Supporting Information

Fluorescent Mimics of Cholesterol that Rapidly Bind Surfaces of Living Mammalian Cells

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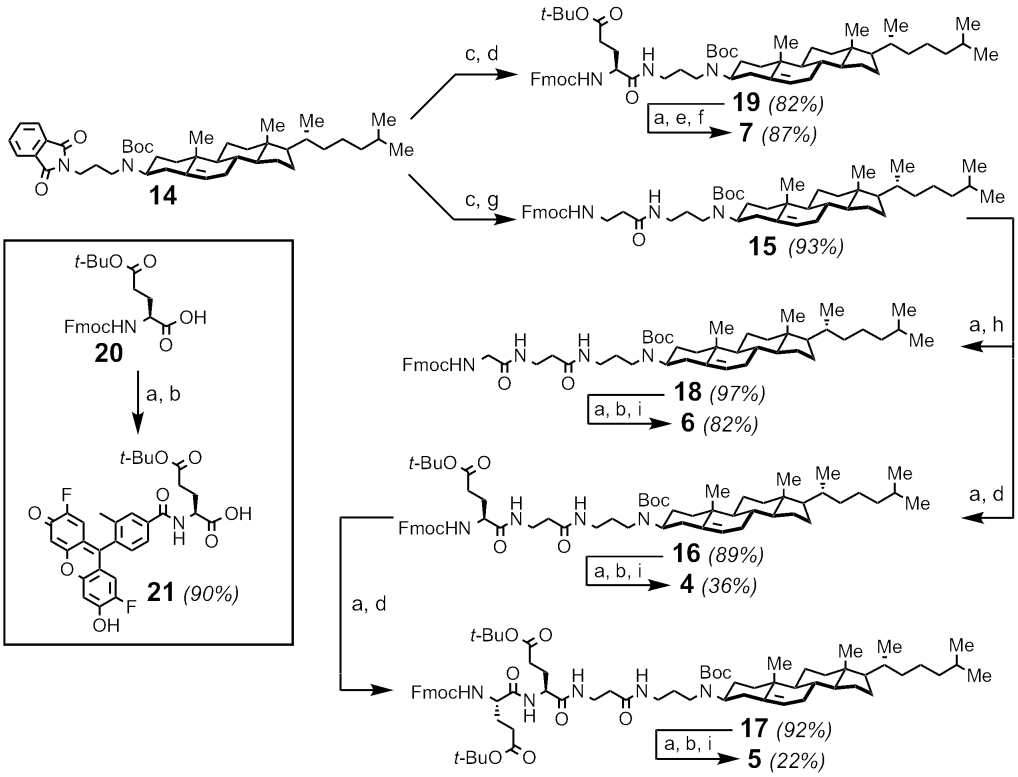
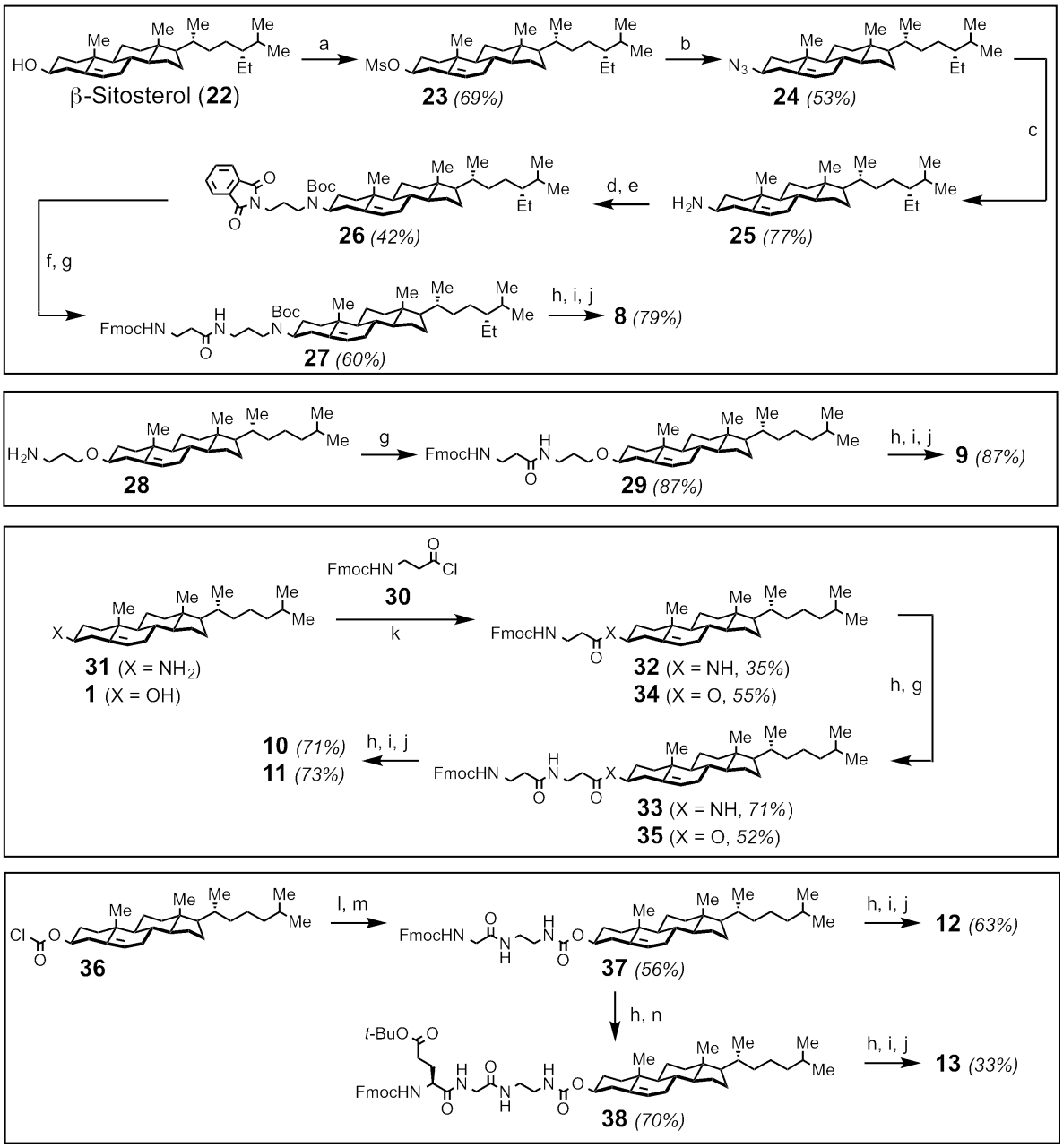
Scheme S1. Synthesis of compounds **4**-**7**.



Reagents and conditions: (a) Piperidine, DMF (1:4), 30 min; (b) 4-Carboxy-Pennsylvania Green NHS Ester, DIEA, DMF, 16 h; (c) H_2NNH_2, EtOH, 50 °C, 4 h; (d) EDC, HOBt, Fmoc-Glu(O*t*-Bu)-OH, 4 °C to 22 °C, 12 h; (e) EDC, HOBt, **21**, DMF, 12 h; (f) TFA, CH_2Cl_2 (1:1), 2 h; (g) EDC, HOBt, Fmoc-β-Ala-OH, 4 °C to 22 °C, 12 h; (h) EDC, HOBt, Fmoc-Gly-OH, 4 °C to 22 °C, 12 h; (i) TFA, CH_2Cl_2 (1.5:8.5), 12 h.

Scheme S2. Synthesis of compounds **8**-**13**.



Reagents and conditions: (a) MsCl, TEA, CH_2Cl_2, 4 °C to 22 °C, 16 h; (b) TMS-N_3, BF_3-OEt_2, CH_2Cl_2, 16 h; (c) $LiAlH_4$, Et_2O, CH_2Cl_2, 4 °C to 22 °C, 2 h; (d) *N*-3-bromopropylphthalimide, K_2CO_3, DMF, 60 °C, 24 h; (e) $(Boc)_2O$, DIEA, CH_2Cl_2, 4 h; (f) H_2NNH_2, EtOH, 50 °C, 4 h; (g) EDC, HOBt, Fmoc-β-Ala-OH, 4 °C to 22 °C, 12 h; (h) piperidine, DMF (1:4), 30 min; (i) EDC, HOBt, **21**, DMF, 12 h; (j) TFA, CH_2Cl_2 (1:1), 2 h; (k) DMAP, CH_2Cl_2, 30 min; (l) excess ethylene diamine, CH_2Cl_2, 2 h; (m) EDC, HOBt, Fmoc-Gly-OH, 4 °C to 22 °C, 12 h; (n) EDC, HOBt, Fmoc-Glu(O*t*-Bu)-OH, 4 °C to 22 °C, 12 h.

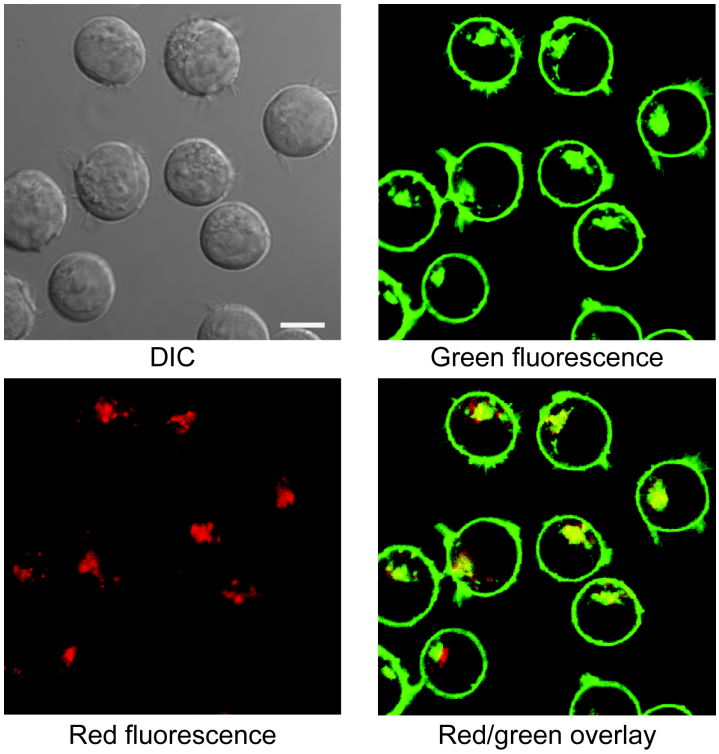


Figure S1. Micrographs of Jurkat cells treated with green fluorescent 4 and red fluorescent transferrin, Alexa Fluor 633 conjugate. Cells were treated with 4 (2 μM) for 1 h to allow trafficking to endosomes followed by addition of transferrin-AF633 (1 μM) for 10 min. Cells were washed with media and imaged by differential interference contrast (DIC) and confocal laser scanning microscopy. Colocalization of red and green fluorescence in early/recycling endosomes, shown as yellow in the overlay image, can be observed. Scale Bar: 7.5 microns.

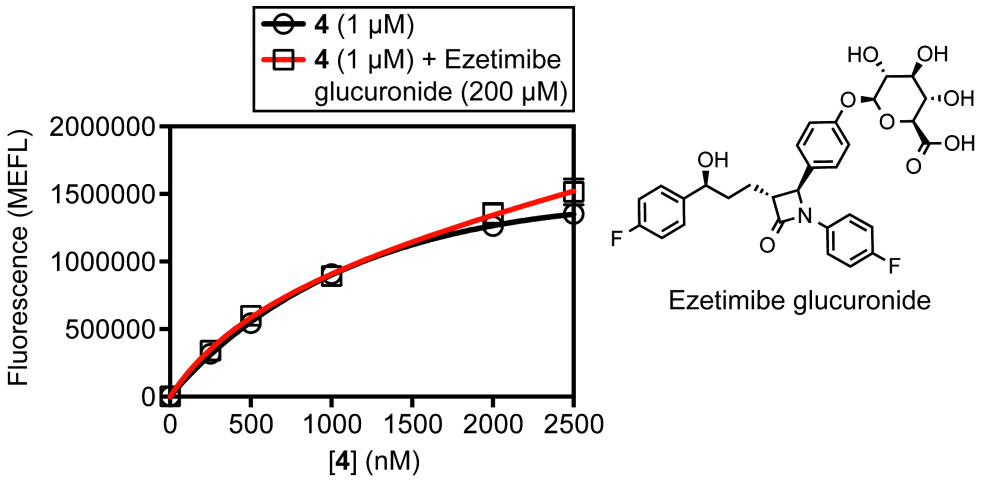


Figure S2. Ezetimibe glucuronide is not a competitive inhibitor of binding of **4** to cell surfaces, providing evidence that NPC1L1 is not a target. Jurkat cells (300,000 cells/mL in RPMI-1640 / 10% FBS) were pre-treated for 30 min with and without ezetimibe glucuronide (200 μM; diluted 1:1000 from a 200 mM DMSO stock, using serial 10-fold dilutions). Cells were incubated with **4** (1 μM) at 37 °C for 5 min, washed once with RPMI-1640 containing 0.5% FBS (to minimize efflux of the probe) and propidium iodide (PI, 3 μM). The green fluorescence of living cells was immediately analyzed by flow cytometry.

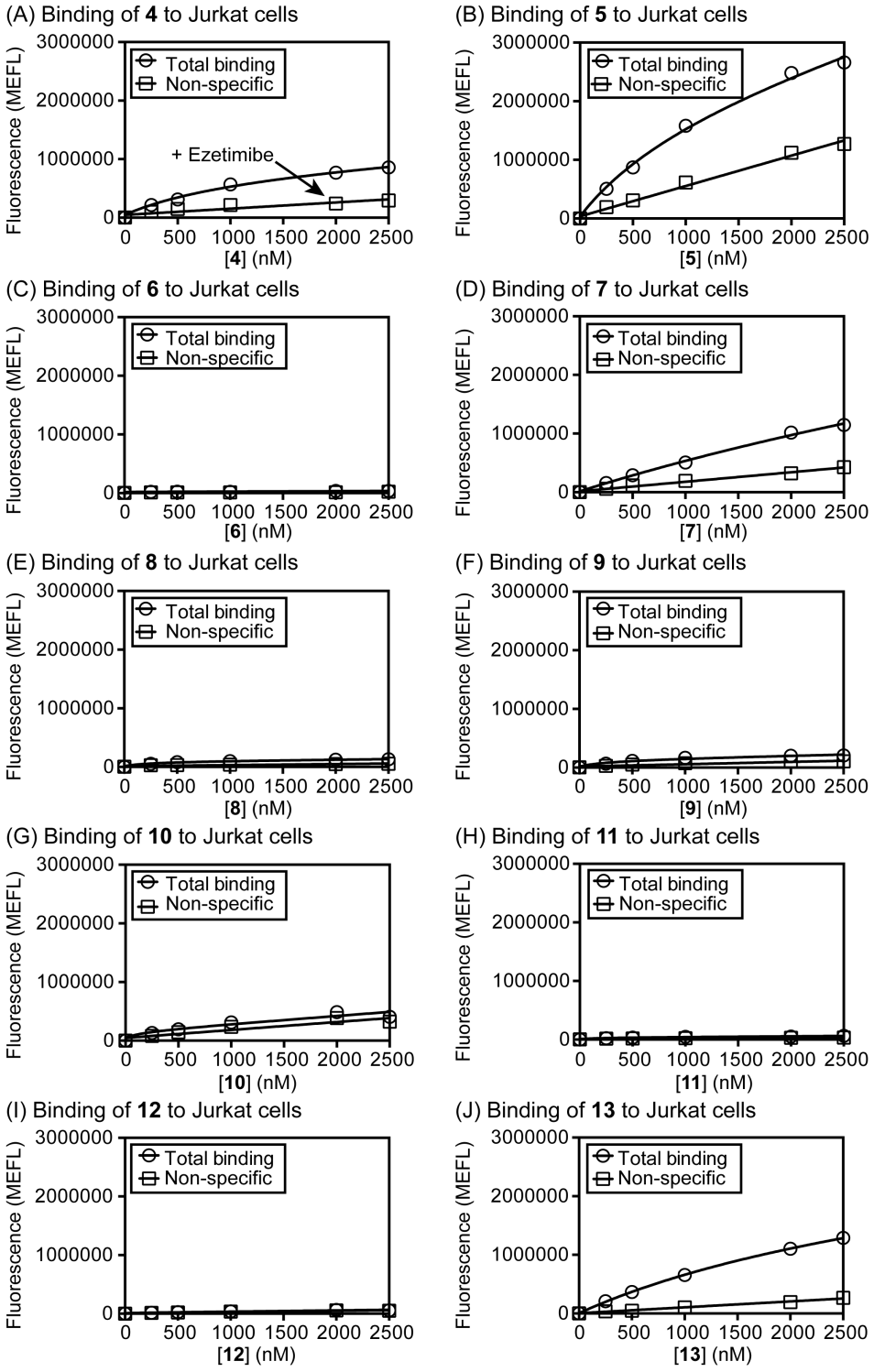
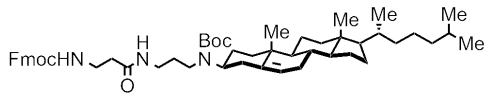


Figure S3. Total and non-specific binding of compounds **4**–**13** to plasma membranes of living Jurkat lymphocytes at 37 °C. Non-specific binding curves were generated by preincubation of cells with ezetimibe (**3**, 200 μM, 0.2% DMSO) for 30 min at 37 °C. Compounds **4**–**13** were added for 5 min to cells with and without ezetimibe (200 μM), cells were washed with fresh media (containing PI (3 μM)), and the green fluorescence of **4**-**13** bound to living cell surfaces was analyzed by flow cytometry. This data was used to calculate the specific binding curves shown in Figure 2 of the main text of the manuscript.

Experimental section

General. Chemical reagents were purchased from Apptec, Acros, Aldrich, Alfa Aesar, EMD Biosciences, or TCI America, and were used without further purification. Solvents were from Aldrich or Fisher Scientific. Ezetimibe (Zetia®), obtained from a local pharmacy, was isolated in pure form by extraction of crushed tablets with CH_2Cl_2/ddw, drying the organic layer over anhydrous Na_2SO_4, and removal of solvent under reduced pressure. Cell culture reagents were from Sigma, Mediatech, BD Biosciences, Gibco, and Invitrogen. 3β-Amino-5-cholestene (**31**),[1] cholesteryloxypropan-1-amine (**28**),[2] 4-Carboxy-Pennsylvania Green NHS ester,[3, 4] **14**,[5] and **19**[5] were synthesized by previously reported methods. Anhydrous solvents were obtained after passage through a drying column of a solvent purification system from GlassContour/SG Waters (Nashua, NH). All reactions were performed under an atmosphere of dry argon or nitrogen. Reactions were monitored by analytical thin-layer chromatography on plates coated with 0.25 mm silica gel 60 F254 (EM Science). TLC plates were visualized by UV irradiation (254 nm) or stained with either phosphomolybdic acid (20%) in ethanol or ninhydrin in ethanol. Column chromatography employed silica gel as a stationary phase (Dynamic Adsorbents, 40-63 μm). Preparative HPLC employed an Agilent 1200 Series preparative pump / gradient extension with a Hamilton PRP-1 (polystyrene-divinylbenzene) reverse-phase preparative column (10-25 μm particle size, 21.5 mm x 25 cm) with a flow rate of 25.0 mL/min. Analytical HPLC traces were acquired using an Agilent 1100 quaternary pump and a Hamilton PRP-1 (polystyrene-divinylbenzene) reverse phase analytical column (7 μm particle size, 4 mm x 25 cm) with UV detection at 254 nm, and elution was achieved with gradients of H_2O / CH_3CN (90:10 to 0:100 containing 0.1% TFA) over 25 min. Melting points were measured with a Thomas Hoover capillary melting point apparatus and are uncorrected. Infrared spectra were obtained with a Perkin Elmer Spectrum 100 FTIR. NMR spectra were obtained with a Bruker Avance-400 or DRX-500 instrument with chemical shifts reported in parts per million (ppm, δ) referenced to either $CDCl_3$ (^{1}H, 7.26 ppm; ^{13}C, 77.16 ppm), CD_3OD (^{1}H, 3.34 ppm; ^{13}C, 49.00 ppm), or $(CD_3)_2S{=}O$ (^{1}H, 2.50 ppm; ^{13}C, 128.06 ppm). High-resolution mass spectra were obtained at the University of Kansas Mass Spectrometry Facility (ESI and EI). Peaks are reported as *m/z*. Basic compounds purified by reverse-phase HPLC were isolated as trifluoroacetic acid salts. Concentrations of all compounds analyzed in bioassays were standardized by absorbance measurements using a Molecular Devices SpectraMax 340PC 96-well plate reader or Agilent 8453 UV/Visible spectrophotometer.

Synthetic procedures and compound characterization data



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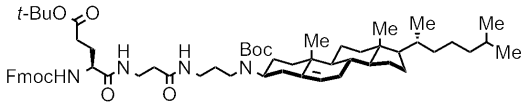
9*H*-Fluoren-9-ylmethyl{3-[(3-{(*tert*-butoxycarbonyl)[3β-cholest-5-en-3-yl]amino}propyl)amino]-3-oxopropyl} carbamate (**15**). To a solution of **14**[5] (1.41 g, 2.1 mmol) in absolute ethanol (50 mL) was added anhydrous hydrazine (350 μL, 11 mmol). The solution was heated to 50 °C and stirred for 4 h. The reaction was cooled to 22 °C, and a white precipitate was removed by filtration. The filtrate was concentrated under reduced pressure, and the residue was dissolved in $CHCl_3$ (100 mL). After insoluble material was removed by filtration, concentration of the filtrate under reduced pressure afforded the phthalimide-deprotected primary amine (1.13 g, 99%), a white solid that was carried forward without further purification. To Fmoc-β-Ala-OH (715 mg, 2.3 mmol) in anhydrous CH_2Cl_2 (50 mL) at 4 °C was added hydroxybenzotriazole (HOBt, 340 mg, 2.5 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)

carbodiimide (EDC, 480 mg, 2.5 mmol). This mixture was stirred at 4 °C for 30 min. The phthalimide-deprotected primary amine in anhydrous CH_2Cl_2 (25 mL) was added dropwise, the reaction was allowed to warm to 22 °C and was further stirred for 12 h. The solution was diluted with CH_2Cl_2 (100 mL) and washed with aqueous NaOH (0.1 M, 100 mL) followed by saturated aqueous NaCl (100 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. Flash column chromatography (hexanes, ethyl acetate, 2:1) afforded **15** (1.62 g, 93%) as a glassy solid, mp 84-86 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.3 Hz, 2H), 7.58 (d, *J* = 7.4 Hz, 2H), 7.39 (m, 2H), 7.30 (m, 2H), 7.05 (br, 1H), 5.82 (br, 1H), 5.33 (s, 1H), 4.34 (d, *J* = 7.2 Hz, 2H), 4.22 (t, *J* = 6.8 Hz, 1H), 3.51-3.24 (m, 6H), 2.60-0.86 (m, 54H), 0.67 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.0, 156.5, 144.0, 143.9, 141.2, 127.6, 127.6, 127.0, 125.1, 125.0, 121.4, 119.9, 77.1, 66.7, 56.7, 56.1, 50.0, 47.2, 42.3, 39.7, 39.5, 37.2, 36.8, 36.5, 36.1, 35.9, 35.7, 31.8, 28.5, 28.2, 27.9, 26.8, 24.2, 23.8, 22.8, 22.5, 20.9, 19.4, 18.7, 11.8 ; IR (film) ν max 3323, 2949, 1717, 1668, 1539, 1449, 1412, 1366, 1251, 1169, 1081, 908, 737 cm^{-1}; HRMS (ESI+) m/z 835.5819 (MNa^+, $C_{53}H_{77}N_3O_5Na$ requires 858.5755).



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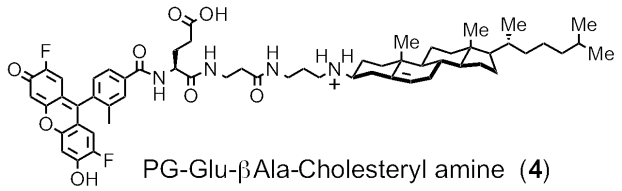
***tert*-Butyl-(15*S*)-5-[3β-cholest-5-en-3-yl]-15-{[(9*H*-fluoren-9-ylmethoxy)carbonyl]amino}-2,2-dimeth yl-4,10,14-trioxo-3-oxa-5,9,13-triazaoctadecan-18-oate(16)**. Compound **15** (303 mg, 0.36 mmol) was added to DMF (2 mL) containing piperidine (20%) and stirred for 30 min at 22 °C. The solvent was removed under reduced pressure to afford the crude primary amine. To a solution of Fmoc-Glu (O-*t*-Bu)-OH (157 mg, 0.37 mmol) in anhydrous CH_2Cl_2 (10 mL) at 4 °C were added HOBt (55 mg, 0.41 mmol) and EDC (79 mg, 0.41 mmol) and the solution was stirred for 30 min. To this solution was added the crude primary amine derived from **15** dissolved in anhydrous CH_2Cl_2 (5 mL). The reaction was allowed to warm to 22 °C and stirred for 12 h. This solution was diluted with CH_2Cl_2 (30 mL) and washed with aqueous NaOH (0.1 M, 30 mL) followed by saturated aqueous NaCl (30 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. Flash column chromatography (CH_2Cl_2, MeOH, 50:1) afforded **16** (361 mg, 98%) as a white solid, mp 120-122 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ7.76 (d, *J* = 7.5 Hz, 2H), 7.60 (d, *J* = 7.5 Hz, 2H), 7.51 (br, 1H), 7.39 (t, *J* = 7.5 Hz, 2H), 7.31 (t, *J* = 7.5 Hz, 2H), 7.09 (br, 1H), 5.88 (d, *J* = 8.0 Hz, 1H), 5.31 (d, *J* = 4.0 Hz, 1H), 4.34 (t, *J* = 7.1 Hz, 2H), 4.21 (t, *J* = 7.1 Hz, 2H), 3.57-3.16 (m, 6H), 2.60-0.85 (m, 67H), 0.66 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.7, 171.4, 171.4, 156.2, 143.9, 143.8, 141.3, 127.7, 127.9, 125.2, 121.4, 119.9, 80.9, 79.9, 67.1, 56.7, 56.1, 54.6, 50.1, 47.2, 42.3, 39.7, 39.8, 39.6, 38.4, 37.1, 36.7, 36.2, 36.0, 35.8, 31.9, 31.5, 28.6, 28.2, 28.1, 28.0, 27.9, 27.0, 24.3, 23.8, 22.8, 22.6, 21.0, 19.5, 18.7, 11.8 ; IR (film) ν max 3295, 3066, 3005, 2935, 2863, 1726, 1652, 1540, 1465, 1451, 1415, 1366, 12523, 1163, 1046, 849, 757, 666 cm^{-1}; HRMS (ESI+) m/z 1043.6853 (MNa^+, $C_{62}H_{92}N_4O_8Na$ requires 1043.6813).



(4*S*)-5-{[3-({3-[3β-Cholest-5-en-3-ylamino]propyl}amino)-3-oxopropyl]amino}-4-{[4-(2,7-difluoro-6-hydroxy-3-oxo-3*H*-xanthen-9-yl)-3-methylbenzoyl]amino}-5-oxopentanoic acid (**4**). Compound **16** (75 mg, 0.074 mmol) was added to DMF (1 mL) containing piperidine (20%) and stirred for 30 min at 22 °C. The solvent was removed under reduced pressure to afford the crude primary amine. The resulting residue was dissolved in anhydrous DMF (5 mL), 4-carboxy-Pennsylvania Green succinimidyl ester[3] (30 mg, 0.062 mmol) was added, followed by diisopropylethylamine (DIEA, 50 μL, 0.24 mmol). The reaction was stirred at 22 °C for 12 h and concentrated under reduced pressure. To the resulting orange residue was added CH_2Cl_2 (5 mL) containing TFA (15%) and the solution was stirred at 22 °C for 12 h. The reaction was concentrated under reduced pressure, the crude product was dissolved in methanol (2 mL), and the product was purified by preparative reverse-phase HPLC (gradient: 90% H_2O, 9.9% MeCN, and 0.1% TFA to 99.9% MeCN and 0.1% TFA over 20 min; retention time = 15.4 min (495 nm) to afford **4** (23 mg, 36.4%) as an orange solid (TFA salt), mp 196-198 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.76-8.72 (m, 3H), 8.19 (s, 2H), 8.14 (s, 1H), 8.05 (d, J = 5.4 Hz, 1H), 7.49 (d, J = 5.7 Hz, 1H), 6.98 (s, 2H), 6.72 (d, J = 8.2 Hz, 2H), 5.45 (s, 1H), 4.53 (d, J = 2.7 Hz, 1H), 3.23 (m, 2H), 3.14 (m, 2H), 3.01 (m, 3H), 2.46-0.92(m, 51H), 0.72 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 174.0, 172.7, 171.2, 170.8, 165.9, 158.0, 149.3, 138.6, 135.9, 135.2, 134.5, 129.8, 129.0, 125.5, 122.4, 105.2, 56.6, 56.1, 55.5, 53.1, 49.3, 41.8, 41.6, 36.4, 36.2, 35.6, 35.3, 35.2, 34.3, 31.2, 30.5, 27.7, 27.4, 26.9, 26.2, 24.3, 23.8, 23.2, 22.6, 22.4, 20.5, 19.2, 18.7, 18.5, 11.6 ; IR (film) ν max 3324, 3071, 2950, 2863, 1671, 1646, 1610, 1539, 1503, 1465, 1373, 1310, 1193, 1136, 836, 759 cm^{-1}; HRMS (ESI+) m/z 1007.5679 (MH^+, $C_{59}H_{77}F_2N_4O_8Na$ requires 1007.5704).

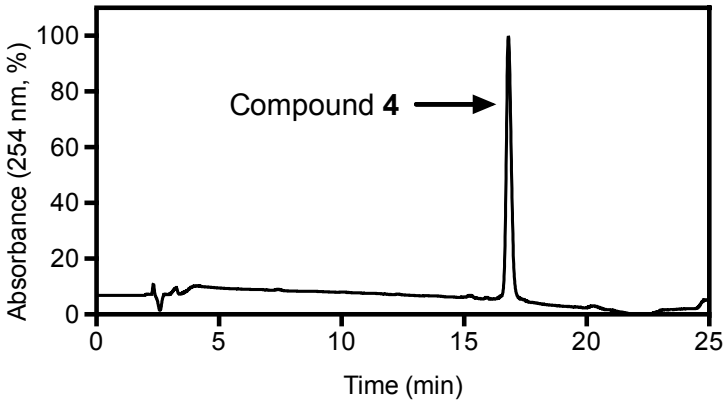
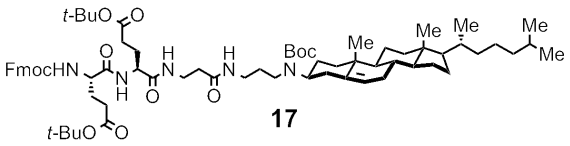


Figure S4. Analytical RP-HPLC profile of **4** after preparative RP-HPLC. Retention time = 16.6 min. Purity > 95%.



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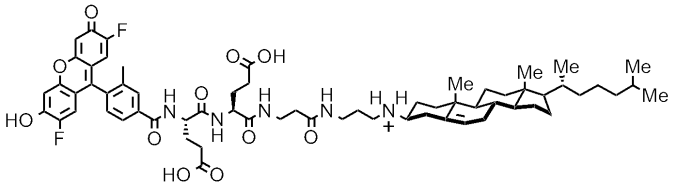
***tert*-Butyl-(15*S*,18*S*)-15-(3-*tert*-butoxy-3-oxopropyl)-5-[3β-cholest-5-en-3-yl]-18-{[(9*H*-fluoren-9-ylmethoxy)carbonyl] amino}-2,2-dimethyl-4,10,14,17-tetraoxo-3-oxa-5,9,13,16-tetraazahenicosan-21-oate** (**17**). Compound **16** (150 mg, 0.15 mmol) was added to DMF (2 mL) containing piperidine (20%) and stirred for 30 min at 22 °C. The solvent was removed under reduced pressure to afford the crude primary amine. To Fmoc-Glu(O-*t*-Bu)-OH (70 mg, 0.16 mmol) in anhydrous CH_2Cl_2 (10 mL) at 4 °C was added HOBt (24 mg, 0.18 mmol) and EDC (35 mg, 0.18 mmol) and the solution was stirred for 30 min. The crude primary amine derived from **16** in anhydrous CH_2Cl_2 (5 mL) was added. The reaction was allowed to warm to 22 °C and stirred for 12 h. This solution was diluted with CH_2Cl_2 (30 mL) and washed with aqueous NaOH (0.1 M, 30 mL) followed by saturated aqueous NaCl (30 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. Flash column chromatography (CH_2Cl_2, MeOH, 30:1) afforded **17** (162 mg, 92%) as a white solid, mp 138-144 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ7.78 (d, *J* = 7.5 Hz, 2H), 7.63 (d, *J* = 7.2 Hz, 2H), 7.59 (br, 1H), 7.41 (t, *J* = 7.4 Hz, 2H), 7.36 (br, 1H), 7.33 (t, *J* = 7.4 Hz, 2H), 7.19 (br, 1H), 5.92 (br, 1H), 5.32 (d, J = 6.4 Hz, 1H), 4.36 (d, *J* = 7.2 Hz, 3H), 4.24 (t, *J* = 7.0 Hz, 2H), 3.58-3.20 (br, 7H), 2.60-0.86 (m, 79H), 0.67 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.9, 172.8, 171.4, 171.1, 156.3, 156.3, 143.9, 143.7, 141.2, 127.7, 127.1, 125.2, 121.3, 120.0, 81.0, 80.9, 77.2, 67.2, 56.7, 56.2, 53.1, 50.1, 47.1, 42.3, 39.8, 39.5, 38.4, 36.7, 36.2, 35.8, 31.9, 28.6, 28.2, 28.0, 27.9, 24.3, 23.8, 22.8, 22.6, 21.0, 19.5, 18.7, 11.8 ; IR (film) ν max 3289, 3066, 3005, 2934, 2868, 1729, 1693, 1679, 1636, 1539, 1450, 1413, 1388, 1367, 1281, 1254, 1158, 1044, 757, 667 cm^{-1}; HRMS (ESI+) m/z 1206.8032 (MH^+, $C_{71}H_{108}N_5O_{11}$ requires 1206.8040).



PG-Glu-Glu-βAla-Cholesteryl amine (**5**)

(4*S*)-5-{[(1*S*)-3-Carboxy-1-({[3-({3-[3β-cholest-5-en-3-ylamino]propyl}amino)-3-oxopropyl]amino}carbonyl)propyl]amino}-4-{[4-(2,7-difluoro-6-hydroxy-3-oxo-3H-xanthen-9-yl)-3-methylbenzoyl]amino}-5-oxopentanoic acid (**5**). Compound **17** (30 mg, 0.025 mmol) was added to DMF (1 mL) containing piperidine (20%) and stirred for 30 min at 22°C. The solvent was removed under reduced pressure to afford the crude primary amine. To the resulting residue in dry DMF (2 mL) was added 4-carboxy-Pennsylvania Green succinimidyl ester[3] (13 mg, 0.027 mmol) followed by DIEA (50 μL, 0.24 mmol). The reaction was stirred at 22 °C for 12 h and concentrated under reduced pressure. The orange residue was treated with CH_2Cl_2 (5 mL) containing TFA (15%) and stirred at 22 °C for 12 h. The reaction was concentrated under reduced pressure, and the crude product was dissolved in methanol (2 mL) and purified by preparative reverse-phase HPLC (gradient: 90% H_2O, 9.9% MeCN, and 0.1% TFA to 99.9% MeCN and 0.1% TFA over 15 min; retention time = 13.5 min (495 nm) to afford **5** (6.2 mg, 22%) as an orange solid (TFA salt), mp 145-148 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.65 (d, J = 7.8 Hz, 1H), 8.37 (s, 2H), 8.07-7.92 (m, 4H), 7.39 (d, J = 8.0 Hz, 1H), 6.85 (s, 1H), 6.62 (d, J = 10.9 Hz, 1H), 5.37 (s, 1H), 4.46 (m, 1H), 4.21 (m, 1H), 3.29-3.20 (m, 4H), 2.90 (m, 3H), 2.46-0.92 (m, 55H), 0.63 (s, 3H); ^{13}C NMR

(100 MHz, DMSO-d_6) δ 174.4, 172.6, 171.8, 171.7, 129.8, 129.0, 125.5, 122.4, 105.2, 57.0, 56.5, 56.0, 49.8, 42.3, 42.1, 39.3, 39.2, 39.1, 36.8, 36.6, 36.1, 36.0, 35.8, 35.6, 33.8, 31.7, 31.0, 30.6, 27.9, 25.8, 24.9, 24.8, 24.3, 23.6, 23.1, 22.8, 19.5, 19.2, 19.0, 12.1 ; IR (film) ν max 3419, 2951, 1682, 1643, 1540, 1438, 1375, 1310, 1205, 1142, 1015, 801, 721.6 cm^{-1}; HRMS (ESI+) m/z 1136.6179 (MH^+, $C_{64}H_{84}F_2N_5O_{11}$ requires 1136.6130).

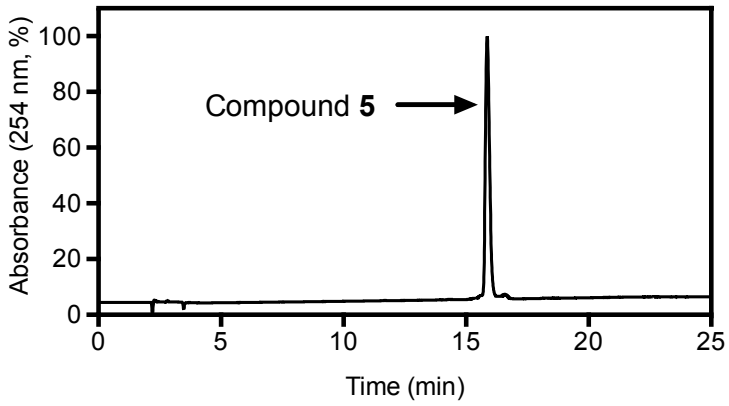
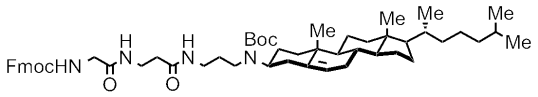
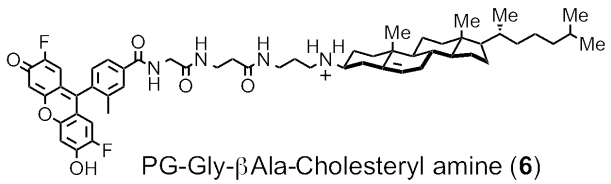


Figure S5. Analytical RP-HPLC profile of **5** after preparative RP-HPLC. Retention time = 15.8 min. Purity > 95%.



18

***tert*-Butyl-3β-cholest-5-en-3-yl[14-(9*H*-fluoren-9-yl)-5,9,12-trioxo-13-oxa-4,8,11-triazatetradec-1-yl]carbamate** (**18**). Compound **15** (150 mg, 0.18 mmol) was added to DMF (2 mL) containing piperidine (20%) and stirred for 30 min at 22 °C. The solvent was removed under reduced pressure to afford the crude primary amine. To a solution of Fmoc-Gly-OH (60 mg, 0.20 mmol) in anhydrous CH_2Cl_2 (10 mL) at 4 °C were added HOBt (30 mg, 0.22 mmol) and EDC (42 mg, 0.22 mmol) and the solution was stirred for 30 min. The primary amine derived from **15** in anhydrous CH_2Cl_2 (5 mL) was added. The reaction was allowed to warm to 22 °C and stirred for 12 h. This solution was diluted with CH_2Cl_2 (30 mL) and washed with aqueous NaOH (0.1 M, 30 mL) followed by saturated aqueous NaCl (30 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. Flash column chromatography (CH_2Cl_2, MeOH, 20:1) afforded **18** (153 mg, 97%) as a white solid, mp 112-114 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 (d, *J* = 7.4 Hz, 2H), 7.60 (d, *J* = 7.0 Hz, 2H), 7.39 (t, *J* = 7.3 Hz, 2H), 7.30 (t, *J* = 7.3 Hz, 2H), 6.99 (br, 1H), 5.78 (br, 1H), 5.31 (s, 1H), 4.38 (d, *J* = 6.9 Hz, 2H), 4.22 (t, *J* = 7.0 Hz, 1H), 3.86 (d, *J* = 4.5 Hz, 2H), 3.56 (d, *J* = 5.0 Hz, 2H), 3.22 (m, 2H), 2.52-0.86 (m, 56H), 0.67 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.5, 169.0, 156.5, 148.3, 143.8, 141.2, 127.7, 127.0, 125.1, 121.4, 119.9, 80.0, 67.1, 56.7, 56.1, 50.0, 47.1, 44.3, 42.3, 41.5, 39.7, 39.5, 38.4, 37.1, 36.7, 36.1, 35.9, 35.7, 31.8, 28.5, 28.2, 27.9, 26.8, 24.2, 23.8, 22.8, 22.5, 20.9, 19.4, 18.7, 11.8 ; IR (film) ν max 3311, 2936, 2868, 1717, 1667, 1543, 1465, 1450, 1413, 1365, 1249, 1168, 1048, 758 cm^{-1}; HRMS (ESI+) m/z 893.6122 (MH^+, $C_{55}H_{81}N_4O_6$ requires 893.6151).



***N*-(2-{[3-({3-[3β-Cholest-5-en-3-ylamino]propyl}amino)-3-oxopropyl]amino}-2-oxoethyl)-4-(2,7-difluoro-6-hydroxy-3-oxo-3H-xanthen-9-yl)-3-methylbenzamide** (**6**). Compound **18** (25 mg, 0.028 mmol) was added to DMF (1 mL) containing piperidine (20%) and stirred for 30 min at 22 °C. The solvent was removed under reduced pressure to afford the crude primary amine. Dry DMF (2 mL) and 4-carboxy-Pennsylvania Green succinimidyl ester[3] (11 mg, 0.023 mmol) were added followed by DIEA (50 μL, 0.24 mmol). The reaction was stirred at 22 °C for 12 h, and solvent was removed under reduced pressure. The orange residue was treated with CH_2Cl_2 (5 mL) containing TFA (15%) and stirred at 22 °C for 12 h. Solvent was removed under reduced pressure, the crude product was dissolved in methanol (2 mL), and purified by preparative reverse-phase HPLC (gradient: 90% H_2O, 9.9% MeCN, and 0.1% TFA to 99.9% MeCN and 0.1% TFA over 15 min; retention time = 13.0 min (495 nm) to afford **6** (18 mg, 82%) as an orange solid (TFA salt), mp 200-203 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.91 (s, 1H), 8.56 (s, 2H), 8.0-7.91 (m, 4H), 7.40 (d, J = 7.6 Hz, 1H), 6.86 (s, 2H), 6.60 (d, J = 11.1 Hz, 2H), 5.38 (s, 1H), 3.87 (m, 2H), 3.14 (m, 4H), 2.93 (m, 3H), 2.30-0.85 (m, 47H), 0.61 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 170.9, 168.8, 166.0, 149.0, 138.5, 136.0, 135.2, 134.8, 129.9, 129.0, 125.3, 122.6, 105.0, 56.5, 56.1, 55.5, 49.3, 42.7, 41.7, 41.5, 36.1, 35.9, 35.6, 35.4, 35.2, 34.2, 31.2, 27.7, 27.4, 26.2, 26.1, 24.4, 23.2, 22.7, 22.4, 20.5, 19.2, 18.7, 18.5, 11.6; IR (film) ν max 3419, 2950, 2115, 1646, 1540, 1465, 1374, 1310, 1194, 1147, 1016, 875 cm^{-1}; HRMS (ESI+) m/z 957.5350 (MNa^+, $C_{56}H_{72}F_2N_4O_6Na$ requires 957.5312).

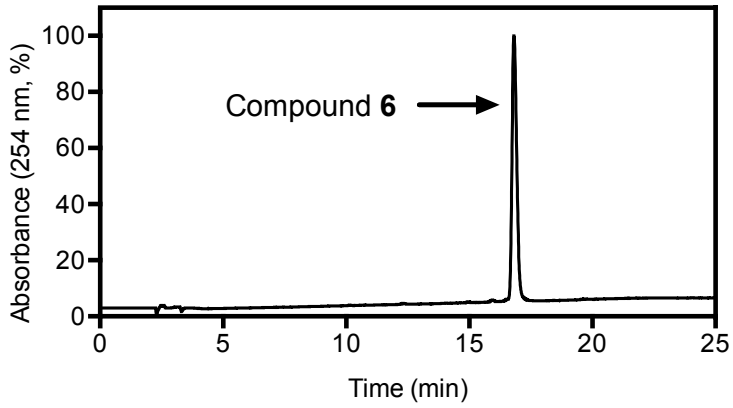
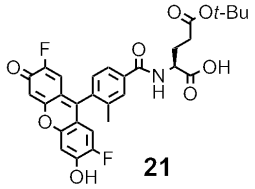
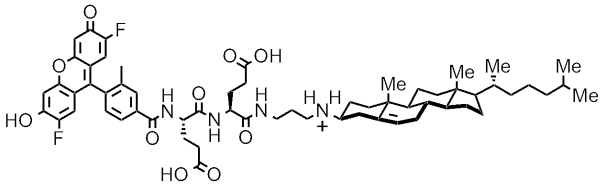


Figure S6. Analytical RP-HPLC profile of **6** after preparative RP-HPLC. Retention time = 16.9 min. Purity > 95%.



21

5-(tert-butoxy)-2-(4-(2,7-difluoro-6-hydroxy-3-oxo-3H-xanthen-9-yl)-3-methylbenzamido)-5-oxopentanoic acid (21) Fmoc-Glu(O-*t*-Bu)-OH (**20**, 67mg, 0.156 mmol) was treated with piperidine (20%) in DMF (1 mL) for 30 min. The solvent was removed under reduced pressure to afford the crude primary amine. 4-Carboxy-Pennsylvania Green succinimidyl ester[3] (25 mg, 0.052 mmol) in DMF (1 mL) was added to the crude primary amine in DMF (1 mL) followed by DIEA (0.25 mL). The reaction was stirred at 22 °C for 16 h, dried under reduced pressure, and purified by flash column chromatography (MeOH, CH_2Cl_2, AcOH, 5:94.9:0.1) to afford **21** (27 mg, 90%) as an orange residue, mp 134-138 °C; ^{1}H NMR (400 MHz, CD_3OD) δ 8.00 (s, 1H), 7.94 (dd, *J* = 7.9, 1.2 Hz, 1H), 7.40 (d, *J* = 7.9 Hz, 1H), 6.98 – 6.89 (m, 2H), 6.81 – 6.77 (m, 2H), 4.70 (dd, *J* = 9.6, 4.9 Hz, 1H), 2.47 (t, *J* = 7.0 Hz, 2H), 2.39 – 2.27 (m, 1H), 2.14 – 2.08 (m, 1H), 2.12 (s, 3H), 1.46 (s, 9H); ^{13}C NMR (101 MHz, CD_3OD) δ 174.95, 173.97, 169.66, 156.19, 138.02, 137.15, 136.55, 131.01, 130.47, 126.62, 112.98, 112.76, 106.32, 81.94, 53.70, 32.95, 28.35 (t), 27.66, 19.60 ; IR (film) ν max 2929, 1678, 1607, 1536, 1371, 1302, 1190, 1139, 953, 842, 801, 750, 724 cm^{-1}; HRMS (ESI+) m/z 568.1779 (MH^+, $C_{30}H_{28}F_2NO_8$, requires 568.1777).



PG-GluGlu-Cholesteryl amine (**7**)

(4S)-5-(((2S)-4-carboxy-1-((3-[3β-cholest-5-en-3-ylamino]propyl)amino)-1-oxobutan-2-yl)amino)-4-(4-(2,7-difluoro-6-hydroxy-3-oxo-3H-xanthen-9-yl)-3-methylbenzamido)-5-oxopentanoic acid (7). Compound **19**, synthesized by a previously reported method,[5] (26.8 mg, 0.028 mmol) was added to DMF (0.5 mL) containing piperidine (20%) and stirred for 30 min at 22 °C. The solvent was removed under reduced pressure to afford the crude primary amine. To a solution of **21** (8.0 mg, 0.014 mmol) in anhydrous DMF (2 mL) at 4 °C was added HOBt (4.0 mg, 0.028 mmol) and EDC (5.4 mg, 0.028 mmol) and the solution was stirred at 22 °C for 30 min. The primary amine derived from **19** in anhydrous DMF (1 mL) was added at 4 °C. The reaction was allowed to warm to 22 °C and stirred for 12 h. The solvent was removed under reduced pressure. The resulting residue was re-dissolved in TFA/CH_2Cl_2 (1:1, 2 mL) and stirred at 22 °C for 2 h. The solvent was removed under reduced pressure, the crude product was dissolved in DMSO (2 mL), and purified by preparative reverse-phase HPLC (gradient: 90% H_2O, 9.9% MeCN, and 0.1% TFA to 99.9% MeCN and 0.1% TFA over 20 min; retention time = 16.5 min (495 nm) to afford **7** (13.1 mg, 87%) as a orange solid (TFA salt), mp 175-178 °C; ^{1}H NMR (500 MHz, CD_3OD) δ 7.93 (s, 1H), 7.87 – 7.84 (m, 1H), 7.45 (t, *J* = 7.5 Hz, 1H), 6.87 – 6.75 (m, 2H), 6.72 – 6.58 (m, 2H), 5.39 (d, *J* = 4.9 Hz, 1H), 4.45 (m, 1H), 4.19 (m, 1H), 3.37 – 3.28 (m, 2H), 3.28 – 3.23 (m, 2H), 3.03 – 2.94 (m, 2H), 2.88 (m, 1H), 2.54 – 0.69 (m, 52H), 0.59 (s, 3H); ^{13}C NMR (126 MHz, CD_3OD) δ 176.85, 176.47, 174.94, 174.34, 169.92, 156.15, 146.38, 140.74, 139.53, 138.09, 136.70, 131.60, 131.47, 131.15, 130.50, 130.09, 126.78 (d, *J* = 13.6 Hz), 124.85 (d, *J* = 4.8 Hz), 122.22, 115.64, 113.74, 106.42, 59.41, 58.03, 57.51, 55.76, 55.01, 51.42, 43.35 (d, *J* = 22.4 Hz), 40.99, 40.69, 38.22, 37.82, 37.35, 37.10, 36.92, 36.38, 32.99 (d, *J* = 14.1 Hz), 31.55, 31.23, 29.22 (d, *J* = 14.1 Hz), 27.82, 27.67, 27.58, 26.27, 25.98, 25.24,

24.96, 23.08 (d, J = 31.7 Hz), 22.06, 19.64 (d, J = 10.6 Hz), 19.22, 12.27 ; IR (film) ν max 3387, 2323, 1675, 1541, 1439, 1202, 1135, 842, 802, 724 cm^{-1}; HRMS (ESI-) m/z 1063.5757 (M^-, $C_{61}H_{77}F_2N_4O_{10}$ requires 1063.5613).

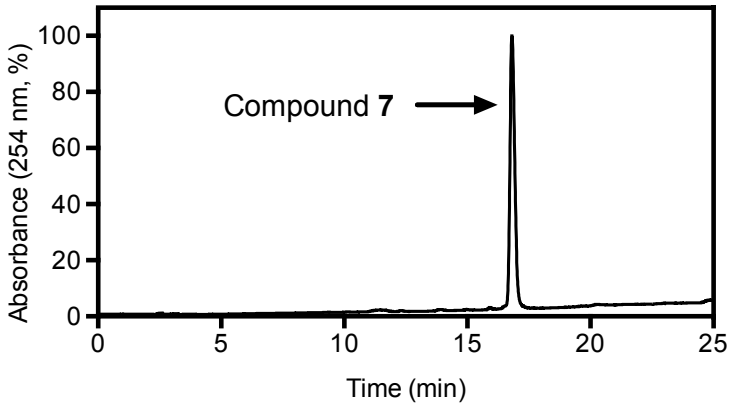
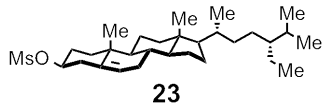
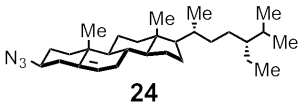


Figure S7. Analytical RP-HPLC profile of **7** after preparative RP-HPLC. Retention time = 16.8 min. Purity > 95%.



23

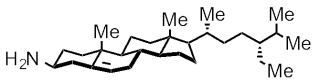
Sitost-5-en-3β-ol, methanesulfonate (23). To a solution of β-sitosterol (**22**, 5.0 g, 12.0 mmol) in anhydrous CH_2Cl_2 (100 mL) at 4 °C was added freshly distilled triethylamine (2.7 mL, 18.1 mmol) followed by a dropwise solution of methanesulfonyl chloride (1.03 mL, 13.25 mmol) in anhydrous CH_2Cl_2 (10 mL). The reaction was maintained at 4 °C for 30 min, warmed to 22 °C, and stirred for 16 h. The solvent was removed reduced pressure, and the resulting residue was purified by flash chromatography (ethyl acetate, hexane, 1:4) to give **23** (4.1 g, 69%) as a white solid, mp 97-100 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.45 – 5.38 (m, 1H), 4.52 (dt, J = 11.9, 3.7 Hz, 1H), 2.99 (s, 3H), 2.51 (ddd, J = 7.2, 4.9, 2.0 Hz, 2H), 2.09 – 0.75 (m, 42H), 0.68 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 138.57, 123.61, 81.82, 56.52, 55.96, 49.86, 45.76, 42.19, 39.55, 39.04, 38.62, 36.79, 36.25, 35.97, 33.84, 31.71 (d, J = 5.5 Hz), 29.12, 28.85, 28.04, 26.09, 24.11, 22.98, 20.89, 19.62, 18.96 (d, J = 9.6 Hz), 18.63, 11.75 (d, J = 13.3 Hz); IR (film) ν max 2958, 2931, 2867, 1465, 1350, 1326, 1168, 946, 865, 802 cm^{-1}; HRMS (EI+) m/z 492.9897 (M^+, $C_{30}H_{52}O_3S$ requires 492.3637).



24

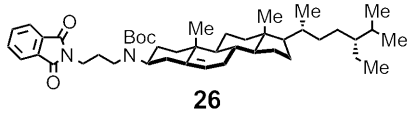
3β-Azido-5-sitostene (24). To a solution of compound **23** (1.7 g, 3.45 mmol) in anhydrous CH_2Cl_2 (30 mL) was added TMS-N_3 (0.68 mL, 5.20 mmol), followed by $BF_3{\cdot}Et_2O$ (0.86 mL, 6.90 mmol). The reaction was stirred at 22 °C for 16 h. The reaction was slowly poured into aqueous NaOH (1.0 M, 30 mL) and stirred for 5 min. The organic phase was separated and the aqueous layer was extracted with CH_2Cl_2 (30 mL). The combined organic extracts were washed with saturated NaCl solution (500 mL), dried over anhydrous Na_2SO_4, and solvent was removed under reduced pressure to give the crude product as light yellow oil. The crude product was purified by flash column chromatography (ethyl acetate, hexane, 2:98) to afforded **24** (800 mg, 53%) as a white solid, mp 74-78 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.42 – 5.35 (m, 1H), 3.20 (tdd, J = 11.5, 7.8, 4.1 Hz, 1H), 2.29 (d, J = 7.9 Hz, 2H), 2.05 – 0.75 (m, 42H), 0.68 (s, 3H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 139.84, 122.55, 61.17, 56.73, 56.07, 50.11, 45.84, 42.32, 39.72, 38.17, 37.59, 36.62, 36.16, 33.95, 31.85 (d, *J* = 6.6 Hz), 29.16, 28.25, 27.96, 26.08, 24.29, 23.08, 21.01, 19.84, 19.29, 19.05, 18.80, 11.93 (d, *J* = 13.3 Hz); IR (film) ν max 2935, 2867, 2090, 1463, 1377, 1259, 750 cm^{-1}; HRMS (EI+) m/z 411.3872 (M-N_2, $C_{29}H_{49}N$ requires 411.3865).



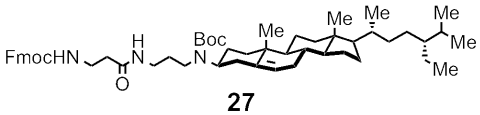
25

3β-Amino-5-sitostene (25). To a solution of **24** (180 mg, 0.44 mmol) in anhydrous diethyl ether (5 mL) at 4 °C was added $LiAlH_4$ powder (25 mg, 0.66 mol) in two equal portions. The reaction was maintained at 4 °C for 30 min, warmed to 22 °C, and stirred for 2 h. The reaction was cooled to 4 °C and carefully quenched by slow dropwise addition of ice-cold water. When evolution of H_2 gas ceased, the solution was poured into water (10 mL). The organic phase was separated, and the aqueous phase was extracted with ethyl acetate (10 mL × 2). The combined organic extracts were washed with saturated NaCl solution (10 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The resulting solid was dissolved in $CHCl_3$ (10 mL) and residual inorganic salts were removed by filtration. Concentration of the filtrate afforded **25** (140 mg, 77%) as a white solid, mp 122-125 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.39 – 5.27 (m, 1H), 2.75 (q, *J* = 7.2 Hz, 1H), 2.49 (d, *J* = 13.0 Hz, 2H), 2.21 – 0.74 (m, 42H), 0.67 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 141.54, 120.89, 56.80, 56.06, 51.90, 50.23, 45.74 (d, *J* = 19.1 Hz), 42.74, 42.32, 39.80, 38.11, 36.56, 36.16, 33.95, 32.14, 31.90, 29.15, 28.26, 26.07, 24.31, 23.07, 21.02, 19.83, 19.45, 19.04, 18.79, 11.93 (d, *J* = 12.1 Hz); IR (film) ν max 3360, 3280, 3160, 2926, 2852, 1587, 1462, 1381, 1022, 957, 836, 799, 738 cm^{-1}; HRMS (ESI+) m/z 414.4074 (MH^+, $C_{29}H_{52}N$ requires 414.4094).



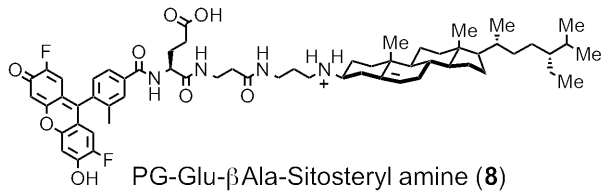
26

***tert*-Butyl-3β-sitost-5-en-3-yl[3-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)propyl]carbamate** (**26**). To DMF (8 mL) was added compound **25** (130 mg, 0.31 mmol), *N*-(3-bromopropyl)phthalimide (103 mg, 0.38 mmol), and K_2CO_3 (108 mg, 0.78 mmol). The solution was heated to 60 °C and stirred for 24 h. The reaction was cooled to 22 °C, and the solvent removed under reduced pressure. To the resulting residue was added CH_2Cl_2 (10 mL). The insoluble material was removed by filtration and washed with CH_2Cl_2 (2 × 5 mL). To the combined filtrate and wash solutions containing the crude secondary amine product was added $(Boc)_2O$ (135 mg, 0.62 mmol) and DIEA (0.17 mL, 1.24 mmol). The reaction was stirred for 4 h at 22 °C and concentrated under reduced pressure. Flash column chromatography (hexane, ethyl acetate, 85:15) afforded **26** (90 mg, 42% over 2 steps) as a white solid, mp 122-124 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.88 – 7.79 (m, 2H), 7.72 (m, 2H), 5.37 – 5.25 (m, 1H), 3.85 (td, *J* = 6.9, 4.9 Hz, 1H), 3.70 (t, *J* = 7.1 Hz, 2H), 3.15 (br, 2H), 2.06 – 0.73 (m, 56H), 0.66 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.34, 134.07, 133.94, 132.11, 123.35, 123.21, 79.42, 56.71, 56.04, 50.12, 45.83, 42.31, 39.75, 36.17, 35.96, 33.94, 31.88, 29.14, 28.47, 28.26, 26.07, 24.29, 23.06, 20.99, 19.83, 19.41, 19.04, 18.78, 11.93 (d, *J* = 12.2 Hz); IR (film) ν max 3340, 2935, 2869, 1713, 1676, 1466, 1365, 1249, 1169, 1143, 907, 730 cm^{-1}; HRMS (ESI+) m/z 723.4980 (MNa^+, $C_{45}H_{68}N_2O_4Na$, requires 723.5071).



27

9*H*-Fluoren-9-ylmethyl{3-[(3-{(*tert*-butoxycarbonyl)[3β-sitost-5-en-3-yl]amino}propyl)amino]-3-oxo propyl}carbamate (**27**). To a solution of **26** (80 mg, 0.11 mmol) in absolute ethanol (3 mL) was added anhydrous hydrazine (17 μL, 0.55 mmol). The solution was heated to 50 °C and stirred for 4 h. The reaction was cooled to 22 °C, and a white precipitate was removed by filtration. The filtrate was concentrated under reduced pressure, and the residue was dissolved in $CHCl_3$ (6 mL). After insoluble material was removed by filtration, concentration of the filtrate under reduced pressure afforded the phthalimide-deprotected primary amine, a white solid that was carried forward without further purification. To Fmoc-β-Ala-OH (69 mg, 0.22 mmol) in anhydrous CH_2Cl_2 (2 mL) at 4 °C were added HOBt (30 mg, 0.22 mmol) and EDC (42 mg, 0.22 mmol) followed by stirring at 4 °C for 30 min. To this solution was added dropwise the phthalimide-deprotected primary amine in anhydrous CH_2Cl_2 (2 mL). The reaction was allowed to warm to 22 °C and stirred for 12 h. The solution was concentrated under reduced pressure, and flash column chromatography (CH_2Cl_2, MeOH, 99:1) afforded **27** (56 mg, 60% over two steps) as a white solid, mp 86-90 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.5 Hz, 2H), 7.60 (d, *J* = 7.5 Hz, 2H), 7.39 (t, *J* = 7.4 Hz, 2H), 7.30 (t, *J* = 7.4 Hz, 2H), 6.99 (br, 1H), 5.79 (br, 1H), 5.33 (d, *J* = 4.6 Hz, 1H), 4.35 (d, *J* = 7.2 Hz, 2H), 4.20 (t, *J* = 7.1 Hz, 1H), 3.56 – 3.46 (m, 2H), 3.25 (m, 4H), 2.44 (m, 2H), 2.07 – 0.73 (m, 56H), 0.67 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 156.50, 144.04, 141.29, 127.64, 127.03, 125.19, 121.47, 119.94, 66.71, 56.75, 56.06, 50.10, 47.27, 45.84, 42.32, 39.75, 38.45, 36.74, 36.16, 35.98, 33.95, 31.88, 29.15, 28.55, 28.26, 26.08, 24.30, 23.07, 21.01, 19.84, 19.47, 19.04, 18.79, 11.93 (d, *J* = 12.7 Hz); IR (film) ν max 3320, 2934, 2869, 1665, 1541, 1450, 1365, 1249, 1167, 1141, 908, 732 cm^{-1}; HRMS (ESI+) m/z 886.5963 (MNa^+, $C_{55}H_{81}N_3O_5Na$, requires 668.6068).



PG-Glu-βAla-Sitosteryl amine (**8**)

(4*S*)-5-{[3-({3-[3β-Sitost-5-en-3-ylamino]propyl}amino)-3-oxopropyl]amino}-4-{[4-(2,7-difluoro-6-hydroxy-3-oxo-3*H*-xanthen-9-yl)-3-methylbenzoyl]amino}-5-oxopentanoic acid (**8**). Compound **27** (24.4 mg, 0.028 mmol) was added to DMF (0.5 mL) containing piperidine (20%) and stirred for 30 min at 22 °C. The solvent was removed under reduced pressure to afford the crude primary amine. To a solution of **21** (8.0 mg, 0.014 mmol) in anhydrous DMF (2 mL) at 4 °C was added HOBt (4.0 mg, 0.028 mmol) and EDC (5.4 mg, 0.028 mmol), and the solution was stirred for 30 min. The primary amine derived from **27** in anhydrous DMF (1 mL) was added. The reaction was allowed to warm to 22 °C and stirred for 12 h. The reaction was concentrated under reduced pressure, TFA/CH_2Cl_2 (1:1, 2 mL) was added, and the mixture was stirred for 2 h. The solvent was removed under reduced pressure and the crude product was dissolved in DMSO (2 mL). Purification by preparative reverse-phase HPLC (gradient: 90% H_2O, 9.9% MeCN, and 0.1% TFA to 99.9% MeCN and 0.1% TFA over 20 min; retention time = 17.5 min (495 nm) afforded **8** (11.4 mg, 79%) as an orange solid (TFA salt), mp 192-195 °C; ^{1}H NMR (500 MHz, CD_3OD) δ 7.93 (s, 1H), 7.85 (d, *J* = 7.4 Hz, 1H), 7.30 (d, *J* = 7.9 Hz, 1H), 6.86 – 6.75 (m, 2H), 6.73 – 6.57 (m, 2H), 5.37 (d, *J* = 4.5 Hz, 1H), 4.44 (dd, *J* = 9.1, 5.1 Hz, 1H), 3.43 (t, *J* = 6.3 Hz, 2H), 2.98 (t, *J* = 7.2 Hz, 2H),

2.89 (d, *J* = 12.1 Hz, 1H), 2.48 – 0.66 (m, 57H), 0.60 (s, 3H); ^{13}C NMR (126 MHz, CD_3OD) δ 176.70, 175.01, 174.05, 169.61, 156.14, 139.52, 138.29 – 137.87 (m), 136.96, 136.67, 131.10, 130.51, 126.76, 124.79, 112.74 (d, *J* = 22.9 Hz), 106.43, 59.30, 58.05, 57.41, 55.45, 51.45, 49.52, 49.35, 49.18, 49.07, 49.07 – 48.88 (m), 48.84, 48.58 (d, *J* = 21.4 Hz), 47.26, 43.40 (d, *J* = 8.9 Hz), 40.99, 38.24, 37.81, 37.41, 37.19 – 36.60 (m), 36.34, 35.07, 32.98 (d, *J* = 13.5 Hz), 31.52, 30.37, 29.32, 27.97 (d, *J* = 9.4 Hz), 27.15, 26.33, 25.24, 24.14, 22.05, 20.21, 19.69, 19.62 – 19.17 (m), 12.32 (d, *J* = 9.0 Hz); IR (film) ν max 3306, 2956, 2869, 1671, 1541, 1302, 1201, 1135, 841, 801, 724 cm^{-1}; HRMS (ESI-) m/z 1033.5874 (M-H$^+$, $C_{61}H_{79}F_2N_4O_8$ requires 1033.5871).

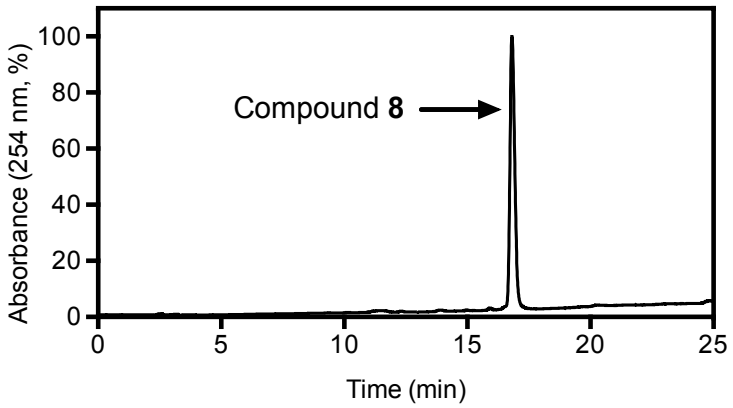
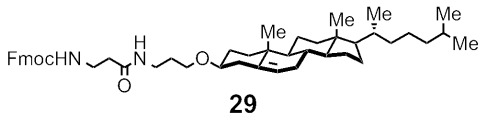
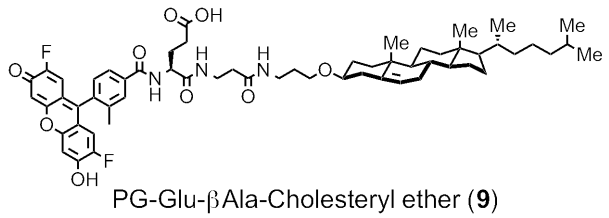


Figure S8. Analytical RP-HPLC profile of **8** after preparative RP-HPLC. Retention time = 16.8 min. Purity > 95%.



29

9H-fluoren-9-ylmethyl{3-[(3-{[3β-cholest-5-en--3-yl]oxy}propyl)amino]-3-oxopropyl}carbamate (29). To Fmoc-β-Ala-OH (380 mg, 1.22 mmol) in anhydrous CH_2Cl_2 (5 mL) at 4 °C was added HOBt (165 mg, 1.22 mmol) and EDC (234 mg, 1.22 mmol), and the mixture was stirred at 4 °C for 30 min. To this solution was added compound **28** (350 mg, 0.81 mmol), synthesized as previously reported,[2] in anhydrous CH_2Cl_2 (5 mL). The reaction was allowed to warm to 22 °C and was stirred for 12 h. The solution was diluted with CH_2Cl_2 (10 mL) and washed with saturated aqueous $NaHCO_3$ (10 mL) and saturated aqueous NaCl (10 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. Flash column chromatography (CH_2Cl_2/MeOH, 99:1) afforded **29** (515 mg, 87%) as a glassy solid, mp 148-150 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.5 Hz, 2H), 7.59 (d, *J* = 7.5 Hz, 2H), 7.39 (t, *J* = 7.4 Hz, 2H), 7.30 (t, *J* = 7.4 Hz, 2H), 6.34 (br, 1H), 5.58 (br, 1H), 5.34 – 5.26 (m, 1H), 4.35 (d, *J* = 7.2 Hz, 2H), 4.20 (t, *J* = 7.1 Hz, 1H), 3.61 – 3.44 (m, 4H), 3.38 (dt, *J* = 11.4, 5.8 Hz, 2H), 3.16 – 3.04 (m, 1H), 2.46 – 0.79 (m, 44H), 0.69 – 0.63 (m, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.19, 156.62, 144.12, 141.42, 140.64, 127.79, 127.17, 125.29, 122.02, 120.09, 79.46, 67.37, 66.90, 56.87, 56.29, 50.27, 47.35, 42.44, 39.89, 39.65, 39.23, 38.86, 37.24, 36.96, 36.33, 35.99 (d, *J* = 15.0 Hz), 32.00 (d, *J* = 5.9 Hz), 29.24, 28.61, 28.36, 28.15, 24.40, 23.96, 22.96, 22.70, 21.17, 19.49, 18.86, 11.99 ; IR (film) ν max 3306, 2934, 2867, 1682, 1639, 1537, 1448, 1376, 1342, 1268, 1240, 1103, 1018, 908, 735 cm^{-1}; HRMS (ESI+) m/z 775.4972 (M+K$^+$, $C_{48}H_{68}N_2O_4K$ requires 775.4811).



PG-Glu-βAla-Cholesteryl ether (**9**)

(4S)-5-{[3-({3-[3β-Cholest-5-en-3-yloxy]propyl}amino)-3-oxopropyl]amino}-4-{[4-(2,7-difluoro-6-hydroxy-3-oxo-3*H*-xanthen-9-yl)-3-methylbenzoyl]amino}-5-oxopentanoic acid (9). Compound **29** (15.0 mg, 0.020 mmol) was added to DMF (0.5 mL) containing piperidine (20%) and stirred for 30 min at 22 °C. The solvent was removed under reduced pressure to afford the crude primary amine. To a solution of **21** (11.3 mg, 0.020 mmol) in anhydrous DMF (2 mL) at 4 °C was added HOBt (5.5 mg, 0.040 mmol) and EDC (8.0 mg, 0.040 mmol) and the solution was stirred for 30 min. The primary amine derived from **29** in anhydrous DMF (1 mL) was added. The reaction was allowed to warm to 22 °C and was stirred for 12 h. The reaction was concentrated under reduced pressure, TFA/CH_2Cl_2 (1:1, 2 mL) was added, and the solution stirred at 22 °C for 2 h. The solvent was removed under reduced pressure and the crude product was dissolved in DMSO (2 mL). Purification by preparative reverse-phase HPLC (gradient: 90% H_2O, 9.9% MeCN, and 0.1% TFA to 99.9% MeCN and 0.1% TFA over 20 min; retention time = 16.5 min (495 nm) afforded **9** (13.1 mg, 87%) as an orange solid, mp 192-194 °C; ^{1}H NMR (400 MHz, CD_3OD) δ 8.01 (s, 1H), 7.94 (d, *J* = 7.9 Hz, 1H), 7.28 – 7.36 (m, 1H), 7.04 – 6.85 (m, 2H), 6.82 – 6.70 (m, 2H), 5.32 – 5.26 (m, 1H), 4.60 – 4.54 (m, 1H), 3.56 – 3.45 (m, 4H), 3.45 – 3.36 (m, 2H), 3.28 – 3.22 (m, 4H), 3.17 – 3.04 (m, 2H), 2.62 – 0.81 (m, 46H), 0.67 (s, 3H); ^{13}C NMR (101 MHz, CD_3OD) δ 190.52, 183.25, 180.16, 173.46, 171.19, 161.26, 155.36, 150.34, 141.55, 136.58, 133.73, 130.91, 126.48, 122.52, 106.27, 80.31, 67.05, 66.56, 57.81, 57.19, 51.31, 43.21, 40.79, 40.41, 39.93, 38.54, 38.11, 37.76 (d, *J* = 16.8 Hz), 37.08, 36.94, 36.78, 36.34, 32.82 (d, *J* = 11.2 Hz), 31.33, 30.49, 29.14 (d, *J* = 15.5 Hz), 28.87, 27.88, 25.06, 24.68, 23.15, 22.89, 21.91, 19.81 (d, *J* = 5.1 Hz), 19.17, 12.28. IR (film) ν max 3369, 2925, 2853, 2076, 1646, 1543, 1464, 1375, 1314, 1193, 1117, 971, 735 cm^{-1}; HRMS (ESI-) m/z 1006.5441 (M-H$^+$, $C_{59}H_{74}F_2N_3O_9$ requires 1006.5399).

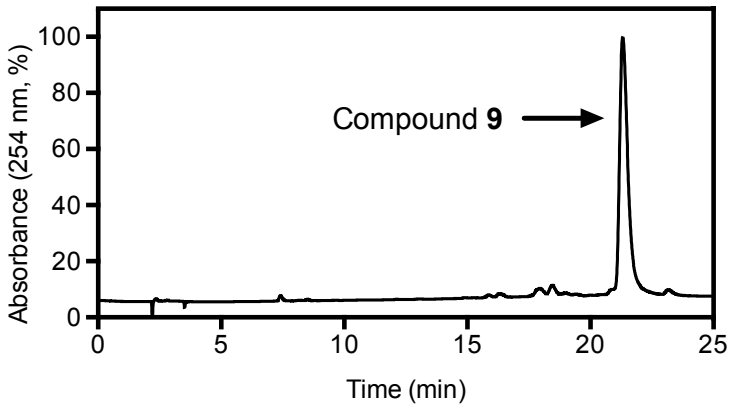
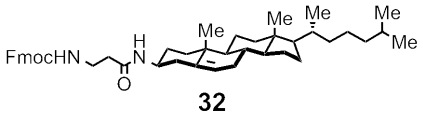
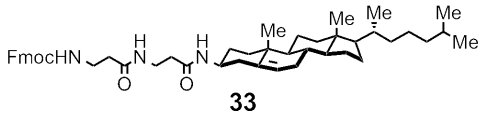


Figure S9. Analytical RP-HPLC profile of **9** after preparative RP-HPLC. Retention time = 21 min. Purity > 95%.



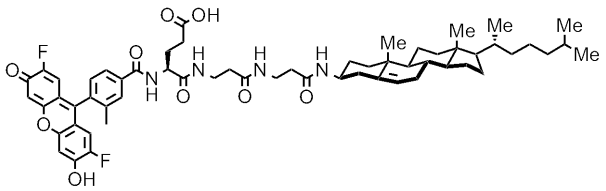
32

(9H-fluoren-9-yl)methyl(3-{[3β-cholest-5-en-3-yl]amino}-3-oxopropyl)carbamate (32). Fmoc-β-Ala-Cl (**30**, 170 mg, 0.54 mmol, prepared by treatment of Fmoc-β-Ala-OH with excess $SOCl_2$ for 1 h followed by removal of $SOCl_2$ under reduced pressure) was dissolved in anhydrous CH_2Cl_2 (5 mL). A solution of 3β-amino-5-cholestene (**31**, 250 mg, 0.65 mmol) and 4-dimethylaminopyridine (DMAP, 15 mg, 0.10 mmol) in anhydrous CH_2Cl_2 was slowly added and the solution was stirred at 22 °C for 30 min. The reaction was diluted with CH_2Cl_2 (25 mL) and filtered to remove precipitated solids. The resulting filtrate was concentrated under reduced pressure and purified via flash column chromatography (CH_2Cl_2, MeOH, 99:1) to afford **32** (128 mg, 35%) as a white solid, mp 124-128 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 (d, *J* = 7.5 Hz, 2H), 7.58 (d, *J* = 7.5 Hz, 2H), 7.39 (t, *J* = 7.4 Hz, 2H), 7.30 (t, *J* = 7.3 Hz, 2H), 5.54 (br, 1H), 5.41 – 5.32 (m, 2H), 4.36 (m, 2H), 4.20 (t, *J* = 6.9 Hz, 1H), 3.69 (m, 1H), 3.48 (m, 2H), 2.39 (m, 2H), 2.29 (dd, *J* = 13.2, 2.6 Hz, 1H), 2.14 – 0.82 (m, 39H), 0.69 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.28, 156.57, 144.00, 141.33, 140.12, 127.65, 127.04, 125.07, 122.07, 119.93, 77.32, 77.00, 76.68, 66.78, 56.74, 56.23, 50.16, 49.86, 47.30, 42.34, 39.78, 39.54, 39.31, 37.86, 37.22, 36.57, 36.22, 35.79, 31.87 (d, *J* = 4.6 Hz), 29.18, 28.20, 27.99, 24.27, 23.86, 22.77, 22.53, 20.99, 19.31, 18.73, 11.86 ; IR (film) ν max 3313, 2933, 2867, 1686, 1636, 1534, 1449, 1267, 1148, 1023, 992, 735 cm^{-1}; HRMS (ESI+) m/z 701.4615 (M+Na$^+$, $C_{45}H_{62}N_2O_3Na$, requires 701.4658).



33

(9H-fluoren-9-yl)methyl(3-((3-{[3β-cholest-5-en-3-yl]amino}-3-oxopropyl)amino)-3-oxopropyl)carbamate (**33**). Compound **32** (30.0 mg, 0.045 mmol) was added to DMF (0.5 mL) containing piperidine (20%) and stirred for 30 min at 22 °C. The solvent was removed under reduced pressure to afford the crude primary amine. To a solution of Fmoc-β-Ala-OH (27.5 mg, 0.090 mmol) in anhydrous DMF (2 mL) at 4 °C were added HOBt (12.0 mg, 0.090 mmol) and EDC (17.0 mg, 0.090 mmol) and the solution was stirred for 30 min. The primary amine derived from **32** in anhydrous DMF (1 mL) was added. The reaction was allowed to warm to 22 °C and stirred for 12 h. The reaction was concentrated under reduced pressure, and flash column chromatography (CH_2Cl_2/MeOH, 98/2) afforded **33** (23.8 mg, 71%) as a white solid, mp 178-180 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.5 Hz, 2H), 7.59 (d, *J* = 7.4 Hz, 2H), 7.39 (t, *J* = 7.4 Hz, 2H), 7.30 (t, *J* = 7.4 Hz, 2H), 6.52 (br, 1H), 5.66 – 5.49 (m, 2H), 5.31 (d, *J* = 11.0 Hz, 1H), 4.35 (d, *J* = 7.0 Hz, 2H), 4.20 (t, *J* = 7.1 Hz, 1H), 3.65 (m, 1H), 3.58 – 3.42 (m, 4H), 2.39 (m, 4H), 2.24 (m, 1H), 2.12 – 0.78 (m, 39H), 0.66 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.64, 156.46, 143.99, 141.30, 139.96, 127.68, 127.05, 125.17, 122.18, 119.97, 66.76, 56.67, 56.13, 49.97 (d, *J* = 13.5 Hz), 47.25, 42.29, 39.62 (d, *J* = 19.0 Hz), 39.21, 37.77, 36.51, 36.19, 35.98, 35.80, 35.54 (d, *J* = 12.7 Hz), 31.80, 29.08, 28.13 (d, *J* = 20.8 Hz), 28.01 – 27.92 (m), 24.27, 23.84, 22.83, 22.57, 20.94, 19.30, 18.72, 11.86 ; IR (film) ν max 3310, 2932, 2867, 1687, 1637, 1536, 1450, 1367, 1273, 1148, 1105, 1025, 739 cm^{-1}; HRMS (ESI+) m/z 772.4980 (M+Na$^+$, $C_{48}H_{67}N_3O_4Na$, requires 772.5024).



PG-Glu-βAla-Cholesteryl amide (**10**)

(4S)-4-(4-(2,7-difluoro-6-hydroxy-3-oxo-3H-xanthen-9-yl)-3-methylbenzamido)-5-((3-((3-{[3β-cholest-5-en-3-yl]amino}-3-oxopropyl)amino)-3-oxopropyl)amino)-5-oxopentanoic acid (10). Compound **33** (21.0 mg, 0.028 mmol) was added to DMF (0.5 mL) containing piperidine (20%) and stirred for 30 min at 22 °C. The solvent was removed under reduced pressure to afford the crude primary amine. To a solution of **21** (8.0 mg, 0.014 mmol) in anhydrous DMF (2 mL) at 4 °C was added HOBt (4.0 mg, 0.028 mmol) and EDC (5.4 mg, 0.028 mmol) and the solution was stirred for 30 min. The primary amine derived from **33** in anhydrous DMF (1 mL) was added. The reaction was allowed to warm to 22 °C and stirred for 12 h. The reaction was concentrated under reduced pressure. TFA/CH_2Cl_2 (1:1, 2 mL) was added and the solution was stirred for 2 h at 22 °C. The solvent was removed under reduced pressure, and the crude product was dissolved in DMSO (2 mL). Purification by preparative reverse-phase HPLC (gradient: 90% H_2O, 9.9% MeCN, and 0.1% TFA to 99.9% MeCN and 0.1% TFA over 20 min; retention time = 19.0 min (495 nm) afforded **10** (10.2 mg, 71%) as a orange solid, mp 192-194 °C; ^{1}H NMR (500 MHz, CD_3OD) δ 8.01 (s, 1H), 7.96 (d, *J* = 6.8 Hz, 1H), 7.33 (d, *J* = 7.7 Hz, 1H), 6.85 (m, 2H), 6.66 (m, 2H), 5.32 (m, 1H), 3.64 – 3.52 (m, 2H), 3.52-3.44 (m, 2H), 2.58 – 0.81 (m, 53H), 0.69 (s, 3H); ^{13}C NMR (126 MHz, CD_3OD) δ 175.61, 173.36, 168.58, 163.08, 155.99, 141.21, 137.35, 136.58, 135.94, 130.61, 130.43, 129.83, 126.05, 122.33, 113.29, 111.80, 106.20, 57.46, 56.87, 50.95, 50.55, 43.30, 42.94, 40.46, 40.17, 39.35, 38.62, 37.24, 36.83, 36.51, 32.51 (d, *J* = 10.1 Hz), 29.19, 28.85, 28.63, 24.84, 24.45, 23.09, 22.83, 21.59, 19.83, 19.64, 19.07, 12.21 ; IR (film) ν max 3305, 2932, 2871, 1646, 1541, 1371, 1306, 1188, 952, 750 cm^{-1}; HRMS (ESI-) m/z 1019.5361 (M-H$^+$, $C_{59}H_{734}F_2N_4O_9$ requires 1019.5351).

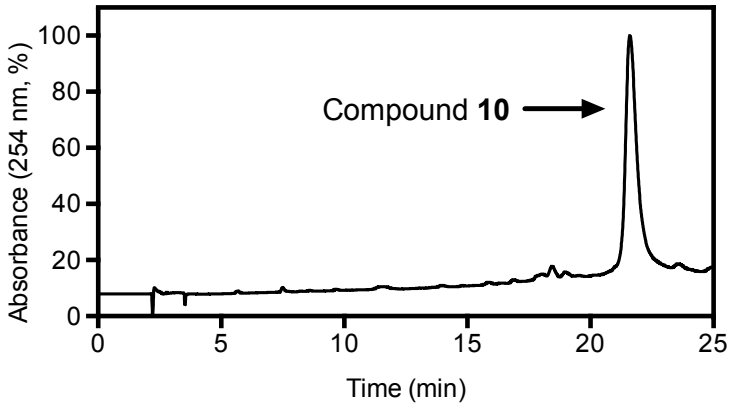
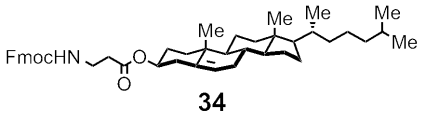
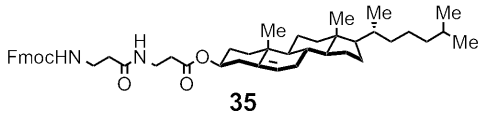


Figure S10. Analytical RP-HPLC profile of **10** after preparative RP-HPLC. Retention time = 21 min. Purity > 95%.



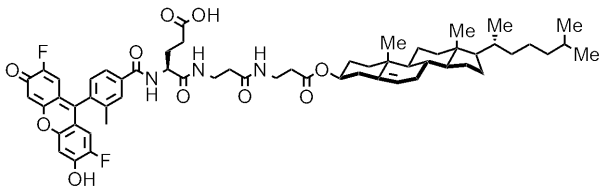
34

Cholesteryl 3-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)propanoate (34). A solution of cholesterol (**1**, 681 mg, 1.76 mmol) and 4-dimethylaminopyridine (60 mg, 0.50 mmol) in anhydrous CH_2Cl_2 (5 mL) was slowly added to Fmoc-β-Ala-Cl (**30**, 500 mg, 1.60 mmol) in anhydrous CH_2Cl_2 (5 mL) and the solution was stirred at 22 °C for 12 h. The reaction was diluted with CH_2Cl_2 (25 mL) and filtered to remove precipitated solids. The resulting filtrate was concentrated under reduced pressure and purified via flash column chromatography (hexane, ethyl acetate, 7:3) to provide **34** (590 mg, 55%) as a white foam, mp 102-104 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.5 Hz, 2H), 7.59 (d, *J* = 6.9 Hz, 2H), 7.40 (t, *J* = 7.4 Hz, 2H), 7.31 (t, *J* = 7.4 Hz, 2H), 5.39 (d, *J* = 3.8 Hz, 1H), 5.39-5.33 (m, 1H), 4.66 (d, *J* = 8.4 Hz, 1H), 4.38 (d, *J* = 7.1 Hz, 2H), 4.21 (t, *J* = 7.0 Hz, 1H), 3.50 – 3.45 (m, 2H), 2.54 (t, *J* = 5.7 Hz, 2H), 2.33 (d, *J* = 7.8 Hz, 2H), 2.07 – 0.80 (m, 38H), 0.69 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.86, 156.32, 143.95, 141.32, 139.48, 127.70, 127.05, 125.09, 122.87, 119.99, 74.52, 66.76, 56.70, 56.15, 50.03, 47.25, 42.33, 39.64 (d, *J* = 19.9 Hz), 38.14, 36.97, 36.60, 36.20, 35.81, 34.72, 31.89 (d, *J* = 6.0 Hz), 28.25, 28.04, 27.82, 24.30, 23.85, 22.85, 22.59, 21.05, 19.33, 18.74, 11.88 ; IR (film) ν max 3356, 2946, 2868, 1721, 1512, 1450, 1376, 1263, 1189, 1006, 735 cm^{-1}; HRMS (ESI+) m/z 702.4473 (M+Na$^+$, $C_{45}H_{61}NO_4Na$, requires 702.4493).



35

Cholesteryl 3-(3-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)propanamido) propanoate (35). Compound **34** (200 mg, 0.30 mmol) was added to DMF (1.0 mL) containing piperidine (20%) and stirred for 30 min at 22 °C. The solvent was removed under reduced pressure to afford the crude primary amine. To a solution of Fmoc-β-Ala-OH (102 mg, 0.33 mmol) in anhydrous DMF (3 mL) at 4 °C were added HOBt (45 mg, 0.33 mmol) and EDC (64 mg, 0.33 mmol) and the solution was stirred for 30 min. The primary amine derived from **34** in anhydrous DMF (3 mL) was added. The reaction was allowed to warm to 22 °C and stirred for 12 h. The reaction was concentrated under reduced pressure, and flash column chromatography (CH_2Cl_2/MeOH, 98/2) afforded **35** (150 mg, 52%) as a white foam, mp 158-160 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 (d, *J* = 7.3 Hz, 2H), 7.59 (d, *J* = 7.3 Hz, 2H), 7.39 (t, *J* = 7.2 Hz, 2H), 7.30 (t, *J* = 7.1 Hz, 2H), 6.24 (br, 1H), 5.60 (br, 1H), 5.35 (m, 1H), 4.62 (m, 1H), 4.35 (d, *J* = 6.5 Hz, 2H), 4.20 (t, *J* = 6.6 Hz, 1H), 3.61 – 3.45 (m, 4H), 2.50 (t, *J* = 6.1 Hz, 2H), 2.45 – 2.35 (m, 1H), 2.29 (d, *J* = 7.3 Hz, 2H), 2.04 – 0.78 (m, 39H), 0.67 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.09, 171.42, 156.61, 144.07, 141.39, 139.49, 127.77, 127.15, 125.25, 122.98, 120.05, 74.71, 66.83, 56.76, 56.23, 50.07, 47.34, 42.39, 39.71 (d, *J* = 18.3 Hz), 38.19, 37.19, 37.00, 36.64, 36.29, 35.97 (d, *J* = 15.7 Hz), 35.03, 34.37, 31.93 (d, *J* = 5.7 Hz), 28.33, 28.12, 27.87, 24.37, 23.94, 22.94, 22.68, 21.11, 19.38, 18.82, 11.95 ; IR (film) ν max 3314, 2937, 1729, 1687, 1640, 1544, 1449, 1377, 1269, 1186, 1027, 739 cm^{-1}; HRMS (ESI+) m/z 773.4793 (MNa$^+$, $C_{48}H_{66}N_3O_4Na$, requires 773.4864).



PG-Glu-βAla-Cholesteryl ester (**11**)

(4S)-4-(4-(2,7-difluoro-6-hydroxy-3-oxo-3H-xanthen-9-yl)-3-methylbenzamido)-5-((3-((3-{[3-cholester-3-yl]oxy} 3-oxopropyl)amino)-3-oxopropyl)amino)-5-oxopentanoic acid (**11**). Compound **35** (21.0 mg, 0.028 mmol) in DMF (0.5 mL) containing piperidine (20%) was stirred for 30 min at 22 °C. The solvent was removed under reduced pressure to afford the crude primary amine. To a solution of **21** (8.0 mg, 0.014 mmol) in anhydrous DMF (2 mL) at 4 °C was added HOBt (4.0 mg, 0.028 mmol) and EDC (5.4 mg, 0.028 mmol) and the solution was stirred for 30 min. The primary amine derived from **35** in anhydrous DMF (1 mL) was added. The reaction was warmed to 22 °C and stirred for 12 h. The reaction was concentrated under reduced pressure. TFA/CH_2Cl_2 (1:1, 2 mL) was added and the solution was stirred at 22 °C for 2 h. The solvent was removed under reduced pressure and the crude product was dissolved in DMSO (2 mL). Purification by preparative reverse-phase HPLC (gradient: 90% H_2O, 9.9% MeCN, and 0.1% TFA to 99.9% MeCN and 0.1% TFA over 20 min; retention time = 17.0 min (495 nm) afforded **11** (10.5 mg, 73%) as an orange solid, mp 180-184 °C; ^{1}H NMR (500 MHz, CD_3OD) δ 8.03 (s, 1H), 7.97 (d, *J* = 7.7 Hz, 1H), 7.36 (d, *J* = 7.9 Hz, 1H), 6.84 (m, 2H), 6.76 (m, 2H), 5.32 (d, *J* = 4.2 Hz, 1H), 4.59 (m, 2H), 3.59 – 3.39 (m, 4H), 2.58 – 2.48 (m, 4H), 2.43 (dd, *J* = 10.6, 6.4 Hz, 2H), 2.29 (d, *J* = 7.7 Hz, 2H), 2.16 (s, 3H), 2.06 – 0.78 (m, 40H), 0.69 (s, 3H); ^{13}C NMR (126 MHz, CD_3OD) δ 174.83, 171.84, 171.03, 167.27, 148.36, 141.22, 139.66, 138.91 (d, *J* = 9.8 Hz), 135.96, 134.86, 134.59, 131.93, 129.58 – 129.33 (m), 129.20 (d, *J* = 21.2 Hz), 128.73, 128.43, 126.53, 125.57 (d, *J* = 2.6 Hz), 124.73 (d, *J* = 14.4 Hz), 123.97, 122.66, 121.83, 119.23, 118.47, 113.50, 112.21, 104.60, 73.79, 56.06, 55.49, 53.34, 49.49, 46.96, 43.56, 41.51, 39.05, 38.74, 37.21, 36.23, 35.77, 35.40, 35.11, 34.63 (d, *J* = 15.8 Hz), 33.33, 31.09 (d, *J* = 12.1 Hz), 29.65, 29.06, 27.39, 27.20, 26.85, 26.11, 23.37, 23.00, 21.47, 21.21, 20.19, 18.07 (d, *J* = 16.6 Hz), 17.48, 10.58 ; IR (film) ν max 3298, 2930, 2868, 1725, 1643, 1610, 1534, 1456, 1374, 1295, 1188, 1024, 952, 838, 732 cm^{-1}; HRMS (ESI-) m/z 1020.5208 (M-H$^+$, $C_{59}H_{73}F_2N_3O_{10}$ requires 1020.5191).

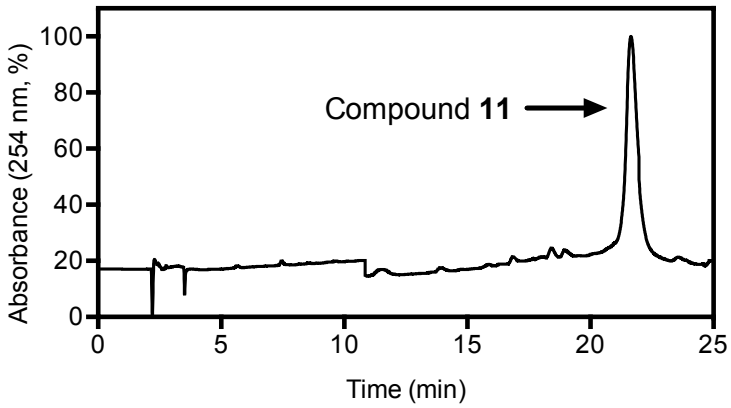
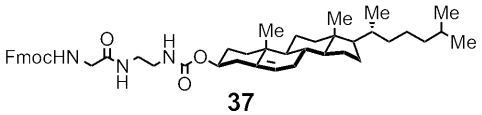
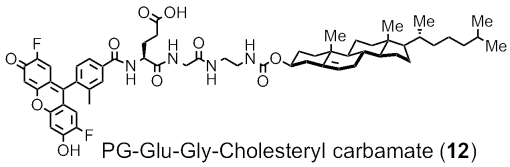


Figure S11. Analytical RP-HPLC profile of **11** after preparative RP-HPLC. Retention time = 21.6 min. Purity > 95%.



37

9H-fluoren-9-ylmethyl(3-((3-((((({[cholester-3-yl]oxy}carbonyl)amino)ethyl)amino)-2-oxoethyl)amino) carbamate (37). A solution of cholesteryl chloroformate (**36**, 500 mg, 1.11 mmol) in CH_2Cl_2 (5 mL) was very slowly added to a solution of ethylenediamine (1.50 mL, 22.26 mmol) in CH_2Cl_2 (10 mL). The reaction was allowed to stir at 22 °C for 2 h. The excess diamine was removed under reduced pressure, the resulting solid was dissolved in CH_2Cl_2 (10 mL), and the organic layer was washed with aqueous NaOH (1 M, 10 mL). The organic phase was dried over anhydrous Na_2SO_4, and solvent was removed under reduced pressure to provide the primary amine product, which was used without further purification. To a solution of Fmoc-Gly-OH (104 mg, 0.35 mmol) in anhydrous CH_2Cl_2 (5 mL) at 4 °C was added HOBt (47 mg, 0.35 mmol) and EDC (67 mg, 0.35 mmol) and the solution was stirred for 30 min. The primary amine derived from **36** (150 mg, 0.32 mmol) in anhydrous CH_2Cl_2 (3 mL) was added. The reaction was allowed to warm to 22 °C and stirred for 12 h. The reaction was concentrated under reduced pressure, and purified by flash column chromatography (CH_2Cl_2/MeOH, 98/2) to afford **37** (130 mg, 56%) as a white foam, mp 82-84 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.5 Hz, 2H), 7.60 (d, *J* = 7.2 Hz, 2H), 7.40 (t, *J* = 7.3 Hz, 2H), 7.31 (td, *J* = 7.5, 1.1 Hz, 2H), 6.74 (br, 1H), 5.46 (br, 1H), 5.31 (m, 1H), 5.04 (br, 1H), 4.43 (d, *J* = 7.2 Hz, 2H), 4.22 (t, *J* = 6.9 Hz, 1H), 3.87 (m, 1H), 3.39 (m, 2H), 3.32 (m, 2H), 2.92 (d, *J* = 32.0 Hz, 2H), 2.38 – 0.73 (m, 40H), 0.65 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.08, 162.84, 156.80, 143.88, 141.40, 139.71, 127.87, 127.22, 125.23, 122.75, 120.12, 74.93, 67.38, 56.72, 56.27, 49.99, 47.24, 44.59, 42.39, 39.72 (d, *J* = 18.3 Hz), 38.59, 36.98, 36.66 (d, *J* = 19.2 Hz), 36.31, 35.91, 31.78 (d, *J* = 19.0 Hz), 28.53 – 28.02 (m), 24.35, 23.97, 22.94, 22.68, 21.11, 19.39, 18.83, 11.95 ; IR (film) ν max 3319, 2937, 2867, 1665, 1527, 1450, 1382, 1252, 1153, 1031, 1009, 737 cm^{-1}; HRMS (ESI-) m/z 786.4638 ($M+Cl^-$, $C_{47}H_{65}N_3O_5Cl$ requires 786.4618).



PG-Glu-Gly-Cholesteryl carbamate (**12**)

(4S)-4-(4-(2,7-difluoro-6-hydroxy-3-oxo-3H-xanthen-9-yl)-3-methylbenzamido)-5-(3-((3-((((({[3-cholester-3-y]oxy}carbonyl)amino)ethyl)amino)-2-oxoethyl)amino)-5-oxopentanoic acid (12). Compound **37** (21.0 mg, 0.028 mmol) was added to DMF (0.5 mL) containing piperidine (20%) and stirred for 30 min at 22 °C. The solvent was removed under reduced pressure to afford the crude primary amine. To a solution of **21** (8.0 mg, 0.014 mmol) in anhydrous DMF (2 mL) at 4 °C was added HOBt (4.0 mg, 0.028 mmol) and EDC (5.4 mg, 0.028 mmol) and the solution was stirred for 30 min. The primary amine derived from **37** in anhydrous DMF (1 mL) was added. The reaction was allowed to warm to 22 °C and stirred for 12 h. The reaction was concentrated under reduced pressure. TFA/CH_2Cl_2 (1:1, 2 mL) was added and the solution was stirred at 22 °C for 2 h. The solvent was removed under reduced pressure and the crude product was dissolved in DMSO (2 mL). Purification by preparative reverse-phase HPLC (gradient: 90% H_2O, 9.9% MeCN, and 0.1% TFA to 99.9% MeCN and 0.1% TFA over 20 min; retention time = 17.0 min (495 nm) afforded **12** (9.0 mg, 63%) as an orange solid, mp 186-190 °C; ^{1}H NMR (500

MHz, CD_3OD) δ 8.06 (s, 1H), 8.01 (d, *J* = 7.0 Hz, 1H), 7.35 (d, *J* = 7.8 Hz, 1H), 7.05 – 6.90 (m, 2H), 6.90 – 6.69 (m, 2H), 5.30 – 5.24 (m, 1H), 4.57 – 4.48 (m, 1H), 4.42 – 4.32 (m, 2H), 4.18 (s, 1H), 3.91 (dd, *J* = 116.3, 16.9 Hz, 2H), 3.41 – 3.33 (m, 2H), 3.31 – 3.25 (m, 2H), 2.62 – 2.51 (m, 2H), 2.40 – 0.78 (m, 43H), 0.68 (s, 3H); ^{13}C NMR (126 MHz, CD_3OD) δ 176.93, 174.60, 171.87, 169.77, 158.73, 150.37, 141.11, 130.75, 128.54, 127.59, 125.98, 124.67, 123.60, 121.25, 120.48, 114.22, 106.58, 75.79, 58.06, 57.50, 51.48, 43.90, 43.52, 41.54 – 41.39 (m), 41.54 – 41.34 (m), 40.98 (dd, *J* = 42.1, 18.4 Hz), 39.75, 38.26, 37.75, 37.42, 37.13, 33.10 (d, *J* = 15.4 Hz), 31.59, 29.56 – 29.12 (m), 27.36, 25.38, 25.01, 23.47, 23.22, 22.20, 20.09 (d, *J* = 17.0 Hz), 19.48, 12.59 ; IR (film) ν max 3312, 2931, 2867, 1644, 1610, 1526, 1463, 1373, 1266, 1188, 1161, 1026, 952, 874, 733 cm^{-1}; HRMS (ESI-) m/z 1021.5169 ($M\text{-}H^+$, $C_{58}H_{71}F_2N_4O_{10}$ requires 1021.5144).

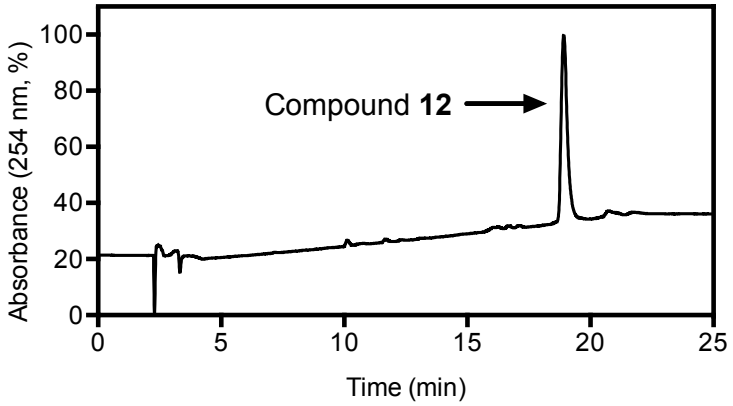
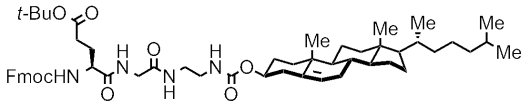


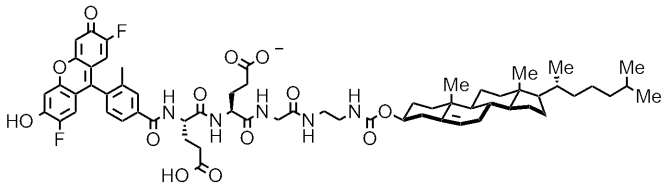
Figure S12. Analytical RP-HPLC profile of **12** after preparative RP-HPLC. Retention time = 19 min. Purity > 95%.



38

***tert*-butyl(*S*)-4-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-5-((2-((2-((((Cholester-3-yl)oxy)carbonyl)amino)ethyl)amino)-2-oxoethyl)amino)-5-oxopentanoate (38).** Compound **37** (100 mg, 0.136 mmol) was added to DMF (2 mL) containing piperidine (20%) and stirred for 30 min at 22 °C. The solvent was removed under reduced pressure to afford the crude primary amine. To a solution of Fmoc-Glu(O-*t*-Bu)-OH (115 mg, 0.272 mmol) in anhydrous CH_2Cl_2 (10 mL) at 4 °C was added HOBt (37 mg, 0.272 mmol) and EDC (52 mg, 0.272 mmol) and the solution was stirred for 30 min. The crude primary amine derived from **37** was added in anhydrous CH_2Cl_2 (5 mL), and the reaction was warmed to 22 °C and stirred for 12 h. This solution was diluted with CH_2Cl_2 (30 mL) and washed with aqueous NaOH (0.1 M, 30 mL) followed by saturated aqueous NaCl (30 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. Flash column chromatography (CH_2Cl_2/MeOH, 50:1) afforded **38** (87 mg, 70%) as a white solid, mp 88-92 °C; ^1H NMR (400 MHz, $CDCl_3$) δ 7.74 (d, *J* = 7.5 Hz, 2H), 7.58 (d, *J* = 7.0 Hz, 2H), 7.38 (t, *J* = 7.5 Hz, 2H), 7.28 (t, *J* = 7.5 Hz, 2H), 7.09 (br, 2H), 6.26 (br, 1H), 5.49 (br, 1H), 5.35 – 5.28 (m, 1H), 4.39 (d, *J* = 6.6 Hz, 2H), 4.20 (t, *J* = 7.0 Hz, 1H), 4.19 – 4.11 (m, 1H), 3.93 (s, 2H), 3.70 – 3.64 (m, 1H), 3.37 – 3.30 (m, 2H), 3.28 – 3.21 (m, 2H), 2.60 – 0.77 (m, 53H), 0.65 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.96, 172.01, 169.39, 156.84, 143.70, 141.29, 139.74, 127.77, 127.13, 125.08, 122.48, 120.00, 81.29, 74.57, 67.24, 56.66, 56.15, 55.35,

49.98, 47.12, 43.16, 42.30, 39.72, 39.52, 38.51, 36.94, 36.50, 36.20, 35.79, 31.83, 28.22, 28.08, 28.01, 24.26, 23.85, 22.82, 22.57, 21.02, 19.30, 18.72, 11.85; IR (film) ν_{max} 3319, 2942, 1681, 1525, 1450, 1367, 1250, 1152, 1032, 843, 735, 702 cm^{-1}; HRMS (ESI+) m/z 959.5876 ($M+Na^+$, $C_{56}H_{80}N_4O_8Na^+$ requires 959.5868).



PG-Glu-Glu-Gly-Cholesteryl carbamate (**13**)

(10*S*,13*S*)-10-(2-carboxylatoethyl)-13-(4-(2,7-difluoro-6-oxido-3-oxo-3*H*-xanthen-9-yl)-3-methylbenz amido)-1-((Cholester-3-yl)oxy)-1,6,9,12-tetraoxo-2,5,8,11-tetraazahexadecan-16-oate (13). Compound **38** (30 mg, 0.027 mmol) was added to DMF (0.5 mL) containing piperidine (20%) and stirred for 30 min at 22 °C. The solvent was removed under reduced pressure to afford the crude primary amine. To a solution of **21** (23 mg, 0.040 mmol) in anhydrous DMF (2 mL) at 4 °C was added HOBt (5 mg, 0.040 mmol) and EDC (8 mg, 0.040 mmol) and the solution was stirred for 30 min. The primary amine derived from **38** in anhydrous DMF (1 mL) was added, and the reaction was warmed to 22 °C and stirred for 12 h. The reaction was concentrated under reduced pressure, CH_2Cl_2 (2 mL) containing TFA (30%) was added, and the solution was stirred at 22 °C for 2 h. The solvent was removed under under reduced pressure and the crude product was dissolved in DMSO (2 mL). Purification by preparative reverse-phase HPLC (gradient: 90% H_2O, 9.9% MeCN and 0.1% TFA to 99.9% MeCN and 0.1% TFA over 20 min; retention time = 19 min (495 nm)) afforded **13** (10 mg, 33%) as an orange solid, mp 182-186 °C; ^1H NMR (500 MHz, CD_3OD) δ 8.02 (s, 1H), 7.99 (d, *J* = 8.1 Hz, 1H), 7.40 (d, *J* = 7.9 Hz, 1H), 6.97 – 6.91 (m, 2H), 6.81 – 6.63 (m, 2H), 5.35 – 5.29 (m, 1H), 4.65 – 4.57 (m, 1H), 4.40 - 4.34 (m, 1H), 4.31 – 4.24 (m, 1H), 4.10 – 3.73 (m, 2H), 3.33 – 3.18 (m, 4H), 2.58 (t, *J* = 8.1 Hz, 4H), 2.53 – 0.80 (m, 48H), 0.68 (s, 3H); ^{13}C NMR (126 MHz, CD_3OD) δ 176.76, 176.34, 174.80, 174.43, 171.80, 169.87, 158.62, 156.09, 153.56, 141.21, 138.02, 136.81, 136.61, 131.33, 130.56, 126.75, 123.53, 115.90, 112.90, 112.74, 106.49, 75.58, 58.05, 57.53, 55.42, 55.23, 51.66, 43.70, 43.43, 41.04, 40.70, 40.50, 39.70, 38.19, 37.64, 37.37, 37.13, 33.06, 32.92, 31.49, 31.13, 30.77, 29.29, 29.18, 28.02, 27.13, 25.24, 24.99, 23.21, 22.96, 22.07, 19.75, 19.69, 19.25, 12.26; IR (film) ν_{max} 3317, 2941, 1644, 1611, 1536, 1465, 1372, 1336, 1307, 1191, 1032, 952, 875, 833 cm^{-1}; HRMS (ESI+) m/z 1174.5601 ($M+Na^+$, $C_{63}H_{79}F_2N_5O_{13}Na^+$ requires 1174.5535).

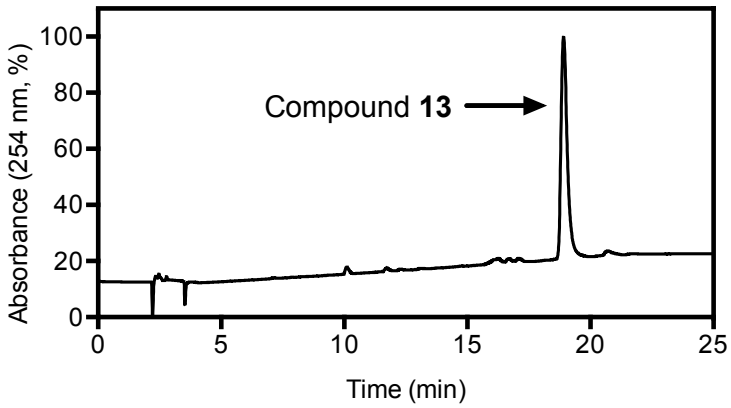


Figure S13. Analytical RP-HPLC profile of **13** after preparative RP-HPLC. Retention time = 19 min. Purity > 95%.

Biological assays and protocols

Cell culture. Jurkat lymphocytes (human acute leukemia, ATCC #TIB-152) were cultivated in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with Fetal Bovine Serum (FBS, 10%), penicillin (100 units/mL), and streptomycin (100 μg/mL). HeLa cells (human cervical adenocarcinoma, ATCC #CCL-2) and HEK293T cells (human embryonic kidney, ATCC #CRL-3216) were cultivated in DMEM supplemented with FBS (10%), penicillin (100 units/mL), and streptomycin (100 μg/mL). All cell lines were grown in a humidified 5% CO_2 incubator at 37 °C. Media used for cell culture and all wash steps contained antibiotics and 10% FBS unless otherwise noted.

Microscopy. An inverted Leica TCS SPE confocal laser-scanning microscope fitted with a Leica 63X oil-immersion objective was employed for imaging. Fluorescent probes were excited with a 488 nm solid-state laser and emitted photons were collected from 495-600 nm. Propidium iodide, used to counterstain dead cells red fluorescent, was excited with a 532 nm solid-state laser and emitted photons were collected from 650-800 nm. To image living cells, 50 μL of cells in media was pipetted to form a small droplet in the center of a coverslip fitted with a press-to-seal silicone isolator (Invitrogen). A microscope slide was added to the top of the coverslip to create a column of media containing living cells. To allow accurate comparisons of differences in cellular fluorescence, laser power and PMT gain settings were identical for all samples shown in a given figure.

Flow Cytometry. Populations of 10,000 cells were analyzed for each sample using either an Accuri C6 or a BD FACSCalibur flow cytometer equipped with 488 nm and 640 nm solid-state lasers. Live cells were gated using both forward scattering (FSC) and side scattering (SSC) dot plots to identify cellular physical properties of size and granularity. For indicated experiments, staining with propidium iodide (3 μM, excitation: 488 nm; emission: 670 nm LP) was used to identify cells with compromised membranes.

Saturation binding studies of fluorescent probes. DMSO was added to fluorescent probes as dry powders to generate 10 mM stock solutions. A 10 mM DMSO stock solution of 4-carboxy-Pennsylvania Green methyl ester (ε = 60,427 M^{-1} cm^{-1} at 520 nm in 1:4 PBS:DMSO) was used to standardize the concentrations of other Pennsylvania Green derivatives. Compound concentrations were calculated from absorbance (520 nm) of stock solutions diluted in triplicate to 20 μM (1:500) in a solution of 1:4 PBS (pH 7.4):DMSO and analyzed on a 96-well plate. Jurkat cells were suspended at 300,000 cells/mL in RPMI media containing 10% FBS. Ezetimibe (200 μM; diluted 1:1000 into media, using serial 10-fold dilutions, from a 200 mM stock solution in DMSO; 0.2% final DMSO concentration) or vehicle control (DMSO at 0.2%) was added in competition/total binding experiments. Cell suspensions containing the competitor or vehicle control were prepared as 1 mL aliquots in 1.5 mL eppendorf tubes. Fluorescent probes were added at concentrations ranging from 0 μM to 2.5 μM (diluted 1:1000 into media, using serial 10-fold dilutions, from stocks in DMSO). Once the probe was added and mixed well, the samples were split into three tubes each containing 330 μL. Cells were incubated at 37 °C for 5 minutes to limit endocytosis. After this incubation, the samples were washed once with RPMI 1640 containing 0.5% FBS (to minimize efflux of the probe from the cell surface) and propidium iodide (3 μM). Samples were immediately analyzed by flow cytometry or confocal laser-scanning microscopy. Immediately after the incubation at 37 °C, and during the analysis, all samples were continuously maintained at 16 °C to minimize endocytosis and compound efflux from the cell surface. Fluorescence values, converted to molecular equivalents of

fluorescein (MEFL) by comparison with fluorescent bead standards from Spherotech, were analyzed as total binding (no competitor added) and a linear non-specific binding component (with added competitor) to calculate specific binding curves. Using GraphPad Prism 6.0 software, these curves were analyzed with a 'One site with hill slope' saturation binding model to determine the relative apparent K_d and B_{max} values.

Kinetic analysis of cellular uptake of fluorescent probes. Jurkat cells were suspended at 300,000 cells/mL in RPMI-1640 media containing 10% FBS. Fluorescent probes were added at concentrations ranging from 0 to 2.5 μM (diluted 1:1000 into media, using serial 10-fold dilutions, from DMSO stocks). Cells were incubated at 22 °C for time points ranging from 1 – 12.5 minutes. After this incubation, the samples were washed once with RPMI-1640 containing 0.5% FBS (to minimize efflux of the probe from the cell surface). Samples were immediately analyzed by flow cytometry. Each probe was analyzed at 6 concentrations over 5 time points. Fluorescence values were converted to MEFL and plotted versus time for each of the 6 concentrations. Linear regression was used to fit the data, and the slope of each line (MEFL/min) was plotted versus concentration. Each assay was performed three times to give average MEFL/min values and SEM for each concentration. These plots were analyzed by non-linear regression using the Michaelis-Menten model in GraphPad Prism 6.0 to generate values for K_M and V_{MAX}.

Analysis of cellular uptake of fluorescent compounds by different cell lines. Jurkat cells were suspended at 300,000 cells/mL in RPMI-1640 media containing 10% FBS with and without ezetimibe (200 μM). HeLa and HEK-293T cells were trypsinized from culture flasks using 0.5% trypsin/EDTA, pelleted by centrifugation, and resuspended at 300,000 cells/mL in DMEM containing 10% FBS with and without ezetimibe (200 μM). Each cell line was allowed to pre-incubate with or without ezetimibe (200 μM) for 30 minutes at 22 °C. Fluorescent probes **4**, **5**, **8**, or **13** were diluted into media to generate 10 × stock solutions (1% DMSO). Each 10 × stock solution was then diluted 1:10 into media containing suspended cells to 0 μM and 2 μM (final DMSO concentration of 0.1%) concentrations. Cells were incubated at 22 °C for 5 min. The cells were then washed once in their respective medias containing 0.5% FBS and analyzed immediately by flow cytometry. Fluorescence values were converted to MEFL by comparison with fluorescent bead standards. Measurements were performed in triplicate and plotted as average background-subtracted fluorescence (MEFL ± SEM).

References for the Supporting Information

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