Supporting Information

Tuned-Affinity Bivalent Ligands for the Characterization of Opioid Receptor Heteromers

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ENTI Synthesis and Biological Assay Procedures

Synthetic procedures for L2, L4, SNC-19, and NTX-19 provided by Nanosyn and Chempartner.

Chemistry. The synthesis of L2 was designed by us and performed by Nanosyn (Santa Clara, CA). L4, NTX-19, and SNC-19 were designed by us and synthesized by ChemPartner (Shanghai, China) using a scheme modified from a known sequence.[1] ENTI[2] was synthesized by us. MA-19[3] was a gift from P. S. Portoghese. Oxymorphone (Mallinckrodt, St. Louis, MO), naltrindole hydrochloride (NTI)(Sigma, St. Louis, MO), SNC80 (Tocris, Ellisville, MO), and naltrexone (Sigma) are commercially available. ^{1}H and ^{13}C NMR spectra were recorded on a Varian 400 spectrometer at 400 and 100 MHz, respectively. Chemical shifts were reported as parts per million (ppm) relative to deuterated solvents. Spectra were analyzed using MestReNova software v. 6.0.3 (Mestrelab Research, Spain). Low-resolution electrospray ionization mass spectra (ESI^{+}-MS) were recorded on a Waters Micromass ZQ 4000 spectrometer. LC/MS (MS: ESI^{+}) was performed on a Waters AllianceHT LC/MS with a flow rate of 0.2 ml min^{-1} (monitored at 210 nm and 260 nm) using an Xterra MS C18 column (Waters). Hi-resolution mass spectra were performed in electrospray mode on a Thermo electron Orbitrap XL by direct infusion. Analytical thin layer chromatography was performed with silica gel 60 F254 glass plates (EMD Chemicals). Flash chromatography was conducted with Geduran Silica Gel 60 (EMD Chemicals). High performance liquid chromatography (HPLC) was performed on a Prostar 210 (Varian) with a flow rate of 10 ml/min (monitored at 210 nm and 260 nm) using a Varian Dynamax Microsorb C18 preparatory column (Varian). For air- and water-sensitive reactions, glassware was oven-dried prior to use and reactions were performed under argon. Solvents were purchased anhydrous or ACS chemical grade (Fisher) and used without further purification. Commercially available starting reagents were used without further purification.

The synthesis of ENTI[2] began with 7'-nitronaltrindole (**11**),[4] which was alkylated with (2-bromoethyl)benzene to give the phenethylether **12**. This compound was then hydrogenated at 40 psi to give the phenethyl ether of 7'-aminonaltrindole (ENTI, **4**).

Phenethyl ether 7'-nitronaltrindole (12). To a solution of 7'-nitronaltrindole (**11**)[4](400 mg, 0.871 mmol) and calcium carbonate (2.75 eq.) in dimethylformamide was added (2-bromoethyl)benzene (1.5 eq.). The reaction was stirred at 90°C for 12 h, diluted with 100 mL EtOAc, washed 2x with H_2O, once with brine, and then dried over Na_2CO_3, filtered, and the solvent removed *in vacuo* to give an orange oil. The residue was purified via silica gel chromatography in 1-2.5% MeOH in CH_2Cl_2 to give the product (327 mg, 67 %). ^{1}H NMR ($CDCl_3$) δ 9.96 (s, N-H), 8.10 (d, *J* = 8.1 Hz, 1H), 7.73 (d, *J* = 7.6 Hz, 1H), 7.27 – 7.15 (m, 5H), 7.10 (t, *J* = 7.9 Hz, 1H), 6.63 (dd, *J* = 20.2, 8.3 Hz, 2H), 5.70 (s, 1H), 4.21 (dt, *J* = 15.1, 7.4 Hz, 1H), 4.11 (dt, *J* = 11.8, 8.9 Hz, 1H), 3.41 (d, *J* = 6.3 Hz, 1H), 3.18 (d, *J* = 18.6 Hz, 1H), 3.02 (t, *J* = 7.5 Hz, 2H), 2.95 (d, *J* = 7.4 Hz, 1H), 2.89 (d, *J* = 4.1 Hz, 1H), 2.85 (d, *J* = 6.5 Hz, 1H), 2.82 – 2.74 (m, 2H), 2.66 (d, *J* = 15.7 Hz, 1H), 2.48 – 2.39 (m, 3H), 2.31 (m, 1H), 2.18 (t, *J* = 2.8

Hz, 1H), 1.85 (d, *J* = 12.7 Hz, 1H), 0.91 (m, 1H), 0.59 (d, *J* = 7.7 Hz, 2H), 0.18 (d, *J* = 4.9 Hz, 2H). ESI-MS [M+H]+ calcd 563.2, found 564.2.

Phenethyl ether 7'-aminonaltrindole (4). To a solution of **12** (327 mg, 0.58 mmol) in anhydrous MeOH (80 mL) was added 5% Pd/C (120 mg). The suspension was hydrogenated at 40 psi for 4 hours, when another 120 mg of Pd/C was added. The hydrogenated was allowed to continue overnight. Filtration through celite and solvent removal in vacuo gave a salmon-colored powder which was loaded on an HPLC and run in a gradient of 35-95 % MeOH in H_2O with 0.1% formic acid over 18 minutes to give the product (186 mg, 85%). ^{1}H NMR (CD_3OD) δ 7.44 (d, *J* = 8.0 Hz, 1H), 7.20 – 7.02 (m, 7H), 6.79 – 6.73 (m, 2H), 5.77 (s, 1H), 4.18 (td, *J* = 9.9, 3.2 Hz, 3H), 3.41 – 3.35 (m, 2H), 3.25 (m, 2H), 3.10 (d, *J* = 7.5 Hz, 1H), 3.04 (s, 1H), 3.01 – 2.93 (m, 2H), 2.86 (dq, *J* = 13.8, 7.1 Hz, 2H), 2.70 (m, 4H), 1.81 (d, *J* = 10.1 Hz, 1H), 1.16 – 1.08 (m, 1H), 0.89 – 0.70 (m, 2H), 0.51 (m, 2H). ^{13}C NMR (CD_3OD) δ 145.1, 143.0, 138.4, 131.1, 129.4, 129.3, 128.8, 128.2, 126.2, 123.4, 119.8, 119.2, 118.0, 109.8, 83.7, 72.4, 62.3, 57.7, 48.3, 48.1, 47.9, 47.7, 47.5, 47.3, 46.8, 35.8, 29.0, 28.5, 23.9, 5.6, 5.1, 2.2. HRMS [M+H]+ calcd 534.2751, found 534.2758.

Molecular Biology. [D-Pen2,D-Pen5]-enkephalin [Tyrosyl-2, 6-^{3}H(N)]-, (^{3}H DPDPE) and [D-Ala2,NMe-Phe4,Gly-ol5]-enkephalin [Tyrosyl-3,5-^{3}H(N)]-, (^{3}H DAMGO) were purchased from Perkin-Elmer. CTAP was purchased from Tocris. All compounds were dissolved in water, with the exception of L2, which was dissolved in 5% dimethyl sulfoxide (DMSO) and 0.01 N HCl, and NTX-19 and SNC-19, which were dissolved in 10% DMSO. Anti-FLAG M2 affinity matrix, albumin from bovine serum (BSA), Triton X-100, and Tween 20 were purchased from Sigma-Aldrich. All other reagents were purchased from Sigma unless otherwise specified. Anti-HA.11 beads were from Covance Research Products (Princeton, NJ).

Cell Culture. HEK293 cells (American Type Culture Collection, Manassas, VA) were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT). N-terminal signal sequence and either HA-DOR or FLAG-MOR-tagged c-DNA murine opioid receptor constructs were stably expressed in HEK293 cells. For generation of clonal stable cell lines, single colonies were chosen and propagated in the presence of selection-containing medium. Cell lines were carefully matched for expression.[5]

Serial Co-IP. HEK293 cells stably expressing both N-terminal FLAG-MOR and HA-DOR were grown to 90% confluence in 10-cm plates coated with poly-D-lysine. Cells were washed twice with PBS and biotinylated with 0.3 mg/ml LC-biotin (Thermo Fisher Scientific, Waltham, MA) at 4°C for 30 min to selectively label a pool of receptors at the cell surface as described.[6] Cells were washed with TBS followed by PBS and then lysed in 0.1% Triton X-100, 150 mM NaCl, 25 mM KCl, and 10 mM Tris-HCl pH 7.4 with protease inhibitors (Roche

Diagnostics, Basel, Switzerland). Lysates were cleared by centrifugation at 10,600 *g* (Eppendorf 5417R; Eppendorf North America, New York, NY) for 10 min at 4°C. The protein concentration was evaluated using a Bradford assay, and 1 mg was carried on. All vials were kept on ice when not incubated. The first immunoprecipitation, overnight at 4°C, used anti-FLAG M2. A control sample of 1 mg was also incubated overnight with IgG–agarose (Sigma). The next day, the samples were pelleted (30 seconds at 10,600 *g*), and the supernatant, which contained DOR homomers only, was transfered to a clean tube ("Sample 1"). The pellet, which contained the FLAG M2 affinity matrix and therefore both MOR homomers and DOR/MOR heteromers, was washed 3x (using "IP buffer": 0.1% Triton X-100, 150 mM NaCl, 25 mM KCl, and 10 mM Tris-HCl pH 7.4) with a 30 second, 10,600 *g* centrifugation between each wash (the IgG supernatant was discarded and the pellet set aside after washing). Then, the pellet was incubated with FLAG peptide for 30 minutes to release all receptors from the affinity matrix. This lysate ("Sample 2") was incubated with HA.11 affinity matrix for 2 hours at 4°C to selectively immunoprecipitate HA-DOR/FLAG-MOR heteromers. At the same time, Sample 1 was also incubated with HA.11 affinity matrix for 2 hours at 4°C, to immunoprecipitate the HA-DOR monomers. After incubation, both samples were pelleted and washed 3x as before. The supernatant from Sample 2 was saved, while the supernatant from Sample 1 was discarded. At this point, the the HA.11 affinity matrix used for Sample 2 contained the DOR/MOR heteromers, whereas the lysate contained MOR homomers. The pellets from Samples 1 and 2 were set aside. Finally, the lysate from Sample 2 was incubated 2 hours at 4°C with anti-FLAG M2 affinity matrix to specifically isolate FLAG-MOR homomers ("Sample 3"). Sample 3 was then pelleted and washed 3x as before (the supernatant was discarded). Finally, all matrix/bead samples were washed 2x with 10 mM Tris, pH 7.5, and precipitates were deglycosylated with peptide *N*-glycosidase F (New England Biolabs, Ipswich, MA) in 10 mM Tris, pH 7.5, for 1 h at 37°C, denatured with SDS sample buffer (no reducing agent), and resolved by SDS-PAGE. Blots were blocked in 5% milk, washed thoroughly (with a 10% Tween in TBS solution), incubated with Vectastain ABC reagent (Vector Laboratories) for 30 min and thoroughly washed again. Blots were developed with enhanced chemiluminescence reagents (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK), scanned, and quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

Radioligand Binding. Competition binding assays were performed in whole cells in 50 mM potassium phosphate buffer with 0.3% BSA with constant concentration of radioligand: 10 nM [^{3}H]-DAMGO (1 mCi/ml; PerkinElmer) or 20 nM [^{3}H]-DPDPE, in a total of 100 mL per reaction, for 2 (DPDPE) or 3 (DAMGO) hours at 25°C in a 96-well plate. The reactions were terminated by rapid filtration over 96-well GF/B glass fiber filters (presoaked 30 minutes in 0.3% BSA phosphate buffer), using a MultiScreenHTS Vacuum Manifold (Millipore). Filters were washed 3 times with 100 µL ice-cold 50 mM potassium phosphate buffer with 0.3% BSA, dried overnight, and then counted using a Packard top counter.

Binding parameters were determined using Prism (GraphPad Software Inc., San Diego, CA). Data are presented as mean ± SEM of triplicate experiments.

K_i's were determined using experimentally evaluated K_d's and the Cheng-Prusoff equation. K_d's were calculated using whole cells in 50 mM potassium phosphate buffer with 0.3% BSA, with a constant concentration of cold ligand (CTAP, DAMGO, or NTI at 10 μM) and varying radioligand concentration over 8 different concentrations (20 – 0.04 nM for DAMGO; 50 – 0.01 nM for DPDPE). Reactions were carried out in a total volume of 100 mL per reaction for 2.5 hours at 25°C in a 96-well plate. The reactions were terminated and analyzed using the competition binding procedure and equipment.

1. Wei, Z. Y.; Brown, W.; Takasaki, B.; Plobeck, N.; Delorme, D.; Zhou, F.; Yang, H.; Jones, P.; Gawell, L.; Gagnon, H.; Schmidt, R.; Yue, S. Y.; Walpole, C.; Payza, K.; St-Onge, S.; Labarre, M.; Godbout, C.; Jakob, A.; Butterworth, J.; Kamassah, A.; Morin, P. E.; Projean, D.; Ducharme, J.; Roberts, E. N,N-diethyl-4-(phenylpiperidin-4-ylidenemethyl)benzamide: A novel, exceptionally selective, potent delta opioid receptor agonist with oral bioavailability and its analogues. *J. Med. Chem.* **2000,** *43*, 3895-3905.
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4. Portoghese, P. S.; Sultana, M.; Nelson, W. L.; Klein, P.; Takemori, A. E. Delta Opioid Antagonist Activity and Binding-Studies of Regioisomeric Isothiocyanate Derivatives of Naltrindole - Evidence for Delta Receptor Subtypes. *J. Med. Chem.* **1992,** *35*, 4086-4091.
5. Waldhoer, M.; Fong, J.; Jones, R. M.; Lunzer, M. M.; Sharma, S. K.; Kostenis, E.; Portoghese, P. S.; Whistler, J. L. A heterodimer-selective agonist shows in vivo relevance of G protein-coupled receptor dimers. *Proc. Natl. Acad. Sci. U.S.A.* **2005,** *102*, 9050-9055.
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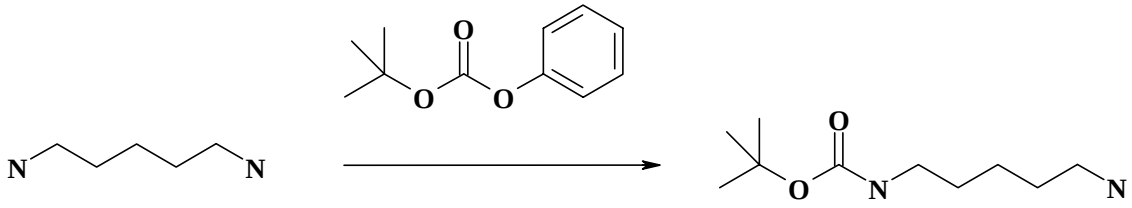
Synthesis of L2 (Nanosyn)

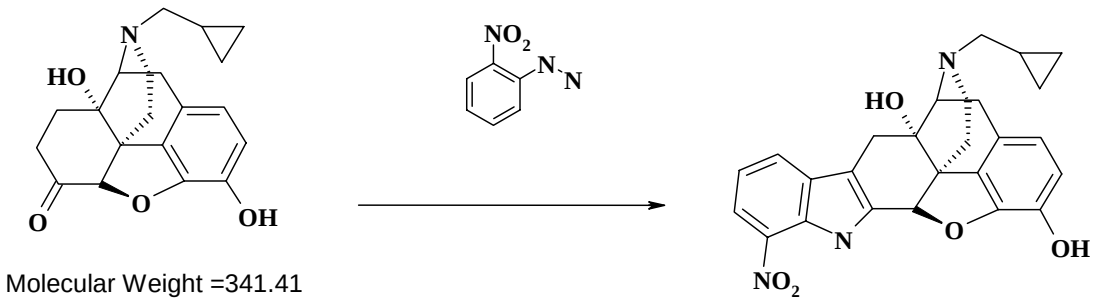
EXPERIMENTALS

Compound A



A solution of 1,5-diaminopentane (12 g, 117 mmol, 1.0 eq) and tert-butyl phenyl carbonate (21.7 mL, 117 mmol, 1.0 eq) in EtOH (70 mL) were heated at 65 °C for 16 h. The reaction mixture was concentrated under reduced pressure. Water (100 mL) was added followed by 2N HCl aq. until pH = 2. Aqueous layer was washed with Et_2O (2x 50 mL), and then basified by 2N NaOH until pH = 11-12. The aqueous layer was extracted with DCM (2 x 50 mL), organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to give the product (12.5 g, 52.8 %) as an yellow oil.

Compound B

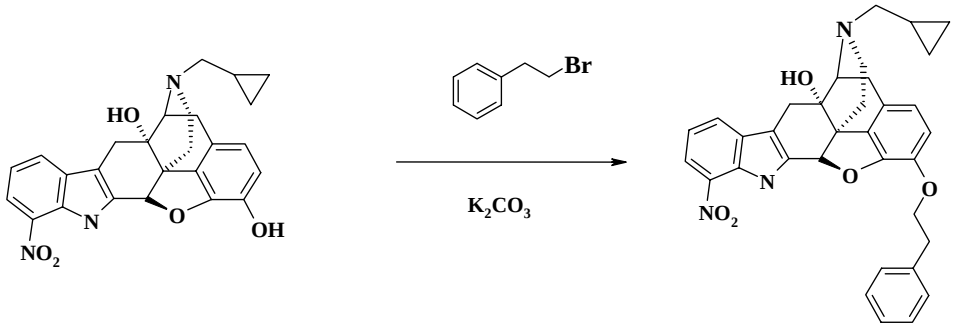


Molecular Weight =341.41

A solution of naltrexone HCl salt (7.54 g, 19.95 mmol, 1.0 eq) and 2-nitrobenzhydrazine (3.05 g, 19.95 mmol, 1 eq) in a mixture of AcOH (100 mL) and HCl conc. (100 mL) was heated in an oil bath at 90 °C with stirring under a nitrogen atmosphere. After 2 h, the mixture was concentrated *in vacuo*.

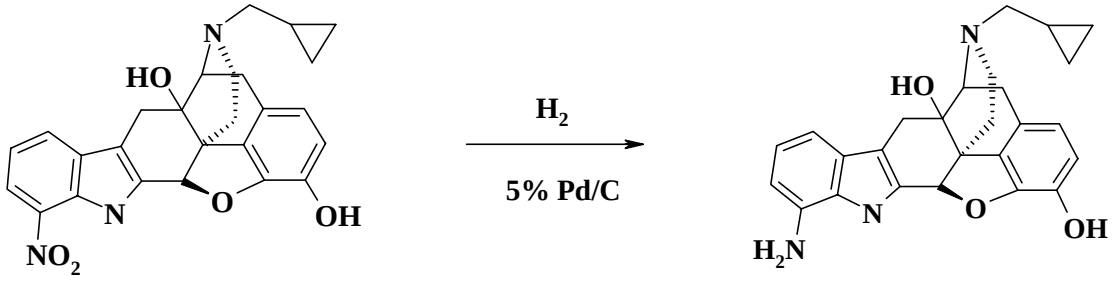
Crude solid product was dissolved in DMSO (20 mL) and purified by preparative scale reverse-phase HPLC [Varian L/L 4002-1 column (50 x 200 cm.; Nanosyn–Packed Microsorb 100-10 C-18), flow rate = 50 ml/min; mobile phase A: 100% water, 0.1% TFA; mobile phase B: 100% MeCN, 0.1% TFA; gradient elution from 10 % to 60% B in 90 min; detection 254 nm]. Fractions containing the desired product were combined and concentrated in vacuum to give 5.7 g (49.8%) of TFA salt as an off-white solid.

Compound C



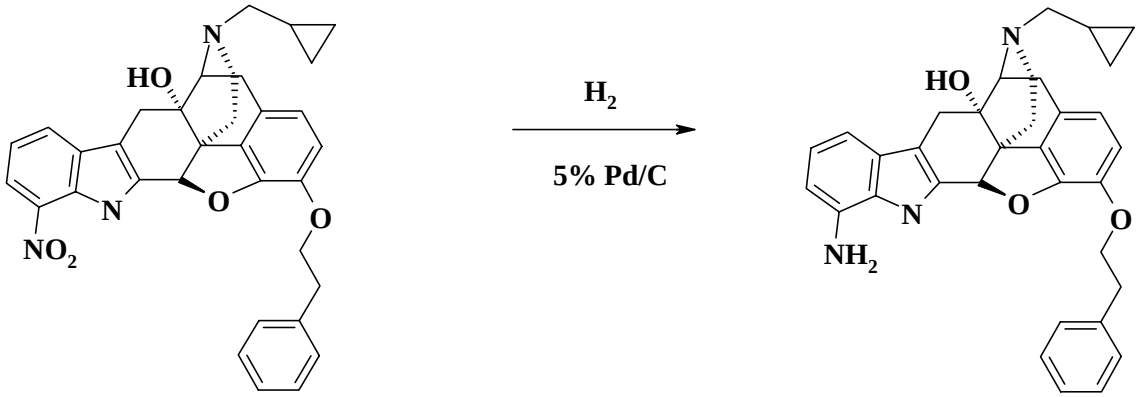
To a solution of compound B (2.5 g, 4.36 mmol, 1.0 eq) and K_2CO_3 (1.65 g, 12 mmol, 2.75 eq) in DMF (15 mL) was added 2-bromoethylbenzene (890mL, 6.39 mmol, 1.46 eq). The reaction mixture was heated at 90 °C with stirring under a nitrogen atmosphere. After 24 h, the mixture was diluted with EtOAc (200 mL) and washed with H_2O (2 x 100 mL), brine (100 mL), then dried over Na_2SO_4 and concentrated under reduced pressure. The residue was Si-gel purified: 120 g silica gel column; 50 mL/min of 0-100% hexanes/ethyl acetate over 55 min. Fractions containing the desired compound were combined and concentrated *in vacuo* to give the purified product (2.25 g, 91.5 %) as a yellow crystalline solid.

Compound D



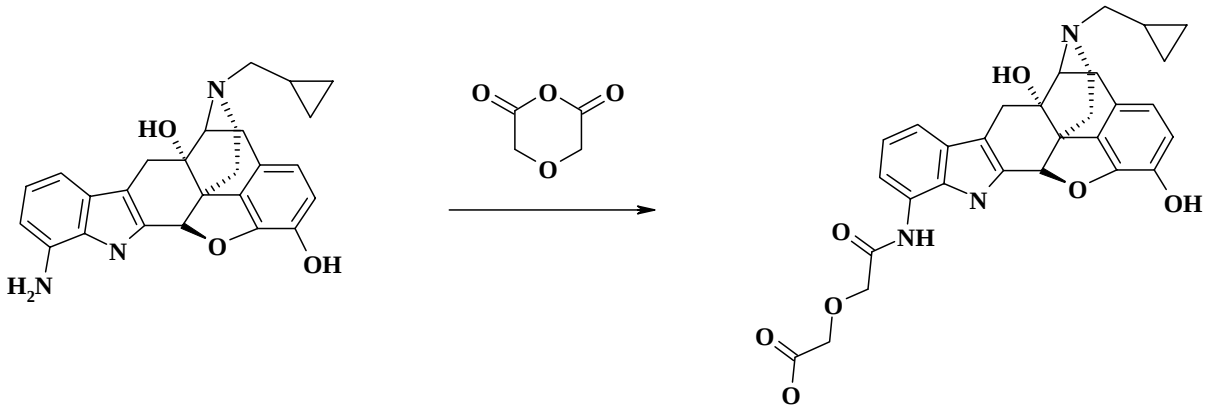
Compound B (1 g, 8.72 mmol) was dissolved in anhydrous MeOH (90 mL) and 5% Pd/C (400 mg) was added. The reaction mixture was hydrogenated on Parr apparatus for 2 h at 40 psi. Pd/C was removed by filtration; MeOH was concentrated *in vacuo* to give the product (0.905 g, 96.7 %) as a white crystalline solid.

Compound E



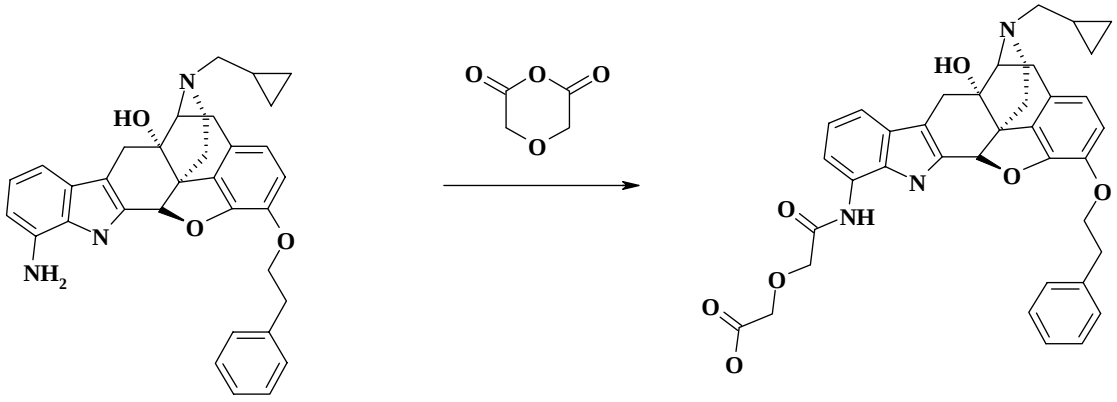
Compound D (1.725 g, 3.06 mmol) was dissolved in anhydrous MeOH (60 mL) and 5% Pd/C (600 mg) was added. The reaction mixture was hydrogenated on Parr apparatus for 1 h at 40 psi. Pd/C was removed by filtration; MeOH was concentrated *in vacuo* to give the product (1. 5 g, 91.8 %) as an off-white crystalline solid.

Compound F



To a solution of compound D (0.9 g, 2.09 mmol, 1.0 eq) in THF (5 mL) was added glycolic anhydride (0.262 g, 2.25 mmol, 1.08 eq). The reaction mixture was stirred under a nitrogen atmosphere. After 1 h, the formed solid was collected by filtration, washed on the filter with THF (10 mL), then with Et_2O (10 mL) to give crude product, which was HPLC purified: [Varian L/L 4002-1 column (50 x 200 cm.; Nanosyn–Packed Microsorb 100-10 C-18), flow rate = 50 ml/min; mobile phase A: 100% water, 0.1% TFA; mobile phase B: 100% MeCN, 0.1% TFA; gradient elution from 10 % to 60% B in 90 min; detection 254 nm]. Fractions containing the desired product were combined and concentrated in vacuum. 1 N HCl (30 mL) was added to the residue and final solution was lyophilized to give 0.47 g (38.6%) of HCl salt as an off-white solid.

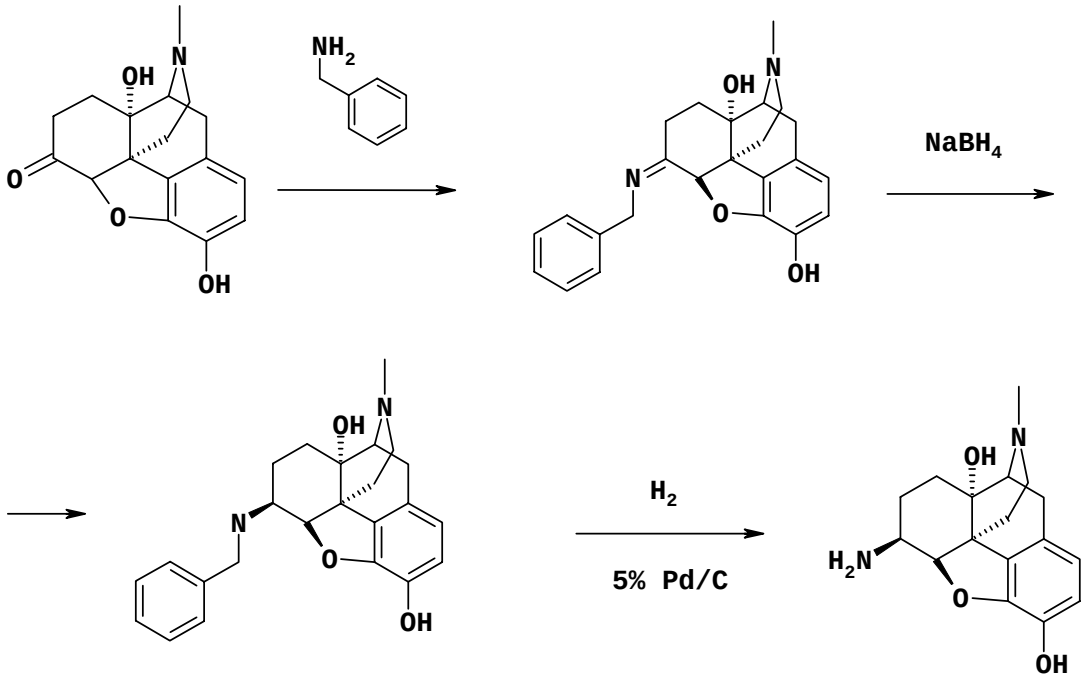
Compound G



To a solution of compound E (1.65 g, 3.05 mmol, 1.0 eq) in THF (35 mL) was added glycolic anhydride (0.445 g, 3.84 mmol, 1.26 eq). The reaction mixture was stirred under a nitrogen atmosphere. After 1 h, the formed solid was collected by filtration, washed on the filter with THF (10 mL), then with Et_2O (10 mL) to give product as an off-white solid, which was used on next step without purification.

Yield: 1.89 g (95.4 %).

Compound H



Oxymorphone HCl salt (11.22 g, 33.2 mmol) was dissolved in H_2O (50 mL) and the solution was basified by conc. Na_2CO_3 aq. solution to pH = 9. After 1 h, the formed solid was filtered, washed with H_2O (10 mL) and dried in high vacuum for overnight to give 9.5 g of free base as a white solid.

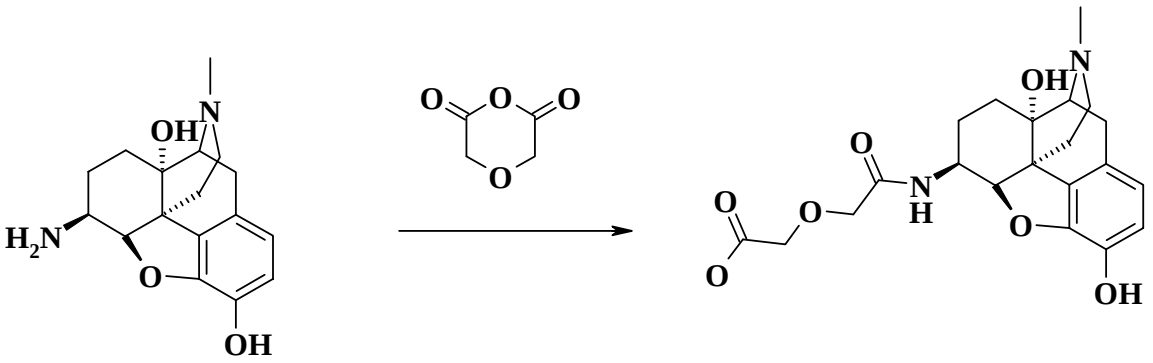
A benzene solution (600 mL) containing oxymorphone free base (9.5 g, 32.52 mmol), benzylamine (3.98 g, 37.2 mmol, 1.15 eq.), and trace (30 mg) of *p*-toluenesulfonic acid was refluxed for 10 h, using Dean-Stark trap for azeotropic removal of water.

The mixture was then concentrated (100 mL) and abs. EtOH (320 mL) was added.

To the stirring mixture $NaBH_4$ (1.2 g, 33.2 mmol) was added in 6 equal portion during 1 h period. After 3 h of stirring, the reaction mixture was concentrated *in vacuo.* The residue was partitioned between EtOAc (200 mL) and H_2O (100 mL), organics were washed with 5 % Na_2CO_3 aq. solution (50 mL), brine (50 mL) and dried over Na_2SO_4. EtOAc concentrated *in vacuo* and residue was dissolved in MeOH (600 mL). The methanolic solution was hydrogenated on Parr apparatus at 40 psi with 3 g of 5% Pd/C for 36 h, then additional 3 g of 5% Pd/C was added for 36 hours more.

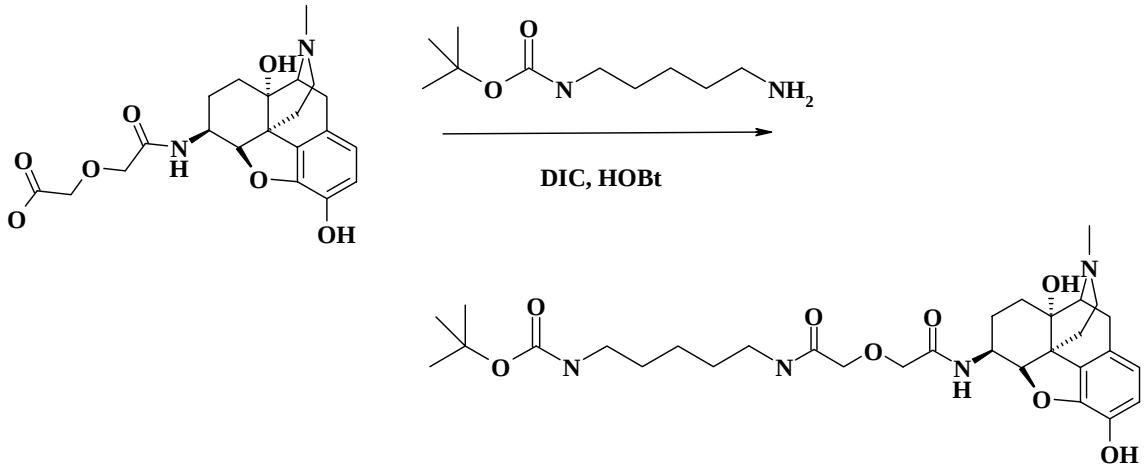
Pd/c was removed by filtration; MeOH was concentrated *in vacuo* to give the product (7.2 g, 71.7 %) as an off-white crystalline solid.

Compound I



To a solution of compound H (7.2 g, 23.8 mmol, 1.0 eq) in THF (50 mL) was added glycolic anhydride (2.76 g, 23.8 mmol, 1.0 eq). The reaction mixture was stirred under a nitrogen atmosphere. After 1 h, the formed solid was collected by filtration, washed on the filter with THF (100 mL), then with Et_2O (50 mL) to give crude product, which was HPLC purified: [Varian L/L 4003-1 column (75 x 300 cm.; Nanosyn–Packed Microsorb 100-10 C-18), flow rate = 100 ml/min; mobile phase A: 100% water, 0.1% TFA; mobile phase B: 100% MeCN, 0.1% TFA; gradient elution from 0 % to 50% B in 75 min; detection 254 nm]. Fractions containing the desired product were combined and concentrated in vacuum. 1 N HCl (100 mL) was added to the residue and final solution was lyophilized to give 3.59 g (33.1%) of HCl salt as an off-white solid.

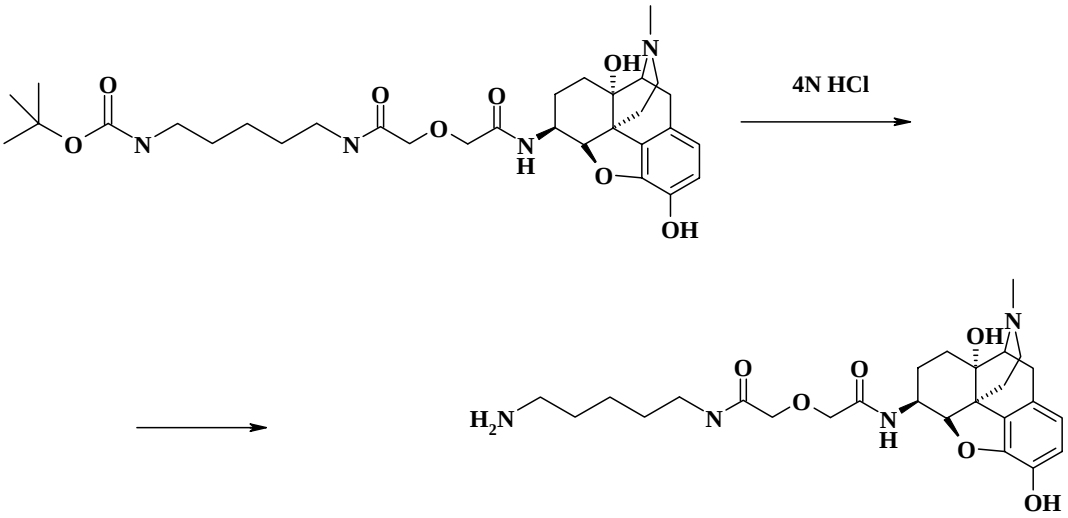
Compound J



To a solution of compound I HCl salt (3 g, 6.59 mmol, 1.0 eq), compound A (1.33 g, 6.59 mmol, 1.0 eq), HOBt (0.89 g, 6.59 mmol, 1.0 eq) and DIEA (2.55 g, 20 mmol, 3 eq) in THF (10mL) under a nitrogen atmosphere, DIC (1.03mL, 6.59 mmol, 1.0 eq) was added with stirring. The reaction mixture was stirred under a nitrogen atmosphere for 2 h at 60 oC. The residue was partitioned between EtOAc (200 mL) and H_2O (100 mL), organics was washed with 5 % Na_2CO_3 aq. solution (50 mL), brine (50 mL) and dried over Na_2SO_4. EtOAc concentrated *in vacuo* and the residue was HPLC purified: [Varian L/L 4002-1 column (50 x 200 cm.; Nanosyn–Packed Microsorb 100-10 C-18), flow rate = 50 ml/min; mobile phase A: 100% water, 0.1% TFA; mobile phase B: 100% MeCN, 0.1% TFA; gradient elution from 10 % to 80% B in 90 min; detection 254 nm]. Fractions containing the desired product were combined and concentrated in vacuum.

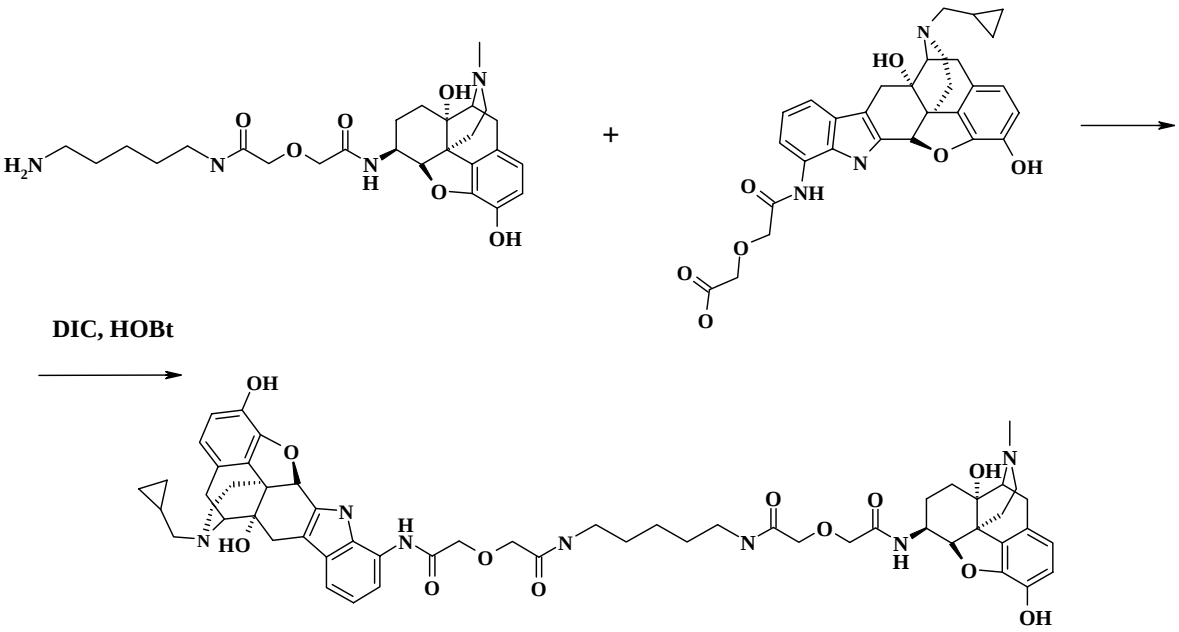
The aq. solution was basified by 5 % Na_2CO_3 aq. solution to pH=9 and extracted by EtOAc (100 mL). Organics was washed with brine (50 mL) and dried over Na_2SO_4 to give 1.19 g (29.9 %) as a white solid.

Compound K



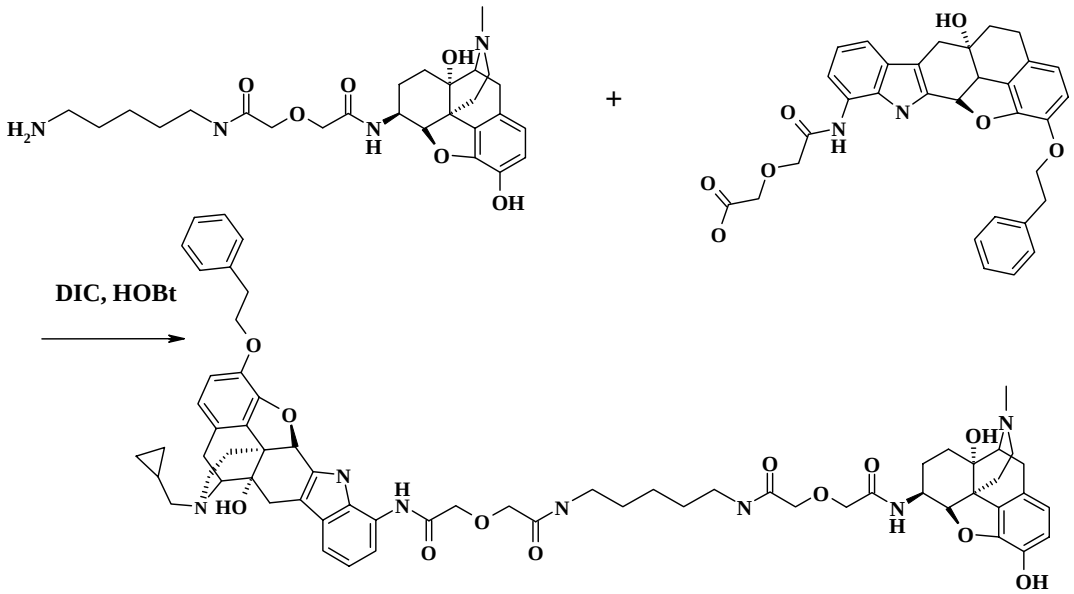
Compound K (1.19 g, 1.97 mmol) was dissolved in dioxane (5 mL) and the 4N HCl solution in dioxane (15 mL) was added. After 2 h of stirring dioxane was removed to dryness go give quantitative yield (1.133 g) of target 2HCl salt product as a white solid

Compound L



To a solution of compound K 2HCl salt (450 mg, 0.78 mmol, 1.0 eq), compound F (0.37 g, 0.68 mmol, 0.87 eq), HOBt (85 mg, 0.7 mmol, 0.89 eq) and DIEA (872 mL, 5 mmol, 6.4 eq) in THF (10mL) under a nitrogen atmosphere, DIC (122 ml, 0.78 mmol, 1.0 eq) was added with stirring. The reaction mixture was stirred under a nitrogen atmosphere for 2 h at 60 oC. The residue was partitioned between EtOAc (100 mL) and H_2O (50 mL), organics was washed with 5 % Na_2CO_3 aq. solution (30 mL), brine (30 mL) and dried over Na_2SO_4. EtOAc concentrated *in vacuo* and the residue was HPLC purified: [Varian L/L 4002-1 column (50 x 200 cm.; Nanosyn–Packed Microsorb 100-10 C-18), flow rate = 50 ml/min; mobile phase A: 100% water, 0.1% TFA; mobile phase B: 100% MeCN, 0.1% TFA; gradient elution from 10 % to 80% B in 90 min; detection 254 nm]. Fractions containing the desired product were combined and concentrated in vacuum.
0.5 N HCl (50 mL) was added to the residue and final solution was lyophilized to give 309 mg (41.2%) of 2HCl salt as an off-white solid.

Compound M



To a solution of compound K 2HCl salt (683 mg, 1.18 mmol, 1.0 eq), compound F (649 g, 1.0 mmol, 0.97 eq), HOBt (135 mg, 1.0 mmol, 0.9 eq) and DIEA (1.14 mL, 6.5 mmol, 6.5 eq) in THF (12mL) under a nitrogen atmosphere, DIC (175 ml, 1 mmol, 0.95 eq) was added with stirring. The reaction mixture was stirred under a nitrogen atmosphere for 3 h at 60 ^{o}C. The residue was partitioned between EtOAc (100 mL) and H_2O (50 mL), organics was washed with 5 % Na_2CO_3 aq. solution (30 mL), brine (30 mL) and dried over Na_2SO_4. EtOAc concentrated *in vacuo* and the residue was HPLC purified: [Varian L/L 4002-1 column (50 x 200 cm.; Nanosyn–Packed Microsorb 100-10 C-18), flow rate = 50 ml/min; mobile phase A: 100% water, 0.1% TFA; mobile phase B: 100% MeCN, 0.1% TFA; gradient elution from 10 % to 80% B in 90 min; detection 254 nm]. Fractions containing the desired product were combined and concentrated in vacuum.

0.5 N HCl (50 mL) was added to the residue and final solution was lyophilized to give 321 mg (26.6 %) of 2HCl salt as an off-white solid.

Synthesis of L4 (Chempartner)

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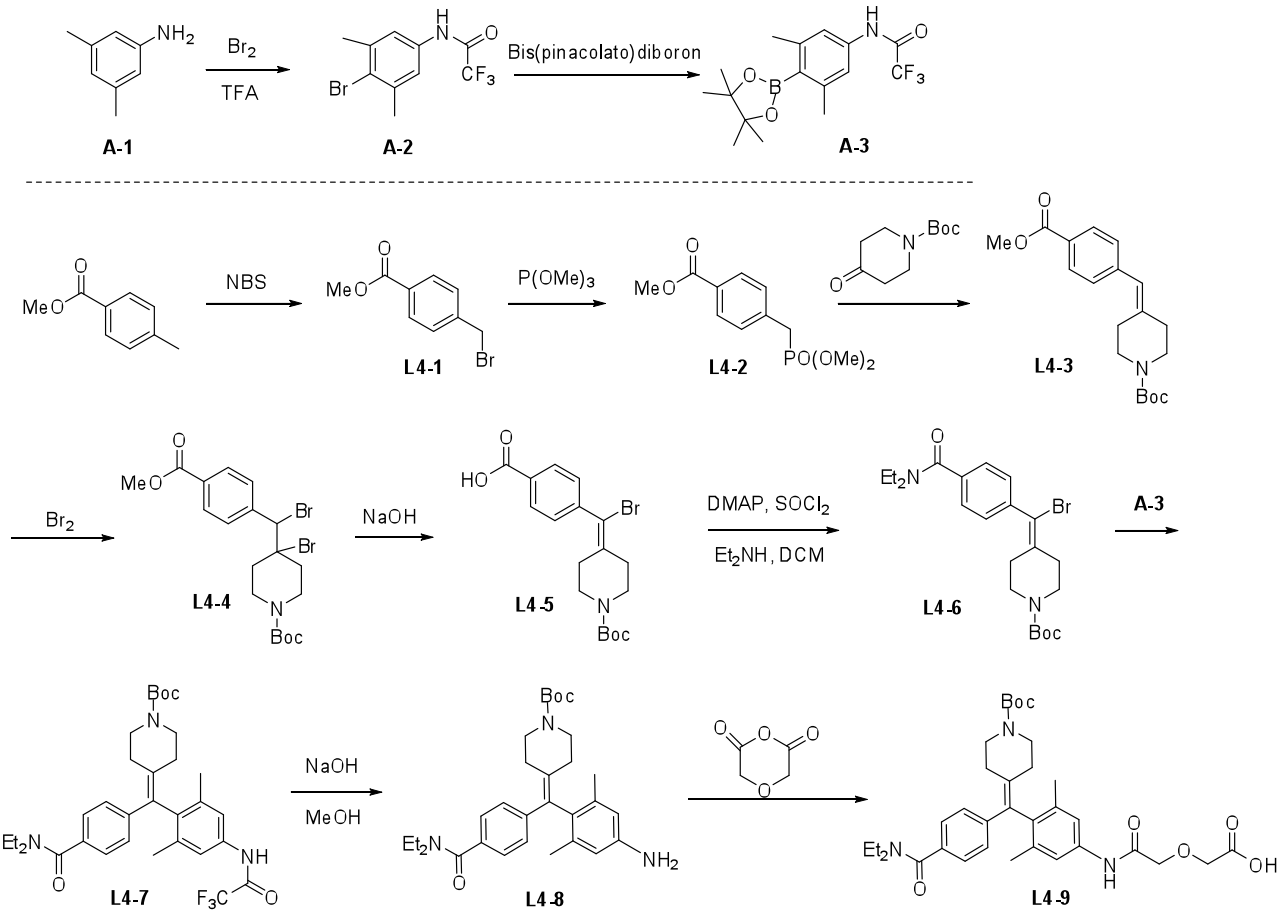
Project Summary

According to the structure of **L4**, the target compound can be prepared through following steps.

The synthesis of A-3 and L4-9



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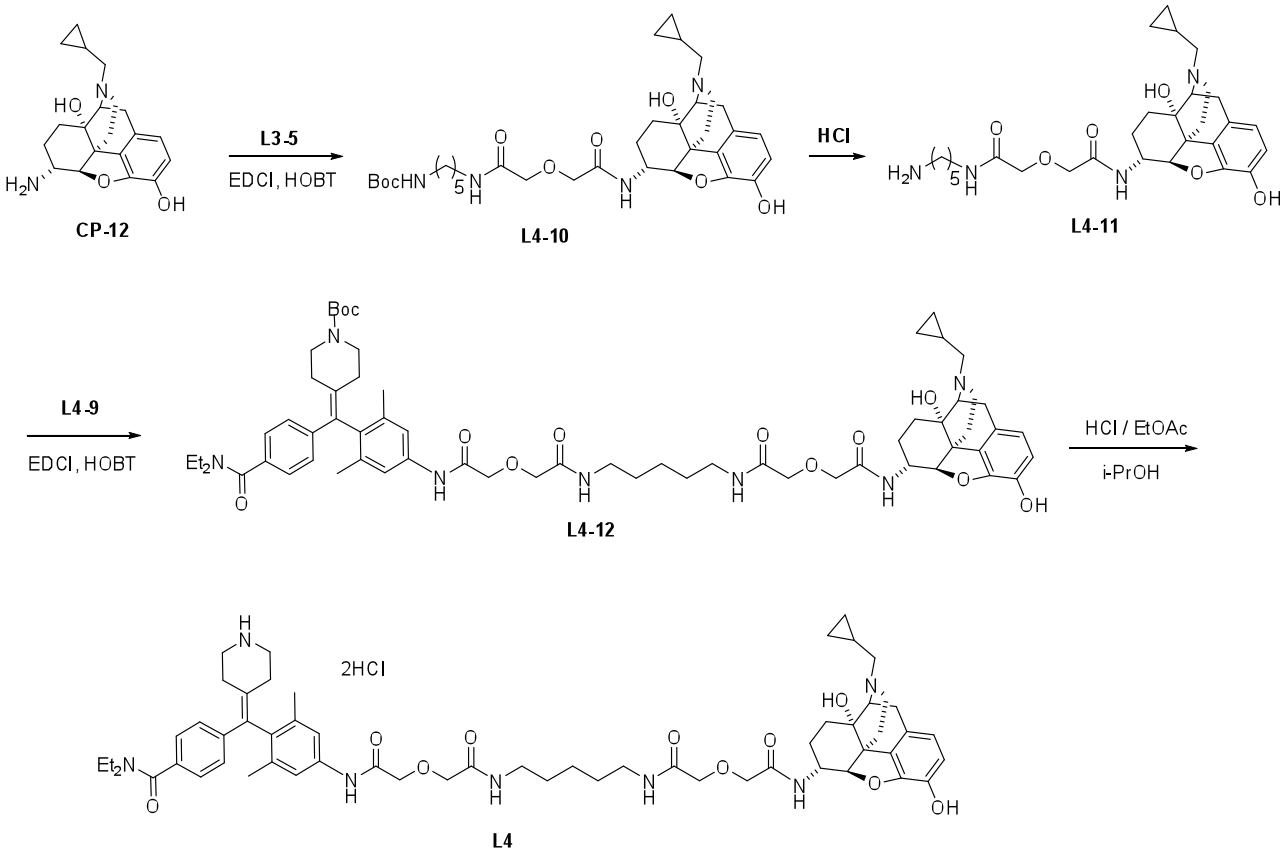
The synthesis of L4



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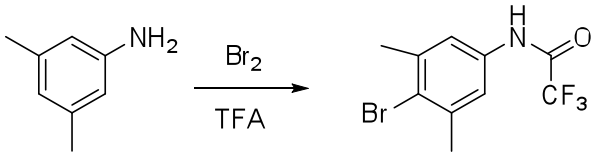
Experimental Section

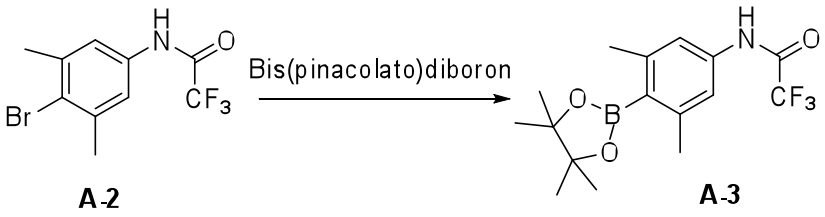
1、The synthesis of **A-2**



A-1 **A-2**

To a solution of **A-1** (5.0 g, 41.3 mmol) in ice cold dichloromethane (200 ml) was added trifluoroacetic anhydride (10.8 g, 51.6 mmol). After 30 minutes, bromine (6.6 g, 41.3 mmol) was slowly added over 10 minutes. After 1h later, the reaction was washed with aqueous $NaHCO_3$, water and concentrated to afford **A-2** (9.4 g) as light yellow oil in 77 % yield.

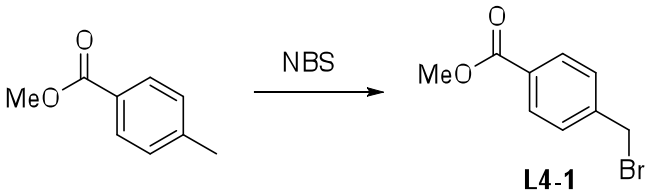
2、The synthesis of **A-3**



A-2 **A-3**

A mixture of **A-2** (5.95 g, 20 mmol), Bis(pinacolato)diboron (6.13 g, 24 mmol), AcOK (5.92 g, 60 mmol) and (dppf)$PdCl_2$ (0.6 g) in DMF (120 mL) was heated at 80 ^{0}C under N_2 for 16 hour. The reaction was poured into water (600 mL) and extracted with EtOAc (2*150 mL). The combined organic layer was washed with brine, dried and concentrated. The residue was purified by column chromatography on silica gel (PE:EA=30:1 to 20:1) to afford **A-3** (2.4 g) as white solid in 34.8 % yield.

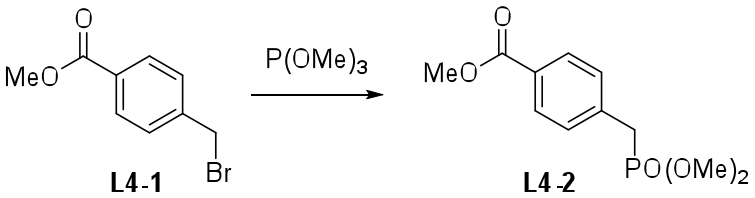
3、The synthesis of **L4-1**



L4-1

A mixture of methyl 4-methylbenzoate (50.0 g, 0.333 mol), NBS (53.4 g, 0.300 mol) and AIBN (5 g) in CCl_4 (1000 mL) was refluxed for 7h. Then the mixture was filtered and concentrated to afford **L4-1** (76.5 g) as light yellow oil in 100 % yield. The crude product was used in the next step without further purification.

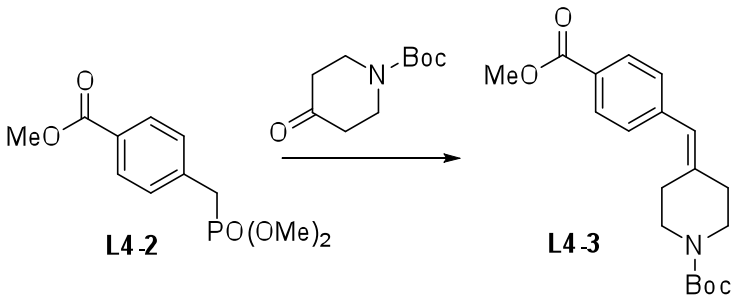
4、The synthesis of **L4-2**



L4-1 **L4-2**

A mixture of **L4-1** (56.5 g, 0.247 mol) and trimethyl phosphite (125 mL) was refluxed under N_2 for 5h. Excess trimethyl phosphite was removed by codistillation with toluene to give **L4-2** (64 g) as light yellow oil in quantitative yield.

5、The synthesis of **L4-3**

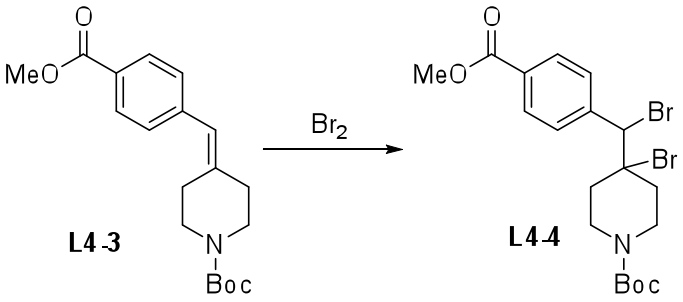


L4-2 **L4-3**

To a solution of **L4-2** (64 g, 0.248 mol) in dry THF (600 mL) was added dropwise LDA (124 mL 2.0 M, 0.248 mol) at -78 °C. The reaction mixture was then allowed to warm to room temperature

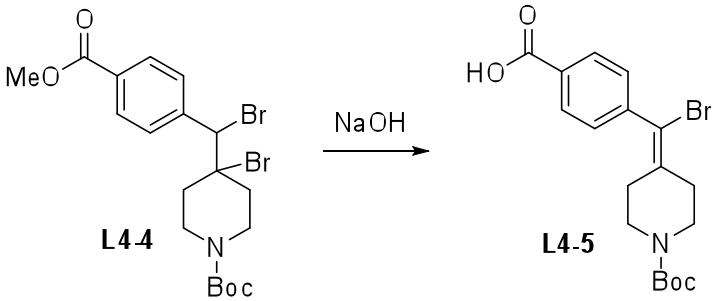
prior to addition of *N-tert*-butoxycarbonyl-4-piperidone (49.4 g, 0.248 mol in 200 mL dry THF). After 12 h, the reaction mixture was quenched with water (1500 mL) and extracted with ethyl acetate (3*400 mL). The combined organic layer was washed with brine, dried over Na_2SO_4 and concentrated. The residue was purified by column chromatography on silica gel (PE:EA=3:0 to 3:1) to afford **L4-3** (30.5 g) as white solid in 37.2 % yield.

6、The synthesis of **L4-4**



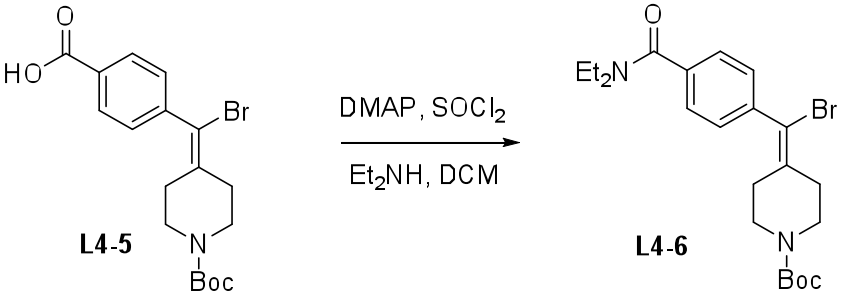
To a mixture of **L4-3** (5.2 g, 16 mmol) and K_2CO_3 (1.0 g) in dry DCM (200 mL) was added a solution of bromine (2.9 g, 18 mmol) in 30 mL DCM at 0 °C. After 1.5 h at room temperature, the solution after filtration was concentrated. The residue was then dissolved in ethyl acetate (200 mL), washed with water (200 mL), 0.5 M HC1 (200 mL) and brine (200 mL), and dried over $MgSO_4$. Removal of solvents provided a crude product, which was recrystallized from methanol to afford **L4-4** as a white solid (6.07 g) in 78 % yeild.

7、The synthesis of **L4-5**



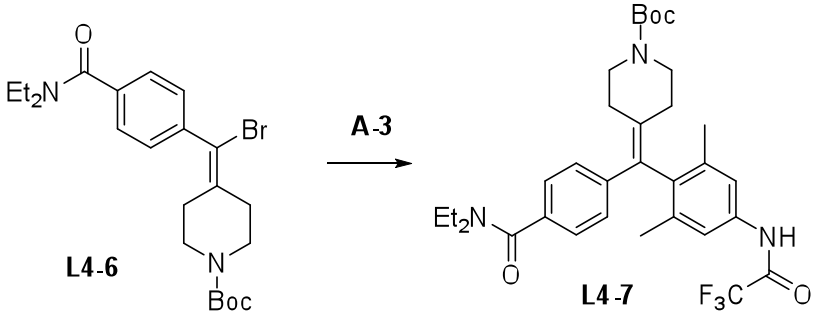
A solution of **L4-4** (5.4g 11 mmol) in methanol (300 mL) and 2.0 M NaOH (100 mL) was heated at 40 °C for 3 h. The solid was collected by filtration and dried overnight under vacuum. The dry salt was dissolved in 40% acetonitrile/water and was adjusted to pH 2 using concentrated HCl. The desired product (**L4-5**; 3.8 g) was isolated as a white powder by filtration in 87 % yield.

8、The synthesis of **L4-6**



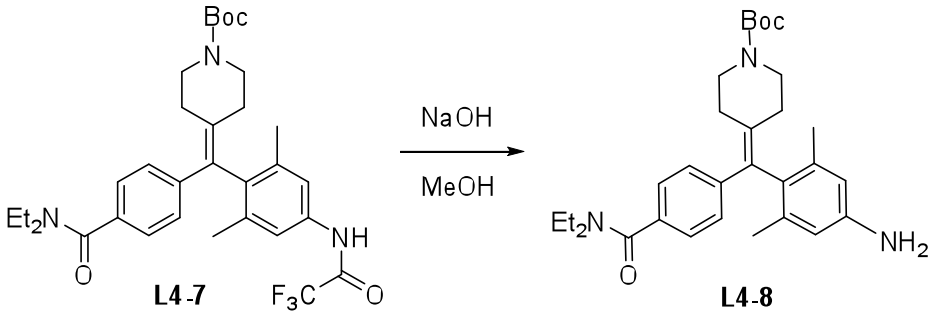
To a solution of DMAP (3.39 g, 27.8 mmol) in DCM (100 mL) was added dropwise $SOCl_2$ (2.55 g, 21.4 mmol) at -20 °C. A precipitate was formed while the mixture was stirred for 15 min. To the slurry, a solution of **L4-5** (5.0 g, 12.6 mmol) in DCM (50 mL) was added and after 15 min at -20 °C the mixture had become a clear solution. Et_2NH (4.43 g, 60.6 mmol) was added and the cooling bath removed. After stirring overnight, the reaction was washed with 1 M citric acid, water, dried and concentrated. The residue was purified by column chromatography on silica gel (PE:EA=3/1 to 3/2) to give **L4-6** (3.9 g) as white solid in 68.4 % yield.

9、The synthesis of **L4-7**



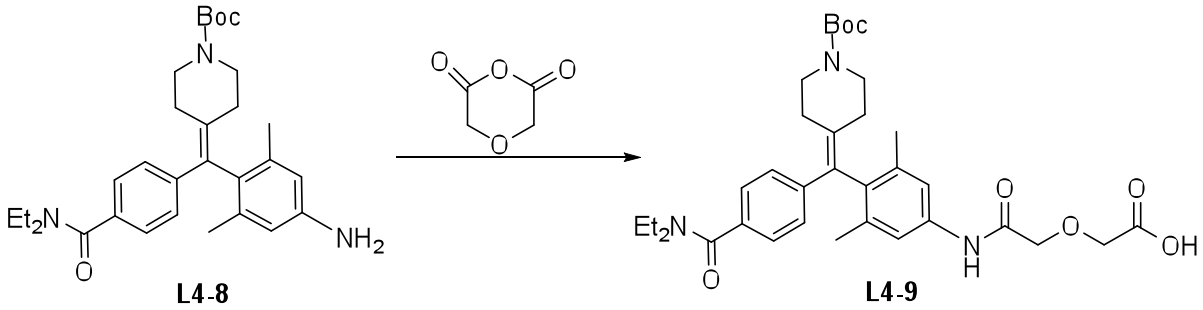
A mixture of **L4-6** (500 mg, 0.66 mmol), **A-3** (500 mg, 0.86 mmol), K_2CO_3 (600 mg, 2.6 mmol) and $Pd(PPh_3)_2Cl_2$ (20 mg) in THF (15 mL) and H_2O (5 mL) was heated at 80 ^{0}C under N_2 for 0.5 hour by microwave. The reaction was poured into water (60 mL) and extracted with EtOAc (2*30 mL). The combined organic layer was washed with brine, dried and concentrated. The residue was purified by column chromatography on silica gel (PE:EA=6:1 to 3:1) to afford **L4-7** (266 mg) as light yellow solid in 40.9 % yield.

10、The synthesis of **L4-8**



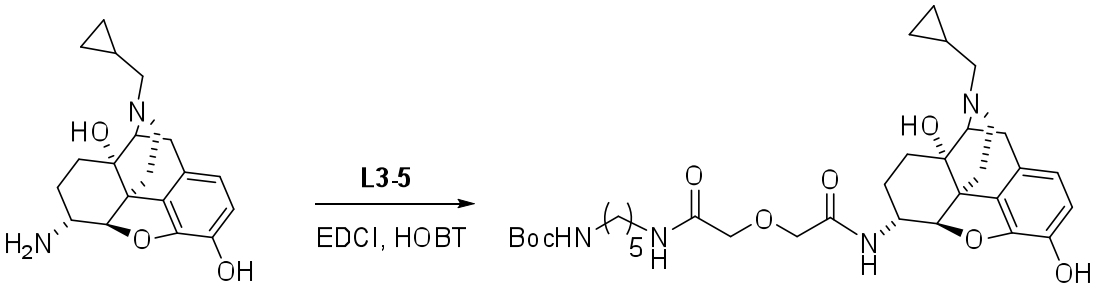
A mixture of **L4-7** (400 mg, 0.68 mmol), NaOH (54 mg, 1.36 mmol) in MeOH (10 mL) and H_2O (10 mL) was stirred at r.t. overnight. The reaction was poured into water (60 mL) and extracted with EtOAc (2*30 mL). The combined organic layer was washed with brine, dried and concentrated to afford **L4-8** (336 mg) as light yellow solid in 100 % yield.

11、The synthesis of **L4-9**



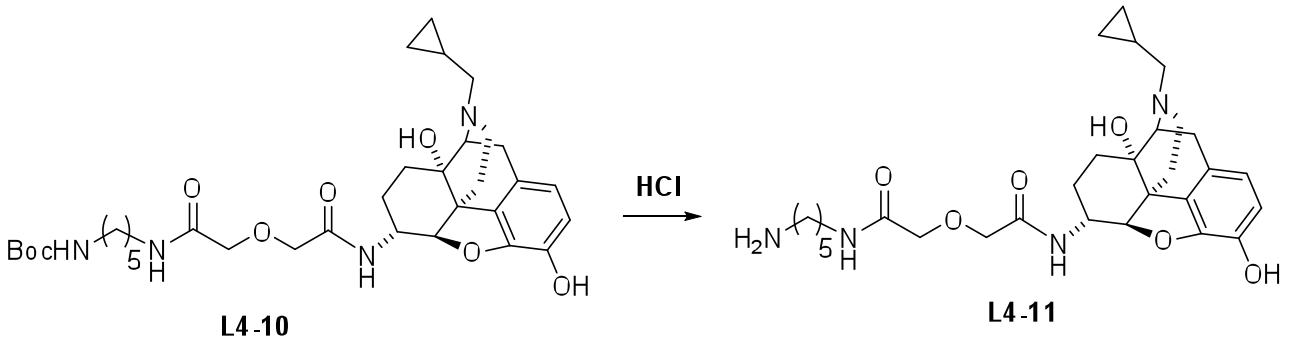
To a solution of **L4-8** (100 mg, 0.0.2034 mmol) in THF (5 mL) was added diglycolic anhydride (33 mg, 0.2847 mmol) one portion under ice-water bath. The reaction was stirred at r.t. overnight. EtOAc (20 mL) was added to the mixture and washed with water (2*15 ml), then brine (15 ml). The organic layer was dried (Na_2SO_4), filtered and concentrated to afford **L4-9** (115 mg) as light yellow solid in 93 % yield.

12、The synthesis of **L4-10**



To a solution of **L3-5** (279 mg, 0.876 mmol), EDCI (168 mg, 0.876 mmol) and HOBT (118 mg, 0.876 mmol) in DMF (6 mL) was added **CP-12** (150 mg, 0.438 mmol). The reaction was stirred at rt overnight. Water (20 mL) was added and then brought to pH 9 with NH_4OH and extracted with EtOAc (3*20 mL). The combined organic layers were dried (Na_2SO_4), filtered and concentrated. The residue was purified by Prep TLC (silica gel, DCM:MeOH=8:1) to afford **L4-10** (230 mg) as light yellow solid in 81.7 % yield.

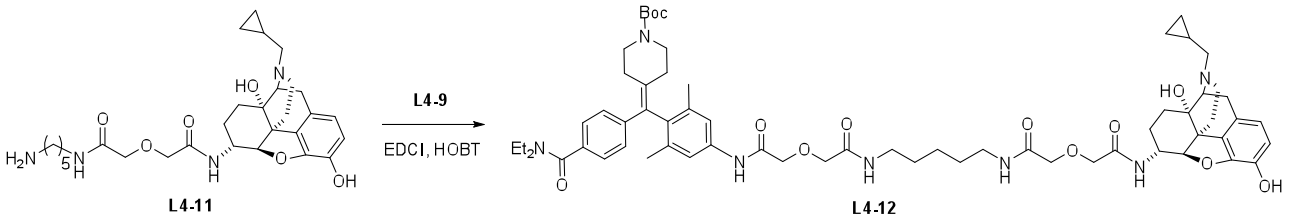
13、The synthesis of **L4-11**



L4-10 L4-11

To a solution of **L4-10** (230 mg, 0.358 mmol) in MeOH (6 mL) was added HCl/EtOAc (6 mL). The reaction was stirred at r.t. overnight and then concentrated to afford **L4-11** (220 mg) as light yellow solid in 100 % yield.

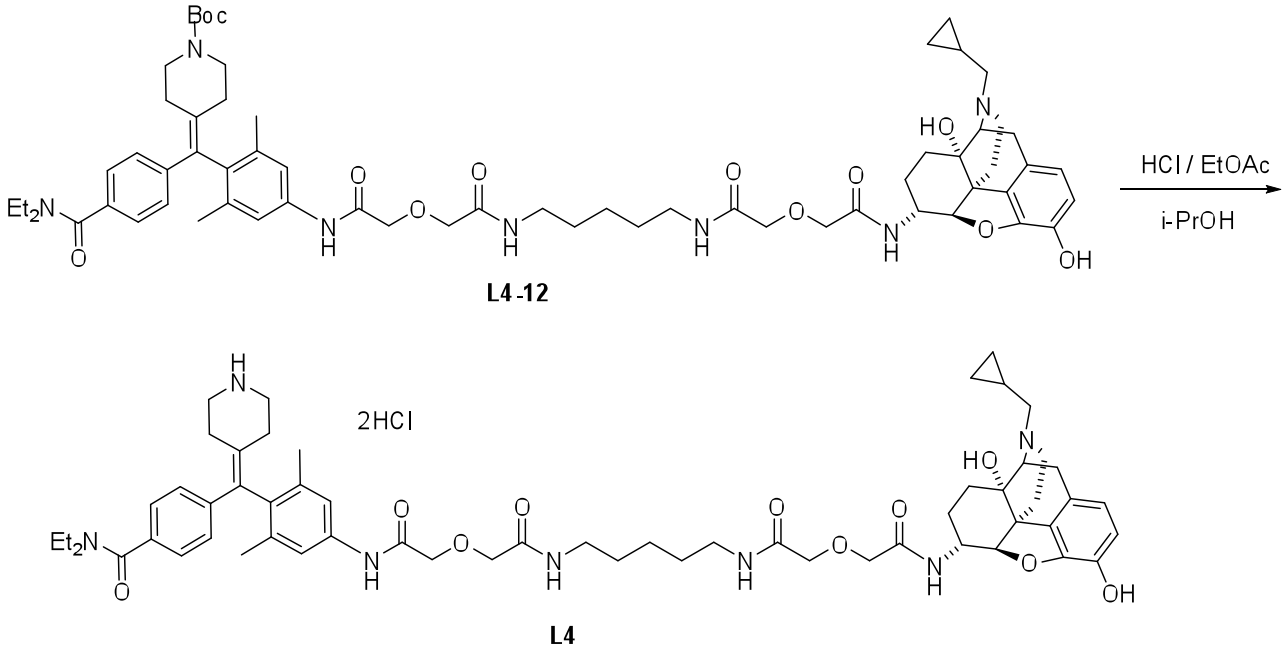
14、The synthesis of **L4-12**



L4-11 L4-12

To a mixture of **L4-11** (220 mg, 0.357 mmol), **L4-9** (217 mg, 0.358 mmol), EDCI (82 mg, 0.429 mmol) and HOBT (58 mg, 0.429 mmol) in DMF (8 mL) was added DIEA (56 mg, 0.429 mmol). The reaction was stirred at rt overnight. Water (20 mL) was added and then brought to pH 9 with NH_4OH and extracted with EtOAc (3*20 mL). The combined organic layers were dried (Na_2SO_4), filtered and concentrated. The residue was purified by Prep TLC (silica gel, DCM:MeOH=10:1) to afford **L4-12** (176 mg) as light yellow solid in 43.6 % yield.

15、The synthesis of **L4**



L4-12

L4

To a solution of **L4-12** (80 mg, 0.0707 mmol) in i-PrOH (10 mL) and EtOAc (6 mL) was added HCl/EtOAc (6 mL). The reaction was stirred at r.t. for about 10 hour and then concentrated the reaction at r.t. Then the product was dried under high vacuum to afford **L4** (78 mg) as light yellow solid in 100 % yield.

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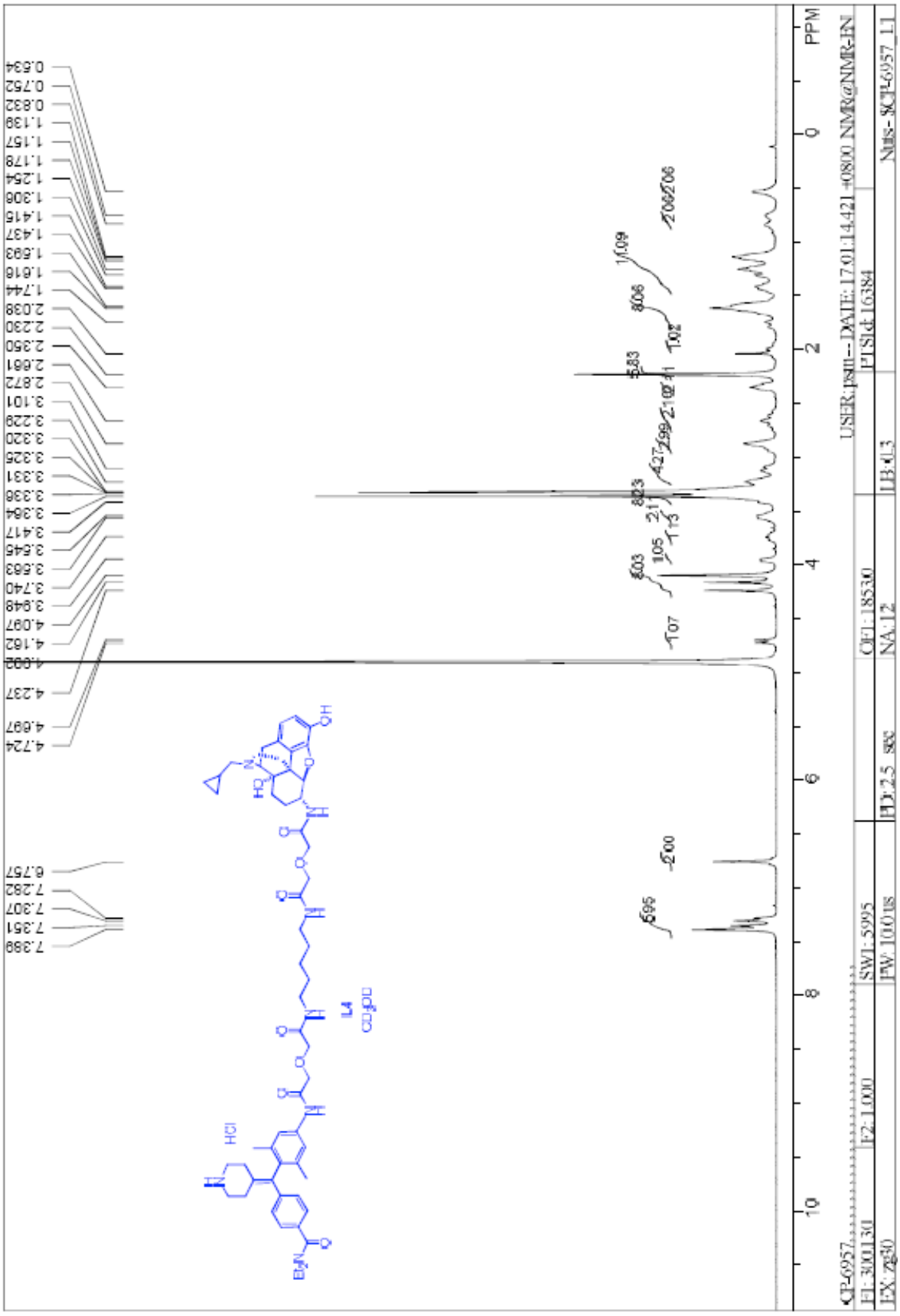
^{1}H-NMR Spectrum (*CD_3OD*) of **L4**



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LCMS of **L4**

```
File D:\DATA\CT20\PRODUCT\L4-15281-LCMSA040.D
Injection Date    : 18 Nov 10   10:37 am +0800       Tgt Mass(EZX) :
Sample Name       : L4                                     Location : P2-E-07
Acq. Operator     : 20product                                 Inj : 1
Spec. Reported    : MS Integration                     Inj Volume : 1.5 ul
Acq. Method       : C:\Chem32\1\METHODS\5-100A5.M
Analysis Method   : C:\CHEM32\1\METHODS\5-100A 3(215).M
Sample Info : Easy-Access Method: '5-100A5'
Method Info : Mobile Phase : A:ACN (0.05%TFA )          B:WATERS (0.05%TFA)
              Graient:  5%-100%,3.0min,
              Flow:1.8ml/min
              Column; X-Bridge C18: 4.6*50,3.5um
              Column Temperature :50C
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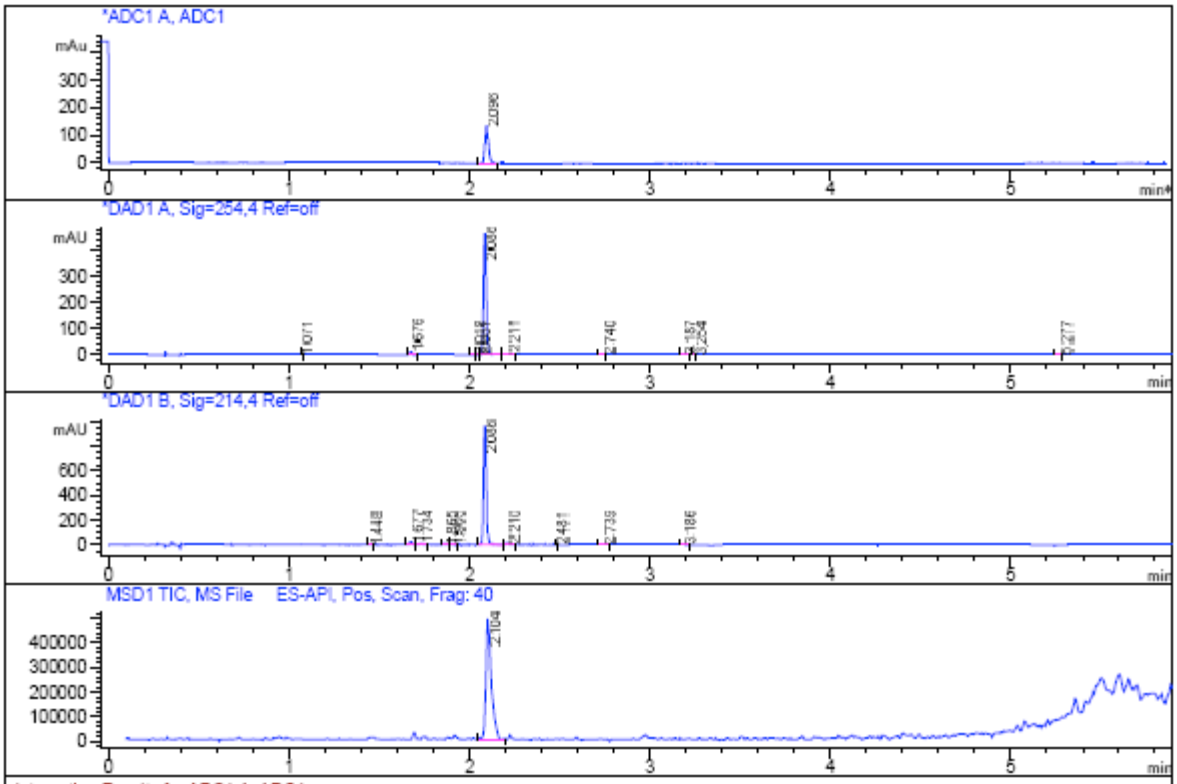

Integration Results for ADC1 A, ADC1

RetTim	Width	Area	Height	Area%
2.10	0.02	198.36	134.31	100.00

Integration Results for DAD1 A, Sig=254,4 Ref=off

RetTim	Width	Area	Height	Area%
1.07	0.00	0.11	0.56	0.02
1.68	0.01	10.77	11.01	2.02
2.02	0.01	1.06	1.16	0.20
2.05	0.01	0.66	0.84	0.12
2.09	0.02	506.68	463.46	94.78
2.21	0.02	7.83	6.71	1.46
2.74	0.02	2.55	1.98	0.48
3.19	0.02	2.98	2.33	0.56
3.25	0.01	0.43	0.50	0.08
5.28	0.03	1.54	0.66	0.29

Integration Results for DAD1 B, Sig=214,4 Ref=off

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1.45	0.01	1.41	1.39	0.13
1.68	0.01	19.19	19.64	1.71
1.73	0.02	10.42	8.17	0.93
1.86	0.02	7.14	5.90	0.64
1.91	0.02	6.62	5.39	0.59
2.09	0.02	1050.17	954.49	93.65
2.21	0.02	13.69	11.79	1.22
2.48	0.00	0.32	1.03	0.03
2.74	0.02	5.71	4.71	0.51
3.19	0.02	6.76	4.93	0.60

RetTim	Width	Area	Height	Area%

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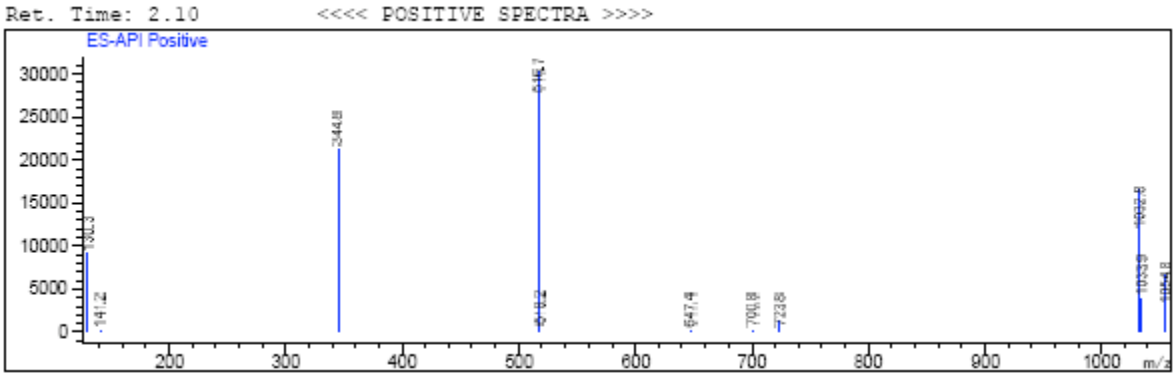

Reference:

1) *JMC, 2000, 3895-3905*

2) *J Label Compd Radiopharm 2003,131-138*

END

Synthesis of SNC-19 (Chempartner)

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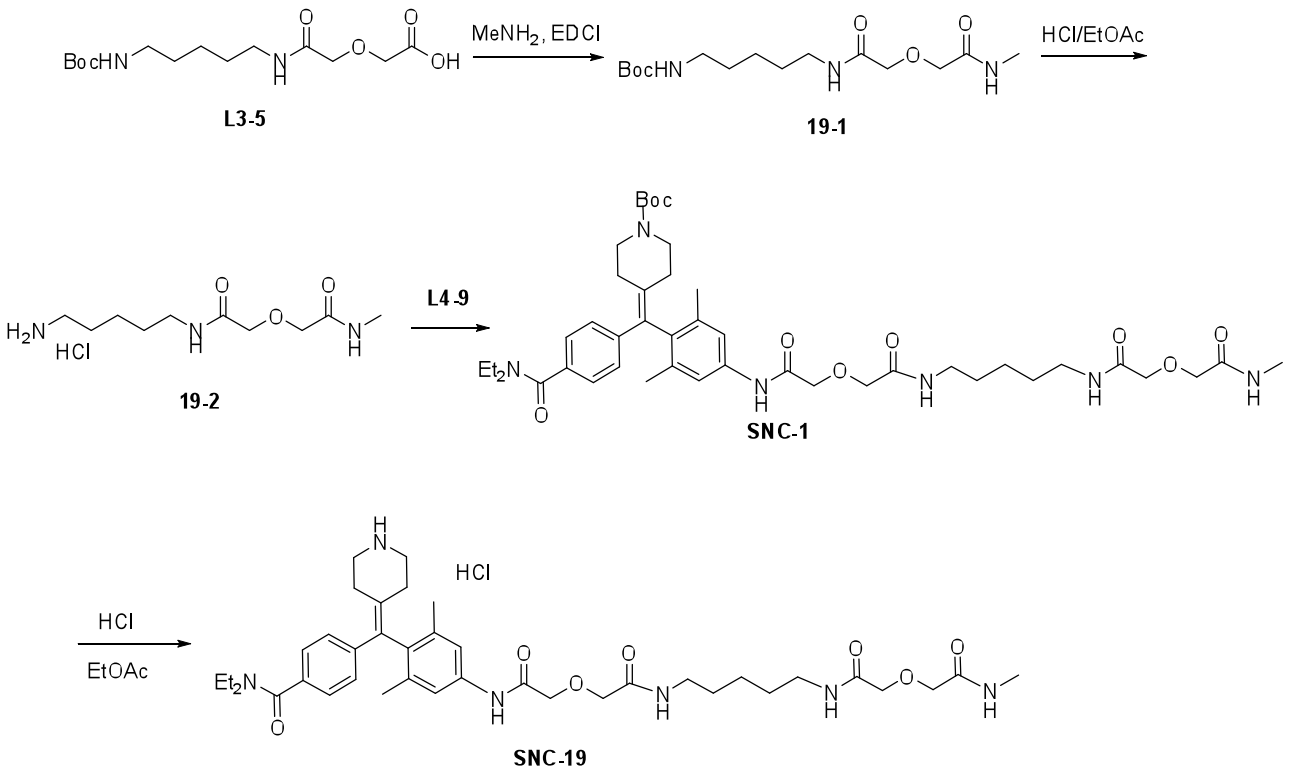
Project Summary

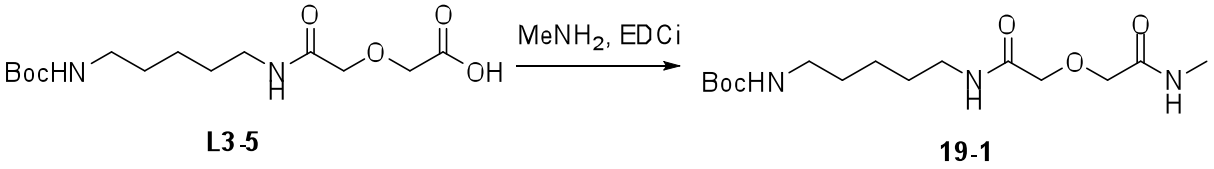
According to the structure of **SNC-19**, the target compound can be prepared through following steps.

The synthesis of SNC-19



Experimental Section

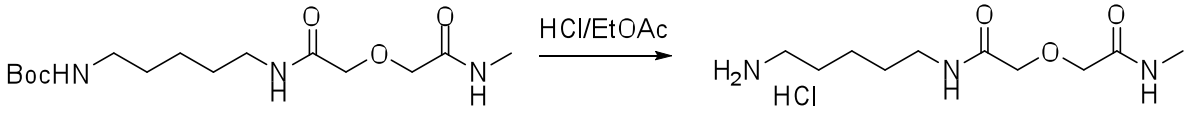
1、The synthesis of **19-1**



L3-5 **19-1**

To a solution of **L3-5** (300 mg, 0.942 mmol) in ice cold dichloromethane (10 ml) was added EDCI (217 mg, 1.13 mmol). After 30 minutes, $MeNH_2$ (MeOH solution, 0.5 mL) was added. After 2h later, the reaction was concentrated. The residue was dissolved in water (20 mL) and extracted with EtOAc (4*15 mL). The combined organic layer was dried and concentrated. The residue was purified by column chromatography on silica gel (PE:EA=2:1 to 1:2) to afford **19-1** (220 mg) as colorless oil in 70.5 % yield.

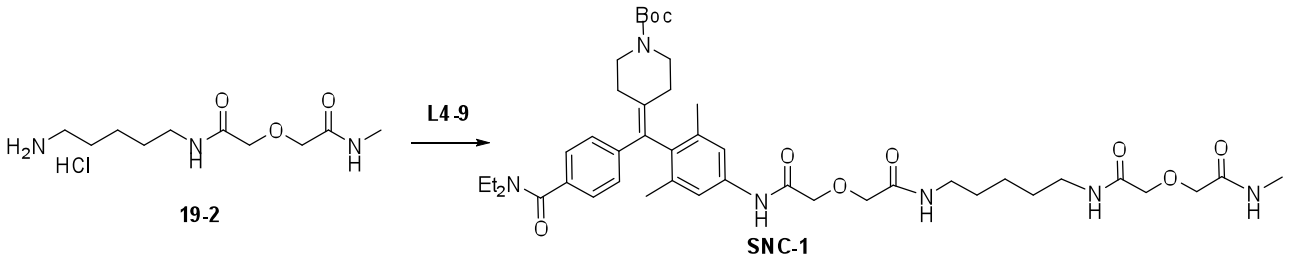
2、The synthesis of **19-2**



19-1 **19-2**

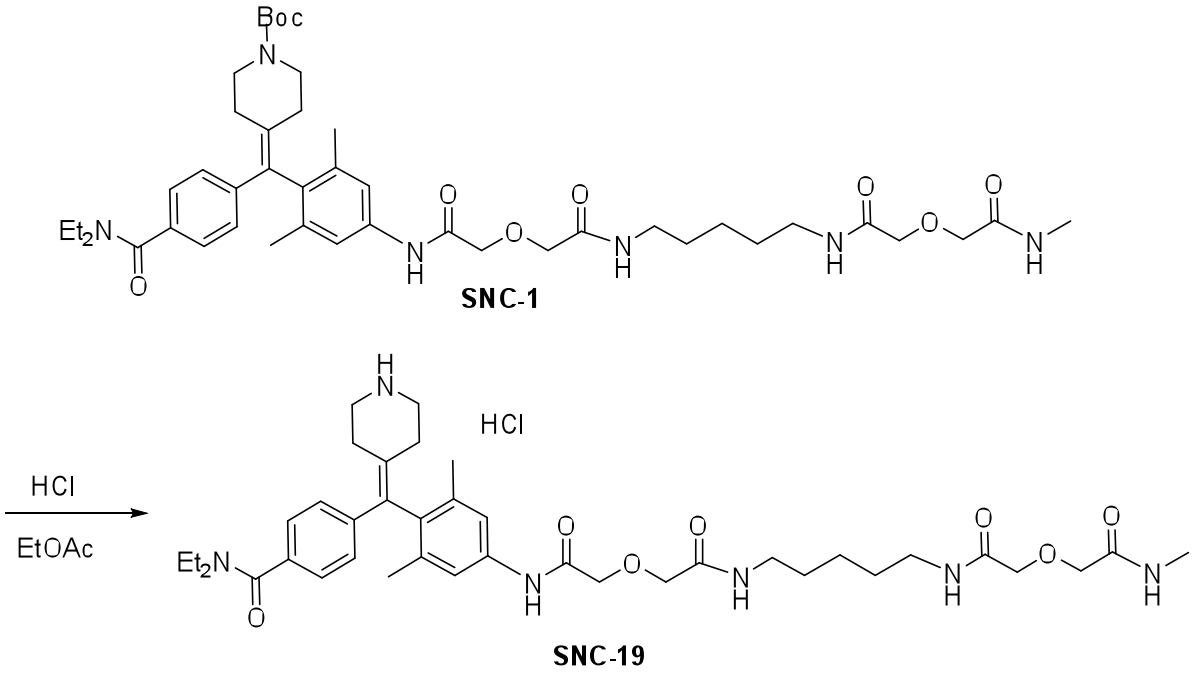
To a solution of **19-1** (220 mg, 0.665 mmol) in MeOH (5 mL) was added HCl/EtOAc (5 mL). The reaction was stirred at r.t. overnight and then concentrated to afford **19-2** (178 mg) as light yellow solid in 100 % yield.

3、The synthesis of **SNC-1**



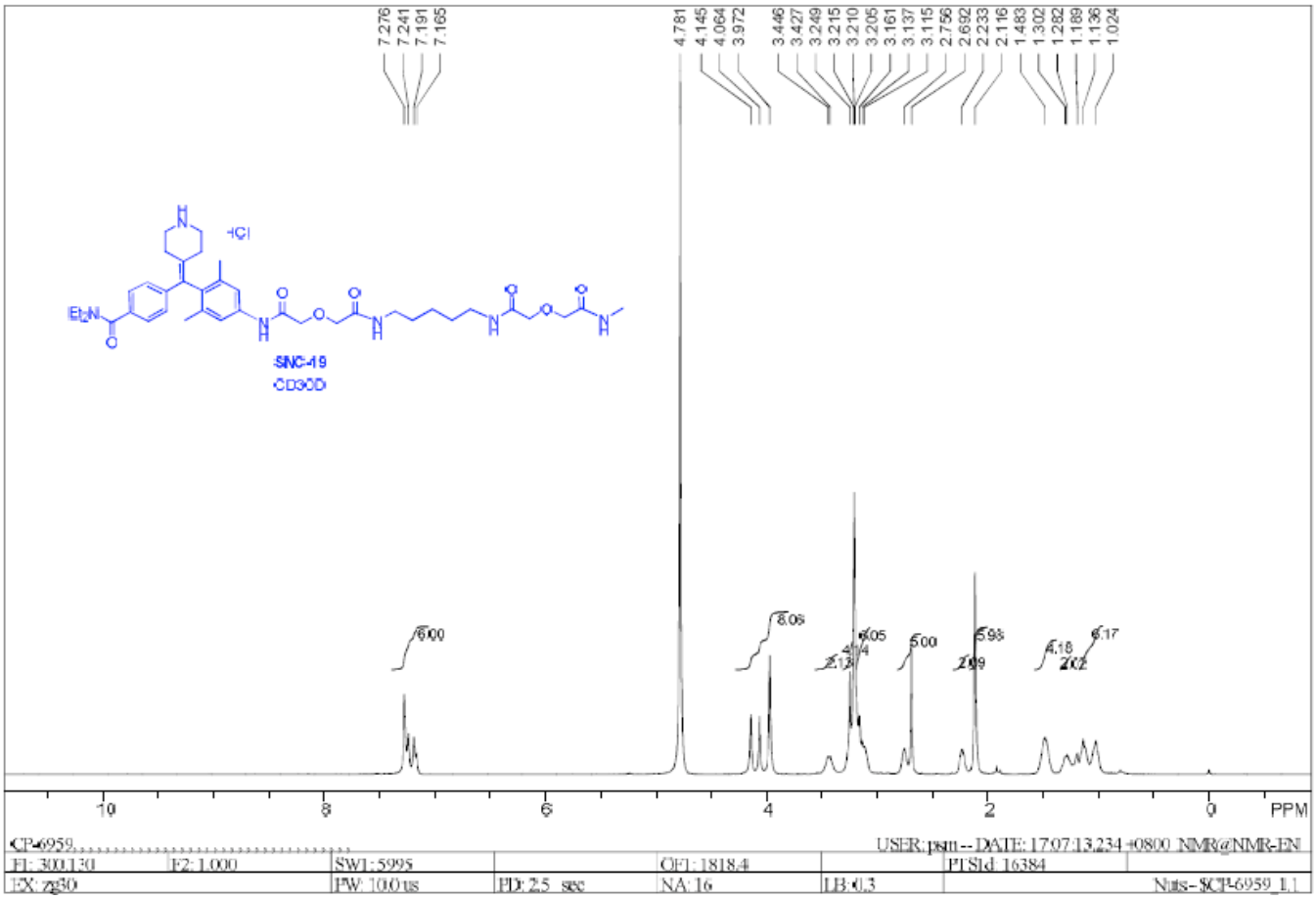
To a mixture of **19-2** (27 mg, 0.0987 mmol), **L4-9** (60 mg, 0.0987 mmol), EDCI (23 mg, 0.118 mmol) and HOBT (16 mg, 0.118 mmol) in DMF (4 mL) was added DIEA (16 mg). The reaction was stirred at rt overnight. Water (20 mL) was added and then brought to pH 9 with NH_4OH and extracted with EtOAc (2*15 mL). The combined organic layers were dried (Na_2SO_4), filtered and concentrated. The residue was purified by Prep TLC (silica gel, DCM:MeOH=9:1) to afford **SNC-1** (48 mg) as light yellow solid in 59.3 % yield.

4、The synthesis of **SNC-19**



To a solution of **SNC-1** (48 mg, 0.0585 mmol) in EtOAc (5 mL) was added HCl/EtOAc (5 mL). The reaction was stirred at r.t. overnight and then concentrated to afford **SNC-19** (44 mg) as light yellow solid in 100 % yield.

^{1}H-NMR Spectrum (*CD_3OD*) of **SNC-19**



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LCMS of **SNC-19**

```
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Injection Date    : 18 Nov 10  10:53 am +0800       Tgt Mass(EZX) :
Sample Name       : SNC-19                               Location : P2-E-09
Acq. Operator     : 20product                               Inj : 1
Spec. Reported    : MS Integration                    Inj Volume : 1.2 ul
Acq. Method       : C:\Chem32\1\METHODS\5-100A5.M
Analysis Method   : C:\CHEM32\1\METHODS\5-100A 3(215).M
Sample Info : Easy-Access Method: '5-100A5'
Method Info : Mobile Phase : A:ACN (0.05%TFA )          B:WATERS (0.05%TFA)
              Graient:  5%-100%,3.0min,
              Flow:1.8ml/min
              Column; X-Bridge C18: 4.6*50,3.5um
              Column Temperature :50C
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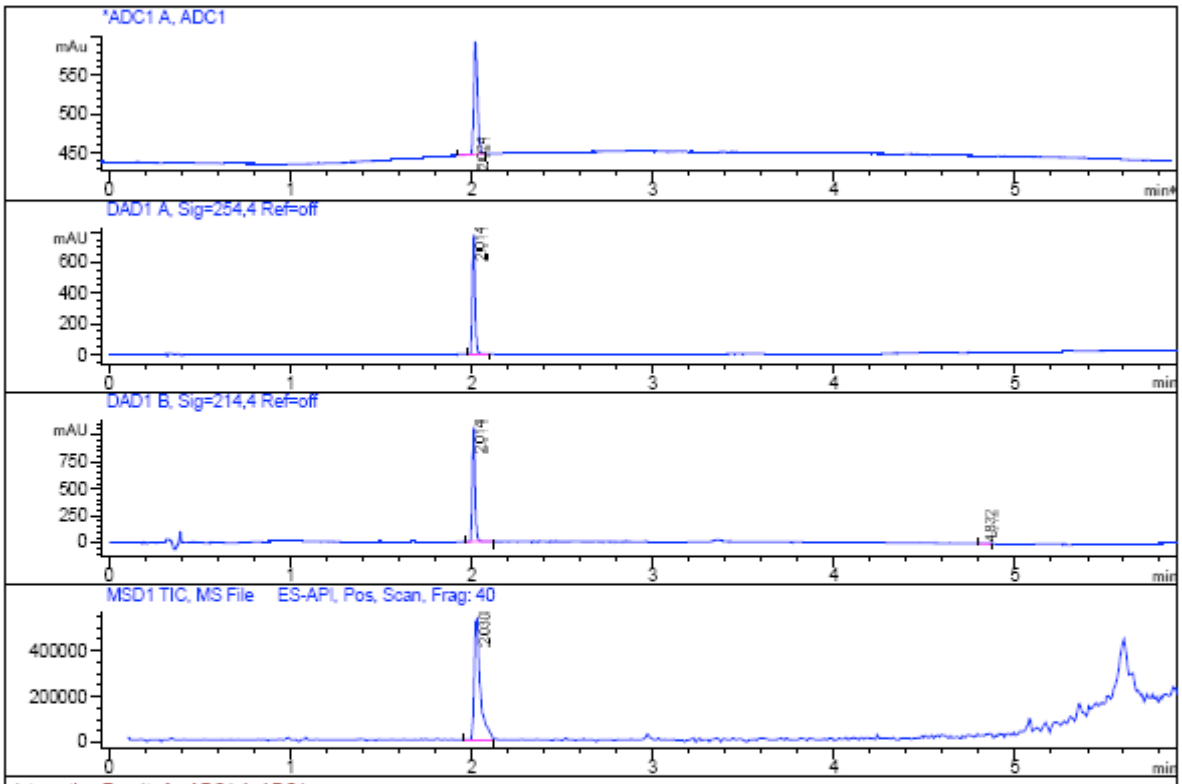

Integration Results for ADC1 A, ADC1

RetTim	Width	Area	Height	Area%
2.02	0.02	198.25	144.61	100.00

Integration Results for DAD1 A, Sig=254,4 Ref=off

RetTim	Width	Area	Height	Area%
2.01	0.02	783.68	783.21	100.00

Integration Results for DAD1 B, Sig=214,4 Ref=off

RetTim	Width	Area	Height	Area%
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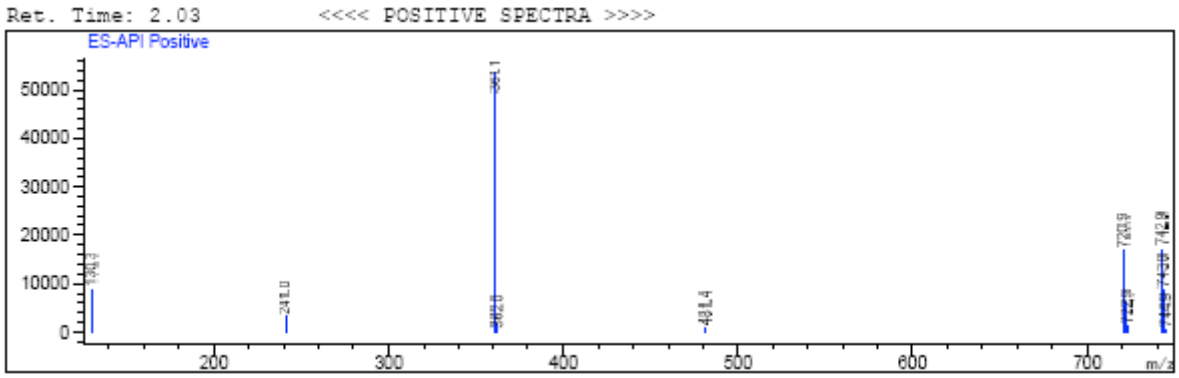
4.83	0.02	5.81	3.96	0.54



Reference:

None

END

Synthesis of NTX-19 (Chempartner)

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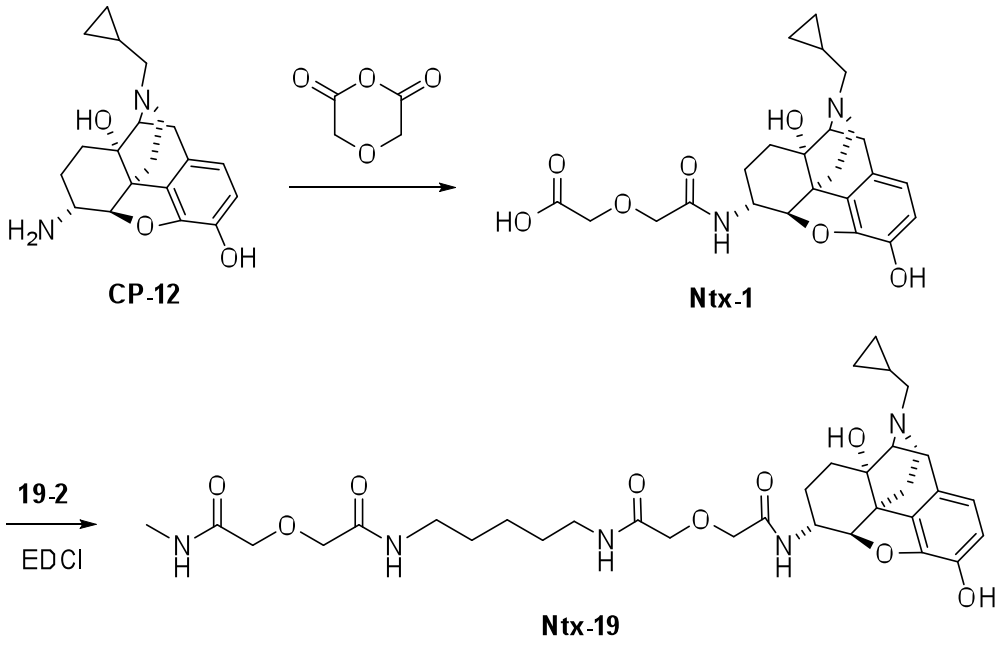
邮编/Zip:201203

Project Summary

According to the structure of **Ntx-19**, the target compound can be prepared through following steps.

The synthesis of Ntx-19



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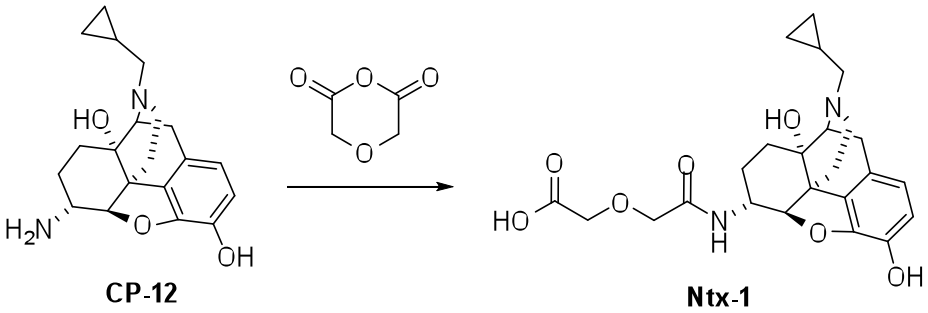
Experimental Section

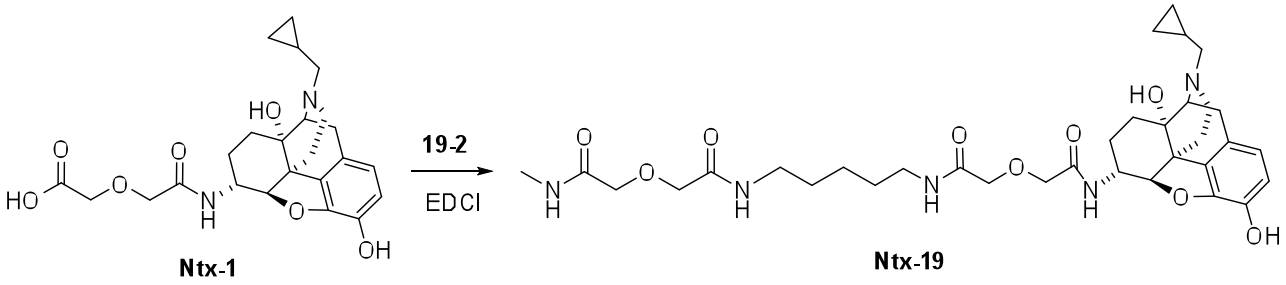
1、The synthesis of **Ntx-1**



CP-12 **Ntx-1**

To a solution of **CP-12** (60 mg, 0.175 mmol) in THF (10 mL) was added diglycolic anhydride (28 mg, 0.245 mmol) one portion under ice-salt bath. The reaction was stirred overnight at r.t. and then concentrated to give **Ntx-1** (82 mg) as gray solid in quantitative yield. The crude product was used in the next step without further purification.

2、The synthesis of **Ntx-19**



Ntx-1 **Ntx-19**

To a mixture of **Ntx-1** (82 mg, 0.179 mmol), **19-2** (48 mg, 0.179 mmol), EDCI (41 mg, 0.215 mmol) and HOBT (29 mg, 0.215 mmol) in DMF (5 mL) was added DIEA (28 mg). The reaction was stirred at rt overnight. Water (20 mL) was added and then brought to pH 9 with NH_4OH and extracted with EtOAc (3*15 mL). The combined organic layers were dried (Na_2SO_4), filtered and concentrated. The residue was purified by Prep TLC (silica gel, DCM:MeOH =8:1) to afford **Ntx-19** (45 mg) as light yellow solid in 37.5 % yield.

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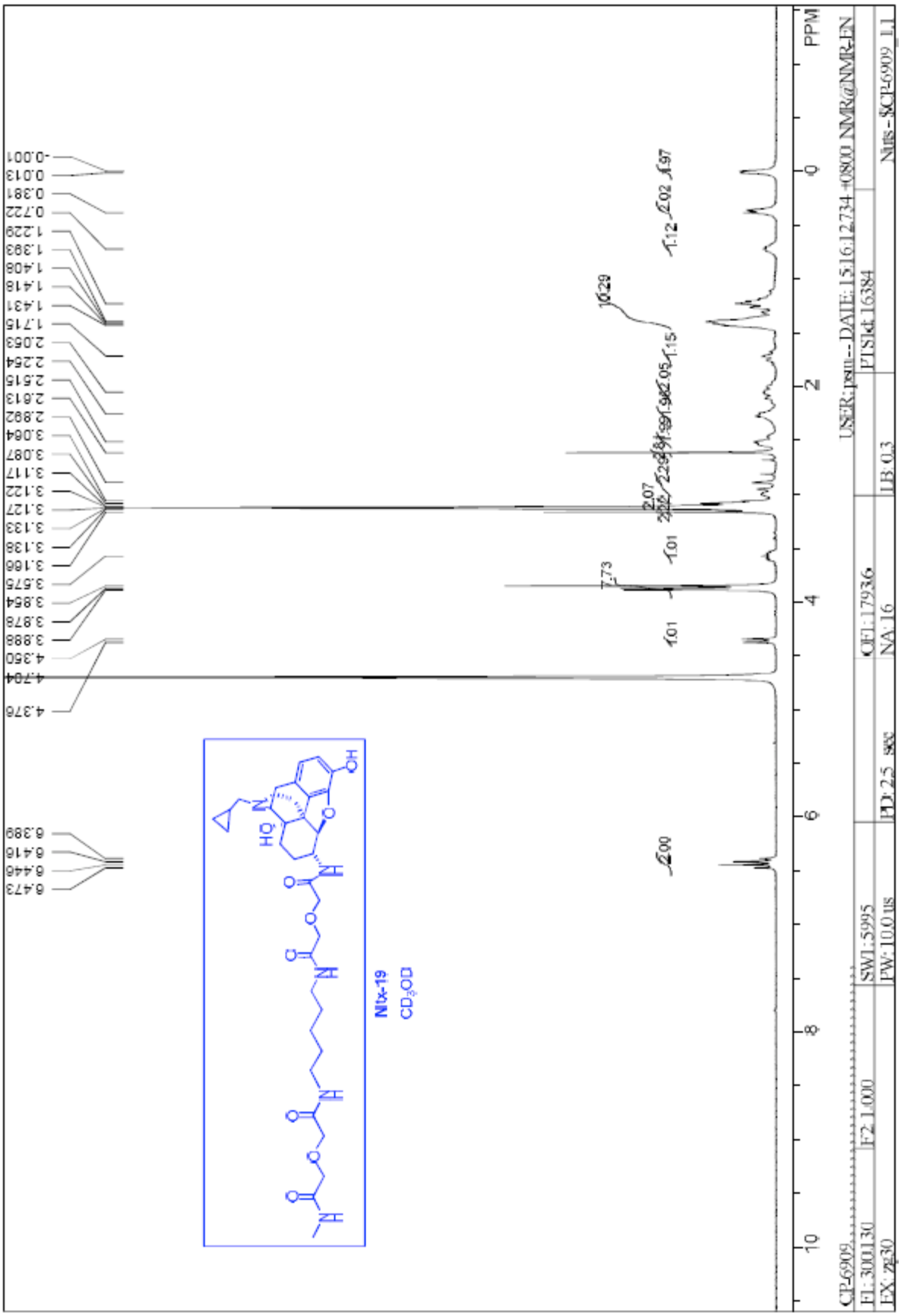
^{1}H-NMR Spectrum (*CD_3OD*) of **Ntx-19**



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LCMS of **Ntx-19**

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Sample Name       : Ntx-19                               Location : P2-E-08
Acq. Operator     : 20product                               Inj : 1
Spec. Reported    : MS Integration                    Inj Volume : 1.5 ul
Acq. Method       : C:\Chem32\1\METHODS\5-100A5.M
Analysis Method   : C:\CHEM32\1\METHODS\5-100A 3(215).M
Sample Info : Easy-Access Method: '5-100A5'
Method Info : Mobile Phase : A:ACN (0.05%TFA )          B:WATERS (0.05%TFA)
              Graient:  5%-100%,3.0min,
              Flow:1.8ml/min
              Column; X-Bridge C18: 4.6*50,3.5um
              Column Temperature :50C
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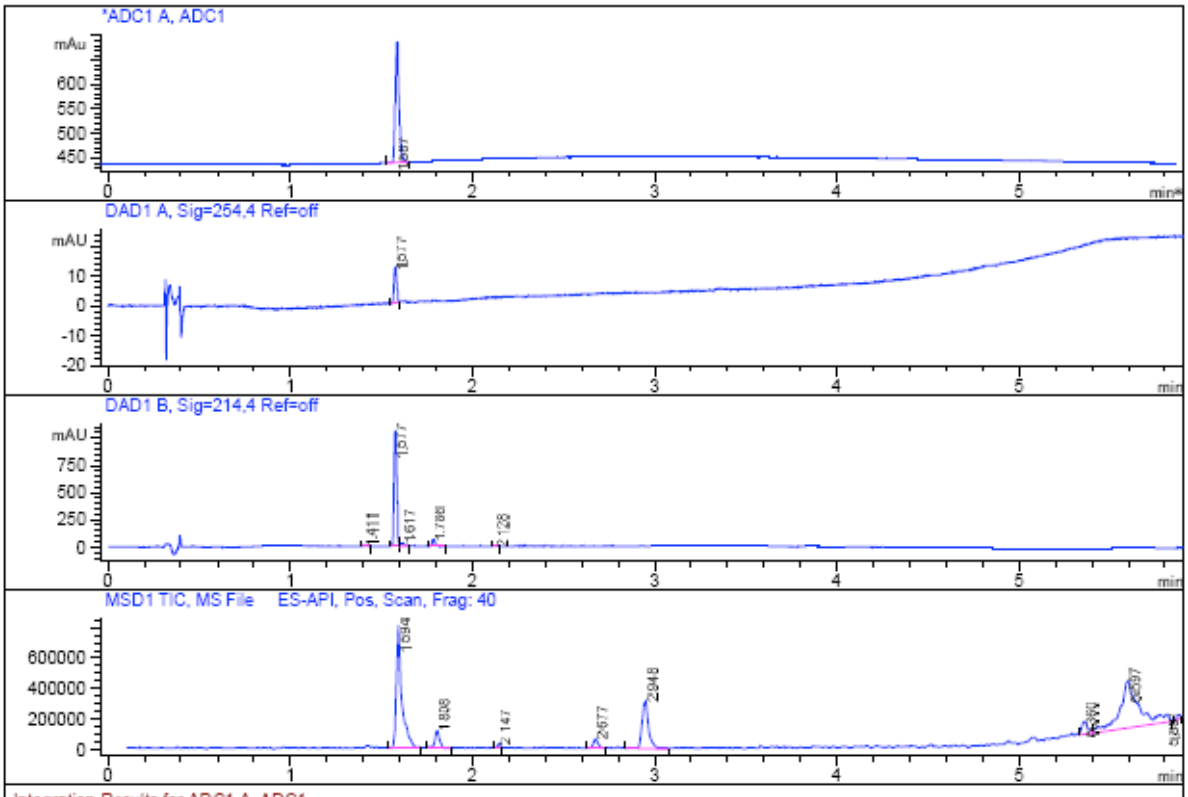


Integration Results for ADC1 A, ADC1

RetTim	Width	Area	Height	Area%
1.59	0.02	364.95	245.42	100.00

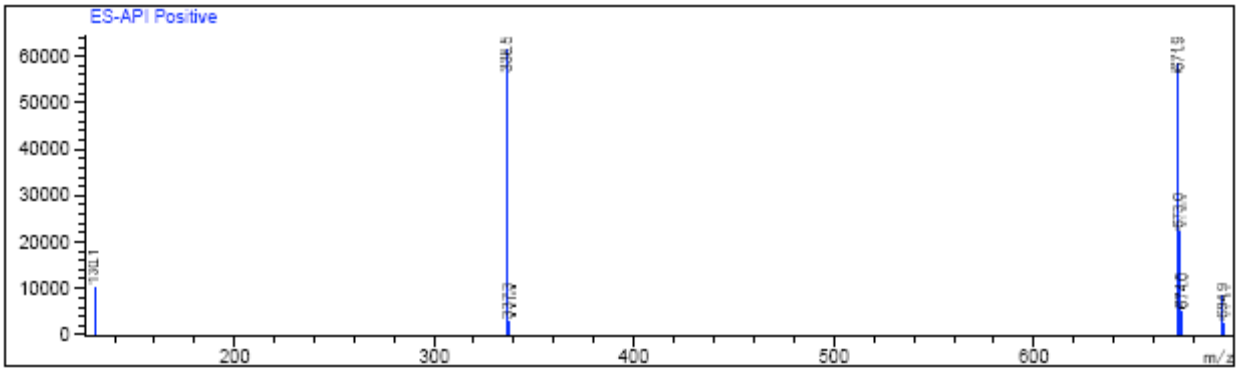
Integration Results for DAD1 A, Sig=254,4 Ref=off

RetTim	Width	Area	Height	Area%
1.58	0.02	12.17	11.90	100.00

Integration Results for DAD1 B, Sig=214,4 Ref=off

RetTim	Width	Area	Height	Area%
1.41	0.02	8.34	7.96	0.69
1.58	0.02	1089.36	1058.20	90.26
1.62	0.02	35.04	33.96	2.90
1.79	0.02	70.14	60.00	5.81
2.13	0.01	4.06	3.88	0.34

Ret. Time: 1.59 <<<< POSITIVE SPECTRA >>>>



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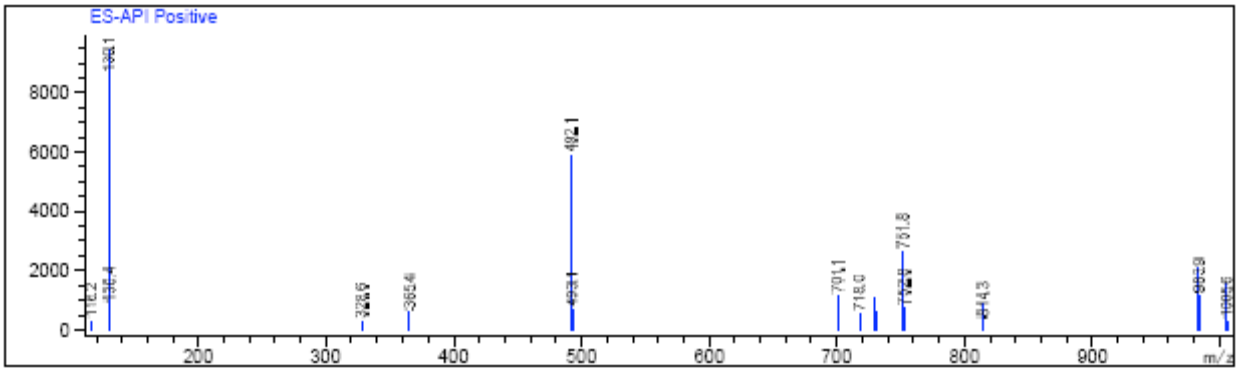

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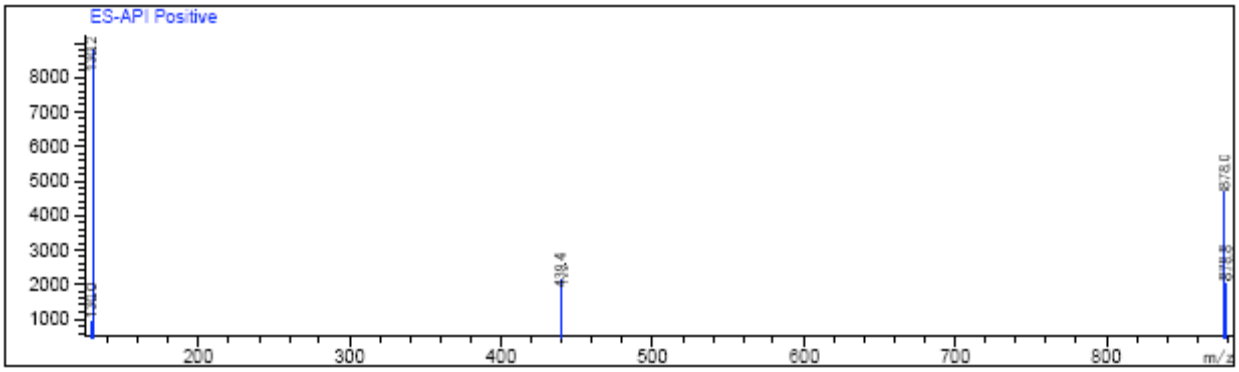

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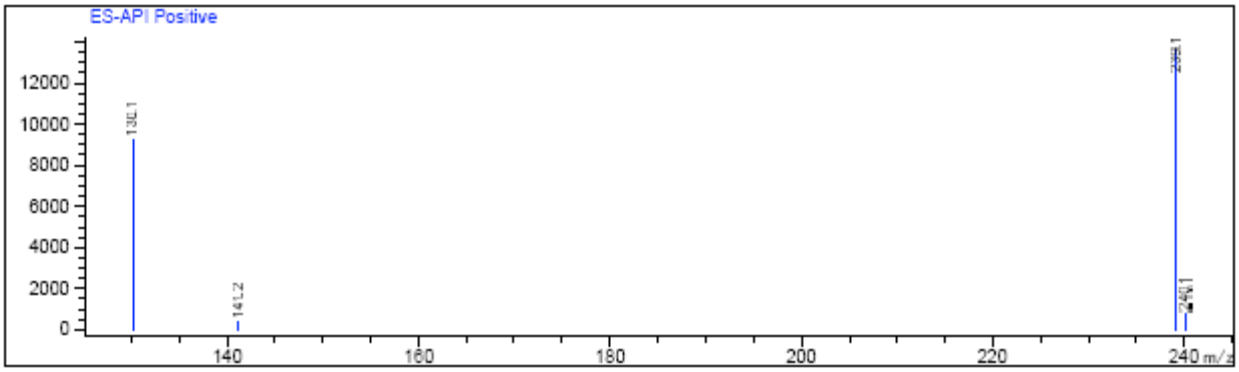

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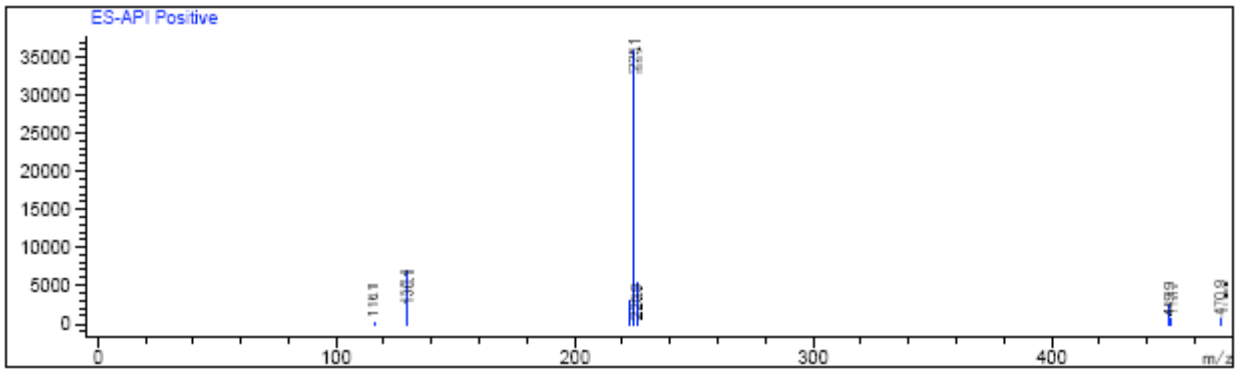
Ret. Time: 2.95



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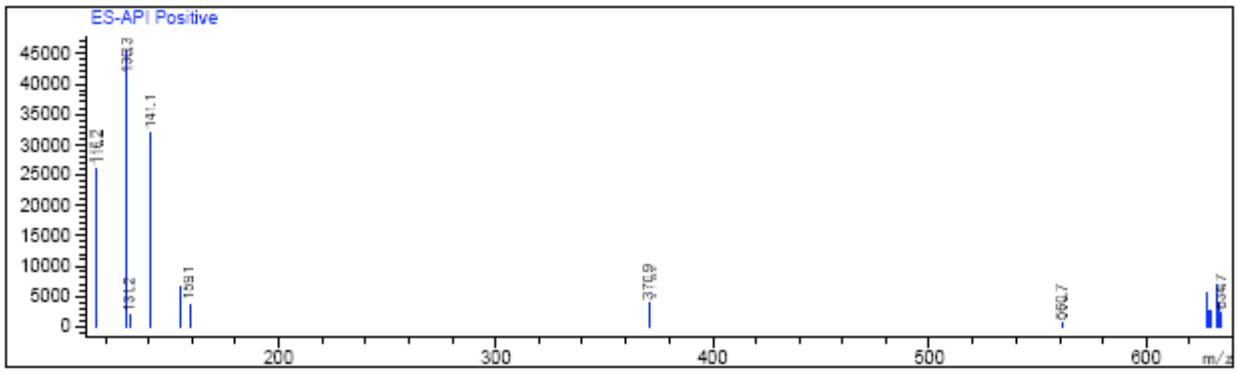

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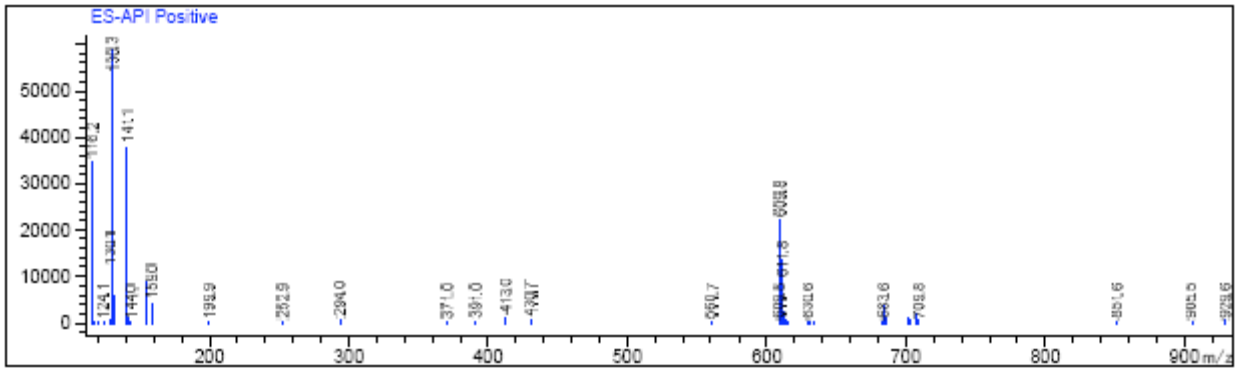

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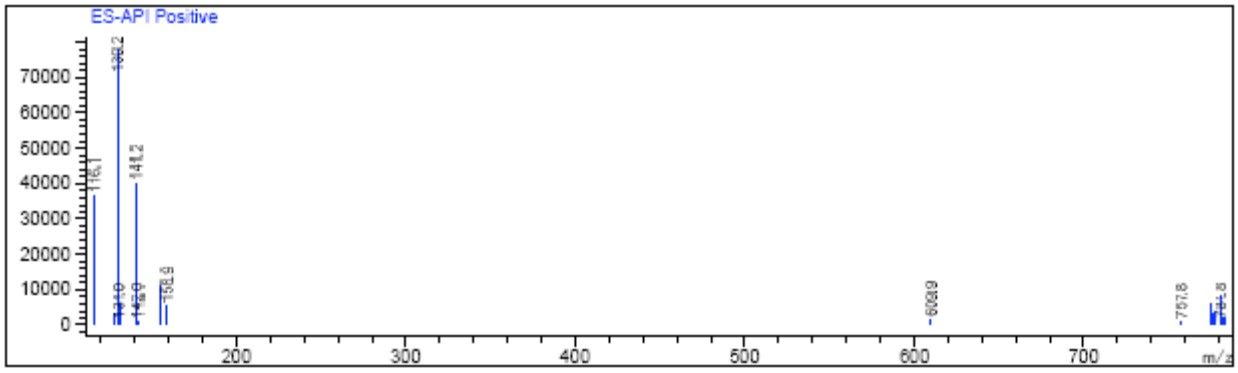

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Reference:

None

END