

Boronic Acid Liposomes for Cellular Delivery and Content Release Driven by Carbohydrate Binding

Supplementary Information

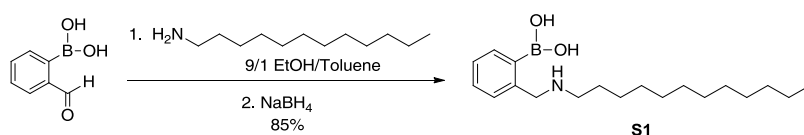
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Scheme S1. Synthesis of single-tail boronic acid lipid **S1**.

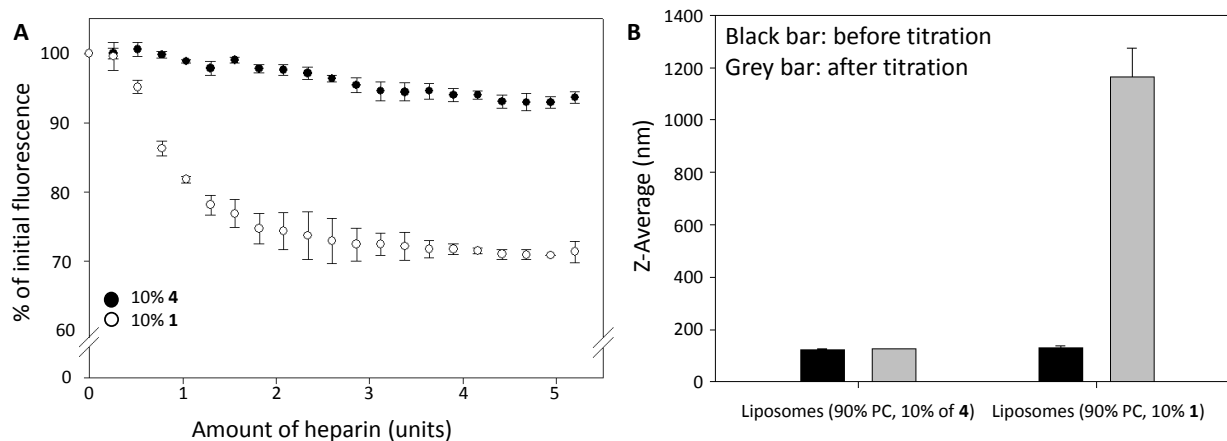


Figure S1. Nile red release and DLS results upon heparin titration using control compound **4**. **A.** Decreases in Nile red fluorescence were much greater for PC liposomes containing 10% of boronic acid lipid **1** compared to those instead containing 10% of control compound **4**. **B.** DLS results show that liposomes containing **1** exhibit large increases size upon heparin treatment while those that instead contain compound **4** do not.

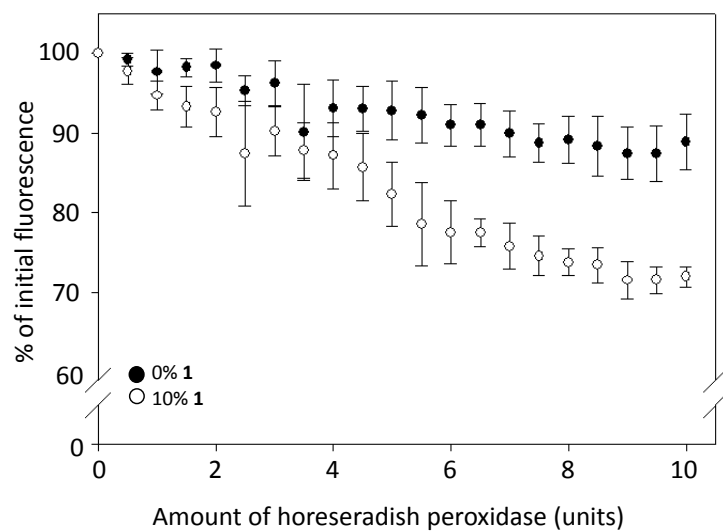


Figure S2. Nile red release titration of liposomes containing 1 with HRP glycoprotein. Decreases in Nile red fluorescence were greater for PC liposomes containing 10% of boronic acid lipid 1 compared to those PC liposomes lacking 1.

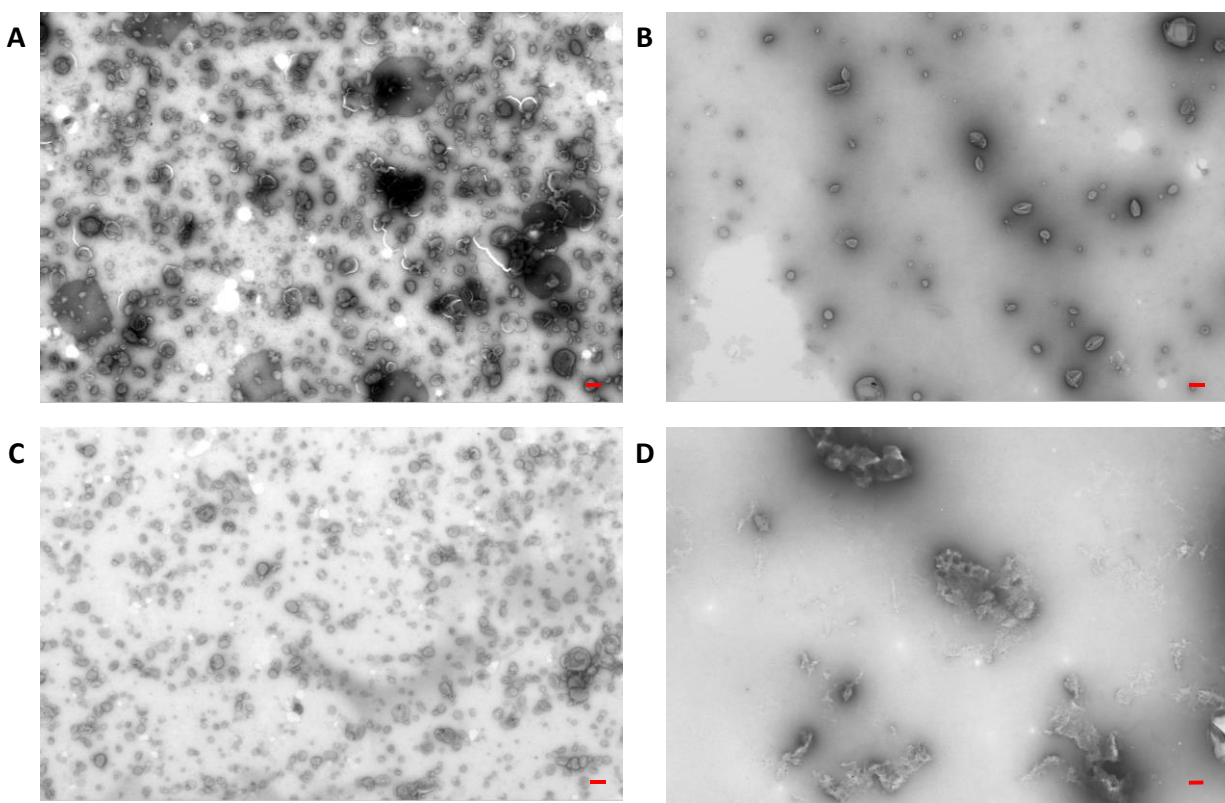


Figure S3. STEM Images. Images are shown for liposomes containing 0% of 1 without (A) and with (B) heparin treatment as well as those containing 10% of 1 without (C) and with (D) heparin treatment. Scale bars correlate with 200 nm.

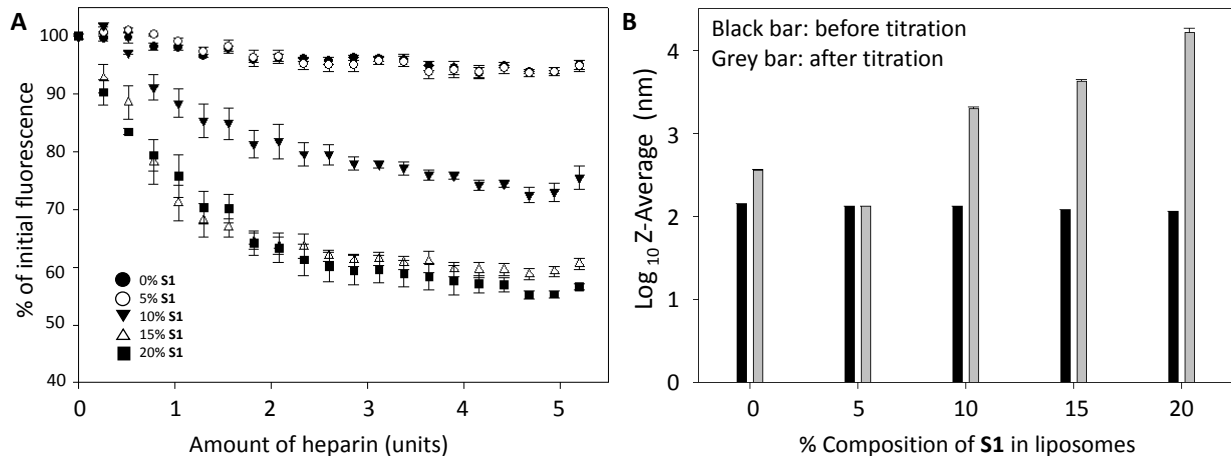


Figure S4. Boronic acid liposome Nile red release and DLS results upon heparin incubation using compound S1. A. Decreases in Nile red fluorescence attributed to release are dependent upon the percentage of boronic acid lipid **S1** in PC liposomes. **B.** DLS results show that untreated liposomes exhibit the expected sizes, while treatment of liposomes containing higher percentages (10-20%) of **S1** with heparin leads to much larger particle sizes. Error bars denote the standard errors of at least three replicates.

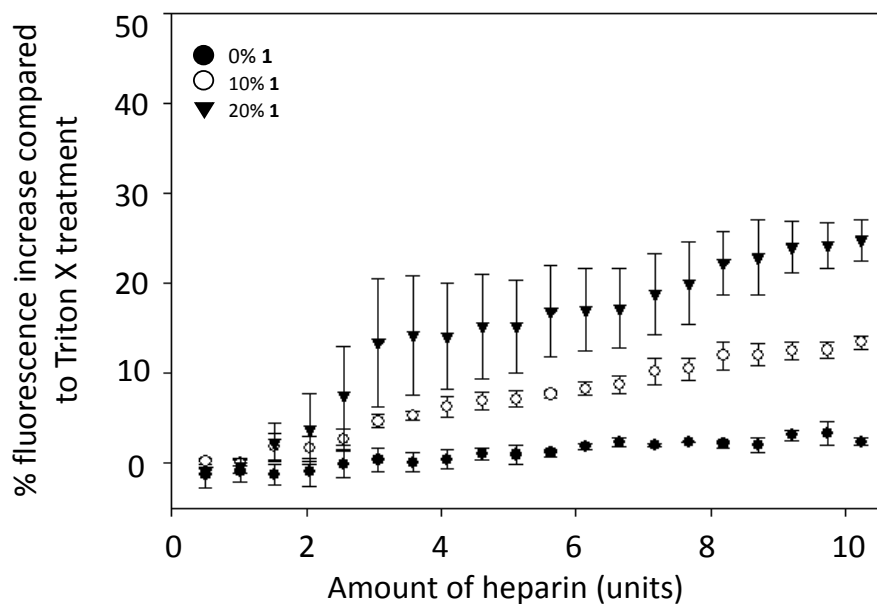


Figure S5. Boronic acid liposome sulforhodamine release upon heparin titration using S1. A. Fluorescence increases upon sulforhodamine B release are shown to correlate with the percentage of **S1** in PC liposomes. Error bars denote the standard errors of at least three replicates.

General experimental. Reagents and solvents were generally purchased from Acros, Aldrich or Fisher Scientific and used as received. Heparin lithium salt from porcine intestinal mucosa and peroxidase from horseradish type VI were purchased from Sigma-Aldrich, Inc. L- α -Phosphatidylcholine (mixed isomers) and L- α -phosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) were purchased from Avanti Polar Lipids, Inc. Dry solvents were obtained from a Pure Solv solvent delivery system purchased from Innovative Technology, Inc. Column chromatography was performed using 230–400 mesh silica gel purchased from Sorbent Technologies. NMR spectra were obtained using Varian Mercury 300 or 500 MHz spectrometers. Mass spectra were obtained with JEOL DART-AccuTOF or Applied Biosystems/ QStar Elite HPLC—QTOF spectrometers. Fluorescence titration experiments were performed using a Cary Eclipse Fluorescence Spectrophotometer from Agilent Technologies. Water for liposome preparation and trigger-release studies was purified using a Milli-Q filtration system. Data were plotted and curve-fitted using SigmaPlot 13. Dynamic Light scattering (DLS) measurements were performed to study the size and stability of liposomes using a Malvern Zetasizer Nano ZS instrument equipped with a He-Ne 633 nm laser and a temperature controller at a scattering angle of 173°. All plots represent the average of at least 3 experiments with error bars denoting +/- the standard error for each set of measurements.

Synthesis

(2-((dodecylamino)methyl)phenyl)boronic acid **S1**

2-Formylphenylboronic acid (0.89 g, 5.929 mmol) and triethylamine (1.09 g, 10.78 mmol) were dissolved in 10 mL of a mixture of ethanol/ toluene (90:10) in a 50 mL round-bottomed flask and then dodecan-1-amine (1 g, 5.39 mmol) was added. A Dean-Stark trap was fixed to the reaction vessel and filled with 10 mL of the same solvent mixture. The reaction was then allowed to stir for 16 h at 100°C, and then cooled using an ice bath. Sodium borohydride (0.82 g, 21.56 mmol) was then added slowly, after which the reaction mixture was allowed to stir for 2 h at 0°C. The solvent was next removed through rotary evaporation and the resulting crude was purified through silica gel column chromatography with an increasing gradient of ethyl acetate/ hexane solvent system from 25% to 100% and then flushed with 100% methanol. The product was then dissolved in dichloromethane and filtered. Then, dichloromethane was removed under reduced pressure to yield compound **S1** as a white gel (1.29 g, 4.03 mmol, 85% yield). ¹H NMR (300 MHz, Chloroform-d) δ 7.53 – 7.41 (m, 2H), 7.35 – 7.28 (m, 2H), 3.88 (s, 2H), 2.67 (t, 2H, J = 6.0 Hz), 1.63 (p, 2H, J = 4.5 Hz), 1.30-1.24 (m, 18H), 0.88 (t, 3H, J = 6.0 Hz). ¹³C NMR (126 MHz, Chloroform-d) δ 140.6, 130.4, 127.3, 126.7, 122.7, 53.5, 49.8, 47.7, 29.5, 29.4, 29.2, 27.0, 22.5, 13.9. ¹¹B NMR (160 MHz, Chloroform-d) δ 13.65. ESI-MS [M+H]⁺: calcd for C₁₉H₃₅BNO₂ 320.2761, found: 320.2318.

2,3-bis(hexadecyloxy)propan-1-amine **4**

2-(2,3-dihydroxypropyl) isoindoline-1,3-dione (**3**) was synthesized as described previously.⁽¹⁾ This compound (260 mg, 1.17 mmol) was dissolved in 15 mL of anhydrous DMF in a 50 mL round-bottomed flask. Sodium hydride (NaH, 112 mg, 4.68 mmol) was added to this solution under a nitrogen stream. The reaction was allowed to stir at -20°C (acetone ice bath) for 15 min and then at rt for 10 min at which point the solution turned yellow. 1-Bromohexadecane (1.56 g, 4.68 mmol) was added to the solution, which was then stirred at rt overnight. The reaction was then quenched by adding 1 mL of water, and the solvent was removed under reduced pressure. The crude was extracted with dichloromethane from saturated sodium chloride solution (3 x 50 mL). The organic layer was dried with magnesium sulfate, filtered and concentrated to yield crude product as the phthalimide-protected version of **4**. Without

further purification, the reaction crude was dissolved in 10 mL ethanol along with 2 mL hydrazine monohydrate and heated to reflux overnight at 70°C. The solvent was then removed under reduced pressure. Thin layer chromatography (TLC) using 10% methanol/ dichloromethane as eluant identified the product spot with an R_f value of 0.4 and Ce/Mo and ninhydrin stain active. The crude was purified by column chromatography using a gradient solvent system of 2-10% methanol/dichloromethane to yield amine **4** as a pale yellowish oil (221 mg, 35% (over 2 steps)). ¹H NMR (500 MHz, Chloroform-d) δ 3.66 – 3.57 (m, 1H), 3.51 – 3.27 (m, 5H), 3.10-3.15 (m, 1H), 2.90 – 2.76 (m, 2H), 1.52 (p, 4H, J = 4.5Hz), 1.36 – 1.17 (m, 52H), 0.88 (t, 6H, J = 6.0 Hz). ¹³C NMR (126 MHz, Chloroform-d) δ 71.9, 71.0, 70.4, 42.7, 31.9, 30.9, 30.1, 29.7, 29.4, 26.1, 22.7, 14.1. DART-MS [M+H]⁺: calcd for C₃₅H₇₄NO₂ 540.5719, found: 540.5948.

(2-(((2,3-bis(hexadecyloxy)propyl)amino)methyl)phenyl)boronic acid **1**

2-Formylphenylboronic acid (55 mg, 0.37 mmol) and triethylamine (51 μL, 0.37 mmol) were dissolved in 5 mL toluene in a 50 mL round-bottomed flask and then amine **4** (100 mg, 0.18 mmol) was added. A Dean-Stark trap was fixed to the reaction vessel and filled with 10 mL toluene. The reaction was then allowed to stir for 16 h at 100°C. Next, the reaction mixture was cooled to 0°C in an ice bath and sodium borohydride (28 mg, 0.74 mM) was added slowly, after which the reaction mixture was allowed to stir for 2 h at 0°C. The solvent was next removed through rotary evaporation and the resulting crude was purified with an increasing gradient of methanol/ dichloromethane solvent system from 10% to 100%. Boronic acid lipid **1** was obtained as a pale yellow oil (33.9 mg, 28% yield). ¹H NMR (500 MHz, Chloroform-*d*) δ 7.84-7.77 (m, 2H), 7.71-7.64 (m, 2H), 4.06 (s, 2H), 3.90 – 3.81 (m, 2H), 3.80-3.75 (m, 1H), 3.63-3.56 (m, 2H), 3.54-3.46 (m, 2H), 3.44-3.38 (m, 2H), 1.51 (q, 4H, J = 3.5 Hz), 1.29-1.14 (m, 52H), 0.86 (t, 6H, J = 6.0 Hz). ¹³C NMR (126 MHz, Chloroform-*d*) δ 138.6, 130.6, 129.3, 128.5, 127.0, 121.0, 71.2, 65.2, 56.0, 51.3, 31.9, 29.6, 29.3, 26.8, 25.8, 22.7, 14.1, DART-MS [M+H]⁺: calcd for C₃₅H₇₄NO₂ 674.6259, found: 674.3933.

Nile red liposome release studies

Liposome preparation was initiated by weighing out samples of PC, boronic acid lipid **1** or **S1**, and Nile red in separate vials to make 32 mM PC, 5 mM BA lipids and 5 mM Nile red stock solutions in ethanol-free chloroform. Phosphate-buffered saline (PBS, 2 mM, pH 8.0) was chosen as the buffer solution. Heparin lithium salt (10 mg) from porcine intestinal mucosa was added into a 2.08 mL PBS buffer to make a 1,040 U/mL (ie 4.79 mg/mL) stock solution. One unit (U) of heparin is defined as the quantity needed to keep 1 mL of cat's blood fluid for 24 h at 0°C. Benzyl alcohol (0.1%) was added in heparin stock to prevent bacterial growth as suggested by manufacturer. After brief vortexing, the appropriate volumes of the stock solution of each lipid component and Nile red were pipetted into a clean vial per calculations on a 5 mM, 240 μL total lipid scale, to obtain the desired molar percentage of each component. As an example, for liposomes composed of 10% **S1** and 85% PC, 5% Nile red, 31.9 μL PC stock (78.6 μg), 24 μL **S1** stock (3.8 μg), and 12 μL Nile red stock (1.91 μg) were combined. After 30 seconds of vortexing, the chloroform solvent was dried with a nitrogen stream, and the lipids were subsequently dried for at least 3 h under vacuum. Then, the lipids were hydrated with 240 μL of PBS buffer, incubated on a 60°C water bath for 3 sets of 20 min, with vortexing after each set. Liposomes were then underwent freeze-thaw cycles between a -80°C dry ice-acetone bath and a 60°C water bath for 10 cycles. Vortexing at low speed was performed during each freeze-thaw cycle. Next, liposomes were extruded through a 200 nm membrane filter for 21 passes using a LiposoFast extruder (Avestin, Inc.), and then added to a fresh vial. DLS scans were performed to confirm the formation of stable liposomes. For fluorescence-based release studies, 100 uL samples of liposome vesicles were added into a sub-micro cuvette cell (Starna Cells, Inc). After an initial fluorescence scan (excitation wavelength

of 553 nm, emission wavelength scanned from 560 nm to 580 nm), 0.5 μ L of heparin stock (0.52 U) was added into the cuvette before each fluorescence scan, which was performed 20 times. Data presented in plots indicate emission values at 621 nm. To account for the dilution caused by the addition of heparin solution, blank samples were run by diluting liposomes with the same volumes of water, which were subtracted from the study samples as follows. From the observed fluorescence during the measurement period, a normalized release function that describes the efflux over the period of the experiment was determined according to the equation below to exclude buffer dilution, where F(tx) and F(bx) are the measured fluorescence at titration times x with heparin and buffer accordingly, and F(t0) and F(b0) are initial fluorescence for 2 different sets, as defined below. HRP titrations were run using the same procedure and 1 unit (U) of HRP is defined by the manufacturer as the amount required to form 1.0 mg of purpurogallin from pyrogallol in 20 sec at pH 6.0 at 20 °C.

$$\% \text{ release} = \frac{F(\text{tx}) - F(\text{bx})}{F(\text{t0}) - F(\text{b0})} * 100$$

STEM Imaging Studies

Liposome solutions (5 mM) containing either 0% or 10% of boronic acid lipid 1, and the remaining portion consisting of PC (100% or 90%, respectively) were prepared as previously described. One portion of liposomes (100 μ L) was mixed with 10 μ L of a 2,170 U/mL heparin stock as described for the Nile red release studies, and was then incubated at rt for 30 min, while another aliquot was mixed with 10 μ L PBS buffer solution and also incubated at rt for the same amount of time. After that, both portions were diluted to 400 μ M solutions with PBS buffer to reach ideal concentrations for scanning transmission electron microscopy (STEM) studies. A drop (5–10 μ L) from each solution was immobilized onto separate carbon films supported by 200 mesh copper grids. The samples were stained with 5% uranyl acetate for 1 h and then washed three times with distilled water for 10 min and stored in a desiccator overnight prior to imaging. Images were collected using a Zeiss Auriga operating in scanning transmission mode. The electron beam energy was set at 30 KeV, and images were detected using an Everhardt-Thornley SE2 detector.

Sulforhodamine B liposome release studies

Stock solutions were initiated by weighing out samples of PC and boronic acid lipid 1 in separate vials to generate separate 32 mM PC and 5 mM 1 or S1 solutions in ethanol-free chloroform. Phosphate-buffered saline (2 mM, pH 8.0) was chosen as the buffer solution. Heparin lithium salt (10 mg) from porcine intestinal mucosa was added into 1 mL of PBS buffer to make a 2,170 U/mL stock solution. Benzyl alcohol (0.1%) was added to heparin stock to prevent bacterial growth as suggested by the manufacturer. Sulforhodamine B sodium salt (116.1 mg) was dissolved in 10 mL PBS buffer to make a 20 mM stock solution. After brief vortexing, proper volumes of each lipid stock solution were pipetted into a clean vial per calculations on a 10 mM, 240 μ L total lipid scale to obtain the desired molar percentage of each component. As an example, for liposomes composed of 15% S1 and 85% PC, 63.8 μ L PC stock (157.1 μ g) and 72 μ L S1 stock (11.5 μ g) were added into a new vial. After 30 seconds of vortexing, the chloroform solvent was dried with a nitrogen stream, and the lipids were subsequently dried for at least 3 h under vacuum. Next, the lipids were hydrated with 240 μ L of the previously described sulforhodamine stock solution and incubated on a water bath at 60°C for 3 sets of 20 min, with vortexing after each set. Liposomes were then underwent freeze-thaw cycling between a -80°C dry ice-acetone bath and a 60°C water bath for 10 cycles. Vortexing at low speed was performed during each freeze-thaw cycle. After that, liposomes were extruded through a 200 nm membrane filter for 21 passes using a LiposoFast extruder (Avestin, Inc.) and then added to a fresh vial. Next, liposomes were

separated from unencapsulated dye using a Sephadex G-50 size exclusion column (SEC). Liposomes came out in the first or second fraction and were detected by the change in fluorescence upon treatment of an aliquot with triton X-100. DLS scans were performed to confirm the formation of stable liposomes. For fluorescence-based release studies, 100 μ L liposome samples were added into a sub-microcuvette cell (Starna Cells, Inc). After an initial fluorescence scan (excitation wavelength at 553 nm, emission wavelength scanned from 560 nm to 660 nm), 0.5 μ L heparin stock (1.08 U) was added for each fluorescence measurement before quenching by adding 1 μ L 1% (v/v) Triton-X 100 in PBS to convert liposomes into mixed micelles and trigger rhodamine release. Data presented in plots indicate emission at 606 nm. To account for the dilution caused by the addition of heparin solution, blank samples were run by diluting liposomes with the same volumes of water, which were subtracted from the study samples as follows. From the observed fluorescence during the measurement period, a normalized release function that describes the efflux over the period of the experiment was determined according to the equation below to exclude buffer dilution where $F(t)$, $F(x)$, $F(0)$ are the fluorescence intensity measured after adding Triton X-100, x addition time of heparin stock and initial fluorescence intensity, accordingly.:

$$\% \text{ release} = \left(1 - \frac{F(t) - F(x)}{F(t) - F(0)} \right) * 100\%,$$

Microplate liposome–heparin binding studies

The wash buffer used in this study was 10 mM PBS at pH 8.0. 10 μ g Heparin–biotin sodium salt purchased for Sigma-Aldrich (\sim 15000 kDa) was added into 10 mL PBS buffer to prepare a 1 μ g/mL stock solution. Liposome solutions (2 mM) consisting of 1% rhodamine L- α -phosphatidylethanolamine (Rd-PE), either 0% or 20% of boronic acid lipid **1**, and the remaining portion consisting of PC (99% or 79%, respectively) were prepared as previously described. Next, opaque streptavidin-coated 96-well microplate wells were washed with 250 μ L buffer for 30 min. After removal of buffer, 100 μ L of heparin–biotin solution was added into study wells. Additional negative controls were run in which wells were treated with the different liposome compositions, but not with heparin–biotin, and experiments were otherwise run the same. After 1 h of incubation, these solutions were removed, and the wells were washed three times with 250 μ L wash buffer for 5 min. Next, aliquots of liposomes were diluted into wash buffer to a total volume of 100 μ L, producing solutions of 0, 20, 40, 80, 100, 200, 400, 500, 600, 800, and 1000 μ M. These solutions were added to separate wells of the microplate functionalized with heparin. After 2h of incubation, these solutions were removed, and the wells were washed with 250 μ L wash buffer three times for 5 min each. Finally, 100 μ L of wash buffer was added to each well, and the microplate was analyzed using a Biotek Synergy 2 microplate reader, and the fluorescence intensity was repeatedly measured at excitation wavelength of 530 \pm 20 nm and emission wavelength of 590 \pm 20 nm.

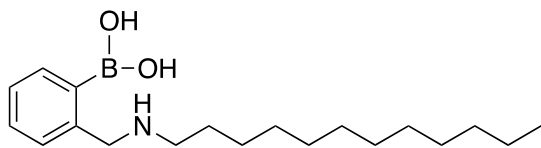
Cell culture and fluorescence microscopy

Melanoma A375 cells, obtained from ATCC[®] (Manassas, VA), were cultured in a humidified incubator under 5% CO₂ in DMEM medium supplemented with 10% of Fetal Bovine Serum, 50 U/mL penicillin, and 50 μ g/ml streptomycin (Invitrogen). Two days prior to the experiment, A375 cells were plated at a seeding density of 1×10^4 cells per well on a glass coverslip. Liposomes containing 0.08% rhodamine L- α -phosphatidylethanolamine (Rd-PE) in the absence or presence of 10% of boronic acid lipid **1**, with the rest of the liposome consisting of PC were incubated with cells at a concentration of 1 mM for 1 h at 37°C. Liposome preparation was conducted under similar conditions as described above, except 10 mM

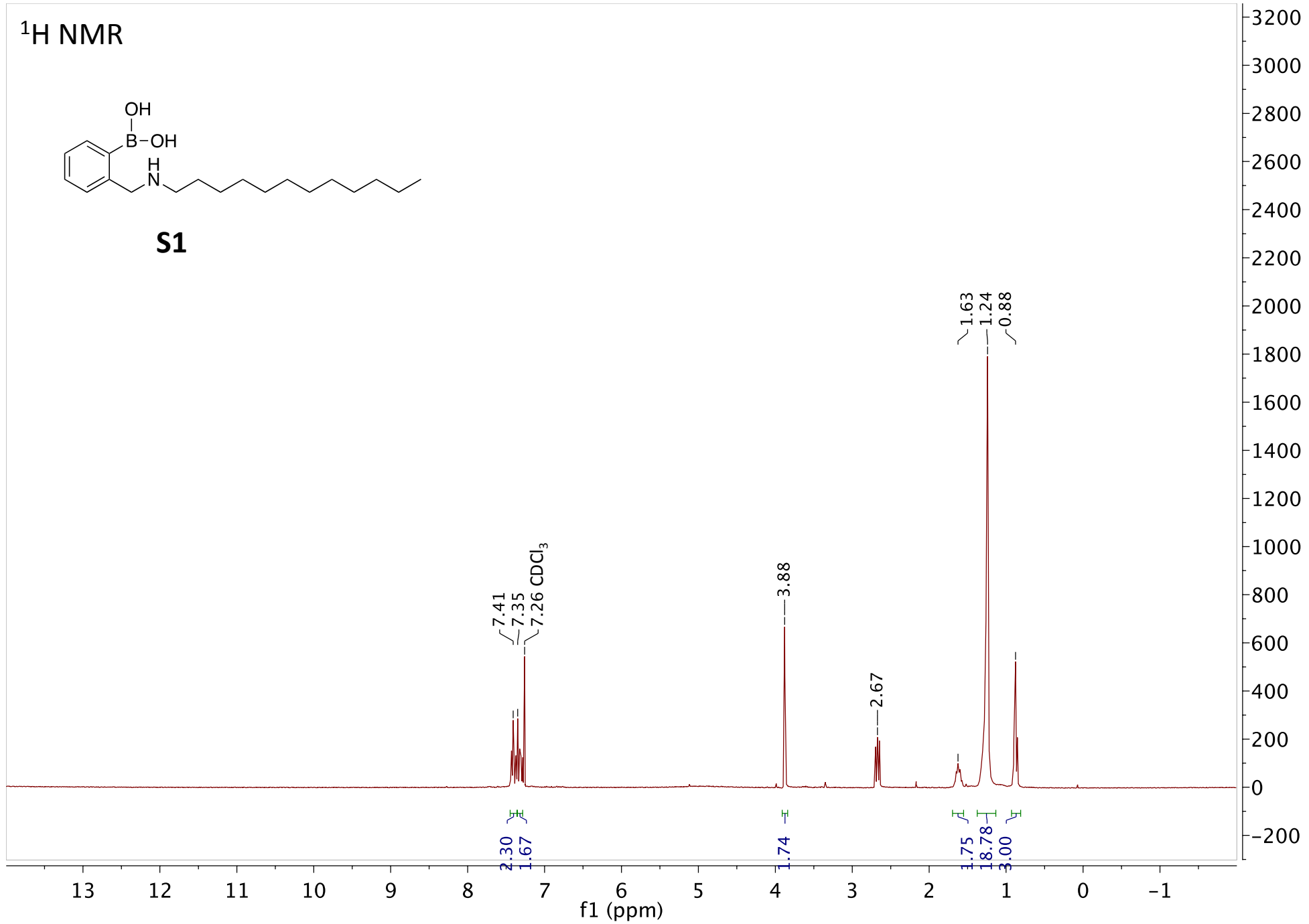
PBS pH 7.4 was used as the buffer. After incubation, cells were washed four times with PBS containing 1 mM MgCl₂ and 100 mM CaCl₂ (PBS⁺⁺), fixed for 30 min in 4% paraformaldehyde and mounted with ProLong Diamond Antifade Mountant with DAPI (Invitrogen). Images were acquired on a confocal laser scanning microscope (Zeiss LSM 710) with 63x objectives using Zen2 blue edition software. Contrast and brightness settings were chosen so that all pixels were in the linear range. Images are the product of fourfold line averaging.

1. Sikoraiová J, Marchalín Š, Dařich A, Decroix B. Acid-mediated intramolecular cationic cyclization using an oxygen atom as internal nucleophile: synthesis of substituted oxazolo-, oxazino- and oxazepinoisoindolinones. *Tet. Lett.* **2002**, 43(27), 4747-51.

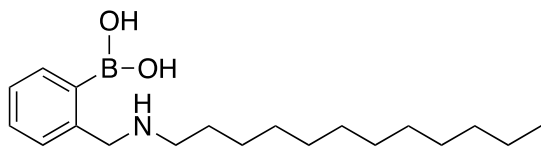
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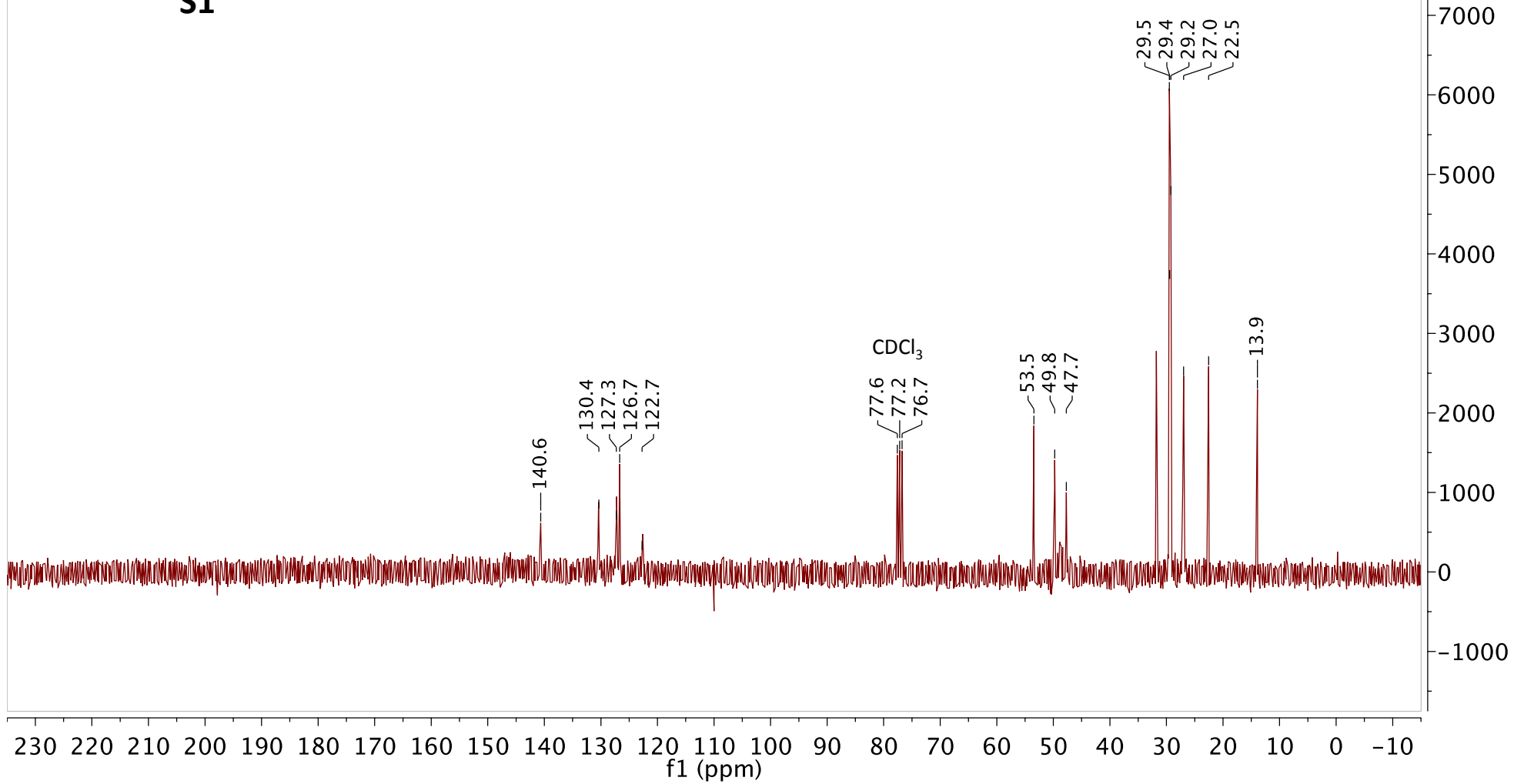
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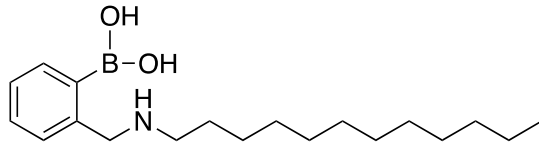
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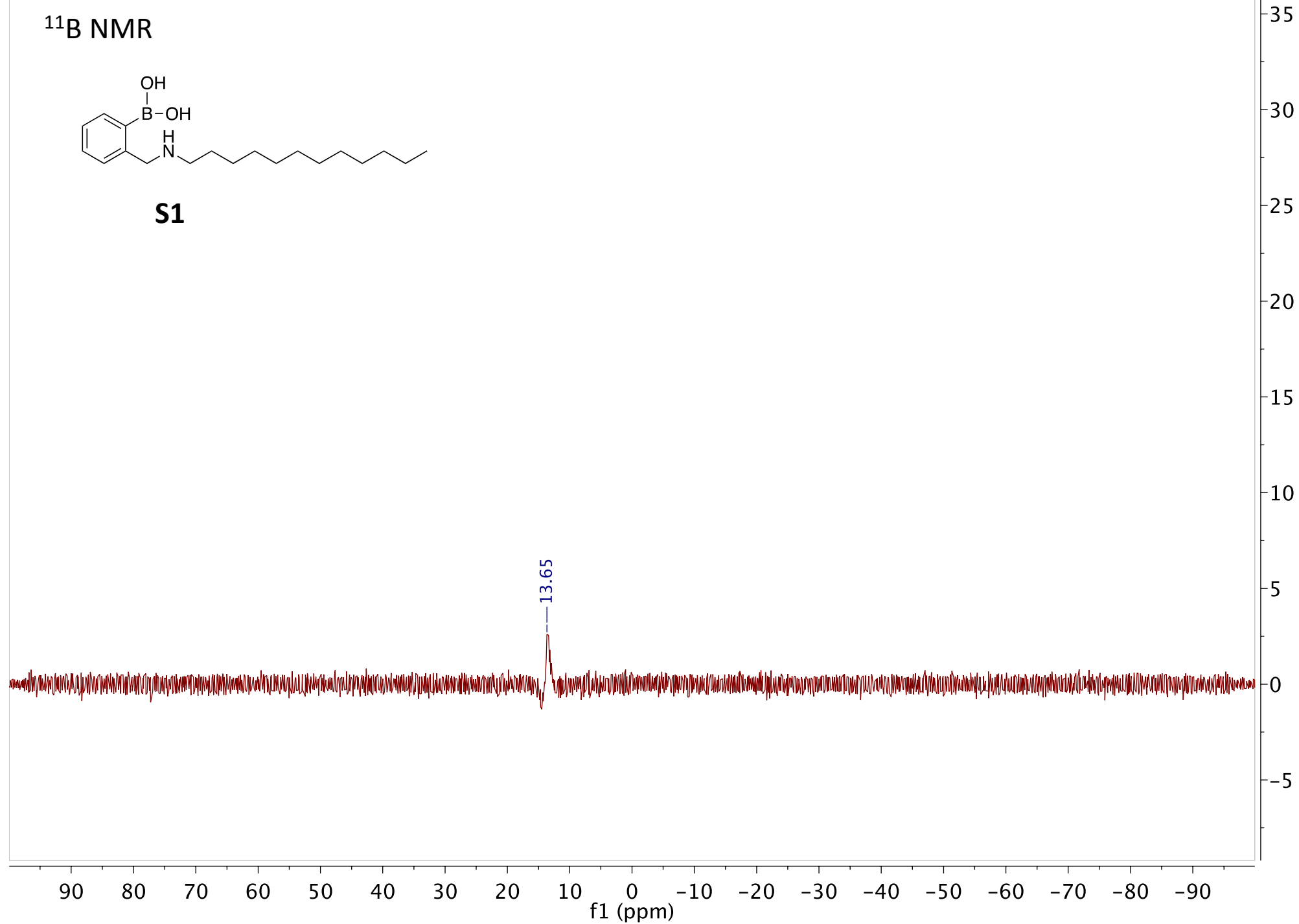
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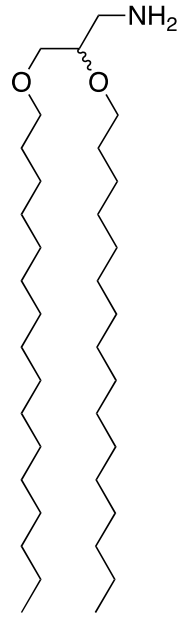
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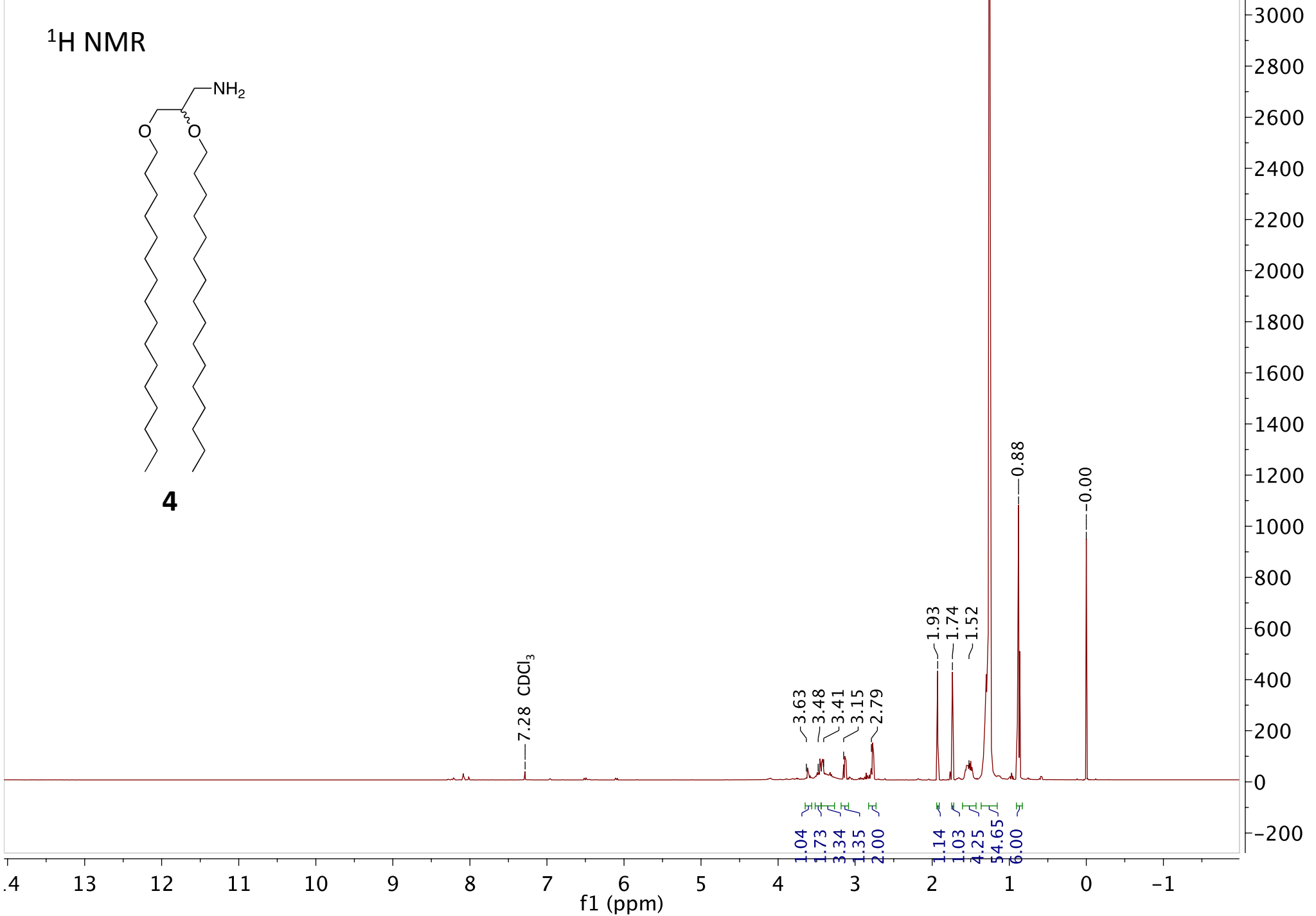
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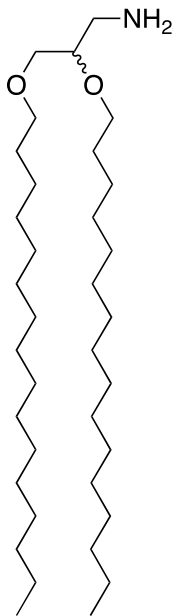
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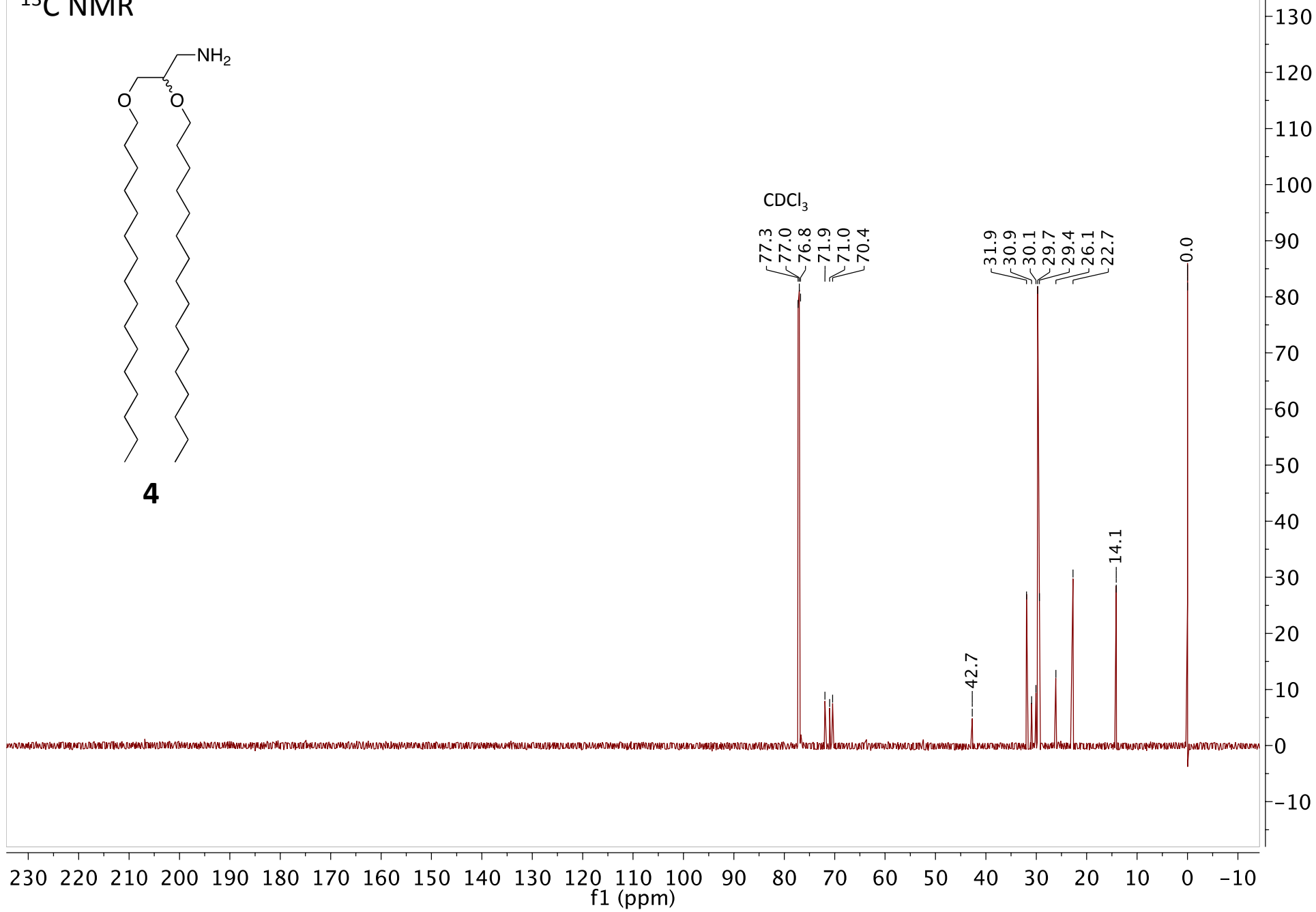
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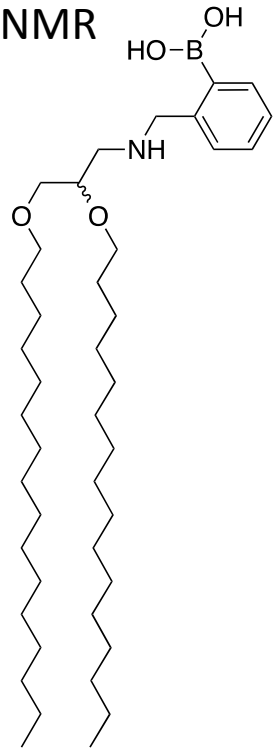
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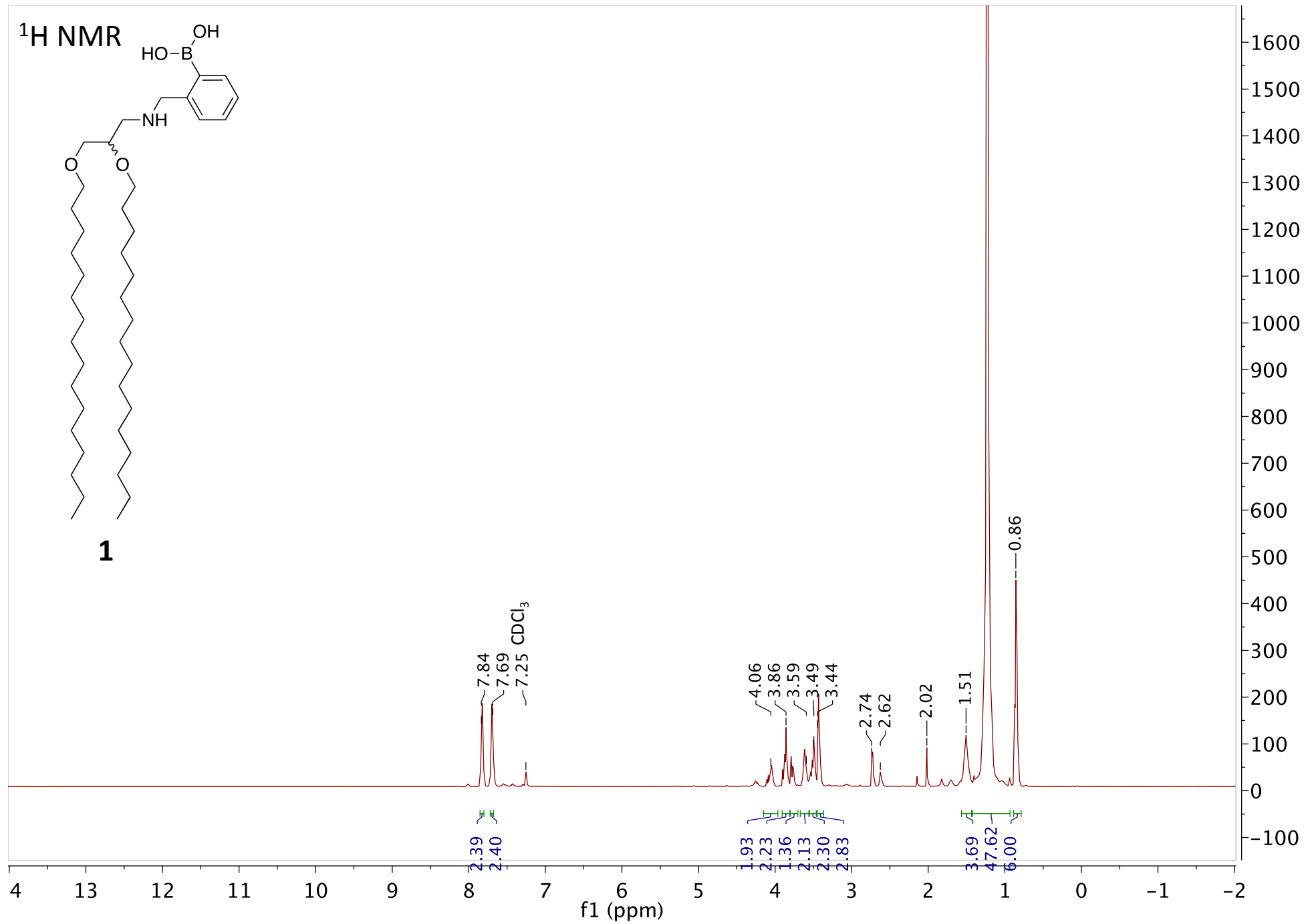
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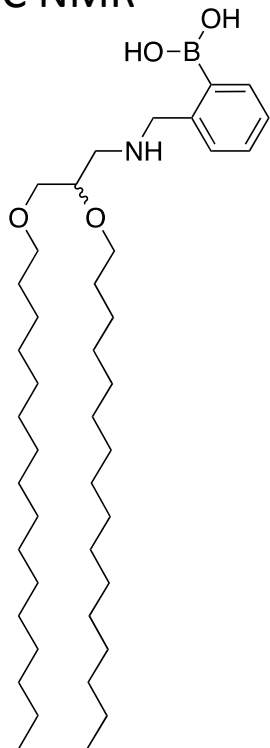
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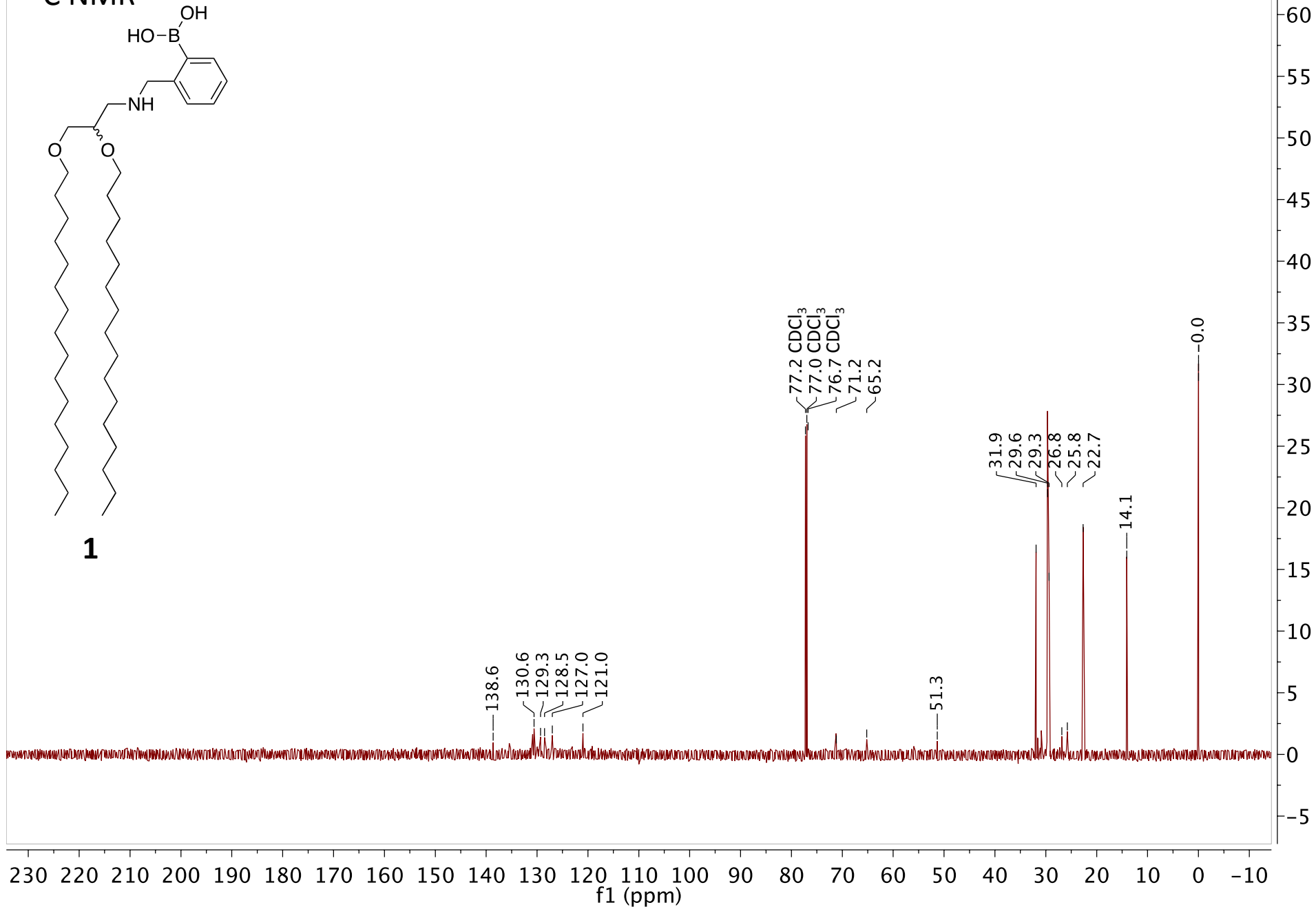
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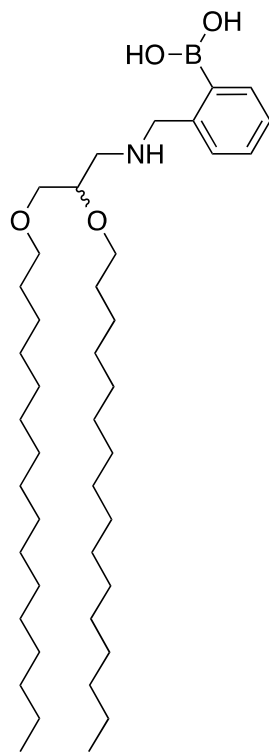
¹³C NMR



1



¹¹B NMR



1

