Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Electronic Supplementary Information

Dimeric benzoboroxoles for targeted activity against *Mycobacterium tuberculosis*

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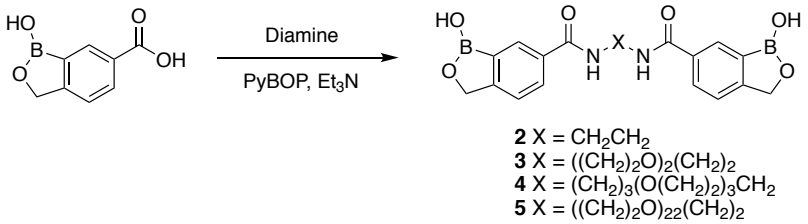
Supplementary Results

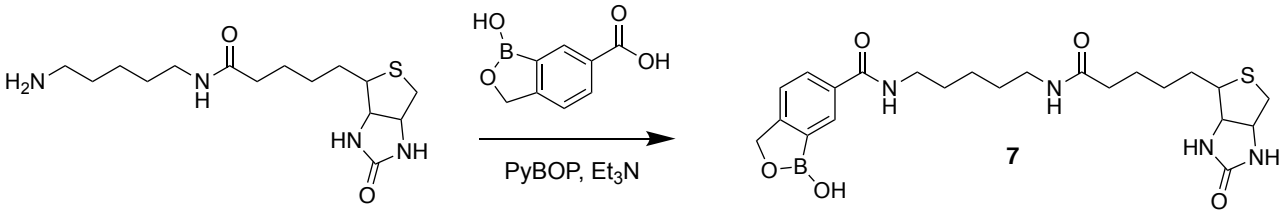
Scheme S1. Synthesis of boroxole dimers **2**-**5**.



2 X = CH_2CH_2
3 X = $((CH_2)_2O)_2(CH_2)_2$
4 X = $(CH_2)_3(O(CH_2)_2)_3CH_2$
5 X = $((CH_2)_2O)_{22}(CH_2)_2$

Reagents and conditions: 0.45 eq. diamine, 1.1 eq. PyBOP, 2 eq. Et_3N, DMF, 16 hours, room temp.

Scheme S2. Synthesis of biotinylated boroxole compound **7**.



Reagents and conditions: 1 eq. 1-Hydroxy-1,3-dihydro-2,1-benzoxaborole-6-carboxylic acid, 1.1 eq. PyBOP, 2 eq. Et_3N, DMF, 16 hours, room temp.

Figure S1. Alizarin red assay. (a) 1,3-Dihydro-1-hydroxy-2,1-benzoxaborole **1**, (b) *N,N′*-(ethylenediamine)bis-(6-(1-hydroxy-1,3-dihydro-2,1-benzoxaborole))amide **2**, (c) *N,N′*-(2,2-(ethylenedioxy)bis-(ethylamine))bis-(6-(1-hydroxy-1,3-dihydro-2,1-benzoxaborole))amide **3**, (d) *N,N′*-(4,7,10-trioxa-1,13-tridecane-diamine)bis-(6-(1-hydroxy-1,3-dihydro-2,1-benzoxaborole))amide **4**.

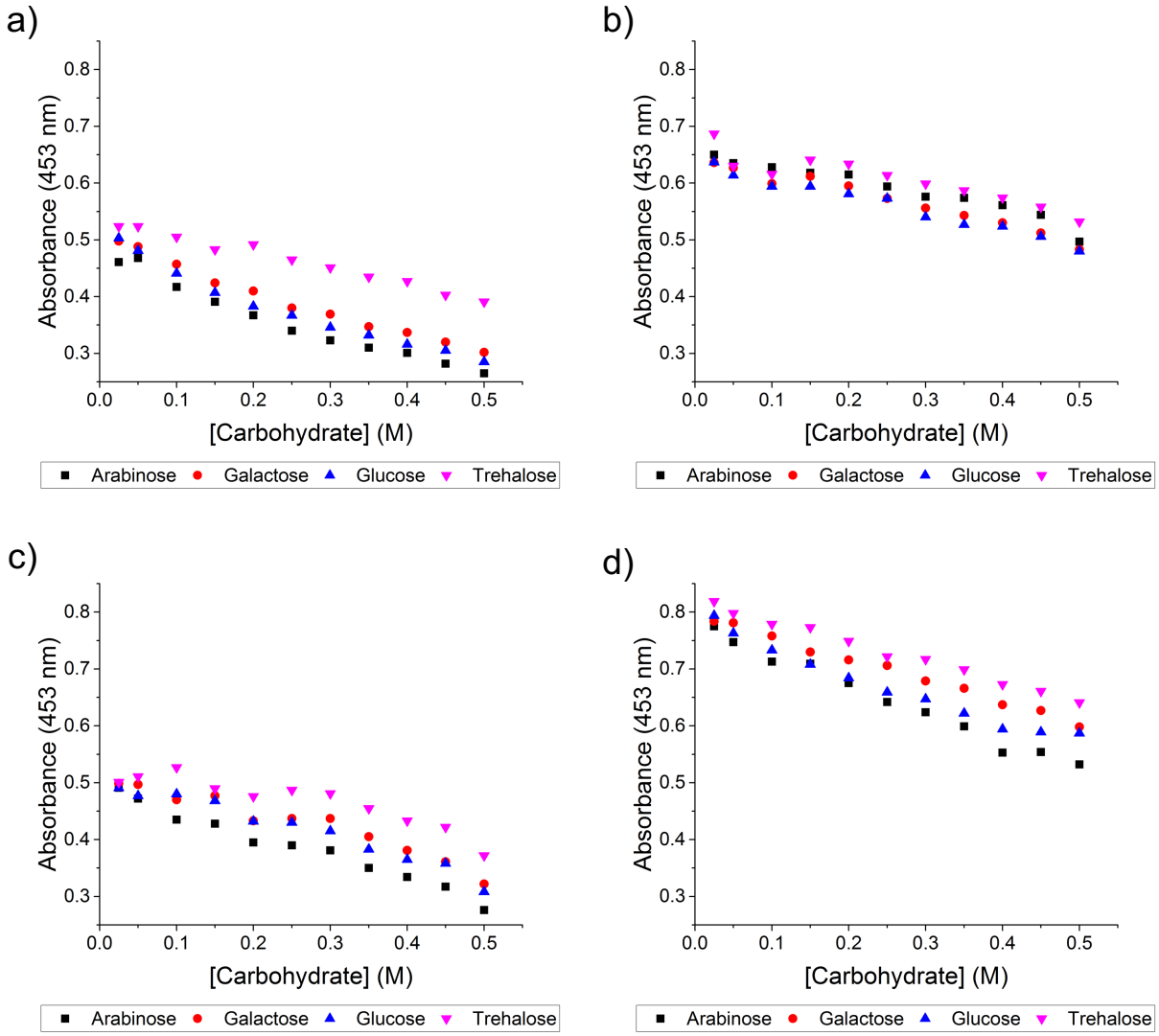
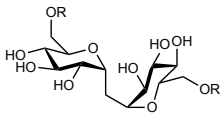


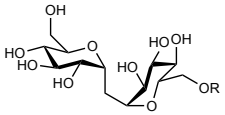
Figure S2. Structures of *Mtb* cell envelope components used in these studies.

Trehalose dimycolate (TDM)



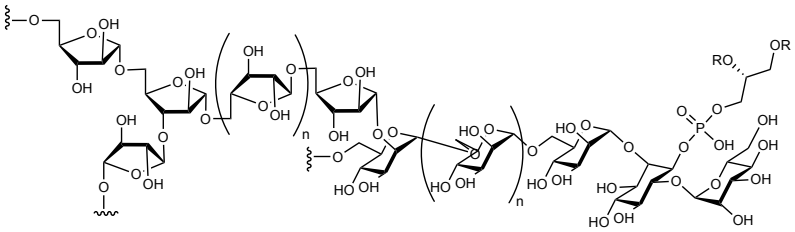
R = mycolic acid

Trehalose monomycolate (TMM)



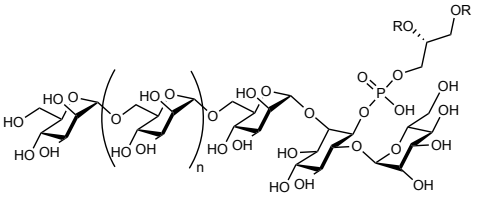
R = mycolic acid

Lipoarabinomannan (LAM)



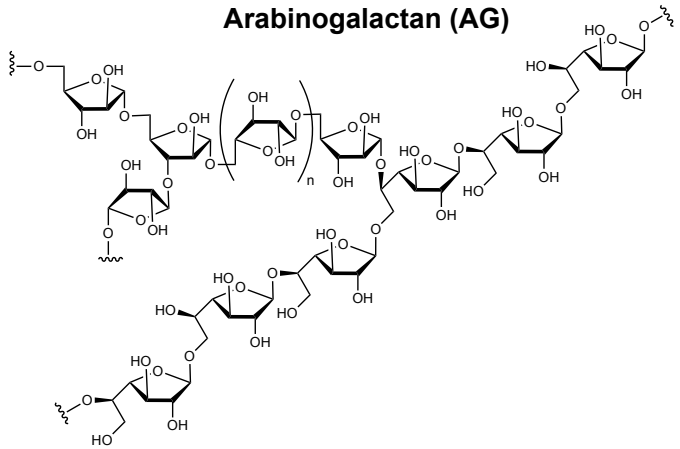
R = Fatty acid chain

Lipomannan (LM)

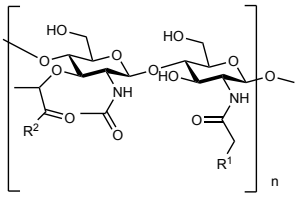


R = Fatty acid chain

Arabinogalactan (AG)



Peptidoglycan (PG)

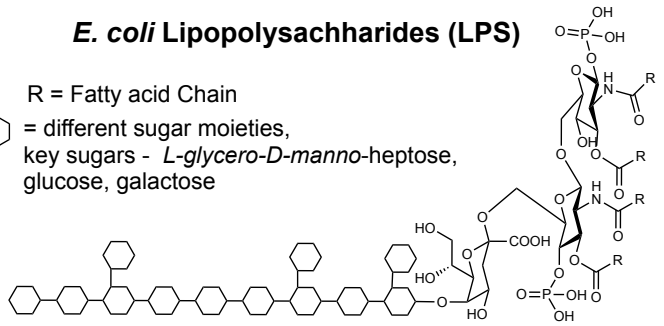


R_1 = H or OH, R_2 = peptide

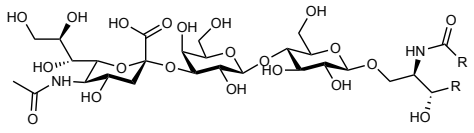
***E. coli* Lipopolysachharides (LPS)**

R = Fatty acid Chain

= different sugar moieties,
key sugars - *L-glycero-D-manno*-heptose,
glucose, galactose



Gangliomannoside-3



R = fatty acid chains

Supplementary Chemical and Synthetic protocols

General Information and Procedures

Unless stated, the chemicals and solvents, including anhydrous solvents, used in these syntheses were used as supplied and without further purification. Chloroform and triethylamine were purchased from Fisher Scientific at laboratory reagent grade. Anhydrous *N,N*-dimethylformamide (DMF) ≥99.8%, deuterium oxide (D_2O) 99.9%, ethylenediamine ≥99%, 2,2-(ethylenedioxy)bis(ethylenediamine) 98%, 4,7,10-trioxa-1,13-tridecanediamine 97% and (benzotriazole-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate ≥97% were purchased from Sigma-Aldrich. 1-Hydroxy-1,3-dihydro-2,1-benzoxaborole-6-carboxylic acid >95% was purchased from Key Organics. Deuteromethanol (MeOD) 99.8% was purchased from Apollo Scientific. Polyoxyethylene bis(amine) MW 1000 was purchased from Alfa Aesar. EZ-link pentylamine-biotin and 100-500 MW dialysis tubing were obtained from Fisher Scientific. Distilled water (H_2O) was used throughout.

All reactions were performed using oven dried glassware and heat transfer was achieved using an oil bath.

Thin Layer Chromatography (TLC) was carried out using Merck aluminium backed sheets coated with 60 F_{254} silica gel. Visualisation of the silica plates was achieved using a UV Lamp (λ = 254 nm), and/or potassium permanganate (1.5 g $KMnO_4$, 10 g K_2CO_3 and 1.25 mL 10% NaOH in 200 mL water) or Alizarin Red S (1 mM alizarin red S in acetone) [4].

Proton (^1H) and carbon (^{13}C) NMR spectra were obtained at 298 K. ^1H NMR were recorded on Bruker DPX-300, DPX-400 and DPX-500 instruments as indicated. ^{13}C DEPT NMR were recorded on Bruker DPX-400 and DPX-500 instruments as indicated. NMR were fully assigned using COSY, HSQC and HMBC. All chemical shifts are quoted in parts per million (ppm), using the residual solvent as the internal standard. (^1H NMR: MeOD = 3.31 and ^{13}C NMR: MeOD = 49.0). Coupling constants (*J*) are reported in hertz (Hz) with the following abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; quin, quintet; m, multiplet; br, broad.

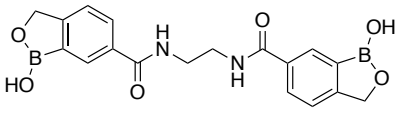
Low resolution mass spectra (LRMS) were recorded on a Bruker Esquire 2000 spectrometer using electrospray ionisation (ESI) and high resolution mass spectra (HRMS) were recorded on either a Bruker HCT or Bruker HCT Ultra spectrometer. M/z values are reported in Daltons.

Infrared (IR) spectra were recorded on a Perkin-Elmer Avatar 320 FTIR spectrometer. Solids were compressed into a thin tablet and oils/liquids were analysed as films over a diamond sensor. Absorption maxima (υ_{max}) are recorded in wavenumbers (cm^{-1}) and classified as strong (s) or broad (br).

In addition to those specified above, the following abbreviations, designations and formulas are used throughout the supporting information: Aromatic (Ar), molecular weight (MW).

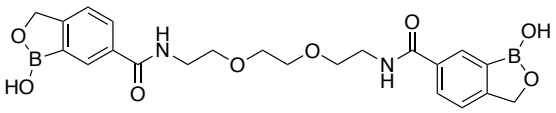
Synthetic procedures

***N,N'*-(ethylenediamine)bis-(6-(1-hydroxy-1,3-dihydro-2,1-benzoxaborole))amide (2)**



1-Hydroxy-1,3-dihydro-2,1-benzoxaborole-6-carboxylic acid (300 mg, 1.69 mmol) was dissolved in anhydrous DMF (50 mL) and (benzotriazole-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (965 mg, 1.85 mmol) and triethylamine (470 μL, 3.37 mmol) were then added at room temperature. The reaction mixture was stirred for 10 mins under nitrogen at room temperature and then ethylenediamine (51 μL, 0.759 mmol) was added. The reaction mixture was then stirred at room temperature overnight before concentrating *in vacuo*. Chloroform (10 mL) was added to the residue and left stirring overnight at room temperature. The resulting precipitate was collected by filtration and washed with chloroform (2 x 10 ml) and water (3 x 10 mL). The resulting solid was dried in desiccator overnight to give the product as a pale orange solid (243 mg, 84%). ^{1}H NMR (300MHz, MeOD) δ_{ppm} 8.63 (2H, br s, NH), 7.99 - 8.34 (2H, m, Ar H), 7.75-7.96 (4H, m, Ar H), 7.44 (2H, t, J = 7.5 Hz, Ar H), 3.64 (4H, m, CONHC$\underline{H}_2$). ^{13}C NMR (100MHz, $CDCl_3$) δ_{ppm} 169.7 (C=O), 136.6 (Ar CH), 133.3 (Ar CC=O), 132.3 (Ar CH), 128.6 (Ar CH), 127.4 (Ar CH), 39.6 (NHC$\underline{H}_2$), (CB(OH_2) not observed). HRMS *m/z* (ES^+): $[M+Na]^+$ calcd. for $C_{16}H_{18}B_2N_2O_6Na^+$, 379.1243; found 379.1242. IR cm^{-1}: 3292 br (O-H), 2936 s (C-H), 1634 s (C=O).

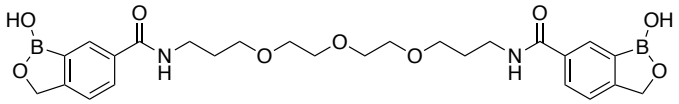
***N,N'*-(2,2-(ethylenedioxy)bis(ethylamine))bis-(6-(1-hydroxy-1,3-dihydro-2,1-benzoxaborole))amide (3)**



1-Hydroxy-1,3-dihydro-2,1-benzoxaborole-6-carboxylic acid (300 mg, 1.69 mmol) was dissolved in anhydrous DMF (50 mL) and (benzotriazole-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (965 mg, 1.85 mmol) and triethylamine (470 μL, 3.37 mmol) were then added at room temperature. The reaction mixture was stirred for 10 mins under nitrogen at room temperature and then 2,2-(ethylenedioxy)bis(ethylenediamine) (111 μL, 0.759 mmol) was added. The reaction mixture was then stirred at room temperature overnight before concentrating *in vacuo*. Chloroform (10 mL) was added to the residue and left stirring overnight at room temperature. The resulting precipitate was collected by filtration and washed with chloroform (2 x 10 ml) and water (3 x 10 mL). The resulting solid was dried in desiccator overnight to give the product as a pale orange solid (319 mg, 90%).

^{1}H NMR (400MHz, MeOD) δ_{ppm} 8.37-8.61 (2H, m, NH), 7.97-7.27 (2H, m, Ar H)), 7.75-7.96 (4H, m, Ar H), 7.31-7.54 (2H, m Ar H), 3.63 – 3.72 (8H, m, OC$\underline{H}_2$), 3.53 – 3.61 (4H, m CONHC$\underline{H}_2$). ^{13}C NMR (100MHz, MeOD) δ_{ppm} 164.8 (C=O), 138.1 (Ar C), 134.8 (Ar CC=O), 133.7 (Ar CH), 130.0 (Ar CH), 128.8 (Ar CH), 71.3 (O$\underline{C}H_2$), 70.6 (O$\underline{C}H_2$), 40.9 (NH$\underline{C}H_2$), (CB(OH_2) not observed). HRMS *m/z* (ES^+): $[M+Na]^+$ calcd. for $C_{20}H_{26}B_2N_2O_8Na^+$, 467.1767; found 467.1773. IR cm^{-1}: 3315 br (O-H), 2934 s (C-H), 1699 s (C=O).

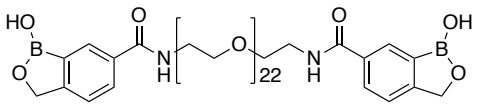
***N,N'*-(4,7,10-trioxa-1,13-tridecanediamine)bis-(6-(1-hydroxy-1,3-dihydro-2,1-benzoxaborole))amide (4)**



1-Hydroxy-1,3-dihydro-2,1-benzoxaborole-6-carboxylic acid (300 mg, 1.69 mmol) was dissolved in anhydrous DMF (50 mL) and (benzotriazole-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (965 mg, 1.85 mmol) and triethylamine (470 μL, 3.37 mmol) were then added at room temperature. The reaction mixture was stirred for 10 mins under nitrogen at room temperature and then 4,7,10-trioxa-1,13-tridecanediamine (166 μL, 0.759 mmol) was added. The reaction mixture was then stirred at room temperature overnight before concentrating *in vacuo*. Chloroform (10 mL) was added to the residue and left stirring overnight at room temperature. The resulting precipitate was collected by filtration and washed with chloroform (2 x 10 ml) and water (3 x 10 mL). The resulting solid was dried in desiccator overnight to give the product as a pale orange solid (242 mg, 59%).

^{1}H NMR (400MHz, MeOD) δ_{ppm} 8.16 (2H, m, Ar H), 7.78-7.94 (4H, m, Ar H), 7.43 (2H, t, J = 7.5 Hz, Ar H), 3.53 – 3.67 (12H, m, OC$\underline{H}_2$), 3.47 (4H, t, J = 6.5 Hz, CONHC$\underline{H}_2$), 1.87 (4H, quin, J = 6.0 Hz, $CH_2C\underline{H}_2CH_2$). ^{13}C NMR (100MHz, MeOD) δ_{ppm} 169.2 (C=O), 136.5 (Ar $\underline{C}$H), 133.6 (Ar $\underline{C}$C=O), 132.1 (Ar $\underline{C}$H), 128.5 (Ar $\underline{C}$H), 127.4 (Ar $\underline{C}$H), 70.1 (O$\underline{C}H_2$), 69.8 (O$\underline{C}H_2$), 69.0 (O$\underline{C}H_2$) 37.4 (NH$\underline{C}H_2$), 29.0 ($CH_2\underline{C}H_2CH_2$) ($\underline{C}B(OH_2)$ not observed). HRMS *m/z* (ES^+): $[M+Na]^+$ calcd. for $C_{24}H_{34}B_2N_2O_9Na^+$, 539.2348; found 539.2397. IR cm^{-1}: 3331 br (O-H), 2871 s (C-H), 1625 s (C=O).

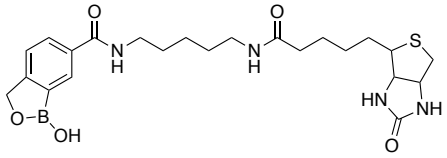
***N,N'*-(polyoxyethylene bis(amine))bis-(6-(1-hydroxy-1,3-dihydro-2,1-benzoxaborole))amide (5)**



1-Hydroxy-1,3-dihydro-2,1-benzoxaborole-6-carboxylic acid (100 mg, 0.562 mmol) was dissolved in anhydrous DMF (15 mL) and (benzotriazole-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (322 mg, 0.618 mmol) and triethylamine (157 μL, 1.12 mmol) were then added at room temperature. The reaction mixture was stirred for 10 mins under nitrogen at room temperature and then poly(ethyleneglycol)bisamine MW1000 (253 mg, 0.253 mmol) was added. The reaction mixture was then stirred at room temperature for 16 hours before concentrating *in vacuo*. The residue was taken up in water (50 mL) and extracted with DCM (4 x 50 mL). The aqueous phase was concentrated *in vacuo* taken up in water (10 mL), dialysed against water (100-500 MW dialysis tubing), and lyophilised to yield the desired product **5** as a colourless oil (231 mg, 69%).

^{1}H NMR (400MHz, MeOD) δ_{ppm} 8.01 – 8.25 (2H, m, Ar H)), 7.61-8.01 (4H, m, Ar H), 7.26 – 7.52 (2H, m, Ar H), 3.49 – 3.84 (90H, m, OC$\underline{H}_2$), 3.39 -3.48 (4H, m, CONHC$\underline{H}_2$). ^{13}C NMR (100MHz, D_2O) δ_{ppm} 171.5 ($\underline{C}$=O), 136.4 (Ar$\underline{C}$H), 132.7 (Ar$\underline{C}$C=O), 131.1 (Ar$\underline{C}$H), 131.1 (Ar$\underline{C}$H), 128.0 (Ar$\underline{C}$H), 71.7, 69.6, 68.9, 68.7 (O$\underline{C}H_2$), 39.5 (NH$\underline{C}H_2$). IR cm^{-1}: 3353 br (O-H), 2880 s (C-H), 1643 s (C=O).

Biotinamido(hexanamido)(6-(1-hydroxy-1,3-dihydro-2,1-benzoxaborole)) (7)



1-Hydroxy-1,3-dihydro-2,1-benzoxaborole-6-carboxylic acid (16 mg, 0.091 mmol), was dissolved in anhydrous DMF (5 mL) and (benzotriazole-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (52 mg, 0.100 mmol) and triethylamine (25 μL, 0.182 mmol) were then added at room temperature. The reaction mixture was stirred at room temperature for 10 min under nitrogen and then EZ link pentylamine-biotin (30 mg, 0.091 mmol) was added. The reaction mixture was then stirred at room temperature for 16 hours before concentrating *in vacuo*. Chloroform (10 mL) was added to the residue and the mixture stirred at room temperature for 2 hours. The resulting precipitate was collected by filtration and washed with chloroform (2 x 10 ml) and water (3 x 10 mL). The resulting solid was dried in desiccator overnight to give the product as an off white solid (25 mg, 56%).

^{1}H NMR (500 MHz, MeOD) δppm 8.12 (1H, d, *J* = 1.5 Hz, ArH), 7.95 (1H, dd, *J* = 8.0, 1.5 Hz, ArH), 7.50 (1H, d, *J* = 8.0 Hz, ArH), 5.15 (2H, s, BOCH_2), 4.50 (1H, dd, *J* = 8.0, 5.0 Hz, SCH_2CHNH), 4.30 (1H, dd, *J* = 8.0, 4.5 Hz, SCHCHNH), 3.41 (2H, t, *J* = 7.0 Hz, ArCONHCH_2), 3.13 – 3.26 (3H, m, SCH + CH_2CONHCH_2), 2.94 (1H, dd, *J* = 13.0, 5.0 Hz, SC$H_a$$H_b$), 2.72 (1H, d, *J* = 12.5 Hz, SC$H_a$$H_b$), 2.20 (2H, t, *J* = 7.5 Hz, COCH_2), 1.54 – 1.77 (8H, m, 4 x CH_2), 1.39 – 1.48 (4H, m, 2 x CH_2). ^{13}C NMR (125MHz, MeOD) δppm 174.6 (NHCOCH_2), 168.9 (ArCONH), 164.7 (NHCONH), 157.2 (ArCCH_2), 133.5 (ArCCO), 129.6 (ArCH), 128.7 (ArCH), 121.1 (ArCH), 70.9 (BOCH_2), 62.0 (SCHCHNH), 60.2 (SCH_2CHNH), 55.6 (SCH), 39.6 (SCH_2), 39.5 (ArCONHCH_2), 38.8 (CH_2NHCOCH_2), 35.4 (CH_2NHCOCH_2), 28.7 (CH_2), 28.7 (CH_2), 28.4 (CH_2), 28.1 (CH_2), 25.5 (CH_2), 23.9 (CH_2), (CB(OH_2) not observed). HRMS m/z (ES+): $[M+Na]^+$ calcd. for $C_{23}H_{33}BN_4O_5SNa^+$, 511.2157; found 511.2159.

Supplementary Biological protocols

Bacterial strains, cell lines, culture conditions and chemicals

Mycobacterium smegmatis mc^2155 (ATCC-700084) was routinely grown at 37 °C in either Middlebrook 7H9 broth (BD Difco) supplemented with 0.2 % glycerol and 0.05 % Tween 80, Tryptic Soy Broth (TSB) supplemented with 0.05 % Tween 80 or on Luria-Bertani (LB) agar. *Mycobacterium bovis* BCG (ATCC-35734) and *Mycobacterium tuberculosis* H37Rv were routinely grown at 37 °C in Middlebrook 7H9 broth supplemented with 0.2 % glycerol, 0.05 % Tween 80 and 10% albumin-dextrose-catalase (ADC) or on Middlebrook 7H10 plates supplemented with 0.5 % glycerol and 10% oleic acid-albumin-dextrose-catalase (OADC) in containment level 2 or 3 laboratories respectively. *Escherichia coli* (Top 10) and *Pseudomonas putida* were routinely cultured in LB medium at 37 °C and 30 °C respectively. Human alveolar basal epithelial A549 cells (Public Health England, ECACC 86012804) were cultured at 37 °C with 5 % CO_2 atmosphere in Ham's F-12K (Kaighn's) Medium (Gibco, UK) supplemented with 10 % fetal-bovine serum, 100 Units/mL penicillin, 100 µg/mL streptomycin, and 250 ng/mL amphotericin B (HyClone). Ovine red blood cells were purchased from TCS Biosciences. PBST is phosphate buffered saline supplemented with 0.05% Tween 80. All chemicals were purchased from Sigma-Aldrich unless otherwise stated. Trehalose dimycolate (TDM) (Sigma-Aldrich #T3034) was from *Mycobacterium bovis.*

Mycobacterium tuberculosis H37Rv reagents

The following reagents were obtained through BEI Resources, NIAID, NIH. All reagents are from *Mycobacterium tuberculosis* H37Rv*:* Purified Lipoarabinomannan (LAM), NR-14848; Purified Lipomannan (LM), NR-14850; Purified Arabinogalactan, NR-14852; Purified Peptidoglycan, NR-14853; Purified Trehalose Monomycolate (TMM), NR-48784.

Alizarin Red S assay

Boroxoles **1-4** (3 mM final concentration) were dissolved in alizarin red S (ARS) (0.144 mM, in sodium phosphate buffer (0.1 M, pH 7.4) with 2% DMSO) and added to the selected carbohydrate (0.5 M final concentration). The carbohydrate was serially diluted with solution A from 0-0.5 M and the absorbance determined at 453 nm (BioTek Synergy HT Microplate Reader).

Determination of minimum inhibitory concentrations

The minimum inhibitory concentrations (MIC) of all compounds were determined using the resazurin reduction microplate assay (REMA) as described previously [5]. *M. smegmatis, M. bovis* BCG, *M. tuberculosis*, *E. coli and P. putida* were grown to mid-log phase and the inoculum standardized to 1 x 10^6 colony forming units (CFU)/mL before addition to the prepared 96-well flat-bottom microtiter plate with 2-fold serial dilutions of each drug in media. An antibiotic control was also added to each plate (rifampicin for *M. smegmatis, M. bovis* BCG and *M. tuberculosis*, ampicillin for *E. coli* and tetracycline for *P. putida*) and the last column of the plate was used as a control without the addition of compound. The plates were incubated without shaking

for 7 days (*M. bovis* BCG and *M. tuberculosis),* 24 hours (*M. smegmatis*), 16 hours (*E. coli* and *P. putida*) before addition of 25 μL resazurin (one tablet of resazurin (VWR) dissolved in 30 mL of sterile PBS). Following a further 24 hour incubation at 37 °C for mycobacteria or 2 hour incubation of *E. coli* and *P. putida* 37 °C and 30 °C respectively the plates were assessed for colour development. The MIC values were determined as the lowest concentration of drug that prevented the colour change of resazurin (blue – no bacterial growth) to resofurin (pink – bacterial growth).

Cytotoxicity assay

The cytotoxicity of the compounds was measured against a human lung epithelial cell line (A549). Briefly, cells were incubated (10^4 cells/well) with 2-fold serial dilutions of compounds **2** - **4** along with relevant DMSO concentration controls in a 96 well plate (Costar #3596) including a cell only control. Following incubation for 24 hours at 37 °C, 5 % CO_2, cell viability was determined by removing compound solutions and adding 100 μL alamarBlue™ (Thermo Fisher #DAL1025) to each well for 4 h at 37 °C, then measuring the absorbance of the resorufin metabolite at 570 nm (BioTek Synergy HT microplate reader). All samples were ran in triplicate.

Haemolysis assay

The agglutination activity of compounds **2-4** were tested against ovine red blood cells. Samples were prepared by dissolving compounds in PBS containing 25% DMSO. Two-fold serial dilutions were then carried out diluting with PBS. A positive control comprised lysis buffer (10 mM Tris, pH 7.8, 0.32 M sucrose, 5 mM $MgCl_2$, 10% Triton X-100), and the negative control comprised PBS (plus relevant % DMSO). 100 μL of ovine blood was incubated with 100 μL compound to give final concentrations of 0.078 - 5 mM for compounds **2** and 0.156 - 10 mM for compounds **3** and **4** and samples incubated at room temperature for 1 hour, after which they were centrifuged for 5 min (2,000 *x g*, 22 °C). 20 μL of the supernatant was added to 750 μL of AHD solution (40 mM Triton X-100, 100 mM NaOH) and 200 μL was then added to 96 well plate and the absorbance read at 580 nm (BioTek Synergy HT Microplate Reader). The percentage lysis was calculated by comparison to the no compound control. All assays were undertaken with 3 independent experimental repeats.

Agglutination assay

The agglutination activity of compounds **2-4** were tested against ovine red blood cells. Samples were prepared by dissolving compounds in PBS containing 25% DMSO. Two-fold serial dilutions were then carried out diluting with PBS. 100 μL of ovine blood was incubated with 100 μL compound to give final concentrations of 0.078 - 5 mM for compounds **2** and 0.156 - 10 mM for compounds **3** and **4**. Separately, 100 μL of 25% polyethylenimine was added as a positive control or 100 μL PBS (plus relevant % of DMSO) as a negative control. Following addition of the compounds, the ovine blood was incubated at room temperature for 1 hour and then the sample (50 μL) was added to a round-bottom 96-well microtiter plate (Corning, #3790) and incubated at room temperature for a further 30 min. The plate was then assessed for signs of agglutination (small red pellet at bottom of wells = no agglutination, red colouring across whole well = agglutination). All

assays were undertaken with three independent experimental repeats.

Determination of compound interactions using a REMA checkerboard assay

A checkerboard assay was used to evaluate whether compound combinations act synergistically, antagonistically, or additively between compounds **3-4** with either meropenem or rifampicin against *M. tuberculosis*[1]. Compounds **3** or **4** were serially diluted two-fold horizontally across the plate to give the final concentrations: 0.098 – 12.5 mM (compound **3**) and 0.049 – 6.25 mM (compound **4**) and either 5.93 – 759 nM (rifampicin) or 1.02 – 130 μM (meropenem) were serially diluted two-fold vertically down the plate. Control wells in which compound **3** or **4** and rifampicin or meropenem alone were tested. *M. tuberculosis* was grown to mid-log phase and the inoculum standardised to 1 x 10^6 colony forming units (CFU)/mL before addition to the plate. The plates were incubated for 7 days at 37 °C without shaking before addition of 25 μL resazurin (one tablet of resazurin (VWR) dissolved in 30 mL of sterile PBS). Following a further 24 hours incubation at 37 °C the plates were assessed for a colour change of the resazurin from blue (no bacterial growth) to pink (bacterial growth). Fractional inhibitory concentrations (FICs) were calculated by use of the following formula: FIC ($X + Y$) = [MIC of compound X in combination with Y]/[MIC of X alone]. The fractional inhibitory index (ΣFIC) was calculated as FIC of compound X + FIC of compound Y to evaluate interaction profiles. ΣFICs of ≤ 0.5 designate synergistic activity, ΣFICs of ≥ 4.0 indicate antagonism, and values in between correspond to additivity, as outlined in previous antibacterial combination studies [1-3].

Generation of spontaneous resistant mutant strains

M. bovis BCG at 1x10^8 CFUs was plated onto Middlebrook 7H10 agar supplemented with 0.5 % glycerol and 10% OADC containing 5 × MIC of compound **1** or **3**. A control in which no compound was added was also performed. The plates were then incubated at 37 °C for 3 months. No colonies were observed on the Middlebrook 7H10 agar plates that contained compound **3**. Colonies observed on the plate with compound **1** were selected individually and grown to an OD_{600} of 0.6 and then streaked onto Middlebrook 7H10 agar supplemented with 0.5 % glycerol and 10% OADC containing 5 × MIC of compound **1**. Colonies that grew were then grown in Middlebrook 7H9 broth supplemented with 0.2 % glycerol and 0.05 % Tween 80 and 2 x MIC of compound **1** to an OD_{600} of 0.8 - 1.0 and the gDNA extracted.

DNA extraction and sequencing

M. bovis BCG wild type and mutant strains BRX1-5 were grown in 10 mL Middlebrook 7H9 broth supplemented with 0.2 % glycerol and 0.05 % Tween 80 and 2 x MIC of compound **1** for the mutant strains to an OD_{600} of 0.8 - 1.0 at 37 °C. The cells were then harvested (2,916 *x g*, 10 mins, 22 °C) and the supernatant discarded. The pellet was resuspended in SET solution (250 μL, 25% sucrose, 50 mM Tris pH 8, 50 mM EDTA), lysozyme solution was added (50 μL, 20 mg/mL lysozyme, 50 mM Tris pH 8) and the sample incubated at 37 °C overnight. RNAse A (10 μL, 10 mg/mL) was then added and the sample incubated at 37 °C for a further 30 mins. Proteinase K solution (250 μL, 400 μg/mL proteinase K, 100 mM Tris pH 8, 1%

SDS) was added and the sample incubated at 55 °C for 2 hours. Phenol:chloroform:isoamyl alcohol (25:24:1, 500 μL) was then added and the sample mixed thoroughly by inversion and the sample centrifuged (16,200 *x g*, 5 mins, 22 °C). The top aqueous layer was taken and added to chloroform:isoamyl alcohol (24:1, 500 μL), mixed thoroughly by inversion and centrifuged (16,200 *x g*, 5 mins, 22 °C). The top aqueous layer was moved to a clean tube and sodium acetate (3 M, 40 μL) was added followed by ethanol (1 mL) and the sample incubated at -20 °C overnight. The sample was then centrifuged (16,200 *x g*, 15 mins, 4 °C), and washed with 500 μL 70% ethanol (16,200 *x g*, 15 mins, 4 °C). The supernatant was removed and the DNA resuspended in 40 μL H_2O and heated to 55 °C for 10 mins, then cooled and stored at -20 °C. The concentration of the gDNA was determined (NanoDrop2000) and the whole genome sequenced at NG Microbes.

Biolayer Interferometry

Polysaccharides and cell wall components were dissolved in water (if necessary DMSO was used to dissolve the compound initially before diluting with water to give a final DMSO concentration of 20%) and serially diluted with water (or water containing 20% DMSO) to give concentrations ranging from 0 - 200 μg/mL. Peptidoglycan did not fully dissolve and formed a suspension and were used at concentrations from 0 - 200 μg/mL. Biolayer Interferometry was carried out on ForteBio Octet Red96 (Forte Bio, USA). Assays were performed in black 96 well plates (Greiner Bio-one #655076). Assays were carried out at 30 °C. The wells were filled with 200 μL of either buffer or sample and agitated at 1,000 rpm. Streptavidin (SA) biosensor tips (Forte Bio, USA) were hydrated in distilled water for at least 10 min prior to use. The tips were functionalized by loading with the addition of 250 μg/mL biotinylated boroxole (in 20% DMSO) **7** for 5 mins followed by a 5 mins wash and equilibration step in water (LAM, LM, PG, GM-3 and LPS) or 20% DMSO (TDM, TMM and AG) to remove unbound **7** and a stable baseline established. Following immobilization of the biotinylated boronic acid **7**, the binding interaction with different concentrations of isolated cell components was carried out which included baseline (5 min), association (10 min), dissociation (10 min).

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1. Lechartier, B., Hartkoorn, R.C. & Cole, S.T. In Vitro Combination Studies of Benzothiazinone Lead Compound BTZ043 against *Mycobacterium tuberculosis*. *Antimicrob Agents and Chemother* **56**, 5790-5793 (2012).
2. Odds, F.C. Synergy, antagonism, and what the chequerboard puts between them. *J Antimicrob Chemother* **52**, 1-1 (2003).
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5. Palomino, J.C. et al. Resazurin microtiter assay plate: Simple and inexpensive method for detection of drug resistance in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* **46**, 2720-2722 (2002).

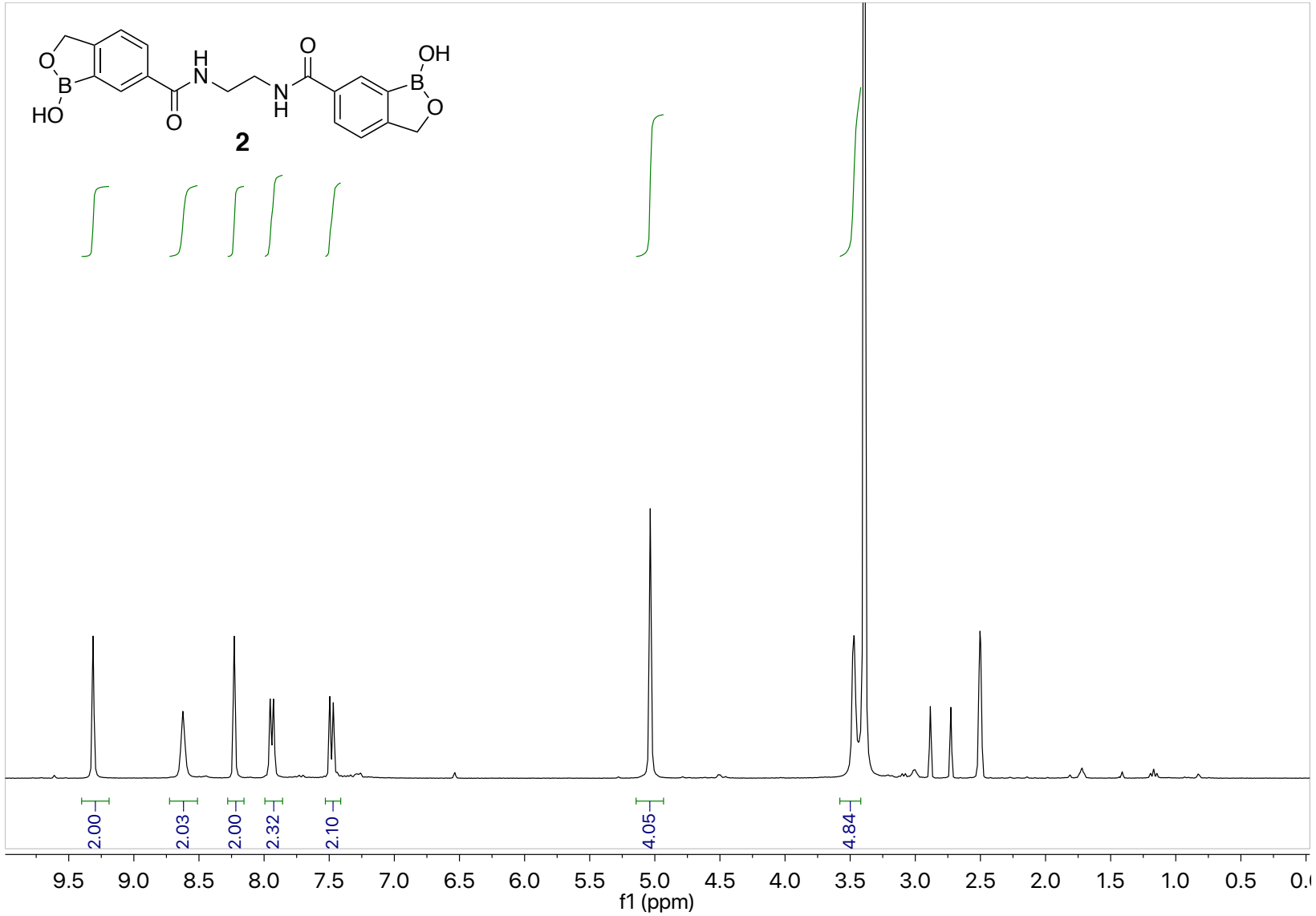

Figure S3. ^{1}H NMR of compound **2**.

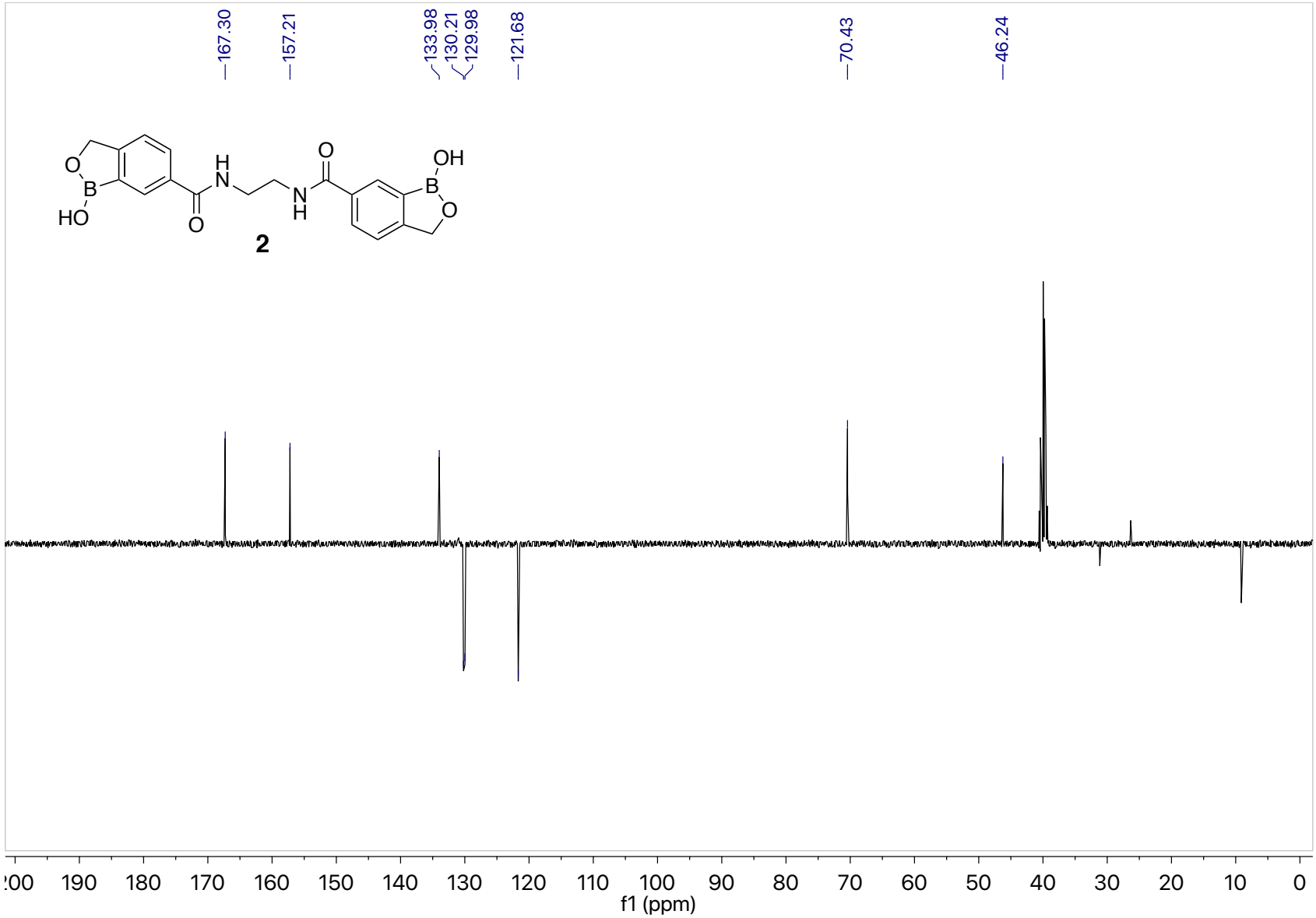

Figure S4. ^{13}C NMR of compound **2**.

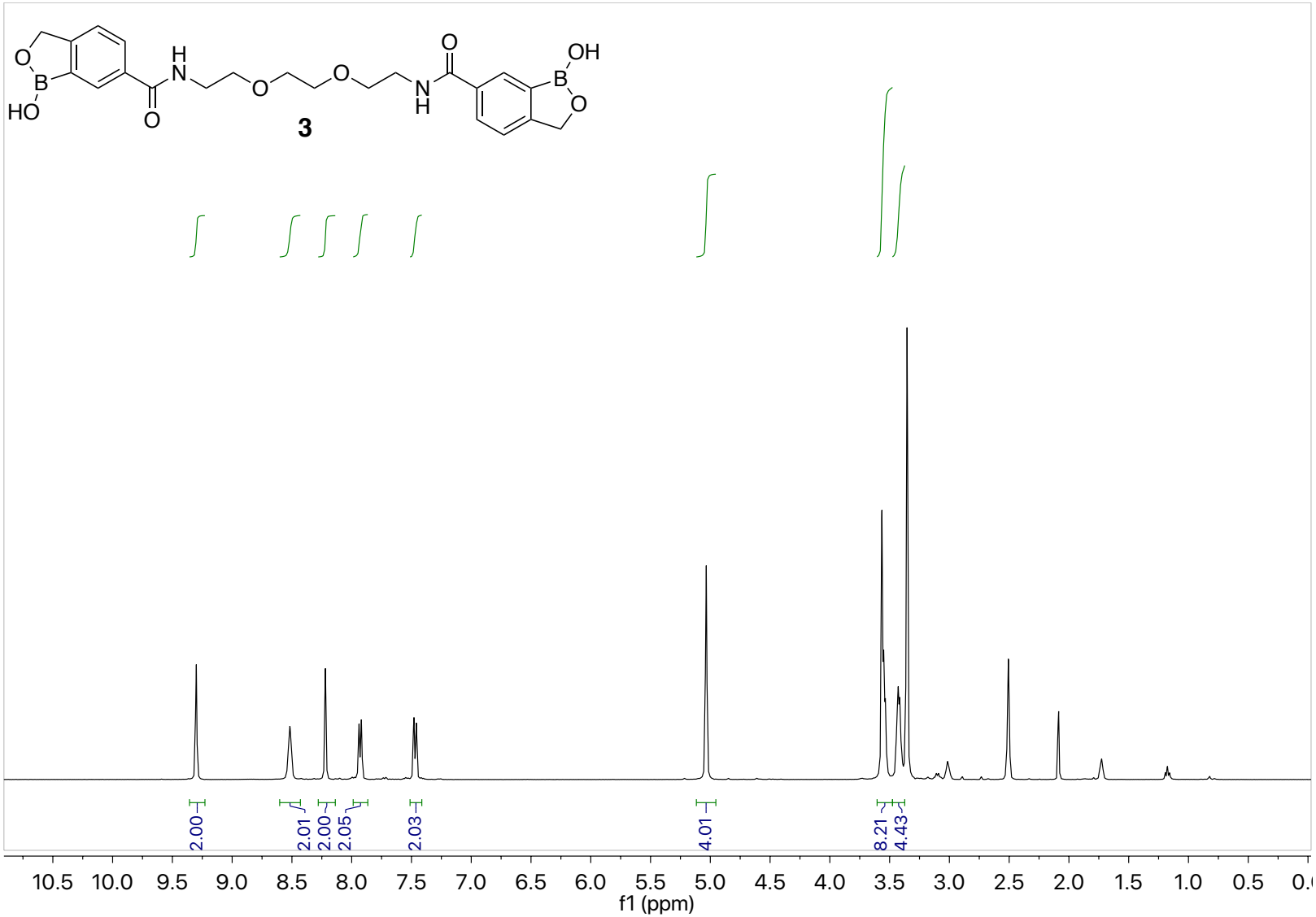

Figure S5. ^{1}H NMR of compound **3**.

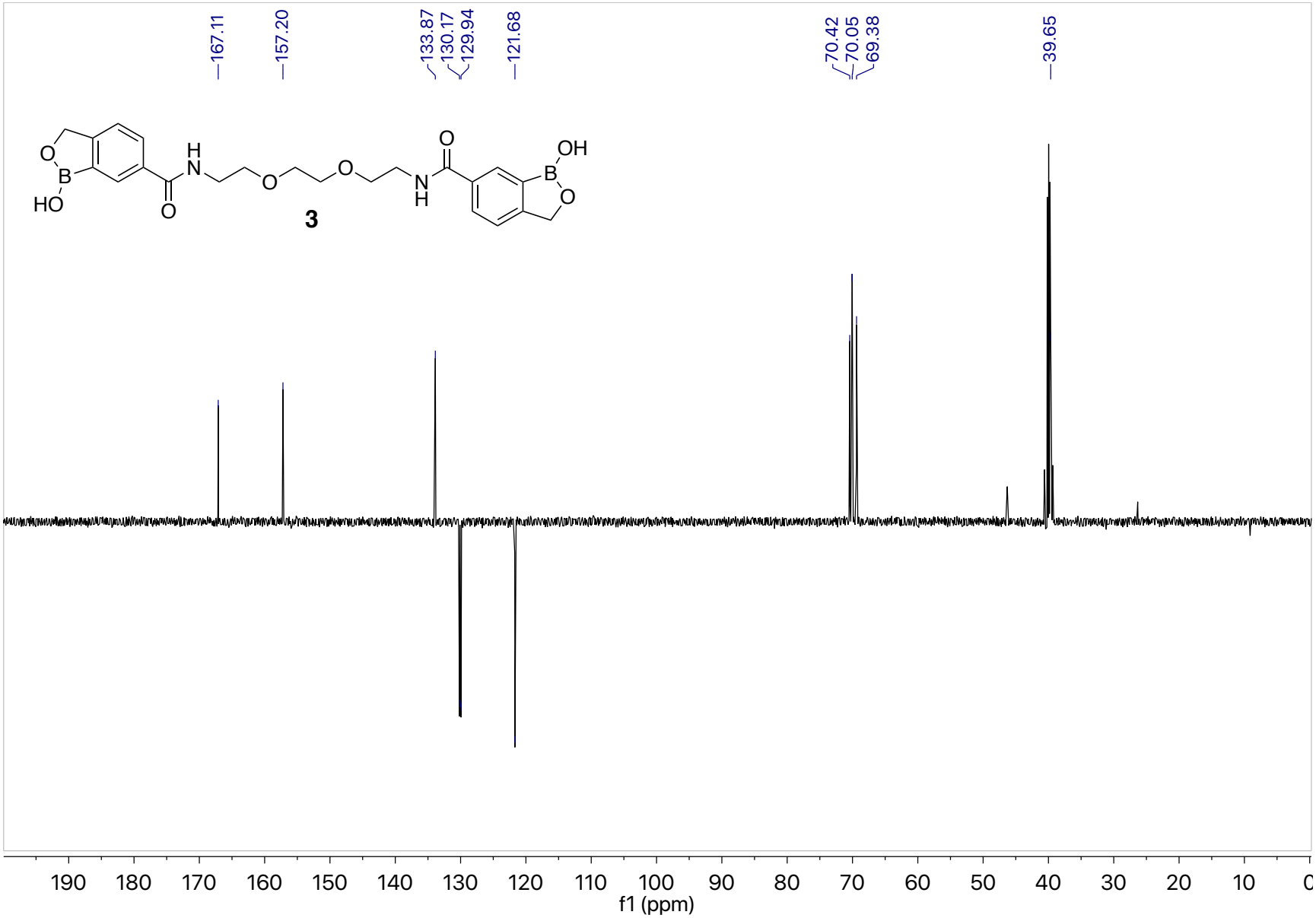

Figure S6. ^{13}C NMR of compound **3**.

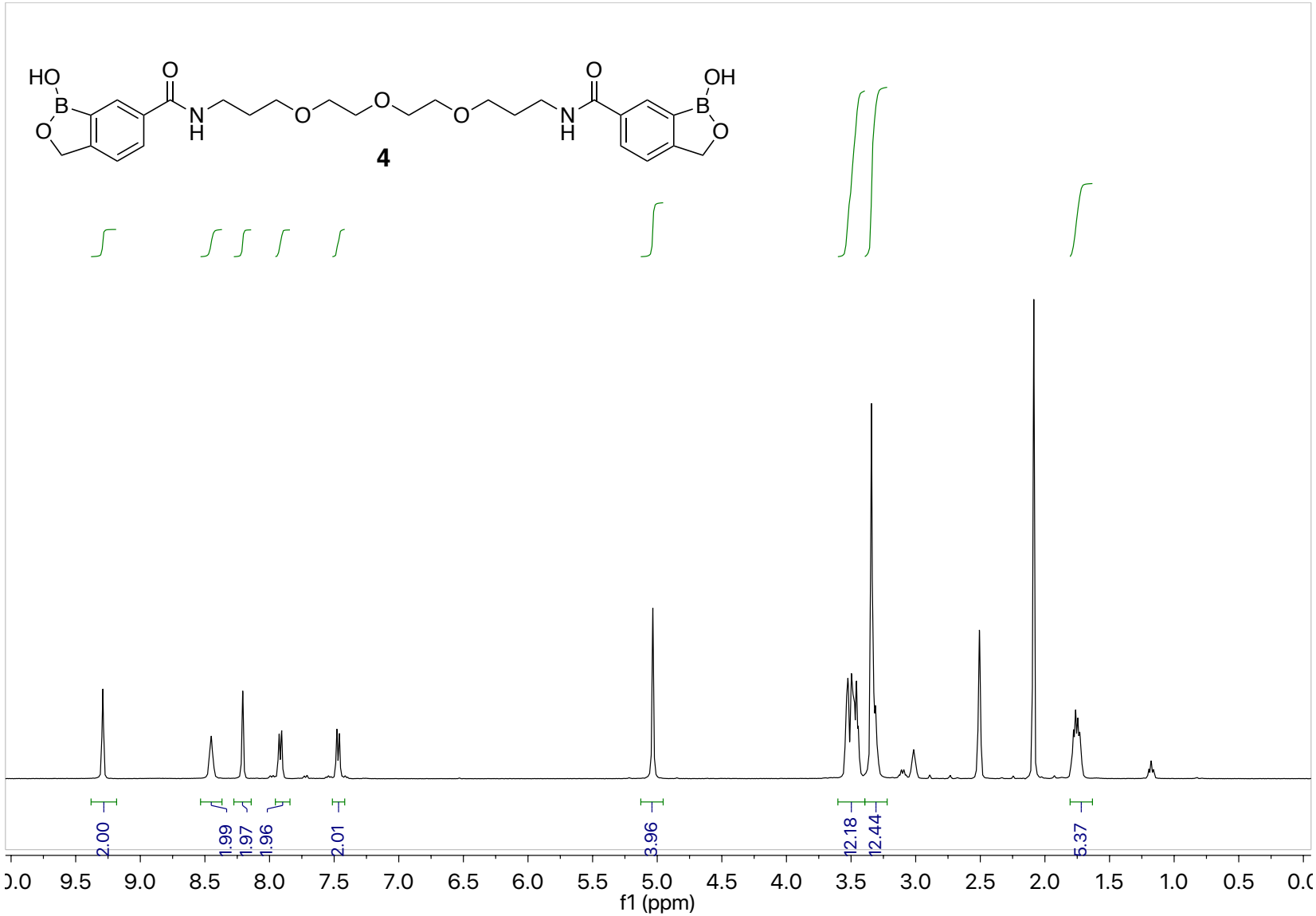

Figure S7. ^{1}H NMR of compound **4**.

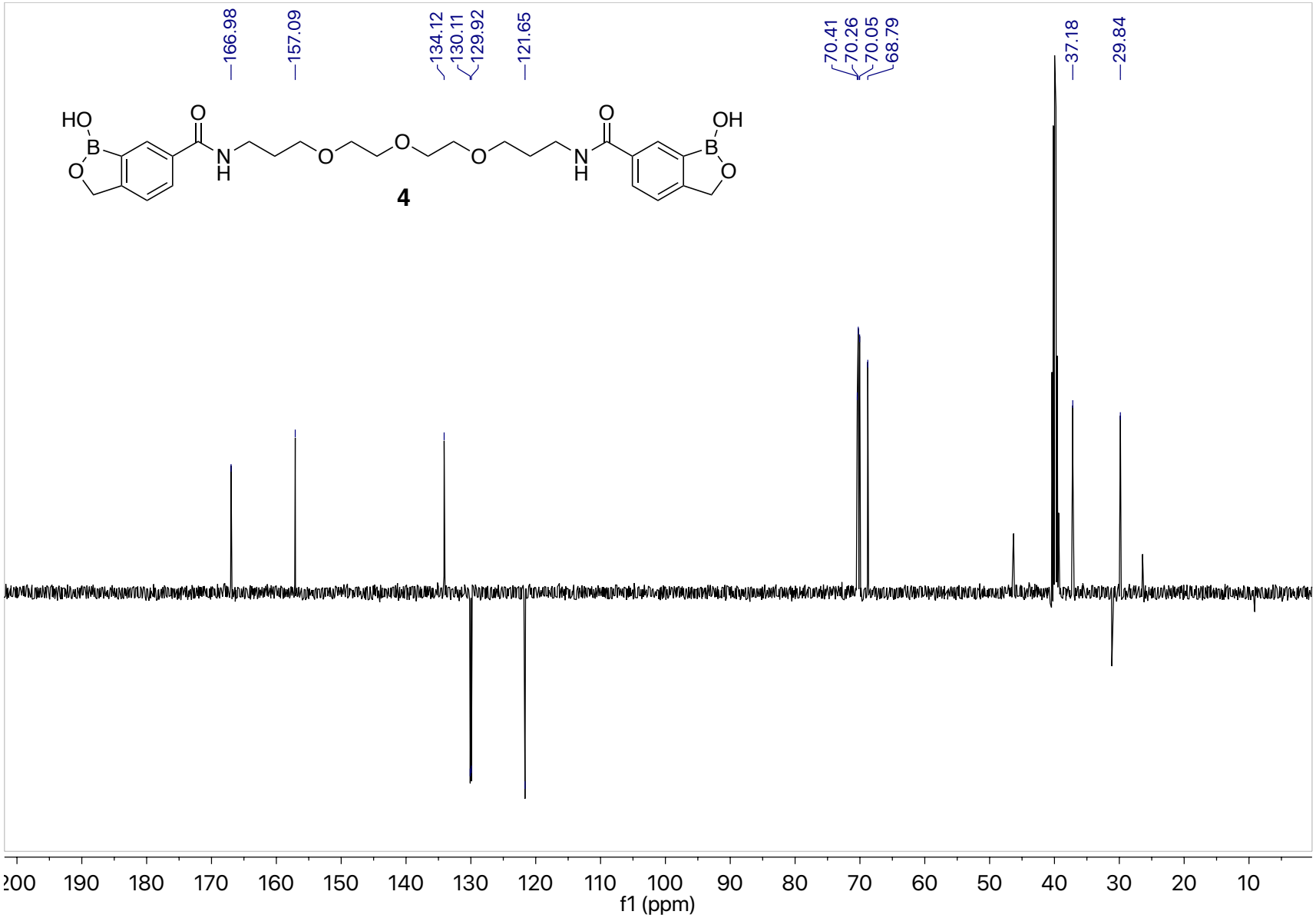

Figure S8. ^{13}C NMR of compound **4**.

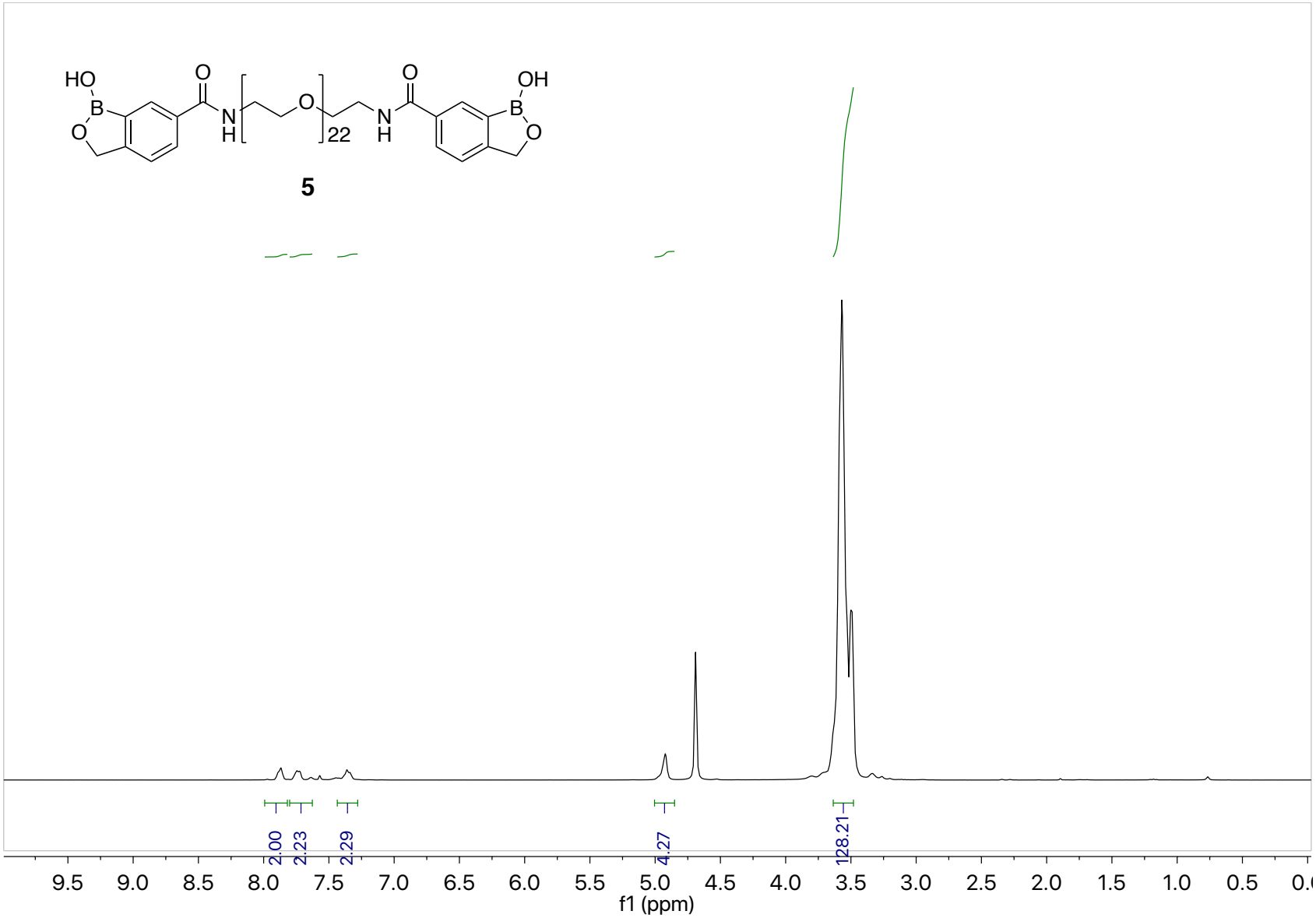

Figure S9. ^{1}H NMR of compound **5**.

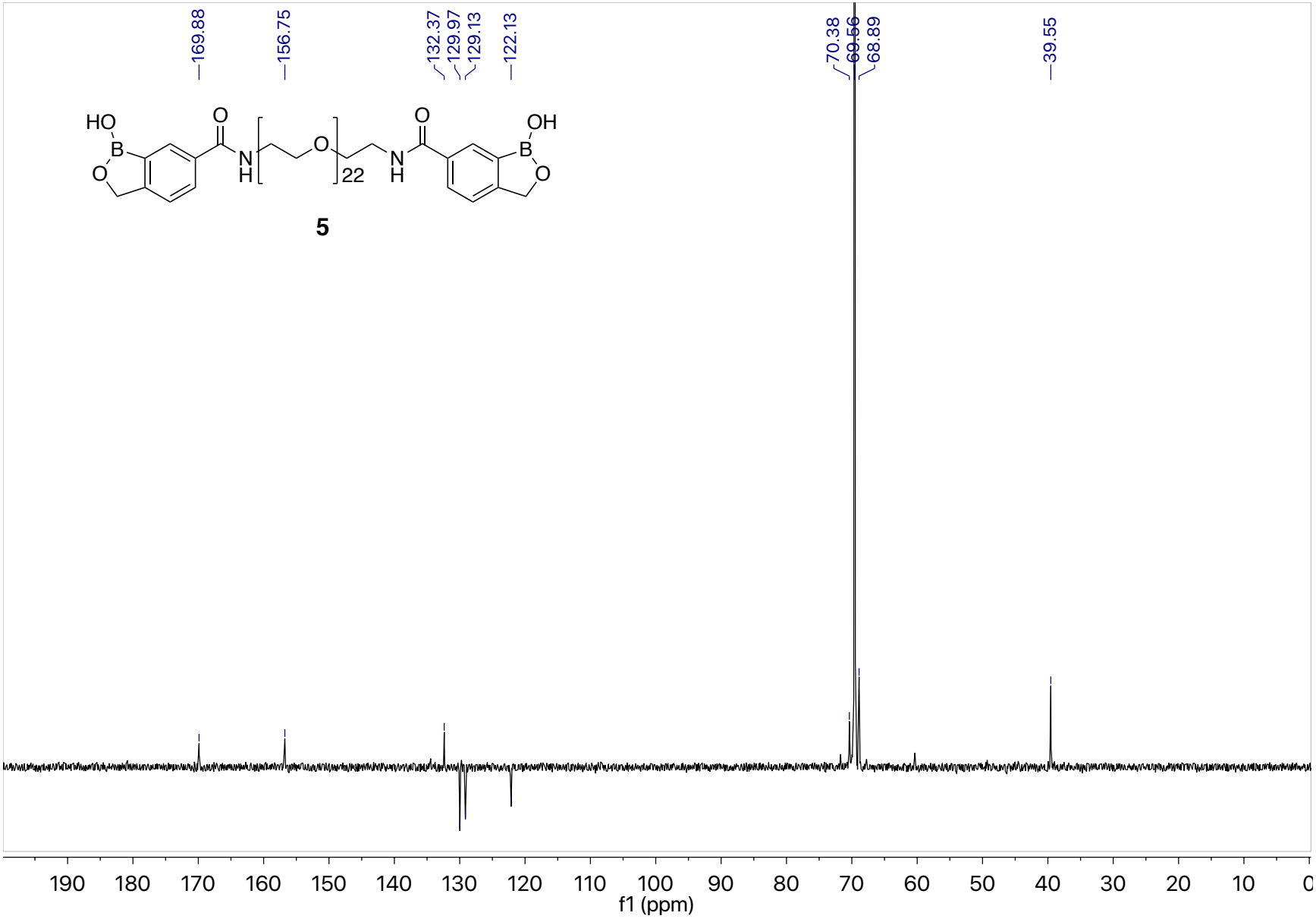

Figure S10. ^{13}C NMR of compound **5**.

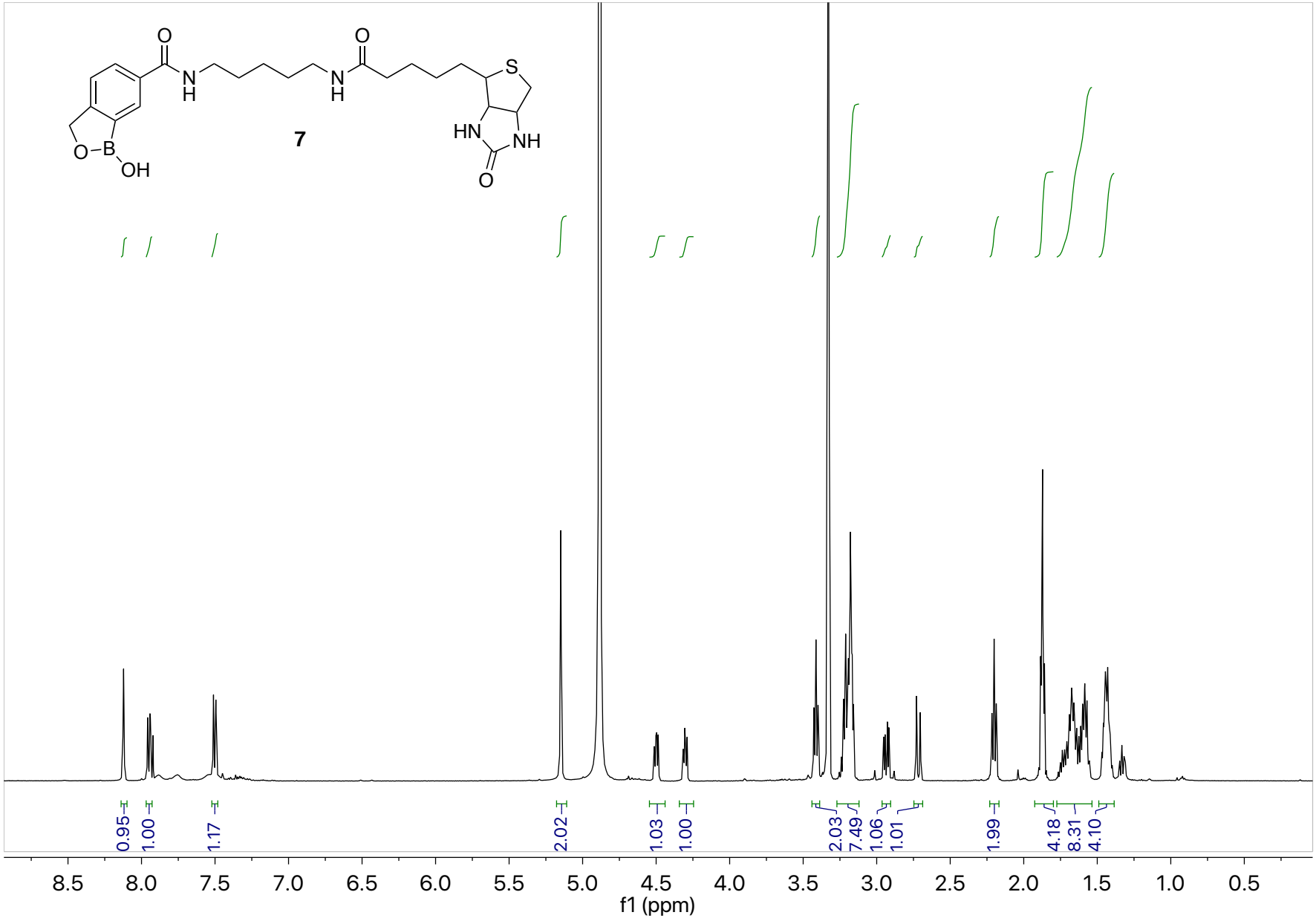

Figure S11. ^{1}H NMR of compound **7**.

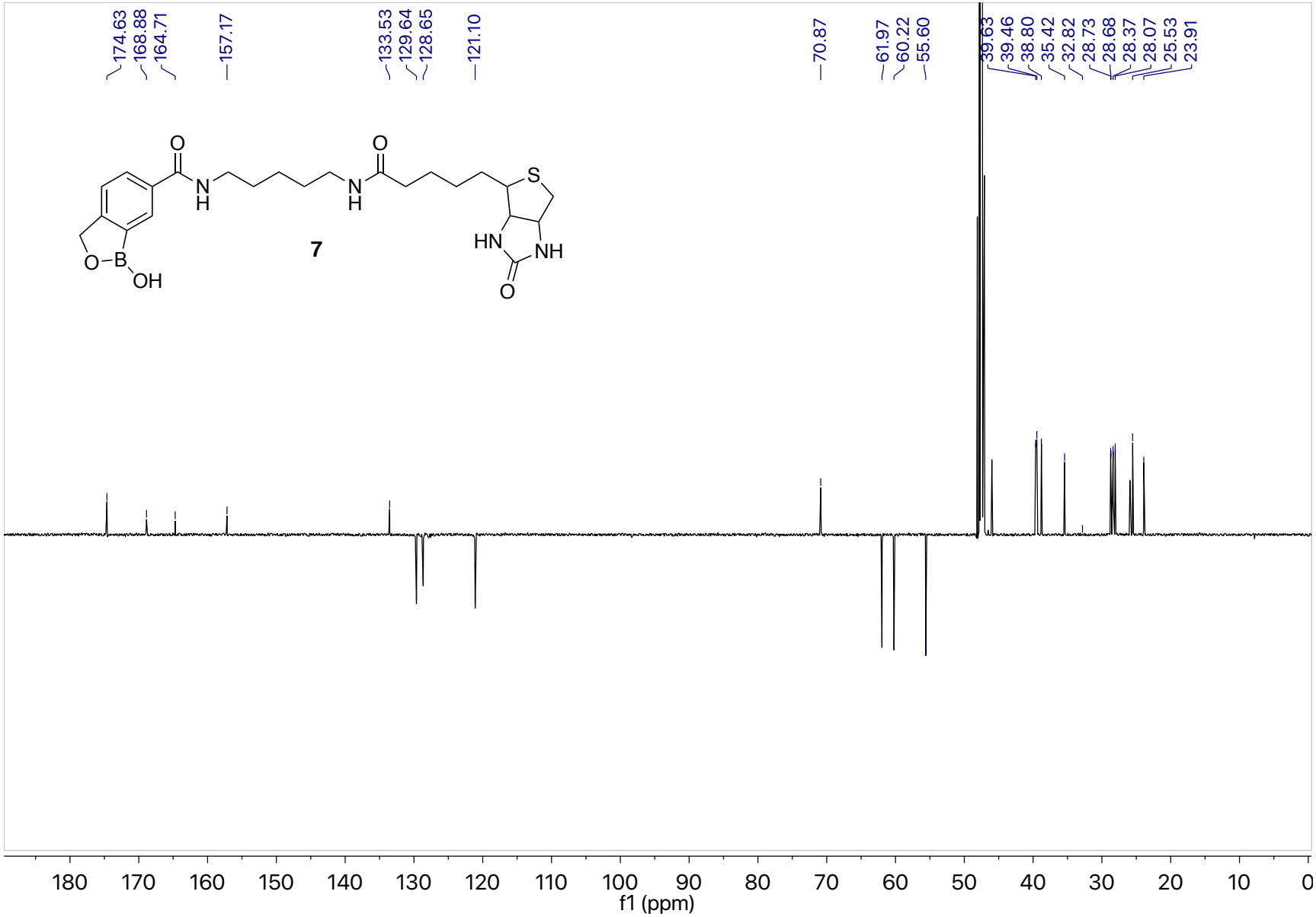

Figure S12. ^{13}C NMR of compound **7**.