Apratoxin S10, a dual inhibitor of angiogenesis and cancer cell growth to treat highly vascularized tumors

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1. General Procedures

All commercial reagents were used without further purification unless otherwise noted. Solvents were purified according to the guidelines in *Purification of Laboratory Chemicals* (5^{th} edition, W. L. F. Armarego, Christina L. L. Chai, Butterworth-Heinemann: Oxford, 2003). Tetrahydrofuran (THF) and diethyl ether were distilled from sodium chips in the presence of small amount of benzophenone; CH_2Cl_2 and toluene were distilled from CaH_2; MeCN, *N*,*N*-dimethylformamide (DMF) were dried with 4Å molecular sieves (MS) and MeOH dried with 3 Å MS; 4 M Hydrochloric acid (HCl) solution in ethyl acetate was prepared by dissolving HCl gas (yielding by dropping aqueous hydrochloric acid (34%) to concentrated sulfuric acid (98%)) to ethyl acetate. All reactions were performed in heat-gun dried flasks (400°C under reduced pressure) under an inert atmosphere of anhydrous Ar unless otherwise noted. Thin layer chromatography was performed on EMD silica gel 60 Å F_{254} glass plates and preparative thin layer chromatography was performed on Whatman silica gel 60 Å F_{254} glass plates (layer thick 1000 μm). Flash column chromatography was performed with Fisher 170-400 mesh silica gel. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Mercury 400 MHz, Bruker Avance II 600, Bruker Avance III 600 MHz or Aligent VNMR 600 MHz spectrometer as indicated in the data list. Chemical shifts for proton nuclear magnetic resonance (^{1}H NMR) spectra are reported in parts per million relative to the signal residual $CDCl_3$ at 7.26 ppm; Chemicals shifts for carbon nuclear magnetic resonance (^{13}C NMR) spectra are reported in parts per million relative to the center line of the $CDCl_3$ triplet at 77.16 ppm; The abbreviations s, d, dd, ddd, dddd, t, q, br and m stand for the resonance multiplicity singlet, doublet, doublet of doublets, doublet of doublet of doublets, doublet of doublet of doublet of doublets, triplet, quartet, broad and multiplet, respectively. Optical rotation was measured on a Perkin-Elmer 341 polarimeter (Na D line) using a microcell of 1-dm path length. High resolution mass spectra (HRMS) data were obtained using an Agilent-LC-TOF mass spectrometer with an APCI/ESI multimode ion source detector. HPLC analyses were carried out at λmax 220 nm or 254 nm using column Luna 4u-RP 250 mm × 4.6 mm × 4 μm on Shimadzu SPD-M20A-PDA system; HPLC purity is reported in area %.

2. Experimental Procedures and Analytical Data

Pyrrolidine-1,2-dicarboxylic acid (2*S*)-2-{(1*S*,3*S*,5*S*)-6-[(1*R*)-3-allyloxycarbonyl-1-(triphenylmethylthio)methyl-propylcarbamoyl]-1-*tert*-butyl-3,6-dimethyl-5-(2,2,2-trichloroethoxycarbonyloxy)hept-1-yl}ester 1-(9*H*-fluoren-9-ylmethyl)ester (4).

To the solution of **3** (71.0 mg, 0.134 mmol) in CH_2Cl_2 (2.0 ml) was added 2,6-lutidine (320.0 µl, 2.755 mmol) and then added trimethylsilyl trifluoro-methanesulfonate (TMSOTf) (400 µl, 2.212 mmol) dropwise at room temperature under argon. After being stirred at the room temperature for 1.5 h, the reaction mixture was quenched with MeOH (3 ml) and water (10 ml) at 0°C, and extracted with CH_2Cl_2 (15 ml × 3). The combined organic layer was washed with brine, dried over anhydrous $MgSO_4$ and evaporated in vacuo to give the crude free amine of **3**, which was used in next step without further purification.

To the crude free amine of **3** in CH_2Cl_2 (2.5 ml) was added acid **2** (74.0 mg, 0.098 mmol), PyAOP (104 mg, 0.197 mmol), DIEA (52 µl, 0.295 mmol) at room temperature. After being stirred overnight at room temperature, the resulting reaction mixture was evaporated in vacuo and purified chromatography column (ethyl acetate/hexane 1: 5) to give product **4** as colorless oil. (104.7 mg, 91.4%). $[\alpha]^{20}_D$: -48.5 (c 0.20, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$, mixture of rotamers, major/minor 6:4): δ 7.78-7.75 (m, 2H), 7.67 (d, J = 7.6 Hz, 0.6H), 7.64-7.61 (m, 1.4H), 7.42-7.37 (m, 8H), 7.35-7.24 (m, 8H), 7.22-7.17 (m, 3H), 5.94 (d, J = 8.4 Hz, 0.4H), 5.92-5.81 (m, 1.6H), 5.30-5.12 (m, 3H), 4.97 (d, J = 12.0 Hz, 0.6H), 4.86 (d, J = 12.0 Hz, 0.4H), 4.79-4.75 (m, 1H), 4.70 (d, J = 12.0 Hz, 0.4H), 4.65 (d, J = 12.0 Hz, 0.6H), 4.54-4.34 (m, 4.4H), 4.29-4.23 (m, 1H), 4.20-4.17 (m, 0.6H), 3.98-3.86 (m, 1H), 3.67-3.48 (m, 2H), 2.41-2.05 (m, 5.6H), 2.04-1.89 (m, 2.4H), 1.87-1.58 (m, 3.6H), 1.47-1.31 (m, 2H), 1.18 (s, 2.4H), 1.16 (s, 3.6H), 1.09-1.02 (m, 1H), 0.98 (d, J = 6.4 Hz, 1.8H), 0.85 (s, 5.4H), 0.82 (s, 3.6H), 0.68 (d, J = 6.4 Hz, 1.2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, mixture of rotamers): δ 174.6, 173.0, 173.0, 172.9, 172.3, 154.7, 154.4, 154.3, 154.2, 144.6, 144.6, 144.4, 144.3, 144.0, 143.8, 141.4, 141.3, 141.3, 141.3, 132.1, 132.1, 129.6, 129.6, 128.1, 128.0, 128.0, 127.7, 127.2, 127.1, 127.1, 126.9, 125.5, 125.4, 125.3, 125.2, 120.0, 120.0, 120.0, 118.5, 118.5, 94.9, 94.8, 81.6, 81.3, 79.8, 79.6, 77.4, 77.0, 67.9, 67.5, 66.7, 66.7, 65.4, 59.9, 59.4, 48.4, 48.4, 47.3, 47.3, 47.1, 46.9, 46.9, 46.4, 38.3, 38.0, 37.2, 36.8, 36.6, 34.8, 34.8, 34.6, 32.0, 31.7, 31.4, 30.9, 30.8, 30.1, 29.8, 29.0, 27.0, 26.7, 25.9, 25.9, 25.4, 24.5, 23.5,

22.9, 22.8, 22.7, 20.5, 20.3, 20.1, 19.6, 14.2 ppm. HRMS (ESI) *m/z* calcd for $C_{64}H_{73}Cl_3N_2O_{10}S$ $[M+Na]^+$ 1189.3944, found 1189.3964.

Pyrrolidine-1,2-dicarboxylic acid (2*S*)-2-{(1*S*,3*S*,5*S*)-6-[(5*R*)-5-(2-allyloxycarbonylethyl)-4,5-dihydro-thiazol-2-yl]-1-*tert*-butyl-3,6-dimethyl-5-hydroxyhept-1-yl}ester 1-(9*H*-fluoren-9-ylmethyl)ester (5).

To the solution of **4** (103.7 mg, 0.089 mmol) in 1, 2-dichloroethane (5 ml) was added $TiCl_4$ (1 M in CH_2Cl_2, 350 µl, 0.356 mmol) at room temperature. The resulting solution was heated to 60°C and stirred at this temperature for 2.0 h. This reaction was monitored by mass spectrum. When the starting material was consumed completely, the reaction was cooled to 0°C and quenched with saturate aq. $NaHCO_3$ (6 ml), stirred at room temperature for another 10 min, extracted with ethyl acetate (20 ml×4), dried over anhydrous $MgSO_4$, filtered and evaporated in vacuo to give crude thiazoline intermediate, which was used in the next step without further purification.

The above crude was dissolved in THF (3.5 ml) and then aqueous NH_4OAc (1 M, 1.1 ml), and zinc powder (freshly activated with 1 M aqueous HCl) (105 mg) were added at room temperature. After being stirred at the same temperature for 1 h, the reaction was added ethyl acetate (10 ml) and brine (10 ml). The aqueous layer was extracted with ethyl acetate (20 ml × 3). The combined organic layer was dried with $MgSO_4$, filtered, concentrated in vacuo, and purified by preparative silica TLC plates (ethyl acetate/hexane 1:2) to give thiazoline ring product **5** (39.0 mg, 60%) as colorless oil. $[\alpha]^{20}_D$ -80.8 (c 0.12, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$, mixture of rotamers, major/minor 7:3): δ 7.77-7.74 (m, 2H), 7.66-7.60 (m, 1.7H), 7.57 (d, J = 7.2 Hz, 0.3H), 7.41-7.37 (m, 2H), 7.32-7.28 (m, 2H), 5.95-5.84 (m, 1H), 5.33-5.19 (m, 2H), 4.89 (dd, J = 11.4, 2.4 Hz, 0.7H), 4.82 (dd, J = 10.0, 2.4 Hz, 0.3H), 4.58-4.54 (m, 2H), 4.52 (d, J = 3.2 Hz, 0.3H), 4.47-4.37 (m, 3H), 4.35-4.19 (m, 2H), 3.69-3.61 (m, 2H), 3.58-3.48 (m, 2H), 3.30-3.24 (m, 1H), 2.85-2.78 (m, 1H), 2.58-2.45 (m, 2H), 2.34-2.19 (m, 1H), 2.12-1.92 (m, 5H), 1.78 (br m, 1H), 1.67-1.55 (m, 2H), 1.49-1.22 (m, 2H), 1.20-1.18 (m, 3.9H), 1.15 (s, 2.1H), 0.96 (d, J = 6.8 Hz, 2.1H), 0.87 (s, 9H), 0.78 (d, J = 6.8 Hz, 0.9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, mixture of rotamers): δ δ 179.0, 178.4, 173.1, 173.0, 172.9, 172.6, 155.0, 154.5, 144.4, 144.3, 144.0, 143.9, 141.4, 141.4, 141.3, 141.3, 141.3, 132.3, 132.3, 127.8, 127.7, 127.7, 127.2, 127.1, 127.1, 125.5, 125.4, 125.3, 125.3, 120.0, 118.4, 118.3, 79.7, 78.8, 77.4, 76.1, 75.9, 74.7, 74.6, 67.9, 67.7, 65.3, 65.3, 59.8, 59.7, 47.3, 47.2, 47.1, 46.1, 45.7, 38.2, 37.9, 37.2, 37.1, 37.0, 36.7, 34.8, 34.7, 32.0, 31.7, 31.6, 31.5, 31.3,

30.2, 30.2, 30.0, 29.8, 29.8, 26.1, 26.1, 26.1, 25.6, 25.4, 24.6, 24.1, 24.0, 23.4, 22.8, 20.6, 20.6, 14.2 ppm. HRMS (ESI) *m/z* calcd for $C_{42}H_{56}N_2O_7S$ $[M+Na]^+$ 755.3700, found 755.3718.

Pyrrolidine-1,2-dicarboxylic acid (2*S*)-2-{(1*S*,3*S*,5*S*)-6-[(5*R*)-5-(2-carboxylethyl)-4,5-dihydro-thiazol-2-yl]-1-*tert*-butyl-3,6-dimethyl-5-hydroxyhept-1-yl}ester 1-(9*H*-fluoren-9-ylmethyl)ester (6).

To a solution of **5** (30.5 mg, 0.0416 mmol) in THF (3.0 ml) were added $Pd(PPh_3)_4$ (4.8 mg, 0.004 mmol) and *N*-methyl aniline (11.4µl, 0.104 mmol) at room temperature under argon. This reaction was protected with aluminum foil. After being stirred at the same temperature for 1 h, the reaction mixture was concentrated in vacuo and purified by preparative TLC (20 cm × 20 cm plate) to give acid **2** (26.7 mg, 95%). $[\alpha]^{20}_D$: -57.7 (c 0.052, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$, mixture of rotamers): δ 7.77-7.74 (m, 2H), 7.65-7.61 (m, 1.7H), 7.58 (d, J = 7.6 Hz, 0.3H), 7.41-7.37 (m, 2H), 7.33-7.28 (m, 2H), 4.90 (dd, *J* = 11.6, 2.4 Hz, 0.7H), 4.84 (dd, *J* = 10.8, 2.0 Hz, 0.3H), 4.52 (dd, *J* = 8.6, 2.8 Hz, 0.3H), 4.45 (dd, *J* = 9.6, 6.0 Hz, 1H), 4.42-4.36 (m, 1.4H), 4.34-4.19 (m, 3.3H), 3.68-3.61 (m, 1.7H), 3.58-3.49 (m, 1.3H), 3.29 (dd, *J* = 11.8, 8.4 Hz, 0.3H), 3.18 (dd, *J* = 11.8, 8.4 Hz, 0.7H), 2.82 (dd, *J* = 10.8, 9.2 Hz, 0.3H), 2.75 (dd, *J* = 10.6, 10.6 Hz, 0.7H), 2.65-2.49 (m, 2H), 2.35-2.16 (m, 1H), 2.09-1.85 (m, 5H), 1.82-1.74 (m, 1H), 1.71-1.59 (m, 2H), 1.50-1.42 (m, 0.3H), 1.35-1.25 (m, 2.7H), 1.21 (s, 0.9H), 1.20 (s, 0.9H), 1.18 (s, 2.1H), 1.17 (s, 2.1H), 0.96 (d, J = 6.4Hz, 2.1H), 0.88 (s, 9H), 0.75 (d, *J* = 6.8 Hz, 0.9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, mixture of rotamers): δ 182.3, 182.2, 180.9, 175.6, 175.6, 173.4, 172.8, 155.1, 154.5, 144.4, 144.2, 144.3, 144.1, 143.8, 141.4, 141.3, 141.3, 127.8, 127.8, 127.7, 127.2, 127.1, 125.5, 125.4, 125.4, 125.3, 120.0, 120.0, 78.8, 77.4, 76.2, 75.8, 75.1, 75.0, 68.0, 67.9, 59.7, 59.6, 47.3, 47.2, 47.1, 46.6, 46.5, 45.9, 38.1, 37.8, 37.6, 37.2, 36.4, 34.8, 34.6, 33.2, 33.2, 32.5, 32.7, 31.7, 31.3, 30.1, 30.0, 29.9, 29.8, 26.1, 26.0, 26.0, 25.3, 24.6, 24.1, 23.5, 23.5, 23.4, 22.8, 20.8, 20.6, 14.3 ppm. HRMS (ESI) *m/z* calcd for $C_{39}H_{52}N_2O_7S$ $[M+Na]^+$ 715.3387, found 715.3397.

Synthesis of cyclic precursors 8.

To a solution of Fmoc protected tripeptide **7** (37.4 mg, 0.056 mmol) in MeCN (1.6 ml) was added diethylamine (0.8 ml) at room temperature. After being stirred at the same temperature for 30 min, the reaction mixture was evaporated in vacuo, then azeotroped with toluene and CH_2Cl_2 two times,

respectively, and dried under reduced pressure for 1 h to give the free amine tripeptide, which was used in the next coupling reaction without further purification.

The above crude free amine tripeptide was dissolved in CH_2Cl_2 (3 ml). To this solution was added acid **2** (0.037 mmol), coupling reagent PyAOP (38.9 mg, 0.074 mmol), DIEA (0.02 ml, 0.115 mmol) at room temperature. After being stirred at the same temperature for 15h, the reaction mixture was concentrated in vacuo and purified by preparative TLC plate (developed by acetone/hexane (2:3, v/v)) to give the precursor **8** as colorless oil (38.5 mg, 92%). $[\alpha]^{20}_{D}$: -130.0 (c 0.10, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$, mixture of rotamers): δ 7.76-7.72 (m, 2H), 7.64-7.56 (m, 2H), 7.40-7.35 (m, 2H), 7.31-7.27(m, 2H), 7.11-7.05 (m, 2H), 6.78-6.71 (m, 3H), 5.92-5.82 (m, 1H), 5.39-5.34 (m, 1H), 5.31-5.19 (m, 2H), 5.17-5.12 (m, 1H), 4.92-4.81 (m, 2H), 4.59-4.54 (m, 3H), 4.48-4.35 (m, 2H), 4.35-4.20 (m, 3H), 3.75-3.73 (m, 3H), 3.68-3.58 (m, 2H), 3.56-3.48 (m, 1H), 3.26-3.19 (m, 1H), 3.05-2.98 (m, 1H), 2.96-2.88 (m, 3H), 2.83-2.65 (m, 3H), 2.62 (s, 2.1H), 2.61 (s, 0.9H), 2.41-2.28 (m, 2H), 2.28-2.21 (m, 1H), 2.15-2.08 (m, 0.7H), 2.05-1.72 (m, 6H), 1.69-1.55 (m, 2H), 1.50-1.44 (m, 0.3H), 1.38-1.21 (m, 4H),1.19-1.17 (m, 6H), 0.99-0.91 (m, 6H), 0.87-0.81 (m, 15H), 0.78 (d, J = 6.4 Hz, 0.9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, mixture of rotamers): δ 179.1, 178.7, 173.1, 172.9, 172.2, 172.0, 171.8, 171.5, 171.3, 171.2, 170.8, 169.7, 158.6, 158.4, 155.0, 154.5, 144.5, 144.3, 144.0, 143.9, 141.4, 141.4, 141.3, 141.3, 131.8, 130.5, 130.5, 130.5, 128.9, 128.4, 128.3, 127.8, 127.7, 127.1, 127.1, 125.5, 125.4, 125.3, 125.2, 120.0, 120.0, 118.8, 114.2, 113.9, 79.7, 78.9, 77.4, 76.1, 75.9, 75.2, 75.1, 68.0, 67.7, 66.1, 65.5, 64.5, 60.5, 59.8, 59.6, 55.3, 50.4, 49.9, 47.3, 47.2, 47.1, 46.6, 45.9, 45.5, 38.2, 37.9, 37.0, 37.0, 36.8, 34.8, 34.7, 33.5, 33.3, 31.7, 31.4, 30.9, 30.9, 30.6, 30.6, 30.3, 29.9, 29.8, 26.1, 26.0, 25.5, 25.4, 25.1, 24.9, 24.6, 24.1, 23.9, 23.5, 23.4, 22.8, 20.8, 20.7, 20.6, 16.2, 15.9, 14.4, 14.2, 11.7, 10.6 ppm. HRMS (ESI) *m/z* calcd for $C_{63}H_{87}N_5O_{11}S$ $[M+Na]^+$ 1144.6015, found 1144.6041.

Synthesis of 1 (apratoxin S10).

To a solution of cyclic precursor **8** (38.0 mg, 33.9 μmol) in THF (3.0 ml) were added $Pd(PPh_3)_4$ (7.8 mg, 6.8 μmol), and *N*-methylaniline (18.5 μl, 0.169 mmol) at room temperature under argon. This reaction was protected with aluminum foil. After being stirred at the same temperature for 1h, the reaction mixture was concentrated in vacuo and purified by preparative TLC plate (developed with MeOH/CH_2Cl_2 1:9, v/v) to give the free acid cyclic precursor.

To the solution of free acid cyclic precursor in MeCN (4.4 ml) were added *N*,*N*-diethylamine (2.2 ml). After being stirred at room temperature for 30 min, the reaction mixture was evaporated in vacuo, azeotroped with toluene (three times) and CH_2Cl_2 (two times) and then dried under reduced pressure for 1h to give the unmasked precursor as foam solid. Then the unmasked precursor was dissolved in CH_2Cl_2 (60 ml). To this solution was added DIEA (60 µl, 0.339 mmol) and PyAOP (53.0 mg, 0.102 mmol) at 0ºC. After being stirred at 0 ºC for 30 min, the reaction mixture was allowed to warm up to room temperature and stirred for additional 15 h. Then the reaction was concentrated in vacuo and purified by semipreparative reversed-phase HPLC (Phenomenex Ultracarb, ODS 250 × 10 mm, 5 µm, 3.0 mL/min, UV detection at 200/220 nm) using an isocratic system of 80% aqueous MeCN for 30 min, 80-100% MeCN for 30-40 min, and 100% MeCN for 40-60 min to afford **1** (apratoxin S10) (22.8 mg, 80% in 3 steps, 95.1% HPLC purity calculated based on peak area and 100% chiral purity). $[\alpha]^{20}_D$ -59.5 (c 0.037, CH_2Cl_2). t_R = 35.0 min. ^{1}H NMR (600 MHz, $CDCl_3$, mixture of rotamers, major and minor (8/2)): δ 7.12 (d, *J* = 8.4 Hz, 0.4H), 7.11 (d, *J* = 8.4 Hz, 1.6H), 6.80 (d, *J* = 9.0 Hz, 1.6H), 6.78 (d, *J* = 8.4 Hz, 0.4H), 6.26 (d, *J* = 8.4 Hz, 0.8H), 5.75 (d, *J* = 9.6 Hz, 0.2H), 5.24 (d, *J* = 11.4 Hz, 0.2H), 5.21 (ddd, *J* = 10.2, 9.9, 5.4 Hz, 0.2H), 5.07 (ddd, *J* = 9.0, 9.0, 4.8 Hz, 0.8H), 4.94 (dd, *J* = 12.6, 2.4 Hz, 0.2H), 4.90 (dd, *J* = 12.6, 3.0 Hz, 0.8H), 4.78 (d, *J* = 11.4 Hz, 0.8 H), 4.58 (q, *J* = 6.6 Hz, 0.8H), 4.55 (d, *J* = 10.8 Hz, 0.2H), 4.47 (dddd, *J* = 9.8, 7.5, 6.6, 4.2 Hz, 0.8H), 4.30 (dd, *J* = 6.6, 1.8 Hz, 0.8H), 4.19-4.13 (m, 1.4H), 4.08 (ddd, *J* = 11.4, 6.6, 4.2 Hz, 0.8H), 3.77-3.73 (m, 3.8H), 3.63-3.57 (m, 1H), 3.37 (dd, *J* = 10.8, 7.8 Hz, 0.8H), 3.35 (dd, *J* = 10.8, 7.8 Hz, 0.2H), 3.26 (q, *J* = 6.6 Hz, 0.2H), 3.08 (dd, *J* = 12.6, 10.2 Hz, 0.2H), 3.05-2.99 (m, 1.6H), 2.91 (s, 2.4H), 2.82 (s, 0.6H), 2.80 (dd, *J* = 10.8, 6.6 Hz, 0.8H), 2.77-2.74 (m, 0.8H), 2.73-2.67 (m, 0.4H), 2.59 (s, 2.4H), 2.44-2.40 (m, 0.2H), 2.29 (t, *J* = 7.2 Hz, 2H), 2.26-2.22 (m, 1H), 2.12-2.08 (m, 1H), 2.05-1.83 (m, 6H), 1.78-1.59 (m, 3H), 1.50 (dt, *J* = 12.3, 3.0 Hz, 0.8H), 1.29-1.24 (m, 1.2H), 1.23 (d, *J* = 6.6 Hz, 0.6H), 1.16 (s, 0.6H), 1.14 (s, 2.4H), 1.11 (s, 0.6H), 1.10 (s, 2.4H), 1.08 (d, *J* = 6.6 Hz, 2.4H), 1.02 (d, *J* = 7.2 Hz, 0.6H), 1.01 (t, *J* = 7.2 Hz, 0.6H), 0.95 (d, *J* = 6.6 Hz, 2.4H), 0.95 (d, *J* = 6.6 Hz, 0.6H), 0.94-0.90 (m, 1H), 0.86 (s, 9H), 0.85 (t, *J* = 7.8Hz, 2.4H), 0.41 (d, *J* = 6.6 Hz, 2.4H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$, mixture of rotamers, major and minor): δ 179.4, 177.5, 172.2, 172.0, 171.9, 171.8, 171.5, 171.2, 170.6, 170.4, 170.3, 170.2, 170.1, 158.9, 158.7, 130.6, 130.5, 128.7, 128.5, 114.3, 114.0, 77.8, 76.3, 75.3, 75.2, 74.2, 60.8, 60.0, 59.6, 58.5, 57.3, 55.5, 55.4, 54.1, 53.6, 51.3, 49.5, 47.7, 47.6, 46.8, 46.1, 41.1, 40.1, 38.6, 37.7, 37.5, 37.2, 36.9, 35.5, 35.2, 35.2, 34.2, 33.9, 33.9, 33.8, 32.7,

30.9, 30.7, 30.5, 30.2, 29.8, 29.4, 29.3, 28.8, 26.3, 26.3, 25.8, 25.6, 25.4, 25.3, 24.8, 24.4, 20.8, 20.2, 19.1, 19.0, 14.6, 14.1, 14.1, 14.1, 10.9, 10.3 ppm. HRMS (ESI) *m/z* calcd for $C_{45}H_{71}N_5O_8S$ $[M+Na]^+$ 864.4916, found 864.4918

Tumor images

Consents have been obtained from patients for the use of their deidentified scan images for research purpose.

Cell culture

Human colon cancer cells (HCT116) were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum at 37 ºC humidified air and 5% CO_2. Human renal carcinoma A498 cells and human neuroendocrine carcinoma NCI-H727 cells were purchased from ATCC (Manassas, VA). Human hepatocellular carcinoma Huh7 cells were provided by Dr. Chen Liu. Human Umbilical Vein Endothelial Cells (HUVEC, cat# CC-2519) were purchased from Lonza. A498, Huh7 and NCI-H727 cells were cultured in Eagle's minimum essential medium, Dulbecco's modified Eagle's medium and RPMI-1640 medium, respectively, supplemented with 10% fetal bovine serum at 37 ºC humidified air and 5% CO_2. HUVEC cells were cultured in EGM (Lonza cat# CC-3124) under the same condition.

Cell Viability Assay (MTT)

A498, Huh7 or NCI-H727 cells were seeded in a 96-well clear bottom plate and 24 h later, cells were treated with various concentrations of the apratoxin S10 (10 pM–1 μM), known RTK inhibitors or solvent control (EtOH for apratoxins and DMSO for RTK inhibitors). After 48 h of incubation, cell viability was detected using MTT according to the manufacturer's instructions (Promega, Madison, WI). Nonlinear regression analysis was carried out using GraphPad Prism software for IC_{50} value calculations.

Antiproliferative evaluation in HCT116 cells was performed in the same way as above except that the cells were treated with various concentrations of the apratoxin analogues (10 pM–1 μM) 24 h post-seeding.

In Vitro Angiogenesis Assay

HUVECs (Lonza) were used at passage 4 for this assay. In vitro Angiogenesis Assay Kit (Chem icon) was used according to the manufacturer's recommendation. Briefly, an ice-cold mixture of ECMatrix was transferred into a precooled 96-well plate. After the matrix solution had solidified (> 1 h incubation at 37 °C, 23,000 cells were mixed with the appropriate inhibitor concentration (in 100 μL EGM) and plated into each well. After incubation at 37 °C for 14 h, images were captured for each well using a Nikon inverted microscope equipped with NIS-Elements software. Branch point counting was used as quantification method. Five random microscope view-fields were counted and the number of branch points was averaged. The number of junctions were analyzed by the Angiogenesis Analyzer plug-in for ImageJ (n = 5 per group).

The cell viability of HUVEC cells was evaluated in the same way as for cancer cells (above), except that 23,000 cells were mixed with the appropriate inhibitor concentration (in 100 μL EGM) and plated into each well of 96-well plate for 48 h before MTT dye was added.

Measurement of Human VEGF-A and IL-6 Secretion

A498, Huh7 or NCI-H727 cells were seeded in a 96-well clear bottom plate. Cells were treated with various concentrations of apratoxins (10 pM–1 μM) or solvent control (EtOH). After 24 h incubation, culture supernatants were collected for detection of VEGF-A or IL-6 using alphaLISA kits (PerkinElmer, Waltham, MA) following the manufacturer's instruction. Briefly, acceptor bead and anti-VEGF-A/ anti-IL-6 antibody were incubated with the supernatants for 60 min firstly, donor beads were added later and incubated for another 30 min. Signal was detected using Envision (PerkinElmer). Levels of VEGF-A/IL-6 (pg/ mL) were calculated using a standard curve and then normalized based on cell numbers.

Immunoblot Analysis

A498, Huh7, NCI-H727 or HUVEC cells were seeded in 6-well clear bottom plate the day before treatment. The next day, cells were treated with apratoxin S10 or solvent control (EtOH). 24 h later, whole cell lysates were collected using PhosphoSafe buffer (EMD Chemicals, Inc, Gibbstown, NJ). Protein concentrations were measured with the BCA Protein Assay kit (Thermo

Fisher Scientific, Rockford, IL). Lysates containing equal amounts of protein were separated by SDSpolyacrylamide gel electrophoresis (4–12%), transferred to polyvinylidene difluoride membranes, probed with primary and secondary antibodies, and detected with the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific). Anti-FGFR4 antibody was obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Anti-VEGFR2, EGFR, Met, IGF1Rβ, β-actin and secondary anti-mouse and rabbit antibodies were from Cell Signaling Technology, Inc (Danvers, MA).

Aqueous stability

Stock solutions of apratoxin S4, S7, S8, S9 and S10 were prepared by dissolving the compounds in acetonitrile to give a 40 µg/mL solution. Serial dilution of the 40 µg/mL solution in acetonitrile gave standard solutions with concentrations of 25, 12.5, 2.5, 0.25, 0.025, and 0 µg/mL. A stock solution of harmine, 35 ng/ml, was prepared in ethyl acetate to serve as the working internal standard solution.

The stability of apratoxin S4, S7, S8, S9 and S10 in aqueous solution was determined in KCl/HCl buffer, pH 2.32. Portions of each solution (100 µL) were spiked with 10 µL of apratoxin solution (25 µg/ml) and allowed to incubate for 0 min, 1 h, 3 h, 8 h, 14 h and 24 h (6 time points). At the end of each incubation period, 400 µL of ethyl acetate was added to each tube, followed by 200 µL of harmine to quench the reaction and extract remaining apratoxin analogues. Samples were further incubated in a thermomixer at 27 °C (750 rpm, 5 min) and later centrifuged for 5 min at 1643 g. The ethyl acetate layer was collected and evaporated to dryness under nitrogen. Samples were reconstituted in 50 µL of acetonitrile. A volume of 10 µL of the reconstituted solution was injected into the HPLC-MS system. HPLC-MS was done on a 3200 QTRAP (Applied Biosystems) equipped with a Shimadzu (Kyoto, Japan) UFLC System.

Analysis of apratoxin analogues was done by using HPLC-MS [column, Onyx Monolithic C18 (3.0 × 100 mm), Phenomenex (Torrance, CA); solvent, water (solvent A) acetonitrile (solvent B); flow rate, 0.5 ml/min; detection by electrospray ionization–MS in positive ion mode (MRM scan)]. A stepwise gradient elution was used starting at 60% B and 40% A, then increasing to 80% B at 5 min and maintained at this condition for 5 min. Parameters were optimized before analysis by using direct syringe infusion. The retention times (t_R, min; MRM ion pair) of the analytes and internal standard are as follows: harmine (1.8; 213.1→ 169.9), apratoxin S4 (4.5; 828.5 → 432.2),

apratoxin S7 (4.5; 814.5→ 418.2), apratoxin S8 (5.7; 842.5 → 446.2), apratoxin S9 (5.5; 828.5→ 432.2), apratoxin S10 (6.3; 842.5→ 446.1). Compound-dependent parameters used were as follows: apratoxins, declustering potential (DP) 51, entrance potential (EP) 12, collision energy (CE) 45, collision cell exit potential (CXP) 6, collision cell entrance potential (CEP) 32; and harmine, DP 46.0, EP 12, CE 12, CXP 4, CEP 12. Source gas parameters used were as follows: curtain gas, 15.0; collision gas low, ionspray voltage 5500; temperature, 600.0; ion source gas 1 50.0; ion source gas 2 60.0.

Calibration curves for apratoxin analogues in the presence of aqueous solution were generated by least-square linear regression analysis of the analyte peak area and internal standard peak area ratio against the nominal concentration of the standard solutions. A linear regression analysis was performed, and the concentration of remaining apratoxins at each time point was determined through interpolation for aqueous stability experiments. All calculations were done by using Analyst 1.4.2 (Applied Biosystems) Quantitate Mode.

3. Supplementary data

Table S1. Summary of Yields of Final Products of Apratoxins

	apratoxin S8	apratoxin S9	apratoxin S10 (**1**)
yield[a] of cyclization (%)	70	45	80
total yield[b] (%)	3.0	2.4	4.5

[a]Yield over 3 steps. [b]Based on the reaction sequence: pivalaldehyde →2 → 4 → 5→ 6→ 8→1

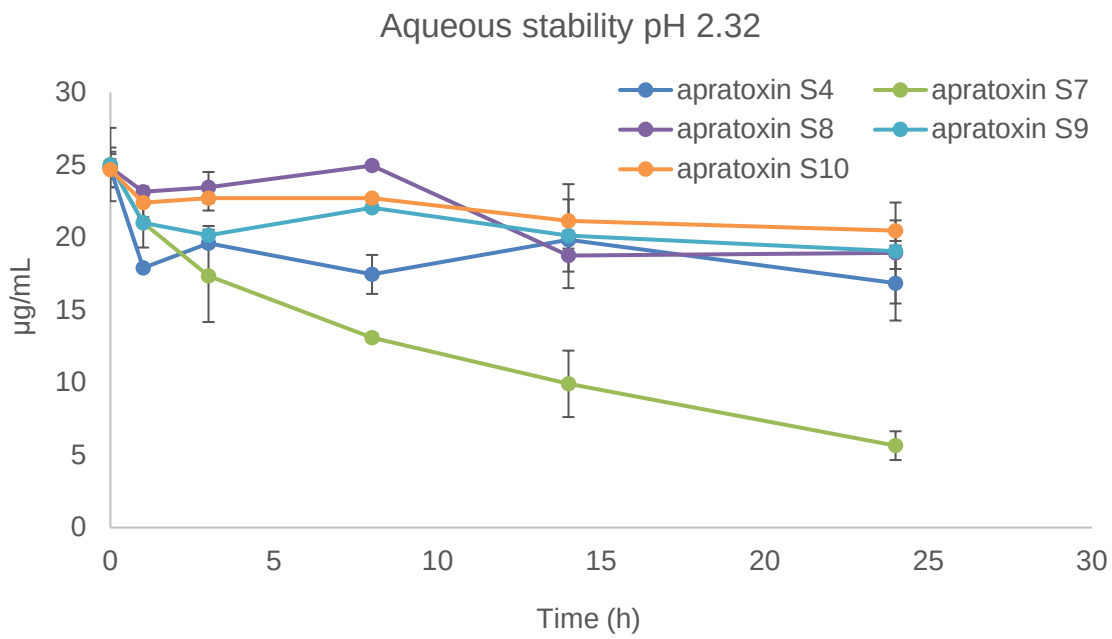
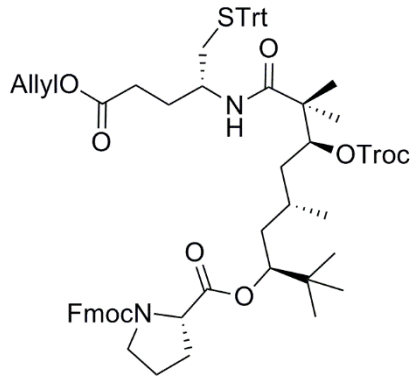


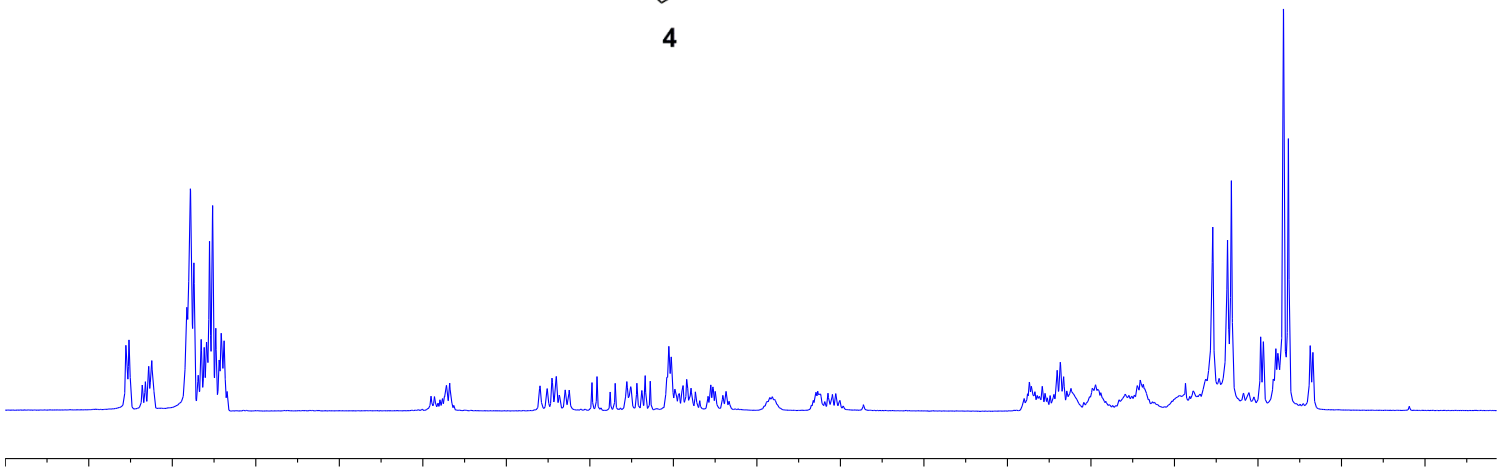
Figure S1. Aqueous stability of apratoxins, pH 2.32.

4. NMR Spectra

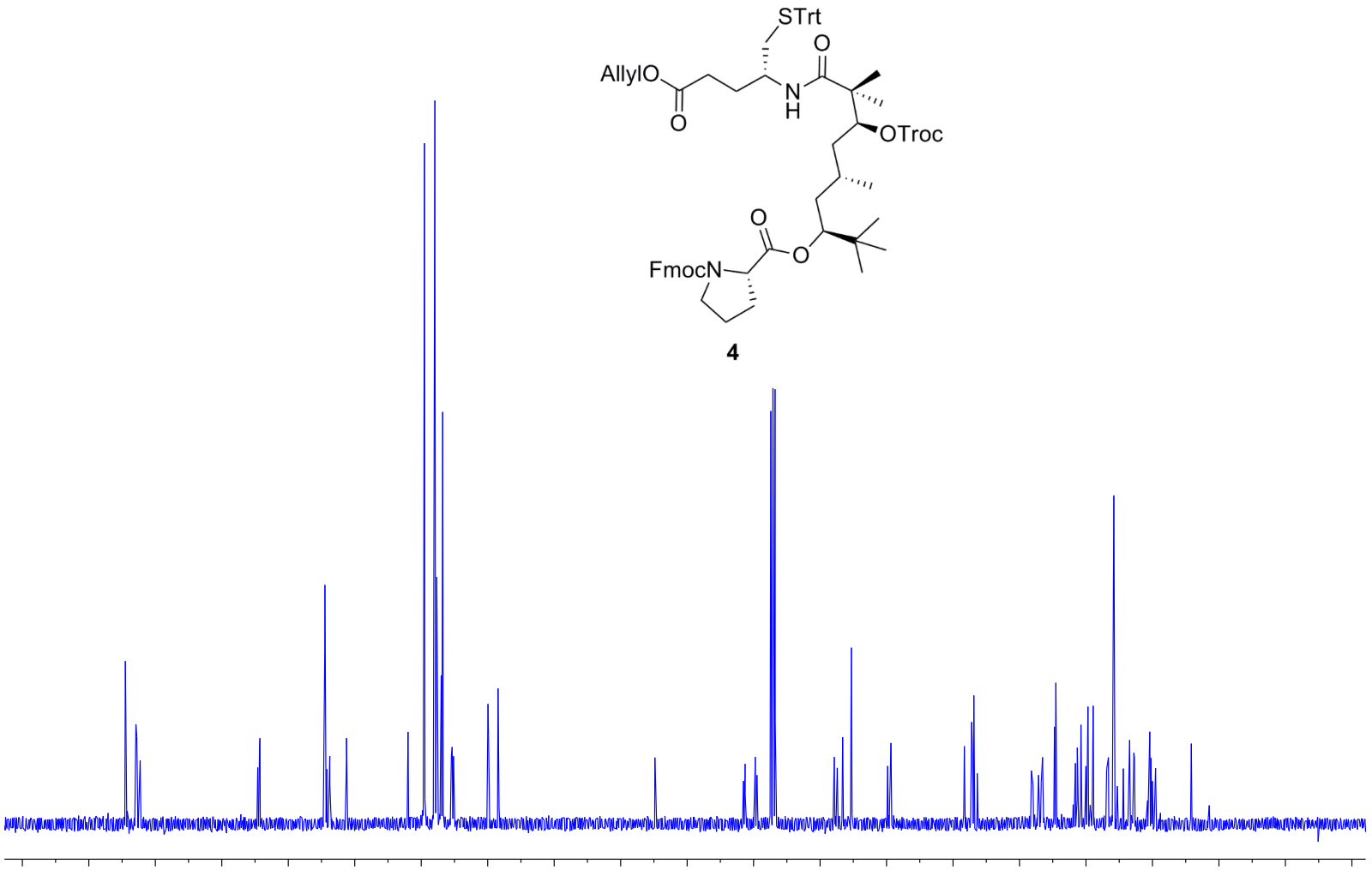
^{1}H NMR Spectrum of **4** in $CDCl_3$ (400 MHz) at 25 °C



4

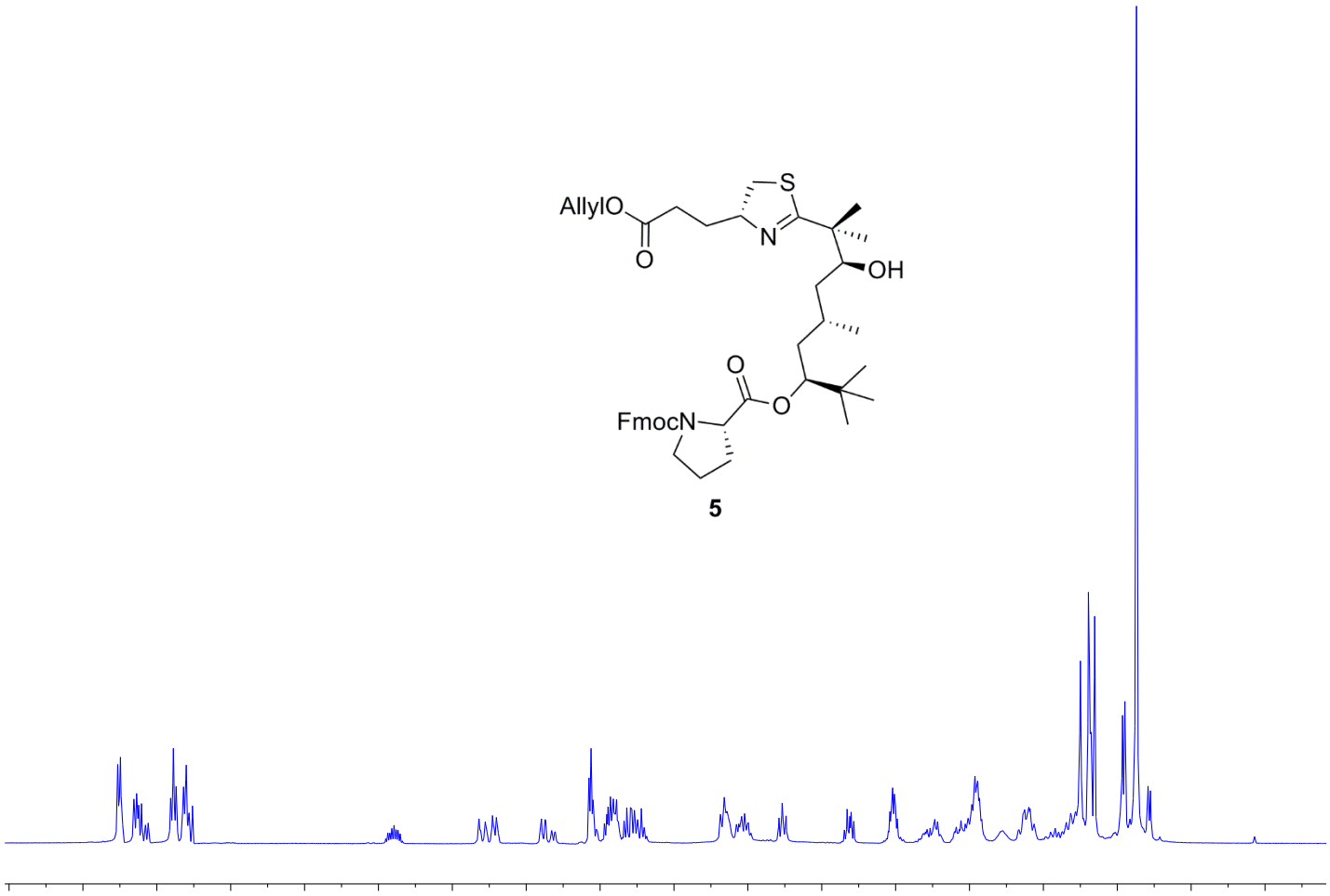


^{13}C NMR Spectrum of **4** in $CDCl_3$ (100 MHz) at 25 °C



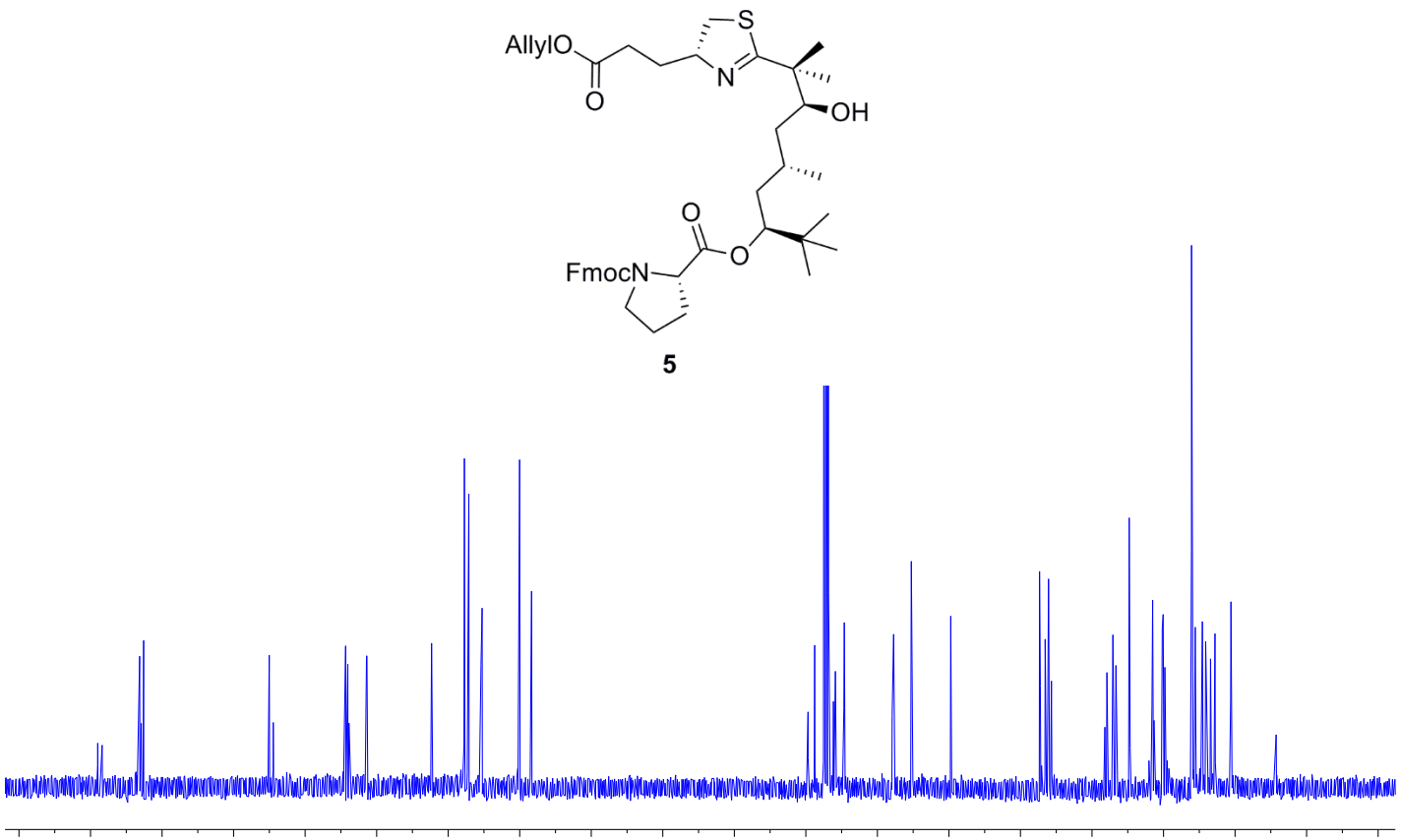
4

^{1}H NMR Spectrum of **5** in $CDCl_3$ (400 MHz) at 25 °C



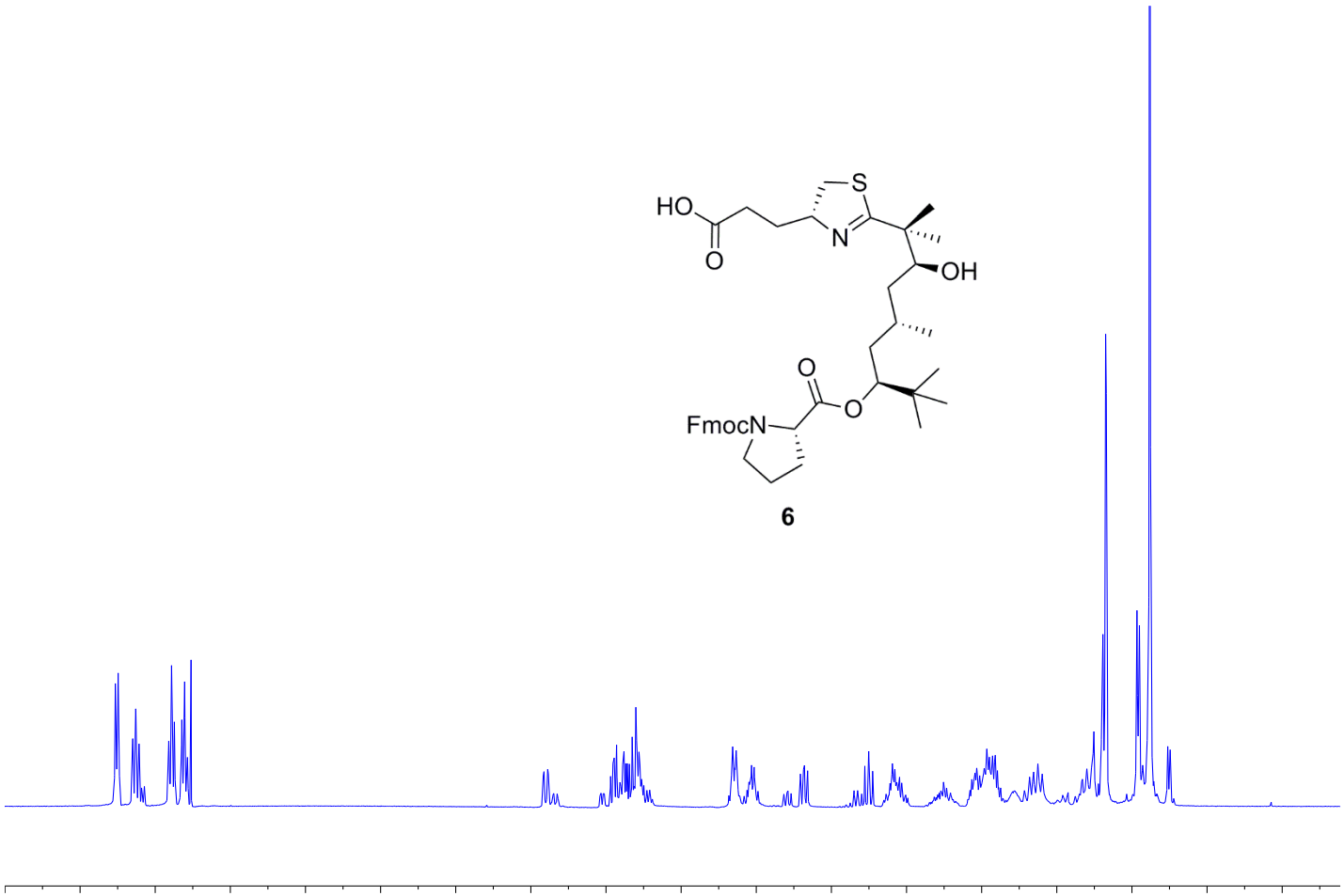
5

^{13}C NMR Spectrum of **5** in $CDCl_3$ (100 MHz) at 25 °C



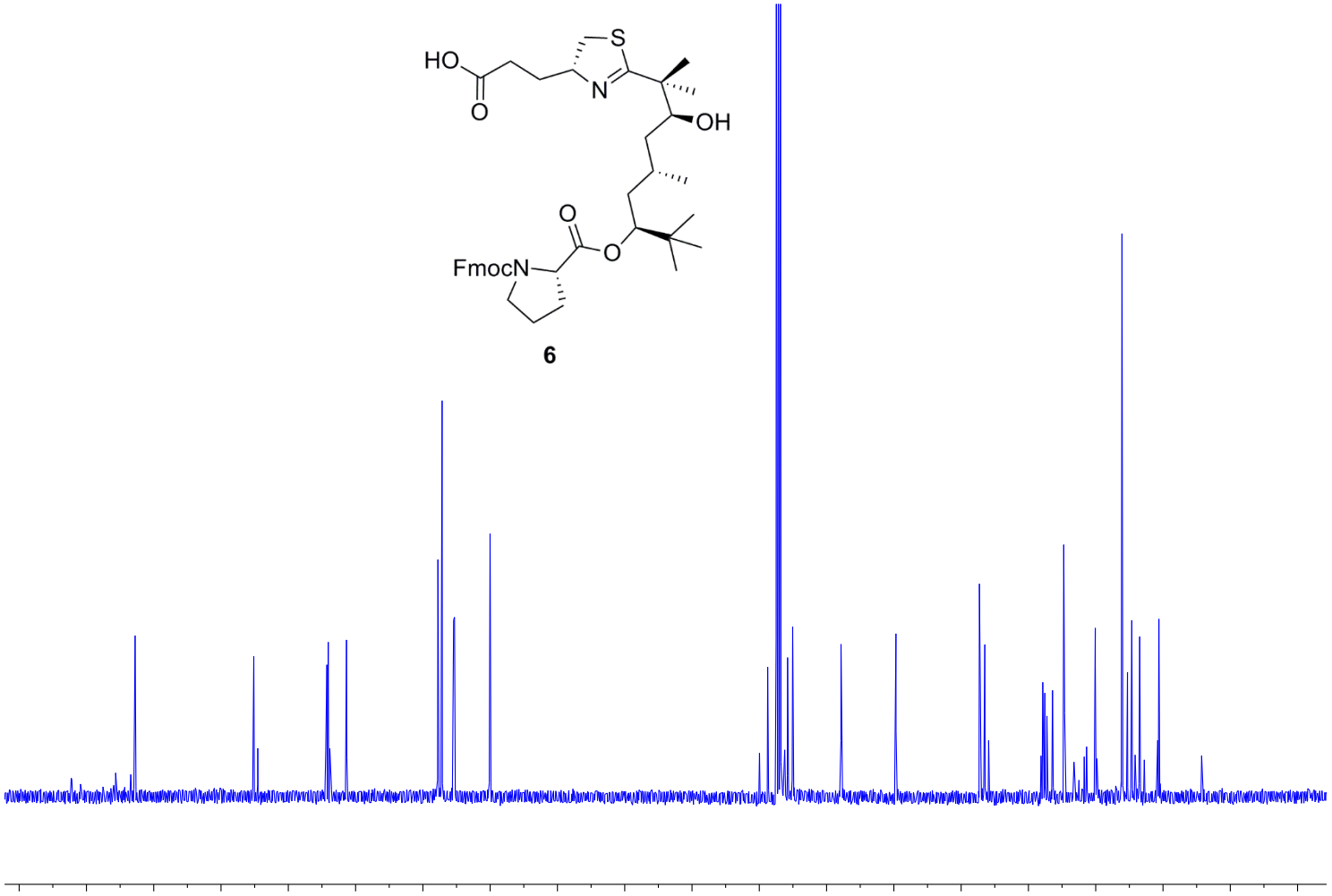
5

^{1}H NMR Spectrum of **6** in $CDCl_3$ (400 MHz) at 25 °C

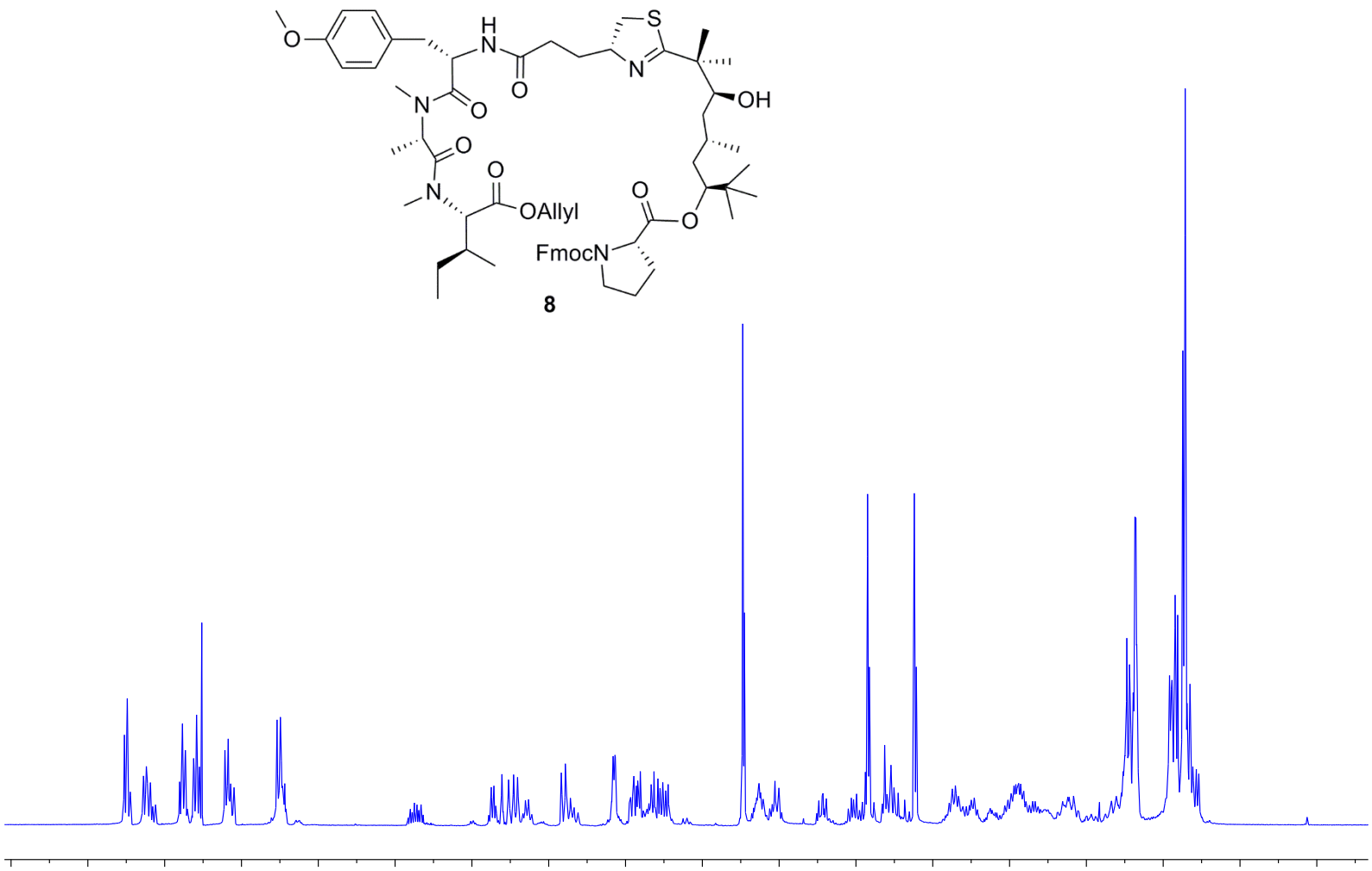


6

^{13}C NMR Spectrum of **6** in $CDCl_3$ (100 MHz) at 25 °C

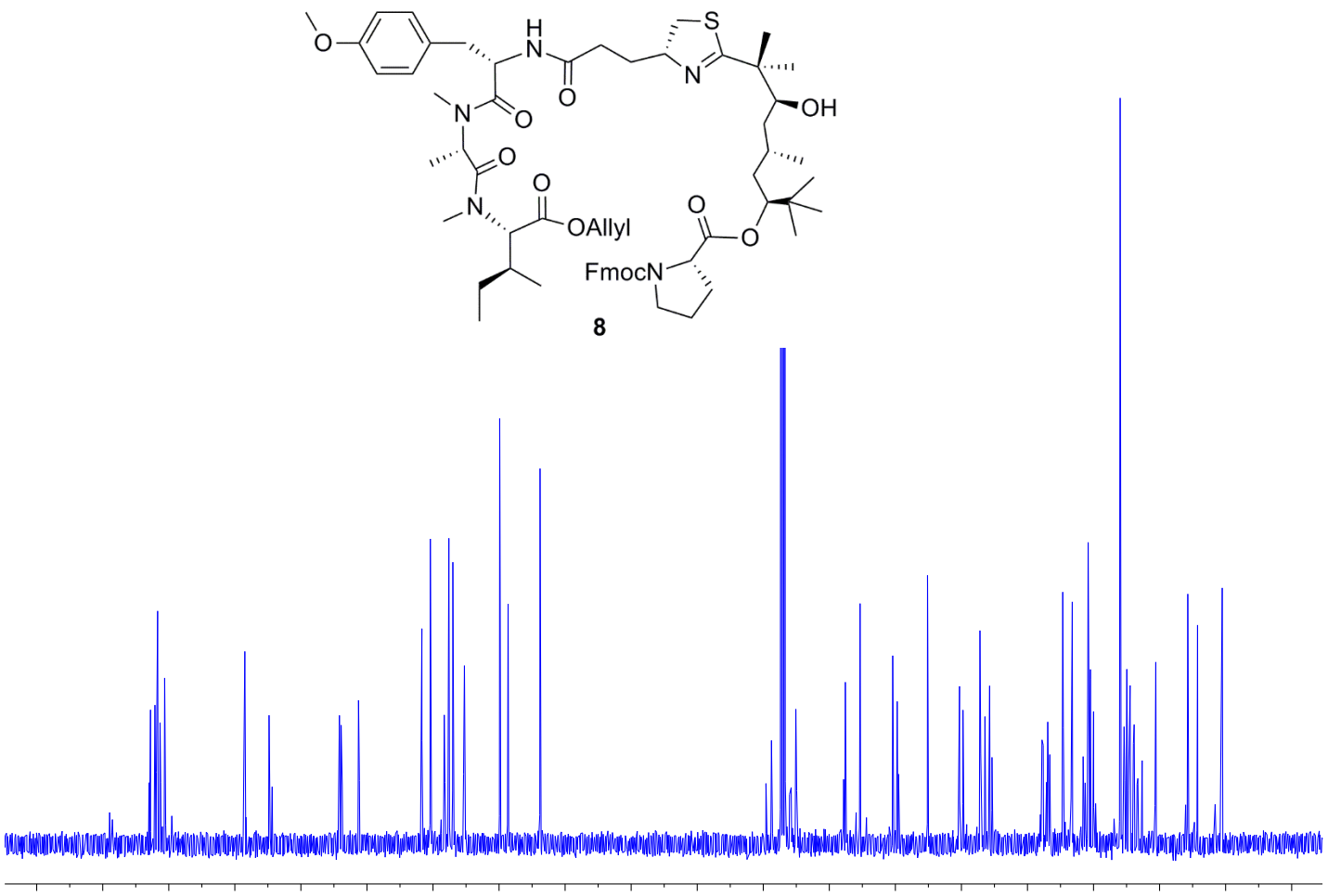


^{1}H NMR Spectrum of **8** in $CDCl_3$ (400 MHz) at 25 °C



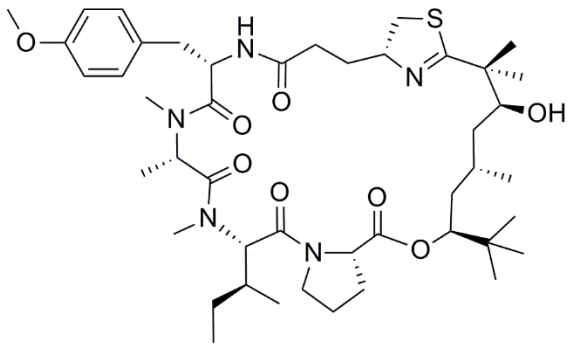
8

^{13}C NMR Spectrum of **8** in $CDCl_3$ (100 MHz) at 25 °C



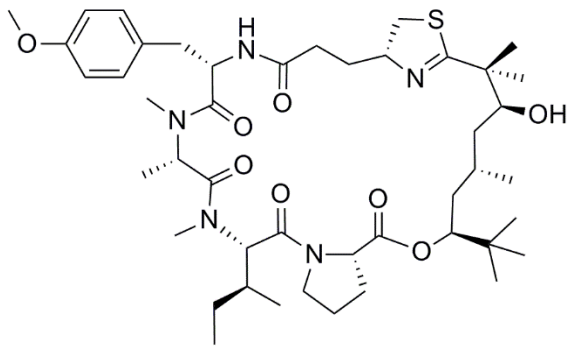
8

^{1}H NMR Spectrum of **1** in $CDCl_3$ (400 MHz) at 25 °C



1 (Apratoxin **S10**)

^{13}C NMR Spectrum of **1** in $CDCl_3$ (100 MHz) at 25 °C



1 (Apratoxin **S10**)