Supporting Information

Efficient Delivery of Streptavidin to Mammalian Cells: Clathrin-Mediated Endocytosis Regulated by a Synthetic Ligand

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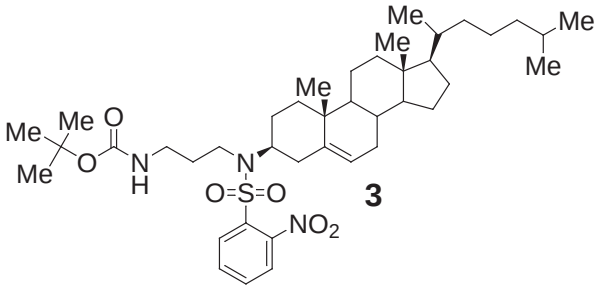
Experimental Section

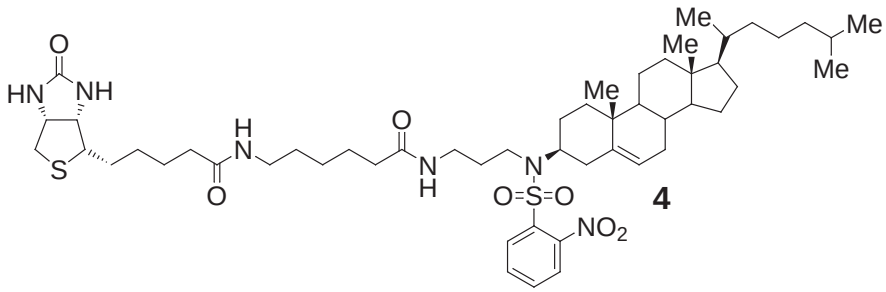
General. Chemical reagents and solvents were obtained from Aldrich, Alfa Aesar, or Fluka. Media and antibiotics were obtained from Gibco BRL. Streptavidin Alexa Fluor 488, Cholera toxin Alexa Fluor 594, and Lysotracker Red were purchased from Molecular Probes. Chlorpromazine and methyl-β-cyclodextrin were obtained from Sigma. Reactions were performed under an atmosphere of dry argon. Methanol (CH_3OH) was spectral-grade. Reactions were monitored by analytical thin-layer chromatography on plates coated with 0.25 mm silica gel 60 F_{254} (EM Science). Flash chromatography employed 230 – 240 mesh silica gel (EM science). TLC plates were visualized by UV irradiation (254 nm) or stained with a solution of phosphomolybdic acid and sulfuric acid in ethanol (1:1:20). Melting points were measured with a Thomas Hoover capillary melting point apparatus and are uncorrected. Infrared spectra were obtained with a Perkin Elmer 1600 Series FTIR. NMR spectra were obtained with a Bruker DRX 400 instrument and are reported in parts per million (ppm, δ) referenced to internal $CHCl_3$ (7.26 and 77.0 ppm). Mass spectra were run by the Penn State University Mass Spectrometry Facility on Perseptive Voyager DE-STR MALDI, Mariner APCI, or Kratos MS50TC FAB instruments. Peaks are reported as *m/z*. Micrographs were captured through a Zeiss Fluar (100 X) objective by a Zeiss Axiocam digital camera interfaced to a Zeiss Axiovert S100TV microscope. Images were processed with Adobe Photoshop 5.0.



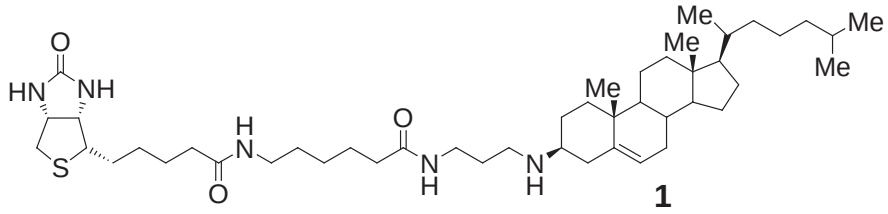
3

***N*-[3-(*tert*-butoxycarbonylamino)propyl]-*N*-cholesteryl-2-nitrobenzenesulfonamide (3).**

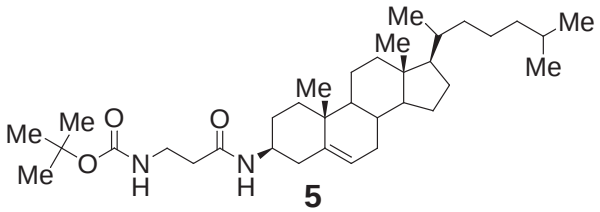
N-cholesteryl-2-nitrobenzenesulfonamide (**2**) (500 mg, 0.87 mmol, prepared as described in *J. Am. Chem. Soc*. **2001**, *123*,12712-12713), potassium carbonate (600 mg, 4.35 mmol) and boc-3-chloropropylamine (252 mg, 1.3 mmol, prepared as described in *J. Am. Chem. Soc*. **1996**, 118, 715 – 721) were added to dimethylacetamide (DMA, 10 mL). This mixture was heated to 120 °C for 12 h. The reaction was filtered to remove excess potassium carbonate and CH_2Cl_2 (50 mL) was added. The solution was washed twice with aqueous NaOH (1 M, 25 mL) and then twice with aqueous citric acid (10%, 25 mL). The organic layer was dried ($NaSO_4$) and removed *in vacuo*. Flash chromatography (2% MeOH in CH_2Cl_2) afforded **3** (595 mg, 94%), mp 158 – 159 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.01 – 7.99 (m, 1H), 7.67 – 7.65 (m, 2H), 7.6 – 7.58 (m, 1H), 5.22 (br s, 1H), 4.85 (m, 1H), 3.64 – 3.57 (t, *J* = 12 Hz, 1H), 3.46 (s, 1H), 3.36 (t, *J* = 7.1 Hz, 2H), 3.18 (m, 2H), 2.44 (t, *J* = 12 Hz, 1H), 2.0 – 1.9 (m, 3H), 1.9 (br s, 1H), 1.84 – 1.74 (m, 5H), 1.71 – 1.65 (m, 2H), 1.58 – 1.48 (m 4H), 1.43 (s, 9H), 1.38 – 1.25 (m, 5H), 1.15 – 1.06 (m, 7H), 0.94 (s, 3H), 0.90 (m, 4H), 0.88 (m, 6H), 0.65 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 156.5, 148.4, 140.4, 134.5, 133.7, 131.9, 131.1, 124.5, 122.8, 79.6, 59.3, 57.0, 56.5, 50.3, 42.6, 42.1, 40.0, 39.9, 38.6, 38.0, 36.9, 36.5, 36.1, 32.5, 32.1, 28.8 (x3), 28.7, 28.6, 28.4, 27.7, 24.6, 24.2, 23.2, 23.1, 23.0, 21.3, 19.7, 19.1, 12.3; IR (film) ν_{max} 3400, 2800, 1700, 1550, 1320 cm^{-1}; MALDIFTMS (DHB) *m/z* 728.4669 (M H$^+$, $C_{41}H_{65}N_3O_6S$ requires 728.4672).



***N*-{6-[3-(*N*-cholesteryl-2-nitrobenzenesulfonamido)propylamino]-6-oxohexyl}biotinamide (4).**
Trifluoroacetic acid (2% in CH_2Cl_2, 10 mL) was added to **3** (50 mg, 0.069 mmol) at 25 °C for 45 min. TLC analysis (2% MeOH in CH_2Cl_2) revealed conversion to the more polar primary amine. Aqueous NaOH (1 M, 20 mL) was added, and the mixture was extracted with CH_2Cl_2 (3 x 10 mL). The organic layer was dried ($NaSO_4$) and removed *in vacuo*. To the remaining residue was added CH_2Cl_2 (10 mL), D-biotinamidocaproate N-hydroxysuccinimidyl ester (38 mg, 0.083 mmol), and diisopropylethylamine (DIEA, 13 µL, 0.076 mmol). This mixture was stirred for 2 h at 25 °C. Aqueous citric acid (10%, 25 mL) was added and the mixture was extracted with CH_2Cl_2 (2 x 15 mL). The organic layer was dried ($NaSO_4$) and removed *in vacuo*. Flash column chromatography (8% MeOH in CH_2Cl_2) afforded **4** (49 mg, 74%), mp 135 – 137 °C; ^{1}H NMR (300 MHz, $CDCl_3/CD_3OD$, (1:1 v/v)) δ 7.99 (m, 1H), 7.7 – 7.68 (m, 2H), 7.62 (m, 1H), 6.66 (m, 2H), 5.2 (br s, 1H), 4.5 (m, 2H), 3.63 (t, *J* = 12 Hz, 1H), 3.36 – 3.14 (m, 6H), 2.94 – 2.7 (m, 3H), 2.46 (t, *J* = 12 Hz, 1H), 2.00 – 1.82 (m, 8H), 1.66 (br m, 8H), 1.5 – 1.31 (m, 12H), 1.24 (s, 3H), 1.1 (m, 8H), 0.94 – 0.84 (m, 20H), 0.64 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3/CD_3OD$, (1:1 v/v)) δ 174.1, 173.9, 162.4, 148.4, 140.4, 134.2, 134.0, 132.1, 130.9, 124.5, 122.8, 88.2, 63.0, 59.5, 57.0, 56.5, 56.0, 53.8, 50.4, 42.7, 41.3, 40.0, 39.9, 39.6, 38.6, 38.0, 36.9, 36.8, 36.7, 36.6, 36.5, 36.2, 32.3, 32.2, 32.1, 29.3, 28.6, 28.4, 28.3, 28.2, 27.9, 26.7, 26.1, 25.6, 24.6, 24.2, 23.2, 22.9, 21.3, 19.8, 19.1, 12.2; IR (film) ν_{max} 3350, 2800, 1710, 1600, 1320 cm^{-1}; MALDIFTMS (DHB) *m/z* 967.5676 (M H$^+$, $C_{52}H_{82}N_6O_7S_2$ requires 967.5764).

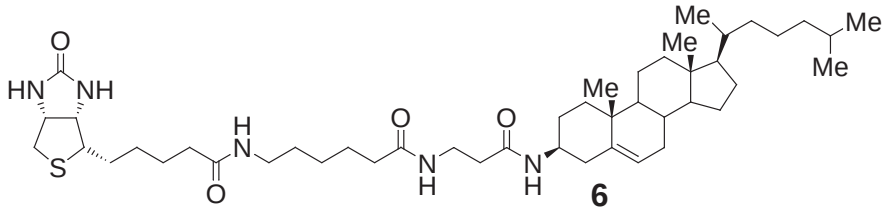


***N*-{6-[3-(cholesterylamino)propylamino]-6-oxohexyl}biotinamide (1).**
Compound **4** (35 mg, 0.036 mmol), thiophenol (15 mg, 0.144 mmol), and K_2CO_3 (50 mg, 0.36 mmol) were added to dimethylformamide (5 mL) and stirred for 18 h at 25 °C. The mixture was filtered and the solvent removed *in vacuo*. Flash chromatography (15% MeOH / 2% triethylamine in CH_2Cl_2) afforded **1** (18 mg, 65%) mp 195 °C, dec.; ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$ (3:1 v/v)) δ 5.14 (br s, 1H), 4.21 (m, 1H), 4.01, (m, 1H), 3.01 (m, 2H), 2.98 (t, *J* = 6 Hz, 2H), 2.87 (m, 4H), 2.62 (m, 3H), 2.43 (m, 1H), 2.09 (m, 2H), 1.89 (m, 4H), 1.71 – 1.56 (m, 7H), 1.55 (m, 3H), 1.4 – 1.18 (m, 10H), 1.1 – 1.08 (m, 12H), 1.06 – 0.90 (m, 8H), 0.89 – 0.8 (m, 3H), 0.55 – 0.54 (m, 12H), 0.38 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3/CD_3OD$ (3:1 v/v)) δ 174.9, 173.6, 164.1, 137.3, 122.7, 61.1, 59.3, 57.0, 55.7, 55.2, 54.8, 49.1, 41.3, 41.0, 39.2, 38.7, 38.5, 38.1, 36.2, 35.8, 35.2, 34.8, 34.7, 34.6, 34.5 (x2), 34.1, 30.8, 27.0, 27.5, 27.2 (x2), 27.0, 25.7, 25.4, 24.7, 24.5, 24.4, 23.2, 22.8, 21.5, 21.2, 20.0, 17.9, 17.5, 10.6; IR (film) ν_{max} 3300, 2850, 1700, 1310 cm^{-1}; MALDIFTMS (DHB) *m/z* 782.5940 (M H$^+$, $C_{46}H_{79}N_5O_3S$ requires 782.5982).



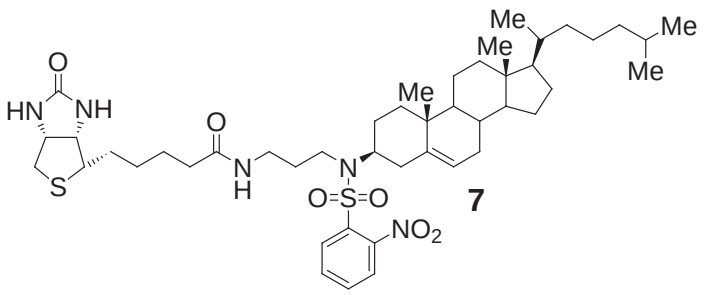
5

3-(*tert*-butoxycarbonylamino)-*N*-cholesterylpropionamide (5).
N-cholesteryl-2-nitrobenzenesulfonamide (**2**) (60 mg, 0.105 mmol), thiophenol (46 mg, 0.421 mmol), and potassium carbonate (142 mg, 1.05 mmol) were added to dimethylformamide (7 mL) and stirred for 18 h at 25 °C. The mixture was filtered and TLC analysis (2% MeOH in CH_2Cl_2) revealed conversion to the more polar cholesterylamine. Aqueous NaOH (1M, 20 mL) was added, and the mixture was extracted with CH_2Cl_2 (3 x 10 mL). The organic layer was dried ($NaSO_4$) and removed *in vacuo*. CH_2Cl_2 (10 mL), N-β-*t*-Boc-β-alanine N-hydroxysuccinimide ester (45 mg, 0.157 mmol), and diisopropylethylamine (DIEA, 40 μL, 0.157 mmol) were added. This mixture was stirred for 2 h at 25 °C. Aqueous citric acid (10%, 25 mL) was added and the mixture was extracted CH_2Cl_2 (2 x 15 mL). The organic layer was dried ($NaSO_4$) and removed *in vacuo*. Flash chromatography (5% MeOH in CH_2Cl_2) afforded **5** (50 mg, 86%), mp 178 – 179 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.75 (s, 1H), 5.34 bs, 1H), 5.18 (bs, 1H), 3.66 (m, 1H), 3.51 (m, 1H), 3.37 (m, 2H), 2.82 (m, 2H), 2.36 (t, *J* = 5.5 Hz, 2H), 2.25 (m, 1H), 2.07 (m, 1H), 1.84 (m, 2H), 1.79 (m, 3H), 1.55 – 1.47 (m, 5H), 1.42 (s, 9H), 1.34 – 1.10 (m, 13H), 1.00 (s, 3H), 0.99 (m, 3H), 0.89 (m, 6H), 0.66 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.8, 169.4, 140.5, 122.3, 79.7, 57.0, 56.5, 50.4, 50.1, 42.6, 40.1, 39.9, 39.6, 38.2, 37.1, 36.9, 36.5, 36.1, 32.5, 32.2, 29.4, 28.8 (x3), 28.6, 28.4, 25.9, 24.6, 24.2, 23.2, 22.9, 21.3, 19.7, 19.1, 12.2; IR (film) ν_{max} 3400, 2850, 2300, 1750, cm^{-1}; MALDIFTMS (DHB) *m/z* 557.4679 (M H^+, $C_{35}H_{60}N_2O_3$ requires 557.4682).

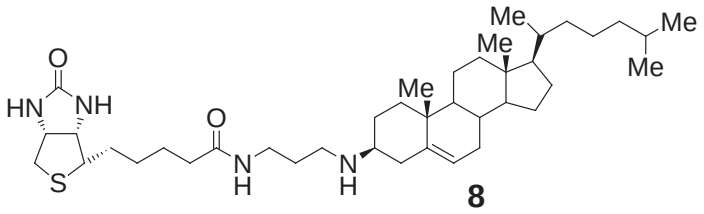


6

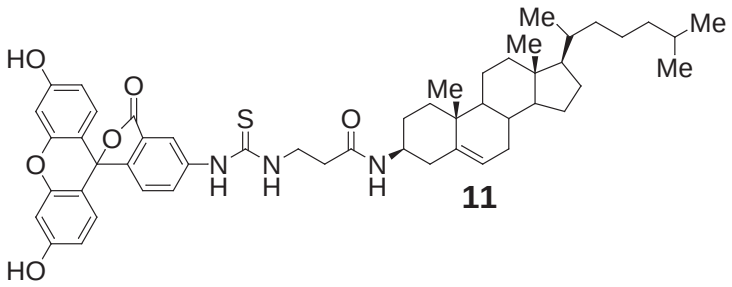
***N*-{6-[3-(cholesterylamino)-3-oxopropylamino]-6-oxohexyl}biotinamide (6).** Compound **5** (40 mg, 0.072 mmol) was treated with trifluoroacetic acid (2% in CH_2Cl_2, 10 mL) at 25 °C for 45 min. NaOH (1M, 20 mL) was added and the mixture was extracted with CH_2Cl_2 (3 x 10 mL). The organic layer was dried ($NaSO_4$) and removed *in vacuo*. CH_2Cl_2 (10 mL), D-biotinamidocaproate N-hydroxysuccinimide ester (49 mg, 0.108 mmol), and diisopropylethylamine (DIEA, 40 μL, 0.157 mmol) were added. This mixture was stirred for 2 h at 25 °C. Aqueous citric acid (10%, 25 mL) was added and the mixture extracted with CH_2Cl_2 (2 x 15 mL). The organic layer was dried ($NaSO_4$) and removed *in vacuo*. Column chromatography (15% MeOH in CH_2Cl_2) afforded **6** (48 mg, 84%) mp 220 °C, dec.; ^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD (1:1 v/v)) δ 4.98 (br s, 1H), 4.10 (m, 1H), 3.89 (m, 1H), 3.00 (m, 1H), 2.95 (t, *J* = 5.9 Hz, 2H), 2.78 (m, 4H), 2.5 (m, 3H), 2.29 (m, 2H), 1.95 (t, *J* = 6.7 Hz, 2H), 1.74 (m, 8H), 1.28 (m, 2H), 1.14 – 0.92 (m, 24H), 0.85 (s, 6H), 0.77 (m, 8H), 0.6 (s, 3H), 0.44 (m, 6H), 0.28 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$/CD_3OD (1:1 v/v)) δ 174.1, 173.9, 170.1, 169.3, 139.8, 120.9, 61.4, 59.7, 56.1, 55.4, 55.0, 49.5, 49.1, 48.3, 41.5, 39.3, 38.7, 38.4, 37.9, 37.2, 36.2, 35.8, 35.4, 35.1 (x2), 34.9, 31.2, 31.1, 28.6, 28.1, 27.8, 27.4, 27.2 (x2), 25.6, 24.9, 24.6, 23.4, 23.0, 21.6, 21.3 (x2), 20.2, 18.2, 17.6, 10.7; IR (film) ν_{max} 3450, 2820, 2100, 1710, cm^{-1}; MALDIFTMS (DHB) *m/z* 796.5756 (M H^+, $C_{46}H_{77}N_5O_4S$ requires 796.5774).



***N*-[3-(*N*-cholesteryl-2-nitrobenzenesulfonamido)propyl]biotinamide (7).**
Compound **3** (50 mg, 0.069 mmol) was treated with trifluoroacetic acid (2% in CH_2Cl_2, 10 mL) at 25 °C for 45 min. TLC analysis (2% MeOH in CH_2Cl_2) revealed conversion to the more polar primary amine. Aqueous NaOH (1M, 20 mL) was added, and the mixture was extracted with CH_2Cl_2 (3 x 10 mL). The organic layer was dried ($NaSO_4$) and removed *in vacuo*. CH_2Cl_2 (10 mL), D-biotin N-hydroxysuccinimide ester (28 mg, 0.083 mmol), and diisopropylethylamine (DIEA, 13 µL, 0.0759 mmol) were added. This mixture was stirred for 2 h at 25 °C. Aqueous citric acid (10%, 25 mL) was added, and the mixture was extracted with CH_2Cl_2 (2 x 15 mL). The organic layer was dried ($NaSO_4$) and removed *in vacuo*. Flash chromatography (8% MeOH in CH_2Cl_2) afforded **7** (46 mg, 79%), mp 131 – 133 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.95 – 7.93 (m, 1H), 7.69 – 7.64 (m, 2H), 7.59 – 7.57 (m, 1H), 5.22 (br s, 1H), 4.47 (m, 2H), 4.29 (m, 2H), 3.61 (m, 1H), 3.33 – 3.26 (m, 4H) 3.11 (m, 1H), 2.87 – 2.81 (m, 3H), 2.78 (m, 2H), 2.48 (t, *J* = 12 Hz, 1H), 2.4 (m, 1H), 2.23 (m, 2H), 1.94 (m, 3H), 1.82 – 1.74 (m, 3H), 1.69 – 1.60 (m, 6H), 1.51 – 1.29 (m, 12H), 1.09 (m, 6H), 0.97 – 0.83 (m, 14H), 0.63 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.5, 169.9, 169.1, 164.6, 148.4, 140.4, 134.2, 133.9, 132.1, 124.4, 62.1, 60.6, 59.5, 57.0, 56.4, 56.1, 55.9, 51.9, 50.3, 42.6, 42.2, 40.9, 40.0, 39.8, 38.6, 37.9, 36.9, 36.7, 36.5, 36.1, 34.1, 32.1, 31.0, 28.4, 27.9, 26.1, 26.0, 24.9, 24.6, 24.2, 23.2, 23.0, 21.3, 19.8, 19.0, 12.3; IR (film) ν_{max} 3325, 2850, 1710, 1620, 1320 cm^{-1}; MALDIFTMS (DHB) *m/z* 854.4986 (M H$^+$, $C_{46}H_{71}N_5O_6S_2$ requires 854.4924).



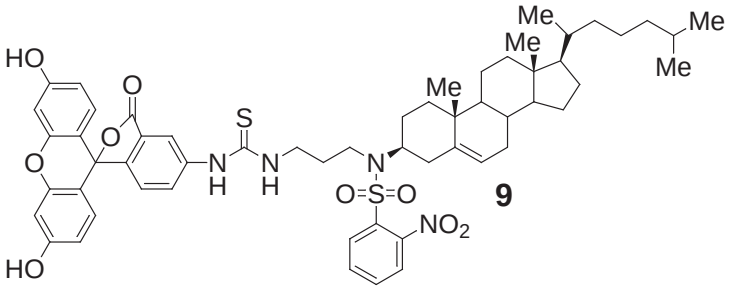
***N*-[3-(cholesterylamino)propyl]biotinamide (8).**
Compound **7** (40 mg, 0.046 mmol), thiophenol (19 mg, 0.184 mmol), and K_2CO_3 (63 mg, 0.46 mmol) were added to dimethylformamide (7 mL) and stirred for 18 h at 25 °C. The mixture was filtered and the solvents removed *in vacuo*. Flash chromatography (15% MeOH / 2% triethylamine in CH_2Cl_2) afforded **8** (22 mg, 71%) mp 160 - 161 °C; ^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD (1:1 v/v) δ 5.14 (br s, 1H), 4.36 (m, 1H), 4.17, (m, 1H), 3.12 (m, 2H), 3.03 (m, 1H), 2.8 – 2.67 (m, 3H), 2.6 (m, 2H), 2.18 (m, 2H), 2.05 (t, *J* = 12 Hz, 1H), 1.89 – 1.66 (m, 8H), 1.53 – 1.18 (m, 20H), 1.06 (m, 8H), 0.96 (s, 3H), 0.87 – 0.7 (m, 12H), 0.54 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$/CD_3OD (1:1 v/v)) δ 179.3, 168.5, 143.2, 127.2, 66.2, 64.4, 62.1, 60.9, 60.4, 59.9, 46.5, 44.6, 43.9, 43.7, 41.6, 41.0, 40.6, 40.5, 40.4, 40.0, 39.9, 36.1, 36.0, 32.7, 32.4, 32.2 (x2), 31.2, 30.4, 29.7, 28.5, 28.3 (x2), 28.0, 27.0, 26.7, 25.5, 23.3, 22.9, 16.1; IR (film) ν_{max} 3310, 2820, 1710, 1300 cm^{-1}; MALDIFTMS (DHB) *m/z* 669.5091 (M H$^+$, $C_{40}H_{68}N_4O_2S$ requires 669.5141).



11

***N*-cholesteryl-3-[3-fluorescein-4'yl)thioureido]propionamide (11).**

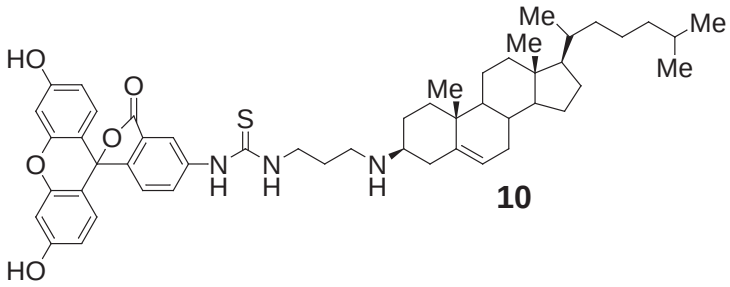
Compound **5** (40 mg, 0.072 mmol) was treated with trifluoroacetic acid (2% in CH_2Cl_2, 10 mL) at 25 °C for 45 min. Aqueous NaOH (1M, 20 mL) was added, and the mixture was extracted with CH_2Cl_2 (3 x 10 mL). The organic layer was dried ($NaSO_4$) and removed *in vacuo*. THF (10 mL), fluorescein isothiocyanate (isomer 1, 42 mg, 0.108 mmol), and diisopropylethylamine (DIEA, 40 µL, 0.157 mmol) were added. This mixture was stirred for 2 h at 25 °C. Aqueous citric acid (10%, 25 mL) was added, and the mixture was extracted with CH_2Cl_2 (2 x 15 mL). The organic layer was dried ($NaSO_4$) and removed *in vacuo*. Flash chromatography (15% MeOH in CH_2Cl_2) afforded **11** (51 mg, 84%) mp 250 °C, dec.; ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$ (1:1 v/v)) δ 8.16 (s, 1H), 7.82 (d, *J* = 10 Hz, 1H), 7.14 (d, *J* = 8 Hz, 1H), 6.66 (m, 3H), 6.52 (d, *J* = 8 Hz, 3H), 5.31 (bs, 1H), 3.89 (m, 2H), 3.63 (m, 2H), 2.58 (t, *J* = 6 Hz, 2H), 2.19 (m, 2H), 1.91 – 1.75 (m, 3H), 1.54 – 1.38 (m, 8H), 1.33 – 1.18 (m, 6H), 1.14 (m, 8H), 1.04 (s, 6H), 0.987 (d, *J* = 14 Hz, 3H), 0.9 (m, 6H), 0.67 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3/CD_3OD$ (1:1 v/v)) δ 181.5, 174.7 (x2), 172.0, 170.3, 160.6, 153.4 (x2), 140.7, 129.5, 129.3, 122.0, 119.4, 114.9, 113.1, 110.6 (x2), 109.9, 105.7, 103, 57.0, 56.4, 49.3, 49.1, 42.5, 40.9, 40.1, 39.8, 39.0, 38.2, 36.8, 36.4, 36.1, 32.1, 29.8, 29.3, 28.9, 28.2, 27.9, 27.1, 24.4, 24.0, 22.8, 22.4, 21.5, 20.7, 19.8, 19.6, 19.3, 18.7, 12.0; IR (film) ν_{max} 3400, 2100, 1710, cm^{-1}; MALDIFTMS (DHB) *m/z* 846.4492 (M H^+, $C_{51}H_{63}N_3O_6S$ requires 846.4516).



9

***N*-cholesteryl-*N*-{3-[3-(fluorescein-4'yl)thioureido]propyl}-2-nitrobenzenesulfonamide (9).**

Compound **3** (50 mg, 0.069 mmol) was treated with trifluoroacetic acid (2% in CH_2Cl_2, 10 mL) at 25 °C for 45 min. TLC analysis (2% MeOH in CH_2Cl_2) revealed conversion to the more polar primary amine. NaOH (1M, 20 mL) was added and the mixture was extracted with CH_2Cl_2 (3 x 10 mL). The organic layer was dried ($NaSO_4$) and removed *in vacuo*. THF (10 mL), fluorescein isothiocyanate (isomer 1, 40 mg, 0.103 mmol), and diisopropylethylamine (DIEA, 13 µL, 0.0759 mmol) were added. This mixture was stirred for 2 h at 25 °C. Aqueous citric acid (10%, 25 mL) was added, and the mixture was extracted with CH_2Cl_2 (2 x 15 mL). The organic layer was dried ($NaSO_4$) and removed *in vacuo*. Flash chromatography (10% MeOH in CH_2Cl_2) afforded **9** (61 mg, 87%), mp 181 – 183 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (m, 1H), 7.69 (m, 1H), 7.46 (m, 1H), 7.39 (m, 1H), 7.33 (m, 1H), 6.89 (m, 1H), 6.37 – 6.28 (m, 3H), 6.23 (m, 2H), 6.15 (m, 2H), 4.88 (bs, 1H), 3.40 (m, 2H), 3.32 – 3.26 (m, 3H), 3.11 (t, *J* = 7 Hz, 2H), 2.17 (m, 1H), 1.71 – 1.57 (m, 5H), 1.55 – 1.24 (m, 4H), 1.23 – 0.92 (m, 8H), 0.86 (m, 5H), 0.77 (m, 4H), 0.66 (s, 3H), 0.56 (m, 6H), 0.53 (m, 6H), 0.33 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 176 (x2), 170.1, 153.4, 153.2 (x2), 148.2, 140.4, 134.3, 133.6, 130.8, 129.5, 129.0, 128.3, 125.6, 125.2, 124.6, 122.5, 115.2, 113.6, 113.0, 110.6, 109.8, 107.1, 103.0, 102.8, 57.6, 56.4, 49.8, 45.6, 43.0, 42.1, 36.8, 36.1, 33.1, 32.9, 32.1, 31.7, 31.2, 30.3, 28.3, 26.1, 25.2, 24.0, 22.9, 22.8, 22.5, 22.1, 21.9, 21.2, 19.6, 19.4, 19.3, 18.6, 17.8, 17.7, 11.7; IR (film) ν_{max} 3410, 2850, 2210, 1720 cm^{-1}; MALDIFTMS (DHB) *m/z* 1017.4482 (M H^+, $C_{57}H_{68}N_4O_9S_2$ requires 1017.4506).



10

1-(3-cholesterylpropyl)-3-(fluorescein-4'-yl)thiourea (10)
Compound **9** (40 mg, 0.039 mmol), thiophenol (19 mg, 0.184 mmol) and K_2CO_3 (63 mg, 0.46 mmol) were added to dimethylformamide (7 mL) and stirred for 18 h at 25 °C. The mixture was filtered and the solvent removed *in vacuo*. Flash chromatography (20% MeOH / 2% HOAc in CH_2Cl_2) afforded **10** (20 mg, 62%) mp 256 °C, dec.; ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$ (1:1 v/v)) δ 8.23 (s, 1H), 7.89 (d, *J* = 10 Hz, 1H), 7.21 (d, *J* = 8 Hz, 1H), 6.71 (m, 3H), 6.59 (d, *J* = 8 Hz, 3H), 5.28 (bs, 1H), 3.8 (m, 2H), 3.41 (m, 2H), 2.5 (t, *J* = 6 Hz, 2H), 2.14 (m, 2H), 1.87 – 1.7 (m, 3H), 1.49 – 1.35 (m, 8H), 1.31 – 1.1 (m, 8H), 1.1 (m, 8H), 0.96 (s, 6H), 0.95 (d, *J* = 14 Hz, 3H), 0.9 (m, 6H), 0.67 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3/CD_3OD$ (1:1 v/v)) δ 181.5, 174.7 (x2), 172.0, 160.6, 153.4 (x2), 140.7, 129.5, 129.3, 122.0, 119.4, 114.9, 113.1, 110.6 (x2), 109.9, 105.7, 103, 57.0, 56.4, 49.3, 49.1, 45.3, 42.5, 40.9, 40.1, 39.8, 39.0, 38.2, 36.8, 36.4, 36.1, 32.1, 29.8, 29.3, 28.9, 28.2, 27.9, 27.1, 24.4, 24.0, 22.8, 22.4, 21.5, 20.7, 19.8, 19.6, 19.3, 18.7, 12.0; IR (film) ν_{max} 3310, 2800, 1720 cm^{-1}; MALDIFTMS (DHB) *m/z* 830.4563 (M H$^+$, $C_{51}H_{65}N_3O_5S$ requires 830.4567).

Micrographs of SA Uptake in Jurkat Lymphocytes Treated with Compounds 6 and 8. A qualitative comparison of Jurkat lymphocytes treated with green fluorescent SA and synthetic compounds are shown in Figure S1. Panels A and B show that the amide analog **6** and the short-chain analog **8** do not appreciably affect cellular fluorescence.

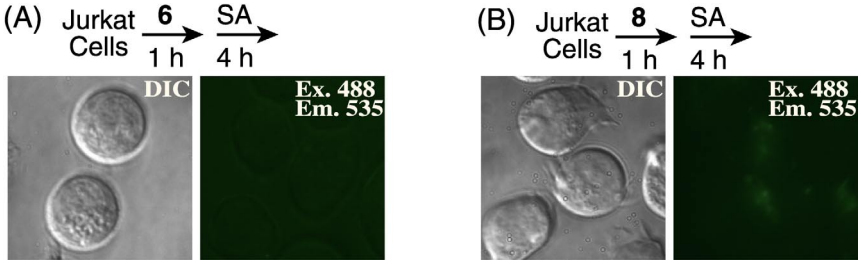


Figure S1. Epifluorescence and differential interference contrast (DIC) microscopy of Jurkat lymphocytes treated with synthetic compounds (10 μM) and SA Alexa Fluor 488 (20 μg/mL). Fluorescence excitation (Ex.) and emission (Em.) wavelengths (nm) are explicitly shown.

Micrographs of SA Uptake in Other Cell Lines. Treatment of other mammalian cell lines with compound **1** and green fluorescent SA engendered ligand-dependent punctate intracellular fluorescence as shown in Figure S2. These results confirmed that **1** enables dose-dependent uptake of SA in all mammalian cell lines examined. No significant cellular fluorescence was observed in the absence of **1** (data not shown).

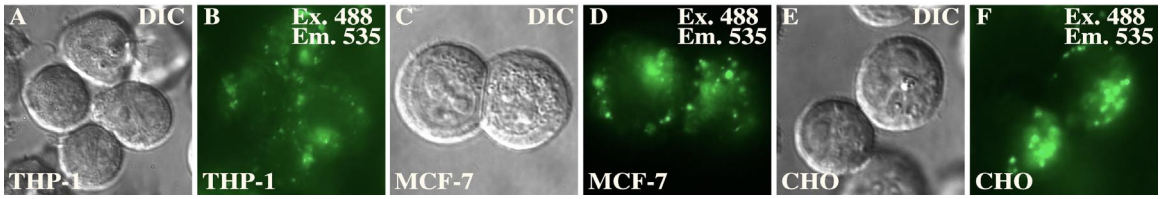


Figure S2. Epifluorescence and differential interference contrast (DIC) microscopy of suspension (THP-1) and adherent (MCF-7 and CHO) cells pretreated with **1** (10 μM) for 1 h, followed by addition of SA Alexa Fluor 488 (20 μg/mL) for 4 h. Adherent cell lines were examined after treatment with trypsin to release cells from flask surfaces. Cell lines and fluorescence excitation (Ex.) and emission (Em.) wavelengths (nm) are explicitly shown.

Micrographs of Jurkat Lymphocytes Treated with Fluorescent Probes 10 and 11. Cells treated with synthetic fluorescent probes are shown in Figure S3. These micrographs revealed that cells treated with compound **10** exhibit qualitatively greater fluorescence intensity at cellular plasma membranes compared with cells treated with compound **11**.

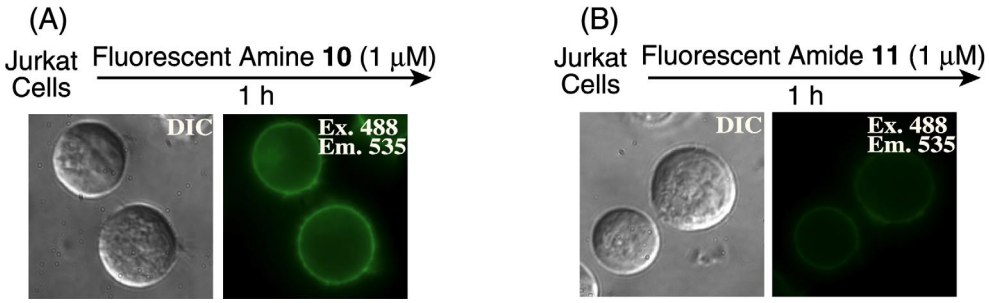


Figure S3. Epifluorescence and differential interference contrast (DIC) microscopy of Jurkat lymphocytes treated with fluorescent probe compounds for 1 h. Fluorescence excitation (Ex.) and emission (Em.) wavelengths (nm) are explicitly shown.

Protein Uptake Assay. Jurkat human T cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 media supplemented with Fetal Bovine Serum (FBS, 10%), penicillin (100 units / mL), and streptomycin (100 μg / mL). CHO cells were maintained in F12K media supplemented with FBS (10%), penicillin (100 units / mL), and streptomycin (100 μg / mL). MCF-7 cells were maintained in DMEM media supplemented with FBS (10%), penicillin (100 units / mL), and streptomycin (100 μg / mL). For cellular analysis, compounds were dissolved in absolute ethanol (EtOH), which was diluted with media to yield a final EtOH concentration of 0.1%. Because cold media blocks endocytic processes such as ligand-regulated uptake of SA, media was maintained at 25 °C for all wash steps. To cells (5 X 10^6) in media (450 μL) was added Streptaphage (**1**) or other related compounds (50 μL of a 10 x stock solution in media containing 1% EtOH) at 37 °C for 1 h. The cells were washed with media (2 x 1 mL) and resuspended in media (98 μL). Streptavidin Alexa Fluor 488 (2 μL, 1 mg / mL) was added to these resuspended cells to yield a final concentration of 0.02 mg / mL. The cells were then incubated at 37 °C for the times indicated, typically 4 h for internalization experiments. Suspension cell lines were analyzed by flow cytometry after washing with media (2 x 1 mL), and resuspension in media (0.75 mL). The adherent cell lines were washed with media (2 x 1 mL), treated with a solution of trypsin (2.5 μg / mL) containing EDTA (0.02%) and analyzed by flow cytometry in this solution. To accurately compare uptake of SA by the larger cell volume adherent cell lines and the smaller cell volume suspension cell lines, Sphero Rainbow Calibration Particles (Spherotech Inc, Libertyville, Il) were employed to normalize the data shown in Figure 3E.

Flow cytometry. Flow analyses were performed on an XL-MCL bench top cytometer (Beckman-Coulter, Miami Lakes, FL) equipped with a 15 mW air cooled argon-ion laser. Fluorescence detection employed excitation at 488 nm (Alexa Fluor 488), splitting the emission with a 550 nm dichroic long pass filter and optical filtering through 530/30-nm band-pass filter. Propidium iodide fluorescence was measured by excitation at 488 nm with optical filtering of fluorescence emission through a 550 nm dichroic and a 610 nm band pass (BP) filter. Forward-scatter (FS) and side-scatter (SSC) dot plots afforded cellular physical properties of size and granularity that allowed gating of live cells. After gating, 10,000 cells were counted.

Confocal Microscopy. Sequential scans were made using an Olympus FV300 laser scanning confocal microscope fitted with a UplanFl objective (100 X). Alexa Fluor 488 was excited with a 488 nm Argon ion laser and emitted photons were collected through 510 nm LP and 530 nm SP filters. Excitation of Alexa Fluor 594 employed a 543 nm HeNe laser, splitting the emission with a 570 nm dichroic mirror, and filtering emitted photons with a 605 nm BP filter. The cells shown in Figure 7, Panels A-D were treated as follows: Jurkat lymphocytes (5 X 10^6) in media (99 μL) were treated with **10** (10 μM final conc.) for 1 h at 37 °C. Cholera toxin Alexa Fluor 594 (1 μL, 0.8 mg / mL in PBS) was added (8 μg / mL final conc.). The cells shown in Figure 7, Panels E-H were treated as follows: Jurkat lymphocytes (5 X 10^6) in media (97 μL) were incubated with **1** (10 μM final conc.) for 1 h at 37 °C. SA Alexa Fluor 488 (2 μL, 1 mg / mL, 20 μg / mL final conc.) and Cholera toxin Alexa Fluor 594 (1 μL, 0.8 mg / mL in PBS) were added. After protein addition, cells were incubated at 37 °C for 5 min, washed with media (2 x 1 mL), the tubes were placed in ice to restrict protein uptake, and cells were immediately analyzed by confocal microscopy.

Analysis of Compound Toxicity. Cells were treated under protein uptake assay conditions (treatment with **1** for 1 h followed by SA Alexa Fluor 488 for 4 h). The cells were washed twice with media and incubated for an additional 24 h at 37 °C. The dead-cell stain propidium ioidide (10 μg / mL) was added to cells prior to analysis, and viability was quantified from flow cytometry forward and side-scatter dot plots.

Lysotracker Red Staining of Cellular Endosomes. The protein uptake assay was performed as described but including Lysotracker Red (Molecular Probes, 1 μL, 50 μM in DMSO, final concentration of dye = 500 nM with1% DMSO) added to cells 3.5 h after addition of SA (30 min prior to analysis).

Inhibition of Uptake Experiments. Cholesterol depletion with methyl-β-cyclodextrin: Cells were suspended in PBS (pH 7.4) containing BSA (1 mg / mL) and methyl-β-cyclodextrin (10 mM) for 30 min prior to the protein uptake assay. Treatment with hypertonic sucrose: Cells were suspended in media containing sucrose (400 mM) for 2 h prior to the protein uptake assay, and sucrose (400 mM) was included in media employed for the protein uptake assay. Chlorpromazine experiments: Cells were treated with media containing chlorpromazine (100 μM) for 2 h prior to the protein uptake assay.