Title: Synthesis and Characterization of D1 Dopamine Receptor Positive Allosteric Modulators

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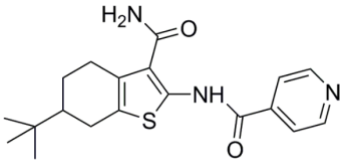
Many pharmacologic agents used to treat CNS disorders, such as schizophrenia, Parkinson's disease and Alzheimer's disease, target the dopaminergic system. Recent evidence suggests an important role for the D1 dopamine receptor (D1R), with signaling enhancement being shown to improve cognitive function. While the clinical adoption of traditional D1R orthosteric agonists has been stymied by acute tolerance and adverse effects, D1R positive allosteric modulators (PAMs) present an innovative approach to amplify D1R signaling without the clinical pitfalls of other D1R agonists. This project aims to synthesize thiophene-based D1R PAMs targeting a novel D1R allosteric site, screen for receptor selectivity, and optimize potentiation.

Abstract
The absence of truly efficacious pharmacologic treatments for many central nervous system disorders, including Alzheimer's disease, schizophrenia, and Parkinson's disease, is an important unmet clinical need. Treatment of the cognitive decline and working memory deficits in these conditions, which is associated with dopamine deficits, would drastically improve patient outcomes and greatly reduce the associated economic and healthcare burden. Clinical and preclinical studies have shown a direct association between D1 dopamine receptor (D1R) signaling deficiencies in the dorsolateral prefrontal cortex (dlPFC) and the working memory defects and cognitive decline in schizophrenia. Therefore, agents targeting the D1R offer an innovative approach to address the cognitive decline and working memory deficits in psychiatric disorders and the normal aging process. While full D1R agonists have shown efficacy in improving working memory, they are limited by a narrow therapeutic window, the development of acute tolerance, and constraining side effects like hypotension and tachycardia.[1,2] On the other hand, D1R positive allosteric modulators (PAMs) present an exciting, new pharmacologic approach by selectively potentiating the D1R response to endogenous DA, thus offering a wider therapeutic window.

We synthesized new D1R PAM analogues based on the promising high-throughput screening (HTS) hit compound, MLS 6585. Synthesized analogues were screened for D1R potentiation, as well as potentiation of the closely related D5 receptor. The Gewald reaction afforded ready access to the core skeleton shared by all analogues, which was then further derivatized to produce the analogue set in this project.

All D1R PAM analogues that were synthesized and screened were found to produce diminished D1R PAM activity compared to the HTS hit MLS 6585, however select analogues did produce D1R silent allosteric modulation, disrupting the PAM activity of MLS 6585 The analogue set generated in this project contributes to the structure–activity relationship studies, reflecting the importance of the amide on the thiophene-based hit compound. This project provides critical guidance to future D1 PAM drug discovery, which will explore substitutions on the cyclohexane and pyridine rings, as well as at the tert-butyl position.

Introduction
The absence of truly efficacious pharmacologic treatment for many central nervous system disorders, including Alzheimer's disease, schizophrenia, and Parkinson's disease, is an important unmet clinical need.



MLS 6585

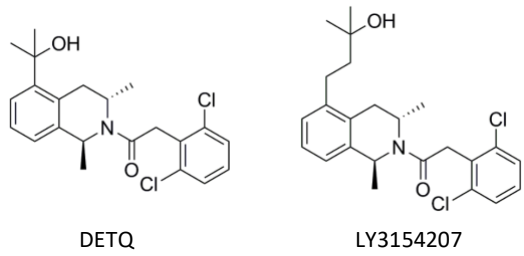
Figure 1. Structure of D1 PAM thiophene hit compound MLS6585. In saturating concentrations (50 nM), MLS6585 increases cAMP production by 8.3 fold.[5]

Current antipsychotics primarily act via D2 dopamine receptor antagonism, implicating a dopamine excess in schizophrenic pathology. However, studies in recent years have also implicated a dopamine deficiency in the pathology of schizophrenic negative symptoms, working memory deficits, and cognitive decline.[3–5] Similarly, the cognitive decline and working memory deficits prevalent in patients living with Parkinson's and Alzheimer's disease is unaddressed by current pharmacologic therapies, leading to poor long-term outcomes with significant economic and healthcare burdens.

Both D1 and D2 receptors are G protein-coupled receptors, with D1 dopamine receptor (D1R) activation coupled to $G_{s\alpha,}$ which activates adenylyl cyclase and increases intracellular cAMP, and D2 coupled to $G_{i\alpha}$, which inhibits adenylyl cyclase and decreases intracellular cAMP.[6] Reduced D1R stimulation, particularly in the prefrontal cortex, plays a major role in the cognitive decline and disruptions of working memory in schizophrenia, as well as other psychiatric conditions.[4,5] The first logical approach to explore this untapped pharmacologic potential was to develop a potent D1 agonist acting at the D1R orthosteric binding site,

where endogenous dopamine binds. The advent of dihydrexidine, the first full D1R agonist, helped reveal both the potential and the limitations of the D1R activation. It was postulated to have a range of clinical utility in psychiatric disorders, but was limited by a poor pharmacokinetic profile (bell-shaped dose-response curve, low oral bioavailability, tachyphylaxis) and dose-limiting adverse effects (hypotension, tachycardia, stereotypy).[1,2,7,8.] In spite of showing early promise in schizophrenia treatment by ameliorating working memory deficits, dihydrexidine's pharmacologic characteristics and narrow therapeutic window have limited further human clinical trials.[9]

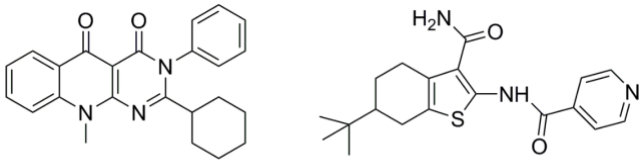
With the orthosteric site of the D1R limited as a pharmacologic target, the allosteric binding sites that enhance dopamine signaling emerged as a rational approach to deftly sidestep the pitfalls of D1R orthosteric agonism, and attain D1R clinical utility. For D1R applications, positive allosteric modulators (PAMs) offer several key pharmacologic benefits including less D1R internalization and desensitization, greater selectivity by binding to less conserved regions of the D1R, and steadier signaling augmentation by relying on endogenous DA, rather than directly stimulating the receptor and over-activating dopaminergic tone, as is seen by the inverted U-shaped dose-response curve with full D1R agonism.[10]



DETQ LY3154207

Figure 2. Structures of Eli Lilly D1 PAM lead compounds DETQ and LY3154207

In recent years, Eli Lilly has produced numerous structurally-similar D1R PAMs. Of particular interest, are DETQ (2-(2,6-dichlorophenyl)-1-((1*S*,3*R*)-3-(hydroxymethyl)-5-(2-hydroxypropan-2-yl)-1-methyl-3,4-dihydroisoquinolin-2(1H)-yl)ethan-1-one) and LY3154207 (2-(2,6-dichlorophenyl)-1-((1*S*,3*S*)-5-(3-hydroxy-3-methylbutyl)-1,3-dimethyl-3,4-dihydroisoquinolin-2(1H)-yl)ethan-1-one) (Figure 2).[11,12] These compounds show promising potential as PAMs for human D1R receptors, with EC_{50} values (in the presence of 12 nM dopamine) of 11.7 nM and 3.7 nM for DETQ and LY3154207, respectively. Both compounds possess useful potentiation, increasing response to dopamine by 21-fold. However, they elicit a maximum agonist response of 12% in the absence of dopamine — ideally, a D1R PAM would possess no agonist activity without dopamine.[7,11] Likewise, the side effect profile of DETQ appears relatively benign, without stereotypies, hypotension, or tachycardia, however D2 receptor potentiation is a possible concern, and has been observed for DETQ. Furthermore, findings in preclinical studies using murine models (with knock-in human D1R) have been auspicious. Novel object recognition memory, which is closely associated with cognitive impairment, was shown to be improved with administration of DETQ after inducing deficits with PCP.[7,11]



MLS 1082 MLS 6585

Figure 3. Structures of D1 PAM lead compounds

The allosteric site on the D1R that DETQ and LY3154207 bind has been established and is relatively well-investigated. Using mutagenesis studies, the binding site was identified as a pocket in the D1R formed by the 2nd intracellular loop (ICL-2) and transmembrane helices 3 and 4, with Arg-130, Lys-134, and Trp-123 as the crucial amino acid interactions for DETQ and LY3154207 binding.[11,12] In order to identify PAMs that bind other allosteric sites reside on the D1R, our team screened the D1R potentiation of DETQ and Bristol-Myers Squibb's Compound B (rel-(9*R*,10*R*,12*S*)-*N*-(2,6-dichloro-

3-methylphenyl)-12-methyl-9,10-dihydro-9,10-ethanoanthracene-12-carboxamide) in combination with two chemically distinctive lead compounds: MLS 1082 and MLS 6585 (Figure 3).[10] Three important observations were made: (1) making a single point mutation in Arg130 on the D1R renders MLS 1082, DETQ, and Compound B ineffective, while not affecting the activity of MLS 6585, (2) co-administration of MLS6585 has additive activity with DETQ or Compound B, but MLS 1082 does not, and (3) simultaneous use of MLS 1082 and MLS 6585 produces additive dopaminergic potentiation. Taken together, these findings indicate that MLS 1082 shares a common binding site with Compound B, DETQ, and, by extension, LY3154207, which is distinct from that of MLS 6585. Thus, the thiophene-based core of lead compound MLS 6585 was used as the initial chemical framework for analogues synthesized in this project.

This project investigated this unique allosteric binding site on the D1R through the synthesis and screening of novel, thiophene-based D1R PAM small molecule probes. To help guide our syntheses, we utilized the HTS hit compound MLS6585, as well as existing preliminary structure–activity relationship studies. Results from analogue screening (Sibley Lab, NINDS) will inform our own optimization efforts, as well as guide future drug discovery of D1R PAMs.[13]

If LY3154207 is not successful in clinical trials, as a result of poor selectivity, off-target effects, or an otherwise unsuitable clinical profile, and the allosteric site it binds is determined to no longer be a viable pharmacologic target, this second, distinct allosteric site will generate a contingency plan for D1R PAM drug development, offering potentially superior potency and a more favorable adverse effect profile due to selectivity. A further rationale for the development of MLS 6585 analogues is the observed synergistic activity when PAMs bind at both allosteric sites. Such a combination therapy approach could be utilized to drastically lower the required dose of each drug, a key consideration for the treatment of chronic conditions.

Clinical and preclinical studies have shown a direct association between D1R signaling deficiencies in the dorsolateral prefrontal cortex (dlPFC) and the working memory deficits and cognitive decline in schizophrenia. Therefore, agents targeting the D1R offer an innovative approach to address the cognitive decline in schizophrenia, as well as in other psychiatric disorders. While full D1R agonists have shown efficacy in improving working memory, they are limited by a narrow therapeutic window, the development of acute tolerance, and constraining side effects like hypotension and tachycardia.[1,2] On the other hand, D1R PAMs present an exciting, new pharmacologic approach by selectively potentiating the response at the D1R to endogenous dopamine, thus offering a wider therapeutic window.

We synthesized a set of six D1R PAM analogues based high-throughput screening (HTS) hit compound (MLS 6585, Figure 1). Synthesized analogues were screened for D1R potentiation, as well as potentiation of the closely-related D5 dopamine receptor (Sibley Lab, NINDS). MLS 6585, a thiophene-based compound, targets a novel allosteric site on the D1R, distinct from that of LY3154207, a D1R PAM in phase 2 clinical trials.[14] While LY3154207 shows promise for D1R pharmacology, it lacks selectivity for the D1 receptor, and, unlike our analogue, potentiates the D2 dopamine receptor, which may impact its long-term clinical and safety profiles. However, by targeting a distinct allosteric site on the D1R, our analogue may stabilize a distinct conformation of the D1R, resulting in a distinct mechanism of D1R potentiation and clinical profile.

Our efforts were guided by the screening results from our initial analogue sets. Modern organic synthesis, purification and spectroscopy techniques (e.g., flash chromatography, IR, HRMS, ^{1}H and ^{13}C NMR) were utilized for the synthesis, purification, and characterization of all analogues. By exploring modifications

that enhance or diminish activity, we gleaned vital information about the relationship between chemical structure and dopaminergic activity that will guide future D1R PAM drug discovery.

Methods

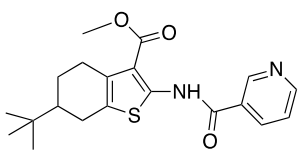
From March 2019 through March 2020, modern medicinal chemistry techniques were utilized for the synthesis of six D1R PAMs (Figure 5). Synthetic techniques including recrystallization, lyophilization, rotary evaporation, microwave reaction conditions, flash chromatography, and thin-layer silica chromatography were used for the synthesis and purification of analogues. Compound characterization, including IR, HRMS, ^{1}H and ^{13}C NMR, was performed for all new, unreported compounds.

Once synthesized and purified, D1R PAM analogues were shipped to our partners at the Sibley Lab and were screened for receptor selectivity and potentiation. D1R potentiation was measured in two complementary assays for D1R activation: (1) a cAMP accumulation assay (to measure G protein-dependent activation) using human embryonic kidney (HEK)-293 cells transfected with human D1 (hD1) receptors, and (2) the DiscoverX PathHunter assay (to measure β-arrestin recruitment), which uses Chinese hamster ovarian (CHO)-K1 cells expressing a combination of modified D1R and β-arrestin components to produce a luminescent signal. A high concentration (50 μM) of the D1 PAM analogue, followed by a range of dopamine concentrations will be added to the cells in individual wells, and cAMP activity will be measured as the percentage of the maximum possible dopamine-mediated response. Radioligand binding assays will be conducted in HEK-293 cells with overexpressed hD1R receptors by incubating varying concentrations of the D1R PAM analogue with and without dopamine to verify the allosteric nature of the analogues. DA receptor selectivity for each of the five dopamine receptor subtypes of the analogues will be evaluated using the analogous DiscoverX PathHunter assays.[10] Subsequently, compounds with initial promising PAM activity will be tested for *in vitro* pharmacokinetic properties, such as solubility, permeability, and stability.

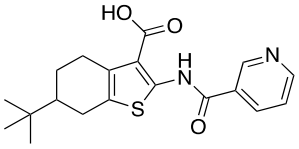
Experimentals for the synthesis of analogues is as follows (see Supplemental Information for NMR Spectra)**:**

General experimental:

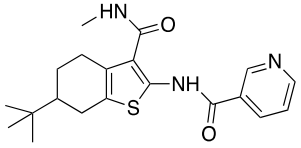
All reagents were used as received from commercial suppliers and used without further purification. The ^{1}H and ^{13}C spectra were recorded on a 400 MHz Varian spectrometer equipped with a broadband observe probe and a 500 MHz Bruker AVIII spectrometer equipped with a dual cryoprobe, respectively. Chemical shifts are reported in parts per million and were referenced to residual proton solvent signals. ^{13}C multiplicities were determined with the aid of an APT pulse sequence, differentiating the signals for methyl (CH_3) and methyne (CH) carbons as “d” (down) from methylene (CH_2) and quarternary (C) carbons as “u” (up). Microwave syntheses were conducted in a Biotage Initiator constant temperature microwave synthesizer. Flash column chromatography separations were performed using the Teledyne Isco CombiFlash Rf using RediSep Rf silica gel columns. TLC was performed on Analtech UNIPLATE silica gel GHLF plates (gypsum inorganic hard layer with fluorescence indicator).



Methyl 6-(*tert*-butyl)-2-(nicotinamido)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate [FK 1-008; UNC 7103A]. To a solution of methyl 2-amino-6-(tert-butyl)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate (0.226 g, 0.845 mmol, 1.0 equiv) in 3.5 ml pyridine was added 3-(chlorocarbonyl)pyridin-1-ium chloride (327 mg, 1.84 mmol, 2.2 equiv). The reaction was stirred at rt for 21 h and the solvent was removed under nitrogen. Flash chromatography (hexanes/ethyl acetate) afforded UNC 7103A as a sandy yellow solid (289.5 mg), which was recrystallized from EtOH, to afford an off-white fluffy powder (227 mg, 60.9 mmol, 72.1% yield). R_f = 0.63 (1:8 ethyl acetates:hexanes); ^{1}H NMR (401 MHz. $CDCl_3$) δ 0.96 (s, 9H), 1.25–1.38 (m, 1H), 1.45–1.55 (m, 1H), 2.00–2.07 (m, 1H), 2.39–2.49 (m, 1H), 2.54–2.66 (m, 1H), 2.74 (dd, *J* = 8.0, 16.0 Hz, 1H), 3.05 (dd, *J* = 8.0, 16.0 Hz, 1H), 3.92 (s, 3H), 7.26 (s, 1H), 7.43–7.49 (m, 1H), 8.26–8.31 (m, 1H), 8.79–8.83 (m, 1H), 9.27 (s, 1H); ^{13}C NMR (100 MHz, APT pulse sequence, $CDCl_3$) δ d (CH, CH_3): 27.3, 45.0, 51.7, 123.6, 135.0, 148.9, 153.0; u (C, CH_2): 24.3, 25.9, 27.4, 32.4, 112.0, 128.31, 128.34, 131.1, 147.6, 161.6, 167.5. HRMS calcd. for $C_{20}H_{25}N_2O_3S$ $[M+H]^+$ 372.1508, found 373.1581; HPLC purity >99%.

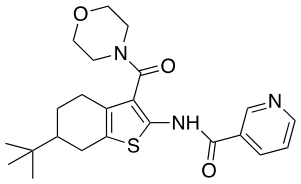


6-(*tert*-Butyl)-2-(nicotinamido)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylic acid (FK 1-012; UNC 7128). To a solution of ethyl 6-(tert-butyl)-2-(nicotinamido)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate (320 mg, 1 Eq, 0.828 mmol, 1 equiv), 6ml THF, 6 ml methanol, and 3 ml of distilled H20 was added lithium hydroxide (122 mg, 5.09 mmol, 6.15 equiv). The reaction was stirred at 50 C for 18 h and flash chromatography (MeOH, H2O) afforded the carboxylic acid-thiophene product (UNC 7128) as a yellow granulated powder (104.6 mg, 0.29 mmol, 35.2% yield). R_f = 0.19 (5% MeOH in DCM); ^{1}H NMR (401 MHz. DMSO-*d*6) δ 0.90 (s, 9H), 1.10–1.23 (m, 1H), 1.36–1.45 (m, 1H), 1.88–1.95 (m, 1H), 2.28–2.51 (m, 6H), 2.55–2.64 (m, 1H), 7.48-7.54 (m, 1H), 8.17-8.23 (m, 1H), 8.64-8.69 (m, 1H), 9.06 (s, 1H); ^{13}C NMR (101 MHz, APT pulse sequence, DMSO-*d*6) δ d (CH, CH_3): 24.3, 26.0, 27.9, 32.4, 59.4, 105.3, 118.2, 132.4, 161.9, 166.5; u (C, CH_2): 14.5, 27.3, 45.2, 50.6. HRMS calc. for $C_{19}H_{24}N_2O_3S$ $[M+H]^+$ 359.1351, found 359.1423; HPLC purity = 96.4%.



N-(6-(*tert*-Butyl)-3-(methylcarbamoyl)-4,5,6,7-tetrahydrobenzo[b]thiophen-2-yl)nicotinamide (FK 1-010; UNC 7129). To a solution of 6-(tert-butyl)-2-(nicotinamido)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylic acid (34.3 mg, 0.096 mmol, 1 equiv) and 2ml DMF was added 2-(3H-[1,2,3]triazolo[4,5-b]pyridin-3-yl)-1,1,3,3-tetramethylisouronium hexafluorophosphate(V) (52.8 mg, 139 µmol, 1.45 equiv) and the reaction was stirred for 10min. Then N-ethyl-N-isopropylpropan-2-amine (26 mg, 35 µL, 0.20 mmol, 2.1 equiv) and methylamine (5.94 mg, 0.1 ml, 0.191 mmol, 2 equiv) were added. The reaction was stirred at rt for 27 h and flash chromatography (H_2O/MeOH) afforded UNC 7129 (12.1 mg, 0.033 mmol, 46.3% yield) as an off-white/yellow solid. ^{1}H NMR (401 MHz. $CDCl_3$) δ 0.95 (s, 9H), 1.28-1.63 (m, 2H), 2.11 (s, 1H), 2.56-3.16 (m, 7H), 6.04 (s, 1H), 7.44 (s, 1H), 8.27 (s, 1H), 8.77 (s, 1H), 9.29 (s, 1H), 13.42 (s, 1H). ^{13}C

NMR (100 MHz, APT pulse sequence, $CDCl_3$) δ d (CH, CH_3): 24.5, 26.0, 27.8, 32.4, 43.7, 114.3, 127.2, 129.2, 145.2, 167.4; u (C, CH_2): 12.6, 17.1, 18.5, 27.2, 44.7, 55.8, 134.8, 149.1, 152.8. R_f = 0.44 (5% MeOH in DCM); HPLC purity >99%.



N-(6-(*tert*-Butyl)-3-(morpholine-4-carbonyl)-4,5,6,7-tetrahydrobenzo[b]thiophen-2-yl)nicotinamide [FK 1-011; UNC 7102A]. To a solution of 6-(tert-butyl)-2-(nicotinamido)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylic acid (27.8 mg, 0.078 mmol, 1 equiv) and 2ml DMF was added 2-(3H-[1,2,3]triazolo[4,5-b]pyridin-3-yl)-1,1,3,3-tetramethylisouronium hexafluorophosphate (V) (59.9 mg, 2.03 equiv, 0.158 mmol, 2.03 equiv.) and the reaction was stirred for 10min. Then morpholine (19 mg, 19 µL, 0.22 mmol, 2.8 equiv) and N-ethyl-N-isopropylpropan-2-amine (31.5 mg, 42.5 µL, 0.244 mmol, 3.14 equiv) were added. The reaction was stirred at rt for 22 h and flash chromatography (DCM/MeOH) afforded the morpholine product (UNC 7102A) as an off-white to yellow powder (22.6mg, 0.05 mmol, 63.8% yield). ^{1}H NMR (401 MHz. $CDCl_3$) δ 0.96 (s, 9H), 1.25 (s, 2H), 1.56 (s, 3H), 1.95-2.07 (m, 1H), 2.32-2.83 (m, 3H), 3.42-3.91 (m, 6H), 7.41-7.47 (m, 1H), 8.14-8.24 (m, 1H), 8.75-8.82 (m, 1H), 9.17 (s, 1H), 10.41 (s, 1H); ^{13}C NMR (100 MHz, APT pulse sequence, $CDCl_3$) δ d (CH, CH_3) 27.3, 45.5, 123.6, 135.0, 148.6, 152.9; u (C, CH_2) 24.4, 25.9, 26.3, 29.7, 32.6, 77.3, 118.2, 129.0, 130.3; R_f = 0.61 (10% MeOH in DCM); HPLC purity = 93.6%.

Results

The Gewald reaction, which produces the 3-ester-2-aminothiophene intermediate in a single step from a ketone and cyanoacetate in the presence of elemental sulfur, readily provides the functional chemical backbone necessary for the entire analogue set.[15] This crucially straightforward and rapid reaction, as shown in the first step in Figure 4, permits the efficient exploration of chemical modifications in a reasonable timeframe, and only required minimal formal chemical synthesis training. The amine group of the methyl ester intermediate is then acylated with aryl acid chloride to provide the aryl- or heteroaryl amides. Next, an ester hydrolysis using lithium hydroxide yields a highly versatile carboxylic acid thiophene intermediate. This carboxylic acid-containing scaffold is then exploited to generate a range of analogues, some of which could include replacing the ester or amide, altering substitutions on the cyclohexane, substituting or replacing the pyridine, separating enantiomers, or even exploring alternatives to the thiophene ring itself to determine which functional groups are integral for binding and potentiating the target D1R PAM allosteric site. Analogues synthesized in this stage of the project specifically targeted amide replacements.

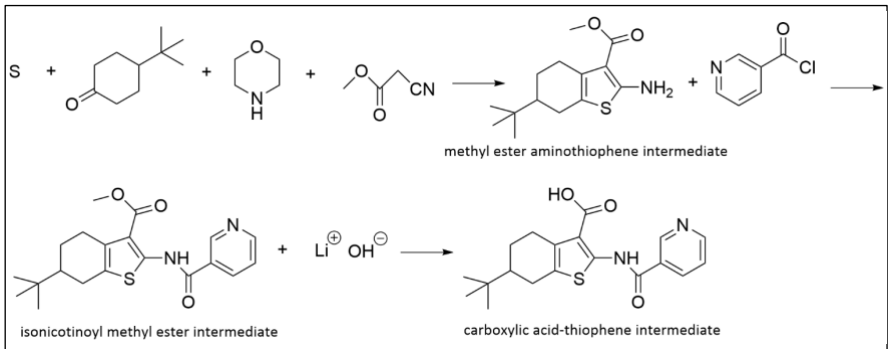


Figure 4. General synthetic route for key thiophene analogue generation

In the course of this project, we successfully prepared a set of four D1 positive allosteric modulator analogues based on the hit thiophene-based hit compound MLS6585 (figure 5).

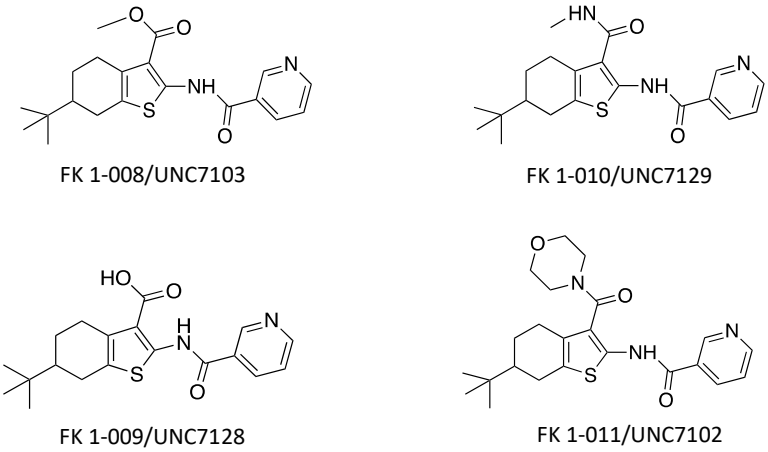
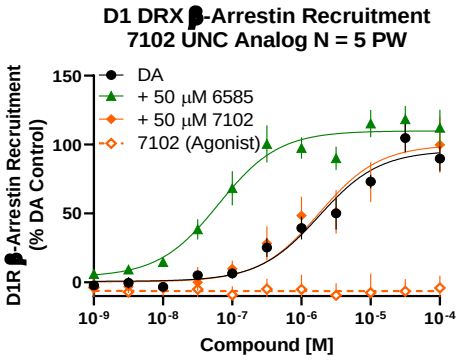
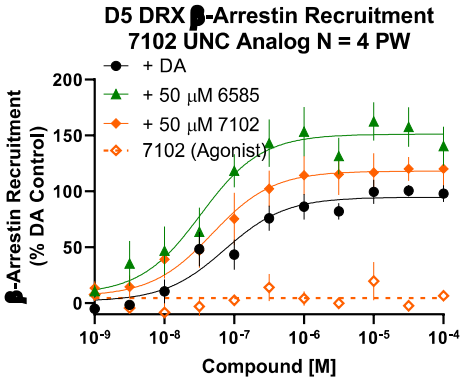
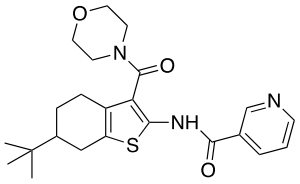


Figure 5. D1R PAM analogues synthesized

Figure 6: Potentiation of dopamine-stimulated activation of the D1 and D5 dopamine receptors by the test compounds shown using the DiscoverX PathHunter assay and D1 and D5 dopamine receptor activation by the test compounds alone







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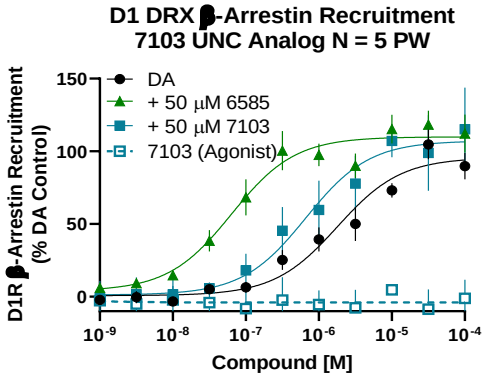

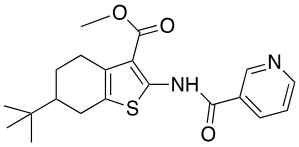

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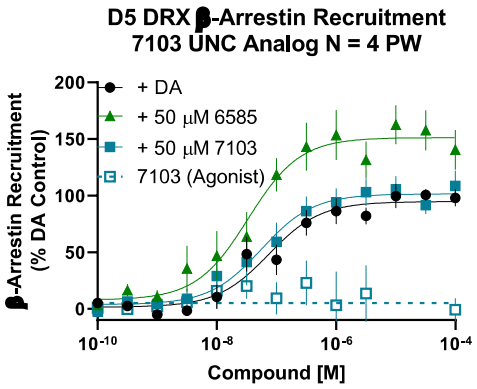

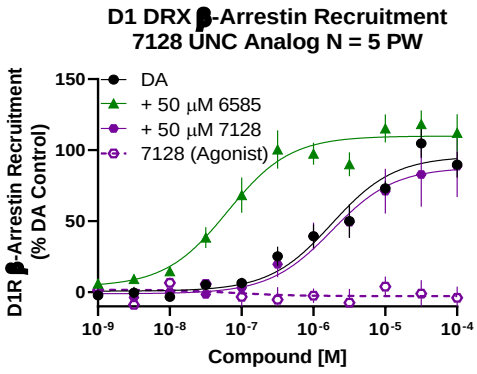

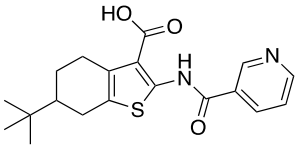

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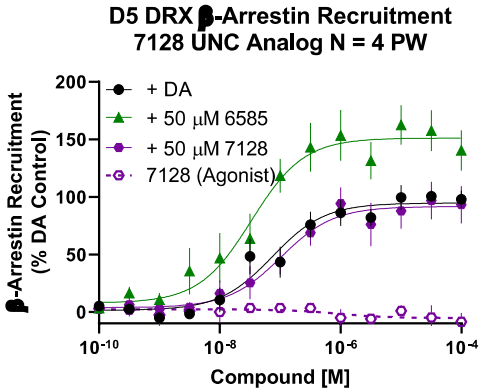

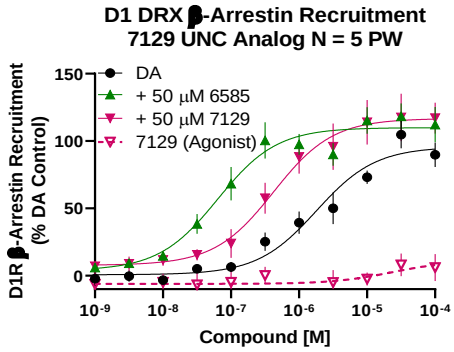

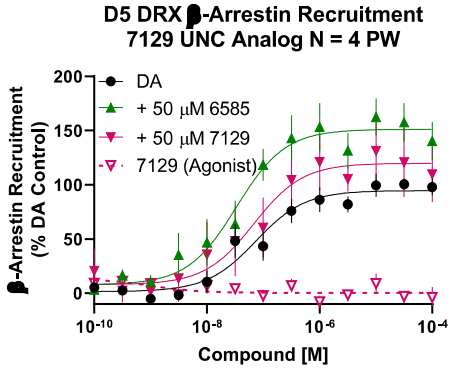

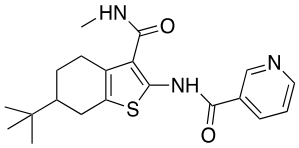

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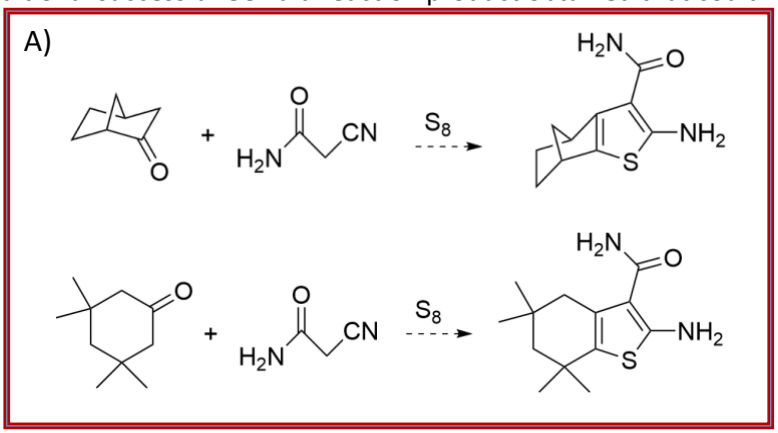
Table 1. Potentiation of dopamine-stimulated activation of the D1 and D5 dopamine receptors[a]

		DA + DMSO	+50 µM 7102	+50 µM 7103	+50 µM 7128	+50 µM 7129	+50 µM 6585
D1R β-arrestin recruitment	E_{max}	95%	98%	106%	87%	109%	106%
	LogEC50	-5.744	-5.824	-6.177	-5.837	-6.374	-7.195
D5R β-arrestin recruitment	E_{max}	93%	143.37%	112%	98%	88%	118%
	LogEC50	-7.131	-7.476	-7.315	-7.254	-6.987	-7.163

[a]concentration-response curves for dopamine-stimulated signaling were generated in the absence (dopamine alone) or presence of 50µM of test compounds using the DiscoverX PathHunter assay

Figure 7: Additional Gewald reactions conducted in this project include: A) reactions that did not afford the target product, which tested the steric limits with the Gewald reaction conditions used, and B) an additional successful Gewald reaction product obtained that could lead to new analogues.



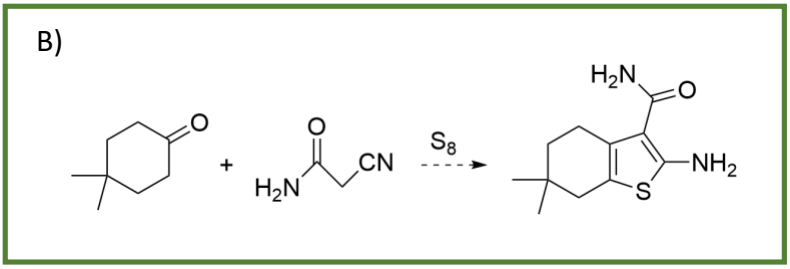
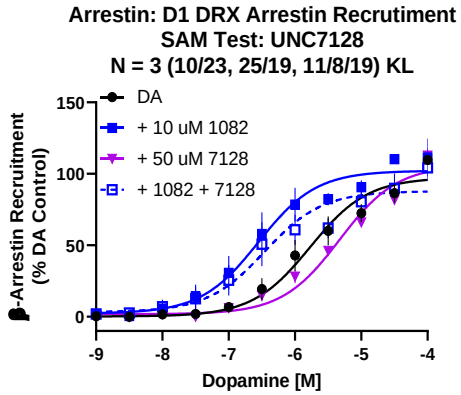
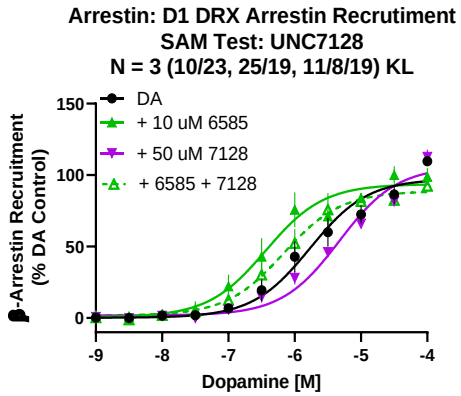
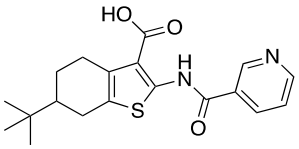


Figure 8: Effect of UNC 7128 on the MLS 6585- or MLS 1082-mediated potentiation of dopamine-stimulated D1R activation. UNC 7128 demonstrated measurable silent allosteric modulation for the D1R as shown by the diminished potentiation of MLS 6585 in the presence of UNC 7128.







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Discussion

Each of the analogues screened, UNC 7102, UNC 7103, UNC 7128, UNC 7129, produced some degree of D1R PAM activity, though all were less potent than the hit compound MLS6585 (see Table 1). As was observed with MLS6585, none of the new analogues produced appreciable inherent D1R agonism in the absence of dopamine.

Conversion of the primary amide of MLS 6585 to a secondary *N*-methyl amide in UNC 7129 produced the most significant D1R PAM out of the analogue set, which is not surprising given that this is a relatively minor structural change from MLS 6585. Additionally, replacement of the amide group with a methyl ester in UNC 7103 produced significant D1R PAM activity, but less than with the *N*-methyl amide moiety. The

log EC_{50} for dopamine following addition of 50 μM of UNC 7103 is -6.177 versus -6.374 for UNC 7129, and -7.195 for MLS 6585. Thus, substitutions at the amide position producing lower, yet still significant D1R PAM activity, which reflects the importance of the amide position in activating the targeted allosteric site. As the primary amide of MLS6585 remained the most potent, future analogues will investigate primary amide isoteres such as sulfonamides, carbamates and triazoles.

On the other hand, UNC 7102 and UNC 7128 produced no observable D1R PAM activity. This is not entirely surprising as these analogues incorporate more drastic structural changes to MLS6585. The much lower D1R PAM activity with these analogues also reveals important clues about the relationship between the chemical structure of these thiophene-based analogues and their potentiation of the D1R. With respect to diminished activity to the morpholine substitution at the amide position in UNC 7102, this suggests either that the sterics from the bulky substitution impair binding at the allosteric site, impairing meaningful potentiation, or that a hydrogen bond donation involving the second amide is required for binding. To probe the latter hypothesis, a dimethyl tertiary analogue could be synthesized in a future SAR round. The diminished D1R potentiation from carboxylic acid substitution in UNC 7128 may indicate that the increased polarity of the carboxylic acid compared with that of the amide interferes with allosteric site binding, or that the hydrogen bond-accepting character of the amide is also critical.

In addition to the final analogues prepared and tested (Figure 5), three other ketone substrates were subjected to the standard Gewald reaction conditions. Figure 7 shows these reaction schemes and the targeted intermediate compounds. The dimethyl intermediate, shown in the green box, was successfully synthesized and carried forward to the nicotinyl amide, however the final analogue could not be purified (minimum purity requirement >95% by HPLC/MS) and tested due to time constraints (compounded by the Covid-19 pandemic). The tetramethyl and norcamphor intermediates, shown in the red box, were not detected in the reaction mixture under the standard Gewald conditions. These intermediate target compounds demonstrate the scope of utility for the Gewald reaction and a notable sensitivity to steric bulk on the ketone substrate (i.e., tetramethyl and norcamphor ketones were unsuccessful, however the dimethyl ketone afforded thiophene product). When synthesizing such analogues, alternatives to the Gewald reaction (or modified Gewald reaction conditions) should be pursued. These limitations of the Gewald reaction will provide valuable guidance to the next iteration of D1R PAM drug discovery efforts.

In canonical pharmacology, the binding affinity of ligands for the receptor correlates with their potency and an observed functional effect. This correlation generally holds true for orthosteric ligands, however ligands binding to allosteric sites can induce nuanced receptor stabilization where this correlation does not necessarily hold. PAM activity may be especially susceptible to this disconnect between binding affinity and functional activity. Silent allosteric modulators (SAMs) describe the activity of compounds that bind to an allosteric site yet do not induce any effect on receptor activation upon binding (neither potentiating, activating nor inhibiting the receptor). SAM compounds can however displace the binding of other allosteric binders at the same allosteric site. Thus, SAM activity is measured by the effect the compound has on known allosteric ligands. Figure 8 shows the effect of UNC 7128 on the PAM effects of both MLS 1082 and MLS 6585. The dopamine-stimulated activation of the D1R in the presence of either known PAM alone or with UNC 7128 shows that the addition of 50 μM of UNC 7128 can diminish PAM activity. While the effect with MLS 1082 is too slight to draw significant conclusions, the rightward shift of the concentration-response curve for MLS6585 indicates the UNC 7128 can partially displace MLS 6585 from binding to the D1R. That these compounds have binding affinity and compete for the same site on the D1R is not entirely surprising given the structural similar between the two compounds. In contrast, the effect on MLS 1082 would be unexpected as we have previously shown that MLS1082 occupies a different allosteric site on the D1R. Taken together, these observations indicate that the allosteric site

targeted by analogues of MLS 6585 can induce or stabilize different conformations of the D1R and produce different effects on dopaminergic signaling. This suggests that MLS 6585 could potentially be further derivatized to produce D1R negative allosteric modulation, which has never been studied or reported in the literature. In fact, it appears that UNC 7128 have produced a small degree of reduced D1R activity (though the effect did not exceed criteria of statistically significant for this n-of-3 experimental set), shifting the concentration-response curve to the right when administered with dopamine alone. This preliminary data could lead to future drug discovery efforts to achieve meaningful D1R negative allosteric modulation and may help further expand our current understanding of D1R pharmacology beyond the paradigm of traditional orthosteric binding.

Strengths of this study are the relatively simple two-step synthetic route, the significant baseline D1R potentiation by the hit compound MLS6585, an absence of D1R agonism with the thiophene-based hit compound scaffold, and potentiation at a novel allosteric site on the D1R. By choosing the thiophene hit compound for optimization and making substitutions based on preliminary structure–activity relationship studies, synthesized analogues were likely to produce significant D1R potentiation.

There were also several key limitations with this project. First, despite promising data and an established hit compound, there was no guarantee that an analogue with improved activity could be developed. Second, the Covid-19 pandemic cut short planned laboratory experiments. In spite of these significant limitations, the potential impact on future D1 drug discovery and our understanding of dopaminergic pharmacology is of such tremendous magnitude, that it is both prudent and necessary to pursue this endeavor.

Conclusion

Numerous drugs target the dopaminergic system for a variety of neurodegenerative conditions, such as Parkinson's disease, Alzheimer's disease, bipolar disorder, and schizophrenia. Despite this, there remains important, unexplored dopamine pharmacologic potential. It is now apparent that D1 receptors, which are the most highly expressed subtype in the body, present a high-value pharmacologic target. Following the general trend towards allosteric therapeutics, seems likely that D1R PAMs will unlock therapeutic utility of the D1R for the treatment of unmet clinical needs of treating working memory deficits and cognitive decline in psychiatric disorders and in the normal aging process. The development of a safe and effective D1R PAM agent could potentially transform patients' lives by restoring their working memory and cognitive function, allowing them to live productive and fulfilling lives. Indeed, D1R PAMs possess the paradigm-shifting potential of improving the lives of countless patients by ameliorating cognitive decline associated prefrontal cortex D1R signaling deficiencies. With such broad clinical applications, and considering the fact that cognitive decline eventually affects virtually all aging adults, the impact of a successful D1R PAM capable of safely improving cognitive decline would be truly immeasurable.

For any patient affected by cognitive decline, a D1R PAM could transform their lives by improving their cognitive deficits, restoring their personal identity, and facilitating their connection to society. From a healthcare perspective, an enormous time burden would be lifted, as patients would avoid seeking care for many of the complications associated with cognitive dysfunction. This would free up an immense amount of precious time for healthcare providers, allowing them to spend more time with other patients. In addition, the financial implications of bringing a D1R PAM to market are staggering. Since the therapeutic applications are so broad with potential use in a variety of neurodegenerative disorders, as well as in the normal aging process, D1R PAMs would enjoy significant and lasting clinical uptake in the ever-growing geriatric patient population affected by cognitive decline.

While the scope of this project was severely limited by time and resources, the synthesis and characterization of novel, thiophene-based D1R PAM analogues provided crucial insight into the relationship between small molecule chemical structure and the novel D1R allosteric site. The various degrees of diminished D1R potentiation for each of the synthesized analogues with substitutions at the amide position reflect the importance of this position for the thiophene-based hit compound MLS 6585 D1R potentiation and discovered silent allosteric modulation activity for select analogues. This project has helped guide future D1R PAM drug discovery by precluding amide substitutions made in this project as viable strategies to increase potentiation. It has also shown that the amide position can be hindered by sterics, which will be a valuable fact to consider when planning prudent analogue exploration. Having established the importance of the amide position, efforts will explore substitutions on the cyclohexane and pyridine rings, as well as at the *tert*-butyl position.

In addition, with Eli Lilly's D1R PAM candidates in preclinical and clinical trials, exploration of this second, distinct D1R allosteric site is invaluable in terms of substantiating the therapeutic opportunities for D1-like allosteric pharmacology. Although the drug development journey is long and fraught with failure, the eventual development of safe, effective D1R PAMs, whether at the allosteric ICL-2 site targeted by the Eli Lilly candidate or by the thiophene-based scaffold, will likely be key to unlocking the awesome therapeutic potential of D1-like pharmacology, allowing it to be harnessed to dramatically improve countless patient outcomes and quality of life.

Acknowledgements
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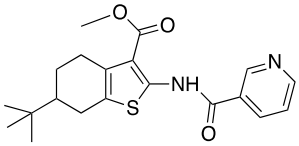
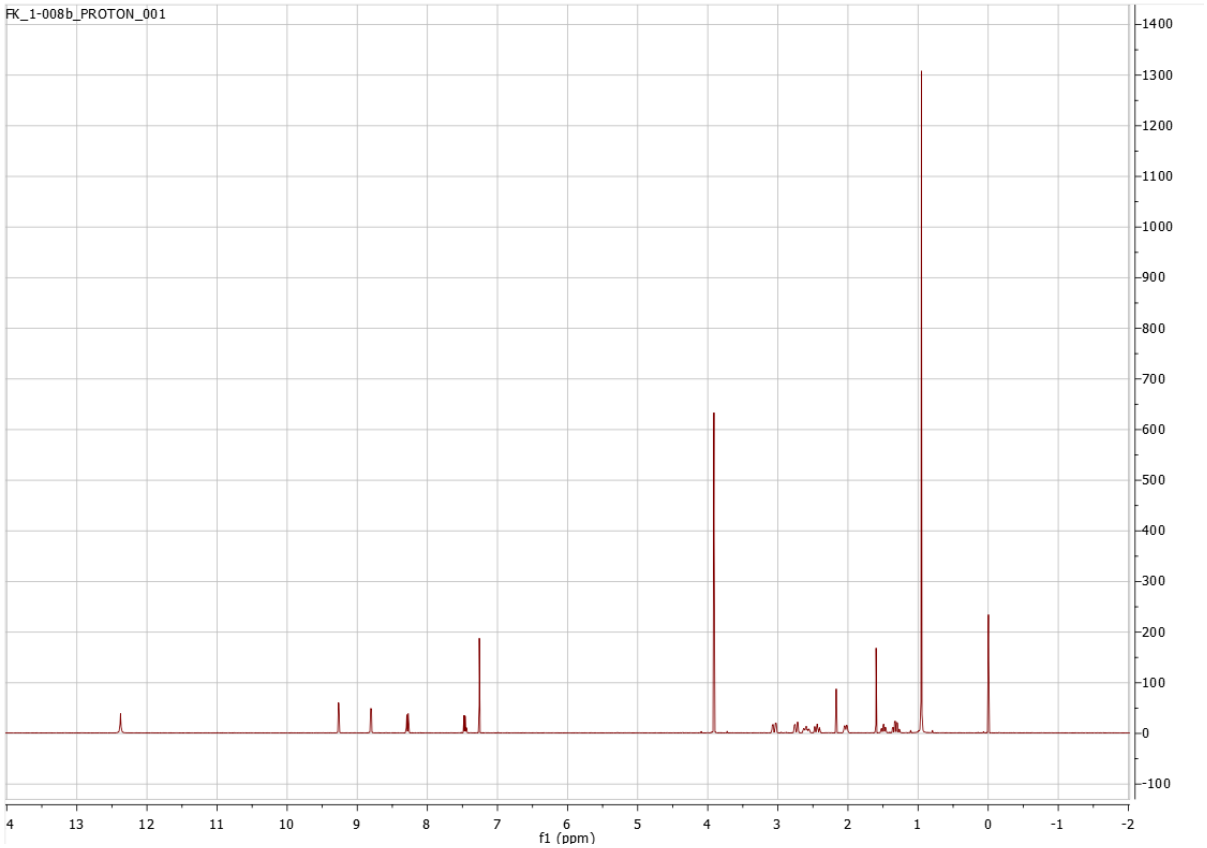
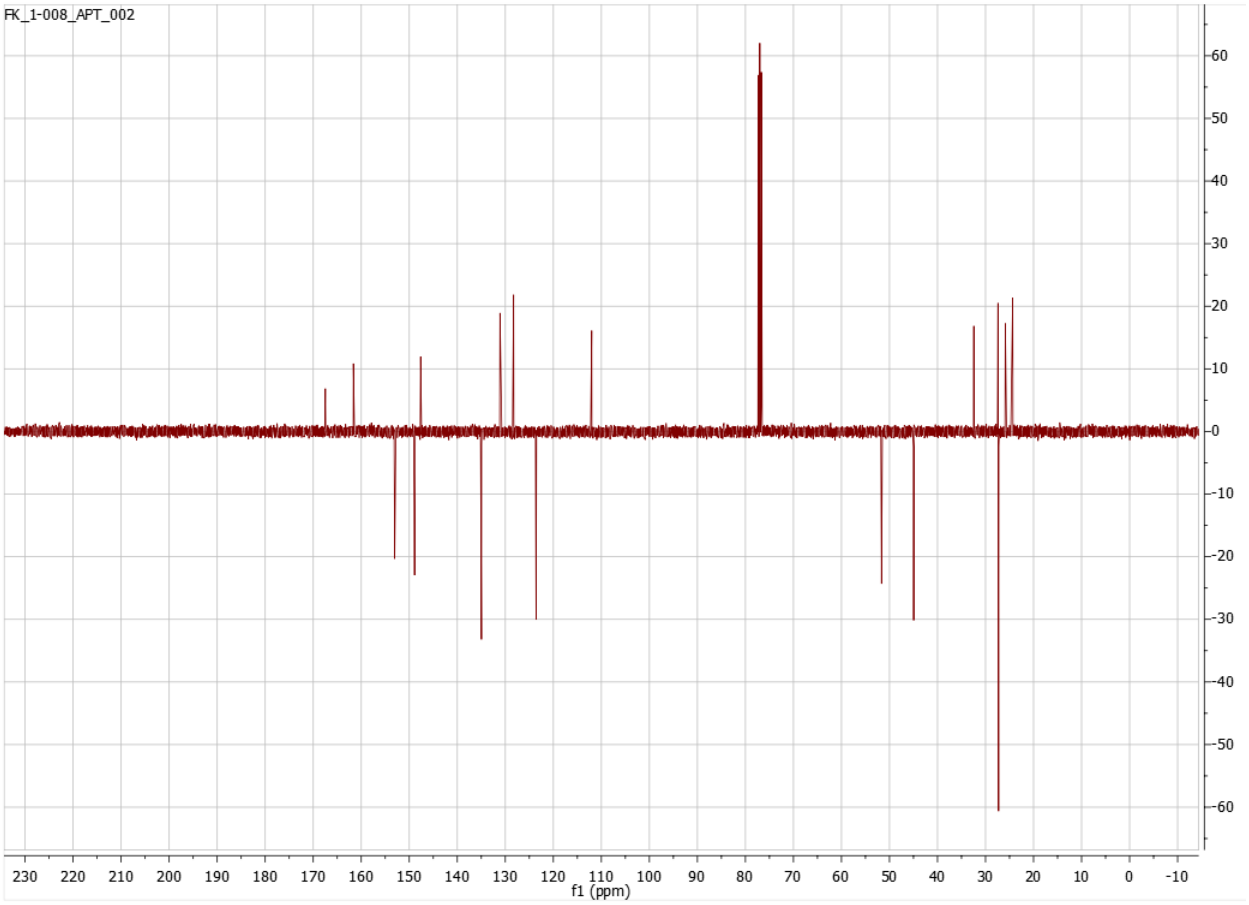
REGULATORY AND ETHICS

Required training includes EHS training for fume hood (4228), laboratory waste management (5521, 5525), and Global Harmonized Systems of hazards (42201). This project does not include human subjects or vertebrae animals.

Supplemental Information



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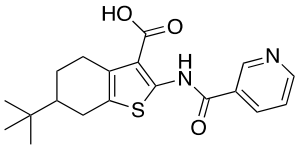
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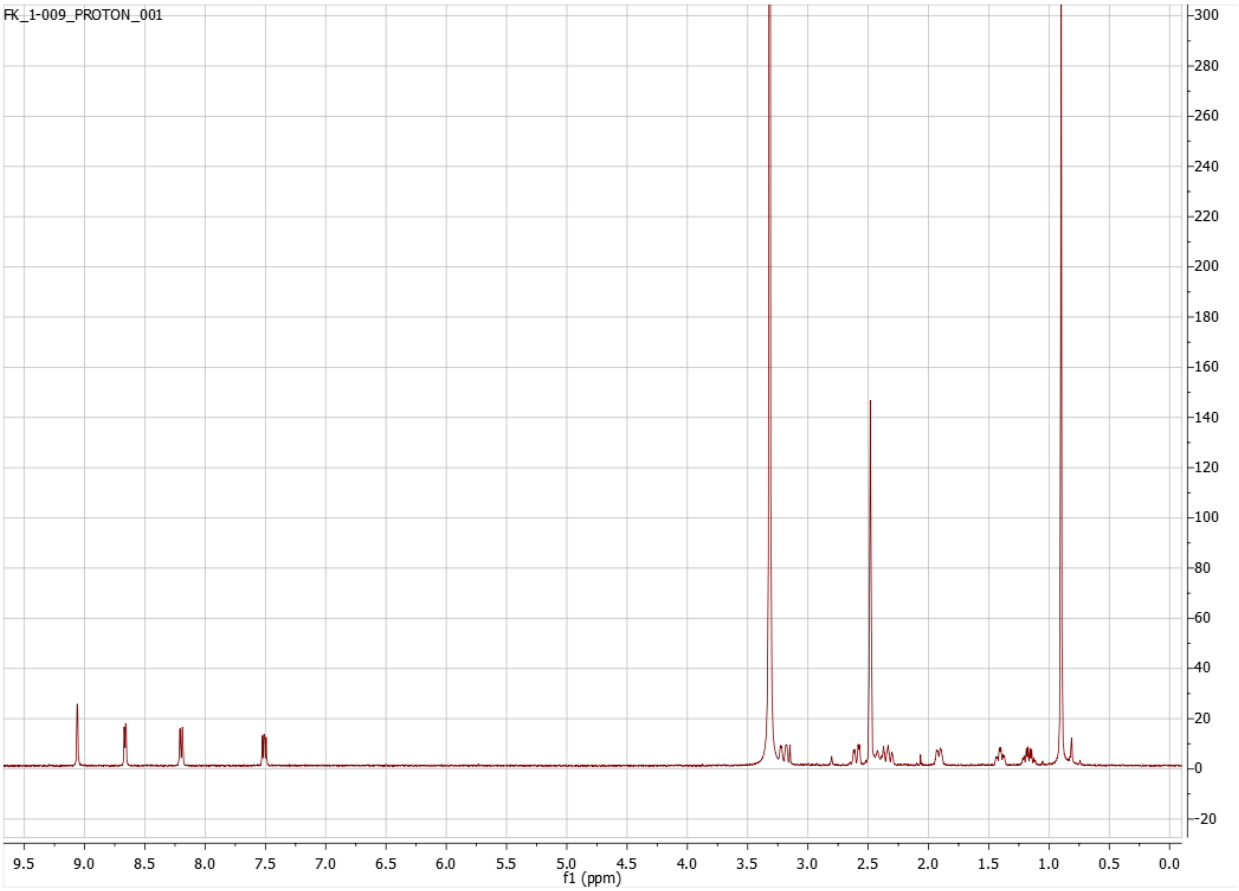
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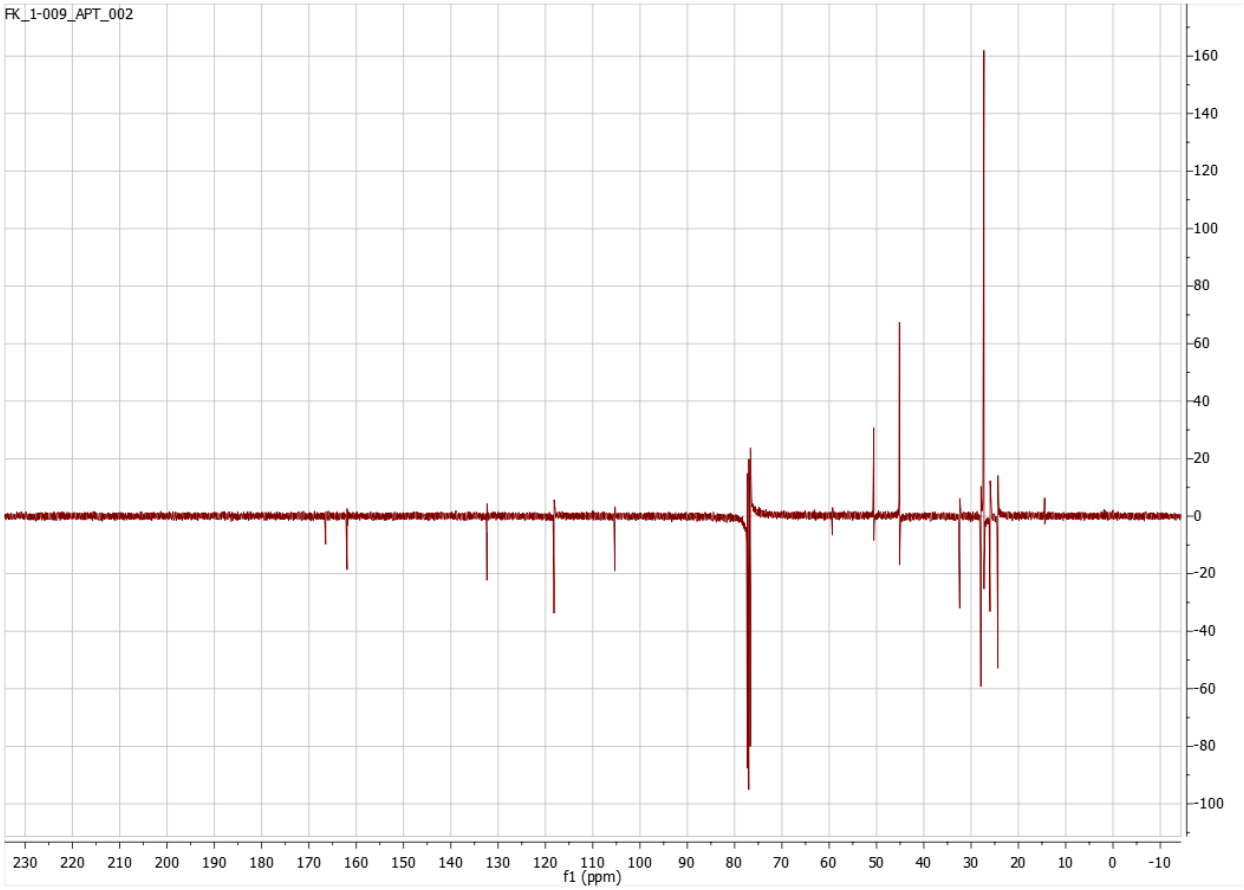
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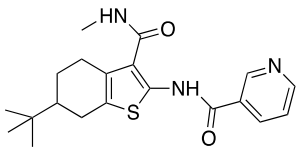
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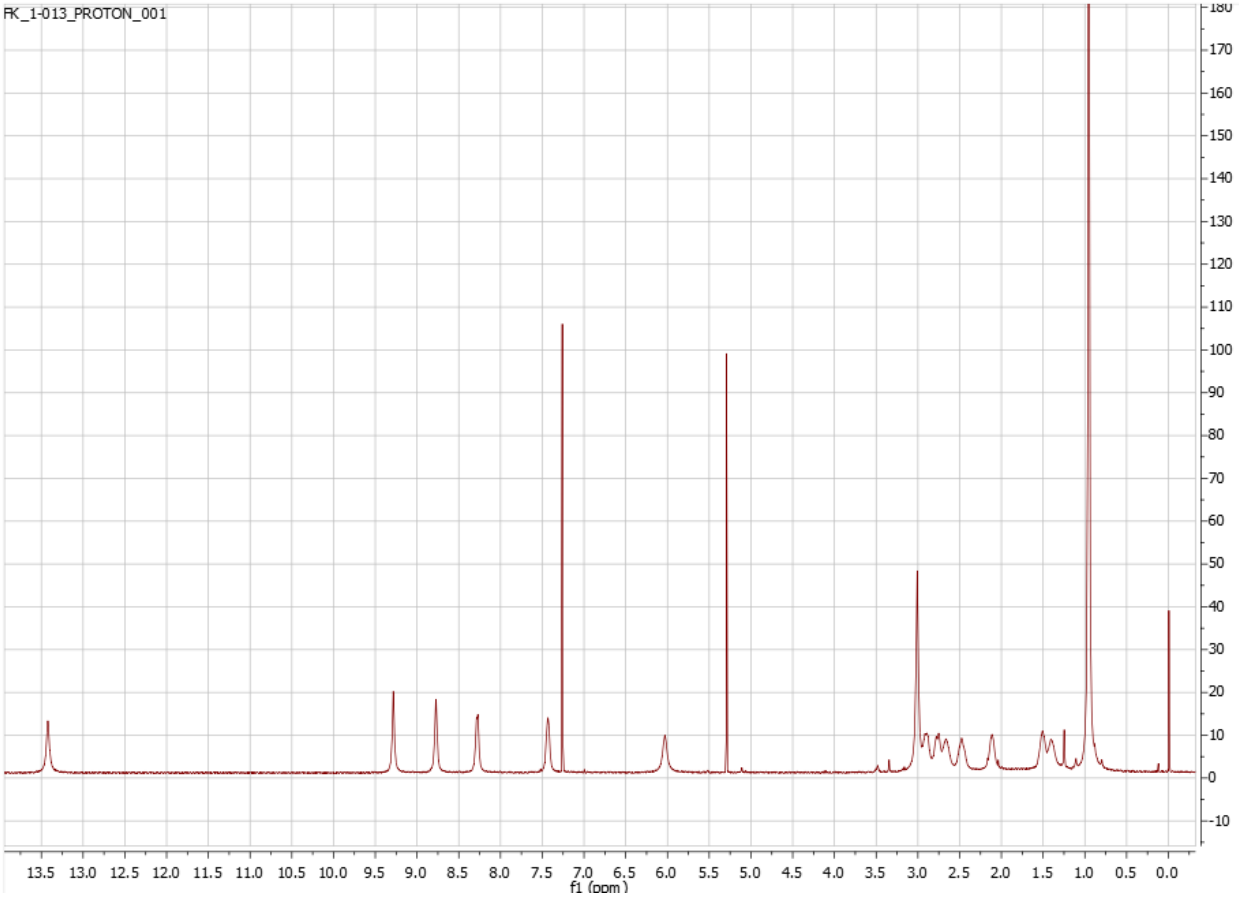
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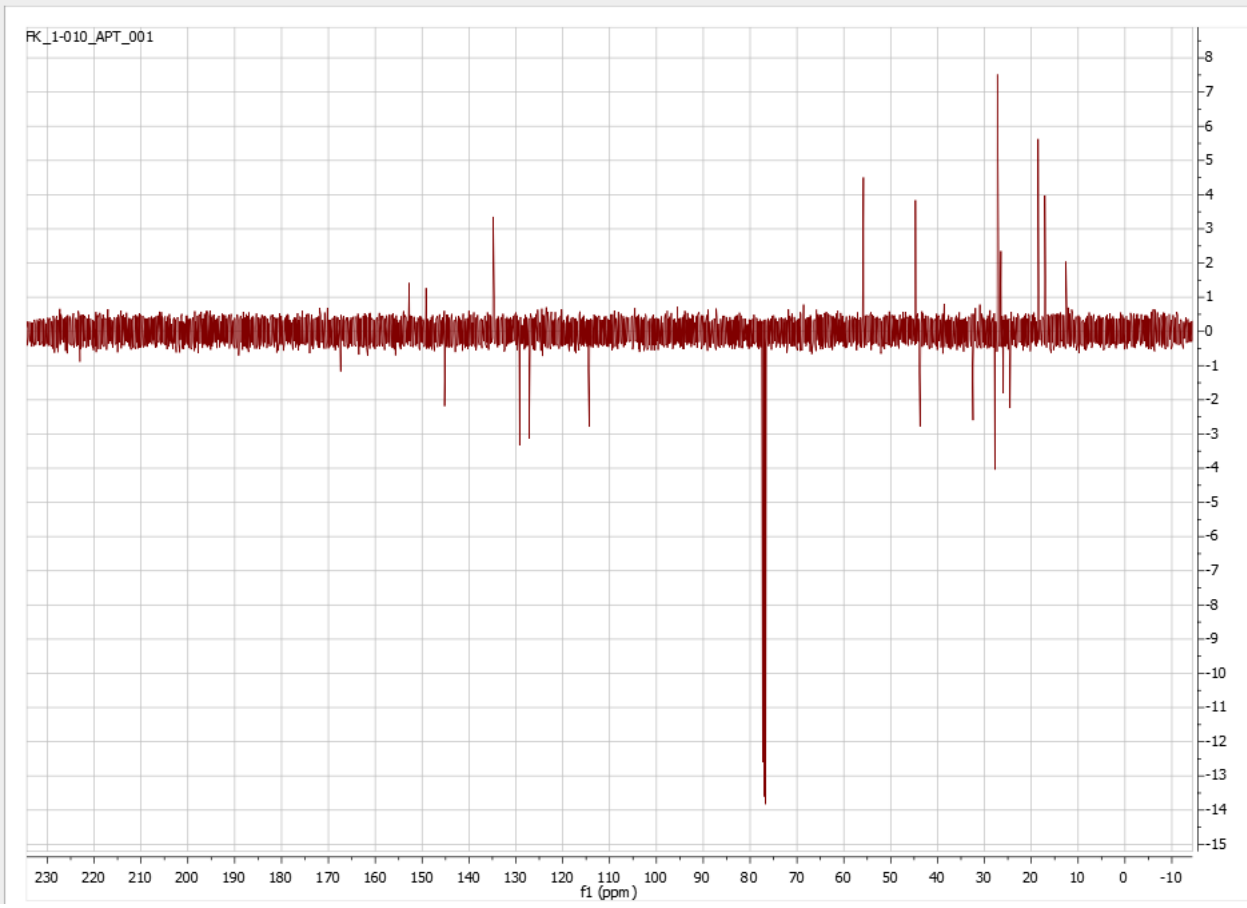
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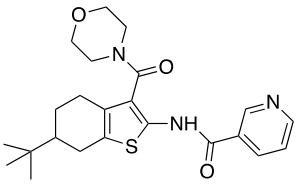
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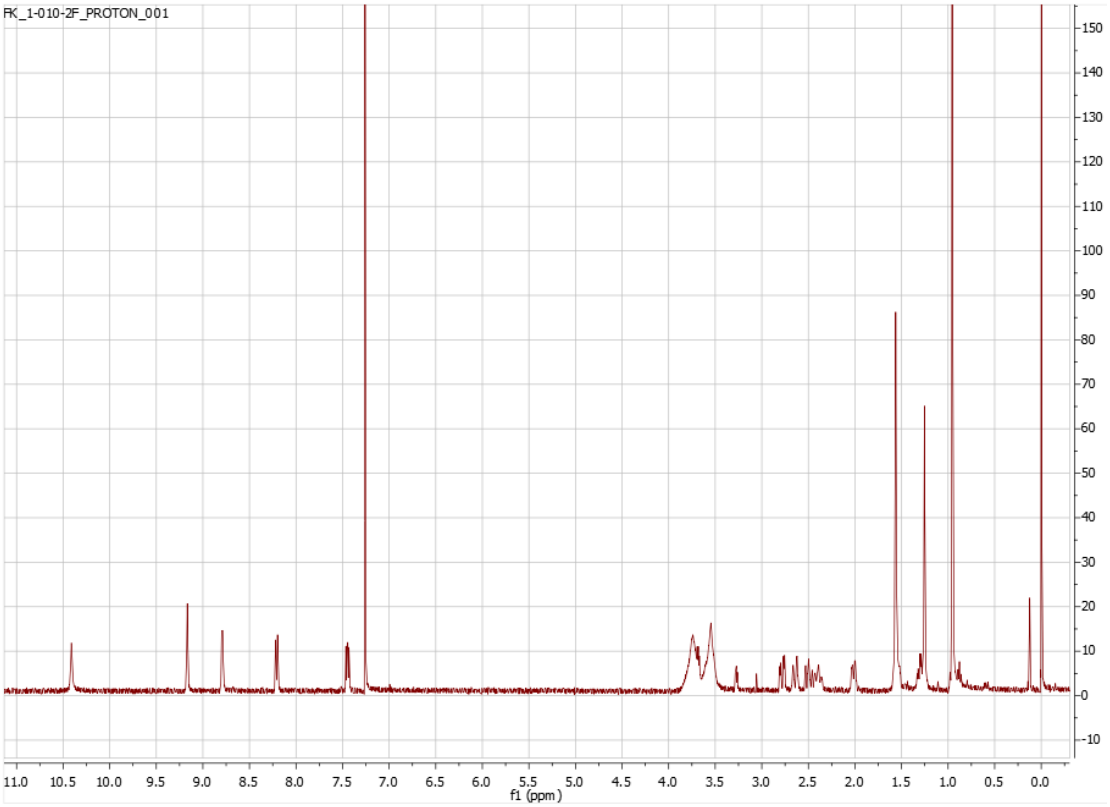
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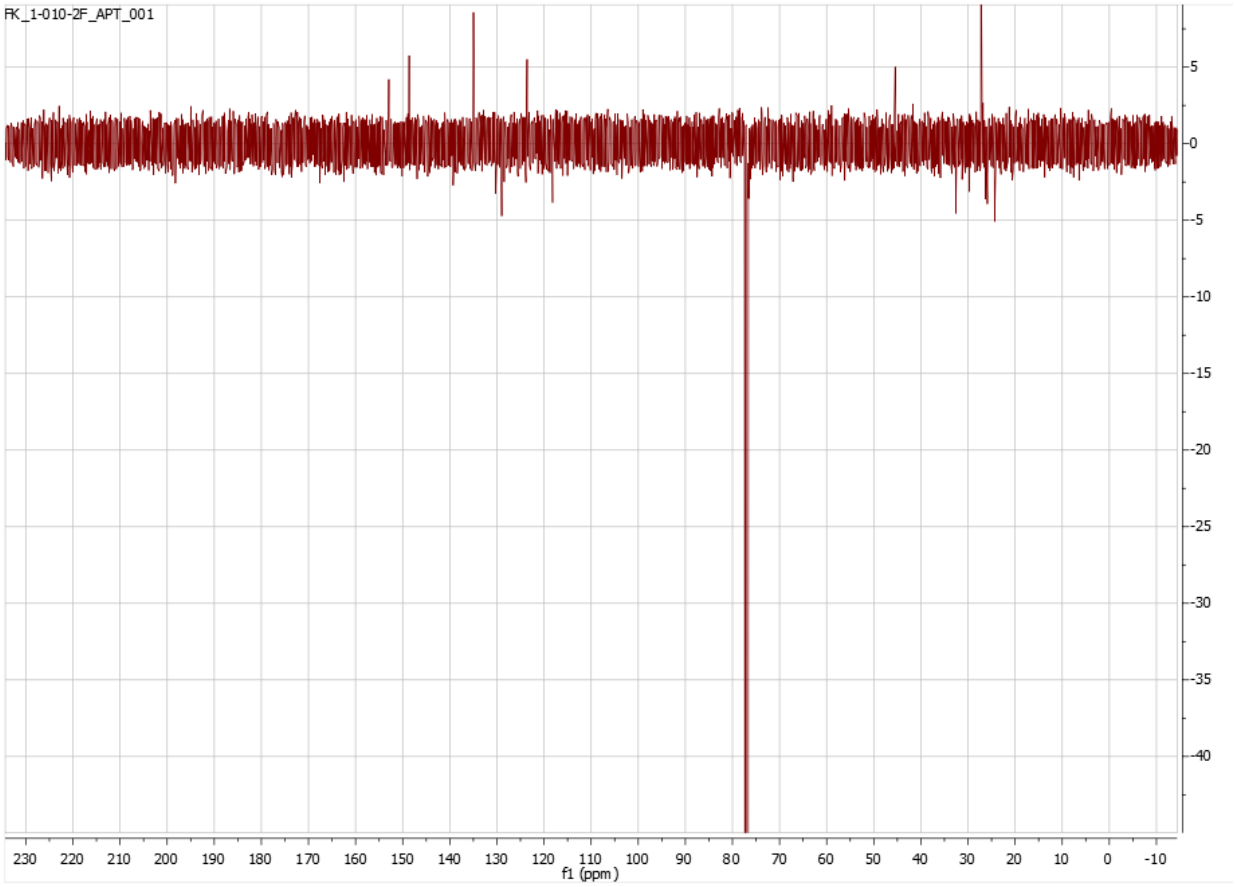
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