Supporting Information

Synthesis of a Cyclic Analogue of Tuv N-Methyl Tubulysin

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General Information

All reactions were performed in flame-dried glassware fitted with a glass stopper under positive pressure of Ar with magnetic stirring, unless otherwise noted. Air- and moisture-sensitive liquids and solutions were transferred via syringe or stainless-steel cannula. TLC was performed on 0.25 mm E. Merck silica gel 60 F254 plates and visualized under UV light (254 nm) or by staining with cerium ammonium molybdenate (CAM), potassium permanganate (KMnO4) or p-anisaldehyde. Flash chromatography was performed on E. Merck 230–400 mesh silica gel 60. All reagents were purchased from commercial suppliers, and used without further purification unless otherwise noted. Solvents were distilled from proper drying agents (CaH2 or Na wire) under Ar atmosphere at 760 mm Hg. All moisture- and/or oxygen-sensitive solids were handled and stored in a glove box under N2. NMR spectra were recorded on Varian Unity 400 instruments at 24 °C. Chemical shifts are expressed in ppm relative to TMS (1H, 0 ppm) or solvent signals: CDCl3 (1H, 7.26 ppm; 13C, 77.2 ppm), CD3OD (1H, 3.31 ppm; 13C, 49.0 ppm) or DMSO-d6 (1H, 2.50 ppm; 13C, 39.5 ppm); coupling constants are expressed in Hz. High resolution mass spectra electrospray ionization (HRMS-ESI) was obtained on an Agilent technologies 6220 TOF LC/MS spectrometer or JEOL JMS-600W 70 eV (Electron Ionization). Chiral HPLC analysis for determination of enantiomeric excess (ee) was performed on a Shimadzu HPLC system (dual LC6AD solvent pumps, an SPD-20AV prominence UV/VIS detector, and SIL-20 prominence auto-sampler) equipped with a chiral Lux 5u Cellulose-1 column (5 μM, 1000 Å, 4.6 × 250 mm). Solvents were eluted at a flow rate of 1 mL/min at room temperature using a binary solvent system (solvent A: Hexane, solvent B: isopropanol, 20–50% B over 20 min, 50% B over 2 min, 50–20% B over 8 min) with UV detection at 254 nm.
Synthetic Procedure

**Synthesis of (R)-ethyl 2-(4-oxo-3,4-dihydro-2H-pyran-2-yl)thiazole-4-carboxylate (4).**

In a one-arm flask, Ti(OiPr)$_4$ (967 μL, 3.28 mmol) was added to a mixture of (R)-BINOL (859 mg, 3.00 mmol) and activated 4Å molecular sieves (7.20 g) in anhydrous toluene (45 mL). After stirring at 35 °C for 1 h, a solution of aldehyde 5 (2.78 g, 15.0 mmol) in anhydrous toluene (15 mL) was added, stirred for 10 min, and cooled to 0 °C. 1-Methoxy-3-trimethylsilyloxy butadiene (5.32 mL, 27.3 mmol) was added to the reaction mixture, stirred at 0 °C for 24 h, and treated with TFA (3.0 mL). After stirring at 0 °C for 15 min, saturated aqueous NaHCO$_3$ (80 mL) was added and the mixture was stirred at room temperature for 10 min. The reaction mixture was filtered through a plug of celite and washed with EtOAc (1 L). The organic layer was separated and the aqueous layer was extracted with EtOAc (3 × 120 mL). The combined organic extracts were dried over anhydrous MgSO$_4$, filtered, and concentrated by rotary evaporation. The residue was purified by column chromatography (1:1 hexane/EtOAc) to afford (R)-2H-pyran 4 (2.94 g, 77%, 95% ee) as a pale yellow solid. The enantiomeric excess was determined by chiral HPLC (Rt 16.5 min for (R)-4, and 20.5 min for (S)-4).

TLC: $R_f$ 0.4 (1:1 hexane/EtOAc). mp: 95–97 °C. [$\alpha$]$^\text{D}_{26.4}$ = –45.8 (c 1.0, CHCl$_3$). IR (KBr, film): 2982, 1725, 1680, 1213 cm$^{-1}$. $^1$H NMR (400 MHz, CDCl$_3$): δ 8.24 (s, 1H), 7.45 (d, 1H, $J$ = 6.4 Hz), 5.81 (dd, 1H, $J$ = 12.4, 4.4 Hz), 5.58 (dd, 1H, $J$ = 6.4, 1.2 Hz), 4.44 (q, 2H, $J$ = 7.2 Hz), 3.09 (ddd, 1H, $J$ = 16.8, 4.4, 1.2 Hz), 3.00 (dd, 1H, $J$ = 16.8, 12.4 Hz), 1.42 (t, 3H, $J$ = 7.2 Hz). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 189.8, 167.7, 161.6, 161.2, 147.8, 128.4, 108.8, 76.1, 61.9, 42.0, 14.5. HRMS (ESI) $m/z$ calculated for C$_{11}$H$_{11}$NO$_4$S 253.0409, found 253.0408.
Synthesis of (R)-ethyl 2-(4-oxotetrahydro-2H-pyran-2-yl)thiazole-4-carboxylate (6).

(R)-2H-Pyranone 4 (2.48 g, 9.79 mmol) and 5% Pd/C (992 mg) were suspended in EtOH (70 mL). The suspension was flushed with H₂ gas, and stirred under a hydrogen atmosphere at room temperature for 24 h. The mixture was filtered through a pad of celite and washed with EtOAc (600 mL). The filtrates were concentrated by rotary evaporation. The residue was purified by column chromatography (1:1 hexane/EtOAc) to afford (R)-4H-pyran 6 (2.21 g, 89%) as a white solid. TLC: Rf 0.43 (1:1 hexane/EtOAc). mp: 93.5–95.5 °C. [α]D²⁵ = +65.4 (c 1.0, CHCl₃). IR (KBr, film): 2980, 1724, 1238, 1209, 1096 cm⁻¹. ¹H NMR (400 MHz, CDCl₃): δ 8.20 (s, 1H), 5.00 (dd, 1H, J = 11.6, 3.2 Hz), 4.48 (ddd, 1H, J = 11.6, 7.6, 2.0 Hz), 4.43 (q, 2H, J = 7.2 Hz), 3.93 (td, 1H, J = 11.6, 3.2 Hz), 3.09 (dd, 1H, J = 14.8, 3.2 Hz), 2.74 (ddd, 1H, J = 15.2, 11.6, 7.6 Hz), 2.69 (dd, 1H, J = 14.8, 11.6 Hz), 2.47 (ddt, 1H, J = 15.2, 3.2, 2.0 Hz), 1.41 (t, 3H, J = 7.2 Hz). ¹³C NMR (100 MHz, CDCl₃): δ 204.0, 170.7, 161.4, 147.3, 128.0, 77.0, 67.0, 61.7, 47.9, 42.0, 14.5. HRMS (ESI) m/z calculated for C11H13NO4S 255.0565, found 255.0565.

Synthesis of ethyl 2-(4-(methylamino)tetrahydro-2H-pyran-2-yl)thiazole-4-carboxylate (3a and 3b).

A mixture of (R)-4H-pyranone 6 (2.11 g, 8.27 mmol), methylamine hydrochloride (2.23 g, 33.0 mol) and 4Å molecular sieves (2.50 g) in EtOH (17 mL) was stirred at room temperature for 2 h. Sodium cyanoborohydride (571 mg, 9.09 mmol) was subsequently added at 0 °C portionwise at 30 minute intervals over a period of 2 h. After stirring at 0 °C for 23 h, the reaction mixture was quenched with saturated aqueous NaHCO₃ (30 mL) and 2 M NaOH (7 mL). Then, 4Å molecular sieves were filtered off. The aqueous layer was extracted with CH₂Cl₂ (4 × 60 mL). The combined organic extracts were dried over anhydrous MgSO₄, filtered, and concentrated by rotary evaporation. The residue was purified by column
chromatography (15:1:0.03 CH₂Cl₂/MeOH/TEA) to afford amine 3a (1.16 g, 52%) and 3b (562 mg, 25%) as colorless sticky oil (combined yield 77%, cis:trans 68:32).

Ethyl 2-((2R,4S)-4-(methylamino)tetrahydro-2H-pyran-2-yl)thiazole-4-carboxylate (3a). TLC: Rf 0.3 (7:1 CH₂Cl₂/MeOH). [α]D²⁷.⁷ = +36.0 (c 0.62, CHCl₃). IR (KBr, film): 3312, 2939, 1729, 1208, 1100 cm⁻¹. ¹H NMR (400 MHz, CDCl₃): δ 8.13 (s, 1H), 4.74 (dd, 1H, J = 12.0, 2.4 Hz), 4.42 (q, 2H, J = 7.2 Hz), 4.22 (ddd, 1H, J = 12.0, 4.8, 1.6 Hz), 3.68 (td, 1H, J = 12.0, 2.0 Hz), 2.76 (m, 1H), 2.63 (dm, 1H, J = 12.0 Hz), 2.47 (s, 3H), 1.89 (dm, 1H, J = 12.8 Hz), 1.49 (qd, 1H, J = 12.8, 4.8 Hz), 1.46 (bri, 1H), 1.40 (t, 3H, J = 7.2 Hz), 1.30 (q, 1H, J = 12.0 Hz). ¹³C NMR (100 MHz, CDCl₃): δ 173.3, 161.7, 147.0, 127.5, 76.7, 67.6, 61.6, 55.8, 39.5, 33.2, 32.9, 14.6. HRMS (ESI) m/z calculated for C₁₂H₁₈N₂O₃S 270.1038, found 270.1039.

Ethyl 2-((2R,4R)-4-(methylamino)tetrahydro-2H-pyran-2-yl)thiazole-4-carboxylate (3b). TLC: Rf 0.43 (7:1 CH₂Cl₂/MeOH). [α]D²⁴.⁵ = +22.0 (c 1.15, CHCl₃). IR (KBr, film): 3334, 2956, 1729, 1208, 1090 cm⁻¹. ¹H NMR (400 MHz, CDCl₃): δ 8.13 (s, 1H), 5.21 (dd, 1H, J = 10.4, 3.2 Hz), 4.41 (q, 2H, J = 7.2 Hz), 4.08 (td, 1H, J = 11.6, 2.4 Hz), 3.87 (ddd, 1H, J = 11.6, 4.4, 3.6 Hz), 3.02 (m, 1H), 2.48 (s, 3H), 2.24 (dm, 1H, J = 13.6 Hz), 1.99–1.88 (m, 3H), 1.63 (dm, 1H, J = 13.6 Hz), 1.40 (t, 3H, J = 7.2 Hz). ¹³C NMR (100 MHz, CDCl₃): δ 174.6, 161.7, 147.0, 127.7, 72.5, 63.3, 61.6, 52.0, 36.6, 34.3, 30.2, 14.6. HRMS (ESI) m/z calculated for C₁₂H₁₈N₂O₃S 270.1038, found 270.1038.

Synthesis of (R)-1-methylpiperidine-2-carboxylic acid (8).

37% Aqueous solution of formaldehyde (1.26 mL, 16.9 mmol) was added to a mixture of (2R)-piperidine-2-carboxylic acid (7) (2.00 g, 15.5 mmol) and 5% Pd/C (500 mg) in MeOH (20 mL). After the reaction mixture was purged with H₂ gas, additional formaldehyde (0.60 mL, 8.06 mmol) was added, and stirred under H₂ atmosphere for 20 h. The reaction mixture was filtered through a pad of celite, and washed with MeOH (200 mL). The filtrate was
concentrated by rotary evaporation to afford analytically pure amino acid **8** (2.23 g, 100%) as a white solid, which was used in the next step without further purification. TLC: \( R_f 0.25 \) (1:1 CH\(_2\)Cl\(_2\)/MeOH). mp: 208–210 °C. \([\alpha]_{D}^{27.1} = +67.6 \) (c 1.27, MeOH). IR (KBr, film): 3407, 2950, 1615, 1399 cm\(^{-1}\). \(^1\)H NMR (400 MHz, CDCl\(_3\)): \( \delta \) 8.22 (brs, 1H), 3.64 (d, 1H, \( J = 12.0 \) Hz), 3.28 (m, 1H), 2.86 (s, 3H), 2.70 (m, 1H), 2.34 (d, 1H, \( J = 14.4 \) Hz), 2.00–1.74 (m, 4H), 1.46 (m, 1H). \(^1^3\)C NMR (100 MHz, DMSO-\( d_6 \)): \( \delta \) 170.4, 68.3, 53.3, 42.3, 28.0, 23.1, 21.8. HRMS (ESI) \( m/z \) calculated for C\(_7\)H\(_{13}\)NO\(_2\) 143.0946, found 143.0947.

### 4.6. Synthesis of (2S,3S)-methyl 2-amino-3-methylpentanoate (10).

Thionyl chloride (1.61 mL, 22.0 mmol) was added to a solution of L-isoleucine (9) (2.62 g, 20.1 mmol) in dry MeOH (40 mL) dropwise at 0 °C. The resulting reaction mixture was then heated at reflux for 98 h. Another portion of thionyl chloride (580 \( \mu \)L, 7.95 mmol) was added dropwise at 0 °C and the mixture was heated at reflux for an additional 86 h. The reaction mixture was treated with saturated aqueous NaHCO\(_3\) (100 mL). The organic layer was separated and the aqueous layer was extracted with CH\(_2\)Cl\(_2\) (3 × 100 mL). The combined organic extracts were dried over anhydrous MgSO\(_4\), filtered, and concentrated by rotary evaporation to afford analytically pure ester **10** (2.18 g, 75%) as a colorless oil, which was used in the next step without further purification. TLC: \( R_f 0.55 \) (7:1 CH\(_2\)Cl\(_2\)/MeOH). \([\alpha]_{D}^{24.8} = +43.6 \) (c = 1.70, MeOH). IR (KBr, film): 2970, 1738, 1033 cm\(^{-1}\). \(^1\)H NMR (400 MHz, CDCl\(_3\)): \( \delta \) 3.72 (s, 3H), 3.36 (d, 1H, \( J = 6.2 \) Hz), 1.75 (m, 1H), 1.51–1.40 (m, 3H), 1.21 (m, 1H), 0.94 (d, 3H, \( J = 6.8 \) Hz), 0.91 (t, 3H, \( J = 7.2 \) Hz). \(^1^3\)C NMR (100 MHz, CDCl\(_3\)): \( \delta \) 176.3, 59.3, 51.8, 39.4, 24.9, 15.9, 11.8. HRMS (ESI) \( m/z \) calculated for C\(_3\)H\(_{15}\)NO\(_2\) 145.1103, found 145.1103.
carboxamido)pentanoate (11).

DIPEA (1.93 mL, 11.0 mmol) was added dropwise to a solution of D-Mep (8) (1.58 g, 11.0 mmol), PyBOP (5.74 g, 11.0 mmol), and HOBT•H₂O (1.69 g, 11.0 mmol) in anhydrous CH₂Cl₂ (60 mL). After stirring at 0 °C for 20 min, a solution of L-Ile methyl ester 10 (1.34 g, 9.23 mmol) in CH₂Cl₂ (22 mL) and DIPEA (1.93 mL, 11.0 mmol) were added sequentially. The reaction mixture was then stirred at 0 °C for 20 min and at room temperature for 5 h. After completion of the reaction, the reaction mixture was treated with saturated aqueous NaHCO₃ (100 mL). The organic layer was separated, and the aqueous layer was extracted with CH₂Cl₂ (4 × 100 mL). The combined organic extracts were dried over anhydrous MgSO₄, filtered, and concentrated by rotary evaporation. The crude residue was purified by column chromatography (15:1:0.03 CH₂Cl₂/MeOH/Et₃N) to afford dipeptide ester 11 (2.20 g, 88%) as colorless oil. TLC: Rf 0.55 (7:1 CH₂Cl₂/MeOH). [α]D²⁶.⁷ = +97.2 (c = 0.93, CHCl₃).

IR (KBr, film): 3388, 2938, 1742, 1681, 1509 cm⁻¹. ¹H NMR (400 MHz, CDCl₃): δ 7.07 (brs, 1H), 4.58 (dd, 1H, J = 9.2, 4.8 Hz), 3.73 (s, 3H), 2.93 (dm, 1H, J = 11.2 Hz), 2.52 (dm, 1H, J = 10.4 Hz), 2.25 (s, 3H), 2.05 (m, 1H), 1.99–1.90 (m, 2H), 1.74 (dm, 1H, J = 12.8 Hz), 1.65 (m, 1H), 1.56 (dm, 1H, J = 12.8 Hz), 1.51–1.40 (m, 2H), 1.28–1.16 (m, 2H), 0.95 (d, 3H, J = 7.2 Hz), 0.93 (t, 3H, J = 7.2 Hz). ¹³C NMR (100 MHz, CDCl₃): δ 174.6, 172.6, 69.8, 56.1, 55.6, 52.2, 45.1, 37.8, 30.7, 25.4, 25.3, 23.4, 16.0, 11.7. HRMS (ESI) m/z calculated for C₁₄H₂₆N₂O₃ 270.1943, found 270.1939.

Synthesis of (2S,3S)-3-methyl-2-((R)-1-methylpiperidine-2-carboxamido)pentanoic acid (12).

To a solution of dipeptide ester 11 (1.15 g, 4.25 mmol) in THF (15 mL) was added 1 M NaOH (6.20 mL, 6.20 mmol) at room temperature. After stirring at room temperature for 3 h, 1 M HCl was added until pH 1–2 and the aqueous layer was washed with EtOAc (30 mL) to
remove organic impurity. The aqueous layer was concentrated by rotary evaporation and the residue was purified through ion-exchange resin (H₂O → 99:1 H₂O/NH₄OH → 98:2 H₂O/NH₄OH → 97:3 H₂O/NH₄OH). The collected fractions were lyophilized to afford dipeptide 12 (998 mg, 92%) as a white solid. TLC: Rᵣ 0.18 (1:1 CH₂Cl₂/Methanol). mp: 72.5–74.5 °C. \[\alpha\]D²⁶⁺⁴ = +23.9 (c = 0.98, Methanol). IR (KBr, film): 3407, 2960, 1673, 1591 cm⁻¹. \(^1\)H NMR (400 MHz, DMSO-d₆): \(\delta\) 7.56 (d, 1H, \(J = 8.0\) Hz), 4.15 (dd, 1H, \(J = 8.0, 5.6\) Hz), 2.87 (d, 1H, \(J = 11.6\) Hz), 2.52 (m, 1H), 2.14 (s, 3H), 1.99 (tm, 1H, \(J = 11.6\) Hz), 1.83 (m, 1H), 1.66 (m, 2H), 1.57 (m, 1H), 1.52–1.35 (m, 3H), 1.24–1.09 (m, 2H), 0.85 (d, 3H, \(J = 6.8\) Hz), 0.84 (t, 3H, \(J = 7.2\) Hz). \(^1\)³C NMR (100 MHz, DMSO-d₆): \(\delta\) 172.8, 172.4, 68.4, 56.0, 54.8, 43.8, 36.4, 29.7, 24.8, 24.7, 22.8, 15.7, 11.2. HRMS (ESI) m/z calculated for C₁₃H₂₄N₂O₃ 256.1787, found 256.1790.

**Synthesis of ethyl 2-((2R,4S)-4-((2S,3S)-N,3-dimethyl-2-((R)-1-methylpiperidine-2-carboxamido)pentanamido)tetrahydro-2H-pyran-2-yl)thiazole-4-carboxylate (2).**

DIPEA (41.8 µL, 240 µmol) was added to a solution of dipeptide 12 (30.8 mg, 120 µmol), PyBOP (62.4 mg, 120 µmol), and HOBT·H₂O (18.4 mg, 120 µmol) in anhydrous CH₂Cl₂ (1.5 mL) at 0 °C. After stirring at 0 °C for 20 min, a solution of amine 3a (27.0 mg, 99.9 µmol) in anhydrous CH₂Cl₂ (0.5 mL) was added and the mixture was stirred at room temperature for 1 h 30 min. The reaction mixture was treated with saturated aqueous NaHCO₃ (6 mL) until pH 9. The organic layer was separated, and the aqueous layer was extracted with CH₂Cl₂ (3 × 10 mL). The combined organic extracts were dried over anhydrous MgSO₄, filtered, and concentrated by rotary evaporation. The crude residue was purified by column chromatography (15:1:0.03 CH₂Cl₂/Methanol/Et₃N) to afford tripeptide 2 (18.5 mg, 37%) as a white solid. TLC: Rᵣ 0.55 (7:1 CH₂Cl₂/Methanol). mp: 49–51 °C. \[\alpha\]D²⁶⁺⁵ = +18.6 (c 0.90, CHCl₃). IR (KBr, film): 3375, 2937, 1638, 1205 cm⁻¹. \(^1\)H and \(^1\)³C NMR confirmed a 5.8:1
mixture of rotamers. Major rotamer: $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 8.15 (s, 1H), 7.13 (d, 1H, $J = 9.2$ Hz), 4.91 (tm, 1H, $J = 12.0$ Hz), 4.81 (dd, 1H, $J = 10.8$, 2.4 Hz), 4.76 (dd, 1H, $J = 9.2$, 6.8 Hz), 4.41 (q, 2H, $J = 4.4$ Hz), 4.25 (dd, 1H, $J = 12.0$, 3.6 Hz), 3.78 (td, 1H, $J = 12.0$, 2.4 Hz), 3.00 (s, 3H), 2.91 (dm, 1H, $J = 12.0$ Hz), 2.49 (dd, 1H, $J = 11.2$, 3.2 Hz), 2.34 (dm, 1H, $J = 12.4$ Hz), 2.24 (s, 3H), 2.20 (m, 1H), 2.02 (t, 1H, $J = 11.2$, 3.6 Hz), 1.88–1.69 (m, 5H), 1.64–1.53 (m, 3H), 1.43 (m, 1H), 1.40 (t, 3H, $J = 7.2$ Hz), 1.26–1.11 (m, 2H), 0.95 (d, 3H, $J = 6.8$ Hz), 0.91 (t, 3H, $J = 7.2$ Hz). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 174.5, 172.5, 171.9, 161.4, 146.9, 127.4, 76.7, 69.7, 67.5, 61.5, 55.4, 53.0, 49.4, 44.9, 37.6, 36.0, 30.6, 29.9, 28.5, 25.1, 24.5, 23.3, 15.8, 14.4, 11.3. Selected signals of the minor rotamer: $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 8.14 (s, 1H), 4.41 (q, 2H, $J = 7.2$ Hz), 4.28 (dd, 1H, $J = 12.0$, 3.6 Hz), 2.84 (s, 3H), 2.51 (dd, 1H, $J = 11.2$, 3.2 Hz), 1.39 (t, 3H, $J = 7.2$ Hz). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 171.7, 171.5, 147.0, 127.5, 76.3, 69.6, 67.2, 61.4, 52.9, 52.6, 45.0, 37.8, 36.5, 30.8, 30.3, 27.9, 24.6, 16.2, 14.3. HRMS (ESI) $m/z$ calculated for C$_{25}$H$_{40}$N$_{4}$O$_{5}$S 508.2719, found 508.2726.


To a solution of tripeptide ester 2 (712 mg, 1.40 mmol) in dioxane (14 mL) was added 1 M NaOH (2.80 mL, 2.80 mmol) dropwise. After the reaction mixture was stirred at 4–8 °C for 7 h, the solvent was evaporated under reduced pressure. The residue was dissolved in water (3 mL), acidified to pH 2 with 1 M HCl, and concentrated by rotary evaporation. The residue was purified by column chromatography (10:1 CH$_2$Cl$_2$ / MeOH $\rightarrow$ 5:1:0.24 CH$_2$Cl$_2$ / MeOH / NH$_4$OH) to afford tripeptide 13 (674 mg, 100%) as a white solid. TLC: $R_f$ 0.35 (7:1 CH$_2$Cl$_2$ / MeOH). $^1$H and $^{13}$C NMR confirmed a 1.3:1 mixture of rotamers. $^1$H NMR (400 MHz, CD$_3$OD): (major and minor) $\delta$ 7.99 (s, 1.3H), 7.96 (s, 1H), 5.21 (d, 1H, $J = 10.0$ Hz), 4.97–4.90 (m, 2.3H), 4.77 (m, 1H), 4.67 (d, 1.3H, $J = 8.4$ Hz), 4.57 (m, 1.3H), 4.25 (dd,
1H, J = 11.6, 3.6 Hz), 4.18 (dd, 1.3H, J = 11.6, 3.6 Hz), 3.94 (t, 1H, J = 11.6 Hz), 3.75 (t, 1.3H, J = 11.6 Hz), 3.08 (s, 3.9H), 3.01 (d, 1.3H, J = 11.2 Hz), 2.86 (s, 3H), 2.77 (d, 1H, J = 11.2 Hz), 2.45 (d, 1H, J = 12.4 Hz), 2.27 (s, 6.9H), 2.35–2.07 (m, 4.9H), 2.04–1.68 (m, 15.8H), 1.64–1.50 (m, 8.2H), 1.40–1.29 (m, 2.3H), 1.26–1.15 (m, 2.3H), 0.97–0.89 (m, 13.8H). \(^{13}\)C NMR (125 MHz, CD\(_3\)OD): (major and minor) \(\delta\) 174.8 (2C), 173.9 (2C), 173.7 (2C), 168.7 (2C), 154.6 (2C), 124.6, 124.1, 77.6, 77.3, 70.1, 68.4, 68.2, 56.7, 56.6, 55.3, 55.1, 54.9, 52.3, 44.5, 44.4 (2C, identified from HSQC), 38.7, 38.5, 38.2, 37.3, 31.3, 31.2, 31.0, 29.6, 28.2, 25.9, 25.8 (2C, identified from HSQC), 25.7, 24.0 (2C, identified from HSQC), 23.9, 15.9, 15.8, 11.7, 11.3. HRMS (ESI) \(m/z\) calculated for C\(_{23}\)H\(_{36}\)N\(_4\)O\(_3\)S 480.2406, found 480.2410.

**Synthesis of (2S,4R)-methyl 4-((2R,4S)-4-((2S,3S)-N,3-dimethyl-2-((R)-1-methylpiperidine-2-carboxamido)pentanamido)tetrahydro-2H-pyran-2-yl)thiazole-4-carboxamido)-2-methyl-5-phenylpentanoate (15).**

Tripeptide 13 (116 mg, 241 \(\mu\)mol) and tubuphenylalanine 14 (93.1 mg, 361 \(\mu\)mol) were dissolved in anhydrous THF (0.73 mL). The reaction mixture was treated sequentially with Et\(_3\)N (118 \(\mu\)L, 844 \(\mu\)mol) and DEPB (144 mg, 482 \(\mu\)mol) at 0 °C. After stirring at 0 °C for 1 h and at room temperature for 4 h, the reaction mixture was quenched with H\(_2\)O (7 mL), saturated aqueous NaHCO\(_3\) (6 mL) and 2 M NaOH (0.3 mL) until pH 9. The organic layer was separated and the aqueous layer was extracted with CH\(_2\)Cl\(_2\) (4 \(\times\) 20 mL). The combined organic extracts were dried over anhydrous MgSO\(_4\), filtered, and concentrated by rotary evaporation. The residue was purified by column chromatography (50:1:0.1 CH\(_2\)Cl\(_2\)/MeOH/Et\(_3\)N) to afford tetrapeptide methyl ester 15 (126 mg, 76%) as a white solid. TLC: \(R_f\) 0.5 (10:1 CH\(_2\)Cl\(_2\)/MeOH), mp: 48–50 °C. \([\alpha]_{D}^{25.8} = +15.4\) (c 0.9, CHCl\(_3\)). IR (KBr, film): 3387, 3296, 2936, 1639, 1541 cm\(^{-1}\). \(^1\)H and \(^{13}\)C NMR confirmed a mixture of rotamers.
Major rotamer: \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 8.03 (s, 1H), 7.27–7.17 (m, 5H), 7.13–7.08 (m, 2H), 4.92 (tm, 1H, \(J = 12.0 \text{ Hz}\)), 4.74 (m, 1H), 4.72 (dd, 1H, \(J = 11.2, 2.4 \text{ Hz}\)), 4.39 (m, 1H), 4.24 (dd, 1H, \(J = 12.0, 4.0 \text{ Hz}\)), 3.76 (td, 1H, \(J = 12.0, 1.2 \text{ Hz}\)), 3.62 (s, 3H), 3.05 (s, 3H), 2.96–2.84 (m, 3H), 2.60 (m, 1H), 2.50 (m, 1H), 2.34 (brs, 3H), 2.19 (dm, 1H, \(J = 11.2 \text{ Hz}\), 2.05–1.98 (m, 3H), 1.95–1.82 (m, 3H), 1.79–1.70 (m, 2H), 1.65–1.53 (m, 5H), 1.33–1.22 (m, 2H), 1.16 (d, 3H, \(J = 7.2 \text{ Hz}\)), 1.08 (d, 3H, \(J = 6.8 \text{ Hz}\)), 0.93 (t, 3H, \(J = 7.2 \text{ Hz}\)). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \(\delta\) 176.7, 172.2, 171.4, 170.8, 160.8, 150.0, 137.8, 129.7, 128.5, 126.7, 123.3, 76.6, 70.0, 67.7, 55.6, 53.3, 51.9, 49.8, 48.6, 45.0, 41.5, 38.0, 37.6, 36.6, 36.1, 30.4, 30.2, 28.8, 28.1, 24.9, 23.4, 18.0, 16.0, 11.4. Selected signals of the minor rotamer: \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 8.03 (s, 1H), 2.88 (s, 3H). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \(\delta\) 137.9, 129.6, 128.6, 123.5, 76.4, 67.4, 51.8, 48.7, 41.7, 38.2, 24.7, 18.1, 16.5, 11.6. HRMS (ESI) \(m/z\) calculated for C\(_{36}\)H\(_{53}\)N\(_5\)O\(_6\)S 683.3717, found 683.3723.

**Synthesis of (2S,4R)-4-(2-(2R,4S)-4-(2S,3S)-N,3-dimethyl-2-((R)-1-methylpiperidine-2-carboxamido)pentanamido)tetrahydro-2H-pyran-2-yl)thiazole-4-carboxamido)-2-methyl-5-phenylpentanoic acid (1).**

To a solution of tetrapeptide methyl ester 15 (86.2 mg, 126 \(\mu\)mol) in dioxane (1.2 mL) was added 1 M NaOH (252 \(\mu\)L, 252 \(\mu\)mol) dropwise at 0 \(^\circ\)C. The reaction mixture was stirred at 4–8 \(^\circ\)C for 29 h. After evaporating the volatiles under reduced pressure, the residue was dissolved in water (0.5 mL), acidified to pH 2 with 1 M HCl, and then concentrated by rotary evaporation. The residue was purified by column chromatography (10:1 \(\text{CH}_2\text{Cl}_2/\text{MeOH} \rightarrow \text{5:1: CH}_2\text{Cl}_2/\text{MeOH}\)) to afford conformationally rigid Tuv \(N\)-methyl tubulysin analog 1 (65.4 mg, 78%) as a white solid. TLC: \(R_f\) 0.43 (7:1 \(\text{CH}_2\text{Cl}_2/\text{MeOH}\)). mp: 104–106 \(^\circ\)C. \([\alpha]_D^{26.7} = -25.6\) (c 0.75, MeOH). IR (KBr, film): 3388, 2961, 1640, 1548 \text{ cm}^{-1}. \(^1\)H and \(^{13}\)C NMR conformed a 1.4:1 mixture of rotamers. \(^1\)H NMR (400 MHz, CD\(_3\)OD): (major and minor) \(\delta\)
8.06 (s, 1H), 8.04 (s, 1.4H), 7.22–7.18 (m, 9.6H), 7.16–7.12 (m, 2.4H), 4.88–4.78 (m, 3.4H),
4.82 (dd, 1.4H, J = 11.2, 2.4 Hz), 4.70 (d, 1.4H, J = 8.4 Hz), 4.62 (tm, 1H, J = 12.0 Hz),
4.36–4.31 (m, 2.4H), 4.28–4.20 (m, 2.4H), 3.87 (td, 1H, J = 12.0, 1.6 Hz), 3.78 (td, 1.4H, J =
12.0, 1.6 Hz), 3.12 (s, 4.2H), 3.10–2.86 (m, 4.4H), 2.89 (d, 1.4H, J = 6.8 Hz), 2.87 (s, 3H),
2.79 (dd, 1.4H, J = 10.8, 2.8 Hz), 2.52–2.47 (m, 2.4H), 2.41 (d, 1H, J = 12.8 Hz), 2.34–2.22
(m, 3.8H), 2.29 (s, 7.2H), 2.11 (m, 1H), 2.01–1.78 (m, 15.8H), 1.72–1.66 (m, 4.8H), 1.65–
1.57 (m, 9.6H), 1.41–1.34 (m, 2.4H), 1.25–1.18 (m, 2.4H), 1.14 (d, 7.2H, J = 6.8 Hz), 0.98 (d,
7.2H, J = 6.8 Hz), 0.94 (t, 3H, J = 7.6 Hz), 0.93 (t, 4.2H, J = 7.6 Hz). ^13^C NMR (100 MHz,
CD$_3$OD): (major and minor) $\delta$ 182.2, 182.0, 174.1, 173.8, 173.7, 173.6, 173.4, 173.1, 163.1,
163.0, 150.7 (2C), 139.7, 139.6, 130.6, 130.5, 129.3, 129.2, 127.4, 127.3, 124.8, 124.7, 77.7,
77.4, 69.8, 69.6, 68.5, 68.2, 56.6, 56.5, 55.2, 54.8, 54.7, 52.0, 51.1, 50.9, 44.3, 44.2, 42.1,
41.9, 39.6, 39.4, 39.2, 39.1, 38.3, 38.2, 37.6, 36.8, 31.2, 31.1, 31.0, 30.9, 29.7, 28.5, 25.8 (2C,
identified from HSQC), 25.7, 25.5, 23.8, 23.7, 18.9, 18.8, 16.2, 15.9, 11.4, 11.3. HRMS (ESI)
$m/z$ calculated for C$_{35}$H$_{51}$N$_5$O$_6$S 669.3560, found 669.3569.
$^1$H-NMR & $^{13}$C-NMR of 4
$^1$H-NMR & $^{13}$C-NMR of 6
$^{1}$H-NMR & $^{13}$C-NMR of 3a
$^{1}H$-NMR & $^{13}C$-NMR of 3b
$^1$H-NMR & $^{13}$C-NMR of 8
1H-NMR & 13C-NMR of 10

Pulse Sequence: standard 1D

![NMR Spectra](image)
$^1$H-NMR & $^{13}$C-NMR of 11
$^1$H-NMR & $^{13}$C-NMR of 12
$^1$H-NMR & $^{13}$C-NMR of 2
$^1$H-NMR & $^{13}$C-NMR of 13
$^1$H-NMR & $^{13}$C-NMR of 15
$^1$H-NMR & $^{13}$C-NMR of 1
HPLC Analytical Chromatogram of 4.

### Peak Table

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1D-NOE Spectra and the identification of the relative stereochemistry of 3a
X-ray crystallographic data of 6.

Crystallographic data for 6 have been deposited to the Cambridge Crystallographic Data Centre. The deposition number is CCDC 1041766. Copies of the data are available free of charge via the Internet at http://www.ccdc.cam.ac.uk. CCDC 12 Union Road Cambridge CB2 1EZ UK (Tel: +44 (0)1223-762911). The thermal ellipsoids are scaled to enclose 50% probability.
Cytotoxicity Assays

Cytotoxicity assays were performed at ReactionBiology Corporation (http://www.reactionbiology.com). Compound 1 and reference compound staurosporine were all dissolved in DMSO in 10 mM stock. Staurosporine was obtained from Sigma-Aldrich (Saint Louis, MI). Cell Titer-Glo® 2.0 Luminescent cell viability assay reagent was obtained from Promega (Madison, WI). CMK human megakaryoblastic leukemia cell line, K562 human chronic myelogenous leukemia cell line, and SW480 human colorectal adenocarcinoma cell line were all obtained from American Type Culture Collection (Manassas, VA). CMK cells were grown in RPMI-1640 medium supplemented with 20% FBS, K562 cells were grown in Iscove's Modified Dulbecco's Medium supplemented with 10% FBS, SW480 cells were grown in Leibovitz's L-15 Medium supplemented with 10% FBS. 100 μg/mL penicillin and 100 μg/mL streptomycin were added to all culture media. Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. Compound 1 and reference compound staurosporine in DMSO solution were added in a source plate. 62.5 nL of compound 1, or 25 nL of staurosporine was delivered from the source plate to each well of the 384-well cell culture plates by Echo 550. 25 μL of culture medium containing 5000 of CMK, K562, or SW480 cells was added to the wells of the cell culture plates. The cells were incubated with the compounds at 37°C, 5% CO₂ for 96 hours. 25 μL of Cell Titer Glo 2.0 reagent was added to each well. The contents were mixed on an orbital shaker for 2 min and incubated at room temperature for 10 min to stabilize luminescent signal. Luminescence was recorded by Envision 2104 Multilabel Reader (PerkinElmer, Santa Clara, CA). The number of viable cells in culture was determined based on quantitation of the ATP present in each culture well. The cell viability data were plotted as histogram graph using the GraphPad Prism 4 program.