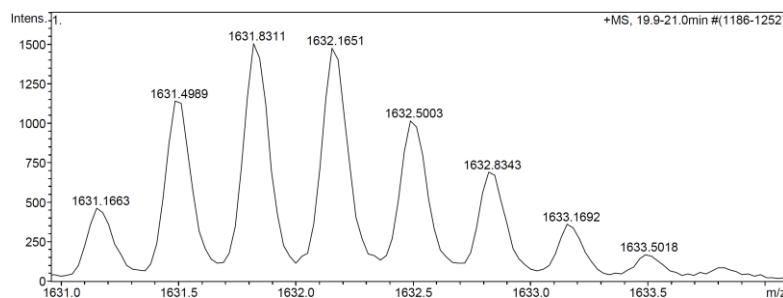
a**b****c****d**

Figure S32. (a) HPLC chart for purification of **50**. Column: Inertsil C8-3 4.6 × 150 mm, eluent A: *n*-PrOH + 0.05% TFA, eluent B: H₂O + 0.05% TFA, linear gradient: A/B = 20/80 to 37/63 over 25 min, flow rate: 0.6 mL/min, detection: UV 220 and 254 nm, temperature: 45 °C. (b) Total ion chromatogram of the crude material. Column: Inertsil C8-3 4.6 × 150 mm, eluent A: *n*-PrOH + 0.05% TFA, eluent B: H₂O + 0.05% TFA, linear gradient: A/B = 20/80 to 37/63 over 25 min, flow rate: 0.6 mL/min, detection: ESI-TOF MS, temperature: 45 °C. (c) MS (ESI-TOF) signals of peak 1 in Figure S32b (m/z = 0–3000) and (d) (m/z = 1631–1634).

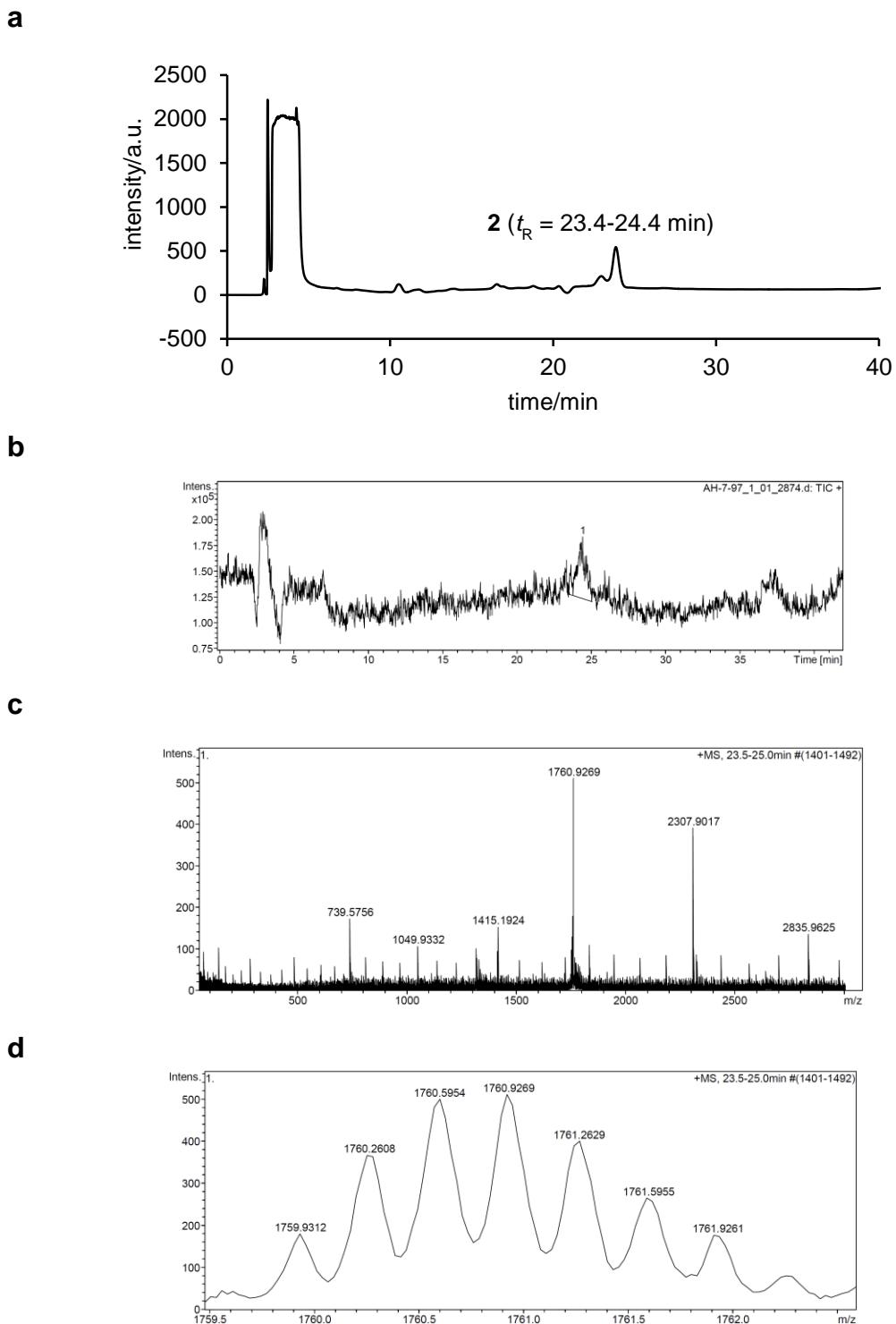
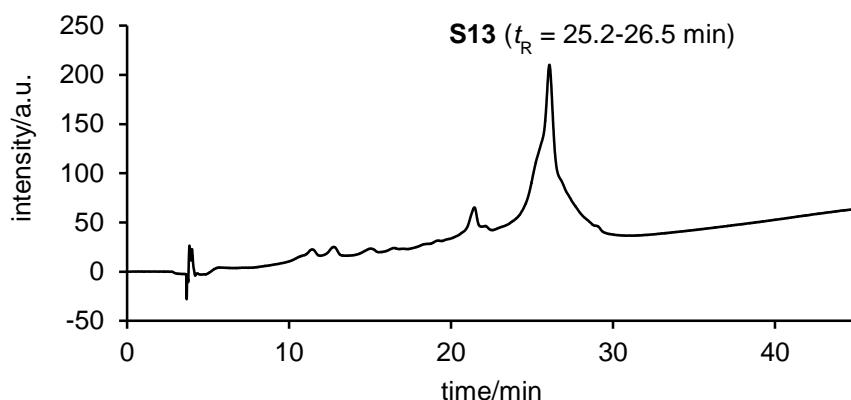


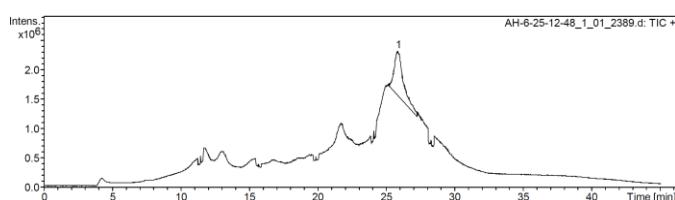
Figure S33. (a) HPLC chart for purification of **2**. Column: Inertsil C8-3 4.6 × 150 mm, eluent A: *n*-PrOH + 0.05% TFA, eluent B: H₂O + 0.05% TFA, linear gradient: A/B = 25/75 to 50/50 over 35 min, flow rate: 0.6 mL/min, detection: UV 220 and 254 nm, temperature: 45 °C. (b) Total ion chromatogram of the crude material. Column: Inertsil C8-3 4.6 × 150 mm, eluent A: *n*-PrOH + 0.05% TFA, eluent B: H₂O + 0.05% TFA, linear gradient: A/B = 25/75 to 50/50 over 35 min, flow rate: 0.6 mL/min, detection: ESI-TOF MS, temperature: 45 °C. (c) MS (ESI-TOF) signals of peak 1 in Figure S33b ($m/z = 0\text{--}3000$) and (d) ($m/z = 1759.5\text{--}1762.5$).

HPLC Charts for Analysis of Synthetic Peptides

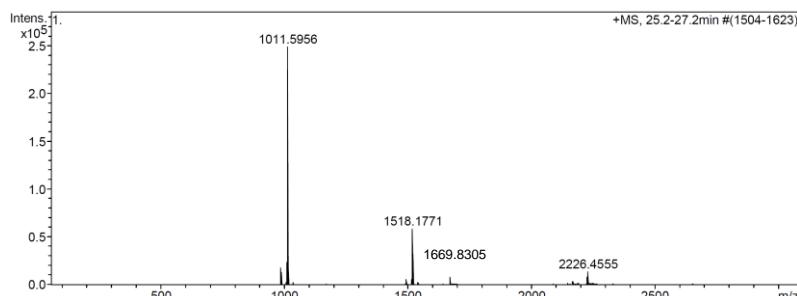
a



b



c



d

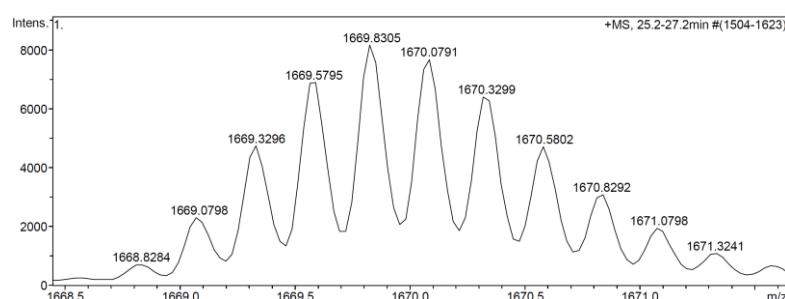


Figure S34. (a) HPLC chart for analysis of **S13**. Column: COSMOSIL Protein-R 4.6 × 150 mm, eluent A: *i*-PrOH + 0.05% TFA, eluent B: H₂O + 0.05% TFA, linear gradient: A/B = 50/50 to 100/0 over 40 min, flow rate: 0.5 mL/min, detection: UV 220 and 254 nm, temperature: 40 °C. (b) Total ion chromatogram of the crude material. Column: COSMOSIL Protein-R 4.6 × 150 mm, eluent A: *i*-PrOH + 0.05% TFA, eluent B: H₂O + 0.05% TFA, linear gradient: A/B = 50/50 to 100/0 over 40 min, flow rate: 0.5 mL/min, detection: ESI-TOF MS, temperature: 40 °C. (c) MS (ESI-TOF) signals of peak 1 in Figure S34b ($m/z = 0\text{-}3000$) and (d) ($m/z = 1668.5\text{-}1671.5$).

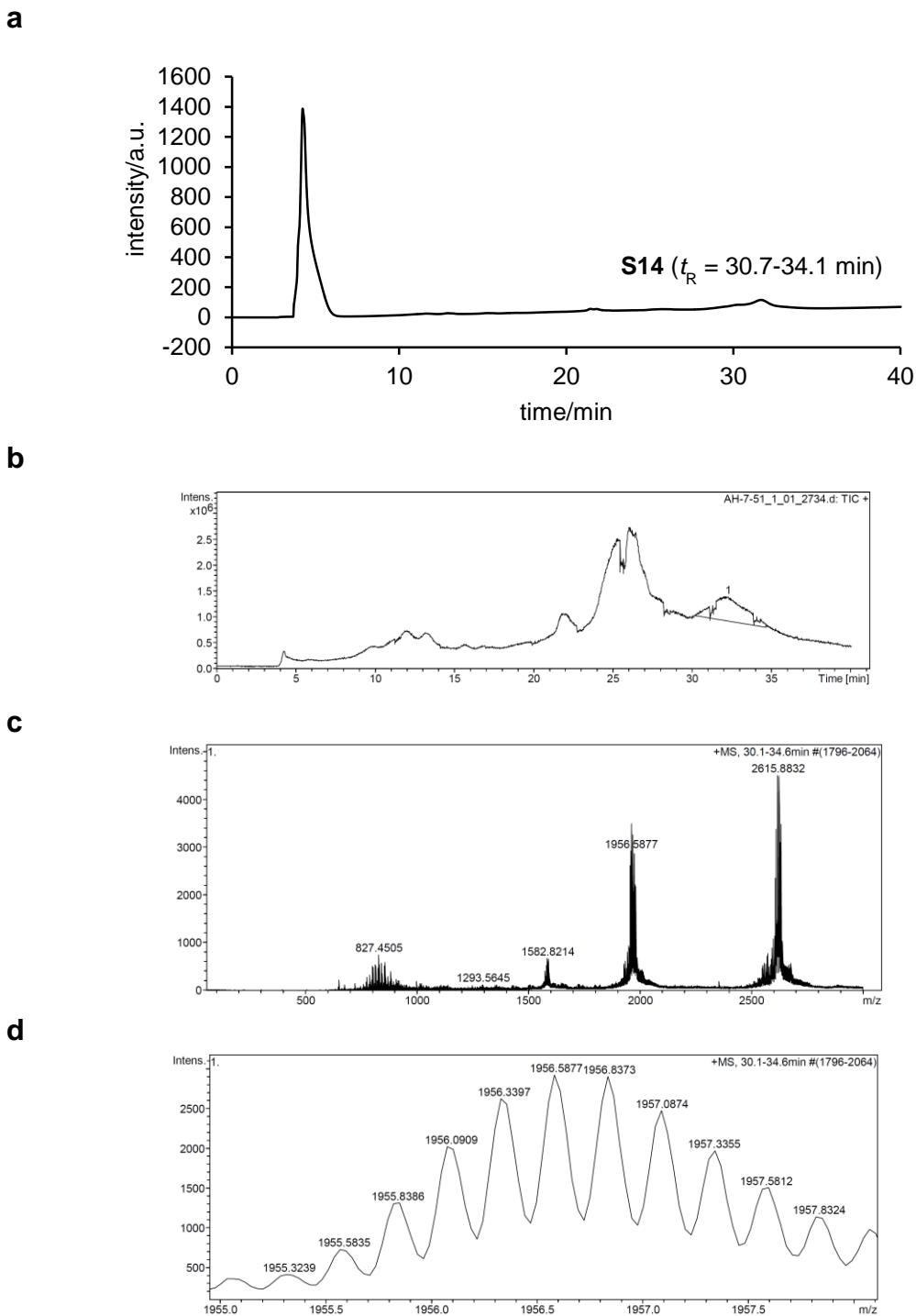


Figure S35. (a) HPLC chart for analysis of **S14**. Column: COSMOSIL Protein-R 4.6 × 150 mm, eluent A: *i*-PrOH + 0.05% TFA, eluent B: H₂O + 0.05% TFA, linear gradient: A/B = 50/50 to 100/0 over 40 min, flow rate: 0.5 mL/min, detection: UV 220 and 254 nm, temperature: 40 °C. (b) Total ion chromatogram of the crude material. Column: COSMOSIL Protein-R 4.6 × 150 mm, eluent A: *i*-PrOH + 0.05% TFA, eluent B: H₂O + 0.05% TFA, linear gradient: A/B = 50/50 to 100/0 over 40 min, flow rate: 0.5 mL/min, detection: ESI-TOF MS, temperature: 40 °C. (c) MS (ESI-TOF) signals of peak 1 in Figure S35b ($m/z = 0\text{-}3000$) and (d) ($m/z = 1955\text{-}1958$).

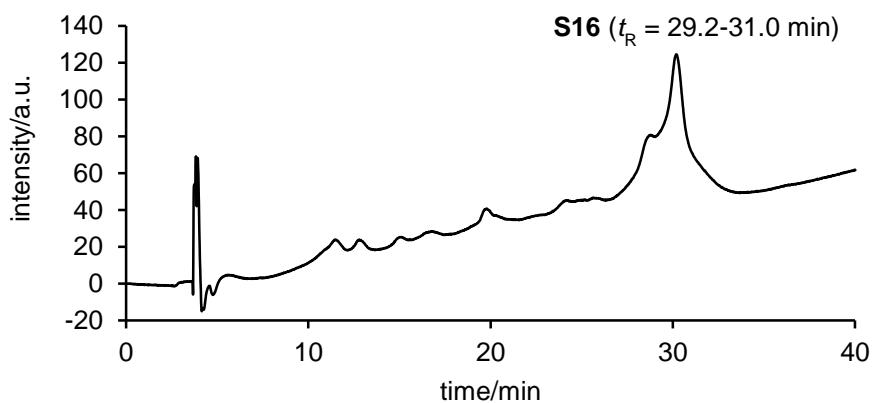
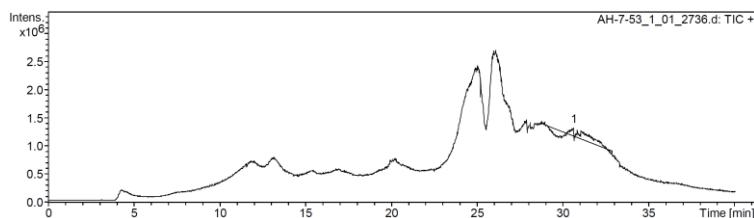
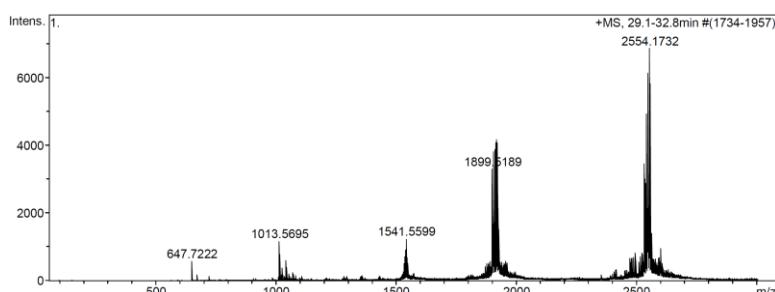
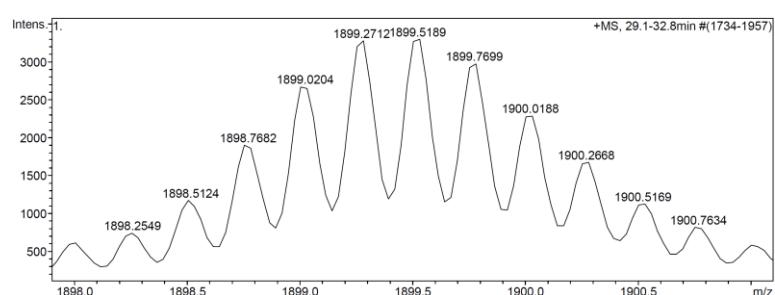
a**b****c****d**

Figure S36. (a) HPLC chart for analysis of **S16**. Column: COSMOSIL Protein-R 4.6 × 150 mm, eluent A: *i*-PrOH + 0.05% TFA, eluent B: H₂O + 0.05% TFA, linear gradient: A/B = 50/50 to 100/0 over 40 min, flow rate: 0.5 mL/min, detection: UV 220 and 254 nm, temperature: 40 °C. (b) Total ion chromatogram of the crude material. Column: COSMOSIL Protein-R 4.6 × 150 mm, eluent A: *i*-PrOH + 0.05% TFA, eluent B: H₂O + 0.05% TFA, linear gradient: A/B = 50/50 to 100/0 over 40 min, flow rate: 0.5 mL/min, detection: ESI-TOF MS, temperature: 40 °C. (c) MS (ESI-TOF) signals of peak 1 in Figure S36b ($m/z = 0\text{--}3000$) and (d) ($m/z = 1898\text{--}1901$).

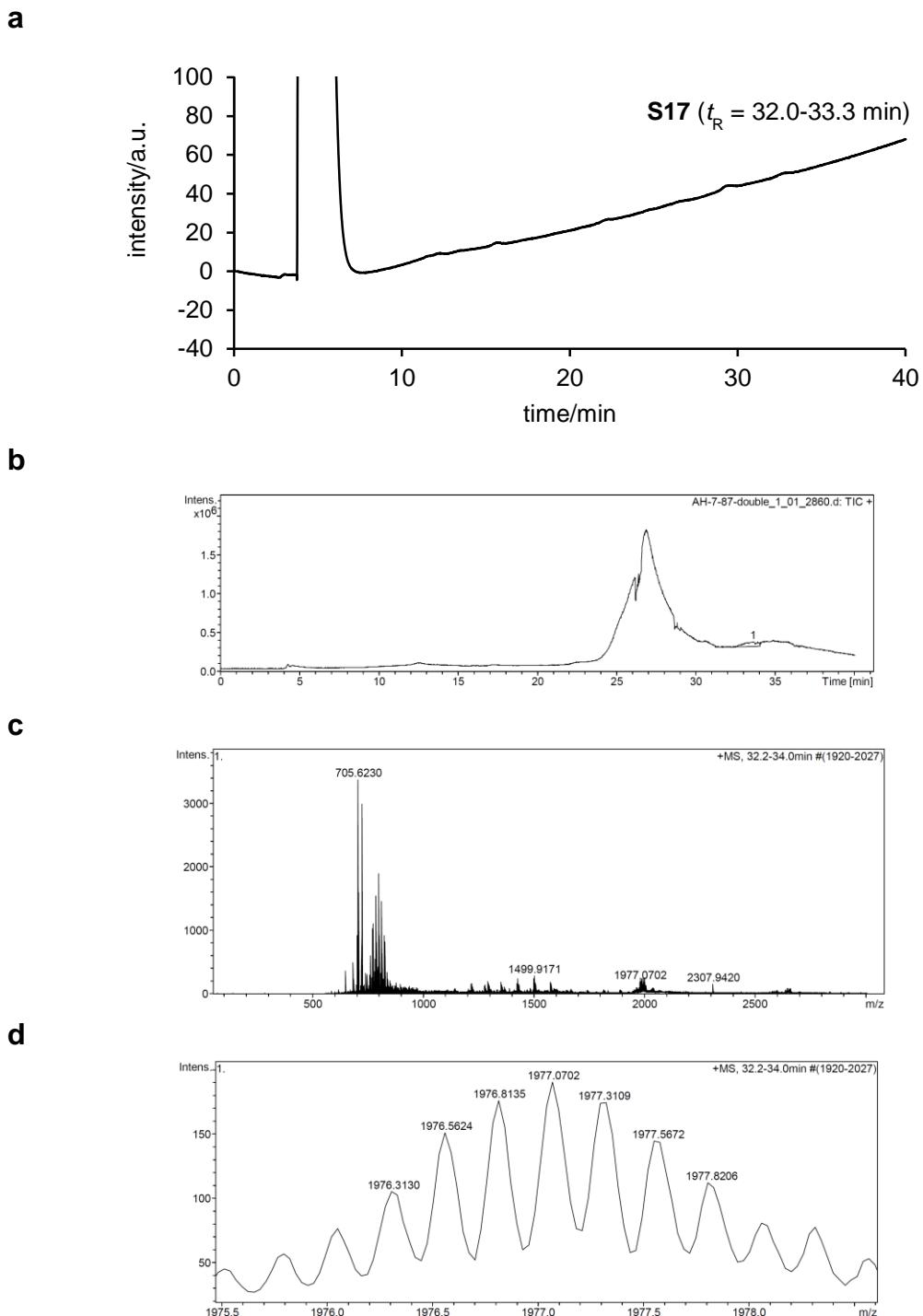


Figure S37. (a) HPLC chart for analysis of **S17**. Column: COSMOSIL Protein-R 4.6×150 mm, eluent A: *i*-PrOH + 0.05% TFA, eluent B: H_2O + 0.05% TFA, linear gradient: A/B = 50/50 to 100/0 over 40 min, flow rate: 0.5 mL/min, detection: UV 220 and 254 nm, temperature: 40 °C. (b) Total ion chromatogram of the crude material. Column: COSMOSIL Protein-R 4.6×150 mm, eluent A: *i*-PrOH + 0.05% TFA, eluent B: H_2O + 0.05% TFA, linear gradient: A/B = 50/50 to 100/0 over 40 min, flow rate: 0.5 mL/min, detection: ESI-TOF MS, temperature: 40 °C. (c) MS (ESI-TOF) signals of peak 1 in Figure S37b ($m/z = 0\text{-}3000$) and (d) ($m/z = 1975.5\text{-}1978.5$).

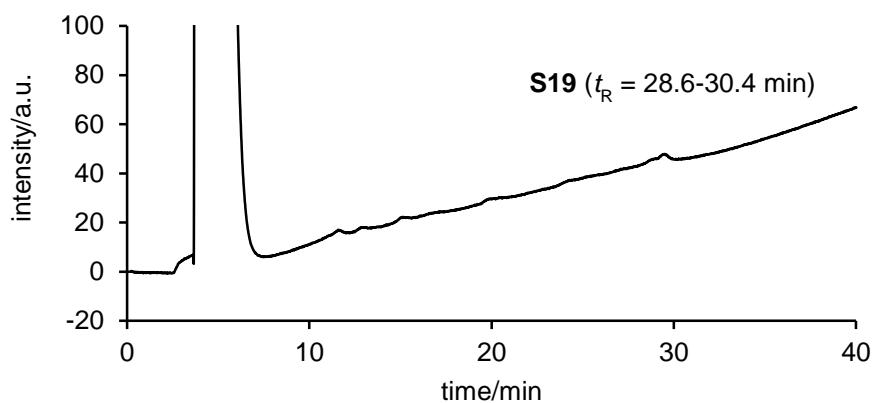
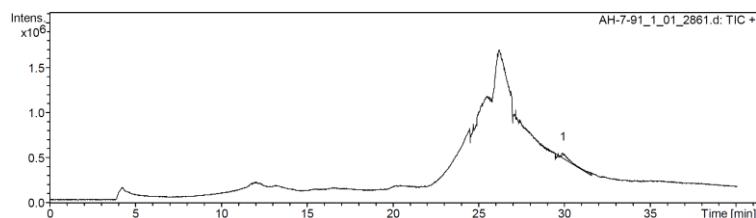
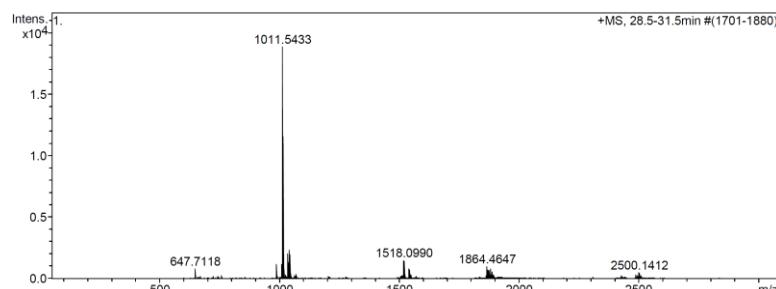
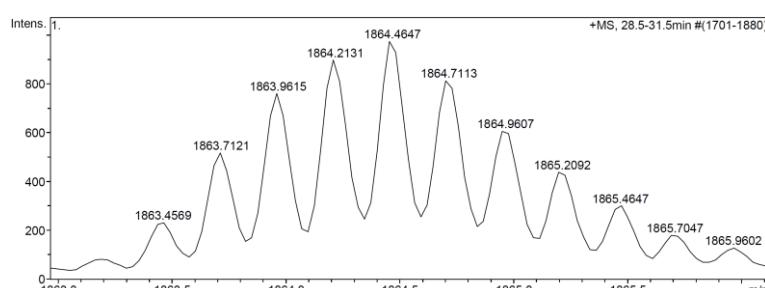
a**b****c****d**

Figure S38. (a) HPLC chart for analysis of **S19**. Column: COSMOSIL Protein-R 4.6 × 150 mm, eluent A: *i*-PrOH + 0.05% TFA, eluent B: H₂O + 0.05% TFA, linear gradient: A/B = 50/50 to 100/0 over 40 min, flow rate: 0.5 mL/min, detection: UV 220 and 254 nm, temperature: 40 °C. (b) Total ion chromatogram of the crude material. Column: COSMOSIL Protein-R 4.6 × 150 mm, eluent A: *i*-PrOH + 0.05% TFA, eluent B: H₂O + 0.05% TFA, linear gradient: A/B = 50/50 to 100/0 over 40 min, flow rate: 0.5 mL/min, detection: ESI-TOF MS, temperature: 40 °C. (c) MS (ESI-TOF) signals of peak 1 in Figure S38b ($m/z = 0\text{--}3000$) and (d) ($m/z = 1863\text{--}1866$).

HPLC Charts of Purified Synthetic Peptides

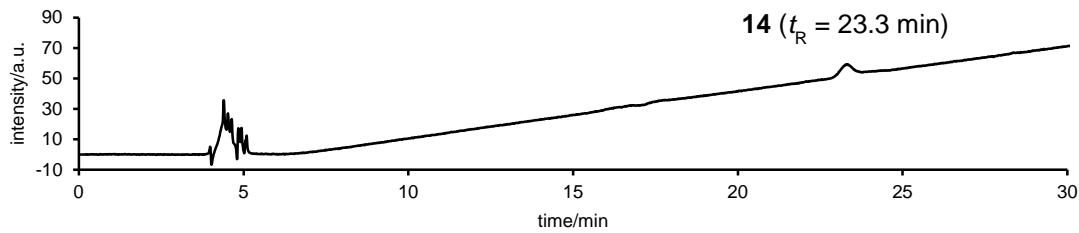


Figure S39. (a) HPLC chart of purified **14**. Column: Intertsil WP300 C8 4.6 × 150 mm, eluent A: *i*-PrOH + 0.05% TFA, eluent B: H₂O + 0.05% TFA, linear gradient: A/B = 20/80 to 100/0 over 40 min, flow rate: 0.5 mL/min, detection: UV 256 nm, temperature: 45 °C.

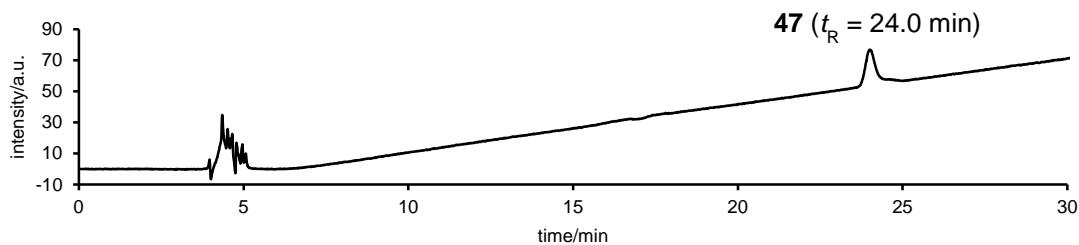


Figure S40. (a) HPLC chart of purified **47**. Column: Intertsil WP300 C8 4.6 × 150 mm, eluent A: *i*-PrOH + 0.05% TFA, eluent B: H₂O + 0.05% TFA, linear gradient: A/B = 20/80 to 100/0 over 40 min, flow rate: 0.5 mL/min, detection: UV 256 nm, temperature: 45 °C.

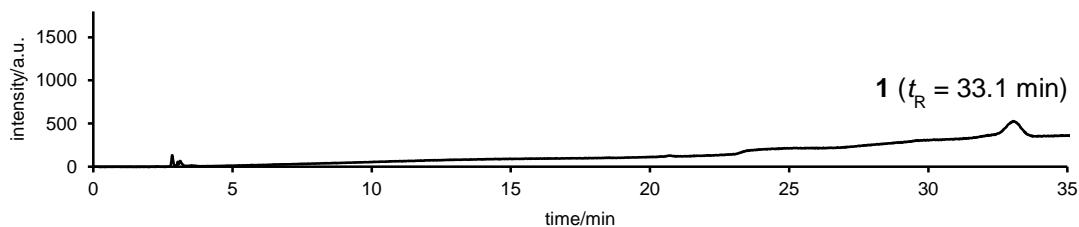


Figure S41. (a) HPLC chart of purified **1**. Column: Inertsil C8-3 4.6 × 150 mm, eluent A: *n*-PrOH + 0.05% TFA, eluent B: H₂O + 0.05% TFA, linear gradient: A/B = 25/75 to 50/50 over 35 min, flow rate: 0.6 mL/min, detection: UV 200 nm, temperature: 45 °C.

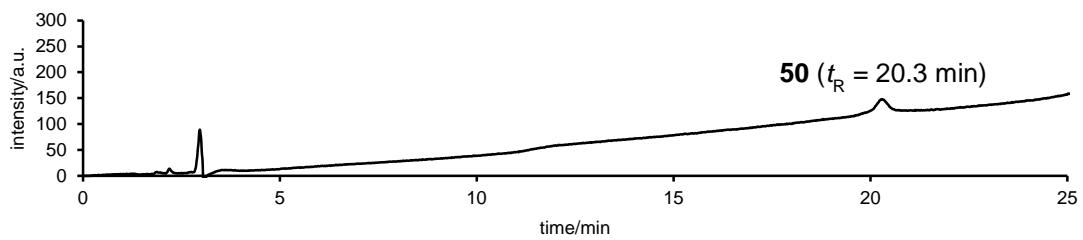


Figure S42. (a) HPLC chart of purified **50**. Column: Inertsil C8-3 4.6 × 150 mm, eluent A: *n*-PrOH + 0.05% TFA, eluent B: H₂O + 0.05% TFA, linear gradient: A/B = 20/80 to 40/60 over 30 min, flow rate: 0.6 mL/min, detection: UV 200 nm, temperature: 45 °C.

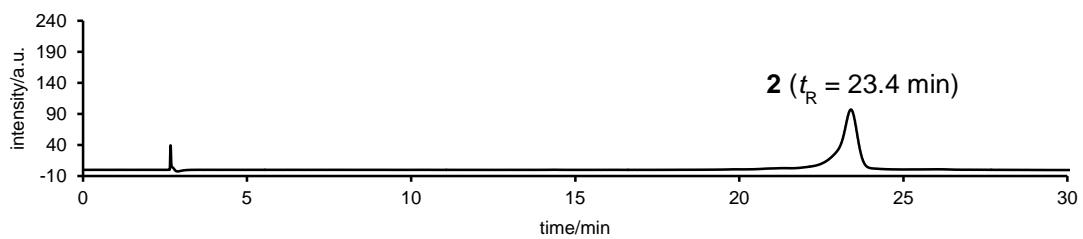


Figure S43. (a) HPLC chart of purified **2**. Column: Inertsil C8-3 4.6 × 150 mm, eluent A: *n*-PrOH + 0.05% TFA, eluent B: H₂O + 0.05% TFA, linear gradient: A/B = 25/75 to 50/50 over 35 min, flow rate: 0.6 mL/min, detection: UV 500 nm, temperature: 45 °C.

References

- S1. Inoue, M.; Shinohara, N.; Tanabe, S.; Takahashi, T.; Okura, K.; Itoh, H.; Mizoguchi, Y.; Iida, M.; Lee, N.; Matsuoka, S. *Nat. Chem.* **2010**, 2, 280–285.
- S2. Ivanov, A. I. *Methods Mol. Biol.* **2008**, 440, 15–33.
- S3. Rasband, W. S. ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <https://imagej.nih.gov/ij/>, 1997-2016.
- S4. Bolte, S.; Cordelières, F. P. *J. Microscopy*, **2006**, 224, 213–232.