SUPPLEMENTARY INFORMATION

New enzymatic and mass spectrometric methodology for the selective investigation of gut microbiota-derived metabolites

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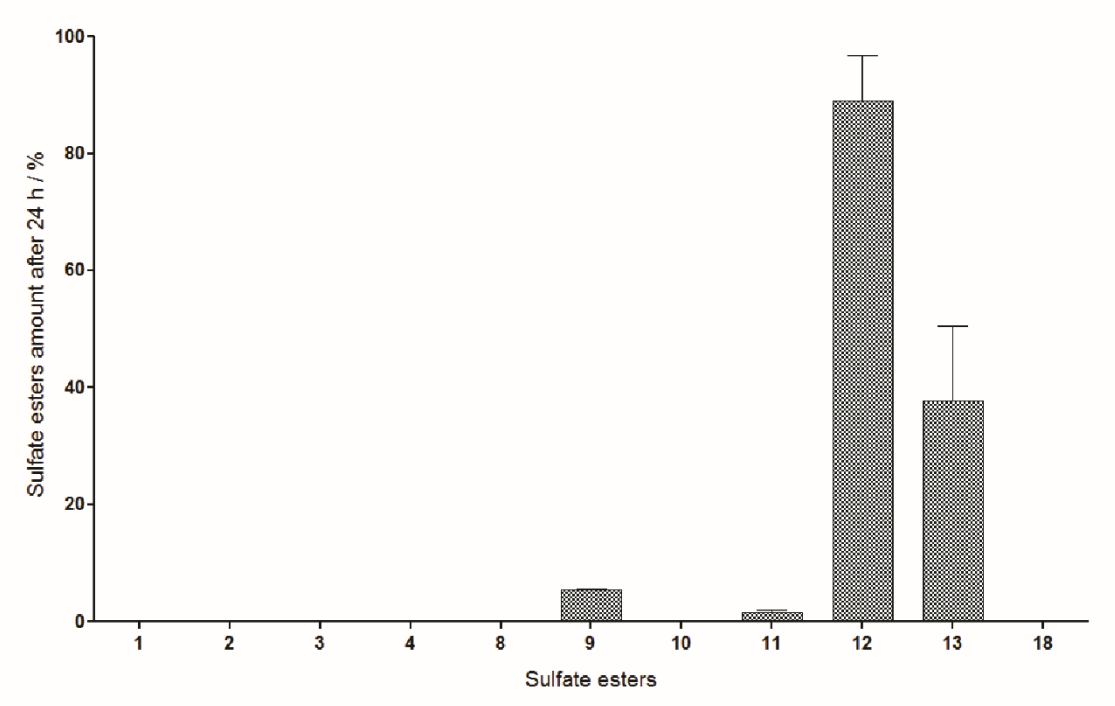


Fig. S1: Percentage of unhydrolyzed sulfate ester substrates after 24 h of enzymatic treatment using 130 U of crude *Helix pomatia* arylsulfatase. Experiments were performed in duplicate and error bars represent SEM. The 12 tested substrates are: indoxyl sulfate (**1**); *p*-cresyl sulfate (**2**); ferulic acid 4-sulfate (**3**); 4-methyl-umbelliferyl sulfate (**4**); *N*-acetyltyramine-*O*-sulfate (**8**); L-tyrosine-*O*-sulfate (**9**); estrone-3-sulfate (**10**); *N*-acetylserotonine-*O*-sulfate (**11**); D-mannose 6-sulfate (**12**); taurolithocholic acid 3-sulfate (**13**); and 4-hydroxy-3-methoxyphenylglycol sulfate (**18**).

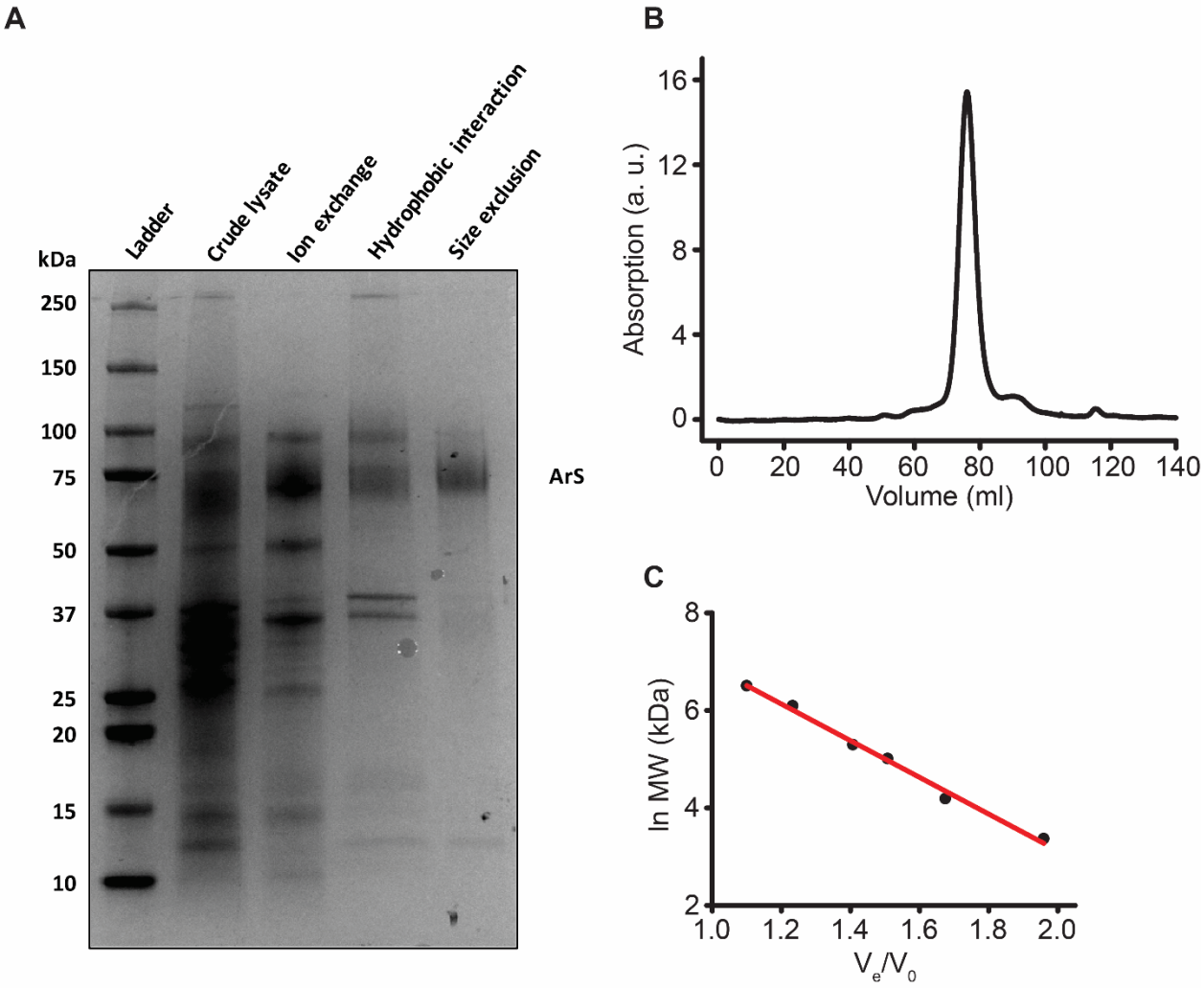


Fig. S2: Purification of the arylsulfatase (ArS); A) SDS-PAGE of the crude *H. pomatia* sulfatase illustrating the purification step-by-step. Lane 1: Ladder; Lane 2: Crude *H. pomatia*; Lane 3: sulfatase mixture after ion exchange chromatography; Lane 4: enriched sulfatase after hydrophobic interaction chromatography; Lane 5: Purified sulfatase after size exclusion chromatography; B) Size exclusion chromatogram of the purified arylsulfatase, using a HiLoad 16/600 Superdex 200 pg; C) Calibration curve for MW determination.

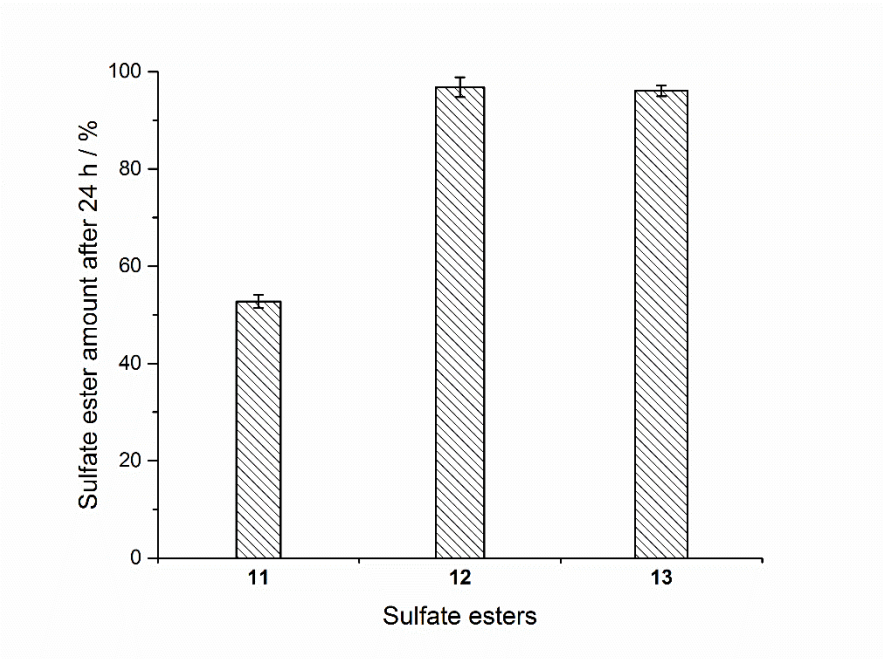


Fig. S3: Percentage of unhydrolyzed sulfate ester substrates after 24 h of enzymatic treatment using 19 U of purified arylsulfatase; *N*-acetylserotonine-*O*-sulfate (**11**); D-mannose 6-sulfate (**12**); taurolithocholic acid 3-sulfate (**13**).

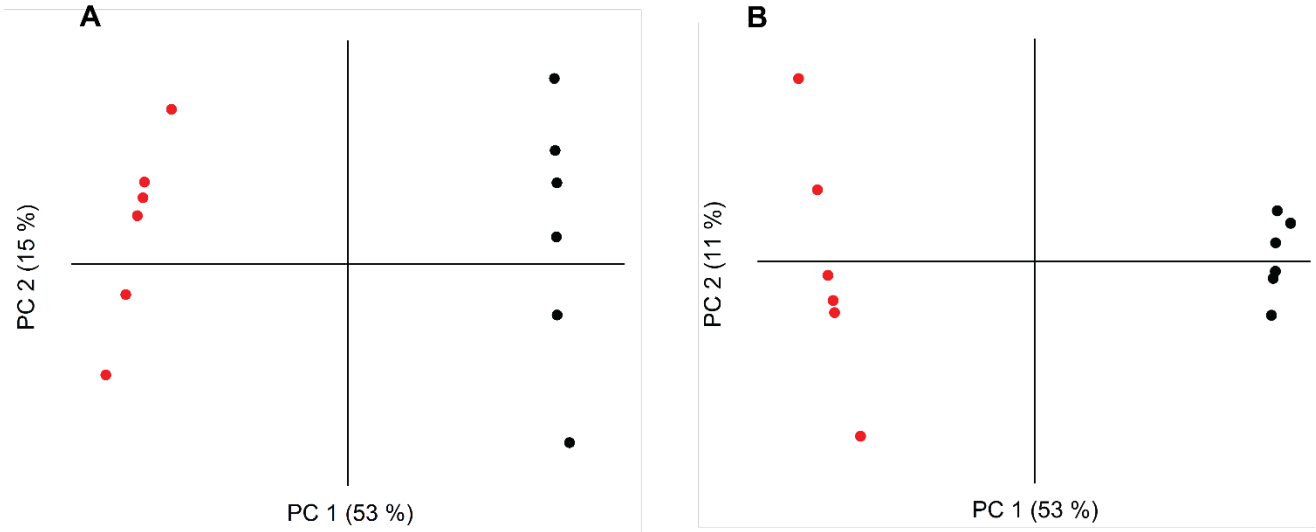


Fig. S4: Multivariate analysis of sulfatase treated (red dots) and untreated (black dots / denatured arylsulfatase) urine sample set; A) Scaled and centred principle component analysis (PCA) plot; B) Partial least square discriminant analysis (PLS-DA) plot.

General

All non-aqueous reactions were performed using flame- or oven dried glassware under an atmosphere of dry nitrogen. All reagents and solvents were purchased from Sigma-Aldrich or Fischer Scientific and were used without further purification. HPLC grade solvents were used for HPLC purification and mass spectrometry grade for UHPLC-ESI-MS analysis. All biochemical reactions were performed with HPLC or LC-MS grade solvents. Solutions were concentrated *in vacuo* on a Heidolph or a IKA rotary evaporator, or in a Speedvac Concentrator Plus System (Eppendorf, Hamburg, Germany). Protein purifications were performed on an ÄKTA Start (GE Healthcare Life Sciences, Chicago, Illinois, USA). Thin Layer Chromatography (TLC) was performed on silica gel 60 F-254 plates. Visualization of the developed chromatogram was performed using fluorescence quenching or staining with CAM (Cerium Ammonium Molybdate), Ninhydrin, Ehrlich reagent (4-(Dimethylamino)benzaldehyde) or Vanillin. Chromatographic purification of products was accomplished using flash column chromatography on Merck silica gel 60 (40−63 μm) or preparative reverse phase HPLC on an Agilent HPLC-1100 series system equipped with a Waters Atlantis T3 preparative column (5 μm, 10×100 mm) at a 2.5 mL/min flow rate. All synthesized compounds were ≥95% pure as determined by NMR. NMR spectra were recorded on an Agilent 400 MHz spectrometer (^{1}H NMR: 399.97 MHz, ^{13}C NMR: 100.58 MHz) or Bruker Ascend™ 600 MHz spectrometer (^{1}H NMR: 600.18 MHz, ^{13}C NMR: 150.92 MHz). Chemical shifts are reported in parts per million (ppm) on the δ scale from an internal standard. Multiplicities are abbreviated as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. High-resolution mass spectra were acquired on a SYNAPT G2-S High Definition Mass Spectrometry (HDMS) using an electrospray ionization (ESI) source with a AQCUITY UPLC I-class system and equipped with a Waters ACQUITY UPLC® BEH C18 column (1.7 μm, 100×2.1 mm) or Waters ACQUITY UPLC® HSS T3 column (1.8 μm, 100×2.1 mm).

Human Samples

Patient fecal and healthy donor urine samples were obtained in accordance with the World Medical Association Declaration of Helsinki and all patients gave written informed consent. Approval for the study was obtained from the ethical committee at Karolinska Institutet Hospital (Ethical approval number: Dnr 2017/290-31). Fecal and urine samples were collected using routine clinical collection protocols and all patient codes have been removed in this publication. All samples were stored at -80 °C.

UHPLC-MS/MS analysis

Mass spectrometric analysis was performed on an Acquity UPLC system connected to a Synapt G2 Q-TOF mass spectrometer, both from Waters Corporation (Milford, MA, USA). The system was controlled using the MassLynx software package v 4.1, also from Waters. The separation was performed on an Acquity UPLC® BEH C18 column (1.7 µm, 100×2.1 mm) and an Acquity UPLC® HSS T3 column (1.8 µm, 100×2.1 mm) from Waters Corporation. The mobile phase consisted of A) 0.1% formic acid in MilliQ water and B) 0.1% formic acid in LCMS-grade methanol. The column temperature was 40 °C and two different gradients were applied: for the T3 column; 0-2 min, 0% B; 2-15 min, 0-100% B; 15-18 min, 100% B; 18-20 min, 100-0% B; 20-25 min, 0% B, with a flow rate of 0.2 mL/min. For the C18 column the gradient was 0-8.5 min, 0-100% B; 8.5-10 min, 100% B; 10-12 min, 100-0% B; 12-17 min, 0% B, with a flow rate of 0.3 mL/min.

The samples were introduced into the q-TOF using negative electrospray ionization. The capillary voltage was set to -2.50 kV and the cone voltage was 40 V. The source temperature was 100 °C, the cone gas flow 50 L/min and the desolvation gas flow 600 L/h. The instrument was operated in MSE mode, the scan range was m/z = 50-1200, and the scan time was 0.3 s. In low energy mode, the collision energy was 10 V and in high energy mode the collision energy was ramped between 25-45 V. A solution of sodium formate (0.5 mM in 2-propanol:water, 90:10, v/v) was used to calibrate the instrument and a solution of leucine-encephalin (2 ng/µl in acetonitrile: 0.1% formic acid in water, 50:50, v/v) was used for the lock mass correction at an injection rate of 30 s.

Arylsulfatase purification

Buffer A	Buffer B	Buffer C	Buffer D
50 mM Tris-HCl buffer, pH 7 20 mM NaCl 10% Glycerol 1 mM DTT	50 mM Tris-HCl buffer, pH 7 500 mM NaCl 10% Glycerol 1 mM DTT	50 mM sodium Phosphate buffer, pH 7 1.5 M ammonium sulfate	50 mM sodium Phosphate buffer, pH 7

Crude preparation

The crude arylsulfatase mixture from *Helix pomatia* (1 g) was dissolved in buffer A and centrifuged; the supernatant was collected and filtered through Zeba™ Spin Desalting Columns (7K MWCO, 5 mL). The resulting solution was then filtered using syringe filters (Whatman™ 30mm SPARTAN™ HPLC certified).

Purification

The crude mixture was purified using to a Hitrap Q HP, 5 × 5 column, at a flow rate of 2 mL/min (ÄKTA start). Four different fractions were collected using isocratic step elution (10%, 20%, 40%, and 100% of buffer B) after equilibration of the column. All fractions were analyzed by SDS-PAGE and tested for sulfatase and glucuronidase activity (Detailed description in the *enzymatic assay* section). The fraction collected at a concentration of 10% buffer B contained the highest sulfatase activity. This fraction was then subjected to a buffer exchange into buffer C using a HiPrep 26/10 desalting column, at a flow rate of 5 mL/min. The solution from the buffer exchange was injected into a HiTrap butyl HP, 5 x 5 column and was eluted over a 50 mL salt gradient (high to low salt concentration). SDS-PAGE and enzymatic assay were used to determine sulfatase and glucuronidase activity. The relevant fractions in the first eluting peak were pooled and buffer exchanged into 50 mM ammonium acetate buffer, pH 7.0. The last purification step for the sulfatase was performed using a HiLoad 16/600 Superdex 200 pg with a 50 mM ammonium acetate buffer, pH 7.0 (Fig. S2). The relevant fractions were pooled and concentrated after SDS-PAGE analysis (Fig. 2A).

Enzymatic assay

At every step of the purification the eluent was tested for sulfatase and glucuronidase activity to determine which fractions contain the purified enzyme, and compared with the crude arylsulfatase mixture. We used 4-methylumbellifery sulfate (**4**) for sulfatase activity and *N*-acetyltyramine-*O,β*-glucuronide (**12**) as standards for sulfatase and glucuronidase activity respectively. For each enzymatic assay, 25 µL of a 1 mM solution of **4** in H_2O, 25 µL of a 1 mM solution of **12** in H_2O, 50 mM ammonium acetate (150 µL, pH 7.0) and 25 µL of each purified fraction were mixed. In parallel, we tested the stability of our standard to hydrolysis in the absence of enzyme, in order to confirm specific enzyme hydrolysis. Five time-points were collected at 0 min, 30 min, 1 h, 2 h, and 24 h for each assay.

At every time-point, 25 µL of the assay mix were collected and the proteins were precipitated with 100 µL of cold methanol for 15 min at 4 °C. After centrifugation at 13400 rpm for 5 min at room temperature, the supernatant was collected and dried *in vacuo*. The pellet was resuspended in 60 µl of a water/acetonitrile mixture (95/5, v/v). The samples were transferred to HPLC-vials and injected into the UPLC-MS/MS system for mass spectrometric analysis.

Determination of sulfatase activity

The arylsulfatase activity assay was based on the assay described for the *Helix pomatia* arylsulfatase (S9626, Sigma-Aldrich). Briefly, for each enzymatic assay, 65 µL of 200 mM sodium acetate buffer pH 5 and 40 µL of a 6.25 mM aqueous solution of 4-nitrocatechol sulfate were mixed. To this mixture were added 5, 7 or 10 µL of 50 times diluted purified arylsulfatase. At the same time, an assay was performed without any enzyme, as a negative control. The mixtures were

incubated for 30 min at 37 °C. After incubation, 500 µL of 1 M NaOH were added to the reaction and the resulting solutions were transferred into a 96-well plate. Their absorbance was measured at a wavelength of 515 nm.

The amount of units in solution was calculated using the following equation:

$$Units/mL = \frac{(A_{\text{Test}} - A_{\text{Blank}}) \times df \times \text{V}_T}{\varepsilon_{515} \times V_E \times \text{t}}$$

Details:
A_{Test} – Absorbance measured for the test solutions at 515 nm
A_{Blank} – Absorbance measured for the blank at 515 nm
t – Time factor correction (Unit definition for 1 hour)
df – Protein dilution factor
V_T – Total volume (in mL) of the assay
ε_{515} – Milimolar extinction coefficient of *p*-nitrocatechol at 515 nm ($\mu m^{-1}cm^{-1}$)
V_E – Volume (in mL) of purified arylsulfatase used

Determination of glucuronidase activity

Glucuronidase activity was tested according to the protocol described by Sigma Aldrich (S9626). In order to calculate the activity of glucuronidase in solution, 65 µL of H_2O were mixed with 50 µL of 75 mM potassium phosphate buffer with 1% (w/v) bovine serum albumin, pH 6.8, 25 µL of 3 mM of phenolphthalein-glucuronide and 10 µL of enzyme test solution. A negative control was also tested, in which no enzyme was added. To stop the reaction, 500 µL of 200 mM glycine buffer, pH 10.4 were added. The resulting solution was transferred to a 96-well plate and the absorbance at 540 nM was measured to monitor the production of phenolphthalein.

At the same time, a phenolphthalein standard curved was prepared, with a ranging quantity of 1-5 µg. The amount of phenolphthalein was plotted against the A_{540} value and test results were based on the measured absorbance.

The amount of units in solution was calculated using the following equation:

$$Units/mL = \frac{(\mu g\ of\ phenolphthalein\ released) \times df}{V_E \times t}$$

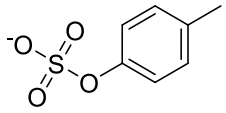
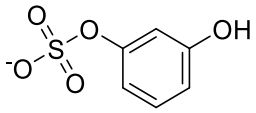
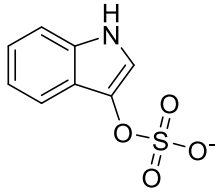
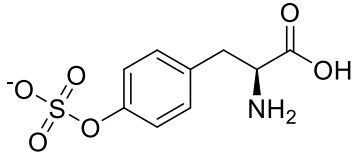
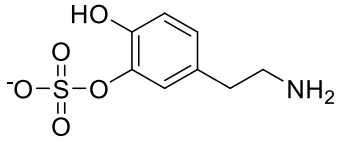
Details:
t – Time factor correction (Unit definition for 1 hour)
df – Protein dilution factor
V_E – Volume (in mL) of purified arylsulfatase used

Data analysis

Data analysis was performed using the XCMS metabolomics software package under R (version 1.1.414), using a script designed to identify features with a *m/z* difference of 79.9568 Da.[1-2] The results were processed using Excel 2016 with applied parameter thresholds to simplify the data set and selectively identify sulfate esters. We applied a 1.5 fold-change in favour of the control group, a p-value cut-off of 0.0001 and an intensity level higher than a 30,000 ion count. The sulfate esters were confirmed by MS/MS fragmentation experiments. In low energy mode, the collision energy was 10 V and in high energy mode the collision energy was ramped from 30-40 V.

Supplementary Tables

Table S1 Validated sulfated metabolites by chemical synthesis and UPLC-MS/MS co-injection in urine samples. Sulfated metabolites previously only described in literature as unsulfated compounds are highlighted with (*).

				m/z			RT /min	
#	Name	Structure	Chemical Formula	Experimental	Theoretical	ppm difference	Urine	Synthesized
1	*p*-Cresyl sulfate (**2**)		$C_7H_7O_4S^-$	187.0072	187.0071	0.5	8.64	8.67
2*	Resorcinol monosulfate		$C_6H_5O_5S^-$	188.9857	188.9863	3.2	5.40	5.40
3	Indoxyl sulfate (**1**)		$C_8H_6NO_4S^-$	212.0016	212.0023	3.3	7.20	7.23
4	L-Tyrosine-*O*-sulfate (**9**)		$C_9H_{10}NO_6S^-$	260.0247	260.0234	5.0	4.82	4.82
5	Dopamine 3-sulfate		$C_8H_{10}NO_5S^-$	232.0285	232.0285	0	4.82	4.82

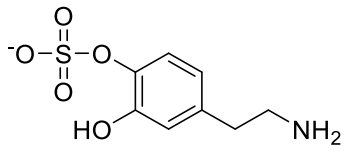
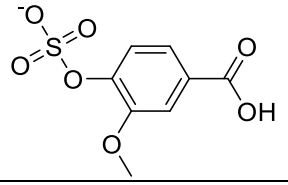
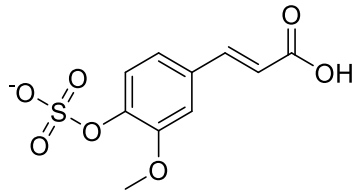
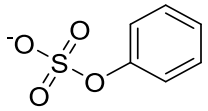
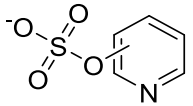
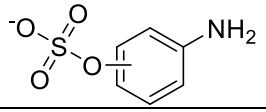
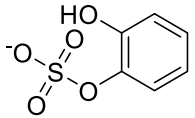
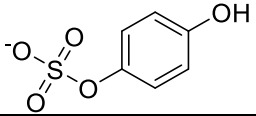
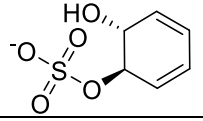
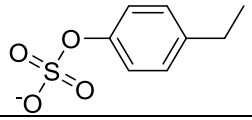
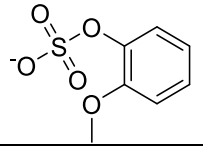
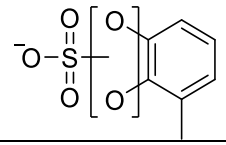
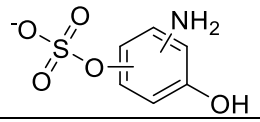
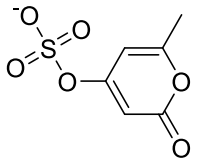
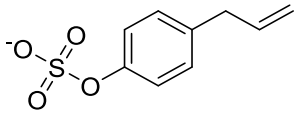
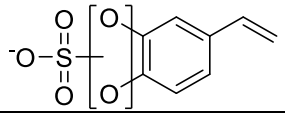
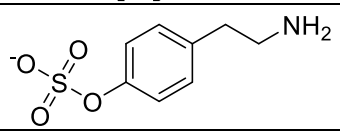
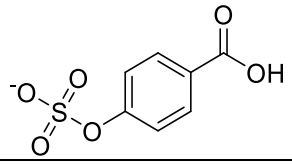
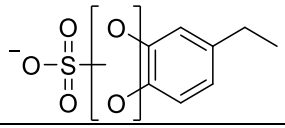
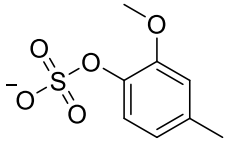
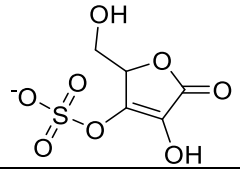
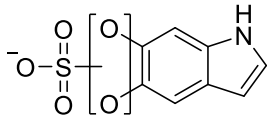
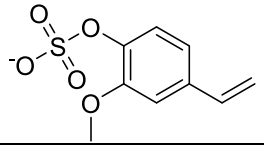
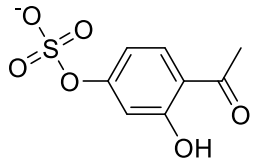
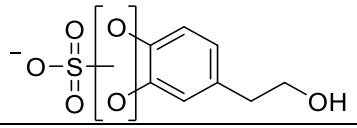
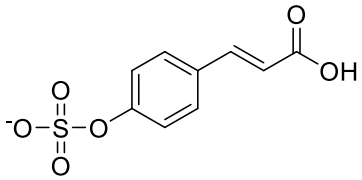
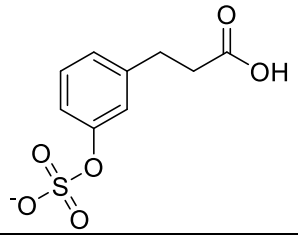
6	Dopamine 4-sulfate		$C_8H_{10}NO_5S^-$	232.0285	232.0285	0	4.98	4.98
7	Vanillic acid 4-sulfate (**15**)		$C_8H_7O_7S^-$	246.9919	246.9918	0.4	6.83	6.83
8	Ferulic acid 4-sulfate (**3**)		$C_{10}H_9O_7S^-$	273.0073	273.0074	0.4	8.05	8.05

Table S2 Tentative structures for sulfate ester identification based on MS/MS fragmentation and database comparison. Sulfated metabolites previously only described in literature as unsulfated compounds are highlighted with (*).

				m/z			
#	Name		Chemical Formula	Experimental	Theoretical	ppm difference	RT /min
1	Phenol sulfate		$C_6H_5O_4S^-$	172.9923	172.9914	5.2	6.57
2	Hydroxypyrine sulfate		$C_5H_5NO_4S^-$	173.9866	173.9867	0.6	2.51
3	Aminophenol sulfate		$C_6H_6NO_4S^-$	188.0010	188.0023	6.9	3.23
4	Pyrocatechol sulfate		$C_6H_5O_5S^-$	188.9860	188.9863	1.6	4.38 6.07
5	Hydroquinone Sulfate			188.9865		1.1	
6	Trans-1,2-dihydrobenzene-1,2-diol monosulfate		$C_6H_7O_5S^-$	191.0016	191.0020	2.1	5.50
7	4-Ethylphenylsulfate (**17**)		$C_8H_9O_4S^-$	201.0226	201.0227	0.5	10.06
8	2-Methoxyphenyl sulfate		$C_7H_7O_5S^-$	203.0024	203.0020	2.0	7.66

9*	2-Hydroxy-3-methylphenyl sulfate		$C_7H_7O_5S^-$	203.0034	203.0020	6.9	7.90
10*	Aminohydroxyphenyl sulfate		$C_6H_6NO_5S^-$	203.9969	203.9972	1.5	2.88
11	Triacetone lactone sulfate		$C_6H_5O_6S^-$	204.9813	204.9812	0.5	6.16
12*	Chavicol sulfate		$C_9H_9O_4S^-$	213.0223	213.0227	1.9	10.56
13*	Vinylbenzene diol sulfate		$C_8H_7O_5S^-$	215.0023	215.0020	1.4	9.08
14	Tyramine sulfate		$C_8H_{10}NO_4S^-$	216.0340	216.0336	1.9	4.97
15	4-Hydroxybenzoic acid sulfate		$C_7H_5O_6S^-$	216.9808	216.9812	1.8	6.80
16*	Ethylcatechol sulfate		$C_8H_9O_5S^-$	217.0186	217.0176	4.6	8.70
17*	Creosol sulfate		$C_8H_9O_5S^-$	217.0176	217.0176	0	9.60

18*	Erythro-ascorbic acid sulfate		$C_5H_5O_8S^-$	224.9705	224.9711	2.7	2.16
19	Dihydroxyindole sulfate		$C_8H_6NO_5S^-$	227.9965	227.9972	3.1	5.75
20*	2-Methoxy-4-vinylphenol sulfate		$C_9H_9O_5S^-$	229.0178	229.0176	0.9	10.16
21	2,4-Dihydroxyacetophenone 5-sulfate		$C_8H_7O_6S^-$	230.9967	230.9969	0.9	7.25
22	Hydroxytyrosol sulfate		$C_8H_9O_6S^-$	233.0147	233.0125	9.4	6.22
23	*p*-Coumaric acid sulfate		$C_9H_7O_6S^-$	242.9970	242.9969	0.4	8.27
24	3-Hydroxyphenylpropionic acid sulfate (**16**)		$C_9H_9O_6S^-$	245.0128	245.0125	1.2	7.79

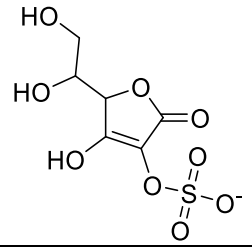
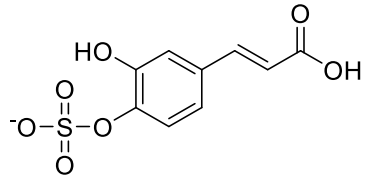
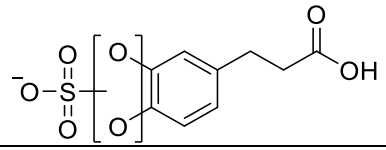
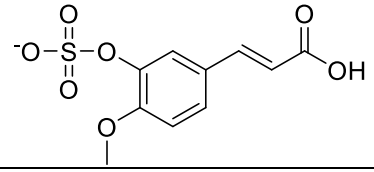
25	Ascorbic acid 2-sulfate		$C_6H_8O_9S^-$	254.9814	254.9816	0.8	2.13
26	Caffeic acid 4-sulfate (**14**)		$C_9H_7O_7S^-$	258.9919	258.9918	0.4	7.52
27	Dihydroxyhydrocinnamic acid sulfate		$C_9H_9O_7S^-$	261.0083	261.0047	14	7.30
28	Isoferulic acid 3-sulfate		$C_{10}H_9O_7S^-$	273.0071	273.0074	1.1	8.36

Table S3 Metabolites containing a validated sulfate ester through MS/MS fragmentation analysis in negative mode.

#	m/z	RT /min
1	161.0456	1.54
2	162.9693	3.78
3	172.9906	6.21
4	173.0078	3.23
5	173.0457	1.47
6	176.9357	1.22
7	177.0402	1.54
8	179.0563	1.54
9	187.0065	6.68
10	187.0069	6.86
11	187.9872	7.82
12	191.0179	2.20
13	191.0185	3.23
14	191.0562	1.48
15	192.9806	3.10
16	192.9812	4.67
17	193.0634	12.8
18	197.9857	7.24
19	199.0067	4.80
20	203.0016	6.30
21	203.0079	6.80
22	204.9808	5.52
23	204.9812	4.67
24	205.0352	3.80
25	206.9965	5.96
26	215.0013	7.44
27	215.0013	7.85
28	215.0016	7.57
29	216.9055	1.19
30	216.9805	6.21
31	217.0173	6.58
32	217.0173	6.91
33	217.0176	7.20
34	218.0119	4.67
35	218.9958	6.80
36	218.9960	7.44
37	218.9968	5.28
38	218.9990	5.35
39	220.9758	5.83
40	222.9914	3.10
41	222.9924	3.50
42	227.9964	6.39
43	228.0645	4.67
44	229.0180	9.01
45	229.0183	9.55
46	230.0120	7.13
47	230.0122	5.99
48	230.9928	7.49
49	230.9968	6.68
50	231.0329	9.74
51	231.0332	10.8
52	233.0119	7.49
53	233.0147	5.92
54	239.9960	6.63
55	242.0121	7.37
56	242.0133	8.00
57	242.0143	8.13
58	242.1210	6.54
59	242.1210	6.73
60	242.9961	7.56
61	242.9963	7.77
62	242.9976	7.30
63	243.9920	5.17
64	243.9920	5.77
65	245.0126	8.74
66	246.0438	5.35
67	246.9940	6.34
68	247.0048	5.35
69	247.0267	7.33
70	247.0279	6.81
71	247.0280	7.23
72	247.0280	7.41
73	247.0287	8.65
74	247.0504	6.53
75	249.0066	5.35
76	251.0014	7.76
77	254.0121	10.1

78	256.9753	7.50
79	258.0076	6.71
80	258.0440	4.98
81	258.0444	7.24
82	258.9915	7.29
83	258.9922	7.69
84	260.0229	7.52
85	261.0074	6.59
86	261.0076	6.91
87	264.9886	14.8
88	269.0129	10.5
89	270.0275	10.5
90	273.0071	9.11
91	273.0078	9.69
92	273.0079	8.74
93	273.0433	9.37
94	273.0442	10.3
95	273.0443	9.77
96	274.0027	5.99
97	274.0390	6.77
98	275.0206	9.63
99	275.0225	9.11
100	275.0225	9.37
101	277.0005	7.84
102	277.0013	7.19
103	277.9980	7.73
104	283.0283	9.47
105	285.0063	8.70
106	287.0222	8.80
107	287.0227	7.85
108	287.0273	7.64
109	290.9995	9.60
110	293.0154	9.65
111	297.0442	9.33
112	305.0340	7.30
113	309.0435	9.79
114	311.0231	9.57
115	313.0490	6.20
116	323.0595	9.39
117	323.0595	9.60
118	330.0295	7.36
119	338.0700	10.6
120	338.0703	10.7
121	340.0800	11.0
122	342.1015	10.4
123	350.0701	11.8
124	351.0734	11.7
125	352.0877	11.5
126	356.1173	11.2
127	366.1017	11.3
128	366.1017	11.4
129	368.0797	9.97
130	368.0812	9.44
131	368.0818	9.25
132	370.0970	9.25
133	372.1135	9.26
134	378.1017	12.6
135	378.1024	12.3
136	387.9949	2.20
137	389.0919	10.7
138	395.0287	6.52
139	398.1271	10.3
140	398.1280	10.7
141	398.1280	10.9
142	400.1436	10.9
143	403.1070	9.61
144	426.1588	11.7
145	445.0807	10.0
146	446.1126	9.75
147	447.0952	10.0
148	451.0549	8.27
149	459.0610	10.3
150	528.1186	9.90
151	532.1497	9.90

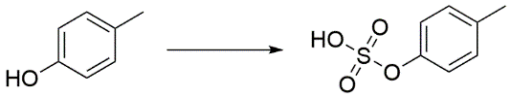
Table S4 List of chemically validated, tentative and sulfate ester containing metabolites in fecal samples analysed in negative mode.

#	Id. molecule	Chemical Formula	m/z	Rt /min
1	Phenyl sulfate	$C_6H_4O_4S^-$	172.9921	6.83
2	*p*-Cresyl sulfate (**2**)	$C_7H_7O_4S^-$	187.0071	4.07
3		$C_6H_5O_5S^-$	188.9860	6.31
4		$C_6H_7N_2O_4S^-$	203.0090	6.50
5		$C_8H_7O_5S^-$	215.0010	7.61
6		$C_8H_7O_5S^-$	215.0020	9.12
7		$C_8H_7O_5S^-$	215.0021	7.92
8		$C_7H_4NO_6S^-$	229.9766	6.19
9	Dihydroxybenzoate sulfate	$C_7H_5O_7S^-$	232.9763	6.13
10		$C_9H_8NO_5S^-$	242.0128	6.90
11		$C_9H_7O_6S^-$	242.9965	7.86
12		$C_9H_9O_6S^-$	245.0123	7.68
13		$C_{10}H_9O_6S^-$, $C_8H_7N_3O_5S^-$	257.0115	8.68
14		$C_9H_7O_7S^-$	258.9907	7.61
15		$C_9H_7O_7S^-$	258.9917	7.89
16	L-Tyrosine-*O*-sulfate (**9**)	$C_9H_{10}NO_6S^-$	260.0234	1.60
17	Ferulic acid 4-sulfate (**3**)	$C_{10}H_9O_7S^-$	273.0066	8.01
18		$C_{10}H_{97}S^-$	273.0066	8.54
19		$C_{12}H_{13}N_2O_4S^-$	281.0599	5.85
20		$C_{12}H_{13}O_6S^-$	285.0432	10.7
21		$C_{11}H_{13}O_9S^-$, $C_{12}H_{10}N_4O_5S^-$	321.0285	7.23
22		$C_7H_6N_3O_{10}S^-$	323.9766	6.32
23		$C_{12}H_{17}N_2O_7S^-$, $C_{13}H_{13}N_6O_3S^-$	333.0757	6.76
24		$C_7H_{11}N_6O_6S_2^-$, $C_{14}H_{11}O_8S^-$, $C_{15}H_7N_4O_4S^-$	339.0180	9.81
25		$C_{14}H_{15}N_2O_6S^-$, $C_{10}H_{11}N_8O_4S^-$	339.0650	6.83
26		$C_{12}H_{13}O_6S^-$	352.0852	11.2
27		$C_{12}H_{13}O_6S^-$	352.0852	11.3
28		$C_{20}H_{13}N_4O_3S^-$, $C_{19}H_{27}O_7S^-$	399.1494	13.5
29		$C_9H_{17}N_6O_8S_2^-$, $C_{16}H_{17}O_{10}S^-$, $C_{17}H_{13}N_4O_6S^-$	401.0547	8.42
30		$C_{21}H_{11}O_6S_2^-$	423.0027	12.1
31		$C_{13}H_9N_{10}O_6S^-$, $C_9H_{17}N_6O_{10}S_2^-$	433.0437	7.04
32		$C_9H_{17}N_6O_{10}S_2^-$, $C_{13}H_9N_{10}O_6S^-$, $C_{17}H_{13}N_4O_8S^-$	433.0439	6.67
33		$C_{13}H_9N_{10}O_6S^-$, $C_9H_{17}N_6O_{10}S_2^-$	433.0445	7.78
34		$C_{19}H_{29}O_9S^-$, $C_{20}H_{26}N_4O_5S^-$	433.1532	6.88

35		$C_{13}H_{22}N_6O_7S_2^-$, $C_{20}H_{23}O_9S^-$, $C_{21}H_{20}N_4O_5S^-$	439.1068	12.0
36		$C_{21}H_{29}O_9S^-$, $C_{22}H_{25}N_4O_5S^-$	457.1532	13.2
37		$C_{19}H_{35}N_8O_5S^-$	487.2448	14.3
38		$C_{24}H_{36}N_3O_8S^-$, $C_{20}H_{32}N_9O_6S^-$	526.2217	15.2
39		$C_{27}H_{38}N_5O_4S^-$, $C_{26}H_{42}NO_8S^-$	528.2643	12.7
40		$C_{27}H_{38}N_5O_4S^-$	528.2690	13.5
41		$C_{23}H_{21}O_{10}S_3^-$, $C_{16}H_{13}N_{10}O_9S_2^-$, $C_{24}H_{17}N_4O_6S_3^-$	553.0301	9.08

Chemical Synthesis

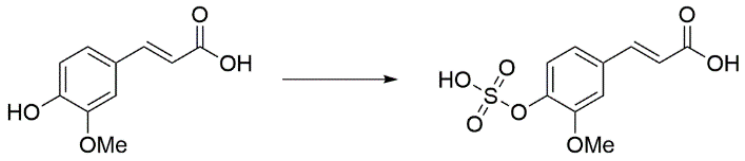
Synthesis of *p*-cresyl sulfate (2)[3]



p-Cresyl sulfate was synthesized according to the procedure by Paulson *et al*. Briefly, to a solution of *p*-cresol (20 mg, 0.19 mmol) in anhydrous pyridine (1 mL) at 0 °C was added dropwise $ClSO_3H$ (17 µL, 0.26 mmol, 1.4 eq) over 10 min. The mixture was stirred at 0 °C for 30 min, warmed up to room temperature and stirred for an additional 1 h. Upon full consumption of all starting material, the reaction mixture was adjusted to slightly basic pH with 1 M NaOH and concentrated *in vacuo*. The crude product was subjected to HPLC purification [r.t. = 10 min, 0-5 min (0% B), 5-20 min (0-100% B) at a flow of 2.5 mL/min; buffer A = ammonium acetate 5 mM (water) and buffer B = ammonium acetate 5 mM (MeOH)] to afford *p*-cresyl sulfate **1** (29 mg, 0.15 mmol, 79%) as a white solid.

^{1}H NMR (400 MHz, CD_3OD) δ (ppm) = 2.30 (3H, s), 7.18 – 7.11 (4H, m); ^{13}C NMR (151 MHz, CD_3OD) δ (ppm) = 19.33, 120.93, 129.03, 133.97, 150.54; HRMS (ESI$^-$) calculated for $C_7H_7O_4S^-$ $(M-H)^-$: 187.0071, found: 187.0072.

Synthesis of ferulic acid 4-sulfate (3)

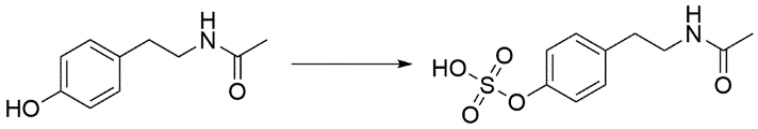


To a solution of ferulic acid (100 mg, 0.51 mmol, 1 eq) and NaOH (61.8 mg, 1.54 mmol, 3 eq) in water (6 mL), was added $NaHCO_3$ (173 mg, 2.06 mmol, 4 eq) and $SO_3{\cdot}NMe_3$ complex (179 mg,

1.29 mmol, 2.5 eq). The reaction mixture was stirred at room temperature for four days and concentrated *in vacuo*. The crude product was subjected to HPLC purification [r.t. = 4 min, 0-5 min (0% B), 5-20 min (0-100% B) at a flow of 2.5 mL/min; buffer A = ammonium acetate 5 mM (water) and buffer B = ammonium acetate 5 mM (MeOH)] to afford ferulic acid 4-sulfate **3** (140 mg, 0.51 mmol, >99%) as a white solid.

^{1}H NMR (400 MHz, CD_3OD) δ (ppm) = 3.88 (3H, s), 6.44 (1H, d, *J* = 15.9 Hz), 7.11 (1H, dd, *J* = 8.3, 2.0 Hz), 7.23 (1H, d, *J* = 1.9 Hz), 7.49 (2H, m); ^{13}C NMR (151 MHz, CD_3OD) δ (ppm) = 55.18, 111.28, 120.08, 122.19, 122.79, 132.99, 140.53, 142.66, 151.93, 172.80; HRMS (ESI^-) calculated for $C_{10}H_9O_7S^-$ $(M-H)^-$: 273.0074, found: 273.0070.

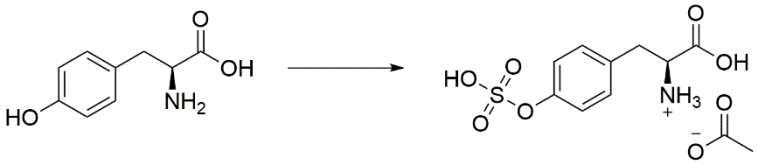
Synthesis of *N*-acetyltyramine-*O*-sulfate (8)



To a solution of previously synthesized *N*-acetyltyramine[4] (20.0 mg, 0.112 mmol, 1.0 eq) and NaOH (13.4 mg, 0.33 mmol, 3.0 eq) in water (1 mL), was added $NaHCO_3$ (37.5 mg, 0.45 mmol, 4.0 eq) and $SO_3{\cdot}NMe_3$ complex (38.8 mg, 0.28 mmol, 2.5 eq). The reaction mixture was stirred at room temperature for 3 days and concentrated *in vacuo*. The crude product was subjected to HPLC purification [r.t. = 11 min, 0-5 min (0% B), 5-20 min (0-100 %B) at a flow of 2.5 mL/min; buffer A = ammonium acetate 5 mM (water) and buffer B = ammonium acetate 5 mM (MeOH)] to afford *N*-acetyltyramine *O*-sulfate **5** (15.5 mg, 0.060 mmol, 54%) as a white solid.

^{1}H NMR (600 MHz, CD_3OD) δ (ppm) = 1.90 (3H, s), 2.77 (2H, t, *J* = 7.2 Hz), 3.38 (2H, t, *J* = 7.2 Hz), 7.26 – 7.10 (4H, m); ^{13}C NMR (151 MHz, CD_3OD) δ (ppm) = 21.08, 34.37, 40.70, 121.22, 129.02, 135.67, 151.15, 171.86; HRMS (ESI^-) calculated for $C_{10}H_{12}NO_5S^-$ $(M-H)^-$: 258.0442, found: 258.0446.

Synthesis of L-tyrosine-*O*-sulfate (9)[5]

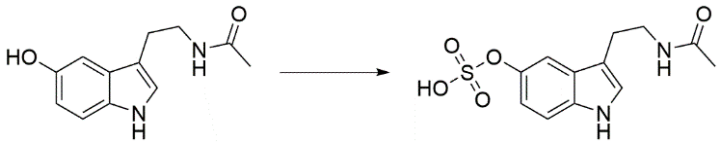


To H_2SO_4 (1.1 mL, 20.6 mmol, 18.7 eq) stirred at -5 °C in a conical-flask, was slowly added L-tyrosine (200 mg, 1.1 mmol) within 10 min. After an additional stirring period of 30 min, the reaction mixture was neutralized in a cold 2 M aqueous solution of NaOH under vigorous stirring

and concentrated *in vacuo*. The crude product was subjected to HPLC purification [r.t. = 5 min, 0-5 min (0% B), 5-20 min (0-100% B) at a flow of 2.5 mL/min; buffer A = ammonium acetate 5 mM (water) and buffer B = ammonium acetate 5 mM (MeOH)] to afford L-tyrosine-*O*-sulfate acetate **6** (10 mg, 0.038 mmol, 4%) as a white solid.

^{1}H NMR (600 MHz, CD_3OD) δ (ppm) = 1.92 (3H, s, CH_3CO_2H counter-ion), 2.92 (1H, dd, *J* = 14.6, 10.0 Hz), 3.38 (1H, dd, *J* = 14.6, 3.9 Hz), 3.75 (1H, dd, *J* = 10.0, 3.9 Hz), 7.28 (4H, s); ^{13}C NMR (151 MHz, CD_3OD) δ (ppm) = 21.18 (CH_3CO_2H counter-ion), 36.29, 56.50, 121.74, 129.63, 132.51, 152.08, 172.20, 176.47 (CH_3CO_2H counter-ion); HRMS (ESI$^-$) calculated for $C_9H_{10}NO_6S^-$ $(M-H)^-$: 260.0234, found: 260.0237.

Synthesis of *N*-acetylserotonine-*O*-sulfate (11)



To a suspension of serotonine hydrochloride (1 g, 4.70 mmol, 1.0 eq) in saturated aqueous $NaHCO_3$ (6.5 mL) was added dropwise acetic anhydride (0.45 mL, 4.8 mmol, 1.0 eq). The reaction mixture was stirred for 5 min and allowed to rest for 10 min before EtOAc extraction. The organic layers were dried over $MgSO_4$, concentrated *in vacuo* and purified by silica gel chromatography (CH_2Cl_2/MeOH 99:1 up to 95:5) affording *N*-acetylserotonine, which was directly consumed in the next reaction. To a solution of *N*-acetylserotonine (20.0 mg, 0.092 mmol, 1.0 eq) and NaOH (11.0 mg, 0.27 mmol, 3.0 eq) in water (1 mL), was added $NaHCO_3$ (30.8 mg, 0.37 mmol, 4.0 eq) and $SO_3{\cdot}NMe_3$ complex (31.9 mg, 0.23 mmol, 2.5 eq). The reaction mixture was stirred at room temperature for 3 d and concentrated *in vacuo*. The crude product was subjected to HPLC purification [r.t. = 12 min, 0-5 min (0% B), 5-20 min (0-100% B) at a flow of 2.5 mL/min; buffer A = ammonium acetate 5 mM (water) and buffer B = ammonium acetate 5 mM (MeOH)] to afford *N*-acetylserotonine *O*-sulfate **8** (7.1 mg, 0.024 mmol, 26%) as a white solid.

^{1}H NMR (400 MHz, CD_3OD) δ (ppm) = 1.91 (3H, s), 2.91 (2H, t, *J* = 7.2 Hz), 3.46 (2H, t, *J* = 7.2 Hz), 7.08 (1H, s), 7.09 (1H, dd, *J* = 8.7, 2.2z Hz), 7.26 (1H, d, *J* = 8.7 Hz), 7.49 (1H, d, *J* = 2.2 Hz); ^{13}C NMR (151 MHz, CD_3OD) δ (ppm) = 21.20, 24.72, 40.03, 110.52, 110.72, 112.33, 116.05, 123.23, 127.41, 134.36, 145.43, 171.89; HRMS (ESI$^-$) calculated for $C_{12}H_{13}N_2O_5S^-$ $(M-H)^-$: 297.0551, found: 297.0554.

References

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4. Globisch, D.; Moreno, A. Y.; Hixon, M. S.; Nunes, A. A. K.; Denery, J. R.; Specht, S.; Hoerauf, A.; Janda, K. D., Onchocerca volvulus-neurotransmitter tyramine is a biomarker for river blindness. *Proc. Natl. Acad. Sci. U. S. A.* **2013,** *110* (11), 4218-4223.
5. Dodgson, K. S.; Rose, F. A.; Tudball, N., Studies on sulphatases. 23. The enzymic desulphation of tyrosine O-sulphate. *Biochem. J.* **1959,** *71* (1), 10-15.

NMR Spectra

Pages S23-S32:

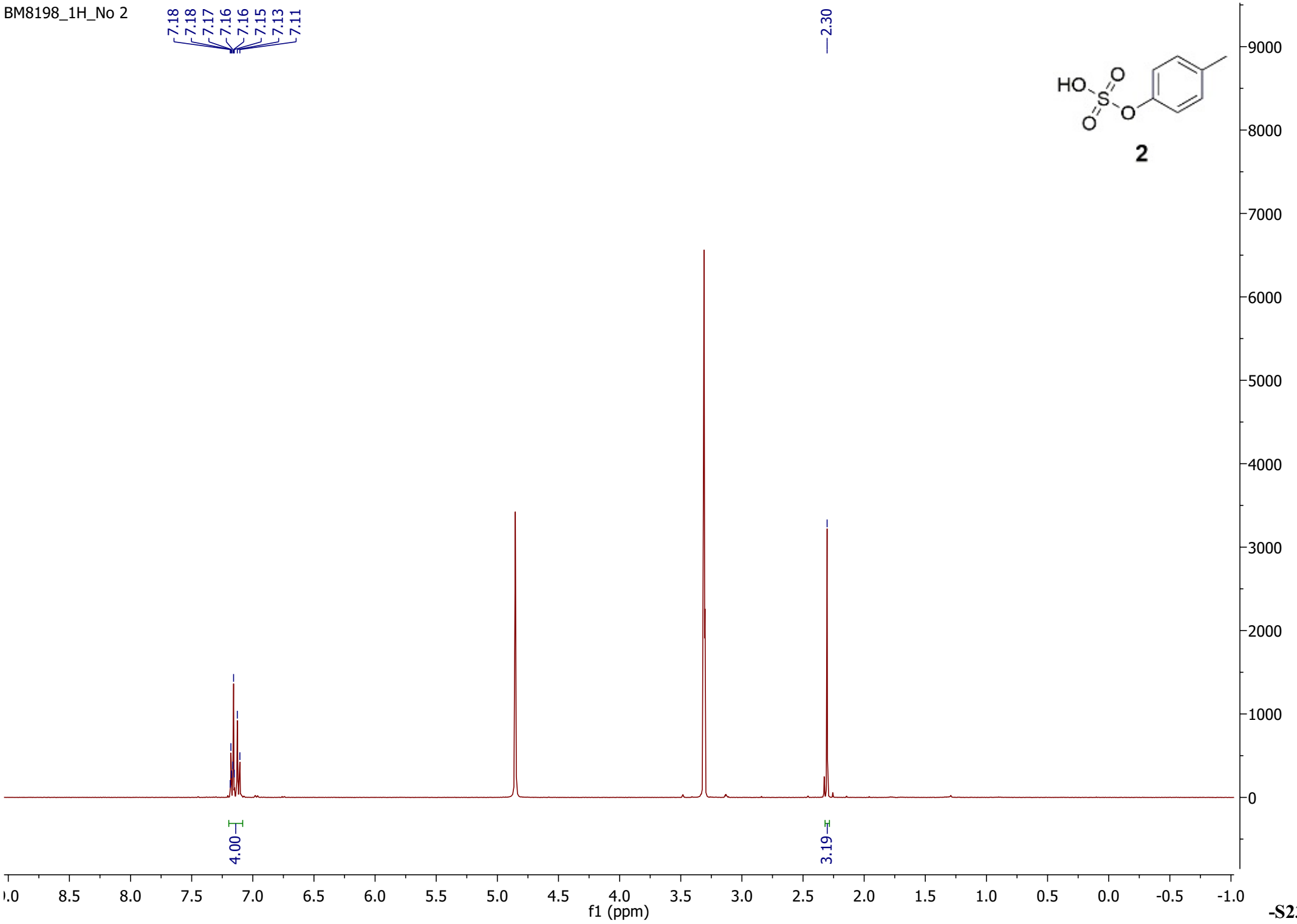
p-cresyl sulfate (**2**)
^{1}H NMR, ^{13}C NMR

Ferulic acid 4-sulfate (**3**)
^{1}H NMR, ^{13}C NMR

N-acetyltyramine-*O*-sulfate (**8**)
^{1}H NMR, ^{13}C NMR

L-tyrosine-*O*-sulfate (**9**)
^{1}H NMR, ^{13}C NMR

N-acetylserotonine-*O*-sulfate (**11**)
^{1}H NMR, ^{13}C NMR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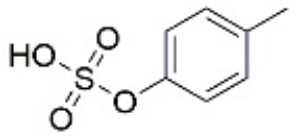
7.17
7.16
7.16
7.15
7.13
7.11
2.30
HO
S
O
O
O
2
4.00
3.19
f1 (ppm)

BM8198_13C_No 2

150.54
133.97
129.03
120.93
19.33



2

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2.8E+11
2.6E+11
2.4E+11
2.2E+11
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1.8E+11
1.6E+11
1.4E+11
1.2E+11
1.0E+11
8.0E+10
6.0E+10
4.0E+10
2.0E+10
0.0E+00
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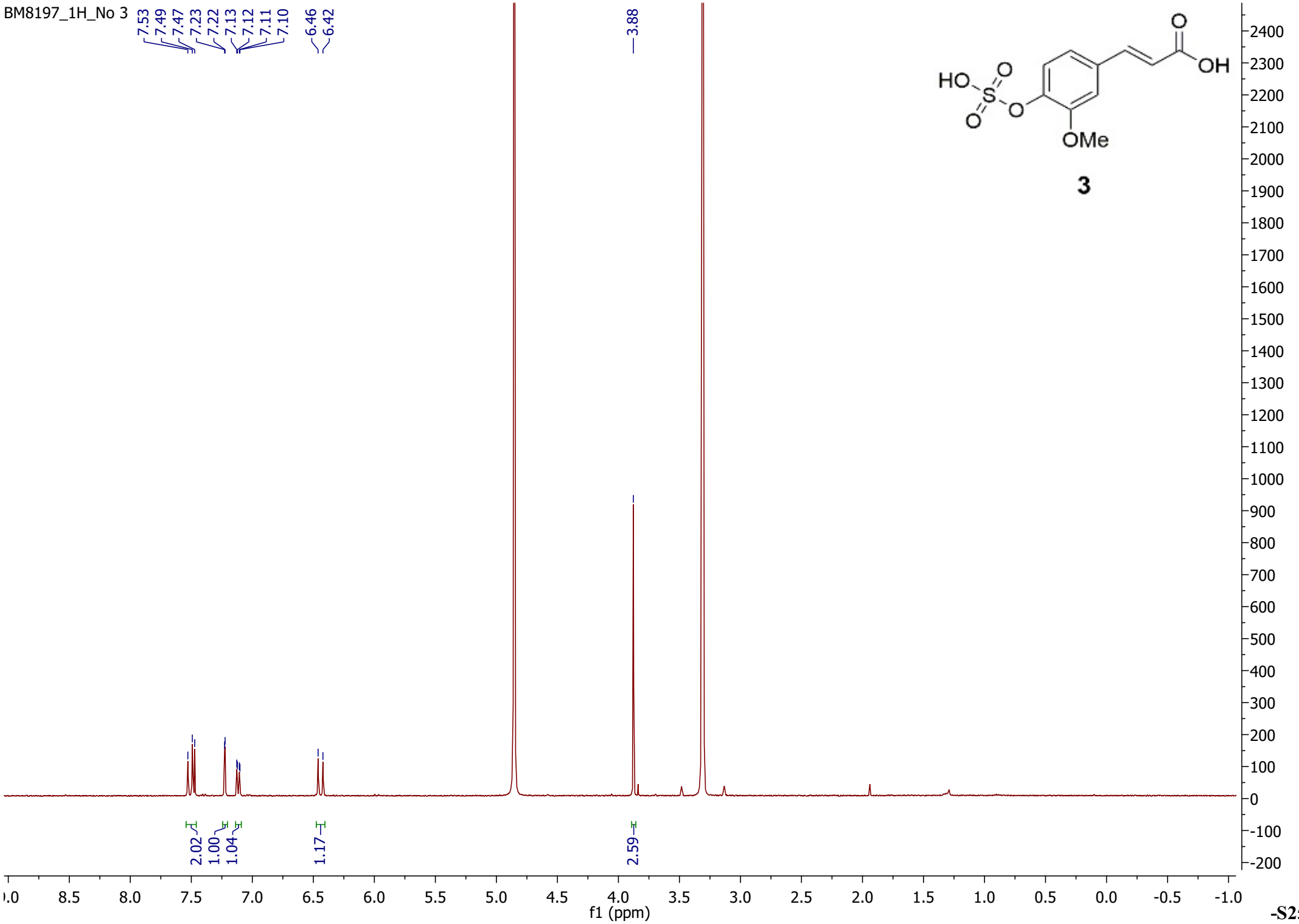
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10

f1 (ppm)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6.42
3.88
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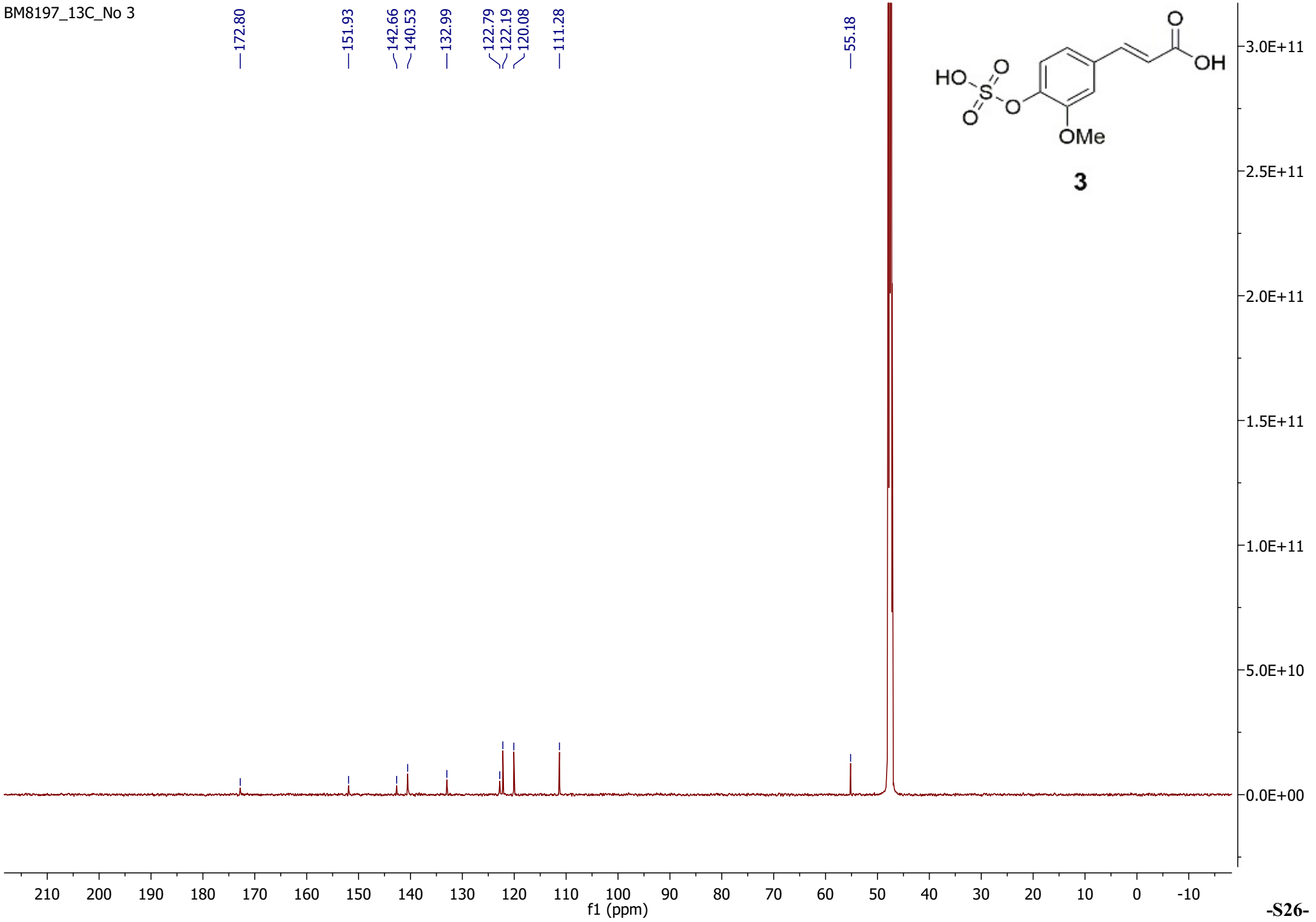
S
O
O
O
O
OH
OMe
3
2.02
1.00
1.04
1.17
2.59
f1 (ppm)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111.28
55.18
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S
O
O
O
OMe
O
OH
3
3.0E+11
2.5E+11
2.0E+11
1.5E+11
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5.0E+10
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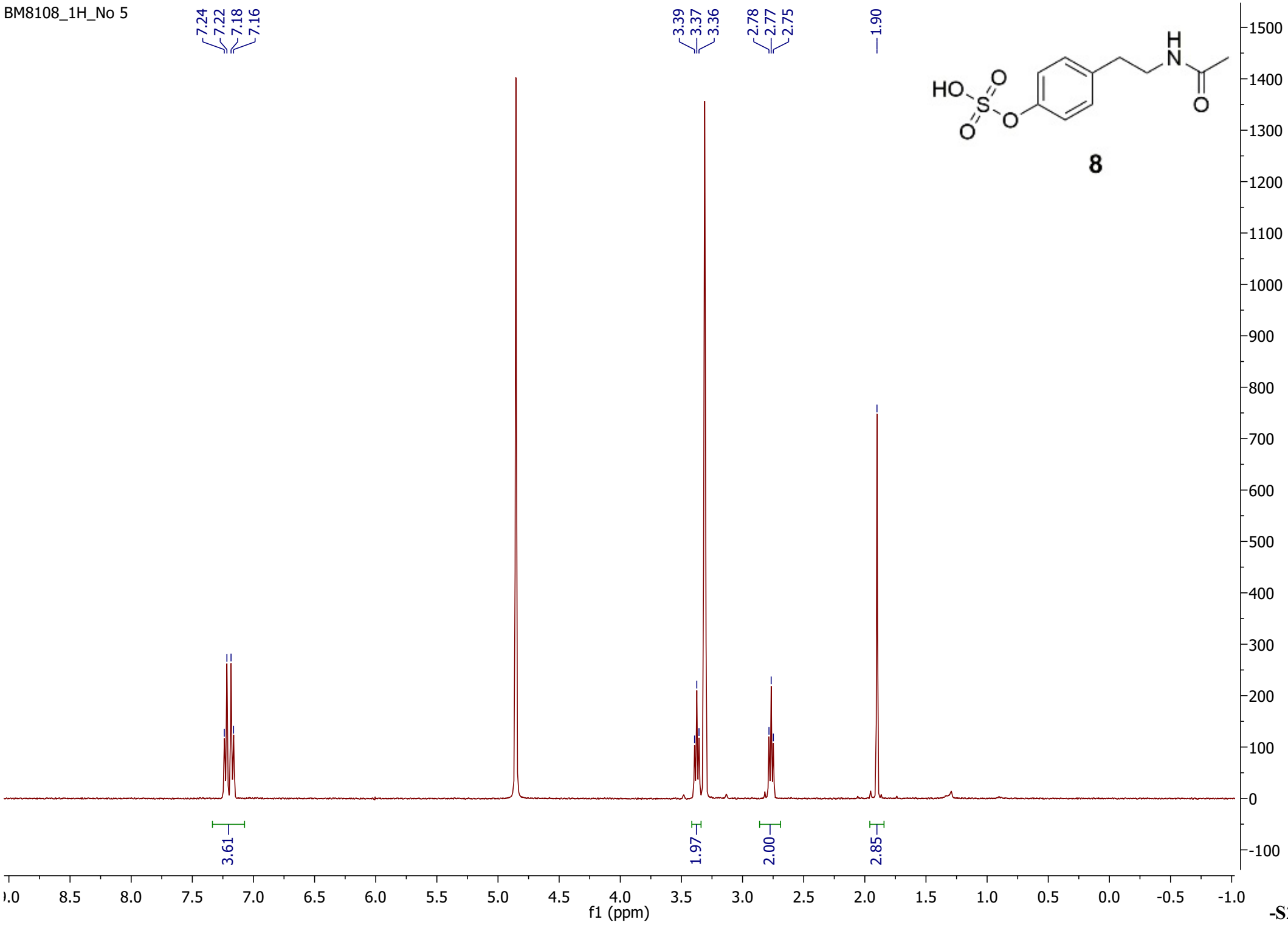
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)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S
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O
O
H
N
O
8
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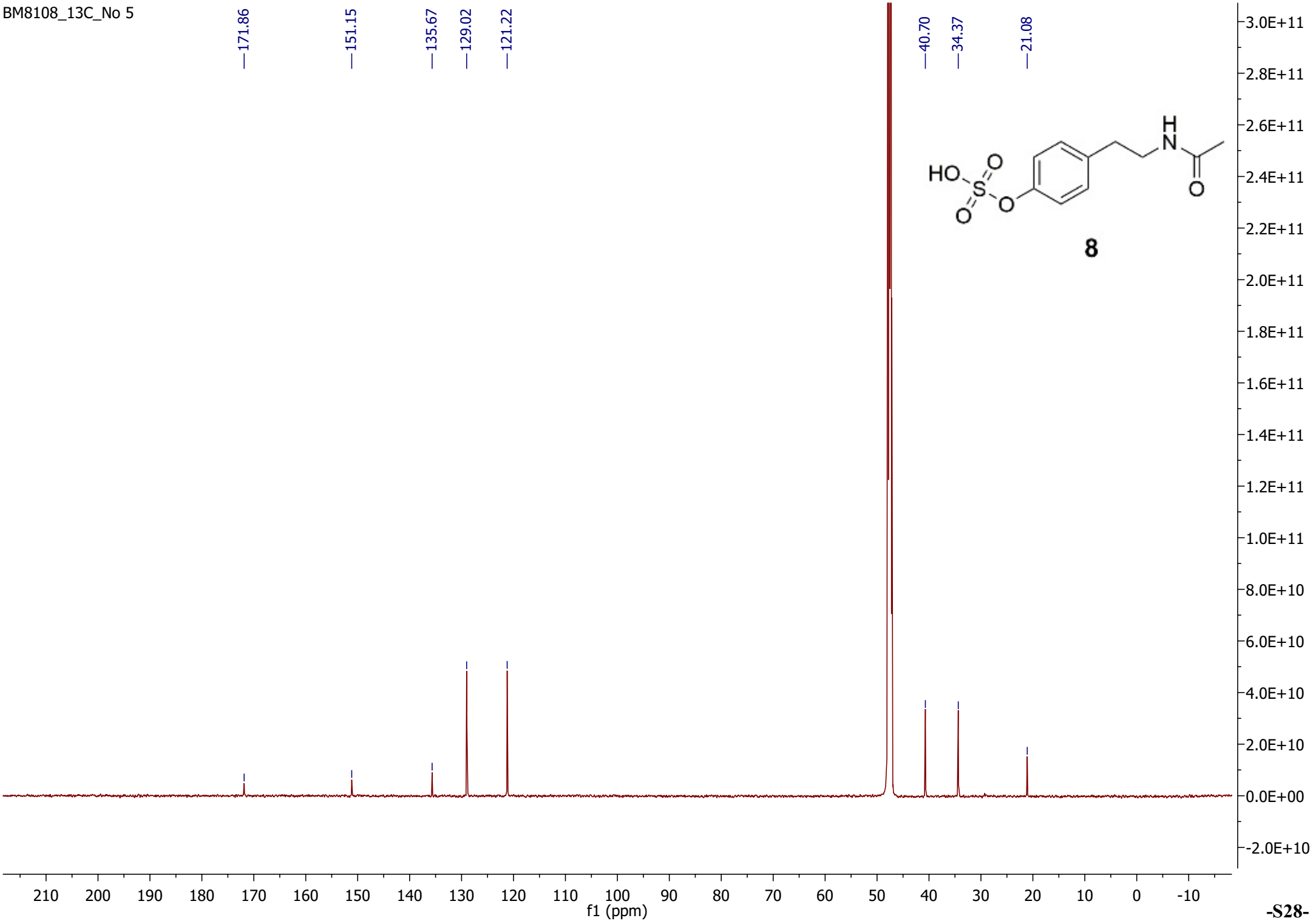
2.00
2.85
f1 (ppm)

BM8108_13C_No 5



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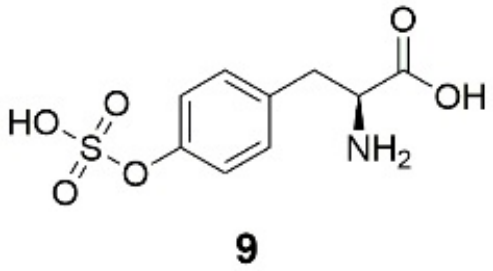

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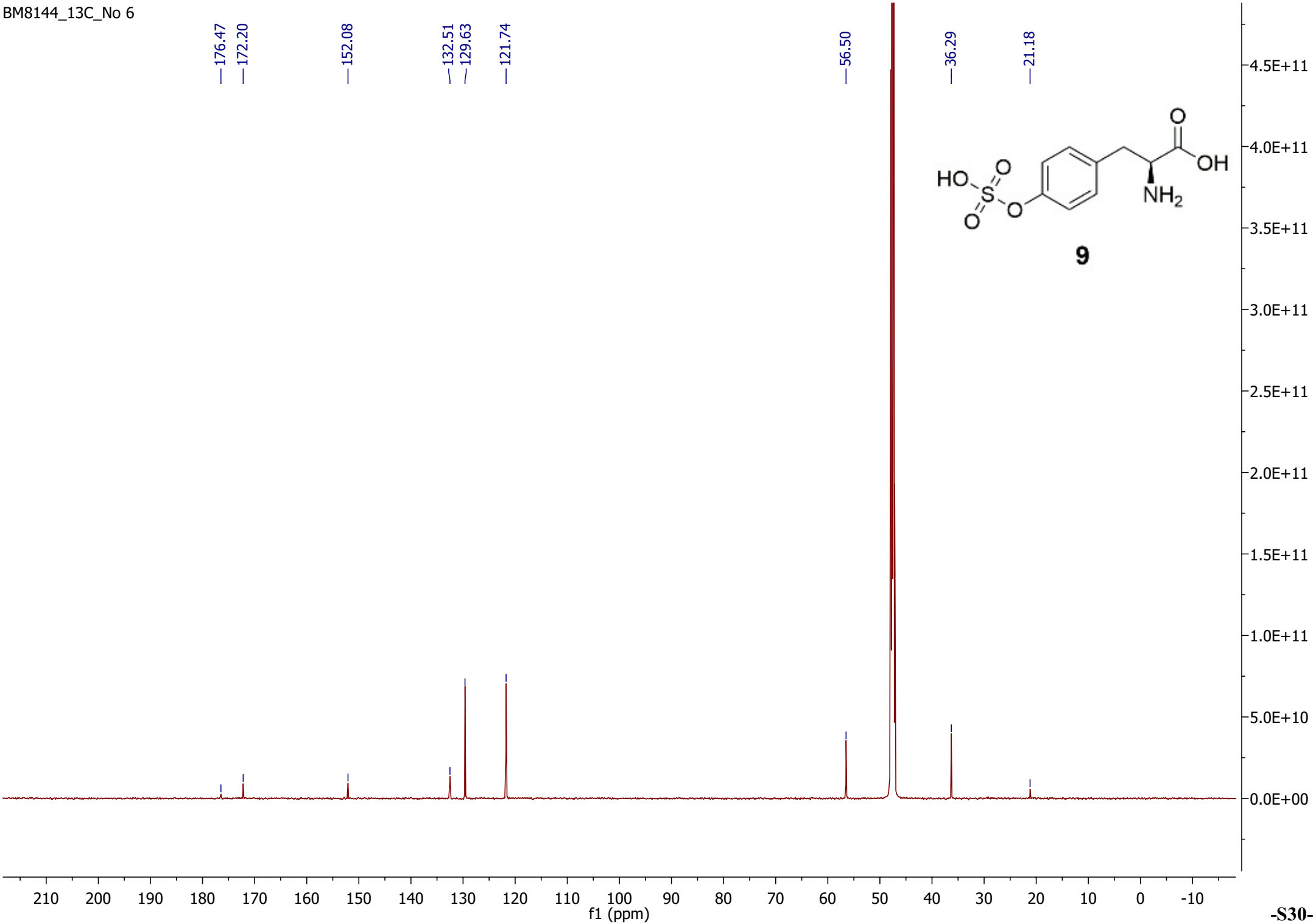

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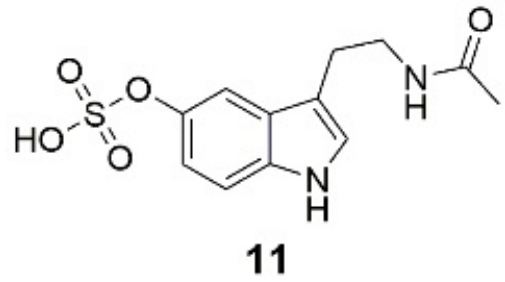

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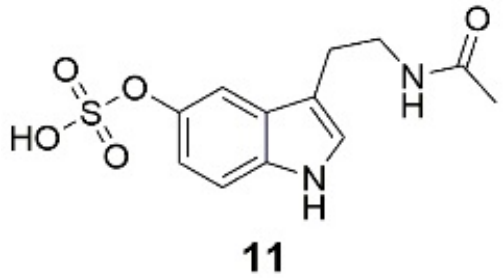

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