A gut-restricted lithocholic acid analog as an inhibitor of gut bacterial bile salt hydrolases

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Supporting Information

Materials and Methods

Reagents. All bile acids and reagents for synthesis were commercially purchased from Steraloids Inc. and Sigma Aldrich. Stock solutions of all bile acids and inhibitors were prepared in molecular biology grade DMSO (Sigma Aldrich) at 1000X concentrations. Solvents used for preparing UPLC-MS samples were HPLC grade. New biological materials reported here are available from the authors upon request. 100 μM bile acid pool consisted of TCA, TβMCA, TUDCA and TDCA (25 μM each).

Bacterial culturing. All bacterial strains were cultured at 37 °C in BHI^{+} (Bacto^{TM} BHI, BD, supplemented with 5 mg l^{-1} hemin, and 2.5 ul l^{-1} Vitamin K_1). All strains were grown under anaerobic conditions in an anaerobic chamber (Coy Lab Products Airlock) with a gas mix of 5% hydrogen and 20% carbon dioxide nitrogen. *Escherichia coli* was grown aerobically at 37 °C in LB medium supplemented with ampicillin to select for the pET21b plasmid.

UPLC-MS analysis. Bile acid profiling by UPLC-MS was performed using a published method.[1] Correction factors for extraction efficiency were used and were determined by extraction of known concentrations of relevant bile acids from buffer or bacterial media and comparison to standard curves. The limits of detection for individual bile acids were determined using commercially available standards/ synthesized compounds solubilized in 1:1 MeOH/water and are as follows: βMCA, 0.03 picomol μl^{-1}; TβMCA, 0.01 picomol μl^{-1}; CA, 0.04 picomol μl^{-1}; TCA, 0.01 picomol μl^{-1}; UDCA, 0.04 picomol μl^{-1}; TUDCA, 0.01 picomol μl^{-1}; DCA, 0.04 picomol μl^{-1}; TDCA, 0.05 picomol μl^{-1}; GCDCA-d4, 0.1 picomol μl^{-1}; CDCA-d4, 0.1 picomol μl^{-1}; 7-oxo-CA, 0.5 picomol μl^{-1}; **AAA-2**, 0.05 picomol μL^{-1}; **AAA-10**, 1.0 picomol μl^{-1}.

Screen of inhibitors in human feces. BSH activity in human stool samples was quantified using a similar method as described above. A fresh human stool samples was collected from a healthy donor, and a small portion (approximately 10-20 mg) was broken into fine particles in buffer (PBS with 0.25 mM TCEP) to obtain a concentration of 1 mg ml^{-1}. Indicated concentrations of inhibitors were added to the fecal slurry and the mixture was incubated at 37 °C for 30. 100 μM glycochenodeoxycholic acid-d4 (GCDCA-*d*4) or taurocholic acid-d4 (TCA-*d*4) was added to the mixture and incubated at 37 °C for 2 h. The tubes were then frozen in dry ice for 5 mins and upon thawing were diluted with an equal volume of HPLC grade methanol. The slurry was centrifuged at 12,500 g for 10 mins. The supernatant was removed into a clean Eppendorf tube and centrifuged again. The supernatant was transferred to MS vials and samples were analyzed as per the method described in "UPLC-MS Analysis". The concentration of product detected from these assays was reported directly.

Protein expression and purification. *B. thetaiotaomicron* recombinant BSH (accession number AAO77193.1) and *B. longum* recombinant BSH (accession number AAF67801.1) were expressed and purified as previously described.[2]

Determination of IC_{50} values of inhibitors against recombinant proteins. 200 nM rBSH was incubated with increasing concentrations of the inhibitors at 37 °C for 1 h in 1 ml PBS buffer containing 0.25 mM TCEP and 5% glycerol at pH 7.5. 100 μM bile acid (TUDCA for *B. theta* BSH and TDCA for *B. longum* BSH) was added to the above solution and incubated at 37 °C for 2 h. The solution was acidified to pH = 1 using 6M HCl and extracted twice with 1 ml ethyl acetate. The combined organic layers were then dried using a Biotage TurboVap LV. The dried extracts were resuspended in 1:1 methanol:water and transferred to mass spectrometry vials.

Samples were analyzed as per the method described in UPLC–MS analysis. The obtained concentrations of bile acids were used to determine percentage deconjugation.

Equation for calculating percent deconjugation.

Percent deconjugation = concentration of deconjugated bile acids detected / (concentration of deconjugated bile acids detected + concentration of conjugated bile acids detected) X 100.

Equation for calculating percent inhibition.

Percent inhibition = (percent deconjugation in control sample - percent deconjugation in inhibitor-treated sample) / percent deconjugation in control sample X 100.

Determination of IC_{50} values of AAA-10 in bacterial cultures. Note that due to slow growth of *B. longum*, *B. adolescentis* was used as a representative of Gram-positive bacteria. Overnight cultures of *B. theta* and *B. adolescentis* were diluted to an OD_{600} of 0.1 in 2 ml fresh CHG media (see "Bacterial Culturing") containing 100 μM TUDCA or TDCA, respectively, and inhibitor at increasing concentrations. *B. theta* and *B. adolescentis* deconjugated TUDCA and TDCA, respectively, to the greatest extent of any of the conjugated substrates, and therefore these substrates were used to determine IC_{50} values. Cultures were then grown anaerobically at 37 °C for 24 h (*B. adolescentis*) or 48 h (*B. theta*). Longer incubation time was required for *B. theta* because for this bacterium, significant BSH activity was only observed during stationary phase. Cultures were extracted and analyzed as per the method described in "BSH Inhibition in Bacterial Cultures" to determine percent deconjugation which was then converted into percent inhibition.

Cell culture. Caco-2 cells and HepG2 cells were obtained from American Type Culture Collection (Manassas, VA). Caco-2 cells were maintained in Minimum Essential Medium

(MEM) supplemented with GlutaMAX. All cell culture media was supplemented with 10% fetal bovine serum (FBS), 100 units ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin (GenClone). Cells were grown in FBS- and antibiotic-supplemented 'complete' media at 37 °C in an atmosphere of 5% CO_2.

Plasmids and transient transfections. For luciferase reporter assays, vectors expressing human reporter constructs were used. The pGL4.29[luc2P/CRE/Hygro] plasmid (Promega Corporation) and the pGL4 [Shp-luc] plasmid obtained from Kristina Schoonjans lab at EPLF was transiently transfected in Caco-2 cells at a concentration of 2 μg ml^{-1} of media each for studying TGR5 and FXR activation respectively. The pGL4.74[hRluc/CMV] plasmid (Promega Corporation) was used as a transfection efficiency control at a concentration of 0.05 μg ml^{-1} of media. All plasmids were transfected using Opti-MEM (Gibco) and Lipofectamine 2000 (Invitrogen, Life Technologies, Grand Island, NY, USA) according to manufacturer's instructions. After overnight incubation, **AAA-10** and/or bile acids were added in complete media. **AAA-10** and/or bile acids were diluted in DMSO and the concentration of DMSO was kept constant. 10 μM of LCA or 10 μM of CDCA was added along with **AAA-10** to study TGR5 and FXR antagonism respectively and incubated overnight. Cells were harvested the next day for the luciferase assay.

Cell viability assay. Cells were treated with indicated compounds diluted in DMSO in complete MEM media. The viability of differentiated Caco-2 cells in transwells was measured using an MTT assay (Abcam, ab211091), while the viability of HepG2 cells was measured using Cell Titer Glo (Promega) according to manufacturer's instructions. The concentration of DMSO was kept constant and used as a negative control. Cells were incubated with the compounds overnight at 37 °C in an atmosphere of 5% CO2. The next day, cell viability was measured. For

differentiated Caco-2 cells, cell culture media was replaced with the MTT reagent and incubated for 3 hours at 37 °C. Following incubation, the MTT reagent was replaced with the MTT solvent and incubated for 15 min, followed by colorimetric analysis. The SpectraMax M5 plate reader (Molecular Devices, San Jose, CA) at the ICCB-Longwood Screening Facility at HMS was used to measure cell viability via luminescence (Cell Titer Glo, HepG2 cells) or absorbance (MTT reagent, Caco-2 cells) at 690 nm. Percentage relative viability was calculated compared to DMSO control.

BSH activity in feces. BSH activity in fecal pellets were quantified using a modified version of a published method.[2,3] Fecal pellets (approximately 10-20 mg) were suspended in buffer (PBS with 0.25 mM TCEP) containing 100 μM (GCDCA-d4) to obtain a concentration of 20 mg ml^{-1}. The fecal pellets were broken into fine particles and the mixture was incubated at 37 °C for 25 mins. Samples were processed and analyzed as per the method described in "Screen of Inhibitors in Conventional Mouse Feces". The concentration of product detected from these assays were reported directly.

Quantification of bile acids and AAA-10 in tissues and plasma. Bile acids and AAA-10 were extracted from tissues and plasma and quantified using a previously published method.[1]

Determination of bile acid abundance in feces over the entire period of the study. The following formula was used to determine the abundance of a particular bile acid over the entire period of the study:

Bile acid abundance over the study = (Picomol of a bile acid of interest at timepoint 1 + picomol of a bile acid of interest at timepoint 2 + …. + picomol of a bile acid of interest at last timepoint) / (total picomol of all fecal bile acids at timepoint 1 + total picomol of all fecal bile acids at

timepoint 2 + …. + total picomol of all fecal bile acids at last timepoint). The obtained value was then divided by the total amount of bile acids detected over the period of study * 100.

In vivo pharmacokinetics of AAA-10 at 4h timepoint. C57BL/6 mice obtained from the facility at Bienta Enamine were maintained under a strict 12 h/12 h light/dark cycle and a constant temperature (22 ± 3 °C) and humidity (40–70%). All experiments were conducted on 10-11 week old male mice. During a seven day of acclimatization period, each animal was kept in a separate cage and had limited access to food (free access twice a day, from 9:00 h to 13:00 h and from 18:00 h to 22:00 h). Mice had no access to food at other times. Mice had free access to acidified boiled tap water. All animals were under observation and only animals without any clinical symptoms of illness were taken into the study. During treatment with **AAA-10,** mice were fed according to the above schedule and treatment was carried out 2 times a day immediately before providing access to food (at 9:00 h and 18:00 h) on the days 1-4 of the experimental period and once a day (9:00 h, before providing access to food) on the terminal (5^{th}) day of the experimental period. Treatment group animals were gavaged with 150 µl of PBS containing 10% captisol (w/v) and **AAA-10** at a concentration of 3.75 mg ml^{-1} while the control group animals were treated with 150 µl of PBS containing 10% captisol (w/v). Plasma and tissue sample collections (liver, kidney, heart, duodenum, jejunum, ileum, cecum and colon) were performed at the terminal sacrifice on the 5^{th} day of the study four hours after the final gavage.

16S rRNA sequencing. 16s rRNA sequencing was performed using a previously published method. [2] Briefly, pre-treatment fecal and post-treatment vehicle and AAA-10 cecal DNA was isolated by using ZymoBIOMICS 96 DNA Kit (ZymoBIOMICS) according to the manufacturer's instructions. The variable region 4 of the 16S rRNA genes was amplified using primers: Forward 5′- TATGGTAATTGTGTGCCAGCMGCCGCGGTAA-3′ Reverse 5′-

AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3′. Quality of the amplified DNA products was checked and roughly 120 ng of each DNA product was pooled together to generate an aggregated library for Illumina MiSeq sequencing. Demultiplexed FASTQ files were generated by the Illumina MiSeq software using default parameters and quality control was performed by the pipeline at the Massachusetts Host-Microbiome Center. The resulting FASTQ sequences were then quality-filtered and analyzed by following QIIME_mothur_DADA2.[4-7] OTUs were picked with 97% sequence similarity. The phylogenetic affiliation of each OTUs were aligned to the Greengenes reference database and 99% ID. Data was analyzed using the online Nephele software by NIH (https://nephele.niaid.nih.gov/).[7]

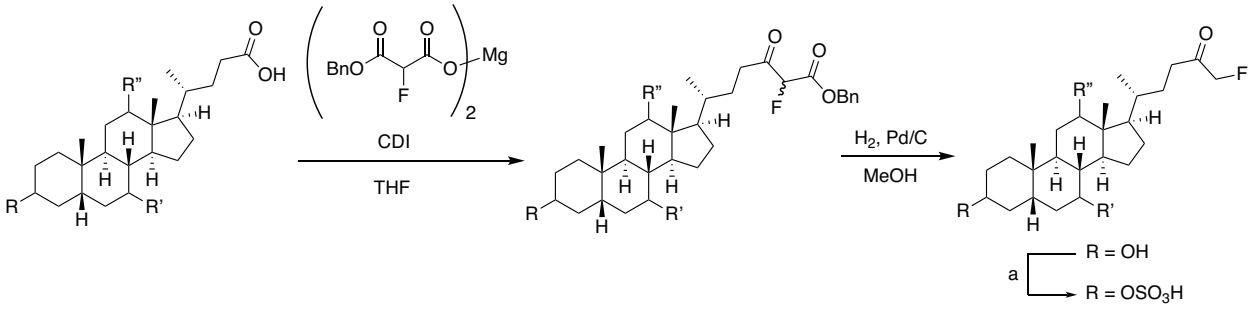
Ethics. For the BSH inhibition in vivo studies, mouse experiments were performed under the approval of the Beth Israel Deaconess Medical Center IACUC. For the in vivo pharmacokinetics studies, protocols were approved by Bienta's Animal Care and Use Committee and experiments were performed in adherence with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (1986). BACUC protocol number - №9-3/2020 dated 30.09.2020. Human stool sample was obtained from a healthy donor and was collected under the protocol "Culturing of Bacterial Strains within Healthy Individuals", reviewed and approved by the Ethical review Board of MIT, the Committee on the Use of Human as Experimental Subjects (COUHES), protocol number 1510271631.

Code availability statement

No custom code or mathematical algorithms were used in this study.

Synthetic procedures

General: All anhydrous reactions were run under a positive pressure of argon or nitrogen. Anhydrous methylene chloride (DCM) and tetrahydrofuran (THF) were purchased from Sigma Aldrich. Silica gel column chromatography was performed using 60 Å silica gel (230−400 mesh). NMR spectra recorded in $CDCl_3$ used residual chloroform or TMS as the internal reference.



Scheme 1: Generic scheme for synthesis of compounds 1-5. CDI = carbonyldiimidazole, a = SO_3.pyridine, pyridine

General procedure for the synthesis of α-fluoromethyl ketone analogs.

The magnesium benzyl fluoromalonate coupling reagent was synthesized according to a reported protocol[8] with modifications.

Step 1. To a 0.3 M solution of the C-24 acid (1.0 equiv.) in anhydrous THF, 1'-carbonyldiimidazole (CDI) (1.0 equiv.) was added and stirred at rt for 1 h. The magnesium benzyl fluoromalonate (2.0 equiv.) was suspended in anhydrous THF (0.3 M) and the above solution was added dropwise. The resultant mixture was stirred at rt for 18 h. The reaction was quenched by the addition of 10 mL of 1M HCl and concentrated using a rotary evaporator. The residue was then partitioned using 10 mL ethyl acetate and 10 mL water. The organic layer was separated and the aqueous layer was extracted with ethyl acetate (2 x 10 mL). The combined

organic layers were then dried over sodium sulfate, filtered and concentrated. The crude compound was then purified by silica gel chromatography (20% ethyl acetate/80% hexanes).

Step 2. To a 0.15 M solution of above compound (1.0 equiv.) in methanol, palladium on carbon (0.05 equiv.) was added. The flask was vacuumed and replaced with a hydrogen balloon. The reaction mixture was stirred at rt for 3 h. The solution was then filtered through a celite bed and the filtrate was concentrated to provide the crude compound. The compound was then purified by silica gel chromatography using a gradient of 50 to 60 % ethyl acetate in hexanes to provide the pure compound.

AAA-1 (compound 7; **1)**. Synthesis and characterization previously described. [2]

AAA-3 (**9**)**.** Yield = 97 mg (19% over 2 steps); TLC R_f = 0.33 (30% ethyl acetate/70% hexanes); ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.79 (d, *J* = 47.6 Hz, 2H), 3.97 (br s, 1H), 3.565-3.57 (m, 1H), 2.63-2.43 (m, 2H), 1.91-1.23 (m, 23H), 1.18-0.94 (m, 6H), 0.91 (s, 3H), 0.68 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 207.53 (d, *J* = 19.2 Hz), 84.91 (d, *J* = 184.3), 73.11 (d, *J* = 11.2 Hz), 71.75, 48.28, 47.21, 46.48, 42.06, 36.44, 36.03, 35.18, 35.12, 34.95, 34.10, 33.69, 30.51, 28.75, 28.59, 27.40, 26.10, 23.60, 23.14, 17.43, 12.74, 12.73; HRMS (*m/z*): [M + HCOO - H]$^-$ calcd. for $C_{26}H_{42}FO_5$, 453.3022; found, 453.3030.

AAA-5 (**10**)**.** Yield = 142 mg (27% over 2 steps); TLC R_f = 0.44 (30% ethyl acetate/70% hexanes); ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.79 (d, *J* = 47.6 Hz, 2H), 3.59 (br s, 2H), 2.62-2.42 (m, 2H), 2.02-1.74 (m, 6H), 1.69-0.92 (m, 26H), 0.68 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 207.59 (d, *J* = 19.2 Hz), 84.92 (d, *J* = 184.2), 71.42 (d, *J* = 8.0 Hz), 71.30, 55.71, 54.87,

43.79, 43.76, 42.44, 40.12, 39.17, 37.32, 36.87, 35.22, 35.17, 34.92, 34.07, 30.35, 28.70, 28.58, 26.86, 23.36, 21.16, 18.50, 12.13, 12.11; HRMS (*m/z*): $[M + HCOO - H]^-$ calcd. for $C_{26}H_{42}FO_5$, 453.3022; found, 453.3025.

AAA-7 (11). Synthesis and characterization previously described. [2]

AAA-9 (7). 38 mg (52% over 2 steps); TLC R_f = 0.45 (40% ethyl acetate/60% hexanes); ^1H NMR (400 MHz, $CDCl_3$) δ 4.77 (d, *J* = 48.0 Hz, 2H), 3.64-3.56 (m, 1H), 2.60-2.39 (m, 2H), 1.95-1.48 (m, 9H), 1.41-0.89 (m, 24H), 0.62 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 207.62 (d, *J* = 19.1 Hz), 84.89 (d, *J* = 184.2), 71.79 (d, *J* = 6.4 Hz), 56.47, 55.89, 42.73, 40.08, 40.42, 40.15, 36.42, 35.83, 35.34, 35.25, 35.18, 34.55, 30.51, 28.70, 28.16, 27.17, 26.40, 24.17, 23.34, 20.80, 18.37, 12.03, 12.01; HRMS (*m/z*): $[M + HCOO - H]^-$ calcd. for $C_{26}H_{42}FO_4$, 437.3073; found, 437.3088.

General procedure for the sulfonation to provide the gut-restricted α-fluoromethyl ketone analogs.

To a 0.1 M solution of the alcoholic precursor (1.0 equiv.) in pyridine, SO_3.pyridine (2.0 equiv.) was added and the resulting solution was stirred at rt for 18 h. The reaction mixture was concentrated using a rotary evaporator. The resulting slurry was resuspended in 5 mL of 10:1 dichloromethane:methanol and 5 mL of a saturated solution of sodium bicarbonate was added. The biphasic solution was concentrated using a rotary evaporator. The resulting crude compound was then purified by silica gel chromatography (80% dichloromethane/20% methanol) to provide the sulfonated compound.

AAA-2' (**3**). Yield = 12 mg (20% di-sulfonated) along with 40 mg (50%) of **AAA-2** was also obtained; TLC R_f = 0.15 (20% methanol/80% dichloromethane); ^{1}H NMR (400 MHz, CD_3OD) δ 4.90 (d, *J* = 47.6 Hz, 2H), 4.47 (d, *J* = 2.4 Hz, 1H), 4.21-4.14 (m, 1H), 2.57-2.26 (m, 4H), 2.15-1.72 (m, 9H), 1.67-1.02 (m, 13H), 0.95-0.94 (m, 6H), 0.69 (S, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 207.69 (d, *J* = 17.0 Hz), 84.51 (d, *J* = 180.9), 79.60, 77.22, 55.71, 49.85, 42.36, 41.72, 39.39, 39.20, 35.54, 35.20, 34.88, 34.39, 34.00, 33.27, 30.27, 28.59, 27.71, 27.62, 22.92, 21.77, 20.32, 17.52, 10.77; HRMS (*m/z*): $[M - H]^-$ calcd. for $C_{25}H_{40}FO_9S_2$, 567.2103; found, 567.2114. Also seen $[M – H/2]^-$ calcd. for $C_{25}H_{39}FO_9S_2$, 283.1015; found, 283.1019.

AAA-2 (GR-7; **2**). Synthesis and characterization previously described. [2]

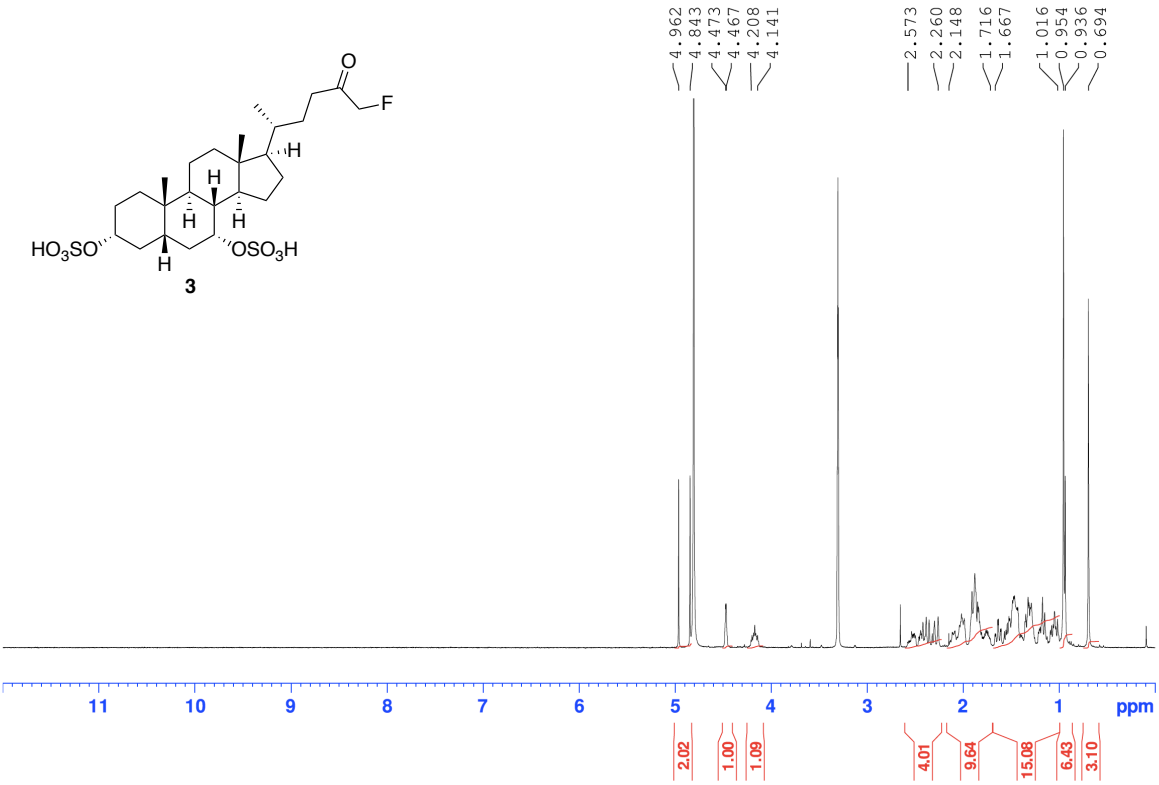
AAA-4 (**4**)**.** Yield = 25 mg (70% brsm); TLC R_f = 0.54 (20% methanol/80% dichloromethane); ^{1}H NMR (400 MHz, CD_3OD) δ 4.91 (d, *J* = 47.6 Hz, 2H), 4.32-4.24 (m, 1H), 3.94 (s, 1H), 2.59-2.39 (m, 2H), 1.99-1.72 (m, 9H), 1.66-1.27 (m, 13H), 1.18-0.98 (m, 6H), 0.93 (s, 3H), 0.70 (s, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 207.77 (d, *J* = 16.8 Hz), 84.51 (d, *J* = 181.1), 79.22, 72.57, 47.83, 46.59, 46.13, 42.32, 36.00, 35.20, 34.94, 34.04, 33.79, 33.33, 33.17, 28.54, 28.47, 27.29, 27.17, 26.87, 25.97, 23.42, 22.16, 16.27, 11.76; HRMS (*m/z*): $[M - H]^-$ calcd. for $C_{25}H_{40}FO_6S$, 487.2535; found, 487.2541.

AAA-6 (**5**)**.** Yield = 13 mg (40% brsm); TLC R_f = 0.36 (20% methanol/80% dichloromethane); ^{1}H NMR (400 MHz, CD_3OD) δ 4.91 (d, *J* = 47.2 Hz, 2H), 4.27-4.19 (m, 1H), 3.51-3.44 (m, 1H), 2.58-2.38 (m, 2H), 2.04-2.01 (m, 1H), 1.92-1.68 (m, 8H), 1.58-1.03

(m, 16H), 0.97-0.94 (m, 6H), 0.71 (s, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 207.72 (d, *J* = 17.0 Hz), 84.51 (d, *J* = 181.1 Hz), 78.56, 70.39, 55.99, 55.01, 43.36, 43.04, 42.71, 40.08, 39.23, 37.06, 35.20, 34.58, 34.09, 33.99, 33.67, 28.62, 28.17, 27.29, 26.50, 22.39, 20.98, 17.61, 11.21; HRMS (*m/z*): $[M - H]^-$ calcd. for $C_{25}H_{40}FO_6S$, 487.2535; found, 487.2553.

AAA-8 (**6**)**.** Yield = 12 mg (40% brsm); TLC R_f = 0.41 (20% methanol/80% dichloromethane); ^{1}H NMR (400 MHz, CD_3OD) δ 4.91 (d, *J* = 47.2 Hz, 2H), 4.18-4.10 (m, 1H), 3.93 (s, 1H), 3.79 (d, *J* = 2.8 Hz, 1H), 2.59-2.39 (m, 3H), 2.31-2.24 (m, 1H), 2.04-1.70 (m, 9H), 1.64-1.51 (m, 5H), 1.44-1.28 (m, 6H), 1.16-0.99 (m, 5H), 0.92 (s, 3H), 0.71 (s, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 207.81 (d, *J* = 15.3 Hz), 84.52 (d, *J* = 180.8 Hz), 79.49, 72.60, 67.54, 46.53, 46.04, 41.88, 41.57, 39.54, 36.29, 35.25, 34.98, 34.39, 34.34, 34.04, 28.57, 28.17, 27.45, 27.19, 26.43, 22.79, 21.63, 16.33, 11.54; HRMS (*m/z*): $[M - H]^-$ calcd. for $C_{25}H_{40}FO_7S$, 503.2484; found, 503.2486.

AAA-10 (**8**)**.** Yield = 0.65 g (34%); TLC R_f = 0.12 (10% methanol/90% dichloromethane); ^{1}H NMR (400 MHz, DMSO-d_6) δ 5.00 (d, *J* = 47.2 Hz, 2H), 3.99-3.91 (m, 1H), 2.48-2.26 (m, 2H), 1.90-1.51 (m, 9H), 1.32-0.97 (m, 16H), 0.92-0.83 (m, 7H), 0.59 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 206.55 (d, *J* = 15.3 Hz), 85.21 (d, *J* = 178.5 Hz), 76.01, 56.43, 55.81, 42.72, 42.04, 40.34, 40.08, 35.81, 35.50, 35.11, 34.56, 34.20, 33.79, 28.74, 28.10, 27.25, 26.52, 24.28, 23.63, 20.84, 18.73, 12.33, 12.31; HRMS (*m/z*): $[M - H]^-$ calcd. for $C_{25}H_{40}FO_5S$, 471.2580; found, 471.2591.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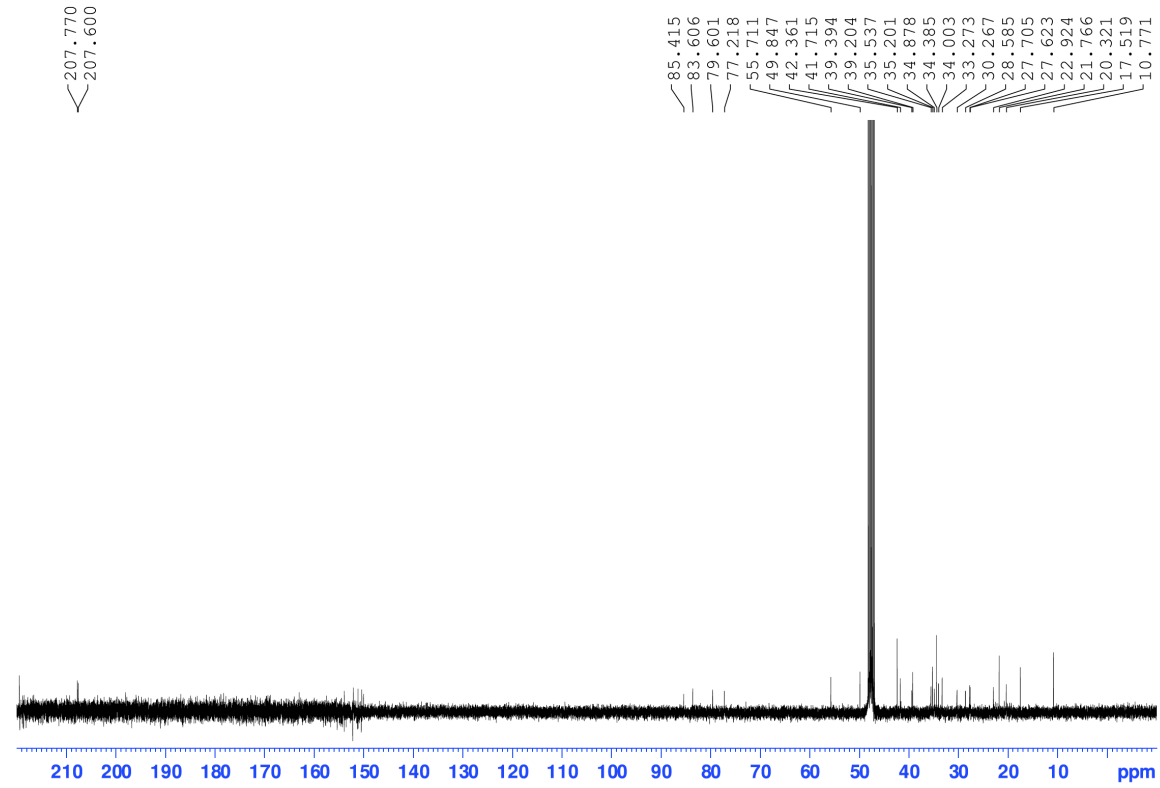
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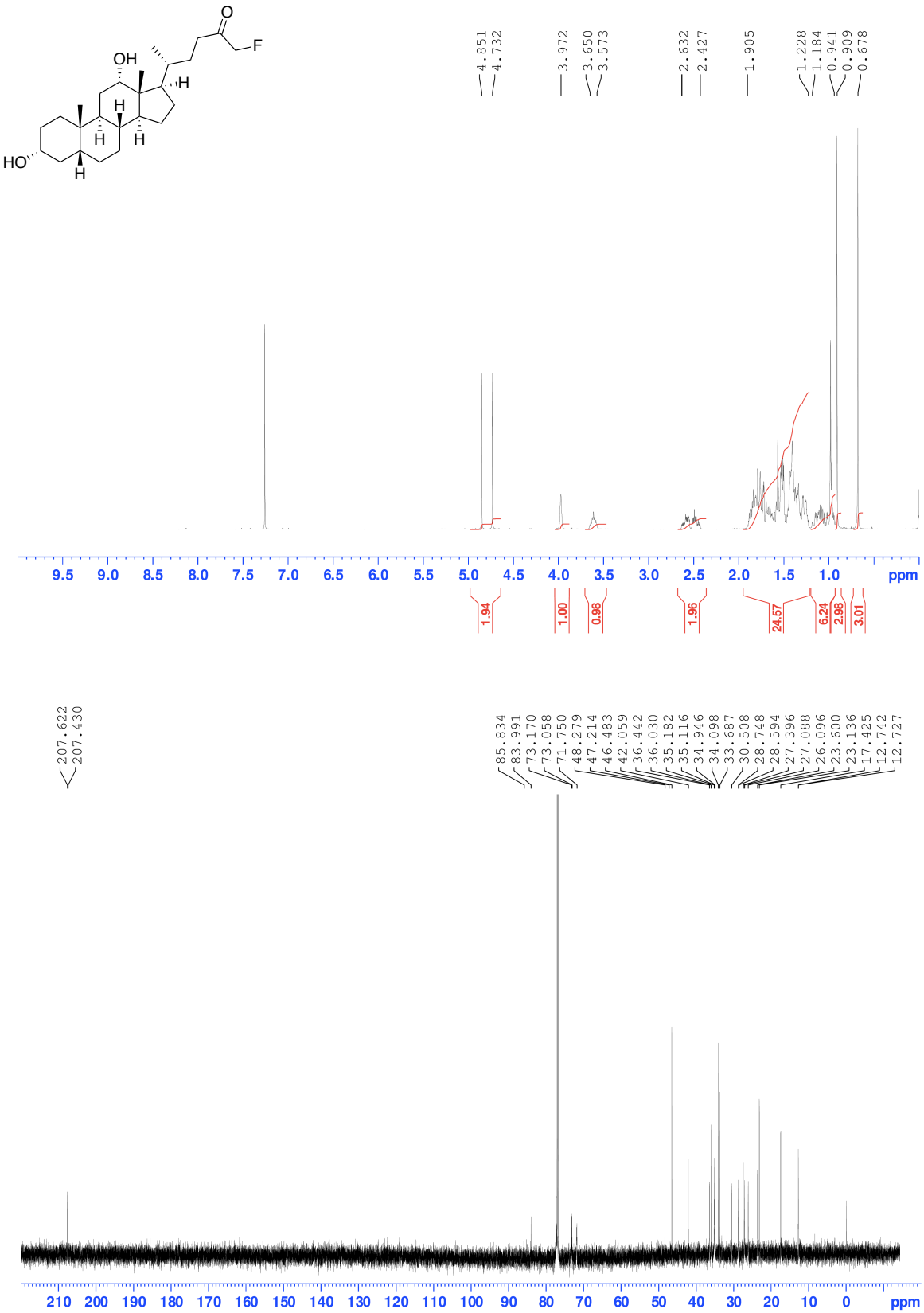
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12.742
12.727
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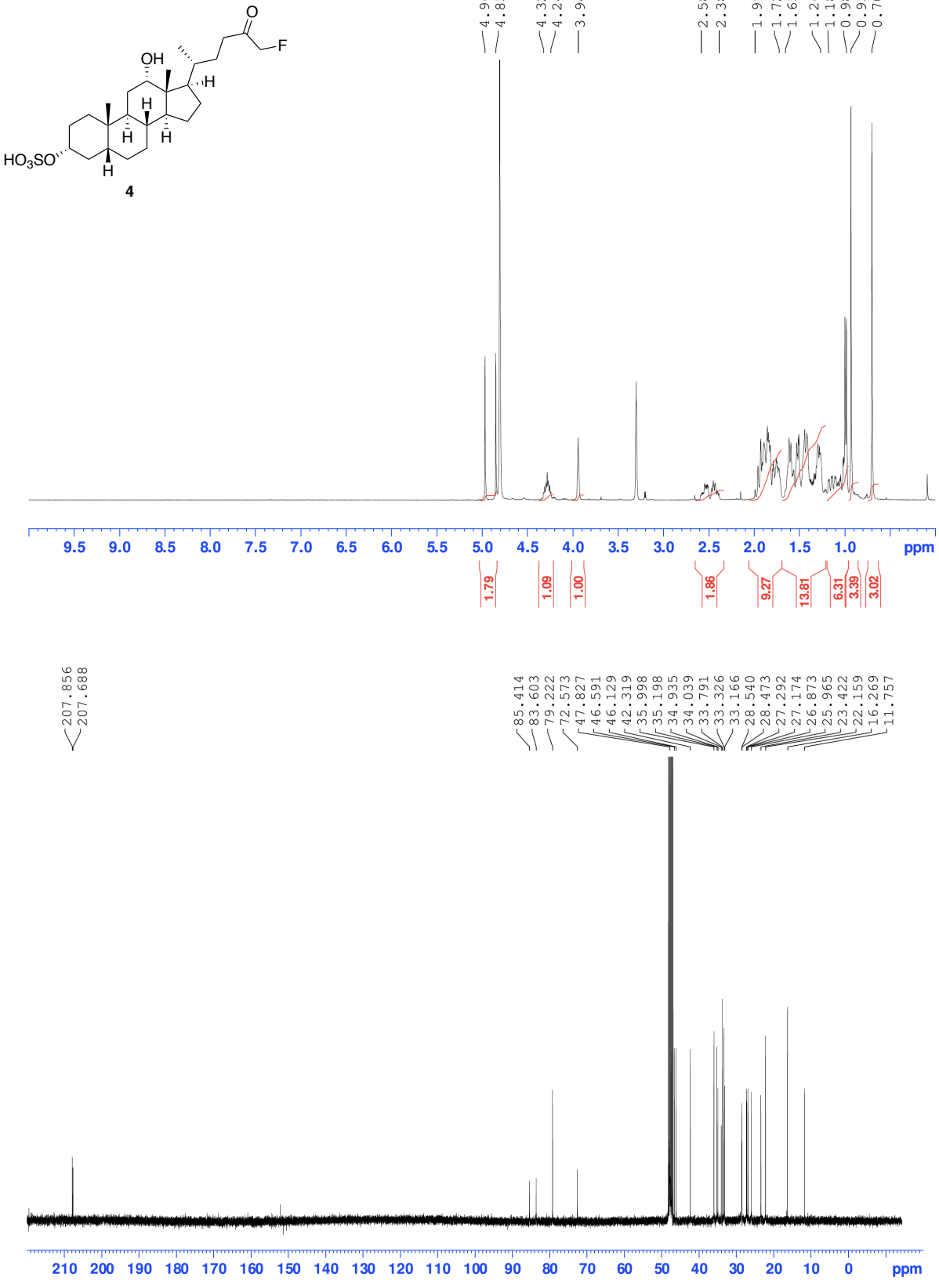
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4.850
4.323
4.243
3.942
2.585
2.386
1.990
1.724
1.655
1.267
1.181
0.983
0.932
0.701
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 ppm
1.79
1.09
1.00
1.86
9.27
13.81
6.31
3.39
3.02
207.856
207.688
85.414
83.603
79.222
72.573
47.827
46.591
46.129
42.319
35.998
35.198
34.935
34.039
33.791
33.326
33.166
28.540
28.473
27.292
27.174
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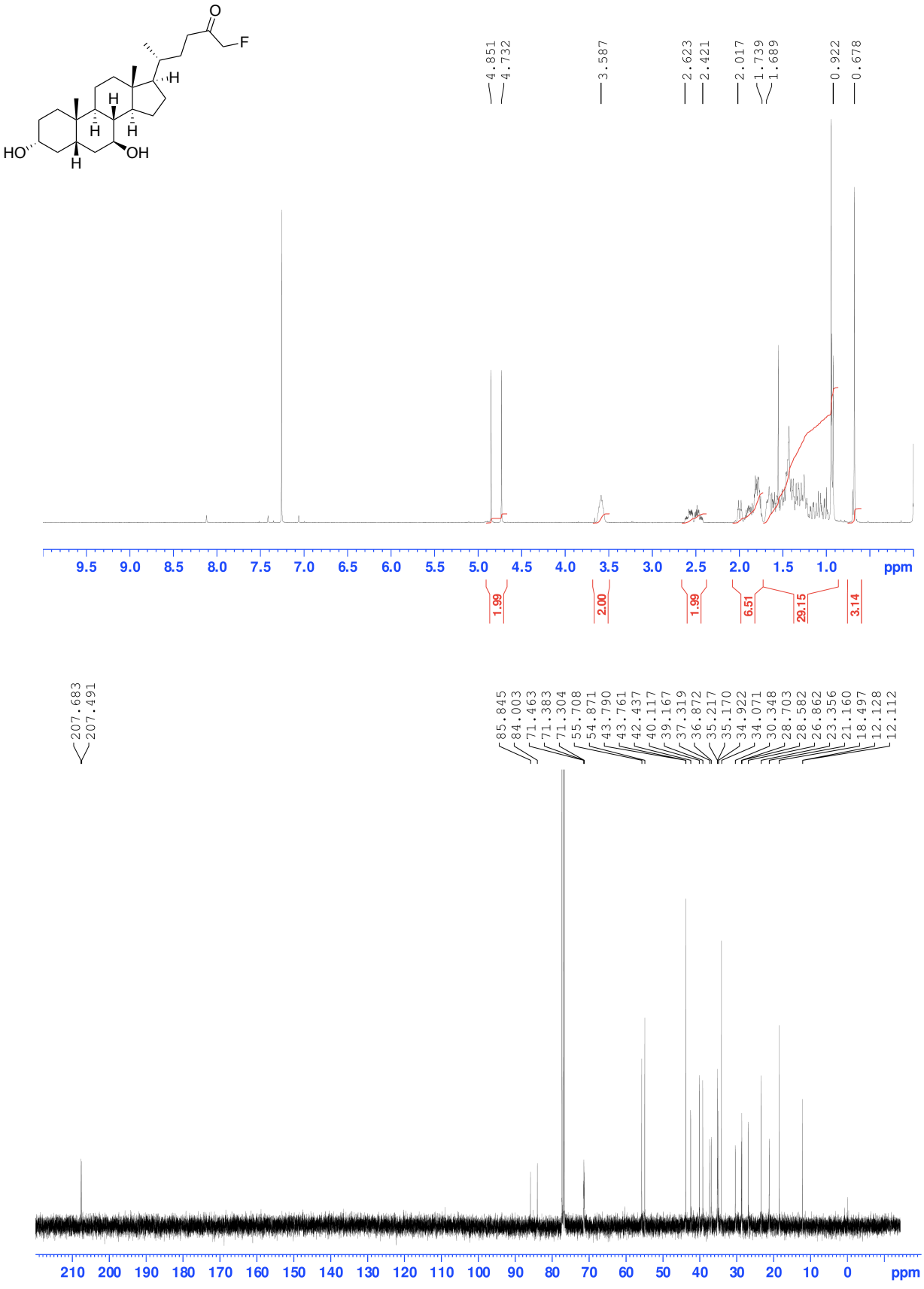
4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F
H
H
H
H
HO
OH
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0.922
0.678
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 ppm
1.99
2.00
1.99
6.51
29.15
3.14
207.683
207.491
85.845
84.003
71.463
71.383
71.304
55.708
54.871
43.790
43.761
42.437
40.117
39.167
37.319
36.872
35.217
35.170
34.922
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30.348
28.703
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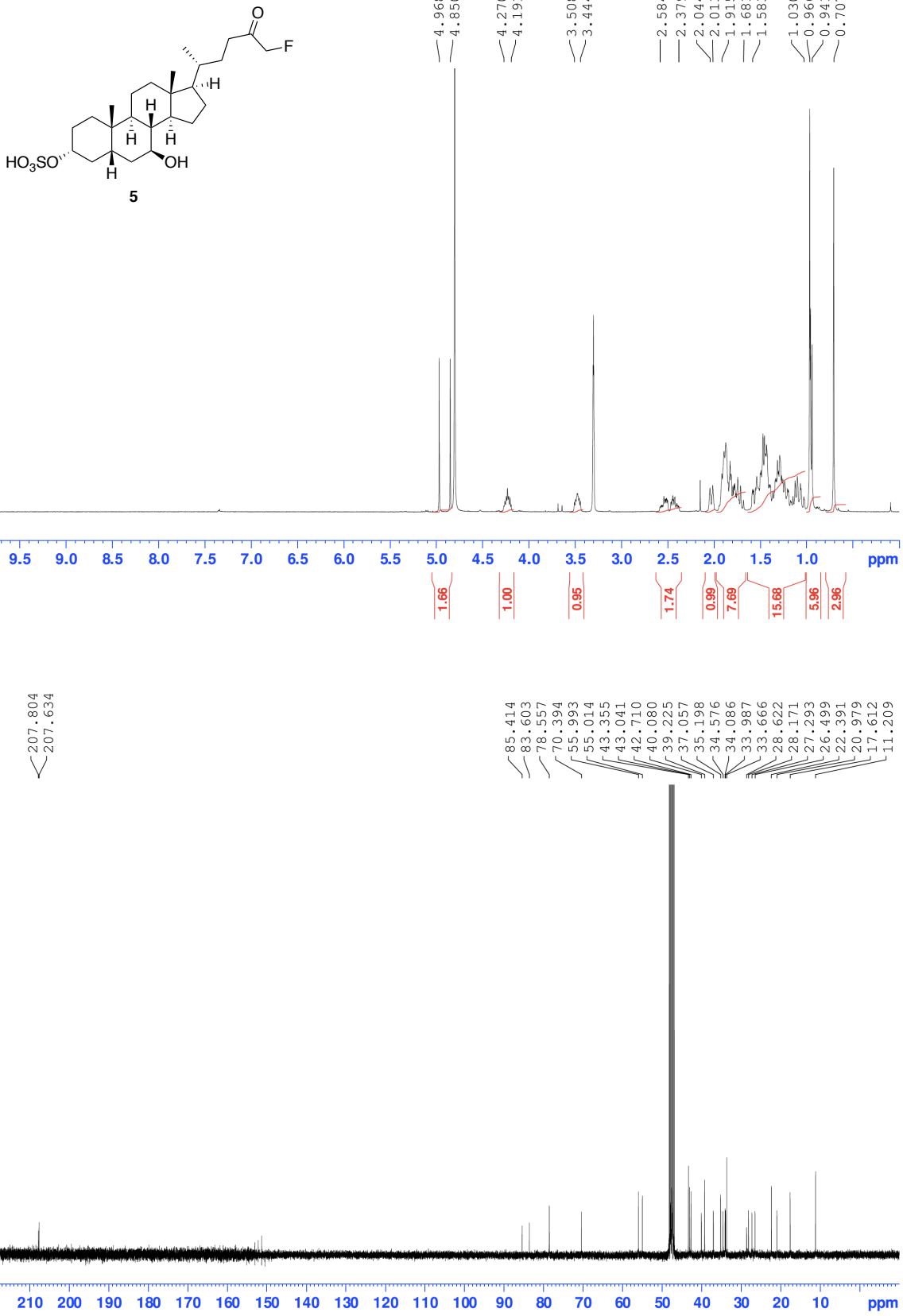
O
F
H
H
H
H
HO3SO
OH
H
5
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4.850
4.270
4.191
3.508
3.444
2.584
2.379
2.044
2.013
1.915
1.681
1.583
1.030
0.966
0.941
0.707
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 ppm
1.66
1.00
0.95
1.74
0.99
7.69
15.68
5.96
2.96
207.804
207.634
85.414
83.603
78.557
70.394
55.993
55.014
43.355
43.041
42.710
40.080
39.225
37.057
35.198
34.576
34.086
33.987
33.666
28.622
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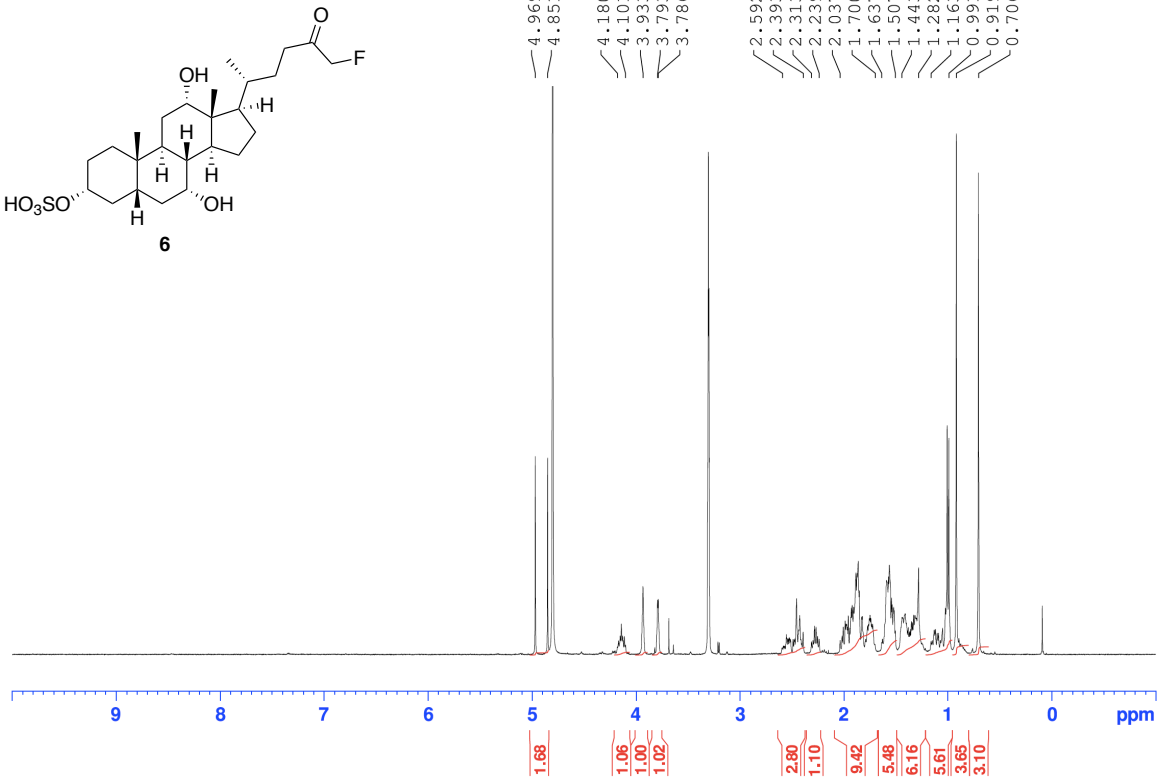
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F
OH
H
H
H
H
HO3SO
OH
H
6
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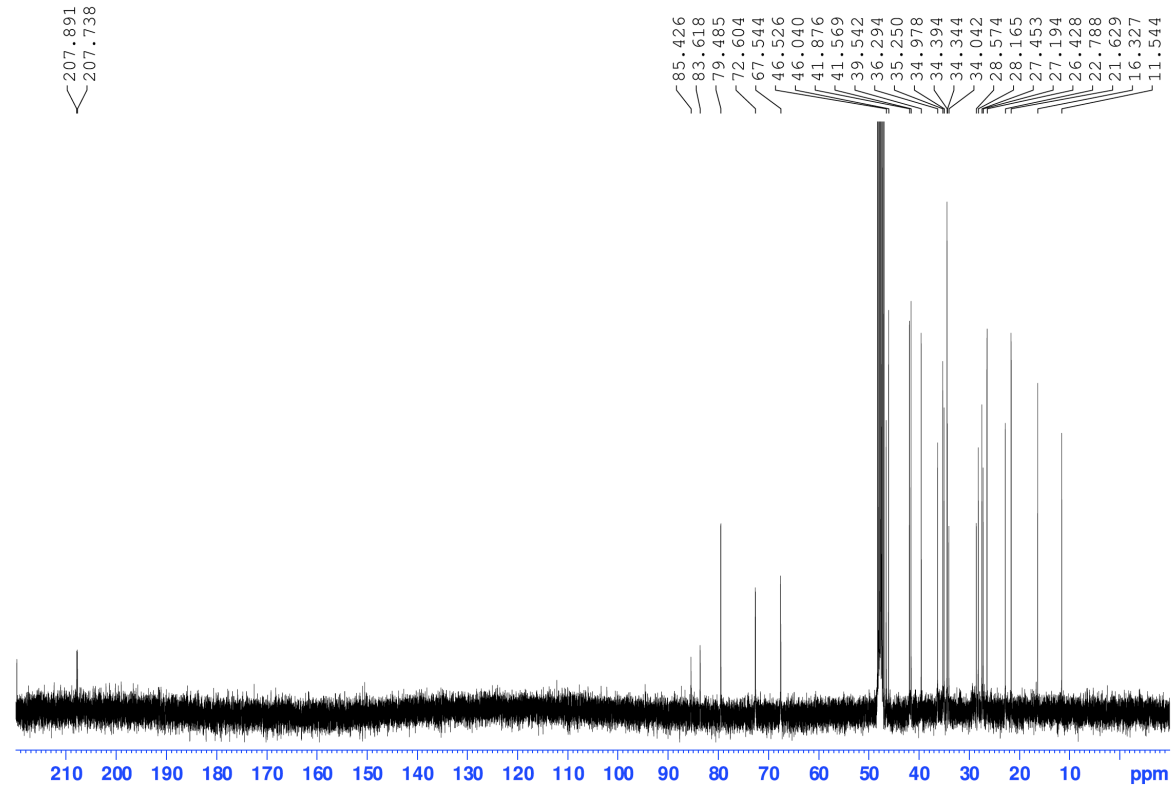
4.851
4.180
4.101
3.933
3.793
3.786
2.592
2.393
2.313
2.239
2.037
1.700
1.637
1.507
1.443
1.282
1.163
0.991
0.919
0.706
9
8
7
6
5
4
3
2
1
0
ppm
1.68
1.06
1.00
1.02
2.80
1.10
9.42
5.48
6.16
5.61
3.65
3.10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35.250
34.978
34.394
34.344
34.042
28.574
28.165
27.453
27.194
26.428
22.788
21.629
16.327
11.544
210
200
190
180
170
160
150
140
130
120
110
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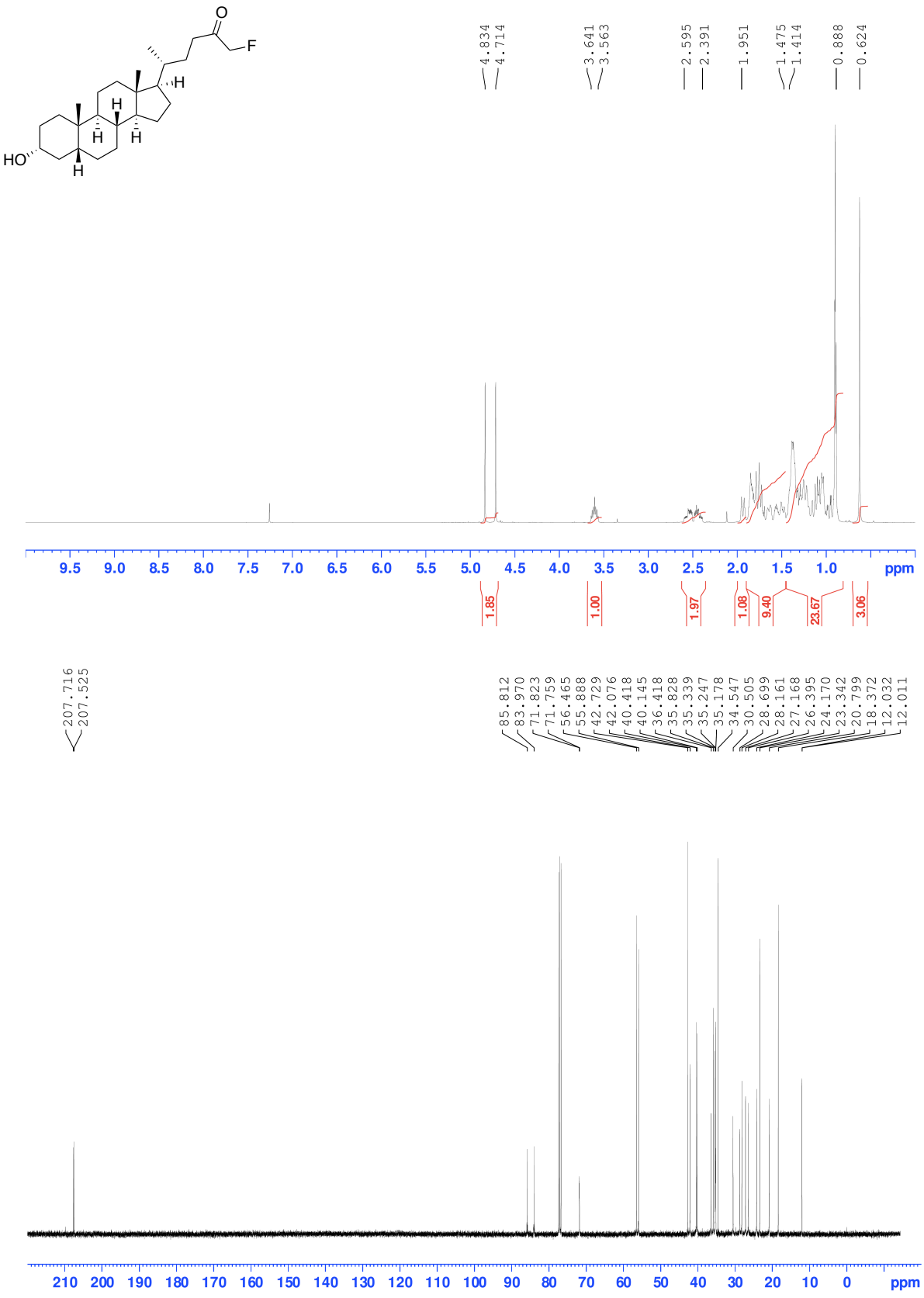
90
80
70
60
50
40
30
20
10
ppm


O
F
H
H
H
H
HO
H
4.834
4.714
3.641
3.563
2.595
2.391
1.951
1.475
1.414
0.888
0.624
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 ppm
1.85
1.00
1.97
1.08
9.40
23.67
3.06
207.716
207.525
85.812
83.970
71.823
71.759
56.465
55.888
42.729
42.076
40.418
40.145
36.418
35.828
35.339
35.247
35.178
34.547
30.505
28.699
28.161
27.168
26.395
24.170
23.342
20.799
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12.032
12.011
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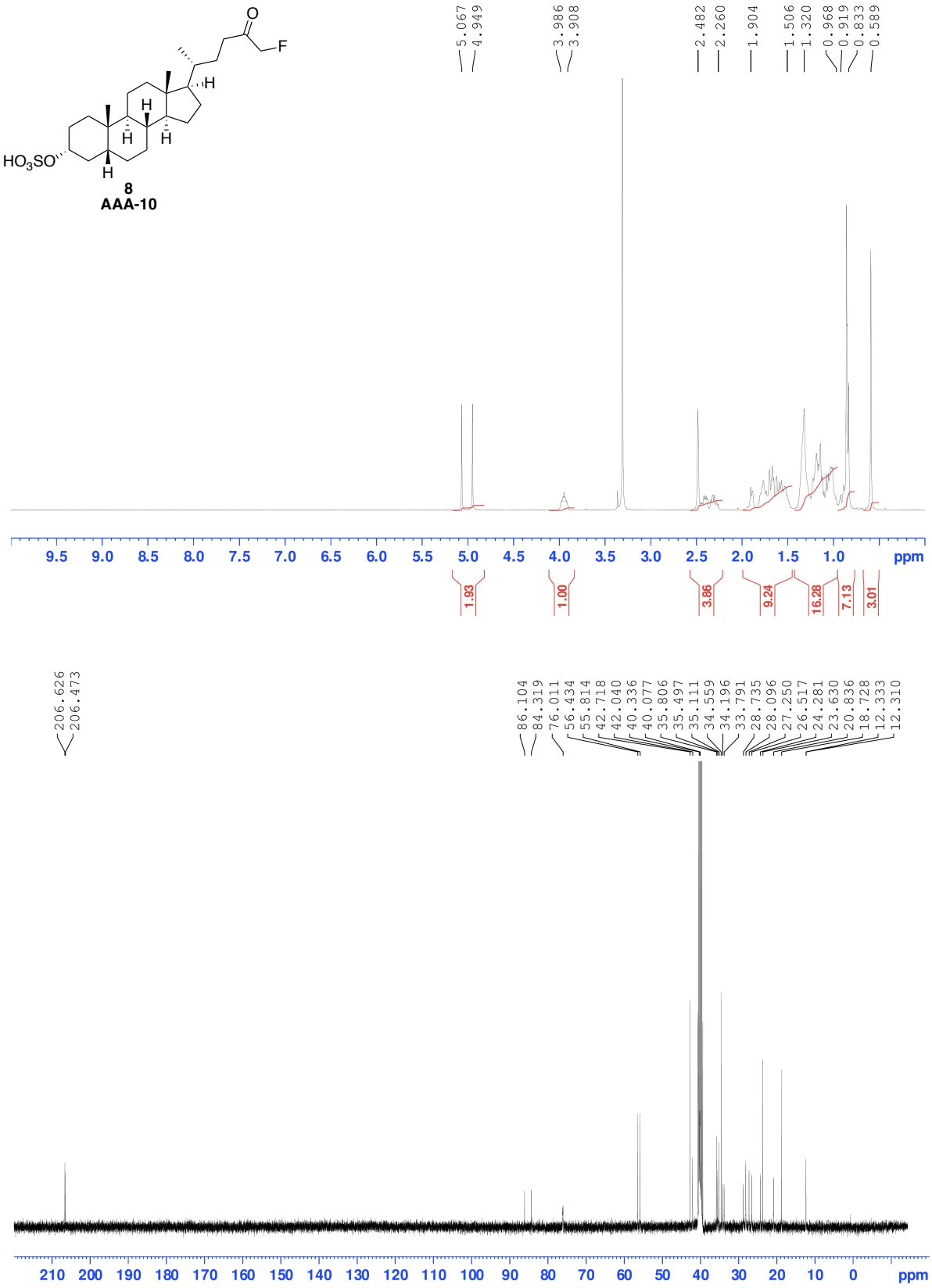

O
F
H
H
H
H
HO3SO
H
8
AAA-10
5.067
4.949
3.986
3.908
2.482
2.260
1.904
1.506
1.320
0.968
0.919
0.833
0.589
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 ppm
1.93
1.00
3.86
9.24
16.28
7.13
3.01
206.626
206.473
86.104
84.319
76.011
56.434
55.814
42.718
42.040
40.336
40.077
35.806
35.497
35.111
34.559
34.196
33.791
28.735
28.096
27.250
26.517
24.281
23.630
20.836
18.728
12.333
12.310
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 ppm

Supporting Tables

Table S1. Comparison of IC_{50} values against recombinant BSHs

Inhibitor	*B. theta* rBSH	*B. longum* rBSH
Compound **7 (AAA-1)**	427 nM	108 nM
GR-**7 (AAA-2)**	2638 nM	1265 nM
AAA-10	10 nM	80 nM

Supporting Figures

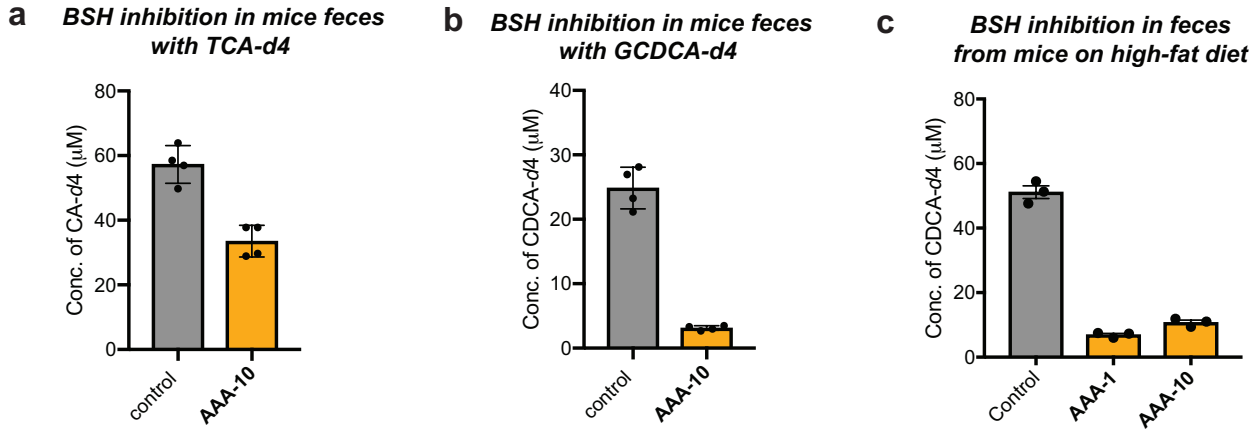


Figure S1. BSH inhibitor efficacy and stability in conventional mouse fecal slurry. a and **b, AAA-10** inhibited BSH activity when either (a) TCA-*d*4 or (b) GCDCA-*d*4 was used as substrate. **AAA-10** was tested at a concentration of 20 μM. **c,** Screen in feces obtained from conventional mice fed a high-fat diet showed that **AAA-10** inhibited BSH activity with similar potency compared to the first-generation unsulfonated inhibitor, **AAA-1**. Inhibitors were tested at a concentration of 20 μM. All assays were performed in biological triplicate, and data are presented as mean ± s.e.m.

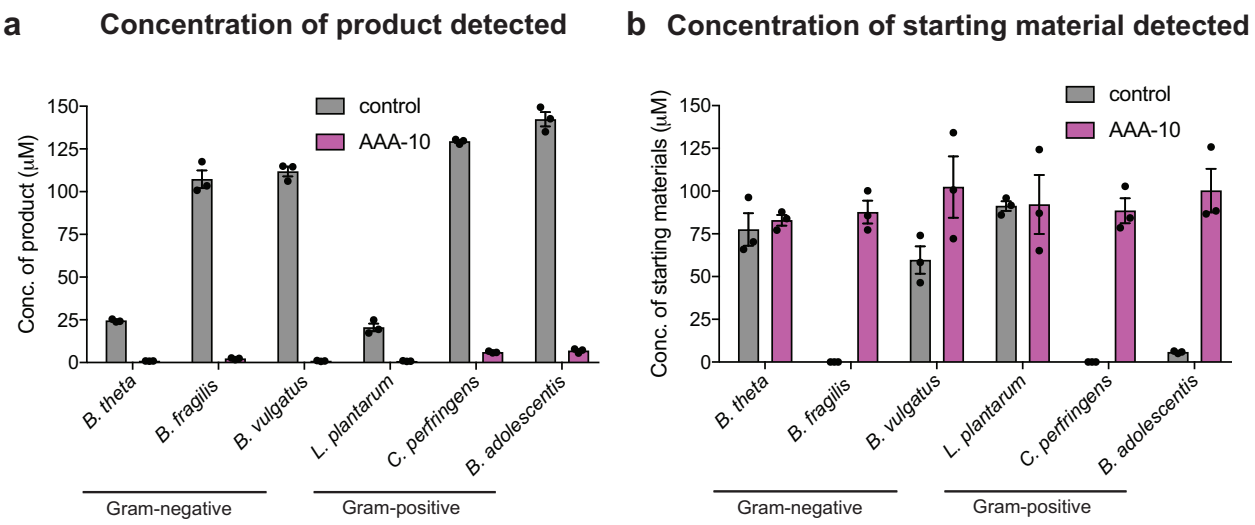


Figure S2. Absolute bile acid concentrations for determining % deconjugation in bacterial culture assays. a, Concentration of products formed (deconjugated bile acids) and **b,** unreacted starting materials in each culture were determined using UPLC-MS. Percent deconjugation for each sample was then determined using the following equation:

% deconjugation = Concentration of products / (Concentration of products + Concentration of starting materials) * 100.
Note that for 5 of the 6 bacteria tested, no bile acids were detected in the cultures other than the starting materials (TCA, TβMCA, TUDCA and TDCA) and their deconjugated products (i.e., CA, βMCA, UDCA and DCA). For *B. fragilis*, these bile acids and one additional compound, 7-oxo-cholic acid (7-oxo-CA), were detected. 7-oxo-CA was quantified and included in the concentration of products in (**a**). Assays were performed in biological triplicate, and all data are presented as mean ± s.e.m.

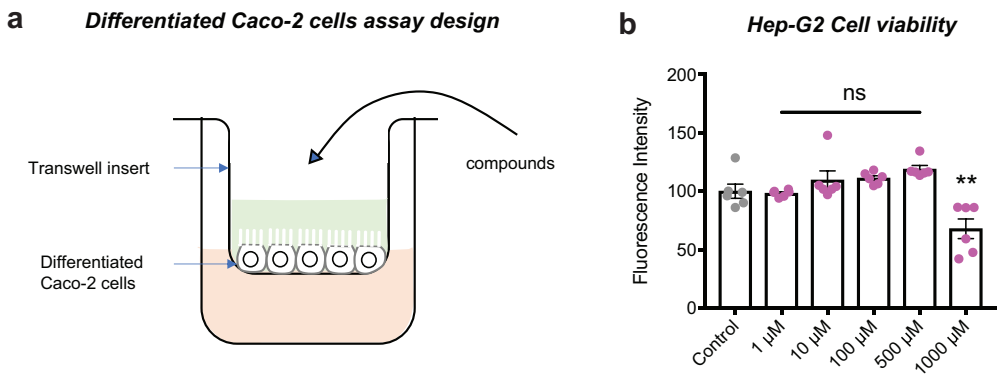


Figure S3. Transwell assay design and AAA-10 toxicity. a, Caco-2 cells were differentiated to form a monolayer with tight junctions. Compounds were then added to the apical side and their transport to the basolateral side was quantified by UPLC-MS. **b,** Incubation of Hep-G2 cells with **AAA-10** did not result in toxicity up to 500 µM. For **b,** one-way ANOVA followed by Dunnett's multiple comparisons test was performed, n=6, and data are presented as mean ± s.e.m.

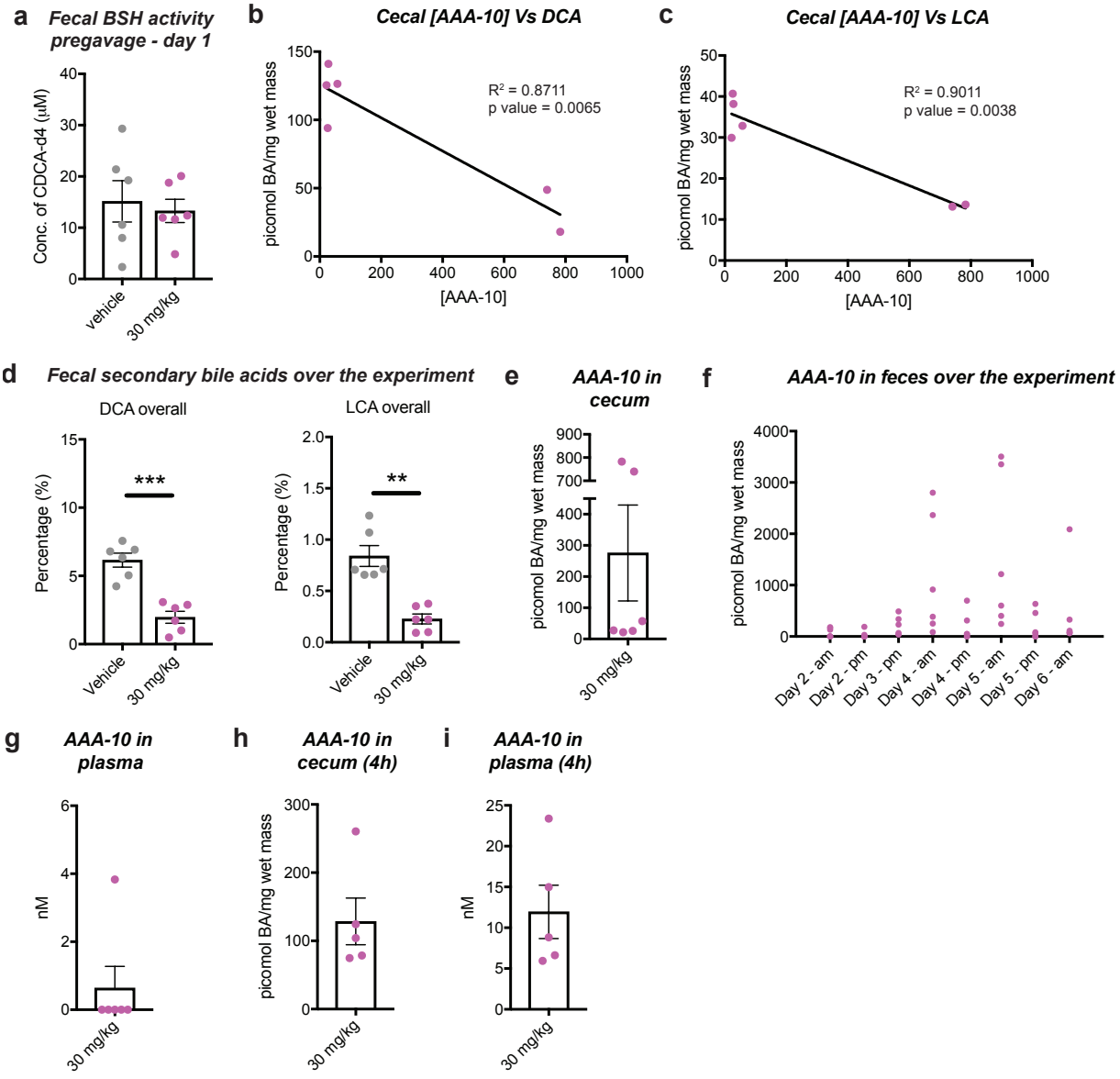


Figure S4. AAA-10 inhibited BSH activity and reduced of secondary bile acid abundance in vivo. a, There was no difference in BSH activity in feces pre-**AAA-10** treatment. **b** and **c,** Cecal **AAA-10** concentration was inversely correlated with the cecal concentrations of the secondary bile acids DCA and LCA. Each dot represents the concentration of **AAA-10** vs bile acid in each mouse. **d,** The abundances of DCA and LCA in feces collected over the entire period of the experiment were significantly lower in AAA-10-treated mice compared to control-treated mice. Each dot represents a mouse. See methods section for the equation used to calculate these overall abundances. **e**, Quantification of **AAA-10** in mouse cecal contents. **AAA-10** was detected in cecum 16 hours after administration. **f, AAA-10** concentration in feces collected over the period of study. Each dot represents the amount of **AAA-10** in each mouse at a particular timepoint. **g**, Quantification of **AAA-10** in mouse plasma 16h after final gavage. **AAA-10** was detected in the plasma of only 1 mouse indicating that **AAA-10** exhibits minimal systemic exposure. **h** and **i,** Quantification of **AAA-10** in a separate mouse experiment 4h post-gavage. While substantial

amounts of **AAA-10** were detected in mouse cecal contents, trace amounts of **AAA-10** were detected in mouse plasma. For **a-g,** n=6 mice/group. For **b** and **c,** linear regression was performed to determine the R^2 and p value. For **h** and **i,** n=5 mice/group. For **a** and **d,** two-tailed Welch's t test was performed. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns = not significant. All data are presented as mean ± s.e.m.

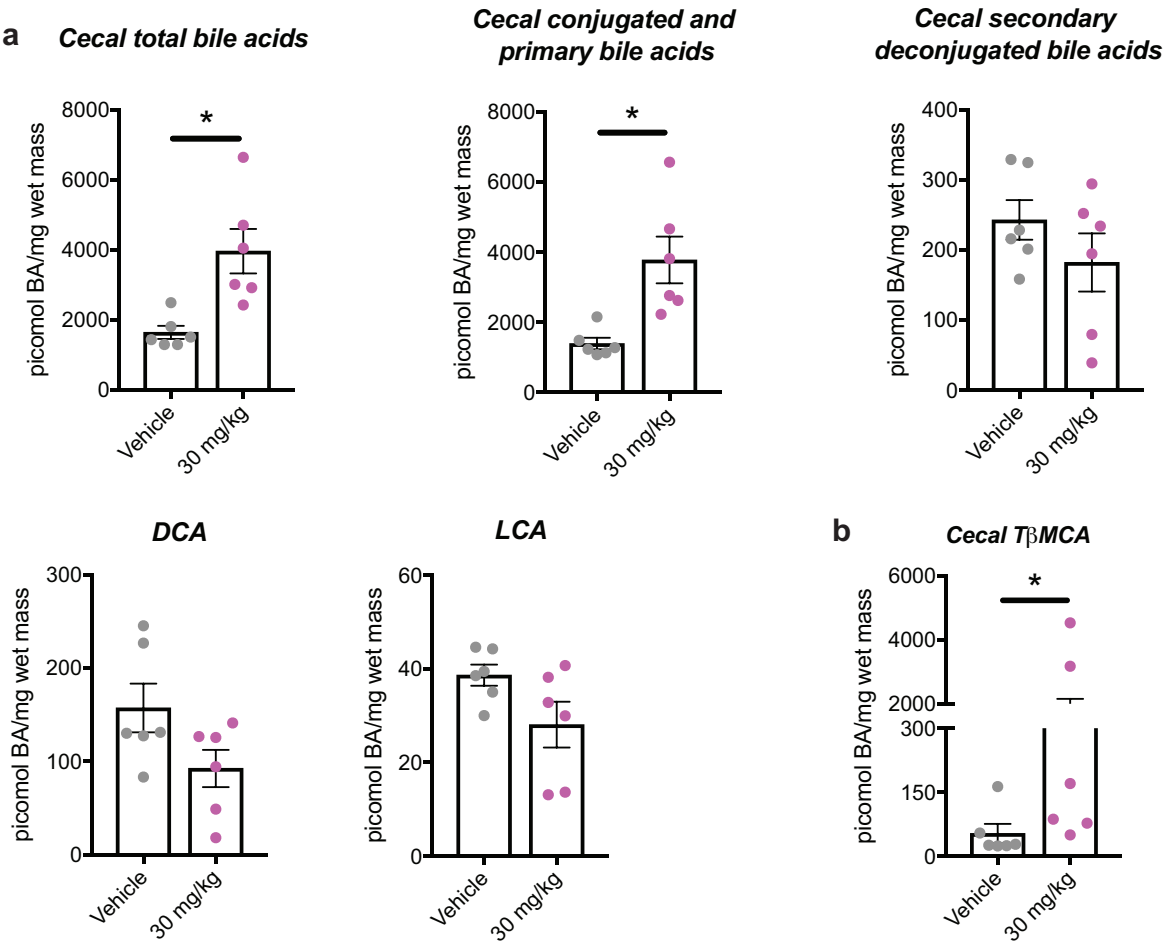


Figure S5. Cecal bile acid concentrations in mice treated with AAA-10. a, Compounds included in the "total bile acid" group were TCA, TβMCA, TωMCA, TUDCA, TCDCA, TDCA, TLCA, CA, βMCA, ωMCA, UDCA, DCA, LCA and CA-7S. Compounds included in the "conjugated and primary bile acids" group were TCA, TβMCA, TωMCA, TUDCA, TCDCA, TDCA, TLCA, CA, βMCA, UDCA, CA-7S. Compounds included in the "secondary deconjugated bile acids" group were wMCA, LCA, DCA. Two-tailed Welch's t tests were performed, *p<0.05, all other comparisons were not significant. **b,** Concentration of TBMCA in cecal contents. Mann-Whitney test was performed because data were non-parametric, *p<0.05. All data are presented as mean ± s.e.m.

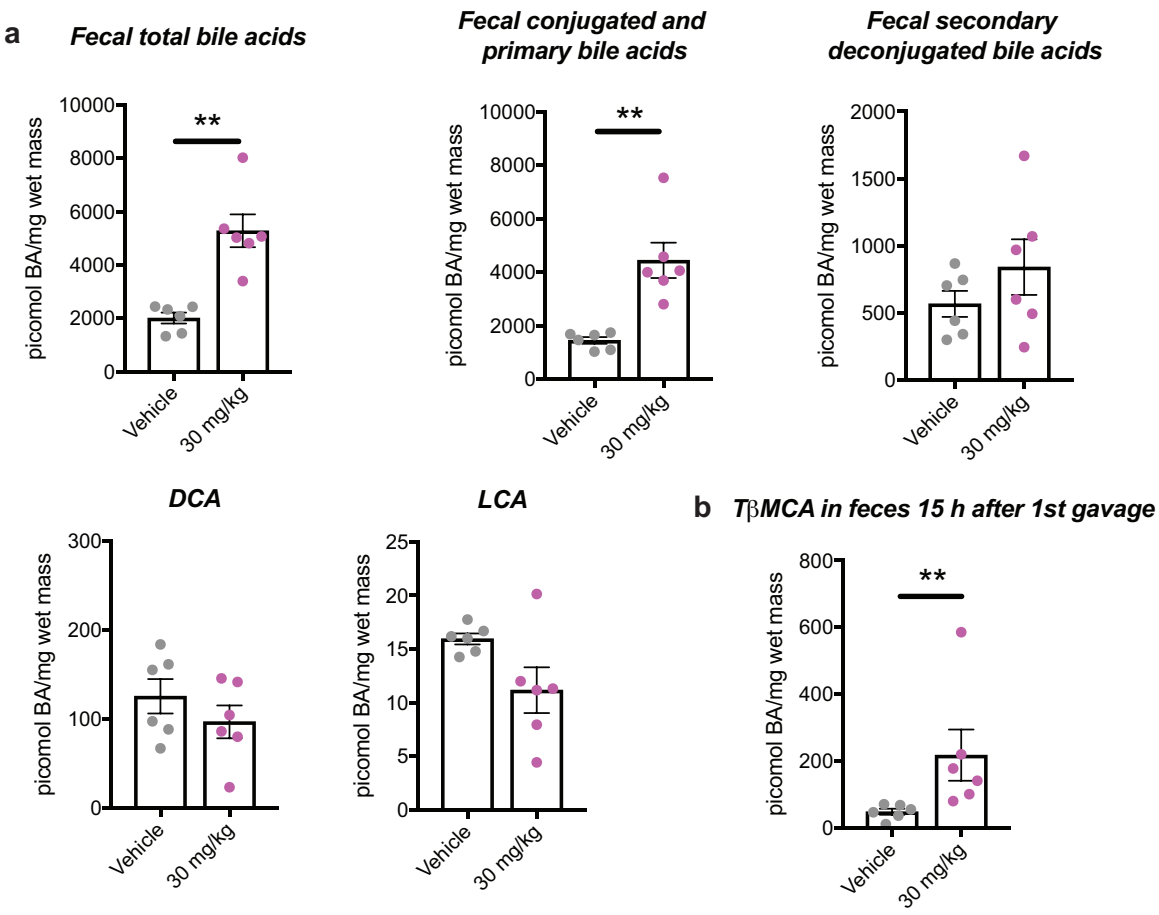


Figure S6. Fecal bile acid concentrations in mice treated with AAA-10. a, Overall fecal bile acid concentrations in mice treated with AAA-10. The bile acids included in the "total bile acids," "conjugated and primary bile acids," and "secondary deconjugated bile acids" groups are the same as listed in Figure S5. See the methods section for the equation used to calculate the overall concentration. Two-tailed Welch's t tests were performed, **p<0.01, all other comparisons were not significant. **b,** Concentration of TβMCA in feces 15 h after the first gavage. Mann-Whitney test was performed because data were non-parametric, **p<0.01. All data are presented as mean ± s.e.m.

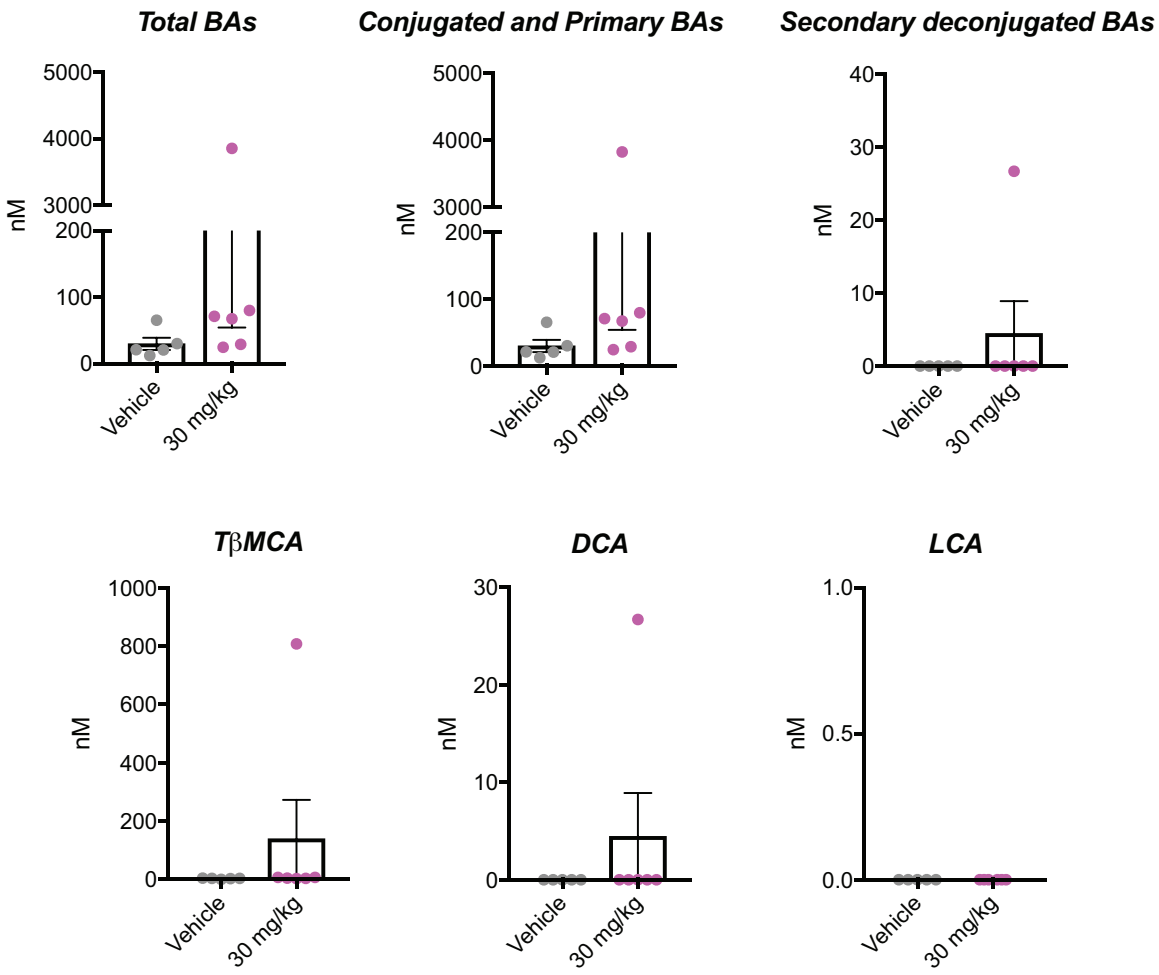


Figure S7. Concentration of bile acids in plasma. No significant differences in plasma bile acid levels were observed between AAA-10-treated and vehicle-treated mice, suggesting that the decrease in cecal and fecal LCA and DCA abundance shown in Figure 6 was not due to increased reabsorption of these bile acids. The bile acids included in the "total bile acids," "conjugated and primary bile acids," and "secondary deconjugated bile acids" groups are the same as listed in Figure S5. Mann-Whitney test was performed because data were non-parametric. All comparisons were not significant. All data are presented as mean ± s.e.m.

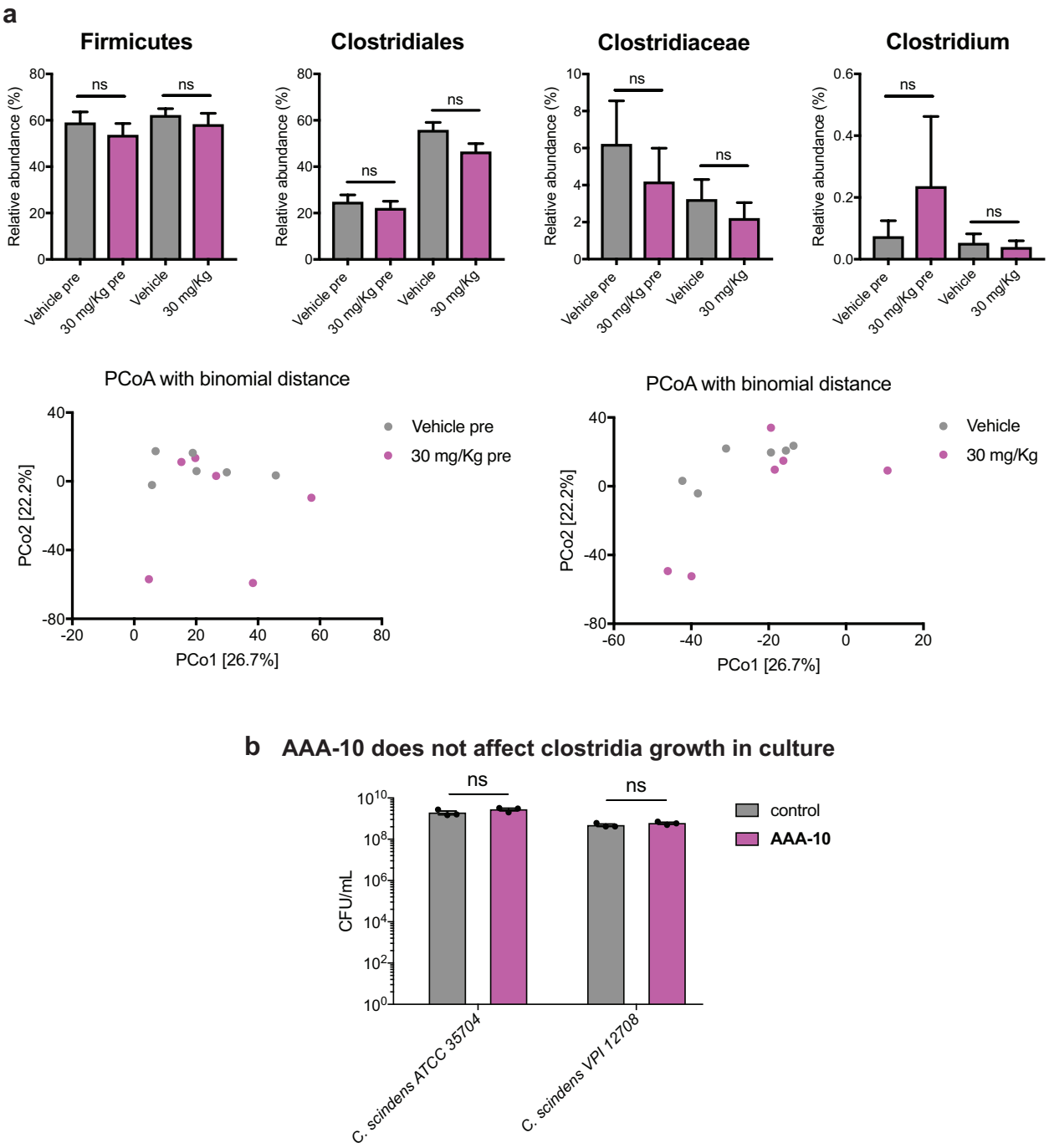


Figure S8. AAA-10 administration in vivo does not result in a shift in the microbiome. a, Relative abundance of phylum Firmicutes, order Clostridiales, family Clostridiaceae, genus *Clostridium* in samples. n=6 mice per group, Vehicle pre and **AAA-10** pre are pre-treatment mouse fecal samples; Vehicle and **AAA-10** are post-treatment mouse cecal samples; ns=not significant, one-way ANOVA followed by Tukey's multiple comparisons test. **b, AAA-10** does not affect cell viability of two *Clostridium scindens* strains which perform 7a-dehydroxylation and produce LCA and DCA. Assay was performed as per the protocol followed for Figure 4b.

For **b,** two-tailed Student's t-test were performed, ns = not significant. Assay was performed in biological triplicate, and data are presented as mean ± s.e.m.

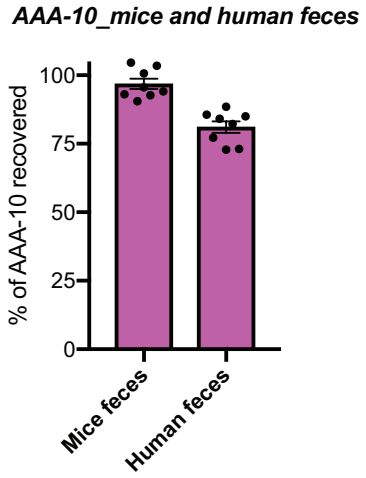
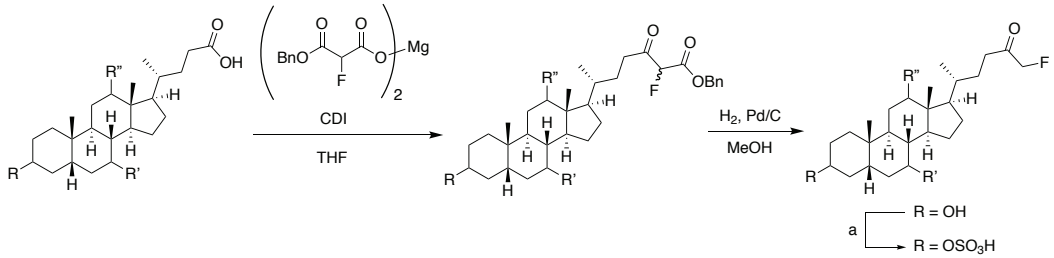


Figure S9. AAA-10 is stable in mice and human feces. AAA-10 was recovered largely unmetabolized after incubation for 18 h in mice (97% mean) and 2 h in human (81% mean) fecal slurry (n = 8 per group). Data are presented as mean ± s.e.m.

Supporting Schemes



Scheme 1: Generic scheme for synthesis of compounds 1-5. CDI = carbonyldiimidazole, a = SO_3.pyridine, pyridine

Scheme S1. Generic scheme for the synthesis of compounds 3-8. CDI – carbonyldiimidazole, a=SO3.pyridine, pyridine.

Supporting References

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