Supplementary Information Table of contents

General Methods

All reagents were purchased from Sigma-Aldrich and used without further purification unless otherwise noted. The TLC plates used for purification were purchased from Agela Technologies (silica 200 × 200 mm, PH = 5, MF = 254, glass back). Other chromatographic purifications were conducted using 40-63 μm silica gel. The microwave reaction was done using a CEM microwave reactor. All mixtures of solvents are given in *v*/*v* ratio. ^{1}H and ^{13}C NMR spectroscopy was performed on a Varian NMR at 400 (^{1}H) or 100 (^{13}C) MHz and a Jeol NMR at 500 (^{1}H) or 125 (^{13}C) MHz. All ^{13}C NMR spectra were proton decoupled. Fluorescence measurements were done using a Perkin Elmer LD-45 spectrophotometer equipped with a single cuvette reader. Ultraviolet absorption data was collected on a Thermo scientific NanoDrop 2000c UV-Vis Spectrophotometer.

Caution! Anhydrous hydrazine (used for all tetrazine syntheses) is highly reactive to oxidizing agents and should be handled with care. **Caution!** 1,2,4,5-tetrazines are nitrogen rich molecules that can be highly reactive. Although we experienced no difficulty in performing the following syntheses at the scales noted, caution should be exercised particularly if attempting to scale up.

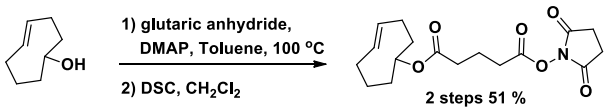
1. Fluorescence assays

All compounds were purified by HPLC and verified by LC-MS prior to quantitative activation experiments. Stock solutions in methanol were diluted into 1.5 mL of the appropriate solvent in a 1cm x 1cm quartz cuvette. Measurements of solvent and pre-activation emission spectra for baseline values were made in at least triplicate, prior to addition of *trans*-cyclooctenol or cyclopropene **4** to initiate the fluorogenic reaction. 3-fold additional excess of *trans*-cyclooctenol and cyclopropene **4** were added for each fluorogenic reaction. Activation ratios were calculated from the peak emission intensity of the reaction product and the corresponding baseline intensity, and all the intensity data was background subtracted. Integration of the area under the emission intensity curves was used to validate the activation ratios. See Supporting **Figure *S11*** for representative emission spectra used to calculate fluorogenic activation ratios.

For quantum yield determinations, fluorescein in 0.1M NaOH was used as a reference for compounds **5, 6, 9** and **10**, with an excitation wavelength of 480nm (ex slit 2.5nm); a value of 0.925 was assigned to the quantum yield of fluorescein (Magde et.al, *Photochem*. *Photobiology*., 2002, 75(4), 327–334), Rhodamine 6G in EtOH was used as a reference for compounds **7** and **8**, with an excitation wavelength of 515nm (ex slit 2.5nm); a value of 0.95 (Magde et.al, *Photochem*. *Photobiology*., 2002, 75(4), 327–334), and calculations made according to the methods described by Crosby and Demas (Chemical Reviews, 1971, 75(8), 991–1024).

2. Live cell imaging experiment

Synthesis of *trans*-cyclooctene NHS ((*E*)-cyclooct-4-en-1-yl (2,5-dioxopyrrolidin-1-yl) glutarate)



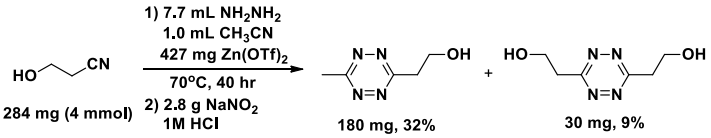
DMAP (6.1 mg, 0.05 mmol) was added to a stirred solution of (*E*)-cyclooct-4-enol (5.0 mg, 0.040 mmol) in toluene (1.0 mL), followed by glutaric anhydride (6.0 mg, 0.05 mmol). The resulting reaction solution was heated to 100°C and stirred at this temperature for 18 hours. After TLC indicated that the reaction had finished the solvent was evaporated and the residue was dissolved in CH_2Cl_2, followed by addition of N, N'-disuccinimidyl carbonate (13.0 mg, 0.05 mmol). After stirring at room temperature for 30 minutes, the reaction solution was evaporated and the residue was purified by preparative TLC (Hexanes : EtOAc = 2:1) to afford 7.0 mg product as a colorless liquid, in 51 % yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.59-1.71 (2H, m), 1.89-2.05 (6H, m), 2.30-2.40 (6H, m), 2.68 (t, J = 10 Hz, 2H), 2.83 (4H, bs), 4.42-4.45 (1H, t, m), 5.46-5.60 (2H, m); ^{13}C (100 MHz, CDCl3) δ 20.05, 25.80, 30.28, 31.18, 32.72, 33.30, 34.46, 38.81, 41.10,

80.64, 133.27, 135.13, 168.32, 169.27, 171.95; HRMS $[M + Na]^+$ m/z calc. for $[C_{17}H_{23}NO_6Na]^+$ 360.1418, found 360.1419.

Cell culture and labeling:
LS174T cells were grown in DMEM media supplemented with 10% fetal bovine serum, 1% *L*-glutamine, 1% penicillin/streptomycin. Cells were incubated in 5.0% carbon dioxide, 95% humidity at 37 °C. The cells were grown in a T-75 tissue culture flask and seeded on a Lab-Tek II chamber slide two days prior to the experiment. A33 antibody (R&D Systems, MN) was modified by incubation with 50 equivalents of *trans*-cyclooctene NHS ester for 2 hours at room temperature. The A33 antibody was washed three times with 0.1M sodium bicarbonate buffer pH 8.3 using a 30 kDa spin filter before adding the antibody to the cells at a final concentration of 200 nM for 1 hour.

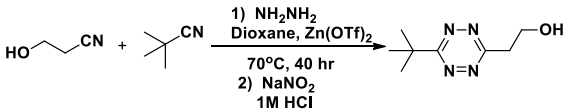
Image acquisition:
All images were acquired on a Yokagawa spinning disk system (Yokagawa, Japan) built around an Axio Observer Z1motorized inverted microscope (Carl Zeiss Microscopy GmbH, Germany) with a 40x, 1.40 NA oil immersion objective. An Evolve 512x512 EMCCD camera (Photometrics, Canada) was used with ZEN imaging software (Carl Zeiss Microscopy GmbH, Germany). Environmental conditions were maintained at 37º C, 5% CO_2 with a heated enclosure and CO_2 controller (Pecon, Germany). Fluorophores were excited with a 488 nm, 100mw green OPSL laser. The media was aspirated, and cells were washed twice with PBS before imaging.

3. Synthesis of 3-methyl-6-hydroxyethyl-1,2,4,5-tetrazine **Tza** and 3, 6-hydroxyethyl-1,2,4,5-tetrazine **Tzd**.



Following a previously developed procedure (*Angew. Chem. Int. Ed*. **2012**, *51*, 5222 –5225), to a 50 mL flask equipped with a stir bar, $Zn(OTf)_2$ (427 mg, 1.2 mmol), 3-hydroxy-propionitrile (285 mg, 4 mmol), acetonitrile (1.0 mL, 20 mmol), and anhydrous hydrazine (7.7 mL, 60 mmol) were added. The reaction was protected with a shield. Under N_2 gas, the mixture was stirred in an oil bath at 70 °C for 40 hours. The reaction solution was cooled with ice water, and sodium nitrite (40 mmol, 2.8 g) dissolved in 20 mL of ice water was slowly added, followed by slow addition of 1M HCl during which time the solution turned bright red, and gas evolved. Addition of 1M HCl continued until gas evolution ceased and the pH value was 3. The mixture was evaporated to remove the water and solvent, EtOAc was added to the residue, and the solid was filtered. The filtrate was evaporated and the residue was purified by silica column chromatography. 6-methyl-3-hydroxyethyl-*s*-tetrazine **Tza** was isolated in 32% yield (Hexane:EtOAc = 1:1, 180 mg) and 3,6-dihydroxyethyl-*s*-tetrazine was isolated in 9% yield (CH_2Cl_2:MeOH = 15:1, 30 mg). For applications requiring the absence of trace metals, we suggest washing the organic phase with an aqueous solution of EDTA prior to purification.
3, 6-dihydroxyethyl-1,2,4,5-tetrazine Tzd: The title product was a red liquid. ^{1}H NMR (500 MHz, Acetone-d_6) δ 3.46 (t, J = 7.5 Hz, 4H), 4.14 (t, J = 10 Hz, 4H). ^{13}C NMR (125 MHz, Acetone-d_6) 38.4, 60.0, 168.8; HRMS $[M+H]^+$ m/z calcd. for $[C_6H_{11}N_4O_2]^+$ 171.0877, found 171.0875.

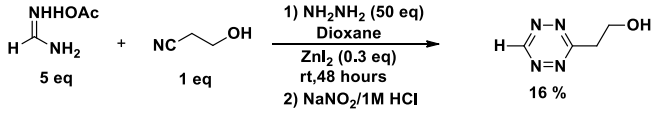
4. Synthesis of 3-*tert*-butyl-6-hydroxyethyl-1,2,4,5-tetrazine **Tzb**.



To a 50 mL flask equipped with a stir bar, $Zn(OTf)_2$ (363 mg, 1.0 mmol), 3-hydroxy-propionitrile (840 mg, 10 mmol), 2,2,2-trimethyl acetonitrile (140 mg, 2 mmol), and anhydrous hydrazine (1.5 mL, 50 mmol) were added. The reaction was protected with a shield. Under N_2 gas, the mixture was stirred in an oil bath at 70°C for 40 hours. Sodium nitrite (20.0 mmol, 1.4 g) in 20 mL of ice water was slowly added to the

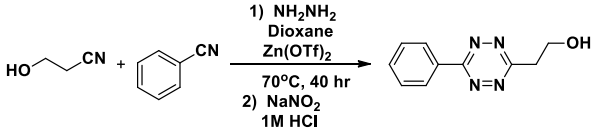
solution, followed by slow addition of 1M HCl during which the solution turned bright red in color and gas evolved. Addition of 1M HCl continued until gas evolution ceased and the pH value was 3. The solution was extracted with EtOAc (50 mL × 3), the combined organic layer was dried over Na_2SO_4 and evaporated. The residue was purified by silica column (Hexanes:EtOAc = 1.5:1) to afford **Tzb** 150 mg product as pink oil, with a yield of 42%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.58 (s, 9H), 3.57 (t, *J* = 5 Hz, 2H), 4.27 (t, *J* = 5 Hz, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 29.2, 37.4, 38.0, 60.0, 167.9, 176.2; HRMS $[M+H]^+$ m/z calcd. for $[C_8H_{15}N_4O]^+$ 183.1240, found 183.1242.

5. Synthesis of 3-*H*-6-hydroxyethyl-1,2,4,5-tetrazine **Tzc**.



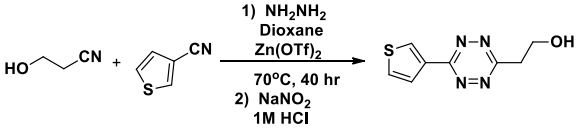
Hydrazine (3.2 g, 100 mmol) was added to a stirring suspension of 3-hydroxy-propionitrile (142 mg, 2 mmol), formamidine acetate (1.01 g, 10 mmol), and ZnI_2 (200 mg, 0.6 mmol) in 1,4-dioxane (3 mL) at room temperature. The reaction was stirred at room temperature for 48 h. Sodium nitrite (20.0 mmol, 1.4 g) in 20 mL of ice water was slowly added to the solution, followed by slow addition of 1M HCl during which the solution turned bright red in color and gas evolved. Addition of 1M HCl continued until gas evolution ceased and the pH value was 3. The mixture was extracted with EtOAc and the organic phase dried over sodium sulfate. The EtOAc was removed using rotary evaporation and the residue was purified by preparative TLC (CH_2Cl_2 : MeOH = 30 : 1) to afford 41 mg compound **Tzc** as a red oil, with the resulting yield of 16 %. ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.61-3.64 (m, 2H), 4.29 (bs, 2H), 10.26 (s, 1H); ^{13}C (125 MHz, $CDCl_3$) δ 38.2, 60.1, 158.5, 171.6. HRMS $[M+H]^+$ m/z calcd. for $[C_4H_7N_4O]^+$ 127.0620, found 127.0618.

6. Synthesis of 3-phenyl-6-hydroxyethyl-*s*-tetrazine **Tze**.



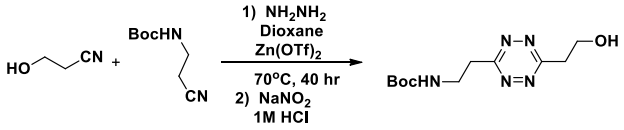
To a 50 mL flask equipped with a stir bar, $Zn(OTf)_2$ (363 mg, 1.0 mmol), 3-hydroxy-propionitrile (430 mg, 6 mmol), benzonitrile (206 mg, 2 mmol), and anhydrous hydrazine (1.5 mL, 50 mmol), dioxane (1 mL) were added. The reaction was protected with a shield. Under N_2 gas, the mixture was stirred in an oil bath at 70°C for 40 hours. Sodium nitrite (20.0 mmol, 1.4 g) in 20 mL of ice water was slowly added to the solution, followed by slow addition of 1M HCl during which the solution turned bright red in color and gas evolved. Addition of 1M HCl continued until gas evolution ceased and the pH value was 3. The solution was extracted with EtOAc (50 mL × 3), the combined organic layer was dried over Na_2SO_4 and evaporated. The residue was purified by silica column (Hexanes:EtOAc = 1.5:1) to afford 143 mg product **Tze** as a pink solid, with a yield of 36%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.65 – 8.50 (m, 2H), 7.70 – 7.53 (m, 3H), 4.30 (t, *J* = 5.8 Hz, 2H), 3.62 (t, *J* = 5.8 Hz, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 168.66, 164.93, 133.17, 131.92, 129.69, 129.69, 128.37, 128.37, 60.44, 37.85. HRMS $[M+H]^+$ m/z calcd. for $[C_{10}H_{11}N_4O]^+$ 203.0927, found 203.0925.

7. Synthesis of 3-(thiophen-3-yl)-6-hydroxyethyl-*s*-tetrazine **Tzf.**



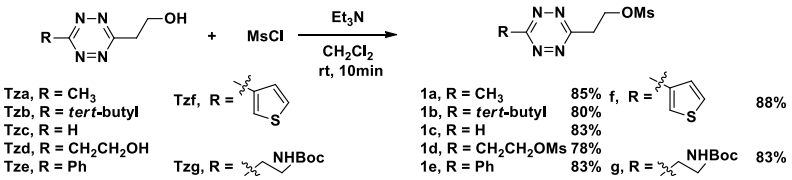
To a 50 mL flask equipped with a stir bar, $Zn(OTf)_2$ (363 mg, 1.0 mmol), 3-hydroxy-propionitrile (430 mg, 6 mmol), 3-Thiophenecarbonitrile (238 mg, 2 mmol), and anhydrous hydrazine (1.5 mL, 50 mmol), dioxane (1 mL) were added. The reaction was protected with a shield. Under N_2 gas, the mixture was stirred in an oil bath at 70°C for 40 hours. Sodium nitrite (20.0 mmol, 1.4 g) in 20 mL of ice water was slowly added to the solution, followed by slow addition of 1M HCl during which the solution turned bright red in color and gas evolved. Addition of 1M HCl continued until gas evolution ceased and the pH value was 3. The solution was extracted with EtOAc (50 mL × 3), the combined organic layer was dried over Na_2SO_4 and evaporated. The residue was purified by silica column (Hexanes:EtOAc = 1.5:1) to afford 147 mg product **Tzf** as a pink solid, with a yield of 35%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.56 (dd, J = 3.0, 1.2 Hz, 1H), 7.99 (dd, J = 5.1, 1.2 Hz, 1H), 7.49 (dd, J = 5.1, 3.0 Hz, 1H), 4.26 (t, J = 5.9 Hz, 2H), 3.57 (t, J = 5.9 Hz, 2H), 3.39 – 2.70 (br, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 167.54, 161.89, 134.37, 130.12, 127.44, 126.26, 59.90, 37.40. HRMS $[M+H]^+$ m/z calcd. for $[C_8H_9N_4OS]^+$ 209.0492, found 209.0493.

8. Synthesis of 3-(2-tert-Butoxycarbonylaminoethyl)-6-hydroxyethyl-*s*-tetrazine **Tzg**.



To a 50 mL flask equipped with a stir bar, $Zn(OTf)_2$ (363 mg, 1.0 mmol), 3-hydroxy-propionitrile (430 mg, 6 mmol), tert-Butyl-2-cyanoethylcarbamate (340 mg, 2 mmol), and anhydrous hydrazine (1.5 mL, 50 mmol), dioxane (1mL) were added. The reaction was protected with a shield. Under N_2 gas, the mixture was stirred in an oil bath at 70°C for 40 hours. Sodium nitrite (20.0 mmol, 1.4 g) in 20 mL of ice water was slowly added to the solution, followed by slow addition of 1M HCl during which the solution turned bright red in color and gas evolved. Addition of 1M HCl continued until gas evolution ceased and the pH value was 3. The solution was extracted with EtOAc (50 mL × 3), the combined organic layer was dried over Na_2SO_4 and evaporated. The residue was purified by silica column (Hexanes:EtOAc = 1.5:1) to afford 318 mg product **Tzg** as a pink solid, with a yield of 59%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.15 (s, 1H), 4.22 (t, *J* = 5.8 Hz, 2H), 3.69 (d, *J* = 6.0 Hz, 2H), 3.54 (t, *J* = 5.8 Hz, 2H), 3.52 – 3.44 (m, 2H), 2.93 (br, 1H), 1.34 (s, 9H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 168.66, 168.59, 156.00, 79.77, 60.16, 38.62, 37.69, 35.73, 28.39. HRMS $[M+Na]^+$ m/z calcd. for $[C_{11}H_{19}N_5O_3Na]^+$ 292.1380, found 292.1382.

9. General procedure for synthesis of **1a-1g**.



In a 50mL flask, 1,2,4,5-Tetrazines **Tza – Tzg** (1.0 eq) were dissolved in CH_2Cl_2, with added Et_3N (1.2 eq), followed by the addition of MsCl (1.2 eq). The reaction solution was stirred at room temperature for 10 min, and checked for completion by TLC. The reaction solution was washed with water. The organic layer was dried over Na_2SO_4 and evaporated. The residues were purified by silica column chromatography to afford products **1a-1g**.

1a: 180 mg of starting material **Tza** yields 238 mg **1a** as a red solid after silica column chromatography (Hexane: EtOAc = 1:1). Yield: 85%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.99 (s, 3H), 3.04 (s, 3H), 3.73 (t, *J* = 7.5 Hz, 2H), 4.83 (dt, *J* = 10, 5 Hz, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 21.4, 34.7, 37.7, 66.4, 166.3, 168.4; HRMS $[M+H]^+$ m/z calcd. for $[C_6H_{11}N_4O_3S]^+$ 219.0546, found 219.0550.

1b: 50 mg of starting material **Tzb** affords 57 mg of **1b** as a red solid after silica column chromatography (Hexane: EtOAc = 2:1). Yield: 80%.^{1}H NMR (500 MHz, $CDCl_3$) δ 1.55 (s, 9H), 3.01 (s 3H), 3.73-3.76 (m,

2H), 4.84-4.87 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 29.2, 34.6, 37.6, 38.1, 66.2, 165.6, 176.4; HRMS $[M+Na]^+$ m/z calcd. for $[C_9H_{16}N_4O_3SNa]^+$ 283.0835, found 283.0838.

1c: 30 mg of starting material **Tzc** affords 40 mg of **1c** as a red solid after silica column chromatography (Hexane: EtOAc = 1:1). Yield: 83%.^{1}H NMR (500 MHz, $CDCl_3$) δ 3.01 (s, 3H), 3.81 (t, J = 10 Hz, 2H), 4.87 (t, J = 7.5 Hz, 2H), 10.27 (s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 35.3, 37.7, 66.0, 158.7, 169.4; HRMS $[M+Na]^+$ m/z calcd. for $[C_5H_8N_4O_3SNa]^+$ 227.0209, found 227.0210.

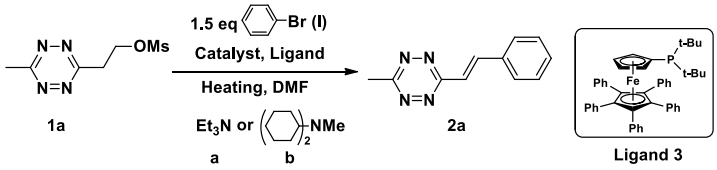
1d: 25 mg of starting material **Tzd** affords 37 mg of **1d** as a red solid after silica column chromatography (Hexane: EtOAc = 1:1). Yield: 78%. ^{1}H NMR (500 MHz, Acetone-d_6) δ 3.10 (s, 6H), 3.81 (t, J = 7.5 Hz, 4H), 4.89 (t, J = 7.5 Hz, 4H); ^{13}C NMR (125 MHz, Acetone-d_6) 34.7, 36.5, 67.2, 167.5; HRMS $[M+Na]^+$ m/z calcd. For $[C_8H_{14}N_4O_6S_2Na]^+$ 349.0247, found 349.0251.

1e: 20 mg of starting material **Tze** affords 24 mg of **1e** as a red solid after silica column chromatography (Hexane: EtOAc = 1:1). Yield: 83%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.69 – 8.55 (m, 2H), 7.62 (ddd, J = 13.1, 7.9, 6.3 Hz, 3H), 4.92 (t, J = 6.2 Hz, 2H), 3.83 (t, J = 6.2 Hz, 2H), 3.04 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.95, 164.66, 132.88, 131.27, 129.24, 129.24, 128.04, 128.04, 65.86, 37.47, 34.51. HRMS $[M+Na]^+$ m/z calcd. For $[C_{11}H_{12}N_4O_3SNa]^+$ 303.0522, found 303.0524.

1f: 21 mg of starting material **Tzf** affords 25 mg of **1f** as a red solid after silica column chromatography (Hexane: EtOAc = 1:1). Yield: 88%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.63 (dd, J = 3.0, 1.2 Hz, 1H), 8.05 (dd, J = 5.1, 1.2 Hz, 1H), 7.53 (dd, J = 5.1, 3.0 Hz, 1H), 4.90 (t, J = 6.2 Hz, 2H), 3.79 (t, J = 6.2 Hz, 2H), 3.04 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.64, 162.45, 134.57, 130.86, 127.84, 126.67, 66.13, 37.73, 34.80. HRMS $[M+Na]^+$ m/z calcd. For $[C_9H_{10}N_4O_3S_2Na]^+$ 309.0087, found 309.0089.

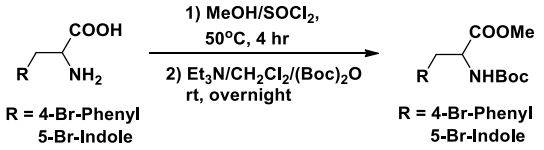
1g: 27 mg of starting material **Tzg** affords 29 mg of **1g** as a red solid after silica column chromatography (Hexane: EtOAc = 1:1). Yield: 83%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.07 (s, 1H), 4.84 (t, J = 5.5 Hz, 2H), 3.74 (t, J = 5.5 Hz, 2H), 3.69 (d, J = 5.8 Hz, 2H), 3.50 (t, J = 5.3 Hz, 2H), 3.00 (s, 3H), 1.33 (s, 9H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 169.00, 166.42, 155.87, 79.61, 66.15, 38.55, 37.53, 35.72, 34.65, 28.36. HRMS $[M+Na]^+$ m/z calcd. For $[C_{12}H_{21}N_5O_5SNa]^+$ 370.1156, found 370.1158.

10. General procedure for screening the heck reaction conditions.



To a 10 mL microwave reaction tube equipped with a stir bar, catalysts (0.03-0.1 eq) and ligands (0.12-0.4 eq), **1a** (1 eq, 6.0 mg, 0.0276 mmol), iodobenzene (1.5 eq, 8.4 mg, 0.0414 mmol) or bromobenzene (1.5 eq, 6.5 mg, 0.0414mmol), and base (3.0 eq, 0.0828 mmol) were dissolved in anhydrous DMF (1.5 mL). The reaction was protected with N_2 gas and then heated in an oil bath (90 °C, 90 min) or by microwave irradiation (50-60 °C, 30-45 min). The reaction solution was cooled to room temperature and EtOAc (20 mL) was added before washing with water (20 mL ×3). The organic layer was dried over Na_2SO_4 and evaporated. The residue was purified by a TLC plate (Agela Technologies, silica 200 × 200 mm, PH = 5, MF = 254, glass back; Hexanes: Et_2O = 3:1) to afford pure **2a** as a pink solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.05 (s, 3H), 7.44 (m, 4H), 7.68 (t, J = 2.5 Hz, 2H), 8.31 (d, J = 20 Hz, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 21.3, 120.6, 128.2, 129.1, 130.3, 135.2, 141.1, 164.8, 166.4; HRMS $[M+H]^+$ m/z calcd. for $[C_{11}H_{11}N_4]^+$ 199.0978, found 199.0976.

11. Synthesis of 4-bromo-N-Boc-DL-phenylalanine methyl ester and 5-bromo-N_2-boc-DL-tryptophan methyl ester.

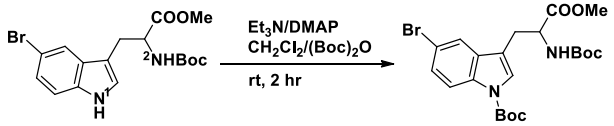


To a 50 mL flask equipped with a stir bar, the amino acid (0.25 mmol) was dissolved in methanol (10 mL) and cooled by ice-water. $SOCl_2$ (90 mg, 0.75 mmol) was added dropwise to the solution and then warmed to 50°C. The reaction was stirred at this temperature for 4 hours and then evaporated to afford the amino acid methyl ester hydrochloride as a white solid. The amino acid methyl ester hydrochloride (0.25 mmol) was dissolved in CH_2Cl_2 (20 mL), and Et_3N (0.6 mmol) was added, followed by $(Boc)_2O$ (65 mg, 0.3 mmol). The resulting solution was stirred at room temperature overnight and then washed with brine (20 mL). The organic layer was dried over Na_2SO_4 and evaporated. The residue was purified by silica column chromatography to afford 4-bromo-N-boc-DL-phenylalanine methyl ester (Hexanes:EtOAc = 2:1, 75 mg, 85% yield) and 5-bromo-N_2-boc-DL-tryptophan methyl ester (Hexanes : EtOAc = 1:1, 90 mg, 91% yield).

4-bromo-N-Boc-DL-phenylalanine methyl ester : ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.40 (s, 9H), 2.97 (dd, *J* = 12, 8 Hz, 1H), 3.07 (dd, *J* = 12, 8 Hz, 1H), 3.70 (s, 3H), 4.56 (t, *J* = 6 Hz, 1H), 5.00 (d, *J* = 6 Hz, 1H), 6.99 (d, *J* = 4 Hz, 2H), 7.40 (d, *J* = 4 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 28.4, 37.9, 52.4, 54.3, 80.2, 121.1, 131.1, 131.7, 135.2, 155.1, 172.2; HRMS $[M+Na]^+$ m/z calcd. for $[C_{15}H_{20}BrNO_4Na]^+$ 397.0763, found 397.0760.

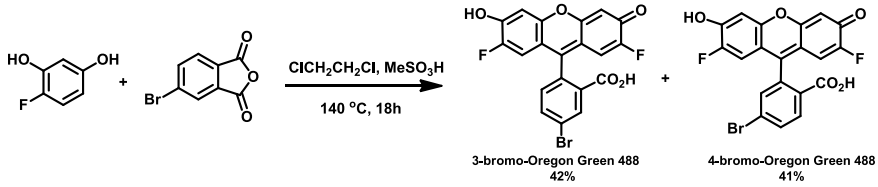
5-bromo-N_2-Boc-DL-tryptophan methyl ester: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.44 (s, 9H), 3.24 (bs, 2H), 3.70 (s, 3H), 4.64 (d, *J* = 8 Hz, 1H), 5.11 (d, *J* = 4 Hz, 1H), 6.97 (s, 1H), 7.18-7.24 (m, 2H), 7.65 (s, 1H), 8.33 (s, 1H) ; ^{13}C NMR (100 MHz, $CDCl_3$) δ 28.2, 28.6, 52.6, 54.4, 80.3, 110.2, 112.9, 113.1, 121.7, 124.3, 125.2, 129.7, 134.9, 155.4, 172.7. HRMS $[M+H]^+$ m/z calcd. for $[C_{17}H_{22}BrN_2O_4]^+$ 380.0468, found 380.0470.

12. Synthesis of 5-bromo-N_1, N_2-boc-DL-tryptophan methyl ester.



In a 50 mL flask, 5-bromo-N_2-boc-DL-tryptophan methyl ester (50 mg, 0.126 mmol) was dissolved in CH_2Cl_2, and Et_3N (13 mg, 0.13 mmol), DMAP (1.5 mg, 0.0126 mmol) was added, and then followed by $(Boc)_2O$ (33 mg, 0.15 mmol). The reaction solution was stirred at room temperature for 2 hours and then washed with brine. The organic layer was dried over Na_2SO_4 and evaporated. The residue was purified with silica column chromatography and afforded 58 mg 5-bromo-N_1,N_2-boc-DL-tryptophan methyl ester as a white solid at 92%. yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.44 (s, 9H), 1.65 (s, 9H), 3.13 (dd, *J* = 15, 10 Hz, 1H), 3.23 (dd, *J* = 15, 5 Hz, 1H), 3.72 (s, 3H), 4.62 (t, *J* = 2.5 Hz, 1H), 5.14 (d, *J* = 10 Hz, 1H), 7.37 (s, 1H), 7.58 (d, *J* = 5 Hz, 2H), 7.97 (bs, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 28.0, 28.4, 28.6, 52.7, 53.9, 84.4, 114.7, 116.2, 117.0, 122.0, 125.5, 127.5, 132.5, 134.3, 149.4, 155.2, 172.3; HRMS $[M+Na]^+$ m/z calcd. for $[C_{22}H_{29}BrN_2O_6Na]^+$ 519.1101, found 519.1102.

13. Synthesis of 3-bromo-2',7'-difluorofluorescein (3-bromo-Oregon Green 488) and 4-bromo-2',7'-difluorofluorescein (4-bromo-Oregon Green 488).

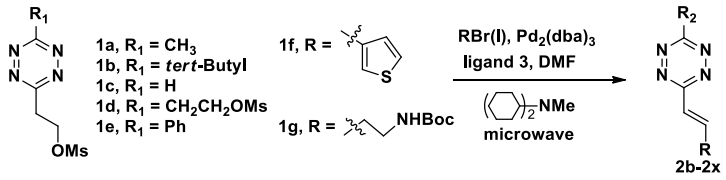


A mixture of 4-Fluoro-1,3-dihydroxybenzene (32 mg, 0.25 mmol), 4-bromophthalic anhydride (28 mg, 0.123 mmol), 1,2-dichloroethane (1 mL) and methanesulfonic acid (2 mL) was heated at 140 °C in seal tube for 18 h. The resultant dark yellow solution was dissolved in EtOAc (100 mL), washed with water, and saturated sodium chloride over Na_2SO_4. The solution was concentrated to give a crude mixture of 3-bromo-Oregon Green 488 and 4-bromo-Oregon Green 488. The isomers were separated by prep-HPLC. Get 3-bromo-Oregon Green 488 23 mg as a yellow solid yield 42%, get 4-bromo-Oregon Green 488 21 mg as a yellow solid yield 41%.

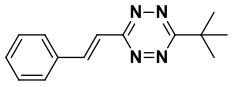
3-bromo-Oregon Green 488: ^{1}H NMR (500 MHz, CD_3OD) δ 7.94 (d, J = 8.2 Hz, 1H), 7.89 (d, J = 8.1 Hz, 1H), 7.45 (s, 1H), 6.84 (d, J = 7.2 Hz, 2H), 6.46 (d, J = 10.9 Hz, 2H). ^{13}C NMR (126 MHz, CD_3OD) δ 168.38, 149.79, 148.68, 147.87, 133.79, 130.10, 127.54, 126.97, 125.81, 113.11, 112.95, 108.57, 104.82. HRMS $[M+Na]^+$ m/z calcd. for $[C_{20}H_9BrF_2O_5Na]^+$ 468.9494, found 468.9486

4-bromo-Oregon Green 488: ^{1}H NMR (500 MHz, DMSO-D6) δ 10.79 (s, 2H), 8.14 (d, J = 1.4 Hz, 1H), 7.95 (dd, J = 8.2, 1.7 Hz, 1H), 7.26 (d, J = 8.2 Hz, 1H), 6.87 (d, J = 7.5 Hz, 2H), 6.65 (d, J = 11.3 Hz, 2H). ^{13}C NMR (126 MHz, DMSO-D6) δ 167.40, 151.08, 149.39, 148.28, 148.17, 147.84, 147.47, 138.90, 128.92, 128.31, 126.49, 123.91, 114.75, 114.58, 108.24, 108.19, 105.25. HRMS $[M+Na]^+$ m/z calcd. for $[C_{20}H_9BrF_2O_5Na]^+$ 468.9494, found 468.9488.

14. General procedure for the synthesis of (*E*)-3-substituted-6-alkenyl-1,2,4,5-tetrazines **2b-2x**.

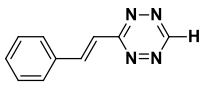


To a 10 mL microwave reaction tube equipped with a stir bar, catalysts (0.03-0.05 eq) and ligand **3** (0.12-0.2 eq), **1a-1g** (1 eq), RBr (RI) (1.1-1.5 eq) and N,N-dicyclohexylmethylamine (3.0-6.0 eq) were dissolved in anhydrous DMF (1.5 mL). The reaction was protected with N_2 gas and then heated by microwave irradiation (50°C, 30-60 min). The reaction solution was cooled to room temperature and EtOAc (20 mL) was added before weashing with water (20 mL ×3). The organic layer was dried over Na_2SO_4 and evaporated. The residue was purified by a prep-TLC plate to afford pure **2b-2x**.



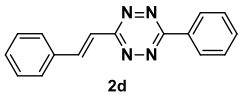
2b

2b: 1.5 eq bromobenzene, 3.0 eq base, $Pd_2(dba)_3$(3%), ligand **3** (12%), microwave irradiation at 50°C for 30 min. 6 mg **1b** affords 5.37 mg **2b** as a pink liquid, in 97% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.60 (s, 9H), 7.41-7.49 (m, 4H), 7.70 (t, J = 5 Hz, 2H), 8.39 (d, J = 15 Hz, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 29.1, 37.8, 120.5, 127.9, 128.9, 135.0, 130.0, 140.7, 164.1, 174.5. HRMS $[M+H]^+$ m/z calcd. for $[C_{14}H_{17}N_4]^+$ 241.1448, found 241.1445.



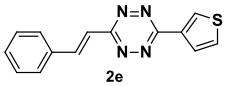
2c

2c: 1.5 eq bromobenzene, 3.0 eq base, $Pd_2(dba)_3$ (5%), ligand **3** (20%), microwave irradiation at 50°C for 30 min. 5.7 mg **1c** affords 3.75 mg **2c** as a pink solid, in 83% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.45-7.50 (m, 4H), 7.71 (dd, J = 5, 2 Hz, 2H), 8.39 (d, J = 15 Hz, 1H), 10.1 (s, 1H. ^{13}C NMR (125 MHz, $CDCl_3$) δ 120.5, 128.4, 129.2, 130.7, 134.9, 142.7, 157.0, 167.3; HRMS $[M+H]^+$ m/z calcd. for $[C_{10}H_9N_4]^+$ 185.0822, found 185.0825.



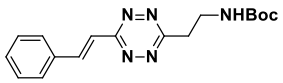
2d

2d: 1.5 eq bromobenzene, 3.0 eq base, $Pd_2(dba)_3$ (3%), ligand **3** (12%), microwave irradiation at 50°C for 35 min. 5.6 mg **1e** affords 5.2 mg **2d** as a pink solid, in 99% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.65 – 8.60 (m, 2H), 8.38 (d, *J* = 16.3 Hz, 1H), 7.72 (dd, *J* = 8.2, 1.3 Hz, 2H), 7.64 – 7.58 (m, 3H), 7.54 (d, *J* = 16.3 Hz, 1H), 7.45 (ddd, *J* = 8.5, 6.4, 3.5 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 164.84, 163.20, 141.33, 135.29, 132.69, 132.04, 130.46, 129.42, 129.42, 129.20, 129.20, 128.30, 128.30, 128.04, 128.04, 120.75; HRMS $[M+H]^+$ m/z calcd. for $[C_{16}H_{13}N_4]^+$ 261.1135, found 261.1162.



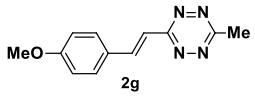
2e

2e: 1.5 eq bromobenzene, 3.0 eq base, $Pd_2(dba)_3$ (3%), ligand **3** (12%), microwave irradiation at 50°C for 35 min. 5.7 mg **1f** affords 4.3 mg **2e** as a pink solid, in 82% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.61 (dd, *J* = 3.0, 1.2 Hz, 1H), 8.33 (d, *J* = 16.3 Hz, 1H), 8.07 (dd, *J* = 5.1, 1.2 Hz, 1H), 7.73 – 7.68 (m, 2H), 7.53 (dd, *J* = 5.2, 3.2 Hz, 1H), 7.50 (d, *J* = 16.3 Hz, 1H), 7.48 – 7.41 (m, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 164.37, 160.87, 140.98, 135.32, 135.16, 130.40, 130.05, 129.19, 129.19, 128.26, 128.26, 127.63, 126.65, 120.84. HRMS $[M+H]^+$ m/z calcd. for $[C_{14}H_{11}N_4S]^+$ 267.0699, found 267.0700.



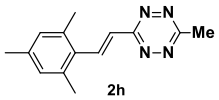
2f

2f: 1.5 eq bromobenzene, 3.0 eq base, $Pd_2(dba)_3$ (3%), ligand **3** (12%), microwave irradiation at 50°C for 45 min. 6.9 mg **1g** affords 5.9 mg **2f** as a pink solid, in 91% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.33 (d, *J* = 16.3 Hz, 1H), 7.69 (dd, *J* = 7.9, 1.4 Hz, 2H), 7.50 – 7.42 (m, 4H), 5.05 (s, 1H), 3.81 – 3.71 (m, 2H), 3.52 (t, *J* = 6.1 Hz, 2H), 1.39 (s, 9H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 167.31, 165.17, 155.92, 141.51, 135.18, 130.48, 129.20, 128.27, 120.59, 79.72, 38.45, 35.63, 28.46. HRMS $[M+Na]^+$ m/z calcd. for $[C_{17}H_{21}N_5O_2Na]^+$ 350.1587, found 350.1588.



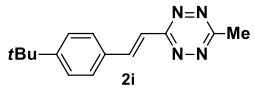
2g

2g: 1.2 eq 4-Bromoanisole, 3.0 eq base, $Pd_2(dba)_3$ (3%), ligand **3** (12%), microwave irradiation at 50°C for 35 min. 4.4 mg **1a** affords 4.2 mg **2g** as a pink solid, in 93% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.26 (d, *J* = 16.2 Hz, 1H), 7.63 (d, *J* = 8.6 Hz, 2H), 7.31 (d, *J* = 16.2 Hz, 1H), 6.96 (d, *J* = 8.8 Hz, 2H), 3.86 (s, 3H), 3.03 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.13, 165.11, 161.49, 140.75, 129.87, 129.87, 128.04, 118.19, 114.61, 114.61, 55.58, 21.29. HRMS $[M+H]^+$ m/z calcd. for $[C_{12}H_{13}N_4O]^+$ 229.1084, found 229.1080.



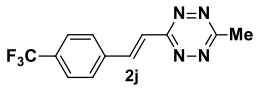
2h

2h: 1.2 eq 2-Bromomesitylene, 3.0 eq base, $Pd_2(dba)_3$ (3%), ligand **3** (12%), microwave irradiation at 50°C for 40 min. 4.4 mg **1a** affords 4.8 mg **2h** as a pink solid, in 97% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.47 (d, *J* = 16.6 Hz, 1H), 7.08 (d, *J* = 16.6 Hz, 1H), 6.95 (s, 2H), 3.06 (s, 3H), 2.44 (s, 6H), 2.05 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.63, 164.67, 139.90, 138.63, 137.21, 137.21, 131.71, 129.49, 125.49, 21.47, 21.31, 21.24, 21.21. HRMS $[M+H]^+$ m/z calcd. for $[C_{14}H_{17}N_4]^+$ 241.1448, found 241.1449.



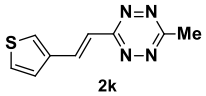
2i

2i: 1.5 eq 1-Bromo-4-tert-butylbenzene, 3.0 eq base, $Pd_2(dba)_3$ (3%), ligand **3** (12%), microwave irradiation at 50°C for 30 min. 4.4 mg **1a** affords 4.8 mg **2i** as a pink solid, in 96% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.30 (d, *J* = 16.2 Hz, 1H), 7.63 (d, *J* = 8.3 Hz, 2H), 7.47 (d, *J* = 8.4 Hz, 2H), 7.42 (d, *J* = 16.3 Hz, 1H), 3.05 (s, 3H), 1.35 (s, 9H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.32, 165.02, 153.97, 141.03, 132.50, 128.06, 128.06, 126.16, 126.16, 119.76, 35.08, 31.32, 21.32. HRMS $[M+H]^+$ m/z calcd. for $[C_{15}H_{19}N_4]^+$ 255.1604, found 255.1606.



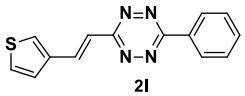
2j

2j: 1.2 eq 4-Bromobenzotrifluoride, 3.0 eq base, $Pd_2(dba)_3$ (3%), ligand **3** (12%), microwave irradiation at 50°C for 40 min. 4.4 mg **1a** affords 4.1 mg **2j** as a pink solid, in 86% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.33 (d, *J* = 16.3 Hz, 1H), 7.79 (d, *J* = 8.1 Hz, 2H), 7.71 (d, *J* = 8.3 Hz, 2H), 7.54 (d, *J* = 16.3 Hz, 1H), 3.08 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.81, 164.47, 143.45, 139.16, 130.62, 129.07, 128.50, 128.25, 128.25, 126.10 (q, *J* = 3.9 Hz), 123.15, 21.36. HRMS $[M+H]^+$ m/z calcd. for $[C_{12}H_9F_3N_4]^+$ 267.0852, found 267.0858.



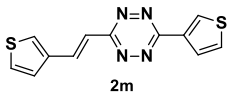
2k

2k: 1.4 eq 3-Bromothiophene, 3.0 eq base, $Pd_2(dba)_3$ (3%), ligand **3** (12%), microwave irradiation at 50°C for 40 min. 4.4 mg **1a** affords 3.5 mg **2k** as a pink solid, in 86% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.30 (d, *J* = 16.2 Hz, 1H), 7.62 – 7.59 (m, 1H), 7.49 (dd, *J* = 5.1, 1.0 Hz, 1H), 7.41 (ddd, *J* = 5.1, 2.9, 0.5 Hz, 1H), 7.28 (d, *J* = 15.0 Hz, 1H), 3.04 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.37, 165.07, 138.49, 134.70, 128.17, 127.32, 125.20, 120.40, 21.31. HRMS $[M+H]^+$ m/z calcd. for $[C_9H_9N_4S]^+$ 205.0542, found 205.0544.



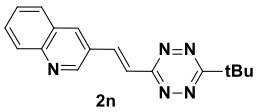
2l

2l: 1.4 eq 3-Bromothiophene, 3.0 eq base, $Pd_2(dba)_3$ (3%), ligand **3** (12%), microwave irradiation at 50°C for 40 min. 5.6 mg **1e** affords 4.4 mg **2l** as a pink solid, in 82% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.61 (dd, J = 7.8, 1.7 Hz, 2H), 8.36 (d, J = 16.1 Hz, 1H), 7.66 – 7.57 (m, 4H), 7.53 – 7.50 (m, 1H), 7.43 (dd, J = 5.1, 2.9 Hz, 1H), 7.34 (d, J = 16.1 Hz, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.03, 163.14, 138.61, 134.88, 132.63, 132.08, 129.40, 129.40, 128.38, 127.99, 127.99, 127.37, 125.25, 120.51. HRMS $[M+H]^+$ m/z calcd. for $[C_{14}H_{11}N_4S]^+$ 267.0699, found 267.0698.



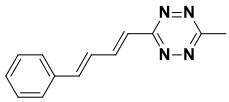
2m

2m: 1.4 eq 3-Bromothiophene, 3.0 eq base, $Pd_2(dba)_3$ (3%), ligand **3** (12%), microwave irradiation at 50°C for 40 min. 5.7 mg **1f** affords 2.8 mg **2m** as a pink solid, in 58% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.60 (dd, *J* = 3.1, 1.2 Hz, 1H), 8.35 – 8.29 (m, 1H), 8.07 (dd, *J* = 5.1, 1.2 Hz, 1H), 7.62 (dd, *J* = 1.7, 1.2 Hz, 1H), 7.53 (dd, *J* = 5.1, 3.0 Hz, 1H), 7.51 (dd, *J* = 5.1, 1.0 Hz, 1H), 7.44 – 7.40 (m, 1H), 7.31 (d, *J* = 16.1 Hz, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 164.56, 160.83, 138.63, 135.19, 134.55, 129.91, 128.26, 127.60, 127.35, 126.63, 125.24, 120.61. HRMS $[M+H]^+$ m/z calcd. for $[C_{12}H_9N_4S_2]^+$ 273.0263, found 273.0264.



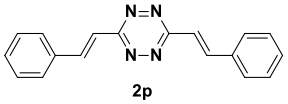
2n

2n: 1.1 eq 3-Bromoquinoline, 3.0 eq base, $Pd_2(dba)_3$ (3%), ligand **3** (12%), microwave irradiation at 50°C for 40 min. 5.7 mg 1**c** affords 2.3 mg 2**n** as a pink solid, in 79% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.26 (d, *J* = 2.1 Hz, 1H), 8.46 (d, *J* = 16.4 Hz, 1H), 8.40 (d, *J* = 1.9 Hz, 1H), 8.15 (d, *J* = 8.4 Hz, 1H), 7.91 (d, *J* = 8.1 Hz, 1H), 7.78 (ddd, *J* = 8.4, 6.9, 1.4 Hz, 1H), 7.71 (d, *J* = 16.4 Hz, 1H), 7.62 (ddd, *J* = 8.1, 6.9, 1.1 Hz, 1H), 1.61 (s, 9H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.19, 164.00, 137.30, 135.55, 131.72, 130.87, 129.42, 129.37, 128.50, 128.27, 127.88, 127.72, 122.84, 38.10, 29.28. HRMS [M+H]+ m/z calcd. for $[C_{17}H_{18}N_5]^+$ 292.1557, found 292.1560



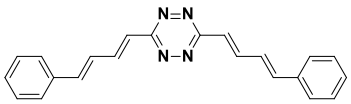
2o

2o: 1.1 eq β-bromostyrene, 3.0 eq base, $Pd_2(dba)_3$ (5%), ligand **3** (20%), microwave irradiation at 55°C for 35 min. 6.0 mg **1a** affords 2.91 mg **2s** as a red solid, in 48% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.03 (s, 3H), 6.98-7.01 (m, 2H), 7.10 (dd, *J* = 15, 10 Hz, 1H), 7.25-7.34 (m, 3H), 7.53 (t, *J* = 5 Hz, 2H), 8.07 (dd, *J* = 15, 10 Hz, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 21.3, 123.9, 127.3, 127.4, 129.0, 129.2, 136.2, 140.2, 141.3, 164.9, 166.0; HRMS $[M+H]^+$ m/z calcd. for $[C_{13}H_{13}N_4]^+$ 225.1135, found 225.1137.



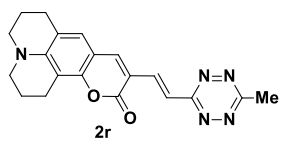
2p

2p: 3.0 eq bromobenzene, 6.0 eq base, $Pd_2(dba)_3$ (6%), ligand **3** (24%), microwave at 50°C for 30 min. 4.3 mg **1d** affords 3.4 mg **2p** as a orange solid, in 89% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.42-7.51 (m, 8H), 7.71 (d, *J* = 5 Hz, 4H), 8.33 (d, *J* = 20 Hz, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 120.9, 128.2, 129.2, 130.4, 135.3, 141.0, 163.9; HRMS $[M+H]^+$ m/z calcd. for $[C_{18}H_{15}N_4]^+$ 287.1291, found 287.1289.



2q

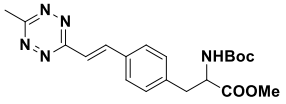
2q: 2.2 eq β-bromostyrene, 6.0 eq base, $Pd_2(dba)_3$ (10%), ligand **3** (40%), microwave at 55°C for 35 min. 5.0 mg **1d** afford 3.0 mg **2q** as a orange solid, in 58% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.99-7.03 (dd, *J* = 15, 5 Hz, 4H), 7.10-7.15 (dd, *J* = 20, 10 Hz, 2H), 7.32-7.34 (m, 2H), 7.37-7.40 (m, 4H), 7.53 (t, *J* = 5 Hz, 4H), 8.07 (dd, *J* = 17.5, 10 Hz, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 124.4, 127.4, 127.7, 129.0, 129.2, 136.3, 140.2, 141.0, 163.6; HRMS $[M+H]^+$ m/z calcd. for $[C_{22}H_{19}N_4]^+$ 339.1604, found 339.1603.



2r

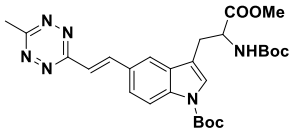
2r: 1 eq 3-iodocoumarin (Xian et.al, *Mol. BioSyst.*, 2009, 5, 918-920), 3.0 eq base, $Pd_2(dba)_3$ (5%), ligand **3** (20%), microwave irradiation at 50°C for 40 min. 4.4 mg **1a** affords 6.0 mg **2r** as a pink solid, in 83% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.15 (d, *J* = 16.0 Hz, 1H), 7.98 (d, *J* = 16.0 Hz, 1H), 7.72 (s, 1H), 6.93 (s, 1H), 3.32 (dd, *J* = 11.7, 6.0 Hz, 4H), 3.02 (s, 3H), 2.91 (t, *J* = 6.5 Hz, 2H), 2.78 (t, *J* = 6.3 Hz, 2H), 1.98 (dd, *J* = 9.9, 4.5 Hz, 4H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.84, 165.52, 160.86, 151.71, 147.41, 144.55,

136.52, 126.09, 121.09, 119.29, 114.33, 108.82, 106.25, 50.33, 49.94, 29.84, 21.41, 21.27, 20.46, 20.31; HRMS $[M+H]^+$ m/z calcd. for $[C_{20}H_{20}N_5O_2]^+$ 362.1617, found 362.1611.



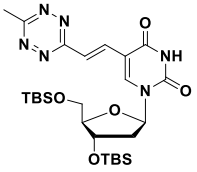
2s

2s: 1.1 eq 4-bromo-N-boc-DL-phenylalanine methyl ester, 3.0 eq base, $Pd_2(dba)_3$ (5%), ligand **3** (20%), microwave irradiation at 50°C for 60 min. 4.4 mg **1a** affords 6.3 mg **2s** as a pink solid, in 76% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.41 (s, 9H), 3.05 (s, 3H), 3.07-3.17 (m, 2H), 3.73 (s, 3H), 4.62 (t, *J* = 10 Hz, 1H), 5.04 (d, *J* = 10 Hz, 1H), 7.21 (d, *J* = 5 Hz, 2H), 7.44 (d, *J* = 20 Hz, 1H), 7.61 (d, *J* = 5 Hz, 2H), 8.27 (d, *J* = 20 Hz, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 21.4, 28.5, 38.6, 52.6, 54.5, 80.3, 120.6, 128.5, 130.3, 134.1, 138.8, 140.8, 155.8, 165.0, 166.5, 172.4; HRMS $[M+H]^+$ m/z calcd. for $[C_{20}H_{25}N_5O_4Na]^+$ 422.1799, found 422.1801.



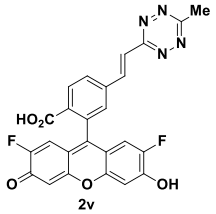
2t

2t: 1.1 eq 5-bromo-N_1, N_2-boc-DL-tryptophan methyl ester, 3.0 eq base, $Pd_2(dba)_3$ (5%), ligand **3** (20%), microwave irradiation at 60°C for 30 min. 4.4 mg **1a** affords 5.9 mg **2t** as a pink solid, in 55% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.42 (s, 9H), 1.67 (s, 9H), 3.04 (s, 3H), 3.21-3.30 (m, 2H), 3.71 (s, 3H), 4.68 (d, *J* = 5 Hz, 1H), 5.18 (d, *J* = 10 Hz, 1H), 7.43 (m, 3H), 7.67 (d, *J* = 10 Hz, 1H), 7.78 (s, 1H), 8.16 (s, 1H), 8.41 (d, *J* = 15 Hz, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 21.4, 28.1, 28.4, 28.5, 52.7, 53.9, 80.4, 84.5, 115.7, 116.1, 119.4, 119.7, 124.4, 125.5, 130.0, 131.3, 136.7, 141.7, 149.5, 155.3, 165.1, 166.3, 172.4; HRMS $[M+Na]^+$ m/z calcd. for $[C_{27}H_{34}N_6O_6Na]^+$ 561.2432, found 561.2433.



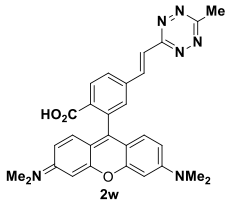
2u

2u: 1.1 eq 2'-deoxy-3'5'-bis-*o*-TBS-5-iodo-uridine (Richert et.al, *Chem. Comm.,* 2011, 47(38), 10824-10826), 3.0 eq base, $Pd_2(dba)_3$ (5%), ligand **3** (20%), microwave irradiation at 50°C for 60 min. 4.4 mg **1a** affords 5.3 mg **2q** as a pink solid, in 47% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.09 (m, 12H), 0.90 (s, 9H), 0.92 (s, 9H), 2.04-2.07 (m, 1H), 2.39-2.41 (m, 1H), 3.02 (s, 3H), 3.79-3.81 (m, 1H), 3.92-3.94 (m, 1H), 4.23 (m, 1H), 4.42 (m, 1H), 6.33 (dd, *J* = 10, 5 Hz, 1H), 7.89 (dd, *J* = 20, 5 Hz, 1H), 8.09 (m, 1H), 8.75 (s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ -5.5, -5.4, -5.0, -4.7, 17.9, 18.4, 21.1, 25.59, 25.62, 25.74, 25.84, 42.07, 63.0, 72.4, 86.2, 88.5, 110.4, 122.1, 132.5, 141.9, 148.8, 161.0, 164.8, 166.1; HRMS $[M+Na]^+$ m/z calcd. for $[C_{26}H_{44}N_6O_5Si_2Na]^+$ 599.2804, found 599.2805.



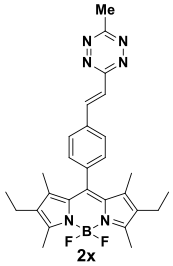
2v

2v: 1.1 eq 4-bromo-Oregon Green 488, 4 eq base, $Pd_2(dba)_3$ (10%), ligand **3** (40%), microwave at 50°C for 40 min. 4.4 mg **1a** afford 5.6 mg **2v** as a dark orange solid, in 58% yield. ^{1}H NMR (500 MHz, DMSO-D6) δ 10.79 (s, 1H), 8.48 (s, 1H), 8.43 (d, *J* = 16.3 Hz, 1H), 8.33 (dd, *J* = 8.2, 1.4 Hz, 1H), 7.88 (d, *J* = 16.4 Hz, 1H), 7.38 (d, *J* = 8.0 Hz, 1H), 6.89 (d, *J* = 7.5 Hz, 2H), 6.61 (d, *J* = 11.2 Hz, 2H), 2.97 (s, 3H). ^{13}C NMR (126 MHz, DMSO-D6) δ 167.96, 166.53, 164.16, 152.66, 147.79, 147.39, 147.02, 138.08, 137.60, 137.43, 136.40, 135.09, 124.95, 124.49, 123.55, 118.18, 116.94, 114.15, 113.98, 105.14, 104.85, 20.98.; HRMS $[M+H]^+$ m/z calcd. for $[C_{25}H_{14}F_2N_4O_5]^+$ 489.1005, found 489.1006.



2w

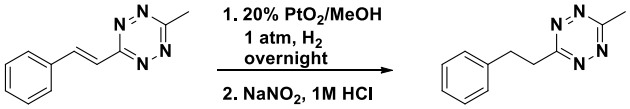
2w: 1.1 eq 9-(5-bromo-2-carboxyphenyl)-3,6-bis(dimethylamino) rhodamine (Peng et.al, *Org. Lett.,* 2013, *15,* 492- 495), 4 eq base, $Pd_2(dba)_3$ (10%), ligand **3** (40%), microwave at 50°C for 40 min. 4.4 mg **1a** afford 5.4 mg **2w** as a dark pink solid, in 53% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.25 (d, *J* = 16.3 Hz, 1H), 8.05 (d, *J* = 8.0 Hz, 1H), 7.85 (dd, *J* = 8.0, 1.3 Hz, 1H), 7.51 – 7.44 (m, 2H), 6.67 (s, 1H), 6.65 (s, 1H), 6.51 (d, *J* = 2.6 Hz, 2H), 6.42 (d, *J* = 2.6 Hz, 1H), 6.40 (d, *J* = 2.6 Hz, 1H), 3.08 (s, 3H), 2.99 (s, 12H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 175.11, 169.32, 163.91, 154.43, 153.04, 152.24, 141.57, 138.95, 129.40, 128.90, 128.61, 125.58, 124.33, 123.11, 108.83, 106.46, 98.69, 40.40, 21.48; HRMS $[M+H]^+$ m/z calcd. for $[C_{29}H_{28}N_6O_3]^+$ 508.2223, found 508.2178.



2x

2x: 1 eq 2,6-diethyl-4,4-difluoro-1,3,5,7-tetramethyl-8-(4-iodophenyl)-4-bora-3a,4a-diaza-sinadcene (Akkaya et.al, *J. Am. Chem. Soc.,* 2006, *128,* 14474-14475), 3 eq base, $Pd_2(dba)_3$ (5%), ligand **3** (20%), microwave at 50°C for 35 min. 4.4 mg **1a** afford 8.2 mg **2x** as a dark orange solid, in 83% yield. ^{1}H NMR (500 MHz, CDCl3) δ 8.38 (d, *J* = 16.3 Hz, 1H), 7.82 (d, *J* = 8.1 Hz, 2H), 7.56 (d, *J* = 16.3 Hz, 1H), 7.39 (d, *J* = 8.2 Hz, 2H), 3.09 (s, 3H), 2.55 (s, 6H), 2.31 (q, *J* = 7.5 Hz, 4H), 1.34 (s, 7H), 0.99 (t, *J* = 7.6 Hz, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 167.03, 165.13, 154.60, 140.48, 139.56, 138.67, 138.30, 136.03, 133.50, 131.02, 129.71, 129.12, 122.05, 21.76, 17.58, 15.13, 13.06, 12.41; HRMS $[M+Na]^+$ m/z calcd. for $[C_{28}H_{31}BF_2N_6Na]^+$ 523.2569, found 523.2569.

15. Synthesis of dialkyl-*s*-tetrazine **11** by hydrogenation of (*E*)-alkenyl-1,2,4,5-tetrazine **2a.**

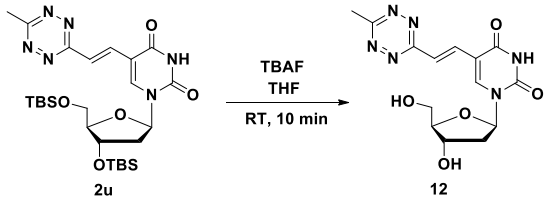


2a 11

PtO_2 (0.46 mg, 0.002 mmol) was added to a stirring solution of compound **2a** (4.0 mg, 0.02 mmol) in methanol (2.0 mL) at room temperature. The reaction solution was hydrogenated under 1 atm hydrogen gas. After 3 hours, TLC indicated that half of the starting material remained and the reaction was proceeding slowly. Another 10% PtO_2 was added and stirring was continued overnight, at which point TLC indicated that the reaction completed. The reaction was worked up by the addition of $NaNO_2$/1M HCl.

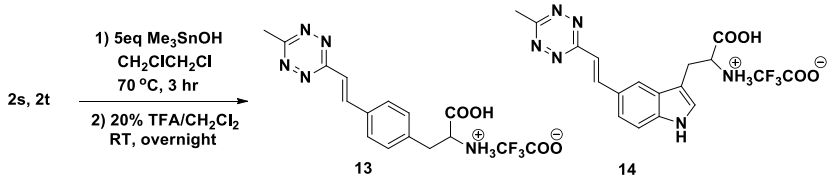
EtOAc (20 mL) was added to the solution and washed with brine (20 mL). The organic layer was dried over Na_2SO_4 and evaporated. The residue was isolated by a TLC plate (Hexanes:Et_2O = 3:1) to afford 3.2 mg **11** as a pink solid, in 81.6% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.03 (s, 3H), 3.28 (t, *J* = 7.5 Hz, 2H), 3.62 (t, *J* = 7.5 Hz, 2H), 7.19-7.30 (m, 5H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 21.2, 34.1, 36.4, 126.6, 128.5, 128.7, 140.0, 167.6, 169.3; HRMS $[M+H]^+$ m/z calcd. For $[C_{11}H_{13}N_4]^+$ 201.1135, found 201.1128.

16. Synthesis of the unnatural deoxyribose **12.**



TBAF (14 µL, 1.0 M in THF) was added to a stirred solution of compound **2u** (8.0 mg, 0.014 mmol) in dry THF (2.0 mL) at room temperature. The reaction solution was stirred at room temperature for 10 min, at which point TLC indicated that the starting material was consumed. The solvent was evaporated and the residue was purified by a TLC plate (EtOAc) to afford 3.7 mg of compound **12** as a pink solid, in 76% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.25-2.35 (m, 1H), 2.93 (s, 3H), 3.76 (dd, *J* = 15, 5 Hz, 1H), 3.86 (dd, *J* = 15, 5 Hz, 1H), 3.94 (dd, *J* = 10, 5 Hz, 1H), 4.06 (m, 1H), 6.27 (t, *J* = 5 Hz, 1H), 7.94 (dd, *J* = 25, 15 Hz, 1H), 8.58 (s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 19.5, 40.6, 61.1, 70.4, 87.8, 87.9, 110.0, 120.4, 133.1, 143.1, 149.8, 162.5, 165.0, 166.2; HRMS $[M+Na]^+$ m/z calcd. for $[C_{14}H_{16}N_6O_5Na]^+$ 371.1074, found 371.1075.

17. Synthesis of unnatural amino acids **13** and **14.**



In a 50 mL flask, **2s** or **2t** (1.0 eq) was dissolved in 1,2-dichloroethane, followed by Me_3SnOH (5.0 eq). The solution was heated to 70°C and stirred for 3 hours. The reaction solution was evaporated and purified by a TLC plate (CH_2Cl_2: MeOH = 20:1) to afford the carboxylic acid intermediate. The intermediate was dissolved in 20% CF_3COOH in CH_2Cl_2 and stirred at room temperature overnight. The reaction solution was evaporated and purified by a TLC plate (CH_2Cl_2 : MeOH : CF_3COOH = 5:1:0.1, the silica was washed with CH_2Cl_2:MeOH:CF_3COOH = 3:1:0.1) to afford the trifluoroacetic acid salt of the unnatural amino acid as a solid.

13: 5.0 mg **2s** afforded 3.0 mg 6 as pink solid, in 85% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.98 (s, 3H), 3.21 (m, 2H), 4.29 (bs, 1H), 7.40 (d, *J* = 5 Hz, 2H), 7.53 (d, *J* = 15 Hz, 1H), 7.76 (d, *J* = 5 Hz, 2H), 8.27 (d, *J* = 15 Hz, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 19.6, 35.9, 120.8, 128.4, 129.9, 134.8, 136.7, 139.6, 164.7, 166.4; HRMS $[M+H]^+$ m/z calcd. for $[C_{14}H_{16}N_5O_2]^+$ 286.1299, found 286.1298.

14: 6.5 mg **2t** afforded 5.1 mg 7 as orange solid, in 95% yield. ^{1}H NMR (500 MHz, CD_3OD) δ 2.95 (s, 3H), 3.38-3.42 (dd, *J* = 15, 10 Hz, 1H), 3.50 (m, 1H), 4.31 (bs, 1H), 7.26 (s, 1H), 7.43 (m, 2H), 7.62 (d, *J* = 5 Hz, 1H), 7.96 (s, 1H), 8.39 (d, *J* = 15 Hz, 1H), 10.93 (bs, 1H). ^{13}C NMR (125 MHz, CD_3OD) δ 21.9, 27.4, 109.1, 113.4, 118.2, 121.1, 122.4, 127.0, 128.2, 128.7, 139.5, 143.8, 166.4, 167.2; HRMS $[M-H]^-$ m/z calcd. for $[C_{16}H_{15}N_6O_2]^-$ 323.1262, found 323.1259.

18. Stability studies of a series of tetrazines **2a**, **2b**, and **2c**.

2a-2c at 1 mM were incubated in 50% DMF, 50% phosphate-buffered saline (PBS) 1x standard buffer pH 7.4 (Sigma Life Science) at 22°C room temperature. A Nanodrop 2000c (Thermo Scientific) UV-Vis absorption scan was used to do the measurements of samples in quartz cuvettes. Disappearance of the

characteristic tetrazine absorption peak intensity at around 520 nm was measured over time and baseline-adjusted (Full 49 h measurements are plotted in Figure S1a. 24 h time point is shown in Figure S1b. 3h in the presence of 1 mM cysteine is shown in Figure S1c). Absorbance peak maxima varied slightly between the samples, and the peak intensity values were averaged at 524-527 nm for **2a**, 535-539 nm for **2b**, and 519-525 nm for **2c**. Baselines were estimated by extrapolating a straight line by using the absorbance intensities immediately preceding and following the tetrazine peak.

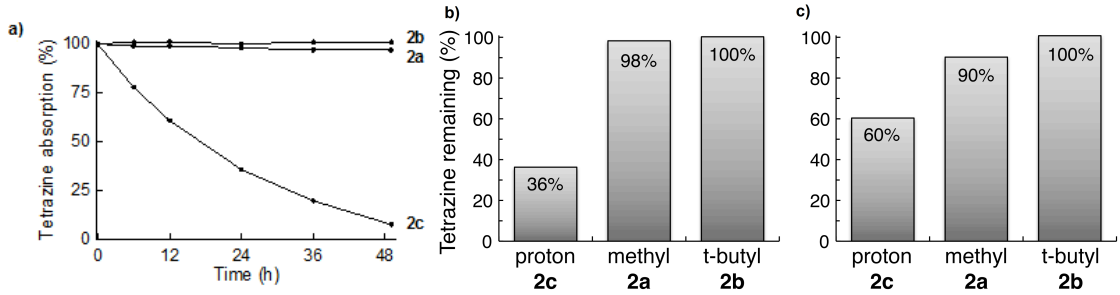


Figure S1. Tetrazines **2a**, **2b**, and **2c** (as indicated by the corresponding curves) at 1 mM were incubated at 22°C. Tetrazine absorption peak intensities at around 520-530 nm were measured over time and are plotted after baseline adjustments as a percent tetrazine remaining. a) Tetrazine decomposition in 1:1 DMF:PBS buffer pH 7.4 over two days. b) Tetrazine stability in phosphate-buffered saline (PBS) buffer after 24 h. Tetrazines **2c**, **2a**, and **2b** at 1 mM were incubated in 50% DMF, 50% PBS 1x pH 7.4 at 22°C, and the fraction remaining was measured as a function of the disappearance of the characteristic 520 nm absorption peak intensity over time. c) Percent tetrazine remaining after 3h in the presence of 1 mM cysteine.

19. Tetrazine kinetic measurements

A Nanodrop 2000c (Thermo Scientific) equipped with a cuvette reader and a stirrer was used in the kinetic measurements, and the disappearance of the tetrazine peak absorption around 520 nm was measured over time. Tetrazines at 1 mM were reacted with an excess 10 mM (*E*)-cyclooct-4-enol with the measurements commencing immediately upon (*E*)-cyclooct-4-enol addition (Figures S2b, S3). Solution conditions were maintained at 1:1 DMF:PBS pH 7.4 buffer (Sigma Life Science), and all the measurements were done at 22°C room temperature. Tetrazine peak intensity at each timepoint was adjusted for background by extrapolation, as in the previous section. Tetrazine peak disappearance is shown as points in Figure 1d, with the connecting lines resulting from the nonlinear fits made with Prism 6.0c. Least squares exponential decay fits were done by using the equation:

$$y = \left(y_0 - \text{Plateau}\right) \times e^{-Kx} + \text{Plateau}$$

in order to determine the pseudo first-order reaction rate constants. Conversion to the second order rate constants was done by using the equation:

$$k_2 = \frac{k_{obs}}{[\text{TCO}]_0}.$$

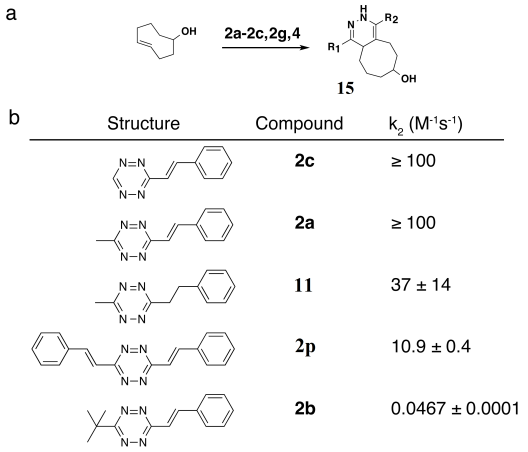


Figure S2. (*E*)-3-substituted-6-styryl-*s*-tetrazine reaction kinetics. a) Reaction scheme of (*E*)-cyclooct-4-enol and the series of tetrazines for kinetic characterization. Only one isomer is depicted for **15**. b) Tetrazine kinetics were determined by reacting 1 mM tetrazine with an excess (10 mM) of (*E*)-cyclooct-4-enol in 50% DMF, 50% PBS 1x pH 7.4 buffer at 22°C. Second-order rate constants derived from the data are listed, and the corresponding curves for **2b**, **2p**, and **11** are shown in Figure S3. Tetrazine **2p** was reacted in 80% DMF, 20% PBS due to its insolubility at lower DMF concentrations.

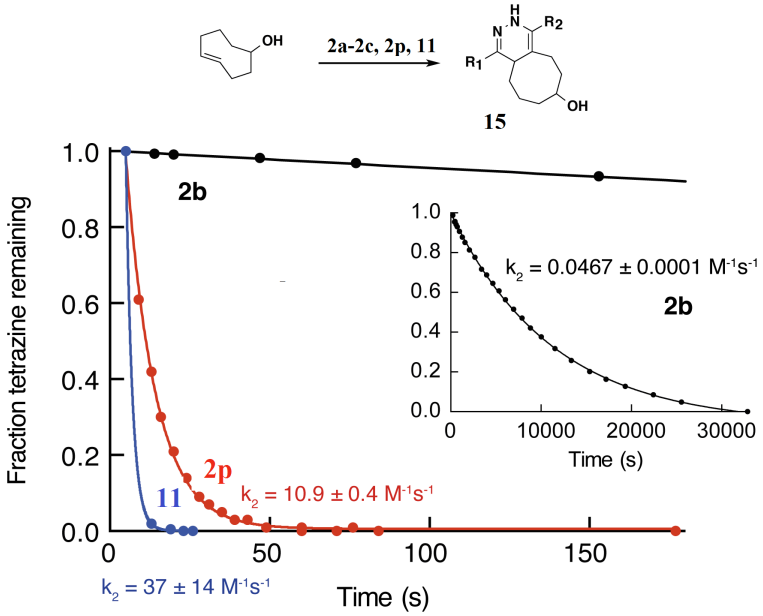
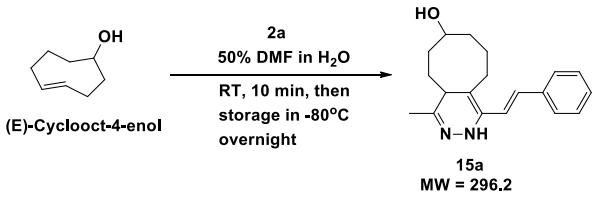


Figure S3. Reaction kinetics of 1 mM tetrazines **2b**, **2p**, and **11** with 10 mM (*E*)-cyclooct-4-enol in 1:1 DMF:PBS pH 7.4 buffer at 22°C. Data was collected under pseudo first-order conditions (data points plotted) and fitted to a single exponential decay (curves indicated). Tetrazine compounds are labelled next to the corresponding data curves (**2b** in black, **2p** in red, and **11** in blue). Inset figure shows extended time points for the reaction of tetrazine **2b** with (*E*)-cyclooct-4-enol.

20. Characterization of the reaction between tetrazine **2a** and (*E*)-Cyclooct-4-enol (TCO).



Tetrazine **2a** (250 mM in DMF, 5 µL) and (*E*)-Cyclooct-4-enol (TCO) (250 mM in DMF, 6 µL) were combined in 125 µL of H_2O and 114 µL DMF at a final concentration of 5 mM for tetrazine **2a** and 6 mM for TCO. The reaction solution was agitated for 10 minutes at room temperature and stored at -80 °C overnight and then analyzed by LC-MS (Figure S4). Note, only one isomer of **15a** is depicted.

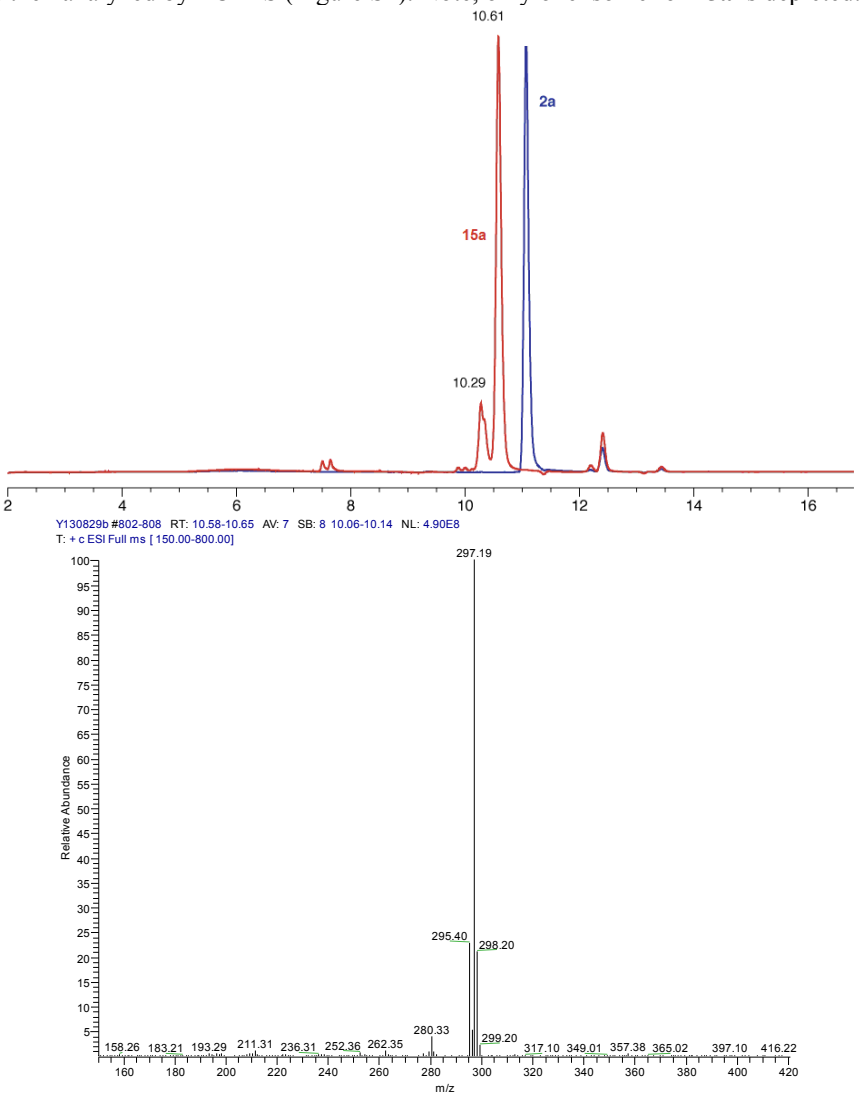
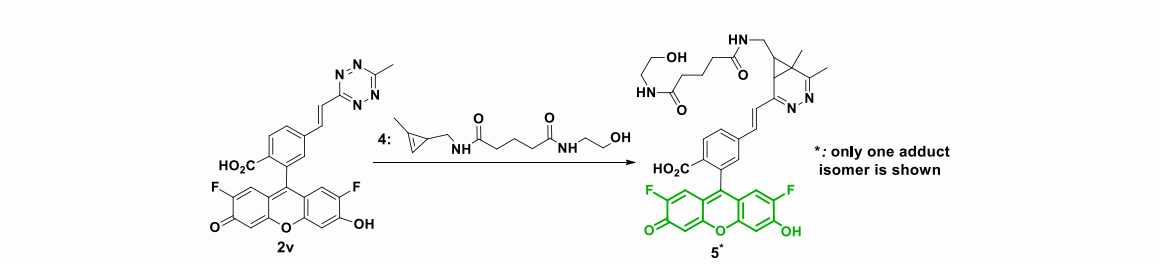


Figure S4. Reaction between tetrazine **2a** and TCO. HPLC trace of tetrazine **2a** (in blue) is shown overlaid with the HPLC trace of the products (in red) of the reaction between **2a** and TCO at 5 mM in 1:1 DMF:H_2O. MS traces of the reaction solution with selected ion monitoring at m/z 297.2 are shown in black (i.e. $[M+H]^+$ peak). Only one isomer of **15a** is depicted.

21. Characterization of the reaction product **5** between tetrazine **2v** and cyclopropene **4**.



Tetrazine **2v** (250 mM in DMF, 5 µL) and cyclopropene **4** (250 mM in DMF, 6 µL) were combined in 50 µL of H_2O and 189 µL DMF at a final concentration of 5 mM for tetrazine **2v** and 6 mM for **4**. The reaction solution was on a shaker for 30 minutes at room temperature then analyzed by LC-MS and HRMS (Figure ***S5***). Note: only one isomer of **5** is depicted.

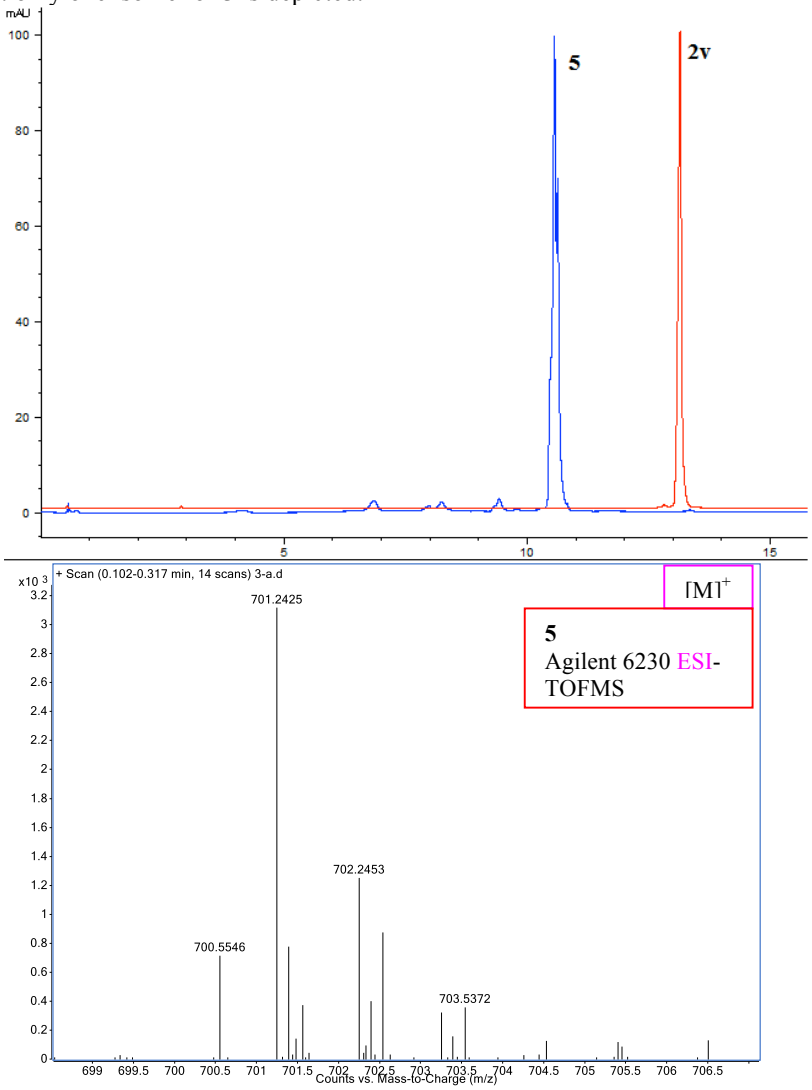
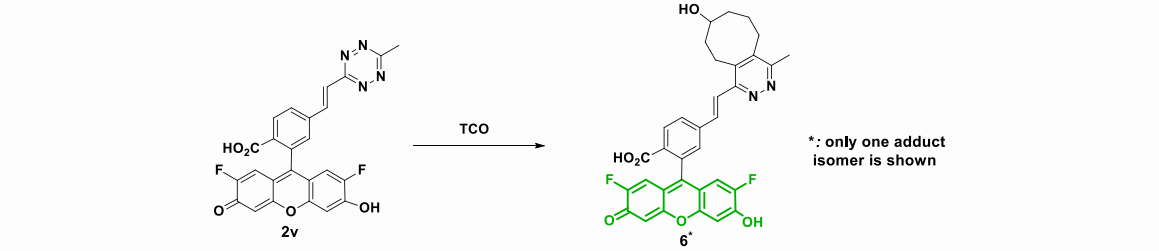


Figure S5: Reaction between tetrazine **2v** and cyclopropene **4**. HPLC trace of tetrazine **2v** (in red) is shown overlaid with the HPLC trace of the reaction products **5** between **2v** and cyclopropene **4** at 5 mM in 4:1 DMF:H_2O (in blue). HRMS traces of the reaction solution was shown below HRMS $[M]^+$ m/z calcd. For $[C_{37}H_{34}F_2N_4O_8]^+$ 701.2417, found 701.2425.

22. Characterization of the reaction between tetrazine **2v** and (*E*)-Cyclooct-4-enol (TCO) product **6**.



Tetrazine **2v** (250 mM in DMF, 5 µL) and TCO (250 mM in DMF, 6 µL) were combined in 50 µL of H_2O and 189 µL DMF at a final concentration of 5 mM for tetrazine **2v** and 6 mM for TCO. The reaction solution was on a shaker for 30 minutes then analyzed by LC-MS and HRMS (Figure ***S6***). Note, only one isomer of **6** is depicted.

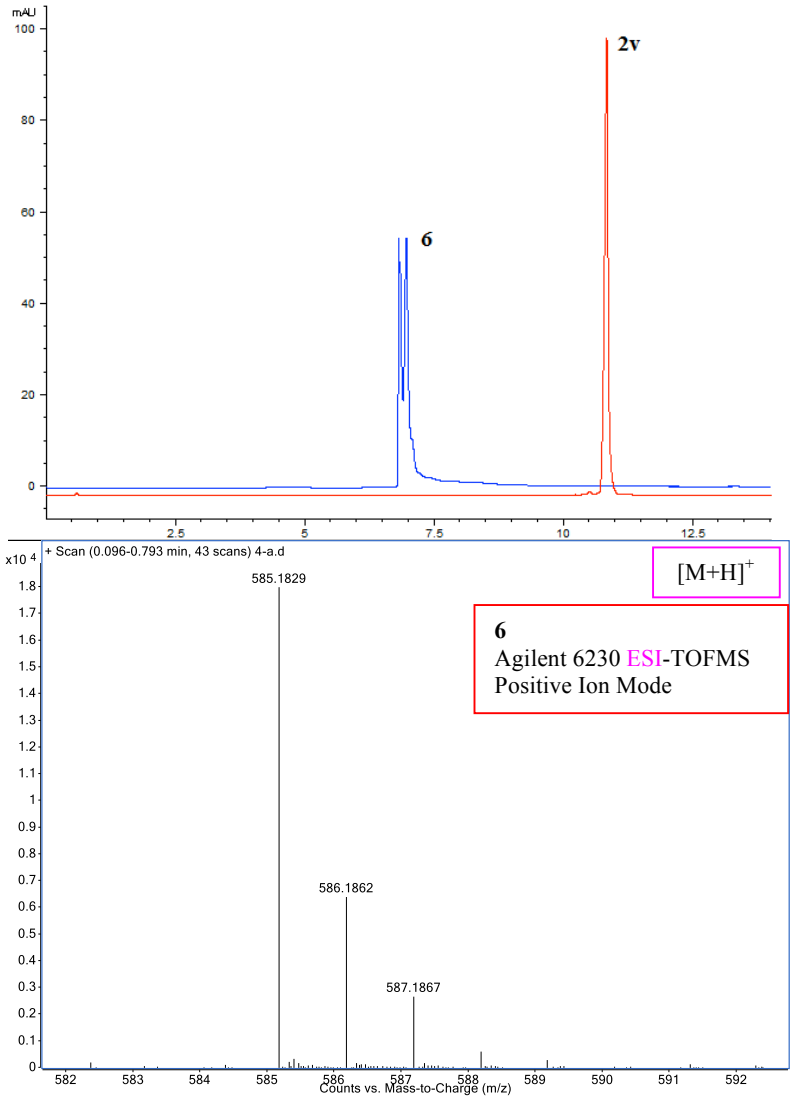
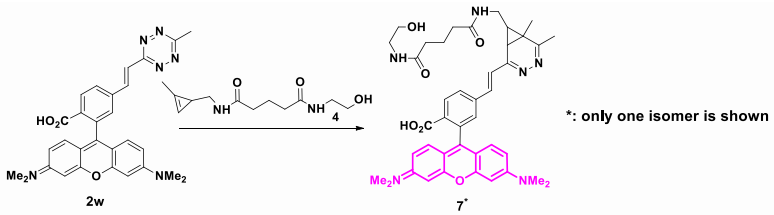


Figure S6: Reaction between tetrazine **2v** and (*E*)-Cyclooct-4-enol (TCO) and oxidation product **6**. HPLC trace of tetrazine **2v** (in red) is shown overlaid with the HPLC trace of the (*E*)-Cyclooct-4-enol (TCO) adduct (in blue). HRMS traces of **6** was shown below HRMS $[M+H]^+$ m/z calcd. For $[C_{33}H_{27}F_2N_2O_6]^+$ 585.1832, found 585.1829.

23. Characterization of the reaction product **7** between tetrazine **2w** and cyclopropene **4**.



Tetrazine **2w** (250 mM in DMF, 5 µL) and cyclopropene **4** (250 mM in DMF, 6 µL) were combined in 50 µL of H_2O and 189 µL DMF at a final concentration of 5 mM for tetrazine **2w** and 6 mM for **4**. The reaction solution was on shaker for 30 minutes at room temperature then analyzed by LC-MS and HRMS (Figure ***S7***). Note, only one isomer of **7** is depicted.

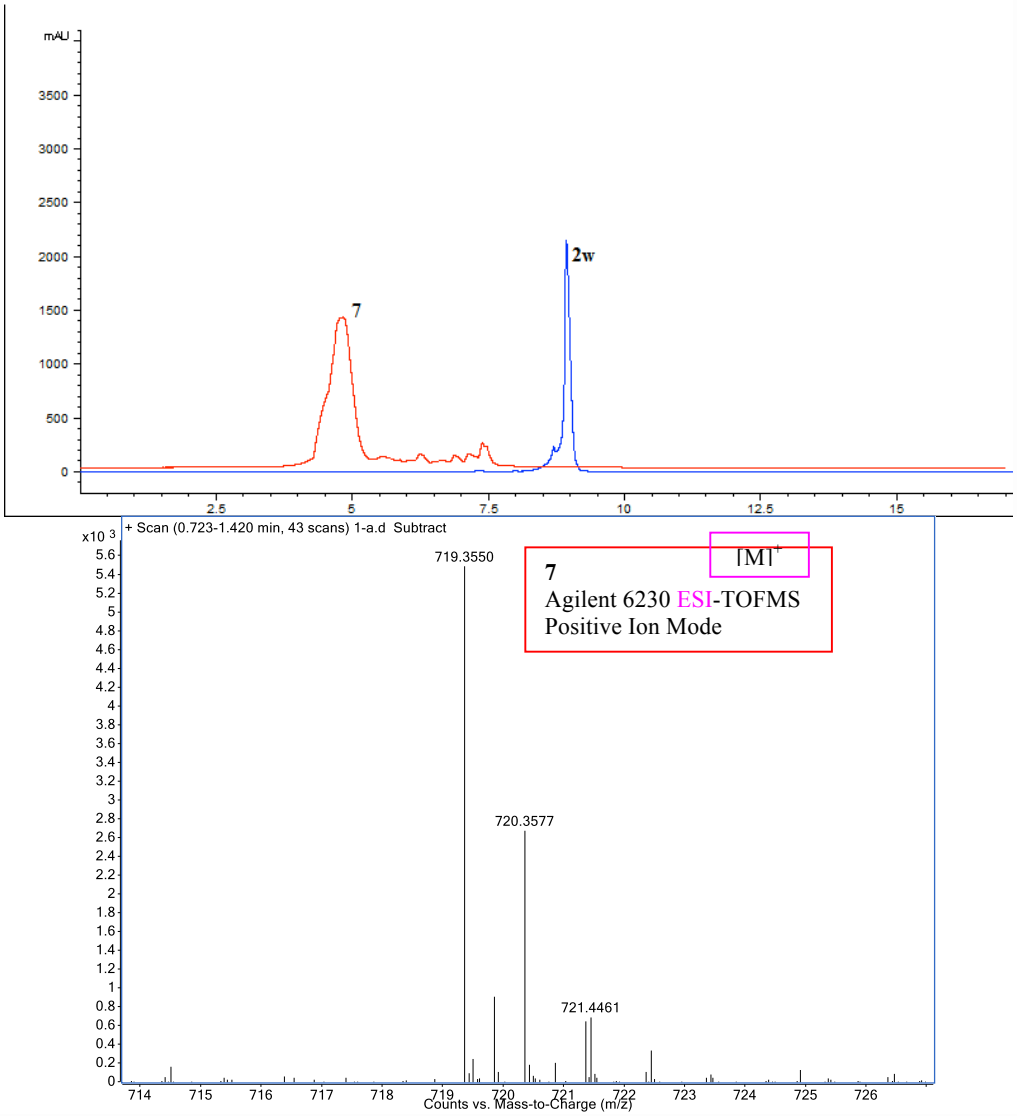
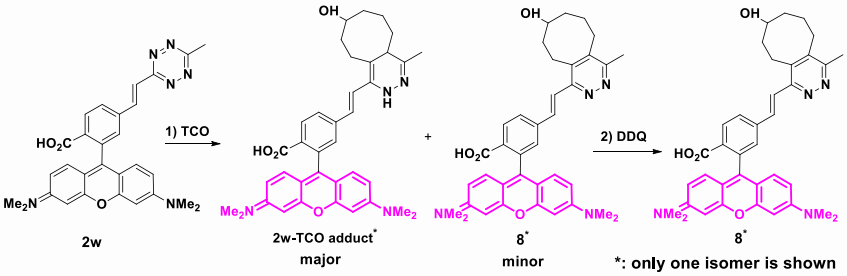


Figure S7: Reaction between tetrazine **2w** and cyclopropene **4**. HPLC trace of tetrazine **2w** (in blue) is shown overlaid with the HPLC trace of the reaction products between **2v** and cyclopropene **4** at 5 mM in 4:1 DMF:H_2O (in red). HRMS traces of the reaction solution was shown below HRMS $[M]^+$ m/z calcd. For $[C_{41}H_{47}N_6O_6]^+$ 719.3557, found 719.3550.

24. Characterization of the reaction between tetrazine **2w** and (*E*)-Cyclooct-4-enol (TCO) and DDQ oxidation product **8**.



Tetrazine **2w** (250 mM in DMF, 5 µL) and (*E*)-Cyclooct-4-enol (TCO) (250 mM in DMF, 6 µL) were combined in 239 µL DMF at a final concentration of 5 mM for tetrazine **2w** and 6 mM for TCO. The reaction solution was on a shaker for 10 minutes at room temperature then 1.25 µmol 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) was added, after 1 minute, 10 µL of water was added to quench the reaction. Both two steps were analyzed by LC-MS and HRMS (Figure ***S8***). Note, only one isomer of **8** is depicted.

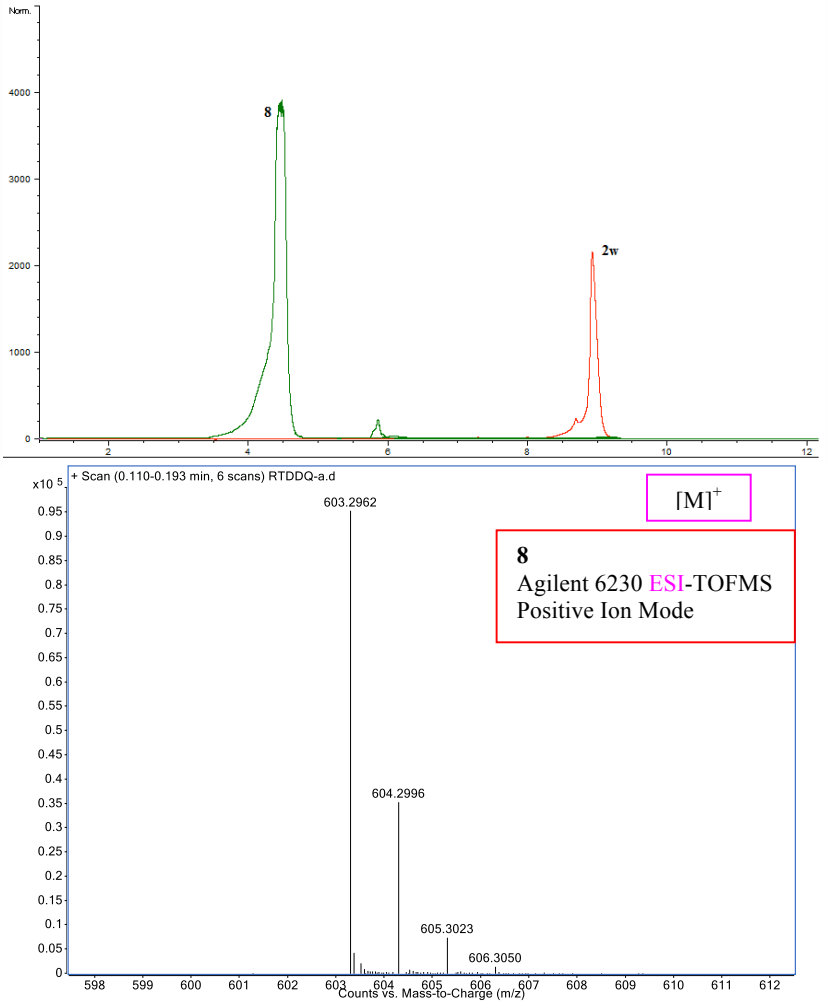
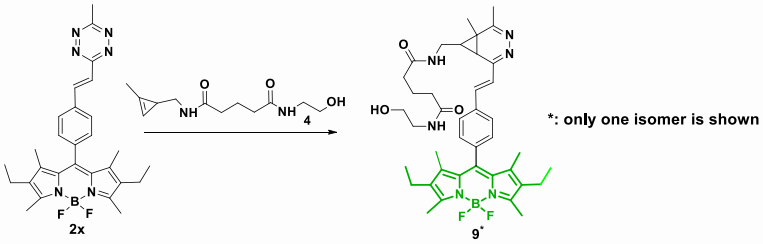


Figure S8: Reaction between tetrazine **2w** and (*E*)-Cyclooct-4-enol (TCO) and oxidation product **8**. HPLC trace of tetrazine **2w** (in red) is shown overlaid with the HPLC trace of the (*E*)-Cyclooct-4-enol (TCO)

adduct after the oxidation (in green). HRMS traces of the **8** was shown below HRMS $[M]^+$ m/z calcd. For $[C_{37}H_{39}N_4O_4]^+$ 603.2966, found 603.2962.

25. Characterization of the reaction product **9** between tetrazine **2x** and cyclopropene **4**.



Tetrazine **2x** (250 mM in DMF, 5 µL) and cyclopropene **4** (250 mM in DMF, 6 µL) were combined in 50 µL of H_2O and 189 µL DMF at a final concentration of 5 mM for tetrazine **2x** and 6 mM for **4**. The reaction solution was on a shaker for 30 minutes at room temperature then analyzed by LC-MS and HRMS (Figure ***S9***). Note, only one isomer of **9** is depicted.

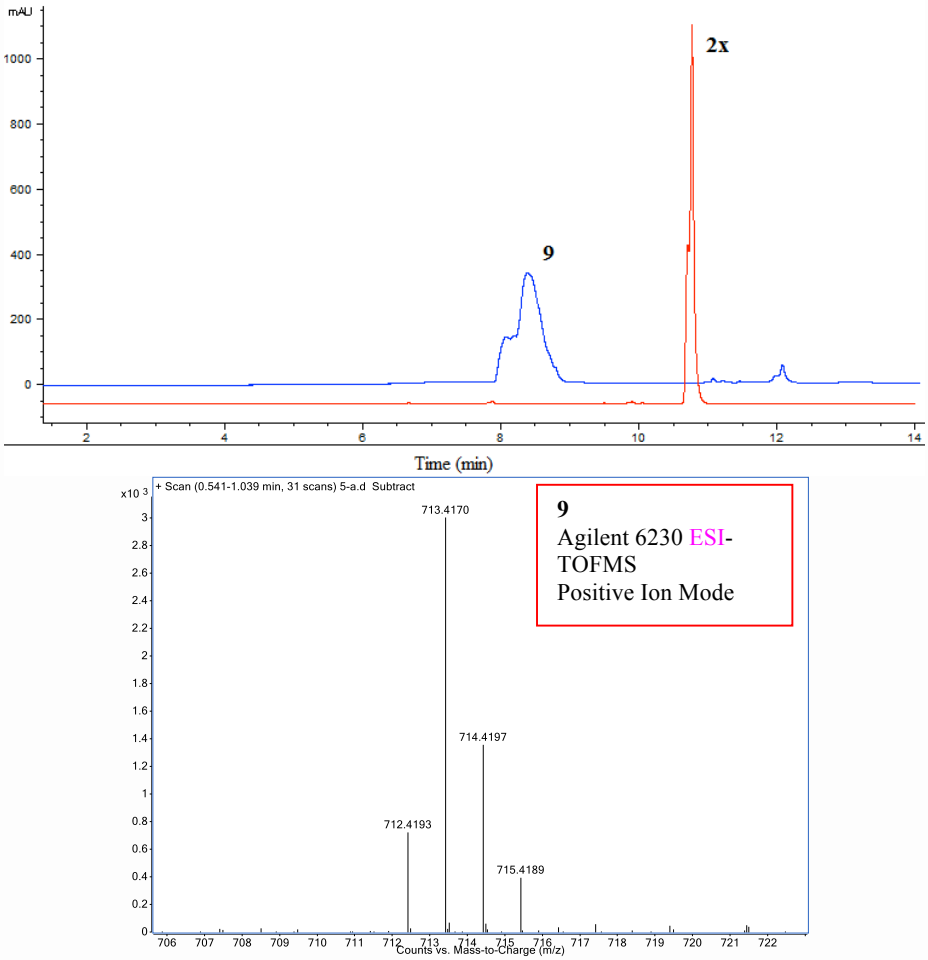
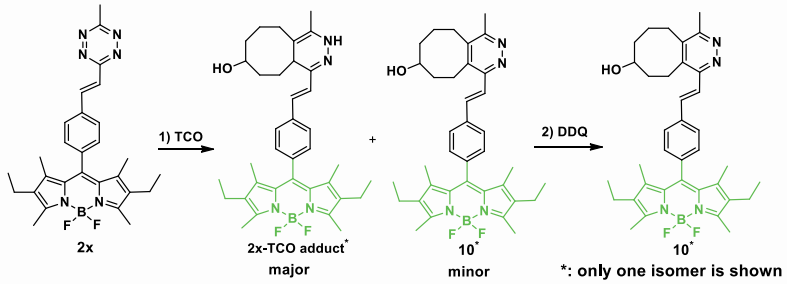


Figure S9: Reaction between tetrazine **2x** and cyclopropene **4**. HPLC trace of tetrazine **2x** (in red) is shown overlaid with the HPLC trace of the products (in blue) of the reaction between **2x** and cyclopropene **4** at 5 mM in 4:1 DMF: H_2O. HRMS traces of **9** was shown below HRMS $[M+H]^+$ m/z calcd. For $[C_{40}H_{52}BF_2N_6O_3]^+$ 712.4193, found 712.4193.

26. Characterization of the reaction between tetrazine **2x** and (*E*)-Cyclooct-4-enol (TCO) and DDQ oxidation product **10**.



Tetrazine **2x** (250 mM in DMF, 5 µL) and (*E*)-Cyclooct-4-enol (TCO) (250 mM in DMF, 6 µL) were combined in 239 µL DMF at a final concentration of 5 mM for tetrazine **2x** and 6 mM for TCO. The reaction solution was on a shaker for 10 minutes at room temperature then 1.25 µmol 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) was added, after 1 minute, 10µL of water was added to quench the reaction. Both steps were analyzed by LC-MS and HRMS (Figure ***S10***). Note, only one isomer of **10** is depicted.

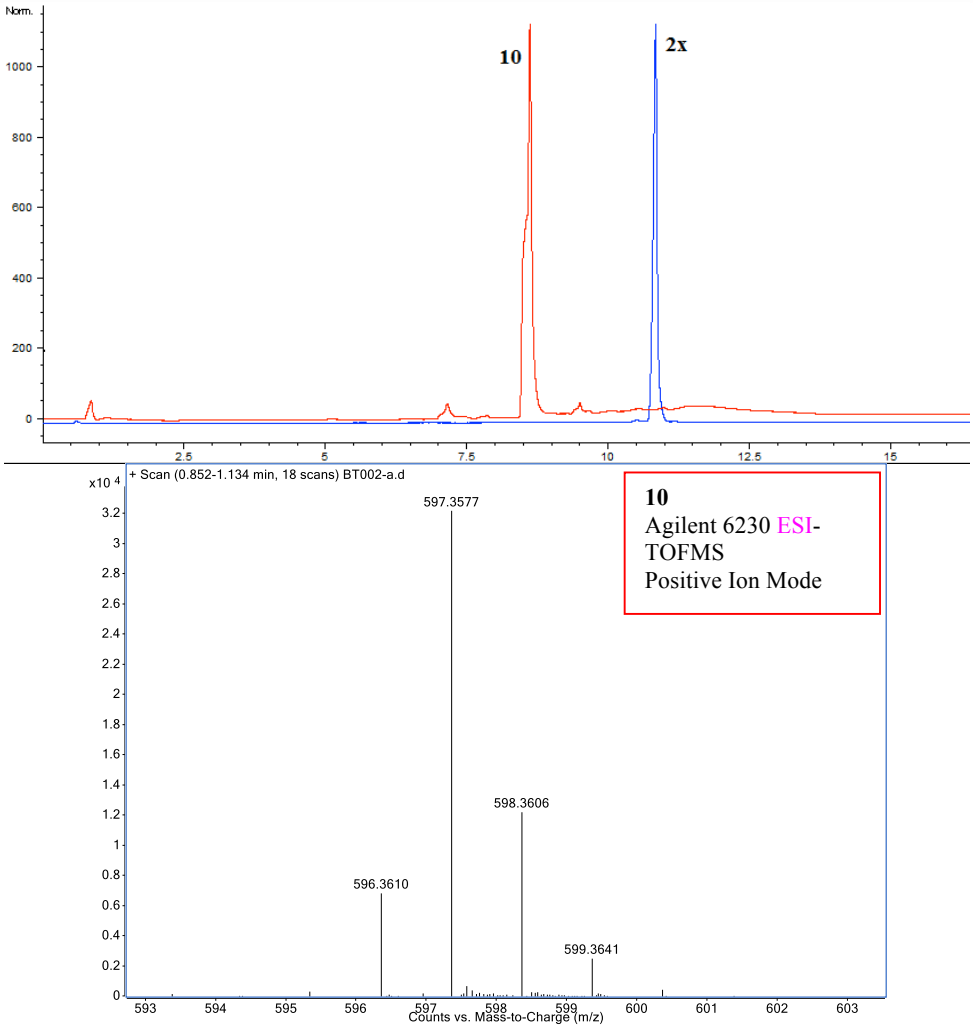


Figure S10: Reaction between tetrazine **2x** and (*E*)-Cyclooct-4-enol (TCO) and oxidation product **10**. HPLC trace of tetrazine **2x** (in blue) is shown overlaid with the HPLC trace of the (*E*)-Cyclooct-4-enol

(TCO) adduct and after the oxidation (in red). HRMS traces of the **10** gwas shown below HRMS [M+H]+ m/z calcd. For $[C_{36}H_{43}BF_2N_4ONa]^+$ 597.3576, found 597.3577.

27. HPLC trace of **2v** (1 mM MeCN) before (blue) and after (red) 24 hours in solution at room temperature

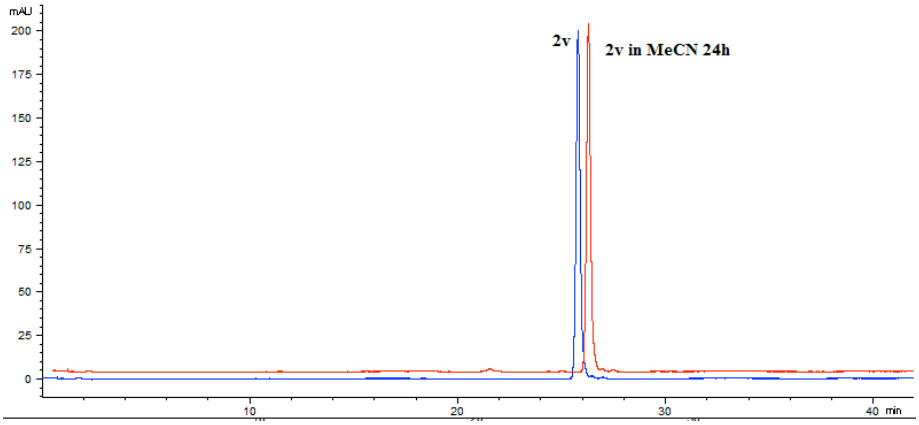
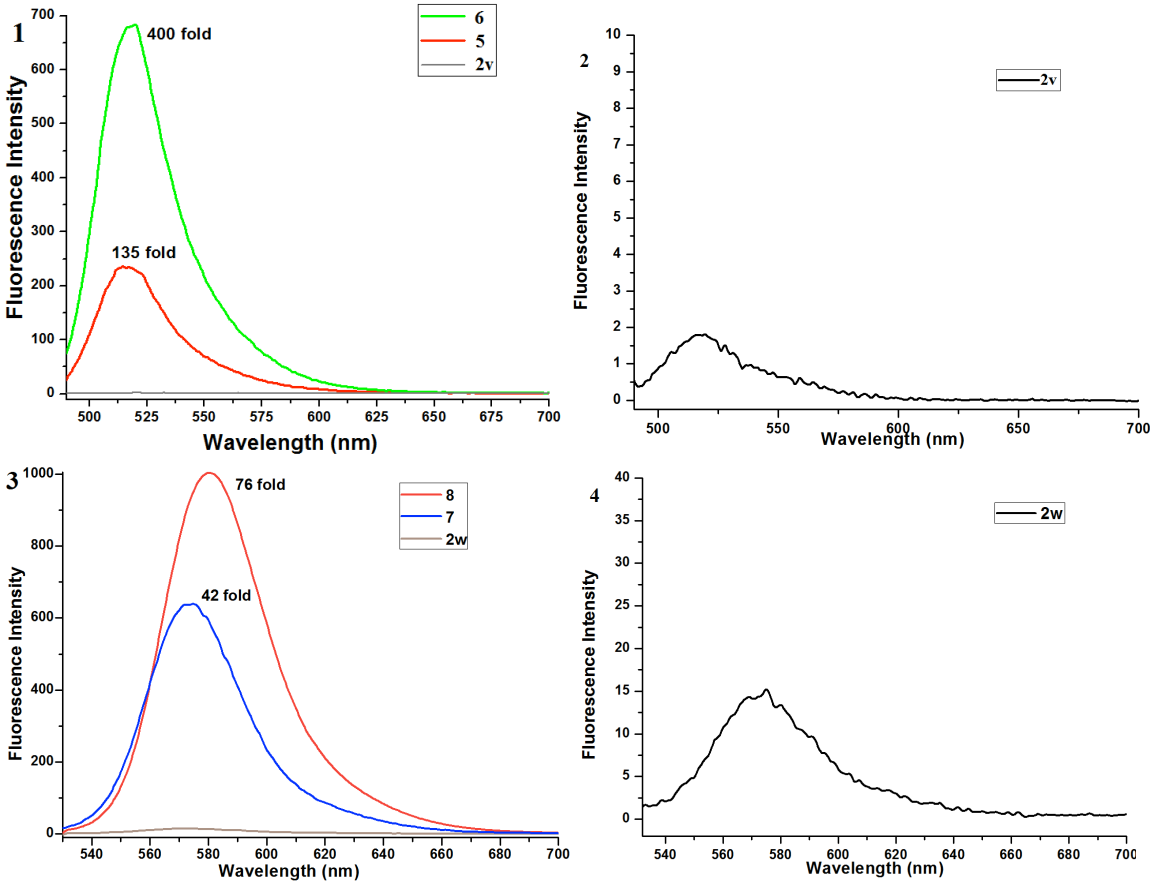


Figure S11: HPLC trace of **2v** (1 mM MeCN) before (blue) and after (red) 24 hours in solution at room temperature

28. Fluorescence emission spectra of turn-on reaction



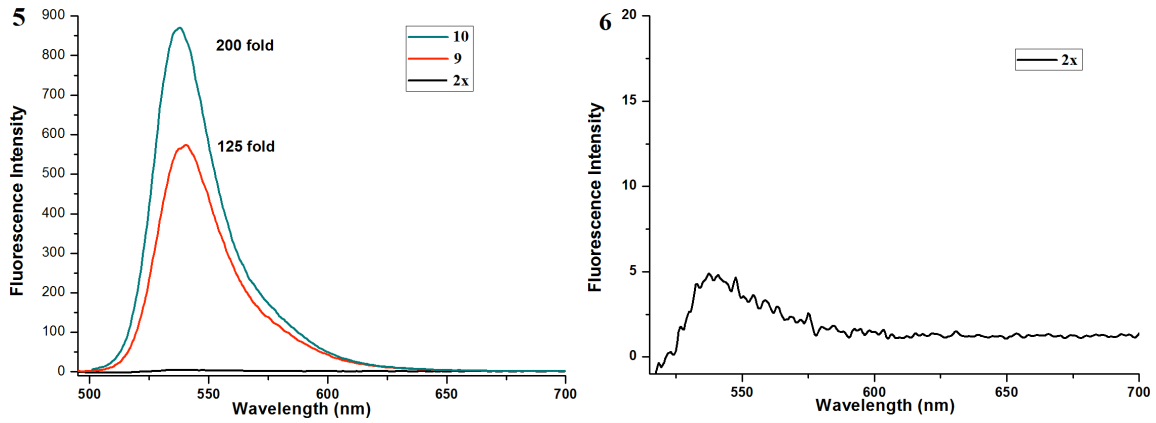
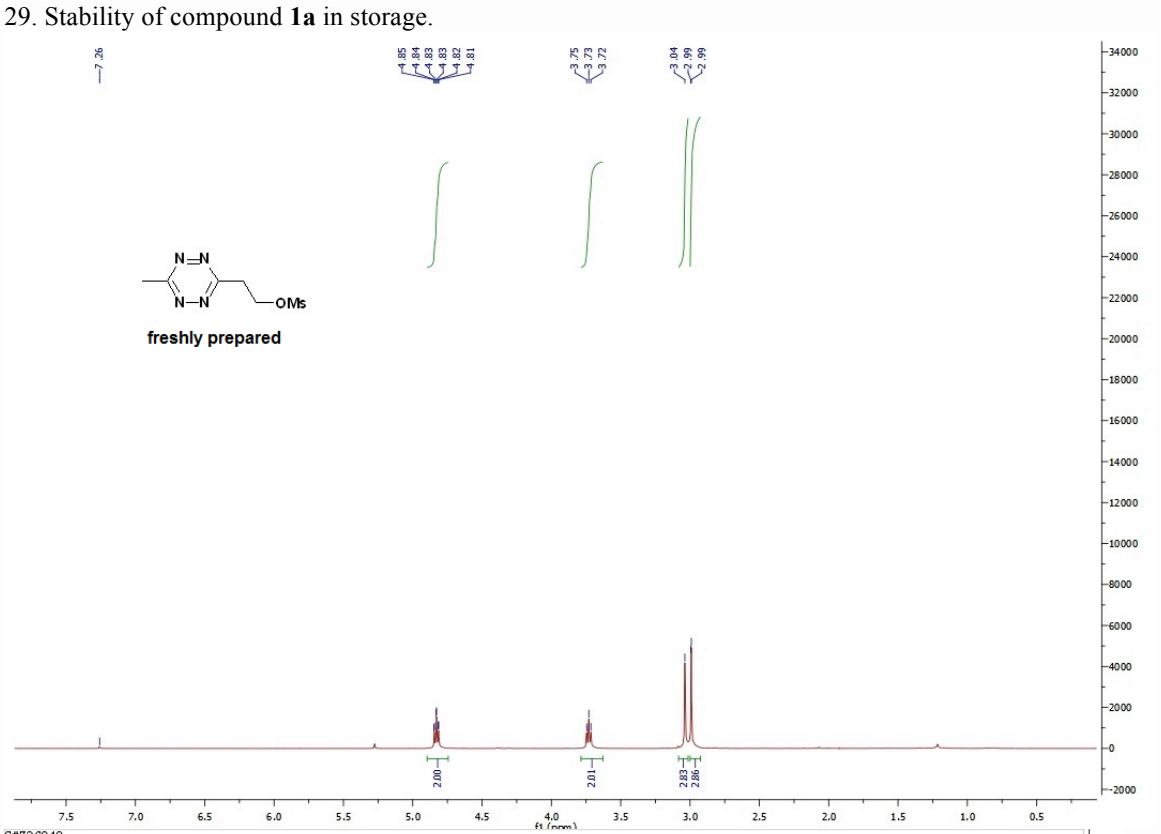
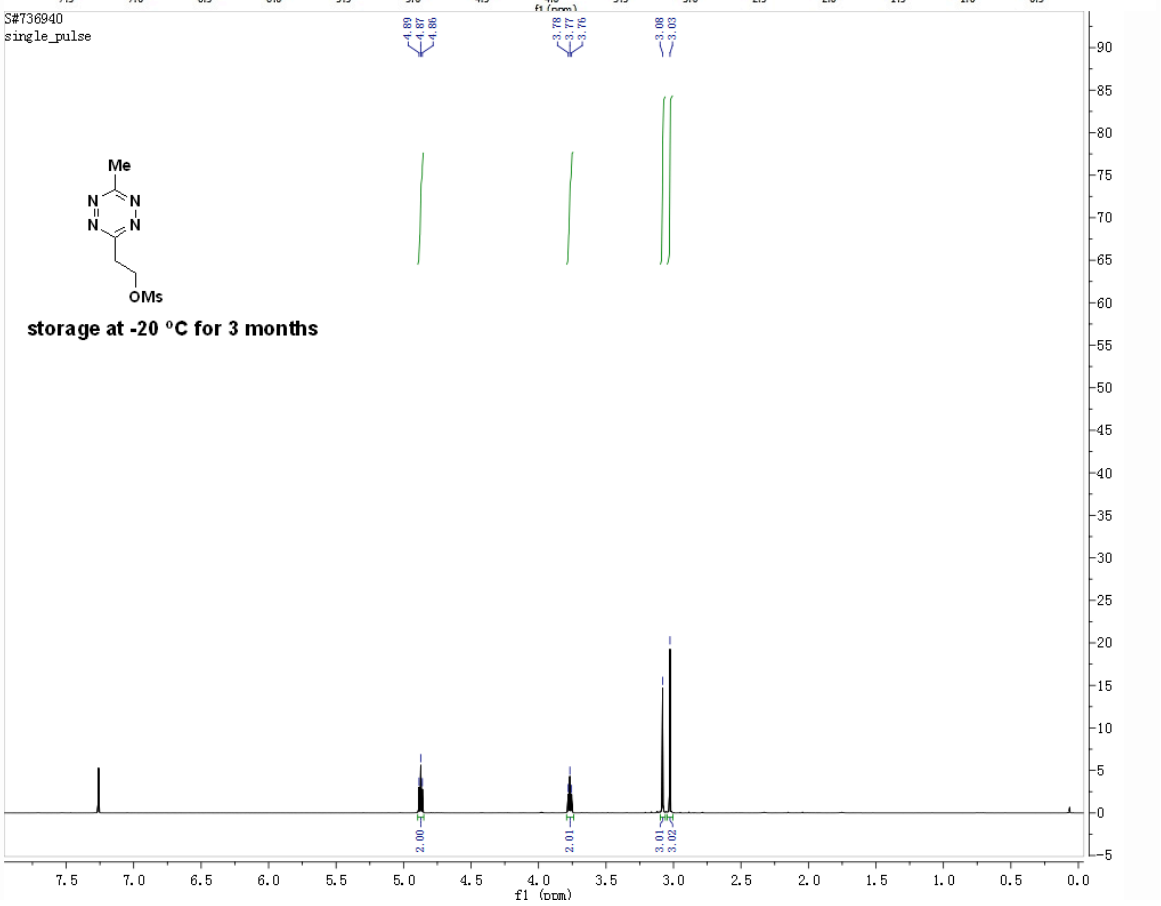
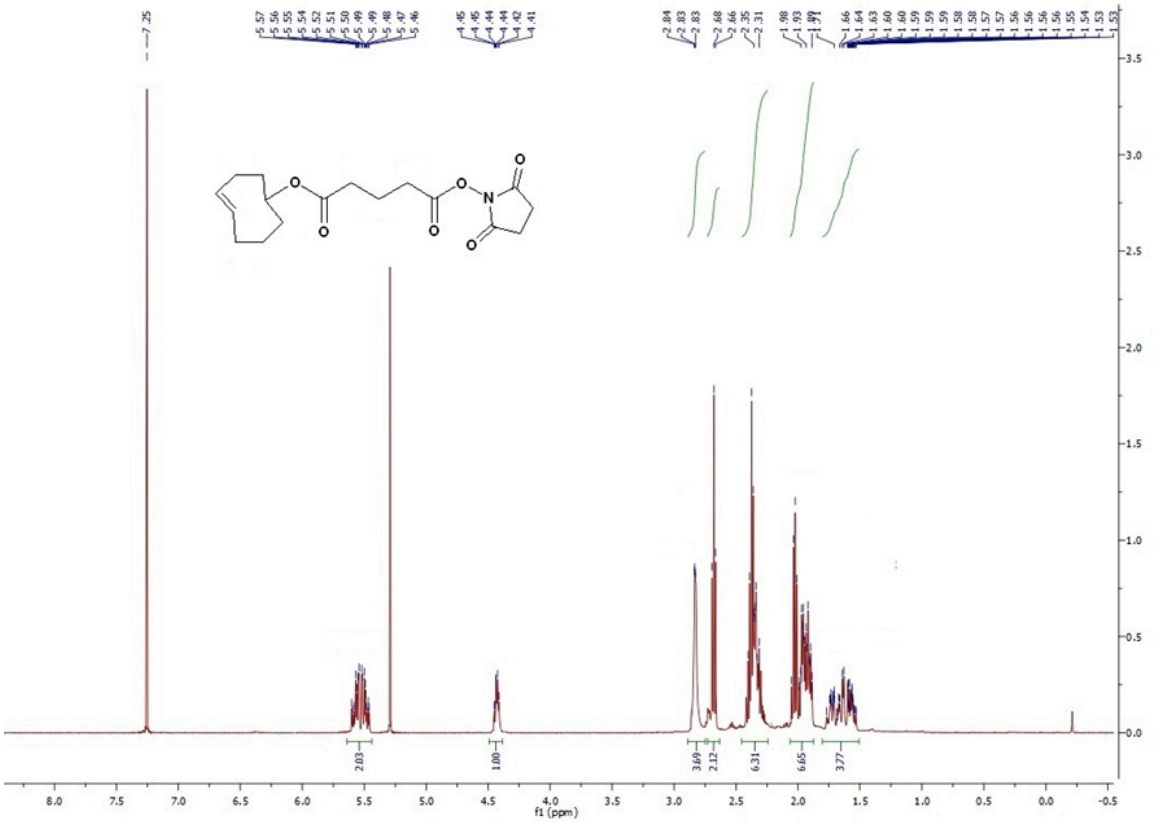


Figure S12: 1) Fluorescence emission of **2v**, **5**, **6**. All three compounds were dissolved in 2 µM phosphate-buffered saline (PBS) at pH 7.4. 2) Fluorescence emission of **2v** in 2 µM phosphate-buffered saline (PBS) at pH 7.4. 3) Fluorescence emission of **2w**, **7**, **8**. All three compounds were dissolved in 2 µM EtOH solution. Compound **2w** and **8** were tested after HPLC purification and **7** was tested directly after reaction. 4) Fluorescence emission of **2w** in 2 µM EtOH solution. 5) Fluorescence emission of **2x**, **9**, **10**. All three compounds were dissolved in 2 µM EtOH solution. Compounds **2x** and **10** were tested after HPLC purification and compound **9** was tested directly after reaction. 6) Fluorescence emission of **2x** in 2 µM EtOH solution.

29. Stability of compound **1a** in storage.






7.25
5.57
5.56
5.55
5.54
5.52
5.51
5.50
5.49
5.49
5.48
5.47
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4.45
4.45
4.44
4.44
4.42
4.41
2.84
2.83
2.83
2.68
2.66
2.35
2.31
1.98
1.93
1.91
1.89
1.66
1.64
1.63
1.60
1.60
1.59
1.59
1.59
1.58
1.58
1.57
1.57
1.56
1.56
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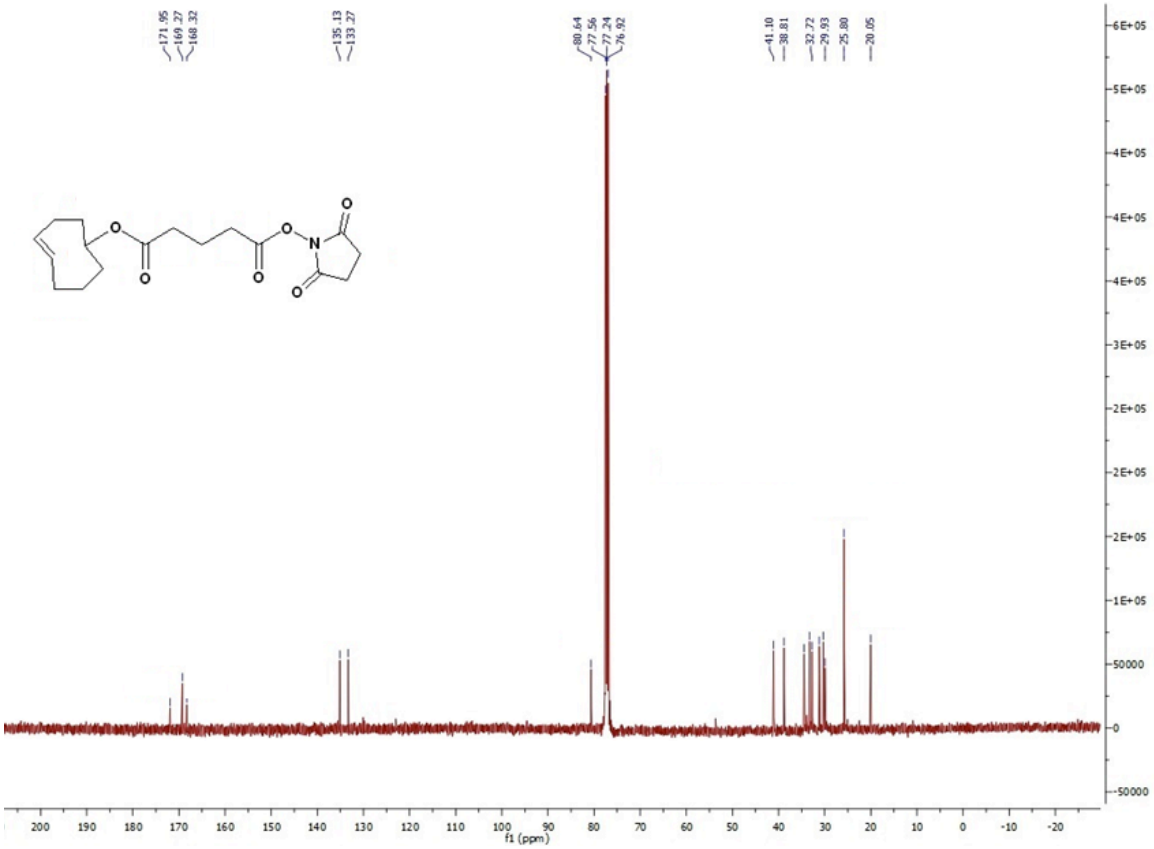
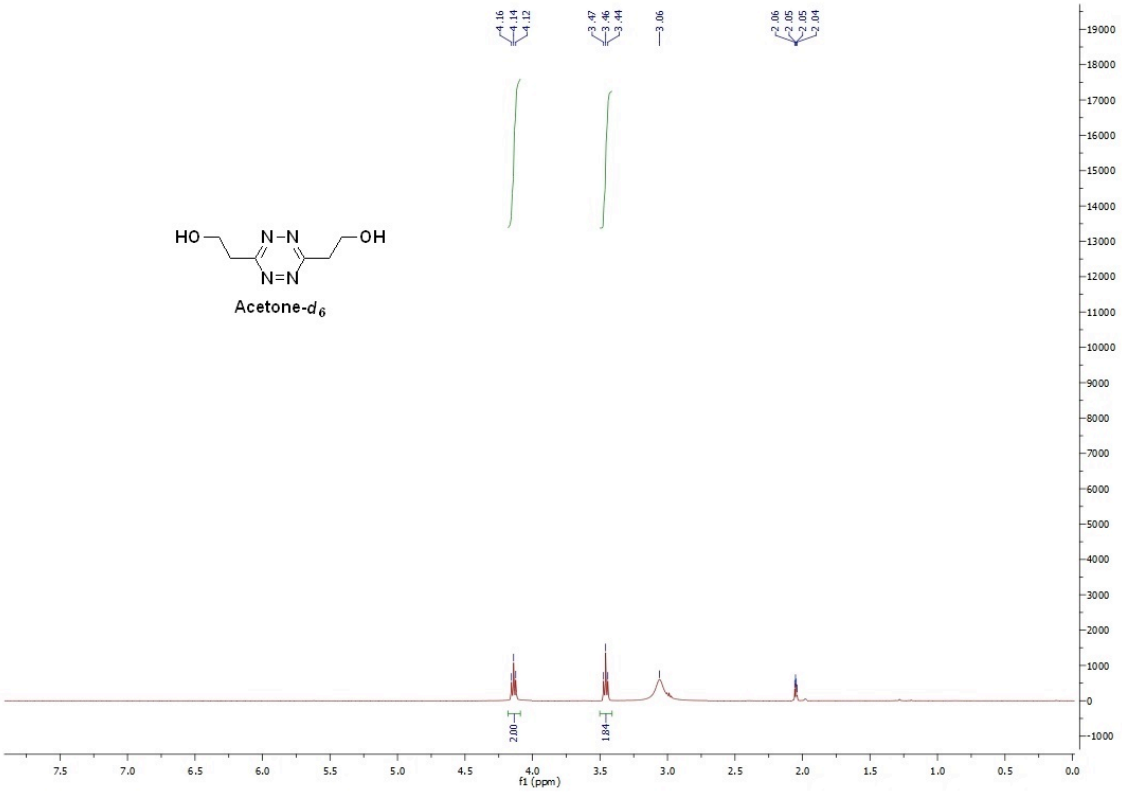
2.12
6.31
6.65
3.77
f1 (ppm)


171.95
169.27
168.32
135.13
133.27
80.64
77.56
77.24
76.92
41.10
38.81
32.72
29.93
25.80
20.05
f1 (ppm)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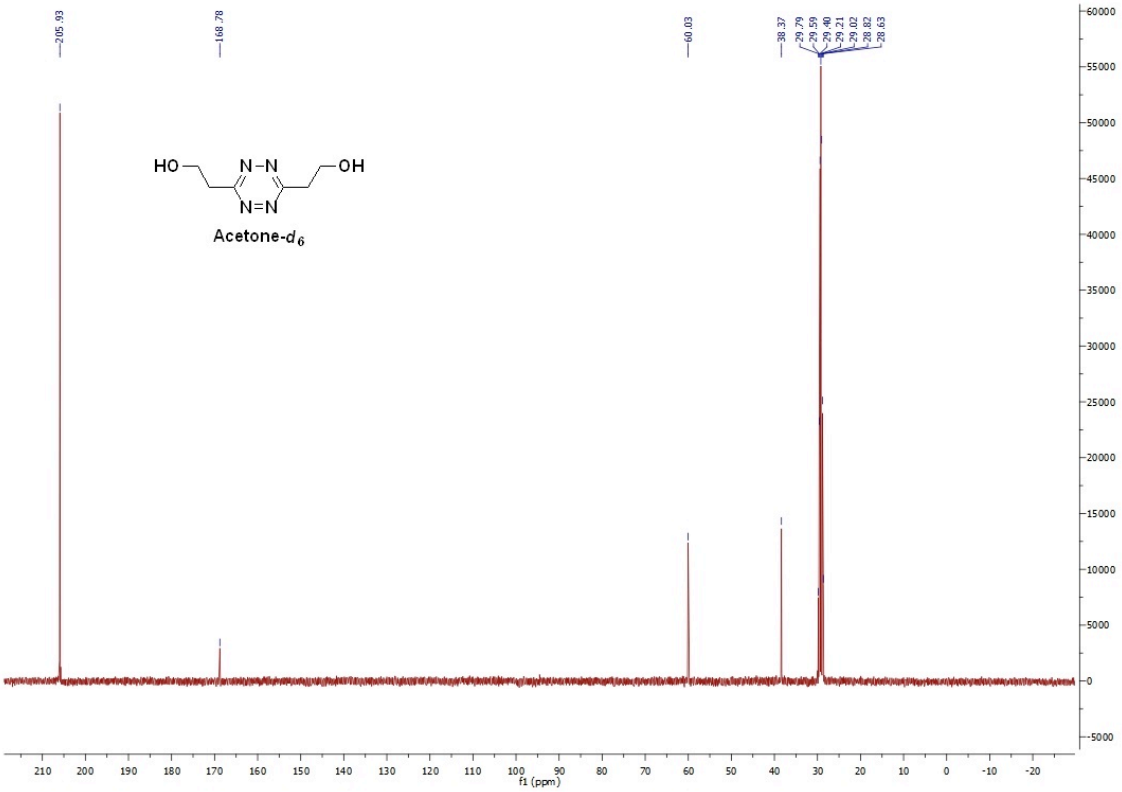
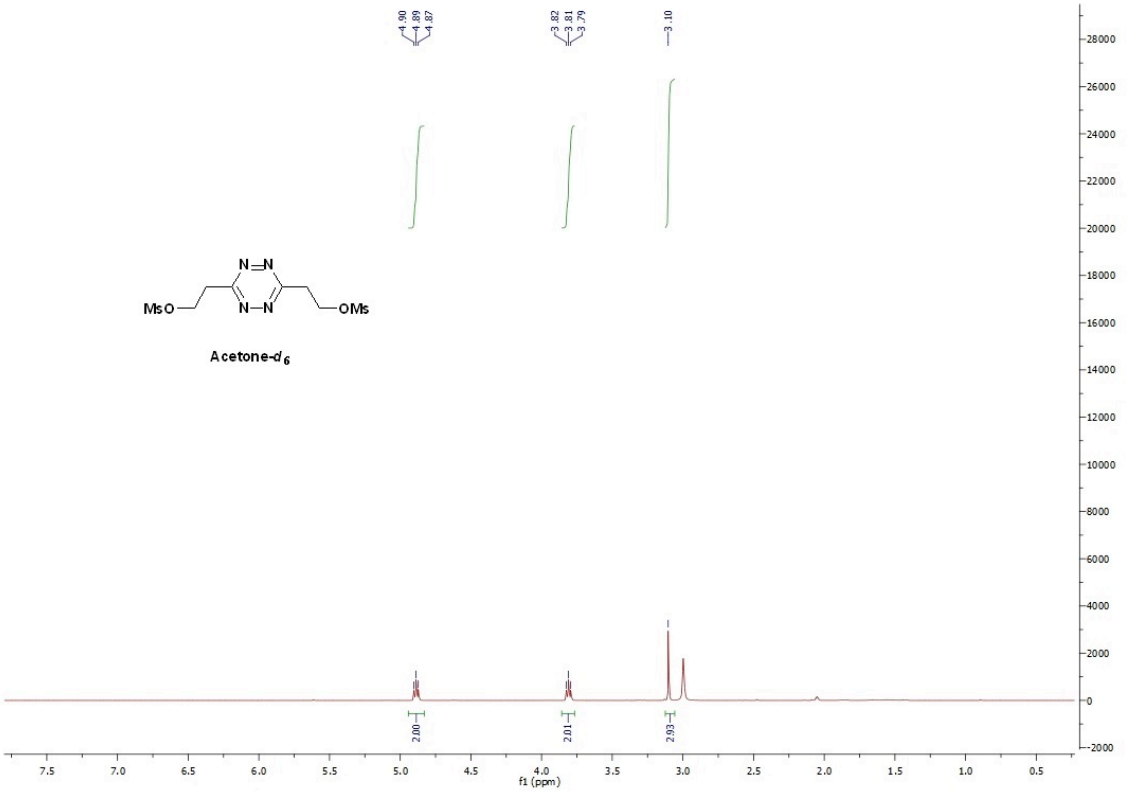
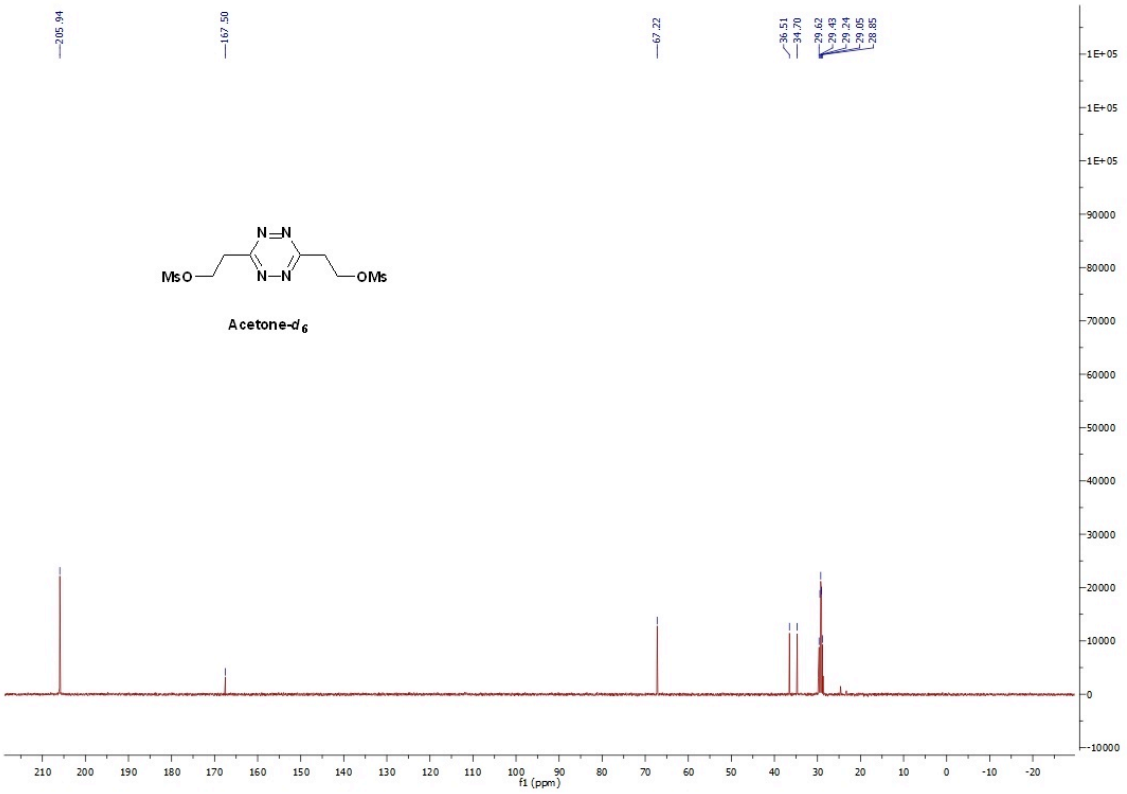
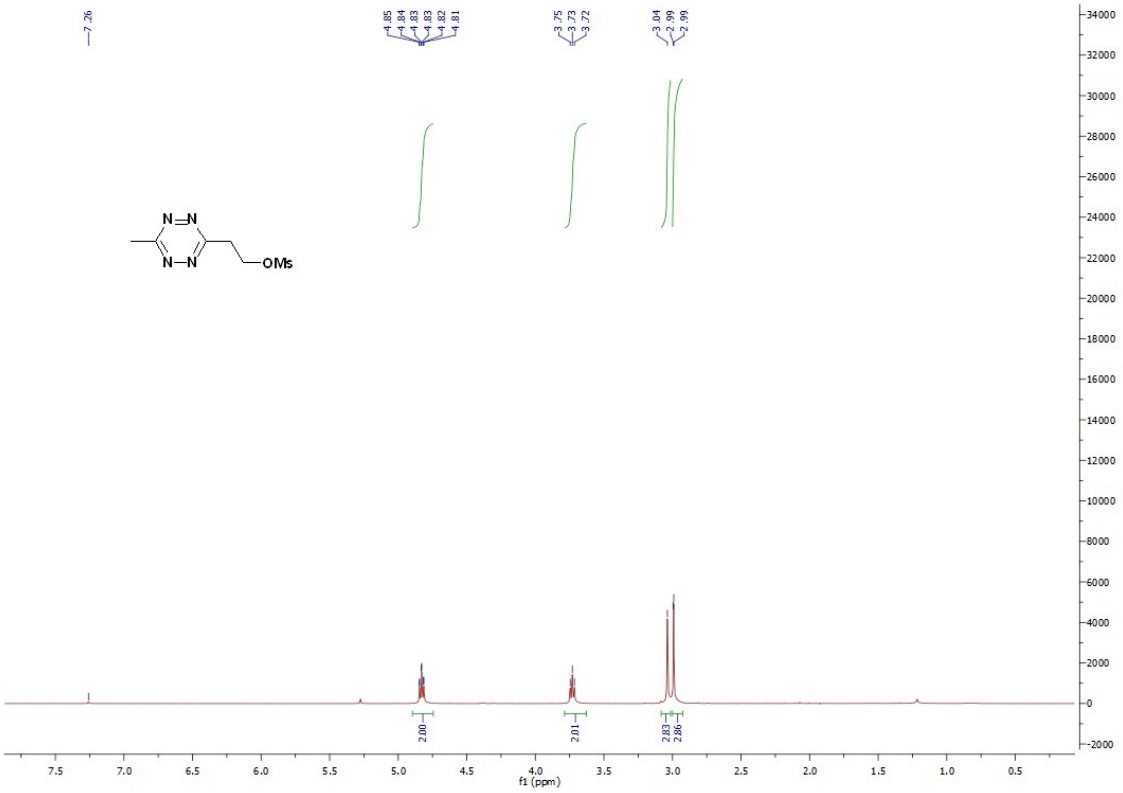
4.14
4.12
3.47
3.46
3.44
3.06
2.06
2.05
2.05
2.04
HO
N–N
OH
N=N
Acetone-d_6
2.00
1.84
f1 (ppm)


205.93
168.78
60.03
38.37
29.79
29.59
29.40
29.21
29.02
28.82
28.63
HO
N–N
OH
N=N
Acetone-d_6
f1 (ppm)


4.90
4.89
4.87
3.82
3.81
3.79
3.10
MsO
OMs
Acetone-d_6
2.00
2.01
2.93
f1 (ppm)


205.94
167.50
67.22
36.51
34.70
29.62
29.43
29.24
29.05
28.85
MsO
OMs
Acetone-d_6
f1 (ppm)


7.26
4.85
4.84
4.83
4.83
4.82
4.81
3.75
3.73
3.72
3.04
2.99
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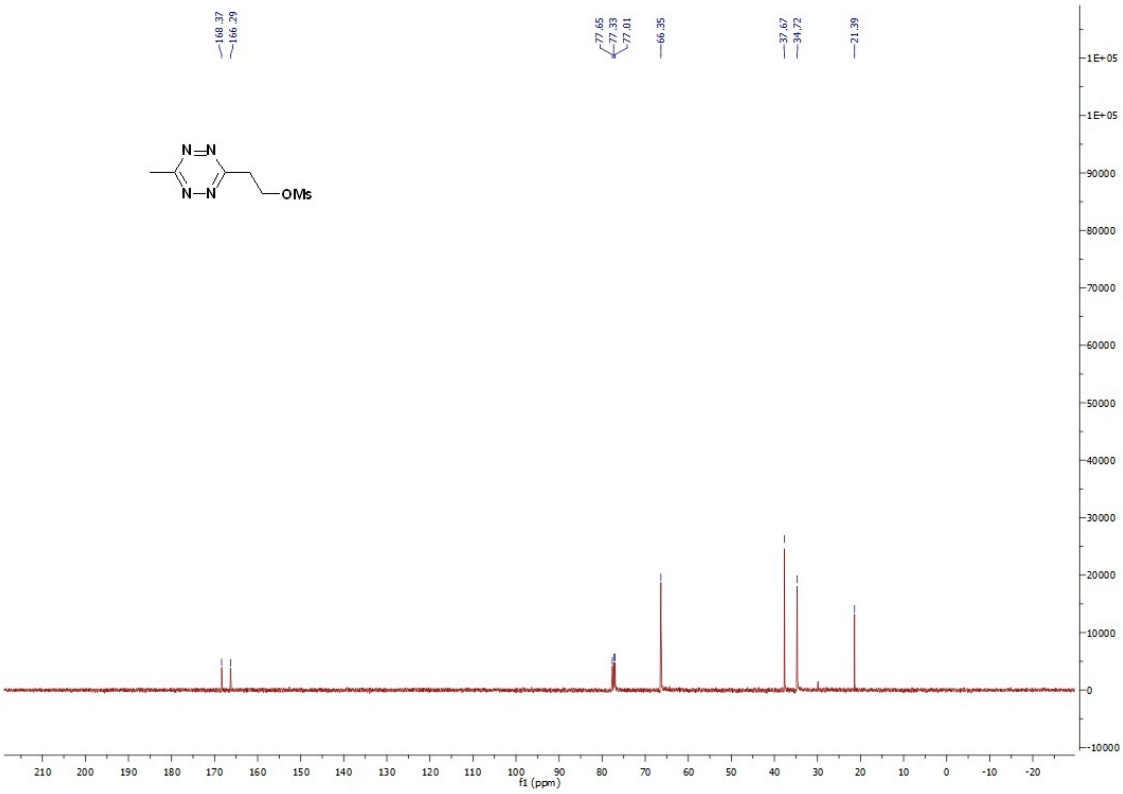
OMs
2.00
2.01
2.83
2.86
f1 (ppm)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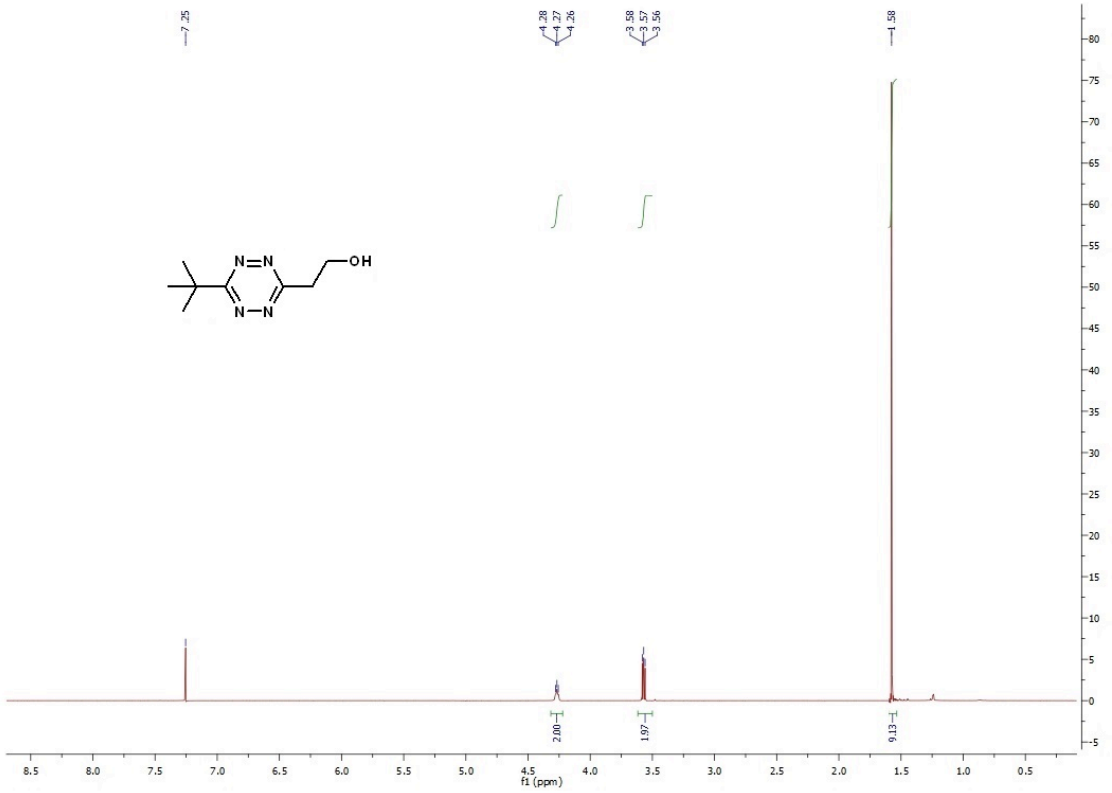
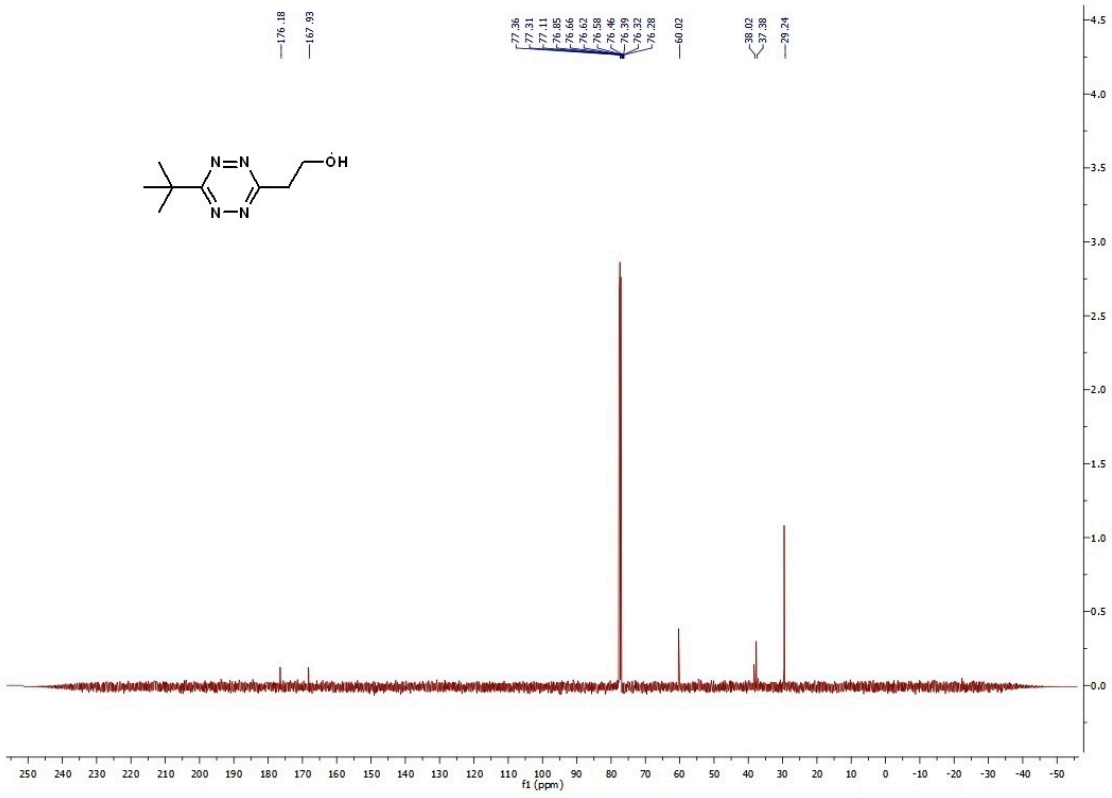
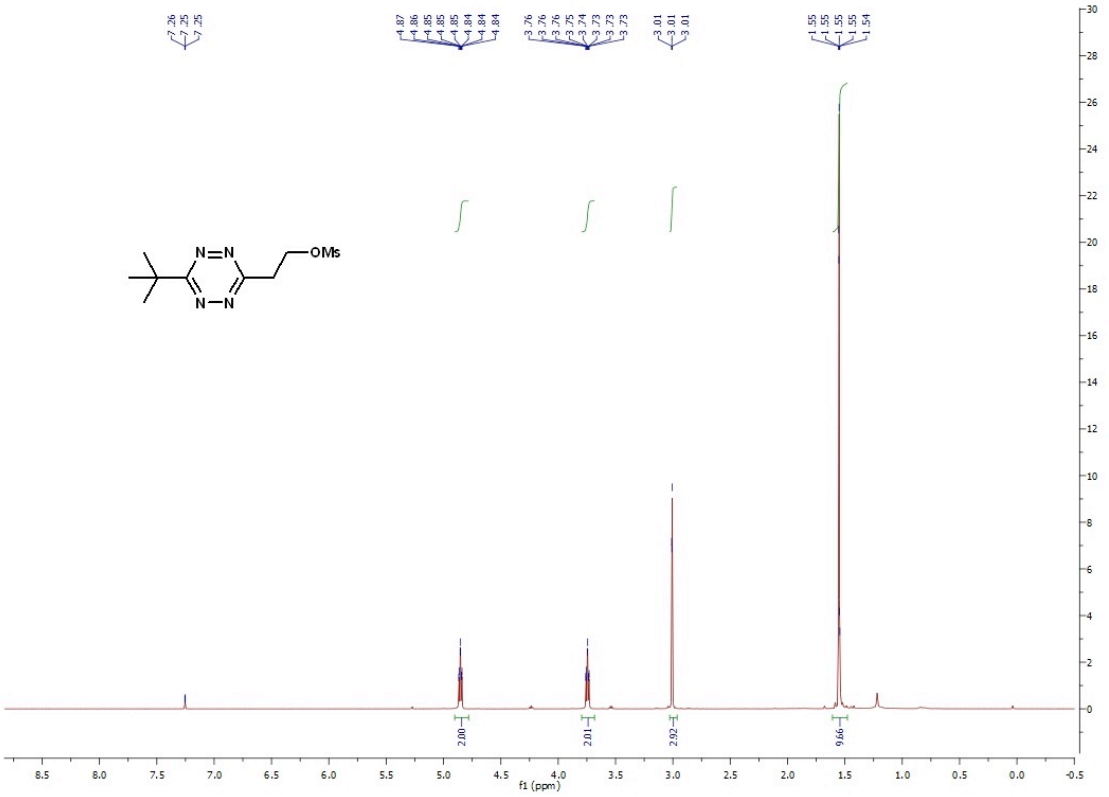
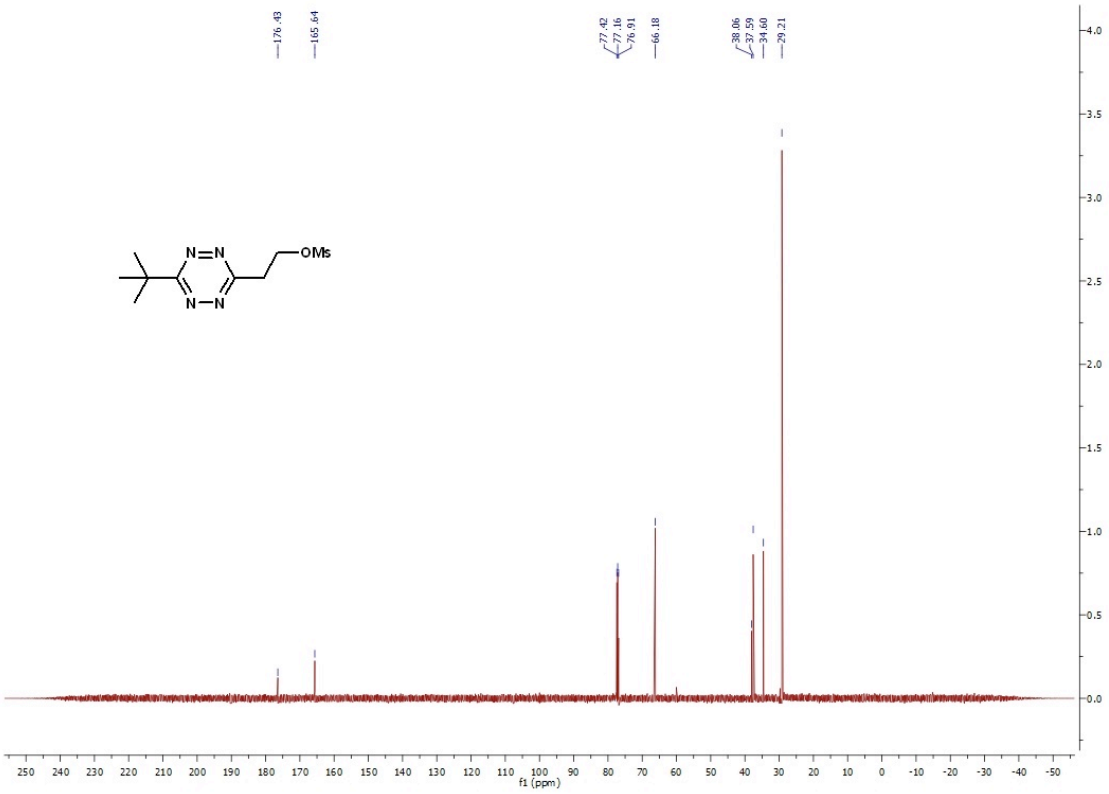
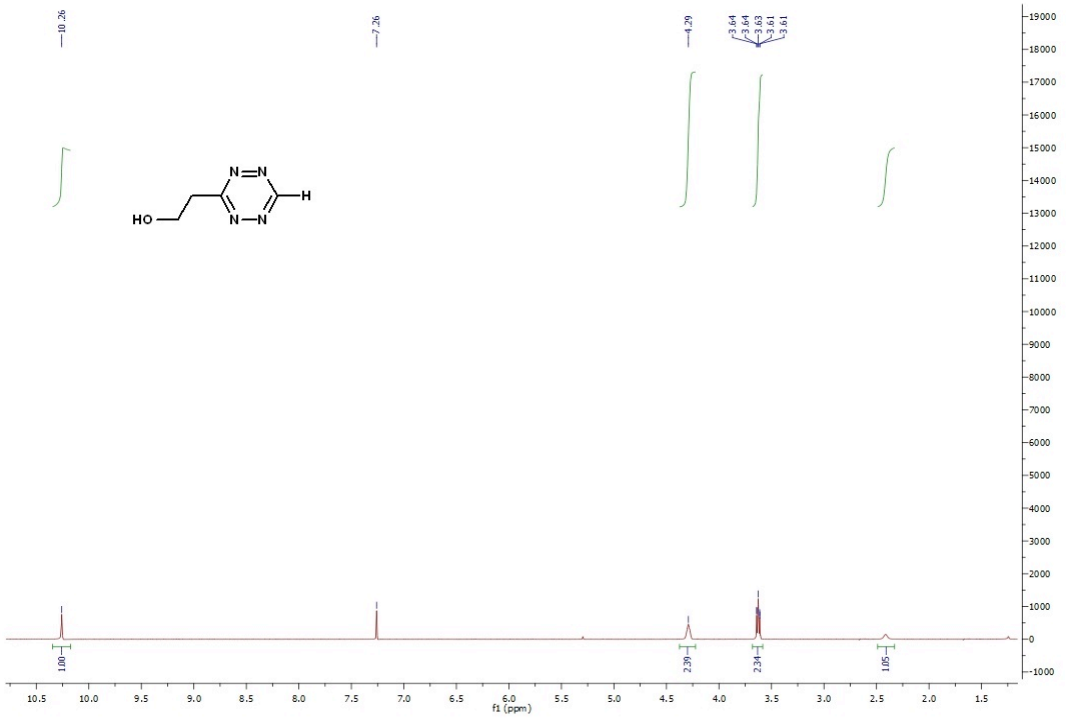
77.01
66.35
37.67
34.72
21.39
OMs
f1 (ppm)

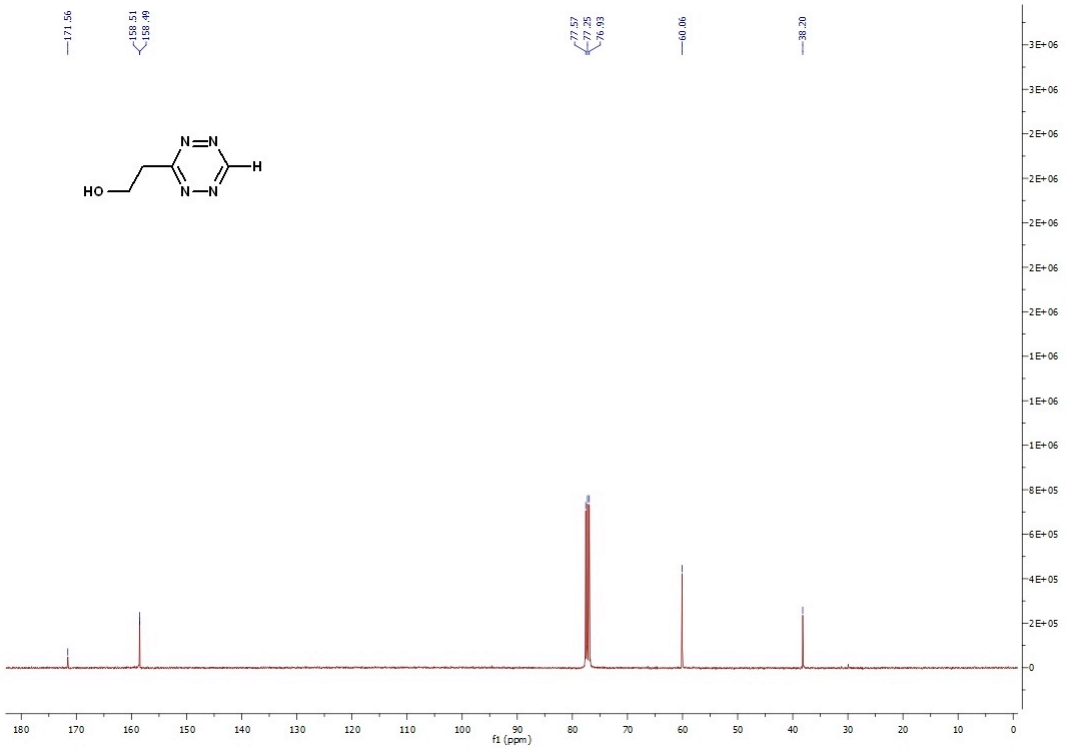

7.25
4.28
4.27
4.26
3.58
3.57
3.56
1.58
OH
2.00
1.97
9.13
f1 (ppm)

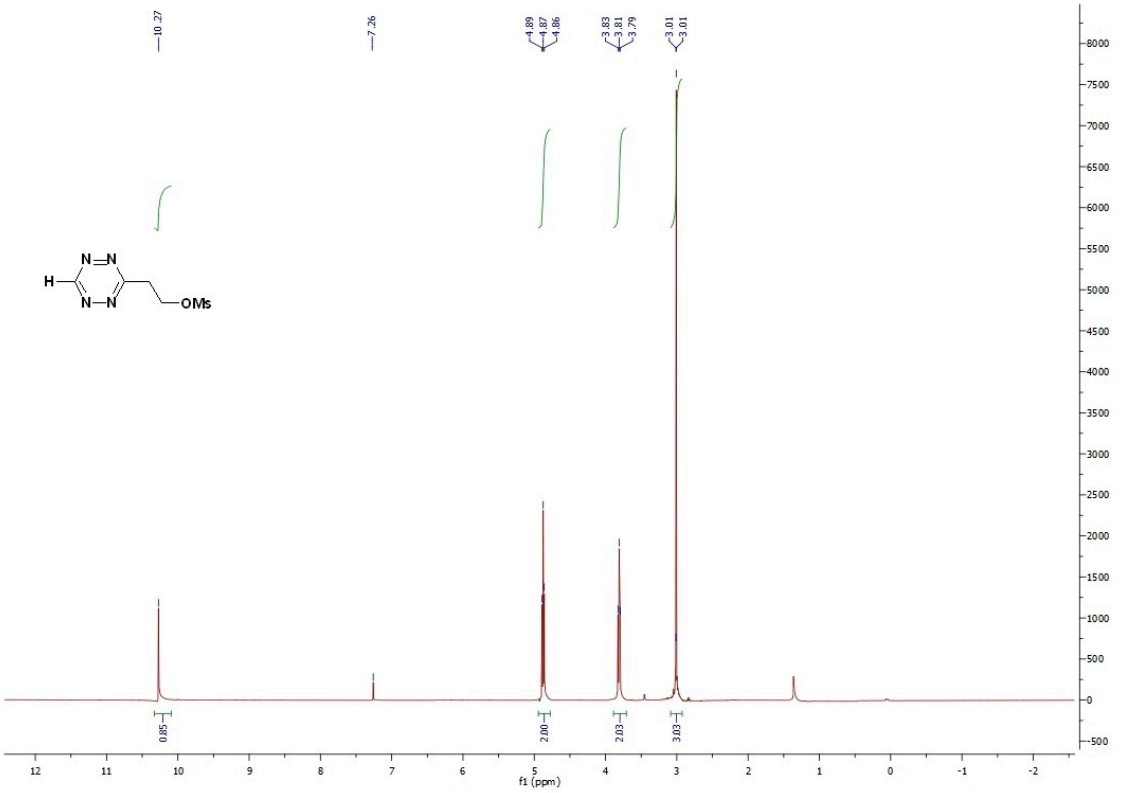

176.18
167.93
77.36
77.31
77.11
76.85
76.66
76.62
76.58
76.46
76.39
76.32
76.28
60.02
38.02
37.38
29.24
OH
f1 (ppm)

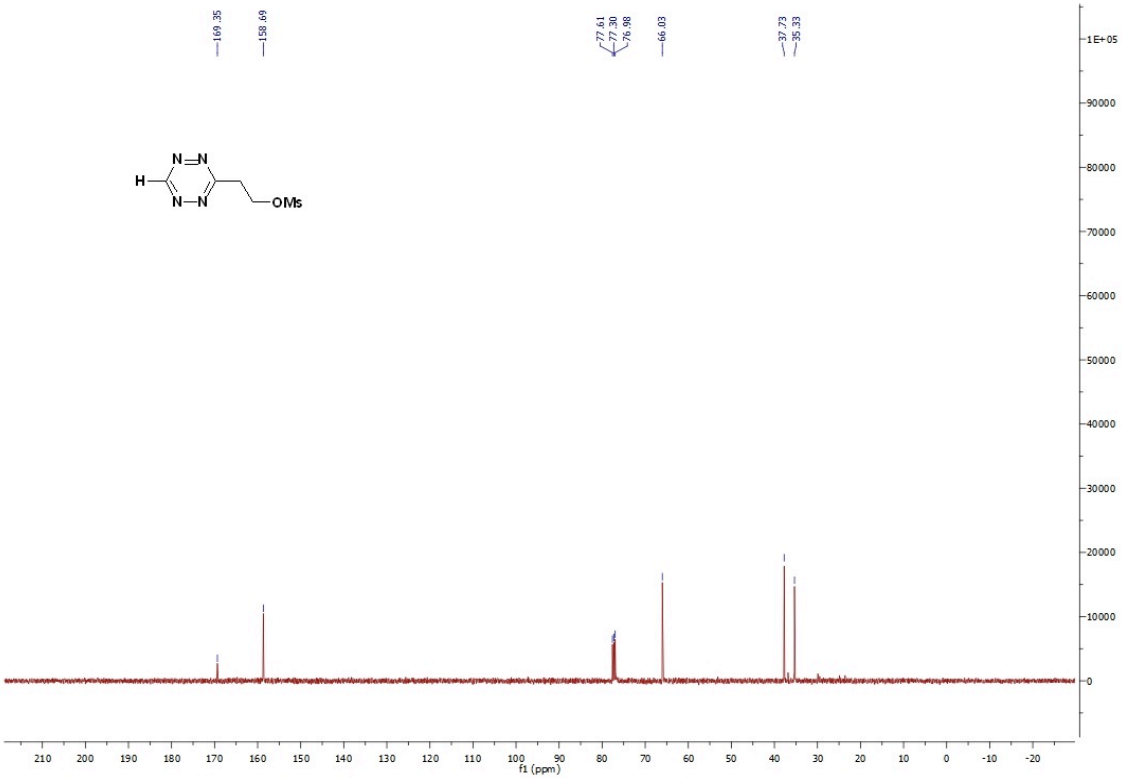

7.26
7.25
7.25
4.87
4.86
4.85
4.85
4.84
4.84
4.84
3.76
3.76
3.76
3.75
3.74
3.73
3.73
3.73
3.01
3.01
3.01
1.55
1.55
1.55
1.55
1.54
OMs
2.00
2.01
2.92
9.66
f1 (ppm)

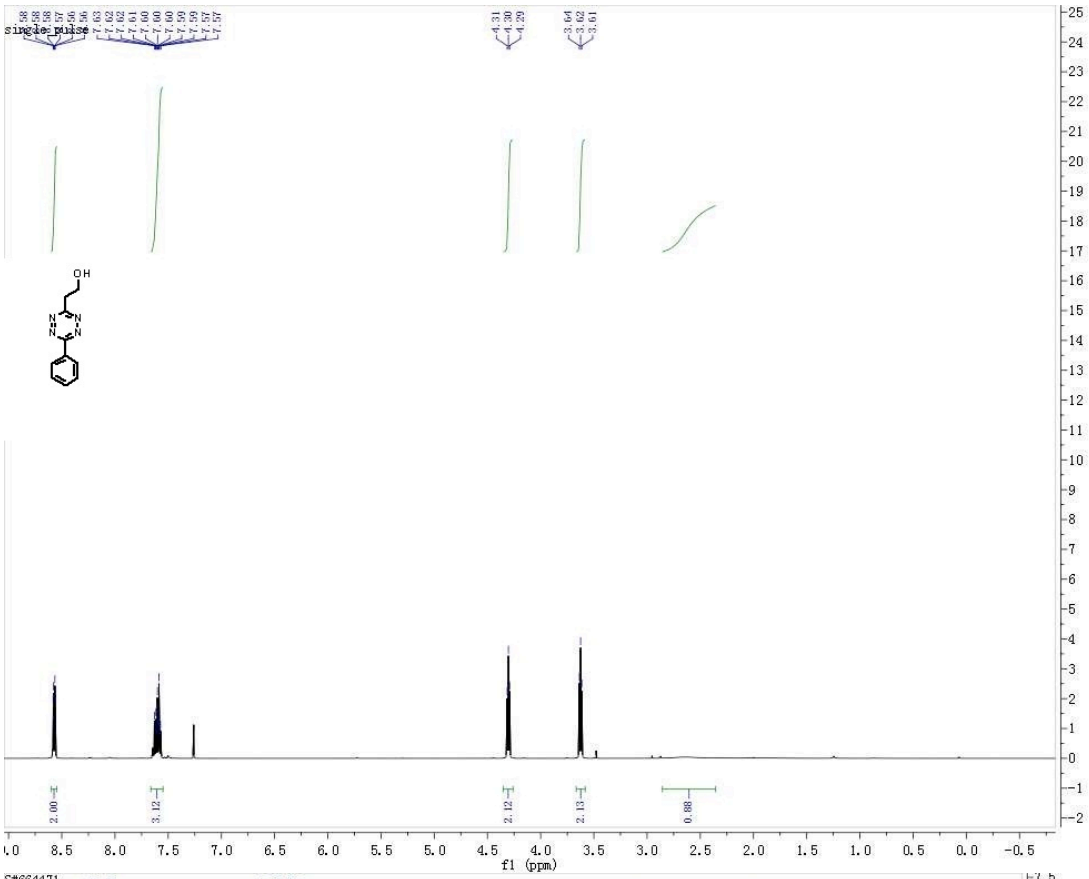

176.43
165.64
77.42
77.16
76.91
66.18
38.06
37.59
34.60
29.21
OMs
f1 (ppm)

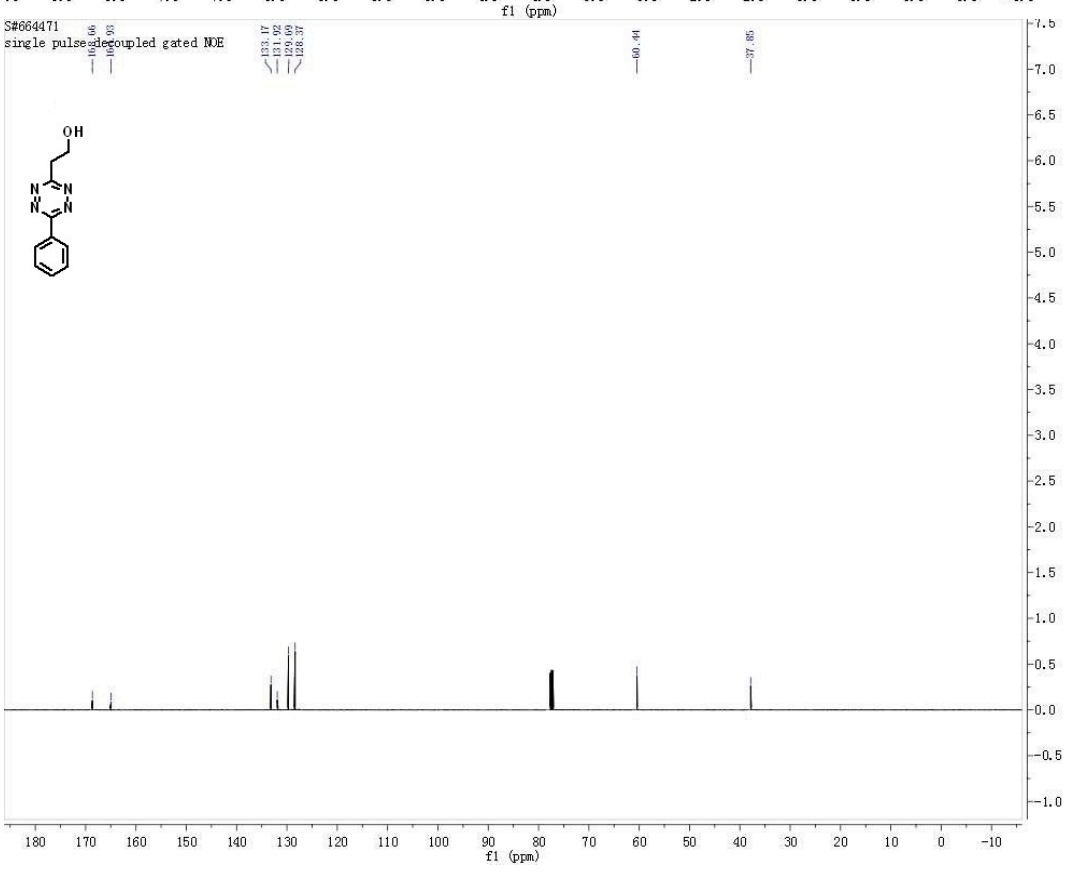

10.26
7.26
4.29
3.64
3.64
3.63
3.61
3.61
HO
N
N
N
N
H
1.00
2.39
2.34
1.05
f1 (ppm)

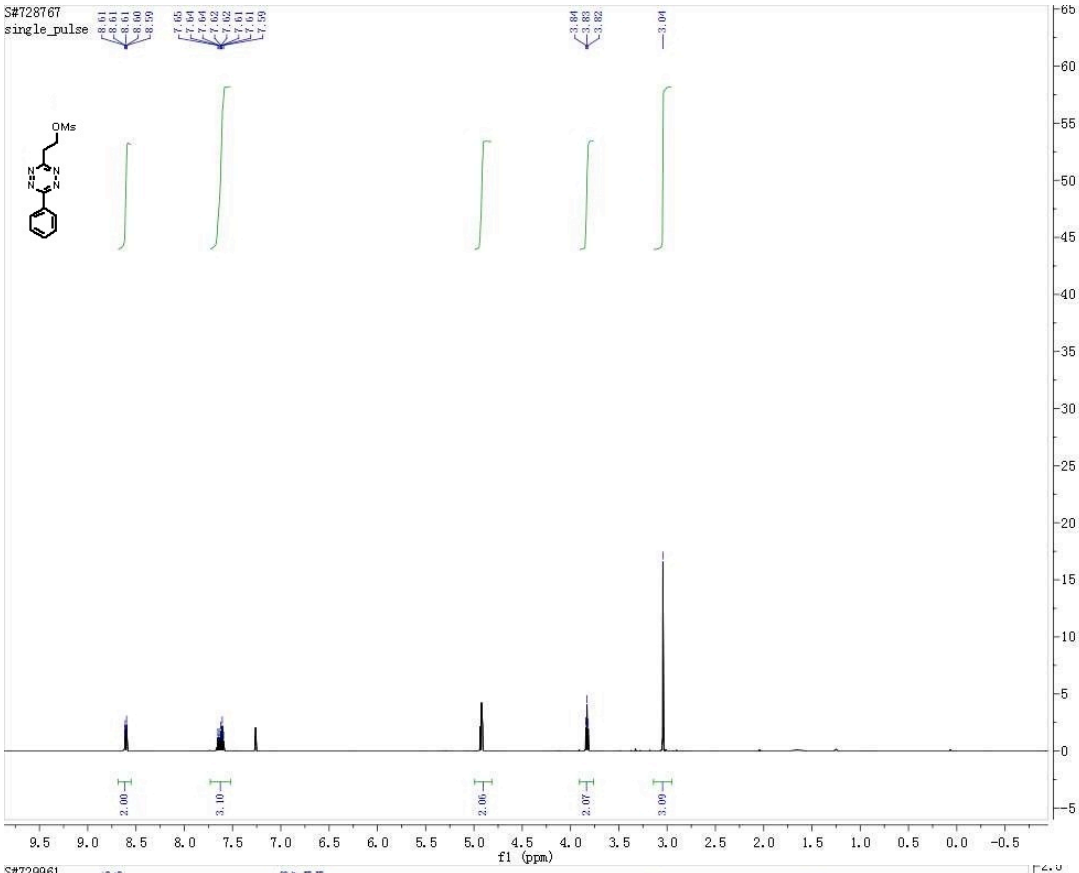

171.56
158.51
158.49
77.57
77.25
76.93
60.06
38.20
HO
N
N
N
N
H
f1 (ppm)

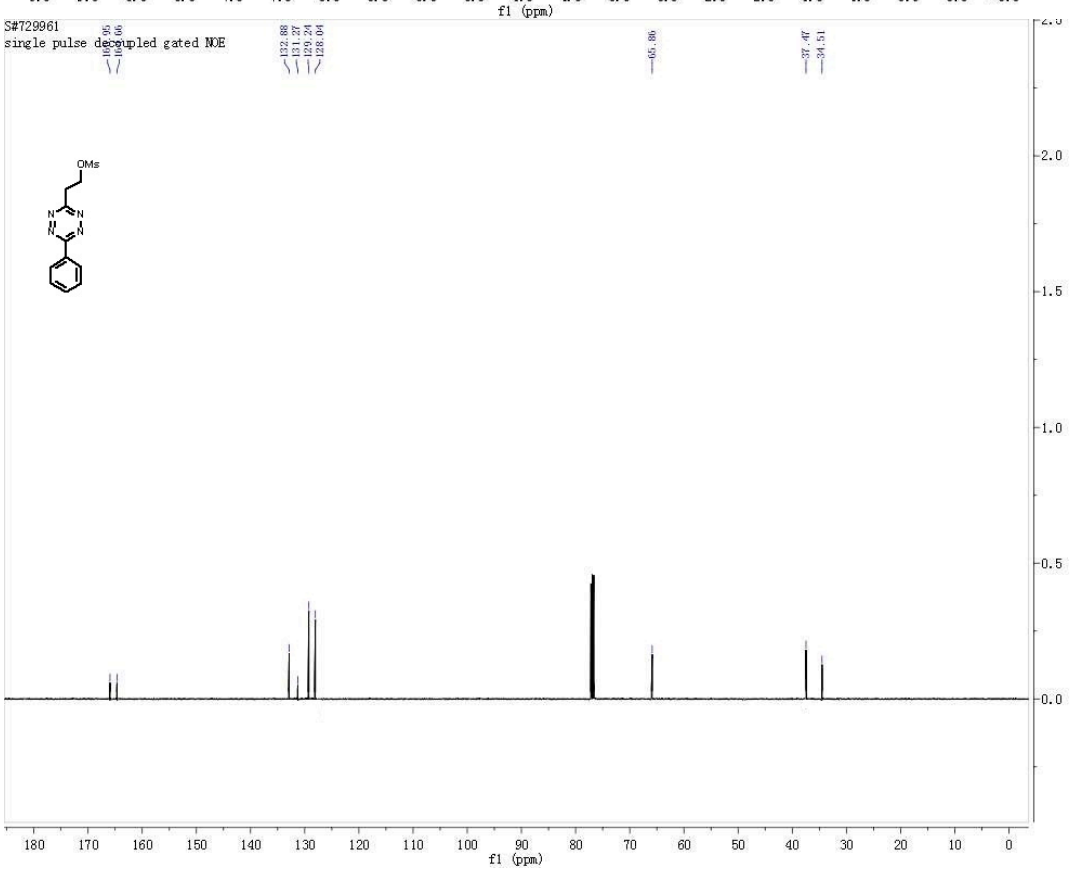

10.27
7.26
4.89
4.87
4.86
3.83
3.81
3.79
3.01
3.01
OMs
0.85
2.00
2.03
3.03
f1 (ppm)

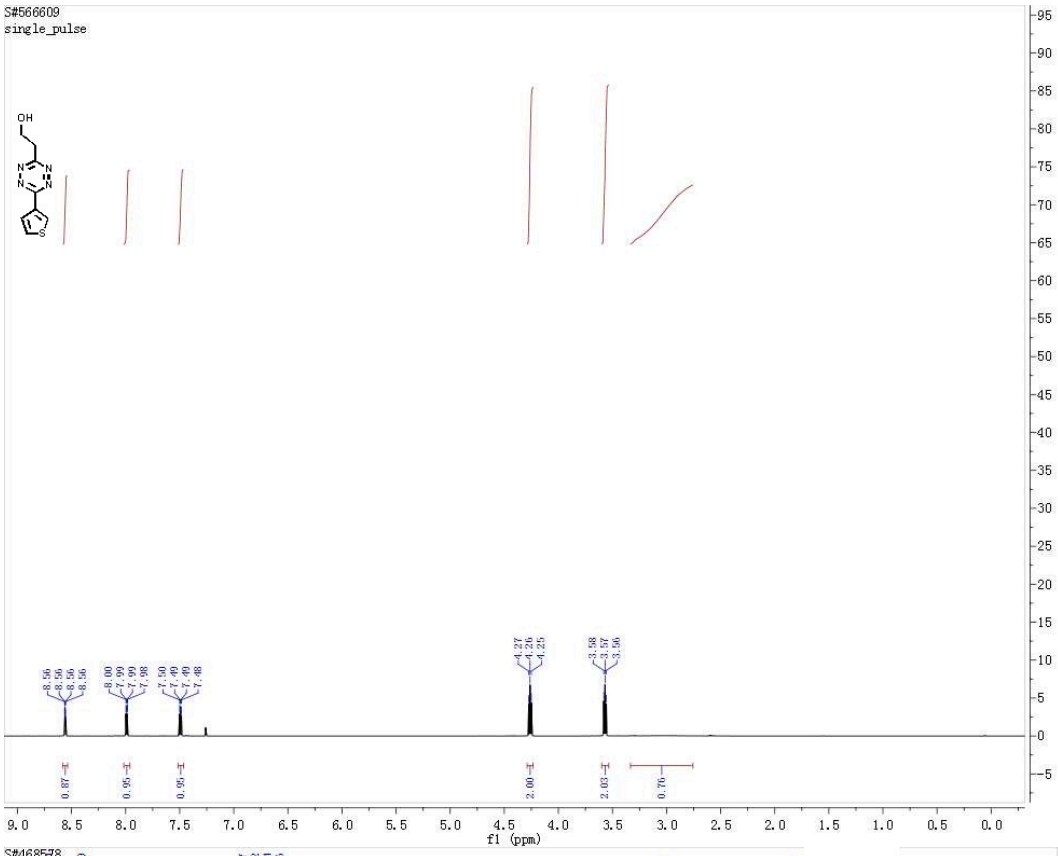

169.35
158.69
77.61
77.30
76.98
66.03
37.73
35.33
OMs
f1 (ppm)

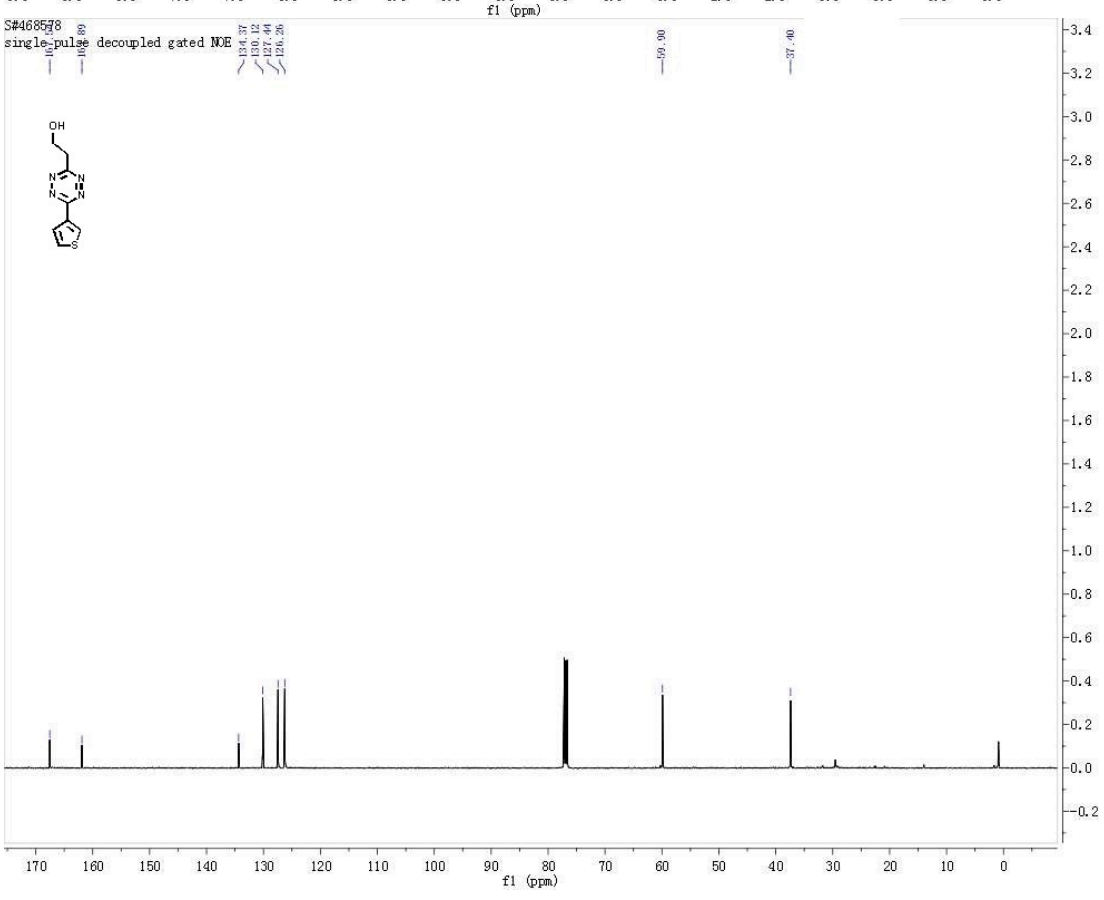

single pulse
f1 (ppm)

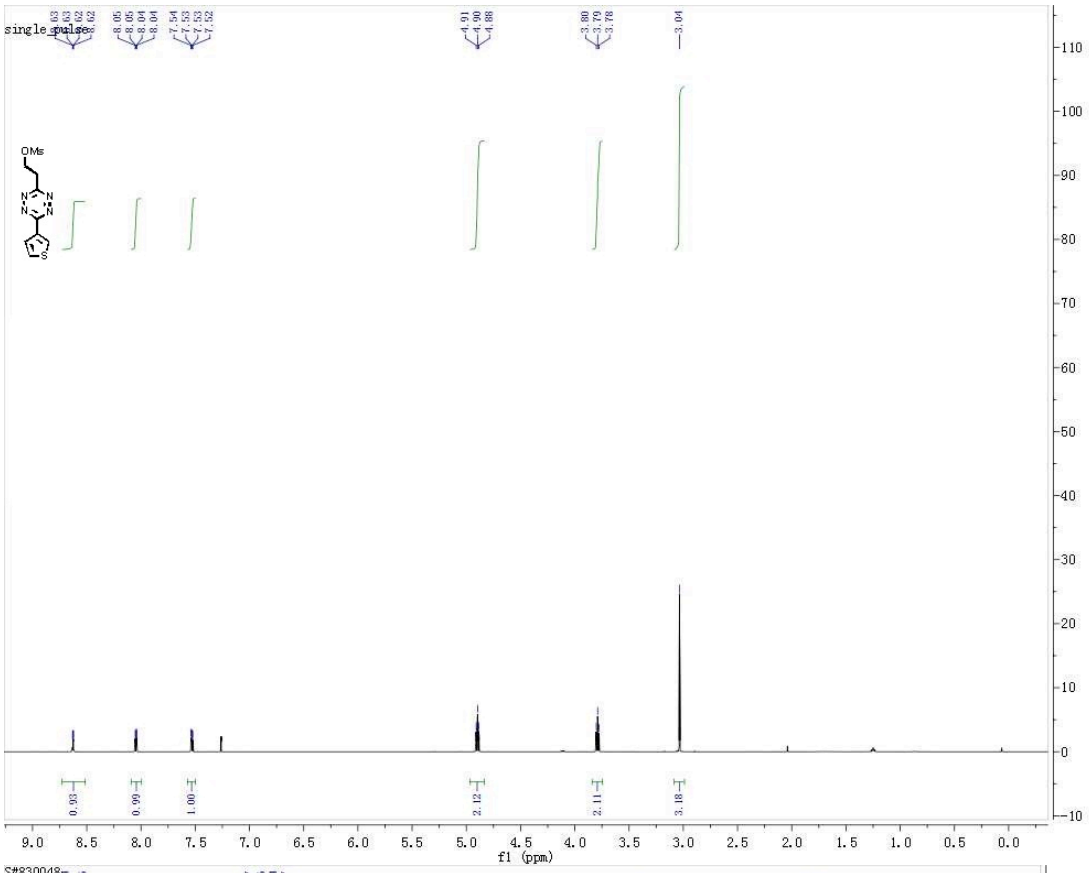

S#664471
single pulse decoupled gated NOE
f1 (ppm)

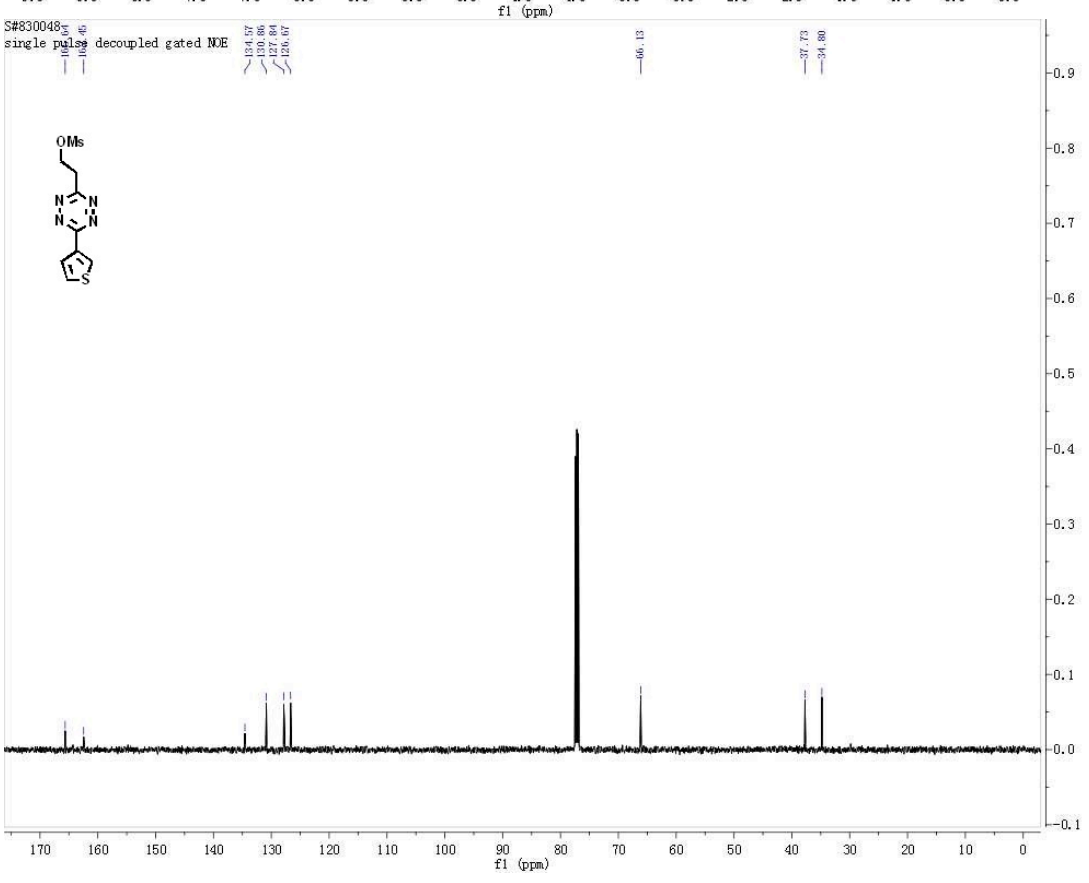

S#728767
single_pulse
OMs
f1 (ppm)

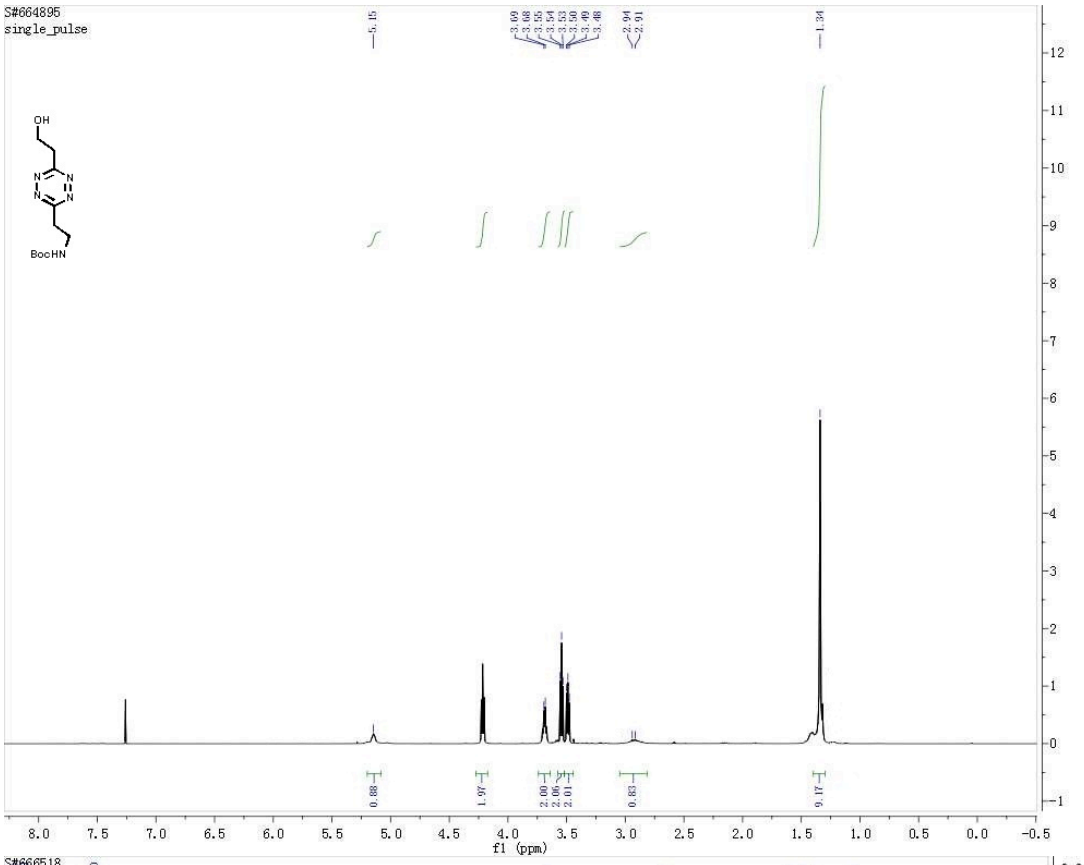

S#729961
single pulse decoupled gated NOE
OMs
f1 (ppm)

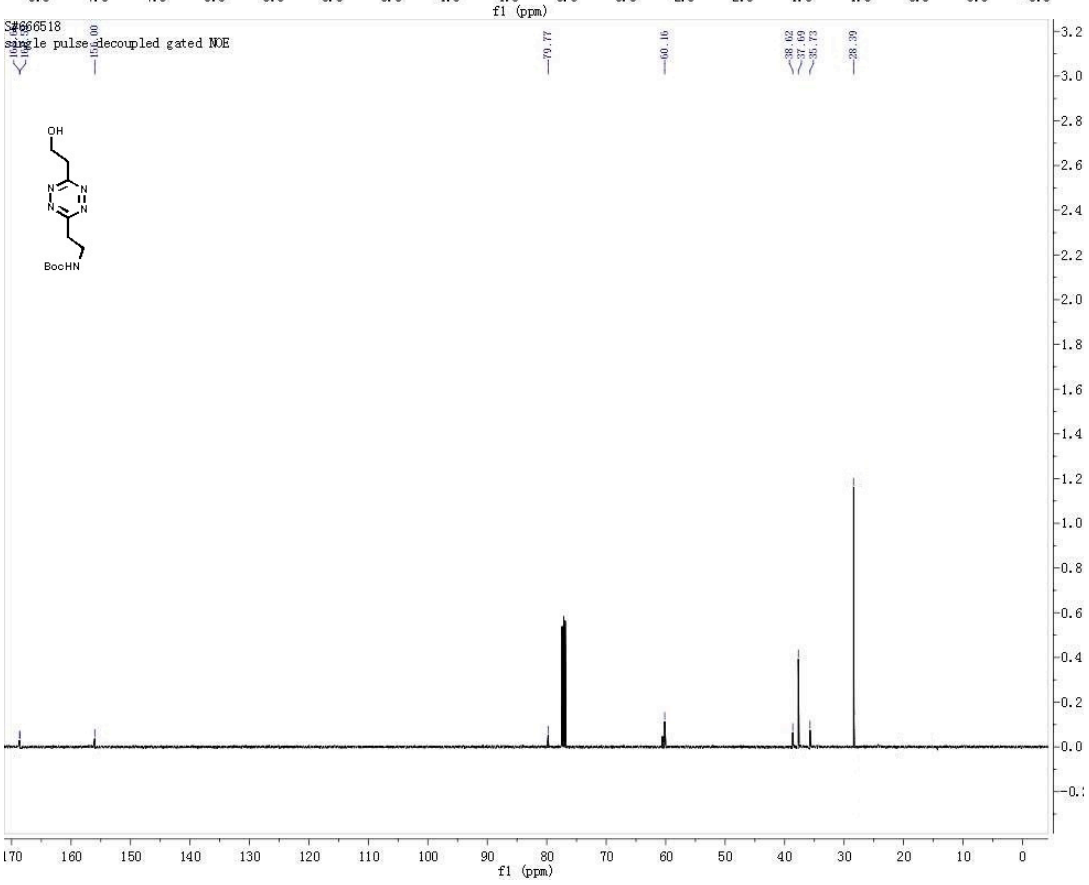

S#566609
single_pulse
OH
8.56
8.56
8.56
8.56
8.00
7.99
7.99
7.98
7.50
7.49
7.49
7.48
4.27
4.26
4.25
3.58
3.57
3.56
0.87
0.95
0.95
2.00
2.03
0.76
f1 (ppm)

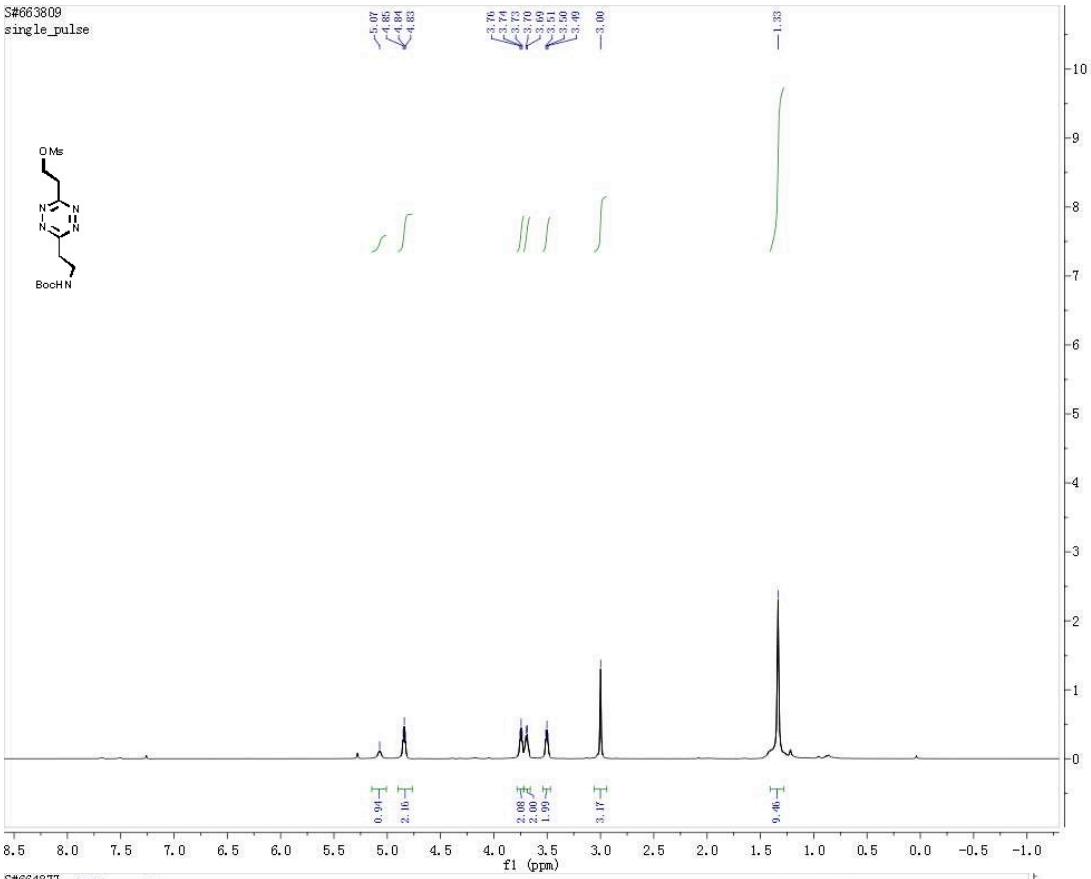

S#468578
single_pulse decoupled gated NOE
161.89
161.69
134.37
130.12
127.44
126.26
59.90
37.40
OH
f1 (ppm)

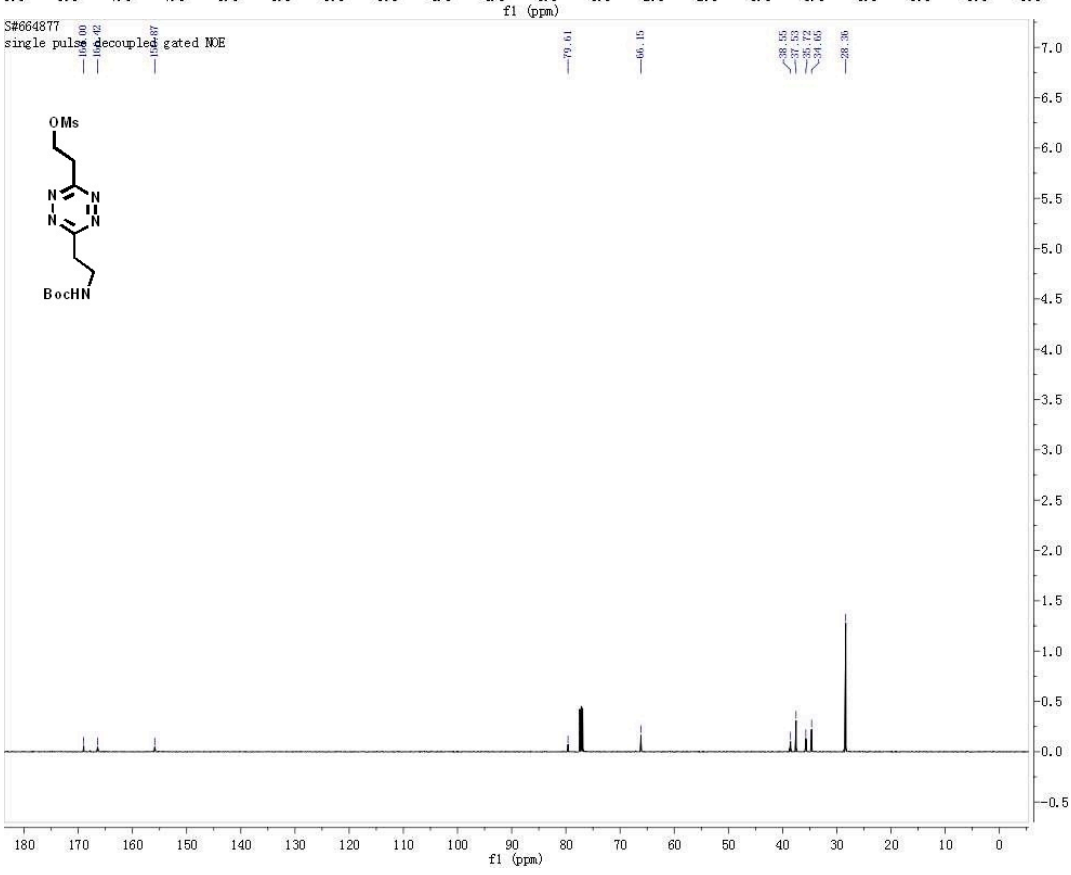

single pulse
OMs
f1 (ppm)

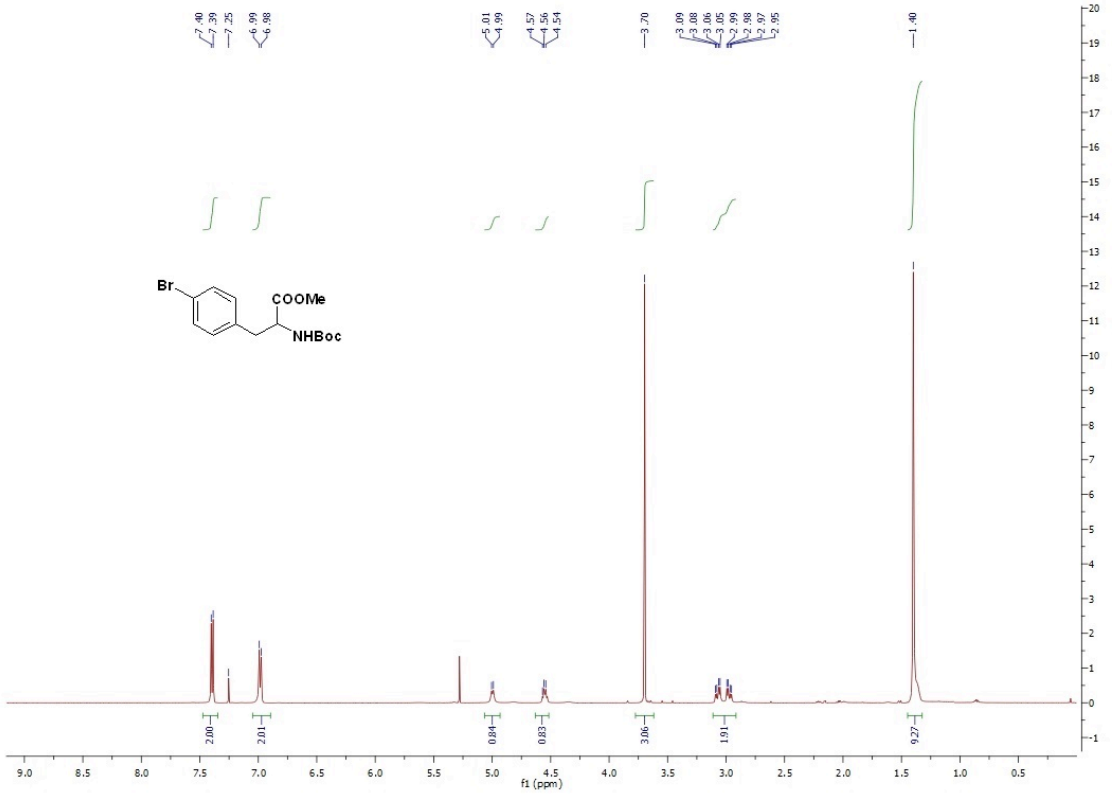

S#830048
single pulse decoupled gated NOE
OMs
f1 (ppm)

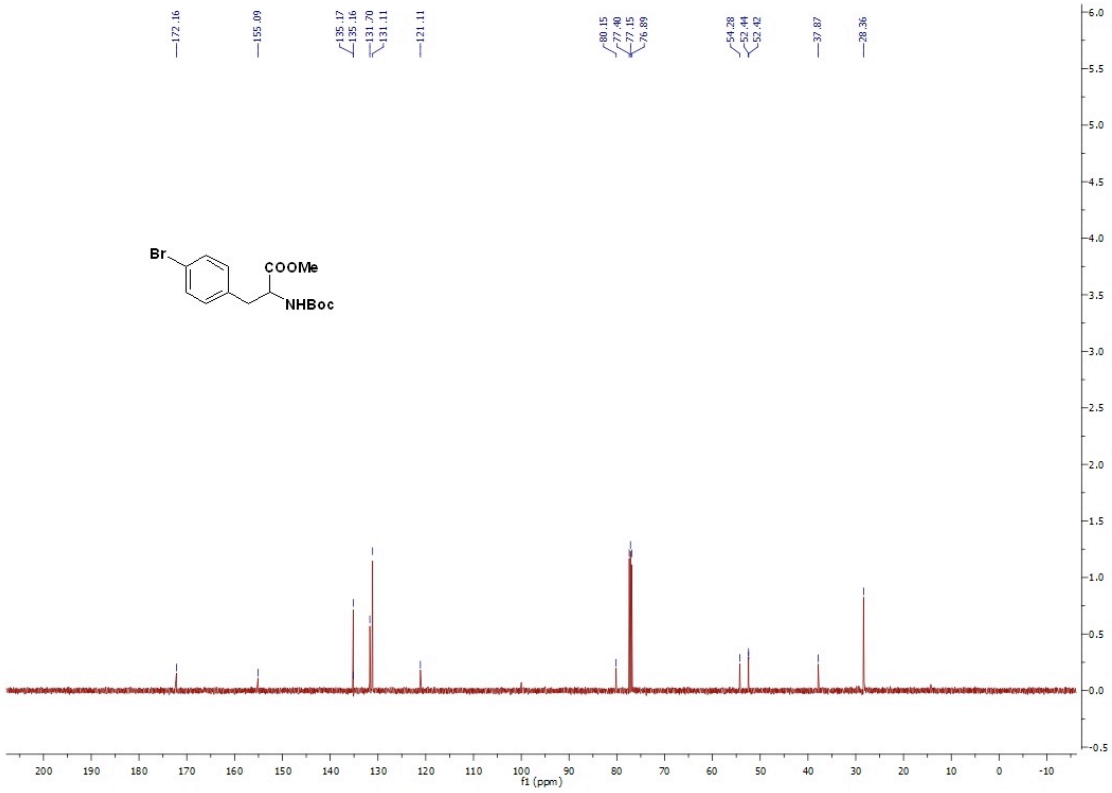

S#664895
single_pulse
OH
BocHN
5.15
3.69
3.68
3.55
3.54
3.53
3.50
3.49
3.48
2.94
2.91
1.34
0.88
1.97
2.00
2.06
2.01
0.83
9.17
f1 (ppm)

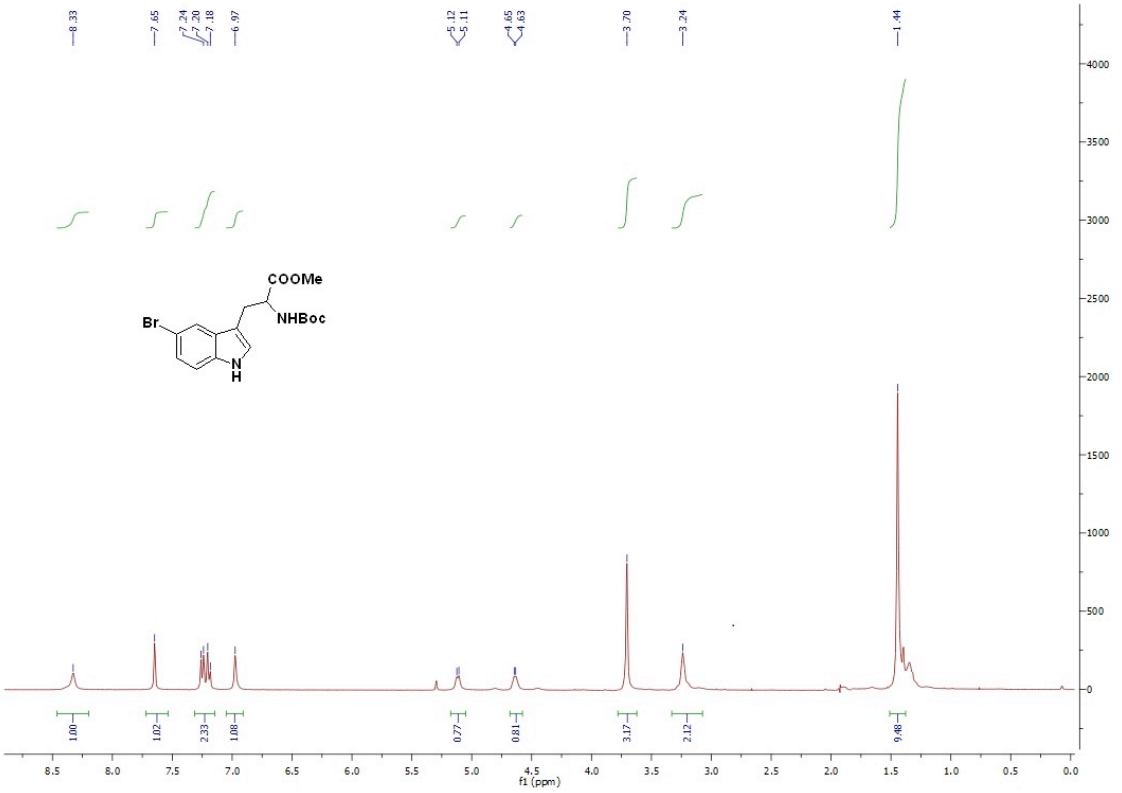

S#666518
single pulse decoupled gated NOE
OH
BocHN
10.00
79.77
60.16
38.62
37.09
35.73
28.39
f1 (ppm)

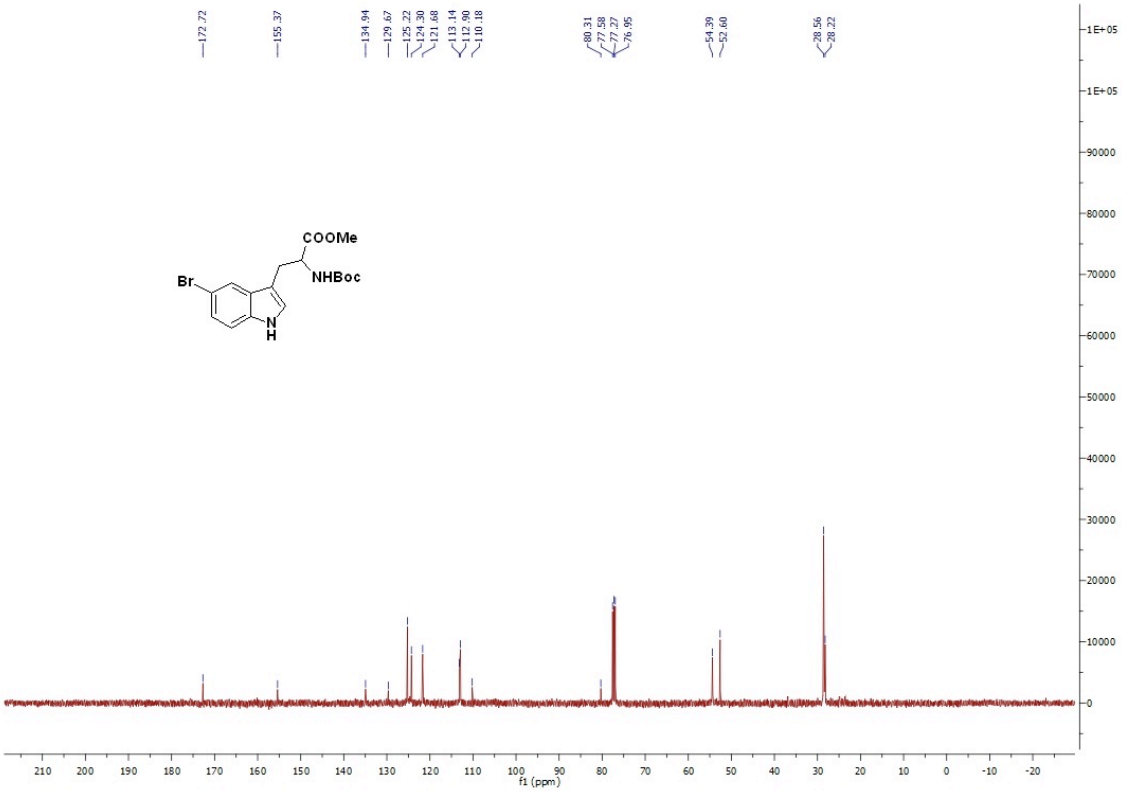

S#663809
single_pulse
OMs
BocHN
f1 (ppm)

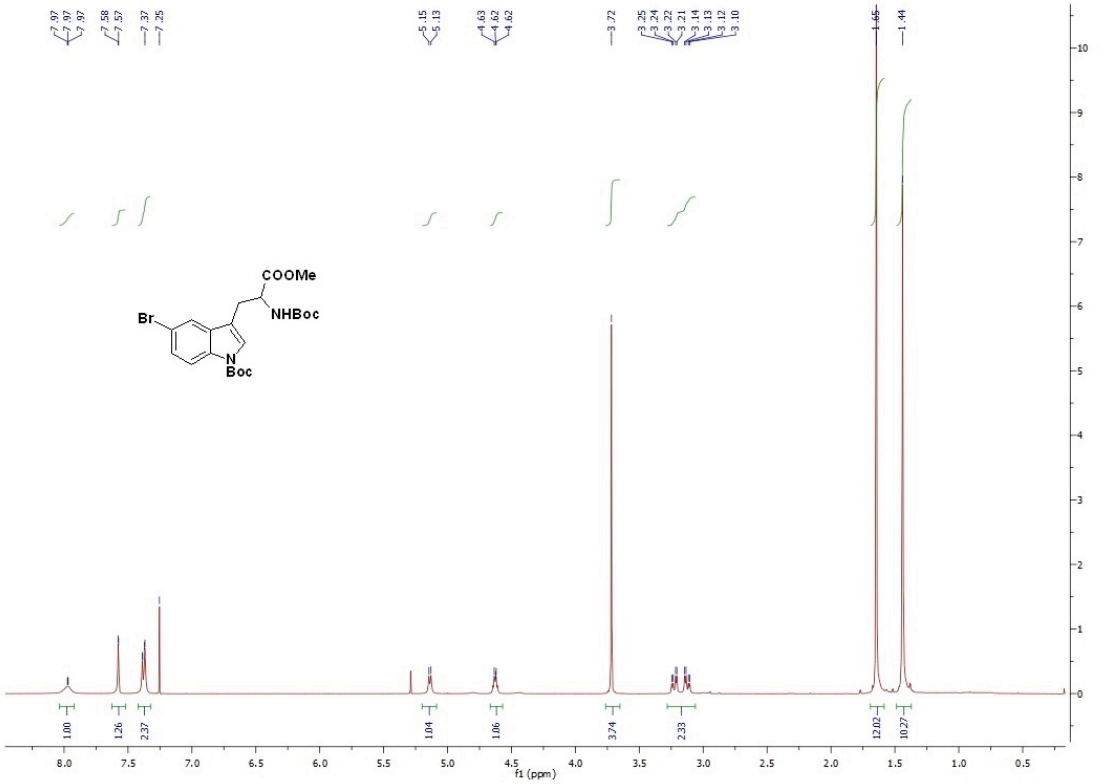

S#664877
single pulse decoupled gated NOE
OMs
BocHN
f1 (ppm)

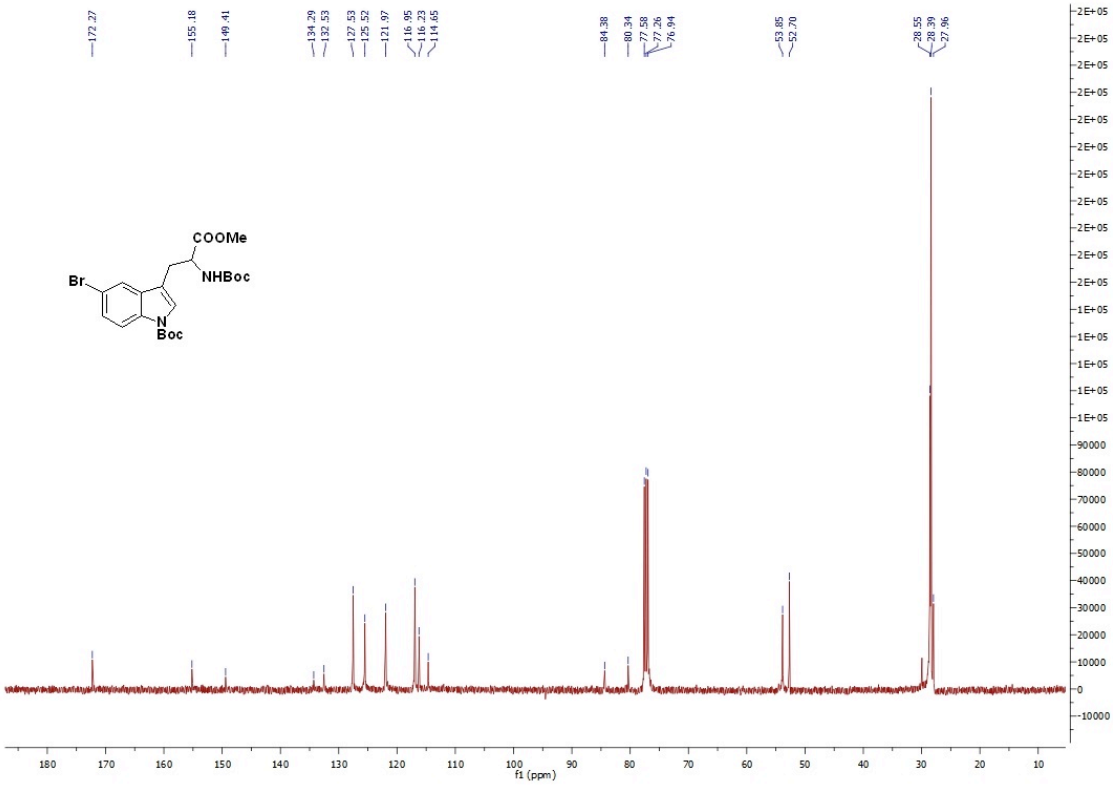

7.40
7.39
7.25
6.99
6.98
5.01
4.99
4.57
4.56
4.54
3.70
3.09
3.08
3.06
3.05
2.99
2.98
2.97
2.95
1.40
Br
COOMe
NHBoc
2.00
2.01
0.84
0.83
3.06
1.91
9.27
f1 (ppm)

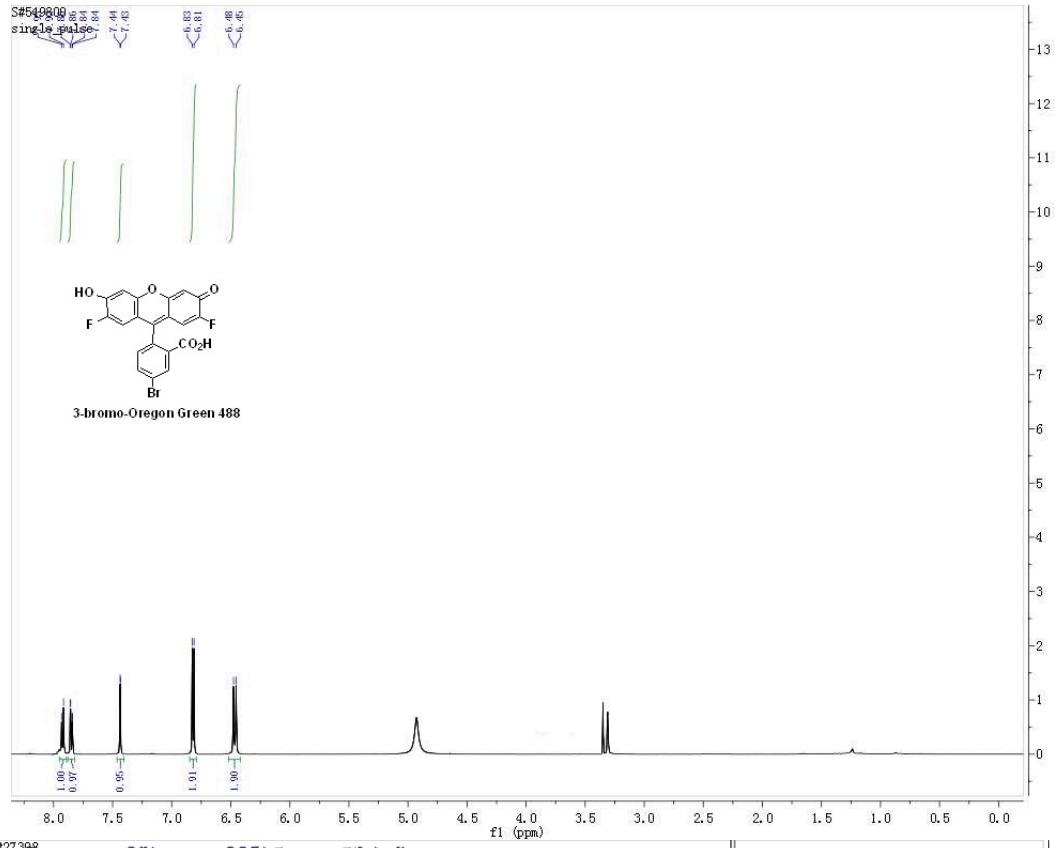

172.16
155.09
135.17
135.16
131.70
131.11
121.11
80.15
77.40
77.15
76.89
54.28
52.44
52.42
37.87
28.36
Br
COOMe
NHBoc
f1 (ppm)

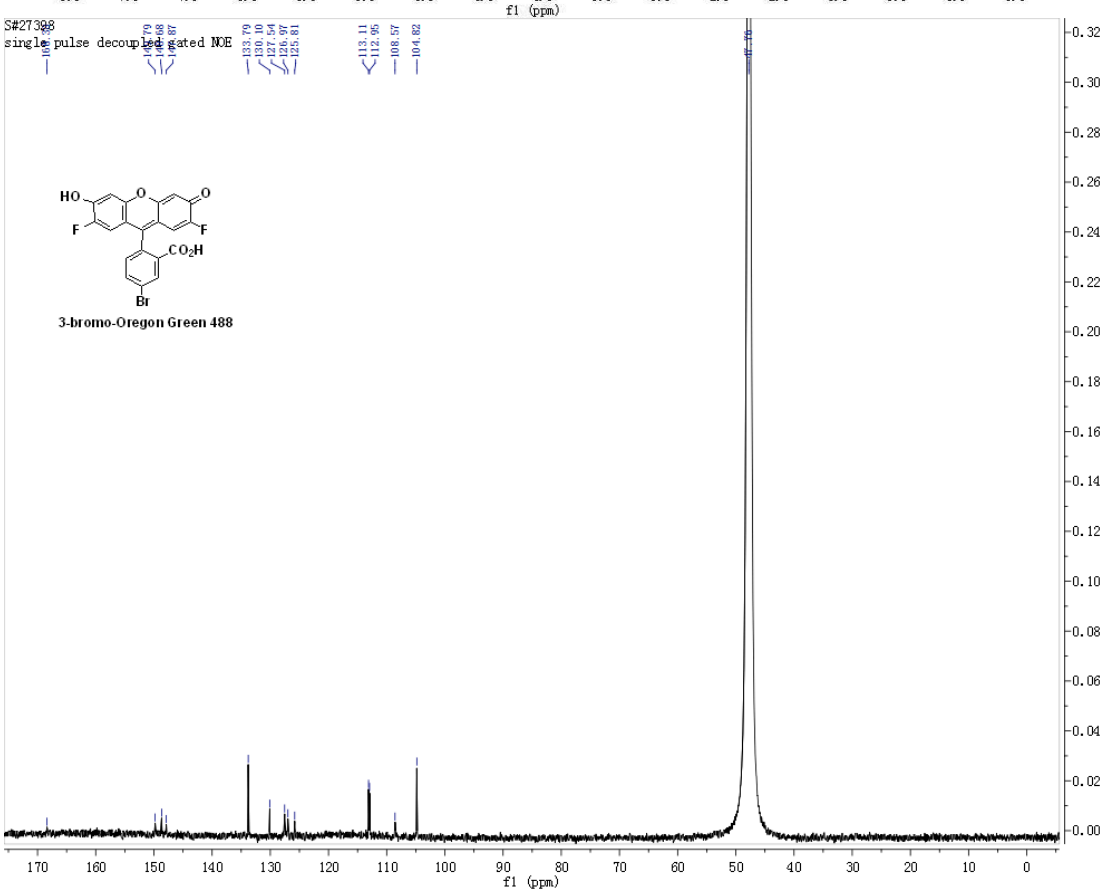

8.33
7.65
7.24
7.20
7.18
6.97
5.12
5.11
4.65
4.63
3.70
3.24
1.44
COOMe
NHBoc
Br
N
H
1.00
1.02
2.33
1.08
0.77
0.81
3.17
2.12
9.48
f1 (ppm)

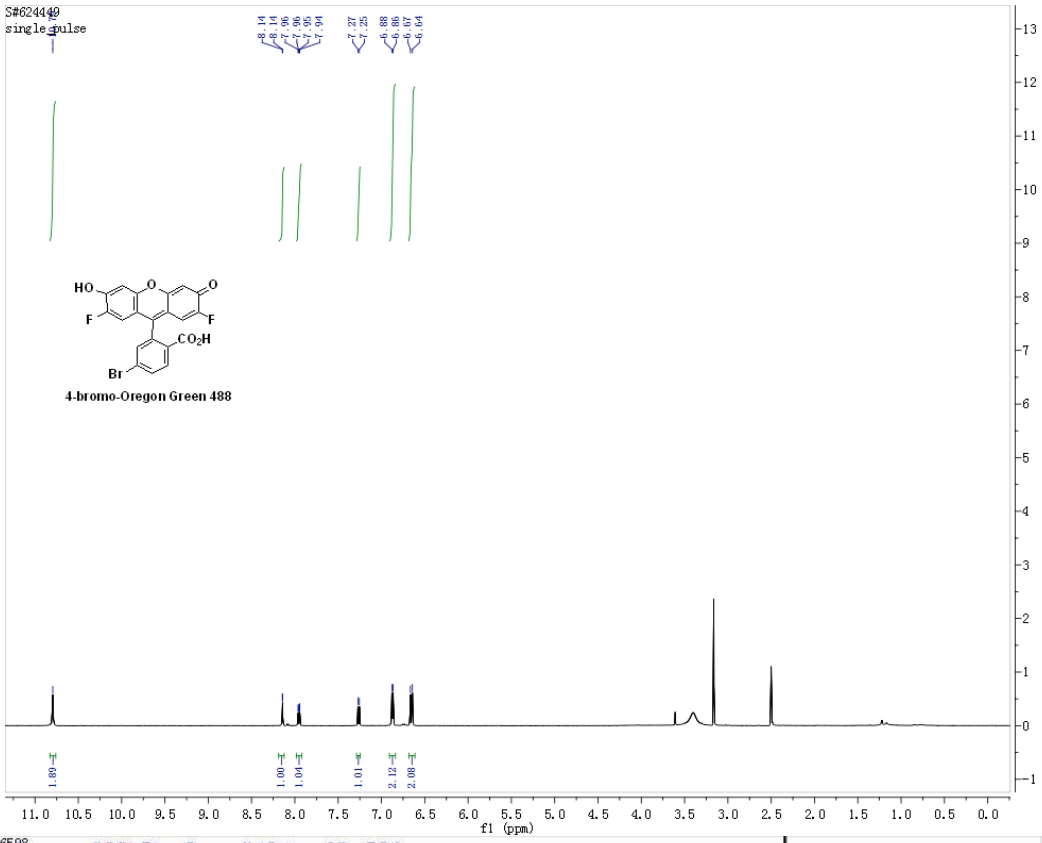

172.72
155.37
134.94
129.67
125.22
124.30
121.68
113.14
112.90
110.18
80.31
77.58
77.27
76.95
54.39
52.60
28.56
28.22
COOMe
NHBoc
Br
N
H
f1 (ppm)

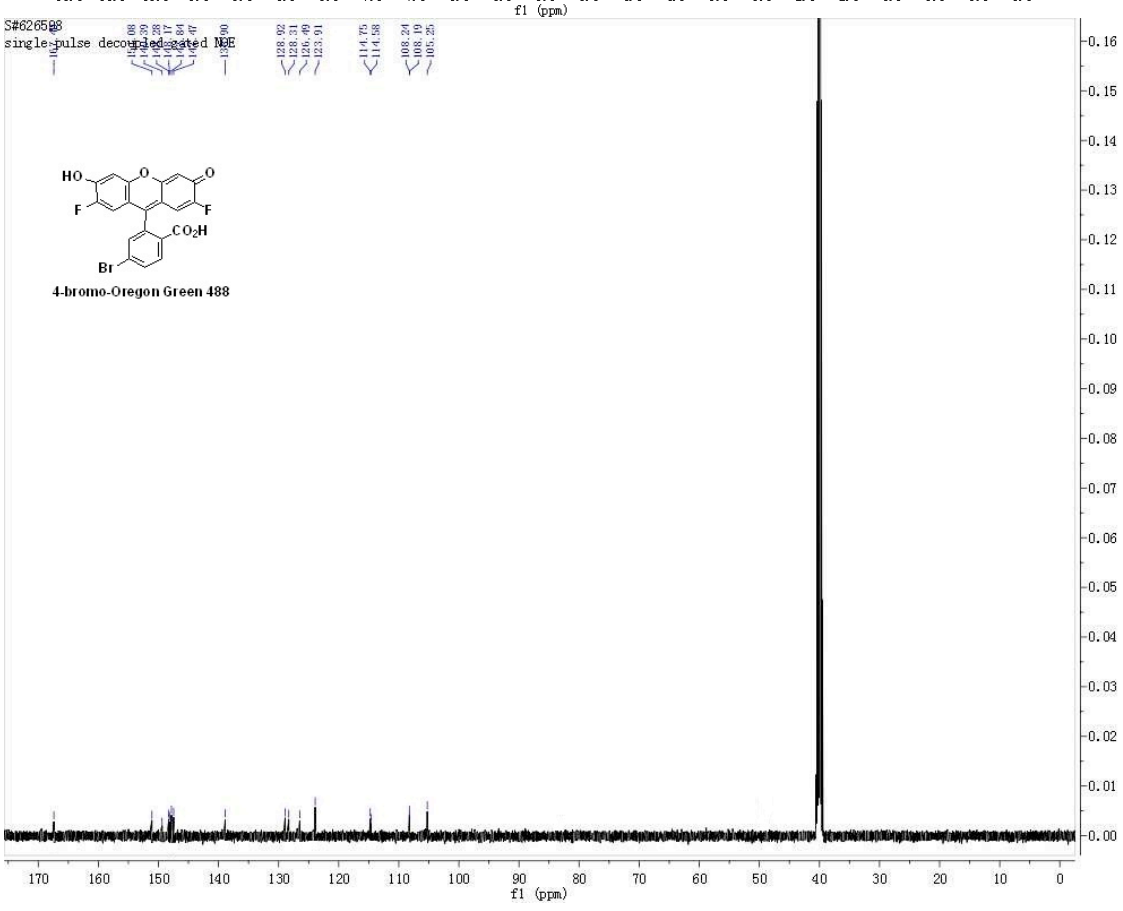

7.97
7.97
7.97
7.58
7.57
7.37
7.25
5.15
5.13
4.63
4.62
4.62
3.72
3.25
3.24
3.22
3.21
3.14
3.13
3.12
3.10
1.66
1.44
COOMe
NHBoc
Br
N
Boc
1.00
1.26
2.37
1.04
1.06
3.74
2.33
12.02
10.27
f1 (ppm)

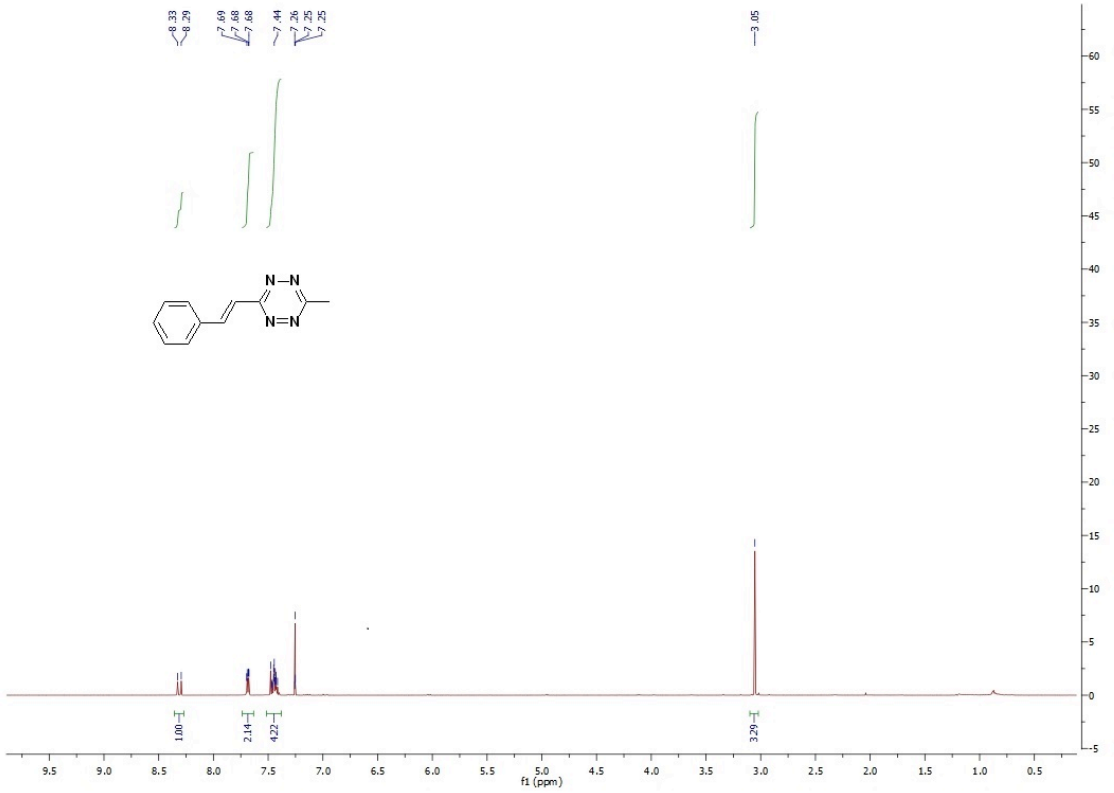

172.27
155.18
149.41
134.29
132.53
127.53
125.52
121.97
116.95
116.23
114.65
84.38
80.34
77.58
77.26
76.94
53.85
52.70
28.55
28.39
27.96
COOMe
NHBoc
Br
N
Boc
f1 (ppm)

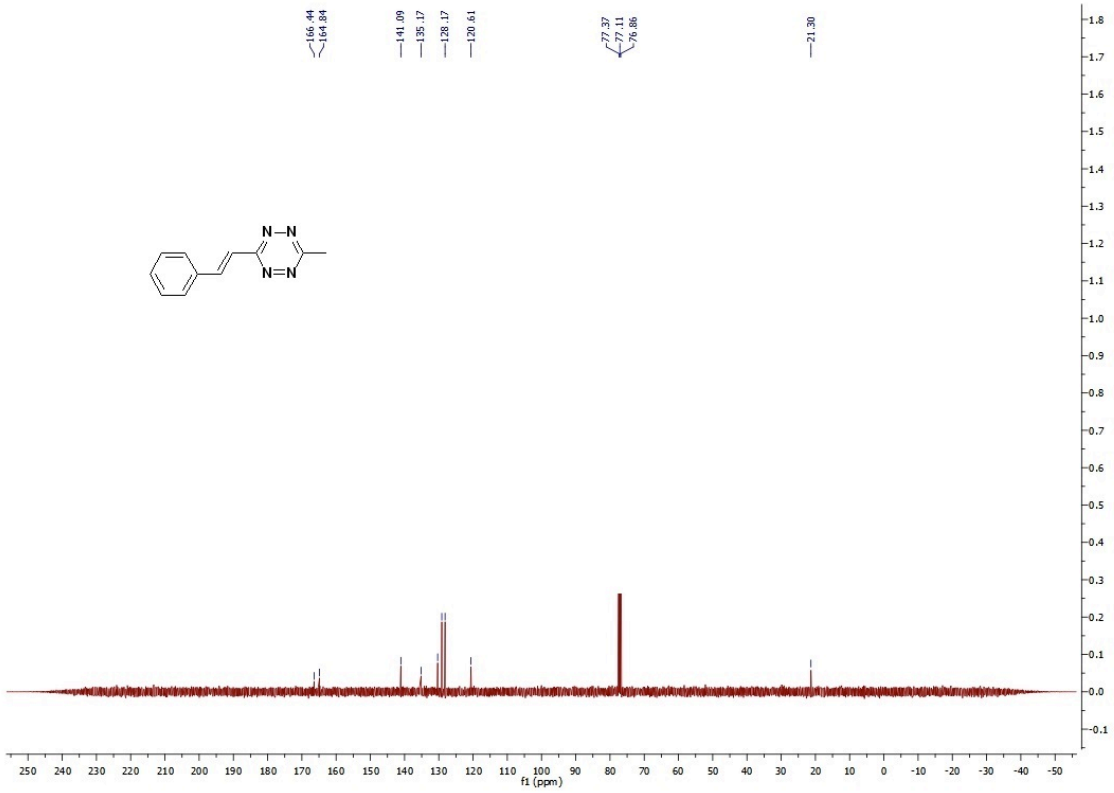

S#540809
single pulse
HO
O
O
F
F
CO2H
Br
3-bromo-Oregon Green 488
f1 (ppm)

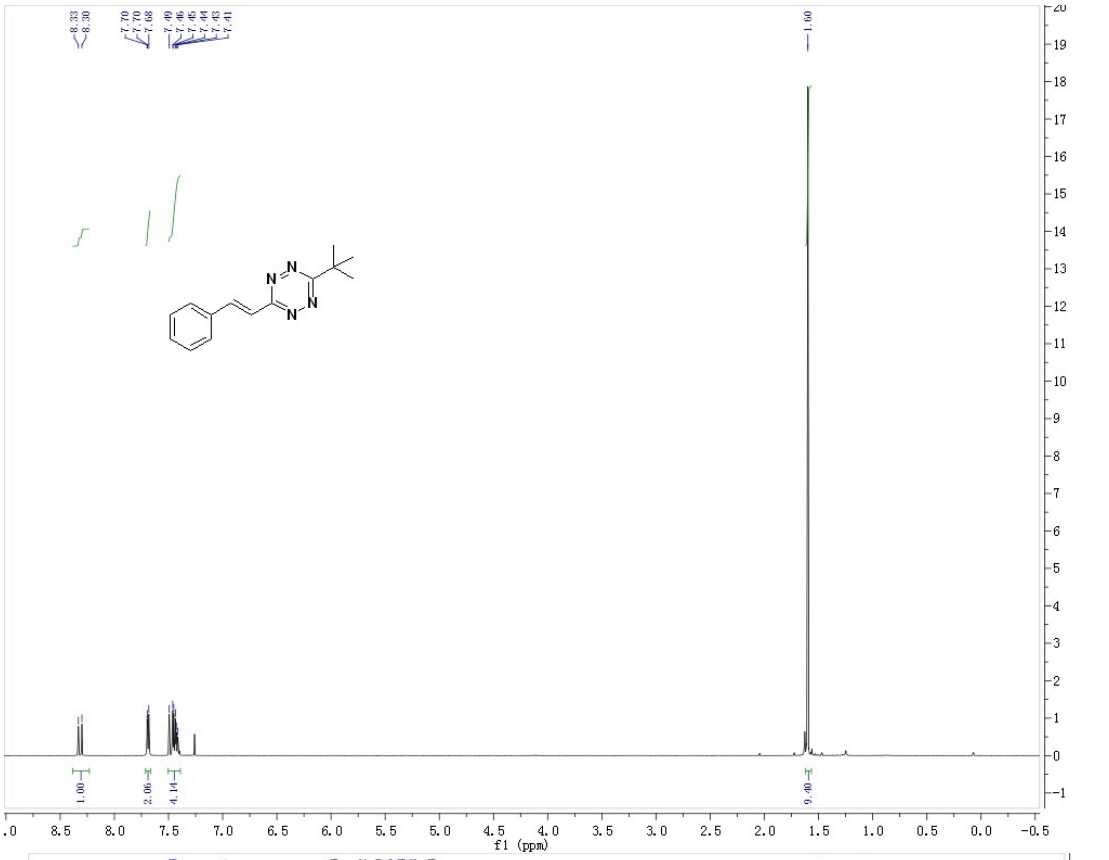

S#27398
single pulse decoupled gated NOE
HO
O
O
F
F
CO2H
Br
3-bromo-Oregon Green 488
f1 (ppm)

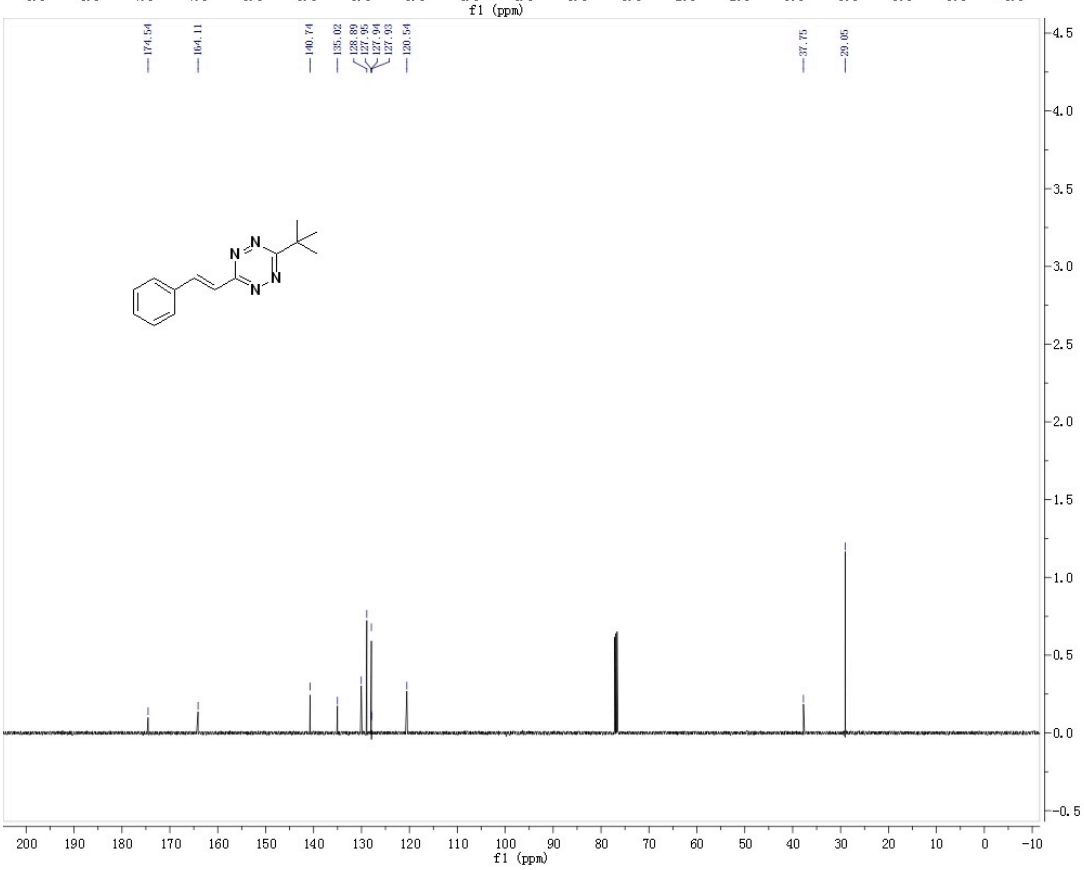

S#624449
single-pulse
4-bromo-Oregon Green 488
f1 (ppm)

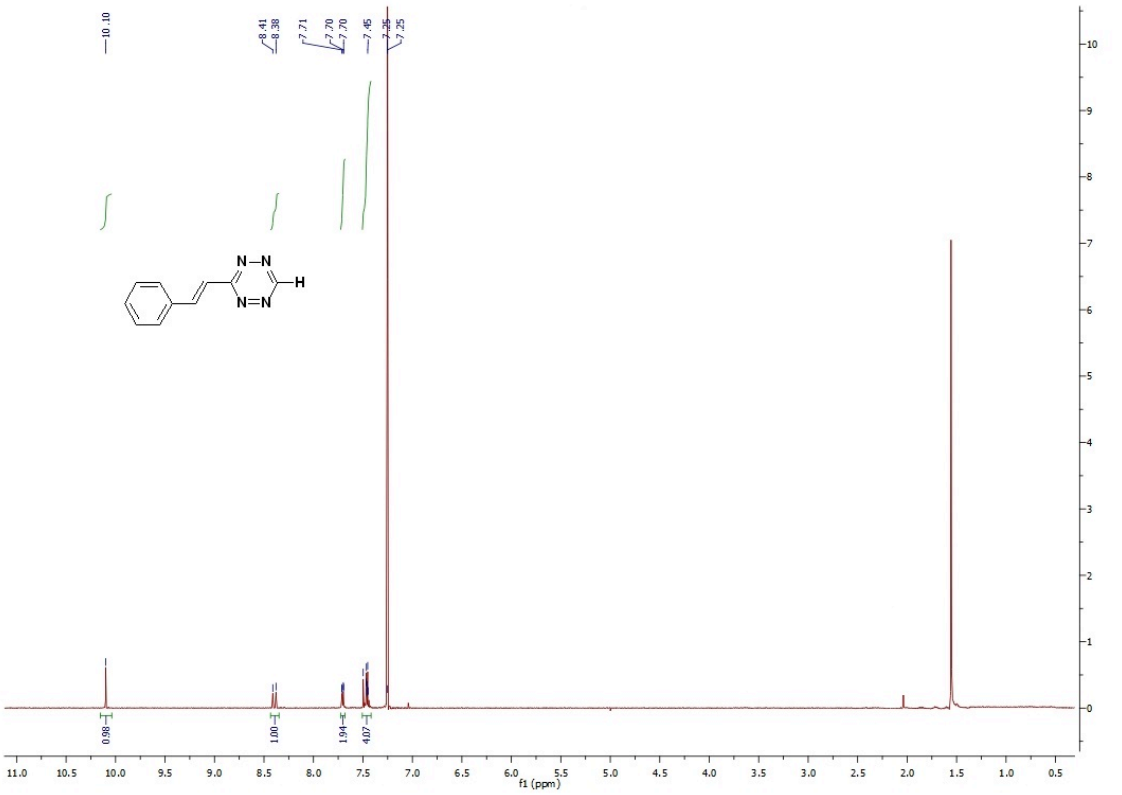

S#626598
single-pulse decoupled-gated NOE
4-bromo-Oregon Green 488
f1 (ppm)

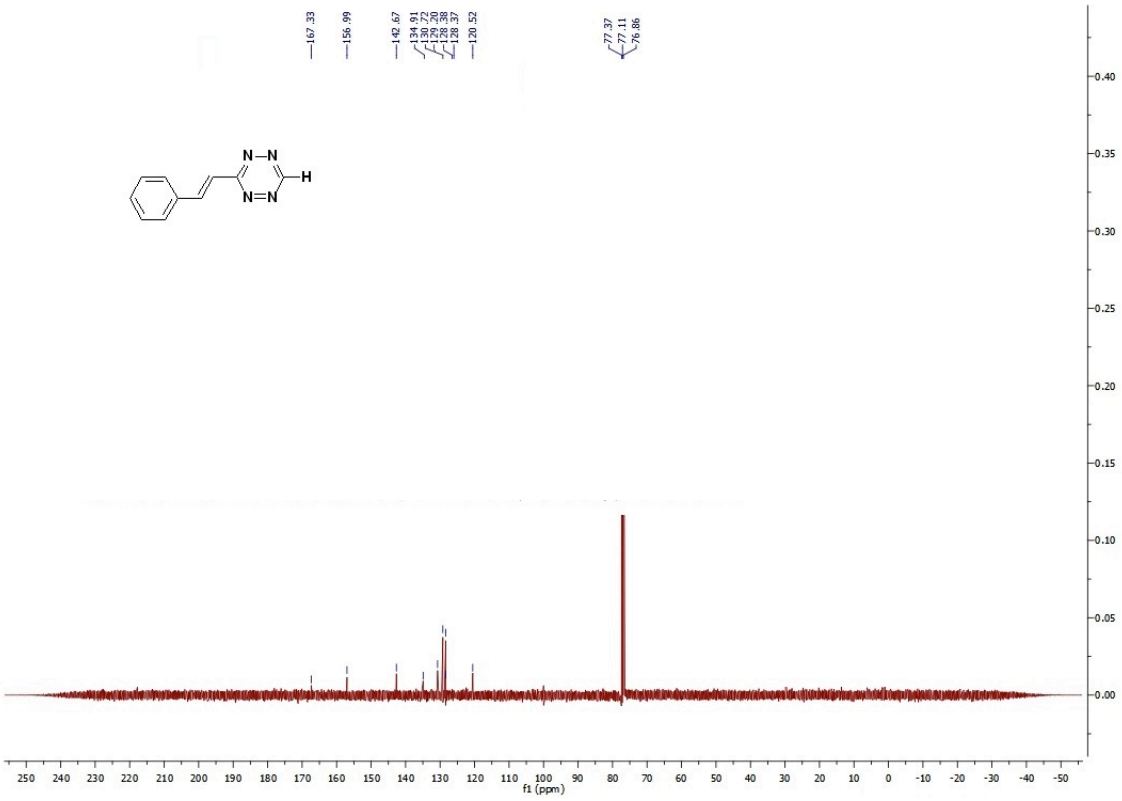

8.33
8.29
7.69
7.68
7.68
7.44
7.26
7.25
7.25
3.05
N–N
N=N
1.00
2.14
4.22
3.29
f1 (ppm)

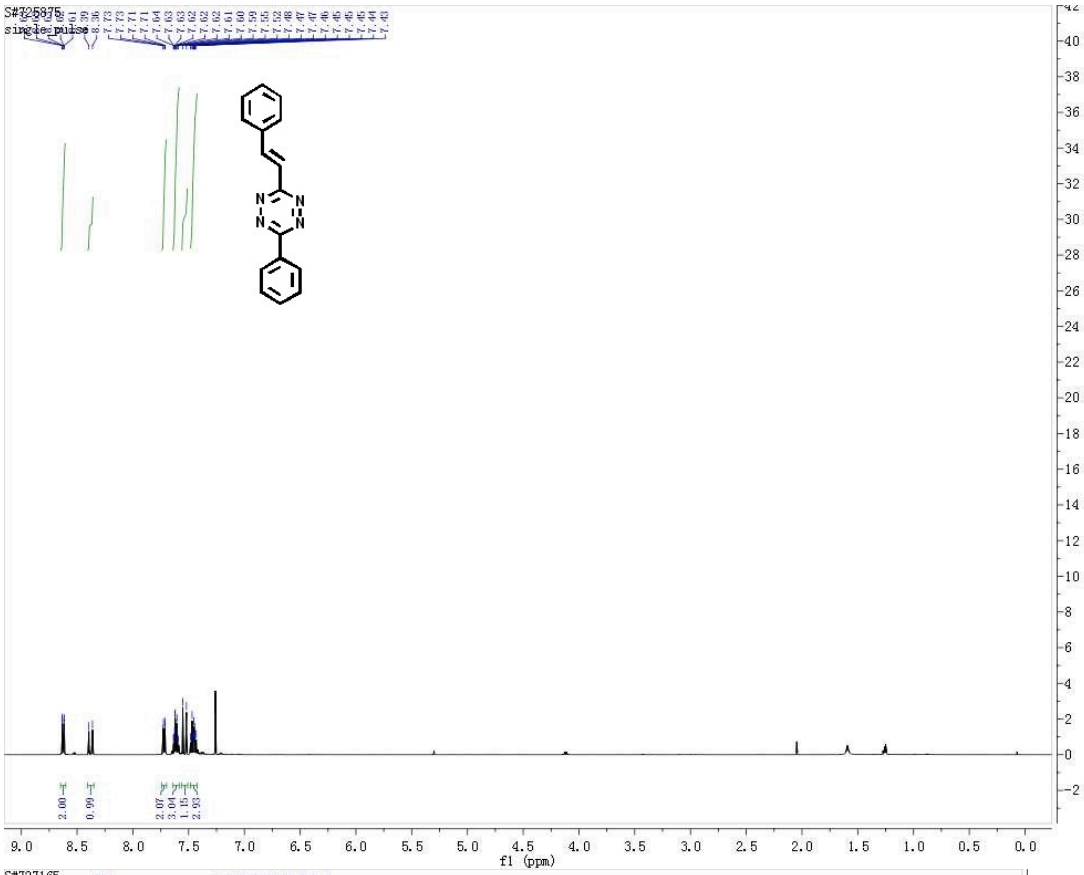

166.44
164.84
141.09
135.17
128.17
120.61
77.37
77.11
76.86
21.30
N–N
N=N
f1 (ppm)

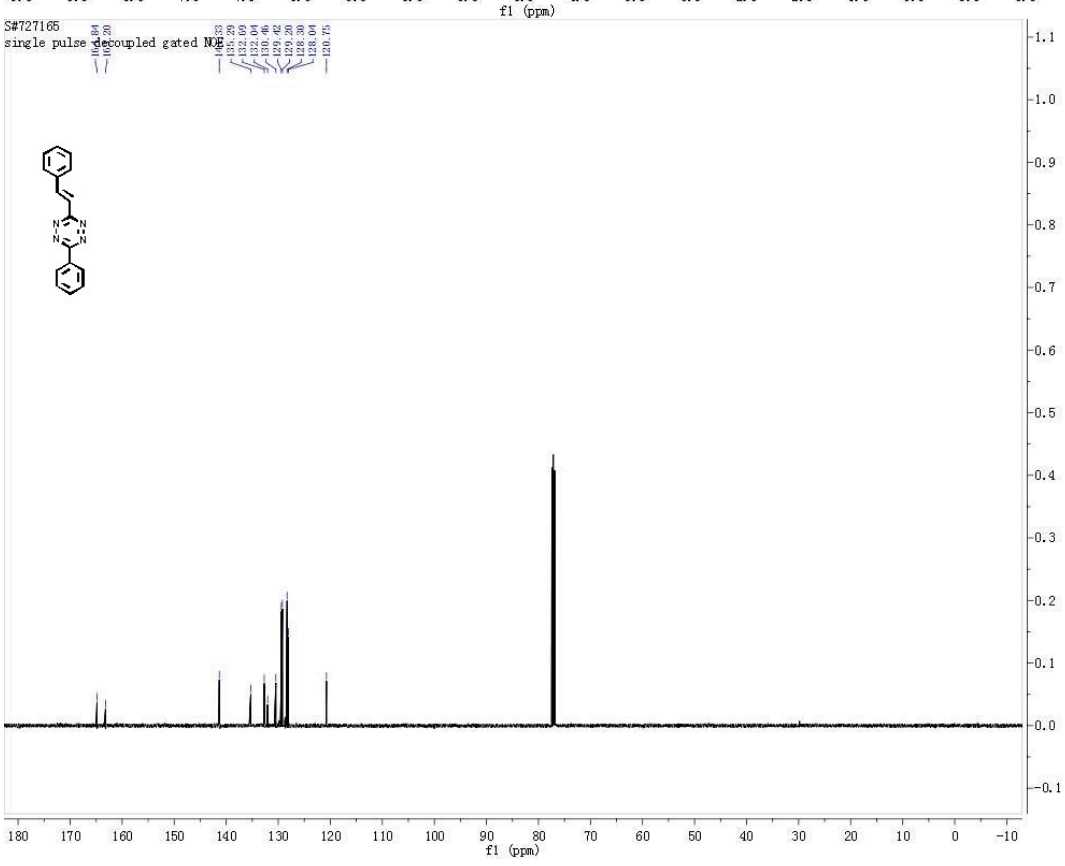

8.33
8.30
7.70
7.70
7.68
7.49
7.46
7.45
7.44
7.43
7.41
1.60
1.00
2.06
4.14
9.40
f1 (ppm)

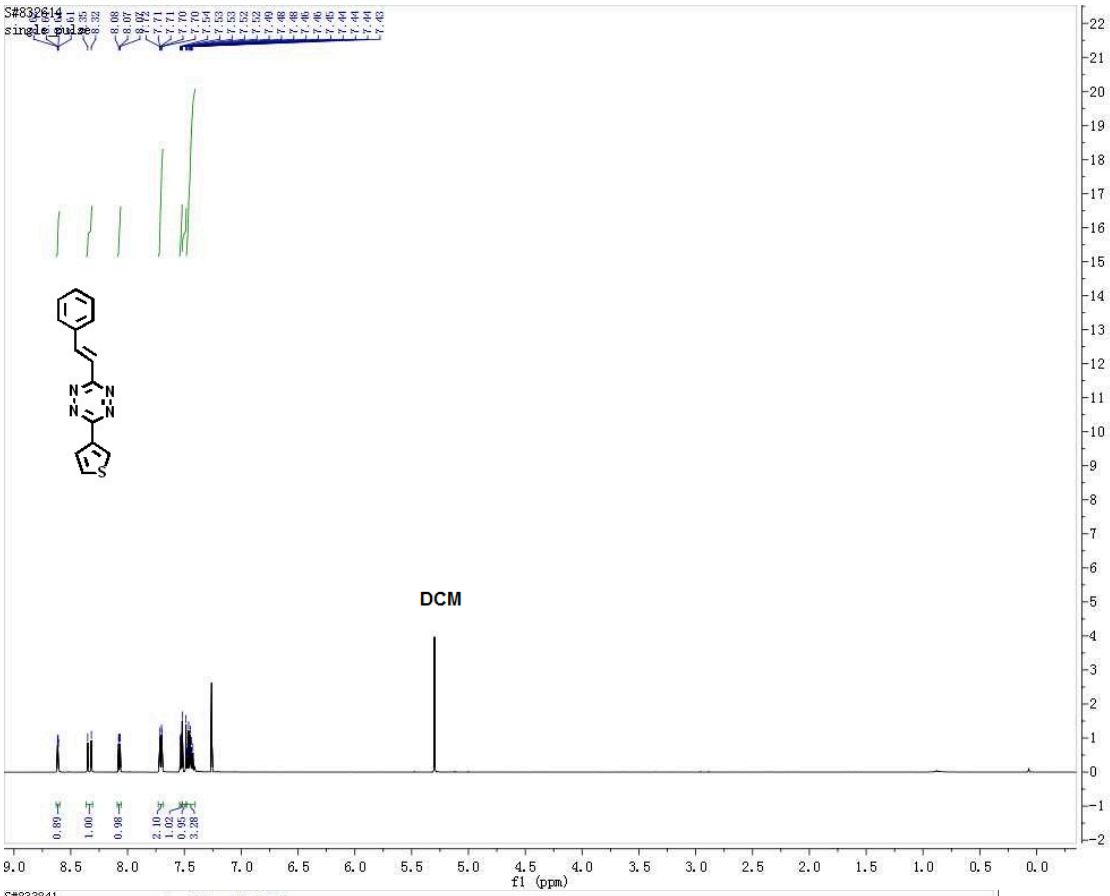

174.54
164.11
140.74
135.02
128.89
127.95
127.94
127.93
120.54
37.75
29.05
f1 (ppm)

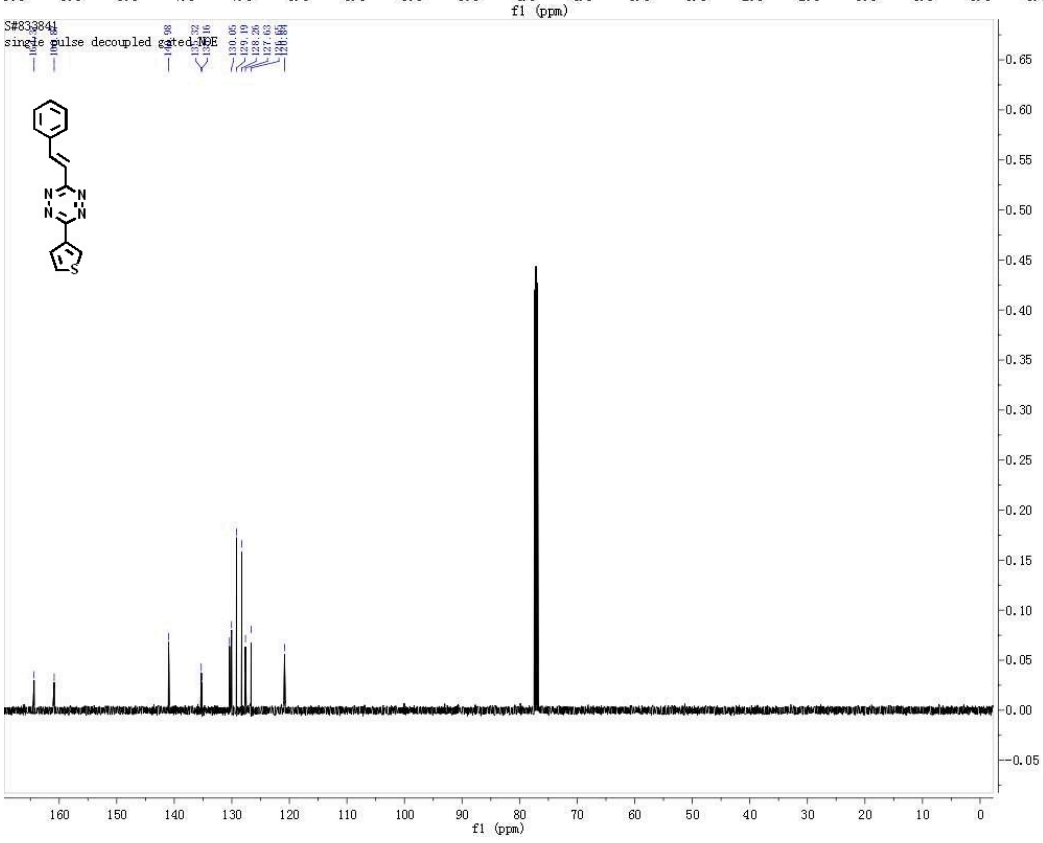

10.10
8.41
8.38
7.71
7.70
7.70
7.45
7.25
7.25
0.98
1.00
1.94
4.07
f1 (ppm)

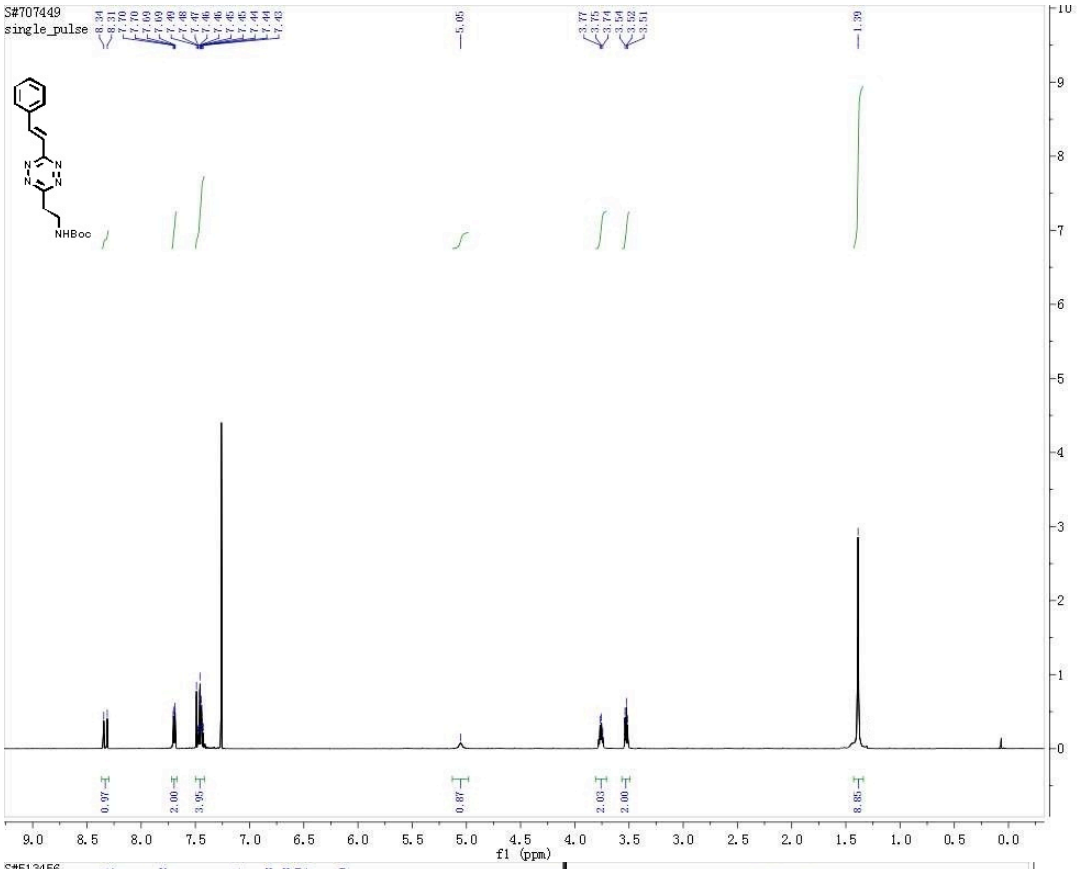

167.33
156.99
142.67
134.91
130.72
129.20
128.38
128.37
120.52
77.37
77.11
76.86
f1 (ppm)

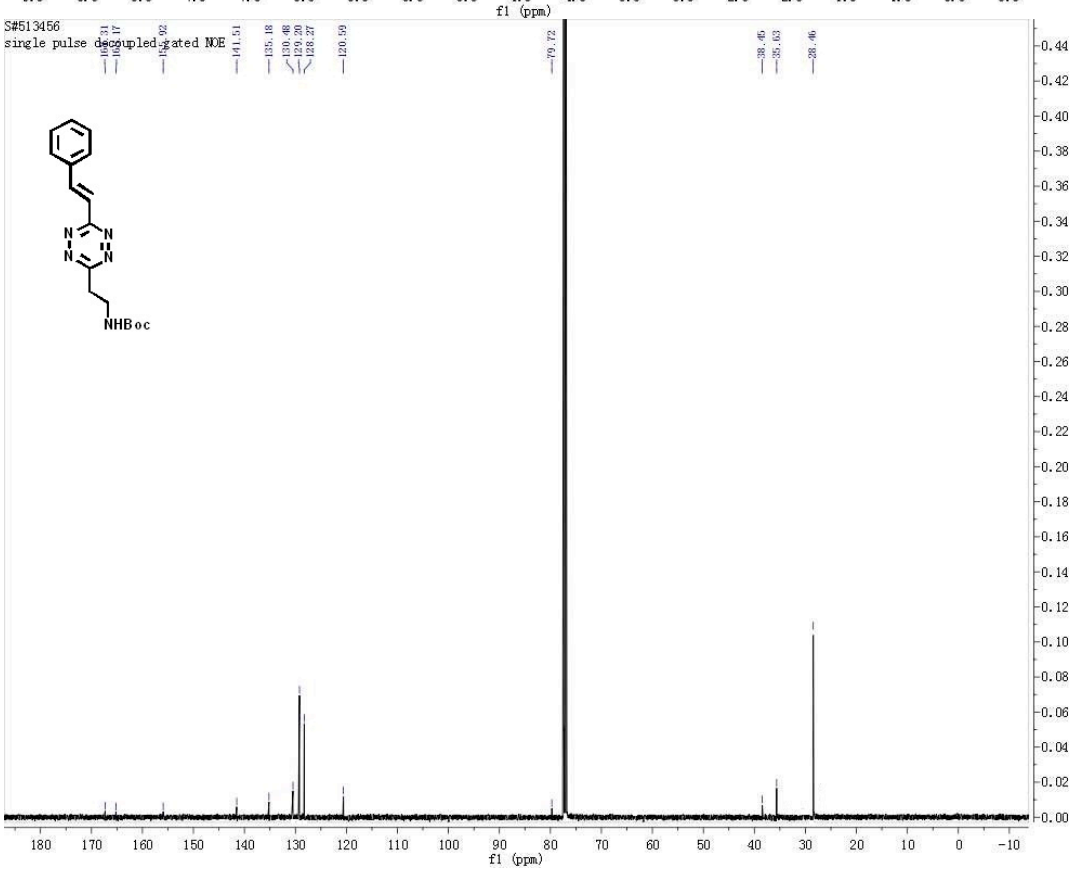

f1 (ppm)

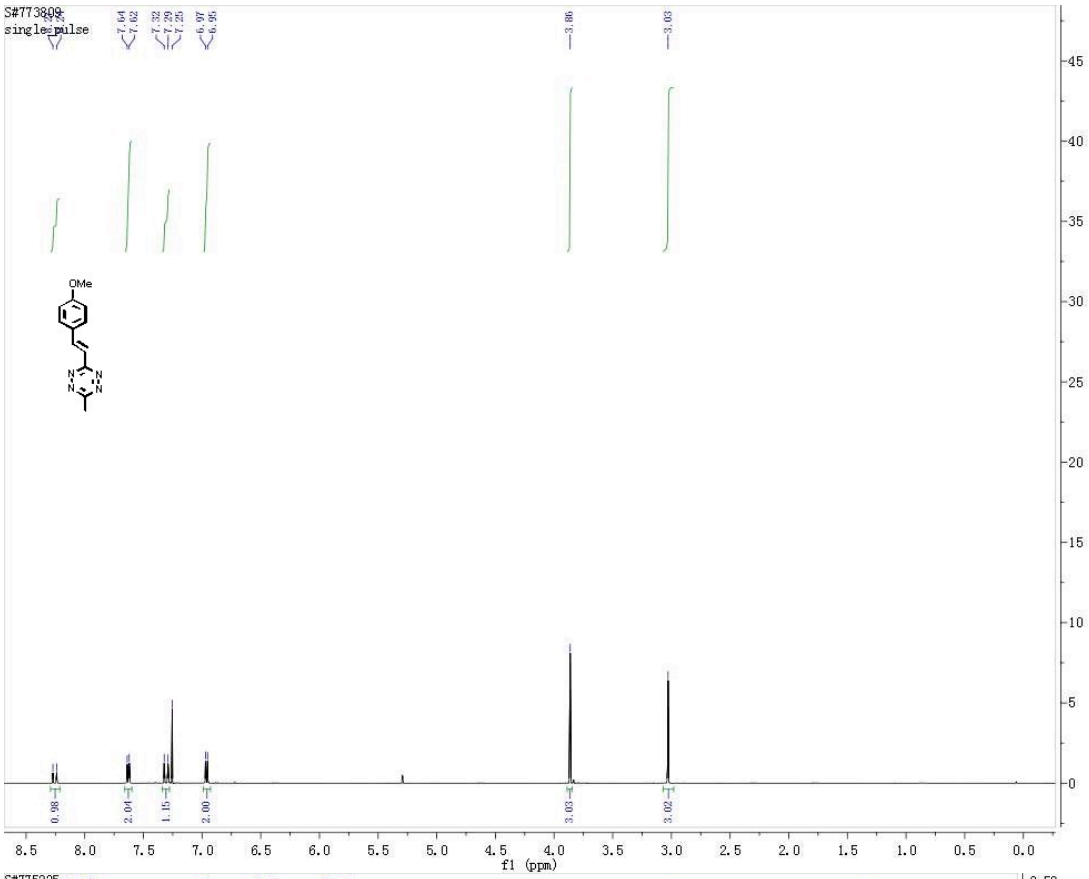

single pulse decoupled gated NOE
f1 (ppm)

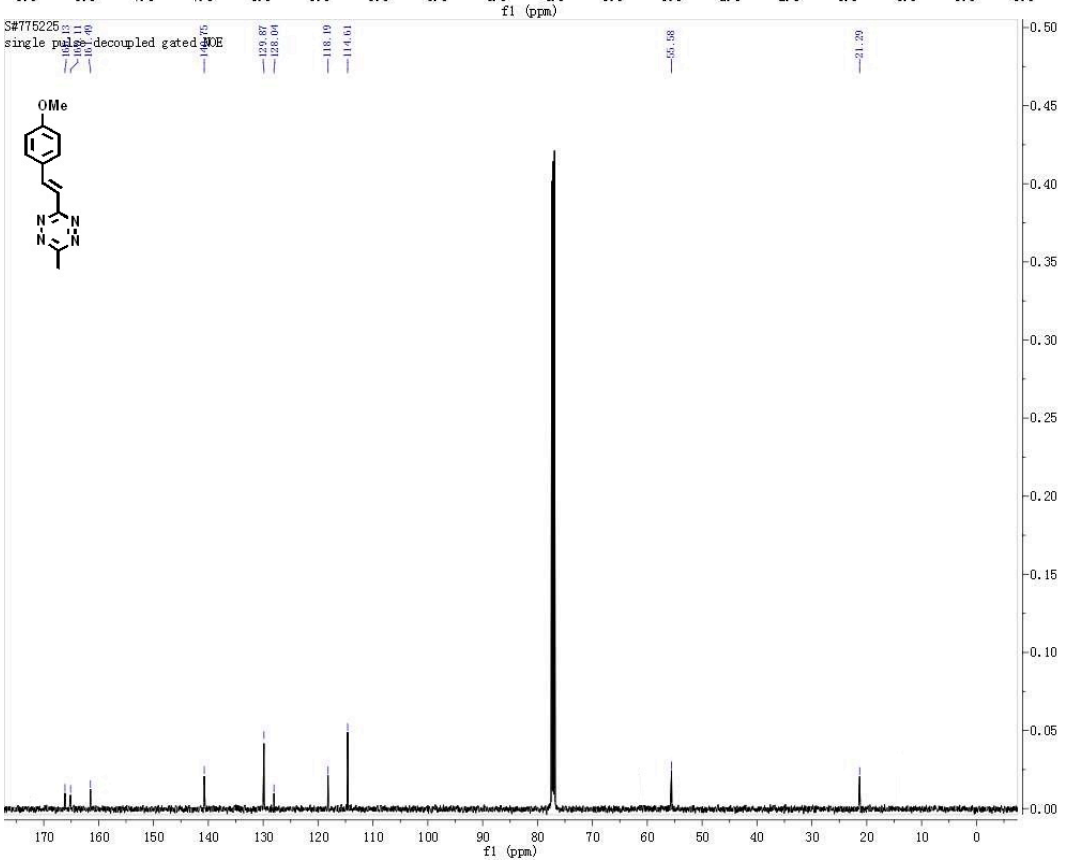

DCM
f1 (ppm)

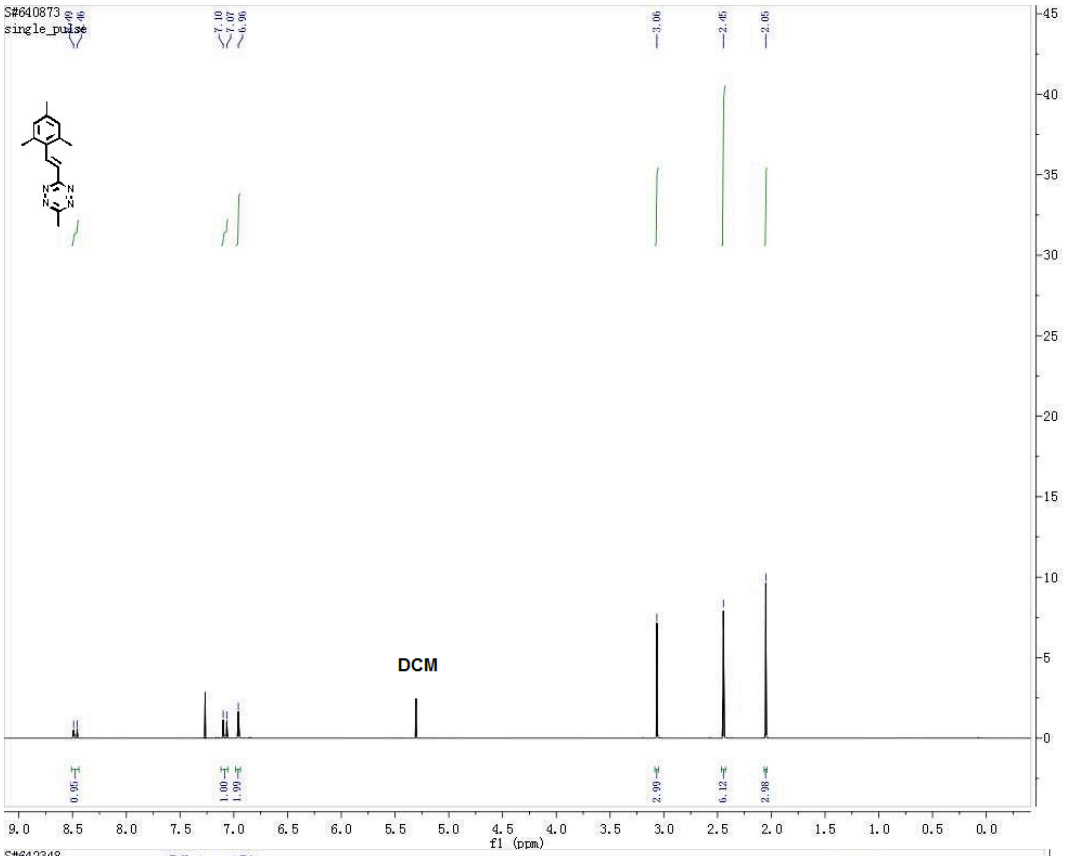

f1 (ppm)

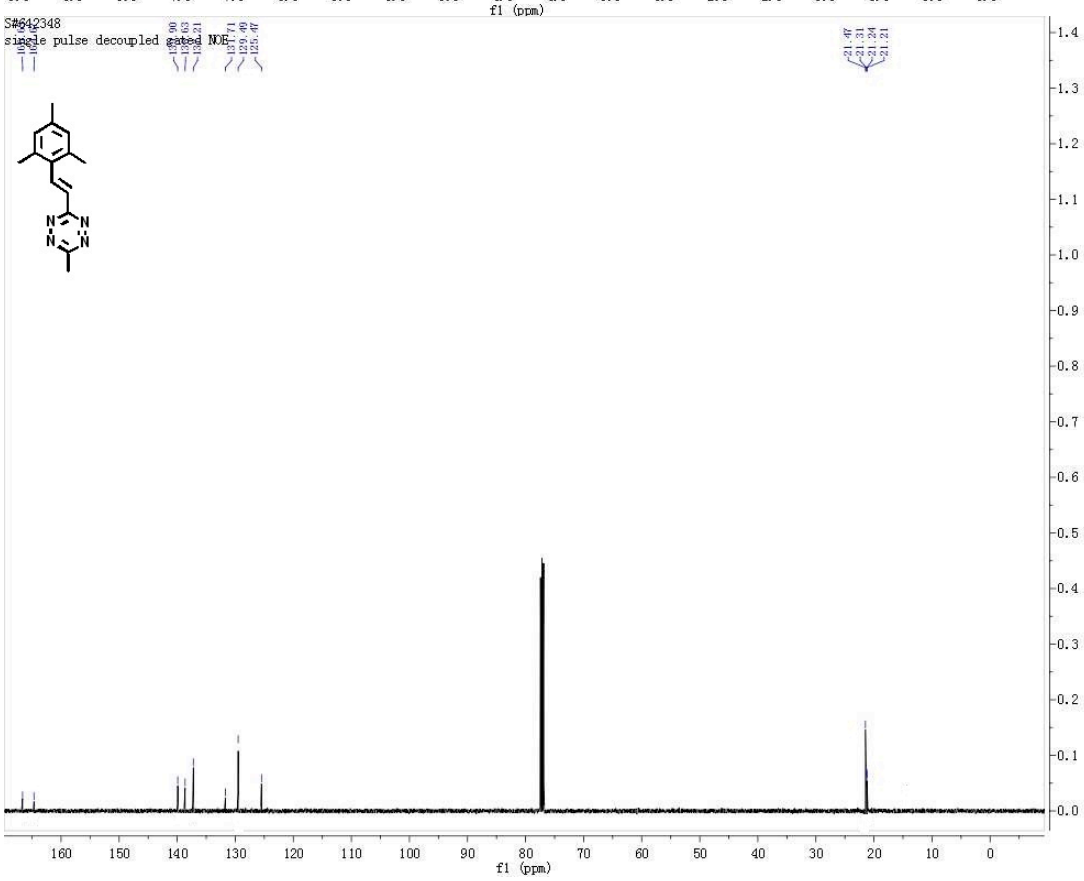

S#707449
single_pulse
NHBoc
f1 (ppm)

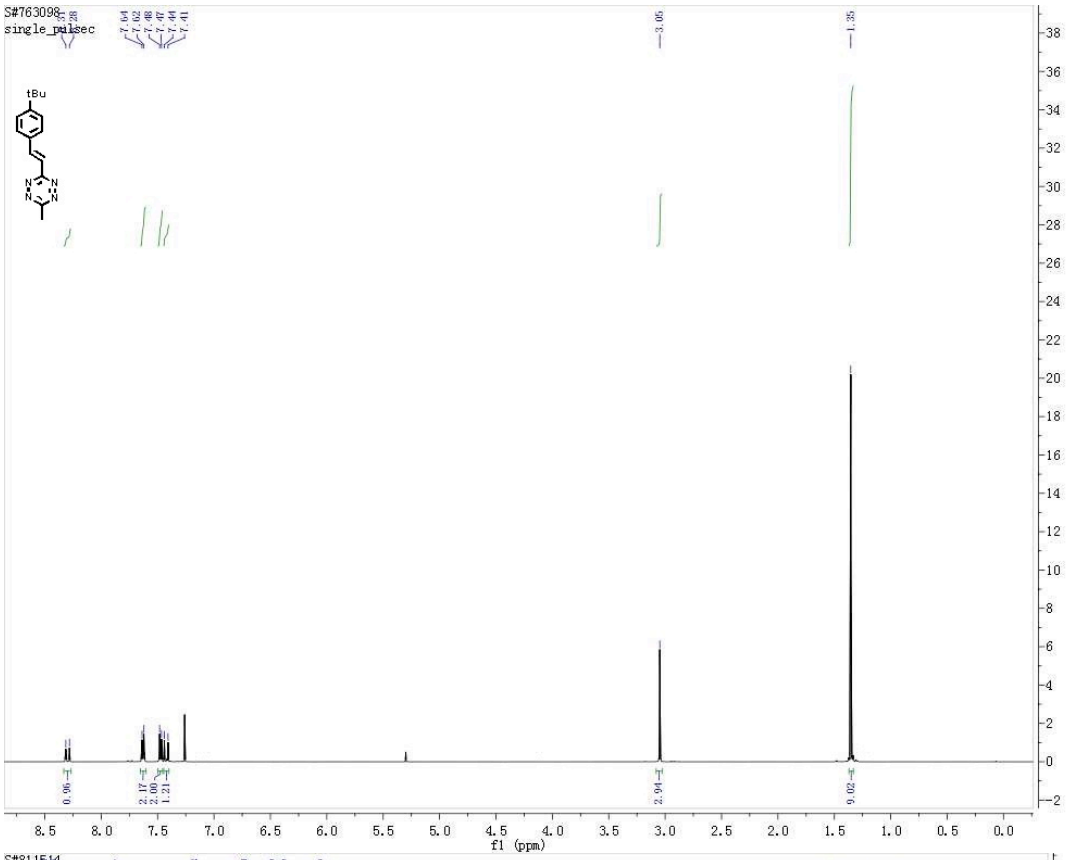

S#513456
single pulse decoupled gated NOE
NHBoc
f1 (ppm)

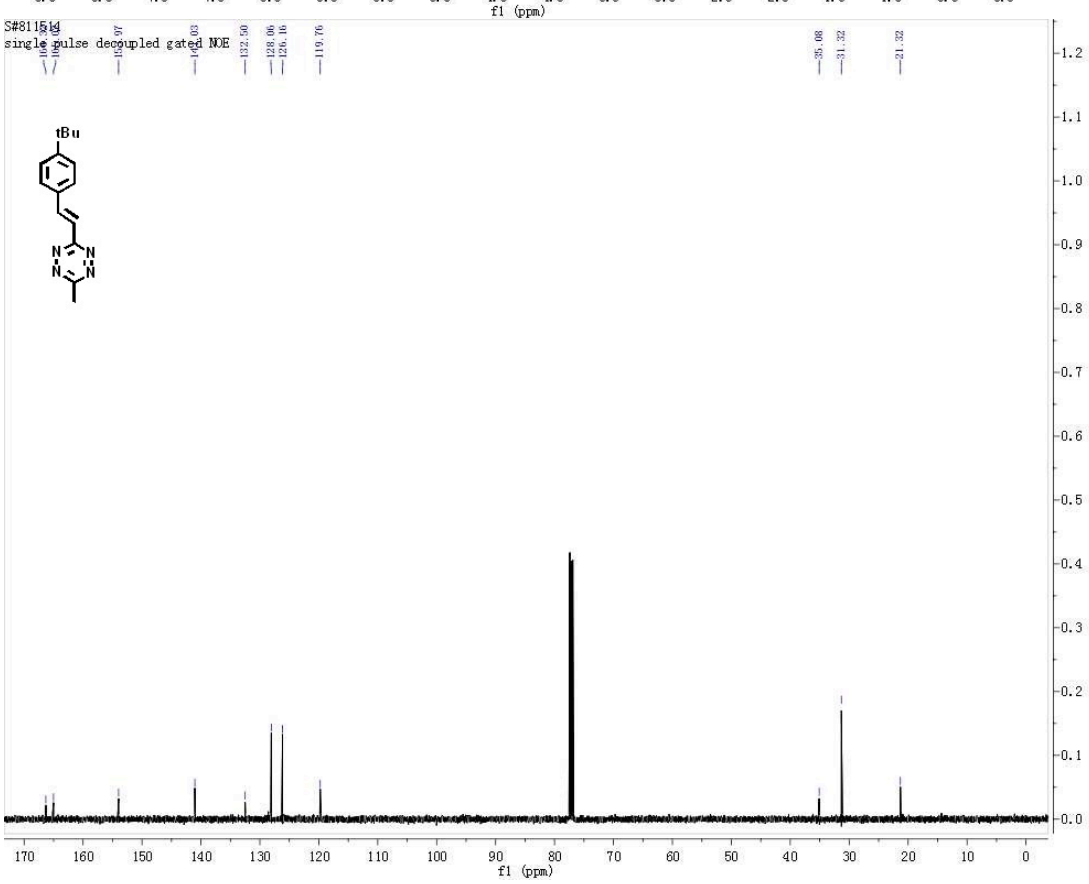

S#773809
single pulse
7.64
7.62
7.32
7.29
7.25
6.97
6.95
3.86
3.03
OMe
0.98
2.04
1.15
2.00
3.03
3.02
f1 (ppm)

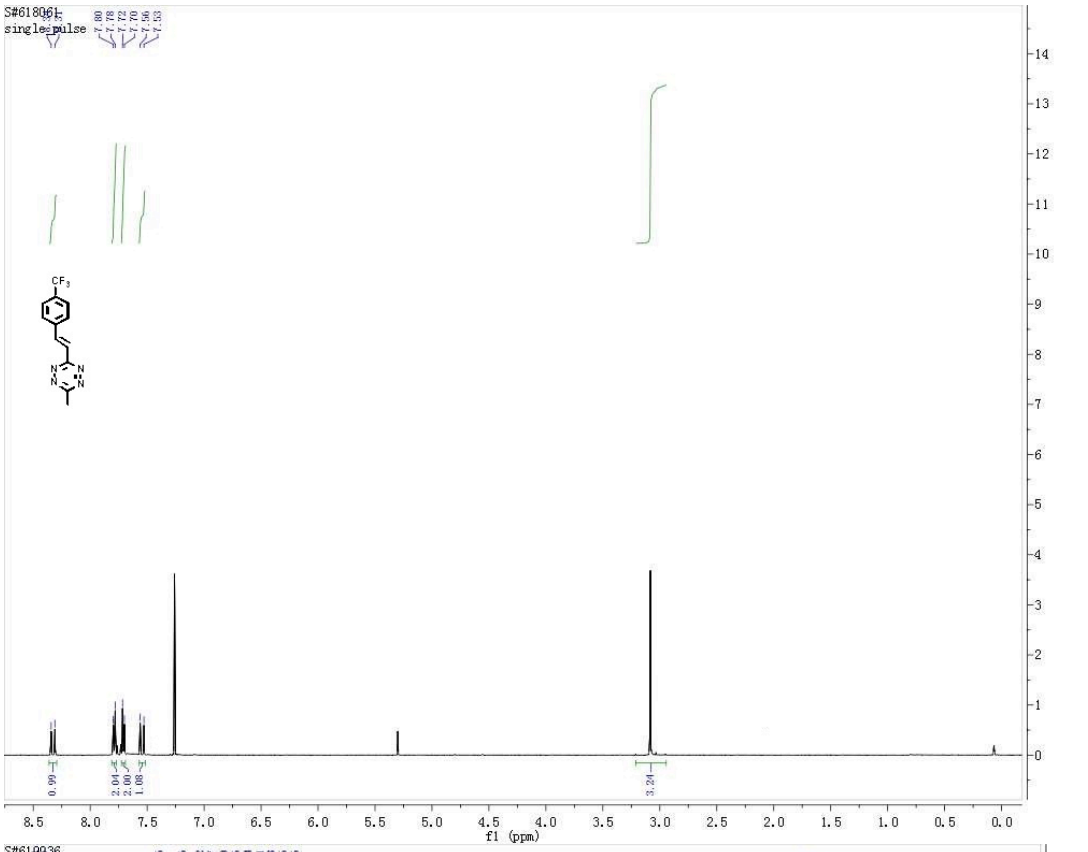

S#775225
single pulse decoupled gated NOE
163.13
161.11
160.49
140.75
129.87
128.04
118.19
114.61
55.58
21.29
OMe
f1 (ppm)

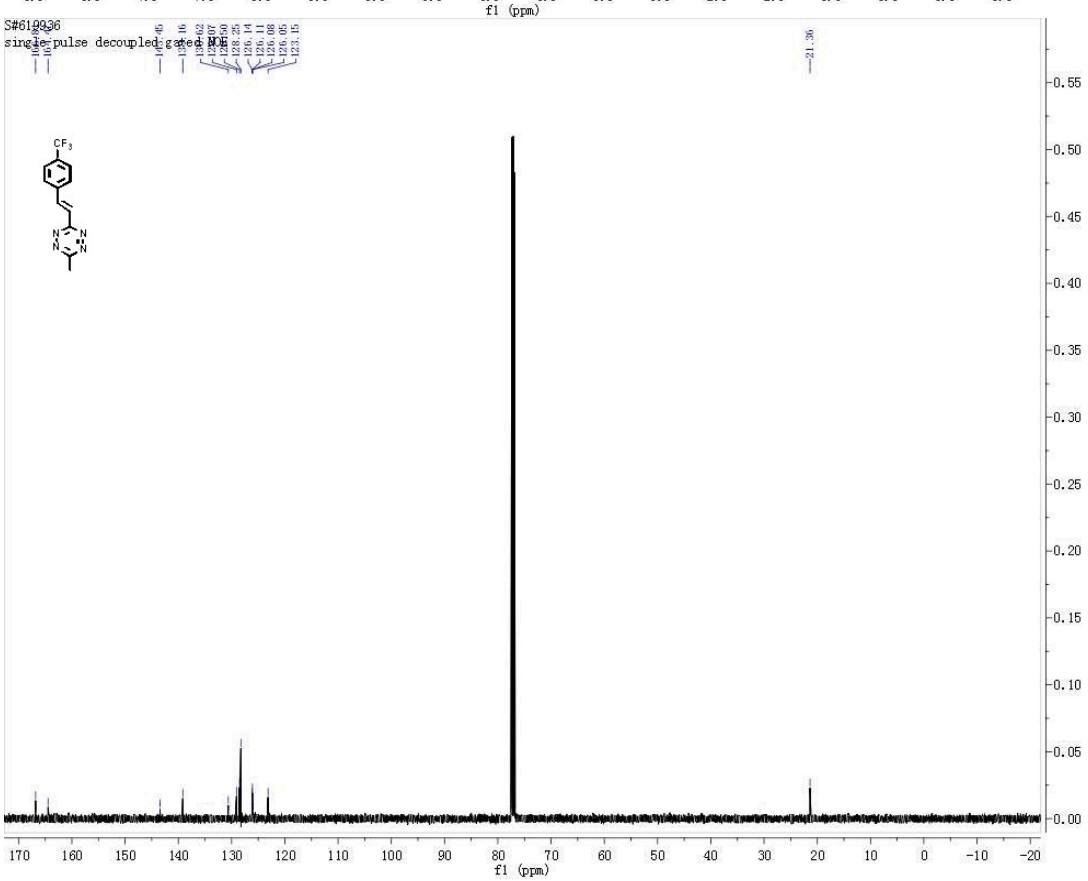

S#640873
single_pulse
8.49
8.46
7.10
7.07
6.96
3.06
2.45
2.05
DCM
0.95
1.00
1.99
2.99
6.12
2.98
f1 (ppm)

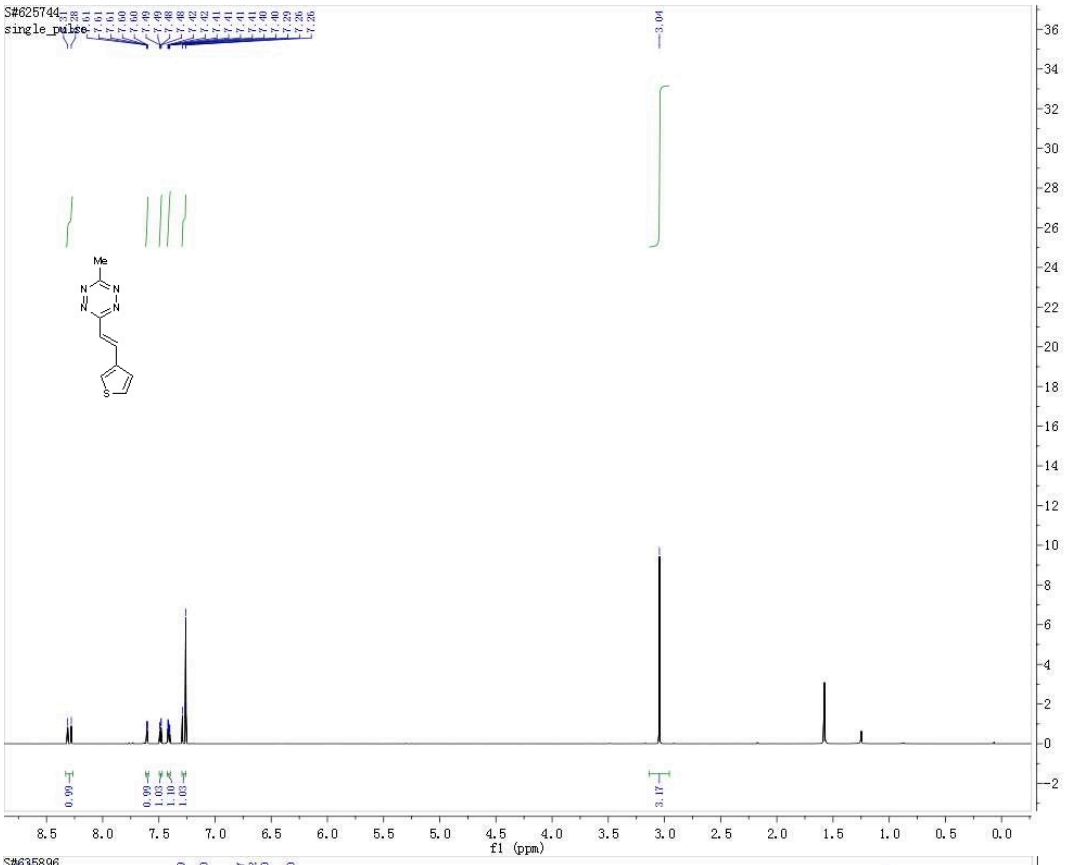

S#642348
single pulse decoupled gated NOE
165.90
163.63
138.21
137.71
129.49
125.47
21.47
21.31
21.24
21.21
f1 (ppm)

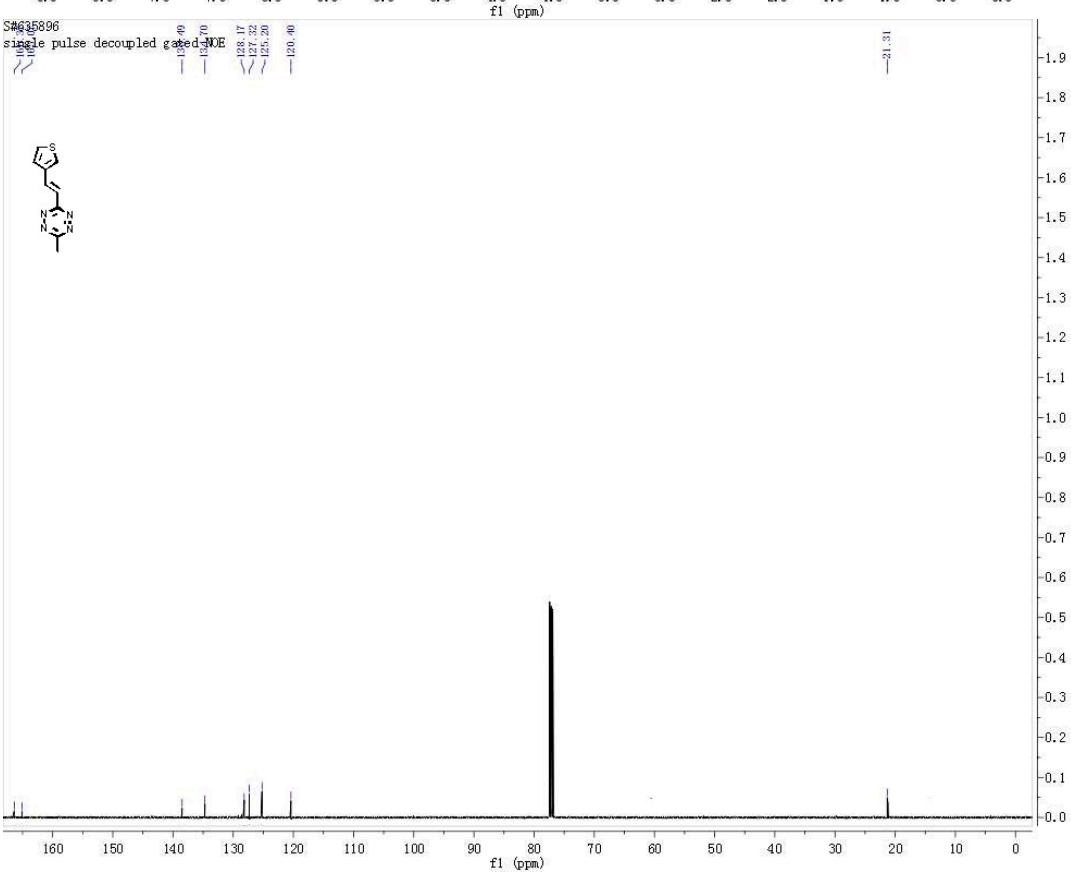

S#763098
single_pulsec
tBu
f1 (ppm)

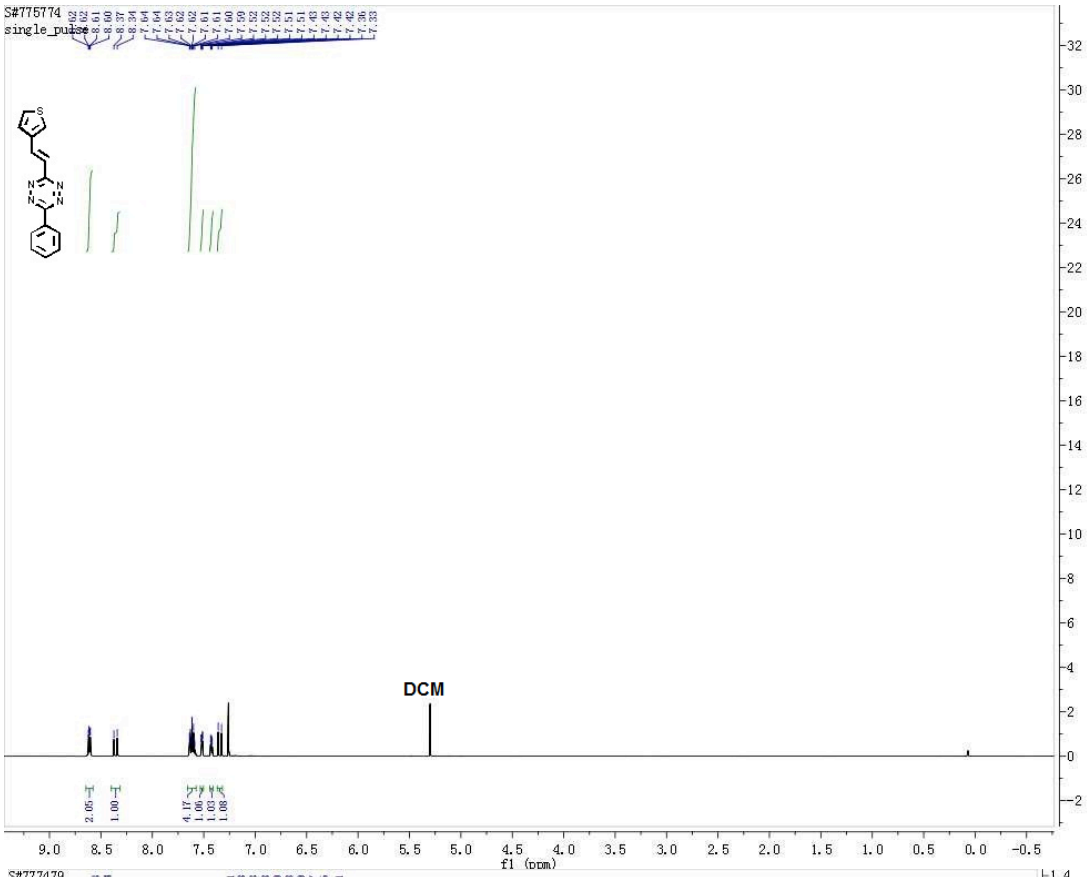

S#811514
single pulse decoupled gated NOE
tBu
f1 (ppm)

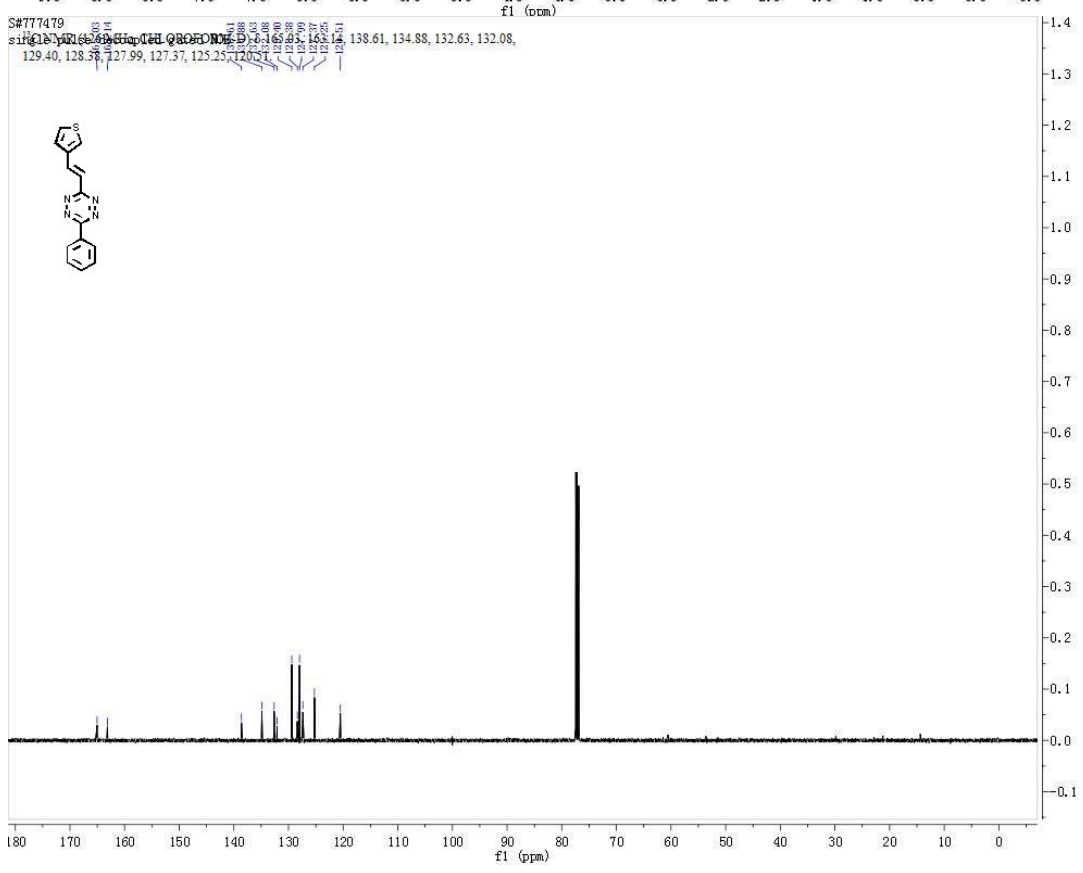

S#618061
single-pulse
7.80
7.78
7.72
7.70
7.56
7.53
CF3
0.99
2.04
2.08
1.08
3.24
f1 (ppm)

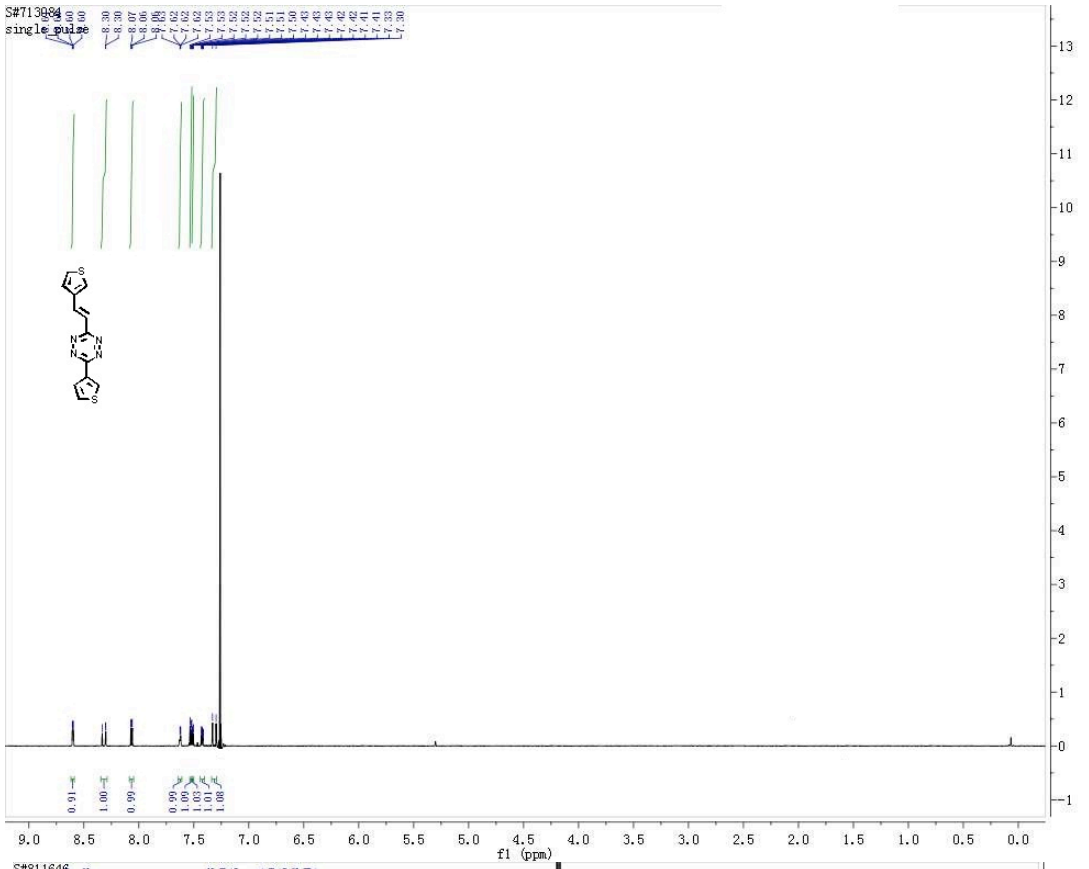

S#619036
single-pulse decoupled gated NOE
CF3
21.36
f1 (ppm)

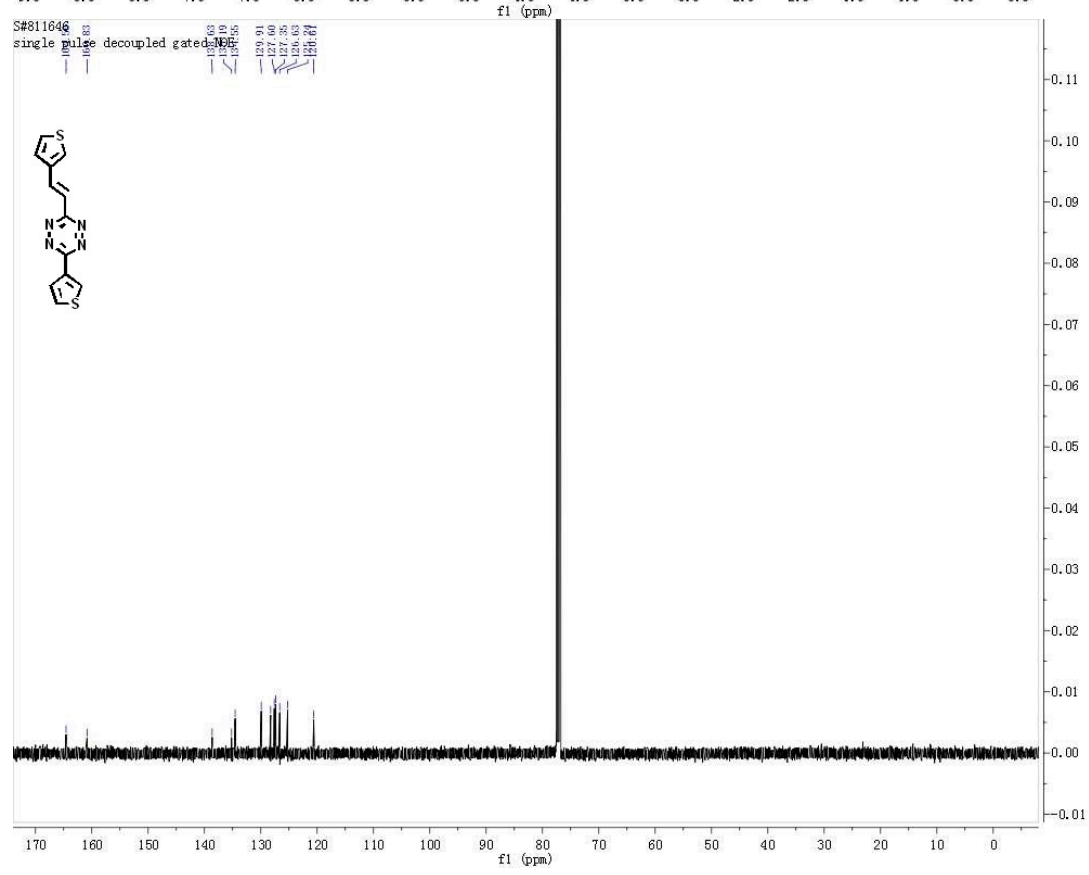

S#625744
single_pulse
Me
f1 (ppm)

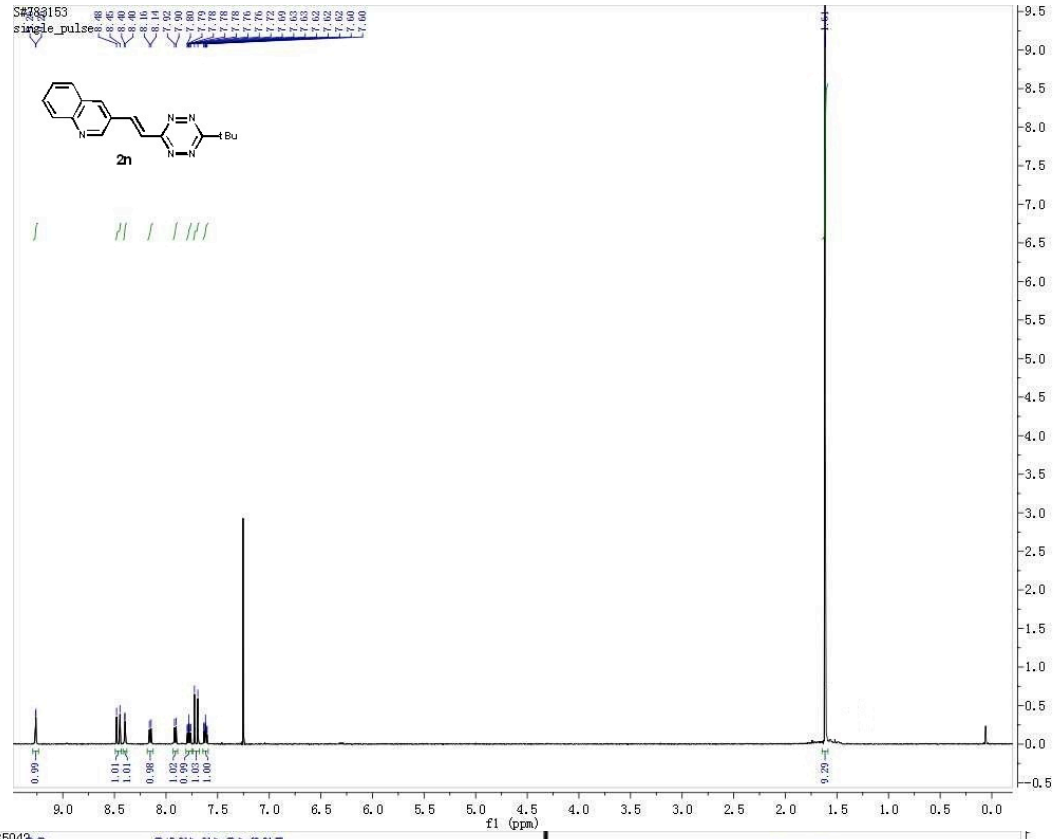

S#635896
single pulse decoupled gated-NOE
f1 (ppm)

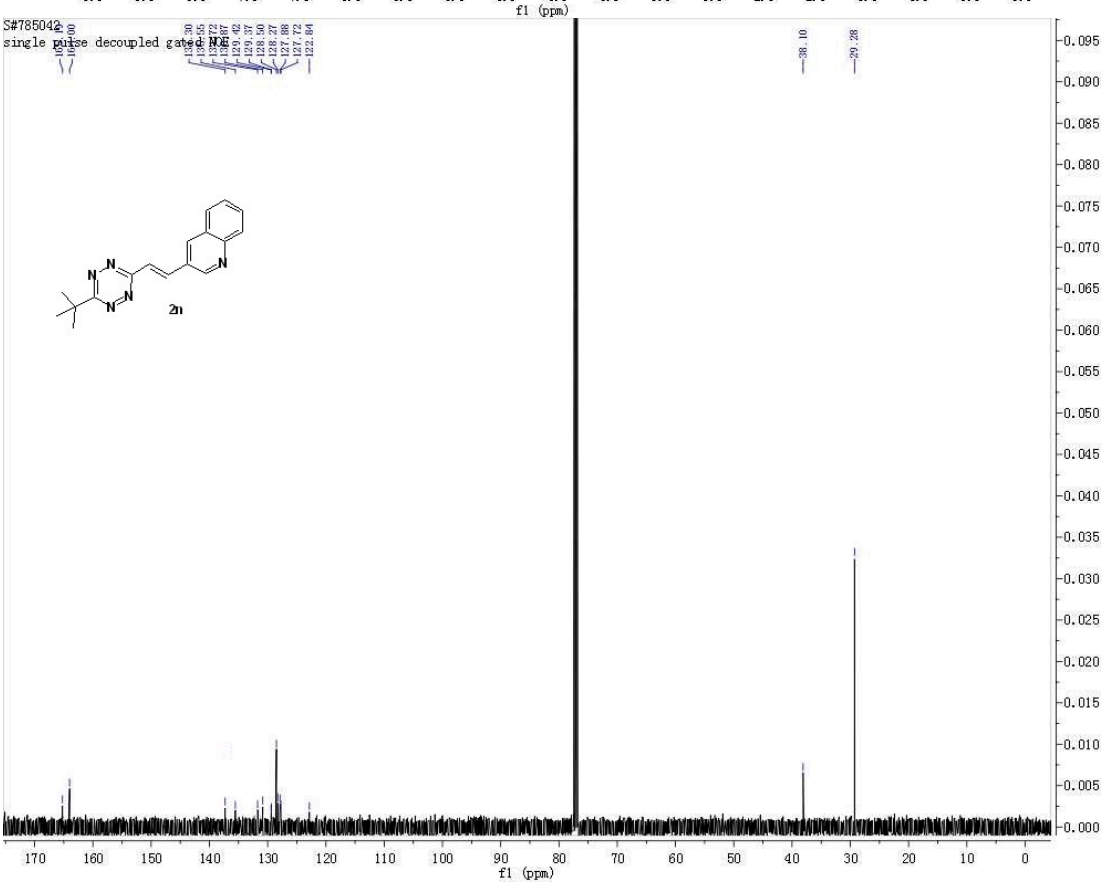

S#775774
single_pulse
DCM
f1 (ppm)

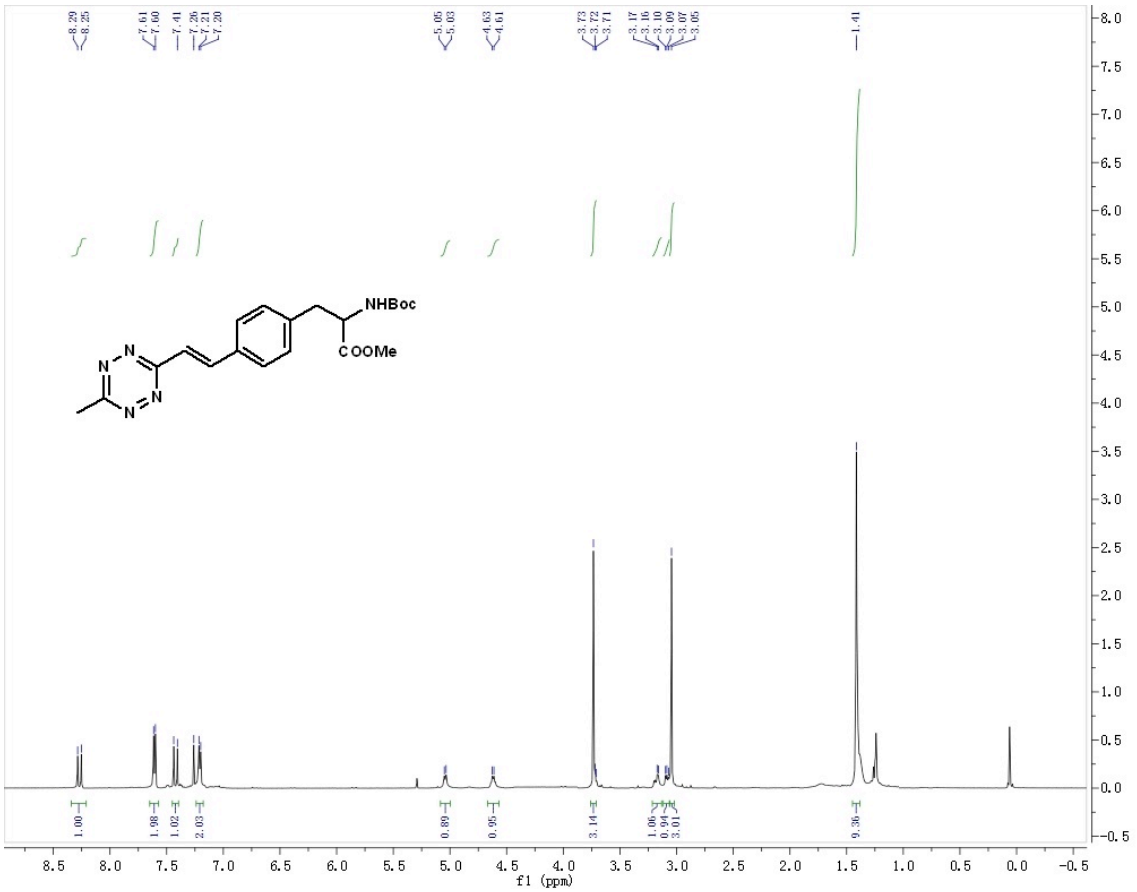

S#777479
f1 (ppm)

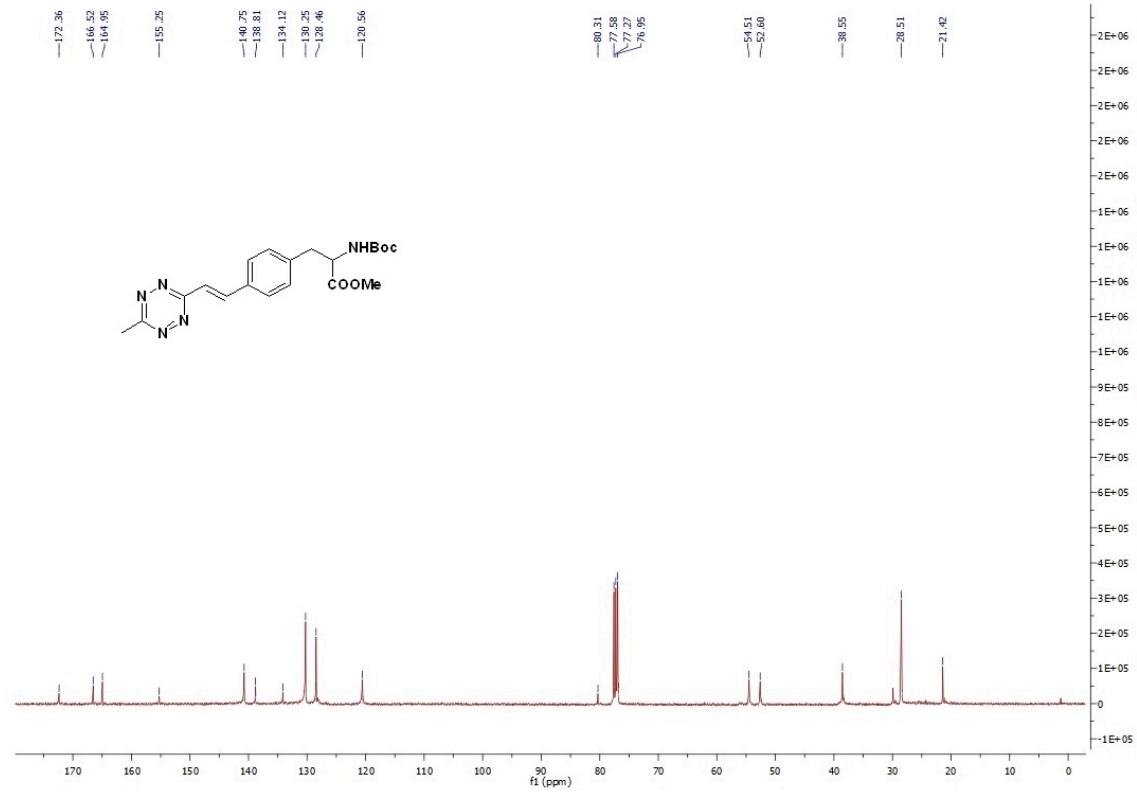

S#713004
single pulse
f1 (ppm)

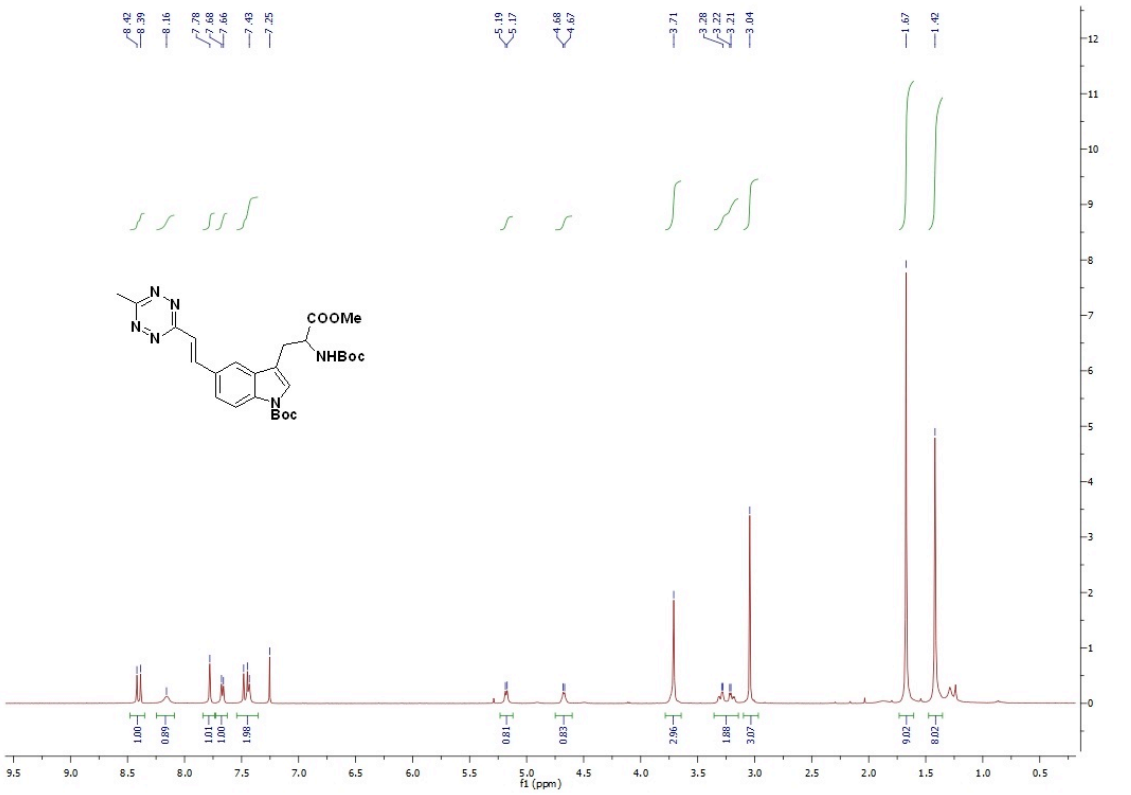

S#811646
single pulse decoupled gated-NOE
f1 (ppm)

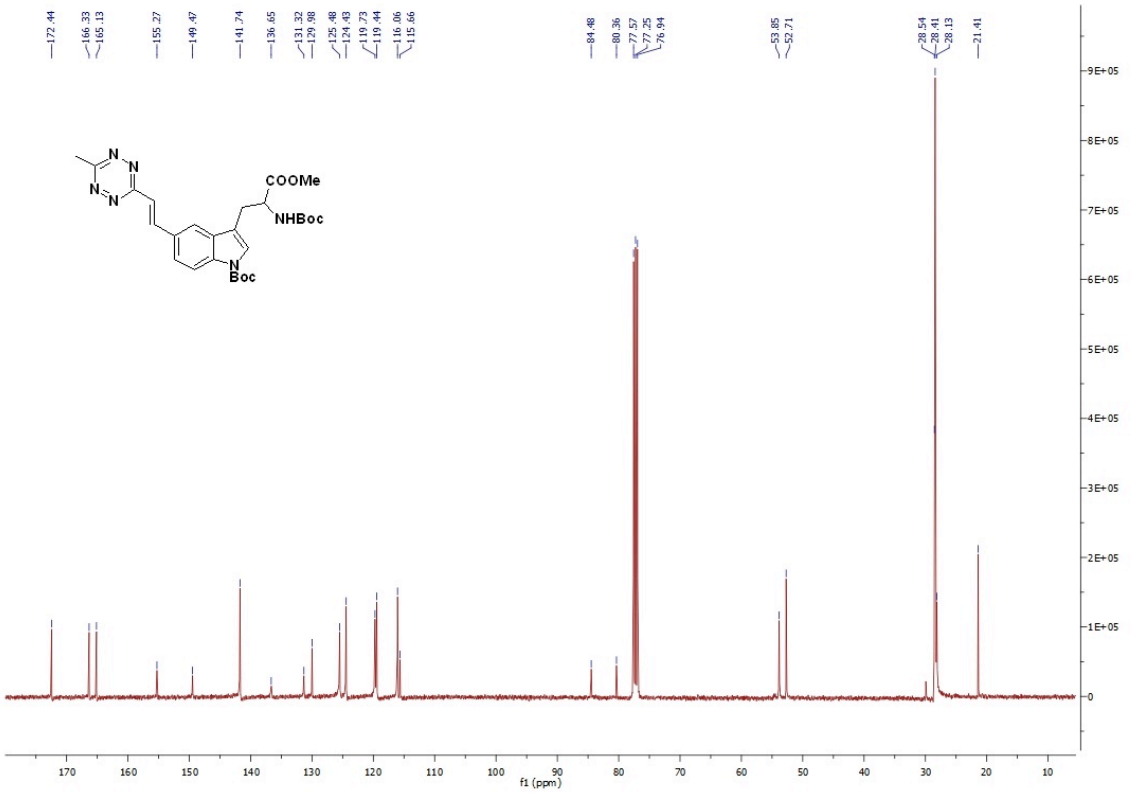

S#783153
single_pulse
2n
f1 (ppm)

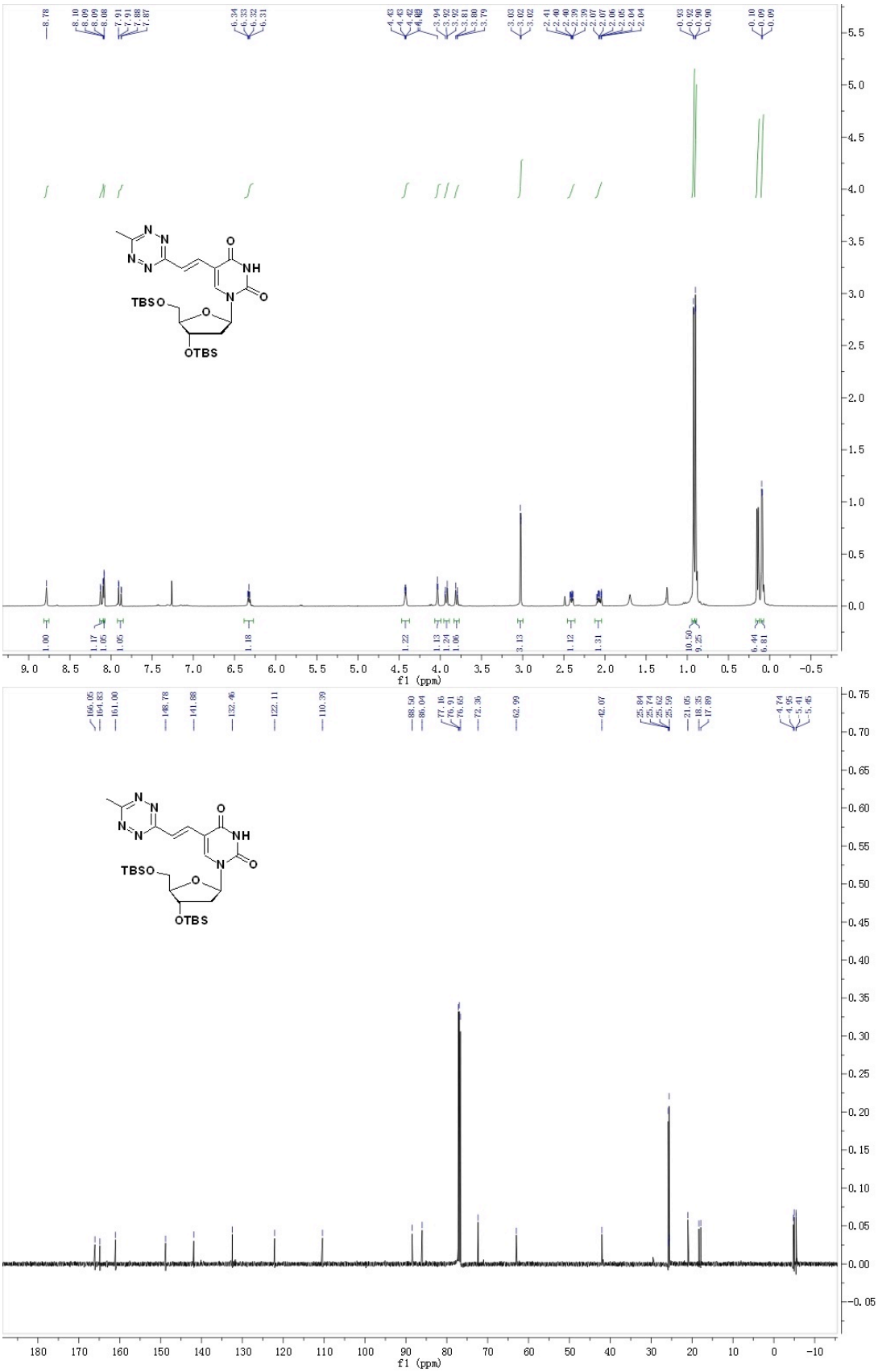

S#785042
single pulse decoupled gated NOE
2n
f1 (ppm)

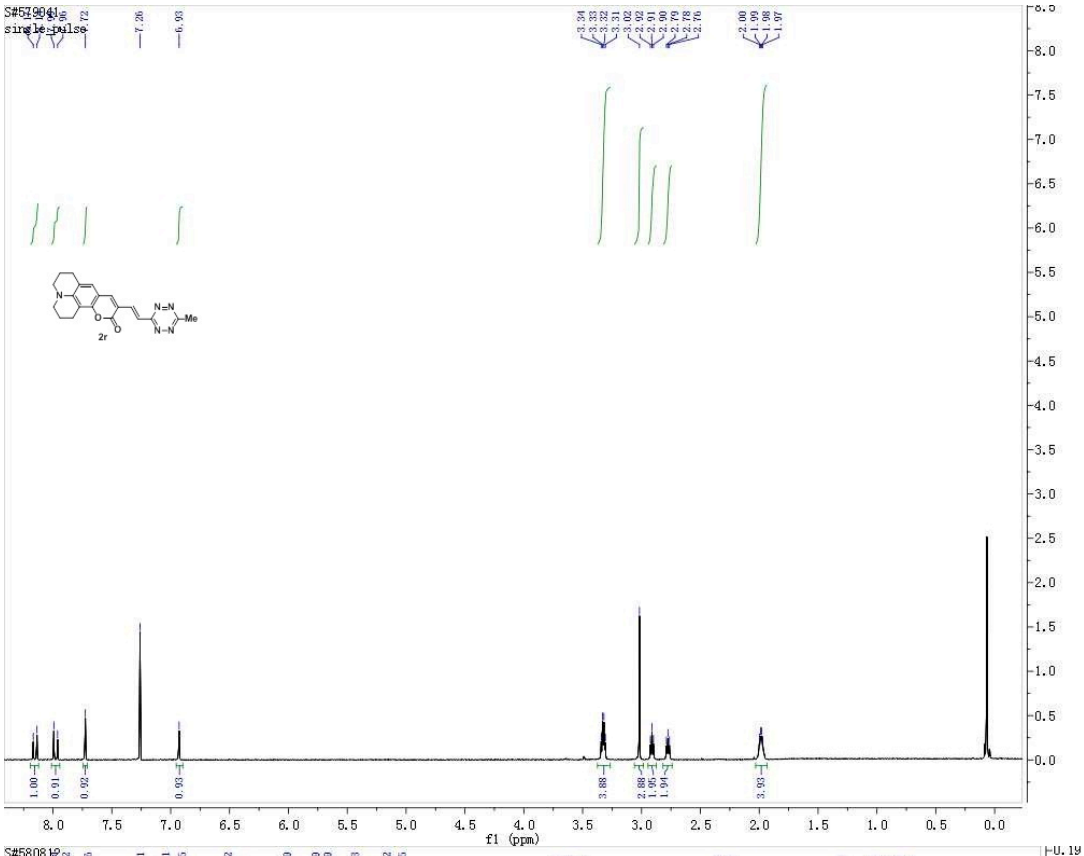

8.29
8.25
7.61
7.60
7.41
7.26
7.21
7.20
5.05
5.03
4.63
4.61
3.73
3.72
3.71
3.17
3.16
3.10
3.09
3.07
3.05
1.41
NHBoc
COOMe
1.00
1.98
1.02
2.03
0.89
0.95
3.14
1.06
0.94
3.01
9.36
f1 (ppm)

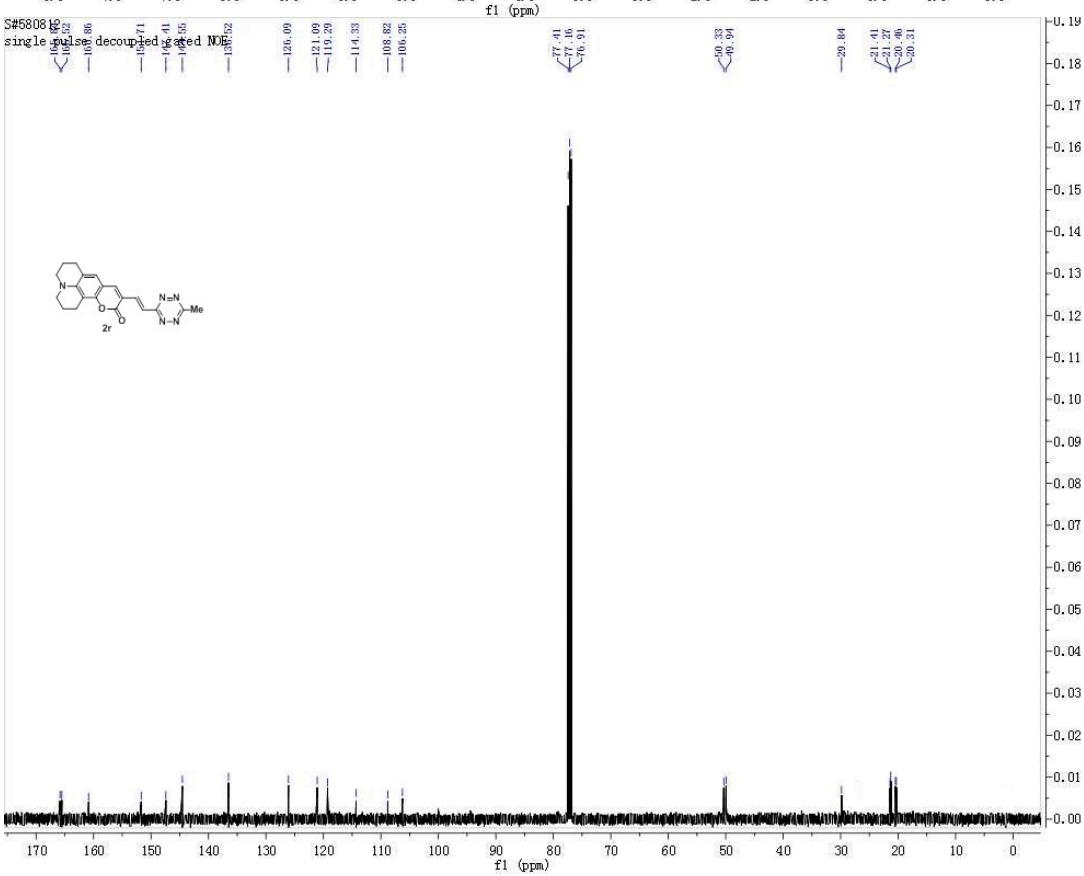

172.36
166.52
164.95
155.25
140.75
138.81
134.12
130.25
128.46
120.56
80.31
77.58
77.27
76.95
54.51
52.60
38.55
28.51
21.42
NHBoc
COOMe
f1 (ppm)

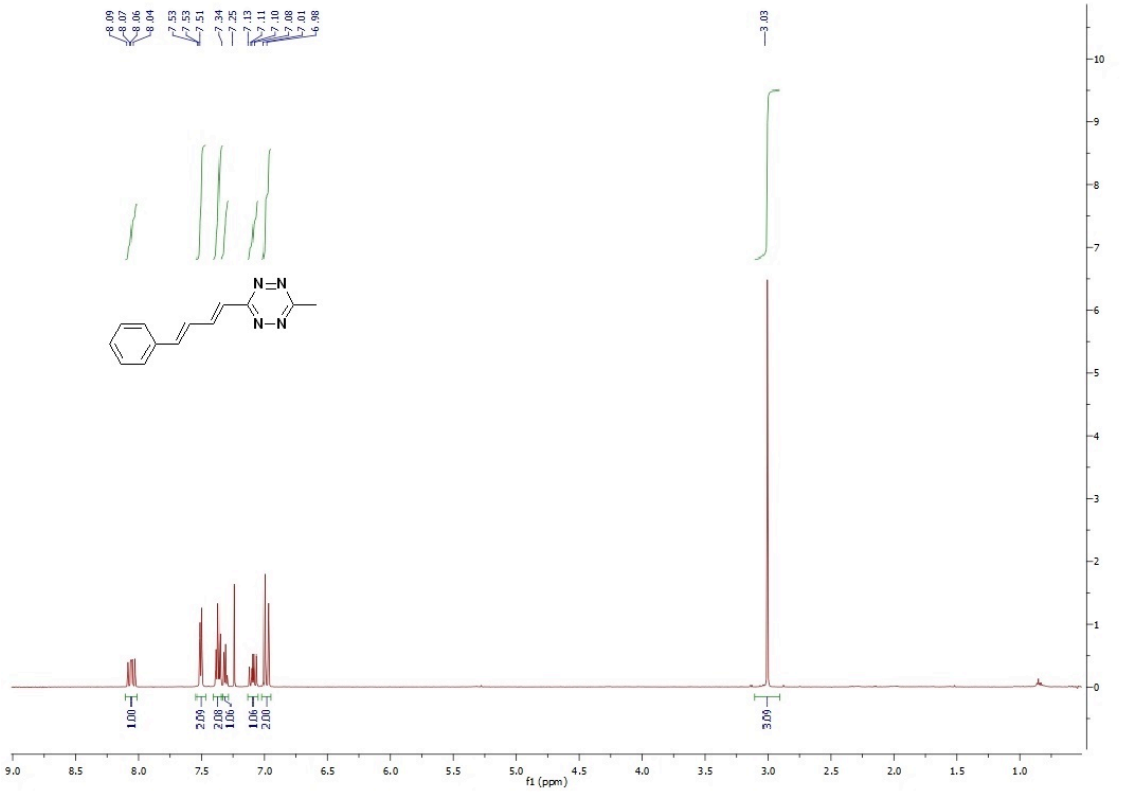

8.42
8.39
8.16
7.78
7.68
7.66
7.43
7.25
5.19
5.17
4.68
4.67
3.71
3.28
3.22
3.21
3.04
1.67
1.42
COOMe
NHBoc
Boc
1.00
0.89
1.01
1.00
1.98
0.81
0.83
2.96
1.88
3.07
9.02
8.02
f1 (ppm)

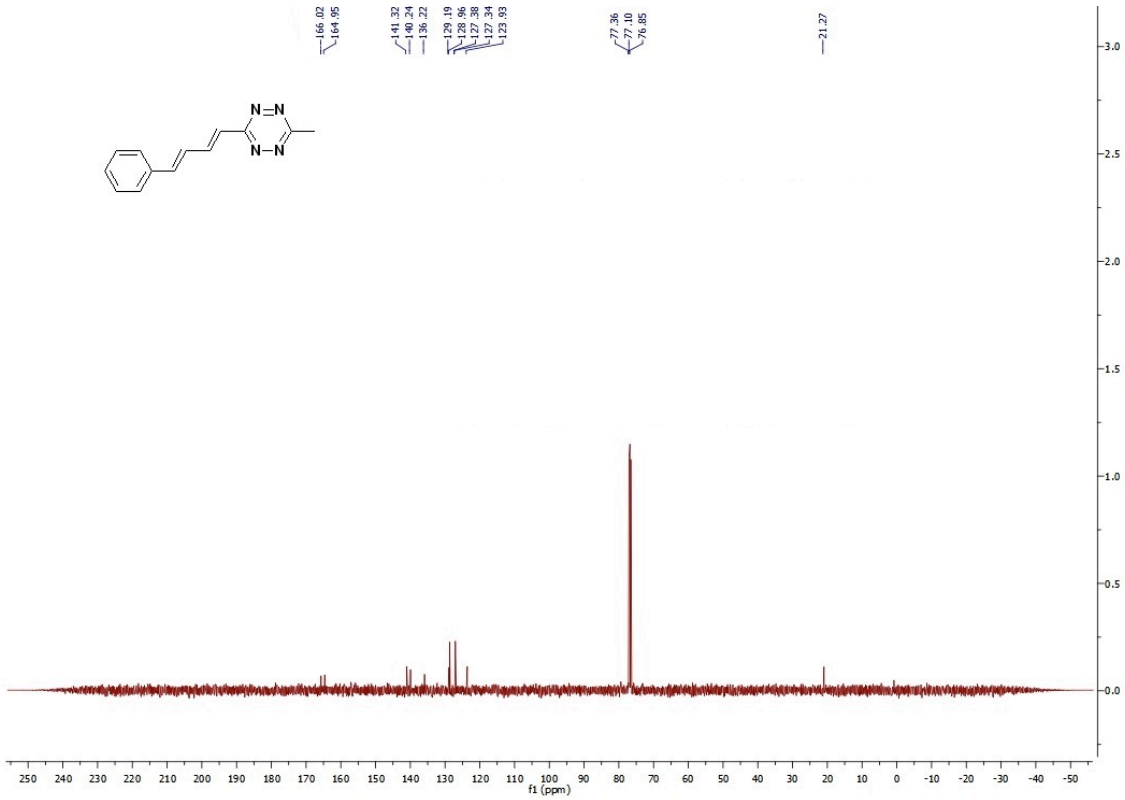

172.44
166.33
165.13
155.27
149.47
141.74
136.65
131.32
129.98
125.48
124.43
119.73
119.44
116.06
115.66
84.48
80.36
77.57
77.25
76.94
53.85
52.71
28.54
28.41
28.13
21.41
COOMe
NHBoc
Boc
f1 (ppm)

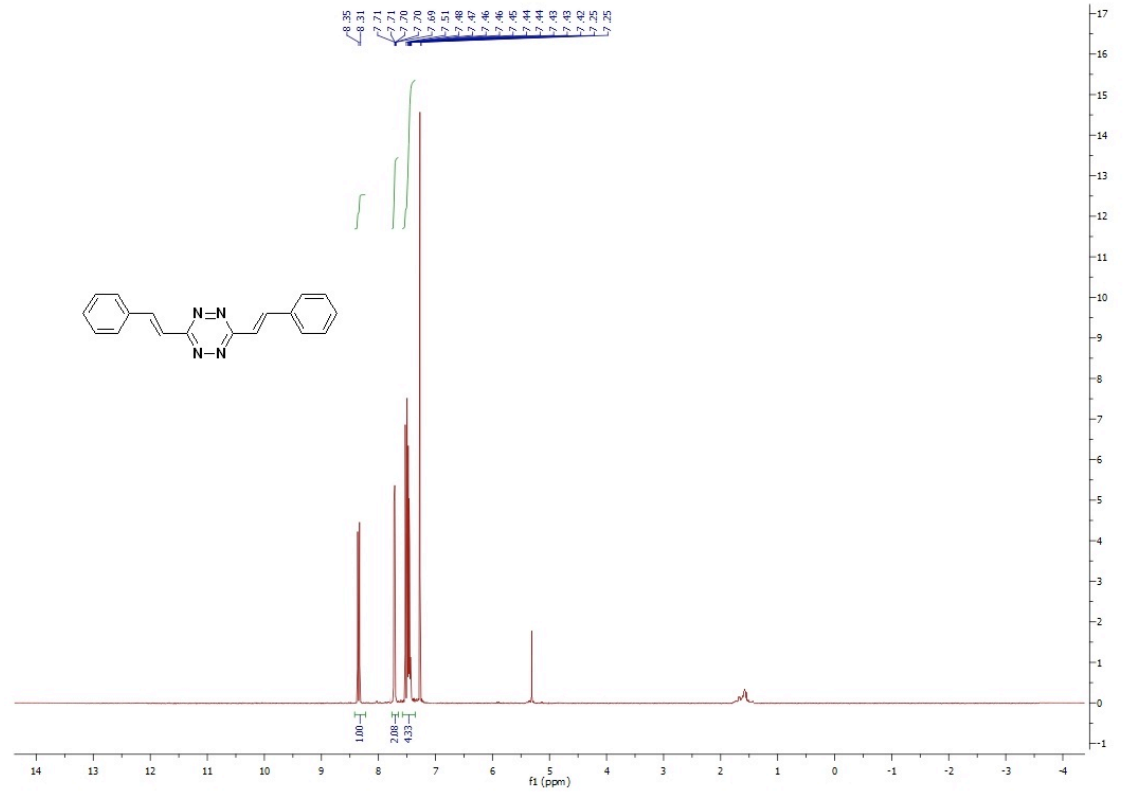

TBSO
OTBS
NH
f1 (ppm)

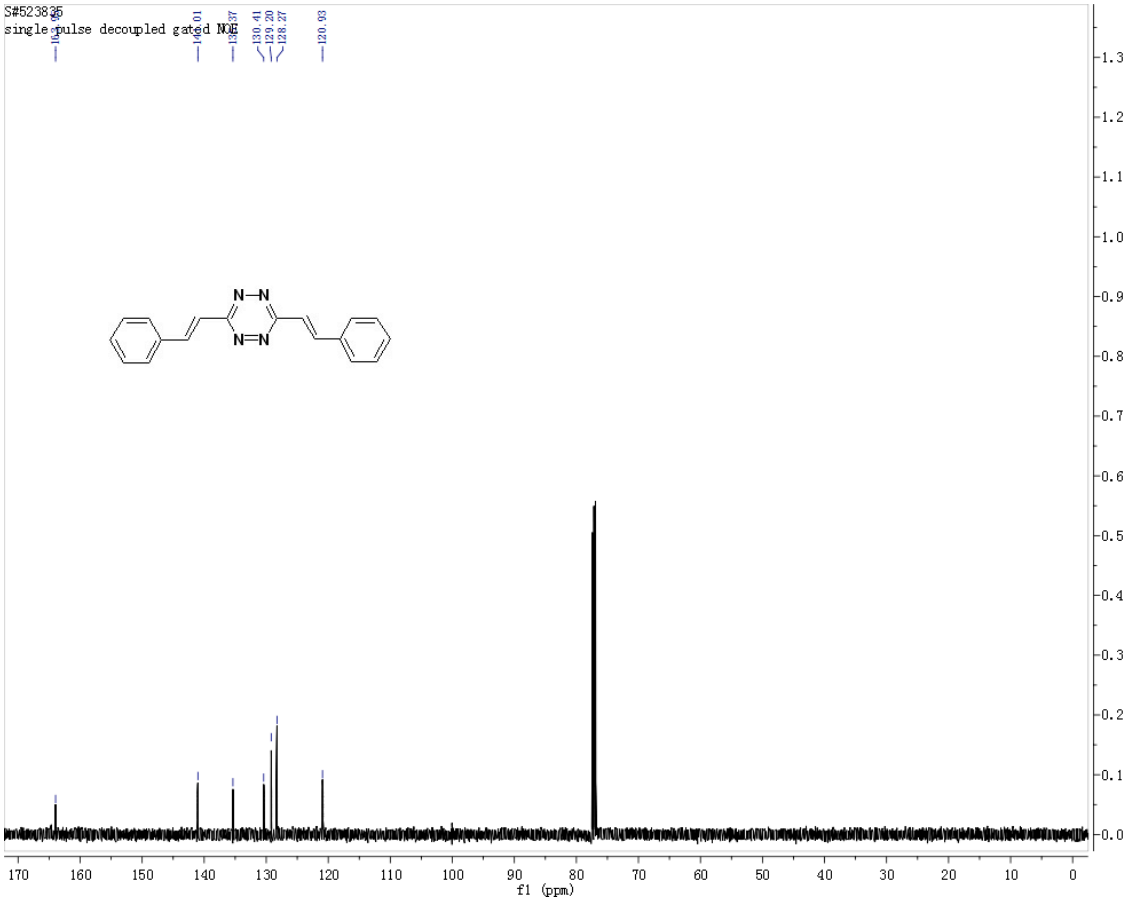

S#519041
single pulse
7.26
6.93
3.34
3.33
3.32
3.31
3.02
2.92
2.91
2.90
2.79
2.78
2.76
2.00
1.99
1.98
1.97
1.00
0.91
0.92
0.93
3.88
2.88
1.95
1.94
3.93
f1 (ppm)
2r
Me

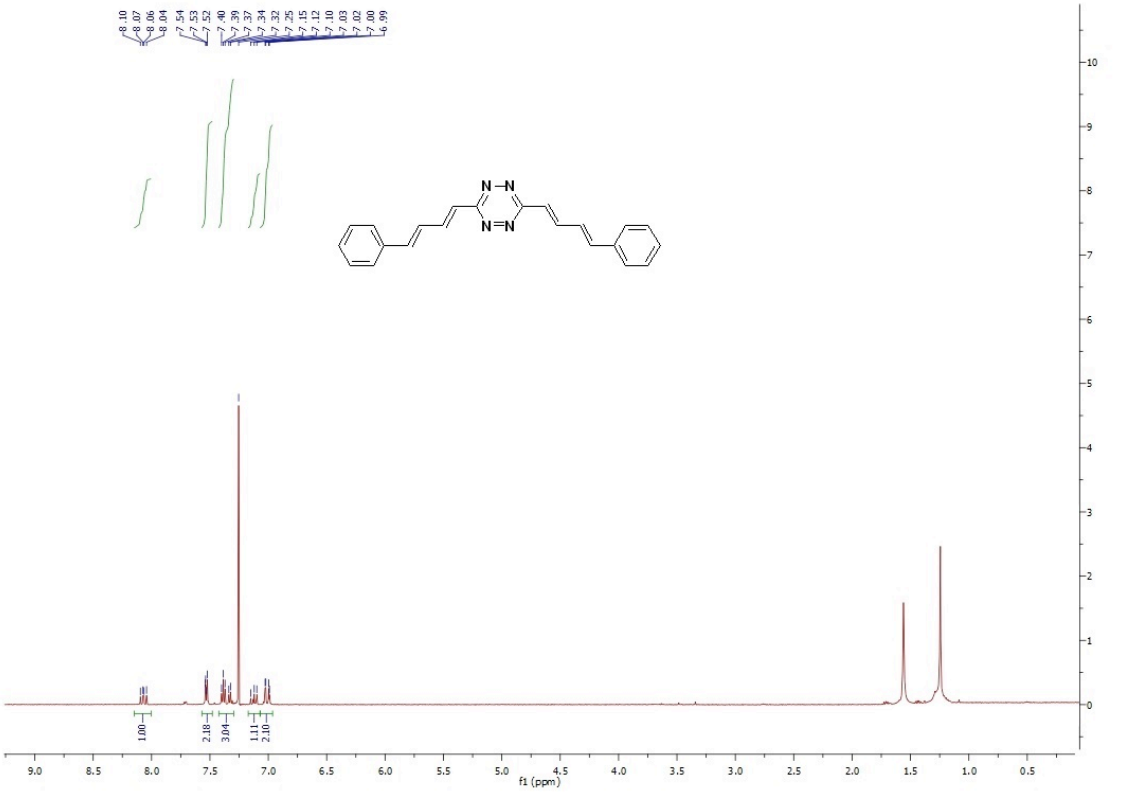

S#580812
single pulse decoupled gated NOE
165.52
165.86
152.71
146.41
146.55
136.52
126.09
121.09
119.29
114.33
108.82
106.25
77.41
77.16
76.91
50.33
49.94
29.84
21.41
21.27
20.46
20.31
f1 (ppm)
2r
Me

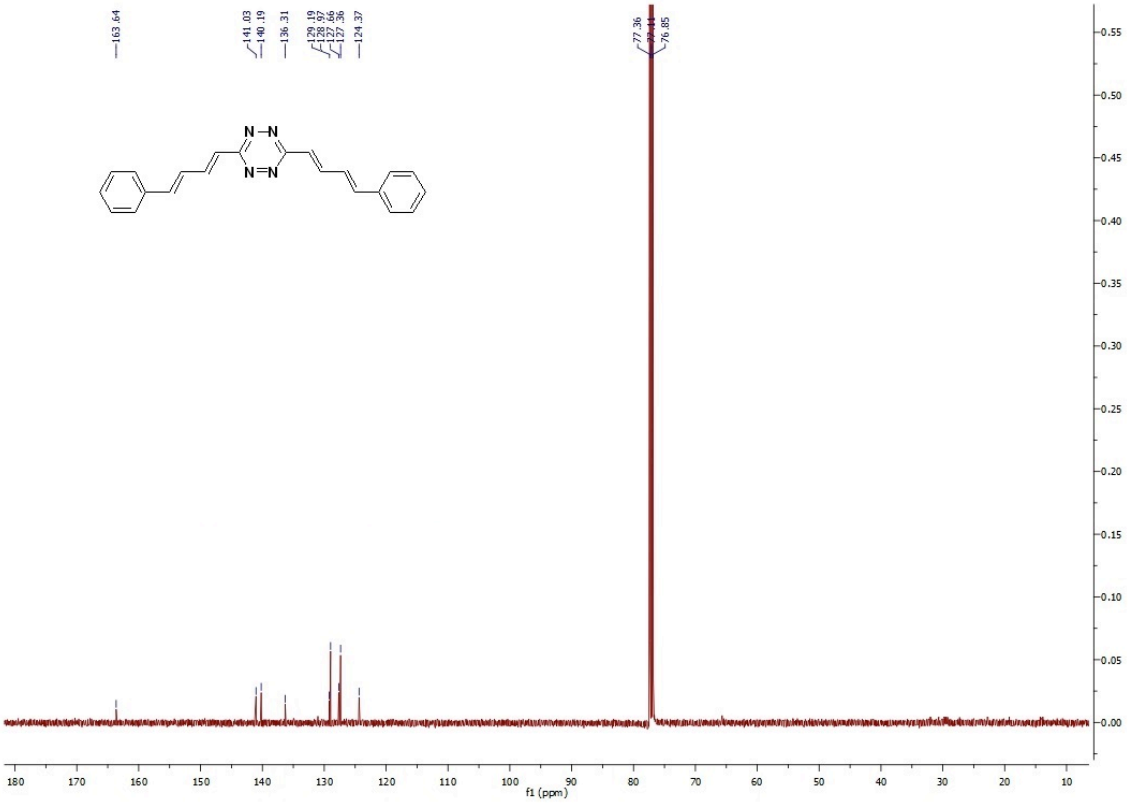

8.09
8.07
8.06
8.04
7.53
7.53
7.51
7.34
7.25
7.13
7.11
7.10
7.08
7.01
6.98
3.03
1.00
2.09
2.08
1.06
1.06
2.00
3.00
f1 (ppm)

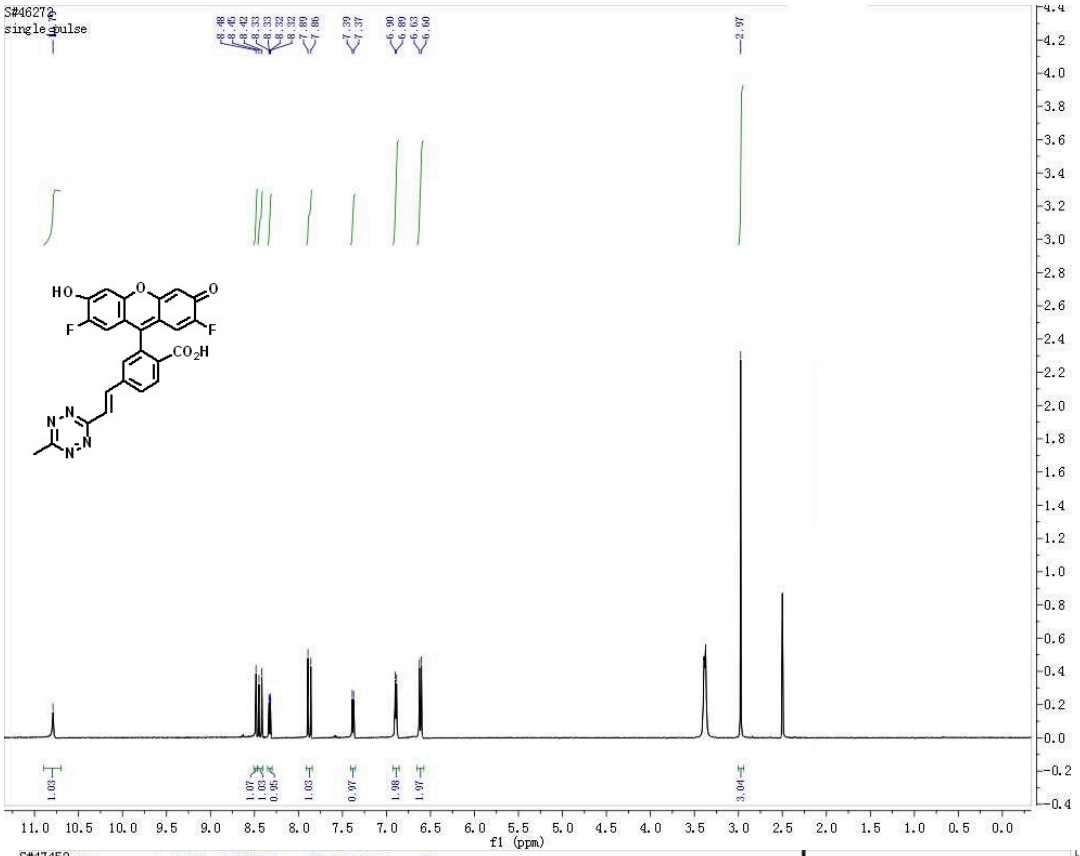

166.02
164.95
141.32
140.24
136.22
129.19
128.96
127.38
127.34
123.93
77.36
77.10
76.85
21.27
f1 (ppm)

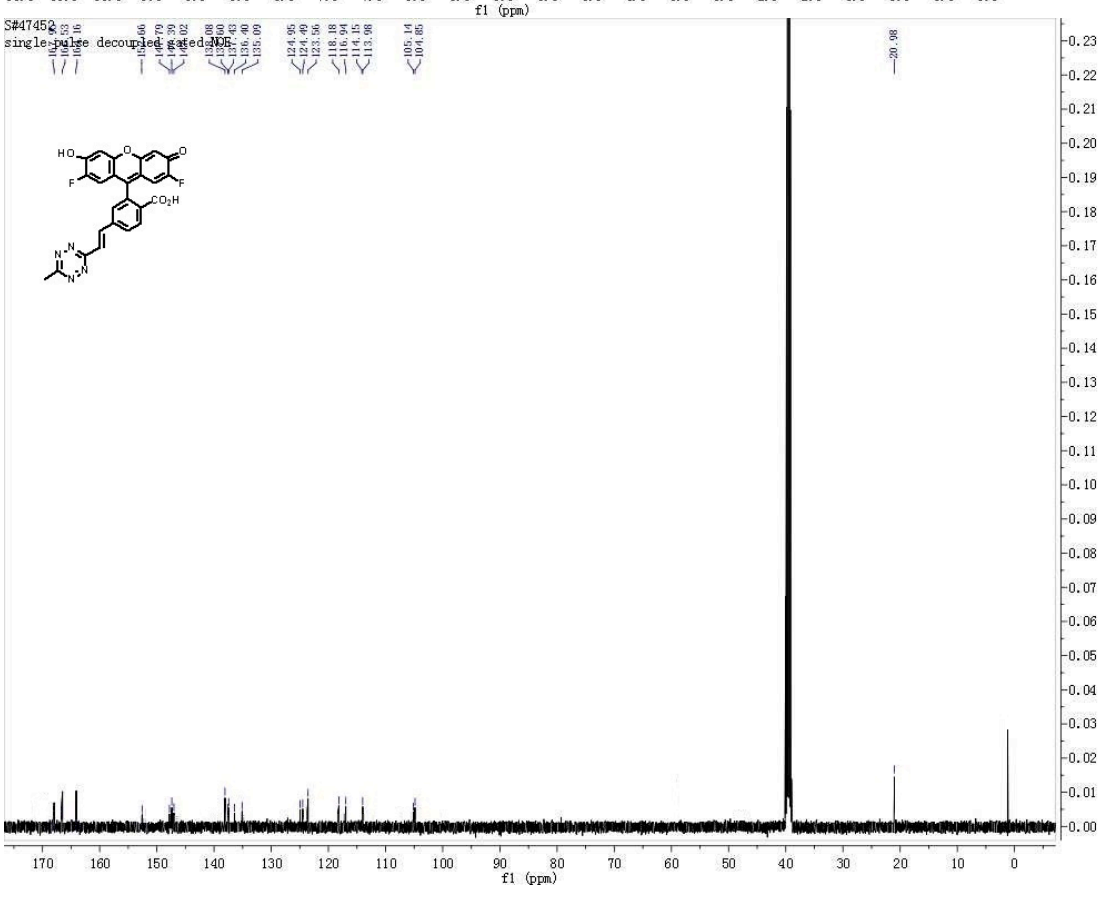

8.35
8.31
7.71
7.71
7.70
7.70
7.69
7.51
7.48
7.47
7.46
7.46
7.45
7.44
7.44
7.43
7.43
7.42
7.25
7.25
1.00
2.08
4.33
f1 (ppm)

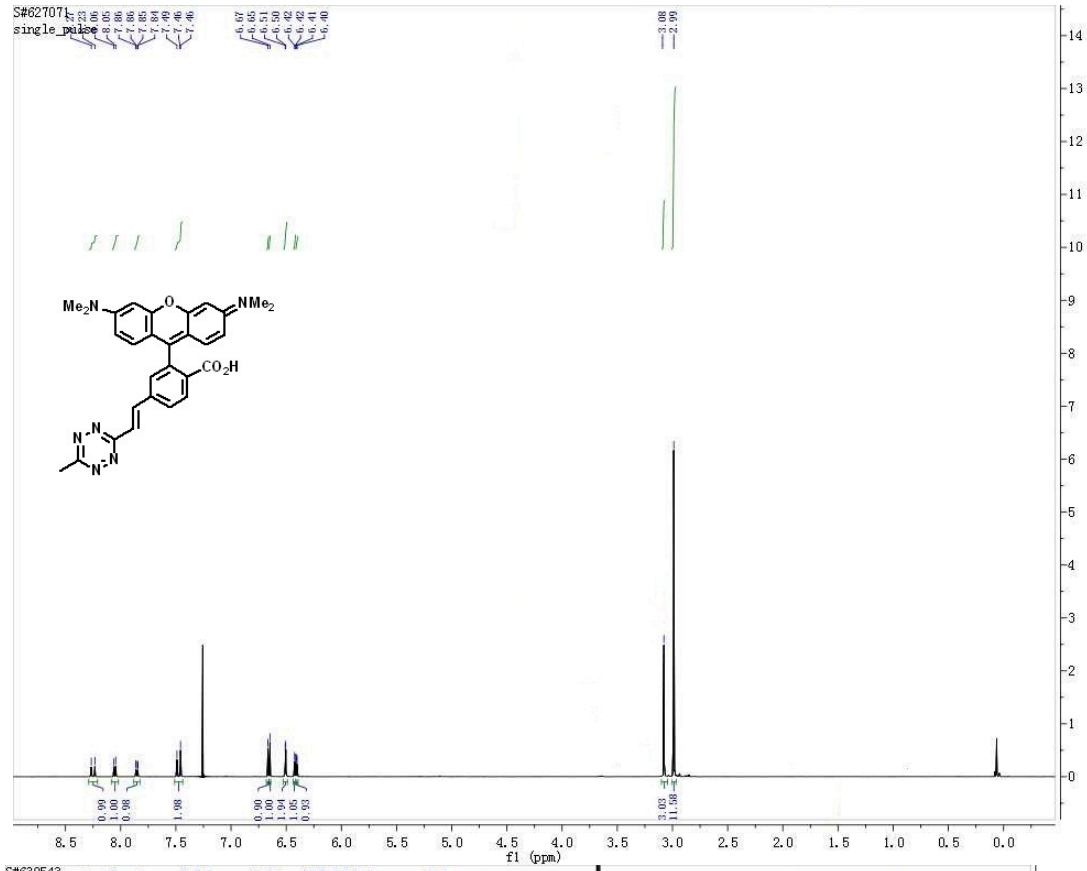

S#523835
single pulse decoupled gated NOE
164.01
130.41
129.20
126.27
120.93
f1 (ppm)

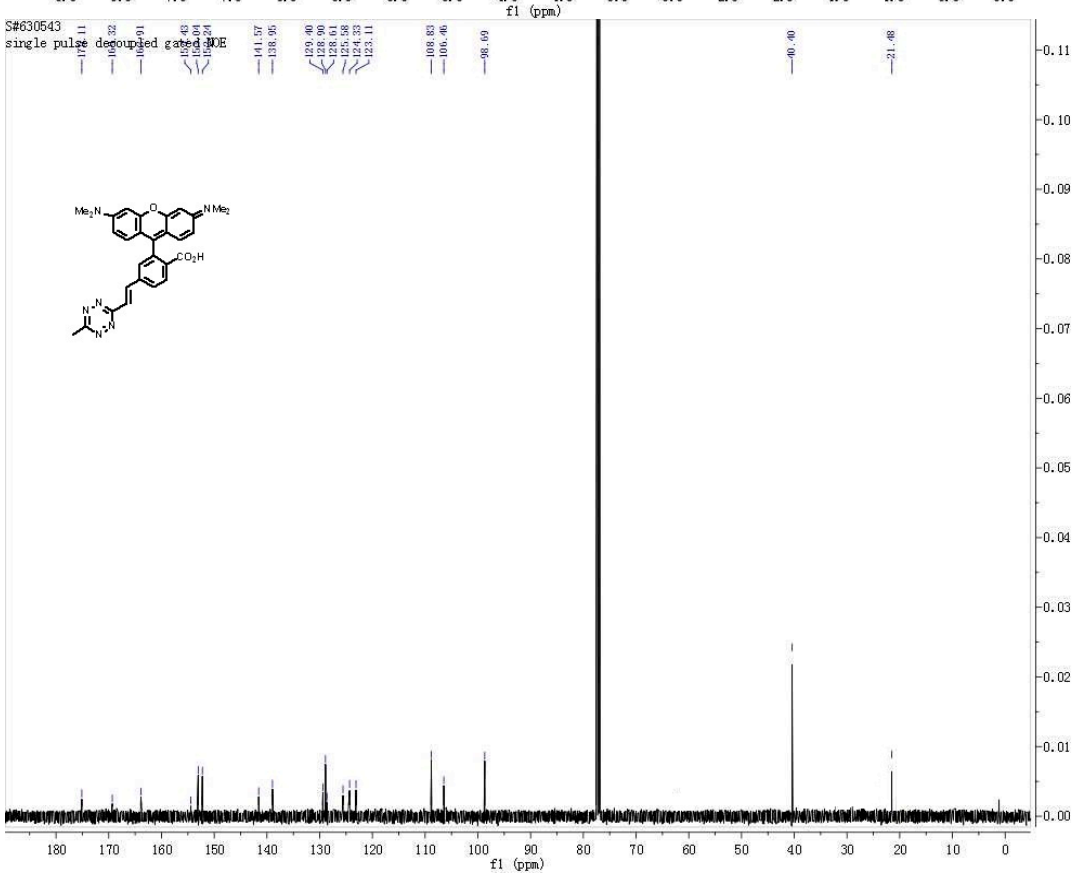

8.10
8.07
8.06
8.04
7.54
7.53
7.52
7.40
7.39
7.37
7.34
7.32
7.25
7.15
7.12
7.10
7.03
7.02
7.00
6.99
N–N
N=N
1.00
2.18
3.04
1.11
2.10
f1 (ppm)

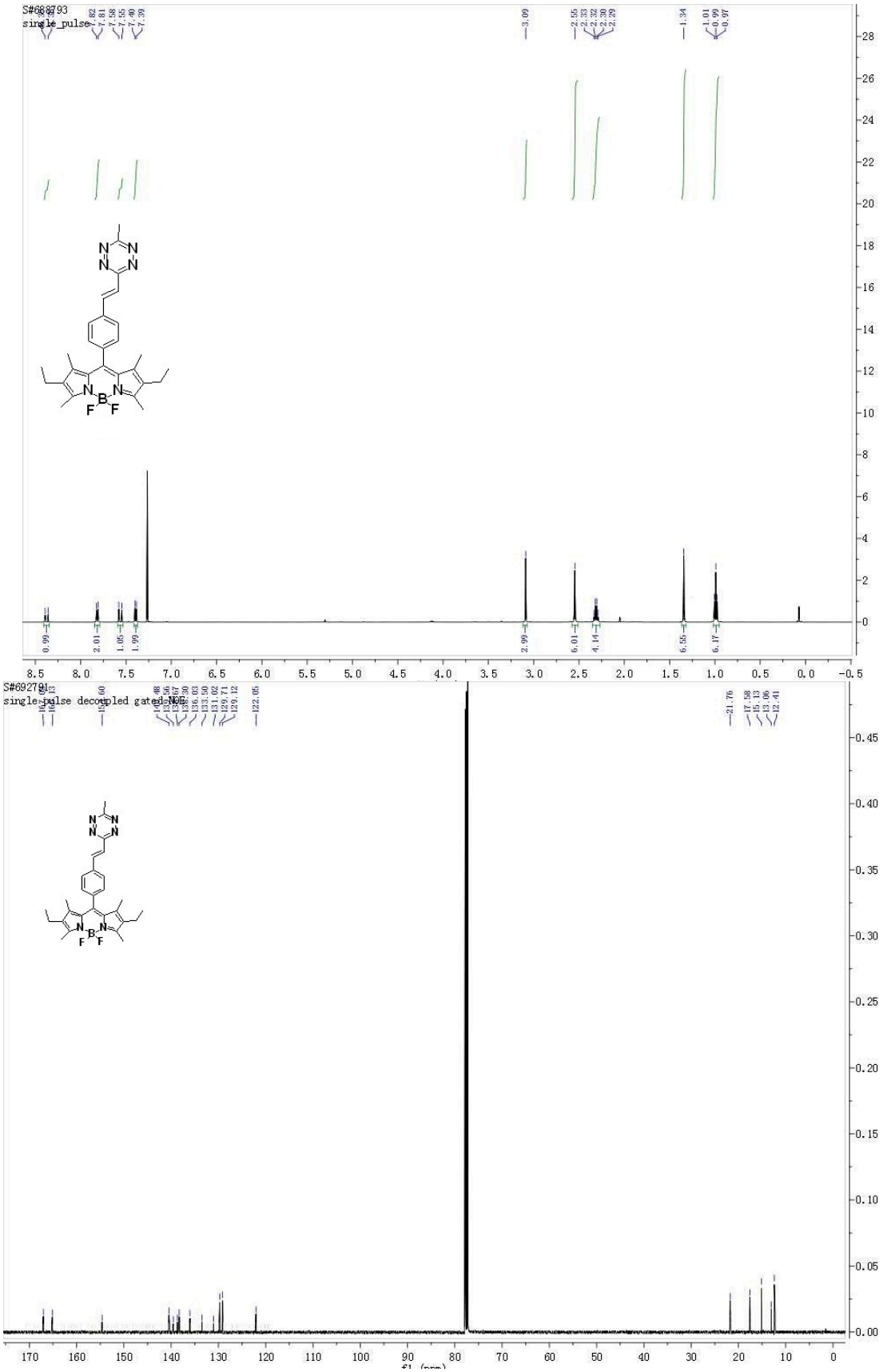

163.64
141.03
140.19
136.31
129.19
128.97
127.66
127.38
124.37
77.36
77.01
76.85
N–N
N=N
f1 (ppm)

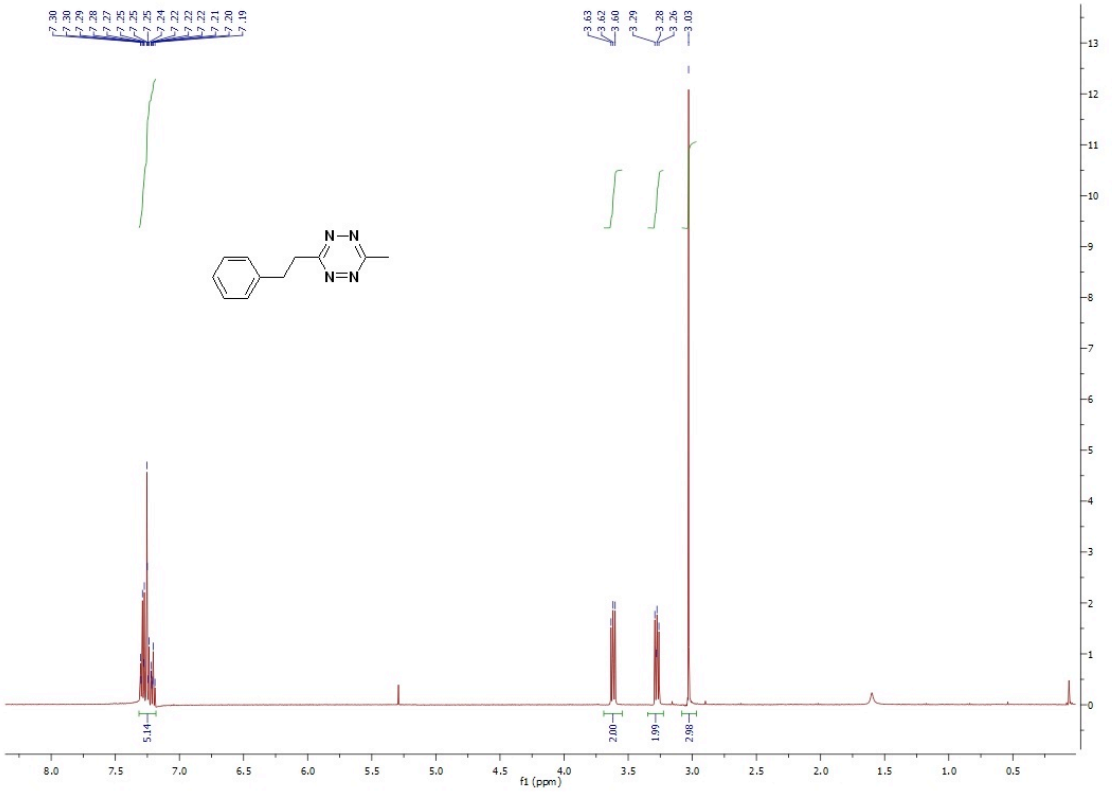

S#46272
single pulse
f1 (ppm)

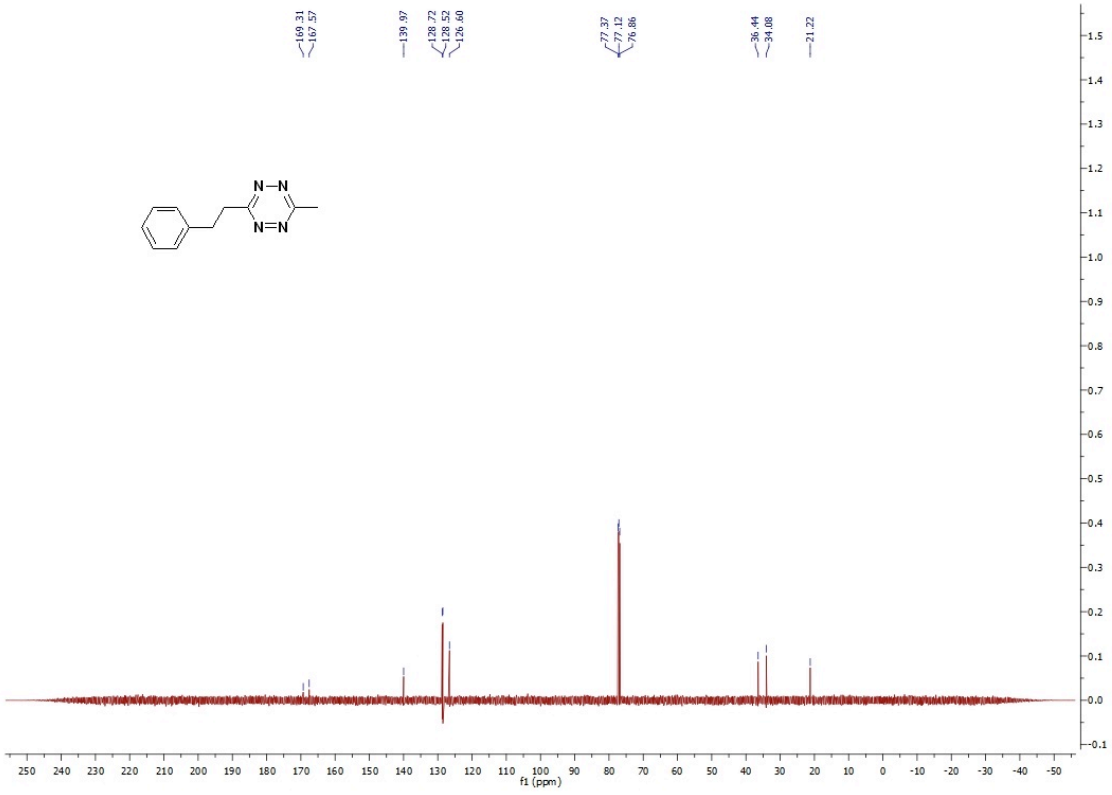

S#47452
single-pulse decoupled gated NOE
f1 (ppm)

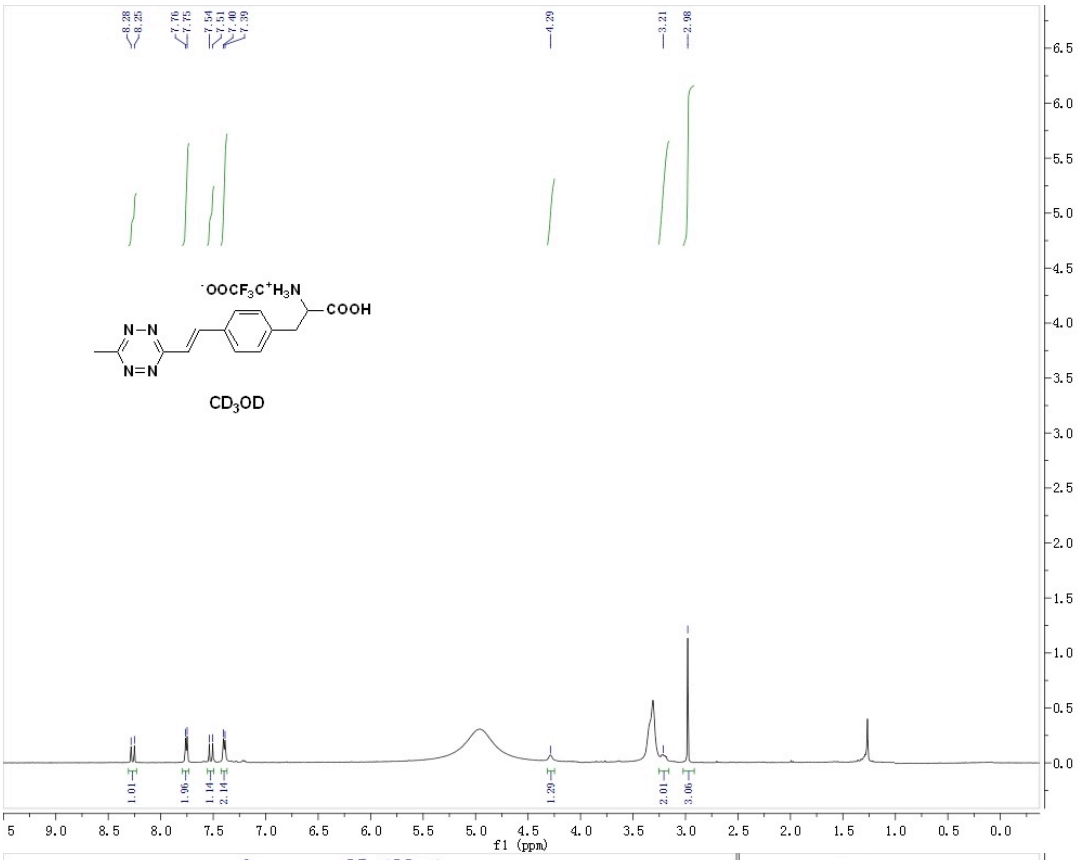

S#627071
single_pulse
Me2N
NMe2
CO2H
f1 (ppm)

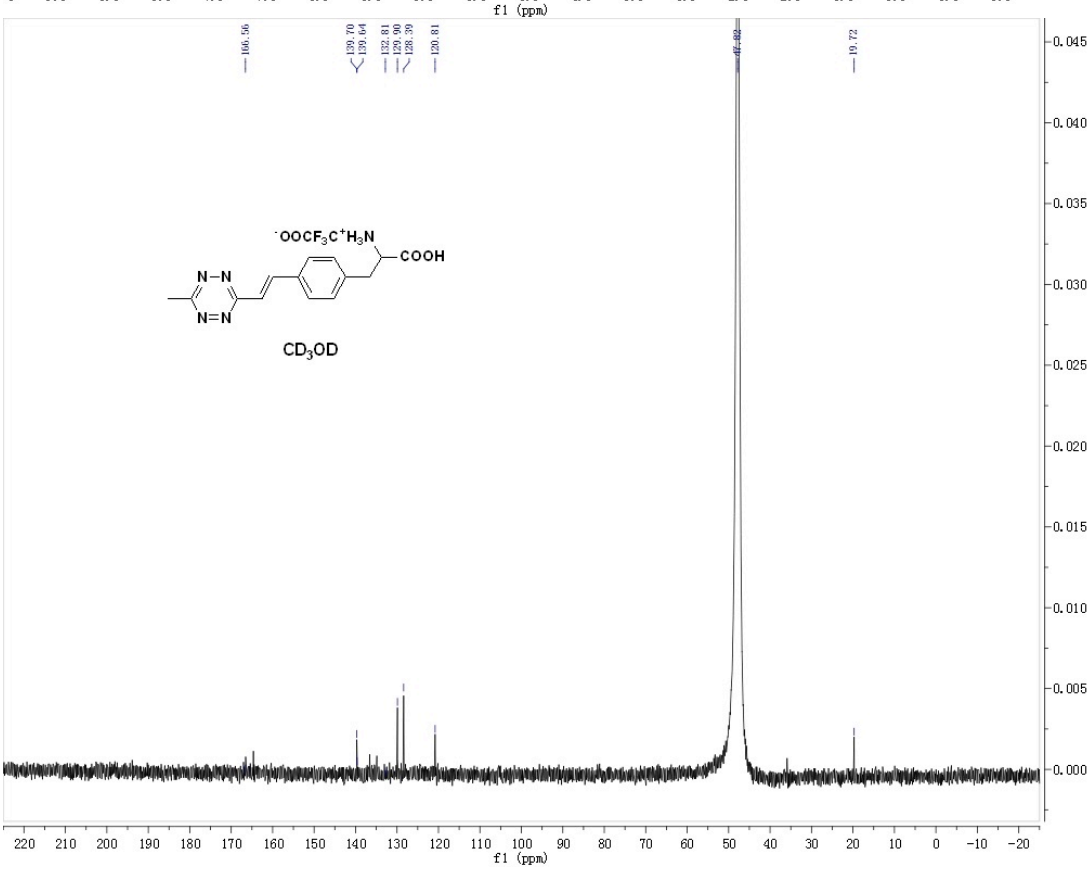

S#630543
single pulse decoupled gated NOE
Me2N
NMe2
CO2H
f1 (ppm)

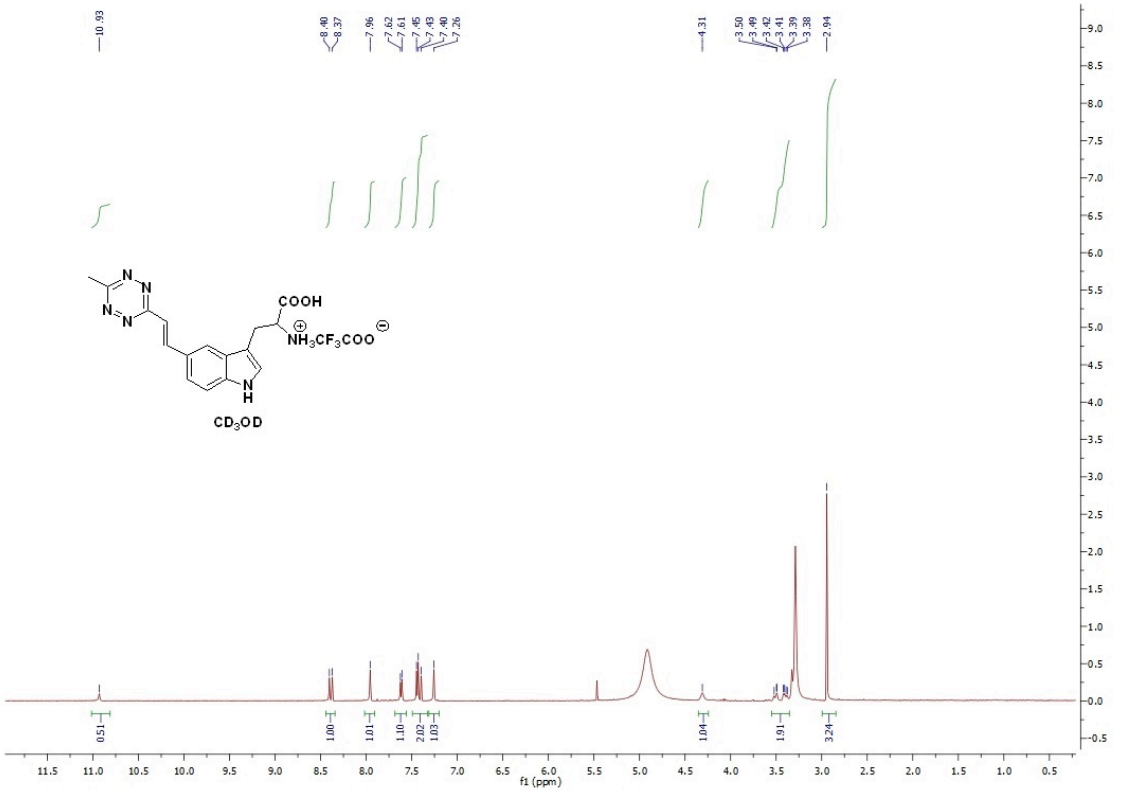

S#692793
single_pulse
7.82
7.81
7.58
7.55
7.40
7.39
3.09
2.55
2.33
2.32
2.30
2.29
1.34
1.01
0.99
0.97
0.99
2.01
1.05
1.99
2.99
6.01
4.14
6.55
6.17
S#692791
single_pulse decoupled gated NOE
170.60
136.03
133.50
131.02
129.71
129.12
122.05
21.76
17.58
15.13
13.06
12.41

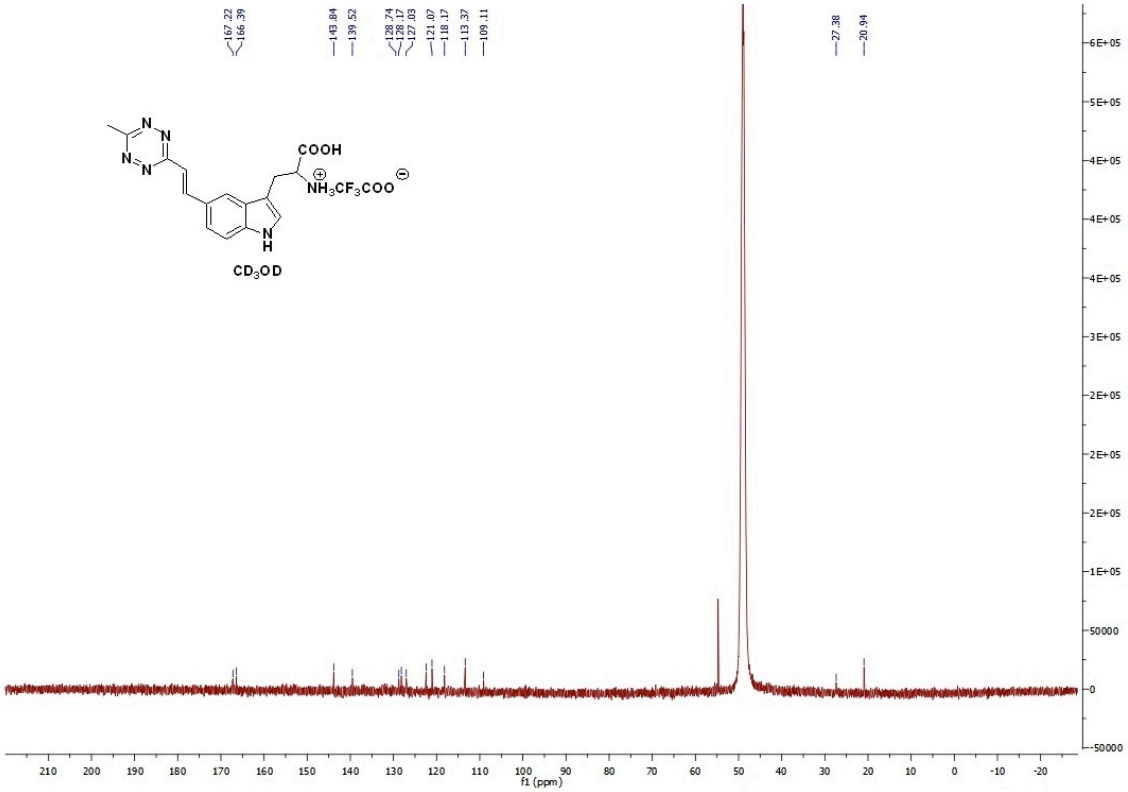

7.30
7.30
7.29
7.28
7.27
7.25
7.25
7.25
7.24
7.22
7.22
7.22
7.21
7.20
7.19
3.63
3.62
3.60
3.29
3.28
3.26
3.03
N–N
N=N
5.14
2.00
1.99
2.98
f1 (ppm)

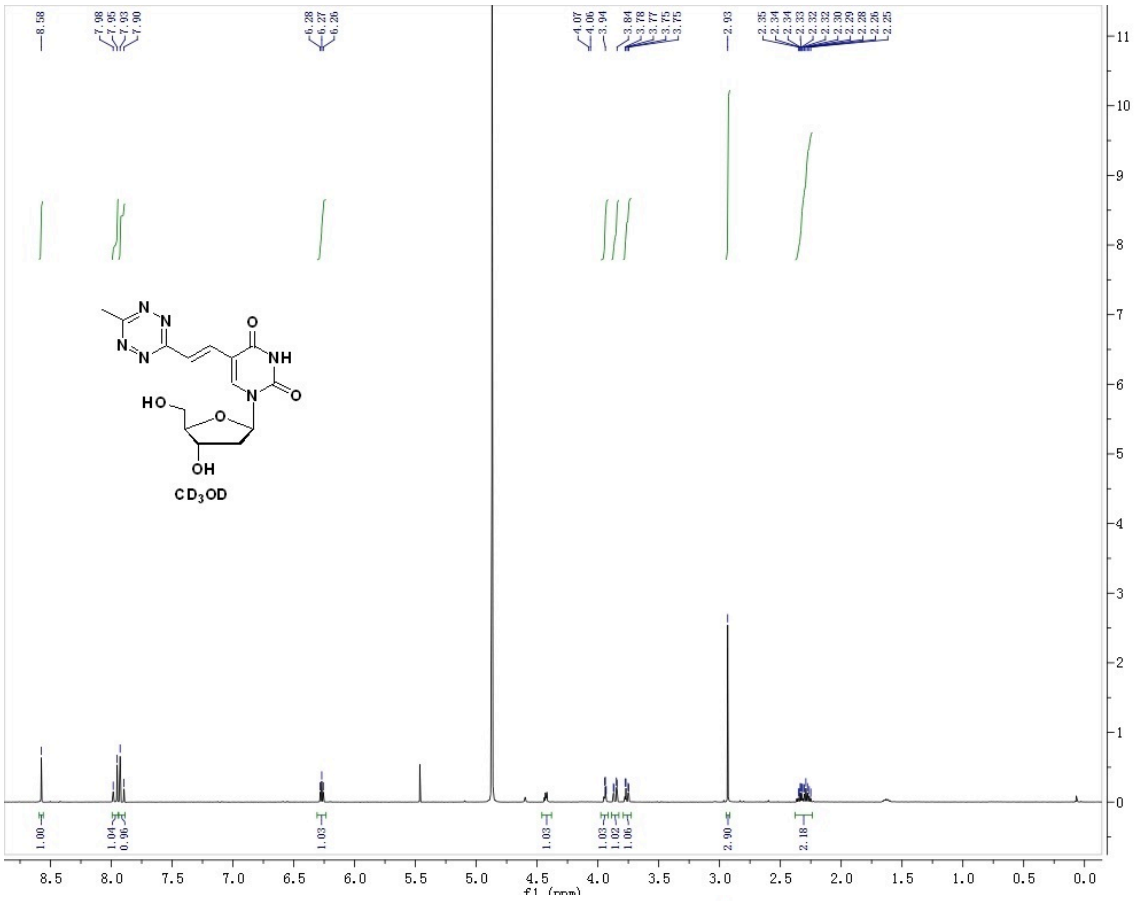

169.31
167.57
139.97
128.72
128.52
126.60
77.37
77.12
76.86
36.44
34.08
21.22
N–N
N=N
f1 (ppm)

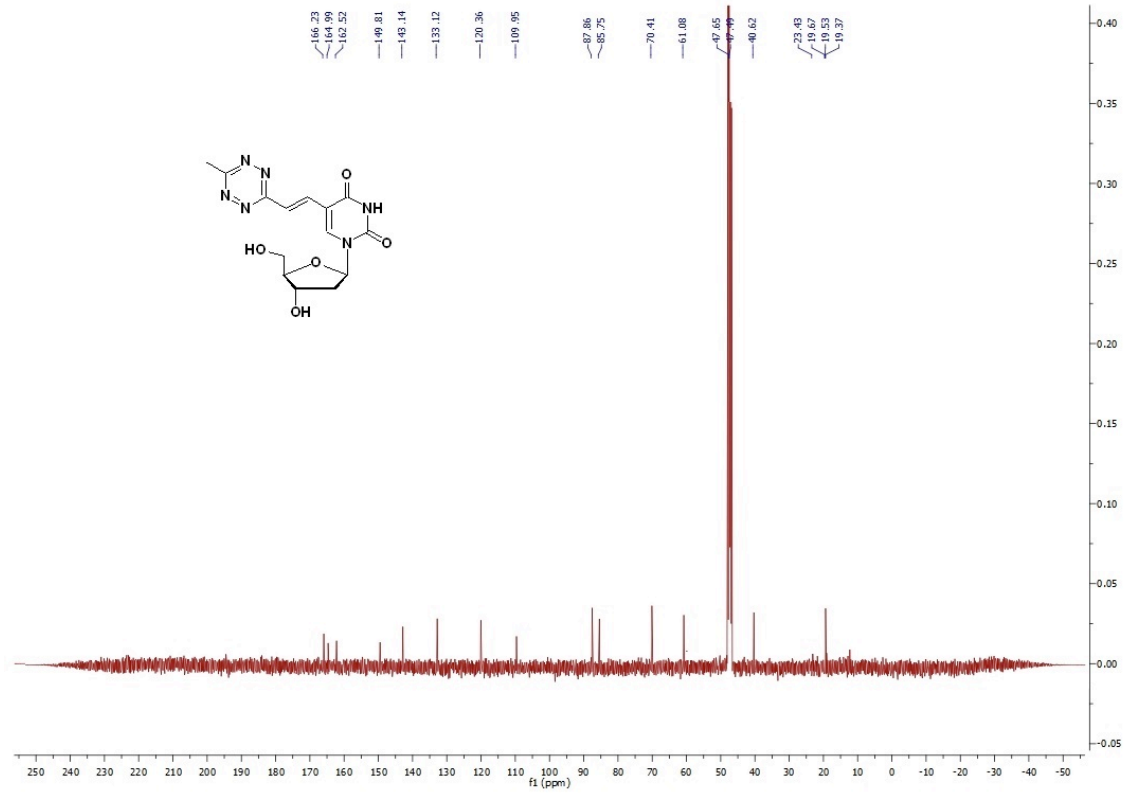

8.28
8.25
7.76
7.75
7.54
7.51
7.40
7.39
4.29
3.21
2.98
·OOCF3C+H3N
COOH
N–N
N=N
CD3OD
1.01
1.96
1.14
2.14
1.29
2.01
3.06
f1 (ppm)


166.56
139.70
139.64
132.81
129.90
128.39
120.81
47.82
19.72
·OOCF3C+H3N
COOH
N–N
N=N
CD3OD
f1 (ppm)


10.93
8.40
8.37
7.96
7.62
7.61
7.45
7.43
7.40
7.26
4.31
3.50
3.49
3.42
3.41
3.39
3.38
2.94
COOH
NH3CF3COO
CD3OD
0.51
1.00
1.01
1.10
2.02
1.03
1.04
1.91
3.24
f1 (ppm)


167.22
166.39
143.84
139.52
128.74
128.17
127.03
121.07
118.17
113.37
109.11
27.38
20.94
COOH
NH3CF3COO
CD3OD
f1 (ppm)


8.58
7.98
7.95
7.93
7.90
6.28
6.27
6.26
4.07
4.06
3.94
3.84
3.78
3.77
3.75
3.75
2.93
2.35
2.34
2.34
2.33
2.32
2.32
2.30
2.29
2.28
2.26
2.25
HO
O
NH
N
O
OH
CD3OD
1.00
1.04
0.96
1.03
1.03
1.03
1.02
1.06
2.90
2.18
f1 (ppm)


166.23
164.99
162.52
149.81
143.14
133.12
120.36
109.95
87.86
85.75
70.41
61.08
47.65
40.62
23.43
19.67
19.53
19.37
HO
O
NH
N
O
OH
f1 (ppm)