Electronic Supplementary Information

Synthesis and characterization of a biocompatible $^{13}C_1$ isotopologue of trityl radical OX071 for in vivo EPR viscometry

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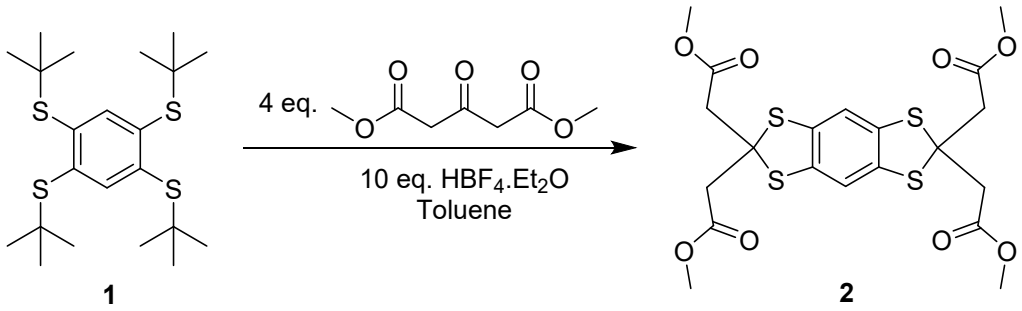
1. General Information

NMR spectra were recorded on a Jeol ECZ 400S NMR spectrometer and data was processed with MestReNova 14. EPR spectra in water and glycerol/water mixtures were recorded at West Virginia University using a Bruker ELEXSYS E580 X-band spectrometer and a Magnettech L-band spectrometer (Germany). Immobilized EPR spectra in trehalose were recorded at the University of Denver on a Bruker ELEXSYS E580 with X-band and Q-Band capabilities. EPR spectra were simulated using EasySpin ver. 5.2.33 and Matlab R2020a. HPLC-MS analyses were carried out using a Water Alliance e2695 separation module, a Water 2998 PDA detector, and a Water SQD2 mass detector. All solvents were purchased from Fisher Scientific. All commercially available reagents were used as received without further purification. Cryogenic conditions were maintained using a Julabo FT 901 immersion cooler. Diethyl ether and THF was purified on an Inert Pure Solv Solvent Purification system from Innovative Technologies, Inc. Diethyl carbonate-(carbonyl-^{13}C) 99 atom % was purchased from MilliporeSigma. Glycerol (99.99%) was purchased from Acros organics. All reactions were carried out under argon in flame-dried glassware and with deoxygenated and anhydrous solvents.

Synthesis of $^{13}C_1$-OX071Na

Benzo[1,2-d:4,5-d']bis(l,3)dithiole-2,2,6,6-tetraacetic acid methyl ester (2)



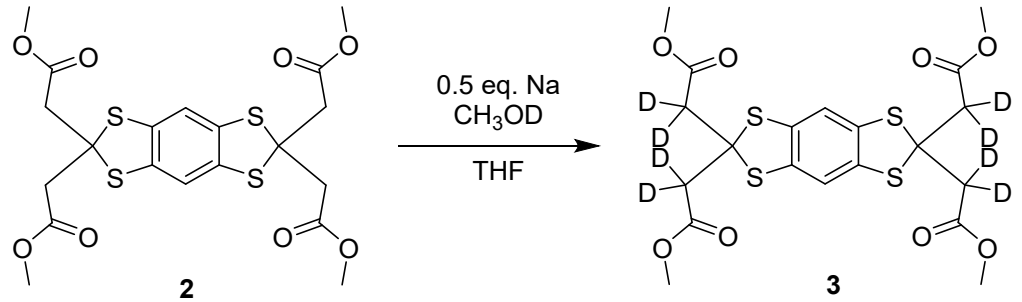
1 **2**

1,2,4,5-Tetra-*tert*-butylthiobenzene **1** (123 g, 0.286 mol) was dissolved in 3 L of toluene. Methyl acetonedicarboxylate (200 g, 1.14 mol, 4 eq.) was added, and the mixture was flushed with argon. $HBF_4.Et_2O$ (54% w/w, 390 mL, 2.85 mol, 10 eq.) was added, and the reaction was stirred vigorously overnight at room temperature. The yellow and heterogeneous solution was filtered, and solid **2** was washed with methanol until it became white (~300 mL). After drying under vacuum, 114 g of **2** was obtained as a white powder (77% yield).

Note: it is important to maintain a vigorous stirring of the biphasic mixture throughout the reaction.

^{1}H-NMR and ^{13}C-NMR match previously reported spectra in reference[1]

Benzo[1,2-d:4,5-d']bis(l,3)dithiole-2,2,6,6-tetraacetic-2-d_2-acid methyl ester (3)

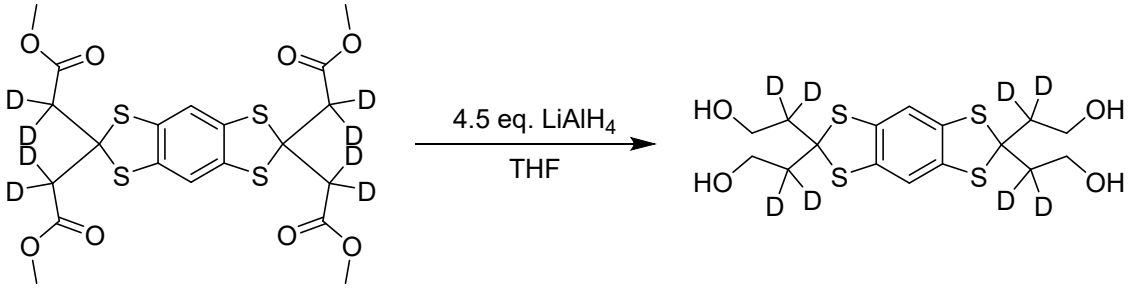


2 **3**

A flame-dried flask was charged with 360 mL anhydrous THF and equipped with a condenser. 603 mL of CH_3OD were added, then 120.6 g of tetraester **2** (232.4 mmol) were added. Metallic sodium (2.67 g, 116

mmol, 0.5 eq.) was added, and the reaction was heated to reflux overnight. The solvent was removed under reduced pressure, then the solid was dissolved in 500 mL of dichloromethane and washed with 500 mL of water. The organic phase was separated and dried over $MgSO_4$ and the solvent evaporated under reduced pressure to afford 104 g of the title compound as a white solid with 85% yield. From ^{1}H NMR, the deuteration percentage was determined to be 80-85%.
^{1}H-NMR and ^{13}C-NMR match previously reported spectra in reference[1]

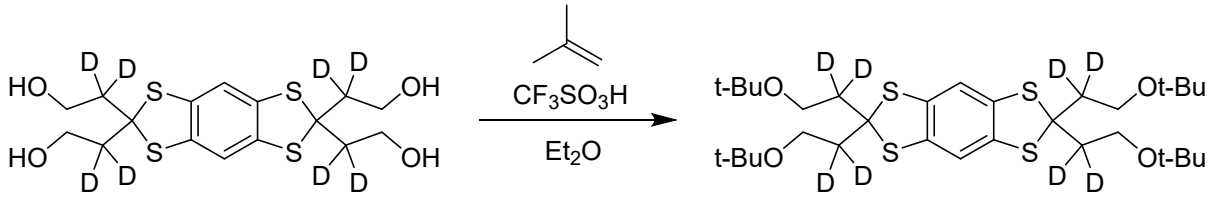
2,2,6,6-Tetra(2-(1-hydroxy-2,2-d$_2$-ethyl))benzo[1,2-d:4,5-d']bis(1,3)dithiole (4)



3 **4**

Tetraester **3** (100 g, 0.19 mol) was dissolved in 4 L of anhydrous THF. $LiAlH_4$ (32.5 g, 0.85 mol, 4.5 eq.) was added by small portions to avoid excessive heating of the reaction (less than 50°C). The reaction was stirred overnight at room temperature and then carefully quenched with 100 mL of methanol. 1 L of methanol, followed by 400 mL of water and 1200 mL of methanol, were added successively. The solution was filtered, and the remaining solid was washed with 1 L of methanol. The washings as well as the previous filtrate were combined and evaporated, then the solid was dissolved in 1 L of 1M hydrochloric acid and heated to 100°C until all the big chunks of solids were disaggregated. The solution was cooled down in an ice bath and filtered. The solid was washed successively with 3x200 mL of water and then 2x200 mL of cold methanol. The remaining white solid was dried under vacuum to afford 46 g of **4** (58% yield).
^{1}H-NMR and ^{13}C-NMR match previously reported spectra in reference[1]

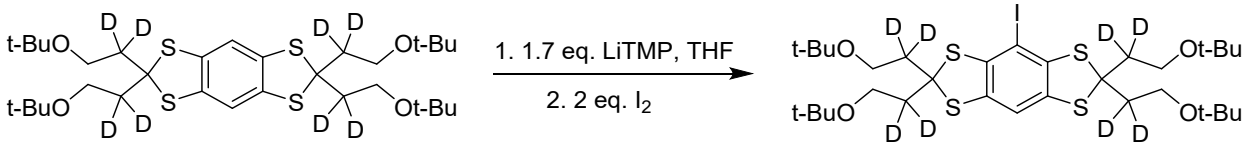
2,2,6,6-Tetra(2-(1-t-butoxy-2,2-d$_2$-ethyl))benzo[1,2-d:4,5-d']bis(1,3)dithiole (5)



4 **5**

The tetraalcohol **4** (30 g, 72.3 mmol) was suspended in 1500 mL of an isobutene solution in diethyl ether (prepared beforehand by bubbling isobutene in diethyl ether cooled down to -30°C, until the volume of the solution increased by approx. 10%) in a 2 L flame-dried flask. Triflic acid (19.1 mL, 216.9 mmol, 3 eq.) was added until all solids were dissolved (ca. 30 min), and the solution turned from pale yellow to light pink. An additional 9.6 mL triflic acid (108.4 mmol, 1.5 eq.) was added. The reaction was stirred for 2.5h at room temperature. Solid sodium bicarbonate was slowly added to the solution until the end of the formation of CO_2 bubbles, leading to a light orange solution. The solids were filtered off from the solution, and the solution was evaporated under reduced pressure. To remove traces of the triple-protected aryl derivative, the residue was treated with 800 mL of ethanol, heated to reflux, then kept at 4°C overnight. The solids were filtered and then dried overnight, affording 38.9 g of the title compound as an off-white powder (84% yield). ^{1}H-NMR and ^{13}C-NMR match previously reported spectra in reference[1]

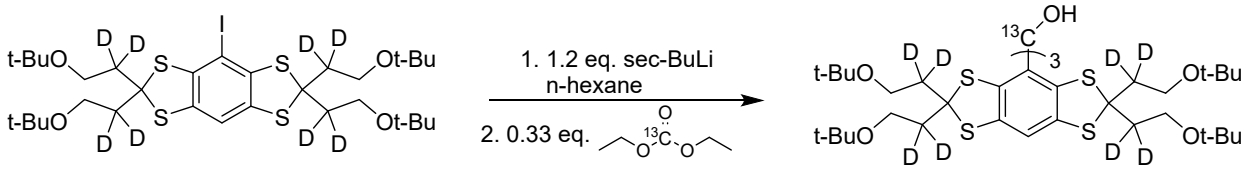
2,2,6,6-Tetra(2-(1-t-butoxy-2,2-d$_2$-ethyl))-4-iodo-benzo[1,2-d:4,5-d']bis(1,3)dithiole (6)



5 **6**

2,2,6,6-Tetramethylpiperidine (17.8 mL, 105.6 mmol, 2 eq.) was dissolved in 240 mL of dry degassed THF and cooled down to -55°C. A solution of *n*-BuLi (39 mL, 2.3M, 89.7 mmol, 1.7 eq.) was added dropwise. The mixture was stirred for 10 minutes at -55°C and 10 minutes at room temperature. The aryl **5** (33.7 g, 52.8 mmol) was dissolved in 1200 mL of dry THF and cooled down to -75°C. The formed LiTMP solution was transferred using a cannula into the compound **5** solution and the reaction was stirred for 2h30 at -75°C. Solid iodine (26.8 g, 105.6 mmol, 2 eq.) was added and the resulting solution was brought to room temperature and stirred for 20 min, turning dark brown to dark red. The reaction was quenched by 600 mL of a saturated solution of $Na_2S_2O_3$ and the reaction was stirred for 10 min until the dark color disappeared. The aryl iodide **6** was extracted twice with 600 mL of ethyl acetate. The combined organic phases were dried over $MgSO_4$ and evaporated under reduced pressure. Flash chromatography using 0-10% ethyl acetate in hexanes afforded 30.2 g of pure iodide **6** as a yellow thick oil (75% yield).
Note: over time, the thick oil may slowly crystallize.
^{1}H-NMR and ^{13}C-NMR match previously reported spectra in reference[1]

Tris(2,2,6,6-tetra(2-(1-t-butoxy-2,2-d$_2$-ethyl))benzo[1,2-d:4,5-d']bis(1,3)dithio-4-yl)methanol-^{13}C (7)

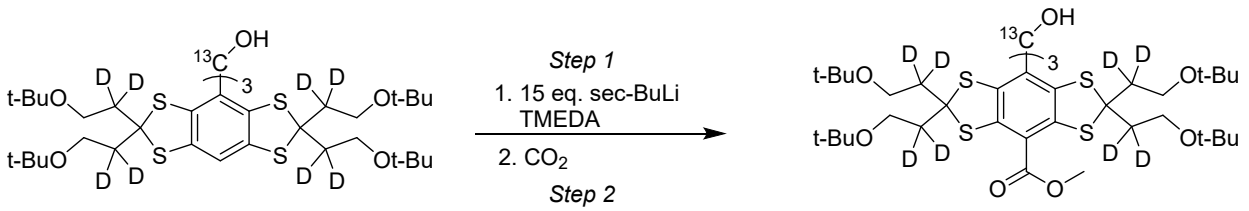


6 **7**

The aryl iodide **6** (1.9 g, 2.5 mmol) was dissolved in 80 mL of degassed, anhydrous *n*-hexane. The solution was cooled to -50°C then a solution of *sec*-BuLi (2.18 mL, 1.4M, 3.0 mmol, 1.2 eq.) was added and stirred 15 minutes at the same temperature, then brought to room temperature, leading to a light yellow and heterogeneous solution. A solution of $^{13}C_1$-diethyl carbonate (100 mg, 0.84 mmol, 0.33 eq.) in 2 mL anhydrous *n*-hexane was added dropwise over 3h using a syringe pump, turning the cloudy solution into a yellow-orange solution. After stirring overnight, the reaction was quenched with 10 mL of a 1M solution of ammonium chloride. The product was extracted twice with 50 mL of dichloromethane. The combined organic phases were dried over $MgSO_4$ and evaporated under reduced pressure. Flash chromatography using 0-20% ethyl acetate in hexanes afforded 1.2 g of the pure trityl alcohol **7** as a yellow foam (74% yield).
Notes: during the addition of the carbonate, the solution turns green and then yellow. The orange color indicates the presence of the diaryl ketone.

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.00 (s, 27H), 1.08 (s, 27H), 1.12 (s, 27H), 1.14 (s, 27H), 3.28-3.51 (m, 24H), 6.55 (d, 1H, *J*=2 Hz, O*H*), 7.03 (s, 3H, H-*Ar*).
^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 27.5, 27.6, 57.9, 58.0, 58.7, 59.3, 68.6, 70.2, 72.6, 72.7, 72.9, 73.1, 83.5 (99% $^{13}C_1$), 117.2, 132.1 (d, *J*=49.6 Hz, *C*-Ar), 137.3 (d, *J*=2.9 Hz, *C*-Ar), 137.4 (*C*-Ar), 137.5 (d, *J*=3.5 Hz, *C*-Ar), 138.7 (d, *J*= 2.9 Hz, *C*-Ar).

Tris(8-methoxycarbonyl-2,2,6,6-Tetra(2-(1-t-butoxy-2,2-d$_2$-ethyl))benzo[1,2-d:4,5-d']bis(1,3)dithio-4-yl)methanol-^{13}C (8)

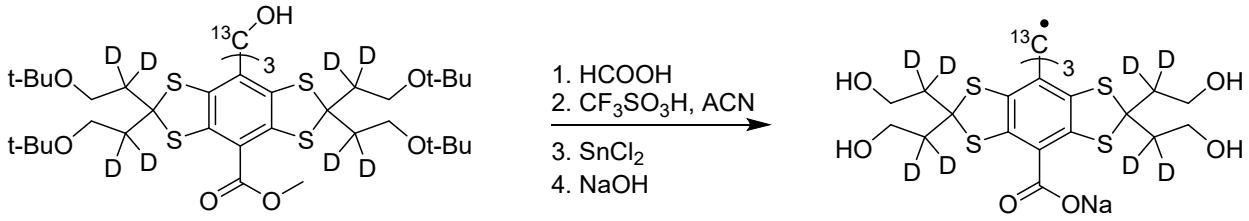


7 **8**

The trityl alcohol **7** (800 mg, 0.41 mmol) was dissolved in 8 mL of degassed, anhydrous TMEDA (ca. 0.05M) and then cooled down to -30°C. *sec*-BuLi (7.4 mL, 1.4M, 10.2 mmol, 25 eq.) was added and the solution was stirred for 150 minutes at -30°C. The color of the solution turned yellow to green when adding the base and slowly turned brown over time. 8 mL of dry TMEDA were loaded in a 50 mL flask under argon, then carbon dioxide was bubbled for 10 min in order to saturate the solution. The trityl solution was cannulated to the CO_2 solution in TMEDA while still bubbling CO_2 through the solution. During addition, the color changed from yellow to dark green and was exothermic. The reaction was stirred for an additional 10 minutes, then HPLC monitoring showed conversion to >75% triacid. The reaction was quenched with MeOH then the solvents were evaporated under reduced pressure. 10 mL of ethyl acetate and 10 mL of 1M HCl were added, then the layers were separated and the organic phase was dried over $MgSO_4$ and the solvent was removed under reduced pressure. The residue was dissolved in 20 mL of anhydrous DMF, iodomethane (154 µL, 2.47 mmol, 6 eq.), and anhydrous Na_2CO_3 (800 mg) were added. The reaction was stirred for 2 hours at 50°C. 10 mL of ethyl acetate were added, and the organic phase was washed with 100 mL of brine. The organic phase was dried over $MgSO_4$, and the solvent was removed under reduced pressure. Separation of the diester and the triester was carried out by flash chromatography using 0-20% ethyl acetate in hexanes to afford 537 mg of the triester **8** (62% yield).

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 0.98 (s, 27H), 1.08 (s, 54H), 1.15 (s, 27H), 3.21-3.52 (m, 24H), 3.86 (s, 9H), 7.06 (s, 1H).
^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 27.4, 27.5, 27.7, 52.1, 57.7, 58.6, 59.0, 66.5, 67.2, 72.7, 72.8, 73.0, 73.1, 84.3 (99% $^{13}C_1$), 120.7, 134.3 (d, *J*=49.5 Hz, *C*-Ar), 138.7 (d, *J*=2.9 Hz, *C*-Ar), 138.8 (*C*-Ar), 141.6 (d, *J*=3.8 Hz, *C*-Ar), 142.3 (d, *J*=2.9 Hz, *C*-Ar), 166.4 (*C*OOMe).

Tris(8-hydroxycarbonyl-2,2,6,6-Tetra(2-(1-t-butoxy-2,2-d$_2$-ethyl))benzo[1,2-d:4,5-d']bis(1,3)dithio-4-yl)methyl-^{13}C radical (OX071)



8 **$^{13}C_1$-OX071Na**

The triester trityl alcohol **8** (47 mg, 0.022 mmol) was dissolved in 1 mL formic acid and heated at 60°C for 60 minutes. The HPLC-MS shows a complete deprotection and subsequent esterification of the 12 alcohol moieties into formyl esters. The solvent was then evaporated under reduced pressure and the residue was dissolved in 1 mL anhydrous acetonitrile under argon. Triflic acid (40 µL, 0.44 mmol, 20 eq.) was added, the solution turned deep green-blue, and the reaction was stirred 30 min at room temperature. $SnCl_2.2H_2O$ (5 mg, 0.022 mmol, 1 eq.) dissolved in 0.5 mL of anhydrous THF was added, and the reaction was stirred for an additional 30 min. HPLC-MS shows the conversion of the trityl alcohol to the radical. 2 mL of a solution of NaH_2PO_4 and NaCl (1 g of NaH_2PO_4 and 0.4 g of NaCl) was added. The trityl radical was extracted with 2 mL of ethyl acetate. The phases were separated then the organic phase was evaporated. The residue was dissolved in 2 mL of a 2.5M solution of NaOH and stirred under argon at 55°C overnight.

The hydrolysis of all esters (formyl and methyl) was verified by HPLC. The pH was adjusted to 2 with trifluoroacetic acid, and the solution was loaded on a Hypersep C18 cartridge (3 cm diameter, 3 cm length) previously conditioned first with 50 mL ACN containing 0.1% TFA then 150 mL water with TFA 0.1%. The cartridge was washed with 80 mL water containing 0.1% of TFA to remove all the salts and the compound was recovered with 10% ACN in water with 0.1% TFA. The fraction was freeze-dried to afford **$^{13}C_1$-OX071** in 90% yield (28 mg). It was dissolved in water, NaOH (2.4 mg, 0.059 mmol, 3 eq.) was added, and the green solution was freeze-dried again to provide **$^{13}C_1$-OX071Na.**

2. Sample preparation in aqueous glycerol

Solutions of **$^{13}C_1$-OX071Na** in water containing various amounts of glycerol were prepared as described in Table S1:

Table S1. Preparation of samples containing the **$^{13}C_1$-OX071Na** probe in water/glycerol.

Glycerol (%V/V)	Glycerol (mg)	**$^{13}C_1$-OX071Na** 10 mM in water (µL)	Water (µL)
0%	0	40	960
12.5%	157	40	835
25%	315	40	710
35 %	441	40	610
45%	567	40	510
67.50%	851	40	285
80%	1008	40	160
85%	1070	40	110
90%	1135	40	60

3. Calibration of X- and L-Band linewidths in various media viscosities

Table S2. Triplicate measurements of X-Band peak-to-peak linewidths of **$^{13}C_1$-OX071Na** in varying amounts of glycerol.

% Glycerol	η (cP) at 22 °C	Low-field Component (G)			Mean (G)	High-field Component (G)			Mean (G)
0	0.98	0.882	0.864	0.873	0.873±0.009	0.972	0.975	0.966	0.971±0.005
12.5	1.46	1.282	1.300	1.325	1.302±0.026	1.443	1.480	1.455	1.459 ±0.019
25	2.34	2.289	2.304	2.301	2.298±0.008	2.500	2.511	2.585	2.532±0.046
35	3.62	3.729	3.654	3.722	3.702±0.041	3.964	3.939	3.964	3.956±0.014
45	6.00	6.616	6.541	6.604	6.587±0.040	7.359	7.483	7.451	7.431±0.064

Table S3. Triplicate measurements of L-Band peak to peak linewidths of **$^{13}C_1$-OX071Na** in varying amounts of glycerol.

% Glycerol	η (cP) at 16°C	Low-field Component (G)			Mean (G)
0	1.11	1.031	1.067	1.091	1.063±0.030
12.5	1.68	1.735	1.794	1.764	1.764±0.029
25	2.74	3.176	3.248	3.200	3.208±0.037
35	4.33	5.266	5.086	5.086	5.146 ±0.104
45	7.36	9.011	9.199	9.198	9.136 ±0.108

For X-Band EPR, 50 µL of each solution was added to gas-permeable Teflon tubes with a diameter of 1.14 mm and a wall thickness of 60 µm (Zeus, Inc., USA) and sealed with clay sealant (Kimble Cha-Seal). The temperature of the samples inside the EPR resonator was maintained at 22°C, and oxygen was removed with a constant nitrogen flow for half an hour. The gas flow and the temperature were maintained using a temperature and gas controller (Noxygen, Germany) throughout the acquisition. EPR acquisition parameters were as follows: microwave power, 1 mW (except 0.1 mW for solutions with 0% glycerol); modulation amplitude, 3 G (except 0.5 G for 0% and 12.5% glycerol, 1G for 25% glycerol, 0.75 G for 25% glycerol and 1 G for 35% glycerol); modulation frequency, 100 kHz; sweep width, 100 G; sweep time, 81.92 s; conversion time, 40.00 ms, number of points, 2048 (4096 points for Figure 6A). Each spectrum was recorded three times.

For L-Band EPR, 500 µL of each solution was placed in a 1.5 mL Eppendorf and placed in the resonator loop. Oxygen was removed by bubbling nitrogen through each solution for 30 minutes. EPR acquisition parameters were as follows: non-saturating power; modulation amplitude, 0.7 G for 0% glycerol, 1.2 G for 12.5% glycerol, 2 G for 25% glycerol, 3 G for 35% glycerol, 7 G for 45% glycerol; modulation frequency, 100 kHz; sweep width, 100 G for full spectra, 1 G for individual peaks with 0% glycerol, 1.2 G for 12.5 glycerol, 2 G for 25% glycerol, 3 G for 35% glycerol; sweep time, 80 s; number of points, 2048; number of scans, 8. Spectra were recorded three times. The temperature of the room (16°C) was monitored in order to calculate the viscosity.

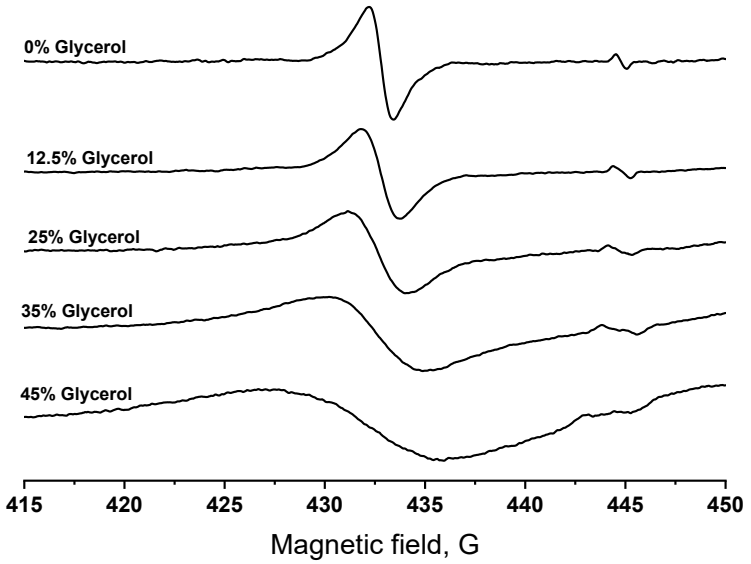


Fig S1 L-band EPR spectra of the low-field peak of **$^{13}C_1$-OX071Na** (400 µM) in water with 0%, 12.5%, 25%, 35%, and 45% (V/V) glycerol at 16°C.

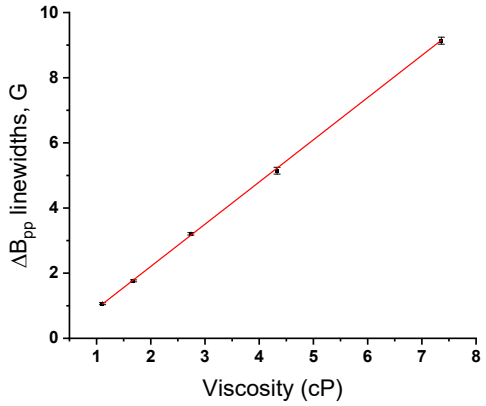
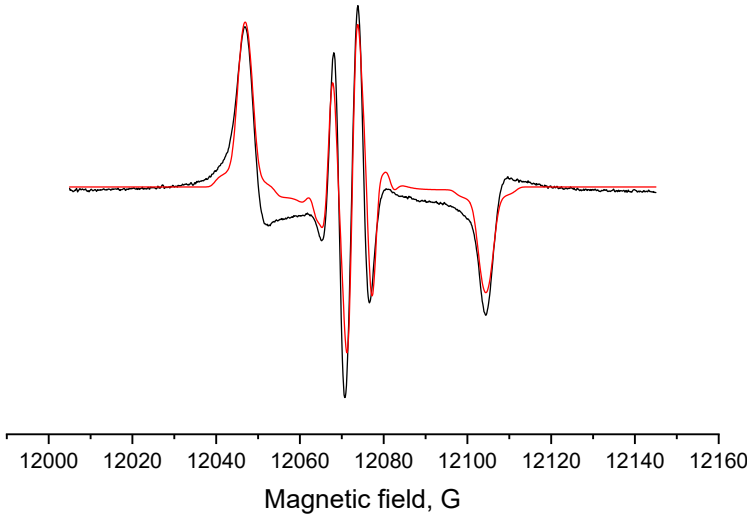


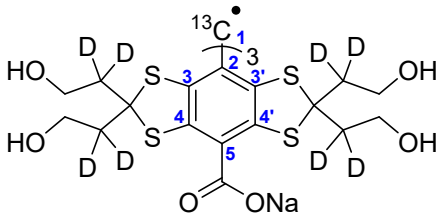
Fig S2 Measured ΔB_{pp} linewidths of **$^{13}C_1$-OX071** (400 μM) for the low-field peaks versus viscosity (cP) in L-band EPR. Linear fit leads to the equation $\Delta B_{pp}(G)=1.29\cdot$viscosity(cP)$-0.38$, $R^2=0.9996$.

4. EPR Spectra of $^{13}C_1$-OX071Na immobilized in Trehalose/Sucrose at X- and Q-Band.

$^{13}C_1$-OX071Na was immobilized in trehalose with a ratio of 2000:1 trehalose to trityl radical and later evacuated to remove oxygen. The sample for X-band experiments was sealed in a 4 mM OD quartz tube under a partial pressure of 200 mTorr of Helium. The X-Band magnetic field calibration was performed with DPPH using a g value of 2.0036 and with LiPc with a g value of 2.0021 for the Q-Band field calibration.

The parameters for acquiring the X-Band CW spectrum were: 9.635 GHz, 3440G center field, 120 G sweep width, 100 kHz modulation frequency, 2 G modulation amplitude, 1024 points, and 56 dB attenuation from 200 mW. To obtain a better signal to noise, 10 scans were accumulated. The Q-Band CW spectrum was acquired with 33.8260 GHz, sweep width 140 G, 1024 points, 99 kHz modulation frequency, 2G modulation amplitude, and 25dB attenuation from 50 mW.





$^{13}C_1$-OX071Na

Fig S3 Q Band spectrum for **$^{13}C_1$-OX071Na** in trehalose at room temperature. black is the experimental data and red is the simulation. No ^{13}C side bands are resolved in the Q Band spectrum. The simulation included the ^{13}C side bands but an equally good simulation can be produced without their inclusion. Simulations parameters are : 80% of A(C_1) = [18.5 19 161]MHz, 10% of A(C_1) +A($C_{2,3,3'}$) = [29 29 30] MHz, 10% A(C_1)+ A($C_{4,4',5}$) = [14 14 13] MHz. Hstrain values for components with 80, 10 and 10% weightings are [7.5 6.5 7], [3 4 7] and [5 8 5]MHz respectively.

5. Comparison of A, g, linewidths, and τ values for various $^{13}C_1$-triarylmethyl radicals

Table S4. Comparison of A, g, linewidths and τ values for **$^{13}C_1$-dFT**[2, 3], **$^{13}C_1$-OX071** and **$^{13}C_1$-PTMTC**[4, 5].

	$^{13}C_1$-dFT[2, 3]	**$^{13}C_1$-OX071 [this work]**	$^{13}C_1$-PCTMTC[4, 5]
A_{iso} (G)	23.35	23.01	29.82
A_x, A_y, A_z (MHz)	18 18 162	18 18 160	26 25 199.5
g_{iso}	2.0031	2.0031	2.0023
g_x, g_y, g_z	2.0033 2.0032 2.00275	2.0033 2.0032 2.0027	2.0015 2.0015 2.0040
Low-field Linewidth (G) at ~1 cP, 0% O_2	0.58	0.87	1.12
High-field Linewidth (G) at ~1 cP, 0% O_2	0.64	0.97	1.06
τ (ns) at ~1 cP	0.27 ns	0.47 ns	0.22 ns

6. NMR spectra

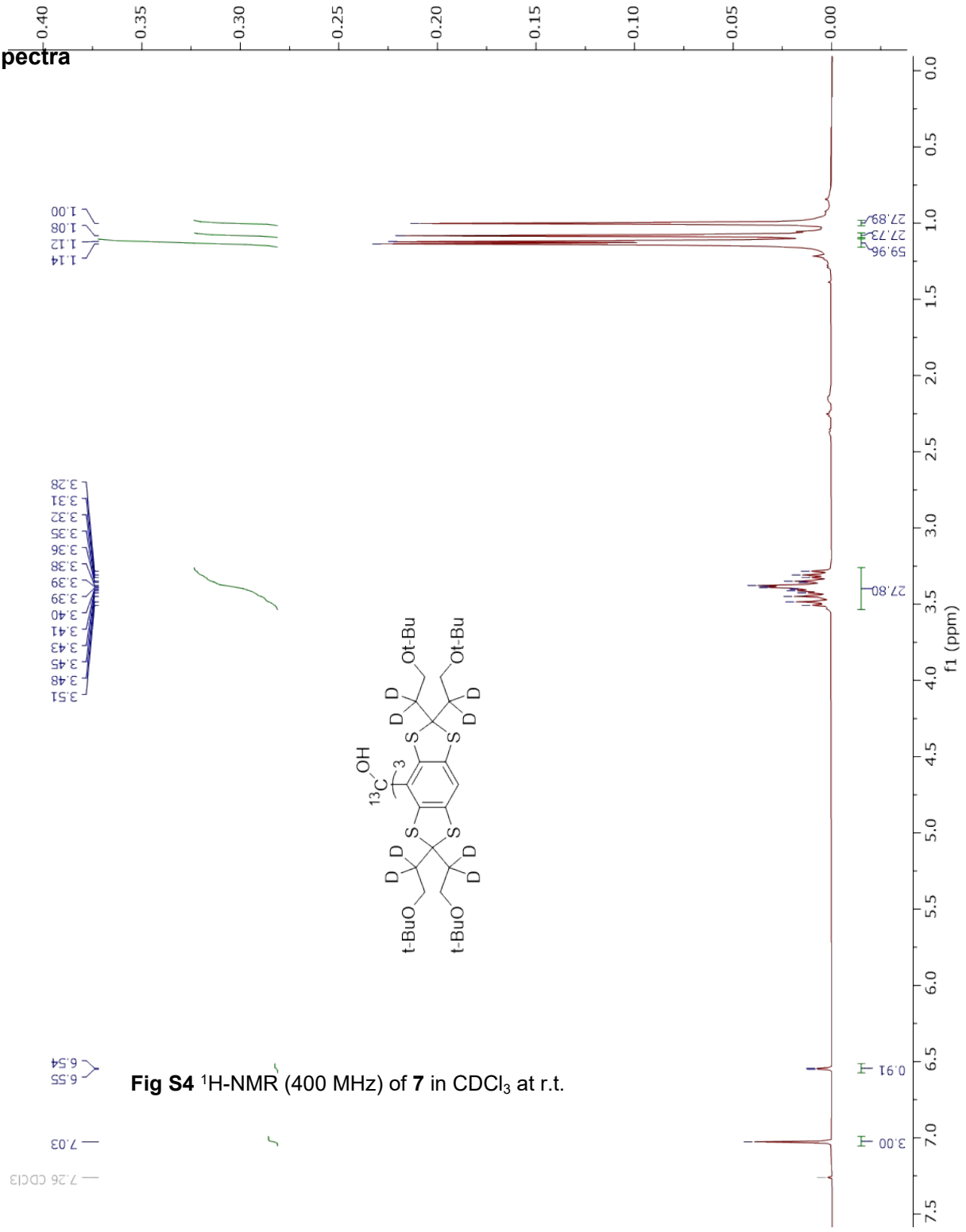


Fig S4 ^{1}H-NMR (400 MHz) of **7** in $CDCl_3$ at r.t.

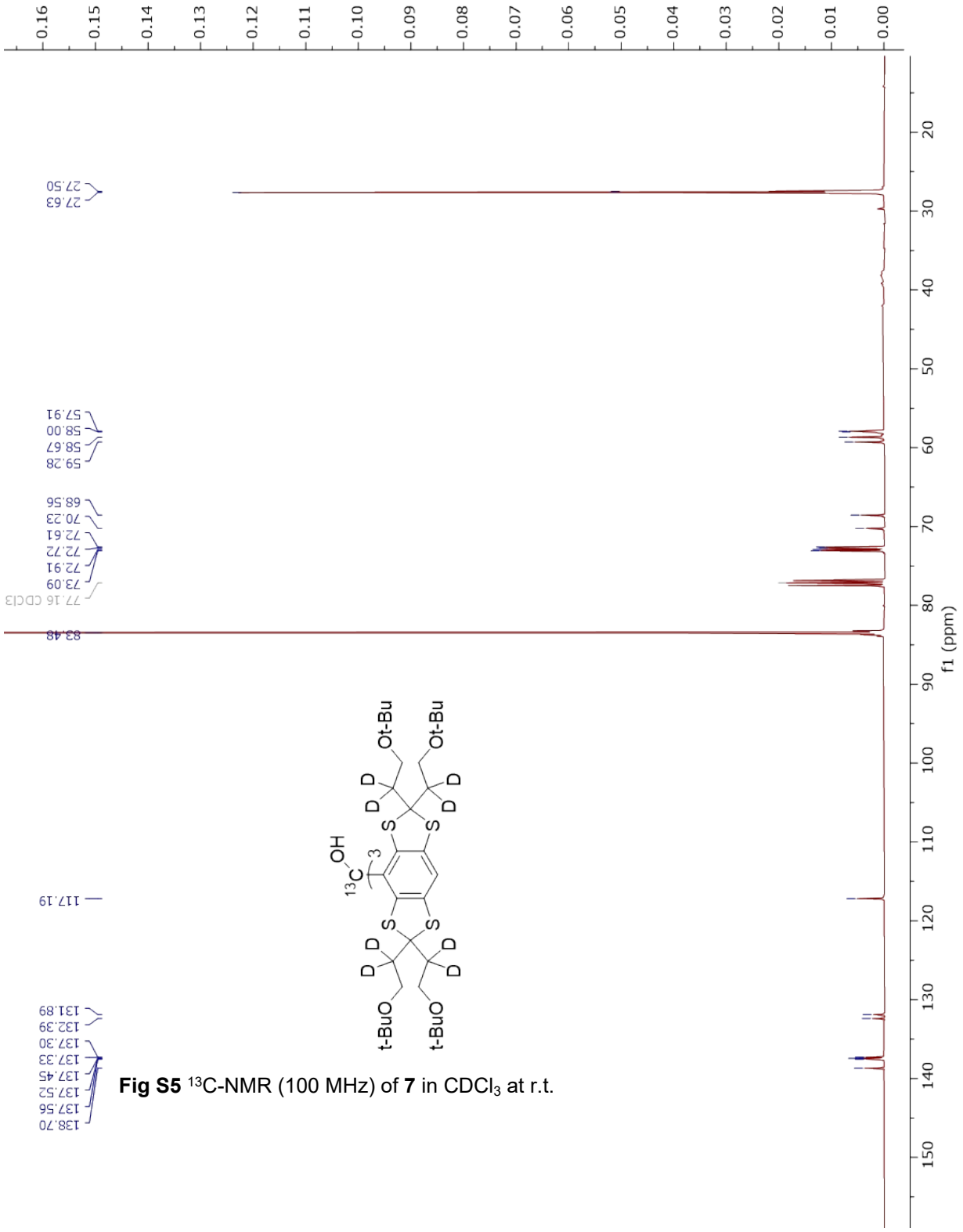


Fig S5 ^{13}C-NMR (100 MHz) of **7** in $CDCl_3$ at r.t.

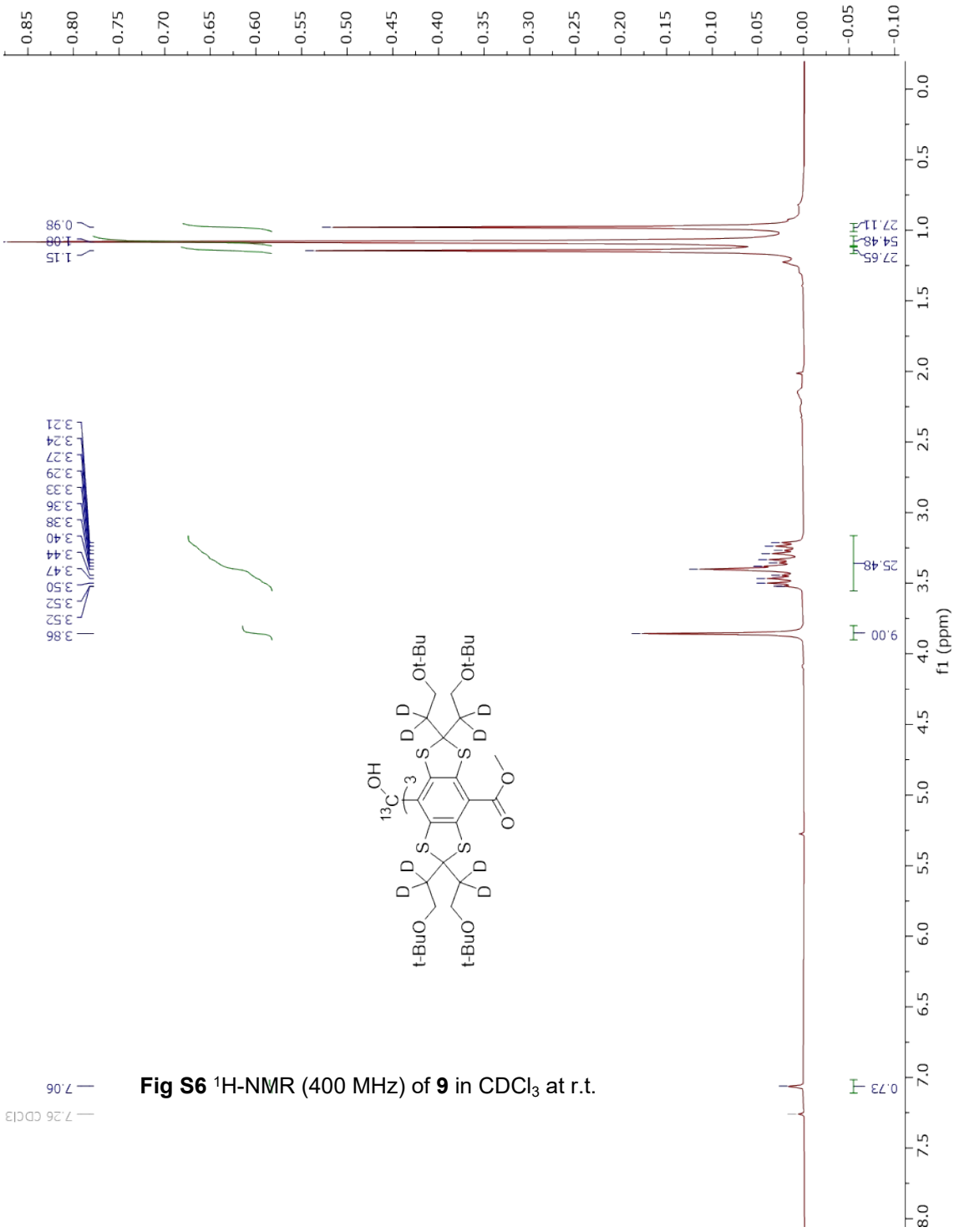


Fig S6 ^{1}H-NMR (400 MHz) of **9** in $CDCl_3$ at r.t.

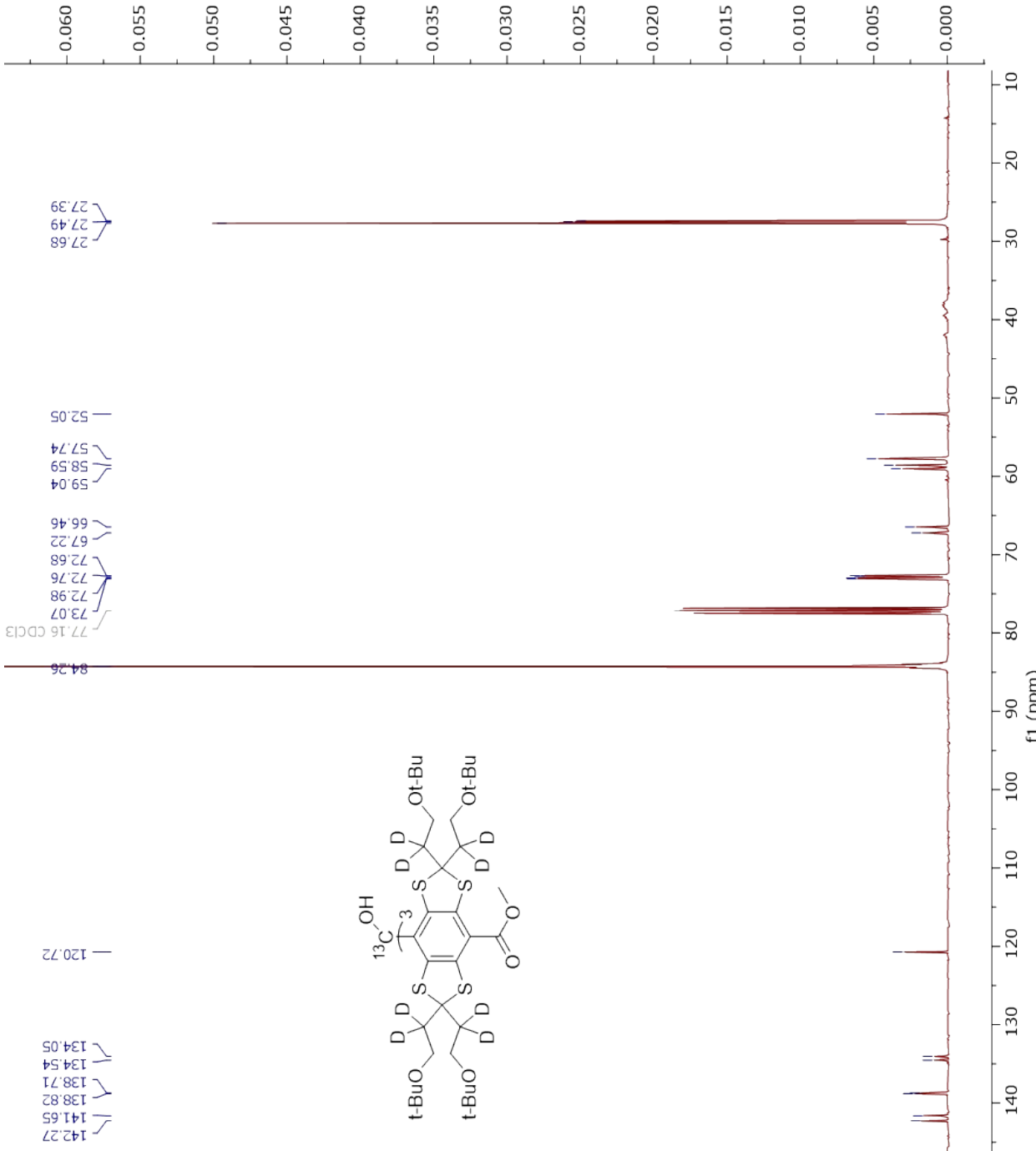


Fig S7 ^{13}C-NMR (100 MHz) of **9** in $CDCl_3$ at r.t.

7. References

1. M. Poncelet, J. L. Huffman, V. V. Khramtsov, I. Dhimitruka and B. Driesschaert, *RSC Adv.*, 2019, **9**, 35073-35076.
2. M. Poncelet and B. Driesschaert, *Angew. Chem. Int. Ed.*, 2020, **59**, 16451-16454.
3. W. Moore, J. E. McPeak, M. Poncelet, B. Driesschaert, S. S. Eaton and G. R. Eaton, *J. Magn. Reson.*, 2020, **318**, 106797.
4. J. L. Huffman, M. Poncelet, W. Moore, S. S. Eaton, G. R. Eaton and B. Driesschaert, *J. Phys. Chem. B.*, 2021, **125**, 7380-7387.

5. W. Moore, J. L. Huffman, B. Driesschaert, S. S. Eaton and G. R. Eaton, *App. Magn. Reson.*, 2022, **53**, 797-808.