# Enhancement of Fluorescent Properties of Near-Infrared Dyes using Clickable Polyglycerol Dendrons

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**General methods.** All reactions requiring anhydrous conditions were performed under an Argon atmosphere. All reactions were carried out at room temperature unless stated otherwise. Chemicals and solvents were either A.R. grade or purified by standard techniques. Thin layer chromatography (TLC): silica gel plates Merck 60 F254: compounds were visualized by irradiation with UV light. Flash chromatography (FC): silica gel Merck 60 (particle size 0.040-0.063 mm), eluent given in parentheses. <sup>1</sup>H-NMR spectra were measured using Bruker Avance operated at 400MHz as mentioned. <sup>13</sup>C-NMR spectra were measured using Bruker Avance operated at 100MHz as mentioned. The chemical shifts are expressed in  $\delta$  relative to TMS ( $\delta$  = 0 ppm) and coupling constants *J* in Hz. The spectra were recorded in CDCl3 as solvent at room temperature unless stated otherwise. All general reagents, including salts and solvents, were purchased from Sigma-Aldrich.

**Abbreviations. DCM**- Dichloromethane, **EtOAc**- Ethylacetate, **Hex**- n-Hexanes, **MeOH**- Methanol, **THF**- Tetrahydrofurane, **PTSA**- *p*-Toluenesulfonic acid, **AcOH**-Acetic acid, **DMF**- Dimethylformamide, **MeCN**- Acetonitrile, **Et**<sub>2</sub>**O**- Diethyl ether, **EtOH**- Ethanol, **Et**<sub>3</sub>**N**-Triethylamine, **CDCl**<sub>3</sub>-Chloroform.

### **Compound 1b**

4-Picoline (3 mmol) was dissolved in 5 ml MeCN. 4-Bromo-1-butyne (9.2 mmol) was added and the reaction mixture was heated to reflux overnight. After completion, the solvent was evaporated and the crude product was precipitated from  $EtO_2$ . The solid was filtered to give compound **1b** (65%) as a brown solid.

<sup>1</sup>H NMR (400 MHz, DMSO  $d_6$ ):  $\delta$  = 8.97 (2H, d, J = 6.8 Hz), 8.04 (2H, d, J = 6.8), 4.7 (2H, t, J = 6.8 Hz), 3.06 (1H, t, J = 2.4 Hz), 2.95 (2H, dt, J = 6.8, 2.4 Hz), 2.62 (3H, s). <sup>13</sup>C NMR (100MHz, CDCl<sub>3</sub>):  $\delta$  = 161.23, 144.78, 129.37, 79.04, 74.87, 59.53, 22.25, 21.63. MS (ES<sup>+</sup>): m/z calc. for C<sub>10</sub>H<sub>12</sub>N<sup>+</sup>: 146.1; found: 147.2 [M+H]<sup>+</sup>.

### Compound 1

A mixture of dialdhyde  $1a^1$  (0.045 mmol), piperidine (0.09 mmol), and picolinium 1b (0.11 mmol) was dissolved in EtOH. The reaction mixture stirred for 30 minutes at 60°C and monitored by RP-HPLC (grad. 10%-90 ACN in water, 20min). After completion, the solvent was evaporated. The crude product was purified by preparative RP-HPLC (grad. 10%-90 ACN in water, 20min) to give compound 1 (97%) as orange solid.

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  = 8.88 (4H, d, *J* = 6.8 Hz), 8.46 (2H, s), 8.31 (2H, d, *J* = 16.4 Hz), 8.26 (4H, d, *J* = 7.2 Hz), 7.58 (2H, d, *J* = 16.4 Hz), 4.72 (4H, t, *J* = 6.4 Hz), 4.45 (2H, q, *J* = 7.1 Hz), 2.99 (4H, dt, *J* = 6.3, 2.5 Hz), 2.59 (2H, t, *J* = 2.4 Hz), 1.45 (3H, t, *J* = 7.2 Hz). <sup>13</sup>C NMR (100MHz, CDCl<sub>3</sub>):  $\delta$  = 166.39, 159.64, 155.29, 144.95, 136.31, 131.41, 125.34, 125.28, 124.69, 123.73, 78.42, 73.99, 61.77, 59.27, 20.97, 14.0. MS (ES<sup>+</sup>): m/z calc. for C<sub>31</sub>H<sub>30</sub>N<sub>2</sub>O<sub>3</sub><sup>+2</sup>: 478.22; found: 478.2 [M]<sup>+</sup>.

### Compound 2

Compuond **1** (0.016 mmol), compound  $2a^2$  (0.039 mmol), sodium ascorbate (0.0157 mmol) and copper(II) sulfate pentahydrate (0.0063 mmol) were dissolved in a mixture of H<sub>2</sub>O and *tert*-butanol (1:1). The reaction mixture stirred overnight at room temperature, and monitored by RP-HPLC (grad. 10%-90 ACN in water, 20min). After completion, the solvent was evaporated. The crude product was purified by preparative RP-HPLC (grad. 10%-90 ACN in water, 20min) to give compound **2** (62%) as yellow solid.

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  = 8.72 (4H, d, *J* = 6.8 Hz), 8.43 (2H, s), 8.27 (2H, d, *J* = 16 Hz), 8.18 (4H, d, *J* = 6.4 Hz), 7.99 (1H, bs), 7.94 (1H, d, *J* = 7.2 Hz), 7.54 (2H, d, *J* = 16.4 Hz), 4.98 (4H, m), 4.44 (2H, q, *J* = 7.2 Hz), 3.97-3.87 (6H, m), 3.76 (1H, m), 3.69 (4H, m),3.55-3.45 (23H, m), 1.45 (3H, t, *J* = 7.2 Hz). <sup>13</sup>C NMR (100MHz, CDCl<sub>3</sub>):  $\delta$  = 147.45, 143.4, 127.31, 127.06, 124.81, 81.41, 76.64, 76.47, 76.39, 74.72, 73.9, 66.88, 65.11, 64.44, 54.78, 45.12, 43.08, 30.55, 30.45. MS (ES<sup>+</sup>): m/z calc. for C<sub>49</sub>H<sub>68</sub>N<sub>8</sub>O<sub>15</sub><sup>+2</sup>: 1008.48; found: 1008.5 [M]<sup>+</sup>.

### Compound 3b

Compound **1** (0.01 mmol), compound  $3a^2$  (0.024 mmol), sodium ascorbate (0.01 mmol) and copper(II) sulfate pentahydrate (0.004 mmol) were dissolved in a mixture of H<sub>2</sub>O and *tert*-butanol (1:1). The reaction mixture stirred overnight at room temperature, and monitored by RP-HPLC (grad. 10%-90 ACN in water, 20min). After completion, the solvent was evaporated. The crude product was used in the next step without further purification.

### **Compound 3**

Compound **3b** was dissolved in methanol and Amberlyst 15 ion-exchange was added. The reaction mixture stirred for 60 min at room temperature. Then,  $Et_3N$  was added and the Amberlyst was filtered out. The crude product was purified by preparative RP-HPLC (grad. 10%-90 ACN in water, 20min) to give compound **3** (30% over two steps) as yellow solid.

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  = 8.72 (4H, bs), 8.45 (2H, s), 8.27 (2H, d, *J* = 16.1 Hz), 8.19 (4H, bs), 8.11 (1H, bs), 8.07 (1H, bs), 7.56 (2H, d, *J* = 16.1 Hz), 4.45 (2H, q, *J* = 7 Hz), 4.09-3.85 (9H, m), 3.73 (13H, m), 3.52 (56H, m), 1.45 (3H, t, *J* = 7 Hz). <sup>13</sup>C NMR (100 MHz, MeOD)  $\delta$  = 172.43, 166.39, 159.49, 154.89, 144.95, 136.07, 131.35, 130.17, 125.39, 124.81, 123.76, 79.32, 78.98, 73.27, 72.17, 71.75, 71.53, 71.30, 70.71, 70.61, 70.29, 69.64, 63.74, 62.20, 61.81, 61.54, 61.26, 60.42, 27.42, 14.05. MS (ES<sup>+</sup>): m/z calc. for C<sub>73</sub>H<sub>116</sub>N<sub>8</sub>O<sub>31</sub><sup>+2</sup>: 1600.77; found: 800.5 [M/2]<sup>+</sup>.

### **Compound 4a**

4-Picoline (10.7 mmol) was dissolved in 5 ml MeCN. 1,3-propanesultone (10.7 mmol) was added and the reaction mixture was heated to reflux for 2.5 hours. After completion, the crude product was precipitated from. The solid was filtered to give compound **1b** (86%) as white solid.

<sup>1</sup>H NMR (400 MHz, DMSO  $d_6$ ):  $\delta$  = 8.81 (2H, d, J = 6.6 Hz), 7.74 (2H, d, J = 6.3), 4.74 (2H, t, J = 7.2 Hz), 2.77 (2H, t, J = 6.4 Hz), 2.58 (3H, s), 2.36 (2H, p, J = 7 Hz). <sup>13</sup>C NMR (100MHz, CDCl<sub>3</sub>):  $\delta$  = 160.69, 144.49, 129.3, 59.77, 27.58, 21.36. MS (ES<sup>+</sup>): m/z calc. for C<sub>9</sub>H<sub>13</sub>NO<sub>3</sub>S: 215.06; found: 216.1 [M+H]<sup>+</sup>.

### **Compound 4**

A mixture of dialdhyde  $1a^1$  (0.45 mmol), piperidine (0.9 mmol), and picolinium 4a (0.9 mmol) was dissolved in EtOH. The reaction mixture stirred for 30 minutes at 80°C and monitored by RP-HPLC (grad. 10%-90 ACN in water, 20min). After completion, the solvent was evaporated. The crude product was purified by preparative RP-HPLC (grad. 10%-90 ACN in water, 20min) to give compound 4 (95%) as purple solid.

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  = 8.42 (4H, d, *J* = 6.8 Hz), 8.12 (2H, d, *J* = 15.9 Hz), 8.06 (2H, s), 7.79 (4H, d, *J* = 6.9 Hz), 7.47 (2H, d, *J* = 15.9 Hz), 4.59 (4H, t, *J* = 7.1 Hz), 4.3 (2H, q, *J* = 7 Hz), 2.81 (4H, t, *J* = 6 Hz), 2.36 (4H, m), 1.35 (3H, t, *J* = 7.1 Hz). <sup>13</sup>C NMR (100MHz, CDCl<sub>3</sub>):  $\delta$  = 156.66, 143.95, 141.49, 134.09, 126.51, 123.51, 120.02, 118.2, 113.95, 60.88, 58.9, 45.1, 23.14, 14.18. MS (ES<sup>-</sup>): m/z calc. for C<sub>29</sub>H<sub>32</sub>N<sub>2</sub>O<sub>9</sub>S<sub>2</sub>: 616.15; found: 615.2 [M-H]<sup>-</sup>.

### Quantum yield calculation for dyes 1-4:

Cresyl-violet-perchlorate was chosen as a reference compound for the determination of dyes **1**-**4** quantum yield. This compound exhibits similar spectral properties such as absorbance around 560 nm and fluorescence at the region of 600-740nm. The measurements were performed at a 96-wells plate at excitation wavelength of 550nm. Cresyl violet was dissolved in methanol and dyes **1**-**4** in PBS 7.4 0.1M. Dyes **1**-**4** quantum yield was calculated by comparison of the ratio between the area under the fluorescence curve of the dye and the reference compound at different concentrations. Conversion to the absolute quantum yield is achieved through the equation given below.

 $\phi_{x} = \phi_{ST}(Grad_{x}/Grad_{ST})(\eta^{2}_{x}/\eta^{2}_{ST})$ 

Where the subscripts ST and X denote standard and test respectively,  $\phi$  is the fluorescence quantum yield, *Grad* the gradient from the plot of integrated fluorescence intensity vs. absorbance, and  $\eta$  the refractive index of the solvents.

 $\phi_{Cresyl-violet}=0.55^3$ .

 $\phi_{\text{Dye 1}} = 0.55\left(\frac{723.13}{8213}\right) \cdot \left(\frac{1.333^2}{1.314^2}\right) = 0.0498$   $\phi_{\text{Dye 2}} = 0.55\left(\frac{857.17}{8213}\right) \cdot \left(\frac{1.333^2}{1.314^2}\right) = 0.059$   $\phi_{\text{Dye 3}} = 0.55\left(\frac{1848.1}{8213}\right) \cdot \left(\frac{1.333^2}{1.314^2}\right) = 0.1273$   $\phi_{\text{Dye 4}} = 0.55\left(\frac{783.54}{8213}\right) \cdot \left(\frac{1.333^2}{1.314^2}\right) = 0.054$ 

**Extinction coefficient calculations:** 



**Figure 1**. linear plots of dye **1** (a), dye **2** (b) and dye **3** (c). The data shown was measured at 1cm cuvette, at the indicated concentrations in PBS 7.4. The measurements were performed at the maximum wavelength of the samples (500nm).

 $\varepsilon_{\text{dye 1}} = 21586[M^{-1}cm^{-1}], \varepsilon_{\text{dye 2}} = 21856[M^{-1}cm^{-1}], \varepsilon_{\text{dye 3}} = 22276[M^{-1}cm^{-1}].$ 

pKa calculation of dye 3:



**Figure 2**. Fluorescence [% RFU] vs. pH of dye **3** [50 $\mu$ M], ( $\lambda_{ex}$ =500 nm,  $\lambda_{em}$ =700 nm).

### Photobleaching of compounds in solution:

Compounds were stored frozen in the dark at a stock concentration of 20 mM in DMSO. To conduct the experiments, stocks were diluted to 100  $\mu$ M in PBS (pH 7.4) and 10 mL were irradiated in a 10 cm diameter cell culture dish without a lid. A UV crosslinker (HL-2000 Hybrilinker, UVP, Upland, CA, USA) was set at the maximum energy (1 Joule/cm<sup>2</sup>). The fluorescence was measured in regular intervals. At each interval, the evaporated water was refilled to 10 mL using dd water. 0.6 mL of compound was taken for measurement and returned afterwards. A cuvette made of quartz suprasil (Hellma Analytics, Müllheim, Germany), 1 cm light path was used. A Spectramax M5 (Molecular Devices) photometer running on the SoftMax Pro 6.3 software was set as follows: 6 flashes per read, basic endpoint Fluorescence, PMT gain automatic, no reference. For the different compounds, the following light sources and filters were used:

Compound	Excitation	Emission	Emission
	wavelength	wavelength	cutoff
Cy5	650	670	665
Cy7	740	760	695
Dye 3	500	700	695

### Photobleaching of Cy7 and Dye 3 after uptake into RAW macrophages:

The murine macrophage cell line RAW 264.7 was grown to confluency on round 12 mm glass cover slides that were placed in 24 well cell culture plates. For cell staining, the wells were carefully rinsed with PBS followed by incubation with 1 mL of the respective compound at 50  $\mu$ M for 30 min. Cells were fixed with 1 mL paraformaldehyde (4%) for 10 minutes and quenched with 1 mL glycine buffer (100 mM glycine in PBS, pH 7.4) for 10 minutes. Afterwards, cells were stained with 4',6-Diamidin-2-phenylindol (DAPI) for 10 minutes. Analysis was performed on a confocal laser scanning microscope (LSM 700, Carl Zeiss) using the software Zen 2009. A 63x/1.4 oil DIC objective was used. For signal enhancement, the "smart setup" function was used. The following laser/filter combinations were used: DAPI: Laser 405 nm, Filter 420-1000, Dye 3: Laser 555 nm, Filter 560-1000, Cy7: Laser 639 nm, Filter LP 640. Pictures were recorded after five and ten scans to analyze the effect of photobleaching on the fluorescence intensity.

# Compound **1b**- <sup>1</sup>H NMR in DMSO- $d_6$





Compound **1b-** MS (ES<sup>+</sup>): *m/z* calc. for C<sub>10</sub>H<sub>12</sub>N<sup>+</sup>: 146.1; found: 147.2 [M+H]<sup>+</sup>.

# Compound 1-<sup>1</sup>H NMR in CDCl<sub>3</sub>



Compound 1-<sup>13</sup>C NMR in CDCl<sub>3</sub>





Compound 1- MS (ES<sup>+</sup>): m/z calc. for C<sub>31</sub>H<sub>30</sub>N<sub>2</sub>O<sub>3</sub><sup>+2</sup>: 478.22; found: 478.2 [M]<sup>+</sup>.

Compound 2-<sup>1</sup>H NMR in CDCl<sub>3</sub>



Compound 2-<sup>13</sup>C NMR in CDCl<sub>3</sub>





Compound 2- MS (ES<sup>+</sup>): m/z calc. for  $C_{49}H_{68}N_8O_{15}$ <sup>+2</sup>: 1008.48; found: 1008.5 [M]<sup>+</sup>.

# Compound 3-<sup>1</sup>H NMR in CDCl<sub>3</sub>



# Compound 3-<sup>13</sup>C NMR in MeOD





Compound **3-** MS (ES<sup>+</sup>): m/z calc. for C<sub>73</sub>H<sub>116</sub>N<sub>8</sub>O<sub>31<sup>+2</sup></sub>:1600.77; found:800.5 [M/2]<sup>+</sup>.

# Compound **4a**- <sup>1</sup>H NMR in DMSO- $d_6$









Compound 4a- MS (ES<sup>+</sup>): m/z calc. for C<sub>9</sub>H<sub>13</sub>NO<sub>3</sub>S: 215.06; found: 216.1 [M+H]<sup>+</sup>

Compound 4-<sup>1</sup>H NMR in CDCl<sub>3</sub>



Compound 4-<sup>13</sup>C NMR in CDCl<sub>3</sub>





Compound 4- MS (ES<sup>-</sup>): *m/z* calc. for C<sub>29</sub>H<sub>32</sub>N<sub>2</sub>O<sub>9</sub>S<sub>2</sub>: 616.15; found: 615.2 [M-H]<sup>-</sup>.

#### Compound **1** – RP-HPLC chromatogram, Wavelength: 353 nm.



#### Compound **2** – RP-HPLC chromatogram, Wavelength: 344 nm.



#### Compound **3** – RP-HPLC chromatogram, Wavelength: 358 nm.



#### Compound **4** – RP-HPLC chromatogram, Wavelength: 347 nm.



#### References

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