Syntheses of Mycobactin Analogs as Potent and Selective Inhibitors of *Mycobacterium tuberculosis*

Supporting Information

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Experimental

Chemistry: General materials and methods. All solvents and reagents were obtained from commercial sources and used without further purification unless otherwise stated. Dichloromethane (CH_2Cl_2) and acetonitrile (CH_3CN) were distilled from CaH_2. Tetrahydrofuran (THF) was distilled from a mixture of sodium metal and benzophenone ketyl. Dimethylformamide (DMF), and diisopropylethylamine (DIPEA), were used from Acros Seal® anhydrous bottles. ^{1}H-NMR and ^{13}C-NMR spectra were obtained on a 500 MHz, or 600 MHz Varian DirectDrive spectrometer and FIDs were processed using ACD/SpecManager version 11. Chemical shifts (δ) are given in parts per million (ppm) and are referenced to residual solvent peaks as internal standards. Coupling constants (*J*) are reported in hertz (Hz). High resolution, accurate mass measurements were obtained with a Bruker micrOTOF II electrospray ionization time-of-flight mass spectrometer in positive ion mode. All reactions were conducted under argon gas unless otherwise noted. Solvents were removed *in vacuo* on a rotary evaporator. All reactions were carried out at ambient temperature (~22 °C) unless stated otherwise. Reactions were monitored by thin layer chromatography (TLC) performed with aluminum-backed Merck 60-F_{254} silica gel plates using a 254 nm lamp, ceric ammonium molybdate (CAM) stain, $FeCl_3$ stain, or ninhydrin stain for visualization. Silica gel column chromatography was performed using Sorbent Technologies silica gel 60 (32-63 μm). Melting points were determined in capillary tubes using a Thomas Hoover melting point apparatus and are uncorrected.

Microbiology: Determination of MIC_{90} values against replicating *Mtb*. Activity against replicating *M. tuberculosis* H_{37}Rv (ATCC 27294, American Type Culture Collection, Rockville, MD) was determined using a fluorescence readout in the Microplate Alamar Blue Assay (MABA)[29] using rifampin and PA-824 as positive controls. Compound stock solutions were prepared in DMSO at a concentration of 128 μM, and the final test concentrations ranged from 128 μM to 0.5 μM. Two fold dilutions of compounds were prepared in glycerol-alanine-salt media in a volume of 100 μL in 96-well microplates (BD Optilux™, 96-well Microplates, black/clear flat bottom) for the GAS assay, in an iron-deficient GAS media with 20% Tween 80 added in the GAST[11] assay, and in Middlebrook 7H12 medium (7H9 broth containing 0.1% w/v casitone, 5.6 μg/mL palmitic acid, 5 mg/mL bovine serum albumin, 4 mg/mL catalase) in a volume of 100 μL in 96-well microplates (BD Optilux™, 96-well Microplates, black/clear flat bottom) for the 7H12 assay. The TB cultures (100 μL inoculums of 2×10^5 CFU/mL) were added to the media, yielding a final testing volume of 200 μL. The plates were incubated at 37 °C. On the seventh day of incubation, 12.5 μL of 20% Tween 80, and 20 μL of Alamar Blue (Invitrogen BioSource™) were added to the wells of test plate. After incubation at 37 °C for 16-24 h, fluorescence of the wells was measured at 530 nm (excitation) and 590 nm (emmision). The MICs are defined as the lowest concentration effecting a reduction in fluorescence of ≥90% relative to the mean of replicate bacteria-only controls.

Low-Oxygen-Recovery Assay (LORA)[31] for the determination of MIC_{90} values against non-replicating *Mtb*. A low-oxygen adapted culture of recombinant H_{37}Rv (pFCA-luxAB), expressing a *Vibrio harveyii* luciferase gene with an acetamidase promoter, was grown in a BiostatQ fermentor. Cells were collected on ice, washed in PBS, and stored at -80 °C. Circa 10^5 CFU/mL of thawed NRP cells were exposed to two-fold serial dilutions of test compound in 7H12 broth in black 96-well plates, which were incubated anaerobically at 37 °C for 10 days. Luminescence readings were obtained following a 28 h recovery in an aerobic environment (5% CO_2). The data was analyzed graphically, and the lowest concentration of test compound preventing metabolic recovery (90% reduction relative to untreated cultures) was determined.

Vero[32] cytotoxicity assay for the determination of IC_{50} values. Samples were dissolved at 12.8 mM in DMSO. Geometric three-fold dilutions were performed in growth medium MEM (Gibco, Grand Island, NY), containing 10% S5 fetal bovine serum (HyClone, Logan, UT), 25 mM *N*-(2-hydroxyethyl)-piperazine-*N*'-2-ethanesulfonic acid (HEPES, Gibco), 0.2% $NaHCO_3$ (Gibco), and 2 mM glutamine (Irvine Scientific, Santa Ana, CA). Final DMSO concentrations did not exceed 1% v/v. Drug dilutions were distributed in duplicate in 96-well tissue culture plates (Becton Dickinson Labware, Lincoln Park, NJ) at a volume of 50 μL per well. An equal volume containing 5×10^5 log phase Vero cells (African green monkey kidney cells, CCL-81; American Type Culture Collection, Rockville, MD) was added to each well and the cultures were incubated at 37 °C in an atmosphere containing 5% of CO_2. After 72 h, cell viability was measured using the CellTiter 96 aqueous non-radioactive cell proliferation assay (Promega Corp., Madison, WI) according to the manufacturer's instructions. Then absorbance at 490 nm was read in a Victor

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multilabel reader (PerkinElmer). The IC_{50} values (inhibition concentration at 50%) were determined using a curve-fitting program.

Antibiotic Susceptibility Testing by the Agar Diffusion Method: General Materials and Methods. All liquids and media were sterilized by autoclaving (121 ºC, 15 min) before use. All aqueous solutions and media were prepared using distilled, deionized, and filtered water (Millipore Milli-Q Advantage A10 Water Purification System). Luria broth (LB) was purchased from VWR. Mueller-Hinton no. 2 broth (MHII broth; cation adjusted) was purchased from Sigma-Aldrich (St. Louis, MO). Mueller-Hinton no. 2 agar (MHII agar; HiMedia Laboratories) was purchased from VWR. McFarland $BaSO_4$ turbidity standards were purchased from bioMérieux, Inc. Sterile plastic Petri dishes (145 mm × 20 mm; Greiner Bio-One) were purchased from VWR. Ciprofloxacin was purchased from Sigma-Aldrich (St. Louis, MO).

Antibacterial activity of the compounds was determined by a modified Kirby-Bauer agar diffusion assay.[33] Overnight cultures of test organisms were grown in LB broth for 18-24 h and standard suspensions of 1.5×10^6 CFU/mL were prepared in saline solution (0.9% NaCl) according to a 0.5 $BaSO_4$ McFarland Standard.[34] Each standardized suspension (0.1 mL) was added to 34 mL of sterile, melted MHII agar tempered to 47-50 °C. After gentle mixing, the inoculated agar media was poured into a sterile plastic Petri dish (145 mm × 20 mm) and allowed to solidify near a flame with the lid cracked for 30 min. Wells of 9.0 mm diameter were cut from the Petri dish agar and filled with exactly 50 µL of the test sample solution. The Petri dish was incubated at 37 °C for 18-24 h, and the inhibition zone diameters were measured (mm) with an electronic caliper after 24-48 h.

Table S1 Antibacterial activity of compounds in the modified Kirby-Bauer agar diffusion assay[a-g]

	Diameter of growth inhibition zones (mm)							
Compound	*B. subtilis* ATCC 6633	*S. aureus* SG511	*M. luteus* ATCC 10240	*M. vaccae* IMET 10670	*M. smegmatis* MC^2155	*P. aeruginosa* K799/wt	*P. aeruginosa* K799/61	*E. coli* X580
13	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	17 V
17	0	0	0	16 M	0	0	0	0
18	0	0	17 U	18	18	0	14	15 P
19	0	0 P	0	24 M, P	0	0	0 P	0 P
28	0	0	0	0	0	0	H	0
29	0	0	0	0	0	0	13 P	0
34	0	0 P	0	0	0	0	0 P	0 P
36	0	0	0	0 P	0	0	0	0 P
40	13	13 U	18 U	0	0	0	0	0

[a]Exactly 50 µL of each compound in solution (2 mM in 10:1, MeOH/DMSO) were added to 9 mm wells in agar media (MHII) inoculated with 5×10^3 CFU/mL. Diameters of growth inhibition zones were measured (mm) after incubation at 37 °C for 24 h, or 48 h for *M. vaccae* and *M. smegmatis*.[33] [b]Ciprofloxacin was used as a standard at 5 µg/mL (0.015 mM) in H_2O. [c]H, hint of inhibition. [d]M, misshapen inhibition zone. [e]P, observed precipitation. [f]U, unclear inhibition zone. [g]V, very unclear inhibition zone.

Table S2 Inhibitory activity against non-replicating *Mtb* and cytotoxicity of mycobactin analogs.[a]

Compound	MIC (µM) LORA[31]	Cytotoxicity (IC_{50} µM) Vero[32]
34	>50	21.50
36	>50	22.37
40	>50	>50

[a]Values reported are the average of three individual measurements.

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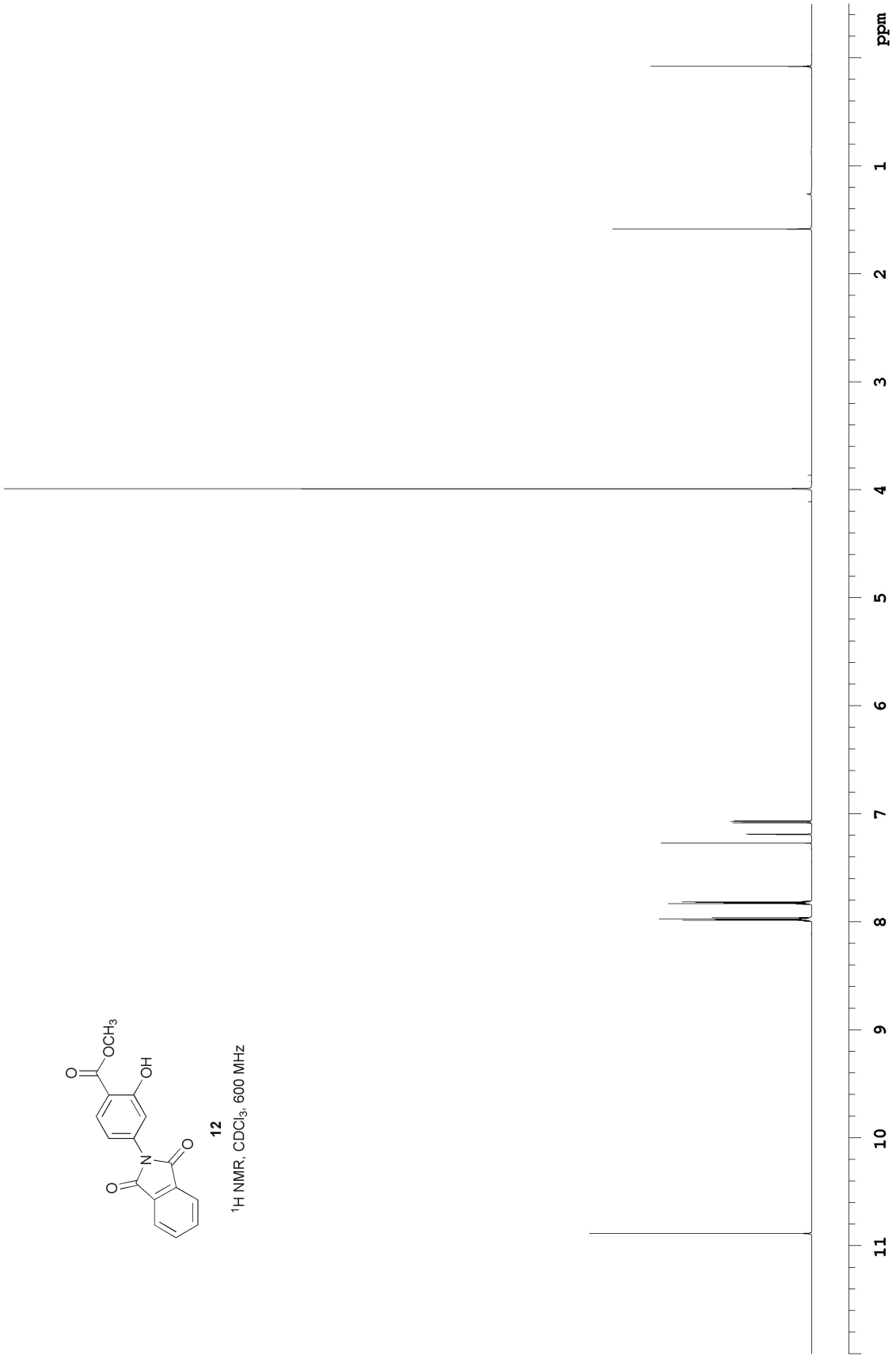

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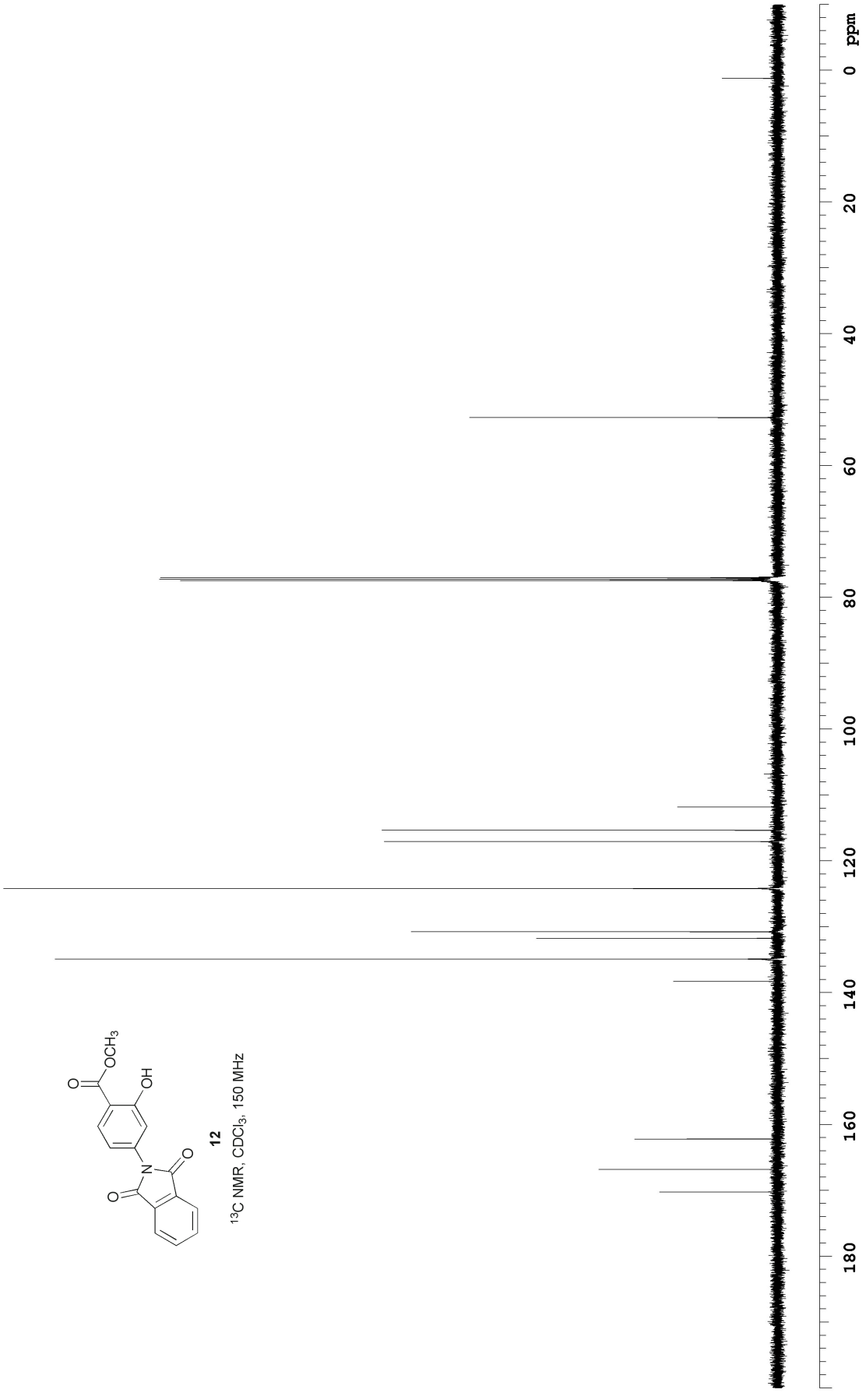

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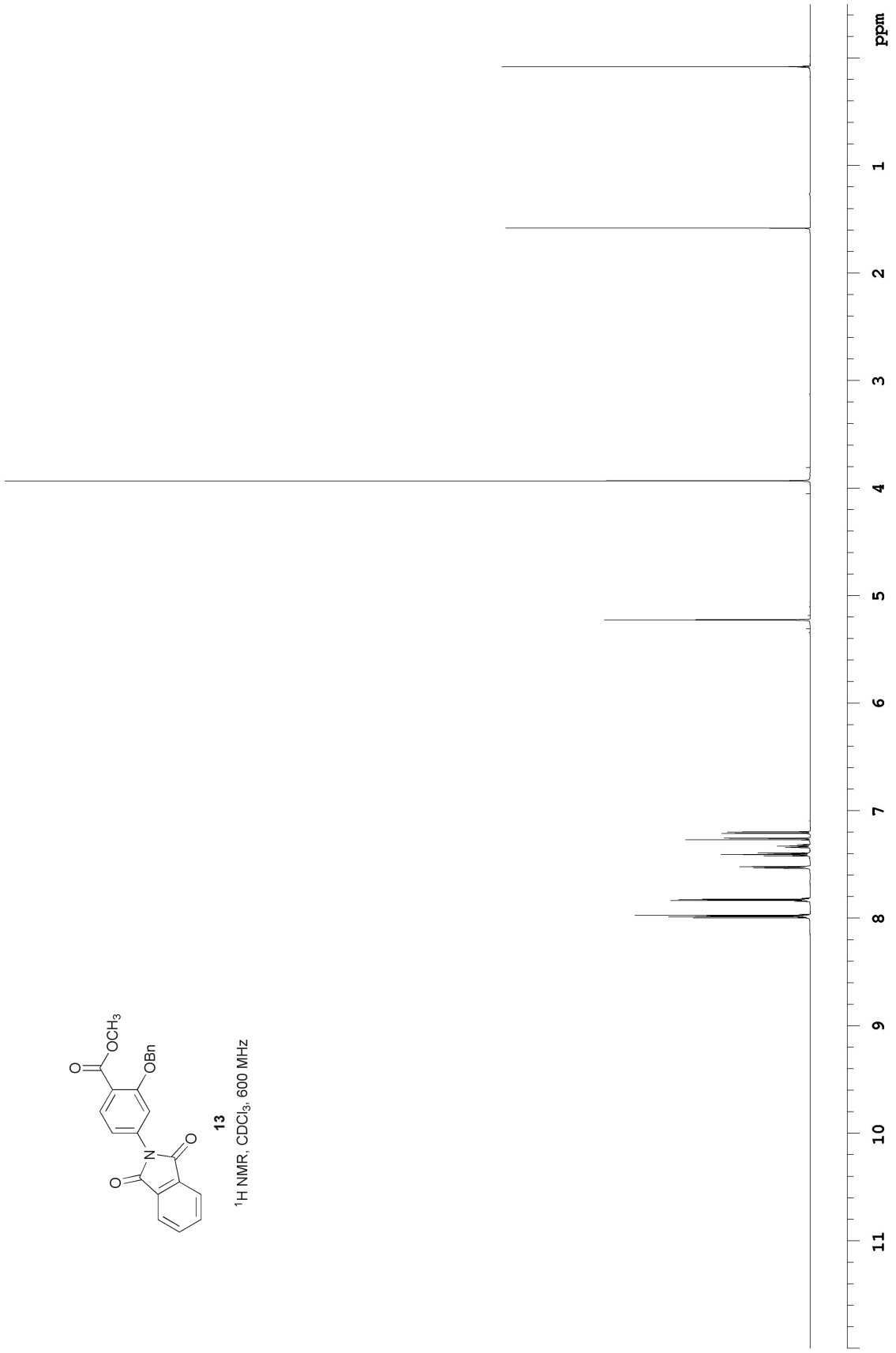

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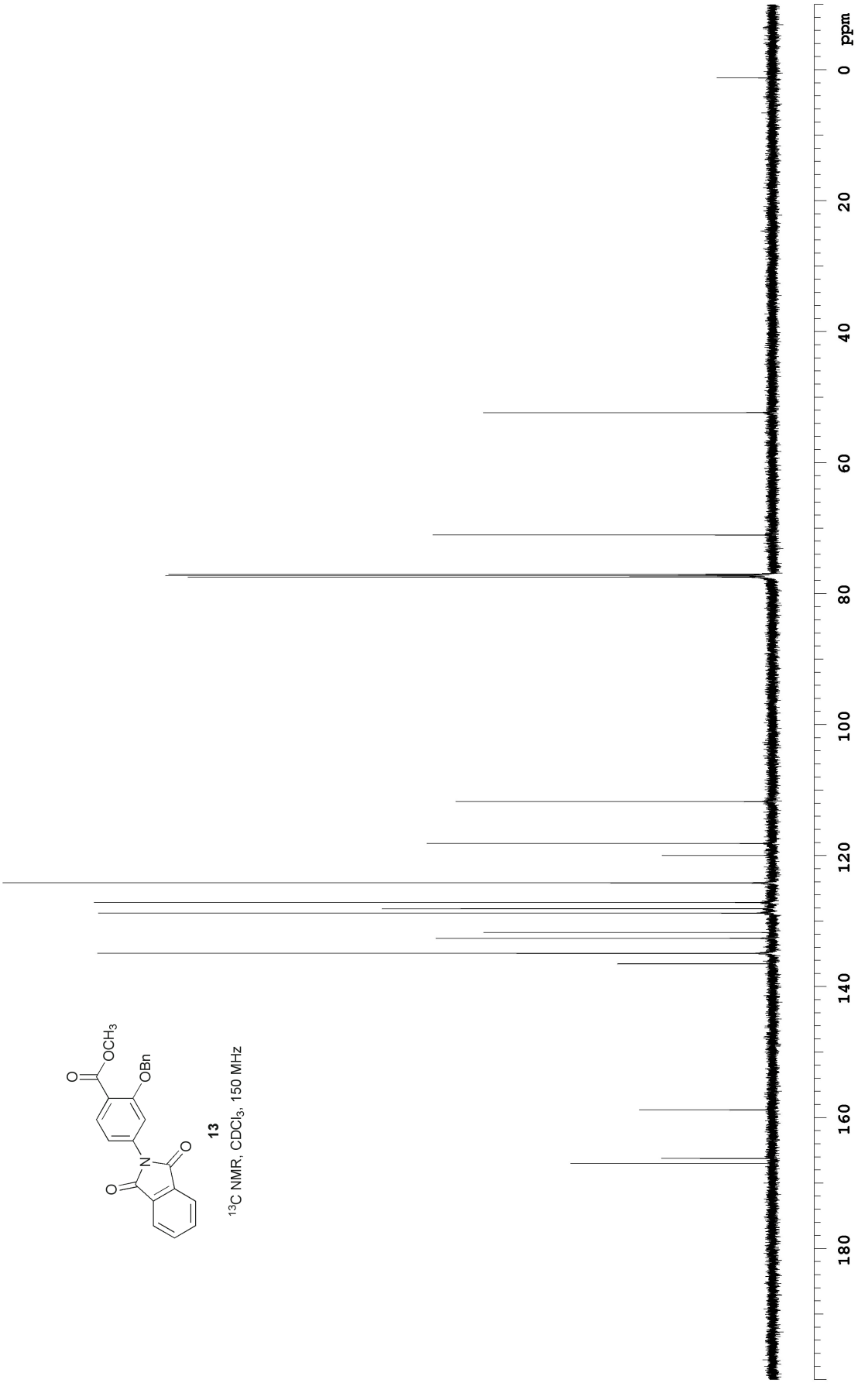

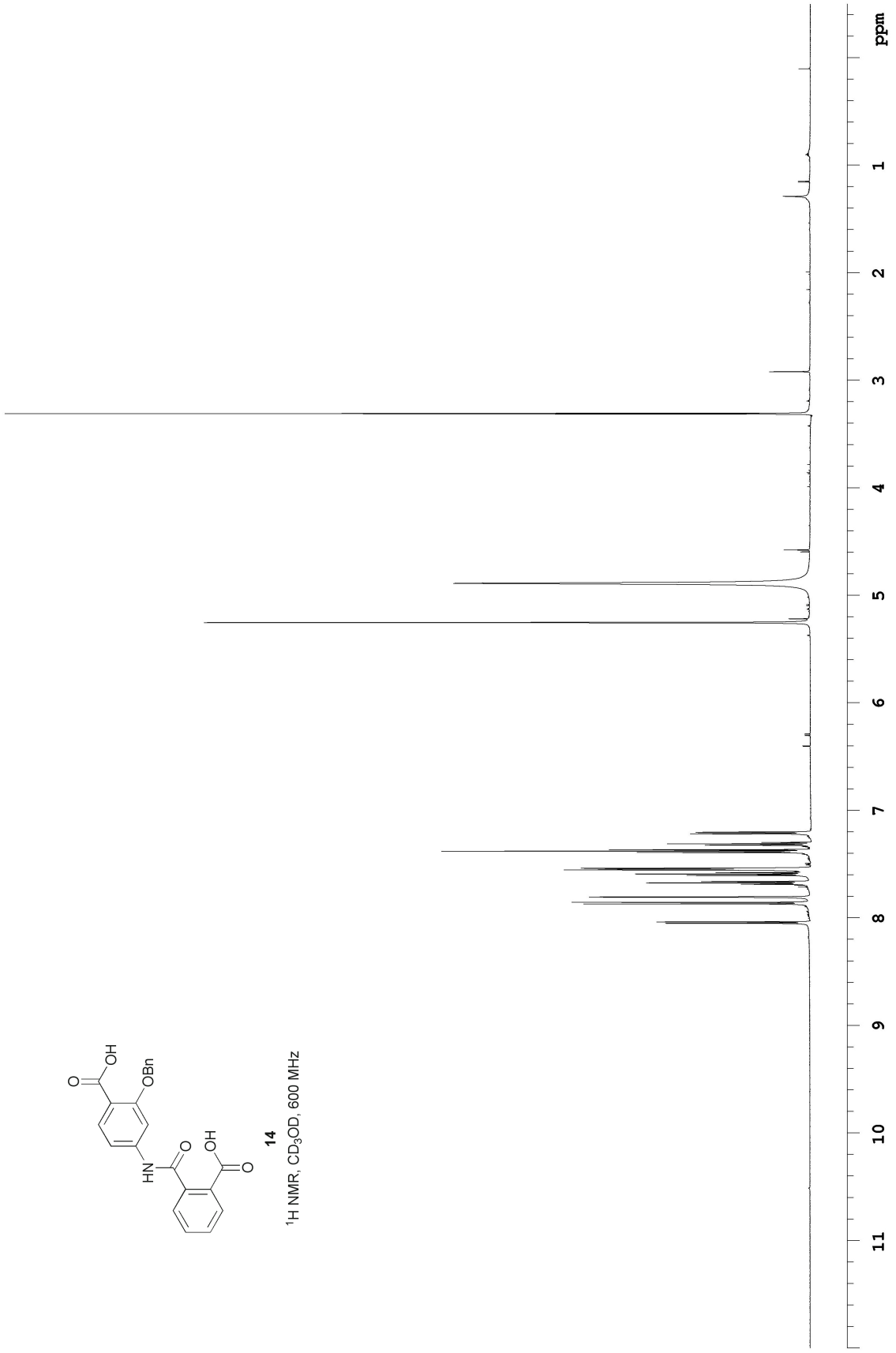

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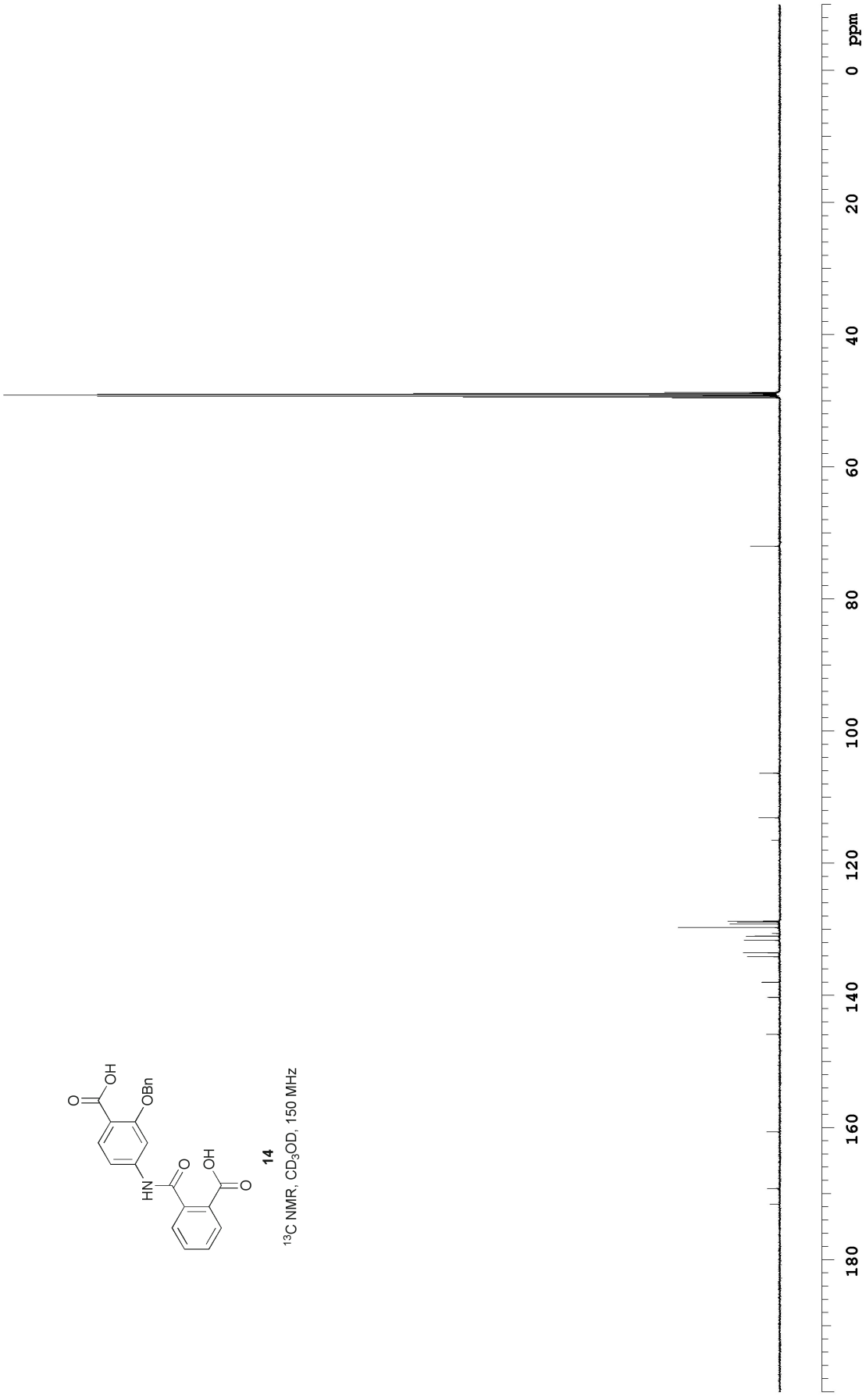

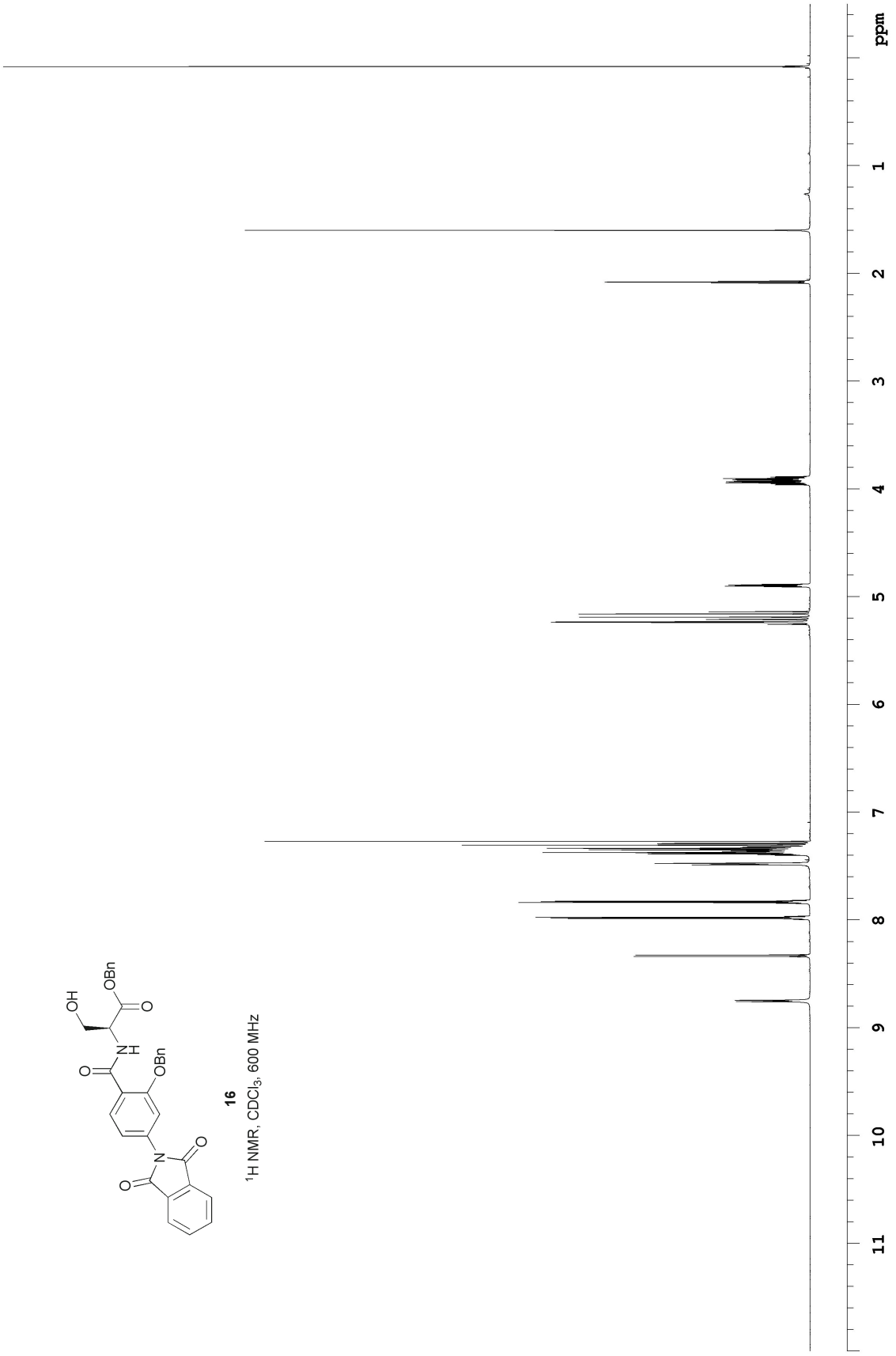

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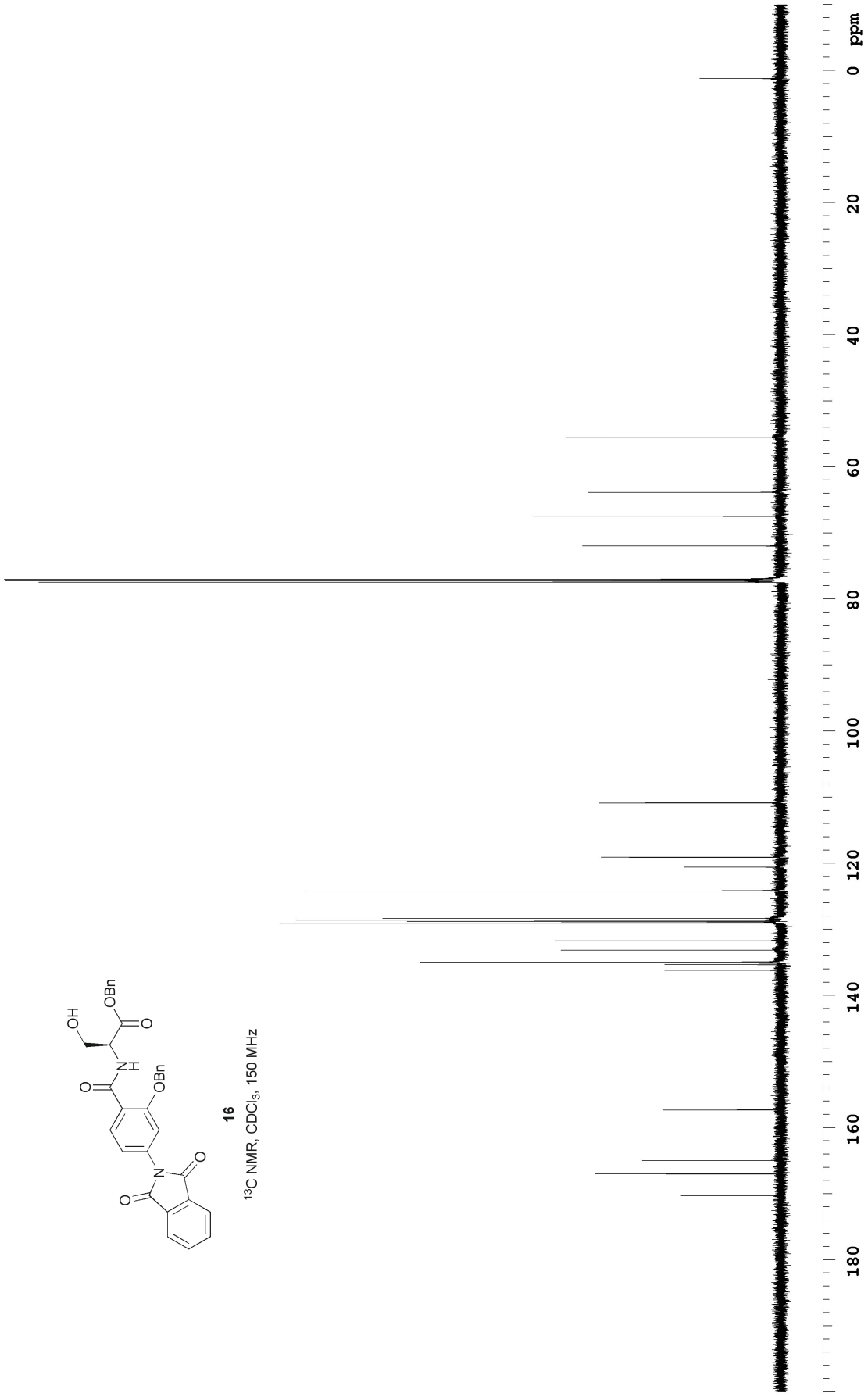

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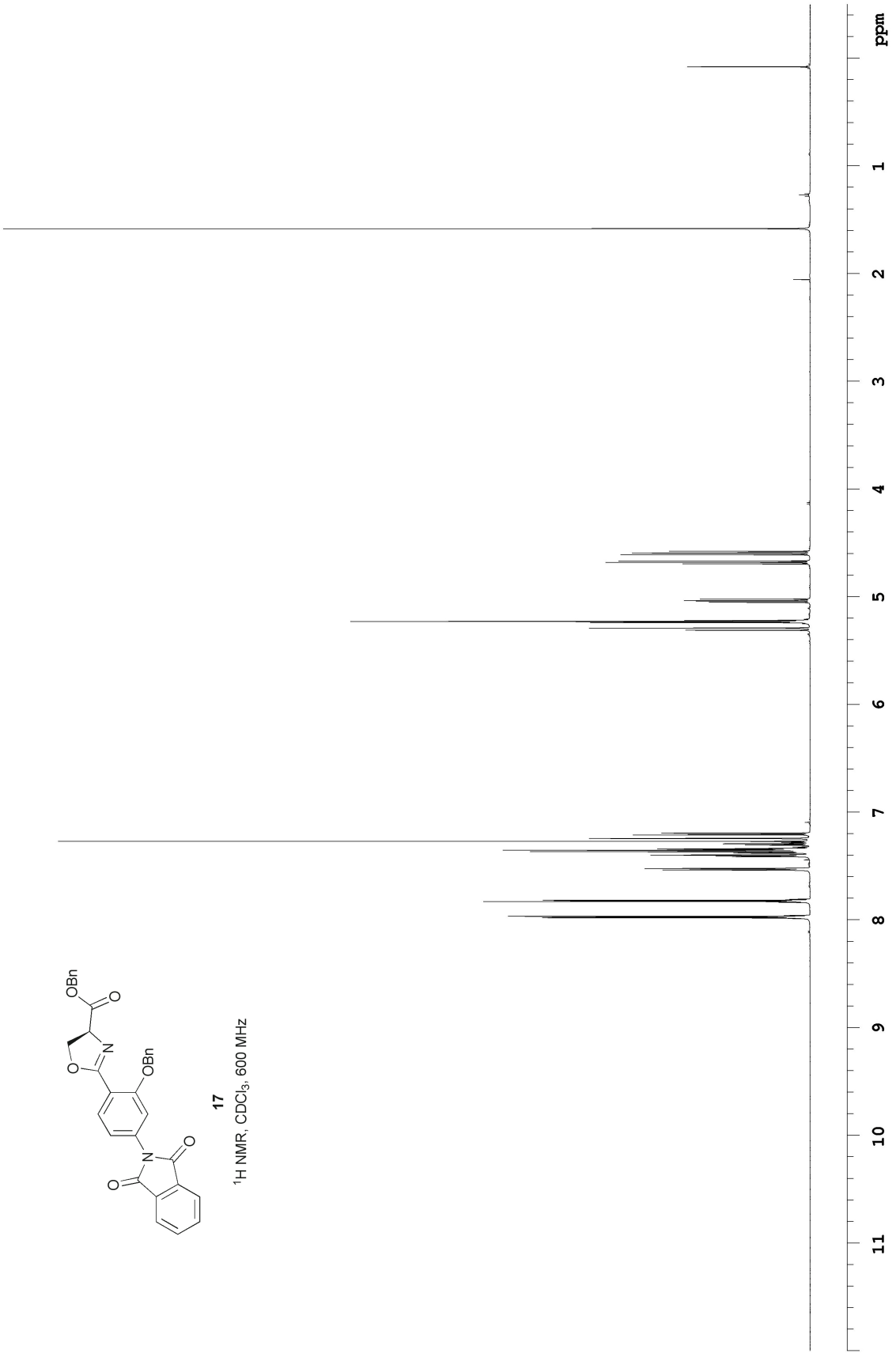

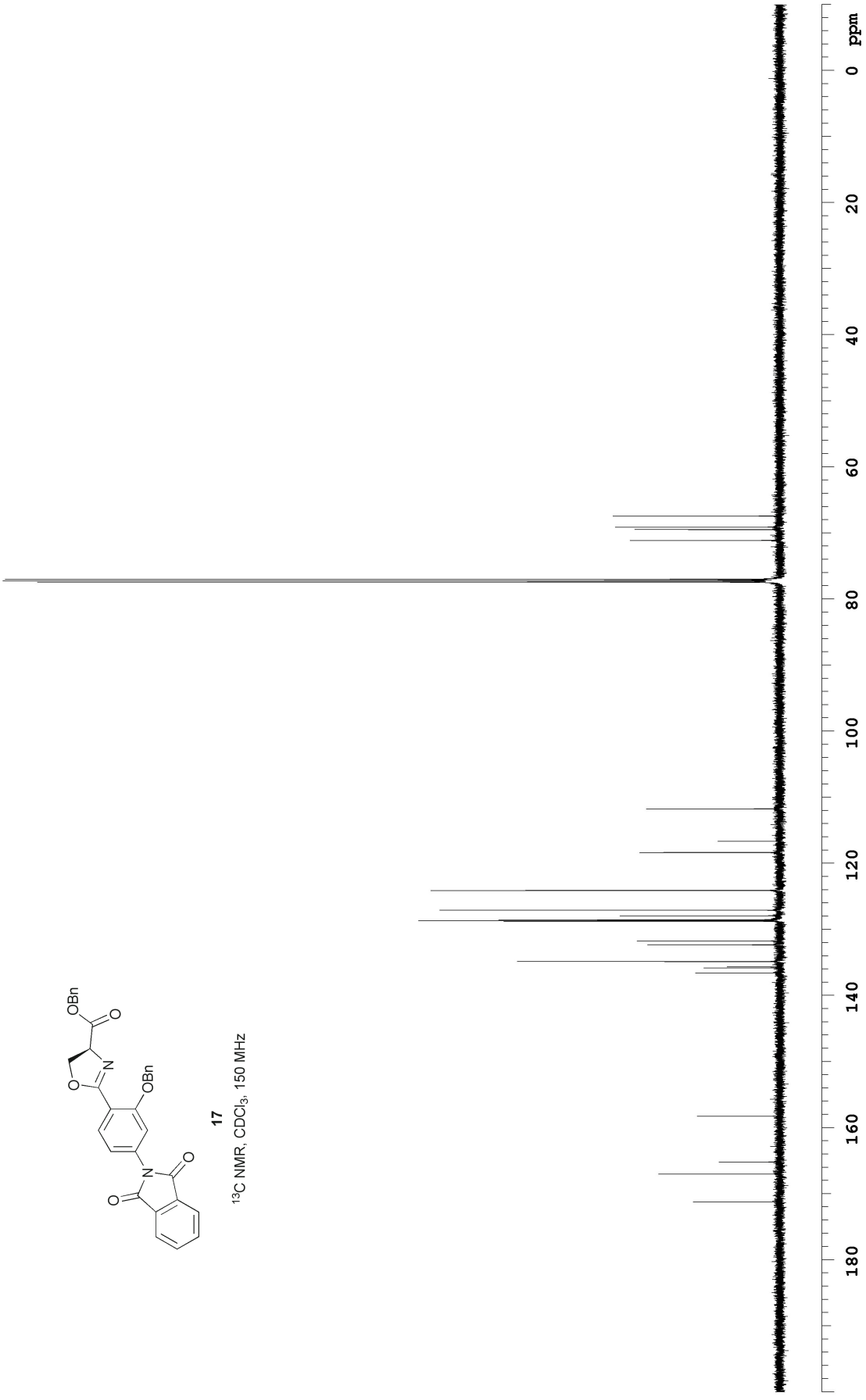

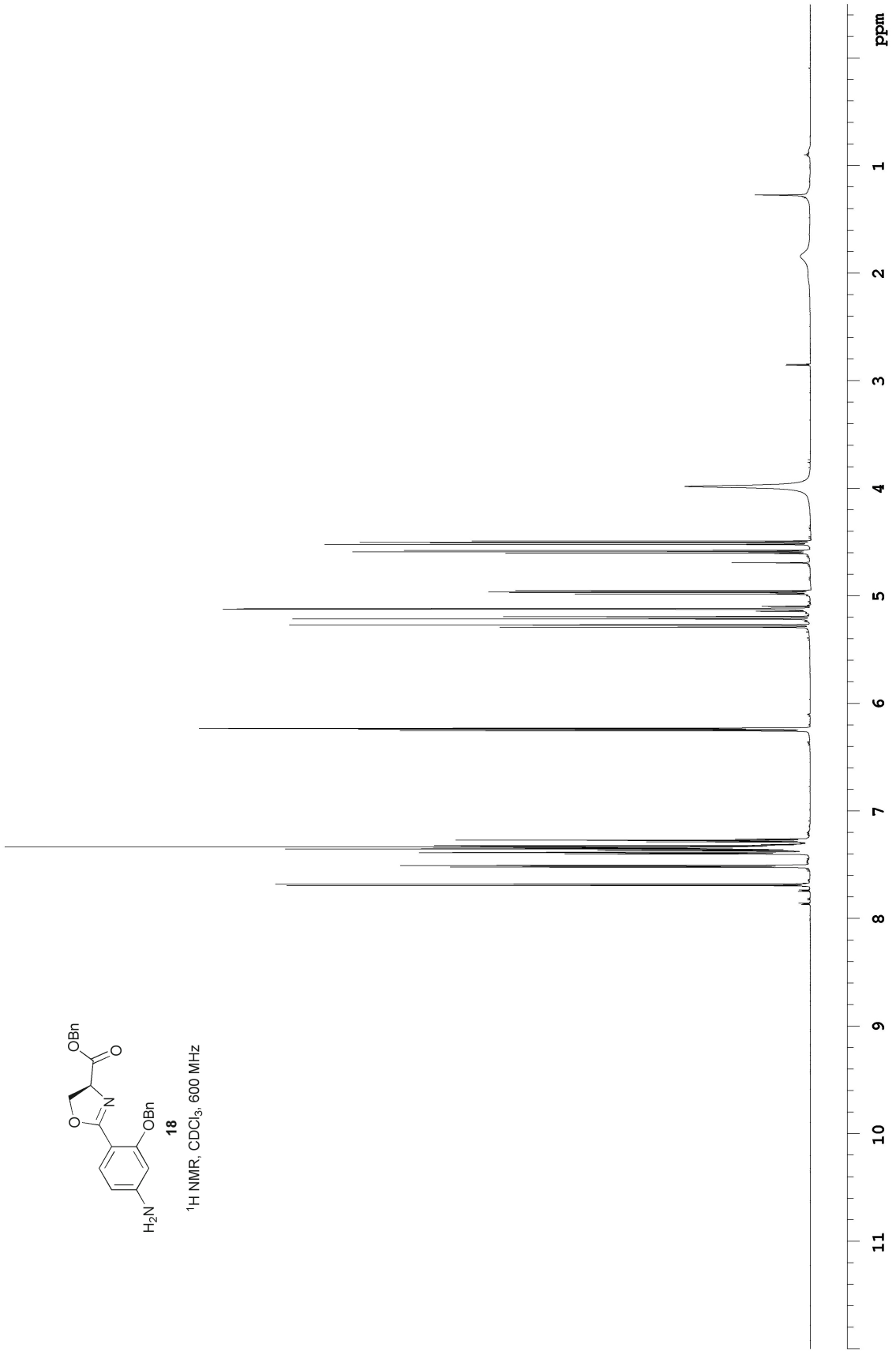

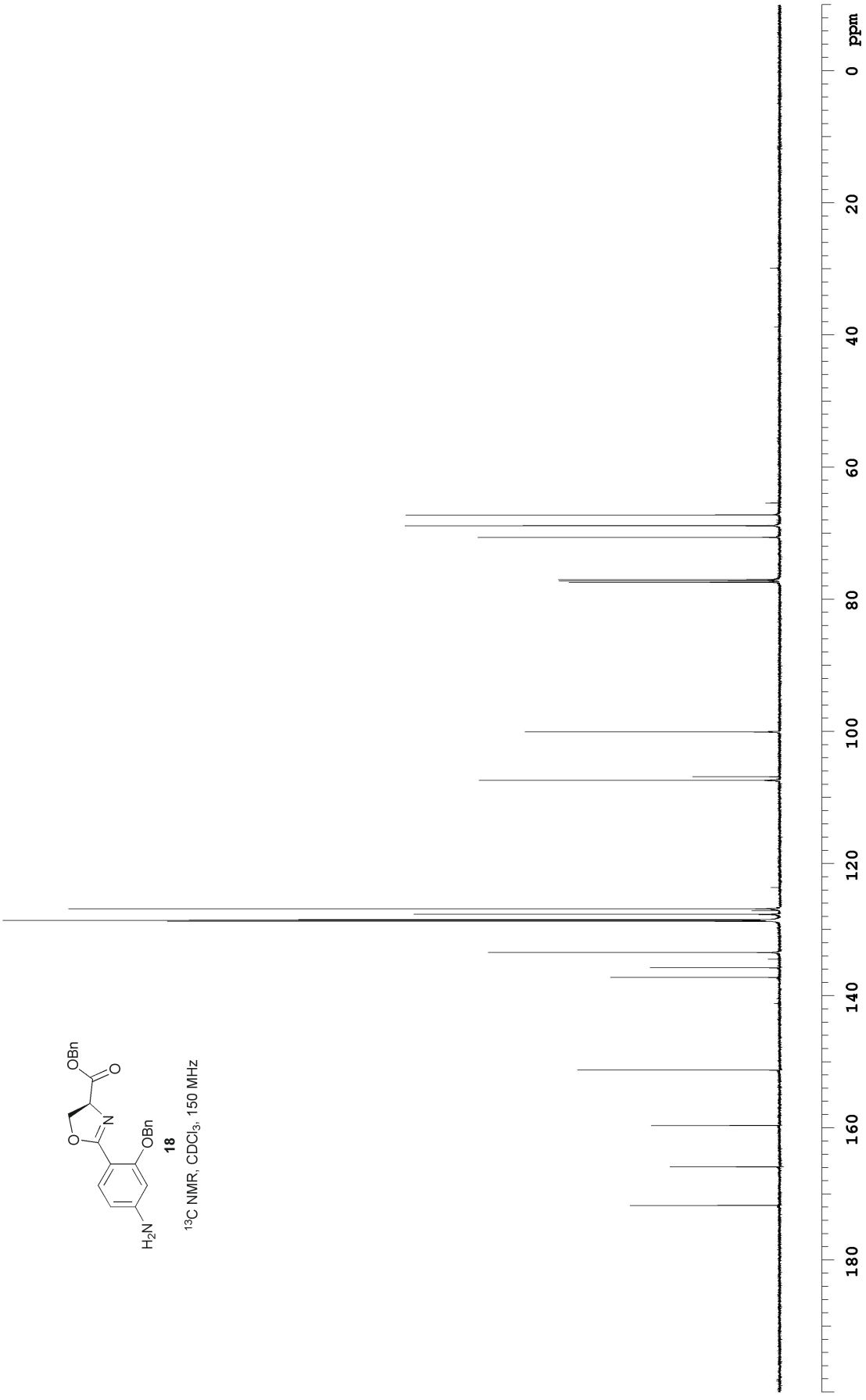

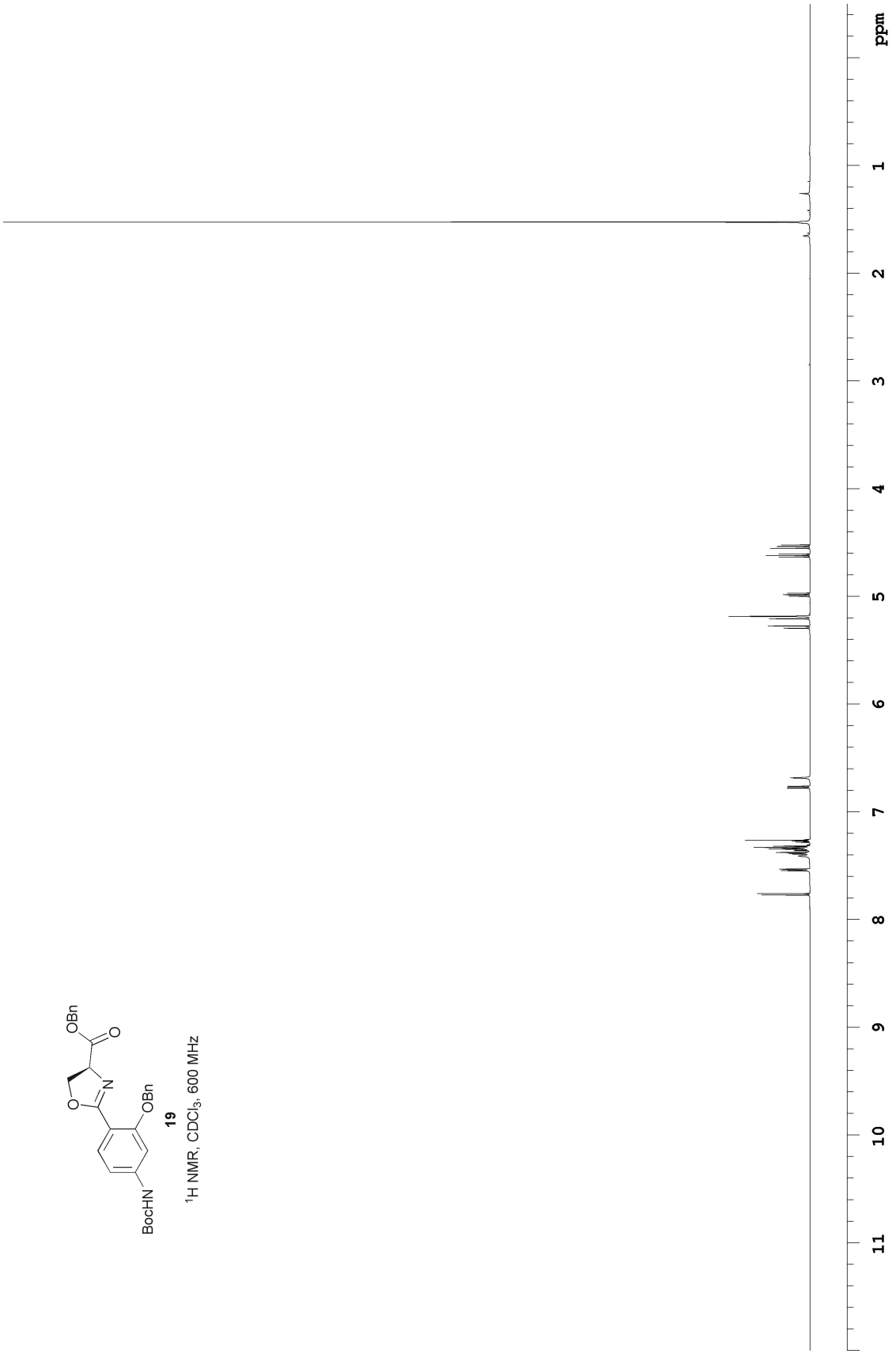

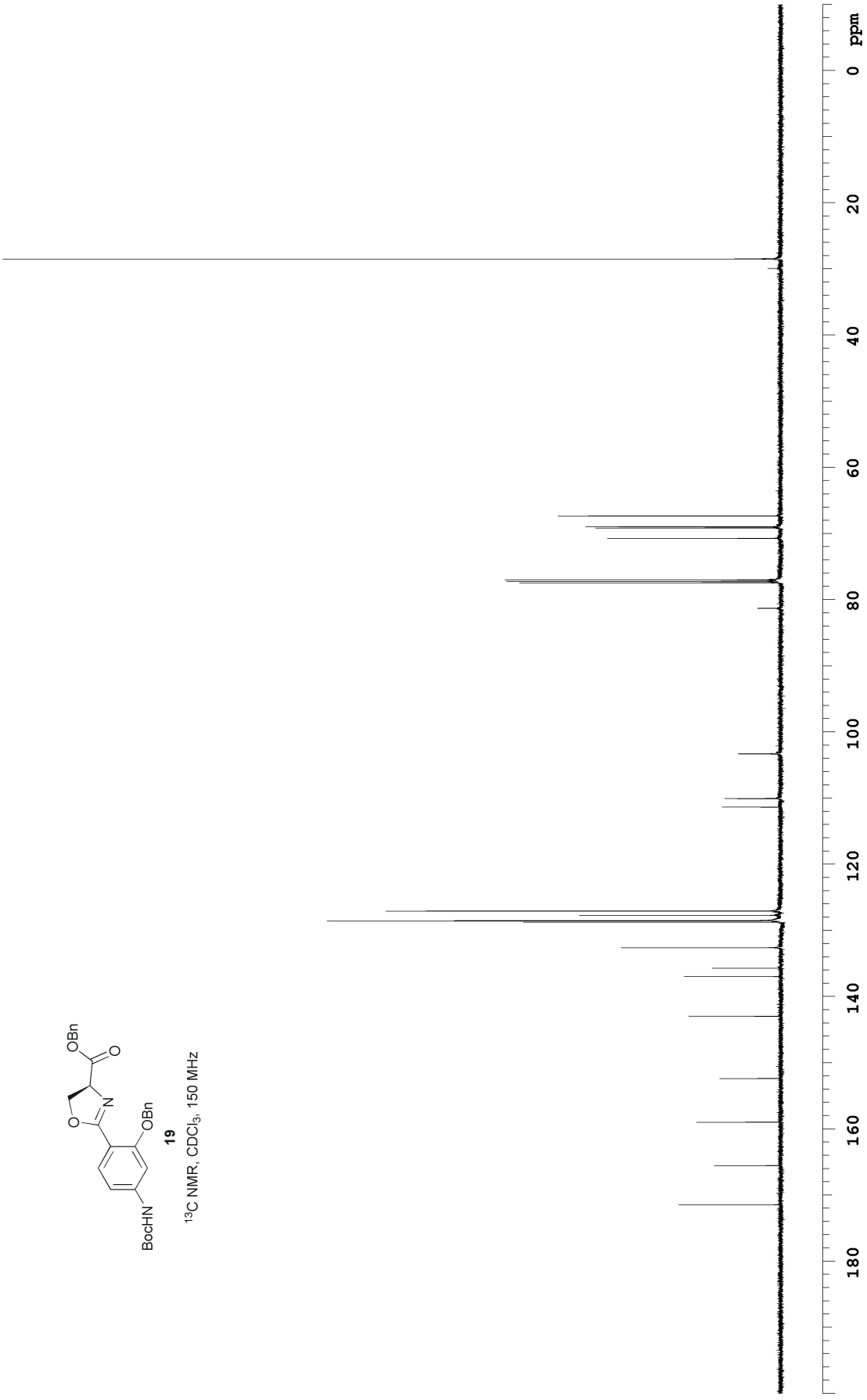

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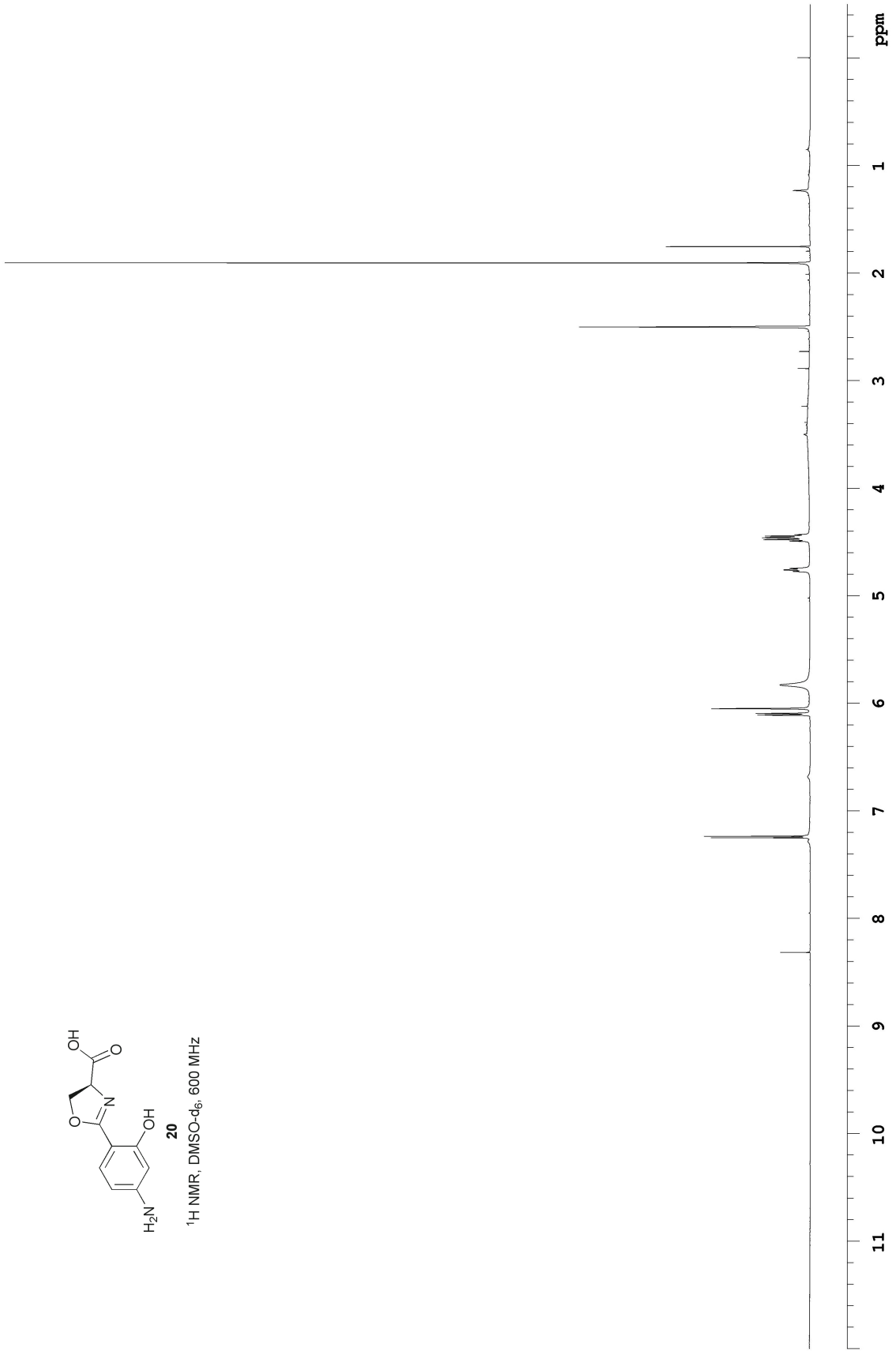

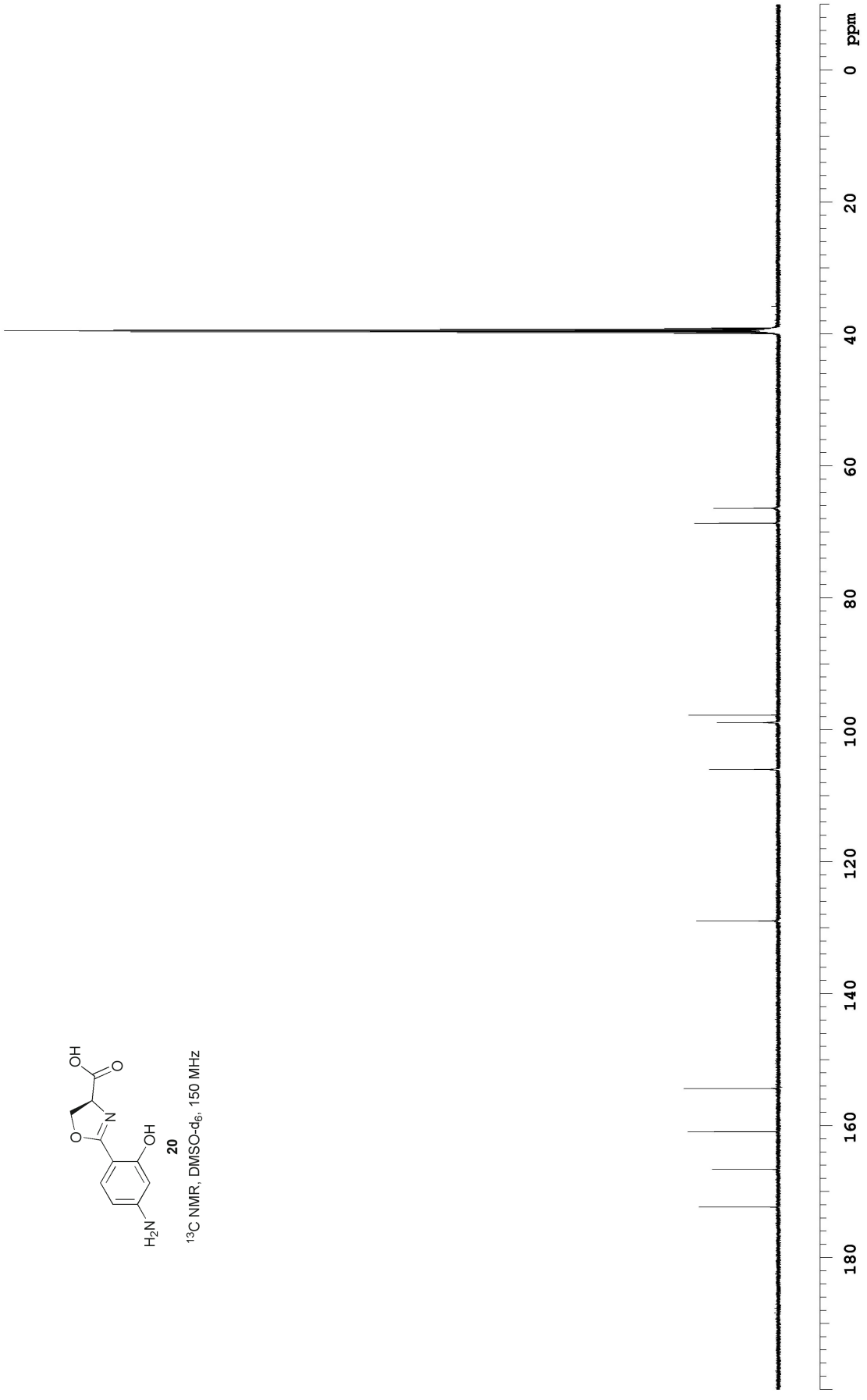

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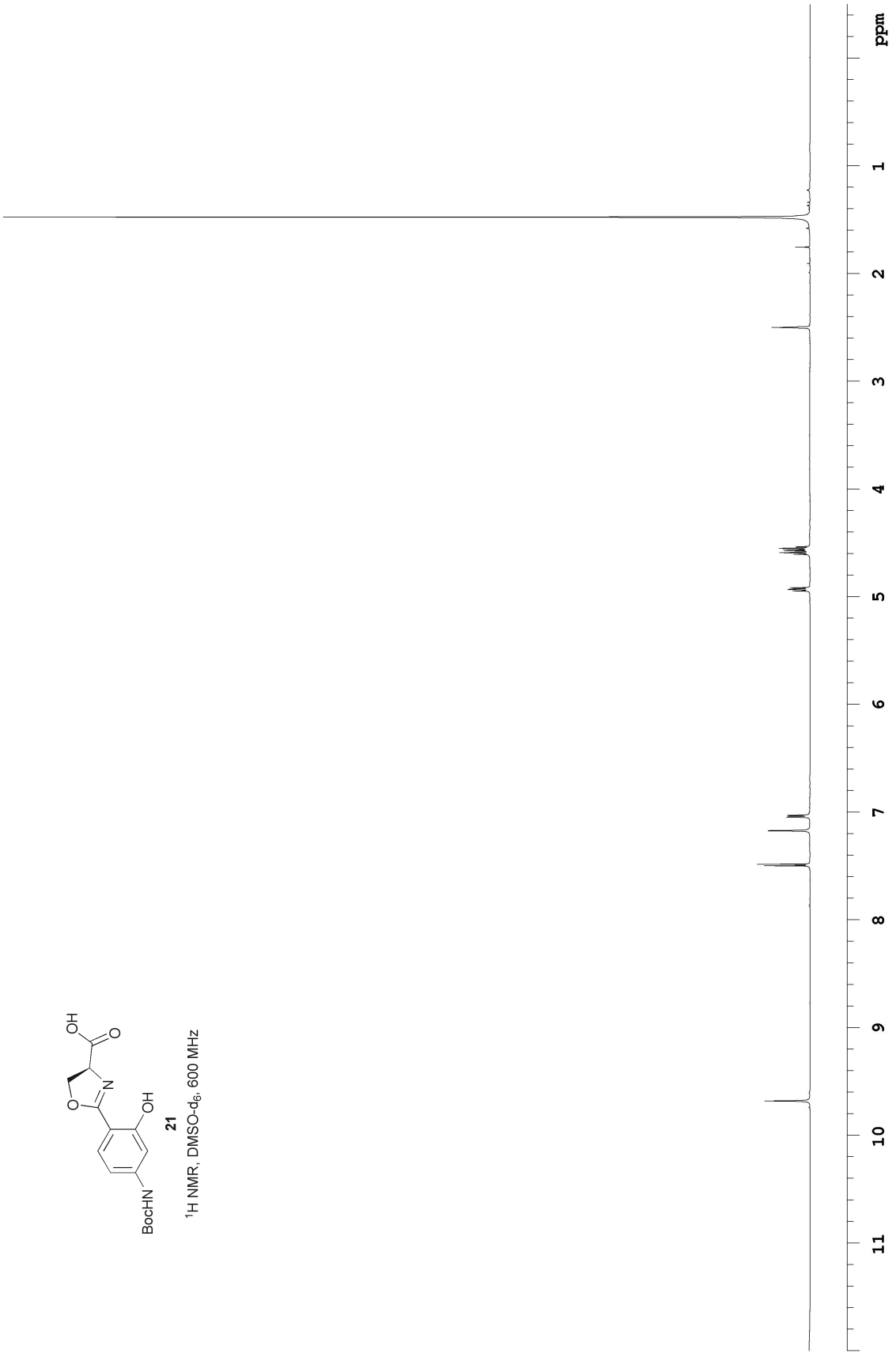

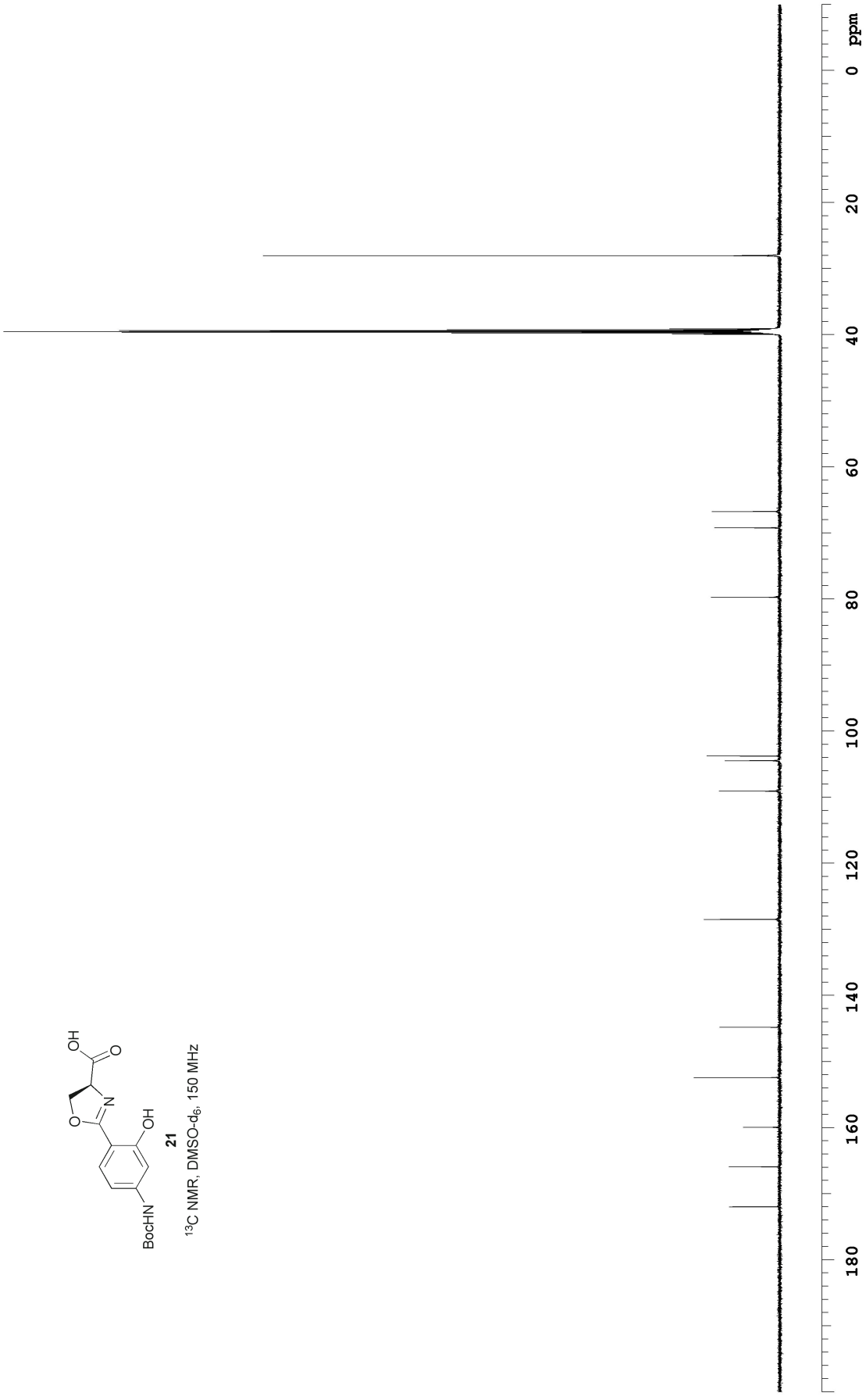

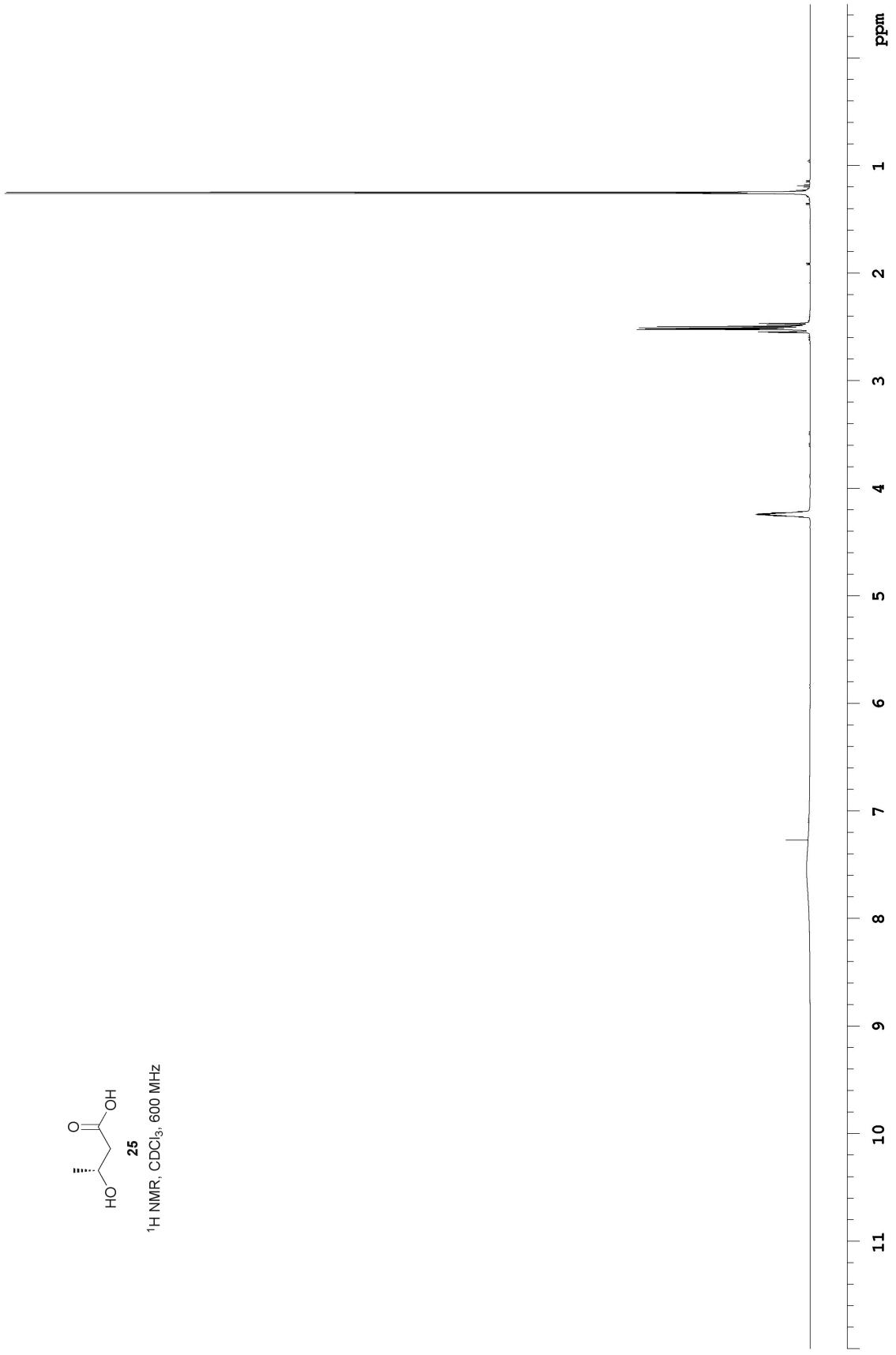

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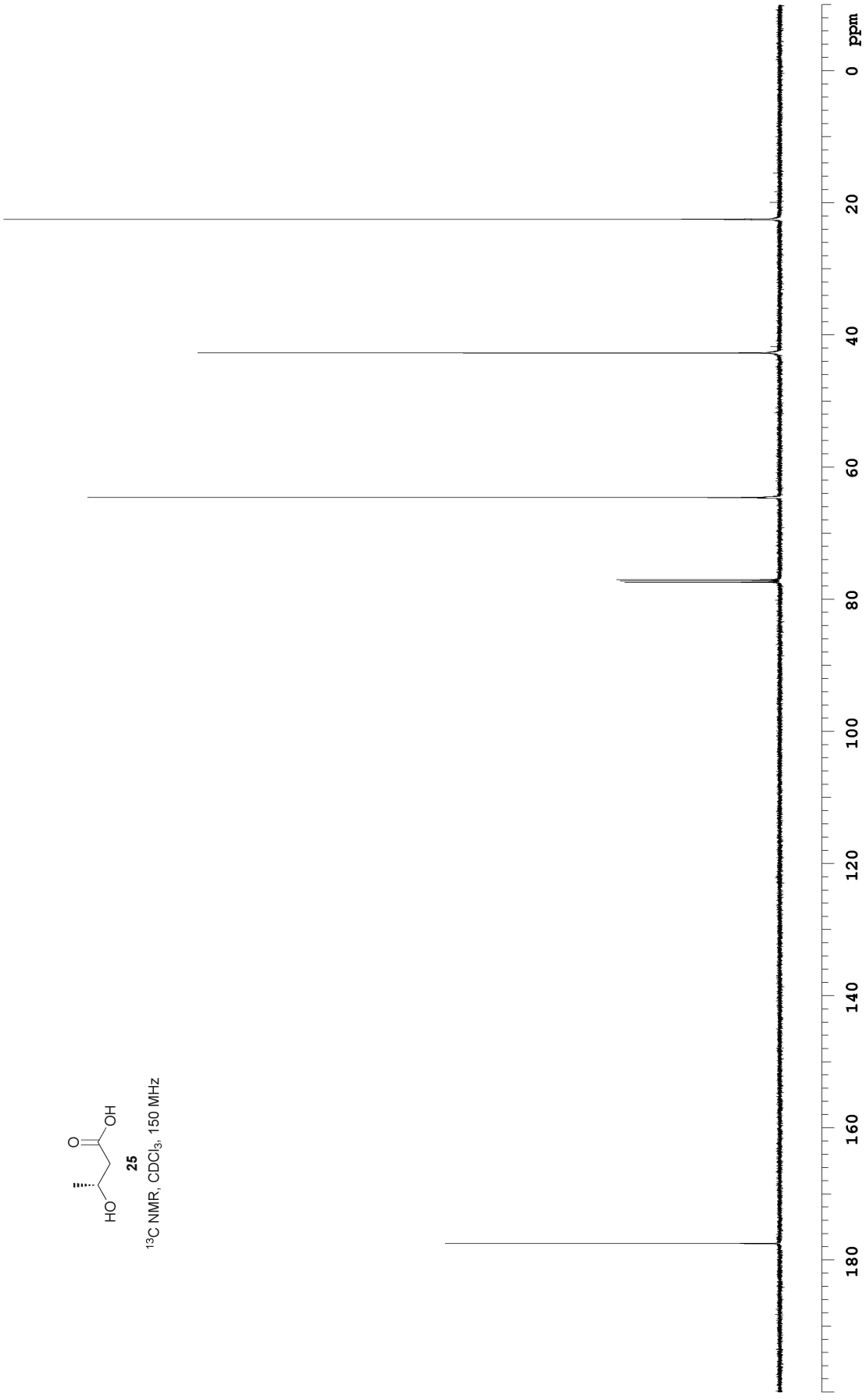

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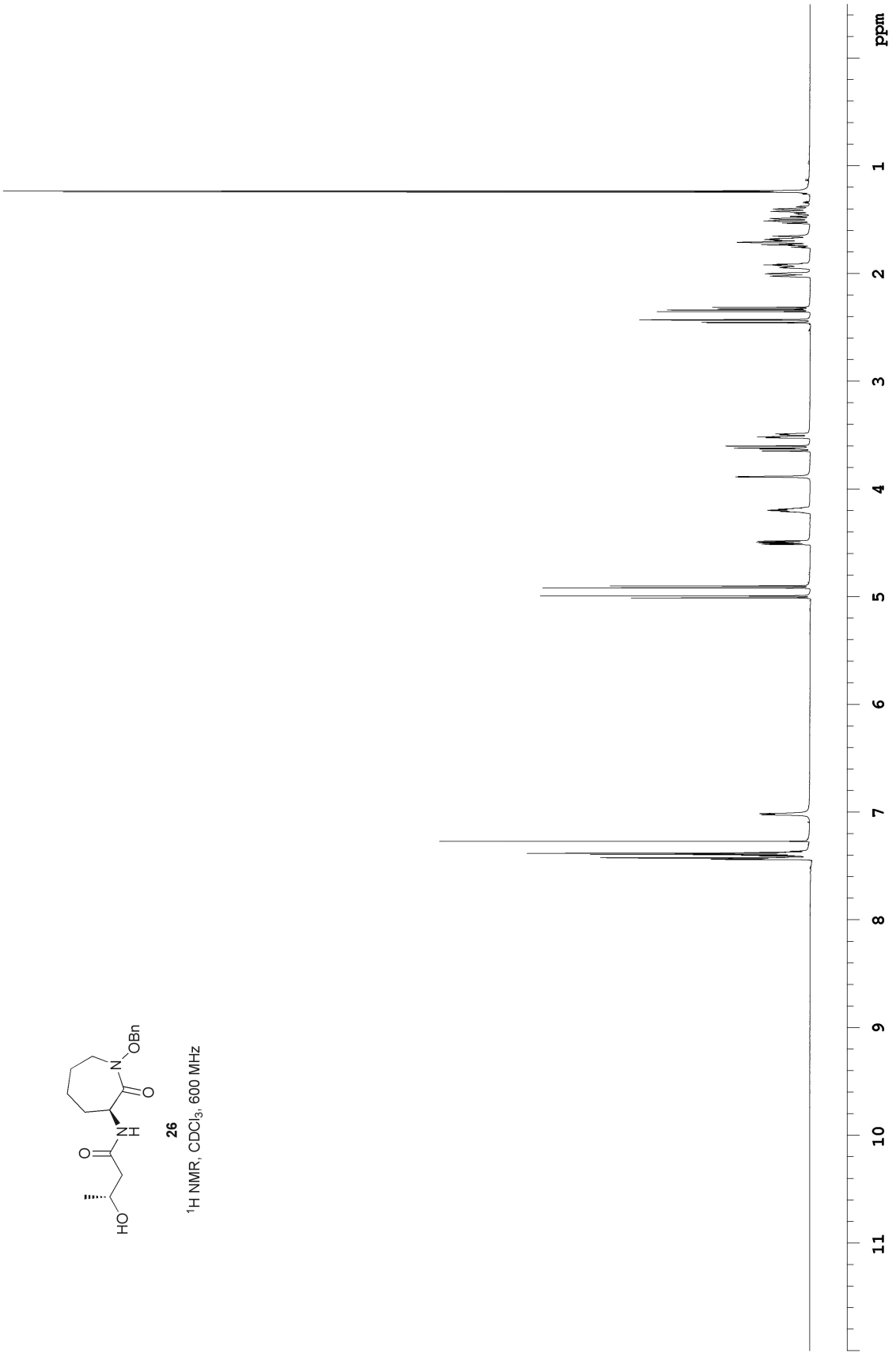

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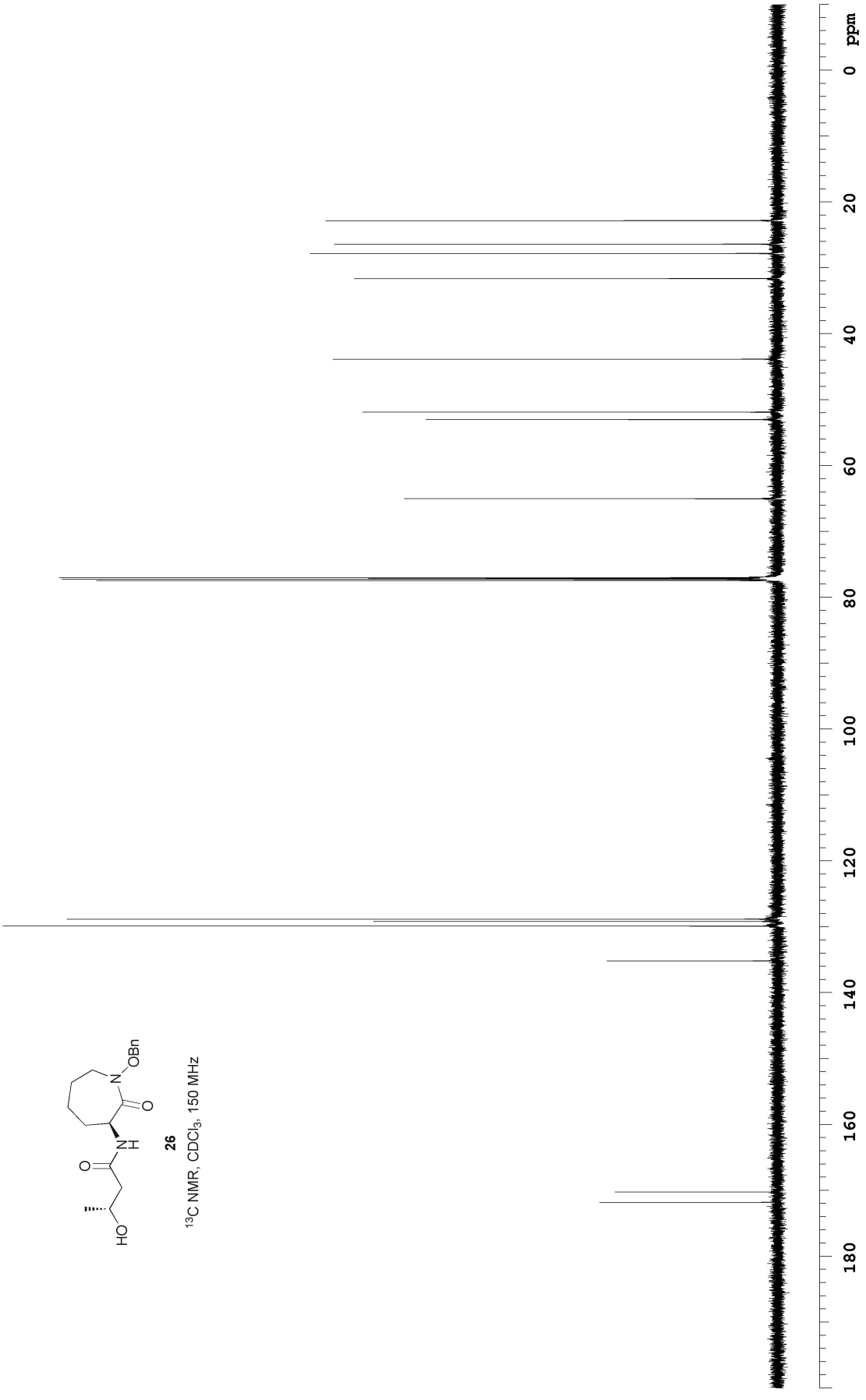

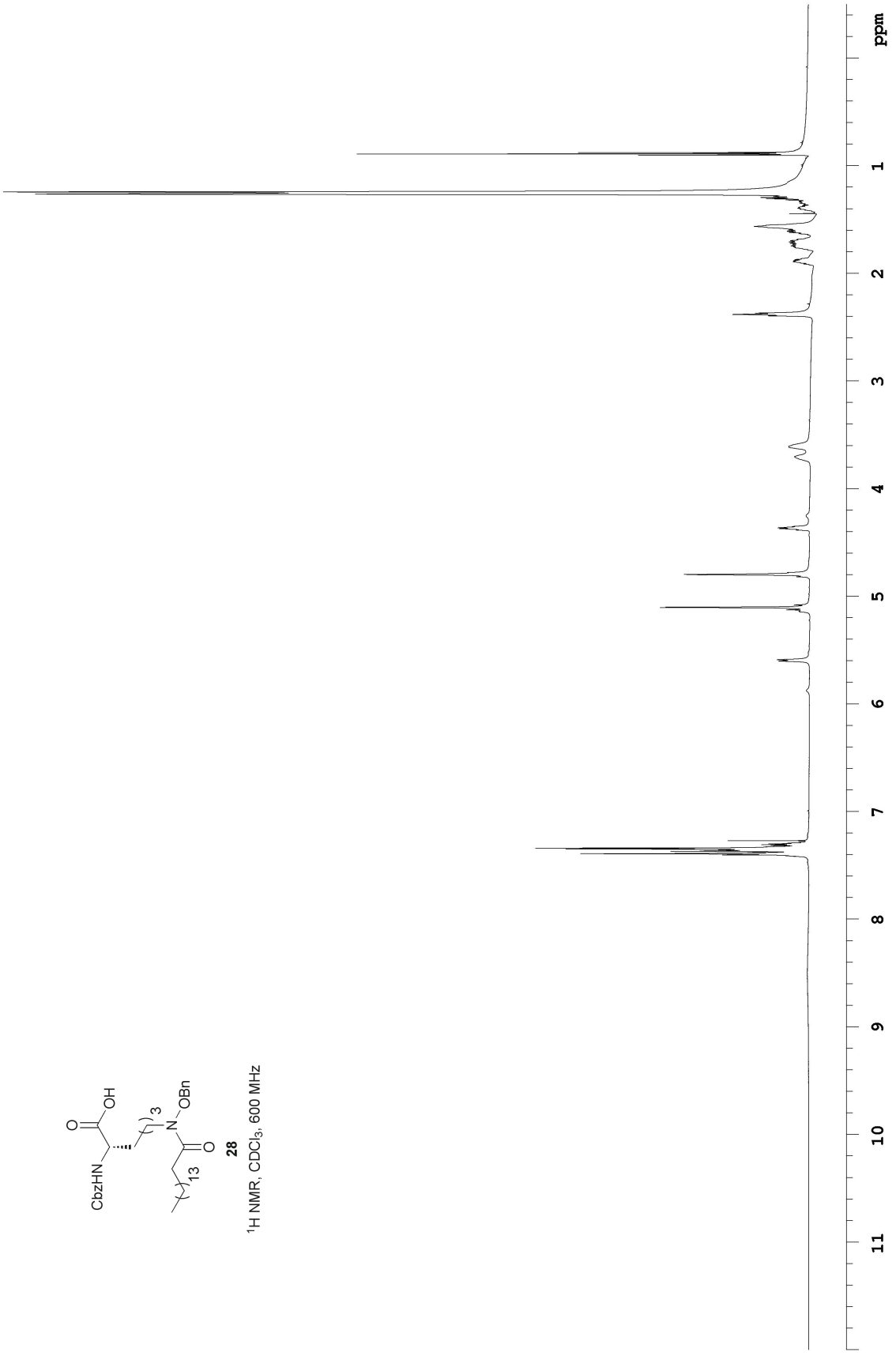

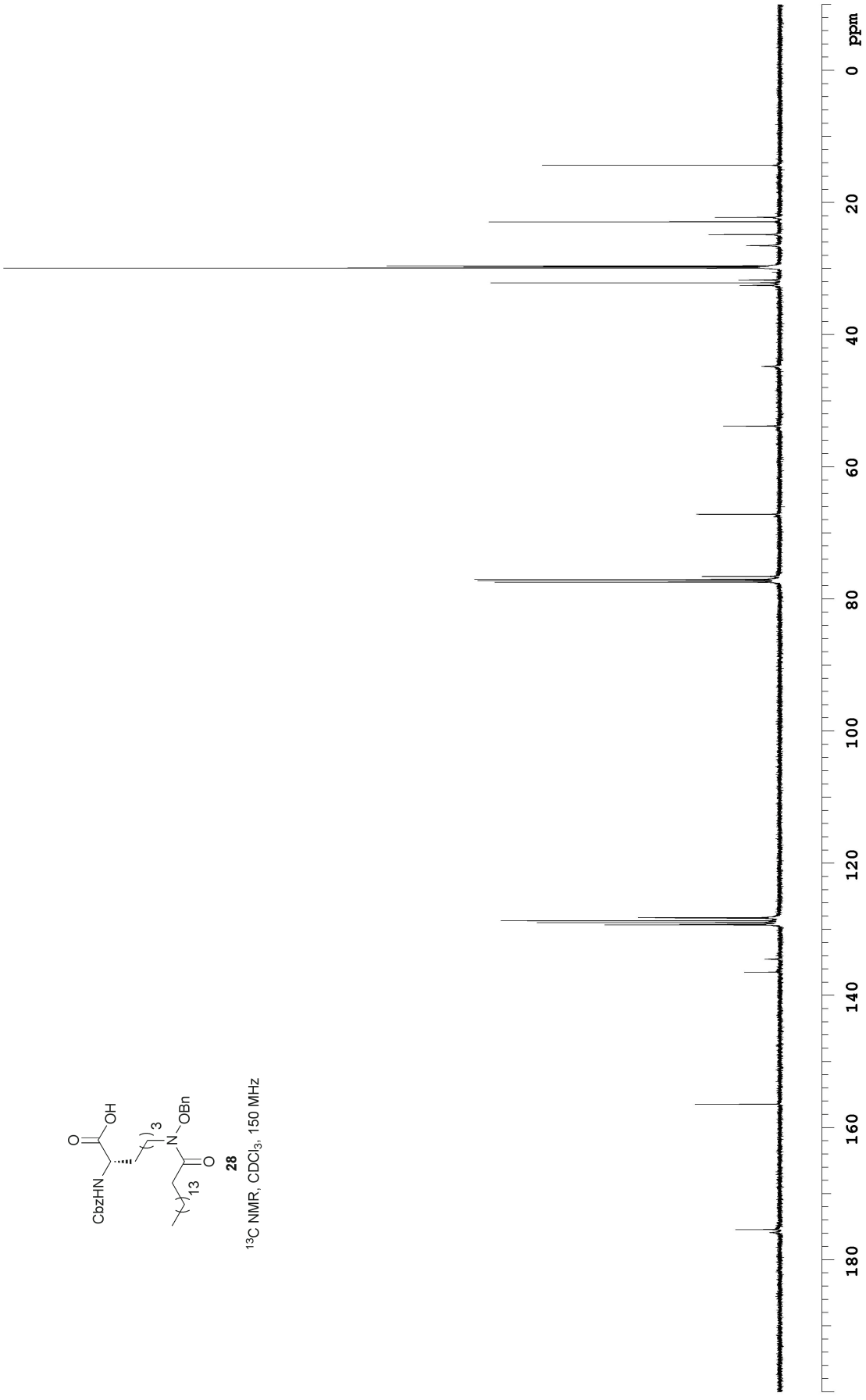

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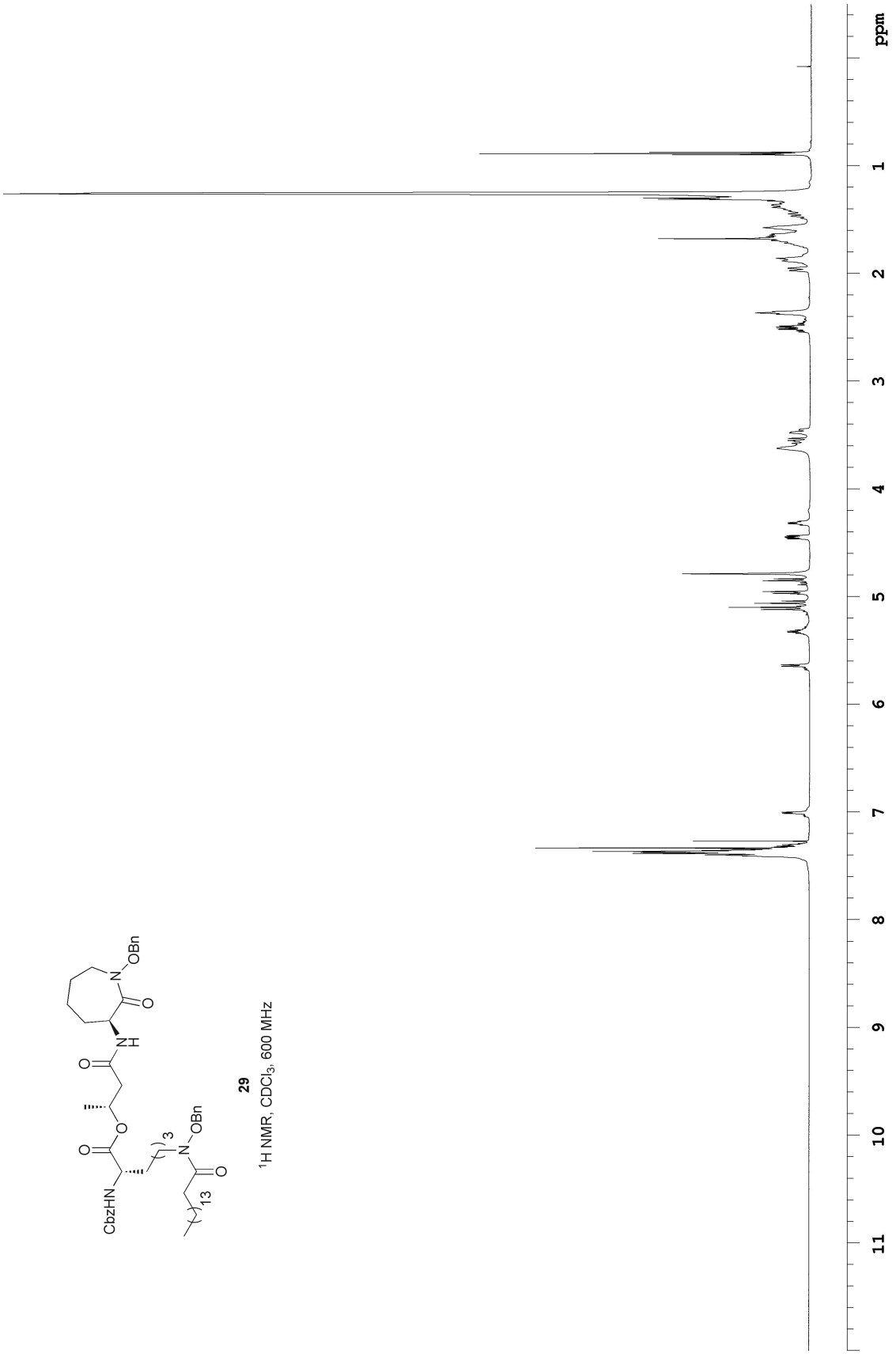

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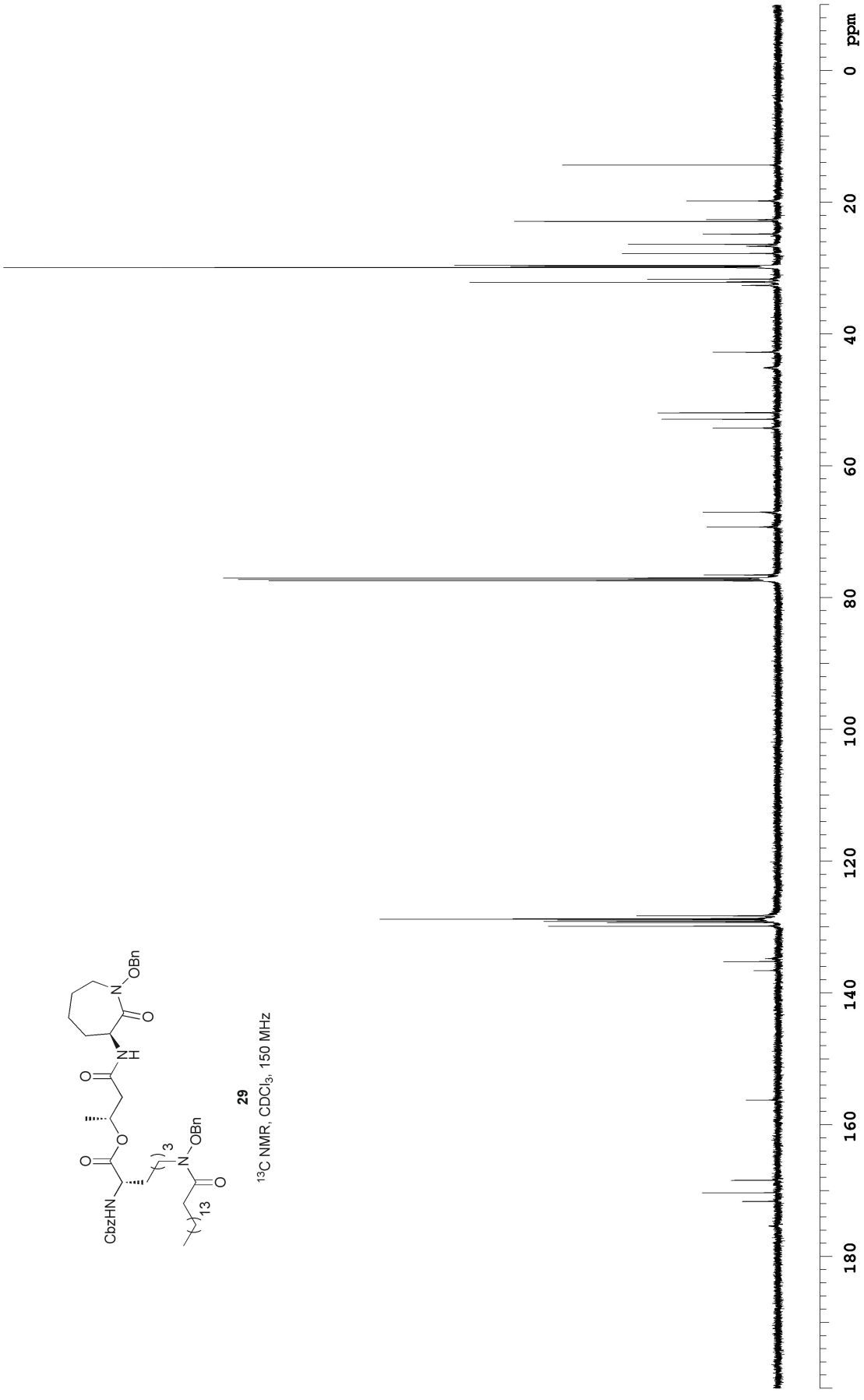

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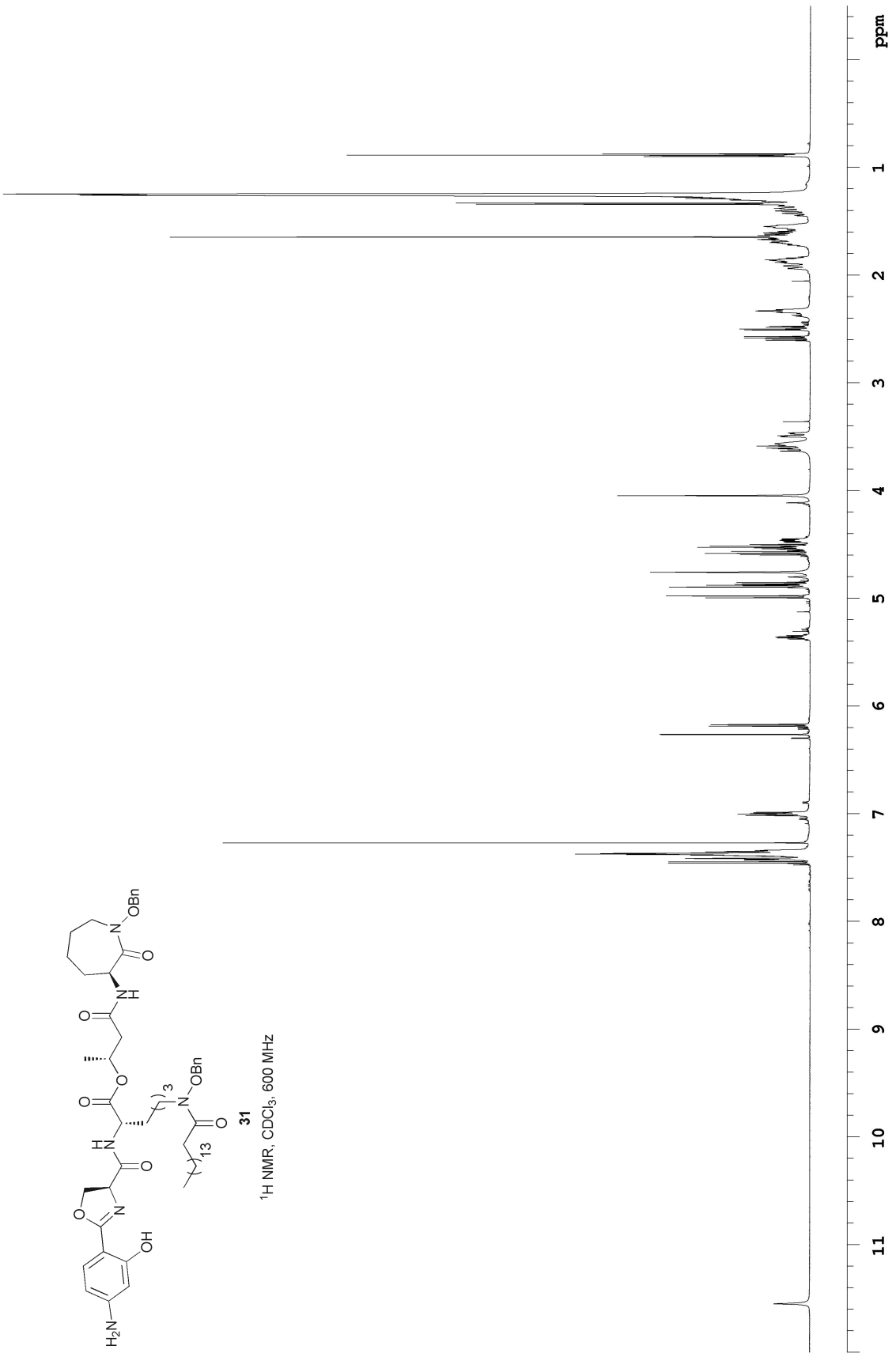

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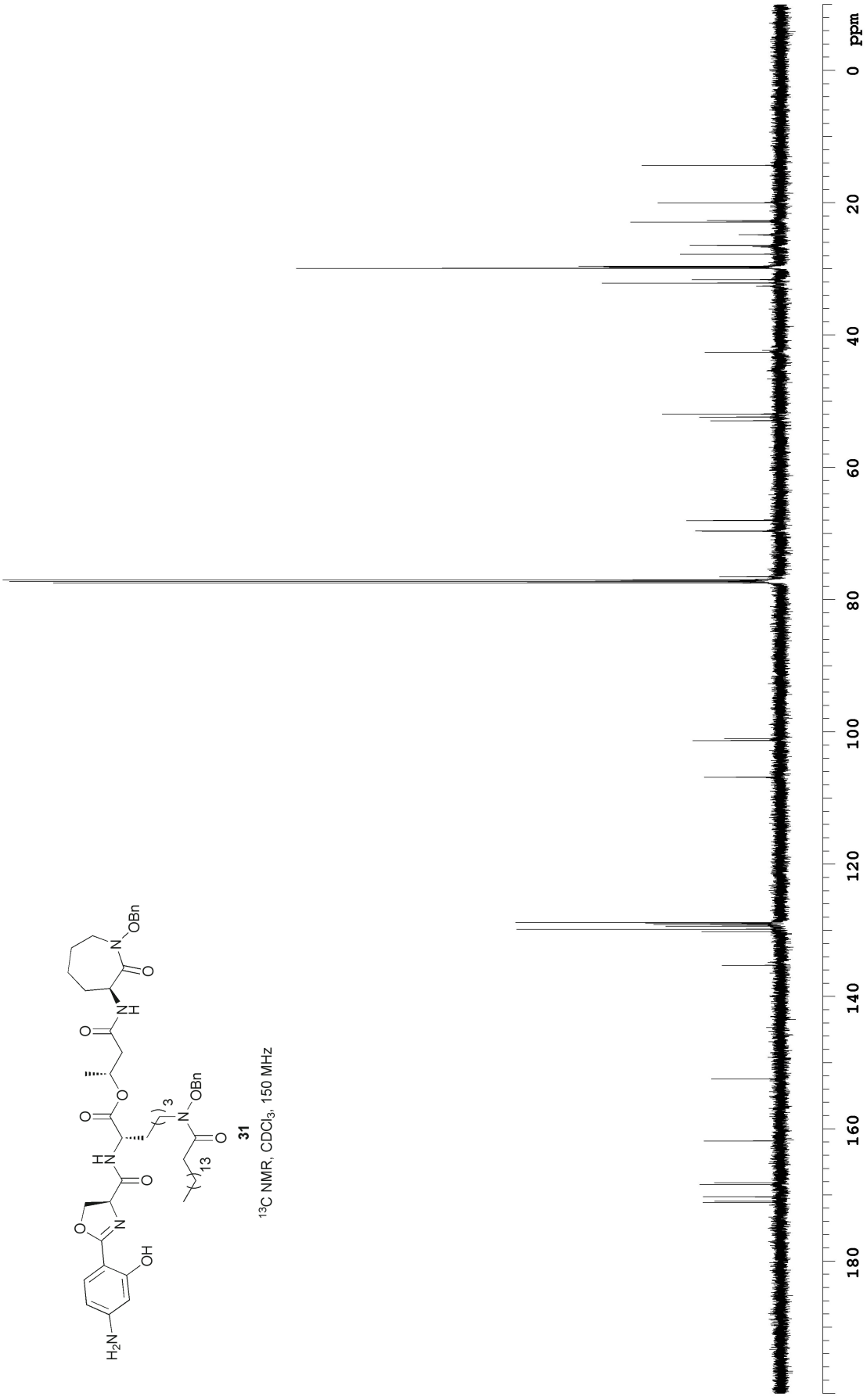

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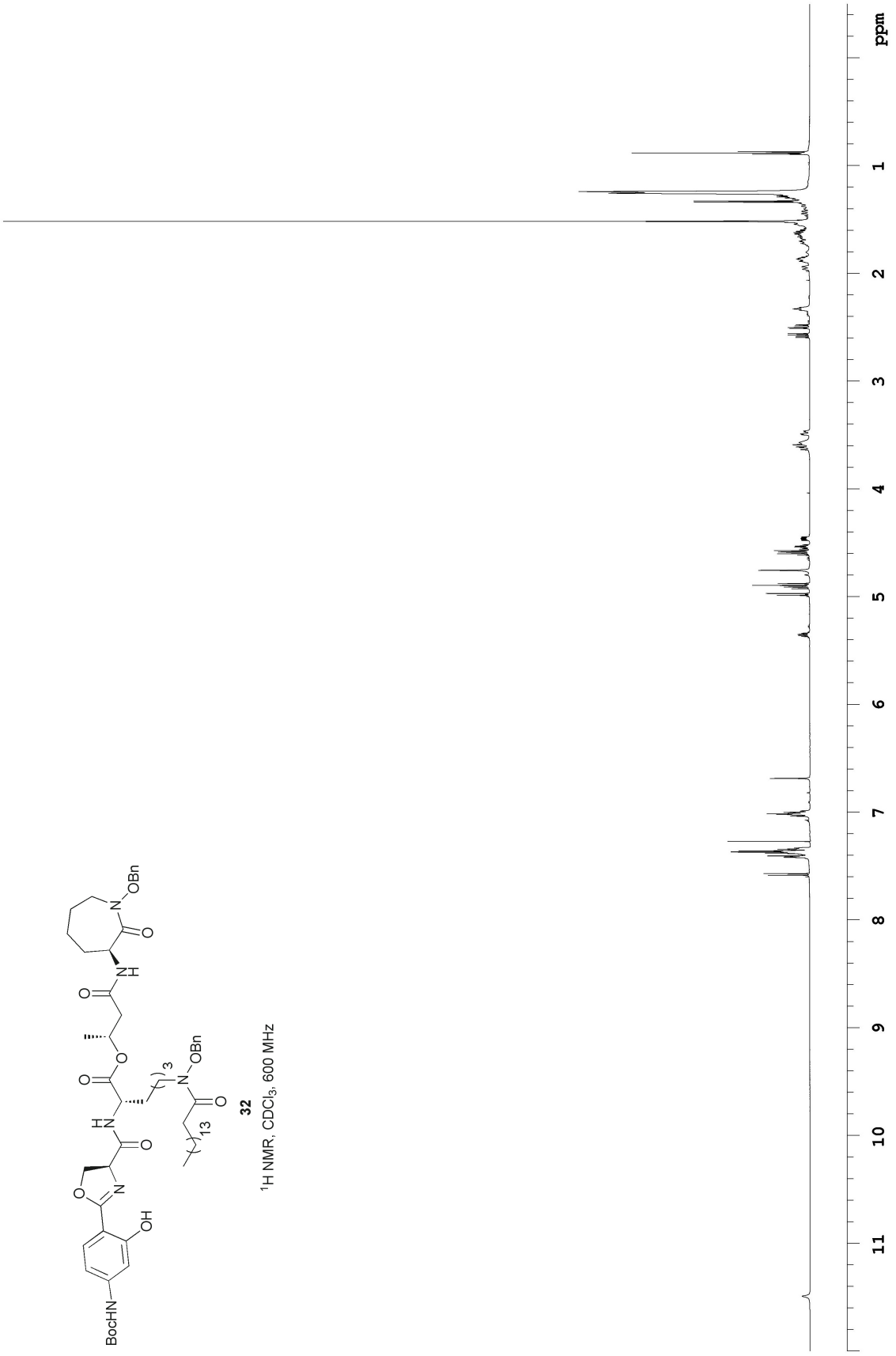

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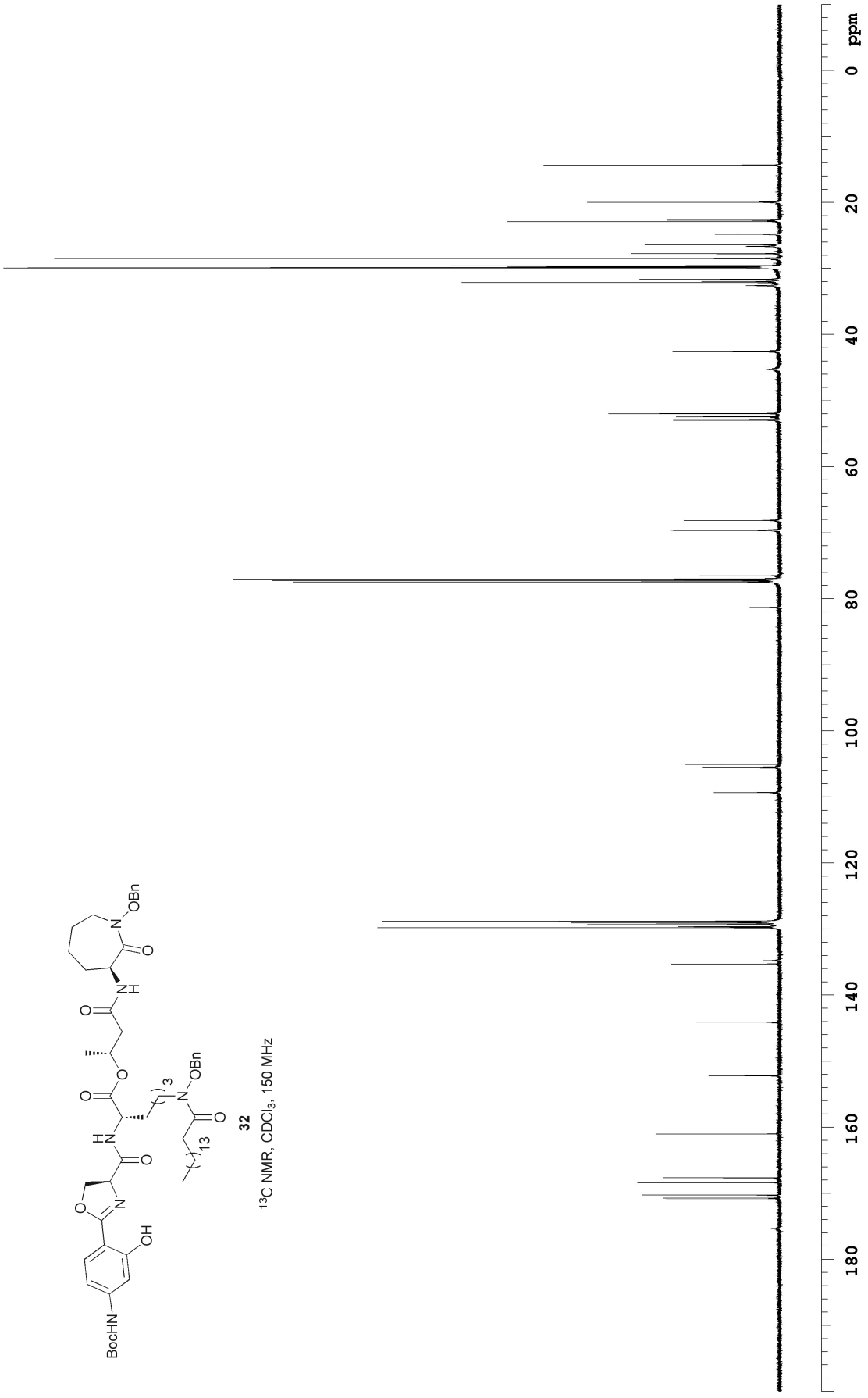

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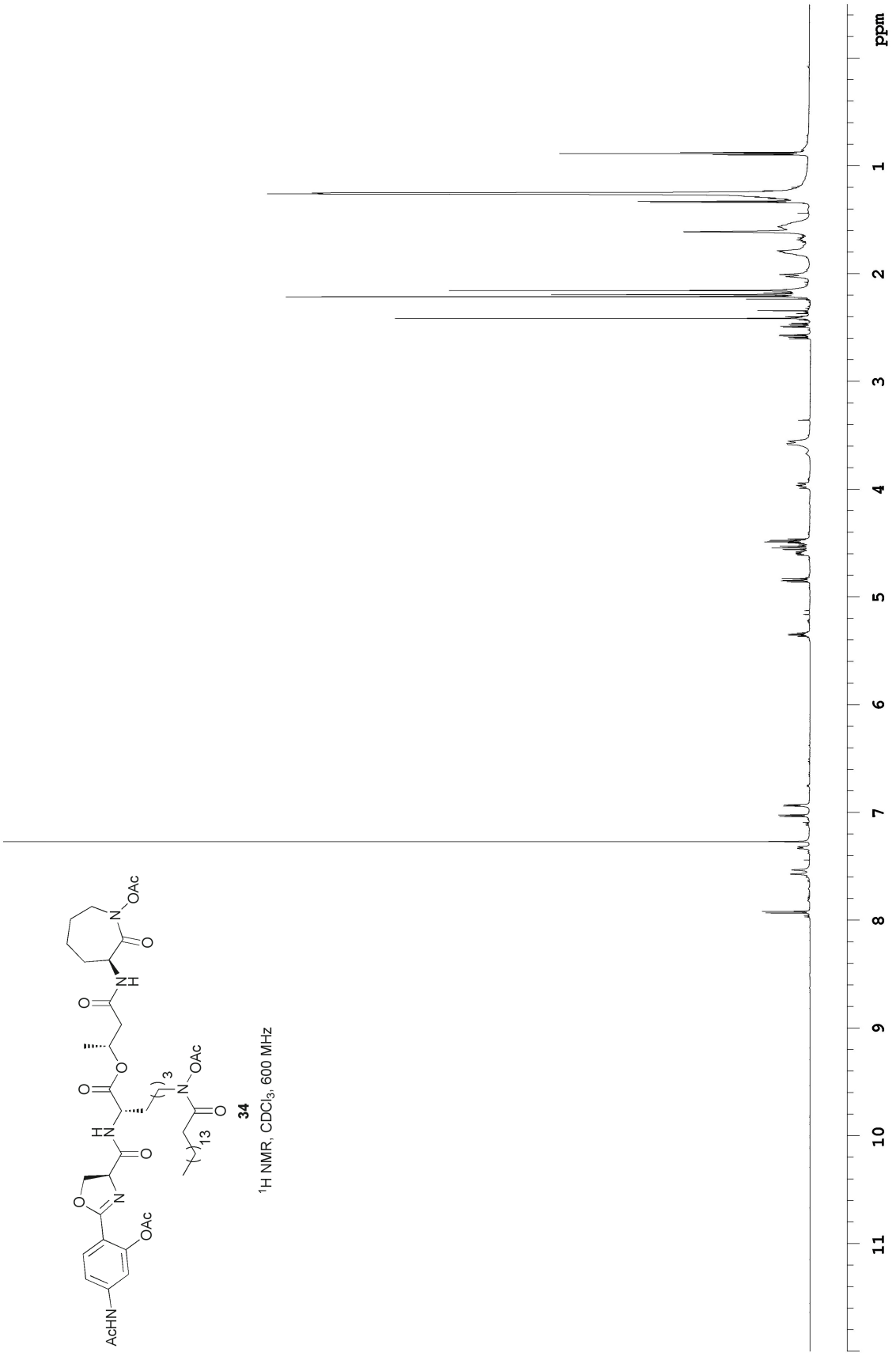

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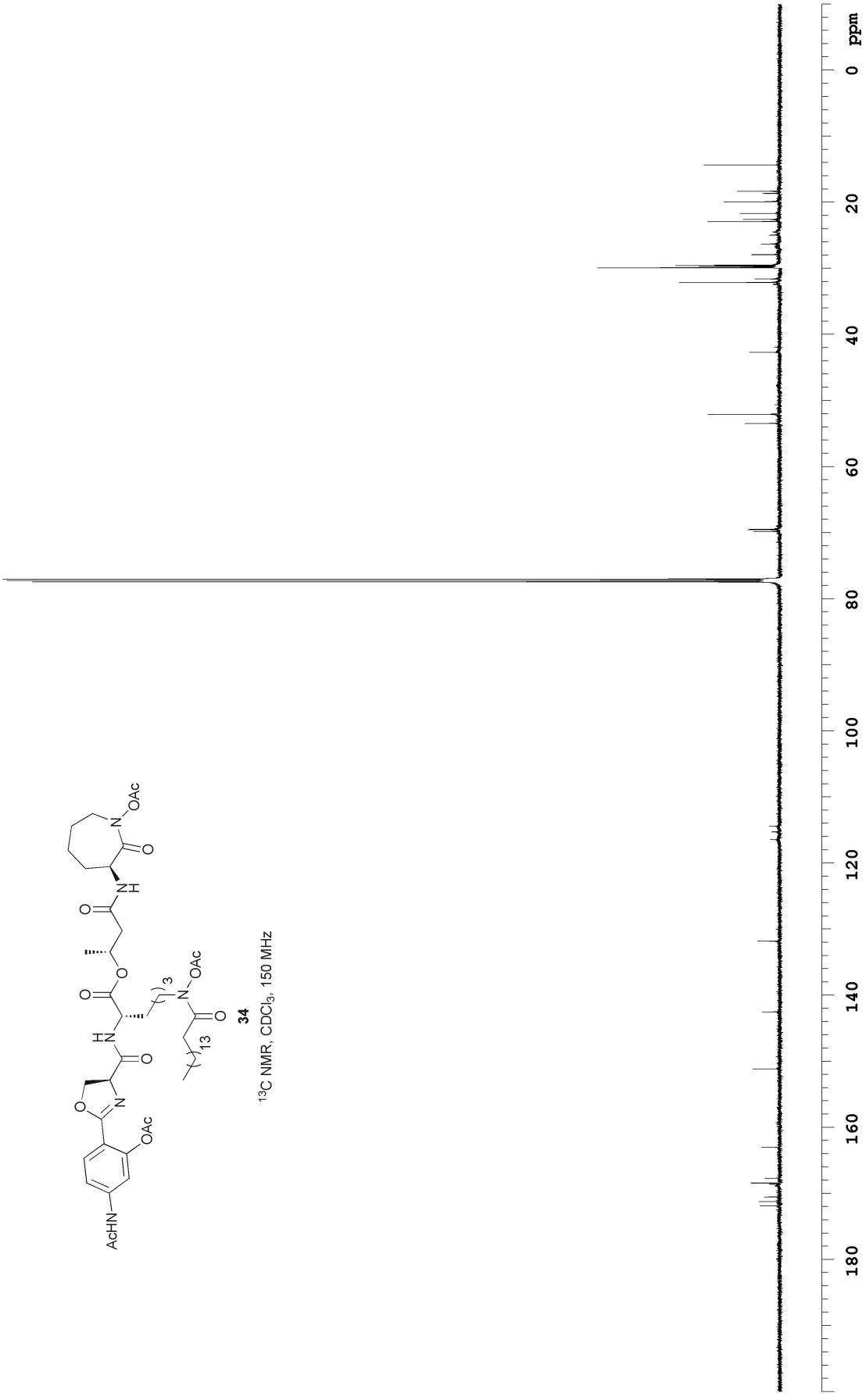

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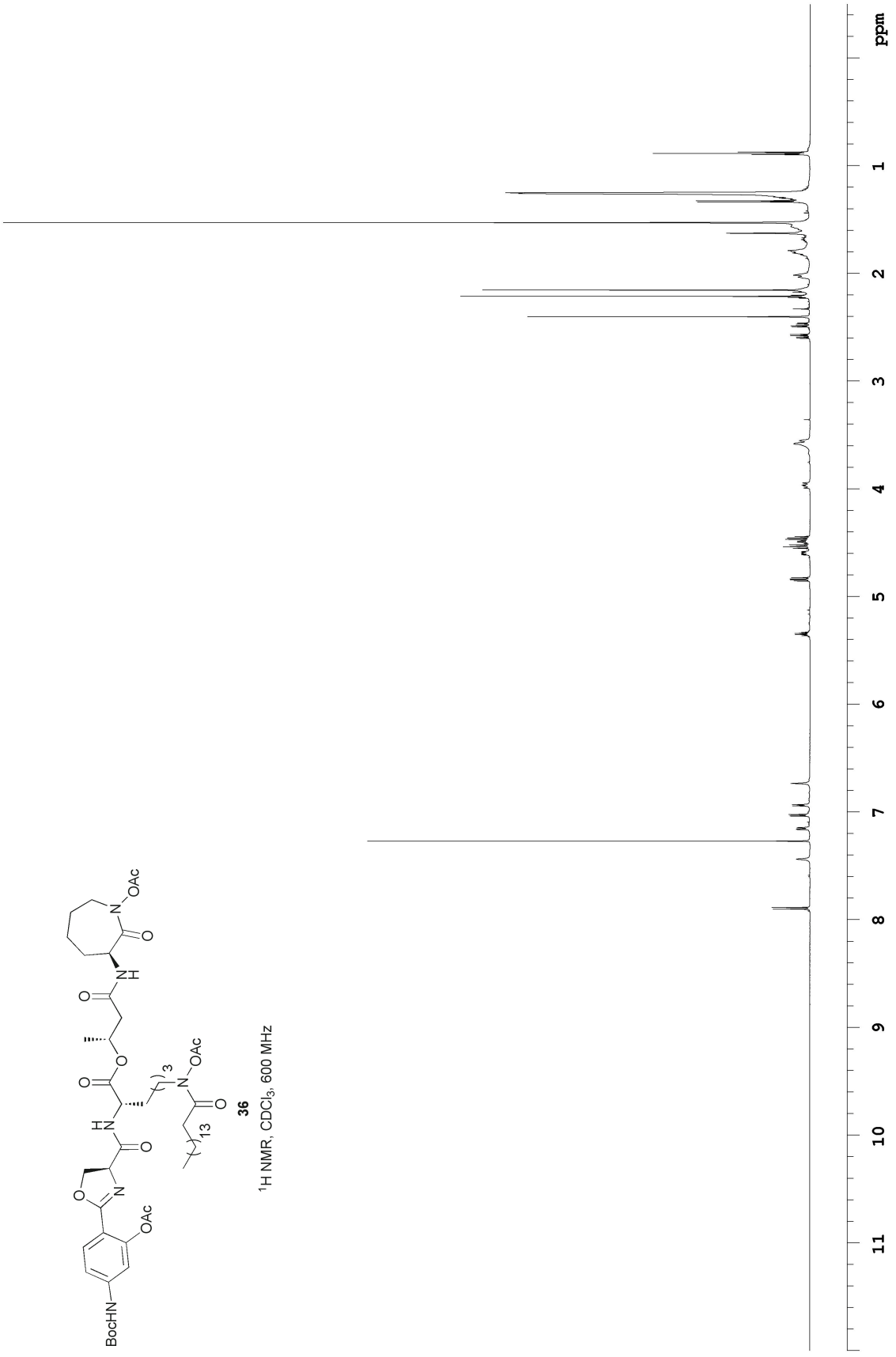

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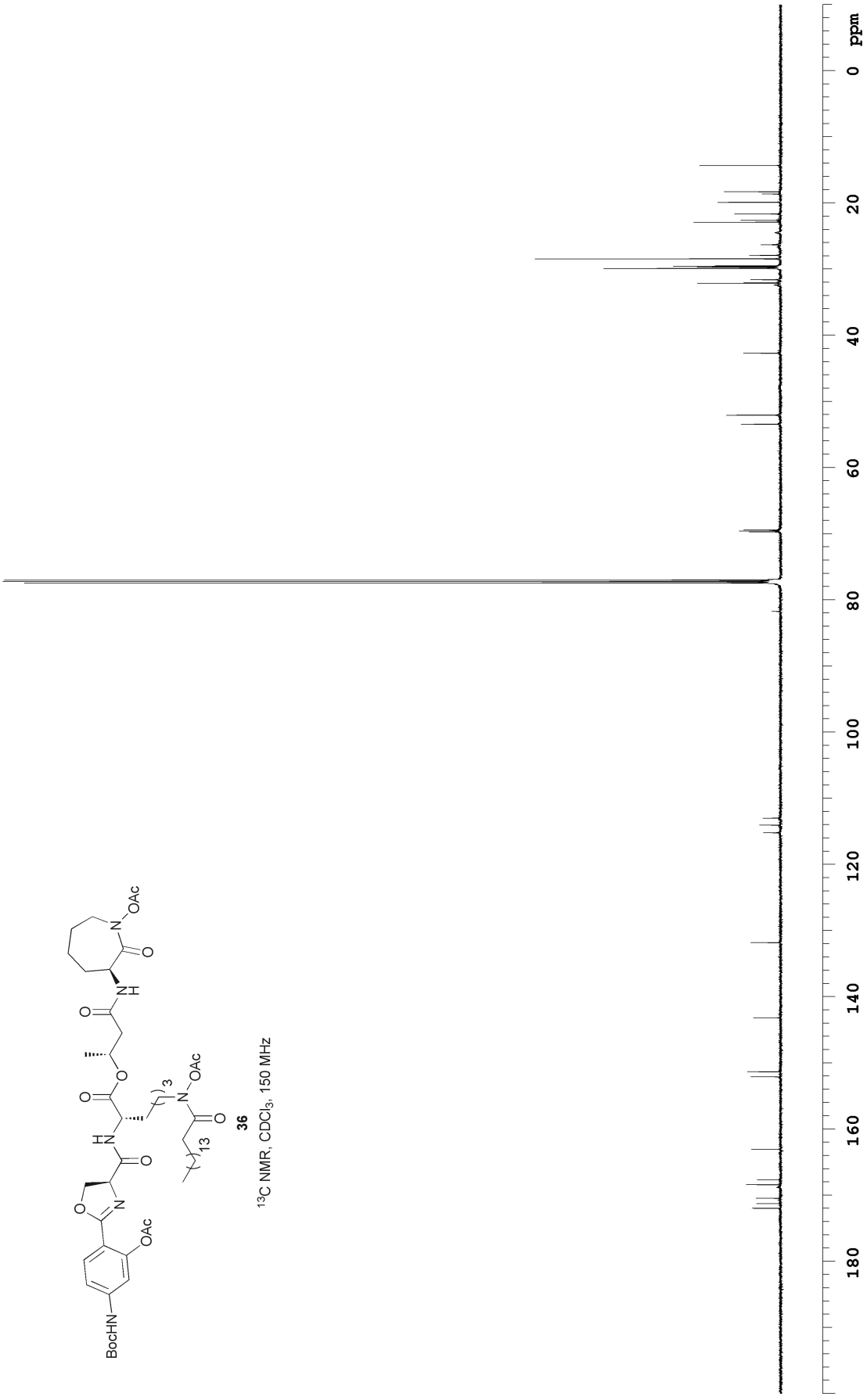

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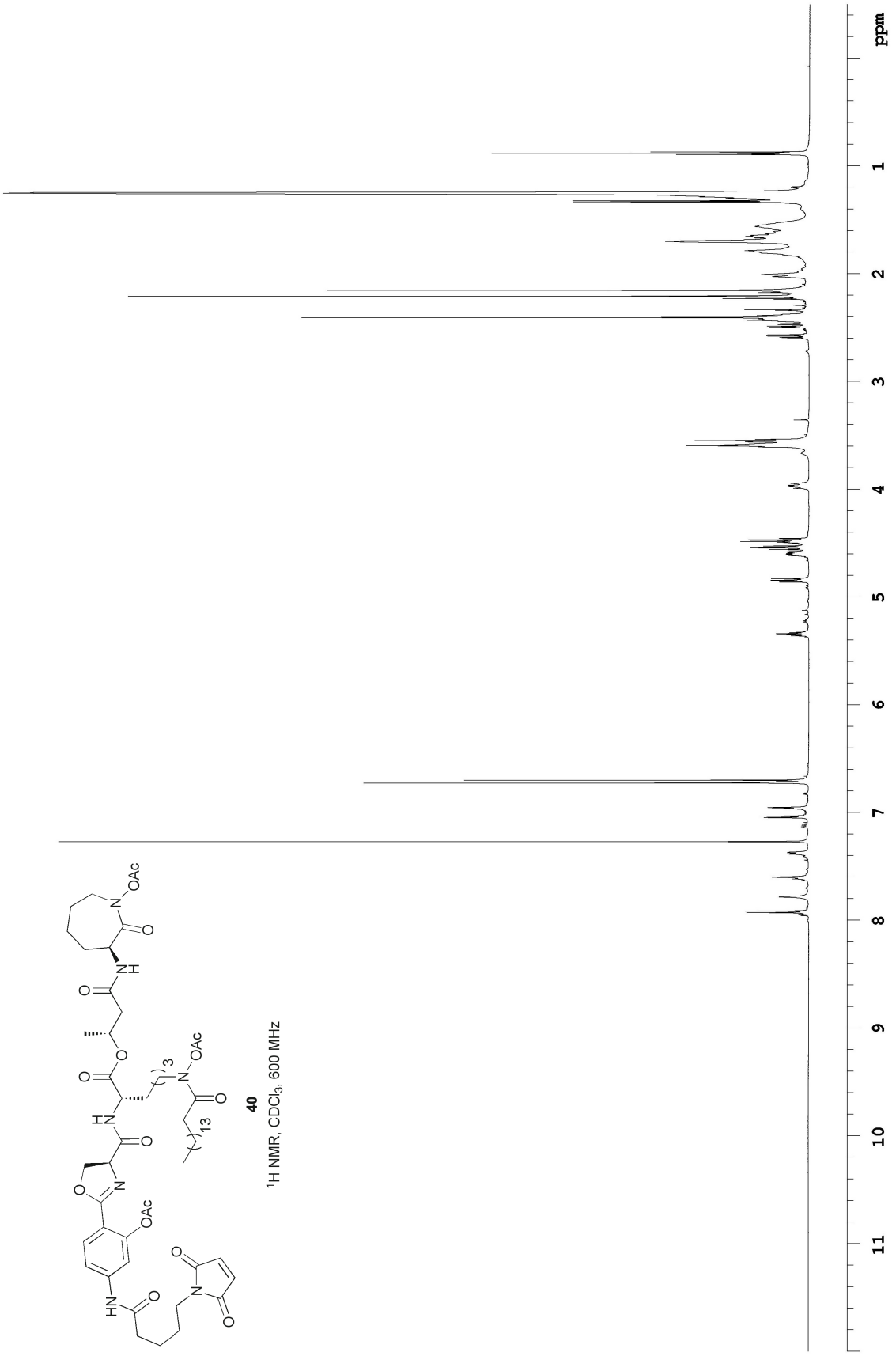

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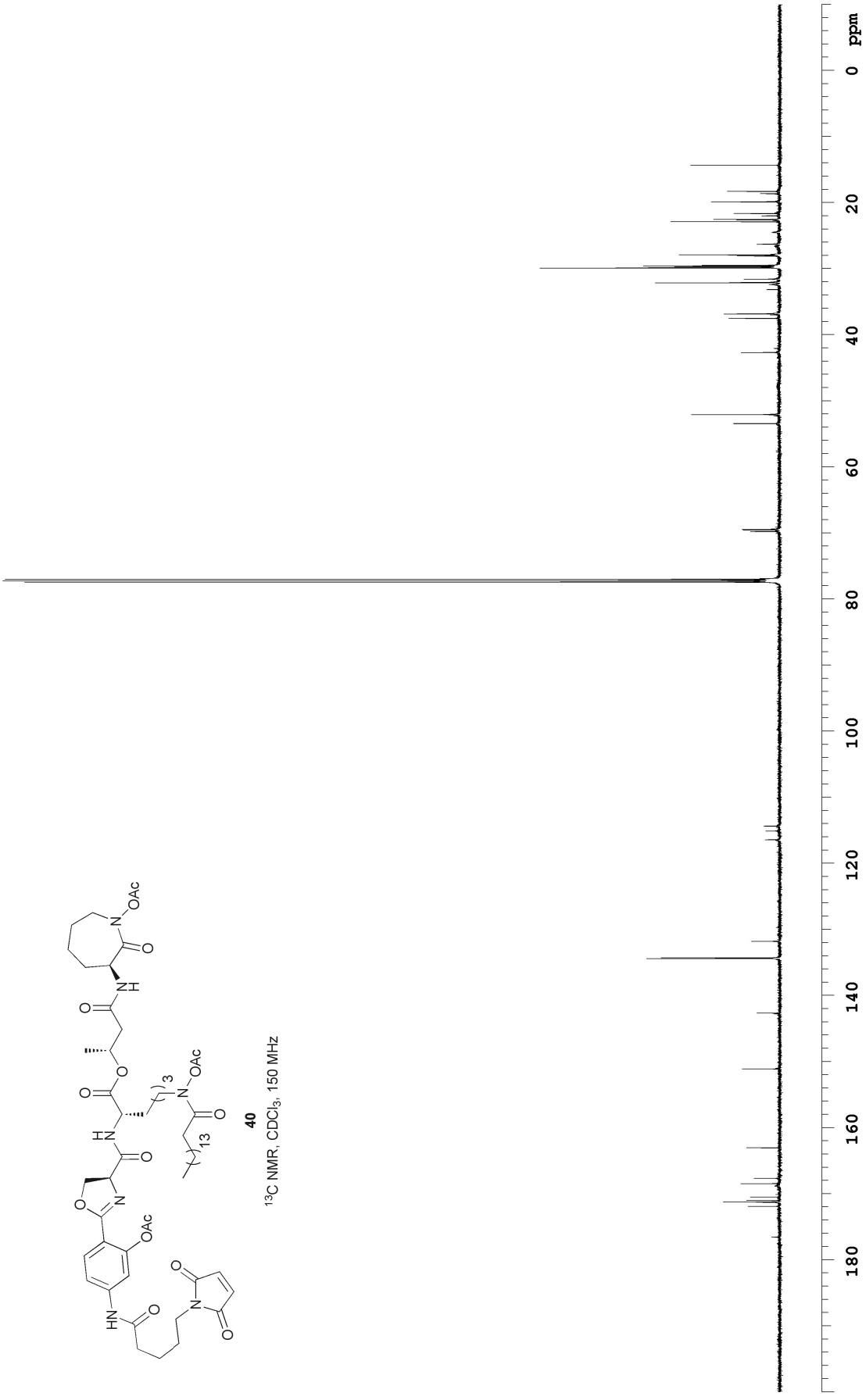

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