Supporting Information

Rapid Bioorthogonal Chemistry Turn-on through Enzymatic or Long Wavelength Photocatalytic Activation of Tetrazine Ligation

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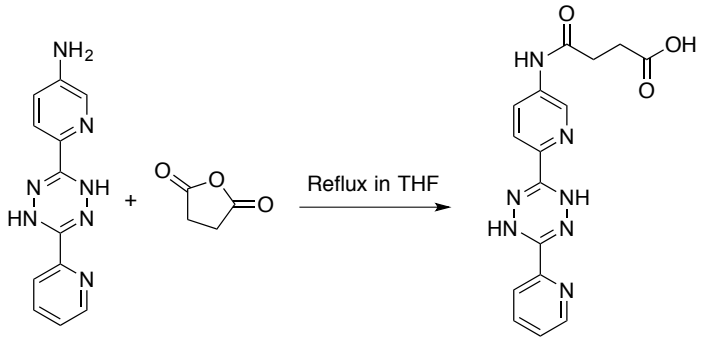
1. Synthetic Procedures

1.1 General Consideration

All reactions were carried out in glassware that was flame-dried under vacuum and cooled under nitrogen. THF was purified by distillation from Na/benzophenone. Flash Chromatography was performed using normal phase Silicycle silica gel (40-63D, 60Å). An APT pulse sequence was used for ^{13}C NMR spectra, where methylene and quaternary carbons appear 'up' (u), and methine and methyl carbons appear 'down' (dn). Other solvents and reagents were purchased from commercial sources without additional purification.

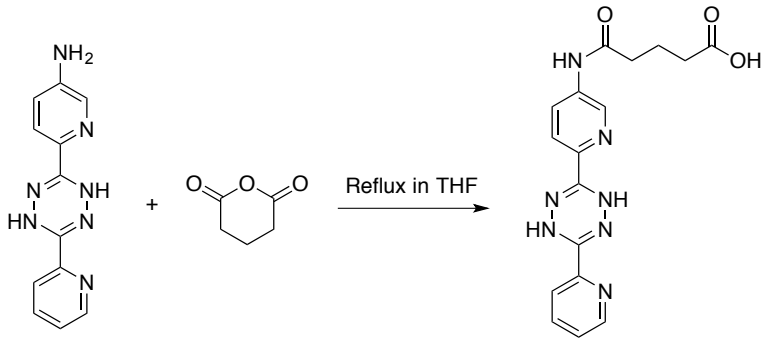
1.2 Synthesis of 4-oxo-4-((6-(6-(pyridin-2-yl)-1,4-dihydro-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)amino)butanoic acid (3a)



3a, 90%

To a dry round-bottom flask was added 6-(6-(pyridin-2-yl)-1,4-dihydro-1,2,4,5-tetrazin-3-yl)pyridin-3-amine (200 mg, 0.79 mmol)[2], succinic anhydride (400 mg, 4.00 mmol) and anhydrous THF (8 mL). The mixture was refluxed for 24 hours at 60 °C and then cooled by an ice bath. The precipitate was filtered and sequentially washed by THF (2 mL) and ethyl acetate (3×3 mL) and dried to yield the title compound (251 mg, 0.71 mmol, 90%) as an orange solid. ^{1}H NMR (400 MHz, DMSO-d_6, δ): 12.20 (br, 1H), 10.47 (s, 1H), 8.95 (s, 1H), 8.89 (s, 1H), 8.82 (d, J = 2.4 Hz, 1H), 8.71 – 8.58 (m, 1H), 8.14 (dd, J = 8.8, 2.5 Hz, 1H), 8.01 – 7.85 (m, 3H), 7.53 (ddd, J = 6.9, 4.8, 1.6 Hz, 1H), 2.63 (t, J = 6.6 Hz, 2H), 2.54 (t, J = 6.9 Hz, 2H). ^{13}C NMR (100 MHz, DMSO-d_6, δ): 173.83 (u, 1C), 171.10 (u, 1C), 148.67 (dn, 1C), 147.36 (u, 1C), 146.42 (u, 1C), 146.15 (u, 1C), 141.43 (u, 1C), 138.75 (dn, 1C), 137.48 (dn, 1C), 137.33 (u, 1C), 126.55 (dn, 1C), 125.38 (dn, 1C), 121.49 (dn, 1C), 121.02 (dn, 1C), 31.07 (u, 1C), 28.63 (u, 1C). HRMS (LIFDI) [M^+]: calcd. for $C_{16}H_{15}N_7O_3^+$, 353.1236 found 353.1277.

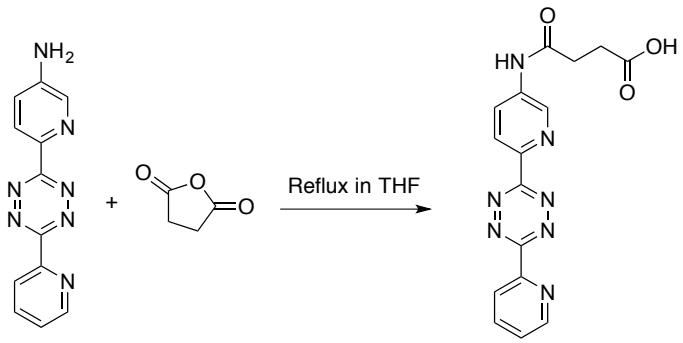
1.3 Synthesis of 5-oxo-5-((6-(6-(pyridin-2-yl)-1,4-dihydro-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)amino)pentanoic acid (3b)



3b, 80%

To a dry round-bottom flask was added 6-(6-(pyridin-2-yl)-1,4-dihydro-1,2,4,5-tetrazin-3-yl)pyridin-3-amine (1.87 g, 7.39 mmol)[2], glutaric anhydride (1.01 g, 8.87 mmol) and anhydrous THF (70 mL). The mixture was refluxed for 24 hours at 60 °C and then cooled by an ice bath. The precipitate was filtered on a Buchner funnel and sequentially rinsed by THF (10 mL) and ethyl acetate (3×10 mL) and dried to yield the title compound (2.16 g, 5.89 mmol, 80%) as a orange solid. ^{1}H NMR (600 MHz, $CDCl_3$, δ): 12.22 (br, 1H), 10.40 (s, 1H), 8.95 (s, 1H), 8.90 (s, 1H), 8.81 (d, *J* = 2.4 Hz, 1H), 8.67 – 8.58 (m, 1H), 8.16 (dd, *J* = 8.8, 2.5 Hz, 1H), 8.02 – 7.85 (m, 3H), 7.53 (ddd, *J* = 6.9, 4.8, 1.6 Hz, 1H), 2.42 (t, *J* = 7.4 Hz, 2H), 2.29 (t, *J* = 7.3 Hz, 2H), 1.89 – 1.76 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6, δ): 174.25 (u, 1C), 171.68 (u, 1C), 148.66 (dn, 1C), 147.35 (u, 1C), 146.42 (u, 1C), 146.15 (u, 1C), 141.45 (u, 1C), 138.91 (dn, 1C), 137.47 (dn, 1C), 137.31 (u, 1C), 126.69 (dn, 1C), 125.37 (dn, 1C), 121.45 (dn, 1C), 121.02 (dn, 1C), 35.36 (u, 1C), 32.96 (u, 1C), 20.24 (u, 1C). HRMS (LIFDI) [M^+]: calcd. for $C_{17}H_{17}N_7O_3^+$, 367.1393 found 367.1403.

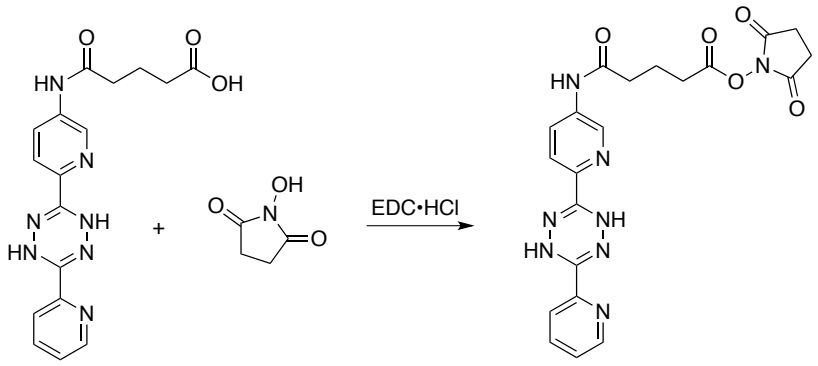
1.4 Synthesis of 4-oxo-4-((6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)amino)butanoic acid (4a)



4a, 46%

To a flame dried flask under nitrogen, 6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-amine (50 mg, 0.20 mmol)[2] and succinic anhydride (114 mg, 1.14 mmol) were added. The flask was charged with tetrahydrofuran (4 mL) and heated to 70°C for 21 hours. The reaction solution was cooled to room temperature before diluting with ethyl acetate (4 mL) and further chilling to 0°C for 15 minutes. Filtering and rinsing with ethyl acetate and diethyl ether (3 × 5 mL) yielded a dark, cherry red powder. The solid was dissolved in 1.5 mL warm dimethylformamide and purified by chromatography (10-100% acetone in hexanes then 1% acetic acid in acetone on 10% triethylamine in hexanes treated silica). After drying in vacuo, the recovered pink solid was rinsed with ice water yielding the title compound (32 mg, 46%). An additional 12 mg (24%) of 6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-amine was recovered during chromatography. ^{1}H NMR (400 MHz, DMSO-d_6, δ) δ 12.19 (s, 1H), 10.65 (s, 1H), 9.05 (d, J = 2.5 Hz, 1H), 8.98 – 8.88 (m, 1H), 8.60 (dd, J = 12.9, 8.3 Hz, 2H), 8.41 (dd, J = 8.7, 2.5 Hz, 1H), 8.15 (td, J = 7.8, 1.8 Hz, 1H), 7.72 (ddd, J = 7.6, 4.7, 1.2 Hz, 1H), 2.69 (dd, J = 7.4, 5.6 Hz, 2H), 2.58 (dd, J = 7.4, 5.7 Hz, 2H). ^{13}C NMR (100 MHz, DMSO-d_6, δ): 173.77 (u, 1C), 171.44 (u, 1C), 163.04 (u, 1C), 162.76 (u, 1C), 150.61 (dn, 1C), 150.20 (u, 1C), 143.79 (u, 1C), 141.13 (dn, 1C), 138.52 (u, 1C), 137.82 (dn, 1C), 126.60 (dn, 1C), 126.02 (dn, 1C), 124.96 (dn, 1C), 124.20 (dn, 1C), 31.16 (u, 1C), 28.61 (u, 1C). HRMS (LIFDI) [MH$^+$]: calcd. for $C_{16}H_{13}N_7O_3^+$, 352.1153 found 352.1136.

1.5 Synthesis of 2,5-dioxopyrrolidin-1-yl 5-oxo-5-((6-(6-(pyridin-2-yl)-1,4-dihydro-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)amino)pentanoate (S1)

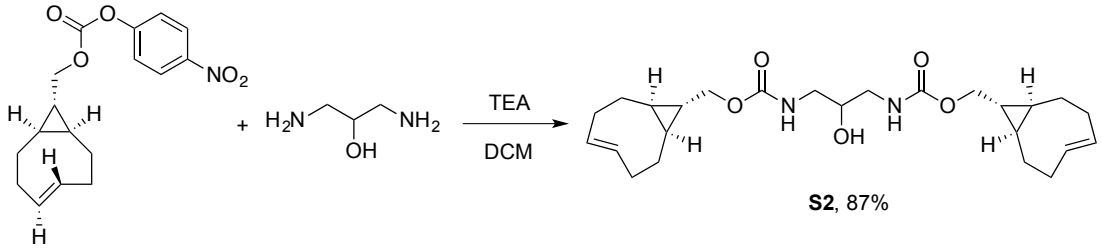


S1, 80%

To a dry round-bottom flask was added **1** (200 mg, 0.54 mmol), *N*-hydroxysuccinimide (125 mg, 1.09 mmol), *N*-(3-Dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (209 mg, 1.09 mmol) and anhydrous DMF (2 mL). The mixture was stirred for 1 hour at room temperature. DMF was removed by rotary evaporation at 50 °C using an efficient vacuum pump (<1 torr). The crude product was dissolved in acetone and then concentrated onto silica gel. Purification by column chromatography using a gradient (10%-70%) of acetone in

hexanes yielded 202 mg (0.44 mmol, 80%) of the title compound as an orange solid. ^{1}H NMR (600 MHz, $CDCl_3$, δ): 8.61 (d, *J* = 2.5 Hz, 1H), 8.57 (dd, *J* = 4.8, 1.4 Hz, 1H), 8.53 (s, 1H), 8.48 (s, 1H), 8.31 (s, 1H), 8.20 (dd, *J* = 8.8, 2.6 Hz, 1H), 8.10 – 7.96 (m, 2H), 7.78 – 7.70 (m, 1H), 7.35 (dd, *J* = 7.5, 4.9 Hz, 1H), 2.97 – 2.90 (m, 4H), 2.76 – 2.70 (m, 2H), 2.55 – 2.50 (m, 2H), 2.28 – 2.19 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$, δ): 170.70 (u, 1C), 169.86 (u, 2C), 168.44 (u, 1C), 148.53 (dn, 1C), 147.60 (u, 1C), 146.91 (u, 1C), 146.48 (u, 1C), 142.80 (u, 1C), 139.32 (dn, 1C), 136.86 (dn, 1C), 136.26 (u, 1C), 127.29 (dn, 1C), 125.02 (dn, 1C), 121.75 (dn, 1C), 121.35 (dn, 1C), 35.47 (u, 1C), 29.94 (u, 1C), 25.80 (u, 2C), 21.41 (u, 1C). HRMS (LIFDI) [M^+]: calcd. for $C_{21}H_{20}N_8O_5^+$, 464.1557 found 464.1541.

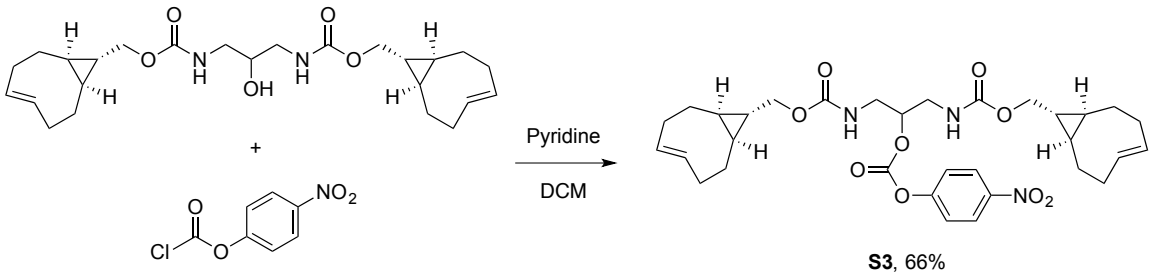
1.6 Synthesis of bis(((E)-bicyclo[6.1.0]non-4-en-9-yl)methyl) (2-hydroxypropane-1,3-diyl)dicarbamate (S2)



S2, 87%

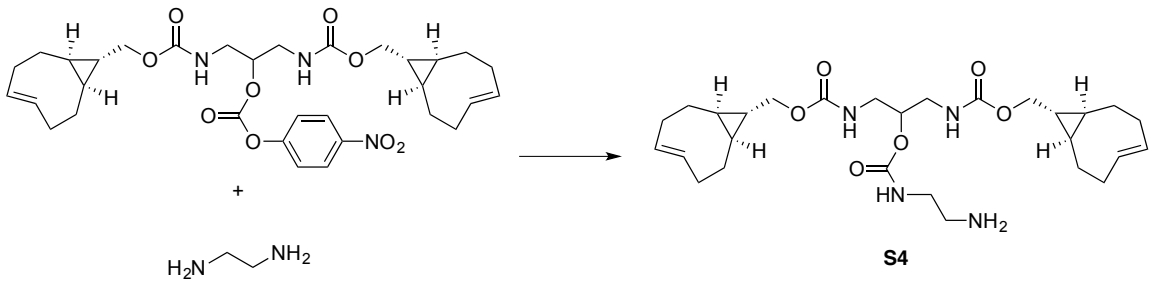
A dry round-bottom flask was sequentially charged *via* syringe with a solution of 1,3-diamino-2-propanol (120 mg, 1.33 mmol) in anhydrous dichloromethane (20 mL) followed by anhydrous triethylamine (744 µL, 5.37 mmol) and (1R,8S,9R,4E)-bicyclo[6.1.0]non-4-en-9-ylmethyl(4-nitrophenyl) carbonate[1] (930 mg, 2.93 mmol). The mixture was stirred overnight at room temperature, diluted with dichloromethane (30 mL) followed by exhaustive aqueous wash (5×50 mL). The organic layer was dried with $MgSO_4$, filtered and then the solvent was removed with a rotary evaporator. Purification by column chromatography first using 10% ethyl acetate in hexanes then switching to 30% acetone in hexanes yielded the title compound (520 mg, 1.16 mmol, 87%) as a colorless oil. ^{1}H NMR (600 MHz, $CDCl_3$, δ): 5.82 (ddd, *J* = 16.1, 9.3, 6.1 Hz, 2H), 5.29 (br, 2H), 5.12 (ddd, *J* = 16.1, 10.6, 3.4 Hz, 2H), 3.94 (d, *J* = 6.3 Hz, 4H), 3.74 – 3.80 (m, 1H), 3.47 (br, 1H), 3.35 – 3.15 (m, 4H), 2.39 – 3.32 (m, 2H), 2.30 – 2.24 (m, 4H), 2.24 – 2.14 (m, 2H), 2.00 – 1.86 (m, 4H), 0.90 – 0.78 (m, 2H), 0.60 – 0.50 (m, 4H), 0.45 – 0.36 (m, 4H).^{13}C NMR (100 MHz, $CDCl_3$, δ): 158.26 (u, 2C), 138.49 (dn, 2C), 131.43 (dn, 2C), 71.10 (dn, 1C), 70.11 (u, 2C), 43.82 (u, 2C), 38.78 (u, 2C), 33.89 (u, 2C), 32.70 (u, 2C), 27.76 (u, 2C), 24.69 (dn, 2C), 22.14 (dn, 2C), 21.12 (dn, 2C). HRMS (LIFDI) [M^+]: calcd. for $C_{25}H_{38}N_2O_5^+$, 446.2781 found 446.2791.

1.7 Synthesis of bis(((E)-bicyclo[6.1.0]non-4-en-9-yl)methyl) (2-(((4-nitrophenoxy)carbonyl)oxy)propane-1,3-diyl)dicarbamate (S3)



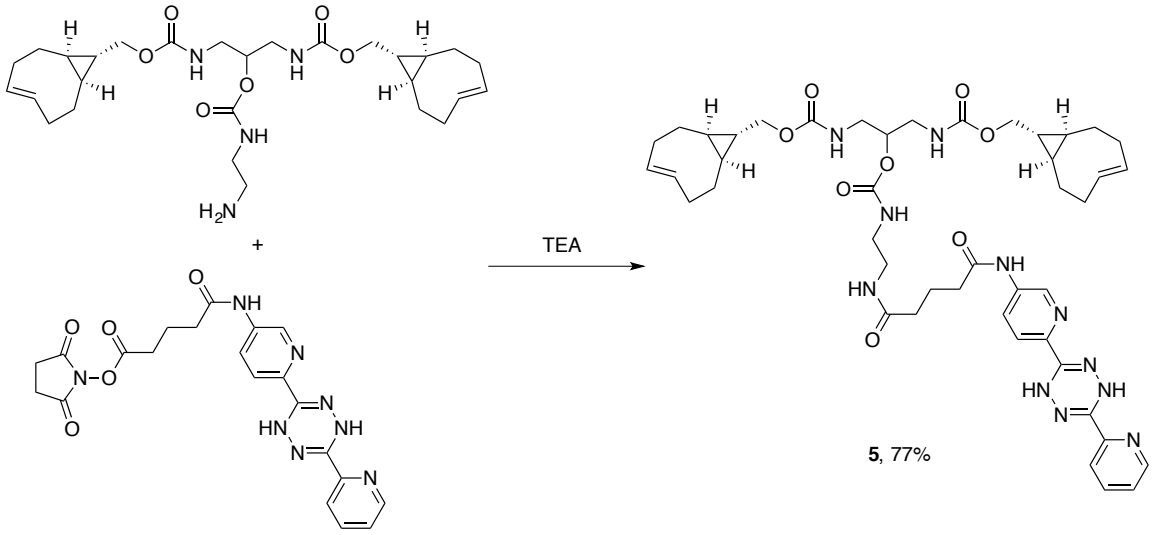
A dry round-bottled flask was charged with **S2** (500 mg, 1.12 mmol). Anhydrous dichloromethane (30 mL) and pyridine (0.23 mL, 2.80 mmol) were added to the flask. A solution of 4-nitrophenylchloroformate (271 mg, 1.34 mmol) in anhydrous dichloromethane (4 mL) was added to the flask via syringe and the solution was stirred for 1 h at room temperature. Saturated aq. NH_4Cl was added to the mixture and the layers were separated, and the aqueous layer was extracted twice with dichloromethane. The organic layers were combined, dried with $MgSO_4$ and filtered, and the solvent was removed using a rotary evaporator. Purification by column chromatography (10% to 30% ethyl actetate/hexanes) yielded 450 mg (0.74 mmol, 66%) of the title compound as a white solid. ^{1}H NMR (600 MHz, $CDCl_3$, δ): 8.28 (d, J = 9.2 Hz, 2H), 7.41 (d, J = 9.2 Hz, 2H), 5.85 (ddd, J = 16.0, 9.3, 6.2 Hz, 2H), 5.41 – 5.22 (m, 2H), 5.11 (ddd, J = 16.8, 10.5, 3.9 Hz, 2H), 4.83 – 4.75 (m, 1H), 3.96 (d, J = 6.5 Hz, 4H), 3.79 – 3.17 (m, 4H), 2.40 – 2.30 (m, 2H), 2.30 – 2.15 (m, 6H), 2.01 – 1.80 (m, 4H), 0.90 – 0.75 (m, 2H), 0.62 – 0.48 (m, 4H), 0.45 – 0.35 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$, δ): 157.49 (u, 2C), 155.51 (u, 1C), 151.85 (u, 1C), 145.59 (u, 1C), 138.45 (dn, 2C), 131.41 (dn, 2C), 125.48 (dn, 2C), 122.00 (dn, 2C), 76.95 (dn, 1C), 70.19 (u, 2C), 40.06 (u, 2C), 38.75 (u, 2C), 33.86 (u, 2C), 32.67 (u, 2C), 27.73 (u, 2C), 24.69 (dn, 2C), 22.14 (dn, 2C), 21.13 (dn, 2C). HRMS (LIFDI) [M^+]: calcd. for $C_{32}H_{41}N_3O_9^+$, 611.2843 found 611.2837.

1.8 Synthesis of bis(((E)-bicyclo[6.1.0]non-4-en-9-yl)methyl) (2-(((2-aminoethyl)carbamoyl)oxy)propane-1,3-diyl)dicarbamate (S4)



A dry round-bottom flask was sequentially charged *via* syringe with ethylenediamine (218 µL, 3.27 mmol) followed by a solution of **S3** (100 mg, 0.16 mmol) in anhydrous dichloromethane (4 mL). The solution was stirred for 1 h at room temperature, diluted with dichloromethane (15 mL) and followed by exhaustive aqueous washes (5×30 mL). The organic layer was dried with $MgSO_4$, filtered and concentrated down with a rotary evaporator to afford the title compound (80 mg, 92% crude yield) as a pale yellow solid. The crude product was carried to the next step of synthesis without further purification.

1.9 Synthesis of bis(((E)-bicyclo[6.1.0]non-4-en-9-yl)methyl) (2-(((2-(5-oxo-5-((6-(6-(pyridin-2-yl)-1,4-dihydro-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)amino)pentanamido)ethyl)carbamoyl)oxy)propane-1,3-diyl)dicarbamate (5)



To a dry round-bottom flask was added **S4** (39 mg, 73.3 µmol), **S1** (25 mg, 53.9 µmol) and a solution of triethylamine (17 µL, 0.12 mmol) in dichloromethane (2 mL). The mixture was stirred for 1 hour under room temperature and then concentrated onto silica gel using a rotary evaporator. Purification by column chromatography using a gradient (20%-70%) of acetone in hexanes yielded the title compound (37 mg, 42.0 µmol, 77%) as a orange solid. ^{1}H NMR (400 MHz, MeOH-d_4, δ): 8.85 (d, J = 2.4 Hz, 1H), 8.64 (dt, J = 5.0, 1.3 Hz, 1H), 8.16 (dd, J = 8.7, 2.6 Hz, 1H), 8.06 (d, J = 7.9 Hz, 1H), 8.01 (d, J = 8.7 Hz, 1H), 7.89 (td, J = 7.7, 1.7 Hz, 1H), 7.48 (ddd, J = 7.5, 4.9, 1.1 Hz, 1H), 5.85 (ddd, J = 16.2, 9.3, 6.2 Hz, 2H), 5.11 (ddd, J = 16.6, 10.2, 3.7 Hz, 2H), 4.75 – 4.65 (m, 1H), 4.02 – 3.79 (m, 4H), 3.43 – 3.27 (m, 4H), 3.27 – 3.12 (m, 4H), 2.54 – 2.44 (m, 2H), 2.39 – 2.28 (m, 4H), 2.28 – 2.11 (m, 6H), 2.08 – 1.97 (m, 2H) 1.98 – 1.80 (m, 4H), 0.94 – 0.79 (m, 2H), 0.68 – 0.49 (m, 4H), 0.49 – 0.35 (m, 4H).^{13}C NMR (100 MHz, MeOH-d_4, δ): 175.67 (u, 1C), 174.17 (u, 1C), 159.44 (u, 1C), 158.42 (u, 2C), 149.82 (dn, 1C), 148.86 (u, 1C), 148.32 (u, 1C), 148.06 (u, 1C), 143.49 (u, 1C), 140.85 (dn, 1C), 139.19 (dn, 2C), 138.51 (u, 1C), 138.26 (dn, 1C), 132.18 (dn,

2C), 128.34 (dn, 1C), 126.36 (dn, 1C), 122.62(dn, 1C), 122.39(dn, 1C), 73.76(dn, 1C), 70.60(u, 2C), 42.28(u, 1C), 41.47(u, 1C), 40.32 (u, 2C), 39.68 (u, 2C), 36.85 (u, 1C), 36.10 (u, 1C), 34.67 (u, 2C), 33.64 (u, 2C), 28.55 (u, 2C), 25.96 (dn, 2C), 23.26 (dn, 2C), 22.63 (u, 1C), 22.21 (dn, 2C). HRMS (ESI) [MH^+]: calcd. for $C_{45}H_{60}N_{11}O_8^+$, 882.4626 found 882.4635.

1.10 N-terminal cysteine-tagged Clover-GFP

The Clover construct sequence[5], coding for this green fluorescent protein with an additional *N*-terminal extension (MGSGS**C**GSGS), was ordered from GeneWiz (http://www.genewiz.com/) and inserted into the pET28a vector via XbaI and NcoI restriction sites. Plasmids were transformed and expressed in BL21(DE3) cells. Cells were grown at 37°C in 2 L of Luria-Bertani medium containing 30 µg/mL kanamycin and induced by the addition of 1.0 mM isopropyl β-D-1-thiogalactopyranoside when the absorbance at 600 nm reached 0.6 AU. Cells were grown for 6 h and collected by centrifugation (at 3,000 g for 10 minutes at 4°C). Cell pellets were resuspended in 50 mM phosphate buffer, pH 7.5, containing 500 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 300 µg/mL lysozyme, and 1 µM leupeptin. Cells were disrupted by two passes through a French pressure cell (at 10,000 psi), and the resulting homogenate was briefly sonicated to shear DNA. The suspension was clarified by centrifugation (at 17,000 g for 30 minutes at 4°C), and the supernatant was rocked with 3 mL of a nickel affinity resin (Sigma HIS-Select Nickel Affinity Gel) for 1 hour at 4°C. The resin-bound protein was loaded into a small column and washed with 40 mL of 50 mM phosphate buffer, pH 7.5, containing 500 mM NaCl, followed by 40 and 20 mL washes of the same buffer with an additional 5 and 20 mM imidazole respectively. The constructs were then eluted from the column with 20 mL of 50 mM phosphate buffer, pH 7.5, containing 500 mM NaCl and 200 mM imidazole, and then dialysed overnight against 4 L of 50 mM phosphate buffer, pH 7.5, containing 1 mM EDTA. The protein was then concentrated to 1 mL and stored at -20°C. The protein construct was >95% pure by SDS-PAGE.

Clover construct protein sequence

The *N*-terminal extension is italicized and the introduced cysteine is bolded. At the *C*-terminus, the hexa-HIS tag with a GSG spacer is underlined.

*MGSGS**C**GSGS*VSKGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGKL
TLKFISTTGKLPVPWPTLVTTFGYGVACFSRYPDHMKQHDFFKSAMPEGYVQE
RTISFKDDGTYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNFNSHNV
YITADKQKNGIKANFKIRHNVEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSHQ
SALSKDPNEKRDHMVLLEFVTAAGITHGMDELYK<u>GSGHHHHHH</u>

Clover construct nucleotide sequence (XbaI and NcoI restriction sites are underlined)

GGG**T**CTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATAATGGGTAGCGGTAGC
TGTGGTAGCGGTTCAGTTAGCAAAGGTGAAGAACTGTTTACCGGTGTTGTTCCGATT
CTGGTTGAACTGGATGGTGATGTTAATGGCCACAAATTTTCAGTTCGTGGTGAAGGC
GAAGGTGATGCAACCAATGGTAAACTGACCCTGAAATTTATCAGCACCACCGGCAA
ACTGCCGGTTCCGTGGCCGACCCTGGTTACCACCTTTGGTTATGGTGTTGCATGTTT
TAGCCGTTATCCGGATCATATGAAACAGCACGATTTTTTCAAAAGCGCAATGCCGGA
AGGTTATGTTCAAGAACGTACCATCTCCTTTAAAGATGATGGCACCTATAAAACCCG
TGCCGAAGTTAAATTTGAAGGTGATACCCTGGTGAATCGCATTGAACTGAAAGGCAT
CGATTTCAAAGAAGATGGTAATATCCTGGGCCACAAACTGGAATATAATTTCAATAG
CCACAACGTGTATATCACCGCAGACAAACAGAAAAATGGCATCAAAGCCAACTTTAA
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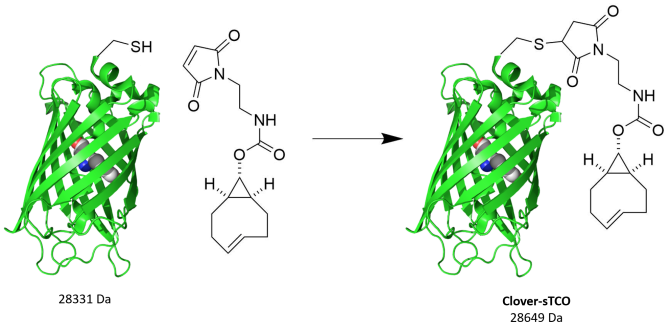
1.11 Preparation of Clover-sTCO



A 0.5 mL solution of 1.02 mM Clover protein solution in phosphate buffer (50 mM, 1 mM EDTA, pH 7.5) was reduced with an aqueous solution of tris(hydroxylpropyl)phosphine (THP) (50 µL of a 100 mM solution, 10 mM final concentration) for 2 hours at room temperature. The reduced protein was loaded onto a desalting column (GE Healthcare PD-10) pre-equilibrated and eluted with phosphate buffer (50 mM, 1 mM EDTA, pH 7.5). Approximately 1.2 mL of pure fractions was collected, containing fully reduced 104 µM Clover protein. The thiol content of the resulting solution was verified by treating a small volume with Ellman's reagent. Concentration was determined by UV-Vis spectrometry using the Clover extinction coefficient of 111,000 $M^{-1}cm^{-1}$. Approximately 1.2 mL of a 104 µM reduced Clover solution in phosphate buffer (50 mM, 1 mM EDTA, pH 7.5) was incubated with a 10 mM DMSO solution of sTCO-Maleimide[1] (62.4 µL, final concentration 520 µM) for 30 minutes. The reaction solution was concentrated using a centrifugal filter (Millipore Ultracel 3k MWCO) at 4,000 rpm for 15 minutes. The concentrated solution was loaded onto a desalting column (GE Healthcare PD-10) pre-equilibrated and eluted with phosphate buffer (50 mM, 1 mM EDTA, pH 7.5). Approximately 1.0 mL of 103 µM conjugated Clover protein was collected.

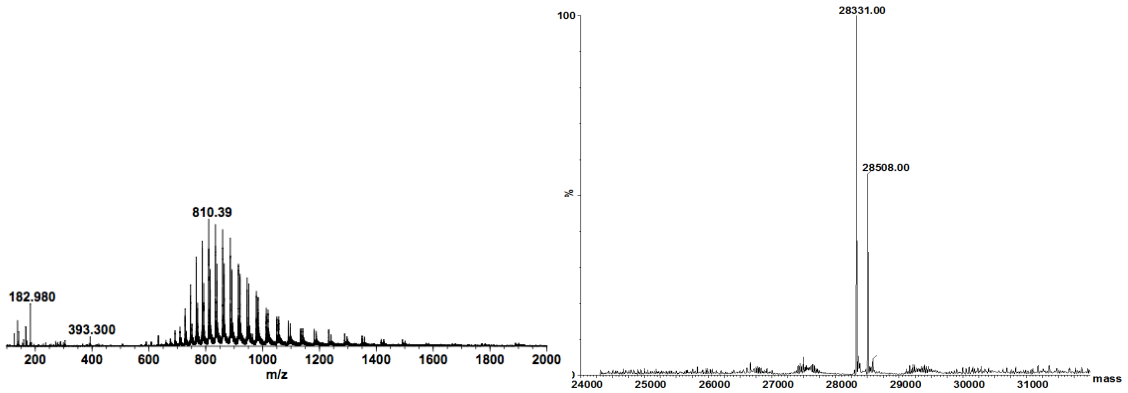


Figure S1a: (left) Positive mode ESI-MS-Tof spectra of Clover-GFP; (right) deconvoluted mass spectrum with a major peak at 28331 Da ± 1 Da and minor peak at 28508 Da ± 1 Da.

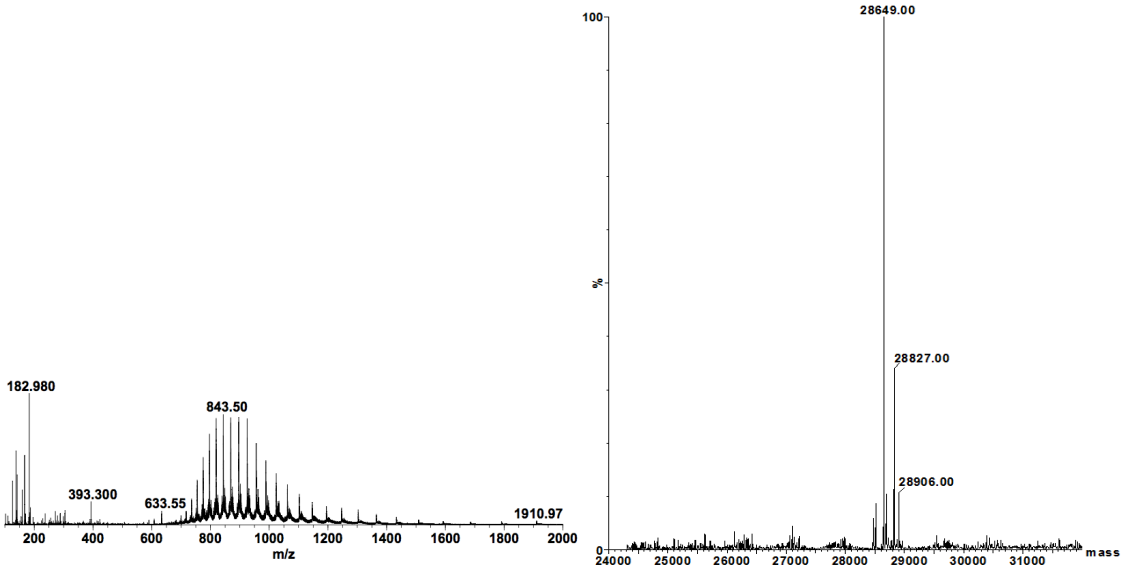
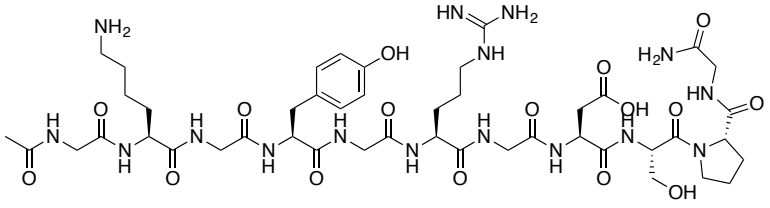


Figure S1b: (left) Positive mode ESI-MS-Tof spectra of Clover-sTCO; (right) deconvoluted mass spectrum with a major peak at 28649 Da ± 1 Da and minor 28827 Da ± 1 Da. This shows an expected molecular weight difference of 319 Da at both the major and minor peaks of Clover-GFP, indicating a successful incorporation of sTCO-Maleimide. Analysis was performed on the Xevo GS-2 QTof (Waters Corp.) system and MaxEnt was used for deconvolution of spectra.

1.12 Synthesis of RGD-sTCO



A cell-adhesive peptide with sequence of GKGYGRGDSPG was prepared on a PS3 peptide synthesizer (Protein technologies Tucson, AZ) using the Rink Amide resin (EMD Millipore, IL) following standard Fmoc solid phase peptide synthesis protocols. The Rink Amide-MBHA resin (0.25 mmol) was swollen in DMF for 15 minutes on the peptide synthesizer before the Fmoc group was removed by a piperidine/DMF solution (20 vol%). After the resin was thoroughly washed with DMF, a 4-fold excess of Fmoc-protected amino acid (1.0 mmol) HBTU (379 mg, 1.0 mmol) and 4-methylmorpholine DMF solution were added to the reaction vessel for standard amine-carboxylic acid coupling. A coupling time of 1 hour was used for all the amino acids. After each coupling step, excess reactants were washed off using DMF, and the Fmoc group was removed before the addition of the next residue. At the end of the peptide synthesis, the amine group at the *N*-terminus was acetylated with acetic anhydride (5 mL, 20% in DMF, with 0.3 mL DIPEA) for 20 min. The peptide was cleaved and deprotected in TFA/H_2O/triisopropylsilane (95/2.5/2.5, v/v) for 3 hours and precipitated in cold diethyl ether, leaving and amide functionality at the *C*-terminus of the peptide product. HPLC purification followed by lyophilization afforded the title compound as dry powder. Analytical UPLC trace of the purified peptide is shown in Figure S2. HRMS (ESI) [MHH^{2+}]: calcd. for $C_{45}H_{72}N_{16}O_{16}^{2+}$, 546.2651 found 546.2638.

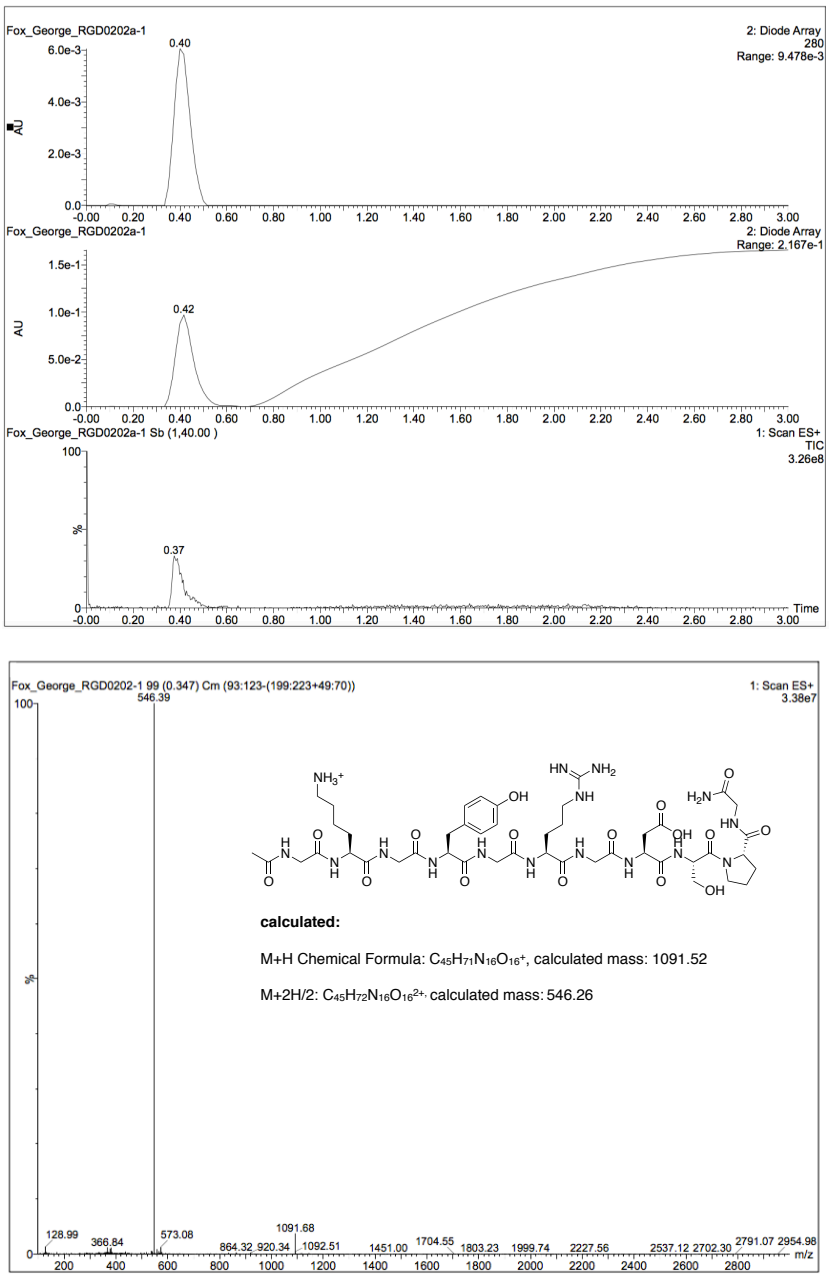
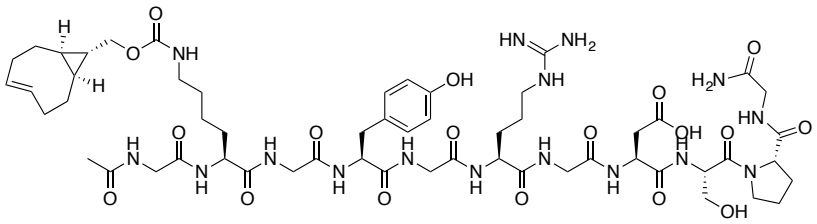


Figure S2. UPLC trace of peptide Ac-GKGYGRGDSPG-NH_2 (C18 column, gradient 95:5 to 5:95 water:ACN in 2.5 minutes and then maintain for half a minute.) Shown are LC traces in the UV channel at 280 nm (top panel), 215 nm (upper middle panel) and MS-TIC (lower middle panel). The mass spectrum of the peak at eluting at 0.37 minutes is shown in the bottom panel.

RGD-sTCO



RGD peptide (42 mg, 38.5 µmol) was dissolved in 550 µL of anhydrous DMF and *N,N*-diisopropylethylamine (17 µL, 97.6 µmol) was added followed by (1R,8S,9R,4E)-bicyclo[6.1.0]non-4-en-9-ylmethyl(4-nitrophenyl) carbonate[1] (18 mg, 56.7 µmol). The reaction was stirred at room temperature for 3 hours. The resulting solution was added dropwise to 35 mL of diethyl ether. The crude product was obtained by precipitation followed by centrifugation (5,000 rpm, 5 minutes). The precipitation/centrifugation procedure was repeated two additional times and then the crude product was purified by HPLC using a gradient of 5% to 95% acetonitrile in pH neutral water (i.e. without TFA or formic acid modifier). Collected fractions were lyophilized and stored in –20°C freezer. HPLC purification afforded 39 mg of sTCO-RGD conjugate (30.8 µmol, 80%). Analytical UPLC trace of the purified peptide is shown in Figure S3. HRMS (ESI) [MH^+]: calcd. for $C_{56}H_{85}N_{16}O_{18}^+$, 1269.2222 found 1269.2268.

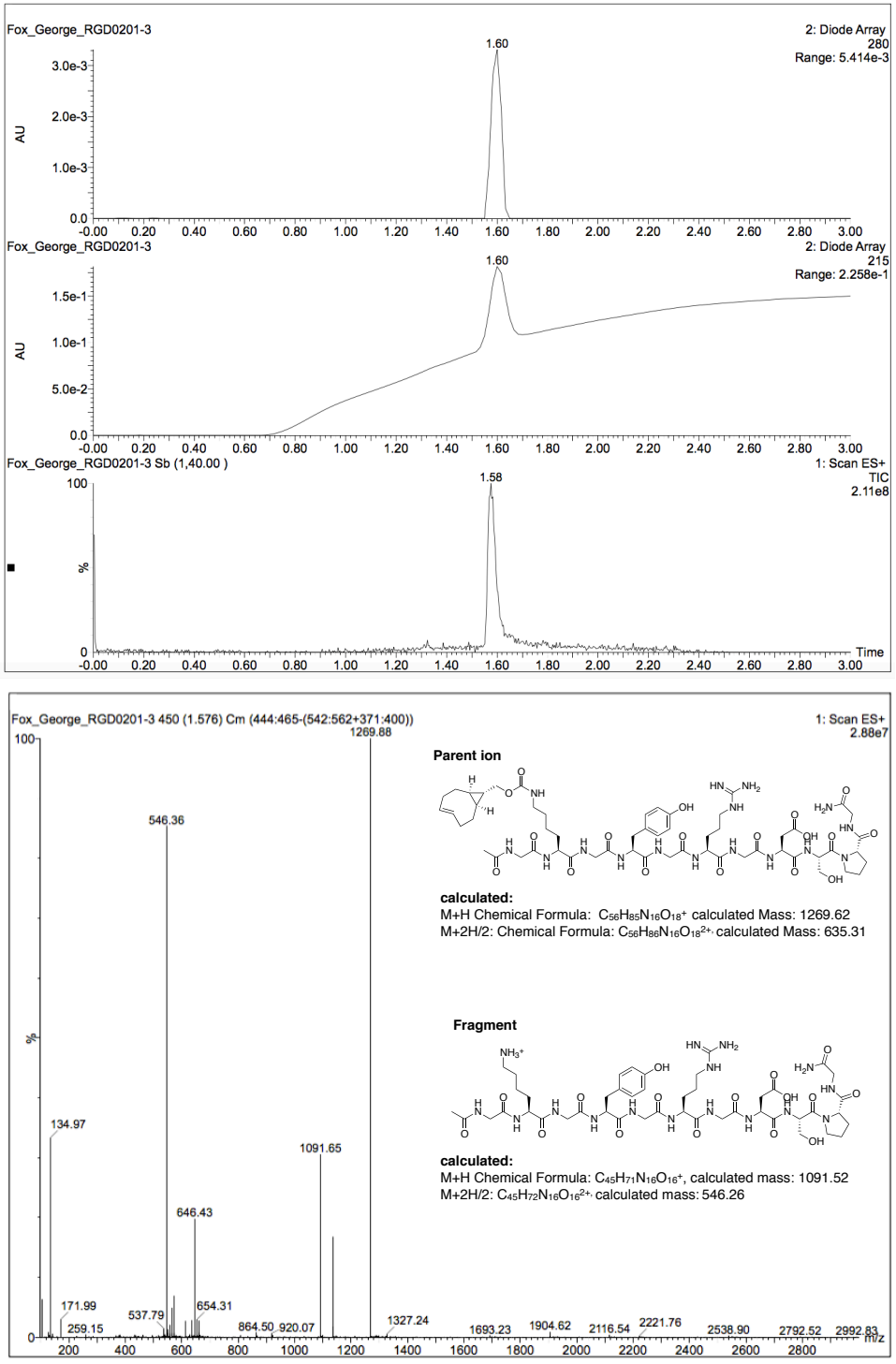


Figure S3. UPLC trace of RGD-sTCO (C18 column, gradient 95:5 to 5:95 water/ACN in 2.5 minutes and then maintain for half a minute.) Shown are LC traces in the UV channel at 280 nm (upper panel), 215 nm (upper middle panel) and MS-TIC (lower middle panel). The mass spectrum of the peak at eluting at 0.37 minutes is shown in the bottom panel.

2. Dihydrotetrazine oxidation experiments

2.1 General consideration

UV-Vis measurements were conducted in quartz cuvettes using either a Hewlett Packard 8453 or 8452A spectrophotometer equipped with temperature controlled, stirring cuvette holders. Glassware was cleaned by rinsing twice with phosphate buffered saline (PBS, pH = 7.4) containing EDTA (2 mM) followed by triple rinsing with PBS buffer free of EDTA. PBS buffer was prepared by adding the following to Milli-Q purified water: NaCl (8 g/L), KCl (0.2 g/L), Na_2HPO_4 (1.42 g/L), KH_2PO_4 (0.24 g/L) and ethylenediaminetetraacetic acid disodium salt (0.672 g/L). The pH of the buffer was then adjusted to 7.4 using either 1M solutions of either HCl or NaOH.

Experiments to study the catalytic photooxidation were conducted at 25°C in a thermostatted UV-cell with stirring capability and a single top-mounted LED. The LED was mounted in a custom, 3-D printed housing that is displayed below. The wavelength of irradiation was varied by exchanging LED bulbs of variable dominant wavelength (DWL). The LEDs were manufactured by CREE and had the following specifications.

Photo Red (CREE XPEPHR-L1), 660 nm LED (650–670 DWL, photo red)

Green (CREE XPEGRN-L1), 528 nm LED (520-535 DWL, green)

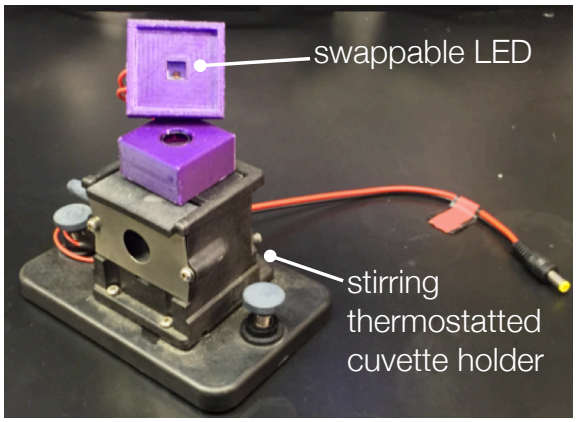
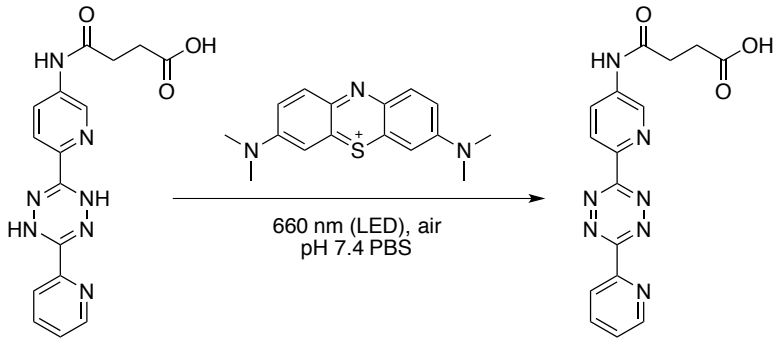


Figure S4. 3-D printed housing used for oxidation experiment

For LEDs, the light intensity was estimated by measuring the light intensity 4 cm from the light source, which is equal to the distance from the LED to the center of the cuvette holder. To measure the intensity of light emitted by the LEDs an International Light IL1400A instrument equipped with a SEL005 sensor was used. The instrument was calibrated to the peak intensity of the LED. All measurements were conducted in a dark room.

2.2 Methylene blue catalyzed photooxidation



Solutions (1 mL) containing **3a** (21 µM) and methylene blue (4 µM) in PBS buffer were prepared in a cuvette from stock solutions. Oxidation of **3a** to **4a** was monitored by recording absorbance at 325 nm every 30 seconds. Cuvettes for these experiments were washed with EDTA/PBS as described in the General Considerations (section 2.1).

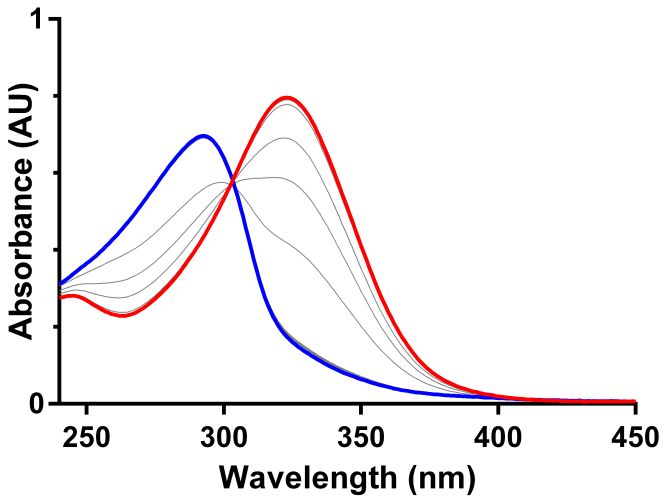


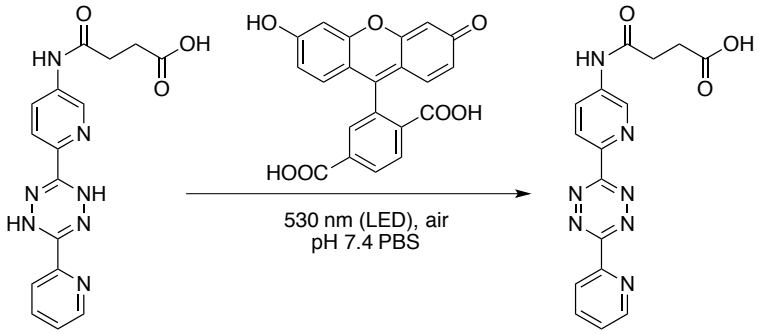
Figure S5. The methylene blue catalyzed photooxidation of **3a** to **4a** displays the same isosbestic point at 303 nm observed in HRP catalyzed oxidations.

Experiment 1 (continuous irradiation, Fig 3B, main manuscript): In a cuvette, a solution of methylene blue (4 µM) and **3a** (21 µM) was irradiated continuously with red light (660 nm, 9.1 mW/cm^2) from an LED until no further oxidation was observed.

Experiment 2 (Toggle experiment, Fig 3C, main manuscript): In a cuvette, a solution of methylene blue (4 µM) and **3a** (21 µM) was irradiated with LED red light (660 nm, 9.1 mW/cm^2) for an interval, and the light was turned off. This pulsing was repeated twice, and then the light was left on until no further oxidation was observed.

Experiment 3 (azide quenching experiment, Fig 3D, main manuscript): In a cuvette, a solution of methylene blue (4 μM) and **3a** (21 μM) was irradiated continuously with red light (660 nm, 2.6 mW/cm^2). Following 60 seconds of irradiation, solid sodium azide (3.9 mg) was directly added to the cuvette, to give a final NaN_3 concentration of 60 mM. Irradiation and monitoring of reaction progress was continued until no further oxidation was observed.

2.3 Carboxyfluorescein catalyzed photooxidation



A solution (1 mL) containing **3a** (19 μM) and fluorescein (7 μM) in PBS buffer with EDTA (2 mM) was prepared in a cuvette from stock solutions. Oxidation of **3a** to **4a** was monitored by recording absorbance at 325 nm every 10 seconds. The cuvette was irradiated with LED green light (660 nm) for an interval, and the light was turned off. This pulsing was repeated twice, and then the light was left on until no further oxidation was observed.

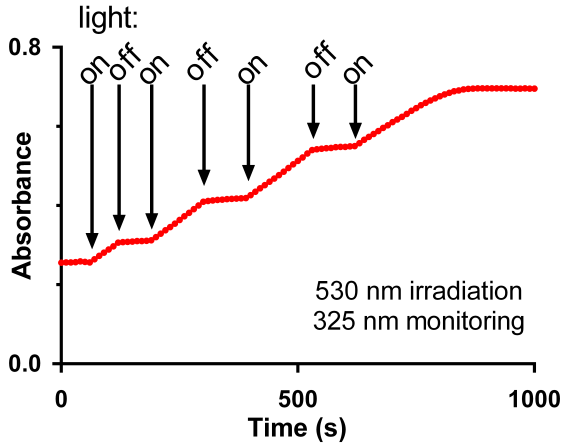
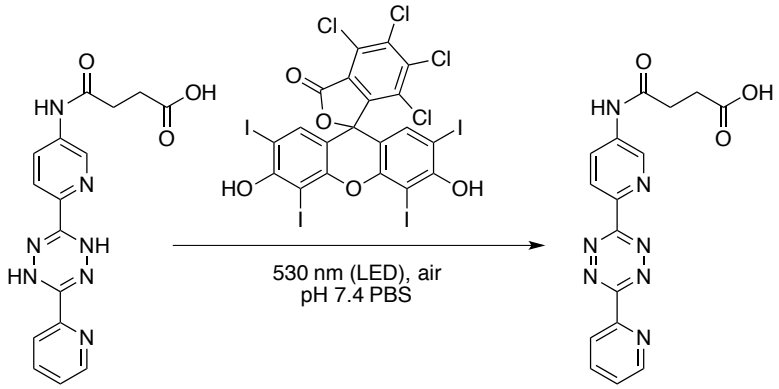


Figure S6. Fluorescein catalyzed photooxidation— 'Toggle' experiment. Irradiation of **3a** (19 μM) in PBS buffer in the presence of fluorescein (7 μM) forms **4a** when light (528 nm) is applied. Reaction progress was monitored every 10 sec at 325 nm (UV maximum of **4a**) and 292 nm (UV maximum of **3a)**. Irradiation with green light (528 nm) in the presence of fluorescein increased formation of **4a**. Turning the light source on and off illustrates that the oxidation requires light and that the reaction stalls in the dark.

2.4 Rose bengal catalyzed photooxidation



Solutions (1 mL) containing **3a** and rose bengal in PBS buffer were prepared in a cuvette from stock solutions. Oxidation of **3a** to **4a** was monitored by recording absorbance at 325 nm every 30 seconds. Cuvettes for these experiments were washed with EDTA/PBS as described in the General Considerations (section 2.1).

Experiment 1 (continuous irradiation): In a cuvette, a solution of rose bengal (9 μM) and **3a** (24 μM) was irradiated continuously with green light (528 nm, 2.2 mW/cm^2) from an LED until no further oxidation was observed.

Experiment 2 (Toggle experiment): In a cuvette, a solution of rose bengal (7 μM) and **3a** (34 μM) was irradiated with LED green light (528 nm, 2.2 mW/cm^2) for an interval and the light was turned off. This pulsing was repeated twice, and then the light was left on until no further oxidation was observed.

Experiment 3 (azide quenching experiment): In a cuvette, a solution of rose bengal (2 μM) and **3a** (32 μM) was irradiated continuously with LED green light (528 nm, 2.2 mW/cm^2). Following 60 seconds of irradiation, solid sodium azide (3.9 mg) was directly added to the cuvette, to give a final NaN_3 concentration of 60 mM. Irradiation and monitoring of reaction progress was continued until no further oxidation was observed.

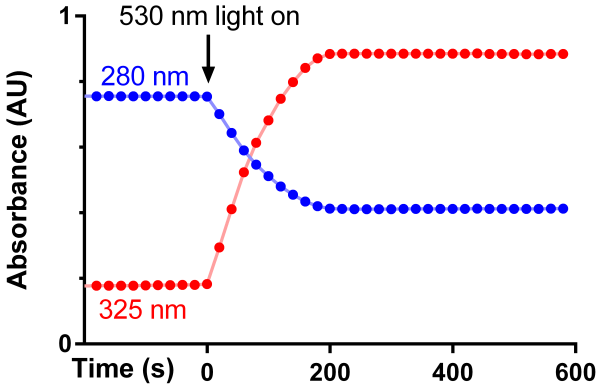


Figure S7. Rose bengal catalyzed photooxidation. Irradiation of **3a** (24 μM) in PBS buffer with green light (528 nm) in the presence of rose bengal (9 μM) resulted in the formation of **4a**. Upon irradiation with 528 nm light (2.2 mW/cm^2), reaction progress was monitored every 20 sec at 325 nm (UV maximum of **4a**) and 280 nm (maximum UV extinction coefficient change for **3a** to **4a**).

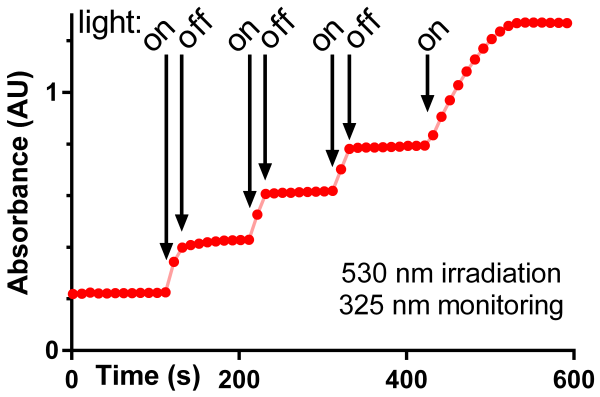


Figure S8. Rose Bengal catalyzed photooxidation — 'Toggle' experiment. Irradiation of **3a** (34 μM) in PBS buffer in the presence of rose bengal (7 μM) forms **4a** when light (528 nm) is applied. Reaction progress was monitored every 10 seconds at 325 nm (UV maximum of **4a**) and 292 nm (UV maximum of **3a)**. Irradiation with green light (528 nm) in the presence of fluorescein increased formation of **4a**. Turning the light source on and off illustrates that the oxidation requires light and that the reaction stalls in the dark.

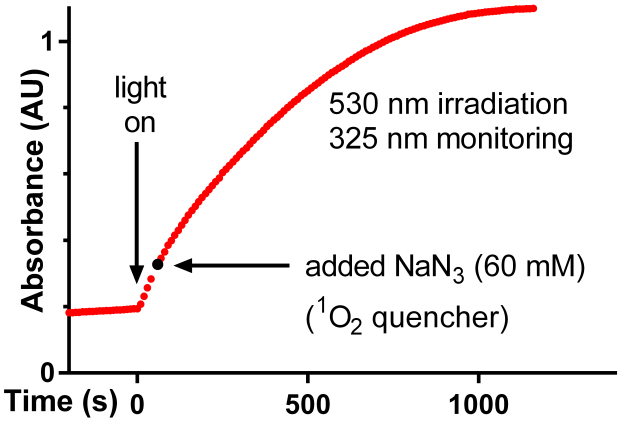
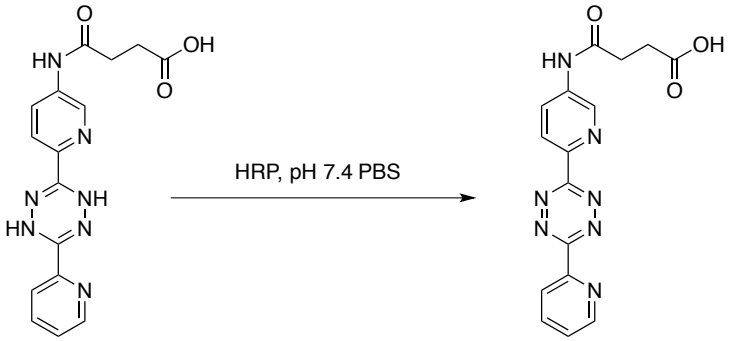


Figure S9. Rose bengal catalyzed photooxidation— NaN_3 addition experiment. Irradiation of **3a** (32 µM) in PBS buffer in the presence of rose bengal (2 µM) forms **4a** when light (528 nm) is applied. Reaction progress was monitored every 10 seconds at 325 nm (UV maximum of **4a**) and 292 nm (UV maximum of **3a)**. After 60 seconds, solid sodium azide was added to achieve a final concentration of 60 mM NaN_3.

2.5 Horseradish peroxidase (HRP) oxidation



Solutions (1 mL) containing **3a** (30 µM) in PBS buffer were prepared in a cuvette from stock solutions. Oxidation of **3a** to **4a** was monitored by recording absorbance at 325 nm every 10 seconds while either hydrogen peroxide (2 mM), HRP (15 nM) or both were added (Figure 4b in the main manuscript). For the superoxide dismutase experiment, HRP (15 nM) was added followed by SOD (770 nM) 40 seconds later (Figure 4c in the main manuscript). For the kinetics experiments, solutions (1 mL) containing **3a** in PBS buffer with EDTA (2 mM) were prepared in the following concentrations of **3a**: 9, 17, 34, 50, 83, 150 and 200 µM. The oxidation rate was determined by observing the conversion of **3a** to **4a** in the first 10 seconds after mixing in HRP (15 nM) and subtracting the background oxidation prior to the addition of HRP. Kinetic parameters were determined using GraphPad's Prism 6 software from the rate data presented in Table S1.

[**3a**] (μM)	Rate of HRP Oxidation of **3a** Rate x 10^{-7} (M/sec)
8	0.34 (+/– 0.04) (3 runs)
17	0.56 (+/– 0.04) (3 runs)
34	0.89 (+/– 0.04) (3 runs)
50	1.2 (+/– 0.2) (3 runs)
83	2.1 (+/– 0.1) (3 runs)
150	2.5 (+/– 0.8) (6 runs)
200	2.6 (+/– 0.4) (3 runs)

Table S1. Rate of oxidation of **3a** by HRP at different substrate concentrations.

2.6 Dihydrotetrazine oxidative stability

Oxidative stability in dark: Solutions (3 mL) containing **3a** (35 μM) in PBS buffer were prepared in cuvettes from stock solutions. Oxidation was monitored by recording solution absorbance at 0, 30 and 150 minutes. Cuvettes for these experiments were stored in the dark between measurements (Figure S10a)

Oxidative stability in ambient light: Solutions (3 mL) containing **3a** (35 μM) in PBS buffer were prepared in cuvettes from stock solutions. Oxidation was monitored by recording solution absorbance at 0, 1 and 2 hours. Cuvettes for these experiments were stored in ambient light between measurements (Figure S10c, S10d, S10e)

Oxidative stability in 10% serum: Solutions (1 mL) containing **3a** (9 μM) in 10% mouse serum in PBS buffer were prepared in cuvettes from stock solutions. Oxidation was measured by recording the absorbance at 325 nm after 1 hour. (Figure S10f)

Comparison of HRP, cytochrome *c* and hemoglobin: Solutions (1 mL) of **3a** (30 μM) in PBS buffer were prepared in a cuvette from stock solutions. To the solutions, either hemoglobin (1.5 μM in protein, 6.0 μM in heme) or cytochrome *c* (9 μM) were added and while oxidation was monitored every 30 seconds by measuring absorbance at 325 nm. (Figure S10b)

All the cuvettes for the experiments above were washed with EDTA/PBS as described in the General Considerations (section 2.1) and the cuvettes were held at 25 °C during measurements

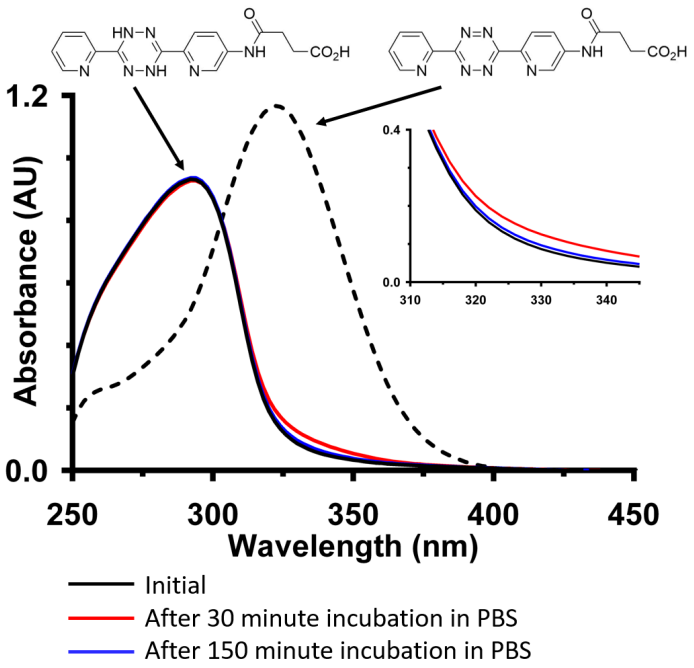


Figure S10a. Oxidative stability of **3a** was monitored by recording absorbance at 0, 30 and 150 minutes. The solution retained 99 and 96% of the dihydrotetrazine oxidation state over 30 minutes and 2.5 hours respectively.

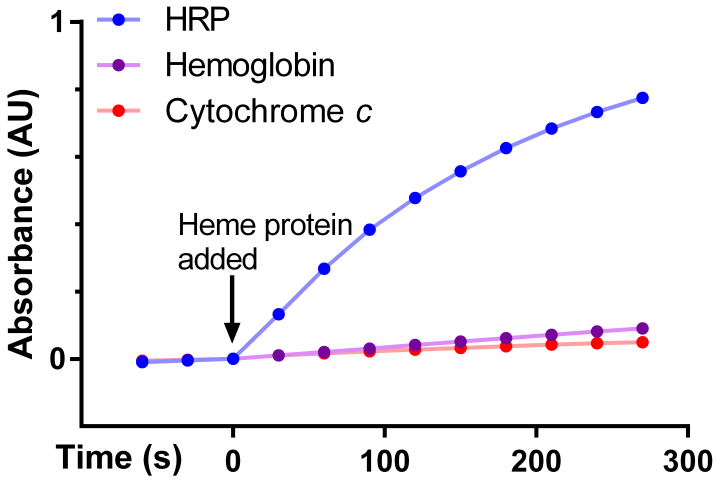


Figure S10b. Heme-containing proteins hemoglobin (1.5 μM in protein, 6.0 μM in heme) or cytochrome *c* (9 μM) do not show significant enzymatic activity towards **3a**. By contrast, HRP (15 nM) is effective at much lower concentration. Oxidation was monitored by measuring absorbance at 325 nm every 30 seconds.

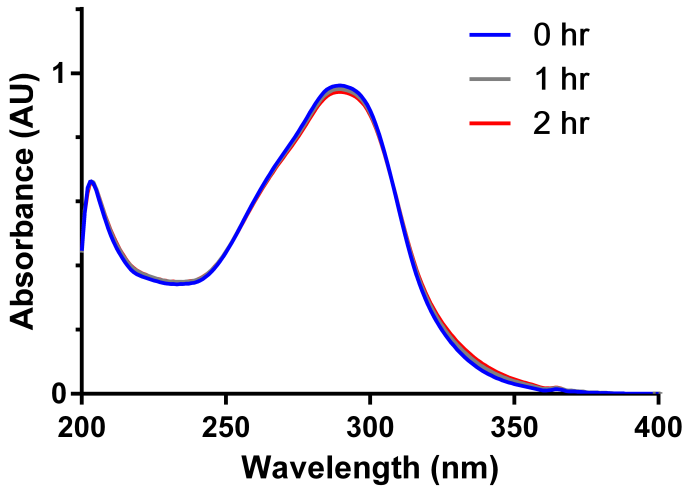


Figure S10c. Oxidative stability of **3a** in ambient light in MeOH solution was monitored by recording absorbance at 0, 1 and 2 hours. The solution retained 99 and 98% of the dihydrotetrazine oxidation state over 1 hour and 2 hours respectively.

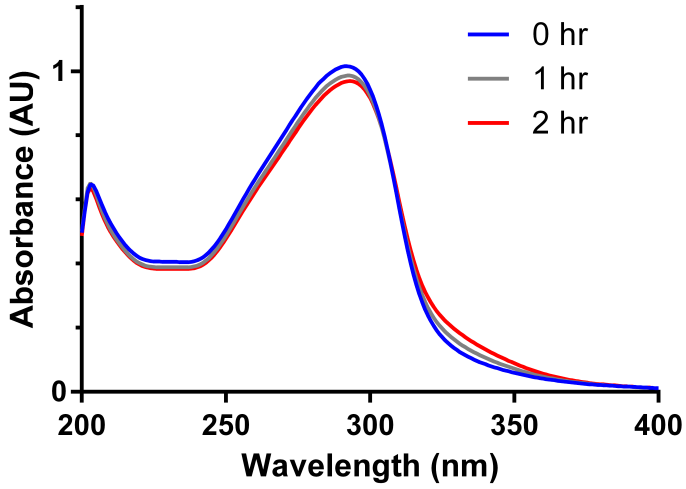


Figure S10d. Oxidative stability of **3a** in ambient light in PBS buffer was monitored by recording absorbance at 0, 1 and 2 hours. The solution retained 97 and 94% of the dihydrotetrazine oxidation state over 1 hour and 2 hours respectively.

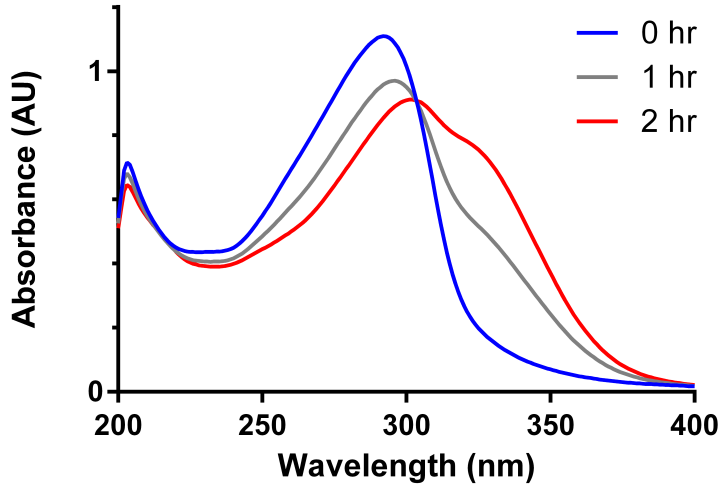


Figure S10e. Methylene blue (4 μM) catalyzed the conversion of **3a** to **4a** in the presence of ambient light in PBS buffer, with 27 and 47% conversion after 1 hour and 2 hours respectively.

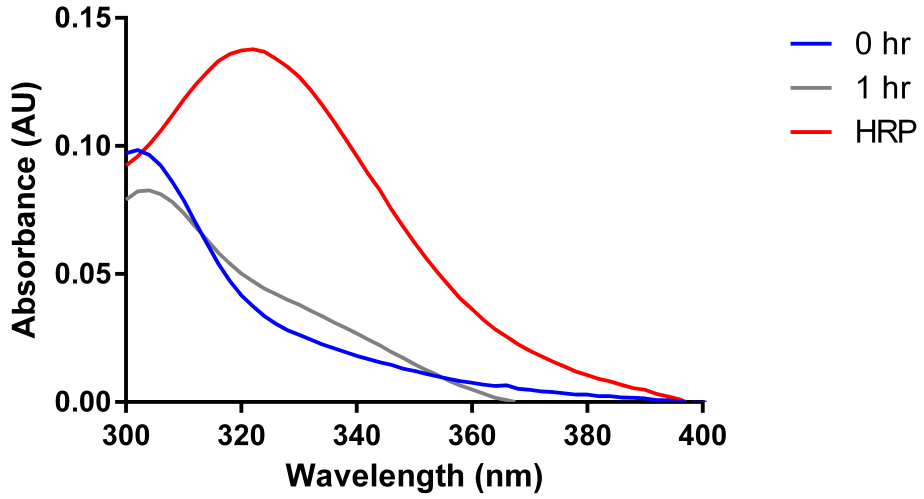


Figure S10f. Oxidative stability of **3a** in PBS buffer containing 10% mouse serum was monitored by recording absorbance at 0 and 1 hour. The solution retained 90% of the dihydrotetrazine oxidation state over 1 hour.

2.7 Tetrazine hydrolytic stability

A solution (1 mL) containing **4a** (800 μM) in PBS buffer was prepared in cuvettes from stock solutions. Tetrazine concentration was measured by recording the absorbance at 525 nm every 20 minutes while holding the cuvette at either 25 for 24 hours. In PBS buffer at 25 °C, tetrazine **4a** (800 μM) shows 98% and 83% fidelity after 2 hours and 24 hours.

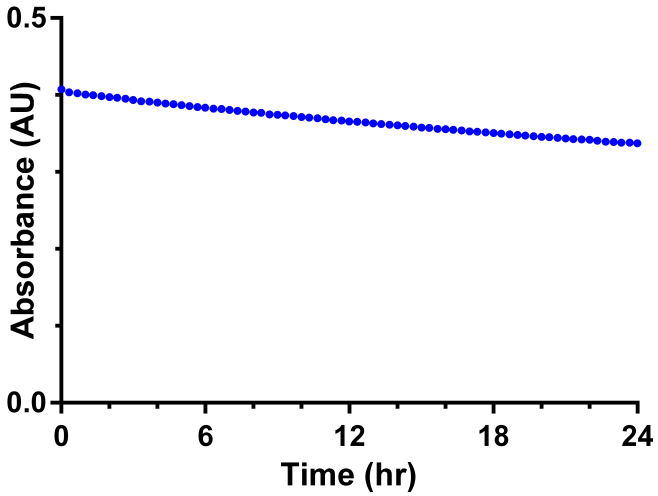
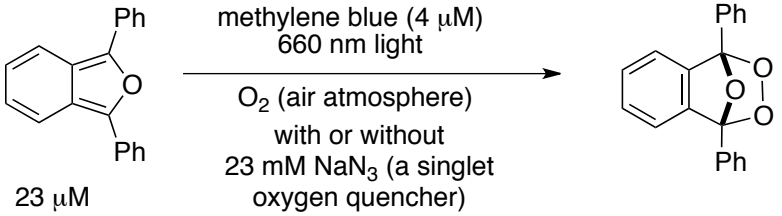


Figure S11. Hydrolytic stability of **4a** in PBS at 25 °C was monitored every 20 minutes at 525 nm

2.8 Azide $^1O_2$ quenching control experiment with 1,3-diphenylisobenzofuran



This experiment provides evidence that photolysis of 4 μM methylene blue solution generates singlet oxygen, and that NaN_3 (23 mM or higher) is an effective quencher of singlet oxygen under these conditions.

A solution (1 mL) containing 1,3-diphenylisobenzofuran (23 μM) and methylene blue (4 μM) in methanol was prepared in a cuvette from stock solutions. Consumption of 1,3-diphenylisobenzofuran was monitored by recording the solution absorbance at 410 nm every 10 seconds while the cuvette was continuously irradiated with red light (centered at 660 nm, 9.1 mW/cm^2) from an LED until completion. In a second, similarly prepared cuvette, sodium azide (23

mM) was added before irradiating with light. The reaction without NaN_3 was approximately 180% faster than the reaction with NaN_3.

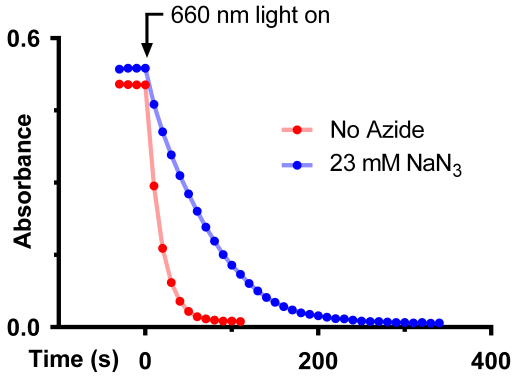


Figure S12. The rate of the reaction between 2,5-diphenylisofuran with $^1O_2$ was greatly reduced when 23 mM NaN_3 was added.

2.9 Electrochemical Measurements

All electrochemistry was performed using a CHI-620D potentiostat/galvanostat. Cyclic voltammetry was performed using a standard three-electrode configuration. CV scans were recorded for quiescent solutions using a platinum disk working electrode (2.0 mm diameter CH Instruments) and a platinum wire auxiliary electrode. All potentials were measured against a Ag/AgCl reference electrode (CH Instruments, 1 M KCl). CV and DPV experiments were performed in a nitrogen saturated 0.1 M potassium phosphate (KH_2PO_4) buffered solution at pH 7.0. The concentration of the analyte was 1.0 mM for all experiments.

2.10 Spectroelectrochemical Measurements.

Controlled potential electrolysis of the analyte was carried out using a CHI-620D potentiostat/galvanostat and a standard three-electrode configuration using a platinum mesh working electrode, a platinum wire auxiliary electrode and a 1M KCl, Ag/AgCl reference electrode. The experiment was performed in a 0.1 cm quartz spectroelectrochemical cell with nitrogen saturated 0.1 M potassium phosphate (KH_2PO_4) buffered solution at pH 7. The concentration of the analyte was 1.0 mM. Absorbance spectra were acquired on a StellarNet CCD array UV-vis spectrometer and acquired every 5 seconds for the duration of the experiment.

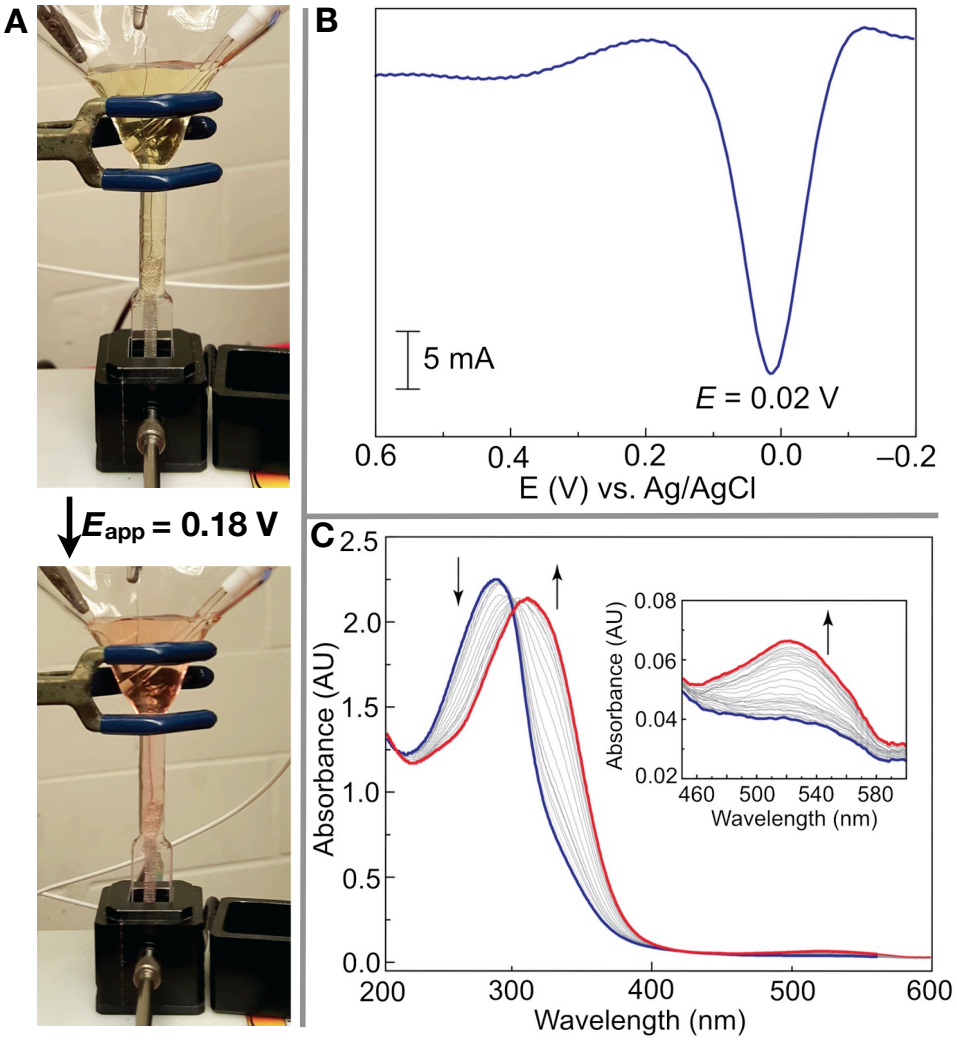
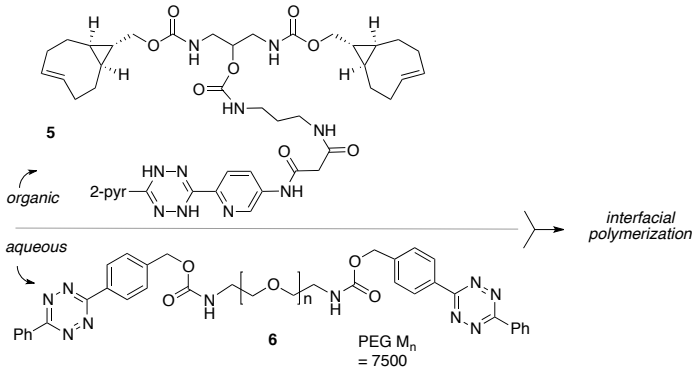


Figure S13. (A) Upon electrochemical oxidation at 0.18 V vs Ag/AgCl, an aqueous solution of 3a (1.1 mM) undergoes a visible change from pale yellow to pink, indicating the formation of tetrazine **4a**. (B) Voltammogram of **3a** (1.1 mM) in 0.1 M KH_2PO_4 at pH 7 buffer solution. A three-electrode setup was used with a Pt disc working electrode, a Pt wire counter electrode and a Ag/AgCl reference electrode which contained a 1M KCl solution. (C) Displays the spectral change during electrochemical conversion of **3a** (blue trace) (1.1 mM) to **4a** (red trace) in a nitrogen saturated 0.1 M KH_2PO_4 at pH 7 buffer solution. A three electrode setup was used with a Pt mesh working electrode, a Pt wire counter electrode and a Ag/AgCl reference electrode which contained a 1M KCl solution. Controlled potential electrolysis was performed at 0.18 V vs Ag/AgCl in a quartz spectroelectrochemical cell with a path length of 0.1 cm.

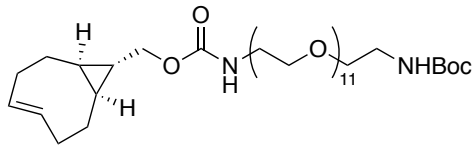
3. DHTz-enriched microfiber fabrication experiments

3.1 Preparation of DHTz-microfibers



Interfacial polymerization was conducted in accord with our previously described procedure[3]. The DHTz-containing bis-sTCO monomer (**5**) was dissolved in ethyl acetate at a concentration of 1.2 mM. The known PEG-based bis-tetrazine monomer **6**[3] was dissolved in water at a concentration of 0.15 mM. To a 60-mm diameter petri dish was added 3 mL of the aqueous solution of the bis-tetrazine monomer **6**. The solution of **5** (3 mL) in ethyl acetate was carefully added over the aqueous phase without disturbing the interface. Upon contact, a polymer thin film formed at the interface. The thin film was grasped gently using sharp tweezers and the fiber that was pulled from the interface was connected to a collecting frame that was constructed of copper wire. The fiber was collected by manually rotating the frame. The microfibers were dried affixed onto precleaned glass slides using adhesive silicon isolators (Purchased from Grace Bio-Labs, product #665301).

To 'cap' any unreacted tetrazine endgroups from the monomer **6**, the fibers were treated with the water soluble sTCO derivative **S5**[4] (shown below). Thus, to a silicon isolator containing DHTz-enriched microfibers was a added PBS solution of **S1** (1 mM). The microfibers were allowed to soak in the solution for 1 minute before the capping solution was removed. The microfibers were then rinsed using PBS solution for 3 times.



S5

Activation and modification of the fibers was conducted inside the silicon isolators following the procedures that are outlined below. All the confocal microscope images were shot at 10X magnification unless noted otherwise.

3.2 Alexa Fluor® 647 tagging experiment and control experiments

To silicon isolator containing DHTz-functionalized microfibers was added a PBS solution of rose bengal (100 μM). The microfibers were allowed to soak in the solution for 5 minutes before rose bengal solution was removed. The microfibers were then rinsed three times with PBS buffer (~3×200 μL). The red microfibers were then immersed in PBS solution and irradiated with a 200-watt incandescent lamp for 5 minutes followed by rinsing with three portions of PBS buffer (~3×200 μL). The microfibers were then treated with a PBS solution of Alexa-sTCO[4] (1 μM) for 1 minute followed by rinsing with three portions of PBS buffer (~3×200 μL). Samples were imaged with a Zeiss LSM 5 Live DuoScan high-speed confocal microscope (Carl Zeiss, Maple Grove, MN). Confocal microscopy images are shown in Figure S14-15.

Control without light: The procedure was identical to that described above, except that the fibers were prepared in a dark room without exposure to light. Confocal images are shown in Figure S16.

Control without sensitizer: The microfibers were immersed in PBS buffer and irradiated with a 200-watt incandescent lamp for 5 minutes and subsequently rinsed three times with PBS buffer (~3×200 μL). The microfibers were then treated with a PBS solution of Alexa-sTCO (1 μM) for 1 minute followed by three rinses with PBS buffer (~3×200 μL). Samples were imaged with a Zeiss LSM 5 Live DuoScan highspeed confocal microscope (Carl Zeiss, Maple Grove, MN). Confocal microscopy images are shown in Figure S17-18.

3.3 Clover protein tagging experiment and control experiment

In a silicon isolator, DHTz-functionalized microfibers were immersed in a PBS solution of methylene blue (100 μM) and irradiated with a 200-watt incandescent lamp for 5 minutes. The microfibers were then rinsed with three portions of PBS buffer (~3×200 μL) and treated with a PBS solution of Clover-sTCO (5 μM) for 1 minute followed by rinsing with three portions of PBS buffer (~3×200 μL). Samples were imaged with a Zeiss LSM 5 Live DuoScan high-speed confocal microscope (Carl Zeiss, Maple Grove, MN). Confocal microscopy images are shown in Figure S19-20.

Control without sensitizer: The microfibers were immersed in PBS solution and irradiated with a 200-watt incandescent lamp for 5 minutes followed by rinsing with three portions of PBS buffer (~3×200 μL). The microfibers were then treated by PBS solution of Clover-sTCO (5 μM) for 1 minute followed by rinsing with three portions of PBS buffer (~3×200 μL). Samples were imaged with a Zeiss LSM 5 Live DuoScan high-speed confocal microscope (Carl Zeiss, Maple Grove, MN). Confocal microscopy images are shown in Figure S21-22.

3.4 Oxidation of DHTz-microfibers by horseradish peroxidase (HRP)

To silicon isolator containing DHTz-microfibers was added a PBS solution of HRP (10 μM). After the microfibers had been immersed in the solution for 1 hour, they were rinsed with three portions of PBS buffer (~3×200 μL). The microfibers were then treated with a PBS solution of Alexa-sTCO (1 μM) for 1 minute followed by rinsing with three portions of PBS buffer (~3×200 μL). Samples were imaged with a Zeiss LSM 5 Live DuoScan high-speed confocal microscope (Carl Zeiss, Maple Grove, MN). Confocal microscopy images are shown in Figure S23.

Control without HRP: The microfibers were submerged in PBS solution for 1 hour followed by PBS solution rinsing for 3 times. The microfibers were then treated by PBS solution of Alexa-sTCO (1 μM) for 1 minute followed by rinsing with three portions of PBS buffer (~3×200 μL). Samples were imaged with a Zeiss LSM 5 Live DuoScan high-speed confocal microscope (Carl Zeiss, Maple Grove, MN). Confocal microscopy images are shown in Figure S24.

3.5 RGD peptide tagging experiment and control experiments

DHTz-microfibers were affixed to a silicone well (9 mm diameter) supported on a poly(2-hydroxyethyl methacrylate) (pHEMA)-coated 1-well Nunc® chamber using silicone isolators (Grace Bio-Labs, product #665301). The fibers were immersed in a solution of methylene blue (100 μM) in PBS, and then irradiated with a 200-watt incandescent lamp for 5 minutes followed by rinsing with three portions of PBS buffer (~3×200 μL). The microfibers were then immersed in a PBS solution of RGD-sTCO (10 μM) for 1 min followed by rinsing with three portions of PBS buffer (~3×200 μL).

Control without sensitizer: In a silicone well, the DHTz-microfibers were immersed in PBS buffer and irradiated with a 200-watt incandescent lamp for 5 minutes followed by rinsing with three portions of PBS buffer (~3×200 μL). The microfibers were then immersed in a PBS solution of RGD-sTCO (10 μM) for 1 min followed by rinsing with three portions of PBS buffer (~3×200 μL).

Control without RGD: The DHTz-microfibers were immersed in a solution of methylene blue (100 μM) in PBS, and irradiated with a 200-watt incandescent lamp for 5 minutes followed by rinsing with three portions of PBS buffer (~3×200 μL).

Control without RGD or sensitizer: The DHTz-microfibers were immersed in PBS and irradiated with a 200-watt incandescent lamp for 5 minutes followed by rinsing with three portions of PBS buffer (~3×200 μL).

3.6. Cell culture and confocal imaging

Fibroblasts (NIH 3T3, ATCC, Manassas, VA) were maintained in DMEM media supplemented with 10% fetal bovine serum (FBS) and 1% Pen-Strep (Invitrogen, Carlsbad, CA). DHTz-microfibers were affixed to a silicone well (9 mm diameter) supported on a poly(2-hydroxyethyl methacrylate) (pHEMA)-coated 1-well Nunc® chamber using silicone isolators (Grace Bio-labs, Bend, OR). The fibers were washed with sterile PBS and cell culture media three times respectively before being sterilized under UV for 15 minutes. A 200 µL suspension of cells with a density of 0.5×10^6 cells/mL was added into each well and cultured at 37°C for 20 hours before confocal imaging under transmitted light. Samples were imaged with a Zeiss LSM 5 Live DuoScan high-speed confocal microscope (Carl Zeiss, Maple Grove, MN). Confocal microscopy images are shown in Figure S25-28.

References

[1] M. T. Taylor, M. L. Blackman, O. Dmitrenko, J. M. Fox, *J. Am. Chem. Soc.* **2011**, *133*, 9646–9649

[2] R. Selvaraj, J. M. Fox, *Tetrahedron Lett.* **2014**, *55*, 4795–4797.

[3] S. Liu, H. Zhang, R. A. Remy, F. Deng, M. E. Mackay, J. M. Fox, X. Jia, *Adv. Mater.* **2015**, *27*, 2783–90.

[4] H. Zhang, K. T. Dicker, X. Xu, X. Jia, J. M. Fox, *ACS Macro Lett.* **2014**, *3*, 727–731.

[5] A. J. Lam, F. St-Pierre, Y. Gong, J. D. Marshall, P. J. Cranfill, M. A. Baird, M. R. McKeown, J. Wiedenmann, M. W. Davidson, M. J. Schnitzer, et al., *Nat. Methods* **2012**, *9*, 1005–12.

Figure S14. Confocal images of Alexa Fluor® 647 tagging experiment: positive sets
Fibers were incubated with rose bengal (100 µM) for 5 min followed by triple PBS rinsing, then irradiated for 5 min with visible light. Activated fibers were triple rinsed with PBS, and incubated with Alexa-sTCO (1 µM) for 1 min, then triple rinsed and imaged with a Zeiss LSM 5 Live DuoScan highspeed confocal microscope

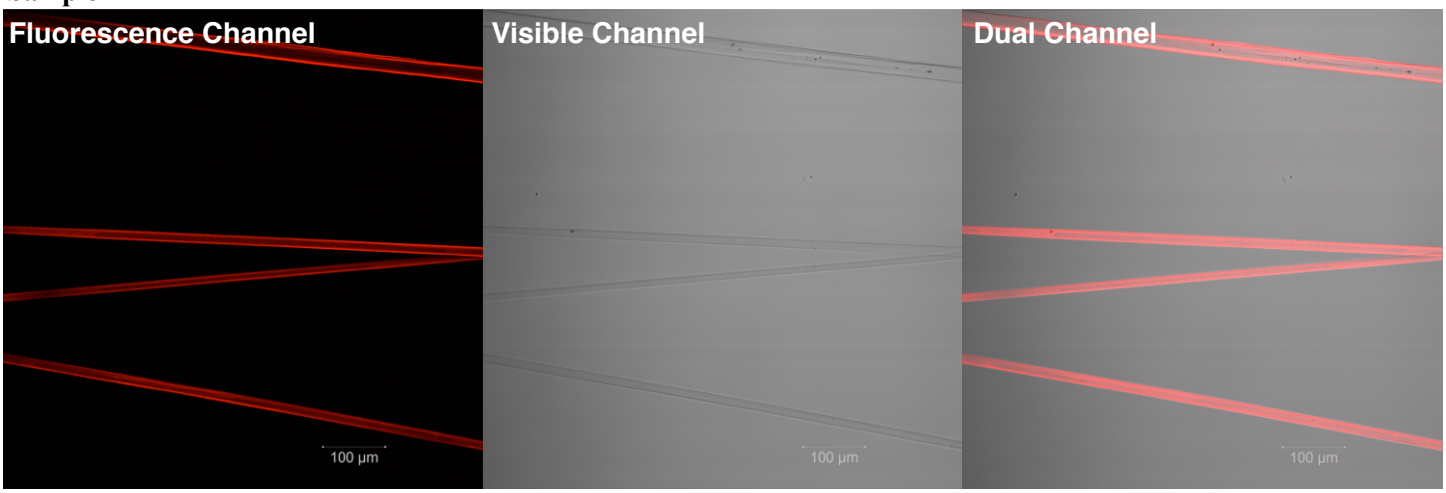
Sample 1

Sample 2

Sample 3

Figure S15. Confocal images of Alexa Fluor® 647 tagging experiment: positive sets (cont)
Fibers were incubated with rose bengal (100 μM) for 5 min followed by triple PBS rinsing, then irradiated for 5 min with visible light. Activated fibers were triple rinsed with PBS, and incubated with Alexa-sTCO (1 μM) for 1 min, then triple rinsed and imaged with a Zeiss LSM 5 Live DuoScan highspeed confocal microscope

Sample 1



Sample 2

Sample 3 (at 40X magnification)

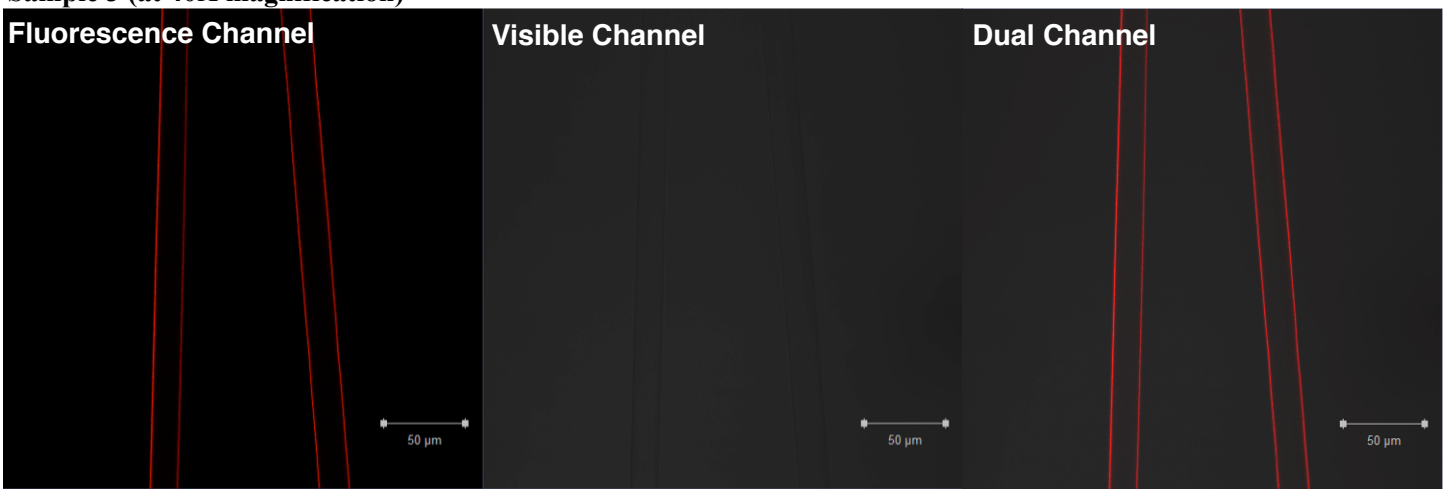


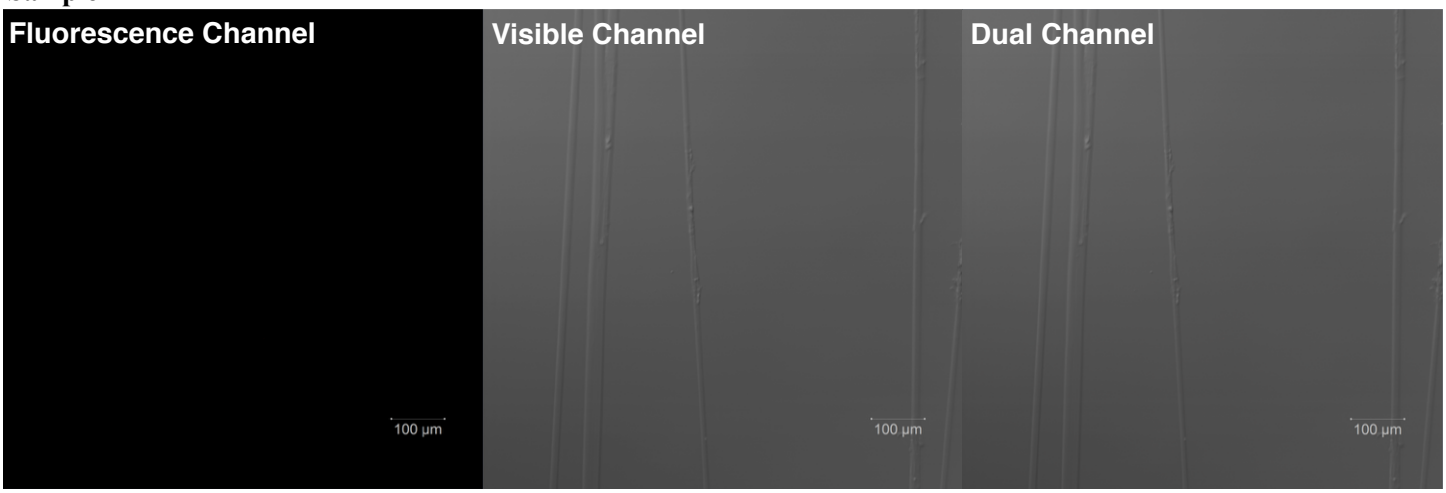
Figure S16. Confocal image of Alexa Fluor® 647 tagging experiment: control without light
In a dark room, fibers were incubated with rose bengal (100 μM) for 5 min followed by triple PBS rinsing, then irradiated in PBS for 5 min. Fibers were triple rinsed with PBS, and incubated with Alexa-sTCO (1 μM) for 1 min, then triple rinsed and imaged with a Zeiss LSM 5 Live DuoScan highspeed confocal microscope

Sample 1

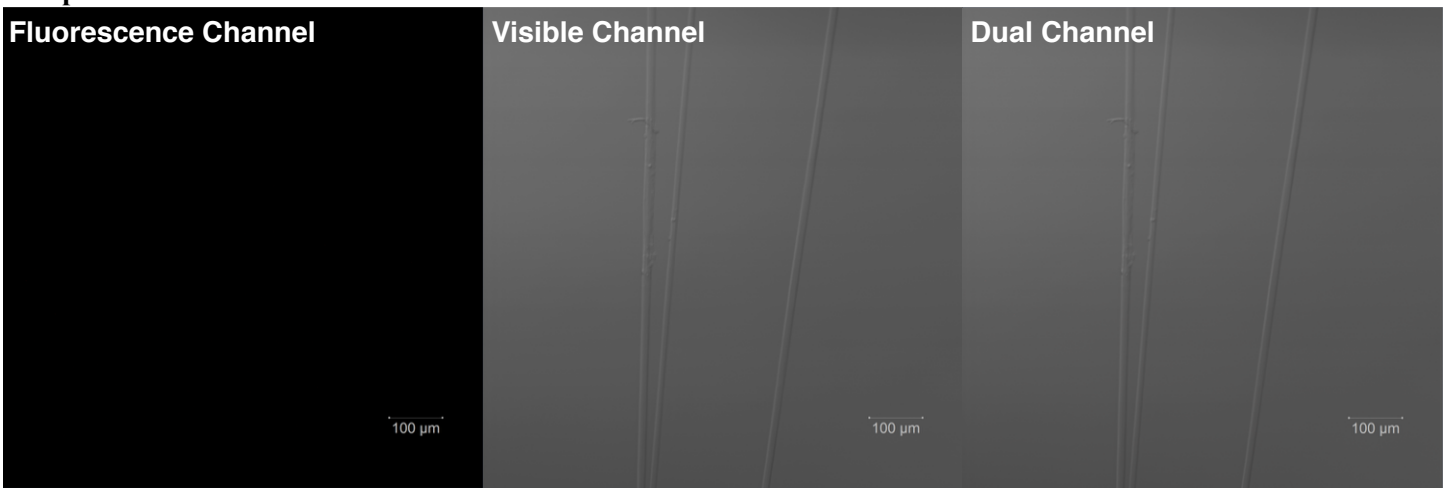
Sample 2

Figure S17. Confocal image of Alexa Fluor® 647 tagging experiment: control without sensitizer
Fibers were incubated with PBS for 5 min followed by triple PBS rinsing, then irradiated in PBS for 5 min. Fibers were triple rinsed with PBS, and incubated with Alexa-sTCO (1 μM) for 1 min, then triple rinsed and imaged with a Zeiss LSM 5 Live DuoScan highspeed confocal microscope

Sample 1



Sample 2



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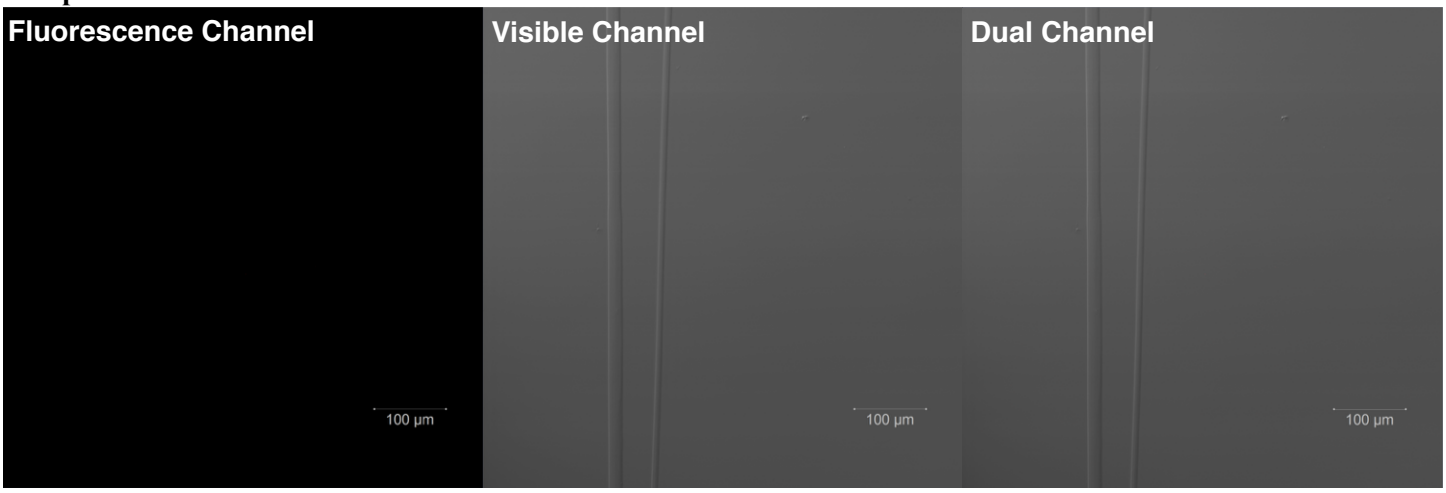
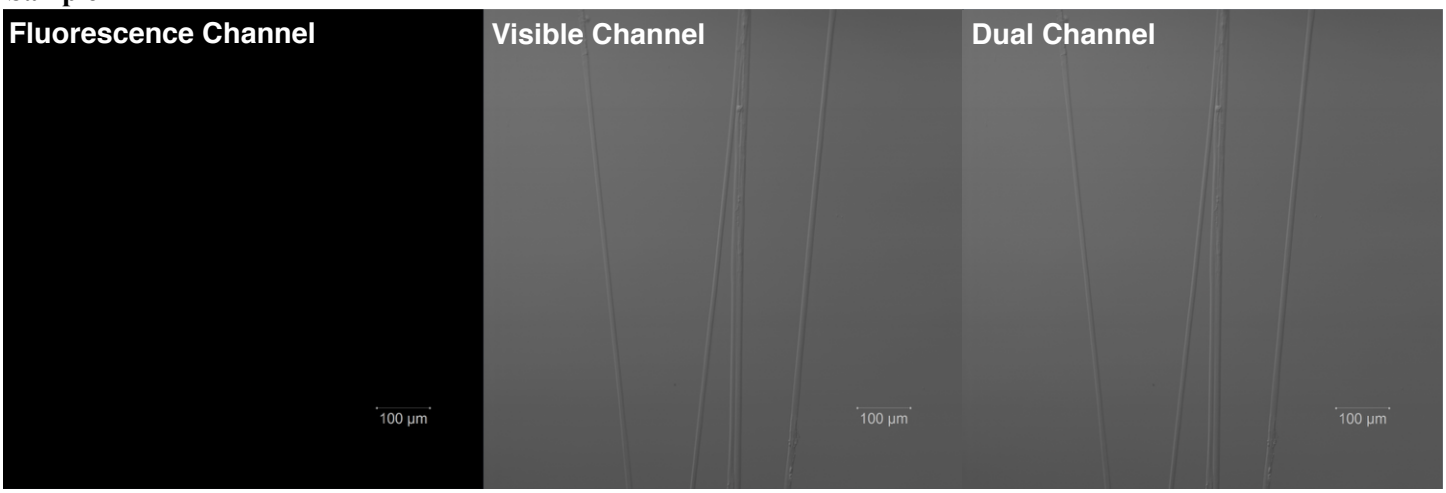


Figure S18. Confocal image of Alexa Fluor® 647 tagging experiment: control without sensitizer (cont)
Fibers were incubated with PBS for 5 min followed by triple PBS rinsing, then irradiated in PBS for 5 min. Fibers were triple rinsed with PBS, and incubated with Alexa-sTCO (1 μM) for 1 min, then triple rinsed and imaged with a Zeiss LSM 5 Live DuoScan highspeed confocal microscope

Sample 1



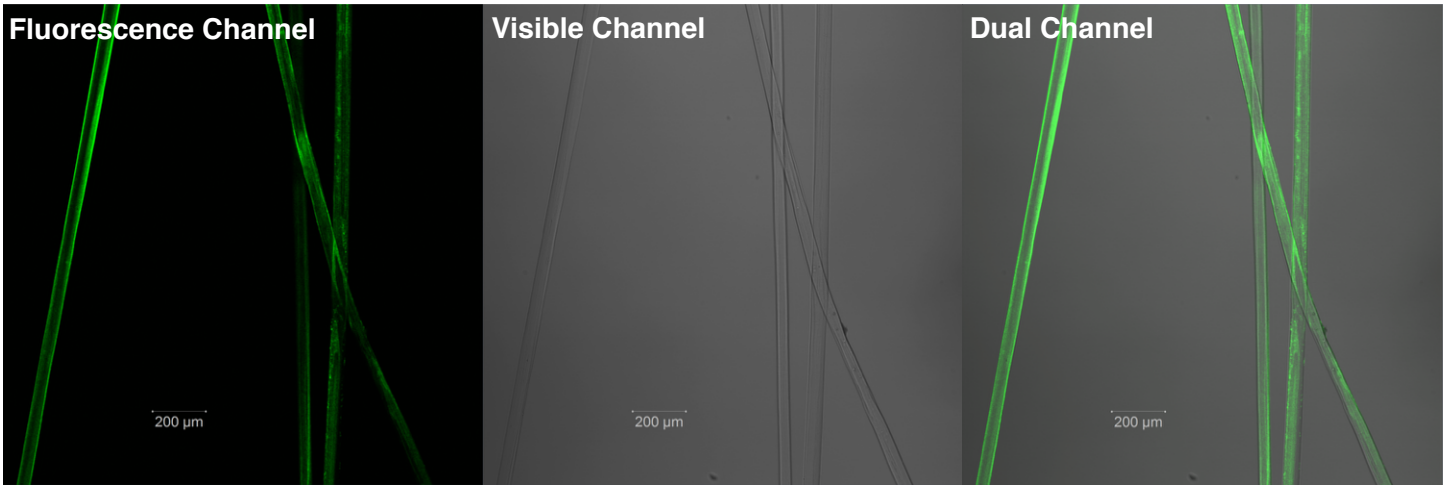
Sample 2

Sample 3

Figure S19. Confocal images of Clover protein tagging experiment: positive sets
Fibers were incubated with methylene blue (100 μM) and irradiated for 5 min with visible light. Activated fibers were triple rinsed with PBS, then incubated with Clover-sTCO (5 μM) for 1 min, then triple rinsed and imaged with a Zeiss LSM 5 Live DuoScan highspeed confocal microscope

Sample 1

Sample 2



Sample 3

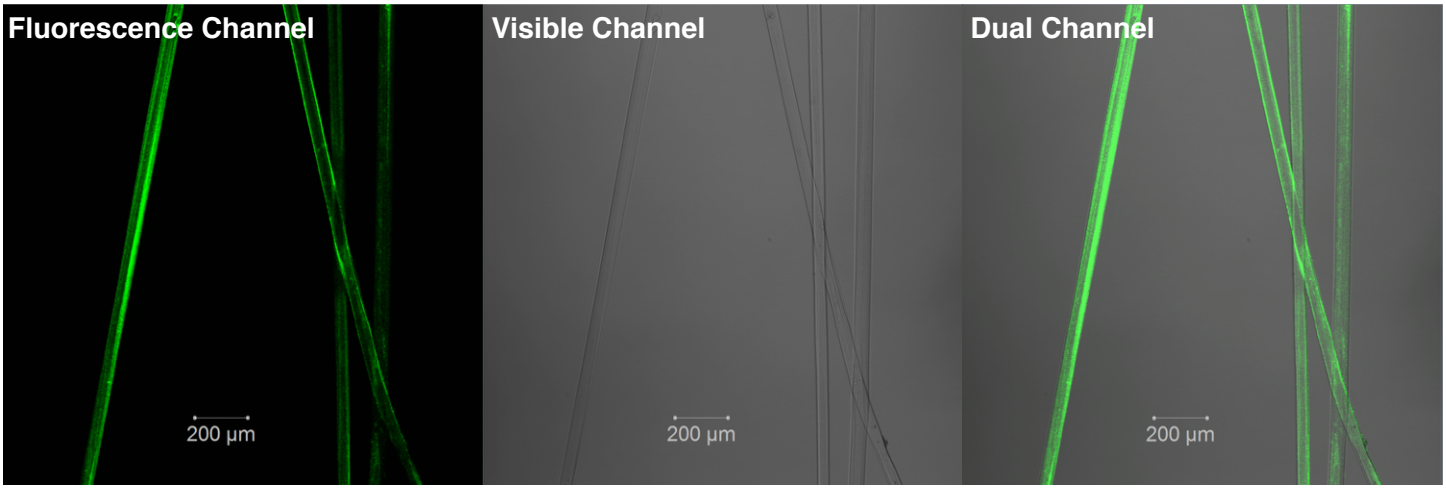
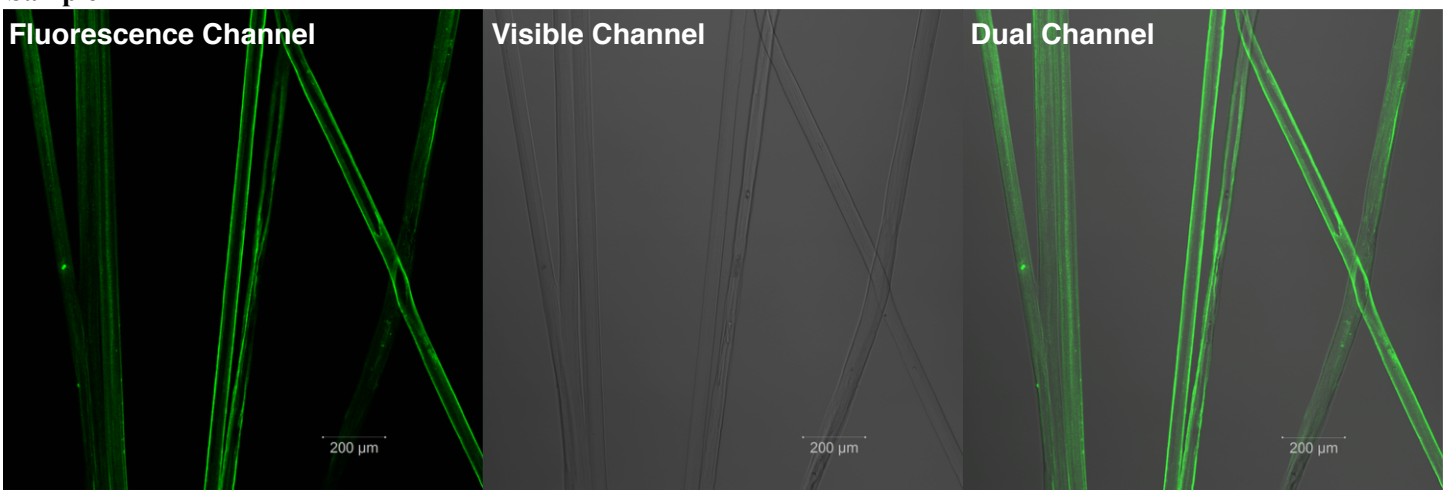


Figure S20. Confocal images of Clover protein tagging experiment: positive sets (cont)
Fibers were incubated with methylene blue (100 μM) and irradiated for 5 min with visible light. Activated fibers were triple rinsed with PBS, then incubated with Clover-sTCO (5 μM) for 1 min, then triple rinsed and imaged with a Zeiss LSM 5 Live DuoScan highspeed confocal microscope

Sample 1



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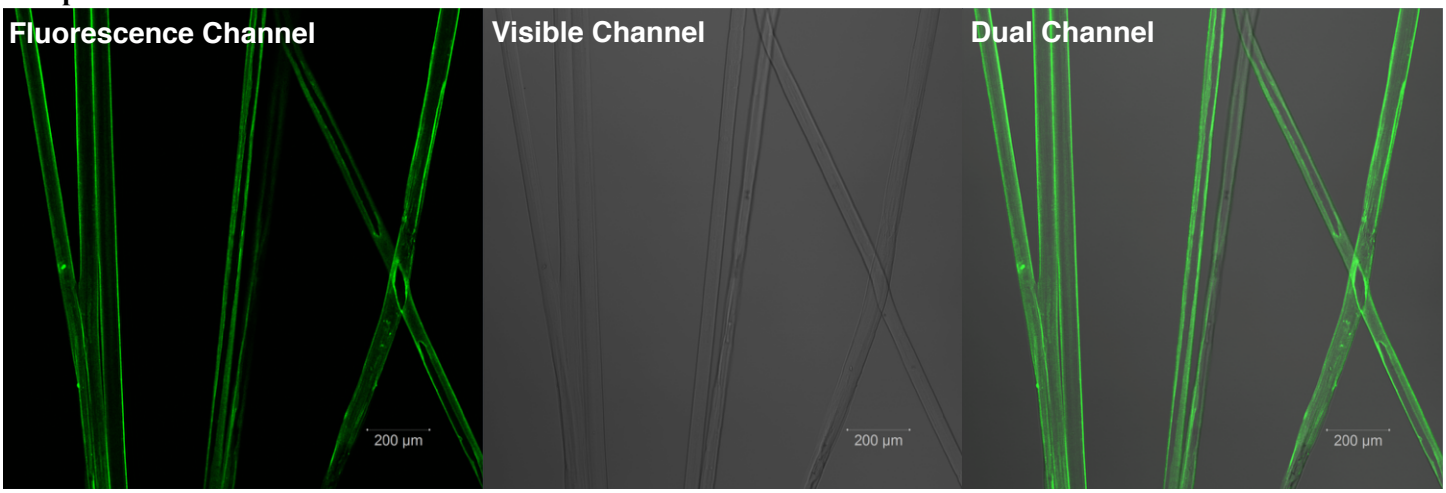

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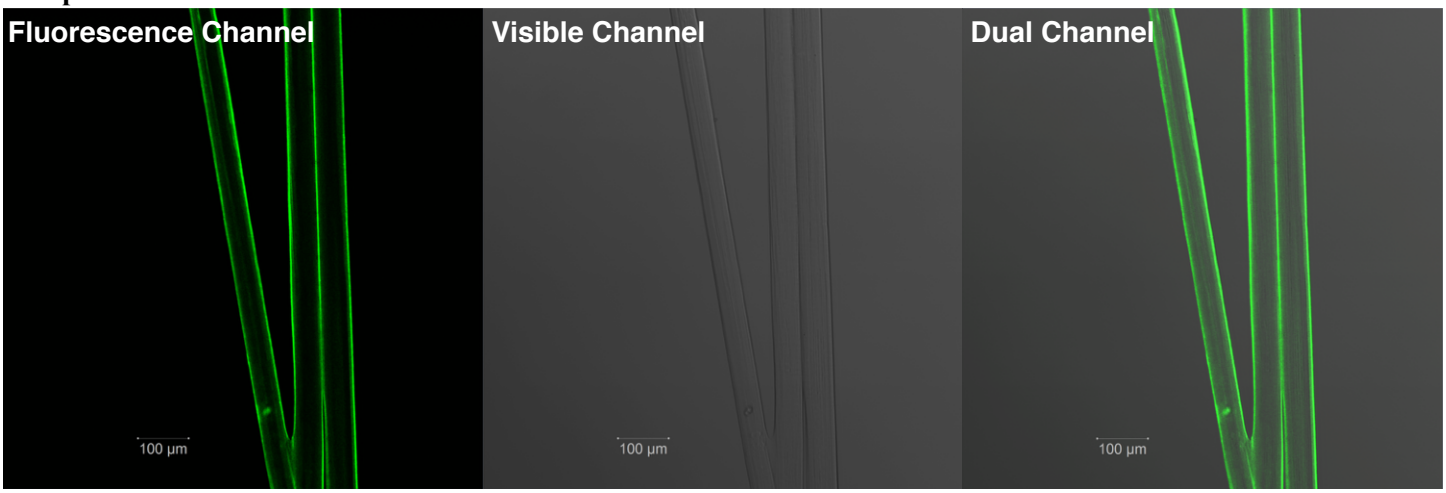
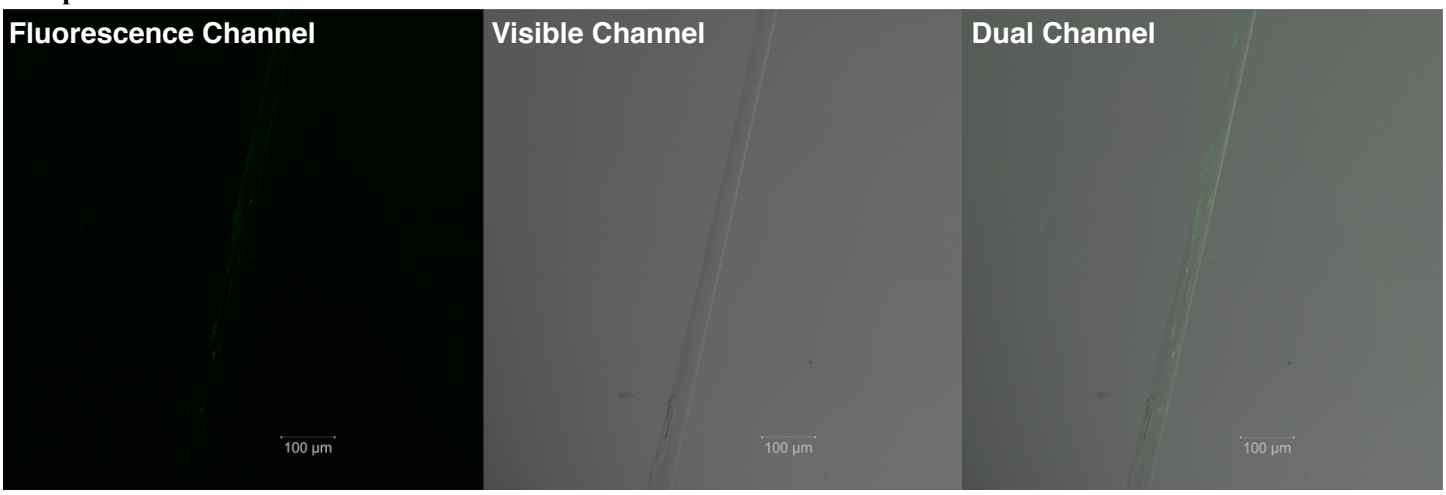
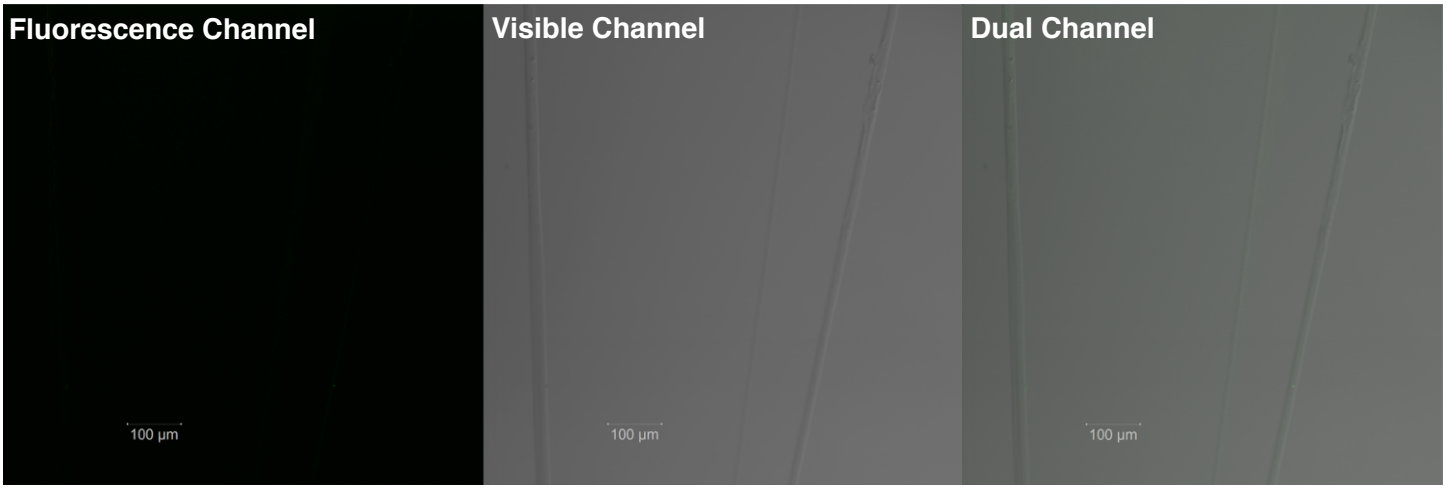


Figure S21. Confocal image of Clover protein tagging experiment: control without sensitizer
Fibers were incubated with PBS for 5 min followed by triple PBS rinsing, then irradiated in PBS for 5 min. Fibers were triple rinsed with PBS, and incubated with Clover-sTCO (1 μM) for 1 min, then triple rinsed and imaged with a Zeiss LSM 5 Live DuoScan highspeed confocal microscope

Sample 1



Sample 2



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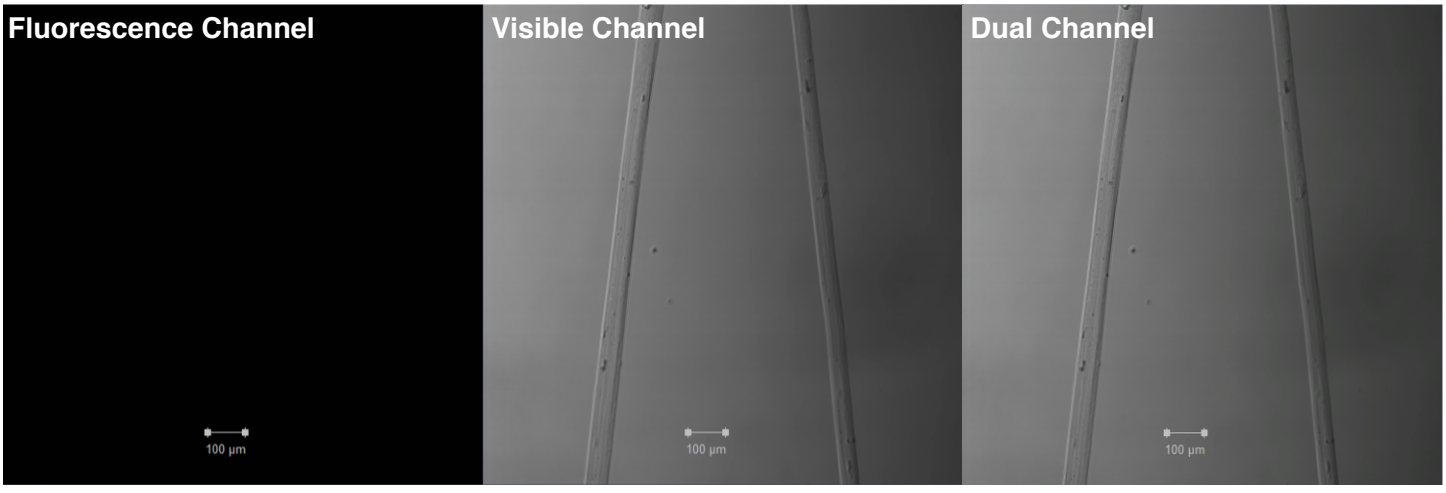


Figure S22. Confocal image of Clover protein tagging experiment: control without sensitizer (cont)
Fibers were incubated with PBS for 5 min followed by triple PBS rinsing, then irradiated in PBS for 5 min. Fibers were triple rinsed with PBS, and incubated with Clover-sTCO (1 μM) for 1 min, then triple rinsed and imaged with a Zeiss LSM 5 Live DuoScan highspeed confocal microscope

Sample 1

Sample 2

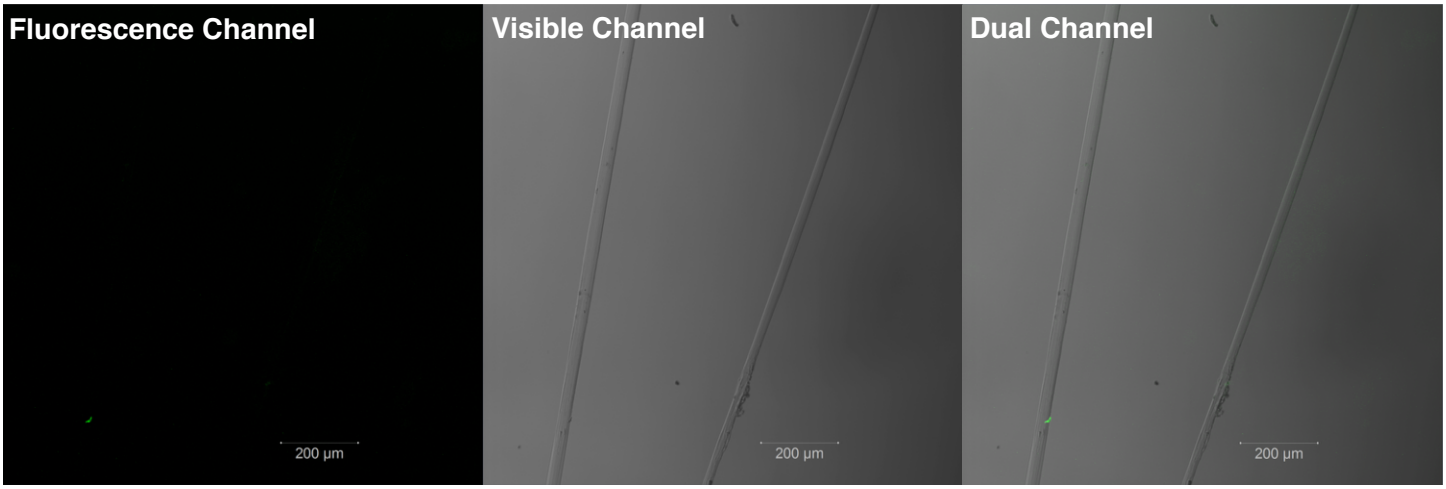
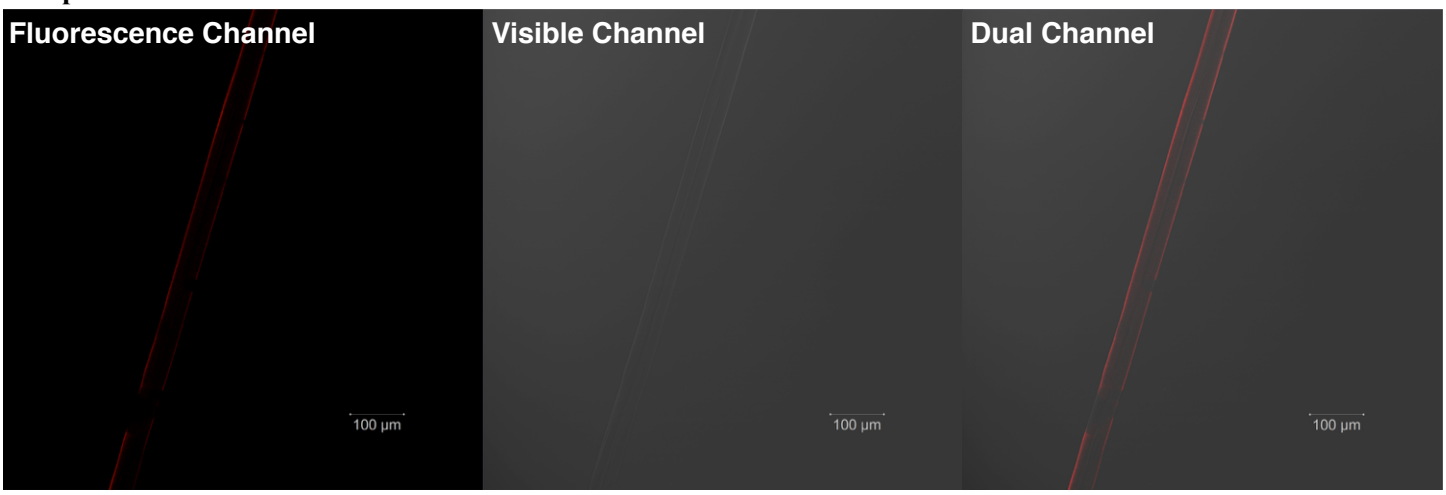


Figure S23. Confocal images of oxidizing DHTz-microfibers by horseradish peroxidase (HRP): positive sets
Fibers were incubated with HRP (10 μM) for 60 min. Activated fibers were triple rinsed with PBS, then incubated with Alexa-sTCO (1 μM) for 1 min, then triple rinsed and imaged with a Zeiss LSM 5 Live DuoScan highspeed confocal microscope

Sample 1



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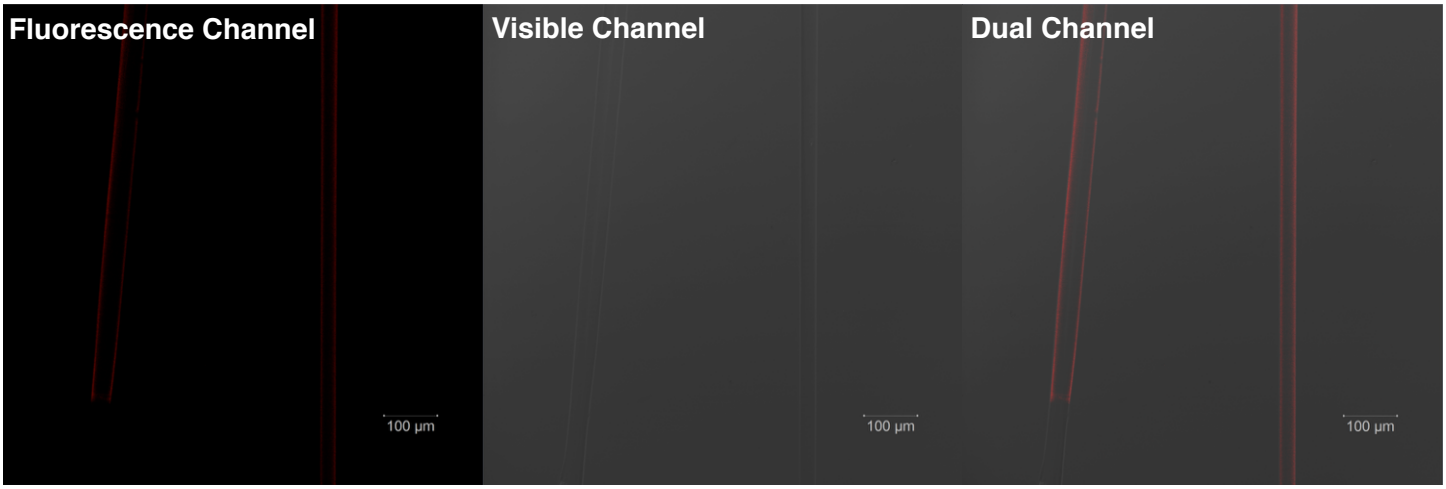

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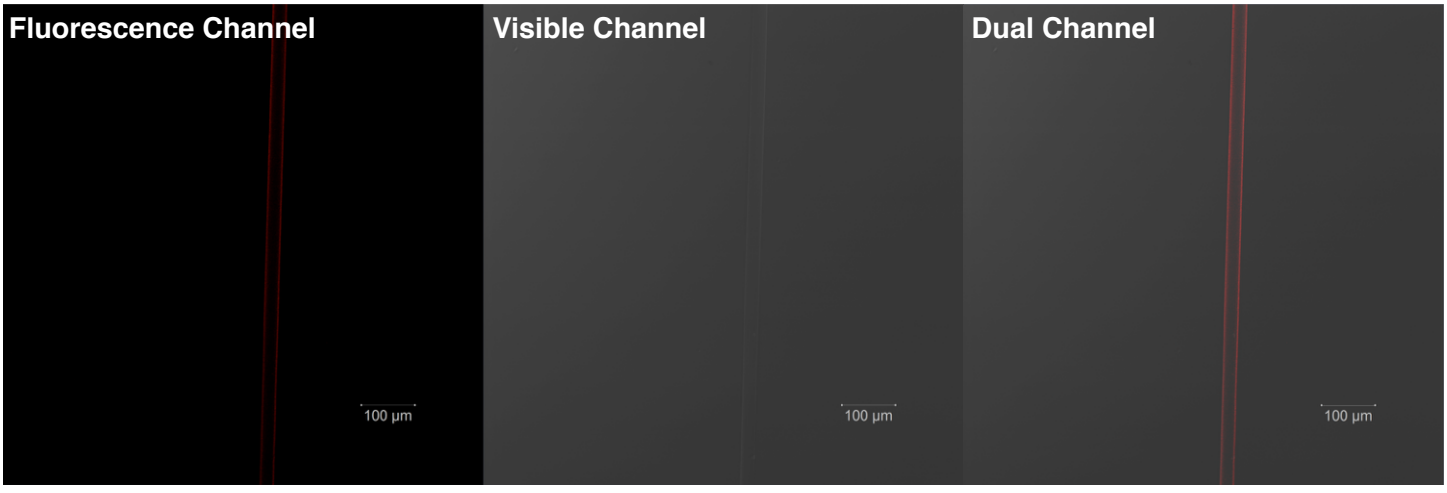
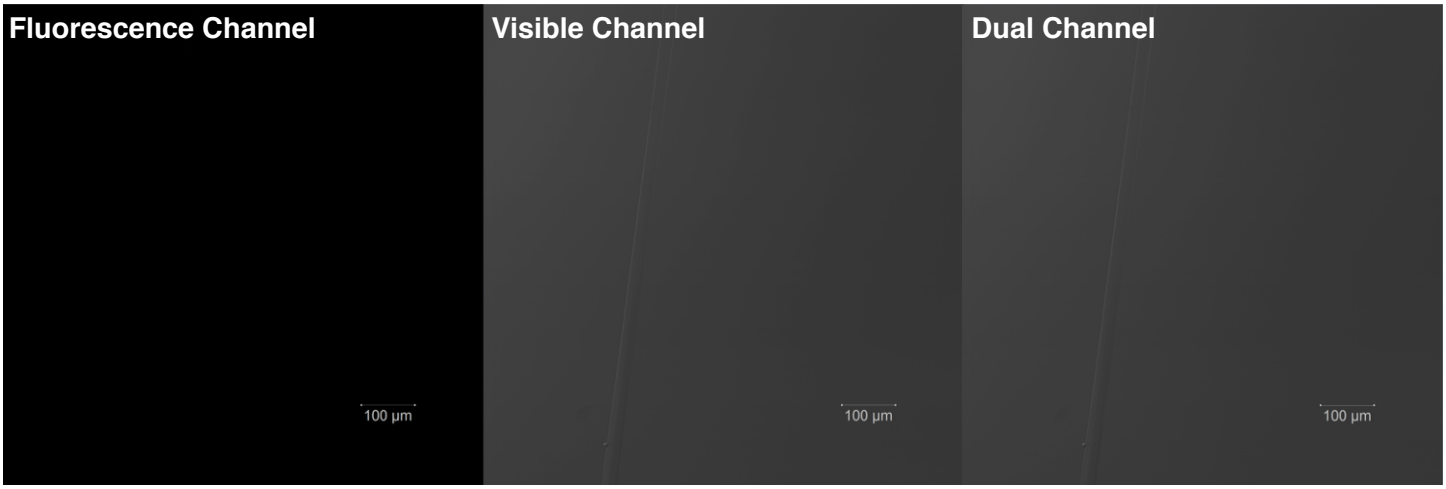
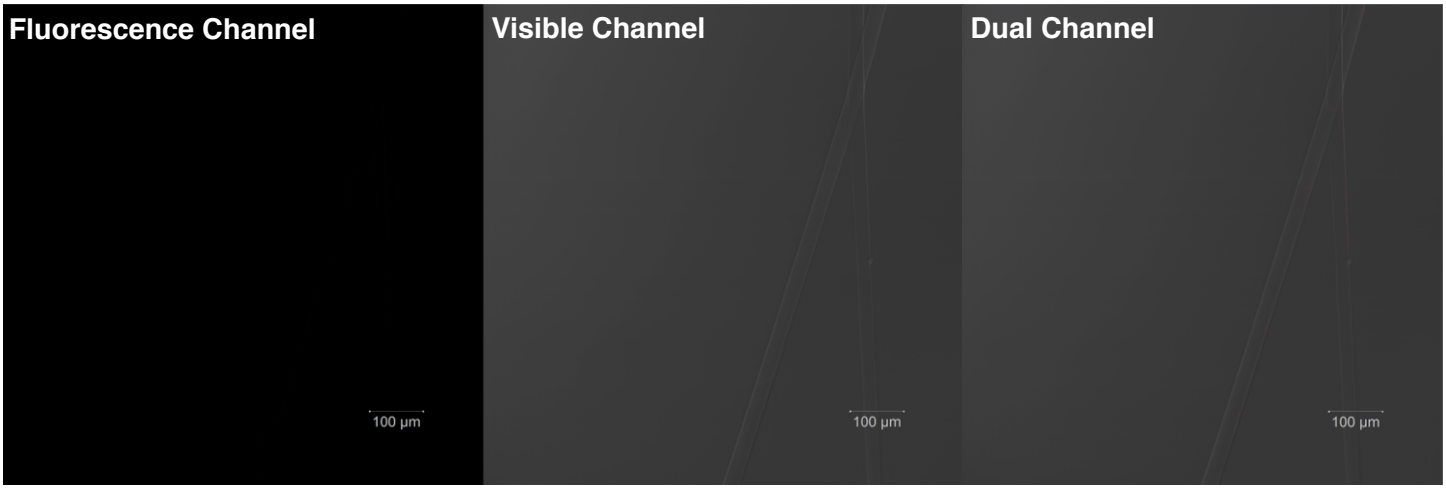


Figure S24. Confocal images of oxidizing DHTz-microfibers by horseradish peroxidase (HRP): control without HRP
Fibers were incubated with PBS for 60 min followed by triple PBS rinsing, then incubated with Alexa-sTCO (1 μM) for 1 min followed by triple PBS rinsing and imaged with a Zeiss LSM 5 Live DuoScan highspeed confocal microscope

Sample 1



Sample 2



Sample 3

Figure S25. Confocal images of RGD peptide tagging experiment: positive sets
Fibers were incubated with methylene blue (100 μM) and irradiated for 5 min with visible light. Activated fibers were triple rinsed with PBS, incubated with RGD-sTCO (10 μM) for 1 min then triple rinsed. The fibers were then washed with sterile PBS and cell culture media three times respectively before being sterilized under UV for 15 minutes. A 200 uL suspension of fibroblasts NIH 3T3 cells with a density of 0.5×10^6 cells/mL was added and cultured at 37°C for 20 hours and imaged with a Zeiss LSM 5 Live DuoScan highspeed confocal microscope under transmitted light

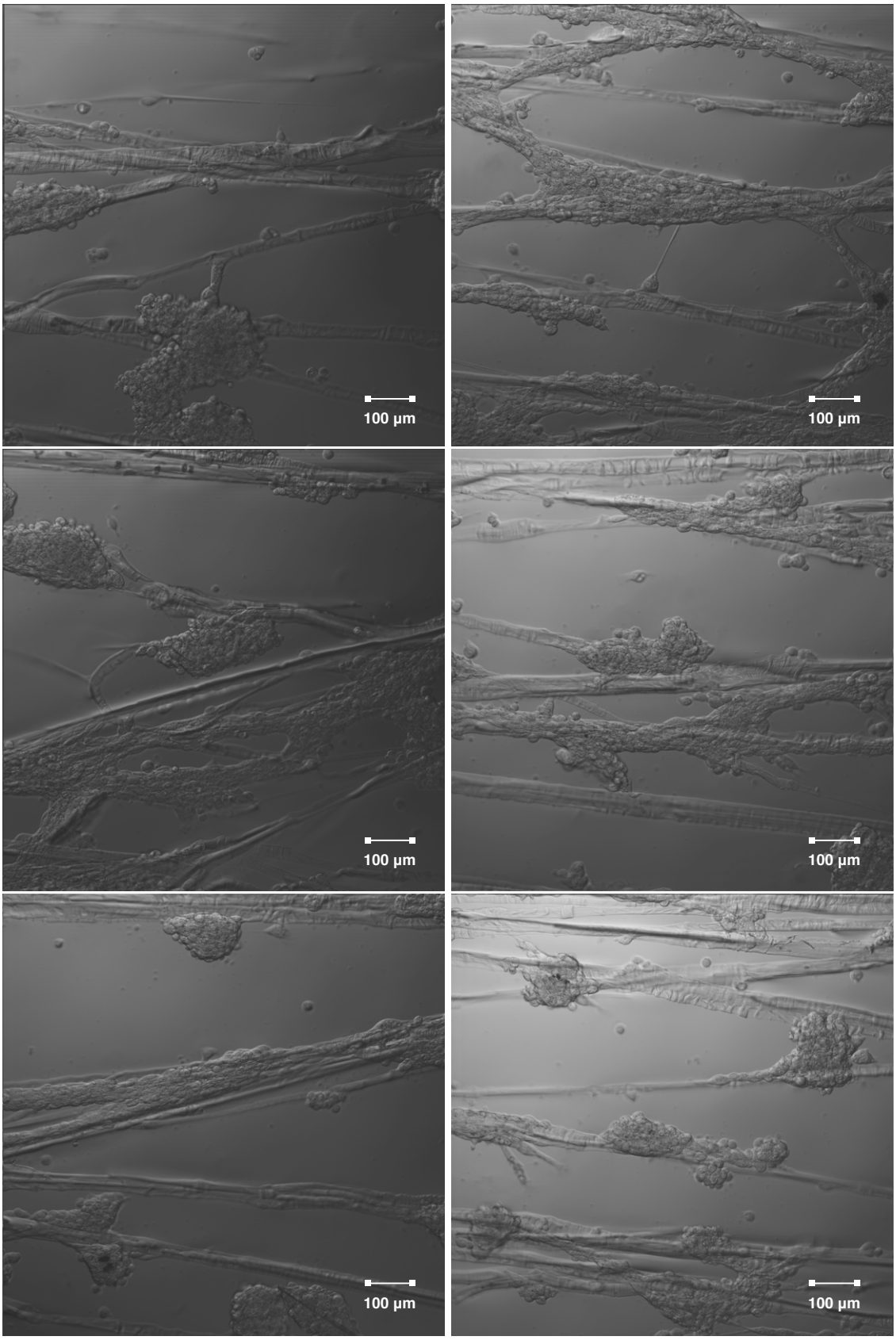


Figure S26. Confocal images of RGD peptide tagging experiment: control without sensitizer
Fibers were incubated with PBS and irradiated for 5 min with visible light. Fibers were then triple rinsed with PBS and incubated with RGD-sTCO (10 μM) for 1 min, then triple rinsed. The fibers were then washed with sterile PBS and cell culture media three times respectively before being sterilized under UV for 15 minutes. A 200 uL suspension of fibroblasts NIH 3T3 cells with a density of 0.5×10^6 cells/mL was added and cultured at 37°C for 20 hours and imaged with a Zeiss LSM 5 Live DuoScan highspeed confocal microscope under transmitted light

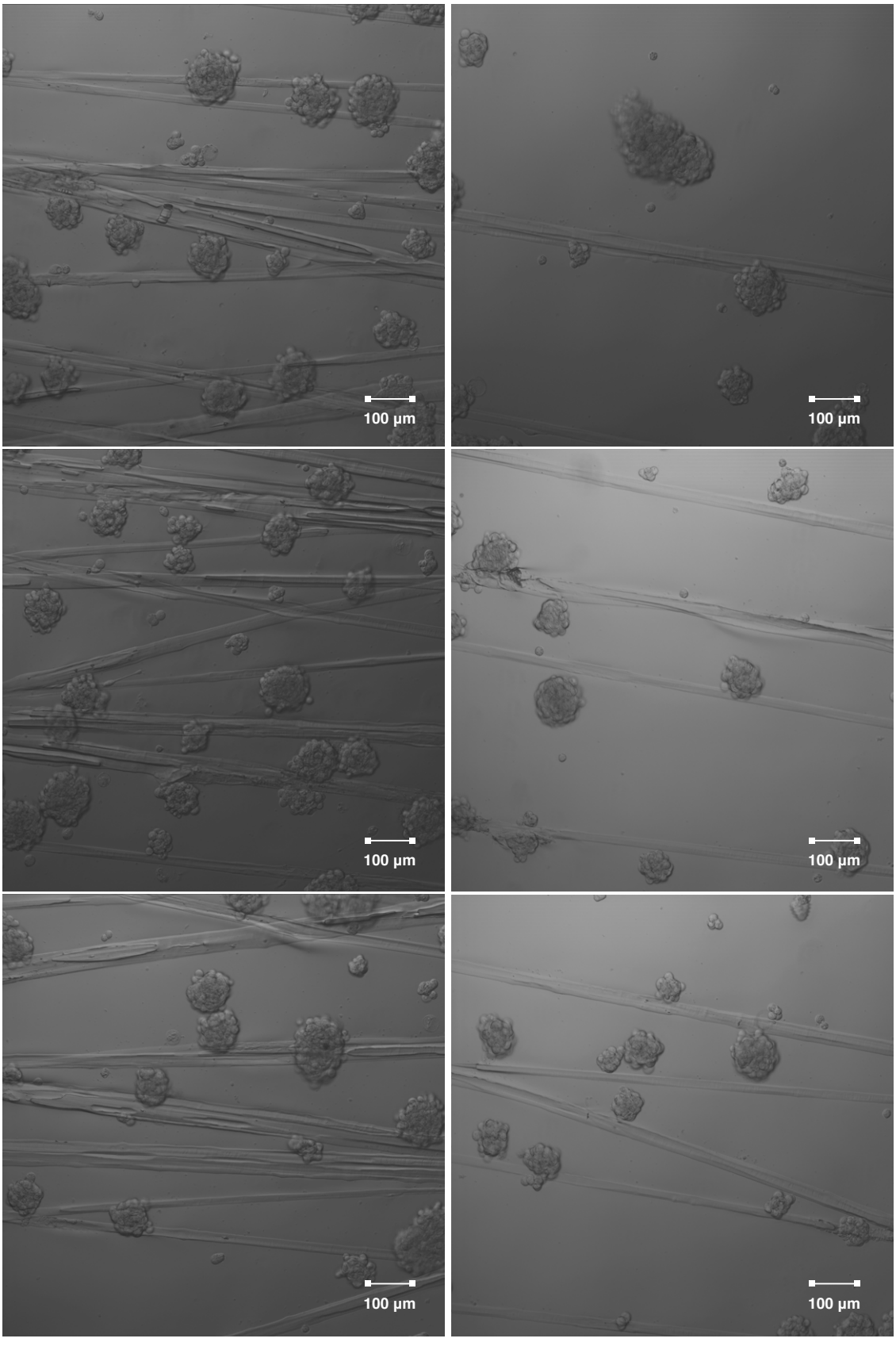


Figure S27. Confocal images of RGD peptide tagging experiment: control without RGD
Fibers were incubated with methylene blue (100 μM) and irradiated for 5 min with visible light. Activated fibers were triple rinsed with PBS, then washed with sterile PBS and cell culture media three times respectively before being sterilized under UV for 15 minutes. A 200 uL suspension of fibroblasts NIH 3T3 cells with a density of 0.5×10^6 cells/mL was added and cultured at 37°C for 20 hours and imaged with a Zeiss LSM 5 Live DuoScan highspeed confocal microscope under transmitted light

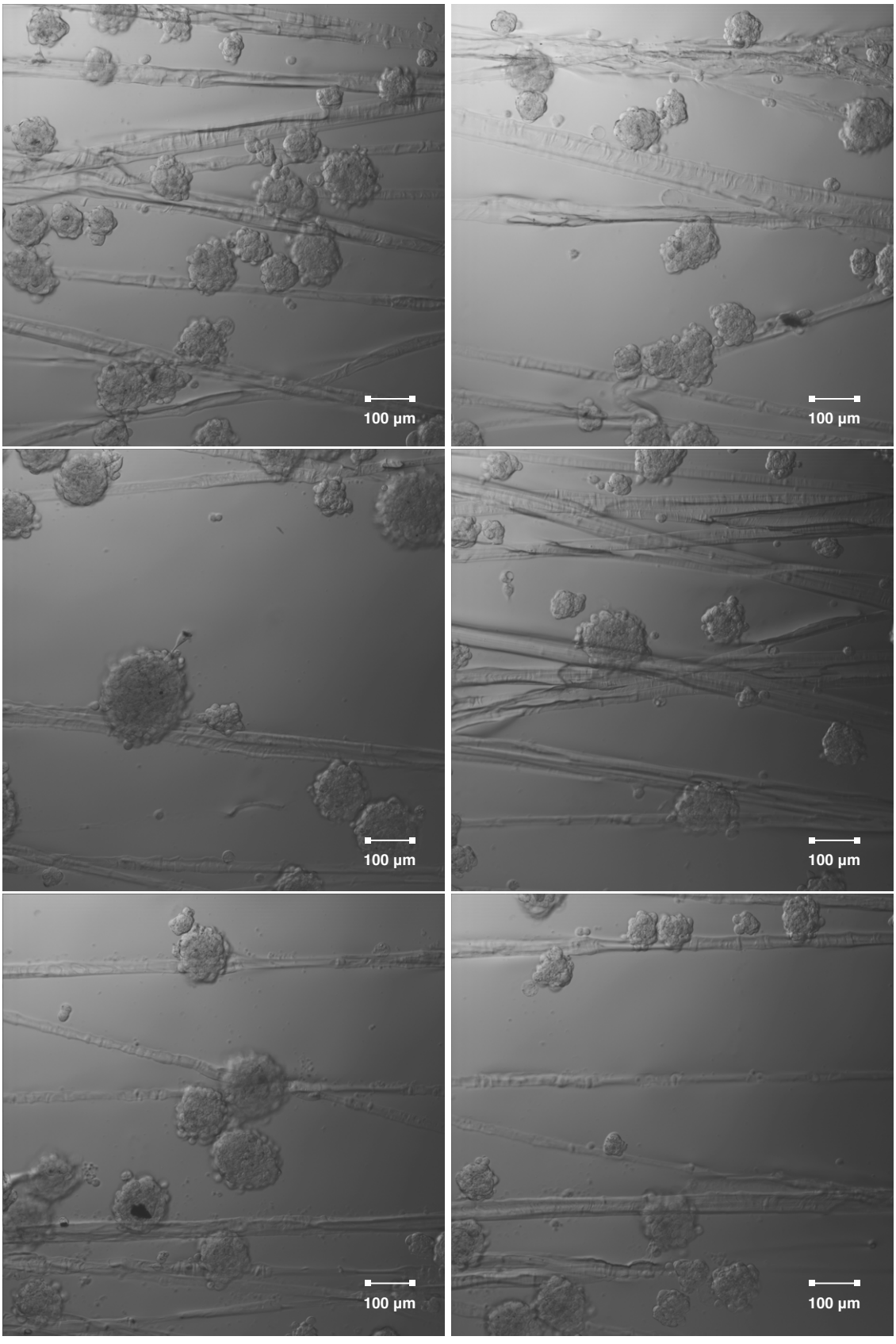
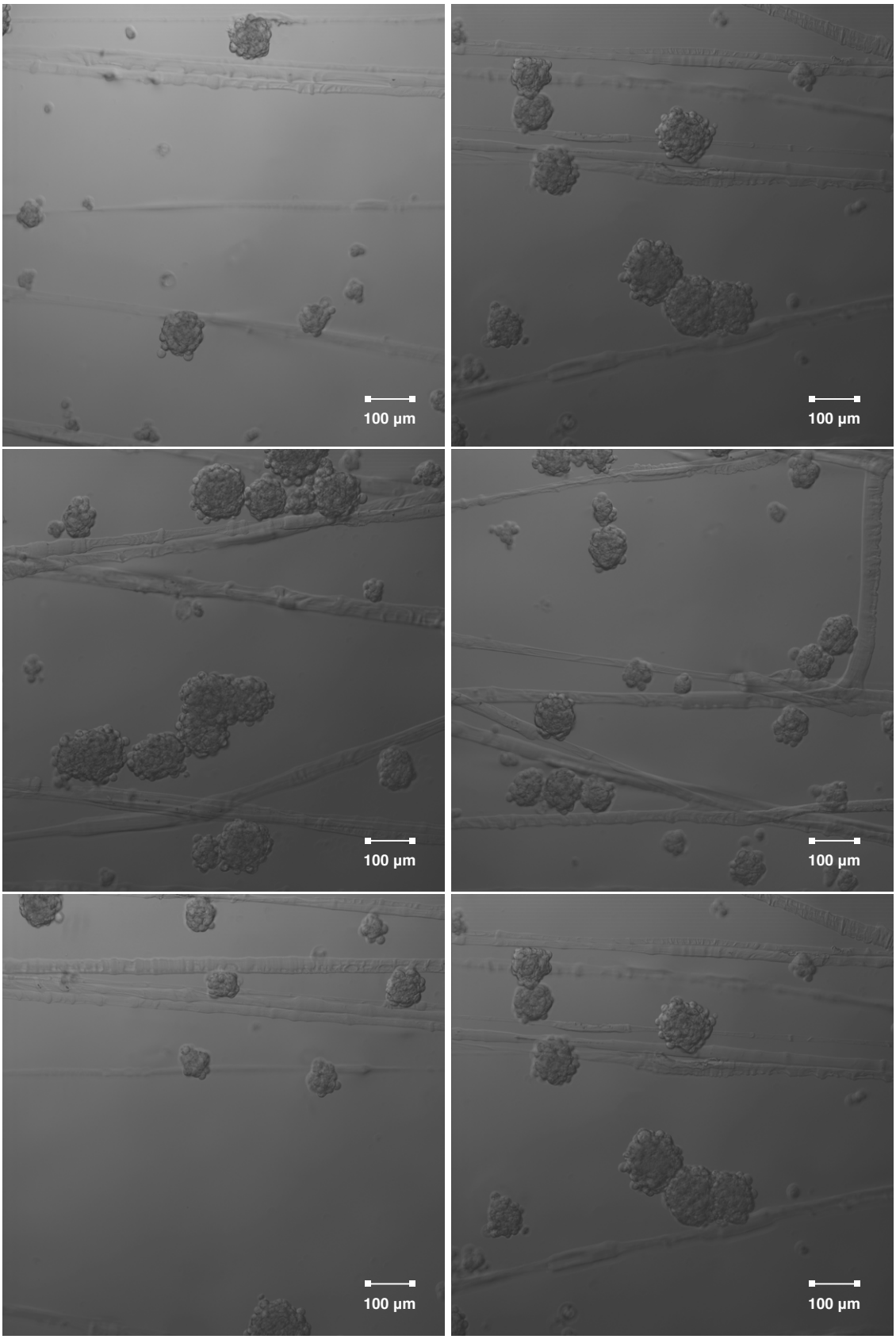


Figure S28. Confocal images of RGD peptide tagging experiment: control without sensitizer or RGD
Fibers were incubated with PBS and irradiated for 5 min with visible light. Fibers were then triple rinsed with PBS. The fibers were then washed with sterile PBS and cell culture media three times respectively before being sterilized under UV for 15 minutes. A 200 uL suspension of fibroblasts NIH 3T3 cells with a density of 0.5×10^6 cells/mL was added and cultured at 37°C for 20 hours and imaged with a Zeiss LSM 5 Live DuoScan highspeed confocal microscope under transmitted light



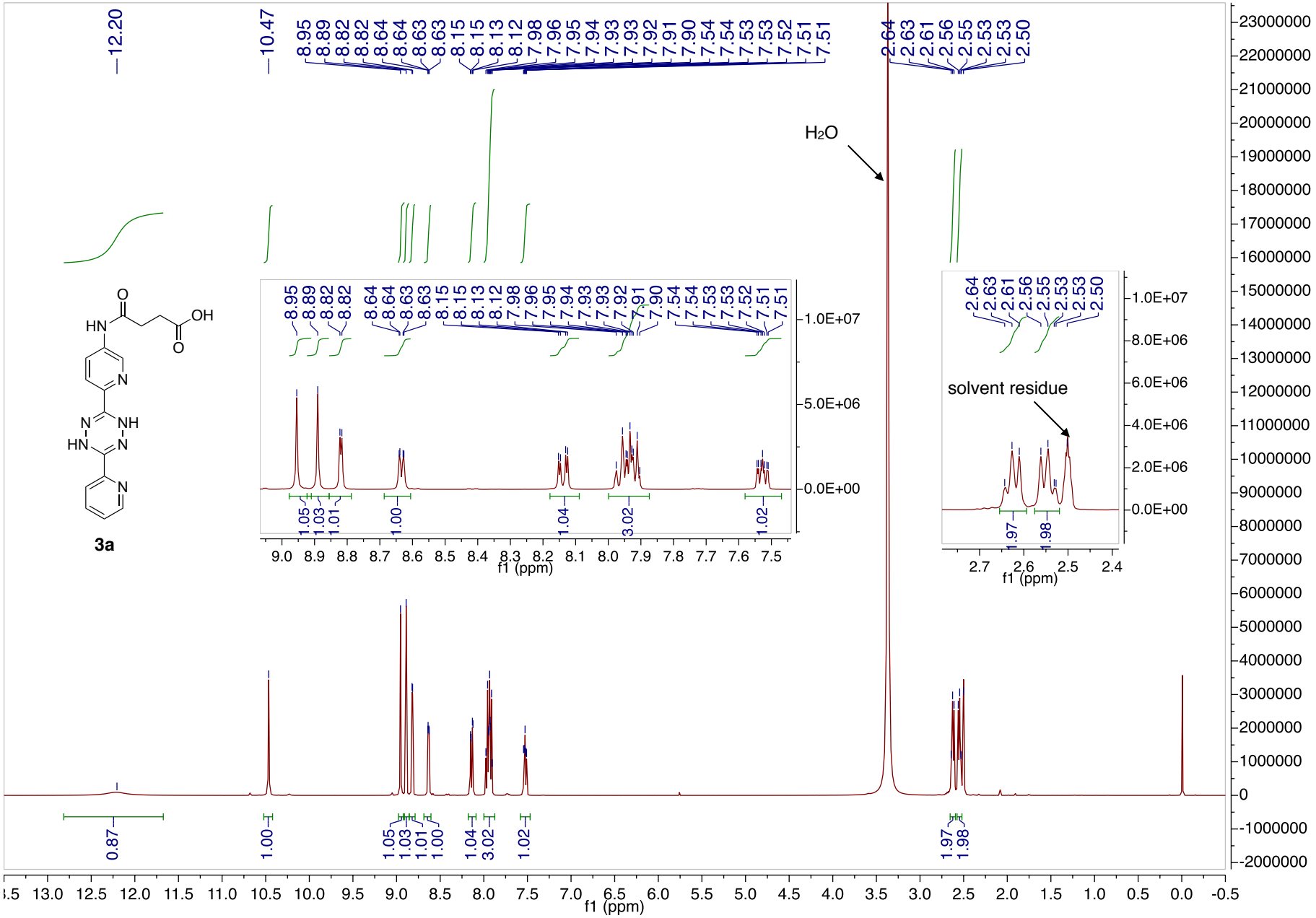


Figure S29. [1]H NMR spectrum of **3a**

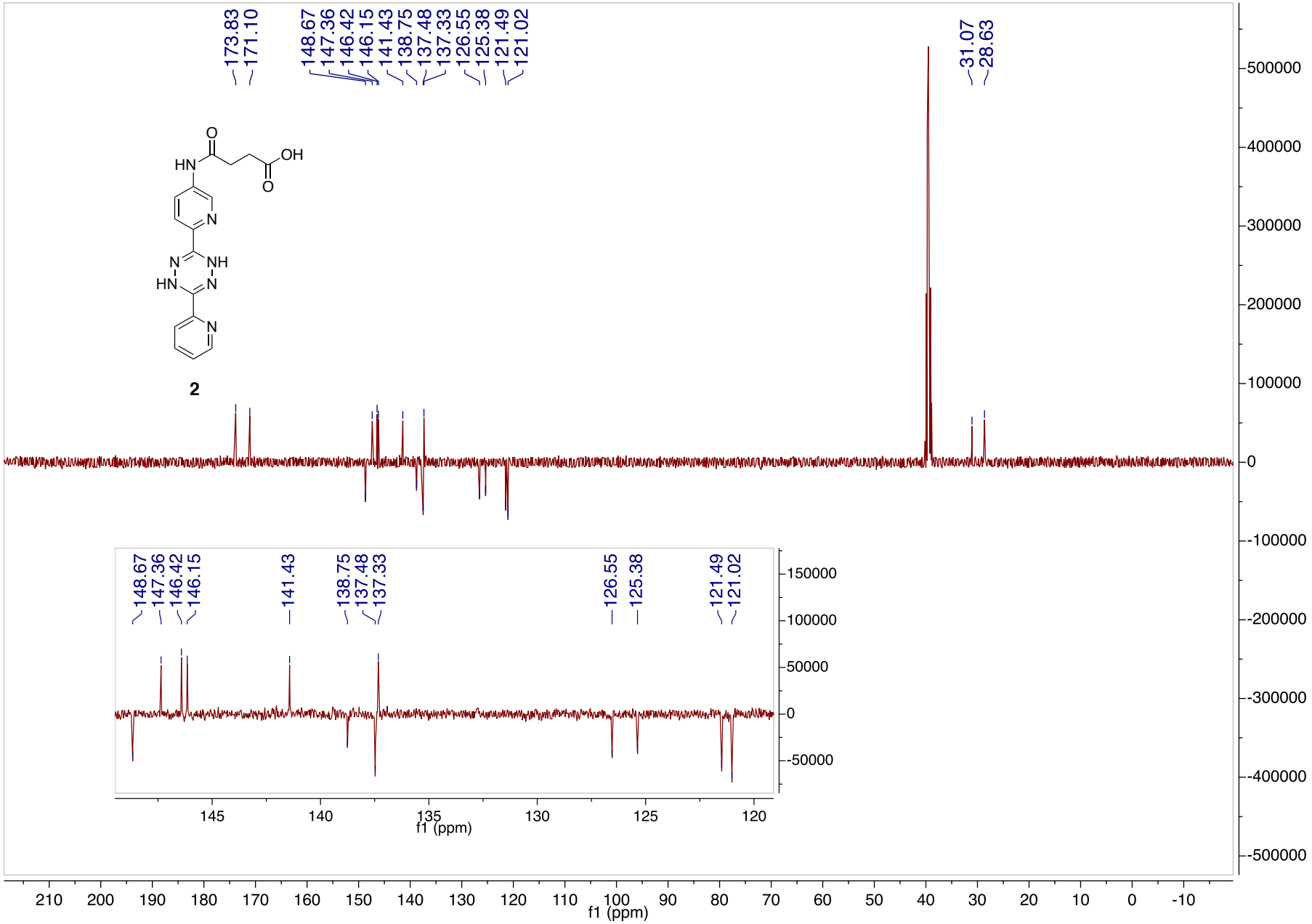


Figure S30. ^{13}C APT NMR spectrum of **3a**

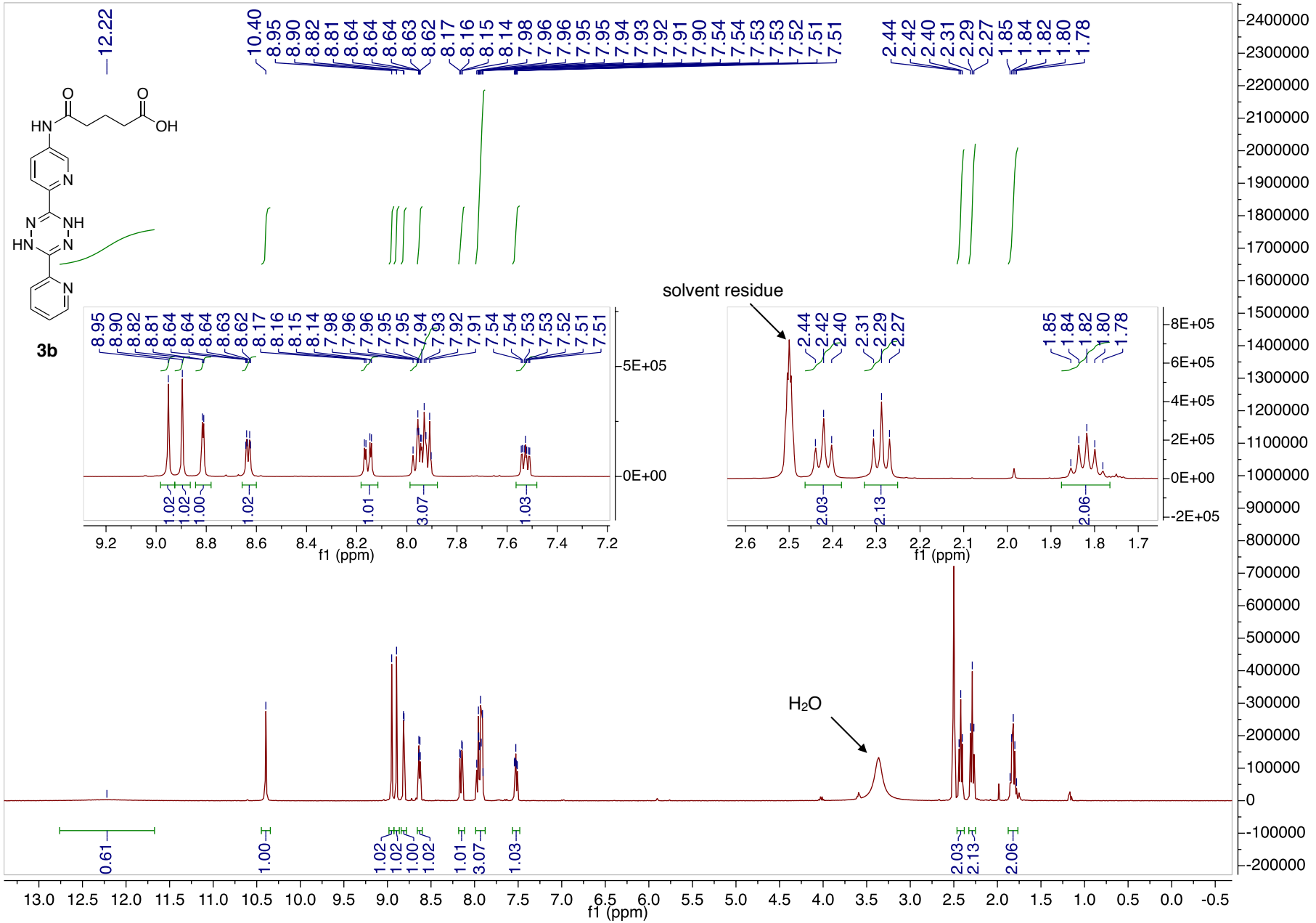


Figure S31. ^{1}H NMR spectrum of **3b**

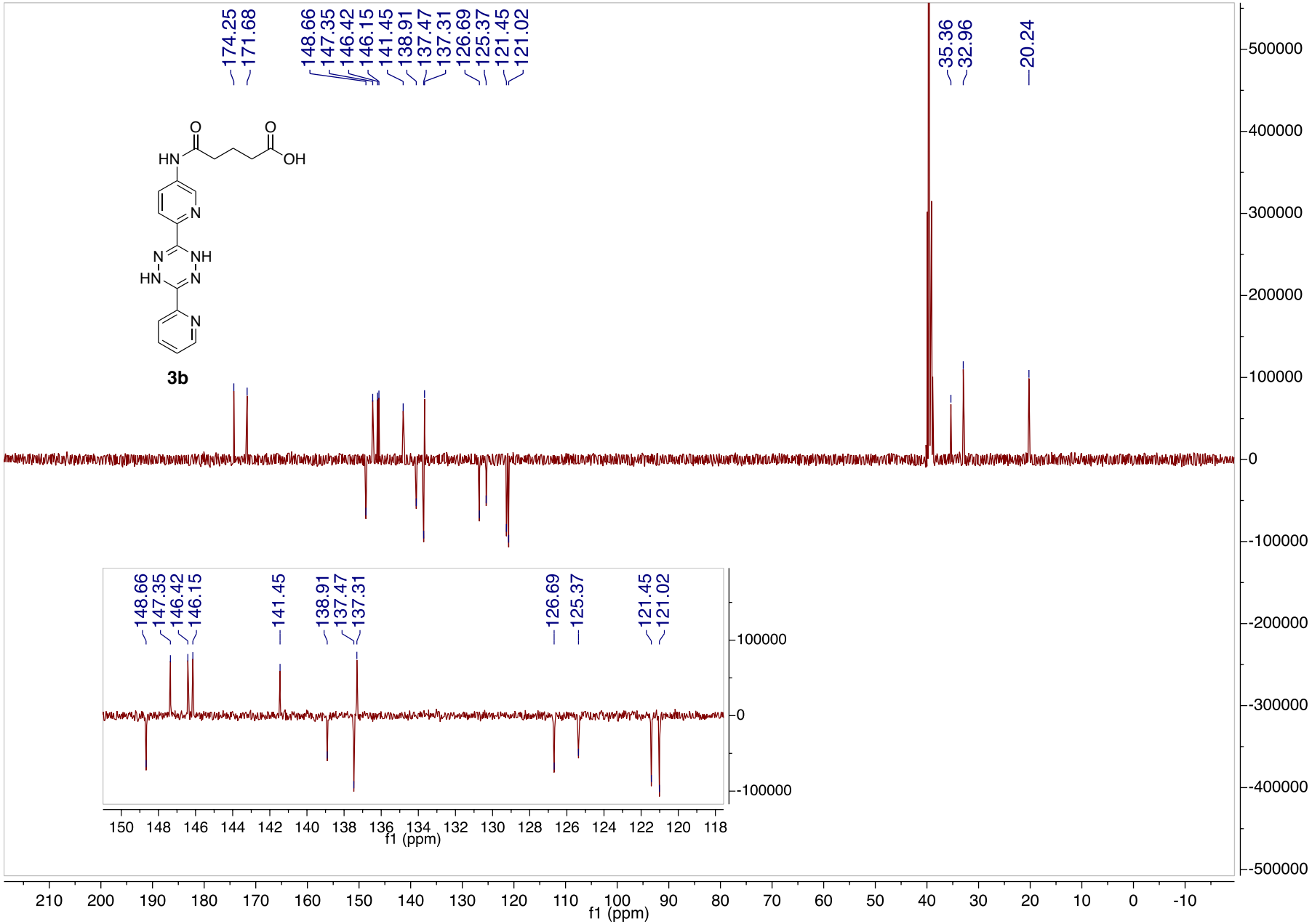


Figure S32. ^{13}C APT NMR spectrum of **3b**

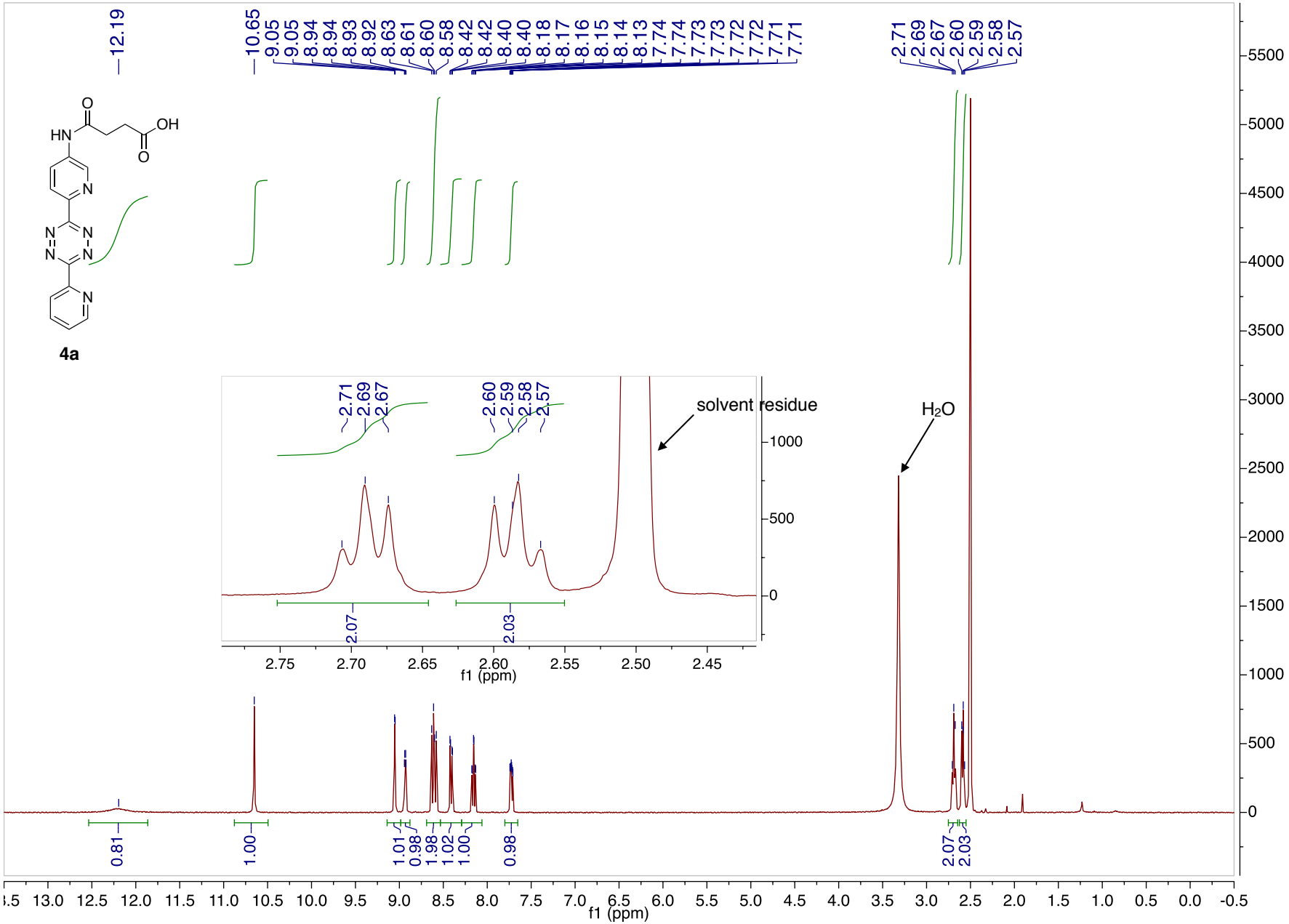


Figure S33. [1]H NMR spectrum of **4a**

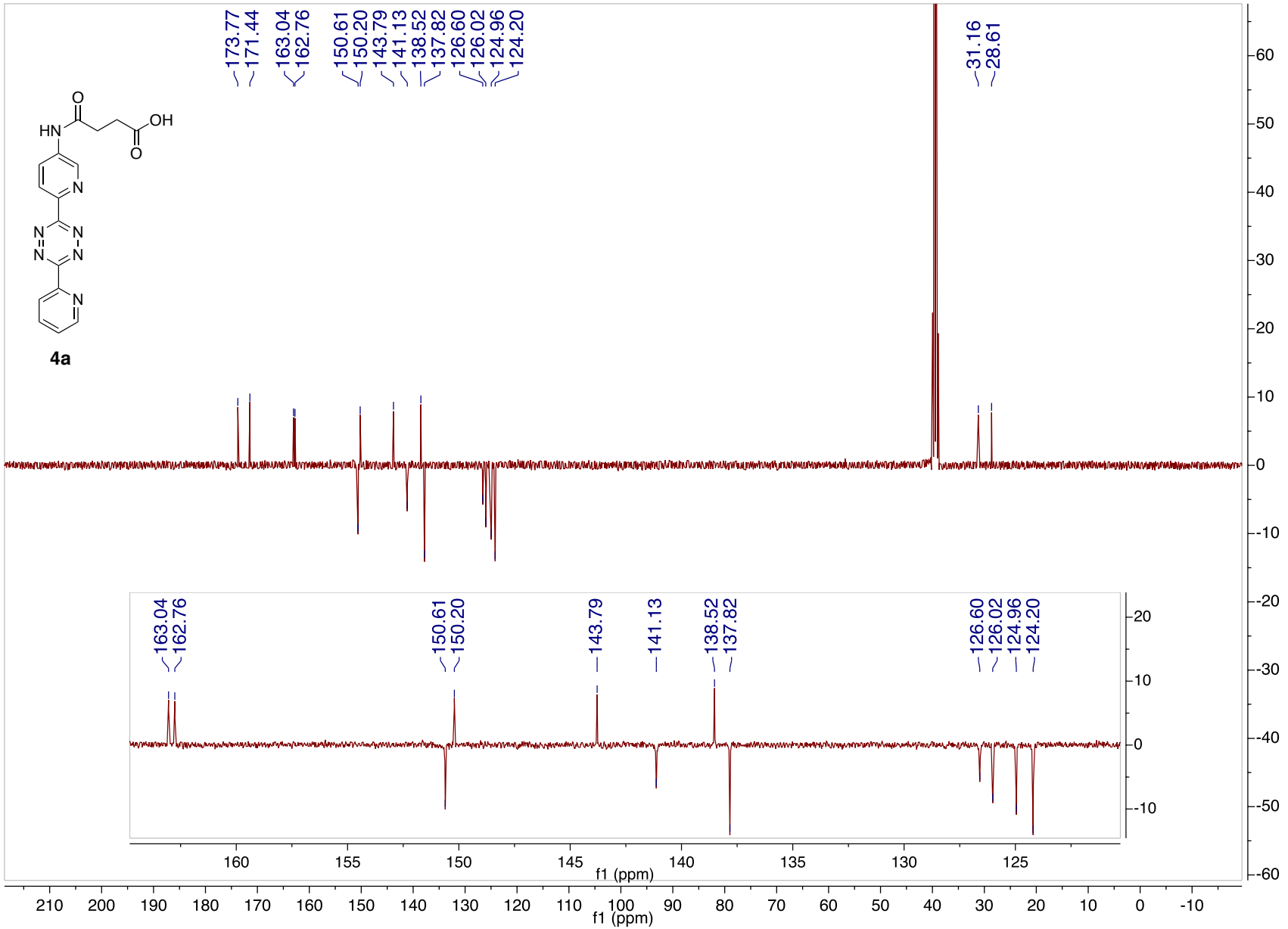


Figure S34. ^{13}C APT NMR spectrum of **4a**

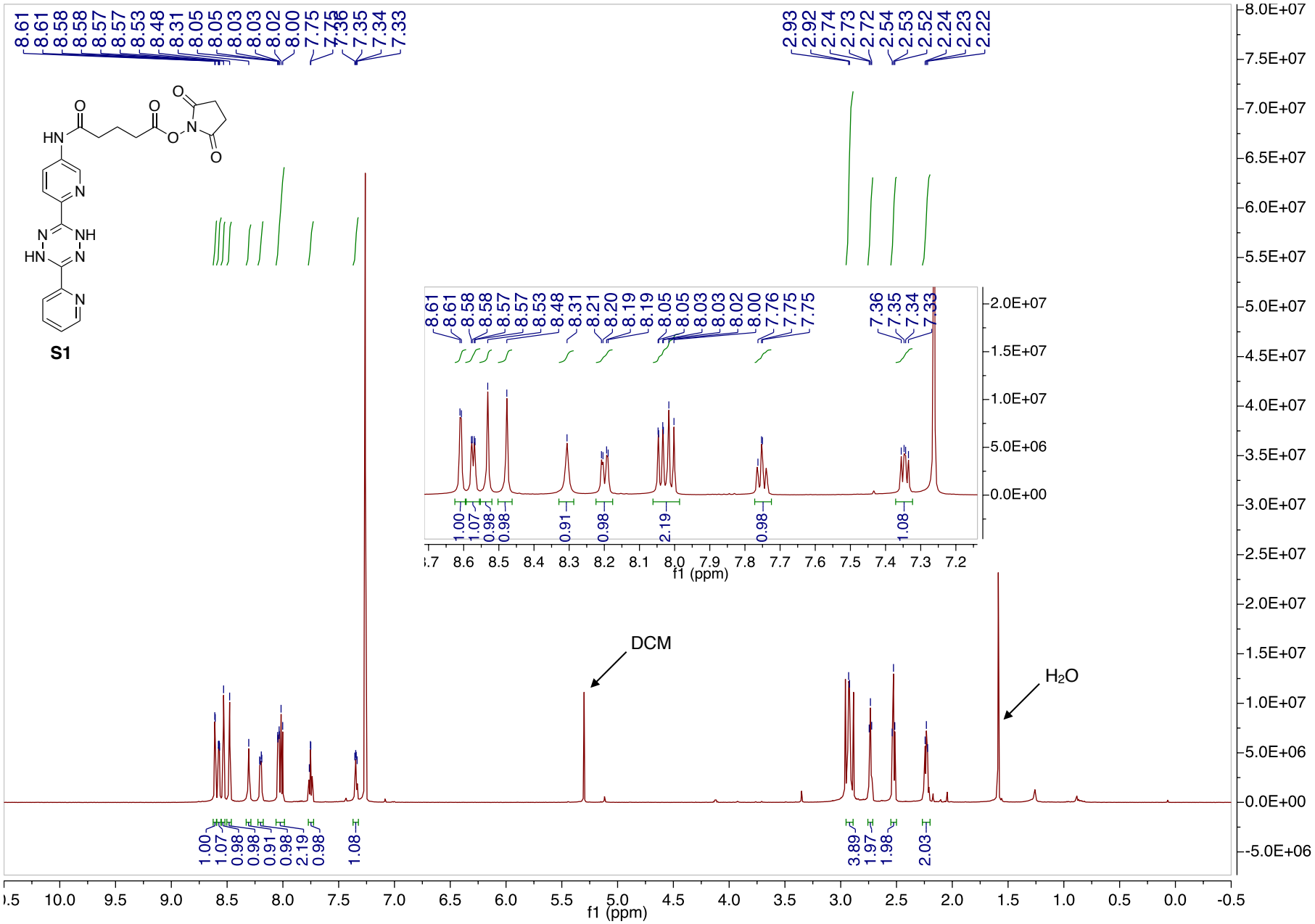


Figure S35. ^{1}H NMR spectrum of **S1**

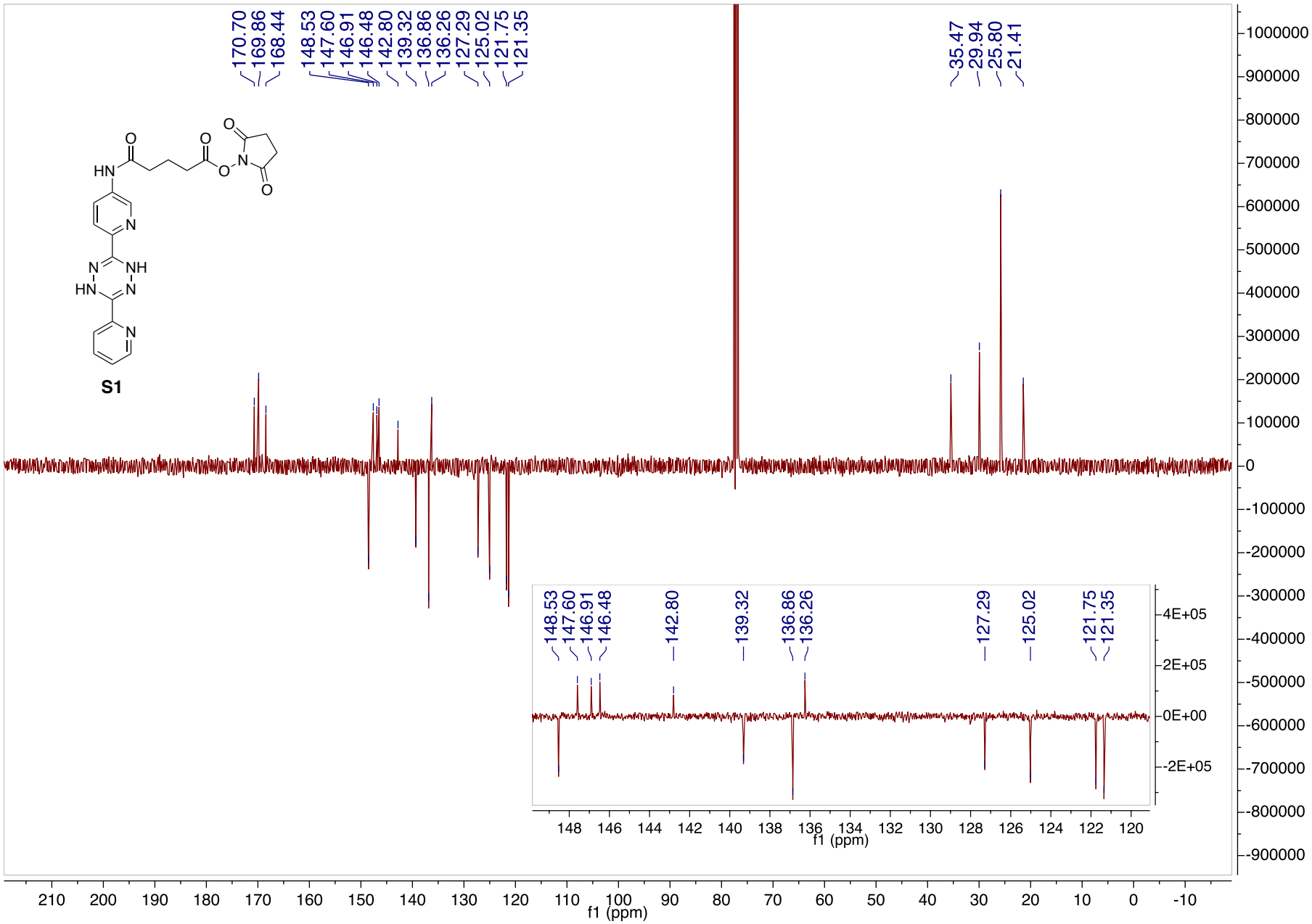


Figure S36. ^{13}C APT NMR spectrum of **S1**

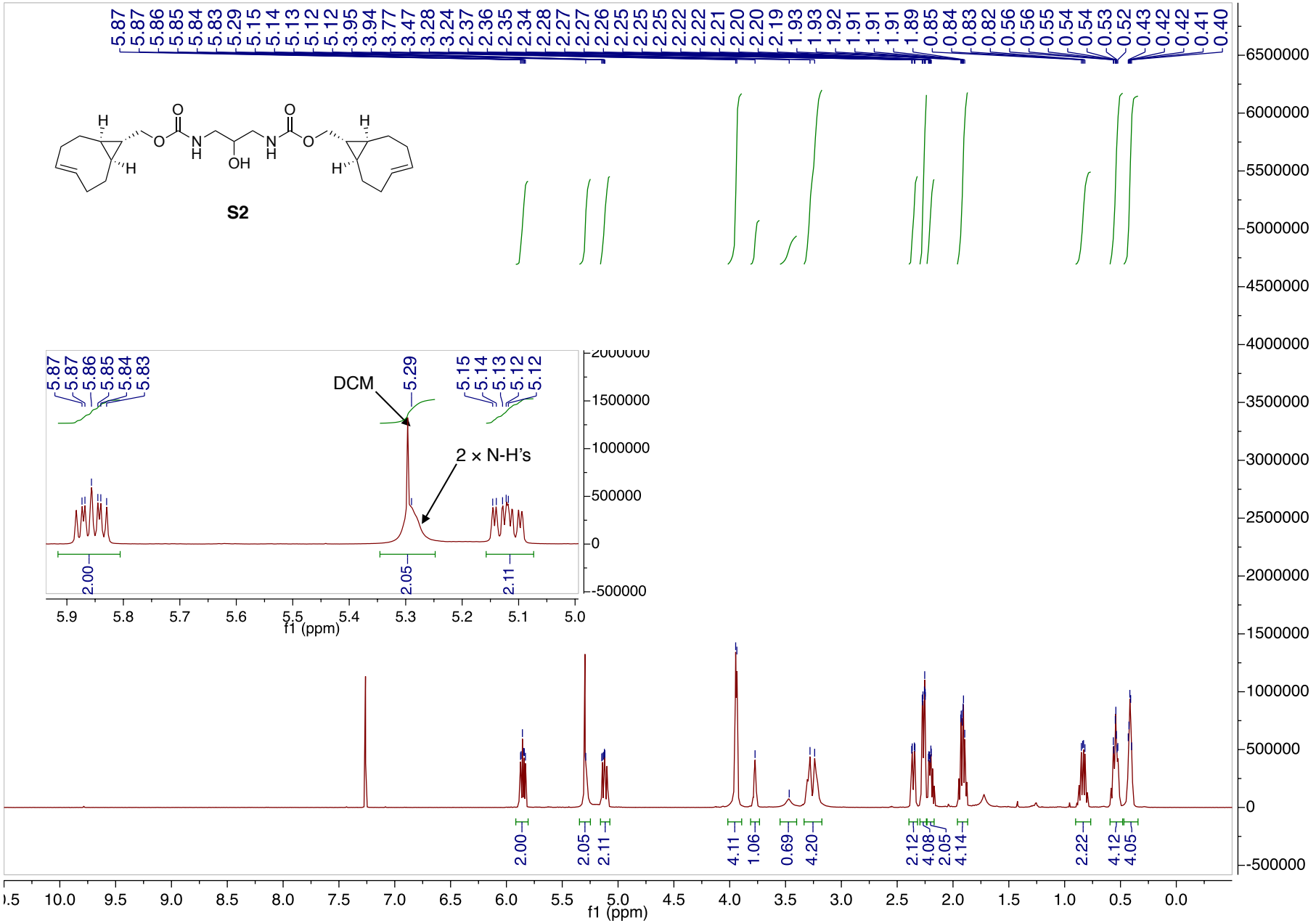


Figure S37. ^{1}H NMR spectrum of **S2**

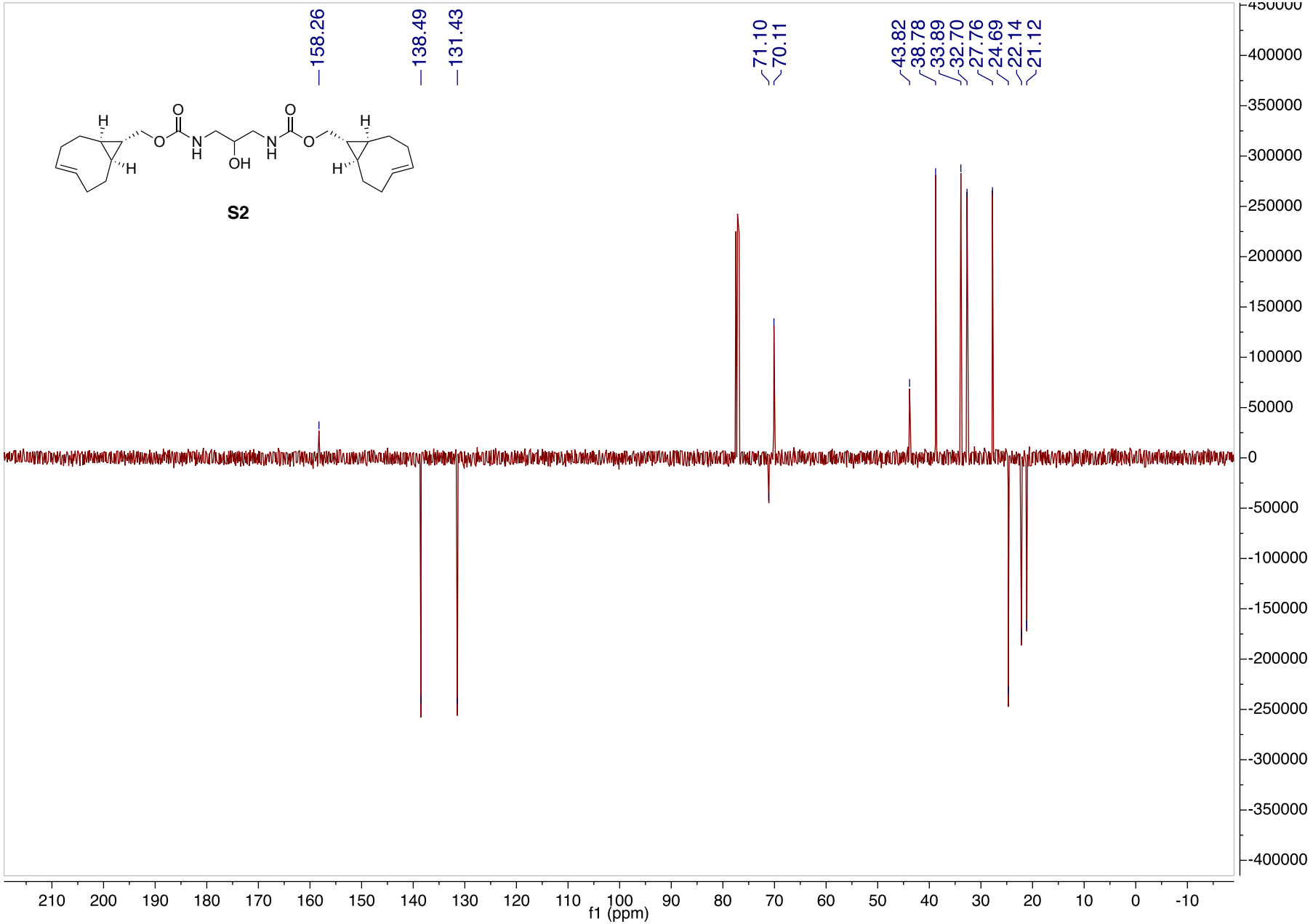


Figure S38. ^{13}C APT NMR spectrum of **S2**

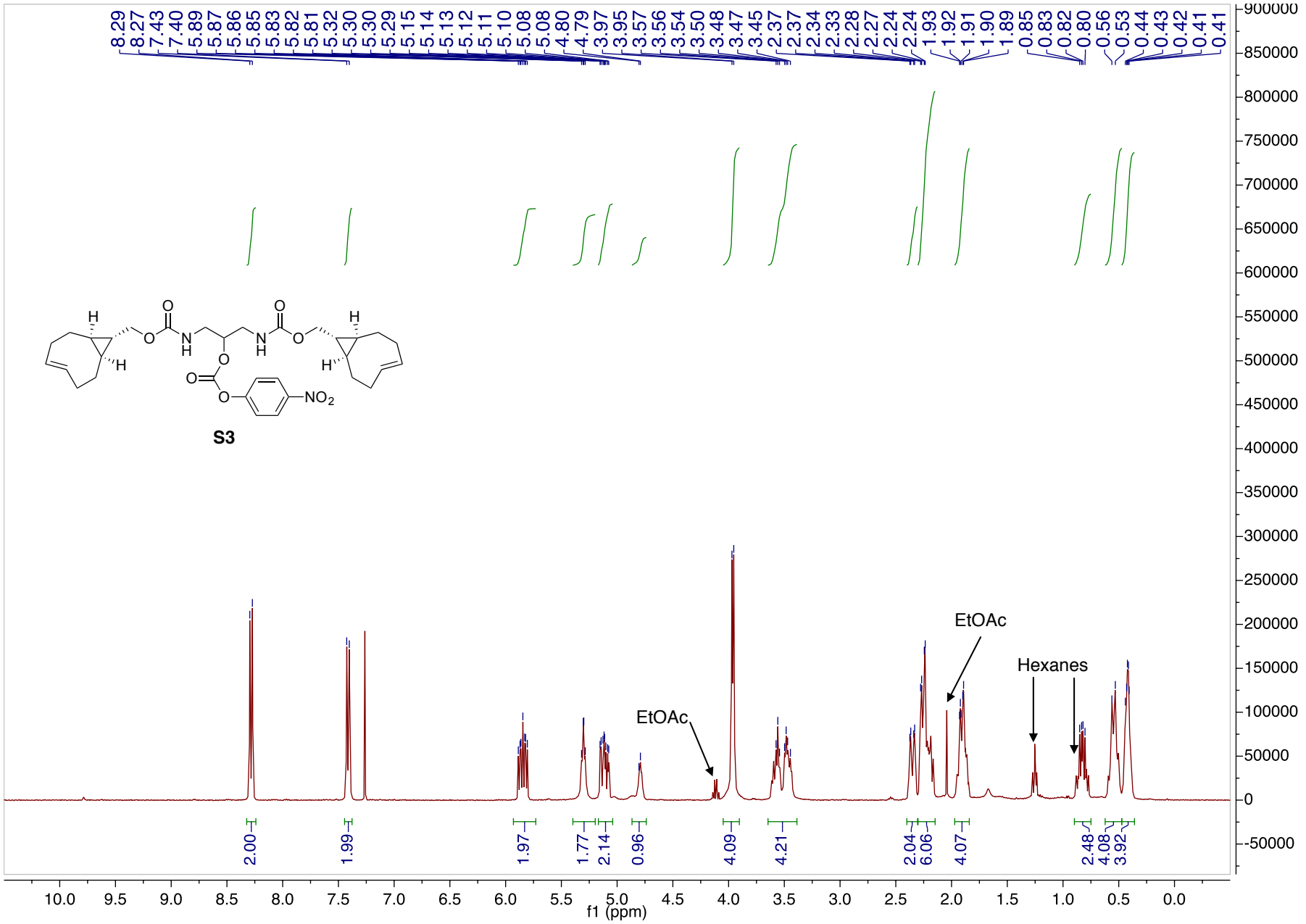


Figure S39. ^{1}H NMR spectrum of **S3**

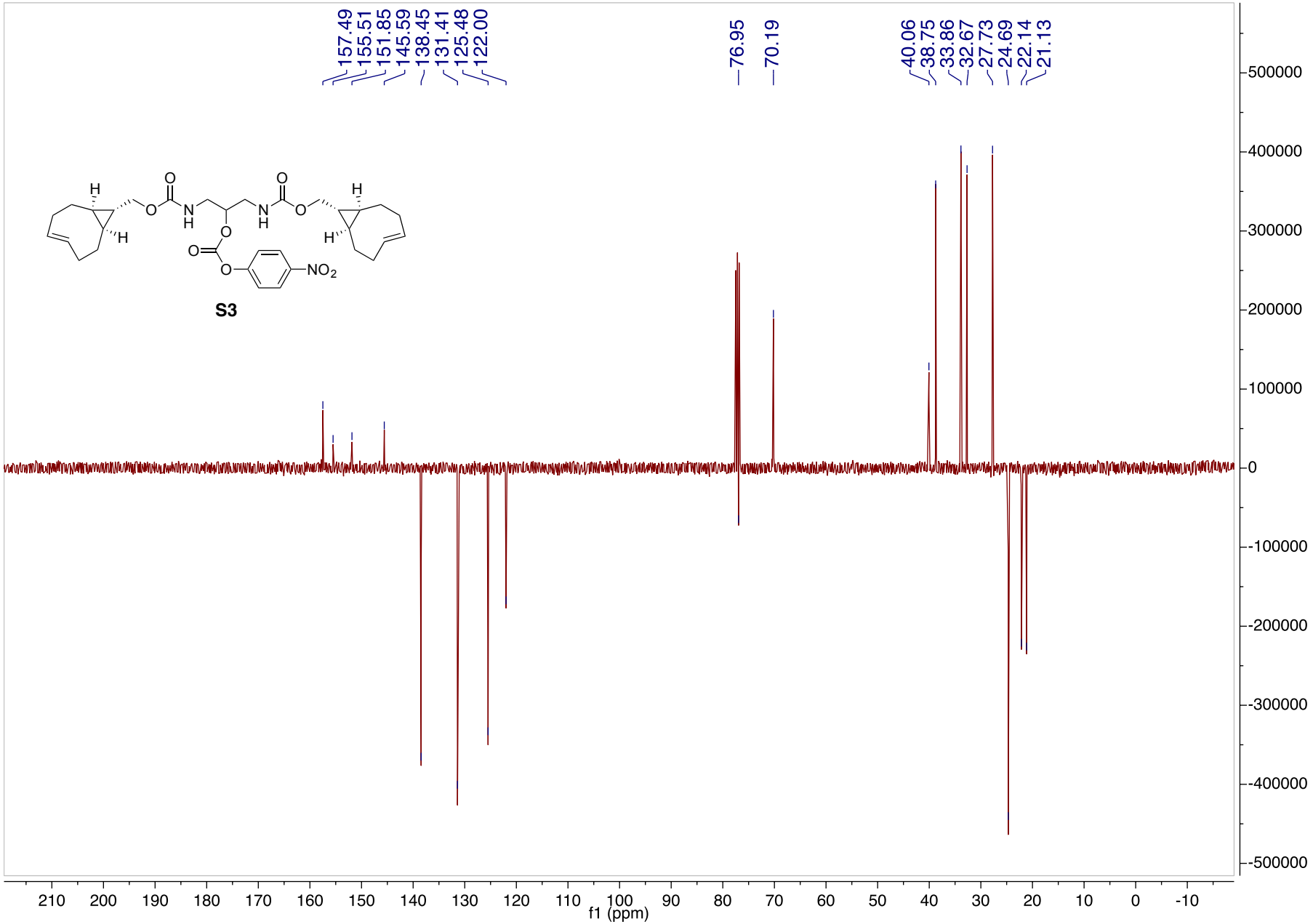


Figure S40. ^{13}C APT NMR spectrum of **S3**

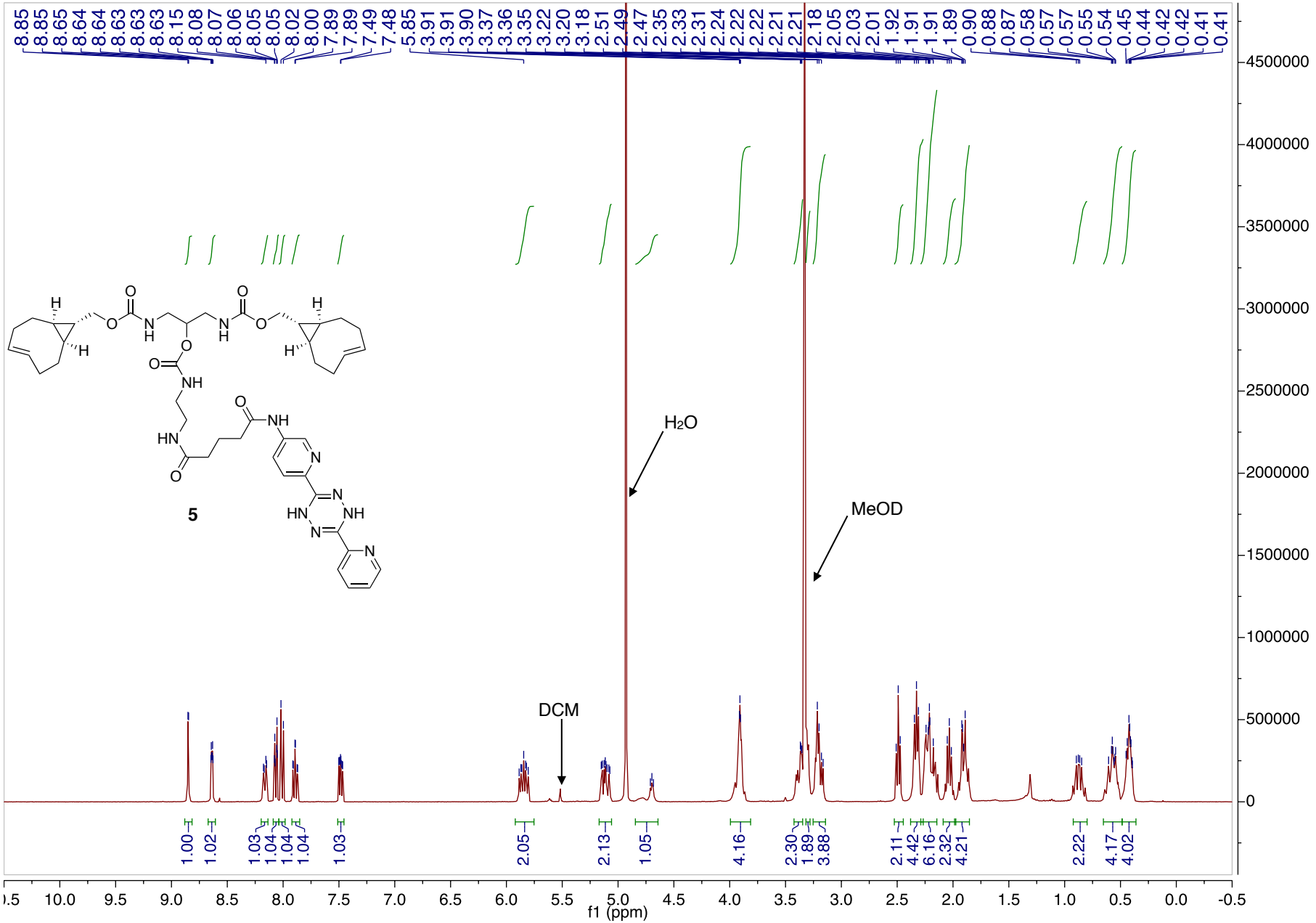


Figure S41. ^{1}H NMR spectrum of **S5**

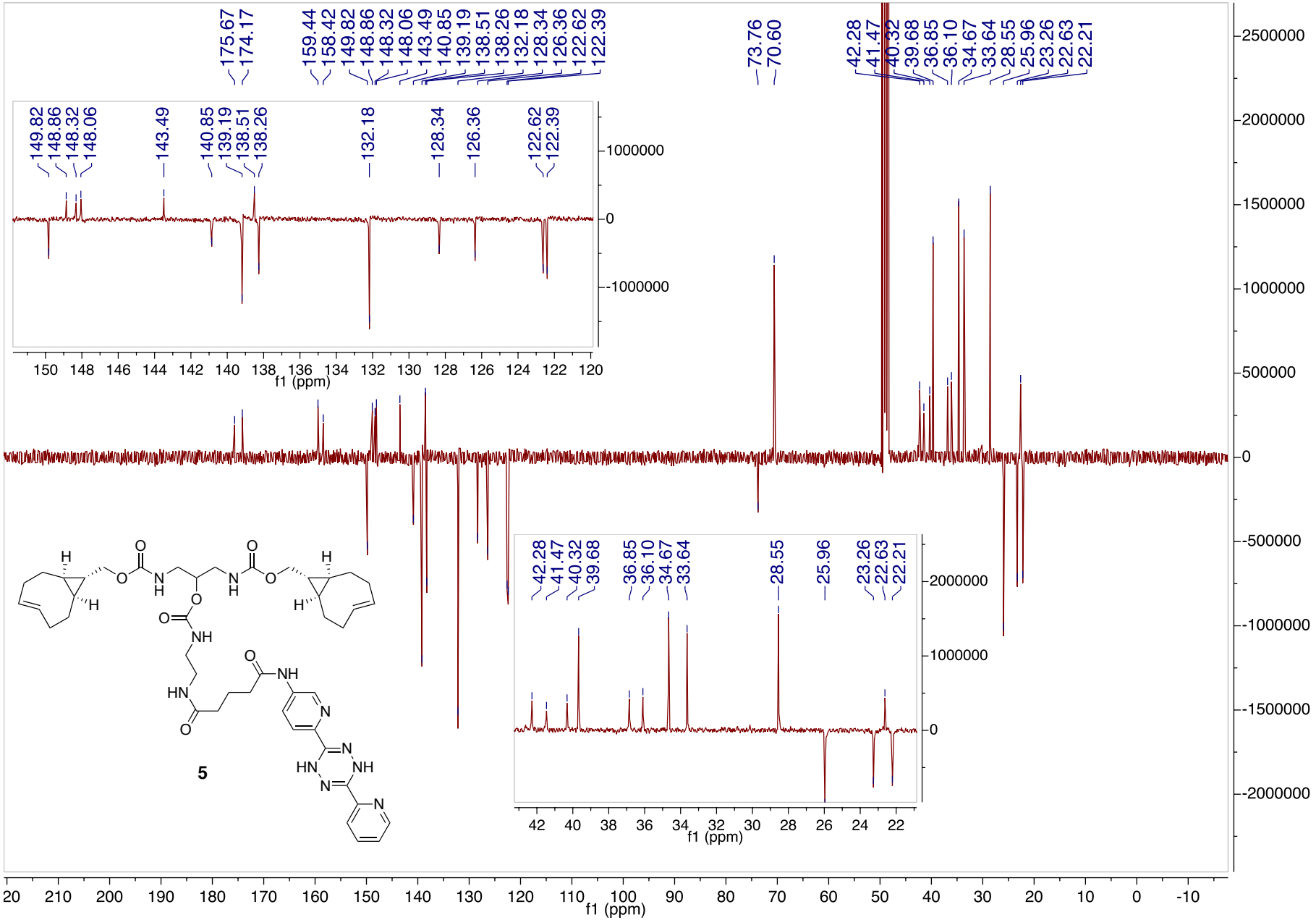


Figure S42. ^{13}C APT NMR spectrum of **S5**