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Supporting Information



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# A Magnetic Gram Stain for Bacterial Detection**

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## *Supporting Information*

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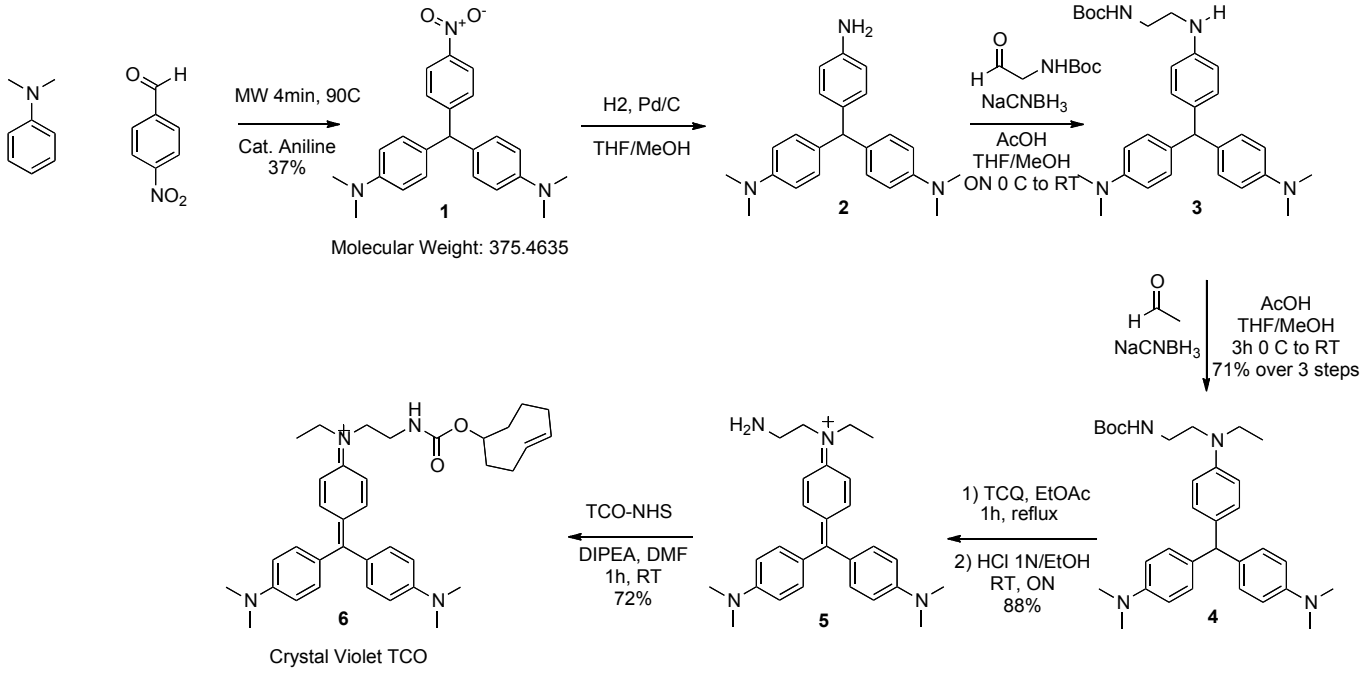
**General experimental procedures.** Unless otherwise noted, reactions were carried out under an atmosphere of nitrogen or argon in air-dried glassware with magnetic stirring. Air- and/or moisture-sensitive liquids were transferred *via* syringe. Organic solutions were concentrated by rotary evaporation at 25 - 60 °C at 15-30 torr. Analytical thin layer chromatography (TLC) was performed using plates cut from glass sheets (silica gel 60 F-254 from Silicycle). Visualization was achieved under a 254 or 365 nm UV light and by immersion in an ethanolic solution of cerium sulfate, followed by treatment with a heat gun. Column chromatography was carried out as "Flash Chromatography" using silica gel G-25 (40-63 μM).

**Materials.** All reagents were obtained from commercial sources and used without further purifications. Dry MeOH, THF and DMF were obtained from Aldrich. (*E*)-cyclooct-4-enyl 2,5-dioxopyrrolidin-1-yl carbonate and magnetofluorescent nanoparticles (MFNPs) were synthesized as described earlier. [1,2]

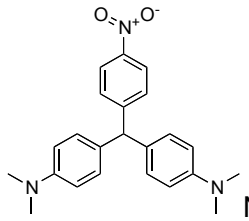
**Instrumentation.** $^{1}H$ and $^{13}C$ NMR spectra were recorded at 23°C on a Varian 400 MHz spectrometers. Recorded shifts are reported in parts per million (δ) and calibrated using residual undeuterated solvent. Data are represented as follows: Chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet, br = broad), coupling constant (*J*, Hz) and integration. LC-ESI-MS analysis and HPLC-purifications were performed on a Waters (Milford, MA) LC-MS system. For LC-ESI-MS analyses, a Waters XTerra® C18 5 μm column was used. For preparative runs, an Atlantis® Prep T3 OBDTM 5 μM column was used (eluents 0.1% TFA (v/ v) in water and MeCN; gradient: 0-1.5 min, 5-100% B; 1.5-2.0 min 100% B). High-resolution electrospray ionization (ESI) mass spectra were obtained on a Bruker Daltonics APEXIV 4.7 Tesla Fourier Transform mass spectrometer (FT-ICR-MS) in the Department of Chemistry Instrumentation Facility at the Massachusetts Institute of Technology. UV absorption measurement were taken on a Tecan Safire$^2$ microplate system (Männedorf, Switzerland). Data were analyzed using Prism 5 for Mac (GraphPad, La Jolla, CA). Transmission electron microscopy was performed using JEM 2011 (Jeol Ltd., Tokyo, Japan). Bright field imaging was performed using a upright Nikon Eclipse 50i Light microscope equipped with 100x objective and captured with a CCD-SPOT RT digital camera (Diagnostic Instruments, Inc.). Confocal imaging was done using a multichannel

upright laser-scanning confocal microscope (FV1000, Olympus) with a 60X water immersion objective lens. All μNMR measurements were performed using the portable NMR system recently developed for point-of-care operations.[12] The polarizing magnetic field was ~0.5 T. Transverse relaxation times were measured on 1-2 μL sample volumes, using Carr–Purcell–Meiboom–Gill pulse sequences with the following parameters: echo time, 3 ms; repetition time, 4 s; number of 180° pulses per scan, 900; number of scans, 7. All measurements were done in triplicate, and data are displayed as mean ± standard error of mean.

***Chemical synthesis***




4,4'-((4-nitrophenyl)methylene)bis(*N*,*N*-dimethylaniline) **1**



N,N'-dimethylaniline (922 μL, 7.28 mmol), 4-nitrobenzaldehyde (500 mg, 3.31 mmol) and aniline (50 mg, 386 mmol, 10 %w/w) were mixed thoroughly in a 10 mL open Pyrex tube containing a Teflon-coated stirring bar. The mixture was irradiated during 4min in a microwave oven at power 100 W at 90 °C. The reaction mixture was purified by silica gel flash chromatography column (hexane/EtOAc90/10) and further purified by recrystallization in hexane/EtOAc. Crystals were filtrated and washed with cold hexane giving **1** (585 mg, 47%) as golden solid.

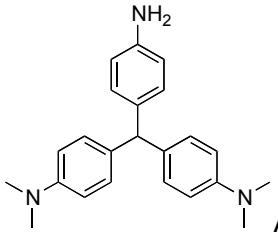
**$^{1}$H NMR (400 MHz, cdcl$_3$)** δ 8.12 (d, *J* = 8.6 Hz, 2H), 7.31 (d, *J* = 8.7 Hz, 2H), 6.96 (d, *J* = 8.6 Hz, 4H), 6.69 (d, *J* = 8.5 Hz, 4H), 5.46 (s, 1H), 2.94 (s, 12H).

**$^{13}$C NMR (101 MHz, cdcl$_3$)** δ 153.58, 149.34, 149.32, 146.31, 130.20, 129.97, 123.49, 112.70, 55.05,

40.75.

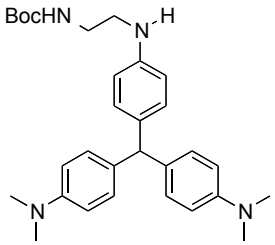
**HRMS** : $[M+H]^+$ m/z calcd 376.2020 for $C_{23}H_{26}N_3O_2$, found 376.2011

4,4'-((4-aminophenyl)methylene)bis(*N*,*N*-dimethylaniline) **2**



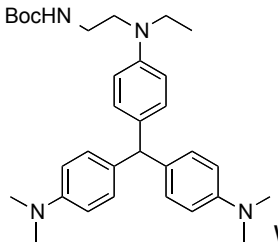
A two neck round bottom flask was charged with compound **1** (100 mg, 0.266 mmol). A nitrogen line was connected to the flask via a syringe needle inserted through one of the septa. A line connected to a second septum via a syringe needle led to an oil-filled bubbler. Nitrogen flow was started and the flask was purge with dry nitrogen. A degassed mixture of dry MeOH/THF 1/2 (3 mL) was added. Pd/C 10% (10 mg) was added and the mixture was further degassed by purging with nitrogen for 10 minutes. The nitrogen flow was stopped and hydrogen was introduce. The mixture was then stirred under hydrogen for 4 hour at room temperature. Complete and clean conversion was detected by HPLC-MS and TLC affording compound **2**.

*tert*-butyl (2-((4-(bis(4-(dimethylamino)phenyl)methyl)phenyl)amino)ethyl)carbamate **3**



The flask was then purge with nitrogen and cooled in an ice bath. N-Boc-2-aminoacetaldehyde (55 mg, 0.346 mmol), sodium cyanoborohydride (22 mg, 0.346 mmol) and acetic acid (20 µL, 0.346) were added to the reaction mixture. After stirring at room temperature overnight with nitrogen flow, an aliquot was examined by TLC and HPLC-MS showing that the starting material had been cleanly converted into the monoalkylated aniline **3**.

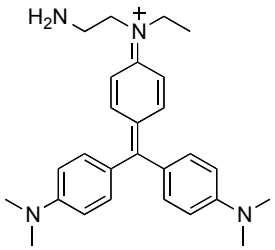
*tert*-butyl (2-((4-(bis(4-(dimethylamino)phenyl)methyl)phenyl)(ethyl)amino)ethyl)carbamate **4**



While stirring under nitrogen flow, the reaction mixture was cooled in an ice bath. Acetaldehyde (30 µL, 0.798 mmol), sodium cyanoborohydride (50 mg, 0.798 mmol) and acetic acid (45 µL, 0.798 mmol) were added and the reaction was stirred at room temperature for 5 hours. An aliquot was examined by TLC and HPLC-MS showing that the starting material had been cleanly converted into the dialkylated aniline **4**. The reaction mixture was then filtrated on celite and washed with MeOH and concentrated. The residue was diluted with water and extracted twice with DCM. Organics were combined and washed with water, dried over $MgSO_4$, filtered and evaporated in vacuo and the resulting residue purified by column chromatography (5 to 30% EtOAc/hexanes) affording **4** (97 mg, 71% over three steps) as a light purple powder.

**$^1$H NMR (400 MHz, cdcl$_3$)** δ 7.02 (d, *J* = 8.6 Hz, 4H), 6.99 (d, *J* = 8.7 Hz, 2H), 6.69 (d, *J* = 8.5 Hz, 4H), 6.65 (d, *J* = 8.6 Hz, 2H), 5.30 (s, 1H), 4.77 (s, 1H), 3.35 (dd, *J* = 14.1, 6.9 Hz, 2H), 3.31 – 3.22 (m, 4H), 2.92 (s, 12H), 1.47 (s, 9H), 1.14 (t, *J* = 7.0 Hz, 3H).
**$^{13}$C NMR (101 MHz, cdcl$_3$)** δ 156.12, 148.92, 146.09, 133.80, 133.32, 130.20, 129.95, 112.67, 112.25, 79.31, 54.11, 50.00, 45.51, 40.91, 38.60, 28.52, 12.32.
**HRMS** : $[M+H]^+$ m/z calcd 517.3537 for $C_{32}H_{45}N_4O_2$, found 517.3530

2-amino-*N*-(4-(bis(4-(dimethylamino)phenyl)methylene)cyclohexa-2,5-dien-1-ylidene)-*N*-ethylethanaminium chloride **5**

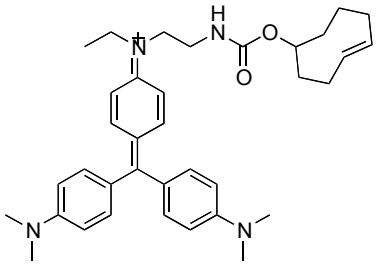


Compound **4** (10 mg, 0.019 mmol) was dissolved EtOAc (3 mL) and tetrachloroquinone (7.1 mg, 0.029 mmol) was added. The solution was stirred at 78 ºC for 1 hour causing the formation of an intense blue indicating the formation of the cationic dye. A 1N HCl (2 mL) solution was then added and the mixture was stirred at room temperature overnight. The reaction mixture was diluted with water and washed four times with EtOAc. The aqueous layer was evaporated in vacuo and the resulting residue purified by column chromatography on neutral alumina (10 to 30%

MeOH/DCM) affording **5** (12 mg, 88%) as an intense green violet powder.

**$^1$H NMR (400 MHz, cd$_3$od)** δ 7.42 – 7.35 (m, 6H), 7.07 (d, *J* = 8.3 Hz, 2H), 7.00 (d, *J* = 8.5 Hz, 4H), 3.87 (t, *J* = 6.3 Hz, 2H), 3.68 (dd, *J* = 12.6, 5.9 Hz, 2H), 3.27 (s, 12H), 3.25 – 3.21 (m, 2H), 1.29 (t, *J* = 6.2 Hz, 3H).
**$^{13}$C NMR (101 MHz, cd$_3$od)** δ 179.85 (s), 157.54 (s), 154.87 (s), 141.19 (s), 140.76 (s), 128.73 (s), 128.08 (s), 113.93 (s), 113.67 (s), 48.43 (s), 46.88 (s), 40.85 (s), 38.09 (s), 12.44 (s).
**HRMS** : $[M]^+$ m/z calcd 415.2856 for $C_{27}H_{35}N_4$, found 415.2857

(*E*)-*N*-(4-(bis(4-(dimethylamino)phenyl)methylene)cyclohexa-2,5-dien-1-ylidene)-2-(((cyclooct-4-en-1-yloxy)carbonyl)amino)-*N*-ethylethanaminium chloride **6**



Compound **5** (1.75 mg, 3.9 µmol), TCO-NHS (1.55 mg, 5.8 µmol) and DIPEA (2 µL, 11.7 µmol) were dissolved in 55 µL DMF and stirred overnight at room temperature. The mixture was purified by HPLC affording **6** ( 1.6 mg, 72%) as a green violet powder.

**HRMS** : $[M]^+$ m/z calcd 567.3694 for $C_{32}H_{45}N_4O_2$, found 567.3685

***Methods***

**Preparation of bioorthogonal nanoparticles** Magnetofluorescent nanoparticles (MFNPs) were synthesized as reported.[2] The nanoparticles had a shell of crosslinked dextran and core of $(Fe_2O_3)_m(Fe_3O_4)_n$ (diameter 3 nm). The hydrodynamic diameter had an average value of 21 nm, with each particle having 22 free amine groups and 8.4 molecules of fluorescein molecules conjugated on the surface. The $r_1$ and $r_2$ relaxivity values were 23 $s^{-1}$ $mM^{-1}$ [Fe] and 51 $s^{-1}$ $mM^{-1}$ [Fe] as measured, respectively. Amine-reactive tetrazine (Tz-NHS) was prepared as described previously.[3] Tetrazine-conjugated magnetofluorescent nanoparticles (MFNP-Tz) were prepared by adding MFNPs in phosphate buffered saline solution (PBS) containing 10 mM sodium bicarbonate, with 500 times molar excess of Tz-NHS dissolved in dimethylsulfoxide (DMSO, 1:9 volume), and allowed to react at room temperature for 4 hours. Unreacted Tz-NHS was removed using Sephadex G-50 (GE Healthcare). The amount of conjugated tetrazine was quantified by reacting the MFNPs with succinimidyl 3-(2-

pyridyldithio)propionate (SPDP), followed by treatment with dithiothreitol (DTT), and measuring the absorbance at 343 nm for traces of the cleaved pyridine-2-thione product. For MFNPs, which have a core of ~2 nm and a hydrodynamic diameter ($D_h$) of ~21 nm, 20 out of 22 amine groups per particle were conjugated with tetrazine molecules. Tetrazine-conjugated gold nanoparticles (GNP-Tz) were prepared similarly by reacting amine-functionalized gold nanoparticles (NANOCS, 10 nm core) with 1000 times molar excess of Tz-NHS in 1:9 DMSO/0.2X PBS solution at room temperature for 4 hours, and washed three times with deionized water using Amicon (Millipore, MWCO 100,000). For GNPs having a core size of 10 nm, 2520 among a total of 3120 amine groups per particle were converted to tetrazine.

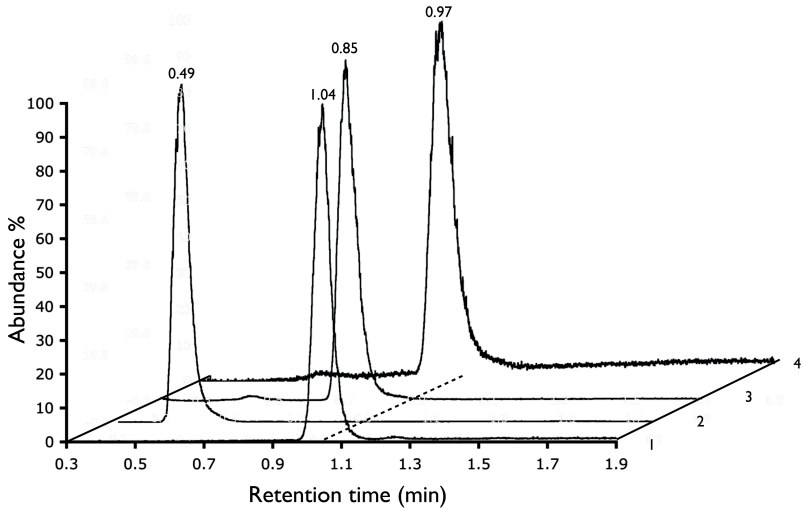
**Bacteria culture** Bacterial strains *Staphylococcus aureus* (*S. aureus*; #25923), *Staphylococcus epidermidis* (S. epidermidis; #29886), *Bacillus subtilis* (*B. subtilis*; #82), *Streptococcus pneumoniae* (*S. pneumoniae*; #6318), *Enterococcus faecalis* (*E. faecalis*; #29212), *Escherichia coli* (*E. coli*; #25922), *Pseudomonas aeruginosa* (*P. aeruginosa*; #142), *Klebsiella pneumoniae* (*K. pneumoniae*; #43816), *Enterobacter aerogenes* (*E. aerogenes*; #13048), and *Citrobacter freundii* (*C. freundii*; #6879) were purchased from ATCC (Manassas, VA). For selective culture, *S. aureus* and *S. epidermidis* were plated in mannitol salt agar (BD Biosciences, Sparks, MD) and colonies were cultured in Staphylococcus broth (BD Biosciences) for growth overnight. *S. pneumoniae* was plated onto selective streptococcus agar and the colony was seeded into Tryptic Soy Broth containing 5% defibrinated sheep blood (Hemostat Laboratories, Dixon, CA) for growth. *P. aeruginosa* was plated on Pseudomonas isolation agar, and *E. faecalis*, *E. coli*, and *K. pneumoniae* were plated onto standard agar plates. For growth, *P. aeruginosa* and *K. pneumoniae* were cultured in Tryptic Soy Broth, *E. faecalis* was cultured in Tryptic Soy Broth containing 5% defibrinated sheep blood, and *E. coli* was cultured in Luria-Bertani (LB) media (BD Biosciences). Bacterial cell numbers were determined by plating onto standard agar plates and counting the number of colony forming units (CFU), and by comparison to optical density measurements, conversion factors were obtained.

**Gram stain** Bacterial cells were smeared onto microscopic slides, and stained with standard crystal violet (20 mM) or CV-TCO (1 mM) in 20% ethanol solution containing 8 mg/ml ammonium oxalate for 3 min. After washing with deionized water, slides were treated with Gram Iodine (BD Biosciences), decolorized with 95% ethanol solution, and counterstained with Gram safranin (BD Biosciences). Slides were observed using a bright field light microscope.

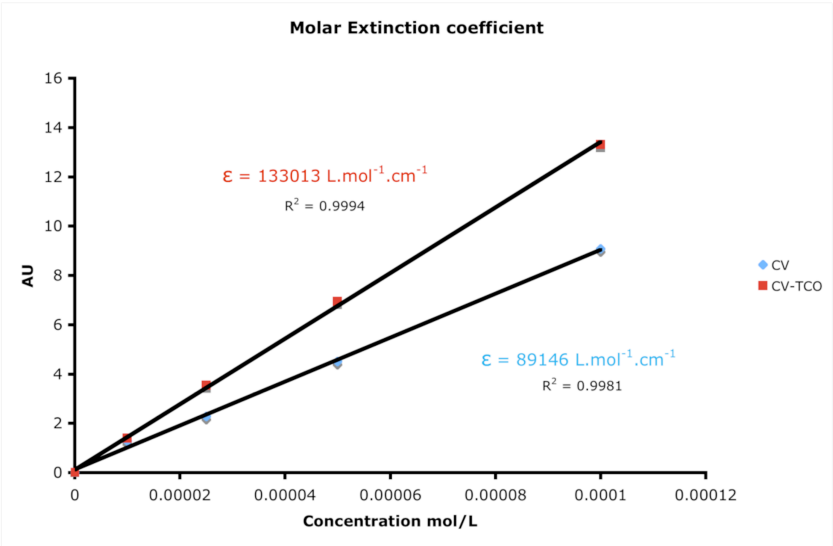
**Bacterial labeling with nanoparticles** Bacterial cells in media were first washed with phosphate buffered saline solution (PBS). For μNMR measurements, absorbance measurements, confocal microscopy, and transmission electron microscopy (TEM), the bacteria ($10^8$ in 100 μl) were stained with 200 μM CV-TCO in 20 % ethanol solution containing 8 mg/ml ammonium oxalate for 5 min at room temperature, and washed with PBS. Then the bacteria were treated with Gram Iodine solution for 1 min

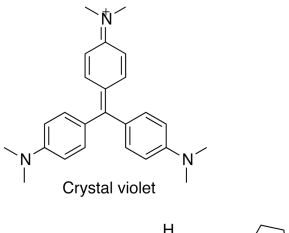
and decolorized with 95% ethanol solution. For μNMR and confocal microscopy, bacteria were incubated with 50 μg/ml MFNP-Tz in PBS for 20 minutes at room temperature, and washed twice with PBS. For confocal microscopy, labeled bacteria were mounted on microscopic slides with Vectashield containing propium iodide (Vector Laboratories), and imaged. For TEM, the stained and decolorized bacteria were incubated with 50 μg/ml GNP-Tz in PBS for 1 h, washed with PBS, and dehydrated with graded ethanol series before applying onto a carbon grid (Ted Pella).

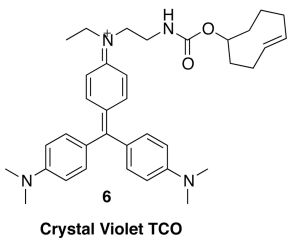
***Supplemental figures***



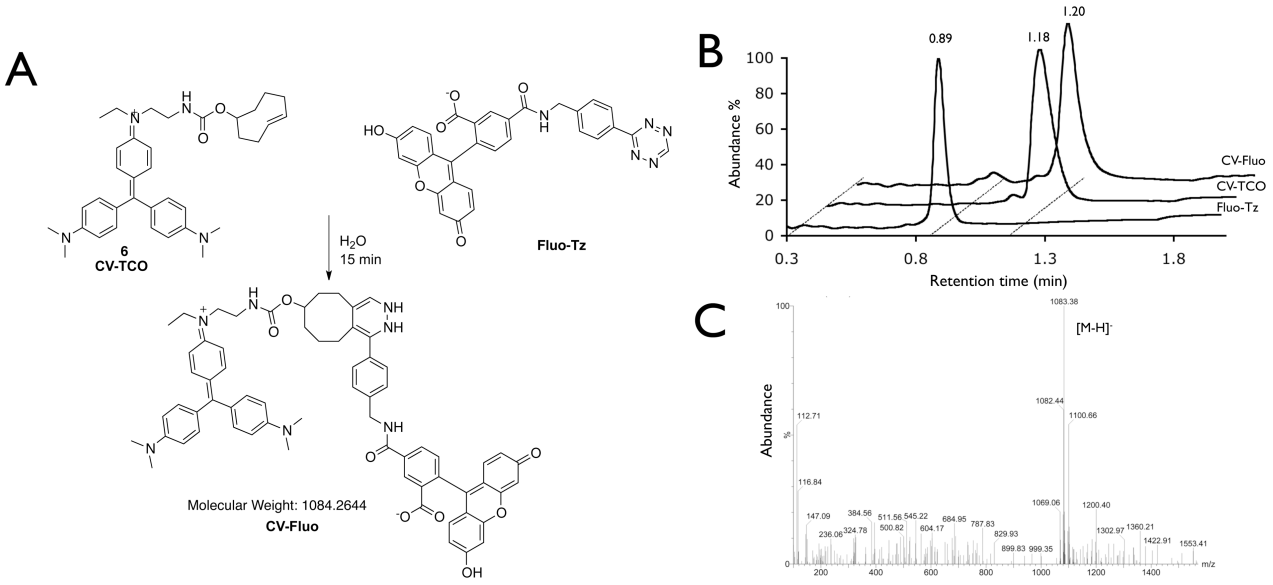

**Supplemental Figure 1**: UV HPLC traces of the multisteps one pot reaction showing purity and quantitative transformation of compound **1** to **4**.



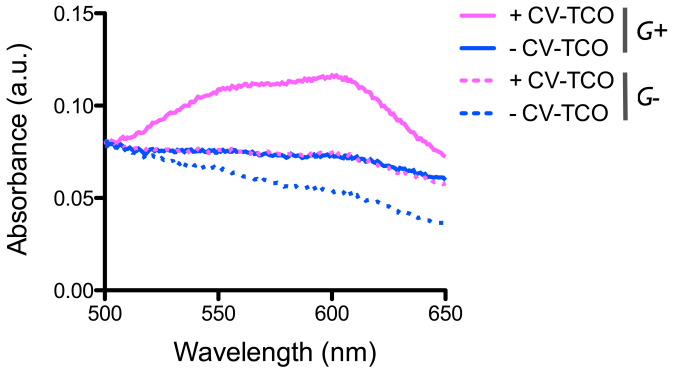



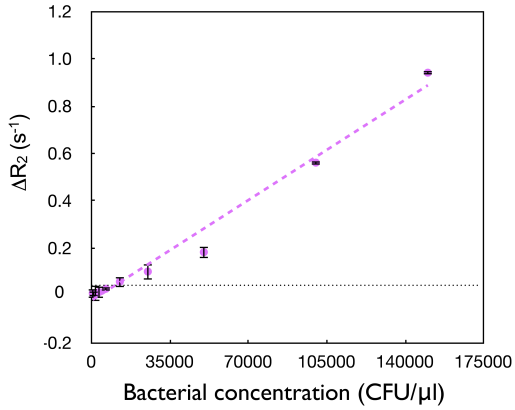

**Supplemental figure 2**: Molar extinction coefficient of crystal violet and crystal violet-TCO



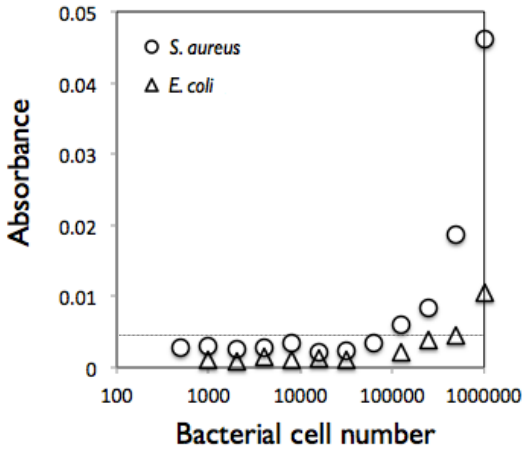

**Supplemental figure 3**: A) Bioorthogonal reaction between CV-TCO and Fluo-Tz (not all isomers shown). The two compounds were mixed at 0.25 mM, stirring for two minutes, and analyzed by high performance liquid chromatography–mass spectrometry (HPLC-MS). B) UV HPLC traces of the starting compounds Fluo-Tz and CV-TCO and clicked compound CV-Fluo (crude reaction mixture in DMSO). C) MS spectrum of the desired compound CV-Fluo.



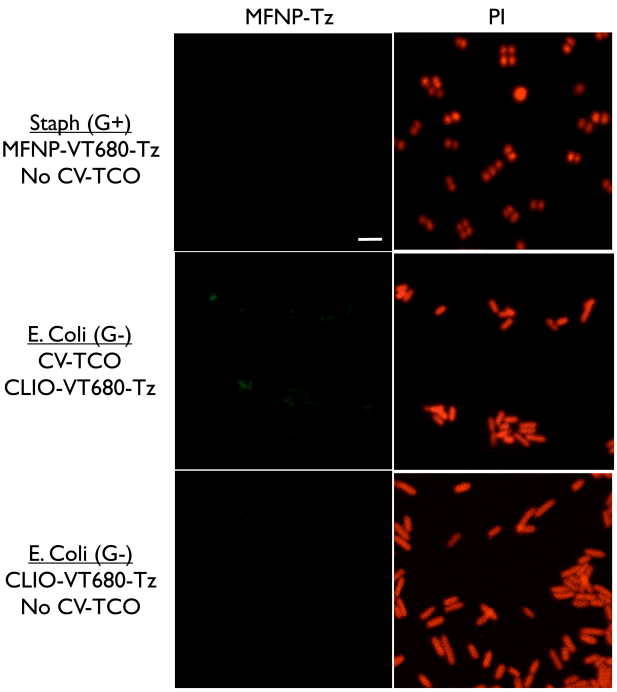

**Supplemental figure 4**: UV absorbance spectra of Gram-positive (*S. aureus*, G+) and Gram-negative (*E. coli*, G-) stained with (+CV-TCO) or without (-CV-TCO) crystal Violet-TCO.



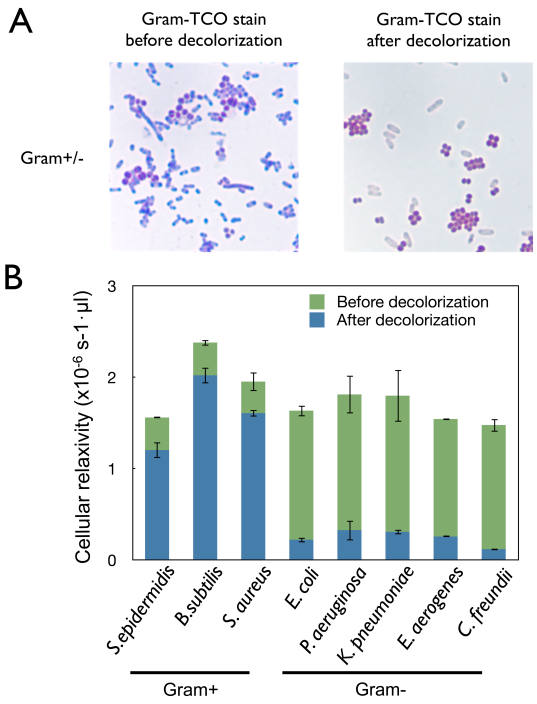

**Supplemental figure 5**: Sensitivity of labeling. $\Delta R_2$ values were calculated by subtracting values of G+ bacteria (*S. aureus*) nonspecifically bound with MFNP-Tz from ones that were specifically targeted (treated with CV-TCO and MFNP-Tz). Dotted line shows threshold of detection. Limit of detection was ~4000 CFU.




**Supplemental figure 6:** Detection sensitivity by absorbance measurements. G+ bacteria (S. aureus) and G- bacteria (E. coli) were labeled with CV-TCO and absorbance at 595 nm was measured. The observed detection limit was ~$10^5$ CFU.

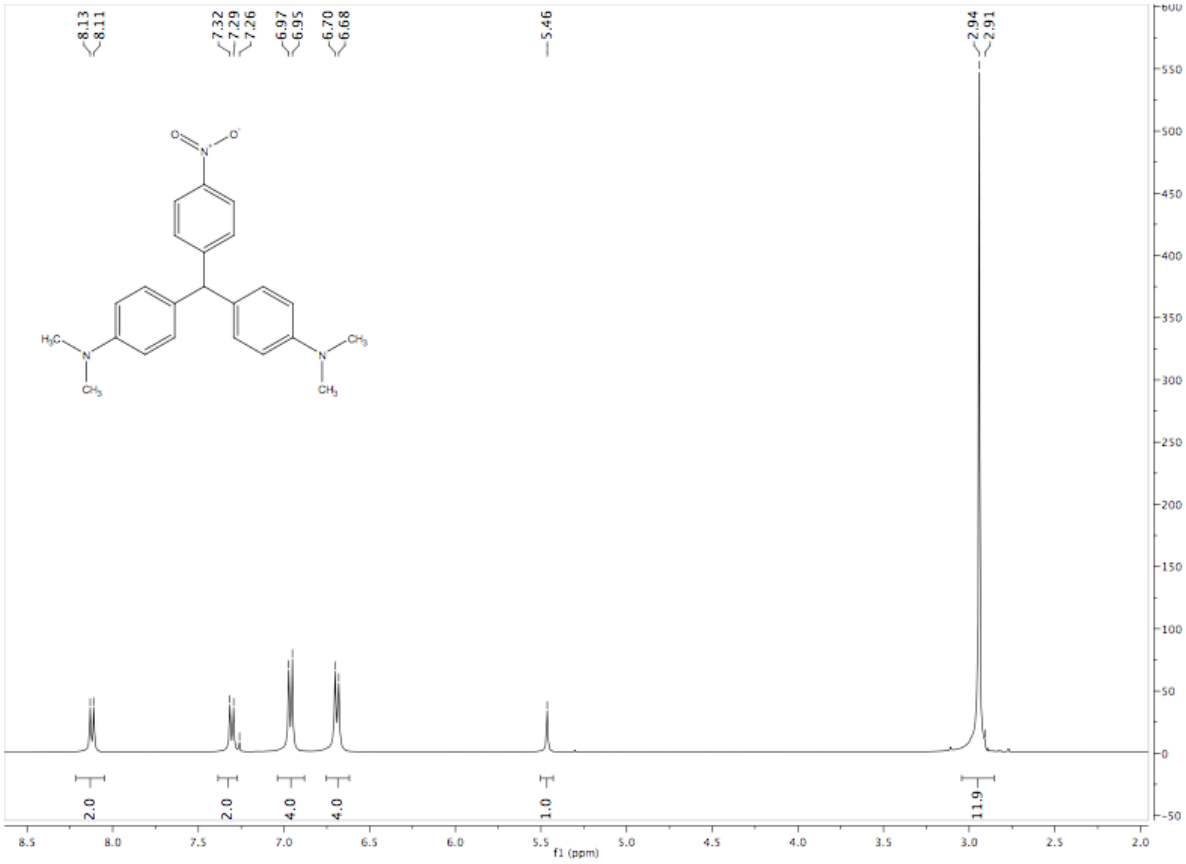


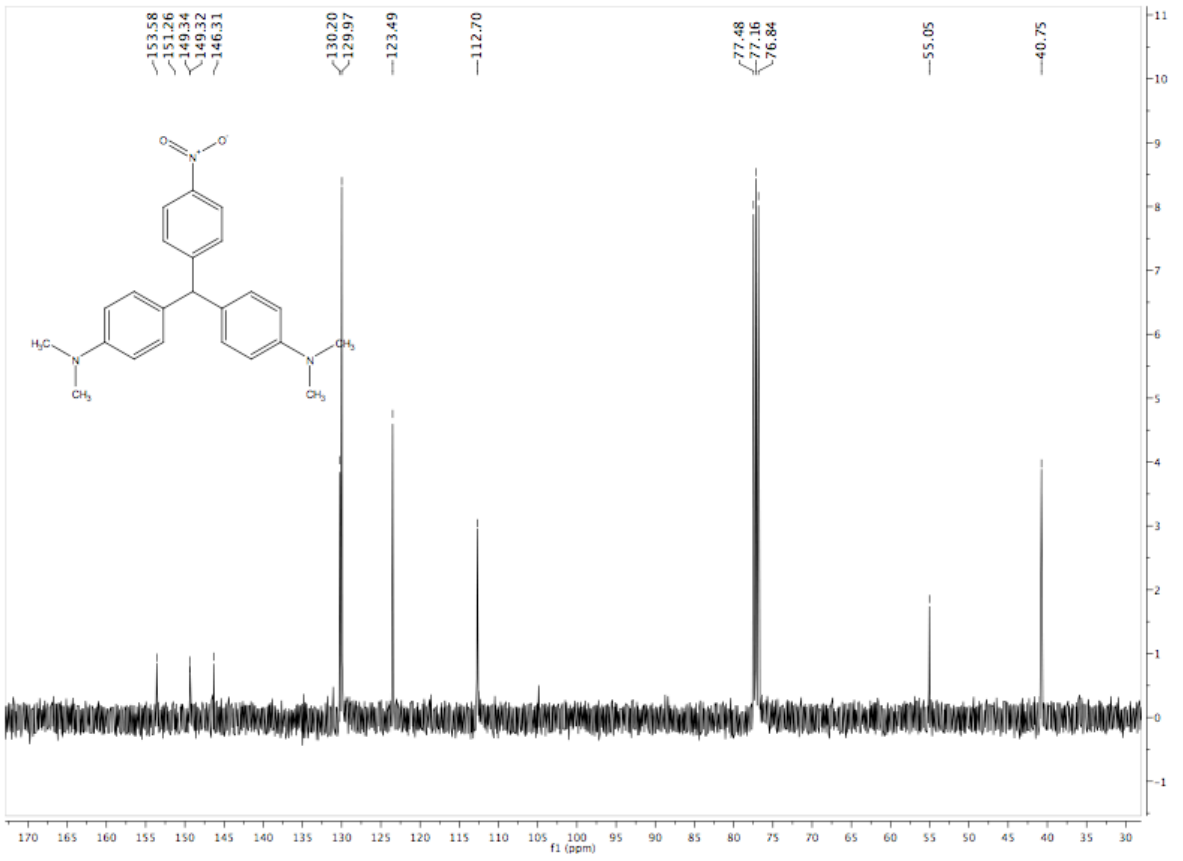

**Supplemental figure 7**: Confocal microscopy for control experiments: *S. aureus* (Gram-positive) labeled with MFNP-Tz alone (top), *E. coli* (Gram-negative) stained with CV-TCO and labeled with MNFP-Tz (middle) *E. coli* labeled with MFNP-Tz alone (bottom) (propidium iodide for nuclear staining; red (left), MNFP-Tz; green (right)) (Scale bar = 5 μm).



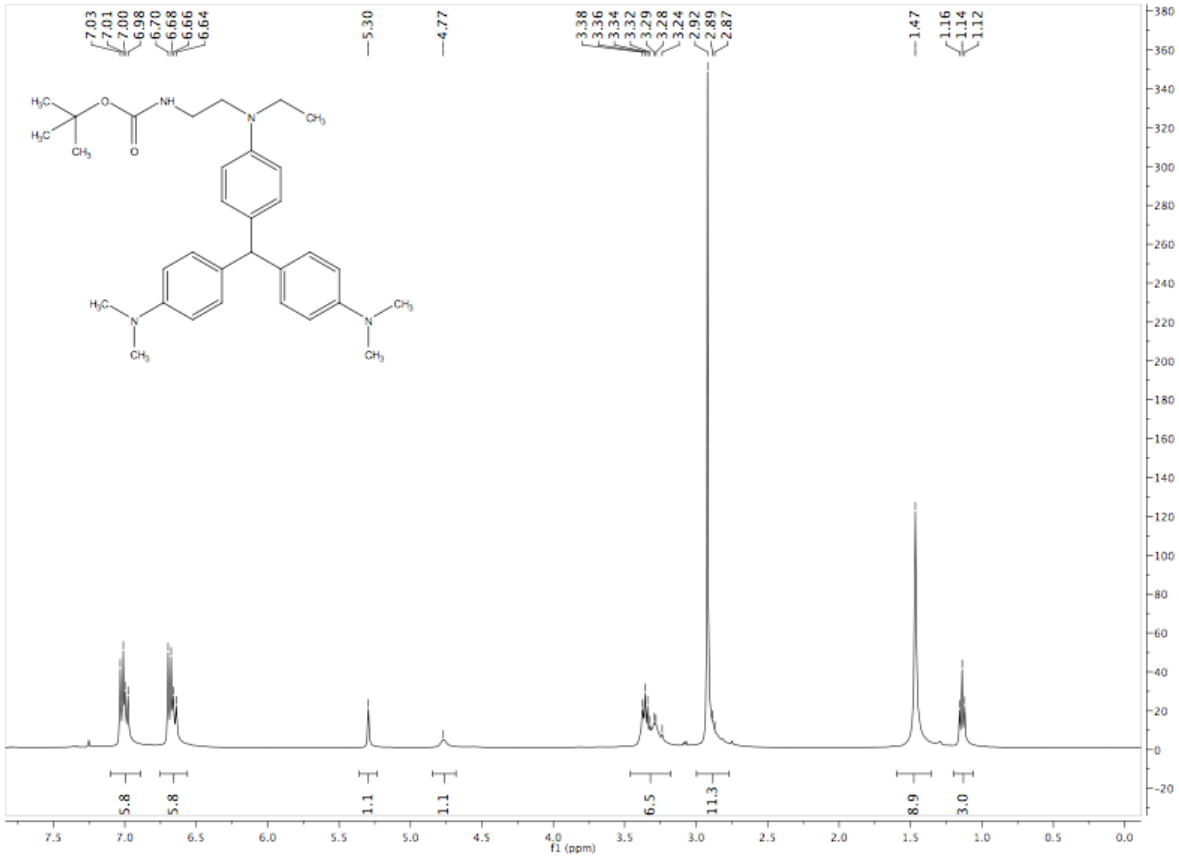

**Supplemental figure 8**: A) Bright field images of a mixture of *S. aureus* (Gram-positive cocci) and *E. coli* (Gram-negative bacilli) after Gram stain using crystal violet-TCO, before (left) and after (right) decolorization. B) μNMR detection of different species of Gram-positive and Gram-negative bacteria before (green + blue bar) and after decolorization (blue bar).

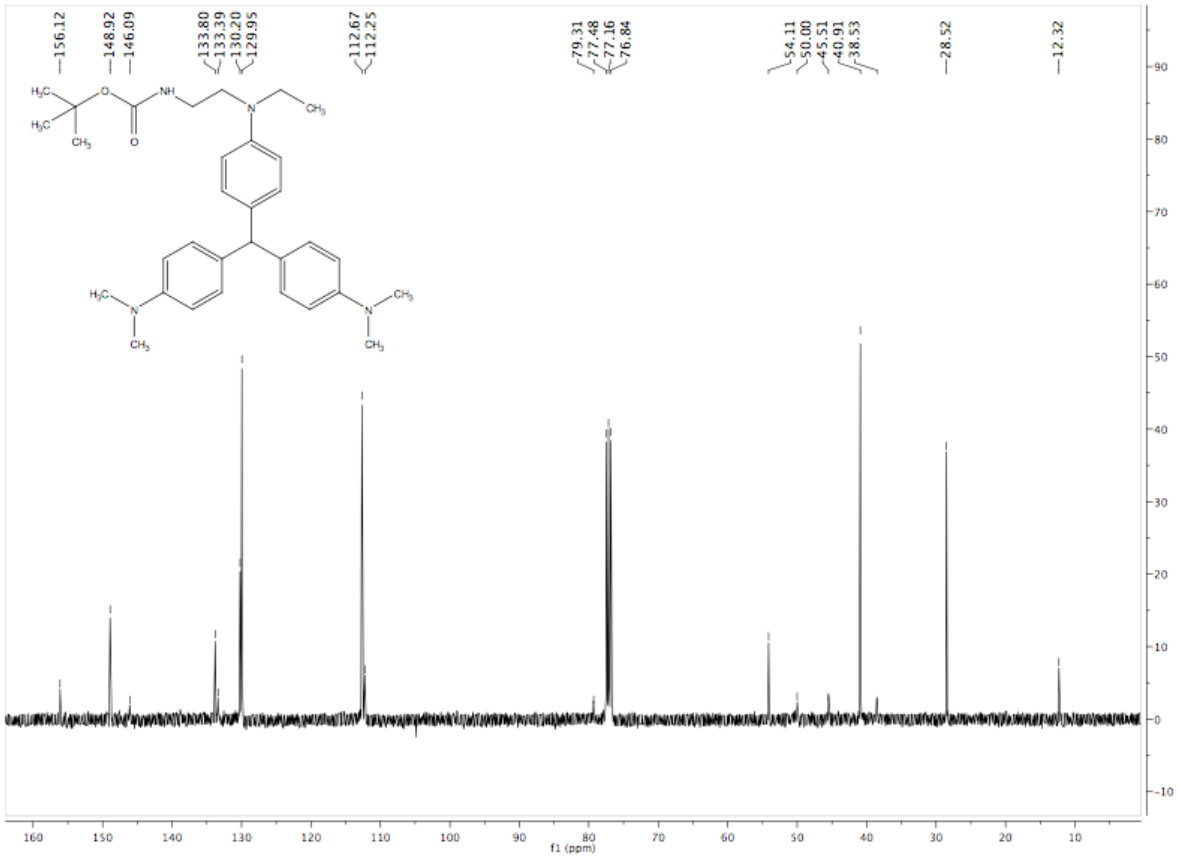
## Spectra $^{1}H$ and $^{13}C$ of compound 1



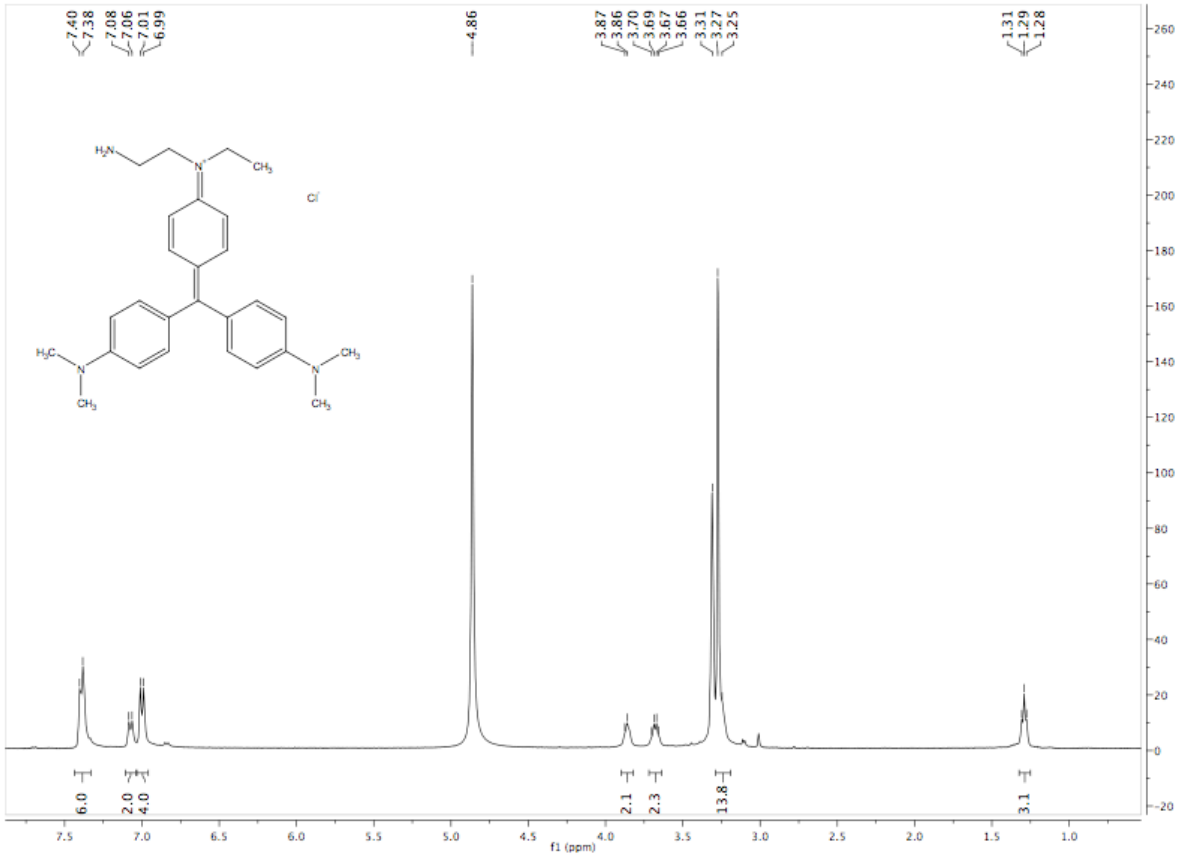



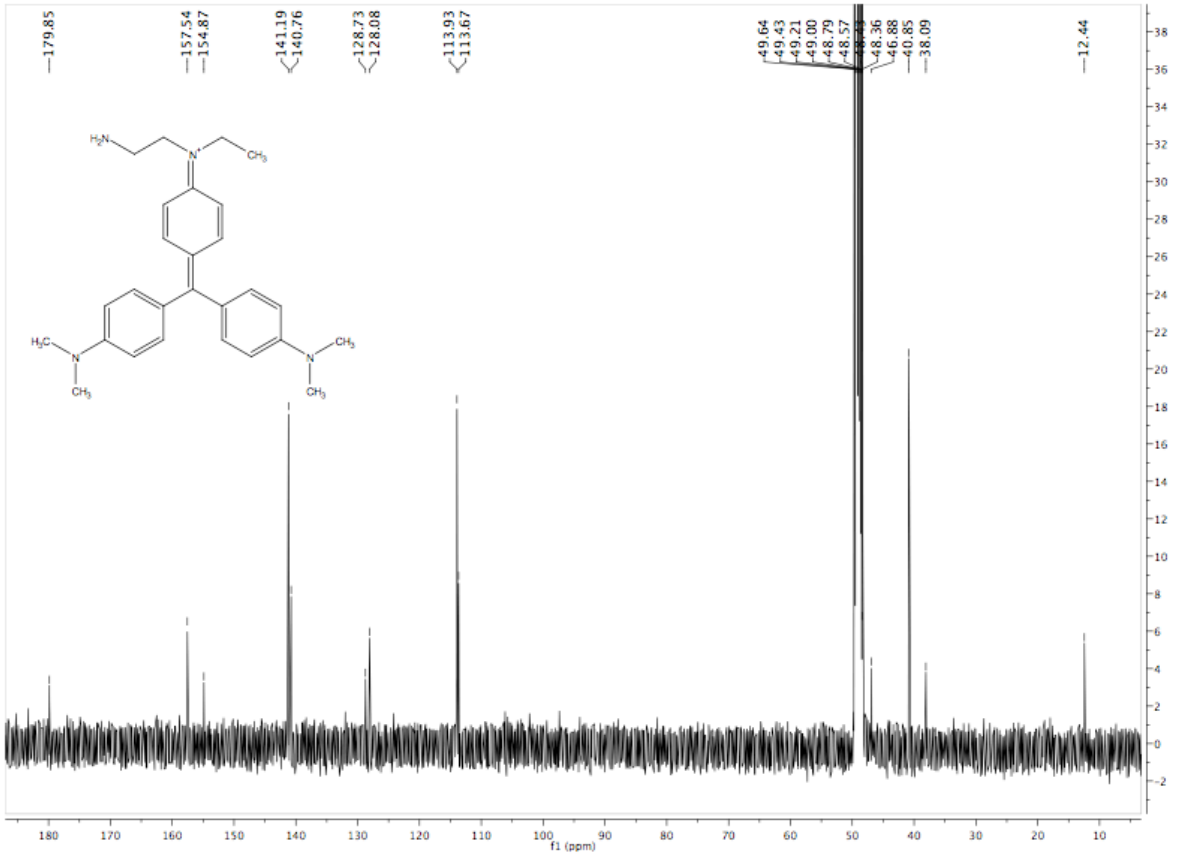
## Spectra $^{1}H$ and $^{13}C$ of compound 4



## Spectra $^{1}H$ and $^{13}C$ of compound 5



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2. Josephson, L.; Tung, C.; Moore, A.; Weissleder, R. High-Efficiency Intracellular Magnetic Labeling with Novel Superparamagnetic-Tat Peptide Conjugates. Bioconjugate Chem. **1999**, *10*, 186–191.

3. Haun et al., 2010, Nat Nanotechnol, 5, 660-66