Supporting Information

Design, Synthesis, and Mechanistic Study of 2-Piperazineone-bearing Peptidomimetics as Novel HIV Capsid Modulators

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Contents

I. Supplemental Results of SPR Assay

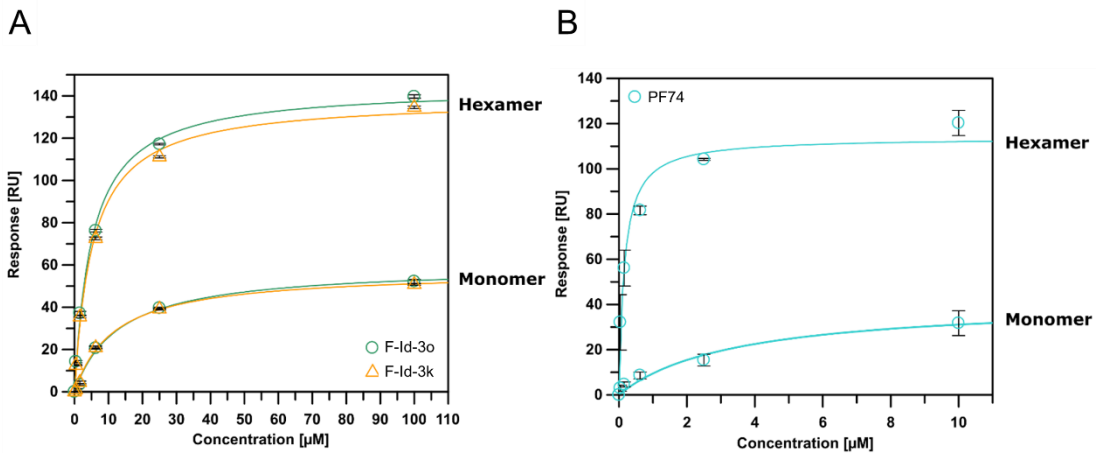


Figure S1. SPR isotherms of **F-Id-3k** and **F-Id-3o** (A) binding to two variants of the CA protein (monomer and disulfide-stabilized hexamer), respectively, with **PF74** (B) as the reference. Isotherms are an average of 3 replicates with error bars represent standard deviation (SD)

II. Supplemental Results of Single-Round Infection Assay

Table S1. Supplemental results of single-round infection assay in early stage

Compounds	Concentration (μM)	% Infection
F-Id-3o	3	69.1 ± 13.1
PF74	0.05	45.8 ± 8.8
DMSO	-	100.0 ± 8.4

III. 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 S2. Compounds information

Compound No.	Compound ID	Batch No.	Exact Mass	Stock Concentration (mM)
1	F-Id-3o	F-Id-3o	609.20	10
2	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 μL of compound and control stock solution (10 mM in dimethyl sulfoxide (DMSO)) were diluted with 495 μL of acetonitrile (ACN) (intermediate solution concentration: 100 μM, 99% ACN).

2.2. NADPH Cofactor Preparation

2.2.1. Materials

NADPH powder: β-Nicotinamide adenine dinucleotide phosphate reduced form, tetrasodium salt; NADPH·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 $MgCl_2$ solution (working solution concentration: 10 mM; final concentration in reaction system: 1 mM).

2.3. Liver Microsomes Preparation

2.3.1. Materials

Table S3. Liver Microsomes Information

Species	Product Information	Vendor	Abbrevation
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 NAPDH 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 NAPDH 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 NAPDH cofactor to incubation plate (T60). Start timing; plate will be incubated at 37°C for 60 min while shaking.

Table S4. 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 µL quenching solution to 'Quenching' plates,

mix once, and serially transfer 60 μL sample from T60 plate per time point to 'Quenching' plates.

Table S5. 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 μL sample from the NCF60 incubation to 'Quenching' plate containing quenching solution at the 60-min time point.

Table S6. 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 μL supernatant into 240 μL 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 T1/2 and CLint(mic) (μL/min/mg).

Equation of first order kinetics:

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

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

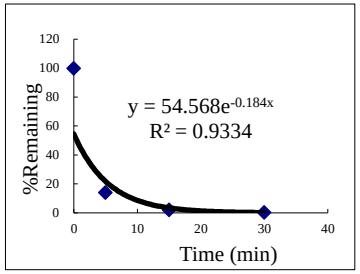
$$T_{1/2} = \frac{Ln2}{k_e} = \frac{0.693}{k_e}$$

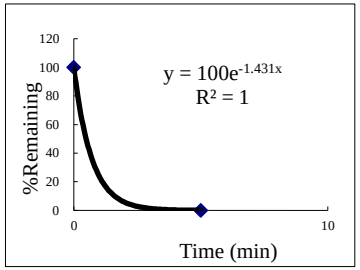
$$CL_{int(mic)} = \frac{0.693}{\text{In vitro } T_{1/2}} \bullet \frac{1}{\text{mg / mL microsomal protein in reaction system}}$$

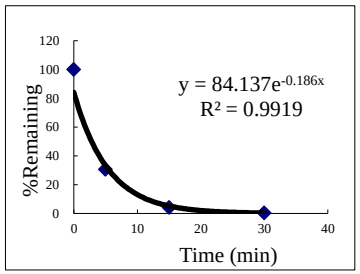
$$CL_{int(liver)} = CL_{int(mic)} \bullet \frac{\text{mg microsomes}}{\text{g liver}} \bullet \frac{\text{g liver}}{\text{kg body weight}}$$

4. Raw Data

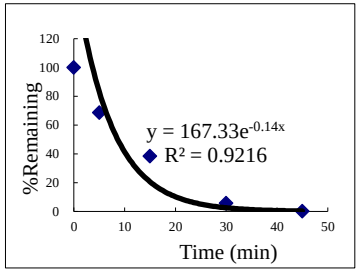
Compound ID	Compound & Species	Time (min)	Analyte Peak Area	IS Peak Area	Analyte/ IS	% Remaining	Time (min)	% Remaining	Ln (%Remaining)		R^2	k_e (min^{-1})	$T_{1/2}$ (min)	$CL_{int(mic)}$ (µL/min/mg)	Remaining (T=60min)	Remaining (NCF=60min)
F2-Id-3o	F2-Id-3oHLM 0.5	Blank	0	109,031	0.000	0.0					0.9520	0.1840	3.8	368.1	0.23%	96.1%
F2-Id-3o	F2-Id-3oHLM 0.5	60	1,522	114,468	0.013	0.2										
F2-Id-3o	F2-Id-3oHLM 0.5	45	1,301	106,444	0.012	0.2										
F2-Id-3o	F2-Id-3oHLM 0.5	30	1,923	110,290	0.017	0.3	30	0.3	-1.2							
F2-Id-3o	F2-Id-3oHLM 0.5	15	12,365	100,738	0.123	2.1	15	2.1	0.7							
F2-Id-3o	F2-Id-3oHLM 0.5	5	81,047	98,665	0.821	14.1	5	14.1	2.6							
F2-Id-3o	F2-Id-3oHLM 0.5	0	597,258	102,666	5.817	100.0	0	100.0	4.6							
F2-Id-3o	F2-Id-3oHLM 0.5	NCF60	606,194	108,444	5.590	96.1										
PF74	PF74HLM 0.5	Blank	0	116,908	0.000	0.0					1.0000	1.4312	0.48	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										
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							

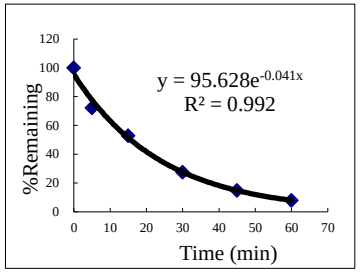






Diclofenac	DiclofenacHLM 0.5	NCF60	83,751	104,298	0.803	96.7										
Propafenone	PropafenoneHLM 0.5	Blank	63	105,722	0.001	0.0					0.9350	0.1397	5.0	279.5	0.0%	93.6%
Propafenone	PropafenoneHLM 0.5	60	63	100,264	0.001	0.0										
Propafenone	PropafenoneHLM 0.5	45	1,024	97,056	0.011	0.2	45	0.2	-1.9							
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										





IV. 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

Compound ID	Batch	MW	FW	Purity (%)	Stock Conc. (mM)	Final Conc.(μM)
F-Id-3o	F-Id-3o	609.6	NA	99.0	10	2
PF74	PF74	425.5	NA	97.1	10	2
Propantheline 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 μL of compound stock solution (10 mM in dimethyl sulfoxide (DMSO)) were diluted with 495 μL of DMSO (Working solution concentration: 100 uM, 100% DMSO).

1.2.2. Propantheline bromide Working solution: 5 μL of Propantheline bromide stock solution (10 mM in H_2O) were diluted with 495 μL of H_2O (Working solution concentration: 100 uM, 100% H_2O).

1.3. 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℃ 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 μL/well of blank plsma 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 μL/well of working solution (100 μM) 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 S7. 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 µL 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 µL 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:

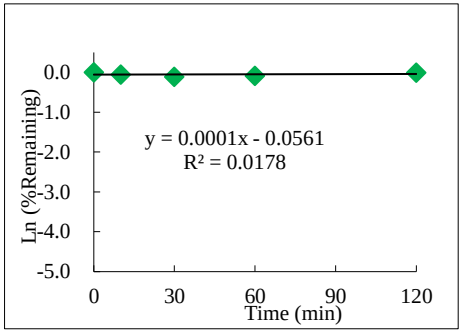
% Remaining= 100 x (PAR at appointed incubation time / PAR at T0 time)

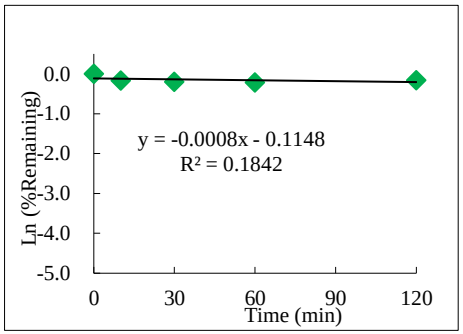
where PAR is the peak area ratio of analyte versus internal standard (IS)

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

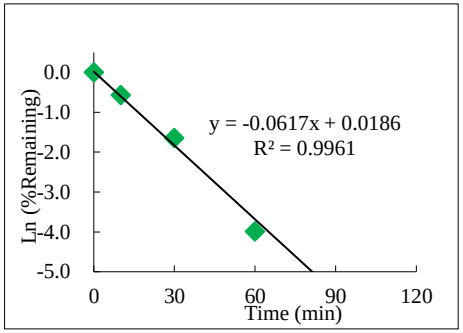
4. Data

Sample_ID	Time (min)	Analyte Peak Area	IS Peak Area	Aa/Ai	% Remaining (n=2)	Ln (% Remaining)			
F2-Id-3o_H_0	0	6.79E+06	1.38E+06	4.9312	100.0	0.00		k=	-0.0001
F2-Id-3o_H_0		6.65E+06	1.37E+06	4.8473				R^2=	0.0178
F2-Id-3o_H_10	10	6.66E+06	1.46E+06	4.5548	95.2	-0.05		t1/2=0.693/k	>289.1
F2-Id-3o_H_10		6.79E+06	1.43E+06	4.7520					
F2-Id-3o_H_30	30	5.86E+06	1.32E+06	4.4486	89.8	-0.11			
F2-Id-3o_H_30		5.88E+06	1.36E+06	4.3339					
F2-Id-3o_H_60	60	6.56E+06	1.46E+06	4.5063	91.6	-0.09			
F2-Id-3o_H_60		7.13E+06	1.60E+06	4.4557					
F2-Id-3o_H_120	120	7.21E+06	1.54E+06	4.6913	99.3	-0.01			
F2-Id-3o_H_120		8.73E+06	1.74E+06	5.0143					
PF74_H_0	0	1.03E+07	1.45E+06	7.1259	100.0	0.00		k=	0.0008
PF74_H_0		1.08E+07	1.56E+06	6.8745				R^2=	0.1842
PF74_H_10	10	9.50E+06	1.59E+06	5.9628	84.7	-0.17		t1/2=0.693/k	>289.1
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					
PF74_H_60	60	8.74E+06	1.42E+06	6.1381	80.7	-0.21			
PF74_H_60		7.65E+06	1.48E+06	5.1652					
PF74_H_120	120	8.79E+06	1.61E+06	5.4640	85.2	-0.16			
PF74_H_120		1.04E+07	1.62E+06	6.4621					
Propantheline bromide_H_0	0	3.32E+06	1.74E+06	1.9093	100.0	0.00		k=	0.0617





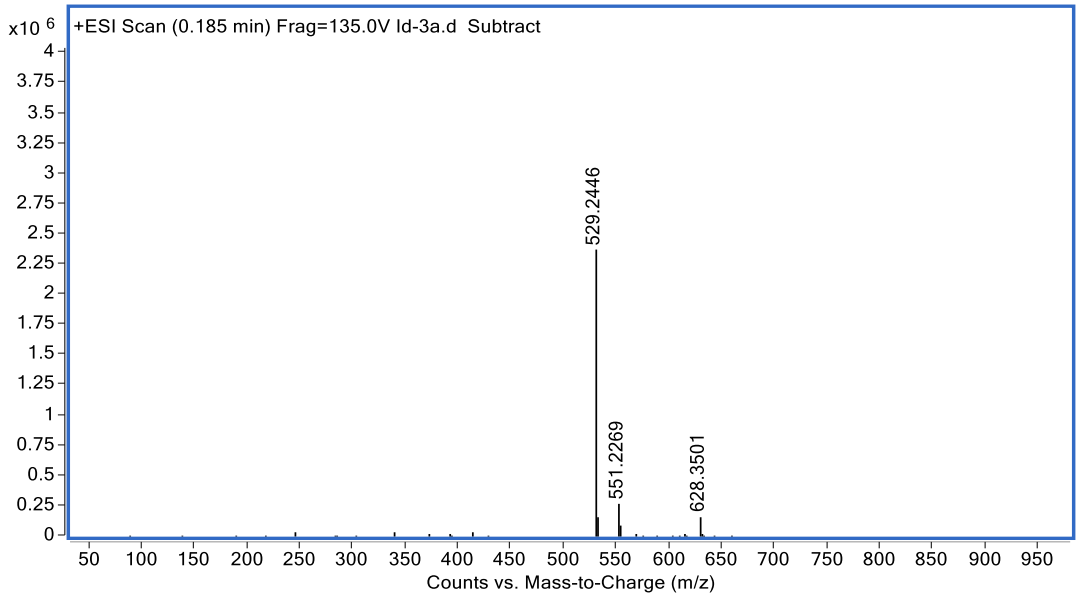
Propantheline bromide_H_0		3.22E+06	1.64E+06	1.9647			R^2=	0.9961
Propantheline bromide_H_10	10	2.01E+06	1.86E+06	1.0818	56.5	-0.57	t1/2=0.693/k	11.2
Propantheline bromide_H_10		1.91E+06	1.73E+06	1.1067				
Propantheline bromide_H_30	30	6.75E+05	1.89E+06	0.3581	19.4	-1.64		
Propantheline bromide_H_30		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				

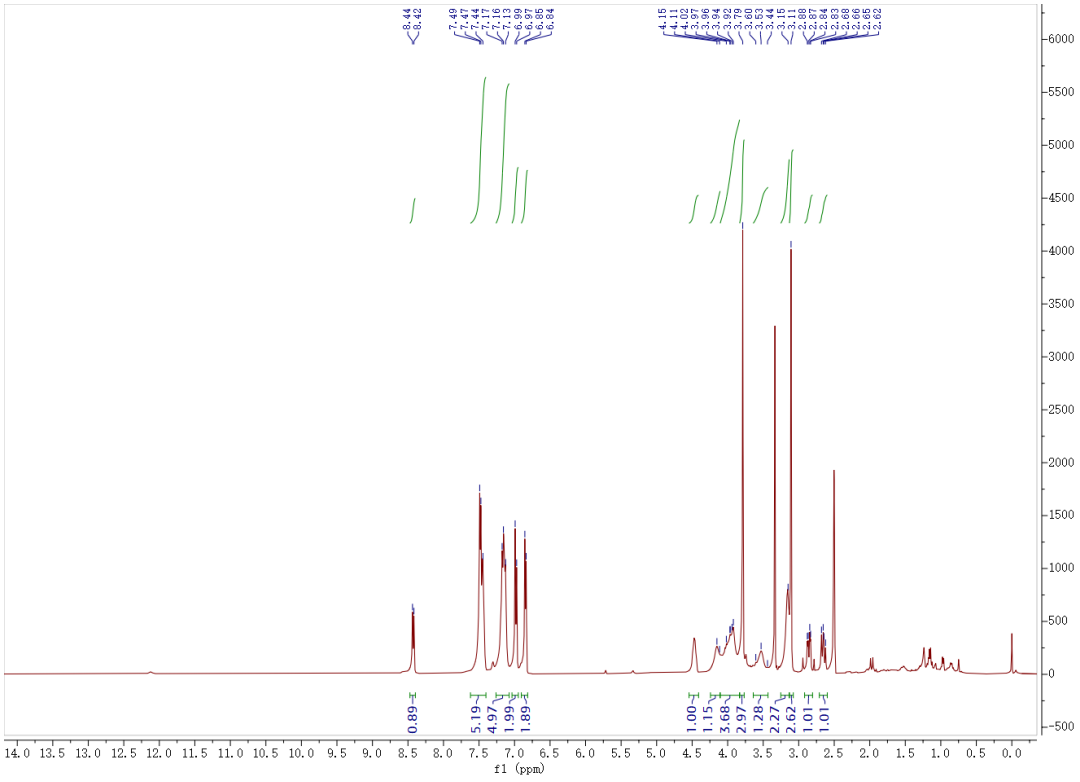


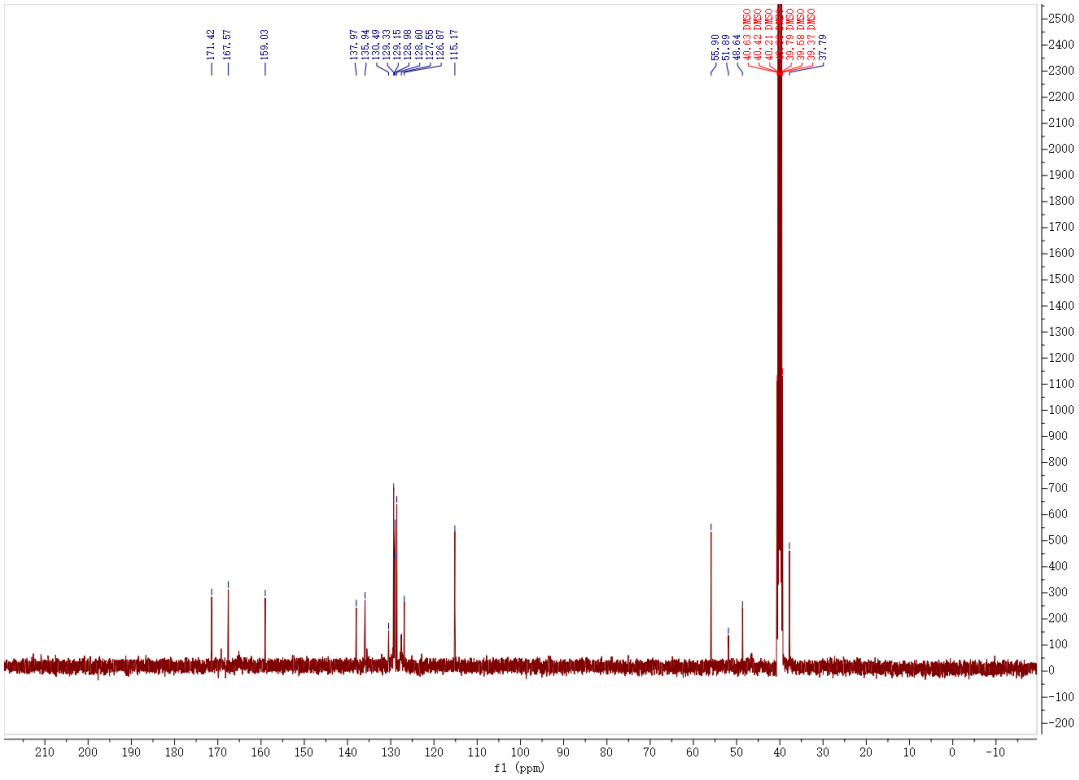
Sample comment: "XXXX_H_0" for example, "H" for Human Plasma, "0" was the sample collected at T=0 incubation.

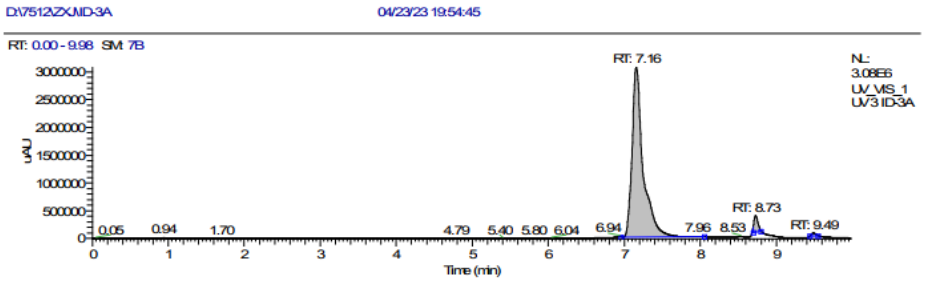
V. HRMS, ^{1}H-NMR, ^{13}C-NMR Spectra and LC-MS for Representative Target Compounds

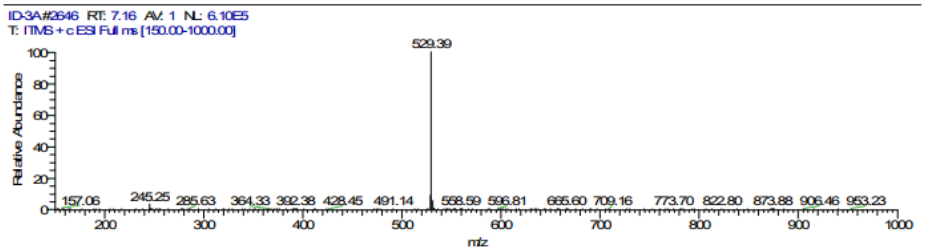
1. HRMS, ^{1}H-NMR, ^{13}C-NMR Spectra and LC-MS for Id-3a











PEAK LIST

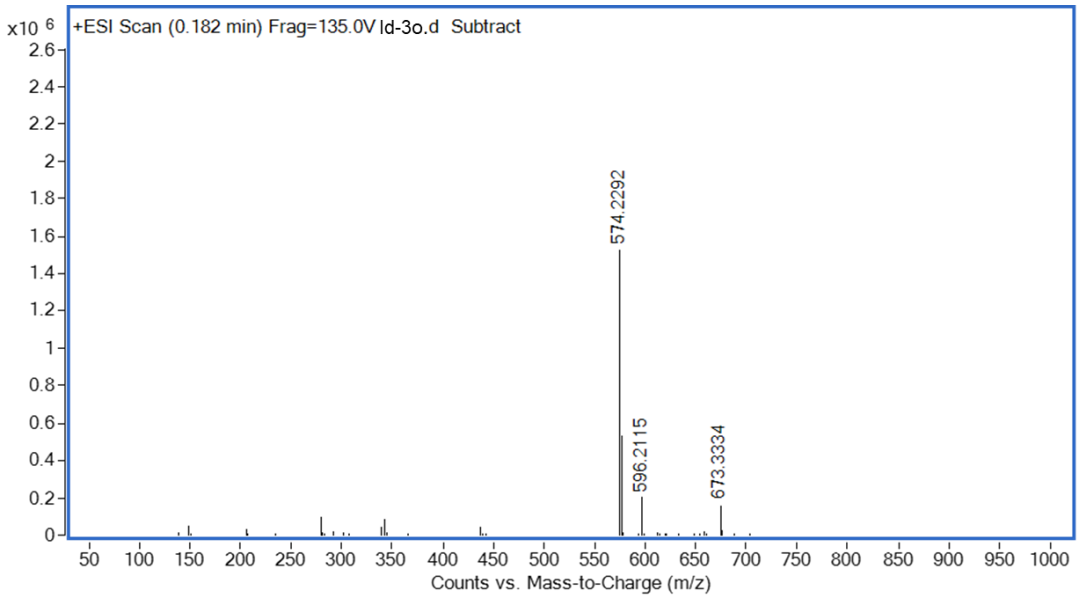
ID-3A.raw

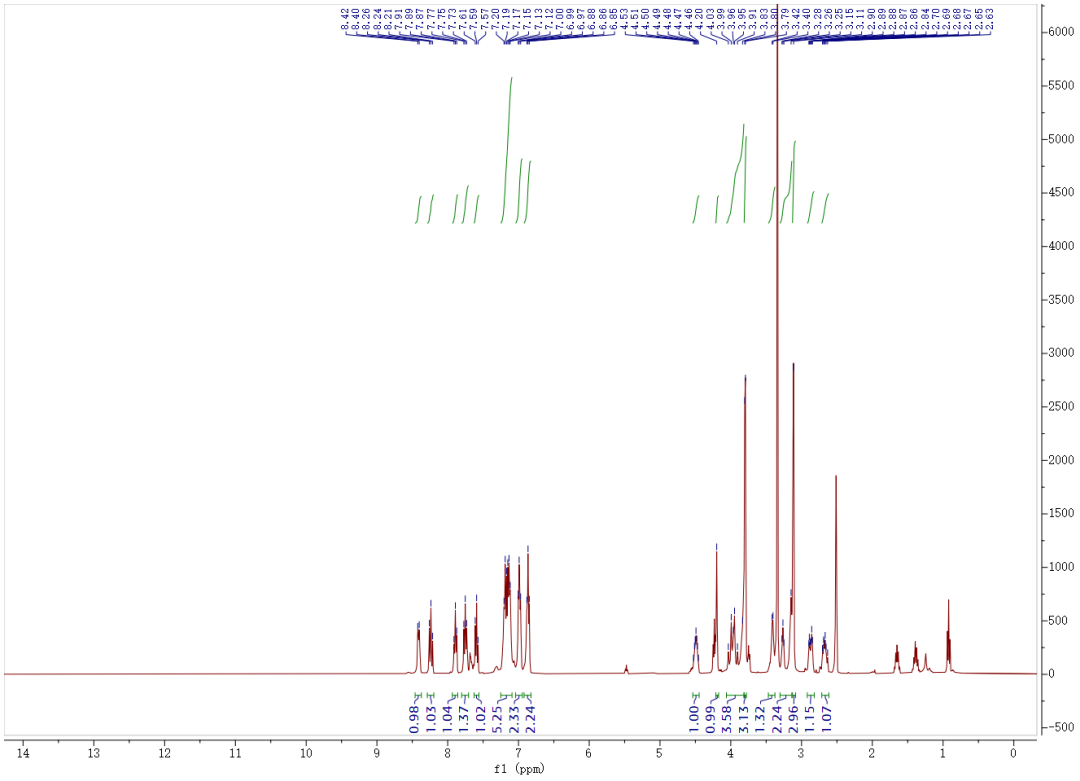
RT: 0.00 - 9.98

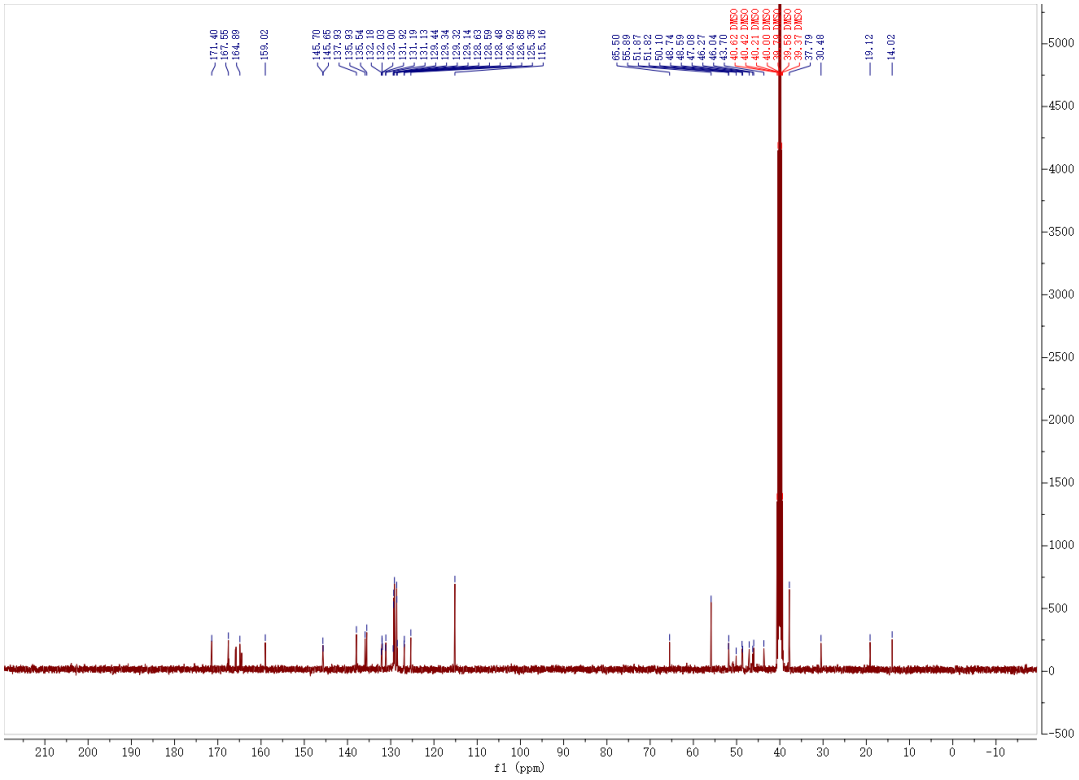
Number of detected peaks: 3

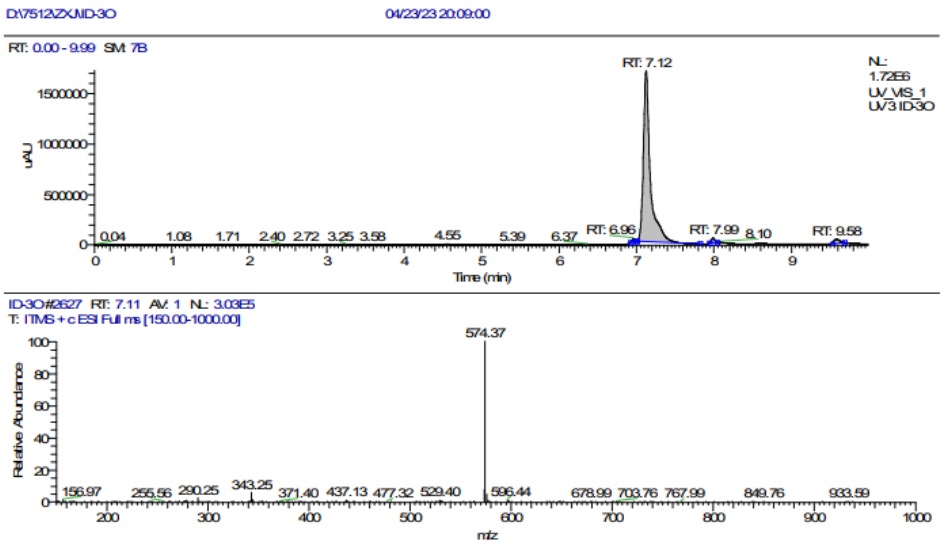
Apex RT		Start RT	End RT	Area	%Area	Height	%Height
7.16	6.95	8.05	31063020.494	96.13	3052643.882	89.84
8.73	8.69	8.79	1074678.634	3.33	298411.093	8.78
9.49	9.44	9.54	174622.138	0.54	46710.272	1.37

2. HRMS, ^{1}H-NMR, ^{13}C-NMR Spectra and LC-MS for Id-3o









PEAK LIST

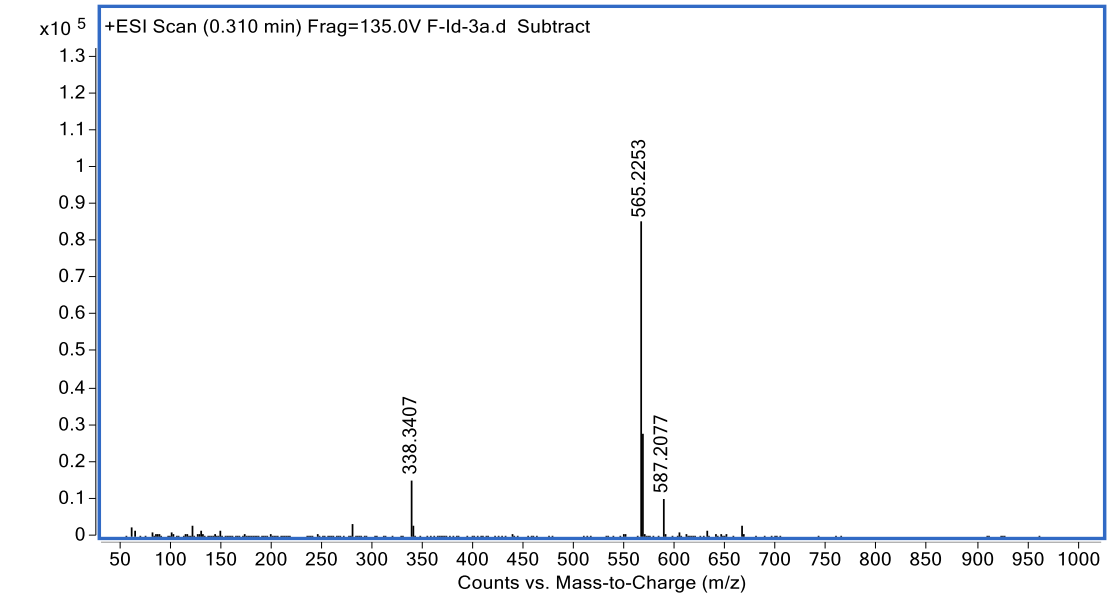
ID-3O.raw

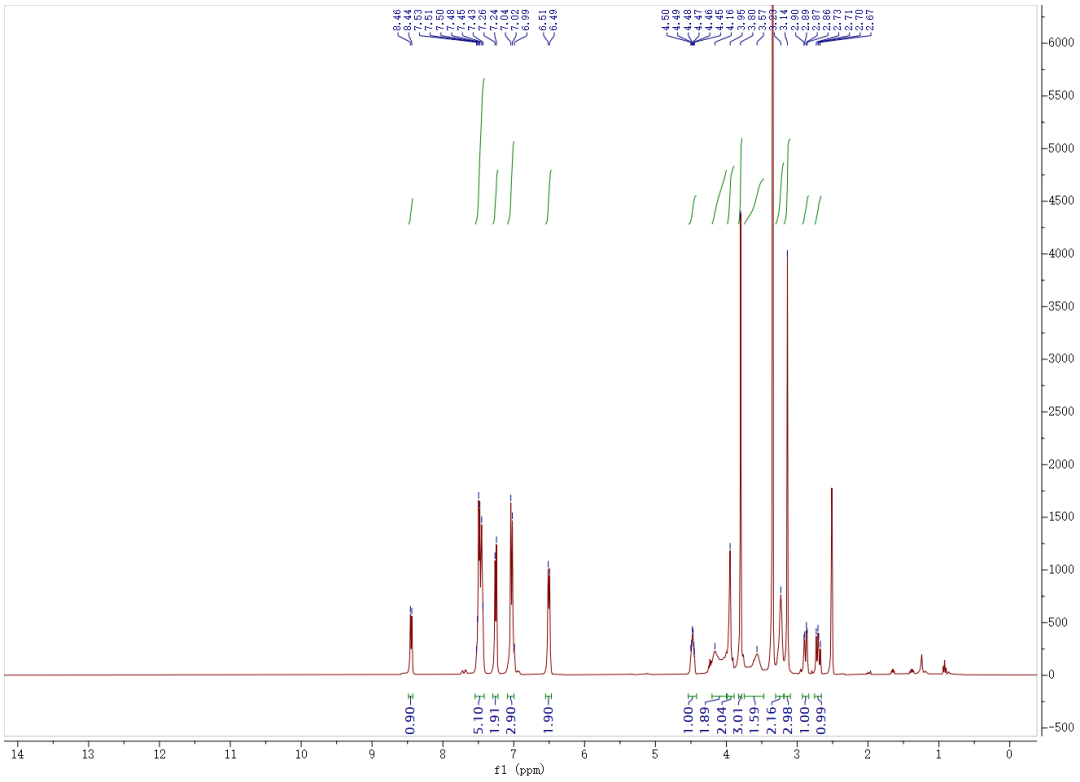
RT: 7.79 - 8.49

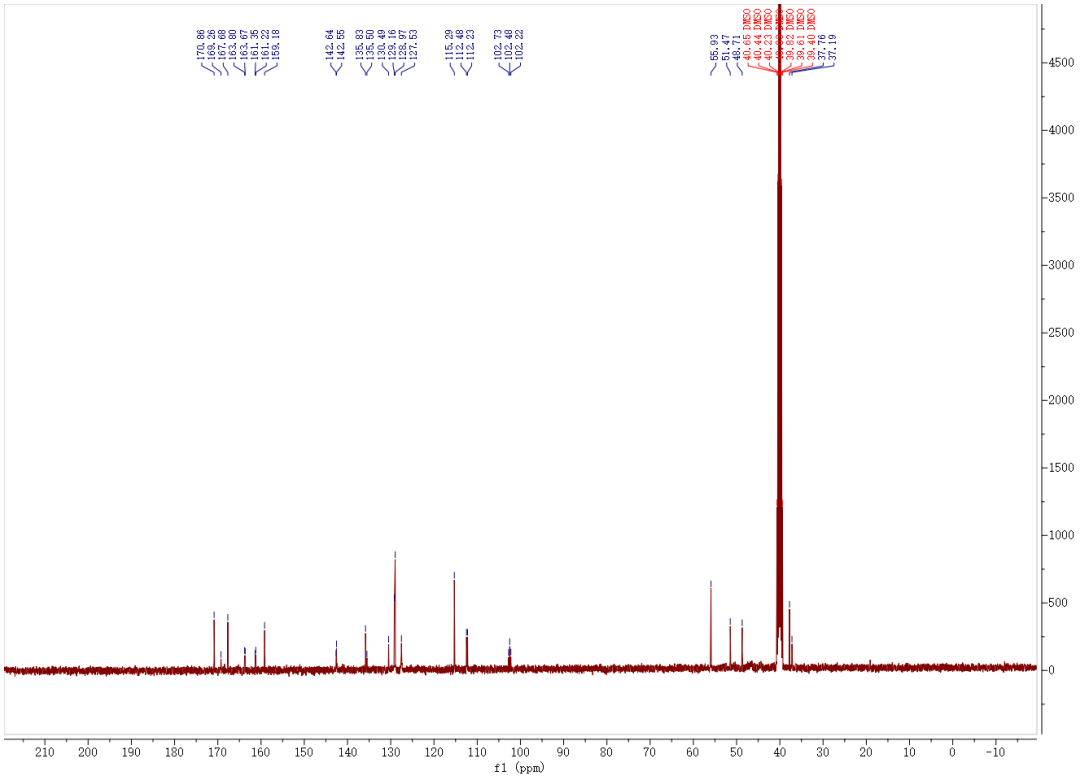
Number of detected peaks: 4

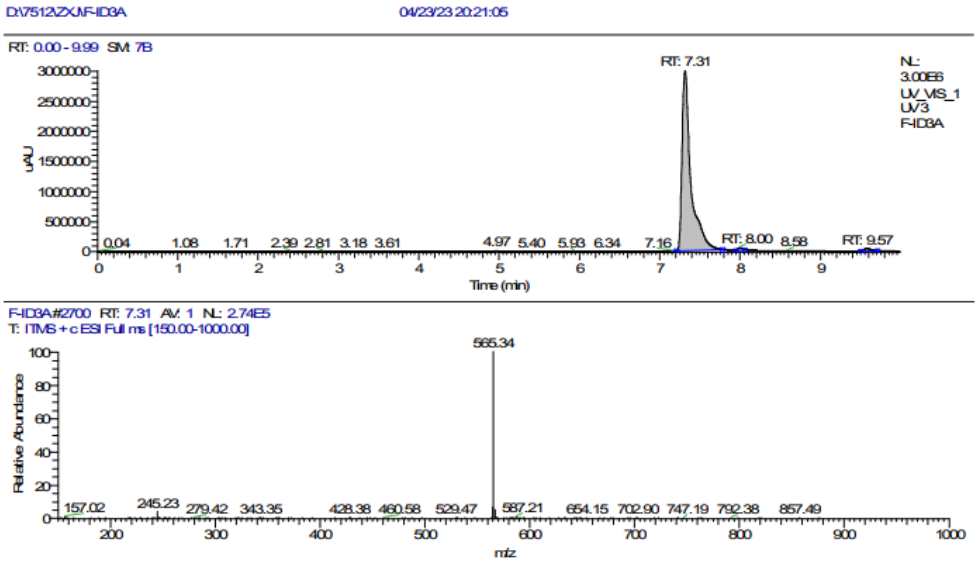
Apex RT		Start RT	End RT	Area	%Area	Height	%Height
6.96	6.92	6.99	81389.400	0.73	29124.202	1.61
7.12	7.01	7.82	10615211.543	95.67	1691932.527	93.46
7.99	7.94	8.04	148923.278	1.34	44289.115	2.45
9.58	9.53	9.69	249972.226	2.25	45029.126	2.49

3. HRMS, ^{1}H-NMR, ^{13}C-NMR Spectra and LC-MS for F-Id-3a









PEAK LIST

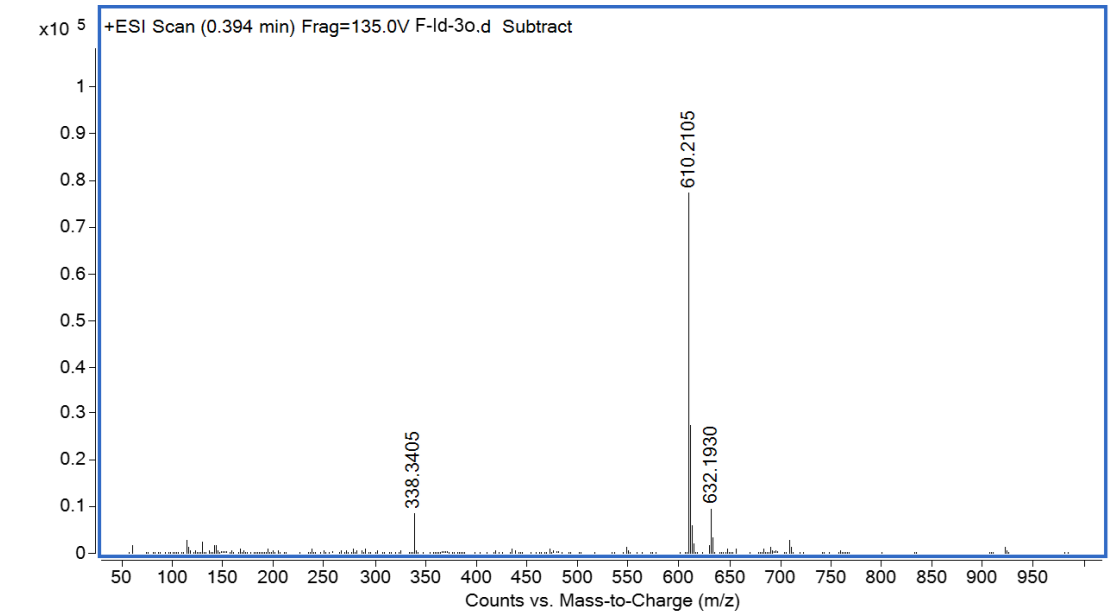
F-ID3A.raw

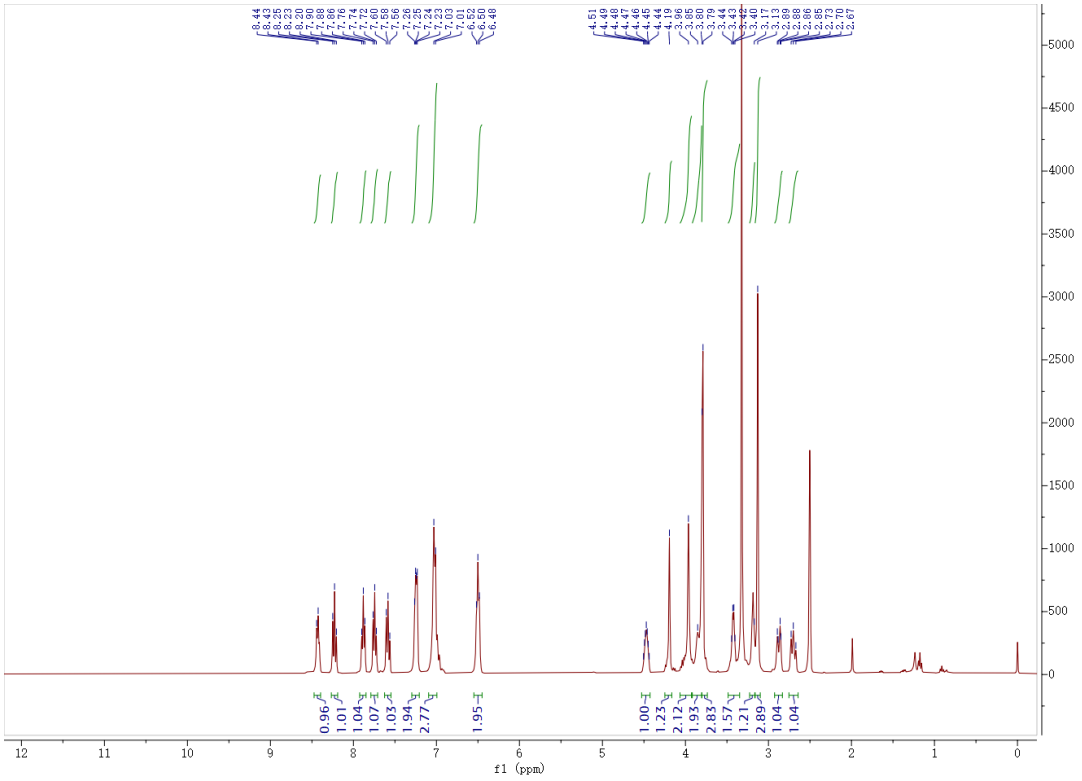
RT: 0.00 - 9.99

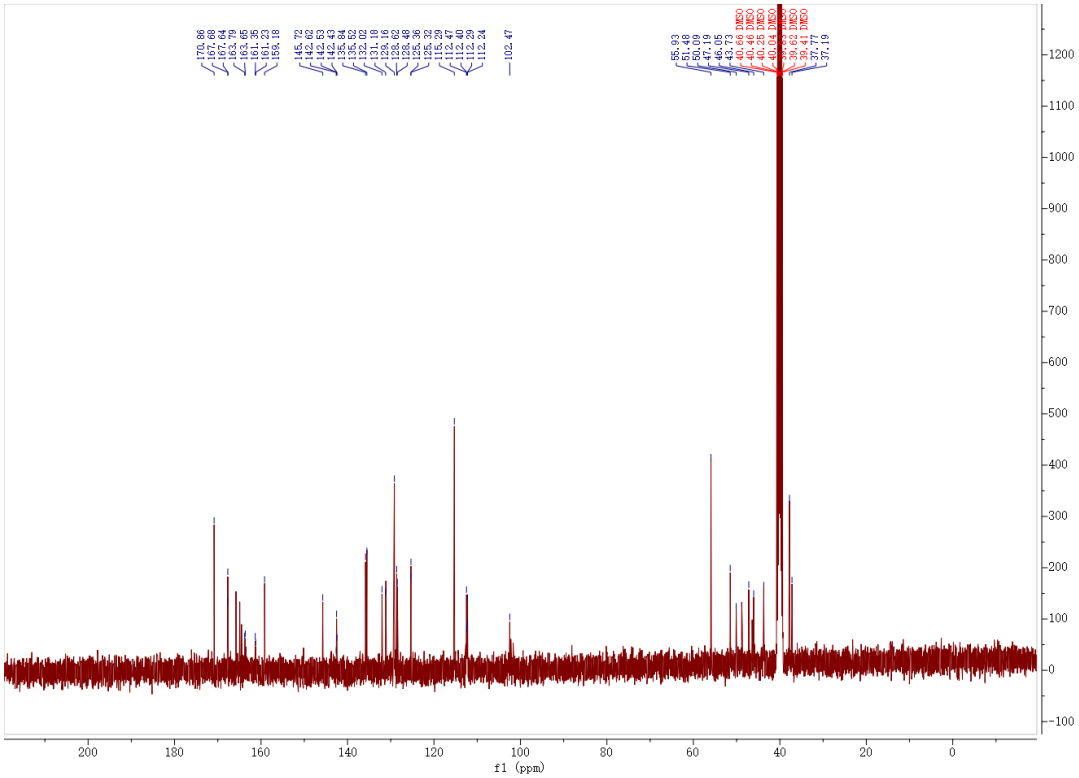
Number of detected peaks: 3

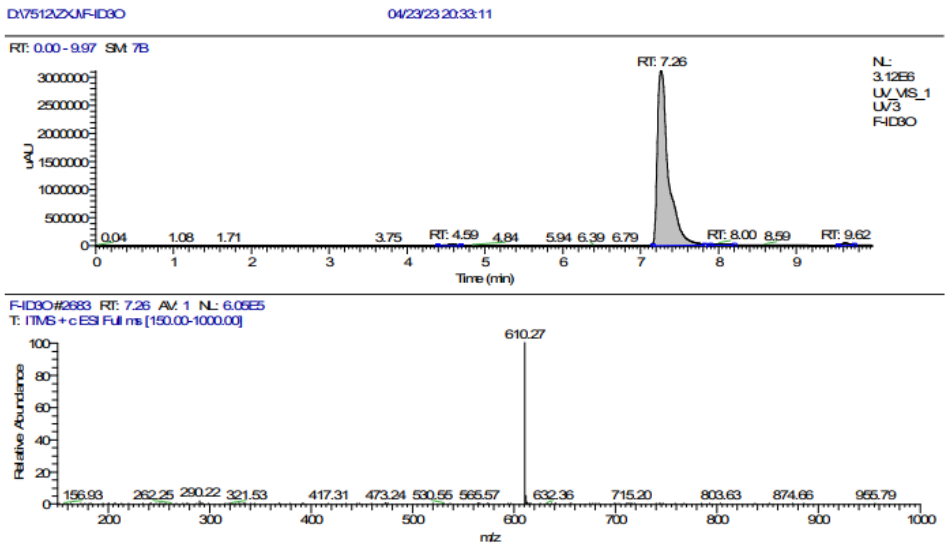
Apex RT		Start RT	End RT	Area	%Area	Height	%Height
7.31	7.21	7.78	22919271.324	97.91	2985790.723	97.02
8.00	7.94	8.06	178632.347	0.76	44876.735	1.46
9.57	9.50	9.70	309662.104	1.32	46943.660	1.53

4. HRMS, ^{1}H-NMR, ^{13}C-NMR Spectra and LC-MS for F-Id-3o









PEAK LIST

F-ID3O.raw

RT: 0.00 - 9.97

Number of detected peaks: 4

Apex RT		Start RT	End RT	Area	%Area	Height	%Height
4.59	4.39	4.69	192337.016	0.60	24231.895	0.75
7.26	7.15	7.82	31020634.565	97.40	3112912.629	96.86
8.00	7.90	8.20	337252.425	1.06	31824.429	0.99
9.62	9.53	9.74	298430.241	0.94	44894.873	1.40