The versatility of asymmetric aminoethyl-tetrazines in bioorthogonal chemistry

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Chapter 4: Simultaneous dual-labeling of live cells using hydrophilic and hydrophobic tetrazine fluorophores

Introduction

In the previous chapter it was shown not to be possible to visualize N-akylcyclopropene mannosamine (ManNAlkCyp **62**, Chapter 3), which was metabolically incorporated in the hydrophilic glycocalyx (hydrophilic glycan rich region on the outside of the cell membrane), using tetrazine-functionalized fluorophores (**23**-**26**, **31**-**34**, Chapter 3). It was postulated that the compounds were not hydrophilic enough when compared to tetrazine-functionalized fluorophores successfully used by others in similar experiments.[1] To overcome this limitation, and not resort to difficult-to-synthesize zwitterionic fluorophores (e.g. disulfo-cyanine dyes or Alexa Fluor dyes), tetrazine-functionalized fluorophores were designed using Boc-Tzm-OH **3** as a starting point. The approach was somewhat inspired by the success of Calfluors[2], with hydrophilic moieties not attached to the fluorophore directly (**Figure 1**). In the first part of this chapter the synthesis of these molecules is described, followed by the successful labeling and fluorescence imaging of metabolically incorporated N-alkylcyclopropene mannosamine (**62**, Chapter 3) on live cells.

Dual-labeling is used to track biomolecules in tandem.[3] Previous studies generally describe the use of multiple techniques to label two biomolecules consecutively. Such approaches may suffer from a number of limitations, such as differences in the concentrations of the biomolecules to be studied, and differences in reaction kinetics of the labeling chemistries employed. In the second part of this chapter, the dual-labeling strategy is employed to simultaneously track both sterculic acid (Compound **64**, Chapter 3) and N-alkylcyclopropene mannosamine (**62**, Chapter 3) using a mixture of tetrazine-functionalized fluorophores comprising of a relative hydrophobic Bodipy-FL (Bodipy-FL **25**, Chapter 3) and relative hydrophilic Bodipy-TMR **7** or Cy5 **8**. Using a fluorophore mixture instead of consecutive labeling allows the number of steps to be reduced increasing the ease of working with sensitive live cells. As is shown here, application of a combination of tetrazines allows low concentrations, gives fast reaction times and therefore enables to swiftly perform dual-labeling of two biomolecules at once.

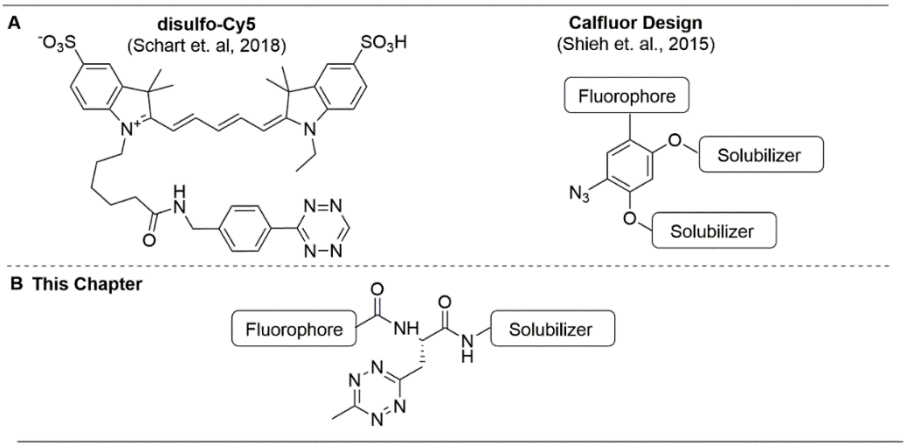


Figure 1: A) Example fluorophores used in literature to label metabolically incorporated N-functionalized mannosamines. B) General design of hydrophilic tetrazine-functionalized fluorophores in this chapter.

Synthesis of hydrophilic tetrazine-functionalized fluorophores

Zwitterionic linker **2** was synthesized in three steps from N,N-dimethyl-1,3-diaminopropane by Boc protection, subsequent reaction with 1,3-propanesultone and a final Boc deprotection under acidic anhydrous conditions (**Figure 2**). Linker **2** was then attached to Boc-Tzm-OH **3**, the synthesis of which is described in Chapter 3. Compound **3** was activated using DCC and NHS and linker **2** was attached at the C-terminal position. The Boc protective group was then removed after which three fluorophores were attached individually to give compounds **6**-**8**.

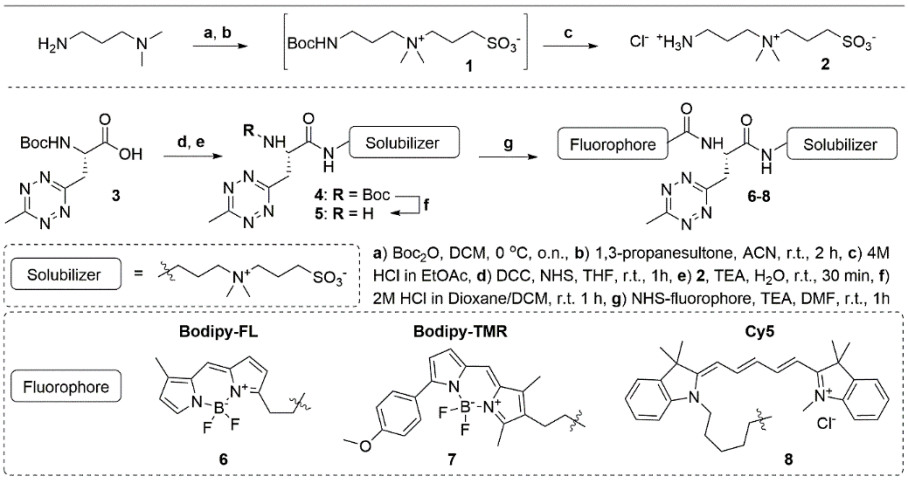


Figure 2: Synthesis of hydrophilic tetrazine-functionalized fluorophores **6**-**8**.

Regiospecific dual-labeling using hydrophilic and hydrophobic fluorophores

Following the synthesis of the hydrophilic tetrazine-functionalized fluorophores above, U2OS cells were incubated with N-alkylcyclopropene mannosamine (**62**, Chapter 3) via similar protocol to the tests performed in Chapter 3. After 2 days, the medium was refreshed, and the fluorophores were introduced for 2 hours before fluorescence imaging (**Figure 3**). The images clearly indicate the labelling of the cell membranes glycocalyx. A clear contrast to the results obtained using tetrazine-functionalized fluorophores (**23**-**26**, **31**-**34**, Chapter 3) from the previous chapter, where these parts of the cell were not visualizable.

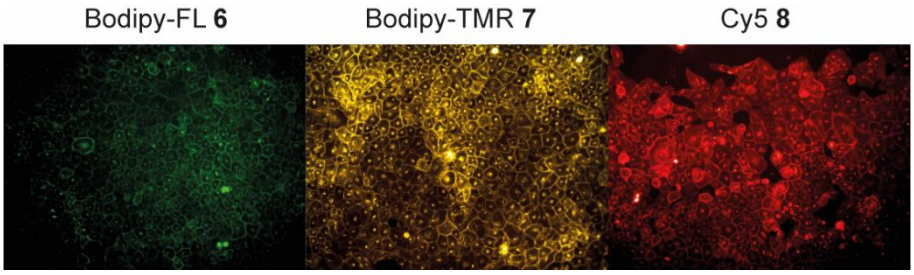


Figure 3: Fluorescence imaging of U2OS cells treated with N-alkylcyclopropene mannosamine (**62**, Chapter 3) and then with Bodipy-FL **6** (left), Bodipy-TMR **7** (middle) and Cy5 **8** (right).

To determine whether each fluorophore was capable of only labeling its respective target selectively purely based on location, instead of the type of bio-orthogonal chemistry used, a dual-labelling was performed . It was already shown that the hydrophobic tetrazine-functionalized Bodipy-FL (**25**, Chapter 3) was not capable of labeling metabolically incorporated mannosamines, due to the inability to reach inside the glycocalyx. It was still unclear whether the hydrophilic tetrazine-functionalized Bodipy-TMR **7** would in turn be unable to cross the cell membrane to reach the inside of the cell; or at least be unable to reach the hydrophobic niches in which sterculic acid had accumulated. HEK cells were therefore incubated with N-alkylcyclopropene mannosamine (**62**, Chapter 3) for 2 days, followed by a one-hour incubation with sterculic acid (**Figure 4**). The cells were washed and a mixture of Bodipy-FL (**25**, Chapter 3) and Bodipy-TMR **7** were added simultaneously, incubated for 2 hours, washed and imaged at the appropriate excitation/emission channels. Bodipy-FL (**25**, Chapter 3), which was postulated to react only in the lipophilic regions of the cell, showed an intracellular distribution. Conversely, the signal from Bodipy-TMR **7** was restricted to the cell membrane. Merged images (**Figure 4**) of the fluorescence from Bodipy FL (**25**, Chapter 3) and Bodipy-TMR **7**, depicted in green and red respectively, indeed show limited overlap (yellow pixels).[4-7] which could also be the result of the poor axial resolution of the widefield fluorescence

microscope used in these experiments. To better confirm the location-dependent labelling the experiment was repeated using confocal microscopy, where the axial resolution is < 1 micron (**Figure 5**). The single Z-resolved images indeed show no overlap between the two signals, supporting the hypothesis that Bodipy-FL tetrazine and **7** can be used for regiospecific labelling with the same chemistry.

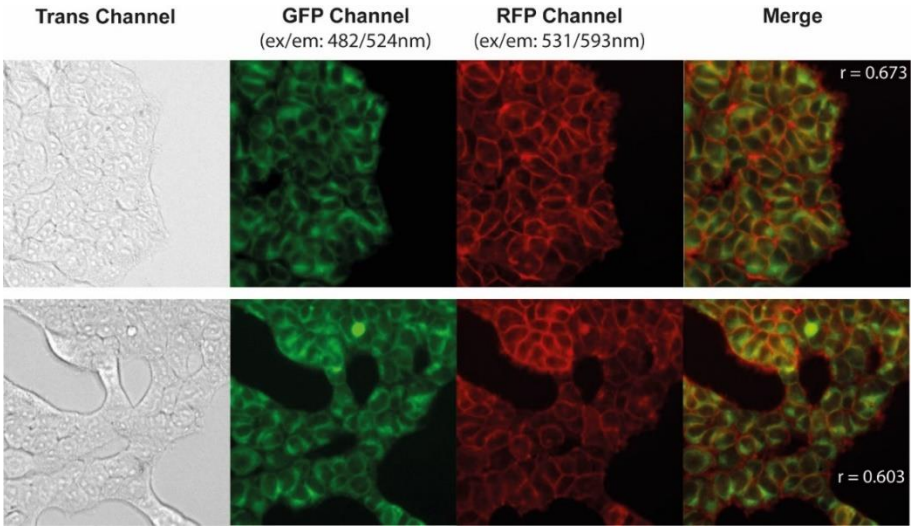


Figure 4: Fluorescence imaging of HEK cells, consecutively incubated with (**62**, Chapter 3) and (**64**, Chapter 3), before simultaneous incubation with Bodipy-FL (**25**, chapter 3) (**GREEN**) and Bodipy-TMR **7** (**RED**). Merged images of both fluorescence channels are included with their respective Pearson's coefficient (**r**).[4-7]

Conclusion

Hydrophilic tetrazine-functionalized fluorophores **6**-**8** were successfully synthesized by attachment of a hydrophilic zwitterionic moiety at the N-terminus, and hporophores to the C-terminus of Boc-Tzm-OH **3**. These fluorophores were capable to enter the glycocalyx coating of the surface of humane cell lines and label metabolically incorporated N-akylcyclopropene mannosamine. Furthermore Bodipy-TMR **7** was used in parallel with Bodipy-FL (**25**, Chapter 3) for the simultaneous regiospecific labeling of strained alkenes, and subsequent fluorescence imaging using confocal-microscopy showing minimal co-localization. These results, to my knowledge, offer the first example regiospecific labelling using parallel identical chemical reactions.

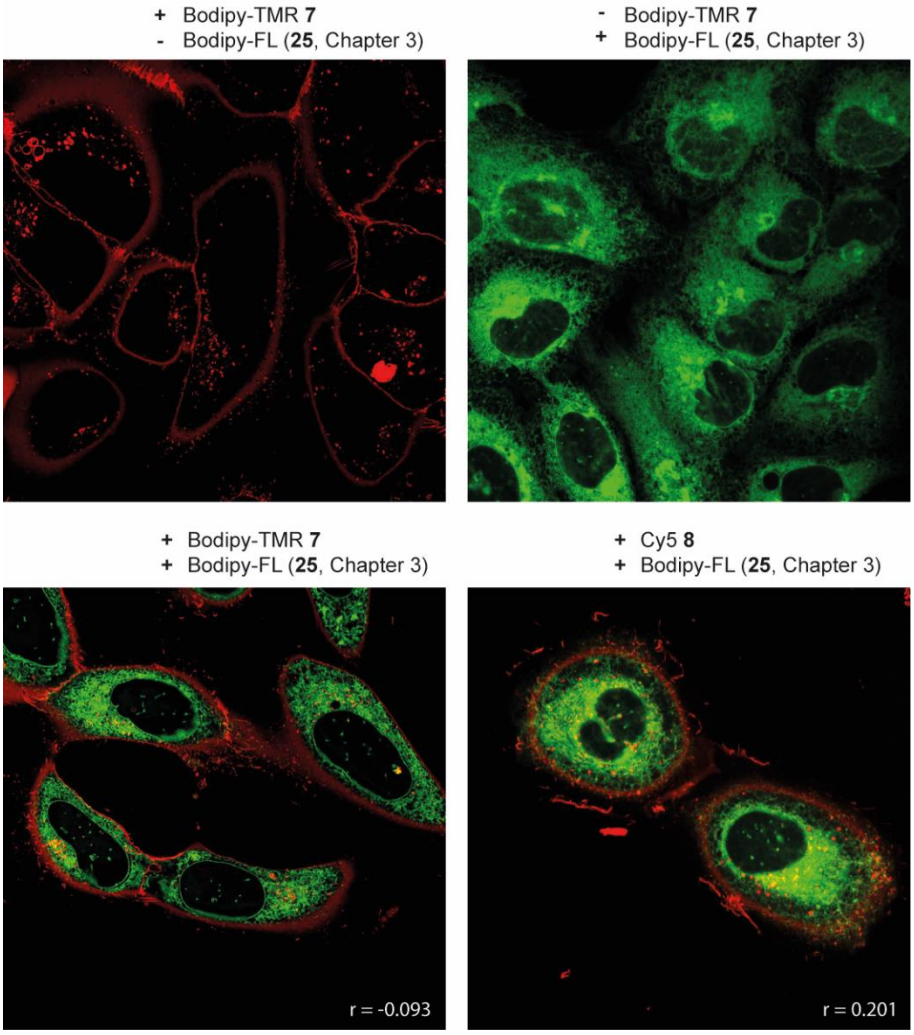


Figure 5: Confocal fluorescence imaging of HEK cells, consecutively incubated with N-alkylcyclopropene mannosamine (**62**, Chapter 3) and sterculic acid (**64**, Chapter 3), before simultaneous incubation with Bodipy-FL (**25**, Chapter 3) (GREEN) and Bodipy-TMR **7** or Cy5 **8** (RED). Merged images of both fluorescence channels are included with their respective Pearson's coefficient (**r**).[4-7]

Cell culturing and imaging procedures

HEK / U2OS cell seeding: HEK / U2OS cells were seeded 2 times a week, at variable 3-4 day intervals. First, the cell culturing flask was removed from the cell culturing incubator, and the cells were inspected using light-microscopy to spot any abmormalities. The old medium was removed, 2.0 mL of trypsin solution was added and the cells were allowed to detatch from the flasks surface for several minutes. 8.0 mL of full medium was added to the flask and the contents were gently homogenized using a serological pipet. Cells were counted, and 100.000 cells were seeded to a new flask after filling the flask with a calculated amount of full medium to obtain a total of 10 mL. The new flask was then returned to the cell culturing incubator.

Human Bone Osteosarcoma Epithelial cells (U2OS) fluorescence microscopy using mannosamine (62, Chapter 3): U2OS cells were cultured using full medium prepared with phenylred-free DMEM, and during seeding a fraction of the homogenized cell solution was diluted and homogenized with phenylred-free DMEM to 25.000 cells / 1 mL. This solution was then devided onto 8 well rectangular plates, (1 cm^2) 200 µL per well, moved to the cell culturing incubator and allowed to adhere and grow overnight. The next day, the old medium was removed, and to each sample well 200 µL of freshly premixed full medium (phenylred-free) containing 100 µM mannosamine **62** (0.2% DMSO). Cells were incubated for 2 days, the medium was removed and the cells were gently washed with pre-warmed medium. Then, 200 µL of full medium (phenylred-free) was added to each well containing freshly premixed 25 µM of tetrazine fluorophore (**6**-**8**) (0.25% DMSO). Cells were incubated for 1 hour, the medium was removed and the cells were gently washed twice with pre-warmed PBS and 200 µL of PBS was added to each well. The plate was analysed using an EVOS fluorescence microscope at 20x amplification, Trans channel filters to verify cell location, before imaging using the GFP/RFP/Cy5 channel filter. Raw images obtained were adjusted using ImageJ software v1.52n (contrast adjusted for **6**: 0-255 to 70-100, **7**: 0-255 to 40-200, **8**: 0-255 to 50-150).

Human Embryonic Kidney cells (HEK) fluorescence microscopy using mannosamine (62, Chapter 3) and sterculic acid (64, Chapter 3): HEK cells were cultured using full medium prepared with phenylred-free DMEM, and during seeding a fraction of the homogenized cell solution was diluted and homogenized with phenylred-free DMEM to 50.000 cells / 1 mL. This solution was then devided onto 8 well rectangular plates, (1 cm^2) 200 µL per well, moved to the cell culturing incubator and allowed to adhere and grow overnight. The next day, the old medium was removed*, and to each sample well 200 µL of freshly premixed full medium (phenylred-free) containing 100 µM mannosamine **62** (0.2% DMSO). Cells were incubated for 2 days, the medium was removed* and the cells were gently washed* with pre-warmed medium. Then, to each sample well 200 µL of freshly premixed full medium (phenylred-free) containing 50 µM sterculic acid **64** (0.5% DMSO). Cells were incubated for 1 hour, the medium was removed* and the cells were gently washed* twice with pre-warmed medium. Then, 200 µL of full medium (phenylred-free) (0.30% DMSO) was added to each well containing freshly premixed 25 µM of tetrazine fluorophore (**7**) and 5 µM of tetrazine fluorophore (**25**, Chapter 3). Cells were incubated for 2 hours, the medium was removed* and the cells were gently washed* twice with pre-warmed medium and 200 µL of pre-warmed medium was added to each well. Plates were analysed using (**A**) An EVOS fluorescence microscope at 20x amplification, Trans channel filters to verify cell location, before imaging using the GFP and

RFP channel filters. Raw images obtained were adjusted using ImageJ software v1.52n (contrast adjusted for Trans-channel: 0-255 to 80-225, GFP-channel: 0-255 to 30-100, RFP-channel: 0-255 to 30-100, 400x400 pixel image selection), or (**B**) Confocal microscope. Raw slices from image sets obtained were selected using ImageJ software v1.52n without adjusting contrast or pixel image selection.

* When removing or old medium or washing cells in this procedure, only around 80% was removed to prevent agitation of the sensitive HEK cells

Pearson's coefficient calculation: The Pearson's coefficient (**r**)[5, 6] was determined in ImageJ using JACoP plugin[4] and applying the Costes[7] automatic thresholds.

Compound Synthesis

Compound 2 (MJ456): 11 mmol (2.4 g) of Boc_2O was dissolved in 10 mL of DCM, 10 mmol (1.26 mL) of N,N-Dimethyl-1,3-diaminopropane was dissolved in 10 mL of DCM, both solutions were cooled 0 °C, added together and stirred overnight at 0 °C. The crude reaction mixture was concentrated using rotary evaporation and used without further purification. Then, 1.45 g (est. 7.5 mmol) of Boc-protected intermediate was dissolved in 10 mL of dry acetonitrile, 10 mmol (1.2 g) of 1,3-propanesultone was added and the reaction mixture was stirred for 2 hours at room temperature. The reaction mixture was concentrated using rotary evaporation and directly purified using silica column chromatography resulting in intermediate **1**. Intermediate **1** was treated with dry 4M HCl in EtOAc and concentrated using rotary evaporation resulting in compound **2**. ^{1}H NMR (101 MHz, D_2O) δ: 3.56 – 3.50 (m, 2H), 3.50 – 3.44 (m, 2H), 3.16 (s, 6H), 3.14 – 3.07 (m, 2H), 3.03 – 2.97 (m, 2H), 2.30 – 2.15 (m, 4H). ^{13}C NMR (101 MHz, D_2O) δ: 62.37, 60.52, 50.78, 47.07, 36.23, 20.49, 18.09.

Compound 4 (AS295, AS284, A268): 0.21 mmol (60 mg) of Boc-Tzm-OH **3** was dissolved in 3 mL dry THF, 0.34 mmol (39 mg) of N-hydroxysuccinimide and 0.28 mmol (58 mg) of N,N'-dicyclohexylcarbodiimide were added and the reaction mixture was stirred for 1 hour at room temperature. Reaction completion was checked by TLC (NHS-intermediate: Rf = 0.8, 1:1 EtOAc/DCM. Boc-Tzm-OH: Rf = 0.4, 1:1 EtOAc/DCM). The reaction mixture was filtered through a pad of celite. Then, 0.38 mmol (100 mg) of solubilizer, 0.38 mmol (53 µL) of TEA and 1.5 mL H_2O were added to the solution and the reaction mixture was stirred for 30 minutes at room temperature. Reaction completion was checked by TLC (Product: Rf = 0.1, 10% H_2O in acetonitrile. NHS-intermediate: Rf = 0.8, 10% H_2O in acetonitrile. Boc-Tzm-OH: Rf = 0.4, 10% H_2O in acetonitrile.) The reaction mixture was concentrated using rotary evaporation and directly purified using silica column chromatography using an 5-13% H_2O in acetonitrile eluent, resulting in 89 mg (0.18 mmol, 86%) of compound **4** as a pink solid. ^{1}H NMR (400 MHz, DMSO) δ: 8.17 (t, *J* = 5.7 Hz, 1H), 7.16 (d, *J* = 8.5 Hz, 1H), 4.50 (td, *J* = 8.5, 5.7 Hz, 1H), 3.48 – 3.37 (m, 3H), 3.25 (dd, *J* = 11.1, 5.6 Hz, 2H), 3.20 – 3.11 (m, 2H), 3.00 (s, 6H), 2.94 (s, 3H), 2.47 (t, *J* = 6.9 Hz, 2H), 2.07 – 1.90 (m, 2H), 1.83 (dd, *J* = 10.8, 5.5 Hz, 2H), 1.29 (s, 9H).^{13}C NMR (101 MHz, DMSO) δ 170.24, 167.00, 166.95, 155.11, 78.54, 62.50, 61.18, 53.29, 50.08, 47.63, 37.20, 35.77, 28.08, 22.32, 20.70, 18.88. ESI-MS (m/z): $[C_{19}H_{35}N_7O_6S+H]^+$ calculated 490.24, found 490.08.

Compound 5 (AS296, AS293, A268): 0.17 mmol (81 mg) of compound **2** was dissolved in 3 mL dry DCM, 2 mL of 4M HCl in dioxane was added and the reaction mixture was stirred for 1 hour at room temperature. The reaction mixture was concentrated using rotary evaporation resulting in 71 mg (0.17 mmol, quant.) of compound **3** as a pink solid. ^{1}H NMR (400 MHz,

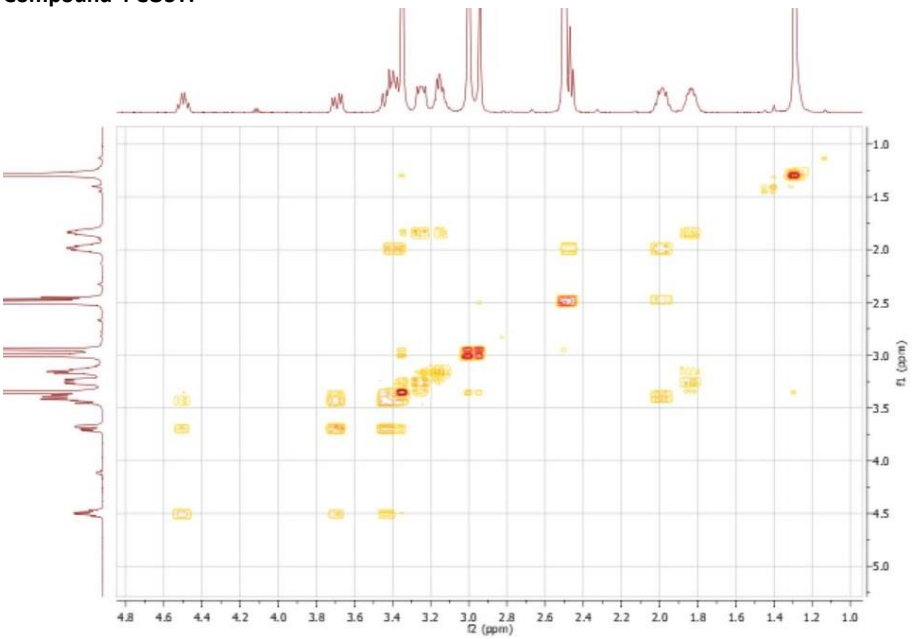
MeOD) δ: 8.72 (t, *J* = 5.9 Hz, 1H), 4.58 (t, *J* = 5.8 Hz, 1H), 3.93 (d, *J* = 5.8 Hz, 1H), 3.74 (d, *J* = 5.0 Hz, 1H), 3.62 – 3.52 (m, 2H), 3.51 – 3.40 (m, 2H), 3.19 (s, 3H), 3.17 (s, 3H), 3.16 – 3.12 (m, 2H), 3.05 (s, 3H), 2.87 (t, *J* = 7.0 Hz, 2H), 2.27 – 2.14 (m, 2H), 2.05 (dd, *J* = 15.4, 11.1 Hz, 2H). ^{13}C NMR (101 MHz, MeOD) δ: 160.19, 159.54, 157.11, 54.25, 53.48, 43.45, 42.37, 40.37, 40.16, 39.95, 39.73, 39.52, 39.31, 39.09, 38.88, 27.98, 27.50, 14.35, 11.91, 10.55. ESI-MS (m/z): $[C_{14}H_{27}N_7O_4S+H]^+$ calculated 390.19, found 390.25.

Compound 6 Bodipy-FL-Tzm-Sol (A270): 26 µmol (10 mg) of Bodipy-FL NHS ester, 026 µmol (10 mg) of compound **5** and 0.21 mmol (30 µL) of TEA were dissolved in 1 mL dry DMF and the reaction mixture was stirred for 1 hour at room temperature. Reaction completion was checked by TLC (Rf = 0.3, 20% H_2O in acetonitrile). The reaction mixture was concentrated using rotary evaporation and directly purified using silica column chromatography using an 2-10% H_2O in acetonitrile eluent, resulting in 1 mg (1.5 µmol, 5.7%) Compound **6** as an orange solid. ^{1}H NMR (400 MHz, MeOD) δ 7.43 (s, 1H), 7.00 (d, *J* = 3.8 Hz, 1H), 6.33 (d, *J* = 3.9 Hz, 1H), 6.23 (s, 1H), 4.96 – 4.91 (m, 2H), 3.79 – 3.67 (m, 2H), 3.56 – 3.47 (m, 2H), 3.43 – 3.34 (m,2H), 3.23 – 3.15 (m, 2H), 3.11 (s, 3H), 3.09 (s, 3H), 2.99 (s, 3H), 2.86 (td, *J* = 6.6, 2.3 Hz, 2H), 2.69 (t, *J* = 7.6 Hz, 2H), 2.51 (s, 3H), 2.29 (s, 3H), 2.22 (dd, *J* = 14.9, 8.0 Hz, 2H), 2.00 (dd, *J* = 15.6, 10.5 Hz, 2H).

Compound 7 Bodipy-TMR-Tzm-Sol (A302): 40 µmol (20 mg) of Bodipy-TMR NHS ester, 38 µmol (15 mg) of compound **5** and 72 µmol (10 µL) of TEA were dissolved in 1 mL dry DMF and the reaction mixture was stirred for 1 hour at room temperature. Reaction completion was checked by TLC (Rf = 0.4, 20% H_2O in acetonitrile). The reaction mixture was concentrated using rotary evaporation and directly purified using silica column chromatography using an 5-20% H_2O in acetonitrile eluent, resulting in 8 mg (10 µmol, 26%) Compound **7**.

Compound 8 Cy5-Tzm-Sol (A294): 50 µmol (30 mg) of Cy5 NHS ester, 77 µmol (30 mg) of compound **5** and 160 µmol (22 µL) of TEA were dissolved in 1 mL dry DMF and the reaction mixture was stirred for 1 hour at room temperature. Reaction completion was checked by TLC (Rf = 0.5, 25% H_2O in acetonitrile). The reaction mixture was concentrated using rotary evaporation and purified using HPLC resulting in Compound **8** as a blue solid. ESI-MS (m/z): $[C_{46}H_{64}N_9O_5S]^+$ calculated 854.47, found 854.40.

Compound 4 COSY:



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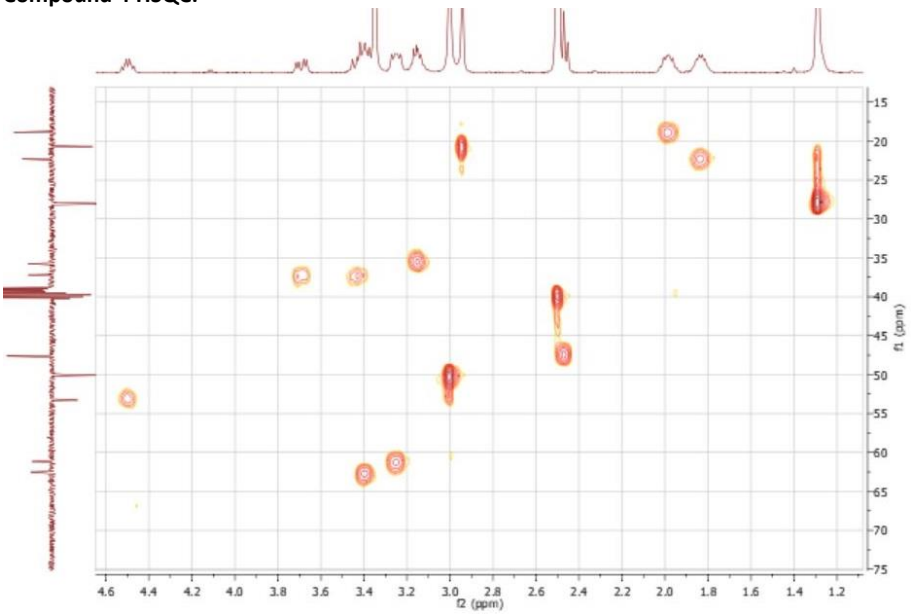

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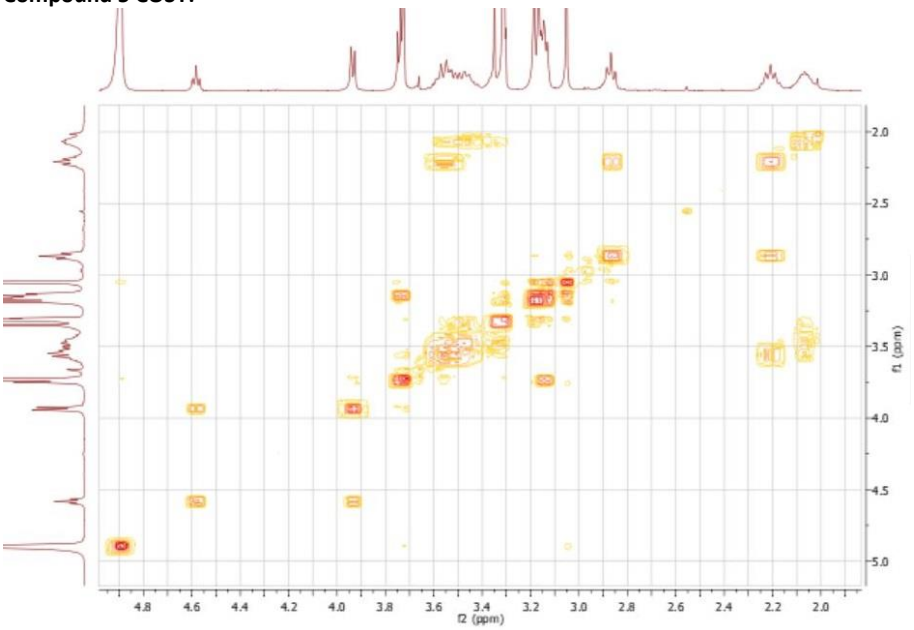

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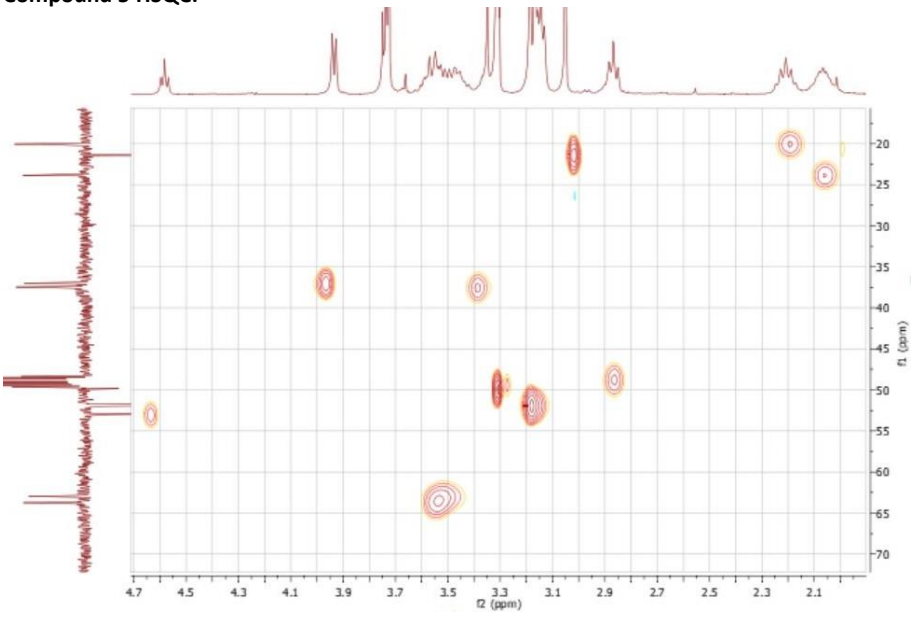

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