Electronic Supplementary Information (ESI) for:

Microwave-assisted slipping synthesis of fluorescent squaraine rotaxane probe for bacterial imaging

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Content :

1. Synthesis

General Information

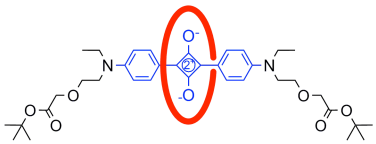
Unless otherwise stated, all starting materials and reagents were purchased from commercial suppliers and used without further purification. ^{1}H and ^{13}C NMR spectra were recorded by using Varian Unity Plus spectrometers. Matrix-assisted laser-desorption/ionization time-of-flight (MALDI-TOF) mass spectra were obtained using a Bruker AutoFlex III equipped with a Nd-YAG Laser. Electrospray ionization time-of-flight (ESI-TOF) mass spectra were obtained using a Bruker micrOTOF II. Mass spectra data was analyzed using Bruker Compass DataAnalysis 4.0. Microwave reaction employed a Synthos 3000 reactor from Anton Paar.

Microwave reaction setting

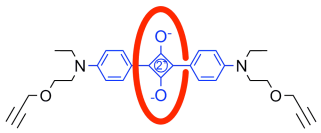
A mixture of squaraine dye and macrocycle is dissolved in dry chloroform (high concentration) and then added to a screw-able Wheaton^{R} glass vial (with a PTFE seal in PEEK screw cap). The vial is then placed into a silicon carbide plate inside a Synthos 3000 microwave instrument. The program is set to the following parameters: rotor type = "4 x 48 - 2", power = 1000 W, ramp time = 5 minutes, hold time = 5 minutes, and IR = 160 ^{o}C.

Methods

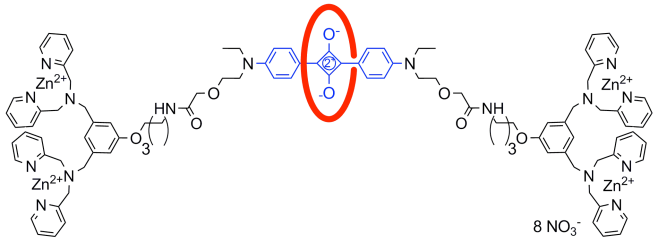
Comounds **2**,[S1] **4**,[S2] **5**,[S1] **6**,[S3] **7**[S4] and **1⊃3**[S2] are previously reported.



2⊃4. A mixture of **4** (0.089 g, 0.14 mmol) and macrocycle **2** (0.10 g, 0.14 mmol) was placed in a vial and dissolved in dry chloroform (1.0 mL). The reaction was placed inside the microwave instrument and operated at 160 °C for 5 minutes. After cooling, the chloroform was evaporated under reduced pressure to quantitatively afford **2⊃4** as a green solid and no further purification was required: δ_H (600 MHz; $CDCl_3$) 9.97 (t, J = 4.2 Hz, 4H), 8.67 (d, J = 7.8 Hz, 4H), 8.23 (t, J = 7.8 Hz, 2H), 7.72 (dd, J = 6.6, 3.0 Hz, 8H), 7.25 (d, J = 9.0 Hz, 4H), 6.67 (dd, J = 6.6, 3.0 Hz, 8H), 5.97 (d, J = 9.0 Hz, 4H), 5.32 (d, J = 4.2 Hz, 8H), 4.06 (s, 4H), 3.77 (t, J = 5.4 Hz, 4H), 3.66 (t, J = 5.4 Hz, 4H), 3.57 (q, J = 7.2 Hz, 4H), 1.50 (s, 18H), 1.28 (t, J = 7.2 Hz, 6H); δ_C (125 MHz; $CDCl_3$) 183.8, 181.8, 169.1, 164.8, 153.0, 149.9, 138.6, 134.3, 130.5, 128.1, 125.8, 125.7, 123.9, 117.2, 110.8, 82.0, 69.1, 68.8, 50.1, 46.2, 37.8, 12.5; MS(MALDI-TOF): calcd. for $C_{82}H_{82}N_8O_{12}$ $[M+H]^+$ 1371.61, Found 1372.15.



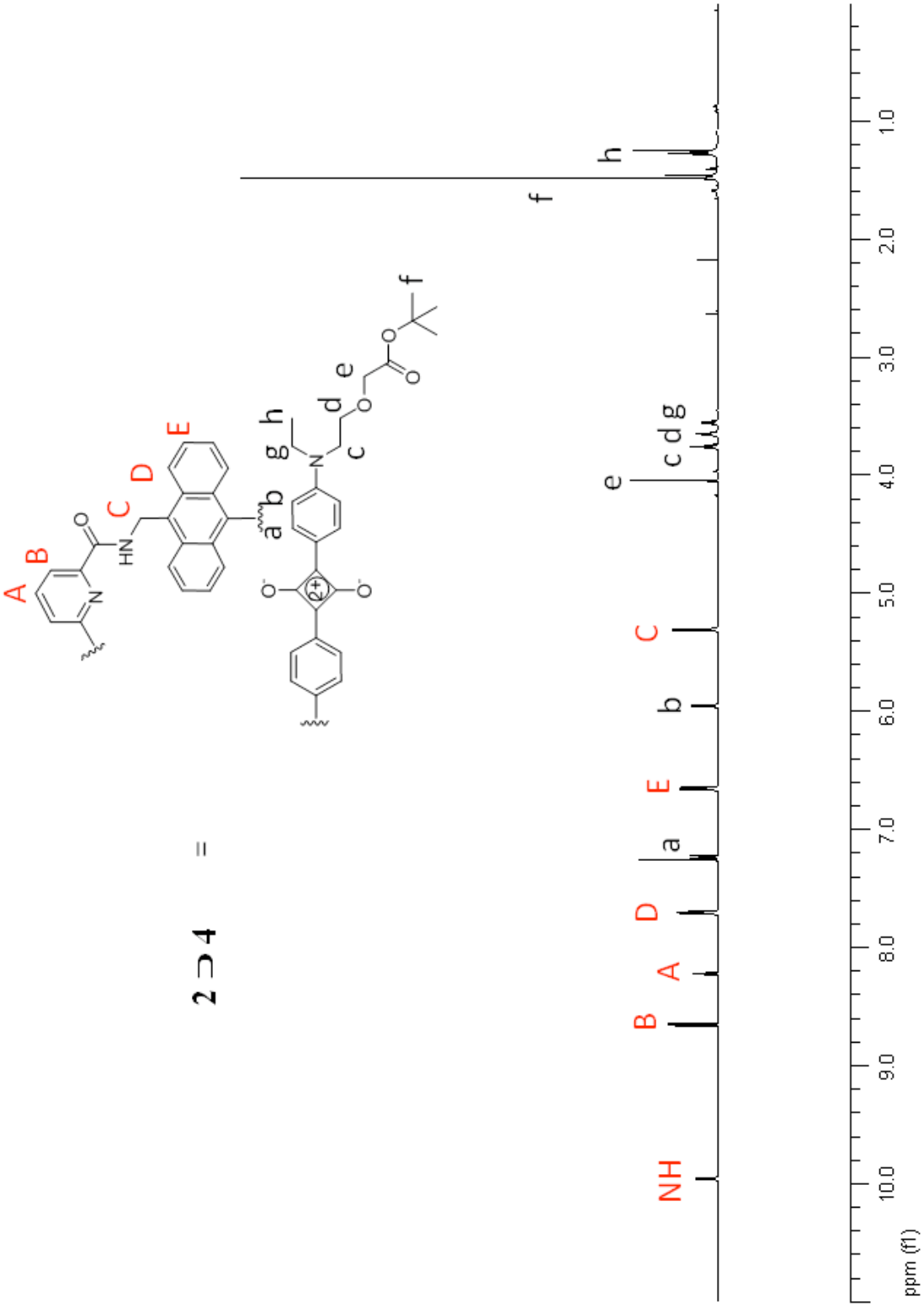
2⊃5. A mixture of **5** (6.3 mg, 0.013 mmol) and macrocycle **2** (9.5 mg, 0.013 mmol) was placed in a vial and dissolved in dry chloroform (1.0 mL). The reaction was placed inside the microwave instrument and operated at 160 °C for 5 minutes. After cooling, the chloroform was evaporated under reduced pressure to quantitatively afford **2⊃5** as a green solid and no further purification was required: δ_H (300 MHz; $CDCl_3$) 9.96 (t, J = 4.2 Hz, 4H), 8.66 (d, J = 8.0 Hz, 4H), 8.23 (t, J = 8.0 Hz, 2H), 7.71 (dd, J = 6.9, 3.2 Hz, 8H), 7.24 (d, J = 9.2 Hz, 4H), 6.66 (dd, J = 6.9, 3.2 Hz, 8H), 5.96 (d, J = 9.2 Hz, 4H), 5.31 (d, J = 4.2 Hz, 8H), 4.25 (d, J = 2.4 Hz, 4H), 3.75 (t, J = 5.7 Hz, 4H), 3.63 (t, J = 5.7 Hz, 4H), 3.53 (q, J =7.2 Hz, 4H), 2.56 (s, 2H), 1.28 (t, J = 7.2 Hz, 6H); δ_C (75 MHz; $CDCl_3$) 183.8, 182.0, 164.8, 153.0, 149.9, 138.6, 134.3, 130.5, 128.1, 125.9, 125.7, 123.9, 117.3, 110.9, 75.5, 67.4, 58.6, 50.1, 46.2, 37.8, 29.7, 12.6; MS(ESI-TOF): calcd. for $C_{76}H_{66}N_8O_8$ $[M + H]^+$ 1219, found 1219. The same synthesis by slipping at 120 °C is described in the supporting information (page S9) for reference S1.

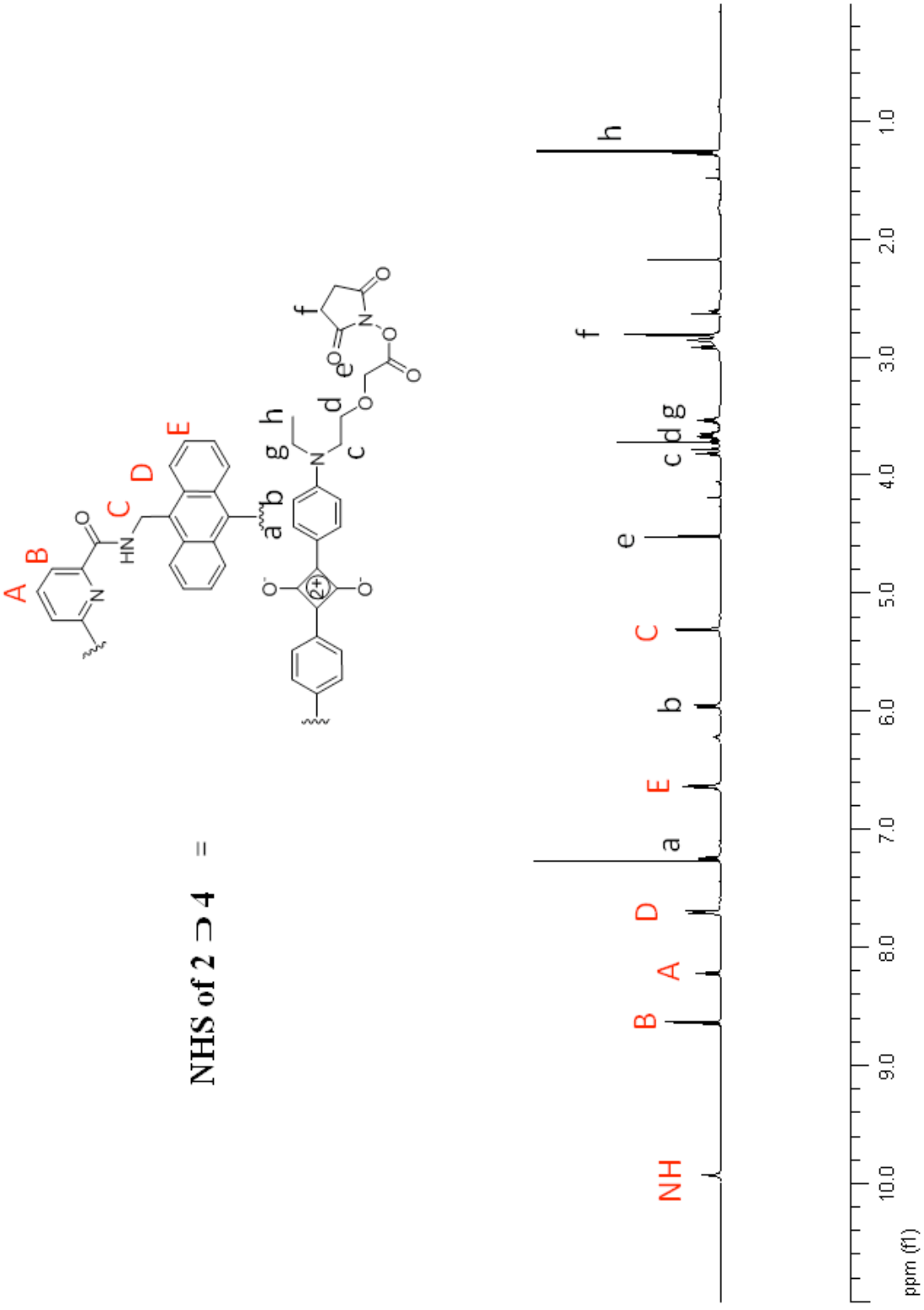


2⊃3. Trifluoroacetic acid (5.0 mL) was added to a solution of **2⊃4** (0.20 g, 0.15 mmol) in CH_2Cl_2 (25 mL). The mixture was stirred for 24 h at room temperature, then the solvent was evaporated under reduced pressure and the residue washed with diethyl ether to remove the remaining TFA. After complete evaporation under high vacuum, EDC (0.17 g, 0.88 mmol) and HOSu (0.10 g, 0.88 mmol) was added and stirred in dry $CHCl_3$ (50 mL) for 4 h at room temperature. The solvent was removed under reduced pressure and the crude product (estimated to be >95 % pure by ^{1}H NMR, with no evidence for rotaxane unthreading) was purified by flash chromatography (CH_3CN:$CHCl_3$ 50:50) to afford NHS ester of **2⊃4** (0.16 g, 0.11 mmol, 77 %) as a green solid: δ_H (600 MHz; $CDCl_3$) 9.93 (t, J = 4.2 Hz, 4H), 8.64 (d, J = 7.8 Hz, 4H), 8.22 (t, J = 7.8 Hz, 2H), 7.71 (dd, J = 6.6, 3.0 Hz, 8H), 7.25 (d, J = 9.0 Hz, 4H), 6.64 (dd, J = 6.6, 3.0 Hz, 8H), 5.97 (d, J = 9.6 Hz, 4H), 5.31 (d, J = 4.2 Hz, 8H), 4.52 (s, 4H), 3.82 (t, J = 6.0 Hz, 4H), 3.67 (t, J = 6.0 Hz, 4H), 3.53 (q, J = 7.2 Hz, 4H), 2.81 (t, J = 3.6 Hz, 8H), 1.27 (t, J = 7.2 Hz, 6H); δ_C (150 MHz; $CDCl_3$) 193.9, 183.7, 182.2, 169.0, 164.7, 152.9, 149.8, 138.6, 134.3, 130.5, 128.1, 125.8, 125.7, 123.9, 117.4, 110.8, 69.5, 66.2, 52.0, 46.2, 37.4, 29.3, 12.6. MS(ESI-TOF): calcd. for $C_{82}H_{72}N_{10}O_{16}$ $[M + H]^+$ 1453.5201, found 1453.5182. A mixture of NHS ester of **2⊃4** (0.044 g, 0.030 mmol) and the amine-DPA shown in reaction step 3 of Scheme 3 (0.057 g, 0.097 mmol) was stirred in dry $CHCl_3$ (20 mL) for 12 h at room temperature. The solvent was removed under reduced pressure and then the crude product (estimated to be >85 % pure by ^{1}H NMR, with no evidence for rotaxane unthreading) was purified by chromatography (NH_4OH: CH_3CN

2:98) to afford pure, isolated apo-**2⊃3** (0.035 g, 0.015 mmol, 49 %) as a green solid: δ_H (600 MHz; $CDCl_3$) 9.90 (t, J = 4.2 Hz, 4H), 8.66 (d, J = 8.4 Hz, 4H), 8.52 (d, J = 4.8 Hz, 8H), 8.22 (t, J = 7.8 Hz, 2H), 7.72 (dd, J = 6.6, 3.6 Hz, 8H), 7.60 (m, 16H), 7.29 (d, J = 9.0 Hz, 4H), 7.13 (m, 6H), 6.85 (s, 4H), 6.84 (t, J = 1.2 Hz, 2H), 6.63 (dd, J = 7.2, 3.0 Hz, 8H), 5.95 (d, J = 9.6 Hz, 4H), 5.32 (d, J = 4.2 Hz, 8H), 4.07 (s, 4H), 4.00 (t, J = 6.0 Hz, 4H), 3.81 (s, 16H), 3.75 (t, J = 6.0 Hz, 4H), 3.66 (s, 8H), 3.63 (t, J = 6.6 Hz, 4H), 3.53 (m, 4H), 3.46 (q, J = 6.6 Hz, 4H), 1.89 (m, 4H), 1.81 (m, 4H), 1.28 (t, J = 7.2 Hz, 6H); δ_C (125 MHz; $CDCl_3$) 183.8, 168.9, 165.0, 160.0, 159.9, 159.3, 153.0, 150.2, 149.3, 140.9, 136.7, 134.7, 130.8, 128.5, 126.0, 124.3, 123.1, 123.0, 122.3, 122.2, 117.8, 113.7, 111.0, 71.1, 69.2, 68.0, 67.5, 60.3, 58.8, 54.6, 50.0, 46.6, 38.0, 26.7, 26.5, 12.9; MS(MALDI-TOF): calcd. for $C_{146}H_{144}N_{22}O_{12}$ $[M+H]^+$ 2398, Found 2398. apo-**2⊃3** (1.0 mg, 0.42 μmol) and zinc nitrate hexahydrate (0.50 mg, 1.7 μmol) were dissolved in methanol (2.0 mL). The mixture was stirred for 60 minutes at room temperature and the solvent was evaporated under reduced pressure to obtain **2⊃3**. This complex was dissolved in deionized water and used for biological experiments.

2. ^{1}H NMR Spectra




NHS of 2 ⊃ 4 =
A
B
C
D
E
HN
N
O
O-
O-
2+
a
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a
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g
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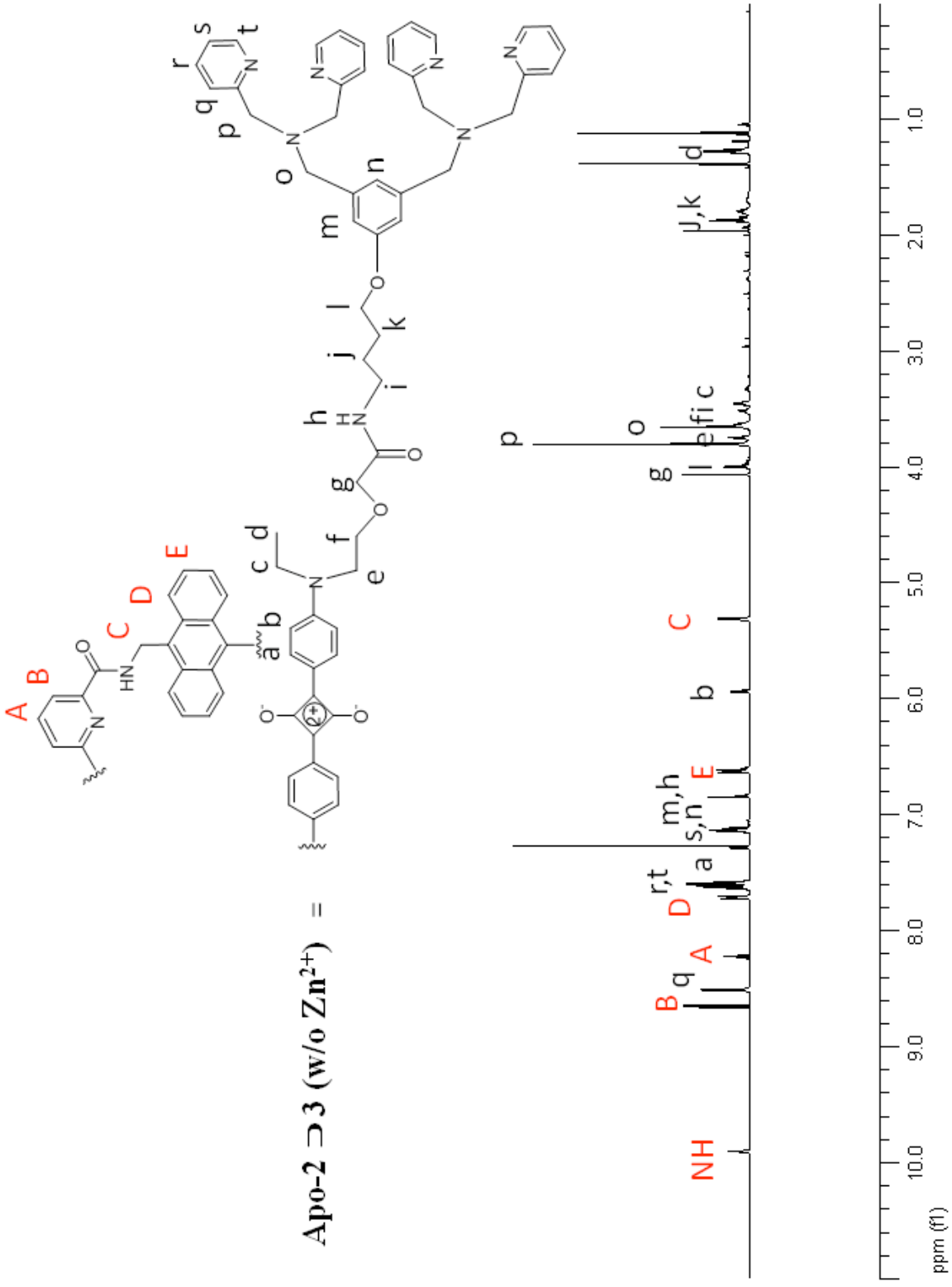
3.0
2.0
1.0
ppm (f1)


Apo-2 ⊃ 3 (w/o Zn2+) =
NH
B
q
A
D
r,t
a
m,h
s,n
E
b
C
p
o
g
l
e
f
i
c
d
j,k
ppm (f1)

3. Bacterial Imaging

Bacterial Cell Culture

Bacteria (*Salmonella typhimurium* AM3, *Staphylococcus aureus* NRS11, or *Escherichia coli* K12) was cultured in Luria-Bertani broth containing 30 μg/mL kanamycin after transfer from a preserved agar plate stored at 4 °C. Cells were allowed to grow overnight by incubating at 37 ºC while shaking at 200 rpm. For imaging, cells were centrifuged at 10000 rpm for 5 minutes. Growth media was removed and ~10^9 cells were resuspended in 5 mM TES buffer (145 mM NaCl buffer, pH 7.4). Cells were then treated with 10 nmol of probe and allowed to incubate in the presence of the dye in the dark for 15 minutes at room temperature. Cells were centrifuged at 10000 rpm for 5 minutes, media was discarded and pellet resuspended in 5 mM TES buffer by vortexing. This washing cycle was repeated.

Fluorescence Microscopy

Fluorescence microscopy was conducted on a Nikon Eclipse TE-2000 U epifluorescence microscope equipped with Cy5 (Exciter: HQ620/60X, Dichroic: 660LP, Emitter: HQ700/75m) filters. Images were acquired using a Photometrics 512 B black and white digital camera at 100 ms acquisition time.

In vitro Image Preparation

Images were obtained using Metamorph v6.2. Images were prepared using ImageJ v1.42q. Files were created from camera as a stack file. Raw image stacks were converted to individual images. Each image was then adjusted using the "Brightness and Contrast" feature and desired images were selected. Images were then background subtracted to 500 pixels and then set to the "Red" pseudocolor. A calibration bar was then added to the images using the "Analyze" followed by the "tools" menus and images were then restacked. Files were saved in tiff format. Lines and letters were added to the images using Adobe Photoshop 7.0.

Animal Preparation

All animal care and procedures were approved by the Notre Dame Institutional Advisory Committee for Animal Care. One ICr strain mouse was anesthetized using 2-3 % v/v isoflurane and hair was removed from the lower quarter of the body by clippers followed by depilatory cream. Bacteria were injected into each calf using a 29 gauge insulin needle. Anesthesia during imaging was maintained at 1.5-2 % v/v isoflurane.

In vivo Fluorescence Imaging

All images were acquired on a Kodak Multispectral *in vivo* Imaging System FX Pro. Fluorescence acquisition was acquired using a 750±18 nm filter (30 seconds exposure for each excitation wavelength, Field-of-view: 120 mm, Binning: 2 x 2, Camera F/stop: 2.5, Focal plane: 12.31 mm). Excitation wavelengths: 550, 570, 590, 610, 630, 650, 670, 690 nm. X-ray acquisition was performed using no filter set (120 second exposure, Field-of-view: 120 mm, Binning: none, F-stop: 2.5, Focal plane: 12.31 mm). Images saved as stacks in BIP format.

In vivo Image Preparation

Image acquisition controlled by Carestream Molecular Imaging Software (v. 5.0.2.30). Spectral unmixing of probes and skin autofluorescence performed by analysis of image stacks file from described acquisition using the Carestream Multispectral software (v. 1.2.0). Files saved as .BIP files and converted to 16-bit TIFF format using Carestream software. Images were prepared using ImageJ v.1.42q. X-ray and respective probe images were cropped and background subtracted to 500 pixels. Files were then combined in a stack and then separated into separate colors using the "Color" followed by "Composite" menus. Channels were placed into pseudocolors as follows: **1⊃3** (green), **2⊃3** (red), X-ray (gray). Files were saved in RGB format. Lines and letters were added using Adobe Photoshop 7.0.

[S1] J. J. Gassensmith, E. Arunkumar, L. Barr, J. M. Baumes, K. M. DiVittorio, J. R. Johnson, B. C. Noll and B. D. Smith, *J. Am. Chem. Soc.*, 2007, **129**, 15054.
[S2] J. R. Johnson, N. Fu, E. Arunkumar, W. M. Leevy, S. T. Gammon, D. Piwnica-Worms and B. D. Smith, *Angew. Chem. Int. Ed.*, 2007, **46**, 5528.
[S3] N. Fu, J. J. Gassensmith and B. D. Smith, *Supramol. Chem.*, 2009, **21**, 118.
[S4] S. Xiao, N. Fu, K. Peckham and B. D. Smith, *Org. Lett.*, 2009, 10.1021/ol902546m.