

Supporting Information

Design, Synthesis, and Mechanistic Investigations of Phenylalanine Derivatives Containing a Benzothiazole Moiety as HIV-1 Capsid Inhibitors with Improved Metabolic Stability

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I. Metabolic Stability in Human Liver Microsomes

The metabolic stability in human liver microsomes of compounds was determined in WuXi AppTec Co. Ltd. (Shanghai), China. The detailed procedure is as follows:

1. Test Compounds

Table S1. Compounds information

Compound No.	Compound ID	Batch No.	Exact Mass	Stock Concentration (mM)
1	LA-7m	LA-7m	496.16	10
2	LA-7u	LA-7u	549.18	10
3	PF74	PF74	425.21	10
Control	Testosterone		288.42	10
Control	Diclofenac		295.14	10
Control	Propafenone		341.44	10

2. Experimental Procedure

2.1. Test Compound and Control Working Solution Preparation

2.1.1. Working solution: 5 μ L of compound and control stock solution (10 mM in dimethyl sulfoxide (DMSO)) were diluted with 495 μ L of acetonitrile (ACN) (intermediate solution concentration: 100 μ M, 99% ACN).

2.2. NADPH Cofactor Preparation

2.2.1. Materials

NADPH powder: β -Nicotinamide adenine dinucleotide phosphate reduced form, tetrasodium salt; NADPH \cdot 4Na (Vendor: Chem-Impex International, Cat. No. 00616).

2.2.2. Preparation Procedure

The appropriate amount of NADPH powder was weighed and diluted into a 10 mM MgCl₂ solution (working solution concentration: 10 mM; final concentration in reaction system: 1 mM).

2.3. Liver Microsomes Preparation

2.3.1. Materials

Table S2. Liver Microsomes Information

Species	Product Information	Vendor	Abbreviation
Human	Cat No. 452117 Lot No. 38295	Corning	HLM

2.3.2. Preparation Procedure

The appropriate concentrations of microsome working solutions were prepared in 100 mM potassium phosphate buffer.

2.4. Stop Solution Preparation

Cold (4°C) acetonitrile (ACN) containing 200 ng/mL tolbutamide and 200 ng/mL labetalol as internal standards (IS) was used as the stop solution.

2.5. Assay Procedure

2.5.1. Pre-warm empty 'Incubation' plates T60 and NCF60 for 10 min minutes.

2.5.2. Dilute liver microsomes to 0.56 mg/mL in 100 mM phosphate buffer.

2.5.3. Transfer 445 uL microsome working solutions (0.56 mg/mL) into pre-warmed 'Incubation' plates T60 and NCF60, then pre-incubate 'Incubation' plates T60 and NCF60 for 10 min at 37°C with constant shaking. Transfer 54 µL liver microsomes to blank plate, then add 6 µL NADPH cofactor to blank plate, and then add 180 µL quenching solution to blank plate.

2.5.4 Add 5 µL compound working solution (100 µM) into 'incubation' plates (T60 and NCF60) containing microsomes and mix 3 times thoroughly.

2.5.5. For the NCF60 plate, add 50 uL of buffer and mix 3 times thoroughly. Start timing; plate will be incubated at 37°C for 60 min while shaking.

2.5.6. In 'Quenching' plate T0, add 180 µL quenching solution and 6 µL NADPH cofactor. Ensure the plate is chilled to prevent evaporation.

2.5.7. For the T60 plate, mix 3 times thoroughly, and immediately remove 54 µL mixture for the 0-min time point to 'Quenching' plate. Then add 44 µL NADPH cofactor to incubation plate (T60). Start timing; plate will be incubated at 37°C for 60 min while shaking.

Table S3. Final Concentration of Each Component in Incubation Medium

Component	Concentration
Microsome	0.5 mg protein/mL
Test Compound	1 µM
Control Compound	1 µM
Acetonitrile	0.99%
DMSO	0.01%

2.5.8. At 5, 15, 30, 45, and 60 min, add 180 μ L quenching solution to 'Quenching' plates, mix once, and serially transfer 60 μ L sample from T60 plate per time point to 'Quenching' plates.

Table S4: Reaction Plates Incubation

Time Point	Start Time	End Time
Blank	1:00:00	0:00:00
T60	1:00:00	0:00:00
T45	0:45:00	0:00:00
T30	0:30:00	0:00:00
T15	0:15:00	0:00:00
T5	0:05:00	0:00:00
T0	mix 3 times and remove out to 'Quenching' plate	

2.5.9. For NCF60: mix once, and transfer 60 μ L sample from the NCF60 incubation to 'Quenching' plate containing quenching solution at the 60-min time point.

Table S5. NCF60 Incubation

Time Point	Start Time	End Time
NCF60	1:00:00	0:00:00

2.5.10. All sampling plates are shaken for 10 min, then centrifuged at 4000 rpm for 20 minutes at 4°C.

2.5.11. Transfer 80 μ L supernatant into 240 μ L HPLC water, and mix by plate shaker for 10 min.

2.5.12. Each bioanalysis plate was sealed and shaken for 10 minutes prior to LC-MS/MS analysis.

3. Data Analysis

3.1. The equation of first order kinetics was used to calculate $T_{1/2}$ and $CL_{int}(\text{mic})$ ($\mu\text{L}/\text{min}/\text{mg}$).

Equation of first order kinetics:

$$C_t = C_0 \cdot e^{-k_e \cdot t}$$

when $C_t = \frac{1}{2} C_0$,

$$T_{1/2} = \frac{\text{Ln}2}{k_e} = \frac{0.693}{k_e}$$

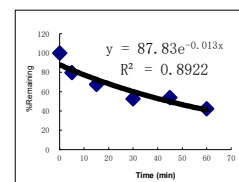
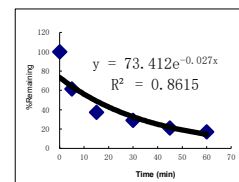
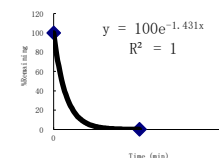
$$CL_{\text{int(mic)}} = \frac{0.693}{\text{In vitro } T_{1/2}} \cdot \frac{1}{\text{mg / mL microsomal protein in reaction system}}$$

$$CL_{\text{int(liver)}} = CL_{\text{int(mic)}} \cdot \frac{\text{mg microsomes}}{\text{g liver}} \cdot \frac{\text{g liver}}{\text{kg body weight}}$$

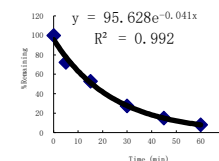
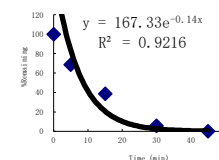
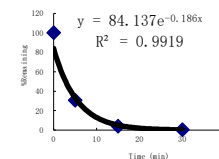
4. Raw Data

Table S6. Raw data of metabolic stability assay in human liver microsomes

Compound ID	Compound & Species	Time (min)	Analyte Peak Area	IS Peak Area	Analyte/IS	% Remaining	Time (min)	% Remaining	Ln (%Remaining)	R ²	k _e (min ⁻¹)	T _{1/2} (min)	CL _{int(mic)} (μL/min/mg)	Remaining (T=60min)	Remaining (NCF=60min)
PF74	PF74HLM 0.5	Blank	0	116,908	0.000	0.0				1.0000	1.4312	0.5	2862.5	0.0%	112.6%
PF74	PF74HLM 0.5	60	0	115,817	0.000	0.0									
PF74	PF74HLM 0.5	45	0	102,033	0.000	0.0									
PF74	PF74HLM 0.5	30	61	106,730	0.001	0.0									
PF74	PF74HLM 0.5	15	212	99,964	0.002	0.0									
PF74	PF74HLM 0.5	5	1,231	104,970	0.012	0.1	5	0.1	-2.6						
PF74	PF74HLM 0.5	0	1,486,739	98,654	15.070	100.0	0	100.0	4.6						
PF74	PF74HLM 0.5	NCF60	1,738,223	102,451	16.966	112.6									
7m	LA-7mHLM 0.5	Blank	0	119,639	0.000	0.0				0.9055	0.0270	25.7	53.9	17.0%	100.1%
7m	LA-7mHLM 0.5	60	163,254	105,807	1.543	17.0	60	17.0	2.8						
7m	LA-7mHLM 0.5	45	223,006	116,578	1.913	21.1	45	21.1	3.1						
7m	LA-7mHLM 0.5	30	285,791	108,878	2.625	29.0	30	29.0	3.4						
7m	LA-7mHLM 0.5	15	363,275	107,176	3.390	37.4	15	37.4	3.6						
7m	LA-7mHLM 0.5	5	575,573	103,231	5.576	61.5	5	61.5	4.1						
7m	LA-7mHLM 0.5	0	1,021,706	112,787	9.059	100.0	0	100.0	4.6						
7m	LA-7mHLM 0.5	NCF60	970,231	106,995	9.068	100.1									
7u	LA-7uHLM 0.5	Blank	1,258	119,368	0.011	0.2				0.9028	0.0127	54.6	25.4	42.2%	92.4%
7u	LA-7uHLM 0.5	60	226,697	112,970	2.007	42.2	60	42.2	3.7						
7u	LA-7uHLM 0.5	45	284,095	111,055	2.558	53.8	45	53.8	4.0						
7u	LA-7uHLM 0.5	30	313,642	125,482	2.499	52.5	30	52.5	4.0						
7u	LA-7uHLM 0.5	15	361,209	112,337	3.215	67.6	15	67.6	4.2						
7u	LA-7uHLM 0.5	5	425,792	112,060	3.800	79.9	5	79.9	4.4						
7u	LA-7uHLM 0.5	0	537,528	112,970	4.758	100.0	0	100.0	4.6						
7u	LA-7uHLM 0.5	NCF60	524,149	119,258	4.395	92.4									



Diclofenac	DiclofenacHLM 0.5	Blank	0	105,690	0.000	0.0					0.9947	0.1860	3.7	372.0	0.0%	96.7%
Diclofenac	DiclofenacHLM 0.5	60	0	104,657	0.000	0.0										
Diclofenac	DiclofenacHLM 0.5	45	0	98,189	0.000	0.0										
Diclofenac	DiclofenacHLM 0.5	30	303	101,980	0.003	0.4	30	0.4	-1.0							
Diclofenac	DiclofenacHLM 0.5	15	3,460	99,988	0.035	4.2	15	4.2	1.4							
Diclofenac	DiclofenacHLM 0.5	5	26,146	102,565	0.255	30.7	5	30.7	3.4							
Diclofenac	DiclofenacHLM 0.5	0	82,212	98,999	0.830	100.0	0	100.0	4.6							
Diclofenac	DiclofenacHLM 0.5	NCF60	83,751	104,298	0.803	96.7										
Propafenone	PropafenoneHLM 0.5	Blank	63	105,722	0.001	0.0					0.9350	0.1397	5.0	279.5	0.0%	93.6%
Propafenone	PropafenoneHLM 0.5	60	63	100,264	0.001	0.0										
Propafenone	PropafenoneHLM 0.5	45	1,024	97,056	0.011	0.2	45	0.2	-1.9							
Propafenone	PropafenoneHLM 0.5	30	40,148	101,776	0.394	5.6	30	5.6	1.7							
Propafenone	PropafenoneHLM 0.5	15	258,004	95,967	2.688	38.5	15	38.5	3.7							
Propafenone	PropafenoneHLM 0.5	5	499,263	104,023	4.800	68.7	5	68.7	4.2							
Propafenone	PropafenoneHLM 0.5	0	659,116	94,347	6.986	100.0	0	100.0	4.6							
Propafenone	PropafenoneHLM 0.5	NCF60	671,724	102,762	6.537	93.6										
Testosterone	TestosteroneHLM 0.5	Blank	223	112,647	0.002	0.2					0.9982	0.0414	16.7	82.8	7.9%	90.7%
Testosterone	TestosteroneHLM 0.5	60	7,607	108,076	0.070	7.9	60	7.9	2.1							
Testosterone	TestosteroneHLM 0.5	45	14,283	107,692	0.133	14.9	45	14.9	2.7							
Testosterone	TestosteroneHLM 0.5	30	25,374	103,996	0.244	27.5	30	27.5	3.3							
Testosterone	TestosteroneHLM 0.5	15	47,238	100,489	0.470	53.0	15	53.0	4.0							
Testosterone	TestosteroneHLM 0.5	5	66,880	104,311	0.641	72.2	5	72.2	4.3							
Testosterone	TestosteroneHLM 0.5	0	90,117	101,541	0.887	100.0	0	100.0	4.6							
Testosterone	TestosteroneHLM 0.5	NCF60	80,291	99,693	0.805	90.7										



II. Stability in Human Plasma

The Stability in Human Plasma of compounds was determined in WuXi AppTec Co. Ltd. (Shanghai), China. The detailed procedure is as follows:

1. Materials

1.1. Test Compounds and Stock Solutions

Table S7. Information of test compounds and stock solutions

Compound ID	Batch	MW	FW	Purity (%)	Stock Conc. (mM)	Final Conc.(μ M)
LA-7m	LA-7m	496.16	NA	98.0	10	2
LA-7u	LA-7u	549.18	NA	99.3	10	2
PF74	PF74	425.5	NA	97.1	10	2
Proprantheline bromide	R000190915	448.39	448.39	97.00	10	2

1.2. Test Compound and Control Working Solution Preparation

1.2.1. Test compound Working solution: 5 μ L of compound stock solution (10 mM in dimethyl sulfoxide (DMSO)) were diluted with 495 μ L of DMSO (Working solution concentration: 100 μ M, 100% DMSO).

1.2.2. Proprantheline bromide Working solution: 5 μ L of Proprantheline bromide stock solution (10 mM in H₂O) were diluted with 495 μ L of H₂O (Working solution concentration: 100 μ M, 100% H₂O).

1.3. Test System

Table S8. Information of test system

Species / Matrix	Minimum No. of Individuals	Anticoagulant Used	Vendor	Cat#	Batch
Human Plasma	3 Male & 3 Female	EDTA-K2	Bioreclamation IVT	HUMANPLK2 P2N	HMN514548

2. Methods

2.1. The pooled frozen plasma was thawed in a water bath at 37°C prior to experiment. Plasma was centrifuged at 4000 rpm for 5 min and the clots were removed if any.

2.2. Using an Apricot automation workstation, 98 μ L/well of blank plasma were added to all 96-well reaction plates. (Blank, T0, T10, T30, T60, and T120)

2.3. An Apricot automation workstation was used to add 2 μ L/well of working solution

(100 μ M) to all reaction plates except Blank. (T0, T10, T30, T60, and T120)

2.4. All reaction plates containing mixtures of compound and plasma were incubated at 37°C in water bath.

2.5. The reaction plates were incubated at 37°C, and timer was started.

Table S9. Reaction Plates Incubation

Time Point	Start Time	End Time
Blank	0:00:00	0:00:00
T120	2:00:00	0:00:00
T60	1:00:00	0:00:00
T30	0:30:00	0:00:00
T10	0:10:00	0:00:00
T0		

2.6. At the end of incubation, added 400 μ L of stop solution (200 ng/mL tolbutamide and 200 ng/mL labetalol in ACN) to precipitate protein. Mixed thoroughly.

2.7. Each plate was sealed and shaken for 20 minutes

2.8. After shaking, each plate was centrifuged at 4000 rpm and 4°C for 20 minutes

2.9. After centrifugation, an Apricot automation workstation was used to transfer 150 μ L supernatant.

2.10. Each bioanalysis plate was sealed and shaken for 10 minutes prior to LC-MS/MS analysis

3. Data Analysis

The % remaining of test compound after incubation in plasma was calculated using following equation:

$$\% \text{ Remaining} = 100 \times (\text{PAR at appointed incubation time} / \text{PAR at T0 time})$$

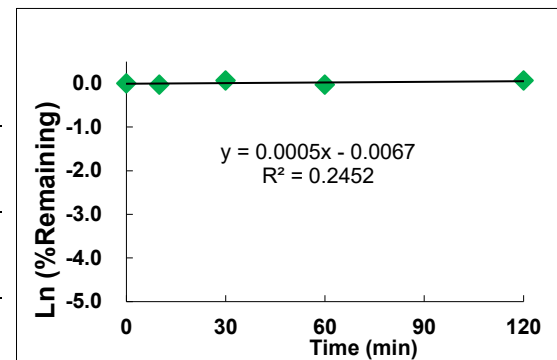
where PAR is the peak area ratio of analyte versus internal standard (IS)

The appointed incubation time points are T0 (0 min), Tn (n=0, 10, 30, 60, 120 min)

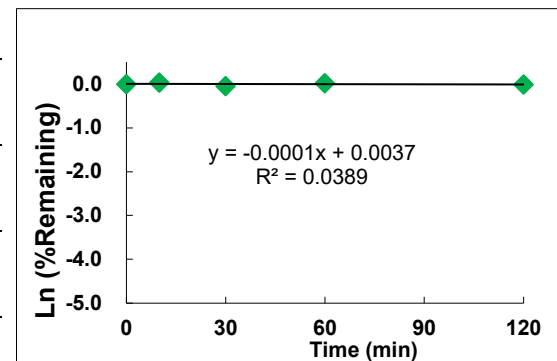
4. Data

Table S10. Raw data of human plasma stability assay

Sample_ID	Time	Analyte Peak Area	IS Peak Area	Aa/Ai	% Remaining (n=2)	Ln (% Remaining)
7m_H_0	0	2.17E+06	1.28E+06	1.6974	100.0	0.00
7m_H_0		2.49E+06	1.34E+06	1.8595		
7m_H_10	10	2.32E+06	1.43E+06	1.6163	97.4	-0.03
7m_H_10		2.52E+06	1.36E+06	1.8487		
7m_H_30	30	3.09E+06	1.53E+06	2.0179	106.6	0.06
7m_H_30		2.37E+06	1.34E+06	1.7732		
7m_H_60	60	2.44E+06	1.39E+06	1.7534	97.0	-0.03
7m_H_60		2.37E+06	1.39E+06	1.6968		
7m_H_120	120	2.73E+06	1.52E+06	1.7941	106.8	0.07
7m_H_120		3.03E+06	1.51E+06	2.0054		
7u_H_0	0	9.40E+05	1.39E+06	0.6771	100.0	0.00
7u_H_0		8.72E+05	1.39E+06	0.6258		
7u_H_10	10	9.18E+05	1.45E+06	0.6342	103.1	0.03
7u_H_10		1.07E+06	1.51E+06	0.7093		
7u_H_30	30	9.23E+05	1.50E+06	0.6135	95.7	-0.04
7u_H_30		9.98E+05	1.58E+06	0.6329		
7u_H_60	60	9.85E+05	1.48E+06	0.6641	101.9	0.02
7u_H_60		1.02E+06	1.54E+06	0.6635		
7u_H_120	120	9.52E+05	1.46E+06	0.6533	98.7	-0.01
7u_H_120		8.91E+05	1.41E+06	0.6327		
PF74_H_0	0	1.03E+07	1.45E+06	7.1259	100.0	0.00
PF74_H_0		1.08E+07	1.56E+06	6.8745		
PF74_H_10	10	9.50E+06	1.59E+06	5.9628	84.7	-0.17
PF74_H_10		8.68E+06	1.47E+06	5.8962		
PF74_H_30	30	8.42E+06	1.54E+06	5.4832	81.7	-0.20
PF74_H_30		8.91E+06	1.50E+06	5.9541		



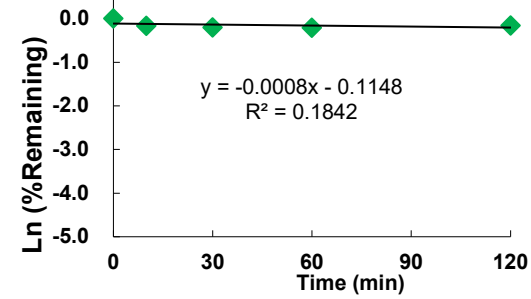
k= -
0.0005
R²= 0.2452
t_{1/2}=0.693/k >289.1



k= 0.0001
R²= 0.0389
t_{1/2}=0.693/k >289.1

k= 0.0008
R²= 0.1842
t_{1/2}=0.693/k >289.1

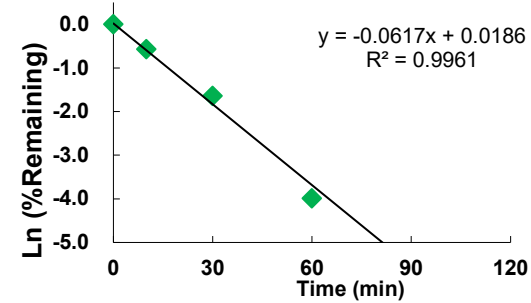
PF74_H_60		8.74E+06	1.42E+06	6.1381		
PF74_H_60	60	7.65E+06	1.48E+06	5.1652	80.7	-0.21
PF74_H_120		8.79E+06	1.61E+06	5.4640		
PF74_H_120	120	1.04E+07	1.62E+06	6.4621	85.2	-0.16
Propantheline bromide_H_0	0	3.32E+06	1.74E+06	1.9093	100.0	0.00
Propantheline bromide_H_0		3.22E+06	1.64E+06	1.9647		
Propantheline bromide_H_10	10	2.01E+06	1.86E+06	1.0818	56.5	-0.57
Propantheline bromide_H_10		1.91E+06	1.73E+06	1.1067		
Propantheline bromide_H_30	30	6.75E+05	1.89E+06	0.3581	19.4	-1.64
Propantheline bromide_H_30		7.44E+05	1.90E+06	0.3922		
Propantheline bromide_H_60	60	6.60E+04	1.77E+06	0.0374	1.9	-3.99
Propantheline bromide_H_60		6.11E+04	1.78E+06	0.0344		
Propantheline bromide_H_120	120	3.75E+03	1.98E+06	0.0019	0.1	-7.29
Propantheline bromide_H_120		1.14E+03	1.52E+06	0.0007		



k= 0.0617

R²= 0.9961

t_{1/2}=0.693/k 11.2

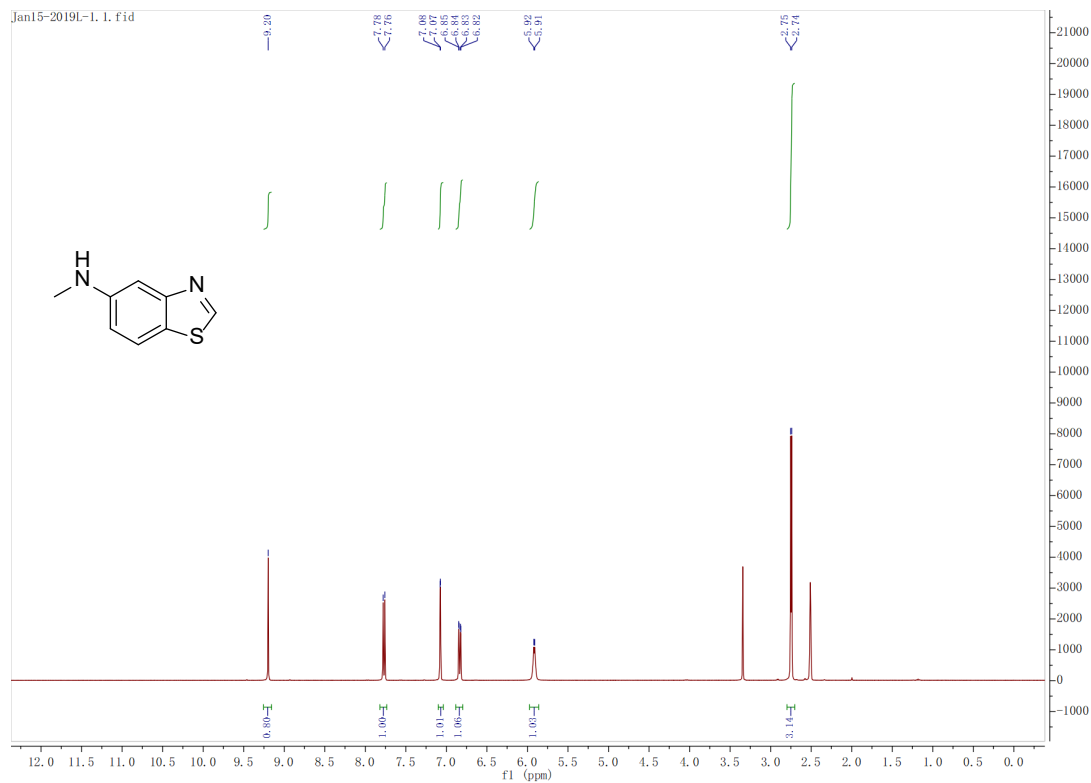


Sample comment: "XXXX_H_0" for example, "H" for Human Plasma, "0" was the sample collected at T=0 incubation.

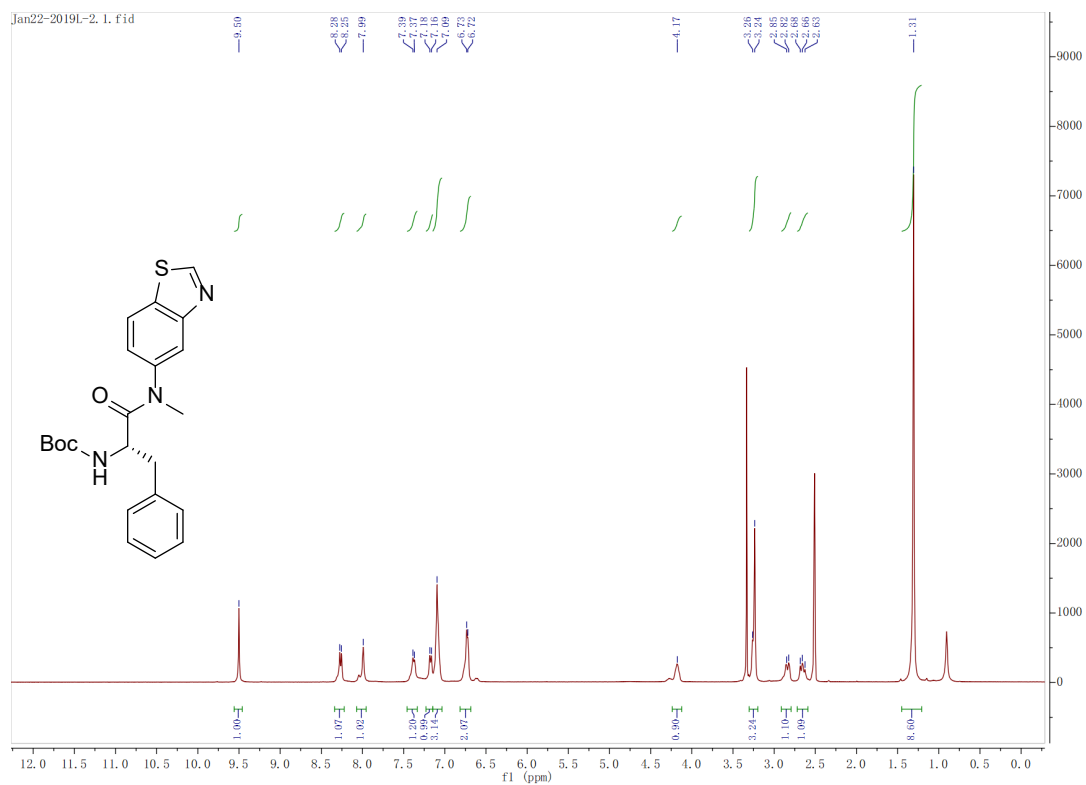
III. NMR Spectra for Intermediates and Target Compounds

Intermediates

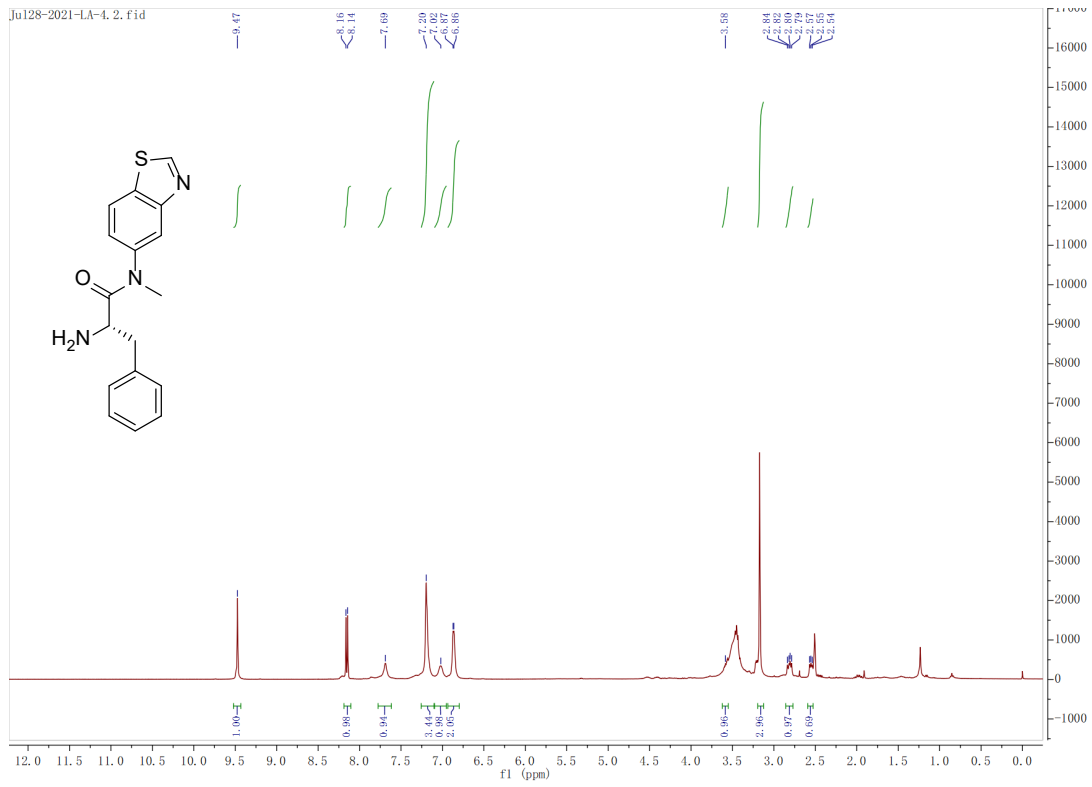
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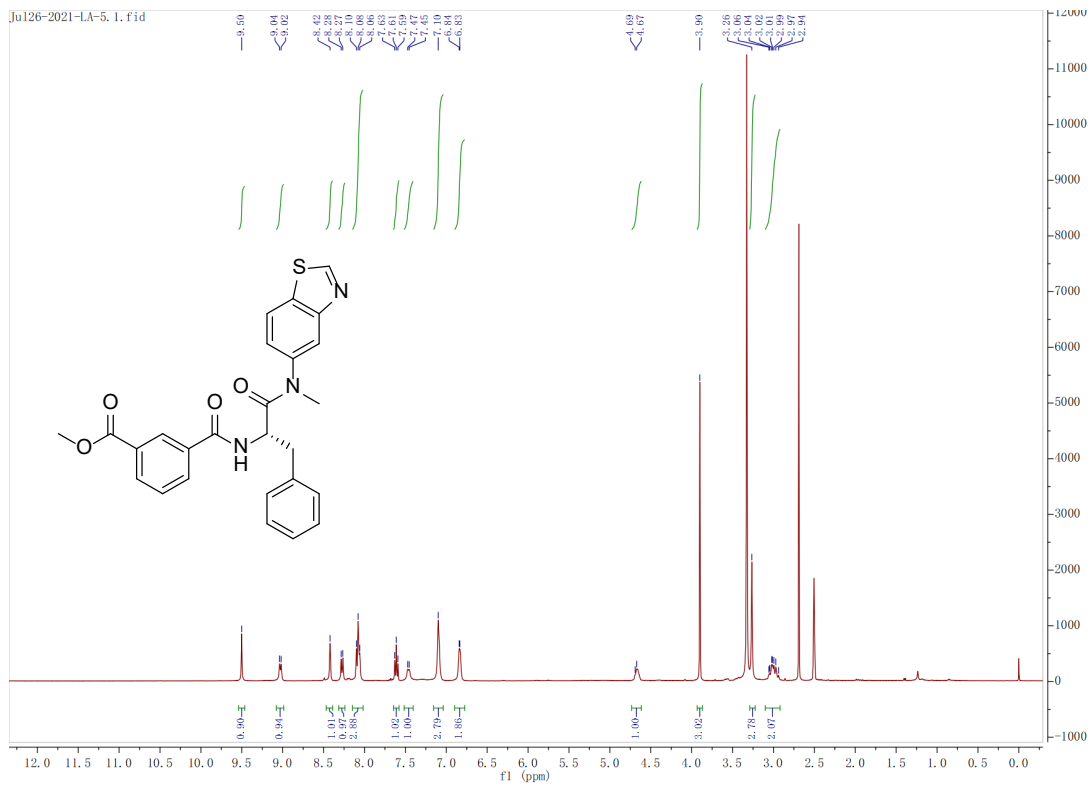
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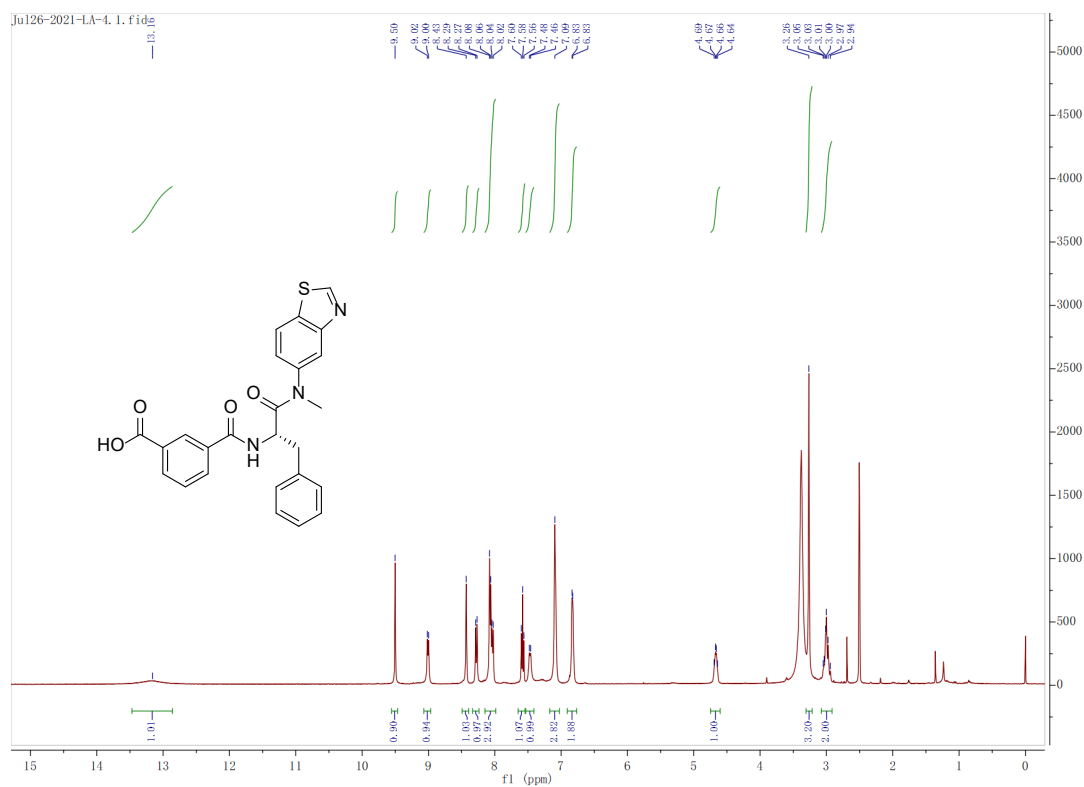
4



5

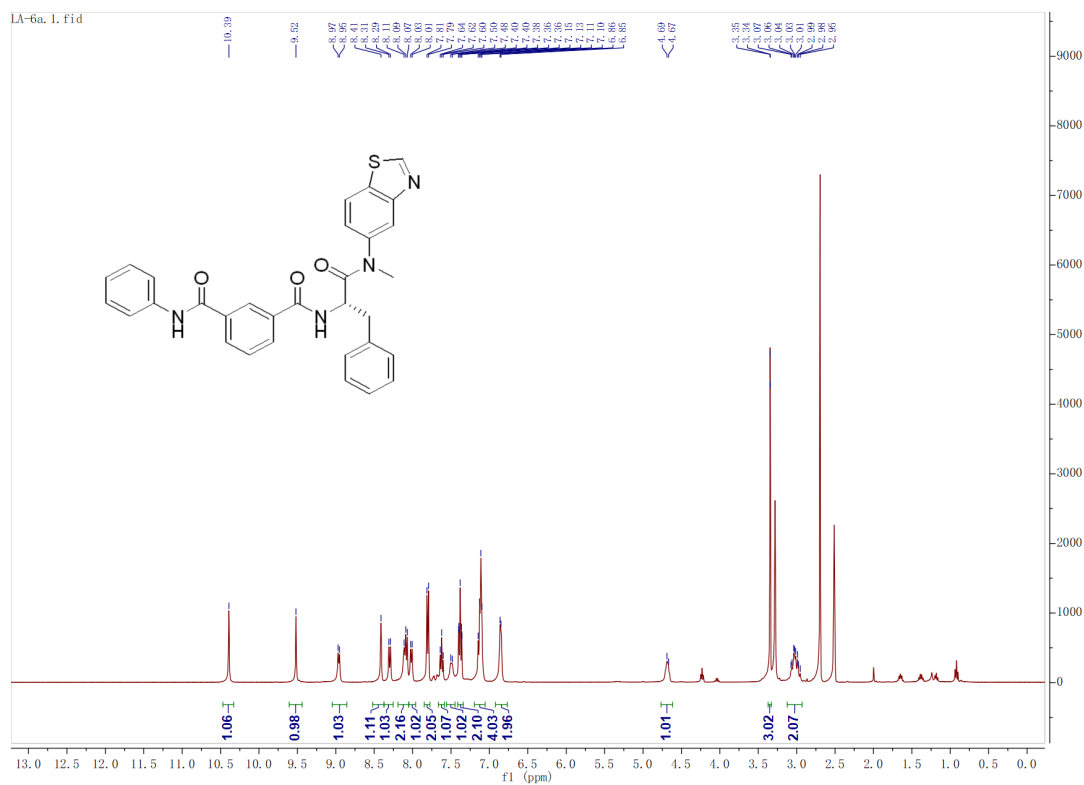


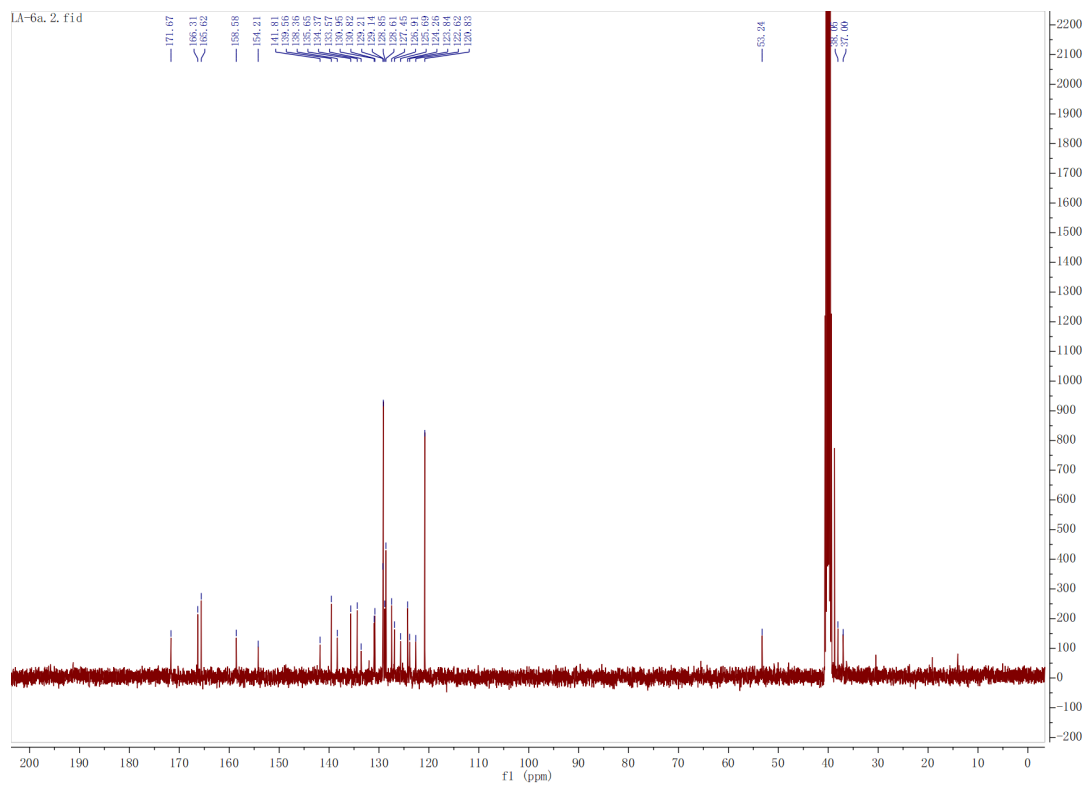
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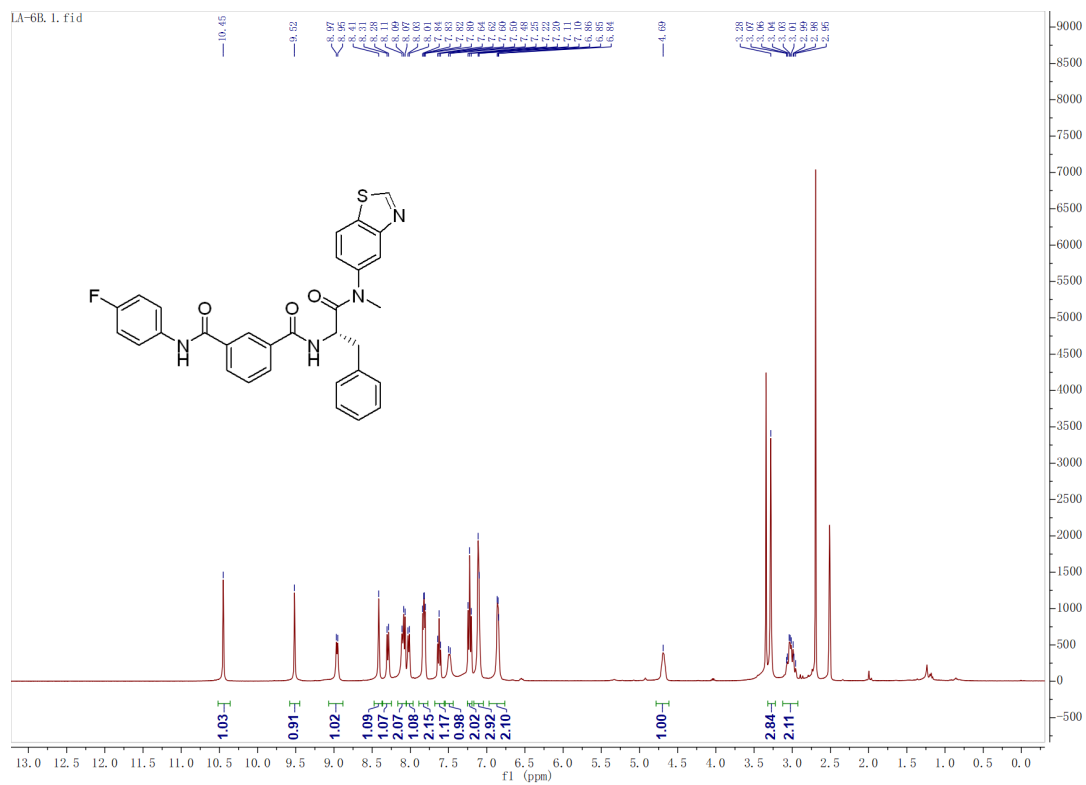
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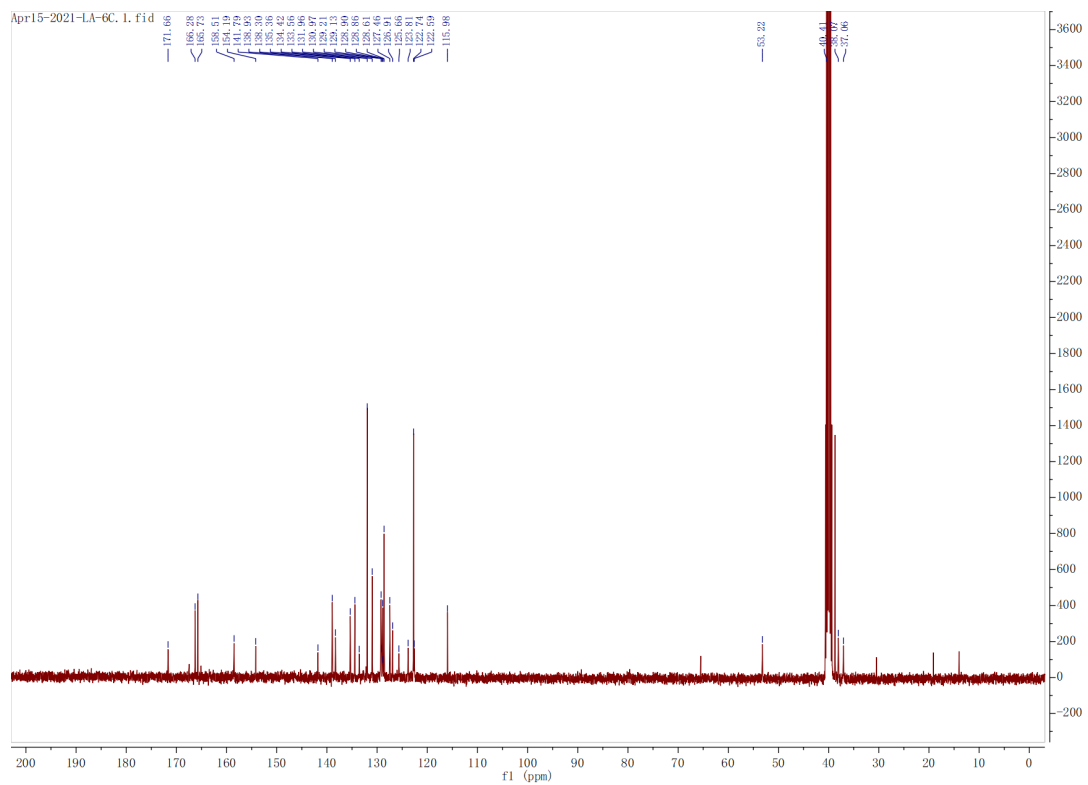
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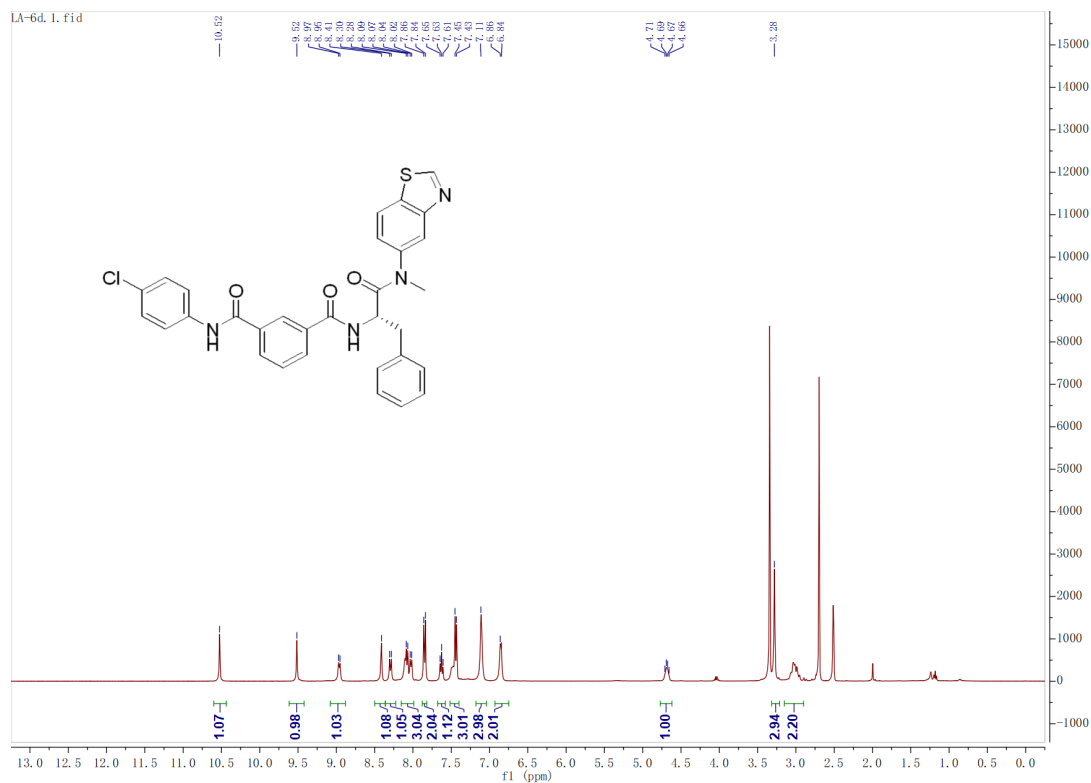


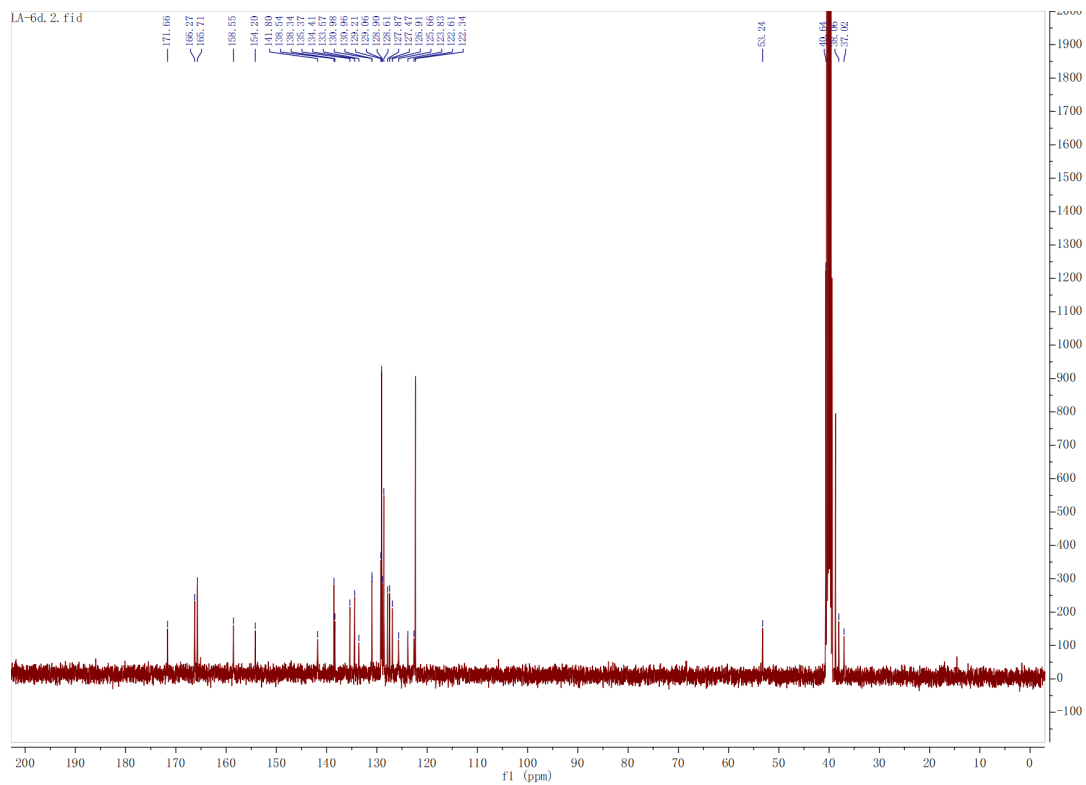
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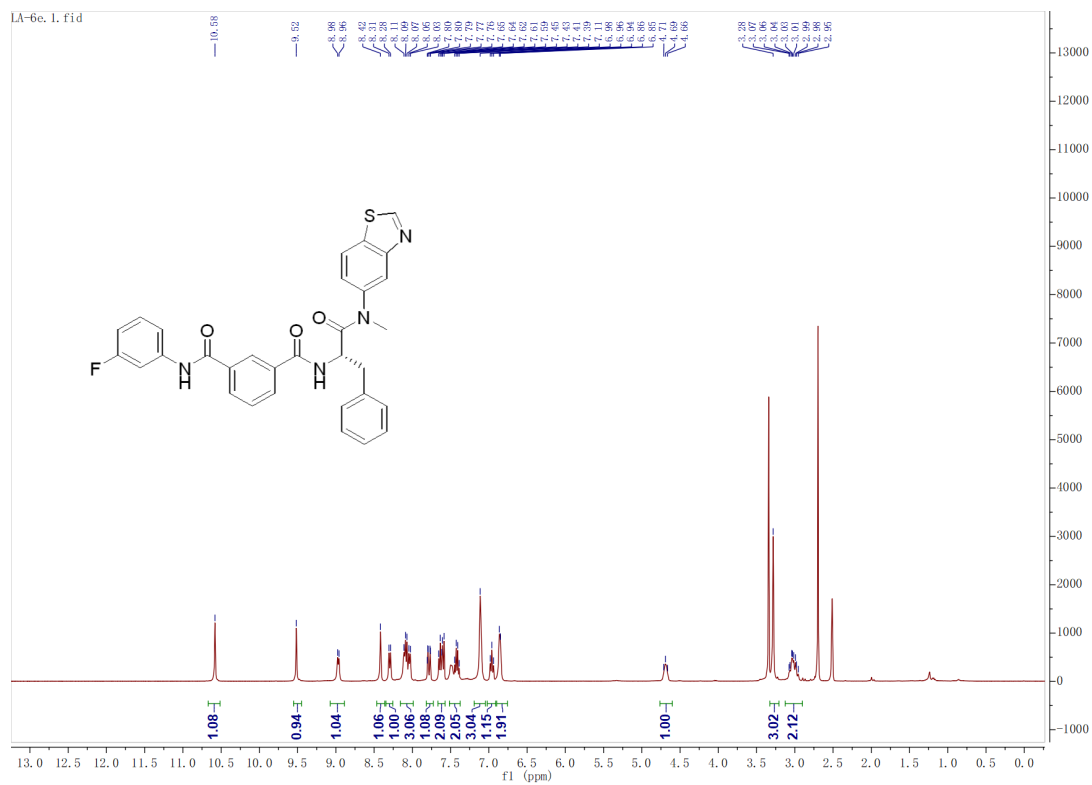


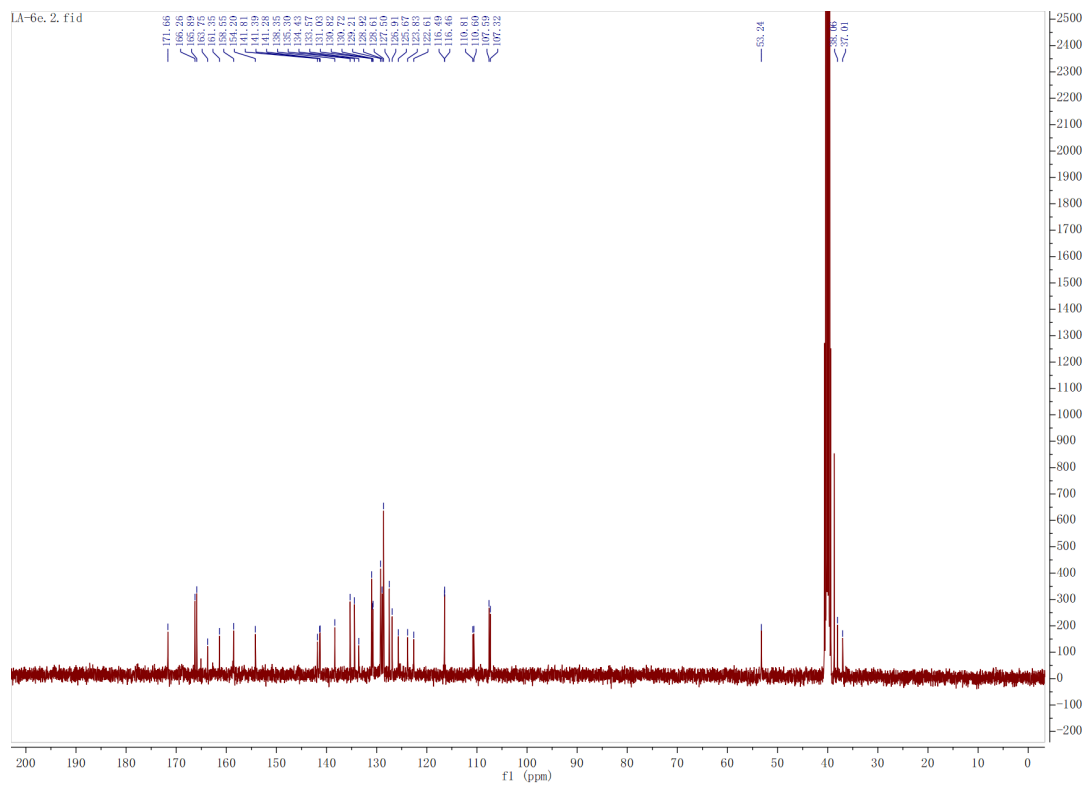
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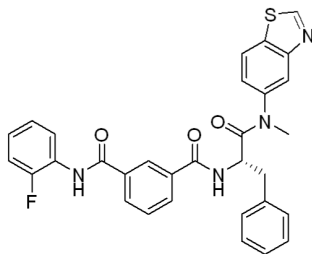
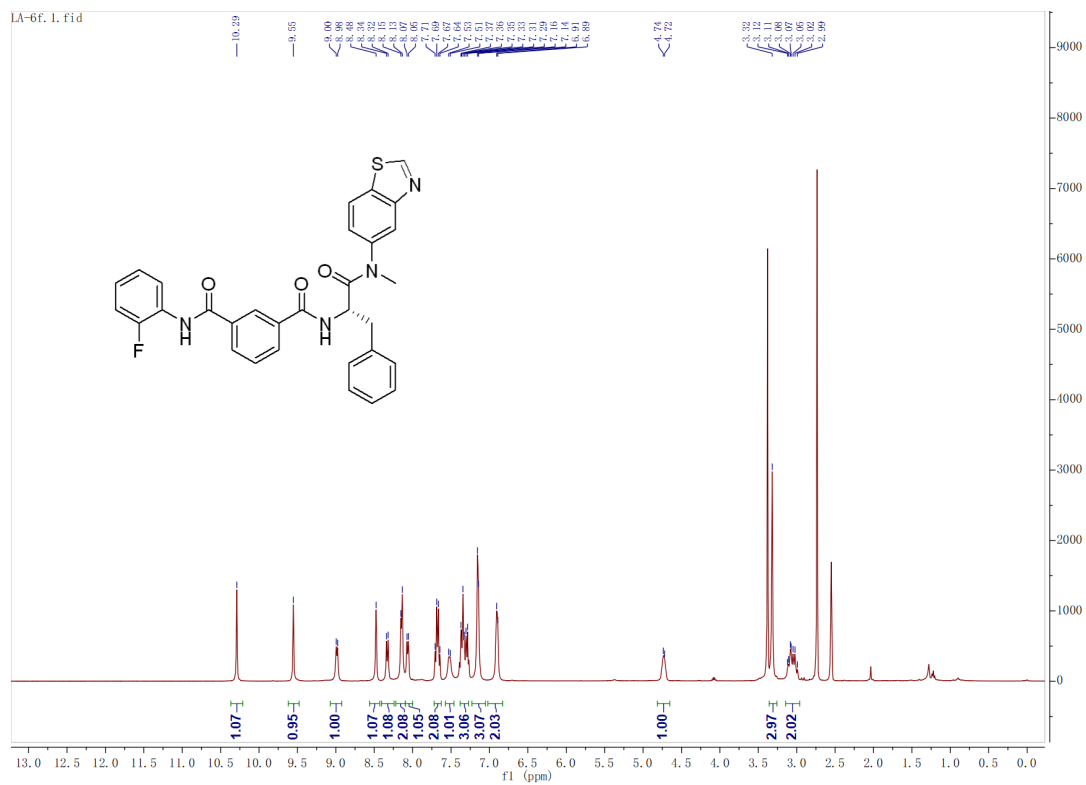


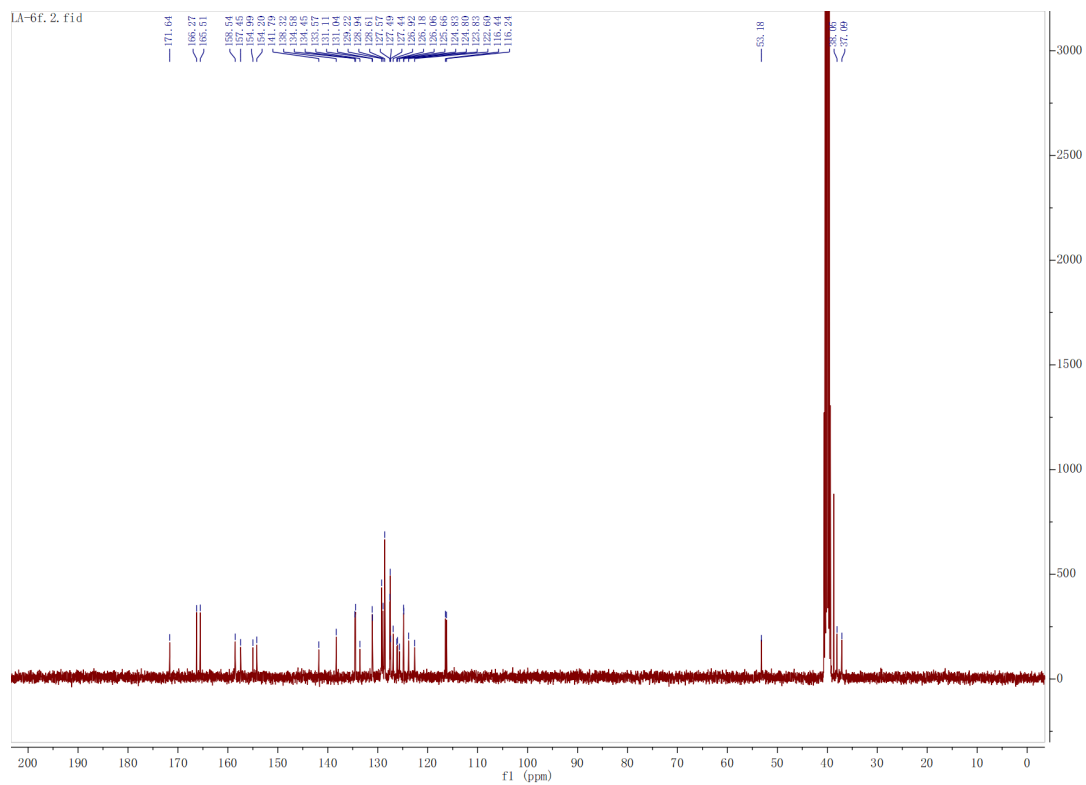
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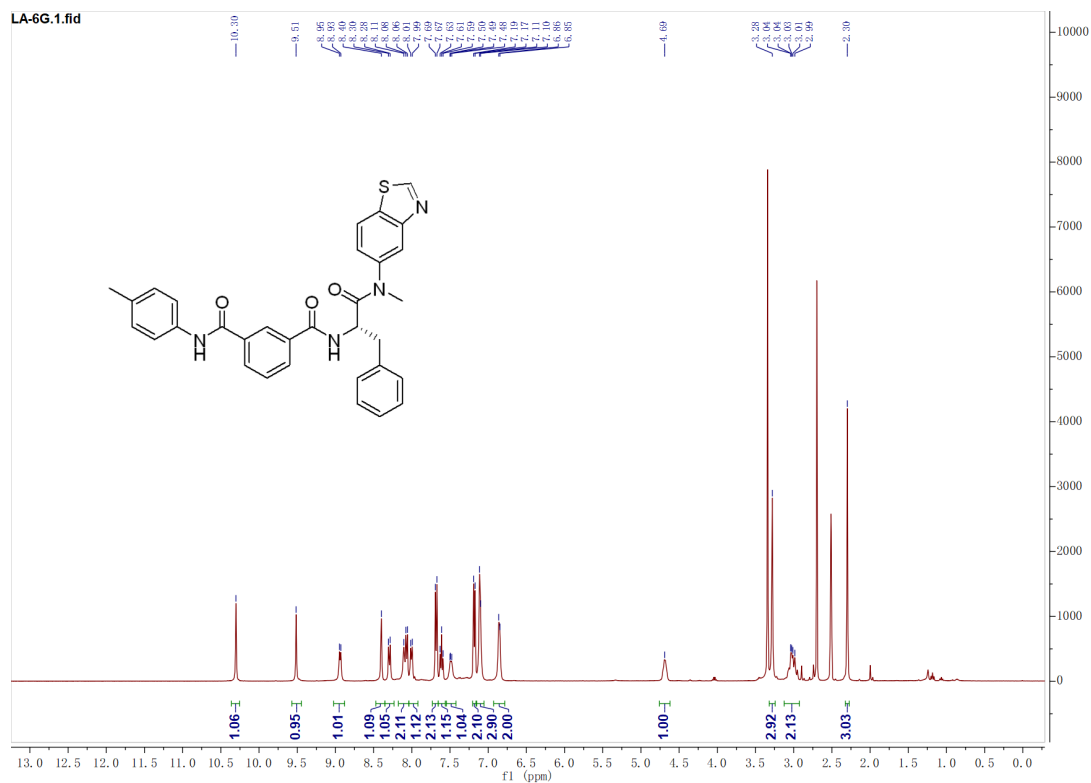


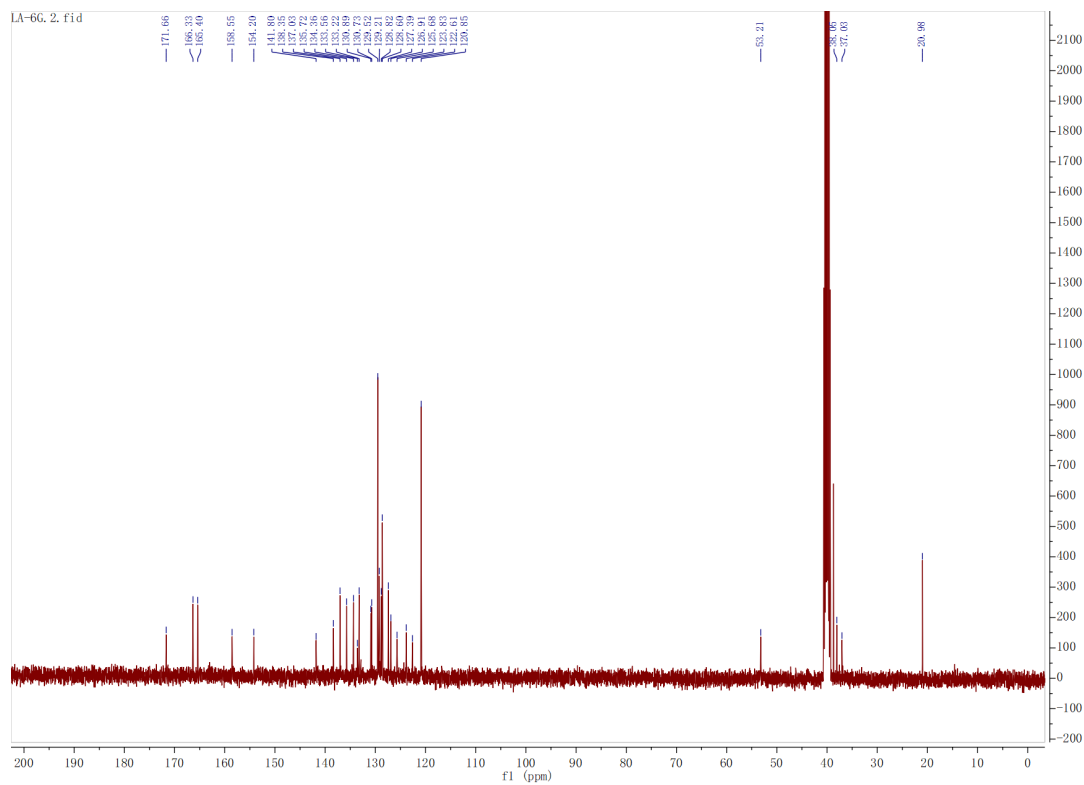
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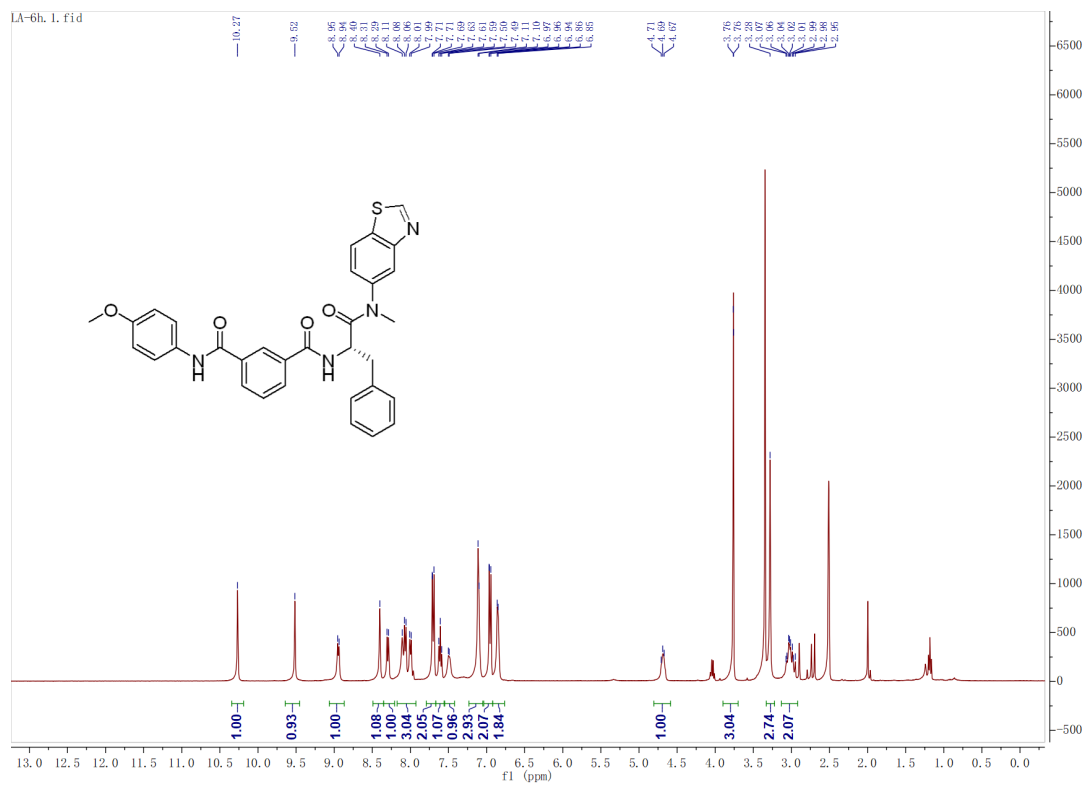


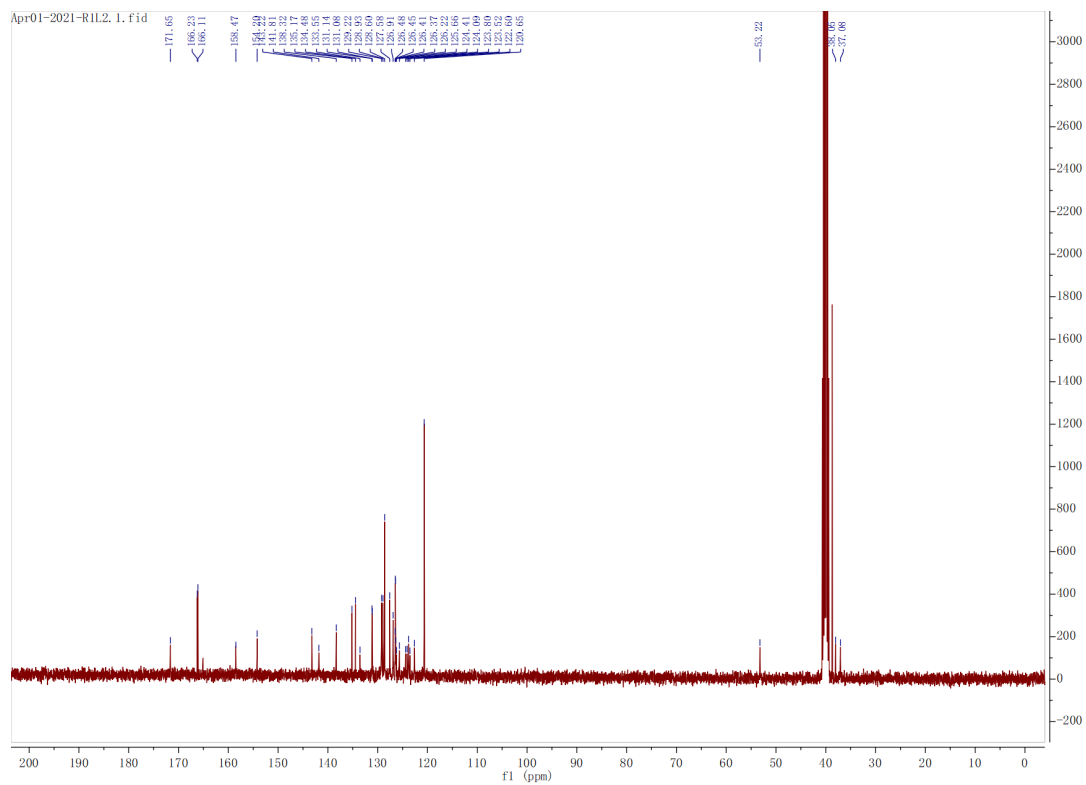
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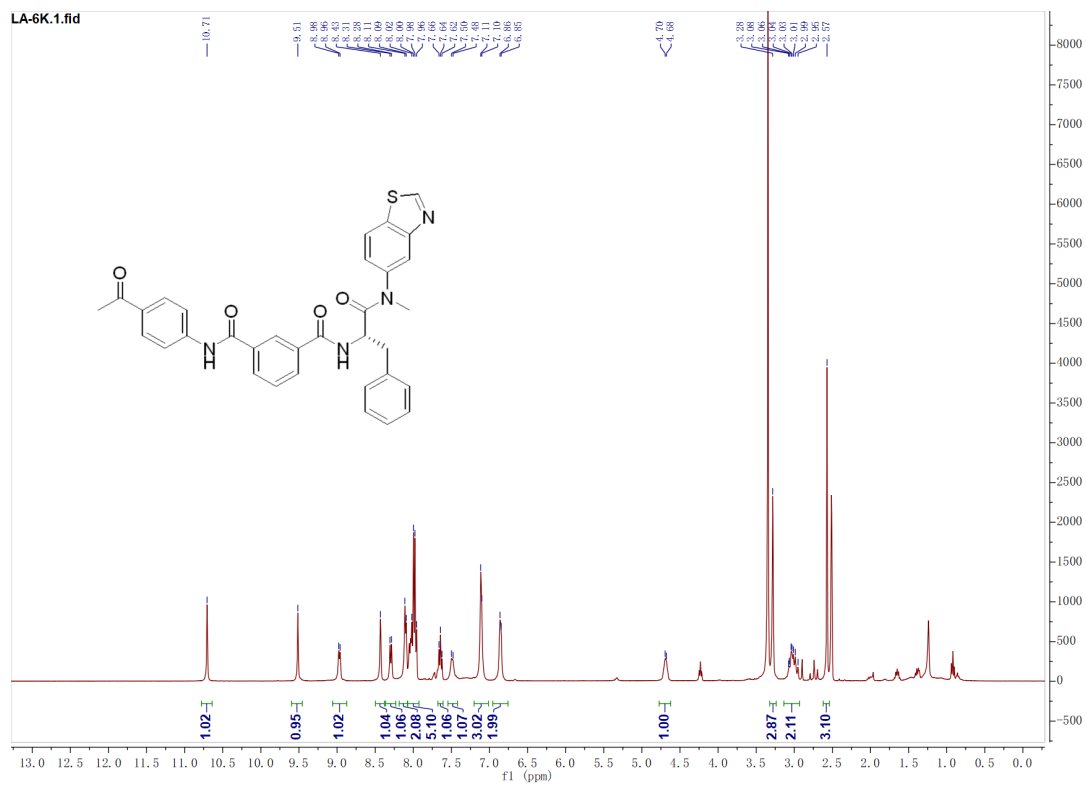


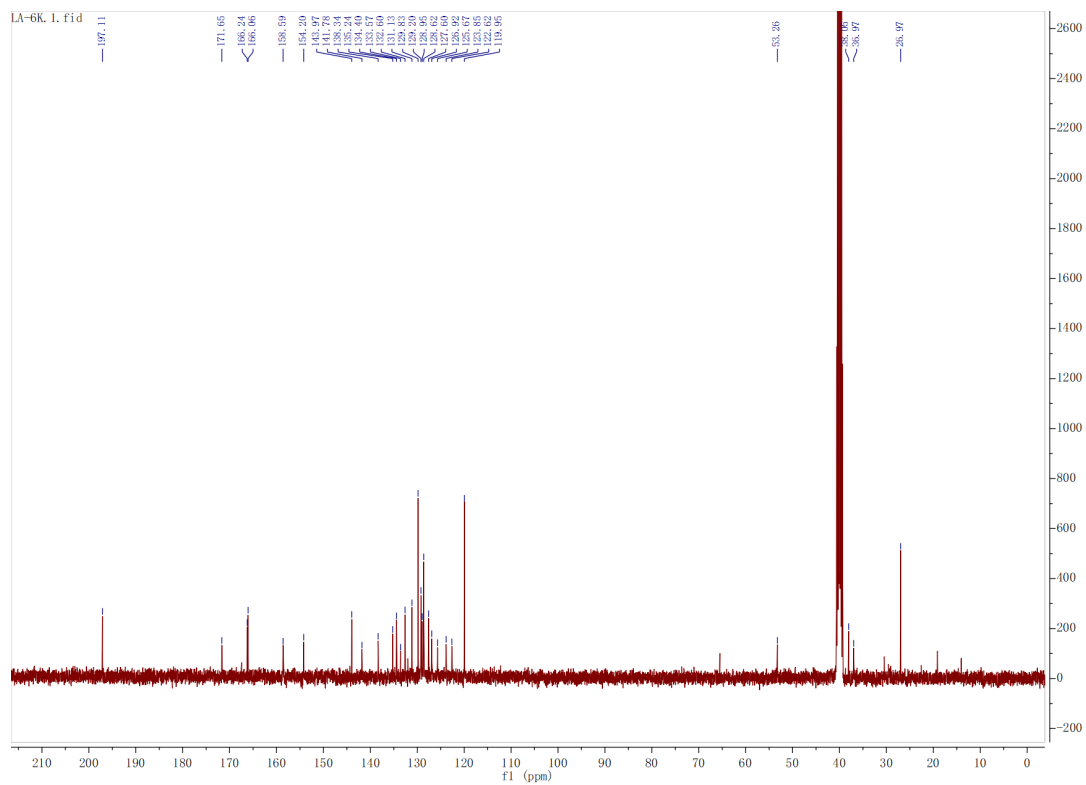
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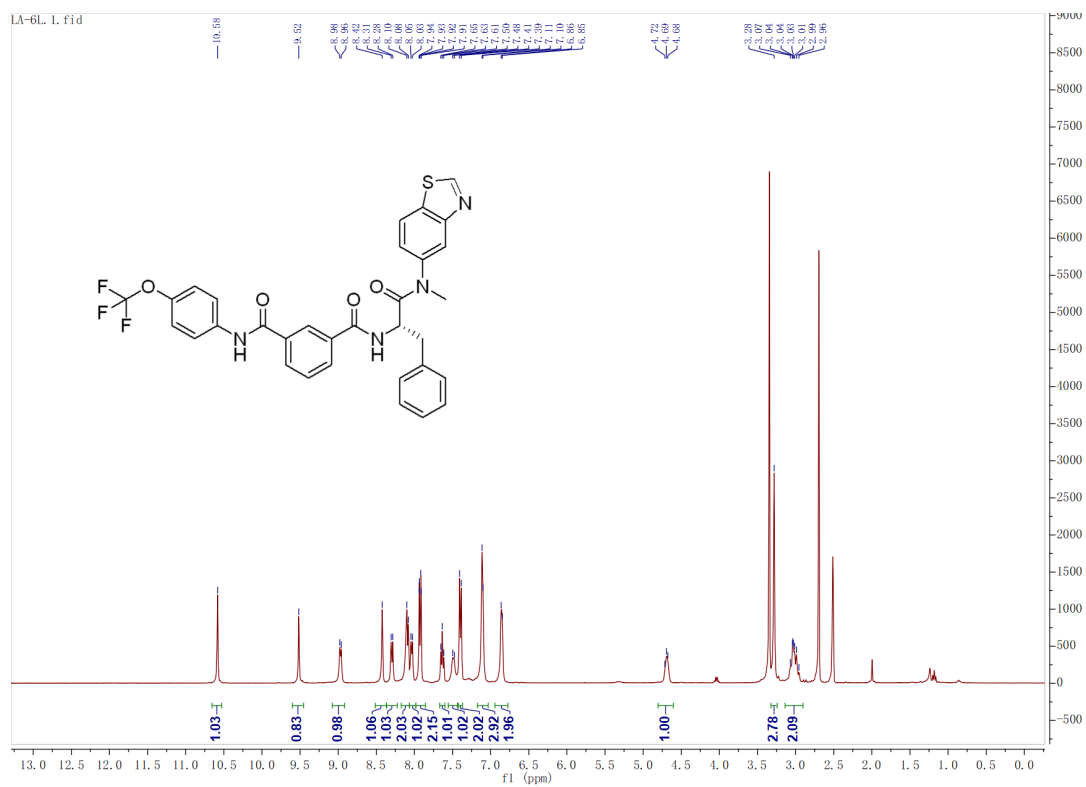


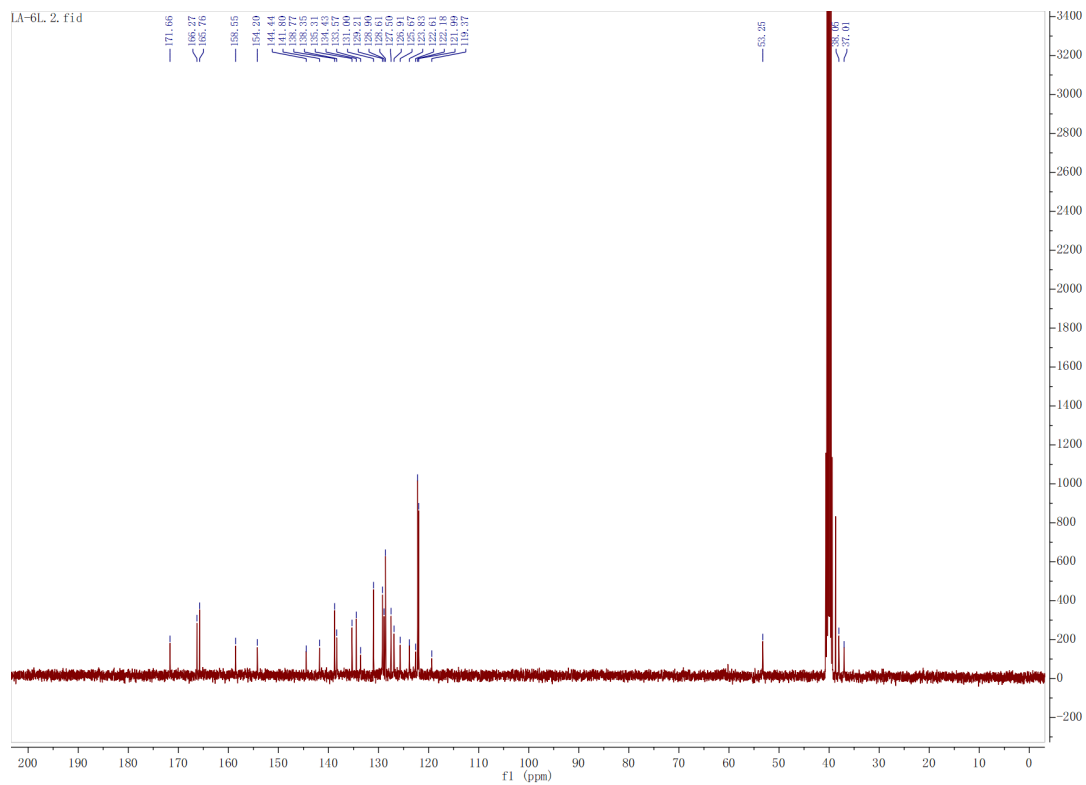
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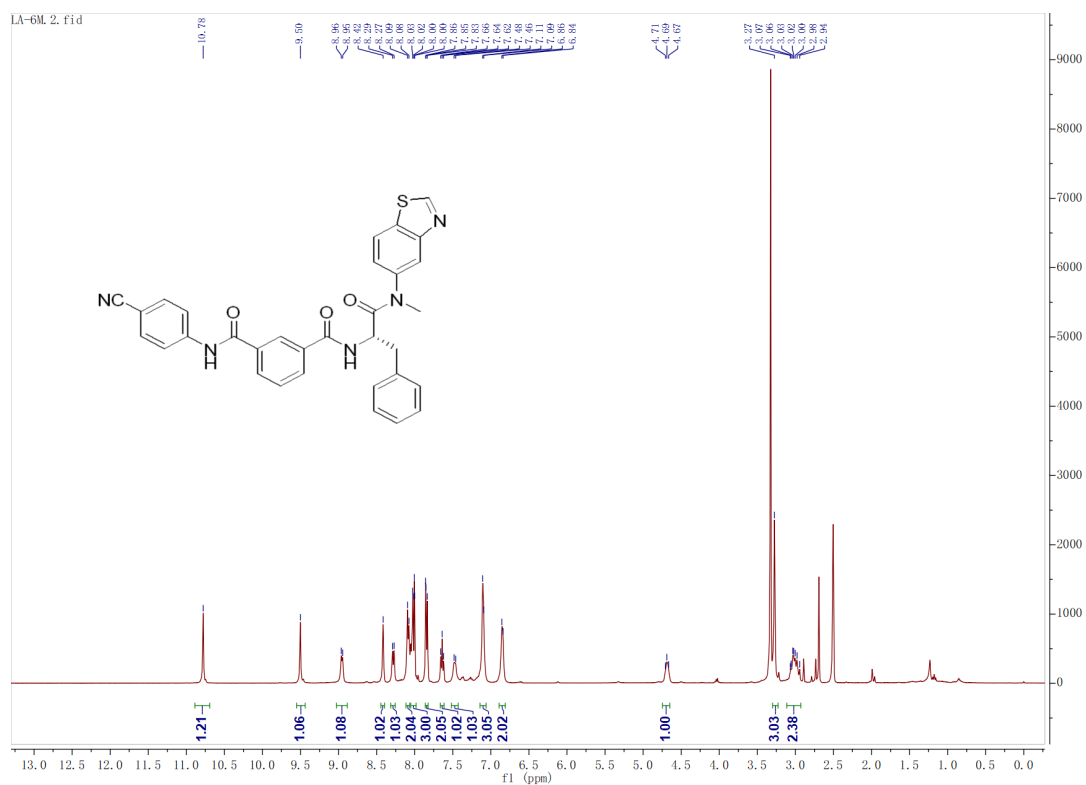


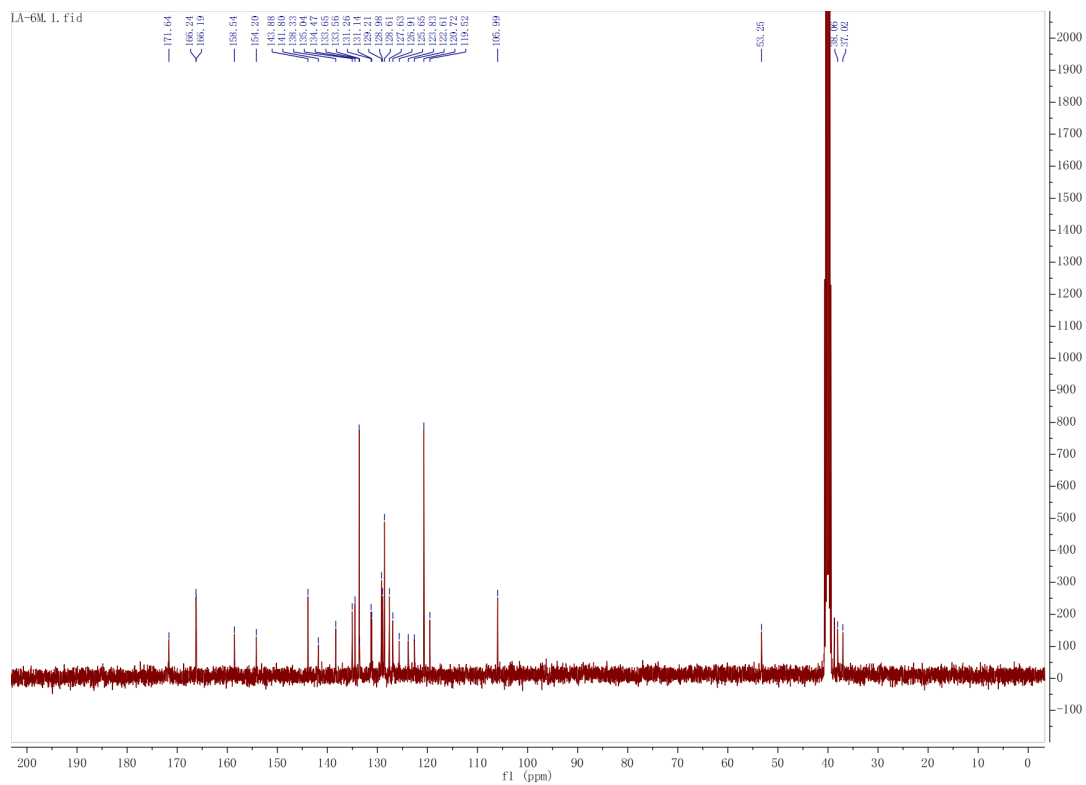
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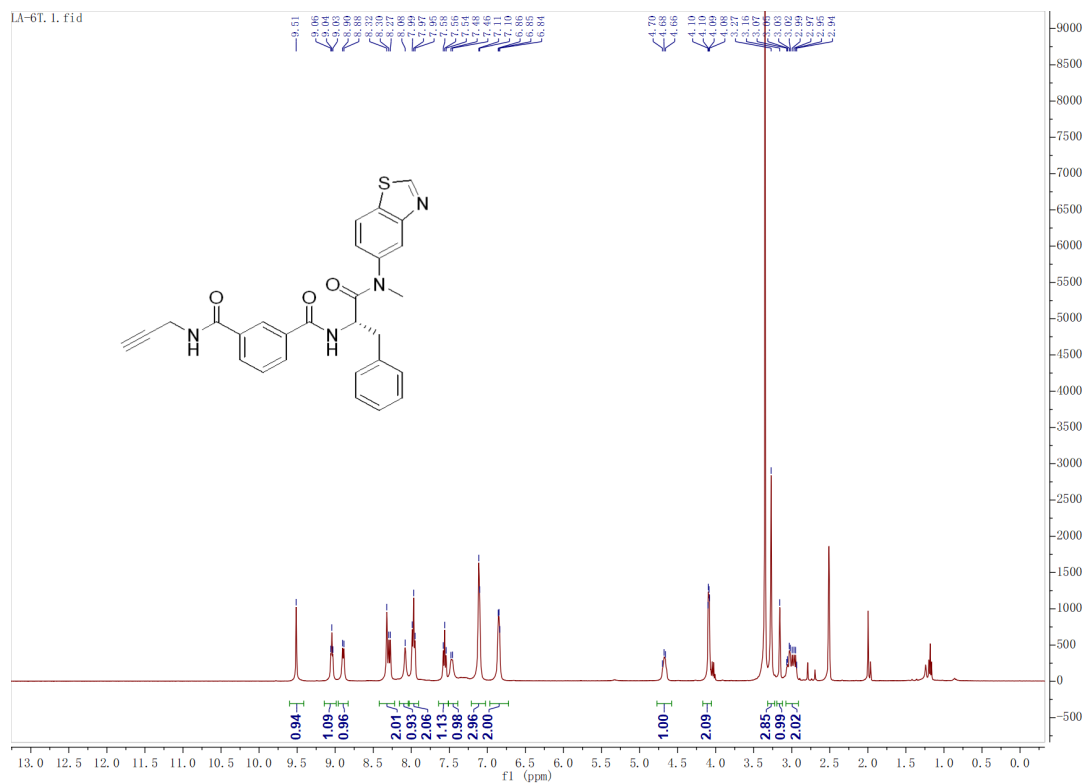


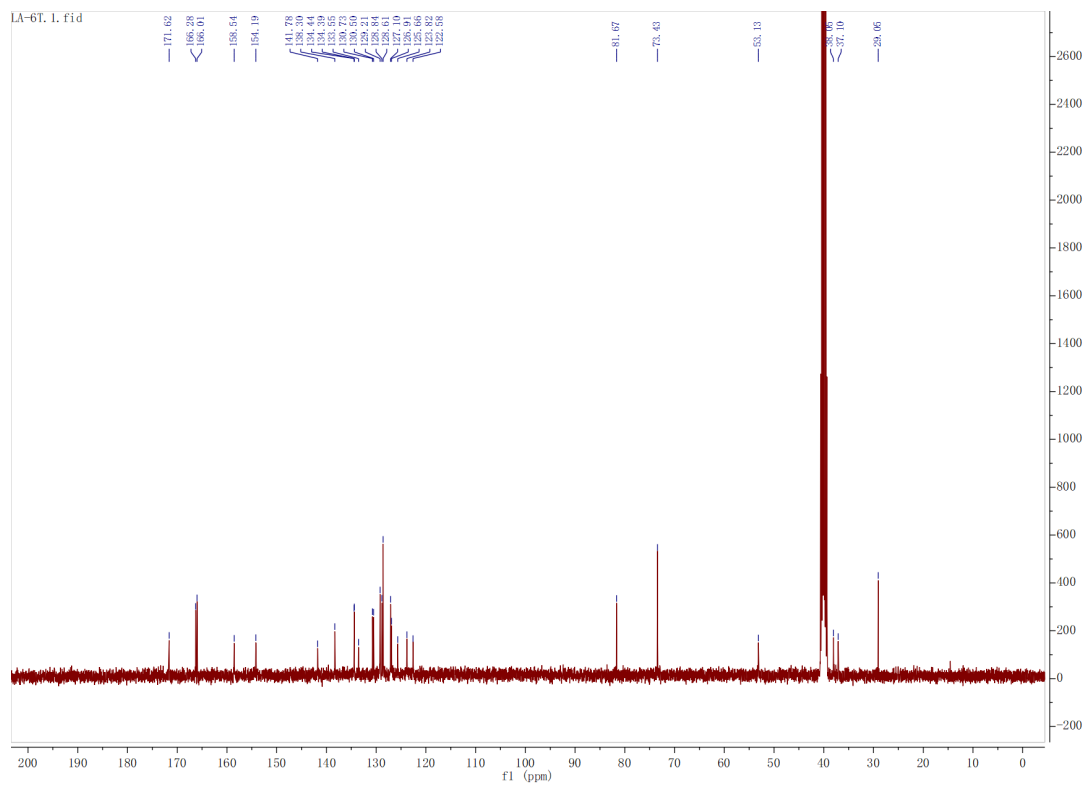
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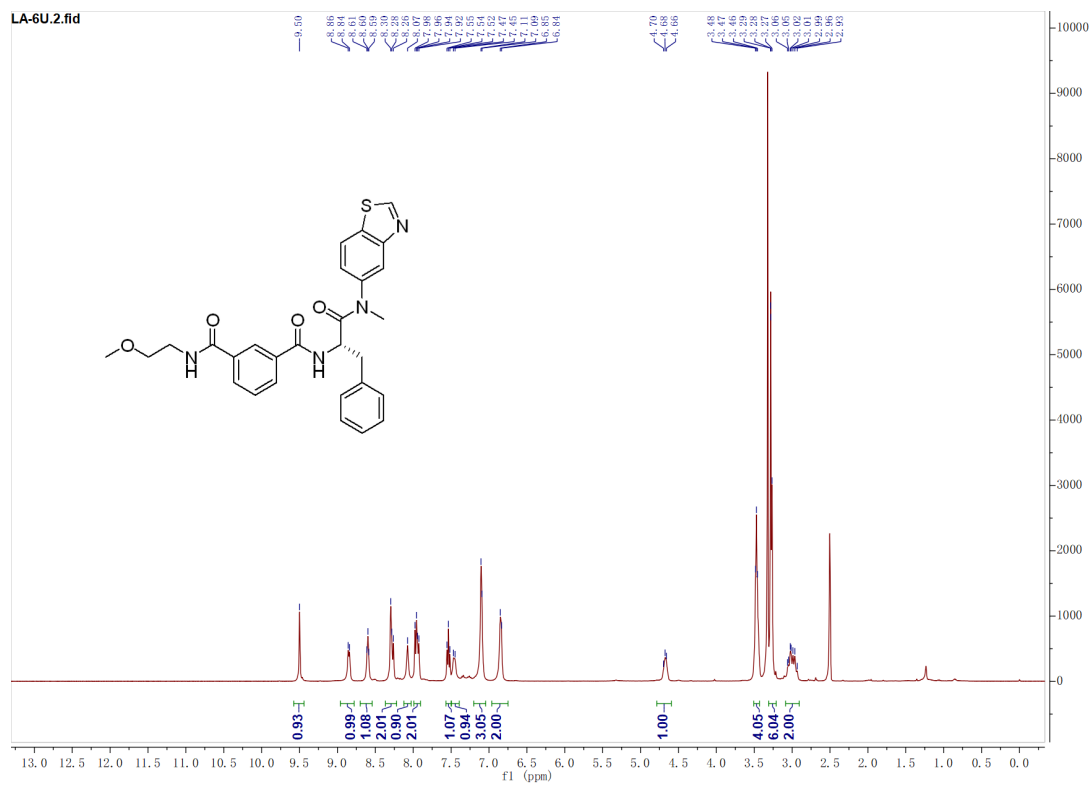


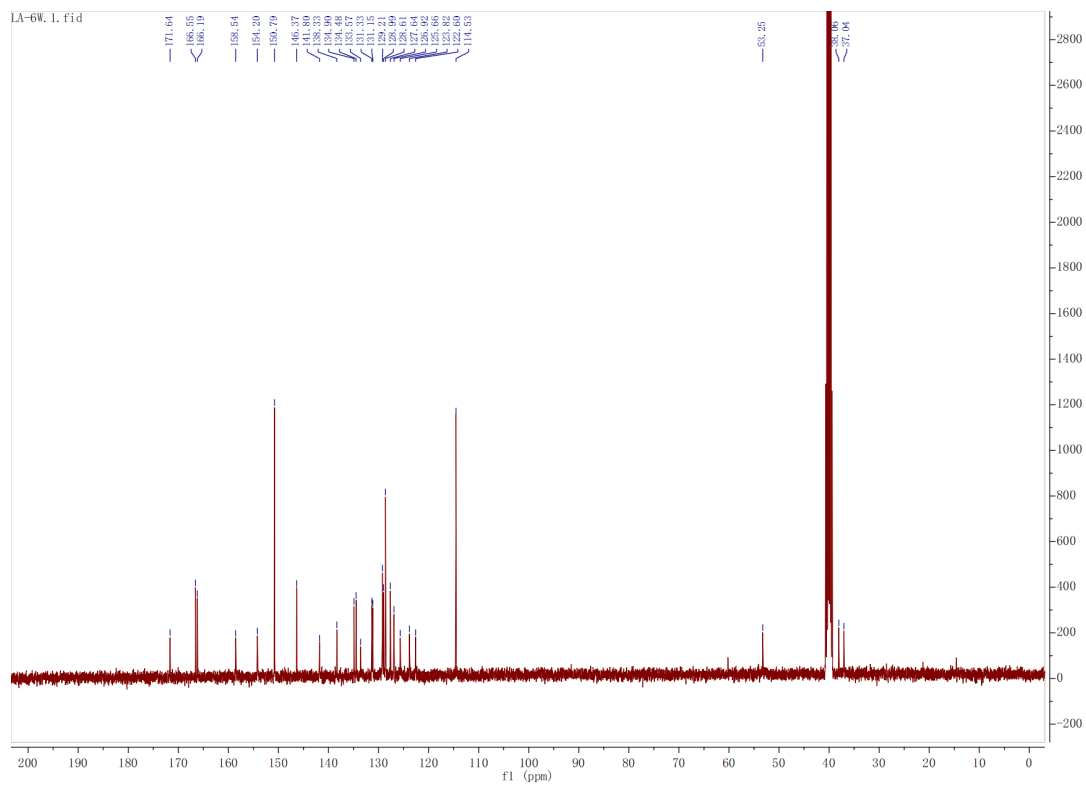
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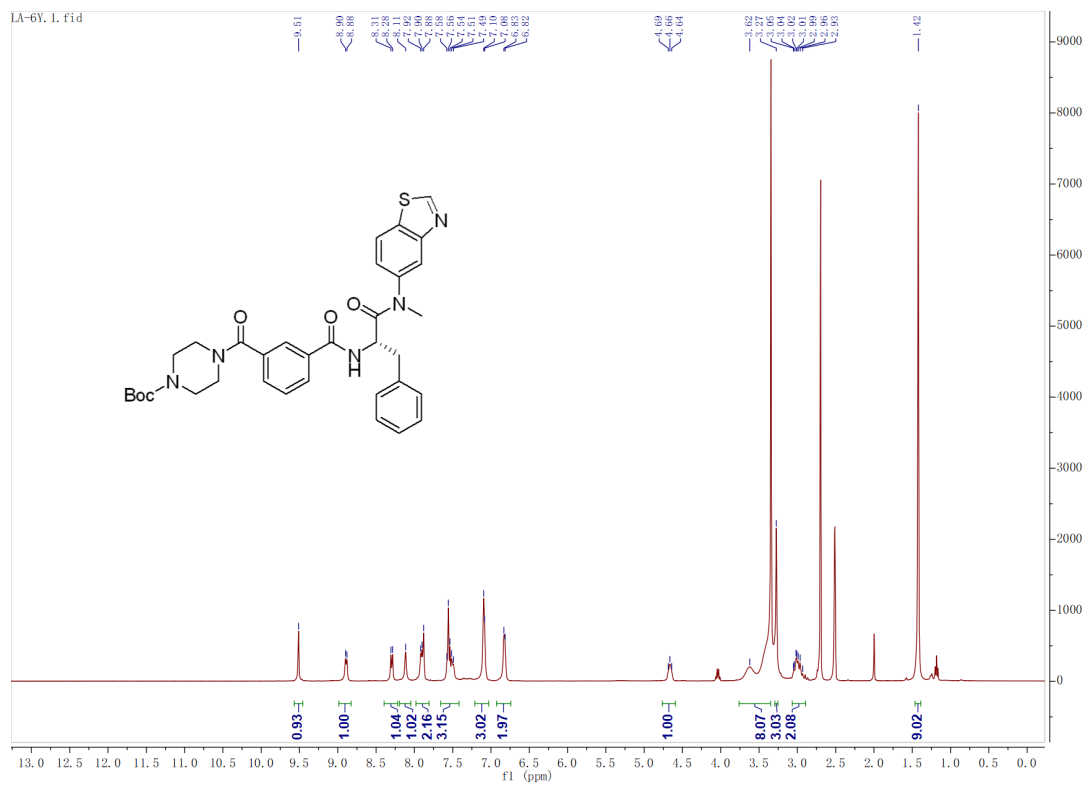


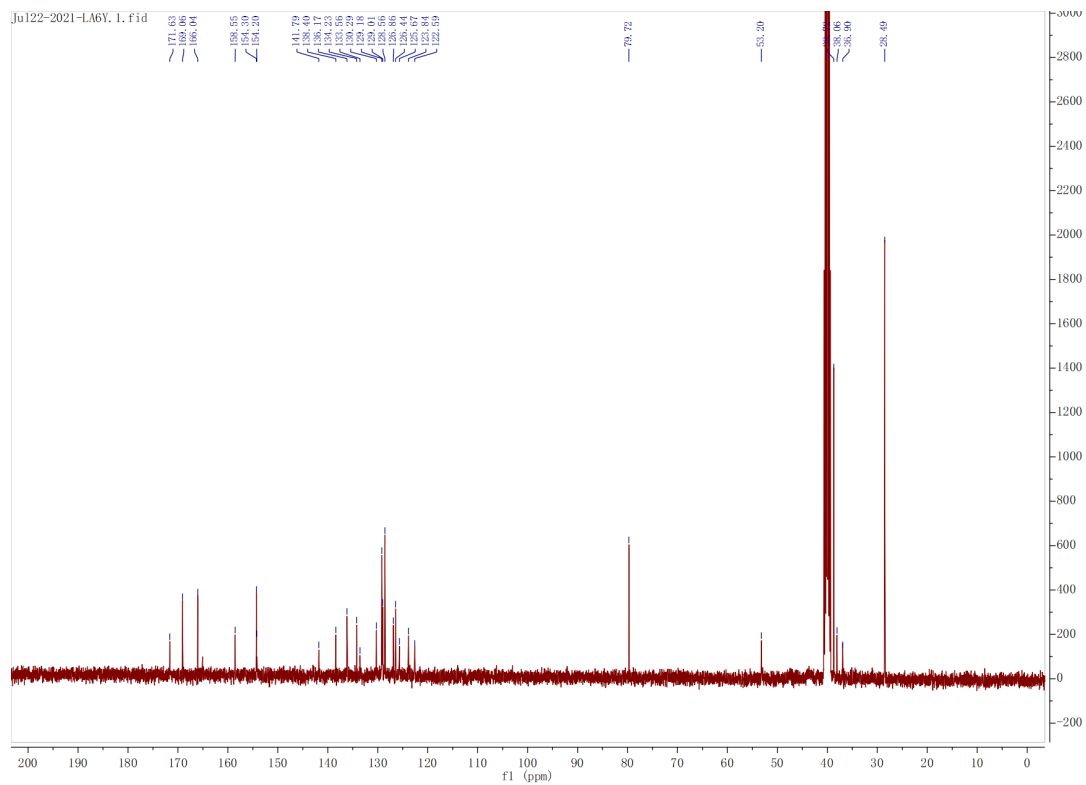
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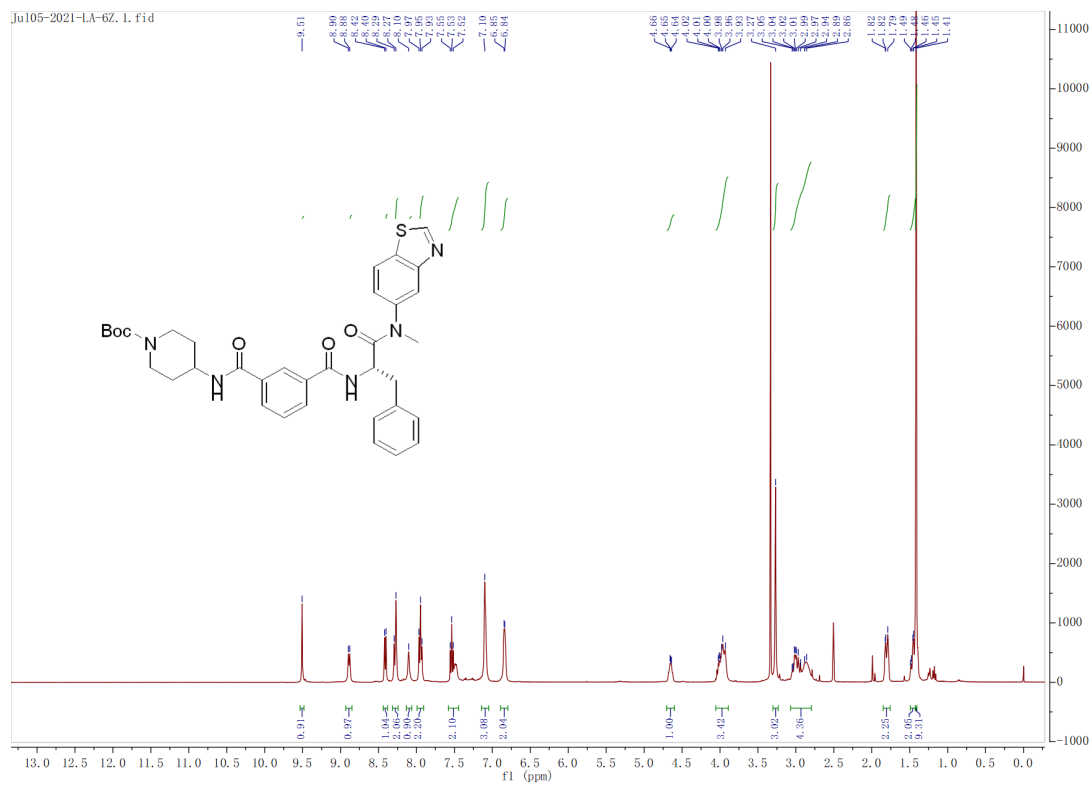


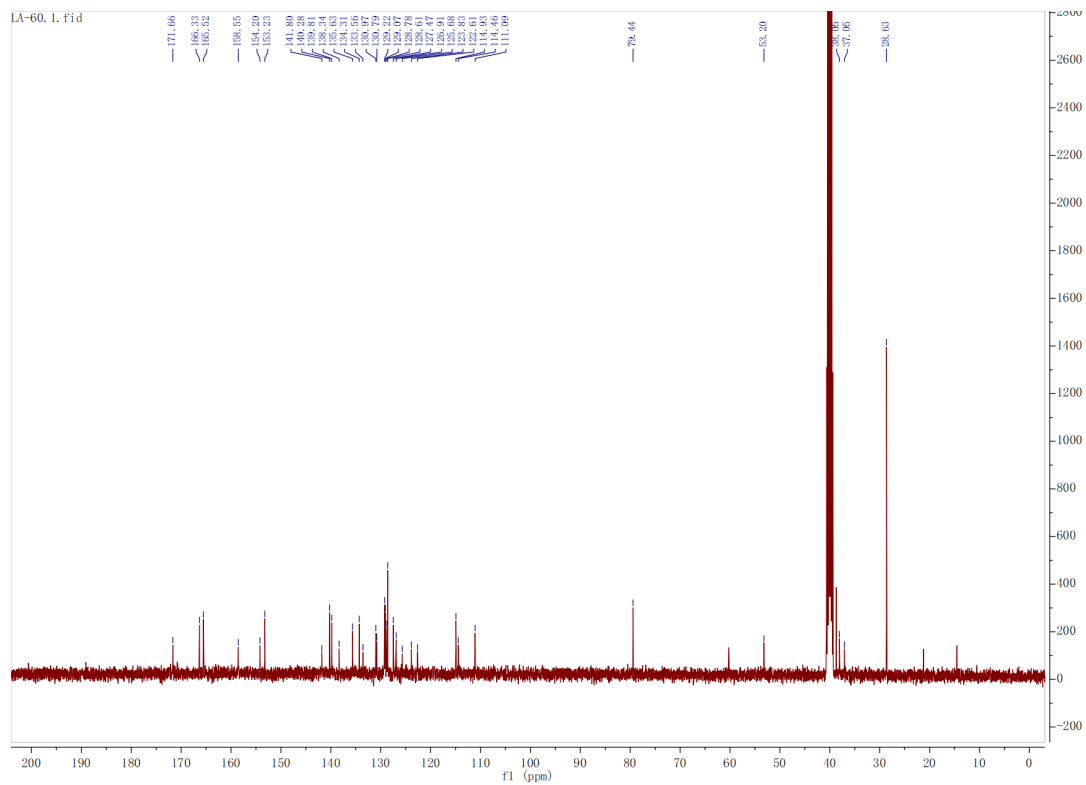
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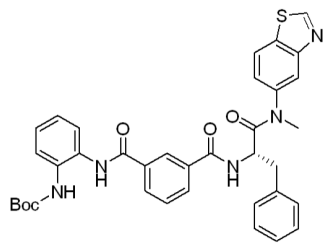
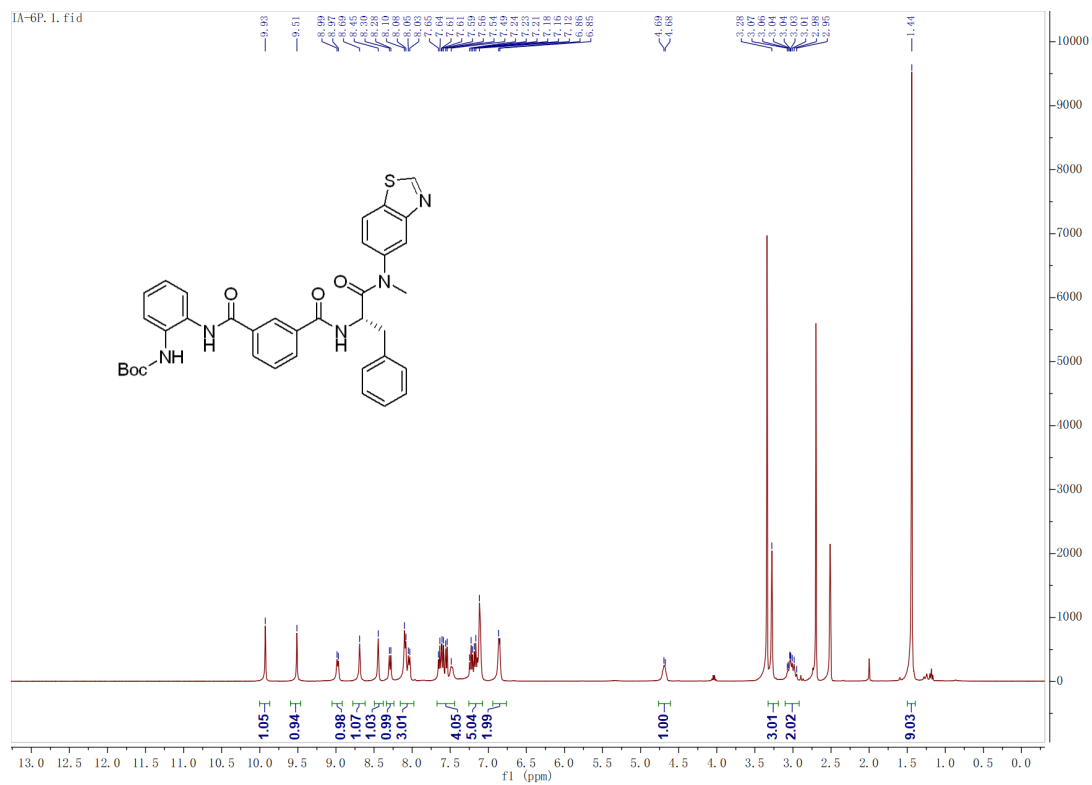


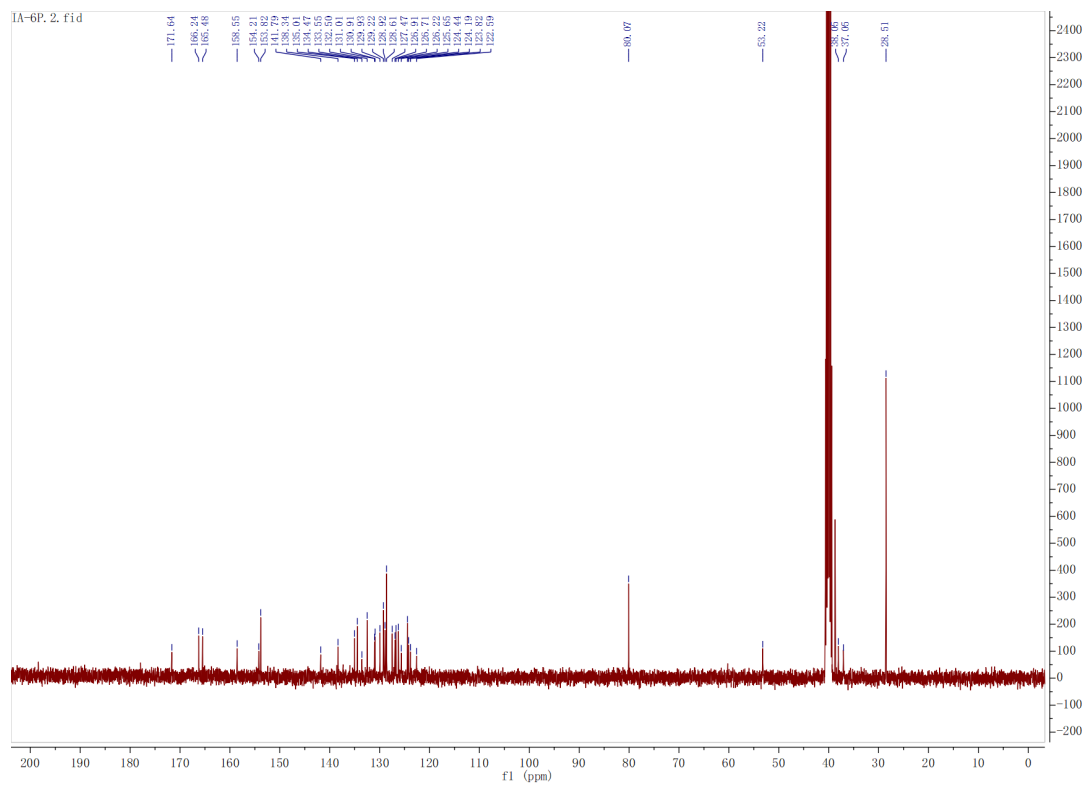
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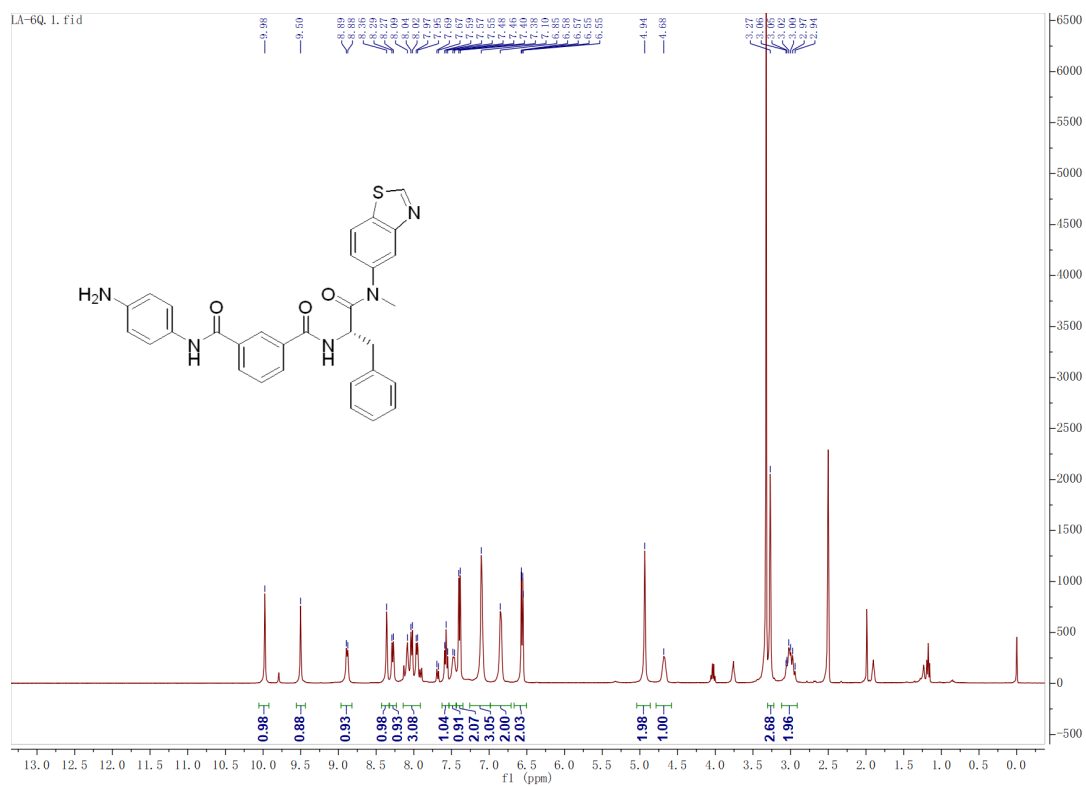


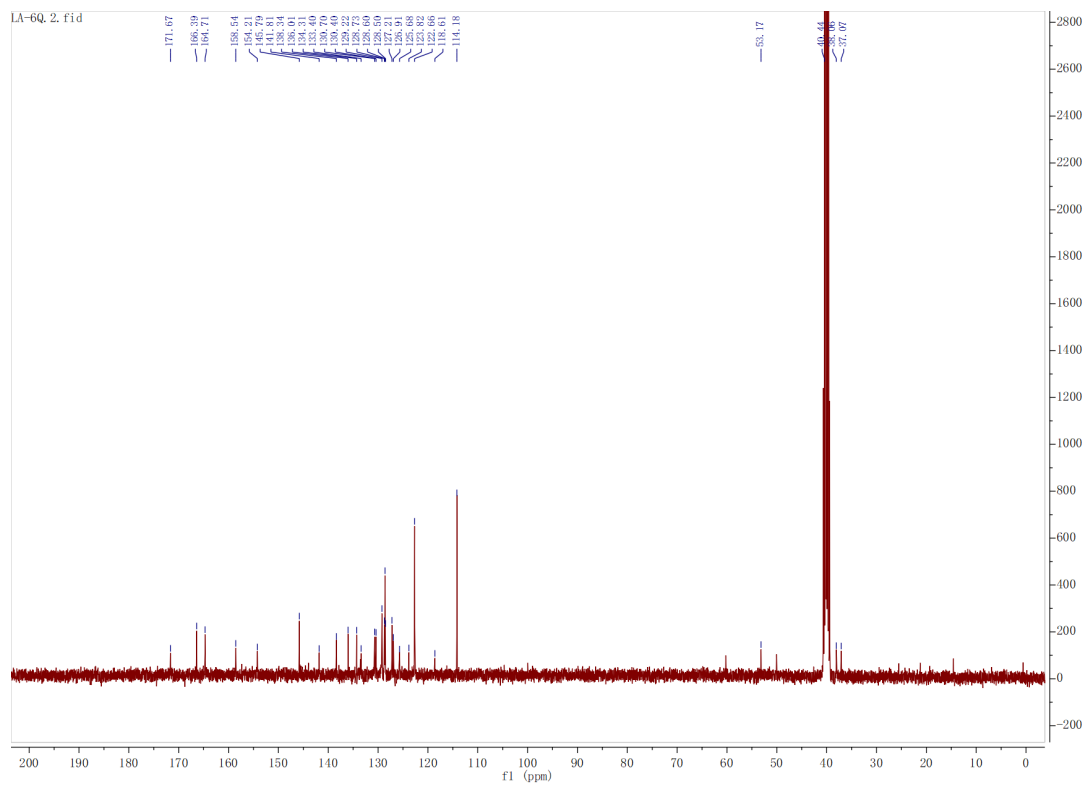
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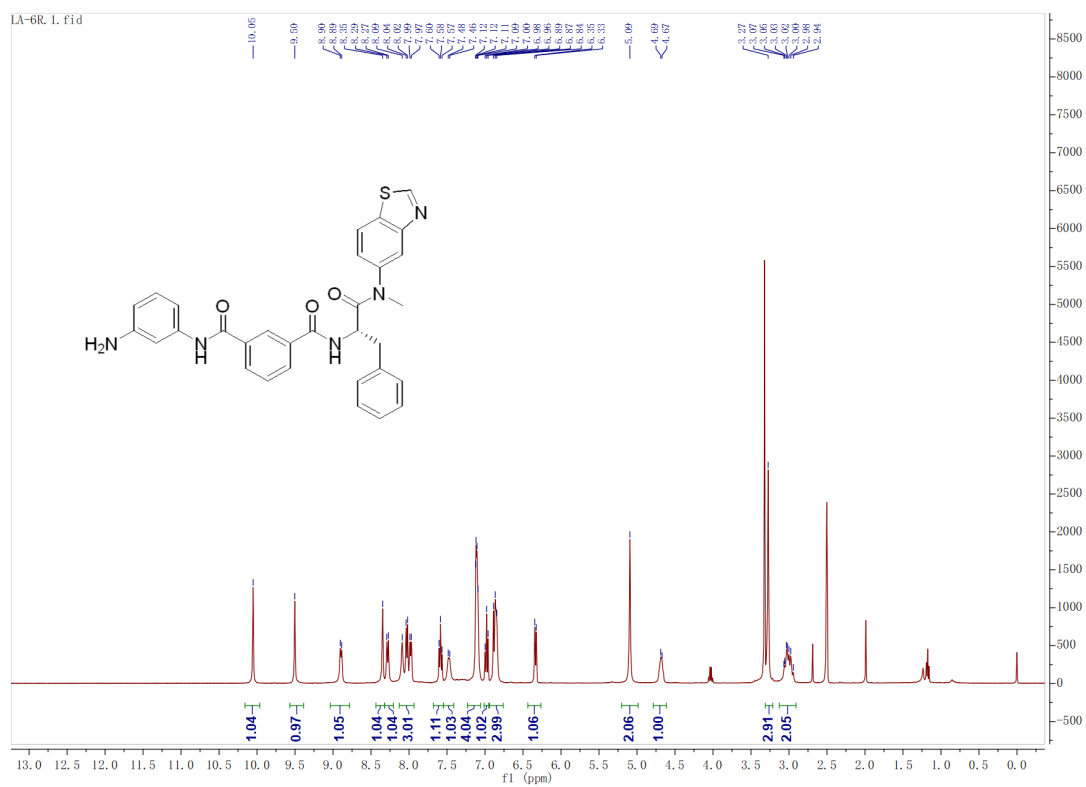


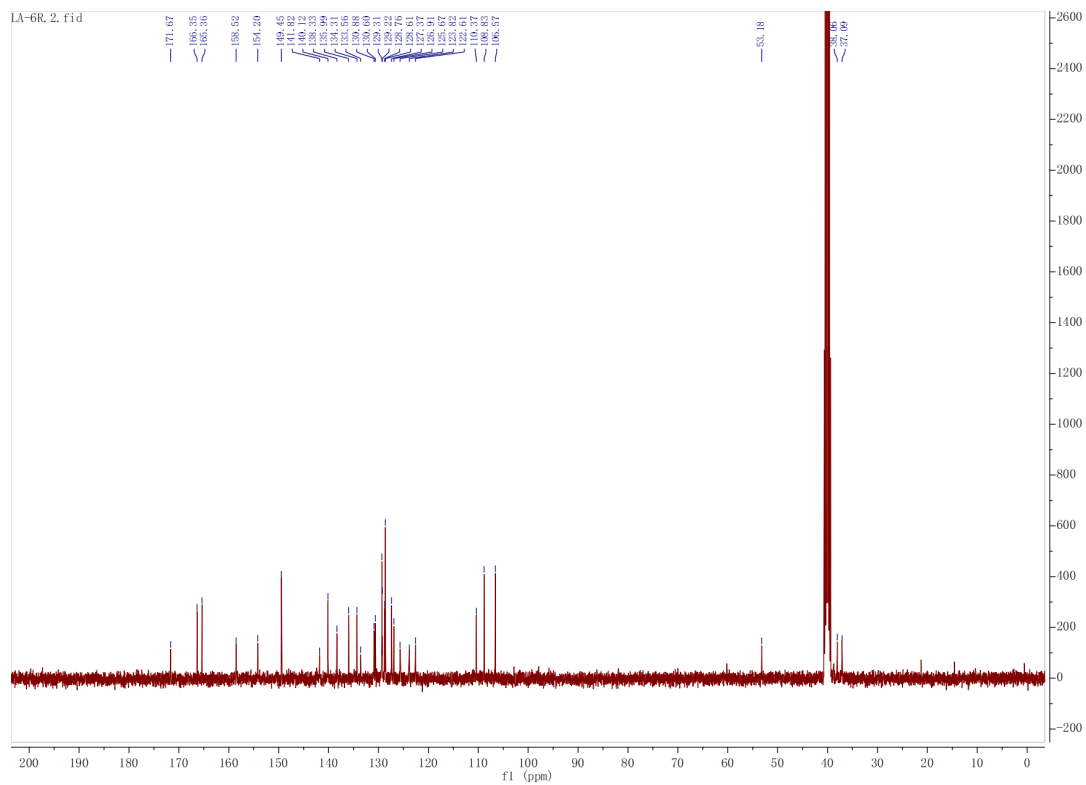
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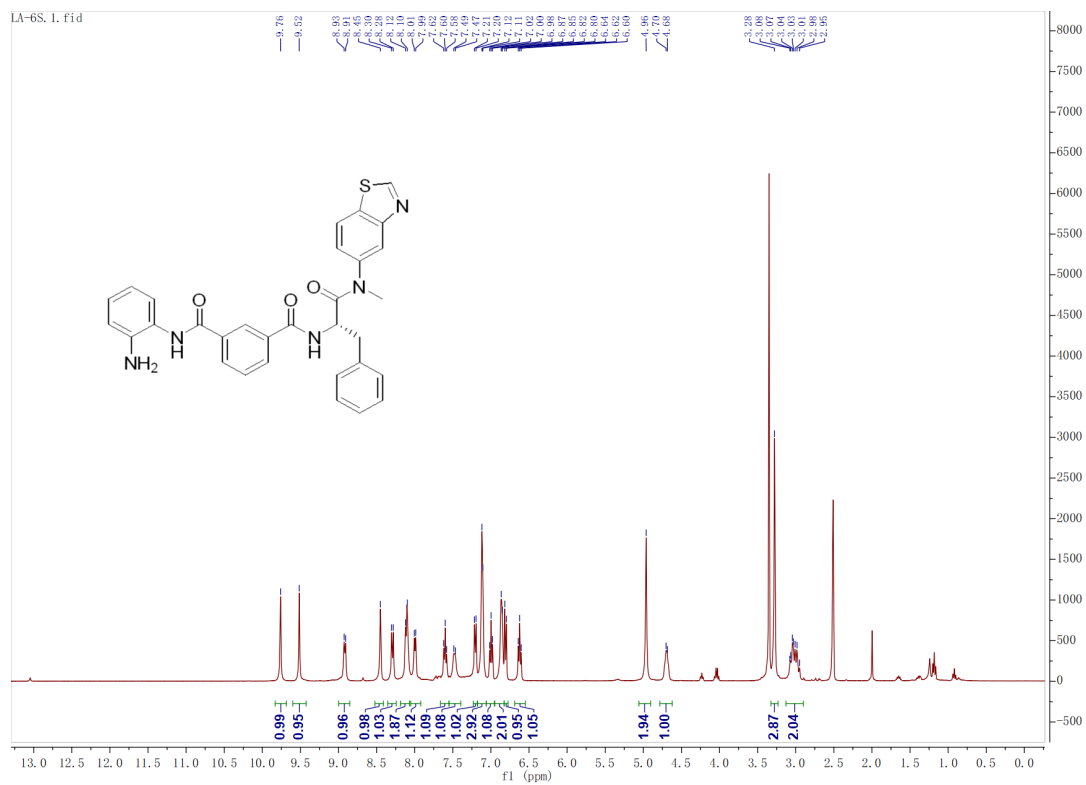


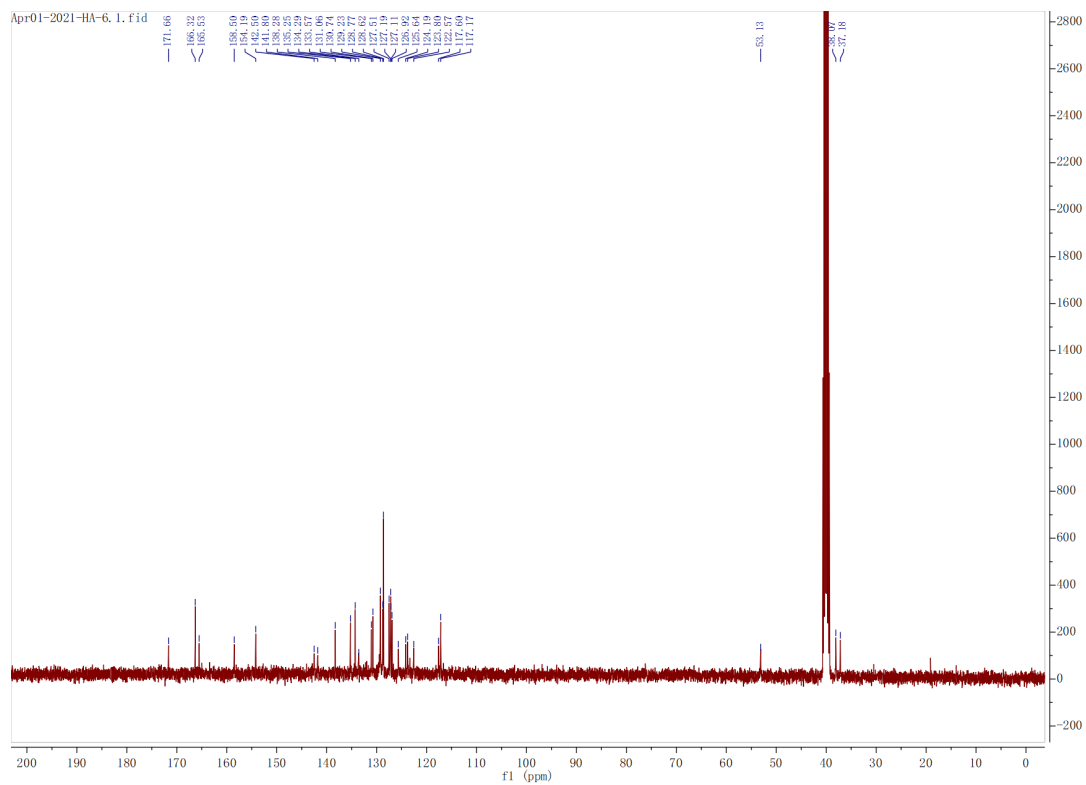
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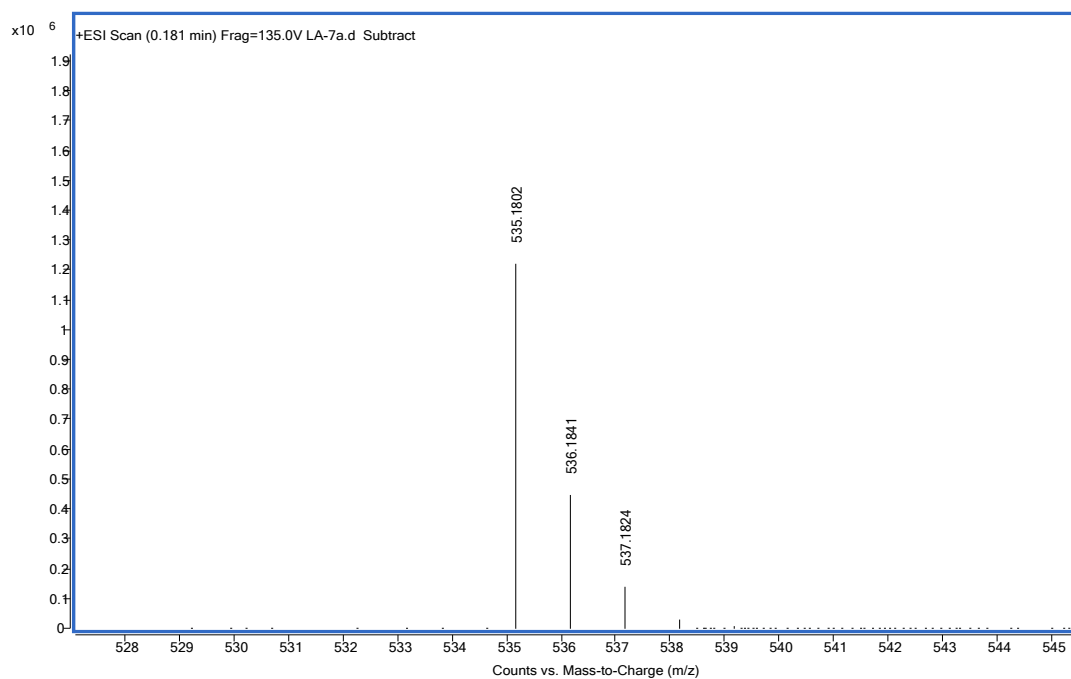
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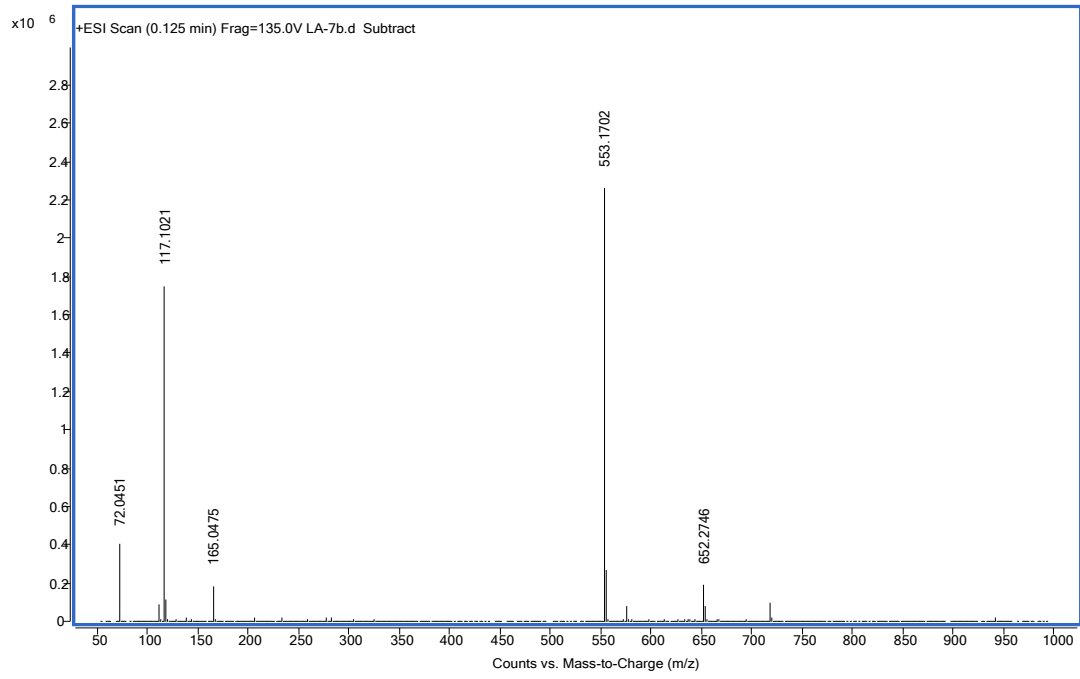


IV. High Resolution Mass Spectrum

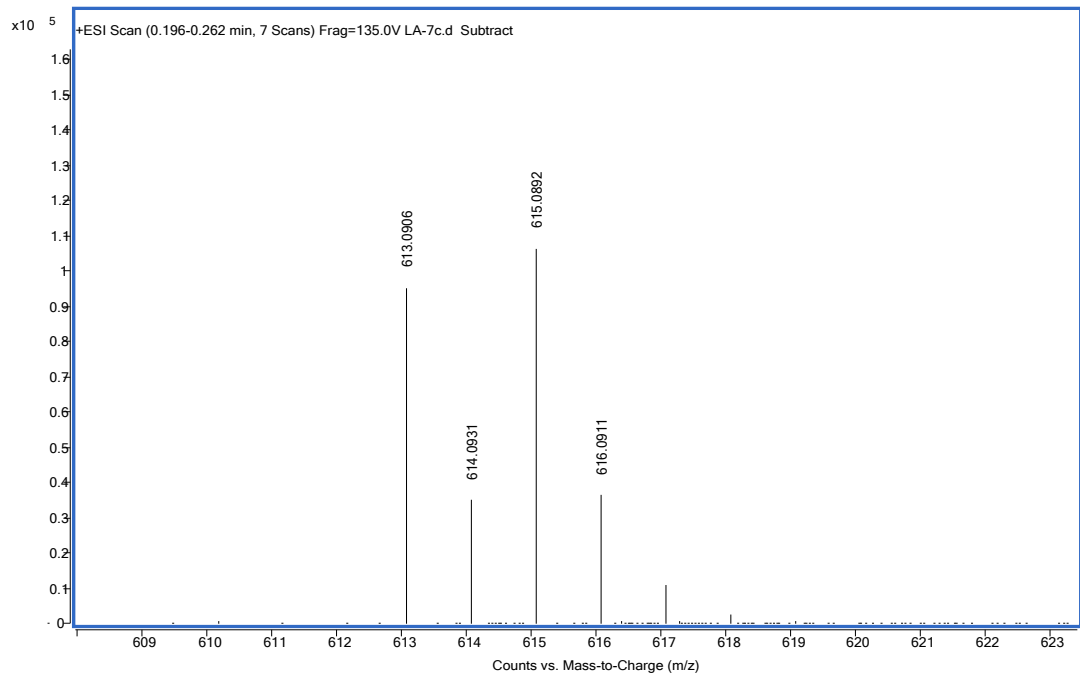
7a



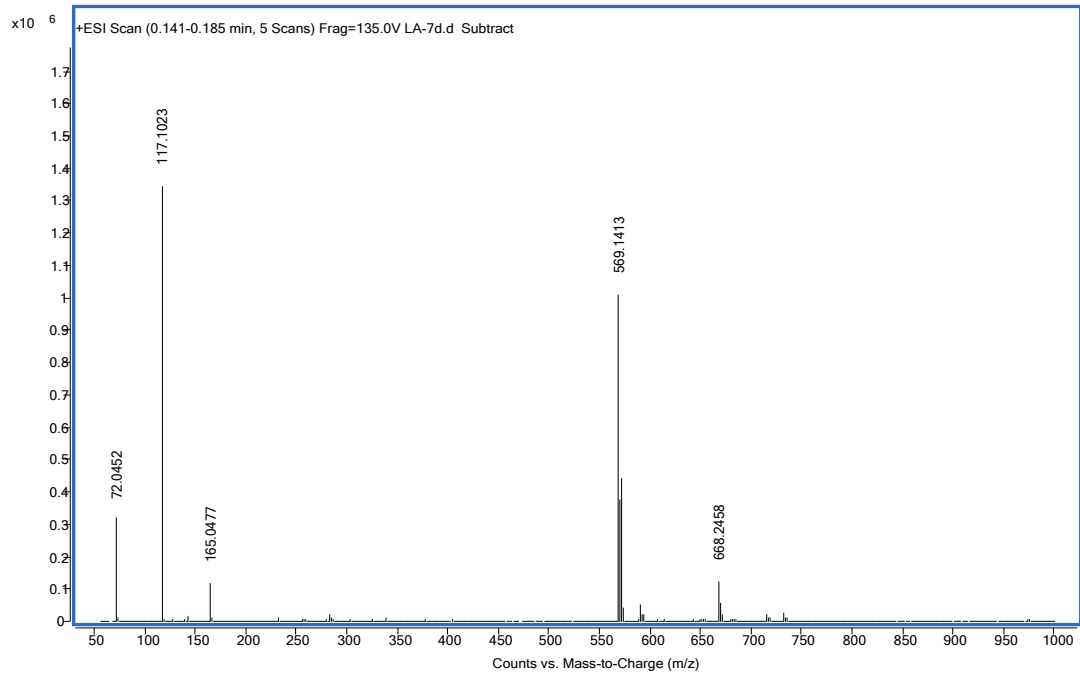
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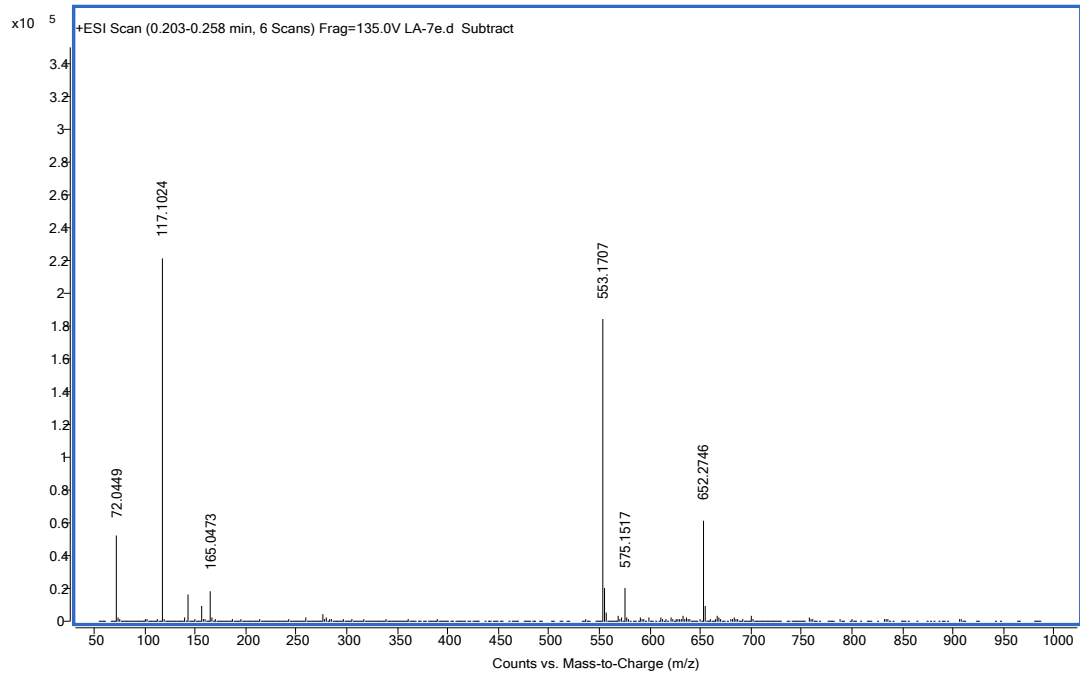
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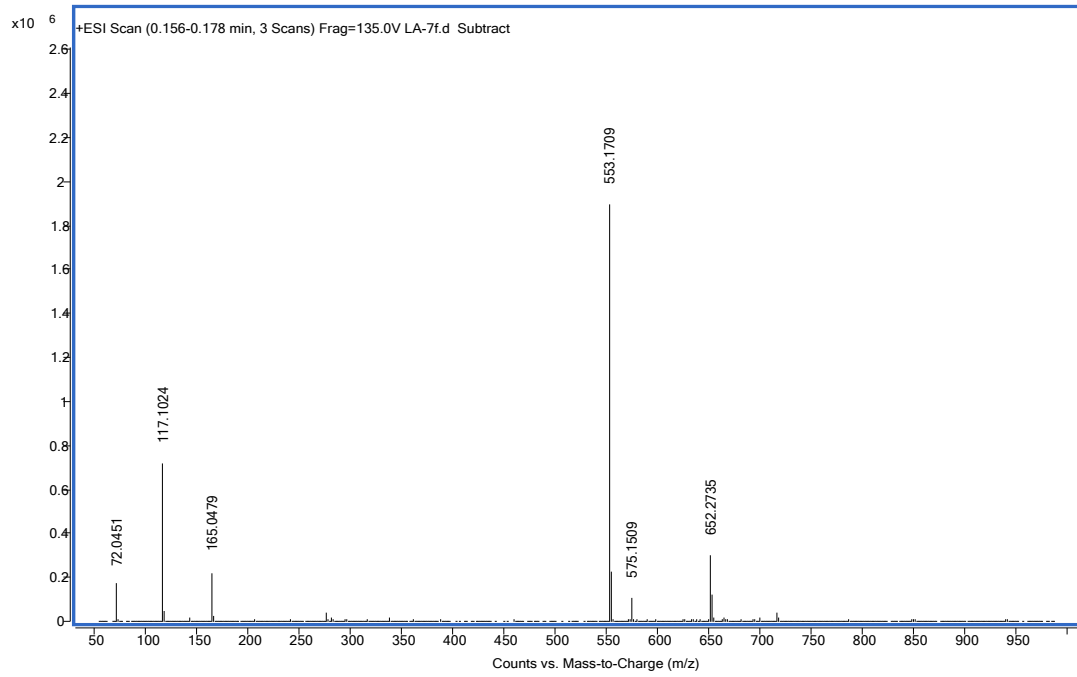
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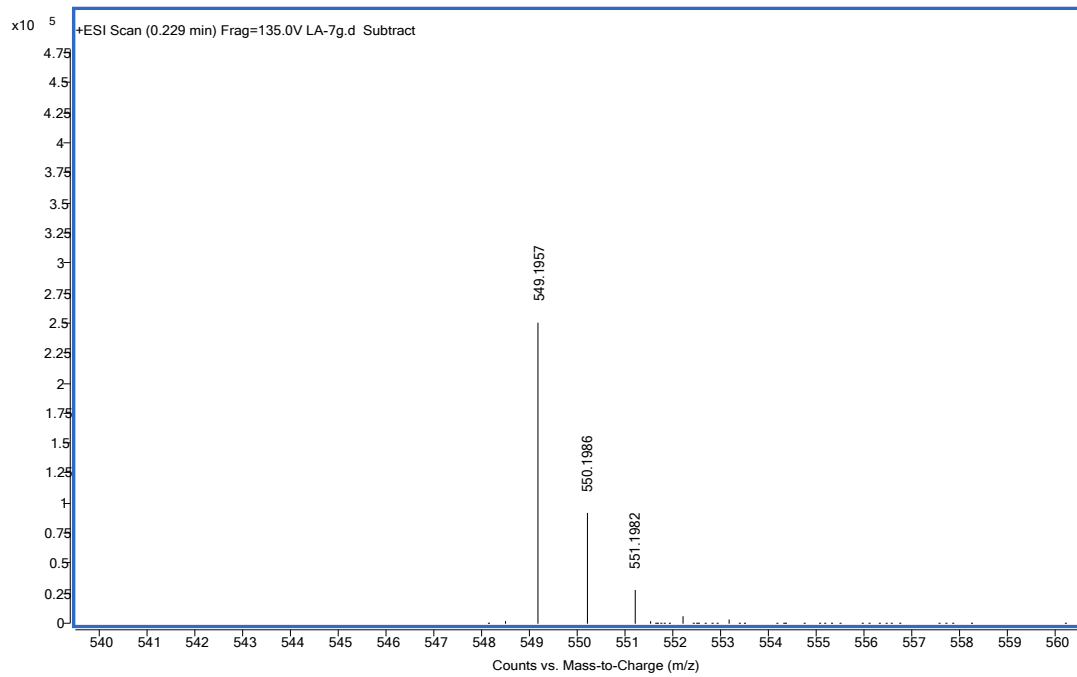
7e



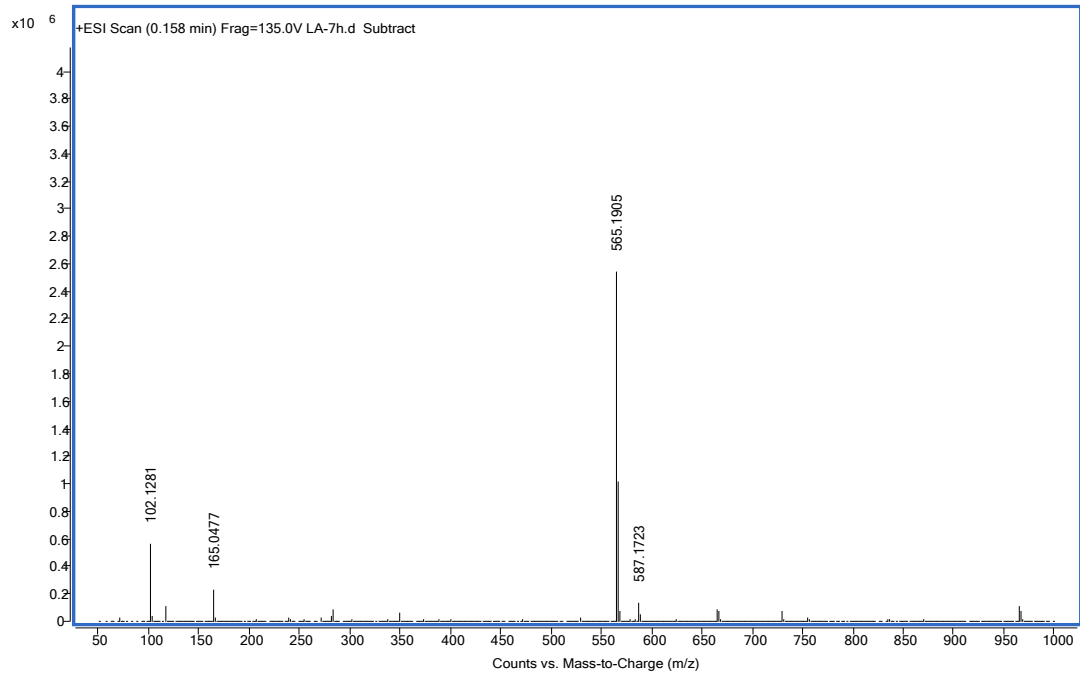
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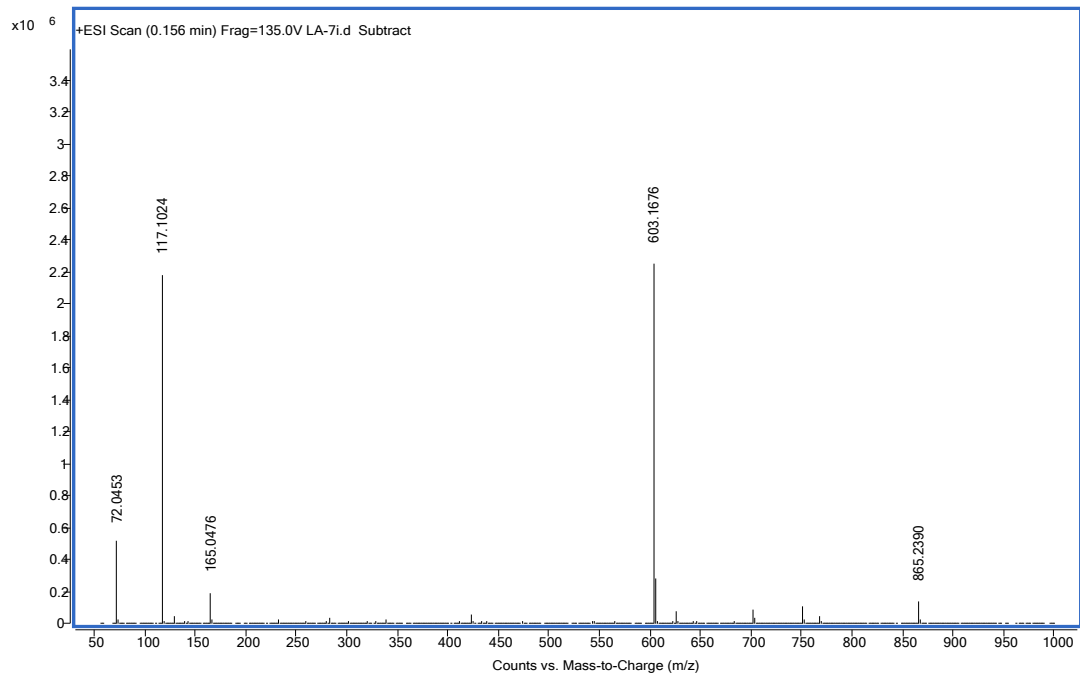
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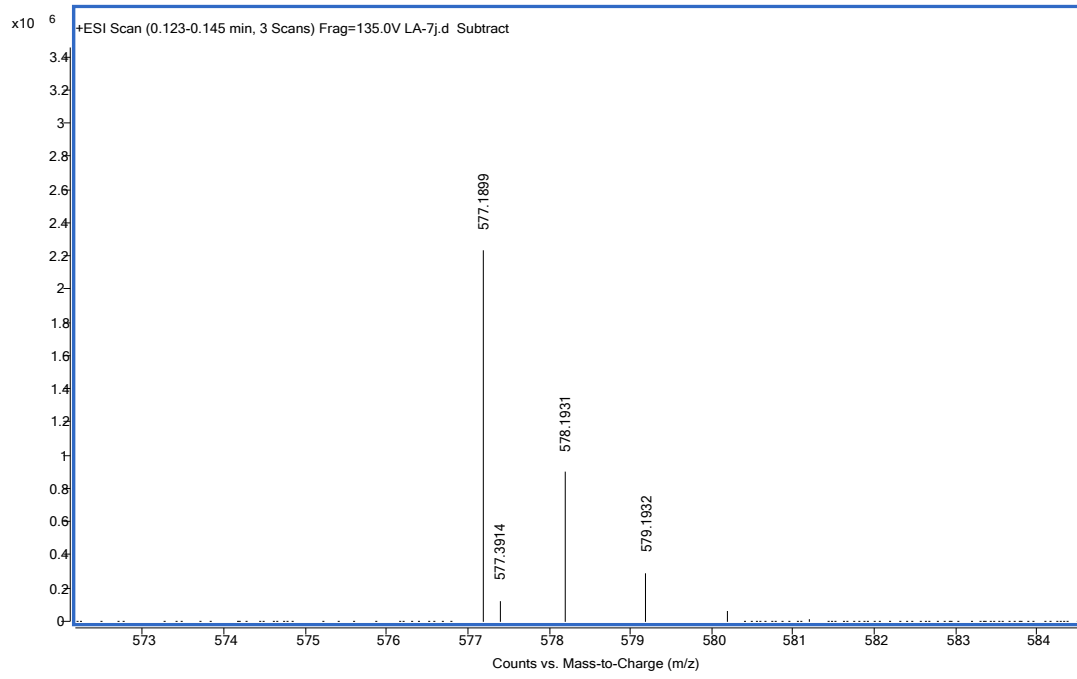
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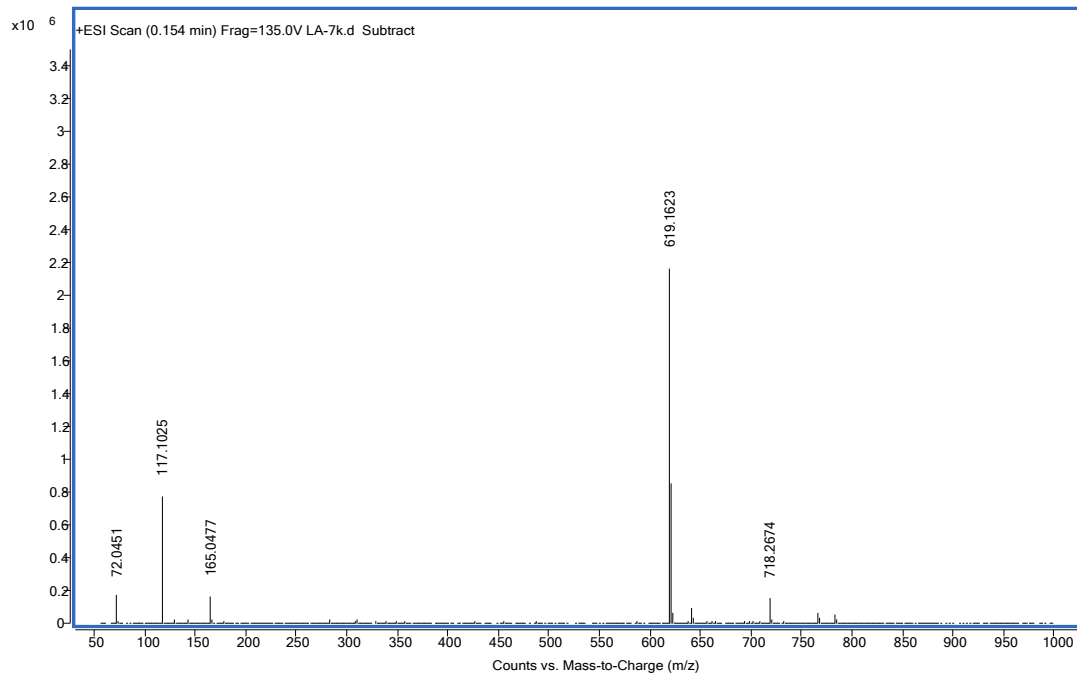
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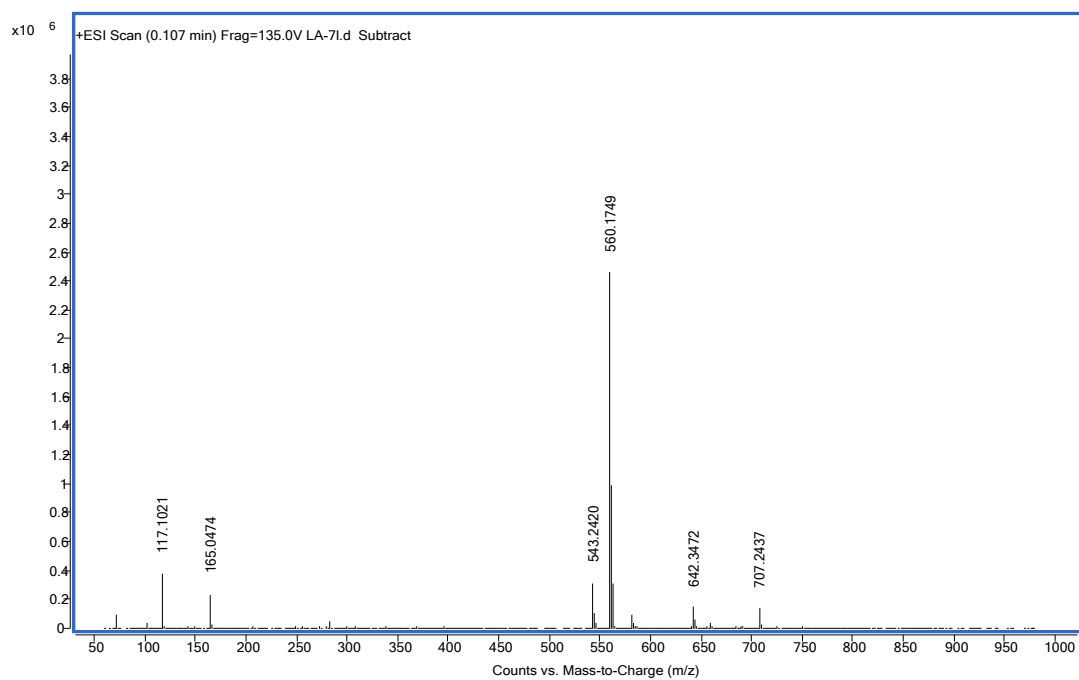
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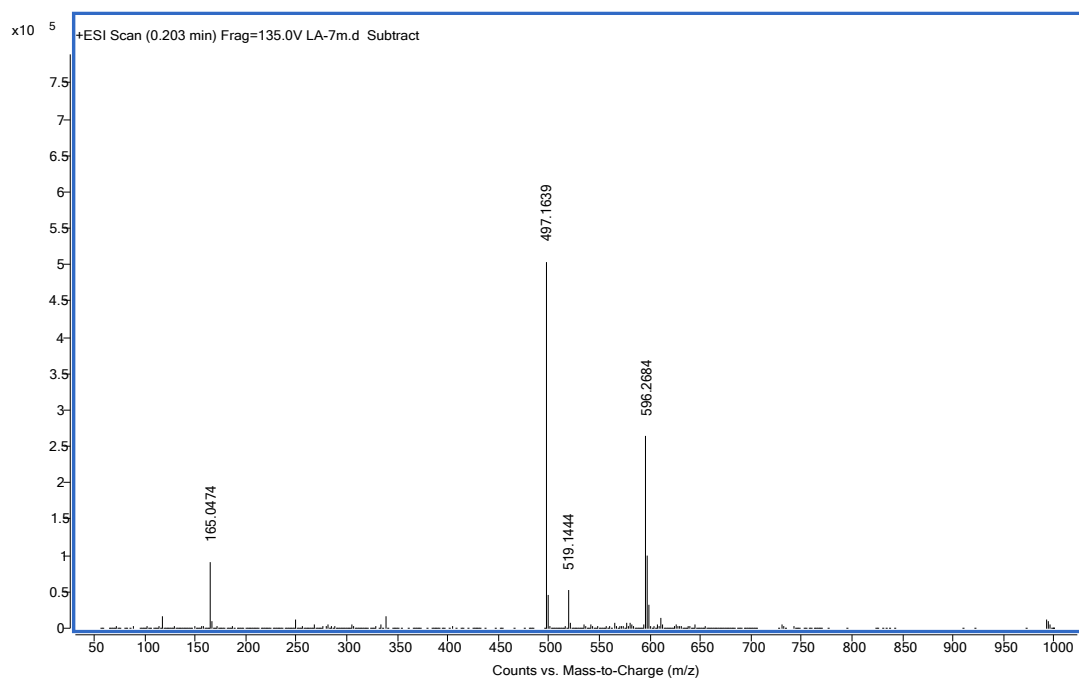
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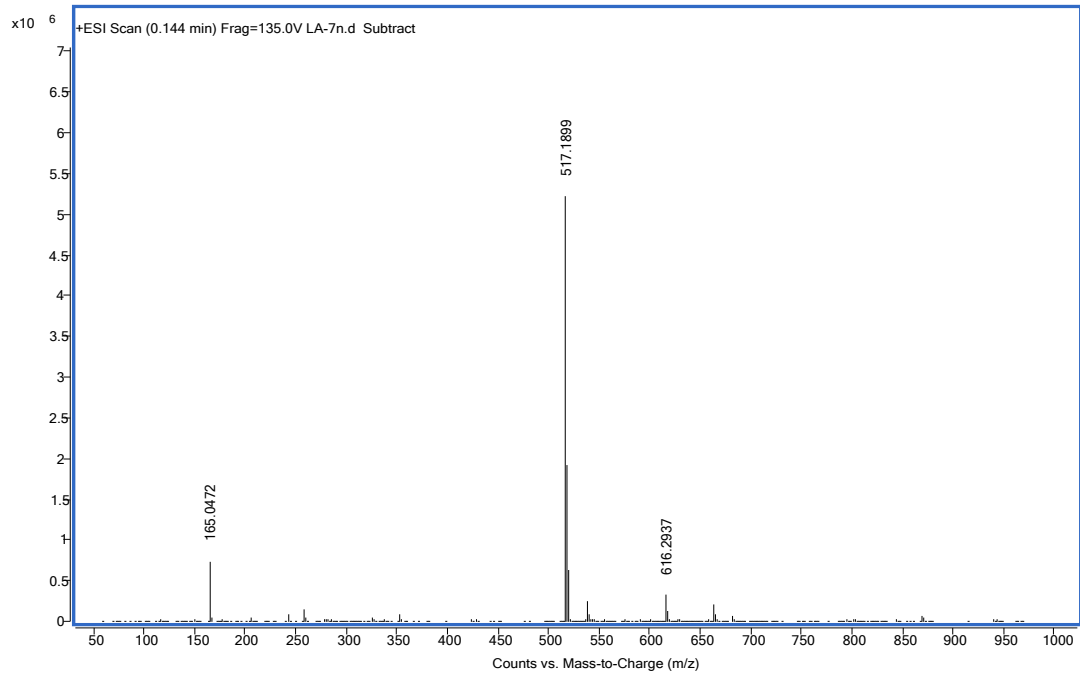
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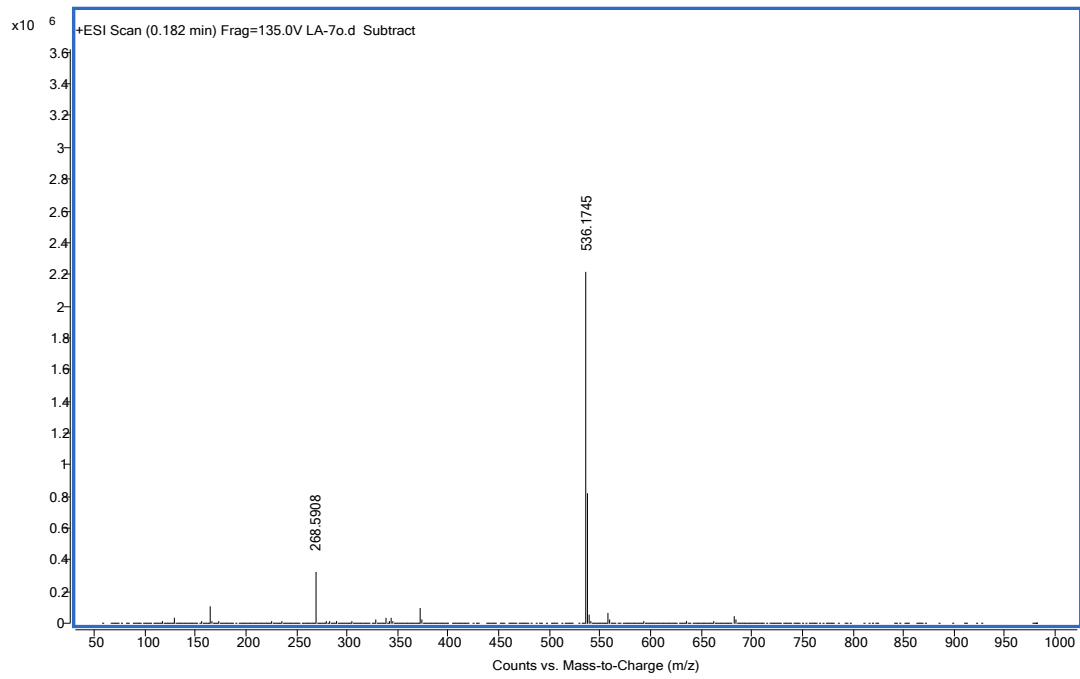
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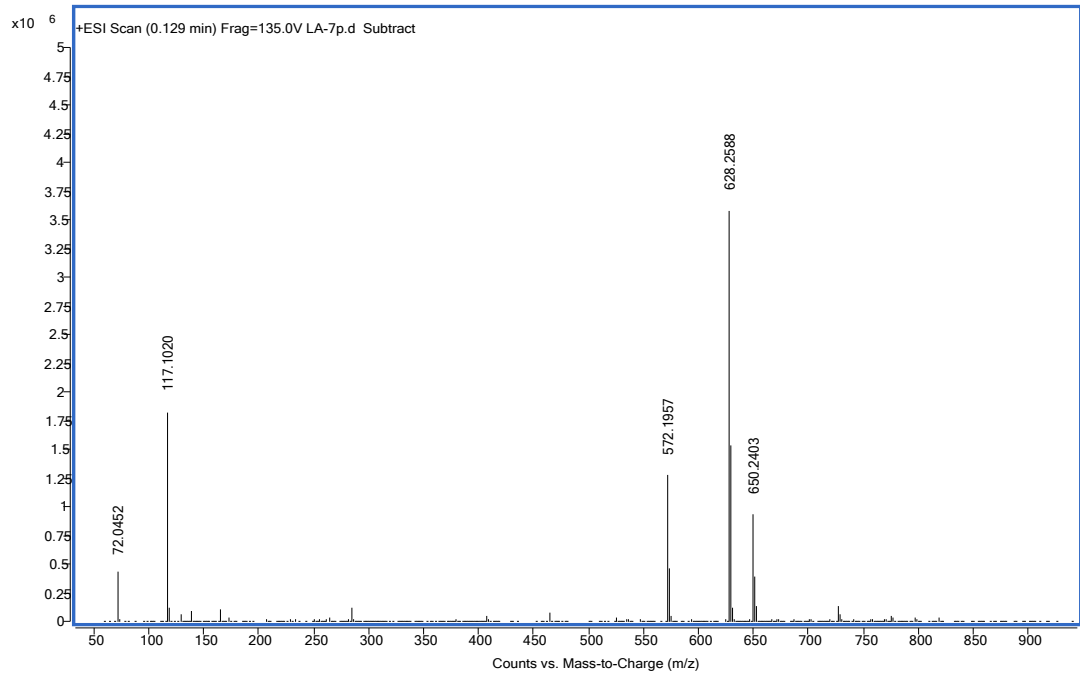
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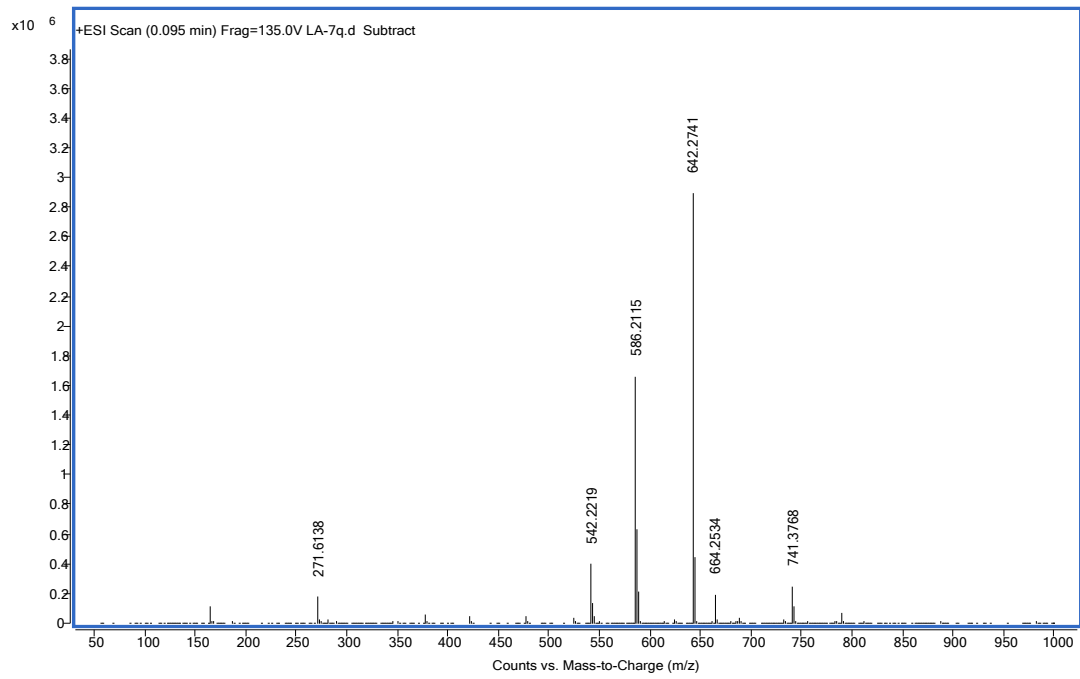
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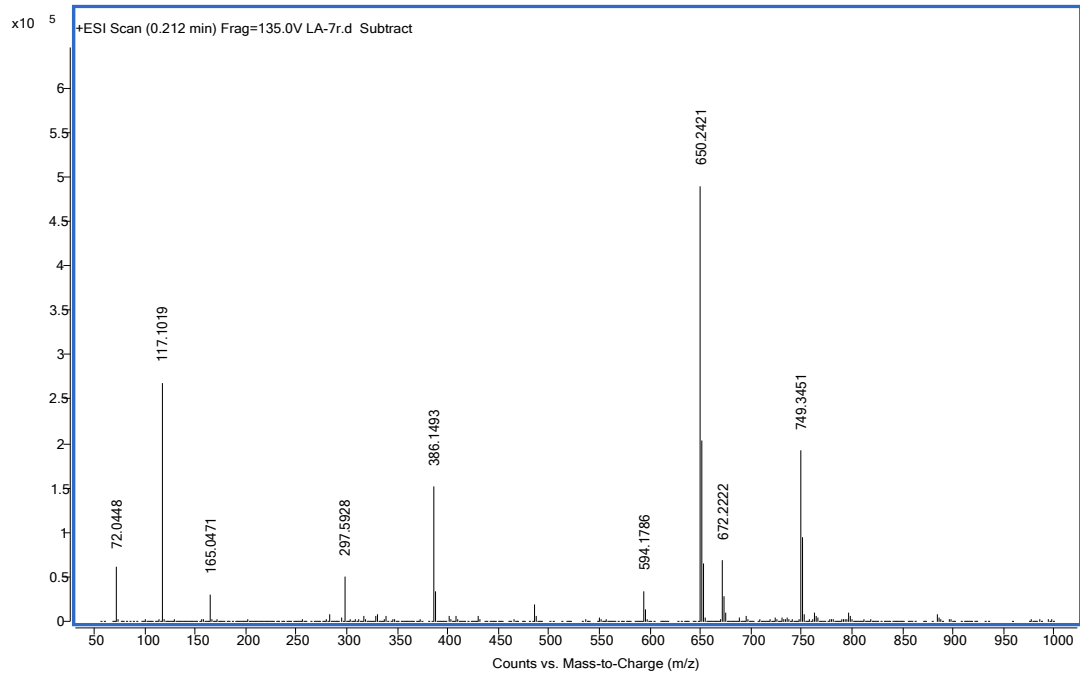
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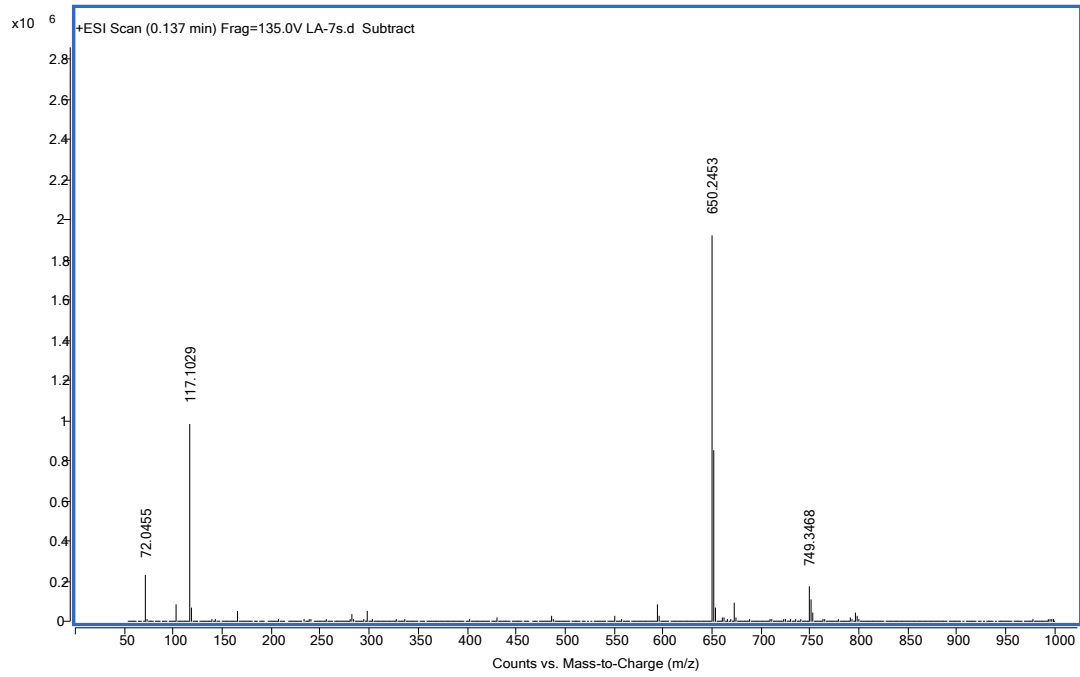
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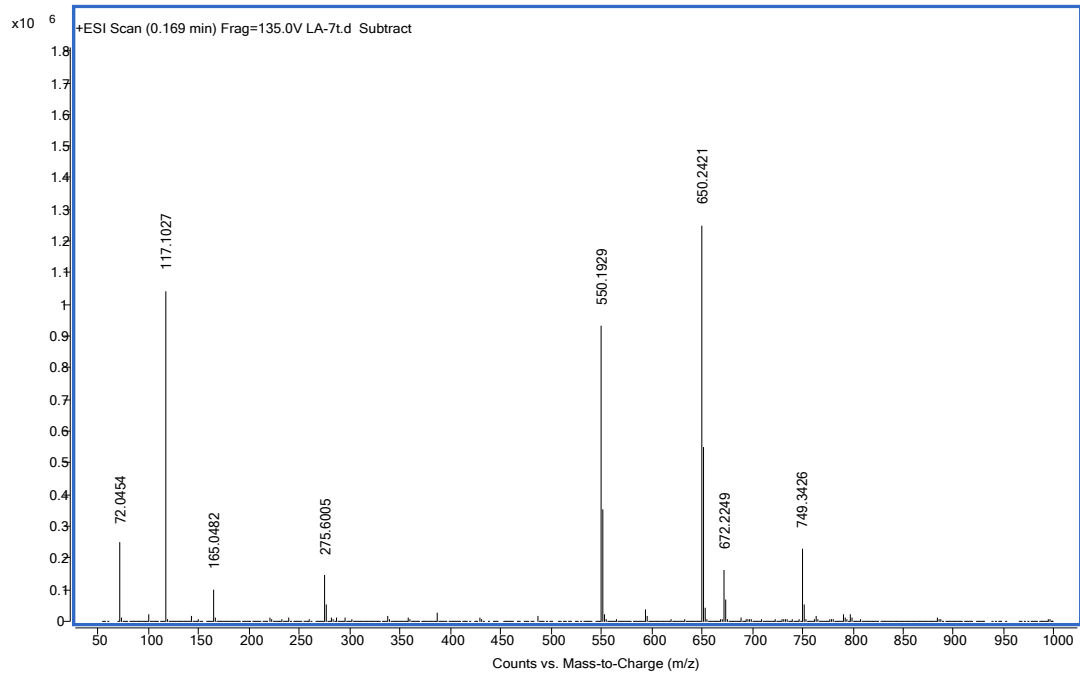
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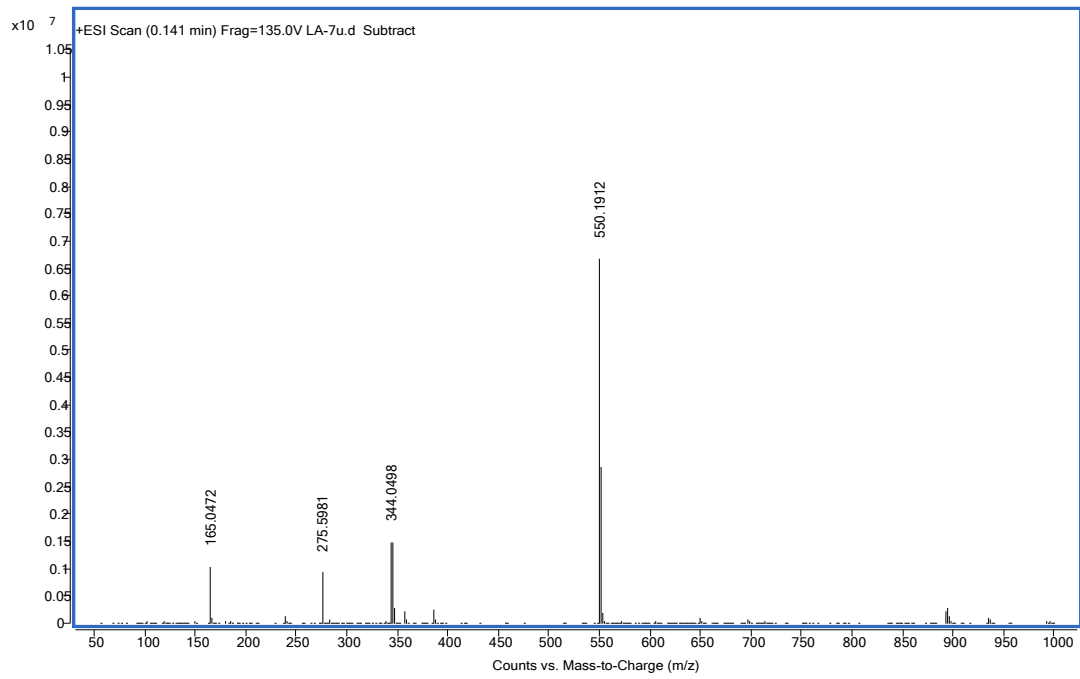
7s



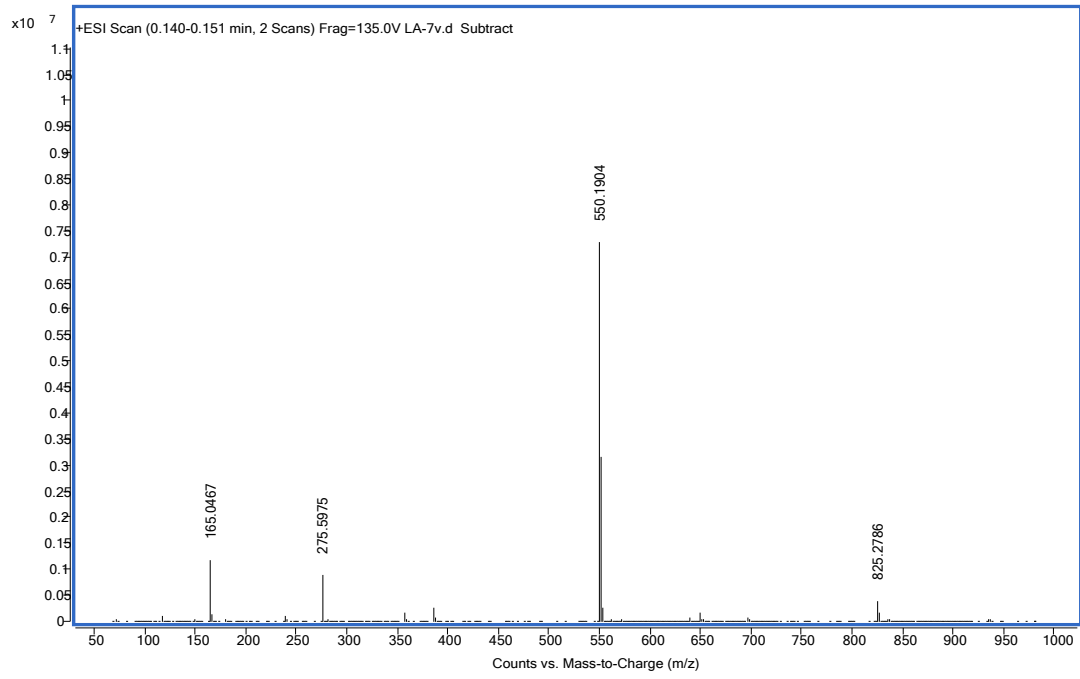
7t



7u



7v



7w

