Design, Synthesis and Cytotoxic Evaluation of Novel Tubulysin Analogs as ADC Payloads

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Experimental Details (Supplemental)

1. General Methods

Synthetic Experimental Procedures:

Experiments were carried out under inert atmosphere (nitrogen or argon), particularly in cases where oxygen- or moisture-sensitive reagents or intermediates were employed. Commercial solvents and reagents were used without further purification, including anhydrous solvents where appropriate (Sure-Seal™ products from the Aldrich Chemical Company, Milwaukee, Wisconsin). Mass spectrometry data is reported from either liquid chromatography-mass spectrometry (LC-MS) or atmospheric pressure chemical ionization (APCI). Unless otherwise noted, purity of all final compounds was 100% by UV (215 nm) and ELSD.

NMR data acquisition and processing:

Chemical shifts for nuclear magnetic resonance (NMR) data are expressed in parts per million (ppm, δ) referenced to residual peaks from the deuterated solvents employed. In some cases, ^{13}C NMR shifts were determined by HMBC correlations and were noted as so in the tabulated form. Samples were prepared in DMSO-*d*6 with a concentration of 5 - 30 mM in 3 mm NMR tubes. NMR data were recorded at 300 K on Bruker AVANCE III 500 MHz spectrometer equipped with DCH cryoprobe. 1D ^{1}H spectra were acquired using standard pulse sequence with 32K data points, 8000 Hz sweep width, 3 s repetition time and 16 scans. 1D ^{13}C spectra were collected with 64 K data points and 2K – 8K scans. Standard 2D NMR methods were used to confirm the structures. DQF-COSY spectra were recorded with spectral width of 10 ppm, 2048 points on F2 and 256 increments on F1. Heteronuclear HSQC and HMBC spectra were obtained with spectral width of 10 ppm on F2, and 150 ppm and 220 ppm on F1 dimension, respectively. The data matrix was 2048 ×128 and zero-filled to 2048 ×512. All data were processed using MestreNova 9.0.

Purification Methods:

Method A: Column: Phenomenex Luna C18 (2), 150 x 21.2 mm I.D., 5 µm; Mobile phase A: 0.1% formic acid in water (v/v); Mobile phase B: 0.1% formic acid in MeCN (v/v); Gradient: variable, increasing gradient of B in A over 10 to 20 minutes. Flow rate: 27 mL/minute. Temperature: not controlled; Detection: DAD 215 nm, 254 nm; MS (+) range 150-2000 daltons; Instrument: Waters FractionLynx.

Method B: Column: Phenomenex Luna C18 (2), 150 x 21.2 mm I.D., 5 µm; Mobile phase A: 0.02% TFA in water (v/v); Mobile phase B: 0.02 % TFA in MeCN (v/v); Gradient: variable, increasing

gradient of B in A over 10 to 20 minutes. Flow rate: 27 mL/minute. Temperature: 45 °C; Detection: DAD 215 nm, 254 nm; MS (+) range 150-2000 daltons; Instrument: Waters FractionLynx.

Method C: Column: Waters Sunfire, C18, 19 x100 mm I.D., 5 µm; Mobile phase A: 0.05% TFA in water (v/v); Mobile phase B: 0.05% TFA in MeCN (v/v); Flow rate 25 mL/minute. Detection: DAD 215 nm; MS (+) range 160-1000 daltons; Instrument: Waters FractionLynx.

Analytical Protocols:

Protocol A: Column: Waters Acquity UPLC HSS T3, C18, 2.1 x 50 mm I.D., 1.7 µm; Mobile phase A: 0.1% formic acid in water (v/v); Mobile phase B: 0.1% formic acid in MeCN (v/v); Gradient: 5% B over 0.1 minute, 5% to 95% B over 2.5 minutes, 95% B over 0.35 minute; Flow rate: 1.25 mL/minute. Temperature: 60 °C; Detection: 200-450 nm; MS (+) range 100-2000 daltons; Injection volume: 5 µL; Instrument: Waters Acquity.

Protocol B: Column: Waters Acquity UPLC HSS T3, C18, 2.1 x 50 mm I.D., 1.7µm; Mobile phase A: 0.1% formic acid in water (v/v); Mobile phase B: 0.1% formic acid in MeCN (v/v); Gradient: 5% B over 0.1 minute, 5% to 95% B over 1.5 minute, 95% B over 0.35 minute; Flow rate: 1.25 mL/minute. Temperature: 60 °C; Detection: 200-450 nm; MS (+) range 100-2000 daltons; Injection volume: 5 µL; Instrument: Waters Acquity.

Protocol C: Column: Phenomenex Luna C18 (2), 150 x 3.0 mm I.D., 5 µm; Mobile phase A: 0.1% formic acid in water (v/v); Mobile phase B: 0.1% formic acid in MeCN (v/v); Gradient: 5% B over 1.5 minutes, 5% to 100% B over 8.5 minutes, then 100% B for 1 minute; Flow rate: 0.75 mL/minute. Temperature: 45 °C; Detection: DAD 215 nm, 254 nm; MS (+) range 150-2000 daltons; Injection volume: 10 µL; Instrument: Agilent 1200 LCMS.

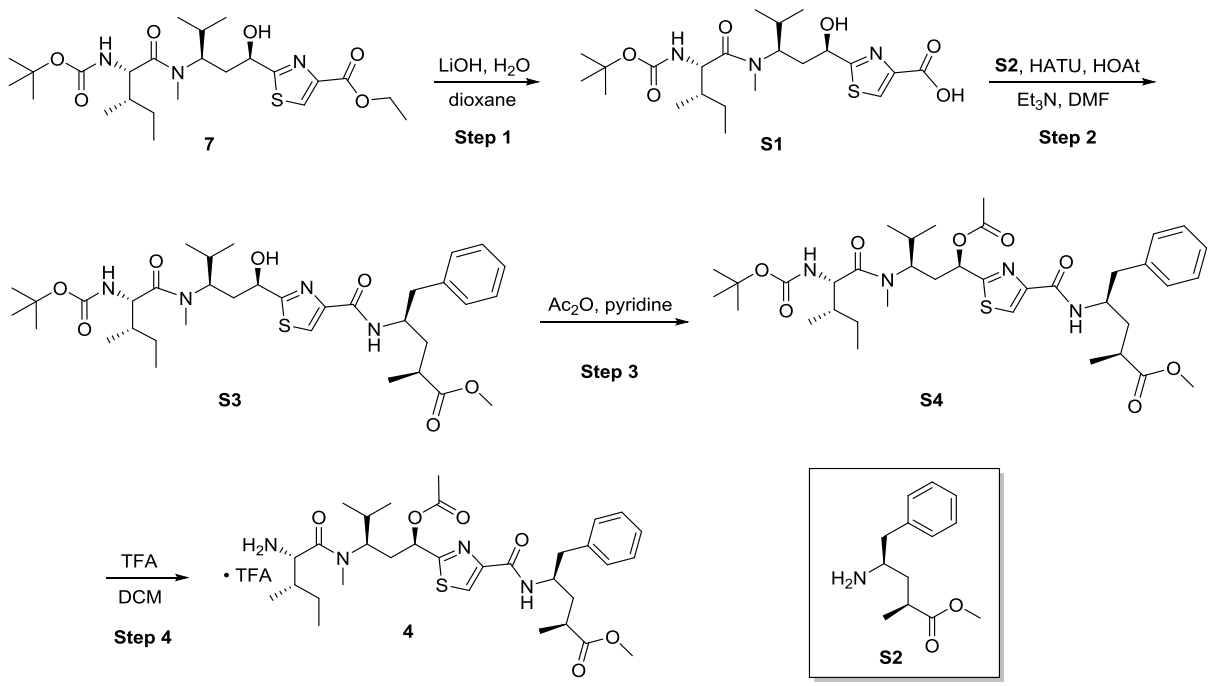
Protocol D: Column: Atlantis dC18, 50 x 4.6 mm I.D., 5 µm; Mobile phase A: 0.05% TFA in water (v/v); Mobile phase B: 0.05% TFA in MeCN (v/v); Gradient: 5% to 95% B over 4.0 minutes, linear; then hold at 95% B over 1 minute. Flow rate: 2 mL/minute. Temperature: room temperature; Detection: DAD 215 nm; MS (+) range 160 -1000 daltons; injection volume 3 uL; Instrument: Waters 996 PDA.

Protocol E: Column: Ultimate XB-C18, 2.1 x 30 mm I.D., 3 µm; Mobile phase A: 0.04% TFA in water (v/v); Mobile phase B: 0.02% TFA in MeCN (v/v); Gradient: 10% to 80% B over 0.9 minutes then hold at 80% B over 0.6 minutes, then 10% B for 0.5 minutes. Flow rate: 1.2 mL/minute. Temperature: 50 °C. Detection: 220 nm; MS (+) range 0 -1000 daltons; injection volume 2 uL; Instrument: Shimadzu LC-MS 2010.

Protocol F: Column: Merck RP-18e, 25-2; Mobile phase A: 0.04% TFA in water (v/v); Mobile phase B: 0.02% TFA in MeCN (v/v); Gradient: 5% to 95% B over 0.7 minutes then hold at 95% B over 0.4 minutes, then 5% B for 0.4 minutes. Flow rate: 1.5 mL/minute. Temperature: 50 °C. Detection: 220 nm; MS (+) range 0 -1000 daltons; injection volume 2 uL; Instrument: Shimadzu LC-MS 2010.

2. Preparation of target compounds

Preparation of methyl (2S,4R)-4-[({2-[(1R,3R)-1-(acetyloxy)-3-{[N-(tert-butoxycarbonyl)-L-isoleucyl](methyl)amino}-4-methylpentyl]-1,3-thiazol-4-yl}carbonyl)amino]-2-methyl-5-phenylpentanoate (4):



Step 1. 2-[(1*R*,3*R*)-3-{[*N*-(*tert*-butoxycarbonyl)-*L*-isoleucyl](methyl)amino}-1-hydroxy-4-methylpentyl]-1,3-thiazole-4-carboxylic acid (S1):

A solution of LiOH monohydrate (839 mg, 20 mmol) in H_2O (50 mL) was added dropwise to a solution of ethyl 2-[(1*R*,3*R*)-3-{[*N*-(*tert*-butoxycarbonyl)-*L*-isoleucyl](methyl)amino}-1-hydroxy-4-methylpentyl]-1,3-thiazole-4-carboxylate (WO 2009 134279) (**7**, 2 g, 4 mmol) in dioxane (50 mL) at 0 °C and the solution was stirred at room temperature for 2 h. The reaction mixture was acidified to pH = 1 and extracted with EtOAc. The organic phase was dried over Na_2SO_4 and concentrated *in vacuo* to afford the title compound **S1** (2.1 g, quantitative yield) as a white solid.

Step 2. methyl (2*S*,4*R*)-4-[({2-[(1*R*,3*R*)-3-{[*N*-(*tert*-butoxycarbonyl)-*L*-isoleucyl](methyl)amino}-1-hydroxy-4-methylpentyl]-1,3-thiazol-4-yl}carbonyl)amino]-2-methyl-5-phenylpentanoate (S3):

HOAt (258 mg, 1.9 mmol) and HATU (722 mg, 1.9 mmol) were added to a solution of 2-[(1*R*,3*R*)-3-{[*N*-(*tert*-butoxycarbonyl)-*L*-isoleucyl](methyl)amino}-1-hydroxy-4-methylpentyl]-1,3-thiazole-4-carboxylic acid (**S1**, 895 mg, 1.8 mmol) and triethylamine (384 mg, 3.8 mmol) in DMF (30 mL) at 0 °C. After stirring for 10 min at 0 °C, methyl (2*S*,4*R*)-4-amino-2-methyl-5-phenylpentanoate hydrochloride (*Org. Biomol. Chem*. **2013**, *11*, 2273-2287) (**S2**, 490 mg, 1.9 mmol) was added and the resulting solution was stirred at rt for 3 h. The reaction mixture was diluted with H_2O (50 mL) and extracted with EtOAc. The organic phase was washed with brine, dried over Na_2SO_4, and concentrated under vacuum. The residue was purified by flash chromatography (7% MeOH in DCM) to afford compound **S3** (1.1 g, 85%) as a yellow oil.

Step 3. methyl (2*S*,4*R*)-4-[({2-[(1*R*,3*R*)-1-(acetyloxy)-3-{[*N*-(*tert*-butoxycarbonyl)-*L*-isoleucyl](methyl)amino}-4-methylpentyl]-1,3-thiazol-4-yl}carbonyl)amino]-2-methyl-5-phenylpentanoate (S4):

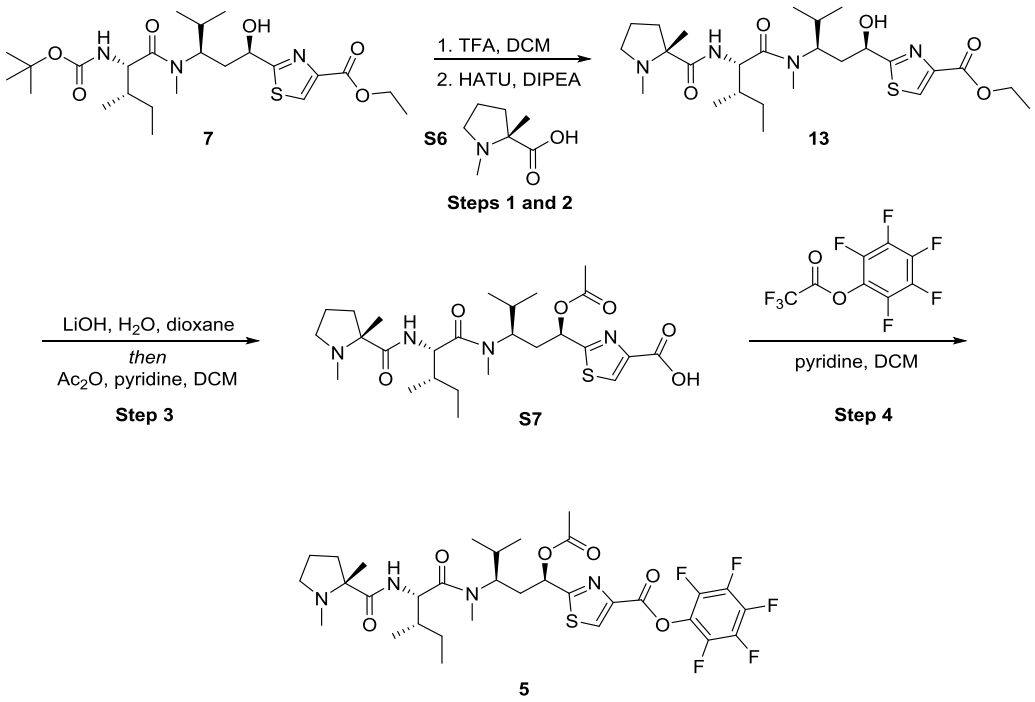
Methyl (2*S*,4*R*)-4-[({2-[(1*R*,3*R*)-3-{[*N*-(*tert*-butoxycarbonyl)-*L*-isoleucyl](methyl)amino}-1-hydroxy-4-methylpentyl]-1,3-thiazol-4-yl}carbonyl)amino]-2-methyl-5-phenylpentanoate (**S3,** 1.4 g, 2.1 mmol), Ac_2O (2 mL) and pyridine (10 mL) was stirred overnight at rt under N_2. The reaction mixture was concentrated under vacuum and the residue was purified by flash chromatography (30% hexanes in EtOAc) to afford the title compound **S4** (1.2 g, 80%) as a white solid. 98.5% *ee*; Column: Chiralcel AS-H

150 x 4.6mm I.D., 5µm, Mobile phase: methanol (0.05% diethylamine) in CO_2 from 5% to 40%; Flow rate: 2.35 mL/min; Wavelength: 220 nm; Retention time = 4.65 min. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.17 (s, 1H), 7.94 (d, *J* = 9.2 Hz, 1H), 7.30 – 7.12 (m, 5H), 6.88 (d, *J* = 9.1 Hz, 1H), 5.55 (dd, *J* = 10.9, 2.8 Hz, 1H), 4.40-4.37 (m, 1H), 4.23-4.18 (m, 2H), 3.51 (s, 3H), 2.97 (s, 3H), 2.91-2.85 (m, 1H), 2.80-2.75 (m, 1H), 2.34 – 2.11 (m, 2H), 2.08 (s, 3H), 1.87-1.66 (m, 4H), 1.54-1.48 (m, 1H), 1.39-1.36 (m, 1H), 1.35 (s, 9H), 1.10-1.01 (m, 1H), 1.06 (d, *J* = 7.1 Hz, 3H), 0.94 (d, *J* = 6.5 Hz, 3H), 0.83-0.72 (m, 9H). ^{13}C NMR (101 MHz, DMSO) δ 175.80, 173.44, 169.72, 169.67, 159.79, 155.56, 149.63, 138.56, 129.09, 128.14, 126.10, 124.19, 77.87, 69.61, 54.67, 51.31, 48.18, 40.55, 37.27, 35.76, 35.11, 34.06, 29.11, 28.13, 24.12, 20.62, 20.15, 19.30, 17.53, 15.29, 10.74. HRMS Calcd. for $[M+H]^+$: 717.3892, Found: 717.3902.

Step 4. methyl (2*S*,4*R*)-4-{[(2-{(1*R*,3*R*)-1-(acetyloxy)-3-[*L*-isoleucyl(methyl)amino]-4-methylpentyl}-1,3-thiazol-4-yl)carbonyl]amino}-2-methyl-5-phenylpentanoate (4):

TFA (1.1 mL) was added to a vial containing methyl (2*S*,4*R*)-4-[({2-[(1*R*,3*R*)-1-(acetyloxy)-3-{[*N*-(*tert*-butoxycarbonyl)-*L*-isoleucyl](methyl)amino}-4-methylpentyl]-1,3-thiazol-4-yl}carbonyl)amino]-2-methyl-5-phenylpentanoate (**S4**, 200 mg, 0.279 mmol) in DCM (20 mL) and the reaction was stirred under an N_2 inlet at rt for 2.25 h. The reaction mixture was concentrated *in vacuo,* azeotroped with DCM/MeOH (1/1, 3 mL), and concentrated under high vacuum overnight to yield title compound **4** (243 mg, quantitative yield) as a solid. The product was carried crude to following steps. LC-MS (Protocol C): *m/z* 617.3 $[M+H]^+$; Retention time = 0.72 min.

<u>Preparation of 1,2-dimethyl-D-prolyl-N-[(1R,3R)-1-(acetyloxy)-4-methyl-1-{4-[(pentafluorophenoxy)carbonyl]-1,3-thiazol-2-yl}pentan-3-yl]-N-methyl-L-isoleucinamide (8):</u>



Step 1. ethyl 2-{(1*R*,3*R*)-1-hydroxy-3-[*L*-isoleucyl(methyl)amino]-4-methylpentyl}-1,3-thiazole-4-carboxylate trifluoroacetic acid salt (S5):

Trifluoroacetic acid (10 mL) was added dropwise to a solution of ethyl 2-[(1*R*,3*R*)-3-{[*N*-(*tert*-butoxycarbonyl)-*L*-isoleucyl](methyl)amino}-1-hydroxy-4-methylpentyl]-1,3-thiazole-4-carboxylate[13] (**7**, 5 g, 10 mmol) in DCM (100 mL) at 0 °C and the solution was stirred at rt for 2 h. The reaction mixture was concentrated under vacuum and the residue was dissolved in EtOAc (100 mL) and basified with saturated aqueous $NaHCO_3$ (50 mL). The organic phase was washed with brine (20 mL), dried over Na_2SO_4, and concentrated under vacuum to afford the title compound **S5** (3.9 g, 100%) as a gum, which was used directly in the next step.

Step 2. 1,2-dimethyl-*D*-prolyl-*N*-{(1*R*,3*R*)-1-[4-(ethoxycarbonyl)-1,3-thiazol-2-yl]-1-hydroxy-4-methylpentan-3-yl}-*N*-methyl-*L*-isoleucinamide (13):

A solution of ethyl 2-{(1*R*,3*R*)-1-hydroxy-3-[*L*-isoleucyl(methyl)amino]-4-methylpentyl}-1,3-thiazole-4-carboxylate trifluoroacetic acid salt (**S5**, 1.2 g, 3 mmol), 1,2-dimethyl- *D*-Proline (*J. Med. Chem*. **2014**, *57*, 10527) (**S6**, 429 mg, 3 mmol), HATU (1.3 g, 3.3 mmol) and DIPEA (1.2 g, 9 mmol) in DMF (20 mL) was stirred at rt overnight. The reaction mixture was diluted with H_2O (50 mL) and extracted with EtOAc. The organic phase was washed with brine, dried over Na_2SO_4, and concentrated under vacuum. The residue was purified by flash silica gel chromatography (15% MeOH in DCM) to afford the title compound **13** (0.9 g, 60%) as a yellow solid. LC-MS (Protocol E): 525.1 $[M+H]^+$; Retention time = 0.98 min; ^{1}H NMR (400MHz, MeOH-d_4) δ 8.31 (s, 1H), 4.77 - 4.72 (m, 1H), 4.70 - 4.64 (m, 1H), 4.57 - 4.43 (m, 1H), 4.41 - 4.33 (m, 2H), 3.15 (s, 3H), 3.13 - 3.07 (m, 1H), 2.81 (s, 1H), 2.61 - 2.53 (m, 1H), 2.36 - 2.31 (m, 3H), 2.25 - 2.14 (m, 1H), 2.05 - 1.88 (m, 3H), 1.87 - 1.76 (m, 1H), 1.71 (m, 3H), 1.43 - 1.34 (m, 3H), 1.20 (s, 3H), 1.09 - 1.04 (m, 1H), 1.03 - 0.95 (m, 7H), 0.94 - 0.88 (m, 3H), 0.80 (s, 3H).

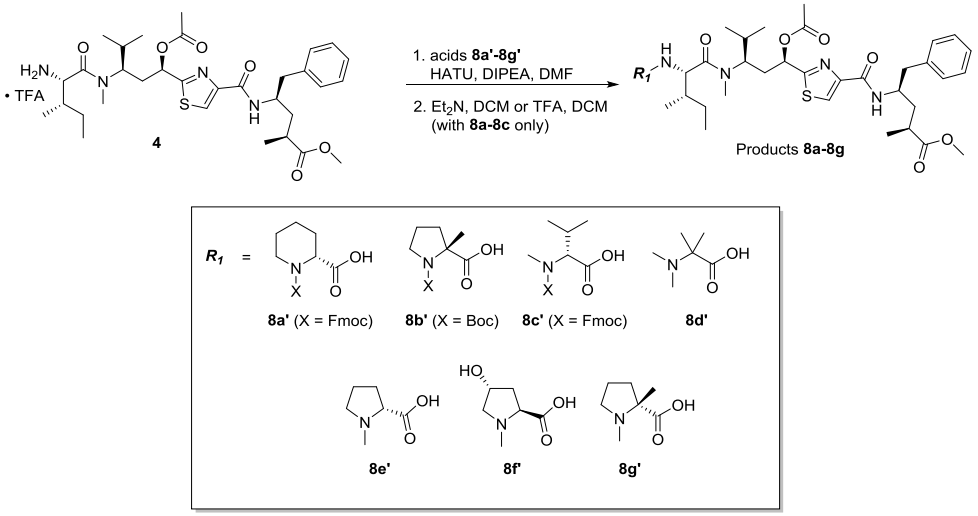
Step 3. 2-((1*R*,3*R*)-3-((2*S*,3*S*)-2-((*R*)-1,2-dimethylpyrrolidine-2-carboxamido)-*N*,3-dimethylpentanamido)-1-hydroxy-4-methylpentyl)thiazole-4-carboxylic acid (S7):

A solution of 1 *M* LiOH in water (1.1 mL) was added to a solution of 1,2-dimethyl-*D*-prolyl-*N*-{(1*R*,3*R*)-1-[4-(ethoxycarbonyl)-1,3-thiazol-2-yl]-1-hydroxy-4-methylpentan-3-yl}-*N*-methyl-*L*-isoleucinamide (**13**, 200 mg, 0.381 mmol) in 1,4-dioxane (2 mL) and the reaction was stirred at rt for 6 h. The reaction was quenched with HOAc (200 µL) and concentrated. The resulting crude acid was re-dissolved in DCM (2.4 mL) and treated with pyridine (2.4 mL) and Ac_2O (0.5 mL). The reaction was stirred under N_2 for 4.5 h then concentrated to a dark oily residue which was re-dissolved in DMSO (2 mL) and purified by medium pressure reverse phase C18 chromatography (10% to 95% MeCN in H_2O over 25 minutes, each solvent containing 0.02% TFA) to afford the title compound **S7** (135 mg, 66% yield). LC-MS (Protocol B): *m/z* 539.5 $[M+H]^+$; Retention time = 0.63 min.

Step 4. 1,2-dimethyl-*D*-prolyl-*N*-[(1*R*,3*R*)-1-(acetyloxy)-4-methyl-1-{4-[(pentafluorophenoxy)carbonyl]-1,3-thiazol-2-yl}pentan-3-yl]-*N*-methyl-*L*-isoleucinamide (5):

DCM (10 mL) and pyridine (130 µL, 0.950 mmol) were added to a vial containing 2-((1*R*,3*R*)-3-((2*S*,3*S*)-2-((*R*)-1,2-dimethylpyrrolidine-2-carboxamido)-*N*,3-dimethylpentanamido)-1-hydroxy-4-methylpentyl)thiazole-4-carboxylic acid (**S7**, 256 mg, 0.475 mmol). The reaction was cooled to 0 °C under N_2 and pentafluorophenyl trifluoroacetate (167 µL, 0.950 mmol) was added. After ~5 minutes, the ice bath was removed and the reaction was stirred under N_2 for 3.5 h, concentrated, re-dissolved in DCM (3 mL), and purified by flash silica gel chromatography (0% to 60% MeOH in DCM) to provide the title compound **5** (283 mg, 85% yield) as a white solid. LC-MS (Protocol A): *m/z* 705.5 $[M+H]^+$; Retention time = 1.64 min. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.77 (s, 1H), 9.07 (s, 1H), 8.73 (d, *J* = 8.7 Hz, 1H), 5.70 – 5.55 (m, 1H), 4.67 – 4.58 (m, 1H), 3.61 – 3.49 (m, 1H), 3.20-3.11 (m, 1H), 3.01 (s, 3H), 2.68 (d, *J* = 4.9 Hz, 3H), 2.31-2.25 (m, 3H), 2.13 (s, 3H), 2.11 – 1.75 (m, 5H), 1.46 (s, 3H), 1.44-1.42 (m, 1H), 1.15 – 1.05 (m, 1H), 0.94 (d, *J* = 6.5 Hz, 3H), 0.90 – 0.79 (m, 6H), 0.71 (d, *J* = 6.5 Hz, 3H). HRMS Calcd. for $[M+H]^+$: 705.2740, Found: 705.2747.

Synthesis of N-terminus Library Analogs



General Method A: Synthesis of analogs 8a and 8c, as exemplified by synthesis of 8a

methyl (2*S*,4*R*)-4-[({2-[(1*R*,3*R*)-1-(acetyloxy)-4-methyl-3-(methyl{*N*-[(2*R*)-piperidin-2-ylcarbonyl]-*L*-isoleucyl}amino)pentyl]-1,3-thiazol-4-yl}carbonyl)amino]-2-methyl-5-phenylpentanoate (8a):

DMF (1 mL) and DIPEA (36 µL, 0.205 mmol) were added to a vial containing (*R*)-*N*-Fmoc-piperidine-2-carboxylic Acid (CAS 101555-63-9) (**8a'**, 17.3 mg, 0.049 mmol) and HATU (19 mg, 0.049 mmol) and the reaction was stirred at rt for 0.5 h. A solution of methyl (2*S*,4*R*)-4-{[(2-{(1*R*,3*R*)-1-(acetyloxy)-3-[*L*-isoleucyl(methyl)amino]-4-methylpentyl}-1,3-thiazol-4-yl)carbonyl]amino}-2-methyl-5-phenylpentanoate (**4**, 30 mg, 0.041 mmol) in DMF (1 mL) was added and the solution was stirred at rt for 19 h. Diethylamine (85 µL, 0.821 mmol) was added and the reaction mixture was stirred for 24 h at rt before concentration. The crude residue was re-dissolved in DMSO (0.9 mL) and purified by high pressure reverse phase C18 chromatography (Method C, 80.0% H_20/20.0% MeCN linear to 40% H_20/60% MeCN in 8.5 min to 0% H_2O/100% MeCN to 9.0 min, HOLD at 0% H_20/100% MeCN from 9.0 to 10.0min.) to afford title compound **8a** (14 mg, 47%). LC-MS (Protocol D): *m/z* 728.4804 $[M+H]^+$; retention time = 2.65 min. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.98 (d, *J* = 11.0 Hz, 1H), 8.66 (d, *J* = 9.1 Hz, 1H), 8.63 – 8.56 (m, 1H), 8.19 (s, 1H), 7.93 (d, *J* = 9.1 Hz, 1H), 7.27 – 7.15 (m, 5H), 5.56 (dd, *J* = 10.9, 2.7 Hz, 1H), 4.68 (dd, *J* = 9.0, 7.3 Hz, 1H), 4.40 (m, 1H), 4.19 (m, 1H), 3.75 (m, *J* = 10.8 Hz, 1H), 3.51 (s, 3H), 3.20 (m, 1H), 2.95 (m, 3H), 2.92 – 2.86 (m, 1H), 2.80-2.76 (m, 1H), 2.31-2.25 (m, 1H), 2.19 – 2.16 (m, 2H), 2.09 (s, 3H), 1.90 – 1.38 (m, 10H), 1.06 (d, *J* = 7.1 Hz, 3H), 1.05-1.02 (m, 2H), 0.96 (d, *J* = 6.5 Hz, 3H), 0.89 – 0.78 (m, 6H), 0.73 (d, *J* = 6.6 Hz, 3H). ^{13}C NMR (126 MHz, DMSO) δ 175.80, 171.60, 169.68, 169.53, 168.32, 159.82, 149.61, 138.55, 129.10, 128.15, 126.11, 124.26, 69.52, 56.88, 53.17, 51.32, 48.22, 43.25, 40.55, 37.33, 36.11, 35.79, 33.86, 29.06, 27.68, 23.43, 21.67, 21.19, 20.61, 20.10, 19.76, 17.55, 15.58, 10.81. HRMS Calcd. for $[M+2H]^{+2}$: 371.7140, Found: 371.7157.

***N*-methyl-*D*-valyl-*N*-[(1*R*,3*R*)-1-(acetyloxy)-1-(4-{[(2*R*,4*S*)-5-methoxy-4-methyl-5-oxo-1-phenylpentan-2-yl]carbamoyl}-1,3-thiazol-2-yl)-4-methylpentan-3-yl]-*N*-methyl-*L*-isoleucinamide (8c):**

Prepared according to General Method A in 55% yield from *N*-(9-fluorenylmethoxycarbonyl)-*D*-valine (CAS 84624-17-9) (**8c'**). Purified by high pressure reverse phase C18 chromatography (Method C, 80.0% H_20/20.0% MeCN linear to 40% H_20/60% MeCN in 8.5 min to 0% H_2O/100% MeCN to 9.0 min, HOLD at

0% H_20/100% MeCN from 9.0 to 10.0min.) LC-MS (Protocol D): *m/z* 730.4859 $[M+H]^+$; retention time = 2.70 min. HRMS Calcd. for $[M+Na]^+$: 752.4027, Found: 752.4041.

Preparation of 2-methyl-D-prolyl-N-[(1R,3R)-1-(acetyloxy)-1-(4-{[(2R,4S)-5-methoxy-4-methyl-5-oxo-1-phenylpentan-2-yl]carbamoyl}-1,3-thiazol-2-yl)-4-methylpentan-3-yl]-N-methyl-L-isoleucinamide (8b):

Step 1. 1-(*tert*-butoxycarbonyl)-2-methyl-*D*-prolyl-*N*-[(1*R*,3*R*)-1-(acetyloxy)-1-(4-{[(2*R*,4*S*)-5-methoxy-4-methyl-5-oxo-1-phenylpentan-2-yl]carbamoyl}-1,3-thiazol-2-yl)-4-methylpentan-3-yl]-*N*-methyl-*L*-isoleucinamide (S8):

DMF (0.8 mL) and DIPEA (48 µL, 0.274 mmol) were added to a vial containing *N*-Boc-α-methyl-*D*-proline (CAS 166170-15-6) (**8b'**, 15.1mg, 0.066 mmol) and HATU (25 mg, 0.066 mmol) and the reaction was stirred at rt for 0.25 h. A solution of methyl (2*S*,4*R*)-4-{[(2-{(1*R*,3*R*)-1-(acetyloxy)-3-[*L*-isoleucyl(methyl)amino]-4-methylpentyl}-1,3-thiazol-4-yl)carbonyl]amino}-2-methyl-5-phenylpentanoate (**4**, 40 mg, 0.055 mmol) in DMF (0.8 mL) was added and the solution was stirred at rt for 19 h. The reaction mixture was directly purified by medium pressure reverse phase C18 chromatography (10% MeCN/90% H_2O for 5 minutes, then 10% MeCN to 95% MeCN in H_2O over 18 minutes, each solvent containing 0.02% HOAc) to afford the title compound **S8** (38 mg, 84%). LC-MS (Protocol B): *m/z* 828.6 $[M+H]^+$; retention time = 1.15 min.

Step 2. 2-methyl-*D*-prolyl-*N*-[(1*R*,3*R*)-1-(acetyloxy)-1-(4-{[(2*R*,4S)-5-methoxy-4-methyl-5-oxo-1-phenylpentan-2-yl]carbamoyl}-1,3-thiazol-2-yl)-4-methylpentan-3-yl]-*N*-methyl-*L*-isoleucinamide (8b):

DCM (1 mL) and TFA (0.1 mL) were added to a vial containing 1-(*tert*-butoxycarbonyl)-2-methyl-*D*-prolyl-*N*-[(1*R*,3*R*)-1-(acetyloxy)-1-(4-{[(2*R*,4*S*)-5-methoxy-4-methyl-5-oxo-1-phenylpentan-2-yl]carbamoyl}-1,3-thiazol-2-yl)-4-methylpentan-3-yl]-*N*-methyl-*L*-isoleucinamide (**S8**, 38 mg, 0.046 mmol) and the reaction was stirred for 4 h at rt under N_2. The reaction was concentrated and crude residue re-dissolved in DMSO (0.9 mL) and purified by high pressure reverse phase C18 chromatography (Method C, 80.0% H_20/20.0% MeCN linear to 35% H_20/65% MeCN in 8.5 min to 0% H_2O/100% acetonitril to 9.0 min, HOLD at 0% H_20/100% MeCN from 9.0 to 10.0 min.) to afford title compound **8b** (25.4 mg, 65%). LC-MS (Protocol D): *m/z* 728.4752 $[M+H]^+$; retention time = 2.67 min. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.27 – 9.18 (m, 1H), 8.75 (m, 2H), 8.14 (s, 1H), 7.91 (d, *J* = 9.1 Hz, 1H), 7.25 – 7.09 (m, 5H), 5.56-5.53 (dd, *J* = 11.0, 2.8 Hz, 1H), 4.61-4.57 (m, 1H), 4.18-4.13 (m, 1H), 3.47 (s, 3H), 3.20-3.15 (m, 2H), 2.95 (s, 3H), 2.86-2.72 (m, 2H), 2.31 – 2.08 (m, 3H), 2.06 (s, 3H), 2.03 – 1.61 (m, 8H), 1.43 (s, 3H), 1.41-1.39 (m, 1H), 1.10 – 1.04 (m, 1H), 1.02 (d, *J* = 7.1 Hz, 3H), 0.91 (d, *J* = 6.5 Hz, 3H), 0.81-0.78 (m, 6H), 0.65 (d, *J* = 6.6 Hz, 3H). ^{13}C NMR (126 MHz, DMSO) δ 175.81, 172.18, 170.36, 169.72, 169.55, 159.83, 149.64, 138.58, 129.10, 128.15, 126.11, 124.25, 69.48, 68.99, 53.69, 51.32, 48.23, 43.85, 40.56, 37.34, 35.79, 35.06, 34.98, 33.82, 29.05, 24.10, 22.52, 20.69, 20.63, 20.05, 19.34, 17.54, 15.13, 10.39. HRMS Calcd. for $[M+H]^+$: 728.4051, Found: 728.4076.

General Method B: Synthesis of analogs 8d-8g and 2 as exemplified by synthesis of 8d

***N*,*N*,2-trimethylalanyl-*N*-[(1*R*,3*R*)-1-(acetyloxy)-1-(4-{[(2*R*,4*S*)-5-methoxy-4-methyl-5-oxo-1-phenylpentan-2-yl]carbamoyl}-1,3-thiazol-2-yl)-4-methylpentan-3-yl]-*N*-methyl-*L*-isoleucinamide (8d):**

A solution of HATU (12.6 mg, 0.033) in DMF was added to a vial containing *N*,*N*,2-trimethylalanine (U.S. Patent 8828401 B2) (**8d'**, 5.5 mg, 0.033 mmol). DIPEA (24 µL, 0.137 mmol) was added and the reaction was stirred at rt for 0.5 h. A solution of methyl (2*S*,4*R*)-4-{[(2-{(1*R*,3*R*)-1-(acetyloxy)-3-[*L*-

isoleucyl(methyl)amino]-4-methylpentyl}-1,3-thiazol-4-yl)carbonyl]amino}-2-methyl-5-phenylpentanoate (**4**, 19 mg, 0.026 mmol) in DMF (0.5 mL) was added and the reaction mixture was stirred at rt for 18 h. The reaction was concentrated, re-dissolved in DMSO (0.9 mL), and purified by high pressure reverse phase C18 chromatography (Method C, 80.0% H_20/20.0% MeCN linear to 40% H_20/60% MeCN in 8.5 min to 0% H_2O/100% MeCN to 9.0 min, HOLD at 0% H_20/100% MeCN from 9.0 to 10.0 min.) to afford title compound **8d** (11.8 mg, 59%). LC-MS (Protocol D): *m/z* 730.5002 $[M+H]^+$; retention time = 2.68 min. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.71 (s, 1H), 8.49 (d, *J* = 8.5 Hz, 1H), 8.18 (s, 1H), 7.94 (d, *J* = 9.1 Hz, 1H), 7.30 – 7.11 (m, 5H), 5.59 (dd, *J* = 11.0, 2.8 Hz, 1H), 4.65-4.61 (m, 1H), 4.26 – 4.12 (m, 1H), 3.51 (s, 3H), 3.00 (s, 3H), 2.90-2.86 (m, 1H), 2.80-2.76 (m, 1H), 2.66-2.63 (m, 6H), 2.33 – 2.12 (m, 2H), 2.10 (s, 3H), 1.99-1.94 (m, 1H), 1.87-1.80 (m, 2H), 1.72-1.66 (m, 1H), 1.53 (s, 3H), 1.49-1.46 (m, 2H), 1.44 (s, 3H), 1.15-1.09 (m, 1H), 1.06 (d, *J* = 7.1 Hz, 3H), 0.95 (d, *J* = 6.5 Hz, 3H), 0.90 – 0.80 (m, 6H), 0.69 (d, *J* = 6.6 Hz, 3H). ^{13}C NMR (126 MHz, DMSO) δ 175.79, 172.20, 169.69, 169.49, 159.81, 149.66, 138.56, 129.09, 128.15, 126.10, 124.23, 69.47, 67.57, 53.91, 51.32, 48.22, 40.55, 38.57, 38.51, 37.32, 35.77, 34.85, 33.85, 28.98, 24.14, 20.62, 20.03, 19.39, 19.34, 19.07, 17.52, 15.05, 10.36. HRMS Calcd. for $[M+H]^+$: 730.4208, Found: 730.4227.

1-methyl-*D*-prolyl-*N*-[(1*R*,3*R*)-1-(acetyloxy)-1-(4-{[(2*R*,4*S*)-5-methoxy-4-methyl-5-oxo-1-phenylpentan-2-yl]carbamoyl}-1,3-thiazol-2-yl)-4-methylpentan-3-yl]-*N*-methyl-*L*-isoleucinamide (8e):

Prepared according to General Method B in 56% yield from *N*-methyl-*D*-proline (CAS 58123-62-9) (**8e'**). Purified by high pressure reverse phase C18 chromatography (Method C, 80.0% H_20/20.0% MeCN linear to 20% H_20/80% MeCN in 8.5 min to 0% H_2O/100% MeCN to 9.0 min, HOLD at 0% H_20/100% MeCN from 9.0 to 10.0 min.) LC-MS (Protocol D): *m/z* 728.4854 $[M+H]^+$; retention time = 2.66 min. HRMS Calcd. for $[M+Na]^+$: 750.3871, Found: 750.3882.

(4*R*)-4-hydroxy-1-methyl-*L*-prolyl-*N*-[(1*R*,3*R*)-1-(acetyloxy)-1-(4-{[(2*R*,4*S*)-5-methoxy-4-methyl-5-oxo-1-phenylpentan-2-yl]carbamoyl}-1,3-thiazol-2-yl)-4-methylpentan-3-yl]-*N*-methyl-*L*-isoleucinamide (8f):

Prepared according to General Method B in 64% yield from (2*S*,4*R*)-4-hydroxy-1-methylpyrrolidine-2-carboxylic acid (CAS 4252-82-8)(**8f'**). Purified by high pressure reverse phase C18 chromatography (Method C, 80.0% H_20/20.0% MeCN linear to 40% H_20/60% MeCN in 8.5 min to 0% H_2O/100% MeCN to 9.0 min, HOLD at 0% H_20/100% MeCN from 9.0 to 10.0 min.) LC-MS (Protocol D): *m/z* 744.4741 $[M+H]^+$; retention time = 2.55 min.

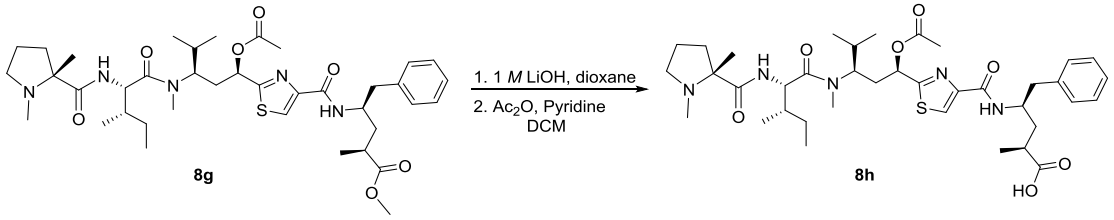
1,2-dimethyl-*D*-prolyl-*N*-[(1*R*,3*R*)-1-(acetyloxy)-1-(4-{[(2*R*,4*S*)-5-methoxy-4-methyl-5-oxo-1-phenylpentan-2-yl]carbamoyl}-1,3-thiazol-2-yl)-4-methylpentan-3-yl]-*N*-methyl-*L*-isoleucinamide (8g):

Prepared according to General Method B in 60% yield from 1,2-dimethyl-*D*-Proline (U.S. Patent 8828401 B2) (**8g'**). Purified by high pressure reverse phase C18 chromatography (Method C, 80.0% H_20/20.0% MeCN linear to 40% H_20/60% MeCN in 8.5 min to 0% H_2O/100% MeCN to 9.0 min, HOLD at 0% H20/100% MeCN from 9.0 to 10.0 min.). LC-MS (Protocol D): *m/z* 742.5098 $[M+H]^+$; retention time = 2.72 min. HRMS Calcd. for $[M+Na]^+$: 764.4027, Found: 764.4040.

methyl (2*S*,4*R*)-4-{[(2-{(1*R*,3*R*)-1-(acetyloxy)-4-methyl-3-[methyl(*N*-{[(2*R*)-1-methylpiperidin-2-yl]carbonyl}-*L*-isoleucyl)amino]pentyl}-1,3-thiazol-4-yl)carbonyl]amino}-2-methyl-5-phenylpentanoate (2):

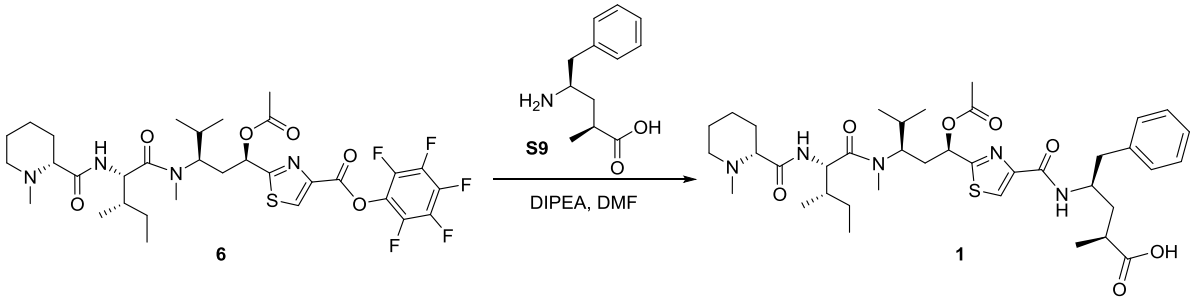
Prepared according to General Method B in 82% yield from (2*R*)-1-methylpiperidine-2-carboxylic acid (CAS 41447-17-0). Purified by high pressure reverse phase C18 chromatography (Method C, 80.0% H_20/20.0% MeCN linear to 40% H_20/60% MeCN in 8.5 min to 0% H_2O/100% MeCN to 9.0 min, HOLD at 0% H_20/100% MeCN from 9.0 to 10.0 min.). LC-MS (Protocol D): *m/z* 742.4959 $[M+H]^+$; retention time = 2.68 min.

Preparation of 1,2-dimethyl-D-prolyl-N-[(1R,3R)-1-(acetyloxy)-1-(4-{[(2R,4S)-4-carboxy-1-phenylpentan-2-yl]carbamoyl}-1,3-thiazol-2-yl)-4-methylpentan-3-yl]-N-methyl-L-isoleucinamide (8h):



1 *M* LiOH (0.4 mL) was added to a vial containing a solution of 1,2-dimethyl-*D*-prolyl-*N*-[(1*R*,3*R*)-1-(acetyloxy)-1-(4-{[(2*R*,4*S*)-5-methoxy-4-methyl-5-oxo-1-phenylpentan-2-yl]carbamoyl}-1,3-thiazol-2-yl)-4-methylpentan-3-yl]-*N*-methyl-*L*-isoleucinamide (**8g**, 29 mg, 0.039 mmol) in dioxane (1.5 mL) and the reaction was stirred at rt for 19 h. Several drops of HOAc was added and the reaction was concentrated. A solution of DCM/pyridine (~1 mL, 1:1) was added to the crude residue followed by Ac_2O (~0.5 mL) and the reaction was stirred at rt for 20 h. After concentration, the residue was re-dissolved in DMSO (0.9 mL) and purified by high pressure reverse phase C18 chromatography (Method C, 70.0% H_20/30.0% MeCN linear to 60% H_20/40% MeCN in 8.5 min to 0% H_2O/100% MeCN to 9.0 min, HOLD at 0% H_20/100% MeCN from 9.0 to 10.0 min.) to afford title compound **8h** (9.2 mg, 34%). LC-MS (Protocol D): *m/z* 728.4948 $[M+H]^+$; retention time = 2.44 min. HRMS Calcd. for $[M+Na]^+$: 751.3904, Found: 751.3902.

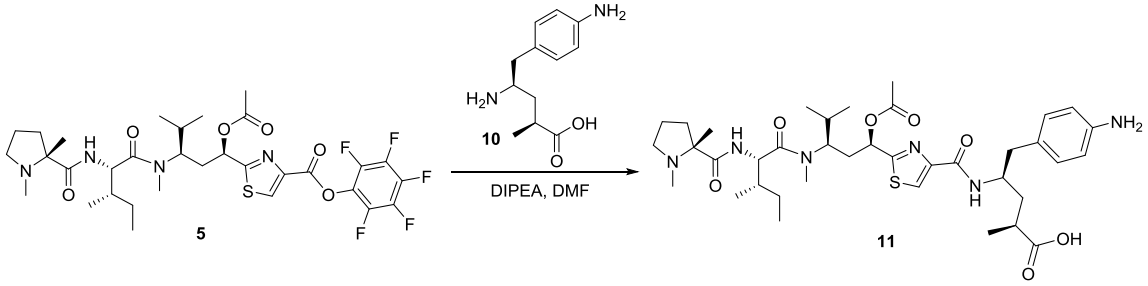
Preparation of (2S,4R)-4-{[(2-{(1R,3R)-1-(acetyloxy)-4-methyl-3-[methyl(N-{[(2R)-1-methylpiperidin-2-yl]carbonyl}-L-isoleucyl)amino]pentyl}-1,3-thiazol-4-yl)carbonyl]amino}-2-methyl-5-phenylpentanoic acid (1):



DMF (0.75 mL) and DIPEA (44 µL, 0.255 mmol) were added to a vial containing (2*S*,4*R*)-4-amino-2-methyl-5-phenylpentanoic acid hydrochloride (*Org. Biomol. Chem*. **2013**, *11*, 2273-2287) (**S9,** 25 mg, 0.102 mmol). After stirring for 10 minutes at rt, a solution of pentafluorophenyl 2-{(1*R*,3*R*)-1-(acetyloxy)-4-methyl-3-[methyl(*N*-{[(2*R*)-1-methylpiperidin-2-yl]carbonyl}-*L*-isoleucyl)amino]pentyl}-1,3-thiazole-4-carboxylate (U.S. patent 2014/197871) (**6**, 60 mg, 0.085 mmol) in DMF (0.75 mL) was added and the reaction was stirred at rt for 18 h. The reaction was concentrated to a crude residue which was re-dissolved in MeOH (2 mL) and purified by high pressure reverse phase chromatography (Method B, 10%

to 95% MeCN in water over 20 minutes, with 0.02% TFA in each phase) to afford title compound **1** as an oil. The oil was re-dissolved in DCM and heptane and concentrated *en vacuo* to yield **1** as a white solid (38 mg, 61%). LC-MS (Protocol B): *m/z* 728.2 $[M+H]^+$; Retention time = 0.75 min.

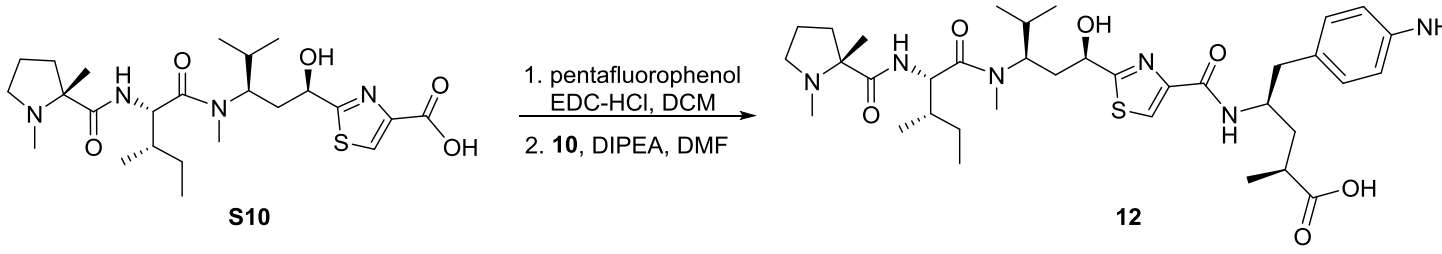
Preparation of 1,2-dimethyl-D-prolyl-N-[(1R,3R)-1-(acetyloxy)-1-(4-{[(2R,4S)-1-(4-aminophenyl)-4-carboxypentan-2-yl]carbamoyl}-1,3-thiazol-2-yl)-4-methylpentan-3-yl]-N-methyl-L-isoleucinamide (11):



DMF (7.3 mL) and DIPEA (270 µL, 1.54 mmol) were added to a vial containing compound **5** (135 mg, 0.192 mmol) and (2*S*,4*R*)-4-amino-5-(4-aminophenyl)-2-methylpentanoic acid hydrochloride (U.S. Patent Number 8394922 B2) (**10,** 57 mg, 0.192 mmol) and the reaction was stirred at rt in a capped vial. After stirring for 17 h at rt, the reaction was concentrated to a crude residue which was re-dissolved in DMSO (2 mL) and purified by medium pressure reverse phase chromatography (10% to 95% MeCN in water with 0.02% TFA in each phase) to afford title compound **11** (115 mg, 80%). LC-MS (Protocol B): *m/z* 743.8 $[M+H]^+$; Retention time = 0.63 min. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.71 (d, *J* = 8.6 Hz, 1H), 8.18 (s, 1H), 8.04 (d, *J* = 9.1 Hz, 1H), 7.16 (d, *J* = 7.8 Hz, 2H), 6.95 (d, *J* = 7.8 Hz, 2H), 5.59 (dd, *J* = 10.7, 3.2 Hz, 1H), 4.63 (t, *J* = 9.1 Hz, 1H), 4.38 (m, 1H), 4.16 (m, 1H), 3.55 (m, 1H), 3.18 (m, 1H), 3.00 (s, 3H), 2.86 – 2.71 (m, 2H), 2.69 (s, 3H), 2.40 (m, 1H), 2.35 – 2.19 (m, 3H), 2.11 (s, 3H), 2.10 – 2.00 (m, 2H), 1.95 (m, 1H), 1.90 – 1.78 (m, 3H), 1.58 (ddd, *J* = 14.1, 9.8, 4.6 Hz, 1H), 1.47 (s, 3H), 1.46 (m, 1H), 1.15 – 1.09 (m, 1H), 1.07 (d, *J* = 7.1 Hz, 3H), 0.96 (d, *J* = 6.5 Hz, 3H), 0.90 – 0.81 (m, 6H), 0.72 (d, *J* = 6.5 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 177.4, 172.6, 170.1, 170.0, 169.9, 160.3, 150.2, 136.4,* 134.5,* 130.5, 130.5, 124.4, 120.0, 120.0, 72.6, 69.9, 55.7,* 54.1, 53.7, 49.4, 40.6, 38.1, 36.5, 36.2, 35.4, 35.4, 35.0,* 29.8,* 29.6, 24.6, 21.3, 20.7, 20.6, 19.9, 18.6, 16.6, 15.8, 11.0. HRMS Calcd. for $[M+H]^+$: 743.4160, Found: 743.4170.

**Value derived by HMBC correlation*

Preparation of 1,2-dimethyl-D-prolyl-N-[(1R,3R)-1-(4-{[(2R,4S)-1-(4-aminophenyl)-4-carboxypentan-2-yl]carbamoyl}-1,3-thiazol-2-yl)-1-hydroxy-4-methylpentan-3-yl]-N-methyl-L-isoleucinamide (12):



Step 1. 1,2-dimethyl-*D*-prolyl-*N*-[(1*R*,3*R*)-1-hydroxy-4-methyl-1-{4-[(pentafluorophenoxy)carbonyl]-1,3-thiazol-2-yl}pentan-3-yl]-N-methyl-*L*-isoleucinamide (S11):

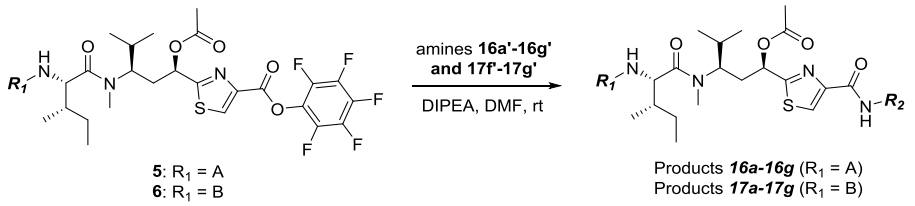
A solution of pentafluorophenol (187 mg, 1.02 mmol) in DCM (1.9 mL) was added to a vial containing **S10** (prepared from **13** according to the procedure for preparation of compound **S1**) (112 mg, 0.226 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC-HCl) (164 mg, 0.894 mmol). DMF (2.8 mL) was added, and the reaction was stirred under N_2 at rt for 4 h. The reaction was concentrated, re-dissolved in DCM (~1.5 mL), and purified by flash silica gel chromatography (5% to 30% MeOH in DCM) to afford the title compound **S11** (117 mg, 77% yield) as a colorless oil. LC-MS (Protocol B): *m/z* 663.7 $[M+H]^+$; Retention time = 0.90 min.

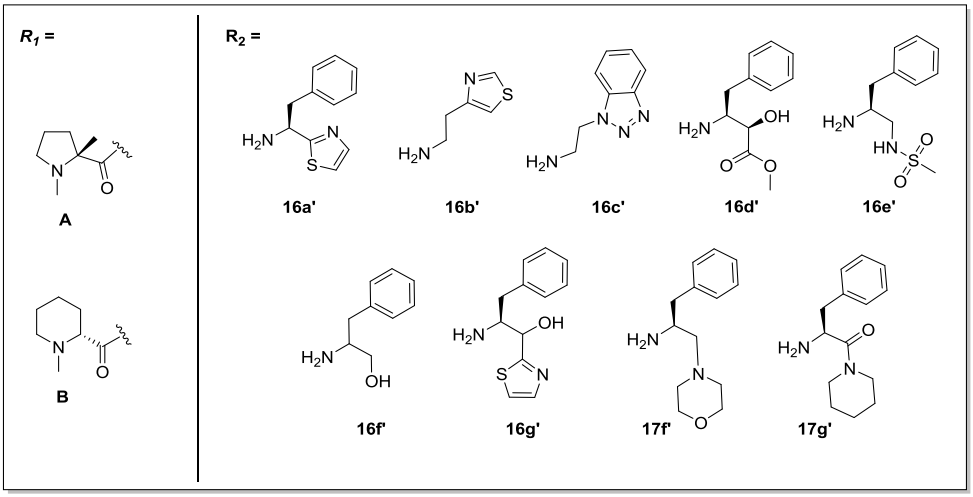
Step 2. 1,2-dimethyl-*D*-prolyl-*N*-[(1*R*,3*R*)-1-(4-{[(2*R*,4*S*)-1-(4-aminophenyl)-4-carboxypentan-2-yl]carbamoyl}-1,3-thiazol-2-yl)-1-hydroxy-4-methylpentan-3-yl]-*N*-methyl-*L*-isoleucinamide (12):

DMF (4.5 mL) and DIPEA (167 µL, 0.95 mmol) were added to a vial containing 1,2-dimethyl-*D*-prolyl-*N*-[(1*R*,3*R*)-1-hydroxy-4-methyl-1-{4-[(pentafluorophenoxy)carbonyl]-1,3-thiazol-2-yl}pentan-3-yl]-N-methyl-*L*-isoleucinamide (**S11**, 84 mg, 0.127 mmol) and (2*S*,4*R*)-4-amino-5-(4-aminophenyl)-2-methylpentanoic acid hydrochloride (U.S. Patent Number 8394922 B2) (**10**, 38 mg, 0.127 mmol) and the reaction was stirred at rt under N_2. After stirring for 17.5 h at rt, the reaction was concentrated to a crude brown residue which was re-dissolved in DMSO (2 mL) and purified by high pressure reverse phase C18 chromatography (Method A) to afford title compound **12** (57 mg, 55%) as an off-white solid. HPLC (Protocol C): *m/z* 702.4 $[M+H]^+$; retention time = 5.89 min. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.71 (d, *J* = 8.4 Hz, 1H), 8.07 (s, 1H), 7.87 (d, *J* = 9.1 Hz, 1H), 7.18 (d, *J* = 7.8 Hz, 2H), 6.99 (d, *J* = 7.8 Hz, 2H), 4.63 (dd, *J* = 10.1, 8.4 Hz, 1H), 4.55 (dd, *J* = 10.0, 2.4 Hz, 1H), 4.54 (m, 1H), 4.16 (m, 1H), 3.56 (m, 1H), 3.18 (m, 1H), 3.06 (s, 3H), 2.87 – 2.72 (m, 2H), 2.69 (s, 3H), 2.40 (m, 1H), 2.30 (m, 1H), 2.20 – 1.94 (m, 4H), 1.87 (m, 4H), 1.55 (m, 1H), 1.52 – 1.45 (m, 1H), 1.48 (s, 3H), 1.13 (dt, *J* = 13.5, 7.8 Hz, 1H), 1.07 (d, *J* = 7.1 Hz, 3H), 0.93 (d, *J* = 6.5 Hz, 3H), 0.88 (d, *J* = 6.7 Hz, 3H), 0.84 (t, *J* = 7.4 Hz, 3H), 0.73 (d, *J* = 6.6 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 178.5, 177.4, 172.4, 170.2, 160.5, 150.2, 135.5,* 135.2,* 130.6, 130.6, 123.3, 120.4, 120.4, 72.7, 68.5, 55.6,* 54.3, 53.8, 49.2, 40.7, 38.1, 37.6, 36.4, 36.1, 35.5, 35.3, 29.9, 29.9, 24.6, 20.6, 20.6, 20.0, 18.5, 16.6, 15.5, 11.0. HRMS Calcd. for $[M+H]^+$: 701.4059, Found: 701.4060.

**Value derived by HMBC correlation*

Synthesis of C-Terminus Library Analogs





General Method C: Synthesis of C-terminus library analogs 16a-16g as exemplified by synthesis of 16e

1,2-dimethyl-*D*-prolyl-*N*-{(1*R*,3*R*)-1-(acetyloxy)-4-methyl-1-[4-({(2*S*)-1-[(methylsulfonyl)amino]-3-phenylpropan-2-yl}carbamoyl)-1,3-thiazol-2-yl]pentan-3-yl}-*N*-methyl-*L*-isoleucinamide (16e):

DMF (0.5 mL) and DIPEA (42 µL, 1.54 mmol) were added to a vial containing 1,2-dimethyl-*D*-prolyl-*N*-[(1*R*,3*R*)-1-(acetyloxy)-4-methyl-1-{4-[(pentafluorophenoxy)carbonyl]-1,3-thiazol-2-yl}pentan-3-yl]-*N*-methyl-*L*-isoleucinamide (**5**, 21 mg, 0.030 mmol) and *N*-[(2*S*)-2-amino-3-phenylpropyl]methanesulfonamide (WO 9746553) (**16e'**, 11.5 mg, 0.036 mmol) and the reaction was stirred at rt in a capped vial. After stirring for 17 h at rt, the reaction was concentrated to a crude residue which was re-dissolved in DMSO (0.9 mL) and purified by high pressure reverse phase C18 chromatography (Method C, 95.0% H_2O/5.0% MeCN linear to 50% H_2O/50% MeCN in 8.5 min to 0% H_2O/100% MeCN to 9.0 min, HOLD at 0% H_2O/100% MeCN from 9.0 to 10.0 min.) to afford title compound **16e** (8.5 mg, 53%). LC-MS (Protocol D): *m/z* 749.5 $[M+H]^+$; retention time = 2.5 min.

1,2-dimethyl-*D*-prolyl-*N*-[(1*R*,3*R*)-1-(acetyloxy)-4-methyl-1-(4-{[(1*S*)-2-phenyl-1-(1,3-thiazol-2-yl)ethyl]carbamoyl}-1,3-thiazol-2-yl)pentan-3-yl]-*N*-methyl-*L*-isoleucinamide (16a):

Prepared according to General Method C in 67% yield from (1*S*)-2-phenyl-1-(1,3-thiazol-2-yl)ethanamine (CAS 130199-65-4) (**16a'**). Purified by high pressure reverse phase C18 chromatography (Method C, 95.0% H_2O/5.0% MeCN linear to 50% H_2O/50% MeCN in 8.5 min to 0% H_2O/100% MeCN to 9.0 min, HOLD at 0% H_2O/100% MeCN from 9.0 to 10.0 min.). LC-MS (Protocol D): *m/z* 725.3 $[M+H]^+$; retention time = 2.7 min.

1,2-dimethyl-*D*-prolyl-*N*-[(1*R*,3*R*)-1-(acetyloxy)-4-methyl-1-(4-{[2-(1,3-thiazol-4-yl)ethyl]carbamoyl}-1,3-thiazol-2-yl)pentan-3-yl]-*N*-methyl-*L*-isoleucinamide (16b):

Prepared according to General Method C in 69% yield from 2-(1,3-thiazol-4-yl)ethanamine (CAS 7728-74-7) (**16b'**). Purified by high pressure reverse phase C18 chromatography (Method C, 95.0% H_2O/5.0%

MeCN linear to 50% H_2O/50% MeCN in 8.5 min to 0% H_2O/100% MeCN to 9.0 min, HOLD at 0% H_2O/100% MeCN from 9.0 to 10.0 min.). LC-MS (Protocol D): *m/z* 325.3 $[M + 2H]^{+2}$; retention time = 2.3 min.

1,2-dimethyl-*D*-prolyl-*N*-[(1*R*,3*R*)-1-(acetyloxy)-1-(4-{[2-(1H-benzotriazol-1-yl)ethyl]carbamoyl}-1,3-thiazol-2-yl)-4-methylpentan-3-yl]-*N*-methyl-*L*-isoleucinamide (16c):

Prepared according to General Method C in 66% yield from 2-(1*H*-benzotriazol-1-yl)ethanamine (CAS 26861-65-4) (**16c'**). Purified by high pressure reverse phase C18 chromatography (Method C, 95.0% H_2O/5.0% MeCN linear to 50% H_2O/50% MeCN in 8.5 min to 0% H_2O/100% MeCN to 9.0 min, HOLD at 0% H_2O/100% MeCN from 9.0 to 10.0 min.). LC-MS (Protocol D): *m/z* 683.3 $[M+H]^+$; retention time = 2.4 min.

1,2-dimethyl-*D*-prolyl-*N*-[(1*R*,3*R*)-1-(acetyloxy)-1-(4-{[(2*S*,3*R*)-3-hydroxy-4-methoxy-4-oxo-1-phenylbutan-2-yl]carbamoyl}-1,3-thiazol-2-yl)-4-methylpentan-3-yl]-*N*-methyl-*L*-isoleucinamide (16d):

Prepared according to General Method C in 61% yield from methyl (2*R*,3*S*)-3-amino-2-hydroxy-4-phenylbutanoate (CAS 139163-87-4) (**16d'**). Purified by high pressure reverse phase C18 chromatography (Method C, 95.0% H_2O/5.0% MeCN linear to 50% H_2O/50% MeCN in 8.5 min to 0% H_2O/100% MeCN to 9.0 min, HOLD at 0% H_2O/100% MeCN from 9.0 to 10.0 min.). LC-MS (Protocol D): *m/z* 730.4 $[M+H]^+$; retention time = 2.6 min. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.77 (s, 1H), 8.72 (d, *J* = 8.7 Hz, 1H), 8.24 (s, 1H), 7.78 (d, *J* = 9.5 Hz, 1H), 7.32 – 7.23 (m, 4H), 7.22-7.19 (m, 1H), 6.04 (br. s, 1H), 5.66 – 5.54 (m, 1H), 4.65-4.53 (m, 1H), 4.55-4.49 (m, 1H), 4.13 (d, *J* = 2.4 Hz, 1H), 3.55 (s, 3H), 3.19-3.13 (s, 1H), 3.01 (s, 3H), 3.01-2.95 (m, 1H), 2.89-2.84 (m, 1H), 2.69-2.68 (m, 3H), 2.33-2.29 (m, 3H), 2.09-1.81 (m, 6H), 2.11 (s, 3H), 1.47-1.42 (m, 1H), 1.49 (s, 3H), 1.13-1.06 (m, 1H), 0.95 (d, *J* = 6.5 Hz, 3H), 0.91 – 0.77 (m, 6H), 0.72 (d, *J* = 6.5 Hz, 3H). ^{13}C NMR (126 MHz, DMSO) δ 172.79, 172.09, 169.93, 169.76, 169.66, 159.51, 148.98, 138.10, 129.12, 128.36, 126.41, 124.81, 72.23, 70.04, 69.54, 53.74, 53.27, 53.01, 51.62, 37.14, 35.78, 34.96, 34.89, 33.91, 29.05, 24.13, 20.63, 20.24, 20.05, 19.33, 16.10, 15.11, 10.35. HRMS Calcd. for $[M+H]^+$: 730.3858, Found: 730.3858.

1,2-dimethyl-*D*-prolyl-*N*-[(1*R*,3*R*)-1-(acetyloxy)-1-{4-[(1-hydroxy-3-phenylpropan-2-yl)carbamoyl]-1,3-thiazol-2-yl}-4-methylpentan-3-yl]-*N*-methyl-*L*-isoleucinamide (16f):

Prepared according to General Method C in 38% yield from 2-amino-3-phenylpropan-1-ol (CAS 16088-07-6) (**16f'**). Purified by high pressure reverse phase C18 chromatography (Method C, 95.0% H_2O/5.0% MeCN linear to 50% H_2O/50% MeCN in 8.5 min to 0% H_2O/100% MeCN to 9.0 min, HOLD at 0% H_2O/100% MeCN from 9.0 to 10.0 min.). LC-MS (Protocol D): *m/z* 672.3 $[M+H]^+$; retention time = 2.5 min.

1,2-dimethyl-*D*-prolyl-*N*-[(1*R*,3*R*)-1-(acetyloxy)-1-(4-{[(2*S*)-1-hydroxy-3-phenyl-1-(1,3-thiazol-2-yl)propan-2-yl]carbamoyl}-1,3-thiazol-2-yl)-4-methylpentan-3-yl]-*N*-methyl-*L*-isoleucinamide (16g):

Prepared according to General Method C in 64% yield from (2*S*)-2-amino-3-phenyl-1-(1,3-thiazol-2-yl)propan-1-ol (CAS 1383049-19-1) (**16g'**). Purified by high pressure reverse phase C18 chromatography (Method C, 95.0% H_2O/5.0% MeCN linear to 50% H_2O/50% MeCN in 8.5 min to 0% H_2O/100% MeCN to 9.0 min, HOLD at 0% H_2O/100% MeCN from 9.0 to 10.0 min.). LC-MS (Protocol D): *m/z* 755.3 $[M+H]^+$; retention time = 2.6 min.

General Method D: Synthesis of C-terminus library analogs 17a-17g, as exemplified by synthesis of 17a

(1*R*,3*R*)-4-methyl-3-[methyl(*N*-{[(2*R*)-1-methylpiperidin-2-yl]carbonyl}-*L*-isoleucyl)amino]-1-(4-{[2-phenyl-1-(1,3-thiazol-2-yl)ethyl]carbamoyl}-1,3-thiazol-2-yl)pentyl acetate (17a):

DMSO (0.25 mL) and DIPEA (25µL, 0.144 mmol) were added to a vial containing (1*S*)-2-phenyl-1-(1,3-thiazol-2-yl)ethanamine (CAS 130199-65-4) (**16a'**, 9.8 mg, 0.048 mmol) and the reaction was stirred for 10 min. A solution of pentafluorophenyl 2-{(1*R*,3*R*)-1-(acetyloxy)-4-methyl-3-[methyl(*N*-{[(2*R*)-1-methylpiperidin-2-yl]carbonyl}-*L*-isoleucyl)amino]pentyl}-1,3-thiazole-4-carboxylate (U.S. patent 2014/197871) (**6**, 34 mg, 0.048 mmol) in DMSO (0.25 mL) was added and the reaction was stirred at rt for 16 h. The reaction was heated to 40 °C for 4 h, cooled to rt, and concentrated to a crude residue which was re-dissolved in DMSO (1 mL) and purified by high pressure reverse phase C18 chromatography (Method C, 85.0% $H_2$0/15.0% MeCN linear to 45% $H_2$0/55% MeCN in 8.5 min to 0% H_2O/100% MeCN to 9.0 min, HOLD at 0% $H_2$0/100% MeCN from 9.0 to 10.0 min.) to afford title compound **17a** (12.9 mg, 37%). LC-MS (Protocol D): *m/z* 725.4 $[M+H]^+$; retention time = 2.51 min. HRMS Calcd. for $[M+2H]^{+2}$: 363.1793, Found: 363.1798.

(1*R*,3*R*)-4-methyl-3-[methyl(*N*-{[(2*R*)-1-methylpiperidin-2-yl]carbonyl}-*L*-isoleucyl)amino]-1-(4-{[2-(1,3-thiazol-2-yl)ethyl]carbamoyl}-1,3-thiazol-2-yl)pentyl acetate (17b):

Prepared according to General Method D in 26% yield from 2-(1,3-thiazol-4-yl)ethanamine (CAS 7728-74-7) (**16b'**). Purified by high pressure reverse phase C18 chromatography (Method C, 95.0% $H_2$0/5.0% MeCN linear to 60% $H_2$0/40% MeCN in 8.5 min to 0% H_2O/100% MeCN to 9.0 min, HOLD at 0% $H_2$0/100% MeCN from 9.0 to 10.0 min.). ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.67 (s, 1H), 8.98 (d, *J* = 8.5 Hz, 1H), 8.51 (t, *J* = 6.0 Hz, 1H), 8.25 (s, 1H), 7.73 (d, *J* = 3.3 Hz, 1H), 7.61 (d, *J* = 3.3 Hz, 1H), 5.57 (dd, *J* = 11.1, 2.7 Hz, 1H), 4.61 (t, *J* = 8.4 Hz, 1H), 4.36 (br. s, 1H), 3.79-3.74 (m, 1H), 3.34 (d, *J* = 12.3 Hz, 1H), 3.27 (t, *J* = 7.2 Hz, 2H), 3.12 – 3.01 (m, 1H), 2.99 (s, 3H), 2.61 (d, *J* = 4.0 Hz, 3H), 2.34-2.28 (m, 1H), 2.25 – 2.15 (m, 1H), 2.08 (s, 3H), 2.07 – 2.00 (m, 2H), 1.86 – 1.73 (m, 4H), 1.73 – 1.52 (m, 2H), 1.50-1.37 (m, 2H), 1.17 – 1.04 (m, 1H), 0.95 (d, *J* = 6.5 Hz, 3H), 0.88 (d, *J* = 6.7 Hz, 3H), 0.84 (t, *J* = 7.4 Hz, 3H), 0.71 (d, *J* = 6.5 Hz, 3H). ^{13}C NMR (126 MHz, DMSO) δ 172.05, 169.73, 169.37, 167.54, 167.27, 160.23, 149.47, 142.20, 124.53, 119.69, 69.25, 65.82, 54.30, 53.54, 41.22, 38.63, 35.54, 33.63, 32.42, 29.01, 28.59, 23.69, 22.26, 21.00, 20.60, 20.02, 19.46, 15.24, 10.64, 1.14. LC-MS (Protocol D): *m/z* 649.4 $[M+H]^+$; retention time = 2.1 min. HRMS Calcd. for $[M+H]^+$: 649.3210, Found: 649.3213.

(1*R*,3*R*)-1-(4-{[2-(1H-benzotriazol-1-yl)ethyl]carbamoyl}-1,3-thiazol-2-yl)-4-methyl-3-[methyl(*N*-{[(2*R*)-1-methylpiperidin-2-yl]carbonyl}-L-isoleucyl)amino]pentyl acetate (17c):

Prepared according to General Method D in 26% yield from 2-(1H-benzotriazol-1-yl)ethanamine (CAS 26861-65-4) (**16c'**). Purified by high pressure reverse phase C18 chromatography (Method C, 80.0% $H_2$0/20.0% MeCN linear to 60% $H_2$0/40% MeCN in 8.5 min to 0% H_2O/100% MeCN to 9.0 min, HOLD at 0% $H_2$0/100% MeCN from 9.0 to 10.0 min. LC-MS (Protocol D): *m/z* 683.5 $[M+H]^+$; retention time = 2.18 min. HRMS Calcd. for $[M+H]^+$: 683.3698, Found: 683.3710.

methyl (2*R*,3S)-3-{[(2-{(1*R*,3*R*)-1-(acetyloxy)-4-methyl-3-[methyl(*N*-{[(2*R*)-1-methylpiperidin-2-yl]carbonyl}-*L*-isoleucyl)amino]pentyl}-1,3-thiazol-4-yl)carbonyl]amino}-2-hydroxy-4-phenylbutanoate (17d):

Prepared according to General Method D in 25% yield from methyl (2*R*,3*S*)-3-amino-2-hydroxy-4-phenylbutanoate) (CAS 26861-65-4) (**16d'**). Purified by high pressure reverse phase C18 chromatography (Method C, 95.0% $H_2$0/5.0% MeCN linear to 50% $H_2$0/50% MeCN in 8.5 min to 0% H_2O/100% MeCN to 9.0 min, HOLD at 0% $H_2$0/100% MeCN from 9.0 to 10.0 min.). LC-MS (Protocol D): *m/z* 730.5 $[M+H]^+$; retention time = 2.38 min. HRMS Calcd. for $[M+H]^+$: 730.3844, Found: 730.3851.

(1*R*,3*R*)-4-methyl-3-[methyl(*N*-{[(2*R*)-1-methylpiperidin-2-yl]carbonyl}-*L*-isoleucyl)amino]-1-[4-({(2*S*)-1-[(methylsulfonyl)amino]-3-phenylpropan-2-yl}carbamoyl)-1,3-thiazol-2-yl]pentyl acetate (17e):

Prepared according to General Method D in 30% yield from *N*-[(2*S*)-2-amino-3-phenylpropyl]methanesulfonamide (WO 9746553) (**16e'**). Purified by high pressure reverse phase C18 chromatography (Method C, 95.0% H_20/5.0% MeCN linear to 50% H_20/50% MeCN in 8.5 min to 0% H_2O/100% MeCN to 9.0 min, HOLD at 0% H_20/100% MeCN from 9.0 to 10.0 min.). LC-MS (Protocol D): *m/z* 749.4 $[M+H]^+$; retention time = 2.28 min. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.65 (br. s, 1H), 8.97 (d, *J* = 8.5 Hz, 1H), 8.20 (s, 1H), 8.08 (d, *J* = 8.5 Hz, 1H), 7.26 (m, 5H), 7.18 (m, 1H), 5.56 (dd, *J* = 11.1, 2.6 Hz, 1H), 4.61 (t, *J* = 8.4 Hz, 1H), 4.35 (br. s, 1H), 4.24-4.22 (m, 1H), 3.78-3.73 (m, 1H), 3.15 (t, *J* = 6.2 Hz, 2H), 3.09-3.02 (m, 1H), 2.99 (s, 3H), 2.93-2.91 (m, 2H), 2.91 (s, 3H), 2.60 (d, *J* = 3.8 Hz, 3H), 2.37 – 2.02 (m, 4H), 2.08 (d, *J* = 10.0 Hz, 3H), 1.87 – 1.34 (m, 7H), 1.14-1.06 (m, 1H), 0.95 (d, *J* = 6.5 Hz, 3H), 0.88 (d, *J* = 6.7 Hz, 3H), 0.83 (t, *J* = 7.4 Hz, 3H), 0.71 (d, *J* = 6.5 Hz, 3H). ^{13}C NMR (126 MHz, DMSO) δ 172.08, 169.74, 169.38, 167.56, 160.04, 149.52, 138.47, 129.11, 128.24, 126.21, 124.44, 69.35, 65.84, 54.33, 53.56, 50.57, 45.28, 41.25, 37.09, 35.56, 33.72, 29.03, 28.62, 23.71, 22.29, 21.01, 20.61, 20.02, 19.47, 15.27, 10.65, 1.16. HRMS Calcd. for $[M+H]^+$: 749.3729, Found: 749.3720.

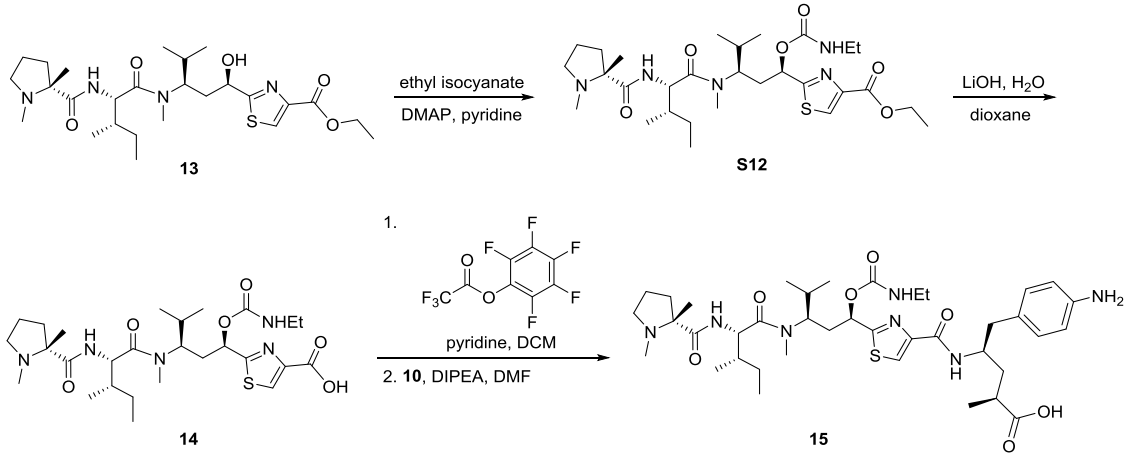
(1*R*,3*R*)-4-methyl-3-[methyl(*N*-{[(2*R*)-1-methylpiperidin-2-yl]carbonyl}-*L*-isoleucyl)amino]-1-(4-{[(2*S*)-1-(morpholin-4-yl)-3-phenylpropan-2-yl]carbamoyl}-1,3-thiazol-2-yl)pentyl acetate (17f):

Prepared according to General Method D in 35% yield from (2*S*)-1-(morpholin-4-yl)-3-phenylpropan-2-amine (*Org. Biomol. Chem.* **2012***, 10*, 7618-7627) (**17f'**). Purified by high pressure reverse phase C18 chromatography (Method C, 95.0% H_20/5.0% MeCN linear to 65% H_20/35% MeCN in 8.5 min to 0% H_2O/100% MeCN to 9.0 min, HOLD at 0% H_20/100% MeCN from 9.0 to 10.0 min. LC-MS (Protocol D): *m/z* 741.5$[M+H]^+$; retention time = 1.92 min. HRMS Calcd. for $[M+H]^+$: 741.4368, Found: 741.4371.

(1*R*,3*R*)-4-methyl-3-[methyl(*N*-{[(2*R*)-1-methylpiperidin-2-yl]carbonyl}-*L*-isoleucyl)amino]-1-(4-{[(2*S*)-1-oxo-3-phenyl-1-(piperidin-1-yl)propan-2-yl]carbamoyl}-1,3-thiazol-2-yl)pentyl acetate (17g):

Prepared according to General Method D in 22% yield from (2*S*)-2-amino-3-phenyl-1-(piperidin-1-yl)propan-1-one (CAS 102292-89-7) (**17g'**). Purified by high pressure reverse phase C18 chromatography (Method C, 85.0% H_20/15.0% MeCN linear to 45% H_20/55% MeCN in 8.5 min to 0% H_2O/100% MeCN to 9.0min, HOLD at 0% H_20/100% MeCN from 9.0 to 10.0 min.). LC-MS (Protocol D): *m/z* 753.6$[M+H]^+$; retention time = 2.7 min. HRMS Calcd. for $[M+H]^+$: 753.4368, Found: 753.4378.

Synthesis of Analog 15



Preparation of 1,2-dimethyl-D-prolyl-N-{(1R,3R)-1-(4-{[(2R,4S)-1-(4-aminophenyl)-4-carboxypentan-2-yl]carbamoyl}-1,3-thiazol-2-yl)-1-[(ethylcarbamoyl)oxy]-4-methylpentan-3-yl}-N-methyl-L-isoleucinamide (15):

Step 1. 1,2-dimethyl-*D*-prolyl-*N*-{(1*R*,3*R*)-1-[4-(ethoxycarbonyl)-1,3-thiazol-2-yl]-1-[(ethylcarbamoyl)oxy]-4-methylpentan-3-yl}-*N*-methyl-*L*-isoleucinamide (S12):

DMF (1.2 mL) and ethyl isocyanate (580 µL, 7.25 mmol) were added to a vial containing 1,2-dimethyl-*D*-prolyl-*N*-{(1*R*,3*R*)-1-[4-(ethoxycarbonyl)-1,3-thiazol-2-yl]-1-hydroxy-4-methylpentan-3-yl}-*N*-methyl-*L*-isoleucinamide (**13**, 85 mg, 0.162 mmol) and DMAP (36 mg, 0.300 mmol). The reaction was stirred in a capped vial at rt for 22.5 h then the reaction was concentrated and the residue was purified by medium pressure reverse phase C18 chromatography (10% to 95% MeCN in H_2O over 25 minutes, each solvent containing 0.02% TFA) to provide title compound **S12** (69 mg, 84%) as a glassy yellow solid. LC-MS (Protocol B): *m/z* 596.7 $[M+H]^+$, Retention time = 0.72 min.

Step 2. 1,2-dimethyl-*D*-prolyl-*N*-{(1*R*,3*R*)-1-(4-carboxy-1,3-thiazol-2-yl)-1-[(ethylcarbamoyl)oxy]-4-methylpentan-3-yl}-*N*-methyl-*L*-isoleucinamide (14):

1 *M* LiOH in water (330 µL) was added to a vial containing a solution of 1,2-dimethyl-*D*-prolyl-*N*-{(1*R*,3*R*)-1-[4-(ethoxycarbonyl)-1,3-thiazol-2-yl]-1-[(ethylcarbamoyl)oxy]-4-methylpentan-3-yl}-*N*-methyl-*L*-isoleucinamide (**S12**, 66 mg, 0.110 mmol) in 1,4-dioxane (5.5 mL). The reaction was stirred at rt for 6 h then quenched with HOAc (60 µL) and concentrated. The crude residue was purified by medium pressure reverse phase C18 chromatography (10% to 95% MeCN in H_2O over 25 minutes, each solvent containing 0.02% TFA) to provide the title compound **14** (53 mg, 84% yield) as a glassy solid. LC-MS (Protocol B): *m/z* 568.6 $[M+H]^+$; Retention time = 0.63 min.

Step 3. 1,2-dimethyl-*D*-prolyl-*N*-[(1*R*,3*R*)-1-[(ethylcarbamoyl)oxy]-4-methyl-1-{4-[(pentafluorophenoxy)carbonyl]-1,3-thiazol-2-yl}pentan-3-yl]-*N*-methyl-*L*-isoleucinamide (S13):

The title compound **S13** was prepared in 58% yield from 1,2-dimethyl-*D*-prolyl-*N*-{(1*R*,3*R*)-1-(4-carboxy-1,3-thiazol-2-yl)-1-[(ethylcarbamoyl)oxy]-4-methylpentan-3-yl}-*N*-methyl-*L*-isoleucinamide (**14**, 0.093 mmol) and pentafluorophenyl trifluoroacetate (0.231 mmol) using the method described above for compound **5**. Purification was performed by flash silica gel chromatography (5% to 20% MeOH in DCM). LC-MS (Protocol B): *m/z* 734.7 $[M+H]^+$; Retention time = 0.88 min.

Step 4. 1,2-dimethyl-*D*-prolyl-*N*-{(1*R*,3*R*)-1-(4-{[(2*R*,4*S*)-1-(4-aminophenyl)-4-carboxypentan-2-yl]carbamoyl}-1,3-thiazol-2-yl)-1-[(ethylcarbamoyl)oxy]-4-methylpentan-3-yl}-*N*-methyl-*L*-isoleucinamide (15):

DMF (0.9 mL) and DIPEA (31 µL, 0.176 mmol) were added to a vial containing 1,2-dimethyl-*D*-prolyl-*N*-[(1*R*,3*R*)-1-[(ethylcarbamoyl)oxy]-4-methyl-1-{4-[(pentafluorophenoxy)carbonyl]-1,3-thiazol-2-yl}pentan-3-yl]-*N*-methyl-*L*-isoleucinamide (**S13**, 16 mg, 0.022 mmol) and (2*S*,4*R*)-4-amino-5-(4-aminophenyl)-2-methylpentanoic acid hydrochloride (U.S. Patent Number 8394922 B2) (**10**, 6.5 mg, 0.022 mmol) and the reaction was stirred at rt under N_2. After stirring for 18 h at rt, the reaction was concentrated to a crude residue which was re-dissolved in DMSO (0.9 mL) and purified by high pressure reverse phase C18 chromatography (Method C, 95.0% H_2O/5.0% MeCN linear to 50% H_2O/50% MeCN in 8.5min to 0% H_2O/100% MeCN to 9.0 min, HOLD at 0% H_2O/ 100% MeCN from 9.0 to 10.0 min.) to afford title compound **15** (10.2 mg, 52%) as an off-white solid. LC-MS (Protocol D): *m/z* 772.5 $[M+H]^+$; retention time = 1.75 min. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.78 (s, 2H), 8.71 (d, *J* = 8.6 Hz, 1H), 8.13 (s, 1H), 8.02 (d, *J* = 9.2 Hz, 1H), 7.45 (m, 1H), 7.29 (d, *J* = 8.0 Hz, 2H), 7.16 (d, *J* = 8.0 Hz, 2H), 5.48 (m, 1H), 4.62 (t, *J* = 9.4 Hz, 1H), 4.5 – 4.3 (m, 1H), 4.20 (m, 1H), 3.57 (m, 1H), 3.17 (m, 1H), 3.03 (s, 3H), 2.98 (q, *J* = 6.8 Hz,

2H), 2.91 – 2.77 (m, 2H), 2.70 (s, 3H), 2.41 (m, 1H), 2.31 (m, 1H), 2.17 (m, 2H), 2.08 (m, 1H), 1.96 (m, 1H), 1.90 - 1.80 (m, 3H), 1.60 (m, 1H), 1.49 (m, 5H), 1.16 – 1.04 (m, 4H), 1.00 (t, *J* = 7.1 Hz, 3H), 0.94 (d, *J* = 6.5 Hz, 3H), 0.90 – 0.80 (m, 6H), 0.74 (d, *J* = 6.7 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 177.4, 172.2, 172.1, 170.1, 160.4, 155.5, 150.1, 138.1,* 131.2,* 130.7, 130.7, 124.0, 122.4, 122.4, 72.7, 70.1, 55.3,* 54.2, 53.6, 49.3, 40.8, 38.1, 36.4, 36.2, 35.6, 35.6, 35.4, 35.0, 29.9, 29.8, 24.6, 20.6, 20.5, 20.0, 18.6, 16.6, 15.7, 15.7, 11.0. HRMS Calcd. for $[M+H]^+$: 772.4426, Found: 772.4430.

**Value derived by HMBC correlation*

3. Cell lines and Cytotoxicity Assay

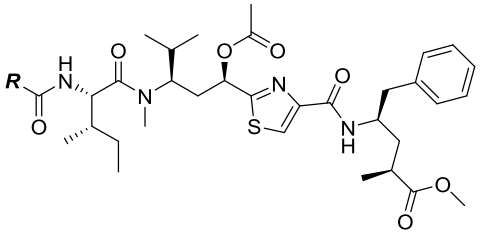
N87 cells were obtained from ATCC (Manassas, VA) and were originally derived from a liver metastasis of gastric carcinoma. MDA-MB-361-DYT2 cells are derived from breast carcinoma and were generously provided by Dr. Dajun Yang of Georgetown University. KB-3-1 (herein referred to as KB) and KB-8-5 (MDR1+) cells were generously provided by Dr. Michael Gottesman at the National Cancer Institute (Rockville, MD) and were generated as described previously (1). Cells were maintained in RPMI (N87 cells), MEM (361 cells), or DMEM (KB cells). KB-8-5 cells were supplemented with 25 nM colchicine during cell maintenance but not during assays. All media were supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% sodium pyruvate. Cytotoxicity assessment was determined as reported previously (2). Briefly, cells were treated with 10-dose dilutions of compounds in duplicate for 4 days, then cell viability assessed with CellTiter® 96 AQueous One MTS Solution (Promega, Madison, WI). IC_{50} values were calculated using a four-parameter logistic model with XLfit (IDBS, Bridgewater, NJ).[1,2]

[1] Akiyama, S.; Fojo, A.; Hanover, J. A.; Pastan, I.; Gottesman, M. M. Isolation and genetic characterization of human KB cell lines resistant to multiple drugs. *Somatic cell and Mol. Gen.* **1985**, *11*, 117-26.

[2] Loganzo, F.; Tan, X.; Sung, M.; Jin, G.; Myers, J. S.; Melamud, E. *et al*. Tumor cells chronically treated with a trastuzumab-maytansinoid antibody-drug conjugate develop varied resistance mechanisms but respond to alternate treatments. *Mol. Cancer Ther*. **2015**, *14*, 952-63.

4. Cytotoxicity Evaluation of Tubulysin Compounds

Table S1: Cytotoxicity Evaluation of N-Terminal Tubulysin Library Compounds

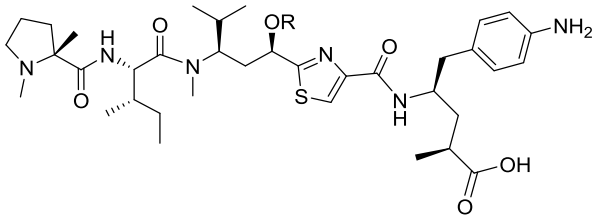


R	ID	N87[1]	MDA-MB-361-DYT2[1]	KB (MDR -ve)[1]	KB 8.5 (MDR +ve)[1]
	**12**	0.46 ± 0.15	0.13 ± 0.04	0.33 ± 0.15	1.10 ± 0.39
	2	0.32 ± 0.18	0.22 ± 0.02	0.22 ± 0.06	0.70 ± 0.02
	**8a**	7.74 ± 4.62	1.68 ± 0.54	2.60 ± 0.55	141 ±45
	**8b**	59 ± 21.1	24 ± 3.9	18.6 ± 3.7	179 ± 27
	**8c**	0.89 ± 0.68	1.06 ± 0.77	1.76 ± 0.45	33.6 ±13
	**8d**	9.76 ± 1.53	3.37 ± 1.03	4.68 ± 0.69	53.9 ±1.0
	**8e**	2.88 ± 1.38	0.67 ± 0.30	1.26 ± 0.76	7.88 ±2.29
	**8f**	106 ± 12	26.9 ± 4.6	22.6 ± 3.70	788 ±27
	**8g**	0.89 ± 0.60	0.45 ± 0.35	1.04 ± 0.15	5.52 ±0.90
	8h[2]	0.13 ± 0.11	0.07 ± 0.03	0.11 ± 0.05	1.02 ± 0.46

1. Reported average IC50 (nM) +/- standard deviation for 2 - 13 independent determinations;

2. Carboxylic acid (Tup) C-terminus.

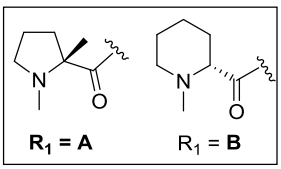
Table S2: Potency of C-11 modified Tubulysins

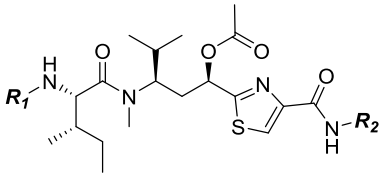


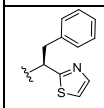
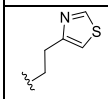
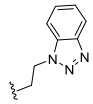
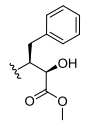
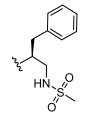
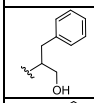
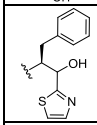
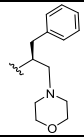
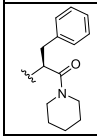
R	ID	N87[1]	MDA-MB-361-DYT2[1]	KB (MDR -ve)[1]	KB 8.5 (MDR +ve)[1]
Ac	**11**	1.11 ± 0.32	0.90 ± 0.61	0.50 ± 0.28	2.77 ± 0.77
H	**12**	>1000	>1000	769	>1000
C(O)NHEt	**15**	1.92 ± 0.59	1.94 ± 0.20	0.77 ± 0.02	4.53 ± 1.70

1. Reported average IC50 (nM) +/- standard deviation for 2 - 13 independent determinations

Table S3: Potency of C-Terminal modified Tubulysins





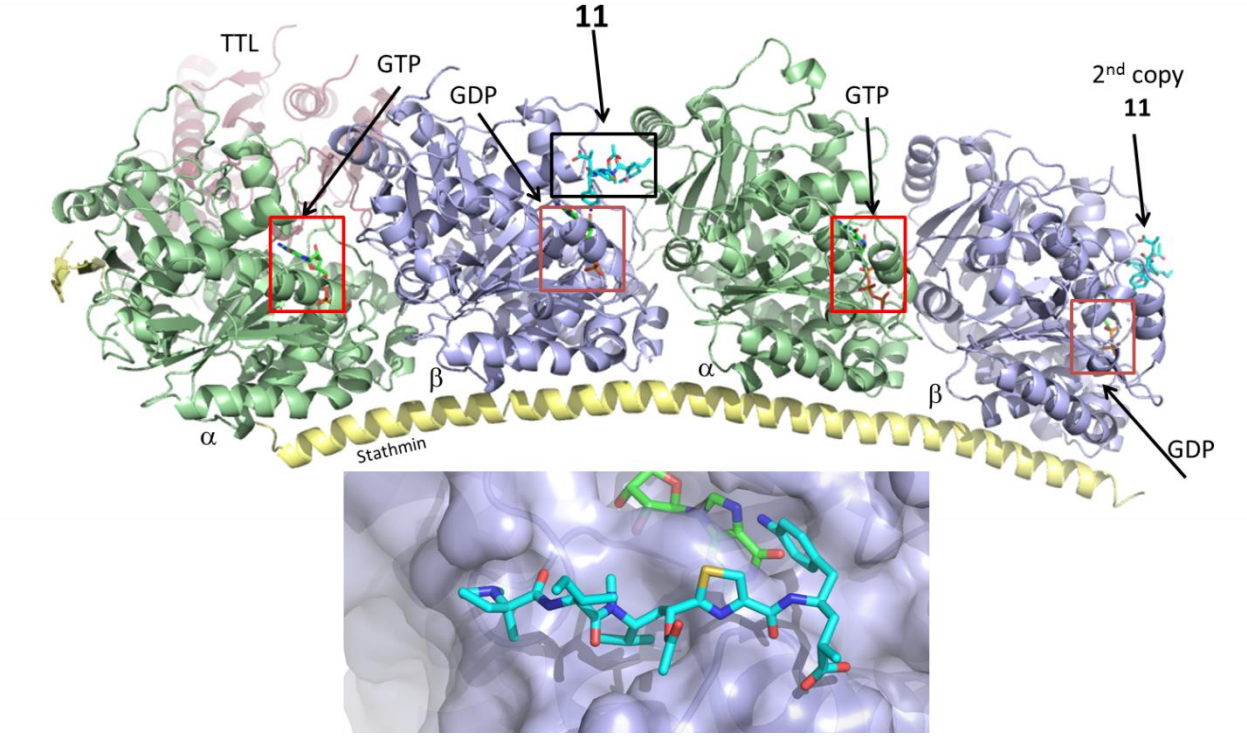
R2	R1	ID	N87[1]	MDA-MB-361-DYT2[1]	KB[1]	KB 8.5[1]
	**A**	**16a**	29.5 ± 15.1	22.0 ± 14.1	37.9 ± 0.9	104 ± 18
	B	**17a**	3.00 ± 2.20	0.95 ± 0.81	3.57 ± 1.34	13.5 ± 6.8
	**A**	**16b**	61.3 ± 37.3	33.2 ± 12.4	86.6 ± 22.3	198 ± 65
	B	**17b**	9.00 ± 3.03	6.45 ± 1.89	3.69 ± 1.15	17.5 ± 4.3
	**A**	**16c**	11.8 ± 0.7	9.05 ± 4.75	11.5 ± 0.9	46.3 ± 4.7
	B	**17c**	1.23 ± 0.37	0.83 ± 0.09	1.44 ± 0.01	15.6 ± 0.2
	**A**	**16d**	9.91 ± 1.71	6.09 ± 2.27	6.28 ± 1.17	28.4 ± 14.0
	B	**17d**	1.00 ± 0.86	0.27 ± 0.05	1.21 ± 0.08	13.4 ± 3.5
	**A**	**16e**	9.24 ±2.18	4.90 ± 1.59	5.32 ±0.79	93.7 ± 4.3
	B	**17e**	0.47 ± 0.13	0.27 ± 0.12	0.89 ± 0.25	31.7 ± 8.2
	**A**	**16f**[2]	29.1 ± 1.4	16.5 ± 6.3	18.0 ± 2.9	69.2 ±26.4
	**A**	**16g**[2]	11.1 ± 2.5	6.39 ± 3.24	8.64 ± 1.70	39.4 ± 4.0
	**B**	**17f**	0.49 ± 0.12	0.31 ± 0.00	1.02 ± 0.10	5.47 ± 0.25
	**B**	**17g**	6.75 ± 3.84	6.32 ± 2.24	3.71 ± 1.56	20.1 ± 5.3

1. Reported average IC50 (nM) +/- standard deviation for 2 - 13 independent determinations;

2. Tested as a 1:1 mixture of diastereomers.

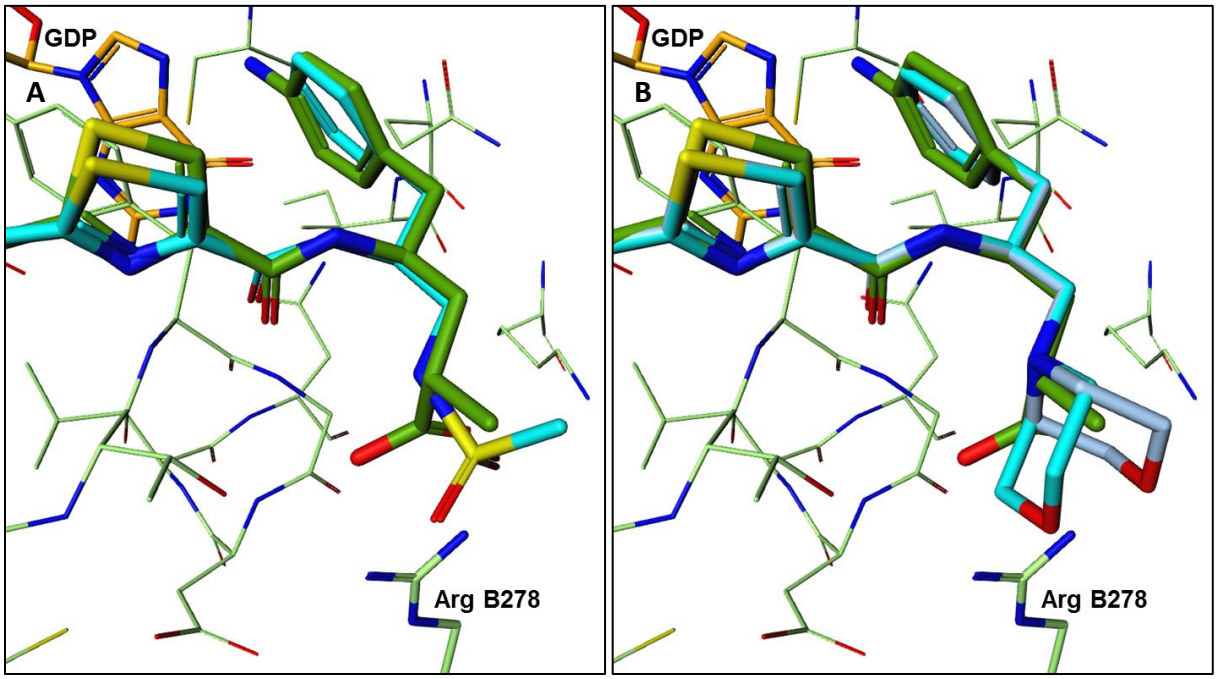
5. Overview of Crystal Structure of Tubulin-Bound Analog 11

Figure S1: Overview of the co-crystal structure of **11** with the T2R-TTL tubulin construct. **11** binds at the α,β-tubulin interface. A second molecule of **11** is also associated with the exterior β-subunit.



6. C-Terminal Shape-Based Overlay Studies of Tubulin-Bound Analog 11

Figure S2: Evaluation of C-terminal replacements using the co-crystal structure of **11** (rendered in green tubes) as the reference compound. Computational shape-based molecular overlays of **17e** and **17f** (rendered in cyan tubes in panels A and B, respectively) show that these replacements could interact with the side chain of residue Arg B278. Note that two possible overlays of **17f** are shown in B.



7. Data for Crystal Structure of Tubulin-Bound Analog 11

Table S4. Data Collection and Refinement Statistics

	11
Data Collection	
Space Group	$P2_12_12_1$
Unit Cell	a=104.79
	b=153.90
	c=185.20
	α=β=γ=90
Resolution (Å)	100 – 2.50 (2.50-2.54)[a]
I/σI	8.8 (0.5)
Completeness (%)	97.8 (78.4)
Redundancy	5.9 (2.6)
Refinement	
Resolution (Å)	38.5 – 2.5
Number of Reflections	101079
R_{work}[b] (%)	19.7
R_{free} (%)	25.1
R.m.s. Deviations from ideal geometry for	
Bonds (Å)	0.010
Angles (°)	1.16
Number of Atoms	
Protein	17421
Ligands/Ions	104
Solvent	121

[a]Values in parentheses are for the highest resolution shell.