"Zipped Synthesis" by Cross-Metathesis Provides a CBS (Cystathionine β-Synthase) Inhibitor that Attenuates Cellular H_2S Levels and Reduces Neuronal Infarction in a Rat Ischemic Stroke Model

Christopher D. McCune[a], Su Jing Chan[b], Matthew L. Beio[a], Weijun Shen[a], Woo Jin Chung[a], Laura M. Szczesniak[a], Chou Chai[c], Shu Qing Koh[b], Peter T.-H. Wong[b]*and David B. Berkowitz[a,]*

Affiliations:

[a]*Department of Chemistry,University of Nebraska, Lincoln Nebraska 68588, United States*

[b]*Department of Pharmacology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore 117597*

[c]*Neurodegeneration Research Laboratory, National Neuroscience Institute, Singapore 308433*

Correspondence:

**co-corresponding authors: dberkowitz1@unl.edu; peter_wong@nuhs.edu.sg*

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I. General Experimental

A. Synthetic Methodology. All reactions were conducted under nitrogen or argon atmosphere using flame or oven-dried glassware, unless otherwise indicated. Methylene chloride was distilled from CaH_2. Toluene, THF and Et_2O were distilled from sodium benzophenone ketyl. Methanol was distilled from Mg, and ethanol from Na-diethyl phthalate. Flash chromatography was performed using Merck silica gel 60 (230-400 mesh). ^{1}H NMR spectra were recorded on Bruker-DRX-Advance 400 MHz, 500 MHz

and 600 MHz instruments with chemical shifts reported relative to residual $CHCl_3$ (7.25 ppm), Benzene (7.16 ppm), Toluene (2.11 ppm, CH_3), H_2O (4.8 ppm), DMSO (3.31 ppm), and MeOH (3.34 ppm). Optical rotation @589 nm was measured at 25 °C in an Autopol polarimeter. Mass spectra were acquired at the Nebraska Center for Mass Spectrometry (University of Nebraska-Lincoln).

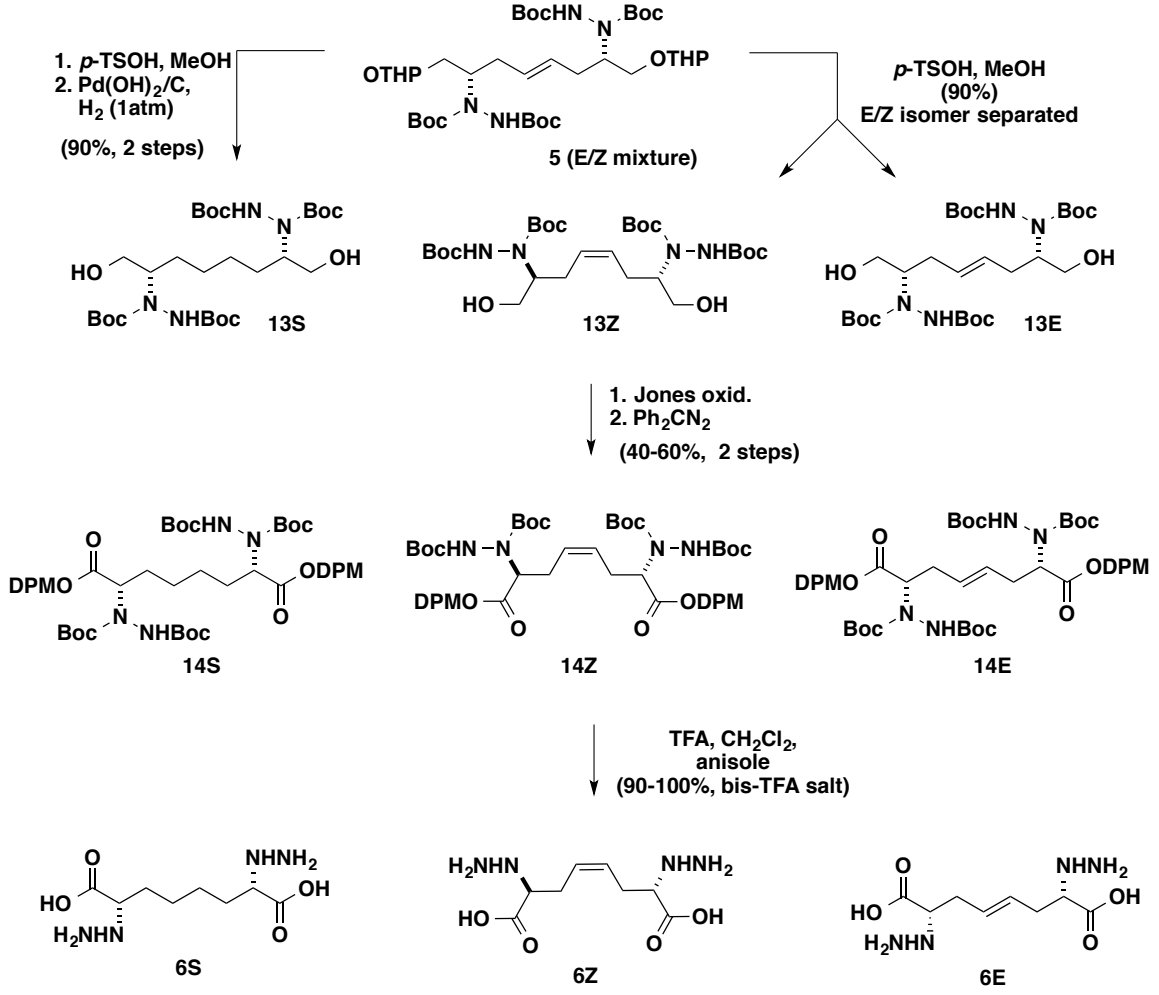
B. Enzymology. *Protein Purification:* All pH measurements were made using a Corning pH-meter equipped with an Orion semi-micro combo pH electrode. Centrifugation was performed using a SorvallRC5B-plus and an Eppendorf-5415C instrument. Protein concentration was measured by the method of Lowry. ***For the activity assay:*** (L,L)-cystathionine and 4,4'-dithioparanitrobenzoinc acid (DTNB) were purchased from Aldrich. A Shimadzu UV-2401PC spectrophotometer equipped with a 16-well microcell with each well holding up to 100μL and thermoelectric temperature control (set at 37 ºC for all experiments reported in this work) was used for the continuous cystathionine lyase activity assays. In general, all enzymatic experiments were performed at 37 ºC with 50 mM Tris buffer, pH 8.0.

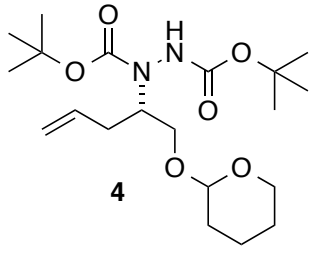
C. Quantification of Inhibitor Concentration by NMR

Stock solutions of all the final inhibitor candidates were prepared in D_2O to give nominal concentration of 30 mM. ^1H NMR was used determined the actual concentration of each stock inhibitor solution. Specifically, 50 μL of each stock inhibitor solution was mixed with 50 μL of 40 mM imidazole (internal standard) in D_2O together with 100 μL of D_2O. The resulting solution (200 μL total volume) was transferred to a 3 mm NMR tube and the ^1H NMR spectrum was obtained from a Bruker Advance 500 MHz spectrometer equipped with a 5 mm cryoprobe. The concentration of the stock solution was determined by comparison from the integrals of imidazole and inhibitors.

II. Synthesis of Inhibitor Library

Figure S1: Hydrazino Acid Series (-N-NH_2)

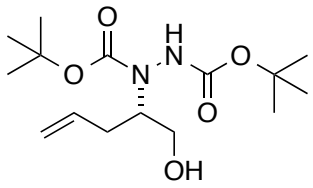




4

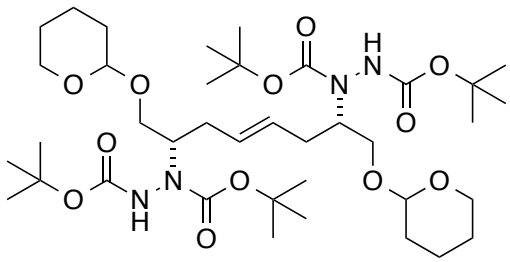
To a solution of THP-protected (S)-homoallylic alcohol **3**[1] (3.96 g, 21.3 mmol, 1.0) in THF (142 mL, 150 mM) was added triphenylphosphine (TTP) (14.0 g, 53.3 mmol, 2.5 eq) and the mixture was cooled to 0 °C. To this was slowly added a solution of Di-*tert*-butylazodicarboxylate (DBAD) (11.8 g, 51.1 mmol, 2.4 eq) in THF (50 mL) via addition funnel. Following the addition of DBAD, the reaction was stirred at 0 °C for 20 minutes, and then warmed to room temperature for an additional hour or until disappearance of the starting material (monitored by TLC). Upon completion, the solvent was evaporated and the crude residue was purified by SiO_2 column chromatography (9:1 hexane:EtOAc) to afford 5.97 g of Mitsunobu product **4** (70%) as an oil. ^{1}H NMR (400 MHz, d_6-DMSO, 353 K) δ 1.38-1.42 (m, 18H), 2.03 (m, 2H), 3.33-3.49 (m, 2H), 4.95-5.03 (m, 2H), 5.64-5.69 (m, 1H), 6.21-6.42 (m, 1H); ^{13}C NMR (rotamer chemical shift in

parenthesis) (100 MHz, $CDCl_3$) δ 28.07, 28.12, 32.92, (57.57, 59.98), (61.86, 62.06), (81.25, 81.93), (82.18, 82.39), (117.06, 117.43), (134.27, 134.65), (155.14, 155.90), (157.96, 158.58); HRMS (FAB, 3-NBA) *m/z* $[m+H]^+$ calcd. for $C_{20}H_{37}N_2O_6$, 401.2652; found, 401.2663.



4 (Free OH)

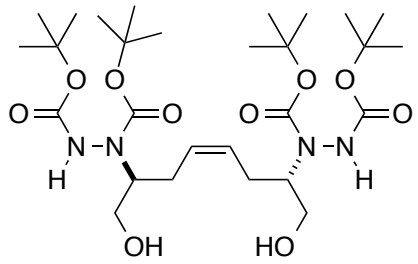
Due to the complex NMR spectrum of compound **4** as a result of both the THP diasteriomers and Boc-rotamers, a small sample of **4** was subjected to standard THP deprotection conditions and the free alcohol was characterized. Specifically, the THP protected alcohol was dissolved in methanol. To this solution was added *p*-toluene sulfonic acid monohydrate (*p*-TsOH). After stirring for 30 minutes at room temperature, the reaction was complete and the mixture neutralized with NEt_3. The crude residue was concentrated and purified by SiO_2 column chromatography to afford the free alcohol. Analytical data is consistent with the THP-deprotected derivative of compound **4**. $[\alpha]_D$ +47.4 (*c* 0.97, CH_2Cl_2); ^{1}H NMR (**4:** Free OH; mixture of rotamers) (400 MHz, $CDCl_3$) δ 1.38-1.42 (m, 18H), 2.03 (m, 2H), 3.33-3.49 (m, 2H), 4.95-5.03 (m, 2H), 5.64-5.69 (m, 1H), 6.21-6.42 (m, 1H); ^{13}C NMR (mixture of rotamers) (100 MHz, $CDCl_3$) δ 28.07, 28.12, 32.92, (57.57, 59.98), (61.86, 62.06), (81.25, 81.93), (82.18, 82.39), (117.06, 117.43), (134.27, 134.65), (155.14, 155.90), (157.96, 158.58); HRMS (FAB, Gly) *m/z* $[m+H]^+$ calcd. for $C_{15}H_{29}N_2O_5$, 317.2076; found, 317.2087.



5 (E/Z mixture)

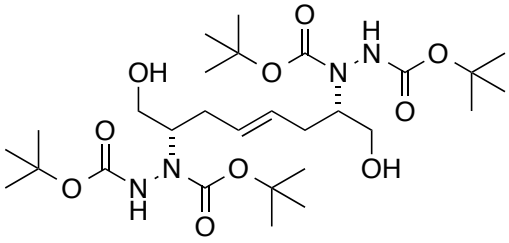
Compound **4** (3.0 g, 7.5 mmol, 1.0 eq) was dissolved in dry CH_2Cl_2 (75 mL, 100 mM) and stirred at room temperature. Next, a solution of 1st Generation Grubbs Catalyst (308 mg, 0.375 mmol, 5 mol%) in CH_2Cl_2 (20 mL) was added slowly via cannulation. The mixture was gently heated to reflux for 48 h and upon disappearance of starting material by TLC the solvent was evaporated and the dark sticky residue was purified by SiO_2 column chromatography (5:1 to 1:1 hexane:EtOAc) to afford 2.4 g (84%) of the cross metathesis product **5** as a mixture of *E/Z* isomers. The *E:Z* ratio (1.2:1) was determined by crude ^{1}H NMR. The NMR spectrum was complicated by a combination of Boc-rotamers, *E/Z* isomers, and THP diastereomers, so the cross methathesis product was directly subjected to THP deprotection.

The bis-THP protected diol **5** (2.4 g, 3.1 mmol, 1.0 eq) was dissolved in methanol (32 mL). To this solution was added *p*-toluene sulfonic acid monohydrate (*p*-TsOH) (361 mg, 1.86 mmol, 0.6 eq) in portions. After stirring for 30 min at room temperature, the reaction was determined complete by TLC and the mixture was neutralized with NEt_3 (0.26 mL, 1.86 mmol, 0.6 eq), concentrated under reduced pressure, and careful SiO_2 column chromatography (3:1 to 1:1 hexane:EtOAc) eluted 767 mg pure *Z*-diol **13Z**, followed by 923 mg of pure *E*-diol **13E** (90% combined).



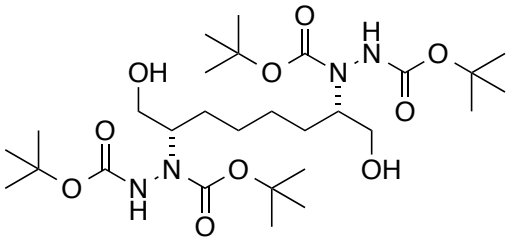
13Z

***Z*-diol (13Z):** $[\alpha]_D$ +12.2 (*c* 1.07, CH_2Cl_2); ^1H NMR (400 MHz, d_6-DMSO, 353 K) δ 1.42 (s, 18H), 1.44 (s, 18H), 2.08 (bs, 2H), 2.16 (bs, 2H), 3.37 (m, 4H), 4.02 (bs, 2H), 4.15 (bs, 2H), 5.45 (bs, 2H), 8.34 (bs, 2H); ^{13}C NMR (100 MHz, d_6-DMSO, 353 K) δ 26.20, 27.53, 27.59, 59.27, 60.77, 79.40, 127.00, 154.30; HRMS (FAB, 3-NBA) *m/z* $[m+H]^+$ calcd. for $C_{28}H_{53}N_4O_{10}$, 605.3762; found, 605.3782.



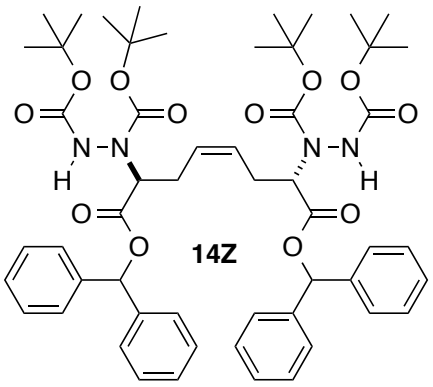
13E

***E*-diol (13E):** $[\alpha]_D$ +10.7 (*c* 1.06, CH_2Cl_2); ^1H NMR (400 MHz, d_6-DMSO, 353K) δ 1.41 (s, 18H), 1.44 (s, 18H), 2.05 (s, 4H), 3.24 (s, 4H), 4.00 (bs, 2H), 4.10 (bs, 2H), 5.48 (bs, 2H), 8.34 (bs, 2H); ^{13}C NMR (100 MHz, d_6-DMSO, 353 K) δ 27.54, 27.60, 31.41, 59.53, 60.68, 79.37, 128.02, 154.26; HRMS (FAB, 3-NBA) *m/z* $[m+H]^+$ calcd. for $C_{28}H_{53}N_4O_{10}$, 605.3762; found, 605.3782.

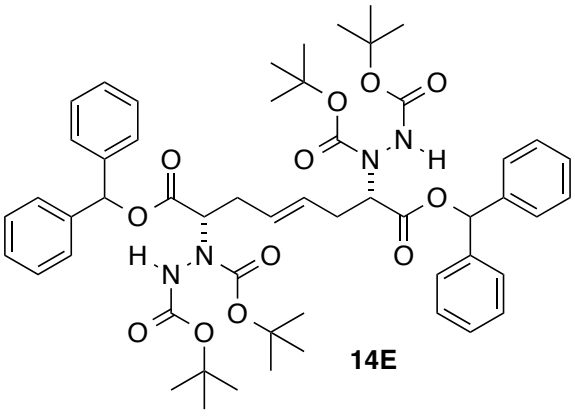


13S

Deprotection of THP was carried out as described above. Briefly, to a cooled solution (0 °C) of the E/Z mixture **5** (3.82 g, 4.94 mmol, 1.0 eq) in MeOH (50 mL, 100 mM) was added *p*-TsOH (564 mg, 2.96 mmol, 0.6 eq) and the solution was warmed to room temperature and stirred for 2 h whereby TLC indicated complete deprotection to the unsaturated E/Z-diol. The crude mixture was quenched by the addition of Et_3N (413 μL, 2.96 mmol, 0.6 eq), concentrated, and dissolved in EtOAc (50 mL, 100 mM). To the crude diol was added 20% $Pd(OH)_2$ /C (764 mg) and the suspension was subjected to an atmosphere of H_2 (1 atm, balloon pressure) and vigorously stirred at room temperature for 3 h whereby complete C=C bond reduction was indicated by crude ^1H NMR. The catalyst was filtered off with a patch of Celite® and the solvent was evaporated to afford 2.70 g (90% - 2 steps) of the saturated diol **13S**. $[\alpha]_D$ +5.8 (*c* 1.04, CH_2Cl_2); ^1H NMR (400 MHz, d_6-DMSO, 353 K) δ 1.26-1.29 (m, 8H), 1.42 (s, 18H), 1.43 (s, 18H), 3.24-3.33 (m, 4H), 3.99 (bs, 2H), 4.11 (bs, 2H), 8.35 (bs, 2H); ^{13}C NMR (100 MHz, d_6-DMSO, 353 K) δ 25.17, 27.55, 27.60, 59.09, 61.18, 79.21, 79.28, 154.51; HRMS (FAB, 3-NBA) *m/z* $[m+H]^+$ calcd. for $C_{28}H_{55}N_4O_{10}$, 607.3926; found, 607.3926.

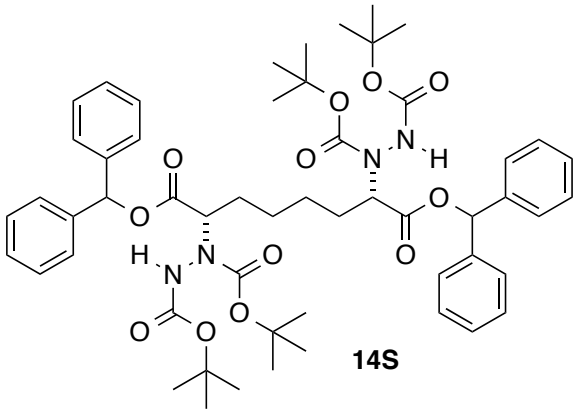


Diol **13Z** (159 mg, 0.263 mmol, 1.0 eq) was dissolved in acetone (10 mL), cooled to 0 °C, and subjected to oxidizing conditions by slow addition of 8N Jones reagent (0.82 mL, 6.58 mmol, 25 eq). The mixture was stirred at 0 °C for 4 h or until TLC (EtOAc: 3% v/v AcOH) indicated complete conversion of diol to bis-acid. Conveniently, the carboxcylic acid functionality could be visually identified in parallel by treatment of the developed TLC plate with bromocresol green (bright yellow color appears). The reaction was quenched by the addition of a minimal amount isopropol alcohol (*i*PrOH) until the solution turned to a blue/green tint, warmed to room temperature, and the crude acid was extracted with EtOAc. The organics were dried over Na_2SO_4, filtered, and concentrated. To facilitate purification, the bis-acid was esterified to the diphenylmethyl ester. Thus, the crude bis-acid was redissolved in EtOAc (10 mL) and to this solution was added diphenyldiazomethane (204 mg, 1.05 mmol, 4.0 eq) which was prepared by the oxidation of benzophenone hydrozone with PbO_2. The dark red reaction mixture was stirred overnight at room temperature and subsequently quenched by drop-wise addition of AcOH until the reaction mixture turned clear. The crude mixture was concentrated under reduced pressure and the residue was purified by SiO_2 column chromatography (3:1 hexane:EtOAc) to afford 127 mg (50% - 2 steps) of pure diphenylmethyl ester **14Z**. $[\alpha]_D$ -2.0 (*c* 1.32, CH_2Cl_2); ^{1}H NMR (400 MHz, $CD_3C_6D_5$, 353 K) δ 1.34 (s, 18H), 1.36 (s, 18H), 2.71 (m, 2H), 2.83 (m, 2H), 4.93 (s, 2H), 5.69 (s, 2H), 6.25 (s, 2H), 6.93 (s, 2H), 7.03 (m, 4H), 7.10 (m, 8H), 7.29 (m, 8H); ^{13}C NMR (100 MHz, $CD_3C_6D_5$, 353 K) δ 27.92, 28.36, 28.49, 78.50, 80.72, 81.76, 127.75, 127.90, 128.19, 128.84, 140.78, 140.90, 155.57, 170.51; HRMS (FAB, 3-NBA) *m/z* $[m+H]^+$ calcd. for $C_{54}H_{69}N_4O_{12}$, 965.4912; found, 965.4926.



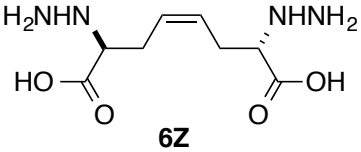
Compound **14E** was synthesized following a procedure identical to that described above for **14Z**. Briefly, **13E** (90 mg, 0.15 mmol, 1.0 eq) in acetone (10 mL) was subjected to 8N Jones reagent (0.47 mL, 3.75 mmol, 25 eq). Following conversion of the diol to the bis-acid the reaction was quenched by the addition of *i*PrOH, the organics were further extracted with EtOAc, dried of over Na_2SO_4, concentrated under reduced pressure, and the crude bis-acid was subsequently treated with diphenydiazomethane (116 mg, 0.60 mmol, 4.0 eq) in EtOAc (10 mL) at room temperature overnight. Following treatment of the reaction mixture with AcOH, the crude mixture was once again concentrated and the residue was purified by SiO_2 column chromatography (3:1 hexane:EtOAc) to afford 81 mg (56% - 2 steps) of the pure diphenylmethyl ester **14E**. $[\alpha]_D$ -1.1 (*c* 1.2, CH_2Cl_2); ^{1}H NMR (400 MHz, $CD_3C_6D_5$,

353 K) δ 1.36 (s, 18H), 1.38 (s, 18H), 2.63 (m, 4H), 4.88 (bs, 2H), 5.66 (m, 2H), 6.30 (s, 2H), 6.94 (s, 2H), 7.20 (m, 4H), 7.11 (m, 8H), 7.29 (m, 8H); ^{13}C NMR (100 MHz, $CD_3C_6D_5$, 353 K) δ 27.75, 27.89, 32.20, 77.77, 80.02, 81.08, 127.25, 127.26, 127.53, 127.59, 128.16, 128.19, 128.71, 140.20, 140.28, 154.92, 169.91; HRMS (FAB, 3-NBA) *m/z* $[m+H]^+$ calcd. for $C_{54}H_{69}N_4O_{12}$, 965.4912; found, 965.4935.



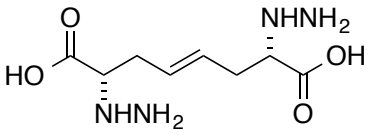
14S

Compound **14S** was synthesized following a procedure identical to that described above for **14Z**. Specifically, **13S** (320 mg, 0.527 mmol, 1.0 eq) in acetone (10 mL) was subjected to 8N Jones reagent (1.65 mL, 13.2 mmol, 25 eq). Upon conversion of the diol to the bis-acid the reaction was quenched by the addition of *i*PrOH, the organics were further extracted with EtOAc, dried of over Na_2SO_4, concentrated under reduced pressure, and the crude bis-acid was subsequently treated with diphenydiazomethane (409 mg, 2.11 mmol, 4.0 eq) in EtOAc (10 mL) at room temperature overnight. Following treatment of the reaction mixture with AcOH, the crude mixture was once again concentrated and the residue was purified by SiO_2 column chromatography (3:1 hexane:EtOAc) to afford 444 mg (87% - 2 steps) of the pure diphenylmethyl ester **13S**. $[\alpha]_D$ -1.28 (*c* 1.08, CH_2Cl_2); ^{1}H NMR (400 MHz, $CD_3C_6D_5$, 353K) δ 1.36 (s, 18H), 1.37 (s, 18H), 1.53 (m, 2H), 1.69 (m, 2H), 1.96 (m, 4H), 4.92 (bs, 2H), 6.24 (s, 2H), 6.77 (s, 2H), 6.93-7.03 (m, 4H), 7.07-7.10 (m, 8H), 7.26-7.28 (m, 8H); ^{13}C NMR (100 MHz, $CD_3C_6D_5$, 353K) δ 26.52, 28.38, 28.50, 29.41, 78.34, 80.58, 81.65, 127.76, 128.22, 128.85, 140.79, 140.90, 155.91, 171.19; HRMS (FAB, 3-NBA) *m/z* $[m+H]^+$ calcd. for $C_{54}H_{71}N_4O_{12}$, 967.5068; found, 967.5090.



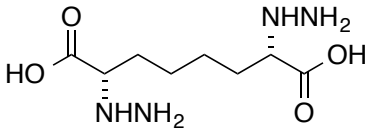
6Z

Starting from ester **14Z** (37 mg, 0.03 mmol, 1.0 eq) in CH_2Cl_2 (0.5 mL) was added anisole (60 μL, 2 mL/mmol) (cation scavenger) followed by the slow addition of trifluoroacetic acid (TFA) (0.3 mL, 10 mL/mmol) and the mixture was stirred at 0 °C until starting material was no longer detected by TLC (~ 1-2 h). The solvent and TFA were gently evaporated under reduced pressure and the resulting oily residue was dissolved in H_2O (0.5 mL) and the organic byproducts were pipetted away with CH_2Cl_2 (3 x 0.5 mL) followed by Et_2O (3 x 0.5 mL), and the aqueous layer was concentrated under reduced pressure to afford 12 mg (90%) of the bis-TFA salt **6Z**. $[\alpha]_D$ +23.0 (*c* 0.70, D_2O); ^{1}H NMR (500 MHz, D_2O) δ 2.60 (app. t，*J* = 6.0, 5.5 Hz, 4H), 3.86 (app. t, *J* = 6.5, 5.5 Hz, 2H), 5.56-5.61 (t, *J* = 5.0 Hz, 2H); ^{13}C NMR (100 MHz, D_2O) δ 27.64, 60.69, 127.02, 174.01; HRMS (FAB, Gly) *m/z* $[m+H]^+$ calcd. for $C_8H_{17}N_4O_4$, 233.1250; found, 233.1245.



6E

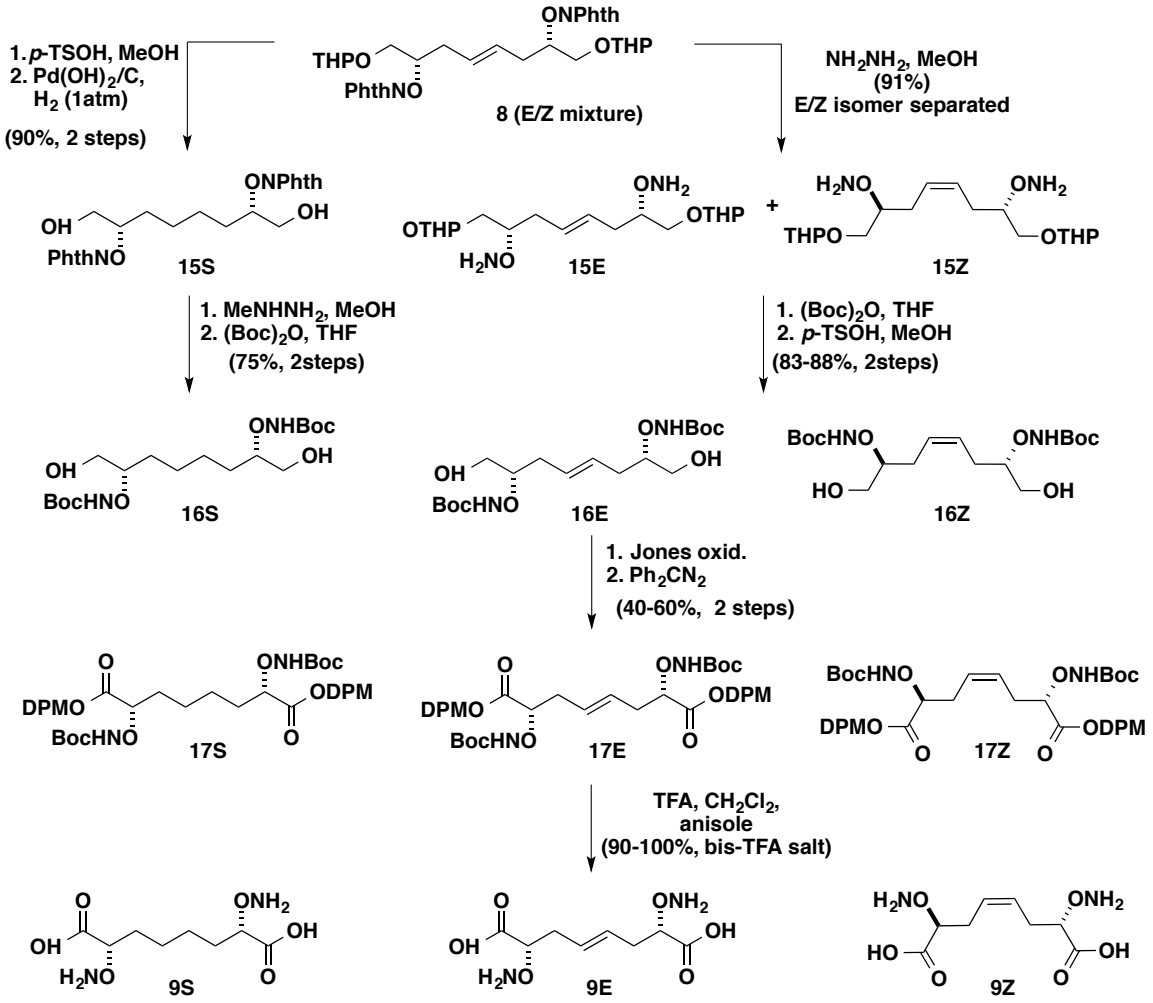
Starting from ester **14E** (41 mg, 0.04 mmol, 1.0 eq) in CH_2Cl_2 (0.5 mL) was added anisole (80 μL, 2 mL/mmol) (cation scavenger) followed by the slow addition of trifluoroacetic acid (TFA) (0.4 mL, 10 mL/mmol) and the mixture was stirred at 0 °C until starting material was no longer detected by TLC (~ 1-2 h). The solvent and TFA were gently evaporated under reduced pressure and the resulting oily residue was dissolved in H_2O (0.5 mL) and the organic byproducts were pipetted away with CH_2Cl_2 (3 x 0.5 mL) followed by Et_2O (3 x 0.5 mL), and the aqueous layer was concentrated under reduced pressure to afford 17 mg (90%) of the bis-TFA salt **6E**. $[\alpha]_D$ -3.60 (*c* 0.74, D_2O); ^1H NMR (500 MHz, D_2O) δ 2.51-2.59 (m, 4H), 3.86 (app t, *J* = 6.5, 5.5 Hz, 2H), 5.56-5.61 (m, 2H); ^{13}C NMR (100 MHz, D_2O) δ 32.63, 60.50, 128.32, 173.77; HRMS (FAB, Gly) *m/z* $[m+H]^+$ calcd. for $C_8H_{17}N_4O_4$, 233.1250; found, 233.1245.

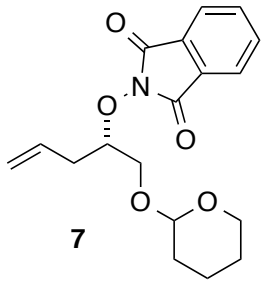


6S

Starting from ester **14S** (40 mg, 0.04 mmol, 1.0 eq) in CH_2Cl_2 (0.5 mL) was added anisole (83 μL, 2 mL/mmol) (cation scavenger) followed by the slow addition of trifluoroacetic acid (TFA) (0.4 mL, 10 mL/mmol) and the mixture was stirred at 0 °C until starting material was no longer detected by TLC (~ 1-2 h). The solvent and TFA were gently evaporated under reduced pressure and the resulting oily residue was dissolved in H_2O (0.5 mL) and the organic byproducts were pipetted away with CH_2Cl_2 (3 x 0.5 mL) followed by Et_2O (3 x 0.5 mL), and the aqueous layer was concentrated under reduced pressure to afford 18 mg (95%) of the bis-TFA salt **6S**. $[\alpha]_D$ -35.6 (*c* 0.59, MeOH) ^1H NMR (500 MHz, D_2O) δ 1.36-1.46 (m，4H), 1.73-1.83 (m, 4H), 3.79 (app. t, *J*= 6.0, 6.5 Hz, 2H); ^{13}C NMR (100 MHz, D_2O) δ 24.14, 28.92, 61.07, 174.35; HRMS (FAB, Gly) *m/z* $[m+H]^+$ calcd. for $C_8H_{19}N_4O_4$, 235.1406; found, 235.1406.

Figure S2: Aminoxy Acids (-O-NH_2) Synthesis

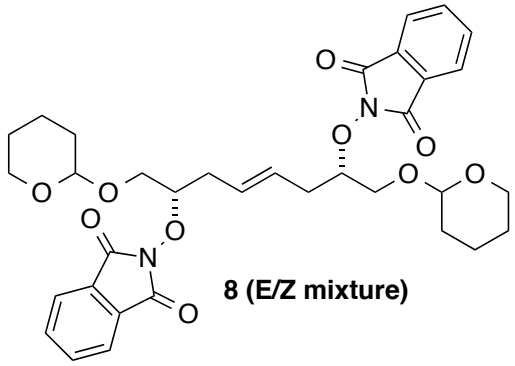




7

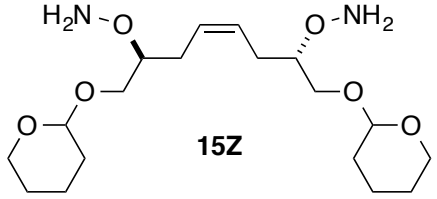
To a cooled (-30 °C) solution of THP-protected (S)-homoallylic alcohol **3** (7.13 g, 38.3 mmol, 1.0 eq), triphenylphosphine (TTP) (18.1 g, 68.9 mmol, 1.8 eq), and N-hydroxyphthalimide (8.12 g, 49.8 mmol, 1.3 eq) in THF (300 mL, ~ 130 mM) was slowly added via syringe pump (0.5 mL/min) Di-isopropylazodicarboxylate (DIAD) (13.5 mL, 68.9 mmol, 1.8 eq). Following the addition the reaction was stirred for an additional 30 min at 0 °C and warmed to rt whereby TLC indicated complete consumption of starting alcohol. The reaction mixture was concentrated under reduced pressure and the crude residue was purified by SiO_2 column chromatography (4:1 hexane:EtOAc) to afford 12.1 g of product **7** (95%) as a mixture of THP diastereomers. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.16-1.66 (m, 6H), 2.47-2.61 (m, 2H), 3.42-3.48 (m, 1H), 3.61-3.79 (m, 2H), 3.87-3.97 (m, 1H), 4.48-4.52 (m, 1H), 4.55-4.62 (m, 1H), 5.10-5.21 (m, 2H), 5.89-6.00 (m, 1H), 7.70-7.75 (m, 2H), 7.78-7.83 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (18.88, 18.93), (25.26, 25.33), (30.12, 30.25), (34.74, 34.99), (61.82, 61.94), (67.54, 69.17), (85.34,

85.56), (98.75, 98.85), (118.06, 118.12), (123.28, 123.38), (129.06, 129.09), (132.81, 133.09), (134.24, 134.34), (163.70, 163.88); HRMS (FAB, 3-NBA) *m/z* $[m+Li]^+$ calcd. for $C_{18}H_{21}NO_5Li$, 338.1580; found, 338.1572.

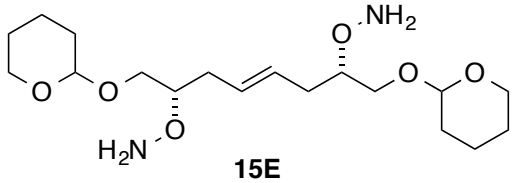


Compound **7** (6.67 g, 20.1 mmol, 1.0 eq) was dissolved in dry CH_2Cl_2 (200 mL, 100 mM) under a stream of N_2 and stirred at room temperature. Next, a solution of 1st Generation Grubbs Catalyst (828 mg, 1.01 mmol, 5 mol%) in CH_2Cl_2 (50 mL) was added slowly via cannulation. The mixture was gently heated to reflux for 48 h and upon disappearance of starting material by TLC the solvent was evaporated and the crude residue was purified by SiO_2 column chromatography (4:1 to 1:1 hexane:EtOAc) to afford 5.10 g (80%) of the cross metathesis product **8** as a mixture of E/Z isomers. The *E:Z* ratio (1.5:1) was determined by crude ^{1}H NMR. ^{1}H NMR (**E/Z mixture**) (400 MHz, $CDCl_3$) δ 1.21-1.64 (m, 12H), 2.49-2.62 (m, 4H), 3.42-3.45 (m, 2H), 3.60-3.76 (m, 4H), 3.87-3.96 (m, 2H), 4.45-4.62 (m, 4H), 5.72-5.75 (m, 2H), 7.69-7.73 (m, 4H), 7.78-7.81 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (18.89, 18.95), (25.27, 25.31, 25.34), (28.52, 28.74), (30.13, 30.25), (33.53, 33.80), (61.77, 61.91), (67.47, 69.06) (85.64, 85.68, 85.73), (85.83, 85.87, 85.94), (98.68, 98.81), (123.25, 123.34), (126.68, 126.75, 126.91, 126.99), (127.93, 127.98, 128.20, 128.26), (129.08, 129.10), (134.20, 134.29), (163.64, 163.67, 163.84); HRMS (FAB, 3-NBA) *m/z* $[m+Li]^+$ calcd. for $C_{34}H_{38}N_2O_{10}Li$, 641.2686; found, 641.2746.

To a solution of clean E/Z mixture **8** (4.6 g, 7.3 mmol, 1.0 eq) in MeOH (73 mL, 100 mM), methylhydrazine (1.2 mL, 21.9 mmol, 3 eq) was slowly added (via syringe) and the reaction was allowed to stir further overnight at room temperature. Upon completion, the reaction mixture was concentrated under reduced pressure, re-dissolved in 3% $Na_2CO_{3(aq)}$ (100 mL), and extracted with diethyl ether (3 x 100 mL). The combined organics were washed with brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. At this stage the E/Z mixture was purified by SiO_2 column chromatography (EtOAc:MeOH, 3% v/v) to afford 1.04 g (38%) of the *Z*-isomer **15Z** (**fraction 1**) as well as 1.56 g (57%) of the *E*-isomer **15E** (**fraction 2**), consistent of a E:Z ratio of 1.5:1. *Note: similar to the hydrazino acid series, the Z-isomer eluted from the column first, followed by the E-isomer.*

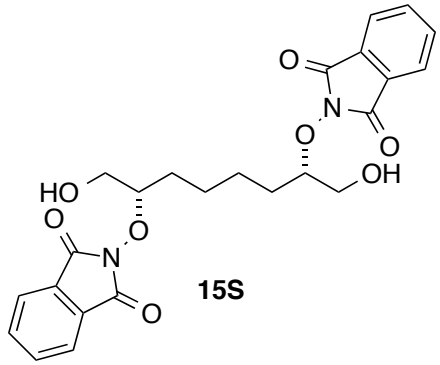


***Z*-alkene (15*Z*)**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.49-1.62 (m, 8H), 1.67-1.73 (m, 2H), 1.79-1.83 (m, 2H), 2.32-2.38 (m, 4H), 3.46-3.53 (m, 4H), 3.74-3.88 (m, 6H), 4.58-4.62 (m, 1H), 5.36 (br, 4H), 5.51-5.55 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (19.42, 19.54), 25.39, (28.31, 28.37), (30.51, 30.59), (62.14, 62.33), (67.70, 68.15), (82.58, 82.83), (98.75, 99.34), (127.29, 127.37), (127.42, 127.50); HRMS (FAB, 3-NBA) *m/z* $[m+H]^+$ calcd. for $C_{18}H_{35}N_2O_6$, 375.2495; found, 375.2507.



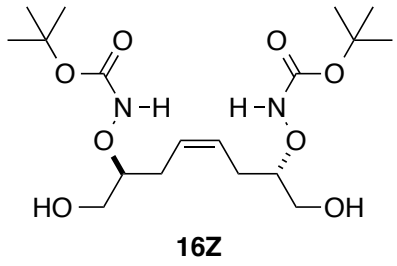
15E

***E*-alkene (15*E*)**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.52-1.62 (m, 8H), 1.68-1.73 (m, 2H), 1.79-1.84 (m, 2H), 2.23-2.34 (m, 4H), 3.46-3.52 (m, 4H), 3.74-3.88 (m, 6H), 4.58-4.61 (m, 2H), 5.35 (br s, 4H), 5.53 (br s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (19.40, 19.53), 25.40, (30.50, 30.60), (33.63, 33.68), (62.10, 62.31), (67.68, 68.19), (82.48, 82.73), (98.70, 99.35), (128.60, 128.63), (128.72, 128.75); HRMS (FAB, 3-NBA) *m/z* $[m+H]^+$ calcd. for $C_{18}H_{35}N_2O_6$, 375.2495; found, 375.2513.



15S

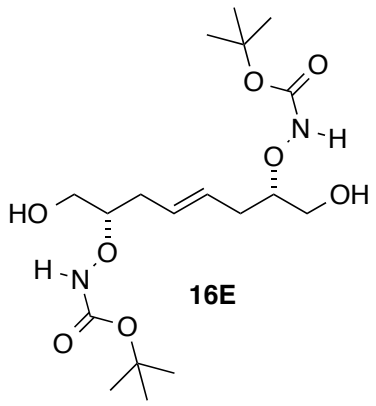
To a solution of clean E/Z mixture **8** (2.55 g, 4.02 mmol, 1 eq) in MeOH (80 mL, 50 mM) was added *p*-TsOH monohydrate (459 mg, 2.41 mmol, 0.6 eq) and the solution was stirred for 30 min at room temperature whereby TLC indicated complete deprotection to the diol. The reaction mixture was quenched by the addition of Et_3N (0.34 mL, 2.41 mmol, 0.6 eq), concentrated, and dissolved in $CHCl_3$ (40 mL, 100 mM). To the crude diol (E/Z mixture) was added 20% $Pd(OH)_2$/C (510 mg) and the suspension was subjected to an atmosphere of H_2 (1 atm, balloon pressure) and vigorously stirred at room temperature for 3 h, upon which time, complete C=C bond reduction was indicated by crude ^{1}H NMR. The catalyst was filtered off with a patch of Celite® and the solvent was evaporated to afford 1.79 g (95%) of the saturated diol **15S**. The product was determined to be analytically pure by NMR and no additional purification was necessary. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.69 (m, 6H), 1.97 (m, 2H), 3.49 (m, 2H), 3.59 (m, 2H), 3.76 (m, 2H), 4.15 (m, 2H), 7.76 (m, 4H), 7.83 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ25.41, 29.36, 61.87, 89.39, 100.51, 123.77, 128.78, 134.76, 164.94; HRMS (FAB, 3-NBA) *m/z* $[m+H]^+$ calcd. for $C_{24}H_{25}N_2O_8$, 469.1611; found, 469.1605.



16Z

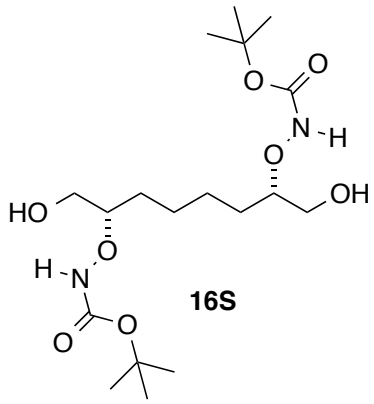
To a solution of **15Z** (260 mg, 0.694 mmol, 1.0 eq) in dry THF (5.0 mL, ~140 mM) was added $(Boc)_2O$ (455 mg, 2.08 mmol, 3.0 eq) followed by the addition of Et_3N (0.30 mL, 2.08 mmol, 3.0 eq) and the reaction mixture was allowed to stir at room temperature for 3 h upon which time no starting material was observed. The reaction was quenched by the addition of 1N HCl (5mL) and diluted with Et_2O (5mL). The organics were extracted with Et_2O (3 x 5 mL), dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The resulting crude residue was directly applied to THP deprotection. Thus, the THP protected diol was dissolved in dry methanol (5.0 mL), followed by the addition of *p*TsOH monohydrate (79.0 mg, 0.416 mmol, 0.6 eq). The reaction was stirred at room temperature for 30 min and subsequently quenched by the addition of Et3N (60 μL). The resulting mixture was concentrated under reduced pressure and the crude

residue was purified by SiO_2 column chromatography (1:1 hexane:EtOAc) to afford 235 mg (83% - 2 steps) of the Z-diol **16Z**. $[\alpha]_D$ -30.3 (*c* 1.20, CH_2Cl_2); ^1H NMR (400 MHz, $CDCl_3$) δ 1.46 (s, 18H), 2.12-2.30 (m, 2H), 2.34-2.41 (m, 2H), 3.50-3.56 (m, 2H), 3.63-3.67 (m, 2H), 3.79-3.85 (m, 2H), 4.42 (br s, 2H), 5.46 (t, *J* = 4.8 Hz, 2H), 7.87 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 27.05, 28.09, 60.91, 82.46, 85.72, 127.34, 159.15; HRMS (FAB, 3-NBA) *m/z* $[m+H]^+$ calcd. for $C_{18}H_{35}N_2O_8$, 407.2393; found, 407.2397.



16E

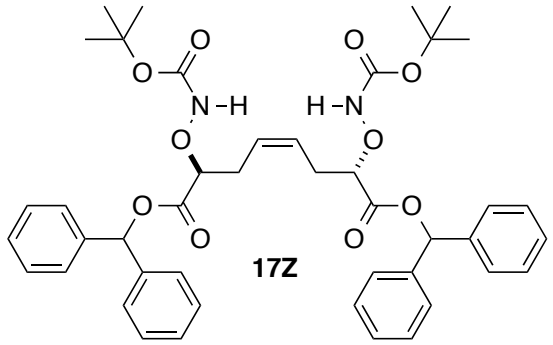
To a solution of **15E** (230 mg, 0.615 mmol, 1.0 eq) in dry THF (5.0 mL, ~120 mM) was added $(Boc)_2O$ (403 mg, 1.85 mmol, 3.0 eq) followed by the addition of Et_3N (0.26 mL, 1.85 mmol, 3.0 eq) and the reaction mixture was allowed to stir at room temperature for 3 h upon which time no starting material was observed. The reaction was quenched by the addition of 1N HCl (5mL) and diluted with Et_2O (5mL). The organics were extracted with Et_2O (3 x 5 mL), dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The resulting crude residue was directly applied to THP deprotection. Thus, the THP protected diol was dissolved in dry methanol (5.0 mL), followed by the addition of *p*TsOH monohydrate (70.0 mg, 0.369 mmol, 0.6 eq). The reaction was stirred at room temperature for 30 min and subsequently quenched by the addition of Et3N (50 μL). The resulting mixture was concentrated under reduced pressure and the crude residue was purified by SiO_2 column chromatography (1:1 hexane:EtOAc) to afford 220 mg (88% - 2 steps) of the E-diol **16E**. mp. 102-104° C; $[\alpha]_D$ -12.9 (*c* 1.38, CH_2Cl_2); ^1H NMR (400 MHz, $CDCl_3$) δ 1.47 (s, 18H), 2.18-2.23 (m, 2H), 2.30-2.38 (m, 2H), 3.50 (dd, *J* = 12.8, 6.4 Hz, 2H), 3.67 (dd, *J* = 12.8, 2.8 Hz, 2H), 3.78-3.84 (m, 2H), 5.56 (m, 2H), 7.45 (br s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 28.13, 33.02, 61.99, 82.82, 86.44, 128.85, 158.74; HRMS (FAB, 3-NBA) *m/z* $[m+H]^+$ calcd. for $C_{18}H_{35}N_2O_8$, 407.2393; found, 407.2400.



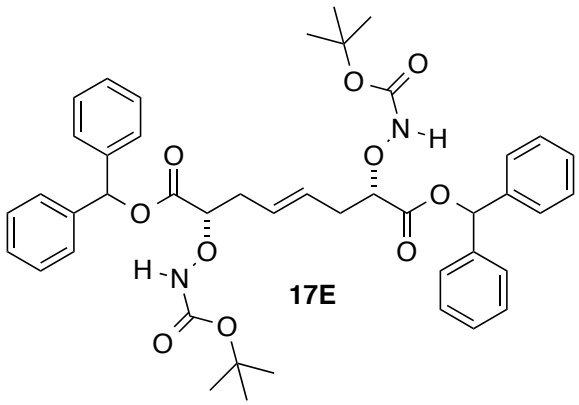
16S

To a solution of saturated diol **15S** (710 mg, 1.52 mmol, 1.0 eq) in $CHCl_3$ (15 mL, 100 mM) was added methylhydrazine (0.40 mL, 7.55 mmol, 5.0 eq) dropwise via syringe and the resulting mixture was allow stir for 2 hr at room temperature. Upon completion a white precipitate was formed (byproduct) and was filtered off from the reaction mixture over a patch of Celite®, washed with $CHCl_3$, and the supernatant was concentrated down and re-dissolved in THF (20 mL). To this solution was added $(Boc)_2O$ (1.0 g, 4.56 mmol, 3.0 eq) followed by the addition of Et_3N (0.64 mL, 4.56 mmol, 3.0 eq) and the reaction mixture was allowed to stir at room temperature for 3 h upon which time no starting material was observed. The reaction was quenched by the addition of 1N HCl (5mL) and diluted with Et_2O (10 mL). The organics were extracted with Et_2O (3 x 10 mL), dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude product was purified by SiO_2 column chromatography (1:1

hexane:EtOAc) to afford 466 mg (75% - 2 steps) of the Boc-protected diol **16S** as a white solid. mp. 106-108°C; $[\alpha]_D$ -17.3 (*c* 1.37, CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.41 (m, 6H), 1.45 (s, 18H), 1.56-1.65 (m, 2H), 3.45 (dd, *J* = 12.7, 5.7 Hz, 2H), 3.64-3.78 (br m, 4H), 4.14 (br s, 2H), 7.52 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 25.44, 28.10, 28.17, 28.85, 62.03, 82.45, 86.65, 158.78; HRMS (FAB, 3-NBA) *m/z* $[m+H]^+$ calcd. for $C_{18}H_{37}N_2O_8$, 409.2550; found, 409.2534.

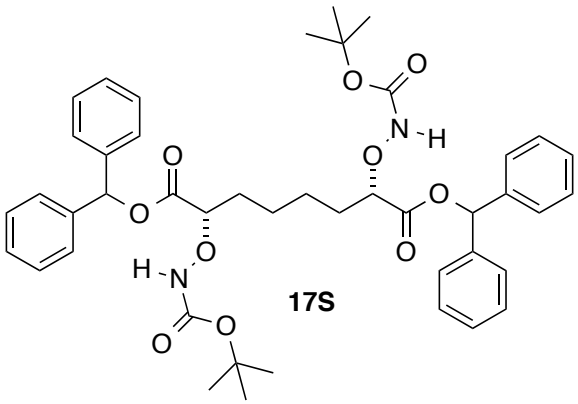


Diol **16Z** (159 mg, 0.39 mmol, 1.0 eq) was dissolved in acetone (10 mL, 40 mM), cooled to 0 °C, and subjected to oxidizing conditions by slow addition of Jones reagent (8N) (1.2 mL, 9.8 mmol, 25 eq) The mixture was stirred at 0 °C until TLC (EtOAc: 3% v/v AcOH) indicated complete conversion of diol to bis-acid (~ 2 hr). Conveniently, the carboxcylic acid functionality could be visually identified in parallel by treatment of the developed TLC plate with bromocresol green (bright yellow color appears). The reaction was quenched by the addition of a minimal amount isopropol alcohol (*i*PrOH) until the solution turned to a blue/green tint, warmed to room temperature, and the crude acid was extracted with EtOAc. The organics were dried over Na_2SO_4, filtered, and concentrated. To facilitate purification, the bis-acid was esterified to the diphenylmethyl ester. Specifically, the crude bis-acid was redissolved in EtOAc (15 mL) and to this solution was added diphenyldiazomethane (300 mg, 1.56 mmol, 4.0 eq). The dark red reaction mixture was stirred overnight at room temperature and subsequently quenched with dropwise addition of AcOH until the reaction mixture turned clear. The crude mixture was concentrated under reduced pressure and the residue was purified by SiO_2 column chromatography (7:3 hexane:EtOAc) to afford 111 mg (37% - 2 steps) of pure diphenylmethyl ester **17Z**. $[\alpha]_D$ -46.2 (*c* 1.02, CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.43 (s, 18H), 2.41-2.53 (m, 4H), 4.48 (dd, *J* = 7.6, 4.8 Hz, 2H) 5.55 (t, *J* = 4.8 Hz, 2H), 6.92 (s, 2H), 7.24-7.35 (m, 20H) 7.45 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 28.16, 28.83, 77.79, 81.94, 82.99, 126.32, 126.98, 127.20, 128.08, 128.21, 128.56, 128.64, 139.46, 139.54, 156.18, 170.18; HRMS (FAB, 3-NBA) *m/z* $[m+H]^+$ calcd. for $C_{44}H_{51}N_2O_{10}$, 767.3544; found, 767.3542.



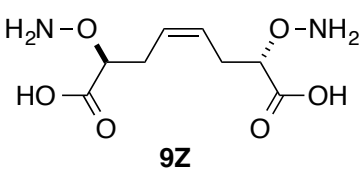
Compound **17E** was synthesized following a procedure identical to that described above for **17Z**. Specifically, **16E** (320 mg, 0.79 mmol, 1.0 eq) in acetone (20 mL) was subjected to Jones oxidation. Upon conversion of the diol to the bis-acid (~ 2 hr) the reaction was quenched by the addition of *i*PrOH, the organics were further extracted with EtOAc, dried of over Na_2SO_4,

concentrated under reduced pressure, and the crude bis-acid was subsequently treated with diphenydiazomethane (606 mg, 3.16 mmol, 4.0 eq) in EtOAc (25 mL) at room temperature overnight. Following treatment of the reaction mixture with AcOH, the crude mixture was once again concentrated and the residue was purified by SiO_2 column chromatography (7:3 hexane:EtOAc) to afford 248 mg (41% - 2 steps) of the pure diphenylmethyl ester **17E**. mp: 153-155° C; $[\alpha]_D$-53.4 (*c* 1.12, CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.44 (s, 9H), 2.38-2.50 (m, 4H), 4.44-4.48 (m, 2H) 5.45 (app t, *J* = 4.0, 3.5 Hz 2H), 6.92 (s, 2H), 7.26-7.35 (m, 20H) 7.54 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 28.18, 33.86, 77.68, 81.88, 83.11, 127.30, 127.49, 128.05, 128.20, 128.54, 128.59, 139.49, 139.58, 156.22, 170.05; HRMS (FAB, 3-NBA) *m/z* $[m+H]^+$ calcd. for $C_{44}H_{51}N_2O_{10}$, 767.3544; found, 767.3529.



17S

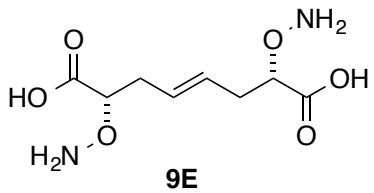
Compound **17S** was synthesized following a procedure identical to that described above for **17Z**. Specifically, **16S** (210 mg, 0.52 mmol, 1.0 eq) in acetone (17 mL) was subjected to Jones oxidation. Upon conversion of the diol to the bis-acid the reaction was quenched by the addition of *i*PrOH, the organics were further extracted with EtOAc, dried of over Na_2SO_4, concentrated under reduced pressure, and the crude bis-acid was subsequently treated with diphenydiazomethane (400 mg, 2.08 mmol, 4.0 eq) in EtOAc (16 mL) at room temperature overnight. Following treatment of the reaction mixture with AcOH, the crude mixture was once again concentrated and the residue was purified by SiO_2 column chromatography (7:3 hexane:EtOAc) to afford 200 mg (50% - 2 steps) of the pure diphenylmethyl ester **17S**. $[\alpha]_D$ -68.4 (*c* 1.14, CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.37 (m, 4H), 1.45 (s, 18H), 1.79 (m, 4H), 4.46 (dd, *J* = 8.0, 4.4 Hz, 2H), 6.94 (s, 2H), 7.32 (m, 20H), 7.53 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 24.65, 28.13, 30.53, 77.58, 81.79, 83.44, 126.88, 127.11, 128.01, 128.18, 128.50, 128.58, 139.41, 139.56, 156.19, 170.73; HRMS (FAB, 3-NBA) *m/z* $[m+H]^+$ calcd. for $C_{44}H_{53}N_2O_{10}$, 769.3700; found, 769.3695.



9Z

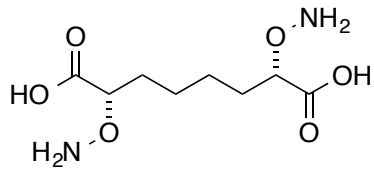
Starting from ester **17Z** (35 mg, 0.05 mmol, 1.0 eq) in CH_2Cl_2 (0.5 mL) was added anisole (92 μL, 2 mL/mmol) (cation scavenger) followed by the slow addition of trifluoroacetic acid (TFA) (0.5 mL, 10 mL/mmol) and the mixture was stirred at 0 °C until starting material was no longer detected by TLC (~ 1-2 h). The solvent and TFA were gently evaporated under reduced pressure and the resulting oily residue was dissolved in H_2O (0.5 mL) and the organic byproducts were pipetted away with CH_2Cl_2 (3 x 0.5 mL) followed by Et_2O (3 x 0.5 mL), and the aqueous layer was concentrated under reduced pressure to afford analytically pure product **9Z** (19 mg, 90%) as the bis-TFA salt. $[\alpha]_D$ -27.5 (*c* 0.80, D_2O) ^{1}H NMR (500 MHz, D_2O) δ 2.64 (t, *J* = 5.5Hz, 4H),

4.67 (app. t, J =6.0, 5.5Hz, 2H), 5.59 (app. t, J =5.0, 4.5Hz, 2H); ^{13}C NMR (100 MHz, D_2O) δ 28.57, 81.68, 126.30, 162.76; HRMS (FAB, Gly) *m/z* $[m+H]^+$ calcd. for $C_8H_{14}D_2N_2O_6$, 238.1134; found, 238.1128.



9E

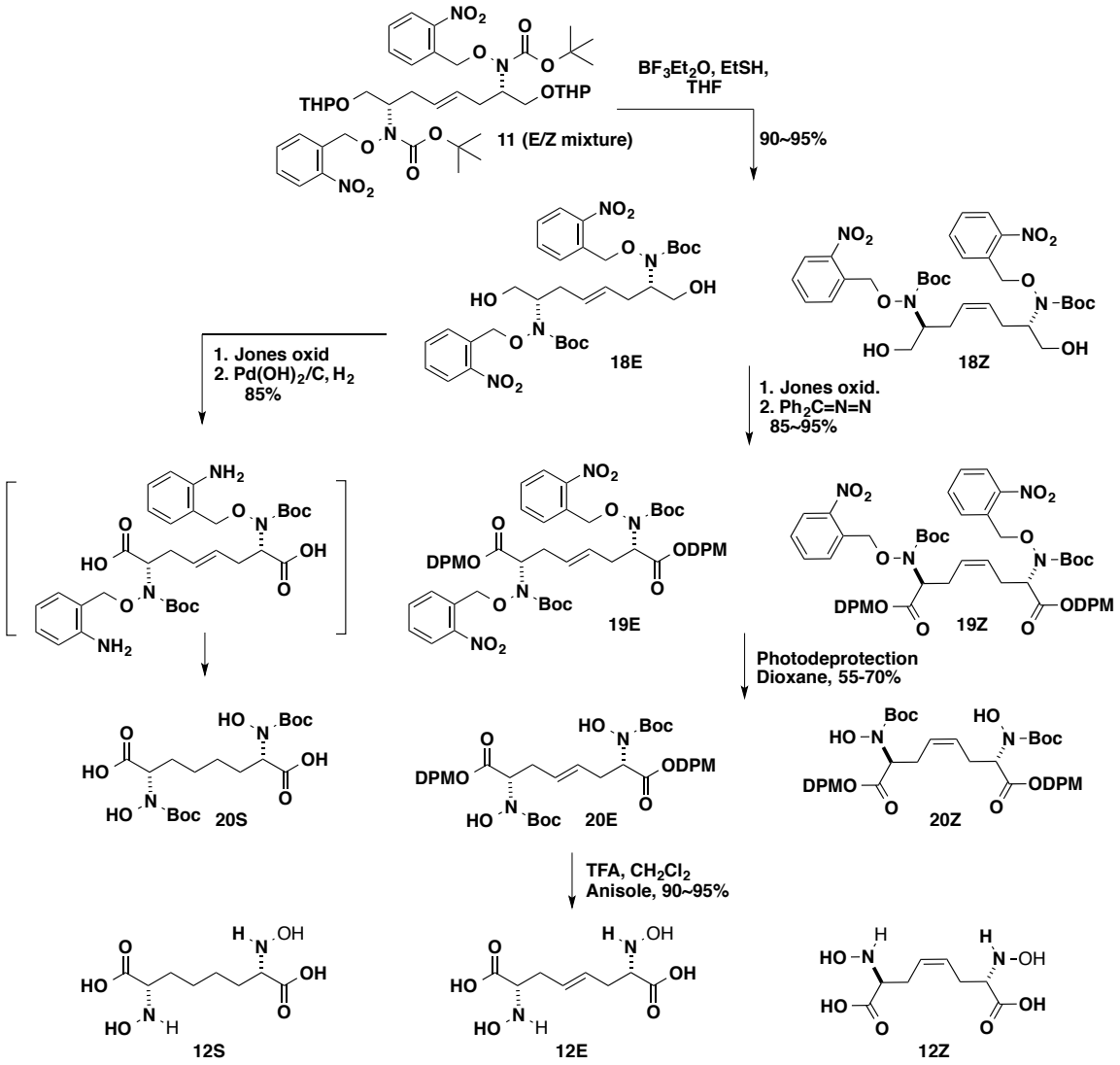
Starting from ester **17E** (110 mg, 0.14 mmol, 1.0 eq) in CH_2Cl_2 (3.0 mL) was added anisole (0.5 mL, 2 mL/mmol) (cation scavenger) followed by the slow addition of trifluoroacetic acid (TFA) (1.4 mL, 10 mL/mmol) and the mixture was stirred at 0 °C until starting material was no longer detected by TLC (~ 1-2 h). The solvent and TFA were gently evaporated under reduced pressure and the resulting oily residue was dissolved in H_2O (0.5 mL) and the organic byproducts were pipetted away with CH_2Cl_2 (3 x 0.5 mL) followed by Et_2O (3 x 0.5 mL), and the aqueous layer was concentrated under reduced pressure to afford 61 mg (95%) of the bis-TFA salt **9E**. $[\alpha]_D$ -29.9 (*c* 0.45, D_2O) ^{1}H NMR (400 MHz, D_2O) δ 2.56-2.69 (m, 4H), 4.66 (dd, J =6.3, 4.6 Hz, 2H), 5.61 (app. t, J =3.8, 3.6 Hz, 2H); ^{13}C NMR (100 MHz, D_2O) δ 33.50, 81.62, 127.48, 173.53; HRMS (FAB, Gly) *m/z* $[m+H]^+$ calcd. for $C_8H_{13}D_2N_2O_6$, 237.1056; found, 237.1061.

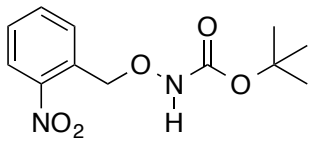


9S

Starting from ester **17S** (38 mg, 0.05 mmol, 1.0 eq) in CH_2Cl_2 (0.5 mL) was added anisole (100 μL, 2 mL/mmol) (cation scavenger) followed by the slow addition of trifluoroacetic acid (TFA) (0.5 mL 10 mL/mmol) and the mixture was stirred at 0 °C until starting material was no longer detected by TLC (~ 1-2 h). The solvent and TFA were gently evaporated under reduced pressure and the resulting oily residue was dissolved in H_2O (0.5 mL) and the organic byproducts were pipetted away with CH_2Cl_2 (3 x 0.5 mL) followed by Et_2O (3 x 0.5 mL), and the aqueous layer was concentrated under reduced pressure to afford 21 mg (90%) of the bis-TFA salt **9S**. $[\alpha]_D$ -54.4 (*c* 1.10, H_2O) ^{1}H NMR (400 MHz, D_2O) δ 1.39-1.42 (m, 4H), 1.74-1.80 (m, 2H), 1.83-1.87 (m, 2H), 4.53 (dd, J = 4.0, 3.2 Hz, 2H); ^{13}C NMR (100 MHz, D_2O) δ 23.57, 29.95, 82.08, 174.37; HRMS (FAB, Gly) *m/z* $[m+H]^+$ calcd. for $C_8H_{16}D_2N_2O_6$, 240.1290; found, 240.1301.

Figure S3: N-Hydroxyamino Acids (-NH-OH) Synthesis





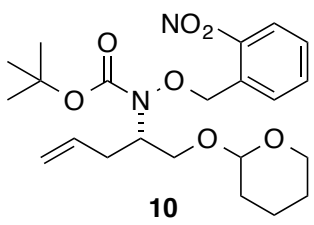
13

Following previously reported procedures[2,3] with some modification, synthesis of Mitsunobu nucleophile **13** was carried out in 3 steps overall. Specifically, to a solution of 2-nitrobenzyl alcohol (7.66 g, 50.0 mmol, 1.0 eq) in THF (200 mL, 250 mM) was added triphenylphosphine (14.4 g, 55.0 mmol, 1.1 eq), and N-hydroxyphthalamide (8.97 g, 55.0 mmol, 1.1 eq) and the mixture was stirred at room temperature. To this mixture was added Di-ethylazodicarboxylate (8.66 mL, 55.0 mmol, 1.1 eq) and the resulting solution was further stirred at room temperature for 1 h. The crude product precipitated out of solution, was filtered, and collected by vacuum filtration. The filtrate was concentrated under reduced pressure, dissolved in THF, cooled for 20 min., and the precipitate once again collected. This process was repeated 2 additional times to yield 11.0 g (74%) of crude 2-nitrobenzylated N-hydroxyphthalimide, which was carried forward without further purification. Spectral

characteristics of the crude product were consistent with previously reported data[2] and carried forward without further purification.

To a solution of crude 2-nitrobenzylated N-hydroxyphthalimide (11.0 g, 36.9 mmol, 1.0 eq) in MeOH (184 mL, 200 mM) was added hydrazine monohydrate (5.37 mL, 111 mmol, 3.0 eq) and the resulting mixture was stirred at room temperature for 1 h. The mixture was concentrated under reduced pressure and re-suspended in a solution of 3% $Na_2CO_{3(aq)}$ and diethyl ether. The organics were extracted with Et_2O (3 × 50 mL), dried over $MgSO_4$, filtered, and concentrated under reduced pressure to yield 6.14 g (99%) of crude *O*-(2-nitrobenzyl)hydroxylamine, which was carried forward without further purification. The crude product was analytically pure by NMR and spectral characteristics were consistent with previously reported data.[3] ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.06 (s, 2H), 5.56 (s, 2H), 7.42-7.46 (m, 1H), 7.61-7.67 (m, 2H), 8.01-8.03 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 74.26, 124.70, 128.22, 129.10, 133.35, 134.22, 148.03.

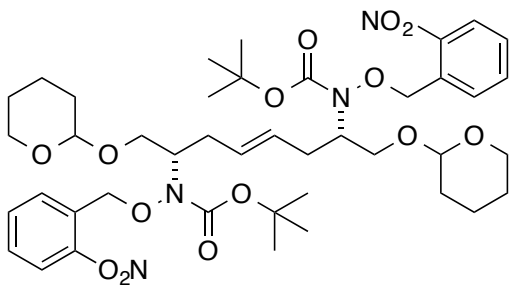
To a solution of crude *O*-(2-nitrobenzyl)hydroxylamine (6.14 g, 36.5 mmol, 1.0 eq) in a THF (183 mL, 200 mM) was added $(Boc)_2O$ (12.0 g, 54.8 mmol, 1.5 eq) and triethylamine (7.64 mL, 54.8 mmol, 1.5 eq) and the resulting solution was stirred at room temperature overnight. The reaction was quenched by the addition of water and the organics were extracted with Et_2O. The organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude residue was purified by SiO_2 column chromatography (4:1 hexane:EtOAc) to yield 9.04 g (90%) of pure compound **13**. mp 97-99 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.46 (s, 9H), 5.26 (s, 2H), 7.29 (br s, 1H), 7.47 (ddd, J =15.2, 8.0, 1.2 Hz, 1H), 7.63 (ddd, J = 15.2, 7.6, 1.2 Hz, 1H), 7.50 (d, J = 7.6 Hz, 1H), 8.04 (dd, J = 8.0, 1.2 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 28.13, 74.85, 82.18, 124.47, 128.74, 129.92, 132.20, 133.51, 147.91, 156.65; HRMS (FAB, 3-NBA) *m/z* $[m+Li]^+$ calcd. for $C_{12}H_{16}N_2O_5Li$, 275.1219; found, 275.1220.



10

To a cooled (0 °C) solution of THP-protected (S)-homoallylic alcohol **3** (6.50 g, 34.9 mmol, 1.0 eq), triphenylphosphine (TTP) (13.7 g, 52.3 mmol, 1.5 eq), and nucleophile **13** (11.2 g, 41.9 mmol, 1.2 eq) in THF (175 mL, 200 mM) was slowly added via syringe pump (0.5 mL/min) a solution of Di-isopropylazodicarboxylate (DIAD) (10.3 mL, 52.4 mmol, 1.5 eq). The resulting mixture was stirred for 0 °C for 1 hr and warmed to room temperature, stirring for an additional 30 min. Following completion, the reaction mixture was concentrated under reduced pressure and the crude residue was purified by SiO_2 column chromatography (5:1 hexane:EtOAc) to afford 10.7 g of product **10** (70%) as a mixture of THP diastereomers. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.42-1.79 (m, 15H), 2.24-2.30 (m, 1H), 2.35-2.46 (m, 1H), 3.41-3.58 (m, 2H), 3.72-3.90 (m, 2H), 4.29-4.37 (m, 1H), 4.56-4.64 (m, 1H), 5.05-5.13 (m, 2H), 5.23-5.35 (m, 2H), 5.76-5.86 (m, 1H), 7.42-7.46 (m, 1H), 7.62-7.66 (m, 1H), 7.83-7.87 (m, 1H), 8.04-8.06 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (18.98, 19.31), 25.40, 28.27, (30.36, 30.46), (33.49, 33.55), (58.88, 59.78), (61.77, 62.20), (66.22, 66.96), (73.55, 73.58), 81.55, (97.99,

99.34), (117.53, 117.56), (124.51, 124.52), (128.18, 128.25), (129.32, 129.30), (132.70, 132.84), (133.54, 133.57), 134.60, (147.17, 147.24), (156.95, 157.00); HRMS (FAB, 3-NBA) *m/z* $[m+H]^+$ calcd. for $C_{22}H_{33}N_2O_7$, 437.2288; found, 437.2283.



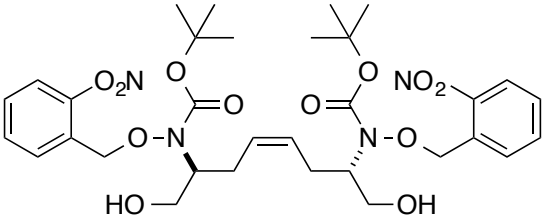
11 (E/Z mixture)

Compound **10** (3.66 g, 8.38 mmol, 1.0 eq) was dissolved in dry CH_2Cl_2 (84 mL, 100 mM) under a stream of N_2 and stirred at room temperature. Next, a solution of 1st Generation Grubbs Catalyst (344 mg, .419 mmol, 5 mol%) in CH_2Cl_2 (21 mL) was added slowly via cannulation. The mixture was gently heated to reflux for 48 h and upon disappearance of starting material by TLC the solvent was evaporated and the crude residue was purified by SiO_2 column chromatography (8:2 to 7:3 hexane:EtOAc) to afford 3.33 g (94%) of the cross metathesis product **11** as a mixture of E/Z isomers. The *E:Z* ratio (1.2:1) was determined by crude ^{1}H NMR. ^{1}H NMR (400 MHz, $CDCl_3$) d 1.44-1.78 (m, 30H), 2.19-2.42 (m, 4H), 3.39-3.55 (m, 4H), 3.67-3.87 (m, 4H), 4.26 (m, 2H), 4.55-4.61 (m, 2H), 5.22-5.31 (m, 4H), 5.51 (m, 2H), 7.39-7.45 (m, 2H), 7.58-7.65 (m, 2H), 7.80-7.86 (m, 2H), 8.00-8.05 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) d (19.09, 19.16) (19.42, 19.45), 25.52, (27.24, 27.27), (28.37, 28.40, 28.48), (30.48, 30.58), 32.65, (59.23, 60.11), (61.83, 61.93), (62.26, 62.30), (66.23, 66.36), (67.05, 67.12), 81.59, (98.08, 98.13), (99.42, 99.48), 124.59, (128.22, 128.31, 128.35, 128.41), (129.10, 129.15), (129.44, 129.52, 129.59), (132.70, 132.77), (133.84, 132.93), (133.67, 133.71, 133.72), (147.25, 147.32, 147.38), (157.03, 157.07, 157.12); HRMS (FAB, 3-NBA) *m/z* $[m+H]^+$ calcd. for $C_{42}H_{61}N_4O_{14}$, 845.4184; found, 845.4199.

THP-Deprotection and E/Z Geometric Isomer Separation:

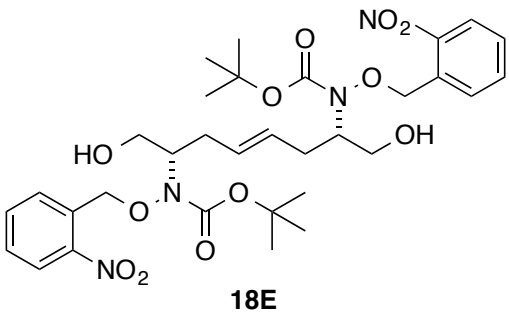
For the N-Hydroxyamino acid series, THP-deprotection was carried out under conditions reported by Nambiar and coworkers[4]. Specifically, to a solution of the E/Z mixture **11** (1.15 g, 1.36 mmol, 1.0 eq) in freshly distilled CH_2Cl_2 (50 mL) containing ethanethiol (1.4 mL, 10% v/v) was added $BF_3 \bullet Et_2O$ (35 μL, 0.27 mmol, 0.2 eq) at -20 °C and the resulting solution was allowed to warm to 0 °C, and stir for an additional 2 h. Upon disappearance of starting material and the mono-deprotected intermediate, indicated by TLC (1:1 hexane:EtOAc), a steady stream of N_2 was introduced to the reaction flask for 20 min by submersion of a glass inlet tube into the reaction mixture, allowing excess thiol to be removed. As an environmental precaution, two traps were connected in series, the first being connected directly to the reaction flask, and the second being charged with commercially available bleach to capture any excess thiol and avoid unpleasant odor to the laboratory. The reaction was then quenched by addition of saturated $NaHCO_3$, the organics were extracted with CH_2Cl_2, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude E/Z diol mixture was purified by SiO_2 column chromatography (1:1 hexane:EtOAc) to yield 394 mg (45%) *Z-isomer* **18Z** (fraction 1) and 438 mg (50%) *E-isomer* **18E** (fraction 2) for a 95% overall isolated yield. Isolated *E:Z* ratio of 1.1:1 was consistent with the 1.2:1 *E:Z* ratio observed following formation of

the THP-protected compound **11**. *Note: similar to the hydrazino and aminooxy acid series, the Z-isomer eluted from the column first, followed by the E-isomer.*



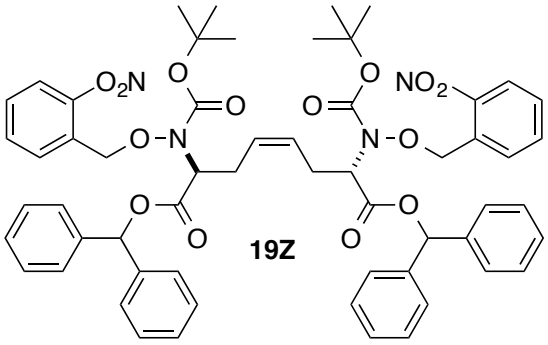
18Z

***Z*-diol (18Z):** $[\alpha]_D$ +14.6 (*c* 1.505, CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.45 (s, 18H), 2.07 (app t, *J* = 6.0, 5.6 Hz, 2H), 2.21-2.26 (m, 2H), 2.37-2.42 (m, 2H), 3.60-3.72 (m, 4H), 4.09-4.13 (m, 2H), 5.22 (s, 2H), 5.49 (t, *J* = 4.8 Hz, 2H), 7.42-7.46 (m, 2H), 7.58-7.63 (dt, *J* = 7.6, 1.2 Hz, 2H), 7.75-7.77 (d, *J* = 6.9 Hz, 2H), 7.97-7.80 (dd, *J* = 8.2, 1.2 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 26.50, 28.19, 62.28, 62.73, 73.83, 82.16, 124.56, 128.07, 128.77, 130.37, 131.67, 133.51, 147.74, 157.61; HRMS (FAB, 3-NBA) *m/z* $[m+H]^+$ calcd. for $C_{32}H_{45}N_4O_{12}$, 677.3034; found, 677.3048.



18E

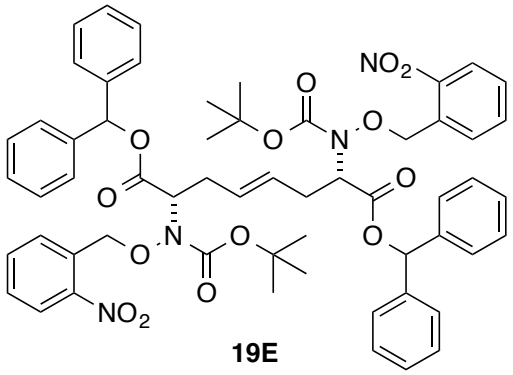
***E*-diol (18E):** $[\alpha]_D$ -3.16 (*c* 0.74, CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.45 (s, 18H), 2.18-2.24 (m, 4H), 2.31-2.36 (m, 2H), 3.62-3.73 (m, 4H), 4.05-4.12 (m, 2H), 5.20-5.28 (m, 4H), 5.50 (app t, *J* = 4.0, 3.6 Hz, 2H), 7.43-7.47 (m, 2H), 7.61-7.65 (m, 2H), 7.76-7.78 (d, *J* = 7.6 Hz, 2H), 8.00-8.02 (dd, *J* = 8.2, 0.92 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 28.19, 31.81, 62.40, 62.53, 73.75, 82.10, 124.57, 128.69, 128.99, 130.11, 131.77, 133.55, 147.60, 157.62; HRMS (FAB, 3-NBA) *m/z* $[m+H]^+$ calcd. for $C_{32}H_{45}N_4O_{12}$, 677.3034; found, 677.3017.



19Z

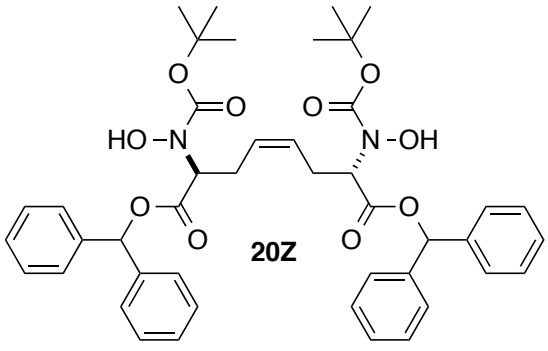
Diol **18Z** (283 mg, 0.417 mmol, 1.0 eq) was dissolved in acetone (10 mL), cooled to 0 °C, and subjected to oxidizing conditions by slow addition of Jones reagent (8N) (1.30 mL, 10.4 mmol, 25 eq). The mixture was stirred at 0 °C until TLC (EtOAc: 3% v/v AcOH) indicated complete conversion of diol to bis-acid (~ 2 h). Conveniently, the carboxcylic acid could be visually identified by treatment of the developed TLC plate with bromocresol green (bright yellow color appears). The reaction was quenched by the addition of a minimal amount isopropanol (*i*PrOH) until the solution turned to a blue/green tint, warmed to room temperature, and the crude acid was extracted with EtOAc. The organics were dried over Na_2SO_4, filtered, and concentrated. To facilitate purification, the bis-acid was esterified to the diphenylmethyl ester. Specifically, the crude bis-acid was redissolved in EtOAc (10 mL) and to this solution was added diphenyldiazomethane (324 mg, 1.67 mmol, 4.0 eq). The dark red reaction mixture was stirred overnight at room temperature and subsequently quenched with dropwise addition

of AcOH until the reaction mixture turned clear. The crude mixture was concentrated under reduced pressure and the residue was purified by SiO_2 column chromatography (7:3 hexane:EtOAc) to afford 390 mg (90% - 2 steps) of pure diphenylmethyl ester **19Z**. $[\alpha]_D$ +8.60 (*c* 0.89, CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.35 (s, 18H), 2.70-2.76 (m, 4H), 4.77 (dd, *J* = 9.5, 5.6 Hz, 2H), 5.22 (d, *J* = 14.0 Hz), 5.29 (d, *J* = 14.0 Hz) 5.61 (app t, *J* = 4.4, 4.0 Hz, 2H), 6.89 (s, 2H), 7.23-7.36 (m, 22H), 7.44 (t, *J* = 7.2 Hz, 2H), 7.55 (d, *J* = 8.0 Hz, 2H), 7.97 (d, *J* = 7.6 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 26.80, 28.80, 62.63, 73.71, 77.98, 82.60, 124.46, 127.14, 127.34, 127.85, 127.92, 127.95, 128.09, 128.33, 128.42, 129.16, 132.39, 133.59, 139.60, 139.62, 146.88, 156.86, 168.85; HRMS (FAB, 3-NBA) *m/z* $[m+H]^+$ calcd. for $C_{58}H_{61}N_4O_{14}$, 1037.4105; found,1037.4178.



19E

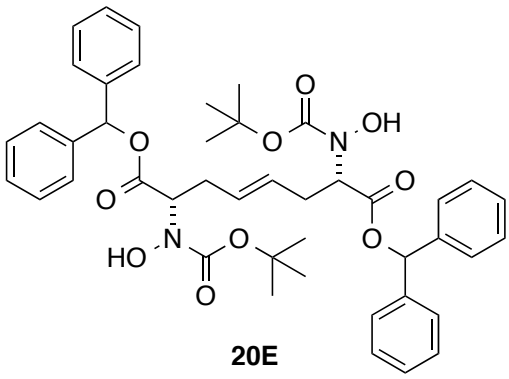
Compound **19E** was synthesized following a procedure identical to that described above for **19Z**. Specifically, **18E** (173 mg, 0.26 mmol, 1.0 eq) in acetone (10 mL) was subjected to Jones oxidation by slow addition of Jones reagent (8N) (0.8 mL, 6.5 mmol, 25 eq) and the mixture was stirred at 0 °C for 2 h. Upon conversion of the diol to the bis-acid, the reaction was quenched by the addition of *i*PrOH, the organics were further extracted with EtOAc, dried of over Na_2SO_4, and concentrated under reduced pressure. The crude bis-acid was subsequently treated with diphenydiazomethane (194 mg, 1.0 mmol, 4.0 eq) in EtOAc (10 mL) at room temperature overnight. Following treatment of the reaction mixture with AcOH, the crude mixture was once again concentrated and the residue was purified by SiO_2 column chromatography (7:3 hexane:EtOAc) to afford 234 mg (90% - 2 steps) of pure diphenylmethyl ester **19E**. $[\alpha]_D$ -7.98 (*c* 1.3, CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.35 (s, 18H), 2.60-2.66 (m, 2H), 2.75-2.79 (m, 2H), 4.73-4.76 (dd, *J* = 9.6, 5.4 Hz, 2H), 5.28 (dd, *J* = 18.0, 13.6 Hz, 4H), 5.63 (app t, *J* = 3.7 Hz, 2H), 6.91 (s, 2H), 7.21-7.37 (m, 22H), 7.50 (td, *J* = 7.6, 1.2 Hz, 2H), 7.59 (d, *J* = 7.3 Hz, 2H), 8.00 (dd, *J* = 8.2, 1.2 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 28.02, 31.74, 62.97, 73.68, 77.97, 82.57, 99.95, 124.48, 127.17, 127.36, 127.88, 127.94, 128.15, 128.35, 128.44, 129.05, 129.35, 132.36, 133.56, 139.64, 147.00, 156.95, 168.92; HRMS (FAB, 3-NBA) *m/z* $[m+Na]^+$ calcd. for $C_{58}H_{60}N_4O_{14}Na$, 1059.4004; found, 1059.3989.



20Z

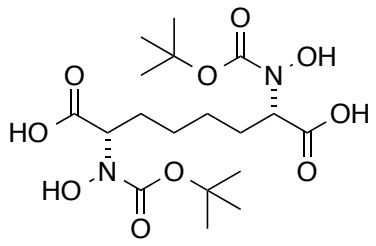
To a pyrex water jacketed flask, a solution of **19Z** (20 mg, 19 μmol, 1.0 eq) in degassed dioxane (10 mL) was stirred at room temperature. Nitrogen gas was bubbled into the reaction flask to facilitate stirring while a Rayonet photochemical reactor (equipped with 8 Southern New England Ultraviolet light bulbs, 300 nm) was employed for the

photodeprotection of the *o*-nitrobenzyl group. After 3 h at rt, TLC (40% EtOAc in hexanes) showed no starting material remaining. The solution was evaporated under reduced pressure and the crude residue was purified by SiO_2 column chromatography to afford 8.0 mg (55%) of pure compound **20Z** (R_f = 0.31 in 40% EtOAc in hexanes). *Note: compound 20Z was also accompanied by an unidentified byproduct (R_f = 0.57 in 40% EtOAc in hexanes).* $[\alpha]_D$ -10.5 (*c* 1.16, CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.39 (s, 18H), 2.74-2.82 (m, 4H), 4.84 (br s, 2H), 5.51-5.53 (app t, *J* = 4.7 Hz, 2H), 6.33 (br s, 2H), 6.89 (s, 2H), 7.27-7.36 (m, 20H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 26.65, 28.05, 61.75, 78.28, 82.43, 127.09, 127.10, 127.78, 128.04, 128.10, 128.53, 128.56, 139.45, 157.27, 170.00; HRMS (FAB, 3-NBA) *m/z* $[m+Na]^+$ calcd. for $C_{44}H_{50}N_2O_{10}Na$, 789.3363; found, 789.3389.



20E

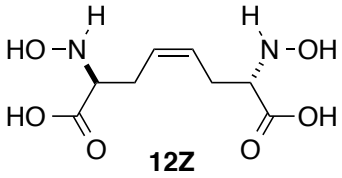
Compound **20E** was synthesized following a procedure identical to that described above for **20Z**. Briefly, a solution of **19E** (20 mg, 19 μmol, 1.0 eq) in degassed dioxane (10 mL) was stirred at room temperature to afford 7.3 mg (50%) of compound **20E**. *Note: compound 20E was also accompanied by an unidentified byproduct (R_f = 0.57 in 40% EtOAc in hexanes).* $[\alpha]_D$ -13.1 (*c* 1.07, CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.39 (s, 18H), 2.67-2.70 (app dd, *J* = 5.2, 4.9, 4H), 4.73-4.76 (m, 2H), 5.56-5.61 (app t, *J* = 3.8 Hz, 2H), 6.25 (s, 2H), 6.89 (s, 2H), 7.26-7.34 (m, 20H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 28.07, 31.79, 61.74, 78.17, 82.54, 127.07, 128.05, 128.10, 128.52, 128.55, 128.83, 139.49, 139.51, 157.29, 169.80; HRMS (FAB, 3-NBA) *m/z* $[m+Na]^+$ calcd. for $C_{44}H_{50}N_2O_{10}Na$, 789.3363; found, 789.3380.



20S

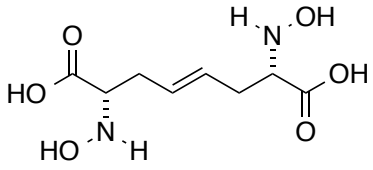
A solution of the E/Z diol **18** (1.91 g, 2.82 mmol, 1.0 eq) in acetone (28 mL, 100mM) was subjected to Jones oxidation protocol by the addition of Jones Reagent (8N) (8.80 mL, 70.5 mmol, 25 eq) and the reaction was stirred for 2 h at 0 °C. Upon conversion of the E/Z diol to the bis-acid, the reaction was quenched by the addition of *i*PrOH, the organics were further extracted with EtOAc, dried of over Na_2SO_4 and concentrated under reduced pressure to afford 1.98 g of the crude bis-acid in quantitative yield. The crude bis-acid was once again dissolved in EtOAc and subjected to hydrogenation (H_2, balloon pressure) over 20 mol% $Pd(OH)_2$/C for an extended period of time to afford 615 mg (50% - 2 steps) of the saturated N-Boc protected hydroxylamine **20S** as a sticky liquid. *Note: Careful TLC and ^{1}H NMR monitoring of the reaction showed that the reaction progressed in the following manner: (i) nitro group reduction, (ii) alkene C=C reduction, and (iii) o-aminobenzyl hydrogenolysis to N-Boc hydroxylamine **20S**.* $[\alpha]_D$ -18.2 (*c* 0.91, CH_2Cl_2); ^{1}H NMR (400 MHz, d_4-MeOH) δ 1.49 (s, 18H), 1.44-1.53 (m 4H), 1.92 (m, 4H), 4.50-4.54 (t, *J* =

7.6 Hz, 2H); ^{13}C NMR (100 MHz, d$_4$-MeOH) δ 27.42, 28.72, 29.30, 63.37, 82.72, 159.66, 175.11; HRMS (FAB, 3-NBA) *m/z* [m+H]$^+$ calcd. for $C_{18}H_{33}N_2O_{10}$, 437.2135; found, 437.2143.



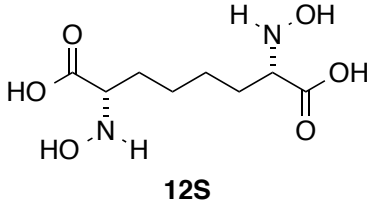
12Z

Starting from ester **20Z** (32 mg, 0.04 mmol, 1.0 eq) in CH_2Cl_2 (0.5 mL) was added anisole (80 μL, 2 mL/mmol) (cation scavenger) followed by the slow addition of TFA (0.4 mL, 10 mL/mmol) and the mixture was stirred at 0 °C until starting material was no longer detected by TLC (~ 1-2 h). The solvent and TFA were gently evaporated under reduced pressure and the resulting oily residue was dissolved in H_2O (0.5 mL) and the organic byproducts were pipetted away with CH_2Cl_2 (3 x 0.5 mL) followed by Et_2O (3 x 0.5 mL) and the aqueous layer was concentrated under reduced pressure to afford 18 mg (97%) of the bis-TFA salt **12Z** as a sticky liquid. $[\alpha]_D$ +4.73 (*c* 0.50, D_2O); ^{1}H NMR (500 MHz, D_2O) δ 2.74-2.84 (m, 4H), 4.07 (t, *J* = 5.5 Hz, 2H), 5.63 (app t, *J* = 5, 4.5 Hz, 2H); ^{13}C NMR (100 MHz, D_2O) *δ* 24.62, 63.74, 126.69, 171.36; HRMS (FAB, Gly) *m/z* [m+H]$^+$ calcd. for $C_8H_{15}N_2O_6$, 235.0930; found, 235.0936.



12E

Starting from ester **20E** (53 mg, 0.07 mmol, 1.0 eq) in CH_2Cl_2 (0.5 mL) was added anisole (114 μL, 2 mL/mmol) (cation scavenger) followed by the slow addition of trifluoroacetic acid (TFA) (0.7 mL, 10 mL/mmol) and the mixture was stirred at 0 °C until starting material was no longer detected by TLC (~ 1-2 h). The solvent and TFA were gently evaporated under reduced pressure and the resulting oily residue was dissolved in H_2O (0.5 mL) and the organic byproducts were pipetted away with CH_2Cl_2 (3 x 0.5 mL) followed by Et_2O (3 x 0.5 mL), and the aqueous layer was concentrated under reduced pressure to afford 27 mg (85%) as the bis-TFA salt **12E** as a sticky liquid. $[\alpha]_D$ +8.34 (*c* 0.86, D_2O); ^{1}H NMR (400 MHz, D_2O) δ 2.71 (app t, *J* = 5.0, 4.5 Hz, 4H), 4.10 (t, *J* = 6.0 Hz, 2H), 5.66 (t, *J* = 3.5 Hz, 2H); ^{13}C NMR (100 MHz, D_2O) δ 29.69, 63.80, 128.12, 170.97; HRMS (FAB, Gly) *m/z* [m+H]$^+$ calcd. for $C_8H_{15}N_2O_6$, 235.0930; found, 235.0937.



12S

Starting from bis-acid **20S** (50 mg, 0.11 mmol, 1.0 eq) in CH_2Cl_2 (0.5 mL) was added anisole (220 μL, 2 mL/mmol) (cation scavenger) followed by the slow addition of trifluoroacetic acid (TFA) (1.1 mL, 10 mL/mmol) and the mixture was stirred at 0 °C until starting material was no longer detected by TLC (~ 1-2 h). The solvent and TFA were gently evaporated under reduced pressure and the resulting oily residue was dissolved in H_2O (0.5 mL) and the organic byproducts were pipetted away with CH_2Cl_2 (3 x 0.5 mL) followed by Et_2O (3 x 0.5 mL), and the aqueous layer was concentrated under reduced pressure to afford 43 mg (85%) of the bis-TFA salt **12S** as a sticky liquid. $[\alpha]_D$ +21.70 (*c* 1.08, D_2O); ^{1}H NMR (500 MHz, D_2O) δ 1.21-1.41 (m, 4H), 1.83-1.87

(m, 4H), 3.96 (t, J = 6.2Hz, 2H); ^{13}C NMR (100 MHz, D_2O) δ 23.94, 26.07, 64.08, 171.33; HRMS (FAB, Gly) *m/z* $[m+H]^+$ calcd. for $C_8H_{17}N_2O_6$, 237.1087; found, 237.1082.

III. Analytical Methods Investigating the Stereocontrol and E/Z Geometrical Preference of the Two Key Synthetic Transformations in the Design of CBS Inhibitor Library.

Figure S4: ^{19}F NMR of Mosher Esters Derived from Mitsunobu Product **7**.

(This trend was consistent with the Mitsunobu products from the other series.)

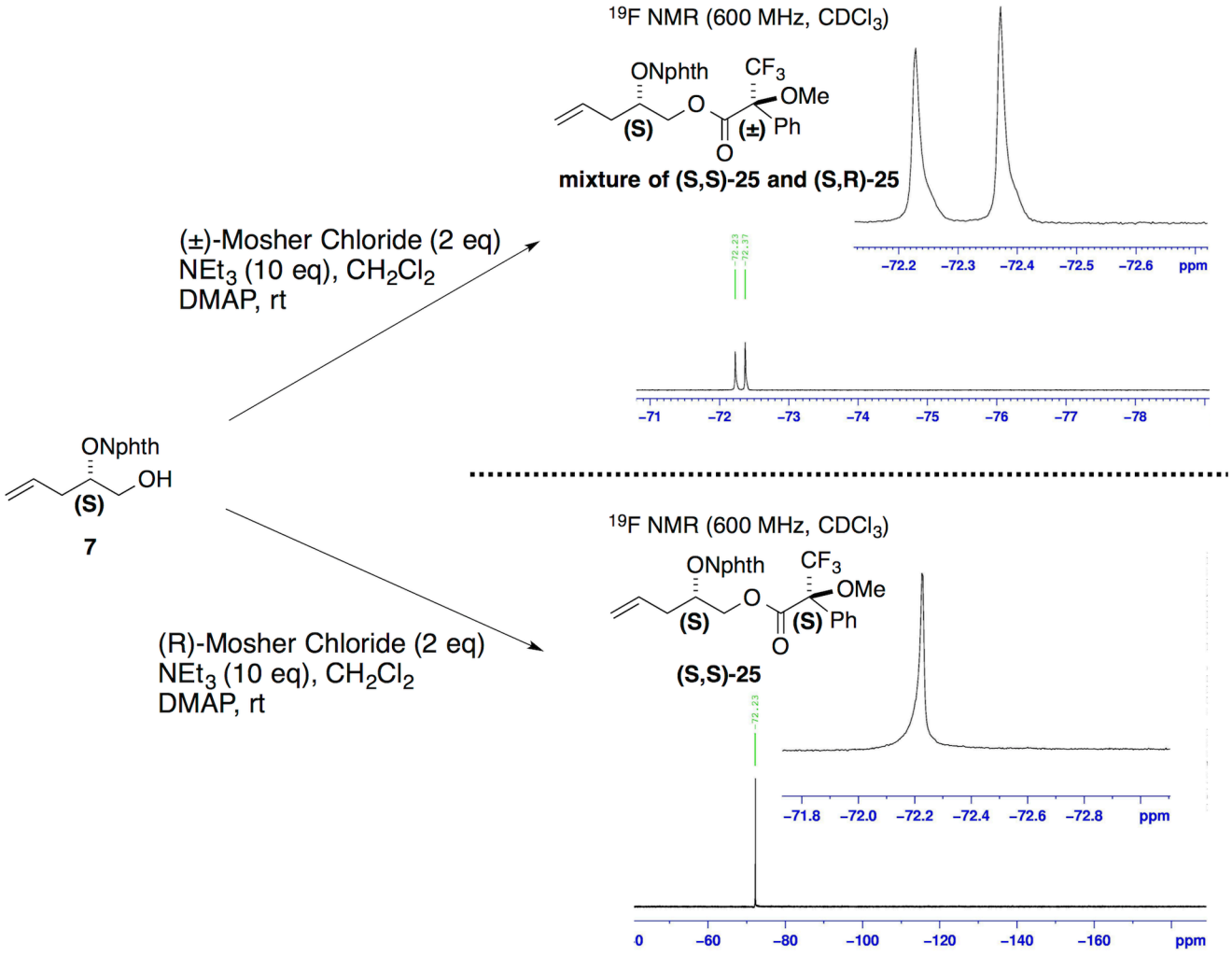


Figure S5: Use of Chiral HPLC to Confirm the Stereospecificity (Inversion) of the Mitsunobu Reaction.

(Data shown for the para-bromobenzoyl ester derived from Mitsunobu product **7**. This trend was consistent with the Mitsunobu products from the other series.)

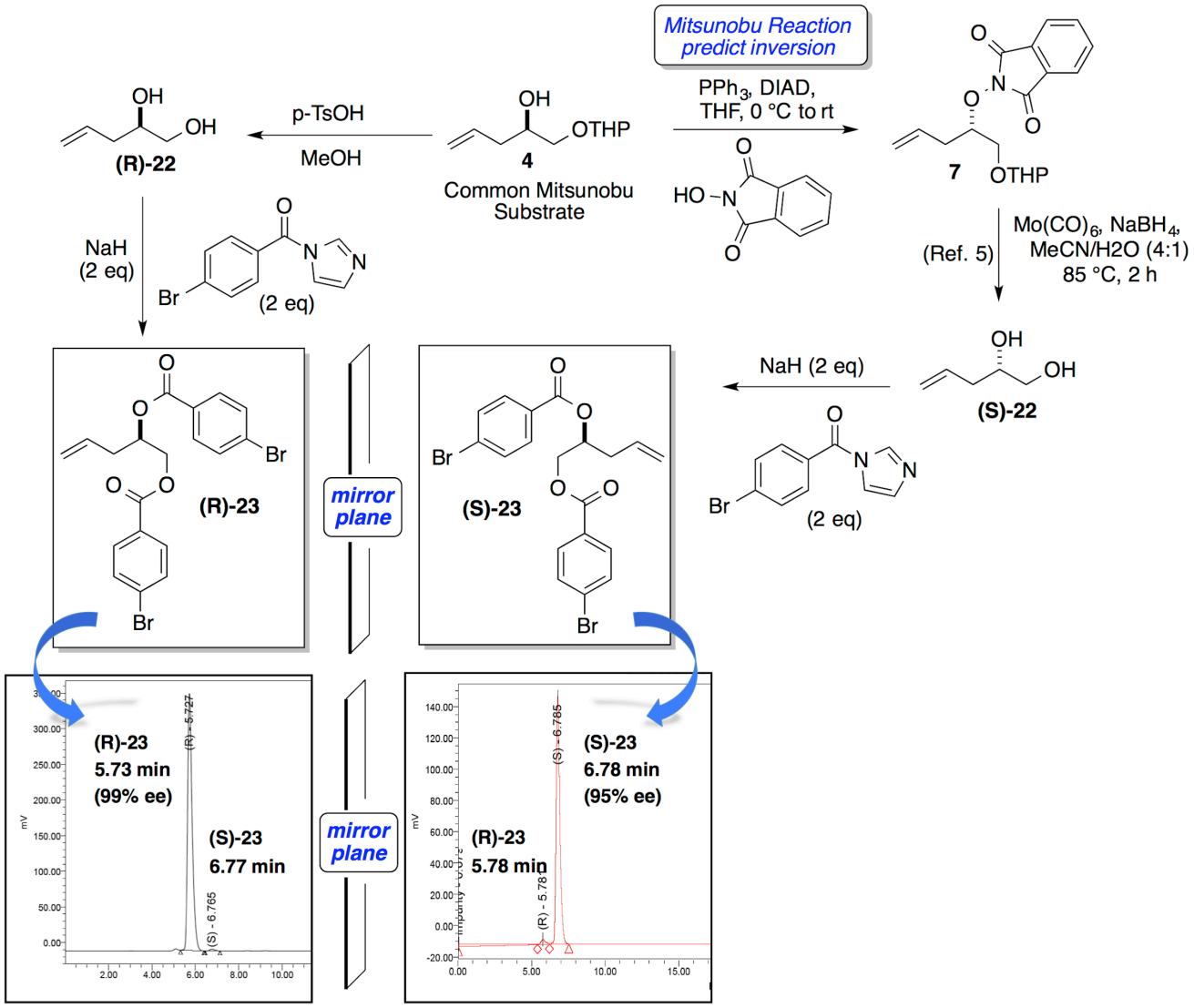


Figure S6: Unambiguous Assignment of Olefin Geometry via ^{13}C NMR. [5]

Unambiguous Assignment of Olefin Geometry following Grubbs Cross Metathesis. The chemical shift of the allylic carbon is upfield relative to the t-Butyl methyl carbons in the Z and downfield of this peak for the E. This trend was consistent for all other series.

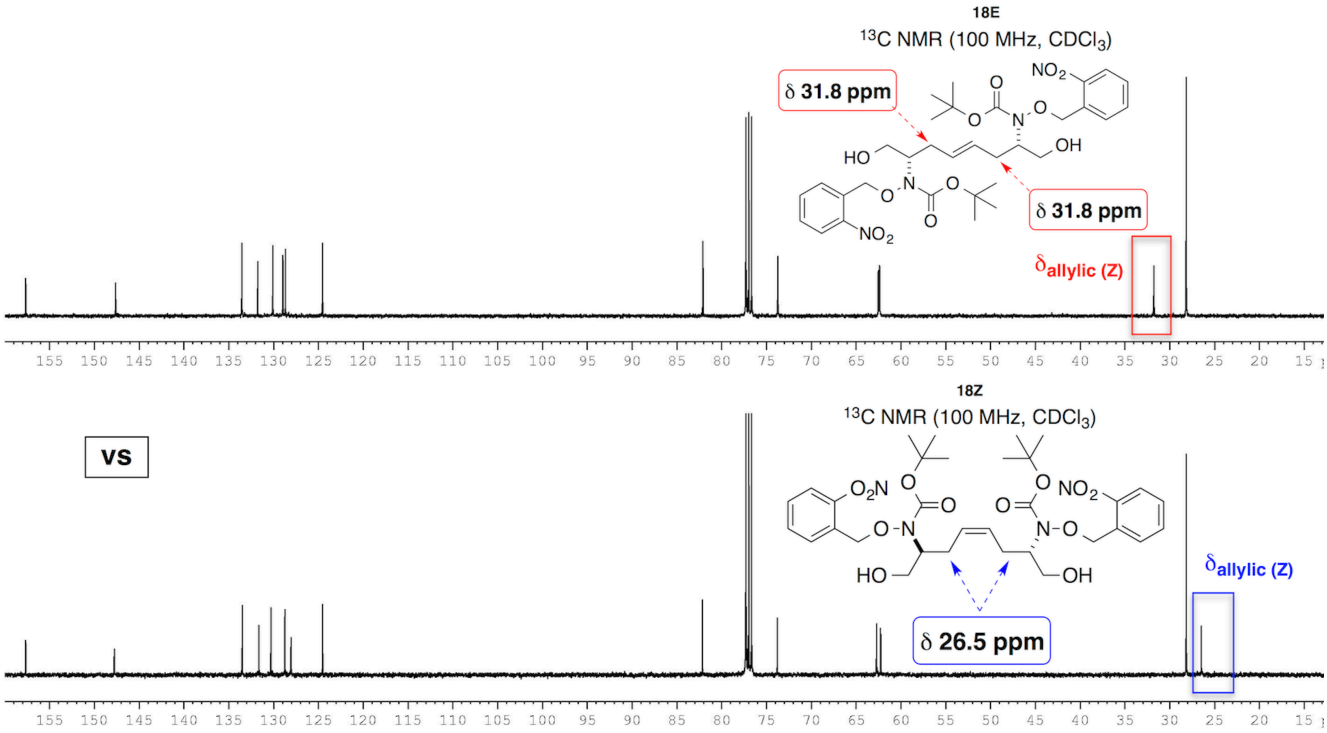
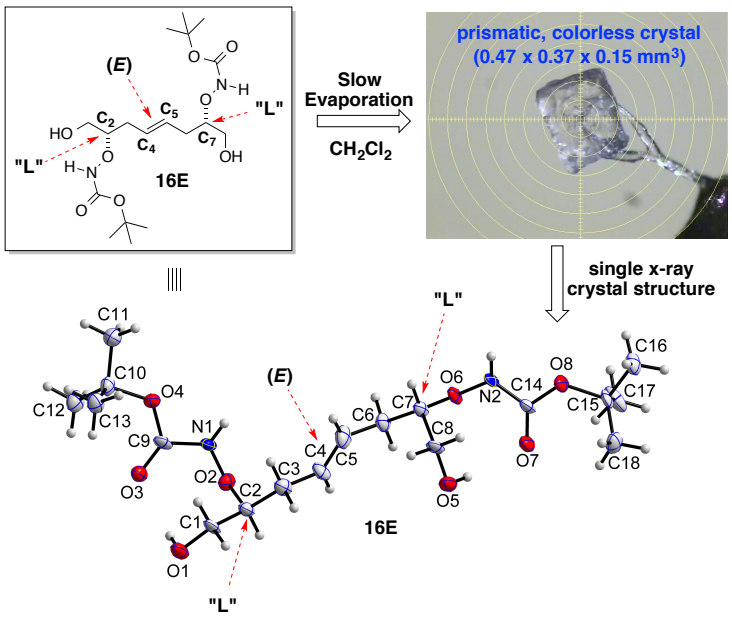


Figure S7: ORTEP Representation of the X-ray Crystallographic Structure of **16E**



IV. Determination of Purity for Lead CBSI 6S by HPLC

Figure S8: UV/Vis Spectrum of CBSI **6S**

UV/Vis spectrum was taken using Spectromax M5 UV/Vis Spectrometer using 30 mM **6S** in 1 mL D_2O, spectrum was compared to blank of D_2O to determine window for HLPC analysis. A wavelength of 230 nm was chosen for visualization of **6S** during HPLC analysis.

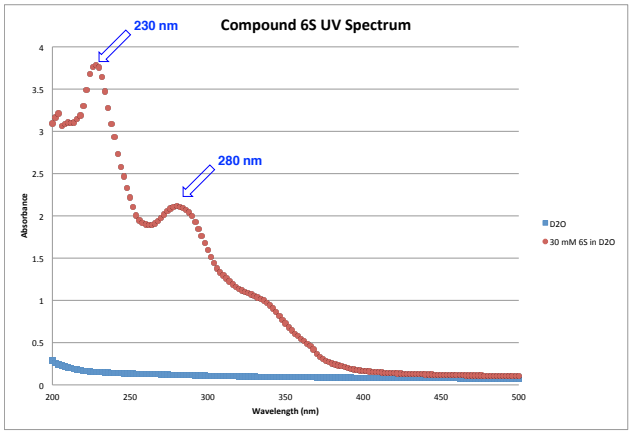
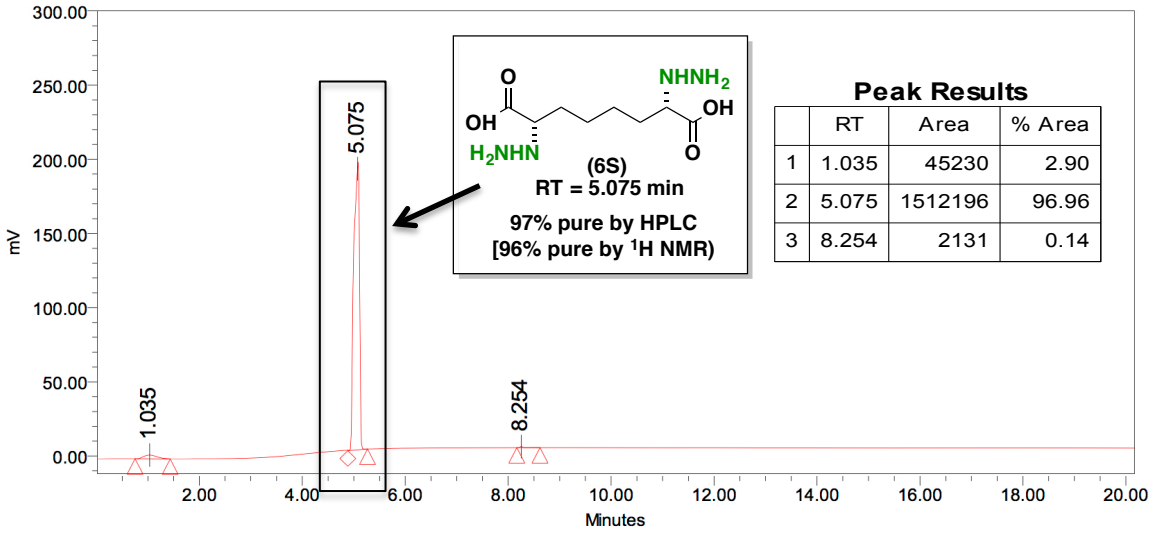


Figure S9: HPLC Trace of CBSI **6S**

HLPC was performed on a Waters 600 Multisolvent Delivery System using the Waters 486 Tunable Absorbance Detector. A 10 µL aliquot of CBSI **6S** (30 mM) was injected into an Ace-121-1546 Reverse Phase C_{18} column (150 X 4.2 mm) at a flow rate of 2 mL/min, eluting with 30:70 Acetonitrile:H_2O (0.1% TFA). CBSI **6S** eluted at a retention time of ~ 5.1 min and the identity of the compound responsible for this major peak was confirmed by ^{1}H NMR to be the CBSI **6S** (97% purity). The purity determined by HPLC agreed with the purity of **6S** estimated by ^{1}H NMR prior to injection (~ 96% purity).

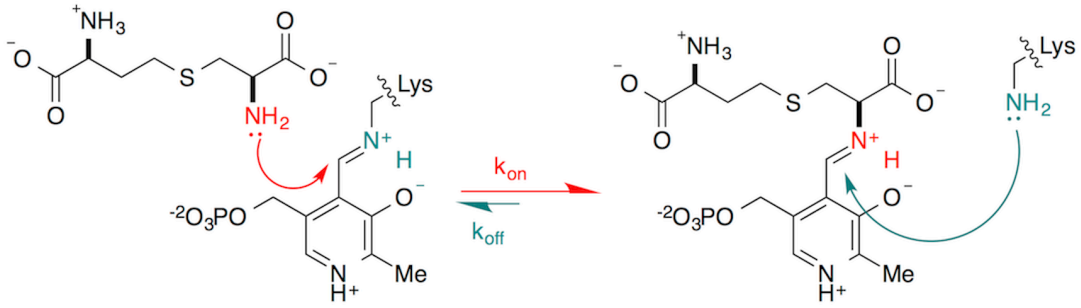


	RT	Area	% Area
1	1.035	45230	2.90
2	5.075	1512196	96.96
3	8.254	2131	0.14

V. Examination of PLP-Imine vs. PLP-Hydrazone Interchange

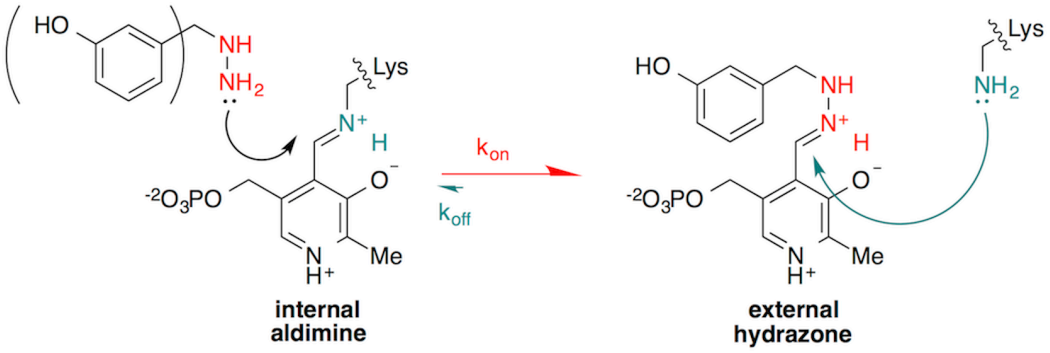
Figure S10: Comparison of Rates for Imine-Imine Interchange vs. Imine-Hydrazone Interchange in Representitive PLP-dependent Enzymes.

Data based on numbers from work from the Banerjee (cysthationine β-synthase)[6], Salvo (serine hydroxymethyltransferase)[7], Kagamiyama (aspartate aminotransferase)[8] and Silverman (GABA-transaminase)[9] labs respectively.



	k_{on}	k_{off}	$t_{(1/2)off}$	$k_{off}/k_{on} = K_d^{eff}$
L-Cystathionine (Cystathionine β-synthase)	$1.5x10^3\ M^{-1}s^{-1}$	$2.3\ s^{-1}$	0.30 s	$1.5x10^{-3}$ M
L-Serine (Cystathionine β-synthase)	$1.4x10^4\ M^{-1}s^{-1}$	$2.1x10^1\ s^{-1}$	0.033 s	$1.5x10^{-3}$ M
L-Serine (Serine Hydroxymethyltransferase Mutant)	$2.9x10^4\ M^{-1}s^{-1}$	$3.9x10^1\ s^{-1}$	0.018 s	$6.2x10^{-4}$ M
L-Glycine (Serine Hydroxymethyltransferase Mutant)	$5.0x10^3\ M^{-1}s^{-1}$	$5.1\ s^{-1}$	0.14 s	$1.0x10^{-3}$ M
vs				
L-Aspartate (Aspartate Aminotransferase)	$6.0x10^2\ M^{-1}s^{-1}$	$0.8\ s^{-1}$	0.87 s	$1.4x10^{-3}$ M

Imine-Hydrazone Interchange Rates for PLP-dependent enzymes



	k_{on}	k_{off}	$t_{(1/2)off}$	$k_{off}/k_{on} = K_d^{eff}$
3-HO(C_6H_4)CH_2-$NHNH_2$	$3.3x10^2\ M^{-1}s^{-1}$	$8.3x10^{-5}\ s^{-1}$	$8.3x10^3$ s = 2.3 h	$2.5x10^{-7}$ M
Me-$NHNH_2$	$3.5x10^1\ M^{-1}s^{-1}$	$7.6x10^{-5}\ s^{-1}$	$9.1x10^3$ s = 2.5 h	$2.2x10^{-6}$ M

VI. Enzyme Cloning, Purification, Characterization, Evaluation

A. Cloning into *E. coli*

The following sequence for the truncated human cystathioinine β-synthase (CBSΔ143) was purchased through genscript in a pGEX-4T1 vector at the BamH1 and Xho1 cut sites. BL21(DE3) Competent Cells were incubated on ice with the pGEX-4T1-CBSΔ143 vector for 30 min in a microcentifuge tube. Subsequently, the tube was transferred to a 45 °C water bath for 45 sec then placed in ice for 2 min. Following the heat shock, cells were transferred to a round bottom culture tube containing 1 mL of LB broth and incubated at 37 °C for 1 h at 250 rpm. Finally, 100 μL of the sample was plated on an ampicillin agar plate and incubated for 12 hours.

GGATCCGGCATTCCGAGCGAAACGCCGCAAGCAGAAGTTGGTCCGACGAGCGCCTGTGGT
GTGTCCCTGGGCATTCCGAGCGAAACGCCGCAGGCCGAAGTTGGTCCGACCGGTTGCCCG
CACCGTAGTGGTCCGCATTCCGCAAAAGGCTCACTGGAAAAAGGTTCGCCGGAAGATAAA
GAAGCCAAAGAACCGCTGTGGATTCGTCCGGACGCACCGAGCCGCTGTACCTGGCAGCTG
GGTCGTCCGGCAAGTGAATCCCCGCATCACCATACGGCCCCGGCAAAATCTCCGAAAATT
CTGCCGGATATCCTGAAGAAAATTGGCGACACCCCGATGGTCCGTATCAACAAAATCGGC
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TCGGTGAAAGATCGTATCAGCCTGCGCATGATTGAAGATGCGGAACGCGACGGCACCCTG
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ATTATCGGTGTTGATCCGGAAGGCAGTATTCTGGCGGAACCGGAAGAACTGAACCAGAC
CGAACAAACCACGTATGAAGTGGAAGGCATCGGTTACGATTTTATTCCGACCGTTCTGGA
TCGCACGGTGGTTGACAAATGGTTCAAATCCAATGACGAAGAAGCTTTTACCTTCGCGCG
TATGCTGATCGCTCAAGAAGGTCTGCTGTGCGGCGGTAGCGCAGGCTCTACGGTCGCTGT
GGCGGTTAAAGCTGCGCAGGAACTGCAAGAAGGCCAGCGTTGTGTCGTGATTCTGCCGGA
TTCCGTGCGCAACTACATGACCAAATTTCTGTCAGACCGTTGGATGCTGCAGAAAGGCTC
CTGAAAGAAGAAGACCTGACCGAAAAGAAACCGTGGTGGTGGCATCTGCGTTGACTCGA
G

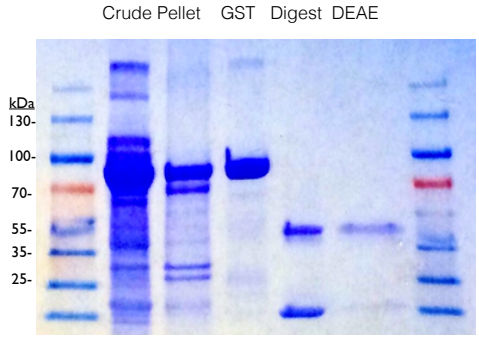
B. Purification and Characterization of CBSΔ143[10,11]

BL21(DE3) Competent Cells containing the pGEX-4T1-CBSΔ143 vector were grown in LB broth to an absobance of 0.8 OD_{600nm} at 37 °C and 250 rpm. IPTG was added at 0.2 mM at temperature was reduced to 25 °C and 300 rpm for 12 hours. Cells were pelleted at 5,000 rpm and frozen.

After thawing, cells were sonicated in 50 mM Tris buffer pH 7.5 containing 10 μM PLP and 0.2 mM PMSF for a 45 sec pulse 5 times. Following sonication, the resulting solution

was centrifuged at 10,000 rpm for 15 minutes. The soluble fraction was poured through a GST column and washed with 2 column volumes of Tris buffer. Column was eluted with 10 mM Glutathione. Purified enzyme was digested with thrombin for 12 h at room temperature. Digested enzyme was poured through a DEAE cellulose column and eluted with a NaCl gradient from 50-300 mM. Activity was calculated from each step via Lead(II)acetate assay and protein concentration was determined by a modified Lowry assay.

Figure S11: CBS Purification Gel and Activity Profile



Step	Volume (mL)	Total Units (U)	Total Protein (mg)	Specific Activity (U/mg)	Purification factor
Crude	20	65.1	9450	0.0069	1
GST-Agarose	10	52.4	10.5	4.9	713.3
DEAE Sepharose	10	44.5	5.8	7.7	1065

C. Evaluation of CBS inhibitor Candidate Fitness

(i) Cystathionine Lyase–Synthase Activity Assay for CBS

The modified cystathionine lyase assay originally developed by S. Aitken and J. Kirsch[12] for yeast cystathionine β-synthase was employed for initial screening of the inhibitor library on the efficacy of cystathionine lyase activity of hCBS. Specifically, the assay mixture consisted of L,L-cystathionine (100 μL of 0.9 mM stock solution), 1 mM DTNB, various concentrations of inhibitors, 50 mM pH 8.0 Tris buffer at 37 ^{o}C. The assay was initiated by the addition of 7.4 μg of hCBS dimer and activity was monitored spectroscopically by the formation of 3-nitro-5-thiol-benzoic acid at 412 nm with a Schimadzu 2401 UV/PC machine equipped with 16 micro well cuvettes. For the 30 min preincubation study: Inhibitors and hCBS (7.4μg) were preincubated in 50mM pH 8.0 Tris buffer at 37^{o}C for 30 min and reaction was initiated by addition of 1mM DTNB and 0.9 mM L,L-cystathionine. For each set of inhibition studies, a background control reaction was performed in the absence of hCBS and the rate (usually within 5 mAbs/min compared to the reaction rate of 120 mAbs/min when there is no inhibitor) was subtracted from the reaction rate.

(ii) Radioactive Assay

Control Assay (no inhibitor):

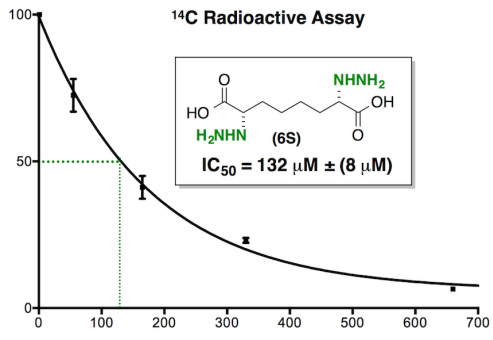
A modified radioactivity based assay originally developed by Mudd et al.[13] was employed to measure ^{14}C-labeled L,L-cystathionine produced by CBS. The reaction mixture, containing 5 mM (D,L)-homocysteine, 3 mM ^{14}C-1 labeled L-serine (specific activity typically 20,000 cpm/μmol), 0.5U CBS, and 5 μM PLP in 100 mM Tris-HCl buffer pH 8.0 with a total volume of 200 μL was incubated at 37 °C for 5 min. The reaction was then terminated by addition of 0.3 mL 10% trichloroacetic acid. The reaction mixture (300 μL) was loaded onto an Econo-column packed with Dowex 50Wx8 cationic exchange resin (2 g dry resin per column), and the column was washed with 20 mL dd water. Then, unreacted L-serine was eluted with 0.6 M HCl (6 x 4 mL). After washing again with 20 mL water, the product was eluted with 3 M NH_4OH (4 x 4 mL). Each basic fraction was diluted with scintillation cocktail (Ecolite, 15 mL) and counted (Packard Model 1900 CA Tricarb scintillation counter), after standing overnight. The control reaction, lacking CBS, was run with every set of assays.

Radioactive Inhibition Assay:

Various concentrations of inhibitors were incubated with hCBS for 15 min in 100 mM Tris (pH 8.0) followed by the addition of 3mM L-serine and 5 mM L-homocysteine to initiate the enzymatic reaction. The reaction was ran for 30 min at 37 °C then quenched by the addition of 10% trichloroacetic acid. 300 μL of reaction sample was loaded onto a Dowex 50Wx8 cation exchange column and the resin was washed with 20 mL of H_2O. The column was then washed with 0.6 M HCL (6 x 4 mL) followed by by 20 mL H_2O. The residual radioactive cystathionine was washed with 3M NH_4OH and the eluent collected into a scintillation vial containing 0.25 mL conc. HCL. The elution with NH_4OH was repeated twice, this time collecting the elution solvent in scintillation vials containing 0.5 mL of conc. HCl. To each vial, 15 mL of scintillation fluid was added and the radioactive counts were measured using a scintillation counter.

Figure S12: IC_{50} Data for Initial Screening of CBS Inhibitor Library.

XY Group	Compound	IC_{50} (μM) Continuous Assay	^{14}C Radioactive Assay	H_2S Signaling Assay
—$NHNH_2$	6S	140 ± (10)	132 ± (8)	122 ± (2)
	6E	200 ± (5)	—	—
	6Z	200 ± (3)	—	—
—ONH_2	9S	520 ± (11)	530 ± (9)	—
	9E	1100 ± (12)	—	—
	9Z	1200 ± (22)	—	—
—NHOH	12S	—	1500 ± (25)	—
	12E	—	9950 ± (42)	—
	12Z	—	1700 ± (28)	—



Dialysis Experiments with CBS Inhibitor 6S:

Dialysis experiments were administered to test the functional irreversibility of the leading CBS inhibitor candidate **6S** where the % remaining activity of the inhibited enzyme (versus control containing no inhibitor) was measured before and after exhaustive dialysis. Furthermore, to examine whether addition of exogenous PLP improved the restoration of enzymatic activity following dialysis, both the control and inhibition experiments were conducted in parallel using two different buffer systems. *Specifically,* ***Buffer A*** *contained 50 mM Tris (pH 8.0) and no exogenous PLP.* ***Buffer B*** *contained 50 mM Tris (pH 8.0) and 50 mM PLP.* All dialysis experiments were ran in D-Tube™ Dialyzers (EMD Millipore) and all experiments were performed in triplicate.

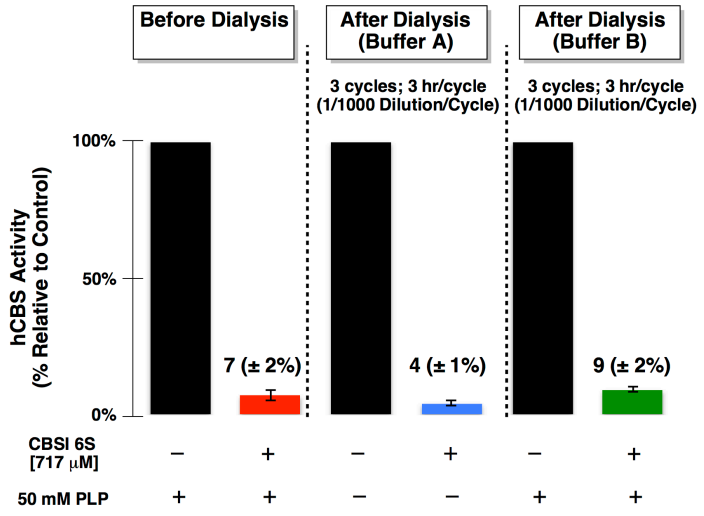
Activity Assay (Prior to Dialysis):

Just prior to dialysis and using a fixed concentration of inhibitor **6S** (717 μM), the ^{14}C radioactive assay previously described herein was carried out to measure the relative % of CBS activity remaining following inhibition (versus control containing no inhibitor). Both the control and inhibition assays prior to dialysis were conducted in 50 mM Tris (pH 8.0) buffer containing 50 μM PLP.

Dialysis and Follow-up Activity Assays:

To an Eppendorf containing a buffered solution (**Buffer A** or **Buffer B**; pH 8.0) of CBS inhibitor **6S** (717 μM) was added hCBSΔC143 and preincubation commenced for 15 min at 37 °C. In parallel, a control experiment (lacking inhibitor **6S**) was performed for both buffer systems, respectively. The mixture was then transferred to a dialysis tube and buffer was added to bring the total volume to 200 μL. Each tube (with or without inhibitor) was placed in a floating rack suspended in **Buffer A** or **Buffer B**, respectively. The buffer was gently stirred for 3 hr per cycle before replacing with fresh buffer (1/1000 dilution per cycle). After completion of three cycles the mixture was centrifuged (10,000 x g for 15 min at 4 °C) and re-suspended to a total volume of 184 μL. The enzymatic reaction was initiated by addition of 3mM of ^{14}C-labeled L-serine and 5 mM L-homocysteine (200 μL total reaction volume) and further incubated for an additional 5 min. Upon completion, the mixture was quenched, passed thru a Dowex cation exchange column, and radioactive counts measured to quantify CBS Activity.

Figure S13: Examination Functional Irreversibility for CBSI **6S**



(iii) Lead(II) Acetate Assay for H_2S Generation

All assays were conducted in 10 mM Phosphate buffer pH 8.0 and 0.4 mM Lead(II) acetate and were spectroscopically visualized at 300 nm. A standard curve for the generation of Pb(II)S was generated using NaSH at concentrations ranging from 10-100 μM. A standard curve for the activity of hCBSΔ143 was generated using constant enzyme and homocysteine (5 mM) while varying the cysteine concentration from 10-100 mM. The results from the study displayed Michaelis-Menten type kinetics. Using the Lead(II) assay established above, various concentrations (100, 125, 150, 175, 200 μM) of the lead inhibitor candidate **6S** were titrated into the assay mixture and cysteine concentration was again varied from 10-100 mM in 5 mM homocysteine. The secondary plot was generated from the slopes of the lineweaver-Burk. Using the y-intercept and the slope, K_i was determined to be 47.9 μM.

Figure S14: H_2S Sensing Assay and CBSI **6S** Inhibition Raw Data

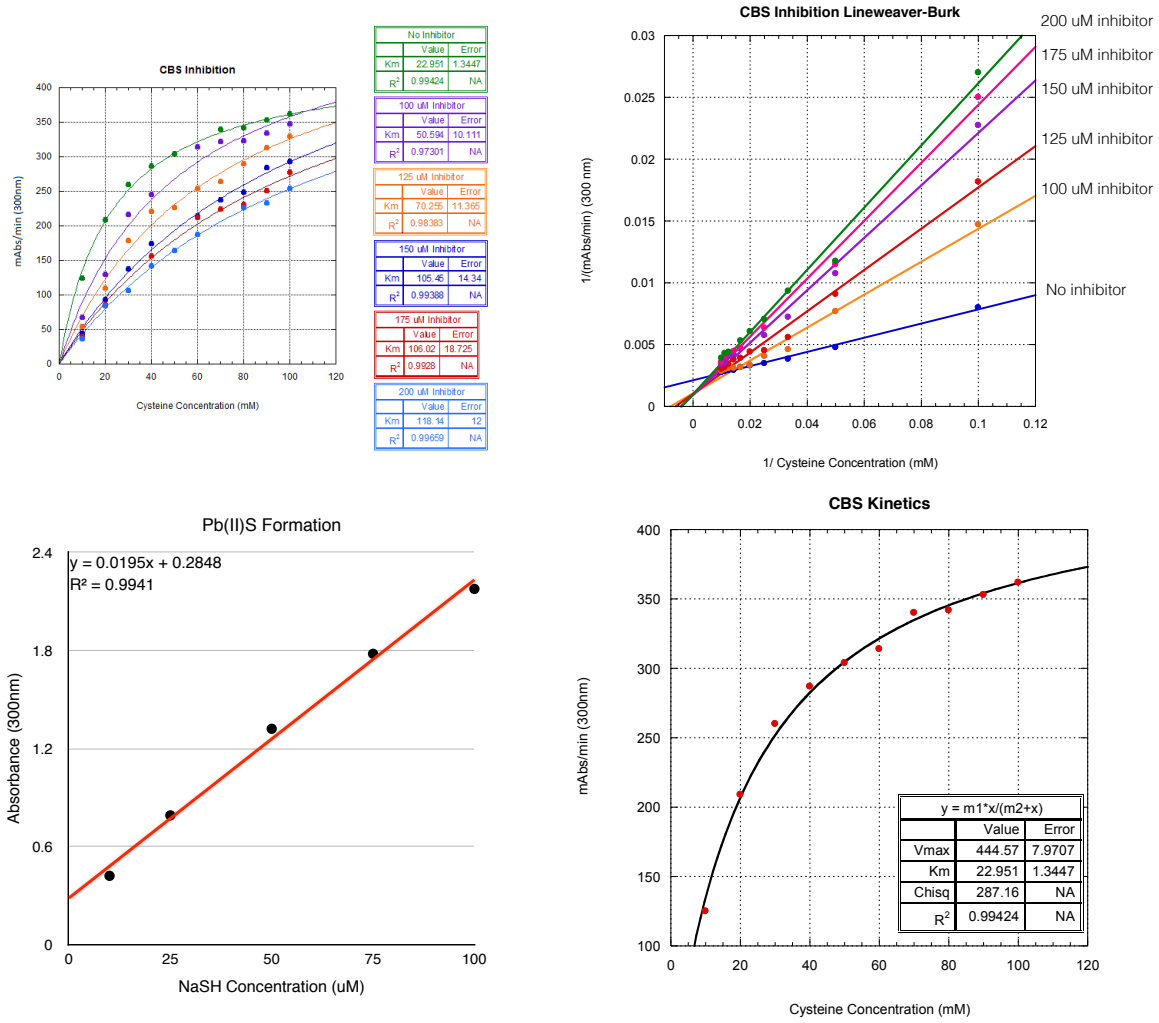
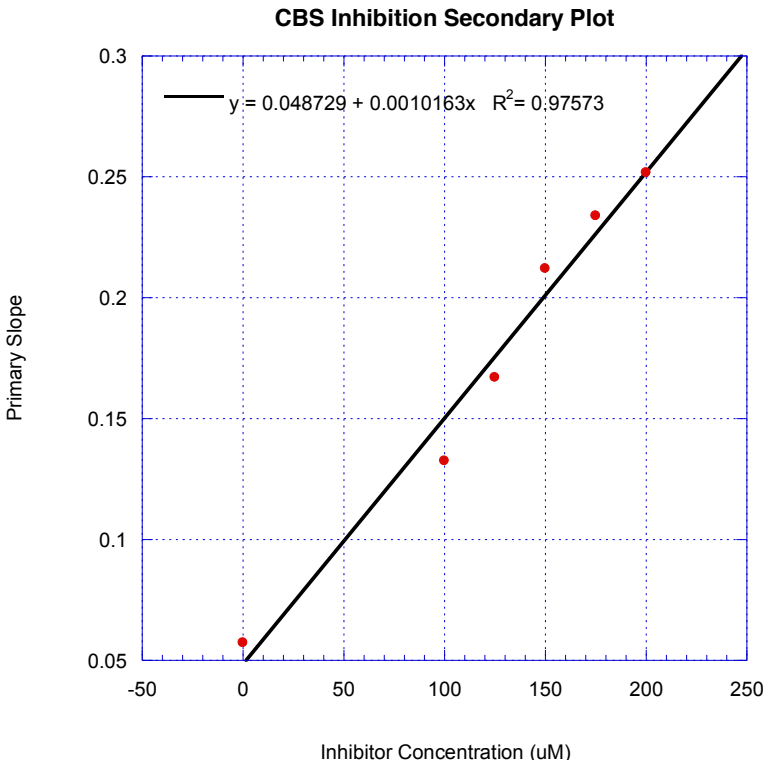


Figure S15: CBSI **6S** Secondary Plot Generated From H_2S Sensing Assay



VII. CBS Cell Expression and Evaluation

A. Construction of Rat CBS Lentiviral Expression Vectors and Cell Transduction

Total RNA was isolated from rat PC12 cells using RNeasy Mini Kit (Qiagen). First strand cDNA was synthesized from total RNA using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas/Thermo Scientific). CBS coding region was isolated by PCR from the cDNA using PrimeStar GXL DNA Polymerase (Clontech) with a SalI site-containing forward (5' CGCGTCGACCATG CCTTCAGGGACATCC 3') and BamHI site-containing reverse (GCGGATCCTA TTTCCGGGTCTGCTCAC) primers. Amplified product was digested with SalI and BamHI and inserted in-frame 3' to a myc-tag sequence in pL6mCWmycIE lentiviral vector to give pL6mCWmycCBSIE. pLenti6mCWmycIE was modified from pLenti6/V5-D-TOPO (Invitrogen) by reengineering the multiple cloning site, insertion of the cPPT and WPRE elements, and insertion of the N-terminal myc tag coding sequence and IRES-EGFP reporter cassette. Lentivirus packaging was performed in 293FT cells according to the protocol provided with the ViraPower™ Lentiviral Directional TOPO® Expression Kit (Invitrogen). Lentivirus particles were harvested from cell culture supernatant according to the protocol of Deiseroth Lab (http://www.stanford.edu/group/dlab/resources/lvprotocol.pdf). Lentivirus carrying the CBS expression constructs was used to transduce undifferentiated SH-SY5Y cells. Prior to transduction, cells were cultured to 90% confluence.

Concentrated virus particle was added to cell culture medium containing 8 μg/ml of Polybrene (Sigma-Aldrich). Where long-term expression of transgene was needed, antibiotic selection was applied by adding Blasticidin S (Invitrogen) at a final concentration of 10 μg/mL to the medium. Expression of transgene was visualized by EGFP fluorescence.

B. Culturing Human Neuroblastoma Cells (SH-SY5Y) and CBS Overexpressed SH-SY5Y Cell Line

Neuroblastoma SH-SY5Y (obtained from American Type Culture Collection, ATCC) with and without CBS overexpression cell lines were maintained in DMEM/F12 (Gibco, Invitrogen, USA) containing 10% fetal bovine serum (FBS, Gibco, Invitrogen, USA) and 1% penicillin–streptomycin (Gibco, Life technologies, USA) at 37°C and incubated in a humidified atmosphere with 95% air/ 5% CO_2.

C. H_2S-Synthesizing Activity Assay in Cells

H_2S-synthesizing activity in cells was measured according to Qu et al.[14] Briefly, cells were homogenized in 0.1 M potassium phosphate buffer, pH 7.4 (0.45 mL) under varying concentrations of L-cysteine and (D,L)-homocysteine (total volume 0.5 mL). The blank was prepared by omitting the substrates. After incubation for 90 min at 37 °C, zinc acetate (1%, 0.25 mL) was added into the reaction mixture and followed by the addition of tricholoracetic acid (10%, 0.25 mL). After centrifugation (13,000 rpm, 10 min) at 4°C, N-dimethyl-p-phenylenediaminesulfate (20 mmol/L prepared in 7.2 mol/L HCl, 0.133 mL) and $FeCl_3$ (30 mM prepared in 1.2 M HCl, 0.133 mL) were added to the supernatant. Absorbance at 670 nm was measured by a microplate reader (Sunrise; TECAN) 20 min later. Absorbance was converted to H_2S concentration through a standard curve obtained by using NaSH as standard.

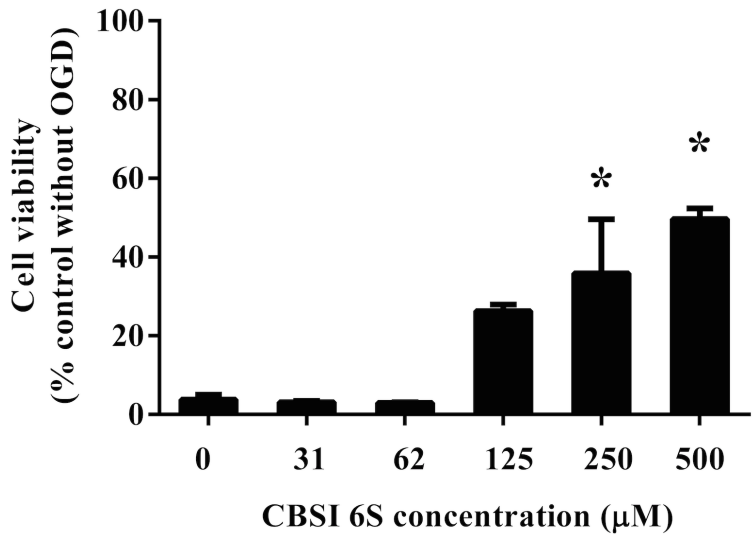
D. Oxygen Glucose Deprivation (OGD)

OGD was achieved by incubating the SH-SY5Y and/or SH-SY5Y CBSOE cells in glucose free DMEM (Gibco, Life technologies) with 1% streptomycin/penicillin in a hypoxia chamber (1% O_2/ 5% CO_2/ 94% N_2) for 24 h at 37 °C in a humidified incubator.

E. Cell Viability Assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) assay was employed to examine the cell viability. Briefly, the cell culture medium was replaced with medium containing MTT at a final concentration of 0.5 mg/mL. After incubation at 37 °C for 2.5 h, the medium was replaced with DMSO. Finally, the absorbance at 570 nm was measured with a reference wavelength at 630 nm by a microplate reader (Sunrise; TECAN) 20 min later.

Figure S16: Dose Dependent Rescue of Cell Viability with CBSI **6S** Under OGD



VIII. *In Vivo* Studies

Ethics Statement

All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the National University of Singapore and all efforts were made to minimize the suffering and number of rats in all experiments. Animals were housed under diurnal lighting conditions and fed standard rat chow and water ad libitum.

A. Transient Middle Cerebral Artery Occlusion (tMCAO) Rat Model Studies

Male Sprague Dawley (SD) rats 270–330g were anesthetized with isoflurane (1.5 unit) with 30% oxygen and 70% nitrous oxide mixture. Body temperature was maintained at 37°C until recovery from anaesthesia. A laser doppler sonography probe was used to monitor and confirm occlusion of the MCA using the intraluminal suture techniques. A small temporal incision was made on the right scalp and the masseter muscle was dissociated from the edge of the skull. Next, the skull was thinned with a dental drill and a laser-doppler sonography probe was mounted on the right parietal bone. The real time relative cerebral blood flow was then monitored throughout the whole operation. To induce cerebral ischemia, animal was placed in a supine position. Briefly, 2cm midline neck incision was made to expose the common carotid artery (CCA). The tissue and nerves surrounding the CCA was carefully dissected. The occipital artery was cauterized and tissues surrounding internal carotid artery (ICA) was carefully dissected to expose pterygopalatine artery. Pterygopalatine artery was permanently ligated with 7-0 silk suture. Temporary ligation was placed in the bifurcation of CCA and external carotid artery (ECA). An incision was made on ECA for monofilament insertion. Next, silicone (CutterSil Mucosa, HeraeusKulzer) coated 4-0 monofilament (0.30 mm to 0.34 mm) was

passed from ECA and inserted into ICA. The filament was advanced to around 19 to 20 mm from the CCA bifurcation. At this point, the MCA was occluded causing a sharp drop in the relative cerebral blood flow to about 70%. After 100 min occlusion, the silicon coated filament was withdrawn and cerebral blood flow was rapidly restored. Finally, the incision was closed and animals were returned to their home cage when awaken from anaesthesia. Free access to food and water was provided.

B. Infarct Volume Assessment

The rats were euthanized at 24 h after tMCAO. The whole cerebrum were cut into five 2-mm coronal sections using a brain-sectioning block (Zivic Miller, USA) and immediately stained by 2% 2,3,5-Triphenyltetrazolium chloride (TTC, Sigma, USA) at room temperature for 15 min. Images for TTC stained brain sections were taken. The infarct volume was measured using the Image J (NIH, USA) and calculated with correction for brain oedema.

C. H_2S-synthesizing Activity Assay in Brain Tissue

H_2S-synthesizing activity was performed in a reaction tube fitted with air-tight rubber septum. A 2-ml eppendorf tube containing a folded 2 cm × 2.5 cm filter paper (Whatman No. 1) was placed inside the reaction tubes. Five hundred microliter of zinc acetate (1%, w/v; dissolved in 12% NaOH) was added into the Eppendorf tube to trap the evolved H_2S. Brain homogenate (300 μL, 50% w/v) in 50 mmol/L potassium phosphate buffer (pH 6.8) was pre-incubated with either vehicle or inhibitor for 10 min at 37°C. Then the reaction was started by the addition of 10 mM L-cysteine, 10 mM (D,L)-homocysteine and 2 mM PLP at a total volume of 1 mL. After 90 min, the reaction was stopped by injecting trichloroacetic acid (TCA, 0.5 ml, 50% w/v) into the reaction mixture through the rubber septum. Another 1 h of incubation was allowed for the complete adsorption of H_2S by the zinc acetate. Then the rubber septum was removed and 0.5 mL of N,N-Dimethyl-p-phenylenediamine sulphate (dissolved in 7.2 M HCl) and 0.5 mL of $FeCl_3$ (dissolved in 1.2 M HCl) were added into the eppendorf tube and incubated at room temperature in the dark for 20 min. Absorbance at 670 nm was measured by a microplate reader (Sunrise; TECAN) which was converted to H_2S concentration through a standard curve obtained by using NaSH as the standard.

D. Immunohistochemistry

Twenty-four hours after tMCAO, the rats were anaesthetized and perfused with 0.1 M phosphate buffer saline (pH 7.2) followed by 4% paraformaldehyde through the heart. Brain were harvested and immersed in 4% paraformaldehyde for overnight at 4°C, and then dehydrated in 10% sucrose at 4°C overnight. Finally, the brains were cryopreserved at 20% sucrose at 4°C. Coronal sections (30 μm) were cut using a cryostat and mounted onto glass slides (Matsunami, Japan) and air dried overnight. Brain slices were stored at -80°C until use. Upon staining, brain slices were air dried in room temperature for 30 min and permealized with 0.1% Triton-X (Sigma, USA, dissolved in 0.1 mol/L phosphate buffer saline) for 10 min. Next, non-specific binding was blocked by incubating the

section in 5% goat serum (Vector laboratories, United Kingdom) for 1 h. Antibodies including OX-42 (1:200, Serotec) and ED-1 (1:200, Chemicon, Millipore Coporation) were applied on coronal brain sections and incubated at 4°C overnight. After washing with PSB, sections were incubated with Alexa 488-conjugated goat anti-rabbit (Molecular probes, USA) or Alexa 555 (Molecular probes, USA) for 1 h at room temperature and cover slipped using ProLong gold antifade reagent (Life technologies, USA). Fluorescent images were captured using QImaging (Canada) and images captured were processed by Image Pro insight (QImaging, Canada).

E. Western Blot Analysis

To 10 mL of RIPA buffer (brain tissue samples, Cell signalling technologies) 1 tablet of protease and phosphatase inhibitors (Roche, Mannheim, Germany) was added before use. Brain tissues or cultured cells were thawed on ice and homogenized in RIPA buffer (100 mg/mL) in a 3-ml glass motor/pestle homogenizer. After shaking on ice for 30 min samples were centrifuged at 12,000 rpm at 4°C for 10 min and supernatant was stored at -80°C until use. Total protein was determined by Bradford protein assay (Biorad, CA, USA) and mixed with laemmli buffer (Biorad, CA, USA). Denaturation of proteins will be performed by heating up of total protein laemmli buffer mixture at 100°C for 5 min. Protein samples were stored at -80°C until use. Proteins were separated by 10% SDS/PAGE and transferred onto a PVDF membrane (Amersham Biosciences, Buckinghamshire, UK). This was followed by non-specific blocking with 10% non-fat milk (Biorad, CA, USA) at room temperature. The membrane was then incubated with OX-42 (1:1000, Serotec) or ED-1 (1:1000, Chemicon, Millipore Coporation) at 4°C overnight. Next, membrane was washed and incubated in HRP-conjugated anti-rabbit or mouse IgG (Chemicon, Millipore Corporation) at room temperature for 1 h. Visualization was carried out using Luminata Forte or Crescendo Western HRP substrate (Millipore Corporation, Billercia, MA, USA) and the chemiluminescence signals were detected using UVIchemi (UVItec, Cambridge, UK).

IX. Selectivity of CBSI 6S vs. AOAA in Inhibiting GABA AT

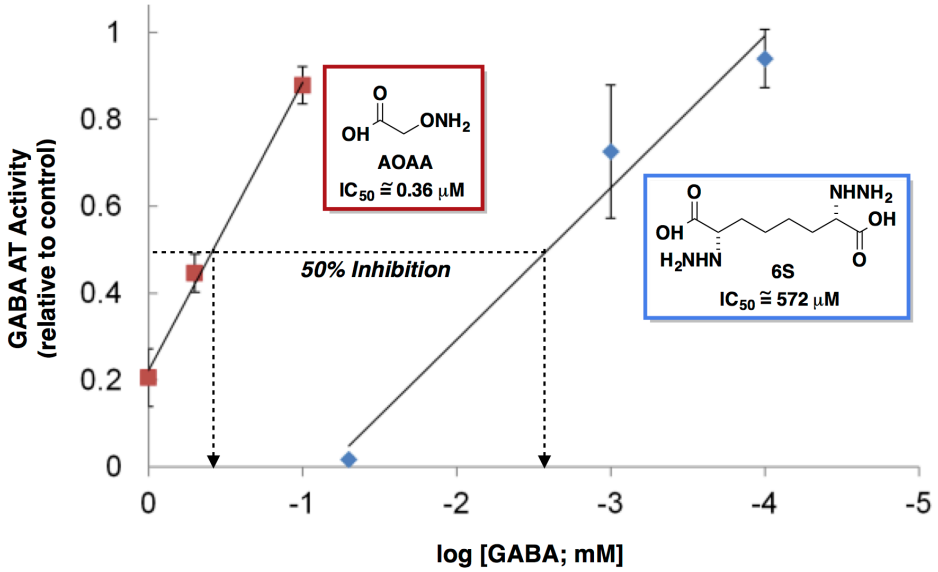
To investigate the selectivity of CBSI **6S** for CBS versus other PLP-dependent enzymes a model study was conducted with GABA aminotransferase, an important enzyme found in the brain. To draw comparisons, a commonly used non-selective inhibitor of PLP-dependent enzymes (aminooxy acetic acid; AOAA) was also tested with GABA AT.

GABA transaminase (GABA AT) Assay

GABA transaminase assay was performed according to Salvador et al.[15] with modification. Rat brains were dissected into cortex and striatum. Brain samples were flash frozen in liquid nitrogen and stored at -80°C until analysis. Frozen rat brains were thawed on ice and homogenized in ice-cold 50 mM potassium phosphate buffer (50% w/v). AOAA or CBSI **6S** at various concentrations were added 10 min prior to the addition of substrates at 37°C. Next, substrate solution contaiinng 50 mM α-ketoglutaric acid (pH 8) and 125 mM γ-aminobutyric acid (pH 8.0) were added to

0.2 mL of rat homogenate (total volume 1 ml). The reaction mixture was further incubated at 37°C for 1 h. Upon completion of incubation, reaction mixture was centrifuged at 3000 × g for 10 min at 4°C. Supernatant (300 µL) was mixed with 0.3 mL of 200 mM of 3,5-diaminobenzoic acid (pH 6.0) followed by 1 h incubation at 60 °C. The fluorescence signal was measured by Varioskan flash multimode reader (Thermo Scientific) at excitation and emission wavelength at 405 and 505 nm respectively.

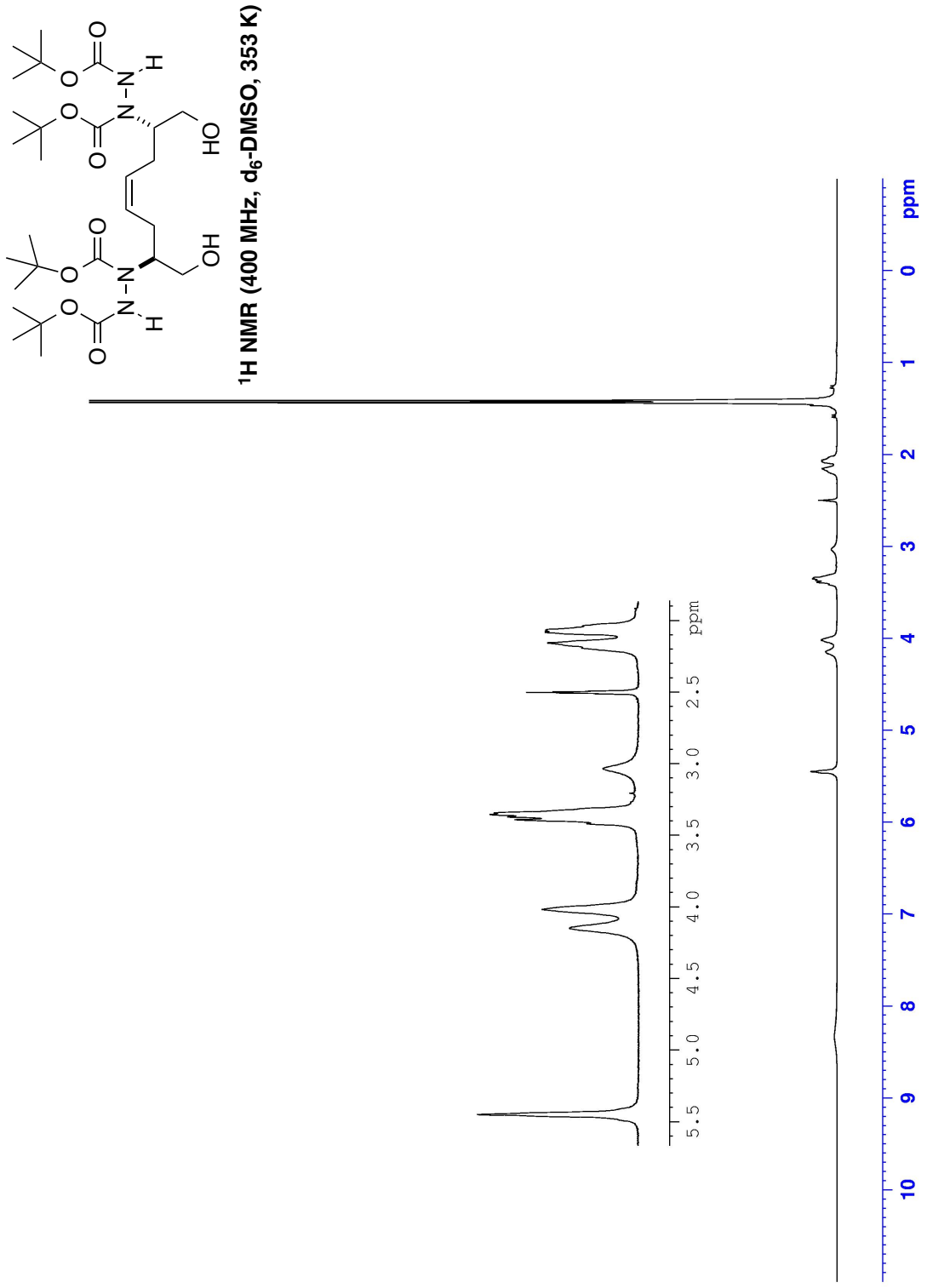
Figure S17: Potency of AOAA versus CBSI **6S** in Inhibiting GABA AT

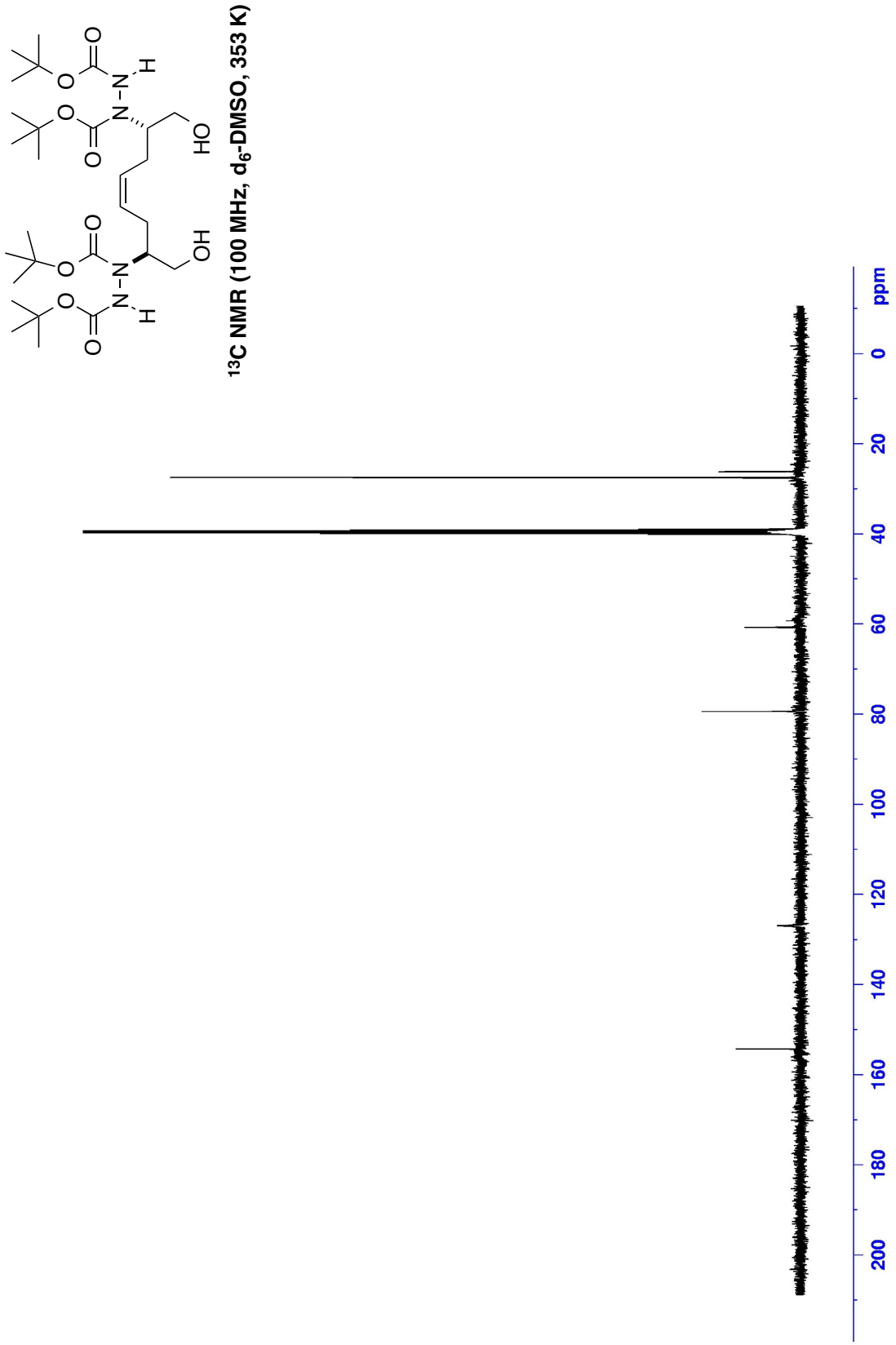


Data Analysis

Multiple group comparisons were performed by one-way analysis of variance (ANOVA) followed by post hoc analysis with Bonferroni correction, while comparison between 2 groups was performed by two-tailed independent t-test using IBM SPSS Statistics 19. Data are expressed as mean ± SEM. Statistical significance is reached when $p < 0.05$.

X. ^{1}H & ^{13}C NMR Spectra

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HO
OH
ppm
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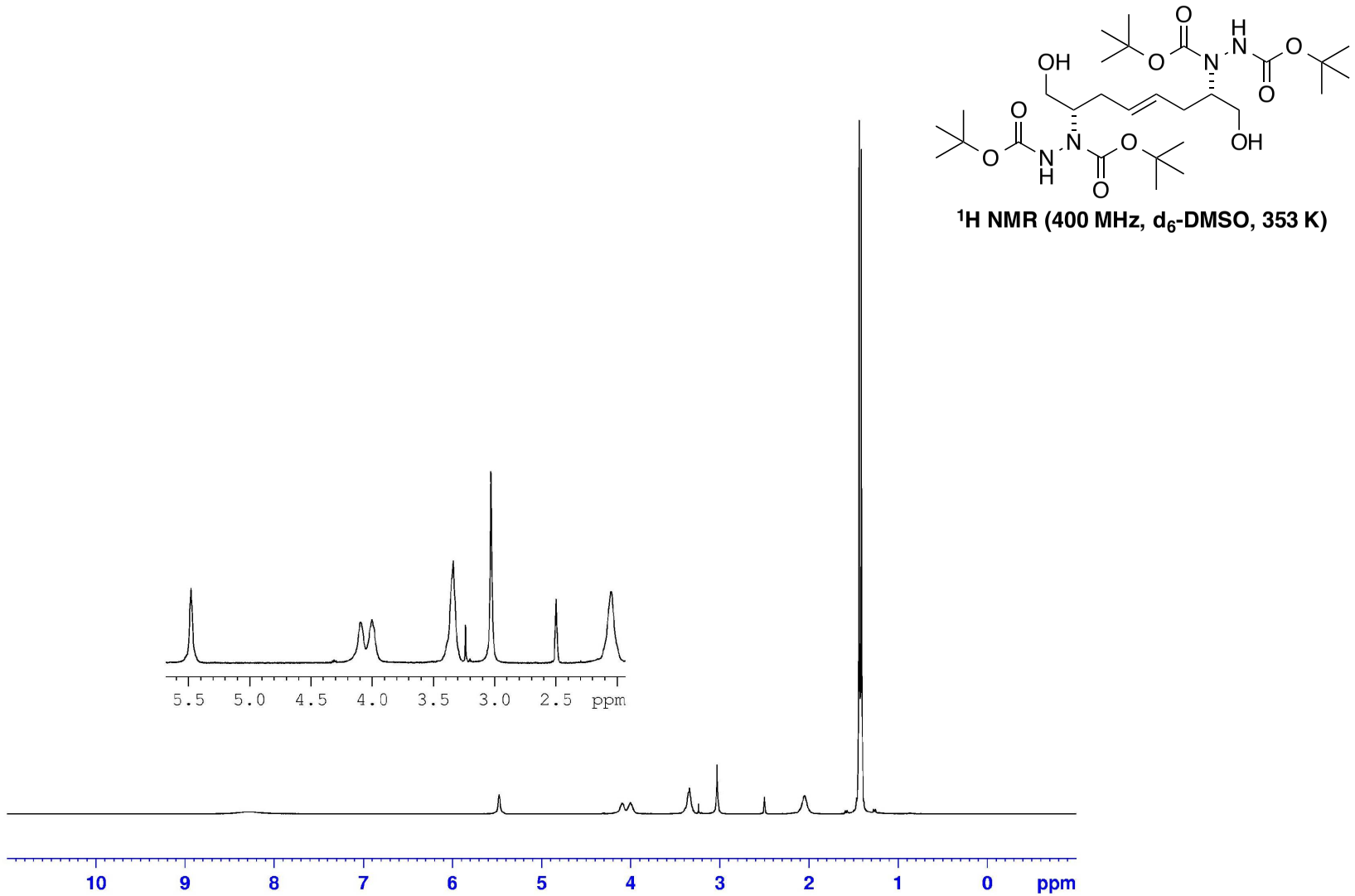
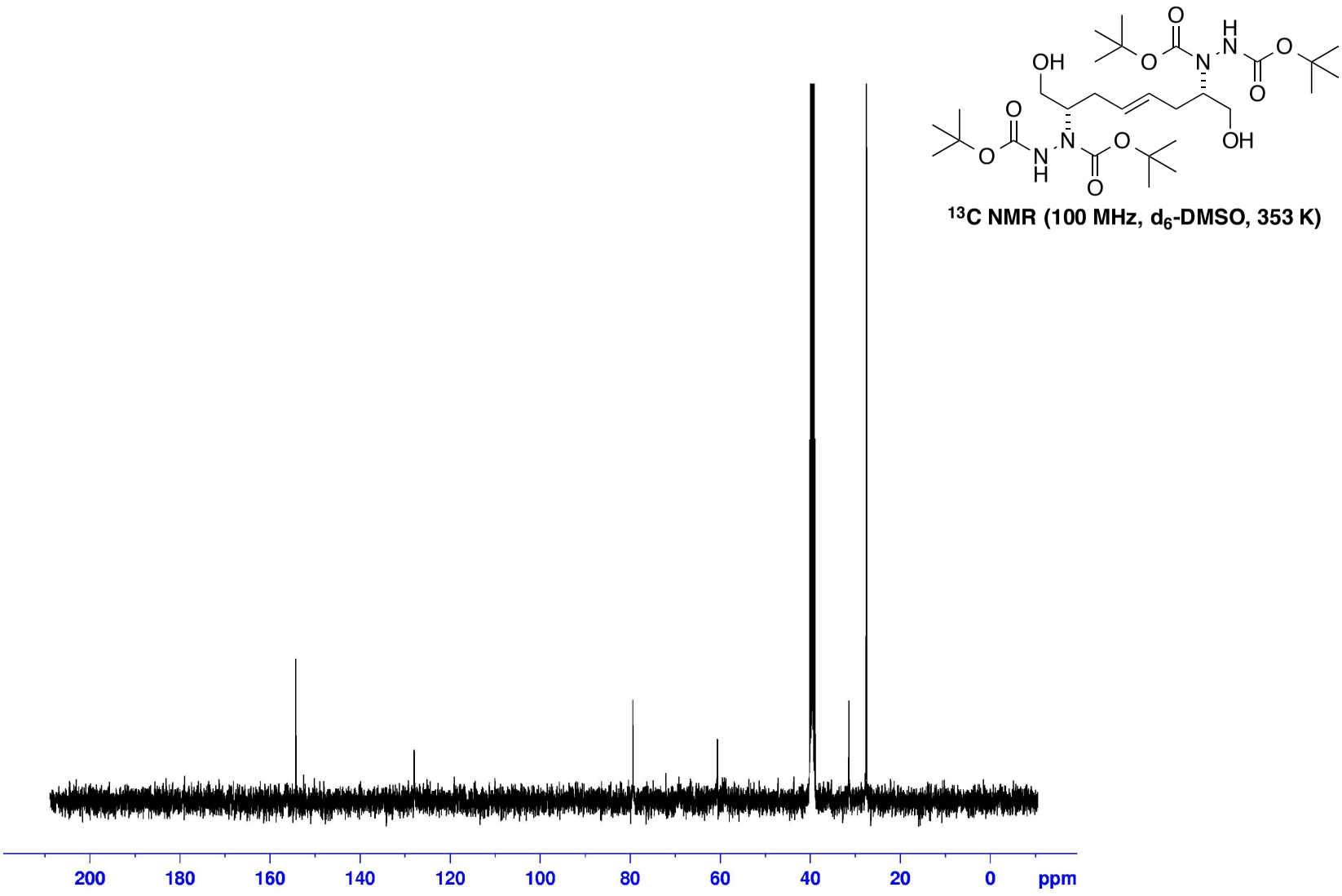
40
60
80
100
120
140
160
180
200



^{1}H NMR (400 MHz, d_6-DMSO, 353 K)



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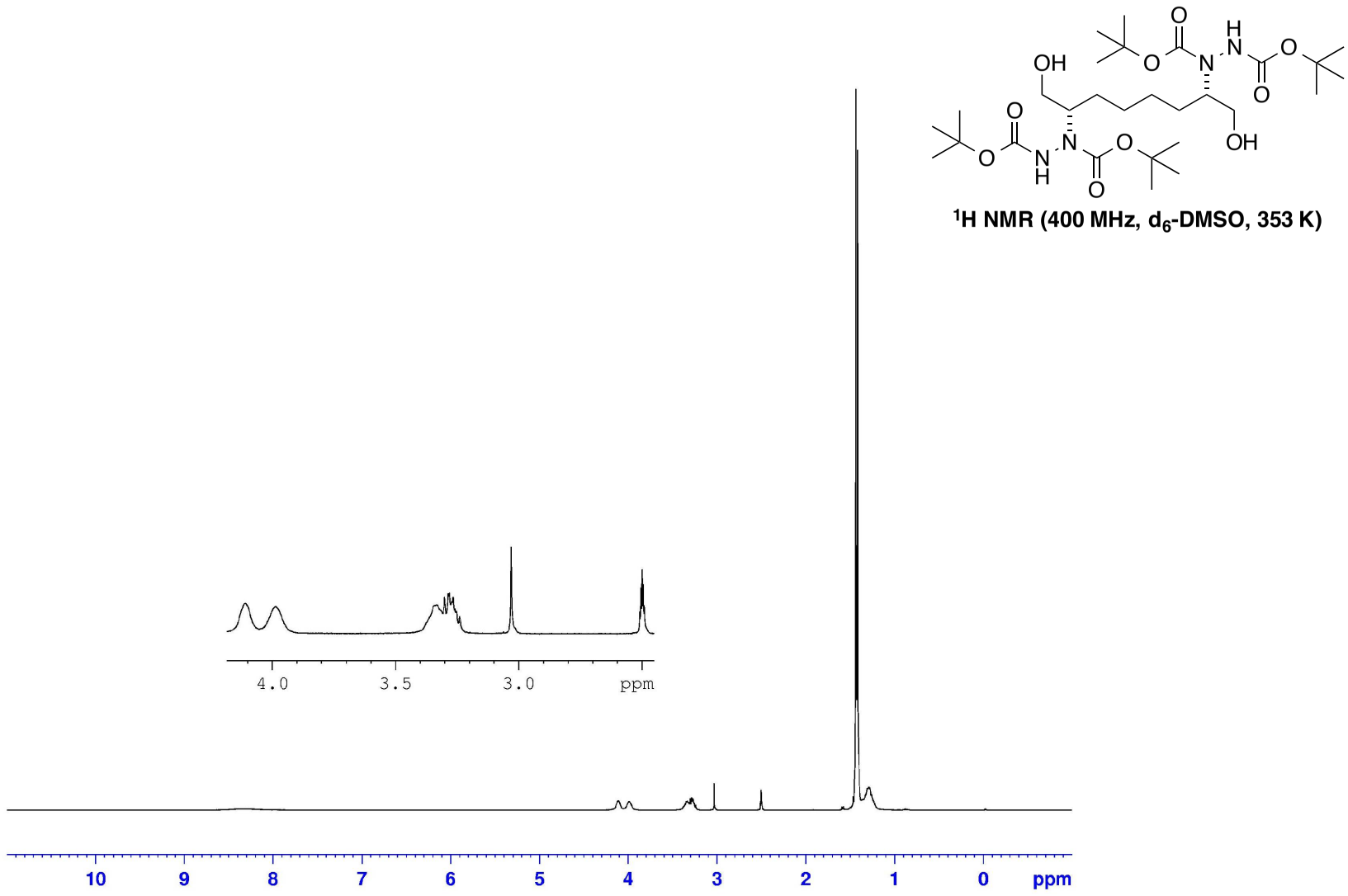

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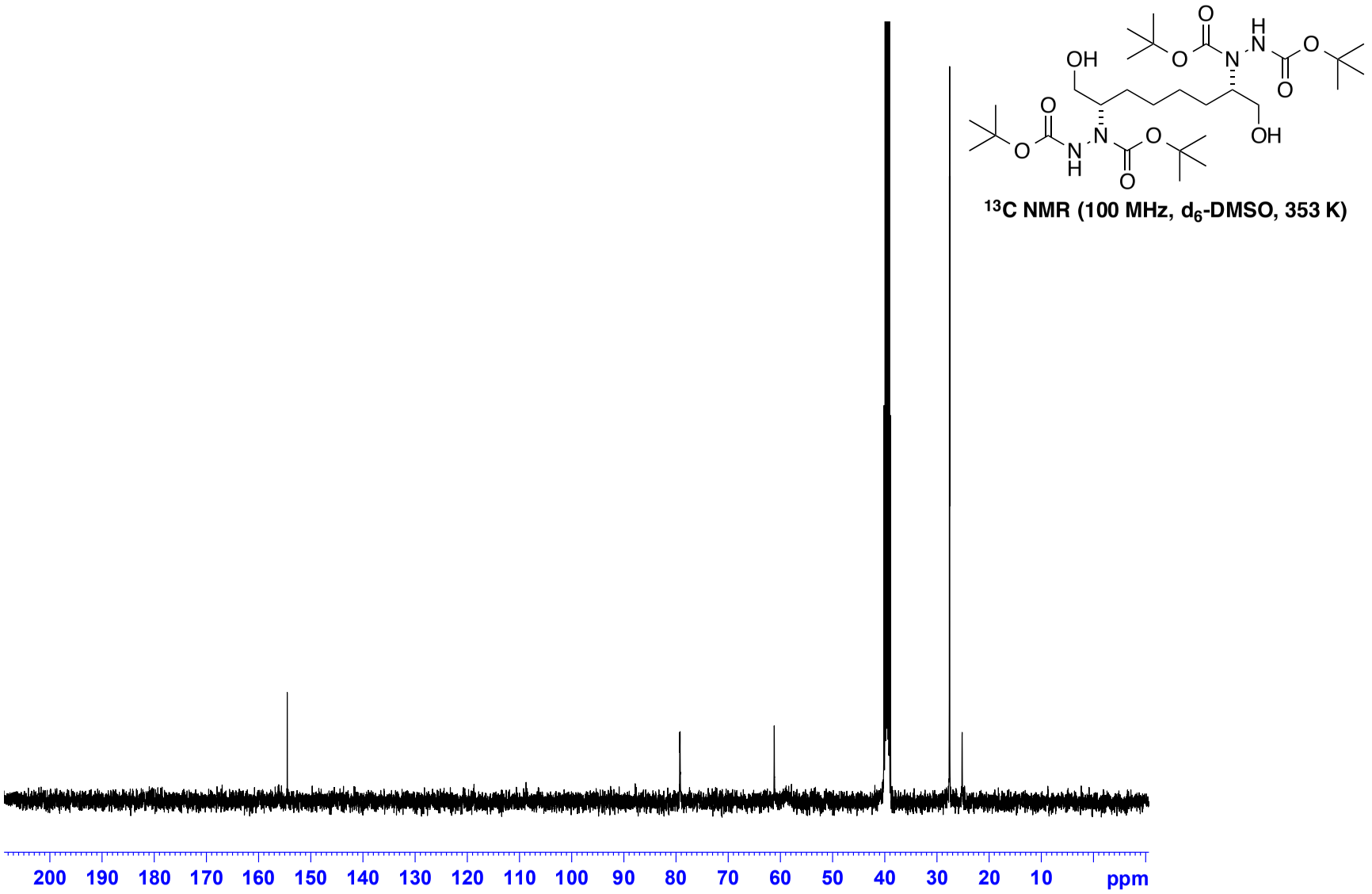

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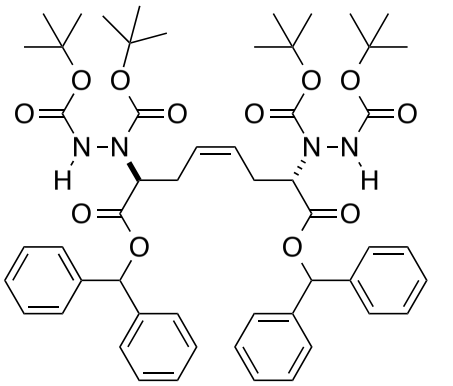
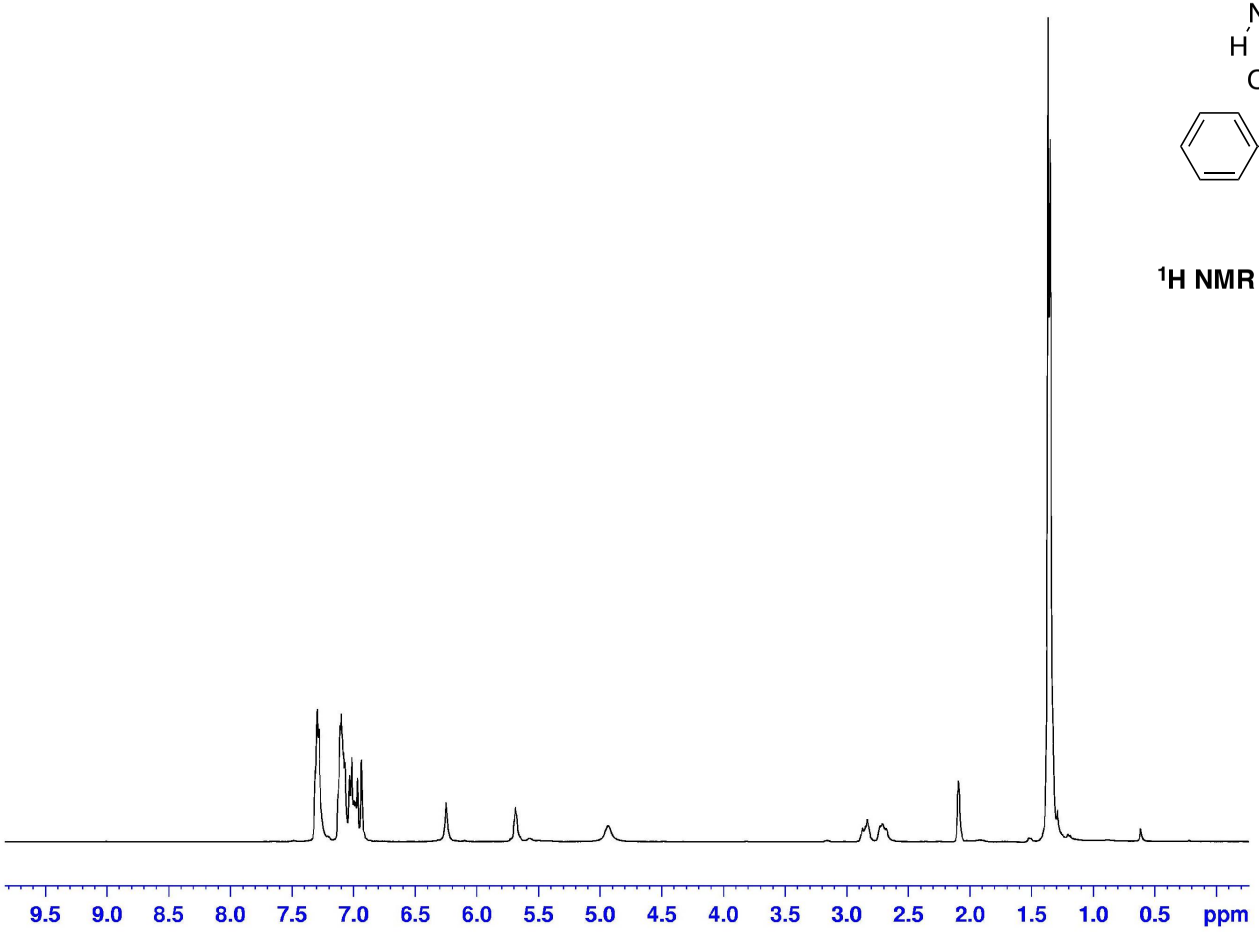
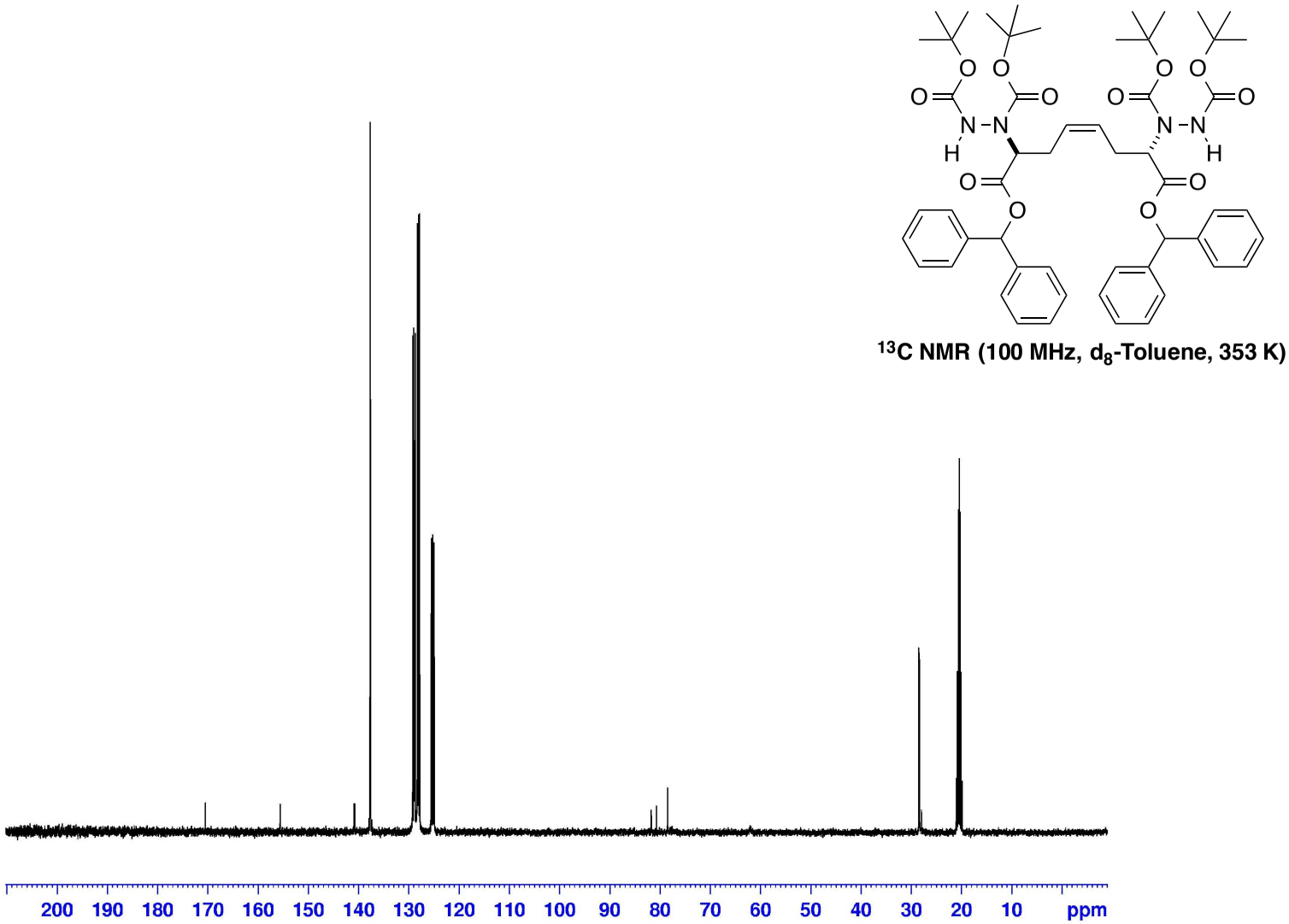


^{1}H NMR (400 MHz, d_8-Toluene, 353 K)





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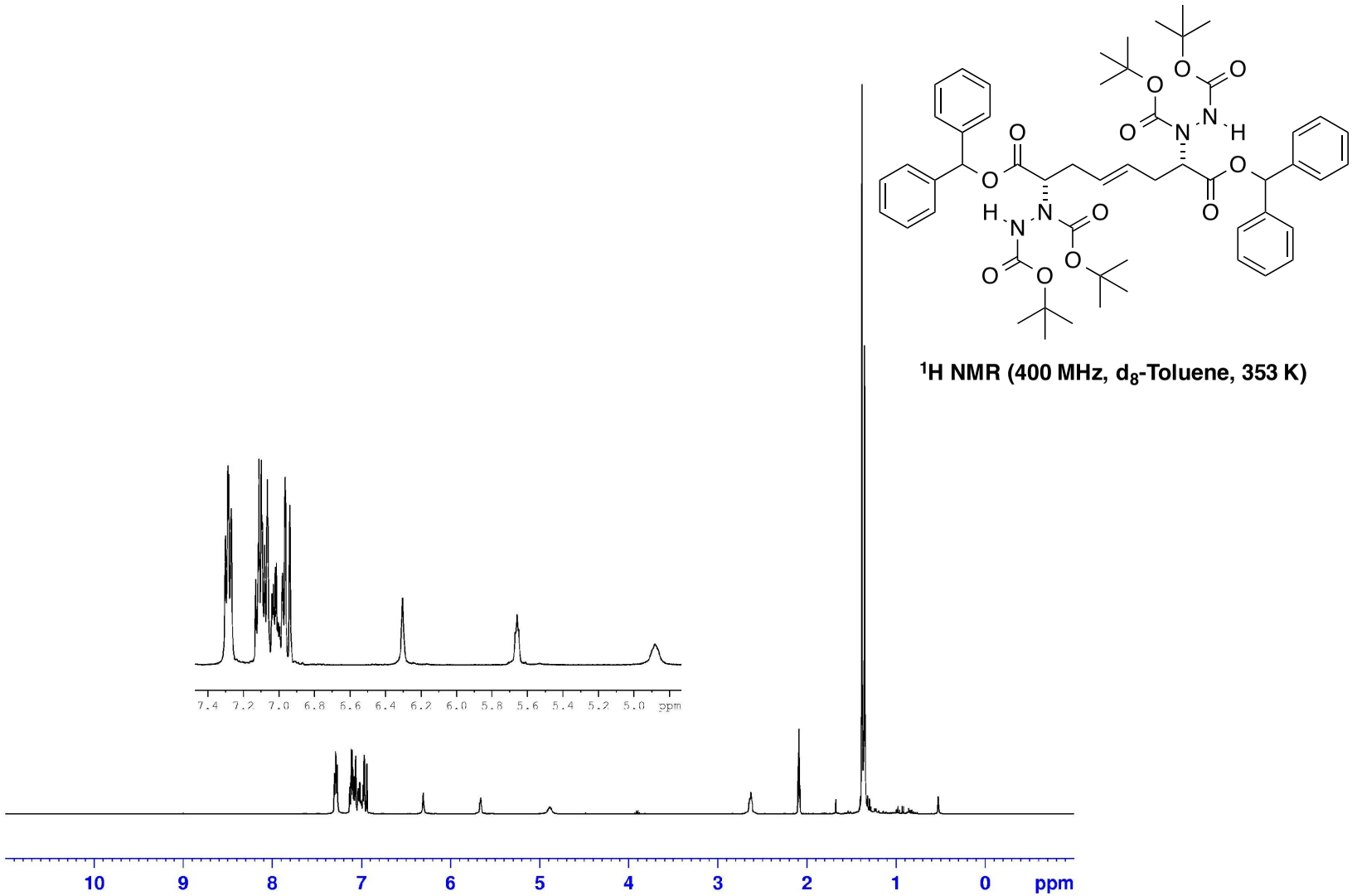

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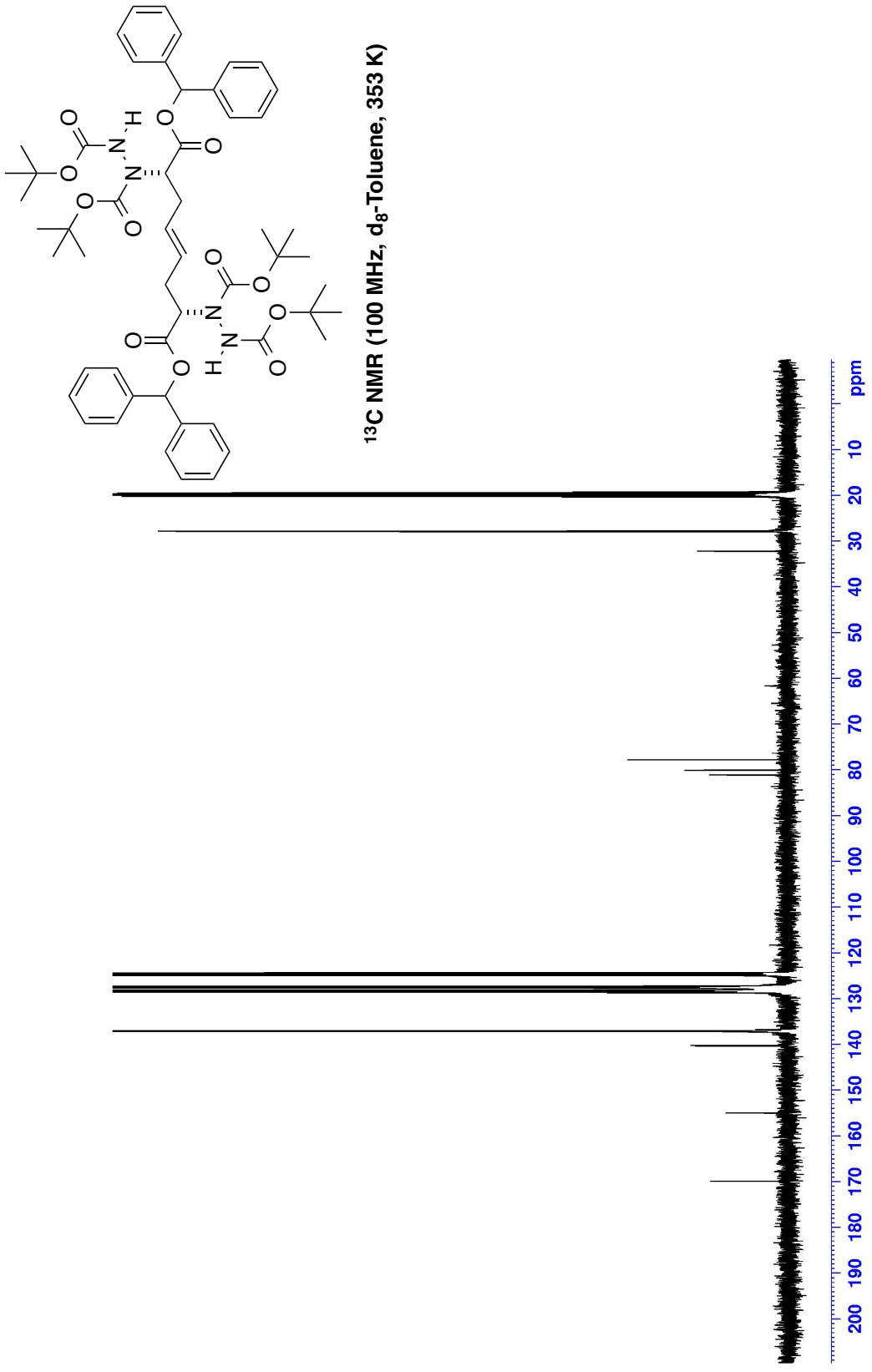
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ppm
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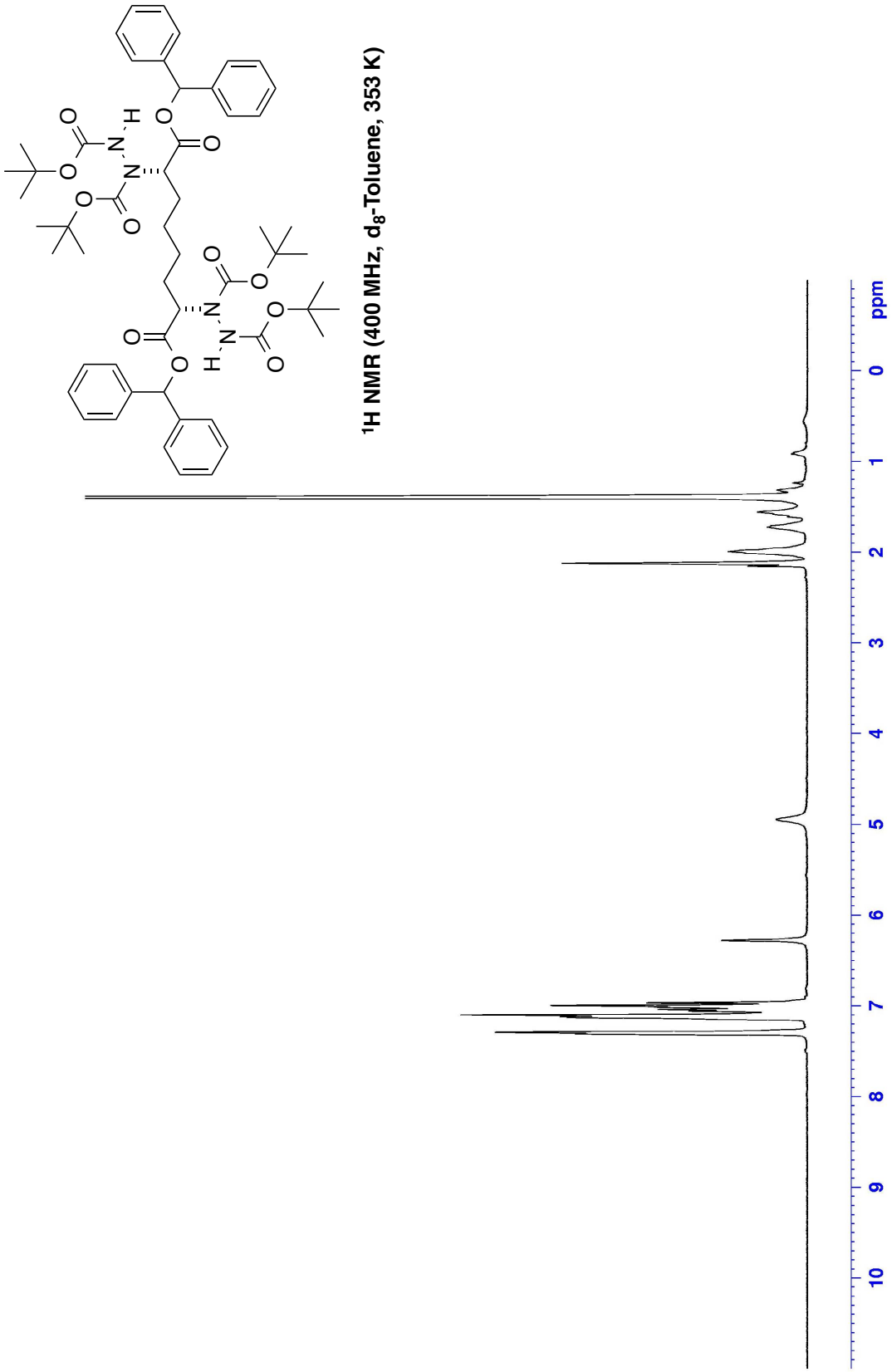
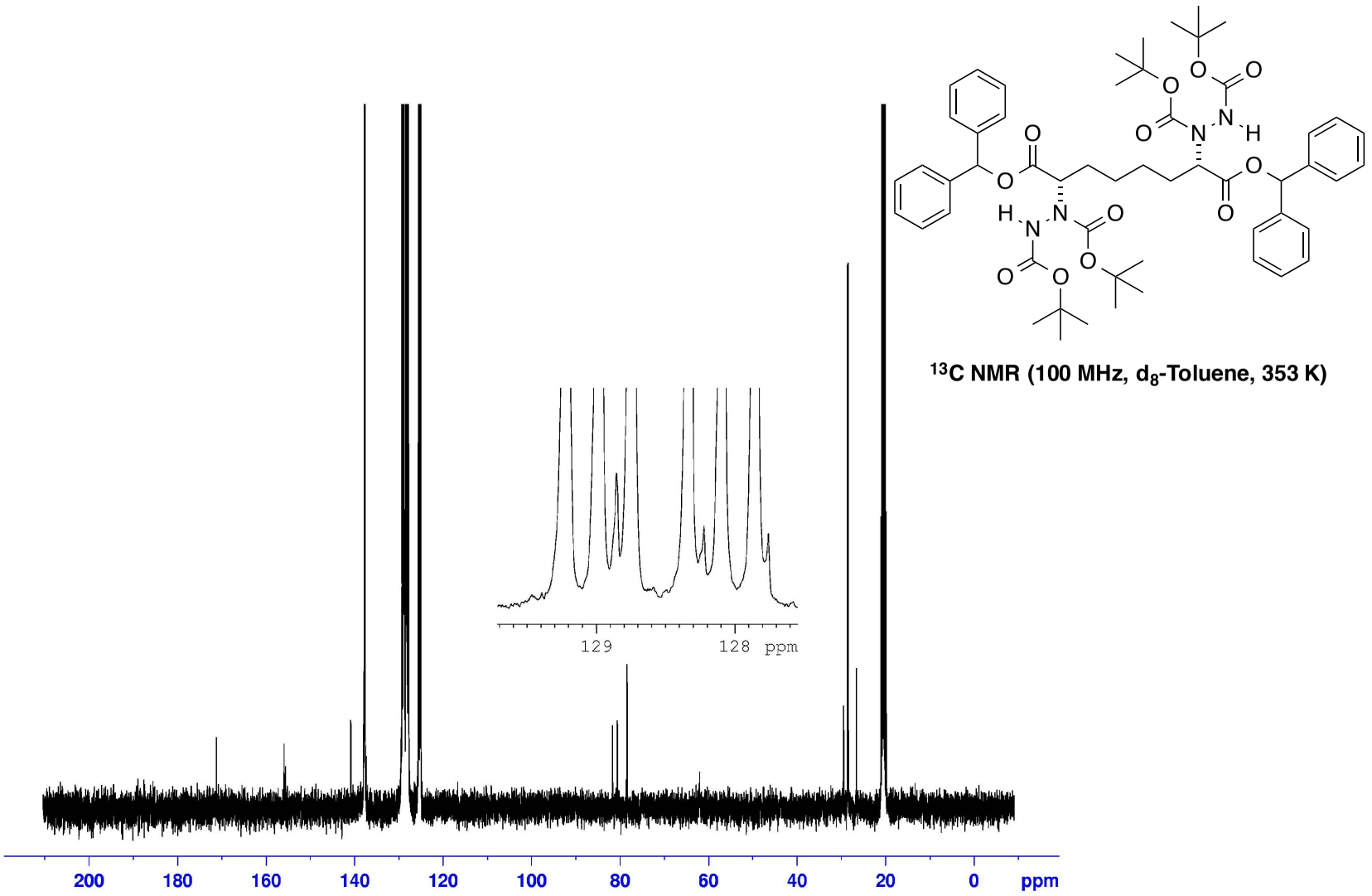

^{1}H NMR (400 MHz, d_8-Toluene, 353 K)
ppm
0
1
2
3
4
5
6
7
8
9
10



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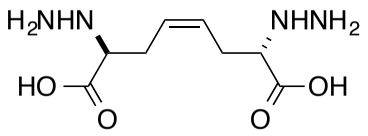

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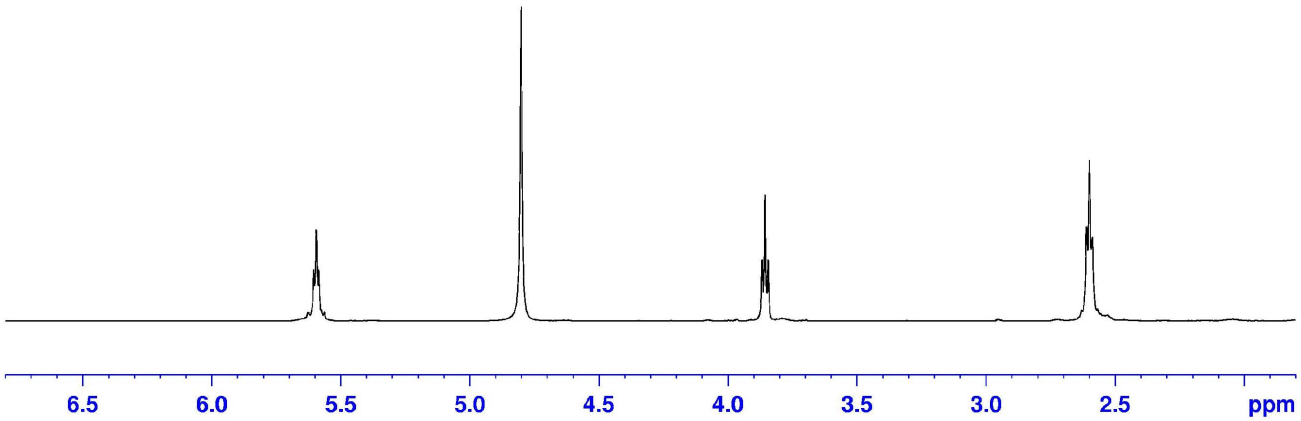

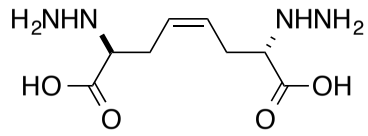

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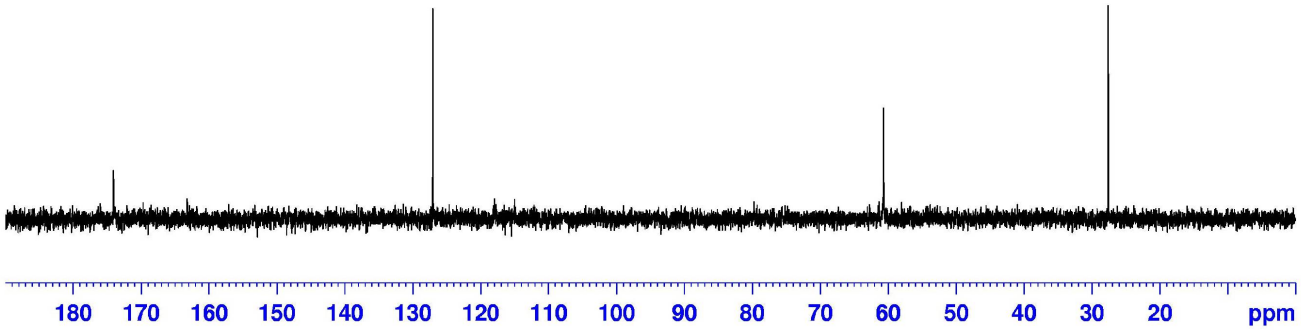

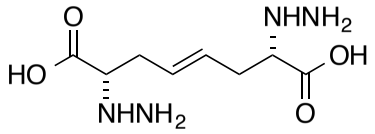

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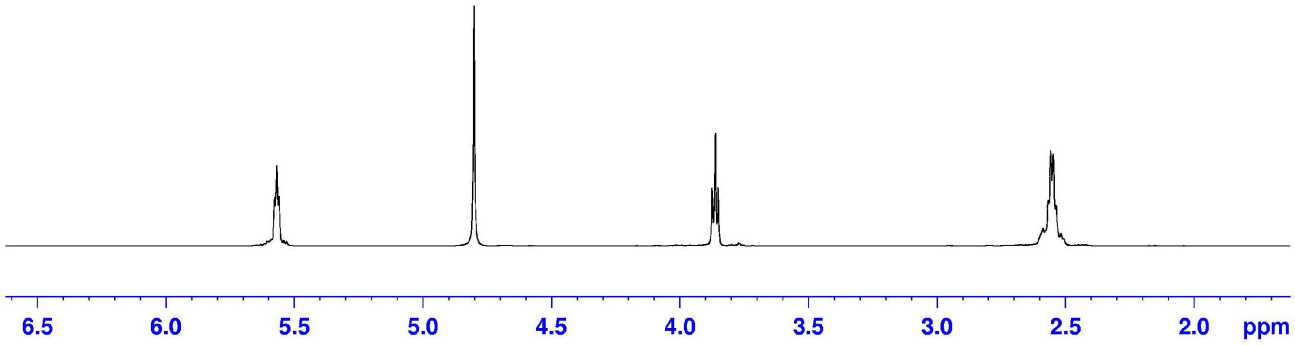

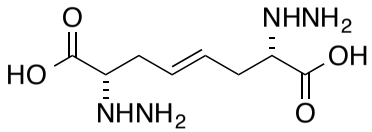

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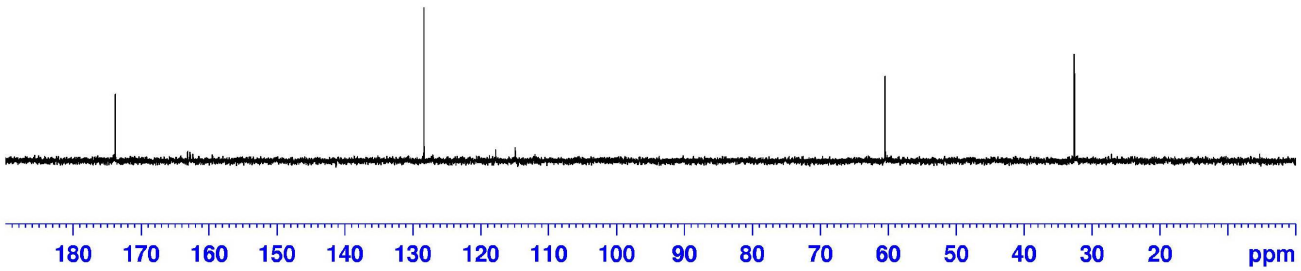

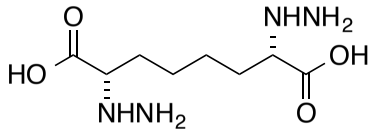

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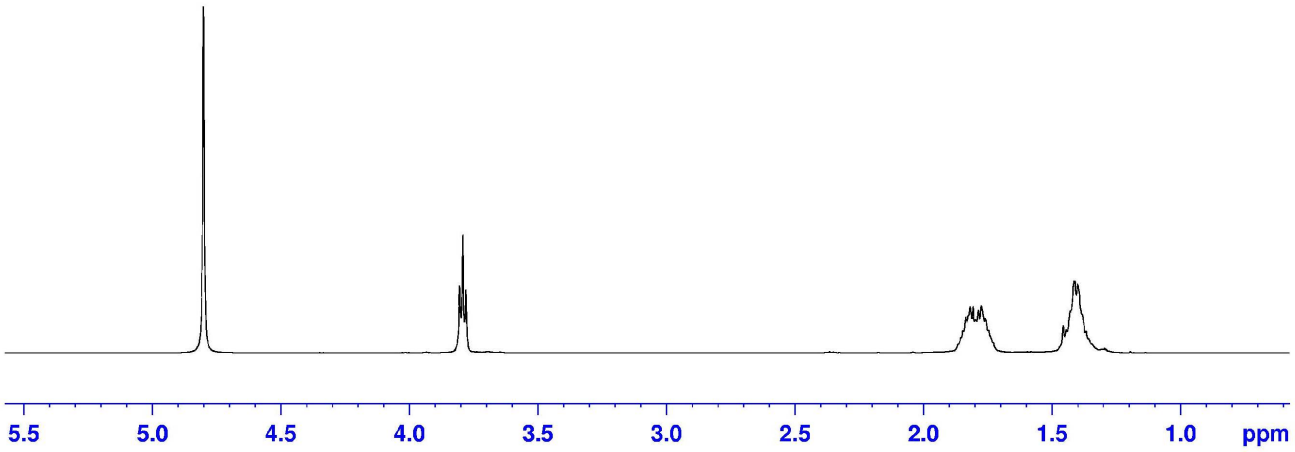

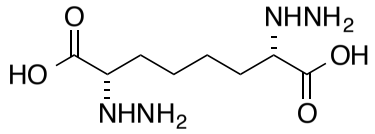

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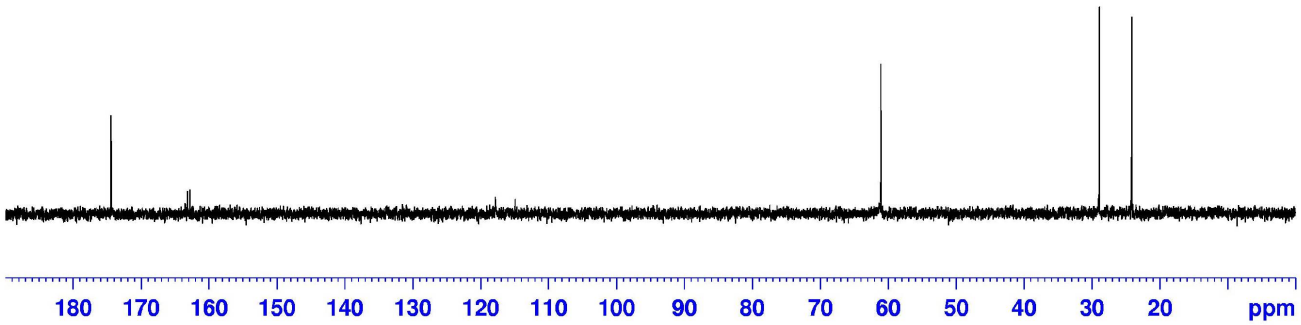
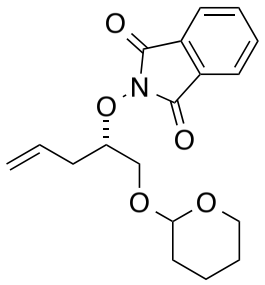
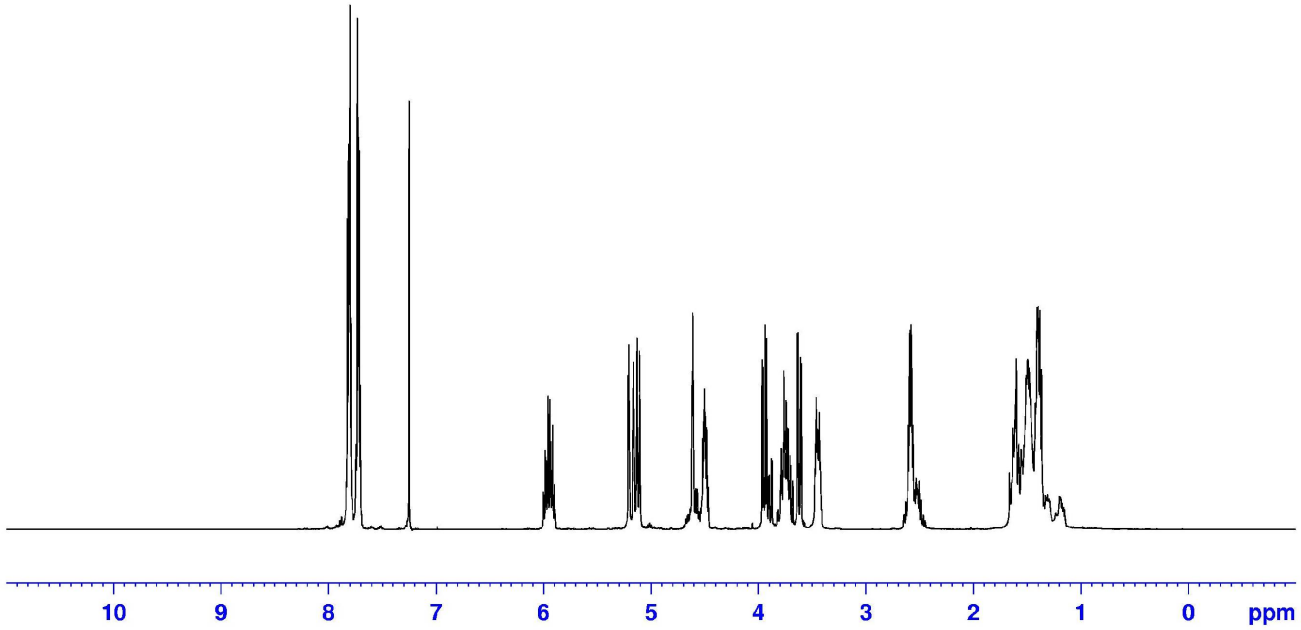
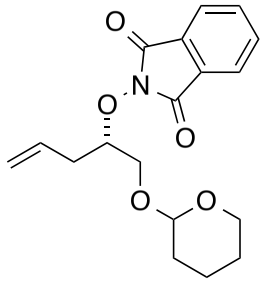


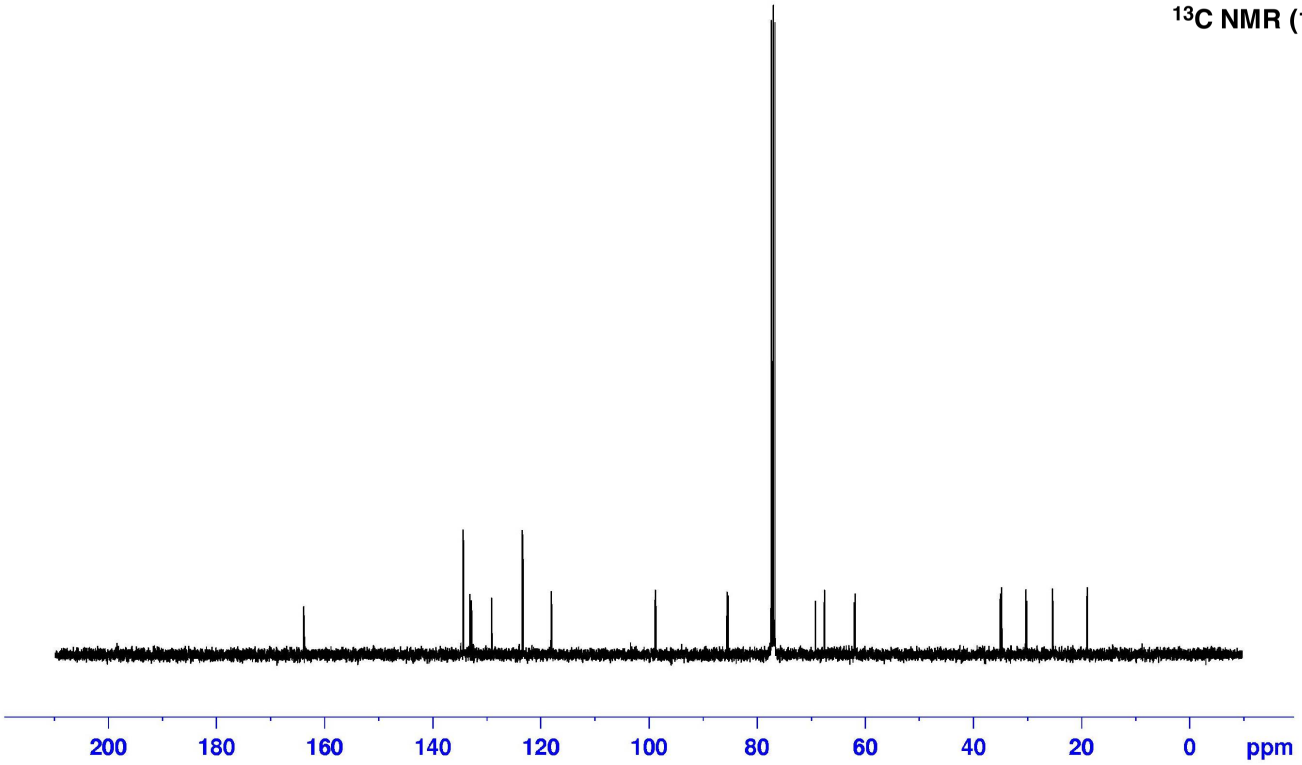


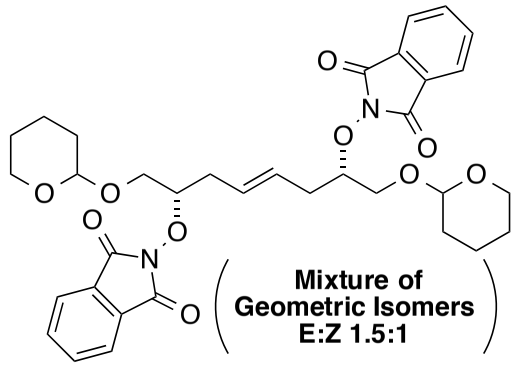
^{1}H NMR (400 MHz, $CDCl_3$)



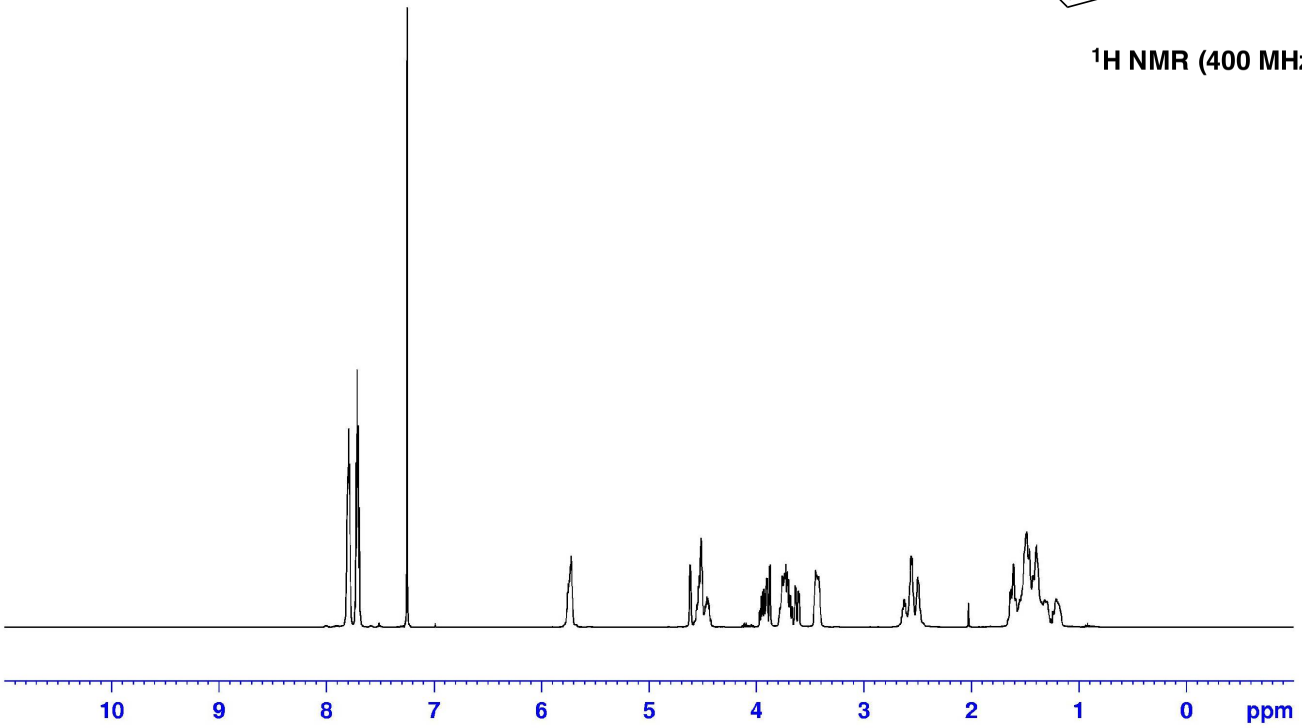


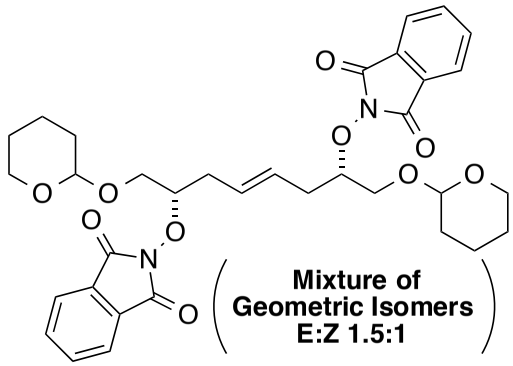
^{13}C NMR (100 MHz, $CDCl_3$)



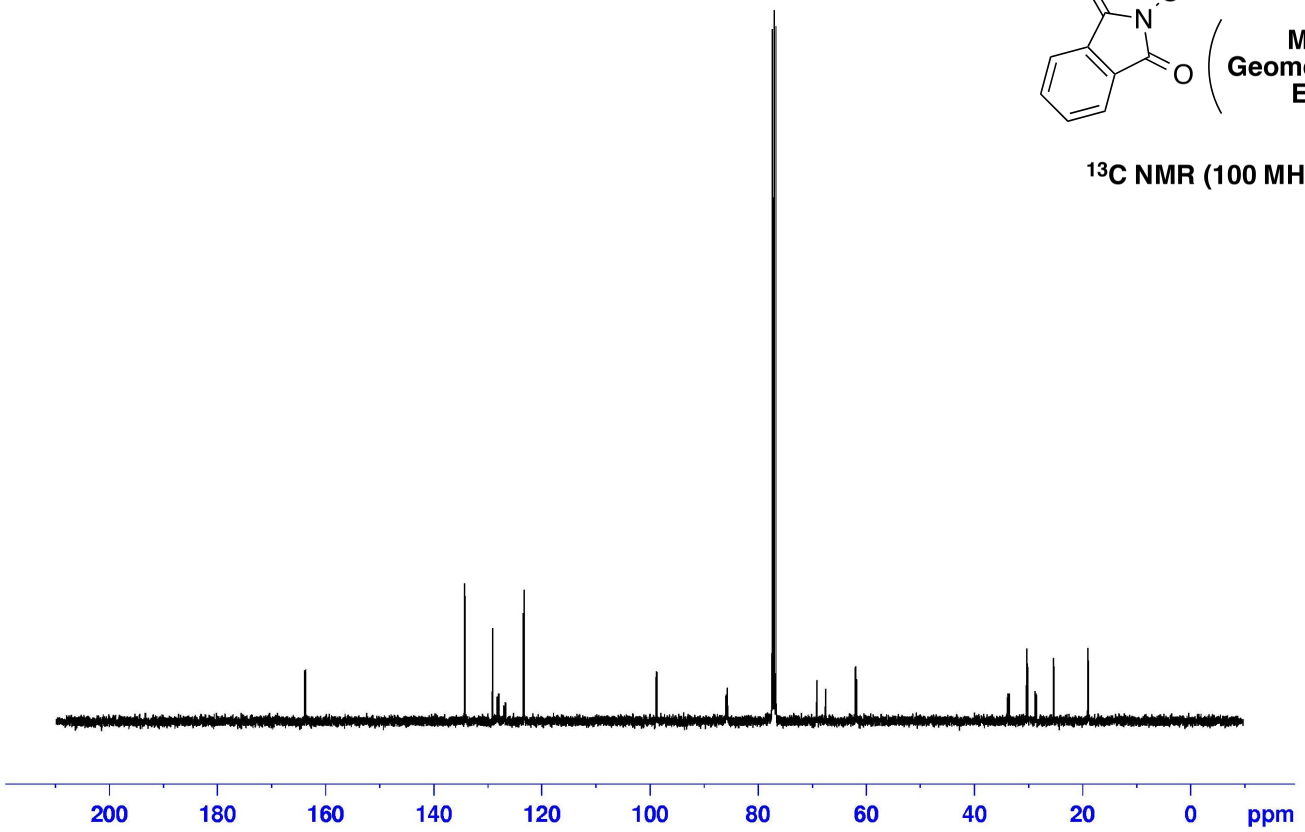


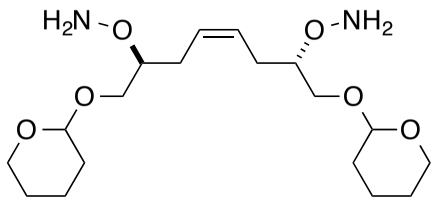
^{1}H NMR (400 MHz, $CDCl_3$)



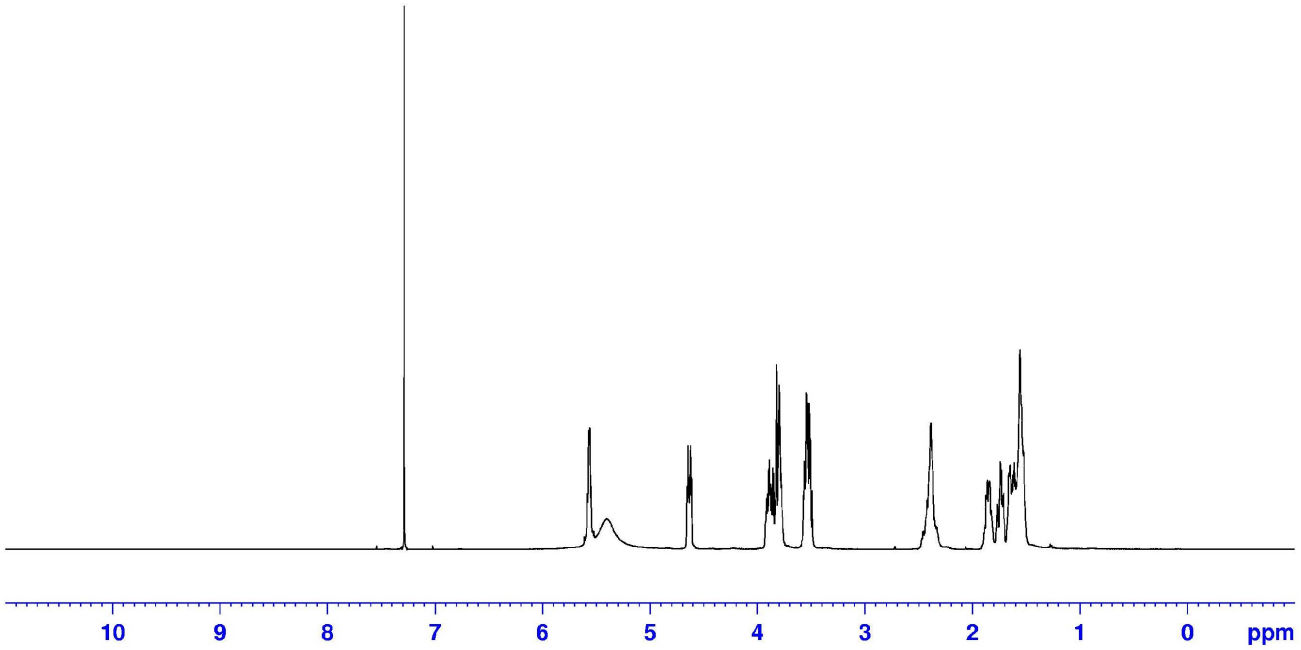


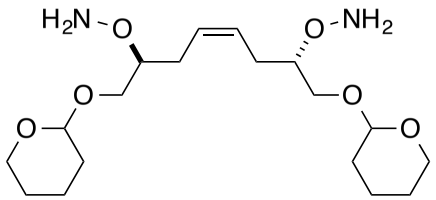
^{13}C NMR (100 MHz, $CDCl_3$)



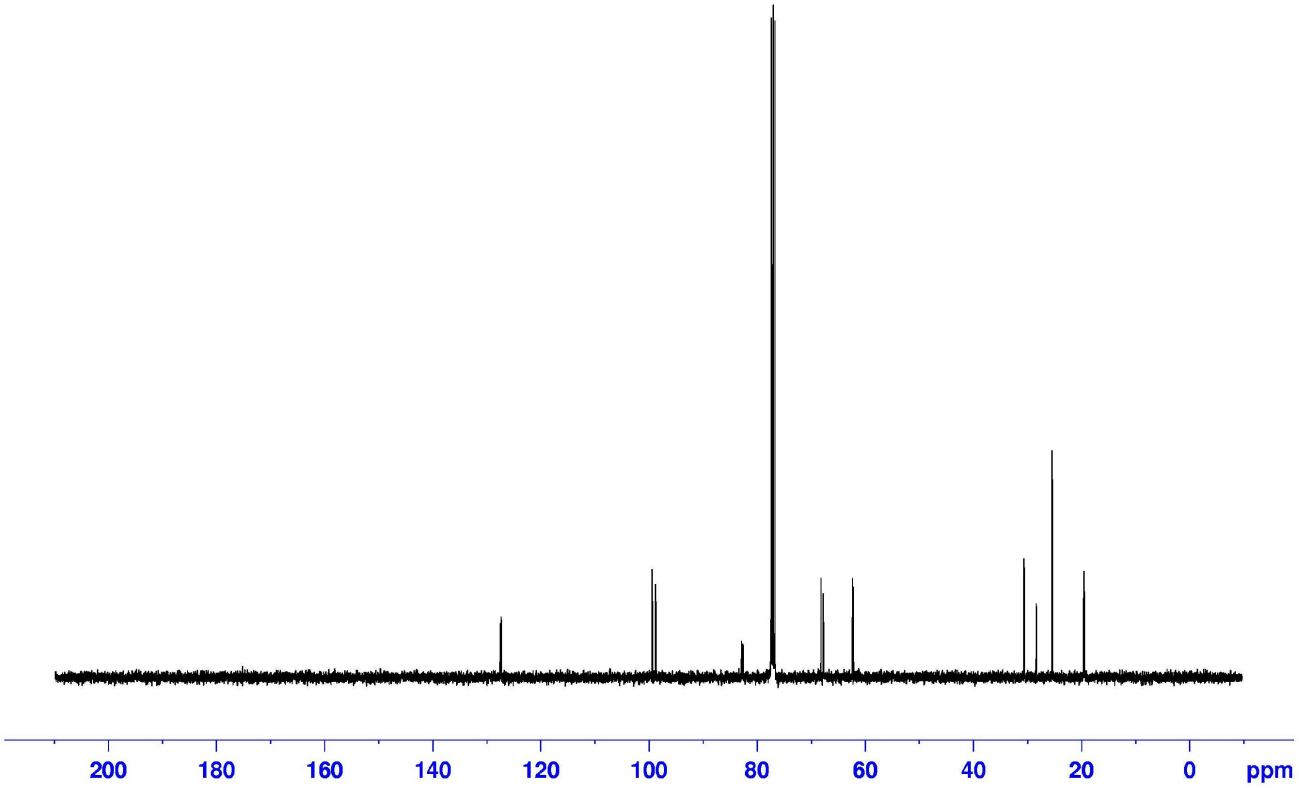


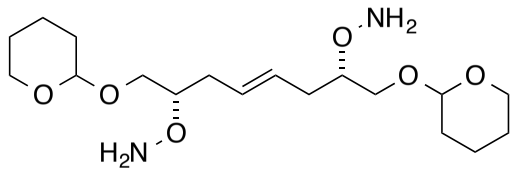
^{1}H NMR (400 MHz, $CDCl_3$)



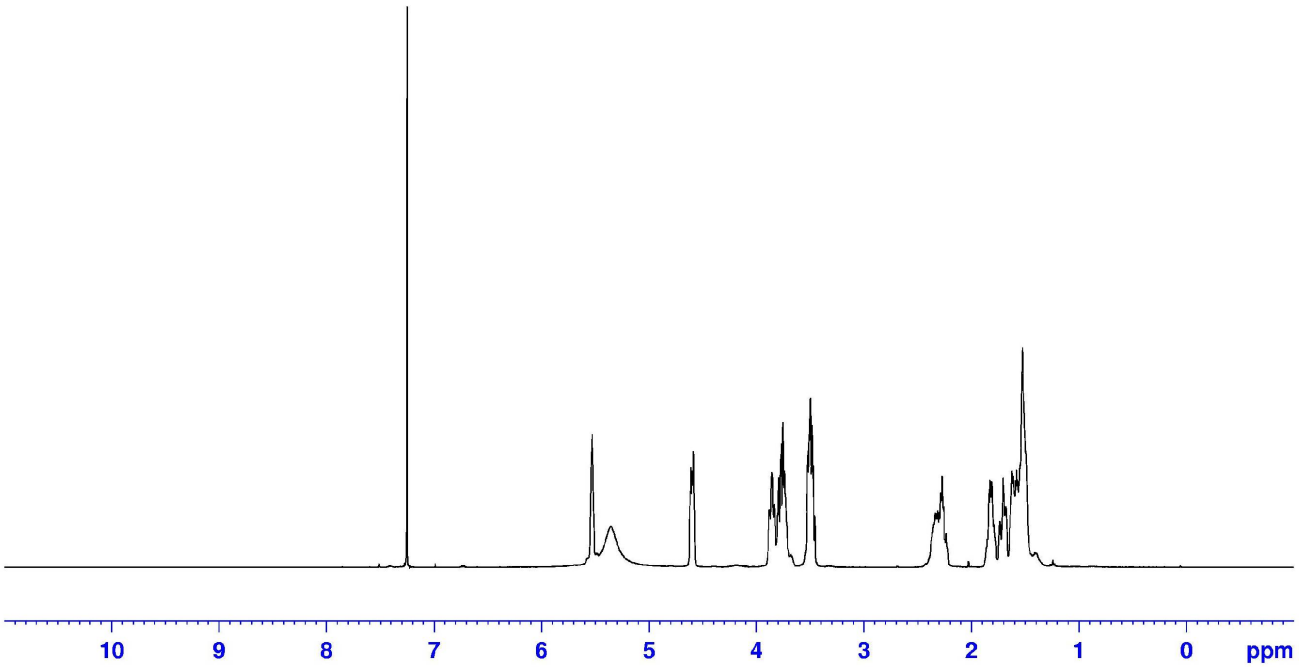


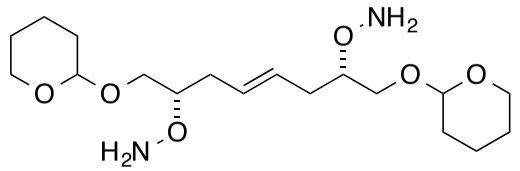
^{13}C NMR (100 MHz, $CDCl_3$)



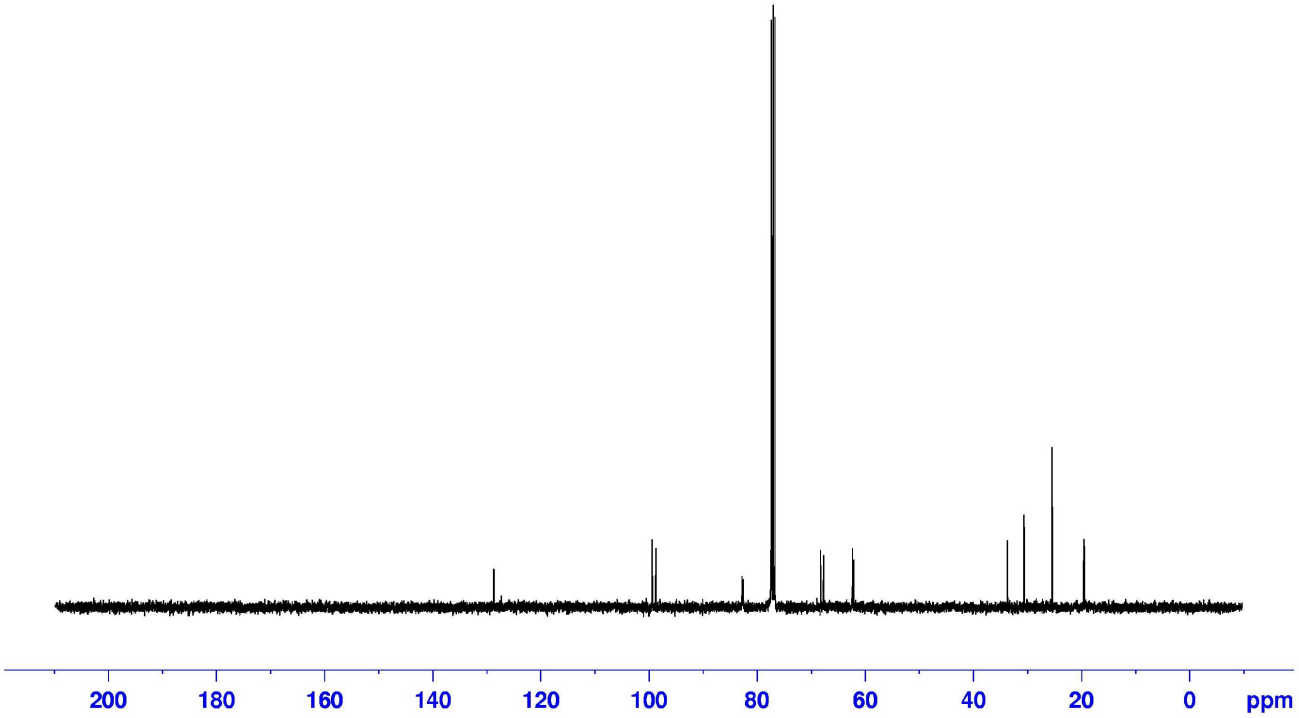


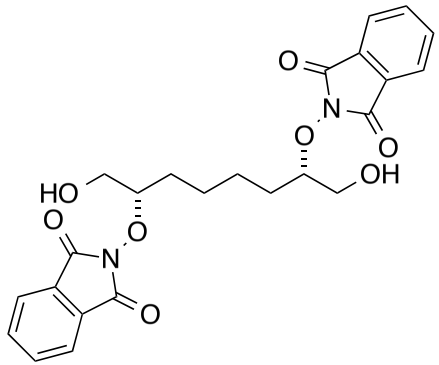
^{1}H NMR (400 MHz, $CDCl_3$)



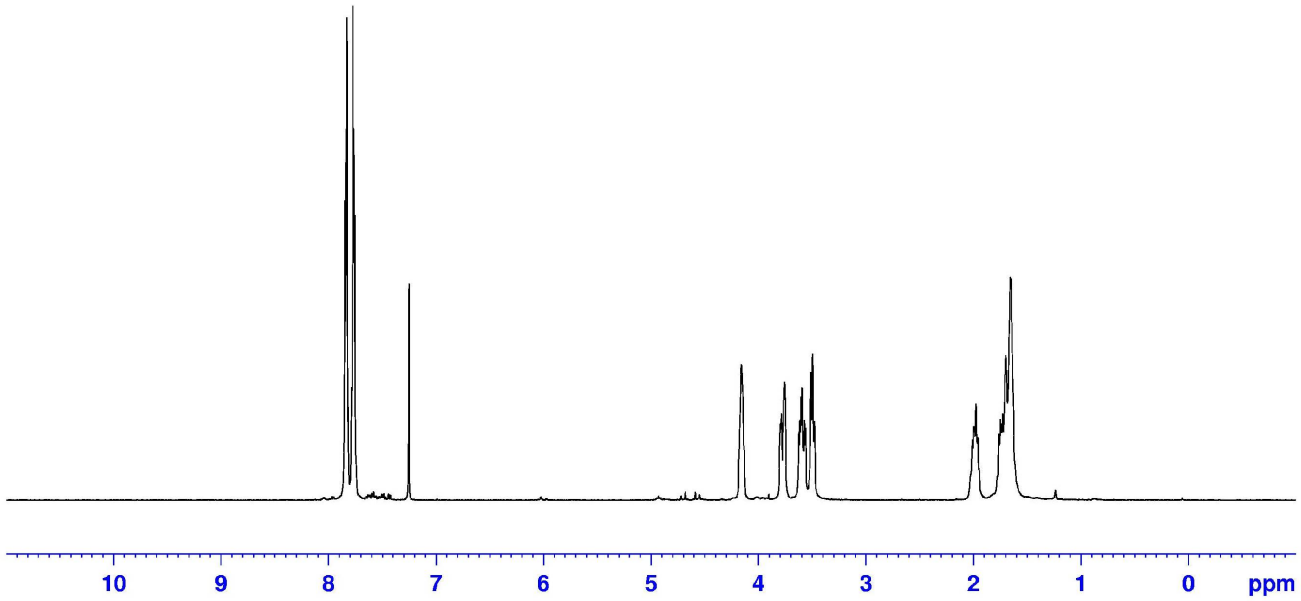


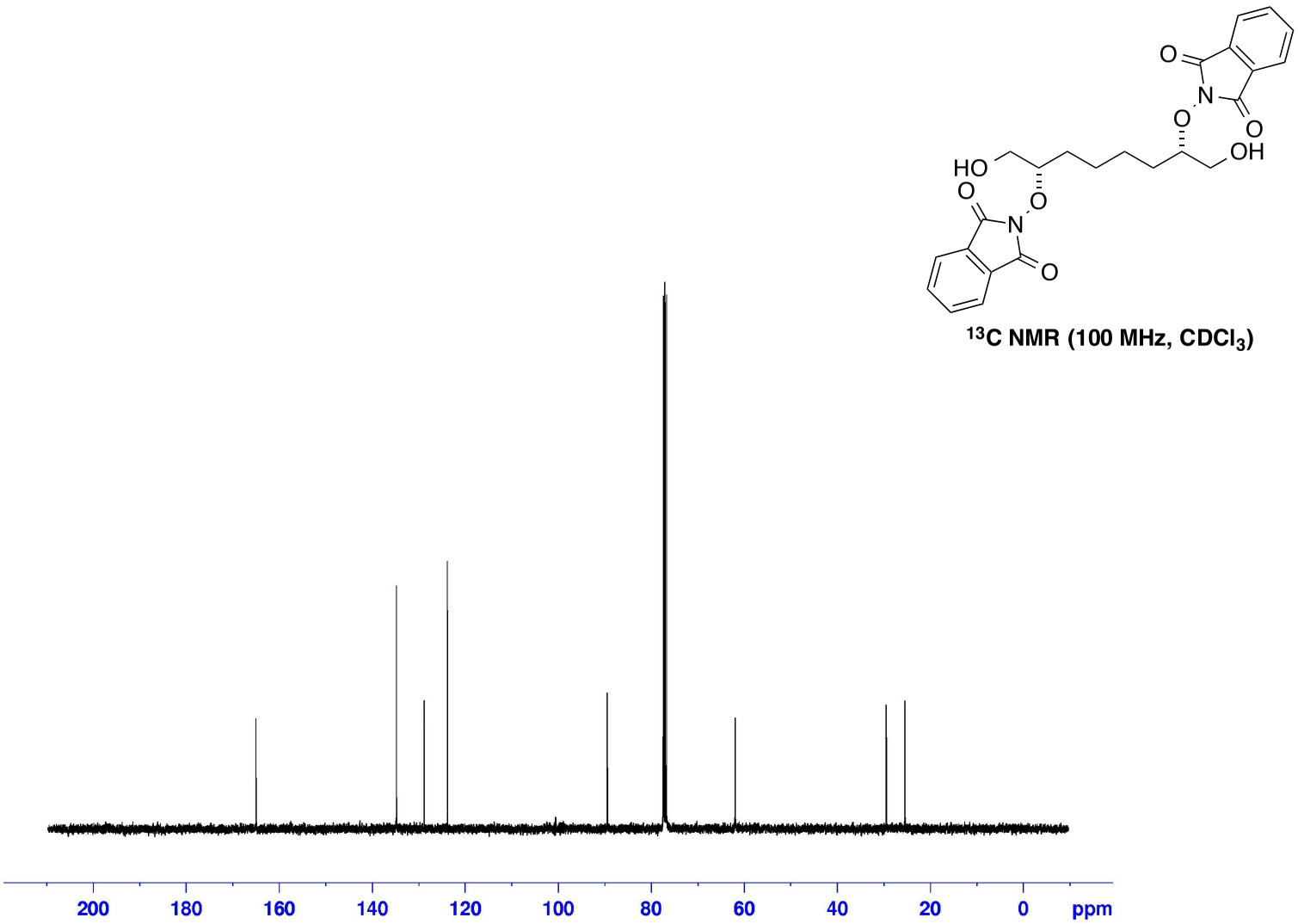
^{13}C NMR (100 MHz, $CDCl_3$)



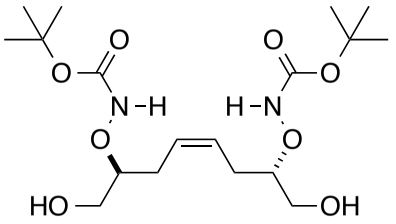


^{1}H NMR (400 MHz, $CDCl_3$)

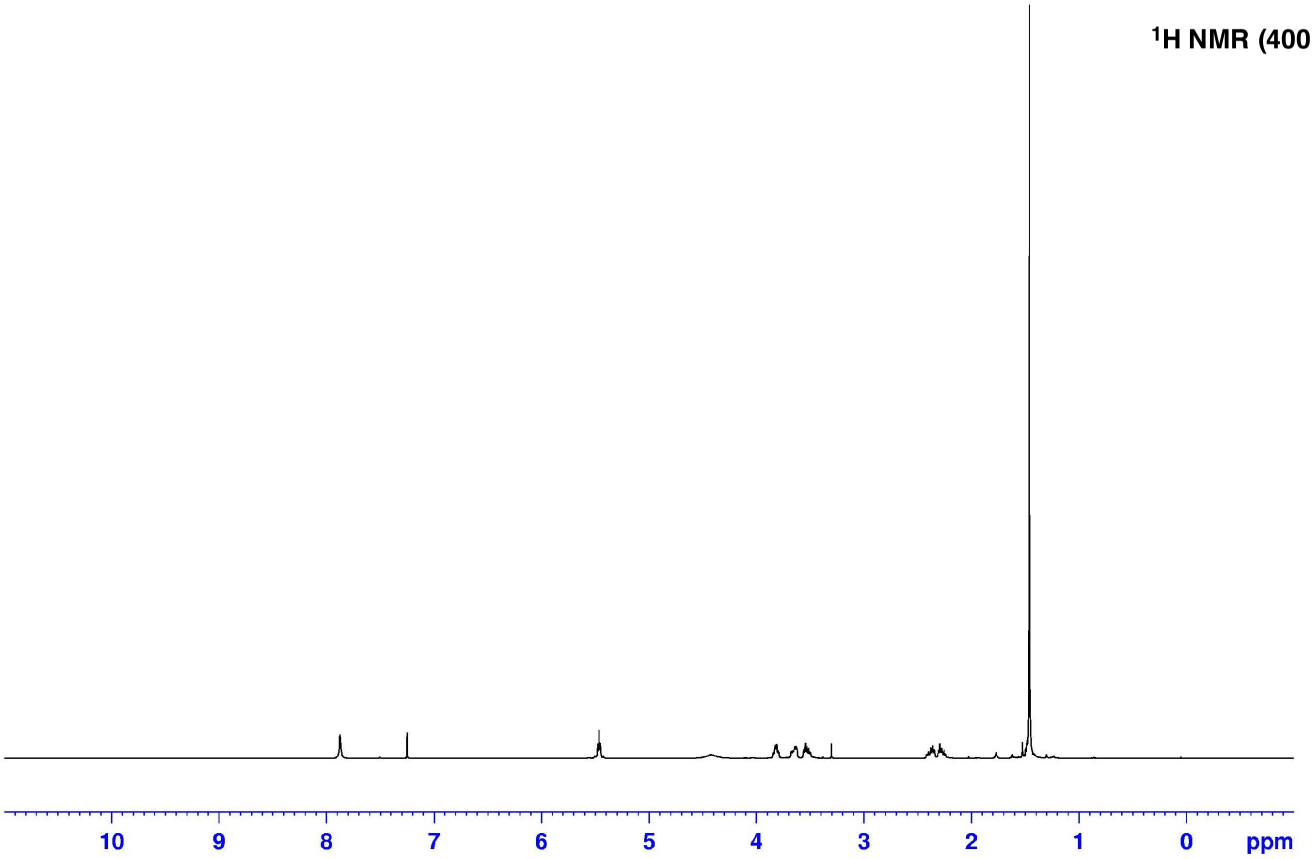


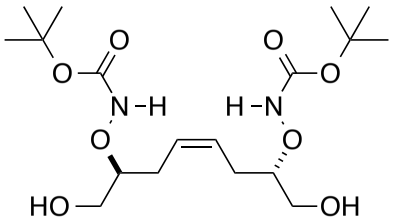


^{13}C NMR (100 MHz, $CDCl_3$)

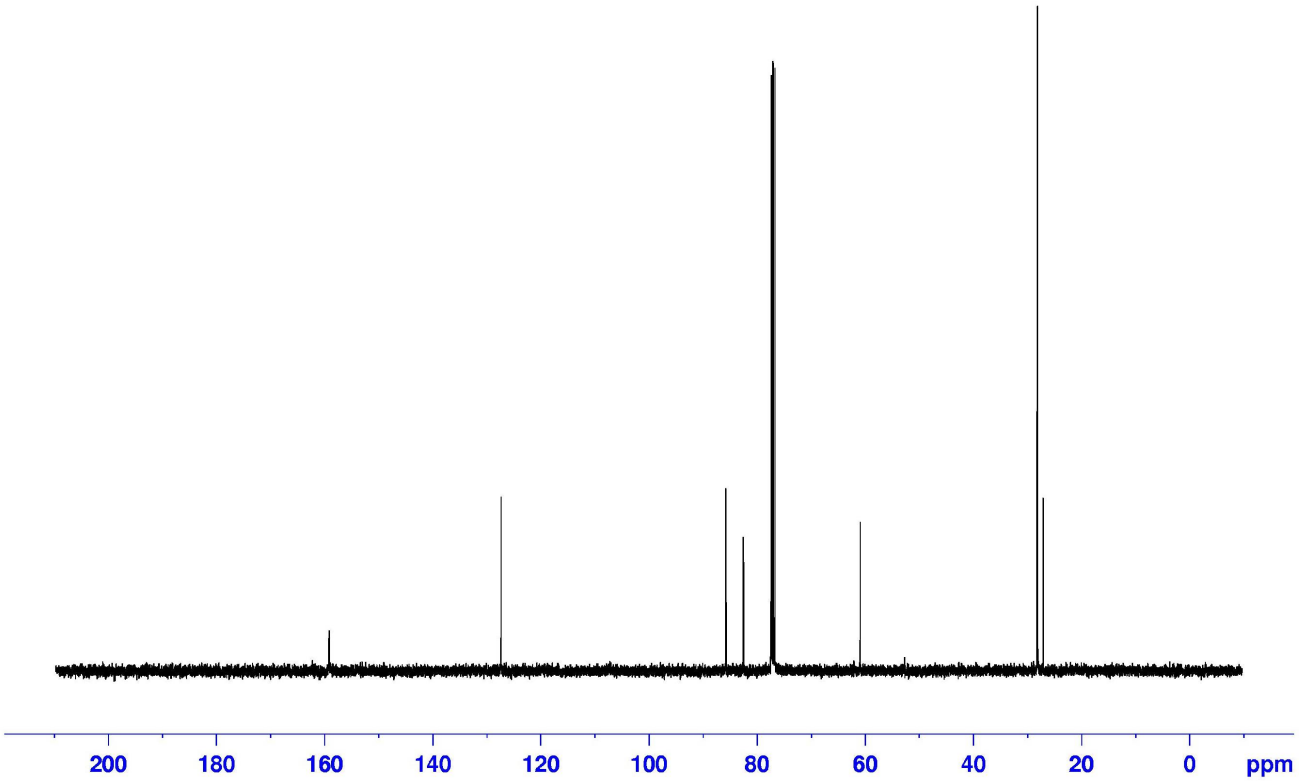


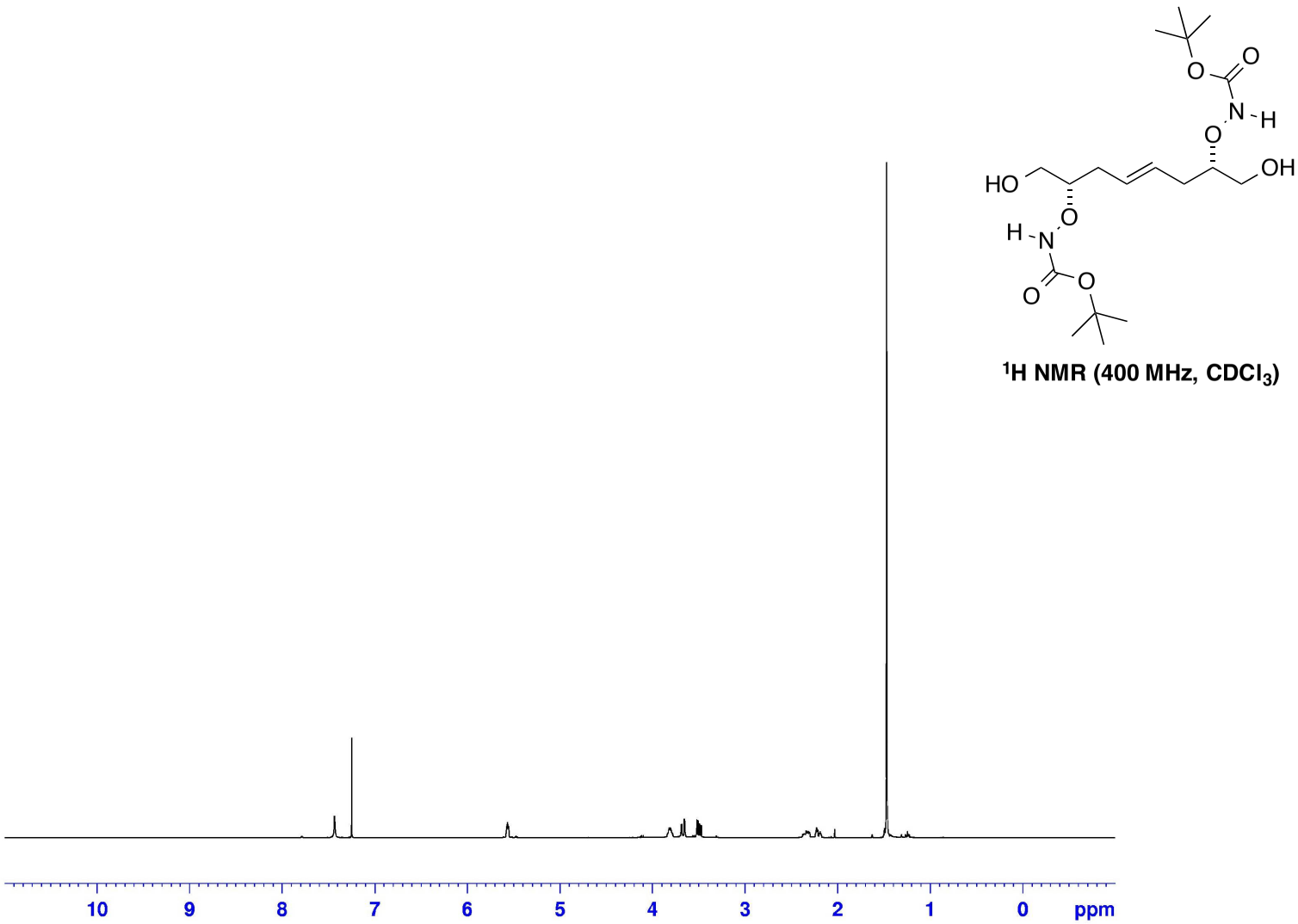
^{1}H NMR (400 MHz, $CDCl_3$)



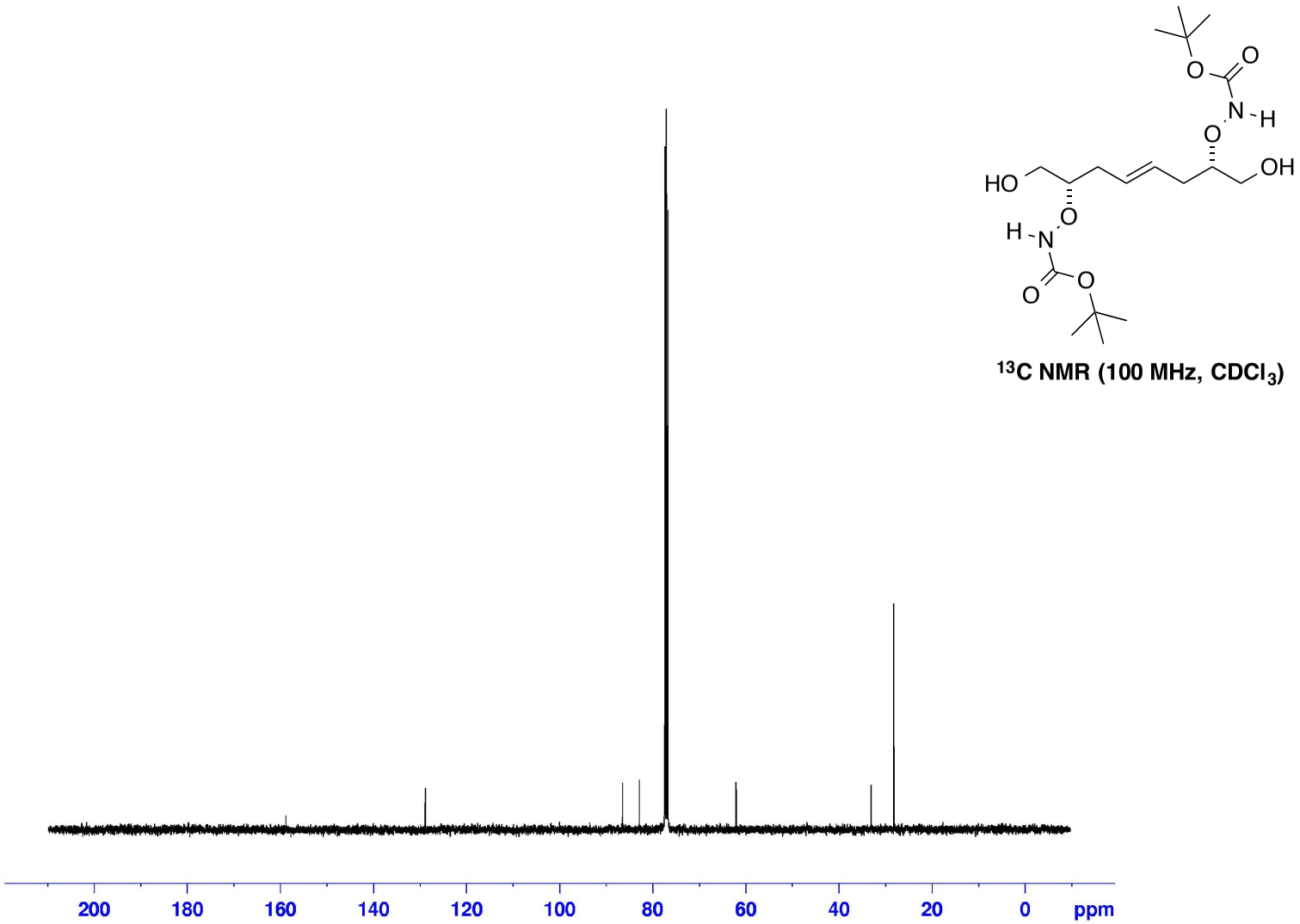


^{13}C NMR (100 MHz, $CDCl_3$)

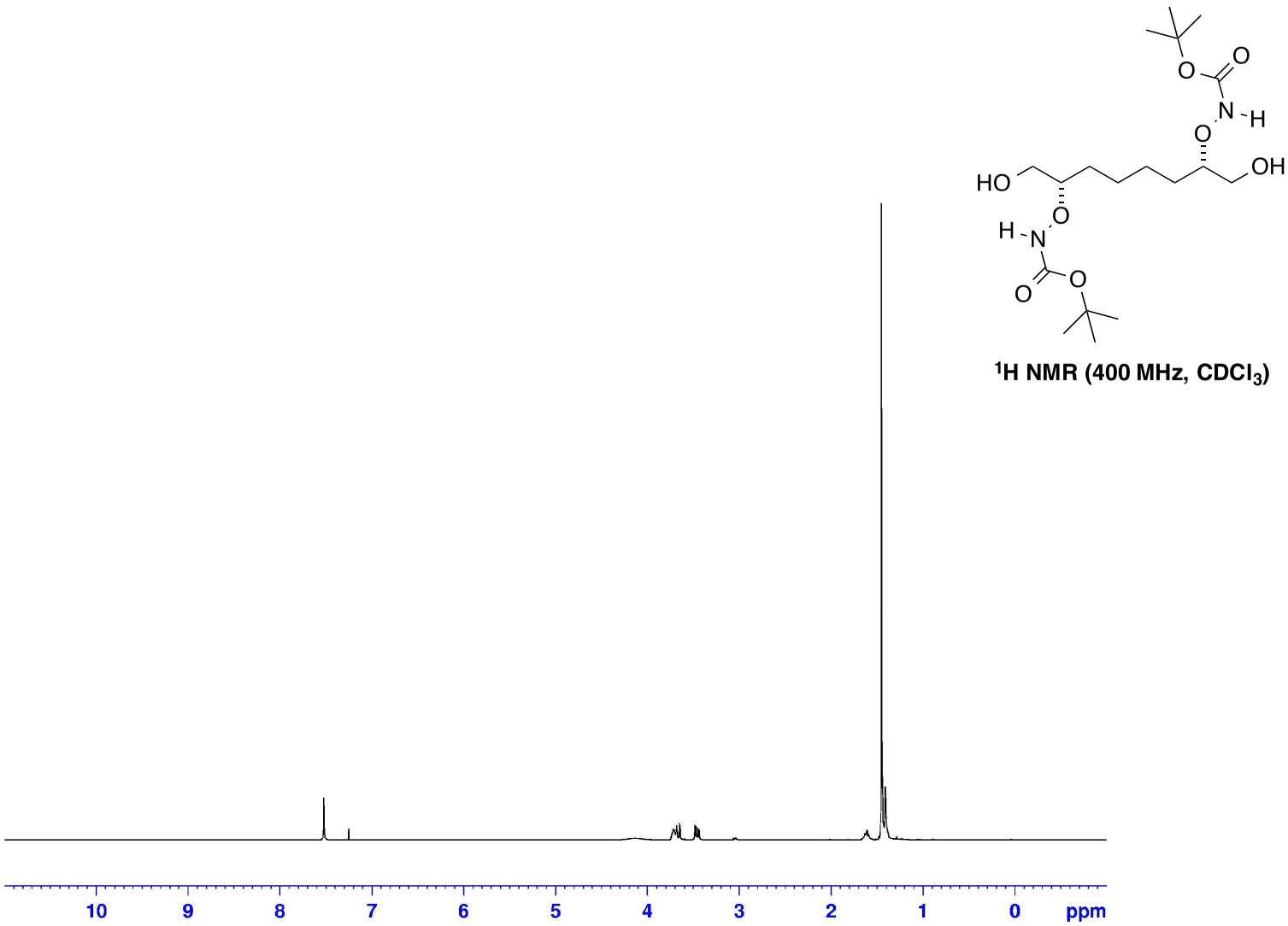




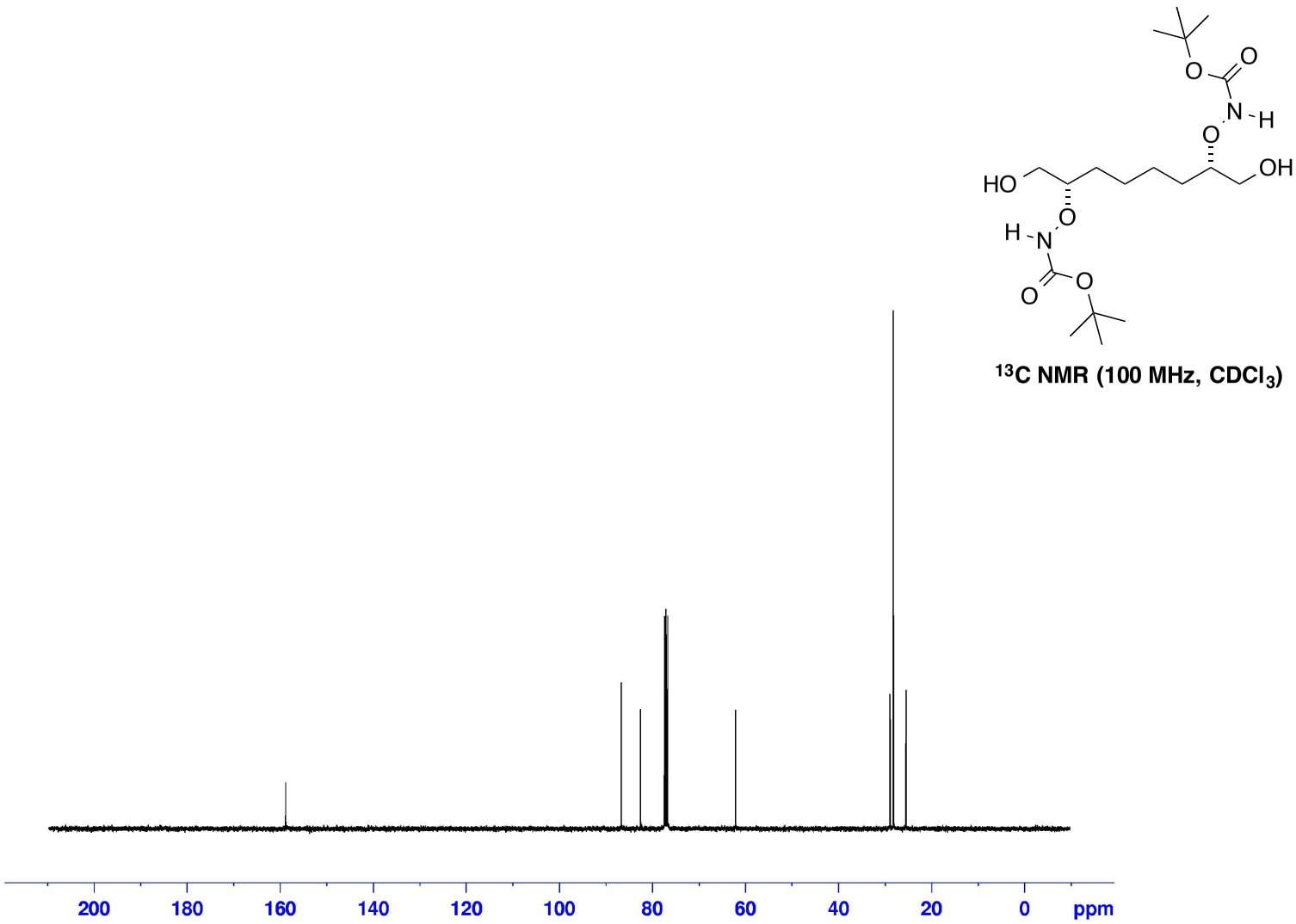
^{1}H NMR (400 MHz, $CDCl_3$)



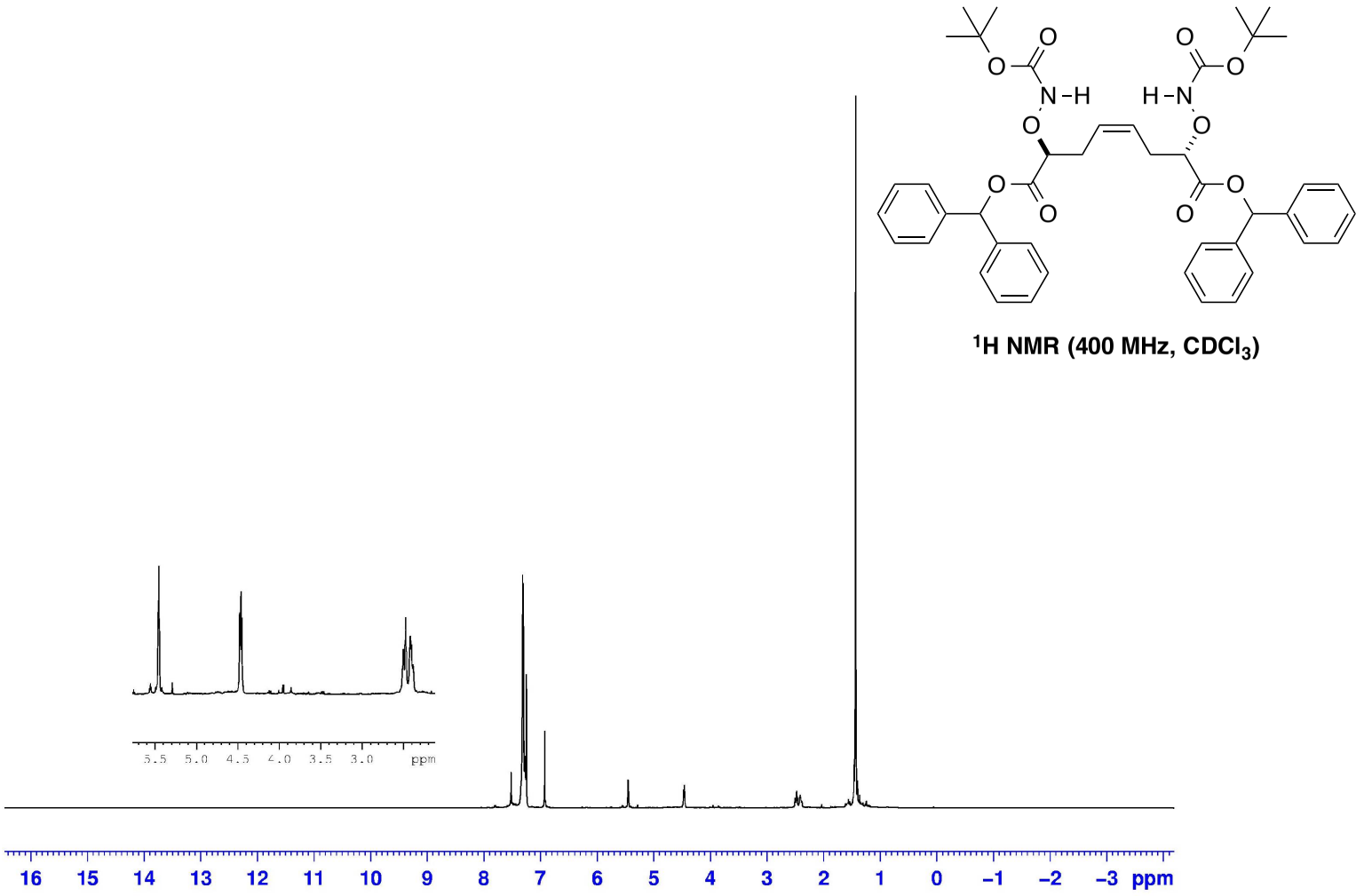
^{13}C NMR (100 MHz, $CDCl_3$)

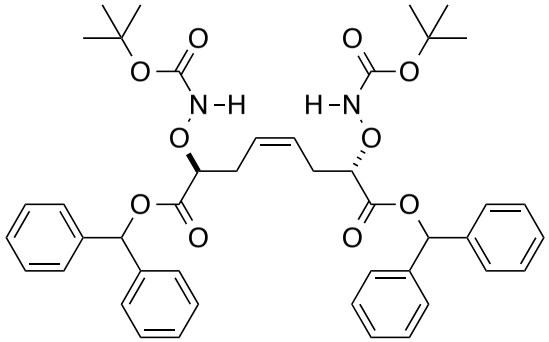


^{1}H NMR (400 MHz, $CDCl_3$)

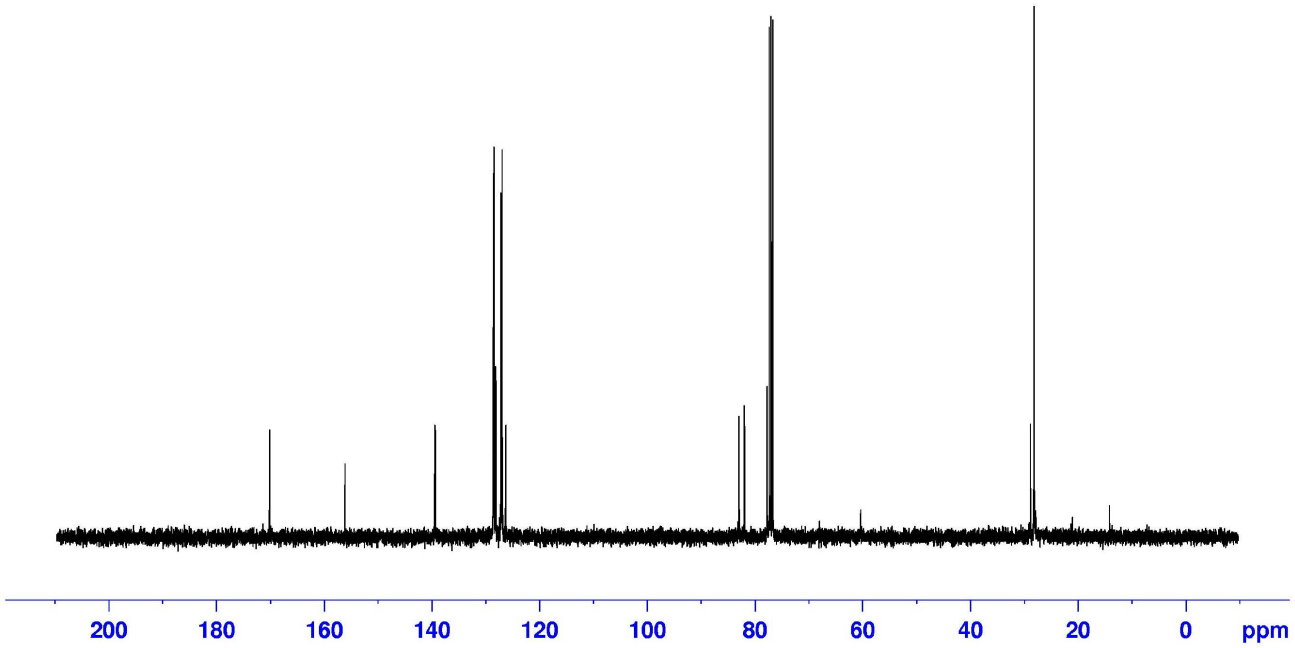


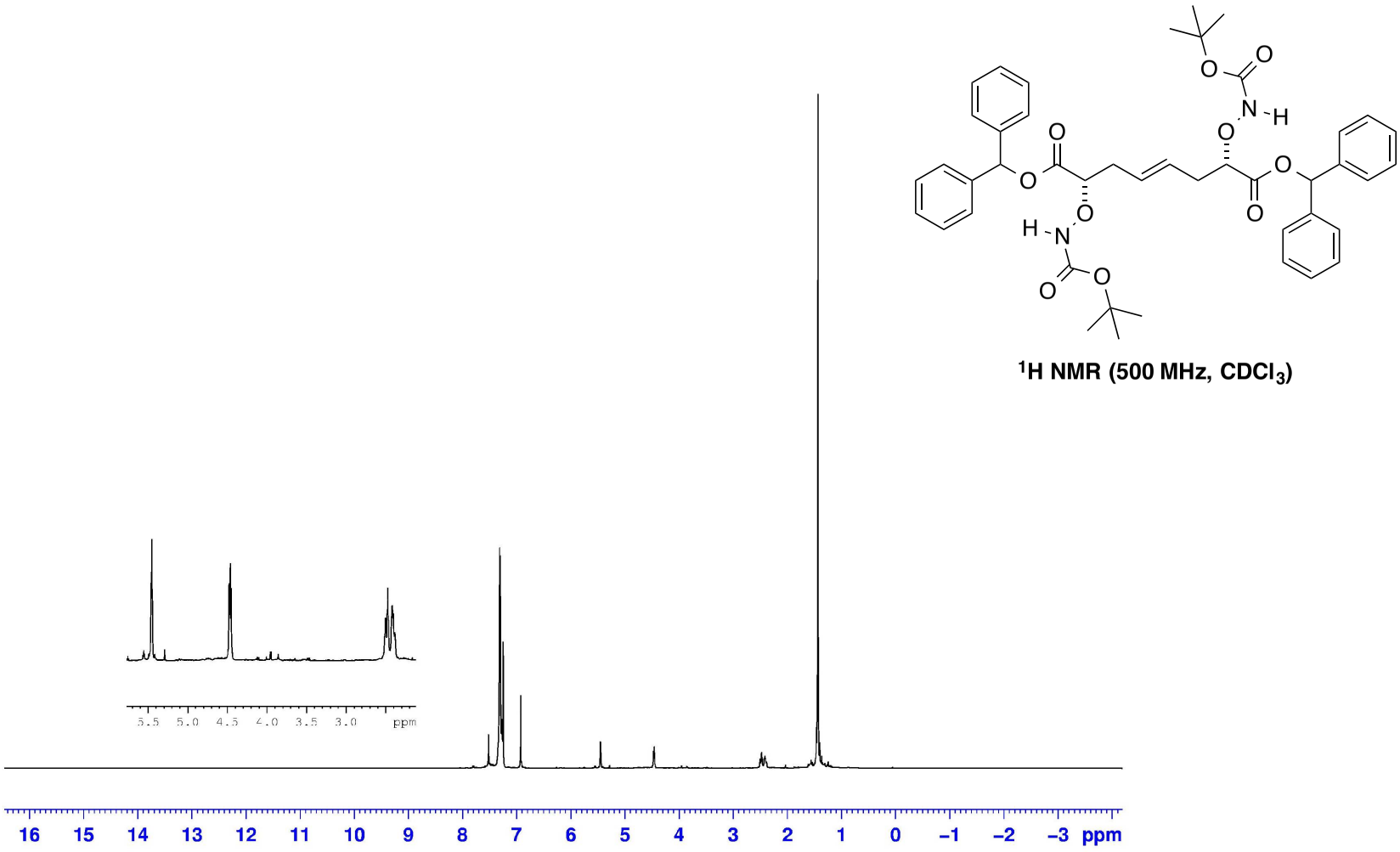
^{13}C NMR (100 MHz, $CDCl_3$)


1H NMR (400 MHz, CDCl3)
16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1 0 −1 −2 −3 ppm
5.5 5.0 4.5 4.0 3.5 3.0 ppm

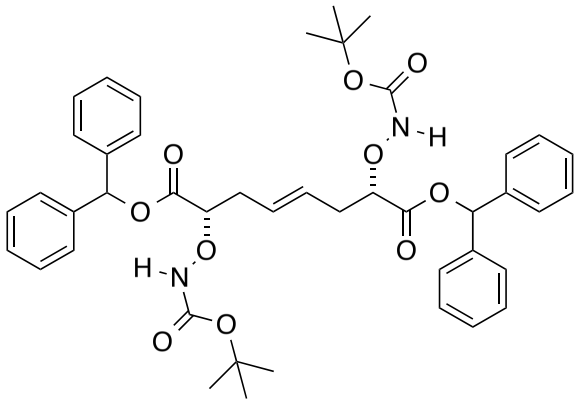


^{13}C NMR (100 MHz, $CDCl_3$)

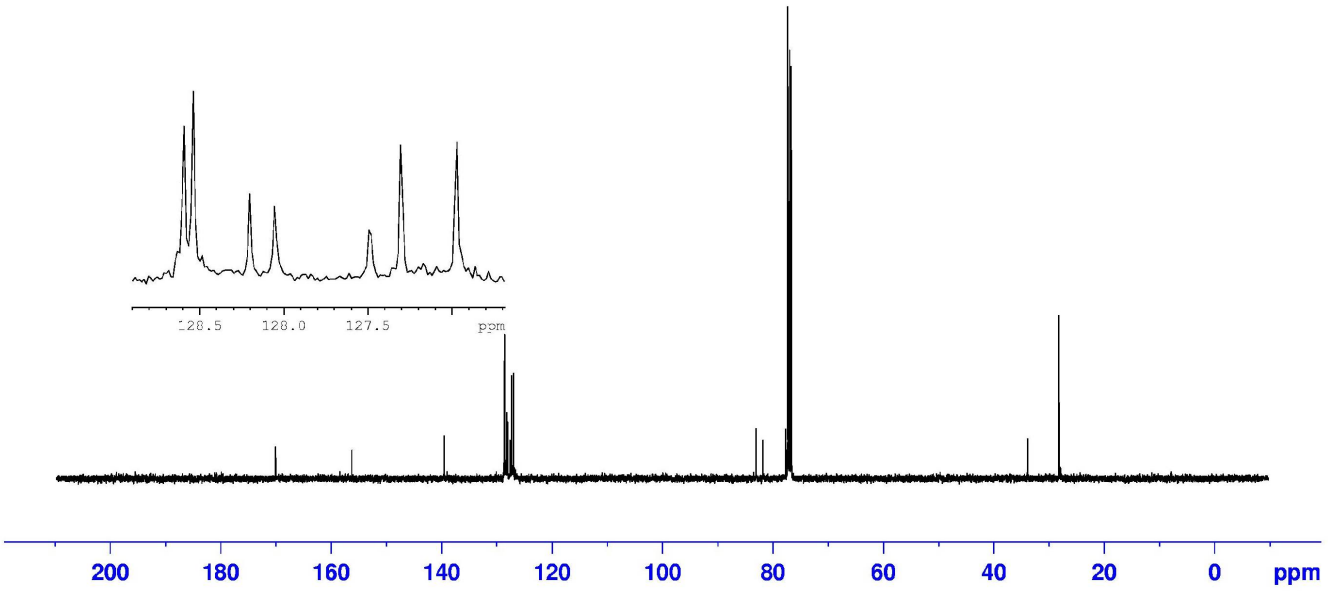


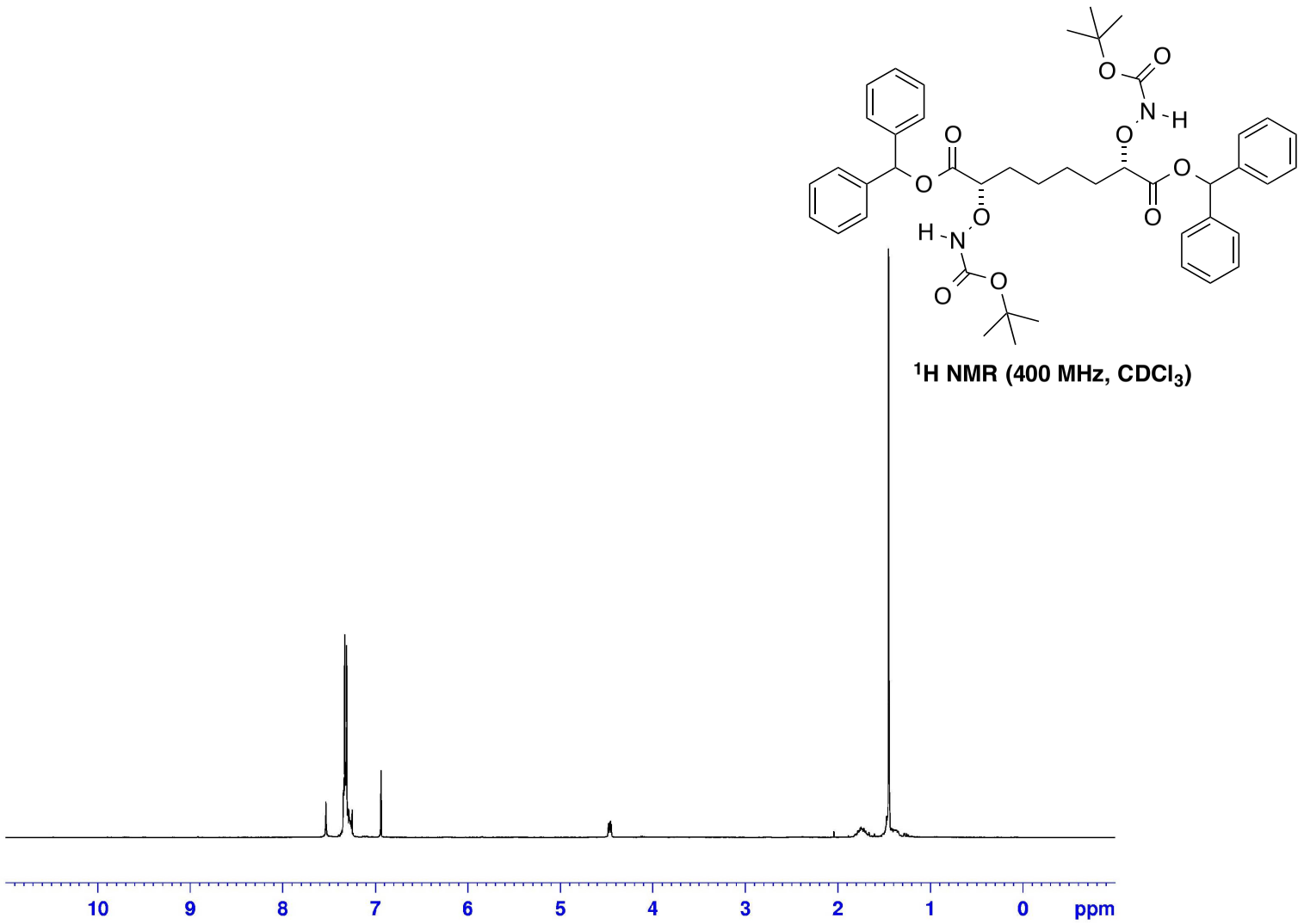


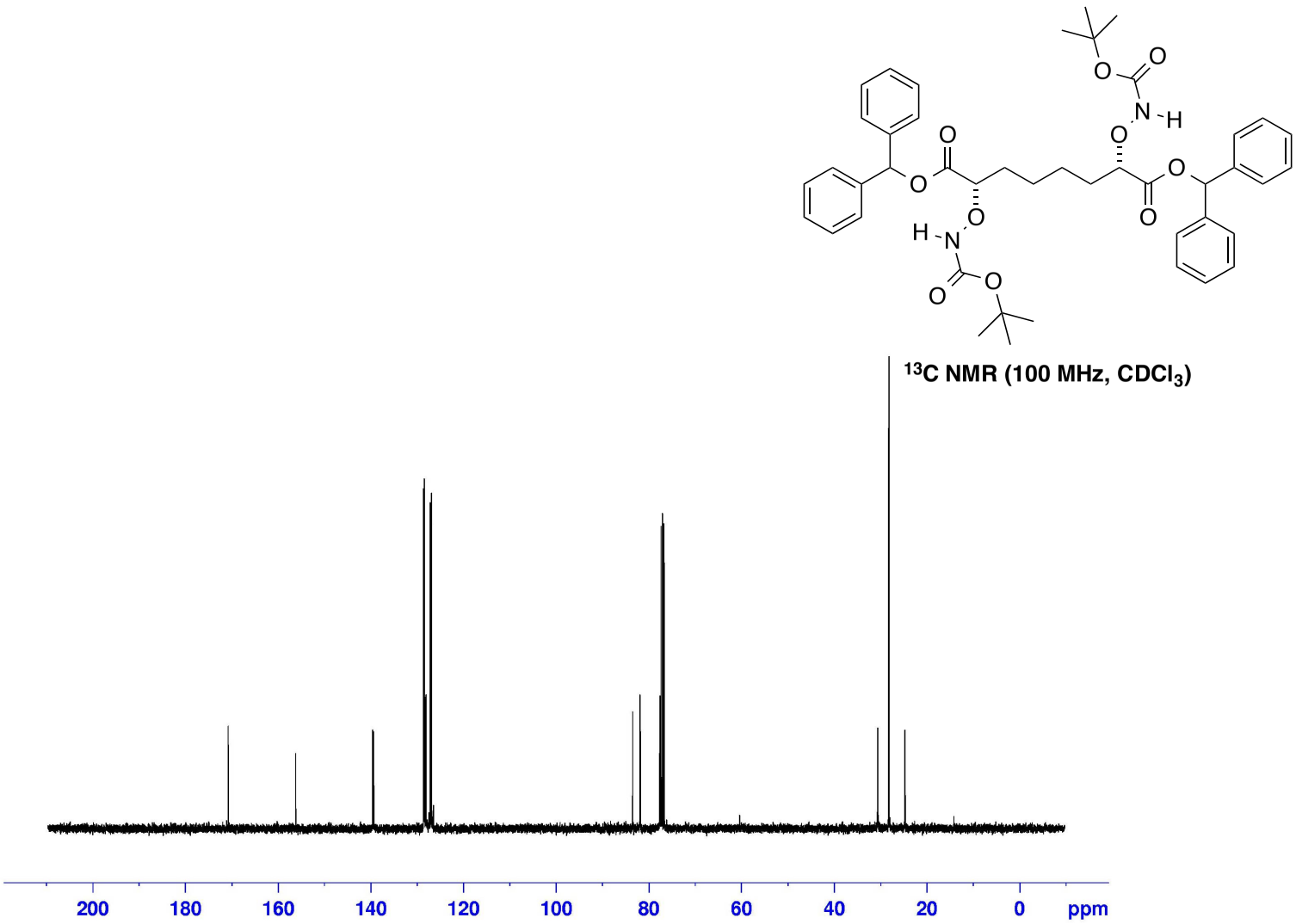
^{1}H NMR (500 MHz, $CDCl_3$)

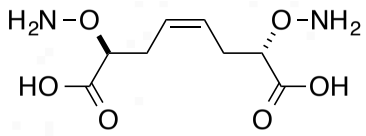


^{13}C NMR (100 MHz, $CDCl_3$)

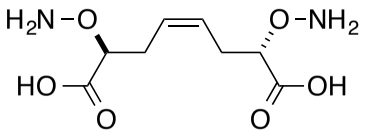



^{1}H NMR (400 MHz, CDCl$_3$)
10
9
8
7
6
5
4
3
2
1
0
ppm

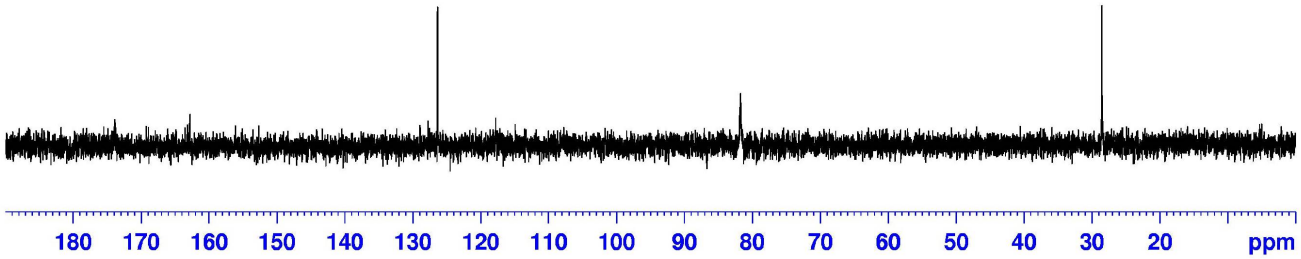

^{13}C NMR (100 MHz, $CDCl_3$)
200
180
160
140
120
100
80
60
40
20
0
ppm

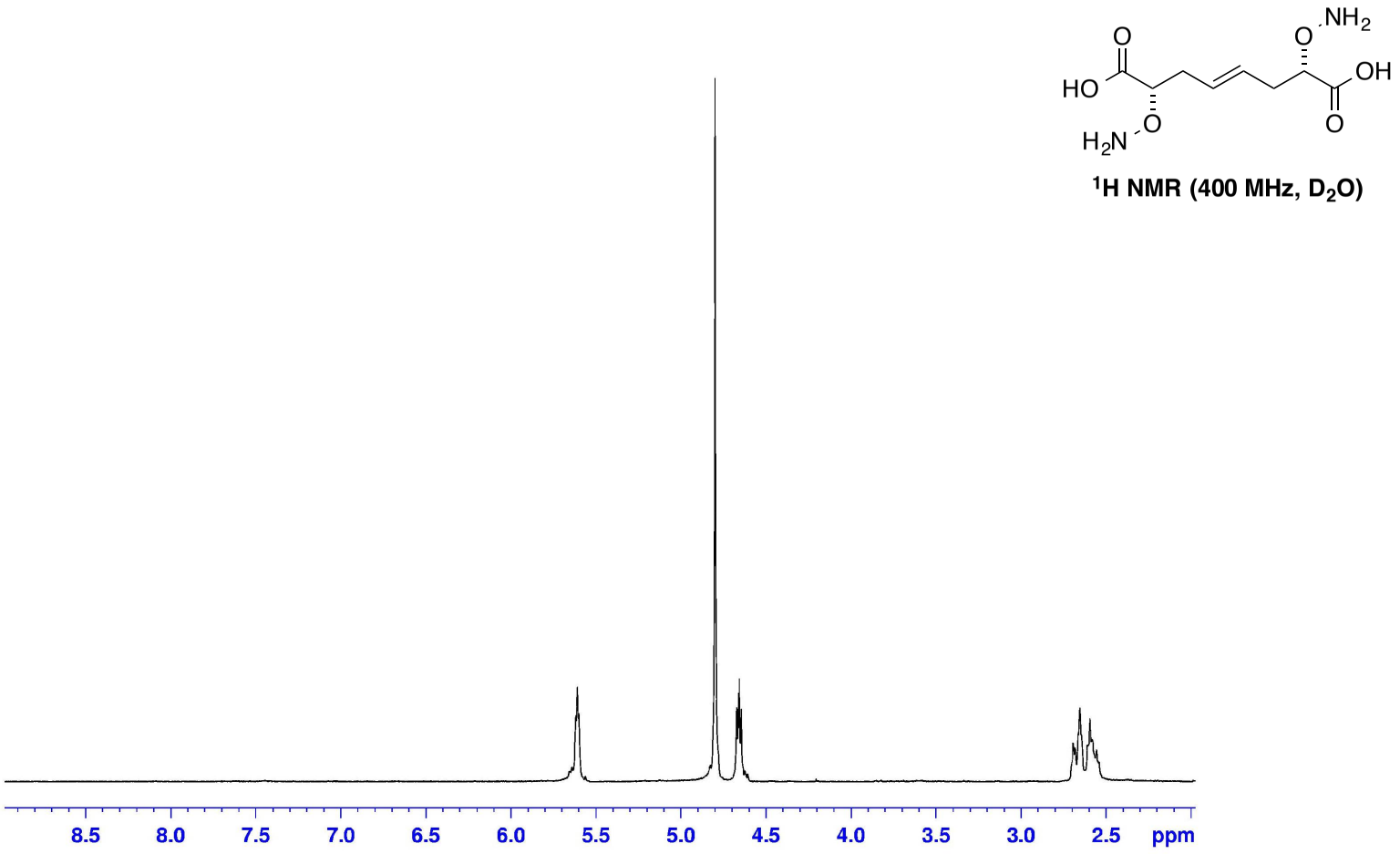


^{1}H NMR (500 MHz, D_2O)

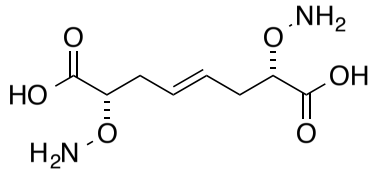


^{13}C NMR (100 MHz, D_2O)

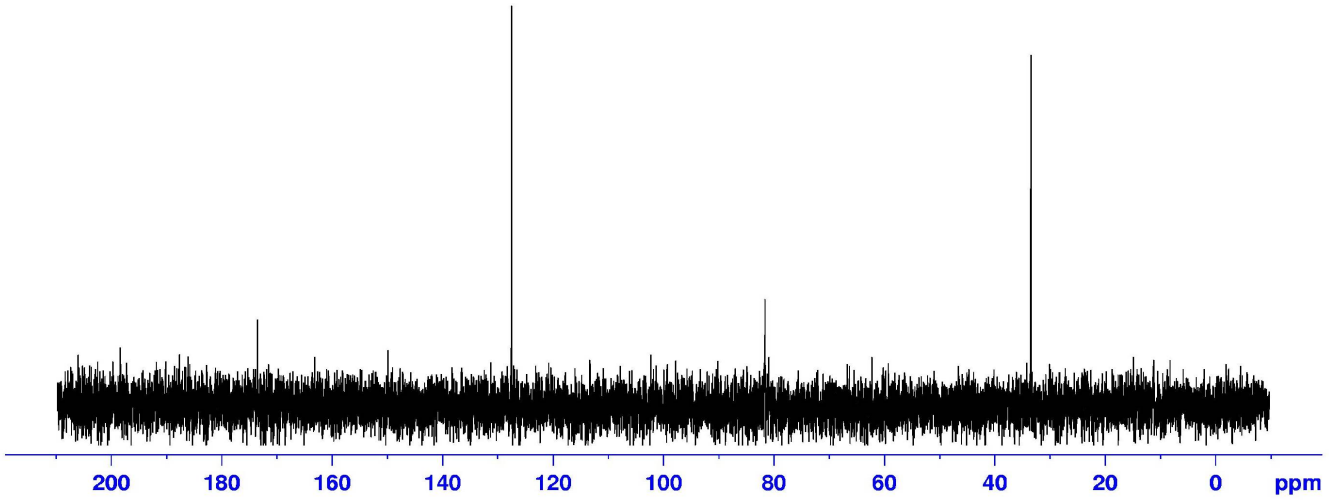


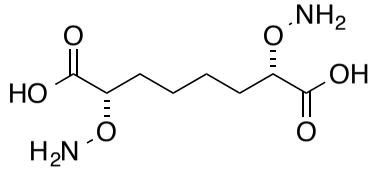


^{1}H NMR (400 MHz, D_2O)

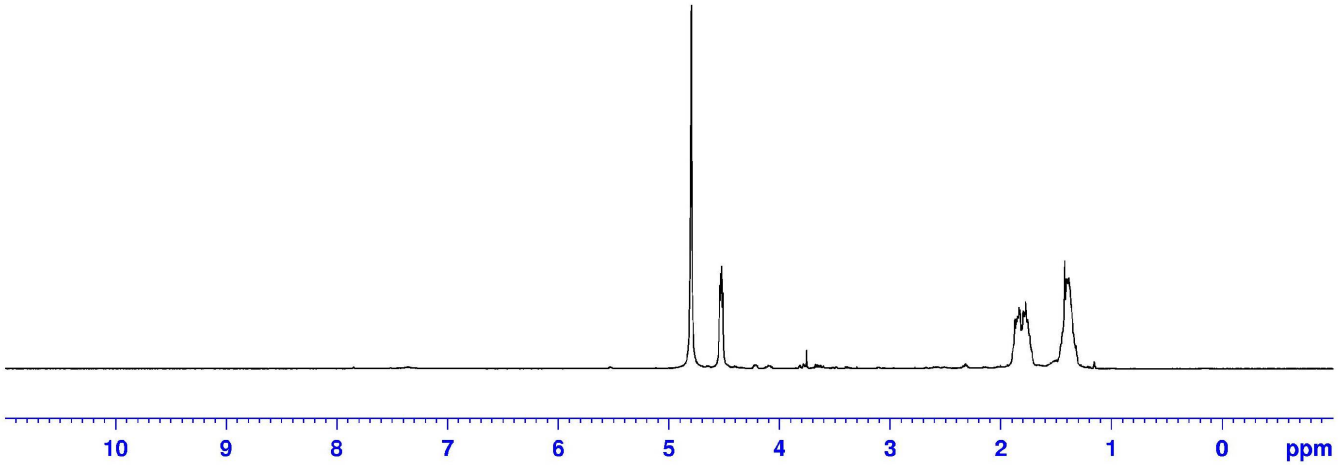


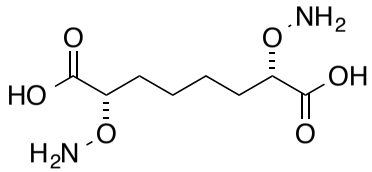
^{13}C NMR (100 MHz, D_2O)



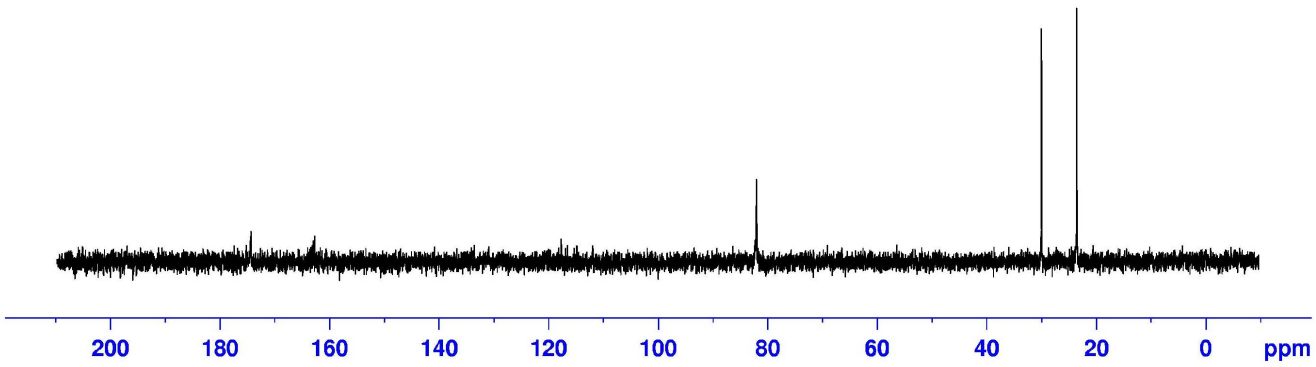


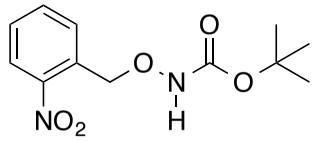
^{1}H NMR (400 MHz, D_2O)



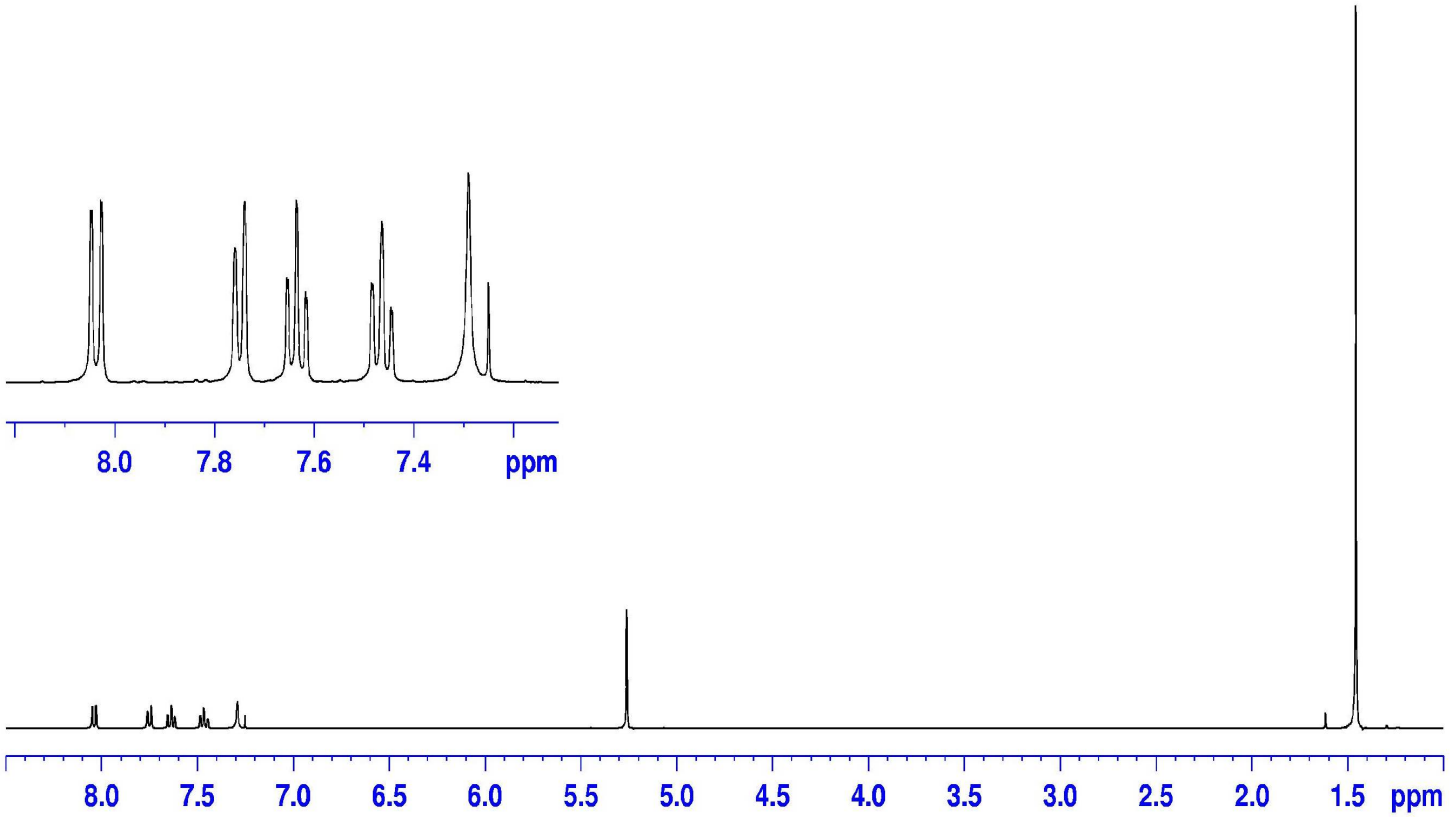


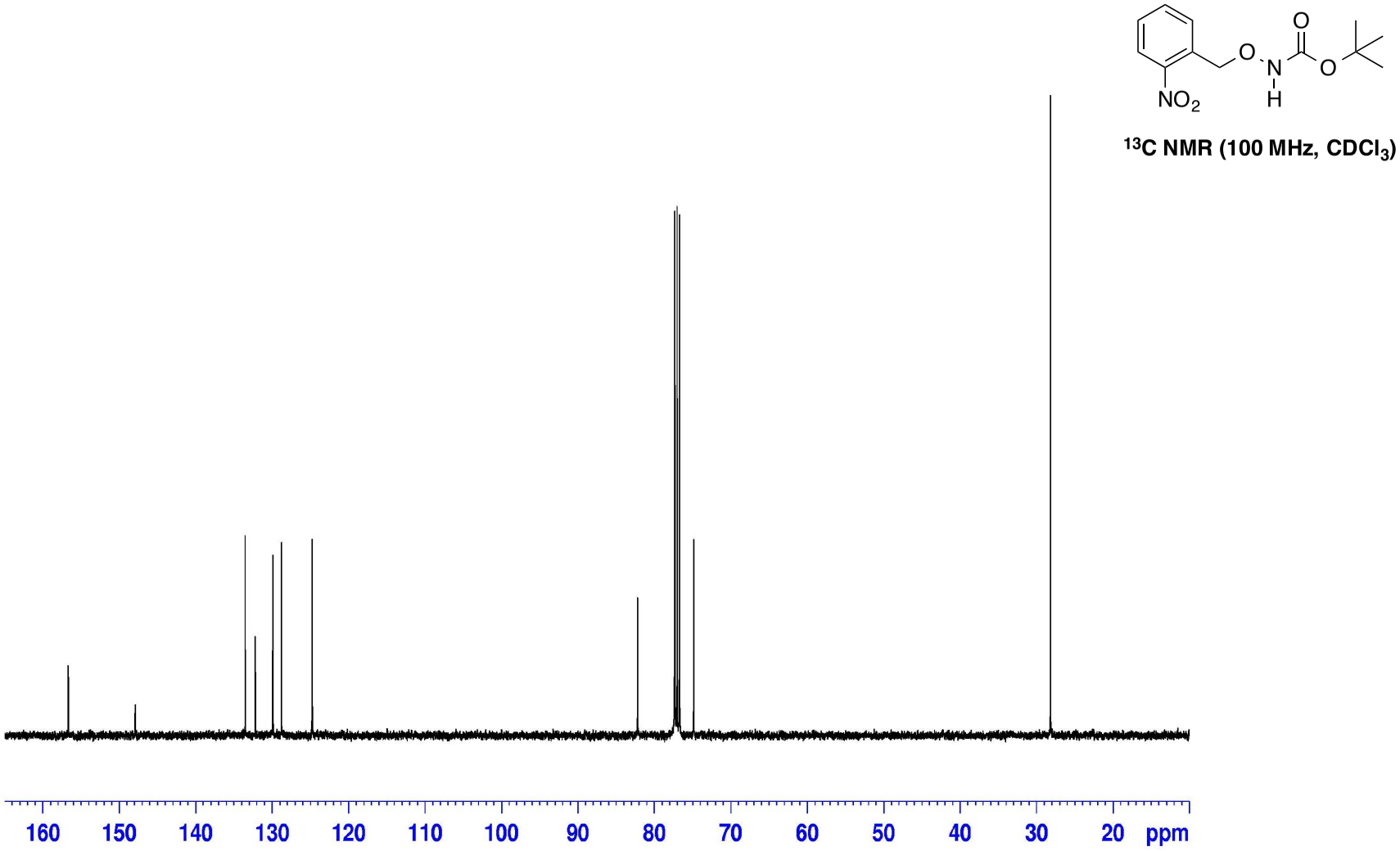
^{13}C NMR (100 MHz, D_2O)

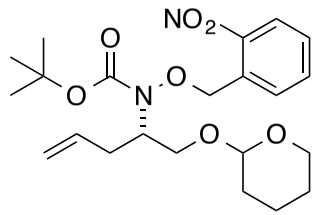




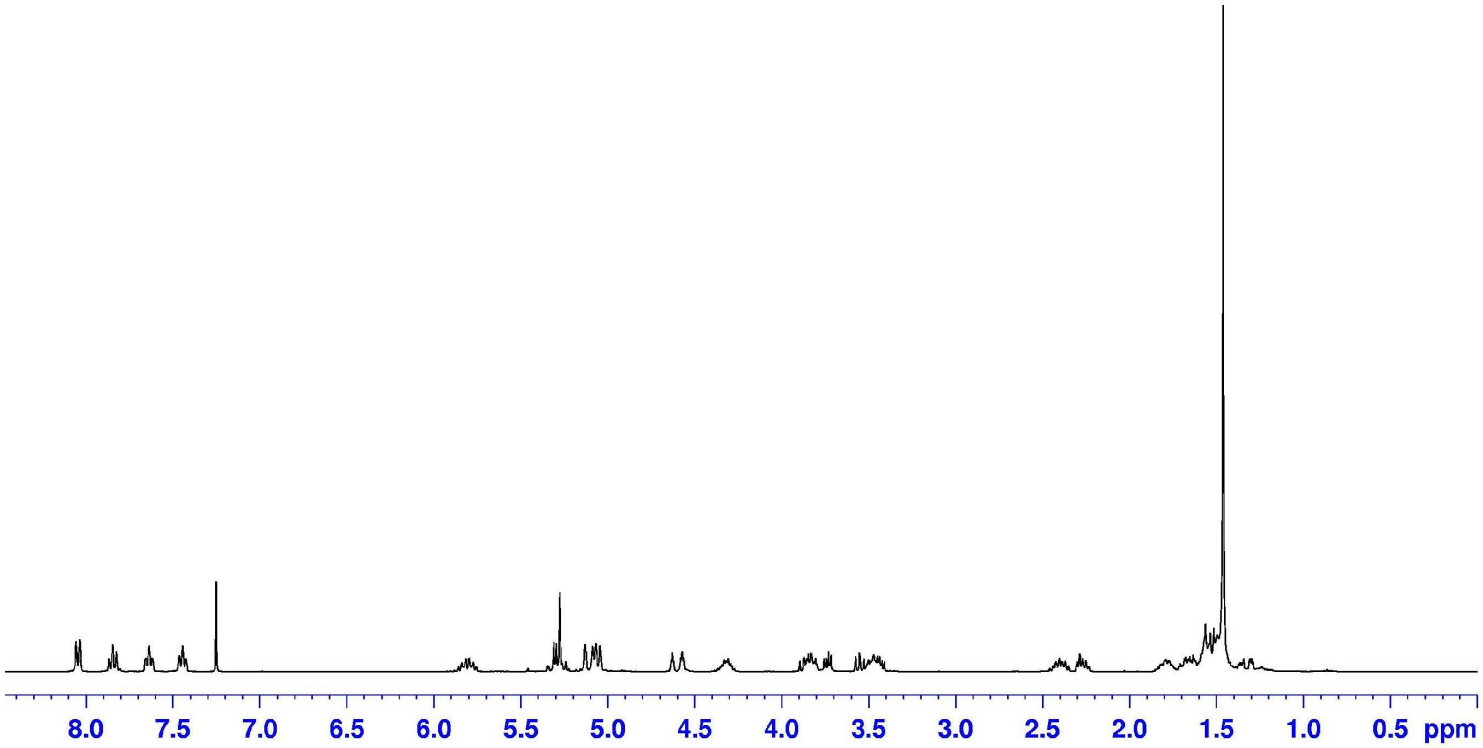
^{1}H NMR (400 MHz, $CDCl_3$)

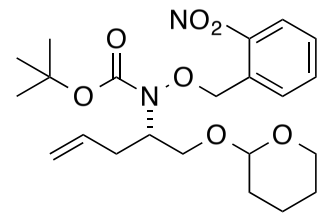



O
O
N
H
O
O
NO2
13C NMR (100 MHz, CDCl3)
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
ppm

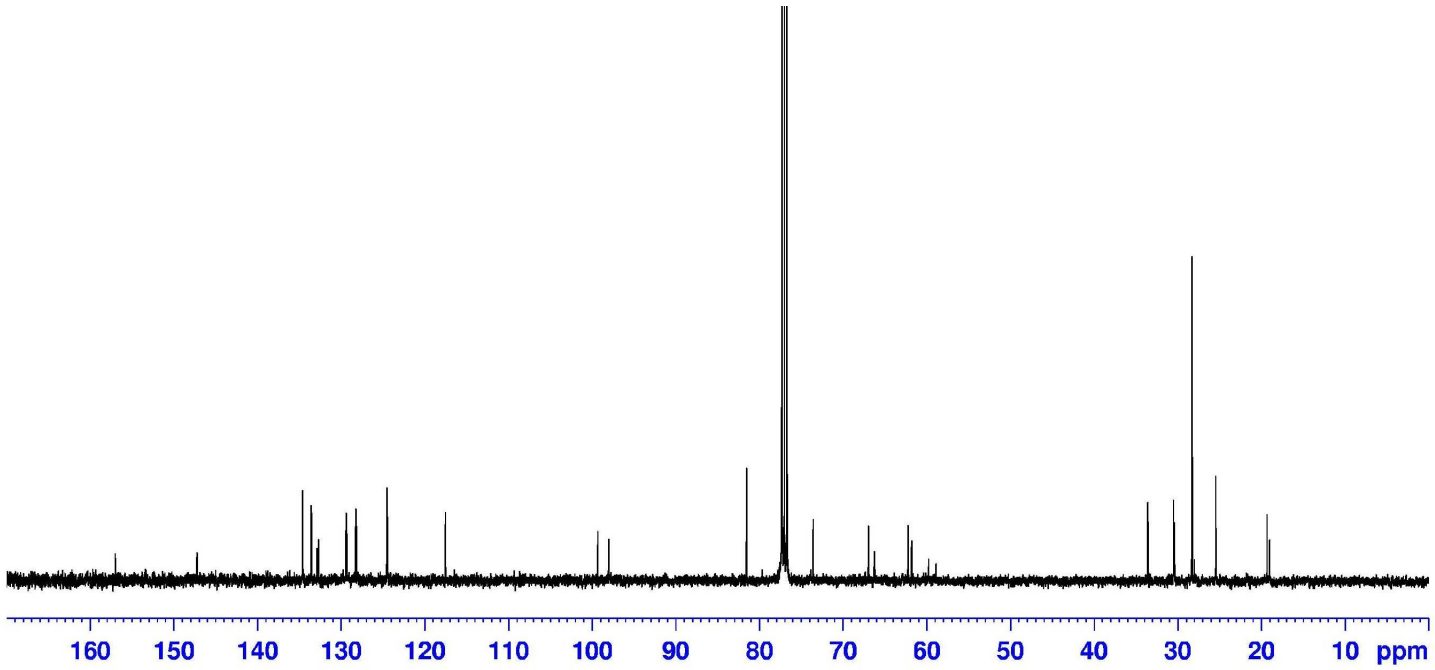


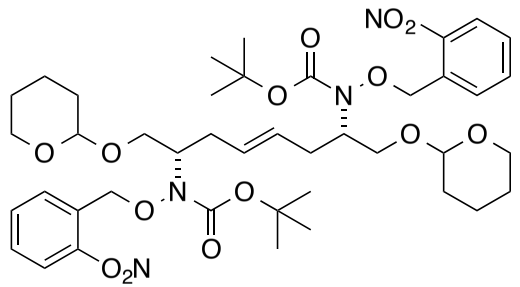
^{1}H NMR (400 MHz, $CDCl_3$)





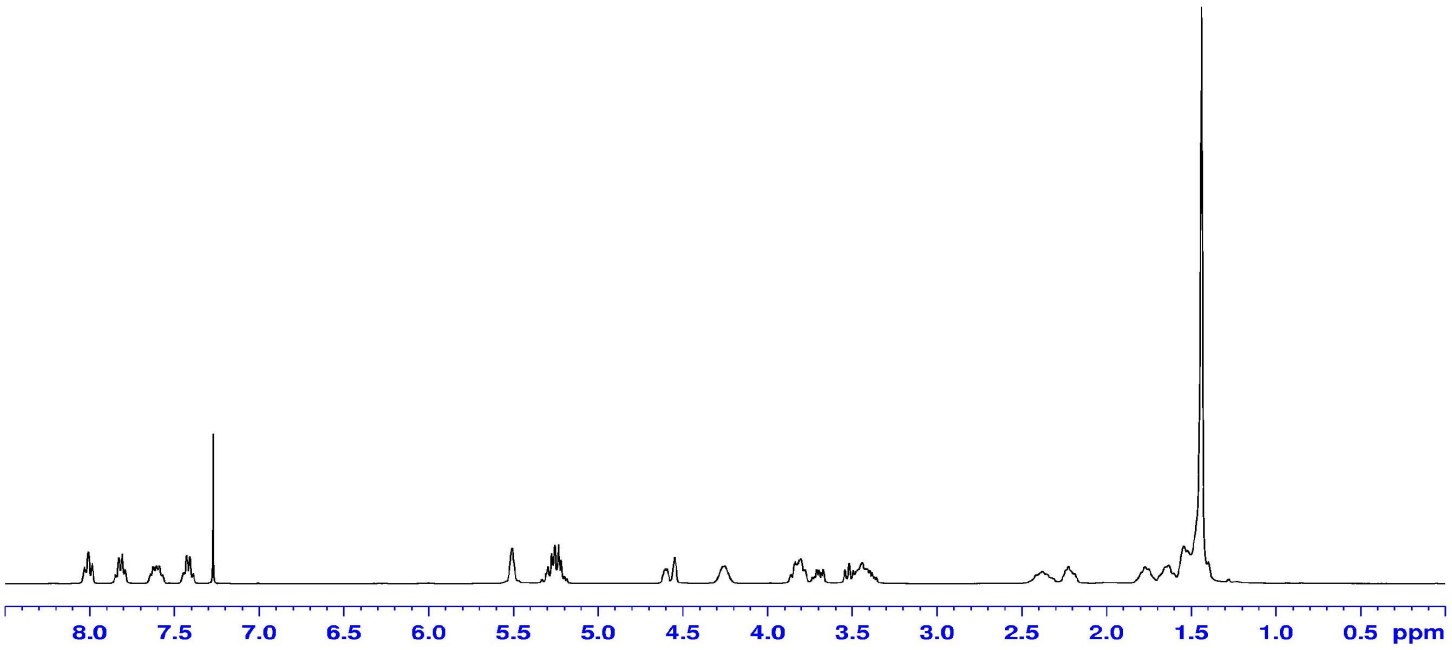
^{13}C NMR (100 MHz, $CDCl_3$)

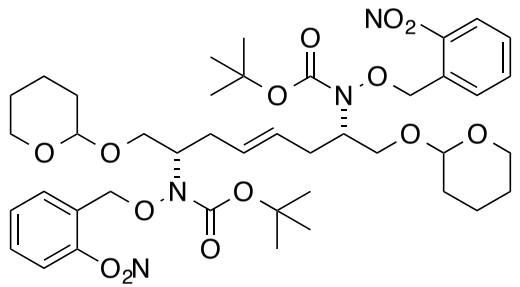




^{1}H NMR (400 MHz, $CDCl_3$)

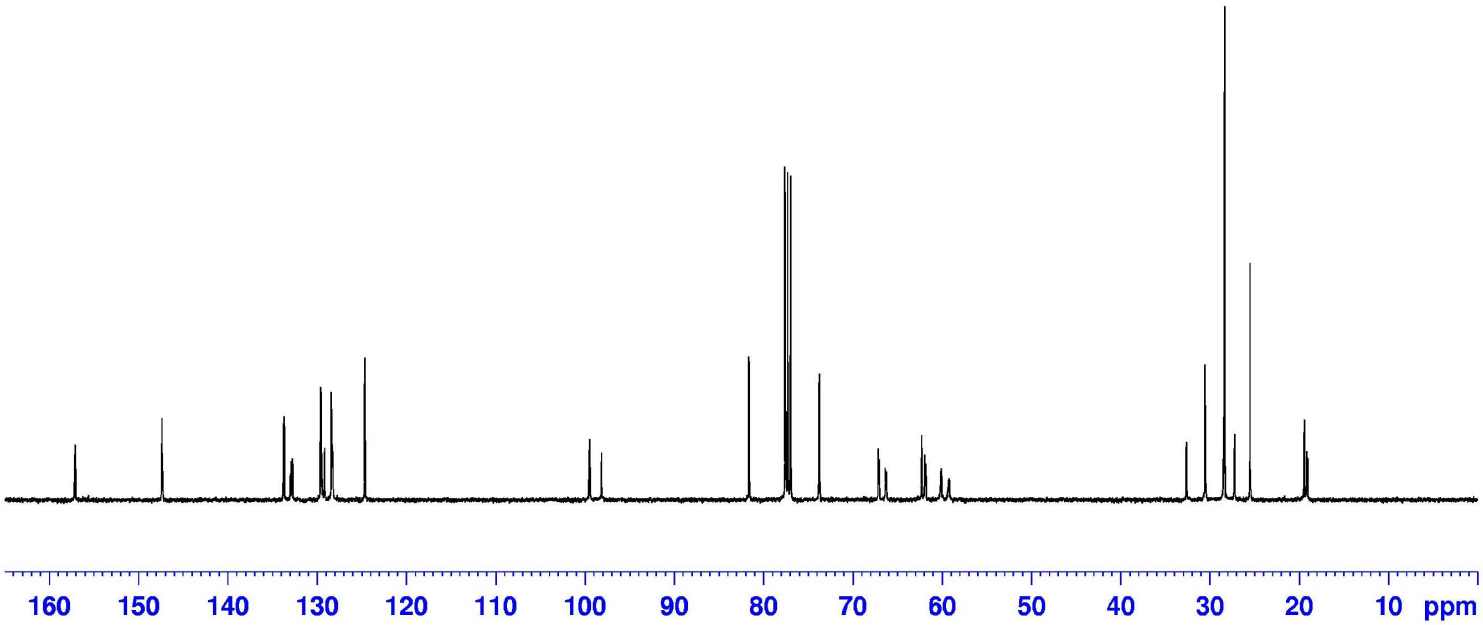
(Mixture of Geometric Isomers E:Z ~ 1.2:1)

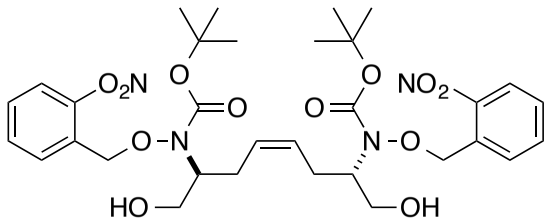




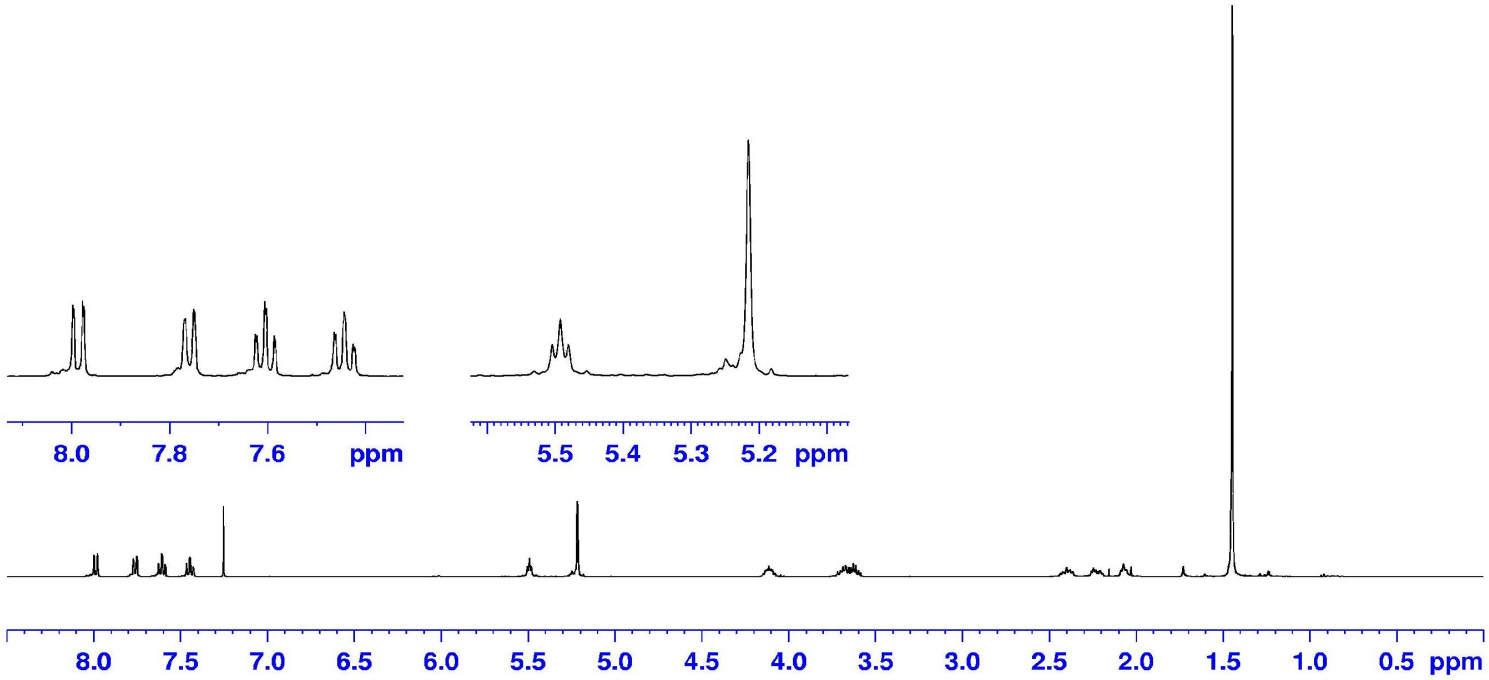
^{13}C NMR (100 MHz, $CDCl_3$)

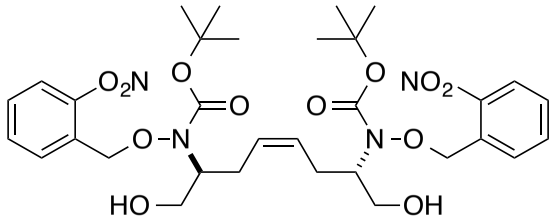
Mixture of Geometric Isomers E:Z ~ 1.2:1



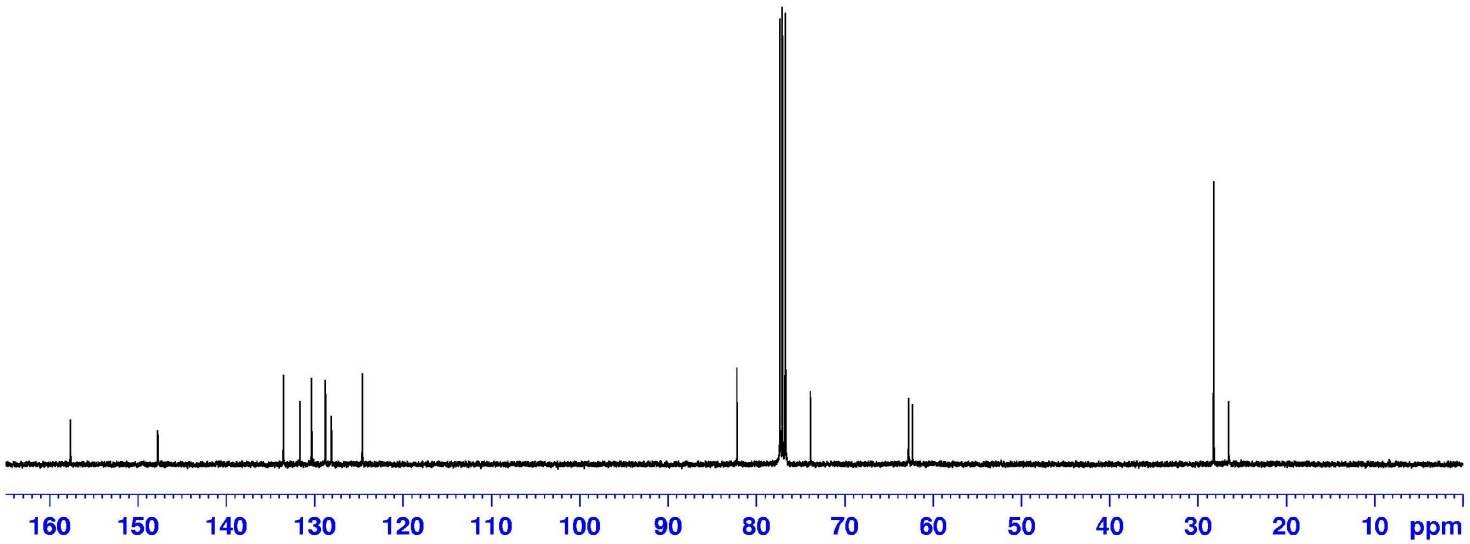


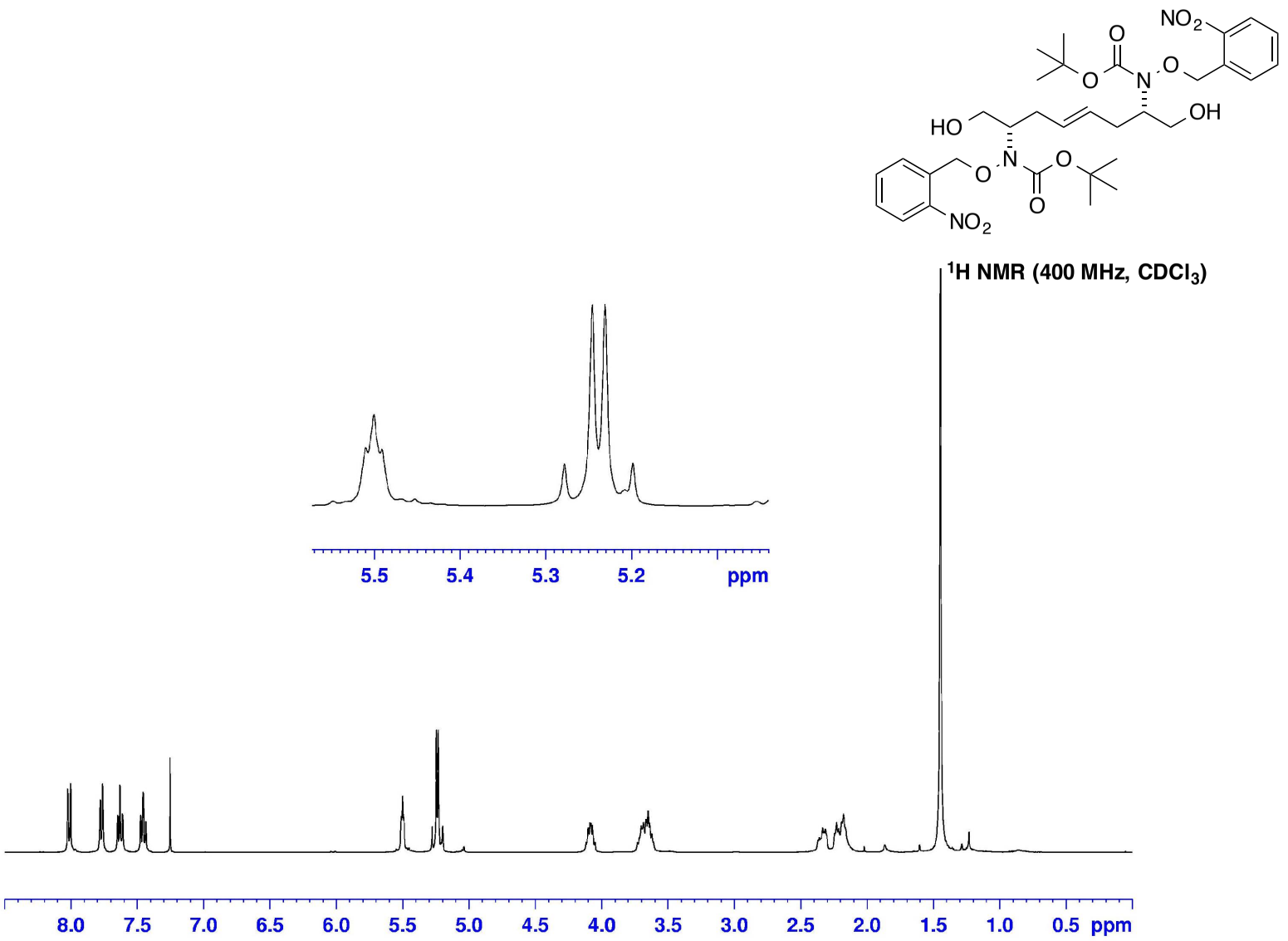
^{1}H NMR (400 MHz, $CDCl_3$)

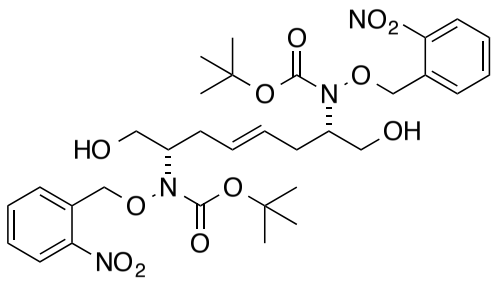




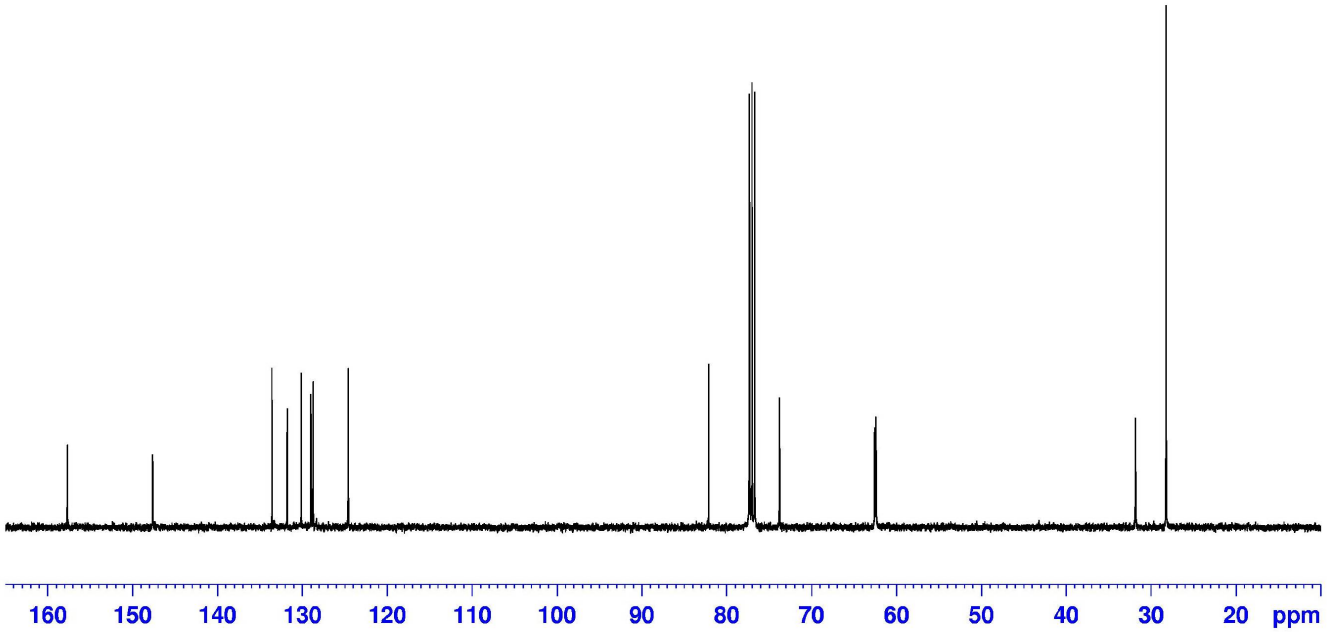
^{13}C NMR (100 MHz, $CDCl_3$)

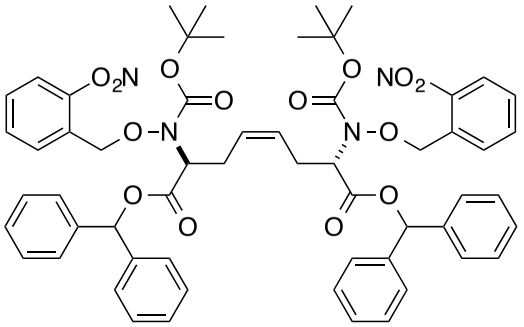



NO2
O
O
N
O
OH
HO
N
O
O
O
NO2
1H NMR (400 MHz, CDCl3)
5.5
5.4
5.3
5.2
ppm
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
ppm

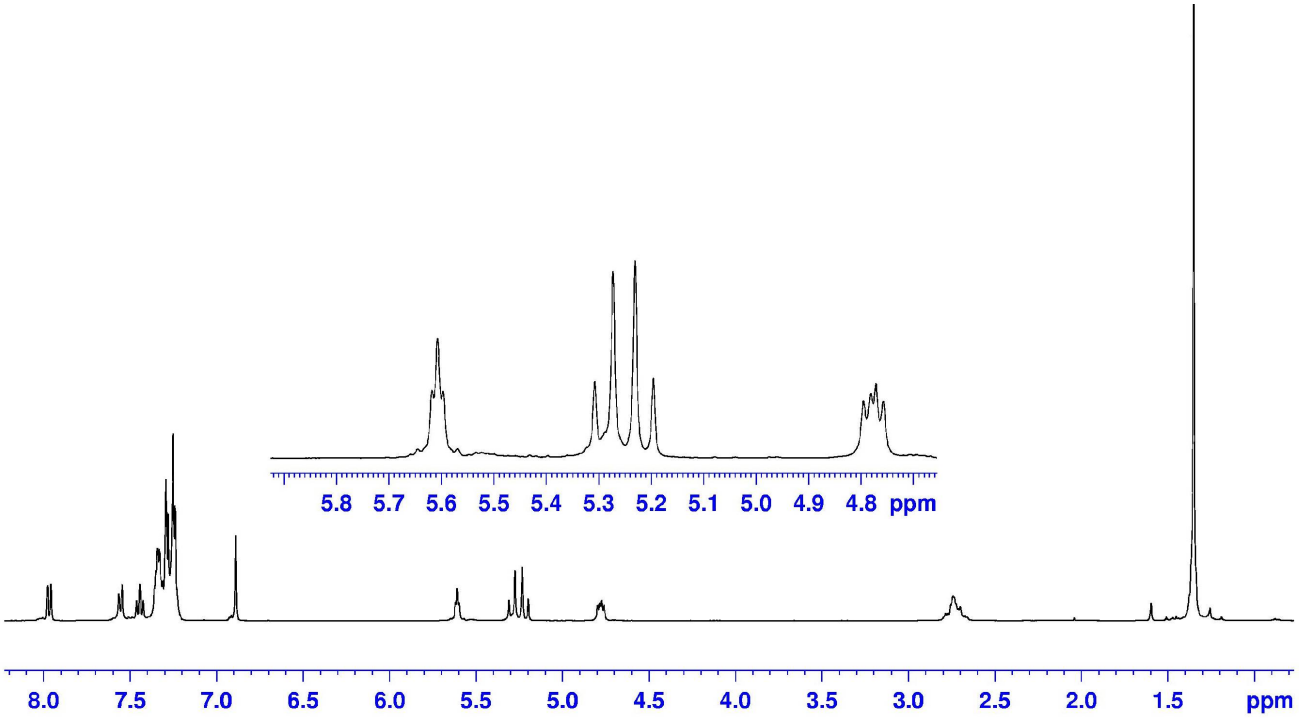


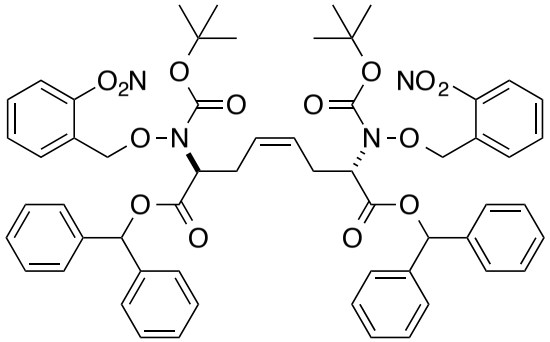
^{13}C NMR (100 MHz, $CDCl_3$)



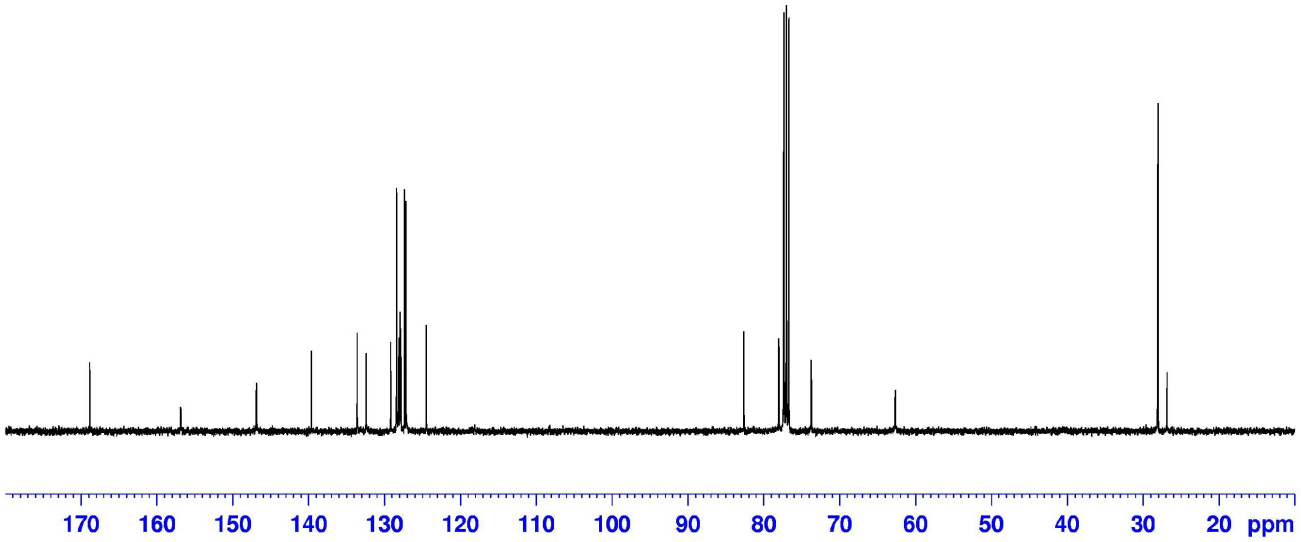


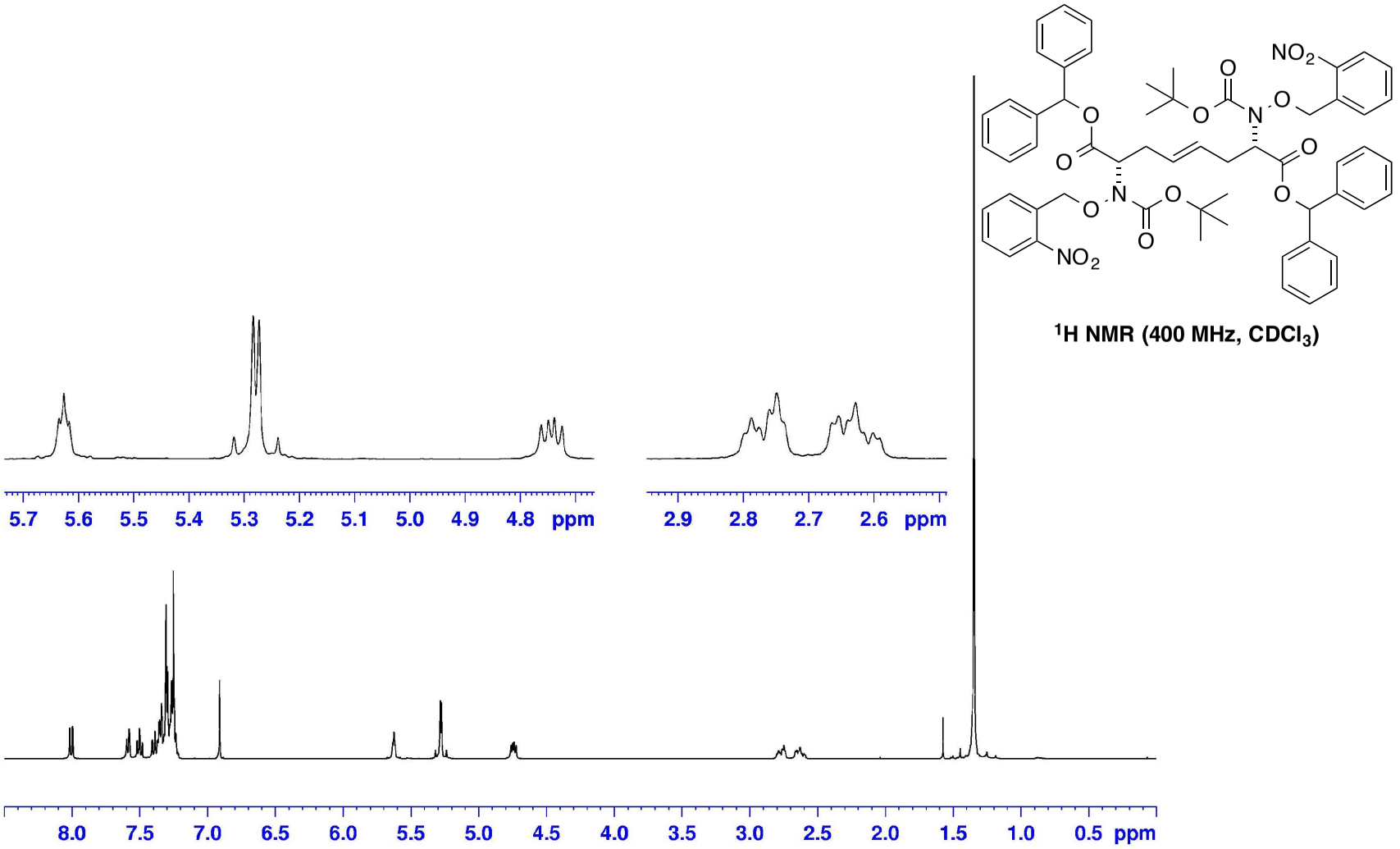
^{1}H NMR (400 MHz, $CDCl_3$)

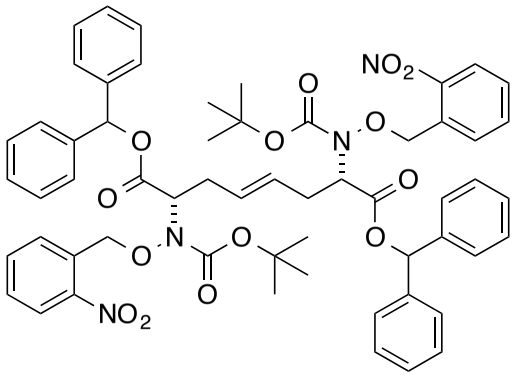




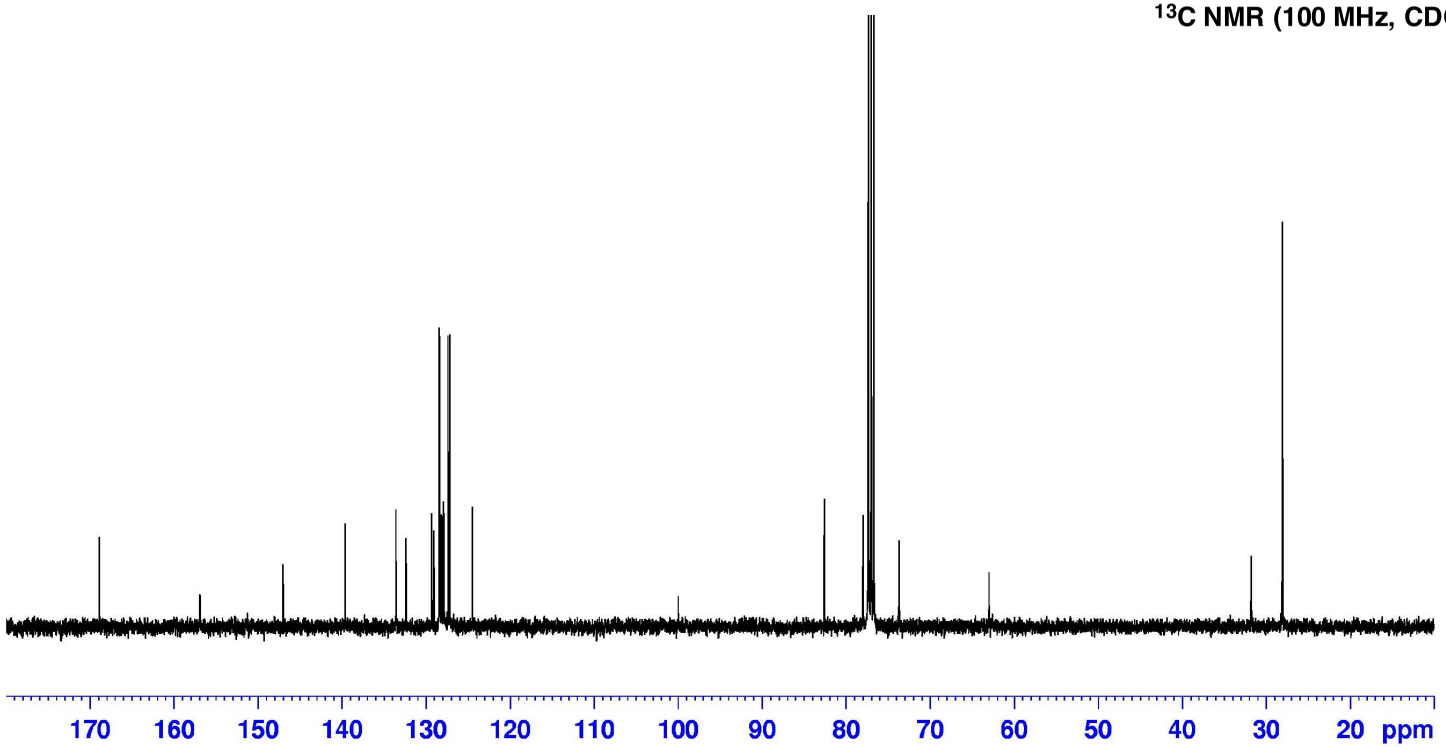
^{13}C NMR (100 MHz, $CDCl_3$)

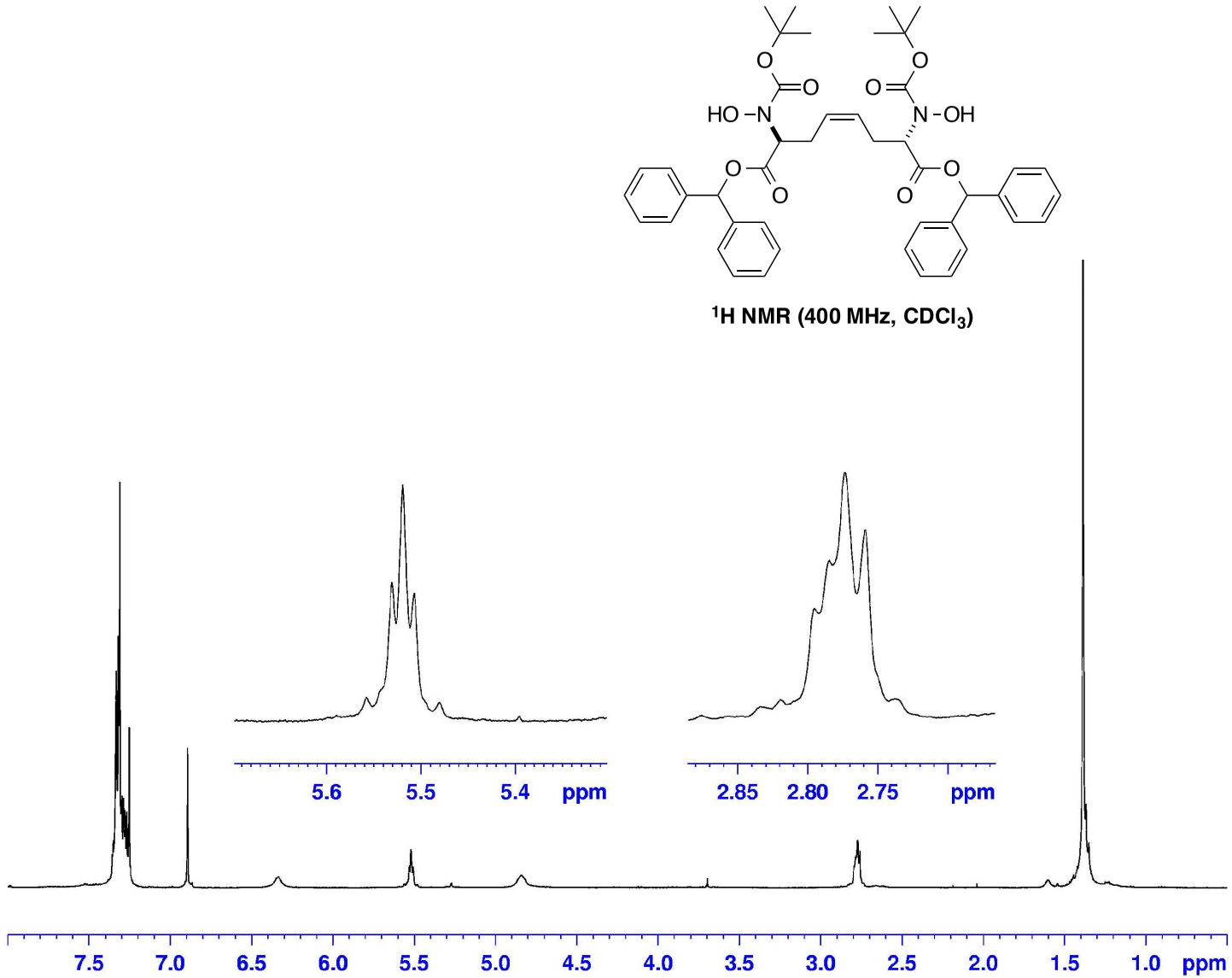


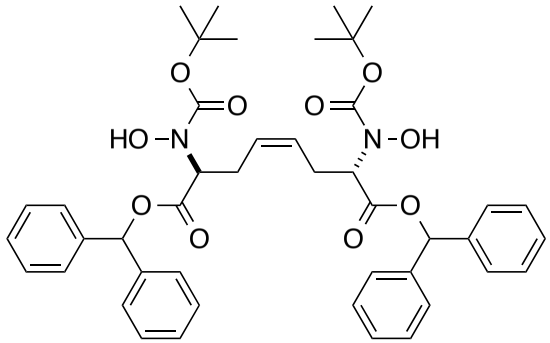

NO2
O
O
O
N
O
O
O
O
O
N
O
O
O
O
NO2
1H NMR (400 MHz, CDCl3)
5.7 5.6 5.5 5.4 5.3 5.2 5.1 5.0 4.9 4.8 ppm
2.9 2.8 2.7 2.6 ppm
8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm



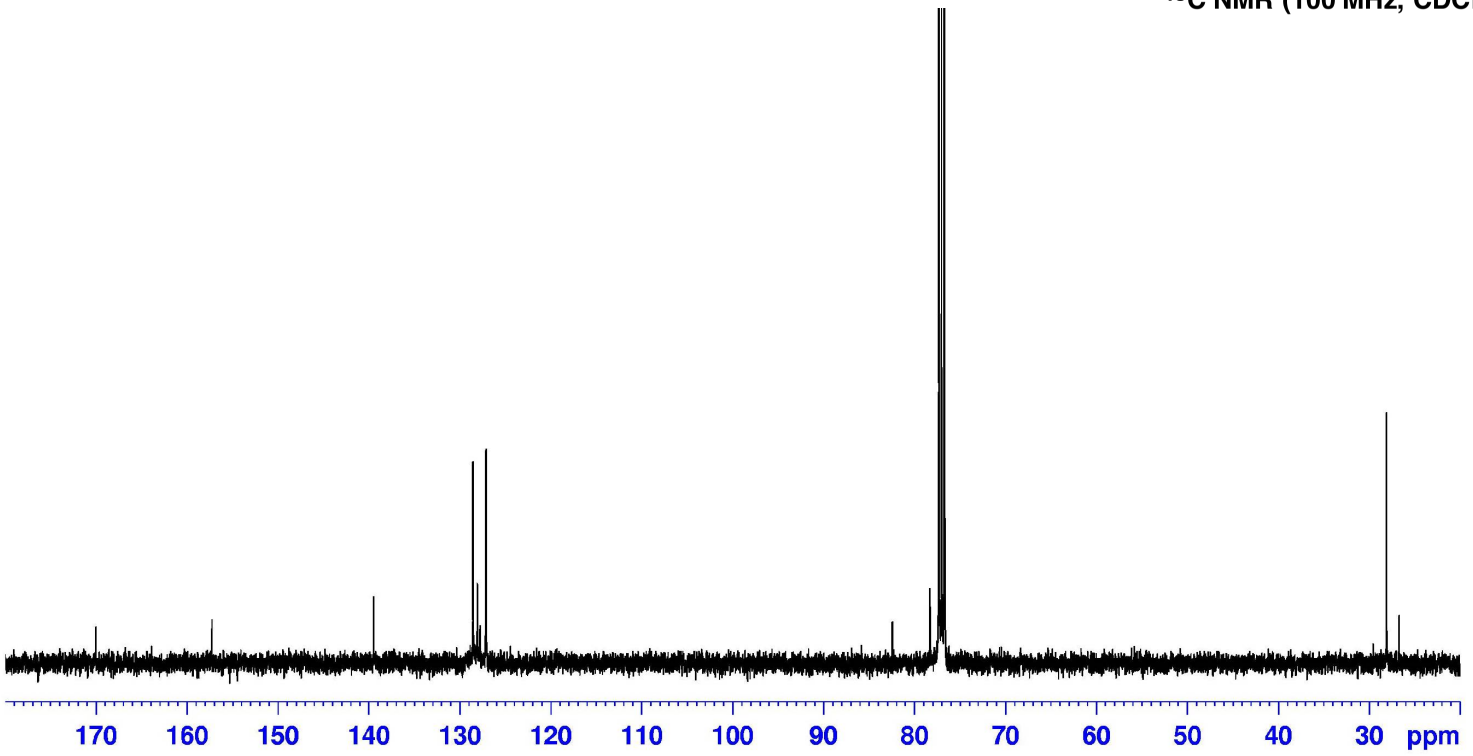
^{13}C NMR (100 MHz, $CDCl_3$)

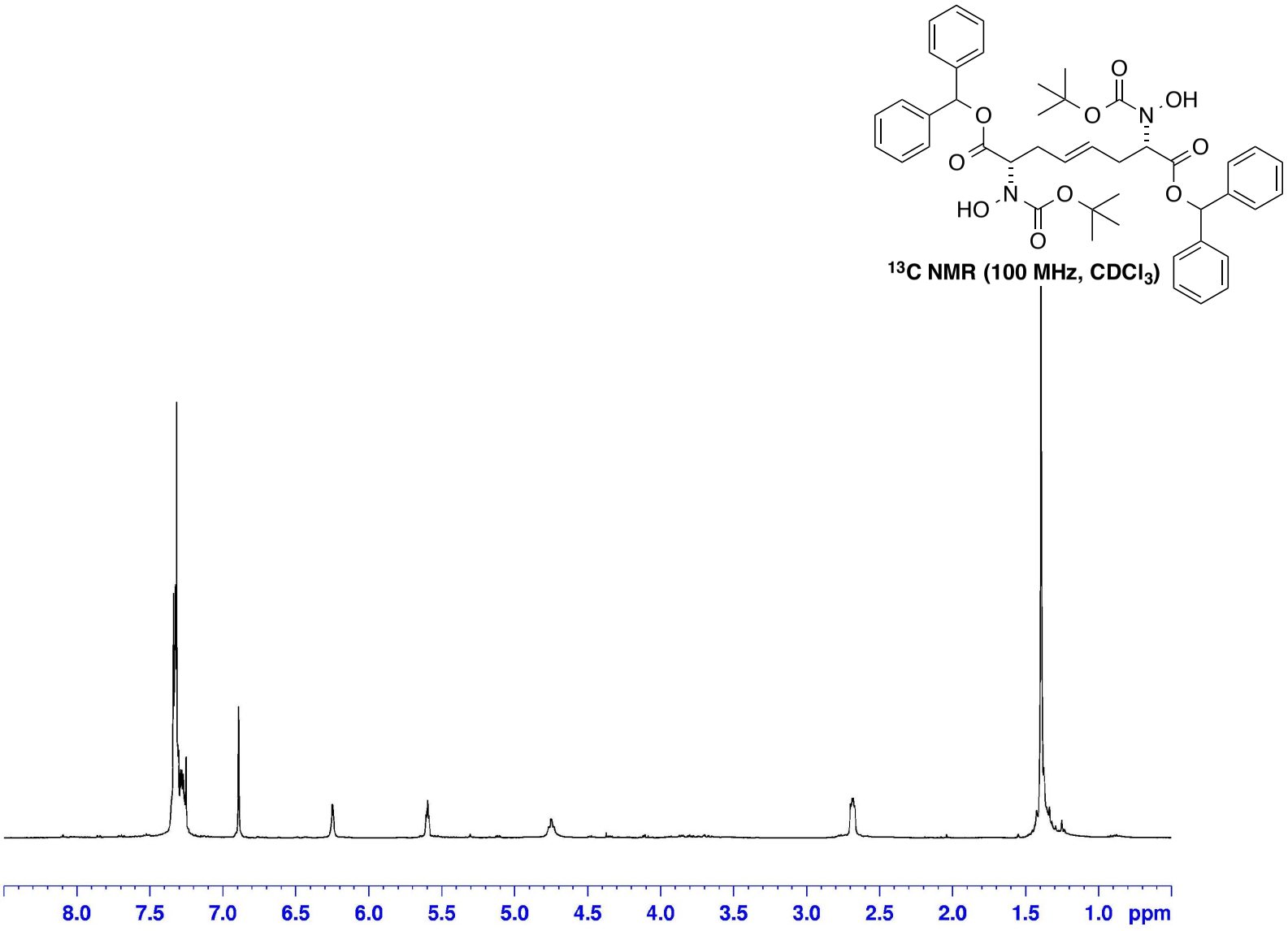


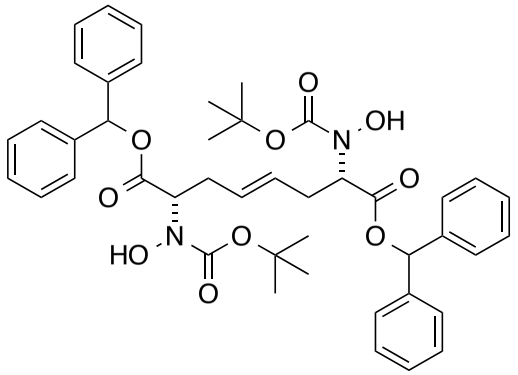

HO
N
O
O
O
O
O
O
N
OH
O
O
O
O
1H NMR (400 MHz, CDCl3)
5.6
5.5
5.4
ppm
2.85
2.80
2.75
ppm
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
ppm



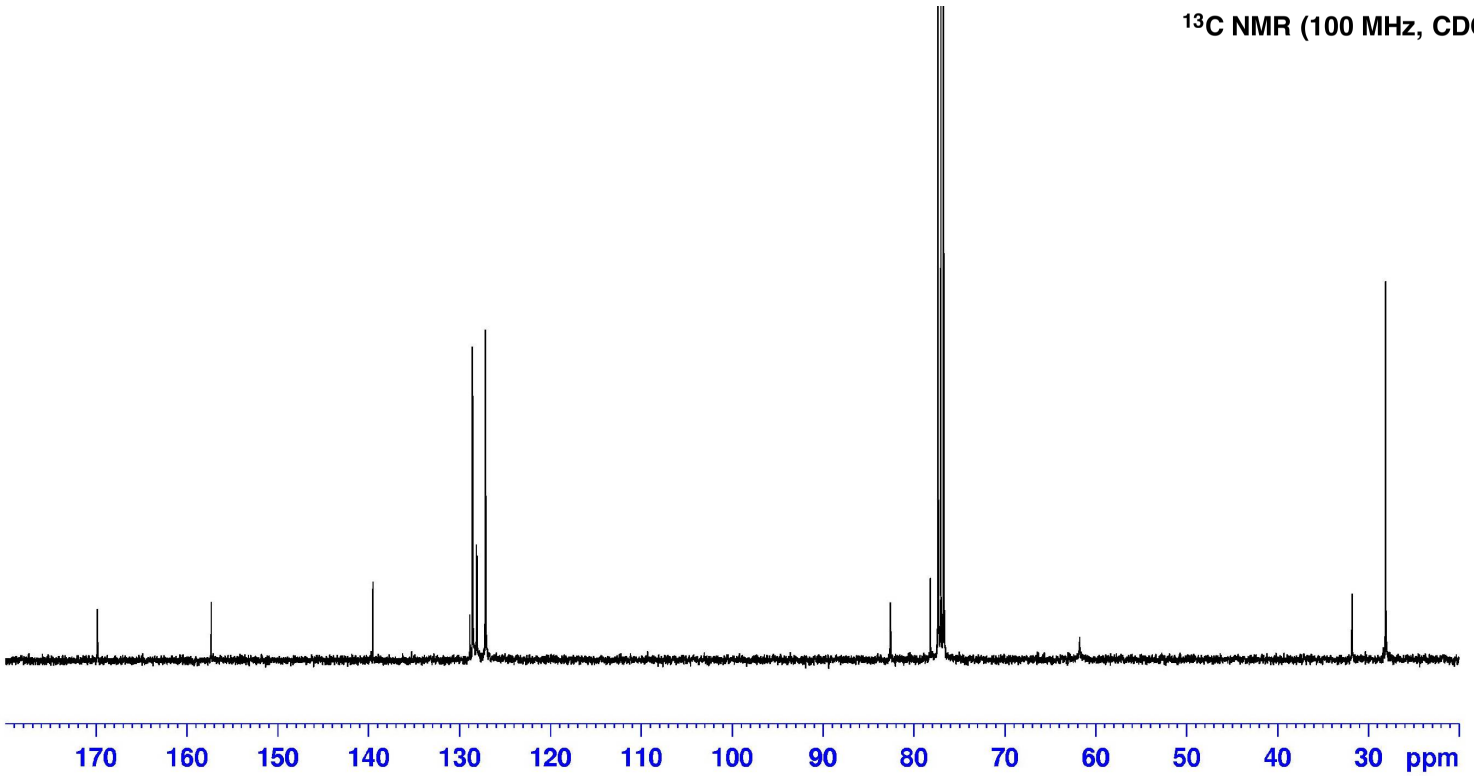
^{13}C NMR (100 MHz, $CDCl_3$)

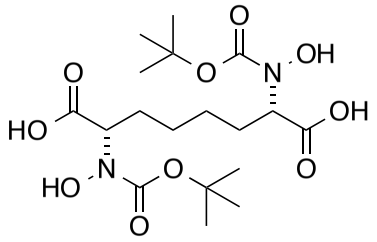



^{13}C NMR (100 MHz, CDCl$_3$)
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
ppm

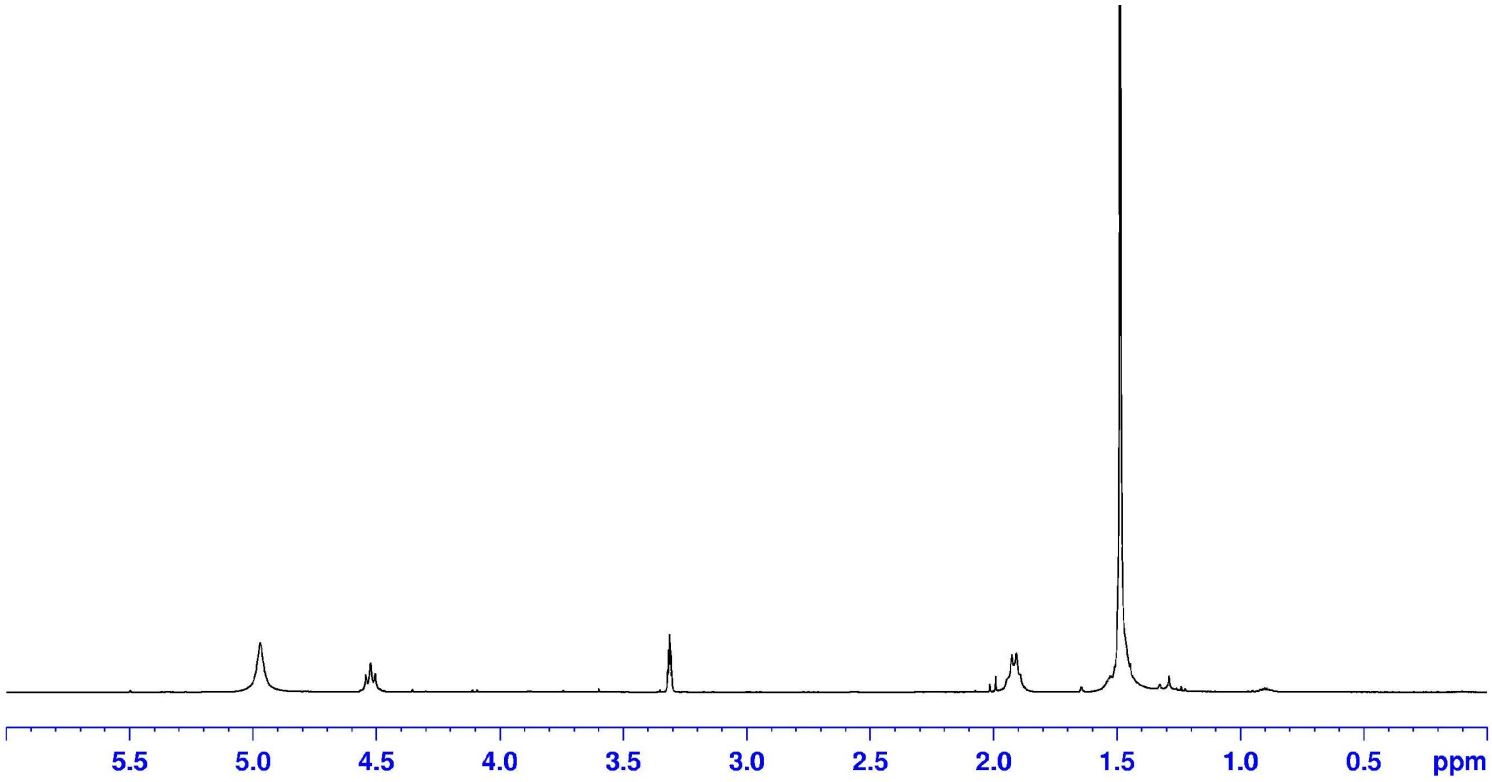


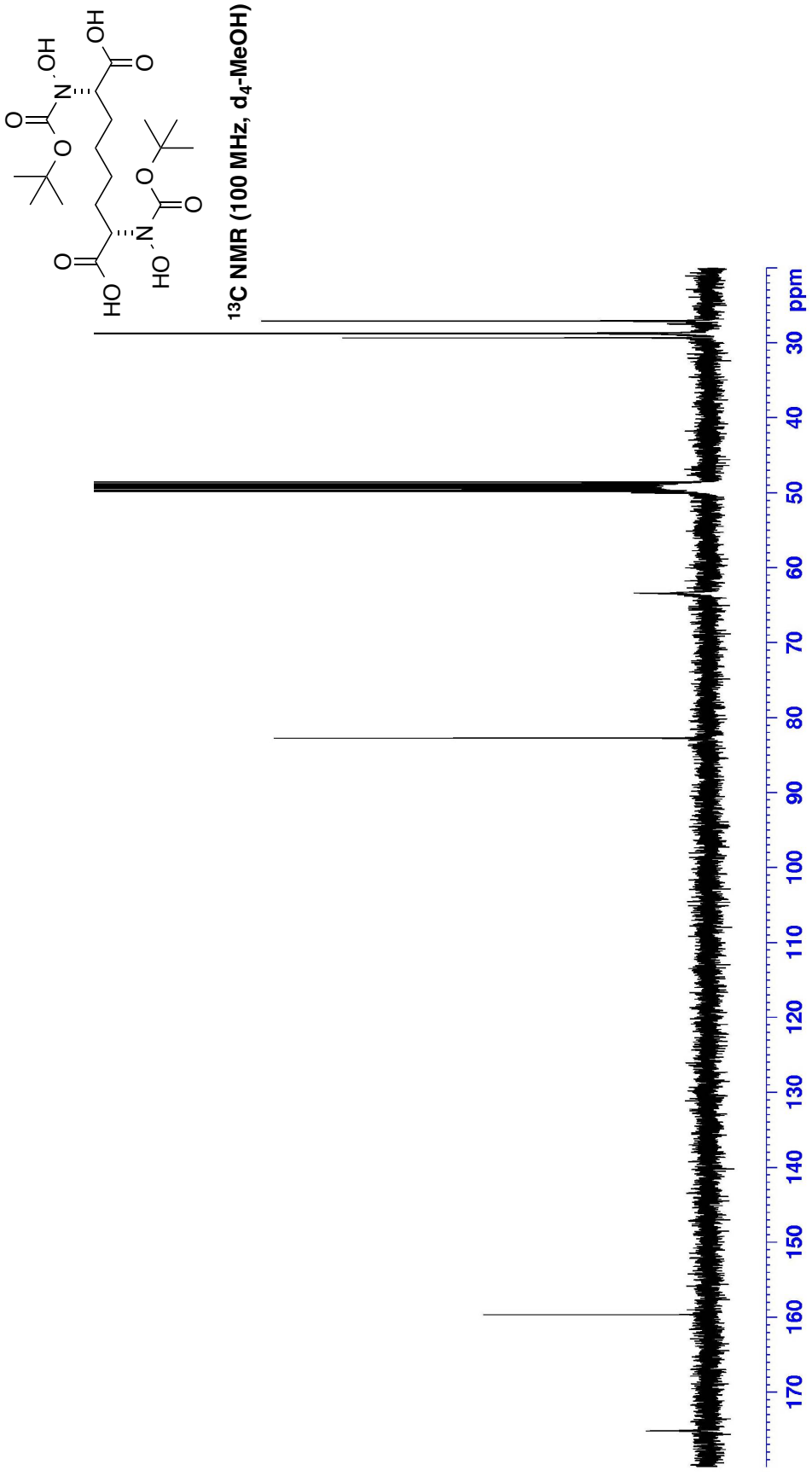
^{13}C NMR (100 MHz, $CDCl_3$)

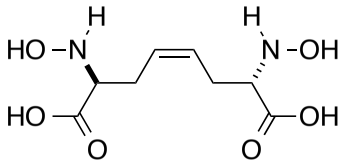




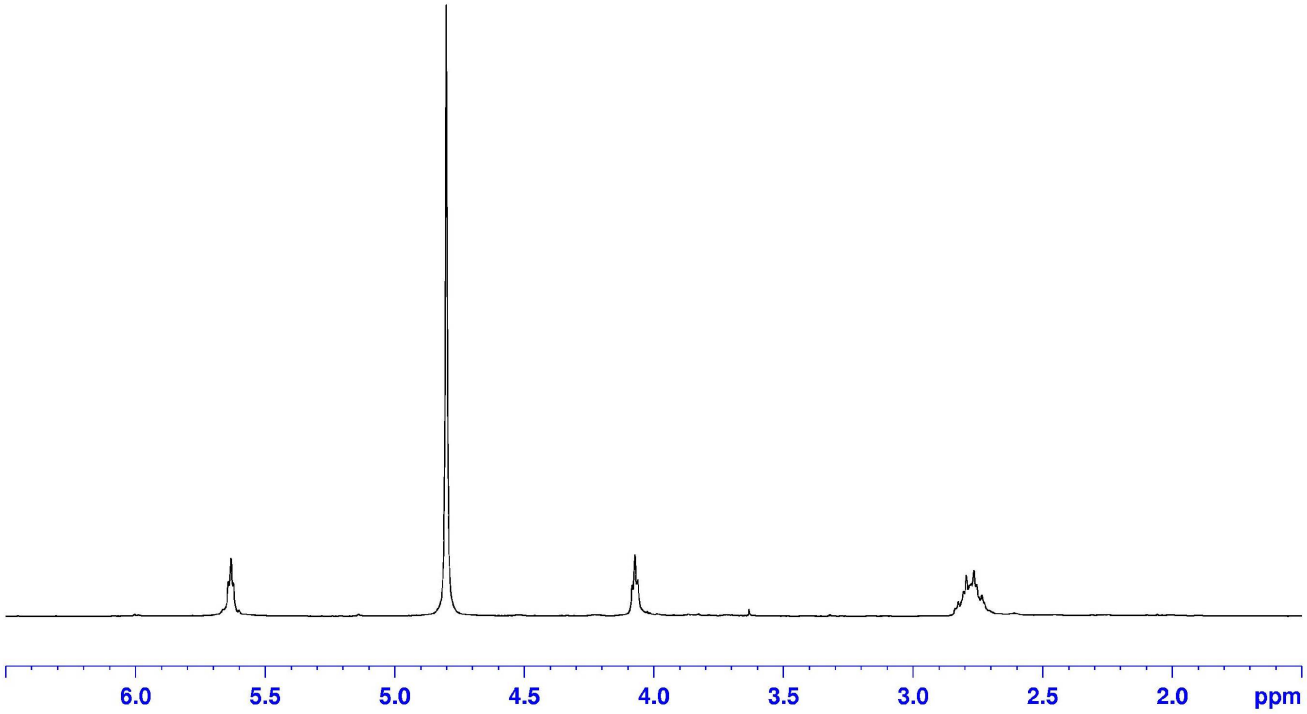
^{1}H NMR (400 MHz, d_4-MeOH)

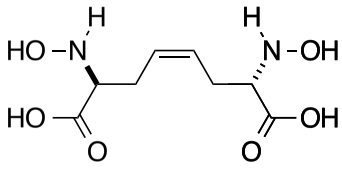



^{13}C NMR (100 MHz, d_4-MeOH)
ppm
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170

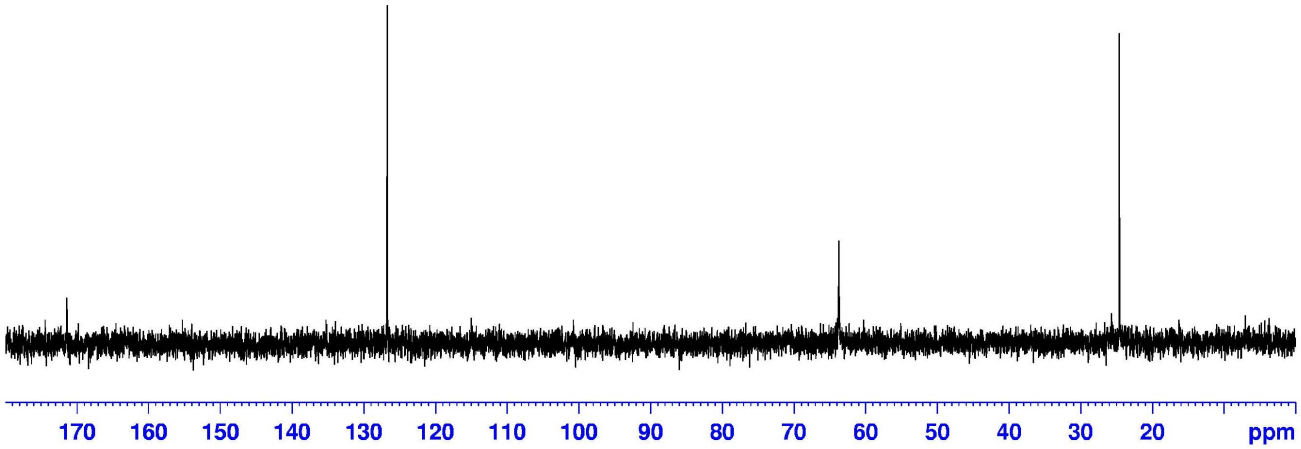


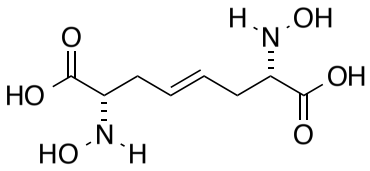
^{1}H NMR (500 MHz, D_2O)



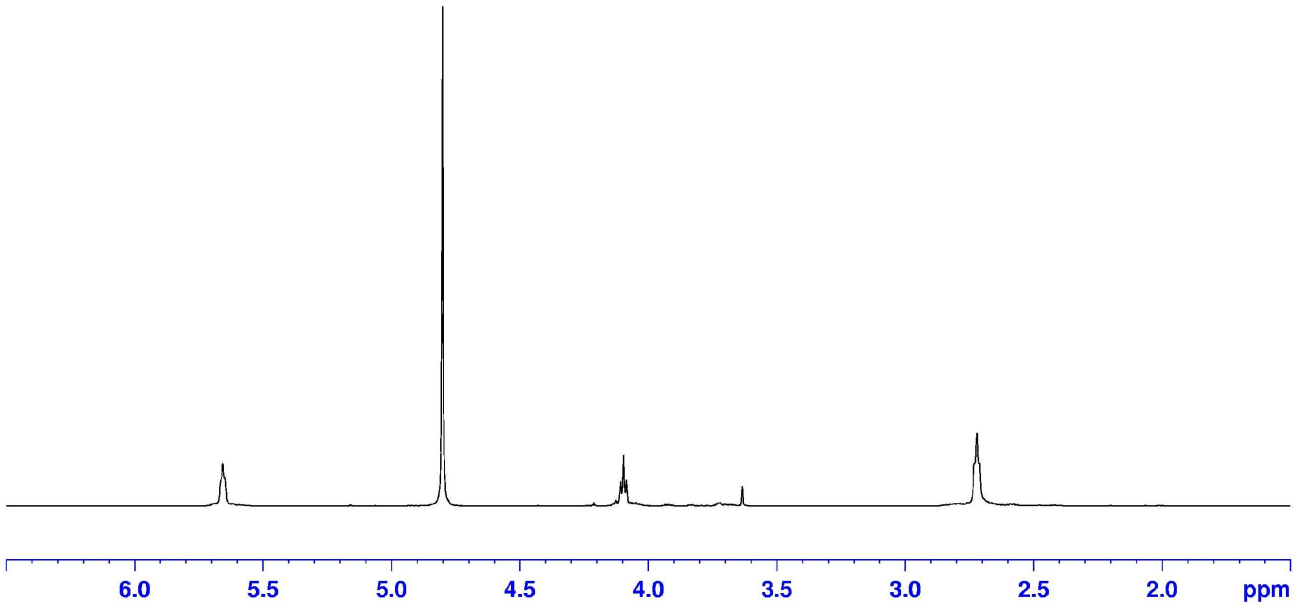


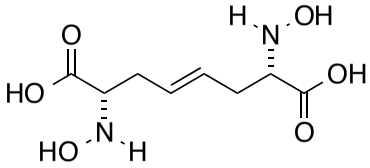
^{13}C NMR (100 MHz, D_2O)



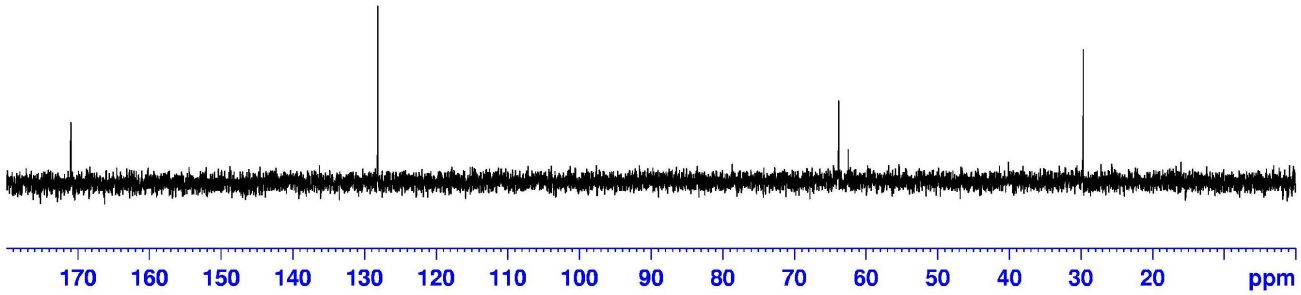


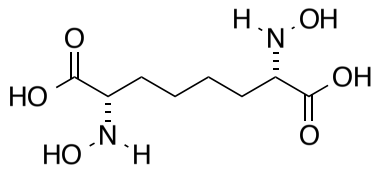
^{1}H NMR (400 MHz, D_2O)



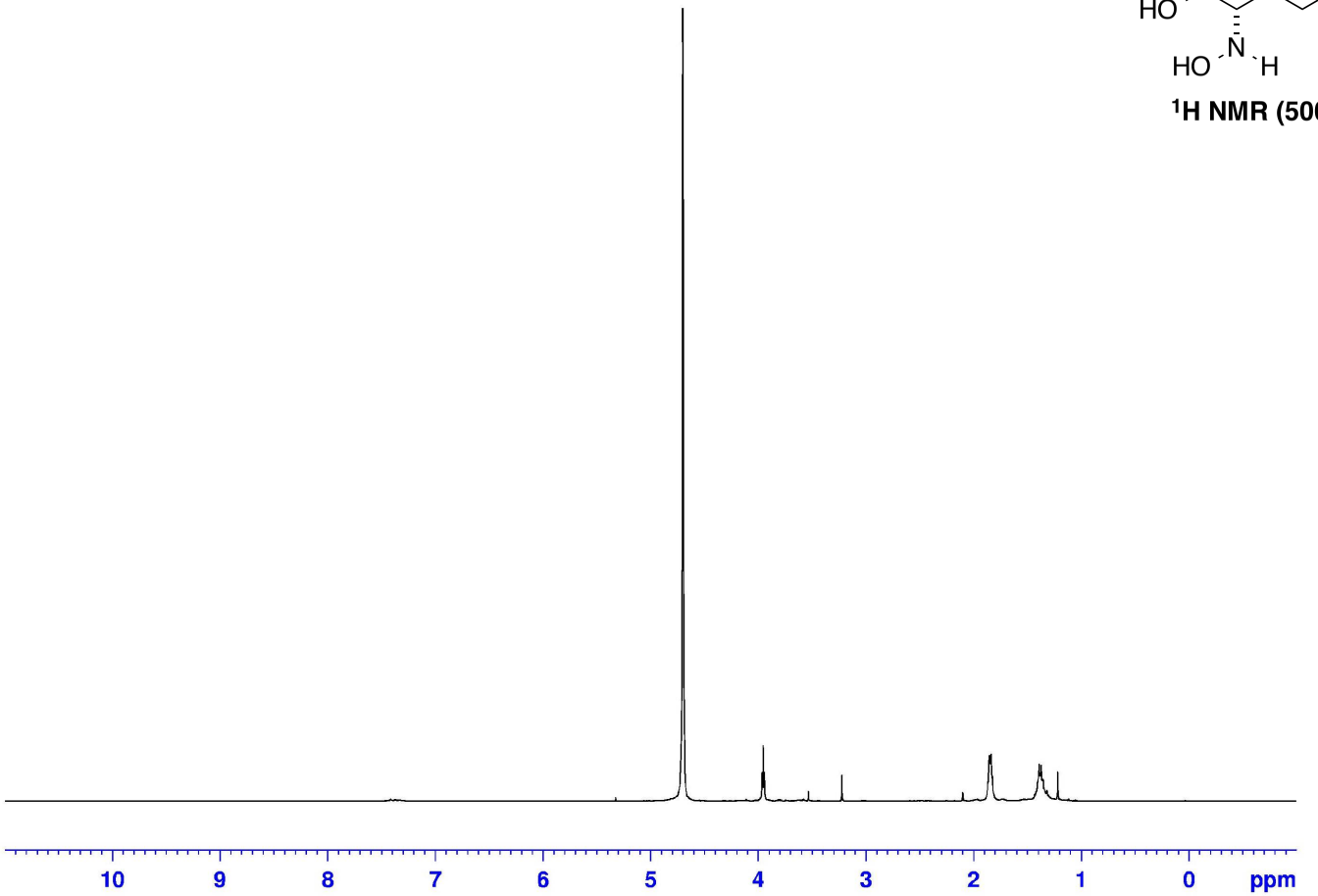


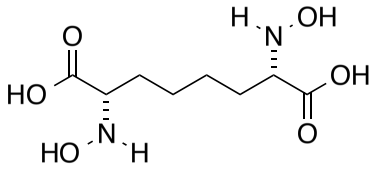
^{13}C NMR (100 MHz, D_2O)



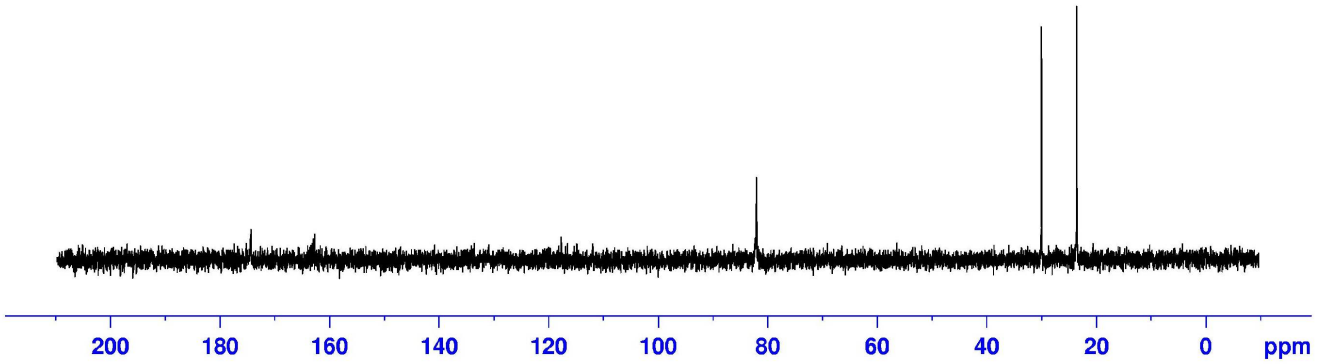


^{1}H NMR (500 MHz, D_2O)





^{13}C NMR (100 MHz, D_2O)



XI: Supporting Information References

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