Supporting Information

Novel FTY720-Based Compounds Stimulate Neurotrophin Expression and Phosphatase Activity in Dopaminergic Cells

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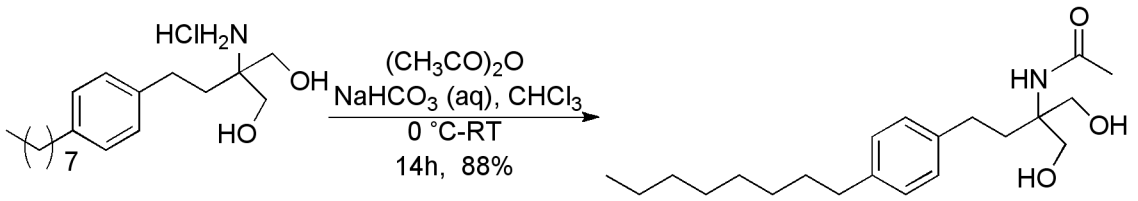
Chemistry

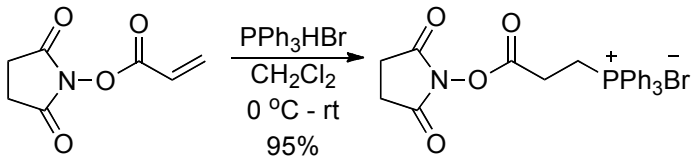
Reagents and solvents were obtained from commercial sources and used without further purification. Preparative chromatography was performed using Sorbent technologies (Norcross, GA, USA) prepacked silica gel columns under medium pressure with ethyl acetate/hexanes (EtOAc/hexanes) or methanol/dichloromethane ($MeOH/CH_2Cl_2$) as eluent. NMR spectra were acquired at ambient temperatures (18 ± 2°C) unless otherwise noted. Reactions were followed by thin-layer chromatography (TLC) on silica gel (60 Å pore size, 5-17 μm) polyester backed sheets that were visualized under a UV lamp, iodine vapor, phosphomolybdic acid, or anisaldehyde. The ^{1}H NMR spectra in $CDCl_3$ were referenced to TMS unless otherwise noted. The ^{13}C {1H} NMR spectra were recorded at 75 or 100 MHz and referenced relative to the ^{13}C {1H} peaks of the solvent. Spectra are reported as (ppm), (multiplicity, coupling constants are given in Hertz (Hz), and number of protons). IR spectra were recorded using Thermo scientific NICOLET iS10 (Waltham, MA, USA). Melting points were determined in open capillary tubes using an Electrothermal Mel-Temp apparatus and are uncorrected. High-resolution mass spectra were obtained using positive electro spray ionization on a Bruker 12 Tesla APEX–Qe FTICR-MS (Billerica, MA, USA) with an Apollo II ion source at the COSMIC Laboratory facility at Old Dominion University, VA.

***N*-(1-hydroxy-2-(hydroxymethyl)-4-(4-octylphenyl)butan-2-yl)acetamide (FTY720-C2):**



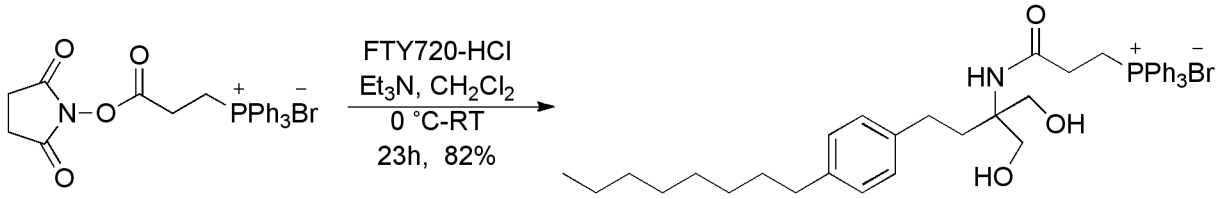
Acetic anhydride (90 µL, 1.2 mmol) was added to fingolimod hydrochloride (FTY720) (0.051 g, 0.300 mmol) in a mixture of chloroform (4.5 mL) and saturated $NaHCO_3$ (2.5 mL) at 0°C. The reaction mixture was allowed to warm to rt and stir for 14 h. The reaction mixture was diluted with chloroform (5 mL), washed with water (5 mL), the organic layer was separated, dried over anhydrous sodium sulfate and volatiles were removed under vacuum. The residue was purified by silica gel column chromatography using MeOH/CH_2Cl_2 (05: 95) to isolate the product (0.092 g, 88%) as a colorless solid. MP: 68-71°C; FT-IR (Neat) 3259, 2924, 1661, 1552, 1436 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.10-7.05 (bs, 4 H), 6.08 (s, 1H), 4.32-4.38 (bs, 2 H), 3.82(d, *J* = 10.55 Hz, 2 H), 3.61 (d, *J* = 10.94 Hz, 2 H), 2.61-2.52(m, 4 H), 1.97-1.90(m, 5 H), 1.52-1.60(m, 2 H), 1.32-1.21 (m, 10 H), 0.87(t, *J* = 6.4 Hz, 3 H); ^{13} C NMR ($CDCl_3$, 75 MHz) δ 171.8, 140.7, 138.5, 128.5, 128.1, 65.6, 61.3, 35.5, 34.4, 31.8, 31.5, 29.4, 29.3, 29.2, 29.1, 23.8, 22.6, 14.05. HRMS (ESI) calcd for $C_{21}H_{35}NO_3Na$ 372.2513 [M + Na], found 372.2507.

3-Triphenylphosphoniumpropanoic acid-N-hydroxysuccinimide ester:



Triphenylphosphine hydrobromide (0.100 g, 0.291 mmol) in CH_2Cl_2 (1 mL) was added to acrylic acid-N-hydroxysuccinimide ester (0.049 g, 0.291 mmol) in CH_2Cl_2 (1 mL) at 0°C and allowed to stir at rt for 3hr. The reaction mixture was concentrated under vacuum to provide the product (0.149 g, 100 %) as a colorless solid that was used in the next step without further purification. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.87-7.69 (m, 15 H), 4.38-4.30 (m, 2H), 3.44 (t, *J* = 6.60 Hz, 1H), 3.38 (t, *J* = 6.60 Hz, 1H), 2.77 (s, 4H); ^{13} C NMR ($CDCl_3$, 75 MHz) δ 168.4, 166.5 (d, $^3J_{CP}$ = 6 Hz), 135.2 (d, $^4J_{CP}$ = 3 Hz), 133.5 (d, $^3J_{CP}$ = 10 Hz), 130.5 (d, $^2J_{CP}$ = 13 Hz), 117.0 (d, $^1J_{CP}$ = 86 Hz), 25.4, 24.55 (d, $^2J_{CP}$ = 4 Hz), 18.0 (d, $^1J_{CP}$ = 54 Hz).

***N*-(1-hydroxy-2-(hydroxymethyl)-4-(4-octylphenyl)butan-2-yl)-3'-triphenylphosphoniumpropanamide (FTY720-Mitoxy):**



To a mixture of fingolimod hydrochloride (FTY720) (0.240 g, 0.700 mmol) and triethylamine (0.14 g, 1.4 mmol) in CH_2Cl_2 (5 mL) was added 3-triphenylphosphoniumpropanoic acid-N-hydroxysuccinimide ester (0.430 g, 0.840 mmol) in CH_2Cl_2 (5 mL) at 0°C and allowed to stir at room temperature for 23hr. The reaction mixture was diluted with CH_2Cl_2 (10mL) washed with water (15mL), the organic layer was separated, dried over anhydrous sodium sulfate and

volatiles were removed under vacuum. The residue was purified by silica gel column chromatography using MeOH/CH_2Cl_2 (05: 95) to isolate the product (0.403 g, 82%) as a white solid. MP: 78-80°C; FT-IR (Neat) 3278, 2921, 1622, 1558, 1461 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.48 (s, 1H), 7.83-7.69 (m, 15 H), 7.07 (d, *J* = 7.81 Hz, 2 H), 7.02 (d, *J* = 7.81 Hz, 2 H), 4.70-4.64 (m, 2 H), 3.88-3.80(m, 2 H), 3.69-3.59 (m, 4 H), 3.03-2.94 (m, 2 H), 2.55-2.48 (m, 4 H), 1.98-1.90 (m, 2 H), 1.59-1.51 (m, 2 H), 1.33-1.20 (m, 10 H), 0.87 (t, *J* = 7.03, 6.6 Hz, 3H); ^{13} C NMR ($CDCl_3$, 75 MHz) δ 170.1 (d, $^3J_{CP}$ = 14.5 Hz), 140.0, 139.4, 135.4 (d, $^4J_{CP}$ = 3 Hz), 133.5 (d, $^3J_{CP}$ = 10 Hz), 130.6 (d, $^2J_{CP}$ = 13 Hz), 128.2, 128.1, 117.4 (d, $^1J_{CP}$ = 86 Hz), 66.4, 62.4, 35.4, 33.2, 31.8, 31.5, 29.4, 29.3, 29.27, 29.2 (d, $^2J_{CP}$ = 4 Hz), 29.17, 22.6, 20.1 (d, $^1J_{CP}$ = 54 Hz), 14.0. HRMS (ESI) calcd for $[C_{40}H_{51}NO_3P]^+$ 624.3606, found 624.3598.

Biology

Recombinant-PP2A Enzymatic Activity Assay

Protein phosphatase 2A catalytic subunit (PP2Ac, Cayman Chemical, Ann Arbor, MI, USA) was incubated for 30 min at 4°C in 4-Nitrophenyl-phosphate, pNPP buffer (50 mM Tris-HCl, pH 7.0, 0.1 mM $CaCl_2$) with or without added a-Syn (0.1- 5 µM; recombinant a-Syn a gift from Dr. J. Rosenberg, University of Pittsburgh), ceramides (**C2**, 2.5 - 10 µM; **C6** , 10 µM; or **C10**, 10 µM; Avanti Polar Lipids, Inc., Alabaster, AL, USA); or 5 µM of FTY- compounds (FTY720; LC Laboratories, Woburn, MA, USA); FTY720-C2, FTY720-Mitoxy (synthesized as described above). Reactions proceeded for 10 min at 30^{o}C with intermittent shaking in the presence of the threonine-phosphopeptide (K-R-pT-I-R-R, New England Peptide LLC, Gardner, MA, USA). Reactions were stopped by placing samples on ice, and aliquots were evaluated by Malachite Green assay with phosphate levels determined relative to a phosphate standard curve read at 630 nm using a Multiskan Spectrum plate reader (Thermo Scientific, Pittsburgh, PA, USA). PP2A activity was expressed as pmol phosphate/min/µg protein.

Cell culture

Low-passage number of rat adrenal gland pheochromocytoma cells (PC12) were grown in DMEM supplemented with 5% horse serum, 5% Bovine Calf Serum and 2 mM glutamine at 37^{o}C in a CO_2 environment as previously described (1-3). The MN9D dopaminergic cell line is a fusion of rostral mesencephalic neurons from embryonic day 14 midbrain C57BL/6J mice with N18TG2 neuroblastoma cells (5). MN9D cells at low-passage number were grown on TPP® plates (LPS, Inc., Rochester, NY, USA) in Dulbecco's modified Eagle's medium (DMEM; D5648; Sigma-Aldrich Co, St Louis, MO, USA) supplemented with 10% of fetal bovine serum (HyClone, Logan, UT, USA) at 37^{o}C in a 5% CO_2 environment (1-3, 6).

Immunoprecipitation and activity of PP2A from cells

Sub-confluent PC12 or MN9D cells were treated with 5 µM FYT720 for 0 – 120 min. PP2A immunoprecipitacion and activity were determined using a non-radioactive kit according to the manufacturer's instructions (catalog # 17-127, EMD Millipore Corporation, Billerica, MA, USA). PC12 cells were homogenized in ice-cold IP buffer containing 20 mM Imidazole-HCl, 2 mM EGTA, and 2 mM EDTA, pH 7.0 with 17 µg/ml aprotinin, 1 mM benzamidine and 1 mM AEBSF (Sigma-Aldrich, San Louis, MO, USA) as described before (1). Protein concentration was determined by the bicinchoninic acid assay (BCA assay) (4) and 0.5-1.0 mg of sample was immunoprecipitated using PP2Ac antibody 1D6 (05-421, EMD Millipore Corporation, Billerica, MA, USA) and the complex (antibody plus PP2Ac) was pull-down with A-Agarose beads (Pierce Biotechnology, Rockford, IL, USA). The enzymatic activity of the immunoprecipitated PP2Ac was determined spectrophotometrically using the Malachite Green assay as previously described.

MTS cell viability assay

A metabolic assay, CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) was used to measure the effect of FYT720, FTY720-C2 or FTY720-Mitoxy from 0.04 to 0.16 µM for 72 hr on the MN9D. Ethanol was used as the solvent for all compounds, thus equal volume of ethanol was used for vehicle controls. Briefly, 20 µl of MTS reagent was added to MN9D cells followed by incubation at 37^{o}C in a CO_2 incubator for 30 min. The reduction of MTS to formazan was determined by reading absorbance at 490 nm with the Multiskan Spectrum plate reader. In addition, 150,000 cells were seeded in a 6-well plate and treated with FTY-compounds for 72 hr, following cells in suspension were stained with Trypan blue and counted using an Automated Cell Counter T10 (Bio-Rad, Hercules, CA, USA).

RNA isolation and qPCR analysis

MN9D cells (0.6 x 10^6 cells) grown as described before but with no antibiotics and were treated with 0.16 µM FTY720, FTY720-C2 or FTY720-mitoxy. After 24 hr treatment, total RNA was extracted using the RNeasy Mini Kit (Qiagen, Redwood City, CA, USA) following the manufacturer's instructions. The mRNA was used to synthesize cDNA using Superscript III First-Strand Synthesis Supermix (Invitrogen, Carslbad, CA, USA) and followed the manufacturer's instructions. Gene expression was analyzed using quantitative real-time PCR (qPCR) TaqMan® Gene Expression Assay kit (Invitrogen, Carlsbad, CA, USA) and the following conditions; 50°C – 2 min, 95°C – 10 min, 95°C – 15 sec & 60°C – 1 min step3 and step 4 repeat for 40 cycles. All steps for cDNA synthesis and Taqman assays were performed using Realplex2 (Eppendorf, NY, USA) thermocycler system. GAPDH expression served as an internal control. Relative expression between FTY-treated and vehicle treated controls was calculated using the comparative Ct method ($2^{-\Delta\Delta Ct}$).

Tumor necrosis factor-α treatment

MN9D (10,000 cells) were seeded in 96-well plates and differentiated for 72 hrs in 200 µl of DMEM media containing 5 mM valproic acid (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and N2 supplement (Life Technologies, Carlsbad, CA, USA) as described (7). After 24 hr of differentiation, a first dose of 0.16 µM FTY720, FTY720-C2 or FTY720-Mitoxy was added to cells. At 48 hr a second dose of FTY-compounds was added to cells. At 64 hr we added 1 ng tumor necrosis factor-α (TNF-α R&D Systems, Inc., Minneapolis, MN, USA) to cells with or without pretreatment with FTY-compounds. Cell viability was assessed by cell counts as described (8). Nuclear morphology of treated MN9D cells was analyzed using a fluorescent stain Hoechst 33342 (10 µg/ml in PBS; Molecular Probes, Inc., Eugene, OR, USA). First, cells were washed with 1X PBS then stained with Hoechst dye (1:1000) for 5 min. For analyzing plasma membrane integrity, cells were also stained with 0.4% Trypan blue (Life Technologies, Carlsbad, CA, USA) for 5 min. Stained cells were fixed with 4% formaldehyde and stored at 4^{o}C in PBS + 0.02% NaN_3 (sodium azide) and analyzed by bright-field and fluorescence microscopy using an inverted microscope (Evos FL, Advanced Microscopy, Life Technologies). Cells were counted randomly selected fields of cells at 20X of magnification. Hoechst stained cells were evaluated under UV light and scored as viable if nuclei exhibited a rounded morphology without fragmentation or condensation. On the other hand, dead cells were scored if nuclei were fragmented or pyknotic. The Trypan blue exclusion assay analyzed cells as viable if dye was excluded or dead if cells were stained blue. Finally, the ratio of live cells/total cells were calculated and presented as % of Control.

Statistical analysis

T-tests or one way ANOVA were performed using Instat (Graphpad Software Inc., San Diego, CA, USA) or Sigma Plot 11 (Systat Software Inc., San Jose, CA, USA) software. Post hoc analyses were performed by

the Tukey-Kramer method for data significant at $P < 0.05$ or better. Data represent the mean ± SEM for all treatments.

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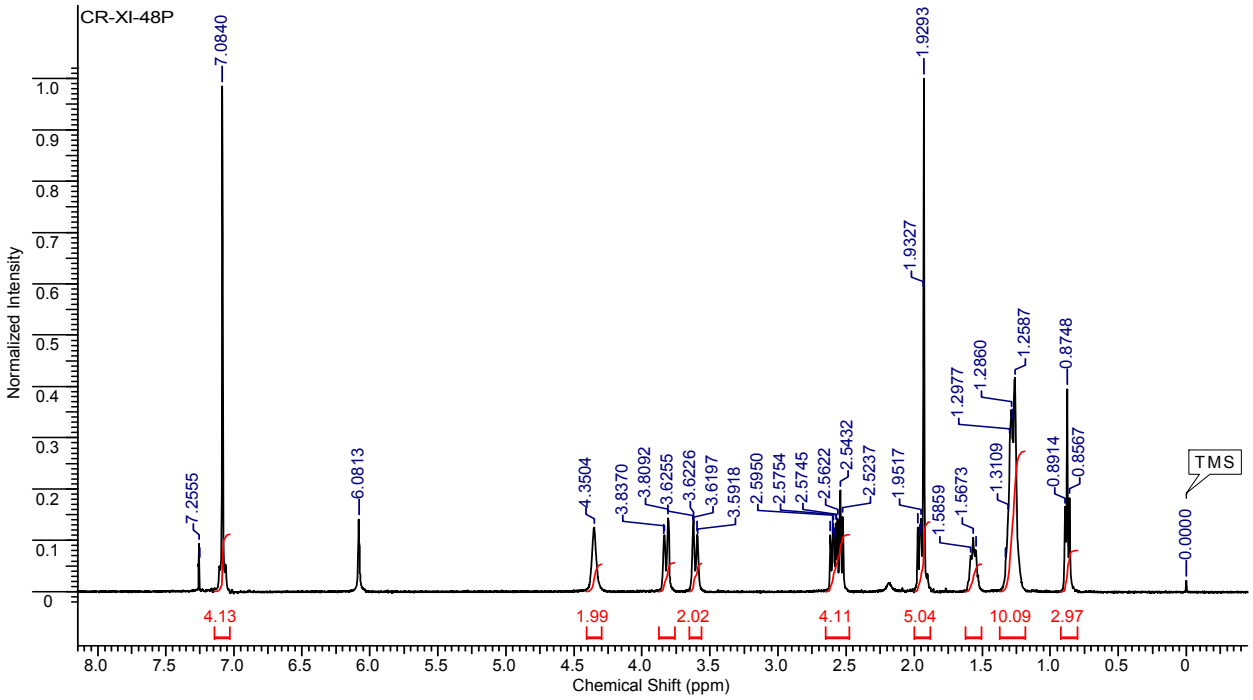
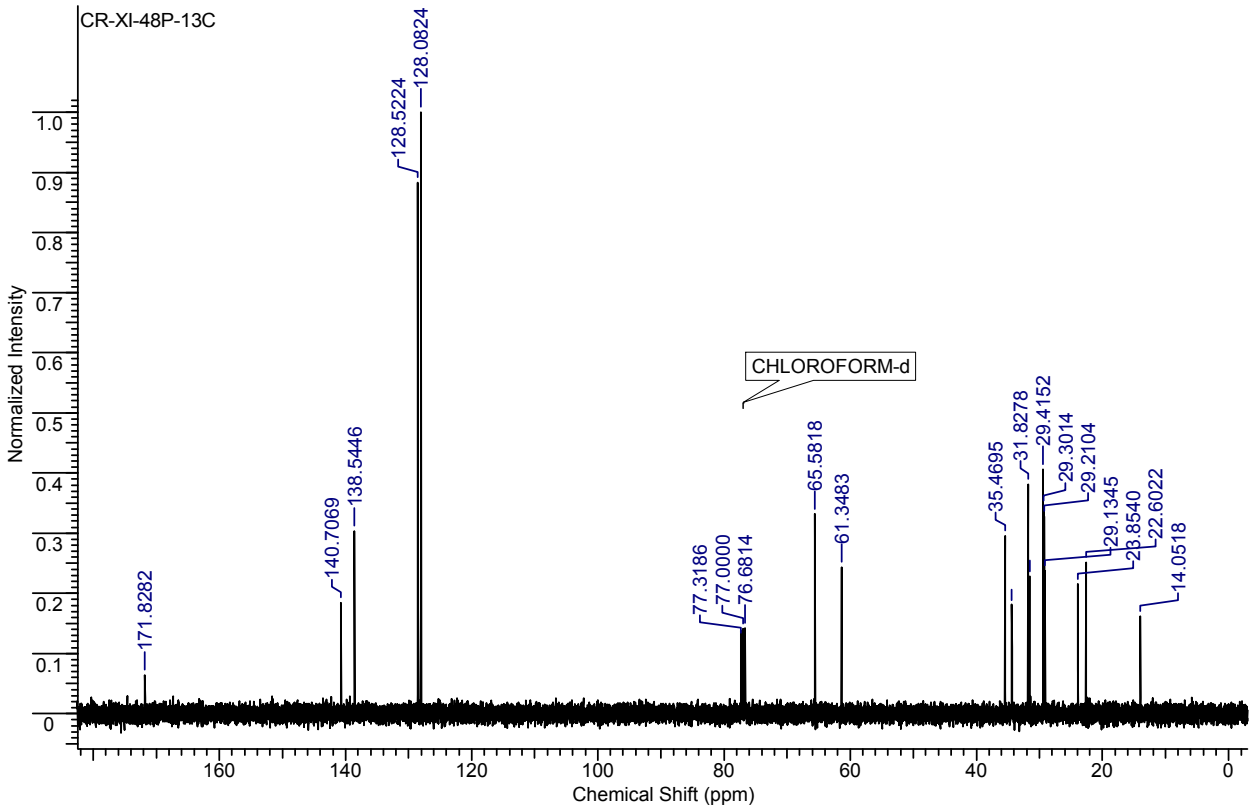
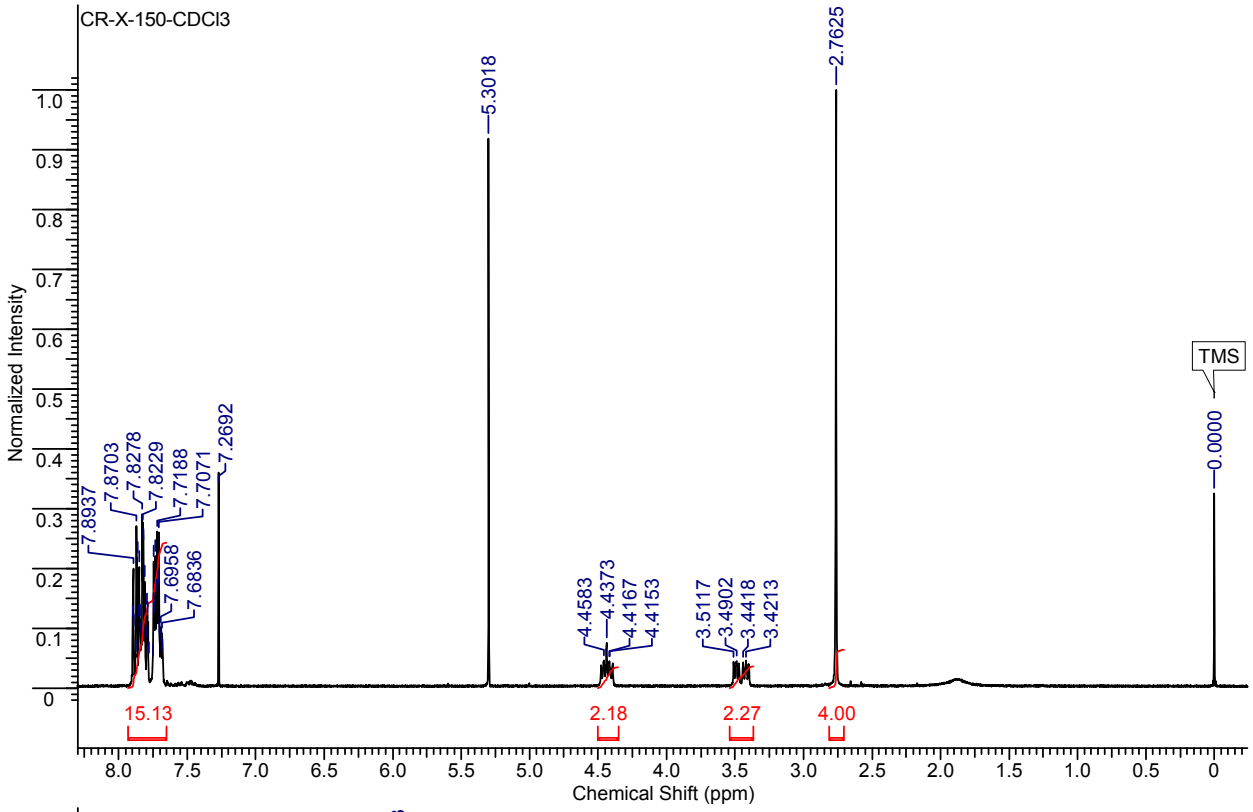
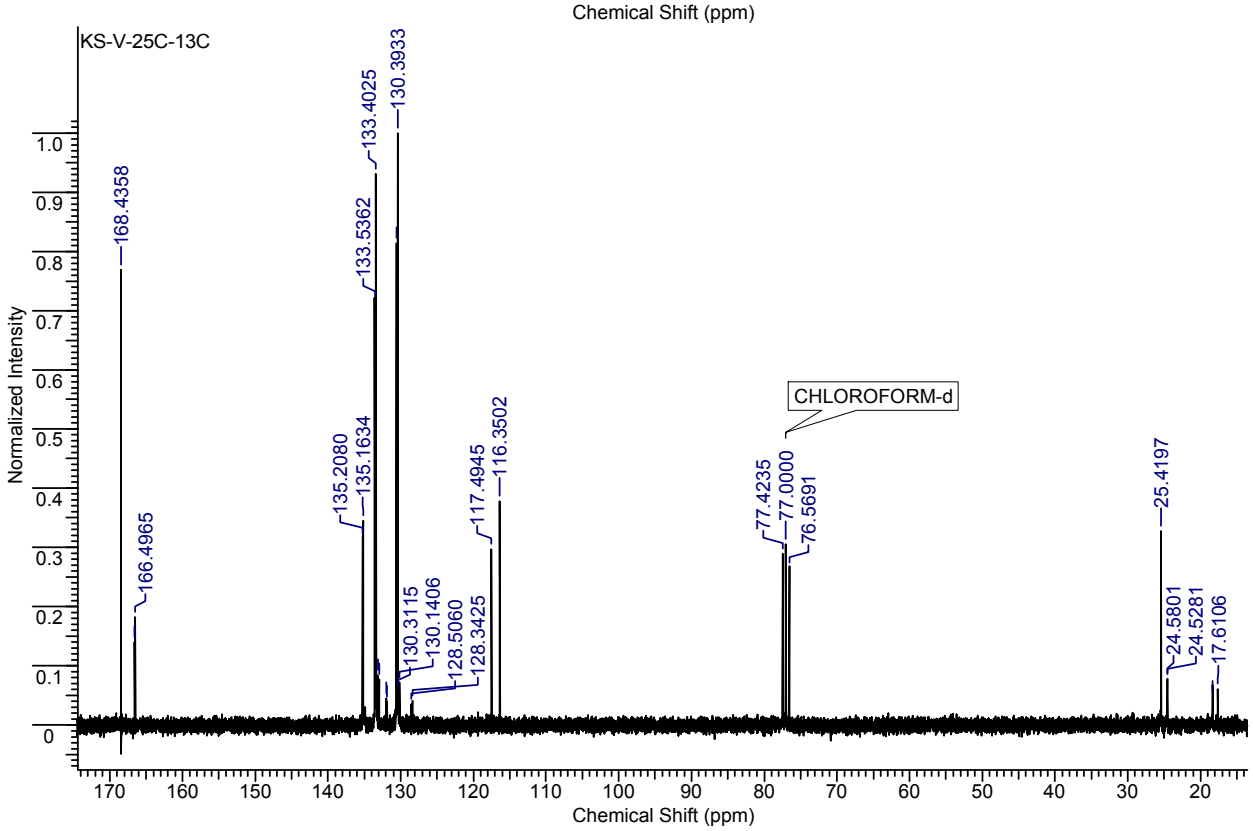
^{1}H NMR and ^{13}C NMR spectrum of FTY720-C2





^{1}H NMR and ^{13}C NMR spectrum of 3-Triphenylphosphoniumpropionic acid-N-hydroxysuccinimide ester bromide





^{1}H NMR and ^{13}C NMR spectrum of FTY720-Mitoxy