Supplementary Information

Gd^{3+}-Gd^{3+} distances exceeding 3 nm determined by very high frequency continuous wave electron paramagnetic resonance

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Content

1. Sample details

Compound	**Content of stock solution in D_2O**	**pH**
Gd-4-iodo-PyMTA	3 mM Gd-4-iodo-PyMTA, 5 mM F_3CCO_2H/F_3CCO_2Na, 15 mM NaCl, 120 mM H_2O	7
$\mathbf{1_0}$	4 mM $\mathbf{1_0}$, 48 mM NaCl, 148 mM H_2O	5
$\mathbf{1_1}$	5 mM $\mathbf{1_1}$, 37 mM NaCl, 100 mM H_2O	8
$\mathbf{2_1}$	5 mM $\mathbf{2_1}$, 0.5 mM F_3CCO_2H/F_3CCO_2Na, 30 mM NaCl, 180 mM H_2O	7
$\mathbf{1_3}$	5 mM $\mathbf{1_3}$, 28 mM NaCl, 100 mM H_2O	8
$\mathbf{2_2}$	5 mM $\mathbf{2_2}$, 30 mM NaCl, 100 mM H_2O	8
3	5 mM **3**, 32 mM NaCl, 122 mM H_2O	

Table S1. Details of the content of stock solutions of Gd-4-iodo-PyMTA and Gd-rulers $\mathbf{1_n}$, $\mathbf{2_n}$, and **3** used to prepare samples for CW-EPR experiments, including pH and the concentrations of additional compounds left over as a result of the synthesis.

Gd-rulers / T (K)	**3**	$\mathbf{1_0}$	$\mathbf{1_1}$	$\mathbf{2_1}$	$\mathbf{1_3}$	$\mathbf{2_2}$
173	1.17 nm	1.42 nm	2.09 nm	3.00 nm	3.41 nm	4.30 nm
215	1.16 nm	1.42 nm	2.08 nm	2.98 nm	3.39 nm	4.27 nm
288	1.16 nm	1.41 nm	2.07 nm	2.96 nm	3.36 nm	4.22 nm

Table S2. Calculated most probable Gd-Gd distances of the Gd-rulers at 173 K, 215 K, and 288 K using the worm-like chain model, as described in the supplementary information of Ref. 48 in the main text. For the measurements at 30 K, the shape of the Gd-rulers became frozen at the glass transition temperature of the mixture of D_2O and glycerol-d_8 (60:40, v:v), which is 173 K. Therefore, the most probably Gd-Gd distances of Gd-rulers at 173 K were calculated for the measurements at 30 K.

2. CW EPR spectra of Gd-4-iodo-PyMTA at 240 GHz and 30 K and corresponding simulation

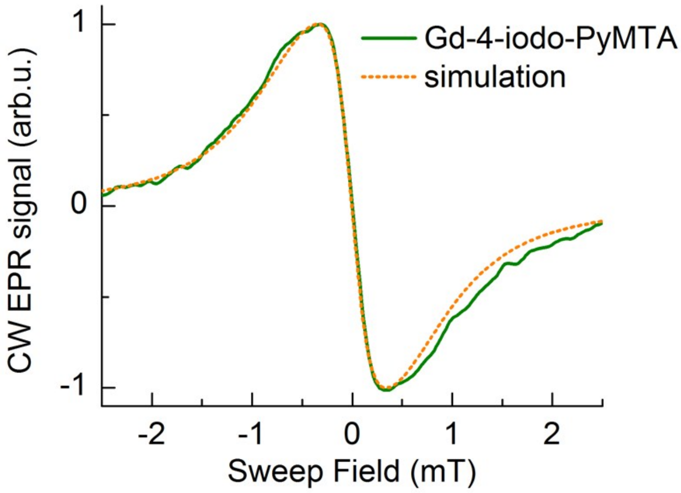


Figure S1. CW EPR spectrum of the central transition of Gd-4-iodo-PyMTA in D_2O/glycerol-d_8 measured at 30 K (dashed green) and simulated spectrum (dotted orange). The simulation consists of an S = 1/2 spin coupled to an S = 7/2 spin at a temperature of 30 K with isotropic g-values of 1.992. The approximate lineshape and peak-to-peak linewidth of Gd-4-iodo-PyMTA was reproduced in the simulation by introducing an artificial g-strain on the S = 1/2 spin of the form of a Lorentzian distribution of g-values centered at g = 1.992 with a FWHM of 0.00028. A small Voigtian convolutional line broadening (0.2 mT Gaussian + 0.5 mT Lorentzian) was additionally included so that the simulated derivative produced a smooth line.

3. 240 GHz CW EPR spectra of Gd-4-iodo-PyMTA and the Gd-rulers at 215 K and 288 K

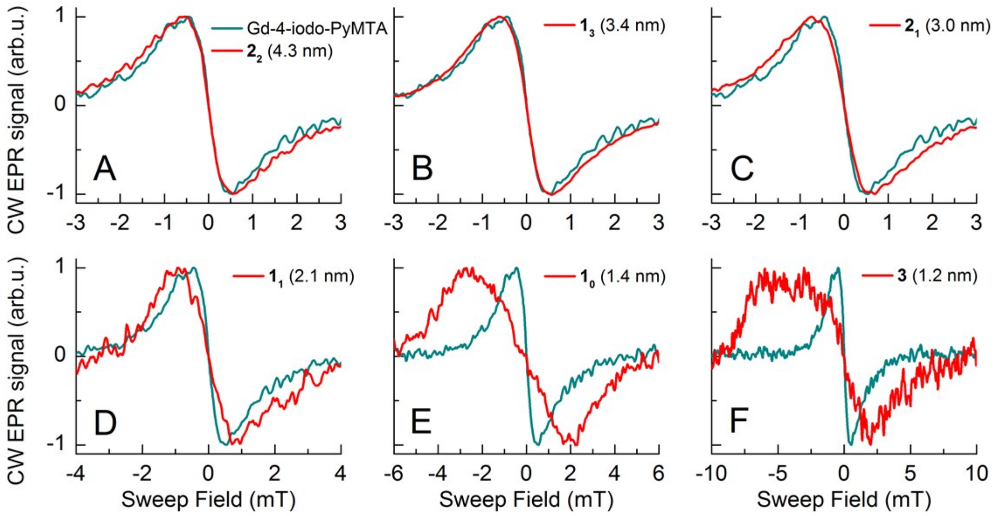


Figure S2. CW EPR spectra of the central transition of Gd-4-iodo-PyMTA and the Gd-rulers $\mathbf{1}_\mathbf{n}$, $\mathbf{2}_\mathbf{n}$, and **3** in D_2O/glycerol-d_8 measured at 215 K. In all cases, the peak-to-peak linewidth is broader than the corresponding measurements at 30 K, but this does not affect the $1/r^3$ dependence of the peak-to-peak broadening with the Gd-Gd distance (Fig. 3, main text).

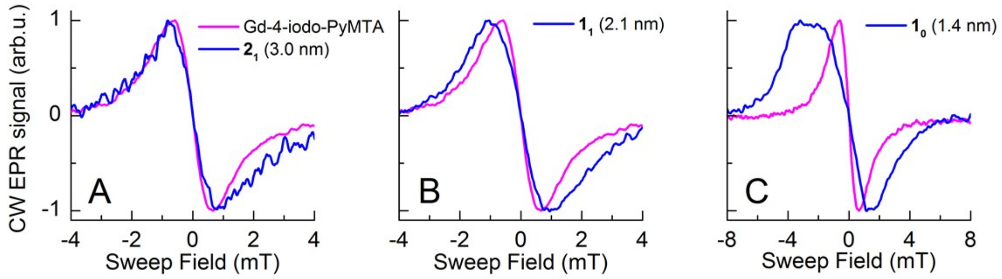


Figure S3. CW EPR spectra of the central transition of Gd-4-iodo-PyMTA and the Gd-rulers $\mathbf{1}_\mathbf{n}$ and $\mathbf{2}_\mathbf{n}$ embedded in glassy trehalose measured at 288 K. Again, the peak-to-peak linewidth is broader at the higher temperature, but this does not affect the $1/r^3$ dependence of the peak-to-peak broadening with the Gd-Gd distance (Fig. 3, main text). Gd-ruler **3** (1.2 nm) was not available at the time of these measurements. For the Gd-rulers $\mathbf{2}_\mathbf{2}$ (4.3 nm) and $\mathbf{1}_\mathbf{3}$ (3.4 nm) it was not possible to record a CW EPR spectrum with sufficient SNR, as discussed in the main text.

4. W-band echo-detected EPR of Gd-4-iodo-PyMTA and Gd-rulers at 10 K

Stock solutions of Gd-rulers $\mathbf{1_n}$ and $\mathbf{2_n}$ in D_2O (Table S3) were diluted with a 7:3 (volume ratio) mixture of D_2O and glycerol-d_8. W-band measurements were carried out at 10 K using a home-built spectrometer, quartz capillaries (0.64 mm i.d., 0.8 mm o.d.), and sample volumes of 2-3 μL. Echo-detected (ED) EPR spectra were recorded using the two pulse echo sequence π/2-τ- π-τ-echo with a 30 ns π/2 pulse, 60 ns the π pulse, and τ = 550 ns. A two-step phase cycling (0, π) was applied over the π/2 pulse.

Compound	**Content of stock solution in D_2O**	**pH**
Gd-4-iodo-PyMTA	2 mM Gd-4-iodo-PyMTA, 2 mM F_3CCO_2H and/or F_3CCO_2Na, 6 mM NaCl, 120 mM H_2O	7
$\mathbf{1_1}$	5 mM $\mathbf{1_1}$, 37 mM NaCl	8
$\mathbf{2_1}$	1 mM $\mathbf{2_1}$, 0.1 mM F_3CCO_2H/F_3CCO_2Na, 6 mM NaCl	7
$\mathbf{1_3}$	5 mM $\mathbf{1_3}$, 28 mM NaCl	8
$\mathbf{2_2}$	5 mM $\mathbf{2_2}$, 30 mM NaCl	8
$\mathbf{1_5}$	5 mM $\mathbf{1_5}$, 38 mM NaCl	8
$\mathbf{1_7}$	5 mM $\mathbf{1_7}$, 30 mM NaCl	8
$\mathbf{1_9}$	2 mM $\mathbf{1_9}$, 20 mM NaCl	8
$\mathbf{1_{11}}$	2 mM $\mathbf{1_{11}}$, 20 mM NaCl	8

Table S3. Details of the content of stock solutions of Gd-4-iodo-PyMTA and Gd-rulers $\mathbf{1_n}$ and $\mathbf{2_n}$ used to prepare samples for W-band EDEPR experiments, including pH and concentrations of additional compounds present as a result of the synthesis.

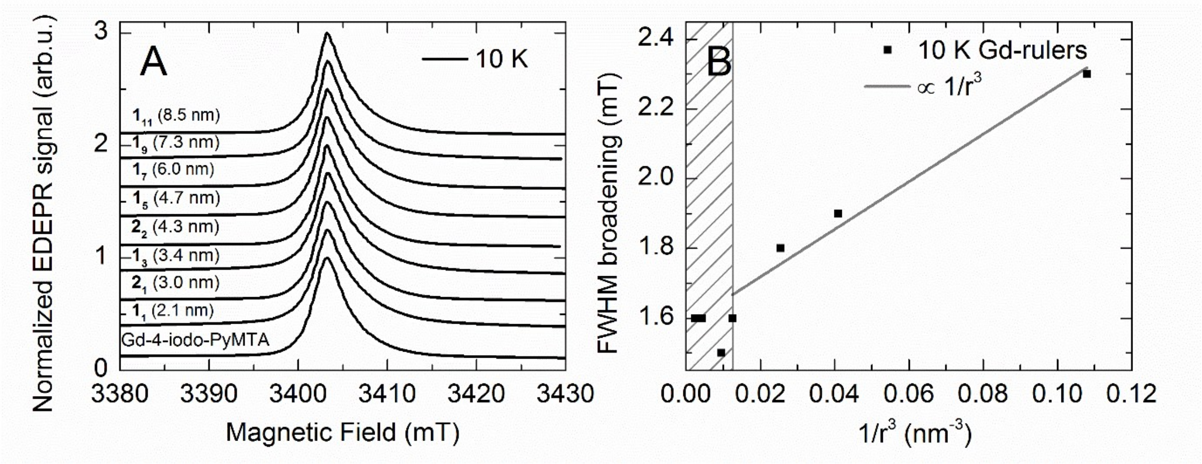
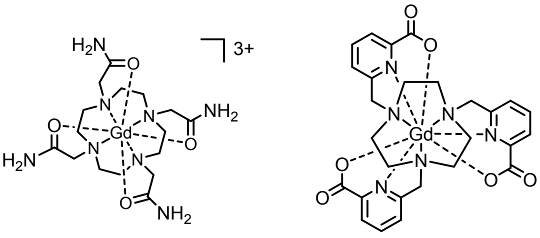


Figure S4. (A) Echo-detected EPR spectra of Gd-rulers in D_2O/glycerol-d_8 measured at W-band and 10 K. (B) Peak-to-peak linewidths taken from the derivative of the EDEPR spectra plotted as a function of the most probable Gd-Gd distance *r*. The relationship is linear in $1/r^3$ for the shorter Gd-rulers. For interspin distances of 4.3 nm and above the linewidth is indiscernible from the peak-to-peak linewidth of Gd-4-iodo-PyMTA (FWHM ~ 1.6 mT).

5. CW EPR spectra of Gd-DOTAM and Gd-NO3Pic (Gd-TPATCN) at 240 GHz and 30 K



Gd-DOTAM

Gd-NO3pic

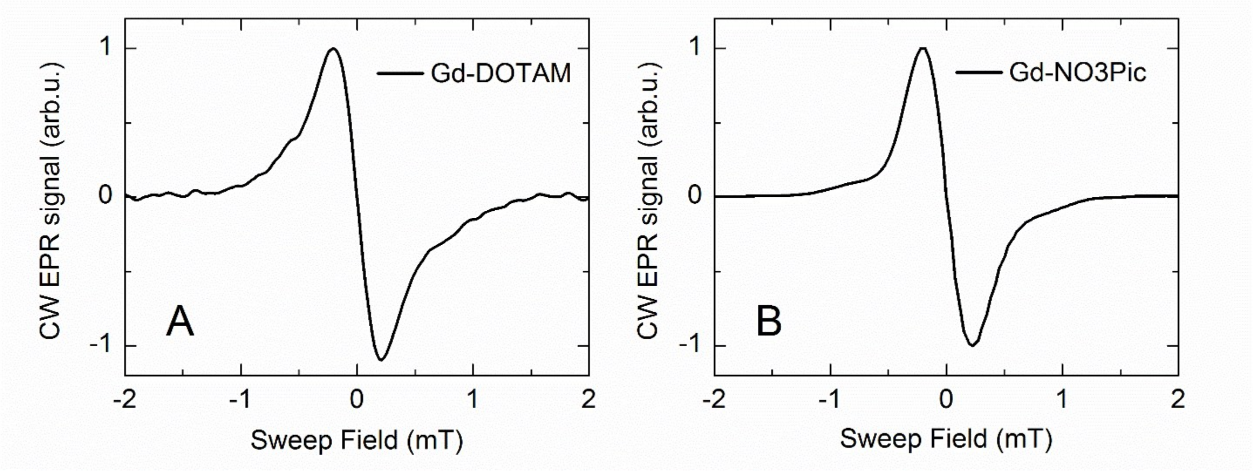


Figure S5. Top: Structural formulae of Gd-DOTAM and Gd-NO3Pic. Bottom: CW EPR spectra of the central transition of (A) Gd-DOTAM and (B) Gd-NO3Pic (Gd-TPATCN) in D_2O/glycerol-d_8 measured at 240 GHz and 30 K. Gd-DOTAM has a peak-to-peak linewidth of ~ 0.53 mT and Gd-NO3Pic has a peak-to-peak linewidth of ~ 0.45 mT.

6. Synthesis of Gd-4-iodo-PyMTA and Gd-rulers 2_1 and 2_2.

General

Unless otherwise stated, reactions were performed in dried glassware under argon using the Schlenk technique and commercial solvents and reagents, except THF (HPLC grade) which was distilled from sodium/benzophenone prior to use. $PdCl_2(PPh_3)_2$ was synthesized according to the literature,[1] however using 2.1 times the given amount of methanol. For the preparation of the aqueous solutions, deionized water was used. The argon was passed through anhydrous $CaCl_2$ prior to use. The solvents used for extraction and chromatography were of technical grade and were distilled prior to their use. The proton-exchange resin (Dowex 50WX4 hydrogen form, 91 g) was subsequently 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).

The temperature given for the reactions refers to the bath temperature. Solvents were removed at a bath temperature of ~40 °C and reduced pressure. The products were dried at room temperature at ~0.05 mbar. The pH/pD values of the solutions were determined using pH indicator strips (resolution: 0.3 pH).

Column chromatography was carried out on silica gel 60 (0.035−0.070 mm) applying slight pressure. In the procedures reported below, the size of the column is given as diameter × length. The material was loaded onto the column dissolved in a small quantity of the eluent. Thin layer chromatography was performed on silica gel 60 containing fluorescent indicator F254. The solid support for the silica gel layer was aluminum foil. Unless otherwise stated, the spots were detected with UV light of λ = 254 and 366 nm. The compositions of solvent mixtures are given in volume ratios.

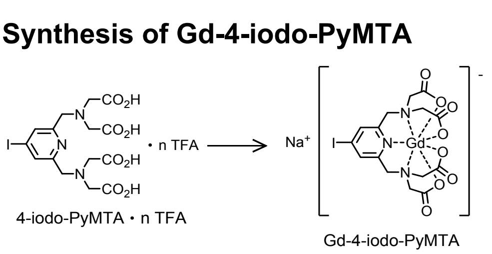
For centrifugation, a centrifuge with a relative centrifugal force of 4000g was used.

NMR spectra were calibrated using the solvent signal as an internal standard [$CDCl_3$: δ (^{1}H) = 7.25, δ (^{13}C{^{1}H}) = 77.0; CD_2Cl_2: δ (^{1}H) = 5.32, δ (^{13}C{^{1}H}) = 53.8; CD_3OD: δ (^{1}H) = 3.31, δ (^{13}C{^{1}H}) = 49.0]. Signal assignments are supported by DEPT-135, COSY, HMBC, and HMQC experiments.

Accurate MS experiments were performed using an FT-ICR mass spectrometer interfaced to an external ESI ion source. Unless otherwise stated, the monoisotopic mass of a compound is reported.

The ratio of the components in a mixture was determined by ^{1}H NMR spectroscopy and is given as molar ratio.

Synthesis of Gd-4-iodo-PyMTA



The reaction was performed under ambient atmosphere. A solution of $GdCl_3 \cdot 6\ H_2O$ in D_2O (50 mM, 222 µL, 11.1 µmol) was added to a solution of 4-iodo-PyMTA • n TFA[2] in D_2O (46.7 mM, 250 µL, 11.7 µmol). Then a solution of NaOD in D_2O (100 mM, 350 µL, 35 µmol) was added to rise the pH of the solution to pH 7. 514 µL of the solution were diluted with D_2O to a total volume of 1000 µL to obtain a 3.0 mM solution of Gd-4-iodo-PyMTA in D_2O containing NaCl and $Na(O_2CCF_3)$. MS (ESI) *m/z* = 648.8 $[M - Na]^-$. Accurate MS (ESI): *m/z* calcd. for $[M - Na]^-$ $C_{15}H_{14}N_3O_8IGd^-$: 648.90721; found 648.90803.

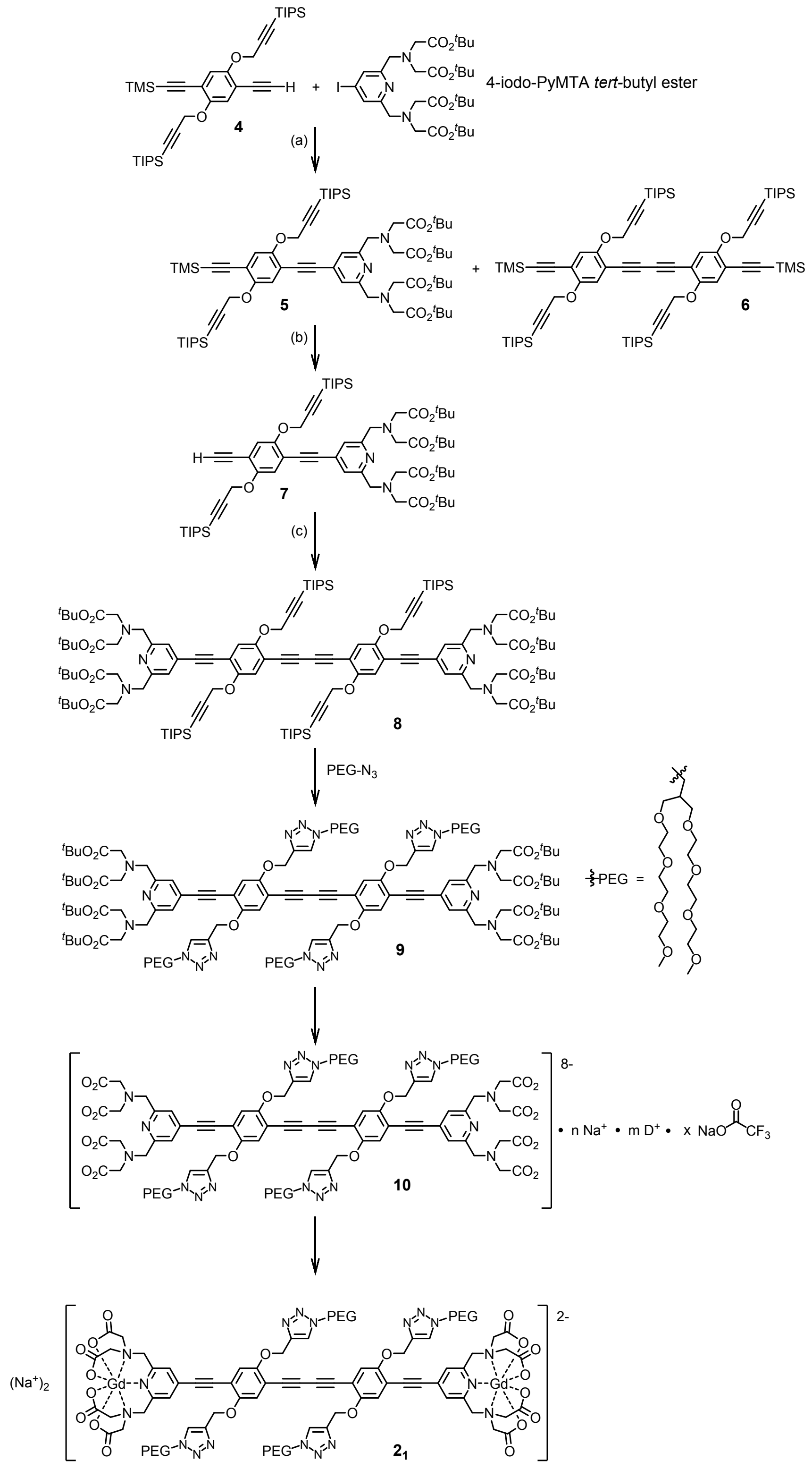
Syntheses of the Gd-rulers 2_1 and 2_2

The final step in the spacer assembly was the oxidative alkyne dimerization (Glaser coupling) to obtain the butadiynes **8** and **14**. As we learned during the project, under the conditions of this reaction, i.e. in case of simultaneous presence of copper ions and oxygen, the aminomethyl substituent at the pyridine is converted to a formyl group.[3] This side reaction is slow and only a trace of the corresponding product was detected to accompany the butadiyne **8**. This was removed through chromatography.

In the experiment in which butadiyne **14** was obtained, the reaction mixture was poor in oxygen because an alkynyl-aryl cross coupling reaction had been intended. Furthermore, the reaction mixture was treated with metal ion scavenger before it was exposed to air. For the reason of the treatment with metal scavenger, see our publication on other Gd-rulers.[3] ^1H NMR spectra give no indication that the above mentioned side reaction had occured.

For the assembly of Gd-ruler $\mathbf{2_1}$ the *tert*-butyl ester of 4-iodo-PyMTA was used and hydrolysed through treatment with trifluoroacetic acid. Nowadays, we would use either the ethyl ester or hydrolyze the *tert*-butyl ester under basic conditions[2] because trifluoroacetic acid harms the spacer backbone in a way not yet identified.[3]

Synthesis of Gd-ruler 2$_1$



TMS protected alkyne 5. The procedure reported for a structurally closely related compound[3] (oligoPPE **18**$_1$ in reference 3) was applied. A solution of alkyne **4**[3] (436 mg, 704 μmol) and 4-iodo-PyMTA *tert*-butyl ester (462 mg, 642 μmol) in THF (8 mL) and piperidine (3.5 mL) was degassed through three freeze−pump−thaw cycles. The solution was brought to room temperature. Then, $PdCl_2(PPh_3)_2$ (3.25 mg, 4.63 µmol) and CuI (1.99 mg, 10.5 µmol) were added, and the reaction mixture was stirred at room temperature. Shortly after the addition of the catalysts, a colorless precipitate formed. After stirring at room temperature for 20 h, 2-methylbut-3-yn-2-ol (18.7 µL, 193 µmol) was added, and the suspension was stirred at room temperature for another 22 h. All volatiles were evaporated. The components of the residual mixture of a yellow viscous liquid and a colorless solid were separated by column chromatography (3.5 cm × 35 cm). Eluting first with pentane/Et_2O 2:1 gave butadiyne **6** (117 mg, 27%; R_f (pentane/Et_2O 2:1) = 0.95; R_f (pentane/Et_2O 1:1) = 0.97). Then, the eluent was changed to pentane/Et_2O 1:1, and trimethylsilyl (TMS) protected alkyne **5** (593 mg, 70%; R_f (pentane/Et_2O 2:1) = 0.35; R_f (pentane/Et_2O 2:1) = 0.78) was obtained as a yellow viscous liquid. Analytical data of TMS protected alkyne **5**: ^1H NMR (500 MHz, CD_2Cl_2): δ = 7.55 (s, 2H, $H_{pyridine}$), 7.27 and 7.21 (2s, 1H each, $H_{benzene}$), 4.82 and 4.78 (2s, 2H each, *benzene*-$OC\underline{H_2}$), 3.97 (s, 4H, *pyridine*-CH_2), 3.45 (s, 8H, $C\underline{H_2}CO_2{}^tBu$), 1.45 (s, 36H, ^tBu), 1.056 and 1.050 (2s, 21H each, TIPS), 0.26 (s, 19H, TMS). ^{13}C NMR (125 MHz, CD_2Cl_2): δ = 170.7 (C=O), 159.6 ($\underline{C}_{pyridine}CH_2$), 153.0 and 152.9 ($C_{benzene}O$), 132.3 ($\underline{C}_{pyridine}C{\equiv}C$), 123.1 ($C_{pyridine}H$), 119.6 and 119.4 ($C_{benzene}H$), 115.2 and 113.8 ($\underline{C}_{benzene}C{\equiv}C$), 102.0, 101.9, 101.2, 100.7, 93.4, 90.7, 90.4, and 89.2 ($\underline{C}{\equiv}\underline{C}$), 81.2 ($O\underline{C}Me_3$), 60.0 (*pyridine*-$\underline{C}H_2$), 58.4 and 58.3 (*benzene*-$O\underline{C}H_2$), 56.1 ($\underline{C}H_2CO_2{}^tBu$), 28.3 ($OC\underline{Me_3}$), 18.71 and 18.70 ($CH\underline{Me_2}$), 11.49 and 11.48 ($\underline{C}HMe_2$), 0.0 ($Si\underline{Me_3}$). MS (ESI): m/z = 1210.7 $[M + H]^+$, 1232.7 $[M + Na]^+$, 1248.6 $[M + K]^+$. Elemental analysis calcd. (%) for $C_{68}H_{107}N_3O_{10}Si_3$: C, 67.45; H, 8.91; N, 3.47; found: C, 66.97; H, 9.09; N, 3.53. Analytical data of butadiyne **6**: ^1H NMR (500 MHz, CD_2Cl_2): δ = 7.23 and 7.19 (2s, 2H each, H_{Ar}), 4.80 and 4.76 (2s, 4H each, CH_2), 1.06 and 1.05 (2s, 42H each, TIPS), 0.25 (s, 18H, TMS). MS (ESI): m/z = 1258.0 $[M + Na]^+$.

Alkyne 7. A solution of *n*-butyllithium in hexanes (1.6 M, 80 µL, 128 µmol) was slowly added to a solution of TMS protected alkyne **5** (393 mg, 325 µmol) in MeOH (8.0 mL) at room temperature. After stirring for 1 h at room temperature the yellow reaction solution was added to a mixture of Et_2O (10 mL) and H_2O (8 mL). The organic phase was separated and the aqueous phase was extracted with Et_2O (2 × 5 mL). To the combined organic phases H_2O (5 mL) and then MeOH (5 mL) were added and the phases were separated. Removal of the solvents from the organic phase gave an orange viscous oil (401 mg). Column chromatography (2 cm × 9 cm, pentane/Et_2O 1:1) of the orange viscous oil (305 mg) gave alkyne **7** (268 mg, 96%) as a pale yellow oil. ^1H NMR (500 MHz, CD_2Cl_2): δ = 7.56 (s, 2H, $H_{pyridine}$), 7.31 and 7.27 (2s, 1H each, $H_{benzene}$), 4.83 and 4.80 (2s, 2H each, *benzene*-$OC\underline{H_2}$), 3.98 (s, 4H, *pyridine*-$C\underline{H_2}$), 3.45 (s, 8H, $C\underline{H_2}CO_2{}^tBu$), 3.41 (s, 1H, $C{\equiv}CH$), 1.45 (s, 36H, ^tBu), 1.04 (s, 42H, TIPS). ^{13}C NMR (125 MHz, CD_2Cl_2): δ = 170.7 (C=O), 159.6 ($\underline{C}_{pyridine}CH_2$), 153.2 and 152.9 ($C_{benzene}O$), 132.2 ($\underline{C}_{pyridine}C{\equiv}C$), 123.1 ($\underline{C}_{pyridine}H$), 119.8 and 119.2 ($C_{benzene}H$), 114.2 and 114.0 ($\underline{C}_{benzene}C{\equiv}C$), 101.9, 101.7, 93.4, 90.7, 90.6, 89.0, 83.3 and 79.7 ($\underline{C}{\equiv}\underline{C}$), 81.1 ($O\underline{C}Me_3$), 60.0 (*pyridine*-$\underline{C}H_2$), 58.3 (*benzene*-$O\underline{C}H_2$), 56.1 ($\underline{C}H_2CO_2{}^tBu$), 28.3 ($OC\underline{Me_3}$), 18.7 ($CH\underline{Me_2}$), 11.5 ($\underline{C}HMe_2$). Accurate MS (ESI): m/z calcd. for $[M + Na]^+$ $C_{65}H_{99}N_3O_{10}Si_2Na^+$: = 1160.67612; found 1160.67664. Elemental analysis calcd. (%) for $C_{65}H_{99}N_3O_{10}Si_2$: C, 68.56; H, 8.76; N, 3.69; found: C, 68.38; H, 8.95; N, 3.77.

Butadiyne 8. The reaction was performed under ambient atmosphere in an open reaction flask. A solution of alkyne **7** (246 mg, 216 µmol), $PdCl_2(PPh_3)_2$ (763 µg, 1.09 µmol), CuI (1.34 mg, 7.03

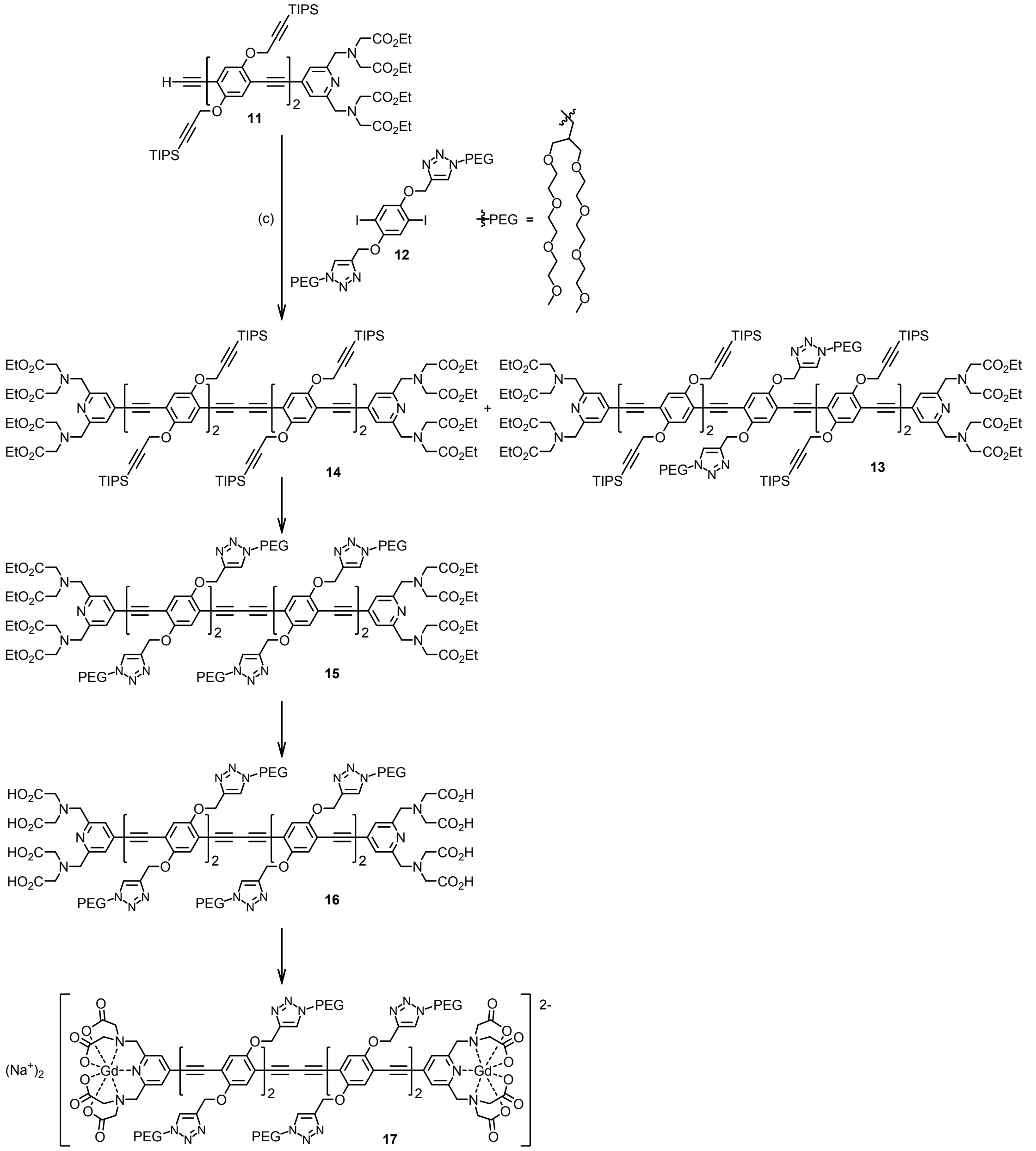
μmol) in $^{i}Pr_2NH$ (1.1 mL) and THF (5 mL) was stirred in air at room temperature for 69 h. A mixture of Et_2O (10 mL) and H_2O (10 mL) was added. The organic phase was separated and the aqueous phase was extracted with Et_2O (3 × 5 mL). The combined organic phases were washed with H_2O (2 × 5 mL) and the solvents were removed. Column chromatography (3 cm × 28 cm, pentane/Et_2O 1:1) of the residual brown oil gave butadiyne **8** (154 mg, 63%). ^1H NMR (500 MHz, CD_2Cl_2): δ = 7.57 (s, 4H, $H_{\textit{pyridine}}$), 7.33 and 7.30 (2s, 2H each, $H_{\textit{benzene}}$), 4.84 (s, 8H each, *benzene*-$OC\underline{H}_2$), 3.98 (s, 8H, *pyridine*-$C\underline{H}_2$), 3.46 (s, 16H, $C\underline{H}_2CO_2{}^{t}Bu$), 1.46 (s, 72H, ^{t}Bu), 1.06 and 1.04 (2s, 42H each, TIPS). ^{13}C NMR (125 MHz, CD_2Cl_2): δ = 170.7 (C=O), 159.7 ($\underline{C}_{\textit{pyridine}}CH_2$), 154.1 and 152.8 ($C_{\textit{benzene}}O$), 132.2 ($\underline{C}_{\textit{pyridine}}C{\equiv}C$), 123.1 ($\underline{C}_{\textit{pyridine}}H$), 119.8 and 119.1 ($C_{\textit{benzene}}H$), 114.9 and 113.6 ($\underline{C}_{\textit{benzene}}C{\equiv}C$), 101.9, 101.7, 94.1, 90.9, 89.1, 79.8 and 79.5 ($\underline{C}{\equiv}\underline{C}$; the signal at 90.9 ppm has about double the intensity as the other $\underline{C}{\equiv}\underline{C}$ signals.), 81.2 ($O\underline{C}Me_3$), 60.0 (*pyridine*-$\underline{C}H_2$), 58.4 and 58.3 (*benzene*-$O\underline{C}H_2$), 56.1 ($\underline{C}H_2CO_2{}^{t}Bu$), 28.3 ($OC\underline{Me}_3$), 18.7 ($CH\underline{Me}_2$), 11.51 and 11.48 ($\underline{C}HMe_2$). MS (ESI): *m/z* = 2296.6 $[M + Na]^+$, 2274.6 $[M + H]^+$, 1159.7 $[M + 2Na]^{2+}$, 1148.8 $[M + Na + H]^{2+}$, 1137.8 $[M + 2H]^{2+}$. Accurate MS (ESI): *m/z* calcd. for $[M + Na]^+$ $C_{130}H_{196}N_6O_{20}Si_4Na^+$: = 2296.34737; found 2296.34948. Elemental analysis calcd. (%) for $C_{65}H_{99}N_3O_{10}Si_2$: C, 68.62; H, 8.68; N, 3.69; found: C, 68.76; H, 8.96; N, 3.77.

Butadiyne 9. Butadiyne **8** (148 mg, 65 μmol) was dissolved in THF (4 mL). A solution of Bu_4NF in THF (1.0 M, 390 μL, 390 μmol) was added upon which the reaction solution changed its color immediately from yellow to brown. The solution was stirred at room temperature for 30 min. A mixture of CH_2Cl_2 (10 mL) and H_2O (10 mL) was added. The organic phase was separated and the aqueous phase was extracted with CH_2Cl_2 (3 × 3 mL). The combined organic phases were filtered through silica gel (2 cm × 3 cm, rinsing with THF/CH_2Cl_2 1:4). Solvent removal gave a yellow viscous oil (151 mg) to which PEG-N_3[3] (141 mg, 333 μmol) and then MeOH (10 mL) were added. This solution was degassed through three freeze-pump-thaw cycles. Degassed aqueous solutions of sodium (*L*)-ascorbate (7.65 mg in 200 μL H_2O, 38.6 μmol) and $CuSO_4{\bullet}5H_2O$ (1.68 mg in 200 μL H_2O, 6.71 μmol) were added successively. A small amount of precipitate formed upon addition of the sodium (*L*)-ascorbate solution. The suspension was stirred at room temperature for 12 d. The reaction was monitored using ^1H NMR spectroscopy. Solvent removal from the reaction mixture gave a viscous brown oil. It was dissolved in CH_2Cl_2 (2 mL) and the resulting solution was washed with H_2O (4 × 2 mL). The washing was performed in a centrifuge tube: The CH_2Cl_2-phase and the aqueous phase were mixed well. Centrifugation of the resulting yellow emulsion at 10 × 10^3 rpm for 2 min separated the mixture into two phases, a yellow CH_2Cl_2 phase and a colorless aqueous phase. The aqueous phase was removed with the help of a glass pipette. After washing, the solvent of the CH_2Cl_2 phase was removed giving a mixture (269 mg) of butadiyne **9**, PEG-N_3, and TIPS-OH as a brown viscous oil. Analytical data of butadiyne **9**: ^1H NMR (500 MHz, CD_2Cl_2): δ = 7.95 and 7.92 (2s, 2H each, $H_{\textit{triazole}}$), 7.55 (s, 4H, $H_{\textit{pyridine}}$), 7.26 and 7.25 (2s, 2H each, $H_{\textit{benzene}}$), 5.27 and 5.24 (2s, 4H each, *benzene*-$OC\underline{H}_2$), 4.51 and 4.49 (2d overlap to a triplett, ^3J = 6.2 Hz, 8H, $N_{\textit{triazole}}C\underline{H}_2CH$), 3.97 (s, 8H, *pyridine*-$C\underline{H}_2$), 3.58 (s, 16H, $C\underline{H}_2CO_2{}^{t}Bu$), 3.58 – 3.41 (m, 96 H, $OC\underline{H}_2C\underline{H}_2$), 3.38 – 3.33 (m, 16H, $CHC\underline{H}_2O$), 3.283 and 3.280 (2s, 12H each, OCH_3), 2.47 (m, 4H, $C\underline{H}CH_2O$), 1.44 (s, 72H, ^{t}Bu). MS (ESI): *m/z* = 1672.0 $[M + 2H]^{2+}$, 1683.0 $[M + H + Na]^{2+}$, 1694.0 $[M + 2Na]^{2+}$. Accurate MS (ESI): *m/z* calcd. for $[M + 2H]^{2+}$ $C_{166}H_{264}N_{18}O_{52}H_2{}^{2+}$: = 1671.93563; found 1671.93132.

Ruler precursor 10. The reaction was performed under ambient atmosphere. Material (52 mg, containing ca. 12.6 µmol of butadiyne **9**) that had been obtained through the desilylation of butadiyne **8** and the reaction of the desilylation product with PEG-N_3 was dissolved in CH_2Cl_2 (0.8 mL). To the solution trifluoroacetic acid (TFA) (200 µL, 2.61 mmol) was added and the yellow solution was stirred at room temperature for 4 h. The volatile components were removed at room temperature lowering the pressure down to 10 mbar giving a brown viscous oil (55 mg). The ^{1}H NMR spectrum of the brown viscous oil showed an incomplete reaction. This brown viscous oil was dissolved in TFA (1 mL) and the solution was stirred at room temperature for 18 h. The volatile components were removed at room temperature lowering the pressure down to 10 mbar giving a brown viscous oil (72 mg). The brown viscous oil was dissolved in D_2O (3 mL). This aqueous solution was washed with Et_2O (4 × 5 mL). Removal of the solvents from the aqueous solution gave a brown viscous oil (51 mg). The brown viscous oil was dissolved in D_2O (3 mL). A solution of NaOD in D_2O (0.1 M, 600 µL, 60 µmol) was added. The aqueous solution was washed with CH_2Cl_2 (3 × 5 mL) using the following procedure: The aqueous solution was transferred into a centrifuge tube. CH_2Cl_2 was added and the two phases were mixed well. The resulting emulsion was centrifuged at 5000 rpm for 5 min. The CH_2Cl_2 phase was removed using a syringe. Removal of the solvents from the aqueous phase gave a brown solid (37 mg). The brown solid was suspended in CD_3OD (1 mL) and the undissolved material was removed through centrifugation. Through removal of the solvent from the yellow solution ruler precursor **10** • n Na^+ • m D^+ • x $Na(O_2CCF_3)$ (36 mg, 86% yield) was obtained as a yellow solid. The content of the ruler precursor **10** was determined by quantitative NMR spectroscopy[2] to be 86.6 wt.%. ^{1}H NMR (500 MHz, CD_3OD): δ = 8.23 (s, 4H, $H_{triazole}$), 7.37 (s, 4H, $H_{pyridine}$), 7.39 and 7.34 (2s, 2H each, $H_{benzene}$), 5.32 and 5.29 (2s, 4H each, *benzene*-OC$\underline{H_2}$), 4.57 (d, ^3J = 5.8 Hz, 8H, $N_{triazole}$C$\underline{H_2}$CH), 3.97 (br s, 8H, *pyridine*-C$\underline{H_2}$), 3.67 – 3.44 (m, 122H, C$\underline{H_2}$CO$_2$^{t}Bu and OC$\underline{H_2}$C$\underline{H_2}$), 3.42 – 3.33 (m, 16H, CHC$\underline{H_2}$O), 3.292 and 3.289 (2s, 12H each, OCH_3), 2.47 (m, 4H, C$\underline{H}$CH$_2$O).

Gd-ruler $\mathbf{2_1}$. The reaction was performed under ambient atmosphere. Ruler precursor **10** • n Na^+ • m D^+ • x $Na(O_2CCF_3)$ (36 mg, containing 10.8 µmol Ruler precursor **10**) was dissolved in D_2O (0.8 mL). A solution of $GdCl_3 \cdot 6\ H_2O$ in D_2O (50 mM, 411 µL, 20.55 µmol) was added. A solution of NaOD in D_2O (0.1 M) was added as much as was needed to rise the pD to 7.0. The solution was diluted with D_2O to a total volume of 2000 µL to obtain a 5.40 mM solution of Gd-ruler $\mathbf{2_1}$ in D_2O. 350 µL of this solution was diluted with D_2O to a total volume of 630 µL to obtain a 3.0 mM solution of Gd-ruler $\mathbf{2_1}$ in D_2O. The pD value of the solution was ca. 7.0. Accurate MS (ESI) of Gd-ruler $\mathbf{2_1}$: *m/z* calcd. for $[M - 2Na]^{2-}$ $C_{134}H_{192}Gd_2N_{18}O_{52}^{2-}$: 1600.57130; found 1600.56714.

Synthesis of Gd-ruler 2_2



Butadiyne 14. Butadiyne **14** was not synthesized on purpose but obtained as a side product when preparing compound **13**[3] (oligoPPE **20_2** in reference 3). A solution of alkyne **11** (146 mg, 94 µmol), diiodobenzene **12** (55 mg, 43 µmol), and $^{i}Pr_2NH$ (1.5 µL) in THF (6 mL) was degassed through three freeze-pump-thaw cycles. The solution was brought to room temperature. Then $PdCl_2(PPh_3)_2$ (806 µg, 1.15 µmol) and CuI (341 µg, 1.79 µmol) were added and the reaction mixture was stirred at room temperature for 20 h. Thin layer chromatography (TLC) (CH_2Cl_2/EtOH 10:1) proved the reaction incomplete. $PdCl_2(PPh_3)_2$ (1.23 mg, 1.60 µmol), CuI (577 µg, 3.03 µmol) and $^{i}Pr_2NH$ (610 µL, not degassed) were added and the solution was stirred at room temperature for 44 h. TLC (CH_2Cl_2/EtOH 10:1) proved the reaction incomplete. The solution was stirred at 50 °C for 22 h, then cooled to room temperature, upon which some precipitate formed. Under argon all volatiles were evaporated and the residue was dissolved in degassed anhydrous CH_2Cl_2 (10 mL). Metal scavenger QuadraPure™ TU (157 mg) was added and the suspension was stirred at room

temperature for 13 h. Metal scavenger QuadraPure^{TM} BzA (5 mg) was added to the suspension. No color change of the metal scavenger QuadraPure^{TM} BzA occurred within 2.5 h of stirring at room temperature, which indicated that there had been no free Cu(I/II) left in solution. The suspension was filtered through a syringe filter (PTFE membrane, 0.45 µm). The solvents of the filtrate were removed. Column chromatography (3.0 cm × 36 cm, $CH_2Cl_2/Et_2O/EtOH$ 10:4:0.5) of the residual yellow-brown oil gave compound **13** (38 mg, 22%; $R_f = 0.33$) as a yellow viscous oil and butadiyne **14** (55 mg, 38%; $R_f = 0.59$) as a yellow viscous oil. Analytical data of butadiyne **14**: ^{1}H NMR (500 MHz, CD_2Cl_2): δ = 7.54 (s, 4H, H$_{pyridine}$), 7.35, 7.32, 7.30, and 7.26 (4s, 2H each, H$_{benzene}$), 4.88, 4.87, and 4.85 (3s, 4H, 8H, and 4H, *benzene*-OC$\underline{H}_2$), 4.15 (q, ^3J = 7.2 Hz, 16H, C$\underline{H}_2$CH$_3$), 4.01 (s, 8H, *pyridine*-CH$_2$), 3.59 (s, 16H, C$\underline{H}_2$CO$_2$Et), 1.26 (t, ^3J = 7.2 Hz, 24H, CH$_2$C$\underline{H}_3$), 1.073, 1.056, 1.053, and 1.049 (4s, 42H each, TIPS). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 171.3 (C=O), 159.2 ($\underline{C}_{pyridine}$CH$_2$), 154.3, 153.1, 152.7, and 152.6 (C$_{benzene}$O), 132.5 ($\underline{C}_{pyridine}$C≡C), 123.4 (C$_{pyridine}$H), 120.5, 120.0, 119.0, and 118.8 (C$_{benzene}$H), 115.9, 115.4, 113.8, and 113.1 ($\underline{C}_{benzene}$C≡C), 102.04, 101.95. 101.89, 101.71, 93.3, 92.2, 91.8, 90.8, 90.7, 89.6, 79.7, and 79.6 ($\underline{C}$≡$\underline{C}$), 60.8 ($\underline{C}$H$_2$Me), 60.3 (*pyridine*-$\underline{C}$H$_2$), 58.66, 58.61, 58.37, and 58.35 (*benzene*-O$\underline{C}$H$_2$), 55.2 ($\underline{C}$H$_2$CO$_2$Et), 18.693 and 18.686 (CH($\underline{C}$H$_3$)$_2$), 14.4 (CH$_2$$\underline{C}H_3$), 11.51, 11.49, and 11.47 ($\underline{C}$HMe$_2$). MS (ESI): *m/z* = 1568.0 [M + 2Na]$^{2+}$, 1557.0 [M + Na + H]$^{2+}$, 1546.0 [M + 2H]$^{2+}$. Accurate MS (ESI): *m/z* calcd. for [M + 2H]$^{2+}$ $C_{178}H_{262}N_6O_{24}Si_8^{2+}$: 1545.88043; found 1545.88245.

Butadiyne 15. Butadiyne **14** (55 mg, 17.8 µmol) was dissolved in THF (4 mL). A solution of Bu_4NF in THF (1.0 M, 178 µL, 178 µmol) was added upon which the reaction solution changed its color immediately from yellow to brown. The solution was stirred at room temperature for 1 h. Afterwards it was filtered through silica gel (1.5 cm × 1.5 cm, rinsing with THF). Solvent removal from the eluate gave a yellow solid (34 mg). This yellow solid and PEG-N$_3$[3] (84 mg, 198 µmol) were dissolved in THF (1.5 mL) and EtOH (1 mL). This solution was degassed through three freeze-pump-thaw cycles. Degassed aqueous solutions of sodium (*L*)-ascorbate (4.58 mg in 120 µL H_2O, 23.1 µmol) and $CuSO_4 \bullet 5H_2O$ (0.1 M, 71 µL, 7.1 µmol) were added successively. A small amount of precipitate formed upon addition of the sodium (*L*)-ascorbate solution. The suspension was heated to 60 °C whereupon the reaction mixture became an orange-brown suspension. The suspension was stirred for 5 d at 60 °C. Then it was cooled to room temperature. Metal scavenger QuadraPure^{TM} TU (175 mg) was added and the suspension was stirred at room temperature for 17 h. Metal scavenger QuadraPure^{TM} BzA (24 mg) was added and the suspension was stirred at room temperature for 3 h. The metal scavenger QuadraPure^{TM} BzA did not change its color, which indicated that there had been no free Cu(I/II) left in solution. The suspension was filtered through silica gel (1.5 cm × 1.5 cm, CH_2Cl_2/EtOH 4:1). Solvent removal from the eluate gave a viscous yellow oil. This oil contained butadiyne **15**, PEG-N$_3$, TIPS-OH and/or TIPS-F, sodium (*L*)-ascorbate, dehydroascorbic acid, and silicone grease. It was dissolved in CH_2Cl_2 (4 mL) and the resulting solution was washed with H_2O (4 × 4 mL). The washing was performed in a centrifuge tube: The CH_2Cl_2 phase and the aqueous phase were mixed well. Centrifugation of the resulting yellow emulsion at 5000 rpm for 5 min separated the mixture into two phases, a yellow CH_2Cl_2 phase and a colorless aqueous phase. The aqueous phase was removed with the help of a glass pipette. After washing, the solvent of the CH_2Cl_2 phase was removed giving a viscous yellow-orange oil (121 mg). ^{1}H NMR spectroscopy revealed that this mixture consisted of butadiyne **15**, PEG-N$_3$, silicone grease, and TIPS-OH. ^{1}H NMR (500 MHz, CD_2Cl_2): Signals assigned to butadiyne **15**: δ = 7.97, 7.94, 7.93, and 7.91 (4s, 2H each, $H_{triazole}$), 7.52 (s, 4H, $H_{pyridine}$), 7.29, 7.28, 7.25, and 7.22 (4s, 2H each, $H_{benzene}$), 5.29, 5.27, 5.23 and 5.20 (4s, 4H each, *benzene*-$OC\underline{H}_2$), 4.52 - 4.42 (m, 16H, $N_{triazole}C\underline{H}_2CH$), 4.12 (q, ^3J = 7.2 Hz, 16H, $C\underline{H}_2CH_3$), 3.98 (s, 8H, *pyridine*-$C\underline{H}_2$), 3.58 (s, 16H, $C\underline{H}_2CO_2Et$), 3.58 – 3.41 (m, 192H, $OC\underline{H}_2C\underline{H}_2$), 3.38 – 3.33 (m, 32H, $CHC\underline{H}_2O$), 3.27 (s, 48H, OCH_3), 2.44 (m, 8H, $C\underline{H}CH_2O$), 1.23 (t, ^3J = 7.2 Hz, 24H, $CH_2C\underline{H}_3$).

Ruler precursor 16. The reaction was performed under ambient atmosphere. Material (121 mg, containing ca. 17.8 µmol of butadiyne **15**) that had been obtained through the desilylation of butadiyne **14** and the reaction of the desilylation product with PEG-N_3 was dissolved in EtOH (0.7 mL) and H_2O (1.5 mL). An aqueous solution of NaOH (2.0 M, 142.2 µL, 288.4 µmol) was added and the solution was stirred at room temperature for 21 h. H_2O (2.8 mL) was added and the solution was washed with CH_2Cl_2 (4 × 4 mL) using the following procedure: The aqueous solution was transferred into a centrifuge tube. CH_2Cl_2 was added and the two phases were mixed well. The resulting emulsion which contained a solid phase was centrifuged at 5000 rpm for 5 min. The mixture separated into three phases - a yellow aqueous phase at the top, a flocculent solid in the middle, and a colorless CH_2Cl_2 phase at the bottom. The CH_2Cl_2 phase was removed using a syringe. The water phase and the flocculent solid were washed three more times with CH_2Cl_2 using the aforementioned procedure. After washing, MeCN (1 mL) was added to dissolve the flocculent solid. Addition of proton exchange resin (126 mg) lowered the pH of the aqueous solution to ca. 3.0. The suspension was filtered through a syringe filter (PVDF membrane, 0.45 µm). Removal of the solvent from the filtrate using freeze-drying and drying the residue over P_4O_{10} at reduced pressure provided ruler precursor **16** (69 mg, 78% over 2 steps starting with butadiyne **14**) as a yellow-orange solid. ^1H NMR (500 MHz, CD_3OD): δ = 8.24, 8.22, 8.16, and 8.15 (4s, 2H each, $H_{triazole}$), 7.53 (s, 4H, $H_{pyridine}$), 7.35, 7.34, and 7.30 (3s, 4H, 2H, and 2H, $H_{benzene}$), 5.33, 5.32, 5.29, and 5.27 (4s, 4H each, *benzene*-$OC\underline{H}_2$), 4.57, 4.56, 4.52, and 4.51(4d, ^3J = 5.8 Hz, 4H each, $N_{triazole}C\underline{H}_2CH$), 4.27 (s, 8H, *pyridine*-$C\underline{H}_2$), 3.71 (s, 16H, $C\underline{H}_2CO_2Et$), 3.64 – 3.44 (m, 192H, $OC\underline{H}_2C\underline{H}_2$), 3.42 – 3.32 (m, 32H, $CHC\underline{H}_2O$), 3.29 (s, 48H, OCH_3), 2.53 – 2.35 (m, 8H, $C\underline{H}CH_2O$).

Gd-ruler $\mathbf{2_2}$. The reaction was performed under ambient atmosphere. Ruler precursor **16** (40 mg, 7.99 µmol) was dissolved in D_2O (500 µL). A solution of $GdCl_3$ in D_2O (100 mM, 155.8 µL, 15.58 µmol) was added. A solution of NaOD in D_2O (1.0 M, 63.93 µL, 63.93 µmol) was added to rise the pD to 8.0. The solution was diluted with D_2O (658.6 µL) to obtain a 5.0 mM solution of Gd-ruler $\mathbf{2_2}$ in D_2O. MS (ESI): *m/z* = 2655.3 $[M - 2Na]^{2-}$. Accurate MS (ESI): *m/z* calcd. for $[M - 2Na + Cl]^{3-}$ $C_{234}H_{356}Gd_2N_{30}O_{88}Cl^{3-}$: 1781.74971; found 1781.74639.

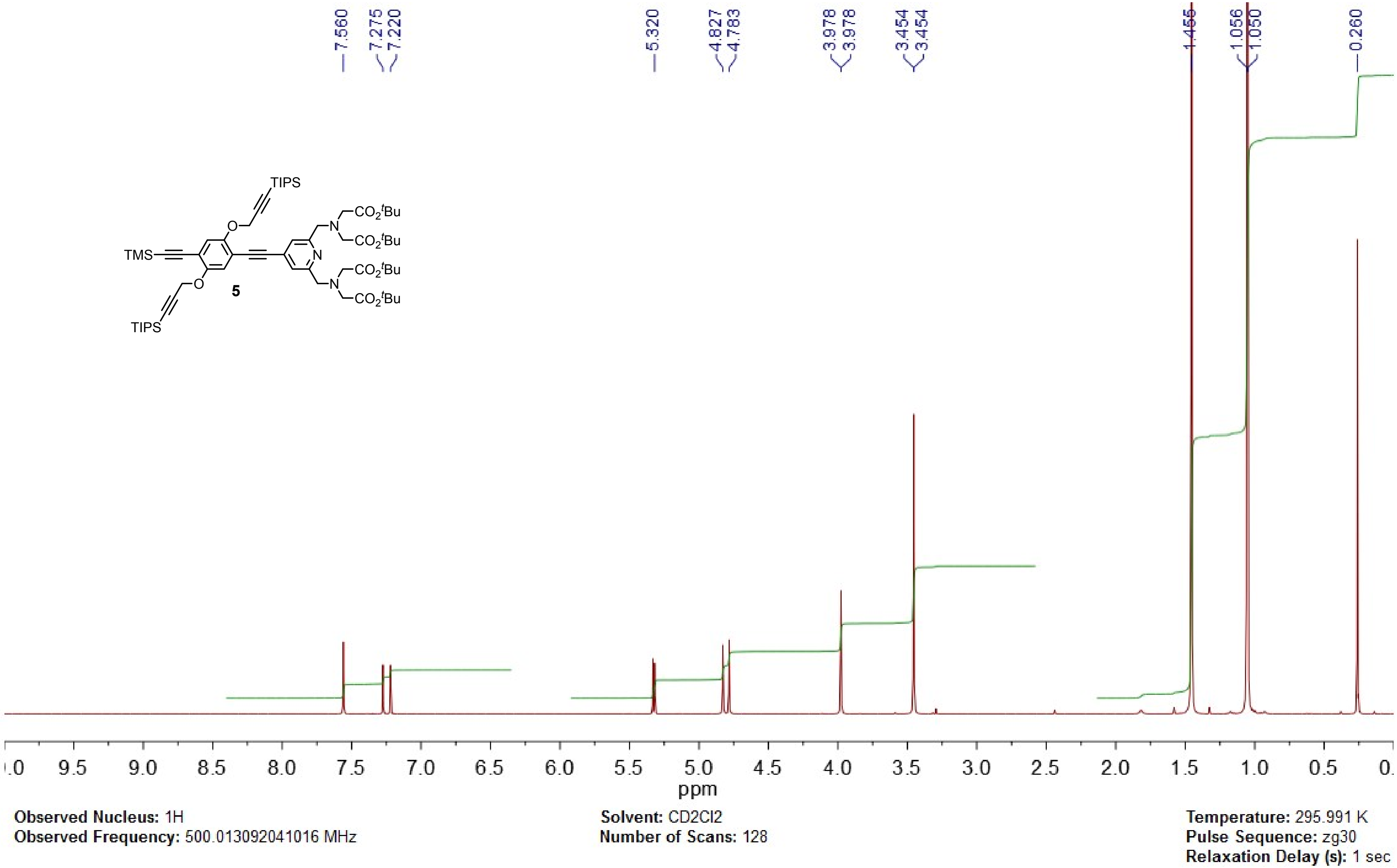
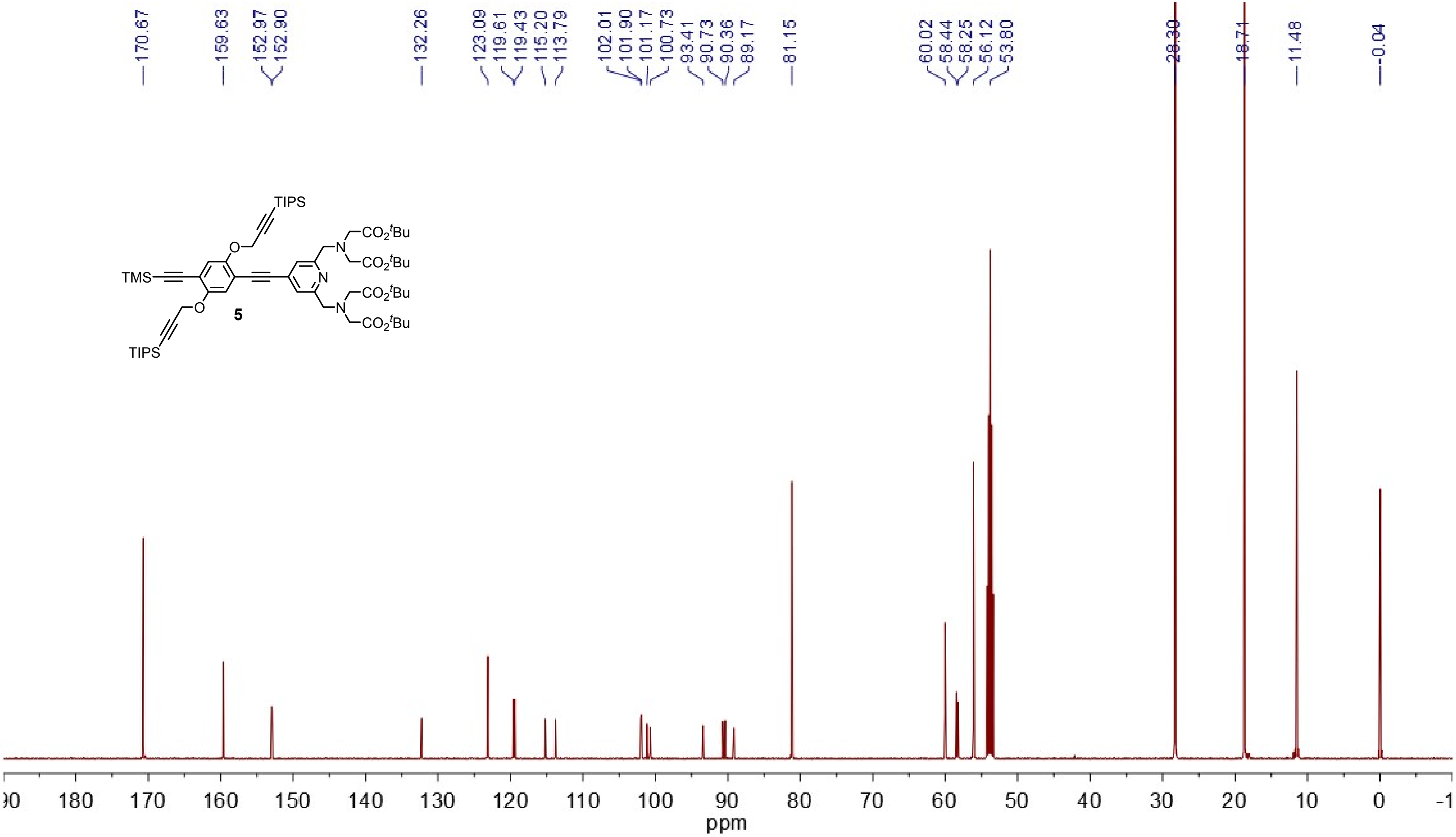


Figure S6. ^{1}H NMR spectrum of TMS protected alkyne **5**.

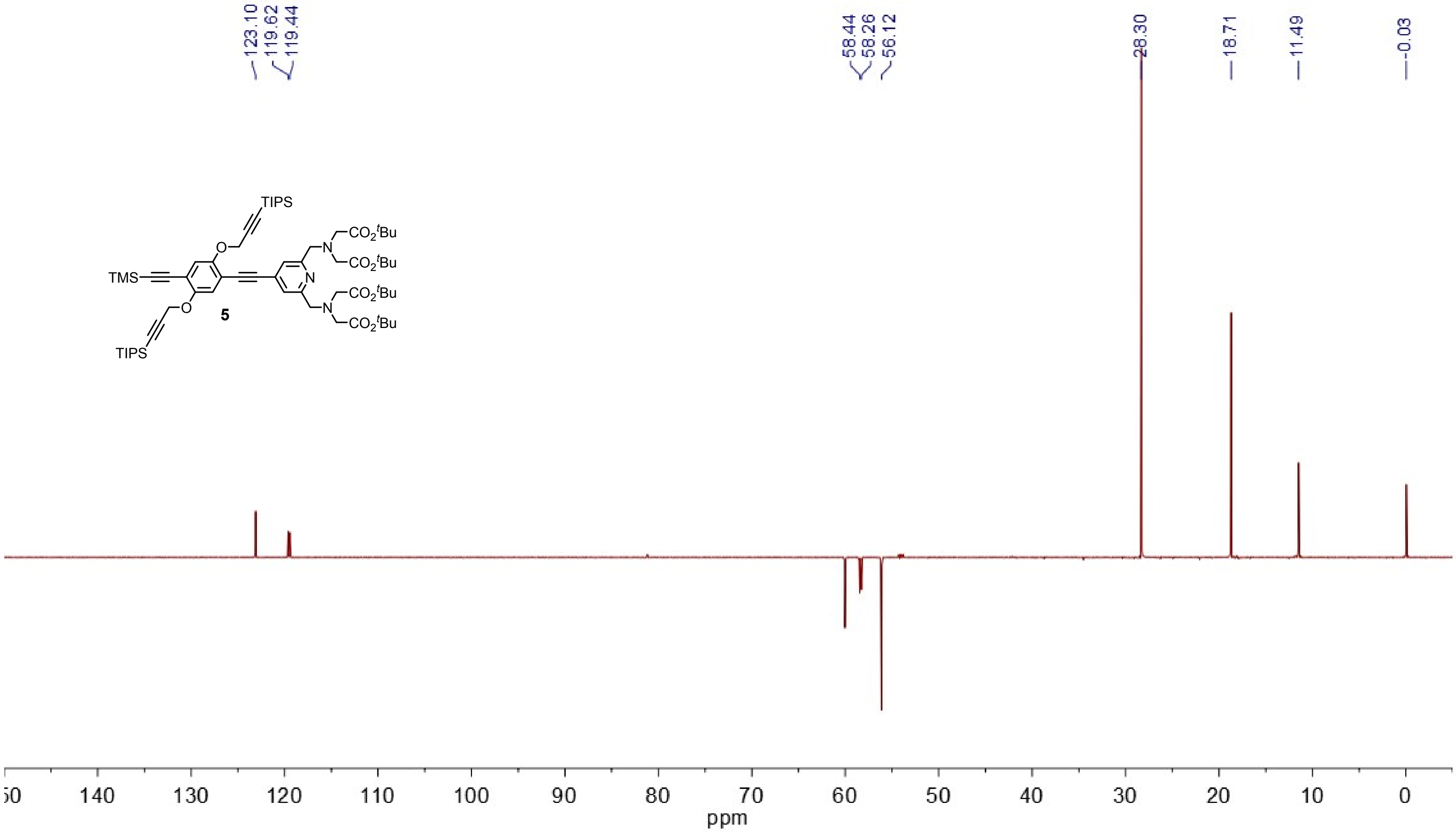


Observed Nucleus: 13C
Observed Frequency: 125.740188598633 MHz

Solvent: CD2Cl2
Number of Scans: 4096

Temperature: 295.991 K
Pulse Sequence: zgpg30
Relaxation Delay (s): 2 sec

Figure S7. ^{13}C NMR spectrum of TMS protected alkyne **5**.



Observed Nucleus: 13C
Observed Frequency: 125.737670898438 MHz

Solvent: CD2Cl2
Number of Scans: 1024

Temperature: 295.991 K
Pulse Sequence: dept135
Relaxation Delay (s): 2 sec

Figure S8. ^{13}C DEPT (135) NMR spectrum of TMS protected alkyne **5**.

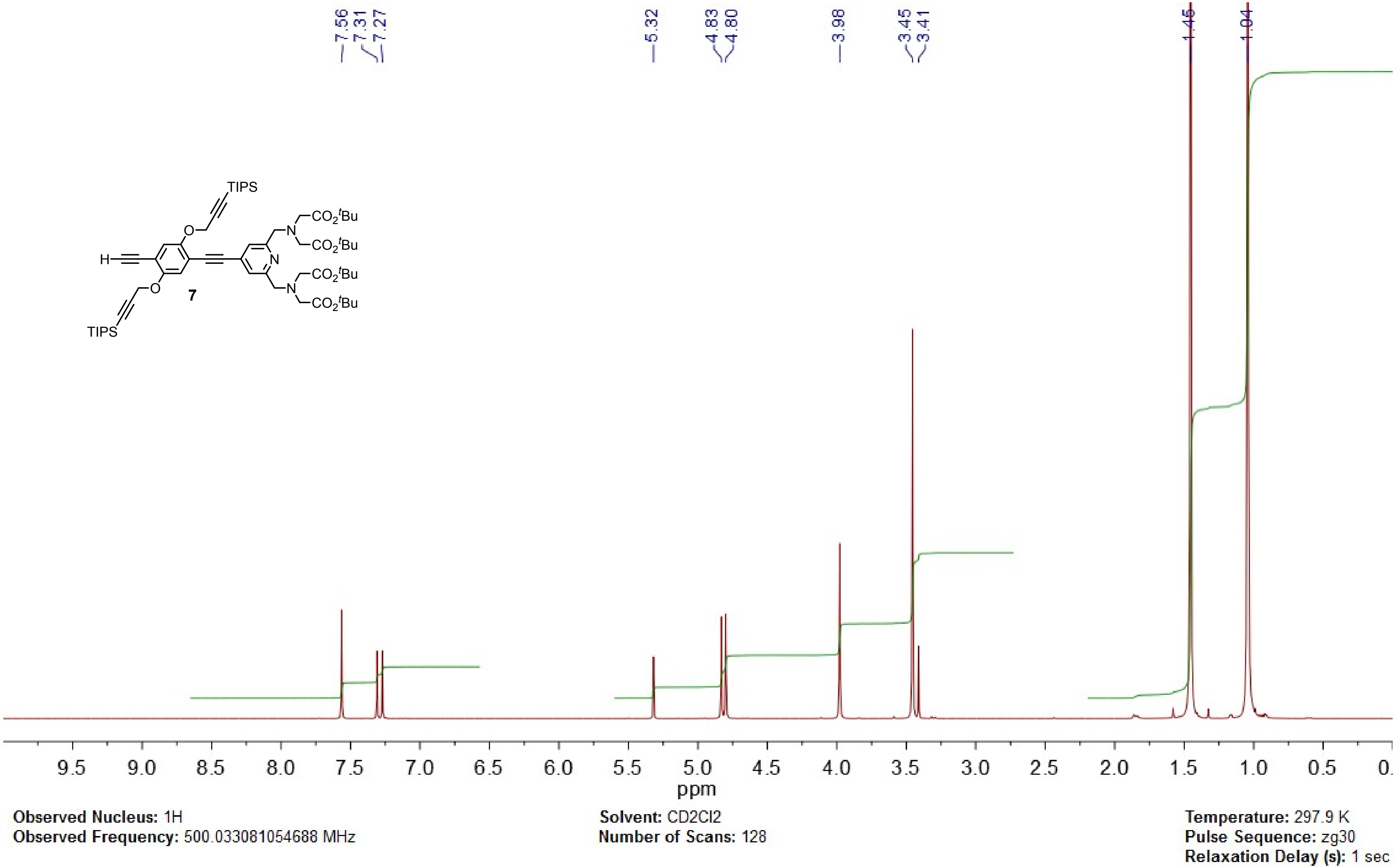


Figure S9. ^{1}H NMR spectrum of alkyne **7**.

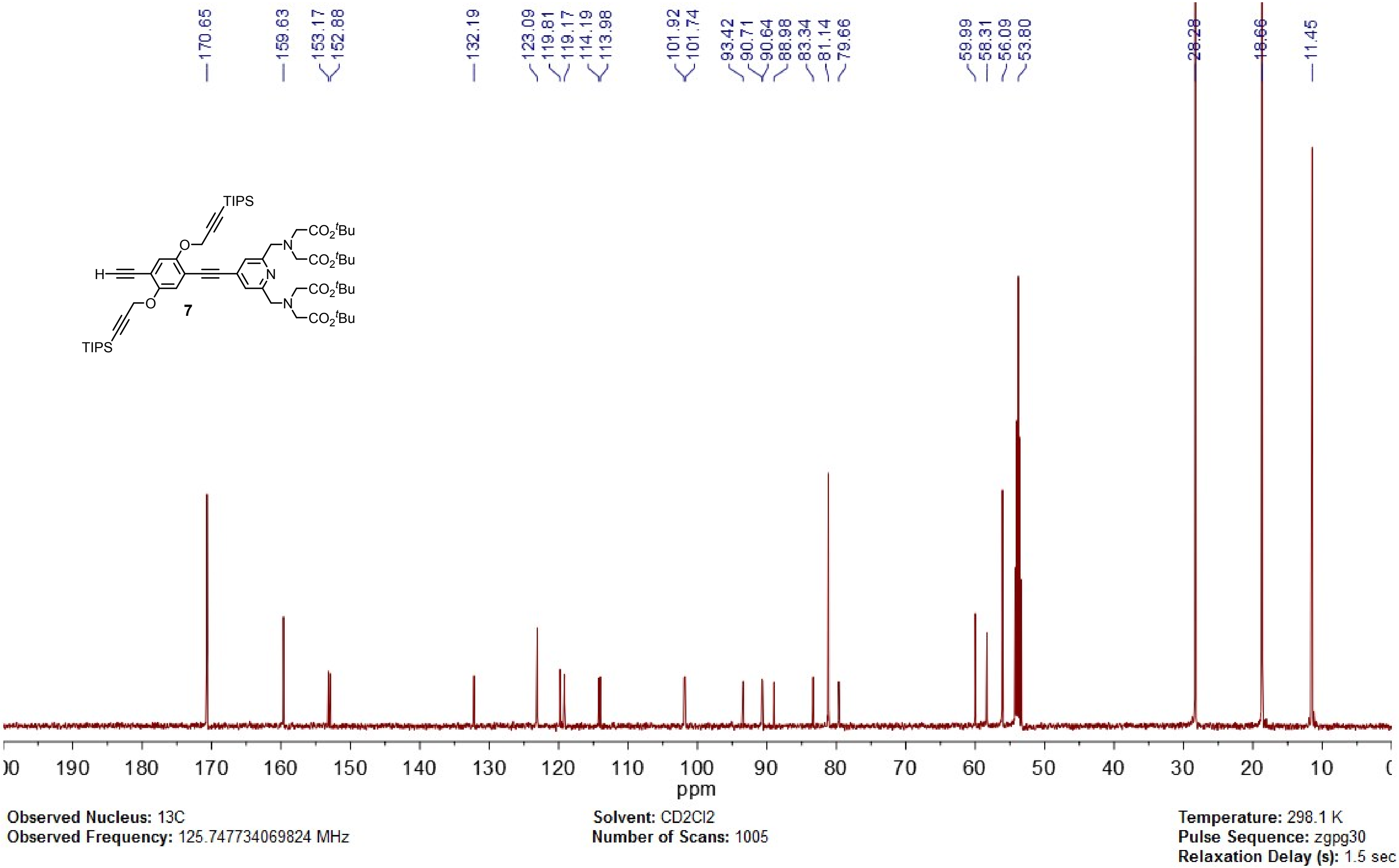
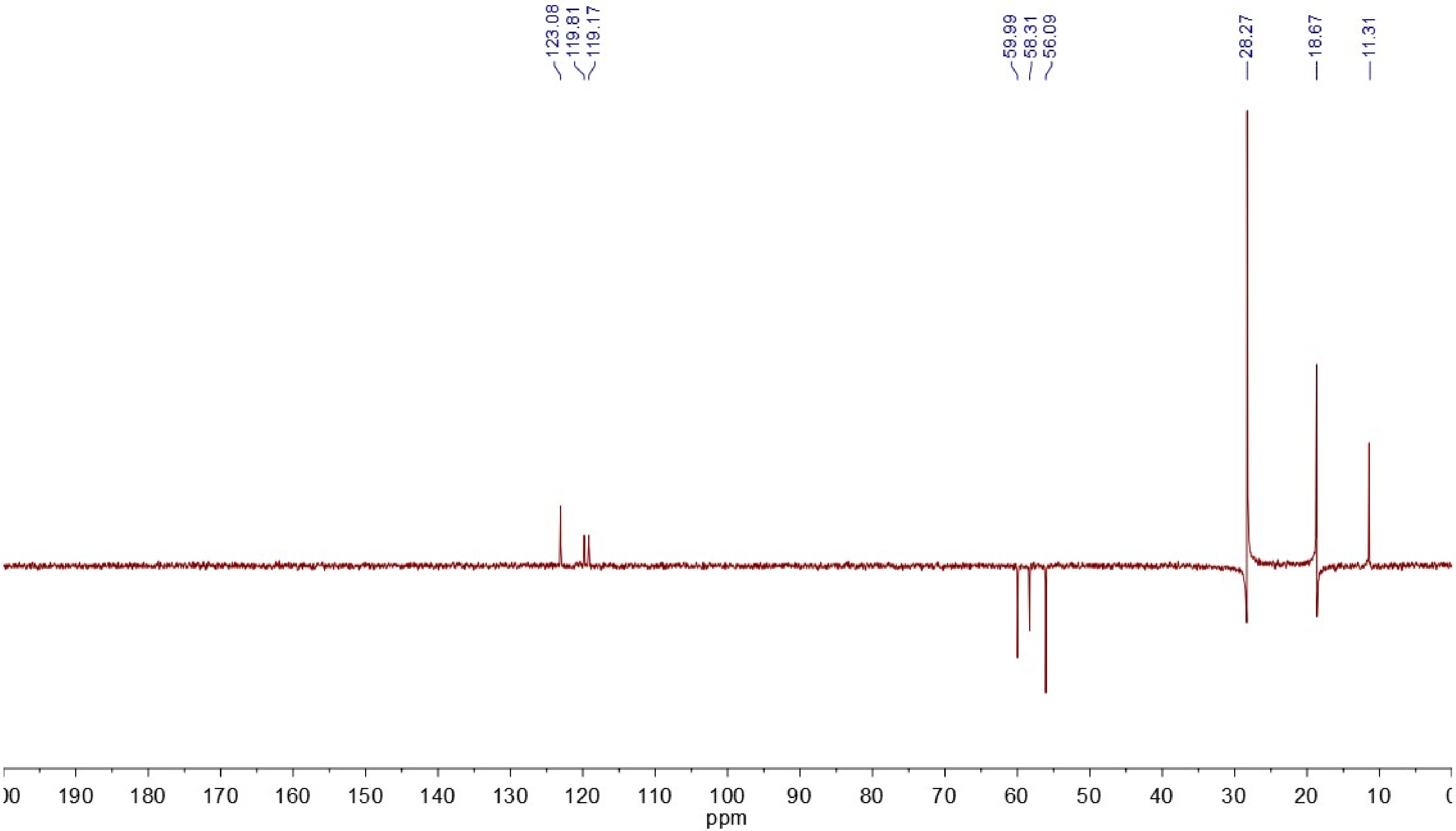


Figure S10. ^{13}C NMR spectrum of alkyne **7**.

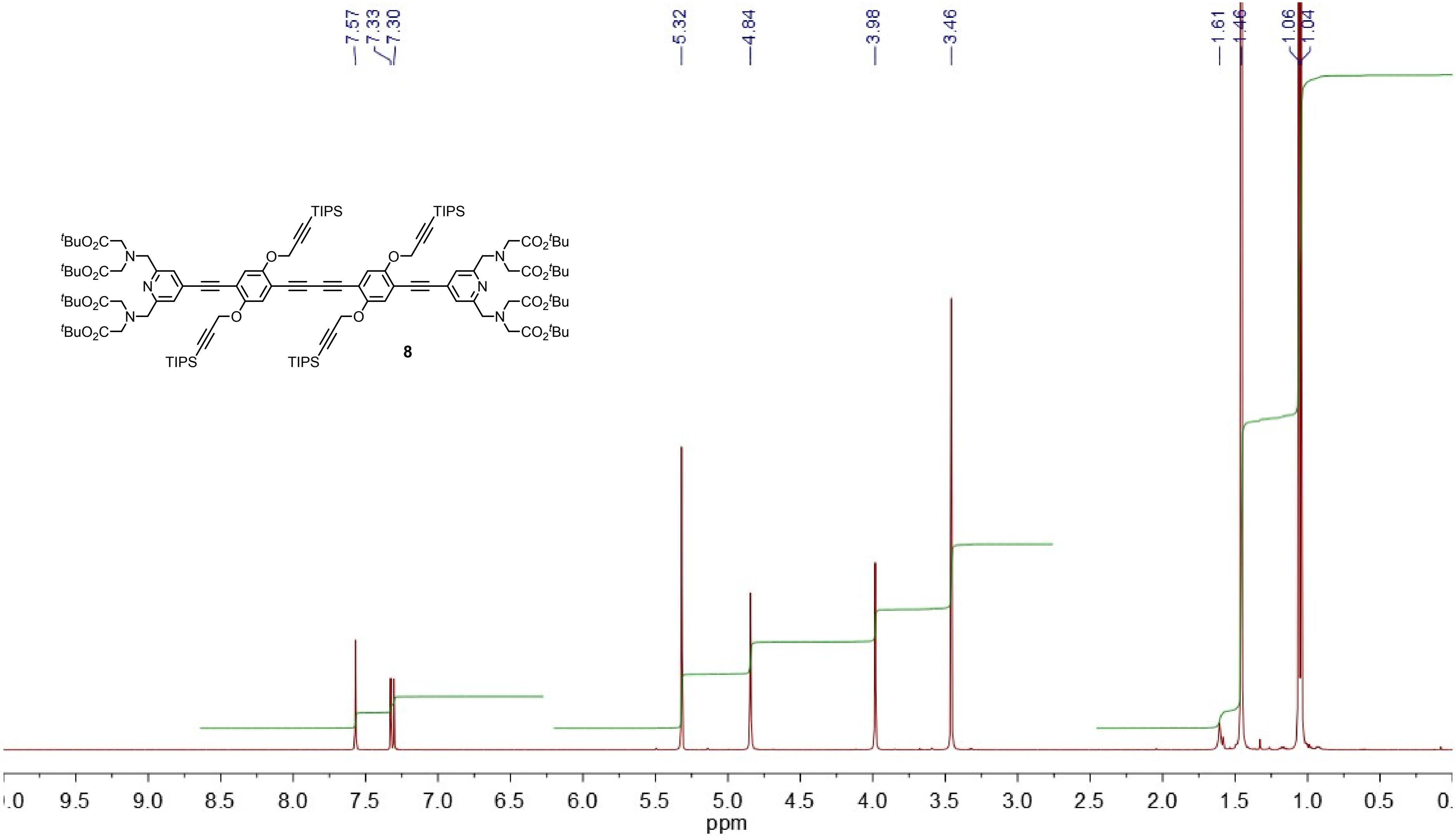


Observed Nucleus: 13C
Observed Frequency: 125.747734069824 MHz

Solvent: CD2Cl2
Number of Scans: 87

Temperature: 298.4 K
Pulse Sequence: dept135
Relaxation Delay (s): 1.5 sec

Figure S11. ^{13}C DEPT 135 NMR spectrum of alkyne **7**.



Observed Nucleus: 1H
Observed Frequency: 500.013092041016 MHz

Solvent: CD2Cl2
Number of Scans: 128

Temperature: 295.991 K
Pulse Sequence: zg30
Relaxation Delay (s): 1 sec

Figure S12. ^{1}H NMR spectrum of butadiyne **8**.

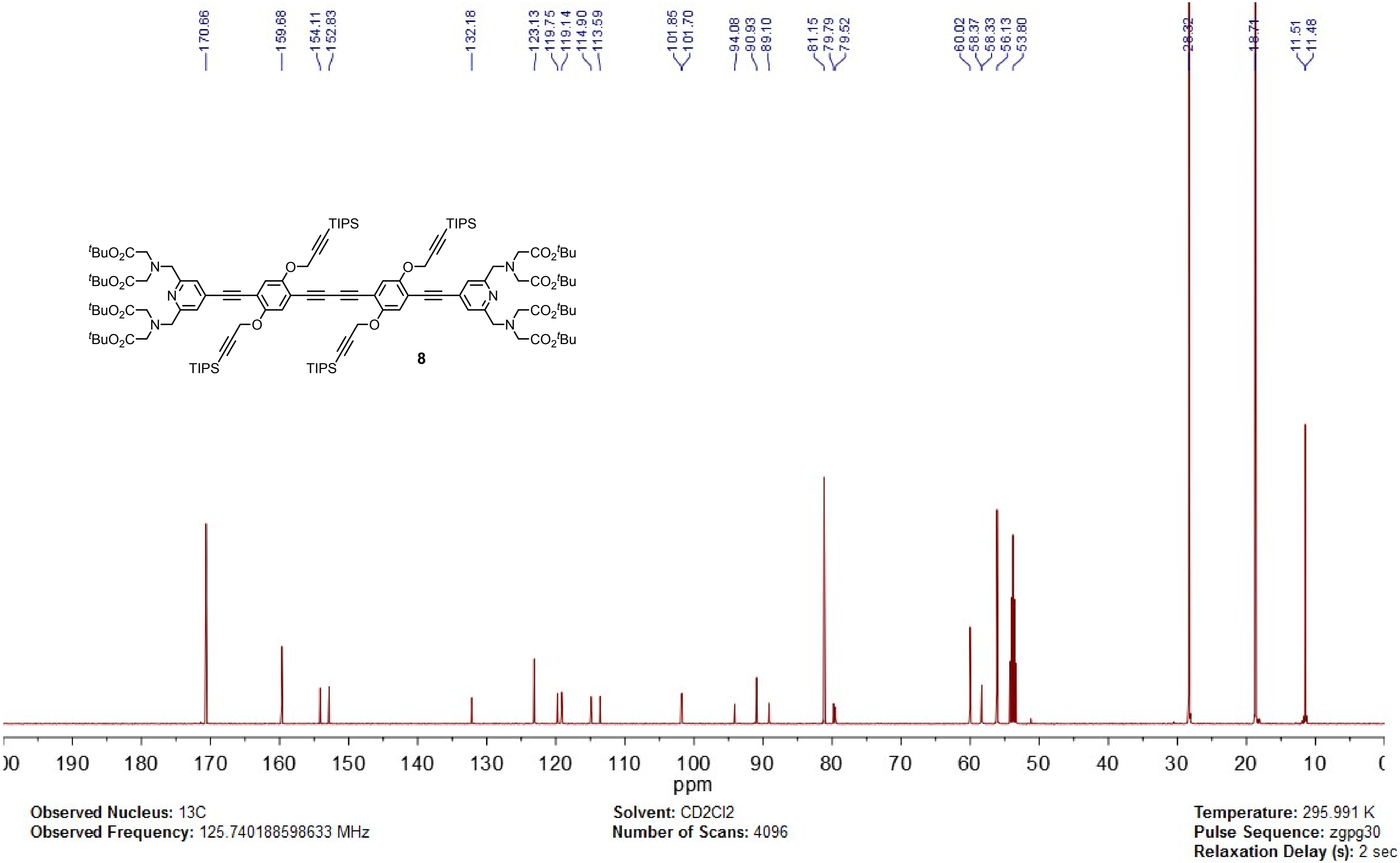
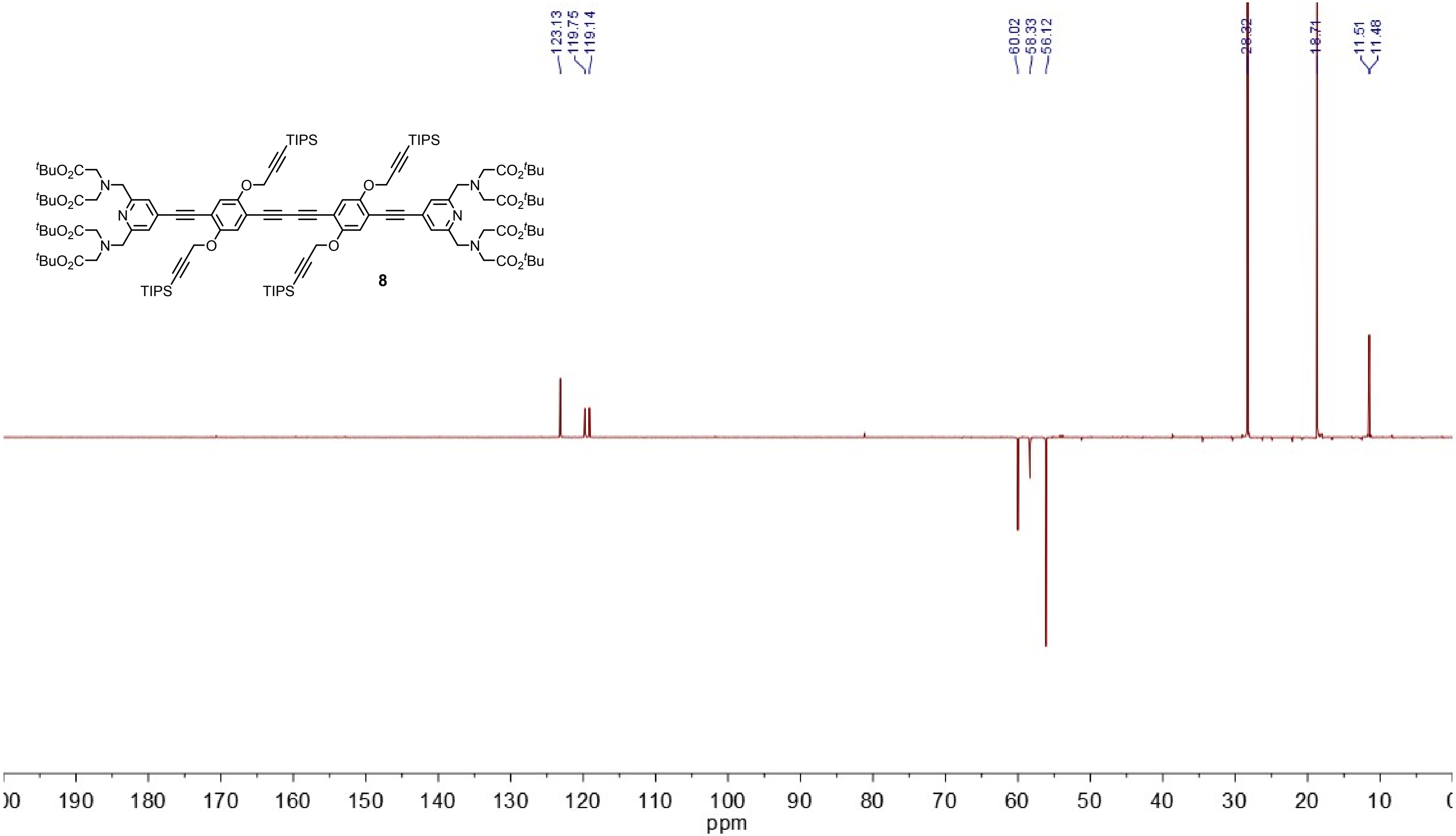


Figure S13. ^{13}C NMR spectrum of butadiyne **8**.

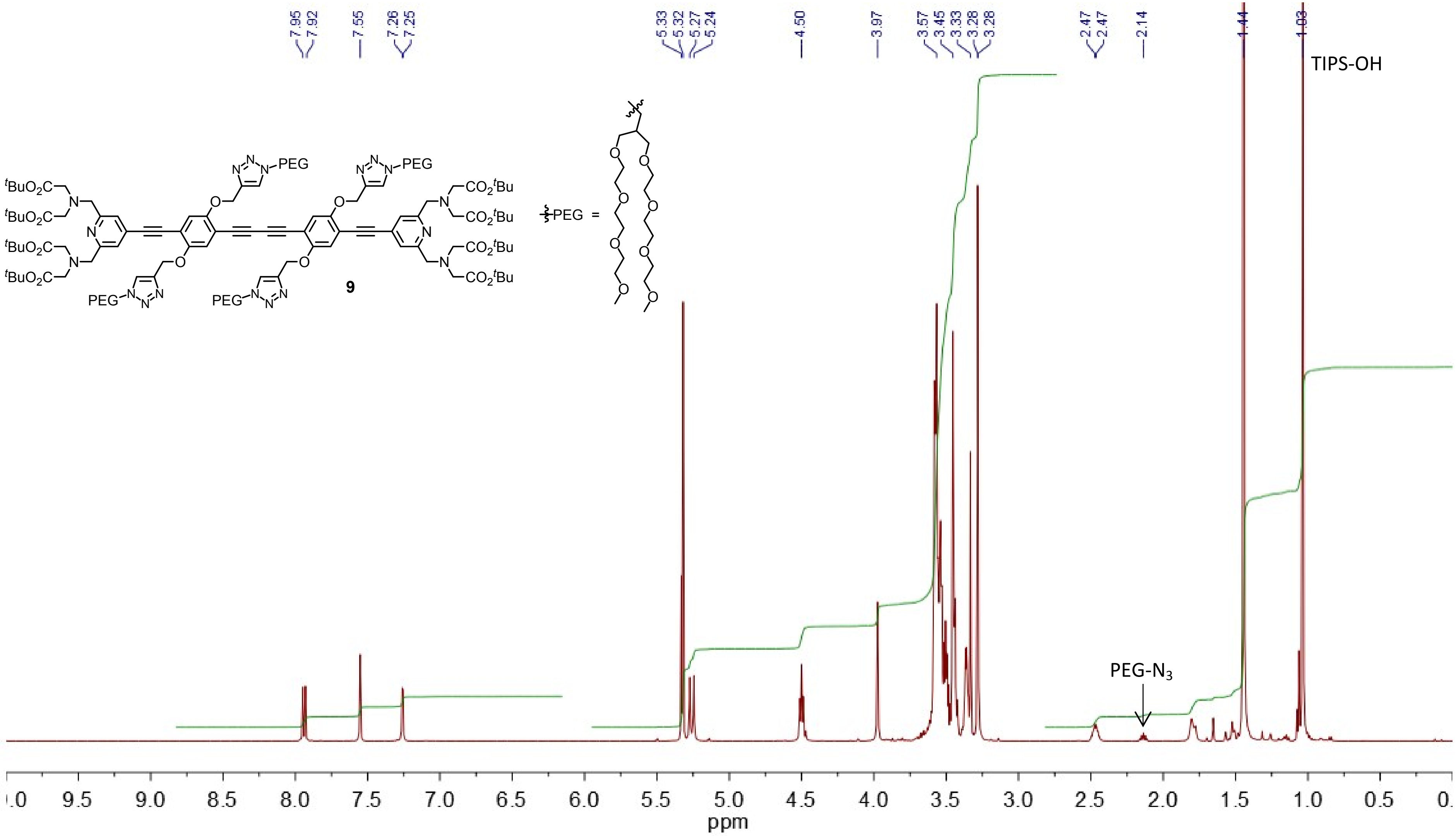


Observed Nucleus: 13C
Observed Frequency: 125.738929748535 MHz

Solvent: CD2Cl2
Number of Scans: 1024

Temperature: 295.991 K
Pulse Sequence: dept135
Relaxation Delay (s): 2 sec

Figure S14. ^{13}C DEPT (135) NMR spectrum of butadiyne **8**.

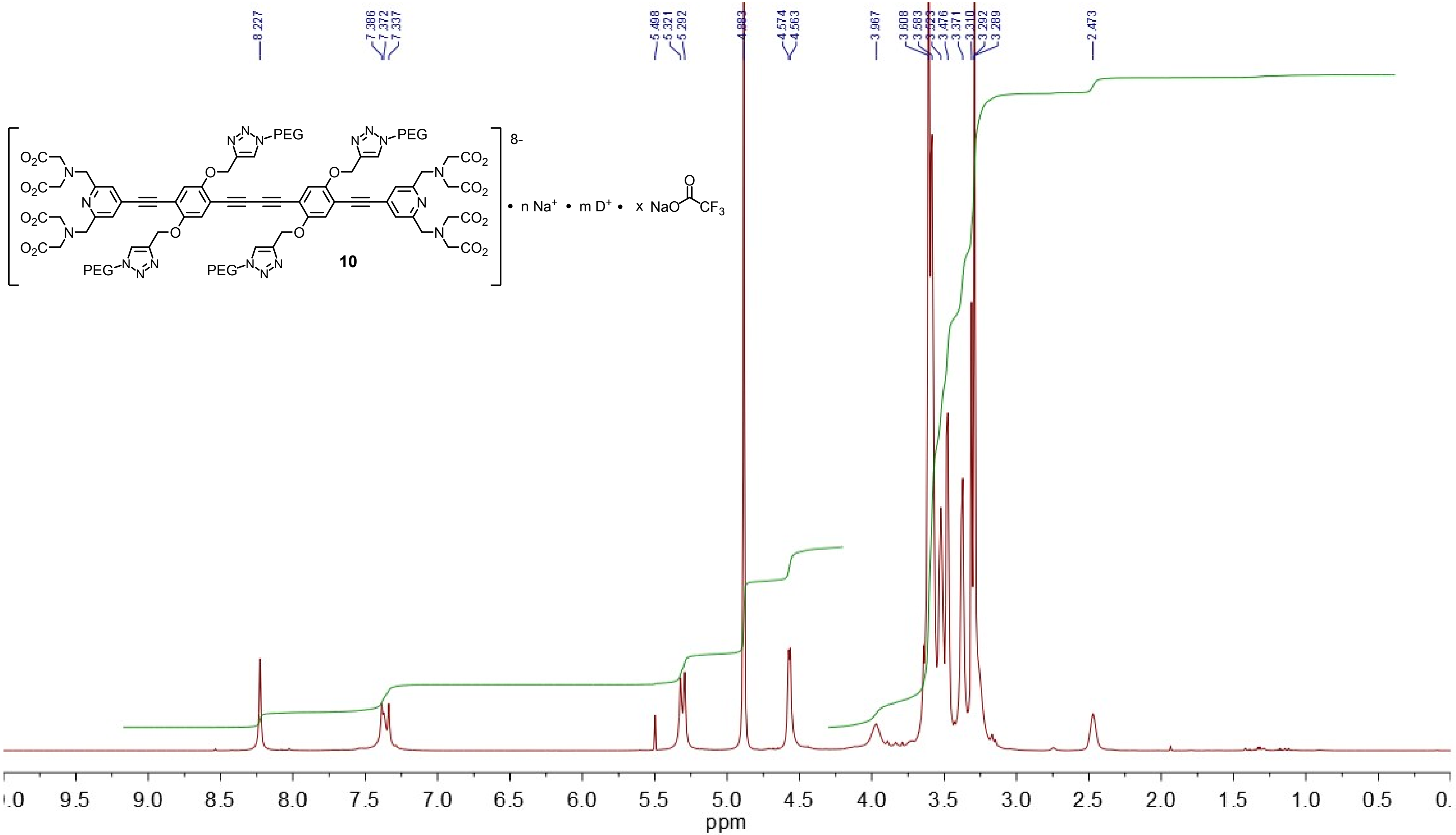


Observed Nucleus: 1H
Observed Frequency: 500.033081054688 MHz

Solvent: CD2Cl2
Number of Scans: 128

Temperature: 297.7 K
Pulse Sequence: zg30
Relaxation Delay (s): 1 sec

Figure S15. ^{1}H NMR spectrum of a mixture of butadiyne **9**, PEG-N$_3$, and TIPS-OH.



Observed Nucleus: 1H
Observed Frequency: 500.033081054688 MHz

Solvent: MeOD
Number of Scans: 128

Temperature: 66 K
Pulse Sequence: zg30
Relaxation Delay (s): 1 sec

Figure S16. ^{1}H NMR spectrum of ruler precursor **10** • n Na^+ • m D^+ • x $Na(O_2CCF_3)$.

]

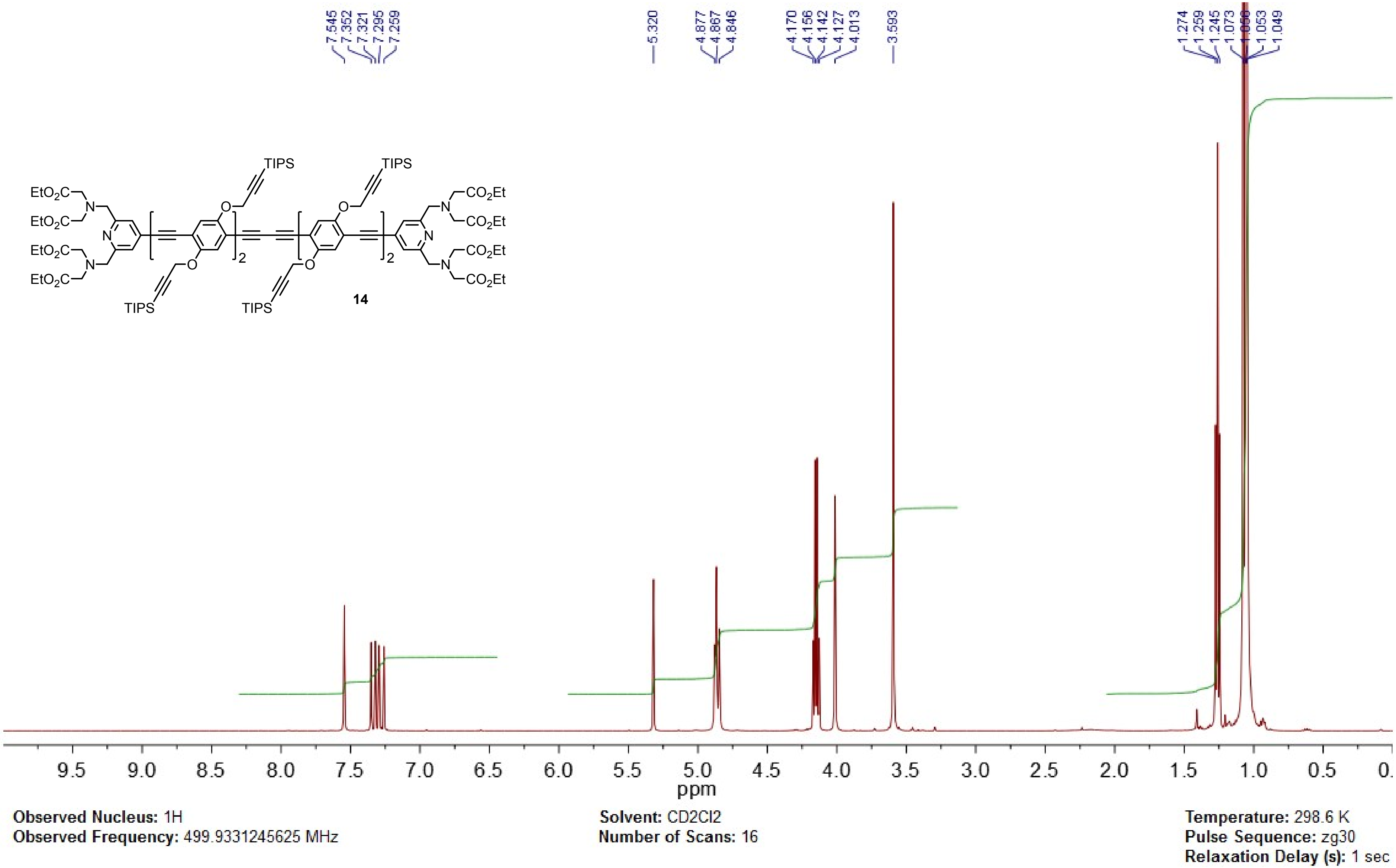
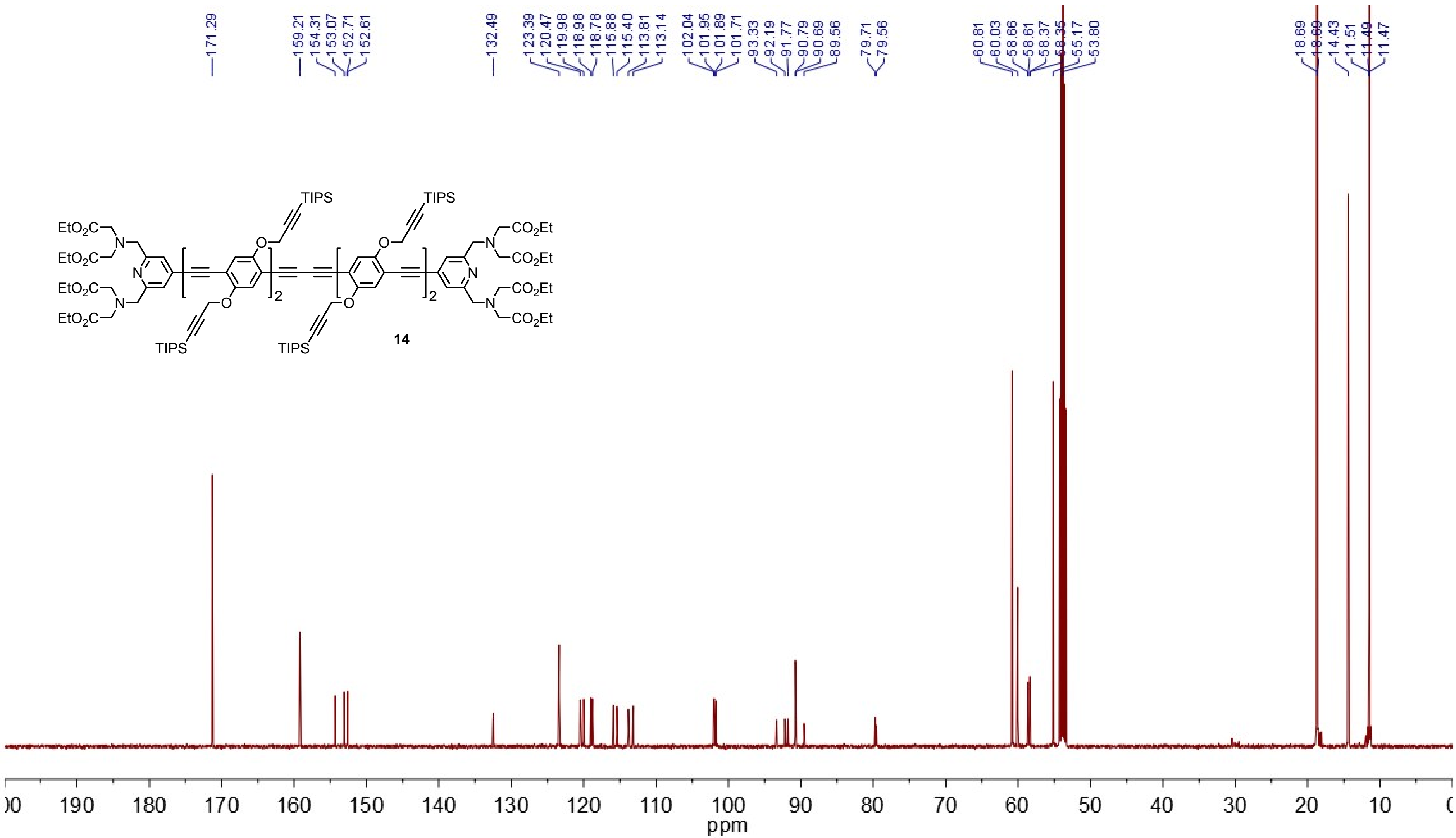


Figure S17. ^1H NMR spectrum of butadiyne **14**.



Observed Nucleus: 13C
Observed Frequency: 125.74018667 MHz

Solvent: CD2Cl2
Number of Scans: 2048

Temperature: 296.0974 K
Pulse Sequence: zgpg30
Relaxation Delay (s): 2 sec

Figure S18. ^{13}C NMR spectrum of butadiyne **14**.

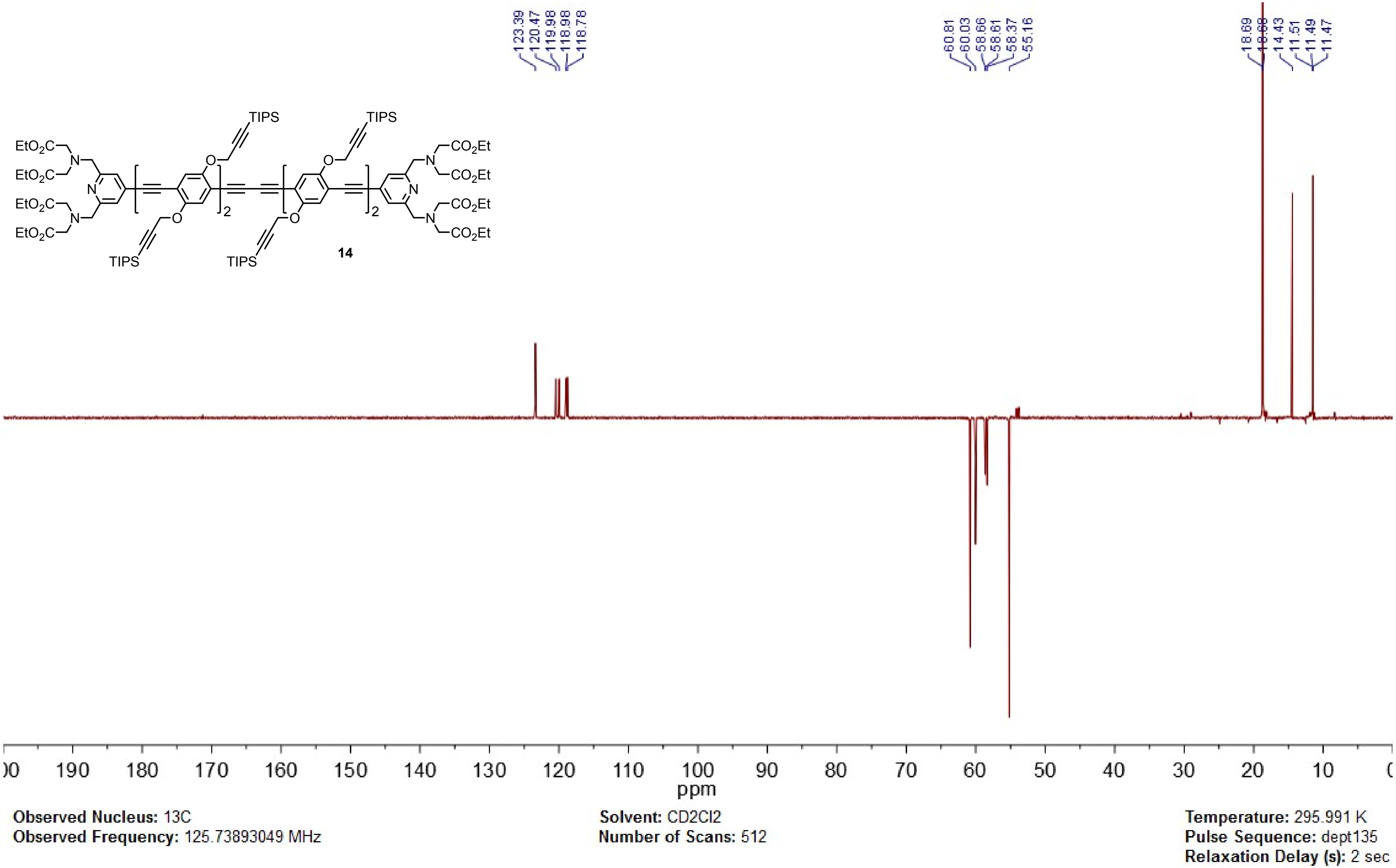


Figure S19. ^{13}C DEPT (135) NMR spectrum of butadiyne **14**.

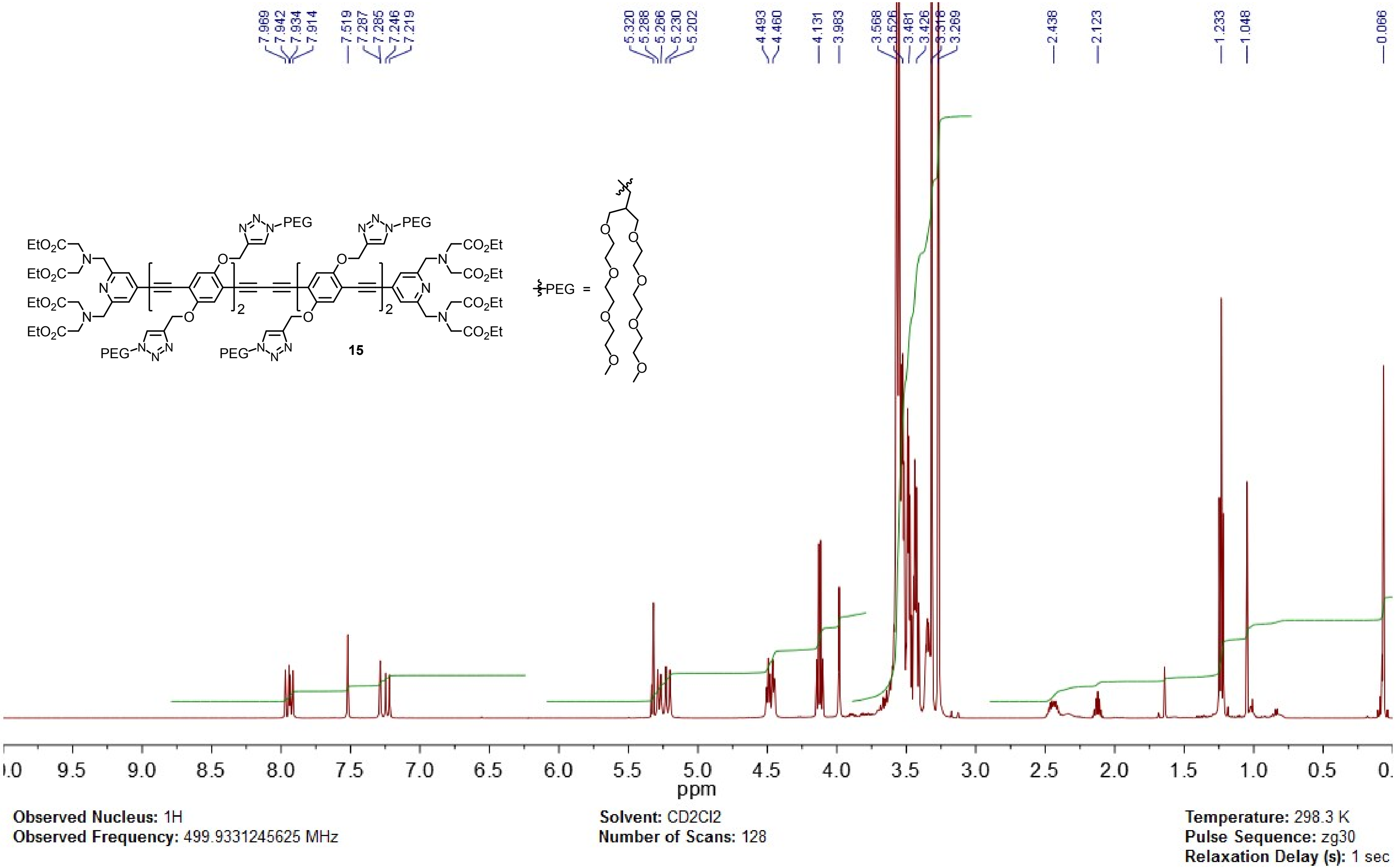
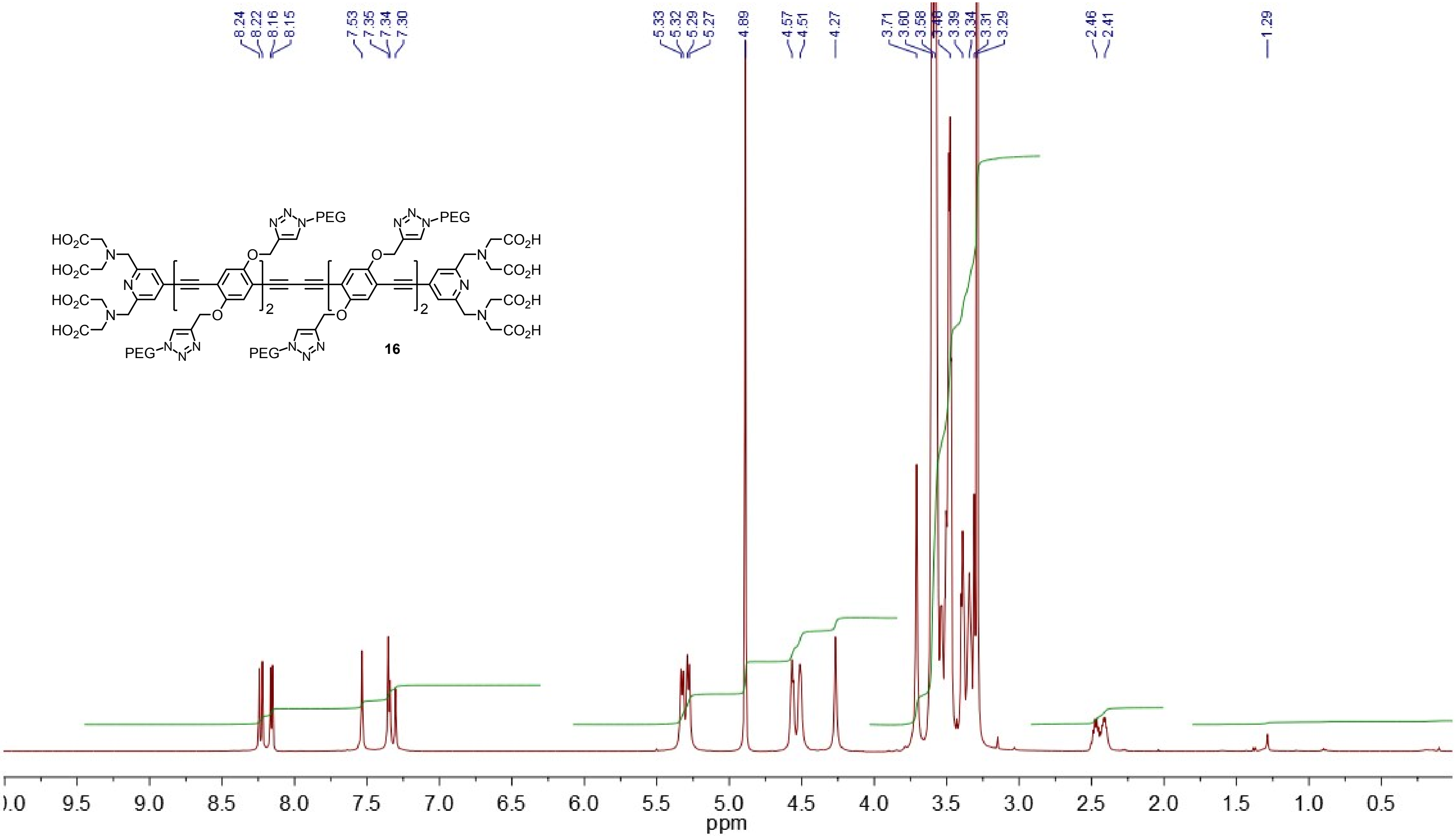


Figure S20. ^{1}H NMR spectrum of butadiyne **15**.



Observed Nucleus: 1H
Observed Frequency: 500.013087766429 MHz

Solvent: MeOD
Number of Scans: 128

Temperature: 295.991 K
Pulse Sequence: zg30
Relaxation Delay (s): 1 sec

Figure S21. ^{1}H NMR spectrum of ruler precursor **16**.

References

1 R. F. Heck, *Palladium reagents in organic syntheses*, Academic Press, London, 1985, p18.

2 M. Qi, M. Hülsmann and A. Godt, *Synthesis*, 2016, DOI: 1055/s-0035-1561660.

3 M. Qi, M. Hülsmann and A. Godt, *J. Org. Chem.*, 2016, **81**, 2549–2571.