

DEVELOPMENT OF A NOVEL ANTIBODY-TETRAZINE CONJUGATE FOR BIOORTHOGONAL PRETARGETING

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SUPPORTING INFORMATION

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1. General Experimental Methods

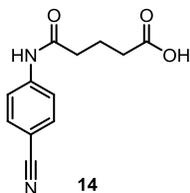
All chemicals and reagents were purchased from commercial suppliers (Sigma-Aldrich, Acros, TCI-Europe, Tractus or BioRad) and used without further purification. All solvents were reagent-grade or higher and used without further purification. Phosphate buffered saline (PBS) solutions were obtained from dilutions of a 0.5 M stock purchased from Gentest Life technology. Fetal bovine serum (FBS), Qualified, E.U.-approved, South America Origin was used in FBS stability studies. Clinical grade trastuzumab (Herceptin[®], Roche) 21 mg/mL was used.

3-(4-Cyanophenyl)-propanoic acid, 3,6-di-2-pyridyl-1,2,4,5-tetrazine (DIPY), 5-(4-(1,2,4,5-tetrazine)benzylamino)-5-oxopentanoic acid **9** and 3,6-bis-(3,5-DMP)-1,2,4,5-tetrazine were purchased from Sigma-Aldrich and Tractus. NHS-fluorescein and disulfo-cyanine3 NHS ester were purchased from Thermo Scientific and Interchim FluoProbes. 3-Methyl-6-thiomethyl-1,2,4,5-tetrazine ¹, 6-thiomethyl-1,2,4,5-tetrazine ¹, 5-(((6-(2-pyridinyl)-1,2,4,5-tetrazine-3-yl)-3-pyridinyl)-amino-5-oxopentanoic acid **10** ², (*E*)-cyclooct-4-enol ³, (*E*)-cyclooct-4-en-1-yl-(4-nitrophenyl) carbonate ⁴ were synthesized according to reported procedures.

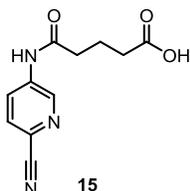
NMR spectra were recorded on a Bruker Avance DRX 400 MHz spectrometer. ¹H and ¹³C spectra are referenced to residual solvent peaks, coupling constants are given in Hz. HRMS analyses were performed using a Q-TOF II instrument (Waters, Manchester, UK). UPLC-MS analyses were performed on a Waters Acquity UPLC system coupled to a Waters TQD ESI mass spectrometer and TUV detector. Kinetics, UV-vis and fluorescence measurements were performed on a Synergy[™] 4Multi-Mode Microplate Reader BioTek. All data obtained from kinetic, UV-vis and fluorescence measurements were analyzed with GraphPad Prism 6 (San Diego, CA).

Chromatographic purifications were performed with a Biotage ISOLERA One flash system equipped with an internal variable dual-wavelength diode array detector (200-400 nm). Preparative HPLC was performed on a Waters 2545 HPLC equipped with a Waters 2998 diode array detector, a Micromass Quattro micro[™] model, a Waters 2767 fraction collector, and a XBridge Prep 5 μm RPC18 column at a flow rate of 21 mL/min.

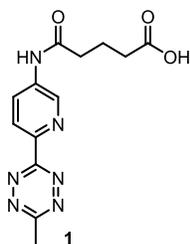
2. Synthetic procedures



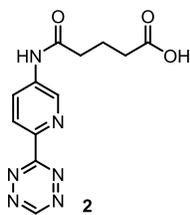
5-((4-Cyanophenyl)amino)-5-oxopentenoic acid (14): 4-Aminobenzonitrile (3g, 25 mmol) was dissolved in anhydrous THF (100 mL). Glutaric anhydride (14 g, 126 mmol) was added and the mixture was refluxed under argon for 48h. After cooling to room temperature the crude product precipitated from the reaction mixture. This was purified by suspending and refluxing it in MeOH followed by filtration. The title compound was obtained as an off-white solid (2.8 g, 47% yield). **¹H NMR** (400 MHz, DMSO): δ 12.09 (s, 1H); 10.33 (s, 1H); 7.78-7.73 (m, 4H); 2.40 (t, J = 7.2); 1.18 (pentet, J = 7.2, 2H) ppm. **¹³C NMR** (100 MHz, DMSO): δ 174.1; 171.6; 143.4; 133.2; 119.0; 104.7; 35.5; 32.8; 20.15 ppm. **MS-ESI:** m/z 233.3 [M+H]⁺.



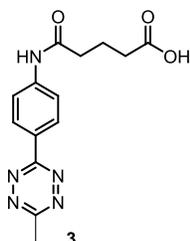
5-((6-Cyano)-3-pyridinyl)-5-oxopentenoic acid (15): The title compound was obtained as an off-white solid (3.7 g, 64% yield) from 5-aminopicolonitrile (3g, 25 mmol) and glutaric anhydride (14 g, 126 mmol) following the procedure reported for the synthesis of compound **14**. **¹H NMR** (400 MHz, CDCl₃): δ 8.47 (dd, J_m = 2.4, J_p = 0.8, 1H), 7.80 (dd, J_o = 8, J_p = 0.8, 1H), 7.63 (dd, J_o = 8, J_m = 2.4, 1H), 2.86 (t, J = 6.4, 4H), 2.15 (pentet, J = 6.4, 2H) ppm. **MS-ESI:** m/z 233.9 [M+H]⁺.



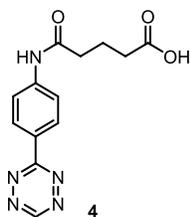
5-((6-(6-Methyl-1,2,4,5-tetrazin-3-yl)-3-pyridinyl)amino)-5-oxopentanoic acid (1): Tetrazine **1** was synthesized according to method A by reacting compound **15** (500 mg, 2.1 mmol), acetamidine hydrochloride (892 mg, 9.4 mmol), and hydrazine hydrate in presence of sulfur (69 mg, 2.1 mmol). The reaction time was 16 h. The title compound was isolated as a fuchsia solid (158 mg, 24% yield). **¹H NMR** (400 MHz, DMSO): δ 12.10 (s, 1H), 10.51 (s, 1H), 8.99 (d, J = 2.8, 1H), 8.49 (d, J = 8.8, 1H), 8.38 (dd, $J_o = 8.8$, $J_m = 2.8$, 1H), 3.01 (s, 3H), 2.47 (t, J = 7.2, 2H), 2.31 (t, J = 7.2, 2H), 1.85 (pentet, J = 7.2, 2H) ppm. **¹³C NMR** (100 MHz, DMSO) δ 174.1, 171.9, 167.1, 162.7, 144.1, 141.1, 138.2, 126.2, 124.2, 35.4, 32.9, 20.9, 20.2 ppm. **HRMS-ESI:** m/z calcd for $[C_{13}H_{15}N_6O_3]^+$ 303.1206, found 303.1206.



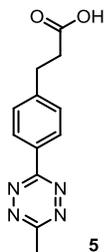
5-((6-(1,2,4,5-Tetrazine-3-yl)-3-pyridinyl)amino)-5-oxopentanoic acid (2): Tetrazine **2** was synthesized according to method A by reacting compound **15** (500 mg, 2.1 mmol), formamidine acetate (554 mg, 9.2 mmol), and hydrazine hydrate in presence of sulfur (69 mg, 2.1 mmol). The reaction time was 16 h. The title compound was isolated as a pink solid (127 mg, 20% yield). **¹H NMR** (400 MHz, DMSO): δ 12.11 (s, 1H), 10.62 (s, 1H), 10.54 (s, 1H), 9.01 (d, $J = 2.8$, 1H), 8.54 (d, $J = 8.8$, 1H), 8.39 (dd, $J_o = 8.8$, $J_m = 2.8$), 2.47 (t, $J = 8$, 2H), 2.32 (t, $J = 8$, 2H), 1.85 (p, $J = 8$, 2H). **¹³C NMR** (100 MHz, DMSO): δ 174.1, 171.9, 164.7, 158.1, 143.9, 141.2, 138.5, 126.1, 124.7, 35.4; 32.9, 20.1. **HRMS-ESI:** m/z calcd for $[C_{12}H_{13}N_6O_3]^+$ 289.1049, found 289.1060.



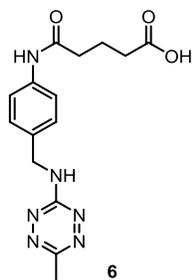
5-((4-(6-Methyl-1,2,4,5-tetrazin-3-yl)phenyl)amino)-5-oxopentanoic acid (3): Tetrazine **3** was synthesized according to method A by reacting compound **14** (116 mg, 0.5 mmol), acetamidine hydrochloride (472 mg, 5 mmol) and hydrazine hydrate in presence of $Zn(OTf)_2$ (91 mg, 0.25 mmol). The reaction time was 16 h. The title compound was isolated as a fuchsia solid (61 mg, 40% yield). **¹H NMR** (400 MHz, MeOD): δ 10.04 (d, $J = 9.6$, 2H), 9.39 (d, $J = 9.6$, 2H), 4.57 (s, 3H), 4.06 (t, $J = 7.2$, 2H), 3.98 (t, $J = 7.2$, 2H), 3.56 (pentet, $J = 7.2$, 2H) ppm. **¹³C NMR** (100 MHz, MeOD): δ 176.9, 174.0, 168.3, 164.9, 144.2, 129.6, 128.4, 121.0, 37.0, 34.2, 21.9, 21.0 ppm. **HRMS-ESI:** m/z calcd for $[C_{14}H_{15}N_5O_3Na]^+$ 324.1073, found 324.1073.



5-((4-(1,2,4,5-Tetrazin-3-yl)phenyl)amino)-5-oxopentanoic acid (4): Tetrazine **4** was synthesized according to method A by reacting compound **14** (116 mg, 0,5 mmol), formamidine acetate (520 mg, 5 mmol) and hydrazine hydrate in presence of $\text{Zn}(\text{OTf})_2$ (91 mg, 0.25 mmol). The reaction time was 48 h. The title compound was isolated as a pink solid (31 mg, 22% yield). $^1\text{H NMR}$ (400 MHz, DMSO): δ 10.51 (s, 1H), 10.35 (bs, 1H), 8.46 (d, $J = 8.4$, 2H), 7.89 (d, $J = 8.4$, 2H), 2.43 (t, $J = 6.8$, 2H), 2.29 (t, $J = 6.8$, 2H), 1.83 (pentet, $J = 6.8$, 2H) ppm. $^{13}\text{C NMR}$ (100 MHz, DMSO): 171.5, 165.1, 157.8, 143.5, 128.7, 125.9, 119.3, 35.6, 33.1, 20.3 ppm. **HRMS-ESI:** m/z calcd for $[\text{C}_{13}\text{H}_{14}\text{N}_5\text{O}_3]^+$ 288.1097, found 288.1104.

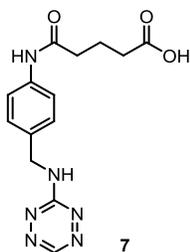


3-(4-(6-Methyl-1,2,4,5-tetrazine-3-yl)phenyl) propanoic acid (5): Tetrazine **5** was synthesized according to method A by reacting 3-(4-cyanophenyl)-propanoic acid (300 mg, 1,7 mmol), acetamidine hydrochloride (394 mg, 6.8 mmol) and hydrazine hydrate in presence of sulfur (55 mg, 1.7 mmol). The reaction time was 16 h. The title compound was isolated as a pink solid (47 mg, 11% yield). $^1\text{H NMR}$ (400 MHz, MeOD): δ 8.46 (d, $J = 8.4$, 2H), 7.49 (d, $J = 8.4$, 2H), 3.04 (t, $J = 8.0$, 2H), 3.02 (s, 3H), 2.69 (t, $J = 8.0$) ppm. $^{13}\text{C NMR}$ (400 MHz, CD₃OD): 176.3, 168.6, 165.3, 147.4, 131.5, 130.4, 128.9, 36.2, 31.9, 21.0. **HRMS-ESI:** m/z calcd for $[\text{C}_{12}\text{H}_{13}\text{N}_4\text{O}_2]^+$ 245.1039, found 245.1050.



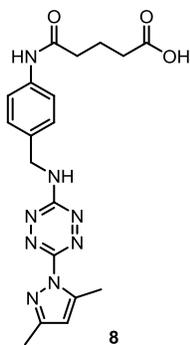
5-(((4-((6-Methyl-1,2,4,5-tetrazin-3-yl)amino)methyl)phenyl)amino)-5-oxopentanoic acid

(6): Tetrazine **6** was synthesized according to method B by reacting 3-methyl-6-thiomethyl-1,2,4,5-tetrazine (159 mg, 1.0 mmol) with 4-aminobenzylamine (129 mg, 1.0 mmol) in methanol for 2 h. After solvent evaporation the crude residue was subjected to column chromatography (gradient *n*-heptane:EtOAc 80:20 to 0:100) to afford *N*-(4-aminobenzyl)-6-methyl-1,2,4,5-tetrazin-3-amine as a red solid (161 mg, 71% yield). ¹H NMR (400 MHz, DMSO): 8.68 (t, J = 6.4, 1H), 7.01 (d, J = 9.6, 2H), 6.05 (d, J = 9.6, 2H), 5.03 (bs, 2H), 4.42 (d, J = 6.4, 2H), 2.66 (s, 3H). ¹³C NMR (100 MHz, DMSO): 161.4, 160.4, 147.6; 128.3; 125.4, 113.8, 43.4, 19.6. **MS-ESI:** m/z 217.3 [M+H]⁺. *N*-(4-aminobenzyl)-6-methyl-1,2,4,5-tetrazin-3-amine (82 mg, 0.4 mmol) was subsequently reacted with glutaric anhydride (216 mg, 1.9 mmol). After purification the title compound was isolated as a red solid (95 mg, 76% yield). ¹H NMR (400 MHz, DMSO): 9.87 (s, 1H), 8.82 (t, J = 6.4, 1H), 7.52 (d, J = 8.4, 2H), 7.27 (d, J = 8.4, 2H), 4.55 (d, J = 6.4, 2H), 2.67 (s, 3H), 2.32 (t, J = 7.2, 2H), 2.25 (t, J = 7.2, 2H), 1.78 (pentet, J = 7.2, 2H). ¹³C NMR (100 MHz, DMSO): 174.2, 170.6, 161.3, 160.6, 138.2, 133.1, 127.7, 119.1, 43.2, 35.3, 33.0, 20.4, 19.5. **HRMS-ESI:** m/z calcd for [C₁₅H₁₉N₆O₃]⁺ 331.1519, found 331.1528.

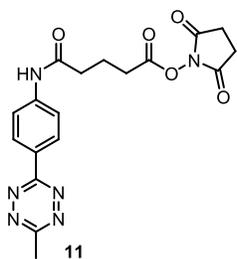


5-((4-(((1,2,4,5-Tetrazin-3-yl)amino)methyl)phenyl)amino)-5-oxopentanoic acid (7):

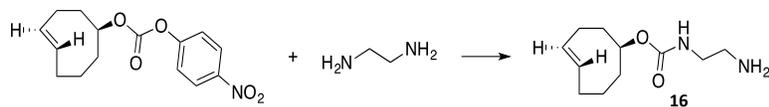
Tetrazine **7** was synthesized according to method B by reacting 6-thiomethyl-1,2,4,5-tetrazine (108 mg, 0.85 mmol), with aminobenzylamine (98 mg, 0.85 mmol) in methanol for 4 h. After solvent evaporation the crude residue was subjected to column chromatography (gradient *n*-heptane:EtOAc 95:5 to 20:80 + Et₃N 2%) to afford *N*-(4-aminobenzyl)-1,2,4,5-tetrazin-3-amine as a red solid (171 mg, quantitative yield). ¹H NMR (400 MHz, MeOD): 9.5 (s, 1H), 7.17 (d, J = 8, 2H), 6.71 (d, J = 8, 2H), 4.57 (s, 2H). ¹³C NMR (100 MHz, MeOD): 164.4, 153.7, 148.1, 129.9, 128.6, 116.6, 45.1. **MS-ESI**: m/z = 203.3 [M+H]⁺. *N*-(4-aminobenzyl)-1,2,4,5-tetrazin-3-amine (171 mg, 0.85 mmol) was subsequently reacted with glutaric anhydride (482 mg, 4.2 mmol). After purification the title compound was isolated as a red solid (202 mg, 76% yield). ¹H NMR (400 MHz, DMSO): 12.1(s, 1H), 9.88 (s, 1H), 9.75 (s, 1H), 9.09 (t, J = 6, 1H), 7.53 (d, J = 8.4, 2H), 7.28 (d, J = 8.4, 2H) 4.57 (d, J = 6H), 2.32 (t, J = 8, 2H), 2.25 (t, J = 8, 2H), 1.79 (p, J = 8, 2H). ¹³C NMR (DMSO, 100 MHz): 174.0, 170.6, 162.6, 152.8, 127.7, 119.0, 43.1, 32.9, 20.3. **HRMS-ESI**: m/z calcd for [C₁₄H₁₆N₆O₃Na]⁺ 339.1182, found 339.1190.



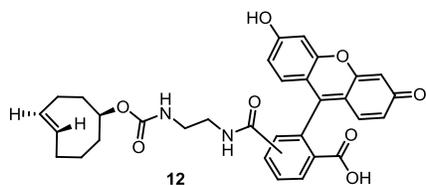
5-(((4-((6-(3,5-Dimethyl-1H-pyrazolyl)-1,2,4,5-tetrazin-3-yl)amino)methyl)phenyl)amino)-5-oxopentanoic acid (8): Tetrazine **8** was synthesized according to method B by reacting 3,6-bis-(3,5-DMP)-1,2,4,5-tetrazine (100 mg, 0.37 mmol) with aminobenzylamine (45 mg, 0.37 mmol) in toluene. After solvent evaporation the crude residue was subjected to column chromatography (gradient DCM:EtOAc 100:0 to 30:70) to afford *N*-(4-aminobenzyl)-6-(3,5-DMP)-1,2,4,5-tetrazin-3-amine as a red solid (100 mg, 91% yield). ¹H NMR (400 MHz, MeOD): 7.19 (d, J = 8.8, 2H), 6.71 (d, J = 8.8, 2H), 6.18 (s, 1H), 4.62 (s, 2H), 2.49 (s, 3H), 2.28 (s, 3H). ¹³C NMR (100 MHz, MeOD): 152.9, 148.4, 143.8, 130.2, 128.6, 116.8, 110.0, 45.8, 13.3, 13.0. **MS-ESI:** m/z 297.4 [M+H]⁺. *N*-(4-aminobenzyl)-6-(3,5-DMP)-1,2,4,5-tetrazin-3-amine (67 mg, 0.23 mmol) was subsequently reacted with glutaric anhydride (129 mg, 1.1 mmol). After purification the title compound was isolated as a red solid (67 mg, 72% yield). ¹H NMR (400 MHz, DMSO): 9.90 (s, 1H), 9.27 (t, J = 7.2, 1H), 7.55 (d, J = 8, 2H), 7.33 (d, J = 8, 2H), 6.18 (s, 1H), 4.62 (d, J = 7.2, 1H), 2.38 (s, 3H), 2.33 (t, J = 7.6, 2H), 2.25 (t, J = 7.6, 2H), 2.21 (3H, s), 1.79 (2H, p, J = 7.6). ¹³C NMR (100 MHz, DMSO): 170.6, 161.2, 156.9, 150.0, 141.3, 138.3, 132.6, 127.8, 119.1, 108.5, 35.4, 43.6, 33.1, 20.5, 13.3, 12.5. **HRMS-ESI:** m/z calcd for [C₁₉H₂₃N₈O₃]⁺ 411.1908, found 411.1908.



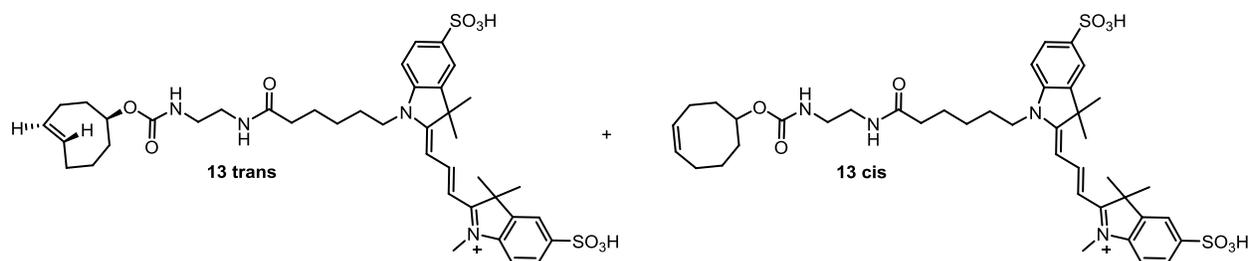
5-((4-(6-Methyl-1,2,4,5-tetrazin-3-yl)phenyl)amino)-5-oxopentanoic acid succinimide ester (11): Tetrazine **3** (80 mg, 0,26 mmol), N-hydroxysuccinimide (NHS) (46 mg, 0.4 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) (66 mg, 0.34 mmol) were placed in a vial equipped with a septum. The vial was filled with argon and DMF was added (dry, 1.5 mL). The mixture was stirred at room temperature for 18 h. Water was added to the reaction mixture until precipitation of the product. The suspension was centrifuged and the supernatant removed (repeated 4 times). The residue was dried under vacuum to afford the title compound as a fuchsia solid (102 mg, 96% yield). **¹H NMR** (400 MHz, DMSO): 10.34 (1H, s), 8.42 (2H, d, J = 9.2), 7.88 (2H, d, J = 9.2), 2.97 (s, 3H), 2.83 (s, 4H), 2.81-2.77 (m, 2H), 2.55-2.52 (m, 2H), 1.97 (pentet, 2H, J = 8). **¹³C NMR** (100 MHz, DMSO): 172.6, 170.9, 170.3, 168.8, 166.6, 162.8, 143.0, 128.5, 119.3, 34.7, 29.6, 25.5, 20.8, 19.9. **MS-ESI:** m/z 399.4 [M+H]⁺. Rotamers are visible both in the ¹H and ¹³C spectra. Only the major peaks were reported.



(E)-cyclooct-4-en-1-yl (2-aminoethyl)carbamate (16): To a stirring solution of ethane-1,2-diamine (0,084 ml, 1,256 mmol) in DCM (2.5 ml) was added (*E*)-cyclooct-4-en-1-yl (4-nitrophenyl) carbonate ⁴ (122 mg, 0,419 mmol) in DCM (2.5 ml) dropwise over 30 minutes. After addition, the crude was diluted with 8 ml DCM and extracted 3 times with H₂O. The organic layer was dried with NaSO₄, filtered and the solvent evaporated to afford a yellow oil (89 mg, 100% yield). ¹H NMR (CDCl₃, 400 MHz) δ 5.59-5.46 (m, 2H), 5.20-4.87 (m, 1H), 4.86 (m, 1H), 3.28-3.19 (m, 2H), 2.87-2.79 (m, 2H), 2.33-2.20 (m, 4H), 2.15-2.04 (m, 1H) 1.87-1.74 (m, 2H), 1.69-1.58 (m, 2H), 1.24-1.13 (m, 2H).

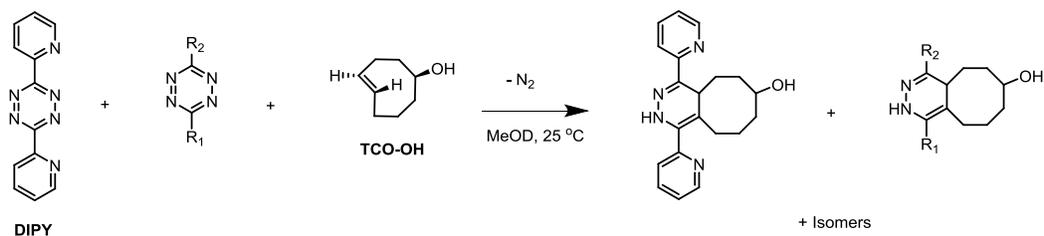


TCO-Fluorescein (12):Compound **12** was synthesized according to to the general procedure for the synthesis of TCO dyes (see experimental procedure) by reacting (*E*)-cyclooct-4-en-1-yl (2-aminoethyl)carbamate **16** (16 mg, 0.076 mmol) and fluorescein -NHS (67 mg, 0.141 mmol). The title compound was isolated as a dark yellow solid (10 mg, 36% yield). ¹H NMR (400 MHz, MeOD) δ 8.45 (s, 3H), 8.19 (d, J = 8.0, 3H), 7.32 (t, J = 11.0, 3H), 6.60 (ddd, J = 15.0, 10.8, 2.1, 16H), 5.71 – 5.47 (m, 6H), 4.82 (d, J = 5.3, 4H), 3.63 – 3.47 (m, 6H), 3.46 – 3.33 (m, 7H), 2.40 – 2.00 (m, 15H), 1.70 (dddd, J = 45.8, 21.1, 12.1, 7.9, 13H), 1.31 (t, J = 23.8, 5H). **HRMS-ESI** [M + H]⁺ m/z calcd for [C₃₂H₃₁N₂O₈]⁺ 571.2080, found 571.2084.

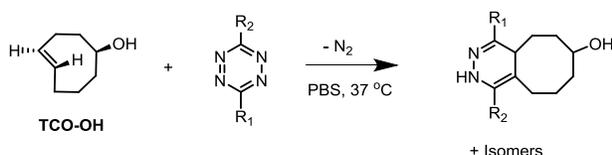


Cy3 dye (13): Compound **13** was synthesized according to the general procedure for the synthesis of TCO dyes (see experimental procedure) by reacting (*E*)-cyclooct-4-en-1-yl (2-aminoethyl)carbamate **16** (9 mg, 0.084 mmol) and Cy3-NHS (20 mg, 0.056 mmol). The title compound was obtained as a mixture of *trans* (TCO-Cy3) and *cis* isomer (CCO-Cy3) in a 1 : 1 ratio (6 mg, 37% yield for the mixture). The isomeric mixture could not be separated by HPLC. **¹H-NMR:** (400 MHz, MeOD) δ 8.57 (t, $J = 14$ 1H), 7.96 (bd, 2H), 7.93 – 7.90 (m, 2H), 7.41 (d, $J = 8.4$, 2H), 6.51 (dd, $J_1 = 14$, $J_2 = 5$) 5.69 - 5.48 (m, 4H), 4.77 - 4.73 (m, 1H), 4.62 (bs, 1H), 4.18 (bt, 2H), 3.71 (s, 3H), 3.46 - 3.35 (m, 2H), 3.27 - 3.02 (m, 6H), 2.40 - 1.99 (m, 13H), 1.90 - 1.81 (m, 4H), 1.79 (s, 12H), 1.76 - 1.43 (m 11H), 1.35 - 1.24 (m, 3H). **HRMS-ESI** $[M + H]^+$ m/z calcd for $[C_{41}H_{55}N_4O_9S_2]^+$ 811.3410, found 811.3373.

3. Tetrazine Reaction Kinetics With (*E*)-cyclooct-4-enol (TCO-OH) (Table 1)



Determination of rate constants via NMR competition experiments (Compounds 1-5): In a general procedure, mesitylene (5 μ L, 36 μ mol) was added to a solution of DIPY and the selected tetrazine (2.1 μ mol) in MeOD (0.7 mL). The mixture was transferred into a NMR tube and the ¹H NMR spectrum recorded. From this spectrum the initial concentrations of the two tetrazines were determined by integration of diagnostic peaks. At this point 21 μ L of a MeOD solution 25 mM in TCO-OH were added directly into the NMR tube. After 5 minutes the ¹H NMR spectra of the mixture was recorded, and the final concentration of the two tetrazines calculated. The final rate constants were obtained using the equation reported by Ingold and Shaw.⁵ Each experiment was repeated in triplicate.



Determination of rate constants via UV-vis spectroscopy (Compounds 6-8): The rate constants were determined under pseudo first order conditions using clear flat-bottomed 96-well plates. Stock solutions of the reagents in DMSO were diluted with PBS pH 7.4 to a final concentration of DMSO of 0.5 %. TCO-OH was used in excess as the dienophile at 0.6, 1.25, 2.25 and 5 mM concentrations with each of the tetrazines (0.05 mM). The decay of the tetrazine absorbance at the selected wavelength (390, 510 and 420 nm for compounds **8**, **9** and **10** respectively) was monitored at 37 °C. The change of absorbance in time was fitted to a first-order exponential, and the pseudo first order rate constant (k_{obs}) determined. The obtained k_{obs} were then plotted vs. the TCO concentration, fitted to a straight line, and the slope taken as the second order rate constant (k_2). Each experiment was repeated in triplicate.

4. Tetrazine Stability in Serum and PBS

DMSO stocks of tetrazines were diluted in PBS pH 7.4 or 100 % fetal bovine serum (FBS) to a concentration of 0.5 and 1 mM respectively, and a final DMSO concentration of 1%. The solutions were incubated at 37 °C under gentle shaking. The decay of the tetrazines absorbance at selected wavelengths (390, 420 or 520 nm) was monitored over 4 days, the percentage of intact compound determined, and the half-life calculated. Each experiment was repeated in triplicate.

5. Antibody Conjugate Reaction Kinetic with TCO-Fluorescein dye **12**

Trastuzumab conjugate bearing 2 tetrazines per antibody (0.4 reactive tetrazines per antibody) was utilized for the determination of the reaction constant in PBS. The measurements were performed under pseudo first order conditions, using a fixed amount of trastuzumab–tetrazine conjugate (0.065 μM) and an excess of TCO-fluorescein dye **12** at 0.65, 3.45 and 6.9 μM concentrations. The solutions were incubated at 37 °C and, at selected time points (1, 2.5, 5, 15 and 30 minutes), 100 μL aliquots were taken and quenched with (*E*)-cyclooct-4-enol (5 μL of a 20 mM solution in DMSO). Each mixture was subjected to size exclusion chromatography (PD MiniTrap G-25, 5K MWCO GE Healthcare) and the fluorescence intensity of the sample measured. The fluorescent intensity with time was fitted to a first-order exponential, and the pseudo first order rate constant (k_{obs}) determined. The obtained k_{obs} were then plotted vs. the TCO dye concentration, fitted to a straight line, and the slope taken as the second order rate constant (k_2) (FigureS6). Each measurement was repeated in triplicate. The k_2 was determined to be $3083 \pm 352 \text{ s}^{-1} \text{ M}^{-1}$.

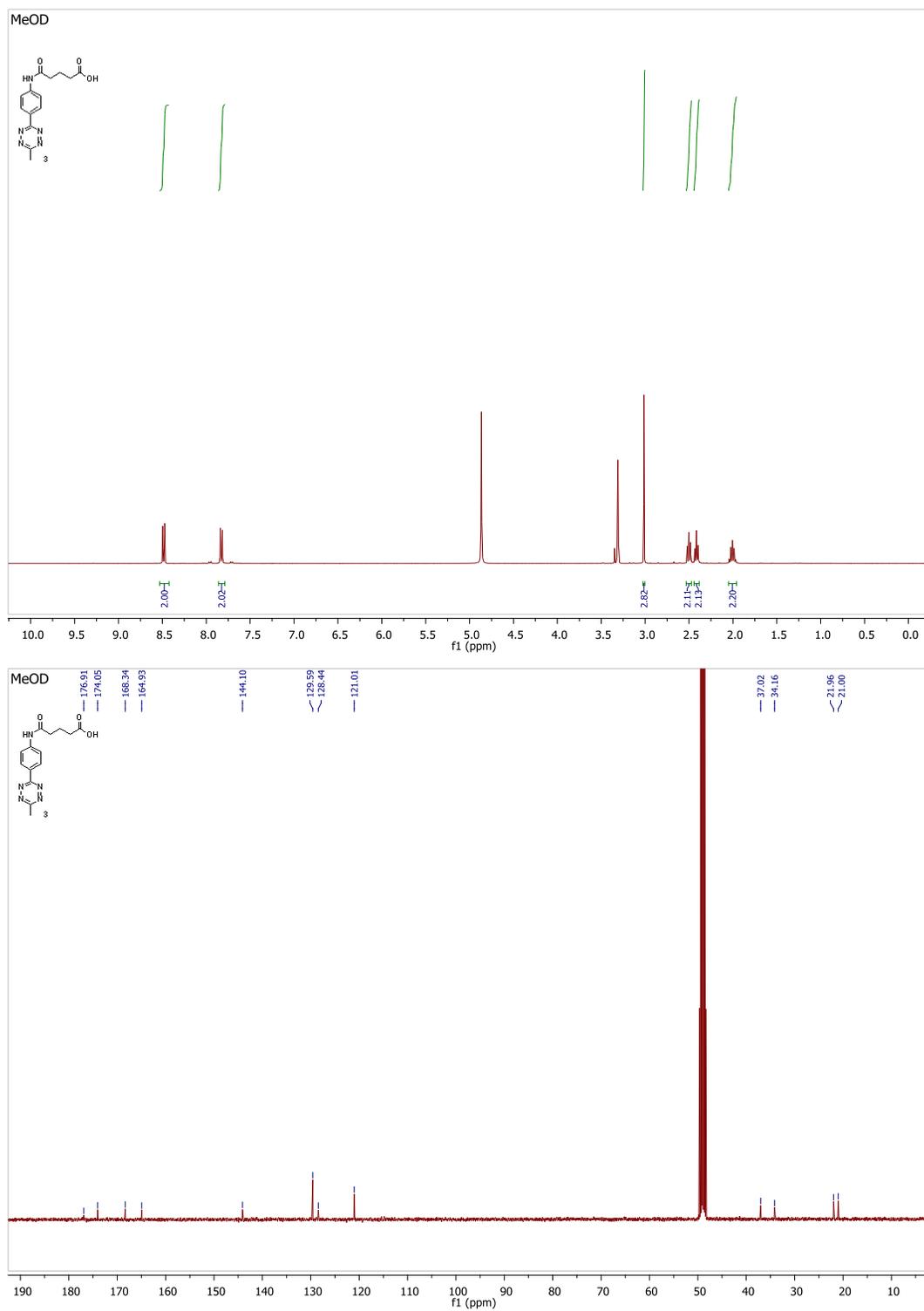
6. References

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- 2 H. Zhou, J. Woo, A. M. Cok, M. Wang, B. D. Olsen, J. A. Johnson, *Proc. Natl. Acad. Sci.*, 2012, **109**, 19119.
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7. Figures

Figure S1 . ^1H and ^{13}C NMR spectra of: (A) tetrazine **3** and (B) tetrazine-NHS ester **11**.

A



B

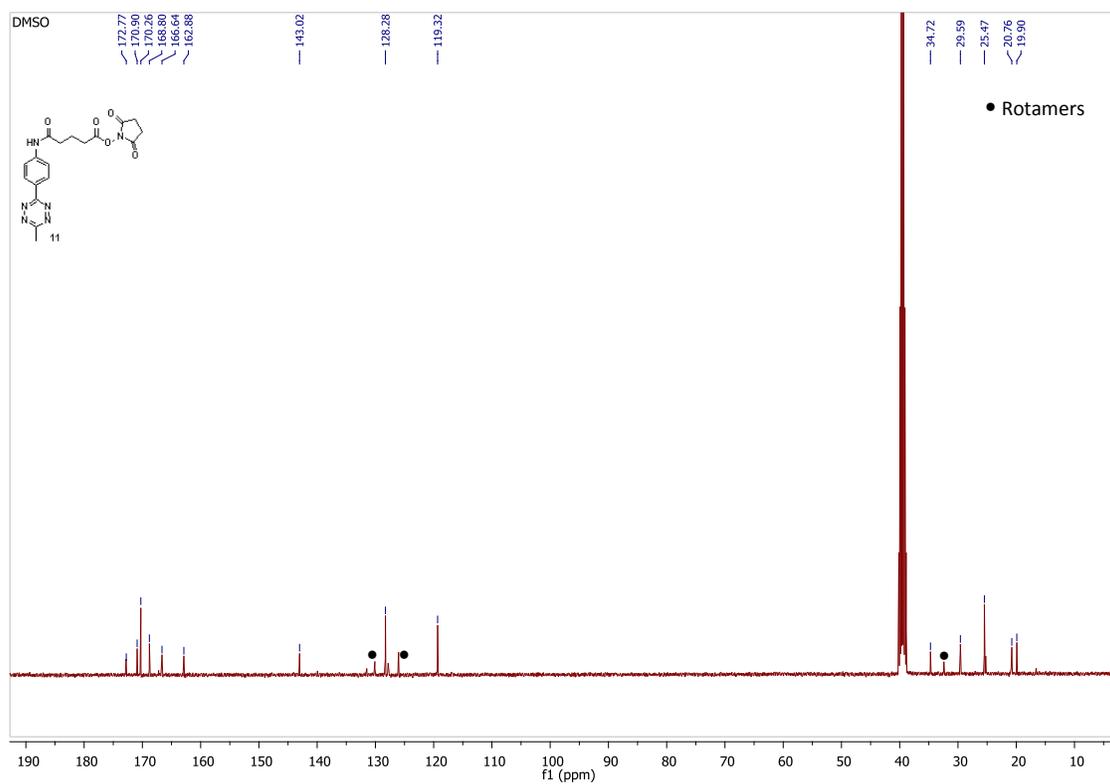
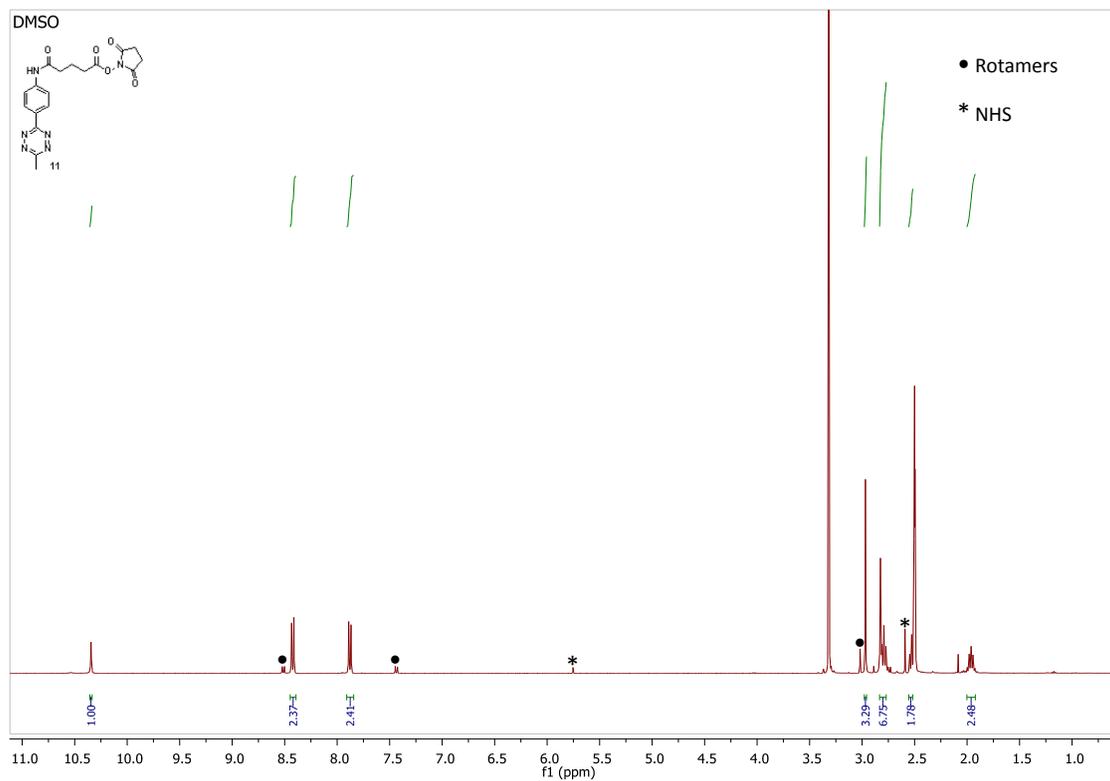


Figure S2. ¹H NMR spectra of dye 12.

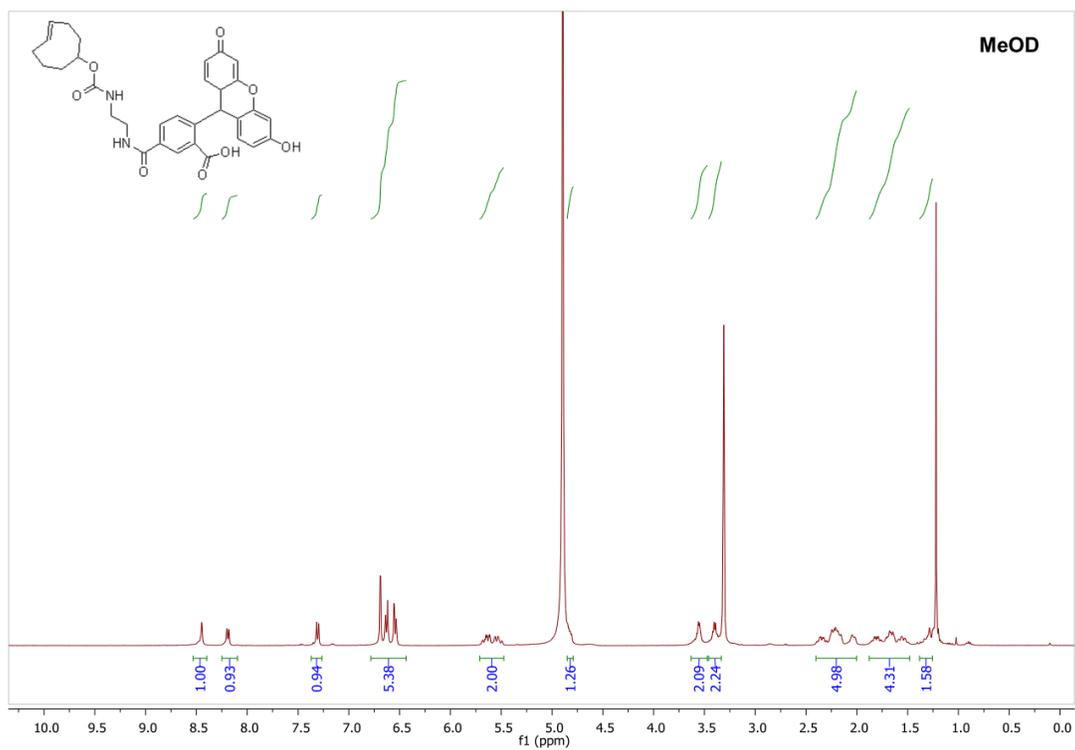
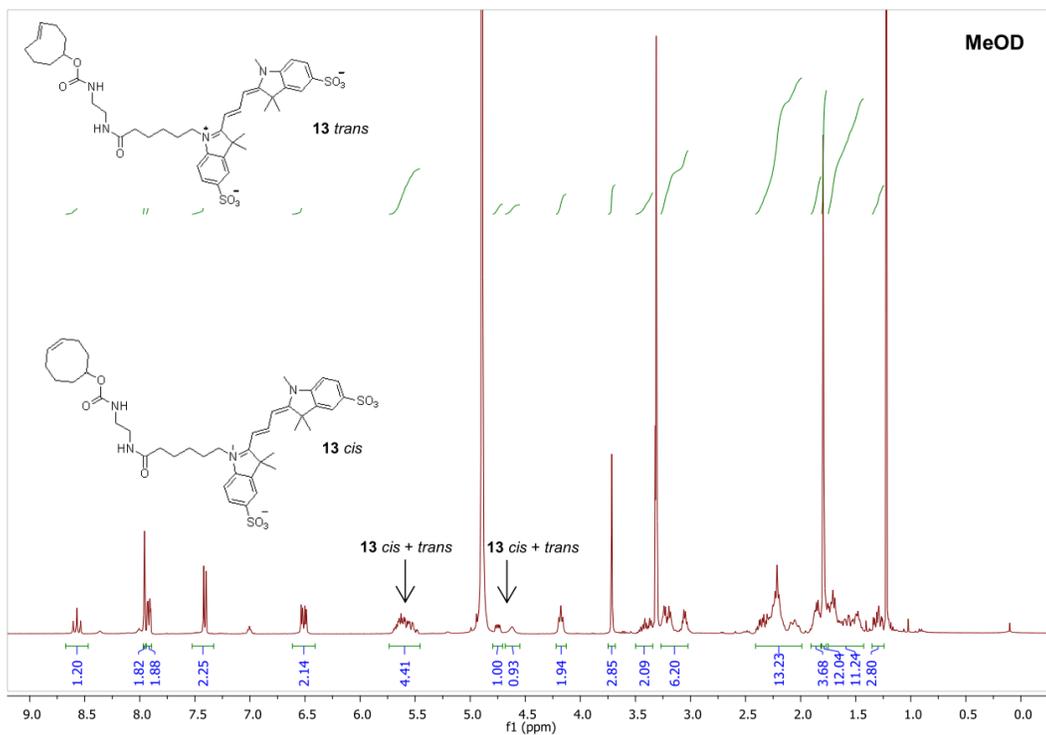


Figure S3. ¹H NMR spectra of dye 13



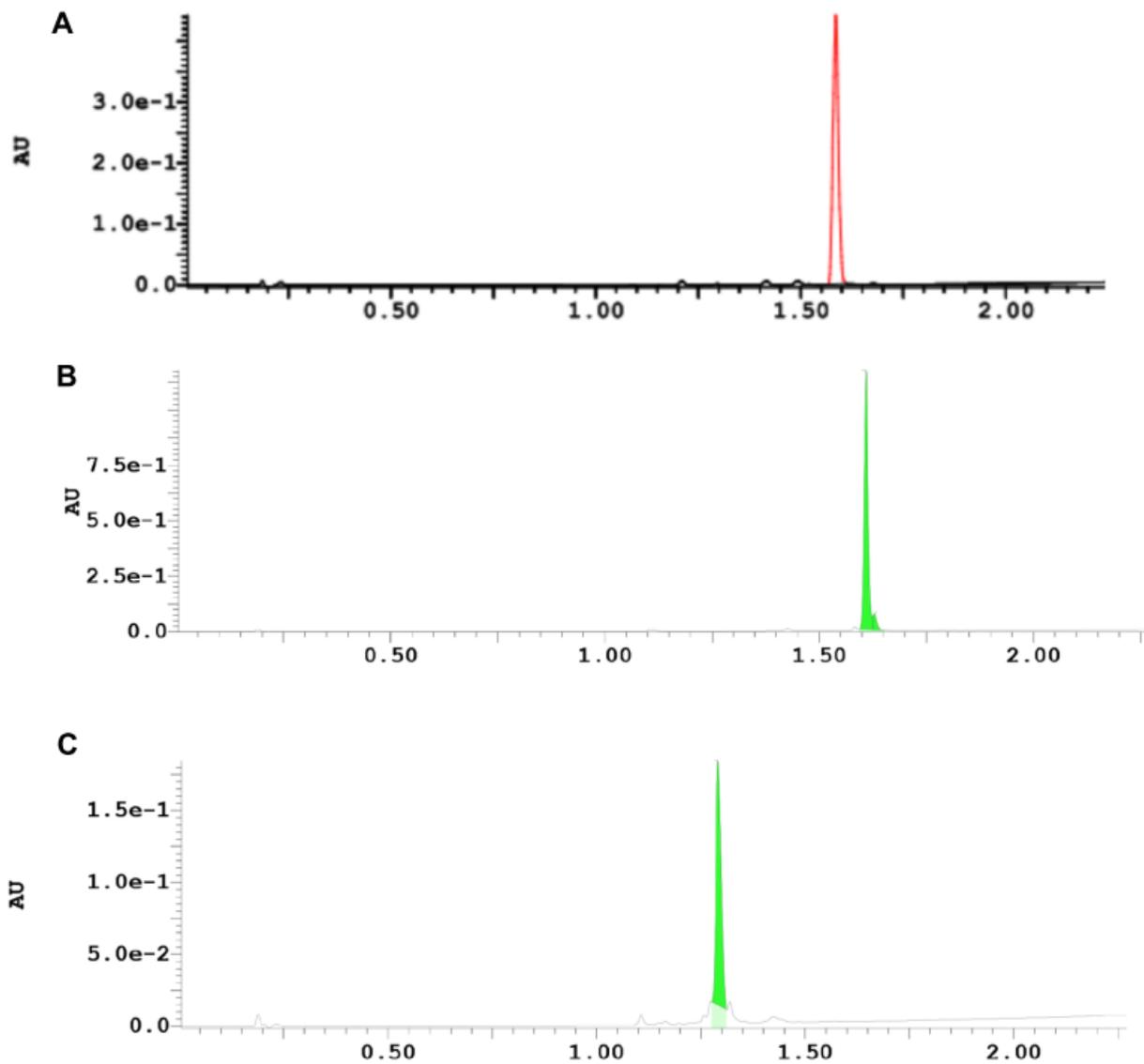


Figure S4. UPLC traces of (A) tetrazine-NHS ester **11** (B) TCO-fluorescein **12**, and (C) TCO-Cy3/CCO-Cy3 **13**. Chromatograms were obtained using a gradient of ACN:H₂O from 5% to 95% containing 1% formic acid with an elution time of 3 minutes.

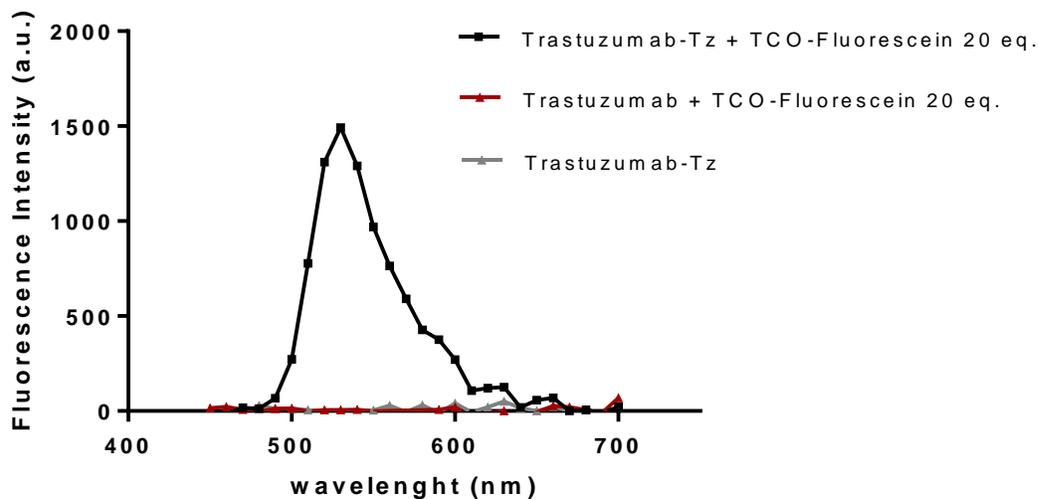


Figure S5. Fluorescence spectra of the trastuzumab-tetrazine (Tz) conjugate (Ab- 100 x Tz) before and after treatment with 20 equivalents (eq.) of dye **12**, and of unmodified trastuzumab after treatment with 20 eq. of dye **12**.

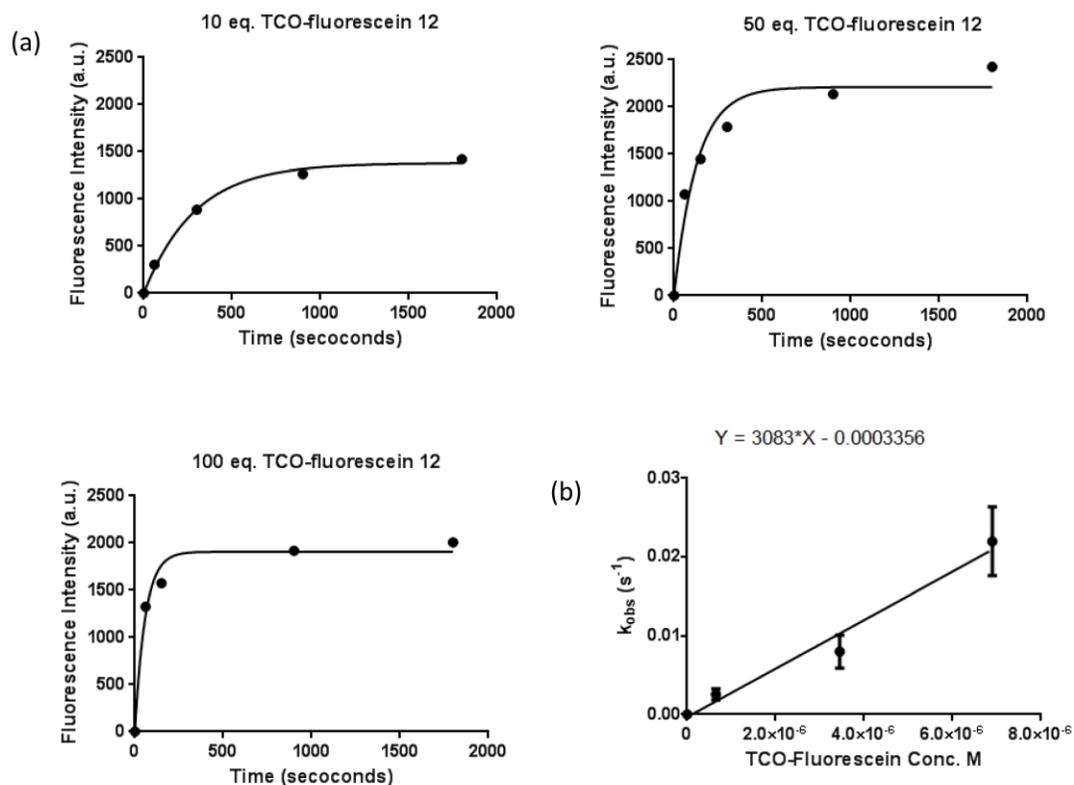


Figure S6. (a) Exponential curve fitting of fluorescence intensity vs. time, (b) linear regression of k_{obs} vs. dye **12** concentration.

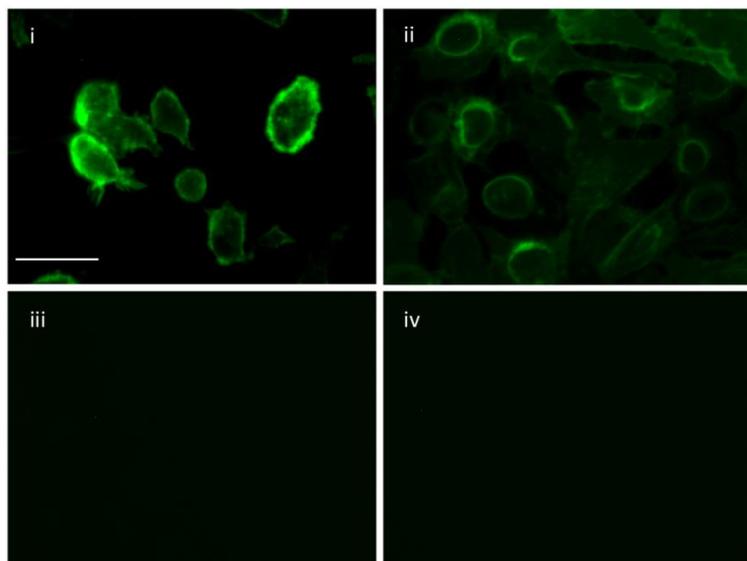
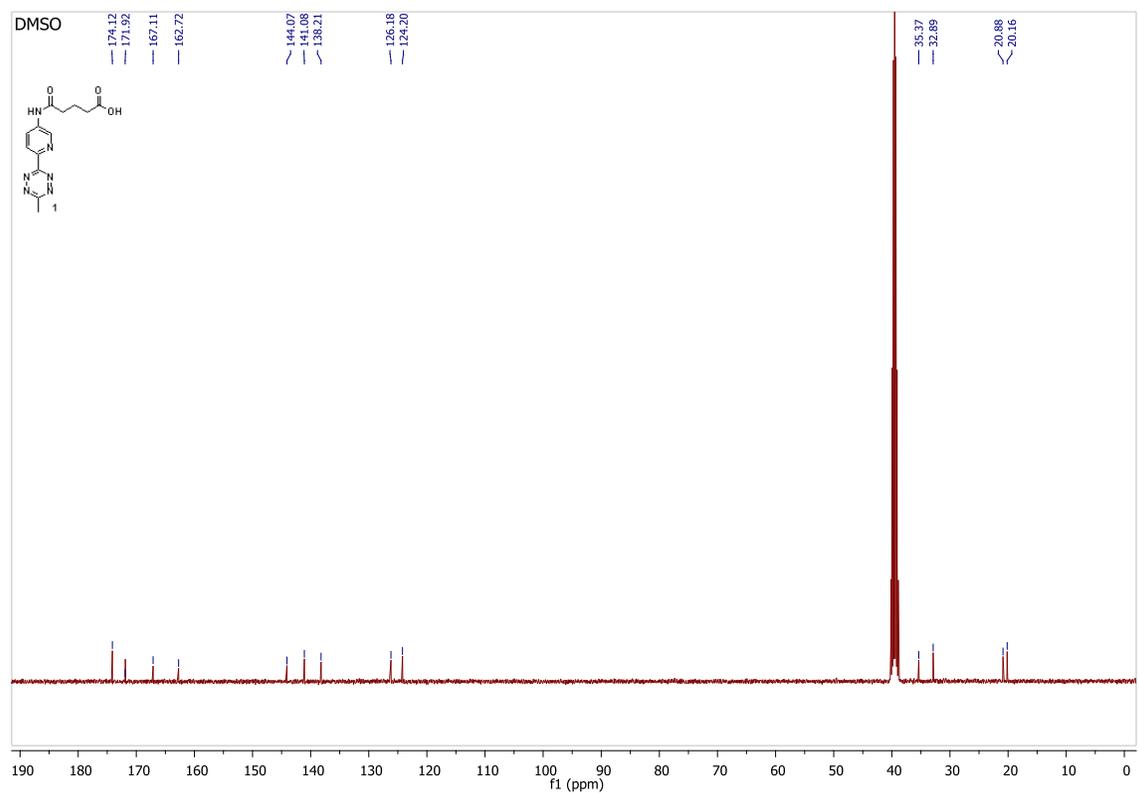
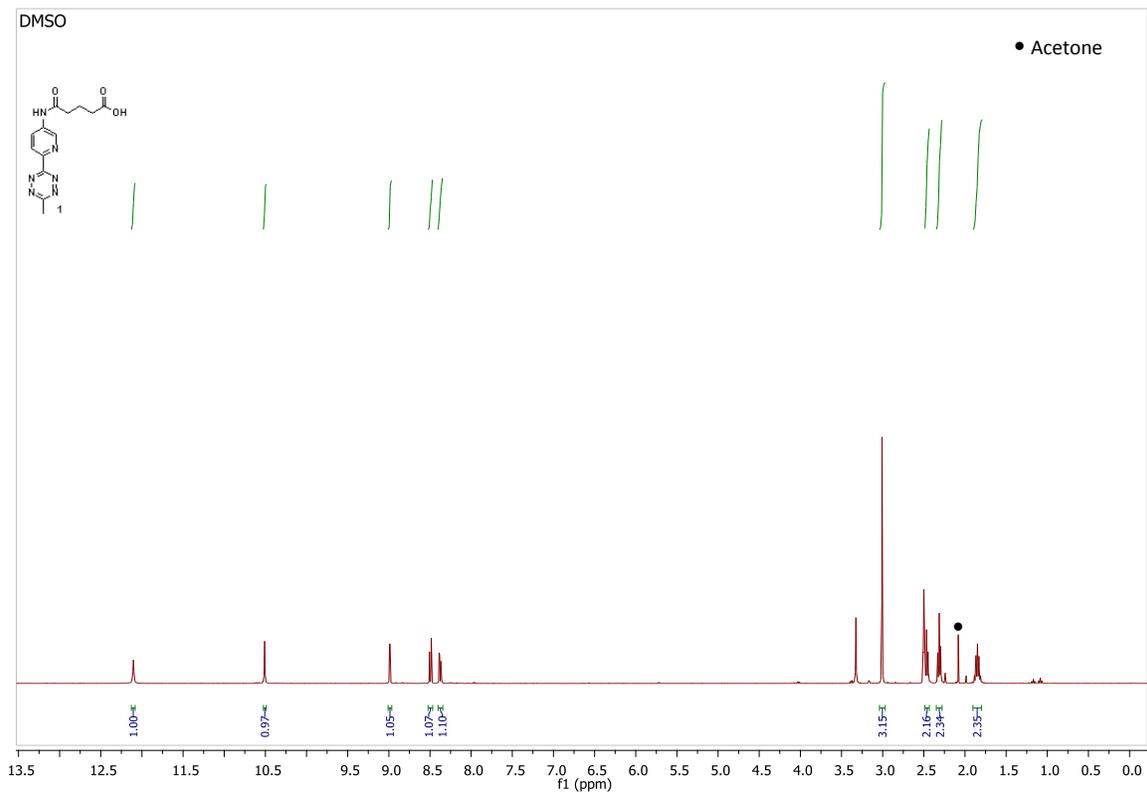
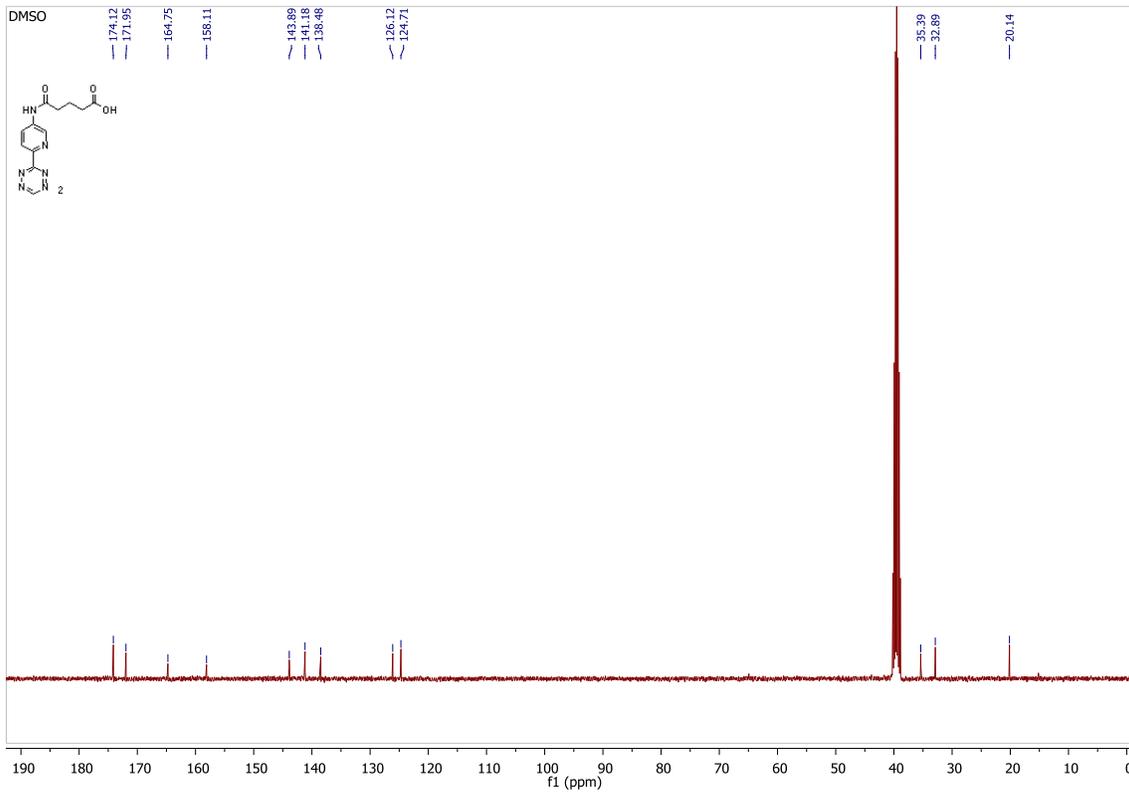
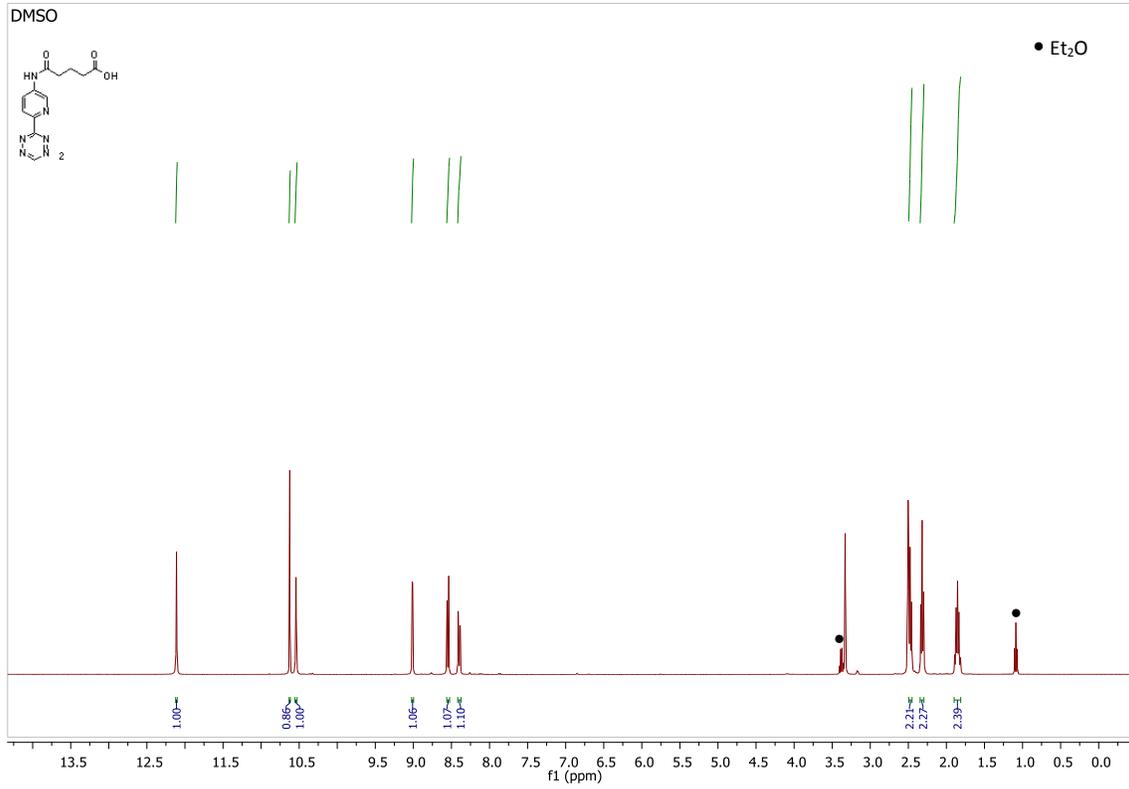
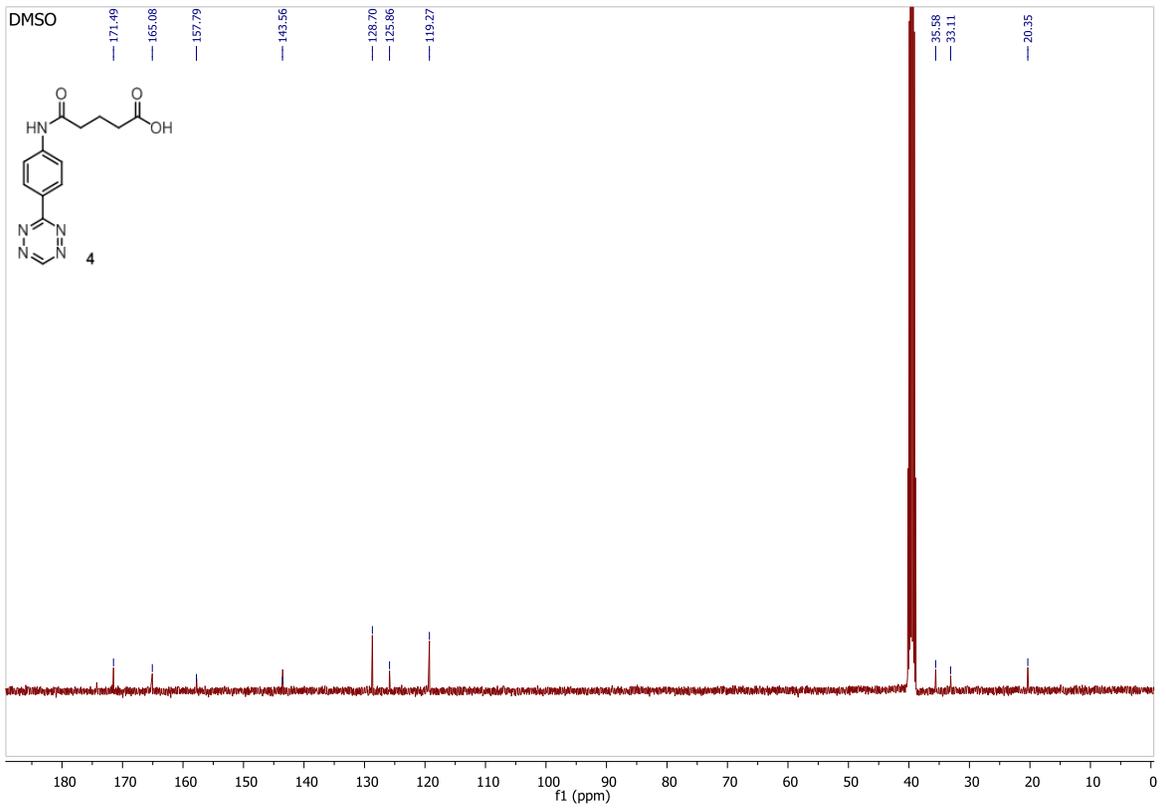
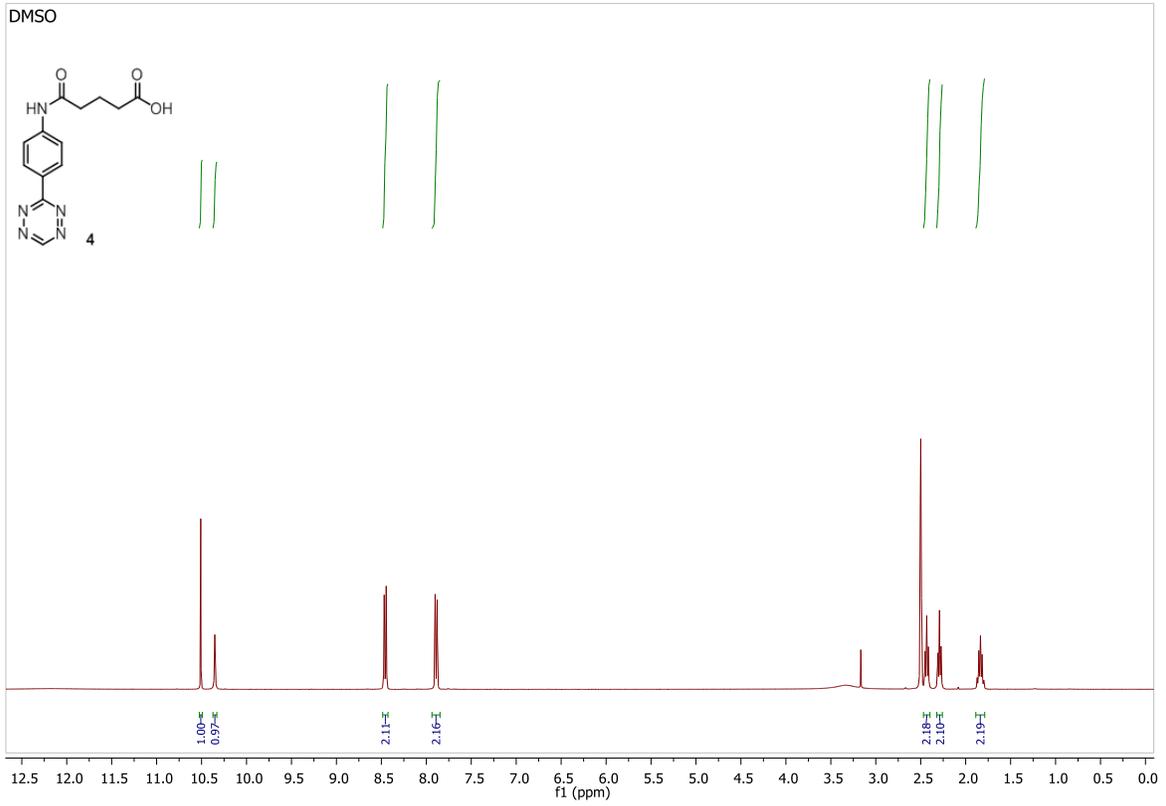


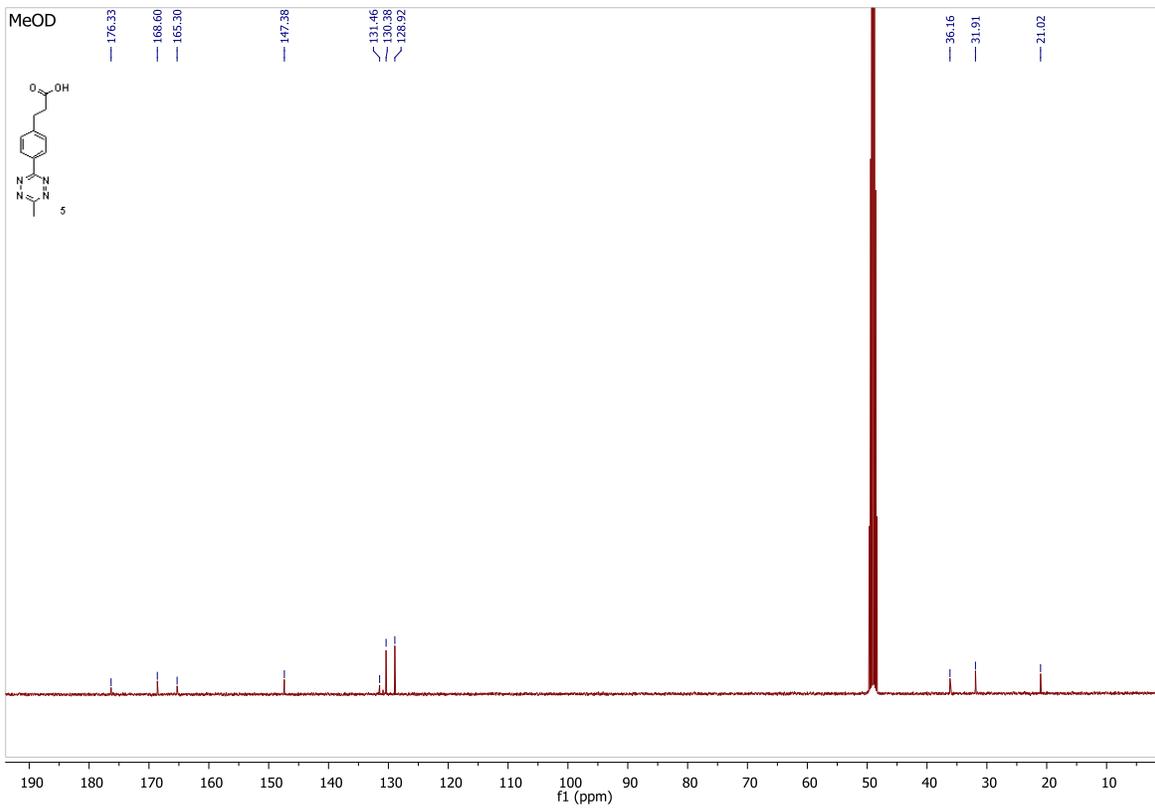
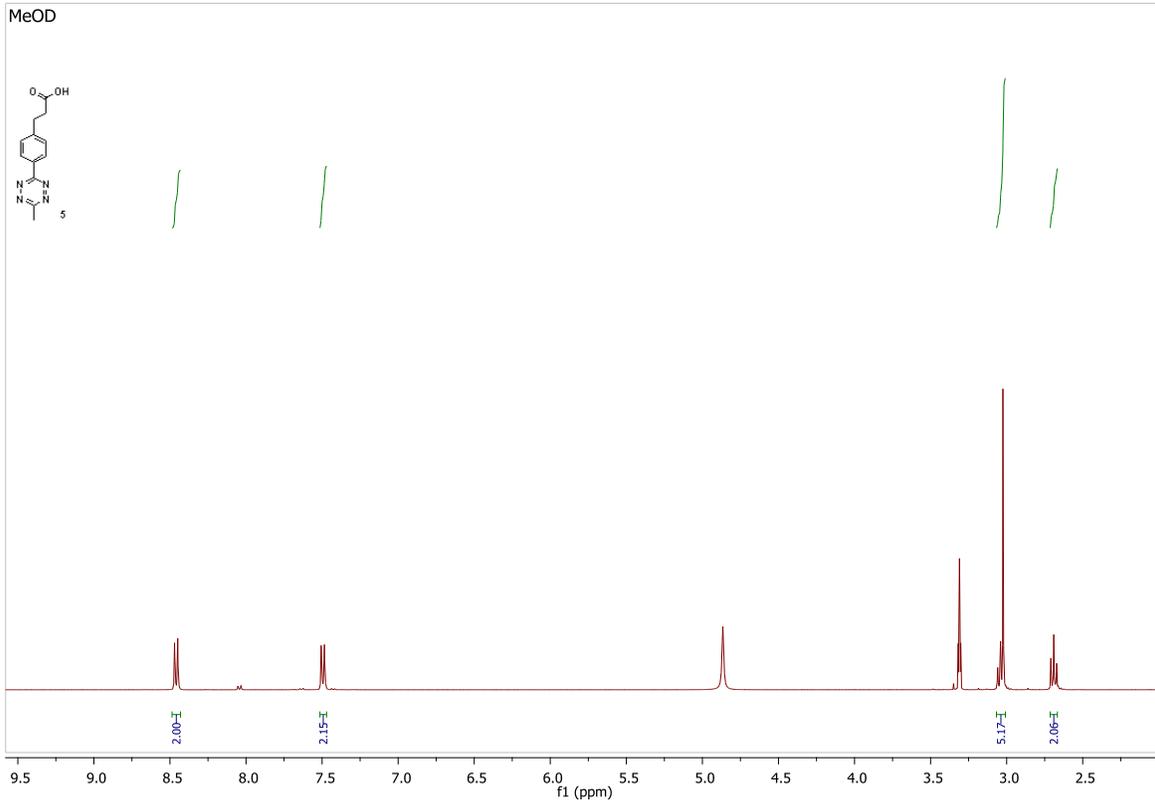
Figure S7, Related to Figure 5. SKOV-3 cells incubated with: (i) trastuzumab-fluorescein (positive control 0.05 μM); (ii) preclicked antibody (trastuzumab-tetrazine-**3**-TCO-fluorescein 0.05 μM); (iii) Cy3 dye **13** (10 μM , negative control); (iv) TCO-fluorescein dye **12** (10 μM , negative control). Scale bar 50 μm .

Figure S8. Additional ^1H and ^{13}C NMR spectra

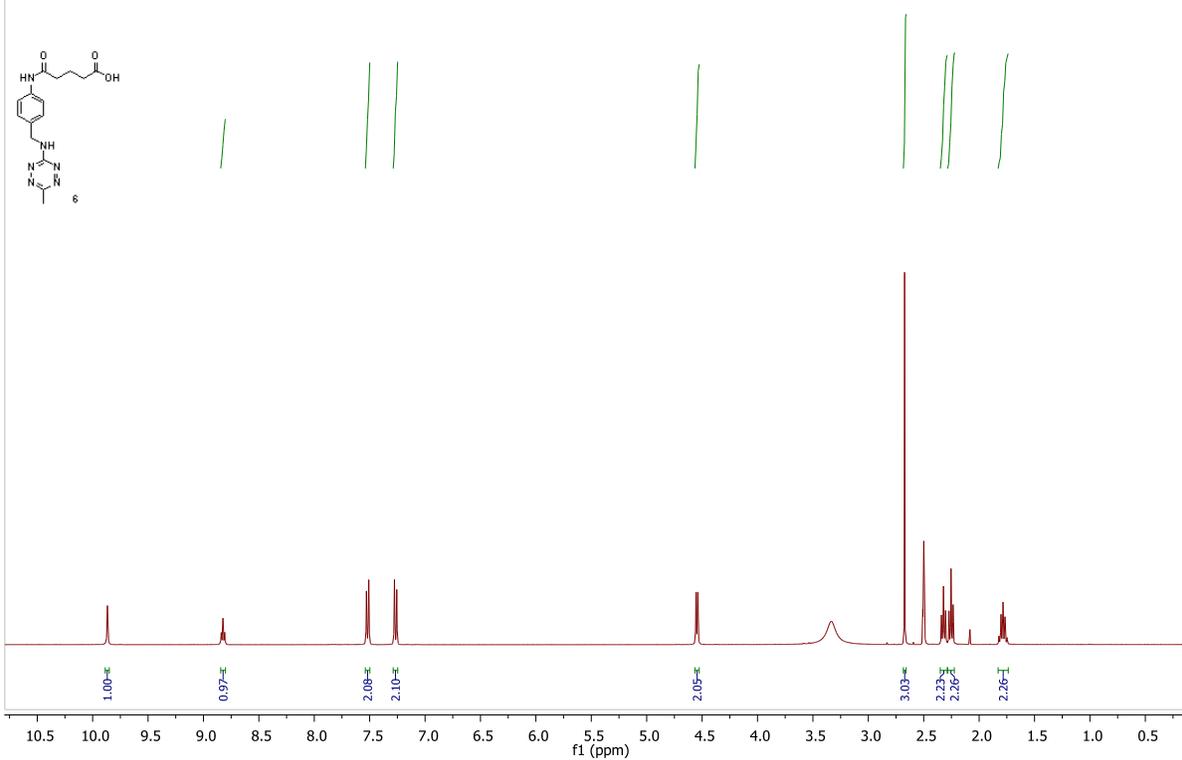
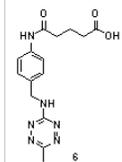








DMSO



DMSO

