Title: Metabolism and Disposition of Siponimod, a Novel Selective S1P1/S1P5 Agonist, in Healthy Volunteers and In Vitro Identification of Human Cytochrome P450 Enzymes Involved in Its Oxidative Metabolism

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Journal title: Drug metabolism and disposition

Supplemental figure legends:

Supplemental Figure 1. Selective metabolites of siponimod by CYP2C9 as compared with human liver microsomes (HLM) and CYP3A4

The pattern of metabolites in 10 µM [^{14}C]siponimod incubations (30 minutes) using recombinant human CYP isoenzyme (30 pmol/mL) were determined by HPLC with radiodetection. The pattern of HLM (108 pmol CYP/mL) is included for comparison.

CYP2C9, cytochrome P450 2C9 (CYP2C9); HPLC, high-performance liquid chromatography;

M, metabolite.

Supplemental Figure 2. Schematic presentation of the synthesis of the M1, M2 and M8

Supplemental Figure 3. MS and NMR data of the authentic reference compounds: Metabolite M1 (A), Metabolite M2 (B), Metabolite M3 (C), Metabolite M4a (D), Metabolite M4b (E), Metabolite M4c (F), Metabolite M4d (G), Metabolite M5 (H), Metabolite M6 (I), Metabolite M7 (K), Metabolite M17

Supplemental table legends:

Supplemental Table 1. Siponimod and related compounds in mouse plasma following a single oral dose of 25 mg/kg [^{14}C]siponimod referring to base

-, Not detected or below lower limit of quantitation. Patterns of metabolites and the parent compound in plasma after a single oral dose of 25 mg base/kg [^{14}C]siponimod hemifumarate were determined by UPLC analysis with offline radiodetection. UPLC, ultra-performance liquid chromatography.

Supplemental Methods

Preparation of reference compounds by biosynthesis

Preparative scale synthesis of hydroxylated metabolites M5, M6 and M7:

Precultures of the three fungi strains ATCC20034 (*Mortierella vinicae*), DSM1344 (*Beauveria bassiana*) and DSM858 (*Staurophoma sp.*) were grown in 100 mL NL148s medium in 500 mL shake flasks at 28°C and 200 rpm for 2, 3 and 4 days, respectively. The NL148s medium consisted of 0.2% soluble starch, 0.8% soya peptone, 0.4% LabLemco, 0.05% yeast extract, 0.15% NaCl, 0.21% MOPS (3-(N-morpholino)propanesulfonic acid),), adjusted to pH 6.5 and trace element B solution (1 mL/L). The trace element B solution consisted of $Na_2MoO_4.2H_2O$ (30 mg/L), $FeSO_4.7H_2O$ (5.5 g/L), $CuSO_4.5H_2O$ (80 mg/L), $MnCl_2.4H_2O$ (180 mg/L), $ZnSO_4.7H_2O$ (4.4 g/L) and H_2SO_4 97% (2 mL/L). Sterile glycerol was added to each preculture to a final concentration of 16.7% (w/v). The precultures were stored at −80°C until usage to inoculate the main cultures. Due to pellet formation, the precultures of ATCC20034 and DSM858 were homogenized using a single-use tube homogenizer (IKA ULTRA-TURRAX Tube Drive) before adding glycerol.

Each of the 10 mL thawed precultures were then used to inoculate 200 mL medium in a 1 L shake flask. These main inoculates were incubated at 28°C and 200 rpm. In total, five shake flasks of each microbial strain were incubated in parallel, resulting in a biotransformation scale of 1 L per microbial strain. The biotransformation substrate (0.1 mg/mL) was added to each flask after 1 day of incubation. In addition, glucose was added to the biotransformation mixtures with DSM1344 and ATCC20034 to a final

concentration of 10 mg/L. The biotransformation process was stopped at 34 h for the DSM1344 and ATCC20034 strains and at 72 h for the DSM858 strain by freezing the whole mixture at −20°C. The mixtures were stored at −20°C until further processing. During the biotransformation process, samples were taken periodically and analyzed by high-performance liquid chromatography (HPLC).

The biotransformation mixtures were thawed at room temperature and approximately 100 g/L NaCl was added to each flask. The aqueous biotransformation mixtures were then acidified using phosphoric acid and extracted three times with ethyl acetate. For each microbial biotransformation, the ethyl acetate extracts were combined and evaporated to a final volume of approximately 20 mL. The concentrate was mixed with diatom granulates (Isolute HM-N, Biotage) in order to absorb the biotransformation substrate and product molecules, and was further evaporated to dryness. The biotransformation products were eluted with 300 mL of methanol/isopropyl alcohol mixture and dried by vacuum evaporation. The residues were reconstituted in 40 mL methanol/H_2O and were applied on a reverse phase chromatography for isolation of hydroxylated siponimod metabolites.

The preparative high-performance liquid chromatography/mass spectroscopy (HPLC/MS) system consisted of a Waters autopurification liquid chromatograph, a DAD UV detector (210-350 nm) and a WATERS ZQ series mass detector equipped with ESI interface. The mass detector was operated in the positive an electrospray ionization (ESI) mode. The components were separated on a Sunfire Prep C18 column (5 µM; 21.0 × 150 mm). Elution was performed with a gradient of aqueous ammonium acetate, pH 4.5 (mobile phase A) and acetonitrile (mobile phase B) at a flow rate of 20.00 mL/min.

Using the ATCC20034 strain, the final isolated yields for M5, M6 and M7 were 34%, 11% and 33%, respectively. The corresponding yields using the DSM1344 strain were 11%, 12% and 2%, respectively. Biotransformation using the DSM858 strain yielded only M5 (33%) and M6 (5%).

Preparation of glucuronide M3:

The hydroxylated metabolite M5 was used as the starting material for the preparative synthesis of the glucuronide M3. To obtain sufficient amounts of starting material, a large scale biosynthesis of M5 was performed in a wave bag fermenter. Biotransformation of siponimod (4 g) using the microbial strain DSM875 (*Beauveria bassiana)* under the same conditions as described before yielded 1.050 g of M5. Glucuronidation of M5 (700 mg) was performed by using a dog liver S9 preparation as the biocatalyst in 160 mmol/L HEPES buffer, pH 8.5. The reaction mixture also consisted of $MgCl_2$ and UDP glucuronic acid at a final concentration of 20 mmol/L and 40 mmol/L, respectively. The biotransformation was performed on a 1 L scale in centrifuge tubes on an orbital shaker for 24 h. After 24 h, the biotransformation mixture was extracted three times with ethyl acetate. The combined ethyl acetate extracts were mixed with Isolute HM-N in order to absorb the biotransformation products and were then evaporated until dryness. The Isolute HM-N was transferred to a cartridge and connected to a HPLC column. The components were separated on a RP18 column using 0.05% trifluoroacetic acid (TFA) and a 25–100% methanol gradient.

The purity of all the metabolites was determined using liquid chromatography mass spectroscopy (LC/MS) and ultra-performance liquid chromatography (UPLC) coupled

with ultraviolet (UV) detection. The structures of the metabolites obtained by biotransformation were determined by NMR (HRMS, 13C HSQC, 1H, ROESY, COSY).

Preparation of sulfates M4a, M4b and M4c:

The sulfates M4a, M4b and M4c were synthesized from the hydroxylated siponimod metabolites M5, M7 and M6, respectively. M5, M6 and M7 were synthesized as described before.

The sulfates were synthesized from the hydroxylated siponimod metabolites according to the following procedure: 0.05 to 0.25 mmol of the starting material were dissolved in 10 to 20 mL dry dimethyl formamide and stirred with a magnetic stirrer in a 60°C water bath. To this solution 3 mol-equivalents of NEt3-SO3 complex were added and the reaction was stirred for 15 to 60 minutes. Then the reaction was quenched with 20 mL MeOH. The quenched reaction was directly pumped onto a RP18 chromatography column representing 20% (v/v) of the total flow, while 80 % (v/v) consisted of ammonia acetate buffer 10 mmol/L (pH 5.5) at a flow rate of 90 mL/min. The conditions for preparative HPLC were: Stationary phase: Self-packed RP18 50*250 mm column; solvent A: aqueous ammonium acetate buffer 10 mmol/L pH 5.5; solvent B: acetonitrile; gradient: 0-5 minutes 35% B, 3 minutes 65% B; room temperature; detection at UV252 nm; and fraction size 50 mL. Fractions containing the product were combined, the solvents were evaporated under reduced pressure to a final volume of about 50 mL and the product was finally dried by lyophilisation overnight. The isolated yields of the sulfates varied between 9 and 50%.

Preparation of reference compounds by chemical synthesis

The schematic presentation of the synthesis of M1, M2 and M8 is presented in **supplemental figure 2.**

Preparation of the metabolite M1

Manganese dioxide (8.8 eq, 49.3 mmol) was added to a solution of siponimod-B8 (1 eq, 5.61 mmol) in heptane (7 ml) at room temperature. The black suspension was then heated at 50°C for 2 h. Subsequently, the mixture was filtered and the resulting filtrate was concentrated to dryness to yield 760 mg of 4-acetyl-2-ethylbenzaldehyde as a yellow oil (yield 73%).

4-acetyl-2-ethylbenzaldehyde (1 eq, 3.56 mmol) and azetidine-3-carboxylic acid (1.2 eq, 4.27 mmol) were stirred in methanol (12 mL) for 1 h at room temperature. Thereafter, sodium triacetoxyborohydride (1.2 eq, 4.26 mmol) was added and the reaction mixture was stirred overnight at room temperature. Purification by reverse phase chromatography using a preparative HPLC system, C18 column, eluting from 5% to 100% of acetonitrile in 25 minutes yielded 480 mg of 1-(4-acetyl-2-ethylbenzyl)azetidine-3-carboxylic acid (M1, Yield 51%).

Preparation of the metabolite M2

A solution of 1-(4-acetyl-2-ethylbenzyl)azetidine-3-carboxylic acid (1 eq, 0.727 mmol) in tetrahydrofuran (3 ml) was mixed with hydroxylamine 50% solution in water (10 eq, 7.3 mmol). The reaction mixture was stirred at room temperature for 4 h. Purification by reverse phase chromatography using a preparative HPLC system, C18 column, eluting from 0 to 100% of acetonitrile in 25 minutes yielded 134 mg of (E)-1-(2-ethyl-4-(1-(hydroxyimino)ethyl)benzyl)azetidine-3-carboxylic acid as a white lyophilisate (TFA salt) (M2, Yield 47%).

Preparation of the metabolite M8

To a solution of 1-(4-acetyl-2-ethylbenzyl)azetidine-3-carboxylic acid (1 eq, 0.727 mmol) in methanol (3 ml) was mixed under argon atmosphere with sodium tetrahydridoborate (2 eq, 1.45 mmol) at room temperature. The reaction mixture was stirred for 1h. Purification by reverse phase chromatography using a preparative HPLC system, C18 column, eluting from 0% to 100% of acetonitrile in 25 minutes yielded 87 mg of 1-(2-ethyl-4-(1-hydroxyethyl)benzyl)azetidine-3-carboxylic acid (M8, Yield 32%).

Preparation of the cholesterol ester conjugate M17

Esterification of siponimod with cholesterol was achieved by N, N'-diisopropylcarbodiimide (DIC) and 4-(dimethylamino)pyridine (DMAP) as coupling agents. The crude reaction product was subjected to RP8-HPLC-purification.

Determination of cholesterol ester conjugate (M17) in human plasma

The presence of M17, a siponimod cholesterol ester conjugate, identified as a major circulating metabolite in mice (Supplemental Table 1), was confirmed in an absolute bioavailability study with a single oral dose of 0.25 mg of siponimod in healthy subjects. Quantitative analysis of this metabolite in human plasma was carried out by using validated LC-MS/MS methods with lower limit of quantitation (LLOQ) of 0.100 ng/mL. Details of the analytical methods are presented below.

Structural characterization of metabolite M17

The structure of M17 was characterized by using LC/MS-MS in the positive ionization mode and comparison with the available authentic standard. A fully validated LC/MS-MS method

was used for the determination of M17 in the human plasma samples collected from this absolute bioavailability study. The overall bias (%) and coefficient of variation (CV; %) values were, respectively, in the range of –2.0 to 1.8% (bias) and 3.5 to 6.6% (CV) for the calibration standards (0.100 to 50 ng/mL). For quality control (QC) samples, the obtained precision and accuracy values ranged, respectively, from 3.8 to 7.3% (CV) and –2.8 to 0.3% (bias) for the QCs at the four concentration levels (0.3, 2.5, 15 and 37.5 ng/mL) evaluated.

Sample preparation and determination using a validated LC- MS/MS method

A plasma sample of 100 µL was added into a 2.2 mL 96-well assay plate (*Eppendorf*). For the double blank and QC0 (zero sample) samples, a 100 µL of blank plasma was added. A 20 µL aliquot of the internal standard working solution (15.0 ng/mL [D7]M17 in acetonitrile/dimethyl sulfoxide (1/1, v/v)) was added to all samples with the exception of the double blanks, to which a 20 µL aliquot of acetonitrile/dimethyl sulfoxide (1/1, v/v) was added. The plate was centrifuged at 2000 rpm for 1 minute at 20°C, then vortex-mixed for 15 minutes. A 50 µL aliquot of 40% formic acid in water (v/v) was added into each well. The plate was centrifuged at 2000 rpm for 1 minute at 20°C, then vortex-mixed for 15 minutes. A 600 µL aliquot of 1% formic acid in acetonitrile (v/v) was added into each well. The plate was vortex-mixed for 10 min, then centrifuged at 4000 rpm for 10 minutes at 4°C. A sample of 500 µL of the resulting supernatant was transferred into a new 2.0 mL 96-well assay plate (Axygen), which was followed by centrifugation at 4000 rpm for 10 minutes at 4°C. A 400 µL sample of the above 500 µL sample extract was transferred into another new 2.0 mL 96-well assay plate (Axygen).

Liquid chromatography experimental conditions

The plasma sample extracts were analyzed by LC-MS/MS via multiple reaction monitoring using positive ESI as the ionization technique. The liquid chromatography was performed on an Agilent 1260 (Agilent Technologies, Santa Clara, CA, USA) system. A Sciex API5000 mass spectrometer (Sciex, Forest City, CA, USA)equipped with an ESI source was used for the MS/MS detection. Gradient chromatographic elution was performed on an ACE 3 C4 column (50 × 2.1 mm). The mobile phase consisted of 5 mM ammonium acetate in water (mobile phase A) and 100% acetonitrile (mobile phase B). The injection volume was 15 µL and the column was maintained at 50°C.

Supplemental results

MS and NMR data of the authentic reference compounds used in the study

The MS and NMR data of the authentic reference compound used in the study are presented in **Supplemental Figure 3**.

Determination of cholesterol ester conjugate (M17) in a mouse ADME study

In a mouse ADME study (single oral dose of 25 mg/kg ^{14}C-siponimod, specific radioactivity 2 megabecquerel [MBq] per mg referring to free base) a nonpolar long-lived metabolite M17 was identified (Supplemental Table 1). The major plasma metabolite was identified as a cholesterol ester of siponimod by LC-MS or LC-MS/MS and the chemical structure was confirmed by comparison of the retention time and mass spectral data with a reference standard.

Determination of metabolite profiles in plasma in the mouse ADME study

For metabolite profiling in mouse plasma, the plasma of three mice per time point were pooled. Each plasma pool was extracted five times with acetonitrile and one time with dimethyl

sulfoxide (DMSO) in order to reach recoveries between 82 and 100%. The extracts were combined and evaporated to dryness. The residues were extracted three times using water/acetonitrile/DMSO mixtures in different ratios. About 90% of the radioactivity could be reconstituted.

The chromatography was performed on an UPLC system (Waters Corporation, Manchester, UK). The components were separated at 40°C on an ACQUITY UPLC BEH300 C4 1.7µm analytical column (2.1 × 100 mm; Macherey-Nagel, Switzerland) protected by a 2.1 × 5 mm guard column of the same stationary phase. Volumes up to 40 µL were injected. Elution was carried out with a gradient of ammonium acetate 25 mM in water (pH 5.4; mobile phase A) and acetonitrile (mobile phase B) at a flow rate of 0.9 mL/min. The HPLC effluent directed into the electrospray LC-MS interface and the larger portion was directed to UV detection followed by online or offline radioactivity detection. For offline radioactivity detection, the Perkin Elmer, Gronningen, The Netherlands). The fractions were evaporated to dryness and the radioactivity was counted in a TopCount NXT microplate scintillation counter (Packard Instruments, Meriden, CT, USA).

Concentrations in plasma of siponimod and its metabolites were estimated from the radiochromatograms, based on the relative peak areas and the concentrations or amounts of radioactivity in the original biological samples, reduced by the losses during sample processing.

Structural characterization of metabolite M17 in the mouse ADME study

The [^{14}C] molecular ion at $[M+H]^+$ 885 had a mass shift of +368 Da compared with siponimod. Accurate mass data indicated a gain of $C_{27}H_{44}$. MS/MS data yielded a fragment at m/z 517 (consistent with the $[M+H]^+$ of siponimod) and m/z 416 (Fragment A) (**Figure 3A)**. The

structure of the metabolite was confirmed to be a cholesterol ester of siponimod by co-injection of reference standard for metabolite M18 and comparison of retention time, mass spectrometry and MS/MS data.

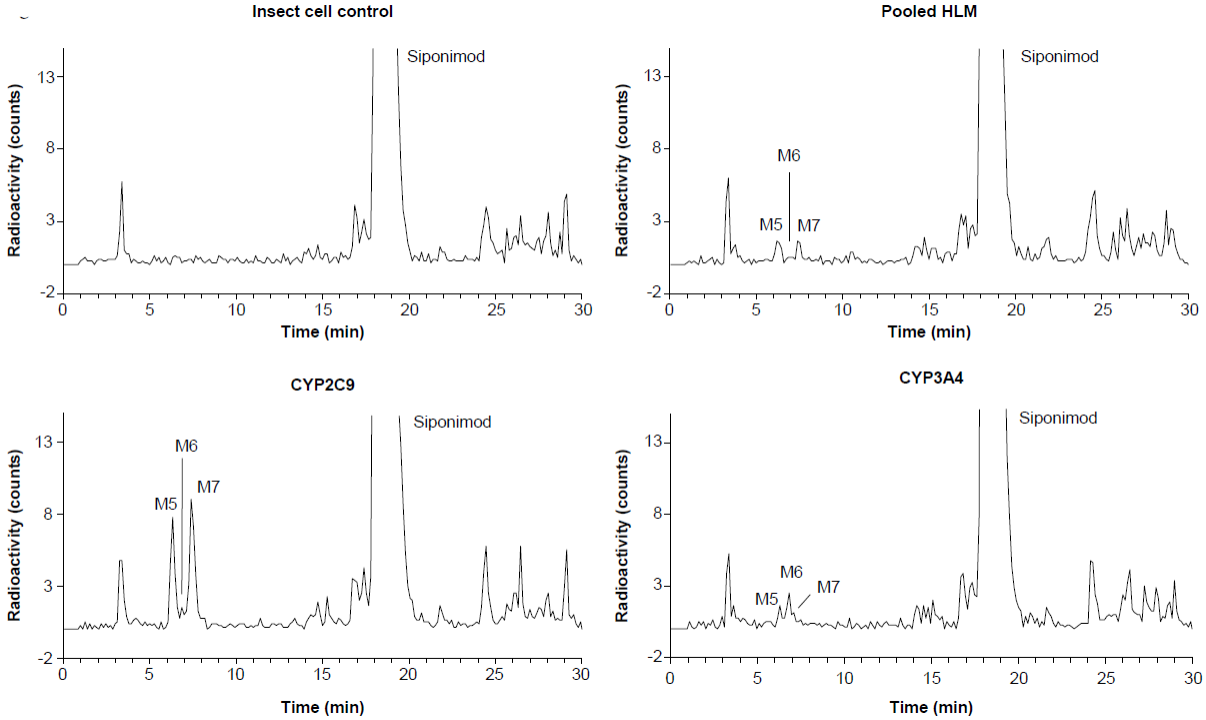
Supplemental Tables

Supplemental Table 1

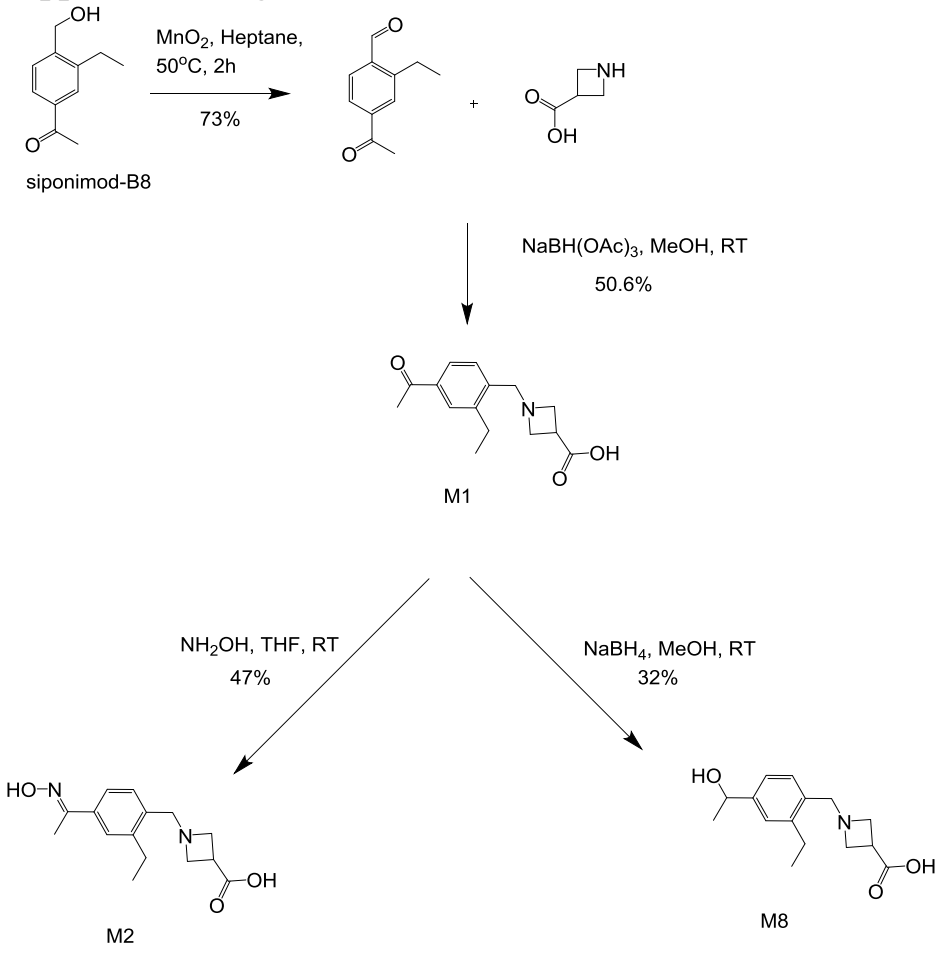
Experiment	Mouse 25 mg/kg single oral dose				
Sample type	Plasma pools (n=3 mice/time point)				
Sample collection time (h)	8	96	168	AUC_{0-168h}	
Concentration		µmol/L		h.µmol/L	(%)
Component					
Siponimod	9.77	0.781	0.285	542	66.1
P73.0	0.0783	0.0563	-	8.26	1.01
P12.3	-	0.0298	-	2.38	0.291
M17	1.60	1.19	0.541	191	23.3
Sum of additional components	0.241	-	-	11.5	1.41
Total detected	11.7	2.05	0.826	755	92.1
Lost during sample processing	0.449	0.457	0.185	64.8	7.90
Total radiolabeled	12.1	2.51	1.01	820	100

Experiment components in original sample	Mouse 25 mg/kg single oral dose

Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3

A. Metabolite M1

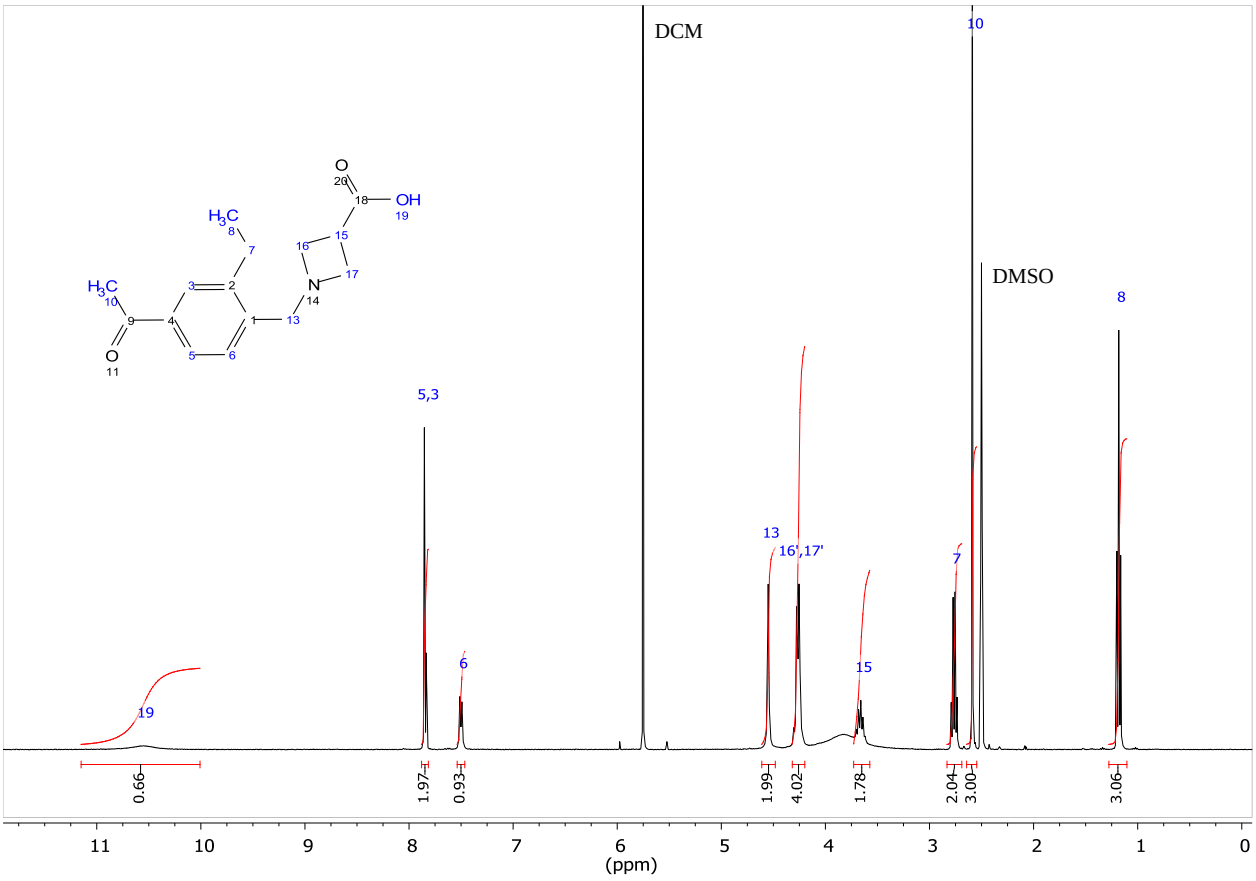
Molecular Formula: $C_{15}H_{19}NO_3$

Average Mass: 261.32

Monoisotopic Mass: 261.13649

High resolution mass spectrometry (HRMS) (ESI/Q-TOF) m/z: $[M + H]^+$ Calculated for $C_{15}H_{20}NO_3$ 262.1438; Found 262.1437.

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.56 (s, 1H), 7.88 – 7.81 (m, 2H), 7.50 (d, *J* = 8.0 Hz, 1H), 4.55 (s, 2H), 4.32 – 4.20 (m, 4H), 3.66 (p, *J* = 8.6 Hz, 1H), 2.76 (q, *J* = 7.5 Hz, 2H), 2.59 (s, 3H), 1.18 (t, *J* = 7.5 Hz, 3H).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(ppm)

B. Metabolite M2

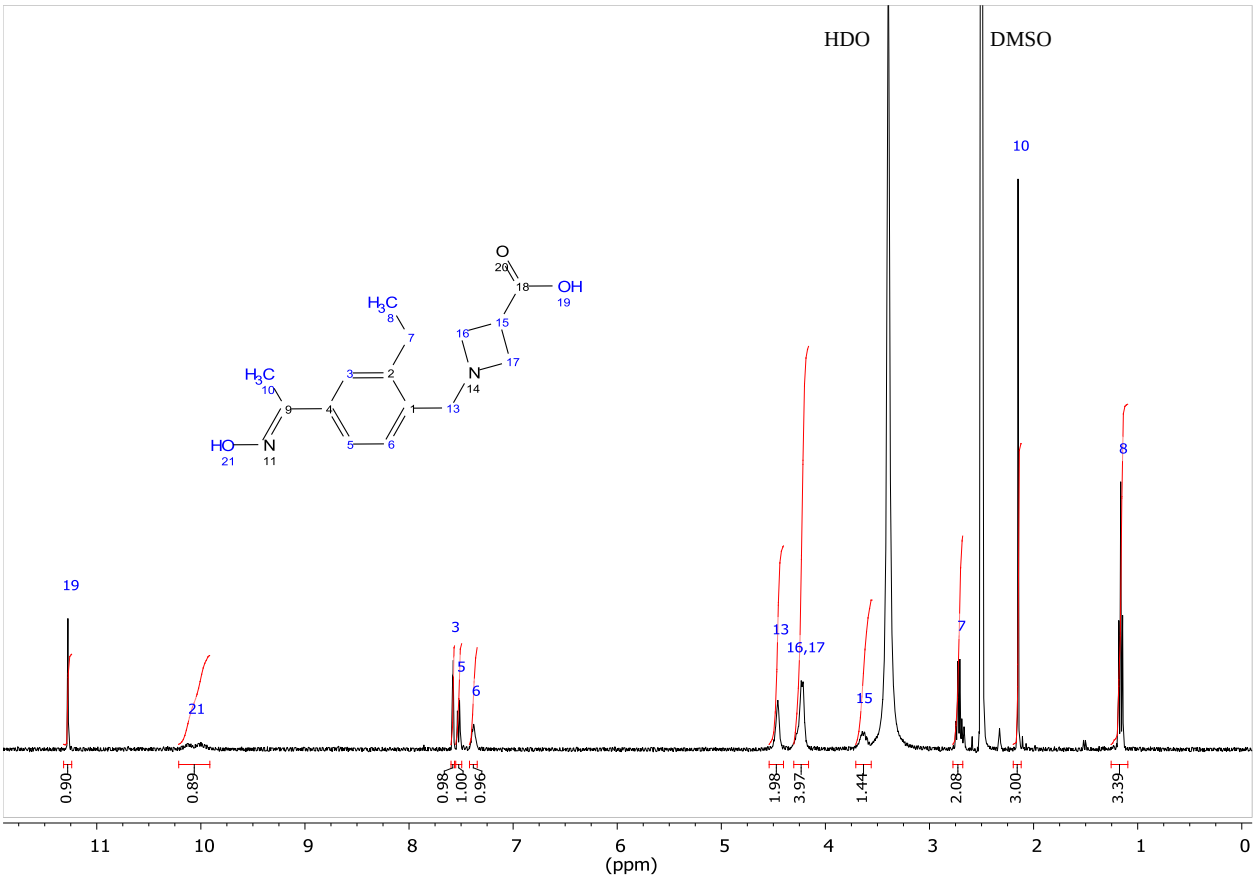
Molecular Formula: $C_{15}H_{20}N_2O_3$

Average Mass: 276.33

Monoisotopic Mass: 276.14739

HRMS (ESI/Q-TOF) m/z: $[M + H]^+$ Calculated for $C_{15}H_{21}N_2O_3$ 277.1547; Found 277.1585.

^{1}H NMR (400 MHz, DMSO-d_6) δ 13.17 (s, 1H), 11.28 (s, 1H), 10.21 – 9.91 (m, 1H), 7.58 (d, J = 1.9 Hz, 1H), 7.52 (dd, J = 8.0, 1.9 Hz, 1H), 7.42 – 7.34 (m, 1H), 4.46 (s, 2H), 4.30 – 4.16 (m, 4H), 3.63 (s, 1H), 2.72 (q, J = 7.5 Hz, 2H), 2.15 (s, 3H), 1.16 (t, J = 7.5 Hz, 3H).



C. Metabolite M3

Molecular Formula: $C_{35}H_{43}F_3N_2O_{10}$

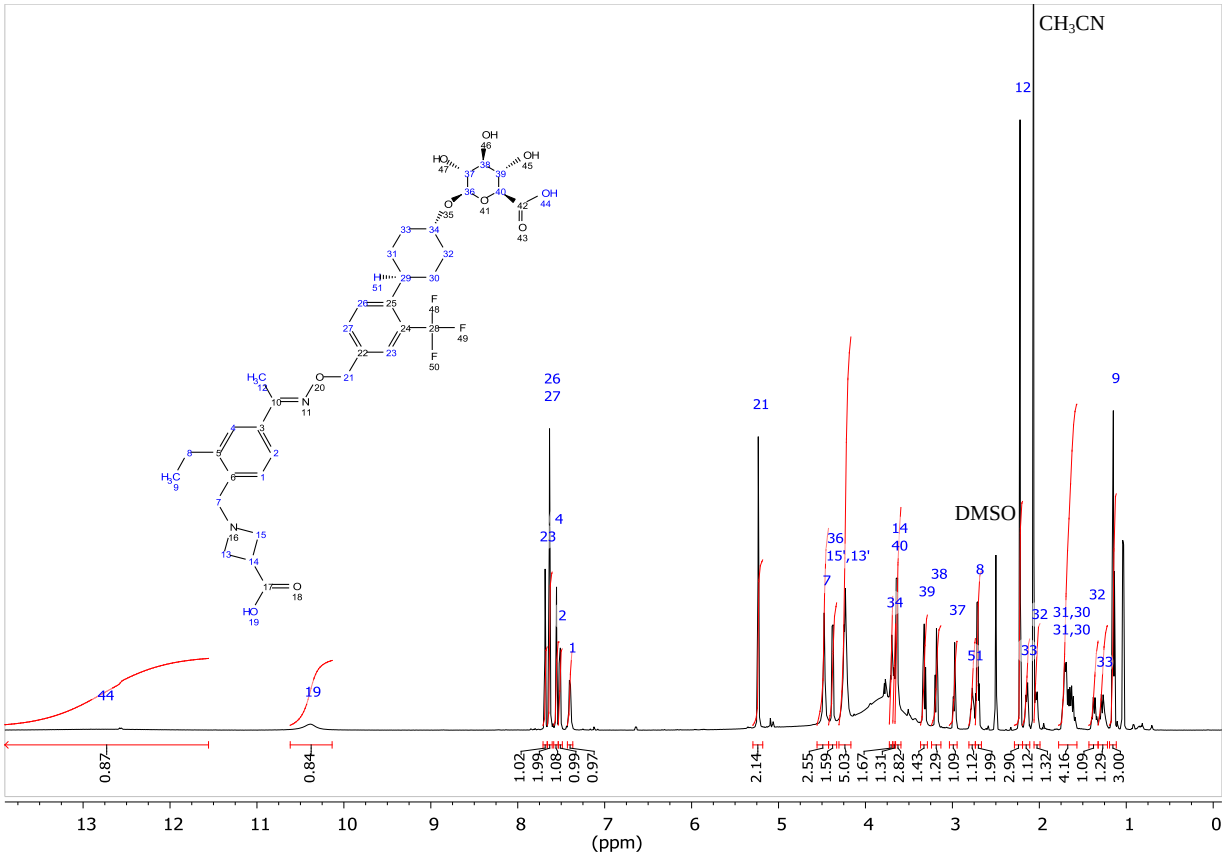
Average Mass: 708.72

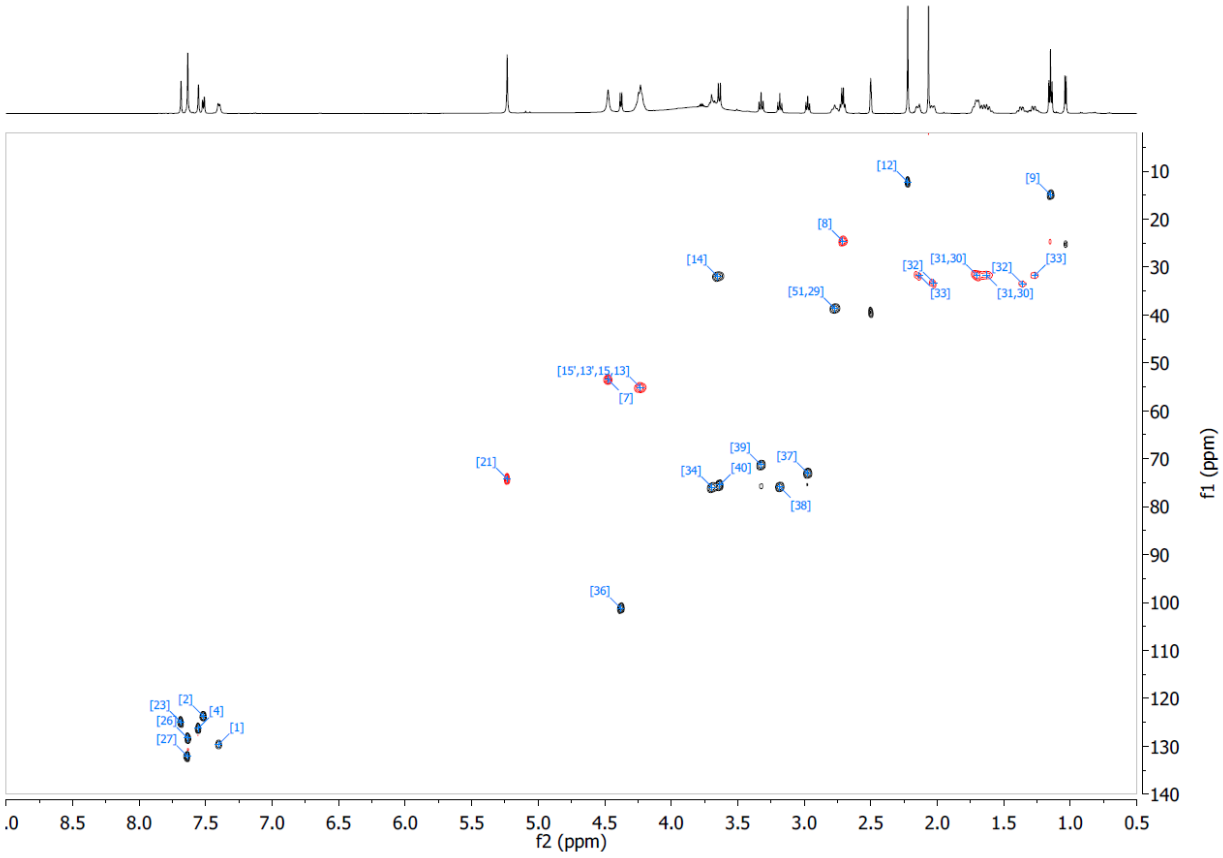
Monoisotopic Mass: 708.28698

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calculated for $C_{35}H_{44}F_3N_2O_{10}$ 709.2943; Found 709.2921.

^{1}H NMR (600 MHz, DMSO-d_6) δ 12.77 (s, 1H), 10.39 (s, 1H), 7.69 (s, 1H), 7.63 (s, 2H), 7.55 (d, J = 1.9 Hz, 1H), 7.52 (dd, J = 8.0, 1.9 Hz, 1H), 7.40 (d, J = 7.9 Hz, 1H), 5.23 (s, 2H), 4.47 (s, 2H), 4.38 (d, J = 7.7 Hz, 1H), 4.24 (dd, J = 10.1, 8.5 Hz, 4H), 3.73 – 3.69 (m, 1H), 3.67 (m, 1H),

3.64 (d, J = 9.6 Hz, 1H), 3.32 (t, J = 9.3 Hz, 1H), 3.18 (t, J = 9.0 Hz, 1H), 2.97 (t, J = 8.4 Hz, 1H), 2.78 (t, J = 11.5 Hz, 1H), 2.71 (q, J = 7.5 Hz, 2H), 2.22 (s, 3H), 2.19 – 2.11 (m, 1H), 2.06 – 1.99 (m, 1H), 1.78 – 1.57 (m, 4H), 1.37 (qd, J = 16.1, 14.2, 6.2 Hz, 1H), 1.28 (tdd, J = 14.9, 9.9, 4.0 Hz, 1H), 1.15 (t, J = 7.5 Hz, 3H).

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f1 (ppm)
f2 (ppm)

D. Metabolite M4a

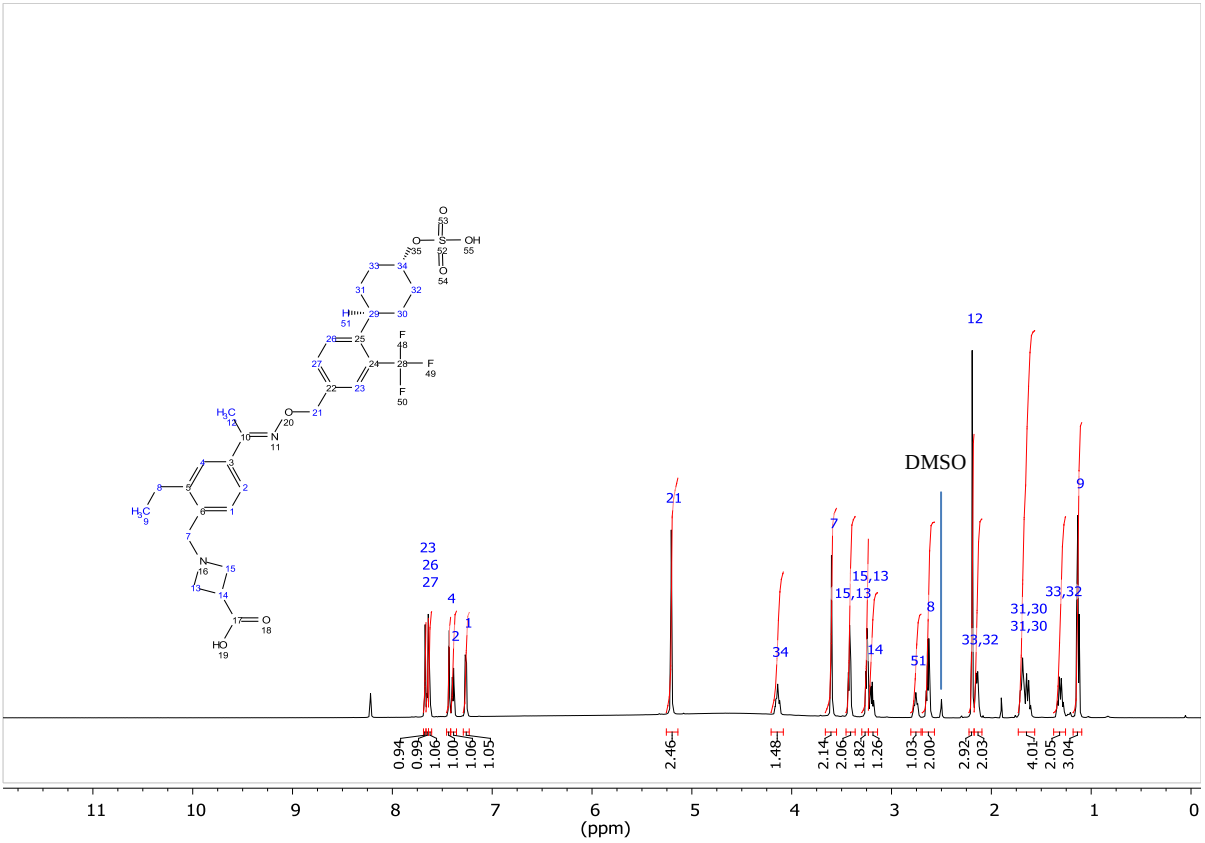
Molecular Formula: $C_{29}H_{35}F_3N_2O_7S$

Average Mass: 612.66

Monoisotopic Mass: 612.21171

HRMS (ESI/QTOF) m/z: $[M + H]_+$ Calculated for $C_{29}H_{36}F_3N_2O_7S$ 613.2190; Found 613.2184.

^{1}H NMR (600 MHz, DMSO-d_6) δ 7.67 (s, 1H), 7.65 (d, *J* = 8.5 Hz, 1H), 7.63 (d, *J* = 10.0 Hz, 1H), 7.43 (d, *J* = 2.0 Hz, 1H), 7.39 (dd, *J* = 7.8, 1.9 Hz, 1H), 7.26 (d, *J* = 8.0 Hz, 1H), 5.21 (s, 2H), 4.14 (tt, *J* = 11.3, 4.3 Hz, 1H), 3.60 (s, 2H), 3.42 (t, *J* = 7.5 Hz, 2H), 3.25 (t, *J* = 6.9 Hz, 2H), 3.19 (p, *J* = 7.5 Hz, 1H), 2.76 (t, *J* = 11.7 Hz, 1H), 2.63 (q, *J* = 7.6 Hz, 2H), 2.19 (s, 3H), 2.17 – 2.09 (m, 2H), 1.73 – 1.57 (m, 4H), 1.31 (qd, *J* = 12.5, 3.9 Hz, 2H), 1.13 (t, *J* = 7.5 Hz, 3H).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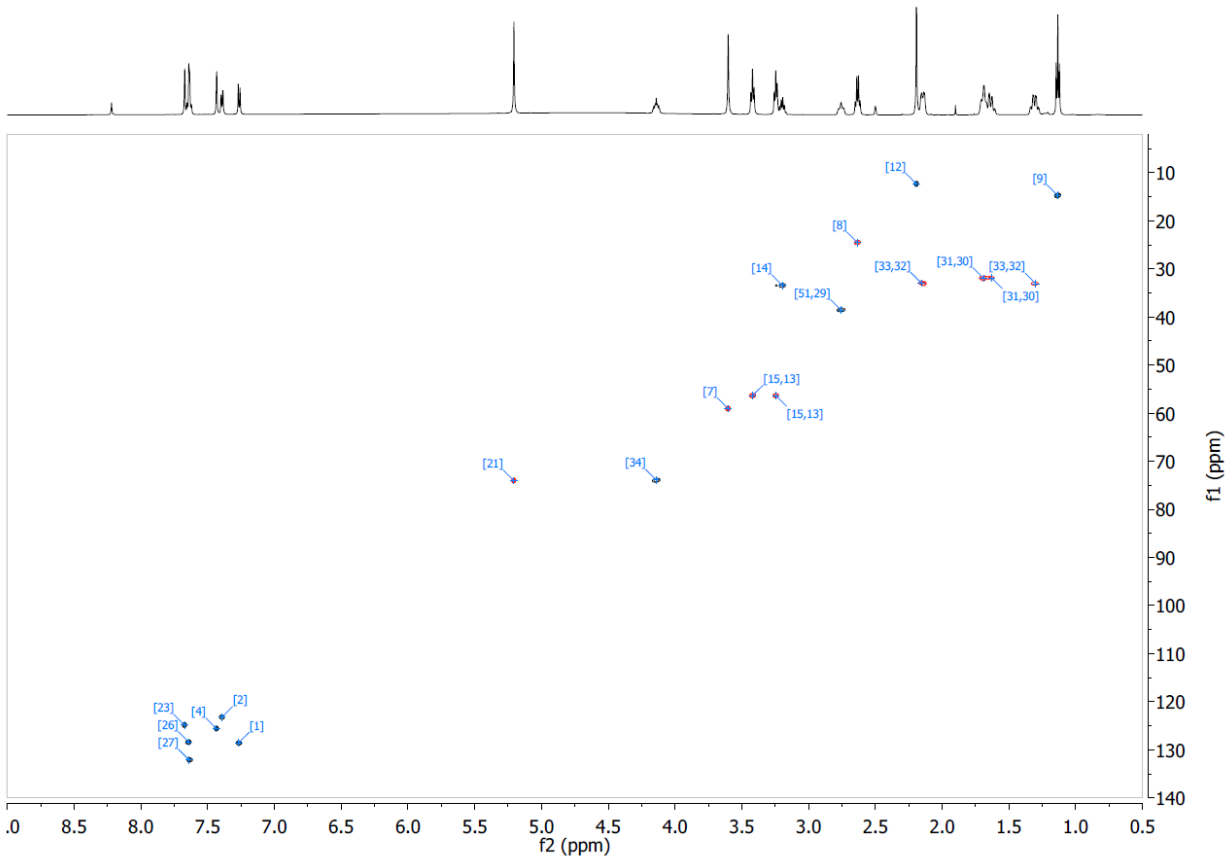
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f1 (ppm)
f2 (ppm)

E. Metabolite M4b

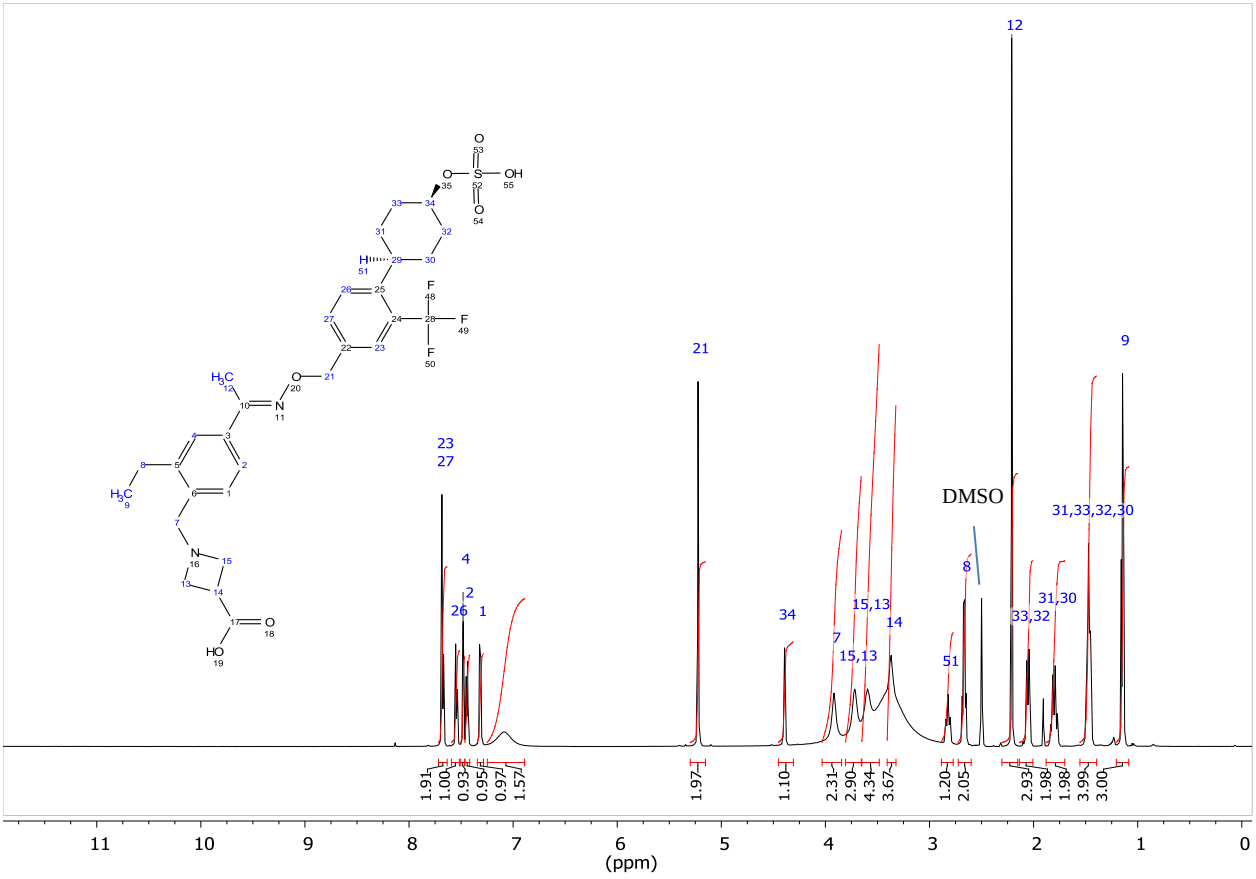
Molecular Formula: $C_{29}H_{35}F_3N_2O_7S$

Average Mass: 612.66

Monoisotopic Mass: 612.21171

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calculated for $C_{29}H_{36}F_3N_2O_7S$ 613.2190; Found 613.2183.

^{1}H NMR (600 MHz, DMSO-d_6) δ 7.68 (d, J = 9.1 Hz, 2H), 7.55 (d, J = 8.0 Hz, 1H), 7.48 (s, 1H), 7.46 – 7.42 (m, 1H), 7.32 (d, J = 8.0 Hz, 1H), 7.09 (s, 2H), 5.22 (s, 2H), 4.39 (t, J = 2.9 Hz, 1H), 3.92 (s, 2H), 3.72 (s, 2H), 3.59 (s, 2H), 3.37 (p, J = 8.5 Hz, 1H), 2.82 (t, J = 12.3 Hz, 1H), 2.67 (q, J = 7.5 Hz, 2H), 2.21 (s, 3H), 2.14 – 2.01 (m, 2H), 1.80 (qd, J = 15.3, 14.5, 3.7 Hz, 2H), 1.55 – 1.39 (m, 4H), 1.15 (t, J = 7.5 Hz, 3H).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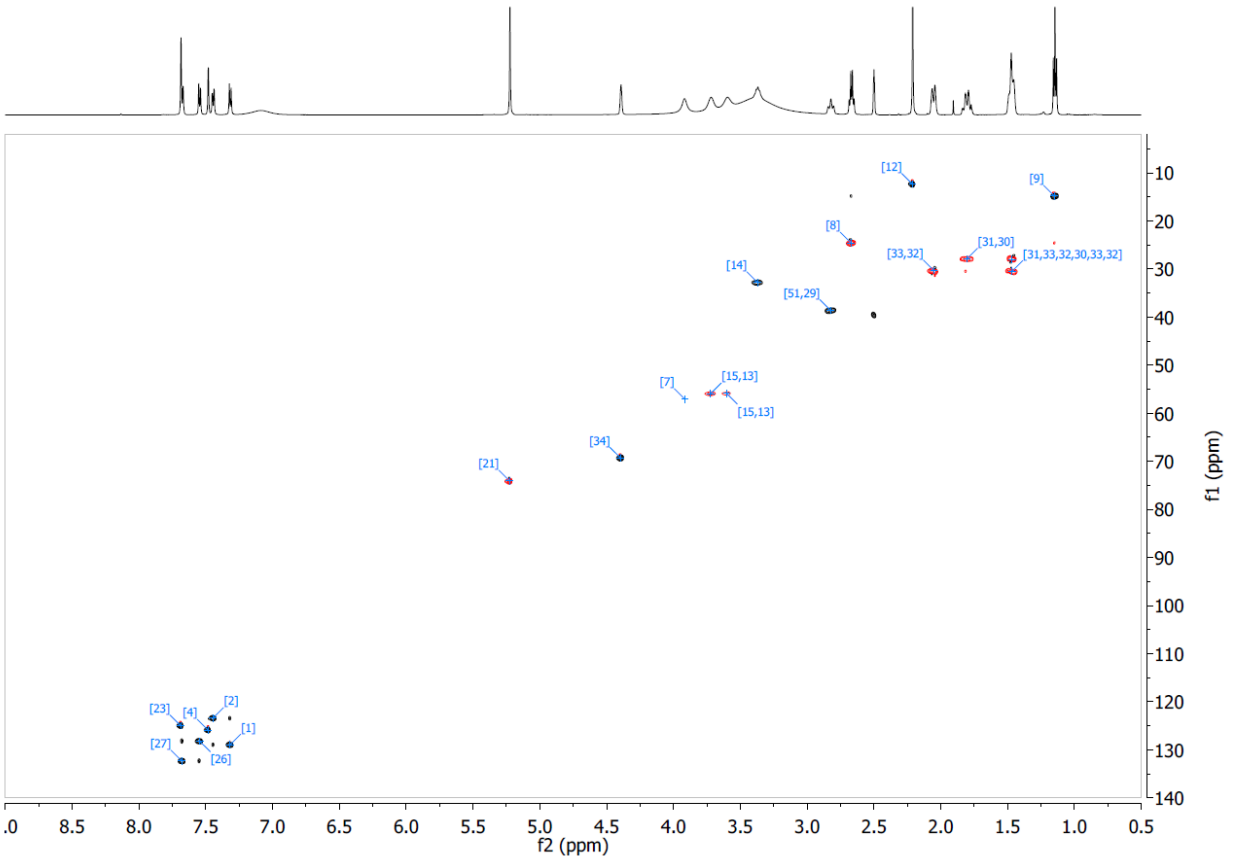
21
9
23
27
4
2
26
1
34
7
15,13
14
15,13
DMSO
8
51
31,33,32,30
33,32
31,30
1.91
1.00
0.93
0.95
0.97
1.57
1.97
1.10
2.31
2.90
4.34
3.67
1.20
2.05
2.93
1.98
1.98
3.99
3.00
11
10
9
8
7
6
5
4
3
2
1
0
(ppm)


[12]
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[33,32]
[31,33,32,30,33,32]
[14]
[51,29]
[7]
[15,13]
[15,13]
[34]
[21]
[23]
[4]
[2]
[1]
[27]
[26]
f1 (ppm)
f2 (ppm)

F. Metabolite M4c

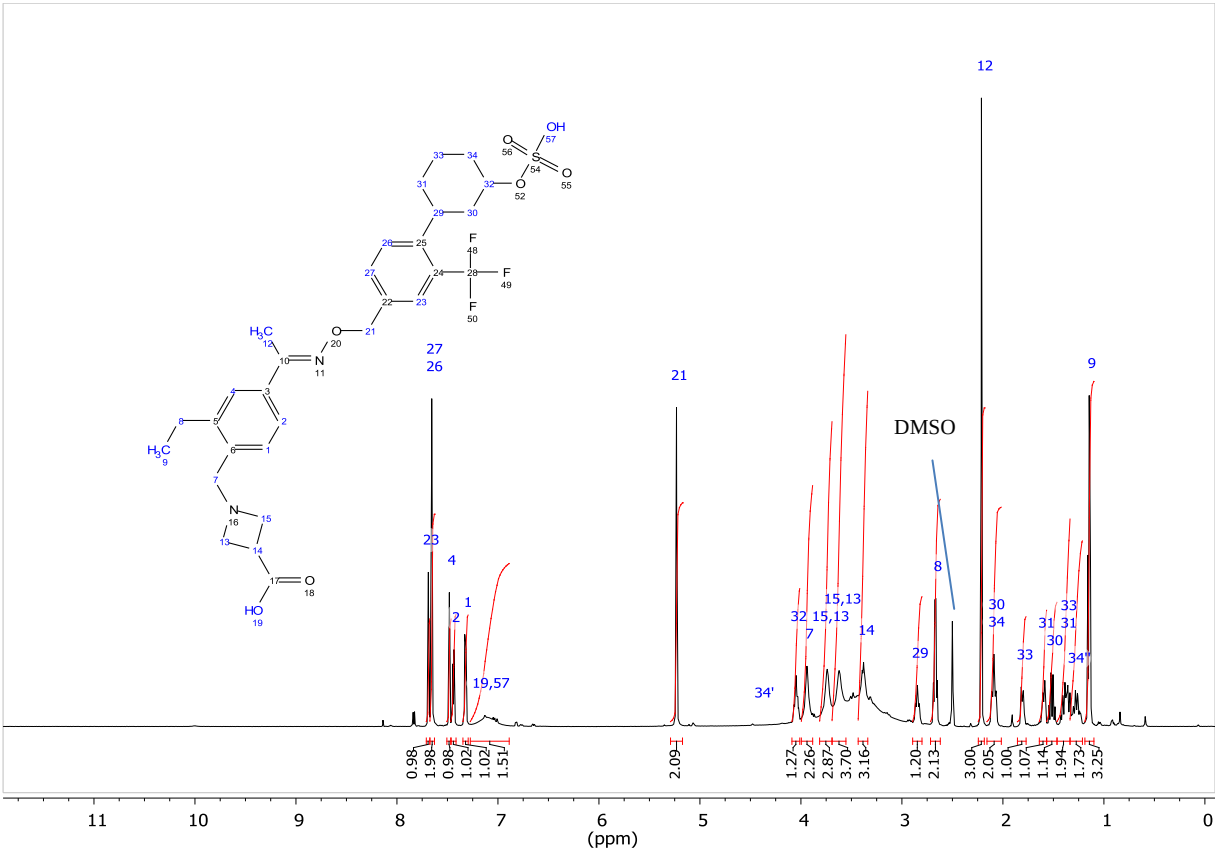
Molecular Formula: $C_{29}H_{35}F_3N_2O_7S$

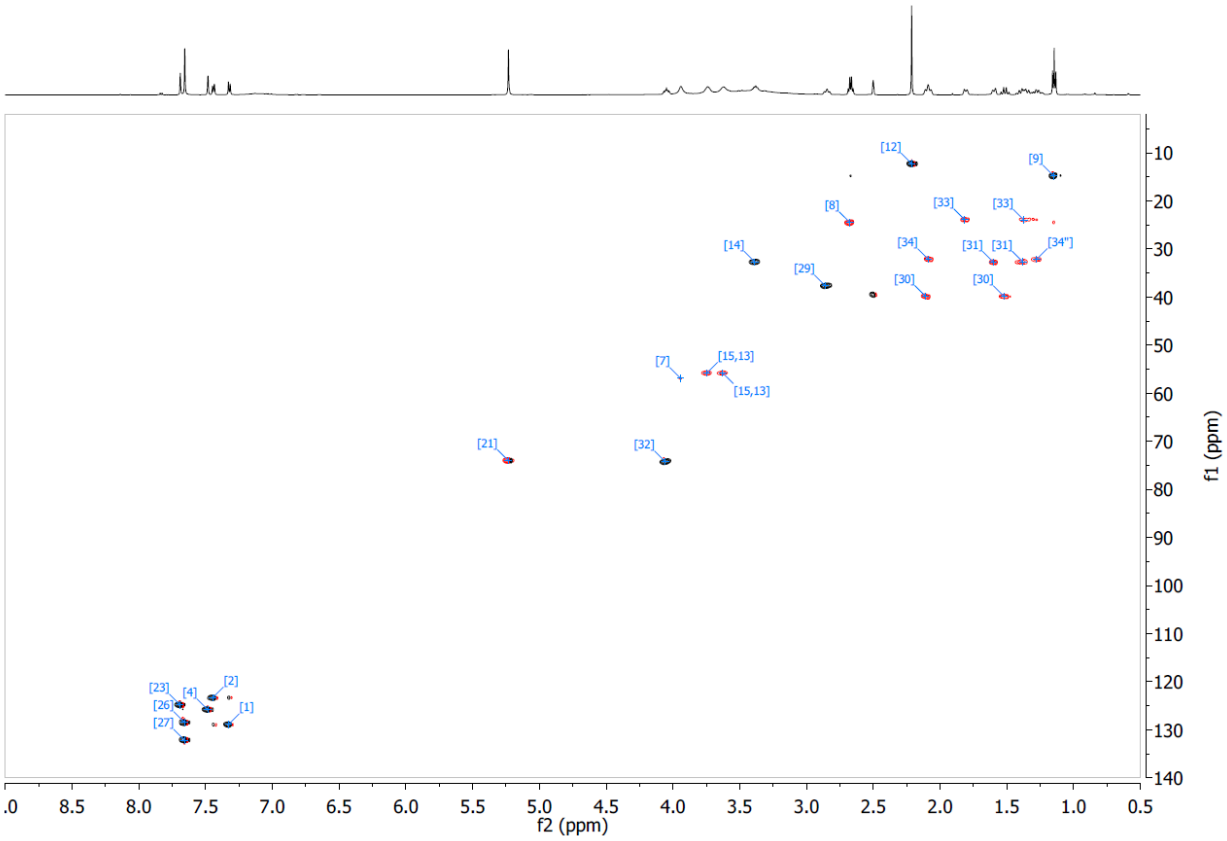
Average Mass: 612.66

Monoisotopic Mass: 612.21171

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calculated for $C_{29}H_{36}F_3N_2O_7S$ 613.2190; Found 613.2188.

^{1}H NMR (600 MHz, DMSO-d_6) δ 7.69 (s, 1H), 7.66 (s, 2H), 7.48 (d, *J* = 1.8 Hz, 1H), 7.44 (dd, *J* = 8.1, 1.9 Hz, 1H), 7.32 (d, *J* = 8.0 Hz, 1H), 7.10 (s, 2H), 5.23 (s, 2H), 4.05 (tt, *J* = 11.0, 4.3 Hz, 1H), 3.94 (s, 2H), 3.74 (s, 2H), 3.62 (s, 2H), 3.38 (p, *J* = 8.0 Hz, 1H), 2.85 (t, *J* = 11.9 Hz, 1H), 2.67 (q, *J* = 7.6 Hz, 2H), 2.21 (s, 3H), 2.09 (td, *J* = 13.7, 12.5, 3.8 Hz, 2H), 1.81 (dt, *J* = 12.5, 3.3 Hz, 1H), 1.60 (d, *J* = 12.0 Hz, 1H), 1.51 (q, *J* = 11.9 Hz, 1H), 1.46 – 1.34 (m, 2H), 1.27 (tdd, *J* = 17.2, 9.5, 6.1 Hz, 1H), 1.15 (t, *J* = 7.5 Hz, 3H).


12
27
26
21
9
DMSO
23
4
1
2
19,57
8
32
15,13
7
15,13
14
30
34
29
33
31
30
33
31
34'
34'
0.98
1.98
0.98
1.02
1.02
1.51
2.09
1.27
2.26
2.87
3.70
3.16
1.20
2.13
3.00
2.05
1.00
1.07
1.14
1.94
1.73
3.25
11
10
9
8
7
6
5
4
3
2
1
0
(ppm)


[12]
[9]
[8]
[33]
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[31]
[34"]
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[30]
[30]
[7]
[15,13]
[15,13]
[21]
[32]
[23]
[4]
[2]
[26]
[1]
[27]
f1 (ppm)
f2 (ppm)

G. Metabolite M5

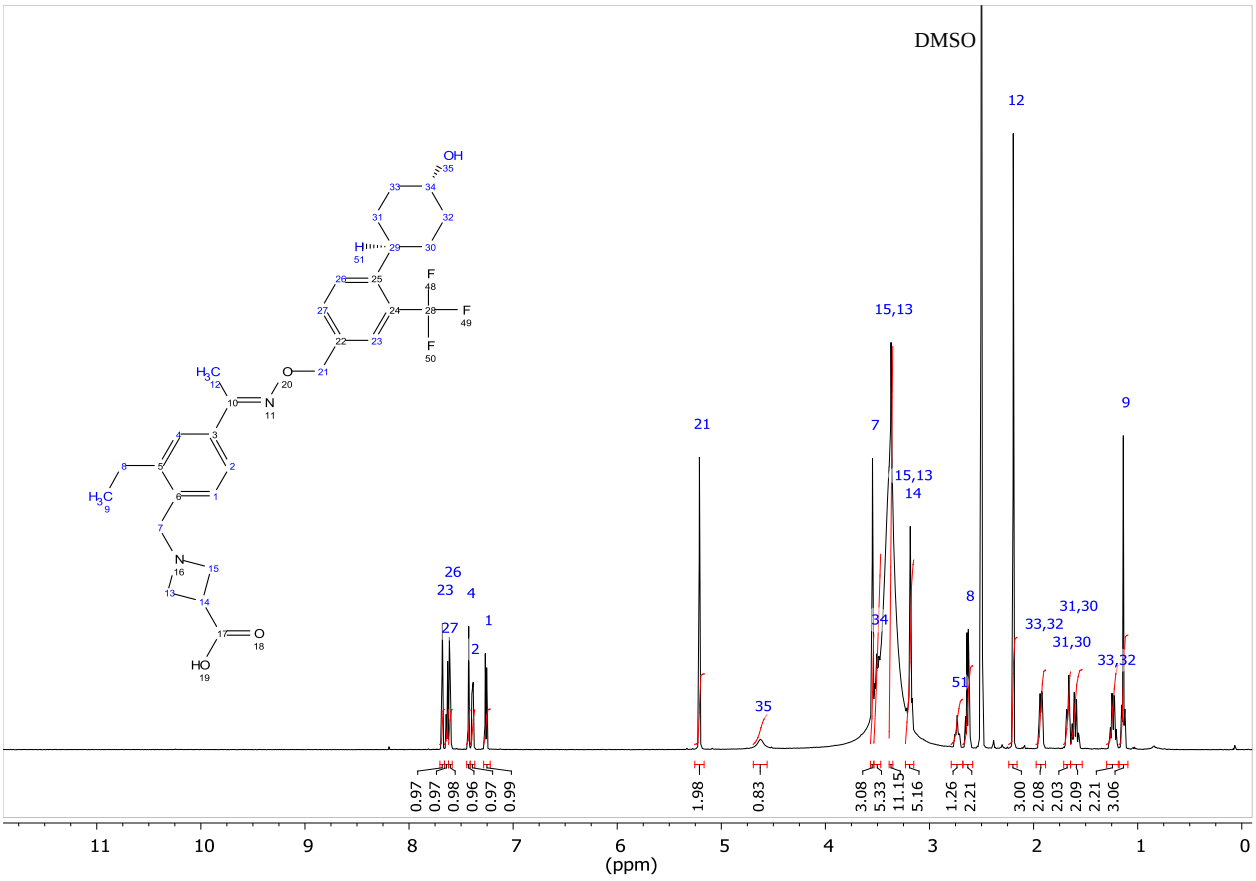
Molecular Formula: C29H35F3N2O4

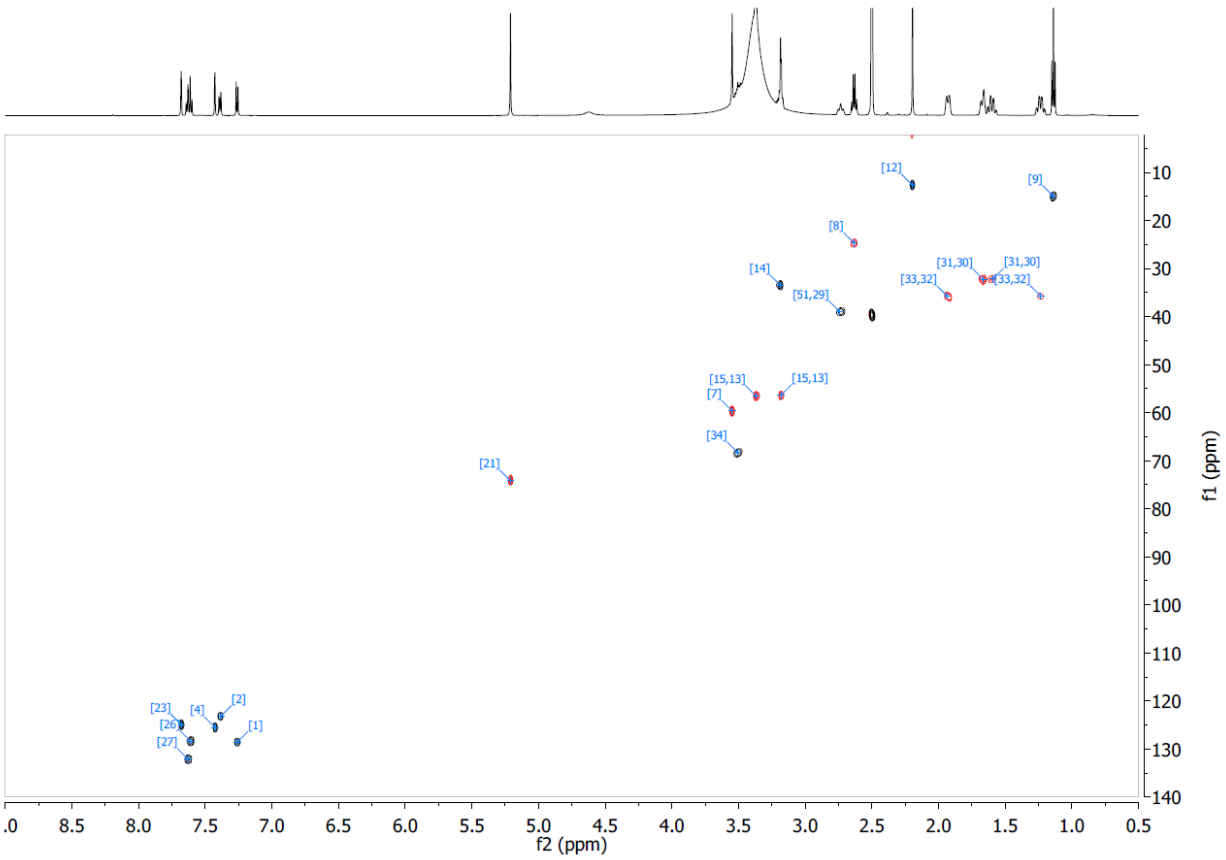
Average Mass: 532.59

Monoisotopic Mass: 532.25489

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calculated for $C_{29}H_{36}F_3N_2O_4$ 533.2622; Found 533.2584.

^{1}H NMR (600 MHz, DMSO-d_6) δ 7.68 (s, 1H), 7.64 (d, *J* = 8.3 Hz, 1H), 7.61 (d, *J* = 8.2 Hz, 1H), 7.43 (d, *J* = 1.8 Hz, 1H), 7.39 (dd, *J* = 8.0, 1.9 Hz, 1H), 7.26 (d, *J* = 8.0 Hz, 1H), 5.21 (s, 2H), 4.62 (s, 1H), 3.55 (s, 2H), 3.51 (tt, *J* = 10.8, 4.4 Hz, 1H), 3.39 – 3.35 (m, 2H), 3.23 – 3.15 (m, 3H), 2.73 (t, *J* = 12.0 Hz, 1H), 2.63 (q, *J* = 7.5 Hz, 2H), 2.20 (s, 3H), 1.93 (dd, *J* = 13.1, 4.0 Hz, 2H), 1.71 – 1.64 (m, 2H), 1.60 (qd, *J* = 13.0, 3.2 Hz, 2H), 1.23 (qd, *J* = 12.8, 3.7 Hz, 2H), 1.14 (t, *J* = 7.5 Hz, 3H)


DMSO
12
15,13
9
21
7
15,13
14
8
31,30
33,32
31,30
33,32
26
23
4
1
27
2
34
51
35
0.97
0.97
0.98
0.96
0.97
0.99
1.98
0.83
3.08
5.33
11.15
5.16
1.26
2.21
3.00
2.08
2.03
2.09
2.21
3.06
11
10
9
8
7
6
5
4
3
2
1
0
(ppm)


[12]
[9]
[8]
[14]
[31,30]
[31,30]
[33,32]
[33,32]
[51,29]
[15,13]
[15,13]
[7]
[34]
[21]
[23]
[4]
[2]
[26]
[1]
[27]
f1 (ppm)
f2 (ppm)

H. Metabolite M6

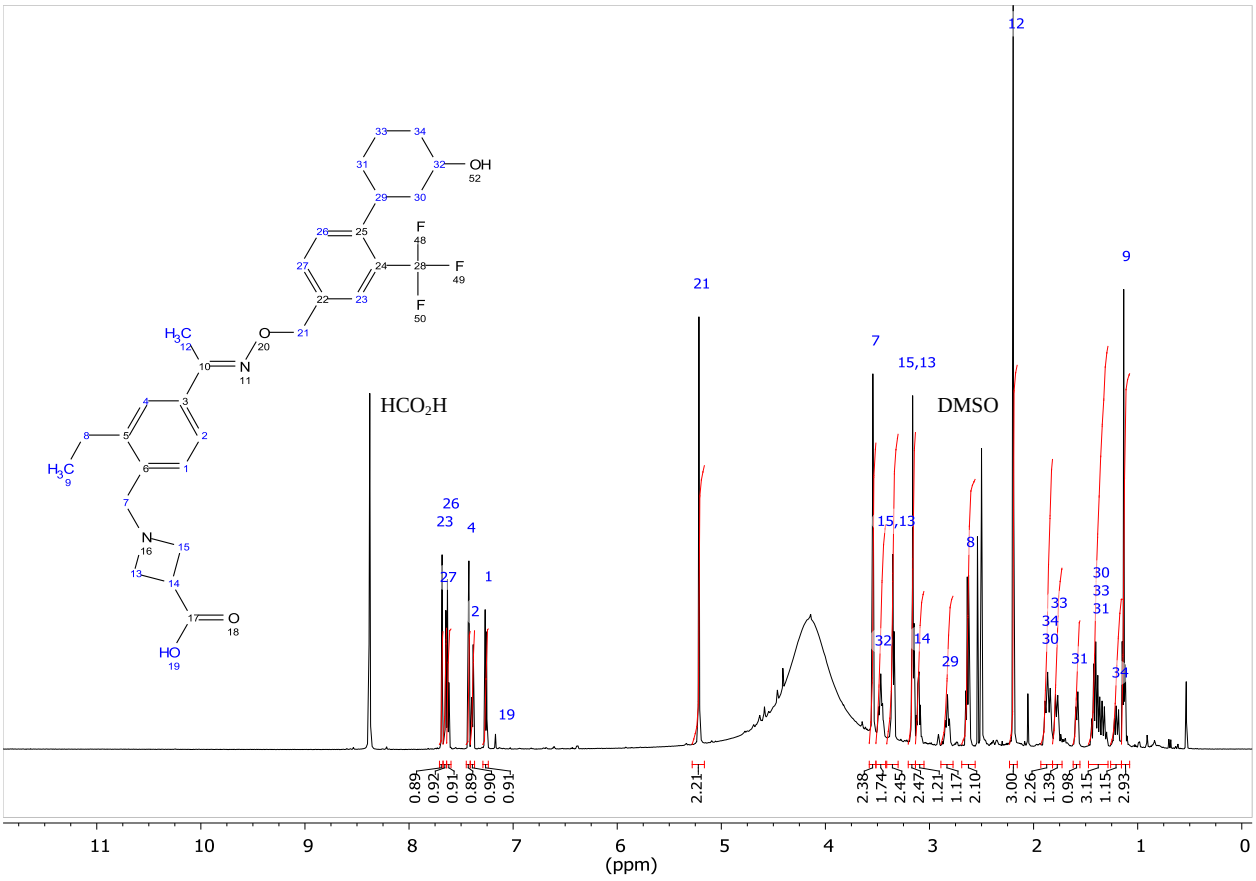
Molecular Formula: C29H35F3N2O4

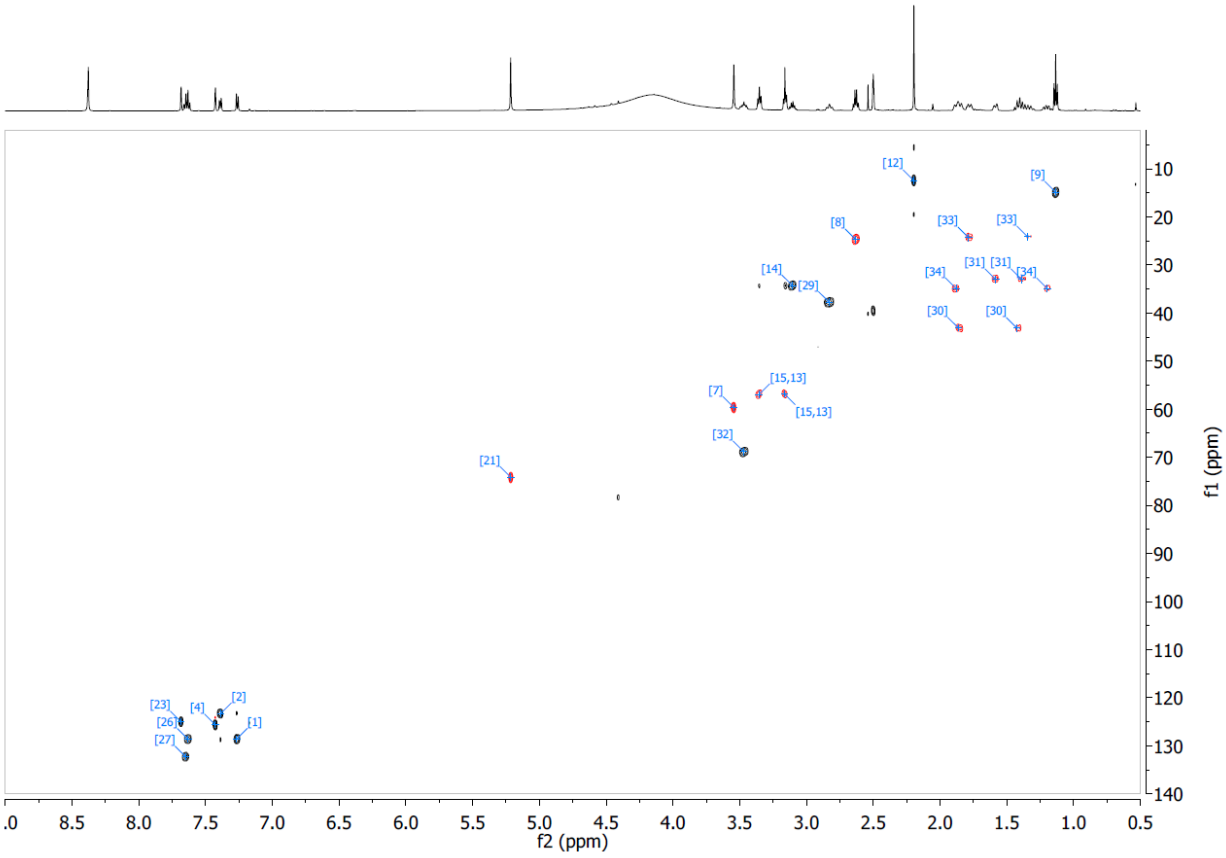
Average Mass: 532.59

Monoisotopic Mass: 532.25489

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calculated for $C_{29}H_{36}F_3N_2O_4$ 533.2622; Found 533.2665.

^{1}H NMR (600 MHz, DMSO-d_6) δ 7.68 (d, *J* = 1.6 Hz, 1H), 7.67 – 7.64 (m, 1H), 7.62 (d, *J* = 8.2 Hz, 1H), 7.43 (d, *J* = 1.8 Hz, 1H), 7.39 (dd, *J* = 8.0, 1.9 Hz, 1H), 7.26 (d, *J* = 8.0 Hz, 1H), 5.22 (s, 2H), 3.54 (s, 2H), 3.47 (tq, *J* = 10.9, 4.3, 3.5 Hz, 1H), 3.35 (t, *J* = 7.3 Hz, 2H), 3.20 – 3.13 (m, 2H), 3.10 (p, *J* = 7.6 Hz, 1H), 2.83 (t, *J* = 12.0 Hz, 1H), 2.63 (q, *J* = 7.6 Hz, 2H), 2.20 (s, 3H), 1.87 (dd, *J* = 18.2, 12.4 Hz, 2H), 1.78 (dq, *J* = 12.9, 3.6 Hz, 1H), 1.58 (d, *J* = 12.2 Hz, 1H), 1.47 – 1.29 (m, 3H), 1.20 (qd, *J* = 12.4, 3.6 Hz, 1H), 1.13 (t, *J* = 7.6 Hz, 3H).


HCO2H
DMSO
(ppm)


[12]
[9]
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[14]
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[30]
[30]
[15,13]
[7]
[15,13]
[32]
[21]
[23]
[4]
[2]
[26]
[1]
[27]
f1 (ppm)
f2 (ppm)

I. Metabolite M7

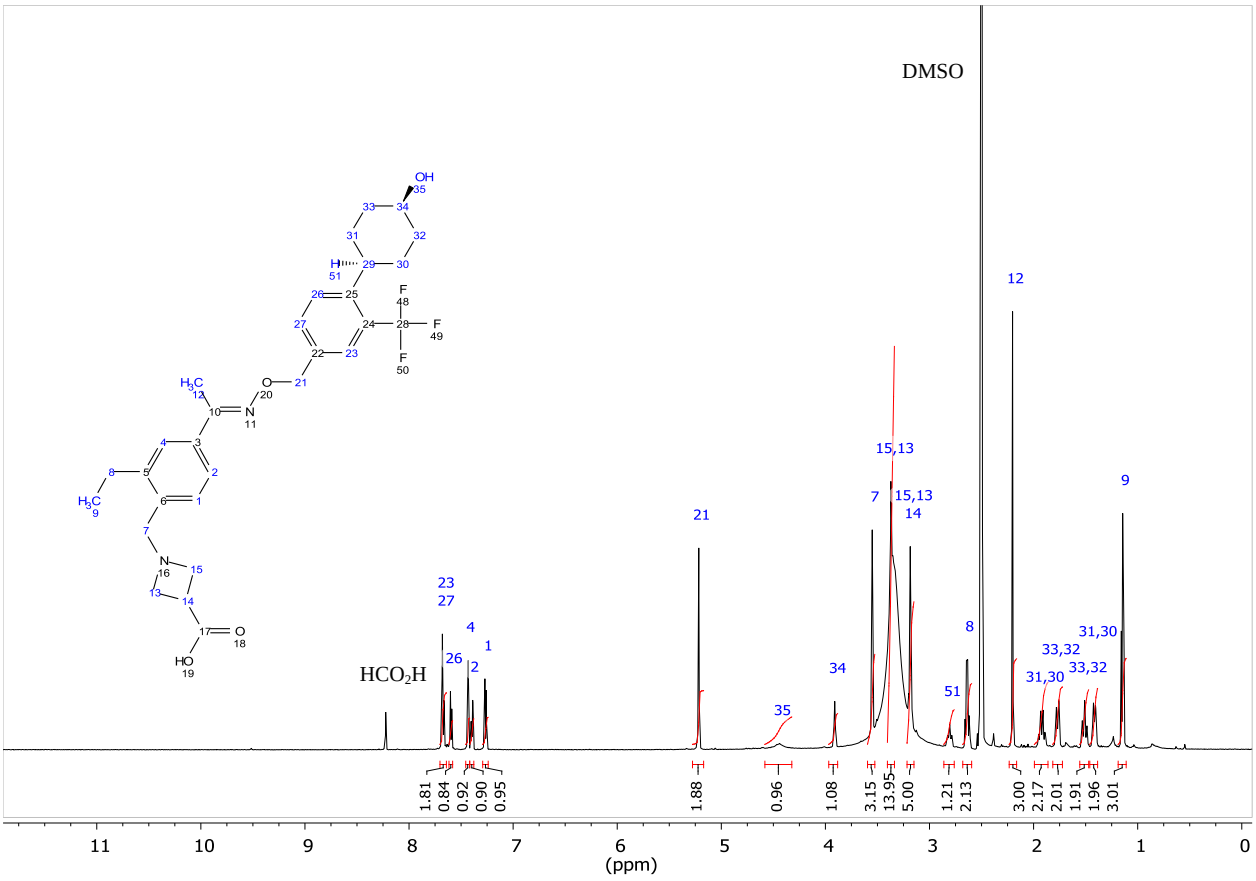
Molecular Formula: C29H35F3N2O4

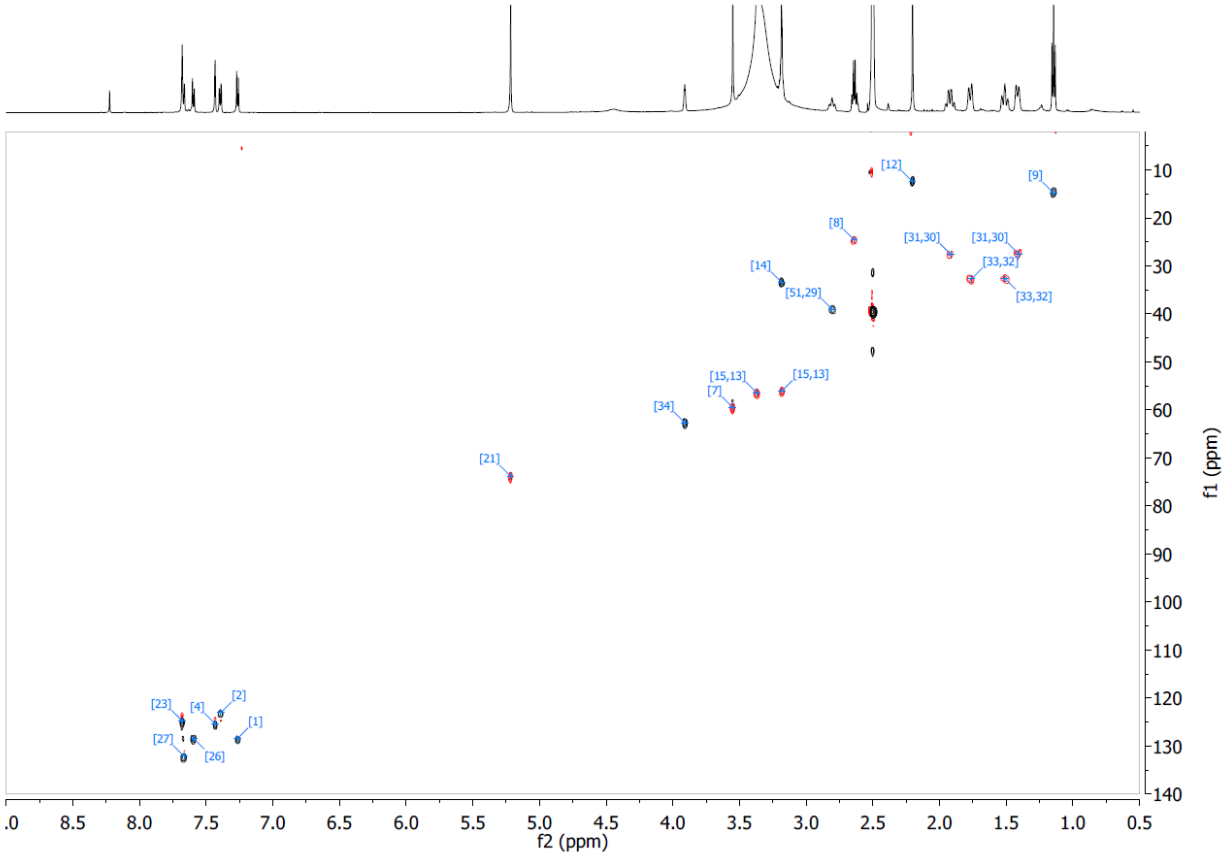
Average Mass: 532.59

Monoisotopic Mass: 532.25489

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calculated for $C_{29}H_{36}F_3N_2O_4$ 533.2622; Found 533.2626.

^{1}H NMR (600 MHz, DMSO-d_6) δ 7.70 – 7.64 (m, 2H), 7.60 (d, *J* = 8.0 Hz, 1H), 7.43 (d, *J* = 1.9 Hz, 1H), 7.39 (dd, *J* = 7.8, 1.8 Hz, 1H), 7.26 (d, *J* = 8.0 Hz, 1H), 5.22 (s, 2H), 4.44 (s, 1H), 3.91 (t, *J* = 2.9 Hz, 1H), 3.55 (s, 2H), 3.37 (m_c, 2H), 3.22 – 3.15 (m, 3H), 2.81 (t, *J* = 12.2 Hz, 1H), 2.64 (q, *J* = 7.6 Hz, 2H), 2.20 (s, 3H), 1.92 (qd, *J* = 12.8, 3.4 Hz, 2H), 1.77 (dd, *J* = 13.0, 3.3 Hz, 2H), 1.51 (tt, *J* = 13.6, 3.3 Hz, 2H), 1.46 – 1.39 (m, 2H), 1.14 (t, *J* = 7.5 Hz, 3H).


DMSO
HCO2H
12
9
15,13
7
15,13
14
21
23
27
4
1
26
2
34
35
51
8
31,30
33,32
31,30
33,32
1.81
0.84
0.92
0.90
0.95
1.88
0.96
1.08
3.15
13.95
5.00
1.21
2.13
3.00
2.17
2.01
1.91
1.96
3.01
11
10
9
8
7
6
5
4
3
2
1
0
(ppm)


[12]
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[8]
[31,30]
[31,30]
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[33,32]
[15,13]
[15,13]
[7]
[34]
[21]
[23]
[4]
[2]
[1]
[27]
[26]
f1 (ppm)
f2 (ppm)

J. Metabolite M8

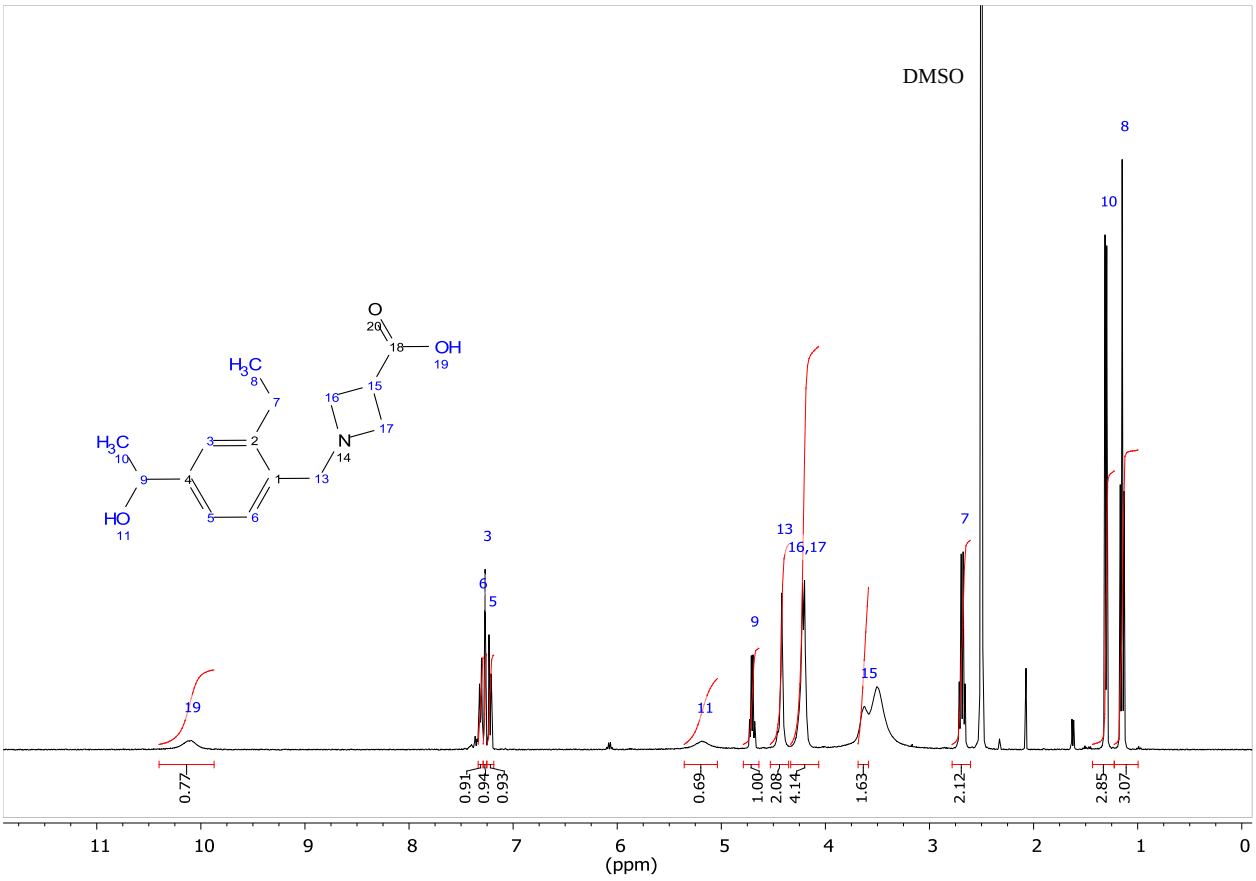
Molecular Formula: $C_{15}H_{21}NO_3$

Average Mass: 263.33

Monoisotopic Mass: 263.15214

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calculated for $C_{15}H_{22}NO_3$ 264.1594; Found 264.1609.

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.11 (s, 1H), 7.31 (d, J = 7.9 Hz, 1H), 7.27 (d, J = 1.6 Hz, 1H), 7.22 (dd, J = 8.0, 1.8 Hz, 1H), 5.18 (s, 1H), 4.70 (q, J = 6.4 Hz, 1H), 4.42 (s, 2H), 4.21 (m_c, 4H), 3.61 (m_c, 1H), 2.69 (q, J = 7.6 Hz, 2H), 1.31 (d, J = 6.4 Hz, 3H), 1.15 (t, J = 7.5 Hz, 3H).


DMSO
8
10
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6
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9
13
16,17
15
7
19
11
0.77
0.91
0.94
0.93
0.69
1.00
2.08
4.14
1.63
2.12
2.85
3.07
11
10
9
8
7
6
5
4
3
2
1
0
(ppm)

K. Metabolite M17

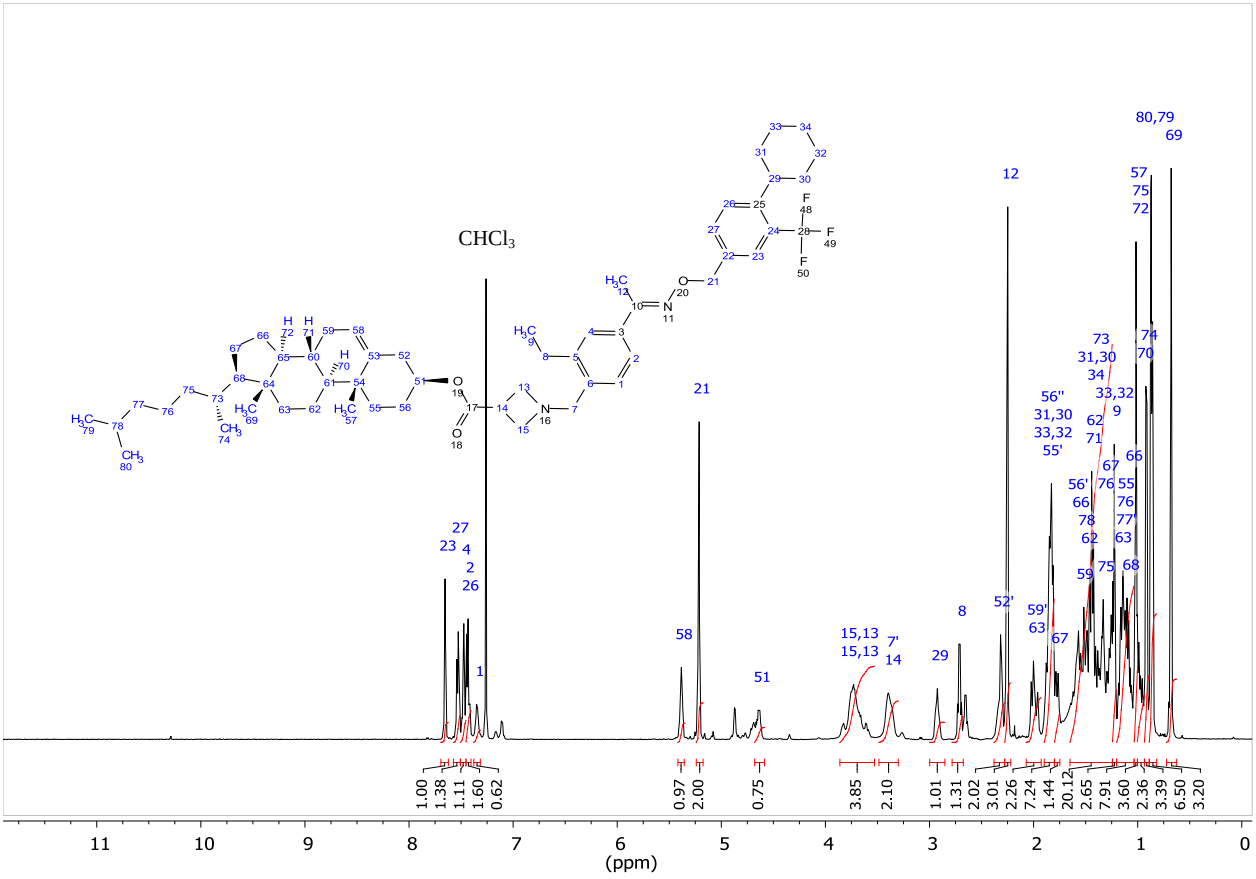
Molecular Formula: $C_{56}H_{79}F_3N_2O_3$

Average Mass: 885.23

Monoisotopic Mass: 884.60428

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calculated for $C_{56}H_{80}F_3N_2O_3$ 885.6116; Found 885.6105.

^{1}H NMR (600 MHz, Chloroform-d) δ 7.65 (s, 1H), 7.53 (d, J = 8.4 Hz, 1H), 7.47 (s, 1H), 7.45 – 7.40 (m, 2H), 7.34 (d, J = 7.7 Hz, 1H), 5.39 (t, J = 5.7 Hz, 1H), 5.21 (s, 2H), 4.63 (td, J = 11.1, 10.6, 6.5 Hz, 1H), 3.86 – 3.53 (m, 4H), 3.38 (d, J = 24.6 Hz, 3H), 2.93 (t, J = 9.9 Hz, 1H), 2.71 (q, J = 7.6 Hz, 2H), 2.38 – 2.28 (m, 2H), 2.25 (s, 3H), 2.07 – 1.93 (m, 2H), 1.90 – 1.80 (m, 6H), 1.77 (d, J = 13.1 Hz, 1H), 1.65 – 1.24 (m, 17H), 1.23 (t, J = 7.5 Hz, 3H), 1.20 – 1.04 (m, 7H), 1.03 – 1.00 (m, 4H), 1.00 – 0.93 (m, 2H), 0.92 (d, J = 6.5 Hz, 3H), 0.87 (d, J = 6.6 Hz, 6H), 0.68 (s, 3H).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55'
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71
66
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56'
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55
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52'
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7'
14
58
51
21
27
23
4
2
26
1
1.00
1.38
1.11
1.60
0.62
0.97
2.00
0.75
3.85
2.10
1.01
1.31
2.02
3.01
2.26
7.24
1.44
20.12
2.65
7.91
3.60
2.36
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6.50
3.20
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10
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8
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6
5
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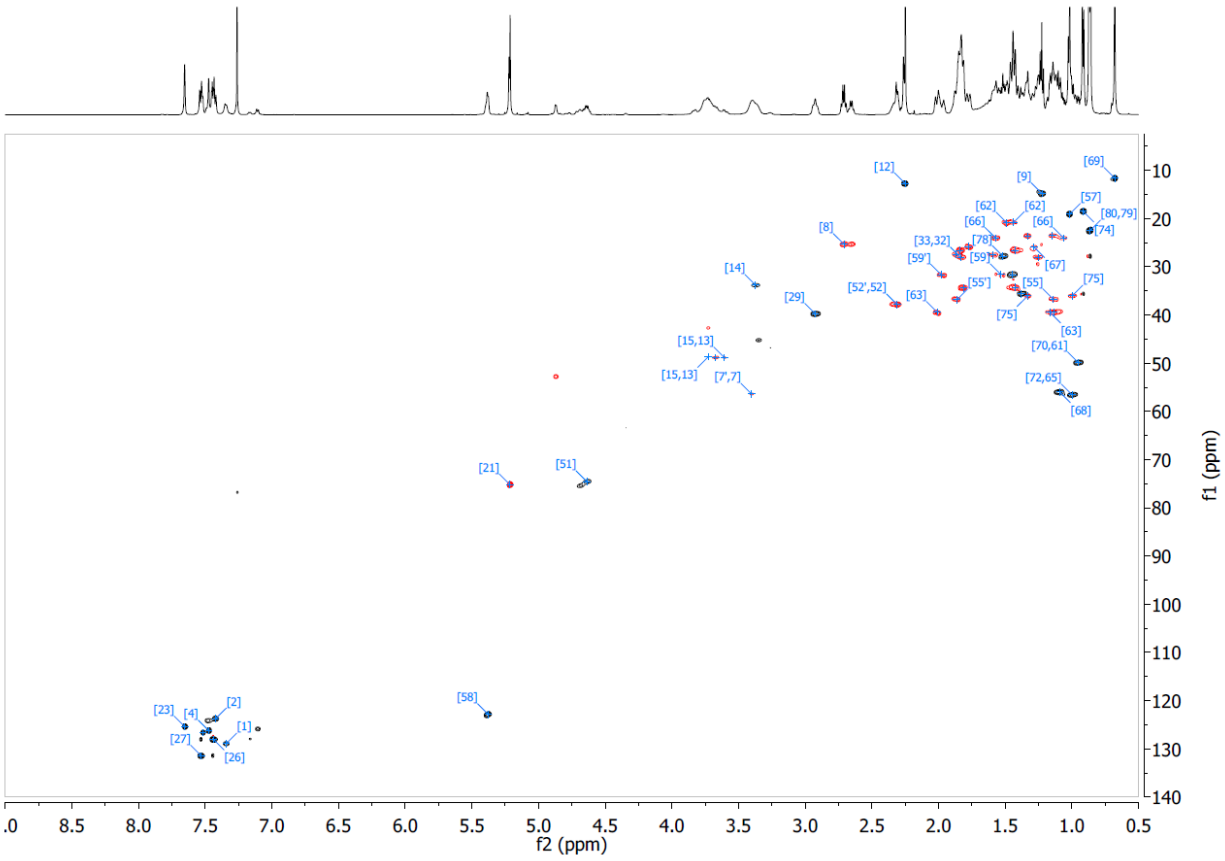
3
2
1
0
(ppm)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[15,13]
[7',7]
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[68]
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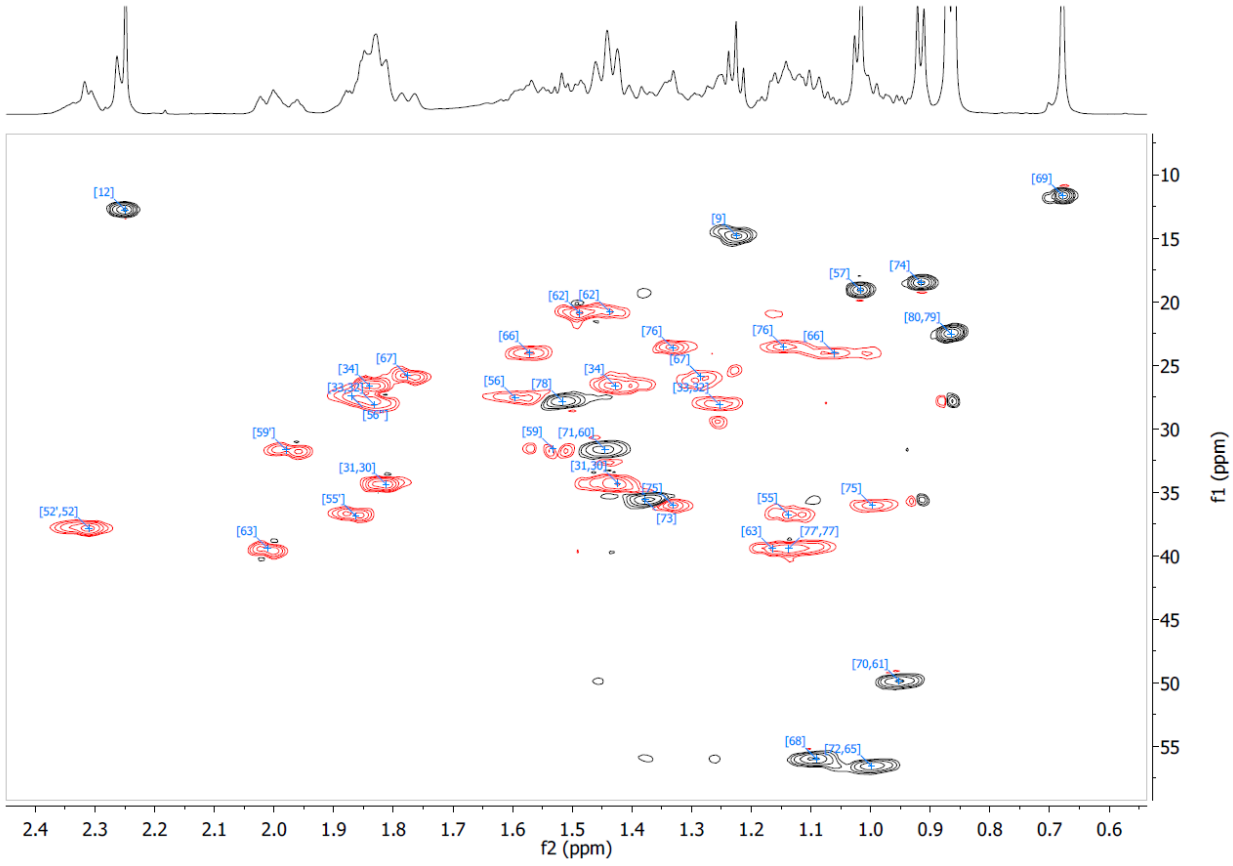
[51]
[58]
[23]
[4]
[2]
[1]
[27]
[26]
f2 (ppm)
f1 (ppm)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[73]
[63]
[63]
[77,77]
[70,61]
[68]
[72,65]
2.4 2.3 2.2 2.1 2.0 1.9 1.8 1.7 1.6 1.5 1.4 1.3 1.2 1.1 1.0 0.9 0.8 0.7 0.6
f2 (ppm)
10 15 20 25 30 35 40 45 50 55
f1 (ppm)