Supporting Information Part II for

Measuring Nanometer Distances in Proteins and Rigid Rulers between ^{19}F and Gd^{3+} by Integration of ^{19}F-ENDOR Signal Intensities

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General

Unless otherwise stated, reactions were performed using commercial solvents and reagents. $PdCl_2(PPh_3)_2$ was synthesized according to the literature.[1] The proton-exchange resin (Dowex® 50WX4 hydrogen form, Sigma-Aldrich, 91 g) was sequentially washed with THF (3 × 200 mL), EtOH (2 × 100 mL), H_2O (2 × 150 mL), and EtOH (200 mL), and then dried over P_4O_{10} at 0.05 mbar for 5 days to obtain a pure and dry proton-exchange resin (30 g). THF (HPLC, VWR) used in reactions were distilled from Na/benzophenone. Solvents used for extraction and chromatography were purchased in technical quality and distilled prior to use. Aqueous solutions were prepared using Milli-Q H_2O. The pH and pD of solutions were determined using pH indicator strips (resolution: 0.2 pH). Since all commercial compounds had a purity of >95%, their amount of substance given in the preparation procedures was calculated using their compound mass without correction through the manufacturer-specified purities.

Argon (Linde, 4.0) was passed through anhydrous $CaCl_2$ prior to use. Degassed solutions were obtained through three freeze−pump−thaw cycles and ventilation of the flask with argon. The temperatures given in the preparative procedures are the bath temperatures. Unless otherwise stated, solvents were removed at ~40 °C and reduced pressure using a rotary evaporator. Traces of remaining solvents were removed at room temperature at <1 mbar. For short path distillations, the reaction flask and the receiver flask were connected by a short, curved glass tube with two ground joints, reduced pressure (<1 mbar) was applied, the liquid in the reaction flask was stirred at 40 °C, and the receiver flask was cooled with liquid nitrogen.

Unless otherwise stated, thin layer chromatography (TLC) was performed on silica gel-coated aluminum foil (Merck, 60 F254), and the spots were detected with UV light (254 nm). Automated preparative flash column chromatography using UV detection at 254 nm was performed on a Pure C-850 FlashPrep instrument (Büchi Labortechnik). The commercial names of the pre-packed, silica gel 60 M filled columns are given in the individual procedures mentioned below. Preparative reverse-phase HPLC was performed with UV detection at 254 nm using a Phenomenex Luna C18(2) column

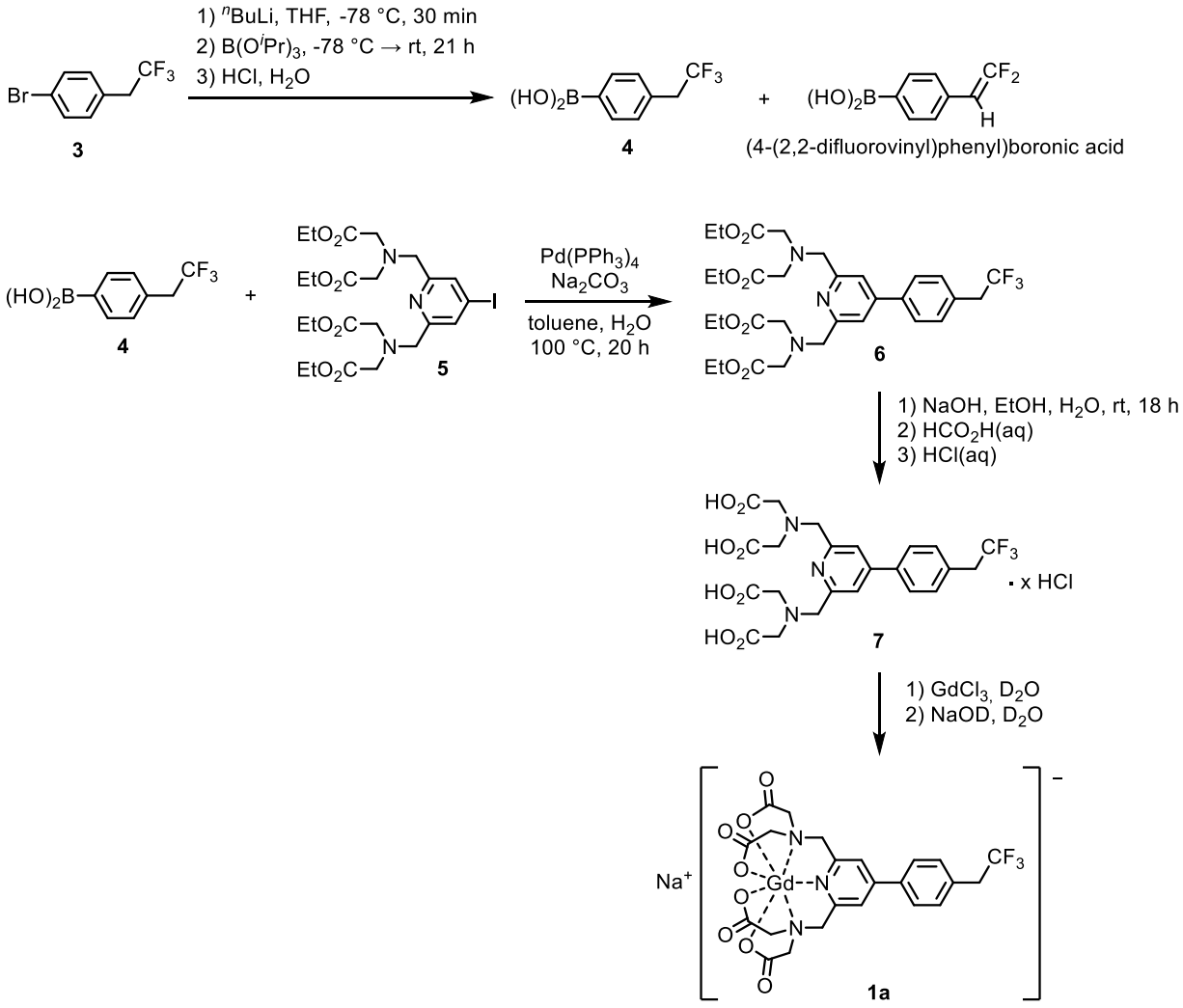
(particle size 5 µm, pore size 100 Å, column size 21.2 mm × 250 mm) at room temperature. The composition of solvent mixtures is given in volume ratios.

Unless otherwise stated, NMR spectra were calibrated using the signal of the solvent as an internal standard [$CDCl_3$: δ(^{1}H of $CHCl_3$)= 7.26 ppm, δ(^{13}C of $CDCl_3$) = 77.16 ppm; DMSO-d_6: δ(^{1}H of DMSO-d5) = 2.50 ppm, δ(^{13}C of DMSO-d6) = 39.52 ppm; D_2O: δ(^{1}H of HDO) = 4.79 ppm]. For the calibration of ^{13}C NMR spectra of compounds in D_2O, a drop of MeOH [δ(^{13}C) = 49.50 ppm] was added. For the calibration of ^{19}F NMR spectra a capillary filled with $CFCl_3$ [δ(^{19}F of $CFCl_3$ = 0.00 ppm)] was added. NMR signal assignment is supported by distortionless enhancement by polarization transfer (DEPT) 135, ^{1}H,^{13}C-heteronuclear multiple quantum coherence (HMQC), and ^{1}H,^{13}C-heteronuclear multiple bond correlation (HMBC) NMR experiments. The contents of ruler precursors were determined by quantitative ^{1}H NMR (qNMR) spectroscopy as described in our previous work.[2] These contents were used to calculate the yield. The given ratio of components in a mixture is a molar ratio and was determined by ^{1}H NMR spectroscopy.

ESI mass spectra were recorded using an Esquire 3000 ion trap mass spectrometer (Bruker Daltonik) equipped with a nano-ESI source. Accurate ESI mass measurements were acquired using a Q-IMS-TOF mass spectrometer Synapt G2Si (Waters, Manchester, UK) in the resolution mode, interfaced with a nano-ESI ion source. Nitrogen, provided by the nitrogen generator NGM 11, served both as the nebulizer gas and the dry gas for nano-ESI. Helium (Linde, 5.0) was used as buffer gas in the IMS entry cell. Nitrogen (Linde, 5.0) was used for ion mobility separation. Samples were introduced by static nano-ESI using emitters pulled in-house from glass capillaries. The mono-isotopic masses of the compounds are reported.

In the names of compounds **1a-e**, **7**, **10**, **11**, **17**, **18**, **22**, **23**, and **28**, the spacer units "ethynylene" and "*para*-phenylene" are abbreviated as "E" and "P", respectively. For example, "Na[{Gd^{III}(PyMTA)}-EP-CH_2CF_3] **1b**" represents "Na[{Gd^{III}(PyMTA)}-ethynylene-*para*-phenylene-CH_2CF_3] **1b**."

Synthesis of ruler 1a



Scheme S-II-1. Synthesis of ruler **1a**.

(4-(Trifluoroethyl)phenyl)boronic acid 4. This reaction was performed under argon. A 1.6 M solution of ^{n}BuLi in hexane (3.4 mL, 5.4 mmol) was added at -78 °C dropwise over 10 min to a colorless solution of 1-bromo-4-(trifluoroethyl)benzene **3** (997 mg, 4.17 mmol) in THF (25 mL). The color of the solution turned from colorless to yellow-orange to dark brown-green. The solution was stirred at -78 °C for 30 min. B(O^{i}Pr)$_3$ (2.9 mL, 12.6 mmol) was added at -78 °C. The cooling bath was removed, and the yellow suspension was stirred at room temperature for 21 h. The yellow suspension was cooled with an ice-water-bath and 2 M aqueous HCl (2.5 mL) was added under cooling. The pH of the reaction mixture was ca. 1.5. The cooling bath was removed, and the reaction mixture was stirred at room temperature for 1.5 h. Et$_2$O (25 mL) and

H_2O (25 mL) were added and the phases were mixed well. The two phases were separated. The aqueous phase was extracted with Et_2O (2 × 10 mL). The combined organic phases were washed with saturated aqueous NaCl solution (10 mL), dried over Na_2SO_4, and filtered. Removal of the solvents gave a yellow solid (528 mg). H_2O (6 mL) was added to this solid. The mixture was heated to boiling, then heating was stopped. After briefly letting the mixture stand, a colorless aqueous solution separated from a yellow slimy-like solid. While the mixture was still hot, the colorless aqueous solution was drawn out using a glass pipette. H_2O (4 mL) was added to the remaining yellow slimy-like solid, the mixture was heated to boiling, then heating was stopped. After briefly letting the mixture stand, a colorless aqueous solution separated from a yellow slimy-like solid. While the mixture was still hot, the colorless aqueous solution was drawn out using a glass pipette. The colorless aqueous solutions from both separations were combined and cooled with an ice-water bath. This resulted in the precipitation of colorless crystals. The colorless crystals were collected through filtration and dried at reduced pressure. A 10:1.0 mixture (127 mg) of (4-(trifluoroethyl)phenyl)boronic acid **4** (571 µmol, 14% yield) and (4-(2,2-difluorovinyl)phenyl)boronic acid (57 µmol, 1.4% yield) was obtained as colorless crystals. ^{1}H NMR signals assigned to (4-(trifluoroethyl)phenyl)boronic acid **4** (500 MHz, DMSO-d6): δ = 8.08 (br s, 2 H, $B(OH)_2$), 7.78 (one half of an AA'XX' spin system, 2 H, $H_{benzene}$ *ortho* to B), 7.31 (other half of the AA'XX' spin system, 2 H, $H_{benzene}$ *meta* to B), 3.63 (q, $^{3}J_{HF}$ = 11.7 Hz, 2H, CH_2CF_3). ^{19}F NMR signals assigned to (4-(trifluoroethyl)phenyl)boronic acid **4** (470 MHz, DMSO-d6): δ = -63.6 (t, $^{3}J_{HF}$ = 11.7 Hz CH_2CF_3). ^{13}C NMR signals assigned to (4-(trifluoroethyl)phenyl)boronic acid **4** (126 MHz, DMSO-d6): δ = 134.3 ($C_{benzene}$ *ortho* to B), 132.1 (q, $^{3}J_{CF}$ = 2.7 Hz, $C_{benzene}$ *para* to B), 129.3 ($C_{benzene}$ *meta* to B), 126.4 (q, $^{1}J_{CF}$ = 277.1 Hz, CF_3), 38.6 (q, $^{2}J_{CF}$ = 28.3 Hz, $\underline{C}H_2CF_3$). ^{1}H NMR signals assigned to (4-(2,2-difluorovinyl)phenyl)boronic acid (500 MHz, DMSO-d6): δ = 7.78 (one half of an AA'XX' spin system, 2 H, $H_{benzene}$ *ortho* to B), 7.33 (other half of the AA'XX' spin system, 2 H, $H_{benzene}$ *meta* to B), 5.78 (dd, $^{3}J_{HF}$ = 28.3 Hz, $^{3}J_{HF}$ = 4.2 Hz, 1 H, $CH=CF_2$). ^{19}F NMR signals assigned to (4-(2,2-difluorovinyl)phenyl)boronic acid (470 MHz, DMSO-d6): -81.3 (dd, $^{3}J_{HF}$ = 28.3 Hz, $^{2}J_{FF}$ = 32.1 Hz, F *trans* to H), -83.5 (dd, $^{3}J_{HF}$ = 4 Hz, $^{2}J_{FF}$ = 31 Hz, F *cis* to H). MS (ESI) *m/z* = 238.7 [**4** + Cl]$^{-}$, 218.8 [(4-(2,2-difluorovinyl)phenyl)boronic acid + Cl]$^{-}$.

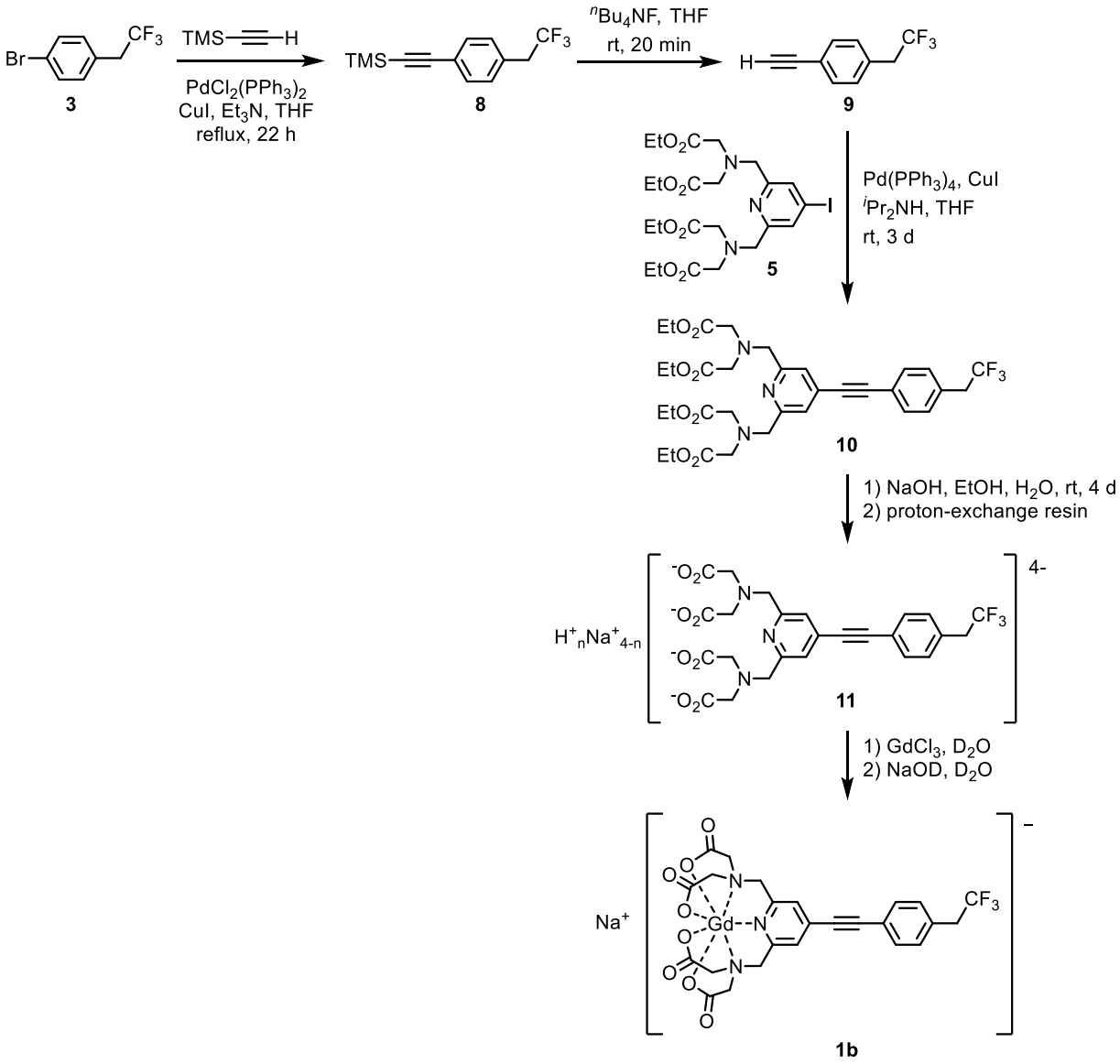
(4-(Trifluoroethyl)phenyl)-PyMTA ester 6. This reaction was performed under argon. A two-phase solution of 4-iodo-PyMTA ester (**5**)[3] (60 mg, 99 µmol), the above described 10:1.0 mixture (30 mg) of boronic acid **4** (135 µmol) and (4-(2,2-difluorovinyl)phenyl)boronic acid (13.5 µmol), and Na_2CO_3 (19 mg, 179 µmol) in toluene (5 mL) and H_2O (2 mL) was degassed. $Pd(PPh_3)_4$ (3.70 mg, 3.20 µmol) was added. The solution was stirred at 100 °C for 20 h. After cooling to room temperature, Et_2O and H_2O were added. The organic and the aqueous phases were separated. The aqueous phase was extracted three times with Et_2O. The combined organic phases were washed three times with H_2O, dried over Na_2SO_4, and filtered. Removal of the solvents gave a green oil (133 mg). Column chromatography of this oil (2 cm × 25 cm, CH_2Cl_2/Et_2O, 5:1) gave a colorless 1.0:0.33:0.10 mixture (R_f = 0.35; 47 mg) of (4-(trifluoroethyl)phenyl)-PyMTA ester **6** (64 µmol, 65% yield), (4-(trifluoroethyl)phenyl)-boronic acid **4** (21 µmol), and triphenylphosphine oxide (6.4 µmol) along with a small amount of other unidentified compounds. ^{1}H NMR signals assigned to (4-(trifluoroethyl)phenyl)-PyMTA ester **6** (500 MHz, $CDCl_3$): δ = 7.83 (br s, 2 H, $H_{pyridine}$), 7.72 (one half of an AA'XX' spin system, 2 H, $H_{benzene}$ *meta* to CH_2CF_3), 7.40 (other half of the AA'XX' spin system, 2 H, $H_{benzene}$ *ortho* to CH_2CF_3), 4.16 (br s, 4 H, pyridine-C$\underline{H}_2$), 4.15 (q, $^3J_{HH}$ = 7.2 Hz, 8 H, C$\underline{H}_2$CH$_3$), 3.64 (s, 8 H, NC$\underline{H}_2$CO$_2$Et), 3.42 (q, $^3J_{HF}$ = 10.7 Hz, 2 H, C$\underline{H}_2$CF$_3$), 1.24 (t, $^3J_{HH}$ = 7.2 Hz, 12 H, CH$_2$C$\underline{H}_3$). ^{1}H NMR signals assigned to (4-(trifluoroethyl)phenyl)boronic acid **4** (500 MHz, $CDCl_3$): δ = 7.79 (one half of an AA'XX' spin system, 4 H, $H_{benzene}$ *meta* to CH_2CF_3), 7.27 (other half of the AA'XX' spin system, 4 H, $H_{benzene}$ *ortho* to CH_2CF_3), 3.42 (q, $^3J_{HF}$ = 10.9 Hz, 2 H, C$\underline{H}_2$CF$_3$). ^{1}H NMR signals assigned to triphenylphosphine oxide (500 MHz, $CDCl_3$): δ = 7.69–7.63 (m, 6 H, $H_{benzene}$ *ortho* to P), 7.58–7.53 (m, 3 H, $H_{benzene}$ *para* to P), 7.49–7.44 (m, 6 H, $H_{benzene}$ *meta* to P). MS (ESI) *m/z* = 640.3 [**6** + H]$^+$, 662.3 [**6** + Na]$^+$, 638.1 [**6** - H]$^-$, 202.9 [**4** - H]$^-$.

H_4[PyMTA-P-CH_2CF_3]•x HCl 7. The above mentioned 1.0:0.33:0.10 mixture (47 mg) of (4-(trifluoroethyl)phenyl)-PyMTA ester **6** (64 µmol), (4-(trifluoroethyl)phenyl)boronic acid **4** (21 µmol), and triphenylphosphine oxide (6.4 µmol) was dissolved in EtOH (2 mL). H_2O (1.5 mL) and 1 M aqueous NaOH solution (588 µL, 588 µmol) were added. The colorless solution was stirred at room temperature for 18 h. 1 M aqueous formic acid (650 µL, 650 µmol) was added. The pH of the solution was approximately 3.5. The components in the solution were separated using preparative reverse-phase HPLC

with a linear gradient elution at a flow rate of 12 mL/min and UV detection at 254 nm. The mobile phase consisted of A (H_2O/MeCN/HCO_2H, 95:5.0:0.10) and B (H_2O/MeCN/HCO_2H, 5.0:95:0.10) with the following percentages of B: 0−1 min, 50%; 1−7 min, 50%−95%; 7−15 min, 95%. The eluate collected between 3.6 and 4.8 minutes was freeze-dried. This gave a 1:50 mixture (51 mg) of H_nNa_{4-n}[PyMTA-P-CH_2CF_3] and sodium formate as a colorless solid. To convert the sodium formate, which could interfere with the complexation of Gd^{III} with [PyMTA-P-CH_2CF_3]$^{4-}$, into sodium chloride, the colorless solid was dissolved in water (750 µL). Then, 1 M aqueous HCl (220 µL) was added. The pH of the solution was approximately 1. Lyophilization of the solution gave a colorless solid (41 mg) consisting of H_nNa_{4-n}[PyMTA-P-CH_2CF_3], sodium formate, and NaCl, with a molar ratio of H_nNa_{4-n}[PyMTA-P-CH_2CF_3] to sodium formate of 1.0:9.2. This solid was dissolved in water (750 µL) giving a solution with a pH of 4. Subsequently, 1 M aqueous HCl (100 µL) was added, and the pH of the solution was approximately 1. Lyophilization of this solution resulted in a colorless solid consisting of H_4[PyMTA-P-CH_2CF_3]•x HCl **7**, sodium formate, and NaCl, with a molar ratio of H_4[PyMTA-P-CH_2CF_3]•x HCl **7** to sodium formate of 1.0:0.44. The colorless solid was dissolved in D_2O (750 µL), and the pD of the solution was approximately 1. The concentration of the structural motif [PyMTA-P-CH_2CF_3]$^{4-}$ was determined by quantitative ^{1}H NMR spectroscopy to be 10.5 mM. The yield of H_4[PyMTA-P-CH_2CF_3]•x HCl **7** was 12%. ^{1}H NMR (500 MHz, D_2O): All signals are slightly broadened. δ = 8.28 (formic acid), 7.97 (s, 2 H, $H_{pyridine}$), 7.85 (one half of an AA'XX' spin system, 2 H, $H_{benzene}$ *meta* to CH_2CF_3), 7.63 (other half of the AA'XX' spin system, 2 H, $H_{benzene}$ *ortho* to CH_2CF_3), 4.82 (s, 4 H, pyridine-C$\underline{H}_2$), 4.28 (s, 8 H, C$\underline{H}_2CO_2H$), 3.67 (q, 8 H, $^3J_{HF}$ = 10.9 Hz, CH_2CF_3). ^{19}F NMR (470 MHz, $CDCl_3$): δ = -65.4 (t, $^3J_{HF}$ = 10.9 Hz). Accurate MS (ESI) *m/z* calcd. for {H_3[PyMTA-P-CH_2CF_3]}$^-$ $C_{23}H_{23}N_3O_8F_3^-$: 526.1443; found: 526.1442.

Na[{Gd^{III}(PyMTA)}-P-CH_2CF_3] 1a. The above mentioned 10.5 mM solution (200 µL) of H_4[PyMTA-P-CH_2CF_3]•x HCl **7** (21.0 µmol) in D_2O, which contained in addition sodium formate and NaCl was mixed with a 100 mM solution of $GdCl_3$•6 H_2O in D_2O (19.9 µL, 1.99 µmol). A 1 M solution of NaOD in D_2O (10 µL, 10 µmol) and a 100 mM solution of NaOD in D_2O (65 µL, 6.5 µmol) were added to rise the pD of the solution to pD 7.0. The solution was diluted with D_2O (101.6 µL) to obtain a 5.0 mM solution of ruler **1a** in D_2O. Accurate MS (ESI) *m/z* calcd. for [M - Na]$^-$ $C_{23}H_{20}N_3O_8F_3Gd^-$: 681.0449; found: 681.0449.

Synthesis of ruler 1b



Scheme S-II-2. Synthesis of ruler **1b**.

Trimethylsilyl (TMS) protected (4-(trifluoroethyl)phenyl)ethyne 8. This reaction was performed under argon. A solution of trimethylsilylacetylene (2.2 mL, 16 mmol), 1-bromo-4-(trifluoroethyl)benzene **3** (1.05 g, 4.39 mmol), triethylamine (3 mL) in acetonitrile (120 mL) was degassed. $PdCl_2(PPh_3)_2$ (155 mg, 221 µmol) and CuI (86 mg, 45 µmol) were added. The solution was heated to reflux for 22 h and then stirred at room temperature for 16 h. H_2O (50 mL), 1 M aqueous HCl (30 mL) and Et_2O (50 mL) were added, the organic and the aqueous phases were separated. The aqueous phase was extracted with Et_2O (2 x 50 mL). The combined organic phases were washed with

saturated aqueous NaCl solution, then dried over Na_2SO_4 and filtered. The solvents were removed. TMS protected (4-(trifluoroethyl)phenyl)ethyne **8** was isolated via automated flash column chromatography (Büchi FlashPure EcoFlex Silica 40 g; irregular silica gel, 40–63 µm, 55–75 Å) using a linear gradient elution at a flow rate of 22 mL/min. The mobile phase consisted of *n*-pentane and CH_2Cl_2, with the following percentages of CH_2Cl_2: 0–4.8 min, 5%; 4.8–19.3 min, 5%–10%; 19.3–25 min, 10%. The eluate between 5.9 and 9.5 min was collected. Solvent removal gave TMS protected (4-(trifluoroethyl)phenyl)ethyne **8** (228 mg, 20%) as an orange solid. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.45 (one half of an AA'XX' spin system, 2 H, $H_{benzene}$ *meta* to CH_2CF_3), 7.23 (other half of the AA'XX' spin system, 2 H, $H_{benzene}$ *ortho* to CH_2CF_3), 3.35 (q, $^3J_{HF}$ = 10.7 Hz, 2H, CH_2CF_3), 0.25 (s, 9 H, TMS). ^{19}F NMR (470 MHz, $CDCl_3$): δ = -65.8 (t, $^3J_{HF}$ = 10.7 Hz, CH_2CF_3).

(4-(Trifluoroethyl)phenyl)ethyne 9. TMS-protected (4-(trifluoroethyl)phenyl)ethyne **8** (228 mg, 980 µmol) was dissolved in THF (7 mL) and a 1 M solution of ^{n}Bu$_4$NF in THF (1.4 mL, 1.4 mmol) was added, whereupon the color of the solution turned from orange to black. After 20 min, the solution was filtered through silica gel (1.5 cm × 6 cm, rinsing with *n*-pentane/Et_2O 91:9.0) and the filtrate was concentrated at reduced pressure. (4-Trifluoroethyl)phenyl)ethyne **9** was isolated via automated flash column chromatography (Büchi FlashPure EcoFlex Silica 25 g; silica gel irregular, 40–63 µm, 55–75 Å) using an isocratic elution with *n*-pentane at a flow rate of 15 mL/min. The eluate between 7.4 and 11.3 min was collected and concentrated at 30 °C and 600 mbar. This gave a 79:21 mixture (114 mg) of (4-(trifluoroethyl)phenyl)ethyne **9** (560 µmol, 57% yield) and *n*-pentane (152 µmol). ^{1}H NMR signals assigned to alkyne **9** (500 MHz, $CDCl_3$): δ = 7.48 (one half of an AA'XX' spin system, 2 H, $H_{benzene}$ *meta* to CH_2CF_3), 7.26 (other half of the AA'XX' spin system, 2 H, $H_{benzene}$ *ortho* to CH_2CF_3), 3.37 (q, $^3J_{HF}$ = 10.7 Hz, 2H, CH_2CF_3), 3.10 (s, 1H, HC≡C). ^{19}F NMR (470 MHz, $CDCl_3$): δ = -65.8 (t, $^3J_{HF}$ = 10.7 Hz, CF_3). ^{13}C NMR signals assigned to alkyne **9** (126 MHz, $CDCl_3$): δ = 132.5 ($\underline{C}_{benzene}$ *meta* to CH_2CF_3), 130.9 (q, $^3J_{CF}$ = 3.0 Hz, $\underline{C}_{benzene}$-CH_2CF_3), 130.3 ($\underline{C}_{benzene}$ *ortho* to CH_2CF_3), 125.7 (q, $^1J_{CF}$ = 277.1 Hz, CF_3), 122.2 ($\underline{C}_{benzene}$ *para* to CH_2CF_3), 83.1 (HC≡$\underline{C}$), 78.0 (H$\underline{C}$≡C), 40.3 (q, $^2J_{CF}$ = 29.9 Hz, $\underline{C}H_2CF_3$).

((4-(Trifluoroethyl)phenyl)ethynyl)-PyMTA ester 10. This reaction was performed under argon. The above mentioned 79:21 mixture (57 mg) of (4-(trifluoroethyl)phenyl)ethyne **9** (280 µmol) and pentane (76 µmol), were dissolved

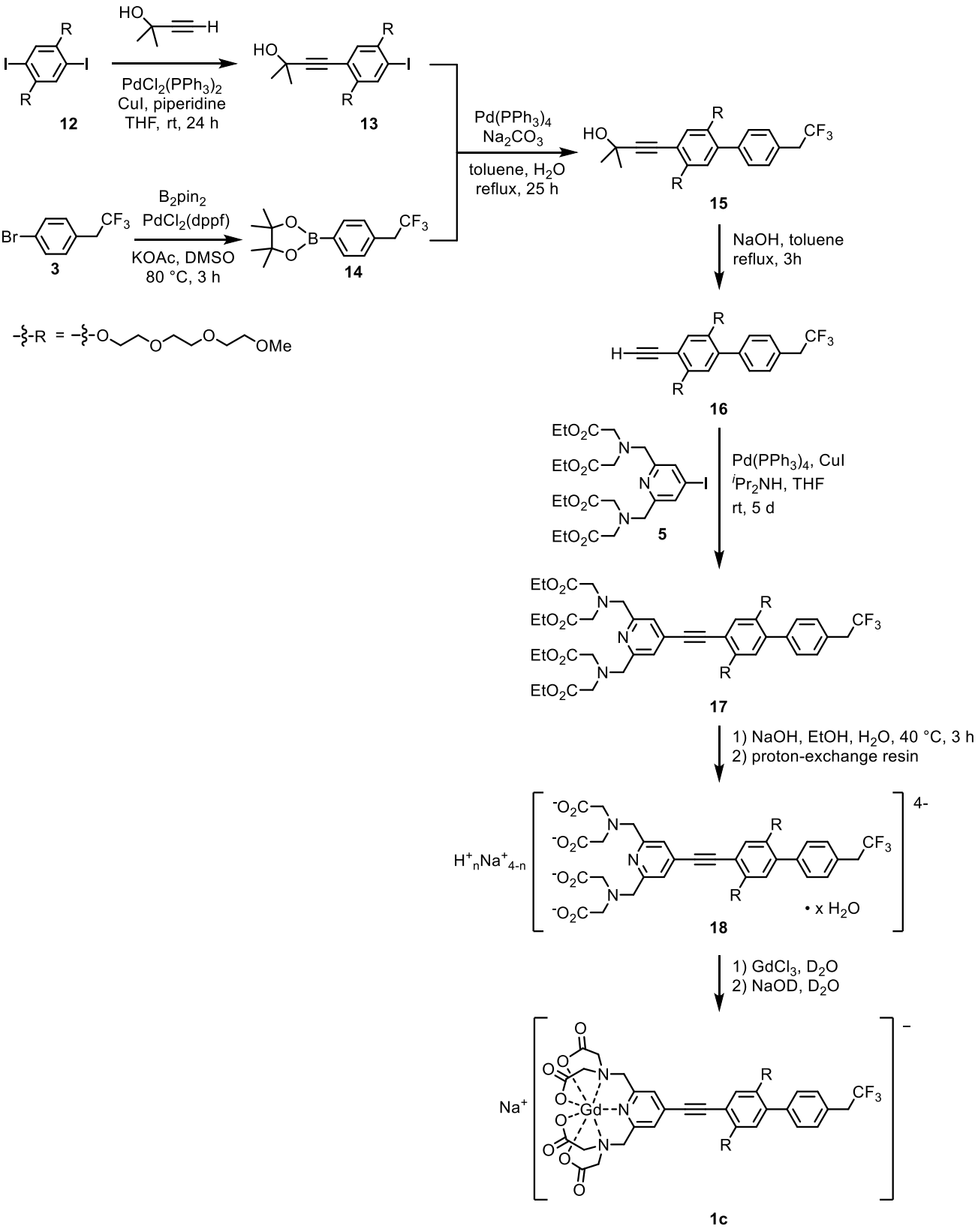
together with 4-iodo-PyMTA ester **5**[3] (126 mg, 207 µmol) and diisopropylamine (1.3 mL, 9.2 mmol) in THF (5 mL). The solution was degassed. $Pd(PPh_3)_4$ (13.7 mg, 11.8 µmol) and CuI (5.34 mg, 28.0 µmol) were added. After a few minutes, a colorless precipitate formed. The yellow suspension was stirred at room temperature for 3 days. H_2O (10 mL), 1 M aqueous HCl (15 mL) and Et_2O (10 mL) were added. The organic and the aqueous phases were separated. The aqueous phase was extracted with Et_2O (3 × 15 mL). The combined organic phases were dried over Na_2SO_4 and filtered. Removal of the solvents gave a yellow viscous oil (174 mg). ((4-(Trifluoroethyl)phenyl)ethynyl)-PyMTA ester **10** was isolated via automated flash column chromatography (Büchi FlashPure EcoFlex Silica 25 g; irregular silica gel, 40–63 µm, 55–75 Å) using a linear gradient elution at a flow rate of 15 mL/min. The mobile phase consisted of *n*-pentane and Et_2O, with the following percentages of Et_2O: 0–3.1 min, 25%–50%; 3.1–19 min, 50%; 19–40 min, 60%. The eluate between 23.1 and 31.9 min was collected. Removal of the solvent yielded ((4-(trifluoroethyl)phenyl)ethynyl)-PyMTA ester **10** (101 mg, 74%) as a colorless viscous oil. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.57 (s, 2 H, $H_{pyridine}$), 7.49 (one half of an AA'XX' spin system, 2 H, $H_{benzene}$ *meta* to CH_2CF_3), 7.28 (other half of the AA'XX' spin system, 2 H, $H_{benzene}$ *ortho* to CH_2CF_3), 4.14 (q, ^3J = 7.1 Hz, 8 H, $OC\underline{H}_2CH_3$), 4.01 (s, 4 H, pyridine-$C\underline{H}_2$), 3.59 (s, 8 H, $NC\underline{H}_2CO_2Et$), 3.36 (q, ^3J = 10.7 Hz, 2 H, CH_2CF_3), 1.24 (t, ^3J = 7.1 Hz, 12 H, $OCH_2C\underline{H}_3$). ^{19}F NMR (470 MHz, $CDCl_3$): δ = -65.7 (t, ^3J = 10.7 Hz, CF_3). ^{13}C NMR (126 MHz, $CDCl_3$): δ = 171.2 ($\underline{C}O_2Et$), 158.8 ($C_{pyridine}$ *ortho* to N), 132.5 ($C_{pyridine}$ *para* to N), 132.1 ($C_{benzene}$ *meta* to CH_2CF_3), 131.0 (q, $^3J_{CF}$ = 3.0 Hz, $\underline{C}_{benzene}$-CH_2CF_3), 130.3 ($C_{benzene}$ *ortho* to CH_2CF_3), 125.6 (q, $^1J_{CF}$ = 277 Hz, CF_3), 123.2 ($C_{pyridine}$ *meta* to N), 122.4 ($C_{benzene}$ *para* to CH_2CF_3), 92.7 (C≡$\underline{C}$-benzene), 88.1 (pyridine-$\underline{C}$≡C), 60.6 ($O\underline{C}H_2CH_3$), 59.8 (pyridine-$\underline{C}H_2$), 55.0 ($\underline{C}H_2CO_2Et$), 40.2 (q, ^2J = 30 Hz, $\underline{C}H_2CF_3$), 14.2 ($OCH_2\underline{C}H_3$). MS (ESI): *m/z* = 686.3 $[M + Na]^+$, 664.3 $[M + H]^+$. Accurate MS (ESI) *m/z* calcd. for $[M+Na]^+$ $C_{33}H_{40}F_3N_3O_8Na^+$: 686.2660; found: 686.2661.

H_nNa_{4-n}[PyMTA-EP-CH_2CF_3] 11. ((4-(Trifluoroethyl)phenyl)ethynyl)-PyMTA ester **10** (101 mg, 152 µmol) was dissolved in ethanol (1.2 mL). 1 M aqueous NaOH (1.2 mL, 1.2 mmol) was added. The solution was stirred at room temperature for 4 days. H_2O (2.5 mL) and proton-exchange resin were added in an amount needed to reduce the pH of the solution to pH 6. The solution was separated from the proton-exchange resin by filtration, and the resin was washed with H_2O and then with EtOH. The solvents of

the combined filtrates were removed. H_nNa_{4-n}[PyMTA-EP-CH_2CF_3] **11** (83 mg, containing 122 µmol of structural motif [PyMTA-EP-CH_2CF_3]$^{4-}$, yield 80%) was obtained as a colorless solid. The content of the structural motif [PyMTA-EP-CH_2CF_3]$^{4-}$ was determined by quantitative ^{1}H NMR spectroscopy. ^{1}H NMR (500 MHz, D_2O): δ = 7.47 (one half of an AA'XX' spin system, 2 H, $H_{benzene}$ *meta* to CH_2CF_3), 7.40 (s, 2 H, $H_{pyridine}$), 7.33 (other half of the AA'XX' spin system, 2 H, $H_{benzene}$ *ortho* to CH_2CF_3), 4.25 (s, 4 H, pyridine-C$\underline{H}_2$), 3.63 (s, 8 H, NCH_2CO_2), 3.49 (q, $^3J_{HF}$ = 11.1 Hz, 2 H, CH_2CF_3). ^{19}F NMR (470 MHz, D_2O): δ = -65.38 (t, $^3J_{HF}$ = 11.1 Hz, CF_3). ^{13}C NMR (151 MHz, D_2O): δ = 173.9 (br s, $\underline{C}O_2$), 153.6 (br s, $C_{pyridine}$ *ortho* to N), 134.2 ($\underline{C}_{benzene}$-CH_2CF_3), 133.0 ($C_{pyridine}$ *para* to N), 132.7 ($\underline{C}_{benzene}$ *meta* to CH_2CF_3), 131.2 ($C_{benzene}$ *ortho* to CH_2CF_3), 126.5 (q, $^1J_{CF}$ = 277 Hz, CF_3), 126.2 ($C_{pyridine}$ *meta* to N), 121.5 ($C_{benzene}$ *para* to CH_2CF_3), 95.4 (C≡$\underline{C}$-benzene), 87.2 (pyridine-$\underline{C}$≡C), 58.9 ($\underline{C}H_2$-pyridine), 58.0 ($\underline{C}H_2CO_2$), 39.5 (q, $^2J_{CF}$ = 30 Hz, $\underline{C}H_2CF_3$). MS (ESI): *m/z* = 550.5 [M - H]$^-$, 274.4 [M - 2 H]$^{2-}$. Accurate MS (ESI) *m/z* calcd. for {H_3[(4-(trifluoroethyl)phenyl)ethynyl)-PyMTA]}$^-$ $C_{25}H_{23}F_3N_3O_8^-$: 550.1443; found: 550.1453.

Na[{Gd^{III}(PyMTA)}-EP-CH_2CF_3] 1b. H_nNa_{4-n}[PyMTA-EP-CH_2CF_3] **11** obtained in the above reported experiment (4.67 mg; containing 6.91 µmol of structural motif [PyMTA-EP-CH_2CF_3]$^{4-}$) was dissolved in D_2O (600 µL) yielding a 11.4 mM solution of [PyMTA-EP-CH_2CF_3]$^{4-}$ in D_2O. A part of this solution (436.5 µL, containing 5.0 µmol of structural motif [PyMTA-EP-CH_2CF_3]$^{4-}$) was taken and a 20 mM solution of $GdCl_3$ in D_2O (237.5 µL, 4.75 µmol) was added, whereupon a colorless precipitate formed. A 0.1 M solution of NaOD in D_2O (150 µL, 15 µmol) was added. Shortly after the precipitate had dissolved completely and a colorless solution had formed. D_2O (176 µL) was added to reduce the concentration of ruler **1b** to 4.75 mM. pD of the solution was ~ 10. MS (ESI): *m/z* = 704.9 [M - Na]$^-$. Accurate MS (ESI) *m/z* calcd. for [M - Na]$^-$ $C_{25}H_{20}F_3N_3O_8Gd^-$: 705.0450; found: 705.0466.

Synthesis of ruler 1c



Scheme S-II-3. Synthesis of ruler **1c**.

Iodobenzene 13. This reaction was performed under argon. A solution of diiodobenzene **12**[4] (2.48 g, 3.79 mmol), 2-methylbut-3-yn-2-ol (0.22 mL, 2.3 mmol) in piperidine (7.5 mL, 75.7 mmol) and THF (10 mL) was degassed. $PdCl_2(PPh_3)_2$ (28.4 mg, 40.5 µmol) and CuI (16.0 mg, 84.0 µmol) were added. The solution was stirred at room temperature for 24 h. During this time a colorless solid formed. H_2O (20 mL), 36 wt.% aqueous HCl (7 mL) and Et_2O (20 mL) were added. The organic and the aqueous phases were separated. The aqueous phase was extracted with Et_2O (5 × 15 mL) and CH_2Cl_2 (3 × 20 mL). The combined organic phases were dried over Na_2SO_4 and filtered. The solvents of the filtrate were removed. Iodobenzene **13** was isolated via automated flash column chromatography (Büchi FlashPure EcoFlex Silica 120 g; irregular silica gel, 40–63 µm, 55–75 Å) using a linear gradient elution at a flow rate of 40 mL/min. The mobile phase consisted of CH_2Cl_2 and EtOH, with the following percentages of EtOH: 0–10 min, 2%–6%; 10–30 min, 6%. The eluate between 15.1 and 17.1 min was collected, and the solvent was removed. This gave iodobenzene **13** (693 mg, 50%) as an orange oil. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.27 (s, 1 H, $H_{benzene}$ *ortho* to I), 6.85 (s, 1 H, $H_{benzene}$ *meta* to I), 4.10, 3.87, and 3.79 (3 m, 4 H each, OCH_2), 3.67 (m, 8 H, OCH_2), 3.55 (m, 4 H, OCH_2), 3.38 and 3.37 (2 s, 3 H each, OCH_3), 3.13 (OH), 1.59 (s, 6H, $C(C\underline{H}_3)_2OH$). ^{13}C NMR (126 MHz, $CDCl_3$): δ = 154.4 ($OC_{benzene}$ *meta* to I), 152.2 ($OC_{benzene}$ *ortho* to I), 124.5 ($HC_{benzene}$ *ortho* to I), 116.9 ($HC_{benzene}$ *meta* to I), 113.8 ($C_{benzene}$ *para* to I), 99.6 ($C\equiv\underline{C}$-$C(CH_3)_2OH$), 87.3 ($IC_{benzene}$), 77.8 ($\underline{C}\equiv$C-$C(CH_3)_2OH$), 72.08, 72.00, 71.29, 71.18, 70.90, 70.78, 70.72, 70.65, 70.13, 69.91, and 69.74 (OCH_2), 65.4 (s, $\underline{C}(CH_3)_2OH$), 59.19 and 59.13 (OCH_3), 31.4 ($C(\underline{C}H_3)_2OH$).

(4-(Trifluoroethyl)phenyl)boronic acid pinacol ester 14. This reaction was performed under argon. A solution of 1-bromo-4-(trifluoroethyl)benzene **3** (573 mg, 2.40 mmol) and KOAc (705 mg, 7.18 mmol) in DMSO (10 mL) was degassed. Bis(pinacolato)diboron (B_2pin_2) (672 mg, 2.65 mmol) and $PdCl_2(dppf)•CH_2Cl_2$ (53.2 mg, 65.1 µmol) were added, and the solution was stirred at 80 °C for 3 h. The solution was cooled to room temperature, and then Et_2O (10 mL) and H_2O (10 mL) were added. The organic and the aqueous phases were separated. The aqueous phase was extracted with Et_2O (3 × 10 mL). The combined organic phases were washed with a saturated aqueous NaCl solution (15 mL) and then with H_2O (2 × 10 mL), dried over Na_2SO_4, and filtered. Removal of the solvents gave a 83:17 mixture (710 mg) of (4-(trifluoroethyl)phenyl)boronic acid pinacol ester **14** (2.10 mmol, yield 79%) and

bis(pinacolato)diboron (430 µmol) as a brown solid. ^{1}H NMR signals assigned to (4-(trifluoroethyl)phenyl)boronic acid pinacol ester **14** (500 MHz, $CDCl_3$): δ = 7.80 (m, one half an AA'XX' spin system, 2 H, $H_{benzene}$ *ortho* to B), 7.30 (m, other half of the AA'XX' spin system, 2 H, $H_{benzene}$ *meta* to B), 3.38 (q, $^3J_{HF}$ = 10.8 Hz, 2 H, CH_2CF_3), 1.34 (slightly broadened s, 12 H, CH_3). ^{1}H NMR signals assigned to bis(pinacolato)diboron (500 MHz, $CDCl_3$): δ = 1.26.

Biphenyl 15. This reaction was performed under argon. Iodobenzene **13** (363 mg, 594 µmol) and the above mentioned 83:17 mixture (261 mg) of (4-(trifluoroethyl)phenyl)boronic acid pinacol ester **14** (772 µmol) and bis(pinacolato)diboron (158 µmol), were dissolved in toluene (6 mL). 2 M Aqueous Na_2CO_3 solution (6 mL) was added. The two-phase solution was degassed. $Pd(PPh_3)_4$ (1.54 mg, 1.32 µmol) was added. The reaction mixture was heated to reflux for 25 h. After cooling to room temperature, CH_2Cl_2 (10 mL) was added. The organic and the aqueous phases were separated. The aqueous phase was extracted with CH_2Cl_2 (3 ×10 mL). The combined organic phases were washed with H_2O (2 × 15 mL), dried over Na_2SO_4, and filtered. The solvents were removed. Biphenyl **15** was isolated from the residue via automated flash column chromatography (Macherey-Nagel CHROMABOND Flash RS 40 SiOH; irregular silica gel, 40–63 µm, 60 Å) using a linear gradient elution at a flow rate of 22 mL/min. The mobile phase consisted of CH_2Cl_2 and EtOH, with the following percentages of EtOH: 0–3 min, 3%; 3–8 min, 3%–6%; 8–25 min, 6%. The eluate between 16.2 and 19.3 min was collected, and the solvent was removed. This gave a 96:4 mixture (67 mg) of biphenyl **15** (102 µmol, 17% yield) and triphenylphosphine oxide (4 µmol) as a brown-yellow oil. ^{1}H NMR signals assigned to biphenyl **15** (500 MHz, $CDCl_3$): δ = 7.53 (one half of an AA'XX' spin system, 2 H, $H_{benzene}$ *meta* to CH_2CF_3), 7.29 (other half of the AA'XX' spin system, 2 H, $H_{benzene}$ *ortho* to CH_2CF_3), 6.98 (s, 1 H, $C_{benzene}$-H *ortho* to C≡C), 6.85 (s, 1 H, $C_{benzene}$-H *meta* to C≡C), 4.14, 4.02, 3.86. 3.81, 3.71, 3.66, 3.62 (7 m, 2 H each, OCH_2), 3.60–3.55 (m, 6 H, OCH_2), 3.54–3.47 (m, 4 H, OCH_2), 3.37 (q, $^3J_{HF}$ = 10.7 Hz, 2 H, $C\underline{H}_2CF_3$), 3.34 (s, 6 H, $OC\underline{H}_3$), 3.19 (s, 1 H, O<u>H</u>), 1.60 (s, 6 H, $C(C\underline{H}_3)_2OH$). ^{19}F NMR (470 MHz, $CDCl_3$): δ = -65.63 (t, $^3J_{HF}$ = 10.8 Hz, CF_3). ^{13}C NMR signals assigned to biphenyl **15** (126 MHz, $CDCl_3$): δ = 154.2 ($OC_{benzene}$ *ortho* to C≡C), 149.8 ($OC_{benzene}$ *meta* to C≡C), 137.7 ($C_{benzene}$ *para* to CH_2CF_3), 131.6 ($C_{benzene}$ *para* to C≡C), 129.79 and 129.77 ($HC_{benzene}$ *ortho* and *meta* to CH_2CF_3), 129.1 (q, $^3J_{CF}$ = 2.7 Hz, $\underline{C}_{benzene}$-CH_2CF_3), 125.8 (q, $^1J_{CF}$ =

277 Hz, CF_3), 118.2 ($HC_{benzene}$ *ortho* to C≡C), 116.1 ($HC_{benzene}$ *meta* to C≡C), 112.8 (C≡C-$\underline{C}_{benzene}$), 99.0 ($\underline{C}$≡C-$C_{benzene}$), 78.1 (C≡$\underline{C}$-$C_{benzene}$), 71.93, 71.90, 71.08, 70.82, 70.71, 70.69, 70.53, 70.52, 69.98, 69.94, 69.65, and 69.15 (OCH_2), 65.3 (HO$\underline{C}(CH_3)_2$), 59.00 and 58.98 (O$\underline{C}H_3$), 40.0 (q, $^2J_{CF}$ = 29.7 Hz, $\underline{C}H_2CF_3$), 31.5 (HOC($\underline{C}H_3)_2$). MS (ESI) *m/z* = 643.1 $[M + H]^+$, 665.0 $[M + Na]^+$. Accurate MS (ESI): *m/z* calcd. for $[M + Na]^+$ $C_{33}H_{45}F_3O_9^+$: 665.2908; found: 665.2917.

Alkyne 16. NaOH powder (22.7 mg, 568 µmol) was added to a solution of the above mentioned 96:4 mixture of biphenyl **15** (102 µmol) and triphenylphosphine oxide (4 µmol) in toluene (5.5 mL). The suspension was heated to reflux for 3 h. After cooling to room temperature, the suspension was filtered through silica gel (rinsing with CH_2Cl_2/EtOH 94:6). Removal of the solvents gave a 49:49:2 mixture (69 mg) of alkyne **16** (101 µmol, 98% yield), toluene (101 µmol), and triphenylphosphine oxide (4 µmol). ^{1}H NMR signals assigned to alkyne **16** (500 MHz, $CDCl_3$): *δ* = 7.56 (one half of an AA'XX' spin system, 2 H, $H_{benzene}$ *ortho* to CH_2CF_3), 7.33 (other half of the AA'XX' spin system, 2 H, $H_{benzene}$ *meta* to CH_2CF_3), 7.08 (s, 1 H, $H_{benzene}$ *ortho* to C≡C), 6.92 (s, 1 H, $H_{benzene}$ *meta* to C≡C), 4.21, 4.05, 3.89. 3.79, 3.74, (5 m, 2 H each, OCH_2), 3.70–3.56 (m, 10 H, OCH_2), 3.55–3.49 (m, 4 H, OCH_2), 3.40 (q, $^3J_{HF}$ = 10.7 Hz, 2 H, C$\underline{H}_2CF_3$), 3.36 (s, 6 H, OCH_3), 3.30 (s, 1 H, H-C≡C). MS (ESI): *m/z* = 585.1 $[M + H]^+$, 607.1 $[M + Na]^+$.

PyMTA ester 17. This reaction was performed under argon. The above mentioned 49:49:2 mixture (69 mg) of alkyne **16** (101 µmol), toluene (101 µmol), and triphenylphosphine oxide (4 µmol) was dissolved together with 4-iodo-PyMTA ester **5**[3] (88.2 mg, 145 µmol) in diisopropylamine (332 µL) and THF (4.5 mL). This solution was degassed and then $Pd(PPh_3)_4$ (1.40 mg, 1.21 µmol) and CuI (0.547 mg, 2.87 µmol) were added. The solution was stirred at room temperature for 5 days. During this time a colorless precipitate formed. All volatiles were removed by a short path distillation. Column chromatography of the residue (2.0 cm × 43.5 cm, CH_2Cl_2/EtOH 20:1) yielded a 94:6 mixture (R_f = 0.35; 76 mg) of PyMTA ester **17** (70 µmol, 69% yield) and triphenylphosphine oxide (4.7 µmol). ^{1}H NMR signals assigned to PyMTA ester **17** (500 MHz, $CDCl_3$): *δ* [ppm] = 7.574 (one half of an AA'XX' spin system, 2 H, $H_{benzene}$ *meta* to CH_2CF_3), 7.567 (s, 2 H, $H_{pyridine}$), 7.32 (other half of the AA'XX' spin system, 2 H, $H_{benzene}$ *ortho* to CH_2CF_3), 7.09 (s, 1 H, $H_{benzene}$ *ortho* to C≡C), 6.95 (s, 1 H, $H_{benzene}$ *meta* to C≡C), 4.23 (m, 2 H, OCH_2), 4.16 (q, $^3J_{HH}$ = 7.1 Hz, 8 H, C$\underline{H}_2CH_3$), 4.08 (m, 2

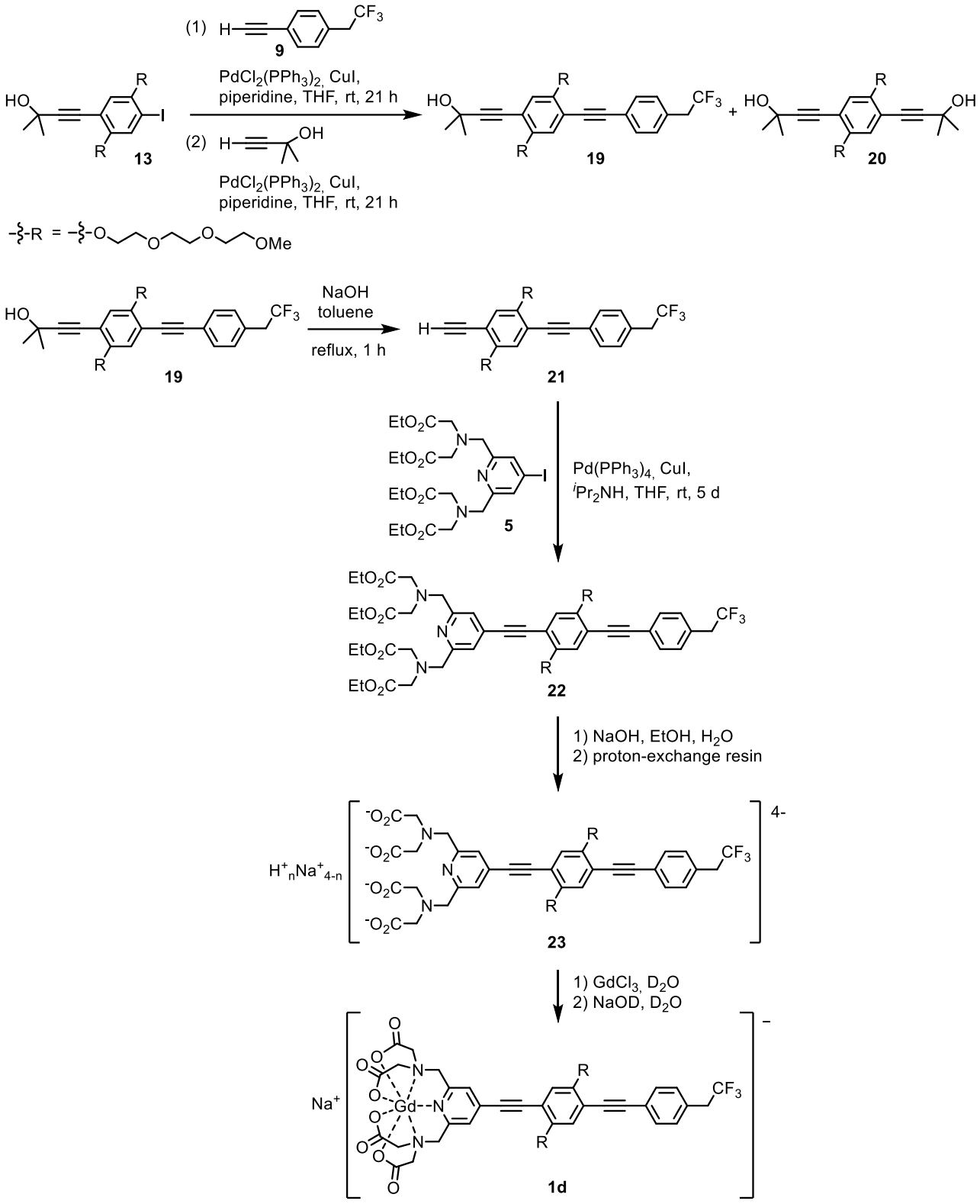
H, OCH_2), 4.03 (s, 4 H, pyridine-$C\underline{H}_2$), 3.92, 3.79, and 3.76 (3 m, 2 H each, OCH_2), 3.62 (s, 8 H, $NC\underline{H}_2CO_2Et$), 3.59 (m, 10 H, OCH_2), 3.50 and 3.47 (2 m, 2 H each, OCH_2), 3.39 (q, $^3J_{HF}$ = 10.7 Hz, 2 H, $C\underline{H}_2CF_3$), 3.34 and 3.31 (2 s, 3 H each, OCH_3), 1.25 (t, ^3J = 7.1 Hz, 12 H, $CH_2C\underline{H}_3$). MS (ESI): *m/z* = 1064.1 $[M + H]^+$, 1086.1 $[M + Na]^+$.

H_nNa_{4-n}[PyMTA-EPP-CH_2CF_3]•x H_2O 18. The above mentioned 94:6 mixture (76 mg) of PyMTA ester **17** (70 µmol) and triphenylphosphine oxide (4.7 µmol) was dissolved in EtOH (2 mL). H_2O (2.0 mL) and 2 M aqueous NaOH (178 µL, 356 µmol) were added. The yellow solution was stirred at 40 °C for 3 h. After cooling to room temperature the yellow solution was washed with Et_2O to remove the triphenylphosphine oxide. The aqueous solution was diluted with H_2O (ca. 10 mL) and EtOH (ca. 10 mL) and proton-exchange resin was added in an amount needed to reduce the pH of the solution to 9.5. The solution was separated from the resin through filtration through a syringe filter (Ø 13 mm, pore size: 0.45 µm, membrane: PVDF) and the resin was washed with H_2O/EtOH 1:1. The solvents of the combined filtrates were removed. This gave H_nNa_{4-n}[PyMTA-EPP-CH_2CF_3]•x H_2O **18** (54.3 mg, containing 32.1 µmol of structural motif [PyMTA-EPP-CH_2CF_3]$^{4-}$, 46% yield) as a yellow solid. The content of the structural motif [PyMTA-EPP-CH_2CF_3]$^{4-}$ was determined by quantitative ^{1}H NMR spectroscopy. ^{1}H NMR (500 MHz, D_2O): *δ* = 7.40 (one half of an AA'XX' spin system, 2 H, $H_{benzene}$ *meta* to CH_2CF_3), 7.27 (other half of the AA'XX' spin system, 2 H, $H_{benzene}$ *ortho* to CH_2CF_3), 7.26 (s, 2 H, $H_{pyridine}$), 7.09 (s, 1 H, $H_{benzene}$ *ortho* to C≡C), 6.72 (s, 1 H, $H_{benzene}$ *meta* to C≡C), 3.99 and 3.92 (2 m, 2 H each, OCH_2), 3.86 (s, 4 H, pyridine-$C\underline{H}_2$), 3.76. 3.70, 3.59, and 3.52 (4 m, 2 H each, OCH_2), 3.50–3.33 (m, 14 H, overlapping signals of CH_2CF_3 and OCH_2), 3.24 and 3.21 (2 s, 3 H each, OCH_3), 3.19 (s, 8H, $NC\underline{H}_2CO_2Et$). ^{19}F NMR (470 MHz, D_2O): *δ* [ppm] = -65.44 (t, $^3J_{HF}$ = 10.9 Hz, CF_3). ^{13}C NMR (126 MHz, D_2O): *δ* = 179.6 ($O_2\underline{C}$-CH_2N), 159.4 ($C_{pyridine}$ *ortho* to N), 154.4 ($OC_{benzene}$ *ortho* to C≡C), 150.0 ($OC_{benzene}$ *meta* to C≡C), 137.4 ($\underline{C}_{benzene}$ *para* to CH_2CF_3), 133.5 ($C_{benzene}$ *para* to C≡C and $\underline{C}_{benzene}$-CH_2CF_3), 133.2 ($\underline{C}_{pyridine}$ *para* to N), 130.5 ($HC_{benzene}$ *ortho* to CH_2CF_3), 130.2 ($HC_{benzene}$ *meta* to CH_2CF_3), 126.8 (q, $^1J_{CF}$ = 276 Hz, CF_3), 124.2 ($HC_{pyridine}$), 118.8 ($HC_{benzene}$ *ortho* to C≡C), 116.1($HC_{benzene}$ *meta* to C≡C), 111.5 (C≡C-$\underline{C}_{benzene}$), 92.2 ($C_{pyridine}$-$\underline{C}$≡C), 91.0 ($C_{pyridine}$-C≡$\underline{C}$), 71.59, 71.58, 70.90, 70.57, 70.34, 70.17, 70.04, 69.93, 69.85, 69.78, 69.65, 69.42 ($O\underline{C}H_2$), 59.6 (pyridine-$\underline{C}H_2$), 58.63 ($O_2C\underline{C}H_2N$), 58.61 and 58.59 ($O\underline{C}H_3$), 39.4(q, $^2J_{CF}$ = 28.9 Hz, $\underline{C}H_2CF_3$). MS (ESI): *m/z* = 952.0 $\{H_4$[PyMTA-EPP-CH_2CF_3] + H$\}^+$, 973.9 $\{H_4$[PyMTA-

EPP-CH_2CF_3] + Na}$^+$. Accurate MS (ESI) *m/z* calcd. for {H_4[PyMTA-EPP-CH_2CF_3] + H}$^+$ $C_{45}H_{56}N_3O_{16}F_3H^+$: 952.3686; found: 952.3689.

Na[{Gd^{III}(PyMTA)}-EPP-CH_2CF_3] 1c. H_nNa_{4-n}[PyMTA-EPP-CH_2CF_3]•x H_2O **18** obtained in the previous above described experiment (54.3 mg; containing 32.1 µmol of structural motif [PyMTA-EPP-CH_2CF_3]$^{4-}$) was dissolved in D_2O (2000 µL) yielding a 16.1 mM pale-yellow solution of [PyMTA-EPP-CH_2CF_3]$^{4-}$ in D_2O. The pD of the solution was ~ 9.5. Part of this solution (93.3 µL, containing 1.5 µmol of structural motif [PyMTA-EPP-CH_2CF_3]$^{4-}$) was taken and a 20 mM solution of $GdCl_3$ in D_2O (71.3 µL, 1.43 µmol) was added, whereupon a colorless precipitate formed. Ultrasonication of the suspension gave a pale-yellow solution of Na[{Gd^{III}(PyMTA)}-EPP-CH_2CF_3] **1c**. D_2O (100 µL) was added to reduce the concentration of ruler **1c** to 4.75 mM. Accurate MS (ESI) *m/z* calcd. for [M - Na]$^-$ $C_{45}H_{52}F_3N_3O_{16}Gd^-$: 1105.2546; found: 1105.2551.

Synthesis of ruler 1d



Scheme S-II-4. Synthesis of ruler **1d**

Alkyne 19. This reaction was performed under argon. A solution of iodobenzene **13** (509 mg, 833 µmol), (4-(trifluoroethyl)phenyl)ethyne **9** (158 mg, 856 µmol), and piperidine (1.7 mL, 17 mmol) in THF (7 mL) was degassed. $PdCl_2(PPh_3)_2$ (6.17 mg, 8.79 µmol) and CuI (3.59 mg, 18.8 µmol) were added. The solution was stirred at room temperature for 21 h. During this time a colorless precipitate formed. H_2O (20 mL), 1 M aqueous HCl (22 mL), and Et_2O (25 mL) were added. The organic and the aqueous phases were separated. The aqueous phase was extracted with Et_2O (4 × 20 mL). The combined organic phases were dried over Na_2SO_4 and filtered. The solvents were removed yielding a 64:36 mixture (520 mg) of alkyne **19** and iodobenzene **13**. The R_f-values of alkyne **19** and iodobenzene **13** are very similar, making their separation via chromatography challenging. To simplify the separation, the mixture was submitted to an alkyne-aryl-coupling with 2-methylbut-3-yn-2-ol, in which iodobenzene **13** was converted into dialkyne **20**. The obtained mixture (520 mg), 2-methylbut-3-yn-2-ol (0.15 mL, 1.5 mmol), and piperidine (0.28 mL, 2.8 mmol) were dissolved in THF (6 mL). After degassing, $PdCl_2(PPh_3)_2$ (3.45 mg, 4.92 µmol) and CuI (2.98 mg, 15.6 µmol) were added. The solution was stirred at room temperature for 21 h. During this time a colorless precipitate formed. H_2O (20 mL), 1 M aqueous HCl (5 mL), and Et_2O (25 mL) were added. The organic and the aqueous phases were separated. The aqueous phase was extracted with Et_2O (3 × 20 mL). The combined organic phases were dried over Na_2SO_4 and filtered. The solvents of the filtrate were removed, yielding a 68:32 mixture (552 mg) of alkyne **19** and dialkyne **20**. Alkyne **19** was isolated via automated flash column chromatography (Büchi FlashPure EcoFlex Silica 80 g; irregular silica gel, 40–63 µm, 55–75 Å) using a linear gradient elution at a flow rate of 20 mL/min. The mobile phase consisted of CH_2Cl_2 and EtOH, with the following percentages of EtOH: 0–12.7 min, 2%–6%; 12.7–42 min, 6%. The eluate between 27.3 and 29.2 min was collected. Removal of the solvents gave an orange oil (281 mg) which consisted substantially of alkyne **19** (417 µmol, 50%) and triphenylphosphine oxide (12 µmol). ^{1}H NMR signals assigned to alkyne **19** (500 MHz, $CDCl_3$): δ = 7.49 (one half of an AA'XX' spin system, 2 H, $H_{benzene}$ *meta* to CH_2CF_3), 7.27 (other half of the AA'XX' spin system, 2 H, $H_{benzene}$ *ortho* to CH_2CF_3), 6.97 (s, 1 H, $H_{benzene}$ *meta* to $C{\equiv}C(CH_3)_2OH$), 6.93 (s, 1 H, $H_{benzene}$ *ortho* to $C{\equiv}C(CH_3)_2OH$), 4.15 and 3.89 (2 m, 4 H each, OCH_2), 3.82 and 3.79 (m, 2 H each, OCH_2), 3.70–3.58 (m, 8H, OCH_2), 3.54 and 3.50 (2 m, 2 H each, OCH_2), 3.37 (q, $^3J_{HF}$ = 10.7 Hz, 2 H, $C\underline{H}_2CF_3$), 3.35 and 3.34 (2 s, 3H each, OCH_3),

3.15 (br s, 1 H, OH), 1.60 (s, 6 H, C(C<u>H</u>$_3$)$_2$OH). ^{19}F NMR (470 MHz, $CDCl_3$): δ = -65.8 (t, ^3J = 10.7 Hz, CF_3). MS (ESI): *m/z* = 689.1 $[M + Na]^+$.

Alkyne 21. Alkyne **19** (281 mg, 421 µmol) was dissolved in toluene (10 mL). NaOH powder (88 mg, 2.2 mmol) was added, and the suspension was heated to reflux for 1 h. After cooling to room temperature, the suspension was filtered through silica gel (rinsing with CH_2Cl_2/EtOH 1:2). Removal of the solvents yielded alkyne **21** as a yellow oil (171 mg, 67%). ^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.51 (one half of an AA'XX' spin system, 2 H, $H_{benzene}$ *meta* to CH_2CF_3), 7.28 (other half of the AA'XX' spin system, 2 H, $H_{benzene}$ *ortho* to CH_2CF_3), 7.03 and 7.01 (2 s, 1 H each, $H_{benzene}$ *ortho* to O), 4.18, 3.89, and 3.79 (3 m, 4 H each, OCH_2), 3.71–3.58 (m, 8 H, OCH_2), 3.54 and 3.51 (2 m, 2 H each, OCH_2), 3.39 (q, $^3J_{HF}$ = 10.7 Hz, 2 H, C<u>H</u>$_2$CF$_3$), 3.37 and 3.35 (2 s, 3 H each, OCH_3), 3.34 (s, 1 H, HC≡C).

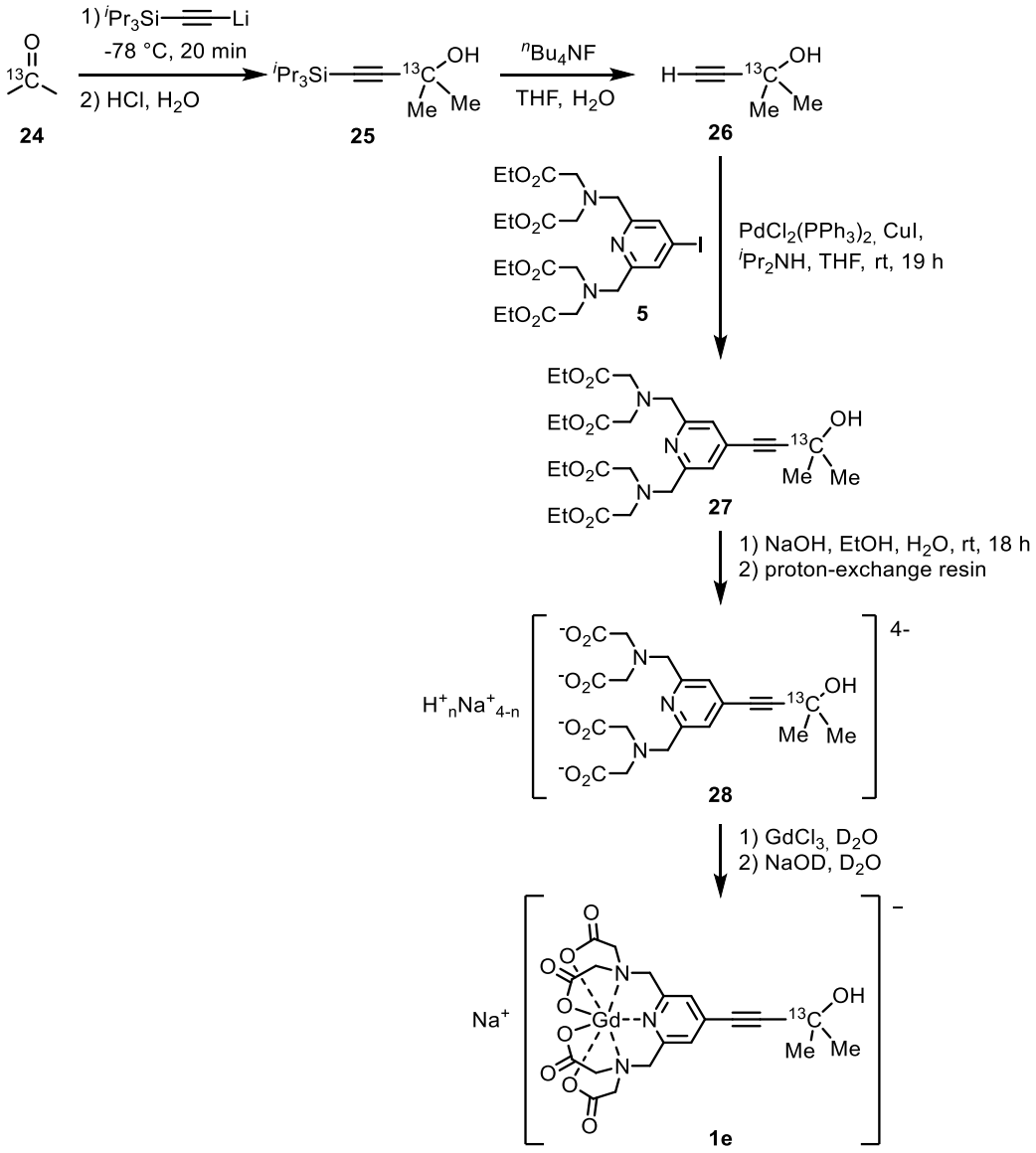
PyMTA ester 22. This reaction was performed under argon. Alkyne **21** (169 mg, 278 µmol) and 4-iodo-PyMTA ester **5** (160 mg, 263 µmol) were dissolved in diisopropylamine (3 mL) and THF (10 mL). This solution was degassed and then $Pd(PPh_3)_4$ (6.03 mg, 1.21 µmol) und CuI (2.08 mg, 10.9 µmol) were added. The orange solution was stirred at room temperature for 5 days. During this time a colorless precipitate formed. H_2O (10 mL) and Et_2O (10 mL) were added. The organic and the aqueous phases were separated. The aqueous phase was extracted with Et_2O (2 × 10 mL). The combined organic phases were washed with 2 M aqueous HCl (3 × 10 mL) dried over Na_2SO_4 and filtered. The solvents of the filtrate were removed. Column chromatography (2.0 cm × 36 cm, CH_2Cl_2/Et_2O/EtOH 10:4.0:0.40) of the residual orange oil (322 mg) gave PyMTA ester **22** (R_f = 0.23; 87 mg, 29%). ^{1}H NMR (500 MHz, $CDCl_3$): δ [ppm] = 7.62 (br s, 2 H, $H_{pyridine}$), 7.52 (one half of an AA'XX' spin system, 2 H, $H_{benzene}$ *meta* to CH_2CF_3), 7.29 (other half of the AA'XX' spin system, 2 H, $H_{benzene}$ *ortho* to CH_2CF_3), 7.08 and 7.04 (2 s, 1 H each, $H_{benzene}$ *ortho* to C≡C), 4.22 (m, 4 H, OCH_2), 4.17 (q, $^3J_{HH}$ = 7.1 Hz, 8 H, C<u>H</u>$_2$CH$_3$), 4.07 (br s, 4 H, pyridine-C<u>H</u>$_2$), 3.93 and 3.82 (2 m, 4 H each, OCH_2), 3.69–3.59 (m, 16 H, overlapping signals of NC<u>H</u>$_2$CO$_2$Et and OCH_2), 3.50 (m, 4 H, OCH_2), 3.39 (q, $^3J_{HF}$ = 10.7 Hz, 2 H, CH_2CF_3), 3.34 and 3.33 (2 s, 3 H each, OCH_3), 1.27 (t, ^3J = 7.1 Hz, 12 H, CH_2C<u>H</u>$_3$). Accurate MS (ESI) *m/z* calcd. for $[M + H]^+$ $C_{55}H_{72}N_3O_{16}F_3H^+$: 1088.4938; found: 1088.4955.

H_nNa_{4-n}[PyMTA-EPEP-CH_2CF_3] 23. PyMTA ester **22** (87 mg, 80 µmol) was dissolved in EtOH (3.0 mL). H_2O (2.4 mL) and 1 M aqueous NaOH solution (640 µL, 640 µmol) were added. The yellow solution was stirred at room temperature for 17 h. H_2O (20 mL) and CH_2Cl_2 (20 mL) were added and the phases were well mixed. Centrifugation of the yellow emulsion at 4000 rpm for 10 min gave two phases, an organic and a yellow aqueous phase. The aqueous phase was well mixed with Et_2O (5 mL). Centrifugation of the resulting yellow emulsion at 4000 rpm for 5 min gave two phases. The organic phase was removed. Then the solvent of the aqueous phase was removed giving a yellow solid (93 mg). This solid was dissolved in H_2O (5 mL) and formic acid (100 µL) was added. The pH of the solution was approximately 3. The components in this solution were separated using preparative reverse-phase HPLC with a linear gradient elution at a flow rate of 12 mL/min and UV detection at 254 nm. The mobile phase consisted of A (H_2O/MeCN/HCO_2H, 95:5.0:0.10) and B (H_2O/MeCN/HCO_2H, 5.0:95:0.10) with the following percentages of B: 0−1 min, 50%; 1−7 min, 50%−95%; 7−20 min, 95%. The eluate between 8.5 and 10.5 minutes was collected and the solvents removed. This yielded a yellow solid (29.6 mg) which was suspended in D_2O (500 µL). 1 M aqueous NaOD was added until the pD of the solution reached 11. The yellow solution was diluted with D_2O to 1400 µL. The concentration of the structural motif [PyMTA-EPEP-CH_2CF_3]$^{4-}$ was determined by quantitative ^{1}H NMR spectroscopy to be 13.5 mM. The yield of H_nNa_{4-n}[PyMTA-EPEP-CH_2CF_3] **23** was 24%. ^{1}H NMR (600 MHz, D_2O): δ = 7.43 (one half of an AA'XX' spin system, 2 H, $H_{benzene}$ *meta* to CH_2CF_3), 7.33 (s, 2 H, $H_{pyridine}$), 7.32 (other half of an AA'XX' spin system, 2 H, $H_{benzene}$ *ortho* to CH_2CF_3), 7.09 and 7.02 (2 s, 1 H each, $H_{benzene}$ *ortho* to O), 4.15 and 3.86 (2 m, 4 H each, OCH_2), 3.80 (s, 4 H, pyridine-C$\underline{H}_2$), 3.74 (m, 4 H, OCH_2), 3.56 (m, 2 H each, OCH_2), 3.53–3.48 (m, 6 H, overlapping signals of CH_2CF_3 and OCH_2), 3.46, 3.41, and 3.37 (3 m, 2 H each, OCH_2) 3.22 and 3.21 (2 s, 3 H each, OCH_3), 3.17 (s, 8H, NC$\underline{H}_2CO_2Et$). ^{19}F NMR (470 MHz, D_2O): δ [ppm] = -65.44 (t, $^3J_{HF}$ = 10.9 Hz, CF_3). ^{13}C NMR (126 MHz, D_2O): δ = 179.8 (CO_2), 159.3 ($C_{pyridine}$ *ortho* to N), 153.9 and 153.6 ($OC_{benzene}$), 133.1 ($C_{pyridine}$ *para* to N), 132.3 ($HC_{benzene}$ *meta* to CH_2CF_3), 132.2 ($\underline{C}_{benzene}$-CH_2CF_3), 131.2 ($HC_{benzene}$ *ortho* to CH_2CF_3), 126.6 (q, $^1J_{CF}$ = 276 Hz, CF_3), 124.4 ($HC_{pyridine}$), 122.7 ($C_{benzene}$ *para* to CH_2CF_3), 118.2 and 118.0 ($HC_{benzene}$ *ortho* O), 115.3 and 113.3 (C≡C-$\underline{C}_{benzene}$ *ortho* to O), 95.9, 93.3, 90.7 and 86.7 (C≡C), 71.60, 71.52, 70.97, 70.95, 70.36, 70.26, 70.03, 69.96, and 69.84 (OCH_2), 59.5 (pyridine-$\underline{C}H_2$), 58.63 ($O_2C\underline{C}H_2N$), 58.61 and 58.57 (OCH_3), 39.5(q, $^2J_{CF}$ = 29.2 Hz, $\underline{C}H_2CF_3$). MS (ESI):

m/z = 974.1 {H_3[PyMTA-EPEP-CH_2CF_3]}$^-$, 996.1 {H_2Na[PyMTA-EPEP-CH_2CF_3]}$^-$. Accurate MS (ESI) *m/z* calcd. for {H_3[PyMTA-EPEP-CH_2CF_3]}$^-$ $C_{47}H_{55}N_3O_{16}F_3H^-$: 974.3540; found: 974.3541.

Na[{Gd^{III}(PyMTA)}-EPEP-CH_2CF_3] 1d. A 100 mM solution of $GdCl_3$ in D_2O (38.9 µL, 3.89 µmol) was added to the above mentioned 13.5 mM solution of H_nNa_{4-n}[PyMTA-EPEP-CH_2CF_3] **23** in D_2O (300 µL, 4.05 µmol), whereupon a colorless precipitate formed. Stirring the suspension for 10 min gave a pale-yellow solution of Na[{Gd^{III}(PyMTA)}-EPEP-CH_2CF_3] **1d** in D_2O. D_2O (438.8 µL) was added to reduce the concentration of ruler **1d** to 5.0 mM. The pD of the solution was 7.5. Accurate MS (ESI) *m/z* calcd. for [M - Na]$^-$ $C_{47}H_{52}F_3N_3O_{16}Gd^-$: 1129.2546; found: 1129.2566.

Synthesis of ruler 1e



Scheme S-II-5. Synthesis of ruler **1e**

2-Methyl-4-(triisopropylsilyl)but-3-yn-2-ol-2-*^{13}C* (25). This reaction was performed under argon. To visualize the spots on the TLC plates, the TLC plates were briefly dipped into a 0.5 wt.% aqueous $KMnO_4$ solution and subsequently heated with a heat gun.

A colorless solution of (triisopropylsilyl)acetylene (4.04 g, 22.1 mmol) in THF (30 mL) was degassed. A 1.6 M solution of ^{n}BuLi in hexane (12.7 mL, 20.3 mmol) was added dropwise at -78 °C over 5 min. Thereby, the color of the solution turned from colorless to pale-yellow. The solution was stirred at -78 °C for 20 min. Then, acetone-2-^{13}C

(1.00 g, 17.0 mmol) was added dropwise at -78 °C over 5 min. After completion of the addition, the cooling bath was removed, and the pale-yellow solution was allowed to warm to 18 °C and was stirred at 18 °C for 2 h. The pale-yellow reaction solution was poured into a mixture of Et_2O (35 mL) and 1 M aqueous HCl (25 mL). After thorough mixing, the organic and the aqueous phases were separated. The aqueous phase was extracted with Et_2O (3 × 20 mL). The combined organic phases were washed with saturated aqueous NaCl solution (20 mL), dried over $MgSO_4$, and filtered. Removal of the solvents gave a mixture (4.94 g) of (triisopropylsilyl)acetylene and 2-methyl-4-(triisopropylsilyl)but-3-yn-2-ol-2-*^{13}C* (**25**) as a pale-yellow liquid. Chromatography (4.5 cm x 23 cm, pentane/Et_2O, 5:1) yielded (triisopropylsilyl)acetylene (R_f = 0.79; 1.15 g) as a colorless liquid and 2-methyl-4-(triisopropylsilyl)but-3-yn-2-ol-2-^{13}C (**25**) (R_f = 0.36; 3.32 g, 81%) as a colorless liquid. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 2.02 (d, $^2J_{CH}$ = 2.7 Hz, 1 H, OH), 1.53 (d, $^2J_{CH}$ = 4.4 Hz, 6 H, C(C<u>H</u>$_3$)$_2$OH), 1.05 (m, 21 H, C<u>H</u>(C<u>H</u>$_3$)$_2$). ^{13}C NMR (126 MHz, $CDCl_3$): δ = 113.0 (d, $^1J_{CC}$ = 68.4 Hz, C≡<u>C</u>-^{13}C), 82.2 (d, $^2J_{CC}$ = 8.4 Hz, <u>C</u>≡C-^{13}C), 65.6 (s, ^{13}<u>C</u>(CH$_3$)$_2$OH), 31.7 (d, $^1J_{CC}$ = 39.7 Hz, ^{13}C(C<u>H</u>$_3$)$_2$OH), 18.7 (s, CH(<u>C</u>H$_3$)$_2$), 11.2 (s, <u>C</u>H(CH$_3$)$_2$). MS (EI, 70 eV): *m/z* (%) = 241.2 (2) $[M]^{+\bullet}$, 198.2 (7) [M - ^{i}Pr]$^+$, 155.1 (32) [M - 2 ^{i}Pr]$^+$, 127.1 (100), 85.0 (16), 75.0 (34), 61.0 (13). Accurate MS (EI, 70 eV) *m/z* calcd. for $[M]^{+\bullet}$ $C_{13}{}^{13}CH_{28}OSi^{+\bullet}$: 241.19375; found: 241.19366.

2-Methylbut-3-yn-2-ol-2-*^{13}C* (26). To visualize the spots on the TLC plate, the TLC plate was briefly dipped into a 0.5 wt.% aqueous $KMnO_4$ solution and subsequently heated with a heat gun.

Under cooling with an ice-water bath, a 1.0 M solution of ^{n}BuNF in THF (12.5 mL, 12.5 mmol) was added dropwise to a solution of 2-methyl-4-(triisopropylsilyl)but-3-yn-2-ol-2-*^{13}C* (**25**) (2.41 g, 9.98 mmol) in THF (40 mL). Subsequently, H_2O (0.5 mL, 27.7 mmol) was added, the cooling bath was removed, and the pale-brown solution was allowed to warm to 18 °C and stirred for 20.5 h. TLC indicated incomplete conversion. The pale-brown solution was stirred at 40 °C for 2 hours. TLC indicated only little increase in conversion. A 1.0 M solution of ^{n}BuNF in THF (10 mL, 10 mmol) was added and the pale-brown solution was stirred at 40 °C for 2 hours. TLC still indicated only little increase in conversion. The solution was cooled to room temperature and stirred for 8 days. During this period, TLC indicated only little increase in conversion and indicated a rather incomplete desilylation. However, an ^{1}H NMR spectrum taken at that stage indicated a nearly complete conversion (see comment at the end of this paragraph).

Et_2O (25 mL), H_2O (25 mL), and 1 M aqueous HCl (10 mL) were added to the reaction solution. After thorough mixing, the organic and the aqueous phases were separated. The aqueous phase was extracted with Et_2O (3 × 25 mL). The combined organic phases were washed with saturated aqueous NaCl solution (25 mL) and concentrated to 20 mL on a rotary evaporator at 35 °C and reduced pressure (first 700 mbar and subsequently 300 mbar). *Caution:* The boiling point of 2-methyl-3-butyn-2-ol is 104 °C,[5] removal of the solvents at a higher temperature or a lower pressure causes loss of material. The components of the concentrate were separated by column chromatography (4.5 cm x 26 cm, pentane/Et_2O, 5:1). The fraction with R_f = 0.12 was collected and concentrated at 35 °C and reduced pressure (first 700 mbar and subsequently 300 mbar for 10 min). The residue was a colorless liquid (1.26 g) consisting of 2-methylbut-3-yn-2-ol-2-^{13}C (**26**) (9.56 mmol, 96% yield), THF (5.41 mmol), Et_2O (0.310 mmol), 2,6-di-*tert*-butyl-4-methylphenol (0.159 mmol), and traces of pentane and TIPS-F/OH. ^{1}H NMR signals assigned to 2-methylbut-3-yn-2-ol-2-^{13}C (**26**) (500 MHz, $CDCl_3$): δ = 2.42 (d, $^2J_{CH}$ = 3.3 Hz, 1 H, C≡CH), 2.01 (br s, 1 H, OH), 1.53 (d, $^2J_{CH}$ = 4.4 Hz, 6 H, $C(CH_3)_2$).

Comment: Obviously, the product content in the reaction mixture was substantially underestimated when applying TLC for reaction monitoring. Therefore, it is recommended to monitor the reaction with ^{1}H NMR spectroscopy instead of TLC. The discrepancy between the results of NMR spectroscopical analysis and TLC analysis may be due to loss of the product 2-methyl-3-butyn-2-ol during the procedure of TLC analysis: During the dipping of the TLC plate into the aqueous $KMnO_4$ solution, some of the 2-methyl-3-butyn-2-ol may have gone into this aqueous solution and/or the subsequent heating of the TLC plate may have caused the product to vaporize because of its low boiling point of ~104 °C.[5]

PyMTA ester 27. This reaction was performed under argon. The above mentioned mixture (35.1 mg) of 2-methylbut-3-yn-2-ol-2-^{13}C (**26**) (266 µmol), THF (151 µmol), Et_2O (8.64 µmol), BHT (4.43 µmol), and traces of pentane and TIPS-F/OH, was dissolved together with 4-iodo PyMTA ester **5** (94.0 mg, 155 µmol) in diisopropylamine (1.5 mL) and THF (5 mL). This solution was degassed and then $PdCl_2(PPh_3)_2$ (2.24 mg, 3.19 µmol) and CuI (1.30 mg, 6.83 µmol) were added. The solution was stirred at room temperature for 23 h. During this time a colorless precipitate formed. Under argon, all volatiles were evaporated by a short path distillation, and the residue was dissolved in

degassed anhydrous CH_2Cl_2 (3 mL). Metal scavenger QuadraPure™ TU (34 mg) was added. The suspension was stirred at room temperature for 19 h. The solution was separated from the resin through filtration through a syringe filter (Ø 13 mm, pore size: 0.45 µm, membrane: PVDF), and the resin was washed with CH_2Cl_2 (3 × 1 mL). All filtrates were combined. Removal of the solvent yielded a yellow solid (156 mg). Column chromatography (2.0 cm × 32 cm, CH_2Cl_2/Et_2O/EtOH 10:4:0.5) of the yellow solid gave PyMTA ester **27** (R_f = 0.22; 67 mg, yield 76%) in mixture with a trace of 2,6-di-*tert*-butyl-4-methylphenol as an orange-brown oil. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.49 (s, 2 H, $H_{pyridine}$), 4.16 (q, $^3J_{HH}$ = 7.2 Hz, 8 H, C$\underline{H}_2$CH$_3$), 4.00 (s, 4 H, pyridine-C$\underline{H}_2$), 3.60 (s, 8 H, NC$\underline{H}_2$CO$_2$Et), 2.01 (d, $^2J_{HC}$ = 2.3 Hz, 1 H, OH), 1.61 (d, $^2J_{HC}$ = 4.4 Hz, 6 H, ^{13}C(CH$_3$)$_2$), 1.27 (t, $^3J_{HH}$ = 7.2 Hz, 12 H, CH$_2$C$\underline{H}_3$).

H_nNa_{4-n}[PyMTA-E-^{13}C(CH$_3$)$_2$OH] 28. PyMTA ester **27** (67 mg, 119 µmol) was dissolved in EtOH (3 mL). H_2O (2 mL) and 1 M aqueous NaOH solution (1.19 mL, 1.19 mmol) were added. The pale yellow solution was stirred at room temperature for 18 h. Solvents were removed. The residue was dissolved in H_2O (3 mL), and this aqueous solution was washed with CH_2Cl_2 (3 × 3 mL) and Et_2O (4 × 2 mL). The washing was performed in a centrifuge tube as follows: The aqueous solution was mixed well with CH_2Cl_2 or Et_2O. Centrifugation of the resulting yellow emulsion at 6500 rpm for 2 min separated the mixture into an organic and an aqueous phase. The organic phase was removed with the help of a glass pipette. To the well-washed aqueous solution, proton-exchange resin was added in an amount sufficient to lower the pH of the solution to ca. 4.5. The solution was separated from the resin through filtration through a syringe filter (Ø 13 mm, pore size: 0.45 µm, membrane: PVDF), and the resin was washed with a mixture of EtOH/H_2O 1:1 (4 × 1 mL). The combined filtrates were combined. Removal of the solvents yielded H_nNa_{4-n}[PyMTA-E-^{13}C(CH$_3$)$_2$OH] **28** (54 mg, containing 86 µmol of structural motif PyMTA-E-^{13}C(CH$_3$)$_2$OH]$^{4-}$, yield 72%) as a beige solid. The content of the structural motif [PyMTA-E-^{13}C(CH$_3$)$_2$OH]$^{4-}$ was determined by quantitative ^{1}H NMR spectroscopy. ^{1}H NMR (500 MHz, D_2O): δ = 7.57 (s, 2 H, $H_{pyridine}$), 4.67 (s, 4 H, pyridine-C$\underline{H}_2$), 3.93 (s, 8 H, NCH$_2$CO$_2$), 1.61 (d, $^2J_{HC}$ = 4.4 Hz, 6 H, ^{13}C(CH$_3$)$_2$). ^{13}C NMR (126 MHz, $CDCl_3$): δ = 170.8 (s, CO$_2$), 151.0 ($C_{pyridine}$ *ortho* to N), 134.6 (d, $^3J_{CC}$ = 1.4 Hz, $C_{pyridine}$ *para* to N), 127.3 ($C_{pyridine}$H), 101.3 (d, $^1J_{CC}$ = 72.8 Hz, C≡$\underline{C}$-^{13}C), 79.3 (d, $^2J_{CC}$ = 11.7 Hz, $\underline{C}$≡C-^{13}C), 65.9 (C≡C-$^{13}\underline{C}$), 58.6 (pyridine-$\underline{C}$H$_2$), 57.7 ($\underline{C}$H$_2$CO$_2$), 30.2 (d, $^1J_{CC}$ = 39.4 Hz, ^{13}C($\underline{C}$H$_3$)$_2$). MS (ESI) *m/z* = 224.8 {H_2[PyMTA-E-

$^{13}C(CH_3)_2OH]\}^{2-}$. Accurate MS (ESI) *m/z* calcd. for $\{H_3[PyMTA\text{-}E\text{-}^{13}C(CH_3)_2OH]\}^-$ $C_{19}{}^{13}CH_{24}N_3O_9^-$: 451.1541; found: 451.1547.

Na[{Gd^{III}(PyMTA)}-E-^{13}C(CH$_3$)$_2$OH] 1e. $H_nNa_{4-n}[PyMTA\text{-}E\text{-}^{13}C(CH_3)_2OH]$ **28** obtained as described above (7.8 mg; containing 12.4 µmol structural motif of $[PyMTA\text{-}E\text{-}^{13}C(CH_3)_2OH]^{4-}$) was dissolved in D_2O (1500 µL) yielding a 8.30 mM solution of $[PyMTA\text{-}E\text{-}^{13}C(CH_3)_2OH]^{4-}$ in D_2O. Part of this solution (190 µL, containing 1.58 µmol of $[PyMTA\text{-}E\text{-}^{13}C(CH_3)_2OH]^{4-}$) was taken and a solution of 50 mM solution of $GdCl_3$•6 H_2O in D_2O (30.0 µL, 1.50 µmol) was added. A 0.10 M solution of NaOD in D_2O (45 µL, 4.5 µmol) was added to rise the pH of the solution to pH 6.8. The solution was diluted with D_2O up to a total volume of 500 µL. This gave a 3.0 mM solution of Na[{Gd^{III}(PyMTA)}-E-^{13}C(CH$_3$)$_2$OH]] **1e** in D_2O. Accurate MS (ESI) *m/z* calcd. for $[M - Na]^-$ $C_{19}{}^{13}CH_{21}N_3O_9Gd^-$: 606.0547; found: 606.0561.

NMR spectra

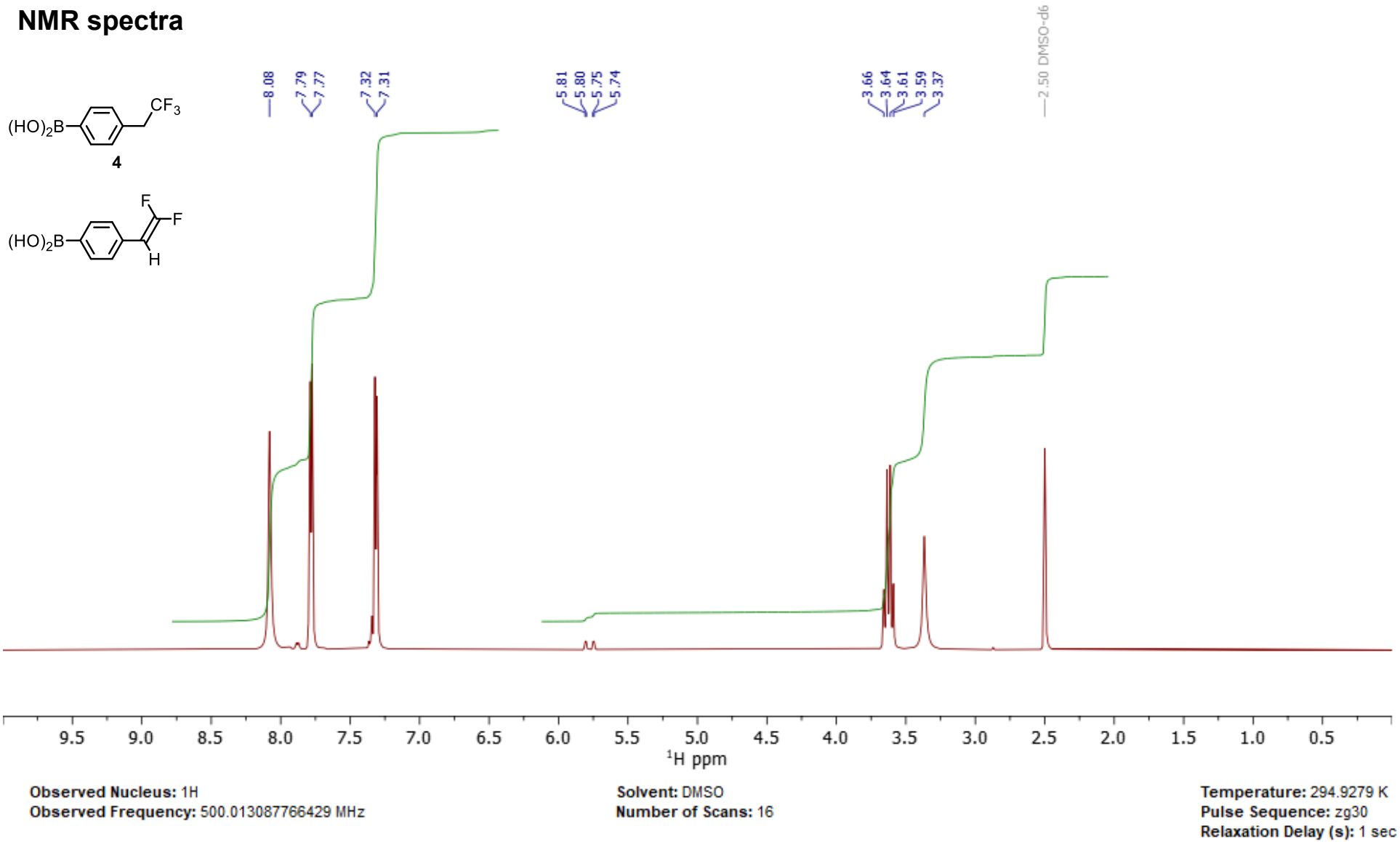


Figure S-II-1. ^{1}H NMR spectrum (500 MHz, DMSO-d6) of the 10:1.0 mixture of (4-(trifluoroethyl)phenyl)boronic acid **4** and (4-(2,2-difluorovinyl)phenyl)boronic acid.

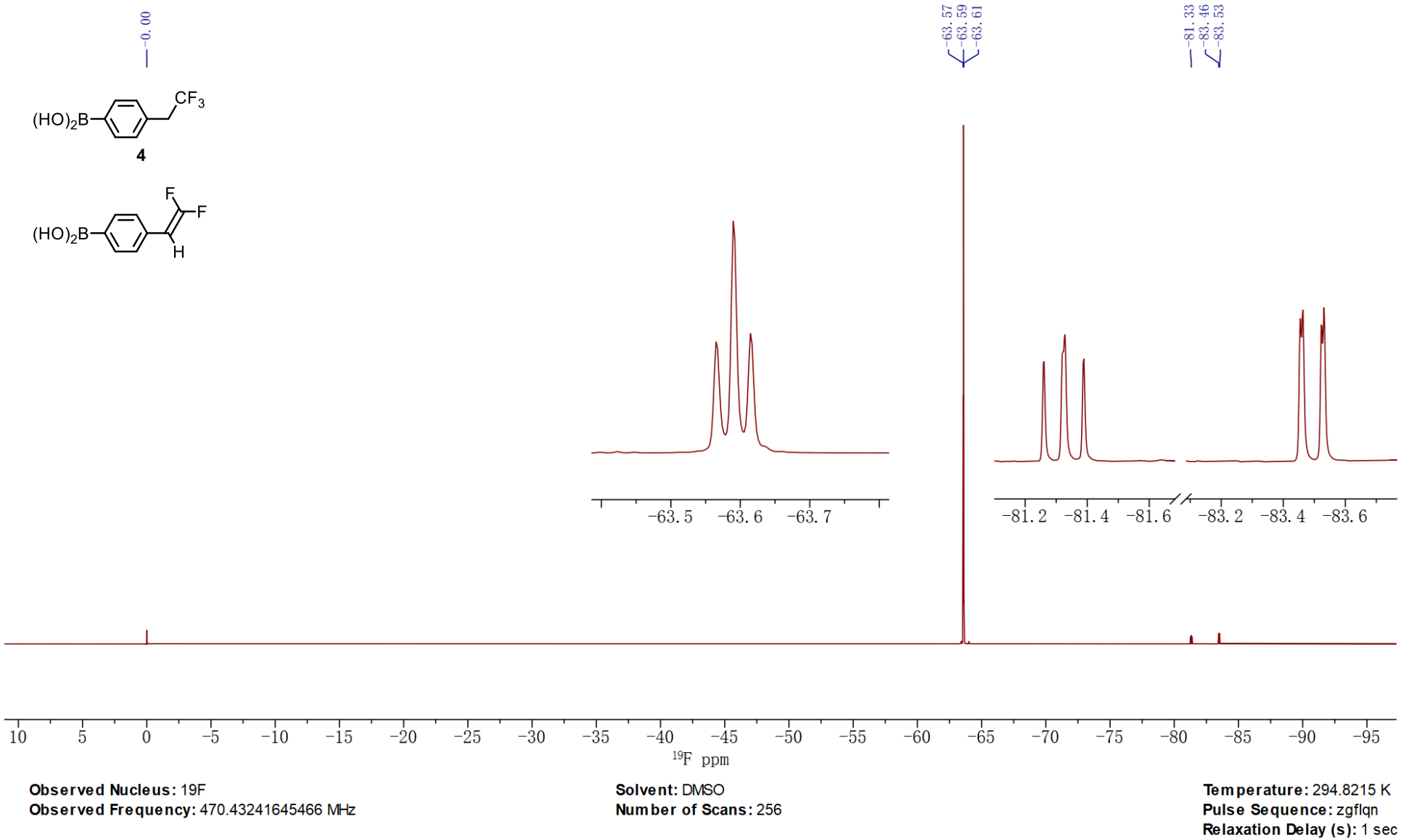


Figure S-II-2. ^{19}F NMR spectrum (470 MHz, DMSO-d6) of the 10:1.0 mixture of (4-(trifluoroethyl)phenyl)boronic acid **4** and (4-(2,2-difluorovinyl)phenyl)boronic acid.

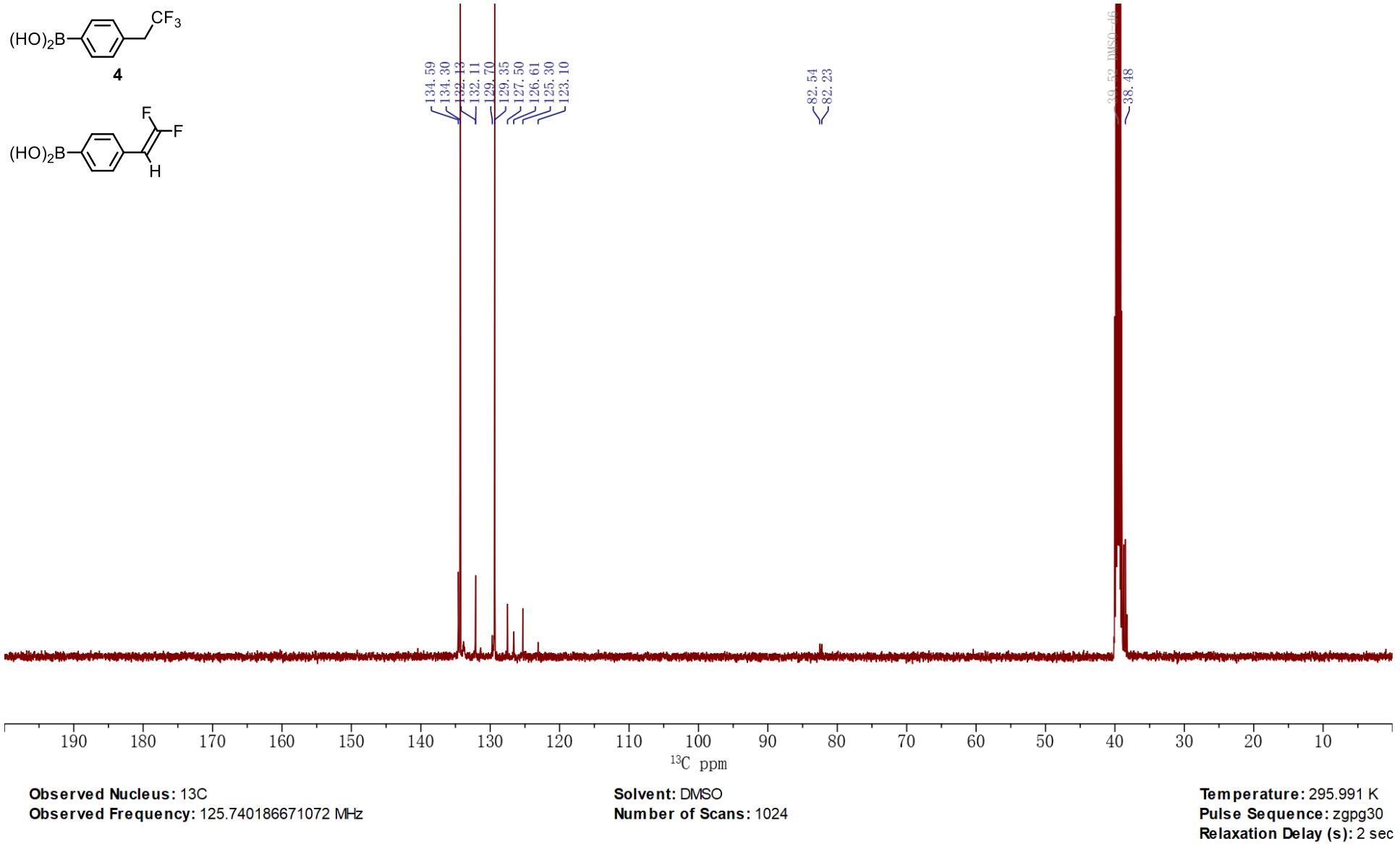


Figure S-II-3. ^{13}C NMR spectrum (126 MHz, DMSO-d6) of the 10:1.0 mixture of (4-(trifluoroethyl)phenyl)boronic acid **4** and (4-(2,2-difluorovinyl)phenyl)boronic acid.

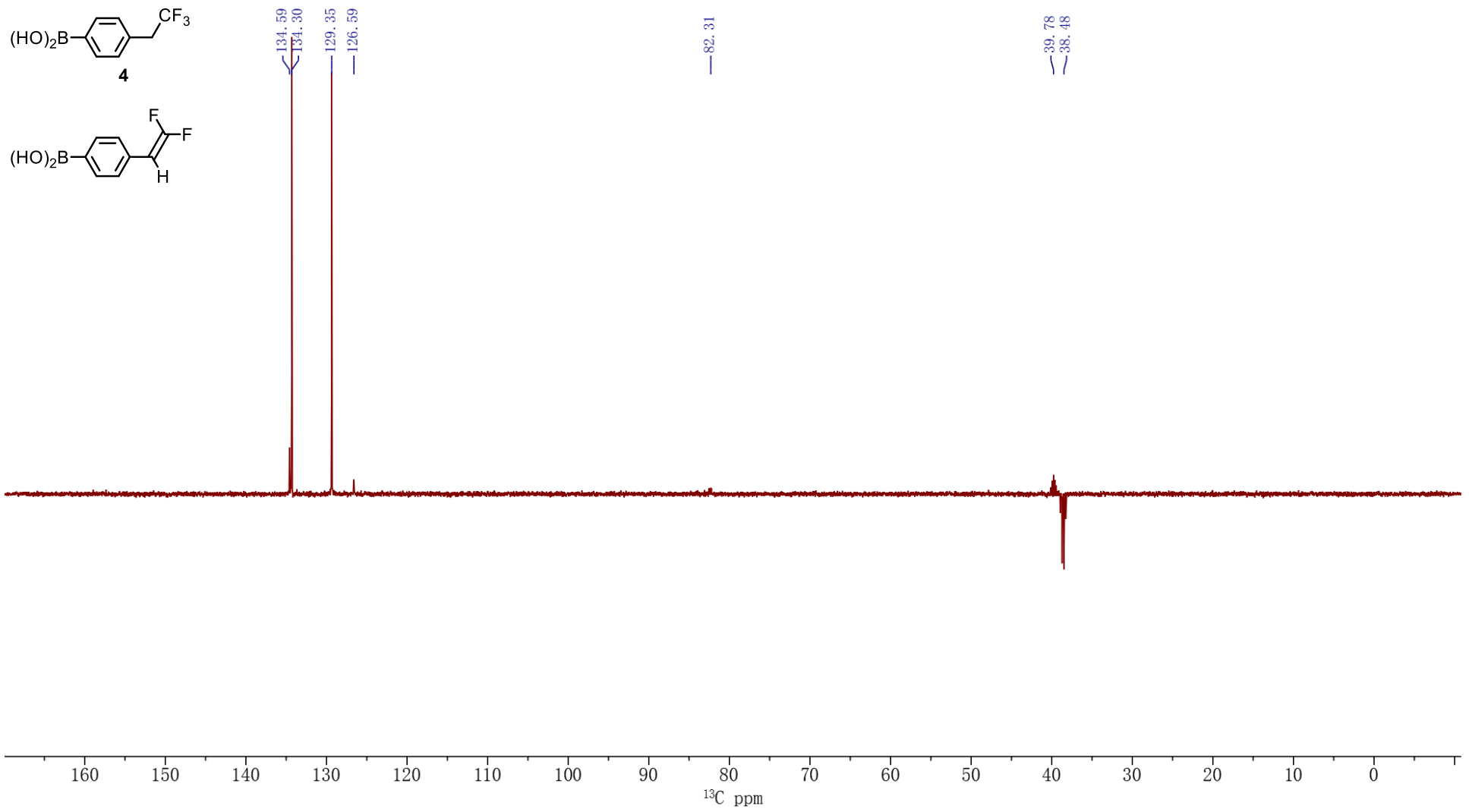


Figure S-II-4. ^{13}C DEPT 135 NMR spectrum (126 MHz, DMSO-d6) of the 10:1.0 mixture of (4-(trifluoroethyl)phenyl)boronic acid **4** and (4-(2,2-difluorovinyl)phenyl)boronic acid.

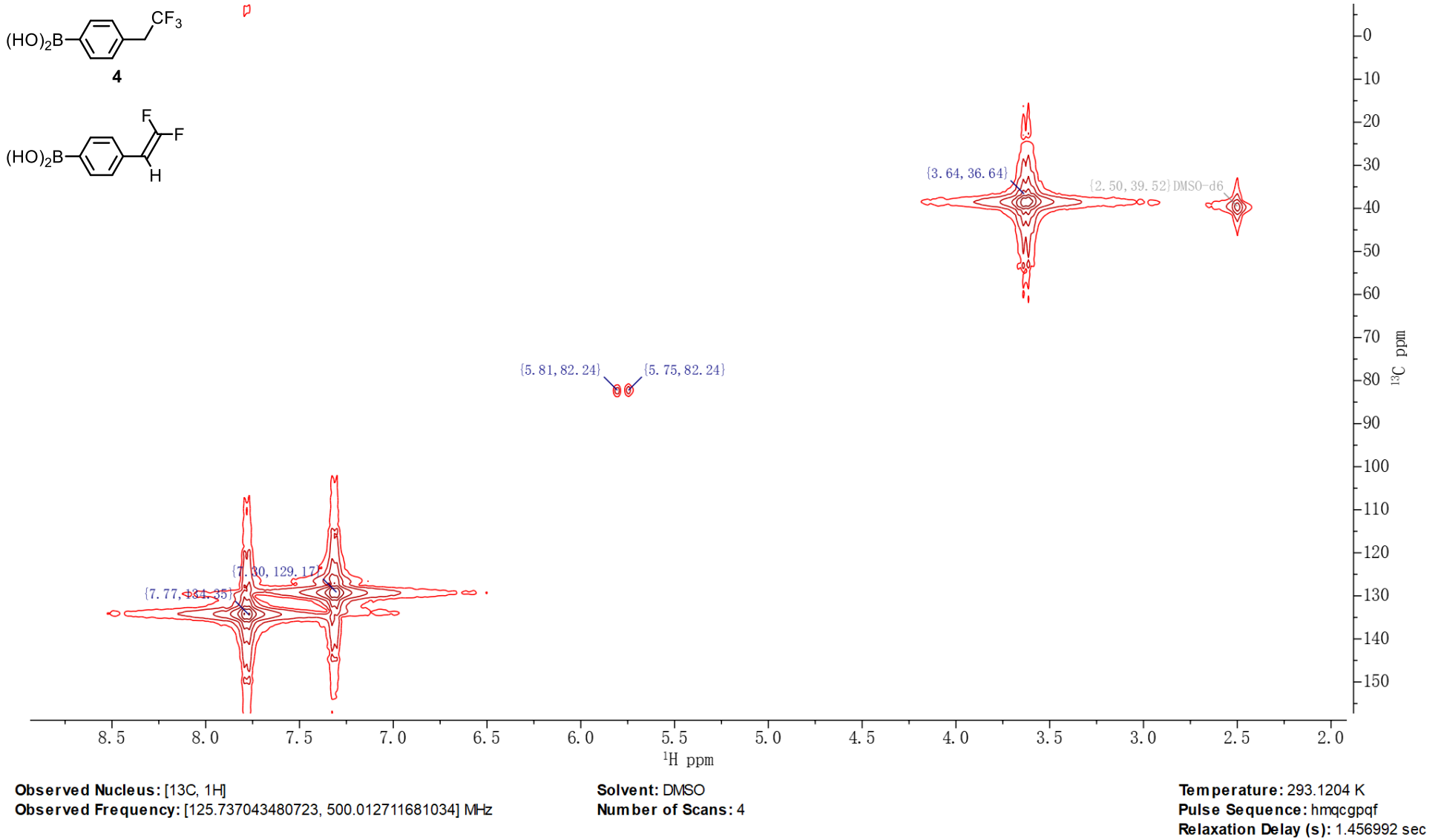


Figure S-II-5. ^{1}H ^{13}C HMQC NMR spectrum (500 MHz, 126 MHz, DMSO-d6) of the 10:1.0 mixture of (4-(trifluoroethyl)phenyl)boronic acid **4** and (4-(2,2-difluorovinyl)phenyl)boronic acid.

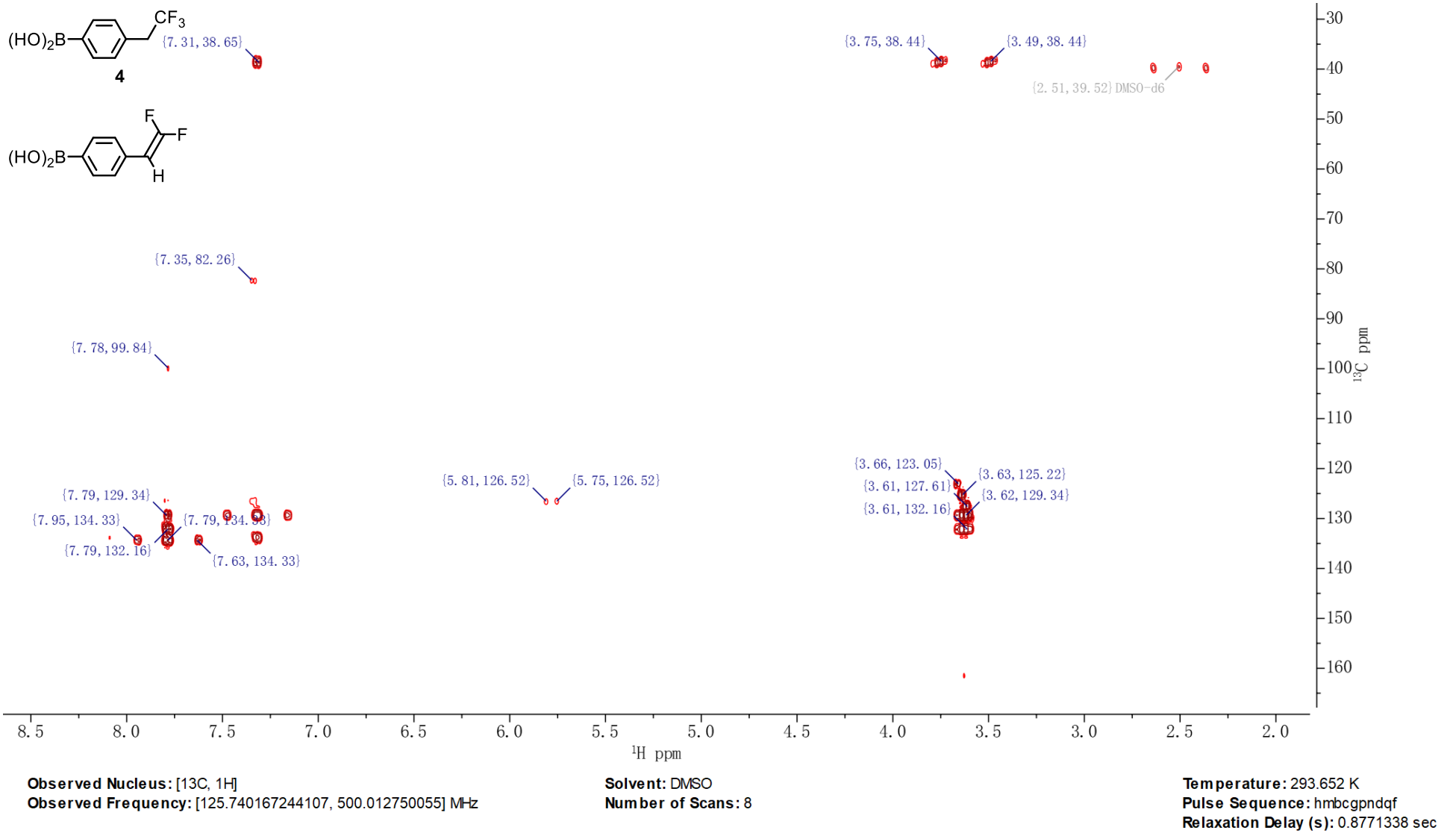


Figure S-II-6. ^{1}H ^{13}C HMBC NMR spectrum (500 MHz, 126 MHz, DMSO-d6) of the 10:1.0 mixture of (4-(trifluoroethyl)phenyl)boronic acid **4** and (4-(2,2-difluorovinyl)phenyl)boronic acid.

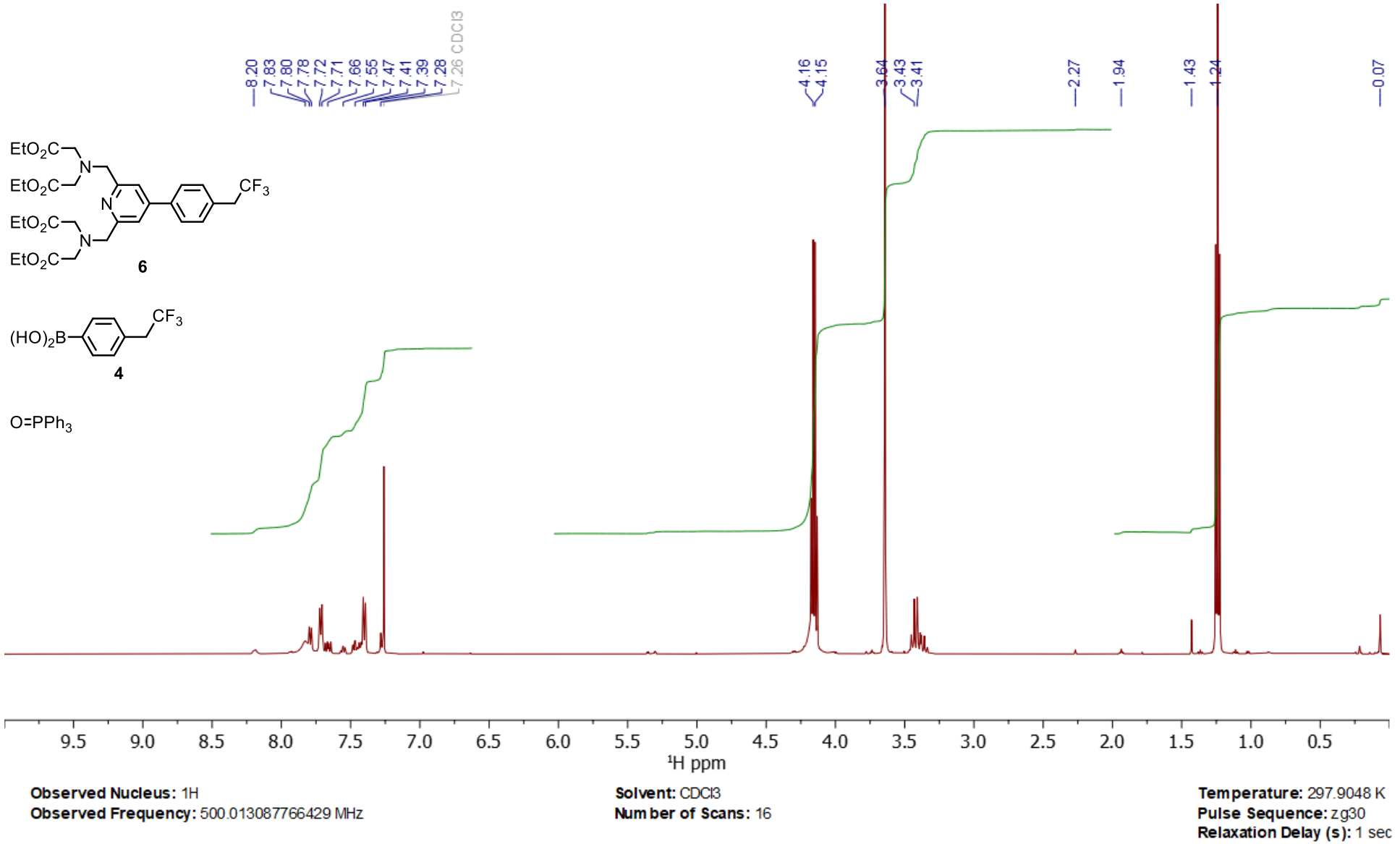


Figure S-II-7. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of the 1.0:0.33:0.10 mixture of (4-(trifluoroethyl)phenyl)-PyMTA ester **6**, (4-(trifluoroethyl)phenyl)boronic acid **4**, triphenylphosphine oxide, and a small amount of other unidentified compounds.

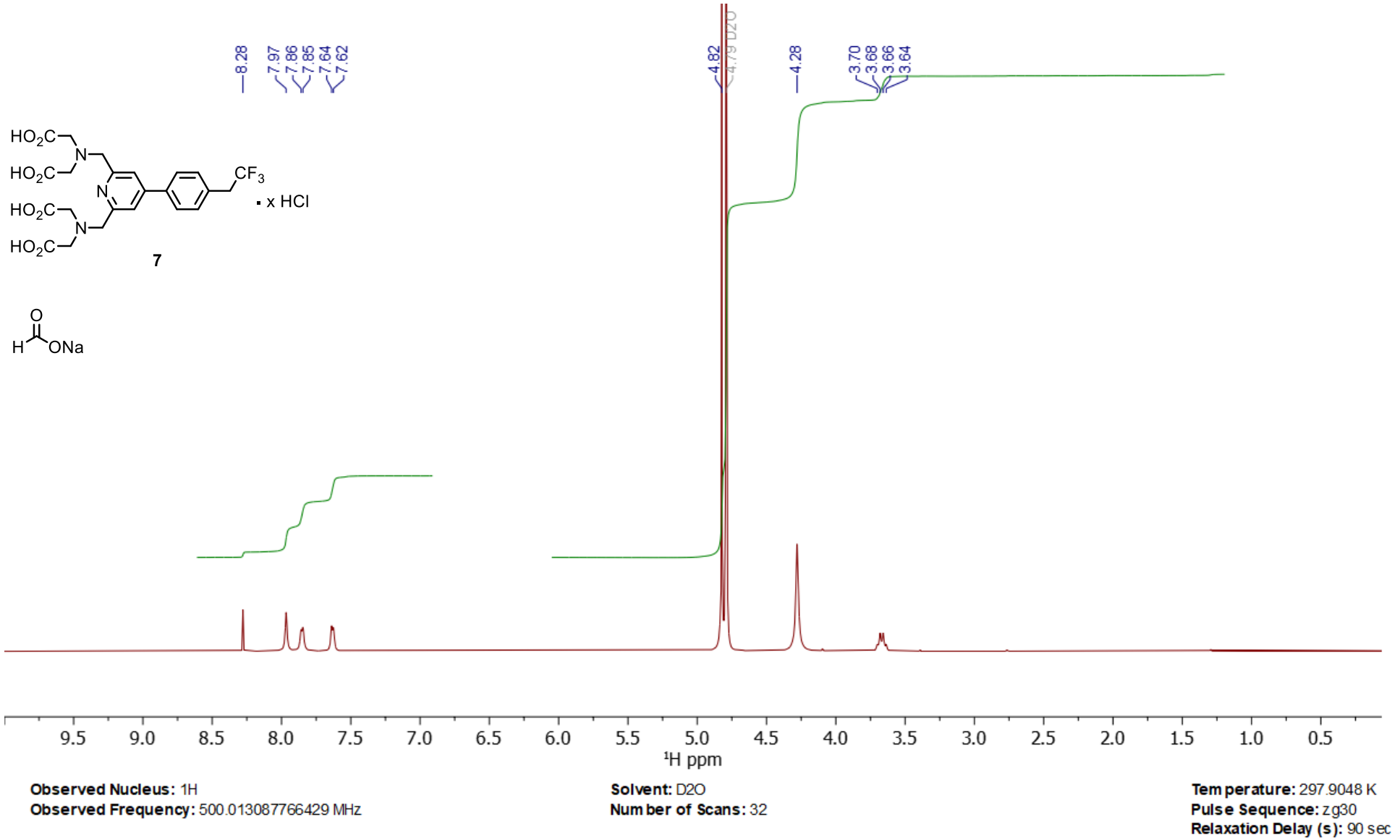


Figure S-II-8. ^{1}H NMR spectrum (500 MHz, D_2O) of the mixture of H_4[PyMTA-P-CH_2CF_3]•x HCl **7**, sodium formate, and NaCl, with a 1.0:0.44 ratio of H_4[PyMTA-P-CH_2CF_3]•x HCl **7** to sodium formate.

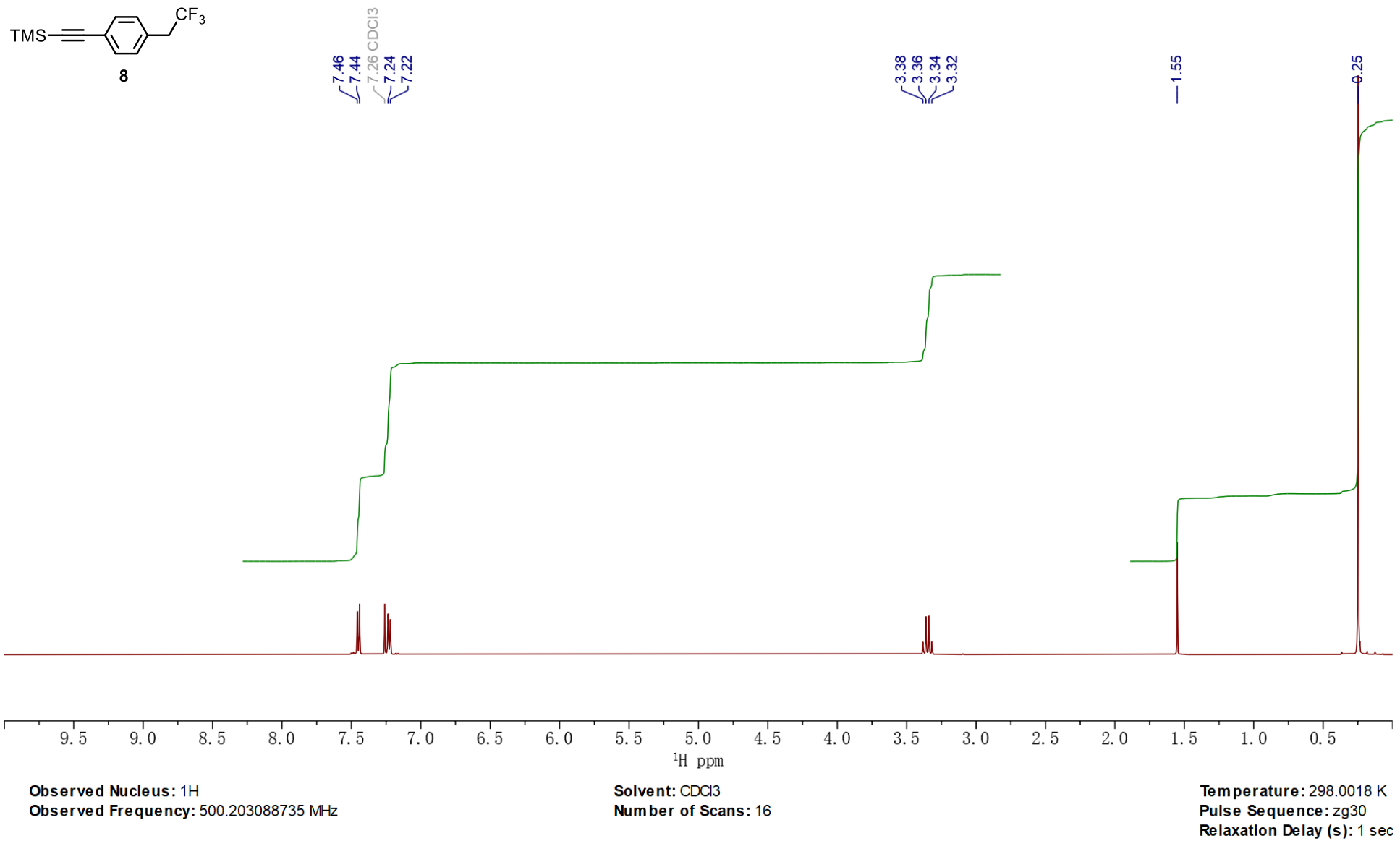


Figure S-II-9. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of (4-(trifluoroethyl)phenyl)ethyne **8**.

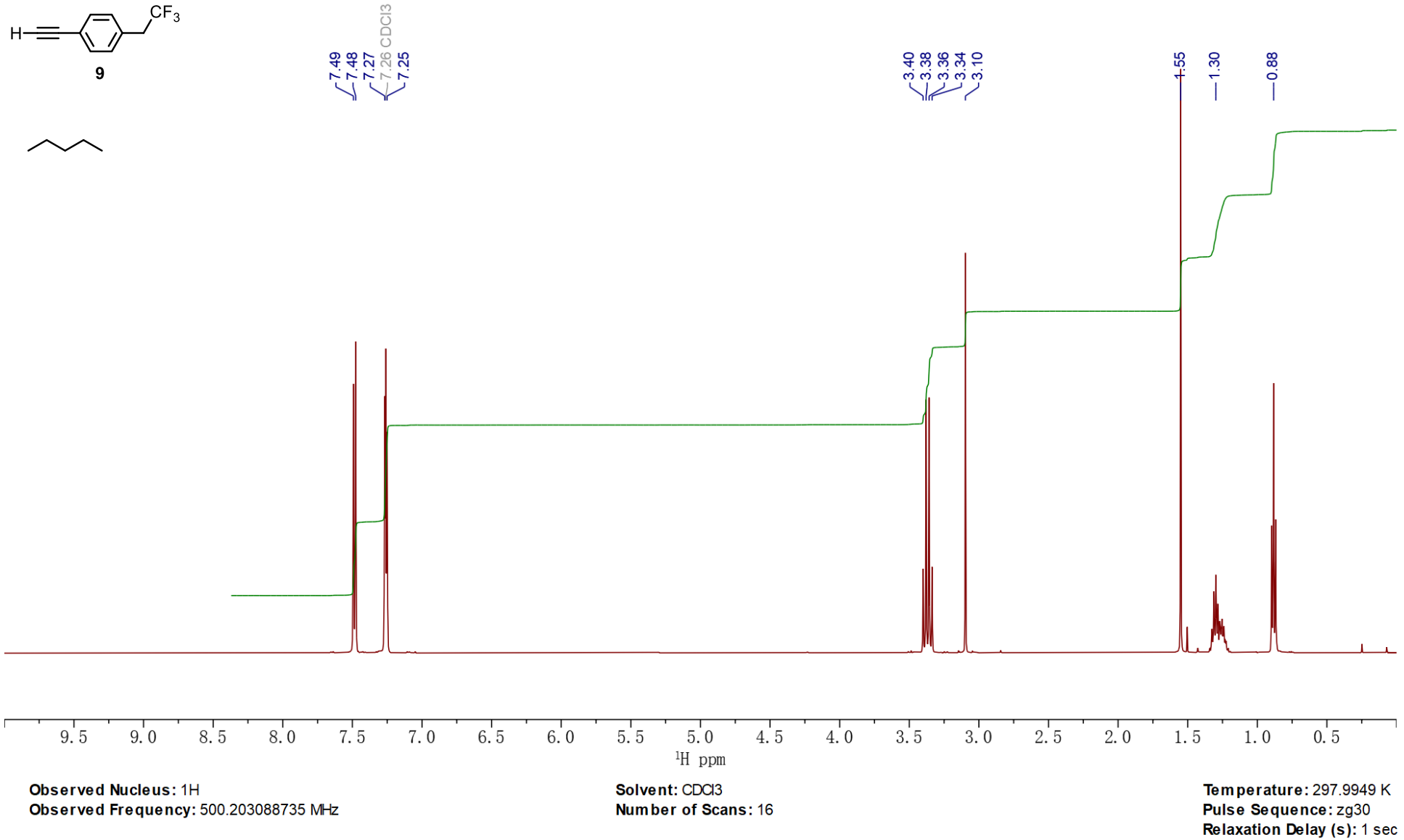


Figure S-II-10. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of the 79:21 mixture of (4-(trifluoroethyl)phenyl)ethyne **9** and *n*-pentane.

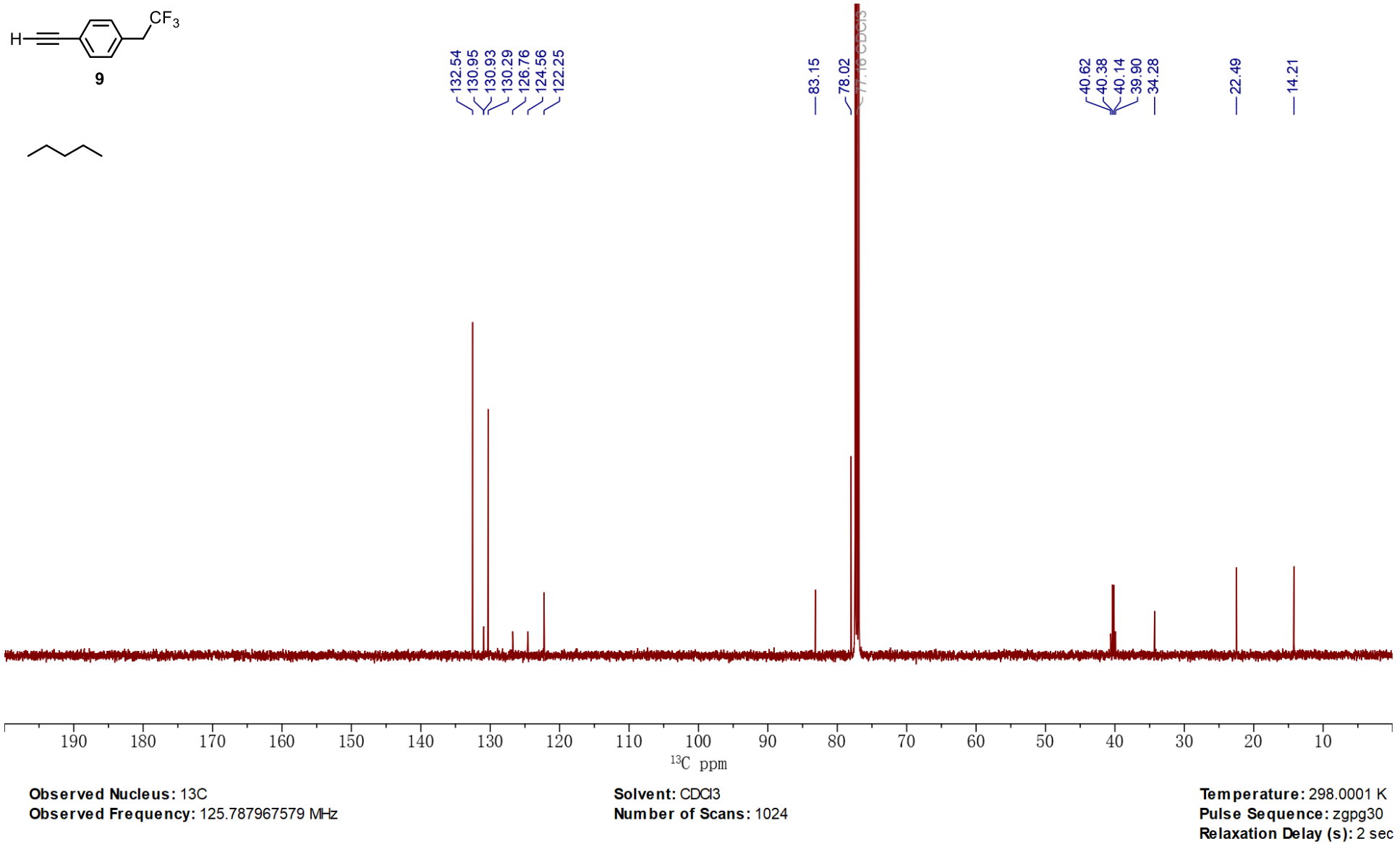


Figure S-II-11. ^{13}C NMR spectrum (126 MHz, $CDCl_3$) of the 79:21 mixture of (4-(trifluoroethyl)phenyl)ethyne **9** and *n*-pentane.

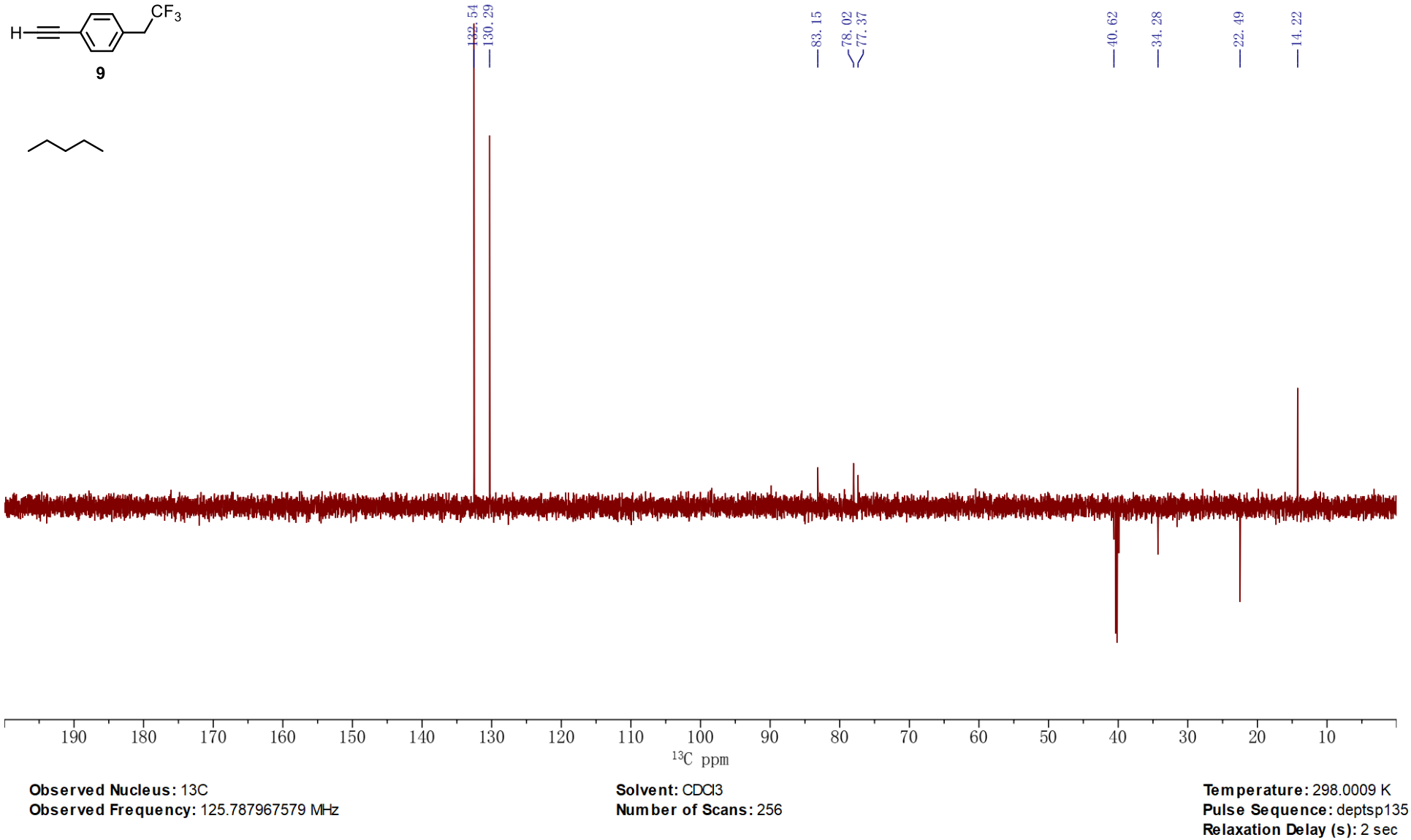


Figure S-II-12. ^{13}C DEPT 135 NMR spectrum (126 MHz, $CDCl_3$) of the 79:21 mixture of (4-(trifluoroethyl)phenyl)ethyne **9** and *n*-pentane.

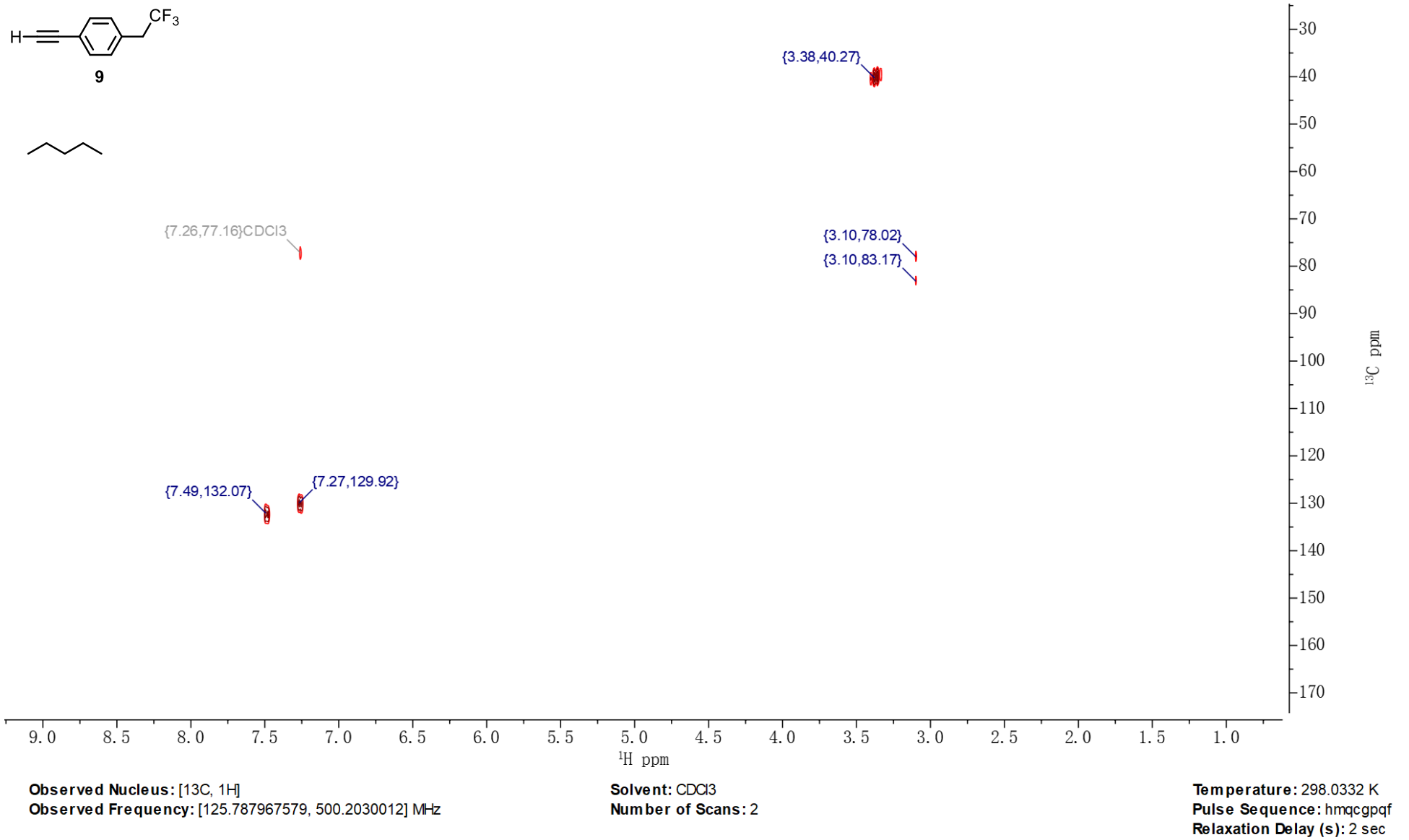


Figure S-II-13. ^{1}H ^{13}C HMQC NMR spectrum (500 MHz, 126 MHz, $CDCl_3$) of the 79:21 mixture of (4-(trifluoroethyl)phenyl)ethyne **9** and *n*-pentane.

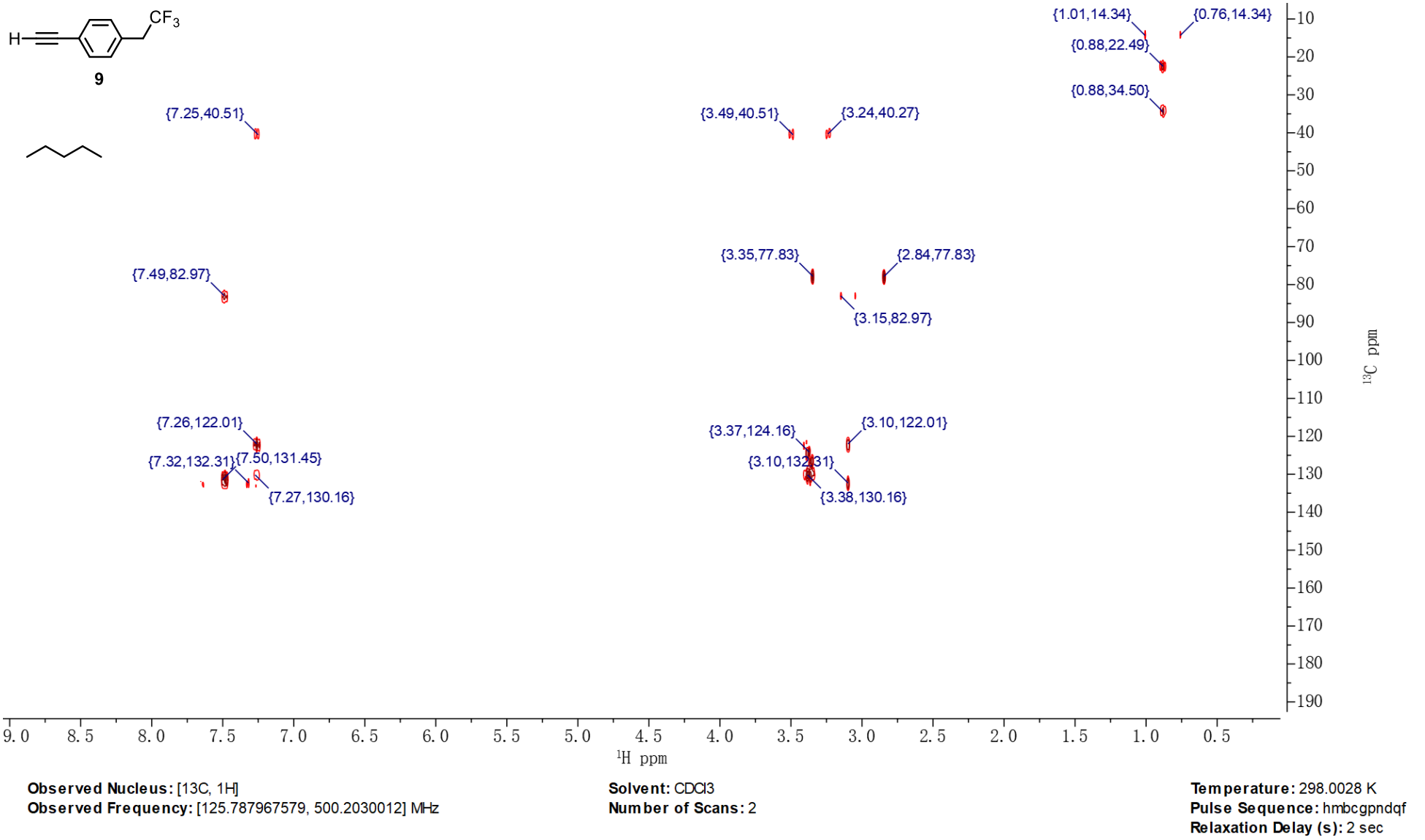


Figure S-II-14. ^{1}H ^{13}C HMBC NMR spectrum (500 MHz, 126 MHz, $CDCl_3$) of the 79:21 mixture of (4-(trifluoroethyl)phenyl)ethyne **9** and *n*-pentane.

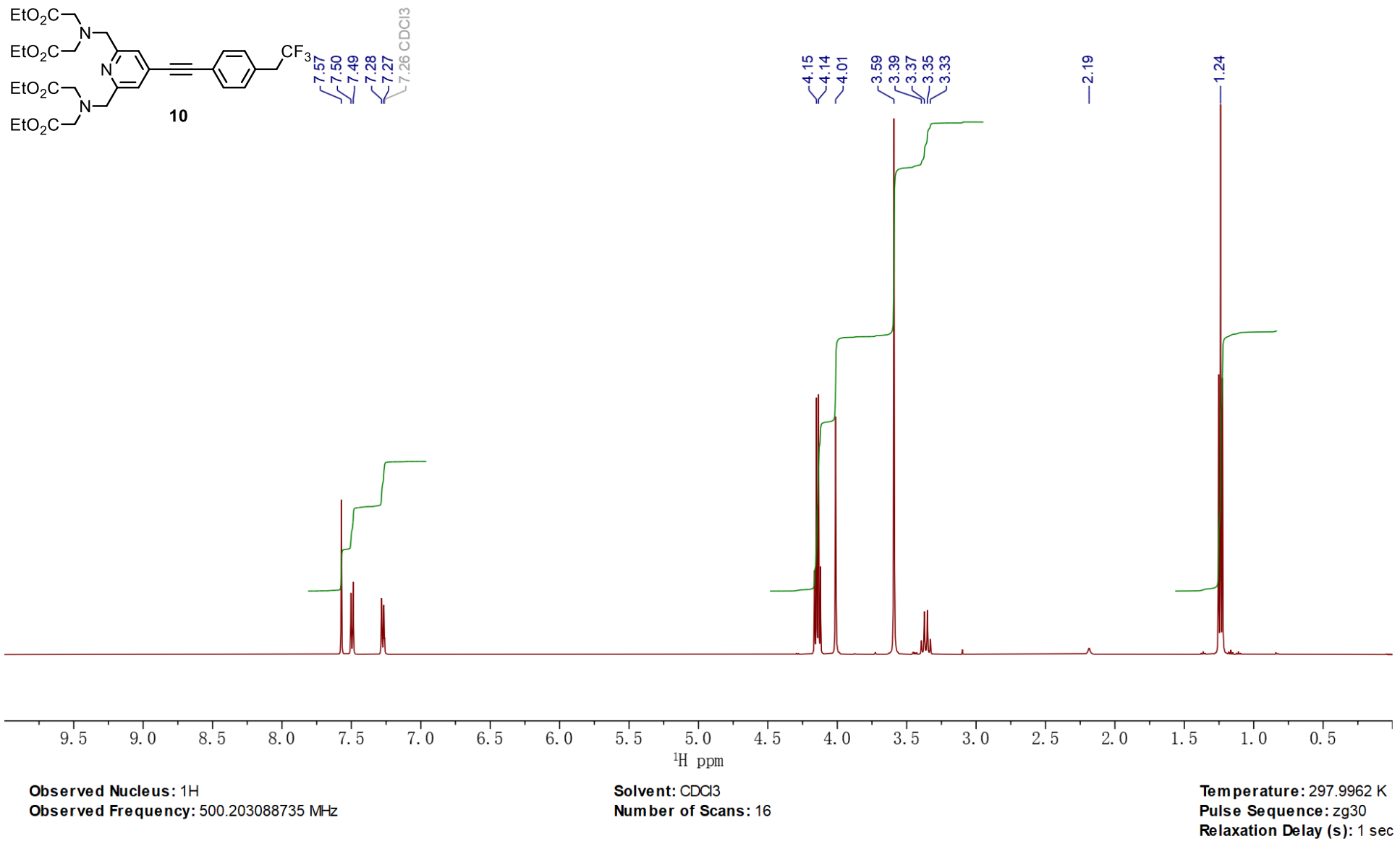


Figure S-II-15. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of ((4-(trifluoroethyl)phenyl)ethynyl)-PyMTA ester **10**.

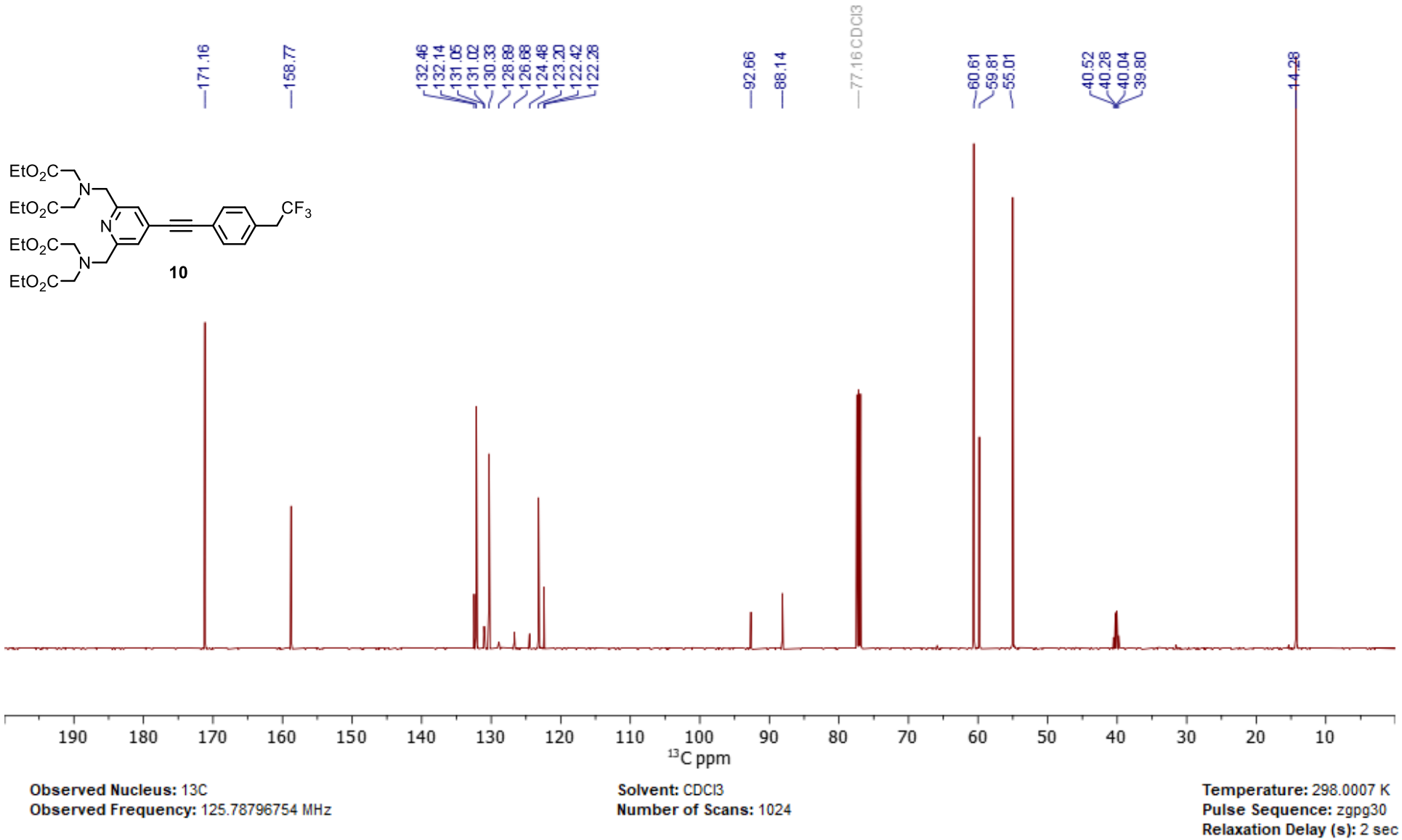


Figure S-II-16. ^{13}C NMR spectrum (126 MHz, $CDCl_3$) of ((4-(trifluoroethyl)phenyl)ethynyl)-PyMTA ester **10**.

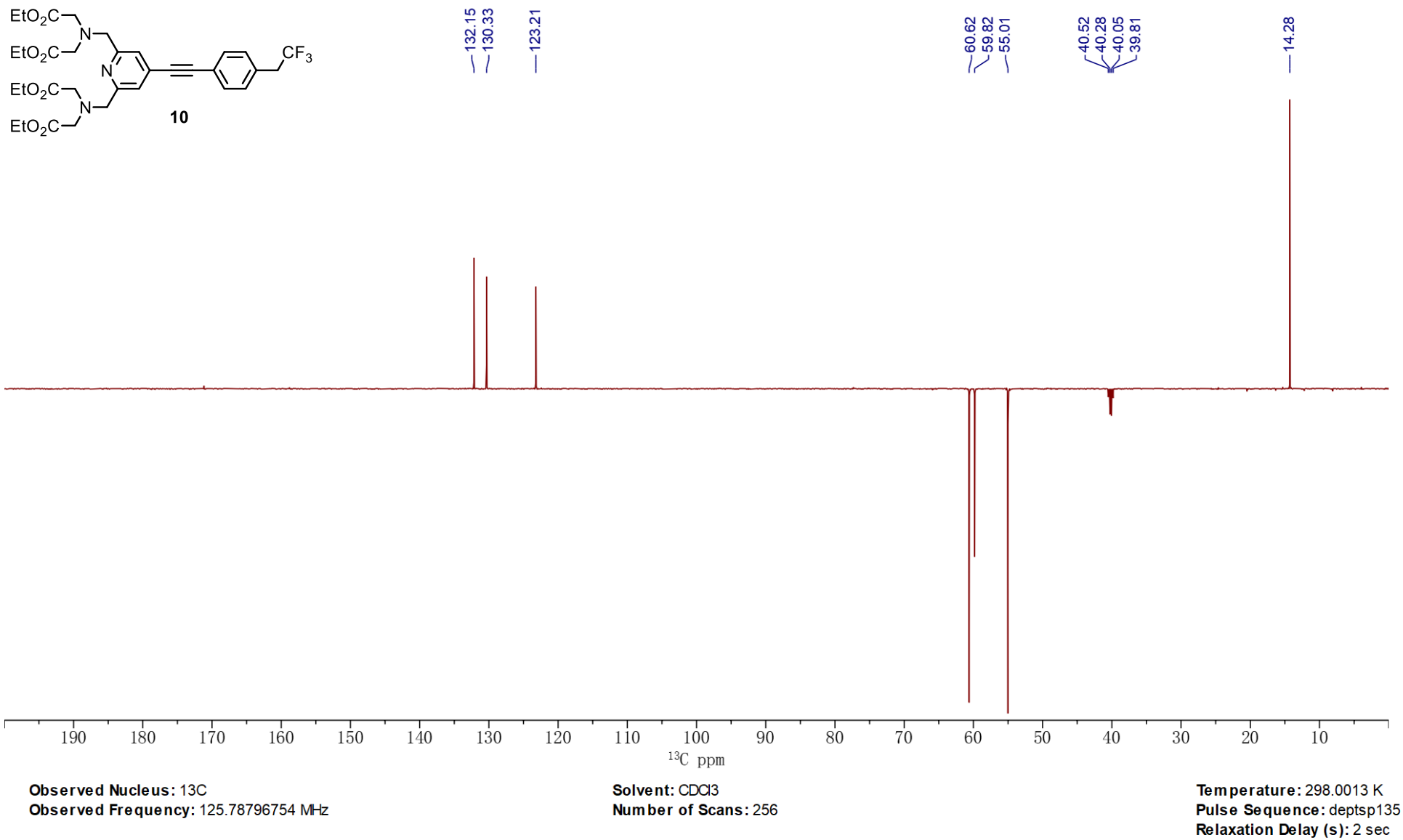


Figure S-II-17. ^{13}C DEPT 135 NMR spectrum (126 MHz, $CDCl_3$) of ((4-(trifluoroethyl)phenyl)ethynyl)-PyMTA ester **10**.

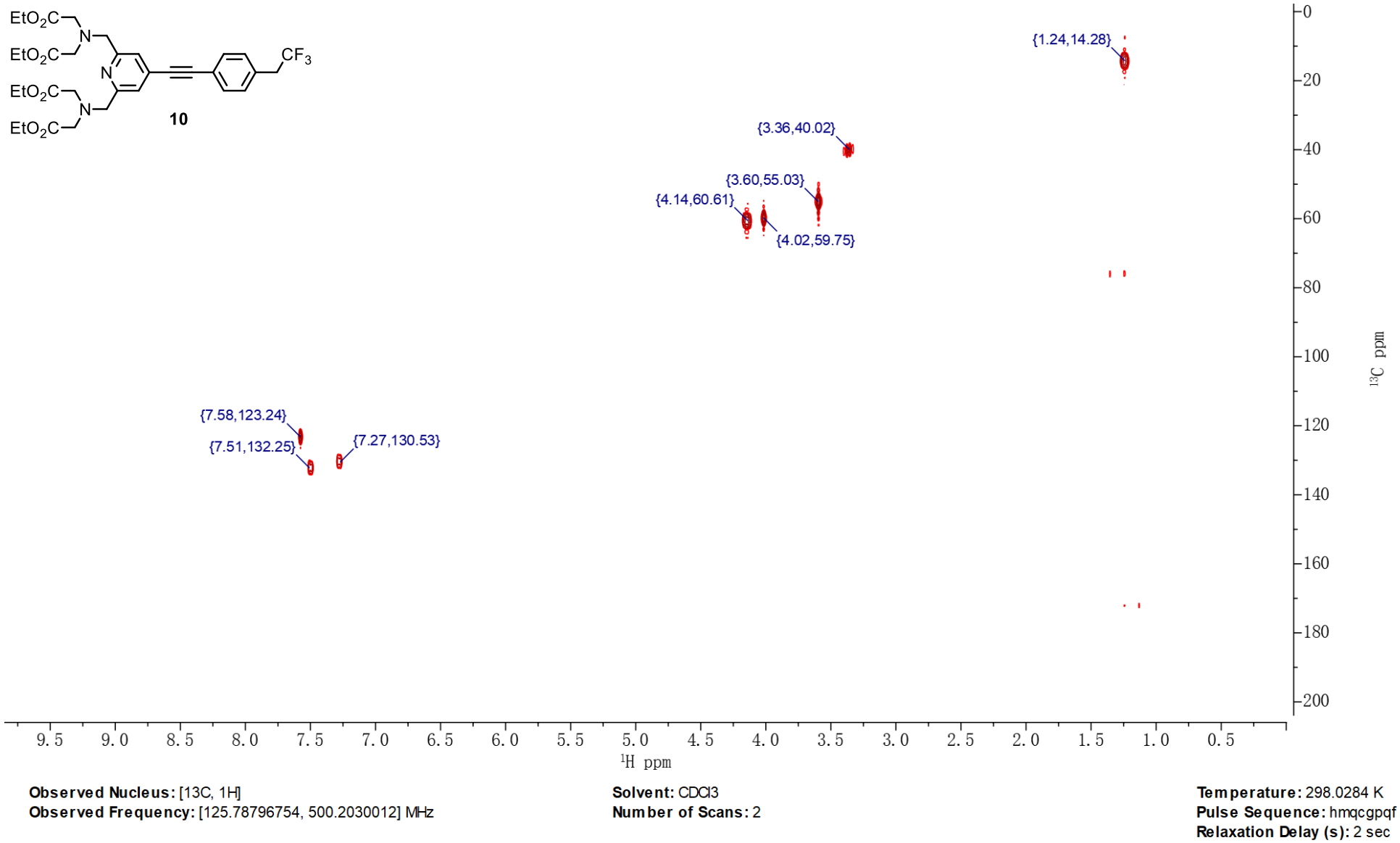


Figure S-II-18. ^{1}H ^{13}C HMQC NMR spectrum (500 MHz, 126 MHz, $CDCl_3$) of ((4-(trifluoroethyl)phenyl)ethynyl)-PyMTA ester **10**.

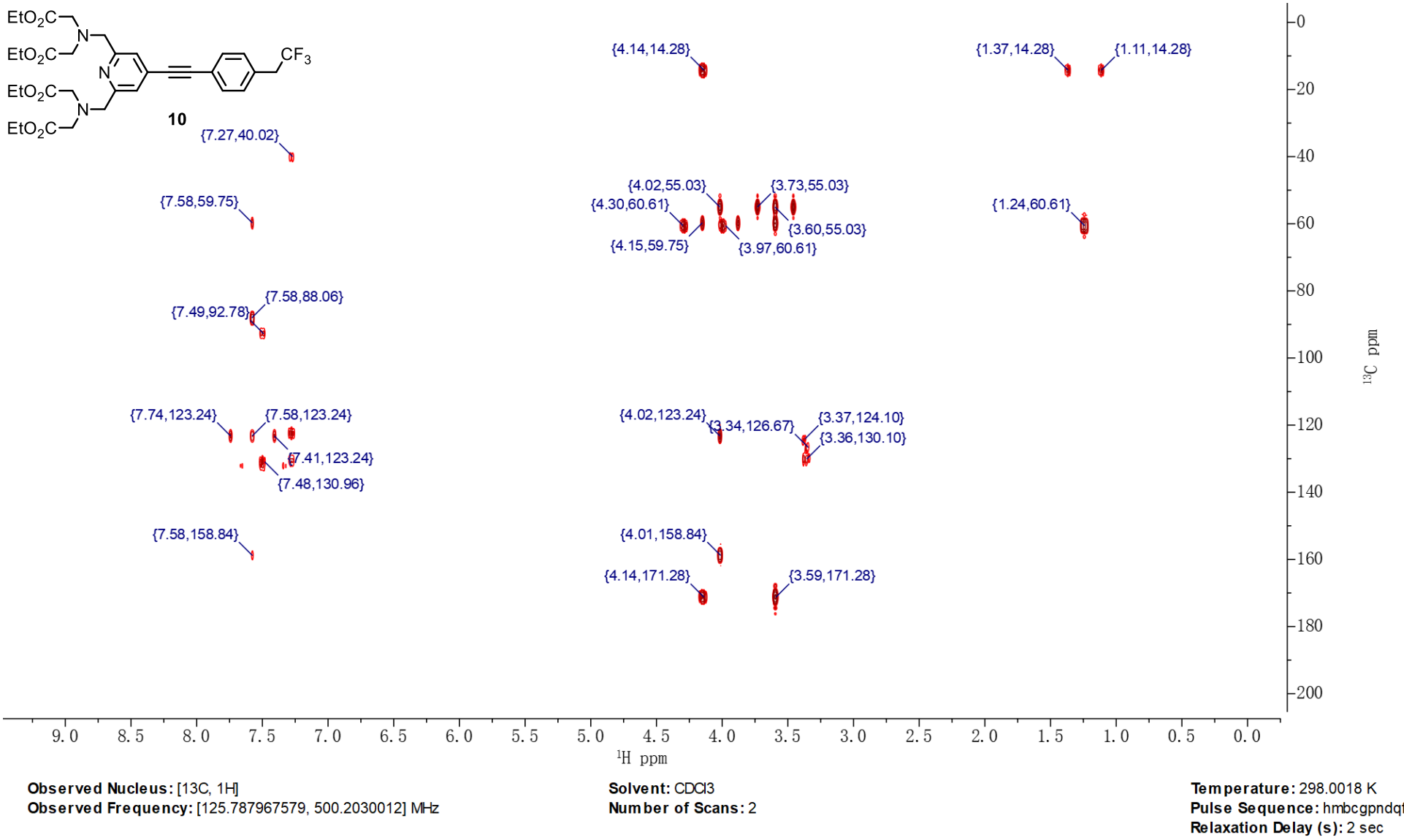


Figure S-II-19. ^{1}H ^{13}C HMBC NMR spectrum (500 MHz, 126 MHz, $CDCl_3$) of ((4-(trifluoroethyl)phenyl)ethynyl)-PyMTA ester **10**.

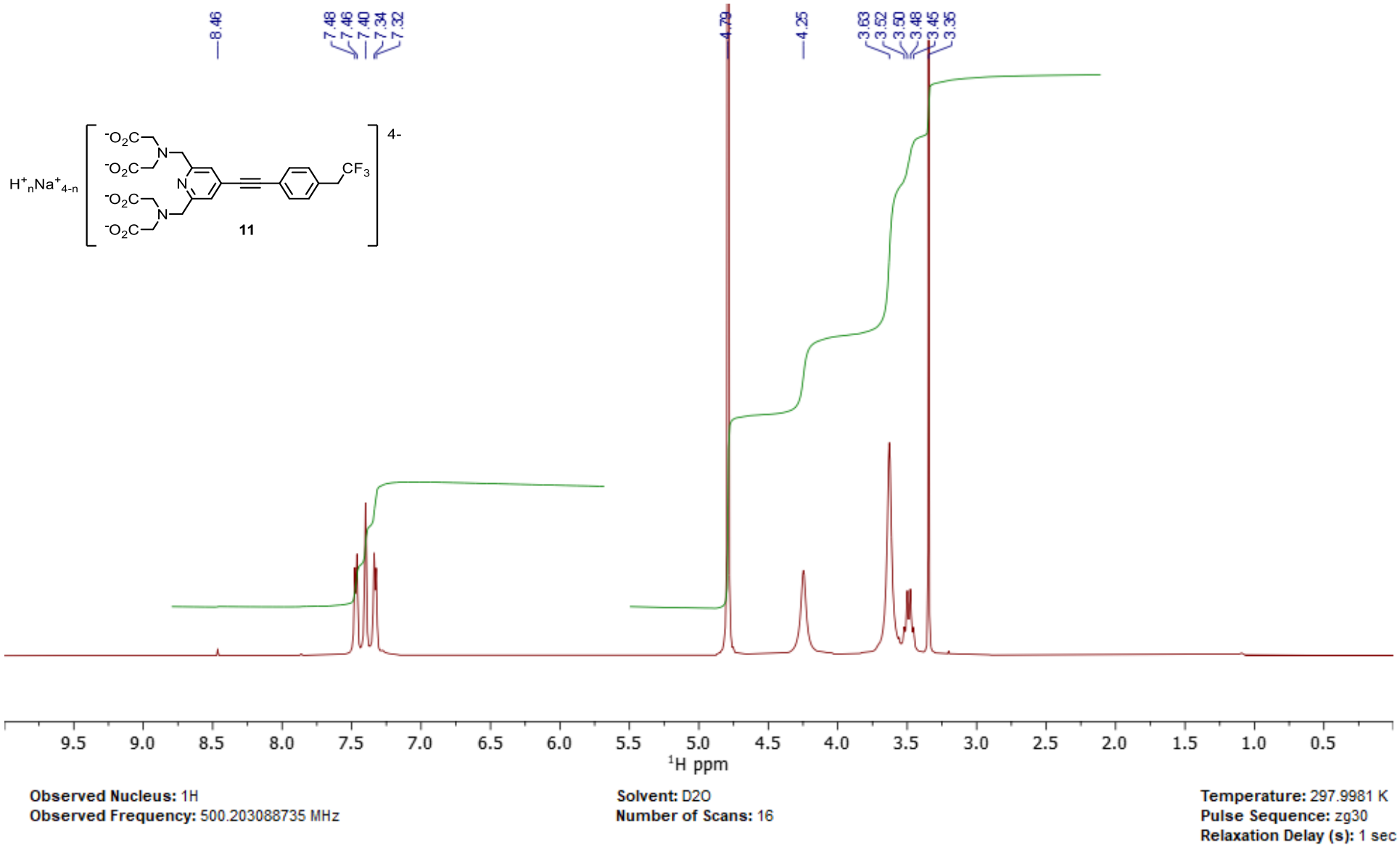


Figure S-II-20. ^{1}H NMR spectrum (500 MHz, D_2O) of H_nNa_{4-n}[PyMTA-EP-CH_2CF_3] **11**, a drop of MeOH was added for calibration of the ^{13}C NMR spectrum.

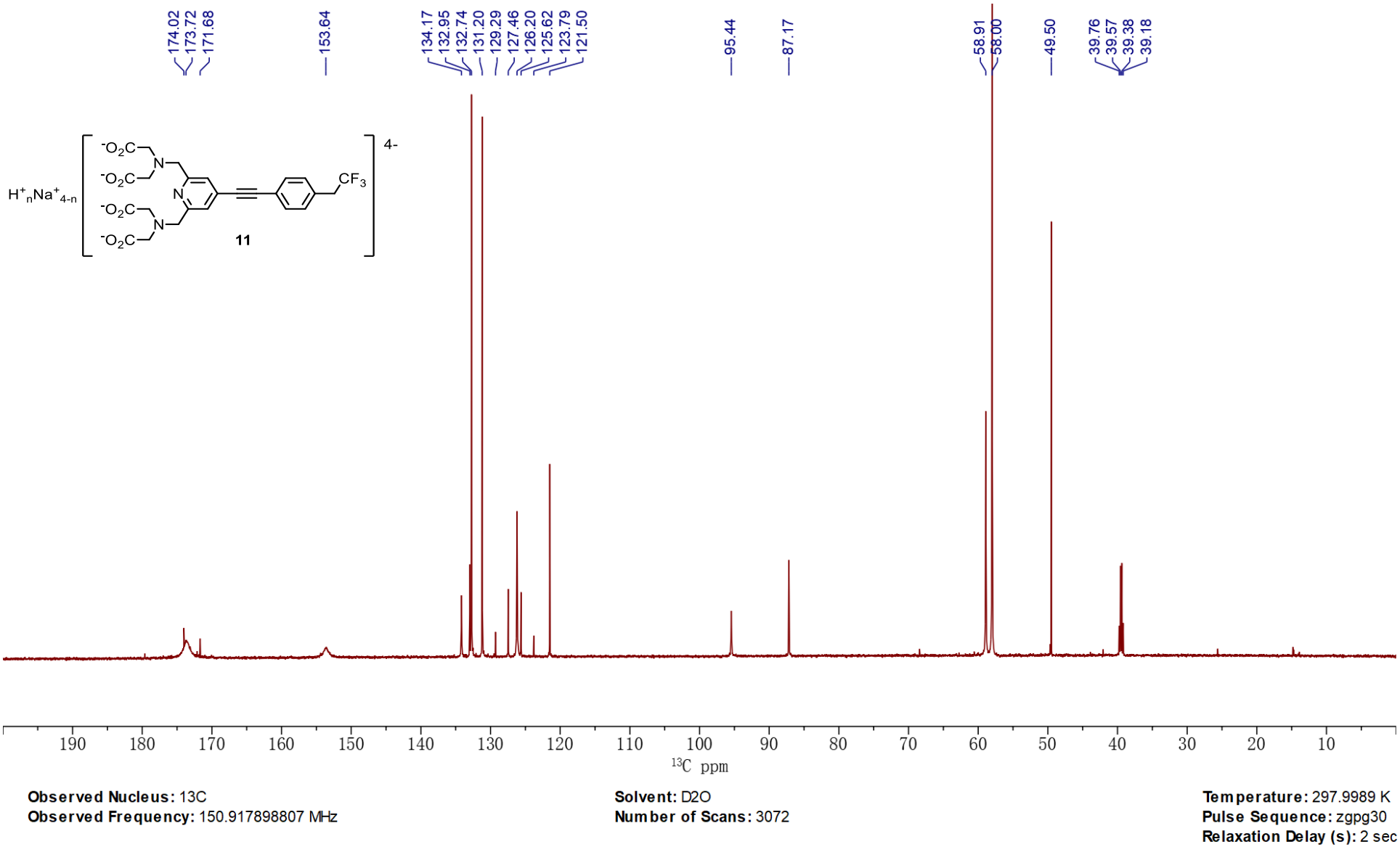


Figure S-II-21. ^{13}C NMR spectrum (150 MHz, D_2O) of H_nNa_{4-n}[PyMTA-EP-CH_2CF_3] **11**, a drop of MeOH was added for calibration.

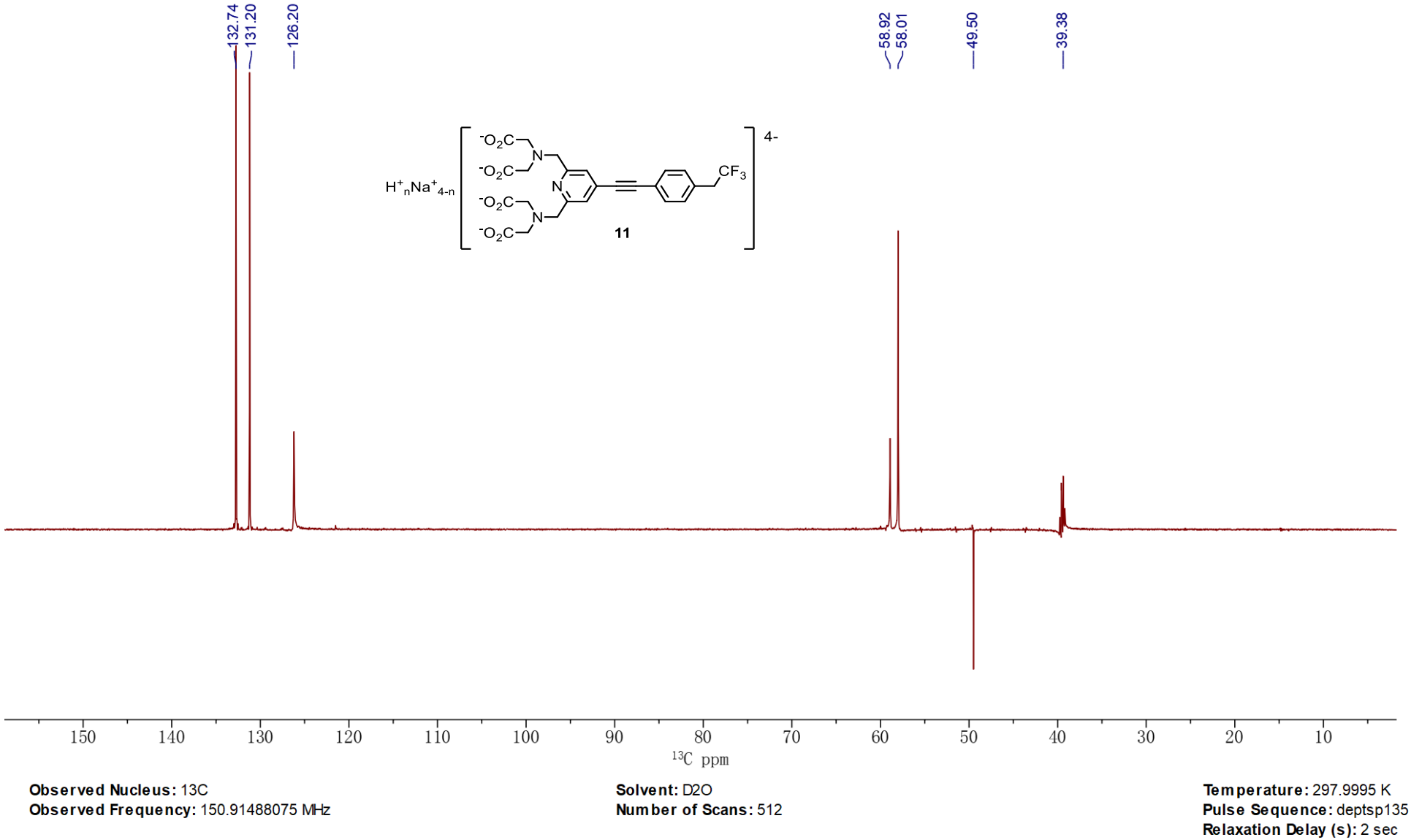


Figure S-II-22. ^{13}C DEPT 135 NMR spectrum (150 MHz, D_2O) of H_nNa_{4-n}[PyMTA-EP-CH_2CF_3] **11**, a drop of MeOH was added for calibration.

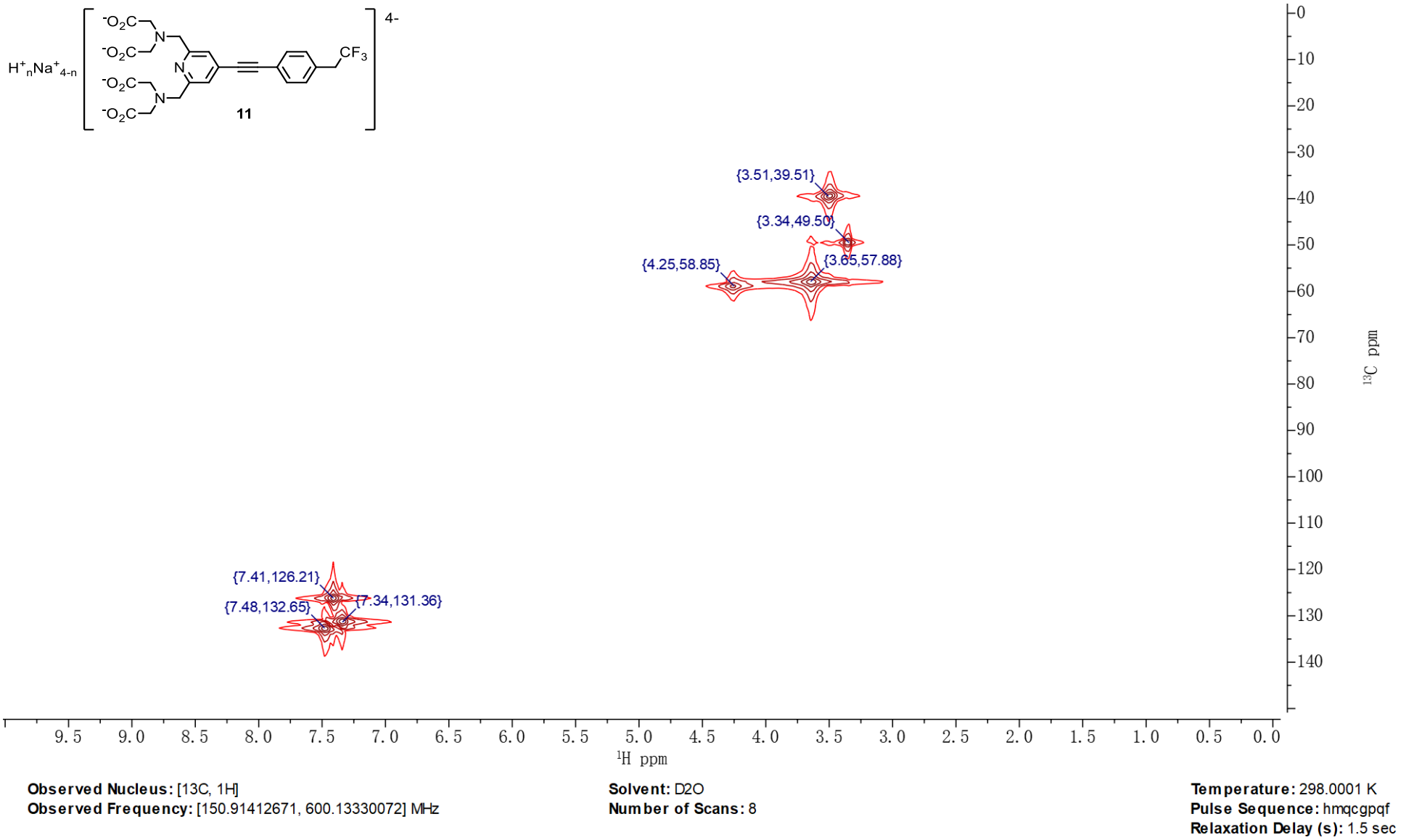


Figure S-II-23. ^{1}H ^{13}C HMQC NMR spectrum (600 MHz, 150 MHz, D_2O) of H_nNa_{4-n}[PyMTA-EP-CH_2CF_3] **11**, a drop of MeOH was added for calibration.

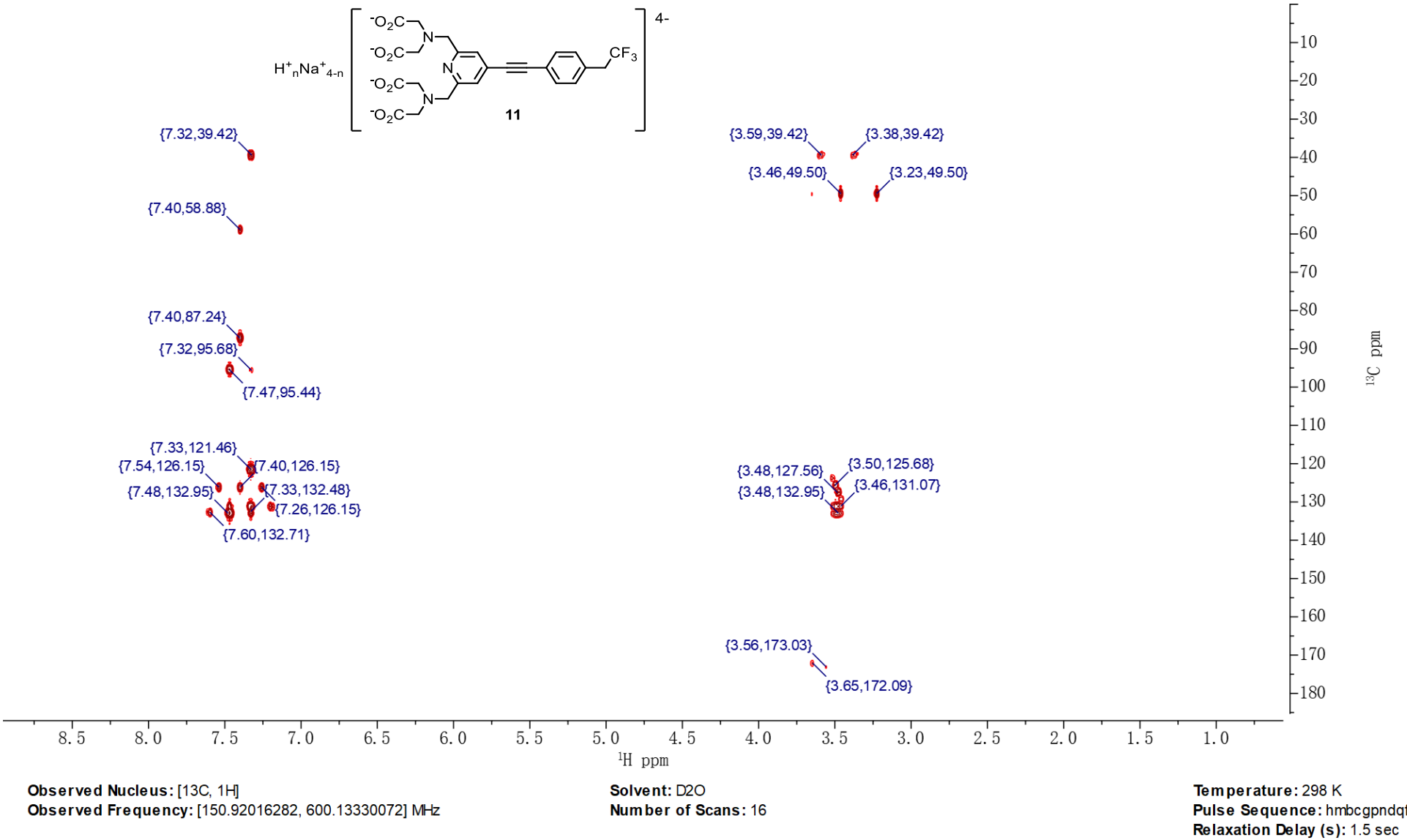


Figure S-II-24. ^{1}H ^{13}C HMBC NMR spectrum (600 MHz, 150 MHz, D_2O) of H_nNa_{4-n}[PyMTA-EP-CH_2CF_3] **11**, a drop of MeOH was added for calibration.

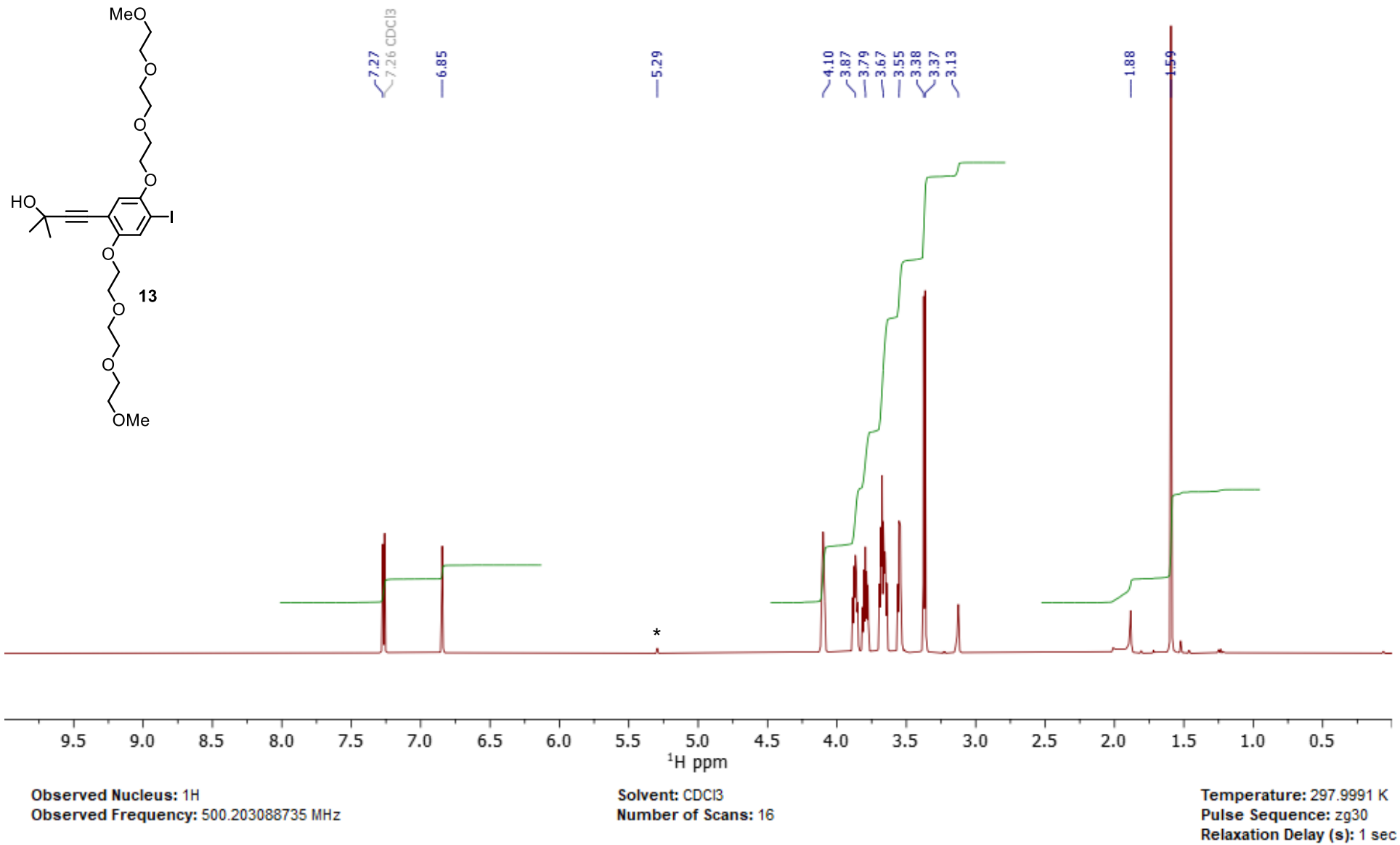


Figure S-II-25. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of iodobenzene **13**. * = CH_2Cl_2.

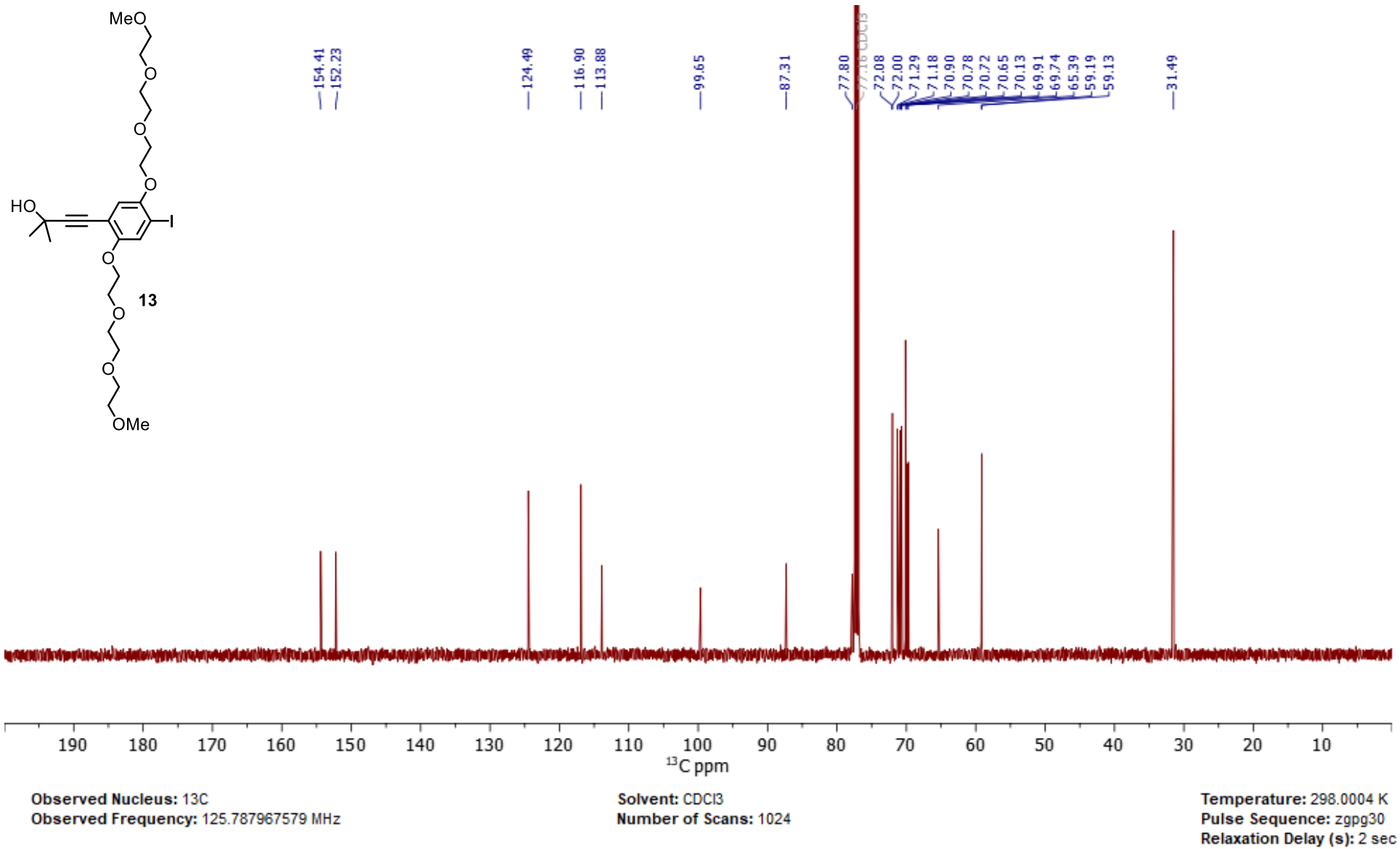


Figure S-II-26. ^{13}C NMR spectrum (126 MHz, $CDCl_3$) of iodobenzene **13**.

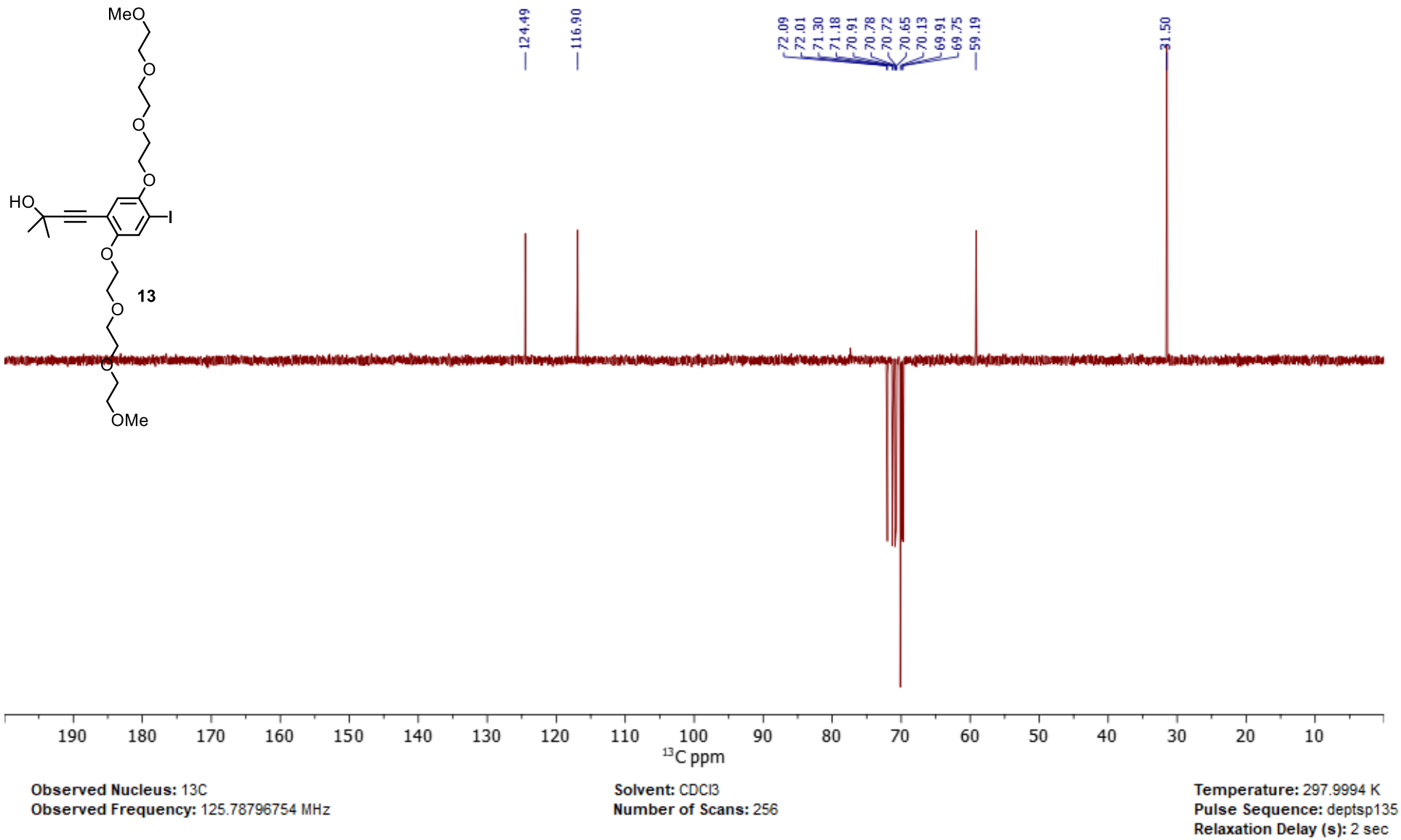


Figure S-II-27. ^{13}C DEPT 135 NMR spectrum (126 MHz, $CDCl_3$) of iodobenzene **13**.

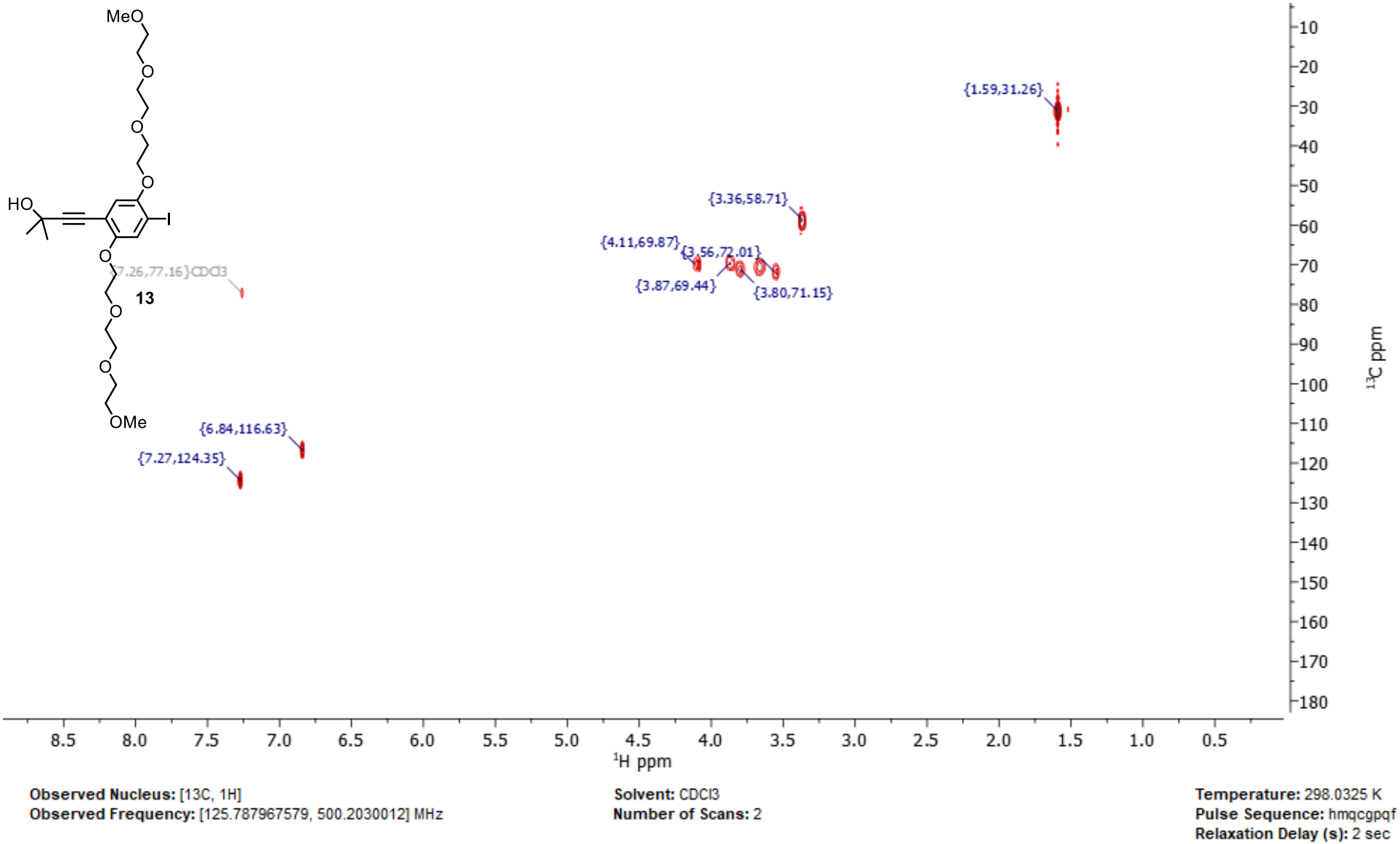


Figure S-II-28. ^{1}H ^{13}C HMQC NMR spectrum (500 MHz, 126 MHz, $CDCl_3$) of iodobenzene **13**.

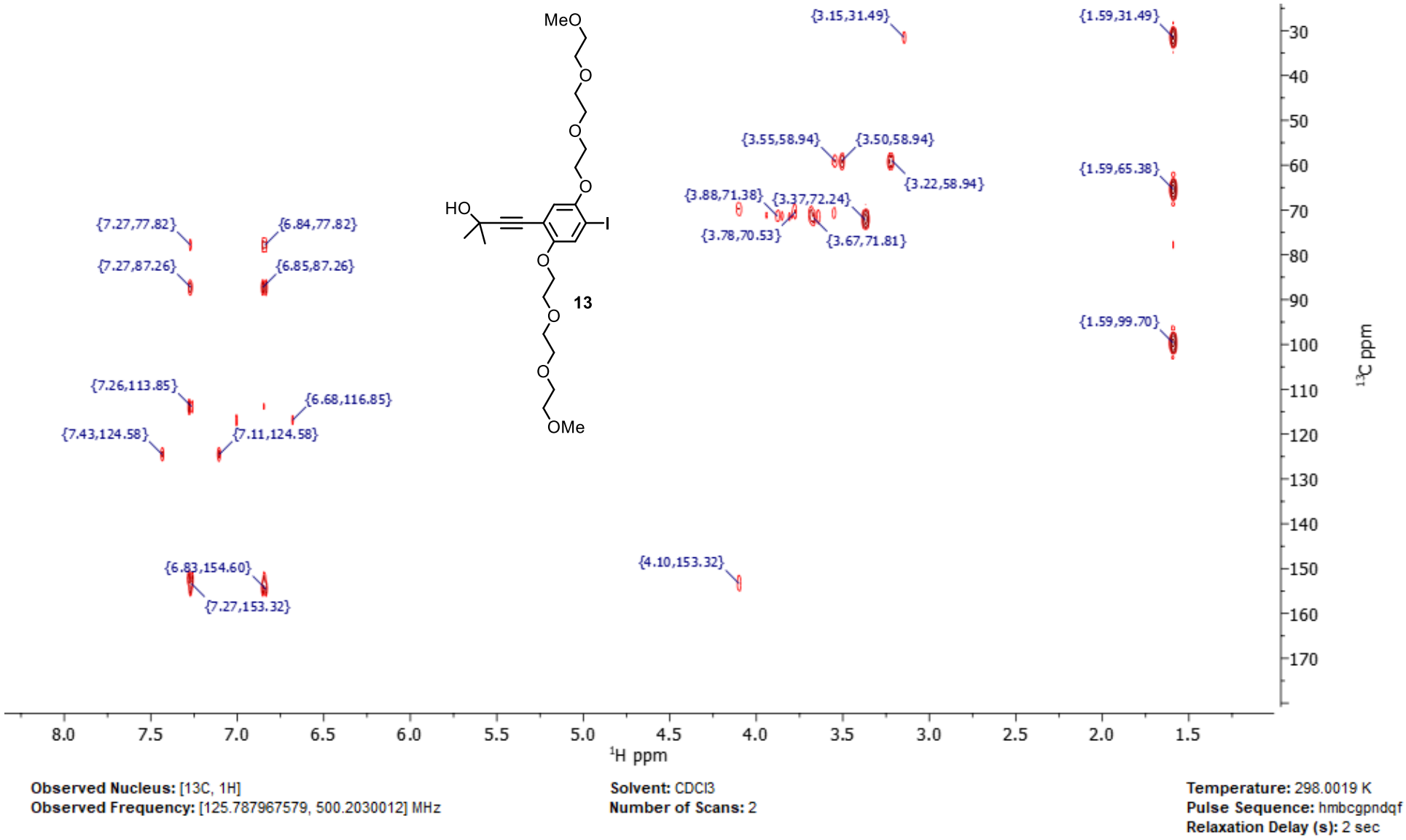


Figure S-II-29. ^{1}H ^{13}C HMBC NMR spectrum (500 MHz, 126 MHz, $CDCl_3$) of iodobenzene **13**.

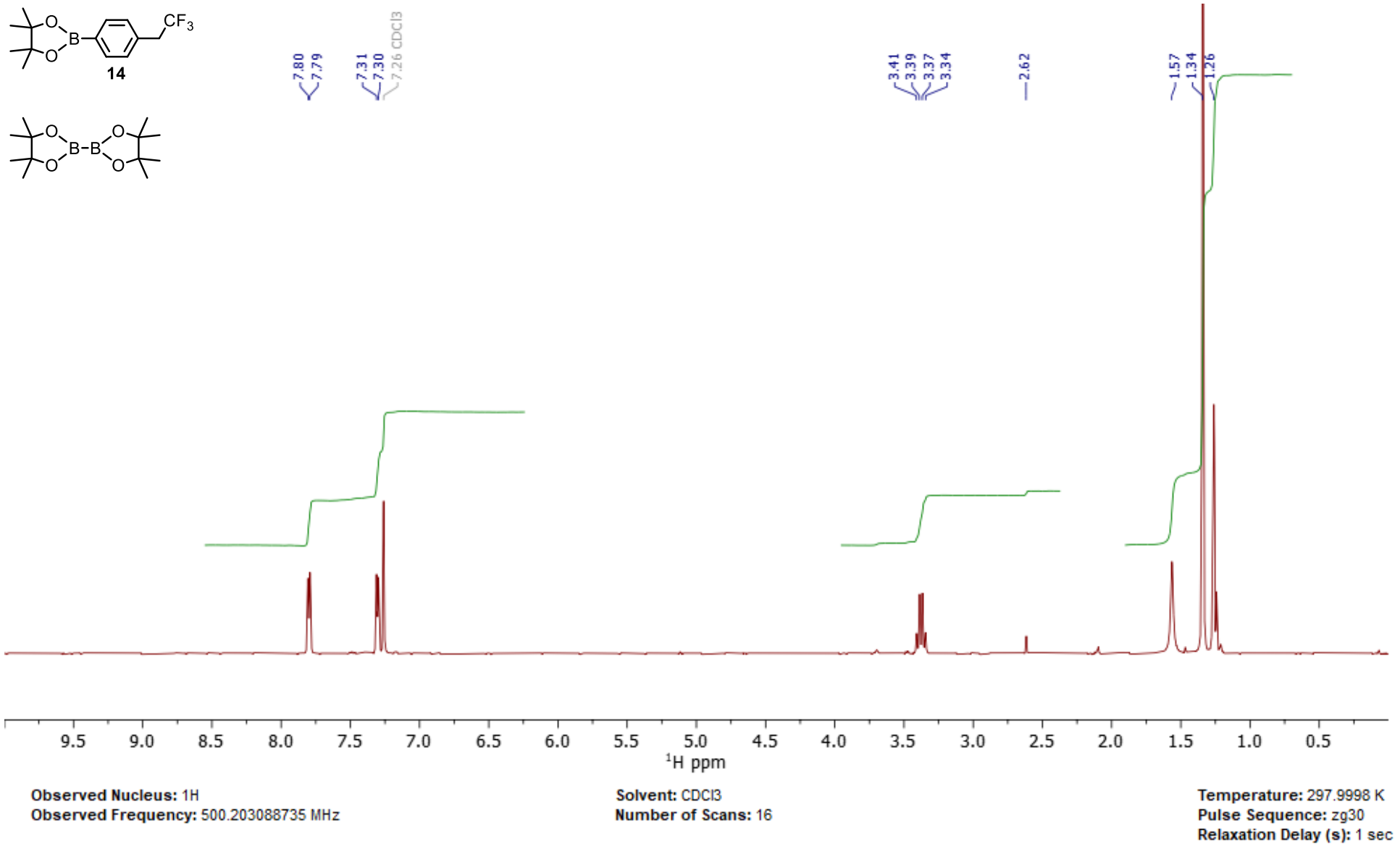


Figure S-II-30. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of the 83:17 mixture of (4-(trifluoroethyl)phenyl)boronic acid pinacol ester **14** and bis(pinacolato)diboron.

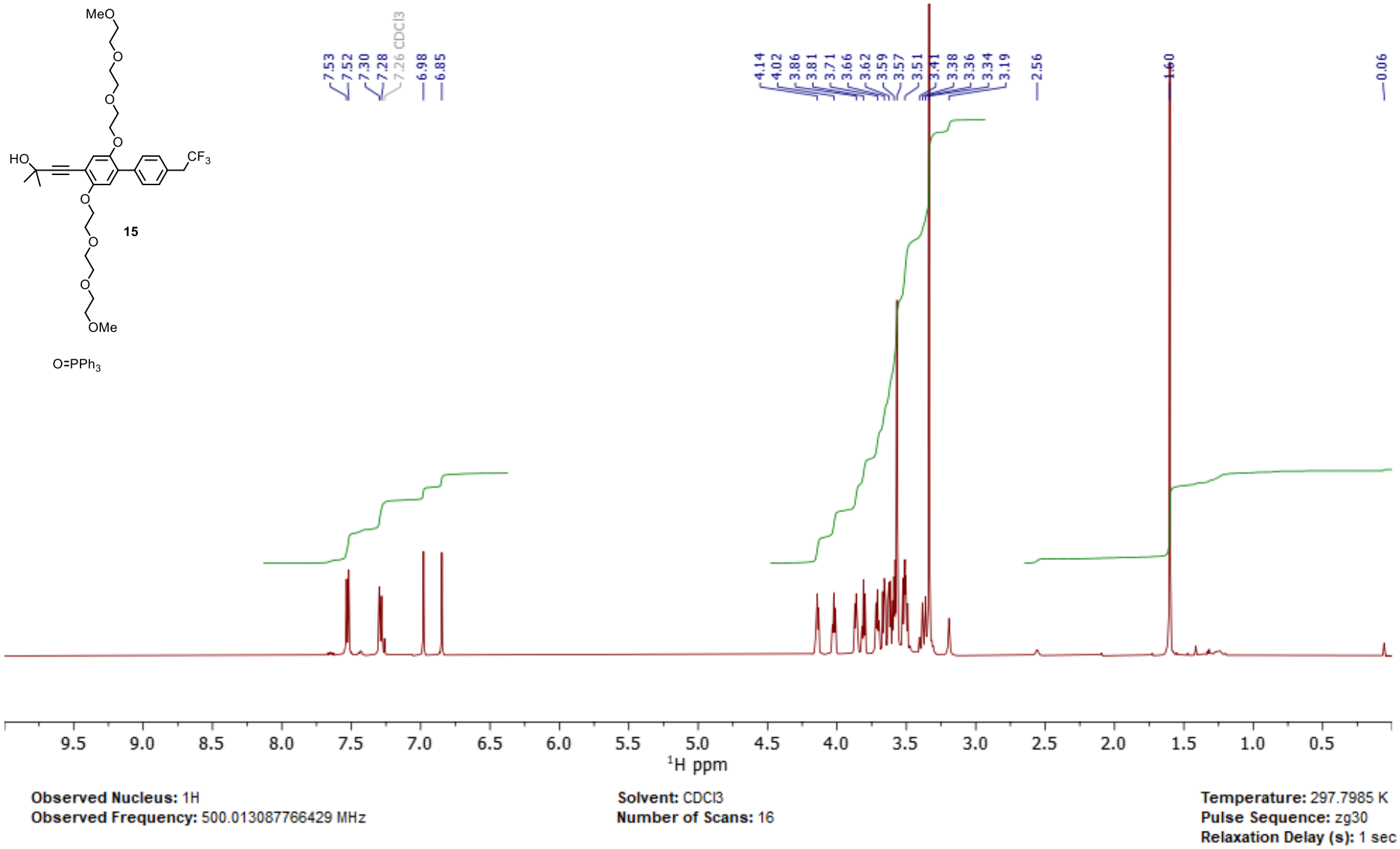


Figure S-II-31. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of the 96:4 mixture of biphenyl **15** and triphenylphosphine oxide.

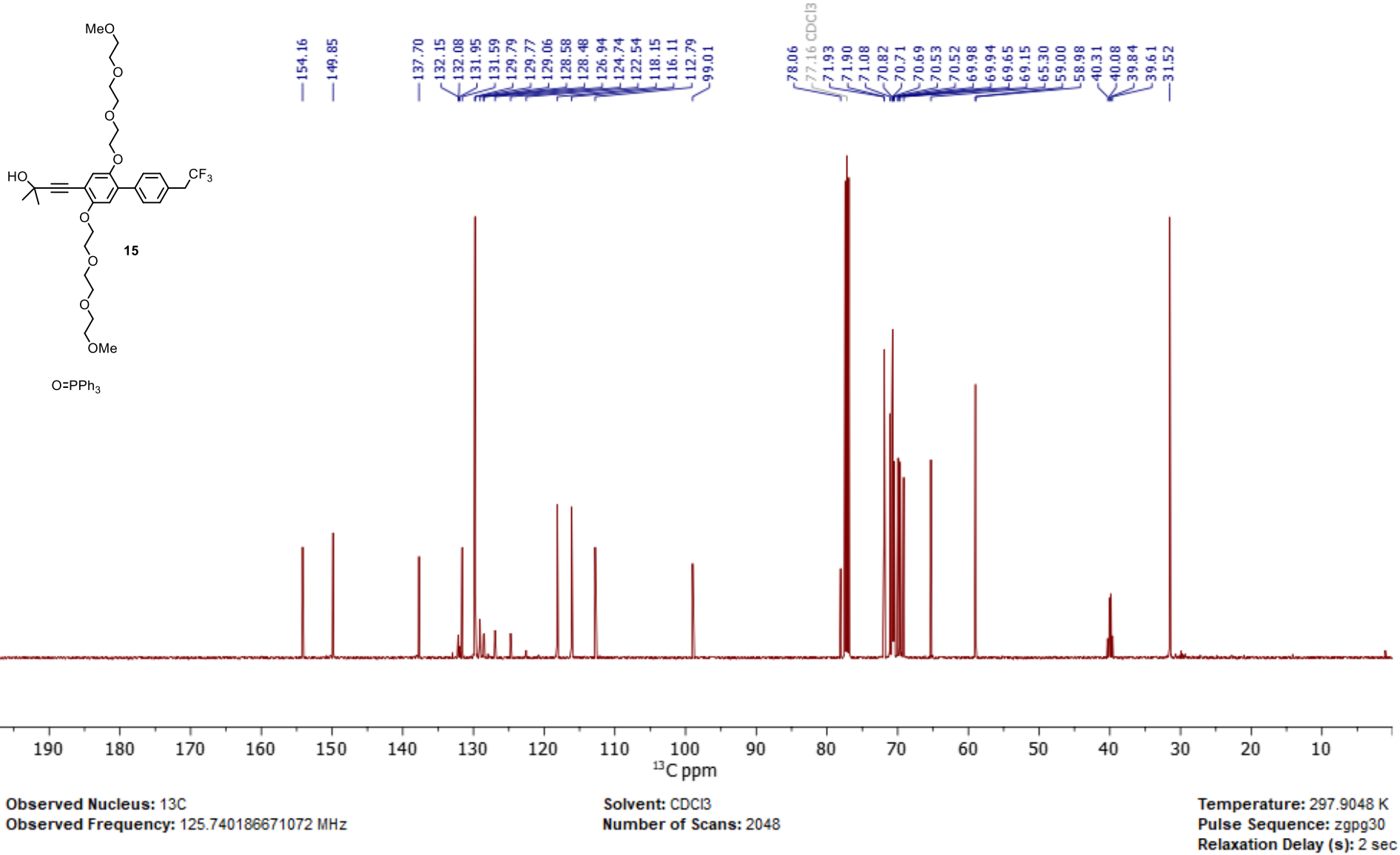


Figure S-II-32. ^{13}C NMR spectrum (126 MHz, $CDCl_3$) of the 96:4 mixture of biphenyl **15** and triphenylphosphine oxide.

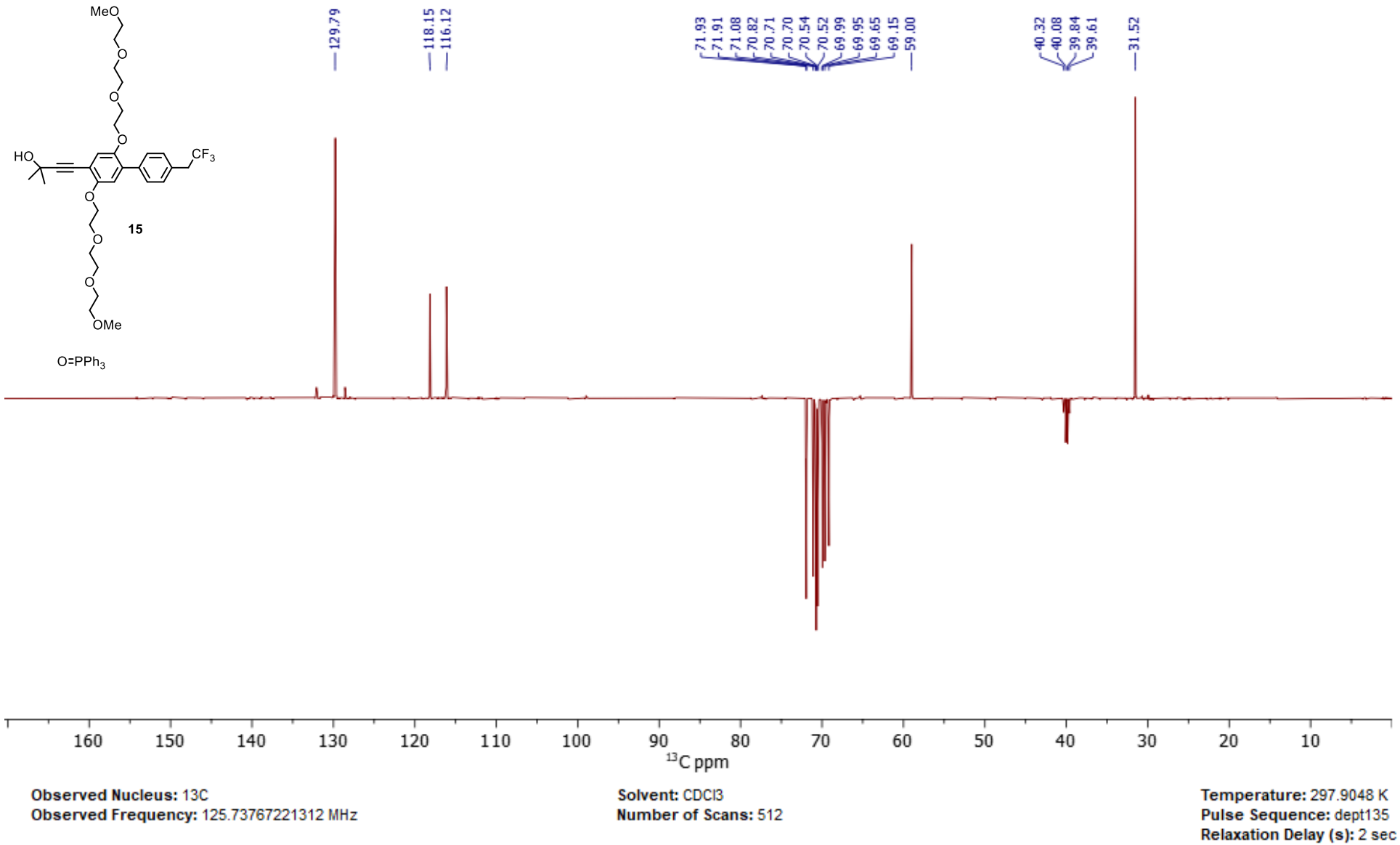


Figure S-II-33. ^{13}C DEPT 135 NMR spectrum (126 MHz, $CDCl_3$) of the 96:4 mixture of biphenyl **15** and triphenylphosphine oxide.

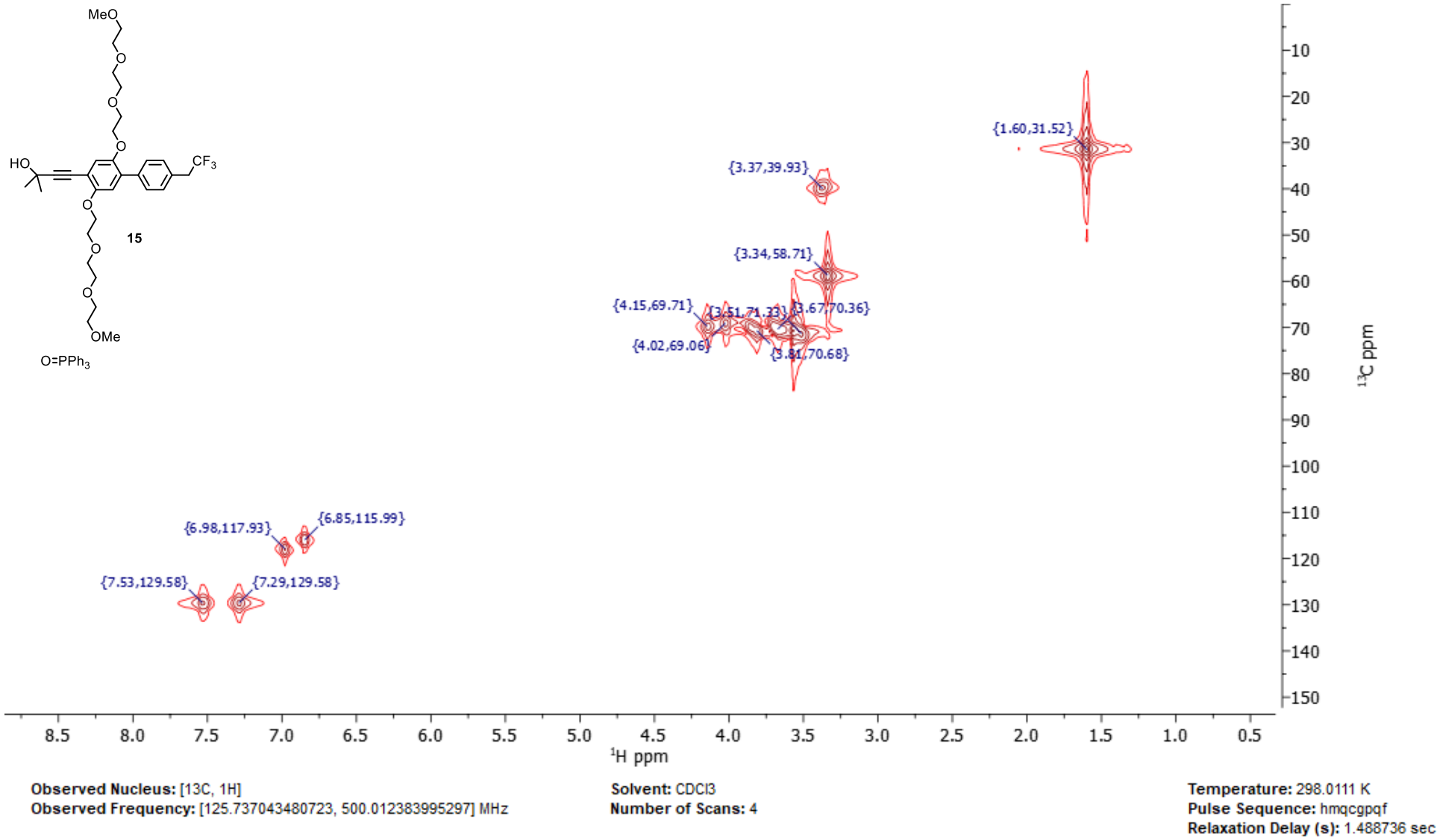


Figure S-II-34. ^{1}H ^{13}C HMQC NMR spectrum (500 MHz, 126 MHz, $CDCl_3$) of the 96:4 mixture of biphenyl **15** and triphenylphosphine oxide.

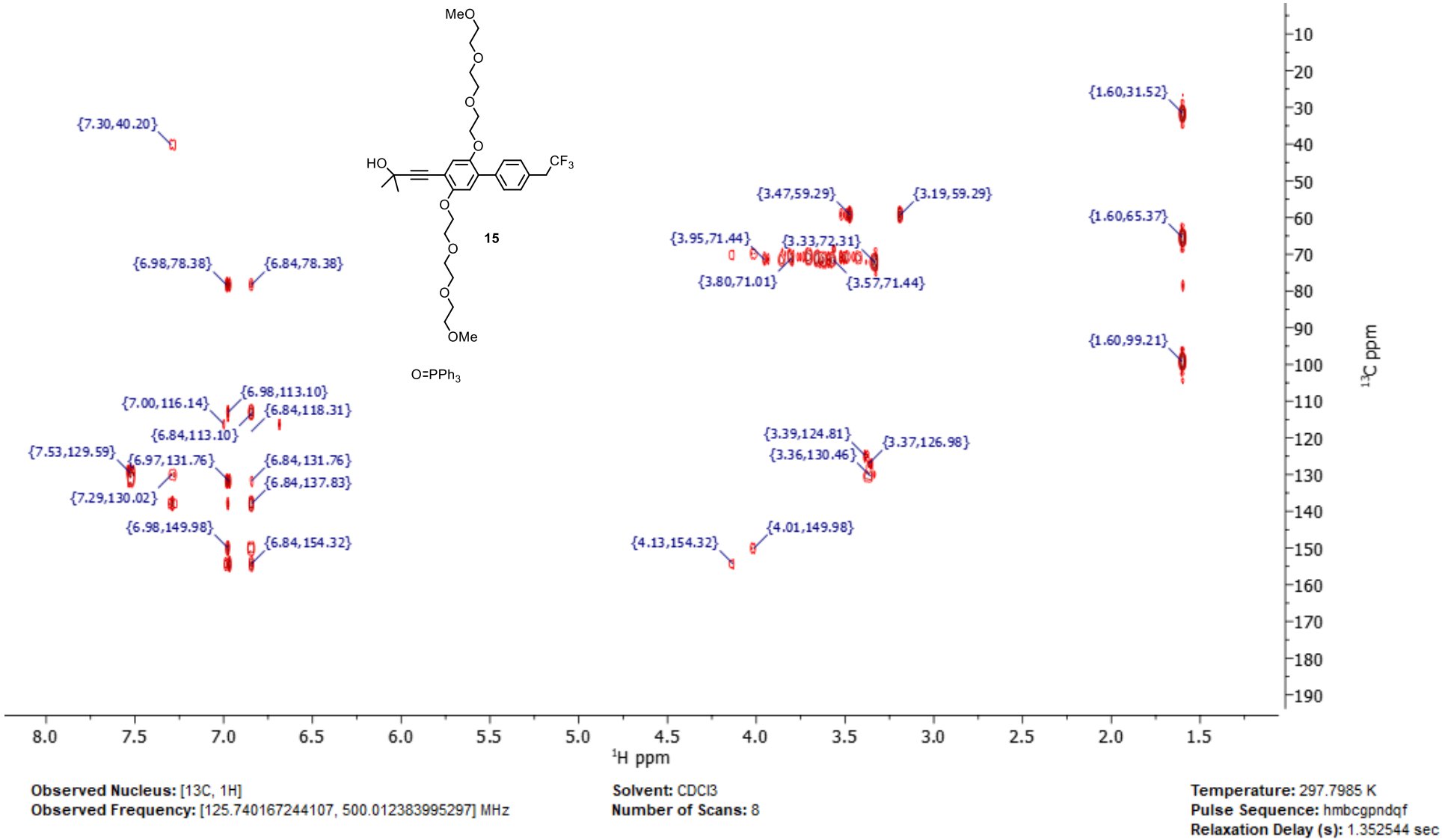


Figure S-II-35. ^{1}H ^{13}C HMBC NMR spectrum (500 MHz, 126 MHz, $CDCl_3$) of the 96:4 mixture of biphenyl **15** and triphenylphosphine oxide.

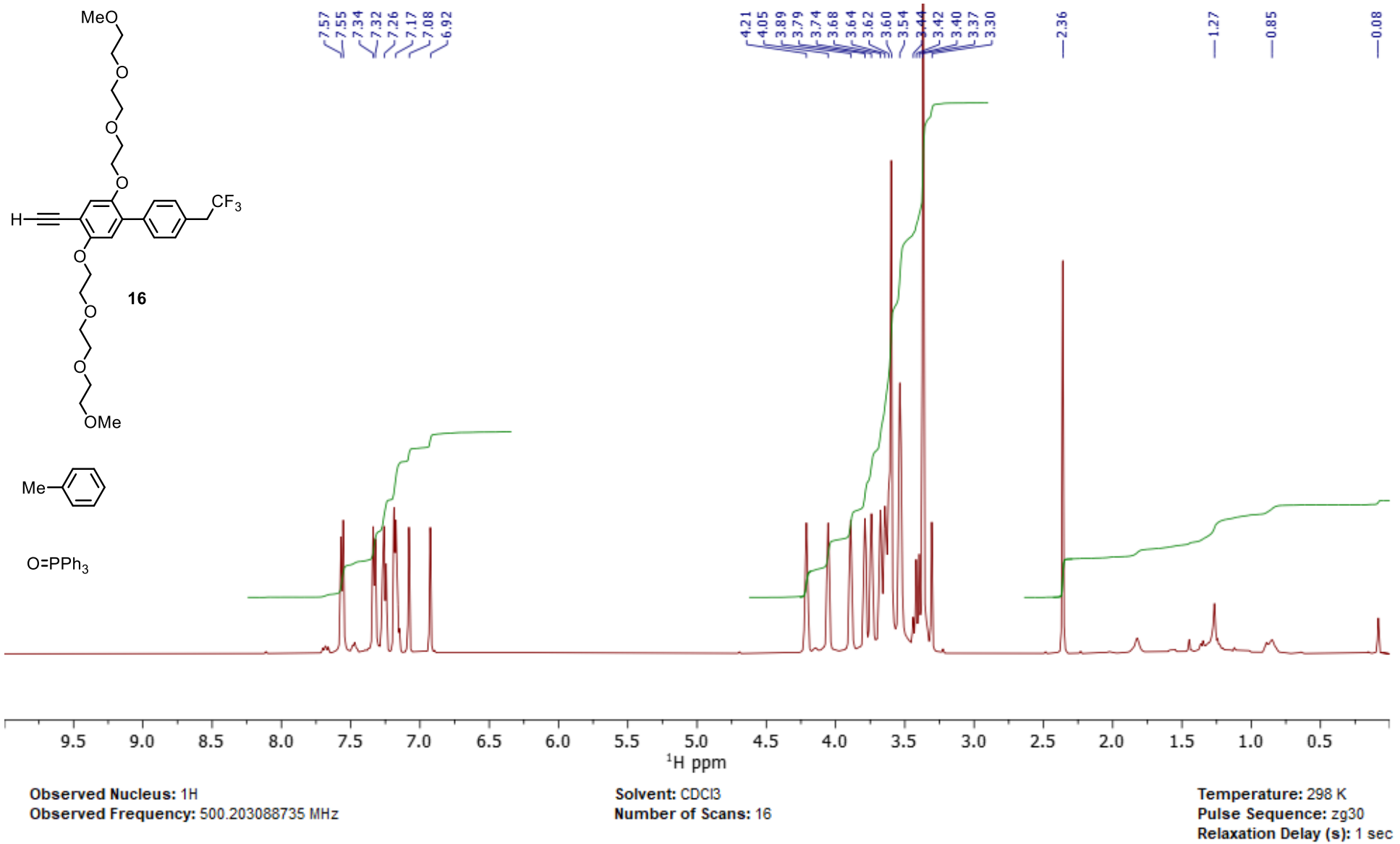


Figure S-II-36. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of the 49:49:2 mixture of alkyne **16**, toluene, and triphenylphosphine oxide.

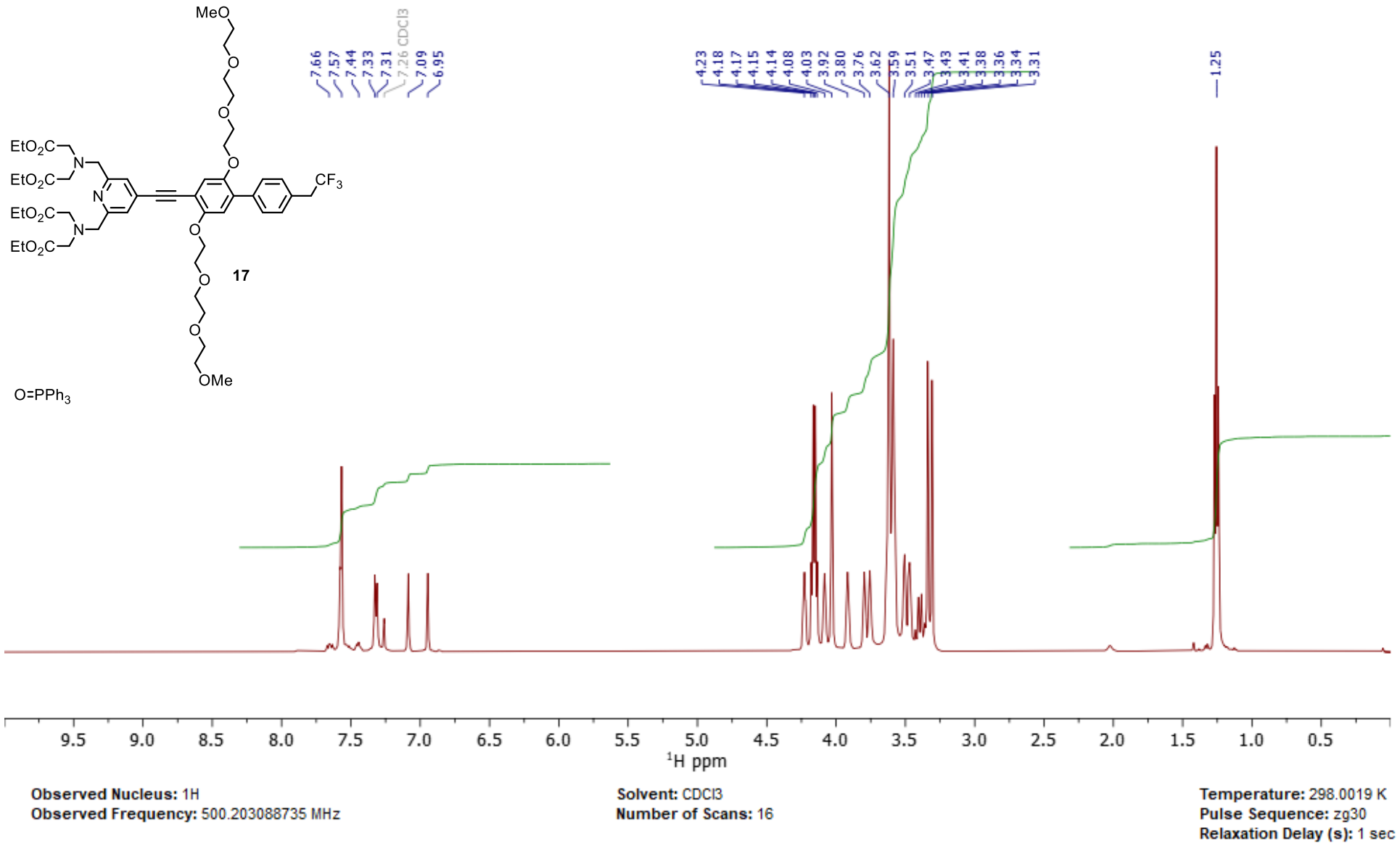


Figure S-II-37. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of the 94:6 mixture of PyMTA ester **17** and triphenylphosphine oxide.

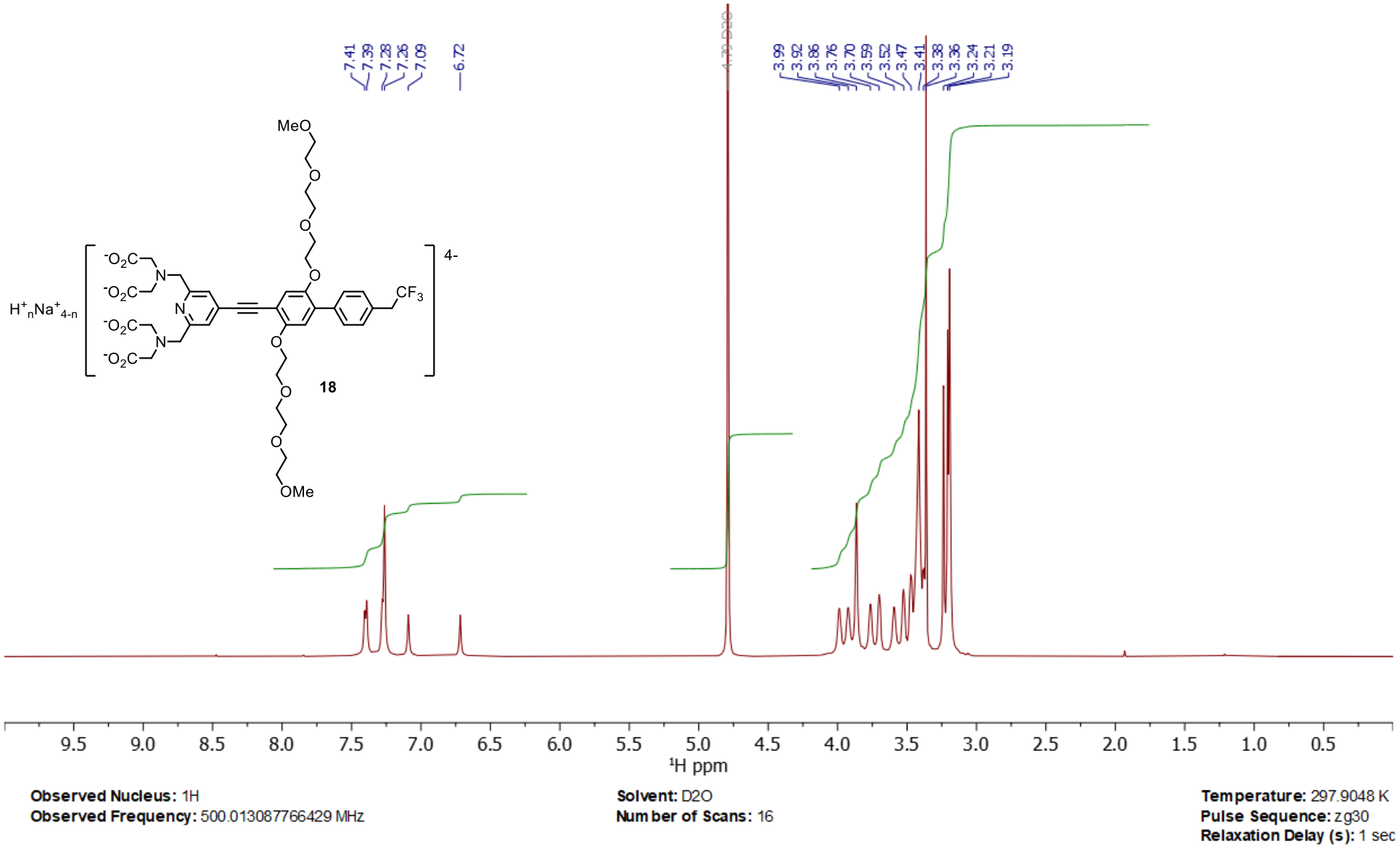


Figure S-II-38. ^{1}H NMR spectrum (500 MHz, D_2O) of H_nNa_{4-n}[PyMTA-EPP-CH_2CF_3] **18**, a drop of MeOH was added for calibration of the ^{13}C NMR spectrum.

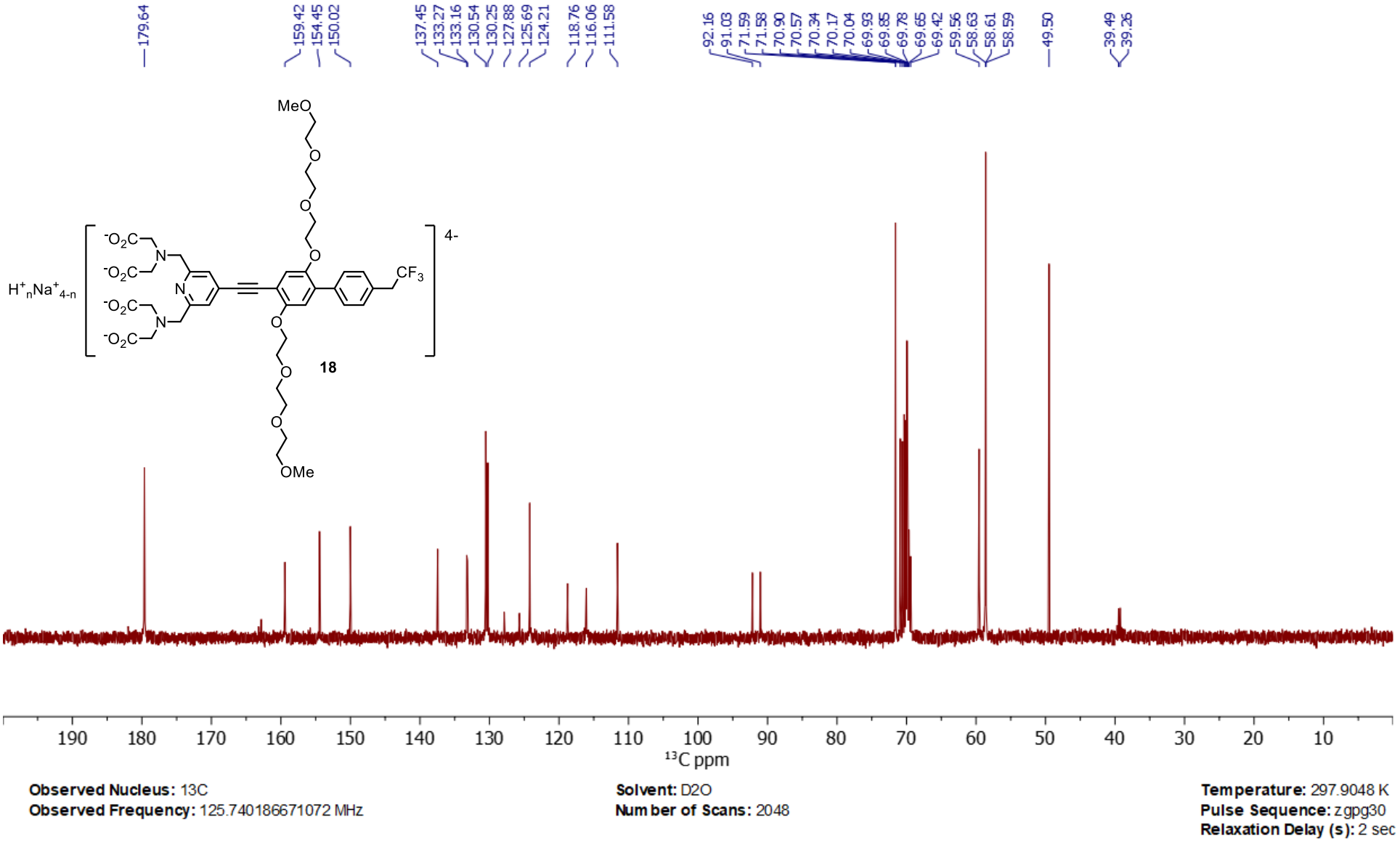


Figure S-II-39. ^{13}C NMR spectrum (126 MHz, D_2O) of H_nNa_{4-n}[PyMTA-EPP-CH_2CF_3] **18**, a drop of MeOH was added for calibration.

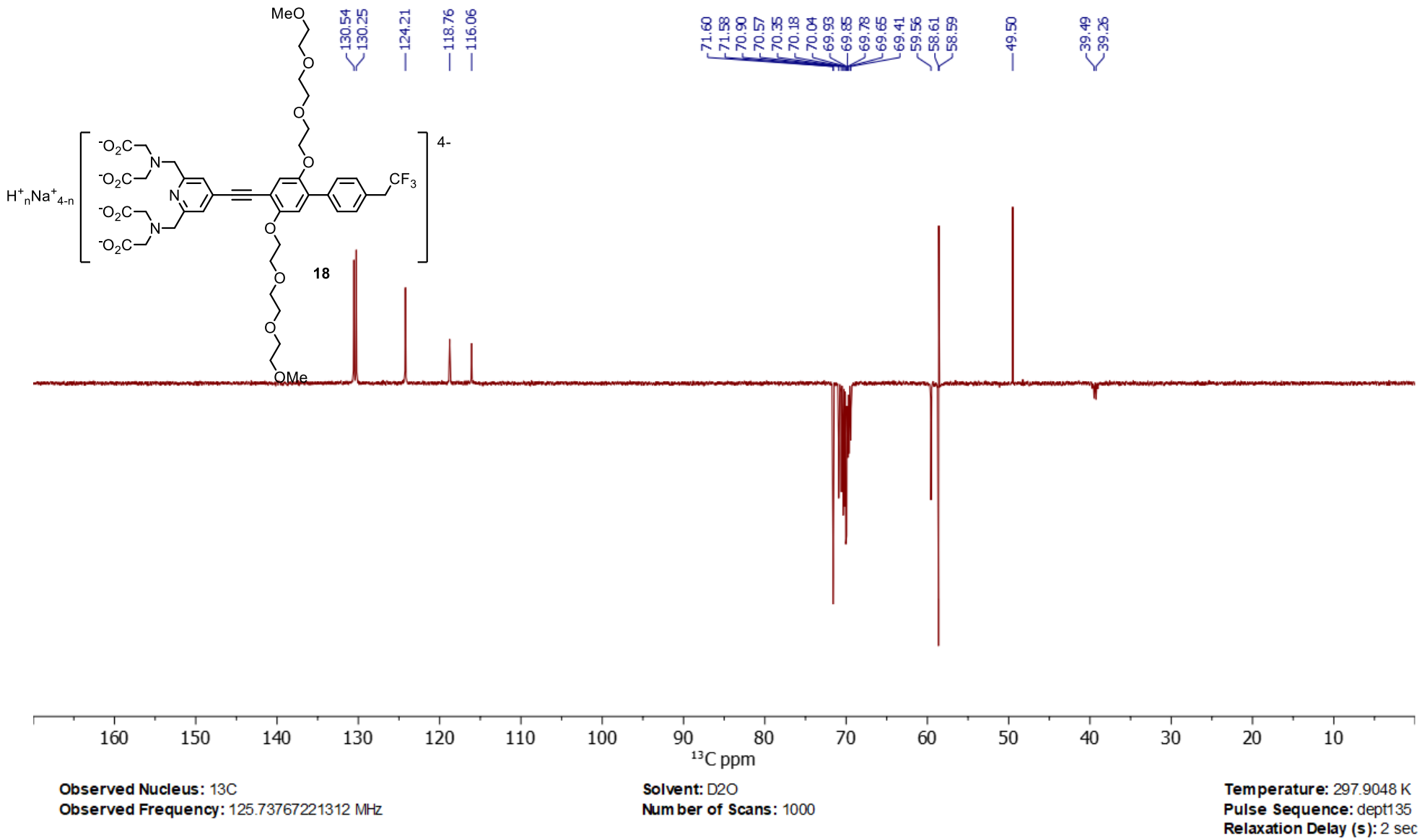


Figure S-II-40. ^{13}C DEPT 135 NMR spectrum (126 MHz, D_2O) of H_nNa_{4-n}[PyMTA-EPP-CH_2CF_3] **18**, a drop of MeOH was added for calibration.

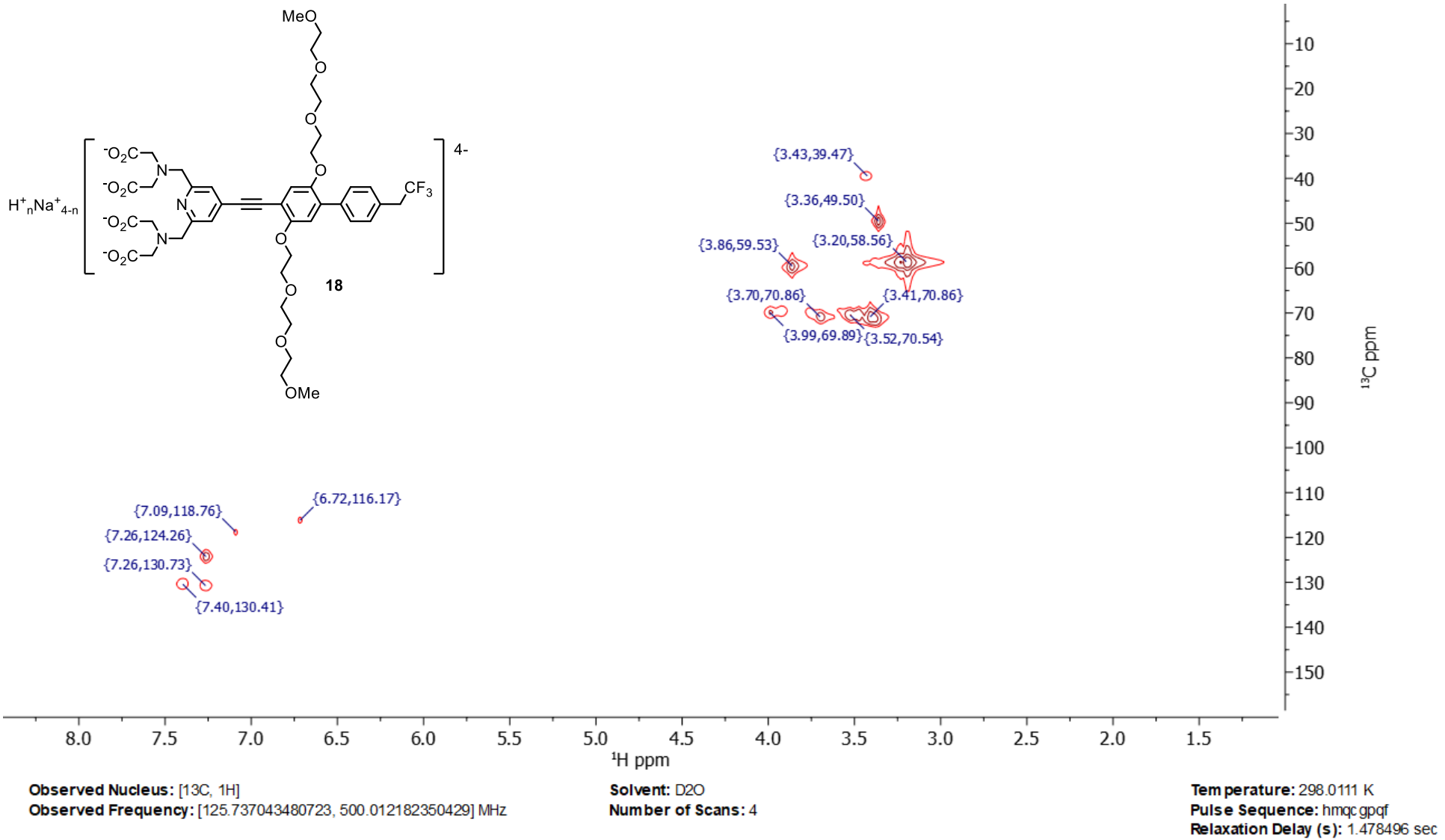


Figure S-II-41. ^{1}H ^{13}C HMQC NMR spectrum (500 MHz, 126 MHz, D_2O) of H_nNa_{4-n}[PyMTA-EPP-CH_2CF_3] **18**, a drop of MeOH was added for calibration.

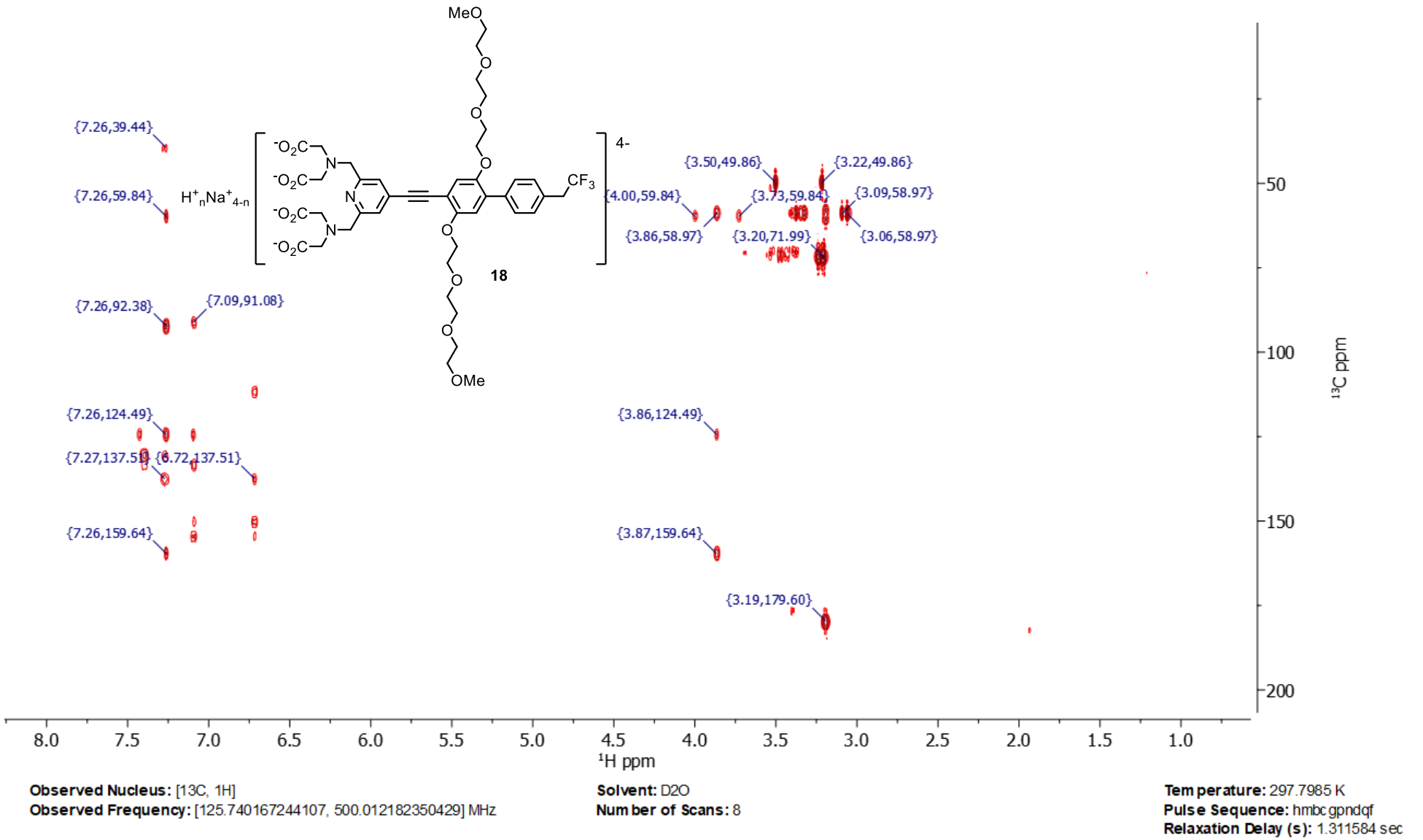


Figure S-II-42. ^{1}H ^{13}C HMBC NMR spectrum (500 MHz, 126 MHz, D_2O) of H_nNa_{4-n}[PyMTA-EPP-CH_2CF_3] **18**, a drop of MeOH was added for calibration.

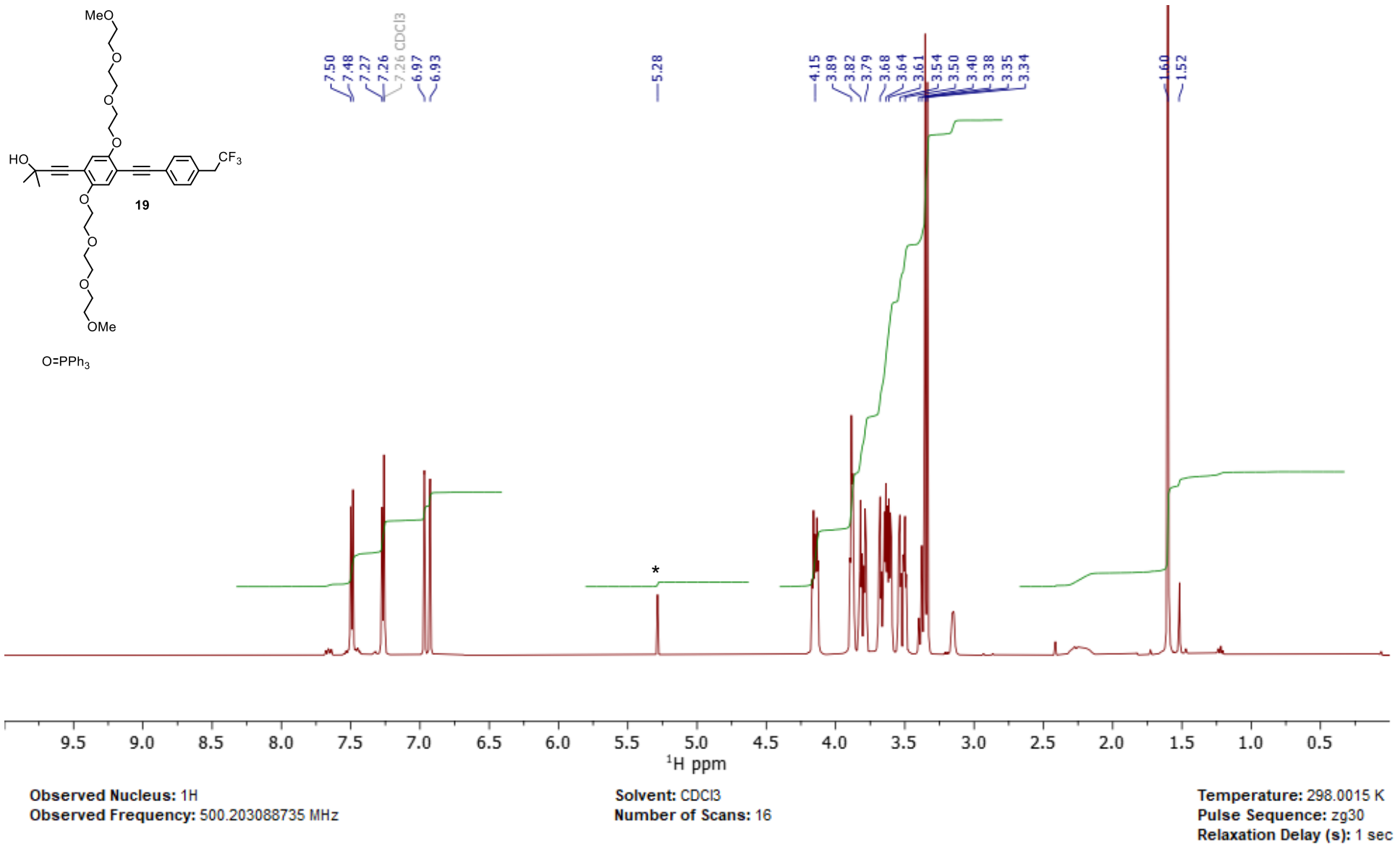


Figure S-II-43. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of the mixture consisting substantially of alkyne **19** and triphenylphosphine oxide in a ratio of 97:3. * = CH_2Cl_2.

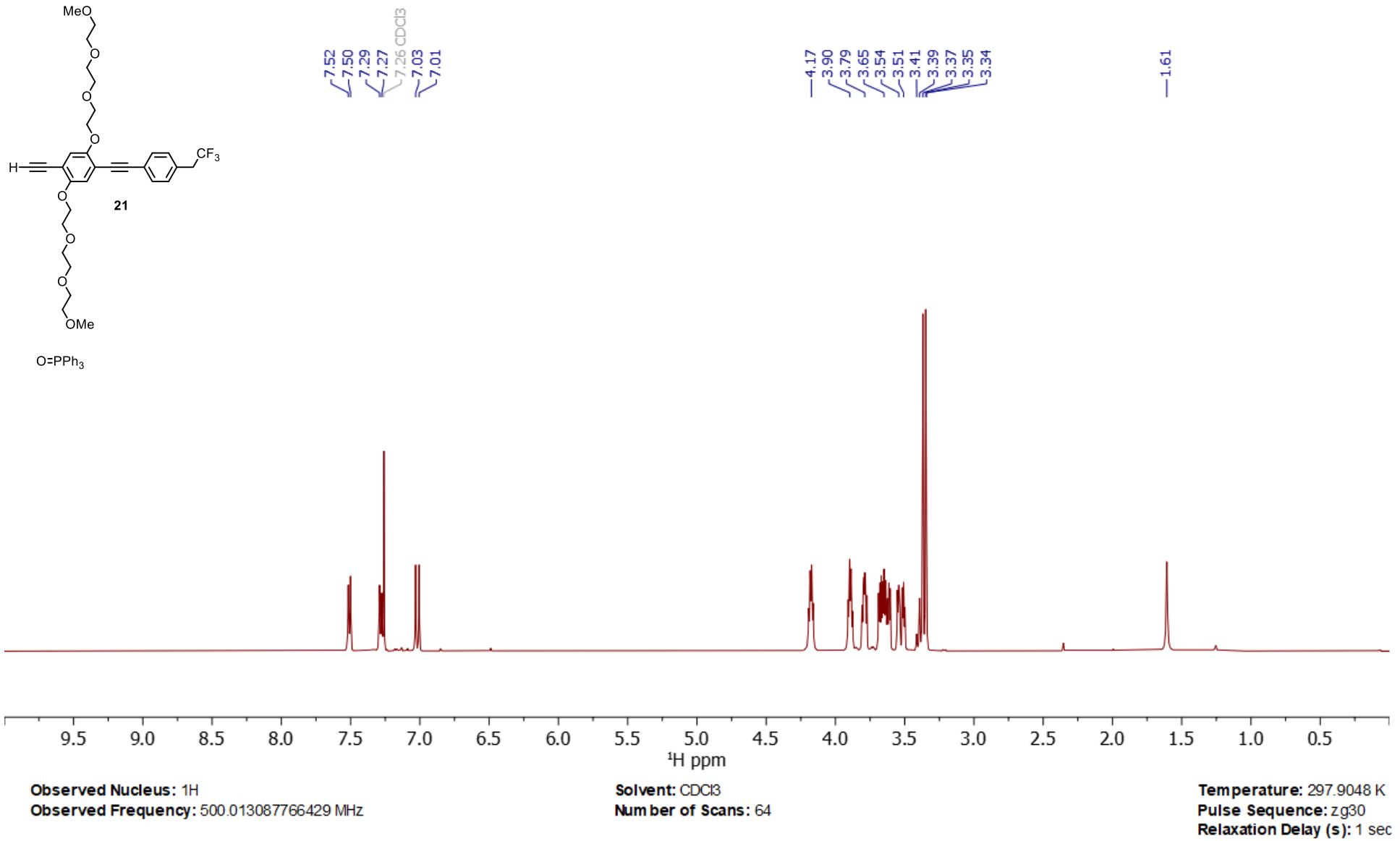


Figure S-II-44. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of alkyne **21** with a very small amount of triphenylphosphine oxide.

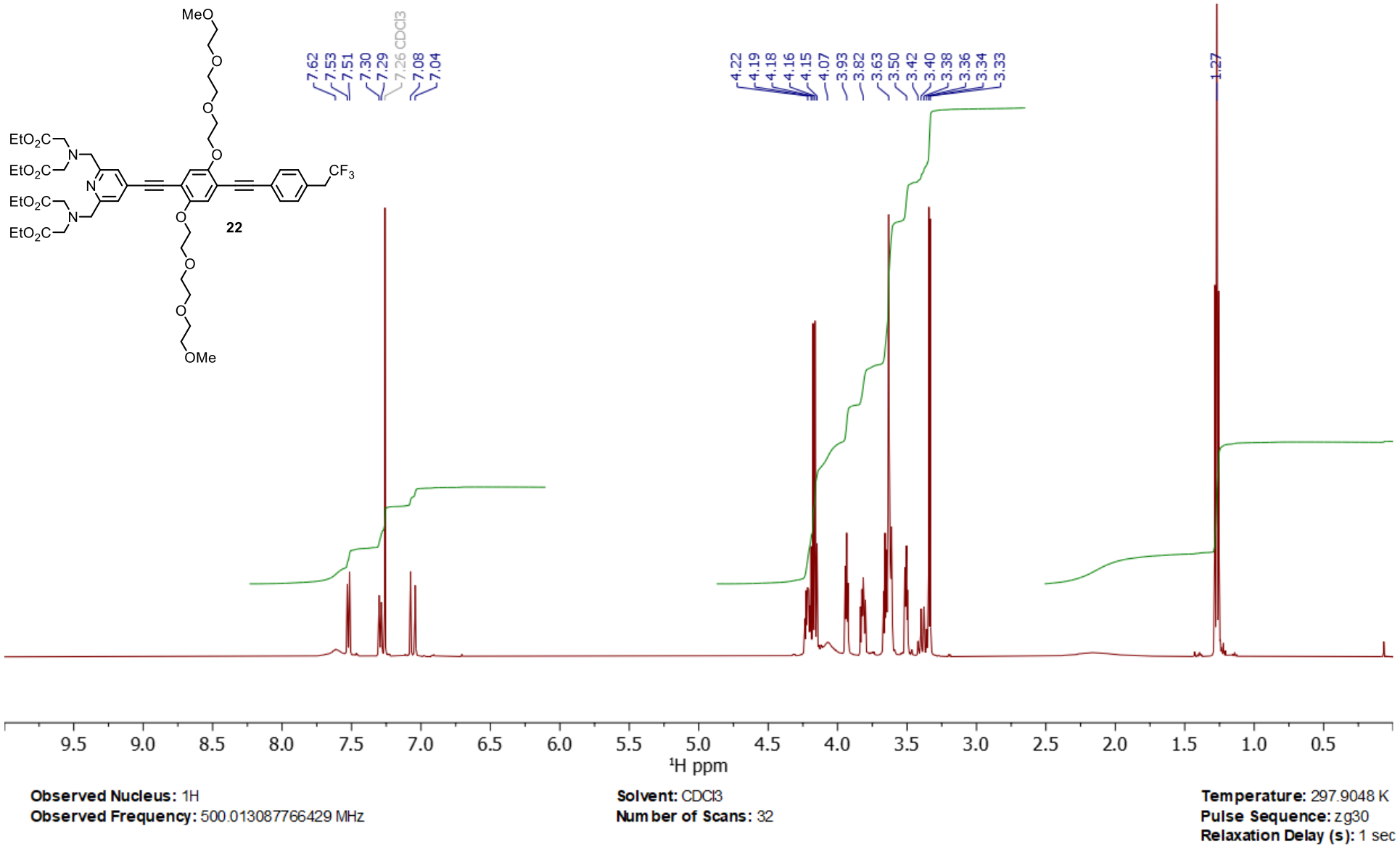


Figure S-II-45. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of PyMTA ester **22**.

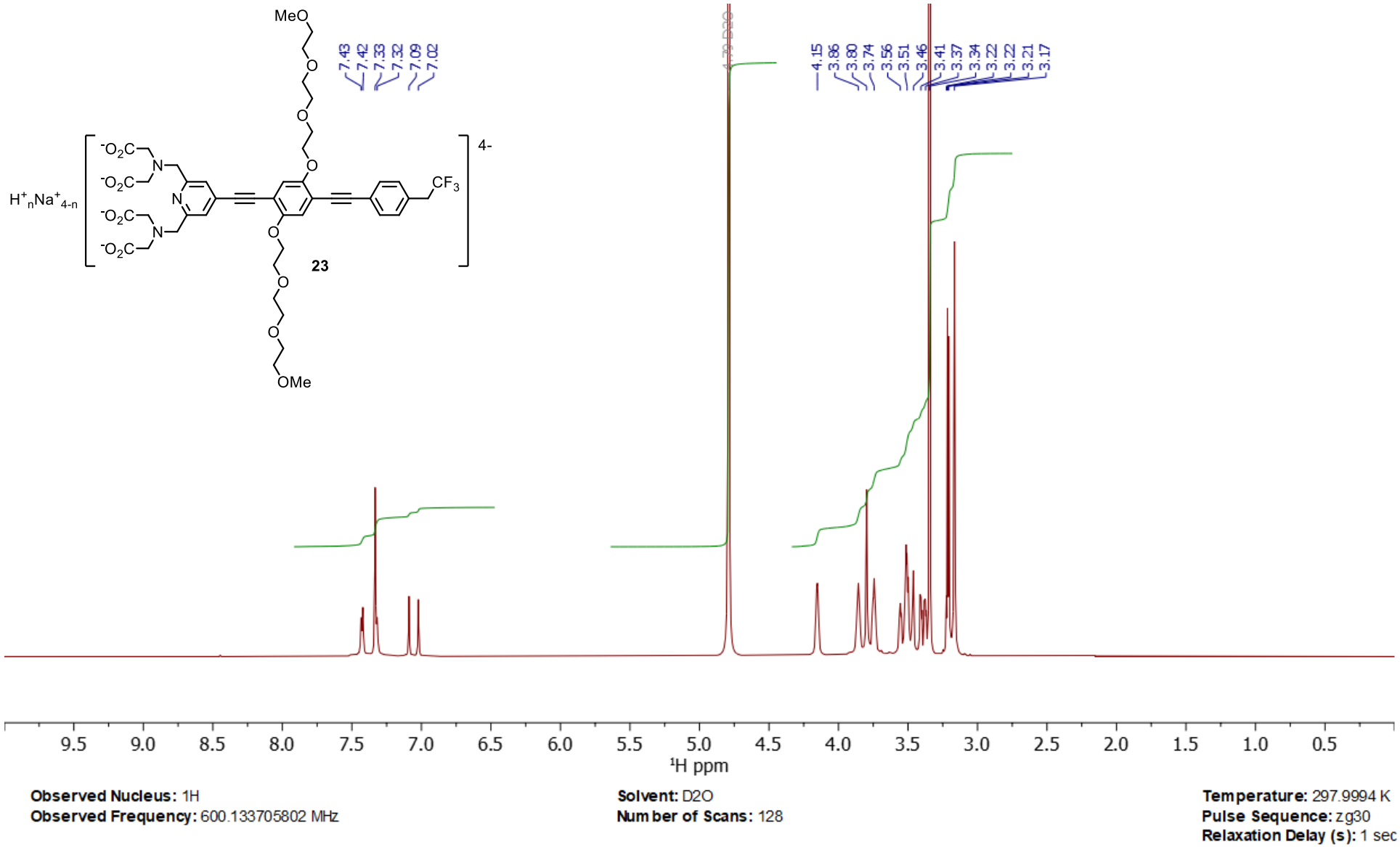


Figure S-II-46. ^{1}H NMR spectrum (600 MHz, D_2O) of H_nNa_{4-n}[PyMTA-EPEP-CH_2CF_3] **23**, a drop of MeOH was added for the calibration of the ^{13}C NMR spectrum.

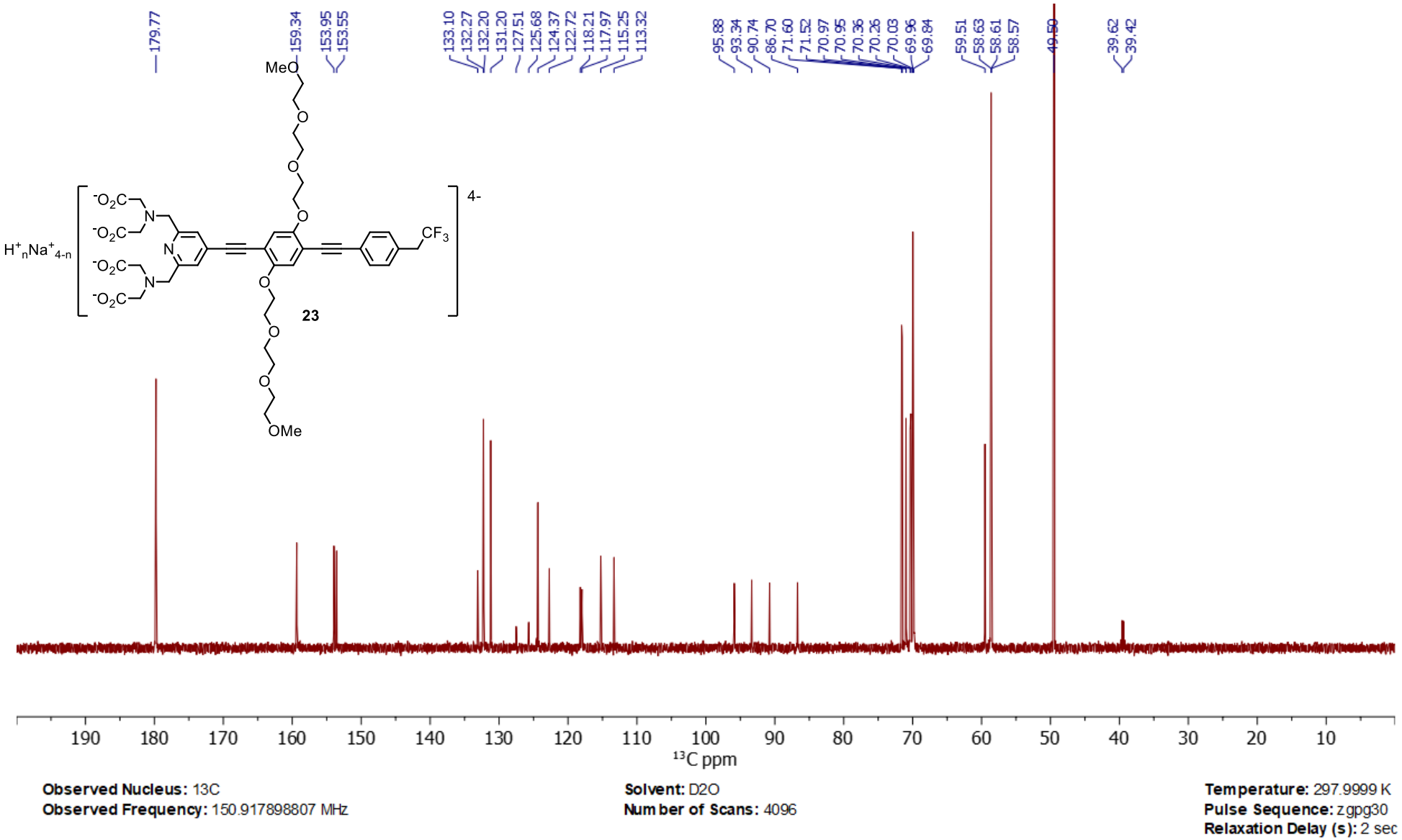


Figure S-II-47. ^{13}C NMR spectrum (150 MHz, D_2O) of H_nNa_{4-n}[PyMTA-EPEP-CH_2CF_3] **23**, a drop of MeOH was added for calibration.

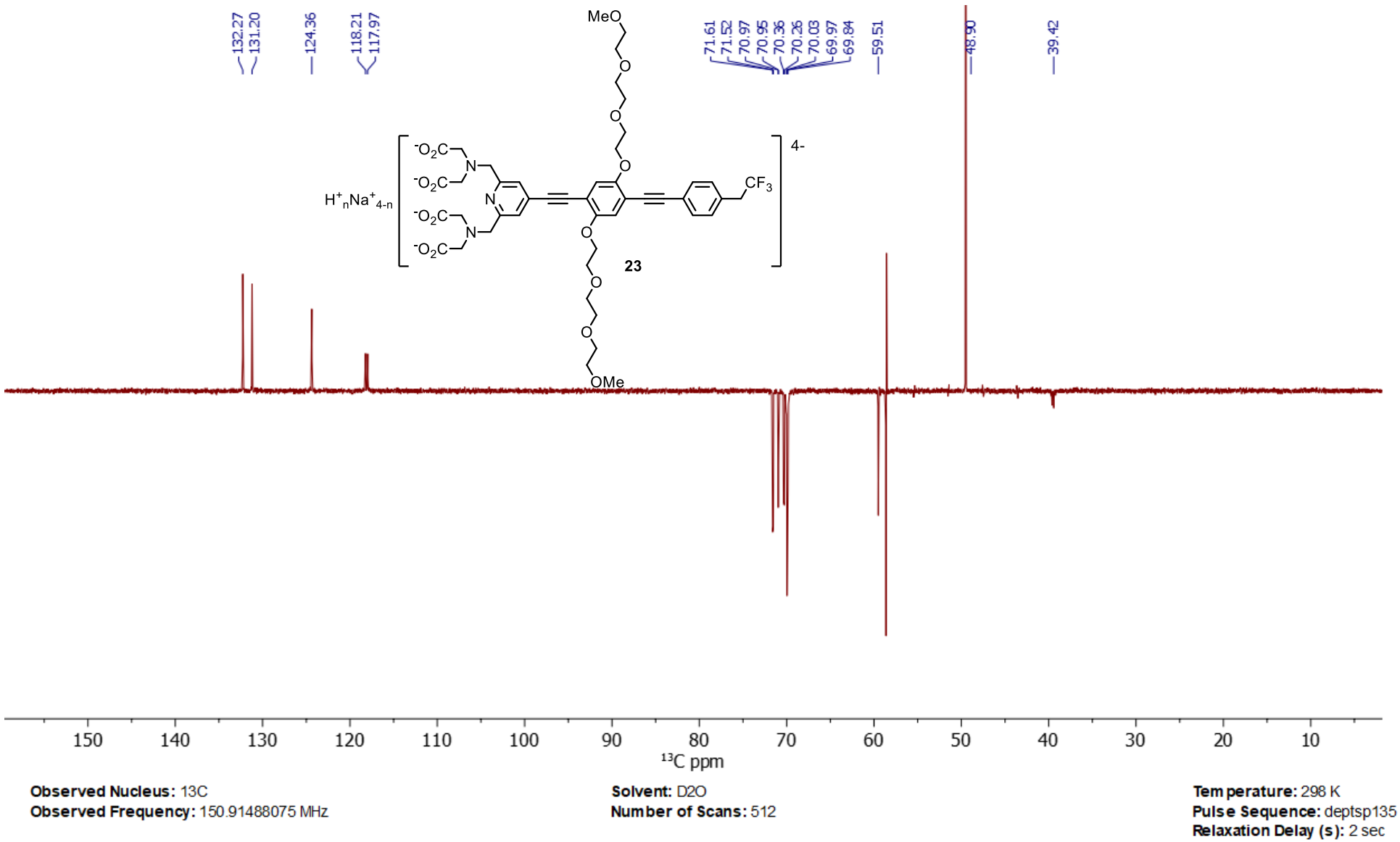


Figure S-II-48. ^{13}C DEPT 135 NMR spectrum (150 MHz, D_2O) of H_nNa_{4-n}[PyMTA-EPEP-CH_2CF_3] **23**, a drop of MeOH was added for calibration.

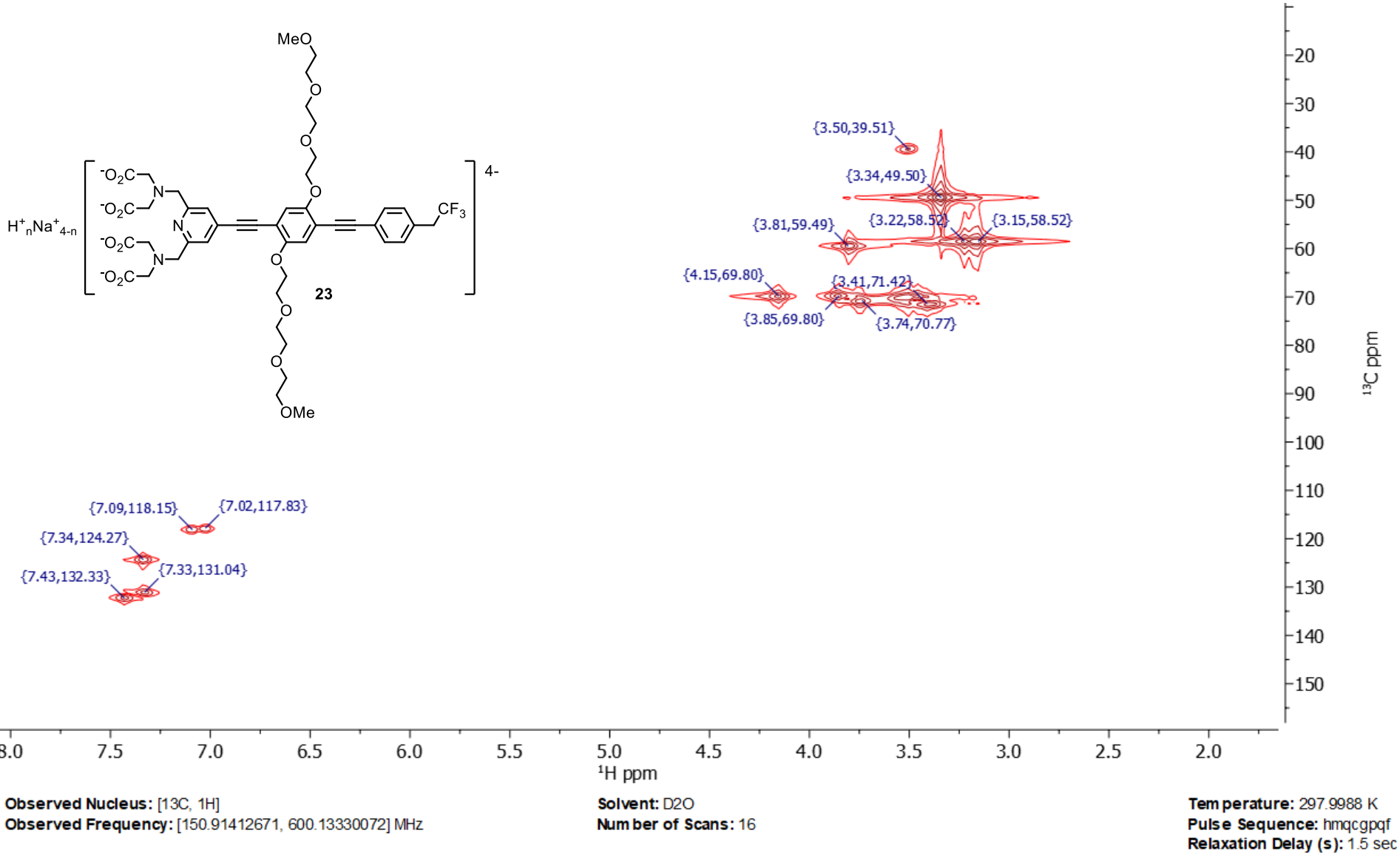


Figure S-II-49. ^{1}H ^{13}C HMQC NMR spectrum (600 MHz, 150 MHz, D_2O) of H_nNa_{4-n}[PyMTA-EPEP-CH_2CF_3] **23**, a drop of MeOH was added for calibration.

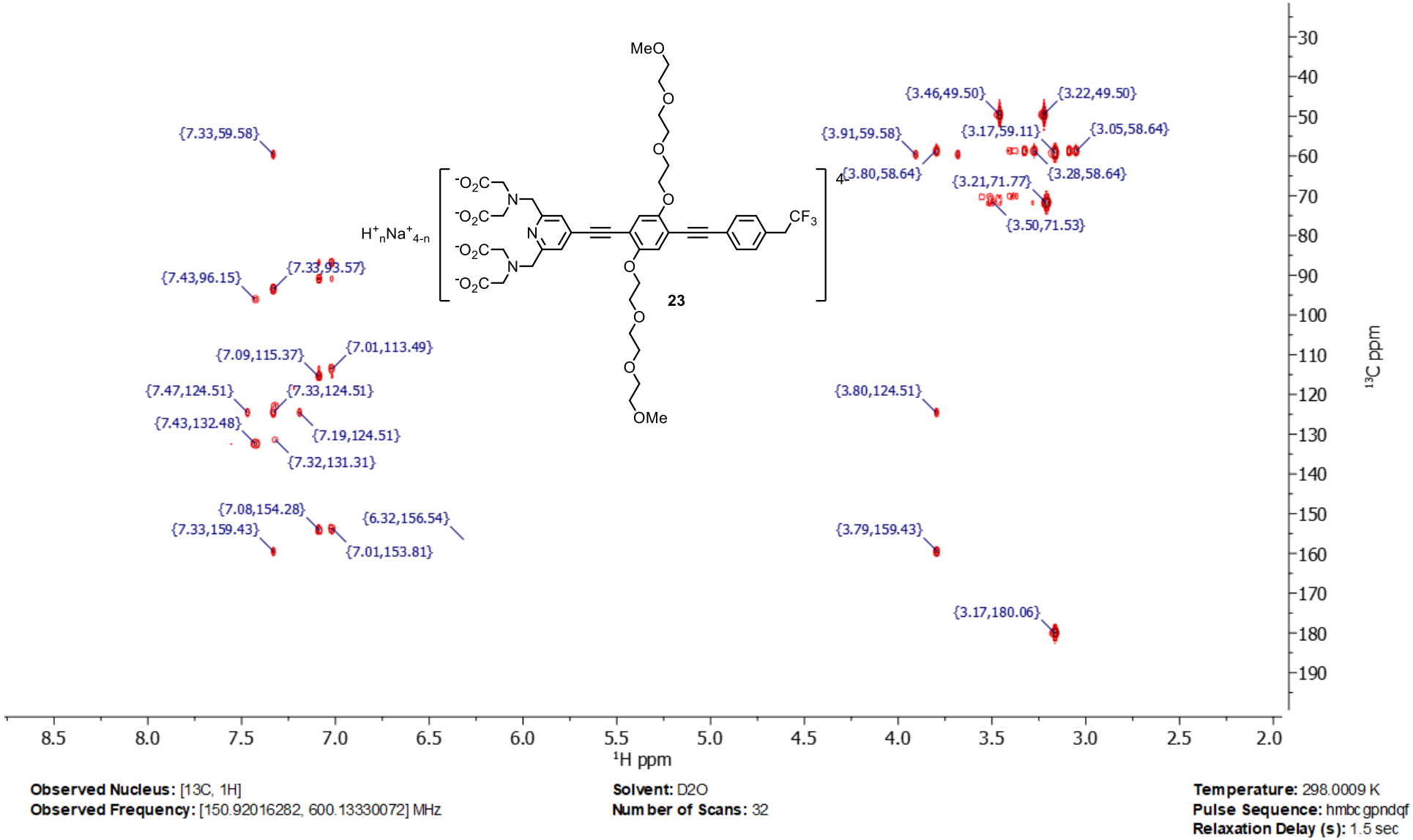
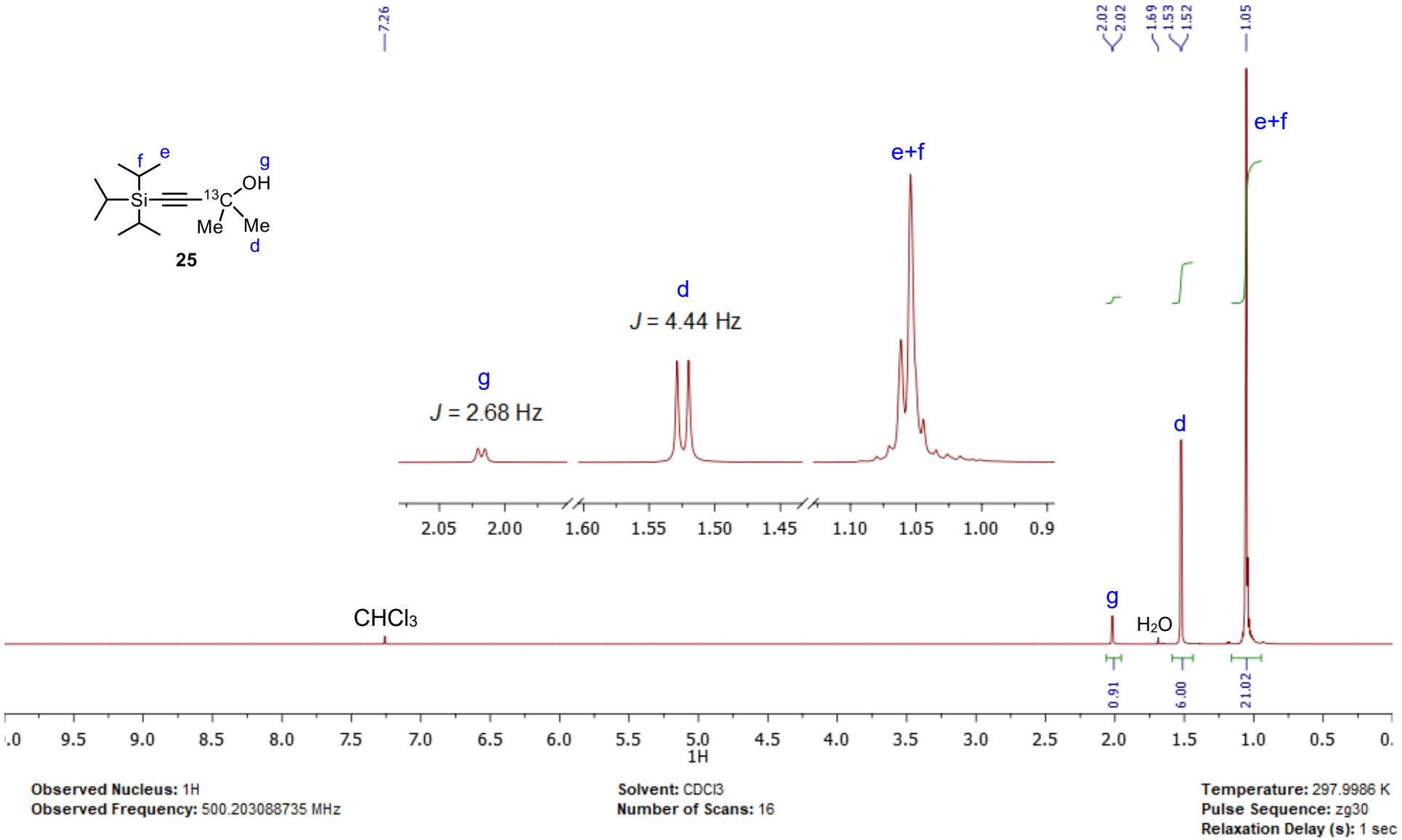


Figure S-II-50. ^{1}H ^{13}C HMBC NMR spectrum (600 MHz, 150 MHz, D_2O) of H_nNa_{4-n}[PyMTA-EPEP-CH_2CF_3] **23**, a drop of MeOH was added for calibration.



25

Figure S-II-51. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of 2-methyl-4-(triisopropylsilyl)but-3-yn-2-ol-2-^{13}C (**25**).

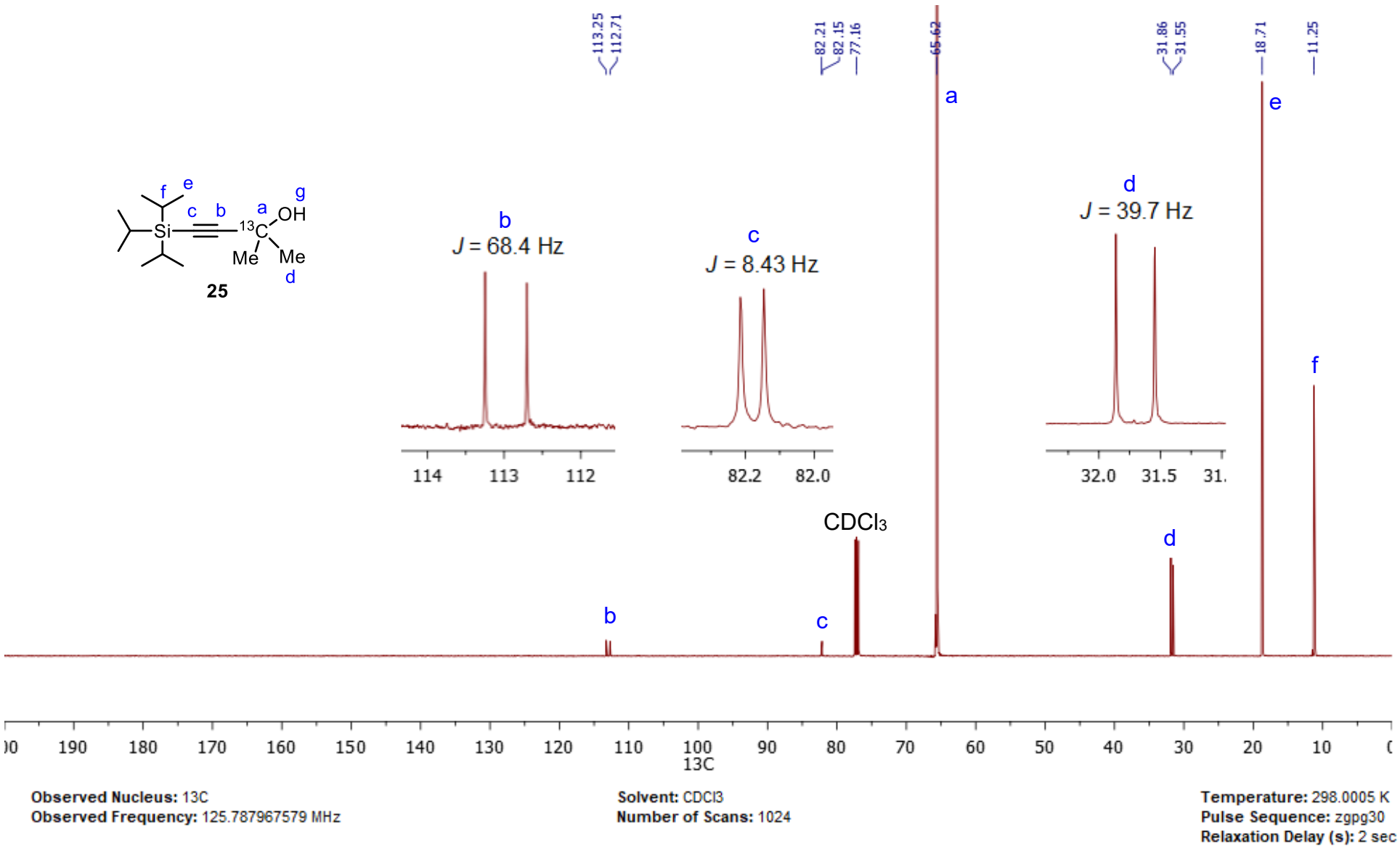


Figure S-II-52. ^{13}C NMR spectrum (126 MHz, $CDCl_3$) of 2-methyl-4-(triisopropylsilyl)but-3-yn-2-ol-2-*^{13}C* (**25**).

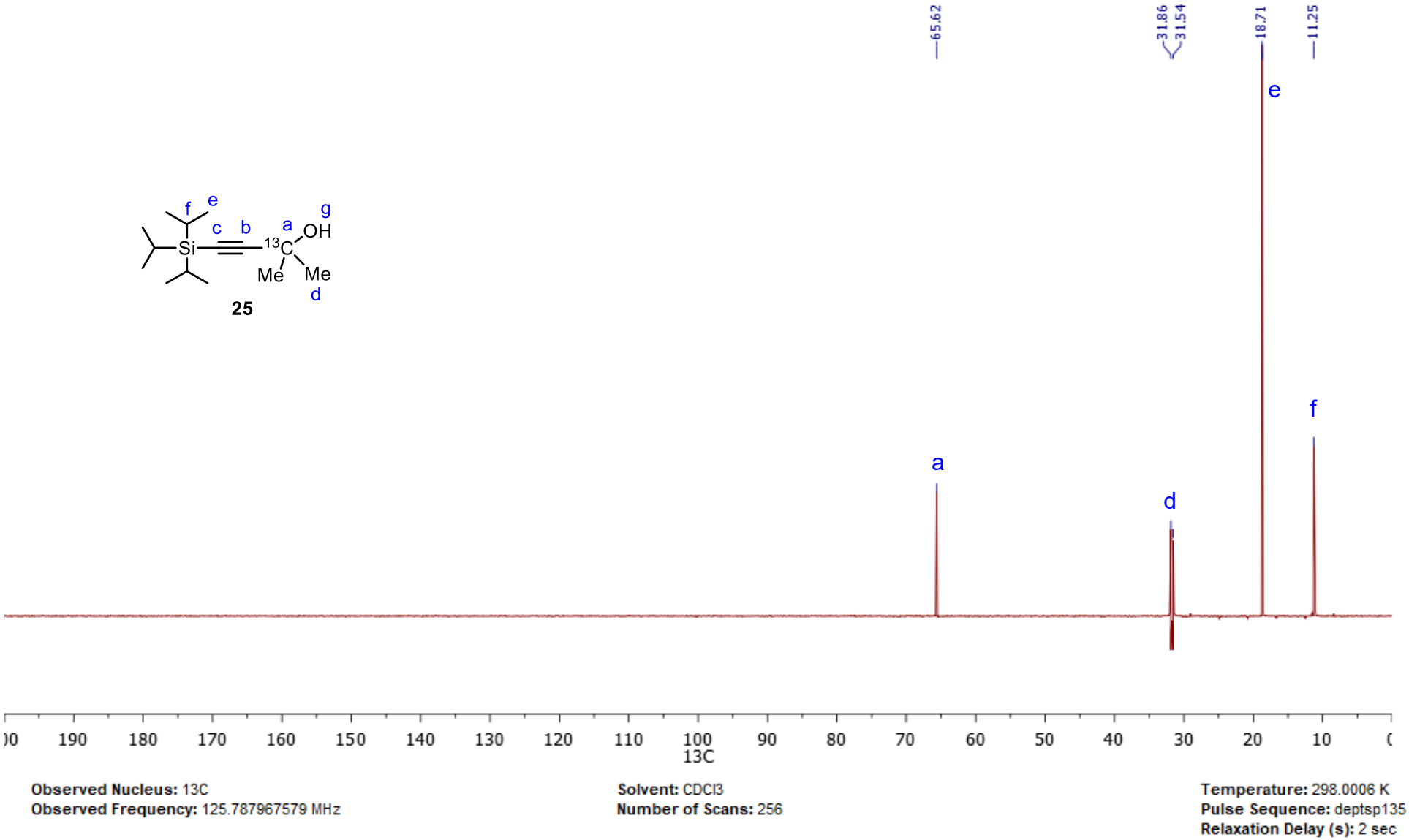


Figure S-II-53. ^{13}C DEPT 135 NMR spectrum (126 MHz, $CDCl_3$) of 2-methyl-4-(triisopropylsilyl)but-3-yn-2-ol-2-*^{13}C* (**25**).

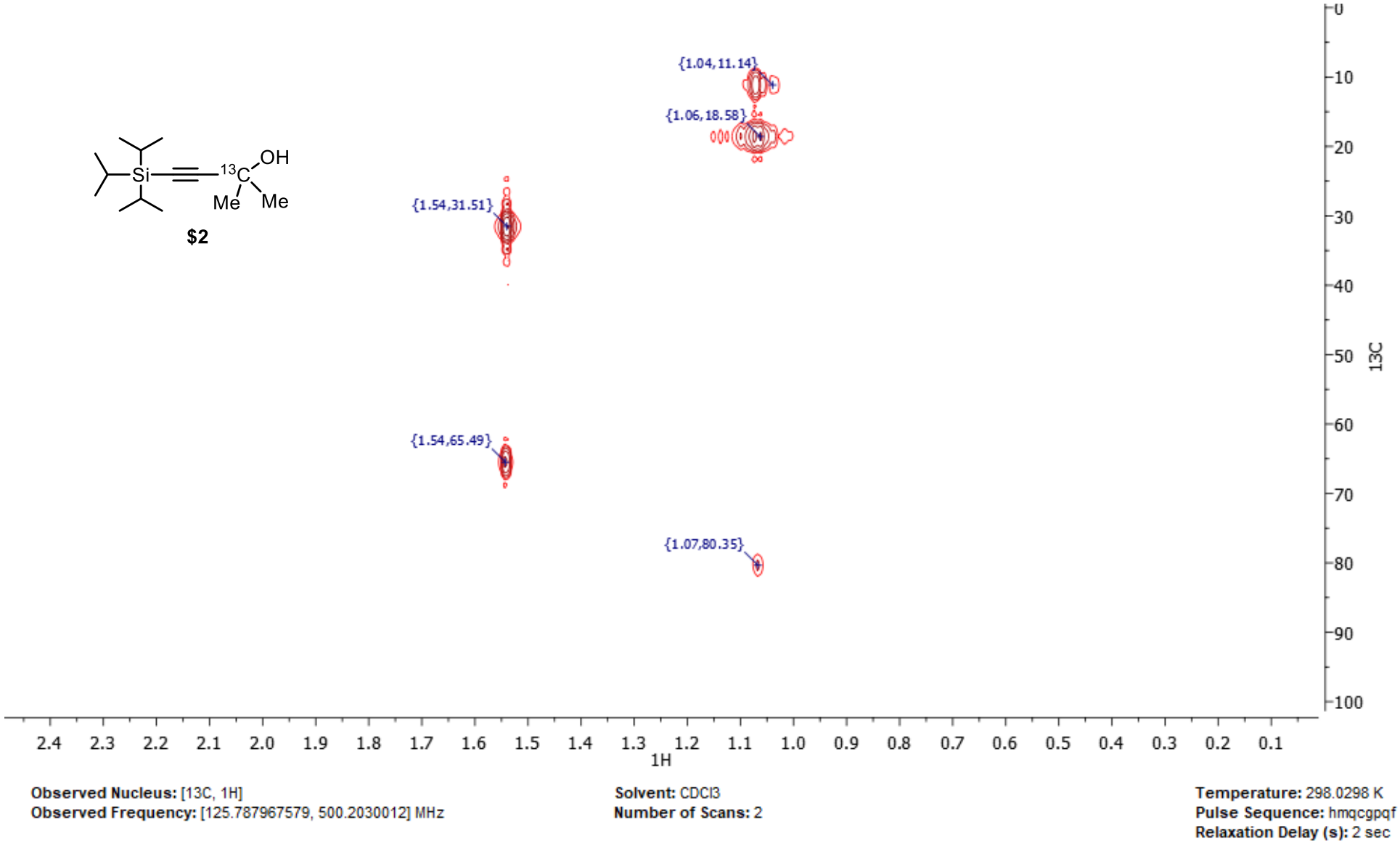


Figure S-II-54. ^{1}H ^{13}C HMQC NMR spectrum (500 MHz, 126 MHz, $CDCl_3$) of 2-methyl-4-(triisopropylsilyl)but-3-yn-2-ol-2-*13C* (**25**).

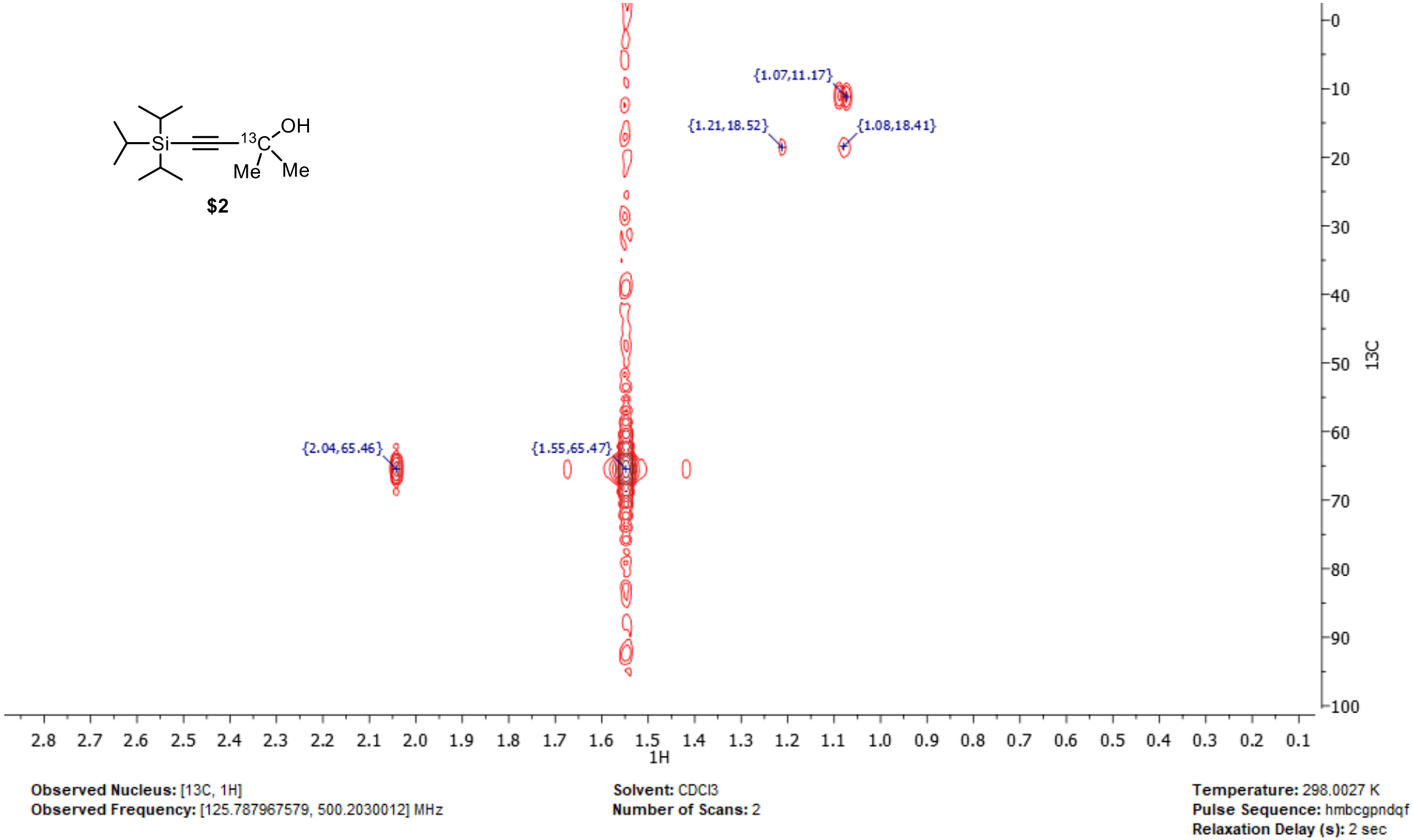


Figure S-II-55. ^{1}H ^{13}C HMBC NMR spectrum (500 MHz, 126 MHz, $CDCl_3$) of 2-methyl-4-(triisopropylsilyl)but-3-yn-2-ol-2-^{13}C (**25**).

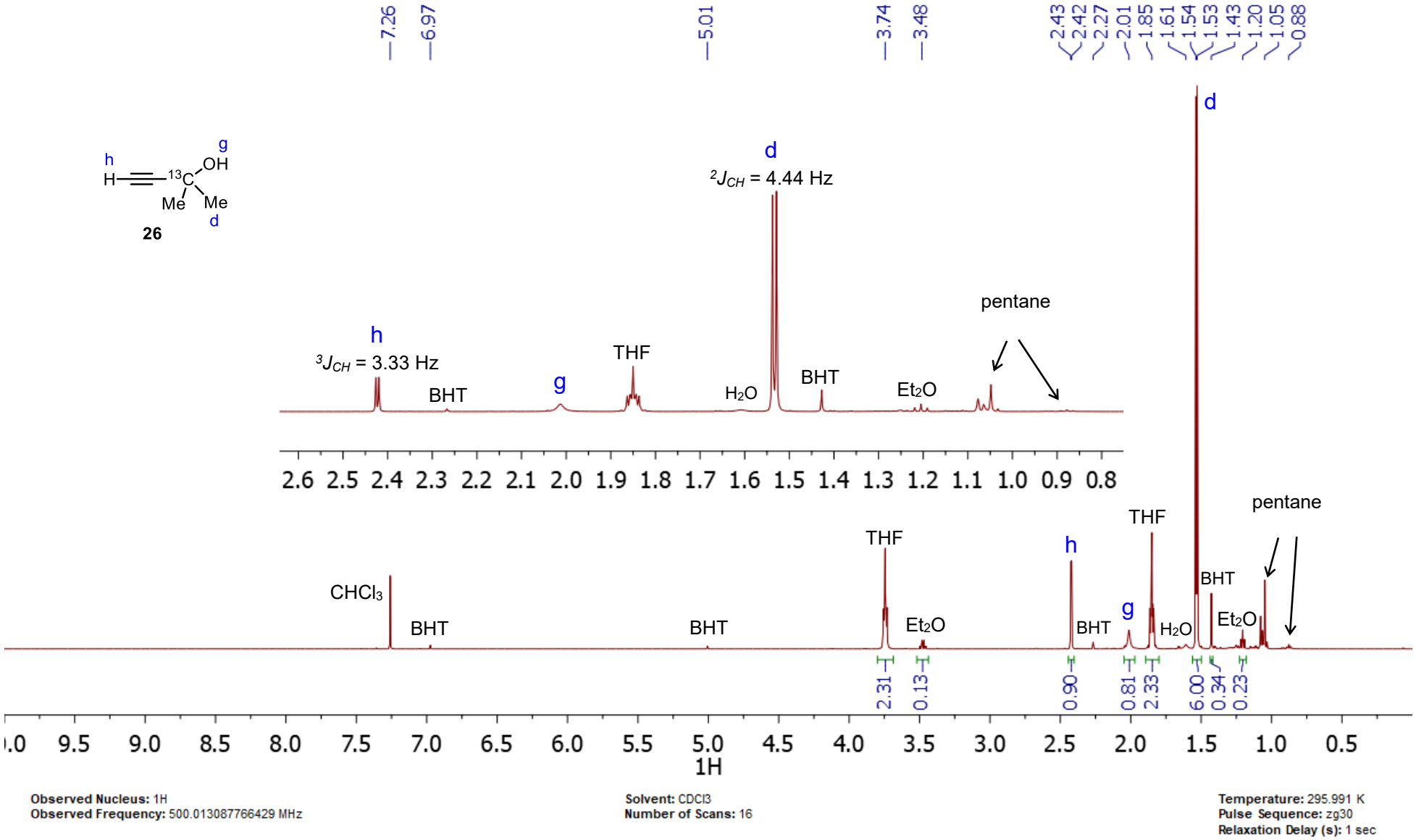


Figure S-II-56. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of the mixture of 2-methylbut-3-yn-2-ol-2-^{13}C (**26**), THF, Et_2O, 2,6-di-*tert*-butyl-4-methylphenol (BHT), and traces of pentane and TIPS-F/OH.

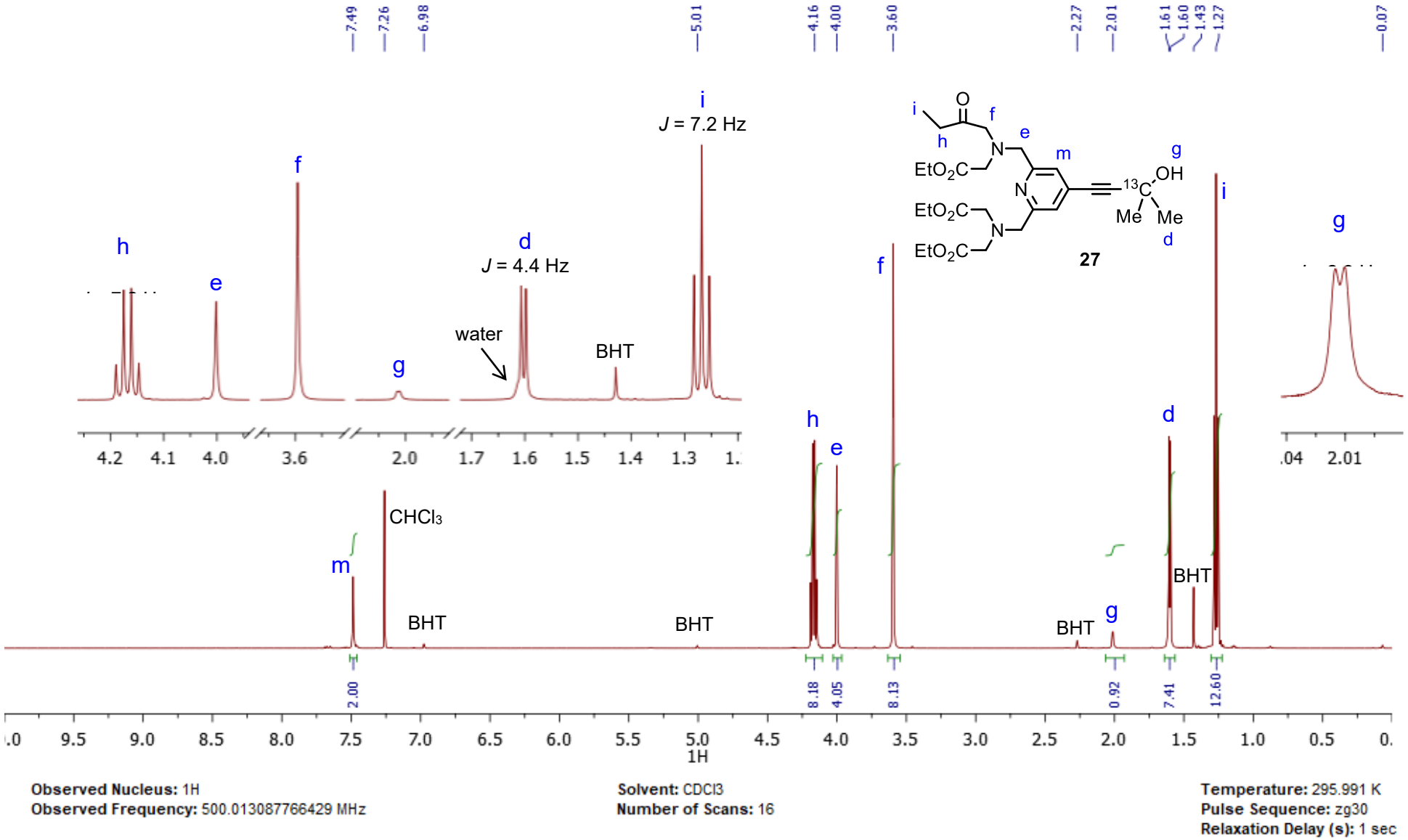
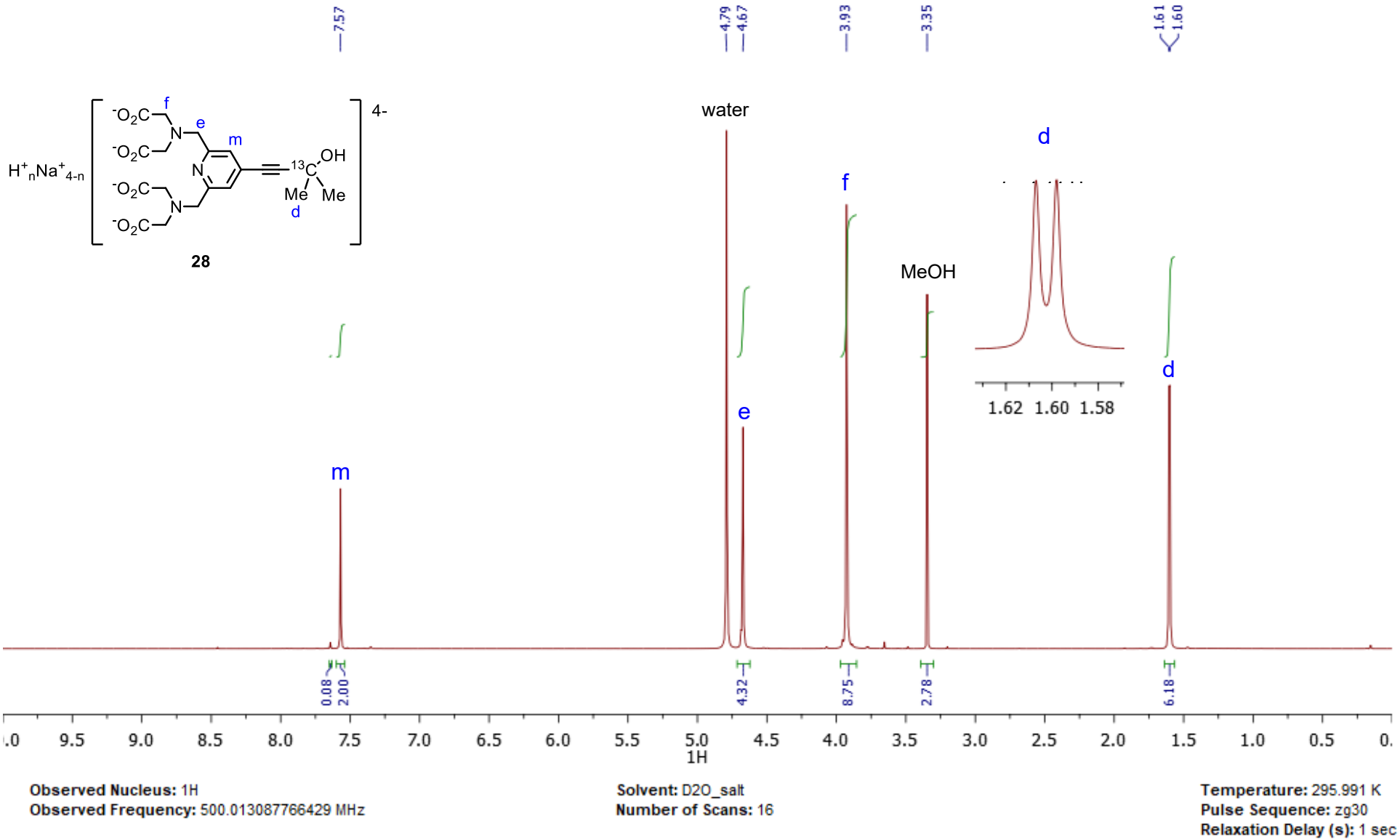


Figure S-II-57. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of PyMTA ester **27** in mixture with 2,6-di-*tert*-butyl-4-methylphenol (BHT).



28

Figure S-II-58. ^{1}H NMR spectrum (500 MHz, D_2O) of H_nNa_{4-n}[PyMTA-E-$^{13}C(CH_3)_2OH$] **28**, a drop of MeOH was added for the calibration of the ^{13}C NMR spectrum.

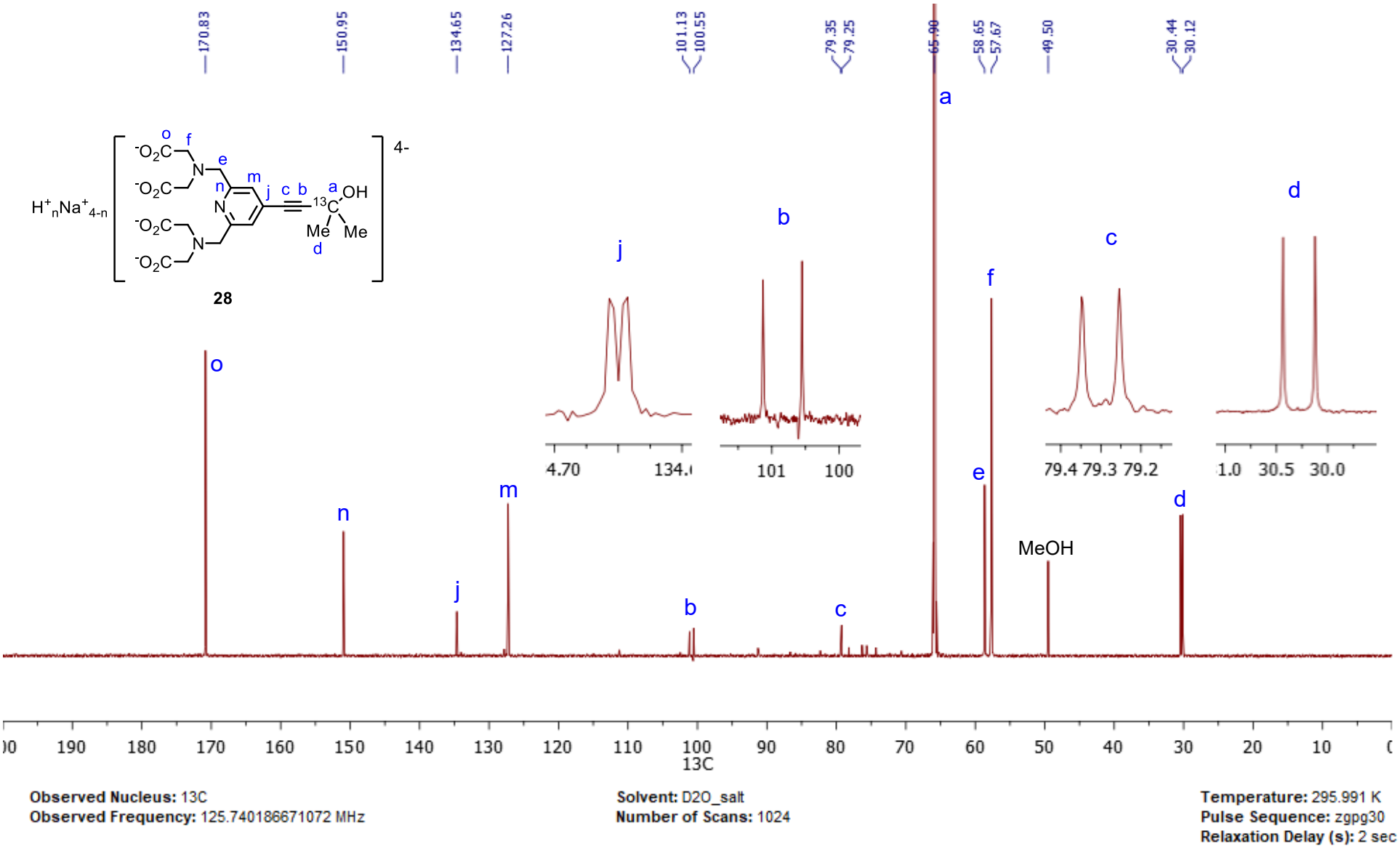


Figure S-II-59. ^{13}C NMR spectrum (126 MHz, D_2O) of H_nNa_{4-n}[PyMTA-E-$^{13}C(CH_3)_2OH$] **28**, a drop of MeOH was added for calibration.

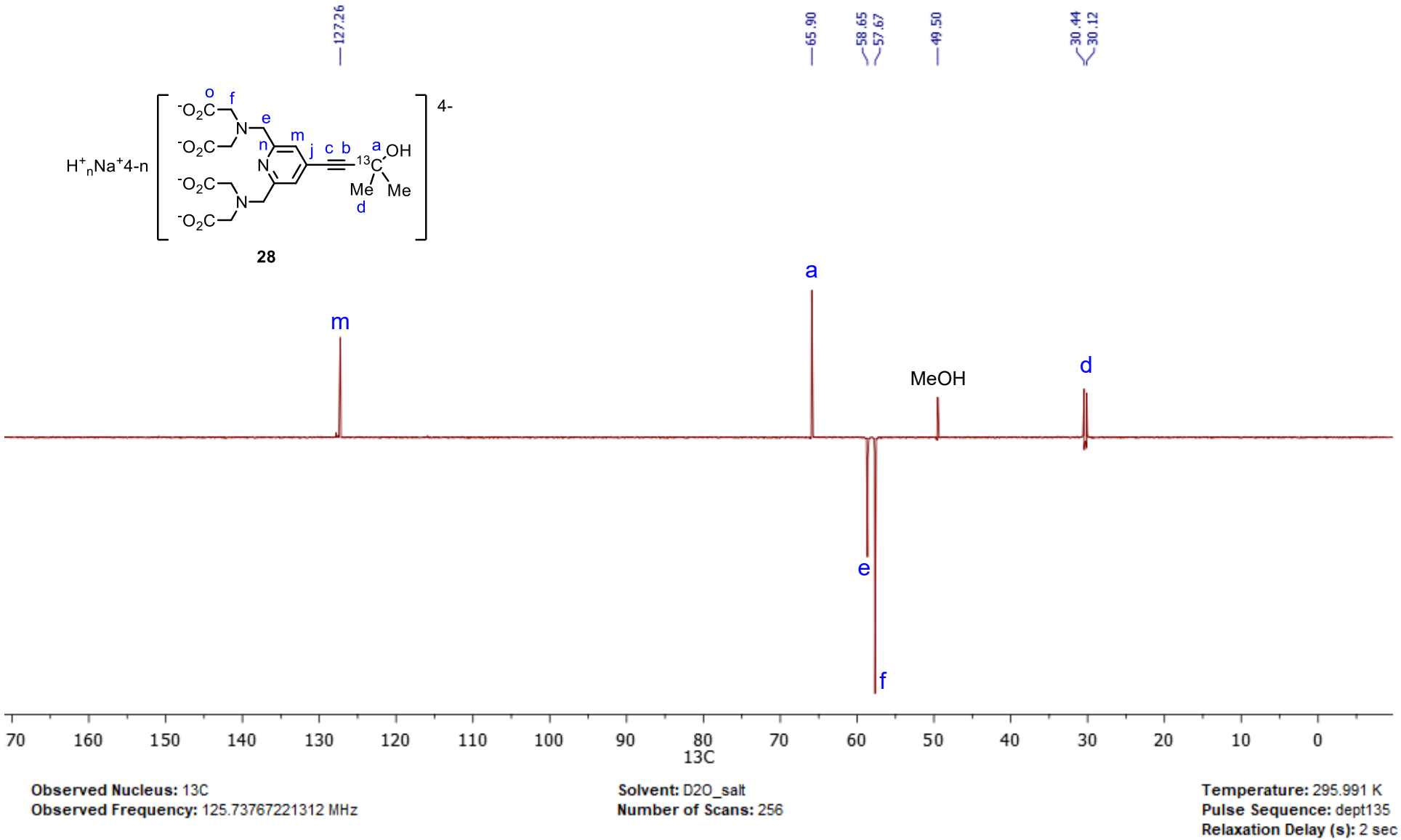


Figure S-II-60. ^{13}C DEPT 135 NMR spectrum (126 MHz, D_2O) of H_nNa_{4-n}[PyMTA-E-$^{13}C(CH_3)_2OH$] **28**, a drop of MeOH was added for calibration.

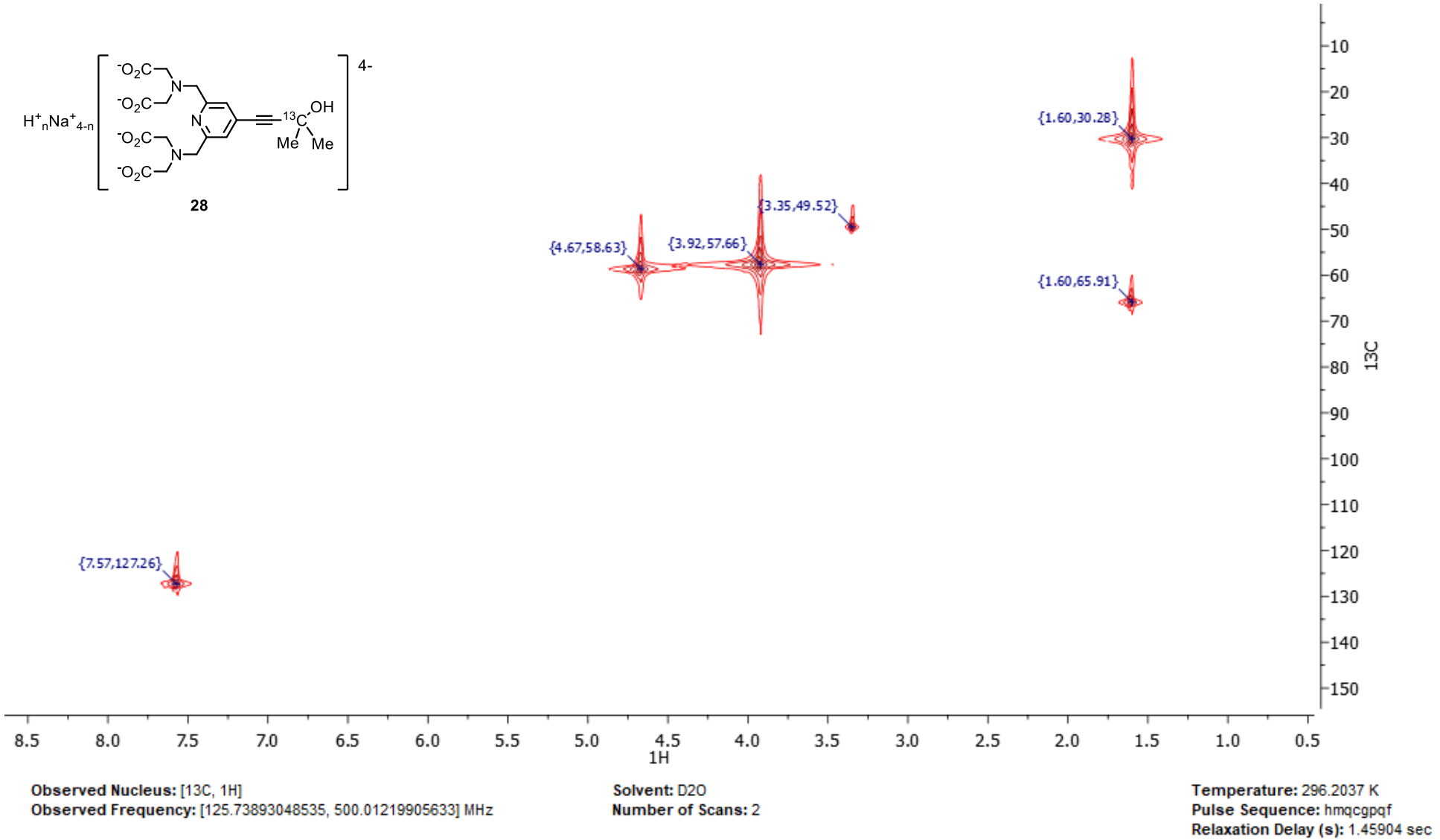


Figure S-II-61. ^{1}H ^{13}C HMQC NMR spectrum (500 MHz, 126 MHz, D_2O) of H_nNa_{4-n}[PyMTA-E-$^{13}C(CH_3)_2OH$] **28**, a drop of MeOH was added for calibration.

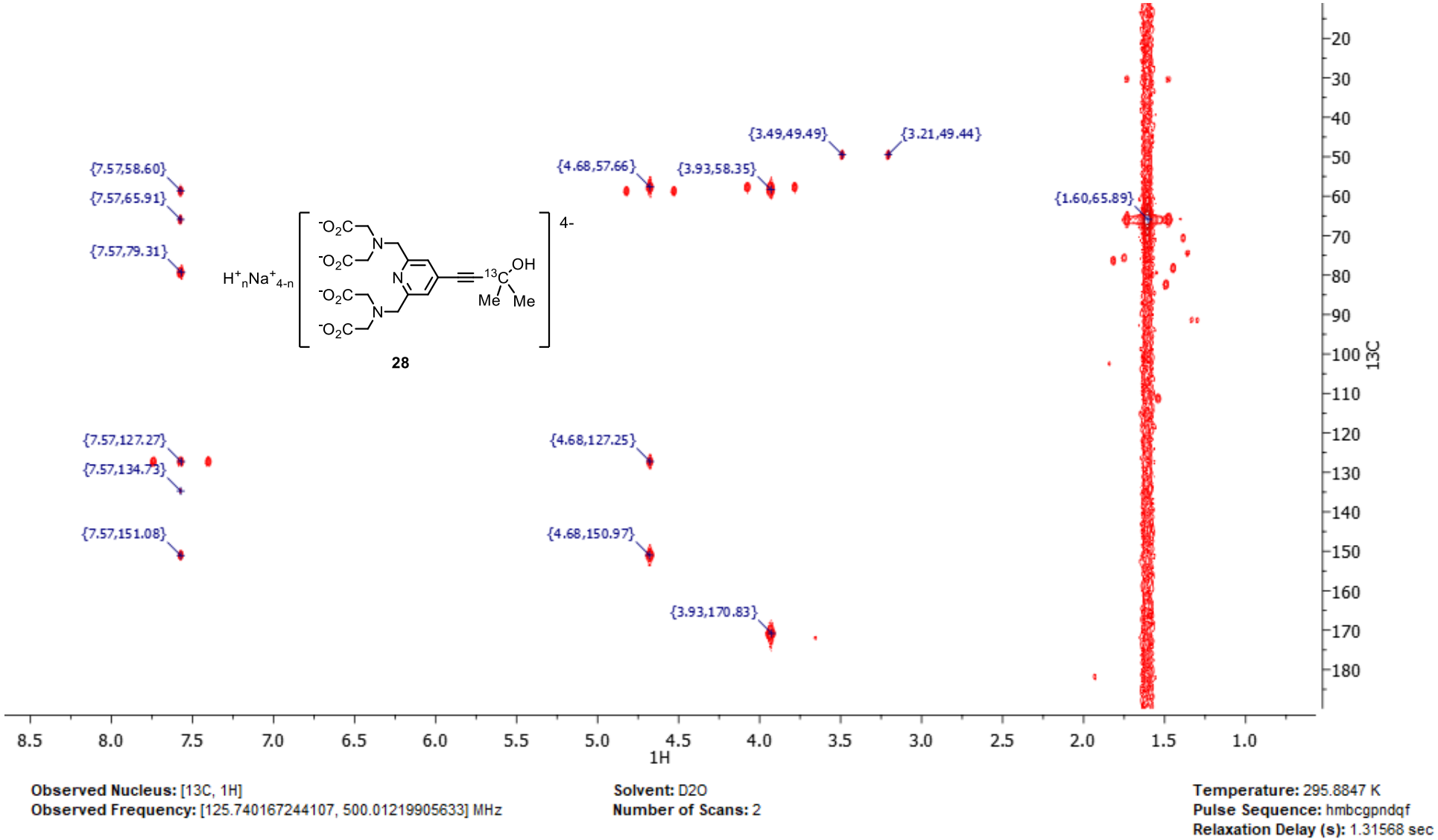


Figure S-II-62. ^{1}H ^{13}C HMBC NMR spectrum (500 MHz, 126 MHz, D_2O) of H_nNa_{4-n}[PyMTA-E-$^{13}C(CH_3)_2OH$] **28**, a drop of MeOH was added for calibration.

MS spectra

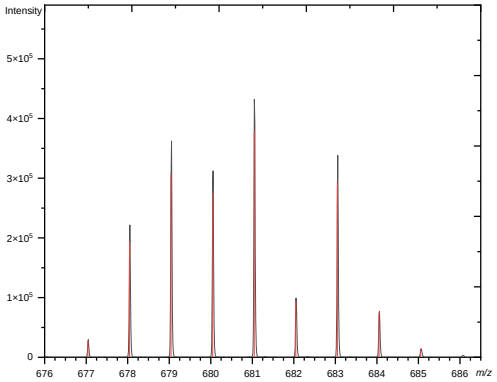


Figure S-II-63. Section of the accurate ESI-MS spectrum (black) of ruler **1a** showing the signal of the [M - Na]$^-$ ion ($C_{23}H_{20}N_3O_8F_3Gd^-$) and the corresponding theoretical isotope distribution (red) that was calculated with the program “Universal Mass Calculator[6] (version 3.14.0.47)”.

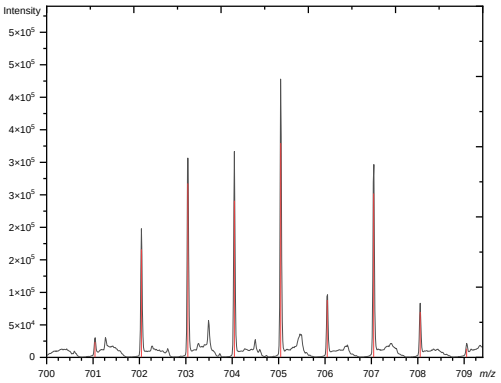


Figure S-II-64. Section of the accurate ESI-MS spectrum (black) of ruler **1b** showing the signal of the [M - Na]$^-$ ion ($C_{25}H_{20}F_3N_3O_8Gd^-$) and the corresponding theoretical isotope distribution (red) that was calculated with the program “Universal Mass Calculator[6] (version 3.14.0.47)”.

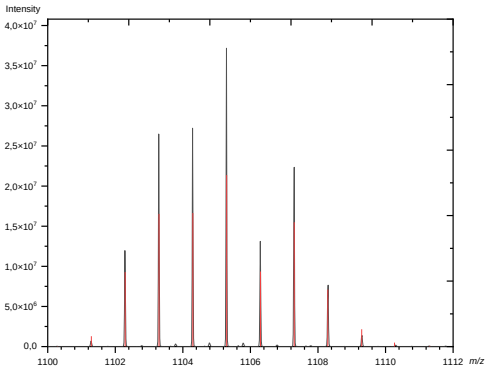


Figure S-II-65. Section of the accurate ESI-MS spectrum (black) of ruler **1c** showing the signal of the [M - Na]$^-$ ion ($C_{45}H_{52}F_3N_3O_{16}Gd^-$) and the corresponding theoretical isotope distribution (red) that was calculated with the program “Universal Mass Calculator[6] (version 3.14.0.47)”.

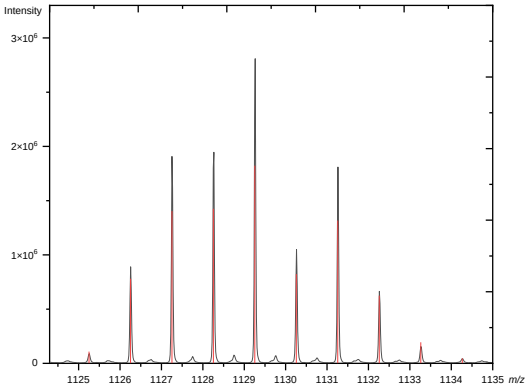


Figure S-II-66. Section of the accurate ESI-MS spectrum (black) of ruler **1d** showing the signal of the [M - Na]$^-$ ion ($C_{47}H_{52}F_3N_3O_{16}Gd^-$) and the corresponding theoretical isotope distribution (red) that was calculated with the program “Universal Mass Calculator[6] (version 3.14.0.47)”.

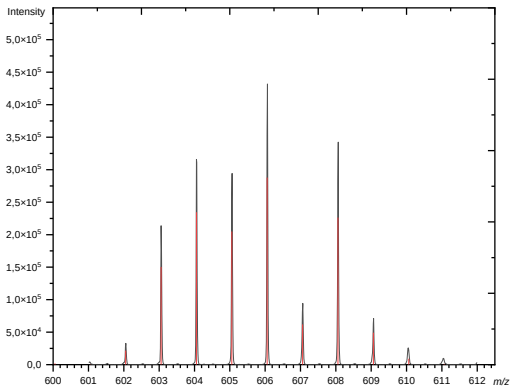


Figure S-II-67. Section of the accurate ESI-MS spectrum (black) of ruler **1e** showing the signal of the $[M - Na]^-$ ion ($C_{19}{}^{13}CH_{21}N_3O_9Gd^-$) and the corresponding theoretical isotope distribution (red) that was calculated with the program "Universal Mass Calculator[6] (version 3.14.0.47)".

SI 2. Synthesis of proteins 2

2.1 Protein expression

The GB1 mutants F52CF$_3$-Phe/A24C1, F52CF$_3$-Phe/K28C1, and F52CF$_3$-Phe/Q32C1 were prepared as reported previously.[7] Samples of the GB1 mutants G14CF$_3$-Phe/K28C1, V21CF$_3$-Phe/K28C1, Q32CF$_3$-Phe/K28C1, A48CF$_3$-Phe/K28C1 and A48SF$_5$-Phe/K28C1 were produced from a construct containing an N-terminal MASMTG tag and a C-terminal His$_6$-tag (Figure S-II-68). The genes encoding different GB1 mutants with the codon for the non-canonical amino acid mutation site changed to the amber stop codon, were cloned between the *Nde*I and *BamH*I restriction sites of a pCDF plasmid.[8]

MASMTGMTYK LILNGKTLK**G** ETTTEA**V**DAA TAEKVFK**Q**YA
NDNGVDGEWT YDD**A**TKTFTV TEHHHHHH

Figure S-II-68 *Amino acid sequence of the* ***GB1*** *construct used. The N-terminal MASMTG tag is shown in red. Residues G14, V21, Q32, and A48 are shown in bold and K28 is underlined.*

GB1 samples containing the fluorinated non-canonical amino acids CF$_3$-Phe and SF$_5$-Phe were produced by co-transforming *E. coli* B-95 ΔAΔ*fabR* cells (https://www.nature.com/articles/srep09699) with the pCDF plasmid containing the GB1 gene and the pRSF-G1pCNPRS plasmid containing the polyspecific G1pCNP tRNA synthetase that can recognize CF$_3$-Phe and SF$_5$-Phe.[9] The transformed cells were cultured at 37 °C in LB medium containing 50 mg/L spectinomycin and 50 mg/L kanamycin. A 2.5 mL overnight culture was used to inoculate 250 mL of LB medium supplemented with 50 mg/L spectinomycin and 50 mg/L kanamycin and 2 mM of the non-canonical amino acid (CF$_3$-Phe or SF$_5$-Phe). The cells were grown at 37 °C until reaching an OD$_{600}$ of 0.6–1.0, at which point the temperature was reduced to 25 °C and protein expression was induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG).

After 16 hours of protein expression, the cells were harvested by centrifugation at 4000 *g* for 15 minutes at 4 °C. The harvested cells were resuspended in buffer A (50 mM Tris-HCl pH 7.5, 300 mM NaCl, 5% glycerol, 10 mM imidazole) and lysed by sonication (ultrasonic homogenizer Omni-Ruptor 4000, Omni International, USA) on ice (50% power and 50% pulse length for 10 min). The cell lysate was clarified by centrifugation for 1 hour at 30000 *g* at 4 °C. The supernatant was loaded onto a 1 mL His GraviTrap column (Cytiva, USA). The column was washed with 20 column volumes of buffer A) and the protein was eluted with 5 column volumes of buffer B (same as buffer A but with 500 mM imidazole). Subsequently, the buffer

was exchanged to phosphate-buffered saline (PBS) using an Amicon ultrafiltration centrifugal tube (Merck Millipore, Germany) with a molecular weight cut-off of 3 kDa.

2.2 Ligation with Gd.C1

For ligation of the GB1 mutants with **Gd.C1**, a 0.1 mM solution of the proteins in PBS, pH 7.4, was incubated with 2 mM dithiothreitol (DTT) for 2 hours at 25 °C. DTT was removed using an Amicon ultrafiltration centrifugal tube with a molecular weight cutoff of 3 kDa. The solution of the reduced protein was slowly added to a solution containing 5 equivalents of **Gd.C1** tag in PBS and the solution was incubated at 25 °C for 16 hours. Mass spectrometry indicated quantitative ligation(

Figure **S**-II-69). Following the labeling reaction, the protein samples were concentrated and buffer-exchanged to 50 mM MES in D_2O, pH 6.5, 50 mM NaCl (pH value is an uncorrected pH meter reading) using Amicon ultrafiltration centrifugal tubes with a 3 kDa molecular weight cut-off. Perdeuterated glycerol 10% (v/v) was added to achieve a final protein concentration of 0.1 mM.

2.3 Intact protein mass spectrometry

Mass spectrometric analysis was done on a Thermo Fisher Scientific UltiMate 3000 HPLC system coupled to an Orbitrap Fusion™ Tribrid™ mass spectrometer (Thermo Fisher Scientific, USA). The HPLC system was equipped with a ZORBAX 300SB-C3, 3.5 µm, 4.6 x 50 mm column (Agilent Technologies, USA). Approximately 30 pmol of the protein sample was injected using a 500 µL/min linear gradient of solvent A (0.1% (v/v) formic acid in water) and solvent B (0.1% (v/v) formic acid in acetonitrile), with solvent B increasing from 5% at 2 min to 80% over 7 min. Data acquisition was performed using an electrospray ionization (ESI) source in positive ion mode. The intact protein mass was determined through deconvolution using Xcalibur 3.0.63 (Thermo Fisher Scientific, USA). The results of the analysis are presented in

Figure **S**-II-69.

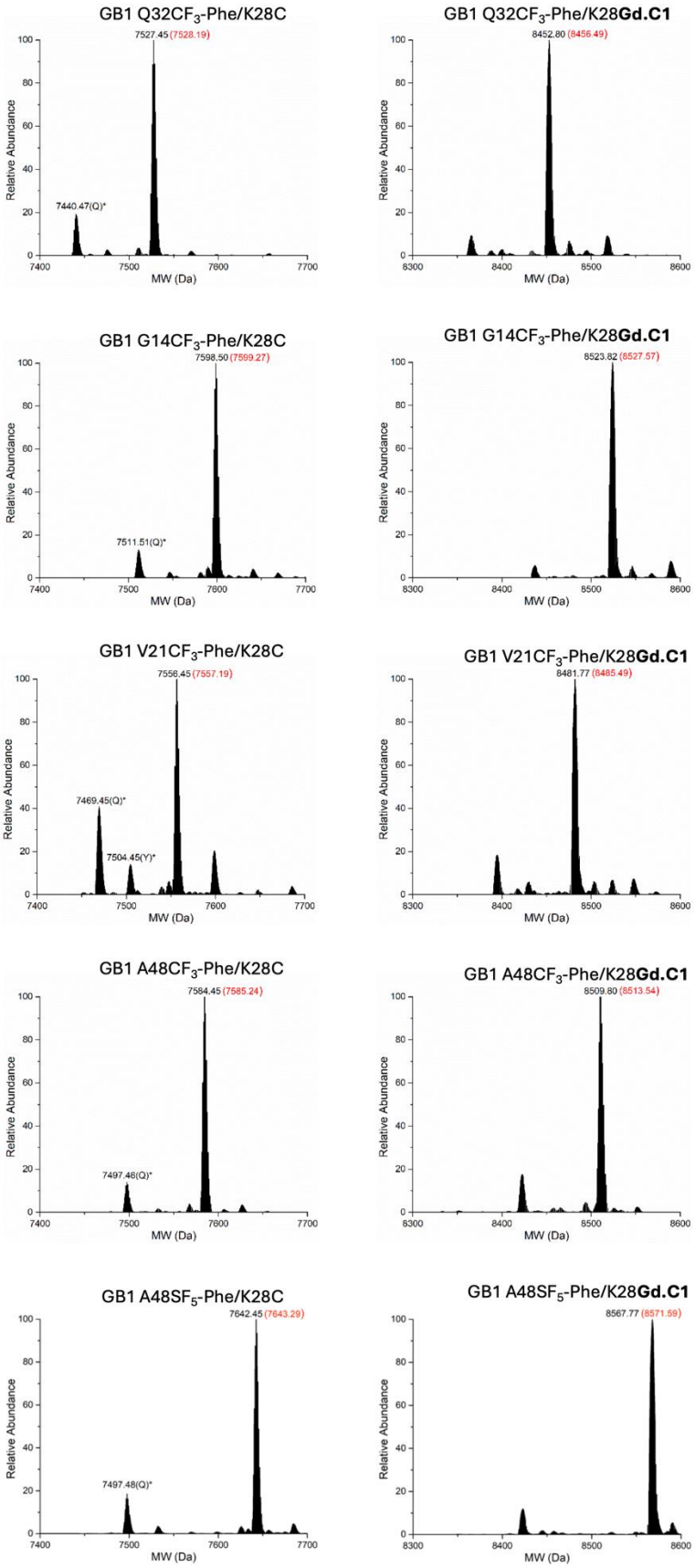


Figure S-II-69 Intact protein mass spectrometric analysis results. The calculated masses are shown in red. The masses marked with an asterisk are the products of amber codon suppression by canonical amino acids (Q = glutamine, Y = tyrosine).

References

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