Type-2 Isopentenyl Diphosphate Isomerase. Evidence for a Stepwise Mechanism

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Supporting Information

SYNTHESIS

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3-(Thiomethyl)prop-2-yn-1-ol (3). Propargyl alcohol (**2-OH**, 5.0 g, 89 mmol) in dry THF was cooled to -78 °C. *n*-Butyllithium (75 mL, 187 mmol) was added drop wise by syringe, and the reaction mixture was allowed to stir for 1 h at -78 °C. Methylthiocyanate (9.1 mL, 134 mmol) was added drop wise via syringe, and the mixture was allowed to stir an additional 2 h at -78 °C. The mixture was warmed to 0 °C in an ice bath, and the reaction was quenched by the slow addition of $NaHCO_3$ (75 mL). The layers were separated, and the organic layer was extracted with ether (10 x 50 mL). The organic layers were combined, dried over $MgSO_4$, filtered and concentrated. The resulting crude oil was purified by flash chromatography on silica using hexanes:ethyl acetate (3:2) to yield 7.6 g (83%) of an orange oil; ^{1}H NMR ($CDCl_3$) δ 1.74 (bs, 1H), 2.39 (s, 3H), 4.36 (s, 2H); ^{13}C NMR (d_6 acetone) δ 19.2, 51.4, 76.4, 93.6; HRMS (EI) calcd for C_4H_6OS 102.0134; found 102.0138.

3-Hydroxypropanethioate (4-OH). To a suspension of **3** (5.0 g, 49 mmol), silica gel (2.0 g) and 1 mL of H_2O in CH_2Cl_2 was added by syringe trifluoroacetic acid (3.6 mL, 49 mmol). The mixture was heated to 40 °C for 1 h, allowed to cool to rt, dried over $MgSO_4$, filtered and concentrated. The residue was chromatographed on silica using hexanes:ether (3:2) to give 1.05 g (18%) of a colorless oil; ^{1}H NMR ($CDCl_3$) δ 2.34 (s, 3H), 2.84 (t, 2H, J = 5.8 Hz), 3.92 (t, 2H, J = 5.3 Hz); ^{13}C NMR ($CDCl_3$) δ 11.8, 46.2, 59.0, 199.8; HRMS (EI) calcd for $C_4H_8O_2S$ 120.0245; found 120.0248.

3-(*tert*-Butyldimethylsilyloxy)propanethioate (4-OTBDMS). A minimal amount of dry THF was used to dissolve *tert*-butylchlorodimethylsilane (1.87 g, 12.5 mmol), which was added to a stirred suspension of **4-OH** (1.0 g, 8.3 mmol), 4-dimethylaminopyridine (cat.) and imidazole (1.13 g, 17 mmol) in dry THF. The mixture was allowed to stir at rt for 18 h. Ether (100 mL) was added and the mixture washed with H_2O. The aqueous layer was extracted with ether (3 x 50 mL). The organic layers were combined, washed with brine, dried over Na_2SO_4, filtered and concentrated. The residue was chromatographed on silica using hexanes:ethyl acetate (95:5) to give 1.84 g (95%) of a light yellow oil; ^{1}H NMR ($CDCl_3$) δ 0.06 (s, 6H), 0.88 (s, 9H), 2.31 (s, 3H), 2.77 (t, 2H, J = 6.3 Hz), 3.93 (t, 2H, J = 6.1 Hz); ^{13}C NMR ($CDCl_3$) δ -5.2, 11.8, 18.5, 26.0, 47.3, 59.6, 199.4; HRMS (FTMS) calcd for $C_{10}H_{23}O_2SSi$ $(M+H)^+$ 235.1183; found 235.1186.

1-(*tert*-Butyldimethylsilyloxy)-3-thiomethyl-3-butene (1-OTBDMS). To a mixture of **4-OTBDMS** (500 mg, 2.1 mmol) and 50 mL of dry toluene was added drop wise dimethyl titanocene (12.1 mL, 6.4 mmol) via syringe. The mixture was heated to 80 °C and allowed to stir in the dark for 8 h. Additional dimethyl titanocene (4.0 mL, 2.1 mmol) was added and the mixture was allowed to stir for an additional 16 h at 80 °C in the dark. The mixture was allowed to cool to rt. Petroleum ether was added (100 mL) and the mixture was allowed to stir for 1 h at rt. The resulting precipitate was filtered over celite and the cake was washed several times with petroleum ether. The filtrates were combined and concentrated. The residue was chromatographed on silica using hexanes:triethylamine (98:2) to give 351 mg (71 %) of an orange oil; ^{1}H NMR (CD_2Cl_2) δ 0.05 (s, 6H), 0.89 (s, 9H), 2.22 (s, 3H), 2.42 (t, 2H, J = 7.2 Hz), 3.75 (t, 2H, J = 6.9 Hz),

4.65 (s, 1H), 5.06 (s, 1H); ^{13}C NMR (CD_2Cl_2) δ -5.3, 14.8, 18.5, 26.0, 41.3, 62.8, 105.6, 144.0; HRMS (FTMS) calcd for $C_{11}H_{25}OSSi$ $(M+H)^+$; 233.1390 found 233.1393; IR: 1603, 1093 cm^{-1}.

3-(Thiomethyl)but-3-en-1-ol (1-OH). To a solution of **1-OTBDMS** (350 mg, 1.5 mmol) in 10 mL of dry ether was added drop wise via syringe tetra-butylammonium fluoride (4.5 mmol). The mixture was allowed to stir at rt for 2 h before addition of saturated Na_2CO_3 (25 mL). The aqueous layer was extracted with ether (3 x 50 mL). The organic layers were combined, dried over Na_2SO_4, filtered and concentrated using a stream of N_2. The residue was chromatographed on silica using hexanes:ether:triethylamine (48:50:2) to give 110 mg (62%) of a pale yellow oil; ^{1}H NMR (CD_2Cl_2) δ 2.25 (s, 3H), 2.47 (t, 2H, *J* = 6.0 Hz), 3.72 (t, 2H, *J* = 6.1 Hz), 4.71 (s, 1H), 5.10 (s, 1H); ^{13}C NMR (CD_2Cl_2) δ 15.0, 41.3, 61.8, 106.2, 114.2; HRMS (MALDI) calcd for $C_5H_{11}OS$ $(M+H)^+$ 119.0525; found 119.0545.

3-(Thiomethyl)but-3-enyl tosylate (1-OTs). To a solution of 4-dimethylaminopyridine (62 mg, 0.5 mmol) and p-TsCl (97 mg, 0.5 mmol) in dry CH_2Cl_2, was added **1-OH** (50 mg, 0.4 mmol) in dry CH_2Cl_2. The mixture was allowed to stir for 18 h at rt. The mixture was concentrated by rotary evaporation and chromatographed on silica using hexanes:ethyl acetate:triethylamine (78:20:2) to give 96 mg (84%) of a pale yellow oil. The concentrated compound decomposed and was maintained in solution until used in the next step. ^{1}H NMR (CD_2Cl_2) δ 2.18 (s, 3H), 2.44 (s, 3H), 2.54 (t, 2H, *J* = 6.6 Hz), 4.13 (t, 2H, *J* = 6.7 Hz), 4.67 (s, 1H), 5.04 (s, 1H), 7.35-7.38 (m, 2H), 7.74-7.71 (m, 2H); ^{13}C NMR (CD_2Cl_2) δ 15.1, 21.9, 37.2, 69.4, 107.4, 128.4, 130.4, 133.4, 142.0, 145.6.

3-(Thiomethyl)but-3-enyl diphosphate (1-OPP). To a mixture of tris(tetra-*n*-butylammonium) hydrogen pyrophosphate (663 mg, 0.8 mmol) in dry acetonitrile was added via syringe **1-OTs** (100 mg, 0.4 mmol) dissolved in a minimal amount of dry acetonitrile. The mixture was allowed to stir under N_2 for 2 h and was concentrated by rotary evaporation. The residue was dissolved in a minimal amount of ion exchange buffer (1:49 isopropanol:25 mM ammonium bicarbonate) and loaded on to the ammonium form of Dowex ion exchange resin. The column was eluted with two column volumes of exchange buffer. The elutant was flash frozen and lyophilized to give a white solid. Inorganic pyrophosphate was removed by dissolving the solid in a minimal volume of 0.1 M ammonium bicarbonate, 15 mL of 1:1 isopropanol:acetonitrile was added, and the suspension was vortexed. The resulting white precipitate was cleared by centrifugation, the supernatant was removed, and the process was repeated three times. The supernatants were pooled, concentrated at reduced pressure and lyophilized to give a white solid. The solid was dissolved in a minimal volume of ammonium bicarbonate and chromatographed on cellulose using 70:30 (isopropanol:0.1 M ammonium bicarbonate) to give 34 mg (28%) of a fluffy white solid; ^{1}H NMR (D_2O) δ 2.28 (s, 3H), 2.37 (t, 2H, *J* = 6.9), 4.08 (dt, 2H, J_1 = 7.0, J_2 = 13.9 Hz), 4.85 (s, 1H), 5.26 (s, 1H); ^{13}C NMR (D_2O) δ 14.4, 38.2 (d, *J* = 7.2 Hz), 65.3 (d, *J* = 5.5 Hz), 107.6, 143.1; ^{31}P NMR (D_2O) δ -6.44 (d, *J* = 23.6 Hz), -10.48 (d, *J* = 21.8 Hz); HRMS (MALDI) calcd for $C_5H_{12}O_7P_2S$ $(M-H)^-$ 276.9706; found 276.9000.

DEUTERIUM EXCHANGE STUDIES

***Thermus thermophilus* IDI-2.** Samples for NMR studies in D_2O were prepared as follows. Sodium phosphate, pH 7.4, $MgCl_2$, and bovine serum albumin (BSA) were dissolved in ddH_2O lyophilized to dryness. The solid was dissolved in D_2O and the process was repeated. The buffer was pipetted into 1 mL portions and D_2O was removed at reduced pressure on a speed vac. The solid samples were stored at -20 ºC until used, at which time they were dissolved in D_2O. Enzyme was exchanged three times with deuterated buffer by centrifugation using a 2 mL Centricon (10,000 MWCO) concentrator. Reactions were run in 0.7 mL of 50 mM sodium phosphate D_2O buffer, pH 7.4, containing 2 mM $MgCl_2$, 0.1 mg/mL BSA, and 25 μM enzyme. The incubation buffer for IDI-2 also contained 2 mM NADPH and 100 μM FMN. A ^1H spectrum was taken of the enzyme and cofactors before addition of substrate. The incubations were initiated by the addition of **1-OPP** (12 mM, final concentration) and a ^1H spectrum was recorded every 5 min for 2 h and then after 18 h. At the end of the incubation, the mixture was assayed using the acid labiality procedure to determine the activity of the enzyme (1).

The k_{cat} for addition of deuterium to **1-OPP** was estimated from the disappearance of the vinyl signal at 5.20 ppm. The rate of decrease of the signal was linear for the first 20 minutes of the reaction, at which time the peak intensity had decreased by 43% corresponding to a 0.036% decrease/second. Based on an initial [**1-OPP**] = 12 mM and [IDI-2] = 25 μM, k_{cat} ~ 0.4 s^{-1} after correcting for the presence of two vinyl hydrogen atoms in the substrate, which require two separate protonation events for complete exchange.

PRODUCT STUDIES WITH FARNESYL DIPHOSPHATE SYNTHASE

Experimental. The incubation mixture consisted of 35 mM HEPES buffer, pH 7.4, containing 10 mM $MgCl_2$, 5 mM βME, 8.5 mM **1-OPP**, 9.4 mM geranyl diphosphate, and 100 μM avian farnesyl diphosphate synthase in a total volume of 500 μL. Assay mixtures were incubated at 4° C or 30° C for 2 h. Samples were diluted with water and chromatographed on a C-18 reverse phase column. The initial elution solvent was 95:5 H_2O:acetonitrile. After 5 min a linear gradient of 5% methanol to 100% methanol was applied over 25 min at a flow rate of 0.2 mL/min. The effluent was analyzed by negative ion ESI.

A sample obtained from an incubation at 4° C was treated with alkaline phosphatase at 4° C as described above for **5-OPP** and analyzed by positive ion HPLC/ESI.

Similar incubations were carried out with 16 mM **1-OPP** and 9 mM DMAPP. Samples were chromatographed on a C-3 reverse phase column. The initial elution solvent was 95:5 H_2O:MeOH. After 5 min a linear gradient of 5% methanol to 100% methanol was applied over 25 min at a flow rate of 0.2 mL/min. The effluent was analyzed by negative ion electrospray MS.

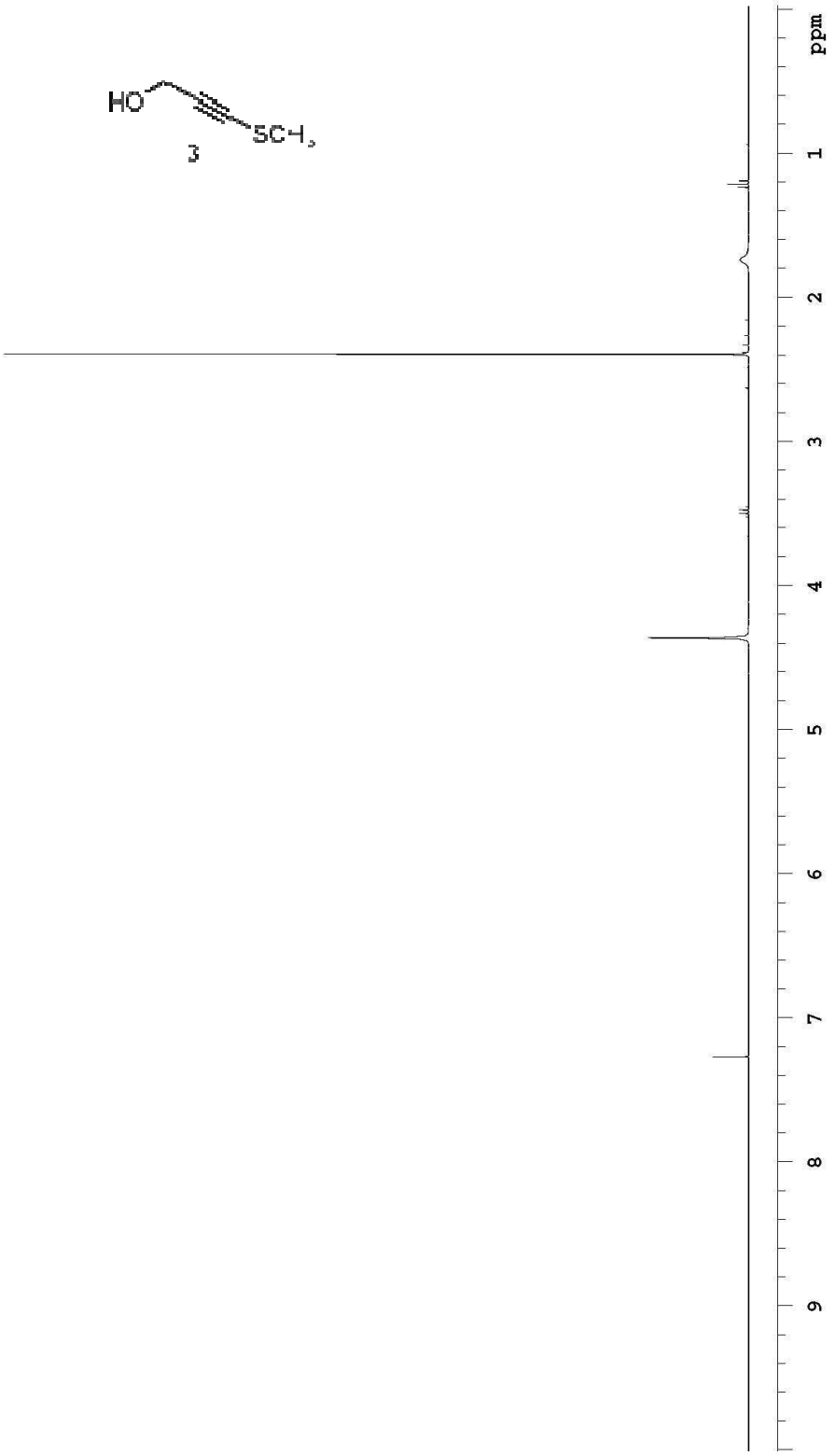
Discussion. Initial attempts to purify chain elongation products from **1-OPP** and DMAPP or GPP for analysis by NMR spectroscopy were unsuccessful because of the reactivity of the allylic thiomethyl diphosphates. We then analyzed for products by LC/MS. **1-OPP** and DMAPP or GPP were incubated at both 4 ^{o}C and 30 ^{o}C. The reactions at 30 ^{o}C did not give detectable amounts of diphosphate products. While a thiomethyl farnesyl diphosphate product was not detected from the incubation with GPP at 4 ^{o}C, a signal for the dithiomethyl analogue from incubation of **1-OPP** and DMAPP was detected by negative ion LC/MS with a diagnostic peak at m/z =445 ($M-H^{+}$). Repeated attempts to detect the thiomethyl analogue from **1-OPP** were unsuccessful. We do not have an explanation for the differences seen in the stabilities of the monothiomethyl and dithiomethyl analogues.

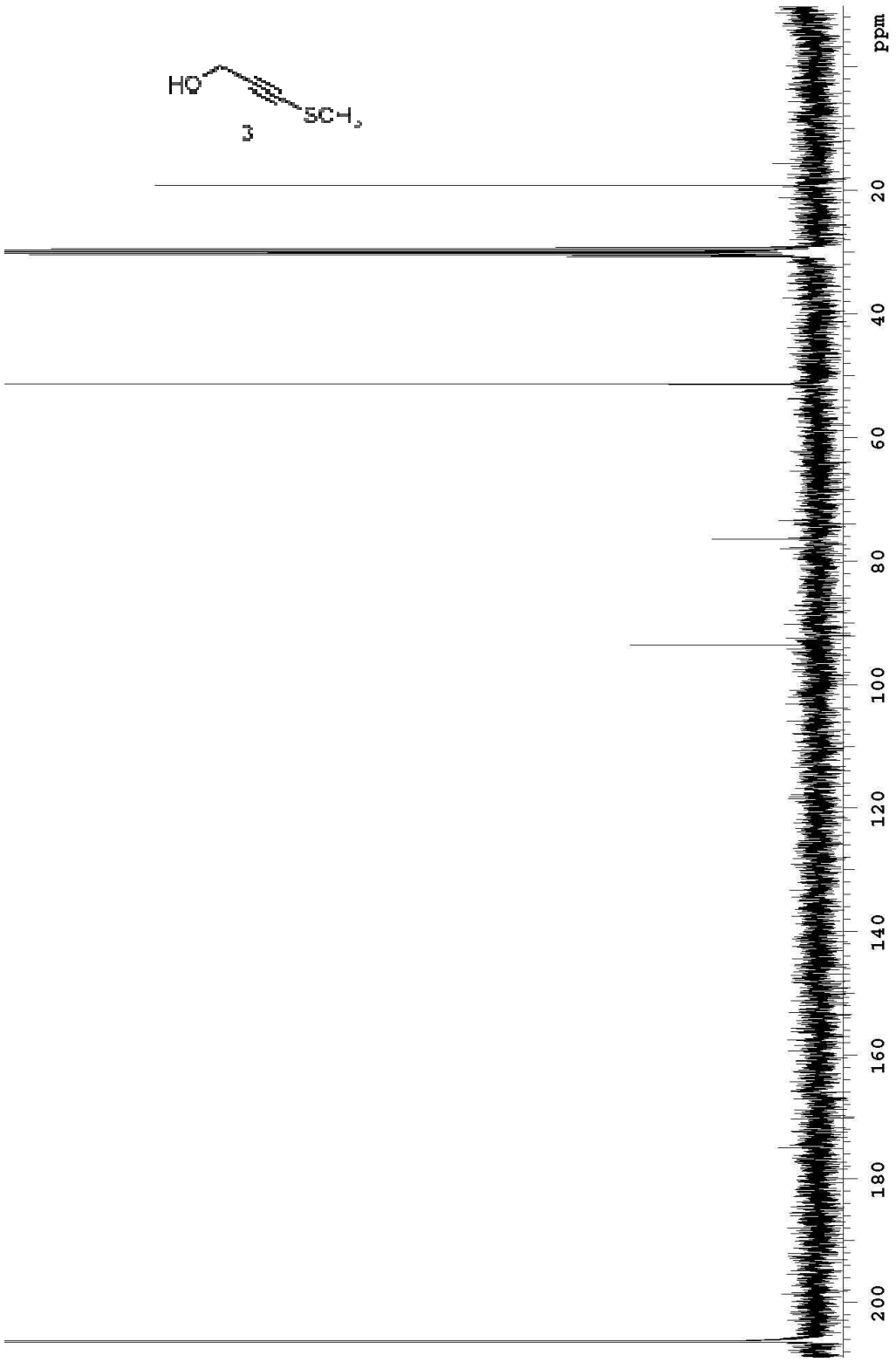
In a separate experiment, the reaction mixture from incubation of **1-OPP** with GPP at 4 ^{o}C was immediately treated with alkaline phosphatase to remove the reactive diphosphate moiety. The methyl t-butyl ether extract of the hydrolysis mixture was analyzed by positive ion MS and gave a peak at m/z – 277 ($M+Na^{+}$) indicative of a sodium adduct for the thiomethyl farnesyl diphosphate analogue.

Reference

1. Laskovics, F.M.; Poulter, C.D. "Prenyltransferase. *Biochemistry* **1981**, *20*, 1893-1901.

NMR SPECTRA

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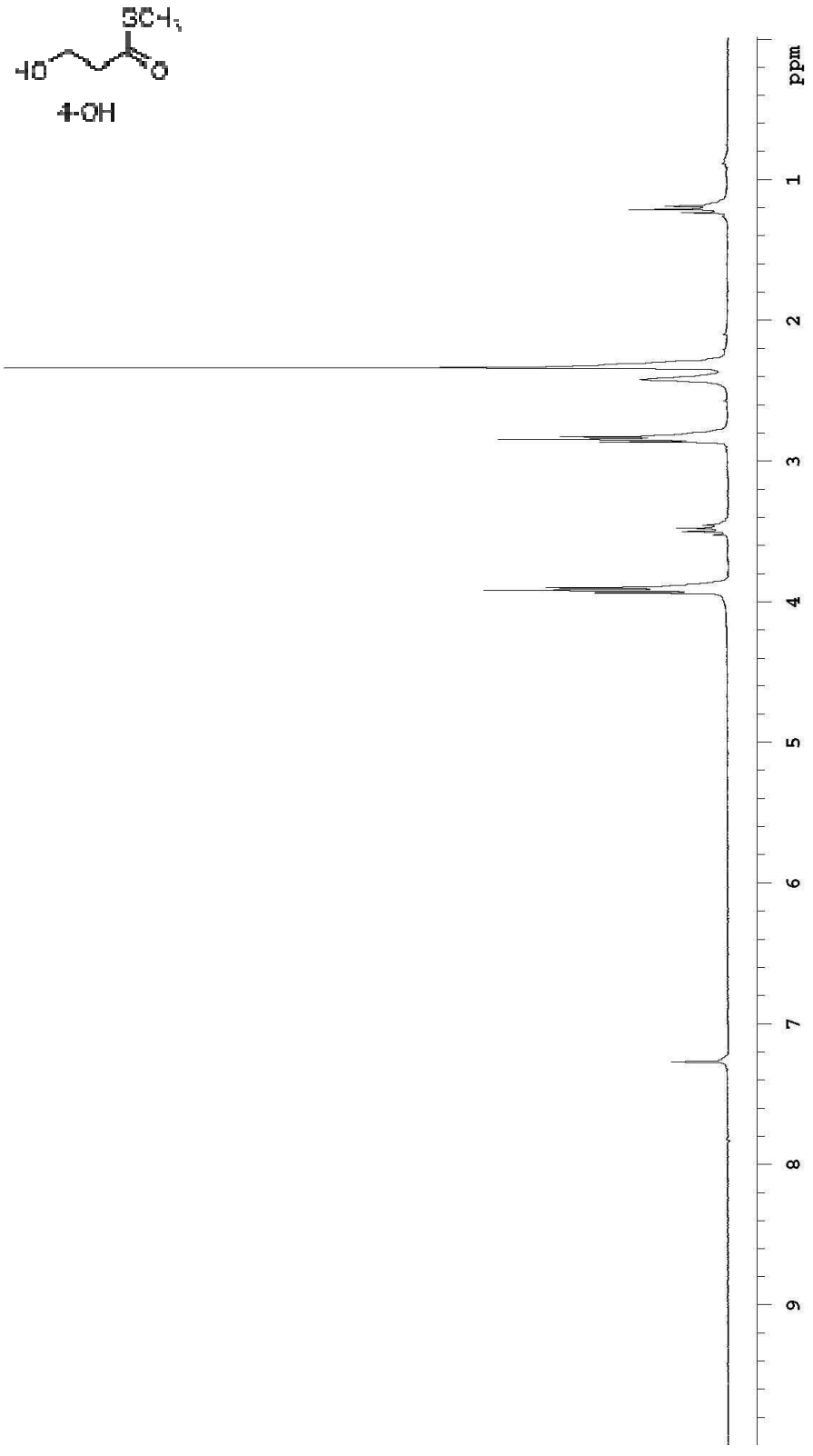
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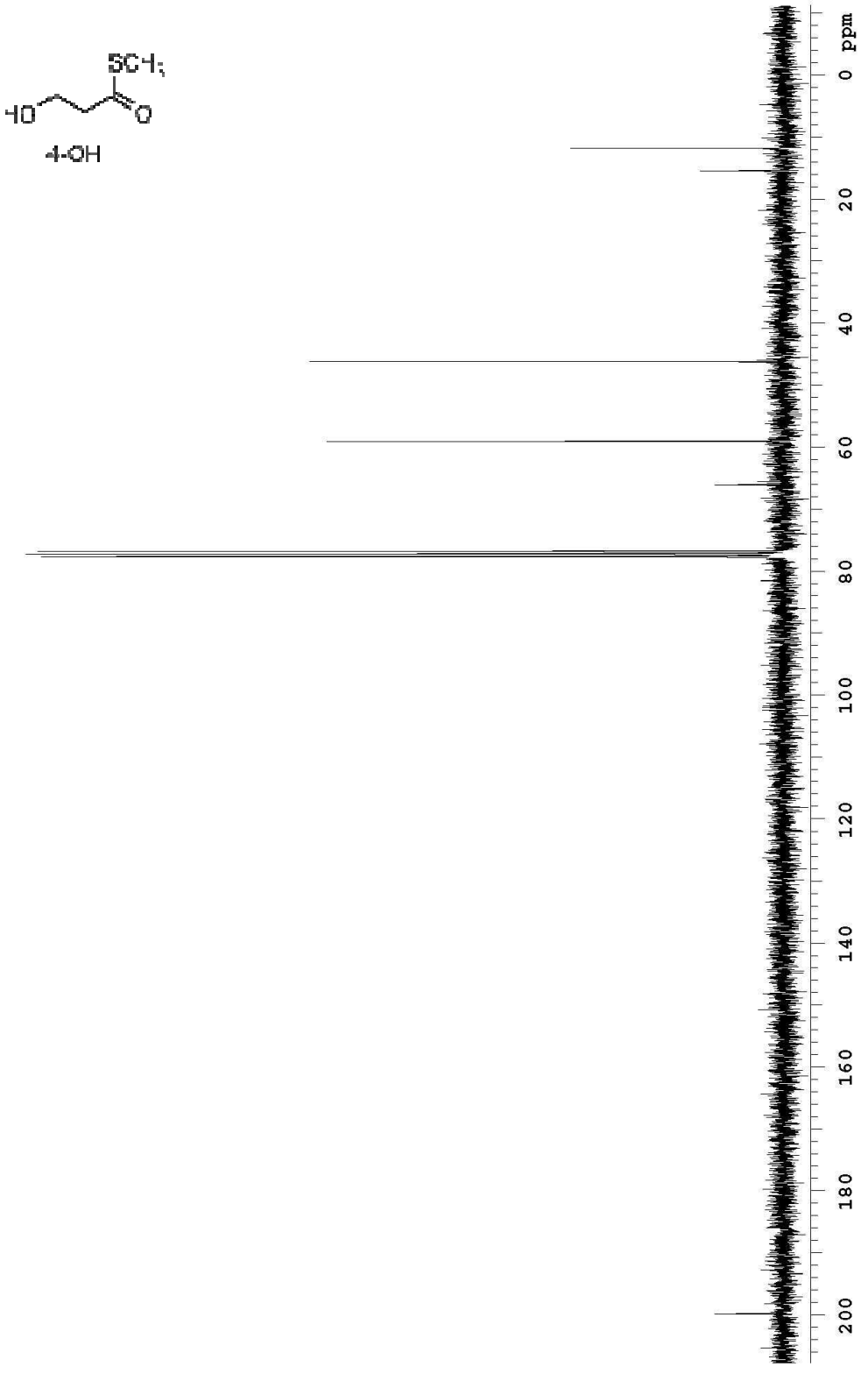
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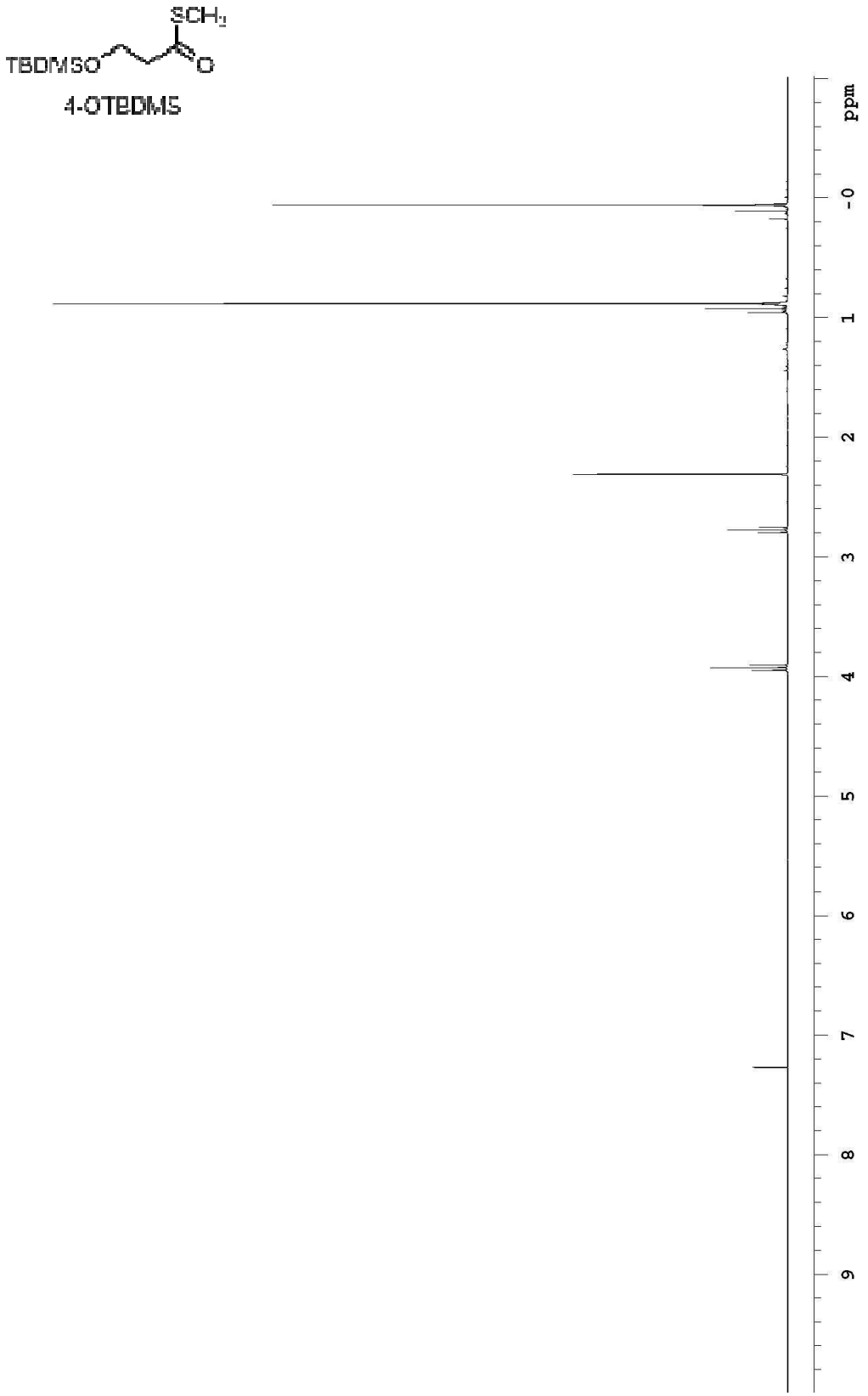
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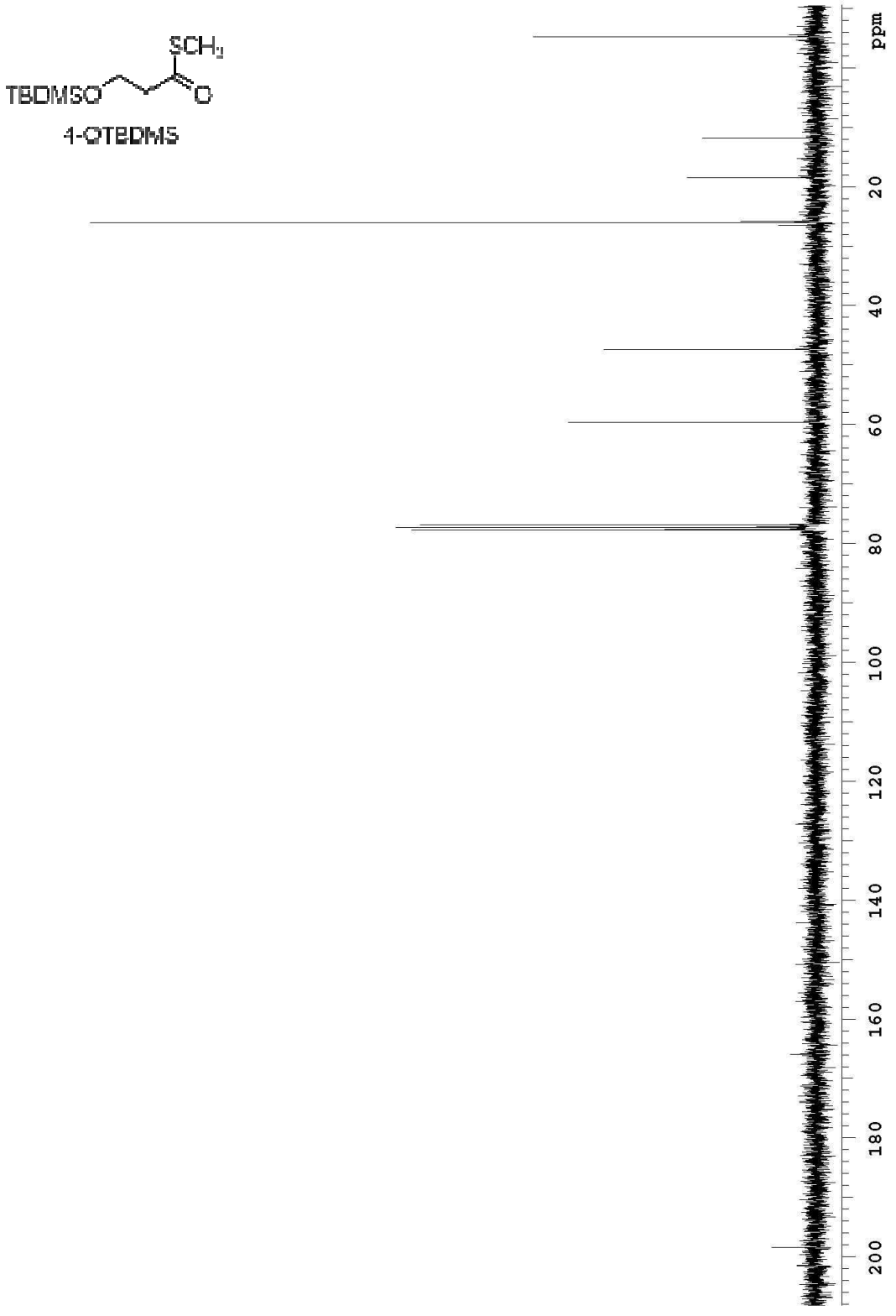
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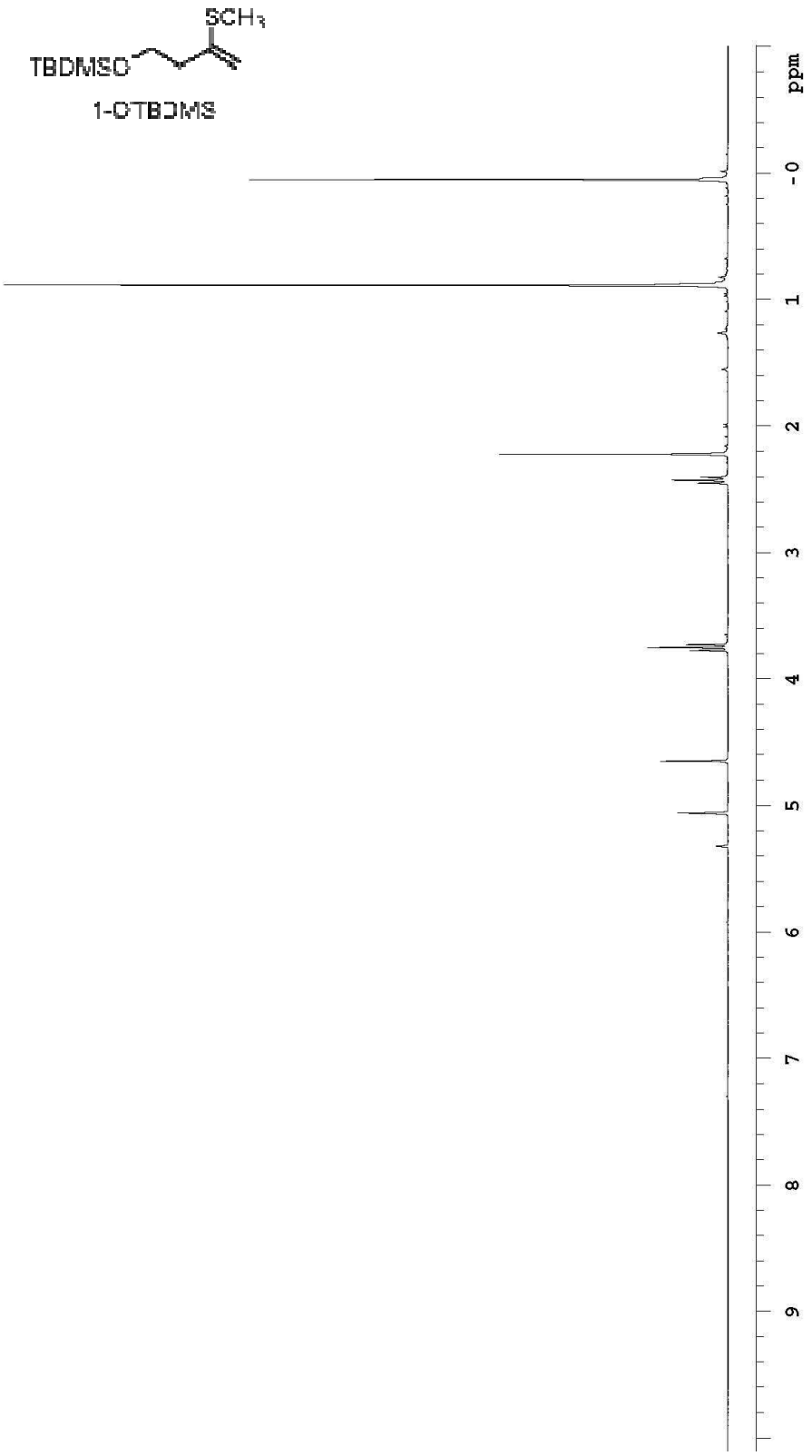
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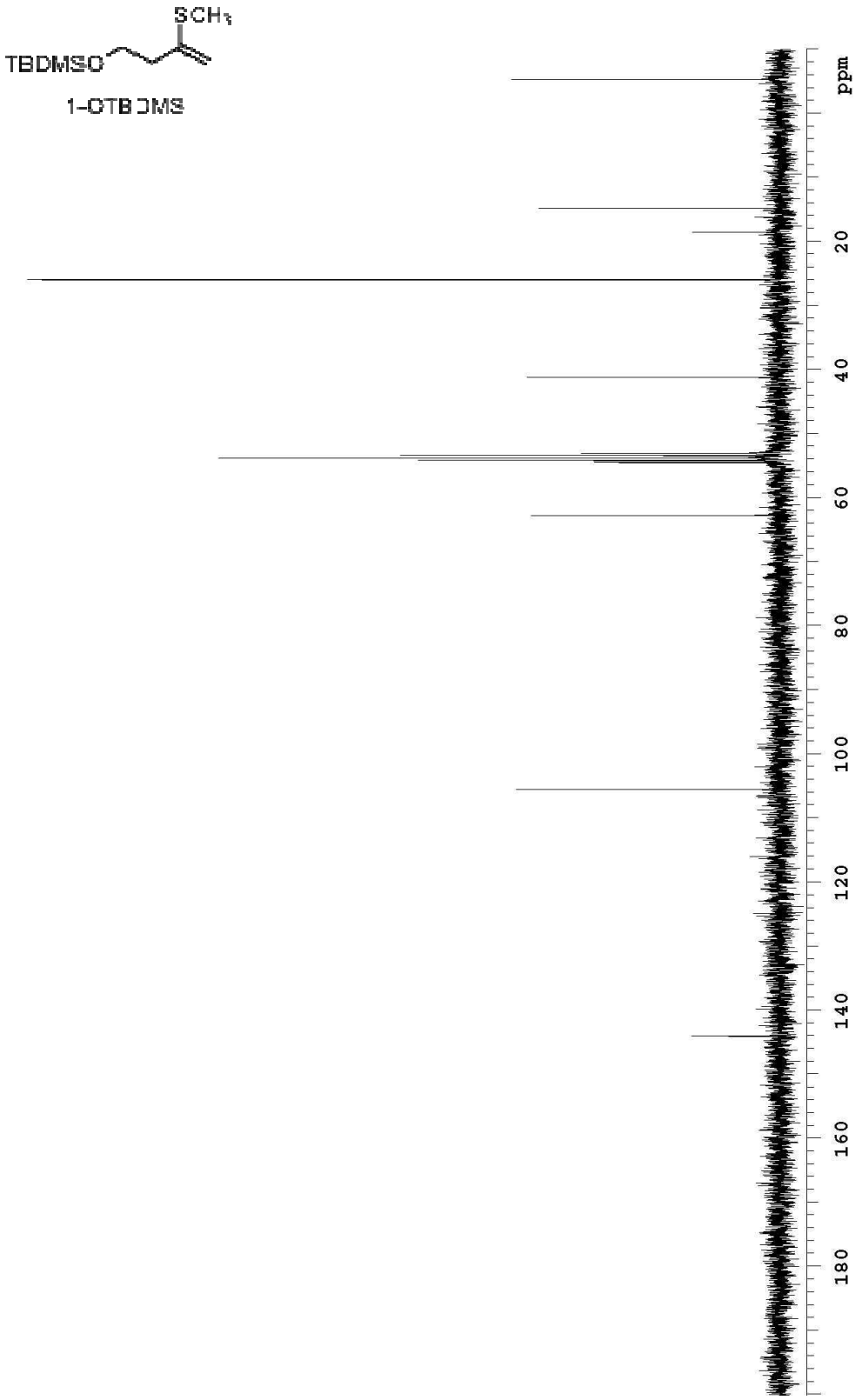
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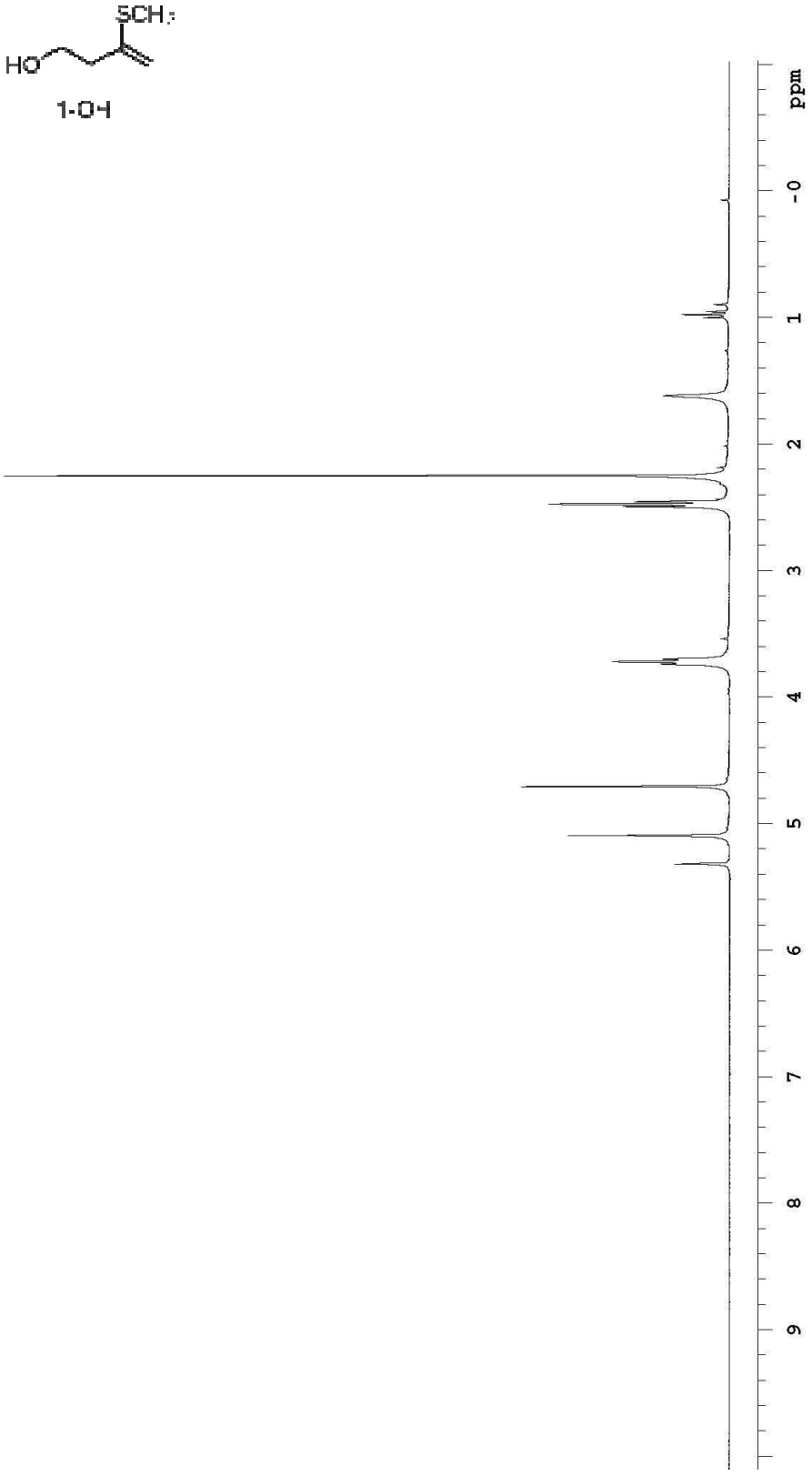
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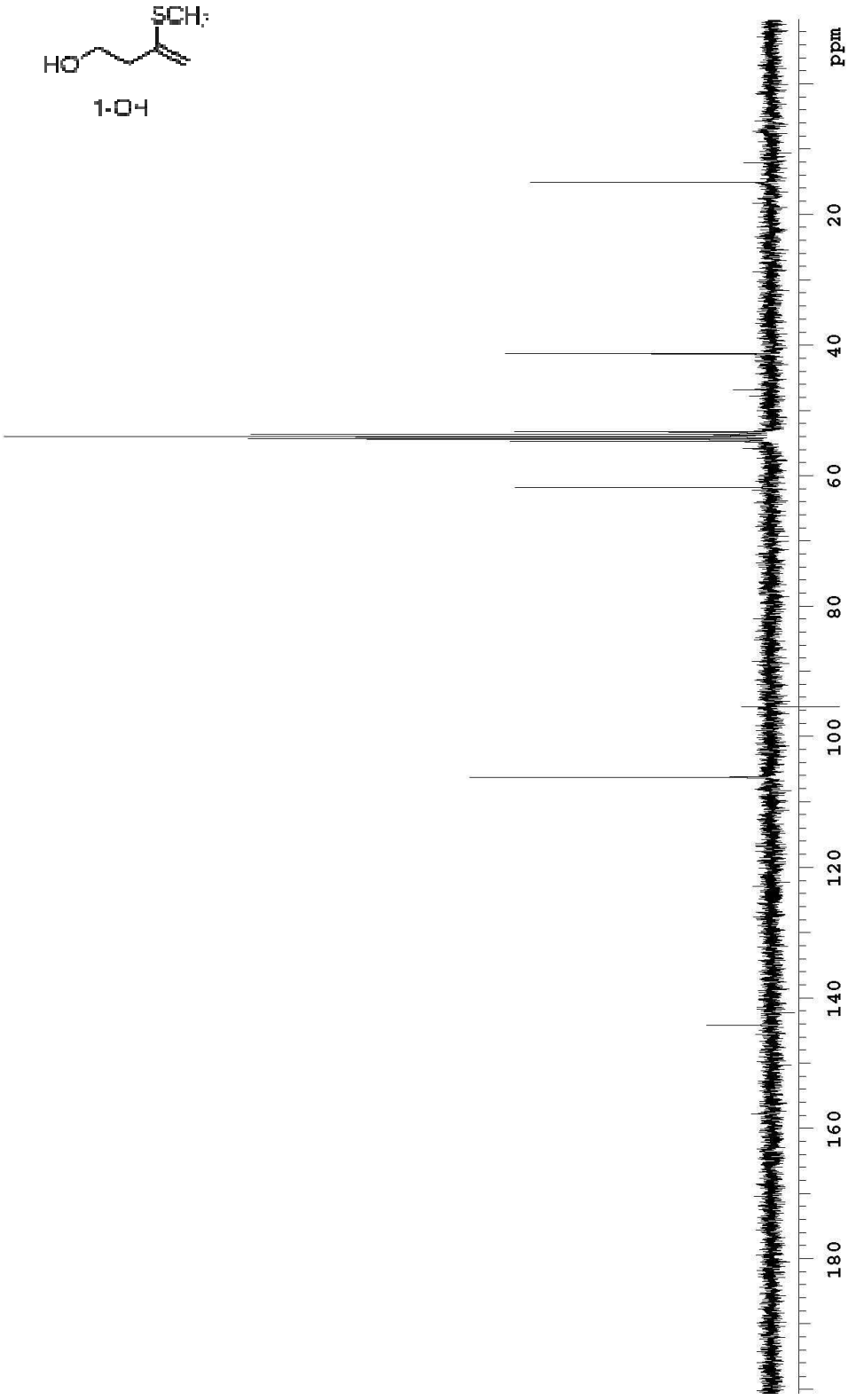
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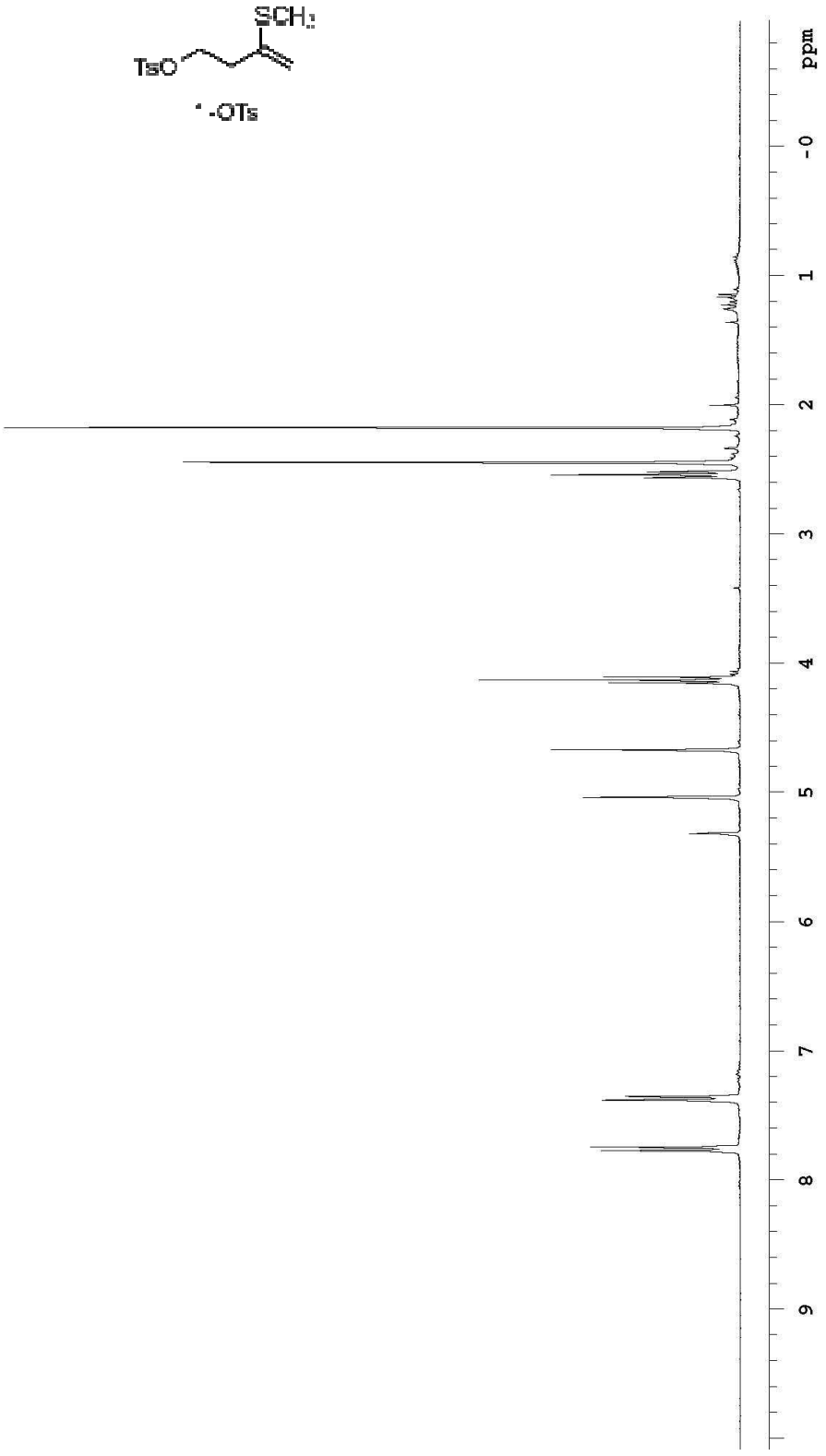
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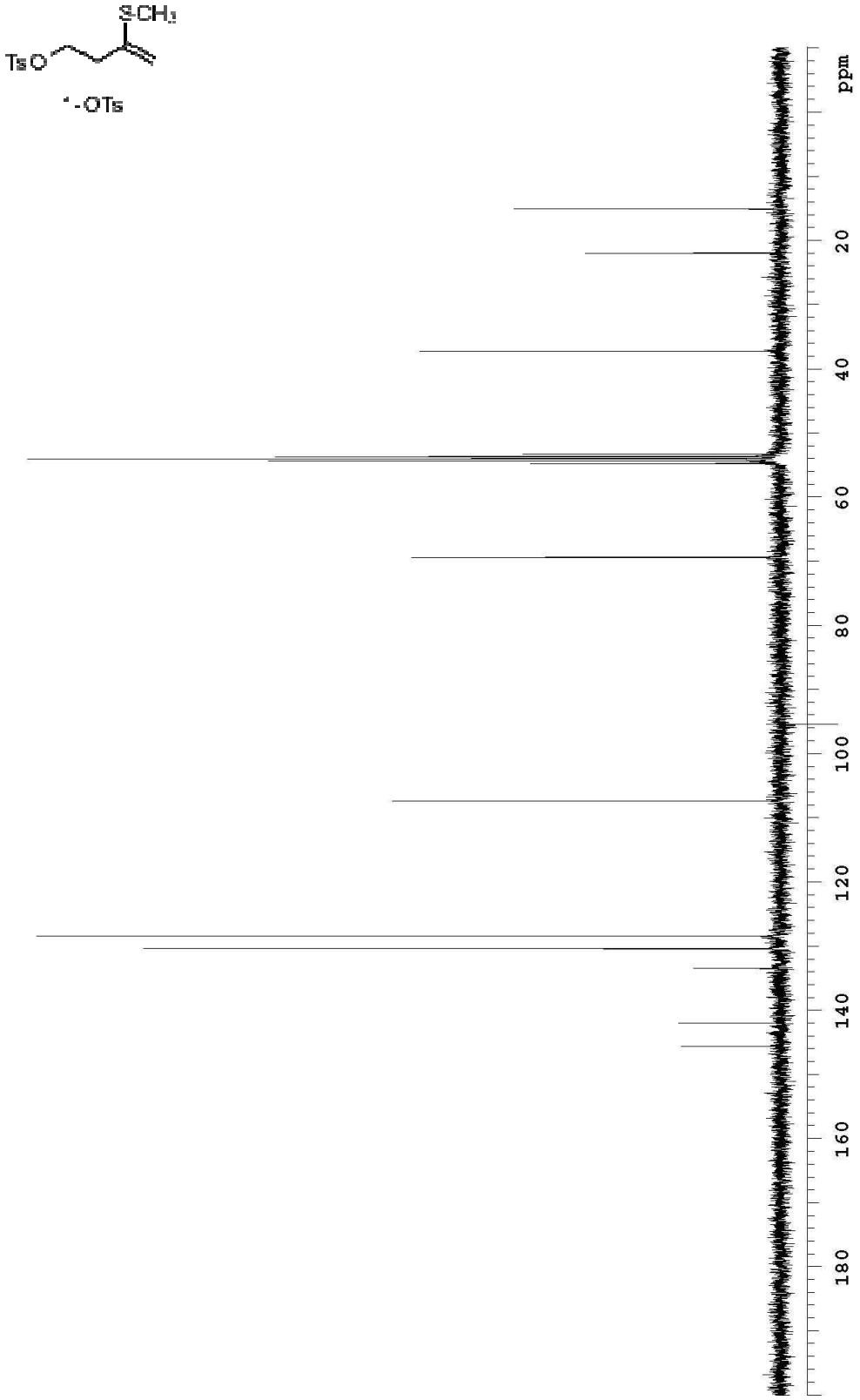
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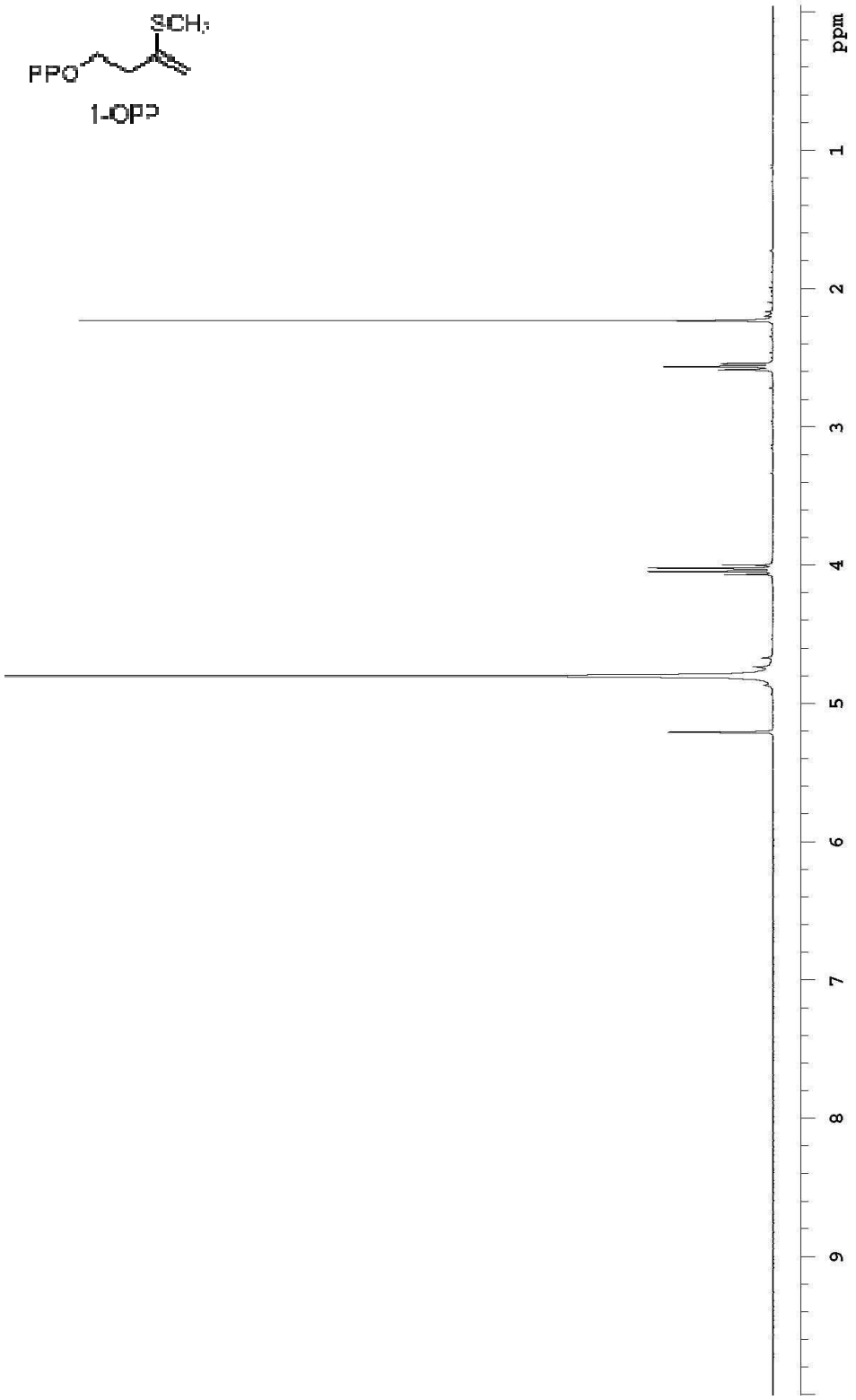
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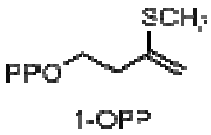

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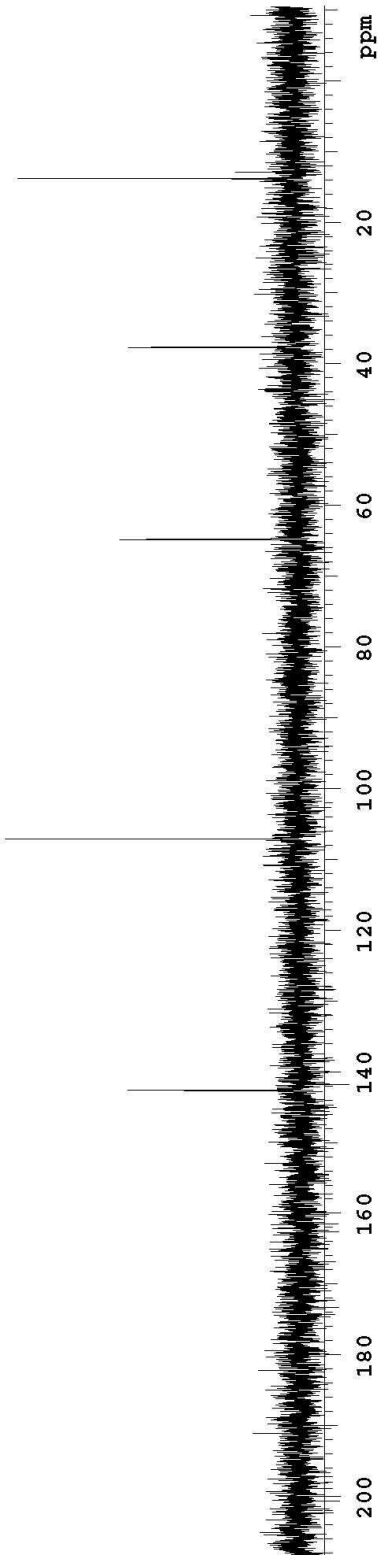

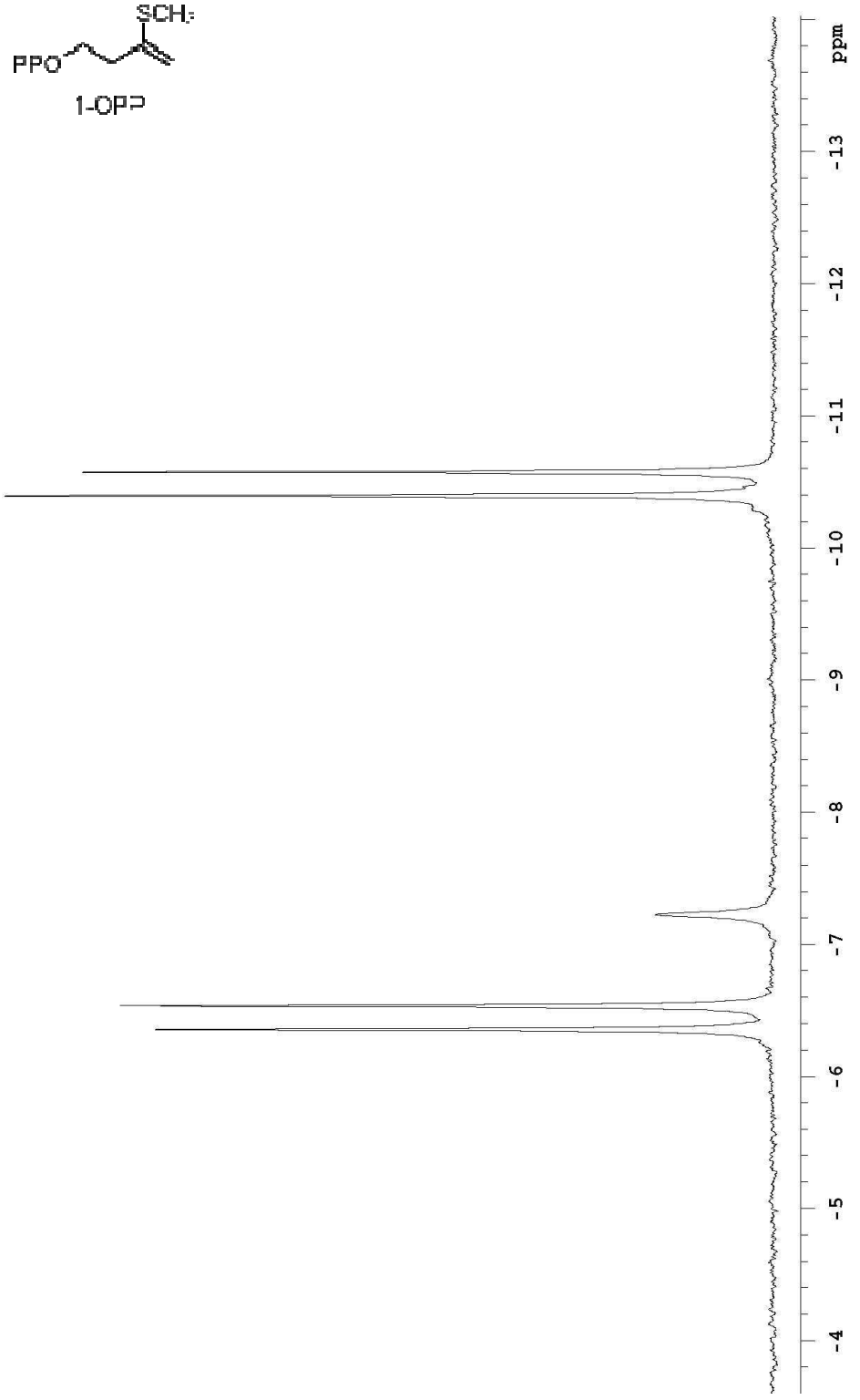
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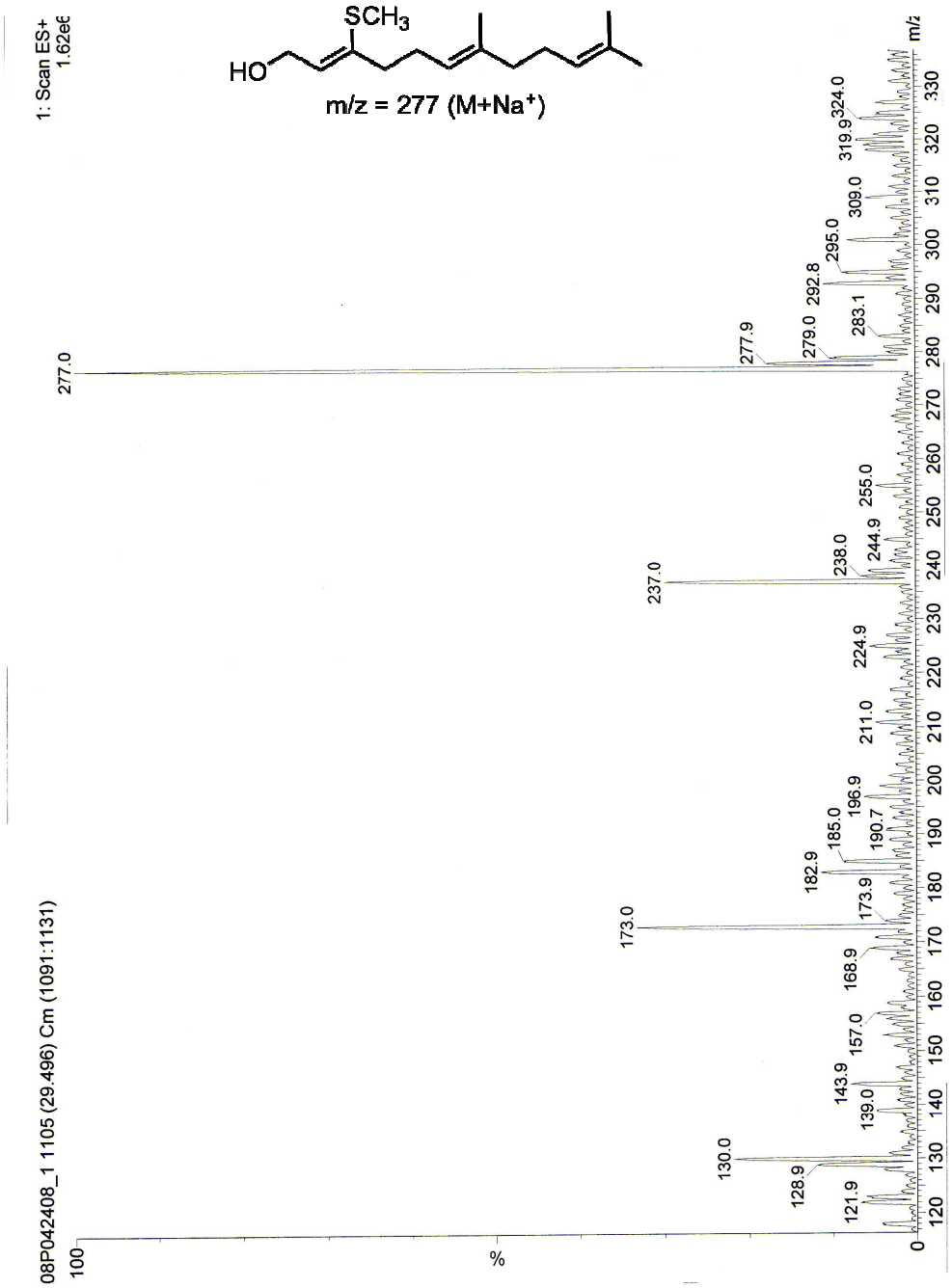
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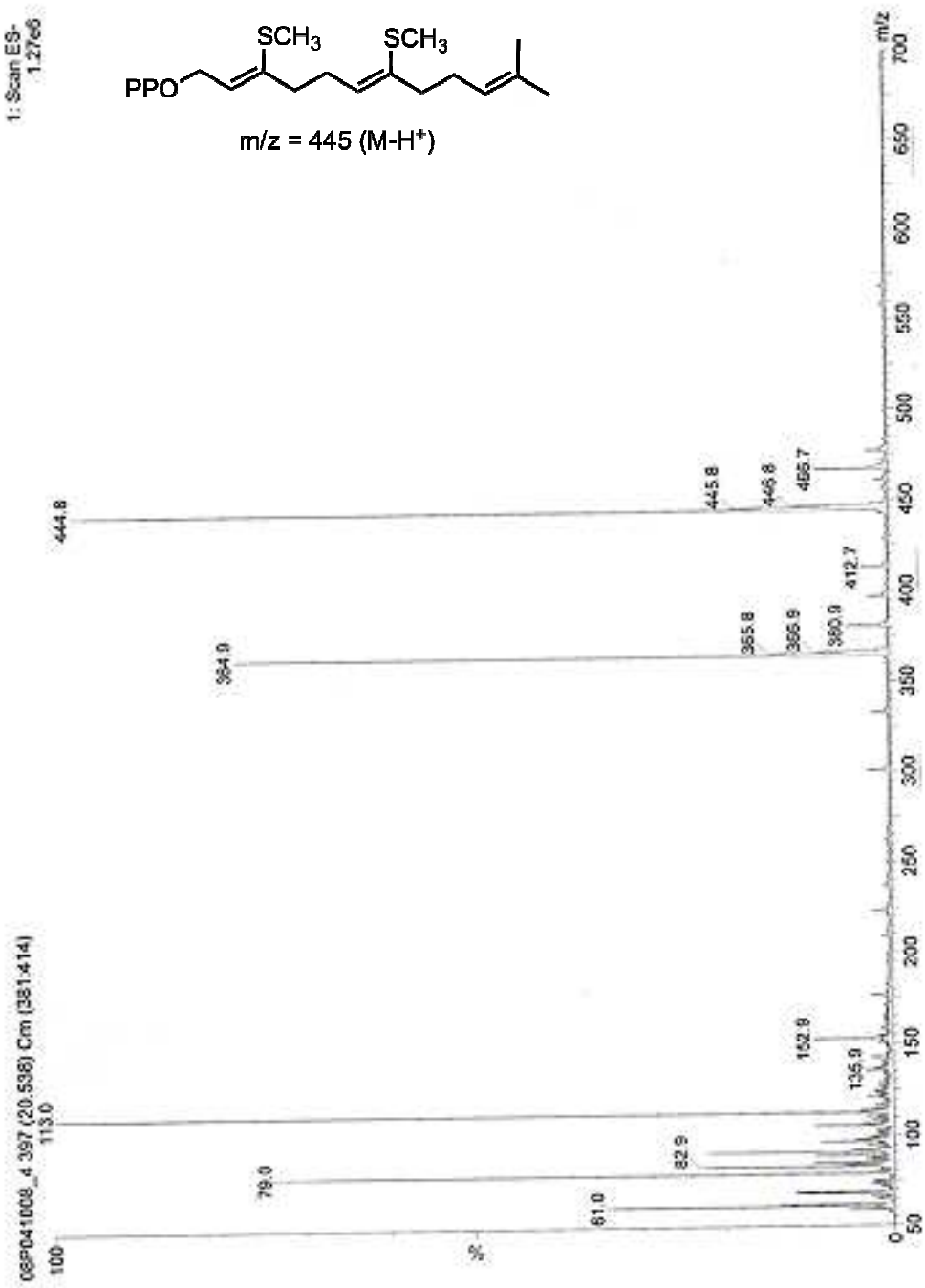
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%
m/z