were visualized with an aqueous solution of 2,4-dinitrophenylhydrazine in 30% H_2SO_4. The ^1H NMR spectra were recorded on a Bruker 300-MHz or 500-MHz multiple-probe instrument. Infrared spectra were recorded on a Nicolet Dx FTIR DX V5.07 spectrometer or a Perkin Elmer 1600 Series FT-IR spectrometer. Low resolution mass spectral data (EI/CI) were obtained on a Hewlett-Packard 5985B gas chromatography-mass spectrometer. High resolution mass spectral data were taken on a VG autospectrometer (Double Focusing High Resolution GC/Mass Spectrometer, UK). Optical rotations were measured on a JASCO DIP-370 polarimeter. Microanalyses were performed on a CE Elantech EA1110 elemental analyzer.

5.1. (3*S*,6*R*)-3-[(1-*tert*-Butyloxycarbonyl-6-methoxy)-3-indoyl]methyl-3,6-dihydro-6-isopropyl-2,5-diethoxypyrazine (12)

To a solution of **10** (3.41 g, 16.1 mmol) in dry THF (60 mL) under nitrogen, *n*-BuLi (2.5 M, 7.08 mL, 17.7 mmol) was added dropwise at −78 °C. The solution which resulted was stirred at −78 °C for 30 min and treated slowly with a solution of crude 3-bromo-methylindole **9a** (4.79 g, 14.1 mmol) in THF (30 mL). The mixture which resulted was stirred at −78 °C for 20 h, and then allowed to slowly warm to rt. The solution was concentrated under reduced pressure and diluted with a saturated aqueous solution of $NaHCO_3$. The aqueous layer was extracted with CH_2Cl_2 (3 × 20 mL). The combined organic layers were washed with brine (30 mL) and dried (K_2CO_3). After removal of solvent under reduced pressure, the residue was purified by flash chromatography (silica gel, hexane/EtOAc, 10:1) to afford **12** as an oil (6.04 g, 91%): $[\alpha]_D^{27}$ +24.7° (*c* 0.9, $CHCl_3$); IR ν_{max} (NaCl) 2970, 1730, 1690 cm^{-1}; ^1H NMR (250 MHz, $CDCl_3$) δ 0.63 (d, 3H, J = 6.8 Hz), 0.92 (d, 3H, J = 6.9 Hz), 1.21 (t, 3H, J = 7.1 Hz), 1.29 (t, 3H, J = 7.1 Hz), 1.62 (s, 9H), 2.15 (m, 1H), 3.13 (d, 2H, J = 4.8 Hz), 3.53 (t, 1H, J = 3.4 Hz), 3.84 (s, 3H), 3.94–4.16 (m, 4H), 4.25 (dd, 1H, J = 3.8 Hz), 6.80 (dd, 1H, J = 2.2 and 8.6 Hz), 7.21 (s, 1H), 7.42 (d, 1H, J = 8.6 Hz), 7.67 (br, s, 1H). ^{13}C NMR (62.90 MHz, $CDCl_3$) δ 14.43, 16.68, 19.04, 28.23, 29.37, 31.72, 55.58, 56.13, 60.42, 60.51, 60.67, 82.93, 99.03, 111.49, 116.74, 120.10, 122.83, 125.28, 136.10, 149.81, 157.67, 162.29, 163.49. EIMS *m/e* (relative intensity) 471 (M^+, 47), 261 (21), 212 (100), 169 (67), 141 (20), 57 (51). Anal. Calcd for ($C_{26}H_{37}N_3O_5$) C, H, N. This material was used directly in a later step.

5.2. (3*S*,6*R*)-3-(1-*tert*-Butyloxycarbonyl-3-indoyl)methyl-3,6-dihydro-6-isopropyl-2,5-diethoxypyrazine (13)

Indole **13** (6.7 g) was prepared in 92% yield from **9b** (5.1 g, 16.4 mmol) and **10** (3.7 g, 17.6 mmol) analogous to the procedure described for the synthesis of **12**. **13**: ^1H NMR (300 MHz, $CDCl_3$) δ 0.66 (d, 3H, J = 6.8, Hz), 0.95 (d, 3H, J = 6.9 Hz), 1.23 (t, 3H, J = 7.1 Hz), 1.32 (t, 3H, J = 7.1 Hz), 1.64 (s, 9H), 2.18 (m,1H), 3.19 (m, 2H), 3.56 (t, 1H, J = 3.4 Hz), 3.96–4.21 (m, 4H), 4.29 (dd, 1H, J = 4.3 Hz), 7.15–7.29 (m, 2H), 7.36 (s, 1H), 7.59 (d, 1H, J = 7.5 Hz), 8.08 (d, 1H, J = 7.6 Hz). ^{13}C NMR (62.90 MHz, $CDCl_3$) δ 14.40, 16.66, 19.01, 28.21, 29.26, 31.60, 31.73, 56.05, 60.56, 60.64, 83.11, 114.89, 116.73, 119.60, 121.97, 123.96, 124.19, 131.42, 135.13, 149.72, 162.25, 163.50. CIMS *m/e* (relative intensity) 442 (M^+, +1). HRMS Calcd for $C_{25}H_{35}N_3O_4$ *m/z* = 441.2628, found *m/z* = 441.2536. This material was used directly in a later step.

5.3. (3*R*,6*S*)-3-[(1-*tert*-Butyloxycarbonyl-6-methoxy)-3-indoyl]methyl-3,6-dihydro-6-isopropyl-2,5-diethoxypyrazine (14)

Indole **14** (5.5 g) was prepared in 92% yield from **9a** (4.4 g, 12.7 mmol) and **11** (3.06 g,14.5 mmol), analogous to the procedure described for the synthesis of **12**. **14**: $[\alpha]_D^{27}$ −25.1° (*c* 0.8, $CHCl_3$); IR ν_{max} (NaCl) 2970, 1730, 1690 cm^{-1}; ^1H NMR (250 MHz, $CDCl_3$) δ 0.63 (d, 3H, J = 6.8, Hz), 0.92 (d, 3H, J = 6.9 Hz), 1.21 (t, 3H, J = 7.1 Hz), 1.29 (t, 3H, J = 7.1 Hz), 1.62 (s, 9H), 2.15 (m, 1H), 3.13 (d, 2H, J = 4.8 Hz), 3.53 (t, 1H, J = 3.4 Hz), 3.83 (s, 3H), 3.94–4.16 (m, 4H), 4.25 (dd, 1H, J = 3.8 Hz), 6.80 (dd, 1H, J = 2.2 and 8.6 Hz), 7.21 (s, 1H), 7.42 (d, 1H, J = 8.6 Hz), 7.67 (br, s, 1H). ^{13}C NMR (62.90 MHz, $CDCl_3$) δ 14.43, 16.68, 19.04, 28.23, 29.37, 31.72, 55.58, 56.13, 60.42, 60.51, 60.67, 82.93, 99.03, 111.49, 116.74, 120.10, 122.83, 125.28, 136.10, 149.81, 157.67, 162.29, 163.49. CIMS *m/e* (relative intensity) 472 (M^+, +1). HRMS Calcd for $C_{26}H_{37}N_3O_5$ *m/z* = 471.2733, found *m/z* = 471.2739. This material was used directly in a later step.

5.4. (3*R*,6*S*)-3-(1-*tert*-Butyloxycarbonyl-3-indoyl)methyl-3,6-dihydro-6-isopropyl-2,5-diethoxypyrazine (15)

Indole **15** (6.6 g) was prepared in 91% yield from **9b** and **11**, analogous to the procedure described for the synthesis of **12**. **15**: ^1H NMR (300 MHz, $CDCl_3$) δ 0.66 (d, 3H, J = 6.8, Hz), 0.95 (d, 3H, J = 6.9 Hz), 1.23 (t, 3H, J = 7.1 Hz), 1.32 (t, 3H, J = 7.1 Hz), 1.64 (s, 9H), 2.18 (m, 1H), 3.19 (m, 2H), 3.56 (t, 1H, J = 3.4 Hz), 3.96–4.21 (m, 4H), 4.29 (dd, 1H, J = 4.3 Hz), 7.15–7.29 (m, 2H), 7.36 (s, 1H), 7.59 (d, 1H, J = 7.5 Hz), 8.08 (d, 1H, J = 7.6 Hz). ^{13}C NMR (62.90 MHz, $CDCl_3$) δ 14.40, 16.66, 19.01, 28.21, 29.26, 31.60, 31.73, 56.05, 60.56, 60.64, 83.11, 114.89, 116.73, 119.60, 121.97, 123.96, 124.19, 131.42, 135.13, 149.72, 162.25, 163.50. CIMS *m/e* (relative intensity) 442 (M^+, +1). HRMS Calcd for $C_{25}H_{35}N_3O_4$ *m/z* = 441.2628, found *m/z* = 441.2634. This material was used directly in a later step.

5.5. (3*S*,6*R*)-3-[(1-*tert*-Butyloxycarbonyl-2-isoprenyl-6-methoxy)-3-indoyl]-methyl-3,6-dihydro-6-isopropyl-2,5-diethoxypyrazine (16)

To a solution of pyrazine **12** (1.94 g, 4.11 mmol) in dry THF (30 mL) at −78 °C under nitrogen, a solution of lithium diisopropylamide (LDA, 1.5 M in THF, 4.2 mL, 6.17 mmol) was added dropwise. The mixture which resulted was stirred at −78 °C for 60 min. The dry (HBr free) 4-bromo-2-methylbutene (1.03 g, 6.91 mmol) was then added dropwise at −78 °C. The mixture was stirred at −78 °C for 1 h and allowed to

warm to rt overnight. The solvent was removed under reduced pressure. The residue was taken up in CH_2Cl_2 and washed with a 5% aqueous solution of $NaHCO_3$. The aqueous layer was extracted with CH_2Cl_2 (3×50 mL). The combined organic layers were dried (K_2CO_3). After removal of the solvent under reduced pressure, the residue was separated by flash chromatography (silica gel, hexane/EtOAc, 15:1) to provide **16** (1.89 g, 85%) as an oil: IR ν_{max} (NaCl) 2970, 1730, 1690 cm^{-1}, ^1H NMR (250 MHz, $CDCl_3$) δ 0.61 (d, 3H, $J = 6.8$ Hz), 0.94 (d, 3H, $J = 6.8$ Hz), 1.18 (t, 3H, $J = 7.1$ Hz), 1.31 (t, 3H, $J = 7.1$ Hz), 1.61 (s, 3H), 1.63 (s, 9H), 1.70 (s, 3H), 2.19 (m, 1H), 2.88 (dd, 1H, $J = 7.4$ and 14.2 Hz), 3.23 (dd, 1H, $J = 3.9$ and 14.3 Hz), 3.56 (t, 1H, $J = 3.4$ Hz), 3.69 (d, 2H, $J = 6.0$ Hz), 3.83 (s, 3H), 3.94–4.22 (m, 5H), 5.16 (t, 1H, $J = 5.8$ Hz), 6.78 (dd, 1H, $J = 2.3$ and 8.5 Hz), 7.37 (d, 1H, $J = 8.6$ Hz), 7.65 (d, 1H, $J = 2.3$ Hz). EIMS *m/e* (relative intensity) 539 (M^+, 65), 439 (11), 328 (16), 272 (58), 228 (100), 212 (55), 169 (31), 141 (16), 57 (48); Anal. Calcd for ($C_{31}H_{45}N_3O_5$) C, H, N. This material was used directly in a later step.

5.6. (3*S*,6*R*)-3-[(1-*tert*-Butyloxycarbonyl-2-isoprenyl)-3-indoyl]-methyl-3,6-dihydro-6-isopropyl-2,5-diethoxypyrazine (17)

Indole **17** (3.4 g) was prepared in 82% yield from **13** under the conditions described above for the preparation of **16**. **17**: ^1H NMR (300 MHz, $CDCl_3$) δ 0.59 (d, 3 H, $J = 6.8$ Hz), 0.91 (d, 3 H, $J = 6.9$ Hz), 1.15 (t, 3 H, $J = 7.1$ Hz), 1.28 (t, 3 H, $J = 7.1$ Hz), 1.60 (s, 9 H), 1.62 (s, 3 H), 1.68 (s, 3 H), 2.09 (m, 1 H), 2.92 (m, 1H), 3.23 (m, 1 H), 3.56 (t, 1 H, $J = 3.4$ Hz), 3.71 (d, 2 H, $J = 6.1$ Hz), 3.98–4.20 (m, 5 H), 5.14 (t, 1 H, $J = 6.0$ Hz), 7.10–7.15 (m, 2 H), 7.49 (dd, 1H, $J = 2.1$ and 6.9 Hz), 7.99 (dd, 1H, $J = 1.3$ and 7.2 Hz). ^{13}C NMR (62.90 MHz, $CDCl_3$) δ 14.34, 14.36, 16.53, 18.13, 19.05, 25.60, 26.10, 28.09, 29.33, 31.41, 56.86, 60.36, 60.40, 60.69, 83.29, 114.98, 115.27, 119.04, 121.80, 122.40, 123.15, 130.54, 131.47, 135.94, 137.83, 150.47, 162.78, 163.23. CIMS *m/e* (relative intensity) 510 (M^+ +1). Anal. Calcd for ($C_{30}H_{43}N_3O_4 0.25H_2O$) C, H, N. This material was used directly in a later step.

5.7. (*S*)-1-*tert*-Butyloxycarbonyl-2-isoprenyl-6-methoxytryptophan ethyl ester (20)

To a solution of 2-prenylpyrazine **16** (1.27 g, 2.36 mmol) in THF (30 mL) at 0 °C was added an aqueous solution of 2 N HCl (10 mL). The reaction mixture was allowed to warm to rt and stirred for 1.5 h. A cold aqueous solution of 15% NH_4OH was added. The solution was concentrated under vacuum and diluted with CH_2Cl_2. The aqueous layer was extracted with CH_2Cl_2. The combined organic layers were dried (K_2CO_3) and the solvent was removed under vacuum. The residue was separated by flash chromatography (silica gel, EtOAc) to provide **20** (0.95 g, 94%) as an oil: $[\alpha]_D^{27}$ +15.2° (*c* 0.92, $CHCl_3$); IR ν_{max} (NaCl) 2975, 1730, 1615 cm^{-1}; ^1H NMR (250 MHz, $CDCl_3$) δ 1.22 (t, 3H, $J = 7.1$ Hz), 1.46–1.55 (m, 2H), 1.63 (s, 9H), 1.66 (s, 3H), 1.71 (s, 3H), 2.82 (dd, 1H, $J = 8.8$ and 14.2 Hz), 3.12 (dd, 1H, $J = 5.0$ and 14.2 Hz), 3.68 (d, 2H, $J = 5.1$ Hz), 3.70 (m, 1H), 3.83 (s, 3H), 4.13 (qd, 2H, $J = 2.1$ and 7.1 Hz), 5.16 (t, 1H, $J = 5.1$ Hz), 6.82 (dd, 1H, $J = 2.3$ and 8.5 Hz), 7.33 (d, 1H, $J = 8.5$ Hz), 7.69 (d, 1H, $J = 2.3$ Hz); ^{13}C NMR (62.90 MHz, $CDCl_3$) δ 14.04, 18.02, 25.48, 26.03, 28.05, 30.35, 55.62, 60.85, 83.47, 100.31, 111.32, 113.98, 118.46, 122.28, 123.66, 131.70, 136.56, 136.95, 150.35, 157.41, 175.02; EIMS *m/e* (relative intensity) 430 (M^+, 3), 272 (36), 228 (100). HRMS Calcd for $C_{24}H_{34}N_2O_5$ *m/z* = 430.2468, found *m/z* = 430.2481. This material was used directly in a later step.

5.8. (*S*)-1-*tert*-Butyloxycarbonyl-2-isoprenyltryptophan ethyl ester (21)

Ester **21** (1.1 g) was prepared in 93% yield from **17** as described above for the preparation of **20**. **21**: $[\alpha]_D^{26}$ +19.7° (*c* 0.8, CH_3OH); ^1H NMR (300 MHz, $CDCl_3$) δ 1.23 (t, 3H, $J = 7.1$ Hz), 1.66 (s, 9H), 1.68 (s, 3H), 1.74 (s, 3H), 1.80 (br s, 2H), 2.89 (dd, 1H, $J = 8.9$ and 14.2 Hz), 3.19 (dd, 1H, $J = 5.0$ Hz and 14.3 Hz), 3.73–3.77 (m, 3H), 4.13–4.19 (m, 2H), 5.19 (t, 1H, $J = 1.4$ Hz), 7.20–7.25 (m, 2H), 7.49 (dd, 1H, $J = 1.0$ and 7.0 Hz), 8.08 (dd, 1H, $J = 1.5$ Hz and 8.8 Hz); ^{13}C NMR (62.90 MHz, $CDCl_3$) δ 14.11, 18.15, 25.60, 25.99, 28.09, 30.27, 54.94, 61.02, 83.72, 114.04, 115.38, 118.09, 121.91, 122.43, 123.66, 129.68, 132.11, 136.00, 138.03, 150.33, 175.09. CIMS *m/e* (relative intensity) 401 (M^+ +1). This material was used directly in a later step.

5.9. (*R*)-1-*tert*-Butyloxycarbonyl-2-isoprenyl-6-methoxytryptophan ethyl ester (22)

Ester **22** (0.86 g) was prepared in 73% yield from **14** as described above for the preparation of **20**. **22**: $[\alpha]_D^{27}$ −15.9° (*c* 0.9, $CHCl_3$); All spectroscopic data were identical to that for **20** (the enantiomer of **22**) reported in the previous experiment except the optical rotation was opposite in sign. This material was used directly in a later step.

5.10. (*R*)-1-*tert*-Butyloxycarbonyl-2-isoprenyltryptophan ethyl ester (23)

Ester **23** (0.77 g) was prepared in 70% yield from **15** as described above for the preparation of **20**. **23**: $[\alpha]_D^{27}$ −19.9° (*c* 0.9, CH_3OH); All spectroscopic data were identical to that for **21** (the enantiomer of **23**) reported in the previous experiment except the optical rotation was opposite in sign. This material was used directly in a later step.

5.10.1. Tryprostation A (1). Fmoc-L-proline (126 mg, 0.374 mmol) was dissolved in thionyl chloride (1 mL). The solution which resulted was stirred overnight at rt. Excess thionyl chloride was removed under reduced pressure. The Fmoc-L-proline chloride **24** which resulted was dissolved in dry $CHCl_3$ (1 mL). This solution was added dropwise at 0 °C to a solution of **20** (107 mg, 0.249 mmol) and triethylamine (63.0 mg, 0.623 mmol) in dry $CHCl_3$ (6 mL). The mixture which resulted was stirred at 0 °C for 0.5 h and then at rt overnight. After

removal of solvent under reduced pressure, a solution of diethylamine (DEA, 2.5 mL) in acetonitrile (2.5 mL) was added in the same flask. The reaction mixture was stirred at rt for 2 h [monitored by TLC (silica gel) until the disappearance of starting material]. Acetonitrile and excess DEA were removed under reduced pressure. Xylene (25 mL) was added into the same reaction vessel and the solution degassed. The reaction mixture was stirred at reflux for 2 d at which time examination by TLC (silica gel) indicated the disappearance of starting material. After removal of xylene under reduced pressure, the residue was subjected to flash chromatography (silica gel, $CHCl_3/CH_3OH$, 95:5) to provide tryprostatin A **1** (78 mg, 82%) as a solid: $[\alpha]_D^{27}$ −65.9° (*c* 0.97, $CHCl_3$) [lit. [7] $[\alpha]_D^{27}$ −69.7° (*c* 0.70, $CHCl_3$)]; ^1H NMR (250 MHz, $CDCl_3$) δ 1.73 (s, 3H), 1.76 (s, 3H), 1.85–2.07 (m, 3H), 2.27–2.34 (m, 1H), 2.89 (dd, 1H, J = 11.4 and 15.0 Hz), 3.41 (d, 2H, J = 7.2 Hz), 3.53–3.72 (m, 3H), 3.81 (s, 3H), 4.05 (dd, 1H, J = 6.9 and 7.7 Hz), 4.32 (dd, 1H, J = 2.7 and 11.1 Hz), 5.28 (dd, 1H, J = 5.8 and 8.6 Hz), 5.61 (s, 1H), 6.74 (dd, 1H, J = 2.2 and 8.6 Hz), 6.81 (d, 1H, J = 2.1 Hz), 7.32 (d, 1H, J = 8.6 Hz), 7.80 (br s, 1H); ^{13}C NMR (62.90 MHz, $CDCl_3$) δ 17.92, 22.63, 25.07, 25.69, 25.71, 28.32, 45.38, 54.56, 55.73, 59.23, 94.87, 104.38, 109.27, 118.33, 119.98, 122.27, 135.14, 135.27, 136.25, 156.31, 165.80, 169.37. EIMS *m/e* (relative intensity) 381 (M^+,4), 228 (100), 212 (14), 198 (9). HRMS Calcd for $C_{22}H_{27}N_3O_3$ *m/z* = 381.2052, found *m/z* = 381.2044. Anal. Calcd for $C_{22}H_{27}N_3O_3 \cdot 1/3H_2O$ (C, H, N). The spectral data for **1** were identical to that reported by Osada et al.[7] in the literature.

5.10.2. Tryprostatin B (2). Indole **2** (300 mg) was prepared in 81% yield under conditions described above for the preparation of tryprostatin A **1**. **2**: $[\alpha]_D^{26}$ −70.9° (*c* 0.80, $CHCl_3$) {lit.[7] $[\alpha]_D^{27}$ −71.1 (*c* 0.63, $CHCl_3$)}; IR ν_{max} (NaCl) 3303, 2971, 1678, 1661 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.76 (s, 3H), 1.79 (s, 3H), 1.75–2.03 (m, 3H), 2.32 (m, 1H), 2.96 (dd, 1H, J = 11.4 and 15.9 Hz), 3.46–3.49 (m, 2H), 3.59–3.72 (m, 3H), 4.06 (dd, 1H, J = 7.5, 8.0 Hz), 4.36 (dd, 1H, J = 3.5, 11.0 Hz), 5.31 (dd, 1H, J = 6.5, 7.0 Hz), 5.62 (br s, 1H), 7.09–7.18 (m, 2H), 7.31 (d, 1H, J = 7.7 Hz), 7.48 (d, 1H, J = 7.7 Hz), 8.00 (br s, 1H), ^{13}C NMR (62.90 MHz, $CDCl_3$) δ 18.37, 23.03, 25.50, 25.98, 26.13, 28.74, 45.80, 54.95, 59.66, 105.03, 111.17, 118.13, 120.07, 120.30, 122.26, 128.37, 135.82, 135.91, 136.80, 166.183, 169.74. CIMS *m/e* (relative intensity) 352 (M^++1, 100), 198 (28). Anal. Calcd for ($C_{21}H_{25}N_3O_2 \cdot 1/4H_2O$) C, H, N. The spectral data for **2** were identical to that reported by Osada et al.[7] in the literature.

5.10.3. Enantiomer of tryprostatin A (3). Enantiomer **3** (75 mg) was prepared in 78% yield from **22** as described above for the preparation of tryprostatin A **1**. The starting material used here was D-tryptophan derivative **22** and Fmoc-D-Pro-Cl **25**. **3**: $[\alpha]_D^{28}$ 70.3° (*c* 1.0, $CHCl_3$). Anal. Calcd for ($C_{22}H_{27}N_3O_3 \cdot 3/5H_2O$) C, H, N. All spectroscopic data were identical to that reported for **1** (the enantiomer of **3**) in a previous experiment except the optical rotation was opposite in sign.

5.10.4. Enantiomer of tryprostatin B (4). Enantiomer **4** (80 mg) was prepared in 83% yield from **23** as described above for the preparation of tryprostatin A **1**. The starting material used here was D-tryptophan derivative **23** and Fmoc-D-Pro-Cl **25**. **4**: $[\alpha]_D^{26}$ 71.9° (*c* 1.1, $CHCl_3$). Anal. Calcd for ($C_{21}H_{25}N_3O_2 \cdot 3/4H_2O$) C, H, N. All spectroscopic data were identical to that for **2** (the enantiomer of **4**) reported in the previous experiment except the optical rotation was opposite in sign. All spectroscopic data were identical to that reported for **2** (the enantiomer of **4**) in a previous experiment except the optical rotation was opposite in sign.

5.10.5. Diastereomer-1 of tryprostatin A (5). Indole **5** (105 mg) was prepared in 84% yield from **22** as described above for the preparation of tryprostatin A **1**. The starting material used here was D-tryptophan derivative **22** and Fmoc-L-Pro-Cl **24**. **5**: $[\alpha]_D^{26}$ −20.0° (*c* 0.12, $CHCl_3$), IR ν_{max} (NaCl) 3269, 2971, 1673, 1650 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.18–1.43 (m, 2H), 1.60–1.71 (m, 1H), 1.74 (s, 3H), 1.78 (s, 3H), 1.99–2.08 (m, 1H), 2.67 (dd, 1H, J = 6.3, 10.7 Hz), 3.08 (dd, 1H, J = 4.5, 14.7 Hz), 3.16 (dd, 1H, J = 2.5, 9.5 Hz), 3.35 (d, 1H, J = 5.0 Hz), 3.39 (d, 2H, J = 7.7 Hz), 3.47–3.57 (m, 1H), 3.81 (s, 3H), 4.23 (dd, 1H, J = 4.3, 8.5 Hz), 5.26 (tt, 1H, J = 1.3, 6.0 Hz), 6.26 (d, 1H, J = 3.6 Hz), 6.74 (dd, 1H, J = 2.3, 8.6 Hz), 6.78 (d, 1H, J = 2.1 Hz), 7.37 (d, 1H, J = 8.6 Hz), 7.92 (br s, 1H), ^{13}C NMR (62.90 MHz, $CDCl_3$) δ 17.88, 21.51, 24.85, 25.74, 28.96, 29.33, 45.03, 55.62, 57.70, 58.62, 94.31, 104.20, 109.08, 118.95, 119.80, 122.70, 135.20, 135.34, 135.73, 155.97, 165.65, 169.23. EIMS *m/e* (relative intensity) 381 (M^+, 15), 228 (100). Anal. Calcd for ($C_{22}H_{27}N_3O_3 \cdot 3/4H_2O$) C, H, N.

5.10.6. Diastereomer-1 of tryprostatin B (6). Indole **6** (77 mg) was prepared in 86% yield from **23** as described above for the preparation of tryprostatin A **1**. The starting material used here was D-tryptophan derivative **23** and Fmoc-L-Pro-Cl **24**. **6**: $[\alpha]_D^{26}$ −41.9° (*c* 0.45, $CHCl_3$). Anal. Calcd for ($C_{21}H_{25}N_3O_2 \cdot 1/5H_2O$) C, H, N. All spectroscopic data were identical to that reported for **8** except the optical rotation was opposite in sign.

5.10.7. Diastereomer-2 of tryprostatin A (7). Indole **7** (48 mg) was prepared in 84% yield from **20** as described above for the preparation of tryprostatin A **1**. The starting material used here was L-tryptophan derivative **20** and Fmoc-D-Pro-Cl **25**. **7**: $[\alpha]_D^{27}$ 21.0° (*c* 0.32, $CHCl_3$). Anal. Calcd for ($C_{22}H_{27}N_3O_3 \cdot 3/8H_2O$) C, H, N. All spectroscopic data were identical to that reported for **5** in a previous experiment except the optical rotation was opposite in sign.

5.10.8. Diastereomer-2 of tryprostatin B (8). Diastereomer **8** (104 mg) was prepared in 79% yield from **21** as described above for the preparation of tryprostatin A **1**. The starting material used here was L-tryptophan derivative **21** and Fmoc-D-Pro-Cl **25**. **8**: $[\alpha]_D^{26}$ 42.8° (*c* 0.65, $CHCl_3$). IR ν_{max} (NaCl) 3266, 2977, 1666 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ 1.30–1.44 (m, 1H), 1.58–1.71 (m, 2H), 1.75 (s, 3H), 1.79 (s, 3H), 2.00–2.09 (m, 1H), 2.69 (dd, 1H, J = 6.3, 16.8 Hz), 3.08–3.19 (m, 2H), 3.37–3.43 (m, 1H), 3.44 (d, 2H, J = 6.3 Hz), 3.48–

3.58 (m, 1H), 4.24 (dd, 1H, J = 4.4, 8.6 Hz), 5.30 (tt, 1H, J = 1.4, 7.3 Hz), 6.09 (d, 1H, J = 3.4 Hz), 7.07–7.13 (m, 2H), 7.07–7.13 (m, 2H), 7.23–7.28 (m, 1H), 7.51 (dd, 1H, J = 2.1, 6.8 Hz), 8.00 (br s, 1H). ^{13}C NMR (62.90 MHz, $CDCl_3$) δ 17.94, 21.53, 24.91, 25.76, 28.99, 29.27, 45.08, 57.74, 58.66, 104.46, 110.37, 118.27, 119.52, 119.67, 121.56, 128.33, 134.97, 135.55, 136.66, 165.59, 169.09. EIMS *m/e* (relative intensity) 351 (M^+, 13), 198 (100). Anal. Calcd for ($C_{21}H_{25}N_3O_2 \cdot 1/8H_2O$) C, H, N.

5.11. (5*R*,2*S*)-3,6-Diethoxy-5-[6-methoxy-2-(triethyl-silyl)-3-indolyl]methyl-2,5-dihydropyrazine (28)

To a three-neck flask (3 L) equipped with an overhead stir were added iodoaniline derivative **26** (150 g), Schöllkopf derivative **27** (265 g), LiCl (2.55 g), Na_2CO_3 (159 g), palladium (II) acetate (1.75 g) and anhydrous DMF (2 L). The mixture was then degassed with a vacuum pump three times at rt with Ar. The suspension which resulted was heated for 36 h at 100 °C under an atmosphere of Ar. At this point TLC (silica gel) indicated **26** had been consumed and the reaction mixture was cooled to rt and the DMF was removed under vacuum (aspirator). Methylene chloride (2 L) was added to the residue and the suspension which resulted was filtered to remove unwanted salts. After removal of the CH_2Cl_2, the crude product was purified by flash chromatography (silica gel, 2% EtOAc in hexane) to give 77% of the desired 6-methoxy substituted indole **28**. IR ν_{max} (NaCl) 3388, 2944, 1683 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.67 (d, 3H, J = 6.8 Hz), 0.85–1.05 (m, 18H), 1.20 (t, 3H, J = 7.1 Hz), 1.30 (t, 3H, J = 7.1 Hz), 2.25 (m, 1H), 2.80 (dd, 1H, J = 13.5 Hz and J = 10.6 Hz), 3.46 (dd, 1H, J = 14.1 Hz and J = 3.1 Hz), 3.84 (s, 3H), 3.88 (t, 1H, J = 3.9), 4.01–4.21 (m, 5H), 6.70 (dd, 1H, J = 8.7 Hz and J = 2.2 Hz), 6.82 (d, 1H, J = 2.1 Hz), 7.60 (d, 1H, J = 8.7 Hz), 7.77 (s, br, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 4.1, 7.9, 14.7, 14.8, 17.1, 19.5, 32.1, 32.5, 56.0, 59.3, 60.9, 61.0, 61.1, 93.9, 109.3, 121.8, 124.4, 124.7, 130.5, 139.5, 157.0, 163.1, 164.2. MS (CI, CH_4) *m/e* (relative intensity) 486 (M^++1, 100), 456 (13), 372 (51), 274 (27). HRMS Calcd for $C_{27}H_{43}N_3O_3Si$ *m/z* = 485.3074, found *m/z* = 485.3055. This material was used directly in a later step.

5.12. N_a-Methyl-(2*S*,5*R*)-3,6-diethoxy-5-[6-methoxy-2-(triethyl-silyl)-3-indolyl]methyl-2,5-dihydropyrazine (29)

Sodium hydride (60% in mineral oil, 0.2 g) in several portions was added to a mixture of **28** (1.5 g, 3.08 mmol), CH_3I (0.65 g, 4.55 mmol) and anhydrous DMF (20 mL) at 0 °C. After this mixture was stirred for 2 h, analysis by TLC (silica gel) indicated the absence of starting material. The reaction solution was quenched with water (1 mL) and then was neutralized with an aqueous solution of NH_4Cl after which it was extracted with EtOAc (3 × 20 mL). The combined organic layers were washed with brine (2 × 30 mL) and dried (K_2CO_3). The solvent was removed under reduced pressure and the residue was subjected to a short wash column (silica gel, EtOAc/hexane, 1:4) to provide the pyrazine **29** (1.6 g, 99%). Mp 91–92 °C; IR ν_{max} (NaCl): 2945, 1688, 1613 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.63 (d, 3H, J = 6.8 Hz), 0.95 (m, 15H), 0.98 (d, 3H, J = 6.9 Hz), 1.14 (t, 3H, J = 7.1 Hz), 1.23 (t, 3H, J = 7.1 Hz), 2.23 (m, 1H), 2.80 (dd, 1H, J = 14.04 Hz and 4.53 Hz), 3.45 (dd, 1H, J = 14.02 and 3.51 Hz), 3.73 (s, 3H), 3.84 (s, 3H), 3.85 (m, 1H), 3.90–4.15 (m, 5H), 6.65 (m, 2H), 7.50 (d, 1H, J = 9.2 Hz); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 4.8, 7.6, 14.3, 14.4, 16.7, 19.1, 31.6, 31.9, 33.1, 55.7, 59.2, 60.4, 60.5, 60.7, 91.9, 108.2, 121.2, 124.2, 124.6, 132.3, 140.6, 156.7, 162.7, 163.9. MS (CI, CH4) *m/e* (relative intensity) 500 (M^++1, 100), 470 (16), 386 (14), 288(21). Anal. Calcd for ($C_{28}H_{45}N_3O_3Si$) C, H, N. This material was used directly in a later step.

5.13. N_a-Isoprenyl-(2*S*,3*R*)-3,6-diethoxy-5-[6-methoxy-3-indolyl]methyl-2,5-dihydropyrazine (30)

Indole **30** (2.1 g) was prepared in 75% yield from **28** and isoprenyl bromide under conditions described above for the preparation of **29**. **30**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.64 (d, 3H, J = 6.78 Hz), 0.92 (d, 3H, J = 6.87 Hz), 1.32 (m, 7H), 1.81 (d, 6H, J = 12.21 Hz), 2.06 (s, 2H), 3.24 (dd, 3H, J = 3.24 Hz and 2,34 Hz), 3.87 (s, 3H), 4.14 (m, 3H), 4.30 (s, 1H), 4.55 (d, 2H, J = 6.93 Hz), 5.34 (s, 1H), 6.74 (m, 2H), 7.50 (d, 1H, J = 2.64 Hz). This material was used directly in a later step.

5.14. N_a-Benzyl-(2*S*,5*R*)-3,6-diethoxy-5-[6-methoxy-2-(triethyl-silyl)-3-indolyl]methyl-2,5-dihydropyrazine (31)

Indole **31** (1.65 g) was prepared from **28** and benzyl bromide in 72% yield under conditions described above for the preparation of **29**. **31**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.88–0.96 (m, 13H), 1.09 (d, 3H, J = 6.87 Hz), 1.21–1.36 (m, 9H), 2.09 (s, 1H), 2.30–2.40 (m, 1H), 3.02 (dd, 1H, J = 14.22 Hz and 9.21 Hz), 3.59 (dd, 1H, J = 16.61 Hz and 5.46 Hz), 3.88 (s, 3H), 3.93 (t, 1H, J = 3.33 Hz), 4.05–4.34 (m, 5H), 5.48 (br, 2H), 6.51 (d, 1H, J = 2.1 Hz), 6.74 (dd, 1H, J = 8.67 Hz and 2.19 Hz), 6.95 (d, 2H, J = 6.96 Hz), 7.20–7.30 (m, 2H), 7.63 (d, 1H, J = 8.67 Hz); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 4.57, 7.52, 14.17, 14.30, 16.62, 19.13, 20.94, 31.34, 31.40, 31.53, 34.62, 49.57, 55.46, 58.71, 60.38, 60.67, 92.98, 108.38, 121.01, 124.67, 125.01, 125.66, 126.80, 127.55, 127.70, 128.40, 128.94, 132.48, 138.49, 140.41, 156.67, 162.76, 163.95. This material was used directly in a later step.

5.15. N_a-Allyl-(2*S*,5*R*)-3,6-diethoxy-5-[6-methoxy-3-indolyl]methyl-2,5-dihydropyrazine (32)

Indole **32** (1.9 g) was prepared from **28** and allyl bromide in 80% yield under conditions described above for the preparation of **29. 32:** ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.64 (d, 4H, J = 6.78 Hz), 0.92 (d, 3H, J = 6.87 Hz), 1.33 (m, 6H), 2.16 (t, 1H, J = 3.60 Hz), 3.32 (m, 3H), 3.86 (s, 3H), 4.16 (m, 3H), 4.60 (m, 2H), 5.02 (s, 1H), 5.18 (d, 1H, J = 1.35), 5.31 (s, 1H), 5.96 (m, 1H), 6.72 (m, 3H), 7.50 (d, 1H, J = 8.58). EIMS *m/e* (relative intensity) 411(M^+, 46). This material was used directly in a later step.

5.16. N_a-Methyl-6-methoxy-L-tryptophan ethyl ester (33)

Ester **33** (700 mg) was prepared in 84% yield from **29** (1.5 g, 3 mmol) as described above for the preparation of **20**. **33**: IR ν_{max} (NaCl) 3374, 3311, 2980, 1736, 1623 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.30 (t, 3H, J = 7.1 Hz), 1.62 (s, br, 2H), 2.99 (dd, 1H, J = 14.4 Hz and 7.7 Hz), 3.24 (dd, 1H, J = 14.3 Hz and 4.7 Hz), 3.65 (s, 3H), 3.79 (dt, 1H, J = 12.6 Hz and 7.4 Hz), 3.89 (s, 3H), 4.18 (q, 2H, J = 7.1 Hz), 6.75–6.83 (m, 3H), 7.48 (d, 1H, J = 8.6 Hz); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 14.1, 30.6, 32.5, 55.2, 55.8, 60.6, 93.1, 108.9, 109.8, 119.6, 122.6, 126.5, 137.8, 156.6, 175.0. EIMS *m/e* (relative intensity) 276 (M^+, 4), 174 (100), 159 (11). Anal. Calcd for ($C_{15}H_{20}N_2O_3$) C, H, N. This material was used directly in a later step.

5.17. N_a-Isoprenyl-6-methoxytryptophan ethyl ester (34)

Ester **34** (850 mg) was prepared from **30** in 85% yield under conditions described above for the preparation of **20**. **34**: ^1H NMR (300 MHz, $CDCl_3$) δ 1.24–1.30 (m, 4H), 1.83 (t, 3H, J = 18.21 Hz), 2.06 (s, 3H), 3.04 (dd, 1H, J = 14.37 Hz and 7.68 Hz), 3.22 (d, 1H, J = 4.8 Hz), 3.87 (s, 3H), 4.13–4.21 (m, 4H), 5.78 (br, 1H), 4.59 (d, 2H, J = 6.54 Hz), 5.37 (s, 1H), 6.82 (m, 3H), 7.48 (d, 1H, J = 8.04 Hz). This material was used directly in a later step.

5.18. N_a-Benzyl-6-methoxytryptophan ethyl ester (35)

Ester **35** (1.0 g) was prepared from **31** in 85% yield under conditions described above for the preparation of **20**. **35**: ^1H NMR (300 MHz, $CDCl_3$) δ 1.16–1.26 (m, 3H), 1.87 (s, 1H), 2.01 (s, 1H), 3.02 (dd, 1H, J = 14.28 Hz and 7.32 Hz), 3.18–3.25 (m, 1H), 3.87 (s, 3H), 4.06–4.18 (m, 3H), 5.17 (s, 1H), 6.71 (s, 1H), 6.77–6.80 (m, 1H), 6.87 (s, 1H), 7.08 (d, 1H, J = 7.38 Hz), 7.22–7.28 (m, 2H), 7.50 (d, 1H, J = 8.61 Hz); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 14.1, 30.6, 32.5, 55.2, 55.8, 60.6, 93.1, 108.9, 109.8, 119.6, 122.6, 126.5, 137.8, 156.6; ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 13.27, 20.06, 29.87, 48.89, 54.26, 54.66, 59.41, 59.92, 92.54, 108.13, 109.53, 118.81, 121.82, 124.99, 125.88, 126.64, 127.82, 136.55, 136.63, 155.62, 174.25. Anal. Calcd for ($C_{21}H_{24}N_2O_3H_2O$) C, H, N. This material was used directly in a later step.

5.19. N_a-Allyl-6-methoxytryptophan ethyl ester (36)

Ester **36** (640 mg) was prepared from **32** in 85% yield under conditions described above for the preparation of **20**. **36**: ^1H NMR (300 MHz, $CDCl_3$) δ 1.23–1.29 (m, 4H), 1.72 (s, 2H), 2.06 (s, 1H), 3.04 (d, 1H, J = 7.56 Hz), 3.87 (s, 3H), 4.12–4.18 (m, 2H), 4.63 (d, 1H, J = 1.5 Hz), 5.18 (dd, 2H, J = 17.2 Hz and 8.7 Hz), 5.98–6.00 (m, 1H), 6.75–6.87 (m, 3H), 7.50 (d, 1H, J = 8.43 Hz). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 14.09, 30.80, 48.55, 55.02, 55.60, 60.45, 93.24, 108.85, 110.06, 117.09, 119.56, 122.53, 125.39, 133.27, 137.10, 156.32, 175.19. This material was used directly in a later step.

5.20. 3-(6-Methoxy-1-methyl-1*H*-indol-3-ylmethyl)-hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione (37)

Indole **37** (280 mg) was prepared as described above for the preparation of **1** in 82% yield. The starting material used here was L-tryptophan derivative **33** (360 mg, 1.3 mmol) and Fmoc-L-pro-Cl **24** (690 mg, 2.05 mmol). **37**: IR ν_{max} (NaCl) 3430, 1610, 1550, 1390 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 2.02–2.05 (m, 4H), 2.89 (dd, 1H, J = 15.03 Hz and 10.92 Hz), 3.59–3.74 (m, 5H), 3.88 (s, 3H), 4.08 (dd, 1H, J = 14.8 Hz and 7.14 Hz), 4.32 (d, 2H, J = 10.92 Hz), 5.78 (br, 1H), 6.77–6.91 (m, 3H), 7.48 (d, 1H, J = 14.0 Hz); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 22.54, 26.66, 28.20, 32.66, 45.29, 54.44, 55.65, 59.13, 93.01, 108.25, 109.36, 119.20, 121.37, 126.71, 138.18, 156.77, 165.49, 169.21. EIMS *m/e* (relative intensity) 327 (M^+, 14), 174 (100). Anal. Calcd for ($C_{18}H_{21}N_3O_3$) C, H, N.

5.21. 3-(6-Methoxy-1-(3-methyl-but-2-enyl)-1*H*-indol-3-ylmethyl)-hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione (38)

Indole **38** (190 mg) was prepared from **34** in 80% yield under conditions described above for the preparation of **1**. **38**: ^1H NMR (300 MHz, $CDCl_3$) δ 1.76–1.85 (m, 3H), 2.00–2.03 (s, 3H), 2.05–2.14 (s, 3H), 2.32 (d, 1H, J = 6.84 Hz), 2.87 (dd, 1H, J = 15.09 Hz and 11.04 Hz), 3.58–3.70 (m, 3H), 3.88 (s, 3H), 4.08 (dd, 1H, J = 14.80 Hz and 7.14 Hz), 4.32 (d, 2H, J = 10.92 Hz), 4.61 (d, 1H, J = 6.87 Hz), 5.38 (t, 1H, J = 1.38 Hz), 5.78 (br, 1H), 6.80–6.82 (m, 2H), 6.90 (s, 1H), 7.45 (d, 1H, J = 6.33 Hz); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 17.99, 22.53, 25.59, 26.82, 28.22, 44.00, 45.29, 53.34, 54.52, 55.63, 59.13, 93.47, 109.22, 119.20, 119.46, 121.66, 125.27, 136.50, 137.47, 156.55, 165.50, 169.19. EIMS *m/e* (relative intensity) 381 (M^+, 16), 160 (100), 228 55. Anal. Calcd for ($C_{22}H_{27}N_3O_3$) C, H, N.

5.22. N_a-Benzyl-3-(6-methoxy-1*H*-indol-3-ylmethyl)-hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione (39)

Indole **39** (130 mg) was prepared from **35** in 74% yield under conditions described above for the preparation of **1**. **39**: mp 124–126 °C; ^1H NMR (300 MHz, $CDCl_3$) δ 1.70–1.81 (m, 4H), 2.03–2.06 (m, 2H), 2.82 (dd, 1H, J = 10.74 Hz and 6.69 Hz), 3.11–3.18 (m, 2H), 3.40 (dd, 1H, J = 14.55 Hz and 5.67 Hz), 3.51–3.61 (m, 1H), 3.82 (s, 3H), 4.22–4.27 (m, 1H), 5.21 (s, 1H), 6.01 (d, 1H, J = 3.09 Hz), 6.72 (s, 1H), 6.80 (d, 1H, J = 8.70 Hz), 6.91 (s, 1H), 7.13–7.16 (m, 2H), 7.28–7.33 (m, 2H), 7.49 (d, 1H, J = 8.70Hz); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 12.41, 28.74, 30.68, 44.97, 49.98, 55.54, 57.77, 58.44, 93.29, 108.48, 109.34, 119.82, 122.03, 126.77, 126.89, 127.69, 128.77, 137.24, 156.53, 165.31, 169.14. Anal. Calcd for ($C_{24}H_{25}N_3O_30.5H_2O$) C, H, N.

5.23. N_a-Allyl-3-(6-methoxy-1*H*-indol-3-ylmethyl)-hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione (40)

Indole **40** (220 mg) was prepared from **36** in 80% yield under conditions described above for the preparation of **1**. **40**: mp 84–86 °C; ^1H NMR (300 MHz, $CDCl_3$) δ 1.20–1.25 (m, 3H), 2.07 (d, 1H, J = 7.56 Hz), 2.25 (d,

1H, J = 22 Hz), 3.24 (d, 2H, J = 5.70 Hz), 3.70 (dd, 1H, J = 13.92 Hz and 7.01 Hz), 3.83 (s, 3H), 4.06–4.13 (m, 3H), 4.60 (m, 3H), 5.18 (d, 1H, J = 3.10 Hz), 5.89–5.94 (m, 1H), 6.70–6.83 (m, 3H), 7.42 (d, 1H, J = 5.61 Hz). Anal. Calcd for ($C_{20}H_{23}N_3O_3$) C, H, N.

5.24. (2*S*,5*R*)-3,6-Diethoxy-2-isopropyl-5-[6-methoxy-3-indolyl]methyl-2,5-dihydropyrazine (41)

A solution of **16** (500 mg, 0.93 mmol) in xylene was stirred at reflux for 3 d at which time examination by TLC (silica gel) indicated the disappearance of starting material. After removal of xylenes under reduced pressure, the residue was subjected to flash chromatography (silica gel, hexane/EtOAc, 10:1) to provide **41** (330 mg, 80%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.61 (d, 3H, J = 6.75 Hz), 0.91 (d, 3H, J = 6.87 Hz), 1.22 (t, 3H, J = 7.08 Hz), 1.35 (t, 4H, J = 7.14 Hz), 1.77 (d, 5H, J = 7.92), 2.15 (s, 1H), 3.24 (m, 3H), 3.42 (d, 2H, J = 7.20 Hz), 3.83 (s, 3H), 3.98–4.30 (m, 4H), 5.29 (s, 1H), 6.73 (m, 2H), 7.42 (t, 1H, J = 8.61 Hz), 7.65 (s, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 14.29, 16.32, 17.71, 18.99, 25.00, 25.67, 28.88, 30.82, 55.58, 57.23, 59.92, 60.24, 60.45, 93.97, 106.77, 108.10, 119.53, 120.94, 123.99, 125.28, 127.67, 134.16, 135.57, 155.45, 162.74, 163.41. EIMS *m/e* (relative intensity) 439 (M^+, 13), 212 (54), 169 (100). Anal. Calcd for ($C_{26}H_{37}N_3O_3$) C, H,N. This material was used directly in a later step.

5.25. N_a-Benzyl-(2*S*,5*R*)-3,6-diethoxy-2-isopropyl-5-[6-methoxy-3-indolyl]methyl-2,5-dihydropyrazine (42)

Indole **42** (550 mg) was prepared in 91% yield from **41** and benzyl bromide as described above for the preparation of **29**. **42**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.62 (d, 3H, J = 6.69 Hz), 0.93 (d, 3H, J = 6.84 Hz), 1.17–1.22 (m, 7H), 1.62 (t, 7H, J = 9.4 Hz), 2.10 (m, 1H), 3.21–3.39 (m, 4H), 3.77 (s, 3H), 4.10 (m, 4H), 5.04 (s, 1H), 5.22 (s, 1H), 6.60 (s, 1H), 6.70 (d, 1H, J = 8.55 Hz), 6.88 (d, 2H, J = 6.87 Hz), 7.27 (m, 3H), 7.48 (d, 1H, J = 8.55 Hz); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 14.28, 16.33, 17.80, 19.08, 24.02, 25.57, 28.07, 29.14, 30.77, 46.43, 55.59, 57.35, 59.96, 60.17, 60.44, 92.95, 107.46, 107.91, 119.77, 122.23, 123.16, 125.67, 126.82, 128.47, 131.87, 136.55, 137.08, 138.30, 155.61, 162.67, 163.3. This material was used directly in a later step.

5.26. N_a-Allyl-(2*S*,5*R*)-3,6-diethoxy-2-isopropyl-5-[6-methoxy-3-indolyl]methyl-2,5-dihydropyrazine (43)

Indole **43** (135 mg) was prepared in 82% yield from **41** (150 mg, 0.34 mmol) and allyl bromide (50 mg, 0.40 mmol) as described above for the preparation of **29**. **43**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.60 (t, 3H, J = 6.75 Hz), 0.91 (t, 3H, J = 6.84 Hz), 1.31 (m, 7H), 1.69 (t, 7H, J = 19.7 Hz), 2.10 (m, 1H), 3.14–3.27 (m, 3H), 3.42 (d, 1H, J = 6.45 Hz), 3.85 (s, 3H), 4.12 (m, 4H), 4.58 (s, 2H), 4.78 (d, 1H, J = 8.67 Hz), 5.09 (d, 2H, J = 9.12 Hz), 5.85 (m, 1H), 6.70 (m, 2H), 7.45 (d, 1H, J = 8.58 Hz); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 14.29, 16.32, 17.83, 18.98, 19.56, 23.87, 25.45, 29.17, 30.79, 45.31, 55.65, 57.41, 59.94, 60.18, 60.43, 92.96, 107.16, 107.67, 115.57, 119.70, 122.45, 123.18, 131.67, 133.62, 136.12, 136.71, 155.46, 162.78, 163.26. EIMS *m/e* (relative intensity) 479 (M^+, 13), 268 (100). This material was used directly in a later step.

5.27. N_a-Benzyl-2-isoprenyl-6-methoxytryptophan ethyl ester (44)

Ester **44** (330 mg) was prepared in 87% yield from **42**, as described above for the preparation of **20**. **44**: IR ν_{max} (KBr) 3054, 2305, 1733, 1422, 1265 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.18–1.32 (m, 3H), 1.60 (d, 8H, J =15.30 Hz), 3.01 (t, 1H, J = 9.30), 3.40 (d, 2H, J = 6.30 Hz), 3.80 (s, 3H), 4.17 (m, 2H), 5.04 (s, 1H), 5.26 (s, 1H), 6.66 (s, 1H), 6.80 (d, 1H, J = 3.96 Hz), 6.92 (d, 2H, J = 7.17 Hz), 7.25 (d, 3H, J = 7.50 Hz), 7.48 (d, 1H, J = 8.61 Hz); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 14.06, 17.86, 24.01, 25.40, 30.64, 46.54, 55.52, 55.67, 60.78, 93.51, 106.85, 108.49, 118.89, 121.57, 122.43, 125.72, 126.98, 128.53, 132.68, 136.34, 137.40, 137.92, 155.99, 175.33. EIMS *m/e* (relative intensity) 420 (M^+, 13), 318 (100). This material was used directly in a later step.

5.28. N_a-Allyl-2-isoprenyl-6-methoxytryptophan ethyl ester (45)

Ester **45** (88 mg) was prepared in 86% yield from **43** (130 mg, 0.27 mmol) as described above for the preparation of **20**. **45**: IR ν_{max} (NaCl) 3365, 1730, 1625 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.26 (m, 3H), 1.78 (t, 10H, J = 10.44), 2.94 (dd, 1H, J = 8.46 and J = 5.82), 3.20 (d, 1H, J = 5.04), 3.44 (d, 1H, J = 6.03), 3.86 (s, 3H), 4.16 (m, 2H), 4.61 (t, 2H, J = 2.43), 4.84 (d, 1H, J = 15.93), 5.12 (m, 2H), 5.90 (s, 1H), 6.75 (t, 2H, J = 6.33), 7.44 (d, 1H, J = 8.55); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 14.05, 17.91, 23.86, 25.49, 30.54, 45.44, 55.47, 55.71, 60.78, 93.50, 106.40, 108.28, 115.91, 118.81, 121.72, 122.40, 132.54, 133.40, 136.18, 136.95, 155.84, 175.30. EIMS *m/e* (relative intensity) 370 (M^++1, 12), 268 (100). Anal. Calcd for ($C_{22}H_{30}N_2O_3$) C, H, N. This material was used directly in a later step.

5.29. N_a-Benzyl-2-isoprenyl-3-(6-methoxy-1*H*-indol-3-ylmethyl)-hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione (46)

Indole **46** (125 mg) was prepared in 87% yield from **44** as described above for the preparation of **1**. **46:** IR ν_{max} (KBr) 3010, 2408, 1735 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.75 (dd, 3H, J = 7.14 Hz and J = 7.41 Hz), 1.55 (s, 2H), 1.64 (d, 4H, J = 11.82 Hz), 2.06 (d, 2H, J = 2.52), 2.28 (d, 2H, J = 2.70 Hz), 2.75 (m, 1H), 3.00 (m, 1H), 3.38 (m, 2H), 3.82 (s, 3H), 4.14 (m, 3H), 5.15 (d, 1H, J = 7.47 Hz), 5.27 (t, 1H, J = 8.88 Hz), 6.60 (s, 1H), 6.86 (m, 2H), 6.91 (d, 2H, J = 6.18 Hz), 7.05 (d, 2H, J = 2.70 Hz), 7.30 (s, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 13.93, 17.83, 21.31, 23.87, 24.32, 25.38, 28.28, 46.44, 55.69, 59.19, 61.22, 64.48, 84.64, 93.94, 108.52, 118.68, 121.60, 126.09, 126.93, 127.21, 127.88, 128.46, 128.68, 129.40, 137.99, 155.72, 169.02. EIMS *m/e* (relative intensity) 472 (M^+, 5), 318 (48) 91 (100). Anal. Calcd for ($C_{29}H_{33}N_3O_3H_2O$) C, H, N.

5.30. N_a-Allyl-2-isoprenyl-3-(6-methoxy-1*H*-indol-3-ylmethyl)-hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione (47)

Indole **47** (80 mg) was prepared as described above for the preparation of **1**. The starting material used here was L-tryptophan derivative **45** (85 mg, 0.23 mmol) and Fmoc-L-pro-Cl **24** (126 mg, 0.37 mmol) in 82% yield. **47**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.27 (s, 1H), 1.51–1.76 (m, 9H), 2.37 (t, 1H, J = 3.60 Hz), 3.00 (s, 1H), 3.29 (t, 1H, J = 4.20 Hz), 3.40 (m, 1H), 3.60 (d, 1H, J = 5.70 Hz), 3.92(s, 3H), 4.23 (d, 1H, J = 9.60 Hz), 4.70 (d, 1H, J = 10.2 Hz), 5.14 (s, 1H), 5.54 (s, 1H), 6.95 (d, 1H, J = 7.71 Hz), 7.18 (s, 1H), 7.24 (d, 1H, J = 5.70 Hz). EIMS *m/e* (relative intensity) 421 (M^+, 14), 268 (100). +TOF MS HRMS Calcd for $(C_{25}H_{31}N_3O_3 + Li)^+ m/z$ = 428.2525, found m/z = 428.2519.

5.31. (5*S*,2*R*)-3,6-Diethoxy-2-bromo-5-[1-*tert*-butyloxycarbonyl-6-methoxy-3-indolyl]methyl-2,5-dihydropyrazine (48)

A solution of NBS (183 mg, 1.03 mmol) which had been dissolved in acetonitrile (10 mL) was syringed into a solution of **28** (500 mg, 1.03 mmol) in acetonitrile (40 mL) at 0 °C. The reaction mixture was allowed to stir at 0 °C for 30 min at which time analysis by TLC (silica gel) indicated the absence of starting material. To this solution was then added 4-dimethylaminopyridine (DMAP, 7 mg, 0.057 mmol) and di-*tert*-butyl-dicarbonate (450 mg, 2.06 mmol) at rt. After the reaction solution was stirred for another 1 h, analysis by TLC (silica gel) indicated the disappearance of the intermediate. The solvent was removed under reduced pressure, and the residue was partitioned between CH_2Cl_2 (100 mL) and H_2O (100 mL). The organic layer was separated, and the aqueous layer was extracted with CH_2Cl_2 (2 × 80 mL). The combined organic layers were dried (Na_2SO_4) and the solvent was removed under reduced pressure. The residue was purified by flash chromatography (silica gel, EtOAc/hexanes, 4:96) to afford **48** (492 mg) as an oil in 87% yield. **48**: IR ν_{max} (NaCl) 2970, 1735, 1690 cm^{-1}; ^{1}H NMR (250 MHz, $CDCl_3$) δ 0.62 (d, 3H, J = 6.8 Hz), 0.97 (d, 3H, J = 6.8 Hz), 1.17 (t, 3H, J = 7.1 Hz), 1.28 (t, 3H, J = 7.1 Hz), 1.67 (s, 9H), 2.20 (m, 1H), 2.92 (dd, 1H, J = 8.1 and 13.9 Hz), 3.28 (dd, 1H, J = 4.6 and 13.9 Hz), 3.68 (t, 1H, J = 3.3 Hz), 3.84 (s, 3H), 3.95–4.25 (m, 5H), 6.80 (dd, 1H, J = 2.2 and 8.6 Hz), 7.38 (d, 1H, J = 8.6 Hz), 7.63 (d, 1H, J = 2.1 Hz); ^{13}C NMR (62.90 MHz, $CDCl_3$) δ 14.31, 14.35, 16.59, 19.09, 28.24, 31.04, 31.46, 55.62, 55.78, 60.49, 60.74, 84.46, 99.59, 107.81,111.61, 119.69, 120.59, 123.66, 137.34, 149.35, 157.65, 162.77, 163.37. EIMS *m/e* (relative intensity) 551 (M^+, 17), 549 (M^+, 17), 470 (64), 414 (61), 370 (58), 341 (32), 240 (49), 238 (49), 212 (72), 169 (100), 141 (39). Anal. Calcd for $(C_{26}H_{36}N_3O_5Br)$ C, H, N. This material was used directly in a later step.

5.32. (2*S*,5*R*)-3,6-Diethoxy-2-5-[1-*tert*-butyloxycarbonyl-2-benzyl-6-methoxy-3-indolyl]methyl-2,5-dihydropyrazine (49)

Indole **49** (800 mg) was prepared in 85% yield from **29** and benzyl bromide, as described below for the preparation of **50**. **49**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.65 (d, 3H, J = 3.75 Hz), 0.98 (d, 3H, J = 6.87 Hz), 1.20 (t, 3H, J = 7.11 Hz), 1.29 (m, 6H), 1.41 (s, 10H), 2.87 (s, 1H), 3.64 (d, 1H, J = 3.36), 3.88 (s, 3H), 4.00 (m, 1H), 4.56 (s, 2H), 6.89 (d, 1H, J = 8.58 Hz), 7.01 (d, 2H, J = 7.08 Hz), 7.20 (m, 3H), 7.52 (d, 1H, J = 8.58 Hz), 7.75 (s, 1H). EIMS *m/e* (relative intensity) 561 (M^+, 14), 461 (11), 212 (100). Anal. Calcd for $(C_{33}H_{43}N_3O_5)$ C, H, N. This material was used directly in a later step.

5.33. (2*S*,5*R*)-3,6-Diethoxy-2-allyl-5-[1-*tert*-butyloxycarbonyl-6-methoxy-3-indolyl]methyl-2,5-dihydropyrazine (50)

A solution of *n*-BuLi (2.5 M in hexane, 0.22 mL, 0.54 mmol) was added dropwise to a solution of 2-bromopyrazine **29** (250 mg, 0.46 mmol) in dry THF (8 mL) at −78 °C under nitrogen. The mixture which resulted was stirred at −78 °C for 30 min and then warmed to 0 °C for 10 min. Then allyl bromide (82.8 mg, 0.69 mmol) was added quickly at 0 °C. The mixture was stirred at 0 °C for 1 h and allowed to warm to rt overnight. The solvent was removed under reduced pressure. The residue was taken up in CH_2Cl_2 and washed with a 5% aqueous solution of $NaHCO_3$. The aqueous layer was extracted with CH_2Cl_2 (3 × 20 mL). The combined organic layers were dried (Na_2SO_4). After removal of the solvent under reduced pressure, the residue was separated by flash chromatography (silica gel, hexane/EtOAc, 15/1) to provide **50** (198 mg, 85%) as an oil. **50**: IR ν_{max} (KBr) 3054, 2306, 1733, 1422 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.65 (d, 3H, J = 6.78 Hz), 0.97 (d, 3H, J = 6.90 Hz), 1.23 (t, 10H, J = 6.54 Hz), 1.66 (d, 8H, J = 7.17 Hz), 2.20 (m, 1H), 3.31 (d, 1H, J = 3.30 Hz), 3.64 (s, 1H), 3.88 (s, 3H), 4.07 (m, 3H), 4.21 (m, 2H), 4.94 (d, 2H, J = 6.36 Hz), 5.98 (m, 1H), 6.84 (d, 1H, J = 2.34 Hz), 7.70 (d, 1H, J = 2.25 Hz), 7.98 (s, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 14.23, 16.46, 18.97, 28.08, 29.19, 30.59, 31.33, 55.54, 60.32, 83.25, 98.73, 99.77, 110.94, 114.75, 119.42, 136.23, 136.70, 157.07. EIMS *m/e* (relative intensity) 511 (M^+, 10), 244 (50), 200 (100). +TOF MS HRMS Calcd for $(C_{29}H_{41}N_3O_5 + H)^+ m/z$ = 512.3124, found m/z = 512.3126. This material was used directly in a later step.

5.34. (5*R*,2*S*)-3,6-Diethoxy-5-[6-methoxy-2-phenyl-3-indolyl]methyl-2,5-dihydropyrazine (51)

A solution of *n*-BuLi (2.5 M in hexane, 0.27 mL, 0.68 mmol) was added dropwise to a solution of 2-bromopyrazine **29** (250 mg, 0.46 mmol) in dry THF (10 mL) at −78 °C under nitrogen. The mixture which resulted was stirred at −78 °C for 30 min and then warmed to 0 °C for 10 min. Then dry anhydrous pure zinc chloride (0.68 mL, 0.69 mmol) was added quickly at 0 °C. The mixture which resulted was stirred at 0 °C for 1 h and iodobenzene was added and this was followed by addition of tri-2-furyl phosphine (21 mg, 0.1 mmol) and $Pd(OAc)_2$ (12 mg, 0.05 mmol). The mixture which resulted was then stirred overnight. The solvent was removed under reduced pressure. The residue was taken up in CH_2Cl_2 and washed with a 5% aqueous solution of $NaHCO_3$. The aqueous layer was extracted with CH_2Cl_2 (3 × 20 mL). The combined organic layers were dried (Na_2SO_4). After removal of the solvent under reduced pressure, the residue was separated by flash chromatography (silica gel, hexane/EtOAc, 15/1) to pro-

vide **51** (170 mg, 65%) as an oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.95 (d, 3H, J = 3.75 Hz), 1.22 (m, 3H), 1.33 (m, 6H), 1.41 (s, 10H), 1.65 (m, 3H), 2.81 (s, 1H), 3.34 (m, 3H), 3.90 (s, 3H), 6.88 (m, 2H), 7.40 (m, 3H), 7.56 (d, 2H, J = 8.58 Hz), 7.71 (s, 1H), 7.52 (d, 1H, J = 2.10 Hz), 7.75 (s, 1H). EIMS *m/e* (relative intensity) 547 (M^+, 56), 236 (100). Anal. Calcd for ($C_{32}H_{41}N_3O_5H_2O$) C, H, N. This material was used directly in a later step.

5.35. (*S*)-1-*tert*-Butyloxycarbonyl-2-benzyl-6-methoxy-tryptophan ethyl ester (52)

Ester **52** (185 mg) was prepared in 87% yield from **49** as described above for the preparation of **20**. **52**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.22 (m, 3H), 1.39 (s, 9H), 1.67 (s, 3H), 2.96 (d, 1H, J = 8.28 Hz), 3.18 (d, 1H, J = 5.46 Hz), 3.89 (s, 3H), 4.10 (m, 2H), 4.49 (s, 2H), 6.90 (d, 1H, J = 2.34 Hz), 7.02 (d, 2H, J = 6.93 Hz), 7.23 (m, 3H), 7.44 (d, 1H, J = 8.58 Hz), 7.80 (s, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 14.10, 27.66, 28.09, 30.13, 32.20, 54.86, 55.58, 64.16, 83.61, 100.01, 111.64, 115.22, 119.38, 122.52, 125.81, 127.44, 128.03, 130.13, 136.79, 139.84, 150.13, 157.60, 174.92. This material was used directly in a later step.

5.36. 2-Benzyl-3-(6-methoxy-1*H*-indol-3-ylmethyl)-hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione (55)

Indole **55** (95 mg) was prepared in 81% yield from **52**, as described above for the preparation of **1**. **55**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.26 (t, 2H, J = 5.16 Hz), 1.64 (s, 1H), 2.01 (m, 2H), 2.33 (m, 1H), 3.03 (t, 1H, J = 11.25 Hz), 3.58–3.73 (m, 2H), 3.83 (s, 3H), 4.09 (m, 2H), 4.32 (d, 1H, J = 10.80 Hz), 5.65 (s, 1H), 6.78 (s, 2H), 7.18 (d, 2H, J = 6.90 Hz), 7.28 (t, 2H, J = 5.70 Hz), 7.40 (d, 2H, J = 9.60 Hz), 7.80 (s, 1H); ^{13}C NMR (75.7 MHz, $CDCl_3$) δ 22.53, 25.65, 28.24, 32.20, 45.32, 54.54, 55.64, 59.15, 94.79, 105.76, 109.56, 118.46, 122.03, 126.48, 128.43, 128.70, 128.89, 136.49, 138.03, 156.53, 165.60, 169.24. EIMS *m/e* (relative intensity) 403 (M^+, 25), 335 (8), 250 (100), 218 (6). Anal. Calcd for ($C_{24}H_{25}N_3O_3$) C, H, N.

5.37. 2-Allyl-3-(6-methoxy-1*H*-indol-3-ylmethyl)-hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione (56)

Indole **56** (88 mg) was prepared in 81% yield from **53**, as described above for the preparation of **1**. **56**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.22 (m, 2H), 1.76 (s, 1H), 2.02 (m, 2H), 2.34 (s, 1H), 3.01 (t, 1H, J = 5.73 Hz), 3.48 (t, 1H, J = 6.30 Hz), 3.67 (s, 2H), 4.05–4.11 (m, 3H), 4.32 (d, 1H, J = 9.60 Hz), 5.15 (m, 1H), 5.88 (m, 1H), 6.78 (t, 2H, J = 3.30 Hz), 7.38 (m, 1H), 8.08 (s, 1H). EIMS *m/e* (relative intensity) 353 (M^+, 8), 200 (100). +TOF MS HRMS Calcd for $(C_{20}H_{23}N_3O_3 + Li)^+$ *m/z* = 360.1899, found *m/z* = 360.1896.

5.38. (2*S*,5*R*)-3,6-Diethoxy-2-isopropyl-5-[1-*tert*-butyloxycarbonyl-2-methyl-acrylate-6-methoxy-3-indolyl] methyl-2,5-dihydropyrazine (58)

Indole **29** (200 mg, 0.36 mmol) and $Pd(PPh_3)_4$ (21 mg, 0.018 mmol) were placed in a round-bottomed flask (50 mL) and purged with argon. Toluene (10 mL) was added and this was followed by methyl acrylate (0.16 mL) and dicyclohexylmethylamine (0.09 mL, 0.43 mmol). The reaction was heated to 95 °C for 48 h, cooled and then filtered through celite. The solvent was removed under reduced pressure and the crude product was purified by flash chromatography to afford **58** (190 mg, 94%). **58**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.77 (d, 3H, J = 6.78 Hz), 0.89 (t, 3H, J = 3.48 Hz), 1.07 (d, 3H, J = 6.87 Hz), 1.25 (m, 6H), 1.67 (s, 9H), 2.18 (m, 1H), 2.89 (t, 1H, J = 9.27 Hz), 3.46 (d, 1H, J = 10.74 Hz), 3.81 (s, 3H), 3.90 (s, 3H), 4.14 (m, 3H), 6.50 (d, 1H, J = 11.46 Hz), 6.86 (d, 1H, J = 2.64 Hz), 7.54 (d, 1H, J = 8.67 Hz), 7.73 (s, 1H), 8.02 (d, 1H, J = 13.53 Hz); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 14.00, 14.15, 14.28, 16.58, 18.97, 22.54, 28.06, 30.30, 31.48, 31.67, 51.34, 55.48, 56.57, 60.53, 60.70, 60.85, 84.17, 99.08, 112.27, 118.04, 119.50, 122.68, 124.06, 131.18, 137.94, 150.24, 158.86, 162.31, 163.29, 167.39. EIMS *m/e* (relative intensity) 555 (M^+, 64), 455 (37), 412 (22), 288 (18), 244 (100), 212 (92). Anal. Calcd for ($C_{30}H_{41}N_3O_7$) C, H, N. This material was used directly in a later step.

5.39. 2-Methyl acrylyl-6-methoxytryptophan ethyl ester (59)

Indole **59** (180 mg) was prepared in 87% yield from **58** as described above for the preparation of **20**. **59**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.23 (m, 3H), 1.58 (s, 2H), 1.67 (m, 10H), 3.04 (d, 1H, J = 8.67 Hz), 3.23 (d, 1H, J = 4.83 Hz), 3.82 (s, 3H), 3.90 (s, 3H), 4.17 (d, 2H), 6.38 (d, 1H, J = 15.0 Hz), 6.91 (d, 1H, J = 2.16 Hz), 7.48 (d, 1H, J = 8.67 Hz), 7.75 (s, 1H), 8.01 (d, 1H, J = 15.09 Hz); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 13.96, 22.09, 28.06, 30.68, 51.55, 55.04, 55.52, 61,13, 84.54, 99.34, 112.66, 118.63, 120.19, 120.88, 123.40, 131.37, 136.29, 137.77, 150.06, 158.99, 164.46, 167.17, 174.88. EIMS *m/e* (relative intensity) 446 (M^+, 35), 244 (100). This material was employed directly in the next step.

5.40. 2-Methyl acrylyl-3-(6-methoxy-1*H*-indol-3-yl-methyl)-hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione (60)

Compound **60** (110 mg) was prepared in 87% yield from **59** as described above for the preparation of **1**. **60**: mp 135–137 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.24 (m, 4H), 3.54 (m, 2H), 3.80 (s, 3H), 3.91 (s, 3H), 4.14 (m, 2H), 6.02 (s, 1H), 6.70 (d, 1H, J = 2.82 Hz), 7.08 (d, 1H, J = 2.79 Hz), 7.38 (s, 1H), 7.68 (d, 1H, J = 12.76 Hz), 8.25 (s, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 14.12, 21.24, 24.35, 28.36, 44.27, 51.64, 55.36, 61.41, 64.01, 93.64, 102.72, 111.40, 119.39, 121.00, 127.26, 128.53, 138.36, 138.61, 159.00, 167.32, 175.08. EIMS *m/e* (relative intensity) 397 (M^+, 34), 324 (77), 293 (100). HRMS Calcd for $C_{21}H_{23}N_3O_5$ *m/z* = 397.1638, found *m/z* = 397.1657.

5.41. 6-Methoxy-L-tryptophan ethyl ester (61)

Ester **61** (700 mg) was prepared in 86% yield from **28** (1.5 g, 31 mmol) as described above for the preparation of **20**. **61**: IR ν_{max} (NaCl) 3365, 1730, 1625 cm^{-1}; ^{1}H

NMR (300 MHz, $CDCl_3$) δ 1.23 (t, 3H, J = 7.1 Hz), 1.60 (s, br, 2H), 3.0 (dd, 1H, J = 14.3 Hz and 7.7 Hz), 3.22 (dd, 1H, J = 14.4 Hz and 4.8 Hz), 3.78 (m, 1H), 3.81 (s, 3H), 4.15 (q, 2H, J = 7.2 Hz), 6.80 (m, 2H), 6.90 (d, 1H, J = 2.0 Hz), 7.45 (d, 1H, J = 8.5 Hz), 8.15 (s, br, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 14.0, 30.7, 54.5, 54.9, 60.8, 94.7, 109.2, 111.0, 119.1, 121.8, 121.9, 136.5, 156.1, 175.2. MS (CI) *m/e* 263 (M^++1, 100). Anal. Calcd for ($C_{14}H_{18}N_2O_3$) C, H, N. This material was used directly in a later step.

5.42. 3-(6-Methoxy-1*H*-indol-3-ylmethyl)-hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione (62)

Indole **62** (280 mg) was prepared as described above for the preparation of **1** in 82% yield. The starting material used here was L-tryptophan derivative **61** (360 mg, 1.37 mmol) and Fmoc-L-pro-Cl **24** (690 mg, 2.05 mmol). mp 133–135 °C; IR ν_{max} (KBr) 3100, 1700, 1400, 1050 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.98–2.06 (m, 3H), 2.29–2.36 (m, 1H), 2.94 (dd, 1H, J = 15.01 and 10.93 Hz), 3.55–3.77 (m, 3H), 3.87 (s, 3H), 4.10–4.15 (m, 1H), 4.36 (d, 1H, J = 11.8 Hz), 5.76 (br, 1H), 6.83 (dd, 1H, J = 8.6 and 2.2 Hz), 6.90 (d, 1H, J = 2.0 Hz), 6.98 (s, 1H), 7.47 (d, 1H, J = 8.6 Hz), 8.03 (br, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 21.41, 28.80, 30.54, 44.94, 55.52, 57.76, 58.19, 94.58, 109.01, 109.58, 119.48, 121.40, 123.12, 136.93, 156.42, 165.66, 169.80. EIMS *m/e* (relative intensity) 313 (M^+, 14), 160 (100). Anal. Calcd for ($C_{17}H_{19}N_3O_3$) C, H, N. This material was used directly in a later step.

5.43. 3-(2-Bromo-6-methoxy-1*H*-indol-3-ylmethyl)-hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione (63)

A solution of NBS (28.5 mg) which had been dissolved in THF (1 mL) was syringed into a solution of **62** (50 mg, 0.16 mmol) in THF (10 mL) at −78 °C. The reaction mixture which resulted was allowed to stir at rt overnight at which time analysis by TLC (silica gel) indicated the absence of starting material. The solvent was removed under reduced pressure. The residue was purified by preparative TLC (silica gel, 5% EtOH/CH_2Cl_2) to afford **63** as a powder in 80% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.76–1.89 (m, 2H), 2.11 (dd, 2H, J = 16.2 Hz and 8.8 Hz), 2.97 (dd, 1H, J = 10.44 and 6.6 Hz), 3.12–3.37 (m, 3H), 3.52–3.60 (m, 1H), 3.83 (s, 3H), 4.27 (d, 1H, J = 4.2 Hz), 6.37 (br, 1H), 6.75–6.84 (m, 2H), 7.39 (d, 1H, J = 8.6 Hz), 8.92 (br, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 21.52, 28.95, 29.77, 45.18, 55.54, 57.78, 58.06, 94.32, 108.61, 108.70, 110.00, 118.88, 121.30, 136.71, 156.59, 165.33, 168.96. EIMS *m/e* (relative intensity) 391 (M^+, 18), 393 (M^+, 18), 154 (100), 240 (78).

5.44. 3-(2-Chloro-6-methoxy-1*H*-indol-3-ylmethyl)-hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione (64)

Indole **64** (55 mg) was prepared in 85% yield from **62** under conditions described above for the preparation of **63**. **64**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.81–1.90 (m, 2H), 2.15 (dd, 2H, J = 16.2 Hz and 8.8 Hz), 2.97 (dd, 1H, J = 10.44 Hz and 6.6 Hz), 3.17–3.32 (m, 3H), 3.52–3.60 (m, 1H), 3.82 (s, 3H), 4.26 (t, 1H, J = 4.2 Hz), 6.43 (br, 1H), 6.74–6.84 (m, 2H), 7.35 (d, 1H, J = 9.4 Hz), 9.08 (s, br, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 18.30, 21.49, 28.96, 45.13, 55.54, 58.00, 58.32, 94.44, 105.26, 109.94, 118.96, 121.12, 121.42, 135.13, 156.57, 165.40, 169.11. EIMS *m/e* (relative intensity) 347 (M^+, 14), 194 (100), 154 (91). +TOF MS HRMS Calcd for ($C_{17}H_{18}N_3O_3$ + Na)$^+$*m/z* = 370.0934, found *m/z* = 370.0935.

5.45. 3-Benzyl-6-(6-methoxy-1*H*-indol-3-ylmethyl)-hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione (66)

Indole **66** (78 mg) was prepared from **61** in 73% yield under conditions described above for the preparation of **1**. **66**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.99–2.05 (m, 2H), 2.60–2.63 (m, 2H), 3.23–3.29 (m, 3H), 3.50 (d, 1H, J = 6.80 Hz), 3.82 (s, 3H), 4.14–4.16 (m, 2H), 4.92 (s, 1H), 6.74–6.83 (m, 3H), 7.22–7.29 (m, 2H), 7.76 (s, 1H), 8.42 (br, 1H). MS (EI) *m/e* (relative intensity) 363 (M^+, 100), 287(62.5). Anal. Calcd for ($C_{21}H_{21}N_3O_3$0.5-H_2O) C, H, N.

5.46. (3*S*,6*S*)-3-Isopropyl-6-[6-methoxy-2-(3-methylbut-2-enyl)-1*H*-indol-3-ylmethyl]piperazine-2,5-dione (67)

Indole **67** (63 mg) was prepared from **20** in 85% yield under conditions described above for the preparation of **1**. **67**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.95–0.97 (d, 3H, J = 6.8 Hz), 1.06–1.08 (d, 3H, J = 7.1 Hz), 1.77–1.80 (d, 6H, J = 9.8 Hz), 2.42 (m, 1H), 2.91–2.99 (dd, 1H, J = 11.0 and 14.4 Hz), 3.43–3.46 (d, 2H, J = 7.2 Hz), 3.58–3.64 (dd, 1H, J = 3.3 and 14.5 Hz), 3.85 (s, 3H), 3.92 (s, 1H), 4.25–4.29 (d, 1H, J = 9.4 Hz), 5.29–5.32 (t, 1H, J = 8.2 Hz), 5.79 (s, 1H), 6.00 (s, 1H), 6.77–6.81 (dd, 1H, J = 8.6 and 2.2 Hz), 6.84 (d, 1H, J = 2.1 Hz), 7.38–7.41 (d, 1H, J = 8.6 Hz), 7.83 (br, 1H). MS (EI) *m/e* (relative intensity) 383 (M^+). +TOF MS HRMS Calcd for ($C_{22}H_{29}N_3O_3$ + H)$^+$*m/z* = 384.2287, found *m/z* = 384.2282.

5.47. (3*S*,6*S*)-3-Benzyl-6-[6-methoxy-2-(3-methylbut-2-enyl)-1*H*-indol-3-ylmethyl]piperazine-2,5-dione (68)

Indole **68** (45 mg) was prepared from **20** in 75% yield under conditions described above for the preparation of **1.68:** ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.81 (d, 6H, J = 9.8 Hz), 1.90–1.98 (m, 1H), 3.12–3.26 (m, 2H), 3.41–3.43 (d, 2H, J = 7.5 Hz), 3.85 (s, 3H), 4.05–4.08 (d, 1H, J = 10.1 Hz), 4.25 (m, 2H), 5.32–5.35 (t, 1H, J = 8.4 Hz), 5.53 (s, 1H), 5.75 (s, 1H), 6.78–6.85 (m, 4H), 7.25–7.28 (m, 3H), 7.43–7.46 (d, 1H, J = 9.3 Hz), 7.83 (br, 1H). MS (EI) *m/e* (relative intensity) 431 (M^+, 100). +TOF MS HRMS Calcd for ($C_{26}H_{29}N_3O_3$+ H)$^+$*m/z* = 432.2287, found *m/z* = 432.2292.

5.48. 2-Isoprenyl-3-(6-nitro-1*H*-indol-3-ylmethyl)-hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione (69)

Tryprostatin B **2** (20 mg, 0.056 mmol) was dissolved in anhydrous THF (5 mL) and trifluoroacetic acid (2 mL) was added, after which the mixture was cooled to −78 °C. A solution of $NaNO_2$ (20 mg, 0.28 mmol) in

TFA (2 mL) was slowly added to the cooled solution via a syringe over a 10 min period. The reaction mixture was stirred for an additional 30 min and then allowed to warm to −20 °C for 30 min, after which CH_2Cl_2 (10 mL) and cold aq NH_4OH (14%) were added until pH 8. The layers were separated and the aqueous layer was washed with CH_2Cl_2 (3 × 10 mL). The combined organic layers were washed with brine (10 mL) and dried (Na_2SO_4). After removal of the solvent under reduced pressure, the residue was purified by flash chromatography (silica gel, CH_2Cl_2/ethanol 20:1) to provide **69** (17.5 mg, 76%) as a yellow powder. **69**: IR ν_{max} (KBr) 3249, 2923, 1650, 1540, 1451, 1304.5 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.60 (s, 3H), 1.76 (s, 3H), 1.84–2.06 (m, 3H), 2.34 (s, 2H), 3.04 (dd, 1H, J = 10.86 Hz and J = 4.32 Hz), 4.12 (m, 2H), 4.35 (d, 1H, 8.74 Hz), 5.34 (s, 1H), 5.53 (s, 1H), 7.53 (d, 1H, J = 8.82 Hz), 8.03 (d, 1H, J = 6.00 Hz), 8.29 (s, 1H), 8.53 (s, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 14.08, 17.96, 22.47, 24.30, 25.66, 28.28, 45.38, 54.57, 59.15, 95.47, 106.08, 107.60, 115.52, 117.38, 118.34, 128.05, 129.42, 132.82, 143.15, 165.78, 169.19. EIMS *m/e* (relative intensity) 396 (M^+, 22), 355 (10), 381 (23), 243 (100). Anal. Calcd for ($C_{21}H_{24}N_4O_4$) C, H, N.

5.49. 2-Isoprenyl-3-(6-amino-1*H*-indol-3-ylmethyl)-hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione (70)

Iron (III) chloride hexahydrate (25 mg, 0.063 mmol) and active carbon (2 mg, 0.16 mmol) were added to a solution of **69** in MeOH (4 mL). After the reaction mixture was heated to reflux, hydrazine monohydrate (0.1 mL) was added dropwise. The reaction mixture was allowed to stir at reflux until analysis by TLC (silica gel) indicated the disappearance of starting material (3 h). The reaction was then cooled to rt and the catalysts were removed by filtration through a pad of celite. The CH_3OH was removed under vacuum and the residue was dissolved in CH_2Cl_2. The organic layer was then washed with water, brine, and dried (Na_2SO_4). After the $CH_2Cl_2$was removed under reduced pressure, the residue was purified by a short wash column (silica gel, CH_2Cl_2/ethanol, 20:1) to provide **70** as an oil (20.6 mg, 88%). **70**: ^1H NMR (300 MHz, $CDCl_3$) δ 1.77 (m, 4H), 1.99–2.06 (m, 5H), 2.33 (s, 1H), 2.89 (t, 1H, J = 14.04 Hz), 3.41 (m, 3H), 3.59–3.67 (m, 4H), 4.12 (s, 1H), 4.34 (d, 1H, J = 9.03 Hz), 5.29 (dd, 1H, J = 4.80 Hz and J = 7.20 Hz), 5.67 (s, 1H), 6.55 (d, 1H, J = 6.60 Hz), 6.64 (s, 1H), 7.28 (d, 1H, J = 3.60 Hz), 7.69 (s, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 17.85, 22.55, 24.94, 25.63, 27.47, 28.26, 45.29, 54.46, 59.17, 96.49, 104.42, 110.30, 118.34, 120.04, 121.27, 134.02, 134.90, 136.74, 142.04, 165.78, 169.20. EIMS *m/e* (relative intensity) 366 (M^+, 10), 198 (62), 165 (100). Anal. Calcd for ($C_{21}H_{26}N_4O_2H_2O$) C, H, N. This material was converted into the hydrochloride salt for storage purposes.

5.50. 2-Isoprenyl-3-(6-isothiocyanato-1*H*-indol-3-ylmethyl)-hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione (71)

6-Aminotryprostatin B **70** (20 mg, 0.055 mmol) was dissolved in $CHCl_3$ (4 mL) and thiophosgene (0.2 mL, 0.003 mmol) was added dropwise at rt. The reaction mixture was stirred for 4 h and then the solution was treated with triethylamine (2 mL). After the solvent was removed under reduced pressure, the residue was purified by a short wash column (silica gel, CH_2Cl_2/ethanol, 20:1) to provide **71** as an oil (20.0 mg, 91%). **71**: IR ν_{max} (KBr) 2961, 2120, 1615 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.59 (s, 3H), 1.75 (s, 3H), 1.82 (s, 3H), 2.34 (m, 1H), 2.97 (dd, 1H, J = 10.86 Hz and J = 11.16 Hz), 3.50 (s, 2H), 3.63 (m, 3H), 4.14 (s, 1H), 4.32 (d, 1H, J = 6.96 Hz), 5.31 (s, 1H), 5.61 (s, 1H), 7.03 (d, 1H, J = 6.72 Hz), 7.28 (s, 1H), 7.42 (d, 1H, J = 8.52 Hz), 8.07 (s, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 17.95, 22.53, 25.13, 25.49, 25.68, 28.31, 29.62, 45.36, 54.49, 59.19, 105.33, 108.07, 118.41, 118.50, 118.94, 124.68, 127.27, 134.81, 136.20, 138.83, 165.37, 169.26. EIMS *m/e* (relative intensity) 408 (M^+, 18), 255 (100). Anal. Calcd for ($C_{22}H_{24}N_4O_2S0.3H_2O$) C, H, N.

5.51. 2-Isoprenyl-3-(6-azido-1*H*-indol-3-ylmethyl)-hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione (72)

6-Aminotryprostatin B **70** (15 mg, 0.041 mmol) was dissolved in CH_2Cl_2 (2 mL). Triethylamine (0.1 mL, 0.72 mmol) and a solution of $CuSO_4$ (2.0 mg, 0.014 mmol) in H_2O (0.05 mL) were added to the reaction mixture. A freshly prepared solution of TfN_3 (21 mg, 0.12 mmol) in CH_2Cl_2 (1 mL) was then added, and the solution which resulted was brought to homogeneity by adding MeOH (1 mL). The solution which resulted was stirred at rt for 2 h. The reaction solution was then poured into a saturated solution of aq $NaHCO_3$ (5 mL) and extracted with CH_2Cl_2 (3 × 10 mL). The combined organic layers were washed with brine (10 mL) and dried (Na_2SO_4). After the solvent was removed under reduced pressure, the residue was purified by a short wash column (silica gel, CH_2Cl_2/EtOH, 20:1) to provide **72** as an oil (14.5 mg, 90%). **72**: IR ν_{max} (KBr) 2960, 2112.2, 1615 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.77 (m, 6H), 2.06 (s, 3H), 2.34 (s, 1H), 3.00 (t, 1H, J = 8.67), 3.47 (s, 2H), 3.60 (m, 3H), 4.12 (t, 1H, J = 7.20), 4.33 (d, 1H, J = 9.63), 5.32 (s, 1H), 5.61 (s, 1H), 6.83 (d, 1H, J = 6.60), 6.99 (s, 1H), 7.38 (d, 1H, J = 12.00), 7.96 (s, 1H). EIMS *m/e* (relative intensity) 392 (M^+, 28). HRMS Calcd for $C_{21}H_{24}N_6O_2$ *m/z* = 392.1961, found *m/z* = 392.1957.

5.52. Topoisomerase II-mediated DNA relaxation assay[52,53]

The DNA relaxation assay tests the ability of a drug to inhibit the Topo II-mediated relaxation of supercoiled DNA. The assay was performed in a total volume of 10 μL and contained 62.5 μg of plasmid pUC18 DNA (from *Escherichia coli*) and 100 μM drug in an incubation assay buffer (0.05 M Tris–HCl, pH 8.0, 0.12 M KCl, 0.01 M $MgCl_2$, 0.5 mM ATP, 0.5 mM DTT, 30 mg/mL BSA). Stock solutions of drug were made up in either DMSO or EtOH and the total percentage of these in the assay mixture was kept to less than 1%. The mixture was allowed to warm to 37 °C and the reaction was initiated by the addition of 2.0 U of Topo II (TopoGEN, Inc.). The reaction was allowed to proceed for 30–45 minutes before being stopped by addition of

2.5 μL decatenation buffer (5% sarkosyl, 25% glycerol, and 0.0025% bromophenol blue). The drugs were then extracted from the incubation with 10 μL of 24:1 $CHCl_3$: isoamyl alcohol and the samples loaded onto a 1% agarose gel to run for 90 min at 90 V. The gel was stained with ethidium bromide and destained with H_2O. The DNA bands were detected on a UV light box and photographed with Polaroid 525 film. Controls were no-enzyme, enzyme, 100 μM *m*-AMSA, and 1% DMSO.

The gels were analyzed qualitatively by looking for the presence of DNA bands that migrate farther down on the gel than the negative controls. Topo II-mediated relaxation of the DNA prevents the band from migrating down the gel as far as one that is still in a supercoiled form. Therefore, DNA incubated with Topo II inhibitors will migrate farther on the gel than the no-enzyme or DMSO controls.

5.53. Microtubule assembly assay[13,54]

Tubulin, containing MAPs (microtubule-associated proteins), was prepared as described in the literature.[59] The tubulin polymerization assay was run at 37 °C by adding to 240 μL of PME buffer (100 mM PIPES, pH 6.9, 2 mM EGTA, 1 mM $MgSO_4$), 8 μL of GTP (50 mM), 32 μL of drug in DMSO, and 120 μL of tubulin (added last). The change in absorbance was measured at 351 nm over 10 min. The sample cuvette was zeroed against a reference cuvette containing 360 μL TBE buffer, 8 μL GTP (50 mM), and 32 μL DMSO. The concentration of the drug solution was varied for different runs to obtain a delta absorbance versus concentration curve. Standard curves were prepared on each batch of separately prepared tubulin using colchicine as the standard. Polymerization assays were conducted on the tryprostatins (**1–8**) and similar derivative (colchicine).

5.54. Cytotoxicity assay

Three human cell lines were purchased from American Type Culture Collection (ATCC) and used in all cytotoxicity assays. The MCF-7 breast adenocarcinoma cells, NCI-H520 lung squamous cell carcinoma cells and PC-3 prostate adenocarcinoma cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were subcultured twice a week in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 0.1 mM non-essential amino acids.

Normally growing cells were plated at 1×10^4 cells/well into 96-well plates and incubated for 24 h at 37 °C. After 24 h, the cells were drugged for initial screening with 100 μM, 50 μM, and 10 μM drug dissolved in a DMSO or EtOH vehicle (less than 1% in culture medium). Any drug showing <50% cell survival at 100 μM was further tested using appropriate drug concentrations to determine its growth inhibition-50% (GI_{50}). Drugs were run in quadruplicate or greater and control wells contained an appropriate percentage of DMSO or EtOH, usually 0.2%. Positive controls were either etoposide (ETOP) or amsacrine (*m*-AMSA).

After incubation with drug for 72 h, the CellTiter 96™ AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega) was used to evaluate cell survival. Cells were treated with a solution of MTS [3-(4,5-dimethylthiozol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium] and an electron coupling reagent, PMS (phenazine methosulfate) diluted in RPMI-1640 medium. MTS (Owen's reagent)[60] was bioreduced by viable cells into formazan, and the amount of formazan present can be measured by reading the absorbance at 490 nm.[61] The amount of formazan present was proportional to the number of living cells in culture. Vehicle control lanes were assumed to have 100% cell survival and the percentage of cells remaining in the drug-treated wells was calculated as a percentage of these control wells. The absorbance of wells containing only the MTS reagent (the plate blank) was subtracted from all wells.

5.55. Cell culture

A temperature-sensitive *cdc2* mutant cell line, tsFT210, which was isolated from the mouse mammary carcinoma cell line FM3A, was a kind gift from Dr. F. Hanaoka (RIKEN).[4] The tsFT210 cells were maintained in RPMI 1640 with 10% fetal calf serum at the permissive temperature of 32 °C.

5.56. Cell cycle analysis

In a synchronous-culture assay, cells were seeded at a density of 1×10^5 cells/mL in 0.5 mL into a 24-well plate and were preincubated at 32 °C for 1 h. Then, 5 μL of each sample solution was added, and the cells were incubated at 32 °C for 18 h. After incubation, morphological characteristics of the cells were examined by microscopic observation. The cells were subjected to flow cytometric analysis as described below to confirm the DNA contents in cells.

Flow cytometric analysis was performed essentially as described by previous reports.[4,58] The harvested cells were stained with solution containing 50 μg/mL propidium iodide, 0.1% sodium citrate, and 0.2% NP-40 and analyzed for DNA contents using a flow cytometer (Coulter Co., Hialeah, FL).

5.57. Cell staining

Carnoy fixation and staining were performed with slight modification. Cells were treated with 0.55% of KCl for 20 min, fixed in Carnoy's solution and dropped onto a wet glass slide. The chromosomes and intact nuclei were stained with 1 mg/mL of Hoechst 33258, and examined by using fluorescent microscopy (Olympus, Tokyo, Japan).

5.58. Proliferation assay

Exponentially growing tsFT210 cells were treated with test compounds at 32 °C for 48 h. The cell number was evaluated by the subsequent color reaction. The 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-

disulfophenyl)-2*H* tetrazolium, mono-sodium salt, WST-8™ (Nakalai Tesque, Kyoto, Japan) was added, and the cells were further incubated for 4 h at 37 °C. The absorbance (A_{450}) of each well was measured by a Wallac 1420 multilabel counter (Amersham Biosciences, Piscataway, NJ).

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Appendix A

Elemental analyses

Compound	Formula	Calculated (%)			Found (%)		
		C	H	N	C	H	N
1	$C_{22}H_{27}N_3O_3 \cdot 1/3H_2O$	68.19	7.20	10.84	68.21	7.16	10.85
2	$C_{21}H_{25}N_3O_2 \cdot 1/4H_2O$	70.86	7.22	11.81	70.88	7.25	11.85
3	$C_{22}H_{27}N_3O_3 \cdot 3/5H_2O$	67.36	7.25	10.71	67.33	7.26	10.75
4	$C_{21}H_{25}N_3O_2 \cdot 3/4H_2O$	69.11	7.32	11.51	69.11	7.28	11.55
5	$C_{22}H_{27}N_3O_3 \cdot 3/4H_2O$	66.90	7.27	10.64	66.92	7.26	10.67
6	$C_{21}H_{25}N_3O_2 \cdot 1/5H_2O$	71.04	7.21	11.84	71.06	7.24	11.79
7	$C_{22}H_{27}N_3O_3 \cdot 3/8H_2O$	70.41	7.25	11.73	70.44	7.26	11.75
8	$C_{21}H_{25}N_3O_2 \cdot 1/8H_2O$	71.31	7.20	11.88	71.34	7.21	11.85
12	$C_{26}H_{37}N_3O_5$	66.22	7.91	8.91	66.23	7.90	8.55
16	$C_{31}H_{45}N_3O_5$	68.99	8.40	7.79	68.69	8.66	7.40
17	$C_{30}H_{43}N_3O_4 \cdot 1/4H_2O$	70.08	8.53	8.17	70.10	8.56	8.15
29	$C_{28}H_{45}N_3O_3Si$	67.29	9.08	8.41	67.49	9.16	8.34
33	$C_{15}H_{20}N_2O_3$	65.18	7.30	10.14	64.96	7.36	10.24
35	$C_{21}H_{24}N_2O_3 \cdot H_2O$	68.11	7.03	7.58	67.71	6.68	7.81
37	$C_{18}H_{21}N_3O_3$	66.04	6.47	12.84	65.80	6.75	13.06
38	$C_{22}H_{27}N_3O_3$	69.27	7.13	11.02	69.03	7.28	11.29
39	$C_{24}H_{25}N_3O_3 \cdot 0.5H_2O$	69.90	6.31	10.19	69.54	5.89	9.87
40	$C_{20}H_{23}N_3O_3$	67.97	6.56	11.89	68.20	6.28	11.63
41	$C_{26}H_{37}N_3O_3$	71.04	8.48	9.56	71.39	8.01	9.82
45	$C_{22}H_{30}N_2O_3$	71.32	8.16	7.56	70.90	7.81	7.89
46	$C_{29}H_{33}N_3O_3 \cdot H_2O$	71.02	7.14	8.57	70.61	6.75	8.28
48	$C_{26}H_{36}N_3O_5Br$	56.73	6.59	7.63	56.97	6.47	7.48
49	$C_{33}H_{43}N_3O_5$	70.56	7.72	7.48	70.23	7.98	7.14
51	$C_{32}H_{41}N_3O_5 \cdot H_2O$	67.96	7.61	7.43	67.51	7.24	7.94
55	$C_{24}H_{25}N_3O_3$	71.44	6.25	10.41	71.09	6.56	10.05
58	$C_{30}H_{41}N_3O_7$	64.85	7.44	7.56	65.22	7.10	7.98
61	$C_{14}H_{18}N_2O_3$	64.12	6.87	10.69	63.96	6.98	10.54
62	$C_{17}H_{19}N_3O_3$	65.16	6.11	13.41	64.93	6.36	13.68
66	$C_{21}H_{21}N_3O_3 \cdot 0.5H_2O$	67.74	5.91	11.29	67.36	5.49	11.73
69	$C_{21}H_{24}N_4O_4$	63.62	6.10	14.13	63.96	5.73	14.47
70	$C_{21}H_{26}N_4O_2 \cdot H_2O$	65.62	7.29	14.58	65.20	14.26	15.02
71	$C_{22}H_{24}N_4O_2S \cdot 0.3H_2O$	63.86	5.95	13.55	63.52	5.54	13.13

High resolution mass spectra (HRMS) (EI)

Compound	Formula	Calculated mass	Found Mass
1	$C_{22}H_{27}N_3O_3$	381.2052	381.2044
13	$C_{25}H_{35}N_3O_4$	441.2628	441.2536
14	$C_{26}H_{37}N_3O_5$	471.2733	471.2739
15	$C_{25}H_{35}N_3O_4$	441.2628	441.2634
20	$C_{24}H_{34}N_2O_5$	430.2468	430.2481
47	$C_{25}H_{31}N_3O_3 + Li^+$	428.2525	428.2519

(*continued on next page*)

Appendix A (*continued*)

Compound	Formula	Calculated mass	Found Mass
50	$C_{29}H_{41}N_3O_5 + H^+$	512.3124	512.3126
56	$C_{20}H_{23}N_3O_3 + Li^+$	360.1899	360.1896
60	$C_{21}H_{23}N_3O_5$	397.1638	397.1657
64	$C_{21}H_{23}N_3O_5 + Na^+$	370.0934	370.0935
67	$C_{22}H_{29}N_3O_3 + H^+$	384.2287	384.2282
68	$C_{26}H_{29}N_3O_3 + H^+$	432.2287	432.2292
72	$C_{21}H_{24}N_6O_2$	392.1961	392.1957

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ORIGINAL ARTICLE

Promotion of glioma cell survival by acyl-CoA synthetase 5 under extracellular acidosis conditions

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Extracellular acidosis (low pH) is a tumor microenvironmental stressor that has a critical function in the malignant progression and metastatic dissemination of tumors. To survive under stress conditions, tumor cells must evolve resistance to stress-induced toxicity. Acyl-CoA synthetase 5 (ACSL5) is a member of the ACS family, which converts fatty acid to acyl-CoA. ACSL5 is frequently overexpressed in malignant glioma, whereas its functional significance is still unknown. Using retrovirus-mediated stable gene transfer (gain of function) and small interfering RNA-mediated gene silencing (loss of function), we show here that ACSL5 selectively promotes human glioma cell survival under extracellular acidosis. ACSL5 enhanced cell survival through its ACS catalytic activity. To clarify the genome-wide changes in cell signaling pathways by ACSL5, we performed cDNA microarray analysis and identified an ACSL5-dependent gene expression signature. The analysis revealed that ACSL5 was critical to the expression of tumor-related factors including midkine (MDK), a heparin-binding growth factor frequently overexpressed in cancer. Knockdown of MDK expression significantly attenuated ACSL5-mediated survival under acidic state. These results indicate that ACSL5 is a critical factor for survival of glioma cells under acidic tumor microenvironment, thus providing novel molecular basis for cancer therapy.

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Introduction

Enhanced lipid biosynthesis occurs selectively in tumor cells and is closely linked with tumorigenesis (Menendez and Lupu, 2007). In tumor cells, the supply of cellular fatty acid is highly dependent on *de novo* synthesis, and several enzymes in the lipid biosynthesis pathways are involved in tumor cell survival (Brusselmans *et al.*, 2005; Hatzivassiliou *et al.*, 2005; Kuhajda, 2006). These observations suggest that mediators of lipid metabolism are newly recognized molecular targets to induce selective tumor cell death.

Acyl-CoA synthetases (ACSs) are enzymes that convert long-chain fatty acids to acyl-CoA. This reaction is a critical step in several lipid metabolic pathways, including phospholipid biosynthesis, lipid modification of cellular proteins and β-oxidation (Coleman *et al.*, 2002). ACSs are overexpressed in a variety of cancers (Cao *et al.*, 2000, 2001; Yamashita *et al.*, 2000; Sung *et al.*, 2003, 2007; Gassler *et al.*, 2005; Liang *et al.*, 2005; Yeh *et al.*, 2006). Moreover, our recent screening identified an ACS inhibitor as a tumor-selective inducer of apoptosis (Mashima *et al.*, 2005; Mashima and Tsuruo, 2005). These data suggest that ACSs are predominantly involved in tumor cell survival.

Acyl-CoA synthetase 5 (ACSL5) is a unique isozyme among the ACS members, as it is the only known ACS isozyme that localizes on mitochondria (Lewin *et al.*, 2001; Coleman *et al.*, 2002). In human glioma, aberrations occur on chromosome 10q25.1–q25.2, on which the ACSL5 gene is located, and ACSL5 is frequently overexpressed (Yamashita *et al.*, 2000). These observations strongly suggest potential functions of the enzyme in the growth or malignancy of glioma. At present, however, the precise functions of ACSL5 in cancer have not been elucidated.

Extracellular acidosis (low pH) is a tumor microenvironmental stressor (Vaupel *et al.*, 1989). Solid tumors are commonly characterized by a unique pathophysiologic microenvironment (Tannock and Rotin, 1989; Vaupel *et al.*, 1989; Tomida and Tsuruo, 1999). This hostile microenvironment activates several intracellular signaling pathways that promote malignant progression and metastatic dissemination (Harris, 2002;

Rofstad *et al.*, 2006; van den Beucken *et al.*, 2006). On the other hand, to survive under such stress conditions, tumor cells must also develop resistance to the microenvironmental stress-induced cytotoxicity (Graeber *et al.*, 1996), although the underlying mechanisms remain unclear.

Midkine (MDK) is a basic heparin-binding growth factor of low molecular weight, a member of the neurite growth-promoting factor family (Kadomatsu and Muramatsu, 2004). MDK shows highly increased expression in a number of malignant tumors (Nakagawara *et al.*, 1995; O'Brien *et al.*, 1996; Mishima *et al.*, 1997; Ye *et al.*, 1999; Ikematsu *et al.*, 2000; Jia *et al.*, 2007; Maeda *et al.*, 2007) and enhances tumor progression by promoting survival, growth, migration and angiogenic activity (Kadomatsu *et al.*, 1997; Takei *et al.*, 2001; Kadomatsu and Muramatsu, 2004; Mirkin *et al.*, 2005; Tong *et al.*, 2007). In human brain tumors, especially MDK is overexpressed during tumor progression, and patients whose tumors express a higher level of MDK have a worse prognosis (Mishima *et al.*, 1997).

In this study, we examined the function of ACSL5 in glioma cell survival under extracellular acidosis conditions. Moreover, the ACSL5-regulated gene signature was analysed. The analysis revealed that ACSL5 is a critical regulator of tumor-related genes including MDK.

Results

ACSL5 promotes human glioma cell survival under extracellular acidosis conditions

To clarify the function of ACSL5 in glioma cell survival, we examined the effect of its overexpression on cell survival under various tumor-related stress conditions. We initially examined the expression of endogenous ACSL5 in human glioma cell lines. As a result, we found two cell lines with low levels of ACSL5, SF268 and U251, and two cell lines with relatively high amounts of ACSL5, SNB78 and A1207 (data not shown; see Figure 2a). We stably transduced SF268 cells with a retroviral vector harboring a human ACSL5 gene with a FLAG tag at its carboxy end. Overexpression of FLAG-tagged ACSL5 in the transduced cells (SF268/ACSL5) was confirmed by immunoblot analysis (Figure 1a). Under normal culture conditions, both SF268/mock and SF268/ACSL5 cells showed similar growth rates (Supplementary Figure 1a). By contrast, SF268/ACSL5 showed markedly enhanced survival under extracellular acidosis conditions (pH 6.5) (Figures 1b and c). Similar results were obtained in another human glioma cell line, U251, when it was stably transduced with ACSL5 (data not shown). The major source of proton ion *in vivo* is lactic acid. Therefore, we also examined cell survival under low pH conditions (pH 6.3–6.5) that were generated by lactic acid. As a result, we found that ACSL5 expression also promoted cell survival under lactic acid-based low pH conditions (Supplementary Figure 1b). Extracellular acidosis (range pH 5.8–7.6) is known as one of the pathophysiologic microenvironmental stresses that are characteristically observed in solid tumors (Tannock and Rotin, 1989; Vaupel *et al.*, 1989; Tomida and Tsuruo, 1999). ACSL5-mediated promotion of survival was selective under acidosis conditions, as SF268/ACSL5 did not show apparent survival advantage under other tumor-related stresses such as hypoxia and low serum conditions (Figure 1d).

We have shown earlier that inhibition of total cellular ACS induces cell death through the activation of caspases, the cysteine proteases that have a central function in apoptosis induction (Mashima *et al.*, 2005). To characterize the molecular mechanisms of the reduced cell viability under low pH, we next examined the involvement of a caspase-mediated pathway. As shown in Supplementary Figure 2a, treatment with a specific caspase inhibitor, Z-VAD-fmk, did not recover the reduced SF268 cell viability under low pH. Consistently, caspase protease activity was not elevated in the cells exposed to extracellular acidosis and neither was it affected by ACSL5 expression (Supplementary Figure 2b). Flow cytometric analysis further revealed that the loss of viability under low pH did not accompany the emergence of the sub-G1 population, a characteristic of apoptotic cells (Supplementary Figure 2c). These results indicate that the reduced cell viability under acidosis is caspase-independent and non-apoptotic.

To confirm the function of ACSL5 under acidic conditions, a loss-of-function study was performed using the small interfering RNA (siRNA) against endogenous ACSL5. We found two ACSL5-overexpressed glioma cell lines, SNB78 and A1207 (Figure 2a), and used these cell lines for the loss-of-function study. When SNB78 cells were transfected with ACSL5-siRNAs (si1 and si2), the level of ACSL5 mRNA was clearly reduced in the ACSL5 siRNA-transfected cells (Supplementary Figure 3a). Consistently, the ACSL5 protein was decreased in the SNB78 cells treated with ACSL5 siRNAs (Figure 2b). We found that the inhibition of ACSL5 expression significantly reduced cell viability under the acidic state (pH 6.5) (Figure 2c, right), whereas it did not influence cell survival under normal conditions (pH 7.3) in SNB78 cells (Figure 2c, left and Supplementary Figure 3b). We observed similar results in A1207 cells (Figures 2b and d), except for slight suppression of A1207 cell growth under normal conditions (pH7.3) by one of the ACSL5 siRNAs (siRNA 1). The growth inhibition by siRNA1 could result from its off-target effect, as the other ACSL5 siRNA (siRNA 2) did not show any growth inhibitory effect under normal conditions. By contrast, the inhibition of ACSL5 expression did not reduce cell viability under low serum conditions (Supplementary Figure 3c). To clarify the function of overexpressed ACSL5 in *in vivo* growth of tumor, we further tested the effect of ACSL5 siRNA treatment on ACSL5-overexpressed tumor. For this study, we chose human glioma A1207 cells, as they overexpress endogenous ACSL5 and are tumorigenic in nude mice (Mishima *et al.*, 2001). As a result, we found that *in vivo* treatment

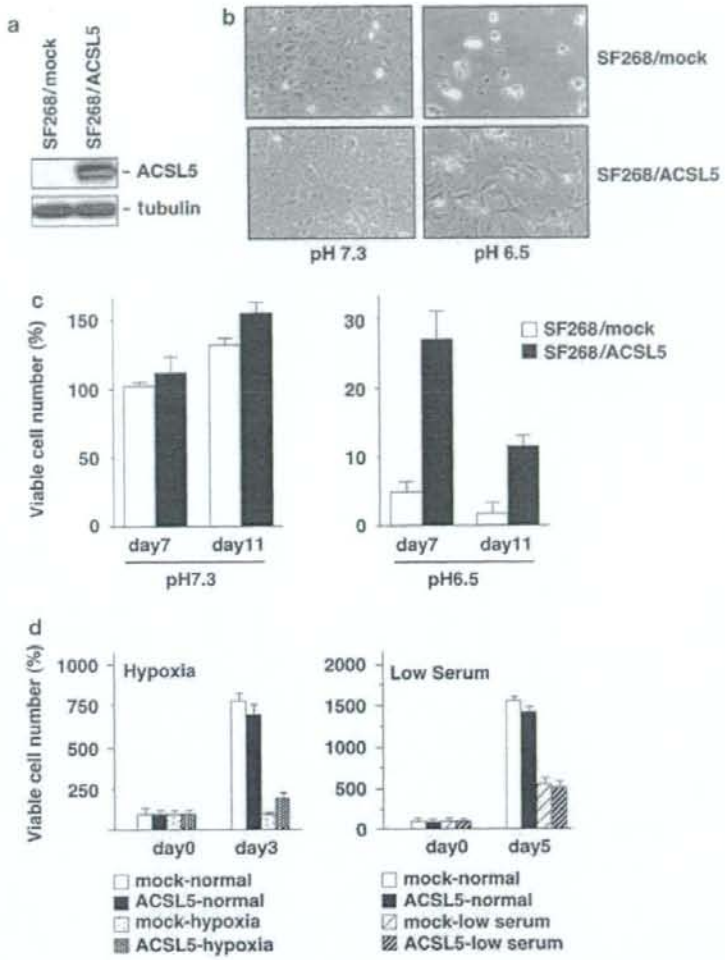

Figure 1 Acyl-CoA synthetase 5 (ACSL5) promotion of survival of human glioma SF268 cells under extracellular acidosis conditions. (**a**) The expression of FLAG epitope-tagged ACSL5 in transduced SF268 cells as revealed by western blot analysis with an anti-ACSL5 antibody. The expressions of α-tubulin were measured as loading controls. (**b** and **c**) Cells were initially seeded on day 0 and maintained under normal (pH 7.3) or acidic (pH 6.5) conditions. Morphologies of the cells on day 7 are shown in (**b**). Cell numbers were counted on days 7 and 11 (**c**). Data are mean values of three independent experiments, and error bars show standard deviations. (**d**) Cells were initially seeded on day 0 and maintained at normal pH levels under hypoxic or low serum (0.1% fetal bovine serum (FBS)) culture conditions. Cell numbers were counted on days 0 and 3 (for hypoxia treatment) or on days 0 and 5 (for low serum treatment). Data are mean values of three independent experiments, and error bars show standard deviations.

with the ACSL5 siRNAs significantly suppressed the growth of A1207 tumor (Supplementary Figure 3d). These results indicate that ACSL5 selectively promotes glioma cell survival under extracellular acidosis and could have a function in tumor survival *in vivo*.

ACSL5 catalytic activity-dependent cell survival under extracellular acidosis conditions

To test whether ACS catalytic activity is required for ACSL5-mediated promotion of survival under acidosis, we constructed an inactive mutant of ACSL5 (ACSL5-MT) (Figure 3a; see Materials and methods). When retrovirally transduced in SF268 cells, the ACSL5-MT protein was expressed stably at a similar level as wild-type ACSL5 (Figure 3b). On the other hand, ACS activity was exclusively elevated in ACSL5-expressed cells but not in ACSL5-MT-expressed cells (Figure 3c), indicating that the ACSL5-MT is actually an inactive mutant. We compared cell survival of these cells under normal and low pH conditions. As shown in Figure 3d, the ACSL5-MT-expressed cells had no survival advantage under acidosis conditions, whereas the wild-type ACSL5-expressed cells did so. These results indicate that

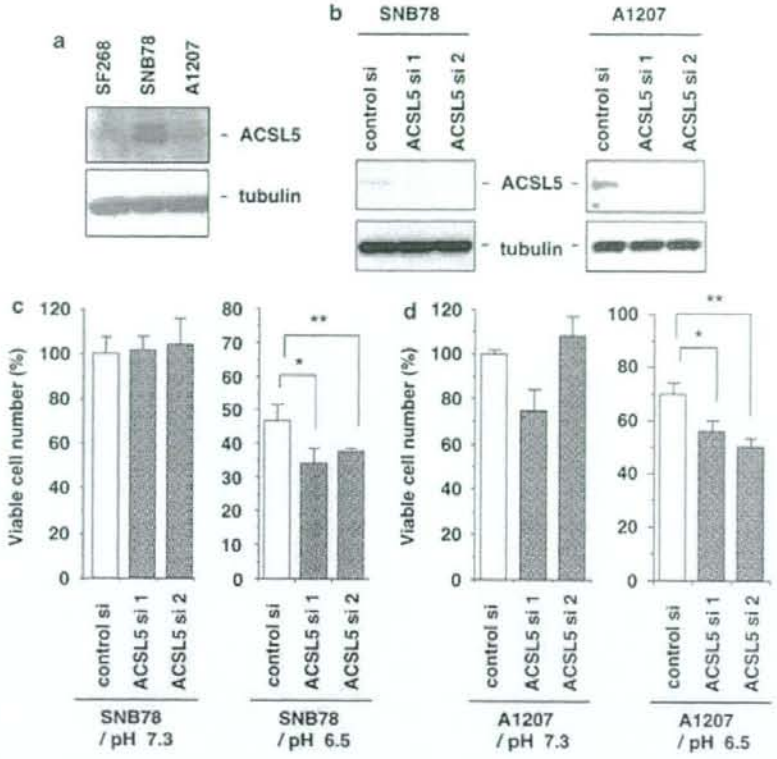

Figure 2 Involvement of endogenously overexpressed acyl-CoA synthetase 5 (ACSL5) in survival of human glioma SNB78 cells under extracellular acidosis conditions. (**a**) Protein expression of endogenous ACSL5 in human glioma cell lines as revealed by western blot analysis with an anti-ACSL5 antibody. The expressions of α-tubulin were measured as loading controls. (**b**) Protein expression of ACSL5 in cells treated with siRNAs. SNB78 and A1207 cells were treated with ACSL5 siRNAs or control siRNA and cultured for 48 h under acidic conditions (pH 6.5). Cell lysates were then prepared, and the expressions of endogenous ACSL5 were detected by an anti-ACSL5 antibody. (**c** and **d**) Viability of SNB78 and A1207 cells after ACSL5 knockdown under normal and acidic conditions. SNB78 and A1207 cells treated with ACSL5-targeted siRNAs or with control siRNA were cultured under normal (pH 7.3) or low pH (pH 6.5) conditions for 4 and 6 days, respectively. Viable cell numbers were counted. Data are mean values of three independent experiments, and error bars show standard deviations. *P*-values (two-sided) were calculated using the Student's *t*-test. *P*-values of <0.05 were considered statistically significant. $^{**}P<0.01$; $^{*}P<0.05$.

ACSL5 promotes survival under low pH conditions through its ACS catalytic activity.

A previous report has shown that ACSL5 selectively promotes the uptake of extracellular palmitic acid. Moreover, palmitic acid enhances the growth of U87MG human glioma cells overexpressed with ACSL5 (Yamashita *et al.*, 2000). Therefore, we examined the involvement of extracellular palmitic acid on cell survival under acidosis. However, palmitic acid treatment did not affect cell viability under acidic conditions in SF268 cells (Supplementary Figure 4a). This result indicates that extracellular palmitic acid is not involved in cell survival under low pH.

ACSL5 localizes on mitochondria and is thought to be involved in β-oxidation of fatty acids (Coleman *et al.*, 2002). As the β-oxidation pathway leads to a cellular energy supply through ATP production, we speculated that the supply of ATP through ACSL5-mediated β-oxidation could be critical for survival promotion under acidic stress. To test this hypothesis, we examined the change in the cellular ATP level after exposure to acidosis. As shown in Supplementary Figure 4b, the ATP level was steeply downregulated under acidosis. This decrease in ATP level was not recovered by ACSL5 overexpression. These results suggest that the ATP level could not be a critical factor for the ACSL5-mediated promotion of glioma cell survival under acidosis.

Upregulation of tumor-related factors by ACSL5 under extracellular acidosis conditions

To clarify the molecular mechanisms of ACSL5-dependent survival, we undertook Affymetrix GeneChip (Human Genome U133 plus 2) analysis and characterized the global program of transcription that reflects the cellular response to extracellular acidosis and the effect of ACSL5 overexpression on it. We hypothesized that extracellular acidosis could either induce a set of cell

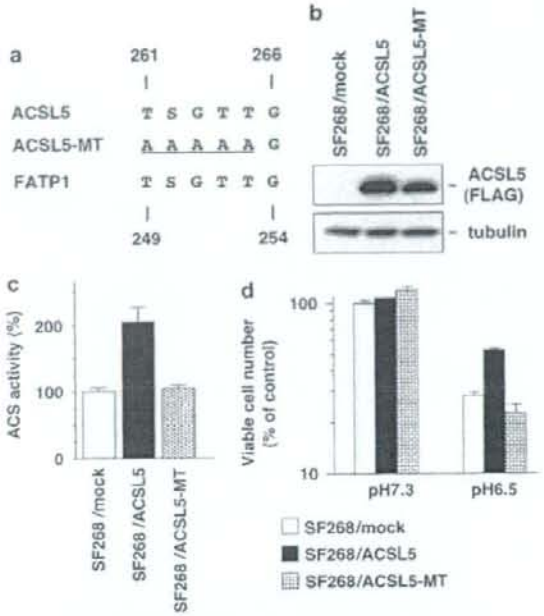

Figure 3 Acyl-CoA synthetase 5 (ACSL5) catalytic activity-dependent cell survival under extracellular acidosis conditions. (**a**) The amino-acid sequences of the putative active site in ACSL5 and FATP1. The amino-acid sequence, TSGTT (261–265), in wild-type ACSL5 was converted to AAAAA in ACSL5-MT. (**b**) The expression of FLAG epitope-tagged ACSL5 or ACSL5-MT in transduced SF268 cells as revealed by western blot with monoclonal anti-FLAG antibody. The expressions of α-tubulin were measured as loading controls. (**c**) ACS activities in ACSL5- or ACSL5-MT-transduced SF268 cells. The ACS assay was performed as described in Materials and methods. (**d**) Cells were seeded as in Figure 1c (day 0) and maintained under normal (pH 7.3) or acidic (pH 6.5) conditions. Cell numbers were counted on day 5. Data are mean values of three independent experiments, and error bars show standard deviations.

death-inducing and growth inhibitory factors or attenuate a set of genes that are required for cell survival. ACSL5 could prevent such genetic alterations. To test these hypotheses, we identified genes that are significantly induced or decreased after low pH treatment of SF268 cells. First, we extracted 229 genes in which the expression levels were altered by more than threefold during the 6-day exposure to extracellular acidosis. Second, we compared the expressions of these genes in SF268/ACSL5 cells with those in SF268/mock cells. Overall, the induction or reduction patterns were similar between the two cell lines (Supplementary Figure 5), suggesting that ACSL5 does not attenuate general stress responses to low pH but rather that some specific signals activated by ACSL5 could be involved in selective survival under low pH conditions. Therefore, we focused on genes in which the expressions were specifically regulated by ACSL5. Because ACSL5 promoted survival under acidosis conditions through its ACS catalytic activity, we tried to identify genes in which induction or decrease by ACSL5 depended on ACS catalytic activity. To determine this, we extracted genes that were up- or downregulated exclusively in SF268/ACSL5 (more than twofold) but not in SF268/ACSL5-MT cells (less than 1.3-fold over control SF268/mock cells) under extracellular acidosis conditions. As shown in Table 1, the expressions of 18 genes were significantly changed by ACSL5 overexpression. Importantly, the genes overexpressed by ACSL5 included two tumor-related genes, MDK and the melanoma cell adhesion molecule (MCAM). MDK is a growth factor frequently overexpressed in malignant tumors, and it promotes cancer cell survival (Kadomatsu and Muramatsu, 2004). MCAM is a cell surface adhesion molecule that is strongly expressed in metastatic melanoma and involved in tumorigenicity and metastasis (Xie *et al*., 1997). Our additional GeneChip analysis further revealed that these two genes were included in a set of genes in which the expressions were significantly reduced in SNB78 cells when treated with ACSL5 siRNAs (data not shown). Meanwhile, there have been no reports that describe tumor-related function of other ACSL5-regulated genes listed here.

ACSL5-dependent expression of MDK supports glioma cell survival under extracellular acidosis conditions

We focused on the MDK and MCAM genes, because our GeneChip analysis showed that their expressions were closely linked with ACSL5, and they have been reported to be associated with the malignant phenotype of cancer. These two genes were clearly induced by ACSL5 under low pH conditions in an ACS catalytic activity-dependent manner (Figure 4a, experiment 1). Time course analysis revealed that MDK was induced by extracellular acidosis, and the expression was strongly enhanced in SF268/ACSL5 cells. On the other hand, MCAM expression was decreased under low pH, and the decrease was prevented by ACSL5 overexpression (Figure 4a, experiment 2). To confirm their expression patterns, we performed reverse transcription–PCR analysis. As shown in Figure 4b, both MDK and MCAM mRNAs were clearly induced by ACSL5 overexpression under acidic conditions. Correspondingly, when endogenous ACSL5 was decreased by specific siRNAs, the expressions of MDK and MCAM were downregulated under low pH. Western blot analysis of protein expression further confirmed that ACSL5 enhances MDK expression, especially under acidic conditions, through its catalytic activity (Figure 4c).

To determine the function of these factors in glioma cell survival under acidosis, we examined the effect of siRNA-mediated knockdown on SF268/ACSL5 cell survival under low pH conditions. As shown in Figures 5a and b, when MDK expression in SF268/ACSL5 cells was attenuated by specific siRNAs, the decrease of MDK protein was also observed. The inhibition of MDK expression markedly reduced cell viability under acidic conditions (pH 6.5) (Figure 5c), whereas it did not influence cell survival under normal conditions (pH 7.3)

Table 1 ACSL5-regulated genes in glioma SF268 cells

Probe set ID	*Gene title*	*Gene symbol*	*Experiment 1 (fold change)*[a]			*Experiment 2 (fold change)*[b]						
			pH 6.5 (day 6)			*Mock (pH 6.5)*			*ACSL5*			
			Mock	*ACSL5*	*ACSL5-MT*	*day 0*	*day 3*	*day 6*	*day 0*	*day 3*	*day 6*	
237411_at	ADAM metallopeptidase with thrombospondin type 1 motif, 6	ADAMTS6	1.00	3.67	1.08	0.66	1.14	1.00	0.93	1.77	2.24	Increased by ACSL5
209087_x_at	Melanoma cell adhesion molecule	MCAM	1.00	2.94	1.24	2.84	1.66	1.00	2.65	2.31	2.10	
209035_at	Midkine (neurite growth-promoting factor 2)	MDK	1.00	2.35	1.02	0.71	0.77	1.00	1.61	1.59	2.60	
205206_at	Kallmann syndrome 1 sequence	KAL1	1.00	2.24	1.30	1.98	1.29	1.00	4.02	3.18	3.08	
219118_at	FK506-binding protein 11, 19 kDa	FKBP11	1.00	2.14	0.95	0.57	0.64	1.00	1.75	2.42	4.23	
205100_at	Glutamine-fructose-6-phosphate transaminase 2	GFPT2	1.00	2.13	0.95	0.40	0.31	1.00	0.65	0.65	2.00	
205304_s_at	Potassium inwardly rectifying channel, subfamily J, member 8	KCNJ8	1.00	2.09	1.27	0.66	1.02	1.00	1.32	2.34	1.98	
220673_s_at	KIAA1622	KIAA1622	1.00	2.09	1.20	1.31	1.36	1.00	2.06	2.28	3.13	
209803_s_at	Pleckstrin homology-like domain, family A, member 2	PHLDA2	1.00	2.05	0.95	0.69	1.12	1.00	1.61	2.26	2.29	
234472_at	GalNAc-T13	GALNT13	1.00	0.48	1.13	1.38	1.03	1.00	0.30	0.34	0.33	Decreased by ACSL5
1555912_at	ST7 overlapping transcript 1 (antisense non-coding RNA)	ST7OT1	1.00	0.48	1.12	0.81	0.66	1.00	0.50	0.42	0.55	
219503_s_at	Transmembrane protein 40	TMEM40	1.00	0.43	1.06	0.73	1.01	1.00	0.14	0.28	0.41	
222892_s_at	Microtubule-associated protein 2	MAP2	1.00	0.42	1.01	0.79	1.06	1.00	0.21	0.29	0.40	
203108_at	G-protein-coupled receptor, family C, group 5, member A	GPRC5A	1.00	0.41	1.06	1.38	1.04	1.00	0.65	0.68	0.63	
212444_at	CDNA clone IMAGE:6025865	—	1.00	0.38	0.76	0.59	0.70	1.00	0.28	0.37	0.50	
214156_at	Myosin VIIA and Rab interacting protein	MYRIP	1.00	0.36	0.84	1.18	1.50	1.00	0.46	0.53	0.49	
235301_at	KIAA1324-like	KIAA1324L	1.00	0.27	1.03	0.86	0.86	1.00	0.38	0.42	0.54	
212094_at	Paternally expressed 10	PEG10	1.00	0.15	1.19	1.40	1.49	1.00	0.44	0.45	0.31	

Abbreviation: ACSL5, acyl-CoA synthetase 5.
[a]In experiment 1, SF268/mock, /ACSL5 and /ACSL5-MT cells were cultured under acidic (pH 6.5) conditions for 6 days. The values of relative expression changes were calculated over mock-transfected SF268 cells as a control.
[b]In experiment 2, SF268/mock and /ACSL5 cells were cultured under acidic (pH 6.5) conditions for 0, 3 and 6 days. The values of relative expression changes were calculated over SF268/mock cells at pH 6.5 at day 6 as a control.

(Supplementary Figure 6a) or under low serum conditions (Supplementary Figure 6b). By contrast, the knockdown of MCAM did not influence cell viability under either normal or acidic pH (data not shown).

Collectively, these results indicate that ACSL5 is functionally involved in glioma cell survival under acidic tumor microenvironment. Our data further revealed that ACSL5-dependent expression of MDK is a critical factor for survival.

Discussion

Extracellular acidosis is an important factor in the malignant progression of tumors (Rofstad *et al.*, 2006), and tumor cells must develop resistance to this stress-induced cytotoxicity. Under tumor microenvironmental stresses, the defect in the p53 tumor suppressor protein is a critical factor for apoptosis resistance and cancer cell survival (Soengas *et al.*, 1999). However, low pH stress inhibits cell growth in a p53-independent manner, suggesting the involvement of other mechanisms (Reichert *et al.*, 2002). Our results suggest that enhanced cell survival by ACSL5 under low pH conditions could have a function in the progression of cancer.

Predominant function for ACSL5 in glioma cell survival

Elevated levels of fatty acid metabolism have a critical function in the malignant growth of tumors (Menendez and Lupu, 2007). Among fatty acid metabolic enzymes, ACS members catalyse an essential step in both the catabolic pathway for fatty acid degradation through the β-oxidation system and the anabolic pathway for cellular lipid synthesis (Coleman *et al.*, 2002). In this study, we showed that ACSL5 was involved in the promotion of glioma cell survival under extracellular acidosis conditions. In human glioma, aberrations are frequently observed on chromosome 10q25.1–q25.2, on which the ACSL5 gene is located and, in fact, the ACSL5 overexpression is highly correlated with malignancy of the tumors (Yamashita *et al.*, 2000). We further sequenced the ACSL5 gene in human glioma cell lines that overexpress ACSL5. We found that wild-type ACSL5 is overexpressed in A1207 and A172 cell lines (unpublished data). In the ACSL5 gene extracted from SNB78 cells, we found one amino-acid difference (M182V) when it was compared with the reported wild-type human ACSL5 gene (data not shown). However, this sequence is not conserved among species, indicating that this amino-acid sequence is not essential for functional ACS activity. These data indicate that