Supporting information

Contents:

1) Supplementary methods

A. Extended experimental procedures

Imaging cells labeled with 19-ethynylcholesterol (eChol) and 19-ethynyl, 25-hydroxycholesterol (25-OH-eChol) by fluorescence microscopy

NIH-3T3 cells were grown in DMEM supplemented with 10% bovine calf serum (BCS), penicillin and streptomycin. For labeling, cells were incubated with 10-50 μM eChol or 25-OH-eChol (added from 20 mM stocks in DMSO) in DMEM with 0.2% BCS. Alternatively, eChol was added to cells at 2-12.5 μM as soluble complex with methyl-β-cyclodextrin (MCD), in DMEM (see below). Cells were fixed with 3.7% formaldehyde in PBS for 30 minutes at room temperature. The fixed cells were washed with Tris-buffered saline (TBS, 10 mM Tris-HCl, 150 mM NaCl, pH 7.5), reacted by CuAAC with 10 μM fluorescein-azide, as described[1], and then washed several times with 0.5 M NaCl and TBS to remove the unreacted azide. Detergents and organic solvents were avoided during the staining procedure, to prevent extraction of eChol or 25-OH-eChol from cell membranes. For staining eChol-labeled cells with TMR-picolyl azide, the CuAAC reaction mix consisted of 10 mM NaHEPES pH 7.5, 150 mM NaCl, 50 μM CuSO4, 50 μM THPTA[2], 5 μM TMR-picolyl azide[3] and 5 mM Na ascorbate. Nuclei were stained with Hoechst and the cells were imaged by epi-fluorescence microscopy on a Nikon TE2000U microscope equipped with an OrcaER digital camera (Hammamatsu), and 20x PlanApo 0.75NA, 40x PlanApo 0.95NA or 100x PlanApo 1.4NA objectives (Nikon). Images were collected using Metamorph image acquisition software (Applied Precision). The cells were also imaged by DIC.

To determine the subcellular localization of eChol, NIH-3T3 cells were transiently transfected with one of the following plasmids encoding red fluorescent protein fusions: pDsRed-Mito (from Clontech Laboratories Inc, to label mitochondria), pmCherry-Sec61β (plasmid #49155 from Addgene, to label the ER), and pmCherry-CAAX[4] (to label the plasma membrane). After transfection, the cells were incubated with 40 μM eChol overnight in DMEM with 0.2% BCS, after which they were fixed, stained with fluorescein-azide and imaged. To determine the detergent sensitivity of the eChol stain, cells labeled with eChol and stained with fluorescein-azide were incubated in TBS with or without 0.5% TritonX-100, for 30 minutes at room temperature. The cells were then washed with TBS and imaged by fluorescence microscopy.

For double labeling with eChol and propargylcholine (PCho), cells were incubated overnight in complete media, in the absence or presence of PCho (100 μM), followed by incubation overnight in the absence or presence of eChol (40 μM in DMEM with 0.2% bovine calf serum). Alternatively, cells could be labeled simultaneously with PCho and eChol in DMEM with 0.2% bovine calf serum. After labeling, the cells were fixed and stained first by CuAAC with Alexa568-azide (10 μM), to detect PCho-labeled phospholipids[5]. The PCho-labeled phospholipids left unreacted after reaction with Alexa568-azide were consumed by performing a CuAAC reaction with 5 mM O-(2-aminoethyl)-O'-(2-azidoethyl)-pentaethylene glycol (Fluka) for 30 minutes at room temperature, as described[5]. The cells were then washed and reacted with fluorescein-azide (10 μM), to detect eChol.

Testing the effect of eChol on cholesterol auxotrophic CHO M19 cells

Chol auxotrophic CHO M19 cells (a gift from T.Y. Chang, Dartmouth) were grown in F12K media[6]. Fetal bovine serum (FBS) was delipidated with diisopropylether, as described[7]. To test if eChol rescues proliferation, CHO M19 cells were grown for 4 days in F12K media supplemented with 10% delipidated FBS and oleic acid (35 μM final concentration, added from 35 mM stock in DMSO), in the absence or presence of eChol (10 μM final concentration of soluble eChol-MCD) or Chol (35 μM final concentration of Chol-MCD). The sterol-MCD complexes were prepared as described[8], and contained sterol and MCD at a molar ratio of 1:10. Representative fields of cells were photographed at 24-hour intervals on days 1, 2, and 3 after plating, by phase contrast microscopy.

Sterol quantification by gas chromatography/mass spectrometry (GC/MS)

To measure sterols by GC/MS, NIH 3T3 cells were incubated for 2 hours in DMEM, in the absence or presence of 12.5 μM eChol-MCD complex. The cells were washed with PBS, and were harvested by scraping into PBS and centrifugation. The cell pellets (average wet weight: 110 mg) were resuspended in 3 mL water, after which the lipids were saponified by addition of 0.3 mL of a 60% solution of potassium hydroxide solution, followed by incubation for 1 hour at 65°C. The lipids were then extracted with hexanes (3 x 5 mL). The combined organic fractions were evaporated under reduced pressure and were dried under nitrogen gas, yielding approximately 1 mg saponified lipids per sample.

GC/MS analysis was performed at the Kansas Lipidomics Research Center. An internal standard, cholesterol-d6 (4.366 nmoles, C/D/N Isotopes Inc), was added to 1/20th of each lipid sample. The samples were then dried under nitrogen, dissolved in 70 μL pyridine, and derivatized by adding 30 μL N-trimethylsilyl-N-methyltrifluoroacetamide with 1% trimethylchlorosilane, followed by incubation at 50°C for 1 hour. The derivatized samples were analyzed by GC/MS, using an Agilent 6890N GC coupled to an Agilent 5970N quadrupole mass selective detector. The GC was fitted with a DB-5ms capillary column with a 5% phenyl methyl siloxane stationary phase (column length: 60 mm, internal diameter: 250 μm, film thickness: 0.25 μm). Helium was used as the carrier gas at a column flow rate of 1 mL/min. The front inlet was operating at a pressure of 24.69 psi and a temperature of 280 °C. The Agilent 7683 autosampler was used to inject 2 μL of the sample in the splitless mode. The GC temperature ramp was operated as follows: initial temperature of 150 °C, ramp 1 at 25 °C/min to 300°C, ramp 2 at 3°C/min to a final temperature of 325°C held for 5 min. The mass spectrometer was operated in the electron impact mode at a ionization energy of 70 eV. The MS quad temperature was 150 °C and the MS source temperature was 250°C. The data were processed with Agilent Chemstation, AMDIS, and MET-IDEA. Each sample was run in triplicate, and the ratio of sterol peak area to the internal standard peak area was used to calculate the amounts of Chol and eChol in the sample. In the case of eChol, a response factor of 0.63 relative to the internal Chol-d6 standard was determined using a sample of pure eChol.

***In vitro* Hedgehog (Hh) processing assays**

A fusion between the maltose-binding protein (MBP) and a fragment of Drosophila Hh encoding amino acids 244 to the C terminus (MBP-DHh) was expressed in bacteria and purified on amylose resin (New England Biolabs) according to the manufacturer's instructions. In vitro Hh processing assays were performed as described[9]. Briefly, recombinant MBP-DHh was diluted to 0.2 mg/mL in 20 mM HEPES pH 7.5, 50 mM NaCl, 0.1% Triton-X100 and 1 mM DTT. Chol or eChol were added to a final concentration of 400 μM (from 20 mM stocks in DMSO). The reactions were incubated at room temperature, aliquots were removed at the indicated times and were boiled in SDS-PAGE sample buffer. The samples were separated by SDS-PAGE and the proteins were visualized by staining with GelCode Blue (Pierce).

Detection of eChol-modified Hh protein on blots

MBP-DHh, in vitro processed with either Chol or eChol, was reacted by CuAAC with biotin-azide. The CuAAC reactions contained 40 ng/μL Chol- or eChol-modified MBP-DHh, 10 μM biotin-azide, 40 μM $CuSO_4$, 40 μM THPTA[10] and 5 mM sodium ascorbate in reaction buffer (12 mM HEPES pH 7.5, 30 mM NaCl, 0.12% TX-100). The reactions were incubated at room temperature overnight and were stopped by boiling in sample buffer with DTT. The samples were separated by SDS-PAGE, blotted onto nitrocellulose, and biotin-modified proteins were detected by probing the blot with anti-biotin-HRP conjugate (Jackson ImmunoResearch).

Assaying Hh processing in cells

The stable human 293T cell line expressing full-length human Sonic Hedgehog (hShh), C-terminally tagged with an HA epitope (hShh-HA) was described before[9]. The 293T cell line was grown in DMEM supplemented with 10% FBS, penicillin and streptomycin. To deplete sterols, cells were washed in DMEM and then incubated for 45 minutes with 0.75% w/v MCD in DMEM, followed by incubation in DMEM supplemented with the HMG-CoA reductase inhibitor pravastatin (20 μM). To rescue sterol depletion, sterols were added back as MCD complexes, in DMEM with pravastatin (20 μM). ER-associated degradation of the C-terminal fragment of hShh (hShh-C) and that of the hShh precursor was blocked by addition of 1 μM of the proteasome inhibitor bortezomib. To assay hShh processing, cells stably expressing hShh-HA were sterol depleted and then incubated with DMEM with bortezomib, pravastatin, and various sterol-MCD complexes, as described[11]. After 3 hours, the cells were washed with PBS, harvested and lysed on ice for 20 min, in TBS with 1% Triton X-100, supplemented with protease inhibitors. The lysate was clarified by centrifugation for 30 min at 4 ^{0}C and 20,000g. The supernatant was mixed with SDS-PAGE sample buffer with DTT (50 mM final), and separated by SDS-PAGE, followed by immunoblotting for HA (rat monoclonal, 3F10, Roche), to detect hShh and hShh-C. Blotting for hShh-N (rabbit monoclonal, Cell Signaling) was used as loading control, by probing the hShh-N portion of the blot in parallel with the portion containing HShh and HShh-C. Ponceau S staining of the blots after electrophoretic transfer to the nitrocellulose membrane provided another loading control.

Hh signaling assays

Hh ligand was produced in 293T cells by transient transfection of an expression plasmid encoding amino acids 1-198 of hShh[12]. Hh pathway activity was assayed in NIH-3T3 cells. Briefly, confluent cultures were starved for 24 hours in DMEM, and were then stimulated with Hh ligand in DMEM. After the desired amount of time, the cultures were analyzed by immunofluorescence to determine the recruitment of endogenous Smoothened (Smo) to primary cilia, by luciferase reporter assays, or by quantitative PCR (qPCR) to measure the transcriptional output of the Hh pathway.

Smo immunofluorescence was performed using affinity-purified rabbit anti-Smo antibodies, as described[12]. Primary cilia were stained with a mouse anti-acetylated tubulin monoclonal antibody (Sigma). For each condition, the presence or absence of Smo at cilia was scored visually for 150 cilia, identified by acetylated tubulin staining. Error bars represent the standard deviation of the mean for groups of 50 cilia counted on different visual fields, on the same coverslip. All experiments showing ciliary counts were repeated independently at least three times. To measure Smo fluorescence intensity, the primary cilium was outlined using acetylated tubulin images (40x PlanApo 0.95NA objective), and the integrated ciliary intensity for Smo was measured using Metamorph software (Applied Precision) in at least 100 cilia for each condition.

Hh-stimulated transcription was measured by luciferase reporter assays in NIH-3T3 Shh-LightII cells (obtained from ATCC), which express firefly luciferase under the control of an artificial Hh-responsive promoter and Renilla luciferase under the control of a constitutive promoter, as described[13]. The activity of the Hh pathway was calculated as the ratio between firefly and Renilla luciferase activities, after background subtraction. Error bars represent standard deviation of the mean for 4 independent experiments.

Activation of endogenous target genes of the Hh pathway, Gli1 and Ptch1, was measured by qPCR, as described[14]. Each qPCR experiment was performed in triplicate, with error bars indicating standard deviation.

Sterol depletion of NIH-3T3 and NIH-3T3 Shh-LightII cells was performed as described[15], with modifications. Confluent cultures cells were starved for 24 hours in DMEM, and were then incubated

with 1% w/v MCD in DMEM for 30 minutes, followed by incubation in DMEM with pravastatin (20 μM, to block Chol synthesis). For rescue experiments, sterols were then added back to cells as sterol-MCD complexes in DMEM supplemented with pravastatin (20 μM). To assay Hh pathway stimulation, the Hh ligand was added to the depleted cells in DMEM with pravastatin (20 μM), with or without sterol-MCD complexes. Undepleted cells, stimulated or not with Hh ligand served as positive and negative controls. The cells were processed for Smo immunofluorescence or for luciferase assays, as described above.

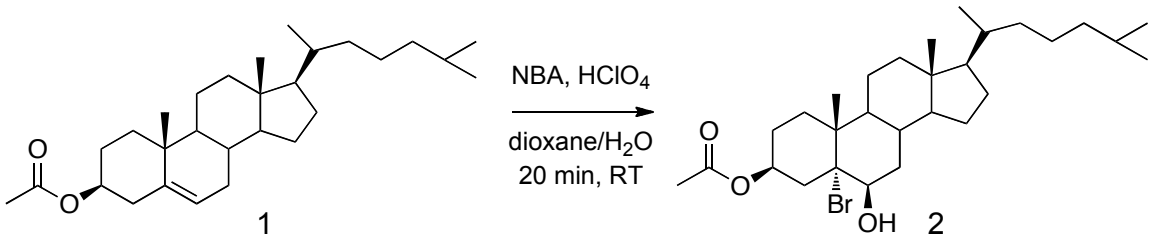
B. Chemicals

Syntheses of 19-ethynylcholesterol and 19-ethynyl, 25-hydroxycholesterol are described below. The following reagents have been described before: propargylcholine[5], fluorescein-azide[1](compound A in Supplementary figure 2), Alexa568-azide (compound C in Supplementary figure 2)[16] and biotin-azide (compound E in Supplementary figure 2)[5]. Tetramethylrhodamine-picolyl azide (TMR-picolyl azide, compound B in Supplementary figure 2) was a gift from Dr Kyle Gee (Life Technologies). Compound D in Supplementary figure 2 was obtained by reacting the succinimidyl ester of Alexa568 carboxylic acid (Molecular Probes) with 2-amino-2'-[6-azido-hexanamide]ethylene glycol diethyl ether, in dry DMSO according to the manufacturer's instructions. Compound F in Supplementary figure 2 was similarly obtained by reacting biotin-succinimidyl ester (Molecular Probes) with 2-amino-2'-[6-azido-hexanamide]ethylene glycol diethyl ether[1]. The following compounds were obtained commercially: SAG from Axxora (≥98%); SANT1 from Calbiochem (≥95%); 20(S)-hydroxycholesterol and 25-hydroxycholesterol from Steraloids (≥98%).

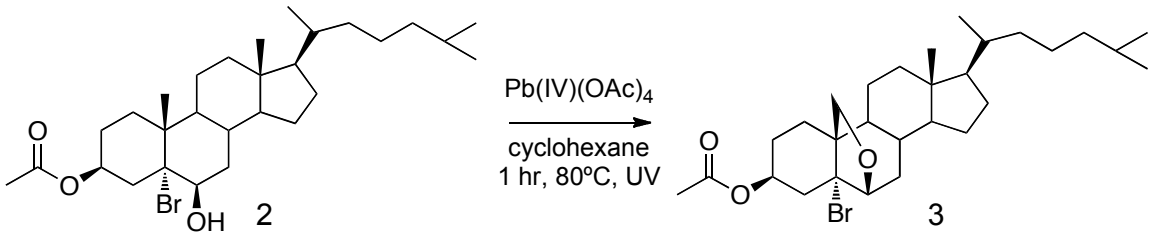
C. Synthesis of 19-ethynylcholesterol (eChol)

All solvents and reagents were obtained from commercial suppliers and were used without further purification. NMR spectra were recorded on a 600 MHz Varian VNMRS or a Varian 400 MHz 400-MR spectrometer, as indicated. NMR chemical shifts are expressed in ppm relative to internal solvent peaks, and coupling constants were measured in Hz. High resolution mass spectrometric data were obtained by the Mass Spectrometry Facility at the University of Texas at Austin, either by electrospray ionization

(ESI) on a Varian IonSpec QFT 9.4T FT-ICR system, or by direct chemical ionization (CI) on a Waters Autospec Ultima sector instrument.

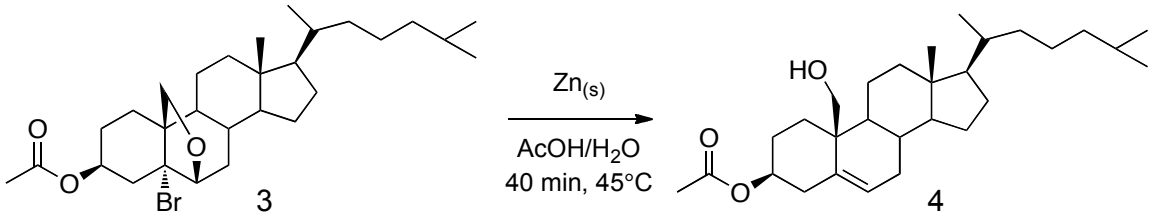


3β-Acetoxy-5α-Bromo-6β-hydroxycholestane (2). 25 g (58.3 mmol) of the acetate was dissolved in 250 mL dioxane while stirring. Then 18.4 mL diluted perchloric acid (5.83 mL 70% perchloric acid, 25 mL water) and 12.5 mL water were added. After slight cooling, 12.5 g (90.6 mmol) N-bromoacetamide was added slowly over 15 minutes and stirred for an additional 20 minutes at room temperature. The flask was cooled to 4°C, the reaction quenched with 150 mL 1% sodium thiosulfate solution and the product (TLC: 20% ethyl acetate/hexane, $R_f = 0.18$, 2% H_2SO_4/EtOH) extracted 3X with diethyl ether. The combined organic layers were dried over Na_2SO_4, filtered, and concentrated. The product was recrystallized (ethyl acetate/hexane) and dried under high vacuum to yield colorless crystals (20 g, 65%). ^{1}H NMR (600 MHz, $CDCl_3$): δ 5.47 (1H, m, 3α-H), 5.30 (DCM), 4.18 (1H, m, 6α-H), 2.03 (3H, s, 3-OAc), 1.32 (3H, s, 19-Me), 0.91 (3H, d, J = 6.6 Hz, 21-Me), 0.86 (6H, m, 26-Me and 27-Me), 0.68 (3H, s, 18-Me), ^{13}C NMR (600 MHz, $CDCl_3$): δ 170.42 (3-O**C**OCH_3), 86.70, 75.78, 72.11, 56.10, 55.71, 18.66 (19-C), 18.02 (21-C), 12.17 (18-C), HR-ESI-MS m/z $[M+Na]^+$ Found: 547.27573, calculated for $C_{29}H_{49}BrO_3Na^+$: 547.27628.

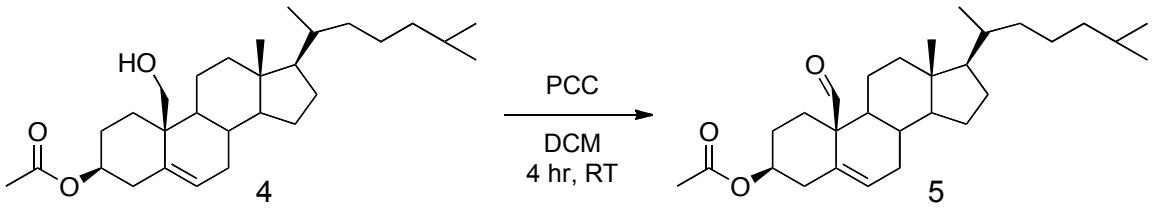


3β-Acetoxy-5α-Bromo-6β-19-Oxidocholestane (3). To a dry flask, 90 g (192 mmol) of 95% lead(IV) tetraacetate and 39 g (390 mmol) of dry calcium carbonate were added and warmed to 80°C in 1800 mL

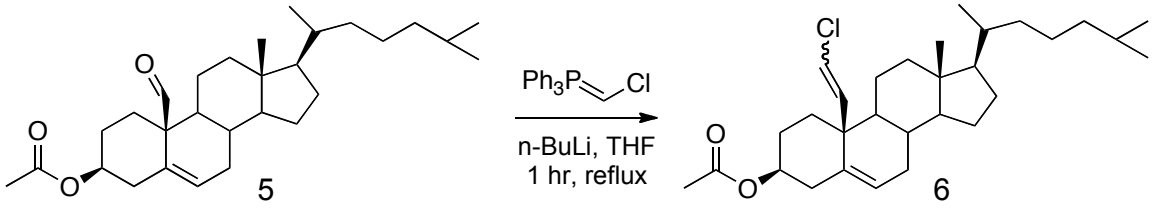
anhydrous cyclohexane. 18.7 g (73.7 mmol) iodine and 17 g (32.3 mmol) bromohydrin **2** were added to the flask. The heat was turned off and the uncapped reaction was exposed to a 500 W long wavelength UV lamp while cooling for 1 hour. The reaction was diluted with diethyl ether, filtered, washed with 10% sodium thiosulfate and water, dried over Na_2SO_4, and concentrated to yield a crude product (TLC: 20% ethyl acetate/hexane, $R_f = 0.31$, 2% H_2SO_4/EtOH), which was recrystallized from methanol/ether/dichloromethane and dried under high vacuum to give colorless needles (16.2 g, 96%). ^{1}H NMR (600 MHz, $CDCl_3$): δ 5.20 (1H, m, 3α-H), 4.05 (1H, d, J = 4.2 Hz, 6α-H), 3.92 (1H, d, J = 7.8 Hz, 19-H), 3.74 (1H, d, J = 8.4 Hz, 19-H), 2.03 (3H, s, 3-OAc), 0.89 (3H, d, J = 6.6 Hz, 21-Me), 0.86 (6H, m, 26-Me and 27-Me), 0.69 (3H, s, 18-Me), ^{13}C NMR (400 MHz, $CDCl_3$): δ 170.39 (3-O**C**$COCH_3$), 82.43, 74.67, 70.10, 67.59, 18.73 (21-C), 12.52 (18-C), HR-ESI-MS m/z $[M+NH_4]^+$ Found: 540.30468, calculated for $C_{29}H_{51}BrO_3N^+$: 540.30539.



3β-Acetoxy-19-Hydroxycholest-5-ene (4). 14 g (26.7 mmol) bromoether **3** was suspended in 450 mL glacial acetic acid and 20 mL water. The flask was heated to 45°C and 92 g (1.4 mol) zinc dust was added in portions while stirring over 15 minutes. After another 40 minutes, the flask was cooled to room temperature, filtered, and concentrated on a rotary evaporator equipped with a KOH trap. The mixture was then dissolved in 5:1 ether/dichloromethane, washed with water, dried over Na_2SO_4, filtered, and concentrated. The product (TLC: 20% ethyl acetate/hexane, $R_f = 0.20$, 2% H_2SO_4/EtOH) was recrystallized from methanol/ether to yield colorless crystals (11.5 g, 97%). ^{1}H NMR (600 MHz, $CDCl_3$): δ 5.77 (1H, m, 6-H, vinylic), 4.64 (1H, m, 3α-H), 3.83 (1H, d, J = 11.4 Hz, 19-H), 3.62 (1H, d, J = 10.2 Hz, 19-H), 2.03 (3H, s, 3-OAc), 0.92 (3H, d, J = 6.6 Hz, 21-Me), 0.86 (6H, m, 26-Me and 27-Me), 0.73 (3H, s, 18-Me), ^{13}C NMR (600 MHz, $CDCl_3$): δ 170.51 (3-O**C**$COCH_3$), 134.52 (5-C), 128.28 (6-C), 73.41, 62.71, 57.55, 56.09, 18.69 (21-C), 12.20 (18-C), HR-ESI-MS m/z $[M+H]^+$ Found: 445.36762, calculated for $C_{29}H_{49}O_3^+$: 445.36795.

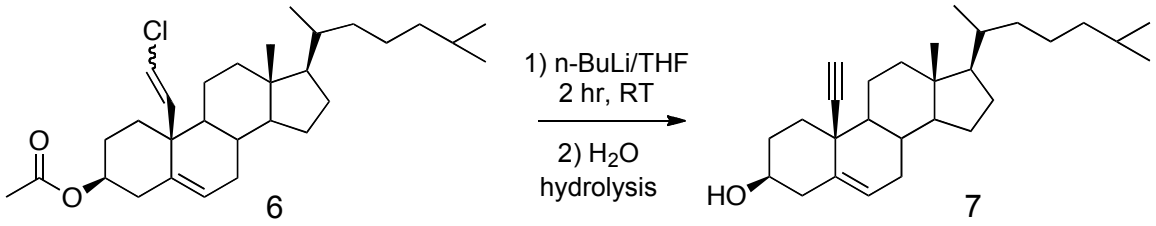


3β-Acetoxy-19-Formylcholest-5-ene (5). 1.45 g (6.75 mmol) pyridinium chlorochromate was added to a mixture of 2.0 g (4.5 mmol) of compound **4**, 1.45 g Celite, and 100 mL anhydrous DCM. The reaction was stirred under Argon at room temperature for 4 hours, was then diluted with diethyl ether, and the insoluble brown residue washed 3X with ether. The combined organic layers were dried over Na_2SO_4, filtered, and concentrated. The product (TLC: 30% ethyl acetate/hexane, $R_f = 0.55$, 2% H_2SO_4/EtOH) was purified by flash chromatography (15% ethyl acetate/hexane) to yield a white solid (1.88 g, 94%). ^{1}H NMR (600 MHz, $CDCl_3$): δ 9.56 (1H, s, 19-H, aldehyde), 5.77 (1H, d, J_{gem} 5.4 Hz, 6-H, vinylic), 4.49 (1H, m, 3α-H), 1.89 (3H, s, 3-OAc), 0.53 (3H, s, 18-Me), ^{13}C NMR (600 MHz, $CDCl_3$): δ 204.31 (19-C), 170.06 (3-O**C**OCH_3), 131.54 (C-5), 128.26 (C-6), 72.56 (3-C), 18.61 (21-C), 11.68 (18-C), HR-ESI-MS m/z $[M+H]^+$ Found: 443.35159, calculated for $C_{29}H_{47}O_3^+$: 443.35252.



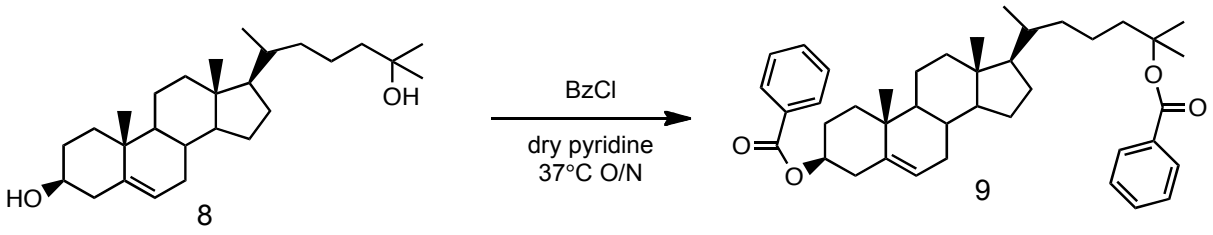
3β-Acetoxy-19-(Chloroalkene)cholest-5-ene (6). To a solution of 7.85 g (22.6 mmol) chloromethyltriphenylphosphonium chloride in 120 mL anhydrous THF was added n-butyllithium (17.9 mmol, 11.2 mL of a 1.6M solution in hexanes) dropwise under Argon. The slurry was stirred at room temperature for 2.5 hrs, after which 1.88 g (4.25 mmol) of the aldehyde **5** dissolved in 20 mL of anhydrous THF was added dropwise. The flask was fitted with a condenser and refluxed under Argon for 1 hour. The reaction was cooled, diluted with saturated ammonium chloride, and the crude product extracted three times with methylene chloride. The combined organic layers were dried over Na_2SO_4, filtered, concentrated, and the product (TLC: 30% ethyl acetate/hexane, $R_f = 0.3$, 2% H_2SO_4/EtOH) was isolated by flash chromatography (30% ethyl acetate/hexane) as a clear oil (1.35 g). During the workup, a significant amount of the product was hydrolyzed to 3β-hydroxy-19-(Chloroalkene)cholest-5-ene,

which was isolated and re-acetylated by refluxing with acetic anhydride in pyridine, to afford a second batch of the product (0.485 g). The total yield of compound **6** was 1.835 g (91%). ^{1}H NMR (600 MHz, $CDCl_3$): δ 6.17 (0.5H, d, J_{trans} 8.4 Hz), 5.78 (1H, d, J_{gem} 1.2 Hz, 6-H, vinylic), 5.59 (0.5H, d, J_{cis} 5.4 Hz), 5.50 (0.5H, d, J_{cis} 5.4 Hz), 5.45 (0.5H, d, J_{trans} 8.4 Hz), 3.55 (1H, m, 3α-C), 0.64 (3H, s, 18-Me), ^{13}C NMR (600 MHz, $CDCl_3$): δ 167.35 (3-O**C**OCH_3), 137.79, 137.35, 136.06, 135.10, 125.08, 123.86, 120.14, 119.26, 71.37, 71.03, HR-CI-MS $[M-H]^{+\cdot}$ Found: 473.3188, calculated for $C_{30}H_{46}ClO_2$: 473.31863.

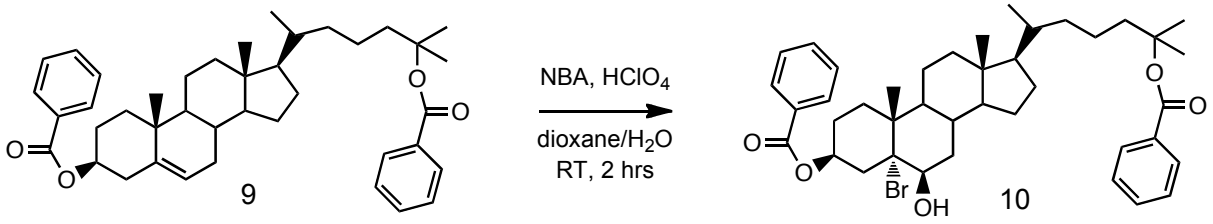


3β-Hydroxy-19-Ethynylcholest-5-ene (7). To a solution of 1.835 g (3.85 mmol) of **6** in 80 mL anhydrous THF was added 32.3 mmol n-butyllithium (20.2 mL of a 1.6M solution in hexanes) dropwise under Argon, and the mixture was allowed to react for 2 hrs. Saturated ammonium chloride was added and the mix was extracted three times with methylene chloride. During the workup, the protective acetate group was quantitatively hydrolyzed, forming the product **7**. The combined organic layers were dried over Na_2SO_4, filtered, and concentrated. The product (TLC: 30% ethyl acetate/hexane, R_f = 0.35, 2% H_2SO_4/EtOH) was purified by flash chromatography (30% ethyl acetate/hexane) to yield a white solid (970 mg, 64%). ^{1}H NMR (600 MHz, $CDCl_3$): δ 5.47 (1H, d, J_{gem} 4.8 Hz, 6-H, vinylic), 3.52 (1H, m, 3α-H), 2.25 (1H, s, 19-CCH, acetylenic), 0.722 (3H, s, 18-Me), ^{13}C NMR (600 MHz, $CDCl_3$): δ 136.84 (5-C), 123.08 (6-C), 87.09 (19-C), 71.97 (19-C**C**H), 70.69 (3-C), HR-ESI-MS m/z $[M+H]^+$ Found: 397.34655, calculated for $C_{28}H_{45}O^+$: 397.34704.

D. Synthesis of 19-ethynyl-25-hydroxycholesterol

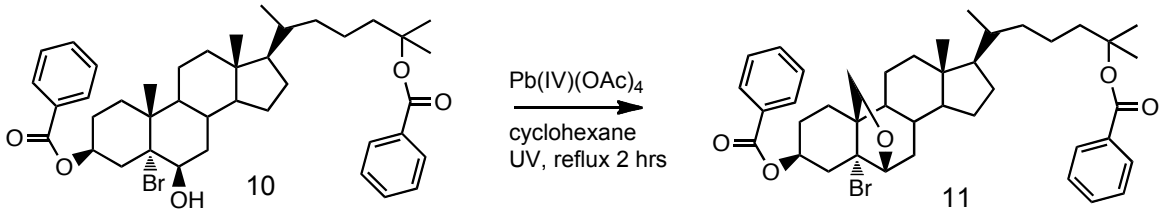


Cholest-5-ene-3β,25-diol 3β,25-Dibenzoate (9). To a solution of 1.5 g (3.73 mmol) of 25-hydroxycholesterol (8) in 60 mL anhydrous pyridine was added 3.5 mL (30 mmol) benzoyl chloride. The reaction was stirred at 37°C overnight under Argon, diluted with 300 mL methanol and 600 mL hexane, washed with 300 mL each of 10% hydrochloric acid, water, saturated sodium bicarbonate, and water, dried over Na_2SO_4, filtered, and concentrated. The product (TLC: 50% ethyl acetate/hexane, R_f = 0.33, 2% H_2SO_4/EtOH) could not be separated from a contaminating benzoyl side product, which had an identical R_f; however, most of the dibenzoate recrystallized upon storage at -20°C. ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.04 (2H, m), 7.99 (2H, m), 7.54 (2H, m), 7.43 (4H, m), 5.42 (1H, m, 6-H, vinylic), 4.86 (1H, m, 3α-H), 1.58 (6H, s, 26-Me and 27-Me), 1.07 (3H, s, 19-Me), 0.93 (3H, d, J = 6.6 Hz, 21-Me), 0.69 (3H, s, 18-Me), ^{13}C NMR (600 MHz, $CDCl_3$): δ 165.98 (3-O**C**OC_6H_5), 165.68 (25-OBz), 139.66 (5-C), 132.89, 132.69, 132.37, 132.09, 130.85, 130.17, 129.56, 129.53, 129.38, 128.34, 128.25, 128.17, 122.75 (6-C), 83.31 (25-C), 74.56 (3-C), 19.37 (19-C), 18.63 (21-C), 11.88 (18-C), HR-ESI-MS m/z $[M+Na]^+$ Found: 633.39047, calculated for $C_{41}H_{54}O_4Na^+$: 633.39143.

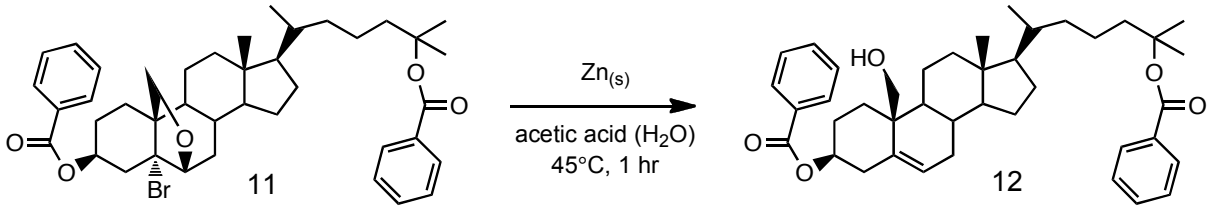


5α-Bromocholestane-3β,6β,25-triol 3β,25-Dibenzoate (10). 1.8 g (3.0 mmol) of dibenzoate 9 was dissolved in 20 mL dioxane while stirring. Then 1.32 mL diluted perchloric acid (0.25 mL 70% perchloric acid, 1.07 mL water) and 0.9 mL water were added. After stirring for 15 minutes, 631 mg

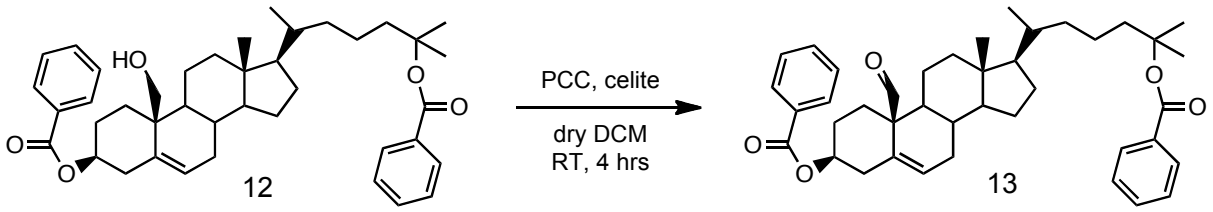
(4.6 mmol) N-bromoacetamide was added and stirred for an additional 2 hours at RT. The flask was cooled to 4°C, the reaction quenched with dilute sodium thiosulfate and the product (TLC: 5% ethyl acetate/hexane, $R_f = 0.33$, 2% H_2SO_4/EtOH) extracted 2X with diethyl ether. The combined organic layers were dried over Na_2SO_4, filtered, and concentrated. The product was purified by flash column chromatography (10% ethyl acetate/hexane) to yield white crystals (1.3 g, 62%). ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.02 (2H, m), 7.99 (2H, m), 7.53 (2H, m), 7.41 (4H, m), 5.73 (1H, m, 3α-H), 4.22 (1H, m, 6α-H), 1.58 (6H, s, 26-Me and 27-Me), 1.37 (3H, s, 19-Me), 0.94 (3H, d, J = 6.6 Hz, 21-Me), 0.68 (3H, s, 18-Me), ^{13}C NMR (600 MHz, $CDCl_3$): δ 165.99 (3-O**C**OC_6H_5), 165.73 (25-O**C**OC_6H_5), 132.80, 132.40, 132.04, 130.61, 129.55, 129.38, 128.27, 128.18, 86.93, 83.34, 75.71 (25-C), 72.88 (3-C), 18.58, 18.06, 12.23 (18-C), HR-CI-MS $[M-H]^+$ Found: 705.3155, calculated for $C_{41}H_{54}O_5Br^+$: 705.3155.



5α-Bromo-6β-19-Oxidocholestane-3β,25-diol 3β,25-Dibenzoate (11). To a dry flask, 4.9 mg (11.0 mmol) of lead(IV) tetraacetate and 2.22 g (22.1 mmol) of dry calcium carbonate were added and warmed to 80°C in 110 mL anhydrous cyclohexane. 1.1 g (4.2 mmol) iodine and 1.3 g (1.8 mmol) bromohydrin (10) were added to the flask and refluxed for 2 hours while being exposed to a 500 W longwave UV lamp under Argon. The reaction was cooled to room temperature, diluted with diethyl ether, filtered, and washed with 10% sodium thiosulfate and water, dried over Na_2SO_4, filtered, and concentrated. The product (TLC: 5% ethyl acetate/hexane, $R_f = 0.5$, 2% H_2SO_4/EtOH) was purified by flash chromatography (5% ethyl acetate/hexane) to yield a clear oil (1.2 g, 93%). ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.02 (2H, m), 7.98 (2H, m), 7.53 (2H, m), 7.42 (4H, m), 5.46 (1H, m, 3α-H), 4.09 (1H, m, 6α-H), 3.99 (1H, d, J = 7.8 Hz, 19-H), 3.78 (1H, d, J = 8.4 Hz, 19-H), 1.58 (6H, s, 26-Me and 27-Me), 0.91 (3H, d, J = 6.6 Hz, 21-Me), 0.70 (3H, s, 18-Me), ^{13}C NMR (600 MHz, $CDCl_3$): δ 165.71 (3-O**C**OC_6H_5), 165.58 (25-O**C**OC_6H_5), 132.86, 132.36, 132.05, 130.44, 129.53, 129.35, 128.29, 128.16, 83.21, 82.33, 74.59, 72.60, 70.60, 67.49, 56.00, 18.53 (21-C), 12.44 (18-C), HR-ESI-MS m/z $[M+Na]^+$ Found: 727.29702, calculated for $C_{41}H_{53}O_5NaBr^+$: 727.29686.

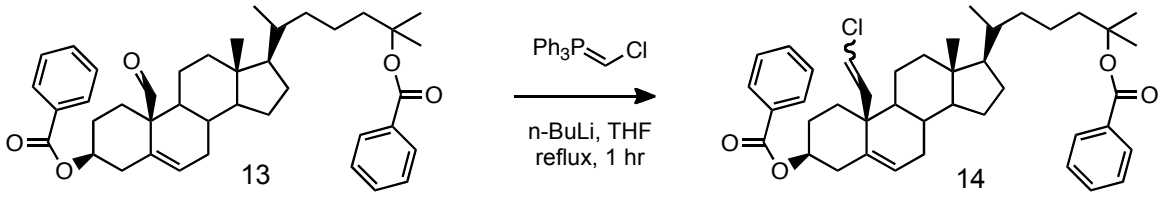


Cholest-5-ene-3β,19,25-triol 3β,25-Dibenzoate (12). 1.2 g (0.1.7 mmol) compound 11 was suspended in 45 mL (784 mmol) glacial acetic acid and 2 mL water. The flask was heated to 45°C and 9.0 g (137 mmol) zinc powder was added in portions while stirring, over 15 minutes. After another hour, the flask was cooled to room temperature, filtered, diluted with water, and extracted 3 times with dichloromethane. The combined organic layers were dried over Na_2SO_4, filtered, and concentrated. The product (TLC: 5% ethyl acetate/hexane, $R_f = 0.25$, 2% H_2SO_4/EtOH) was purified by flash chromatography (20% ethyl acetate/hexane) to yield a white solid (0.7 g, 66%). ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.03 (2H, m), 7.98 (2H, m), 7.53 (2H, m), 7.42 (4H, m), 5.81 (1H, m, 6-H, vinylic), 4.91 (1H, m, 3α-H), 3.89 (1H, d, J = 11.4 Hz, 19-H), 3.67, m, 19-H), 1.58 (6H, s, 26-Me and 27-Me), 0.93 (3H, d, J = 6.6 Hz, 21-Me), 0.74 (3H, s, 18-Me), ^{13}C NMR (600 MHz, $CDCl_3$): δ 165.94 (3-O**C**OC_6H_5), 165.68 (25-O**C**OC_6H_5), 134.58 (5-C), 132.80, 132.37, 132.08, 130.66, 129.53, 129.37, 128.36 (6-C), 128.28, 128.17, 83.31 (25-C), 73.98 (3-C), 62.77 (19-C), 18.61 (21-C), 12.23 (18-C), HR-ESI-MS m/z $[M+H]^+$ Found: 627.40445, calculated for $C_{41}H_{55}O_5^+$: 627.40440.

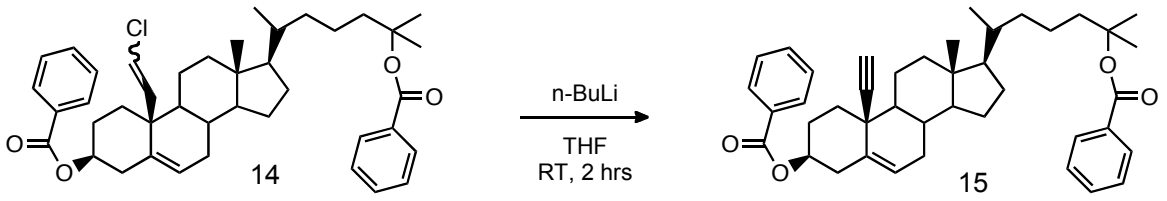


19-Formylcholest-5-ene-3β,25-diol 3β,25-Dibenzoate (13). 311 mg (1.44 mmol) pyridinium chlorochromate was added to a mixture of 0.7 g (0.96 mmol) of compound 12, 315 mg Celite, and 100 mL anhydrous dichloromethane. The reaction was stirred under Argon at room temperature for 4 hours, diluted with diethyl ether and filtered. The insoluble brown residue was washed 3 times with ether, and the combined organic layers were dried over Na_2SO_4, filtered, and concentrated. The product (TLC: 25% ethyl acetate/hexane, $R_f = 0.42$, 2% H_2SO_4/EtOH) was purified by flash chromatography (10%

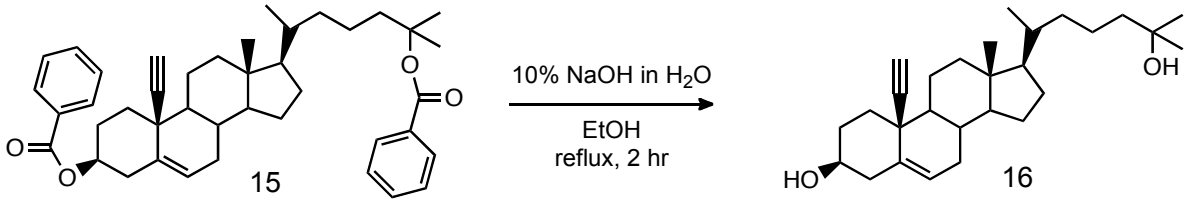
ethyl acetate/hexane) to yield a white solid (0.57 g, 95%). ^{1}H NMR (600 MHz, $CDCl_3$): δ 9.66 (1H, d, 1.2 Hz, aldehydic), 7.99 (4H, m), 7.49 (2H, m), 7.38 (4H, m), 5.88 (1H, m, 6-H, vinylic), 5.24 (DCM), 4.84 (1H, m, 3α-H), 1.55 (6H, s, 26-Me and 27-Me), 0.88 (3H, d, J = 6.6 Hz, 21-Me), 0.60 (3H, s, 18-Me), ^{13}C NMR (600 MHz, $CDCl_3$): δ 204.81 (19-C), 165.78 (3-O**C**OC_6H_5), 165.56 (25-O**C**OC_6H_5), 132.79, 132.36, 132.04, 131.48, 130.53, 129.52, 129.35, 128.62, 128.59, 128.25, 128.16, 83.18 (25-C), 73.26 (3-C), 18.57 (21-C), 11.80 (18-C), HR-ESI-MS m/z $[M+Na]^+$ Found: 647.37052, calculated for $C_{41}H_{52}O_5Na^+$: 647.37070.



19-(Chloroalkene)cholest-5-ene-3β,25-diol 3β,25-Dibenzoate (14). To a solution of 1.55 g (4.45 mmol) chloromethyltriphenylphosphonium chloride in 50 mL anhydrous THF was added n-butyllithium (3.0 mL, 4.7 mmol) dropwise under Argon. The slurry was stirred at RT for 2.5 hrs under Argon, after which 570 mg (0.91 mmol) of the aldehyde 13 was added dropwise in 20 mL anhydrous THF. The flask was equipped with a condenser and the mix was refluxed under Argon for 1 hour. The reaction was cooled, diluted with aqueous ammonium chloride, and the crude product extracted with dichloromethane. The combined organic layers were dried over Na_2SO_4, filtered, and concentrated. The product (TLC: 2.5% ethyl acetate/hexane, R_f = 0.4, 2% H_2SO_4/EtOH) was purified by flash chromatography (2.5% ethyl acetate/hexane) to yield a clear oil (0.3 g, 50%). ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.04 (2H, m), 7.99 (2H, m), 7.52 (2H, m), 7.41 (4H, m), 6.22 (0.5H, m), 5.83 (0.5H, m), 5.65 (0.5H), 5.58 (1H, m, 6-H, vinylic), 5.47 (0.5H, m), 4.90 (1H, m, 3α-H), 1.58 (6H, s, 26-Me and 27-Me), 0.93 (3H, d, J = 4.2 Hz, 21-Me), 0.65 (3H, s, 18-Me), ^{13}C NMR (600 MHz, $CDCl_3$): δ 165.88 (3-O**C**OC_6H_5), 165.59 (25-O**C**OC_6H_5), 137.69, 136.34, 124.95, 132.75, 132.36, 132.09, 130.79, 130.55, 129.38, 128.17, 126.18, 124.96, 120.31, 119.53, 83.21 (25-C), 74.02, 73.73, 64.76, 18.63 (21-C), 11.88 (18-C), HR-CI-MS $[M-H]^+$ Found: 655.3558, calculated for $C_{42}H_{52}O_4Cl^+$: 655.3554.

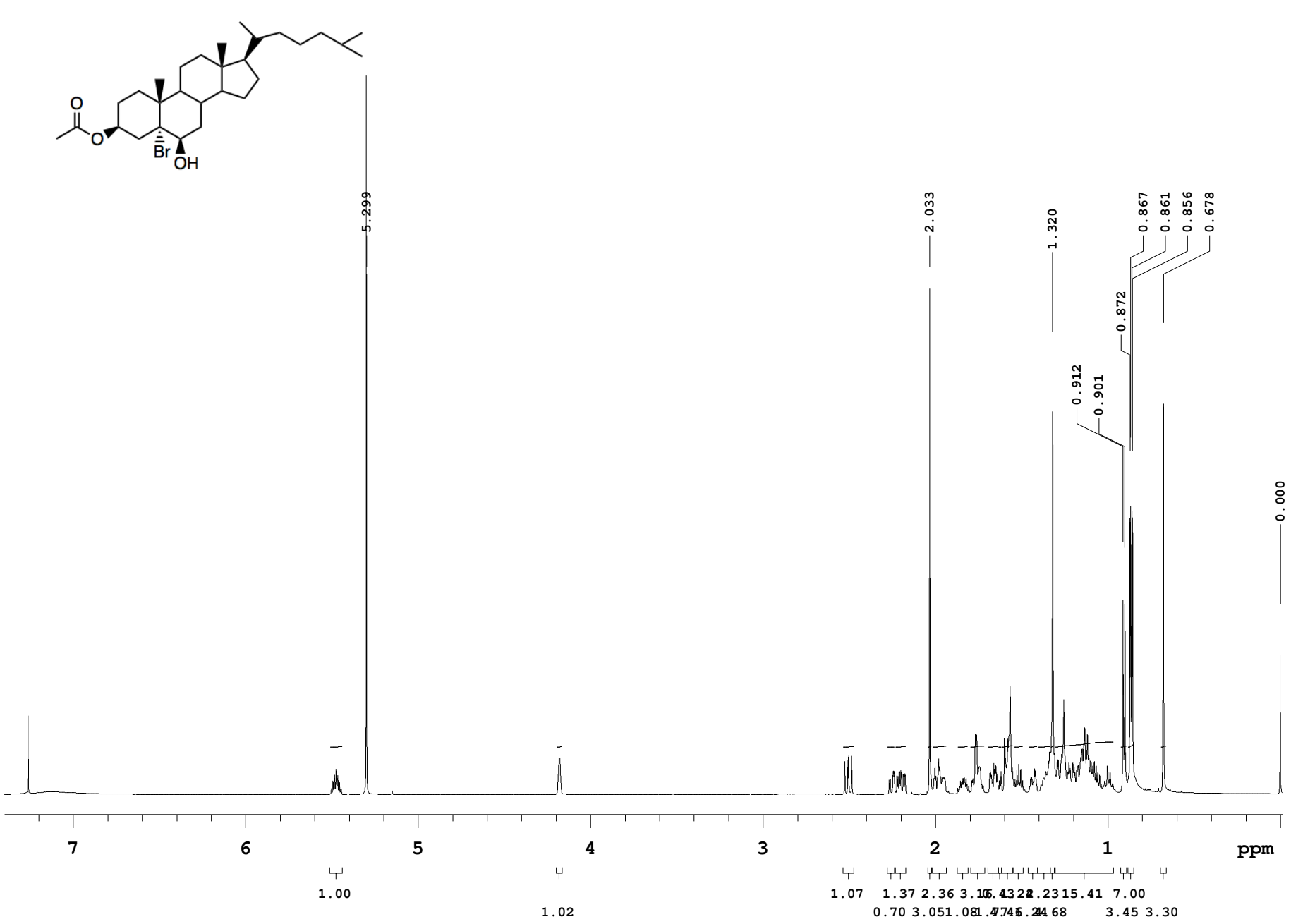


19-Ethynylcholest-5-ene-3β,25-diol 3β,25-Dibenzoate (15). To a solution of 300 mg (0.46 mmol) compound 14 in 10 mL anhydrous THF was added 250 mg (3.9 mmol) n-BuLi dropwise under Argon, and the mixture was allowed to react for 2 hours. Saturated ammonium chloride was added and the product was extracted with dichloromethane. The combined organic layers were dried over Na_2SO_4, filtered, and concentrated. The product (TLC: 50% ethyl acetate/hexane, $R_f = 0.7$, 2% H_2SO_4/EtOH) was purified by flash chromatography (50% ethyl acetate/hexane) to yield a clear oil (100 mg, 35%). ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.99 (2H, m), 7.71 (2H, m), 7.52 (4H, m), 5.46 (1H, m, 6-H, vinylic), 3.50 (1H, m, 3α-H), 2.23 (1H, s, 19-CCH, acetylenic), 1.20 (6H, s, 26-Me and 27-Me), 0.93 (3H, d, J = 6.6 Hz, 21-Me), 0.71 (3H, s, 18-Me), ^{13}C NMR (600 MHz, $CDCl_3$): δ 167.80 (3-O**C**OC_6H_5), 136.79 (5-C), 123.20 (6-C), 87.10, 71.97, 71.11, 70.82, 65.87, 18.68 (21-C), 11.69 (18-C).



19-Ethynylcholest-5-ene-3β,25-diol (19-ethynyl-25-hydroxycholesterol, 16). 100 mg (0.16 mmol) of compound 15 was dissolved in 50 mL ethanol and 7.5 mL 10% aqueous sodium hydroxide, and the mix was refluxed for 2 hours. The reaction was cooled to room temperature, the product (TLC: 25% ethyl acetate/hexane, $R_f = 0.33$, 2% H_2SO_4/EtOH) extracted with dichloromethane, and the organic layers were combined and washed with dilute acid and water, dried over Na_2SO_4, filtered, and concentrated, yielding a white solid (65 mg, 98%). ^{1}H NMR (600 MHz, $CDCl_3$): δ 5.47 (1H, m, 6-H, vinylic), 3.52 (1H, m, 3α-H), 2.22 (1H, s, 19-CCH, acetylenic), 1.22 (6H, s, 26-Me and 27-Me), 0.94 (3H, d, J = 6.6 Hz, 21-Me), 0.72 (3H, s, 18-Me), ^{13}C NMR (600 MHz, $CDCl_3$): δ 136.78 (5-C), 123.25 (6-C), 87.10 19-C**C**H), 71.97 (25-C), 71.10 (19-**C**CH), 70.86 (3-C), 60.39, 18.67 (21-Me), 11.70 (18-C), HR-ESI-MS m/z $[(M+H)^2+H]^+$ Found: 827.69227, calculated for $C_{56}H_{91}O_4^+$: 827.69119.

E. NMR spectra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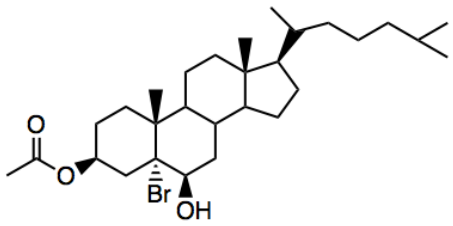
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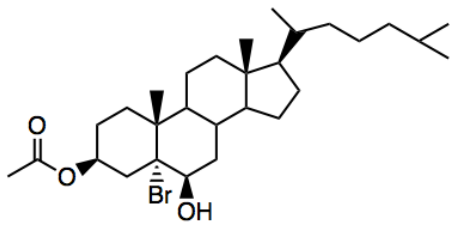
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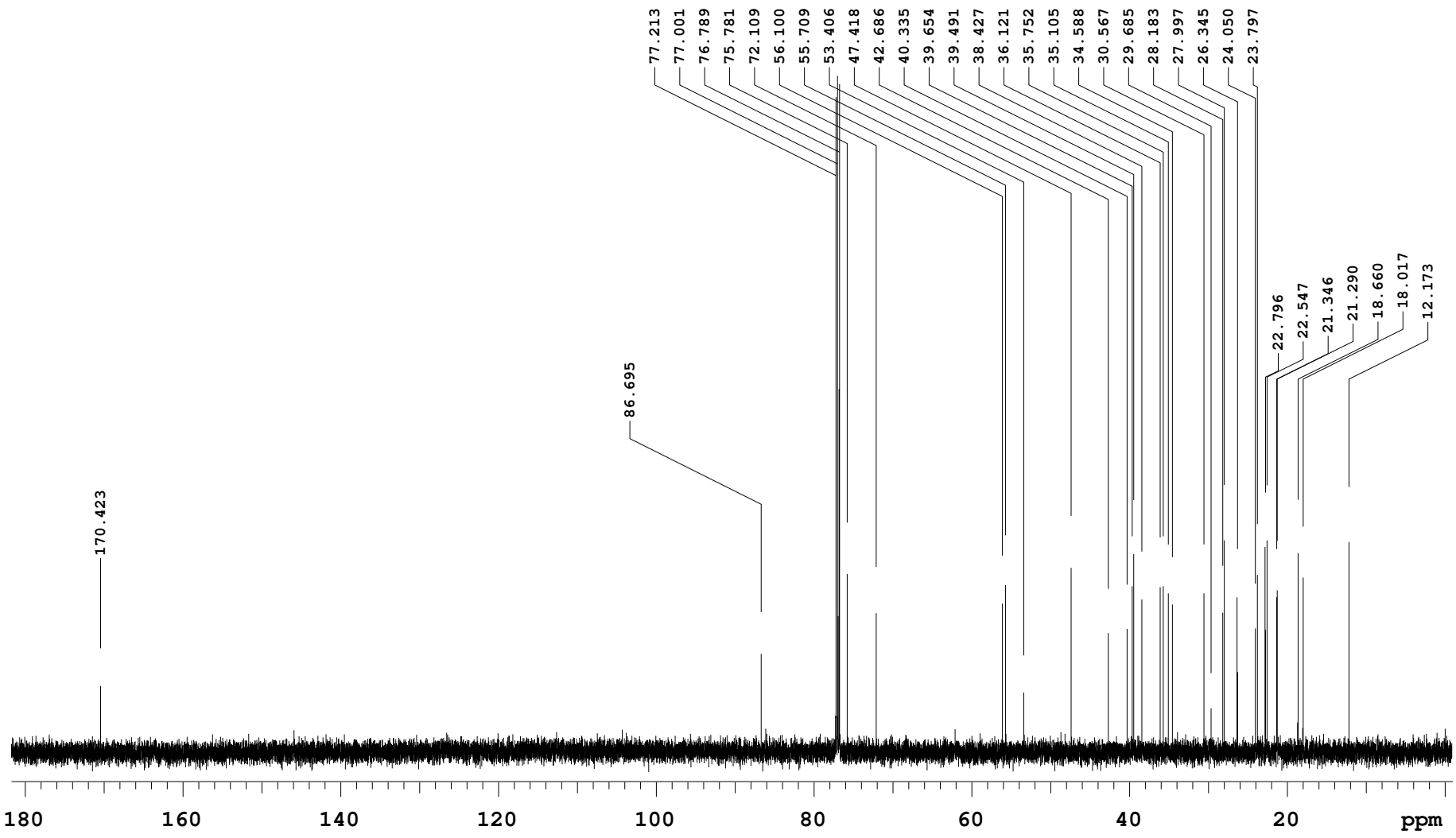
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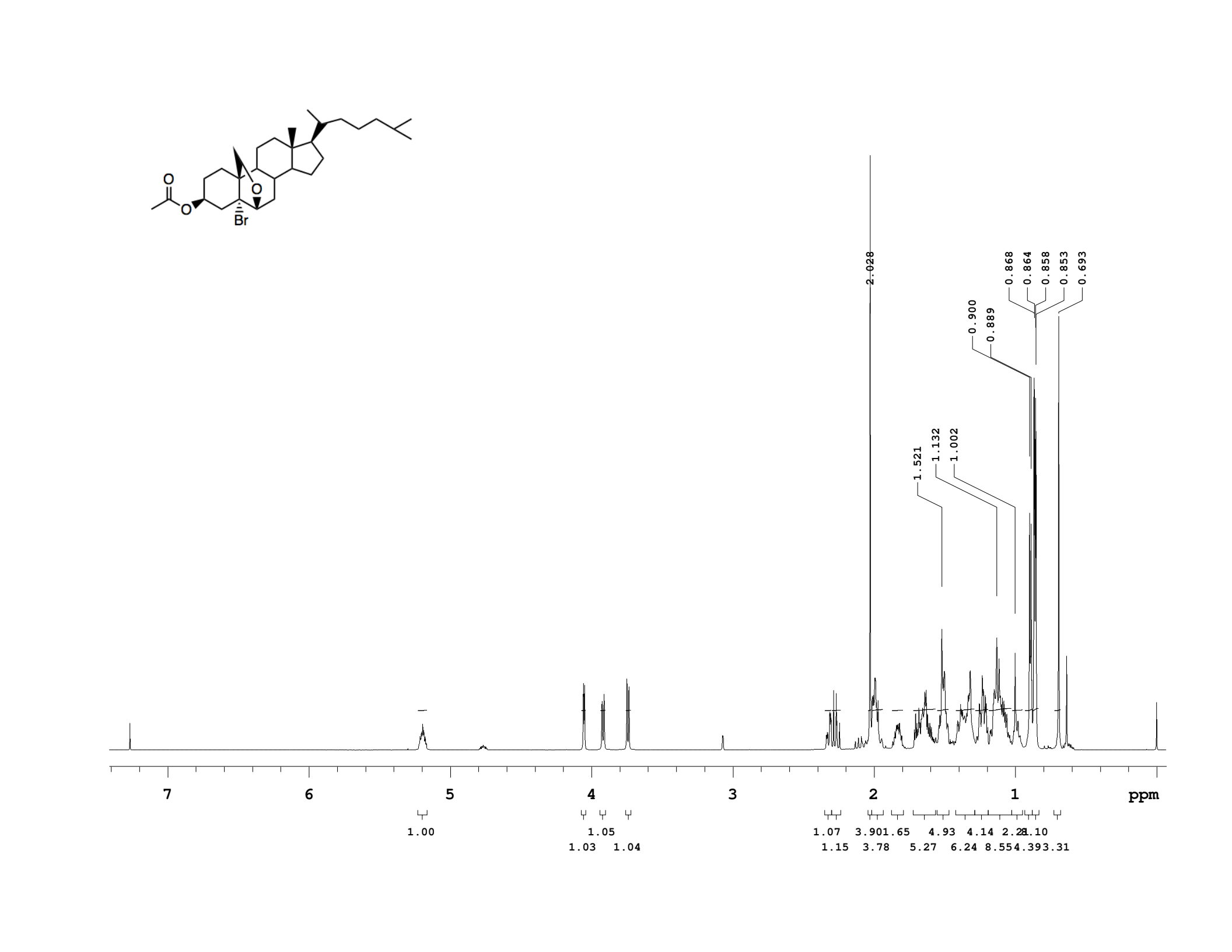
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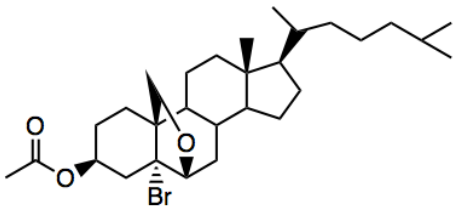
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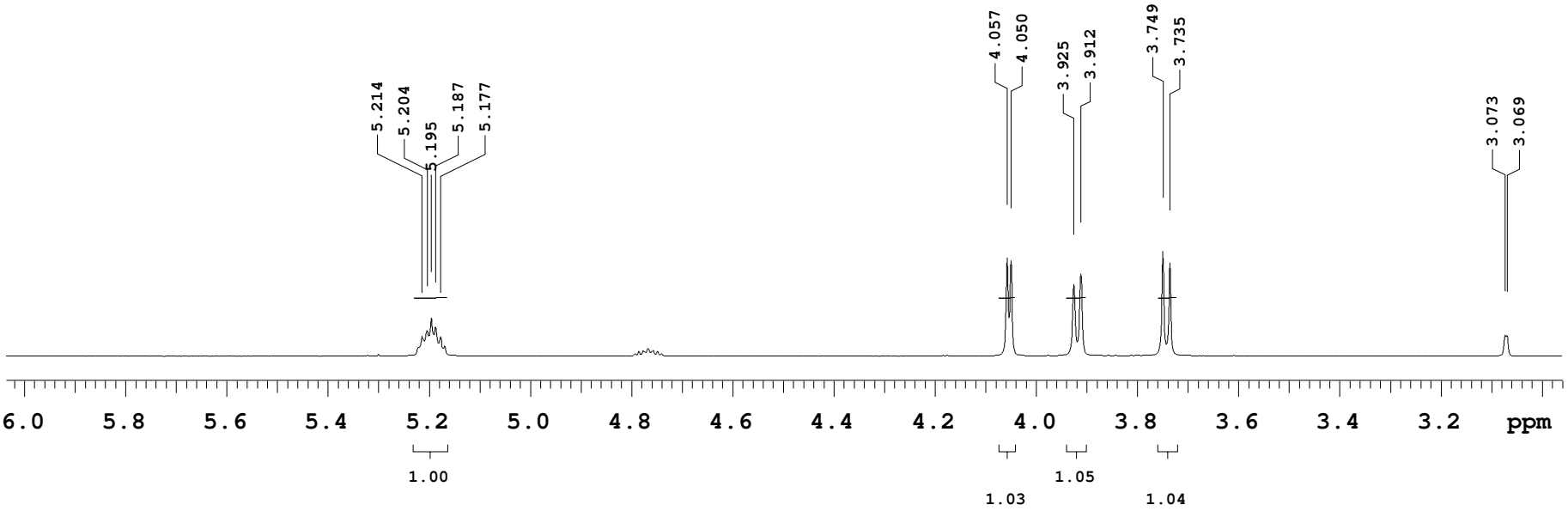
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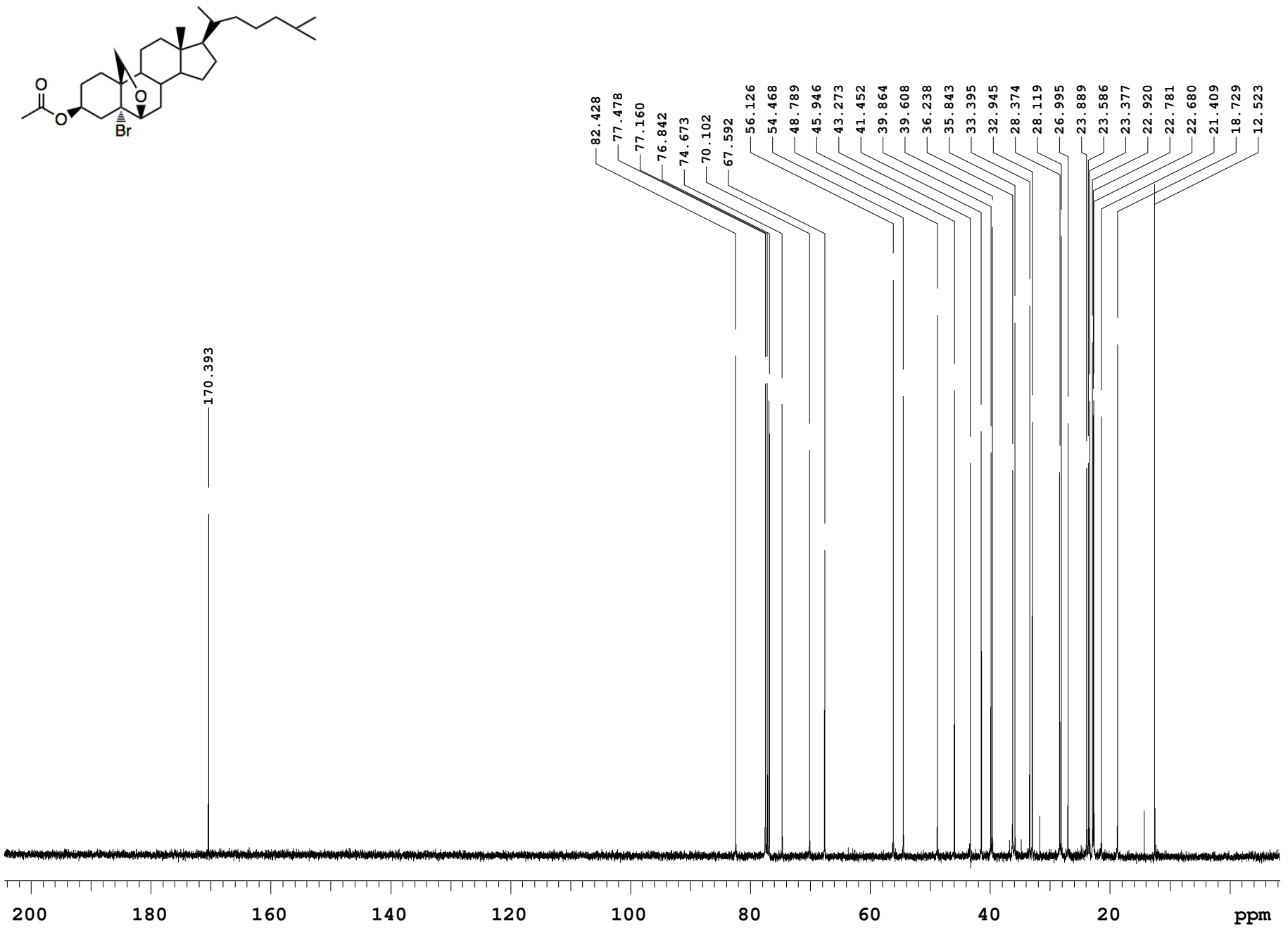
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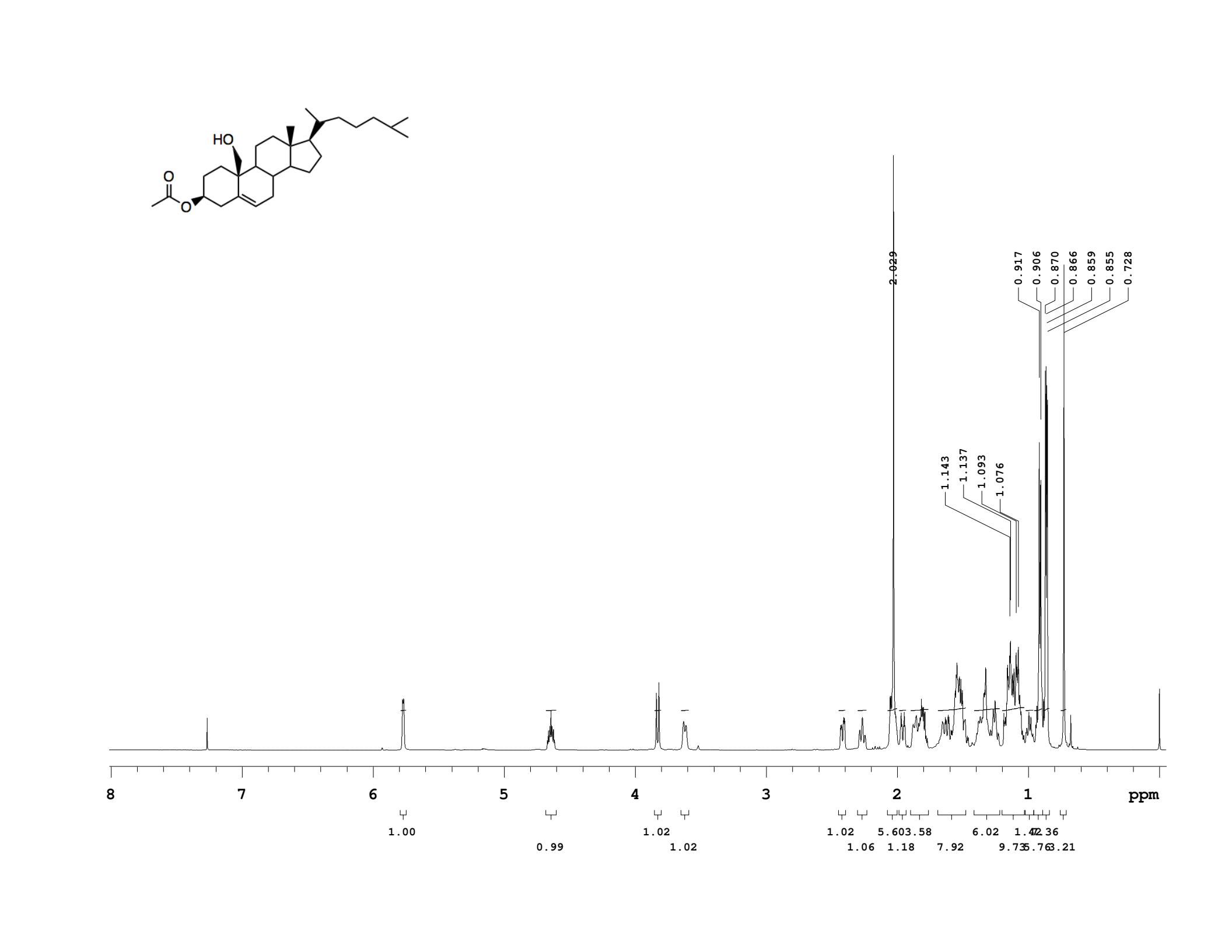
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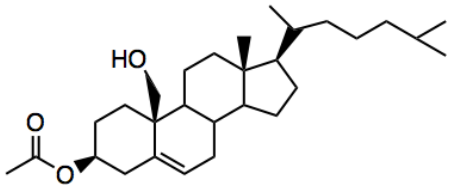
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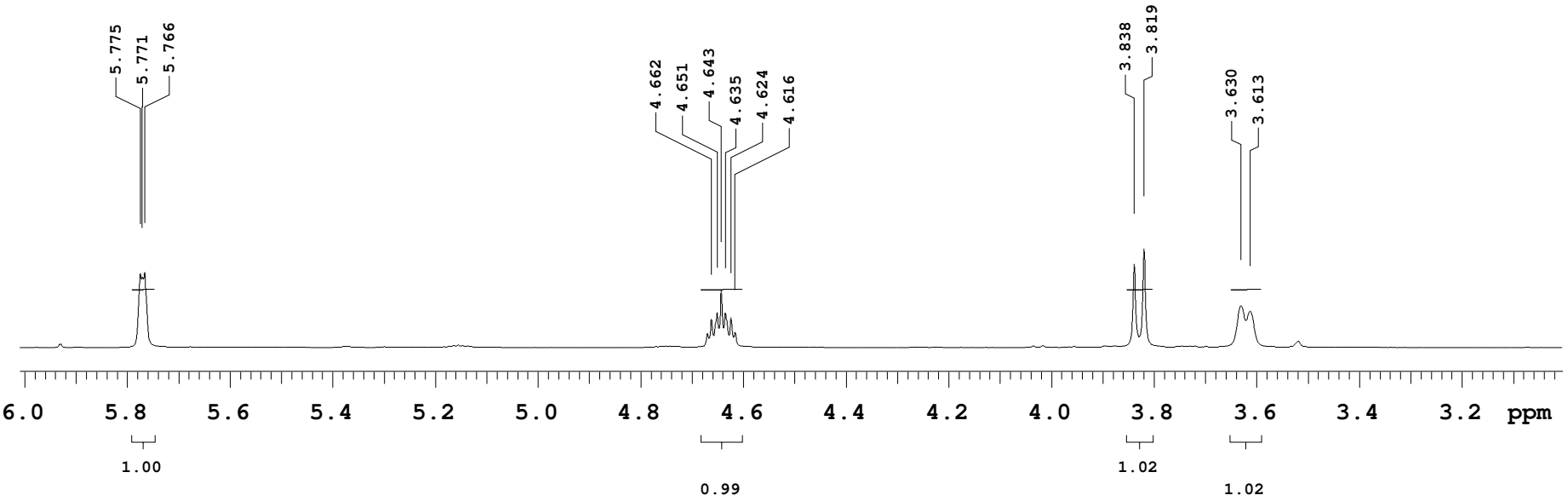
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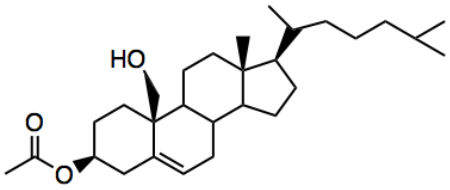
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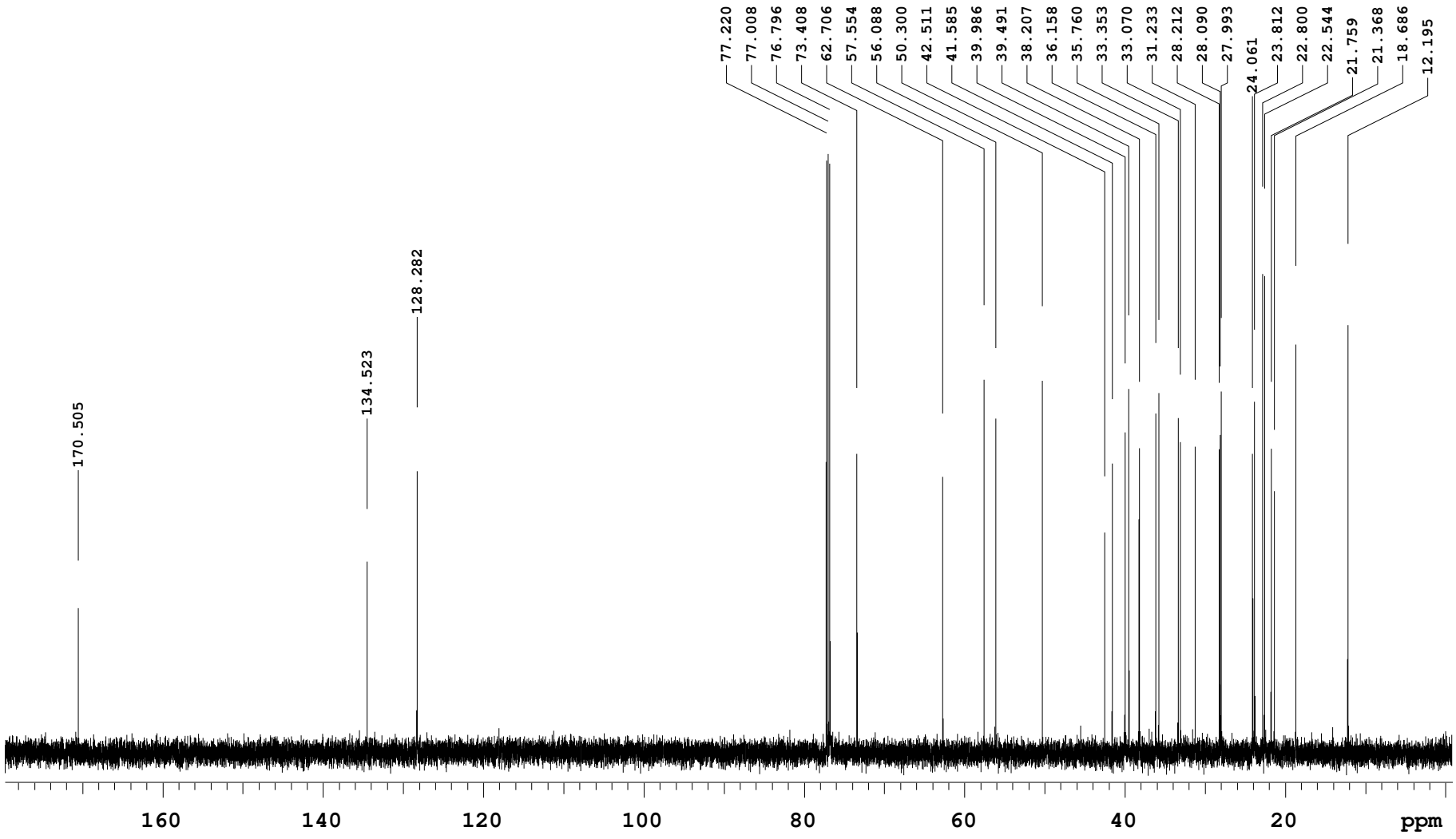
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38.207
36.158
35.760
33.353
33.070
31.233
28.212
28.090
27.993
24.061
23.812
22.800
22.544
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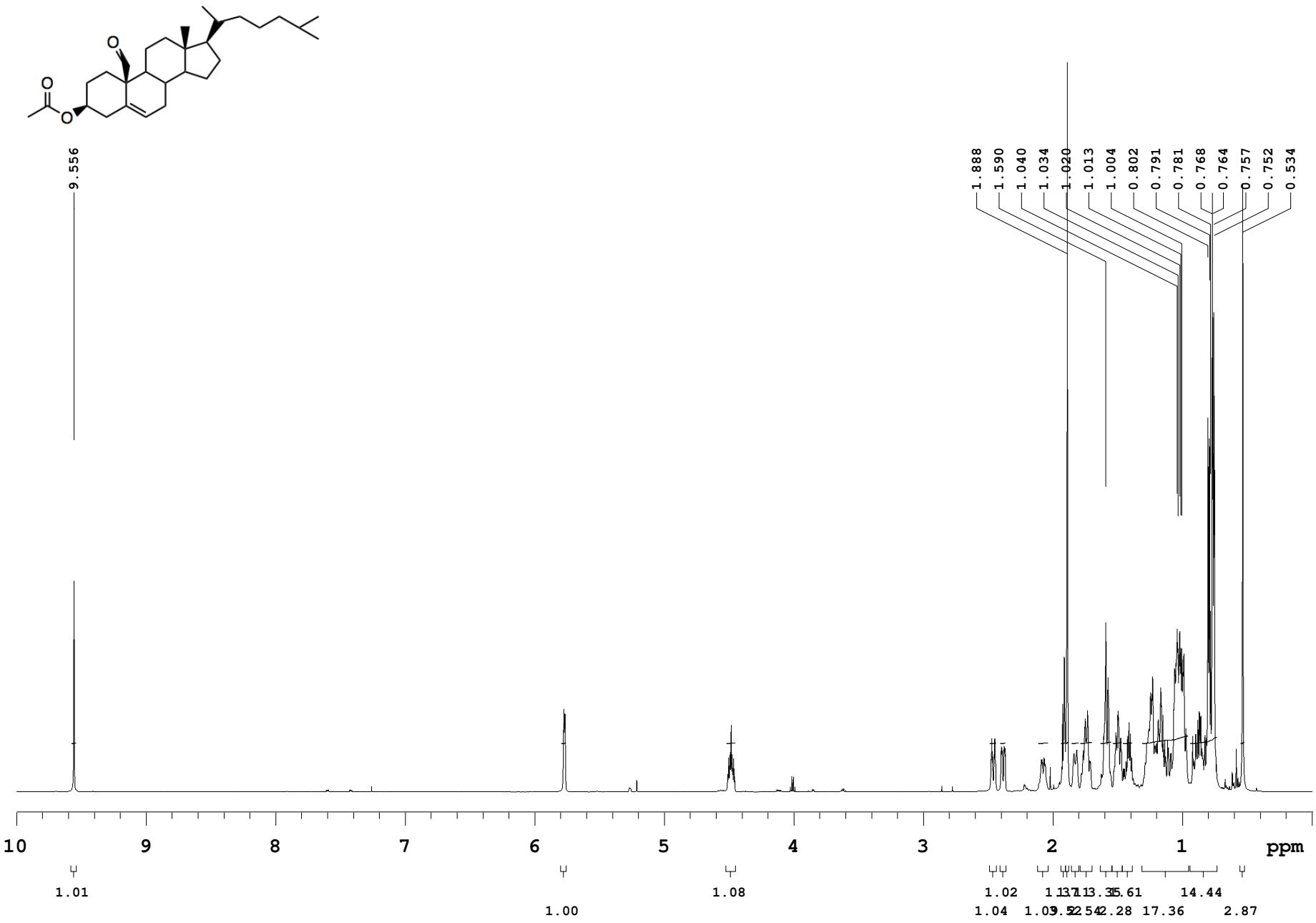
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1.590
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1.004
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0.791
0.781
0.768
0.764
0.757
0.752
0.534
10
9
8
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2
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ppm
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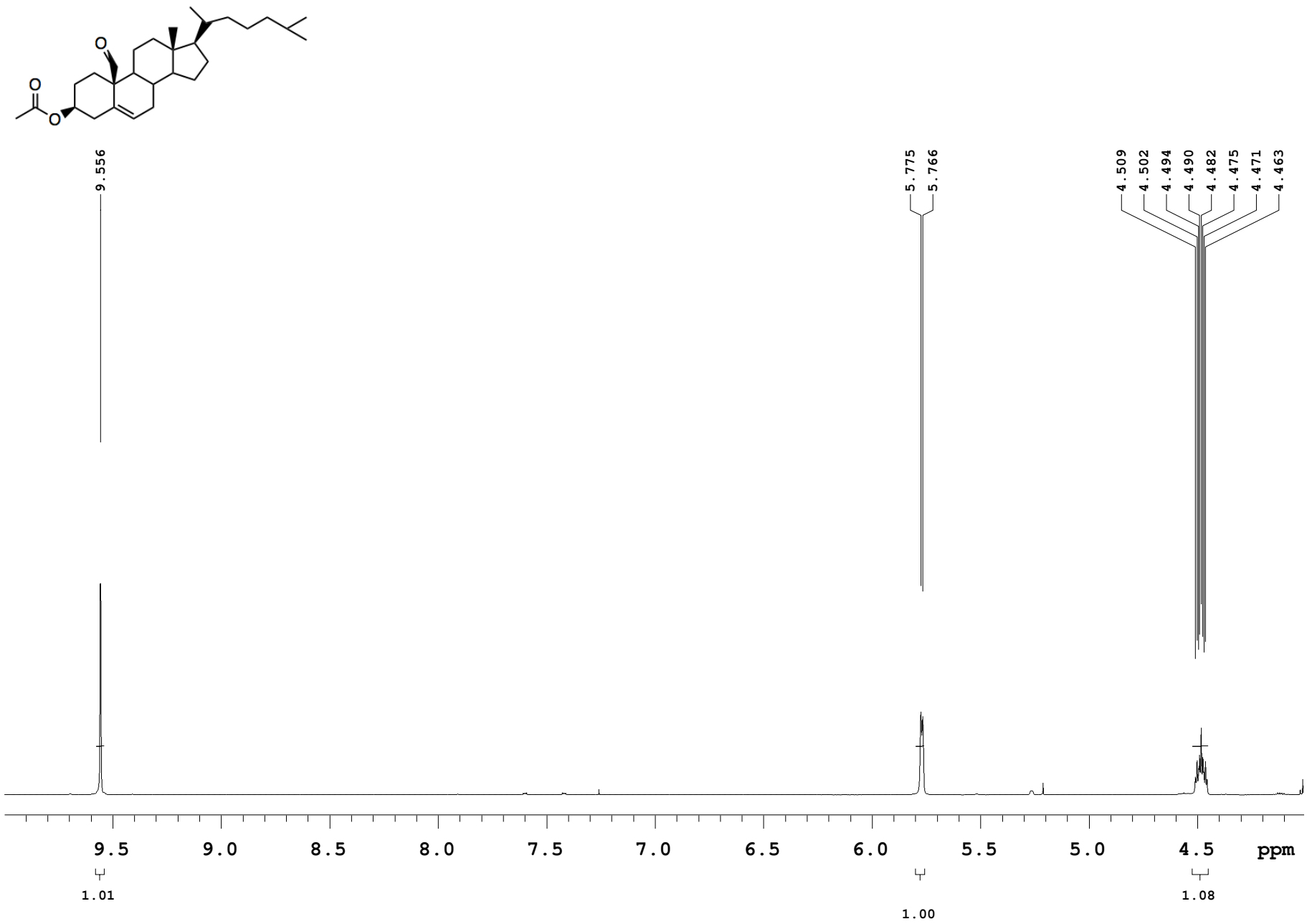
1.00
1.08
1.02
1.04
14.44
2.87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5.775
5.766
4.509
4.502
4.494
4.490
4.482
4.475
4.471
4.463
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
ppm
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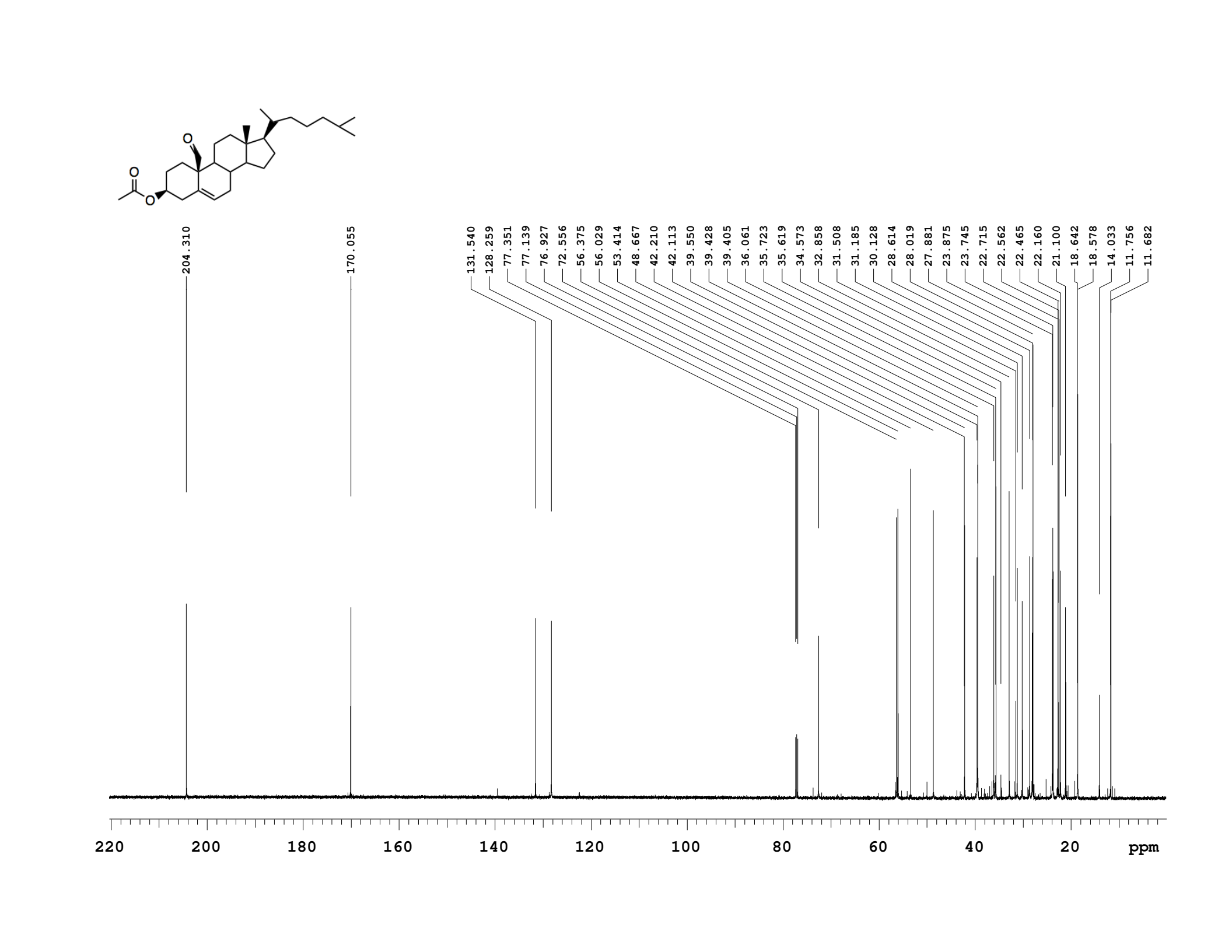
1.00
1.08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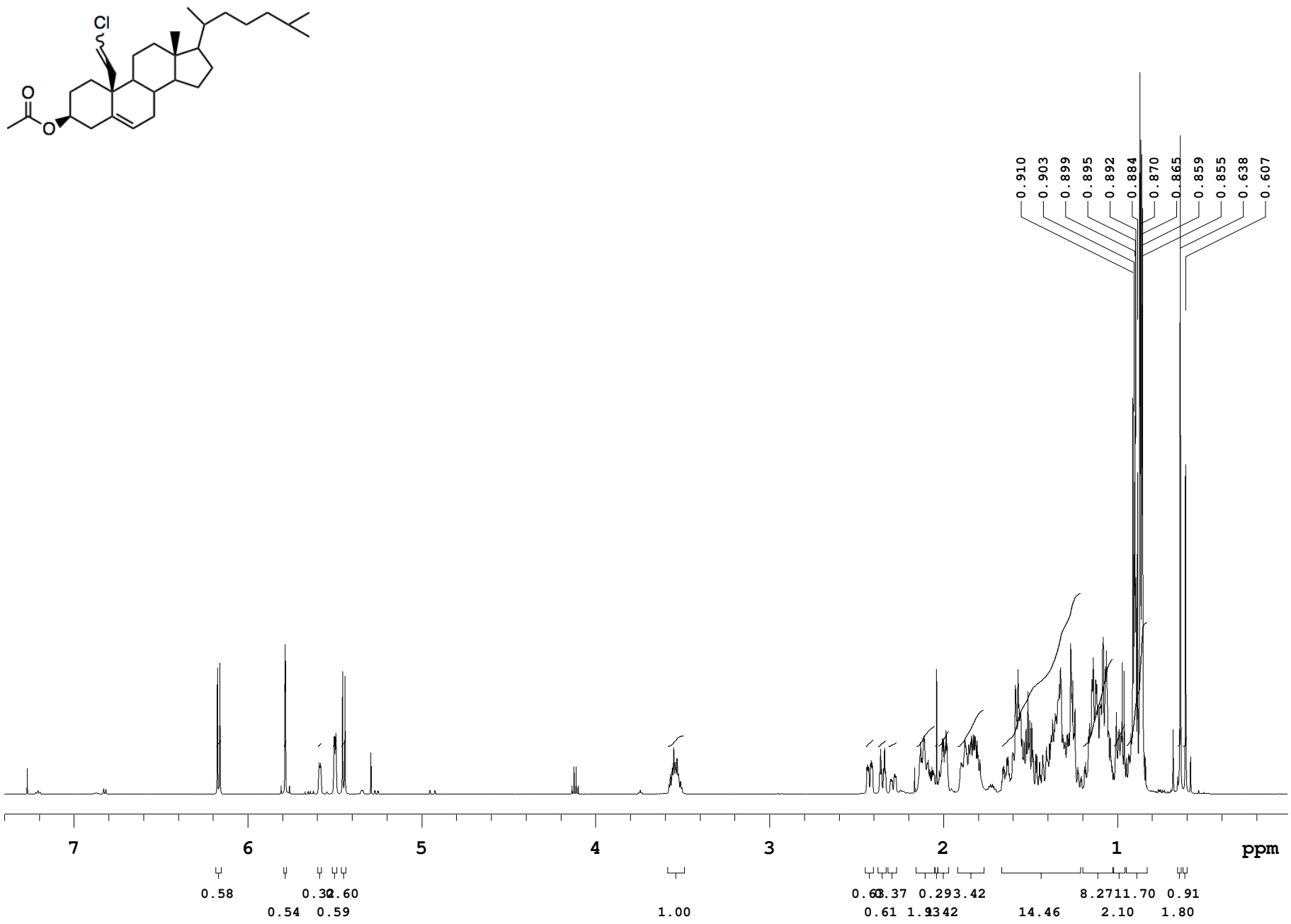
77.139
76.927
72.556
56.375
56.029
53.414
48.667
42.210
42.113
39.550
39.428
39.405
36.061
35.723
35.619
34.573
32.858
31.508
31.185
30.128
28.614
28.019
27.881
23.875
23.745
22.715
22.562
22.465
22.160
21.100
18.642
18.578
14.033
11.756
11.682
220
200
180
160
140
120
100
80
60
40
20
ppm


Cl
O
O
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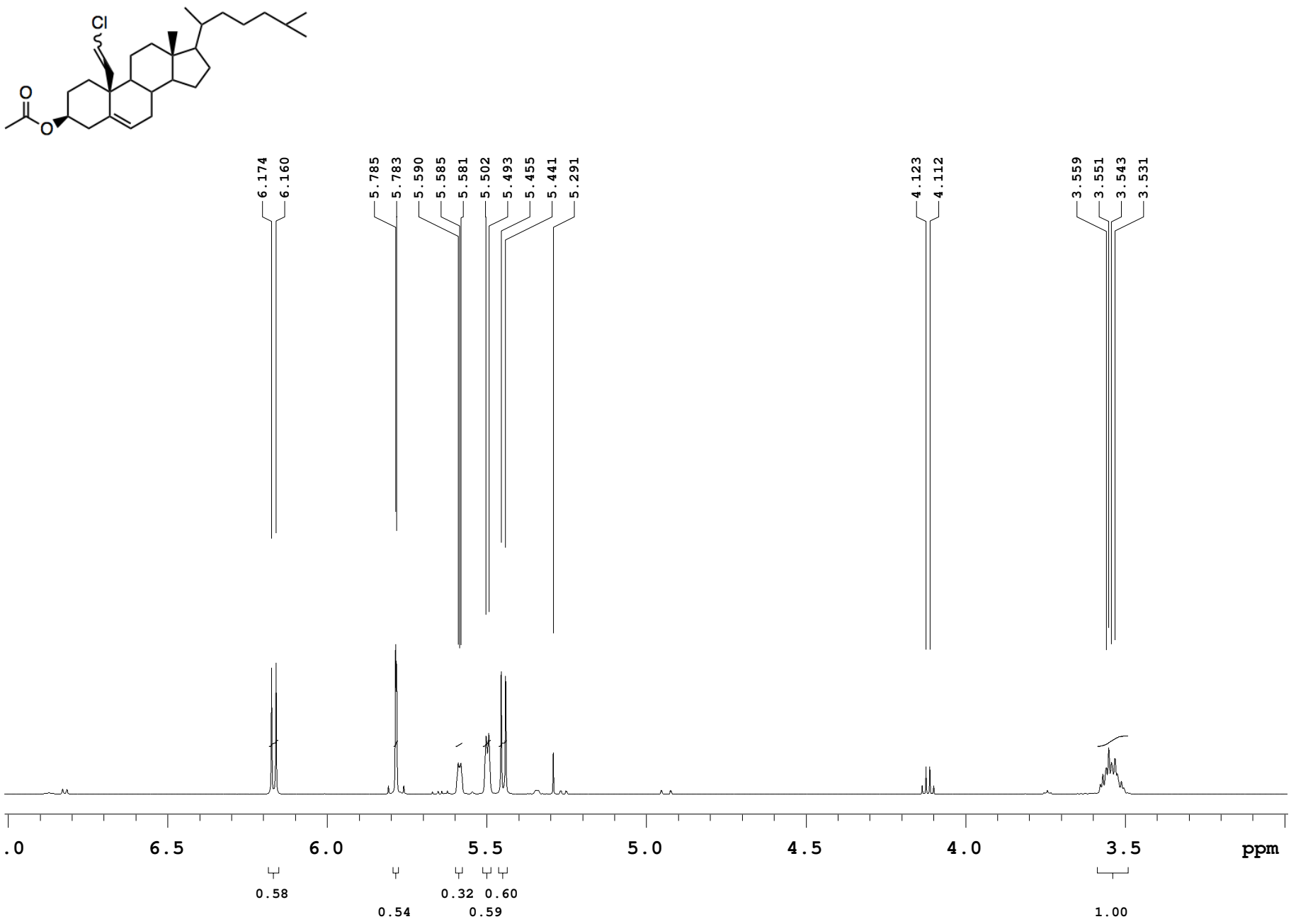
0.903
0.899
0.895
0.892
0.884
0.870
0.865
0.859
0.855
0.638
0.607
7
6
5
4
3
2
1
ppm
0.58
0.54
0.59
1.00
0.61
14.46
2.10
1.80


Cl
O
O
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6.160
5.785
5.783
5.590
5.585
5.581
5.502
5.493
5.455
5.441
5.291
4.123
4.112
3.559
3.551
3.543
3.531
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
ppm
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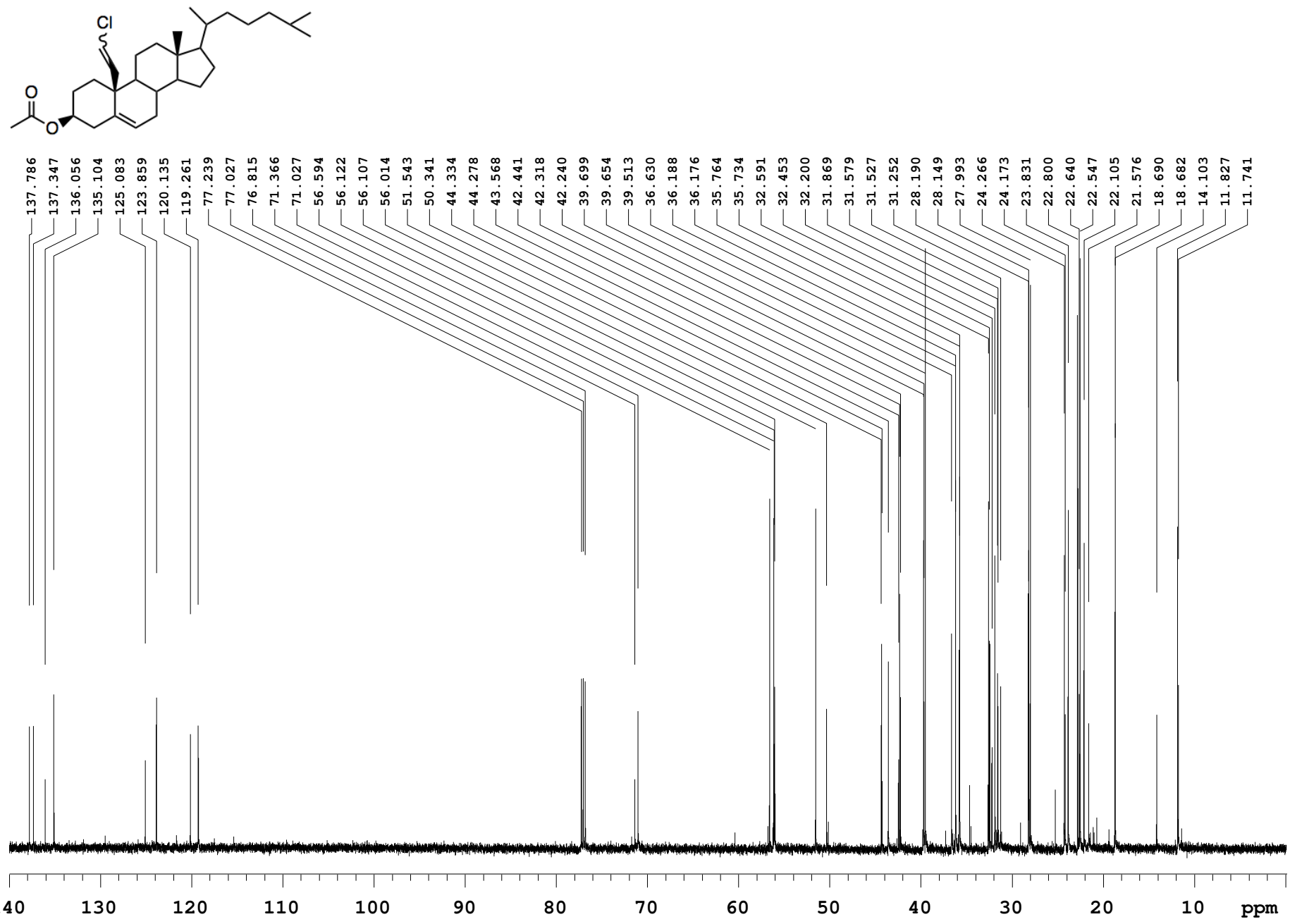
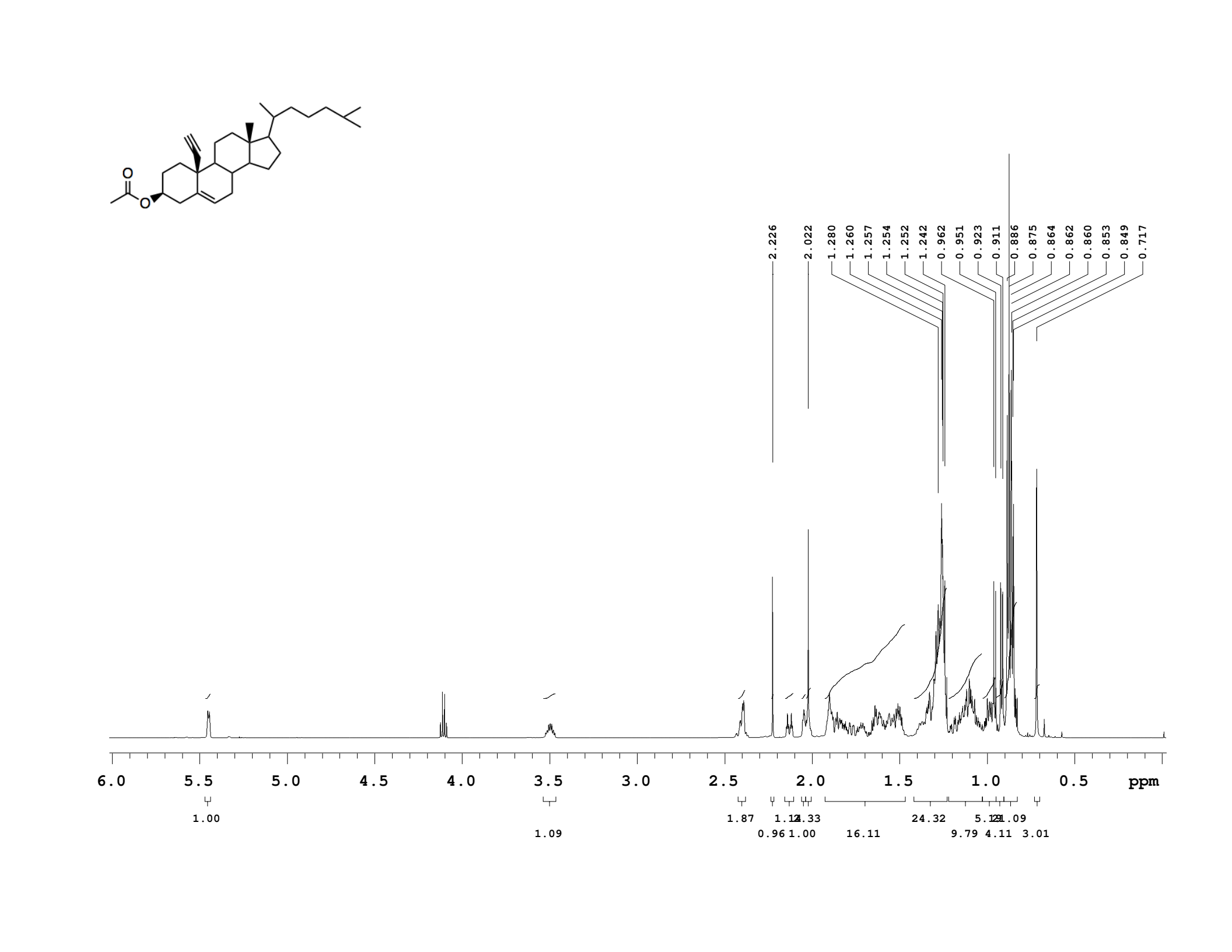
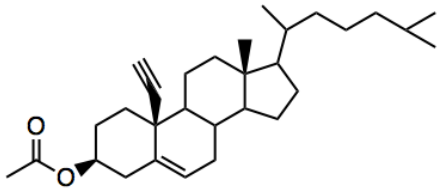
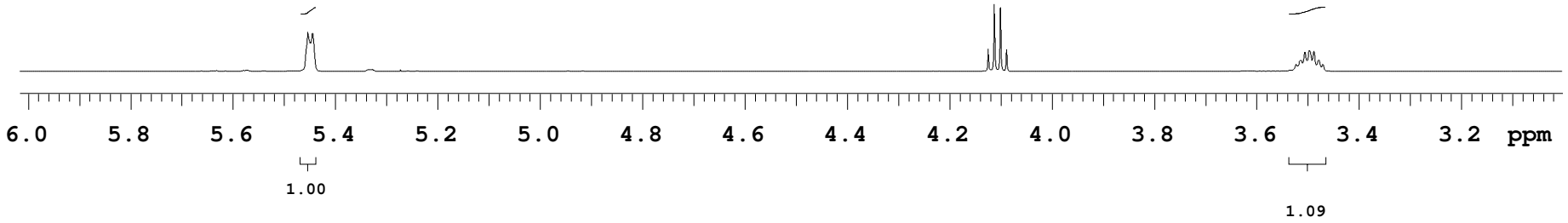
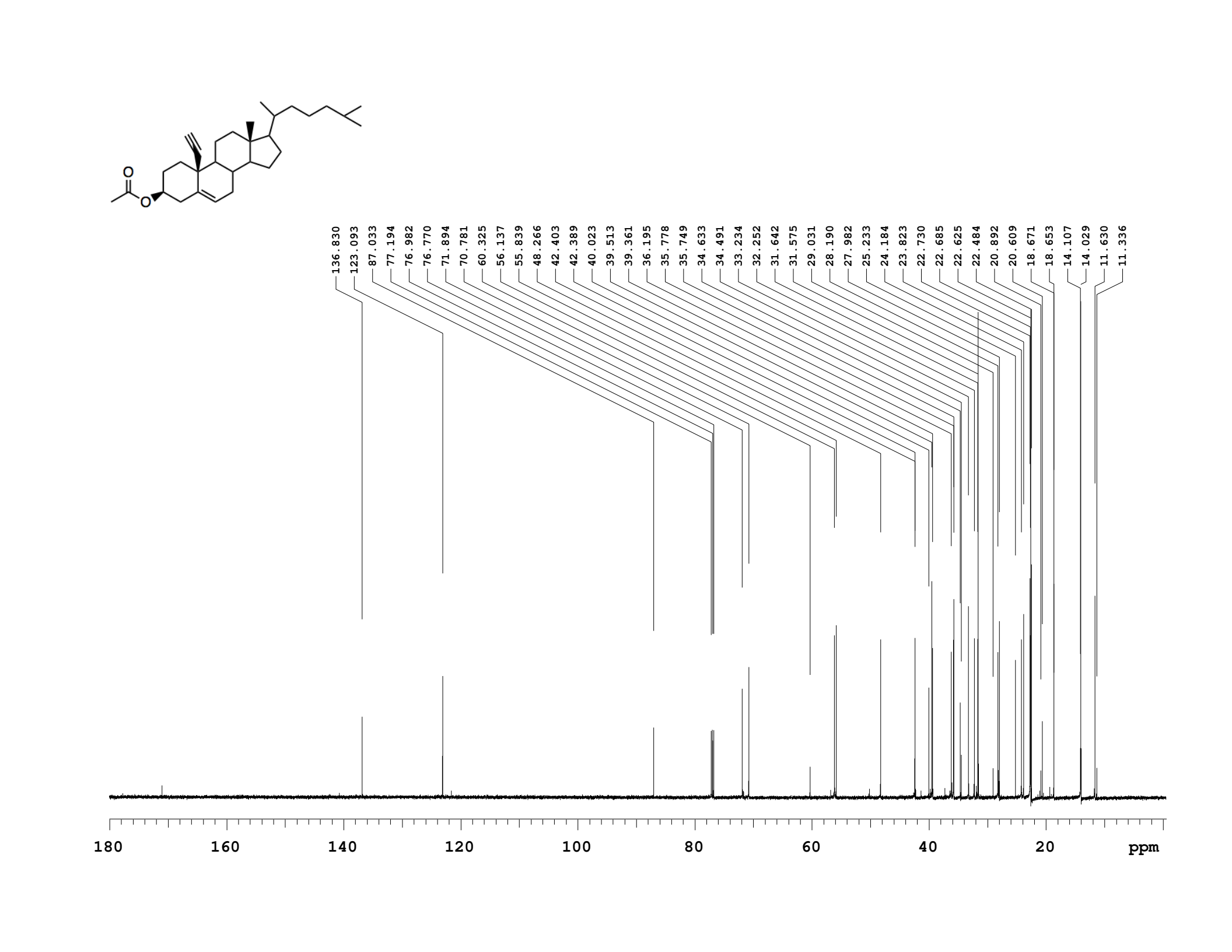
0.54
0.32
0.60
0.59
1.00

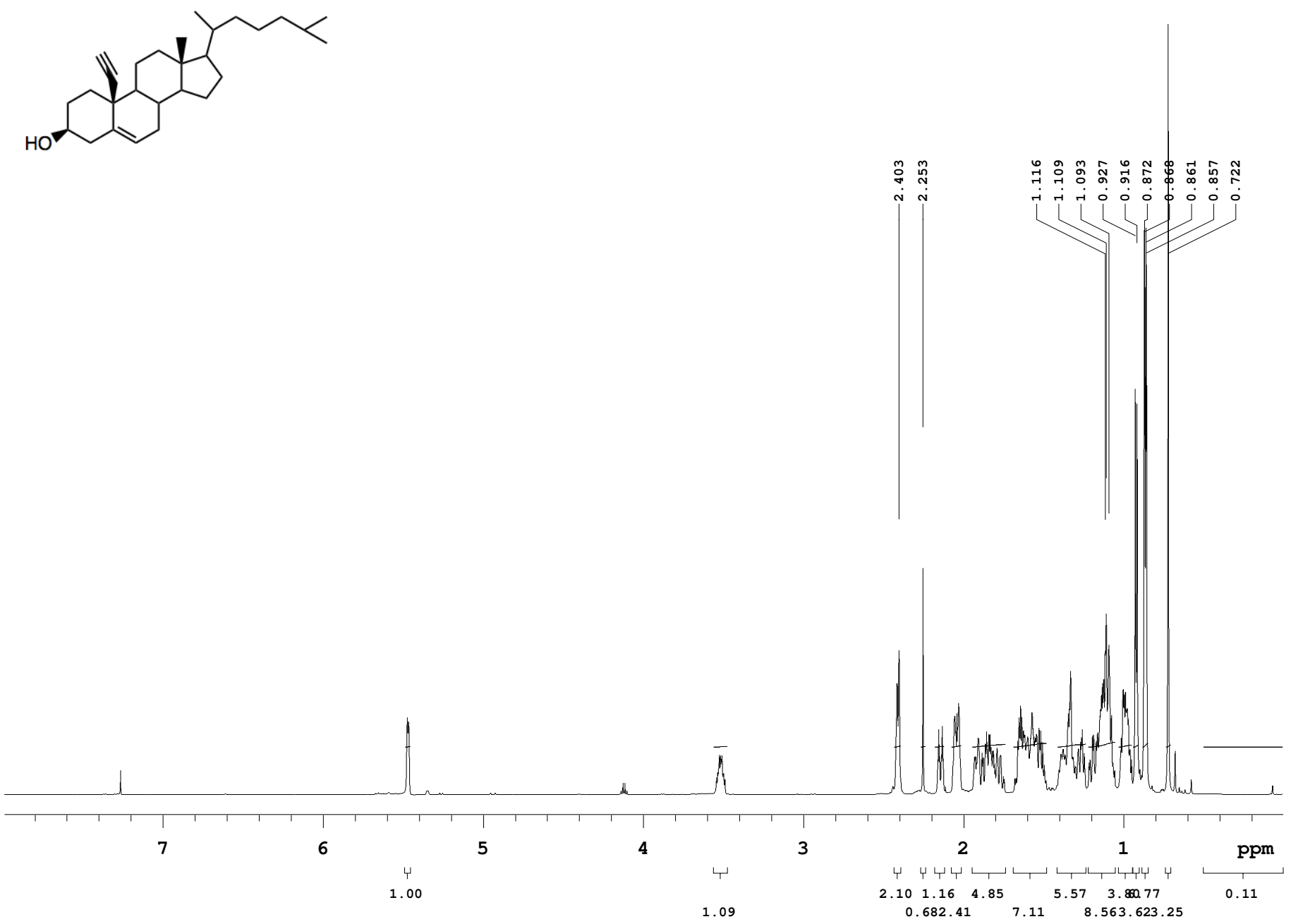

Cl
O
O
137.786
137.347
136.056
135.104
125.083
123.859
120.135
119.261
77.239
77.027
76.815
71.366
71.027
56.594
56.122
56.107
56.014
51.543
50.341
44.334
44.278
43.568
42.441
42.318
42.240
39.699
39.654
39.513
36.630
36.188
36.176
35.764
35.734
32.591
32.453
32.200
31.869
31.579
31.527
31.252
28.190
28.149
27.993
24.266
24.173
23.831
22.800
22.640
22.547
22.105
21.576
18.690
18.682
14.103
11.827
11.741
140
130
120
110
100
90
80
70
60
50
40
30
20
10
ppm

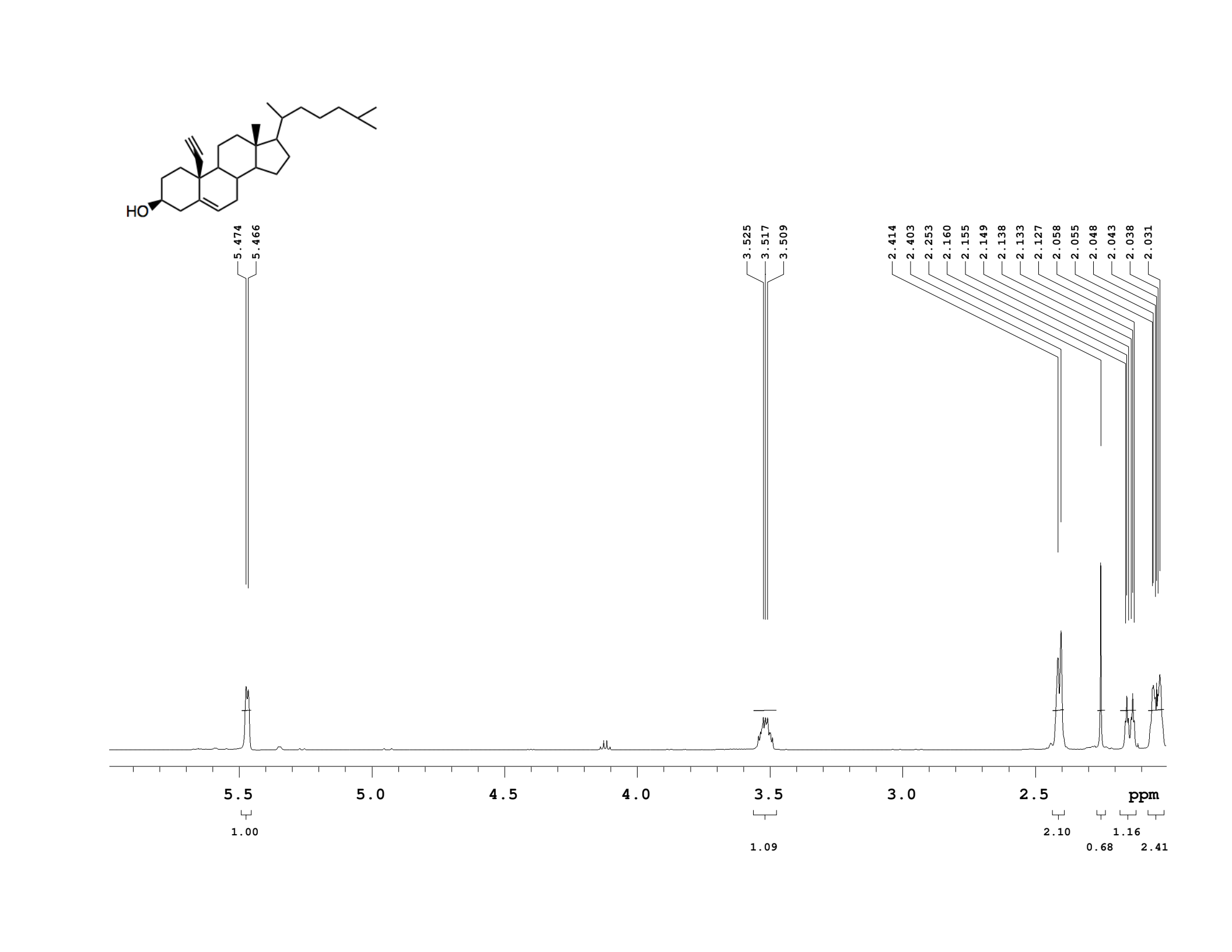

O
O
2.226
2.022
1.280
1.260
1.257
1.254
1.252
1.242
0.962
0.951
0.923
0.911
0.886
0.875
0.864
0.862
0.860
0.853
0.849
0.717
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
ppm
1.00
1.09
1.87
1.12
4.33
0.96
1.00
16.11
24.32
5.12
1.09
9.79
4.11
3.01

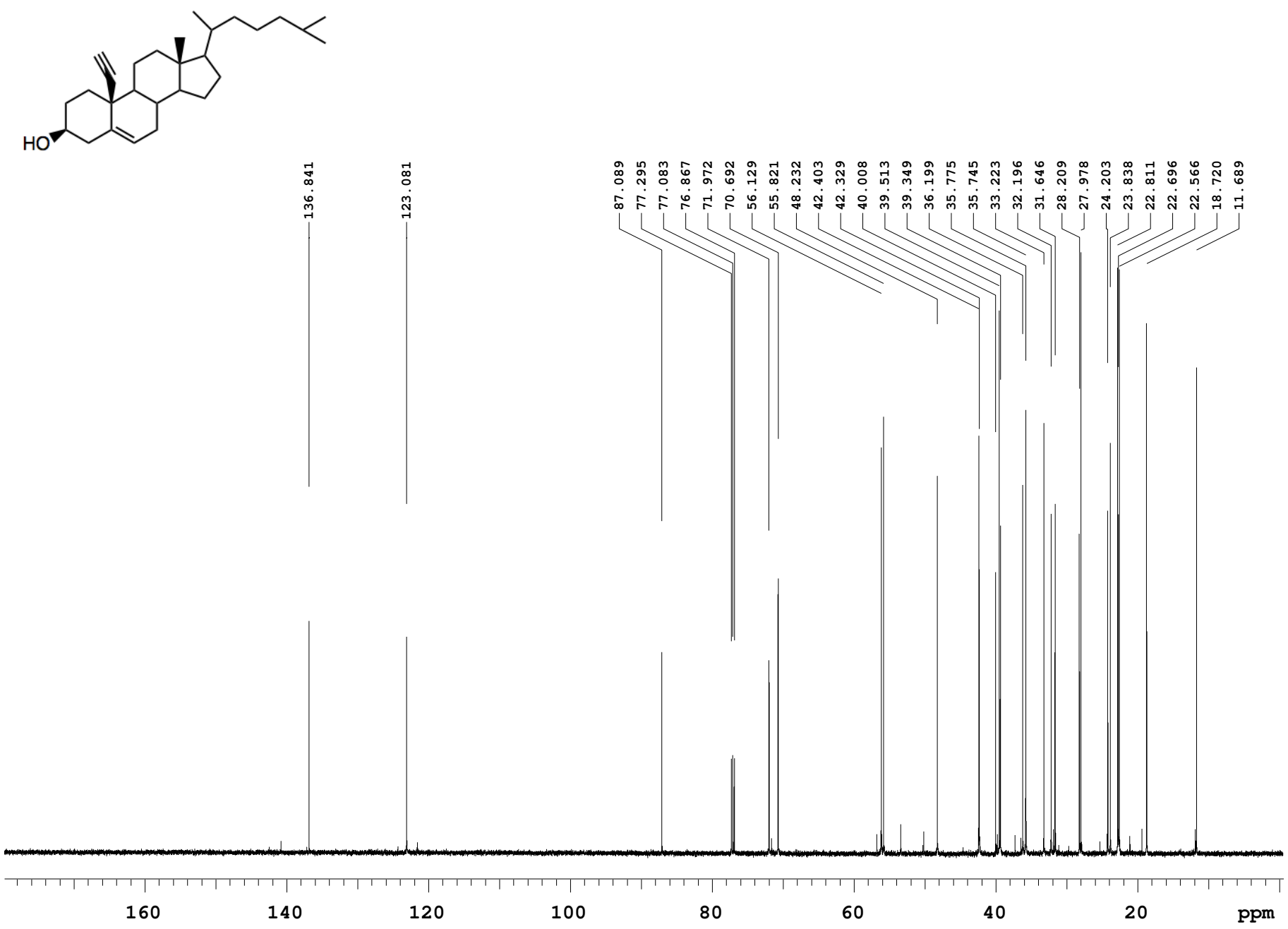

O
O


6.0
5.8
5.6
5.4
5.2
5.0
4.8
4.6
4.4
4.2
4.0
3.8
3.6
3.4
3.2
ppm
1.00
1.09


136.830
123.093
87.033
77.194
76.982
76.770
71.894
70.781
60.325
56.137
55.839
48.266
42.403
42.389
40.023
39.513
39.361
36.195
35.778
35.749
34.633
34.491
33.234
32.252
31.642
31.575
29.031
28.190
27.982
25.233
24.184
23.823
22.730
22.685
22.625
22.484
20.892
20.609
18.671
18.653
14.107
14.029
11.630
11.336
180
160
140
120
100
80
60
40
20
ppm


HO
2.403
2.253
1.116
1.109
1.093
0.927
0.916
0.872
0.868
0.861
0.857
0.722
7
6
5
4
3
2
1
ppm
1.00
1.09
2.10
1.16
4.85
5.57
0.11
0.68
2.41
7.11
8.56
3.62
3.25


HO
5.474
5.466
3.525
3.517
3.509
2.414
2.403
2.253
2.160
2.155
2.149
2.138
2.133
2.127
2.058
2.055
2.048
2.043
2.038
2.031
5.5
5.0
4.5
4.0
3.5
3.0
2.5
ppm
1.00
1.09
2.10
0.68
1.16
2.41


HO
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77.295
77.083
76.867
71.972
70.692
56.129
55.821
48.232
42.403
42.329
40.008
39.513
39.349
36.199
35.775
35.745
33.223
32.196
31.646
28.209
27.978
24.203
23.838
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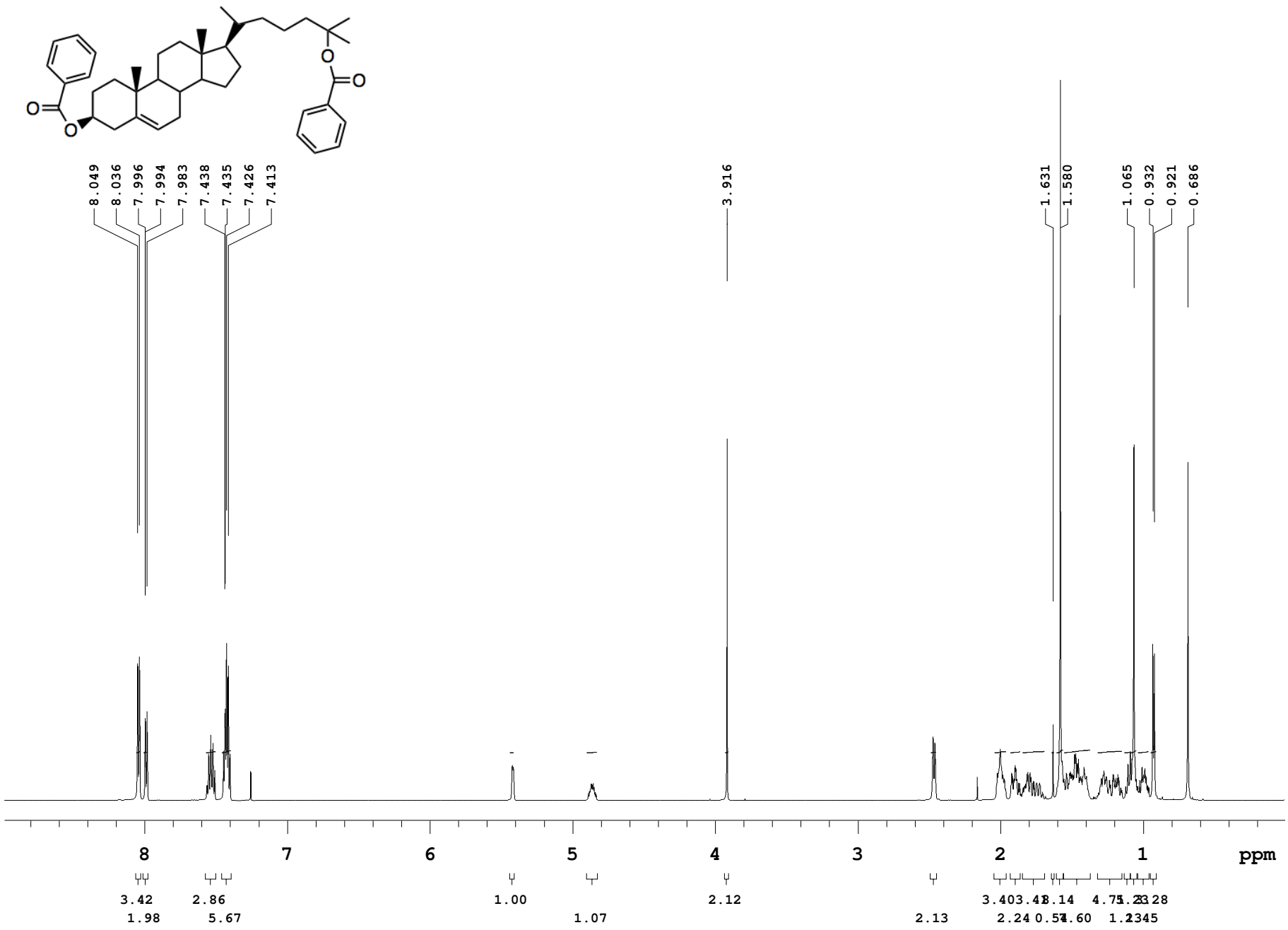
22.696
22.566
18.720
11.689
160
140
120
100
80
60
40
20
ppm


8.049
8.036
7.996
7.994
7.983
7.438
7.435
7.426
7.413
3.916
1.631
1.580
1.065
0.932
0.921
0.686
8
7
6
5
4
3
2
1
ppm
3.42
1.98
2.86
5.67
1.00
1.07
2.12
2.13
3.40
2.24
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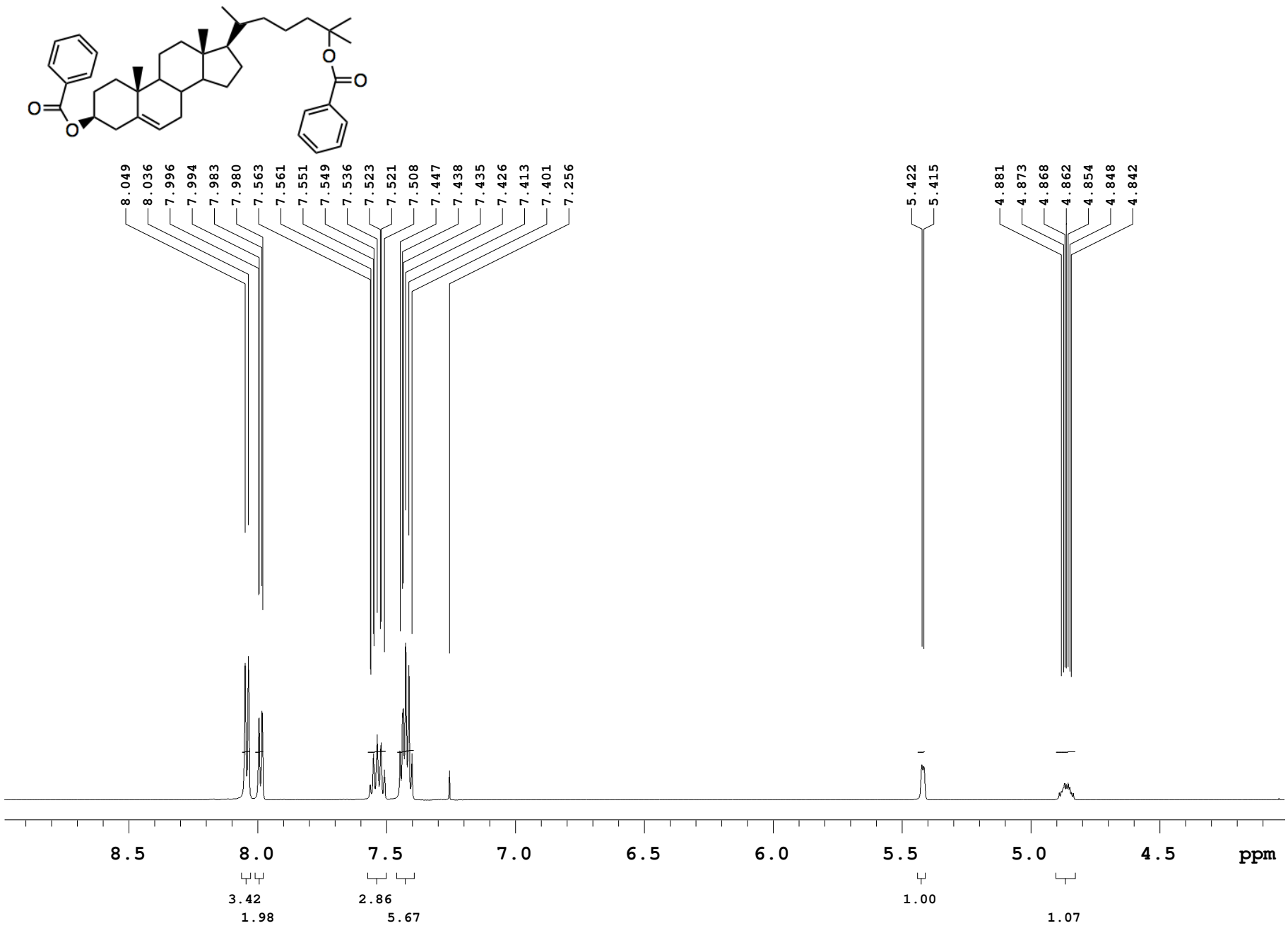
4.60
1.23
0.28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7.996
7.994
7.983
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7.563
7.561
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7.549
7.536
7.523
7.521
7.508
7.447
7.438
7.435
7.426
7.413
7.401
7.256
5.422
5.415
4.881
4.873
4.868
4.862
4.854
4.848
4.842
8.5
8.0
7.5
7.0
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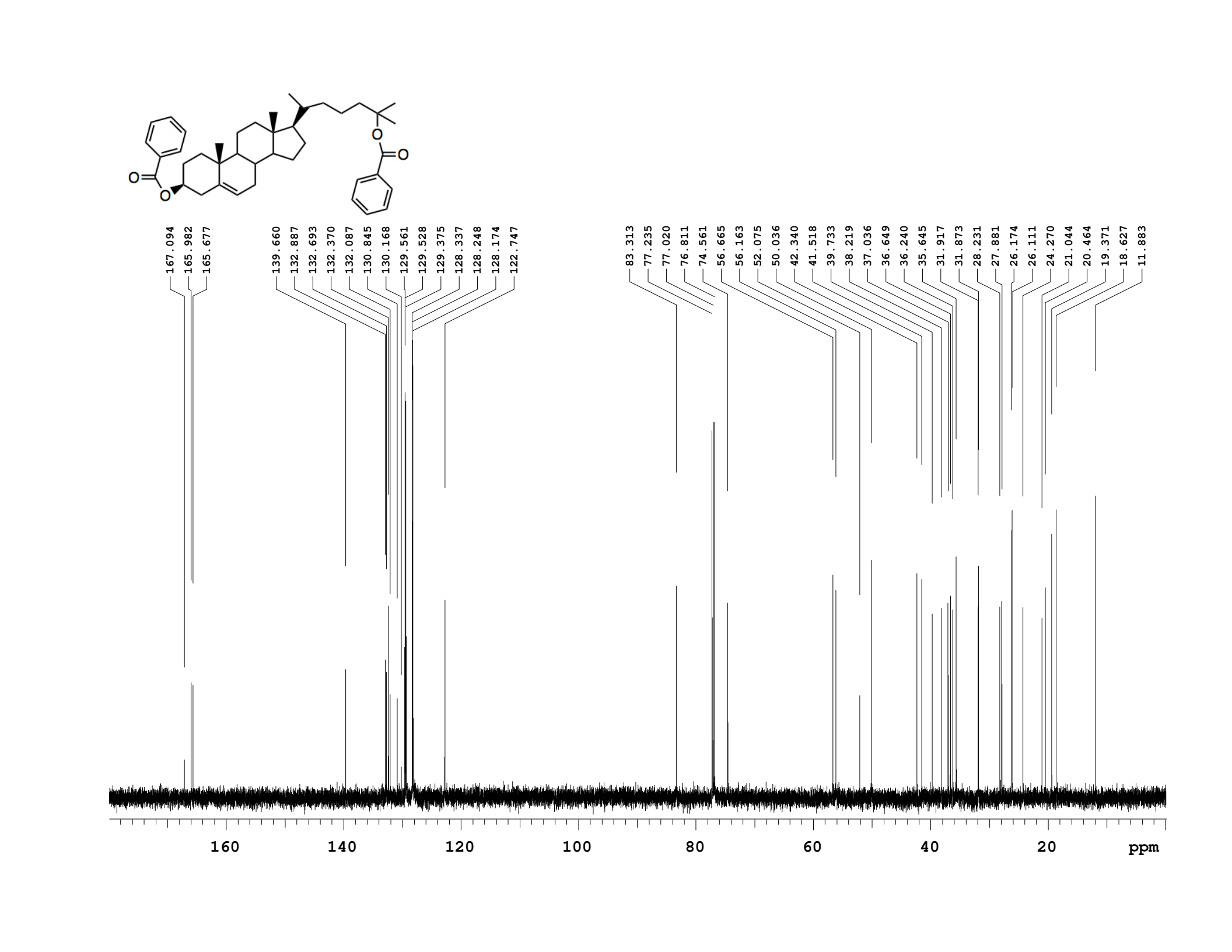
5.5
5.0
4.5
ppm
3.42
1.98
2.86
5.67
1.00
1.07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O
O
O
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132.370
132.087
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130.168
129.561
129.528
129.375
128.337
128.248
128.174
122.747
83.313
77.235
77.020
76.811
74.561
56.665
56.163
52.075
50.036
42.340
41.518
39.733
38.219
37.036
36.649
36.240
35.645
31.917
31.873
28.231
27.881
26.174
26.111
24.270
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20.464
19.371
18.627
11.883
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140
120
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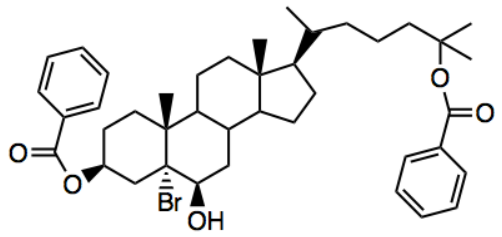
80
60
40
20
ppm


O
O
Br
OH
O
O

1.578
1.373
0.884
0.682
9
8
7
6
5
4
3
2
1
ppm
2.04
2.17
2.23
4.11
0.98
1.00
1.04
1.22
2.12
1.12
0.32
0.97
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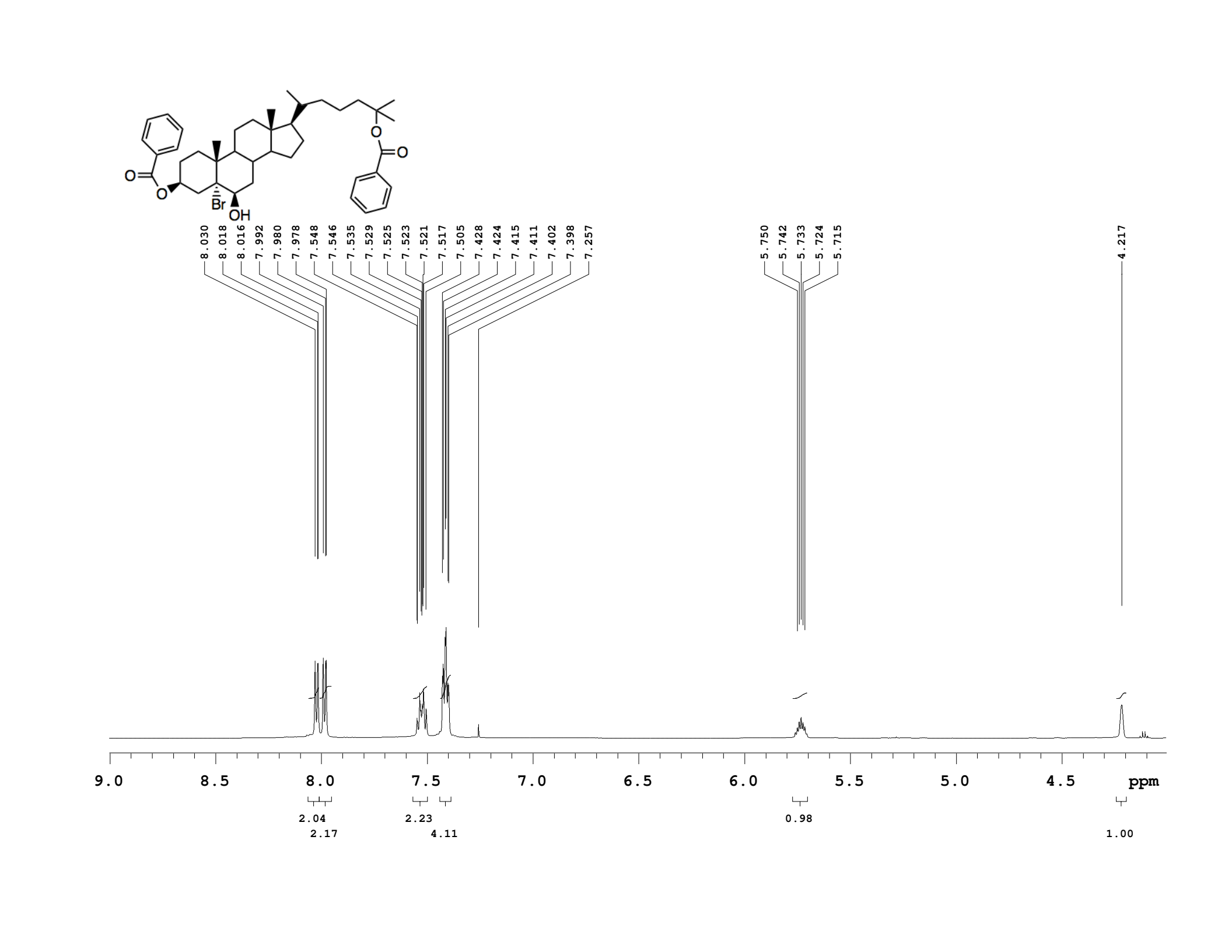
1.70
3.26
7.98
3.00
8.74
7.88
0.59
7.92
3.04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8.016
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7.980
7.978
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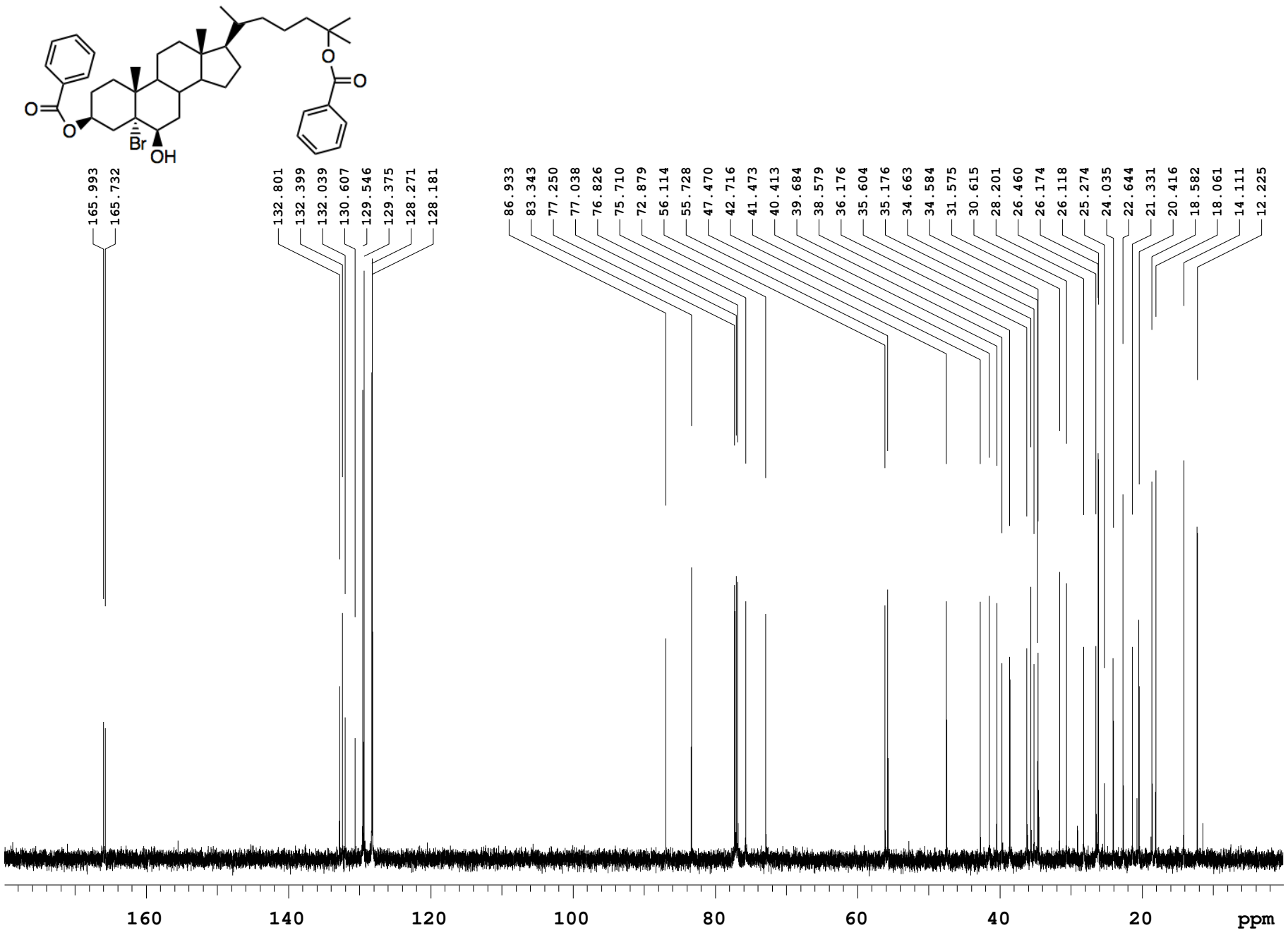
7.546
7.535
7.529
7.525
7.523
7.521
7.517
7.505
7.428
7.424
7.415
7.411
7.402
7.398
7.257
5.750
5.742
5.733
5.724
5.715
4.217
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
ppm
2.04
2.17
2.23
4.11
0.98
1.00


O
O
Br
OH
O
O
165.993
165.732
132.801
132.399
132.039
130.607
129.546
129.375
128.271
128.181
86.933
83.343
77.250
77.038
76.826
75.710
72.879
56.114
55.728
47.470
42.716
41.473
40.413
39.684
38.579
36.176
35.604
35.176
34.663
34.584
31.575
30.615
28.201
26.460
26.174
26.118
25.274
24.035
22.644
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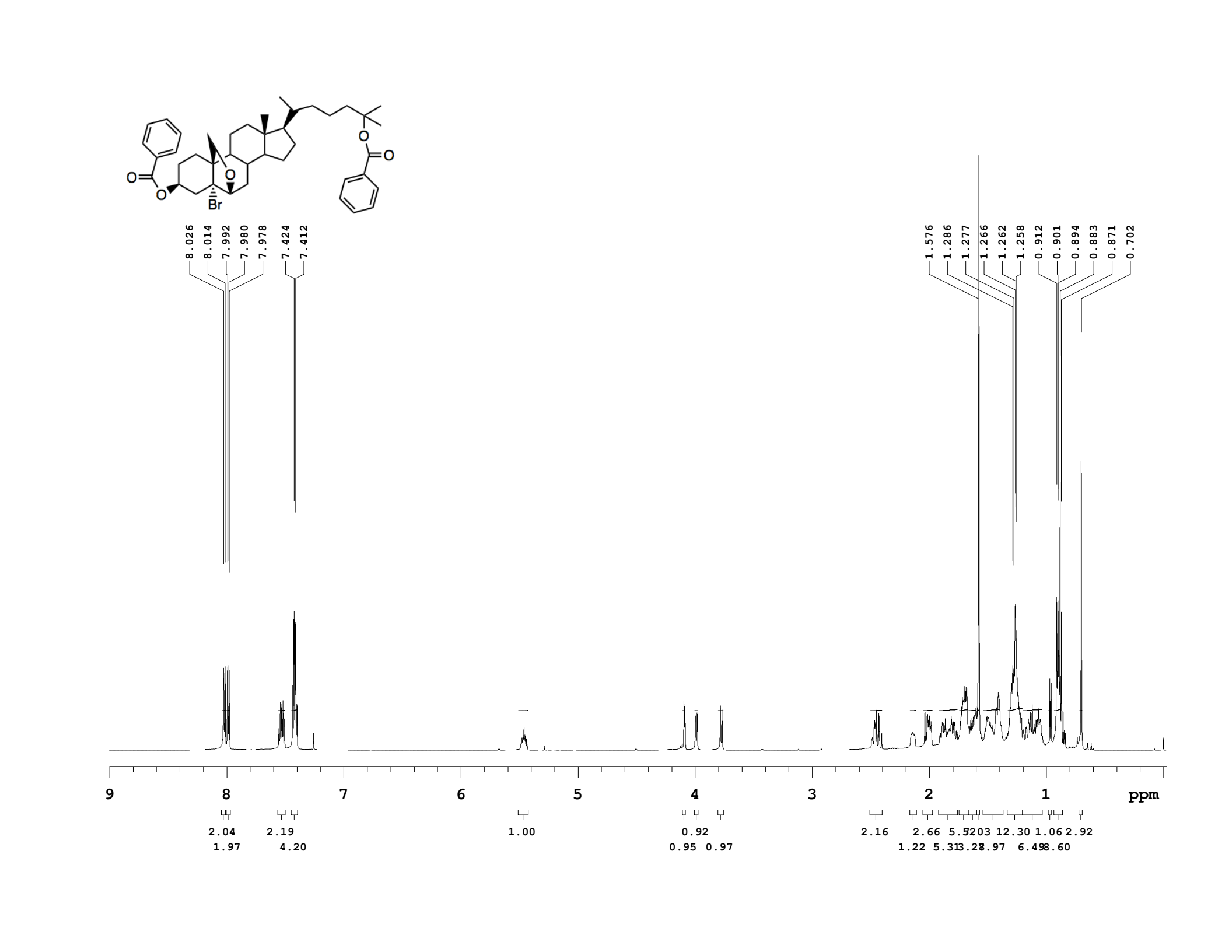
18.582
18.061
14.111
12.225
160
140
120
100
80
60
40
20
ppm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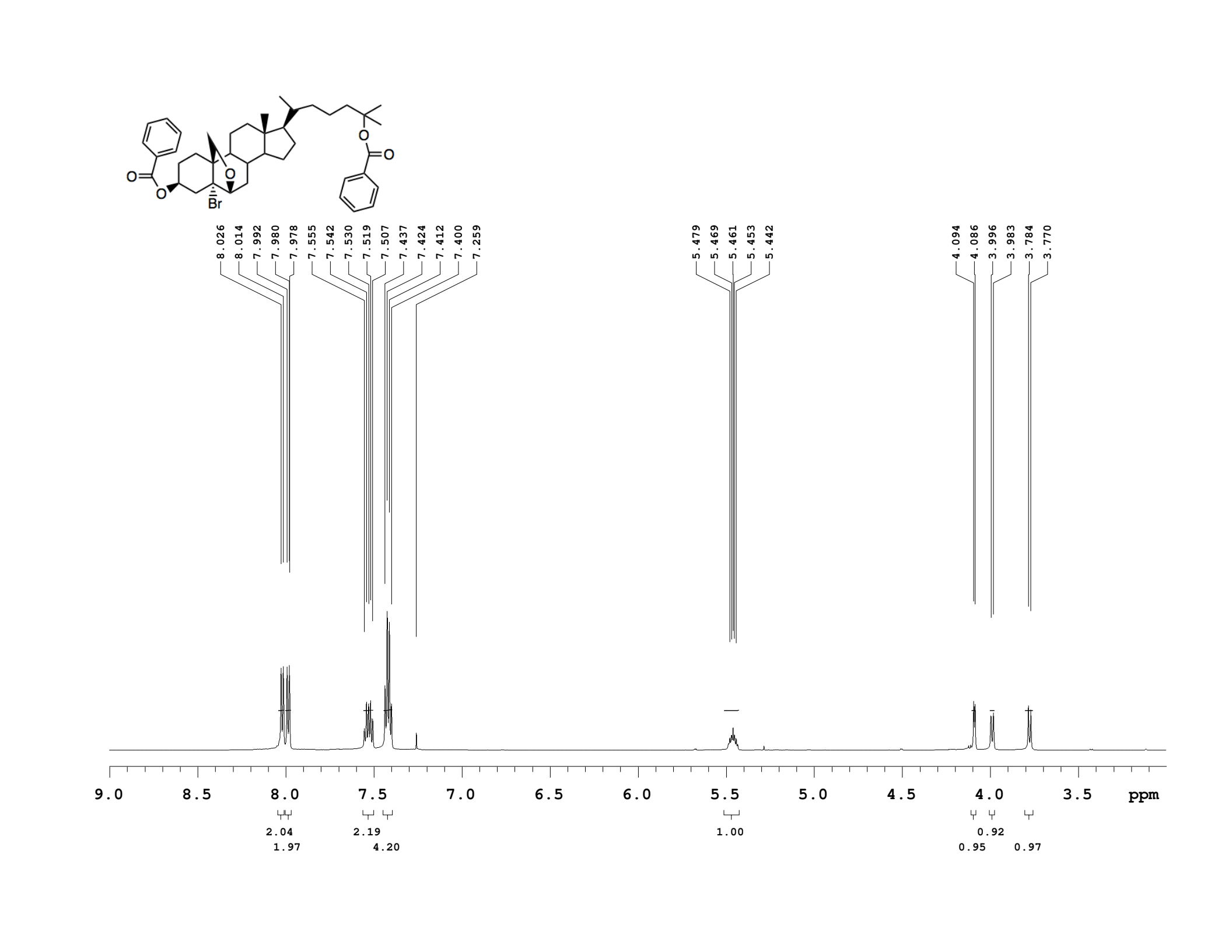
7.992
7.980
7.978
7.424
7.412
1.576
1.286
1.277
1.266
1.262
1.258
0.912
0.901
0.894
0.883
0.871
0.702
9
8
7
6
5
4
3
2
1
ppm
2.04
1.97
2.19
4.20
1.00
0.92
0.95
0.97
2.16
2.66
1.22
5.31
5.52
3.28
03
8.97
12.30
6.49
1.06
8.60
2.92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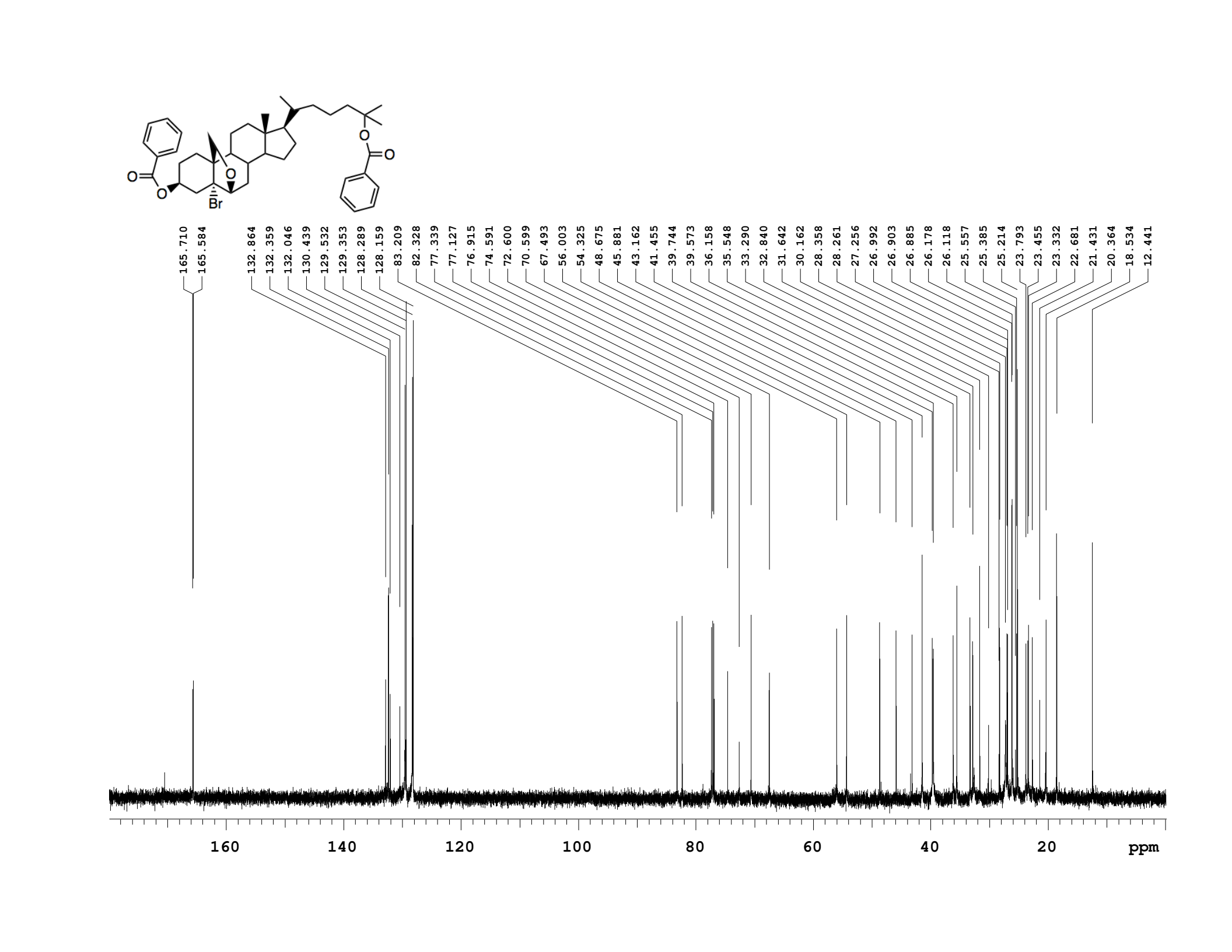
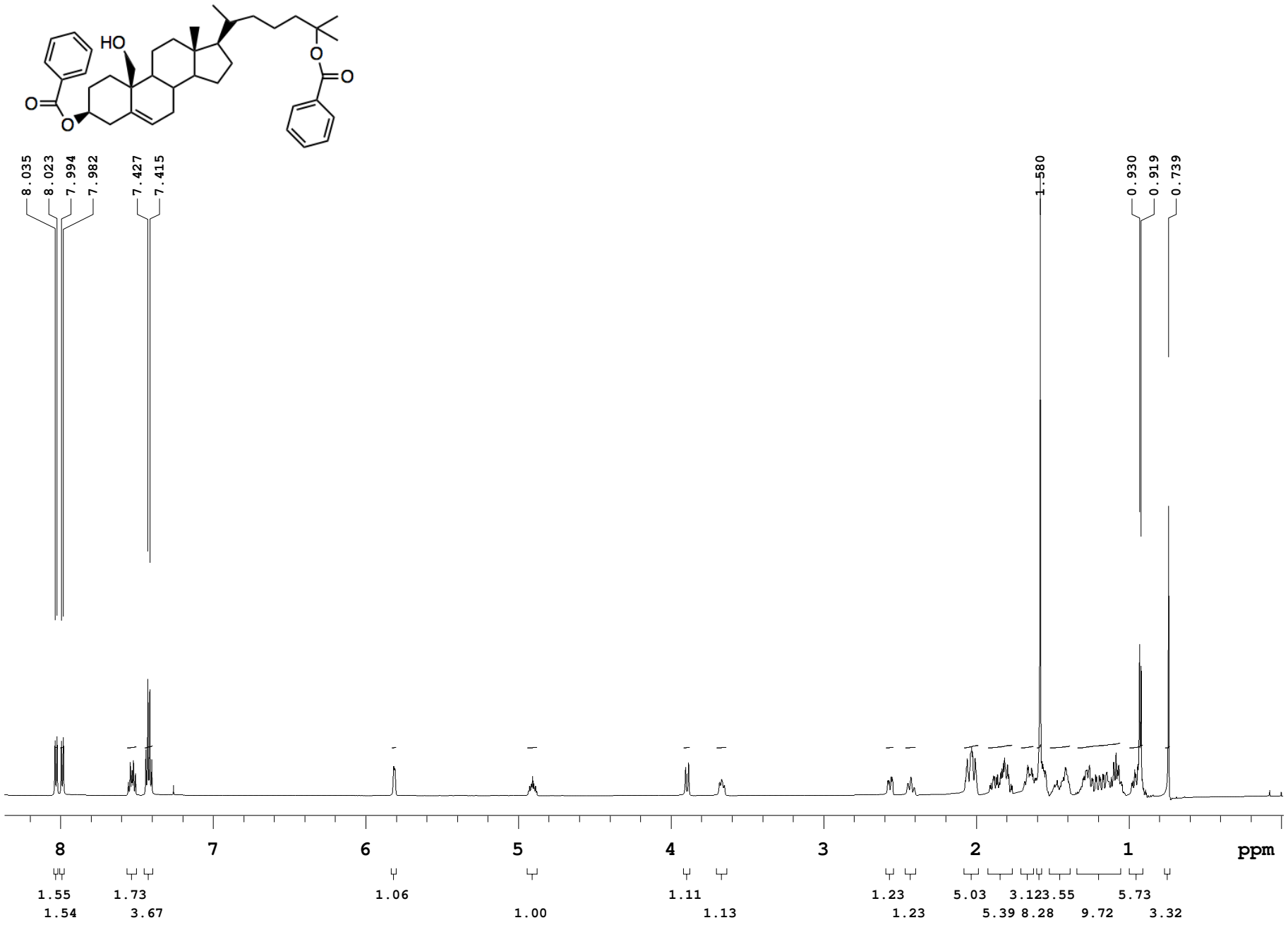
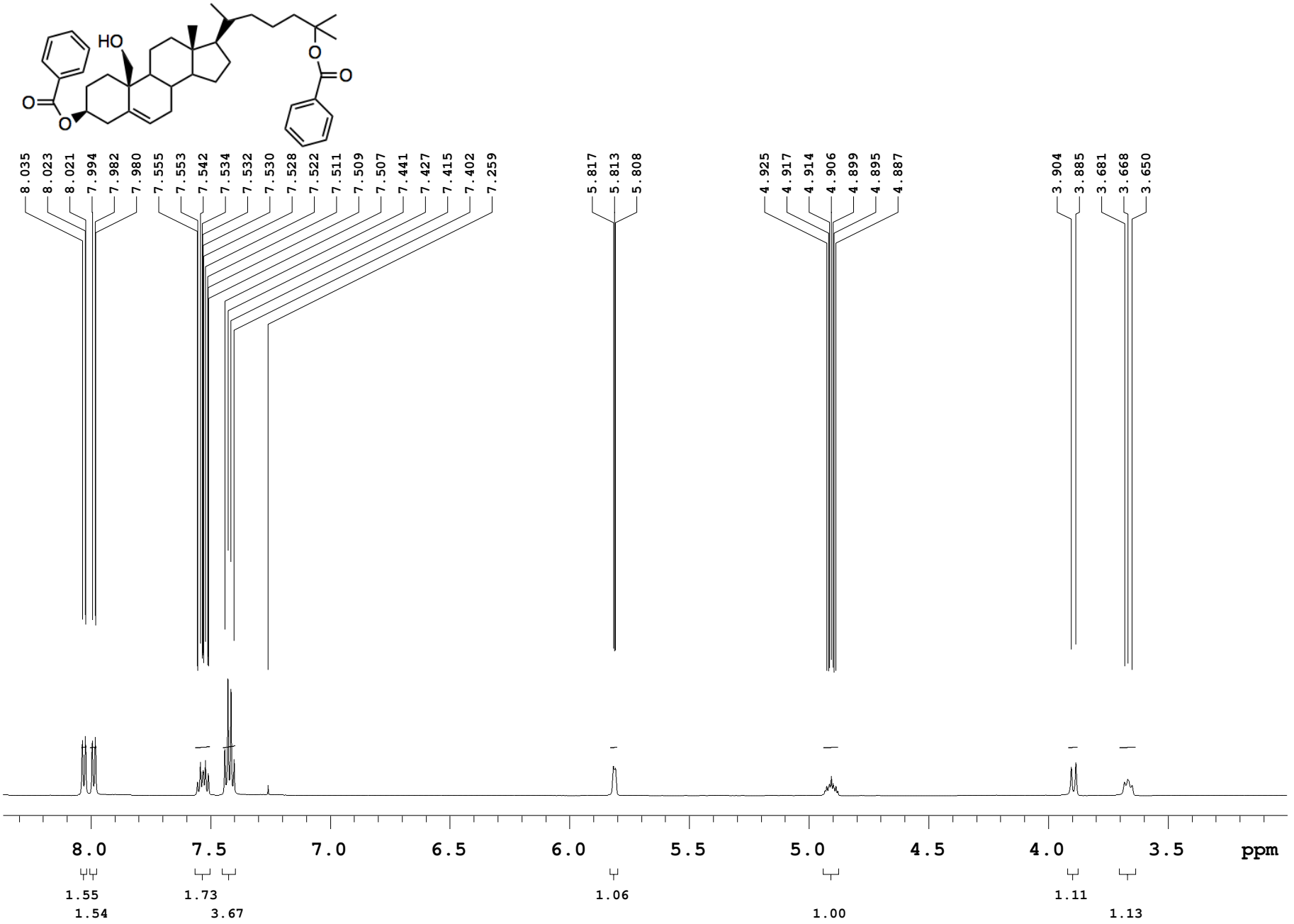
8.014
7.992
7.980
7.978
7.555
7.542
7.530
7.519
7.507
7.437
7.424
7.412
7.400
7.259
5.479
5.469
5.461
5.453
5.442
4.094
4.086
3.996
3.983
3.784
3.770
Br
O
O
O
O
O
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
ppm
2.04
1.97
2.19
4.20
1.00
0.92
0.95
0.97


O
O
O
O
O
Br
165.710
165.584
132.864
132.359
132.046
130.439
129.532
129.353
128.289
128.159
83.209
82.328
77.339
77.127
76.915
74.591
72.600
70.599
67.493
56.003
54.325
48.675
45.881
43.162
41.455
39.744
39.573
36.158
35.548
33.290
32.840
31.642
30.162
28.358
28.261
27.256
26.992
26.903
26.885
26.178
26.118
25.557
25.385
25.214
23.793
23.455
23.332
22.681
21.431
20.364
18.534
12.441
160
140
120
100
80
60
40
20
ppm


HO
O
O
O
O
8.035
8.023
7.994
7.982
7.427
7.415
1.580
0.930
0.919
0.739
8
7
6
5
4
3
2
1
ppm
1.55
1.54
1.73
3.67
1.06
1.00
1.11
1.13
1.23
1.23
5.03
5.39
3.12
8.28
3.55
9.72
5.73
3.32


HO
O
O
O
O
8.035
8.023
8.021
7.994
7.982
7.980
7.555
7.553
7.542
7.534
7.532
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7.528
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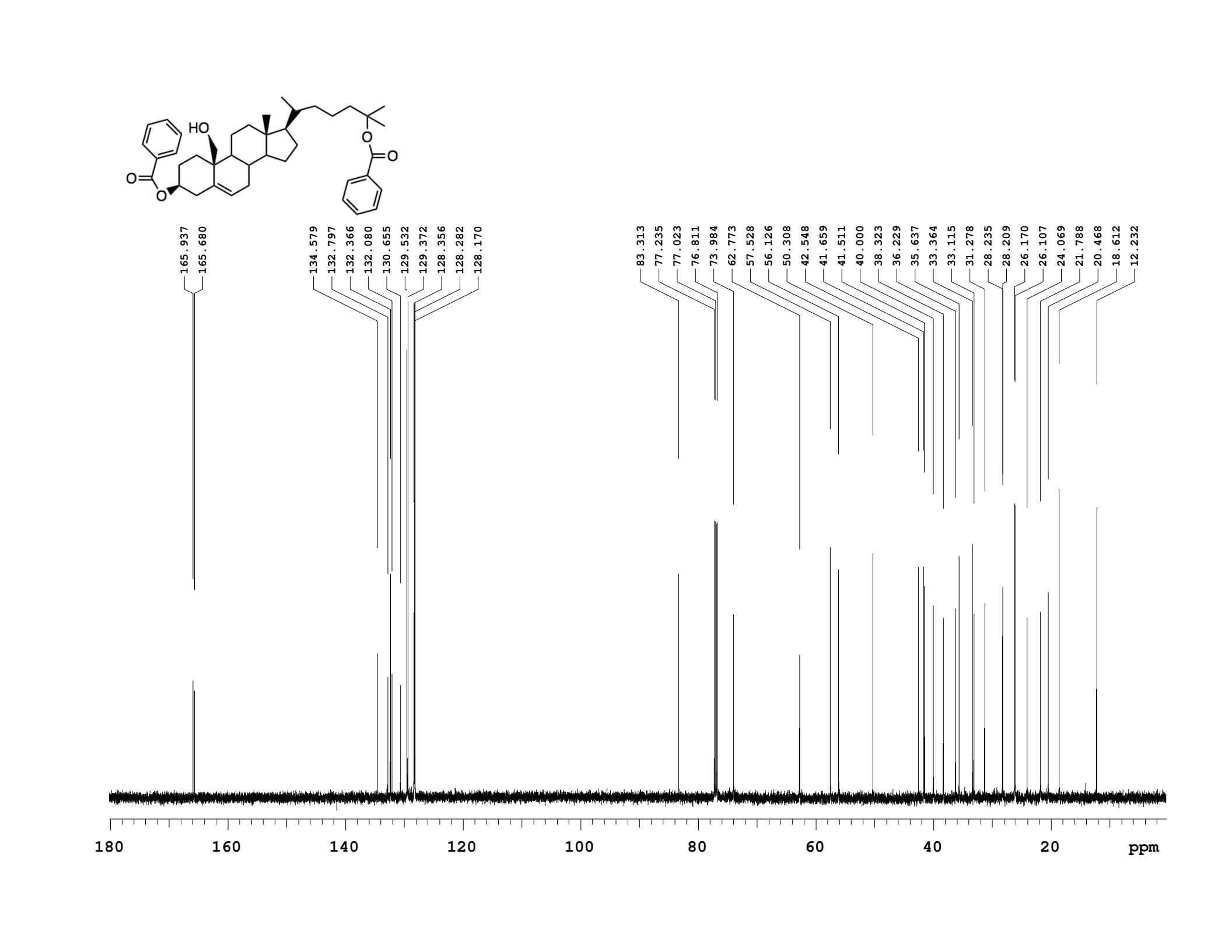
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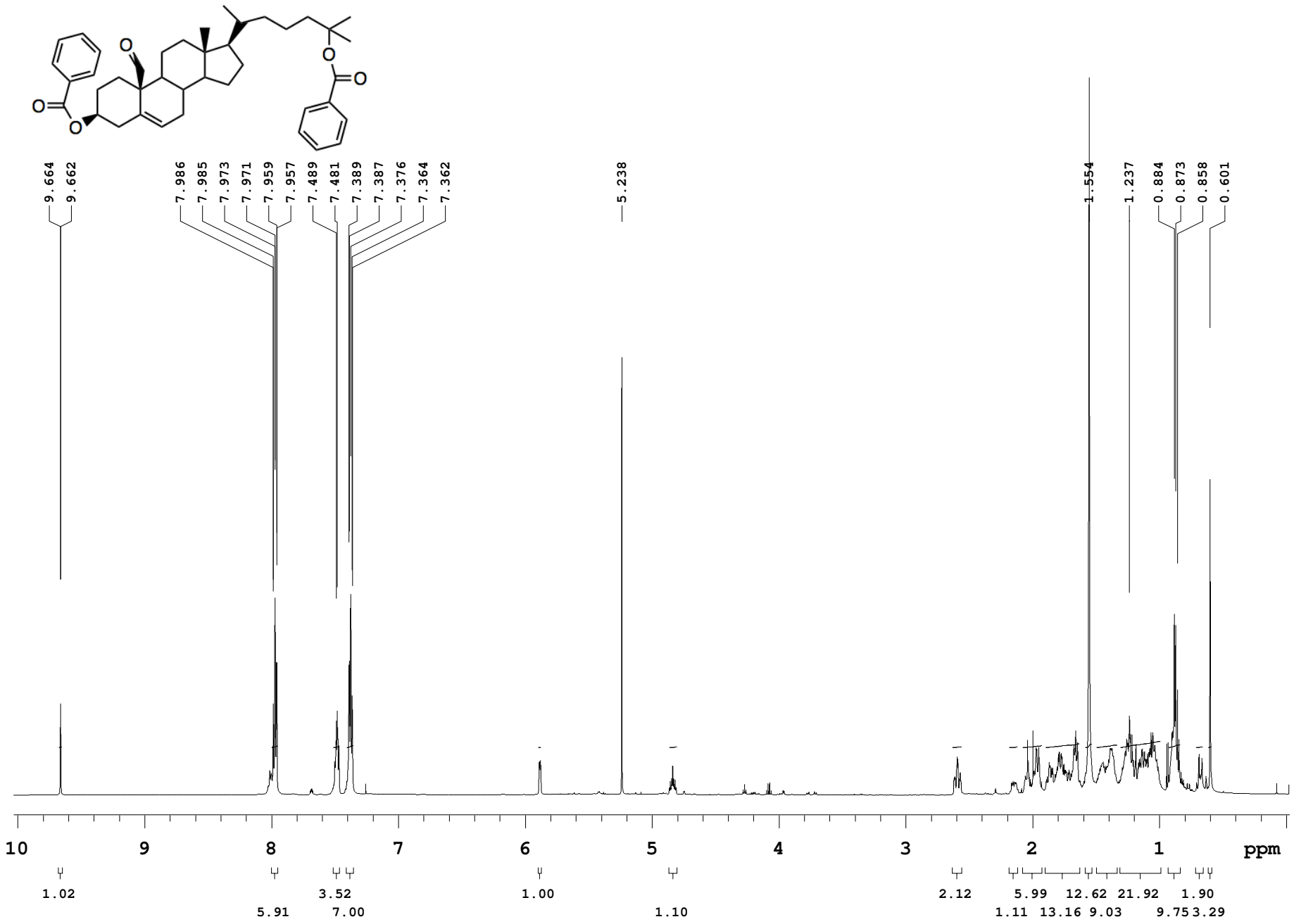
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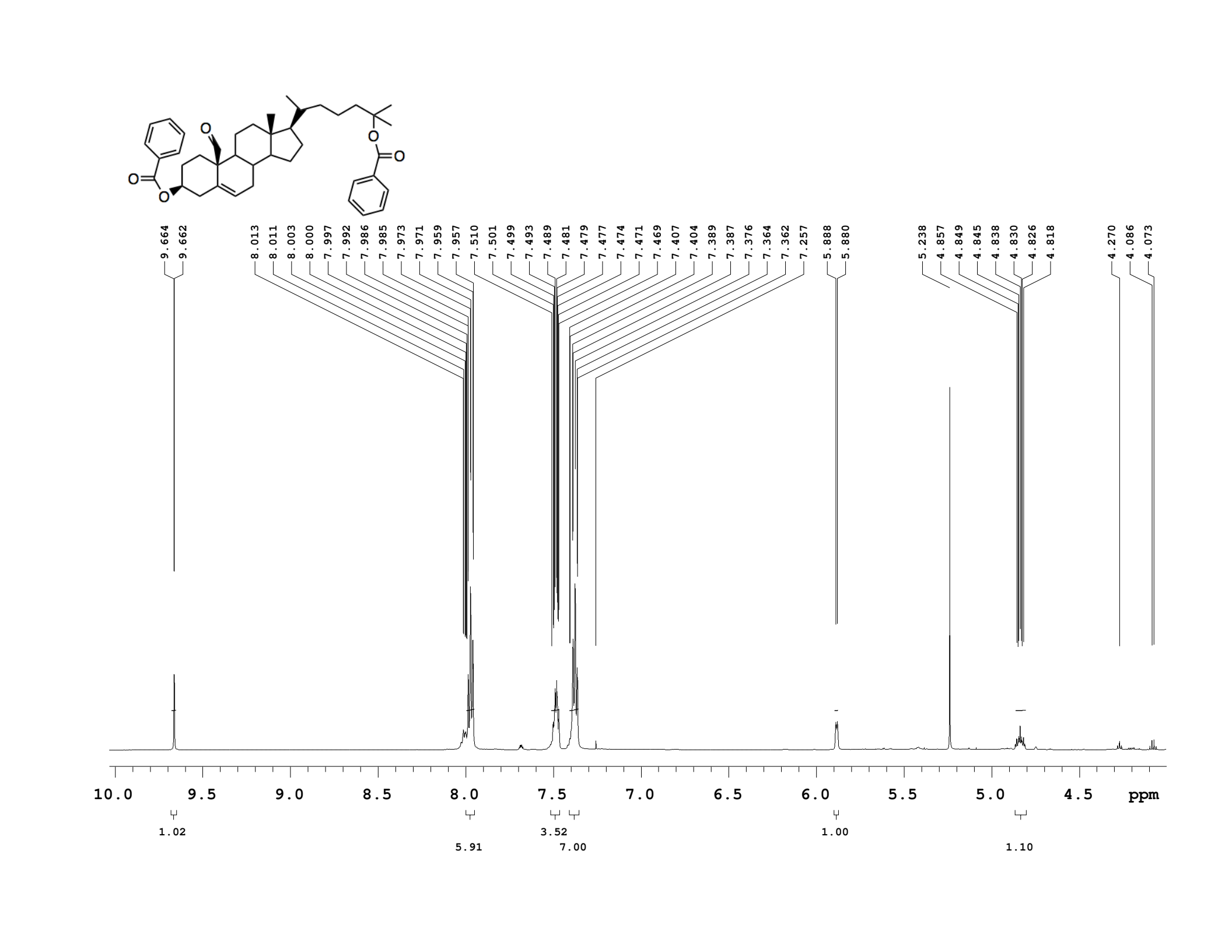
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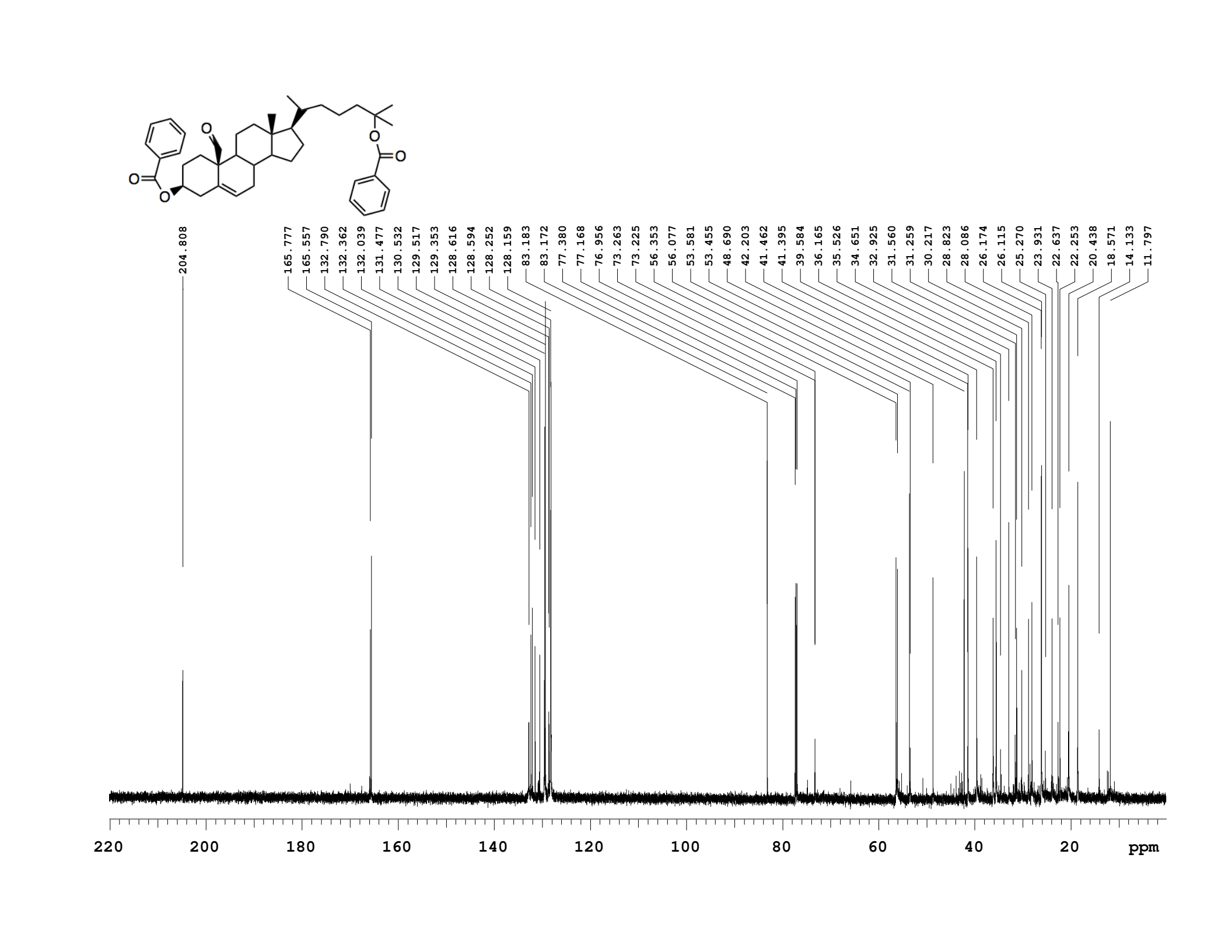
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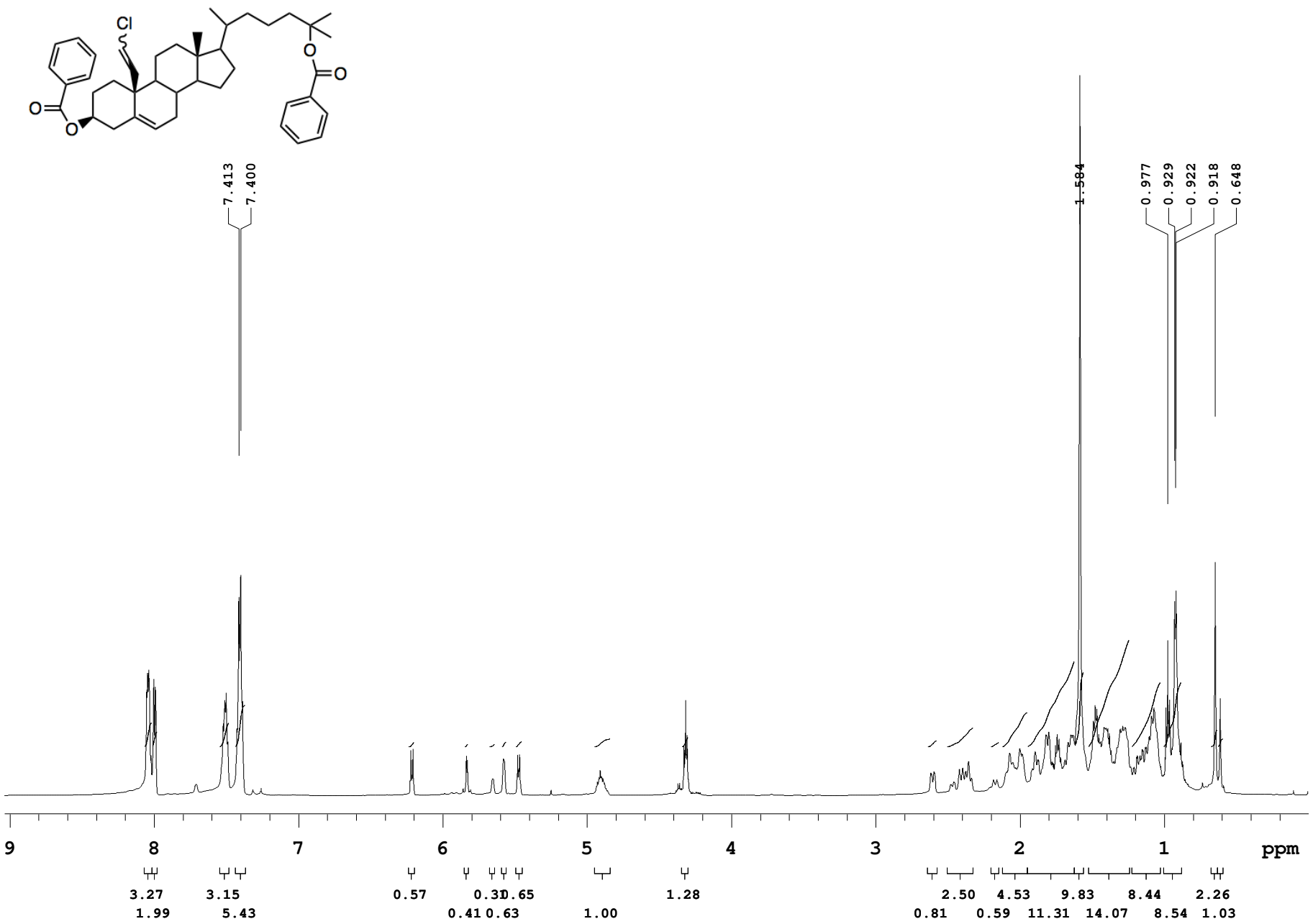
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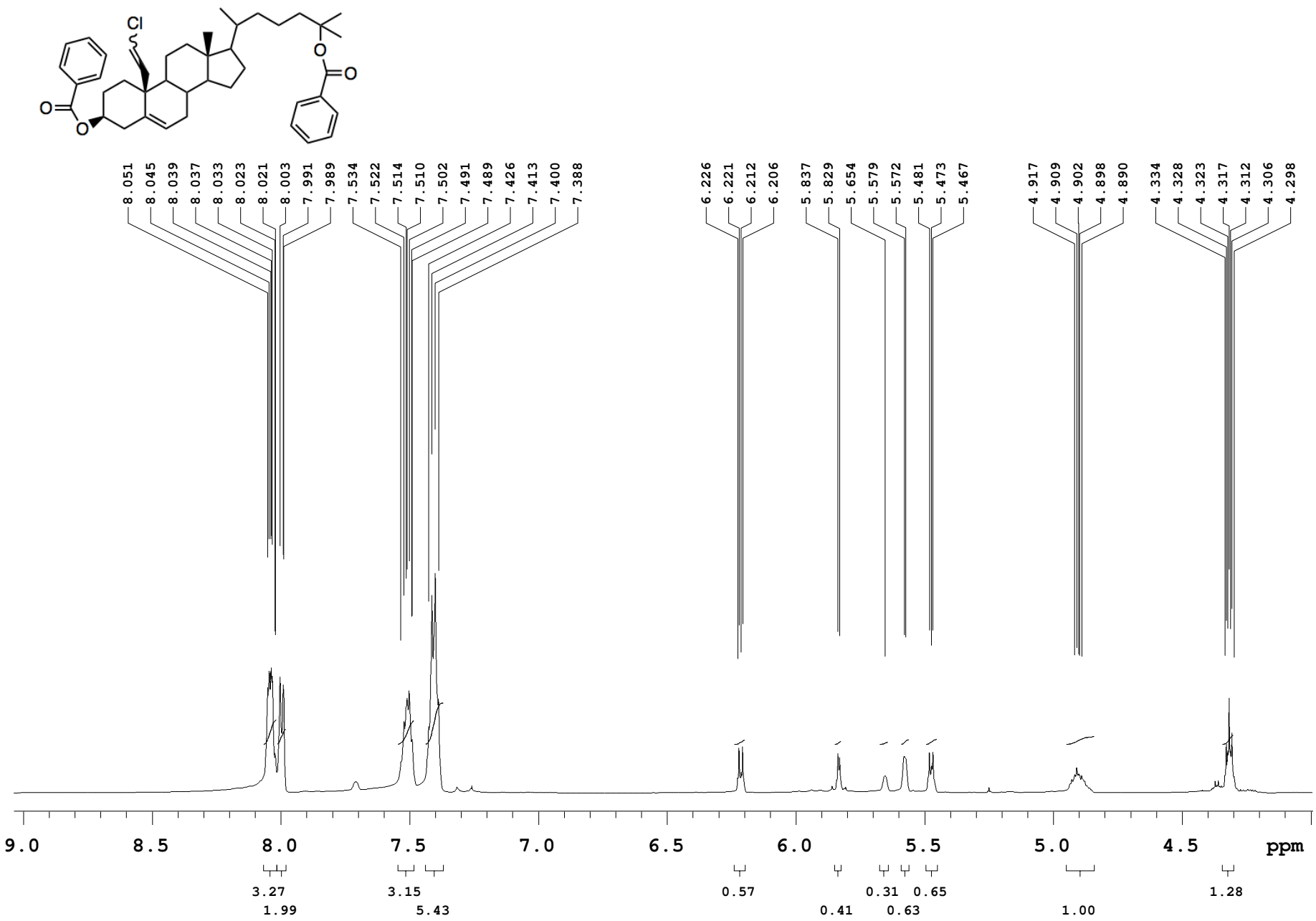
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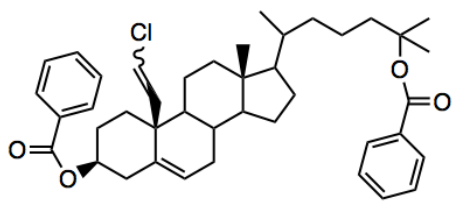

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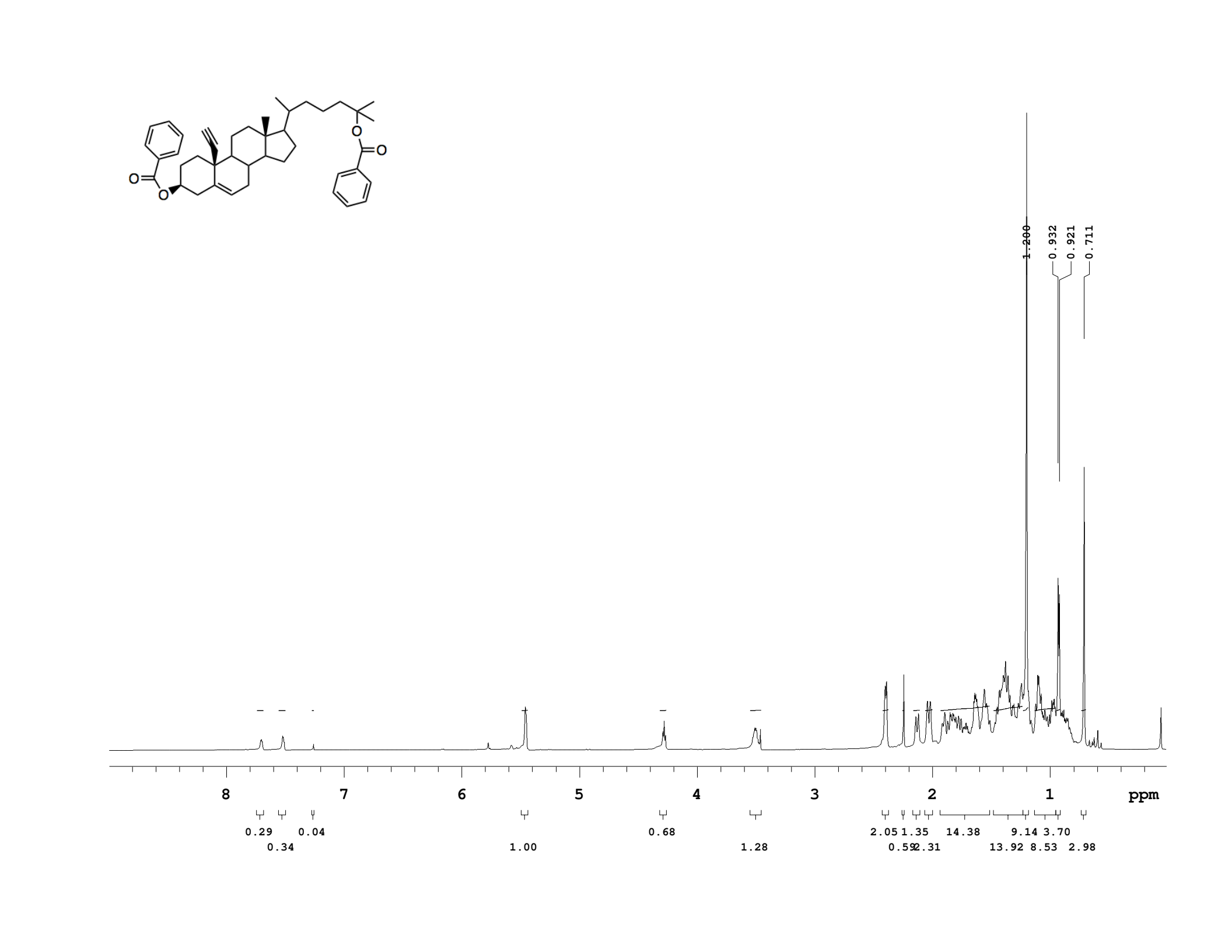
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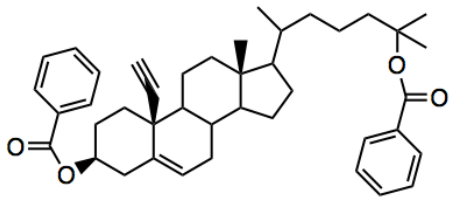

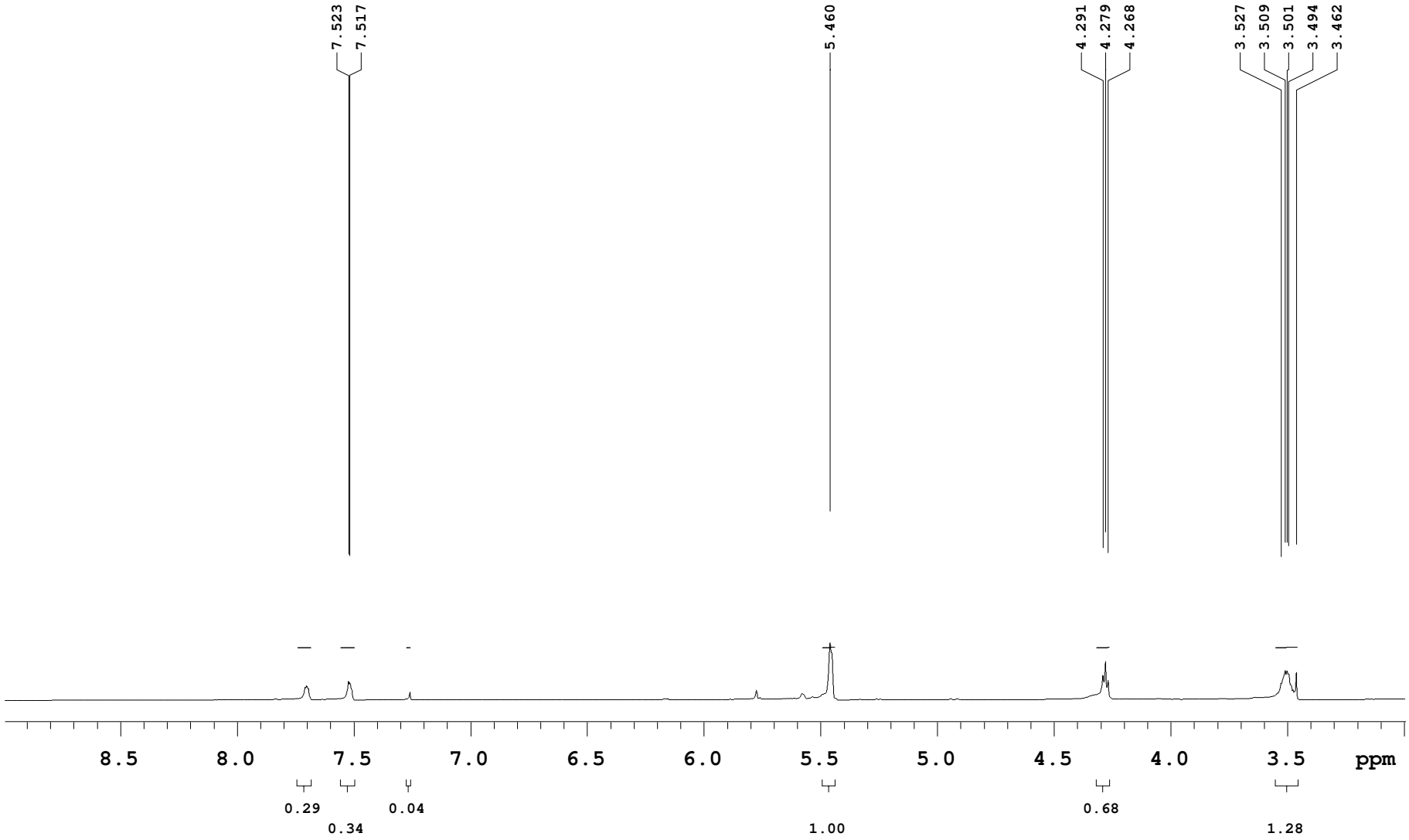
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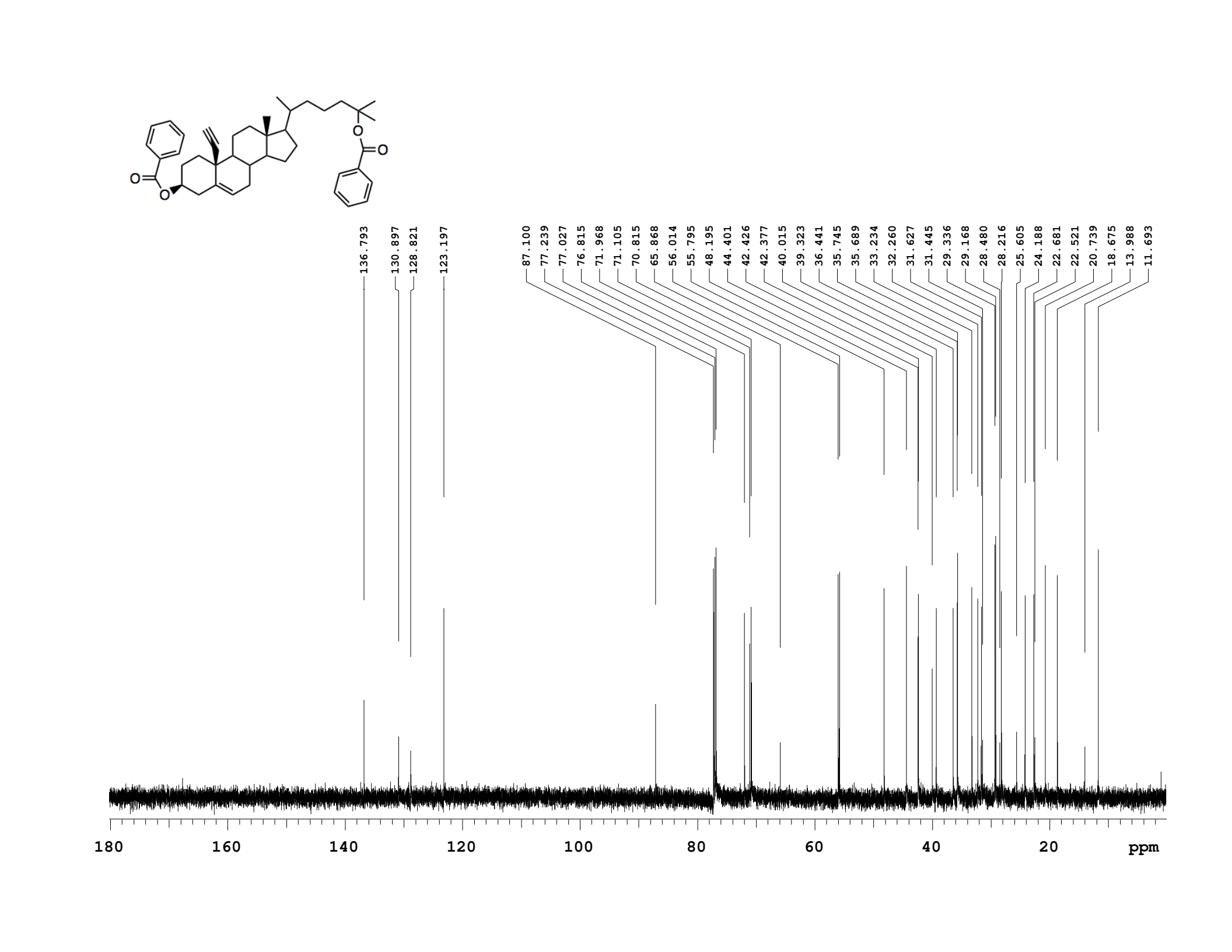
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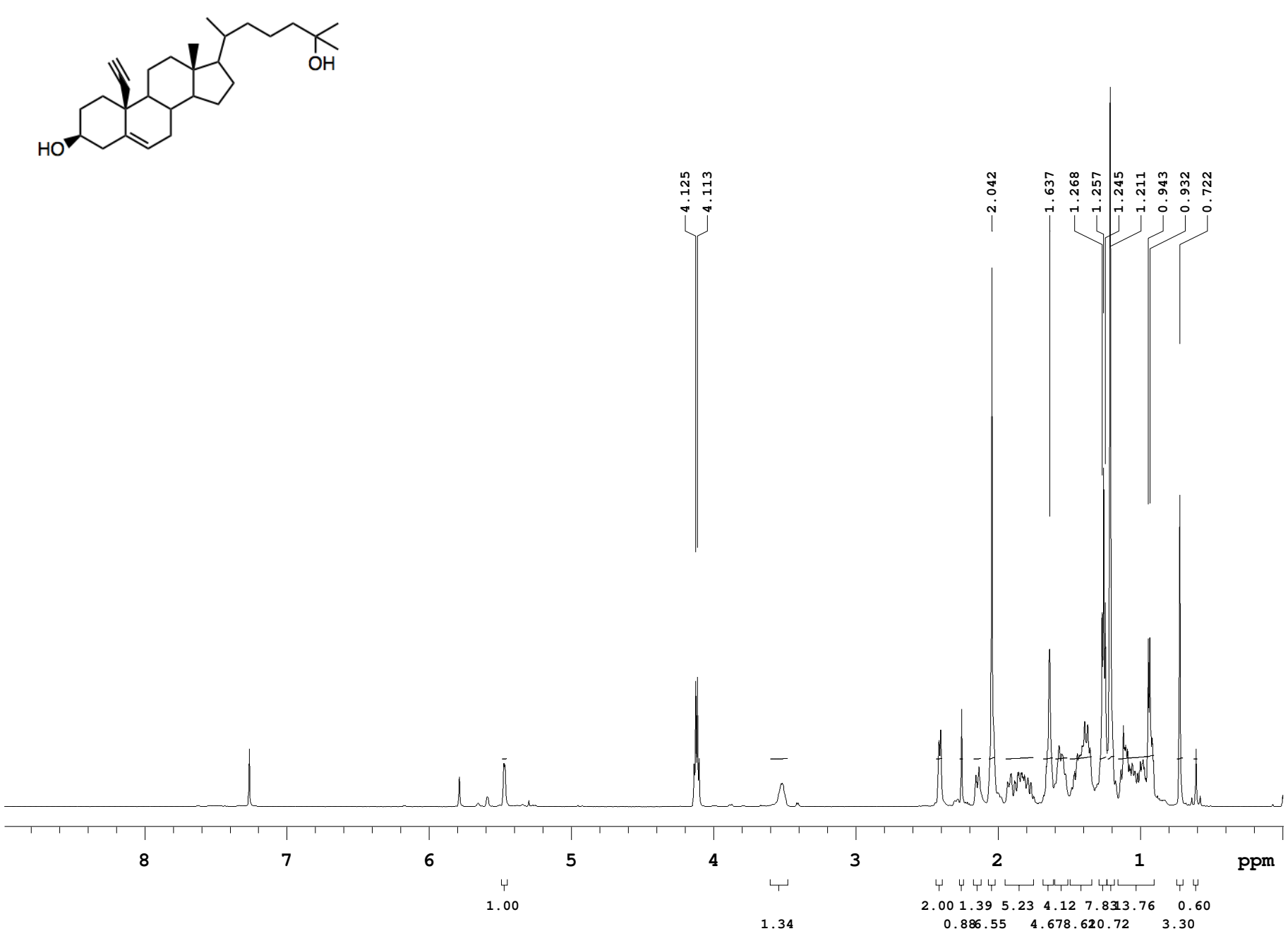
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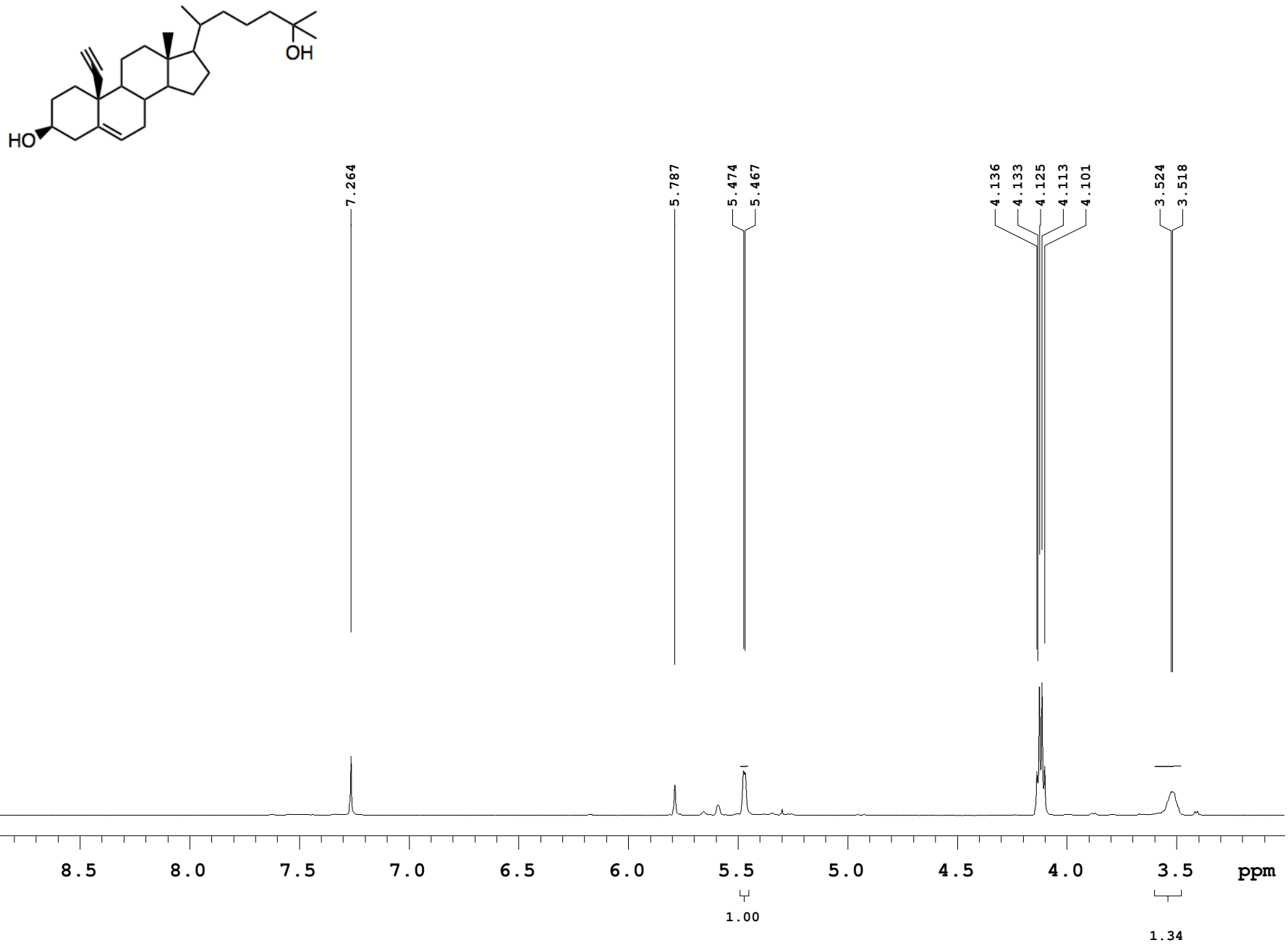
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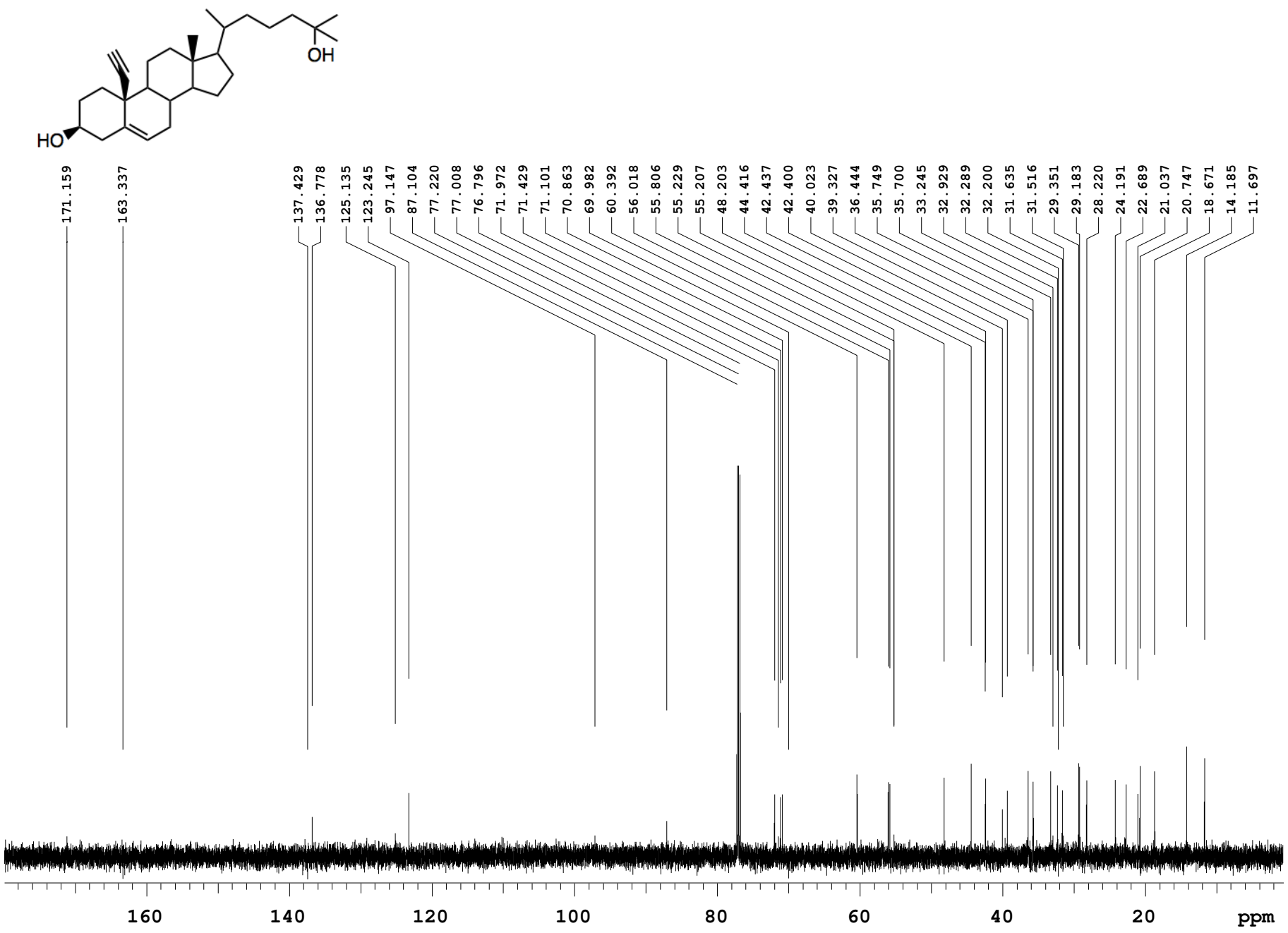
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21.037
20.747
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14.185
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140
120
100
80
60
40
20
ppm

2) Supplementary results

A. Supplementary figure legends

Supplementary figure 1

EChol efficiently supports modification of the Hh ligand, *in vitro* and in cells

(A) Quantification of eChol delivery to cells using soluble eChol-methyl β-cyclodextrin (MCD) complexes. NIH-3T3 cells were incubated for 2 hours in serum-free media, in the absence or presence of 12.5 μM eChol-MCD. Total lipids were extracted and saponified, and Chol and eChol levels were measured by gas chromatography/mass spectrometry (GC/MS). Samples were analyzed in triplicate; the error bars represent standard deviation of the mean. Labeling for 2 hours results in a cellular concentration of eChol that is 27% of the concentration of endogenous Chol.

(B) *In vitro* processing of the purified Hh ligand precursor is promoted by eChol. A fusion between maltose binding protein (MBP), the last 15 amino acids of the N-terminal fragment of Drosophila Hh, and the entire C-terminal fragment of Drosophila Hh (MBP-DHh) was purified from bacteria[9]. MBP-DHh was incubated at room temperature with 1 mM dithiothreitol (DTT), in the absence or presence of 400 μM eChol or Chol. The samples were analyzed by reducing SDS-PAGE and Coomassie staining. MBP-DHh-N and DHh-C are the N- and C-terminal fragments generated by sterol-dependent MBP-DHh processing. Both eChol and Chol stimulate Hh processing with similar efficiency.

(C) Chol- and eChol-modified MBP-DHh-N were generated as in (A), followed by CuAAC reaction with biotin-azide. The samples were separated by SDS-PAGE and biotinylated proteins were detected by immunoblotting with anti-biotin antibodies. Biotin-azide specifically detects eChol-modified MBP-DHh-N. The Ponceau S-stained blot serves as loading control.

(D) EChol rescues human Sonic Hedgehog (HShh) processing in sterol-depleted cells. HShh C-terminally tagged with a hemagglutinin tag (HShh-HA) was stably expressed in human embryonic kidney 293T cells[9, 11]. Cells were incubated in the absence or presence of 0.75% MCD in DMEM for 45 minutes (to deplete sterols), followed by incubation with 20 μM pravastatin, to block Chol synthesis. Sterols were added back to depleted cells in the pravastatin-containing media as soluble MCD complexes, at the following concentrations: Chol (100 μM), epicholesterol (100 μM) and eChol (5 μM). To block degradation of the HShh precursor and the C-terminal fragment (HShh-C), the media was also supplemented with 1 μM of the proteasome inhibitor bortezomib. After 3 hours, cells were harvested and cellular extracts were analyzed by SDS-PAGE and immunoblotting with anti-HA antibodies (to

detect HShh and HShh-C) and anti-HShh antibodies (to detect HShh-N, which serves as loading control). This experiment was performed together with the experiment we published in Figure 1C of Supplementary reference 11, and shares the control samples (left panels) with the published experiment[11].

Supplementary figure 2

Differential staining of eChol-labeled cellular membranes by various azides

Fluorescein-azide (5(6)-azido-fluorescein, A) and tetramethylrhodamine-picolyl azide (B) stain strongly eChol-labeled cells; by contrast, Alexa568-azides C and D do not stain eChol-labeled cells. Azides similar to C and D but in which Alexa568 is replaced by other fluorophores (Alexa488, Alexa594 or tetramethylrhodamine) also do not stain eChol in cells. The biotin-azides E and F do not stain eChol in cells but react efficiently with eChol attached to Hh protein in vitro (see Supplementary figure 1C). All azides above strongly stain propargylcholine-labeled phospholipids[5] or ethynyl-deoxyuridine-labeled DNA[1] in cells. We speculate that only azides A and B can gain access to the ethynyl group when eChol is embedded in the membrane bilayer. All azides were used at 5-20 μM in CuAAC staining reactions.

Supplementary figure 3

Detergent sensitivity of the eChol stain

NIH-3T3 cells were incubated for 12 hours in the presence or absence of eChol (40 μM, added from DMSO stock), after which the cells were fixed and stained with fluorescein-azide, followed by incubation for 30 minutes in the presence or absence of 0.5% Triton-X100. The cells were then washed and imaged by fluorescence microscopy.

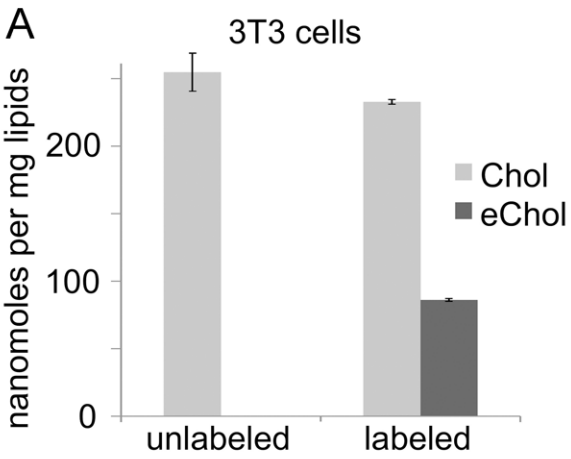
Supplementary figure 4

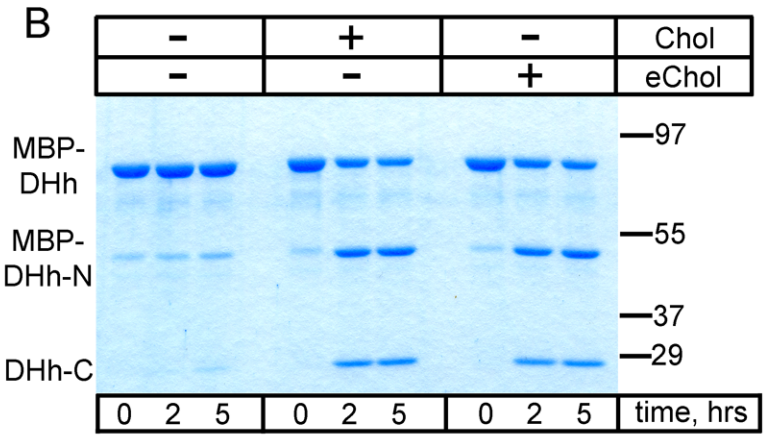
17-ethynyl-25-hydroxycholesterol (25-OH-eChol) activates Hh signaling at the level of Smo

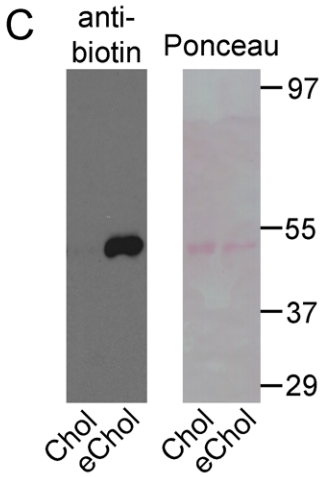
Hh-responsive Shh-LightII cells were incubated overnight with 25-OH-eChol (16 μM), in the presence or absence of the Smo inhibitor, SANT1 (1 μM). The synthetic Smo agonist, SAG (1 μM) and the Smo-activating oxysterol, 20(S)-hydroxycholesterol (20-OHC, 16 μM) were used as positive controls. Relative luciferase counts were normalized to those of SAG-stimulated cells (100% activation). Error bars represent the standard deviation for 4 independent experiments. Smo inhibition blocks activation of Hh signaling by 25-OH-eChol.

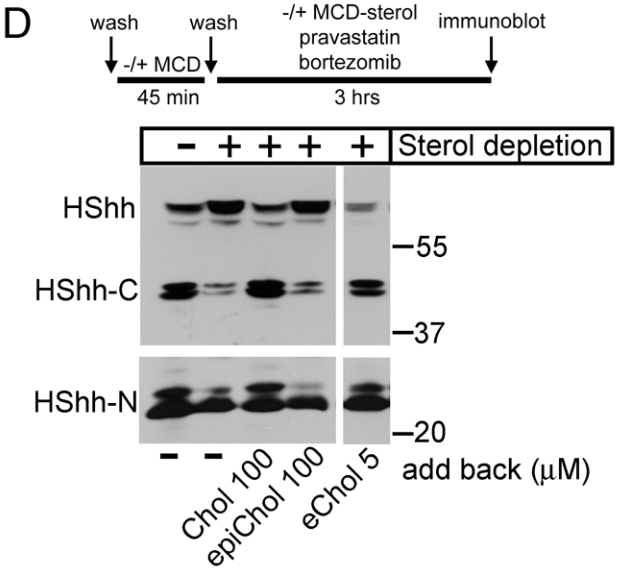
B. Supplementary figures

Supplementary figure 1



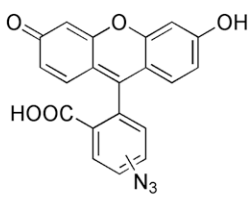






Supplementary figure 2

Azides that stain eChol-labeled cellular membranes



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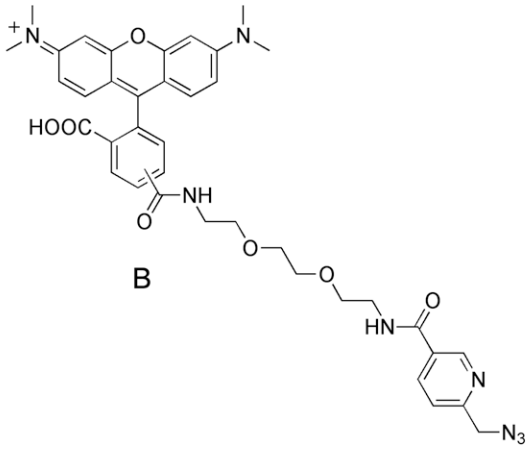
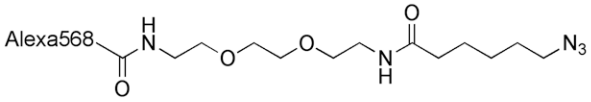


B

Azides that do not stain eChol-labeled cellular membranes



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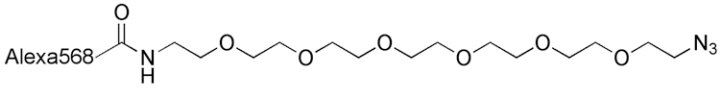
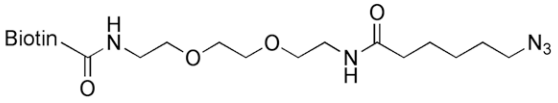


D

Azides that do not stain eChol-labeled cellular membranes but react with eChol-modified Hedgehog protein in vitro



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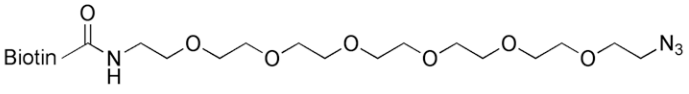
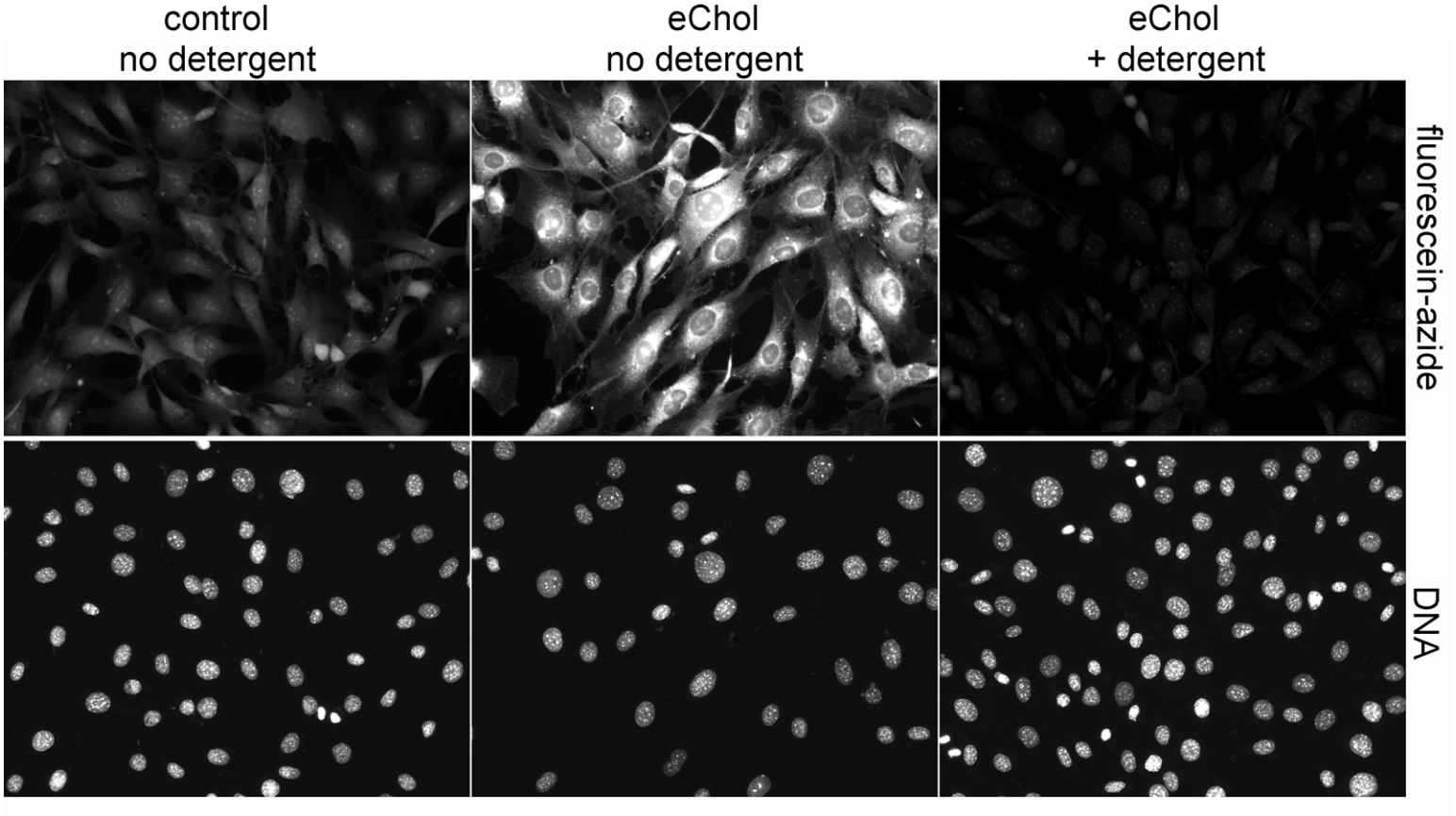
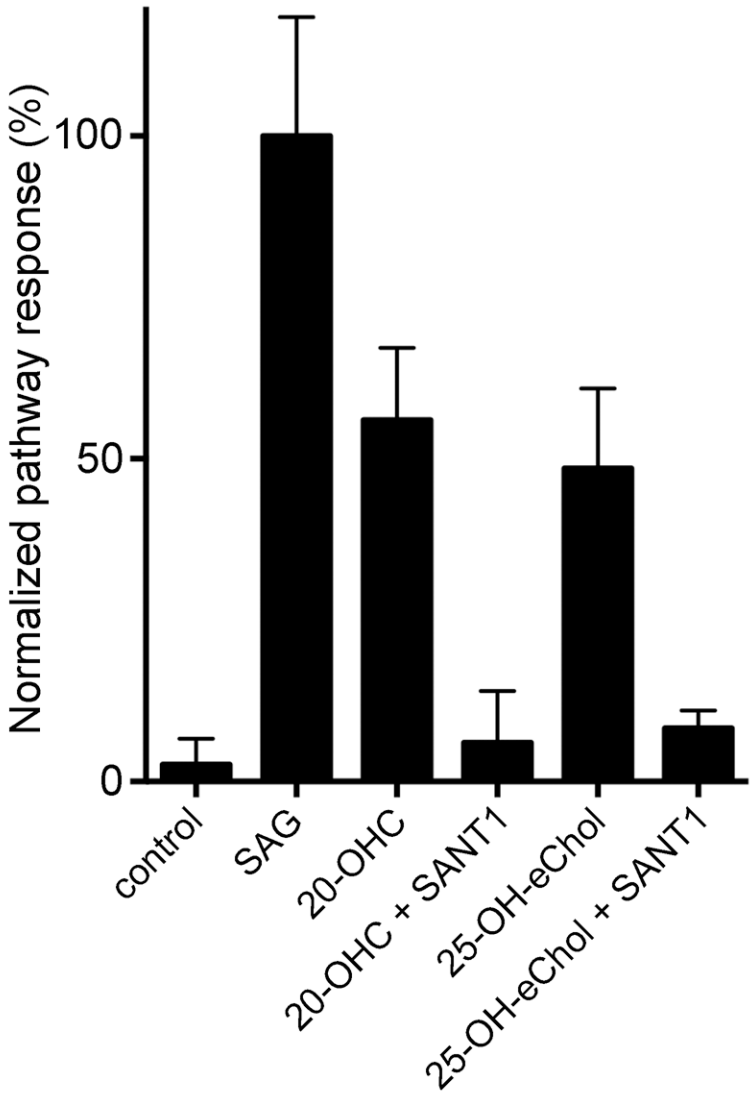


F

Supplementary figure 3



Supplementary figure 4



3) Supplementary references

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