Supporting Information for:

Imaging Cell Surface Glycans with Bioorthogonal Chemical Reporters

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Experimental procedures

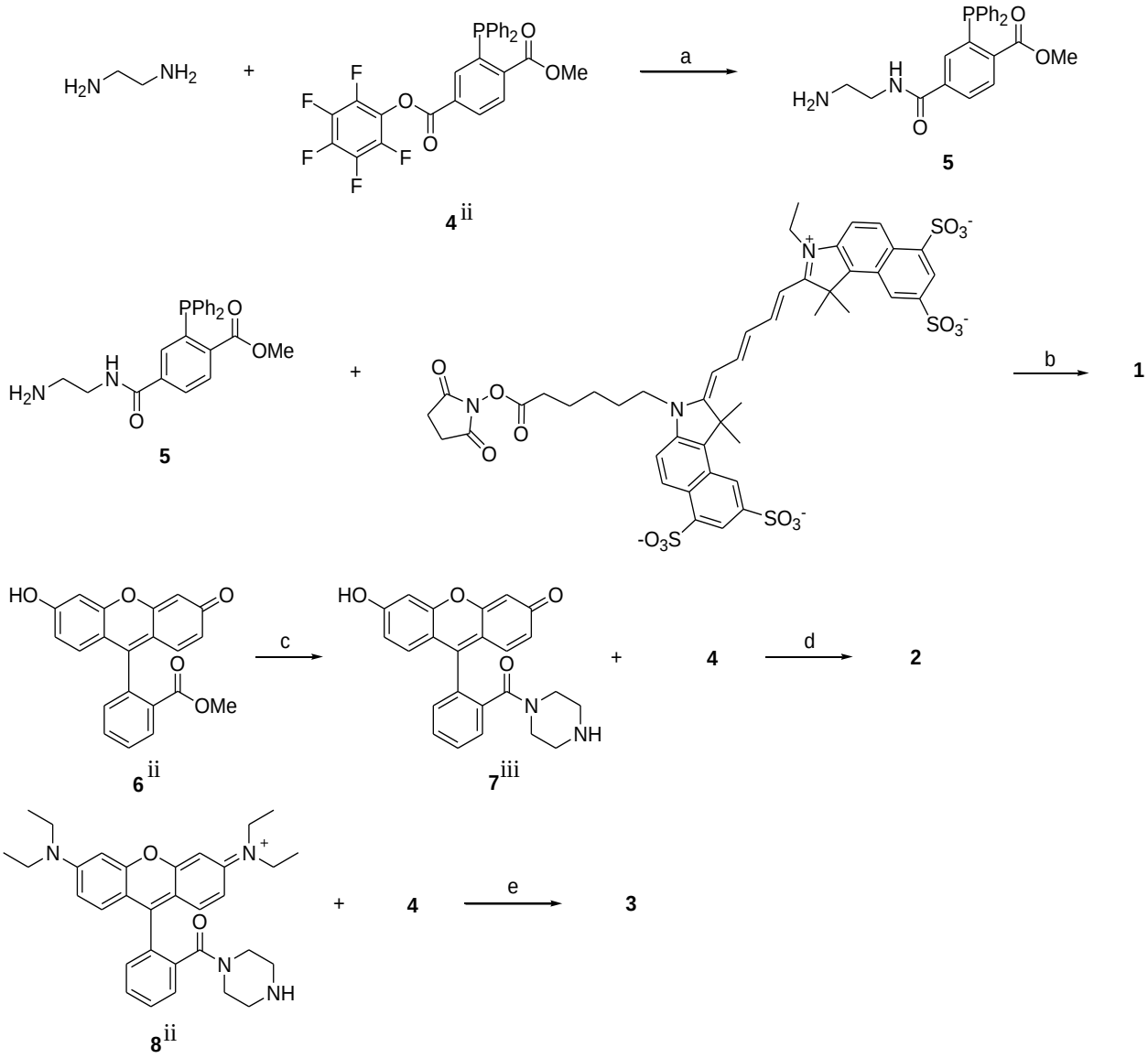
Materials and methods.

All chemical reagents were of analytical grade, obtained from commercial suppliers, and used without further purification unless otherwise noted. Cyanine 5.5 was purchased as its mono-N-hydroxysuccinimidyl (NHS) ester from GE Healthcare. Air-sensitive reactions were performed under Ar using standard Schlenk techniques, and all solvents were degassed by three freeze-pump-thaw cycles. Reversed-phase HPLC was performed using a Rainin Instruments Dynamax SD-200 system equipped with a Varian UV-Vis detector (model 345) and a Microsorb C18 analytical column (4.6 x 250 mm) at a flow rate of 1 mL/min, a semipreparative column (10 x 250 mm) at a flow rate of 4 mL/min, or a preparative column (21.4 x 250 mm) at a flow rate of 20 mL/min. HPLC samples were filtered with a Pall Life Sciences Acrodisc CR 13 mm syringe filter equipped with a 0.2 μm PTFE membrane prior to injection. Flash chromatography was performed using Merck 60 Å 230-400 mesh silica gel. Analytical thin layer chromatography (TLC) was performed on glass-backed Analtech Uniplate GHLF silica gel plates, and compounds were visualized by staining with ceric ammonium molybdate, 5% H_2SO_4 in ethanol, ninhydrin, and/or the absorbance of UV light (λ = 254 nm or 356 nm). Dichloromethane (CH_2Cl_2) and triethylamine (TEA) were dried over CaH_2 and distilled under a N_2 atmosphere prior to use. Anhydrous *N*,*N*-dimethylformamide (DMF), dimethylsulfoxide (DMSO), and *N*,*N*-diisopropylethylamine (DIPEA) were used from commercial sources without further purification.

NMR spectra were acquired using a Bruker AVQ-400, AVB-400, or AV-300 spectrometer. ^{1}H NMR spectra were obtained at 400 MHz and ^{13}C NMR spectra were obtained at 75 or 100 MHz and are referenced to residual solvent peaks. ^{31}P NMR spectra were obtained at 162 or 202 MHz and are referenced relative to 85% H_3PO_4 in D_2O. IR spectral data were obtained using a Perkin-Elmer 1600 FT-IR spectrometer. Low and high-resolution fast atom bombardment (FAB) mass spectra were obtained at the UC-Berkeley Mass Spectrometry Laboratory. ESI-FT/MS was performed at the HHMI Mass Spectrometry Laboratory at UC Berkeley. Uncorrected melting points were determined using a Mel-Temp 3.0 melting point apparatus.

Synthetic procedures.

Scheme 1[i]



[i]Reagents: (a) DIPEA, CH_2Cl_2, under Ar, 91%; (b) TEA, DMSO, under Ar, 36%; (c) Trimethyl aluminum (2.0 M in toluene), piperazine, CH_2Cl_2, reflux, 88%; (d) DIPEA, DMF, under Ar, 30%; (e) DIPEA, CH_2Cl_2, under Ar, 75%.
[ii]Compound **4**[1], **6**[2], **8**[3] were synthesized as previously described.
[iii]Compound **7** was synthesized with modifications to procedures previously described.[4]

Phosphine ethylenediamine conjugate (5). To a solution of ethylenediamine (411 mg, 6.84 mmol) in CH_2Cl_2 (100 mL) was added DIPEA (1.3 mL, 7.5 mmol). Compound **4** (3.3 g, 6.2 mmol) in CH_2Cl_2 was added dropwise over 10 min. The resulting mixture was stirred for 6 h under Ar. The crude reaction mixture was concentrated *in vacuo* and purified by flash chromatography on silica gel (9:1 CH_2Cl_2:MeOH) to give 2.3 g (91%) of a yellow solid. IR (thin film): 3313, 3242, 3064, 3003, 2957, 2917, 2849, 1696, 1630 cm^{-1}. ^1H NMR (400 MHz, CD_3OD): δ 3.01 (app t, *J* = 6.0 Hz, 2H), 3.51 (app t, *J* = 6.0 Hz, 2H), 3.69 (s, 3H), 7.24-7.36 (m, 10H), 7.52 (dd, *J* = 1.6, 4.0 Hz, 1H), 7.83 (dd, *J* = 1.6, 8.0 Hz, 1H), 8.04 (dd, *J* = 3.6, 8.4 Hz, 1H); ^{13}C NMR (100 MHz, CD_3OD): δ 40.4, 42.1, 51.2, 126.3, 128.2, 128.3, 128.7, 130.1, 130.2, 133.1, 133.6, 133.8, 136.7, 136.9, 137.1, 137.2, 137.5, 140.8, 141.1, 167.0, 168.3; ^{31}P NMR (162 MHz, MeOD): δ –4.06; HRMS (FAB): Calcd for $C_{23}H_{24}N_2O_3P$ $[M + H]^+$ 407.1525, found 407.1520.

Cyanine 5.5 phosphine conjugate (1). Cyanine 5.5 mono-NHS ester (10.0 mg, 8.90 μmol) was dissolved in DMSO (110 μL). Compound **5** (7.23 mg, 17.8 μmol) was added to the solution followed by addition of TEA (75.0 μL, 534 μmol). The reaction mixture was agitated under Ar for 6 h at rt in the dark. The sample was then diluted three-fold with water and purified by reversed-phase HPLC (C18 semipreparative column) using a gradient of 25%-35% CH_3CN/0.1% TFA over 60 min. The purified product was lyophilized to dryness to yield 4.2 mg of a blue solid (36% yield). mp: 200-202 °C (dec); ^1H NMR (500 MHz, 1:1 CD_3OD:$CDCl_3$): δ 1.39 (m, 2H), 1.44 (t, *J* = 7.5 Hz, 3H), 1.64 (m, 2H), 1.78 (m, 2H), 2.00 (s, 12H), 2.16 (m, 2H), 3.31 (m, 2H, under CD_3OD signal), 3.35 (t, *J* = 4.0 Hz, 2H), 3.70 (s, 3H), 4.06 (m, 2H), 4.20 (m, 2H), 7.31-7.38 (m, 8H), 7.54-7.61 (m, 8H), 7.88 (dd, *J* = 1.5, 8.0 Hz, 1H), 8.10 (dd, *J* = 4.0, 8.0 Hz, 1H), 8.19 (m, 2H), 8.56 (s, 1H), 8.59 (s, 1H), 8.73 (s, 1H), 8.75 (s, 1H), 9.05 (d, *J* = 9.0, 1H), 9.08 (d, *J* = 9.0, 1H); ^{31}P NMR (202 MHz, 1:1 CD_3OD:$CDCl_3$): δ -6.58; HRMS (ESI-FT): Calcd for $C_{64}H_{66}N_4O_{16}PS_4$ $[M+H]^+$ 1305.302, found 1305.324.

Fluorescein piperazine core (7). A 2.0 M solution of trimethyl aluminum in toluene (22 mL, 45 mmol) was added dropwise to a solution of piperazine (7.7 g, 0.090 mol) in 35 mL of CH_2Cl_2. After 4 h, a white precipitate formed. A solution of **6**[2] (7.8 g, 23 mmol) in 15 mL CH_2Cl_2 was added dropwise to the heterogeneous solution and gas evolution was observed. After stirring at reflux for 18 h in the dark, the reaction was cooled to rt and 200 mL of MeOH was added. Then, 1 M HCl (500 mL) was added slowly and gas evolution was observed. The heterogeneous solution was stirred in the dark until gas evolution ceased. The reaction mixture was concentrated *in vacuo* and purified by flash chromatography on silica gel (4:1 CH_2Cl_2:MeOH) to yield 7.9 g (88%) of a red-orange solid. R_f = 0.23 (6:1 CH_2Cl_2:MeOH, UV); mp 281-283 °C (dec); ^1H NMR (400 MHz, CD_3OD): δ 3.14 (m, 4H), 3.62 (m, 2H), 3.84 (m, 2H), 7.27 (dd, *J* = 2.0, 9.2 Hz, 2H), 7.34-7.37 (m, 2H), 7.61-7.62 (m, 1H), 7.65-7.68 (d, *J* = 9.2 Hz, 2H), 7.84-7.86 (m, 3H); ^{13}C NMR (100 MHz, CD_3OD): δ 39.9, 41.8, 44.4, 45.6, 103.7, 118.4, 121.6, 129.4, 132.0, 132.0, 132.2, 132.4, 134.9, 135.6, 161.1, 166.2, 169.2, 173.3; HRMS (ESI): Calcd for $C_{24}H_{21}N_2O_4$ $[M+H]^+$ 401.1501, found 401.1491.

Fluorescein phosphine conjugate (2). Compound **4** (368 mg, 0.695 mmol) was added to a solution of **7** (278 mg, 0.695 mmol) and DIPEA (0.250 mL, 1.44 mmol) in 5 mL of DMF. The reaction was stirred under Ar overnight at rt in the dark. The resulting reaction mixture was concentrated *in vacuo* and purified by flash chromatography on silica gel (20:1 to 4:1

CH_2Cl_2:MeOH) to yield 155 mg (30%) of a bright orange solid. Due to facile phosphine oxidation, the solid was further purified by HPLC (reversed-phase C18 preparative column, 10%-80% CH_3CN over 60 min) prior to use. R_f = 0.82 (10:1 CH_3CN:H_2O, UV); mp 100-102 °C; ^{1}H NMR (400 MHz, CD_3OD): δ 2.99 (m, 4H), 3.47 (m, 4H), 3.66 (s, 3H), 6.82, (m, 1H), 7.14-7.29 (m, 12 H), 7.47-7.58 (m, 5H), 7.73-7.82 (m, 4H), 8.04-8.07 (m, 1H); ^{13}C NMR (100 MHz, CD_3OD): δ 41.2, 41.3, 41.6, 46.3, 51.2, 102.2, 116.5, 120.2, 127.2, 127.7, 128.3, 128.4, 128.5, 128.7, 130.1, 130.3, 130.4, 130.7, 130.8, 131.3, 131.4, 132.0, 132.1, 133.1, 133.4, 133.6, 134.8, 135.7, 135.9, 137.0, 137.1, 137.7, 159.2, 162.9, 166.6, 167.7, 169.6, 172.3; ^{31}P NMR (162 MHz, acetone-d_6): δ -4.79; HRMS (MALDI-TOF): Calcd for $C_{45}H_{36}N_2O_7P$ $[M+H]^+$ 747.2260, found 747.2270.

Rhodamine phosphine conjugate (3). Compound **8**[3] (122 mg, 0.223 mmol) was added to a solution of **4** (131 mg, 0.245 mmol) in CH_2Cl_2 (10 mL), followed by 120 μL (0.86 mmol) of DIPEA. The solution was stirred overnight under Ar in the dark. The reaction solution was concentrated *in vacuo*, and the resulting crude product was purified by flash column chromatography (12:1 CH_2Cl_2:MeOH) to provide 151 mg (75%) of a dark red solid. ^{1}H NMR (400 MHz, CD_3OD): δ 1.18 (t, *J* = 6.8, 12H), 2.92 (br s, 2H), 3.19-3.37 (m, 5H), 3.55-3.62 (m, 10H) 6.74 (br s, 1H), 6.89-7.01 (m, 4H), 7.11-7.30 (m, 12H), 7.36-7.42 (m, 2H), 7.50-7.61 (m, 2H), 7.65-7.72 (m, 2H), 7.95 (dd, *J* = 3.6, 8.0, 1H); ^{13}C NMR (100 MHz, CD_3OD): δ 11.4, 45.5, 51.2, 95.9, 113.4, 114.0, 127.5, 128.4, 128.4, 128.7, 129.9, 130.3, 130.7, 130.7, 131.8, 132.0, 133.4, 133.6, 134.9, 137.1, 137.2, 137.7, 155.5, 155.8, 157.8, 166.6, 168.1, 169.5; ^{31}P NMR (162 MHz, CD_3OD): δ –4.60; HRMS (FAB): Calcd. for $C_{53}H_{54}N_4O_5P$ (M^+) 857.3832, found: 857.3813.

Photophysical characterization.

Absorption spectra were collected on a Varian Cary-100 UV-Vis spectrophotometer using 1 cm quartz cells or a Molecular Devices UV-Vis spectrophotometric microtiter plate reader. Fluorescence spectra were recorded on a Photon Technology International Quanta Master 4 L-format scanning spectrofluorometer equipped with a LPS-220B 75-W xenon lamp and power supply, A-1010B lamp housing with integrated igniter, switchable 814 photon-counting/analog photomultiplier detection unit, and MD5020 motor driver.

Extinction coefficients were determined by plotting a standard Beer-Lambert plot (A = clε). Relative quantum yields were measured according to the methods of Fery-Forgues *et al*.[5] Cyanine 5.5[6], fluorescein[7], and rhodamine 101[8] were used as standards for compounds **1**, **2**, and **3**, respectively. Briefly, the sample and standard were weighed on a microbalance and dissolved in Dulbecco's phosphate-buffered saline (PBS), pH 7.4 (**1** and **3**) or 0.1 N NaOH (**2**), such that the absorbance value was ~0.4 at the excitation wavelength. The sample and standard were diluted ten-fold, and the emission spectra were recorded and integrated.

Protein labeling with phosphine probes.

Wild-type murine dihydrofolate reductase (mDHFR) and azido mDHFR[9] (400 ng) were labeled with compounds **1-3** (10 μM final concentration in 20 μL of 8 M urea) for 12 h at rt in the dark. The samples were then diluted with 4X SDS-PAGE loading buffer and heated for 3 min at 95 °C before being loaded onto a polyacrylamide gel (Criterion pre-cast gel, 4-12% Bis-Tris).

After electrophoresis, the gel was visualized on a Typhoon 9410 Variable Mode Imager (fluorescence mode) to detect protein labeling and then stained with Coomassie Blue to verify equal protein content.

Cell surface azide labeling and detection by flow cytometry.

All media and reagents were obtained from commercial suppliers and used without further purification or alteration unless otherwise noted. Jurkat cells were cultured in RPMI 1640 media (GIBCO) supplemented with 10% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin. The cells were maintained at 37 ˚C and 5% CO_2 and counted using a Z2 Coulter cell counter. Centrifugation of mammalian cells was performed in a Sorvall centrifuge from Kendro Laboratory Products equipped with a SH3000 rotor. Flow cytometry data were acquired on a FACSCalibur flow cytometer (BD Biosciences Immunocytometry Systems) equipped with a 635 He-Ne red diode array laser and standard 488 Ar laser. Data were collected in triplicate using at least 10,000 live cells for all data points.

Jurkat cells were seeded at a density of 2.0 x 10^5 cells/mL in culture flasks containing 10 mL media and incubated for 3 d in untreated media or media containing peracetylated *N*-azidoacetylmannosamine (Ac_4ManNAz, 25 μM final concentration). After incubation, the cells were transferred to a 96-well V-bottom plate and pelleted by centrifugation at 3500 rpm for 3 min. The medium was decanted, and the cells were resuspended in 200 μL DPBS (pH 7.4) containing 1% FBS (FACS buffer). Centrifugation was repeated and cells were washed a second time. After pelleting, the supernatant was decanted, and the cells were resuspended with 100 μL of compounds **1**-**3** in FACS buffer (10 μM, 100 μM, and 1 mM final concentrations) and incubated at rt for 1 h in the dark. As a negative control, the cells were also treated with an oxidized version of **1** (**1-ox**, 1 mM final concentration).

Following the labeling reaction, the samples were maintained at 4 ˚C until analysis in order to slow recycling of the plasma membrane. The cells were pelleted, the supernatant decanted, and the cells were then resuspended in 200 μL of FACS buffer. This step was repeated six additional times to wash away the excess unreacted probe. The cells were then resuspended in 200 μL of FACS buffer and transferred into an additional 200 μL of FACS buffer for flow cytometry analysis.

Cell labeling and detection by fluorescence microscopy.

Chinese hamster ovary (CHO) cells were cultured in Ham's F-12 media (GIBCO) supplemented with 10% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin and counted using a hematocytometer. A Zeiss Axiovert 200M inverted microscope equipped with a 63 × 1.4 numerical aperture Plan-Apochromat oil immersion lens was used for imaging. A 175 W Xenon lamp housed in a Sutter DG4 illuminator linked to the microscope by an optical fiber assured shuttering and illumination. Image stacks containing 20-40 sections spaced 0.5 μm apart were acquired by using a CoolSNAP HQ charged-coupled device camera (Roper Scientific). SLIDEBOOK software (Intelligent Imaging Solutions) was used to control the microscope and the camera. The image stacks were digitally deconvolved by using the nearest-neighbor algorithm of SLIDEBOOK.

Cells were seeded on slides mounted with 8 tissue culture wells (Lab-Tek) with either untreated media or media containing Ac_4ManNAz (100 μM final concentration) for 3 d. The

cells were labeled with **1** (200 μM) for 2 h at 37 ˚C. The cells were washed three times with media, then fixed and permeabilized with cold MeOH (-20 ˚C) for 5 min. The cells were washed with PBS (3 times, 10 min each). The cells were blocked in PBS with 1% BSA for 15 min, followed by the addition of the primary antibody (rabbit polyclonal anti-Giantin, 1:750 dilution, Abcam®) diluted in blocking buffer. After incubation at rt for 2 h, the cells were washed three times, blocked for 10 min, and incubated with the secondary antibody (FITC-conjugated goat anti-rabbit, 1:400 dilution, Invitrogen) diluted in blocking buffer for 1 h. After three washes, the cells were mounted with Vectashield containing the nuclear stain DAPI (Vector Laboratories) before imaging.

Cell surface azide and ketone labeling and detection by flow cytometry.

Jurkat cells were seeded at a density of 2.5 x 10^5 cells/mL in culture flasks containing 10 mL of media and incubated for 2 d in untreated media, media containing peracetylated *N*-azidoacetylgalactosamine (Ac_4GalNAz, 150 μM final concentration), peracetylated *N*-levulinoylmannosamine (Ac_4ManLev, 50 μM final concentration) or both Ac_4GalNAz (150 μM final concentration) and Ac_4ManLev (50 μM final concentration). The cells were then treated with biotin hydrazide (Sigma Aldrich, 1 mM in DPBS with 1% FBS pH 6.5) and compound **1** (100 μM final concentration in DPBS with 1% FBS pH 6.5) for 2 h at rt in the dark.

The cells were pelleted, the supernatant was decanted, and the cells were then resuspended in 200 μL of FACS buffer. This step was repeated two additional times. After the final wash, the samples were resuspended FACS buffer containing FITC-avidin (Sigma Aldrich, 5 μg/mL final concentration). The cells were incubated with FITC-avidin for 15 min in the dark, washed three times with FACS buffer, and then resuspended in 200 μL of FACS buffer. The labeling with FITC-avidin and wash steps were repeated once more. After the second labeling, the cells were resuspended in 200 μL of FACS buffer and transferred into 200 μL of additional FACS buffer for flow cytometry analysis.

As a negative control, Jurkat cells treated with both Ac_4GalNAz and Ac_4ManLev were labeled with **1-ox** (100 μM final concentration in DPBS with 1% FBS pH 6.5), followed by FITC-avidin as described above (Figure S4).

It should be noted that the unnatural sugars used in the above experiments show no adverse effects toward cell viability. We have addressed this issue in previous work.[10-12] These published studies have established that concentrations up to 50 μM Ac_4ManLev and Ac_4ManNAz, and up to 200 μM Ac_4GalNAz, are not toxic to a majority of mammalian cells as assessed by exclusion of trypan blue and propidium iodide and confirmed by normal growth of the cells (based on expected doubling times during cell division). We have also quantified the amount of cell surface glycans that are metabolically labeled with Ac_4ManNAz. Our results indicate that 40% of natural sialic acids are replaced with SiaNAz upon Ac_4ManNAz treatment of Jurkat cells, whereas the substitution efficiency of CHO cell sialosides under similar conditions is about 30%.[13]

Supplementary Data

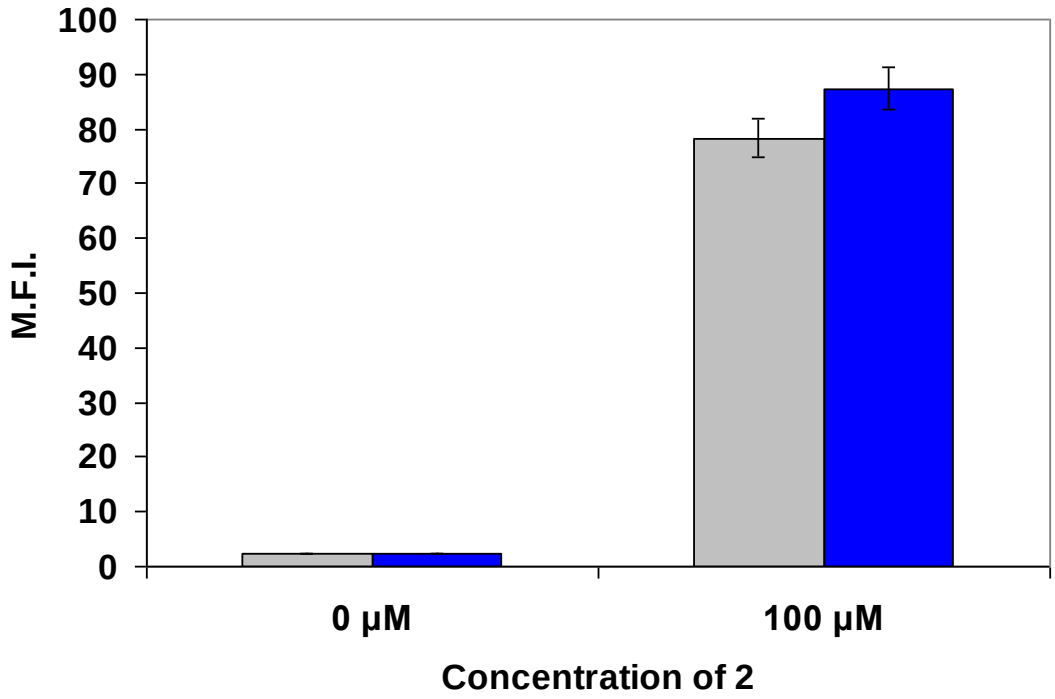


Figure S1. Flow cytometry analysis of Jurkat cells labeled with **2**. The cells were first incubated for 3 d in the presence (blue bars) or absence (gray bars) of $Ac_4ManNAz$ (25 μM) and then incubated with **2** for 1 h at rt at various concentrations. M.F.I. = mean fluorescence intensity (arbitrary units).

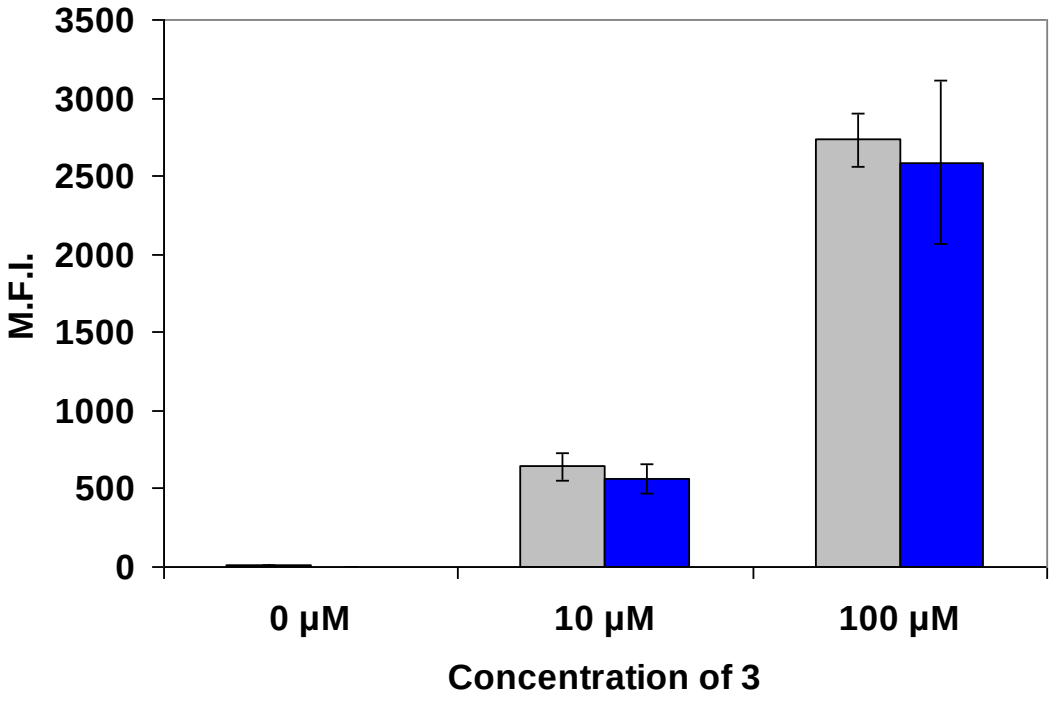


Figure S2. Flow cytometry analysis of Jurkat cells labeled with **3**. The cells were first incubated for 3 d in the presence (blue bars) or absence (gray bars) of Ac_4ManNAz (25 μM) and then incubated with **3** for 1 h at rt at various concentrations. M.F.I. = mean fluorescence intensity (arbitrary units).

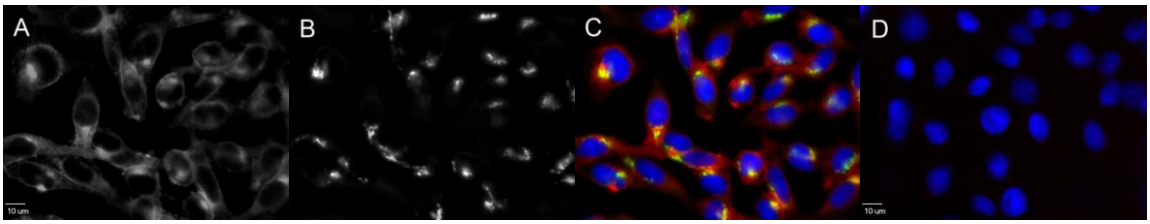


Figure S3. Fluorescence microscopy of CHO cells labeled with **1**. Cells incubated for 3 d in the presence (A–C) or absence (D) of Ac_4ManNAz (50 μM) were treated with **1** (200 μM) for 2 h at 37 °C. The cells were fixed and permeabilized with MeOH and then stained with a primary antibody specific for the Golgi apparatus (rabbit polyclonal anti-Giantin, Abcam®) and a secondary antibody (FITC-conjugated goat anti-rabbit, Invitrogen). The cells were stained with DAPI and imaged. (A) Cy5.5 channel. (B) FITC channel. (C) DAPI, FITC, and Cy5.5 channels. (D) DAPI and Cy5.5 channels. Green = FITC. Red = Cy5.5. Blue = DAPI. Scale bar = 10 μm.

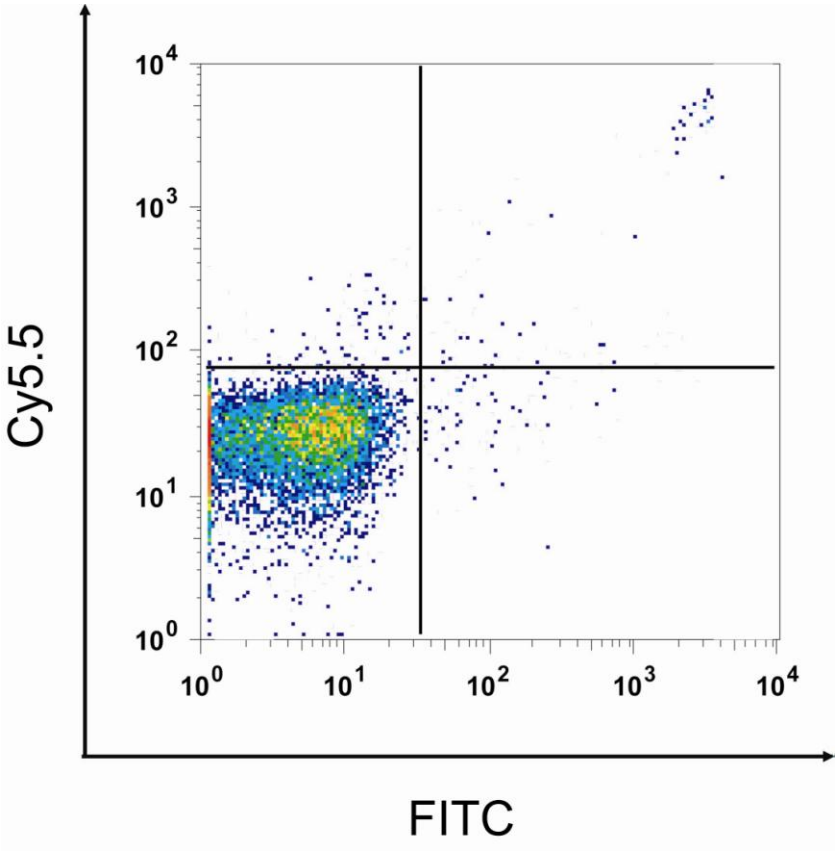
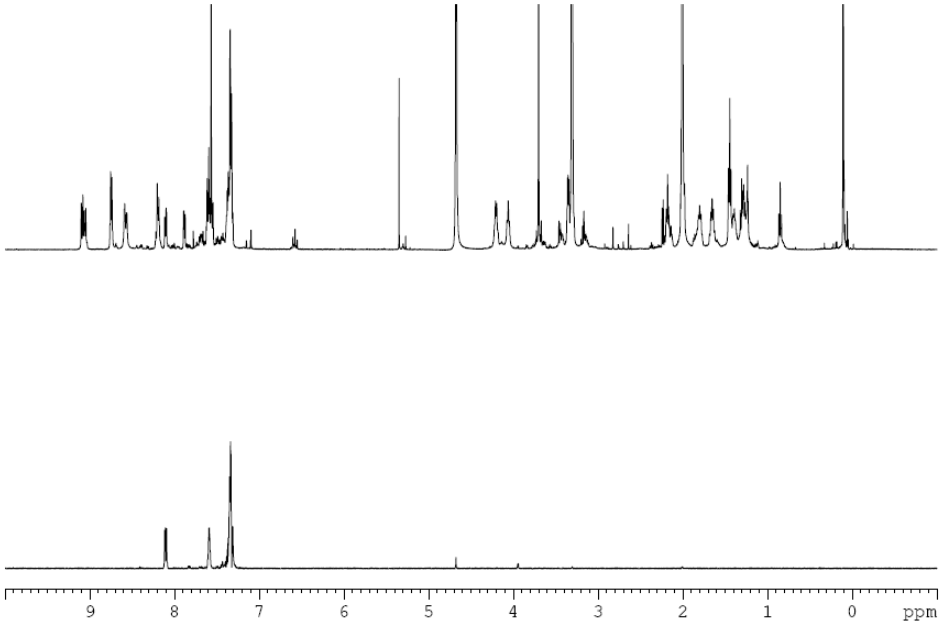


Figure S4. Labeling of live cells bearing two bioorthogonal chemical reporters. Jurkat cells were incubated with both Ac_4ManLev (50 μM) and Ac_4GalNAz (150 μM) for 2 d and then labeled with **1-ox** (100 μM) and FITC-avidin (5 μg/mL). The fluorescence of the sample was measured by flow cytometry. x-axis: FITC fluorescence; y-axis: Cy5.5 fluorescence.

A



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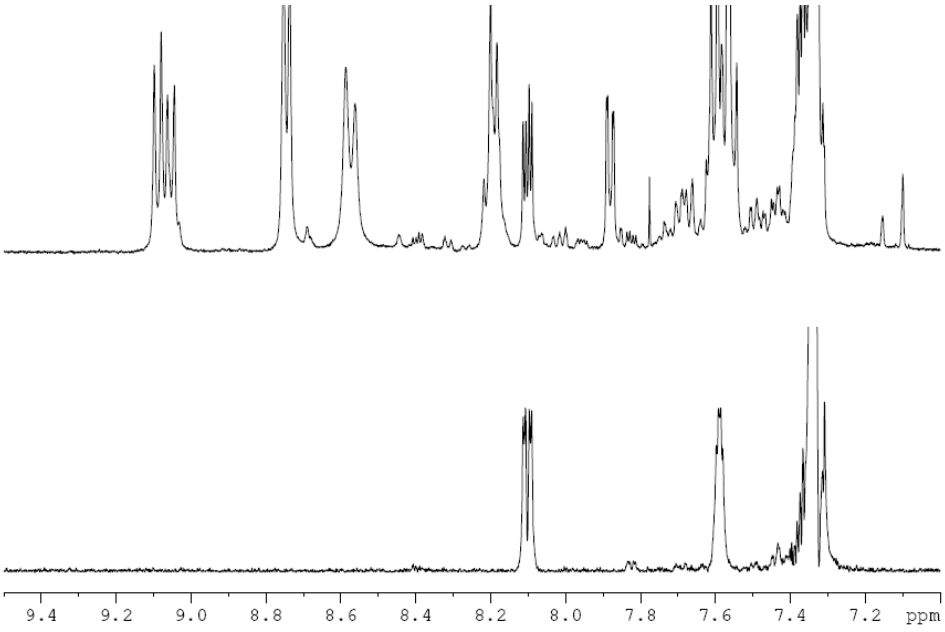


Figure S5. A ^{31}P-filtered ^{1}H NMR spectrum of compound **1** (1:1 CD_3OD:$CDCl_3$) was recorded by a 1D gs-HMQC experiment, without ^{31}P-decoupling during acquisition, using the Bruker pulse program 'hmqcgpnd1d.' The spectrum was acquired in 352 scans, with the delay optimized for a $^{3}J_{PH}$ coupling of 7.6 Hz. ^{31}P Chemical shifts are referenced to external 85% H_3PO_4 in D_2O. (A) ^{1}H NMR spectrum (top). ^{1}H signals that couple with ^{31}P (bottom). (B) Same spectra (expanded from 7.0 – 9.5 ppm)

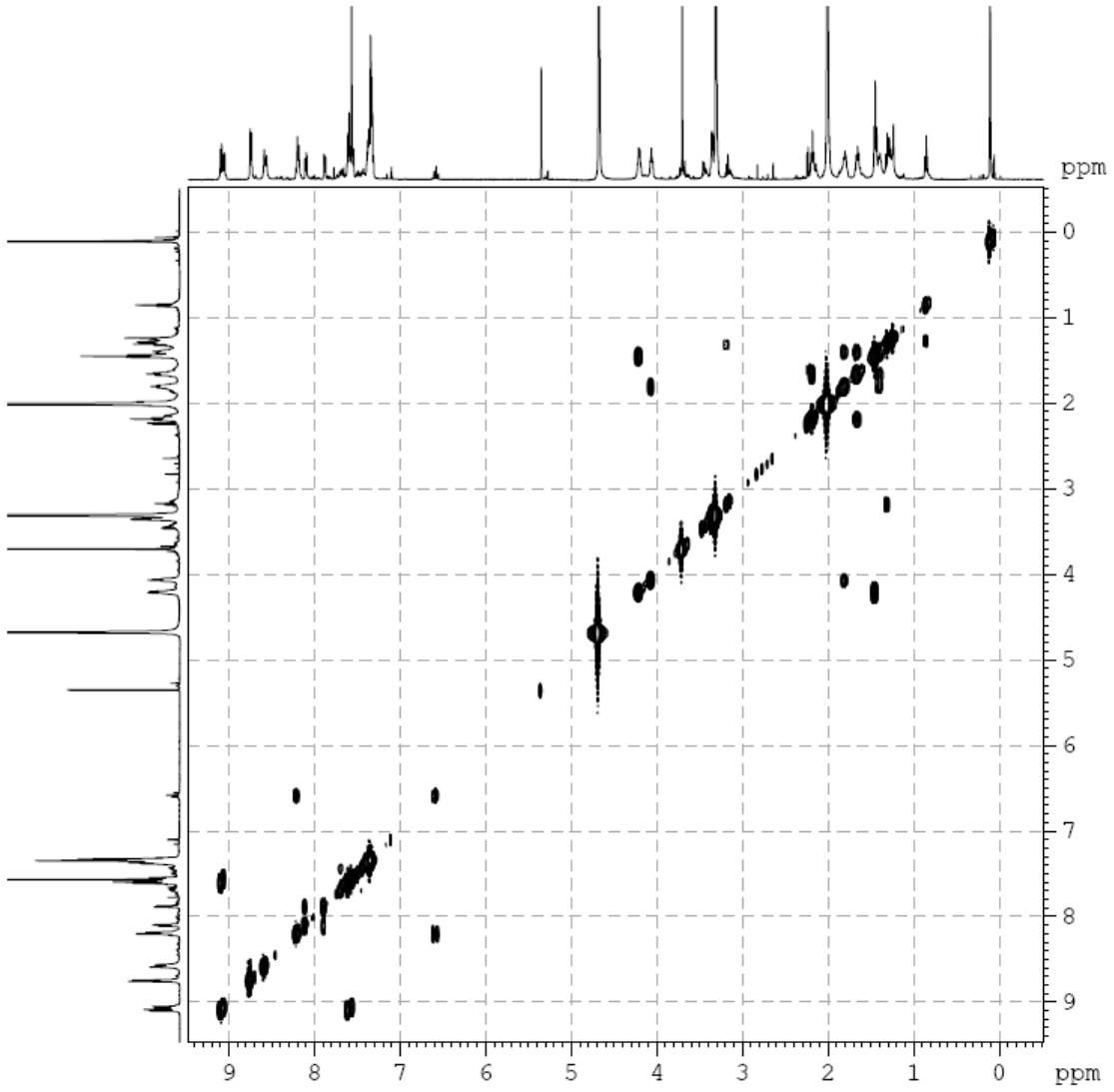
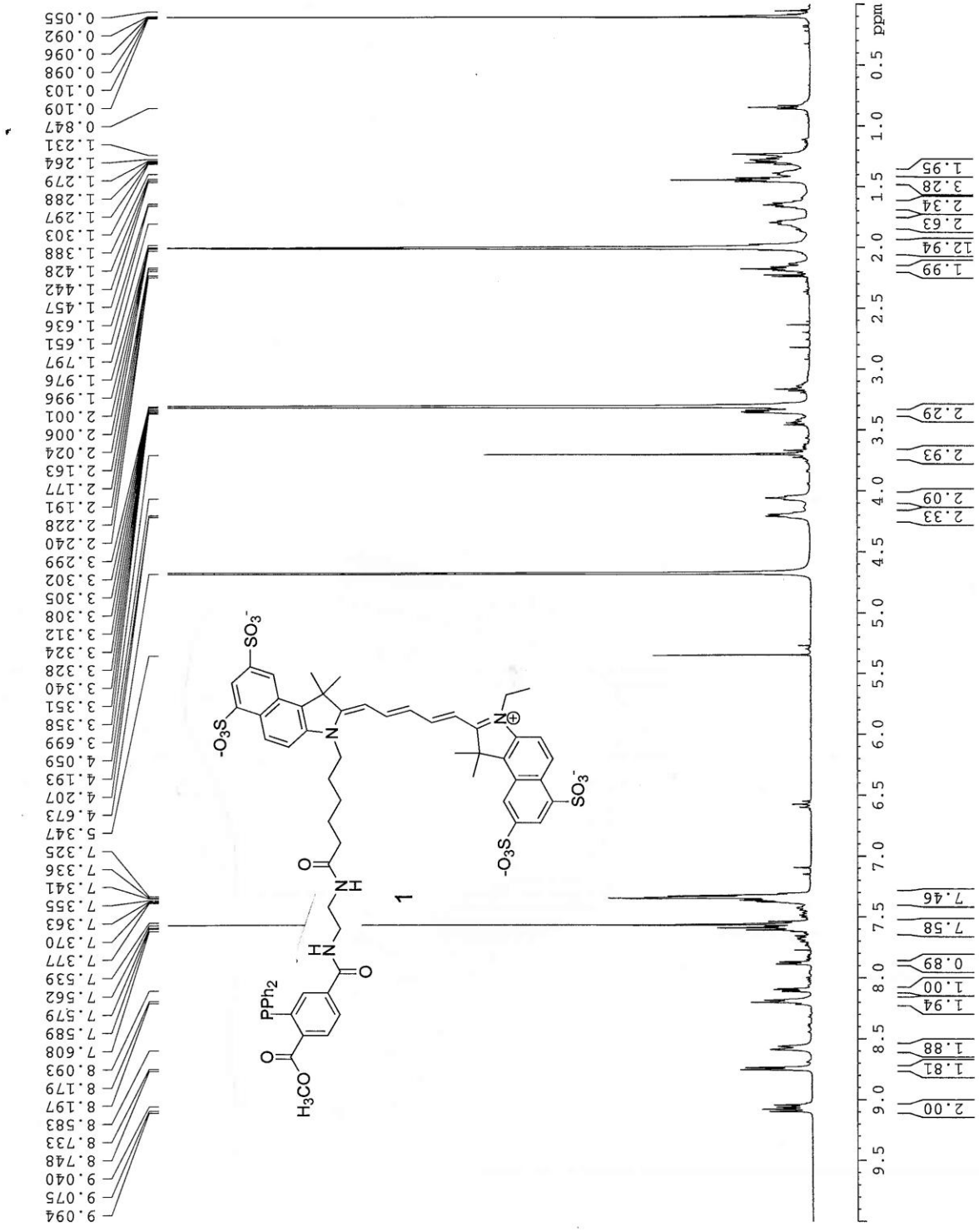


Figure S6. A 2D {^{1}H,^{1}H} gs-COSY (1:1 CD_3OD:$CDCl_3$) experiment of compound **1** was performed in magnitude mode, using the program 'cosygpqf' in the Bruker pulse program library; 128 experiments were conducted, each consisting of 32 scans. The data were processed with sine bell functions applied in both the t_2 and the t_1 dimensions, along with zero-filling in the t_1 dimension. ^{1}H chemical shifts are reported relative to TMS, but were actually measured by reference to internal CD_2HOD (a residual solvent peak from methanol) at δ 3.310 ppm, with accuracy of 0.005 ppm.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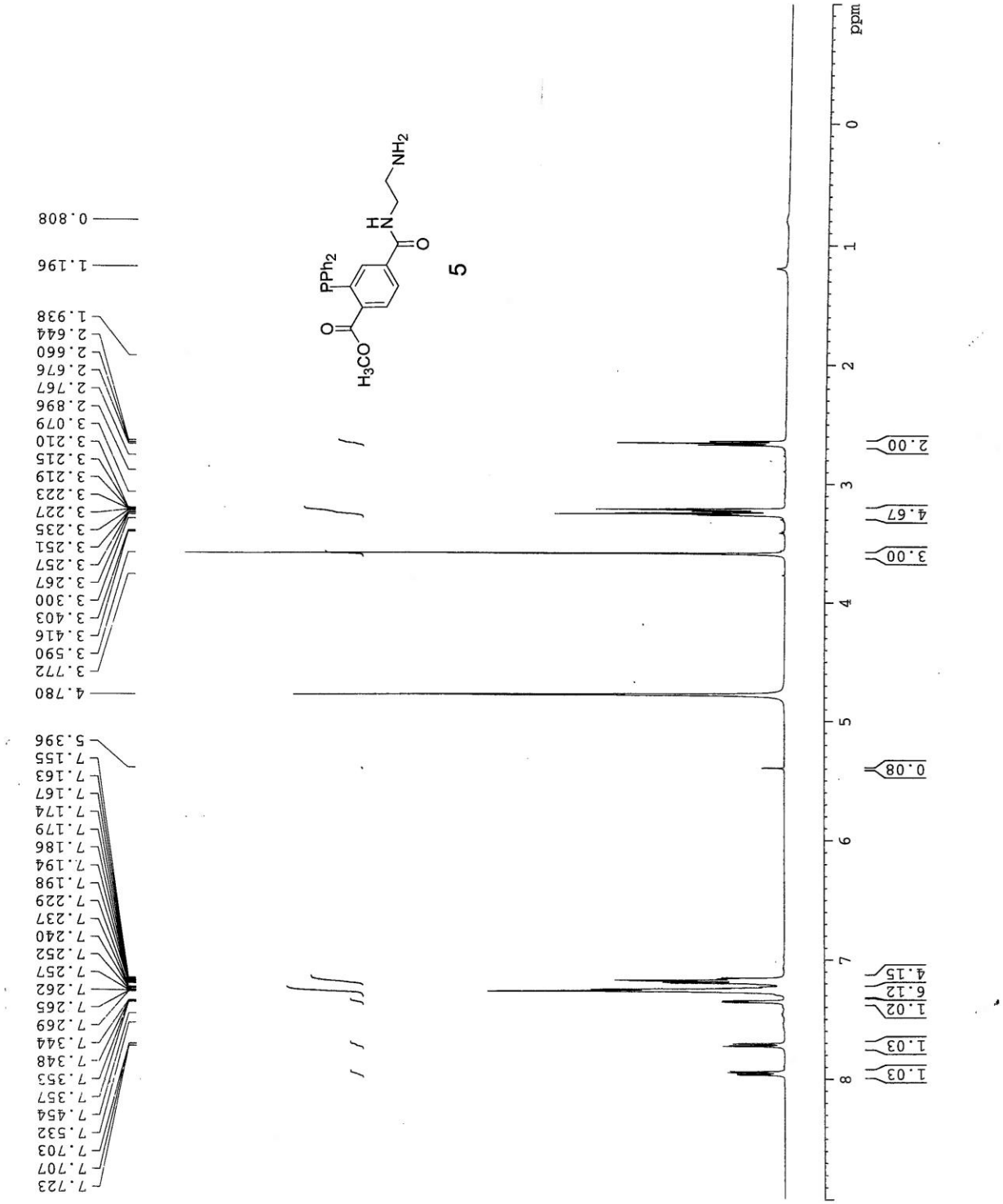
H3CO
Ph2P


5
PPh2
NH2
H3CO
ppm
0
1
2
3
4
5
6
7
8
2.00
4.67
3.00
0.08
1.03
1.03
1.02
6.12
4.15
0.808
1.196
1.938
2.644
2.660
2.676
2.767
2.896
3.079
3.210
3.215
3.219
3.223
3.227
3.235
3.251
3.257
3.267
3.300
3.403
3.416
3.590
3.772
4.780
5.396
7.155
7.163
7.167
7.174
7.179
7.186
7.194
7.198
7.229
7.237
7.240
7.252
7.257
7.262
7.265
7.269
7.344
7.348
7.353
7.357
7.454
7.532
7.703
7.707
7.723

7
HO
O
O
O
N
NH
7.857
7.843
7.676
7.653
7.622
7.610
7.475
7.361
7.356
7.288
7.283
7.265
7.260
4.925
3.836
3.617
3.331
3.296
3.292
3.288
3.138
3.03
1.99
1.01
2.03
2.00
4.10
4.02
ppm

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