# Electronic Supplementary Information (ESI)

# Synthesis and Immunological Effects of C14-linked 4,5-Epoxymorphinan Analogues as Novel Heroin Vaccine Haptens

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**Experimental**

## Materials and Methods

All reactions were performed in glassware containing a Teflon coated stir bar. All reagents were obtained from commercial sources and used without further purification. Melting points were determined on a Thomas Hoover Melting Point Apparatus and are uncorrected. Proton nuclear magnetic resonance ($^{1}$H NMR, 400 MHz) and carbon nuclear magnetic resonance ($^{13}$C NMR, 100 MHz) spectra were recorded on a Varian Gemini-400 wide-bore spectrometer in $CDCl_3$ (unless otherwise noted) with the values given in ppm and *J* (Hz) assignments of $^{1}$H resonance coupling. For $^{1}$H NMR spectra ($CDCl_3$), the residual solvent peak was used as the reference (7.27 ppm) while the central solvent peak was used as the NMR reference (77.0 ppm in $CDCl_3$). The high-resolution electrospray ionization (ESI) mass spectra were obtained on a Waters LCT Premier Time-of-flight (TOF) mass spectrometer. Thin-layer chromatography (TLC) was performed on 0.25 mm Analtech GHLF silica gel and used to determine the completion of the reaction. Gas chromatography (GC) was performed on an Agilent Technologies 6850 Series system equipped with Agilent Technologies 7683B series injector and Agilent Technologies 5975C VL MSD Triple-Axis detector. Flash column chromatography was performed with Bodman silica gel LC 60 A. Elemental analyses were performed by Robertson Microlit Laboratories, Ledgewood, NJ, and were within 0.4% for C, H, and N. The NHS-$(PEG)_2$-maleimide crosslinker [(SM-$(PEG)_2$], spin desalting columns (Zeba™, 7K MWCO), dialysis cassettes (Slide-A-Lyzer G2™, 10K MWCO), Pierce™ bicinchoninic acid (BCA) protein assay kit, and the bovine serum albumin (BSA) that was used for coupling reactions were purchased from Fisher Scientific (Rockford, IL). Tetanus toxoid (TT) was purchased from Mass Biologics (Mattapan, MA). Dulbecco's phosphate-buffered saline (DPBS, pH 7.4) was purchased from

Quality Biological Inc. (Gaithersburg, MD). Lipids used to prepare liposomal adjuvant, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), synthetic monophosphoryl lipid A (3D-PHAD®) (MPLA), and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL), Alhydrogel® was purchased from Brenntag (Reading, PA). Peroxidase-linked sheep anti-mouse IgG (γ-chain specific) was purchased from The Binding Site (San Diego, CA). The 2,2'-Azino-di(3-ethylbenzthiazoline-6-sulfonate) (ABTS) peroxidase substrate system was purchased from KPL, Inc. (Gaithersburg, MD). Mass spectrometry grade water and acetonitrile (ACN), methanol (MeOH), and rapid equilibrium dialysis (ED) plates (12 kDa MWCO) were purchased from Fisher Scientific (Rockford, IL). Sodium fluoride was purchased from Sigma-Aldrich (Milwaukee, WI). Mass spectrometry standards, heroin•HCl, heroin-$d_3$, 6-acetylmorphine•HCl, 6-acetylmorphine-$d_3$, morphine•HCl, and morphine-$d_3$ were from Lipomed Inc. (Cambridge, MA). Heroin•HCl that was used for animal challenge experiments was from Cayman Chemical (Ann Arbor, MI).

## Synthesis

**2,2,2-Trichloroethyl (4*R*, 7*R*, 7a*R*,12b*R*)-7,9-dimethoxy-3-methyl-1,2,3,4,7,7a-hexahydro-7,4a-(epoxyimino)-4,12-methanobenzofuro[3,2-e]isoquinoline-15-carboxylate (4).** A solution of thebaine (12.62 g, 40.5 mmol, 1 equiv) in in EtOAc (403 mL) was charged with a buffer solution of 0.5M aq NaOAc (201 mL, pH adjusted to 6 with conc HCl) and stirred for 5 min. To this biphasic mixture was added $NaIO_4$ (13.01 g 60.8 mmol, 1.5 equiv) and the mixture was stirred at 25 °C for 5 min then cooled to 0 °C. Once cool, 2,2,2-trichloroethyl *N*-hydroxycarbamate (12.68 g, 60.8 mmol, 1.5 equiv) was added in three portions over a 15 min period. The mixture was allowed to stir at 0 °C open to atmosphere for 1 h until conversion was

complete by TLC (90:9:1 $CHCl_3$: MeOH: $NH_4OH$). The red suspension was then basified to pH 8.5 with satd aq $NaHCO_3$ (300 mL). The EtOAc layer was washed successively with $Na_2S_2O_3$ (400 mL) and brine (400 mL). The EtOAc layer was dried over $Na_2SO_4$, decanted, and concentrated under vacuum. The resulting brown oil was crystallized from MeOH. The solid was collected via vacuum filtration to give **4 (**8.00 g, 15.7 mmol) as a white solid. A second crop of **4** (4.04 g, 7.9 mmol) was obtained by crystallization of the filtrate from MeOH. The combined filtrates from the crystallizations were purified via $SiO_2$ column chromatography with 5% $NH_4OH$ in MeOH/$CHCl_3$ (gradient, 1→10%) to give additional **4 (**7.91 g, 14.9 mmol). This procedure afforded **4** (19.95 g, 38.9 mmol, 95% combined yield) as a tan solid. $^1$H-NMR (400 MHz; DMSO-$d_6$): δ 6.73 (d, *J* = 8.2 Hz, 1H), 6.59 (d, *J* = 8.2 Hz, 1H), 6.31 (dd, *J* = 8.9, 1.1 Hz, 1H), 6.22 (d, *J* = 8.9 Hz, 1H), 4.95 (d, *J* = 12.2 Hz, 1H), 4.79 (d, *J* = 12.2 Hz, 1H), 4.45-4.41 (m, 2H), 3.70 (s, 3H), 3.53 (d, *J* = 8.0 Hz, 3H), 3.28 (d, *J* = 18.8 Hz, 1H), 2.64 (dd, *J* = 18.8, 6.9 Hz, 1H), 2.55-2.51 (m, 1H), 2.37 (dd, *J* = 12.3, 3.4 Hz, 1H), 2.32 (d, *J* = 4.6 Hz, 3H), 2.13 (td, *J* = 12.8, 5.4 Hz, 1H), 1.83 (dd, *J* = 13.0, 1.7 Hz, 1H); $^{13}$C NMR (101 MHz; DMSO-$d_6$): δ 147.3, 141.4, 130.6, 127.6, 122.6, 119.8, 114.6, 104.5, 94.9, 89.5, 74.5, 57.8, 56.1, 52.5, 47.1, 44.3, 42.6, 32.9, 23.0; mp: 176-178 ˚C; HRMS (ESI): Calc. [M+H]+: 517.0700, Found: 517.0704; Analysis for $C_{22}H_{23}Cl_3N_2O_6$: Calc: C, 51.03; H, 4.48; N, 5.41; Found: C, 50.96; H, 4.60; N, 5.33.

***tert*-Butyl ((4*R*,4a*S*,7a*R*,12b*R*)-9-methoxy-3-methyl-7-oxo-1,2,3,4,5,6,7,7a-octahydro-4a*H*-4,12-methanobenzofuro[3,2-*e*]isoquinolin-4a-yl)carbamate. (*N*-Boc-14β-Aminodihydrocodeinone) (5). 4 (**7.58 g, 14.5 mmol) was dissolved in MeOH 200 mL (Note: It was necessary to warm the MeOH to ~ 40 ˚C to achieve this, then cool back to 25 ˚C). A separate solution of NaOAc (3.60 g, 43 mmol), AcOH (4.2 mL, 73 mmol) and $H_2O$ (28 mL) was prepared (Note: It is important that all solids are completely dissolved). These two solutions

were combined in a 500 mL Parr hydrogenation flask. To this mixture was added 10 % Pd/C (1.4 g, 0.1 g/mmol **4**). The flask was evacuated and then placed under 60 psi $H_2$ overnight while being shaken on a Parr apparatus. The catalyst was removed via filtration through Celite and the filter cake was washed with 2N AcOH (1 x 100 mL). The filtrate was basified to pH 9 with conc $NH_4OH$ and the mixture was extracted with $CHCl_3$ (4 x 100 mL). The combined organic layers were dried over $Na_2SO_4$, the solid was removed via vacuum filtration and the solvent was removed under reduced pressure. A silica gel filter column ($R_f$ : 0.62, 90:9:1 $CHCl_3$:MeOH:$NH_4OH$) gave 4.3 g of a slightly yellow oil that was an inseparable mixture of the desired 14β-aminodihydrocodeinone and the C-5 cleavage product. This mixture was dissolved in $CHCl_3$ (5 mL) and 1:1 THF:$H_2O$ (50 mL) was added. This was followed by the addition of $NaHCO_3$ (3.60 g, 43 mmol) and the mixture was cooled to 0 ˚C. A solution of $Boc_2O$ (3.8 mL, 16 mmol) in THF (10 mL) was prepared and then added dropwise to the biphasic mixture. The mixture was allowed to stir and warm to 25 ˚C overnight. The mixture was diluted with $H_2O$ (50 mL) and subsequently extracted with $Et_2O$ (4 x 100 mL). The combined organic layers were dried over $MgSO_4$, the solid removed via vacuum filtration, and the solvent was removed under reduced pressure. Purification via flash chromatography (1:1 hexanes: EtOAc) gave **5** (3.20 g, 7.7 mmol, 53% yield over 2 steps) as a white foam. $^1$H-NMR (400 MHz; DMSO-$d_6$): δ 6.92 (s, 1H), 6.75 (d, *J* = 8.1 Hz, 1H), 6.67 (d, *J* = 8.1 Hz, 1H), 4.96 (s, 1H), 3.77 (s, 3H), 3.70 (d, *J* = 5.3 Hz, 1H), 3.05 (d, *J* = 18.5 Hz, 1H), 2.66-2.57 (m, 1H), 2.42-2.35 (m, 2H), 2.31-2.20 (m, 5H), 2.07 (d, *J* = 15.7 Hz, 1H), 1.98-1.91 (m, 1H), 1.44 (d, *J* = 15.7 Hz, 9H), 1.40-1.33 (m, 1H), 1.19-1.16 (m, 1H); $^{13}$C NMR (101 MHz; DMSO-$d_6$): δ 208.3, 154.7, 144.4, 141.9, 128.9, 126.5, 119.1, 114.5, 88.8, 78.0, 58.2, 56.24, 56.21, 48.1, 45.0, 42.5, 36.1, 28.6, 28.3, 28.0, 20.6; HRMS (ESI): Calc. [M+H]+: 415.2233, Found: 415.2228; Analysis for $C_{23}H_{30}N_2O_5 \cdot 0.1\ H_2O$ : Calc: C, 66.36; H, 7.31; N, 6.73; Found: C, 66.06; H, 7.24; N, 6.42.

***tert*-Butyl ((4*R*,4a*S*,7*S*,7a*R*,12b*R*)-7-acetamido-9-methoxy-3-methyl-1,2,3,4,5,6,7,7a-octahydro-4a*H*-4,12-methanobenzofuro[3,2-*e*]isoquinolin-4a-yl)carbamate (6). 5 (**0.83 g, 2 mmol) was dissolved in 2M $NH_3$ in EtOH (6 mL) under Ar. To this was added $Ti(OiPr)_4$ (1.3 mL, 4.4 mmol) dropwise. The solution was allowed to stir at 25 ˚C for 6 h. Subsequently, $NaBH_4$ (0.16 g, 4.2 mmol) was added and the solution was allowed to stir at 25 ˚C overnight. To the solution was added 2N $NH_4OH$ (50 mL) and the mixture was stirred at 25 ˚C for 15 min. The solid was removed via vacuum filtration. The aq mixture was extracted with EtOAc (4 x 100 mL). The combined organic extracts were dried over $MgSO_4$, filtered to remove the solid and the solvent removed under reduced pressure. The resulting residue was dissolved in $CH_2Cl_2$ (100 mL). To this was added $Et_3N$ (0.9 mL, 6.4 mmol) and $Ac_2O$ (0.4 mL, 4.2 mmol) under Ar. The solution was heated to 40 ˚C overnight. The solution was cooled to 25 ˚C and diluted with $H_2O$ (75 mL). The mixture was extracted with $CH_2Cl_2$ (3 x 100 mL). The combined organic extracts were dried over $Na_2SO_4$, the solid was removed via vacuum filtration and the solvent was removed under reduced pressure. Purification via flash chromatography (90:9:1 $CHCl_3$:MeOH:$NH_4OH$, $R_f$ = 0.95) gave **6** (0.74 g, 1.6 mmol, 81% yield) as a pale yellow oil. $^1$H-NMR (400 MHz; DMSO-$d_6$): δ 7.46 (d, *J* = 7.6 Hz, 1H), 6.75 (d, *J* = 7.8 Hz, 2H), 6.59 (d, *J* = 8.2 Hz, 1H), 4.67 (d, *J* = 3.2 Hz, 1H), 4.35-4.30 (m, 1H), 3.78 (s, 4H), 2.97 (d, *J* = 18.6 Hz, 1H), 2.46-2.40 (m, 2H), 2.25 (dd, *J* = 24.0, 9.0 Hz, 2H), 2.18 (s, 3H), 2.02-1.96 (m, 1H), 1.86 (s, 3H), 1.42 (s, 9H), 1.33-1.27 (m, 1H), 1.10-1.08 (m, 2H), 0.85-0.79 (m, 1H); $^{13}$C NMR (101 MHz; DMSO-$d_6$): δ 168.6, 154.4, 145.9, 140.8, 130.6, 127.2, 118.2, 114.0, 87.6, 77.6, 57.6, 56.1, 54.7, 45.6, 44.7, 44.3, 43.0, 31.6, 28.4, 25.3, 22.7, 20.94, 20.80; HRMS (ESI): Calc. [M+H]+: 458.2655, Found: 458.2663.

***N*-((4*R*,4a*S*,7*S*,7a*R*,12b*R*)-7-Acetamido-9-hydroxy-3-methyl-1,2,3,4,5,6,7,7a-octahydro-4a*H*-4,12-methanobenzofuro[3,2-*e*]isoquinolin-4a-yl)-3-(tritylthio)propenamide (1)**. **6** (0.20 g, 0.4 mmol) was dissolved in $CH_2Cl_2$ (25 mL ) in a 50 mL round-bottom flask. The solution was cooled to -78 ˚C under Ar. To this solution was added neat $BBr_3$ (0.5 mL, 5 mmol). The solution was stirred -78 ˚C for 10 min then allowed to warm to 25 ˚C and stir for 3 h. After cooling back to -78 ˚C, the solution was quenched with MeOH (10 mL). The solvent was removed on the rotary evaporator and the quench procedure was repeated three times. The resulting oil was dissolved in $CH_2Cl_2$ (25 mL) and $Et_3N$ (0.4 mL, 2.8 mmol), *S*-tritylthiopropanoic acid (0.51 g, 1.4 mmol) and TBTU (0.60 g, 1.8 mmol) were added subsequently. The solution was allowed to stir at 25 ˚C overnight. The solution was diluted with satd $NaHCO_3$ (25 mL). The mixture was extracted with 9:1 $CHCl_3$:MeOH (3 x 100 mL). The organic extracts were dried over $Na_2SO_4$, the solid removed via vacuum filtration and the solvent removed under reduced pressure to give a white foam. The foam was taken up in MeOH (50 mL) and $K_2CO_3$ (0.5 g) was added in order to hydrolyze any bis-alkylated product. The mixture was stirred for 2.5 h at 25 ˚C. The mixture was extracted with 9:1 $CHCl_3$:MeOH (3 x 100 mL). The combined organic extracts were dried over $Na_2SO_4$, the solid removed via vacuum filtration and the solvent removed under reduced pressure. Purification via flash chromatography (90:9:1 $CHCl_3$:MeOH:$NH_4OH$; $R_f$: 0.2) gave **1** (0.085 g, 0.12 mmol, 29 % yield) as an off-white foam. $^1$H-NMR (400 MHz; DMSO-$d_6$): δ 8.86 (s, 1H), 7.61 (s, 1H), 7.44-7.40 (m, 1H), 7.42-7.37 (m, 10H), 7.28 (d, *J* = 2.2 Hz, 3H), 6.62 (dd, *J* = 7.9, 1.0 Hz, 1H), 6.50 (d, *J* = 7.9 Hz, 1H), 4.68 (d, *J* = 2.9 Hz, 1H), 4.33-4.29 (m, 1H), 3.87 (d, *J* = 5.9 Hz, 1H), 3.37 (s, 2H), 2.92 (d, *J* = 18.4 Hz, 1H), 2.48-2.23 (m, 7H), 2.07 (d, *J* = 19.9 Hz, 4H), 1.91 (s, 3H), 1.33-1.27 (m, 1H), 1.20 (d, *J* =

12.1 Hz, 1H), 1.14-1.08 (m, 1H), 0.84-0.81 (m, 1H); $^{13}$C NMR (101 MHz; DMSO-$d_6$): δ 169.8, 168.4, 145.2, 144.5, 138.0, 130.1, 129.1, 128.0, 126.6, 125.6, 118.3, 117.2, 87.2, 79.2, 65.8, 57.2, 55.3, 45.5, 44.9, 44.5, 43.1, 35.0, 31.6, 28.1, 24.9, 22.8, 21.0, 20.7; HRMS (ESI): Calc. [M+H]+: 674.3053, Found: 674.3062; Analysis for $C_{41}H_{43}N_3O_4S \cdot 1.0\ CHCl_3$ : Calc: C, 63.59; H, 5.59; N, 5.30; Found: C, 63.77; H, 5.73; N, 5.22.

**(4a*S*,7a*R*,12b*R*)-4a-Amino-9-methoxy-3-methyl-2,3,4,4a-tetrahydro-1H-4,12-methanobenzofuro[3,2-e]isoquinolin-7(7aH)-one** (**7**)**.** A 1 L 2-neck round-bottomed flask was charged with of 4 Å mol. sieves (3.8 g) and a magnetic stir bar. The flask was flame-dried under vacuum and upon cooling to room temperature charged with 10-camphorsulfonic acid (22.38 g, 96.3 mmol, 2.5 equiv). The flask was evacuated and backfilled with argon three times and charged with ethylene glycol (584 mL) via cannula and stirred for 5 min at room temperature. To this mixture was added a solution of **4 (**19.95 g, 38.9 mmol) in $CH_2Cl_2$ (77 mL) via syringe. The mixture was allowed to stir at room temperature for 2 h and then charged with ammonium carbonate (9.26 g, 96.32 mmol, 2.5 equiv) slowly in three portions over 30 min. A reflux-condenser was attached to the round-bottomed flask and activated zinc dust (15.12 g, 231.2 mmol, 6 equiv) was added and the suspension was heated at 100 °C for 5 h until conversion was complete by TLC ($R_f$: 0.41; 95:4.5:0.5 $CHCl_3$:MeOH:$NH_4OH$). The reaction mixture was filtered hot through a pad of Celite® and the Celite® pad washed with $CHCl_3$ (2 x 400 mL). The filtrate was poured into a separatory funnel containing satd aq $NaHCO_3$ (500 mL). The aq phase was extracted with $CHCl_3$ (3 x 500 mL) and the extracts washed with brine (1 x 1.5 L), dried over $MgSO_4$, filtered, and concentrated under vacuum affording dark oil. The oil was dissolved in 2:1 MeOH:$H_2O$ (347 mL) and charged with 6 N HCl (12.5 mL). The reaction was heated under reflux for 1 h until conversion was complete by TLC ($R_f$: 0.30; 95:4.5:0.5

$CHCl_3$:MeOH:$NH_4OH$). The reaction was cooled to room temperature and basified to pH 9-9.5 with 10% w/v aq NaOH. The bulk of the MeOH was stripped off under vacuum and the aq mixture was extracted with $CHCl_3$ (3 x 400 mL). The combined organic layers were washed with brine (1 x 1.2 L), dried over $Na_2SO_4$, decanted, and concentrated under vacuum affording a dark oil. Purification via $SiO_2$ column chromatography with 5% $NH_4OH$ in MeOH/$CHCl_3$ (gradient, 1→10%) afforded a yellow solid. Recrystallization from MeOH afforded **7 (**5.61 g, 17.9 mmol, 47%) as tan needles. $^1$HNMR (400 MHz; DMSO-$d_6$): δ 6.84 (d, *J* = 10.0 Hz, 1H), 6.70 (d, *J* = 8.2 Hz, 1H), 6.62 (d, *J* = 8.2 H, 1H), 5.94 (d, *J* = 10.0 Hz, 1H), 4.68 (s, 1H), 3.71 (s, 3H), 3.12 (d, *J* = 18.6 Hz, 1H), 2.88 (d, *J* = 5.3 Hz, 1H), 2.47 (s, 3H), 2.47 – 2.39 (m, 1H), 2.37 – 2.28 (m, 4H), 2.04 (td, *J* = 11.8, 3.4 Hz, 1H), 1.40 (d, *J* = 12.7 Hz, 1H); $^{13}$CNMR (101 MHz; $CDCl_3$): δ 194.4, 153.1, 143.8, 141.5, 131.5, 131.2, 126.2, 119.3, 113.8, 87.4, 63.3, 56.0, 52.2, 46.3, 45.2, 42.4, 28.3, 21.6; HRMS-ESI (m/z): [M+H]+ calcd for $C_{18}H_{21}N_2O_3$ 313.1552, found: 313.1553.

**(4a*S*,7*S*,7a*R*,12b*R*)-4a-Amino-9-methoxy-3-methyl-2,3,4,4a,7,7a-hexahydro-1H-4,12-methanobenzofuro[3,2-e]isoquinolin-7-ol (8).** A solution of **7 (**2.00 g, 6.4 mmol, 1 equiv) in MeOH (1.2 L) was charged with $NaBH_4$ (3.33 g, 88.0 mmol, 13.8 equiv) added carefully in three portions over 30 min at room temperature. The reaction was stirred at room temperature for 3.5 h until conversion was complete by TLC ($R_f$: 0.24; 95:4.5:0.5 $CHCl_3$:MeOH:$NH_4OH$). The reaction was concentrated under vacuum and the crude residue was taken up in $CHCl_3$ (250 mL) and poured into a separatory funnel containing $H_2O$ (250 mL). The aq layer was extracted with $CHCl_3$ (3 x 500 mL) and the combined organic layers were washed with brine (1 x 1.5 L), dried over $Na_2SO_4$, decanted, and concentrated under vacuum affording **8** (2.02 g, 6.4 mmol, 99%) as a white solid. $^1$HNMR (400 MHz; DMSO-$d_6$): δ 6.58 (d, *J* = 7.4 Hz, 1H), 6.46 (d, *J* = 7.8 Hz, 1H), 5.57 (d, *J* = 9.3 Hz, 1H), 5.44 (d, *J* = 9.0 Hz, 1H), 4.72 (s, 1H), 4.64 (s, 2H), 3.72 (s, 3H),

3.32 (s, 1H), 3.04 (d, $J$ = 18.5 Hz, 1H), 2.77 (s, 1H), 2.42 (d, $J$ = 9.5 Hz, 1H), 2.37 – 2.26 (m, 4H), 2.24 – 2.11 (m, 2H), 2.02 (s, 2H), 1.45 (d, $J$ = 11Hz, 1H); $^{13}$CNMR (101 MHz; DMSO-$d_6$): δ 146.0, 141.4, 135.4, 133.0, 131.7, 126.6, 117.8, 113.1, 90.9, 64.8, 64.3, 56.0, 52.7, 46.7, 45.3, 42.7, 30.7, 21.2; HRMS-ESI (m/z): [M+H]+ calcd for $C_{18}H_{22}N_2O_3$ 315.1709, found 315.1703.

**(4a*S*,7*S*,7a*R*,12b*R*)-4a-Amino-9-methoxy-3-methyl-2,3,4,4a,5,6,7,7a-octahydro-1H-4,12-methanobenzofuro[3,2-e]isoquinolin-7-ol (9).** A solution of **8** (0.40 g 1.27 mmol, 1 equiv) in MeOH (61 mL) was added to a 1 L Parr hydrogenation flask containing 5% w/w wet palladium on carbon (0.1 g). The flask was evacuated and then placed under 50 psi $H_2$ for 2.5 h while being shaken on a Parr apparatus until conversion was complete by TLC ($R_f$: 0.20; 95:4.5:0.5 $CHCl_3$:MeOH:$NH_4OH$). The crude reaction mixture was filtered through a pad of Celite® and the filter cake was washed with MeOH (2 x 150 mL). The filtrate was concentrated under vacuum and purified via $SiO_2$ column chromatography with 5% $NH_4OH$ in MeOH/$CHCl_3$ (gradient, 1→10%) affording **9** (0.33 g, 1.0 mmol, 81%) as a white solid. $^1$HNMR (400 MHz; $CDCl_3$): δ 6.71 (d, $J$ = 8.2 Hz, 1H), 6.61 (d, $J$ = 8.2 Hz, 1H), 4.66 (d, $J$ = 4.4 Hz, 1H), 4.44 – 4.40 (m, 1H), 3.87 (s, 3H), 3.13 (d, $J$ = 17.5 Hz, 1H), 2.58 – 2.47 (m, 2H), 2.45 – 2.38 (m, 1H), 2.33 (s, 3H), 2.25 – 2.12 (m, 3H), 2.08 – 1.80 (brs, 2H), 1.77 – 1.71 (m, 1H), 1.56 – 1.45 (m, 2H), 1.26 (dt, $J$ = 14.8, 7.7 Hz, 1H), 1.11 – 1.00 (m, 1H); $^{13}$CNMR (101 MHz; $CDCl_3$): δ 146.4, 141.5, 132.0, 127.0, 118.7, 113.5, 90.9, 66.8, 66.2, 56.5, 52.2, 46.4, 45.4, 43.5, 33.0, 30.4, 24.2, 21.7; HRMS-ESI (m/z): [M+H]+ calcd for $C_{18}H_{24}N_2O_3$ 317.1865, found 317.1866.

**(4a*S*,7*S*,7a*R*,12b*R*)-4a-Amino-3-methyl-2,3,4,4a,5,6,7,7a-octahydro-1H-4,12-methanobenzofuro[3,2-e]isoquinoline-7,9-diol (10)**: A solution of **9** (1.42 g, 4.5 mmol, 1.0 equiv) in $CH_2Cl_2$ (90 mL) was cooled to 0 °C and charged dropwise with a cold solution of neat

$BBr_3$ (2.56 mL, 27.0 mmol, 6 equiv) in $CH_2Cl_2$ (27 mL) over 15 min at 0 °C. The reaction mixture was gradually allowed to warm up to room temperature over 5 h until TLC ($R_f$: 0.5; 90:9:1 $CHCl_3$:MeOH:$NH_4OH$) analysis indicated the full consumption of starting material. The reaction was cooled to 0 °C and quenched by the careful addition of MeOH (18 mL) followed by 4N aq NaOH (24 mL) affording a red biphasic mixture. The crude reaction mixture was transferred to a separatory funnel and the organic layer was washed successively with 2N aq NaOH (2 x 20 mL) and $H_2O$ (1 x 90 mL). Concentration of the organic layer under vacuum afforded unreacted **9** (0.14 g, 0.4 mmol, 10%) as a tan solid. The combined alkaline aq extracts were acidified to pH 1 with concentrated HCl and then basified to pH 9 with concentrated $NH_4OH$. The aq layer was extracted with 9:1 $CHCl_3$:MeOH (6 x 100 mL), dried over $Na_2SO_4$, decanted, and concentrated under vacuum affording **10 (**1.14 g, 3.8 mmol, 84%) as a tan solid which was used without further purification. $^1$HNMR (400 MHz; DMSO-$d_6$): δ 8.71 (s, 1H), 6.52 (d, *J* = 8.0 Hz, 1H), 6.41 (d, *J* = 8.0 Hz, 1H), 4.40 – 4.31 (m, 2H), 4.19 (d, *J* = 5.7 Hz, 1H), 2.97 (d, *J* = 18.0 Hz, 1H), 2.47 (s, 1H), 2.42 (d, *J* = 18.1, 6.0 Hz, 1H), 2.33 (d, *J* = 6.6 Hz, 1H), 2.25 (s, 3H), 2.10 (brs, 1H), 2.04 (Apq, *J* = 10.9 Hz, 2H), 1.44 – 1.33 (m, 1H), 1.32 – 1.20 (m, 3H), 0.95 – 0.80 (m, 1H); $^{13}$CNMR (101 MHz; DMSO-$d_6$): δ 145.8, 137.7, 131.5, 125.1, 117.6, 116.7, 90.4, 65.9, 64.5, 51.5, 45.9, 44.8, 43.1, 32.8, 29.7, 23.4, 21.0; HRMS-ESI (m/z): [M+H]+ calcd for $C_{17}H_{22}N_2O_3$ 303.1709, found 303.1707.

**N-((4a*S*,7*S*,7a*R*,12b*R*)-7,9-Dihydroxy-3-methyl-1,2,3,4,5,6,7,7a-octahydro-4aH-4,12-methanobenzofuro[3,2-e]isoquinolin-4a-yl)-3-(tritylthio)propenamide (2)**. A solution of 3-(tritylthio)propanoic acid (0.52 g, 1.5 mmol, 3 equiv) and TBTU (0.48 g , 1.5 mmol, 3 equiv) in DMF (3 mL) was cooled to 0 °C and charged with $Et_3N$ (0.42 mL, 3.0 mmol, 6 equiv) dropwise via syringe. The reaction mixture was stirred for 20 min, then charged with a solution of **10 (**0.15

g, 0.5 mmol, 1 equiv) in DMF (2 mL). The reaction was allowed to gradually warm to room temperature over 3 h until conversion was complete by TLC ($R_f$: 0.33; 90:9:1 $CHCl_3$:MeOH:$NH_4OH$). DMF was removed under high vacuum at 60 °C and the resulting brown oil was taken up in 1:1:1 THF:EtOH: 10% aq. KOH (6 mL) and refluxed for 15 min. After cooling to room temperature, $H_2O$ (20 mL) was added and the aq layer was extracted with 9:1 $CHCl_3$:MeOH (4 x 40 mL). The combined organic layers were washed with brine (1 x 200 mL), dried over $Na_2SO_4$, decanted, and concentrated under vacuum. The crude reaction mixture was purified via $SiO_2$ column chromatography with 5% $NH_4OH$ in MeOH/$CHCl_3$ (gradient, 1→10%) affording 0.143 g (0.23 mmol, 45%) of **2** as a white foam. $^1$HNMR (400 MHz; DMSO-$d_6$): δ 8.73 (s, 1H), 7.36 (s, 1H), 7.33 – 7.26 (m, 12H), 7.25 – 7.17 (m, 3H), 6.52 (d, *J* = 7.9 Hz, 1H), 6.39 (d, *J* = 7.9 Hz, 1H), 4.52 (d, *J* = 3.8 Hz, 1H), 4.27 (d, *J* = 6.3 Hz, 1H), 4.08 – 3.99 (m, 1H), 3.79 (d, *J* = 5.9 Hz, 1H), 2.85 (d, *J* = 18.6 Hz, 1H), 2.35 – 2.14 (m, 8H), 2.02 (s, 3H), 1.97 (td, *J* = 11.9, 3.2 Hz, 1H), 1.30 (t, *J* = 10.8 Hz, 1H), 1.15 (d, *J* = 11.5 Hz, 1H), 1.09 – 0.98 (m, 1H); $^{13}$CNMR (101 MHz; DMSO-$d_6$): δ 169.3, 145.6, 144.4, 137.8, 130.2, 128.9, 127.9, 126.5, 125.3, 117.7, 117.0, 109.4, 88.8, 65.7, 64.5, 57.2, 55.6, 45.7, 44.4, 43.0, 34.8. 31.6, 28.1, 24.8, 23.5, 20.6; HRMS-ESI (m/z): [M+H]+ calcd for $C_{39}H_{41}N_2O_4S$ ($M+H^+$) 633.2795, found 633.2787. Anal. Calc for $C_{38}H_{40}N_2O_4S$ ● 0.6$CHCl_3$ C, 66.96%; H, 5.91%; N, 4.05%. Found C, 67.13%; H, 5.60%; N, 3.89%.

**(4a*S*,7*S*,7a*R*,12b*R*)-9-Hydroxy-3-methyl-4a-(3-(tritylthio)propanamido)-2,3,4,4a,5,6,7,7a-octahydro-1H-4,12-methanobenzofuro[3,2-e]isoquinolin-7-yl acetate (3).** A solution of **2** (0.327 g, 0.52 mmol, 1 equiv), DMAP (0.032g, 0.26 mmol, 0.5 equiv) in $CH_2Cl_2$ (26 mL) was charged with $Et_3N$ (0.43 mL, 3.12 mmol, 6 equiv) and cooled to 0 °C. $Ac_2O$ (0.2 mL, 2.08 mmol, 4 equiv) was added dropwise via syringe and the reaction was allowed to gradually warm to

room temperature overnight. Conversion to the diacetate was complete by TLC ($R_f$: 0.53; 95:4.5:0.5 $CHCl_3$:MeOH:$NH_4OH$). The reaction was concentrated under vacuum and the residue was taken up in EtOAc (30 mL). The organic phase was washed with satd $NaHCO_3$ (2 x 30 mL), brine (1 x 30 mL), dried over $MgSO_4$, filtered, and concentrated under vacuum affording a tan solid. The solid was transferred to a 35 mL pressure tube and dissolved in 9:1 MeOH/$CHCl_3$ (10 mL). Concentrated $NH_4OH$ (0.2 mL) was added and the tube was purged with argon, sealed, and stirred at room temperature overnight. Additional $NH_4OH$ (0.1 mL) was added after this time to drive the reaction to completion. The reaction was concentrated under vacuum and purified via $SiO_2$ column chromatography ($CHCl_3$/MeOH/$NH_4OH$ 97:2.5:0.5) affording **3** (0.136 g, 0.20 mmol, 39%) as a white foam. $R_f$ : 0.19 (95:4.5:0.5 $CHCl_3$:MeOH:$NH_4OH$); $^1$HNMR (400 MHz; DMSO-$d_6$): δ 8.99 (s, 1H), 7.49 (s, 1H), 7.35 – 7.29 (m, 12H), 7.28 – 7.21 (m, 3H), 6.58 (d, *J* = 8.0 Hz, 1H), 6.47 (d, *J* = 8.1 Hz, 1H); 5.21 (m, 1H), 4.79 (d, *J* = 4.79 Hz, 1H), 3.79 (d, *J* = 5.4 Hz, 1H), 2.91 (d, *J* = 18.4 Hz, 1H), 2.36 (dd, *J* = 18.3, 5.6 Hz, 1H), 2.32 – 2.11 (m, 7H), 2.08 (s, 3H), 1.97 (td, *J* = 11.5, 3.1 Hz, 1H), 1.89 (s, 3H), 1.67 – 1.55 (m, 1H), 1.25 – 1.07 (m, 3H); $^{13}$CNMR (101 MHz; DMSO-$d_6$): δ 169.7, 169.4, 145.0, 144.5, 138.3, 129.9, 129.0, 128.0, 126.6, 124.6, 118.2, 116.9, 84.8, 67.6, 65.8, 57.5, 56.4, 45.6, 44.9, 42.9, 35.0, 30.6, 28.1, 23.5, 21.6, 20.7, 20.6; HRMS-ESI (m/z): [M+H]+ calcd for $C_{41}H_{42}N_2O_5S$ ($M+H^+$) 675.2893, found 675.2882. Anal. Calc for $C_{41}H_{42}N_2O_5S$ ● 0.9$CHCl_3$ C, 64.33%; H, 5.53%; N, 3.58%. Found C, 64.38%; H, 5.31%; N, 3.59%.

## Synthesis of TT-hapten conjugates and vaccine formulation

A reaction based on thiol-maleimide chemistry[1, 2] was used to conjugate the haptens to TT. Briefly, TT was reacted with $SM(PEG)_2$ in DMSO at a protein:linker ratio of 1:1600 for 2 h at 25 °C in BupH 7.2 (100 mM sodium phosphate, 150 mM sodium chloride, pH 7.2). Excess

linker was removed by spin column (Zeba™, 7K MWCO) and the flow through containing TT-maleimide was reacted with the deprotected hapten at a protein: hapten molar ratio of 1:300 for 2 h at 25 °C in BupH 7.2. Hapten deprotection was performed following the published procedures.[1, 3] The hapten concentration was measured by Ellman's assay.[1] The reaction products were transferred to dialysis cassettes (Slide-A-Lyzer G2™, 10K MWCO) and repeatedly dialyzed overnight against DPBS, pH 7.4 at 4 °C. Protein content was quantified using Pierce™ BCA assay kit following manufacturer's instructions. Hapten density was measured using MALDI-TOF MS in an AXIMA MegaTOF (Shimadzu Scientific Instruments, Columbia, MD) as described.[1, 3, 4] Each final vaccine formulation of 50 µL contained 10 µg TT-hapten conjugate (based on protein content of the protein-hapten conjugate), 20 µg 3D-PHAD® in ALF43, and 30 µg aluminum (Alhydrogel®) in DPBS pH 7.4. ALF43 contained DMPC:DMPG:cholesterol:3D-PHAD® at a molar ratio of 9:1:7.5:1.136; the molar ratio of phospholipids:3D-PHAD® was 8.8:1. ALF43, derived from small unilamellar vesicles (SUVs), was prepared as lyophilized powder following the detailed procedures as previously described.[5, 6] The total concentration of phospholipids in the reconstituted ALF43A was 2.29 mM. The ALF that contains 43% cholesterol in our previous works[4, 5] and the ALF43 described here are the same. Our older works[7-9] used liposomes containing large amounts of phospholipids and cholesterol.

## Animal studies

All animal studies were conducted under an approved animal use protocol in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALACi)-accredited facility in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals. Experiments involving animals adhered to the principles stated in the Guide for the Care and Use of Laboratory Animals, 8th edition[10]. Briefly,

~7 weeks old female Balb/c mice ($n$=10 per group) (Jackson Laboratories, Bar Harbor, ME) were immunized *via* intramuscular (*i.m.*) route at alternate rear thighs with 50 µL of vaccine formulation on weeks 0, 3, and 6, and 14. Mice were bled at weeks 0, 3, 6, 9, 14, and 20 for serum collection. Heroin challenge (1.0 mg/kg) was performed at week 16 *via* subcutaneous (*s.c.*) route. Antinociception was measured 15 min post-heroin injection.

## Antinociception assay

The tail-immersion assay was performed as described.[3] Briefly, the mouse tail was immersed in a water bath set at 54 °C (IITC Life Science, Woodland Hills, CA). The latency times were measured with a cutoff time of 8 sec to prevent tail injury. Antinociception, measured as % Maximum Potential Effect (%MPE) was calculated using equation (1):

$$\%MPE = \frac{Post\ injection\ latency\ time - baseline\ latency\ time}{Cutoff\ latency - baseline\ latency\ time} x\ 100 \quad (1)$$

## Drug sequestration assay

Serum binding was measured using equilibrium dialysis (ED) as before.[3, 11] To enable multiple measurements of the limited amount of samples, sera were pooled and diluted. Mouse sera from week 14 were diluted with 0.05% BSA in Dulbecco's phosphate-buffered saline (DPBS), pH 7.4 (ED buffer) containing 5 nM of a drug. For heroin serum binding analysis, the ED buffer was added with 3 mg/mL of sodium fluoride to prevent spontaneous hydrolysis.[12] An aliquot (100 µL) was seeded into sample chambers of rapid equilibrium dialysis plate and the buffer chamber was filled with 300 µL of ED buffer. The plate was incubated at 4 °C and 300 rpm for 24 h in a thermomixer. Aliquots (90 µL) from sample and buffer chambers were pipetted out and analyzed by LC-MS/MS.

The instrument parameters used in the LC-MS/MS analysis are based on previous works.[3, 13, 14] The column was maintained at 65 °C at a flow rate of 500 µL/min. The injection

volume was 10 µL using a full-loop injection mode using the gradient shown in **Table S1**. The autosampler needle was rinsed with a weak wash (600 µL, 10 % MeOH in $H_2O$) and a strong wash (200 µL, 90 % ACN in $H_2O$) before each injection. All data were acquired using positive electrospray ionization (ESI) in multiple reaction monitoring (MRM) mode. The electrospray and source settings were as follows: 0.7 kV (capillary voltage), 120 °C (source temperature), 500 °C (desolvation temperature), 900 L/h (desolvation gas flow, $N_2$), and 60 L/h (cone gas flow, $N_2$). The collision gas (Ar) flow in the collision cell was maintained at 0.3 mL/min. MRM transitions are provided in **Table S2**. Data were processed using external calibration with $1/X^2$ weighting in TargetLynx™ application of MassLynx™ version 4.2 software (Waters, Milford, MA).

**Table S1. LC-MS/MS gradient**

| Time (min) | % A (10 mM $NH_4HCOO$ with 0.1% HCOOH) | % B (MeOH with 0.1% HCOOH) |
|---|---|---|
| 0 | 100 | 0 |
| 0.50 | 100 | 0 |
| 2.70 | 90 | 10 |
| 3.30 | 80 | 20 |
| 4.60 | 20 | 80 |
| 4.61 | 0 | 100 |
| 5.20 | 0 | 100 |
| 5.21 | 100 | 0 |
| 8.00 | 100 | 0 |

**Table S2. MRM transitions, cone voltage, and collision energy settings**

| Analyte | Ret. Time (min) | MRM[a] transition (*m/z*) | Cone voltage (V) | Collision energy (V) | Dwell time (msec) |
|---|---|---|---|---|---|
| Heroin-$d_3$ | 4.46 | 373>165 | 40 | 40 | 328 |
| 6-Acetylmorphine-$d_3$ | 3.96 | 331>165 | 40 | 35 | 328 |
| Morphine-$d_3$ | 2.06 | 289>165 | 40 | 40 | 328 |
| Naloxone-$d_5$ | 3.47 | 333>212 | 35 | 30 | 328 |
| Methadone | 4.99 | 310>105 | 30 | 30 | 328 |
| Buprenorphine | 4.69 | 468>101 | 40 | 50 | 328 |
| Naltrexone-$d_3$ | 3.82 | 345>212 | 40 | 45 | 328 |

[a]All ions were detected as $[M+H]^+$

## Enzyme-linked immunosorbent assay

The IgG endpoint titers against haptens **1**, **2**, and **3** were measured using the sera collected on weeks 0, 3, 6, 9, 14, and 20. Plates were coated with either BSA-**1**, BSA-**2**, or BSA-**3** (0.1 μg/0.1 mL in PBS, pH 7.4) and incubated overnight at 4 °C. ELISA was performed the next day as noted.[3, 4, 8] Synthesis of the coating agents was performed following published procedures.[1]

## Data analysis

The 3D molecular modeling of compounds described in this study was performed in ChemDraw 19.1. Structures were energy minimized using the built-in molecular mechanics 2 (MM2) method. Data processing and analyses were performed using Prism 8 (GraphPad Inc., San Diego, CA). In competition ED LC-MS/MS, $IC_{50}$ was interpolated from the linear regression of % inhibition as a function of log-transformed concentrations of a competitive inhibitor. Statistical comparisons between the control and the immunized group employed ordinary one-way ANOVA. In comparing serum binding data, a two-tailed, unpaired *t*-test was used. Statistical significance was defined as $p \leq 0.05$.

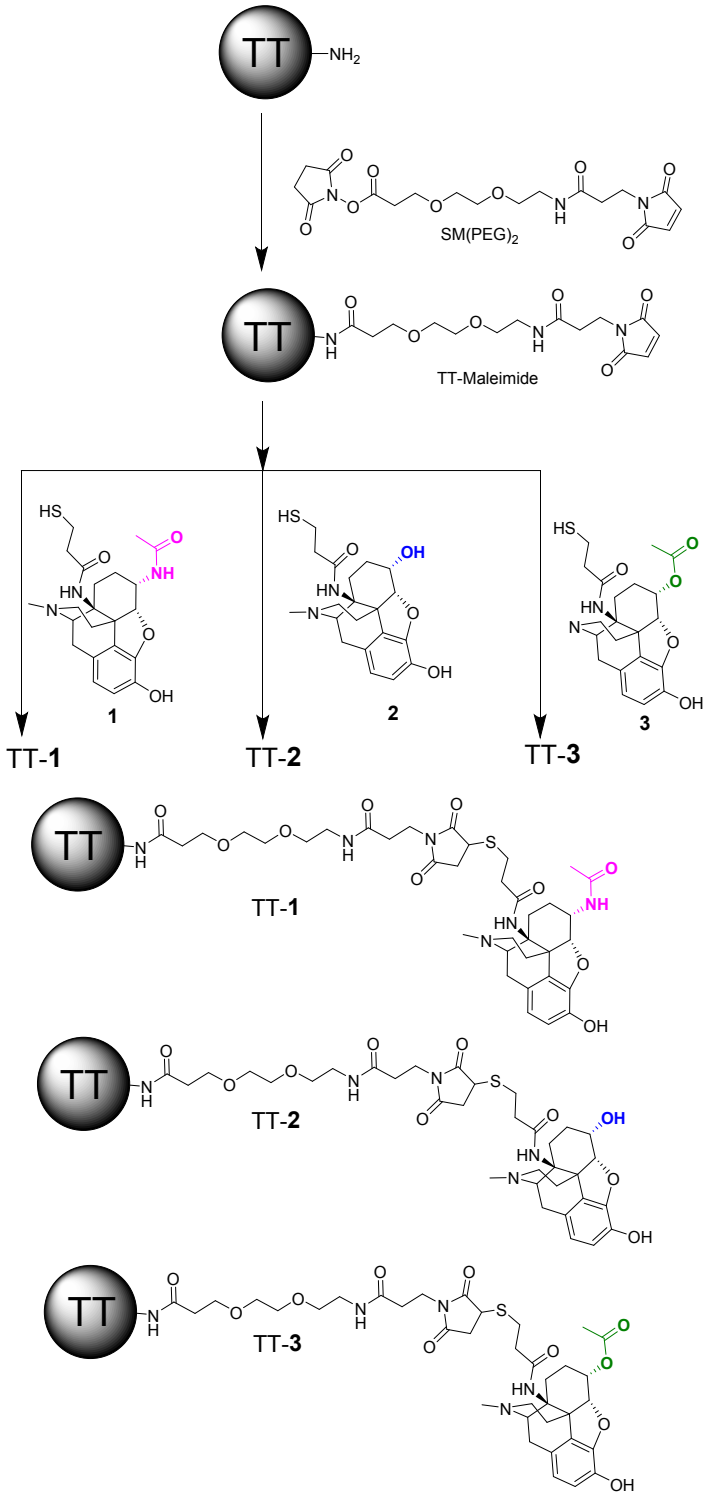



**Fig. S1 Conjugation of SM(PEG)2 linker and hapten to TT carrier protein.**

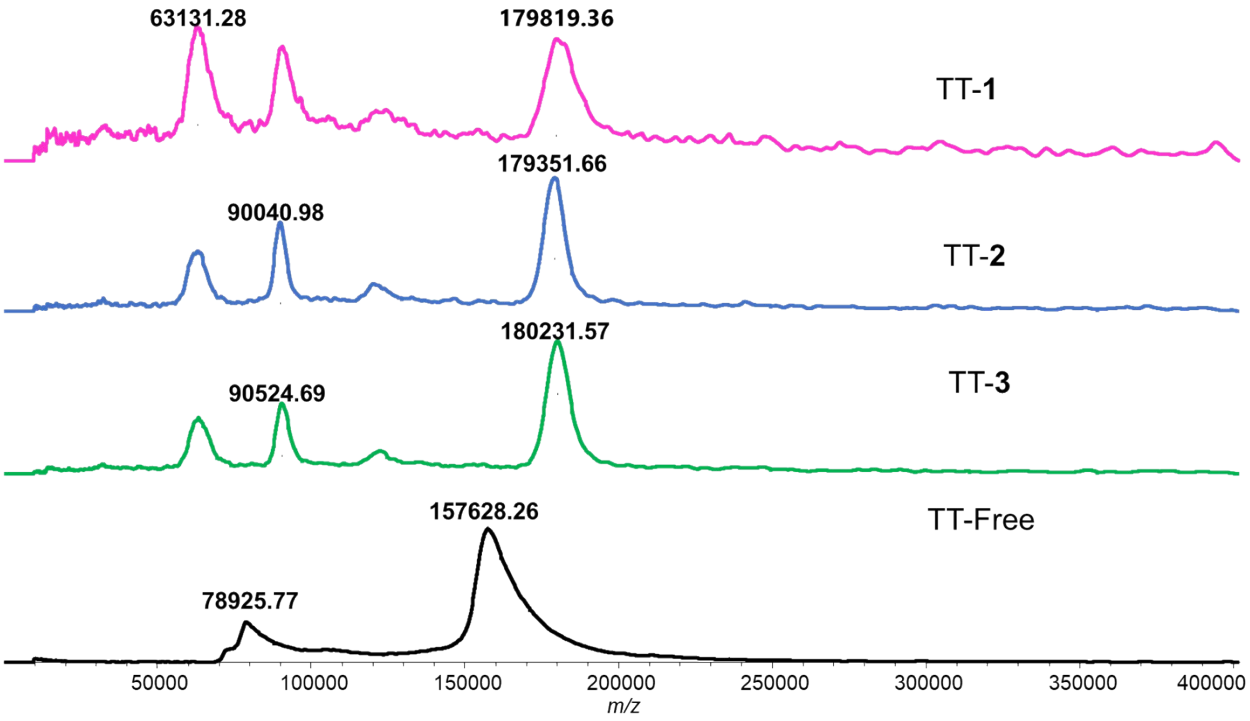



**Fig. S2 Representative MALDI-TOF MS spectra of TT-hapten conjugates.** Samples were desalted and co-crystallized with sinapinic acid on a MALDI steel plate. Spectra were smoothed using the Gaussian method, and masses were assigned using the threshold apex peak detection method.

Hapten density was calculated as described,[1, 4] according to the following equation:

$$Hapten\ density = \frac{mass_{TT-hapten\ conjugate} - mass_{unconjugated\ TT}}{mass_{linker+hapten}}$$

Sample calculation is shown in **Table S3.**

**Table S3. Sample calculation of hapten density**

| Sample | *m/z* | Hapten MW | Hapten density |
|---|---|---|---|
| TT-**1** | 179819.36 | 431.55 | 29.92 |
| TT-**2** | 179351.66 | 390.5 | 31.01 |
| TT-**3** | 180231.57 | 433.54 | 30.39 |

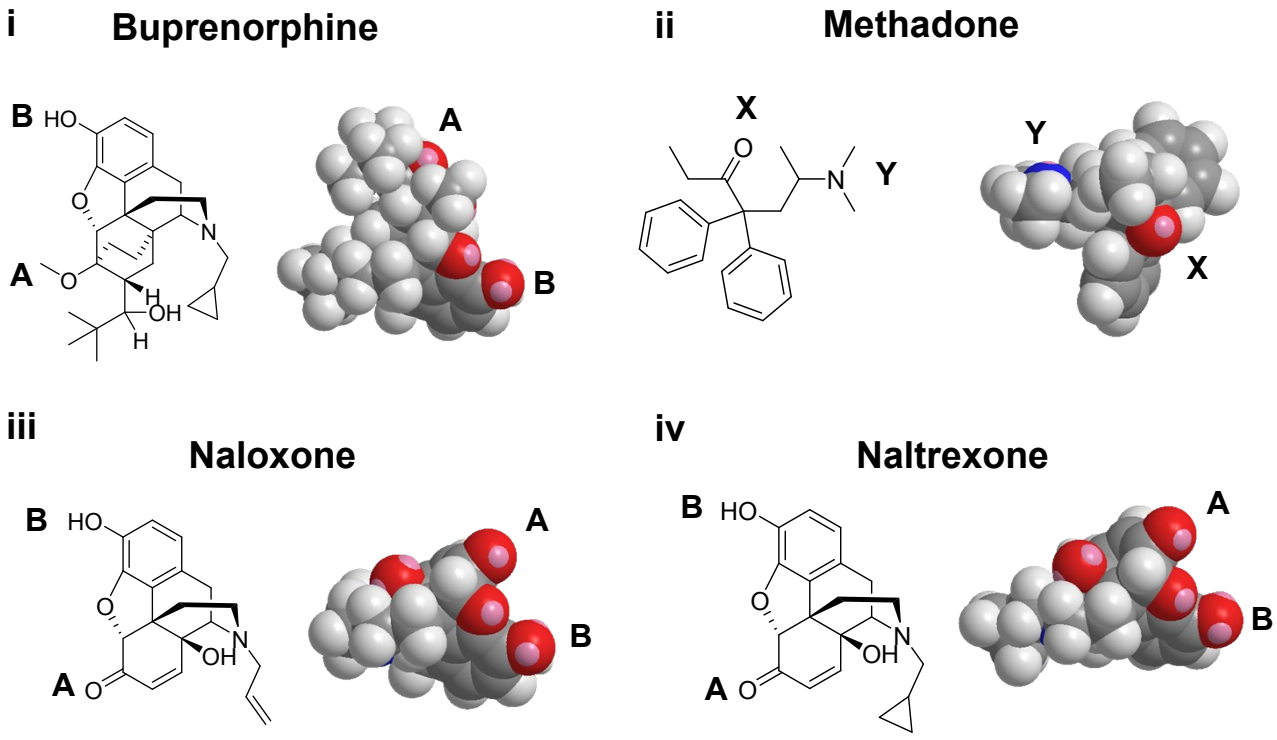
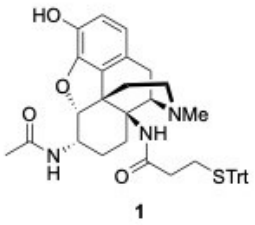



**Fig. S3 Space-filling models of therapeutic drugs.** A) Buprenorphine. B) Methadone. C) Naloxone. D) Naltrexone. Structures were drawn in ChemDraw 19.1 and minimized using the built-in MM2 method.

**NMR Spectra**

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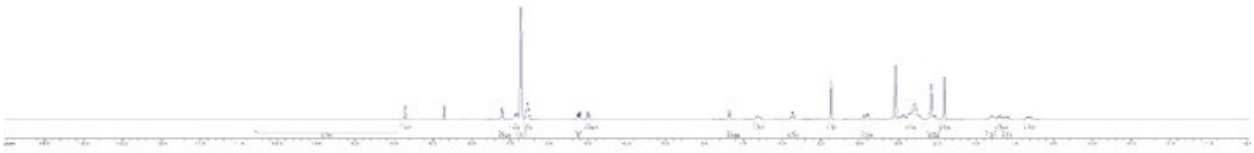

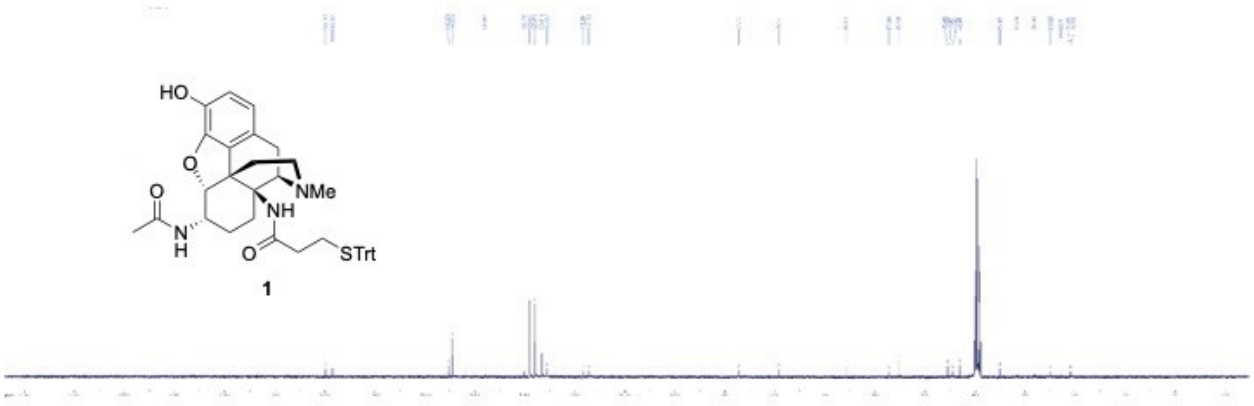


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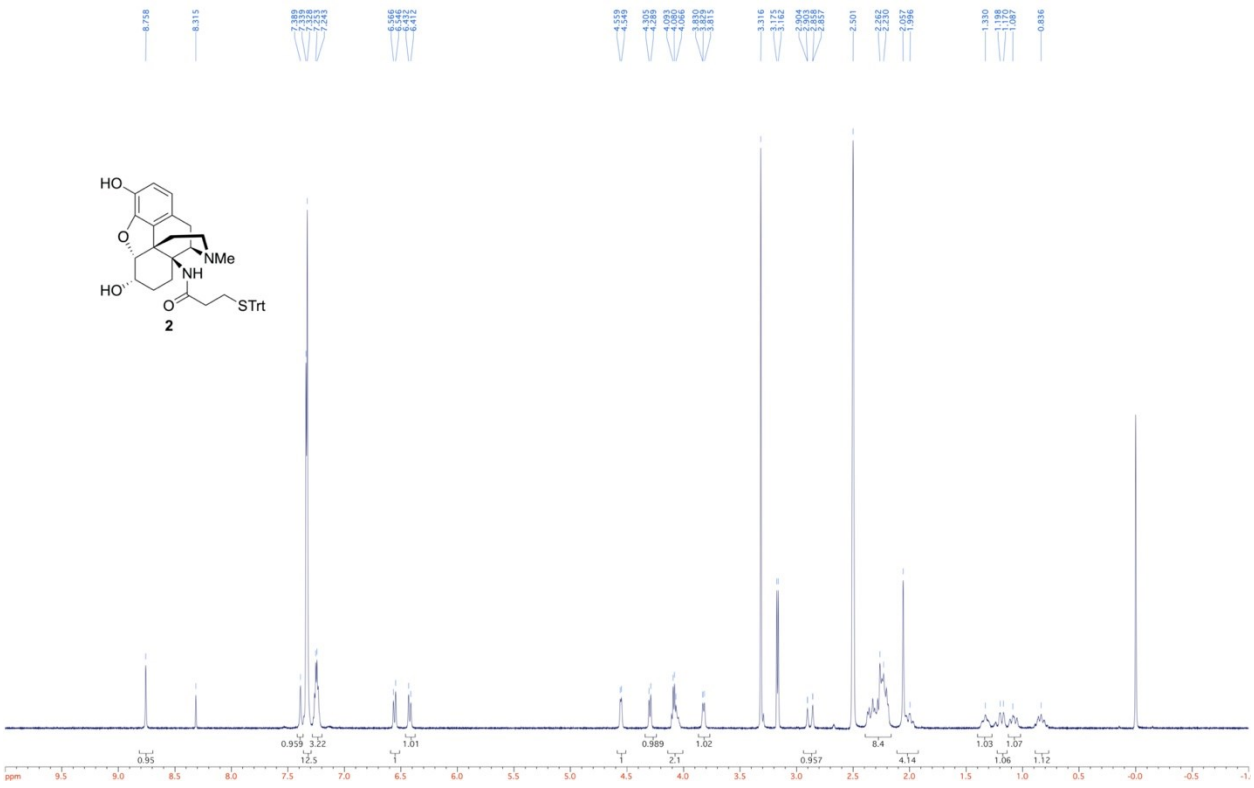
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HO
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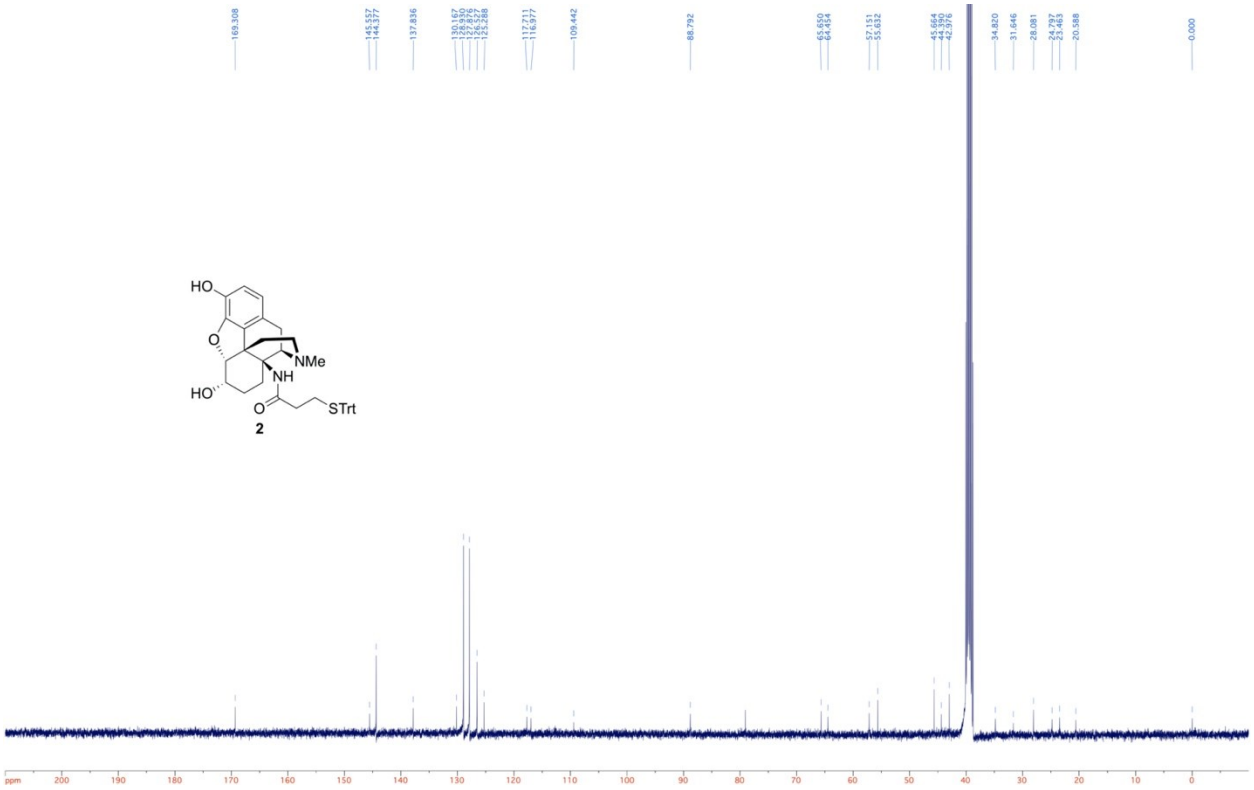
STrt
2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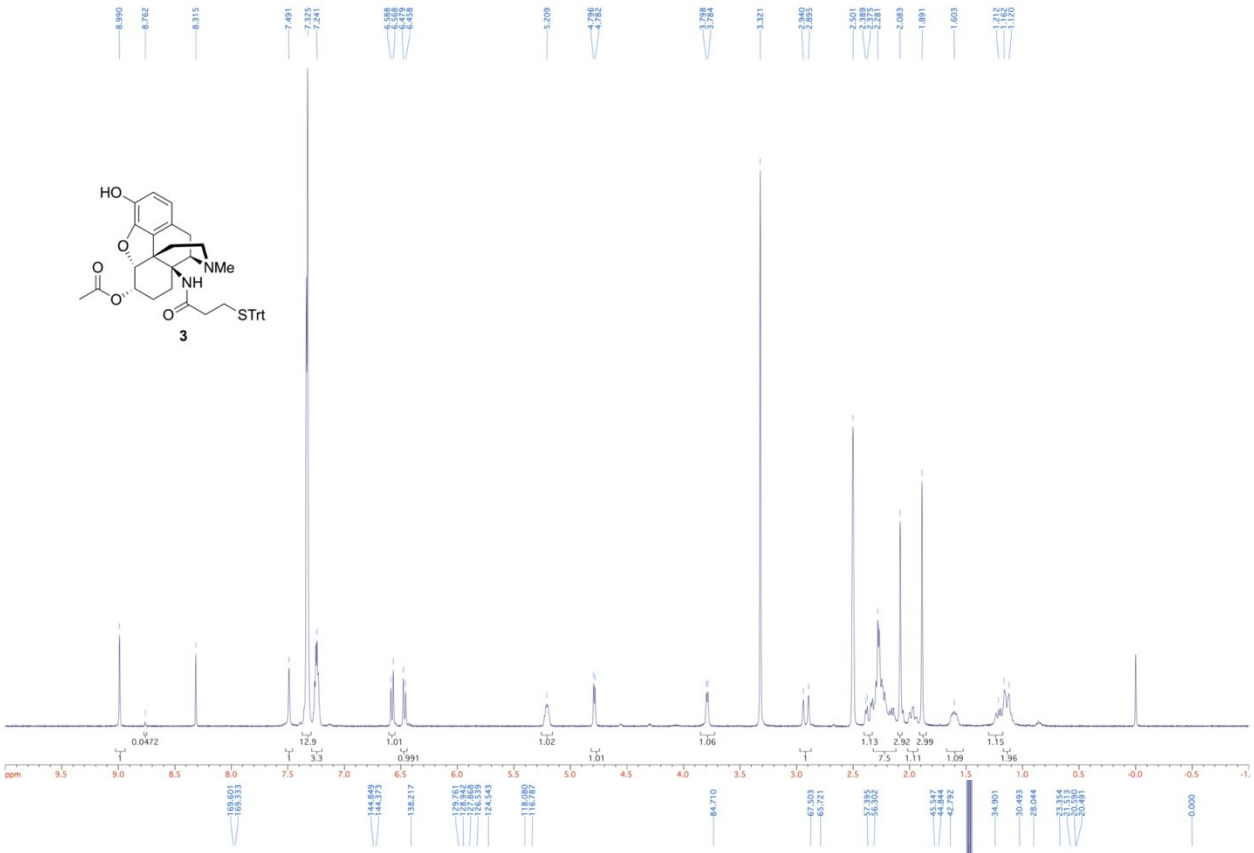
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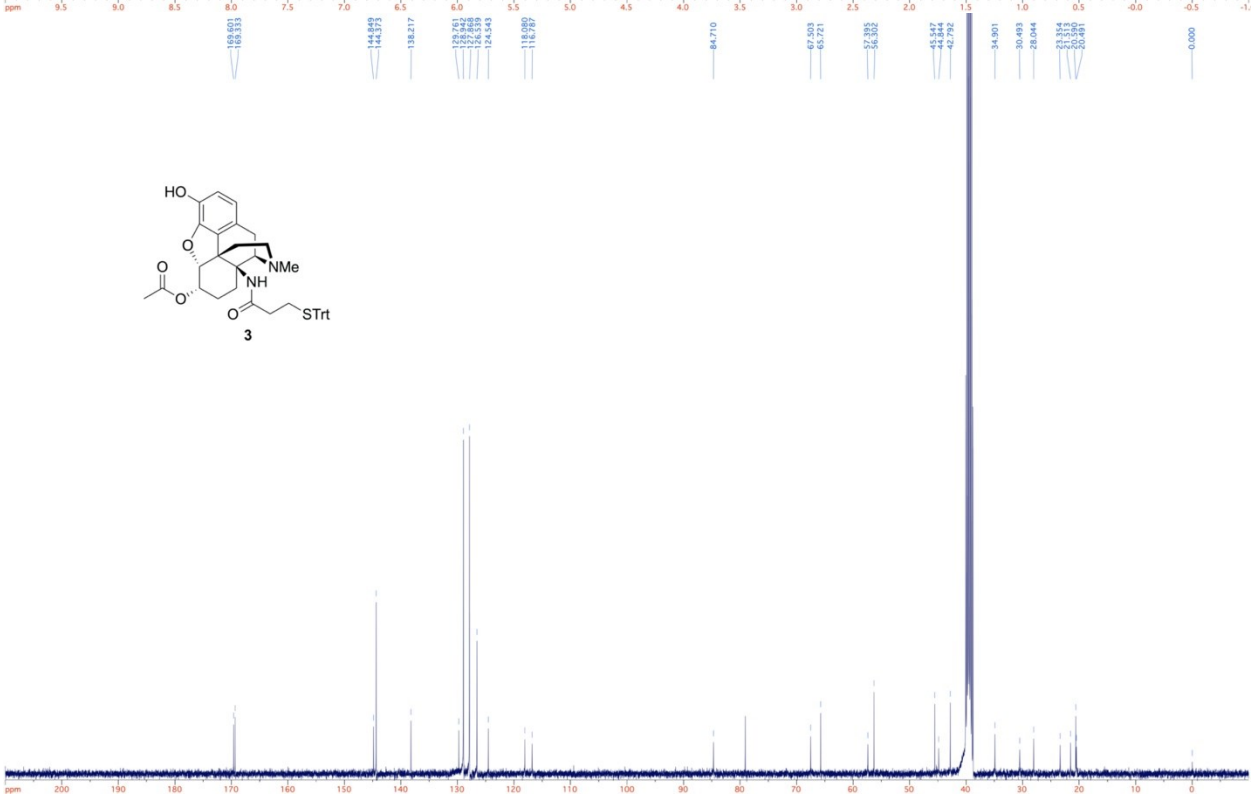
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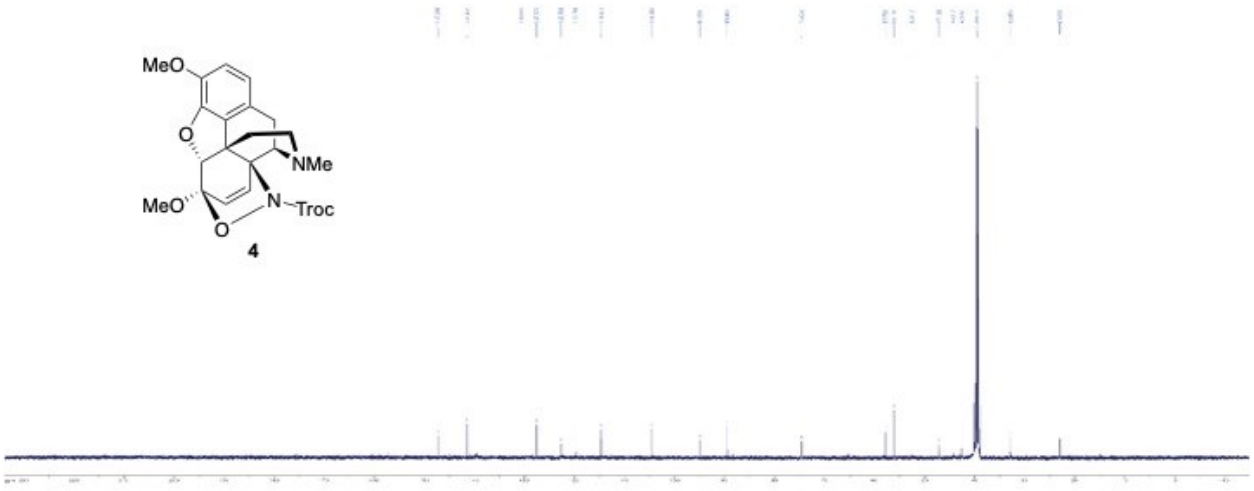
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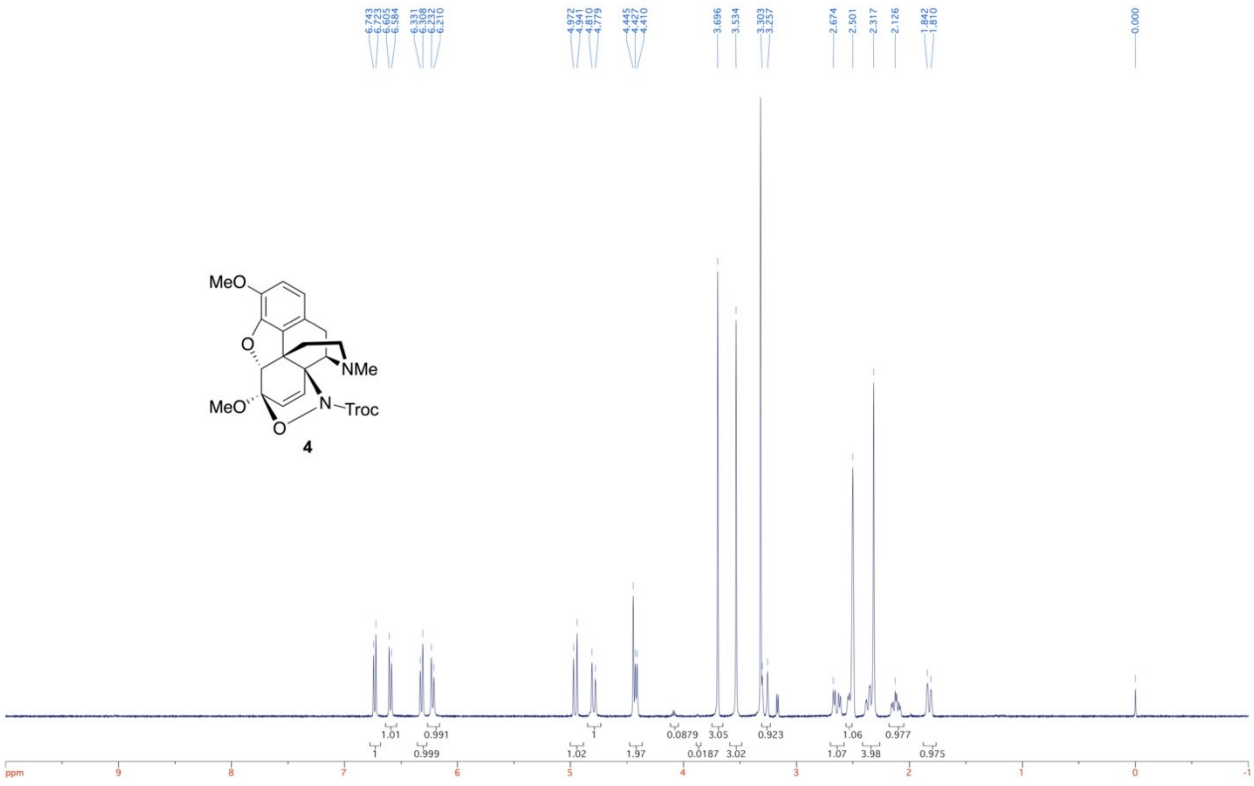
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3.696
3.534
3.303
3.257
2.674
2.501
2.317
2.126
1.842
1.810
0.000
MeO
O
NMe
MeO
N
Troc
O
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0.991
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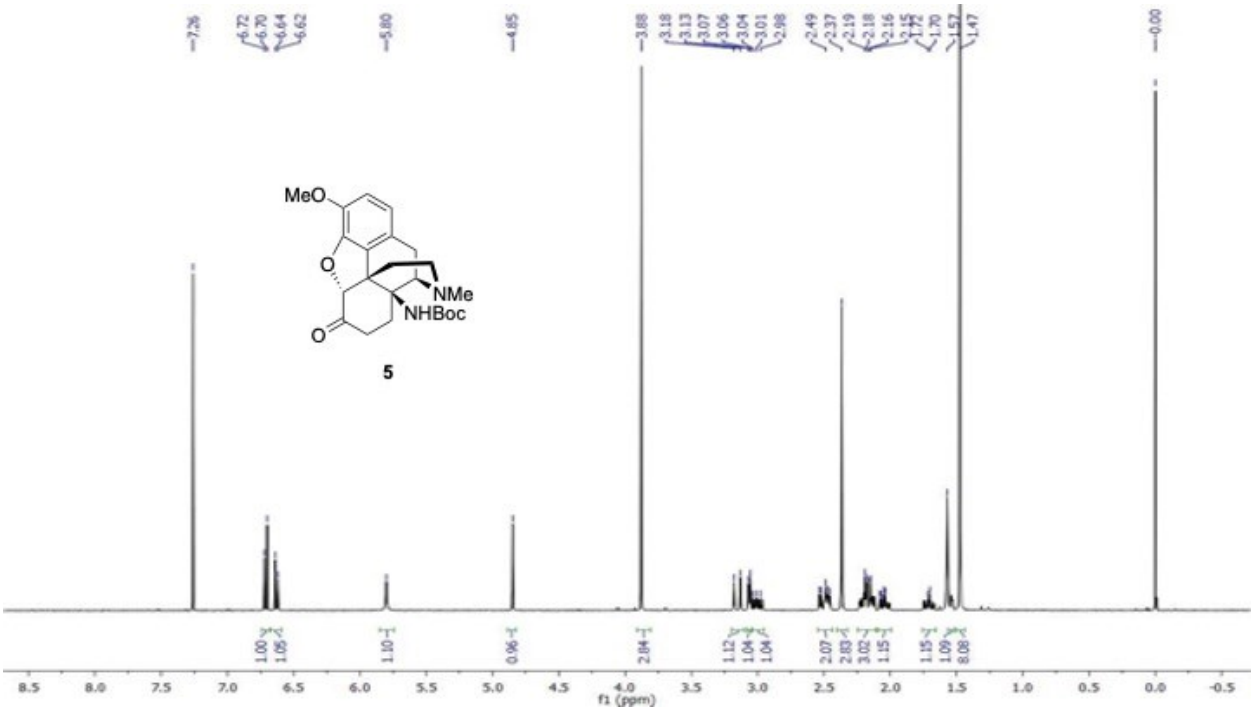
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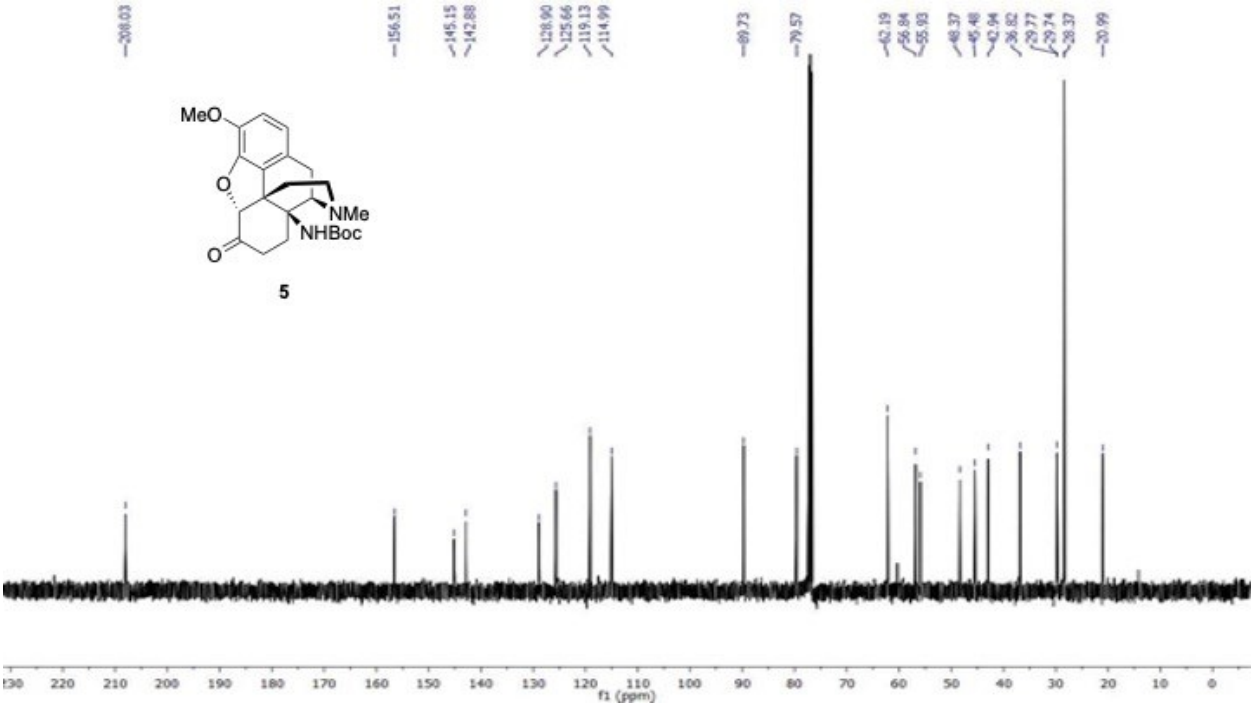
f1 (ppm)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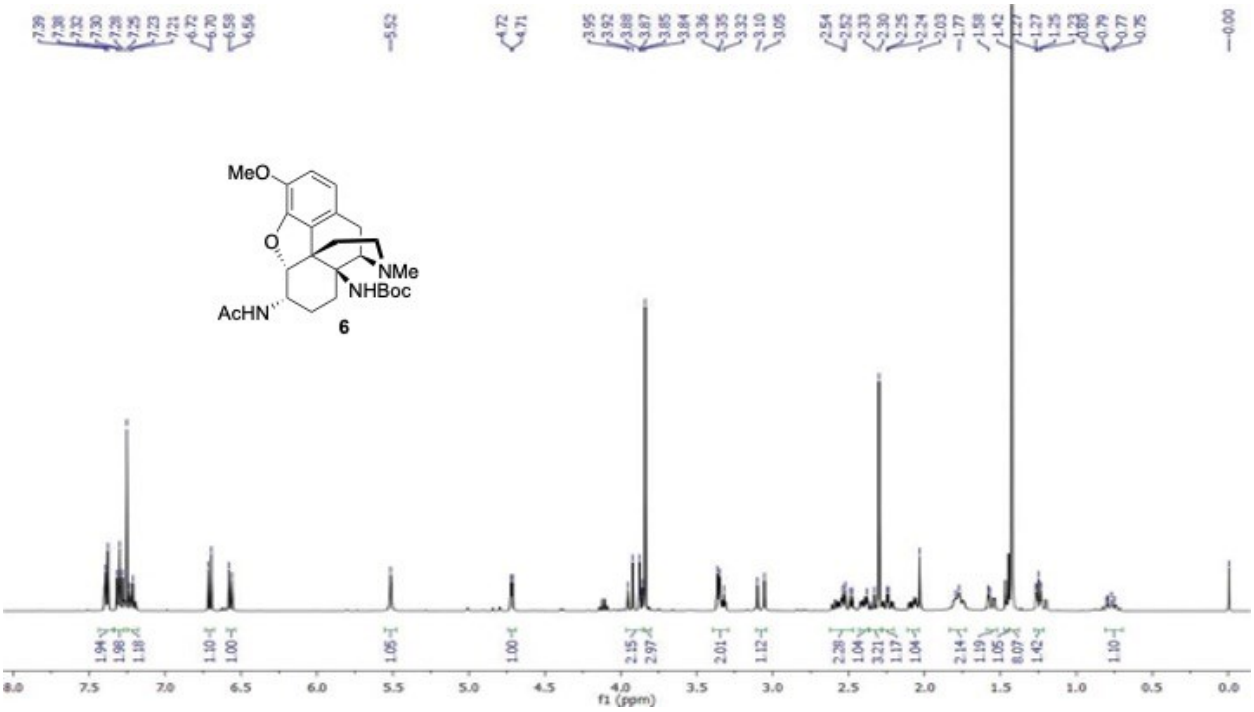
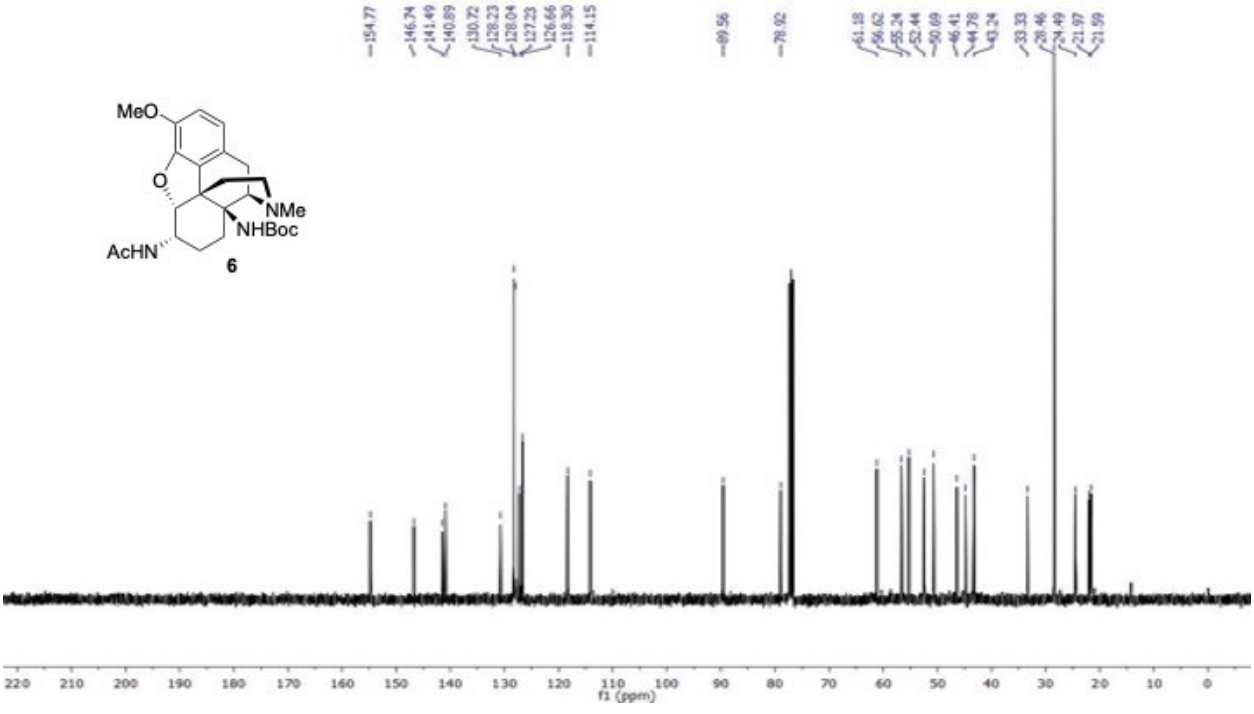
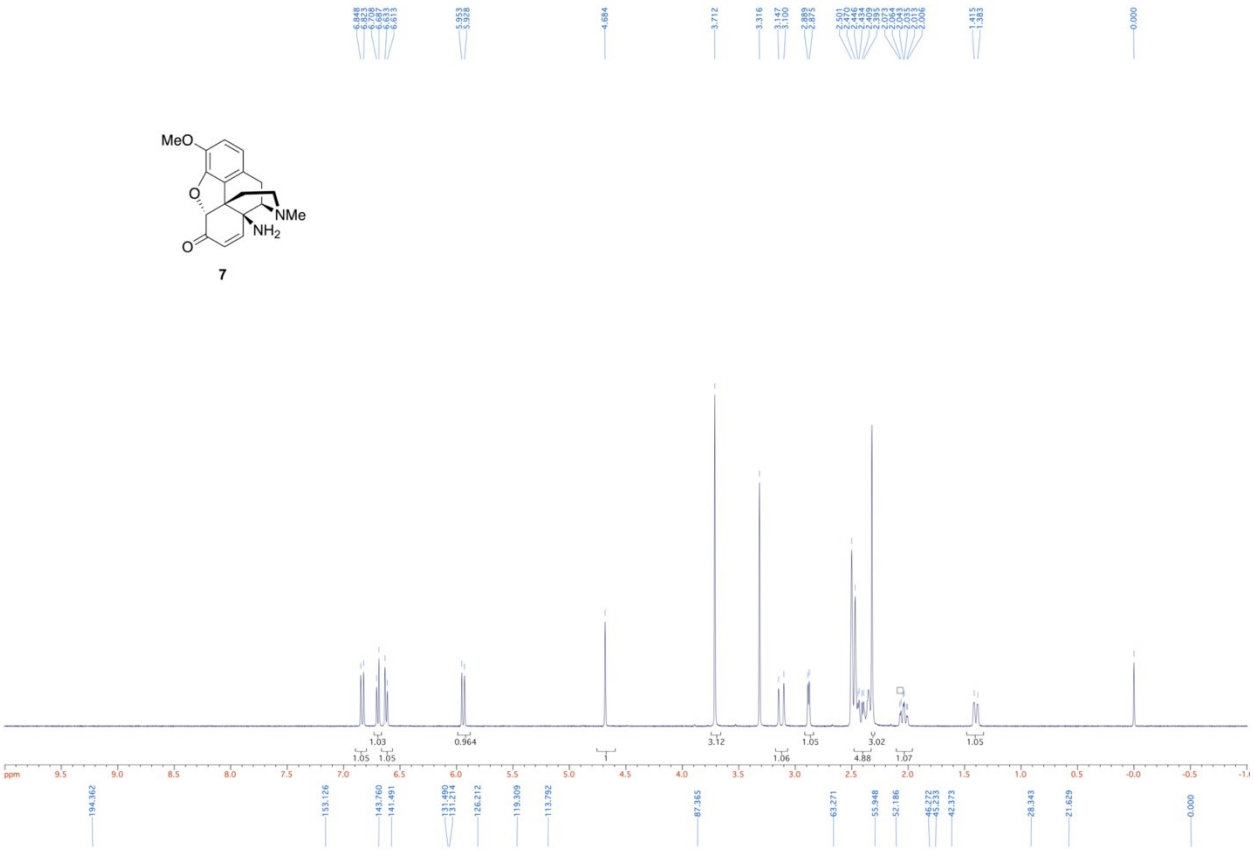
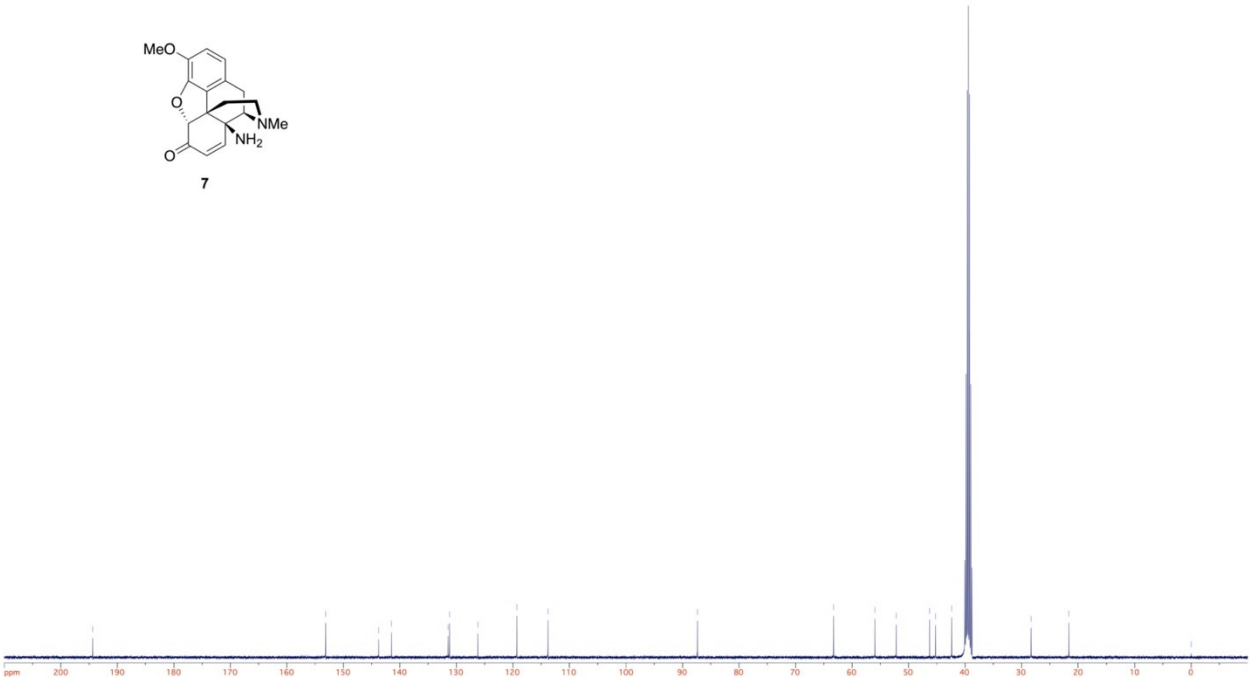
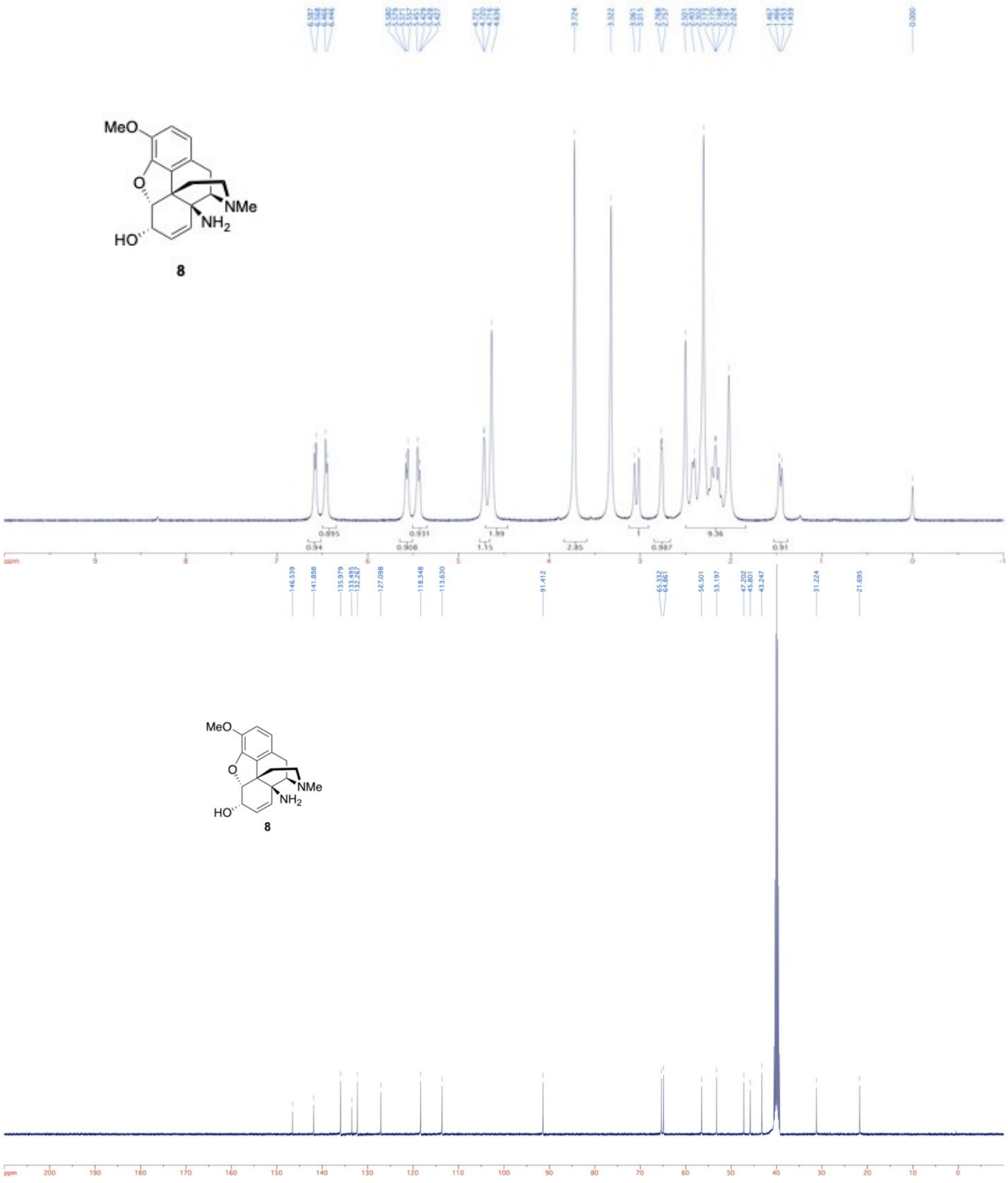
5
f1 (ppm)

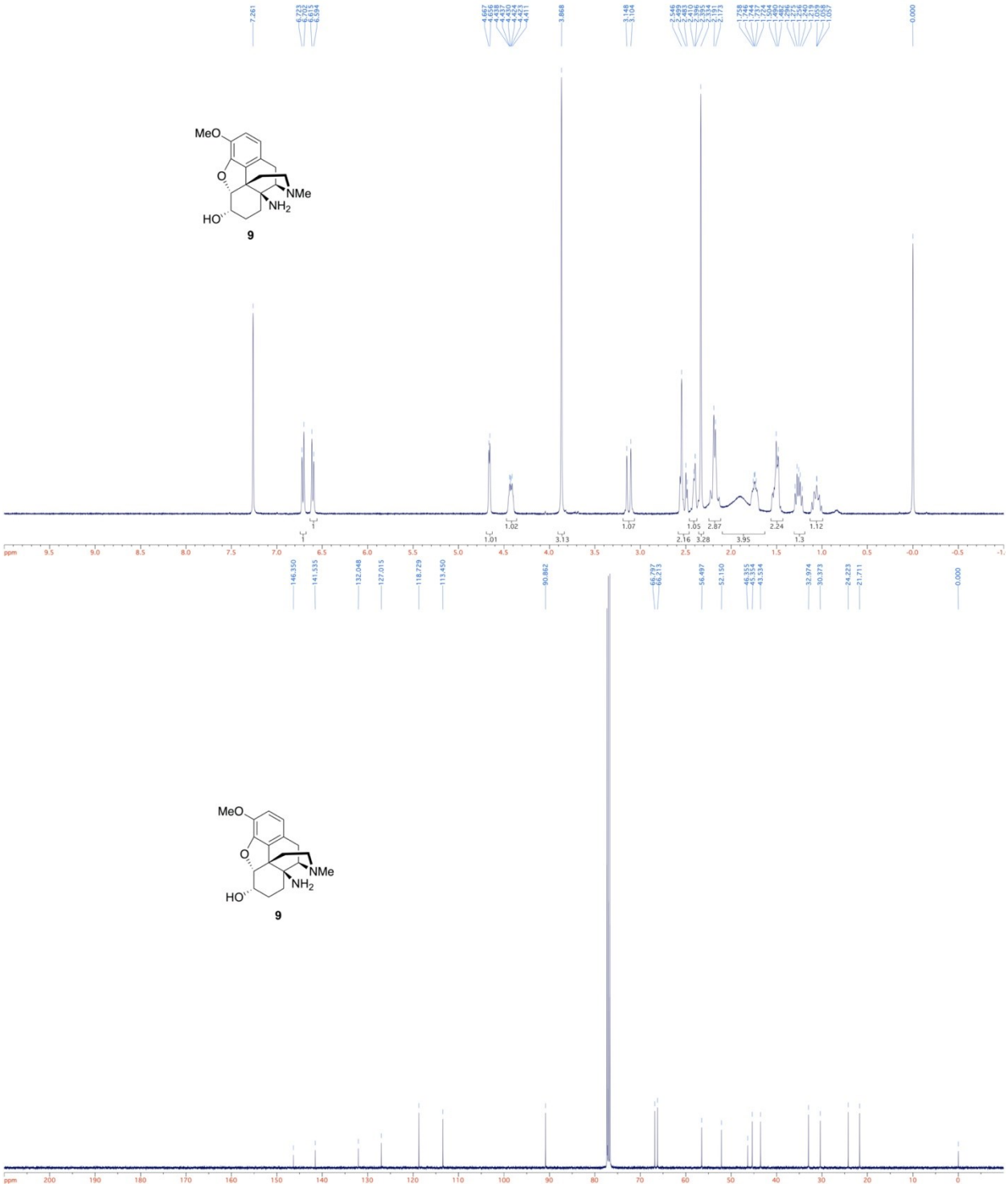

MeO
O
NMe
NHBoc
AcHN
6
f1 (ppm)

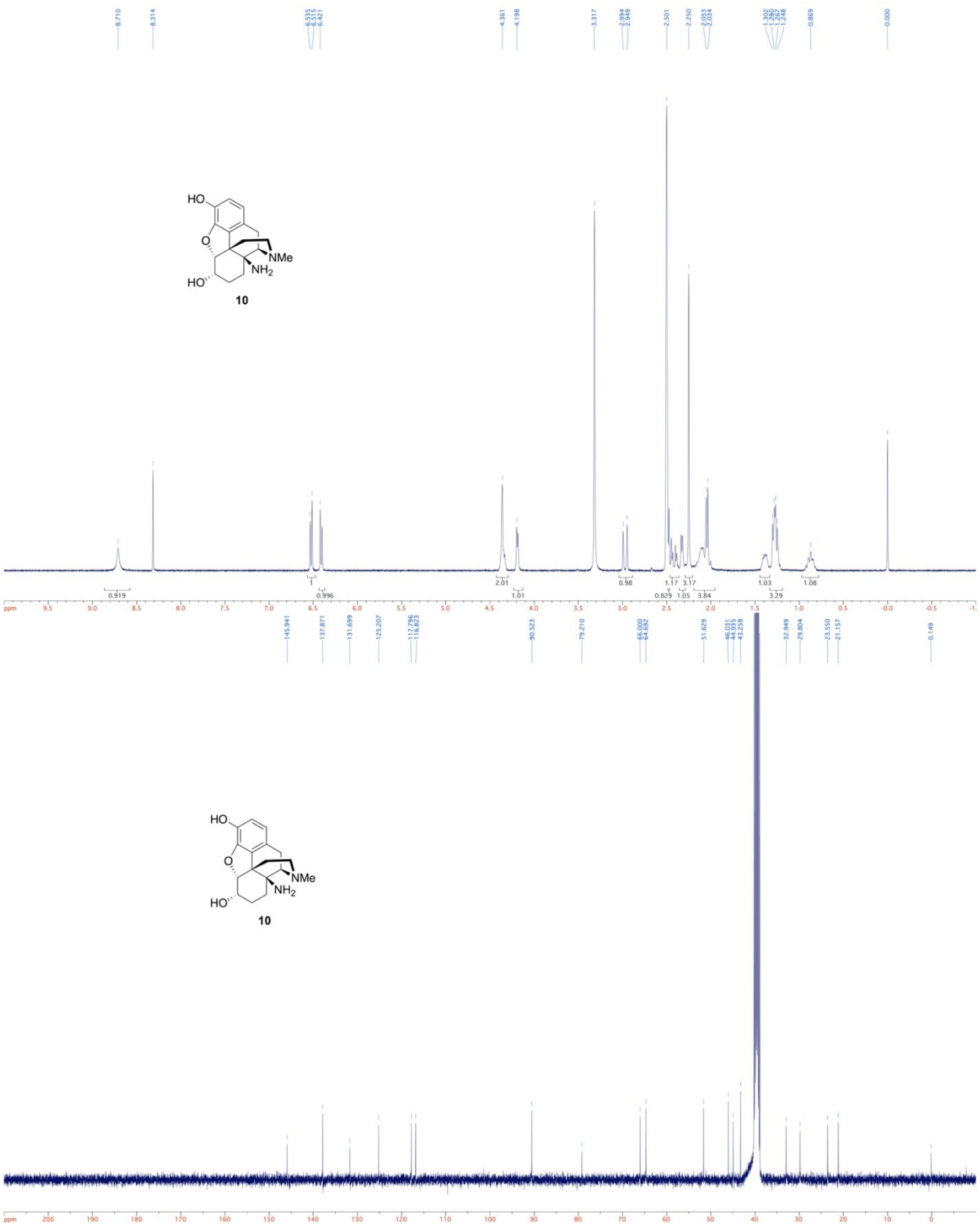

MeO
O
NMe
NHBoc
AcHN
6
f1 (ppm)


MeO
O
NMe
NH2
O
7
1.03
1.05
1.05
0.964
1
3.12
1.06
1.05
4.88
3.02
1.07
1.05
ppm


MeO
O
NMe
NH2
O
7
ppm


MeO
O
NMe
NH2
HO
8
MeO
O
NMe
NH2
HO
8


MeO
O
NMe
NH2
HO
9


HO
O
NMe
NH2
HO
10

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