Supporting Information.

Table of Contents

Materials and Methods	**S2-S12**
Table S1	**S13**
Figures S1-S8	**S14-S17**

Experimental Methods.

1.0 Materials and analyses.

Unless otherwise noted, chemicals were obtained from Sigma-Aldrich, Inc., peptides were obtained from AnaSpec, Inc., and cell culture reagents were purchased from Invitrogen, Inc. H2B-eDHFR was provided by Prof. V.W. Cornish, and pcDNA3-TagRFP-T was obtained from Prof. R.Y. Tsien. MDCK cells were provided by Prof. J.R. Turner, HeLa and NIH3T3 cells were provided by Prof. W. Cho, and HEK293 cells were obtained from the American Type Culture Collection.

Electrospray ionization (ESI) high-resolution mass spectra (HRMS) were obtained by the Mass Spectrometry Facility, College of Chemistry, University of California, Berkeley, CA. MALDI-TOF mass spectra were obtained by the Mass Spectrometry Facility, Dept. of Chemistry, Universiy of Illinois at Urbana. Flash chromatography was performed using EM Science Silica Gel 60 (230- 400 mesh). NMR spectra were obtained using either Bruker AM-300 or DRX-500 spectrometers operating at 300 (75) MHz and 500 (125) MHz for 1H (or 13C) respectively. 1H (or 13C) chemical shifts are reported in parts per million (ppm) relative to the solvent resonances, taken as δ 7.26 (δ 77.0) and δ 2.49 (δ 39.5) for $CDCl_3$ and $(CD_3)_2SO$, respectively. For the Lumi4 intermediates (**9**, **10**, **15**, **16**), the observed NMR spectra were very complicated due to the presence of differing conformers/isomers in solution, and are not reported.[1] Analytical HPLC was performed on an Agilent 1200 instrument (Agilent, Santa Clara, CA) equipped with a diode array detector (λ = 254, 280, 315 and 340 nm, 600 nm reference), a thermostat set at 25 °C, and a Zorbax Eclipse XDB-C18 column (4.6 x 150 mm, 5 µm, Agilent, Santa Clara, CA). The mobile phase of a binary gradient (2-40% B/20 min; solvent A, 0.1% TFA; solvent B, ACN) at a flow rate of 1 mL/min was used for analytical HPLC. UV absorption measurements were recorded on a Varian Cary 300 double beam spectrometer using quartz cells of 1 cm path length. Emission spectra of **1**, **3**, and **4** were measured using a HORIBA Jobin Yvon Fluorolog-3 spectrofluorometer equipped with an IBH TBX-04-D detector. Emission spectra of **2**, **11**, and **17** were measured using a HORIBA Jobin Yvon Fluoromax-3 spectrofluorometer.

2.0 Synthesis.

2.1 Preparation of Lumi4-L-Arg9 1, Lumi4-D-Arg9 2, and Lumi4-L-TAT[49-57] 3 (Chart S1).

A resin-bound nonapeptide with sequence H_2N-L-$[Arg]_9$-$CONH_2$ (after cleavage from support) was purchased from a commercial vendor (Anaspec, Inc., San Jose, CA). A portion of this resin (41.92 mg, 3.51 µmol) was placed in an O-ring microfuge tube, diethylether (1 mL) was added and allowed to stand for 5 minutes to swell the resin. The resin was centrifuged briefly, ether was decanted, and the resin was allowed to dry with a stream of nitrogen. The N-hydroxysuccinimide derivative of Lumi4® (**Lumi4-NHS**, 71%, 3.63 mg, 1.73 µmol) was dissolved in DMF (200 µL) and added to the resin. Diisopropylethylamine (3 µL, 17 µmol) was added, the suspension was blanketed with nitrogen, and shaken at 800 rpm overnight. The resin was centrifuged briefly, the supernatant was decanted using a pipette, and the resin was washed with DMF (6 x 1 mL) and MeOH (6 x 1 mL). The resin was allowed to dry using a stream of air, whereupon a solution of 5% triisopropylsilane in trifluoroacetic acid (600 µL) was added. The suspension was blanketed with nitrogen, and shaken at 800 rpm for 6 hours. The resin was centrifuged briefly, vortexed, then filtered using a medium fritted funnel. The resin was washed with additional 5% triisopropylsilane in trifluoroacetic acid (3 x 300 µL) and the combined filtrate and washes transferred to four microfuge tubes. Diethylether (1.2 mL per tube) was added, and the crude peptide allowed to stand overnight at 4 °C. The precipitate was centrifuged briefly, washed with ether (1.2 mL) and allowed to air dry. The crude peptide was dissolved in sterile water (150 µL) and purified by HPLC using a reversed phase column and 0.1% trifluoroacetic acid and acetonitrile gradient. Fractions containing product were combined, lyophilized, and the purified peptide conjugate **1** was dissolved in sterile water. Lumi4 absorbance at 340 nm (ε_{340} = 26,000, pH 7.4) was used to quantify the product (280 nmol, 16.2%). FTMS pEI: calc. for $C_{115}H_{193}N_{50}O_{23}$ $[M+5H]^{5+}$, 528.5088, found, 528.5102. MALDI-TOF MS: calc. for $C_{115}H_{189}N_{50}O_{23}$ $[M+H]^{+}$, 2638.51, found, 2638.60. The preceding protocol was repeated using a resin-bound nonapeptide of

identical sequence except for the configuration of the arginine residues, H_2N-D-[Arg]$_9$-$CONH_2$ to prepare compound **2**. MALDI-TOF MS: calc. for $C_{115}H_{189}N_{50}O_{23}$ $[M+H]^+$, 2638.51, found, 2638.52. The preceding protocol was repeated using a resin-bound nonapeptide of sequence H_2N-L-ArgLysLysArgArgGln[Arg]$_3$-$CONH_2$ to prepare compound **3** (294 nmol, 32.5%). FTMS pEI: calc. for $C_{114}H_{187}N_{44}O_{24}$ $[M+3H]^{3+}$, 852.1583, found, 852.1614. MALDI-TOF MS: calc. for $C_{114}H_{186}N_{44}O_{24}$ $[M+H]^+$, 2554.46, found, 2554.47.

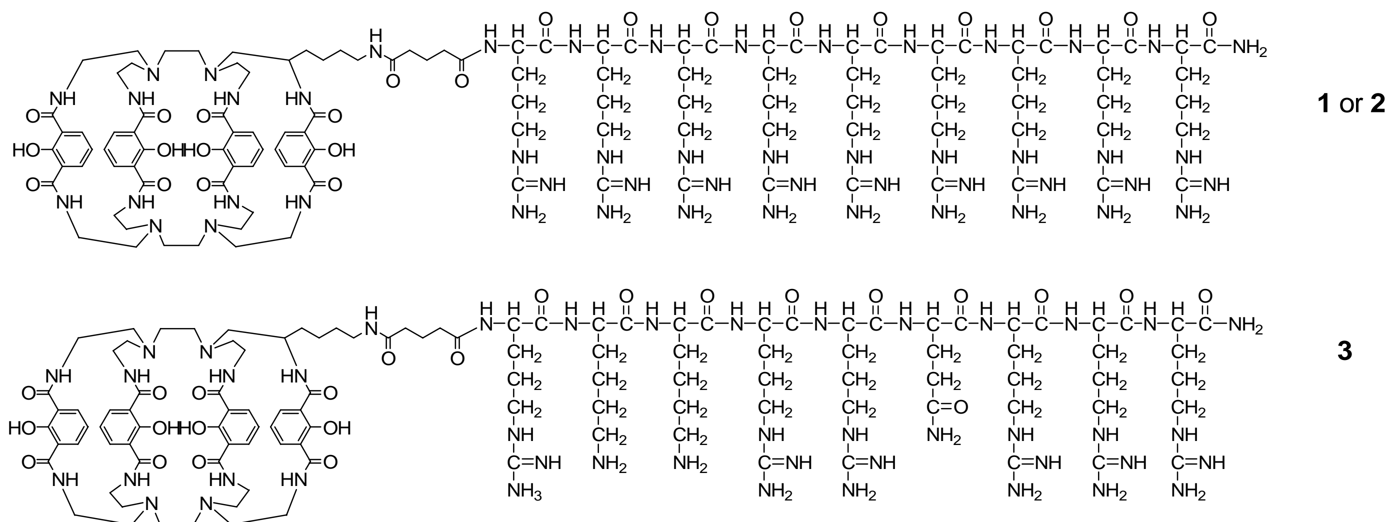
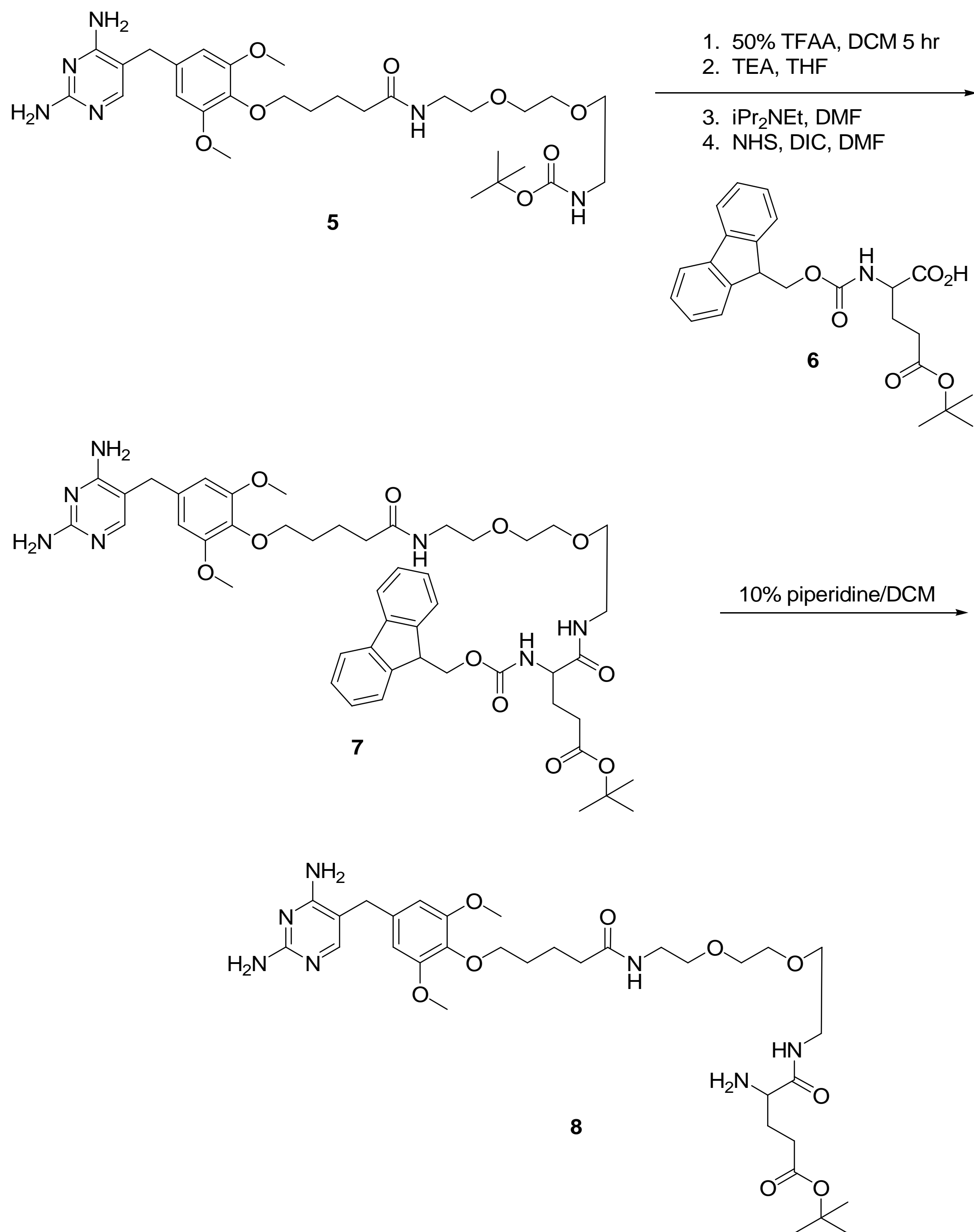


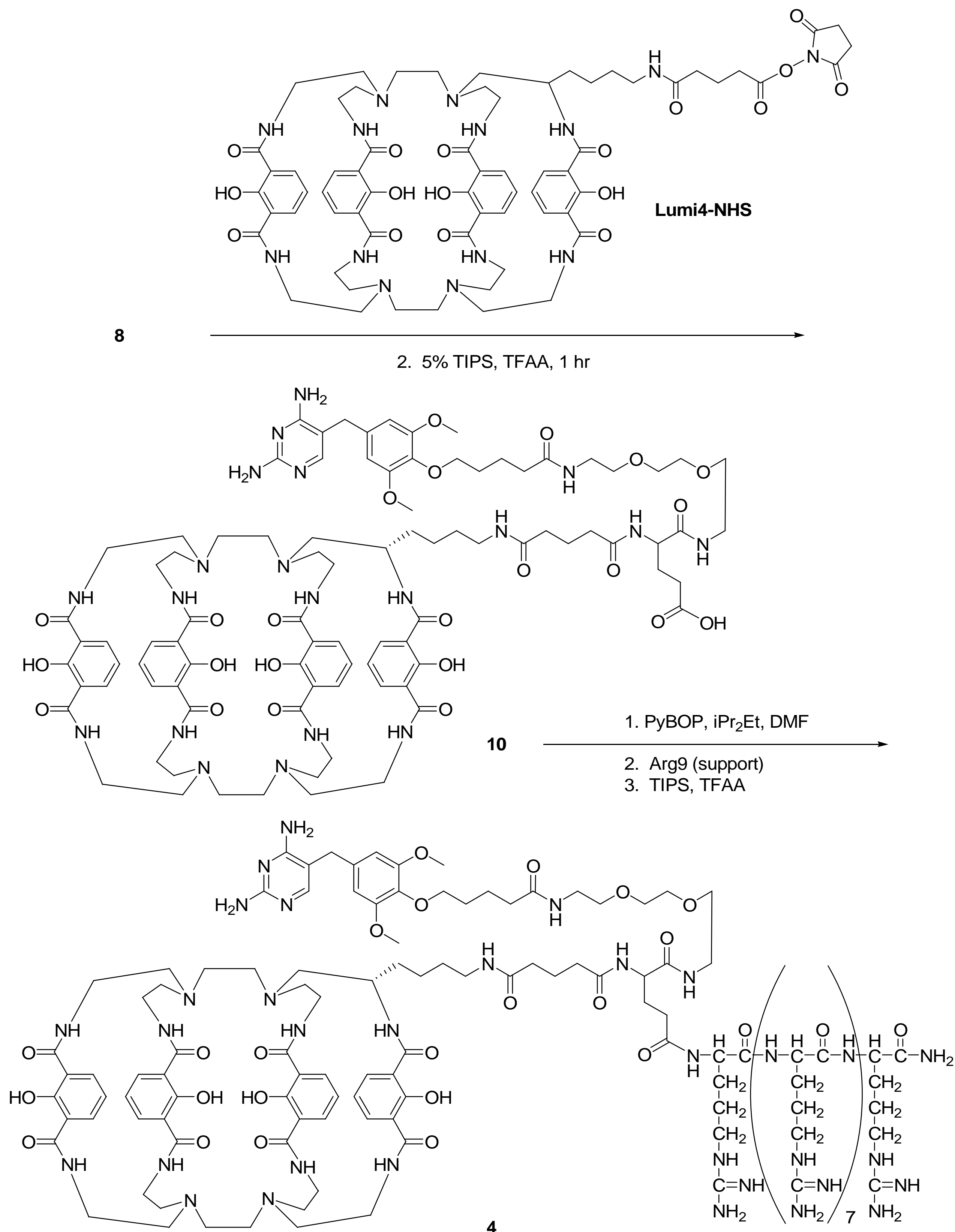
Chart S1. Structures of Conjugates **1**-**3**.

2.2 Preparation of Lumi4-glutamic amide of TEGTMP, Arg$_9$ derivative 4.

N-FMOC-glutamic amide of TEGTMP, δ-tert-butyl ester **7** (Scheme S1)**.** TEGBocTMP **5** was prepared as described previously.[2] TEGBocTMP (74 mg, 122 μmol) was treated with trifluoroacetic acid: dichloromethane (1:1, 4 mL) for 6 hr. Solvents were removed in vacuo, and the residue was dissolved in THF (1 mL) and TEA (200 μL). After stirring for 5 minutes, solvents were removed in vacuo, and the residue dissolved in dimethylformamide (400 μL) and diisopropylethylamine (25 uL, 144 μmol). In a separate flask, N-hydroxysuccinimide (18.09 mg, 157 μmol) and N-FMOC-glutamic acid δ-tert-butyl ester **6** (62.47 mg, 146.8 μmol, Anaspec, Inc.) were dried together in vacuo. Dimethylformamide (600 μL) and diisopropylcarbodiimide (30 μL, 194 μmol) were added, and the resulting solution was allowed to stir under nitrogen for 3 hr, whereupon the solution was transferred via syringe to the dimethylformamide solution prepared above from TEGBocTMP. After 20 hr, the solution was diluted with dichloromethane (10 mL) and washed with deionized water (3 x 10 mL). Solvents were removed under reduced pressure, and the residue purified by silica gel chromatography using 5 – 7.5% methanol in dichloromethane as eluent. Fractions containing product were combined, solvent was removed under reduced pressure, and the residue dried in vacuo to provide N-FMOC-glutamic amide of TEGTMP, δ-tert-butyl ester **7** (77 mg, 69.1%). 1H NMR (300 MHz, $CDCl_3$): δ = 7.75 (s, 1H, ArH), 7.73 (s, 2H, ArH), 7.56 (d, 2H, ArH), 7.38 (t, 2H, ArH), 7.28 (t, 2H, ArH), 6.34 (s, 2H, ArH), 4.92 (s, 2H, NH_2), 4.77 (s, 2H, NH_2), 4.36 (m, 2H, CH_2O), 4.18 (m, 1H, ArCH), 3.91 (t, 2H, $ArOCH_2$), 3.74 (s, 6H, $ArOCH_3$), 3.60-3.42 (m, 14H, $ArCH_2Ar$ + CH_2O), 2.40-2.20 (m, 4H, CH_2CONH, CH_2CO_2), 2.20-2.00 (m, 1H, $C\underline{H}CONH$), 1.80-1.70 (m, 6H, $CHC\underline{H}_2$, $C\underline{H}_2C\underline{H}_2$), 1.42 (s, 9H, $O(CH_3)_3$). 13C NMR (300 MHz, $CDCl_3$): δ = 173.3, 172.6, 171.5, 162.7, 162.1, 156.5, 153.7, 143.7, 141.2, 135.8, 133.8, 127.7, 127.0, 125.0, 119.9, 106.3, 105.0, 80.9, 72.8, 70.2, 70.1, 69.9, 69.5, 67.0, 56.1, 54.4, 47.1, 39.2, 39.1, 36.1, 34.6, 31.6, 29.6, 29.2, 28.0, 22.4. FTMS pEI: calculated for $C_{48}H_{64}N_7O_{11}$ $[MH]^+$, 914.4658, found, 914.4630.



Scheme S1. Preparation of glutamic amide of TEGTMP, δ-tert-butyl ester **8**



Scheme S2. Preparation of N-Lumi4-glutamic amide of TEGTMP, Arg9 derivative **4**

Glutamic amide of TEGTMP, δ-tert-butyl ester **8** (Scheme S1). N-FMOC-glutamic amide of TEGTMP, δ-tert-butyl ester **7** (77 mg, 84 µmol) was dissolved in dichloromethane (4.5 mL). Piperidine (0.5 mL) was added, and the resulting solution was stirred at ambient temperature for 4.5 hr. Solvents were removed under reduced

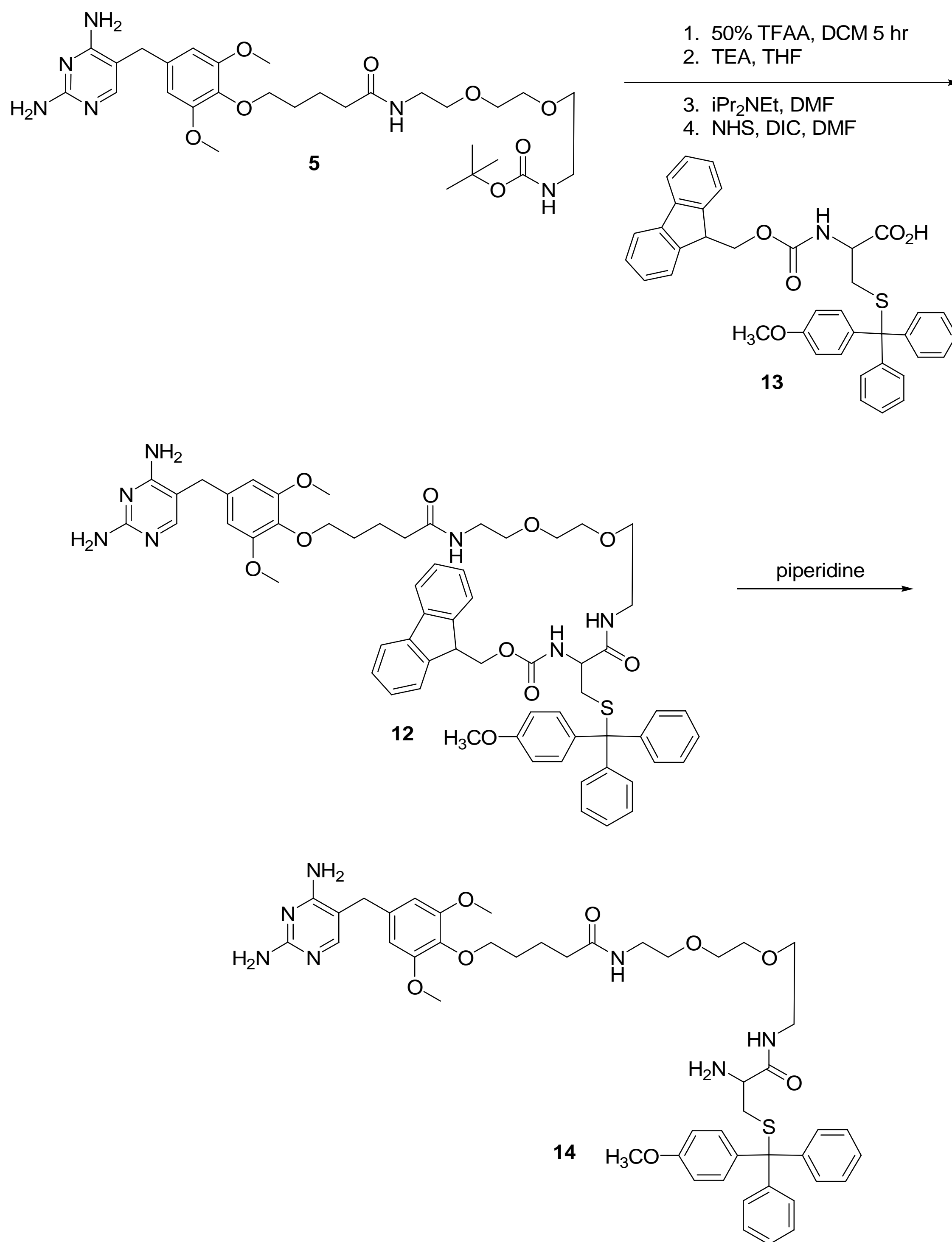
pressure, and the residue dried in vacuo. The residue was purified by silica gel chromatography using 10 – 20% methanol in dichloromethane as eluent. Fractions containing product were combined, solvent was removed under reduced pressure, and the residue dried in vacuo to provide the glutamic amide of TEGTMP, δ-tert-butyl ester **8** (50 mg, 86%). 1H NMR (300 MHz, $CDCl_3$): δ = 7.67 (s, 1H, ArH), 7.59 (t, 1H, NH), 6.59 (t, 1H, NH), 6.35 (s, 2H, ArH), 5.21 (s, 2H, NH_2), 5.04 (s, 2H, NH_2), 3.92 (t, 2H, $ArOCH_2$), 3.76 (s, 6H, $ArOCH_3$), 3.61-3.39 (m, 14H, $ArCH_2Ar$ + CH_2O), 2.35-2.25 (m, 4H, CH_2CONH, CH_2CO_2), 2.10-2.01 (m, 1H, C$\underline{H}$CONH), 1.81-1.72 (m, 6H, CHC$\underline{H}_2$, C$\underline{H}_2$C$\underline{H}_2$), 1.41 (s, 9H, $O(CH_3)_3$). 13C NMR (300 MHz, $CDCl_3$): δ = 175.0, 173.7, 173.1, 163.1, 162.0, 155.7, 154.0, 136.1, 134.0, 106.7, 105.4, 80.9, 73.2, 70.4, 70.3, 70.1, 56.4, 54.9, 50.6, 39.4, 39.1, 36.4, 34.8, 32.2, 30.6, 29.6, 28.3, 22.7. FTMS pEI: calculated for $C_{33}H_{54}N_7O_9$ $[MH]^+$, 692.3978, found, 692.3966.

N-Lumi4-glutamic amide of TEGTMP, δ-tert-butyl ester **9** (Scheme S2**).** The glutamic amide of TEGTMP, δ-tert-butyl ester **8** (15 mg, 22 µmol) was dissolved in dimethylformamide (1 mL) and diisopropylethylamine (15 µL, 86 µmol). The resulting solution was added to the NHS ester of Lumi4 (15 mg, 8.22 µmol). The resulting solution was stirred at ambient temperature for 5 hrs, whereupon solvents were removed in vacuo. The residue was dissolved in methanol (500 µL), the solution transferred to 2 tared, O-ring capped microcentrifuge tubes, and ether was added (1 mL per tube). After allowing sufficient time for mixing, the tubes were centrifuged for 2 minutes at 12,000 rpm, and the supernatent was decanted. This process was repeated five times. The residue was air dried, then dissolved in methanol (300 µL), isopropyl alcohol (900 µL) was added, the tubes were centrifuged, the supernatant decanted, and the residue was dried in vacuo to provide compound **9** (8.16 mg, 52%). FTMS pEI: calculated for $C_{94}H_{132}N_{20}O_{23}$ $[M+2H]^{2+}$, 954.4882, found, 954.4903.

N-Lumi4-glutamic amide of TEGTMP **10** (Scheme S2). N-Lumi4-glutamic amide of TEGTMP, δ-tert-butyl ester **9** (6.2 mg, 3.2 µmol) was treated with 5% solution of triisopropylsilane in trifluoroacetic acid (200 µL) in an O-ring microcentrifuge tube. The tube was shaken at 800 rpm for 1 hr, ether (1 mL) was added, and after allowing sufficient time for mixing, the tube was centrifuged at 12,000 rpm for 2 minutes. The supernatant was decanted, the pellet was washed with ether (1 mL), and allowed to air dry. The residue was dissolved in methanol (200 µL), ether (1 mL) was added, and, after allowing sufficient time for mixing, the tube was centrifuged at 12,000 rpm for 2 minutes. The supernatant was decanted, the pellet was washed with ether (1 mL), and allowed to air dry. The residue was dissolved in methanol (200 µL), triethylamine (10 µL) was added, and the solution was vortexed briefly. Ether was added, and, after allowing sufficient time for mixing, the tube was centrifuged at 12,000 rpm for 2 minutes. The supernatant was decanted, the pellet was washed with ether (1 mL), and allowed to air dry. The residue was dried in vacuo to provide compound **10** (3.67 mg, 61%). FTMS pEI: calculated for $C_{90}H_{123}N_{20}O_{23}$ $[MH]^+$, 1851.9064, found, 1851.9075.

N-Lumi4-glutamic amide of TEGTMP, Arg_9 derivative **4** (Scheme S2). The resin-bound nonapeptide with sequence H_2N-L-$[Arg]_9$-$CONH_2$ described above (48.3 mg, 4.05 umol) was placed in an O-ring microfuge tube, diethylether (1 mL) was added and the suspension was shaken for 1.5 hr at 800 rpm to swell the resin. The resin was centrifuged briefly, ether was decanted, and dry dimethylformamide (1 mL) was added. The resin was shaken for 5 minutes, centrifuged, and the supernatant decanted. The N-Lumi4-glutamic amide of TEGTMP **10** (779 nmol) and benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP, 1.55 mg, 2.98 µmol) were dissolved in DMF (100 µL) and added to the resin. Diisopropylethylamine (2 µL, 11 µmol) was added, the suspension was blanketed with nitrogen, and shaken at 800 rpm overnight. The resin was centrifuged briefly, the supernatant was decanted using a pipette, and the resin was washed with DMF (5 x 1 mL), MeOH (5 x 1 mL), and ether (1 mL). The resin was allowed to air dry, whereupon a solution of 5% triisopropylsilane in trifluoroacetic acid (600 µL) was added. The suspension was blanketed with nitrogen, and shaken at 800 rpm for 8 hours. The resin was centrifuged briefly, vortexed, then filtered using a coarse fritted funnel. The resin was washed with additional 5% triisopropylsilane in trifluoroacetic acid (3 x 300 µL) and the combined filtrate and washes transferred to four microfuge tubes. Diethylether (1.2 mL per tube) was added, and the crude peptide allowed to stand overnight at 4 ^oC. The precipitate was centrifuged briefly, washed with ether (1.2 mL) and allowed to air dry. The crude peptide was dissolved in sterile water (200 µL) and purified

by HPLC using a reversed phase column and 0.1% trifluoroacetic acid and acetonitrile gradient. Fractions containing product were combined, lyophilized, and the purified peptide conjugate was dissolved in sterile water. Lumi4 absorbance at 340 nm (ε_{340} = 26,000, pH 7.4) was used to quantify the compound (176 nmol, 23%). FTMS pEI: calc. for $C_{144}H_{236}N_{57}O_{31}$ $[M+5H]^{5+}$, 651.9723, found, 651.9755. MALDI-TOF MS: calc. for $C_{144}H_{232}N_{57}O_{31}$ $[M+H]^{+}$, 3255.83, found, 3256.36.



Scheme S3. Preparation of cysteine-S-monomethoxytrityl ether, TEGTMP amide **14**.

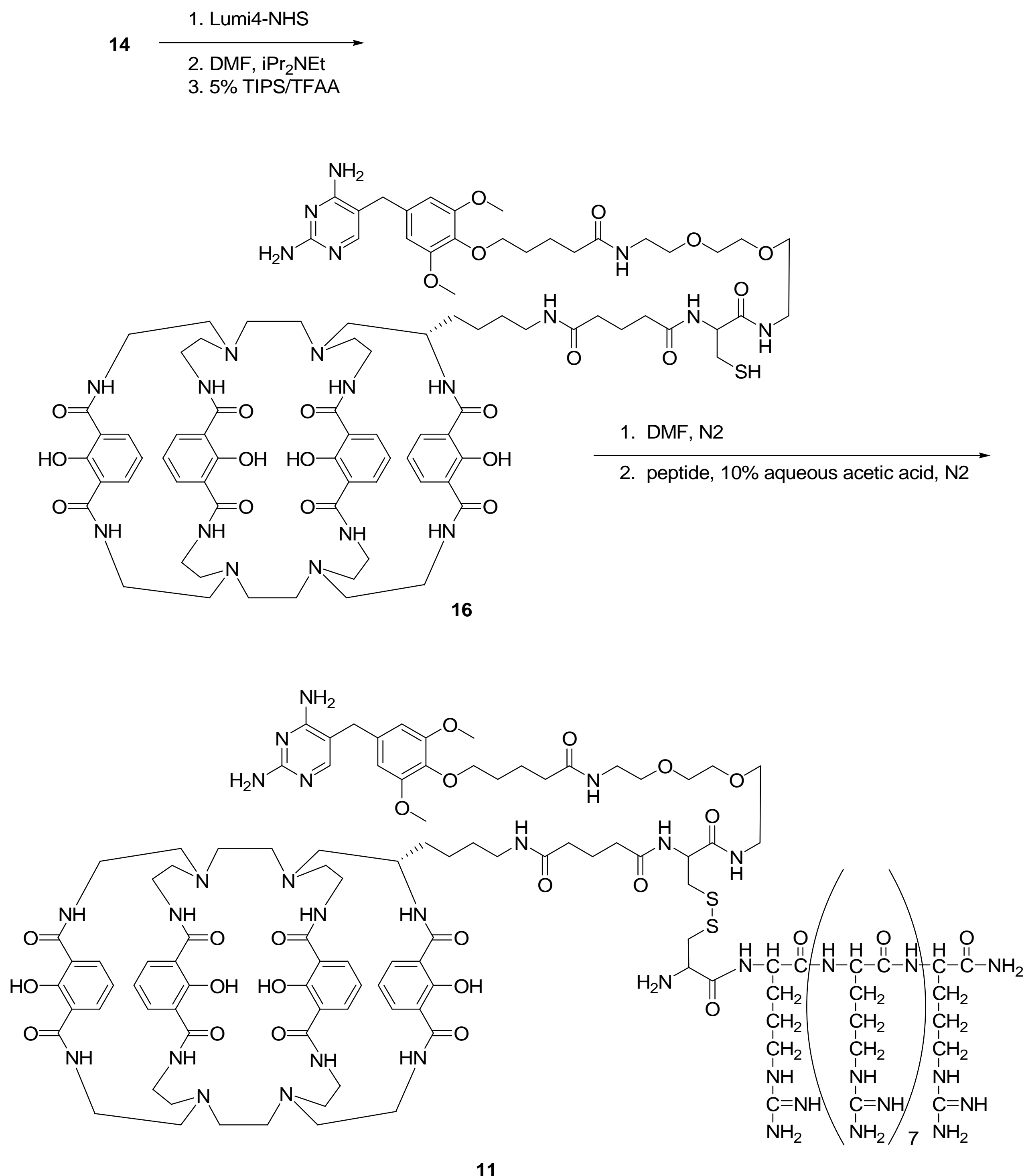
2.3 Preparation of N-Lumi4-cysteine amide of TEGTMP, $CysArg_9$ derivative 11.

N-FMOC-cysteine-S-monomethoxytrityl ether, TEGTMP amide **12** (Scheme S3). TEGBocTMP **5** (75 mg, 124 µmol) was treated with trifluoroacetic acid: dichloromethane (1:1, 4 mL) for 6 hr. Solvents were removed in vacuo, and the residue was dissolved in THF (1 mL) and TEA (200 µL). After stirring for 5 minutes, solvents were removed in vacuo, and the residue dissolved in dimethylformamide (400 µL) and diisopropylethylamine (30 uL, 172 µmol). In a separate flask, N-hydroxysuccinimide (17.1 mg, 149 µmol) and N-FMOC-cysteine S-monomethoxytrityl ether **13** (62.47 mg, 146.8 µmol, Anaspec, Inc.) were dried together in vacuo. Dimethylformamide (600 µL) and diisopropylcarbodiimide (30 µL, 194 µmol) were added, and the resulting solution was allowed to stir under nitrogen for 6 hr, whereupon the solution was transferred via syringe to the dimethylformamide solution prepared above from TEGBocTMP. After 22 hr, the solution was diluted with dichloromethane (10 mL) and washed with deionized water (3 x 10 mL). Solvents were removed under reduced pressure, and the residue purified by silica gel chromatography using 5 – 7.5% methanol in dichloromethane as eluent. Fractions containing product were combined, solvent was removed under reduced pressure, and the residue dried in vacuo to provide N-FMOC-cysteine-S-monomethoxytrityl ether, TEGTMP amide **12** (31 mg, 23%). 1H NMR (300 MHz, $CDCl_3$): δ = 7.76-7.17 (m, 21H, ArH), 6.79 (d, 2H, ArH), 6.35 (s, 2H, ArH), 4.98 (s, 2H, NH_2), 4.83 (s, 2H, NH_2), 4.34 (m, 2H, CH_2O), 4.17 (m, 1H, ArCH), 3.93 (t, 2H, $ArOCH_2$), 3.78 (s, 3H, $ArOCH_3$), 3.75 (s, 6H, $ArOCH_3$), 3.60-3.44 (m, 14H, $ArCH_2Ar$ + CH_2O), 2.67-2.57 (m, 3H, $C\underline{H}CONH$, CH_2S), 2.25 (t, 2H, CH_2CONH), 1.81-1.59 (m, 4H, $C\underline{H_2}C\underline{H_2}$). 13C NMR (300 MHz, $CDCl_3$): δ = 173.3, 170.4, 162.7, 161.9, 158.1, 156.1, 153.6, 144.6, 143.6, 141.2, 136.3, 135.8, 133.7, 130.7, 129.4, 128.0, 127.7, 127.0, 126.8, 125.0, 119.9, 113.2, 106.3, 105.0, 72.8, 70.1, 70.0, 69.9, 69.4, 67.0, 66.7, 56.0, 55.2, 50.6, 47.0, 39.3, 39.0, 36.0, 34.6, 29.2, 22.4. FTMS pEI: calculated for $C_{62}H_{70}N_7O_{10}S$ $[MH]^+$, 1104.4899, found, 1104.4910.

Cysteine-S-monomethoxytrityl ether, TEGTMP amide **14** (Scheme. S3). N-FMOC-cysteine-S-monomethoxytrityl ether, TEGTMP amide **12** (31 mg, 28 umol) was dissolved in tetrahydrofuran (1 mL). Piperidine (0.25 mL) was added, and the resulting solution was stirred at ambient temperature for 4 hr. Solvents were removed under reduced pressure, and the residue dried in vacuo. The residue was purified by silica gel chromatography using 10 – 20% methanol in dichloromethane as eluent. Fractions containing product were combined, solvent was removed under reduced pressure, and the residue dried in vacuo to provide compound **14** (50 mg, 86%). 1H NMR (300 MHz, $CDCl_3$): δ = 7.72 (s,1H, ArH), 7.42-7.17 (m, 12H, ArH), 6.79 (d, 2H, ArH), 6.35 (s, 2H, ArH), 4.95 (s, 2H, NH_2), 4.78 (s, 2H, NH_2), 3.93 (t, 2H, $ArOCH_2$), 3.77 (s, 3H, $ArOCH_3$), 3.76 (s, 6H, $ArOCH_3$), 3.62-3.33 (m, 14H, $ArCH_2Ar$ + CH_2O), 2.67-2.57 (m, 1H, $C\underline{H}CONH$), 2.71-2.52 (m, 2H, CH_2S), 2.26 (t, 2H, CH_2CONH), 2.07 (s, 2H, NH_2), 1.81-1.75 (m, 4H, $C\underline{H_2}C\underline{H_2}$). 13C NMR (300 MHz, $CDCl_3$): δ = 173.2, 162.8, 158.1, 155.6, 153.7, 144.8, 136.6, 135.8, 133.6, 130.7, 129.4, 127.9, 126.7, 113.2, 105.0, 72.8, 70.1, 70.0, 69.7, 66.4, 56.1, 55.2, 54.0, 39.1, 38.8, 37.4, 36.1, 34.6, 29.3, 22.4. FTMS pEI: calculated for $C_{47}H_{60}N_7O_8S$ $[MH]^+$, 882.4219, found, 882.4192.

N-Lumi4-cysteine-S-monomethoxytrityl ether, TEGTMP amide **15** (Scheme S4). Cysteine-S-monomethoxytrityl ether, TEGTMP amide **14** (18 mg, 20 µmol) was dissolved in dimethylformamide (1 mL) and diisopropylethylamine (15 µL, 86 µmol), then added to the NHS ester of Lumi4 (15 mg, 8.22 µmol). The resulting solution was stirred at ambient temperature for 27 hrs, whereupon solvents were removed in vacuo. The residue was dissolved in methanol (600 µL), the solution transferred to 2 tared, O-ring capped microcentrifuge tubes, and ether was added (0.75 mL per tube). After allowing sufficient time for mixing, the tubes were centrifuged for 2 minutes at 12,000 rpm, and the supernatent was decanted. This process was repeated. The residue was dissolved in dimethylformamide (75 µL/tube), ether was added (400 µL/tube). After allowing sufficient time for mixing, the tubes were centrifuged for 2 minutes at 12,000 rpm, and the supernatent was decanted. The residue was dissolved in methanol (250 µL/tube), ether was added (750 µL/tube). After allowing sufficient time for mixing, the tubes were centrifuged for 2 minutes at 12,000 rpm, and the supernatent was decanted. The residue was dissolved in methanol (250 µL/tube), isopropyl alcohol was added (750 µL/tube). After allowing sufficient time for mixing, the tubes were centrifuged for 2 minutes at 12,000 rpm, and the supernatent was decanted. The residue was air dried, then dissolved in methanol (300 µL), ether (900

µL) was added, the tubes were centrifuged, the supernatant decanted, and the residue was dried in vacuo to provide compound **15** (14.42 mg, 84%). FTMS pEI: calculated for $C_{108}H_{138}N_{20}O_{22}S$ $[M+2H]^{2+}$, 1049.5002, found, 1049.5035.



Scheme S4. Preparation of N-Lumi4-cysteine amide of TEGTMP, CysArg9 derivative **11**.

N-Lumi4-cysteine, TEGTMP amide **16** (Scheme S4). A solution of 5% triisopropylsilane in trifluoroacetic acid (150 µL) was added to N-Lumi4-cysteine-S-monomethoxytrityl ether, TEGTMP amide **15** (3.1 mg, 1.5 µmol). The resulting solution was blanketed with nitrogen, and shaken at 600 rpm for 1 hour, whereupon ethyl ether

(0.9 mL) was added. The suspension resulting upon mixing was stored briefly at 4 °C, and centrifuged at 12,000 rpm for 2 minutes. The supernatant was removed and the solids were washed with ethyl ether (1.2 mL). The suspension was centrifuged and supernatant was removed. The resulting solids were air dried briefly then dried in vacuo to provide the trifluoroacetate salt of compound **16** (3.6 mg, 100%). Compound **16** was stored under nitrogen. FTMS pEI: calculated for $C_{88}H_{122}N_{20}O_{21}S$ $[M+2H]^{2+}$, 913.4402, found, 913.4442.

N-Lumi4-cysteine amide of TEGTMP, $CysArg_9$ derivative **11** (Scheme S4). The peptide with sequence H_2N-L-Cys(NPys)-$[Arg]_9$-$CONH_2$, (0.5 mg, 298 nmol,) was dissolved in a solution of 10% acetic acid in sterile water (75 μL) and degassed with nitrogen. A solution of N-Lumi4-cysteine, TEGTMP amide **16** (0.57 mg, 250 nmol) in dimethylformamide (10 uL), degassed with nitrogen, was added and the resulting solution was mixed at 400 rpm. The progress of the reaction was monitored using reversed phase HPLC. After 21 hours, the crude peptide was purified by HPLC using a reversed phase column and 0.1% trifluoroacetic acid and acetonitrile gradient. Fractions containing product were combined, lyophilized, and the purified peptide conjugate was dissolved in sterile water. Lumi4 absorbance at 340 nm (ε_{340} = 26,000, pH 7.4) was used to quantify the compound (83 nmol, 33%). FTMS pEI: calc. for $C_{145}H_{239}N_{58}O_{31}S_2$ $[M+5H]^{5+}$, 670.5670, found, 670.5667. MALDI-TOF MS: calc. for $C_{145}H_{235}N_{58}O_{31}S_2$ $[M+H]^+$, 3348.80, found, 3348.78.

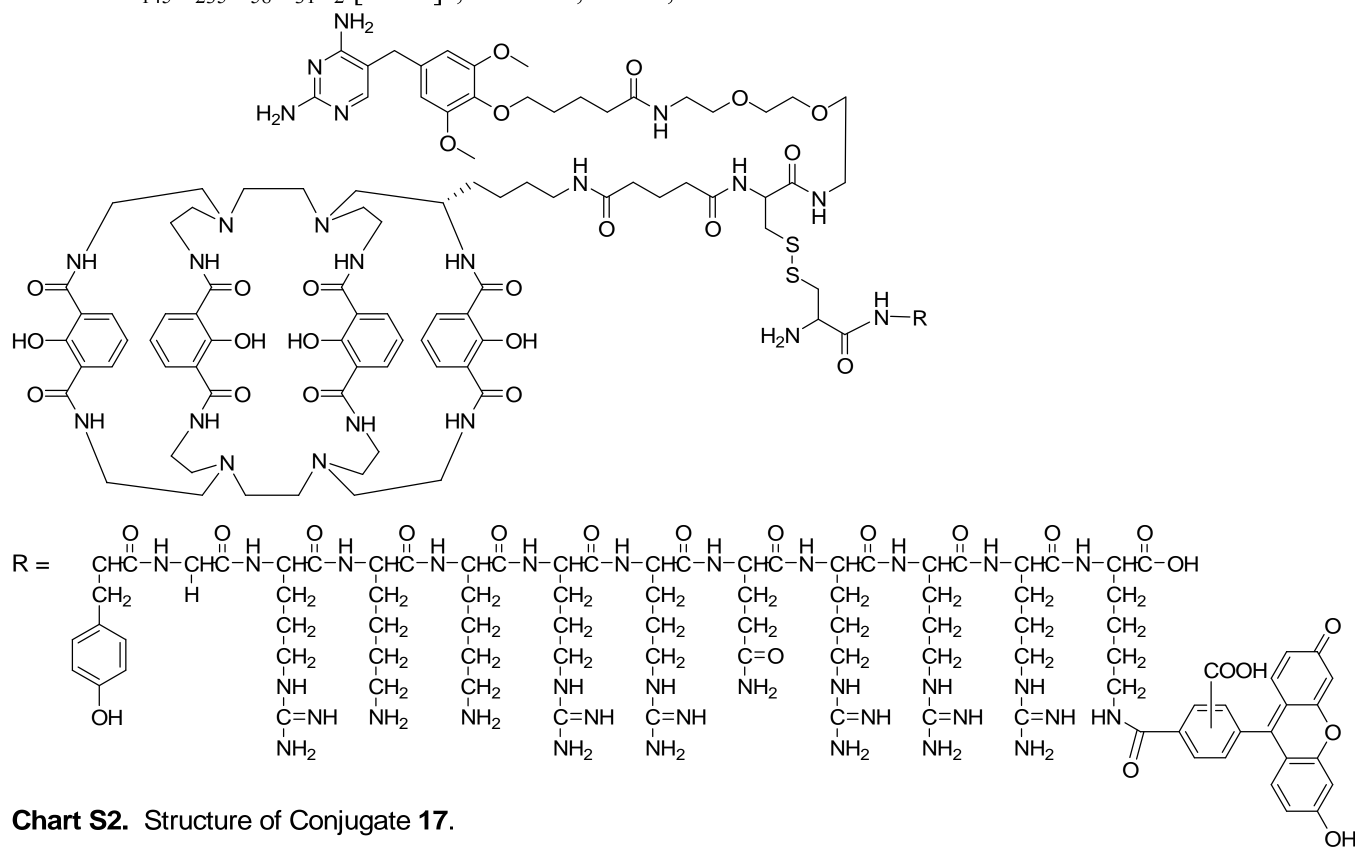


Chart S2. Structure of Conjugate **17**.

2.4 N-Lumi4-cysteine amide of TEGTMP, Cys-TAT(47-57)-LysFAM derivative 17 (Chart S2).

The peptide with sequence H_2N-L-Cys(NPys)-Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Lys(FAM)-$CONH_2$, (0.5 mg, 217 nmol,) was dissolved in a solution of 10% acetic acid in sterile water (100 μL) and degassed with nitrogen. A solution of N-Lumi4-cysteine, TEGTMP amide **16** (0.57 mg, 250 nmol) in dimethylformamide (10 uL), degassed with nitrogen, was added and the resulting solution was mixed at 400 rpm. The progress of the reaction was monitored using reversed phase HPLC. After 3 days, the crude peptide was purified by HPLC using a reversed phase column and 0.1% trifluoroacetic acid and acetonitrile gradient. Fractions containing product were combined, lyophilized, and the purified peptide conjugate was dissolved in sterile water. Lumi4 absorbance at 340 nm (ε_{340} = 26,000, pH 7.4) was used to quantify the compound (35 nmol, 16%). FTMS pEI: calc. for $C_{182}H_{269}N_{56}O_{42}S_2$ $[M+5H]^{5+}$, 795.0015, found, 795.0013. MALDI-TOF MS: calc. for $C_{182}H_{265}N_{56}O_{42}S_2$ $[M+H]^+$, 3970.97, found, 3971.16.

2.5 Metallation with terbium and conjugate luminescence characterization.

Lyophilized conjugates were dissolved in sterile water (conc. = 1 mM) in 1.5 mL microfuge tubes. $TbCl_3$ (~1.2 equiv.) in water was added, and the samples were vortexed ~5 min. and allowed to rest at RT for ~30 min. The fluorescence excitation spectrum (**1**) and emission spectra (**1**, **2**, **3**, **4** and **11**) were essentially the same as the spectra for the parent Lumi4-Tb complex (Fig. S1).[1] Conjugate **17** exhibited an expected mix of Tb^{3+} and fluorescein emission (Fig. S1). All samples exhibited bright green luminescence following dissolution in water and addition of terbium when exposed to light from a hand-held UV lamp.

3.0 Plasmids.

The gene encoding TagRFP-T was subcloned from plasmid TagRFP-T pcDNA3[3] to pRSETb-EGFP-eDHFR[2] to generate pRSETb-TagRFPT-eDHFR. A *BamHI* to *Acc65I* fragment encoding TagRFP-T was prepared by PCR from TagRFP-T pcDNA3 using the primers 5'-GCA TAC GTC GGA TCC GAT GGT GTC TAA GGG CGA -3' (*BamHI*, coding strand) and 5'-GCA TAC GTC GGT ACC CCT TGT ACA GCT CGT CCA T-3' (*Acc65I*, non-coding strand). This fragment was inserted between the *BamHI* site and the *BsrGI* site in pRSETb-GFP-eDHFR to give to pRSETb-TagRFPT-eDHFR. The gene encoding TagRFPT-eDHFR was subcloned from plasmid pRSETb-TagRFPT-eDHFR to H_2B-eDHFR[4] to generate H_2B-TagRFPT-eDHFR. A *BamHI* to *NotI* fragment encoding TagRFPT-eDHFR was prepared by PCR from pRSETb-TagRFPT-eDHFR using the primers 5'- T CGT GAT CTG GAT CCC GCT TCT GCT TCG TCT TCG ATG GTG TCT AAG GGC -3' (*BamHI*, coding strand) and 5'- GCT TTG TTA GCG GCC GCA CCA TGC TTA GAA TCC TTA-3' (*NotI*, non-coding strand). This fragment was inserted between the *BamHI* site and the *NotI* site in H_2B-eDHFR to give to H_2B-TagRFPT-eDHFR. Plasmid integrity was confirmed by direct sequencing.

4.0 Cell culture, transfection and labeling.

MDCK, NIH3T3, HEK293 and HeLa cells were cultured in DMEM (+) (Dulbecco's Modified Eagle Media supplemented with 10% FBS, 2 mM L-glutamine, 100 unit/mL penicillin and 100 µg/mL of streptomycin) at 37 °C and 5% CO_2. MDCK cells were seeded at 10^5 cells per well into a 6-well plate. After ~18 h incubation at 37 °C and 5% CO_2, adherent cells (~80% confluent) were transfected with 2 µg of the pH2B-TagRFPT-eDHFR using Lipofectamine2000™ (Invitrogen) according to manufacturer's instructions. Approximately 6 h after transfection, cells were trypsinized and reseeded at 15,000 cells/well into 8-well chambered coverglasses (Nunc™, 12-565-470) and incubated at 37 °C and 5% CO_2 overnight.

Cells growing in DMEM (+) in 8-well chambered coverglasses were washed 1X with PBS and re-immersed in culture medium (with or without FBS) at the desired temperature (37 °C or 4 °C) containing peptides at the desired concentration. Cells were then incubated at 37 °C and 5% CO_2 (on ice in ambient atmosphere for 4 °C studies), washed 2X in PBS and reimmersed in DMEM (+) supplemented with 1 mM Patent Blue™ dye to quench extracellular luminescence. Following incubation and washing, cells were maintained at 37 °C and 5% CO_2 until imaging.

5.0 Microscopy, image processing and analysis.

Imaging of adherent live cells was performed using a previously described, modified epifluorescence microscope (Zeiss Axiovert 200).[5] All images were acquired using a EC Plan Neofluar, 63X, 1.25 N.A. objective (Carl Zeiss, Inc.) Filter cubes containing the appropriate excitation and emission filters and dichroics allowed for wavelength selection. Bright field and continuous wave fluorescence images were acquired using an Axiocam MRM CCD camera (Carl Zeiss, Inc.). For time-gated luminescence, pulsed excitation light from a UV LED (λ_{em} = 365 nm, illumination intensity = 0.6 W/cm^2 at the sample plane) was synchronized with the

intensifier component of an ICCD (Mega-10EX, Stanford Photonics, Inc.) such that a 10 μs delay was inserted between the end of the LED pulse and the intensifier start-time. For each acquisition, the signal from multiple excitation/emission events was accumulated on the ICCD sensor and read out at the end of the camera frame. The source/camera timing parameters were the same for all of the time-gated images and data presented here: excitation pulse width = 1500 μs, pulse period = 3000 μs, delay time = 10 μs, intensifier on-time = 1390 μs. The sensitivity of time-gated imaging is dependent on the number of excitation/detection events integrated on the CCD during a single camera frame and on the intensifier gain voltage. Frame summing was used to increase the signal-to-noise ratio and to remove ion-feedback noise from the intensifier. Each frame summed effectively increases the bit depth of the resulting image in increments of 1024 (i.e., 1 frame yields bit depth equal to 1024, 2 frames, 2048, etc.). Table S1 lists the number of excitation/detection events, frame length, number of frames, total acquisition time and intensifier gain voltage used to acquire all time-gated images and data reported here.

ICCD images (tagged image file format, .TIF) were captured with Piper control software (v2.4.05, Stanford Photonics, Inc.) and Axiocam images (.ZVI) were captured with Zeiss AxioVision software (v4.6). All images were cropped and adjusted for contrast using NIH ImageJ (v1.34). Table S1 provides the image processing parameters applied to each time-gated image in the paper, including pixel dimensions, pixel (bit) depth and contrast level (minimum and maximum gray values).

For quantitative analysis of Tb^{3+}-to-fluorescein LRET (Fig. 3c), two sets of time-gated images (λ_{em} = 520 nm and λ_{em} = 540 nm) were acquired for the same fields of view under identical exposure settings (Table S1). Cells were selected for analysis from fields of view where the continuous wave fluorescein signal was either completely diffuse or completely punctate (2 separate experiments and 4 fields of view for each condition). The threshold tool in NIH ImageJ was used to select regions of interest (ROI) in the time-gated images corresponding to the nuclei of luminescent cells. The emission signal intensity was calculated according to the equation: $S = (\mu_{signal}-\mu_{bckg})$, where, μ_{signal} is equal to the mean pixel gray value in an ROI corresponding to a luminescent nucleus, and μ_{bckg} is equal to the mean pixel gray value in a similarly sized ROI in a part of the image with no cells. The donor normalized LRET signal was defined as the ratio (S_{520}/S_{540}) of mean gray values from corresponding ROIs in each image pair. The mean and standard deviation of the LRET ratios for numerous cells representing each condition (diffuse and punctate) were then calculated.

6.0 References.

1 J. Xu, T. M. Corneillie, E. G. Moore, G. L. Law, N. G. Butlin, K. N. Raymond, *J Am Chem Soc* **2011**, *133*, 19900-19910.

2 H. E. Rajapakse, D. R. Reddy, S. Mohandessi, N. G. Butlin, L. W. Miller, *Angew Chem Int Ed Engl* **2009**, *48*, 4990-4992.

3. N. C. Shaner, M. Z. Lin, M. R. McKeown, P. A. Steinbach, K. L. Hazelwood, M. W. Davidson, R. Y. Tsien, *Nat Methods* **2008**, *5*, 545-551.

4. R. Wombacher, M. Heidbredder, S. van de Linde, M.P. Sheetz, M. Heileman, V.W. Cornish, M. Sauer *Nat. Methods* **2010**, *7*, 717-719.

5. N. Gahlaut, L. W. Miller, *Cytometry A* **2010**, *77*, 1113-1125.

Table S1. Detection and image processing parameters for all time-gated images and data presented in the paper.

image or data	λ_{em} (nm)	excitation events	frame length (ms)	frames	acquisition time (ms)	intensifier gain (V)	pixel dimensions	pixel depth	contrast (min./max.)
1a, DMEM(+) **1** (5 μM)	530-550	220	667	4	2667	778	900X900	4096	348/2069
1a, DMEM(+) **2** (5 μM)	530-550	110	333	8	2667	778	900X900	8192	374/2504
1a, DMEM(+) **4** (5 μM)	530-550	110	333	4	1333	833	900X900	4096	120/700
1a, DMEM(+) **1** (20 μM)	530-550	44	133	16	2133	833	900X900	16384	709/15382
1a, DMEM(+) **2** (20 μM)	530-550	66	200	8	1600	889	900X900	8192	370/2944
1a, DMEM(+) **4** (60 μM)	530-550	22	67	8	533	833	900X900	8192	211/5822
1b, **1** (5 μM) + **Arg9** (30 mM)	530-550	660	2000	4	8000	778	900X900	4096	240/2350
1b, **1** (20 μM)	530-550	22	67	4	267	778	900X900	4096	240/2350
2b, LRET	598-612	220	667	8	5333	889	900X900	8192	700/4500
2b, LRET + TMP	598-612	220	667	8	5333	889	900X900	8192	700/4500
3a, TGL (top)	530-550	660	2000	4	8000	833	900X900	4096	400/4076
3a, LRET (top)	510-530	660	2000	4	8000	833	900X900	4096	400/4076
3a, TGL (bottom)	530-550	660	2000	4	8000	833	900X900	4096	250/3500
3a, LRET (bottom)	510-530	660	2000	4	8000	833	900X900	4096	250/3500
3b	*	660	2000	4	8000	833	N/A	N/A	N/A
S2, top **1** (5 μM)	530-550	66	200	8	1600	833	900X900	8192	396/5708
S2, top **2** (10 μM)	530-550	22	67	4	267	889	900X900	4096	370/2944
S2, top **4** (10 μM)	530-550	110	333	8	2667	889	900X900	8192	233/7180
S2, bottom, **1** (10 μM)	530-550	22	67	4	267	833	900X900	4096	126/2770
S2, bottom, **2** (10 μM)	530-550	44	133	8	1067	889	900X900	8192	271/7211
S2, bottom, **4** (10 μM)	530-550	220	667	8	5333	889	900X900	8192	862/5417
S3	530-550	220	667	8	5333	833	900X900	8192	493/8184
S4, top	530-550	66	200	16	3200	778	900X900	16384	522/15097
S4, middle	530-550	66	200	8	1600	778	900X900	8192	281/5375
S4, bottom	530-550	66	200	4	800	833	900X900	4096	190/3098
S5, LRET	598-612	44	133	4	532	778	600X600	4096	200/2500
S5, TGL	530-550	44	133	4	532	778	600X600	4096	200/2500

* For quantitative analysis of donor-normalized LRET signals (presented in Fig. 3b), 2 images were acquired using the indicated source/camera parameters: acceptor emission (510-530 nm) and donor emission + acceptor emission (530-550 nm).

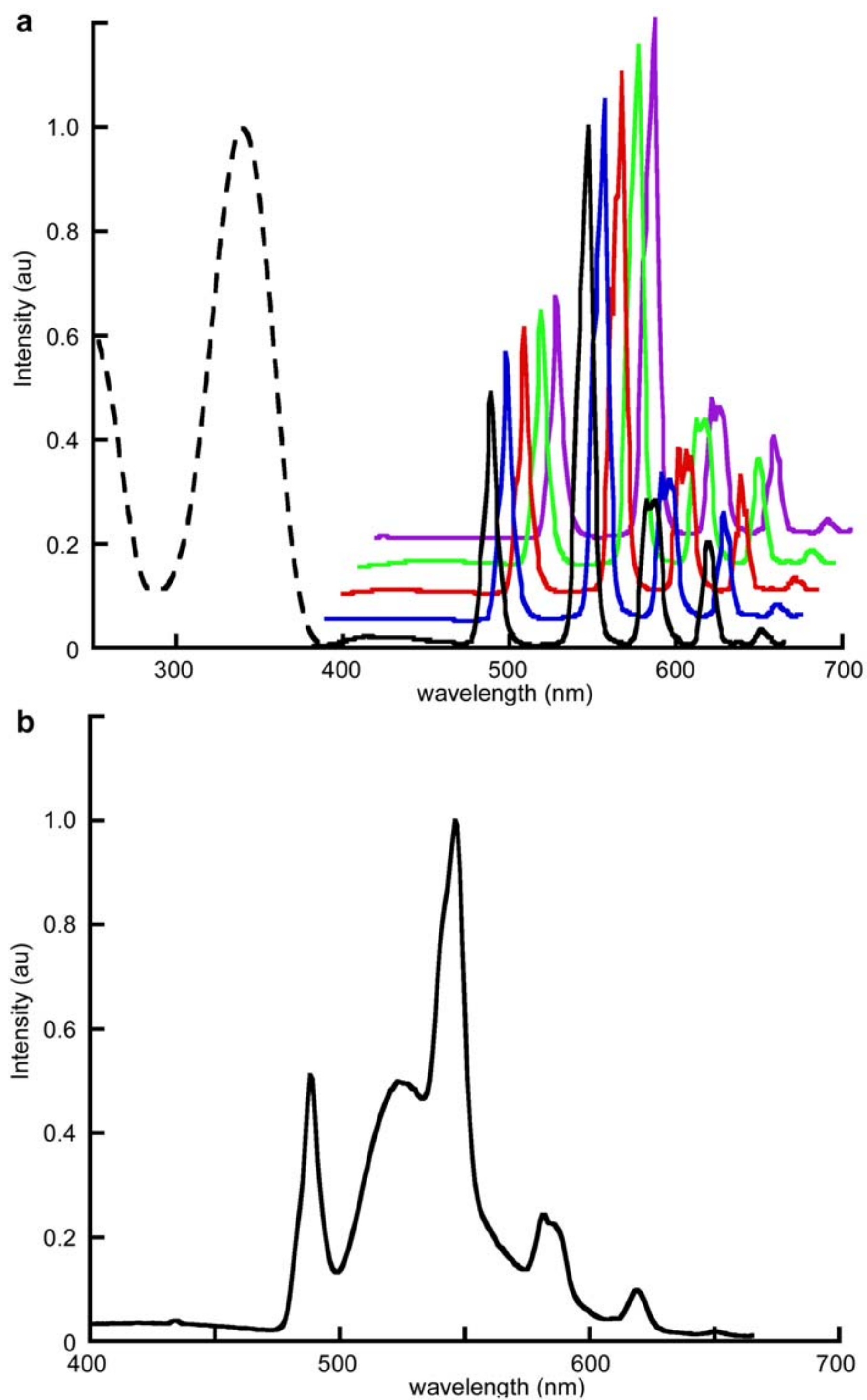


Figure S1. **a**, Excitation spectrum of **1** (black, dotted) and emission spectra (solid) of **1** (black), **2** (blue), **3** (red), **4** (green) and **11** (purple). Emission spectra are vertically offset (0.05 a.u.) and horizontally offset (10 nm) for clarity. **b**, Emission spectrum of **17** showing mix of Tb^{3+} and fluorescein emission.

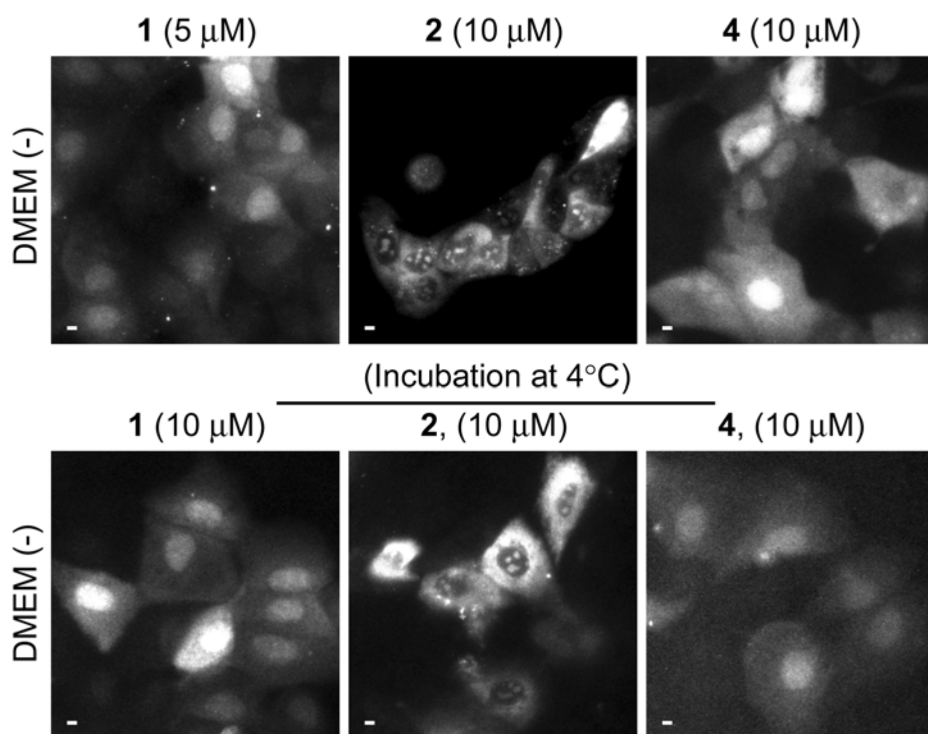


Figure S2. Effects of serum and temperature on uptake and distribution. Micrographs: time-gated luminescence (delay = 10 μs, λ_{ex} = 365 nm, λ_{em} = 540 ± 10 nm) Scale bars, 10 μm. MDCK cells were incubated for 30 min. at 37 °C (top) or 4 °C (bottom) in Dulbecco's modified eagle medium without fetal bovine serum (DMEM (-)) that contained indicated concentrations of peptides **1**, **2**, or **4**.

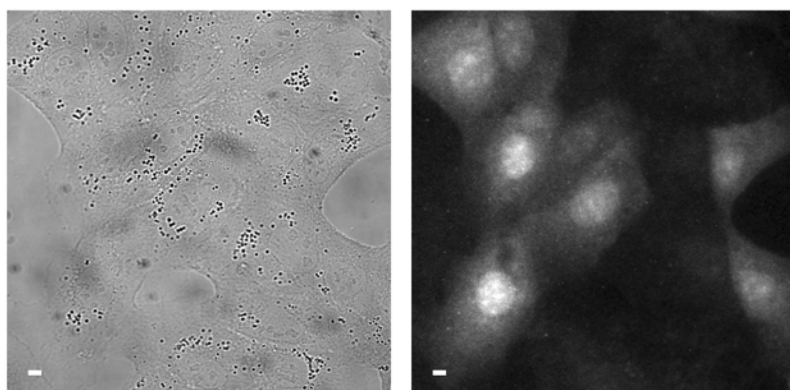
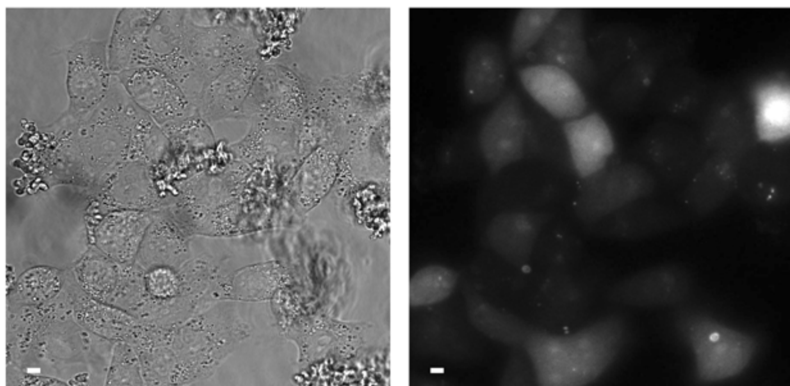
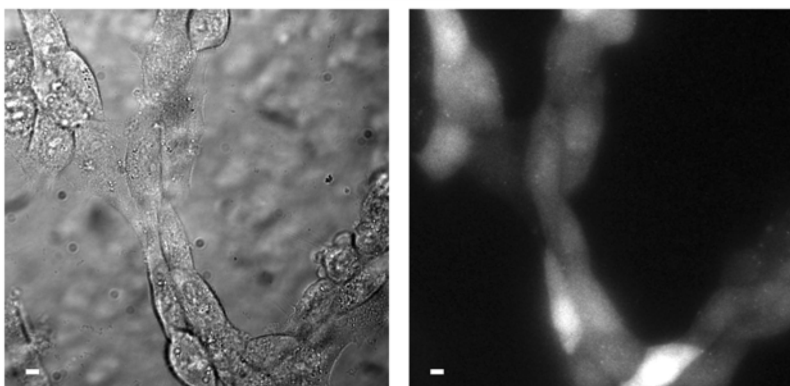


Figure S3. Cytoplasmic delivery of Lumi4(Tb)-Tat(49-57) (3**) in MDCK cells.** Micrographs: left, bright field; right, time-gated luminescence (delay = 10 μs, λ_{ex} = 365 nm, λ_{em} = 540 ± 10 nm) Scale bars, 10 μm. MDCK cells were incubated for 30 min. at 37 °C in DMEM (-) containing **3** (10 μM), washed 2X in PBS and reimmersed in DMEM (+) containing 1 mM Patent Blue™ prior to imaging.

HeLa



NIH3T3



HEK293

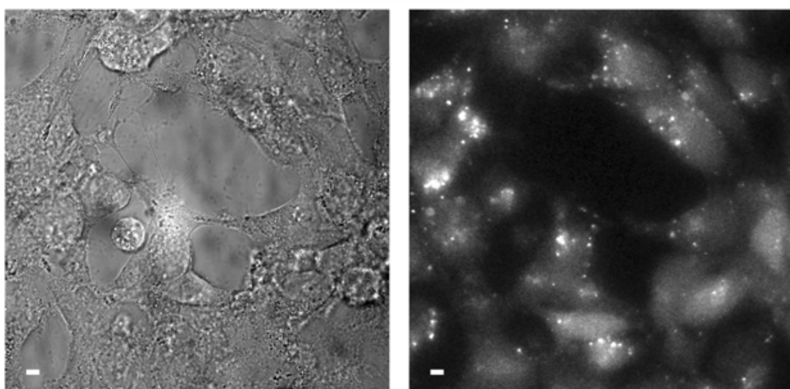


Figure S4. Cytoplasmic delivery of Lumi4-(L)-Arg9 (1**) in various cell types.** Micrographs: left, bright field; right, time-gated luminescence (delay = 10 μs, λ_{ex} = 365 nm, λ_{em} = 540 $\pm$ 10 nm) Scale bars, 10 μm. Cells were incubated for 30 min. at 37 °C in DMEM (+) containing **1** (10 μM), washed 2X in PBS and reimmersed in DMEM (+) containing 1 mM Patent Blue™ prior to imaging. **(top**) HeLa cells, (middle) NIH3T3 cells, (bottom) HEK293 cells.

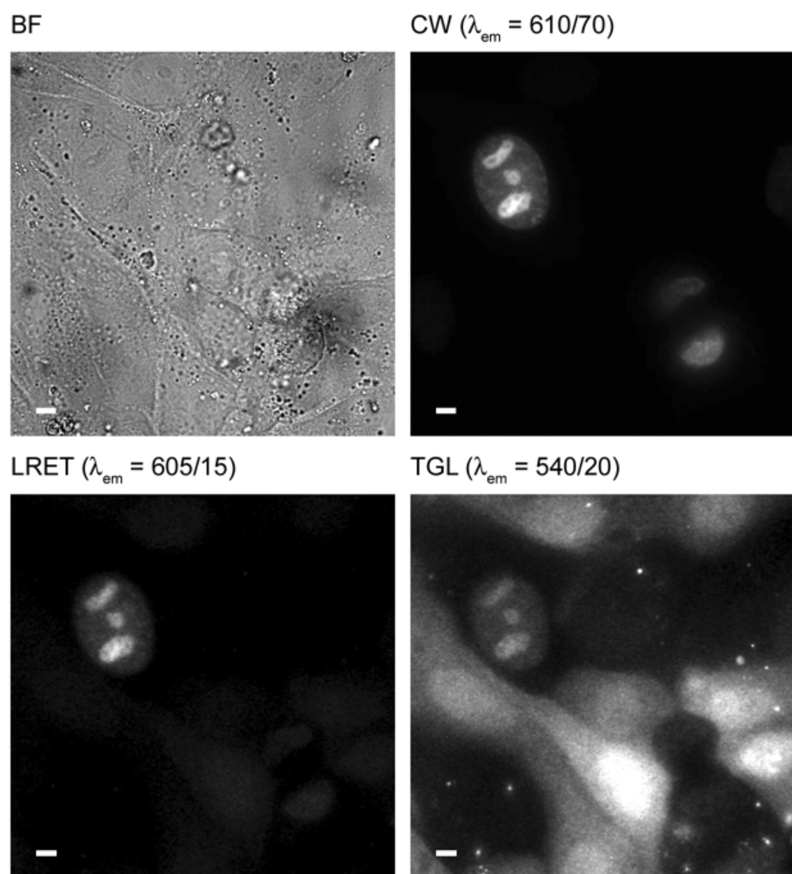


Figure S5. Arg9 mediates cytoplasmic delivery of 11 **and specific labeling of H2B-TagRFPT-eDHFR as evidenced by time-gated LRET imaging of Tb^{3+}-to-TagRFPt sensitized emission**. MDCK cells transiently expressing H2B-TagRFPT-eDHFR were incubated for 30 min. at 37 °C in DMEM (-) containing **11** (10 μM), washed 2X in PBS and reimmersed in DMEM (+) containing 1 mM Patent Blue™ prior to imaging. Micrographs: BF, bright field; CW, continuous wave fluorescence (λ_{ex} = 570 ± 40 nm, λ_{em} = 610 ± 70 nm); LRET, time-gated luminescence (delay = 10 μs, λ_{ex} = 365 nm, λ_{em} = 605 ± 15 nm); TGL, time-gated luminescence (delay = 10 μs, λ_{ex} = 365 nm, λ_{em} = 540 ± 10 nm). Scale bars, 10 μM.