Synthesis and μ-opioid activity of the primary metabolites of carfentanil

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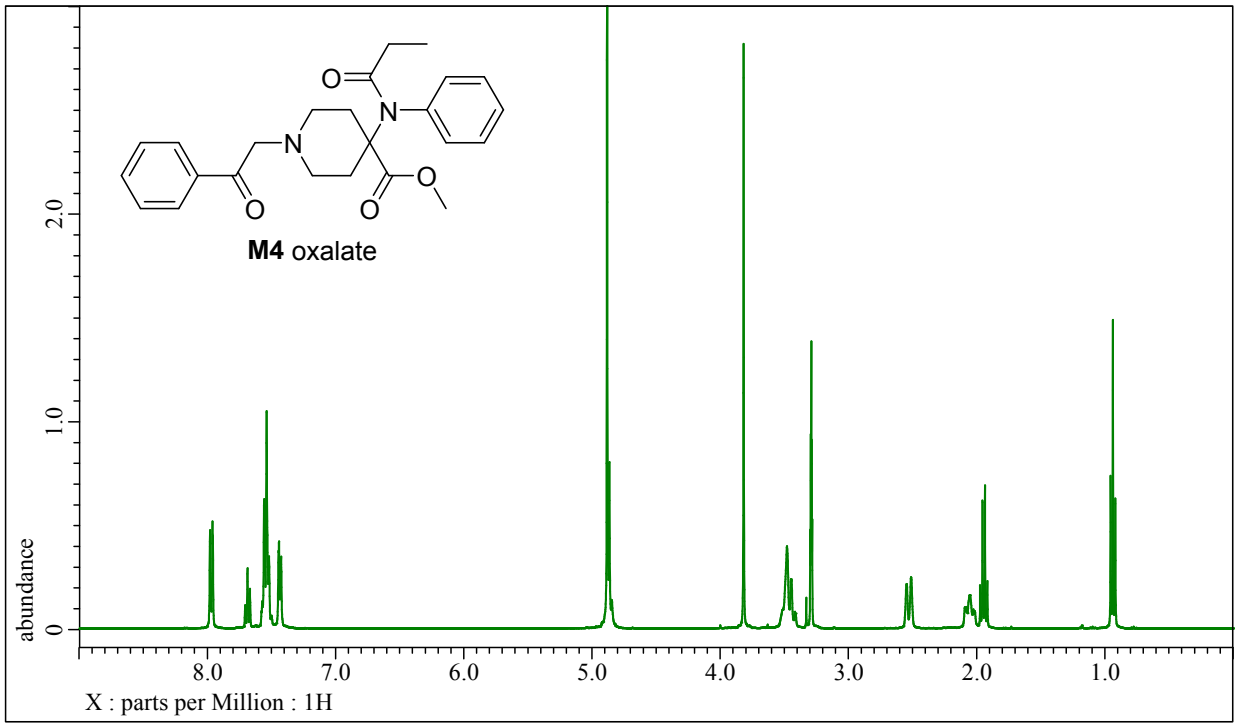
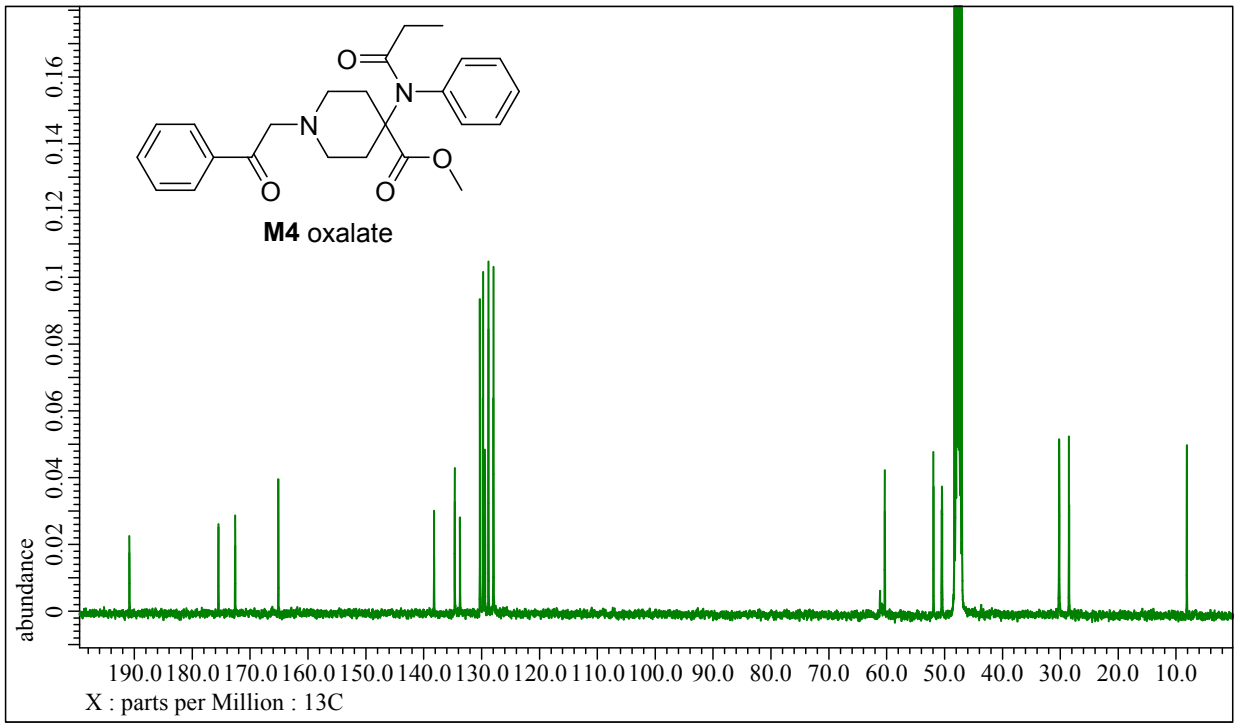


Supporting Figure 2. ^{13}C NMR spectrum of **M4** oxalate in D4-METHANOL.



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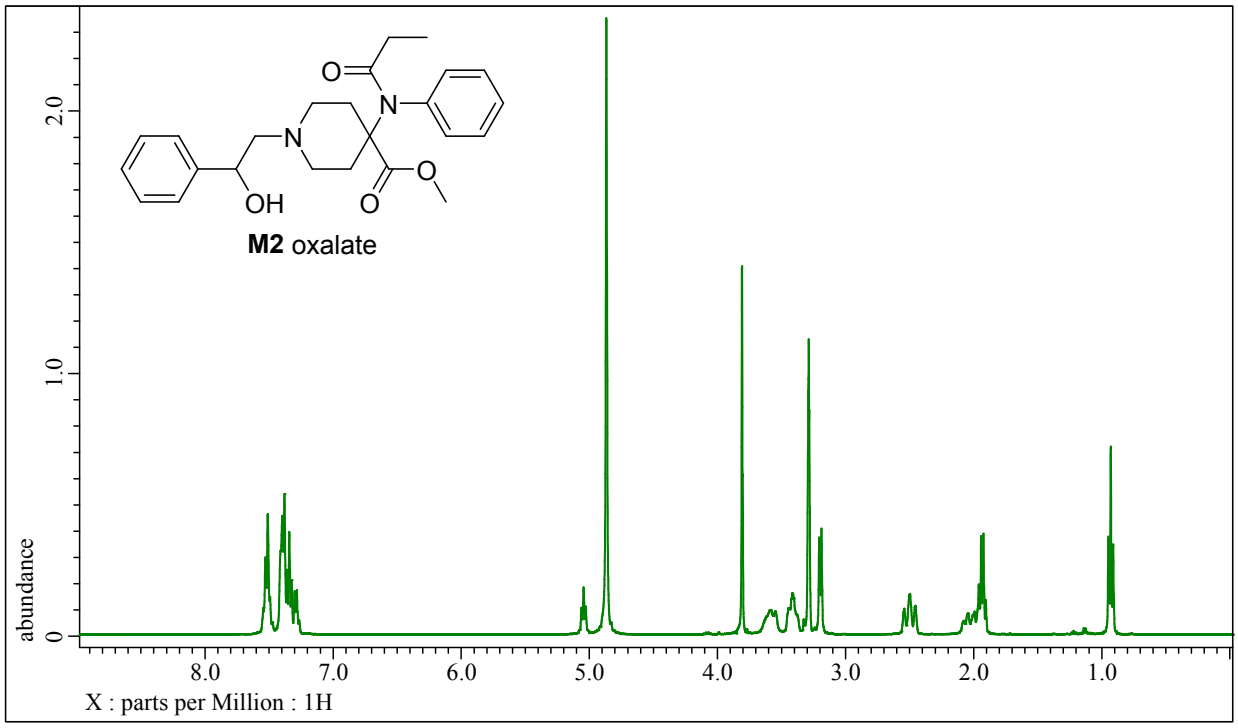
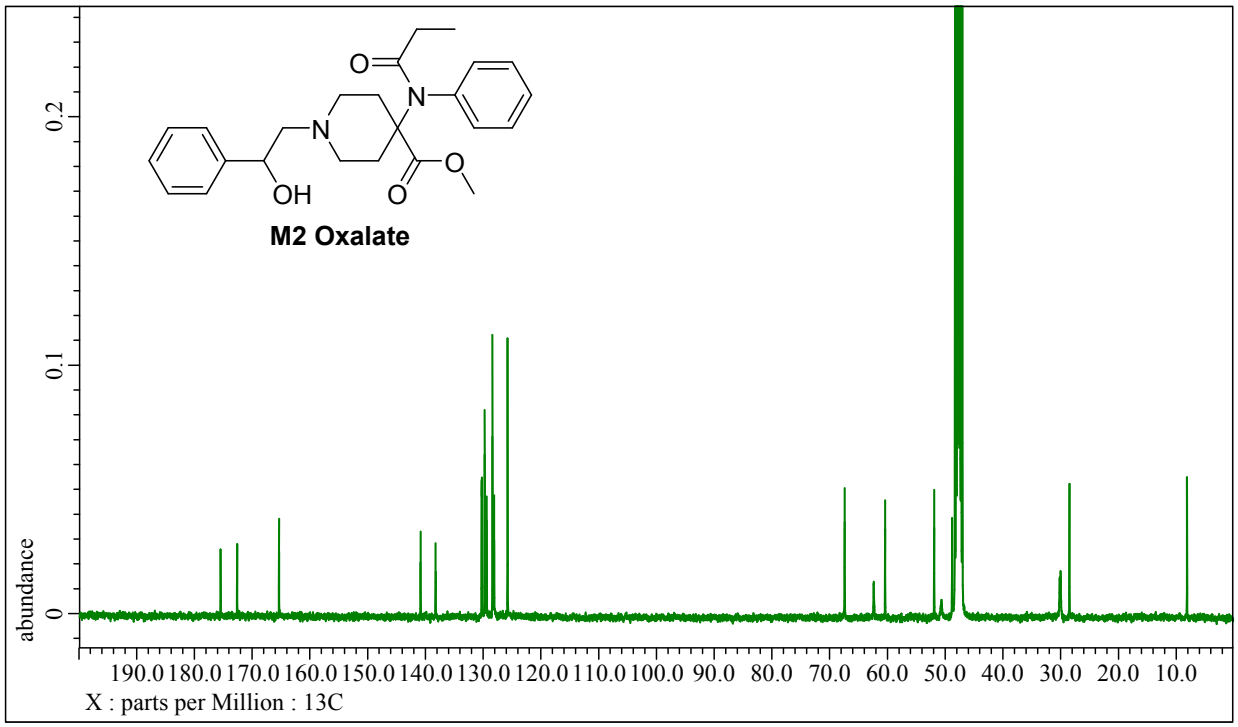


Supporting Figure 4. ^{13}C NMR spectrum of **M2** oxalate in D4-METHANOL.



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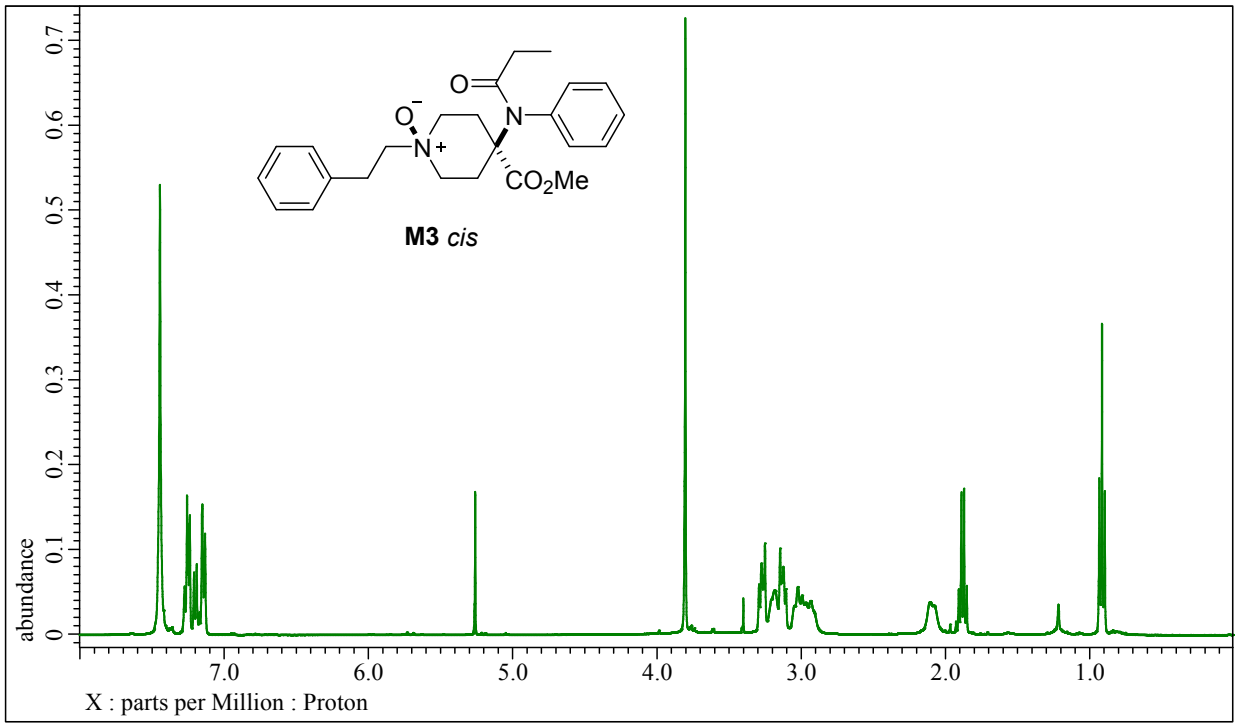
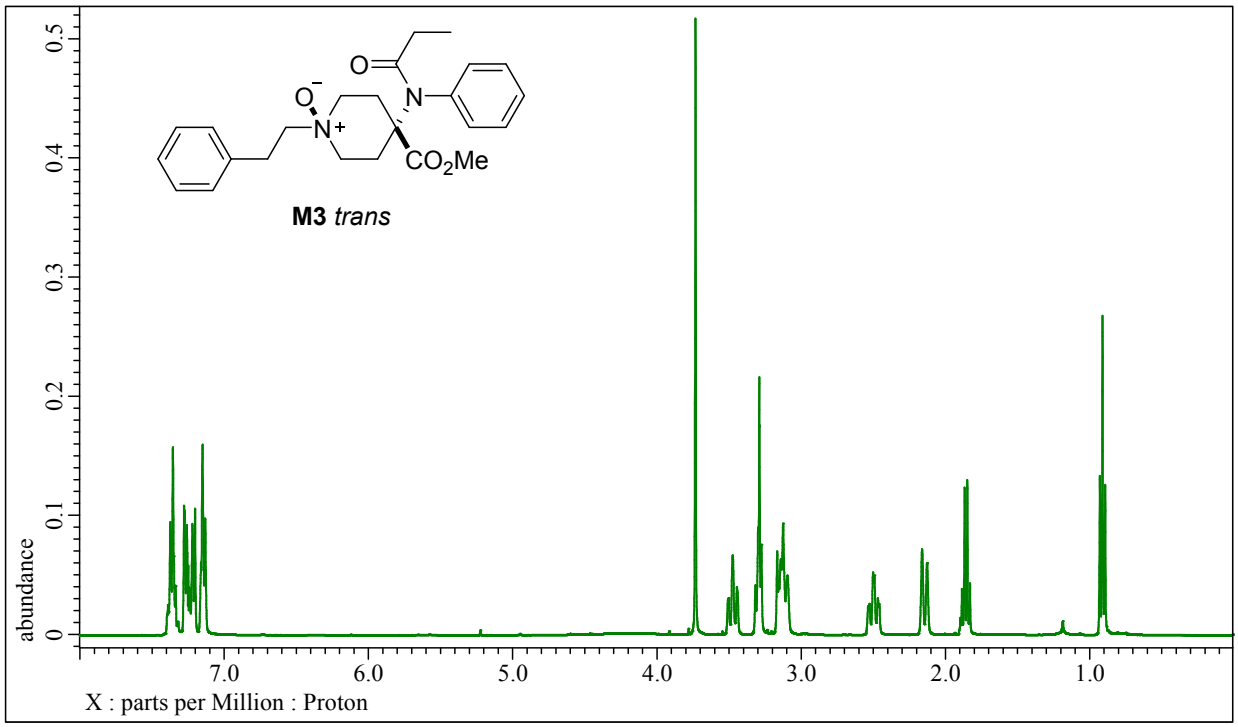
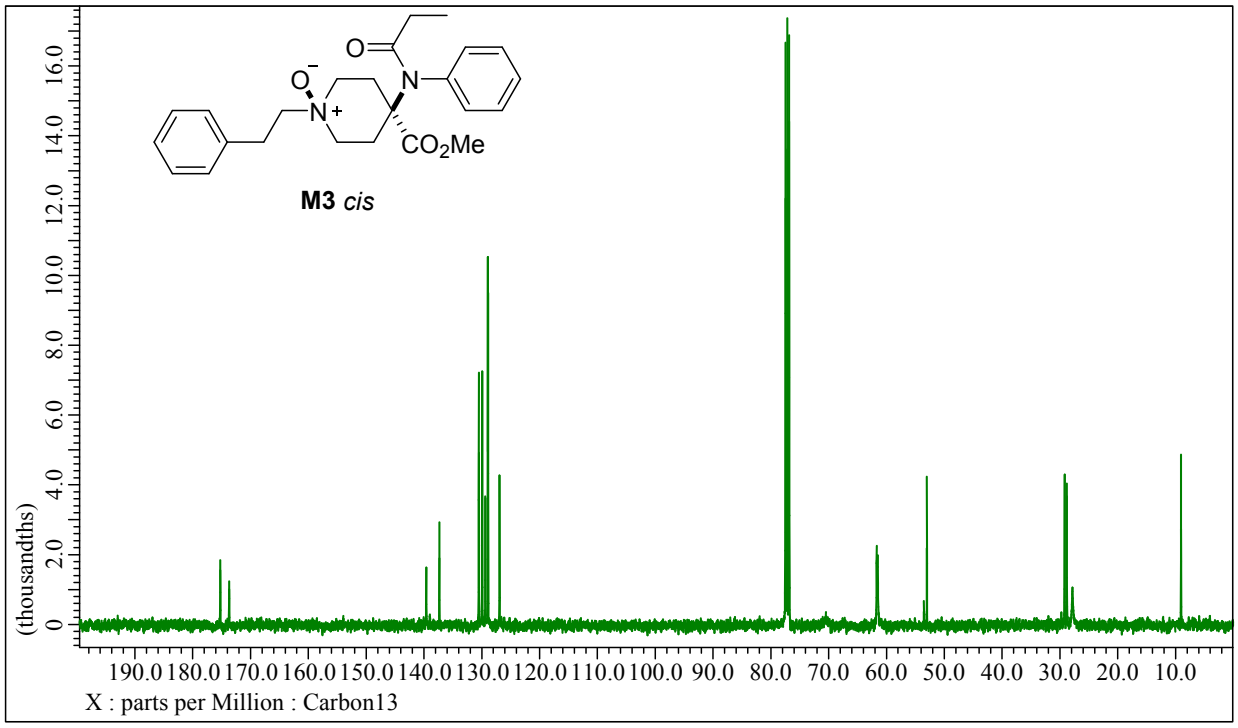
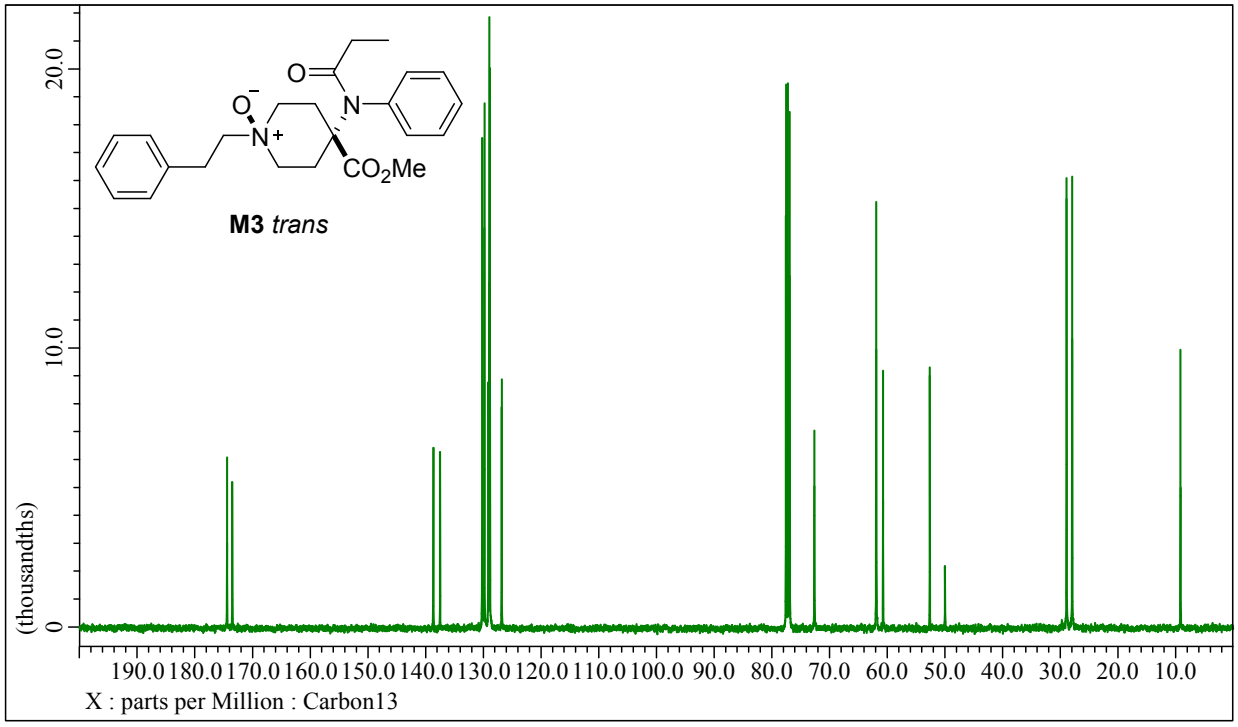


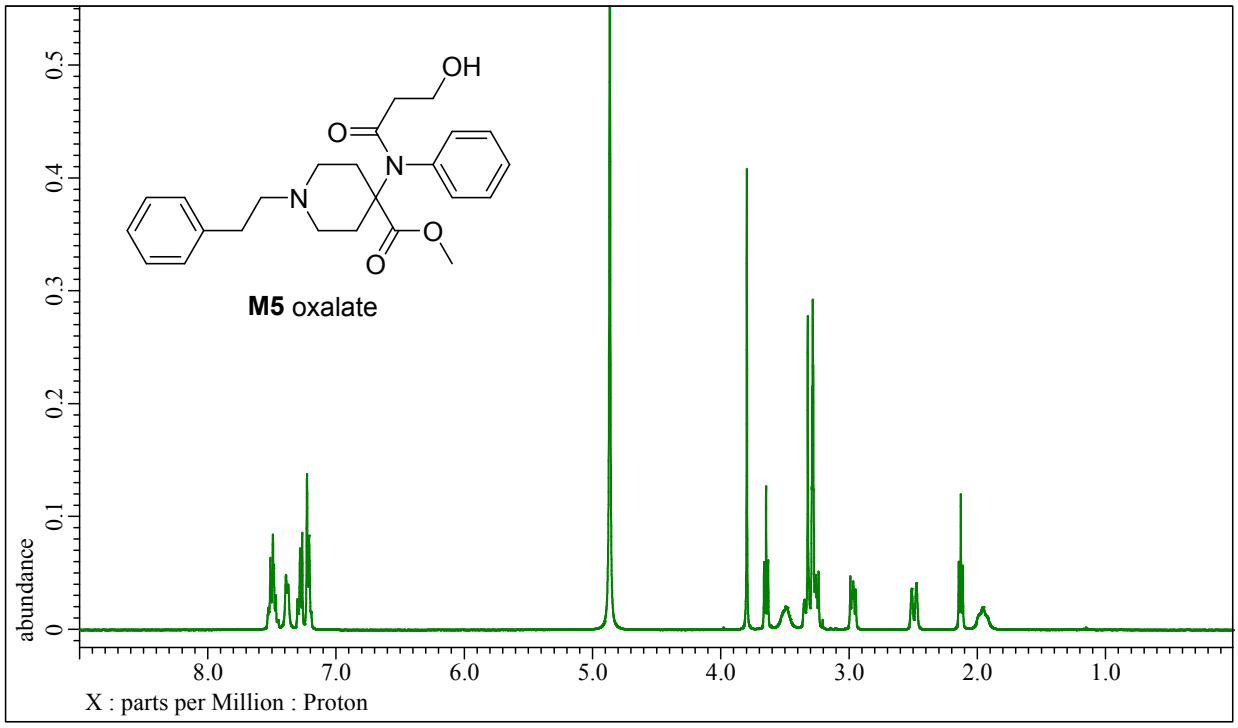


Supporting Figure 6. ^{13}C NMR spectrum of **M3** *cis* and **M3** *trans* in D-CHLOROFORM.





Supporting Figure 7. ^{1}H NMR spectrum of **M5** oxalate in D4-METHANOL.



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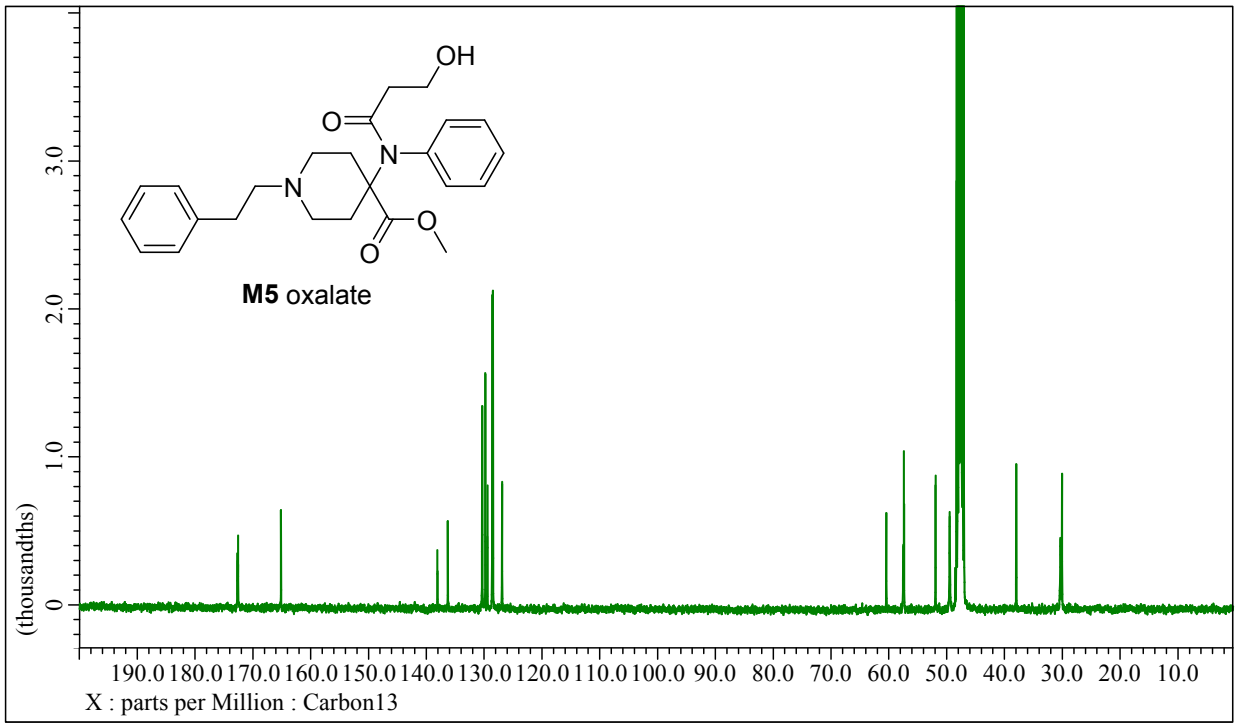
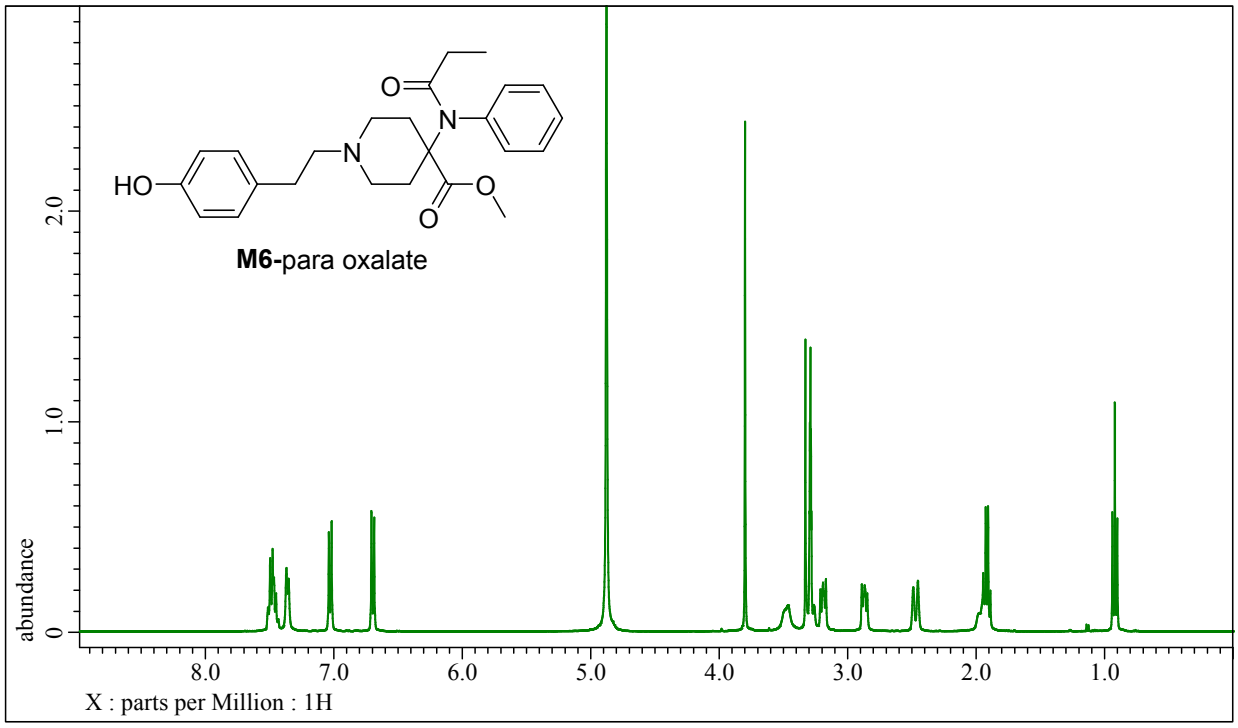
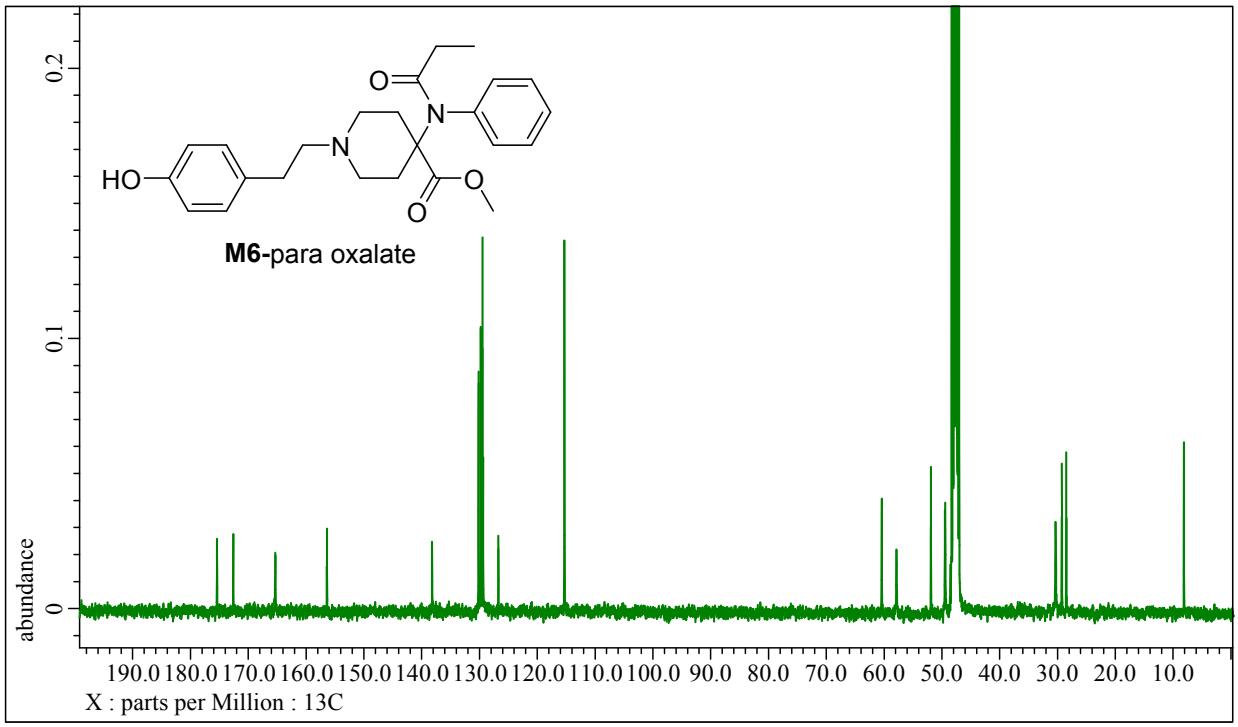


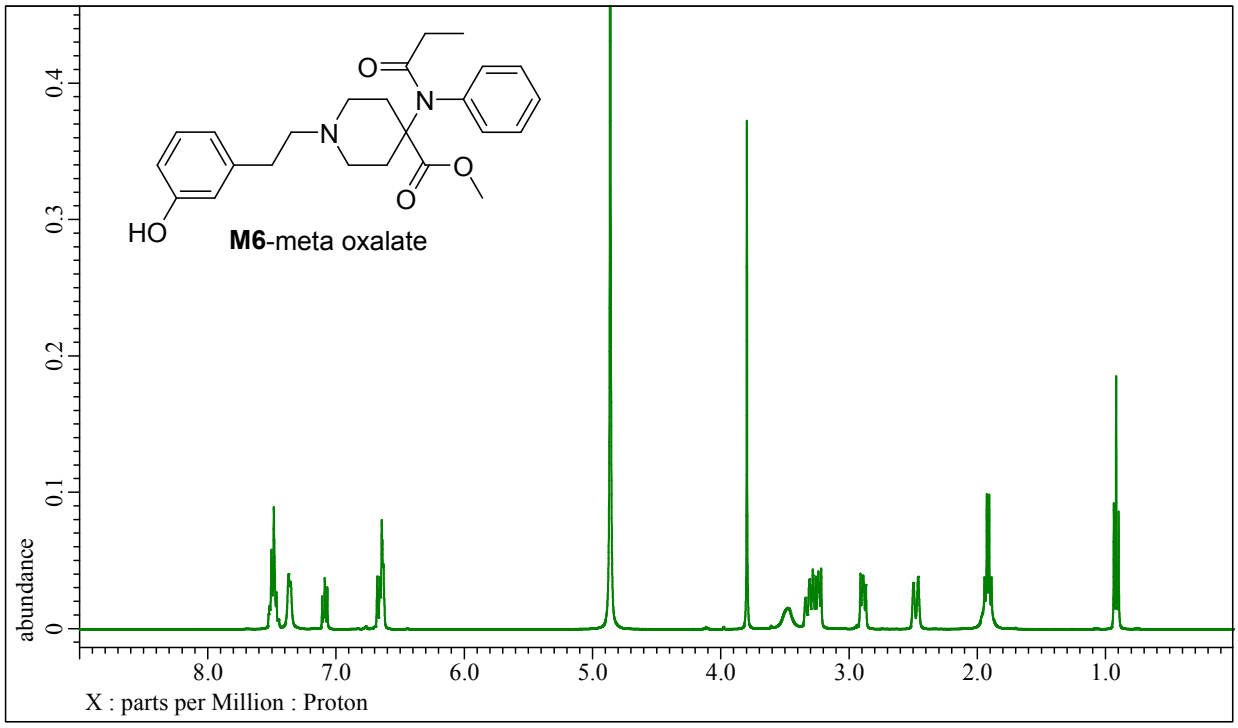
Supporting Figure 9. ^{1}H NMR spectrum of **M6**-para oxalate in D4-METHANOL.



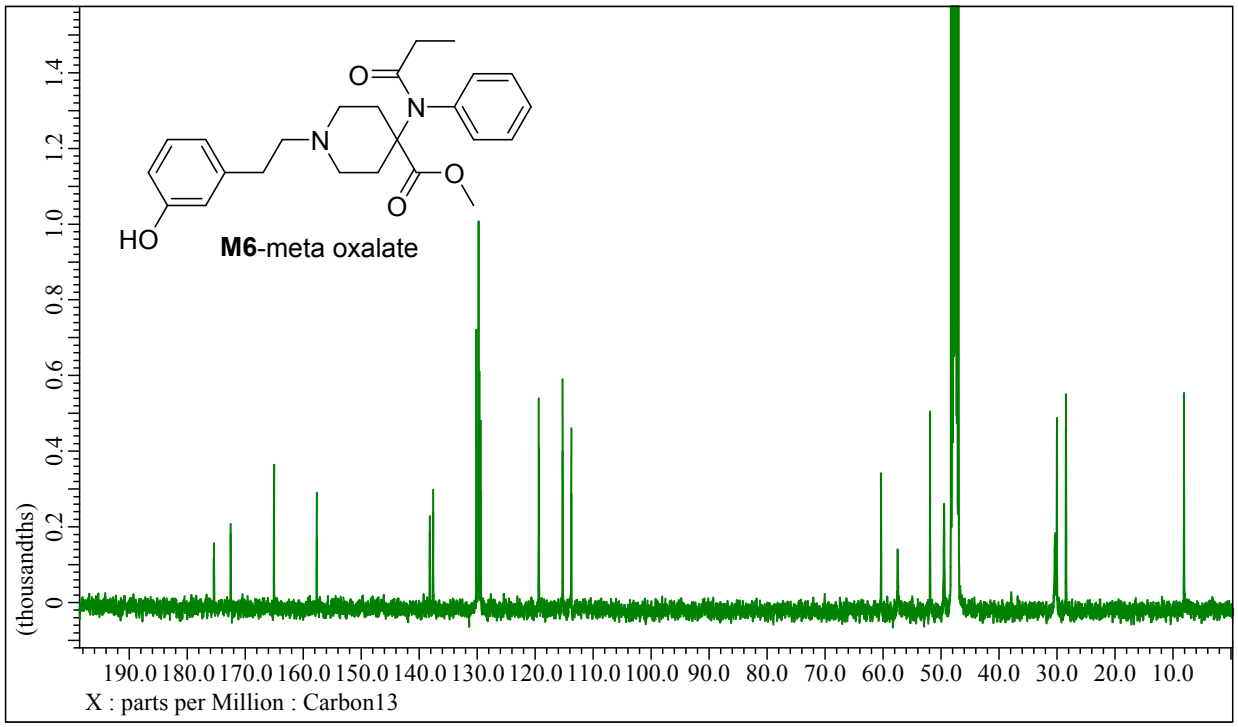
Supporting Figure 10. ^{13}C NMR spectrum of **M6**-para oxalate in D4-METHANOL.



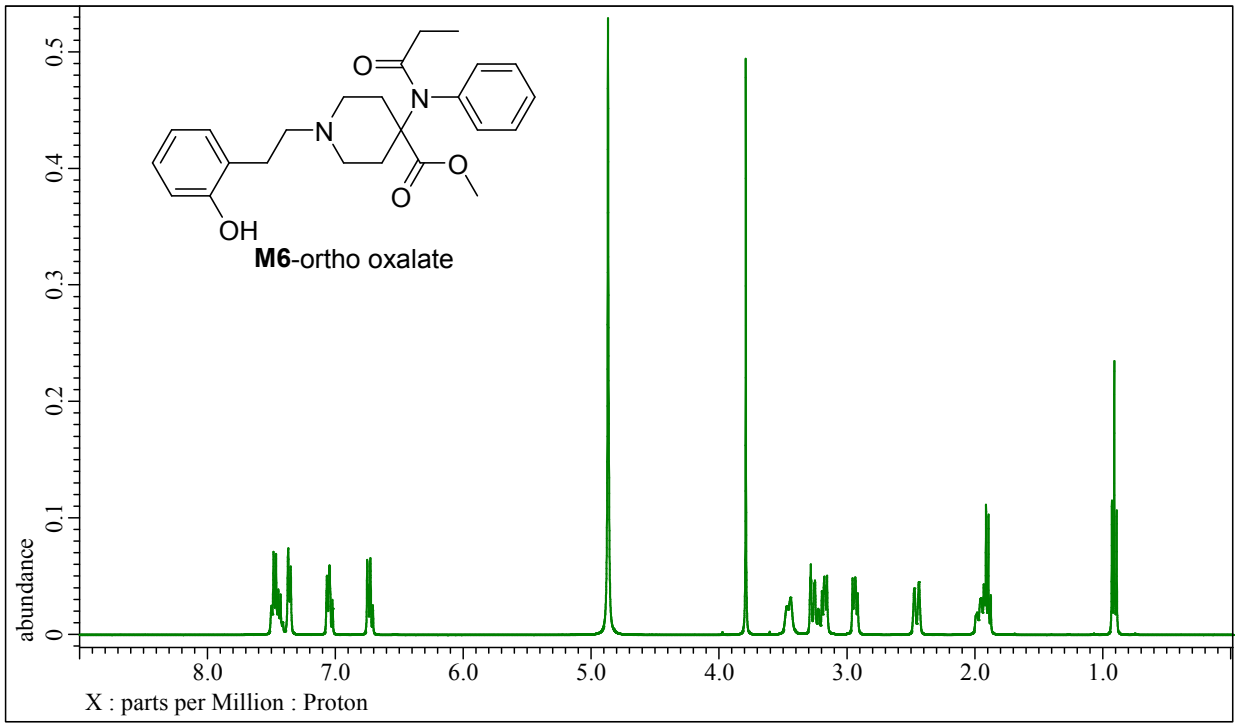
Supporting Figure 11. ^{1}H NMR spectrum of **M6**-meta oxalate in D4-METHANOL.



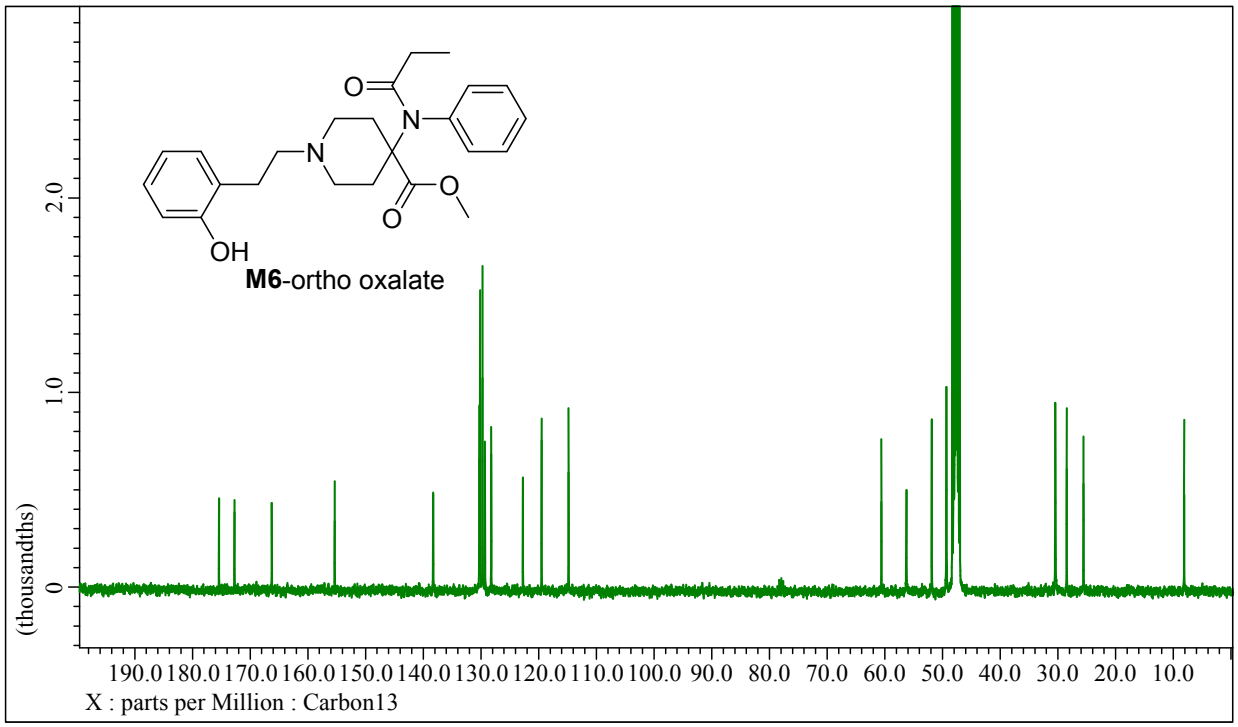
Supporting Figure 12. ^{13}C NMR spectrum of **M6**-meta oxalate in D4-METHANOL.



Supporting Figure 13. ^{1}H NMR spectrum of **M6**-ortho oxalate in D4-METHANOL.

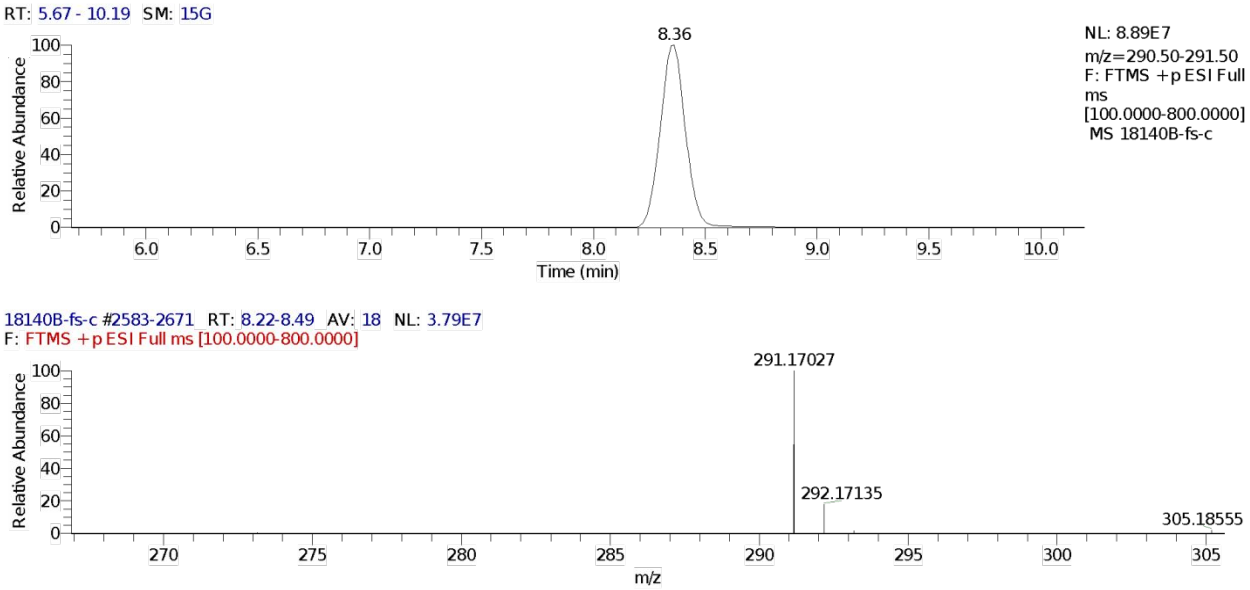


Supporting Figure 14. ^{13}C NMR spectrum of **M6**-ortho oxalate in D4-METHANOL.

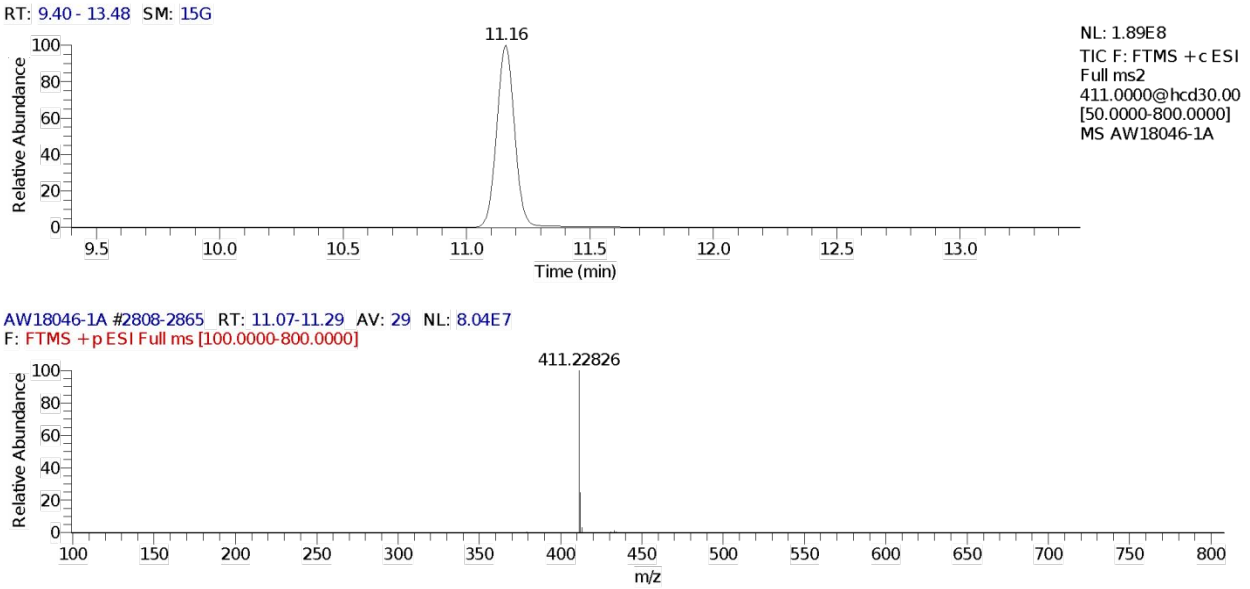


Supporting Figure 15. Mass spectra for synthesized compounds.

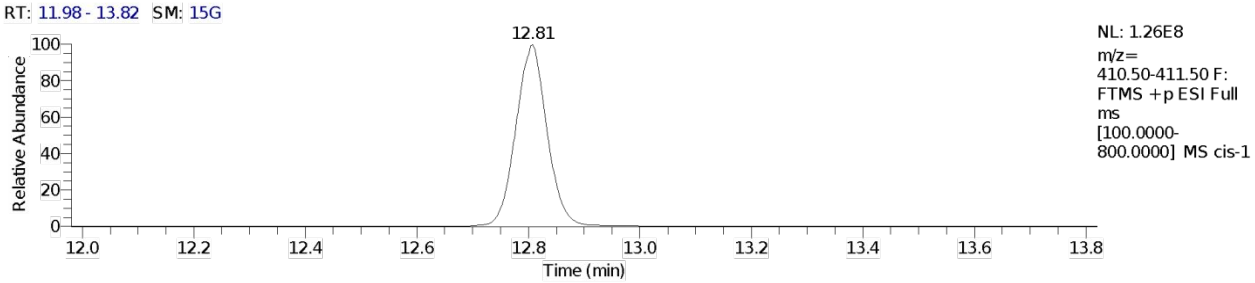
M1 (m/z 291)

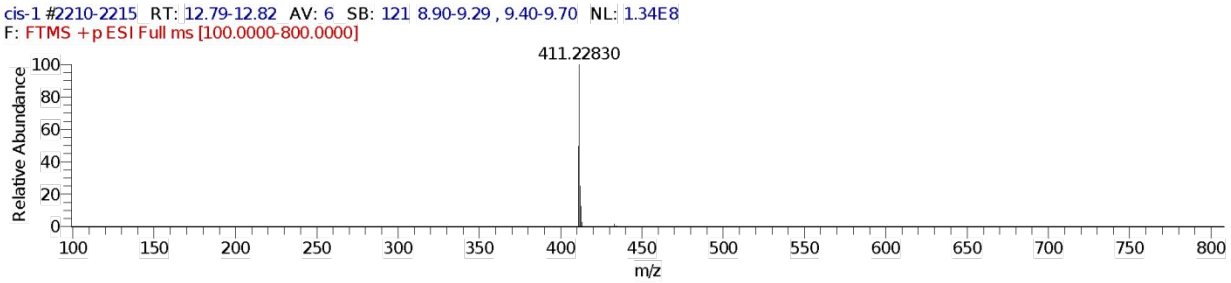


M2 (m/z 411)



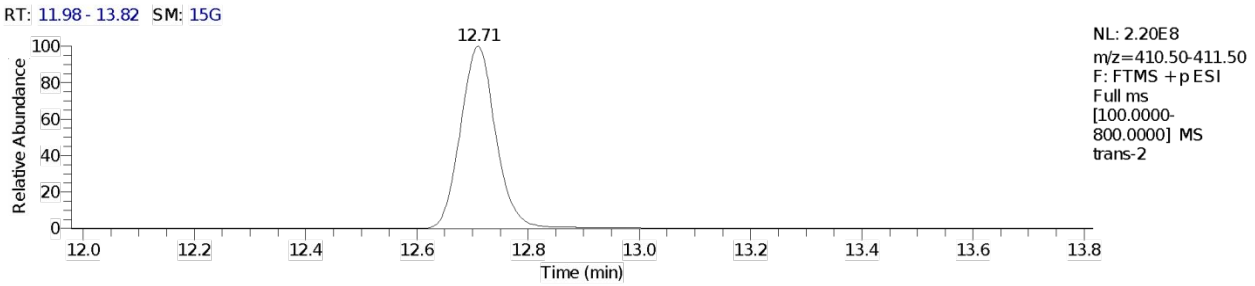
M3 *cis* **(m/z 411)**

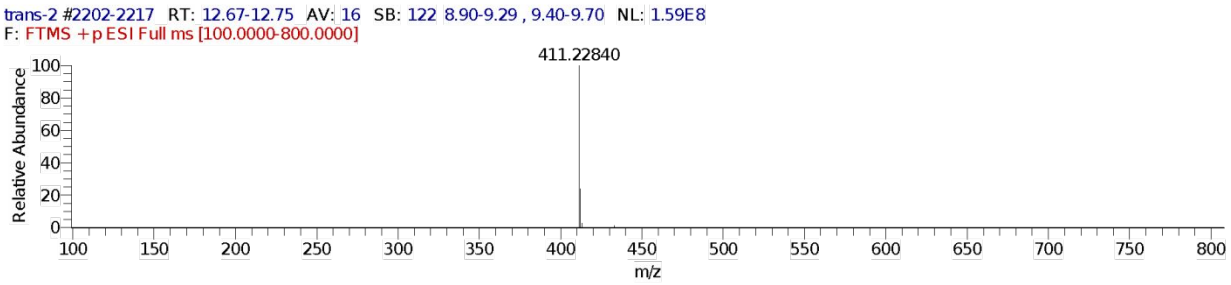




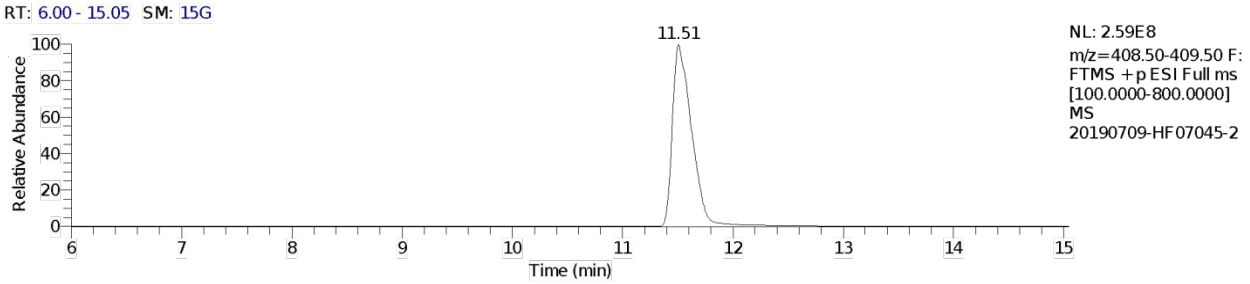
M3 *trans* **(m/z 411)**

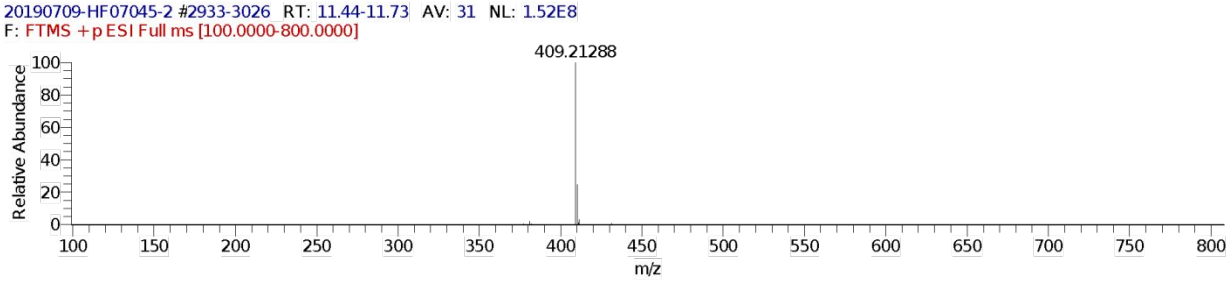
Trans



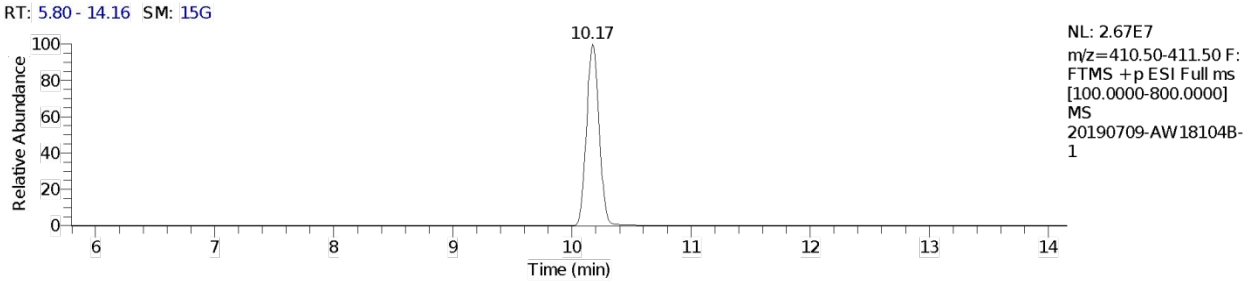


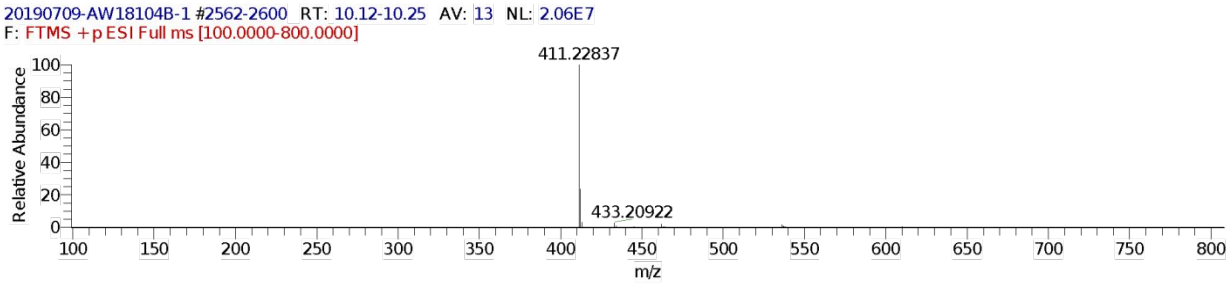
M4 (m/z 409)



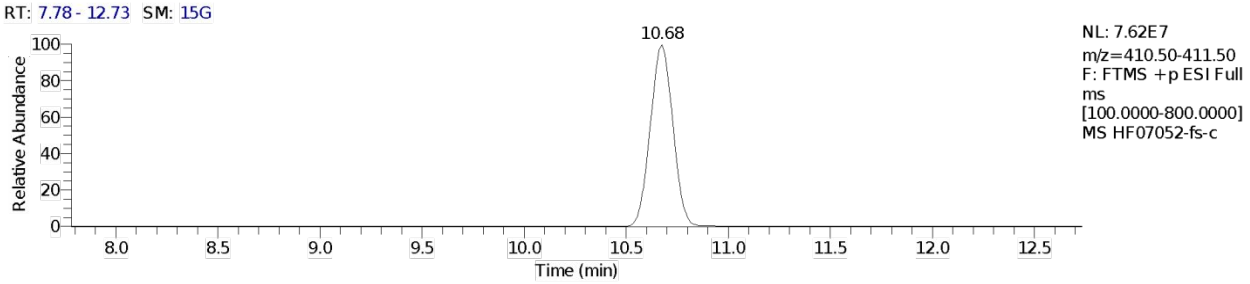


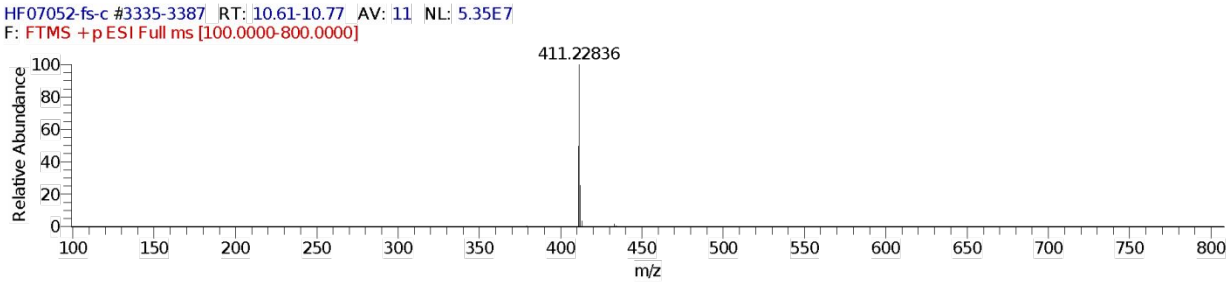
M5 (m/z 411)



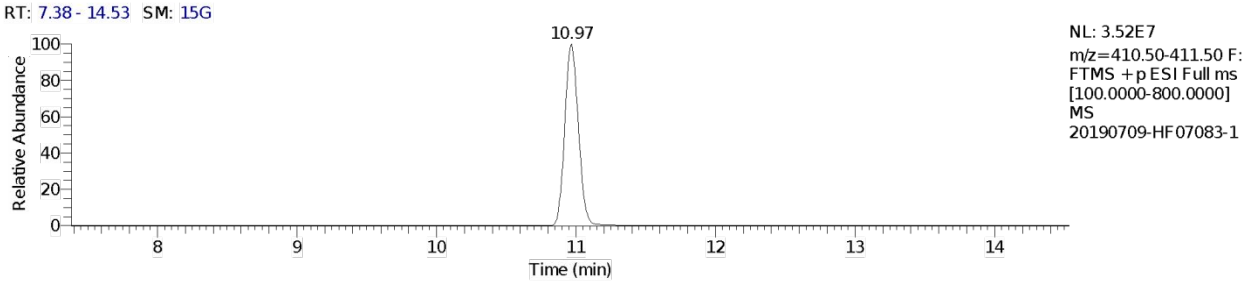


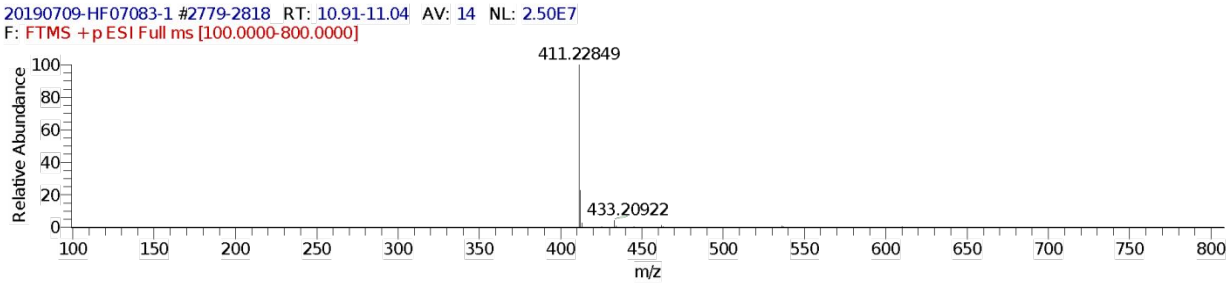
M6-para **(m/z 411)**



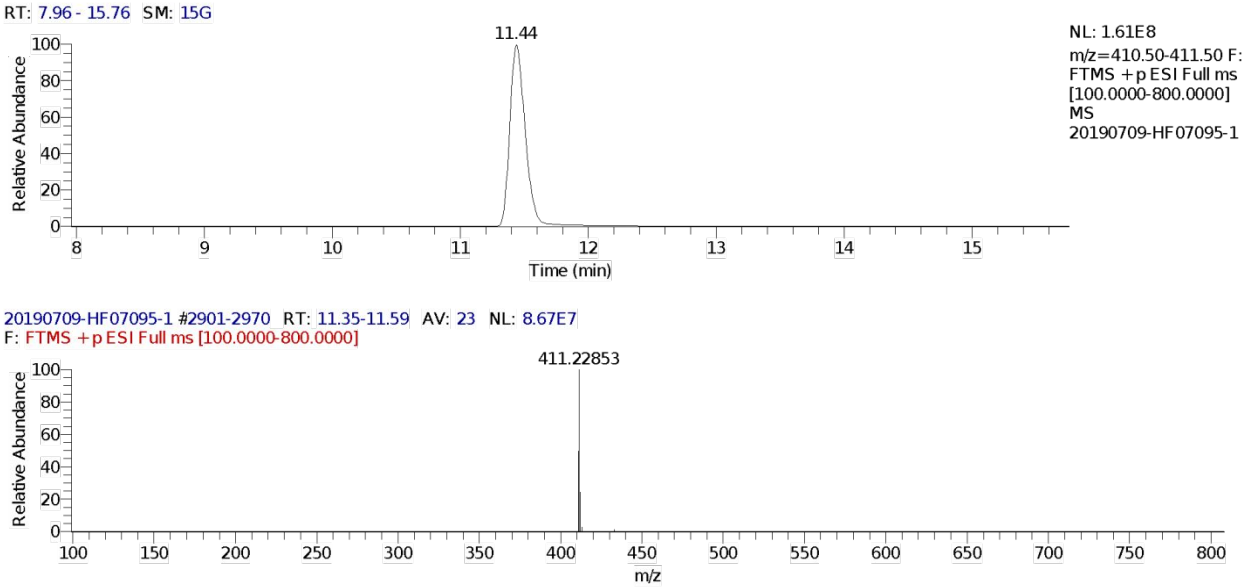


M6-meta **(m/z 411)**





M6-ortho **(m/z 411.22853)**



Supporting Figure 16. Metabolism, UHPLC and mass spectral methods and data for synthesized standards and metabolites.

Metabolism chemicals and reagents

NADPH (β-Nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate), magnesium chloride, ammonium formate, and formic acid (FA) were from Sigma-Aldrich (St. Louis, MO, USA). OmniSolv® LC-MS acetonitrile, high purity water (B & J brand) were from VWR International (Radnor, PA, USA). Pooled mixed gender human liver microsomes were from Sekisui XenoTech, LLC (Kansas City, KS, USA) and were stored at -80 °C until use. All other chemicals were of the highest grade available.

Incubation of carfentanil with pooled human liver microsomes

Carfentanil (10 µM) was incubated in 200 µL of a reaction solution containing 0.1 M potassium phosphate buffer (pH 7.4), 3 mM $MgCl_2$, 2 mg/mL human liver microsomal protein. After 5 min at 37 °C, 2 mM NADPH was added to initiate the reaction. The reactions were performed in triplicate and a control reaction, without liver microsomes, was performed in duplicate. Each reaction was run for 3, 7, 13, 20, 30, 45, 60 and 120 min then quenched by the addition of an equal volume of ice-cold acetonitrile containing internal standard (1 µM carfentanil-d_5). The incubation mixtures were vortexed then centrifuged at 2250 x *g* for 10 min. The supernatants were frozen and stored at -80 °C until analysis.

Ultra-high performance liquid chromatography-high resolution mass spectrometry (UHPLC-HRMS) conditions

UHPLC-HRMS was performed on a Thermo-Fisher Scientific Ultimate 3000 HPLC system coupled to an Orbitrap Fusion™ Tribrid™ mass spectrometer (Orbitrip Fusion TMS, Thermo-Fisher Scientific, Waltham, MA, USA). Metabolite separation was achieved by using a Kinetex® EVO C18 column (100 x 2.1 mm, 1.7 µm particle size, 100 Å pore size, Phenomenex, CA, USA) at a flow rate of 280 µL/min. Mobile phase A was 10 mM ammonium formate with 0.1% FA and mobile phase B was acetonitrile with 0.1% FA. The elution gradient was as follows: 0-1 min, an isocratic elution of 5% B; 1-10 min, a linear gradient to 40% B; 10-14 min, a linear gradient to 95% B; 14-15.7 min, an isocratic elution of 95% B; 15.8 min, a gradient back to 2% B. The total run time was 17 min. The injection volume was 5 µL. The auto sampler chamber was maintained at 4 °C with a column temperature of 30 °C.

Heated electrospray ionization (HESI) was utilized in the positive ion mode and carfentanil and its metabolites were analyzed from *m/z* 100 to 800. Orbitrap Fusion TMS has two fragmentation techniques, collision induced dissociation (CID) and higher-energy collisional dissociation (HCD). In this study, HCD was used. The parameters used for the mass spectrometer were: spray voltage, 4100 V; sheath gas flow rate, 40 respective arbitrary unit; aux gas flow rate, 20 respective arbitrary units; ion transfer tube temperature, 325 °C; vaporizer temperature, 300 °C; MS^1 detector, Orbitrap; MS^1 resolution, 120,000; MS^1 scan range, 100-800; MS^1 maximum injection time, 100 ms; MS^1 automated gain control (AGC) target, 100,000; S-lens RF level, 60 V; MS^2 HCD collision energy, 30%; MS^2 detector, Orbitrap; MS^2 resolution, 60,000; MS^2 AGC target, 50,000; MS^2 maximum injection time, 35 ms; MS^2 start mass, 50. In all experiments, active internal mass calibration was employed during the analysis. *Xcalibur* Qual and Quan Browser software were used for the qualitative and quantitative analysis.

METABOLITES	% of initial carfentanil (2h incubation)	RT (min)	Calculated mass (molecular ion and major fragment ions)	Measured mass	Mass error (ppm)	Std measured mass	Mass error (ppm)
M1		8.36-m/z 291	291.17032	291.17015	0.58	291.17027	0.17
			113.05971	113.05976	-0.44	113.05976	-0.44
			142.08626	142.08627	-0.07	142.08627	-0.07
	40%		146.09643	146.09642	0.07	146.09642	0.07
			175.12297	175.12297	0.00	175.12297	0.00
			231.14919	231.14912	0.30	231.14912	0.30
Potential M7		9.37-m/z 411	411.22783	411.22773	0.24		
			113.05971	113.05976	-0.44		
			170.08117	170.08115	0.12		
			262.14377	262.14364	0.50		
			295.18049	295.18033	0.54		
			317.18597	317.18575	0.69		
			351.20670	351.20653	0.48		
M5		10.17-m/z 411	411.22783	411.22757	0.63	411.22837	-1.31
			113.05971	113.05973	-0.18	113.05972	-0.09
			134.09643	134.09625	1.34	134.09640	0.22
			246.14886	246.14871	0.61	246.14870	0.65
			279.18558	279.18518	1.43	279.18537	0.75
			351.20670	351.20644	0.74	351.20643	0.77
M6-para		10.68-m/z 411	411.22783	411.22831	-1.17	411.22836	-1.29
			113.05971	113.05964	0.62	113.05970	0.09
			150.09134	150.09130	0.27	150.09125	0.60
			202.12264	202.12266	-0.10	202.12253	0.54
			262.14377	262.14365	0.46	262.14347	1.14
			295.18049	295.18022	0.91	295.18004	1.52
			351.20670	351.20646	0.68	351.20618	1.48
M2		11.16-m/z 411	411.22783	411.22764	0.46	411.22826	-1.05
			113.05971	113.05975	-0.35	113.05973	-0.18
			162.09134	162.09133	0.06	162.09128	0.37
	12%		202.12264	202.12264	0.00	202.12256	0.40
			232.13321	232.13314	0.30	232.13307	0.60
			262.14377	262.14365	0.46	262.14356	0.80

			295.18049	295.18031	0.61	295.18017	1.08
			351.20670	351.20647	0.65	351.20633	1.05
M3-trans		**12.71-m/z 411**	411.22783	411.22769	0.34	411.22840	1.73
			105.06988	105.06993	-0.48	105.06991	-0.19
			132.08078	132.08076	0.15	132.08074	-0.15
			150.09134	150.09131	0.20	150.09129	-0.13
			202.12264	202.12164	4.95	202.12257	4.60
			230.11756	230.11750	0.26	230.11742	-0.35
	2.50%		244.13321	244.13328	-0.29	244.13304	-0.98
			247.14410	247.14396	0.57	247.14391	-0.20
			262.14377	262.14357	0.76	262.14352	-0.19
			274.14377	274.14361	0.58	274.14353	-0.29
			303.17032	303.17007	0.82	303.16998	-0.30
			355.20162	355.20141	0.59	355.20128	-0.37
M3-cis		**12.81-m/z 411**	411.22783	411.22763	0.49	411.22830	1.63
			105.06988	105.06993	-0.48	105.06990	-0.29
			134.09643	134.09643	0.00	134.09638	-0.37
			150.09134	150.09131	0.20	150.09127	-0.27
			202.12264	202.12221	2.13	202.12255	1.68
			230.11756	230.11746	0.43	230.11736	-0.43
			244.13321	244.13426	-4.30	244.13301	-5.12
			262.14377	262.14361	0.61	262.14352	-0.34
			274.14377	274.14363	0.51	274.14353	-0.36
			303.17032	303.17010	0.73	303.16998	-0.40
			335.21179	335.21198	-0.57	335.21145	-1.58
			379.20162	379.20138	0.63	379.20118	-0.53
M4		**11.51-m/z 409**	409.21218	409.21203	0.37	409.21288	-1.71
			113.05971	113.05972	-0.09	113.05984	-1.15
			146.09643	146.09637	0.41	146.09658	-1.03
	1.90%		158.09643	158.09639	0.25	158.09650	-0.44
			202.12264	202.12254	0.49	202.12273	-0.45
			260.12812	260.12743	2.65	260.12808	0.15
			349.19105	349.19064	1.17	349.19087	0.52

Supporting Figure 17. MOR assay experimental methods.

Pharmacological assaying materials

Hank's Balanced Salt Solution (1X), cell growth media (Ham's F-12, MEM Non-Essential Amino Acids Solution, FBS, and G418 selective antibiotic), non-enzymatic dissociation solution (Versene™ Solution), and HEPES were purchased from Invitrogen (Gathersburg, MD, USA). Forskolin and IBMX were purchased from Sigma-Aldrich (St. Louis, MO, USA). Optiplate 384 white opaque 384-well microplates, TopSeal™-A adhesive film, and the Lance Ultra cAMP kit were purchased from Perkin Elmer (Waltham, MA, USA). Chinese Hamster Ovary (CHO) K1 cells stably expressing the human mu opioid receptor gene (OP3; MOR) were purchased/licensed from ChanTest Corporation, now Charles River Laboratories (Wilmington, MA, USA).

Cell culture and transfections

CHO-K1 cells expressing the human MOR were cultivated in Ham's F12 medium, supplemented with 10% FBS, 1X NEAA for 24 h post-thaw, and media replaced at 24 h by similar media with 0.4 mg/mL geneticin (G418) selection antibiotic until ready to assay. Subconfluent cells (~70%) were used for the assay.

Lance *Ultra* cAMP assay

The assay procedure was followed exactly as described in the manufacturer's Assay Development Guidelines and has been reported in the literature.[1-3] In brief, on the day of the assay, ligand solutions (10 mM stocks in dimethylsulfoxide) were diluted into assay buffer and added to the wells of a 384-well Optiplate in triplicate. Cell suspensions (1,000 cells/well) were added and incubated with the ligands for 30 min, followed by the addition of the assay tracer (Eu-cAMP) and antibody (*Ulight™*- anti-cAMP) solutions prepared immediately before plating and incubation in the dark for 1 h at room temperature covered by adhesive film. The plate was then read on a Molecular Devices FlexStation III by exciting the wells with light of 340 nm and measuring the emission at 615 nm and 665 nm.

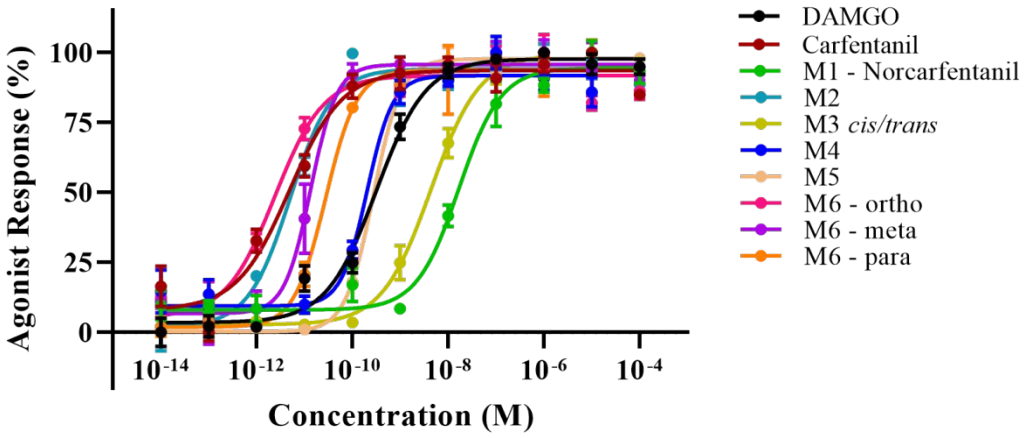
Data analysis

The measured fluorescence energy transfer ratio (665 nm/615 nm) was plotted against metabolite concentration in GraphPad Prism v8.1.2 (San Diego, CA, USA) and normalized to the control agonist's DAMGO response. Concentration-response curves were fitted by non-linear regression [agonist] vs. response – variable slope (four parameter) equation:

$$Y = Bottom + (X^{Hillslope}) \times (Top - Bottom)/(X^{HillSlope} + EC_{50}^{Hillslope})$$

Supporting Figure 18. Lance Ultra cAMP assay concentration-response curves.

Data for carfentanil and the eight metabolites synthesized. Data are normalized to the reference compound DAMGO for percent response and are shown as means (error bars represent SEM) of a single representative experiment performed in triplicate.



References:

(1) Livingston, K. E.; Mahoney, J.; Manglik, A.; Kobilka, B.; Sunahara, R.; Traynor, J. R. Efficacy at the Mu Opioid Receptor: Insights from Orthosteric and Allosteric Ligands. *Faseb J.* **2016**, *30*, 1.

(2) Perkin Elmer. Lance *Ultra* cAMP Assay Development Guidelines. Version2. Catalogue Number TRF0263: 10,000 assay point kit. 2010.

(3) LANCE *Ultra* cAMP: A New, Two-Component TR-FRET cAMP Assay for HTS of G_s- and G_i-coupled Receptors. SBS 16th Annual Conference (2010).