Supplemental Online Materials for

Near-instant surface-selective fluorogenic protein quantification using sulfonated triarylmethane dyes and fluorogen activating proteins

Qi Yan[1], Brigitte F. Schmidt[2], Lydia A. Perkins[1], Matharishwan Naganbabu[3], Saumya Saurabh[3], Susan Andreko[2], Marcel P. Bruchez[1,2,3]

1. Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, PA
2. Molecular Biosensors and Imaging Center, Carnegie Mellon University, Pittsburgh, PA
3. Department of Chemistry, Carnegie Mellon University, Pittsburgh, PA

Supplemental Figure S1. K_D determination of MG-B-Tau and other MG analogs.

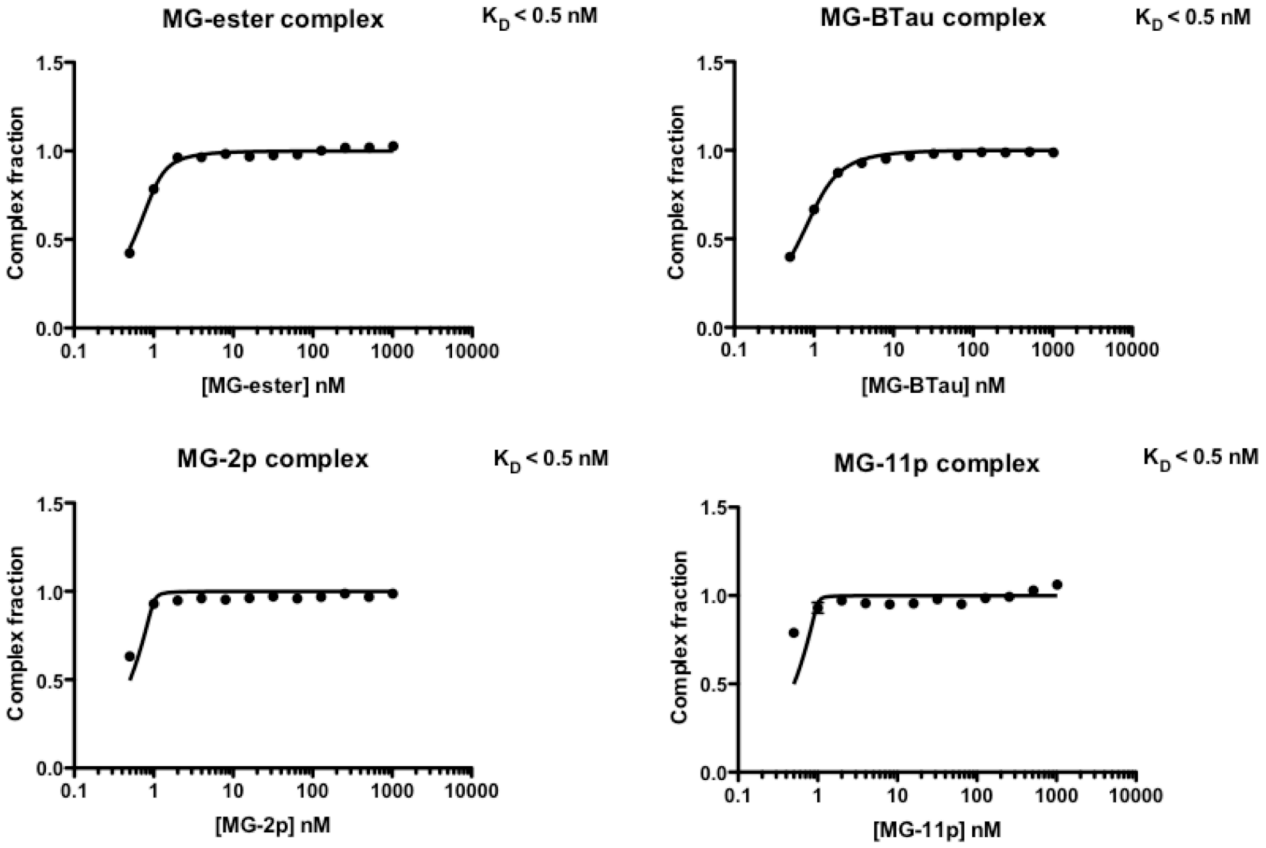
Supplemental Figure S2. Actin labeling with cell-excluded dyes.

Supplemental Figure S3. Nonspecific dye activation on living cells.

Supplemental Figure S4. Labeling protocol comparison for antibody labeling and fluorogen labeling methods.

Supplemental Figure S5. Amino acid sequence of dL5-ADRB2 construct.

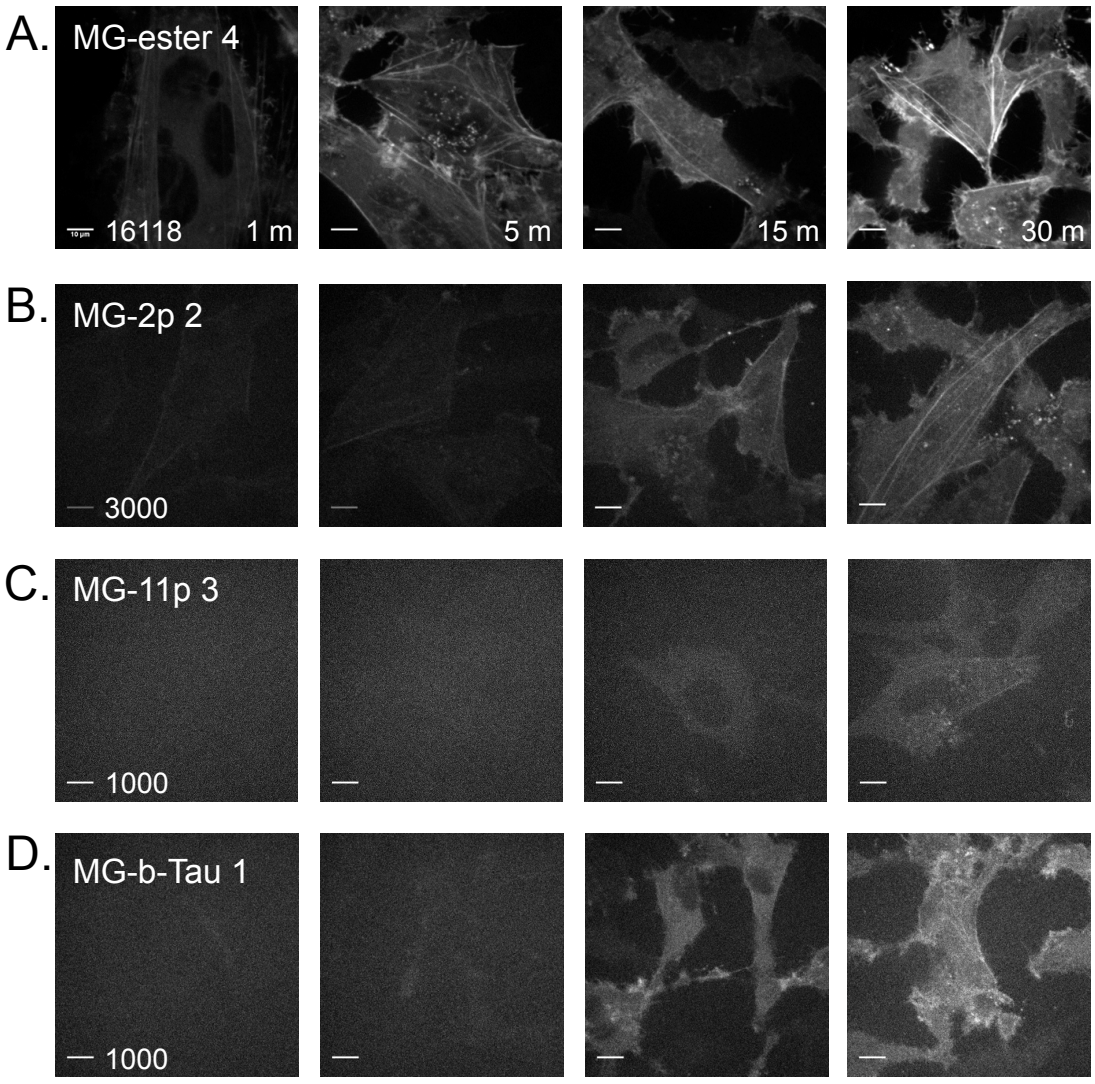
Supplemental Experimental Procedures: Synthesis of the MG-B-Tau (1) dye.



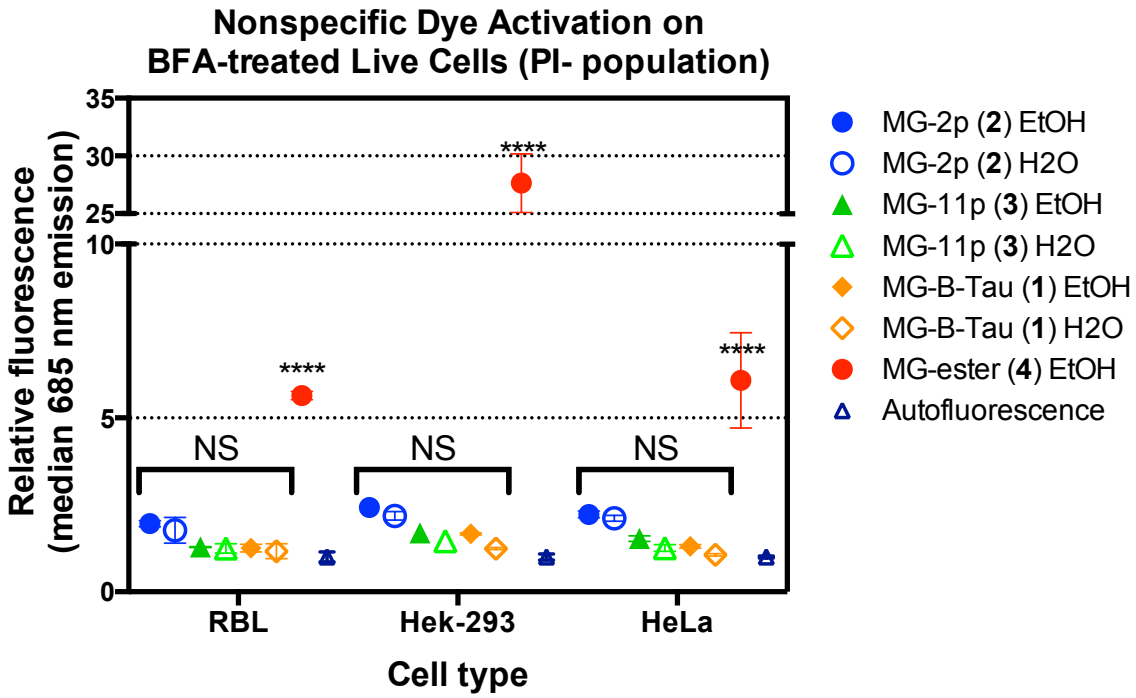
Supplemental Figure S1. K_d measurement of various MG dyes using purified recombinant dL5** protein. 1 nM dL5 was incubated with a serial dilution of 1024 nM to 0.5 nM of the respective fluorogen dissolved in PBS with 0.1% Pluronic F127. The FAP+dye complex fluorescence was corrected by subtracting the fluorescence of a dye only sample, and then normalized to the maximum signal at saturation to establish the fractional occupancy. The K_D was determined by fitting the data with a ligand depletion single-site binding model in Graphpad Prism 5.0.

The ligand depletion model assumes that changes in complex formation are associated with complementary changes in free ligand and free receptor, and are a typical model for ligand-receptor interactions when one has to work at protein concentrations that are near the K_d value. In the equation below, X is the added concentration of dye, R is the added receptor concentration (1 nM), and K_d is the adjustable parameter used to determine the equilibrium dissociation constant.

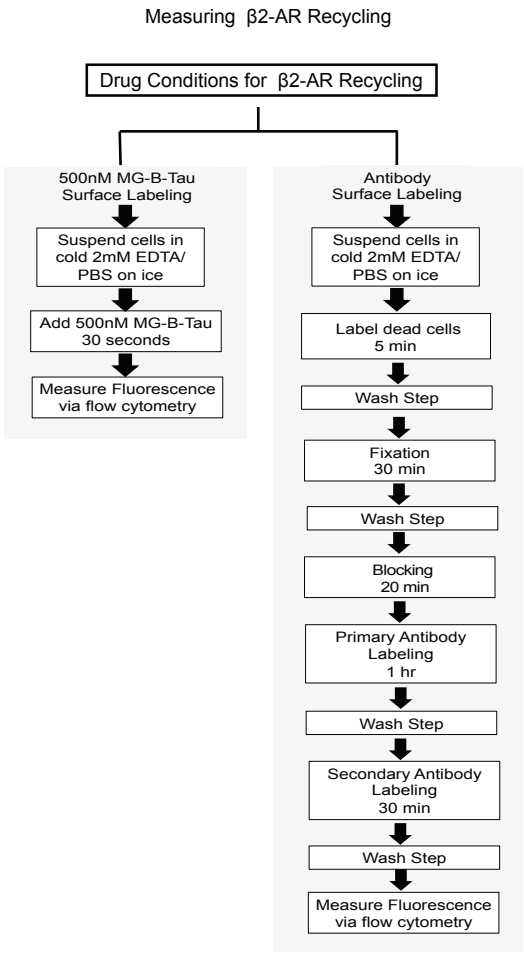
$$Y = \frac{X+K_d+R-\sqrt{-(X+K_d+R)^2-4XR}}{2}$$



Supplemental Figure S2. Actin labeling with cell-excluded dyes. Time-lapse images of cell-permeant MG-ester 4 (100 nM), and cell impermeant MG-2p 2, MG-11p 3 and MG-B-Tau 1 (all at 500 nM) over 30 minute incubation in the presence of dye (unwashed). Images are displayed on lookup tables to maximally display the relatively low signals with the maximum pixel value indicated in the bottom of the first panel of each image series. Scalebar = 10 μm.



Supplemental Figure S3. Nonspecific dye activation on living cells. Cells that were not expressing any FAP were treated with Brefeldin A to induce apoptosis followed by 30 minute incubation with dyes at 500 nM (MG-B-Tau **1**, MG-11p **3**, and MG-2p **2**) or 100 nM (MG-ester **4**) prepared from the indicated stock solution. Propidium iodide negative (e.g. non-apoptotic) cells were selected and analyzed for associated MG fluorescence due to nonspecific activation (633 nm laser excitation with 685/70 nm emission filter). Fluorescence intensity was normalized to autofluorescence of cells (no MG dye applied) for each cell type, and then plotted as mean (marker) and range (bars) of independent duplicate experiments on separate days. The only samples that are significantly different from the autofluorescent cells by ANOVA test for multiple comparisons Graphpad Prism 7.0) are the MG-ester (**4**) labeled cells, which are also significantly different from each of the other dyes. No statistically significant differences are seen among the other dye comparisons on living cells.



Supplemental Figure S4. Comparison of protocols for fluorogenic dye labeling and antibody surface labeling approaches. The fluorogenic dye is added to cells immediately prior to measurements and then, essentially within the mixing time, is ready for analysis. In contrast, antibody methods require extensive sample preparation, incubation and washing steps to achieve robust surface-selective labeling.

Fri Dec 05, 2014 13:26 EST
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From 1468 to 3606.
Translation 712 a.a. MW=77330.01000000014

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 T   G   A   V   T   S   G   H   Y   A   N   W   F   Q   Q   K   P   G   Q   A   P   R   A   L   I   F   D   T   D   K

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 E   R   L   Q   T   V   T   N   Y   F   I   T   S   L   A   C   A   D   L   V   M   G   L   A   V   V   P   F   G   A

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 I   N   C   Y   A   N   E   T   C   C   D   F   F   T   N   Q   A   Y   V   I   A   S   S   I   V   S   F   Y   V   P

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GTC CAG AAC CTT AGC CAG GTG GAG CAG GAT GGG CGG ACG GGG CAT GGA CTC CGC AGA TCT TCC AAG TTC TGC TTG AAG GAG CAC AAA GCC
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 G   T   V   P   S   D   N   I   D   S   Q   G   R   N   C   S   T   N   D   S   L   L   *
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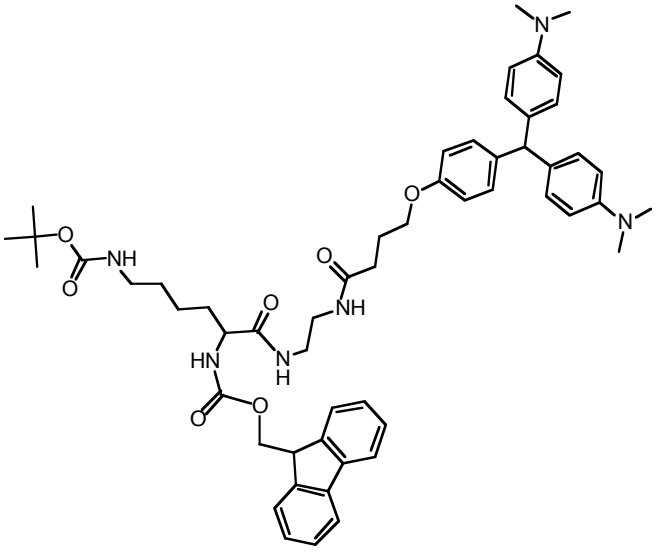
Supplemental Figure S5. Nucleotide and amino acid sequence of dL5-ADRB2 construct. Color Coded as follows: Ig-κ leader sequence (cleaved), Influenza Hemagglutinin epitope (HA), dL5** FAP sequence, c-myc epitope tag, ADRB2 coding sequence.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Synthesis of MG-B-Tau (1)

Chemistry Unless otherwise noted, all chemicals were obtained from commercial suppliers and used without further purification. MG[H]-EDA **5** was prepared by a previously described procedure (Szent-Gyorgyi et al, 2010). Fmoc-Lys(Boc)OSu was purchased from Bachem (Switzerland). Dicyclohexylcarbodiimide (DCC) was purchased from AAPPTEC (Louisville, KY). N-Hydroxysuccinimide (HO-SU), piperidine and Diethylisopropylamine (DIEA) were purchased from Sigma-Aldrich (St. Louis, MO), Taurine, succinic anhydride, anhydrous DMF and anhydrous CH_2Cl_2 were purchased from AlfaAesar (Ward Hill, MA). Methanol, acetonitrile, ethyl acetate, chloroform, petroleum ether and hexane were obtained from Fisher Scientific (Fairlawn NJ). Solvents were removed using a Buchi rotary evaporator under reduced pressure. Reaction progress was monitored using thin-layer chromatography on SiliaPlate Aluminium-backed TLC plates (Silicycle, Quebec). Ultra-High-performance liquid chromatography (UPLC) analysis was performed on a Waters Acquity system with a Waters UPLC BEH C18 1.7mm, 2.1x50 mm analytical column using PDA detection. Preparative separation on RP-18 (Separation Methods Technologies, Newark) was performed on a MPLC Buchi Sepacore Chromatography system. Mass spectra were measured with a Thermo-Fisher LCQ ESI/APCI Ion Trap. ^{1}H-NMR and ^{13}C-NMR analysis was performed on a Bruker Avance-300 and Bruker Avance 500. For proton and carbon assignments COSY, HMBC and HMQC experiments were performed on the Bruker Avance 500. Chemical shifts are reported as δ values relative to internal chloroform (δ 7.27) and methanol (3.35) and expressed in ppm.

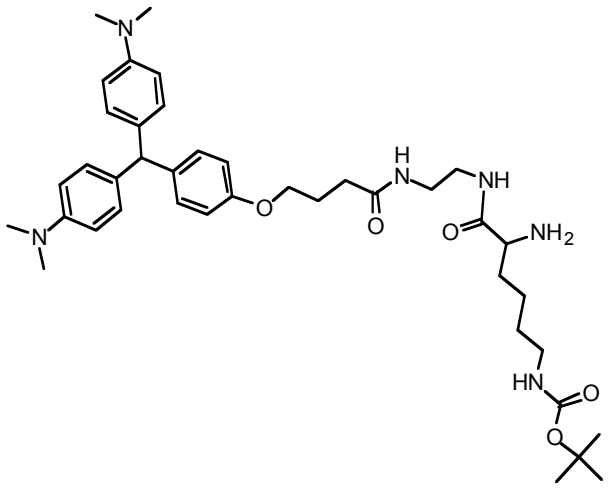
(9*H*-Fluoren-9-yl)methyl *tert*-butyl (6-((2-(4-(4-(bis(4-(dimethylamino)phenyl) methyl)phenoxy)butanamido)ethyl)amino)-6-oxohexane-1,5-diyl)dicarbamate [MG[H]EDA-Lys(Boc)Fmoc] 6



Fmoc-Lys(Boc)OSu (3g, 5.3 mmol) was dissolved in 30 mL of dry methylene chloride and added dropwise to a solution of MG[H]EDA **5** (2.51 g, 5.3 mmol) and DIEA (0.93 mL, 5.3 mmol). The reaction mixture was stirred overnight. The product started to crystallize from the reaction mixture. Hexane (10 mL) was added and the precipitate was filtered off. The product was recrystallized from boiling methanol. Yield: 4g (81%). $C_{55}H_{68}N_6O_7$. Monoisotopic mass: 924.51; Monoisotopic experimental mass (ESI, m/z): 925.4

^{1}H-NMR (300 MHz; $CDCl_3$) 7.72 (2H,d, *Fmoc*), 7.56 (2H,d, *Fmoc*), 7.36 (2H,t, *Fmoc*), 7.27 (2H,t, *Fmoc*), 6.98 (2H,d), 6.93 (4H,d), 6.71 (2H,d), 6.63 (4H,d), 6.29 (1H, m, *NH*); 5.49 (1H,m, *NH*), 5.27 (1H,s), 4.64 (1H,m, *EDA-NH*), 4.39 (2H,d, *Fmoc*), 4.17 (1H,t, *Fmoc*), 4.02 (1H,m, *NH*), 3.88 (2H,t, *MG-O-CH$_2$*), 3.34 (4H,m, *EDA*), 3.05 (2H,m, *Lys*), 2.88 (12H,s, *NMe*), 2.31 (2H,t, *MG-O-CH$_2$-CH$_2$-CH$_2$*), 2.03 (2H,t, *MG-O-CH$_2$-CH$_2$-CH$_2$*), 1.78 (2H,m, *Lys*), 1.62 (1H,m, *Lys*), 1.42 (1H,m, *Lys*), 1.41 (9H,s, *Boc*), 1.32 (2H,m, *Lys*).

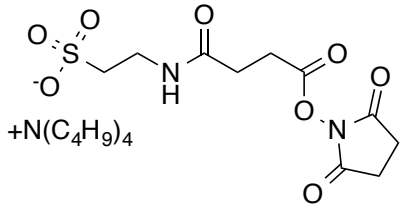
***Tert*-butyl (5-amino-6-((2-(4-(4-(bis(4-(dimethylamino)phenyl)methyl)phenoxy) butanamido)ethyl)amino)-6-oxohexyl)carbamate [MG[H]-EDA-Lys(Boc)] 7**



MG[H]-EDA-Lys(Boc)Fmoc **6** (2g, 2.16 mmol) was dissolved in a mixture of chloroform/ 20% piperidine (5mL) and stirred for 1 hr at control. The reaction was monitored by TLC on silicagel (eluent: ethyl acetate/20% methanol). The reaction mixture was added dropwise under stirring to petroleum ether (75 mL). The product precipitated. Stirring was continued until the supernatant became clear. The organic phase was decanted and discarded. Petroleum ether (50 mL) was added to the residue and the mixture was heated to reflux. The reaction mixture was filtered to give 1.2 g (79%) of a pale green solid. $C_{40}H_{59}N_6O_5$ Monoisotopic mass:702.45, monoisotopic experimental mass (ESI, m/z): 702.4

^{1}H-NMR (300 MHz; MeOD) 6.93 (2H,d), 6.87 (4H,d), 6.75 (2H,d), 6.66 (4H,d), 5.23 (1H,s), 3.92 (2H,t, *MG-O-CH$_2$*), 3.29 (4H,m, *EDA*), 2.98 (2H,m, *Lys*), 2.83 (12H,s, *NMe*), 2.34 (2H,t, *MG-O-CH$_2$-CH$_2$-CH$_2$*), 2.01 (2H,t, *MG-O-CH$_2$-CH$_2$-CH$_2$*), 1.62 (2H,m, *Lys*), 1.42 (2H,m, *Lys*), 1.39 (9H,s, *Boc*), 1.32 (2H,m, *Lys*).

Tetrabutylammonium 2-({4-[(2,5-dioxopyrrolidin-1-yl)oxy]-4-oxobutanoyl}amino)ethanesulfonate 8

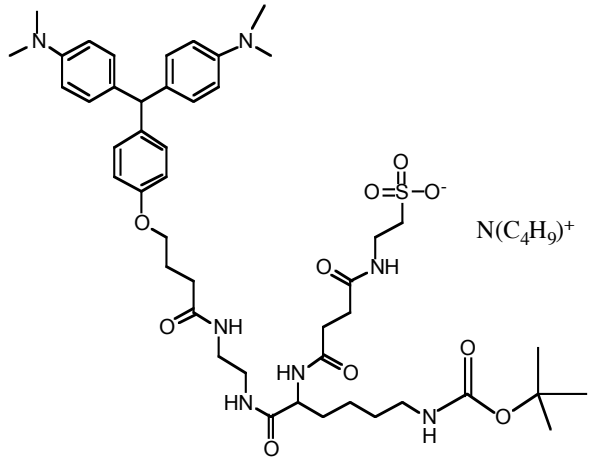


Tetrabutylammonium 2-aminoethylsulfonic acid (Mathews and Holan, 2003) (3.8 g, 10 mmol) was dissolved in acetonitrile (20 mL). Solid succinic anhydride (1.1g, 11 mmol) was added under stirring. Stirring was continued for 30 min. The reaction mixture was filtered. The filtrate was concentrated to give tetrabutylammonium 2-(3-carboxypropanamido)ethanesulfonate as a colorless oil:^{1}H-NMR (300 MHz, $CDCl_3$) 7.62 (1H,t, *NH*), 3.60 (2H,m, *CH$_2$-NH*), 3.20 (8H,m, *Nbut*), 2.86 (2H,m, *CH$_2$-SO$_3$*) 2.49 (4H,m, *Succ*), 1.60 (8H,m, *Nbut*), 1.39 (8H,m, *Nbut*), 0.97 (12H,t, *Nbut*).

The tetrabutylammonium salt of 4-oxo-4[(2-sulfoethyl)amino]butanoic acid (467 mg, 1 mmol) was dissolved in dry acetonitrile (5 mL). N-Hydroxysuccinimide (126 mg, 1.1

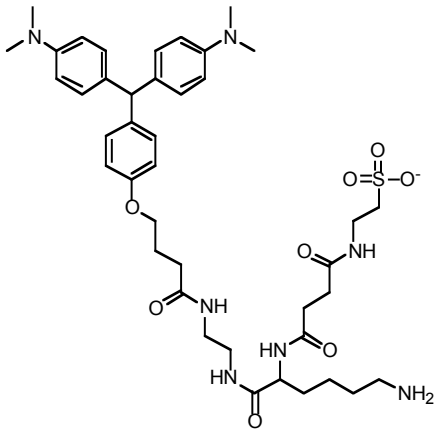
mmol) was added followed by dicyclohexylcarbodiimide (226 mg, 1.1 mmol). The reaction mixture was stirred overnight. The precipitate was filtered off and washed with 5 mL of acetonitrile. The filtrate containing tetrabutylammonium 2-({4-[(2,5-dioxopyrrolidin-1-yl)oxy]-4-oxobutanoyl}amino)ethanesulfonate **8** was used as such in the next step**.**

Tetrabutylammonium 10-((2-(4-(4-(bis(4-(dimethylamino)phenyl)methyl)phenoxy) butanamido)ethyl)carbamoyl)-2,2-dimethyl-4,12,15-trioxo-3-oxa-5,11,16-triazaoctadecane-18-sulfonate [MG-EDA-Lys(Boc)TAU] 9



The combined filtrate of **8** was added to a solution of MG[H]-EDA-Lys(Boc)-a-amine (703 mg, 1 mmol). The reaction mixture was stirred overnight at rt. The reaction mixture was concentrated and separated by column chromatography on silica gel to give 550 mg (60%) of a resinous solid . Eluent: ethyl acetate/methanol. $C_{46}H_{69}N_7O_{10}S^-N(but)^+$ Monoisotopic mass: 908.46 Monoisotopic experimental mass (ESI, m/z):908.5

^{1}H-NMR (300 MHz; $CDCl_3$) 7.73 (1H,M, *NH-EDA*), 7.35 (2H,M, *NH-EDA*), 6.96 (2H,d), 6.93 (4H,d), 6.74 (2H,d), 6.62 (4H,d), 5.27 (1H,s), 5.00 (1H,m, *NH-TAU*), 4.44 (1H,m, *CH-Lys*), 3.91 (2H,t, *MG-O-CH$_2$*), 3.66 (1H,m, *NH-TAU*), 3.52 (2H,m, *TAU*), 3.31 (4H,m, *EDA*), 3.19 (8H,m, *Nbut*), 3.04 (2H,m, *Lys*), 2.93 (2H,m, *TAU*), 2.87 (12H,s, *NMe*), 2.58 (2H,m, *Succ*), 2.36 (2H,m, *Succ*), 2.34 (2H,t, *MG-O-CH$_2$-CH$_2$-CH$_2$*), 2.03 (2H,t, *MG-O-CH$_2$-CH$_2$-CH$_2$*),), 1.80 (1H,m, *Lys*), 1.69 (1H,m, *Lys*), 1.60 (8H,m, *Nbut*); 1.40 (2H,m, *Lys*), 1.40 (9H,s, *Boc*), 1.38 (8H, qu, *Nbut*), 0.97 (12H,t, *Nbut*).



Tetrabutylammonium 10-((2-(4-(4-(bis(4-(dimethylamino)phenyl)methyl)phenoxy) butanamido)ethyl)carbamoyl)-2,2-dimethyl-4,12,15-trioxo-3-oxa-5,11,16-triaza-octadecane-18-sulfonate (MG[H]-EDA-Lys-taurine tetrabutylammonium salt) 10

MG[H]-EDA-Lys(Boc)taurine tetrabutylammonium salt **9** (568 mg, 0.5 mmol was dissolve in 1N HCl (2 mL)and stirred overnight. The solvent was removed under vacuum and the residue used as such in the next reaction step.

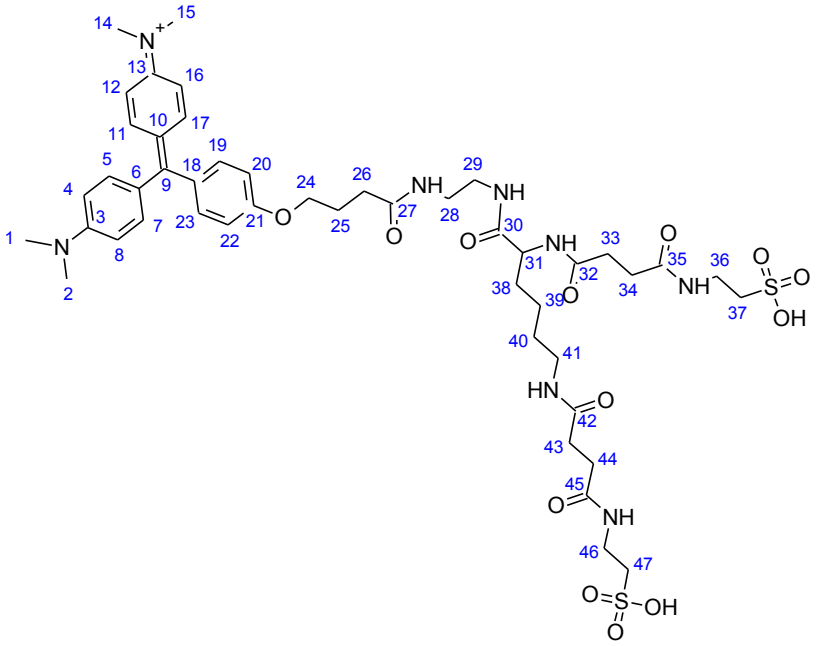
^{1}H-NMR (300 MHz; MeOD) 7.67 (4H,d), 7.33 (4H,d), 7.00 (2H,d), 6.86 (2H,d), 5.71 (1H,s), 5.00 (1H,m, *NH-TAU*), 4.22 (1H,m, *CH-Lys*), 3.97 (2H,t, *MG-O-CH$_2$*), 3.55 (2H,m, *TAU*), 3.31 (4H,m, *EDA*), 3.27 (12H,s, *NMe*), 3.23 (8H,m, *Nbut*), 2.93 (2H,m, *Lys*), 2.93 (2H,m, *TAU*), 2.52 (4H,m, *Succ*), 2.40 (2H,t, *MG-O-CH$_2$-CH$_2$-CH$_2$*), 2.03 (2H,m, *MG-O-CH$_2$-CH$_2$-CH$_2$*),), 1.8 (1H,m, *Lys*), 1.63 (3H,m, *Lys*), 1.60 (8H,m, *Nbut*); 1.40 (2H,m, *Lys*), 1.40 (8H, qu, *Nbut*), 1.00 (12H,t, *Nbut*). MS 808.6

9-((2-(4-(4-((4-(dimethylamino)phenyl)(4-(dimethyliminio)cyclohexa-2,5-dien-1-ylidene)methyl)phenoxy)butanamido)ethyl)carbamoyl)-4,7,15,18-tetraoxo-3,8,14,19-tetraazahenicosane-1,21-disulfonate [MG-EDA-Lys-bis(taurine) in short MG-BTAU] 1

Crude MG[H]-EDA-Lys-taurine **10** (0.5 mmol) was dissolved in DMF/10% DIEA (2mL). Tetrabutylammonium 2-(4-((2,5-dioxopyrrolidin-1-yl)oxy)-4-oxobutanamido) ethanesulfonate **8** dissolved in dry acetonitrile (5 mL) was added. The reaction mixture was stirred overnight at rt. Acetonitrile (15 mL) was added and the reaction mixture was heated up to reflux. Tetrachlorobenzoquinone (147 mg, 0.6 mmol) dissolved in 5 mL of hot acetonitrile was added. Reflux was continued for 1 hr. The reaction mixture was cooled to rt and concentrated. The product was taken up in diluted sulfuric acid and separated by MPLC on RP-18. Eluent: water, 0.1%TFA/acetonitrile 20% to 30% gradient.

UPLC analysis of the product fractions was performed. The mobile phase contained 0.1% trifluoroacetic acid in H_2O and acetonitrile. At a flow rate of 0.5 mL/min with 0.5 min of H_2O/0.1% TFA followed by a linear gradient of 0%-100% acetonitrile over 2.5 min, the product elutes at 2.246 min. The product fractions that show a 99.5% purity (calculation

based on the 280 nm and 610 nm UV absorption chromatogram) were combined and concentrated to yield 75 mg of product. $C_{47}H_{69}N_7O_{13}S_2^{2-}$ monoisotopic mass:1013.41; monoisotopic experimental mass (ESI, m/z): 1013.6.



^{1}H-NMR (500 MHz; D_2O) 6.89 (4H,d/ *5,7,11,17*), 6.81 (2H,d/ *20,22*), 6.75 (2H,d/ *19,23*), 6.61 (4H,d/ *4,8,12,16*), 4.04 (1H,m/ *31*), 3.95 (4H,m/ *37,47*), 3.91 (2H,t/ *24*), 3.45 (4H,m/ *36,46*), 3.22 (4H,m/ *28,29*), 3.08 (14H,s/*1,2,14,15*), 2.99 (2H,t/*41*), 2.46 (2H,m/*44*), 2.45 (2H,m/*43*), 2.36 (4H,m/*33,34*), 2.30 (2H,t/*26*), 1.96 (2H,quint/*25*), 1.63 (1H,m/*38*), 1.56 (1H,m/*38*), 1.34 (2H,m/*40*), 1.28 (1H,m/*39*), 1.18 (1H,m/*39*).

^{13}C-NMR (500 MHz; D_2O) 175.30(*27*), 174.83(*9*), 174.30(*30,45*), 174.17(32,*35,42*), 163.16(*21*), 156.26(*3,13*), 140.19(*5,7,11,17*), 137.37(*20,22*), 131.07(*18*), 125.*82*(*6,10*), 114.62(*19,23*), 113.04(*4,8,12,16*), 67.84(*24*), 53.13(*31*), 49.75(*37,47*), 40.1(*1,2,14,15*), 38.97(*41*), 38.67(*29*), 38.60(*28*), 35.13(*36,46*), 32.29(*26*), 31.36(*33*), 31.28(*34*), 30.93(*43*), 30.77(*44*), 30.57(*38*), 27.96(*40*), 24.7(*25*), 22.54(*39*).

SUPPLEMENTAL REFERENCES

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