Supplementary Information

Discovery of new imidazole[1,2-a] pyridine derivatives as CDK9 inhibitors: Design, synthesis and biological evaluation

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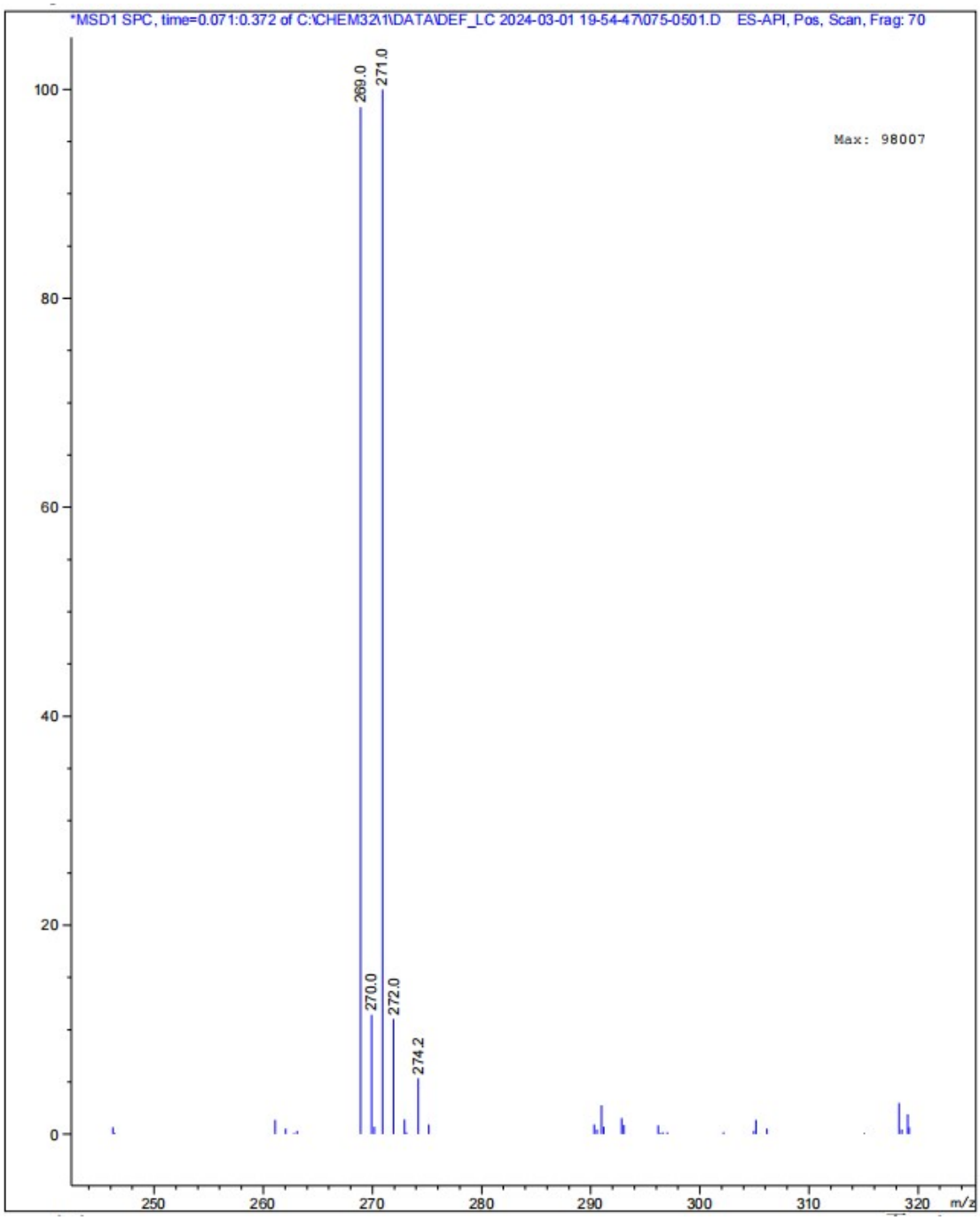


Figure S1. ESI-MS of intermediate **2a**

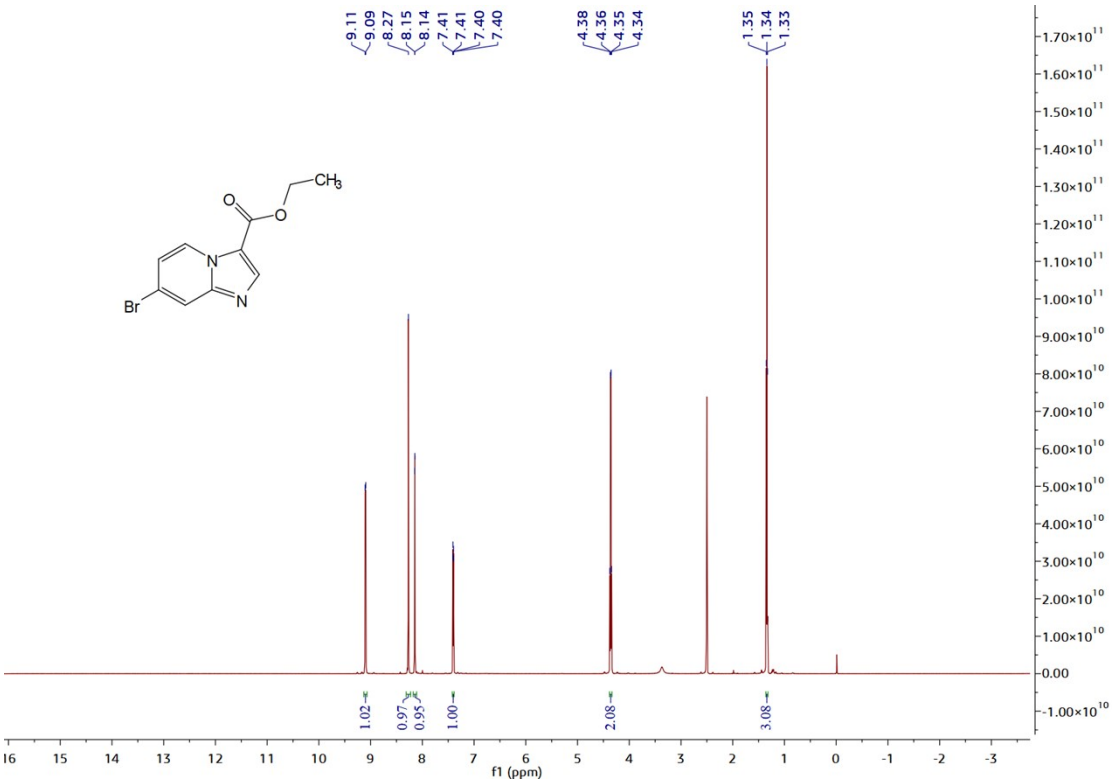


Figure S2. ^{1}H NMR Spectrum of compound **2a**

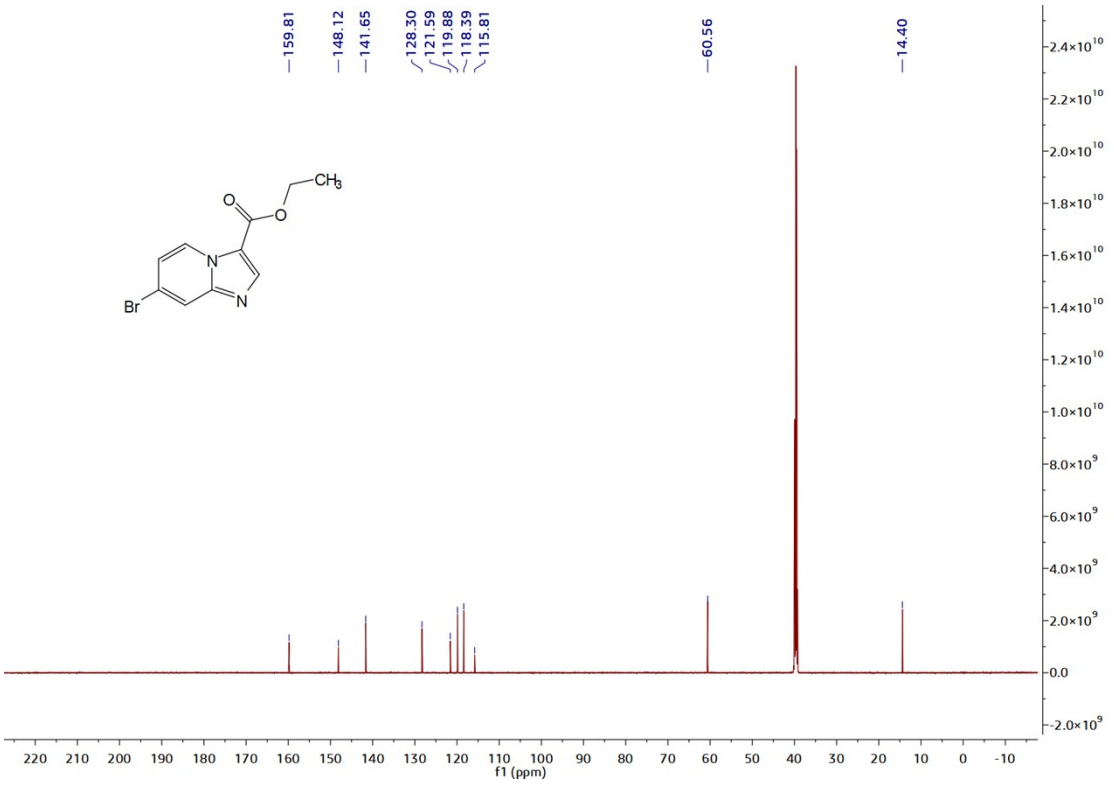


Figure S3. ^{13}C NMR Spectrum of compound **2a**

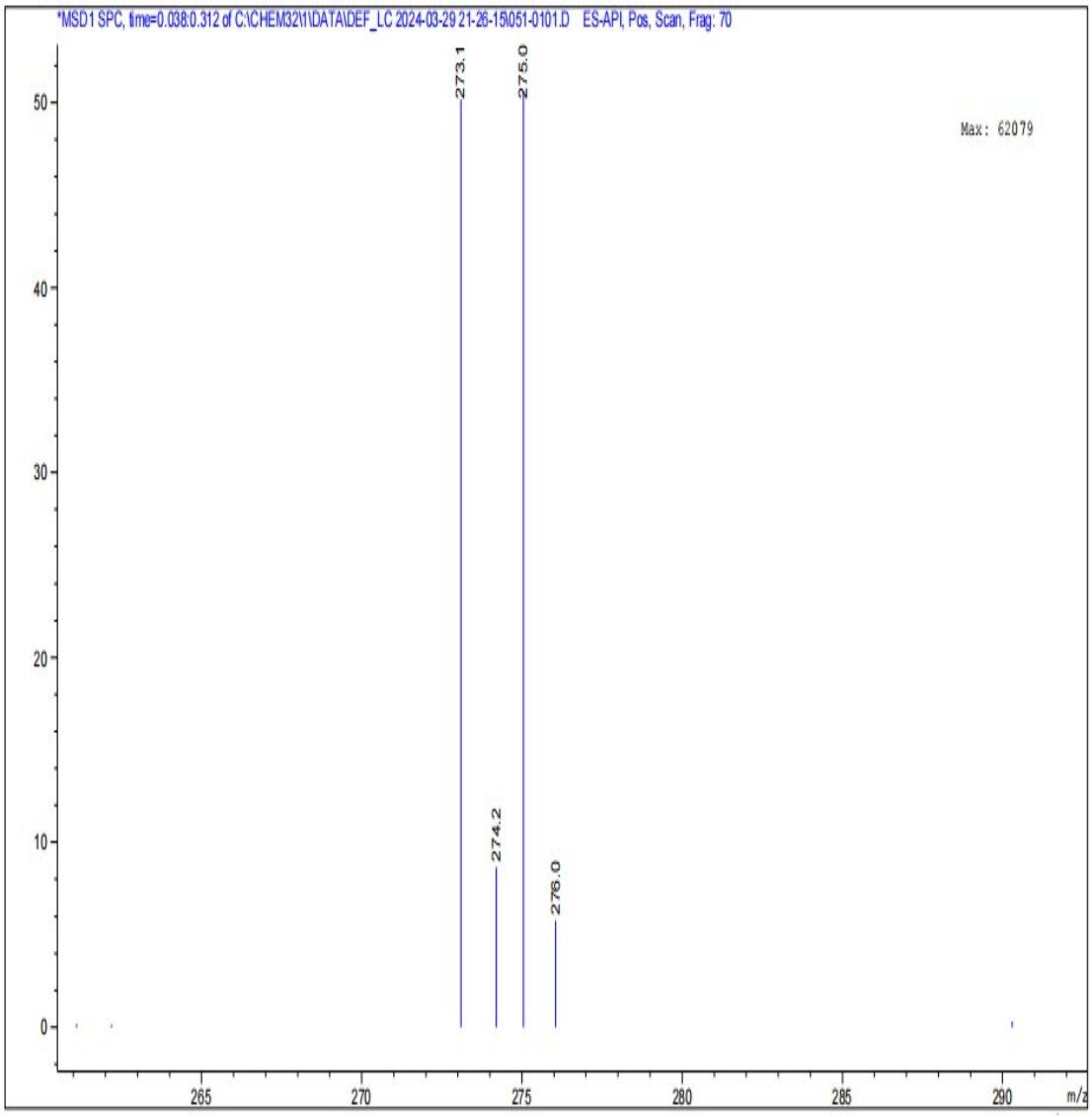


Figure S4. ESI-MS of intermediate **3a**

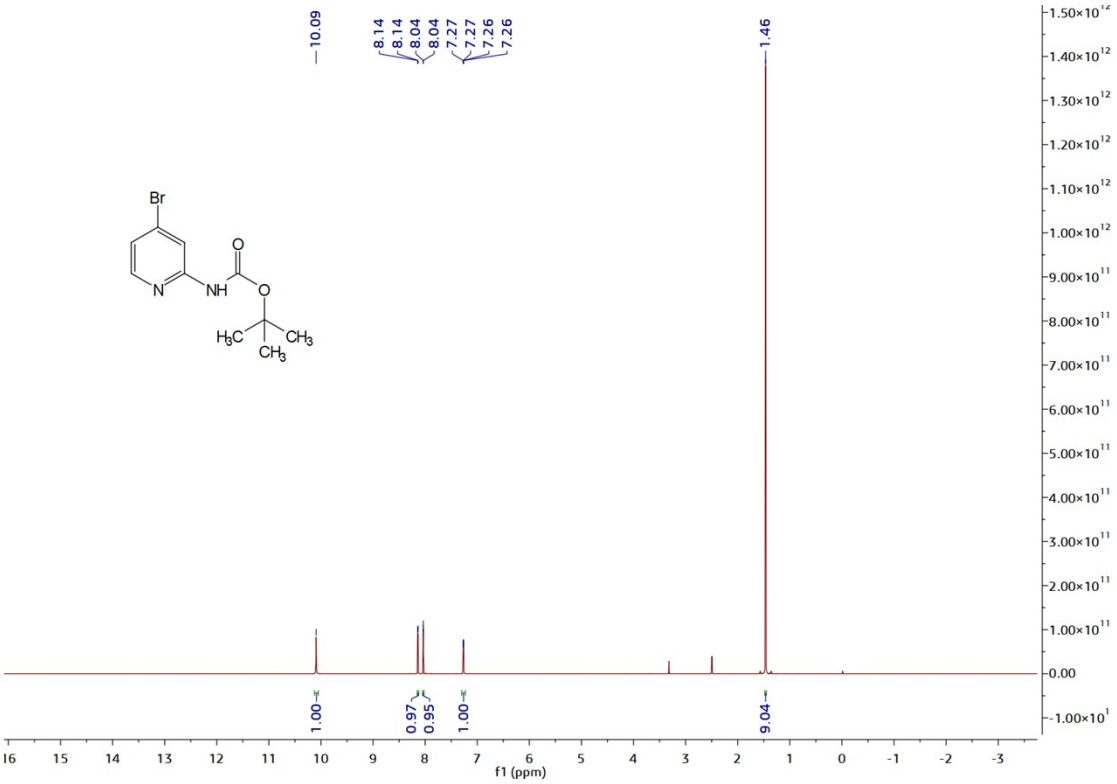


Figure S5. ^{1}H NMR Spectrum of compound **3a**

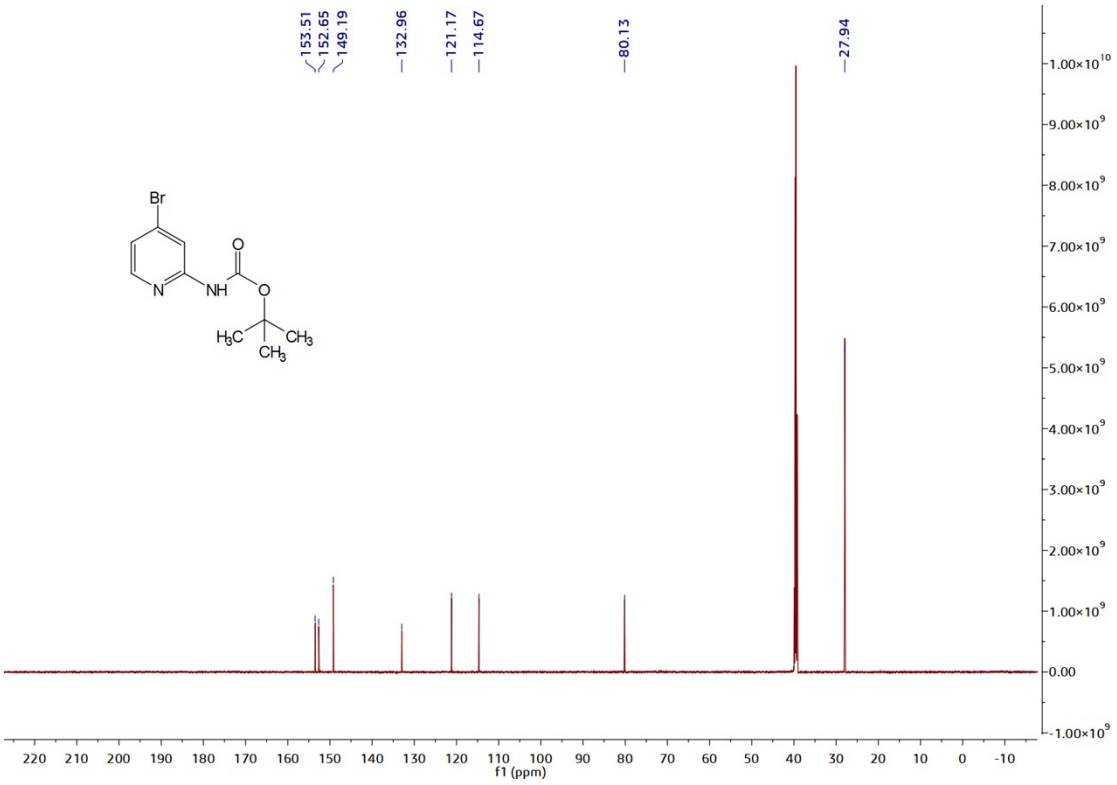


Figure S6. ^{13}C NMR Spectrum of compound **3a**

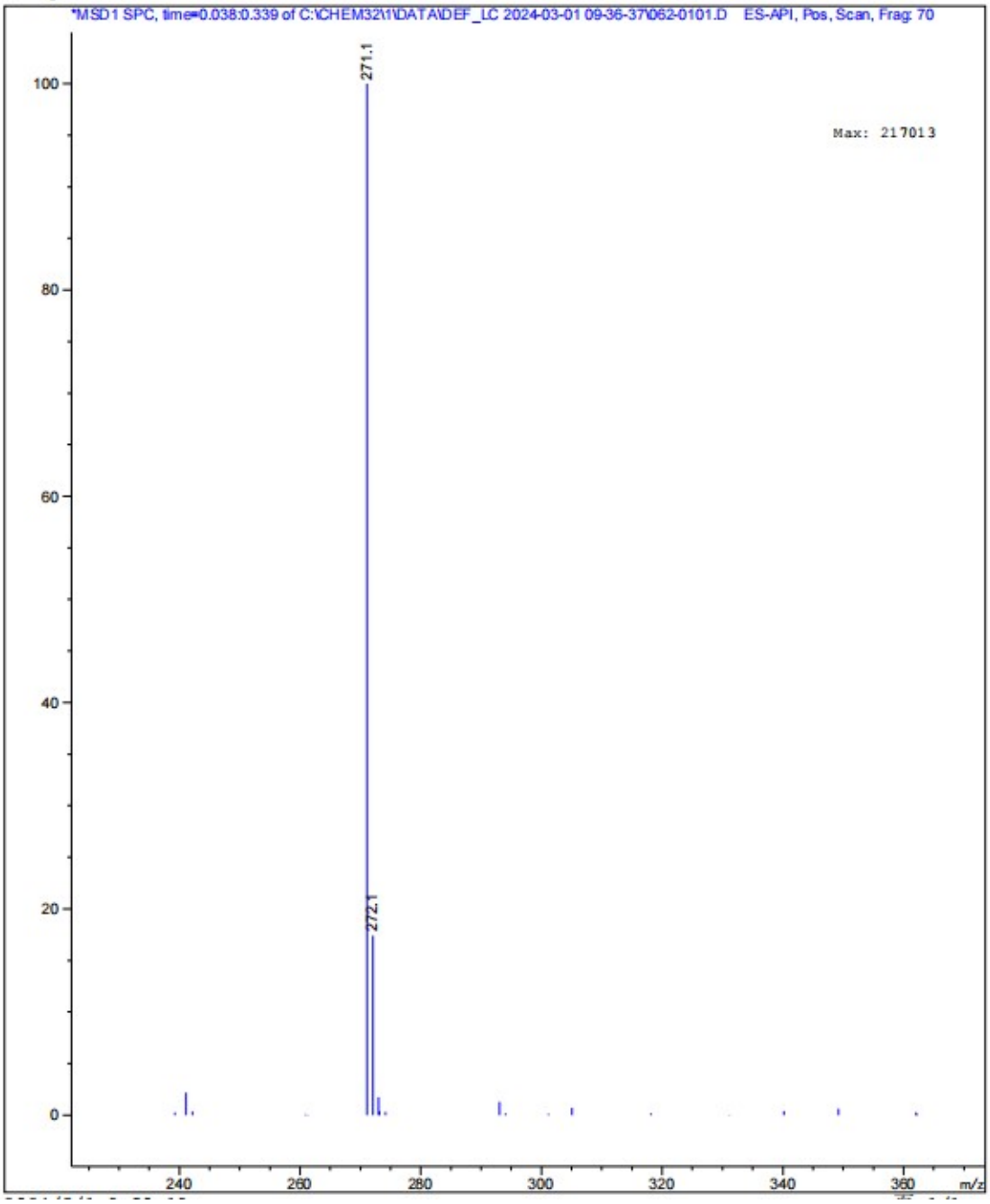


Figure S7. ESI-MS of intermediate **6a**

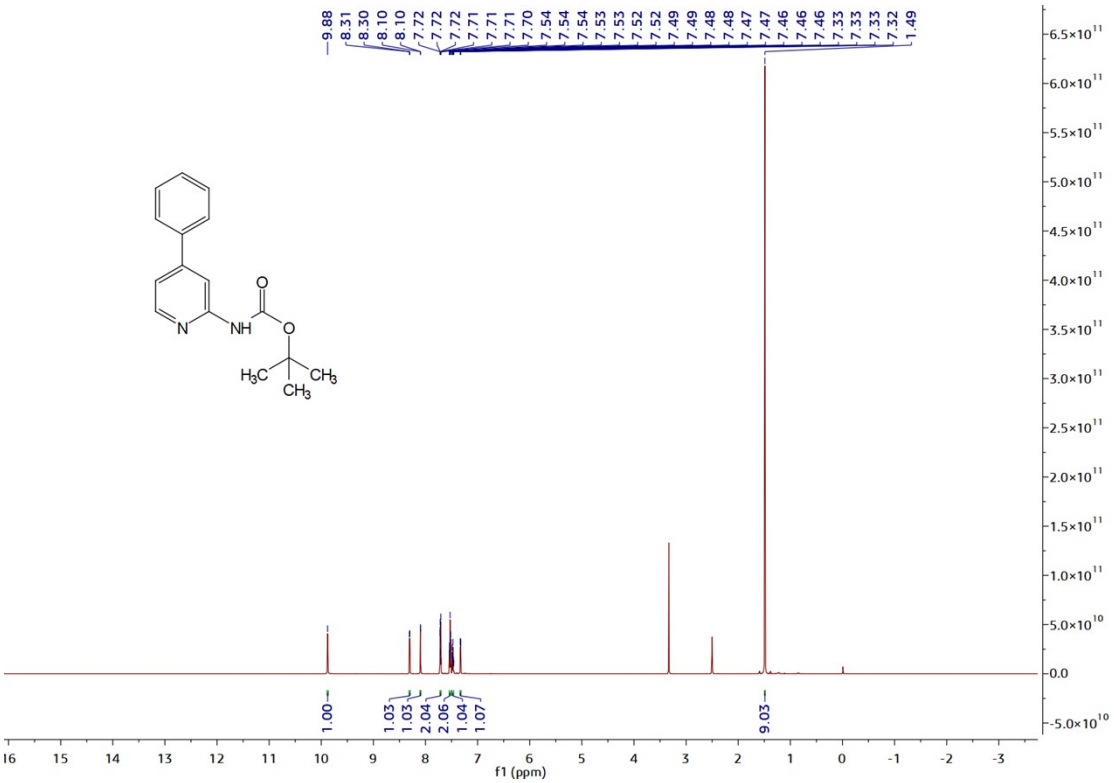


Figure S8. ^{1}H NMR Spectrum of compound **6a**

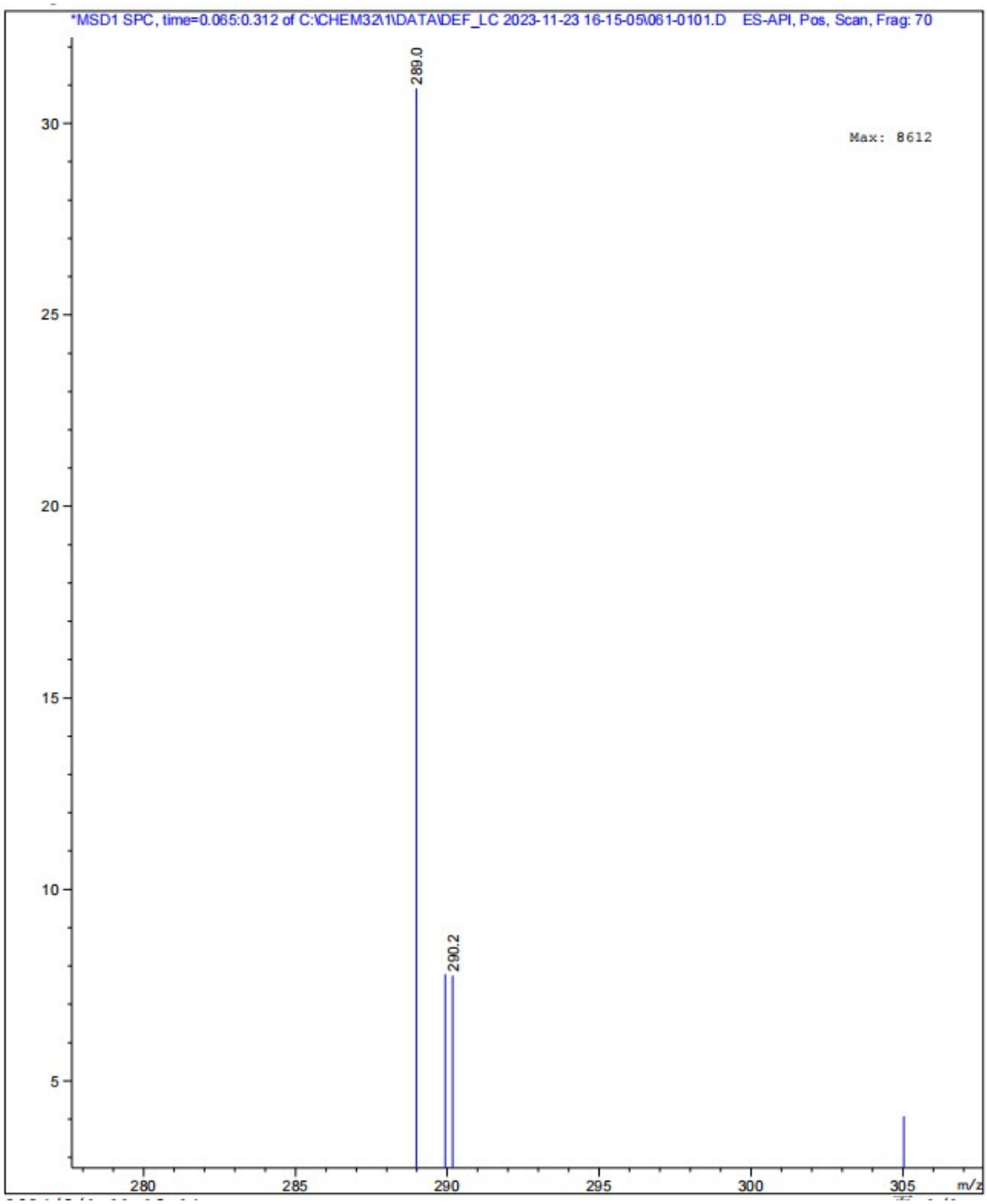


Figure S9. ESI-MS of intermediate 6b

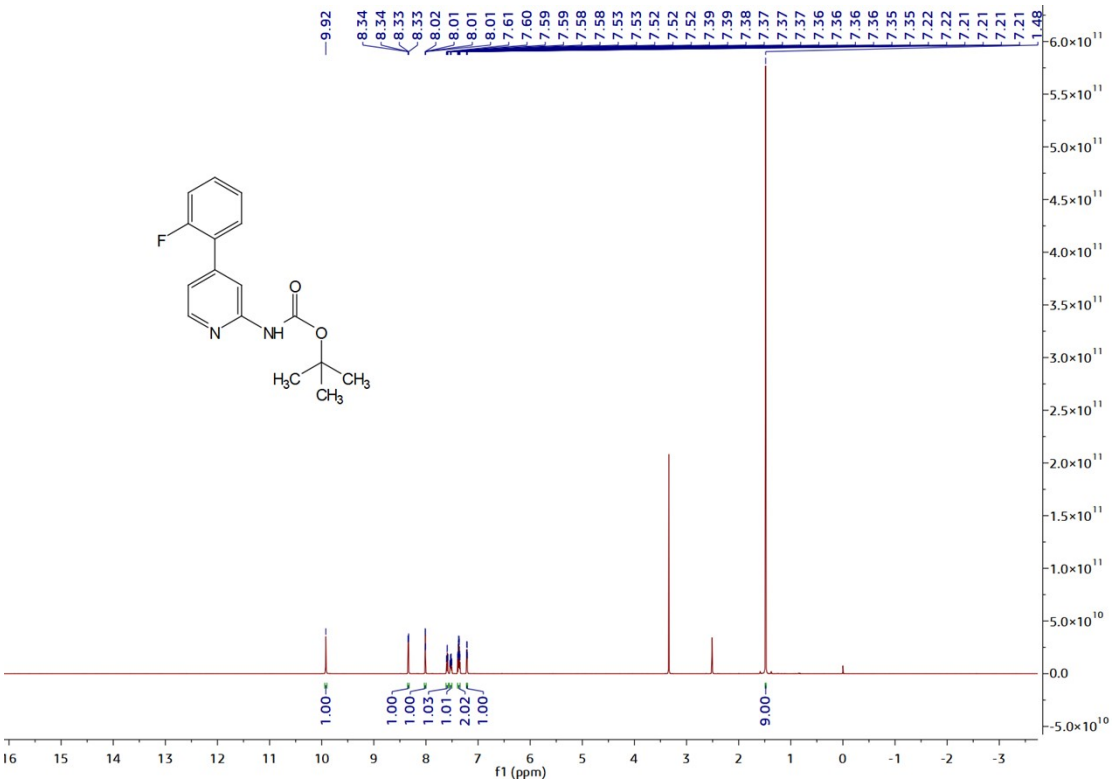


Figure S10. ^{1}H NMR Spectrum of compound 6b

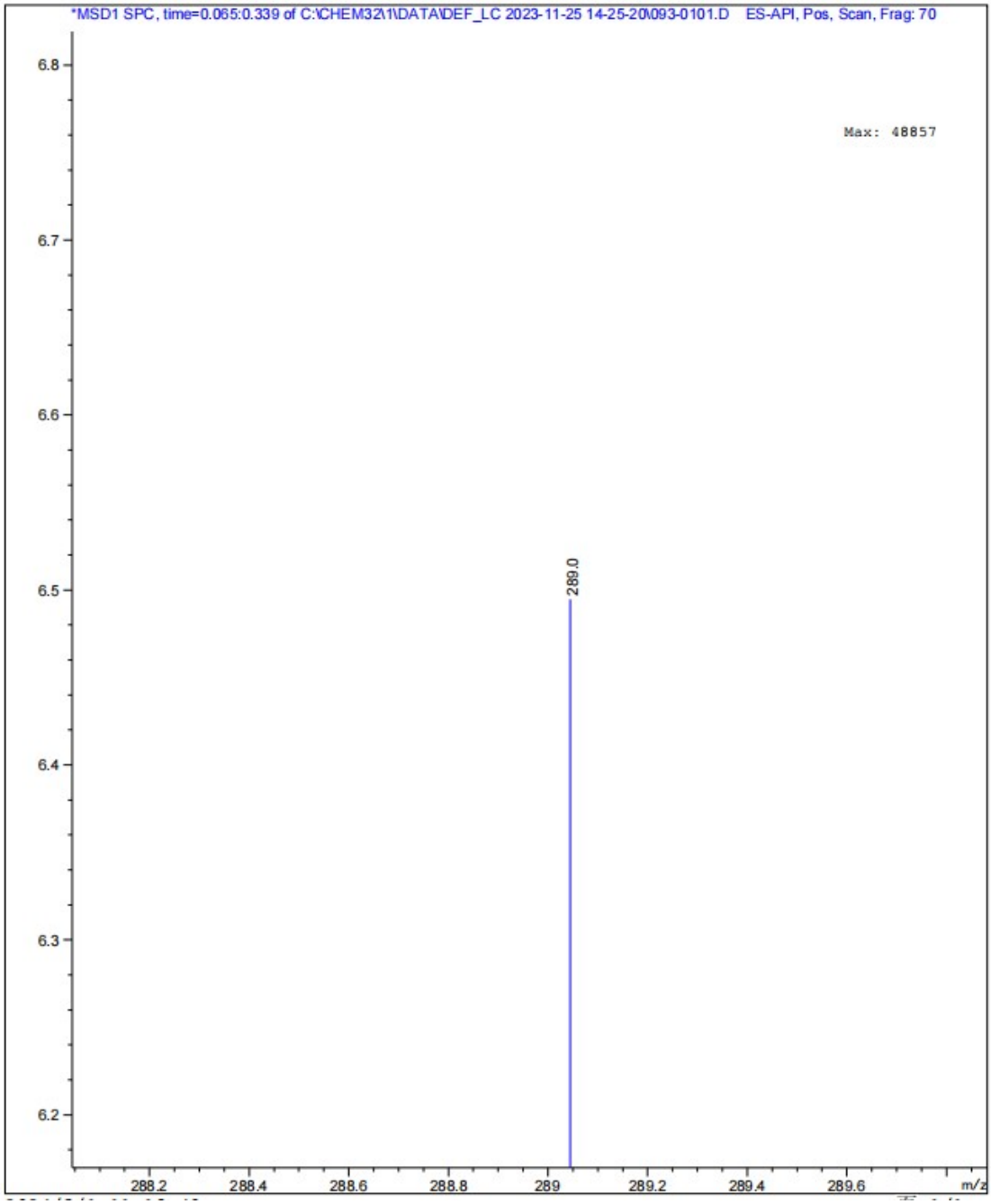


Figure S11. ESI-MS of intermediate 6c

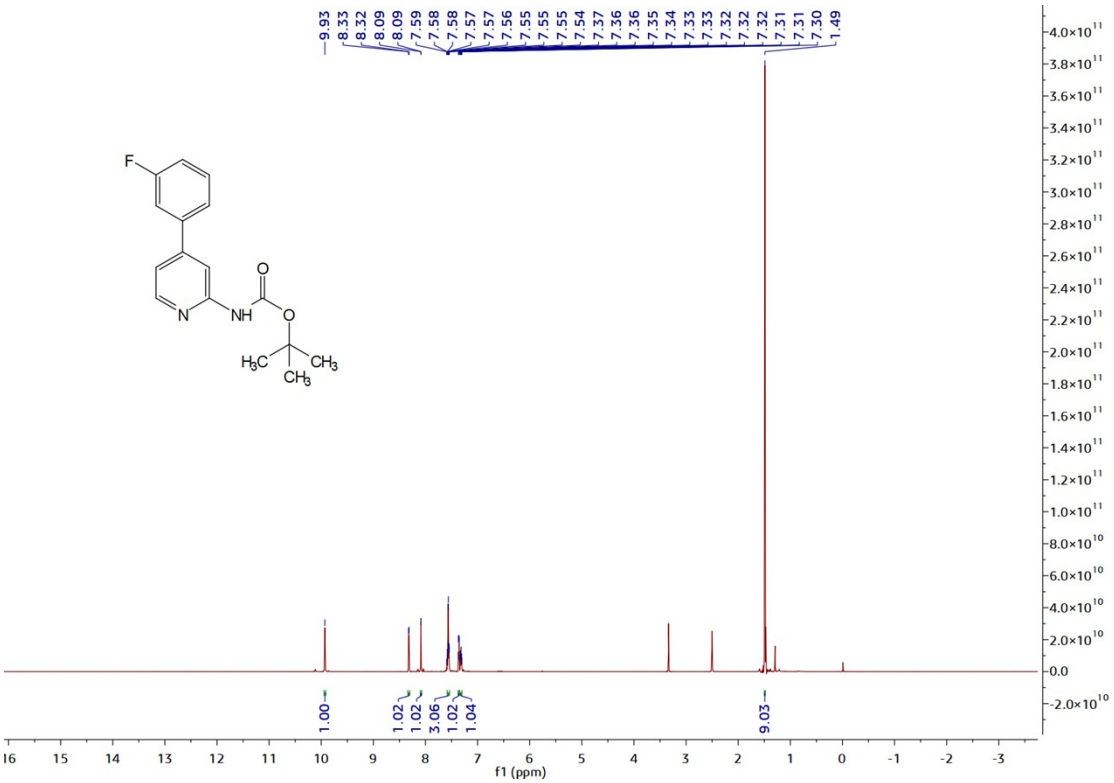


Figure S12.[1]H NMR Spectrum of compound 6c

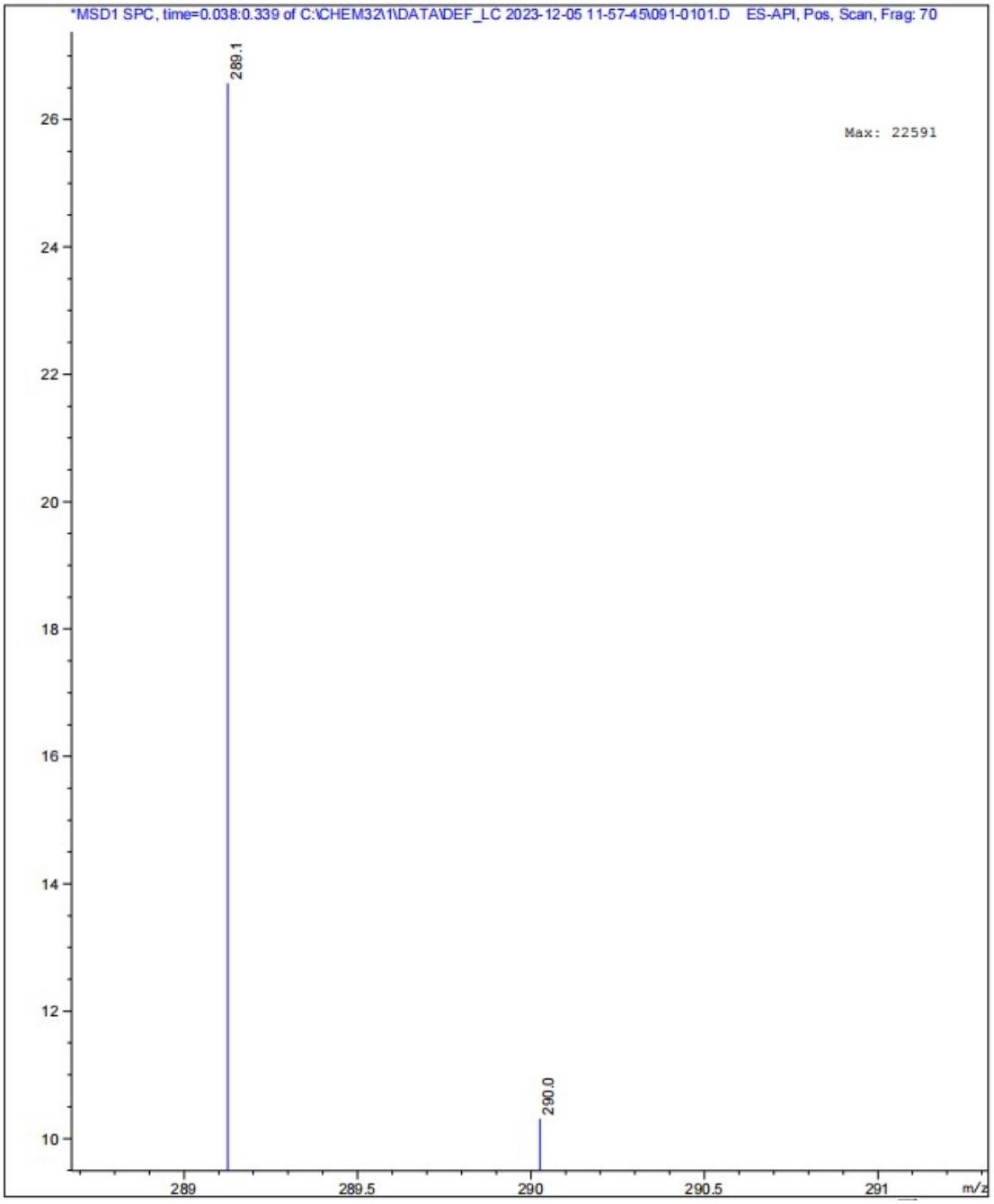


Figure S13.ESI-MS of intermediate 6d

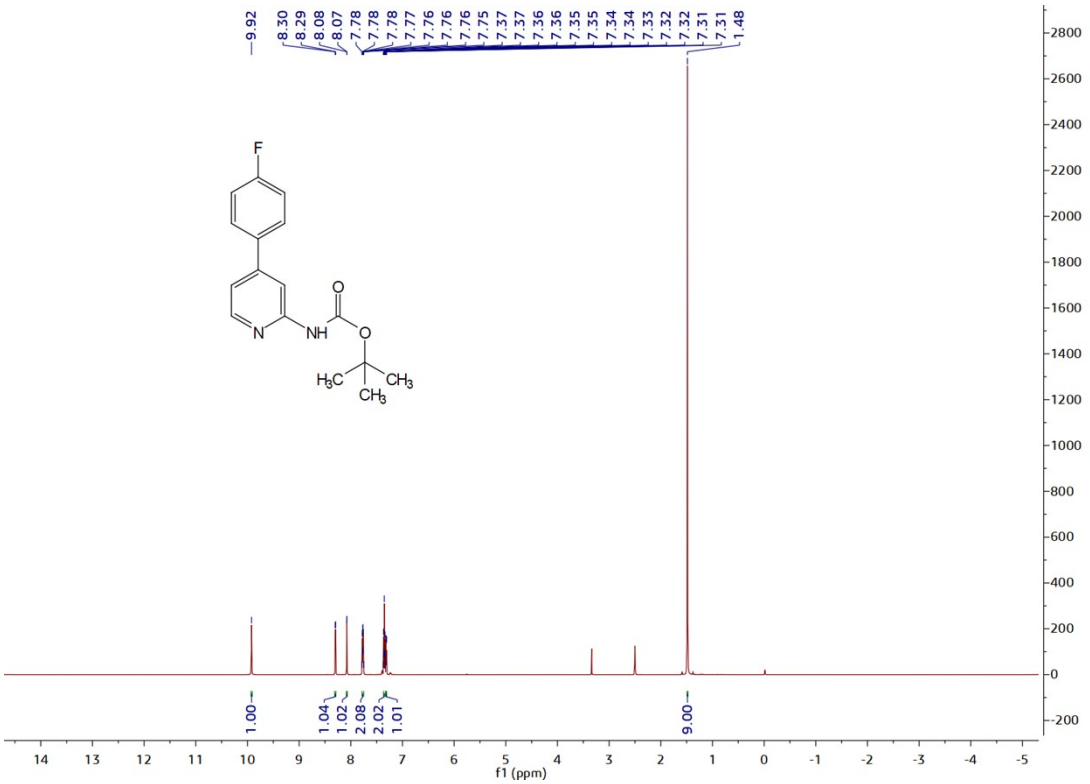


Figure S14. ^{1}H NMR Spectrum of compound 6d

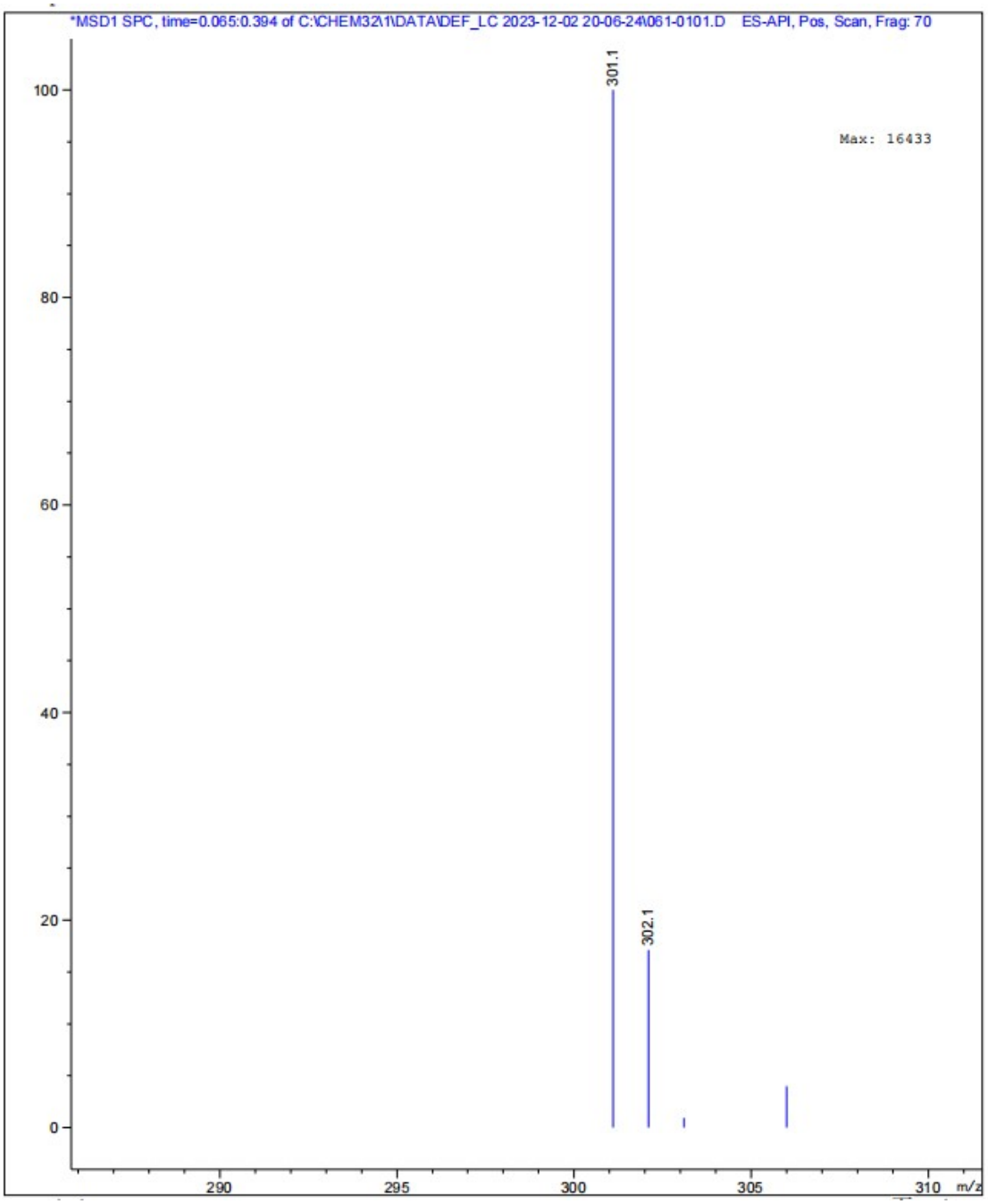


Figure S15. ESI-MS of intermediate 6e

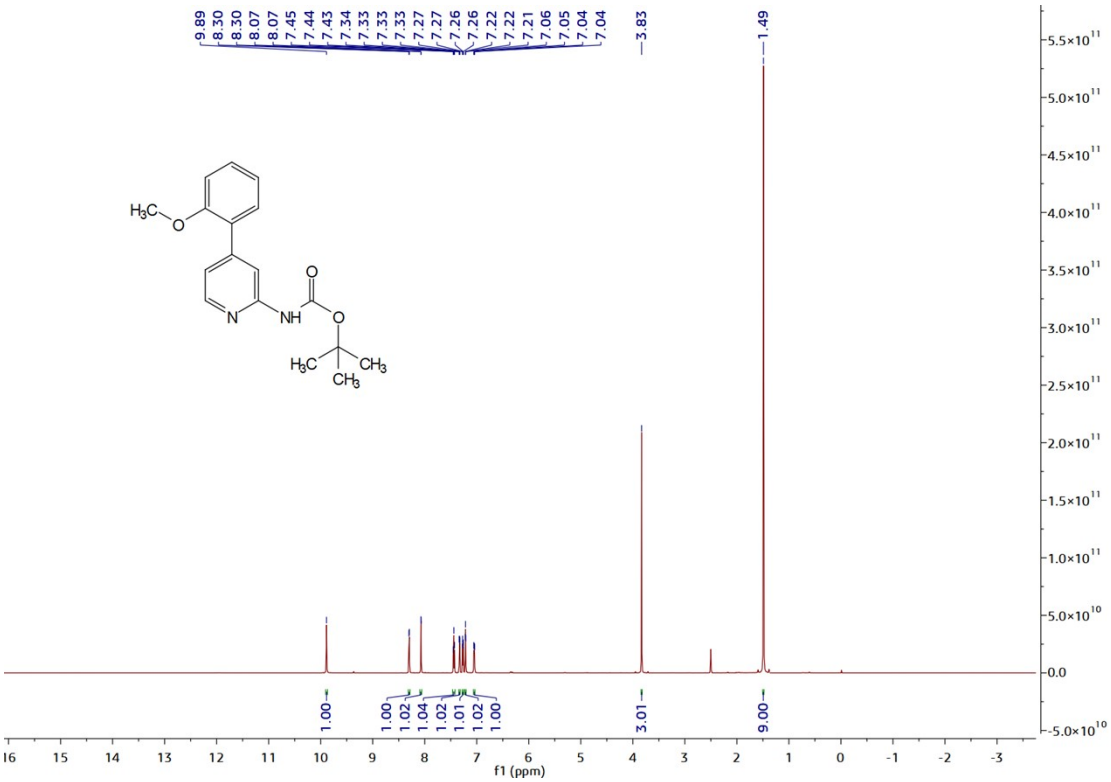


Figure S16. ^{1}H NMR Spectrum of compound 6e

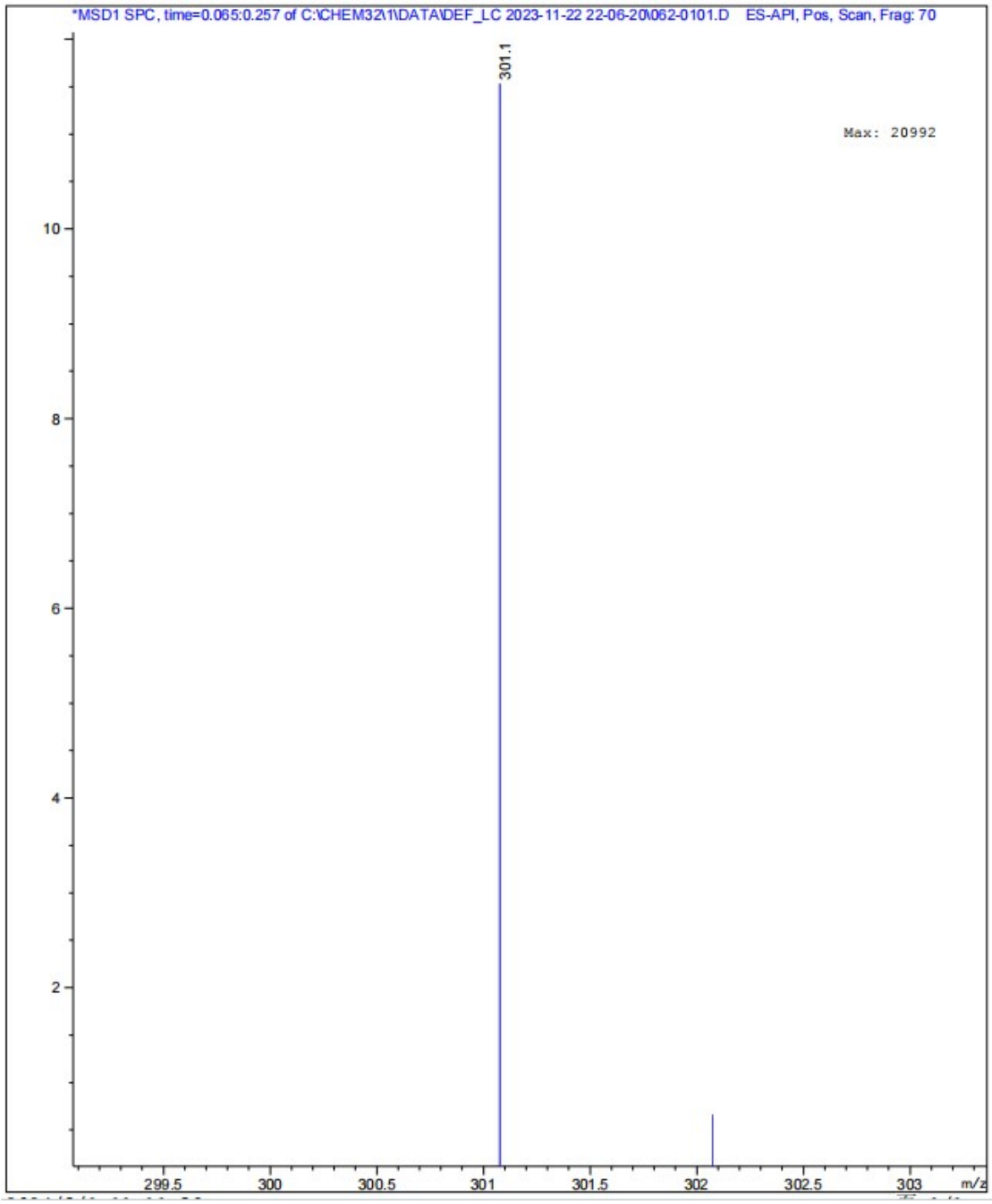


Figure S17. ESI-MS of intermediate 6f

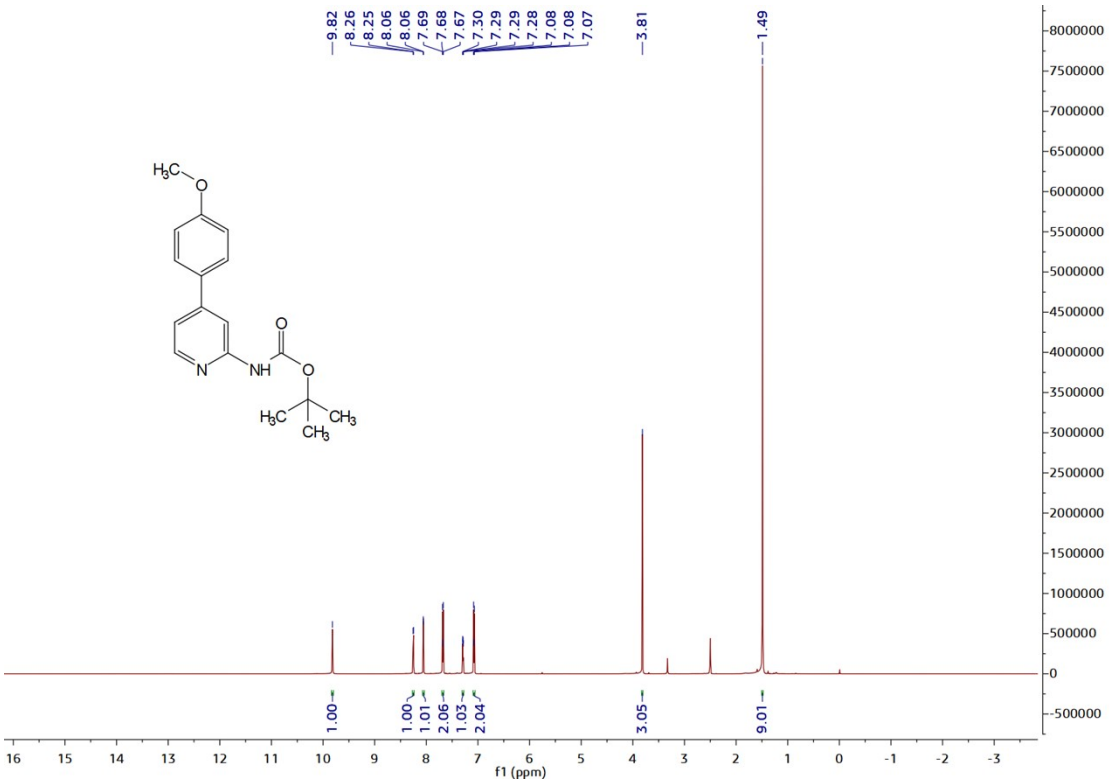


Figure S18. ^{1}H NMR Spectrum of compound 6f

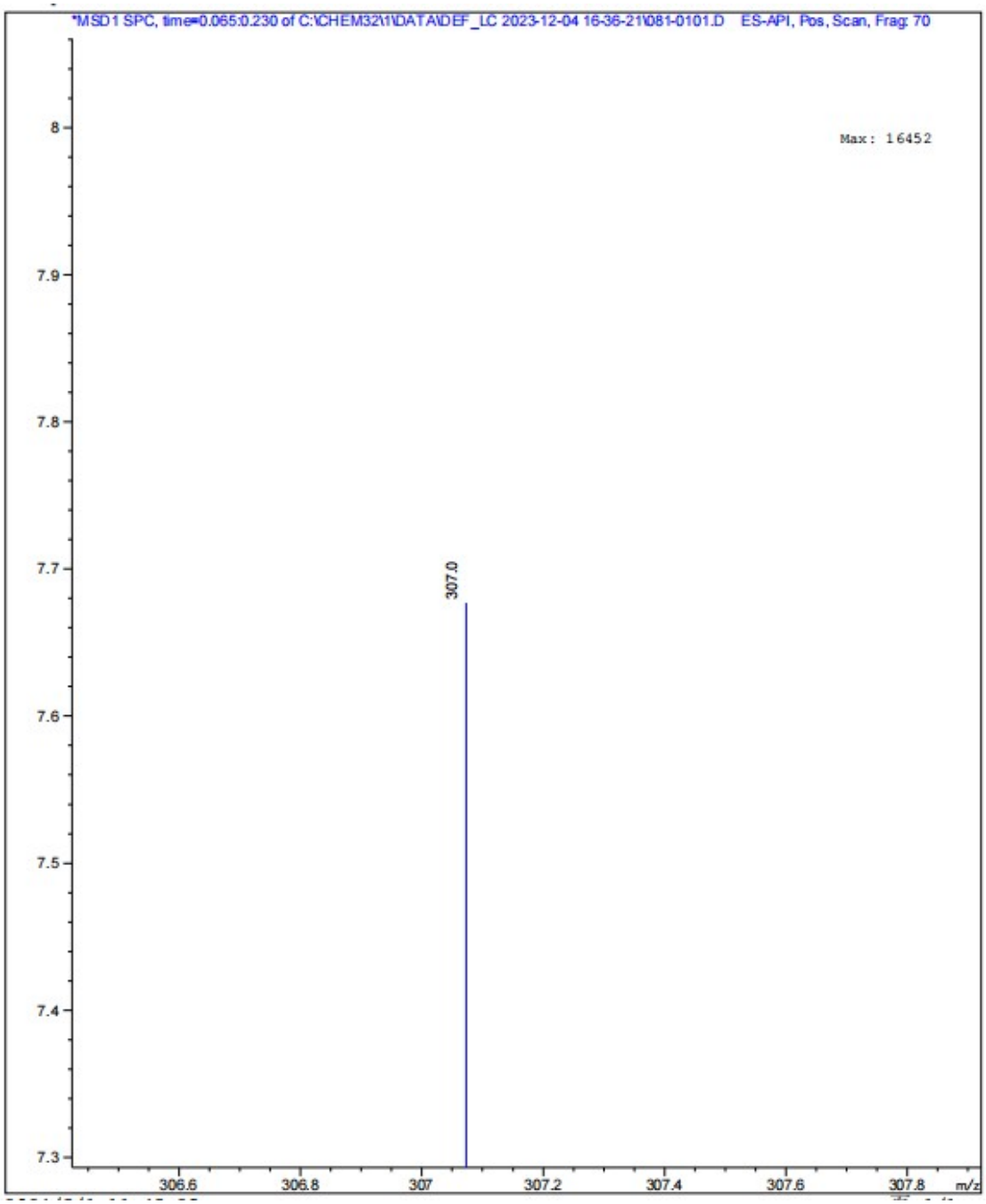


Figure S19. ESI-MS of intermediate 6g

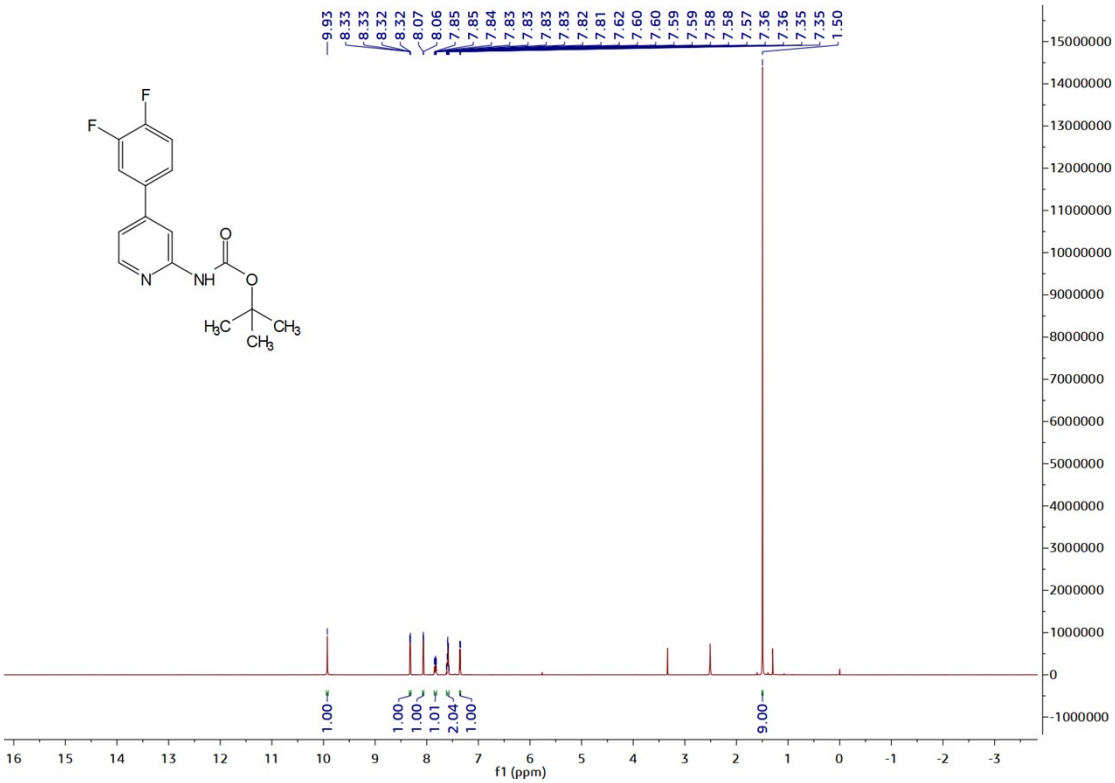


Figure S20. ^{1}H NMR Spectrum of compound 6g

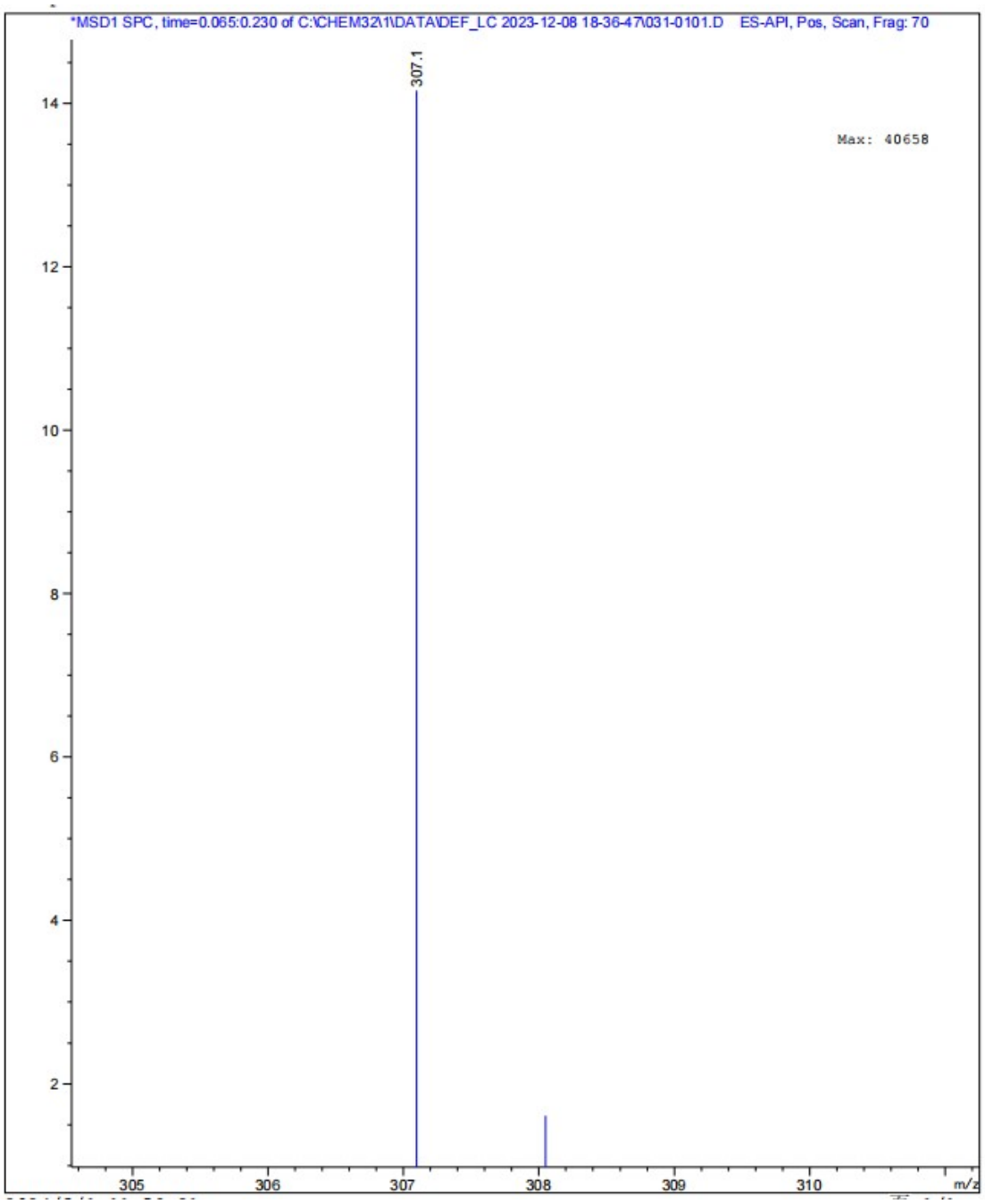


Figure S21. ESI-MS of intermediate 6h

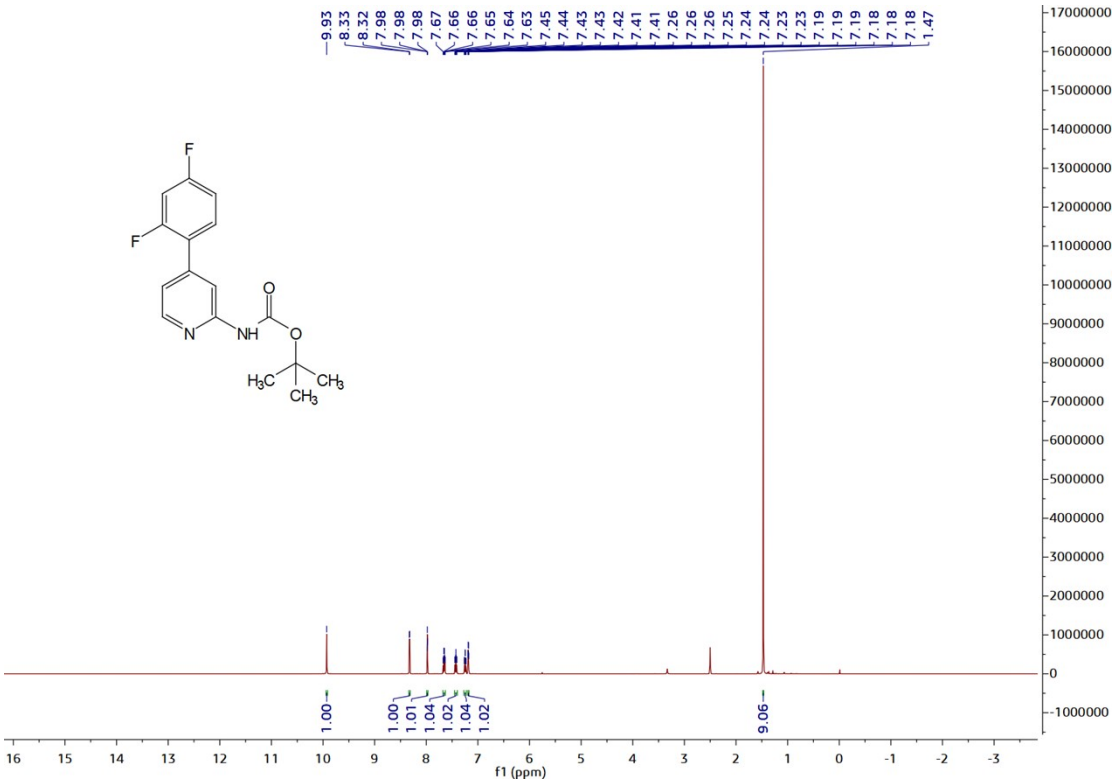


Figure S22. ^{1}H NMR Spectrum of compound 6h

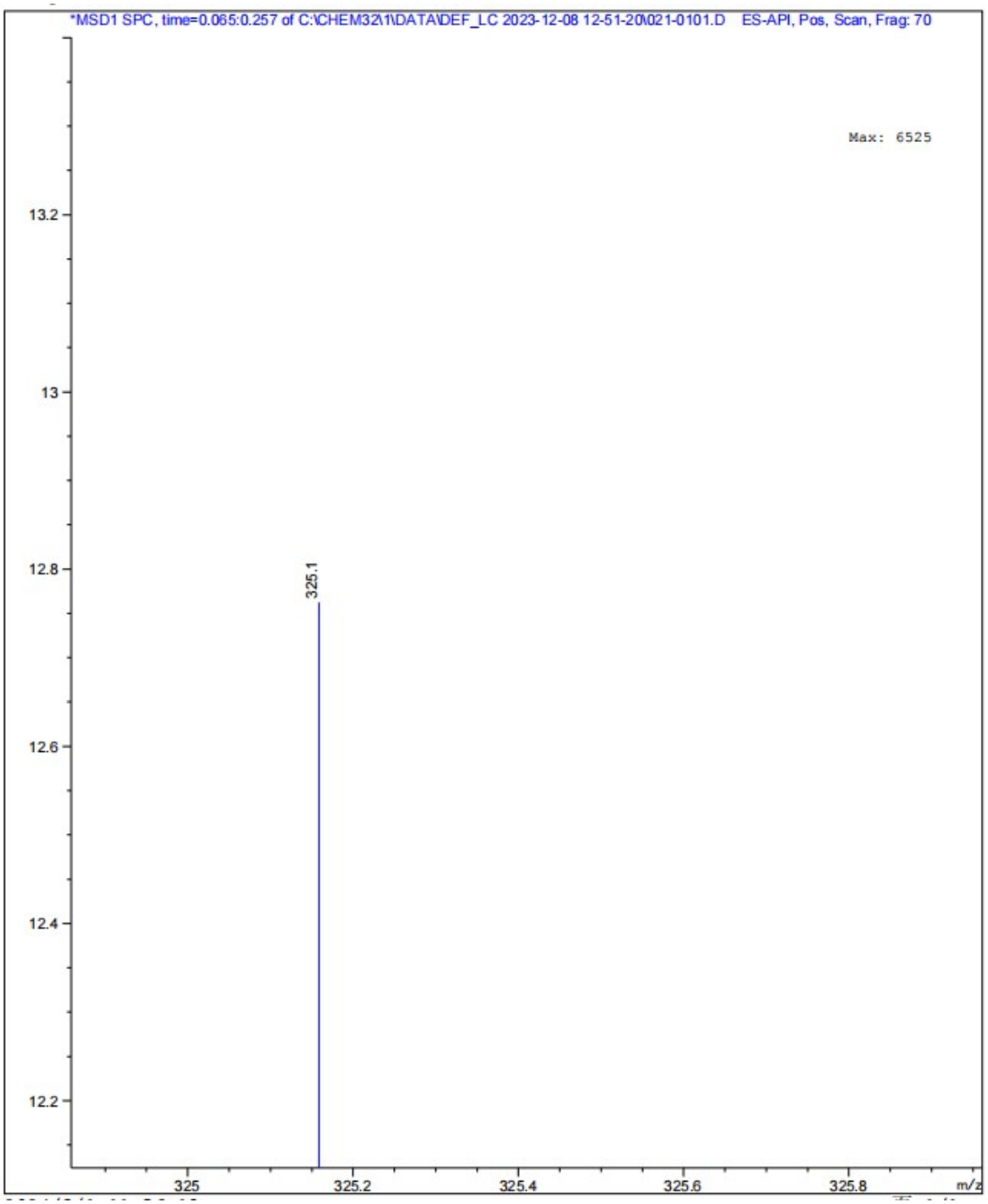


Figure S23. ESI-MS of intermediate 6i

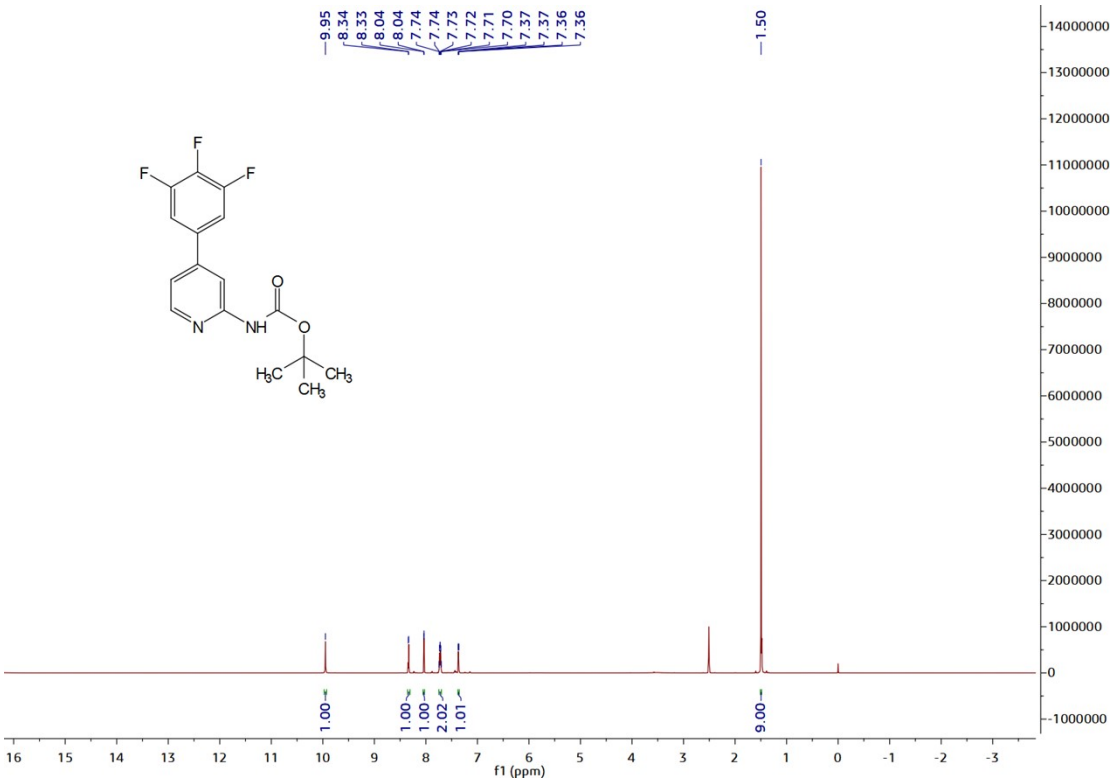


Figure S24. ^{1}H NMR Spectrum of compound 6i

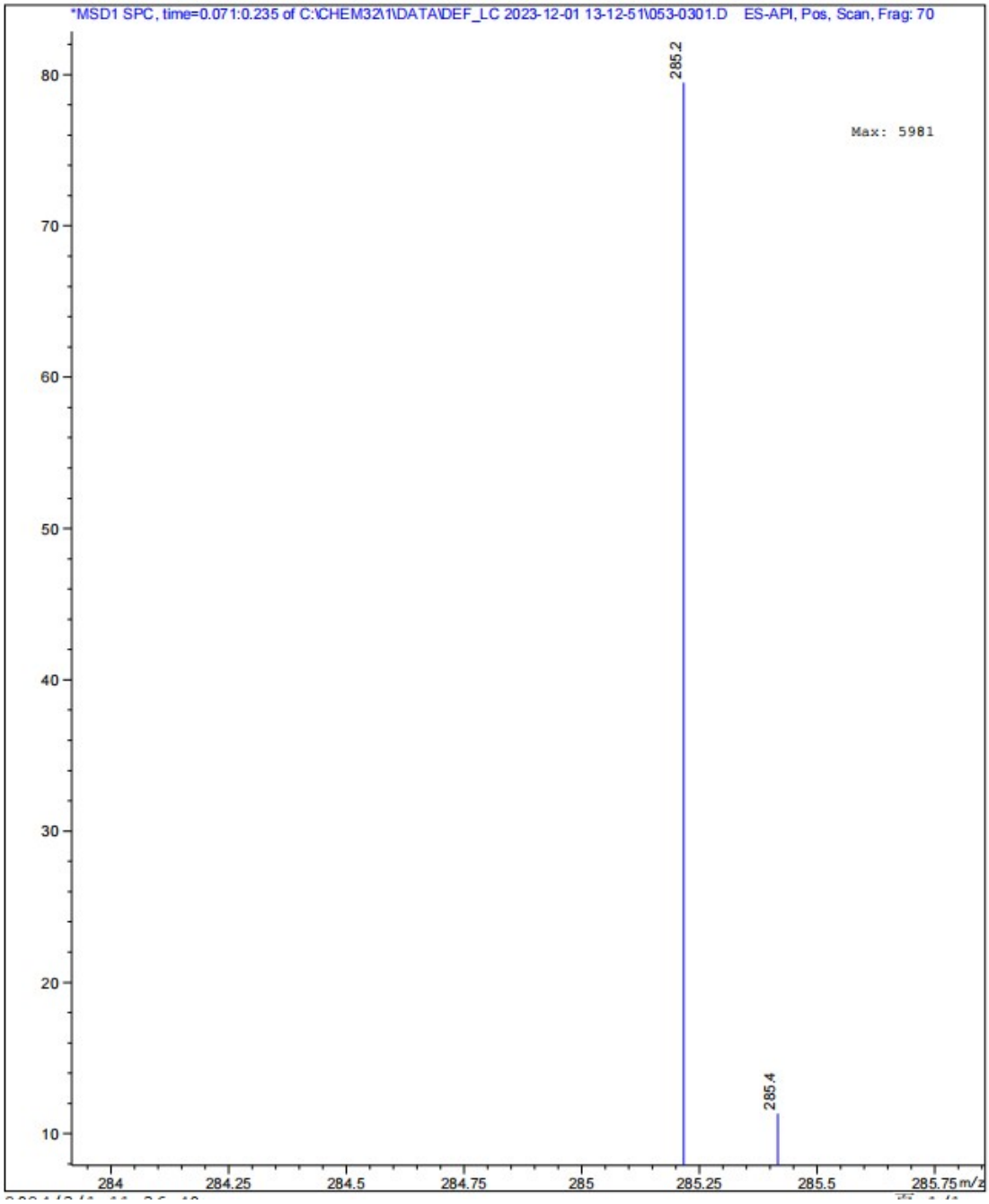


Figure S25. ESI-MS of intermediate 6j

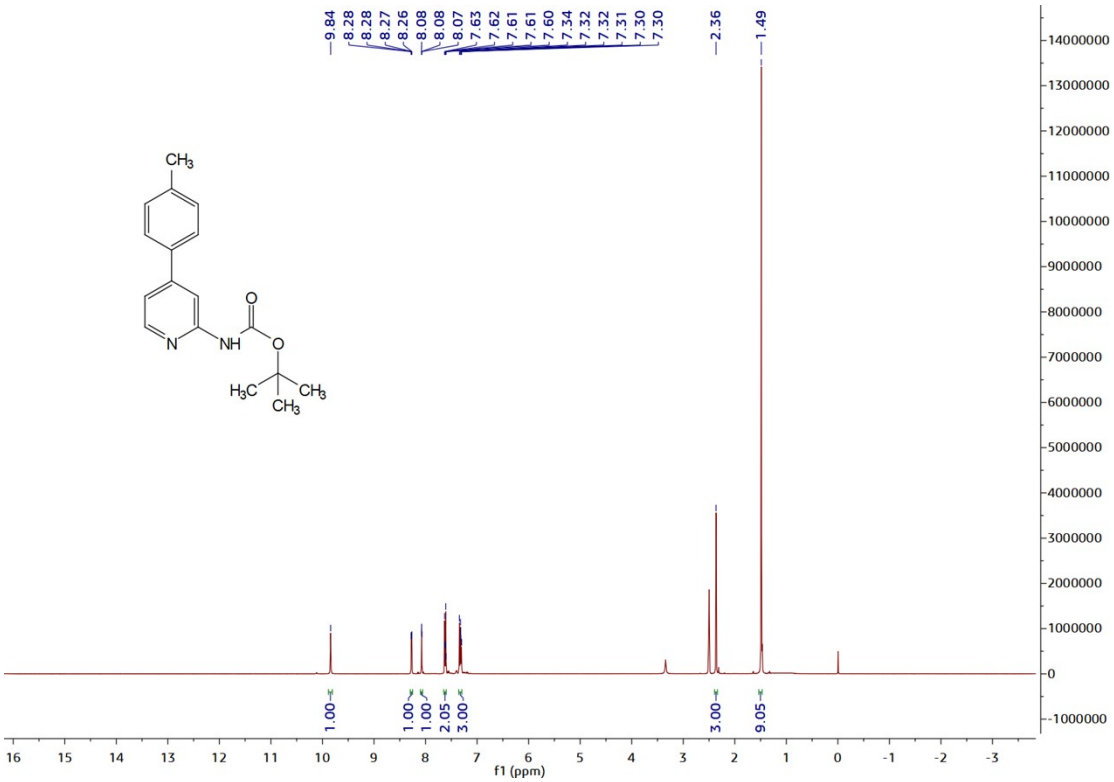


Figure S26. ^{1}H NMR Spectrum of compound 6j

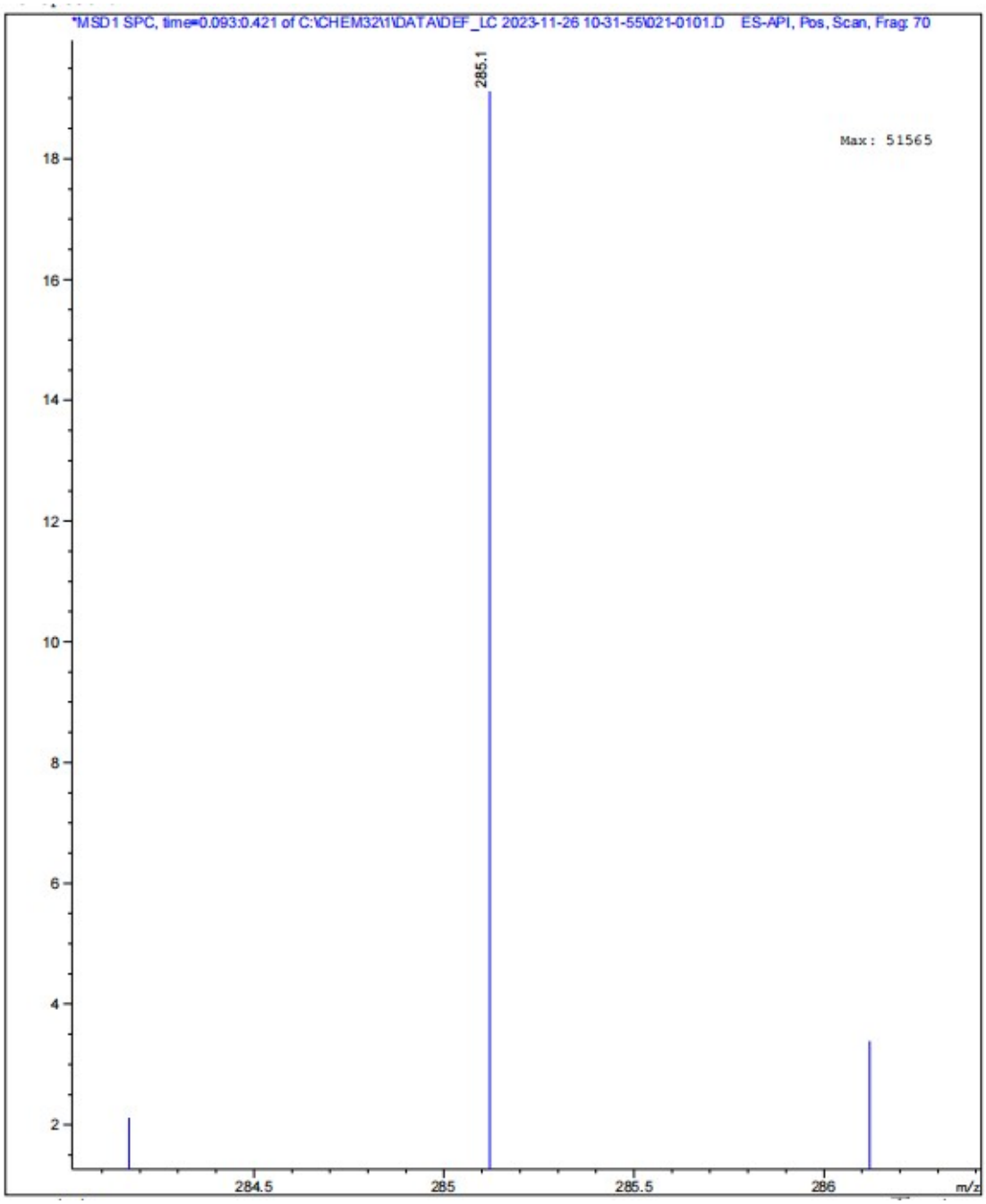


Figure S27. ESI-MS of intermediate 6k

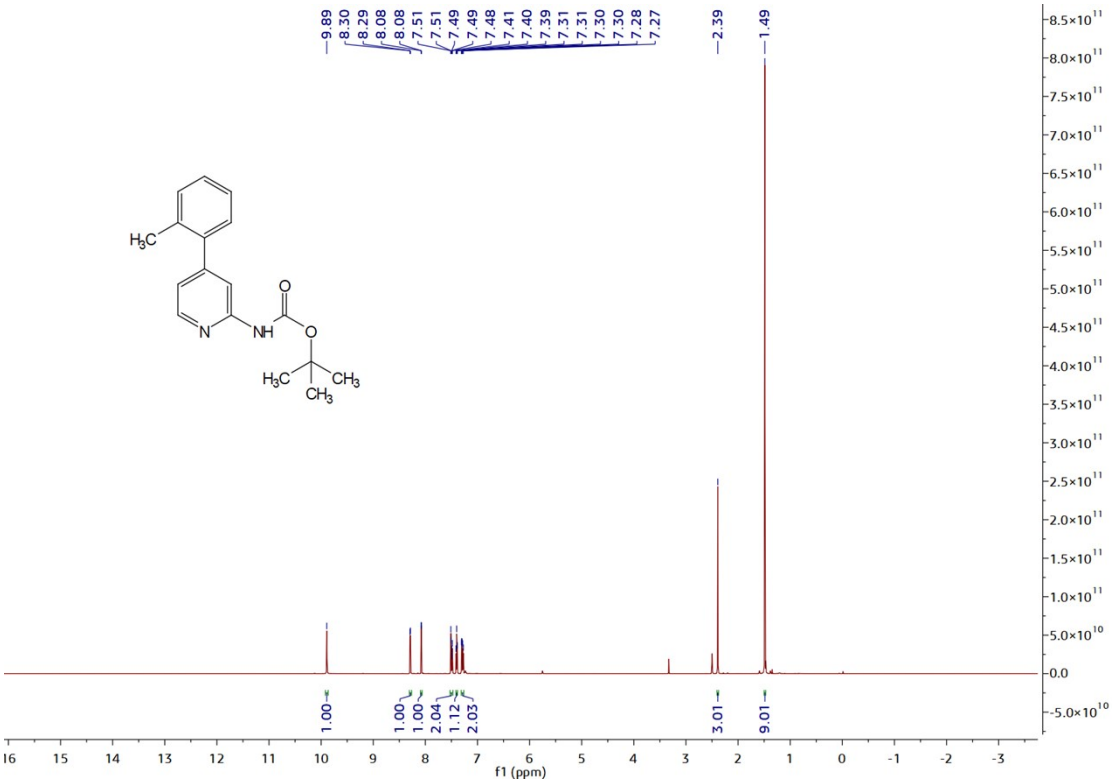


Figure S28. ^{1}H NMR Spectrum of compound 6k

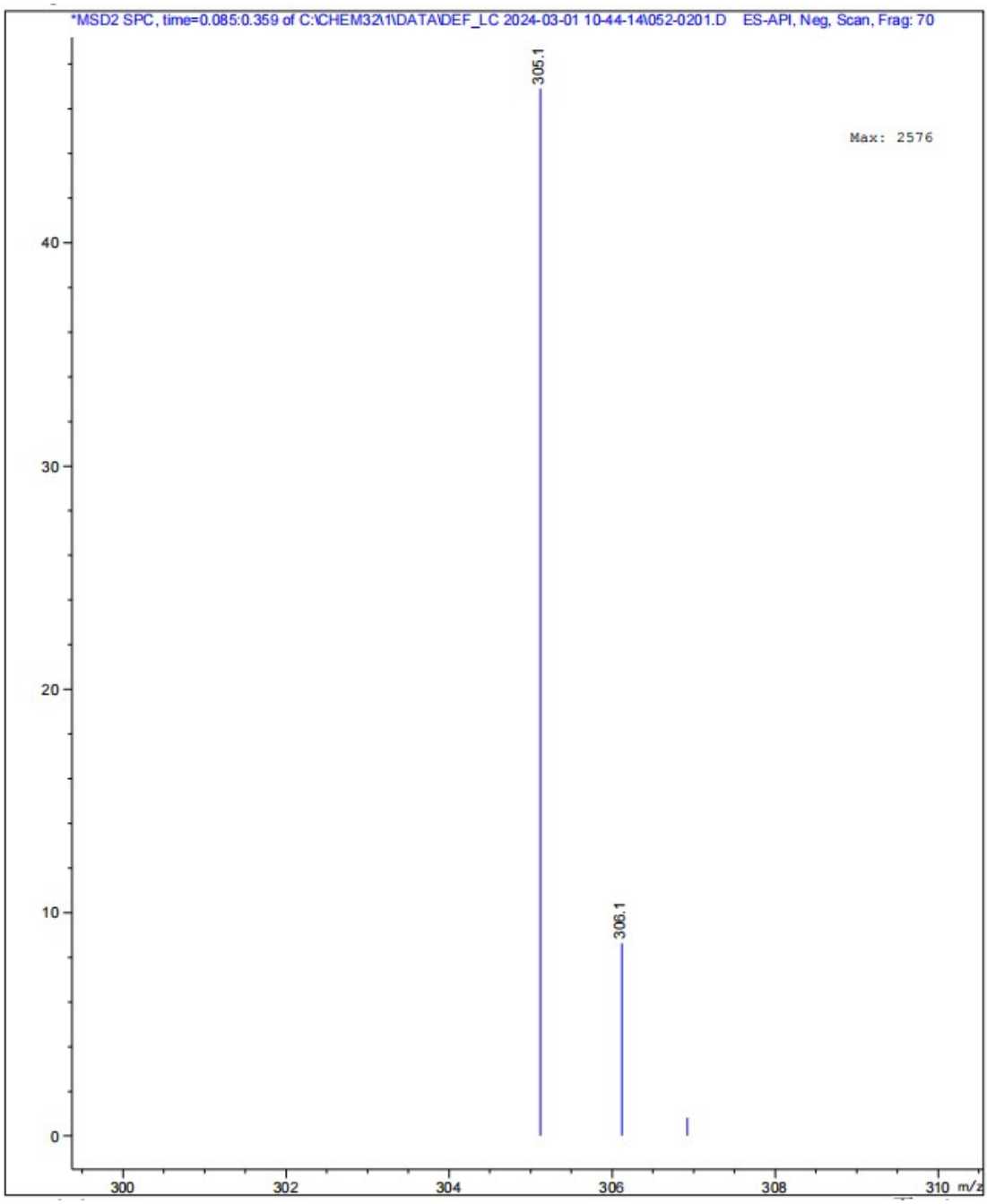


Figure S29. ESI-MS of intermediate 6l

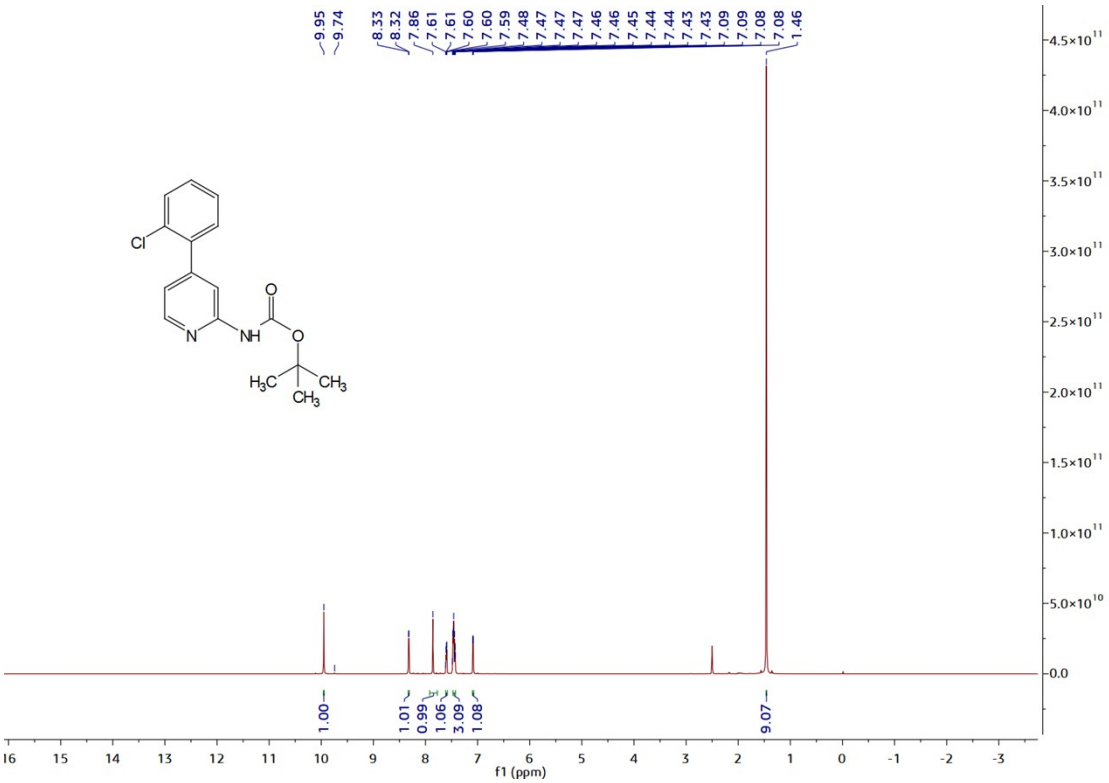


Figure S30. ^{1}H NMR Spectrum of compound 6l

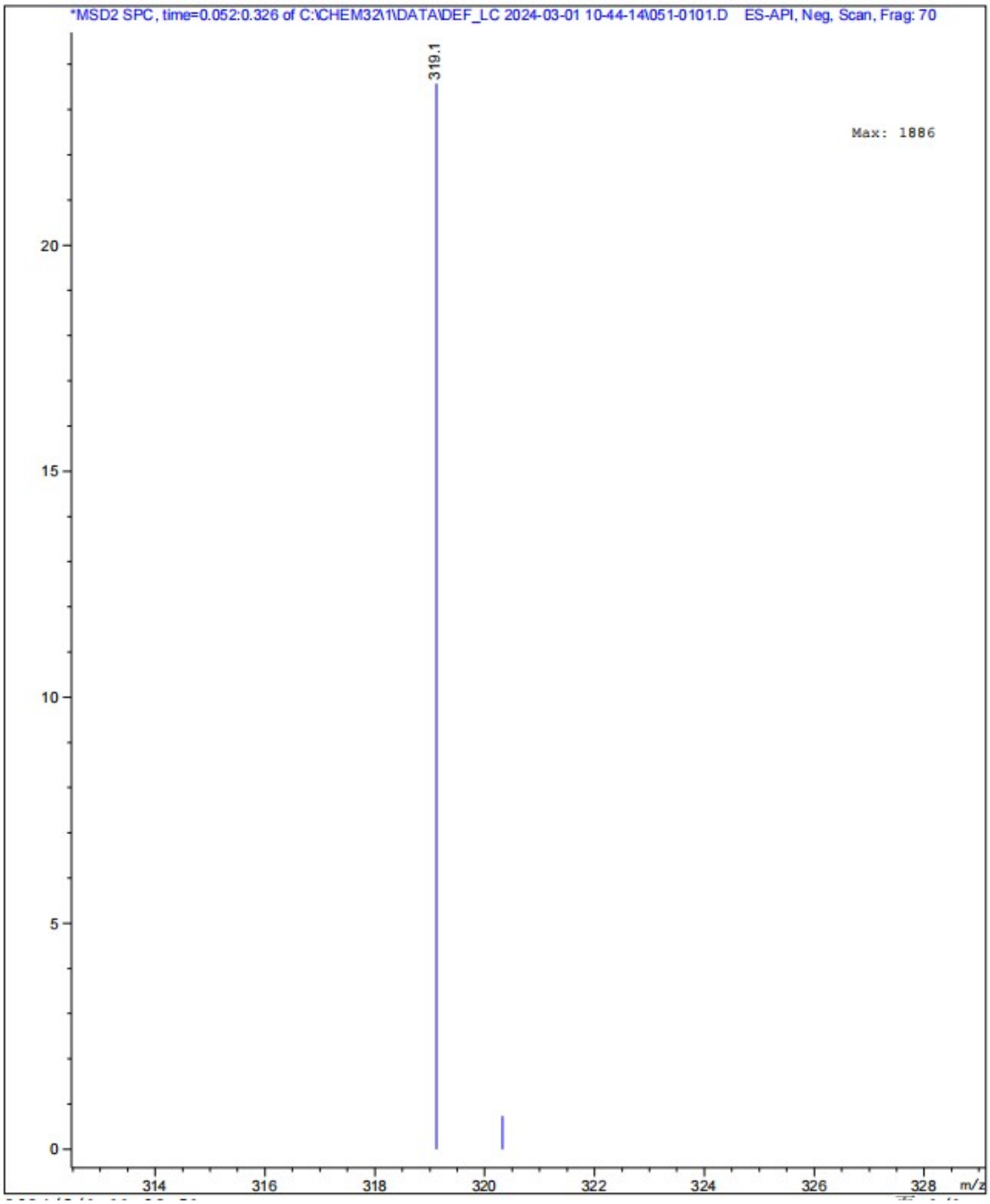


Figure S31. ESI-MS of intermediate 6m

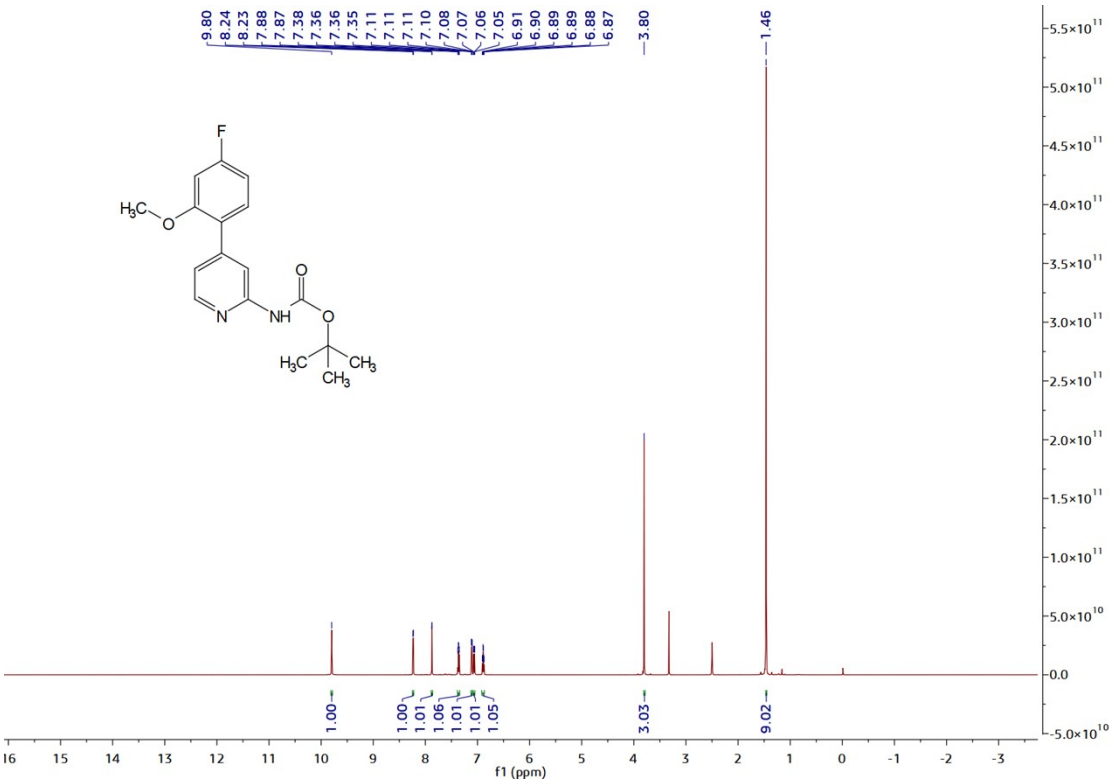


Figure S32. ^{1}H NMR Spectrum of compound 6m

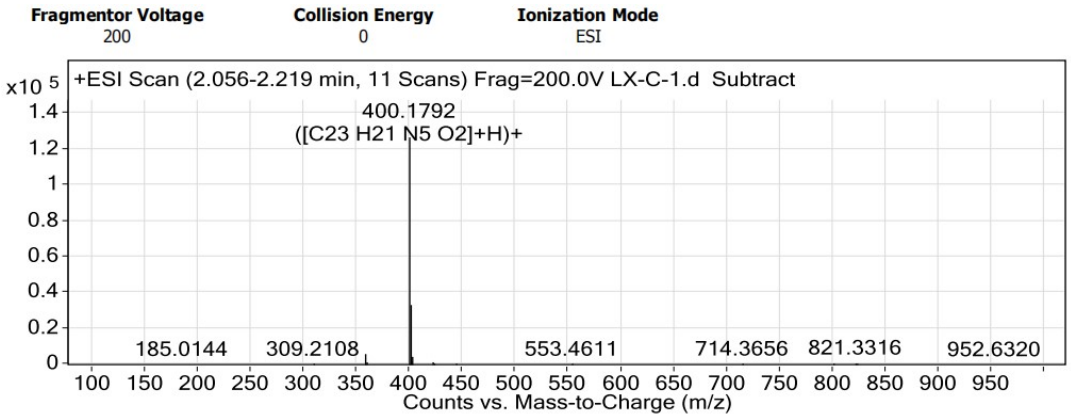


Figure S33. HRMS of compound LA-1

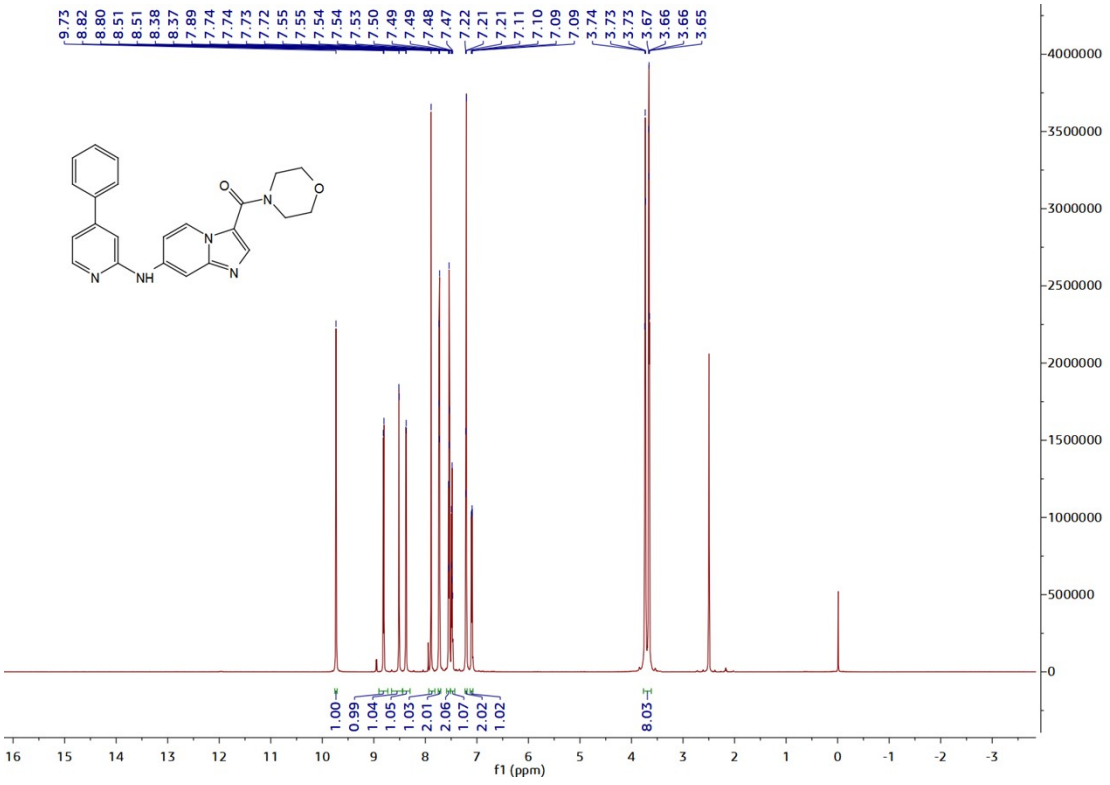


Figure S34. ^{1}H NMR Spectrum of compound LA-1

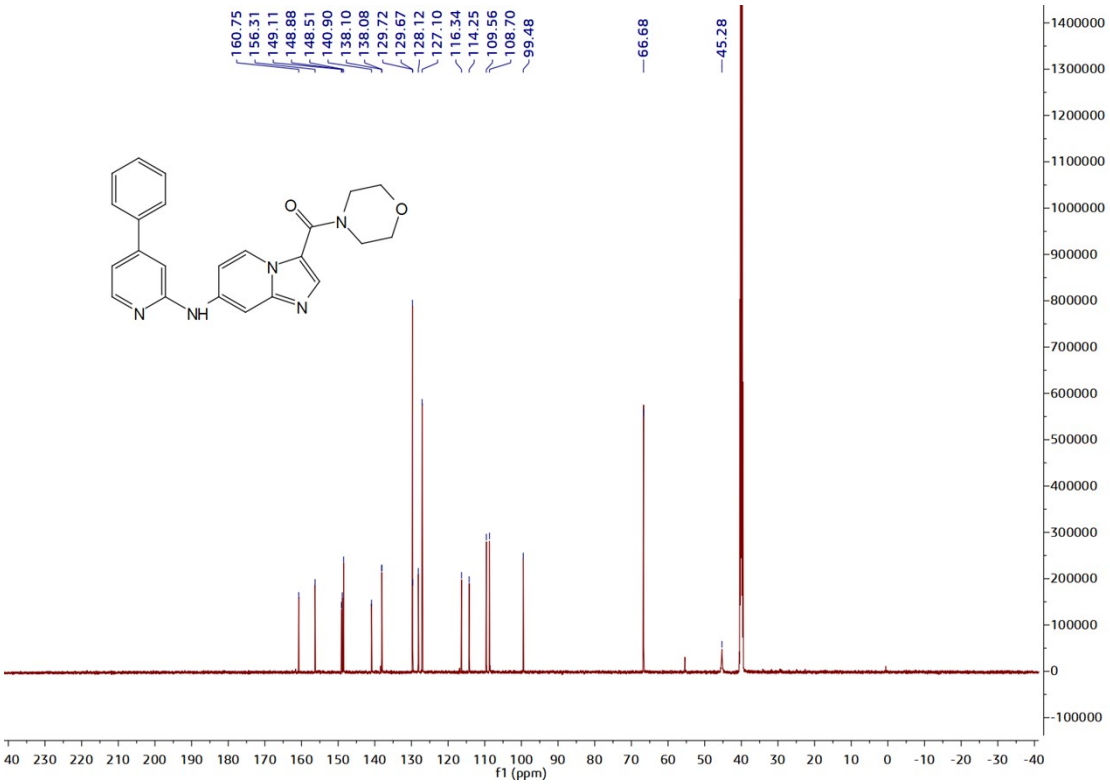


Figure S35. ^{13}C NMR Spectrum of compound LA-1

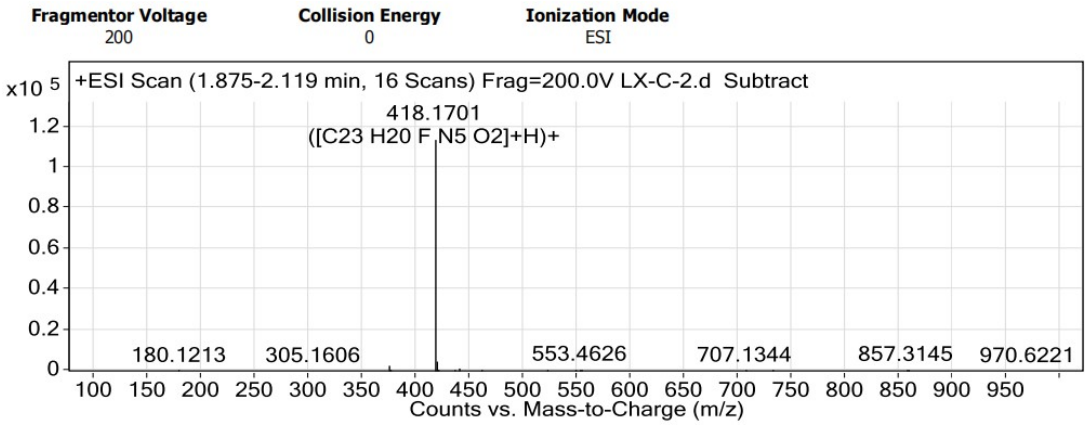


Figure S36. HRMS of compound LA-2

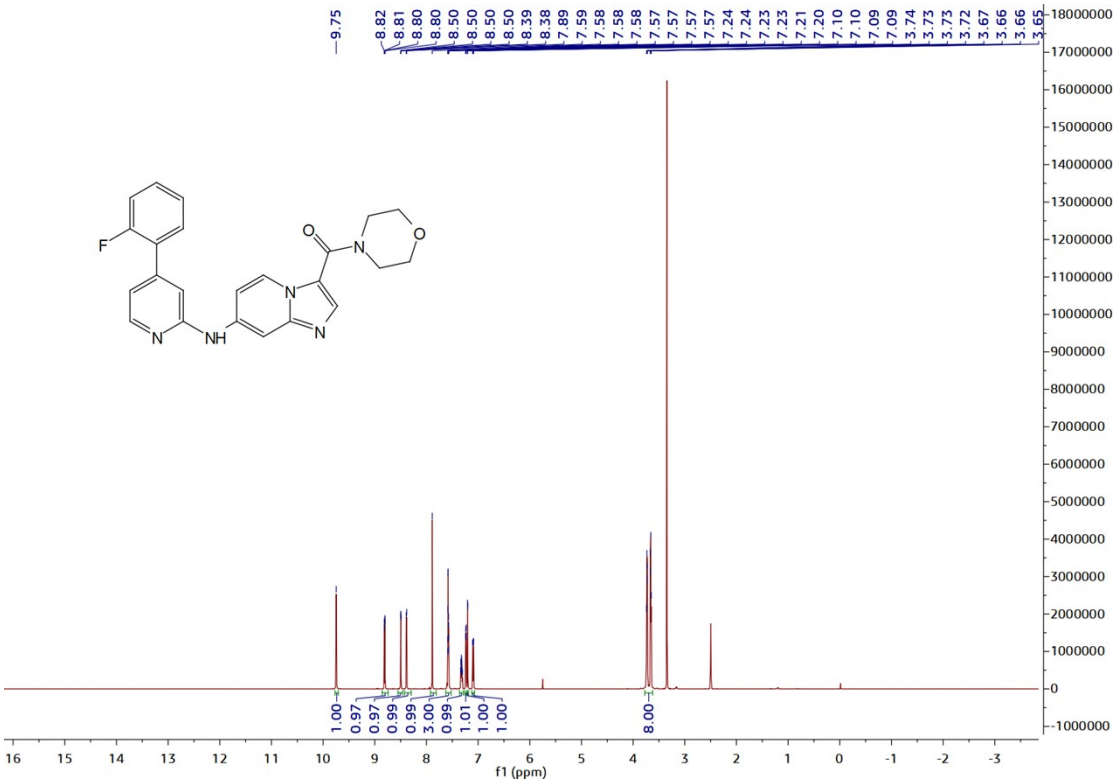


Figure S37. ^{1}H NMR Spectrum of compound LA-2

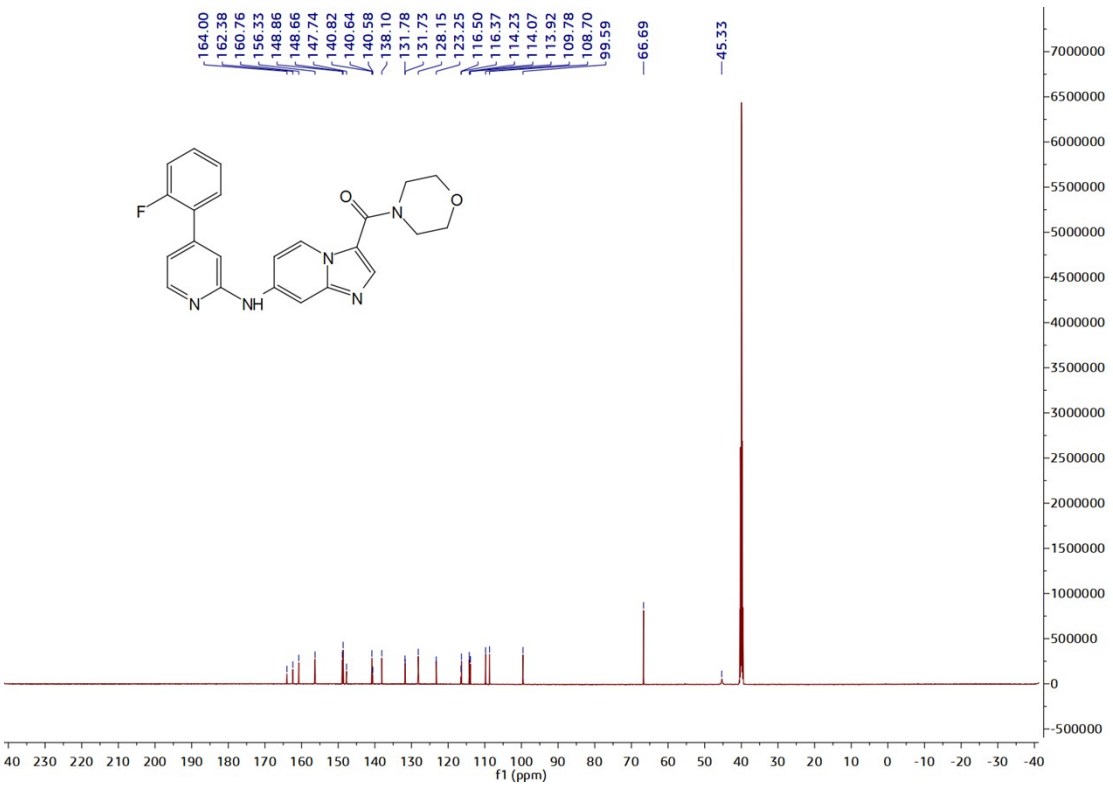


Figure S38. ^{13}C NMR Spectrum of compound LA-2

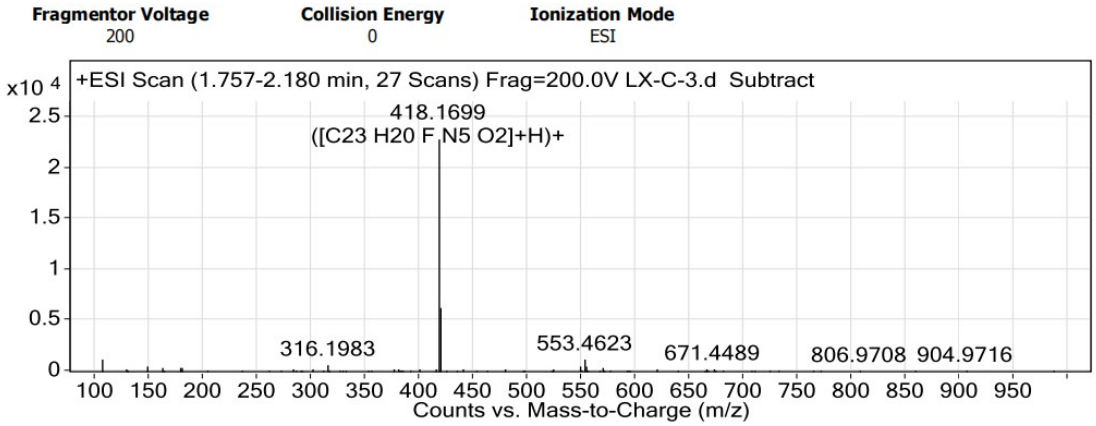


Figure S39. HRMS of compound LA-3

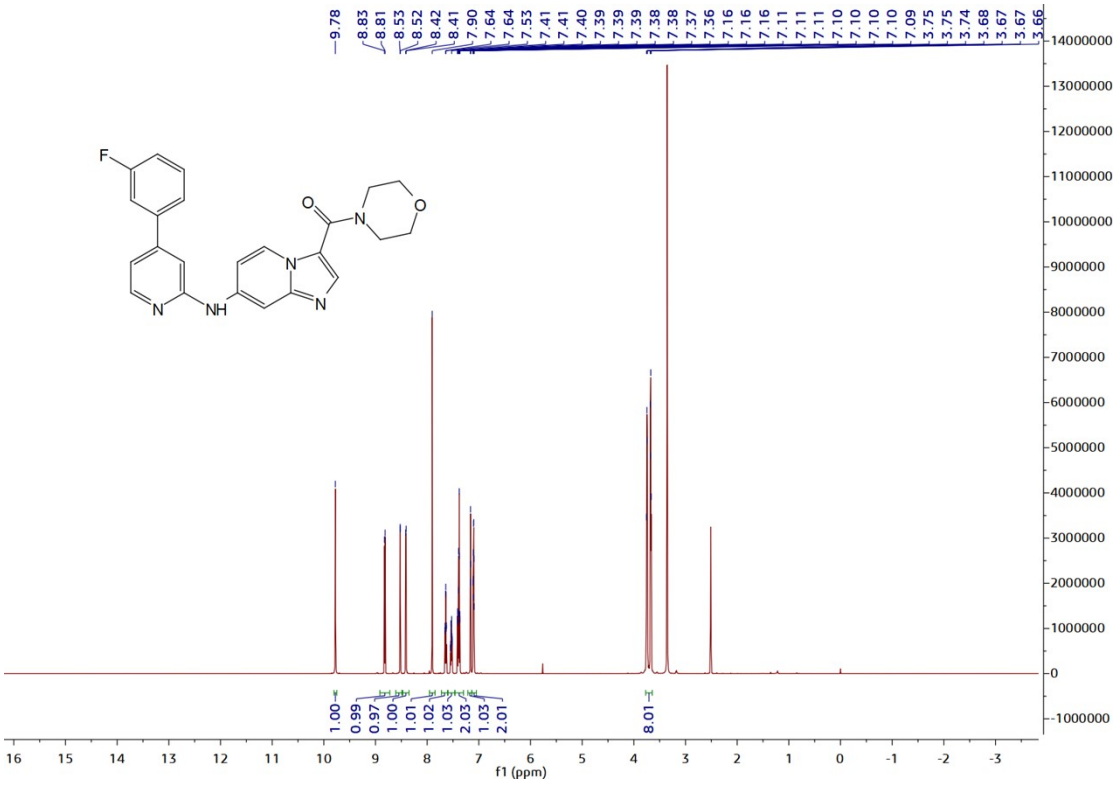


Figure S40. ^{1}H NMR Spectrum of compound LA-3

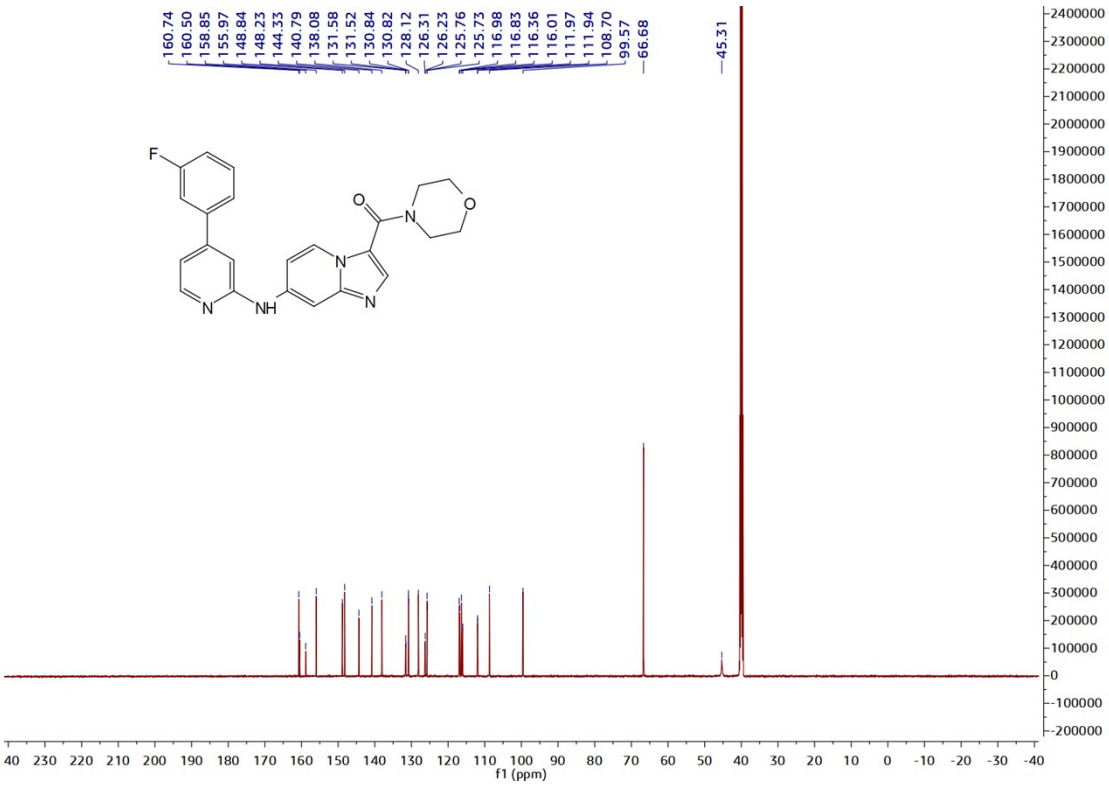


Figure S41. ^{13}C NMR Spectrum of compound LA-3

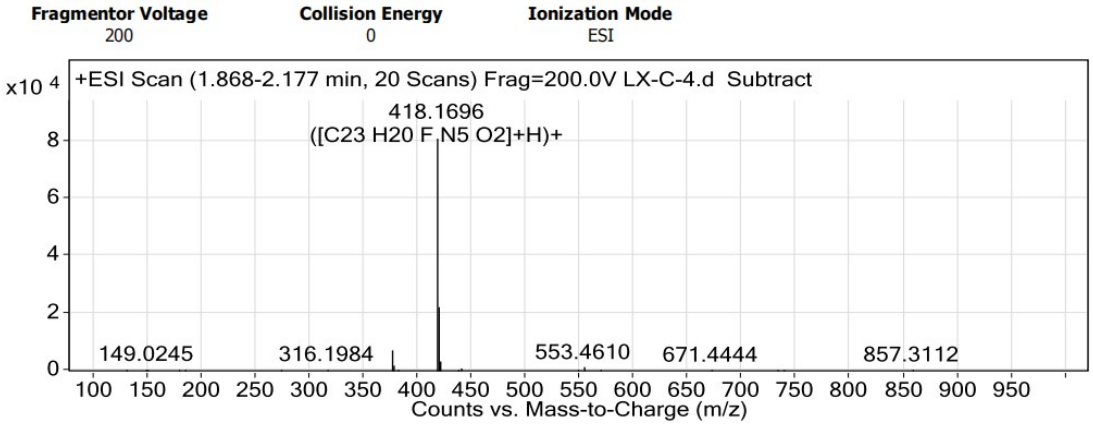


Figure S42. HRMS of compound LA-4

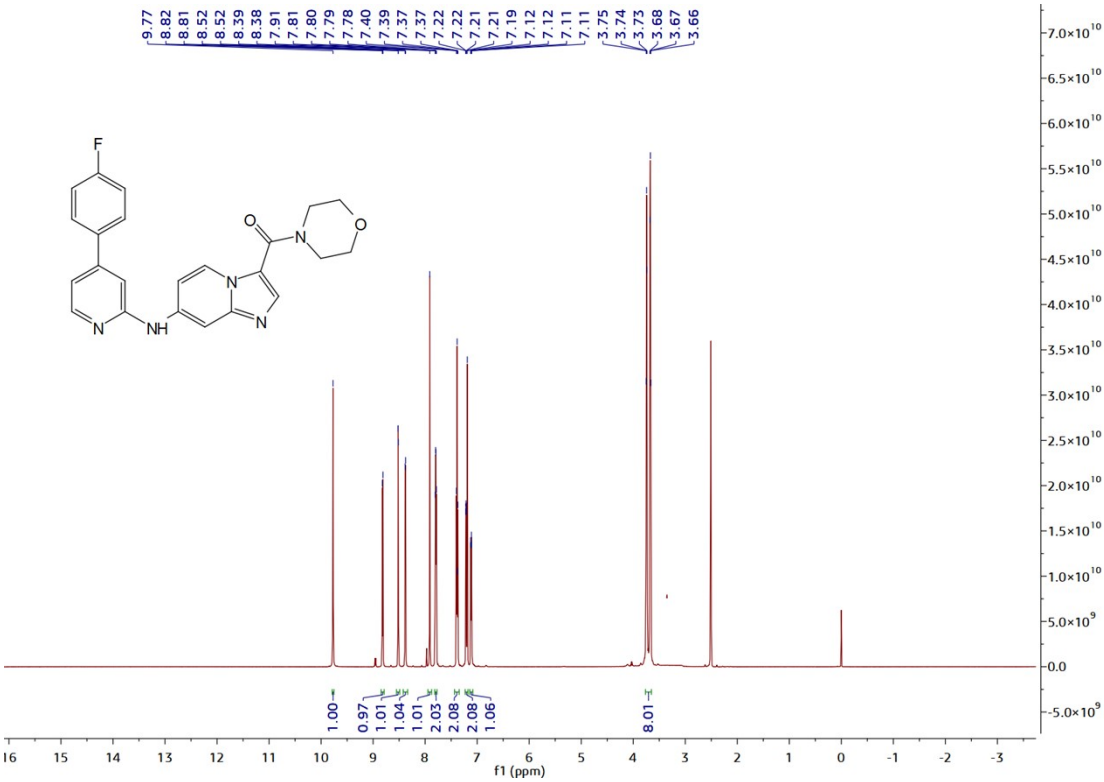


Figure S43. ^{1}H NMR Spectrum of compound LA-4

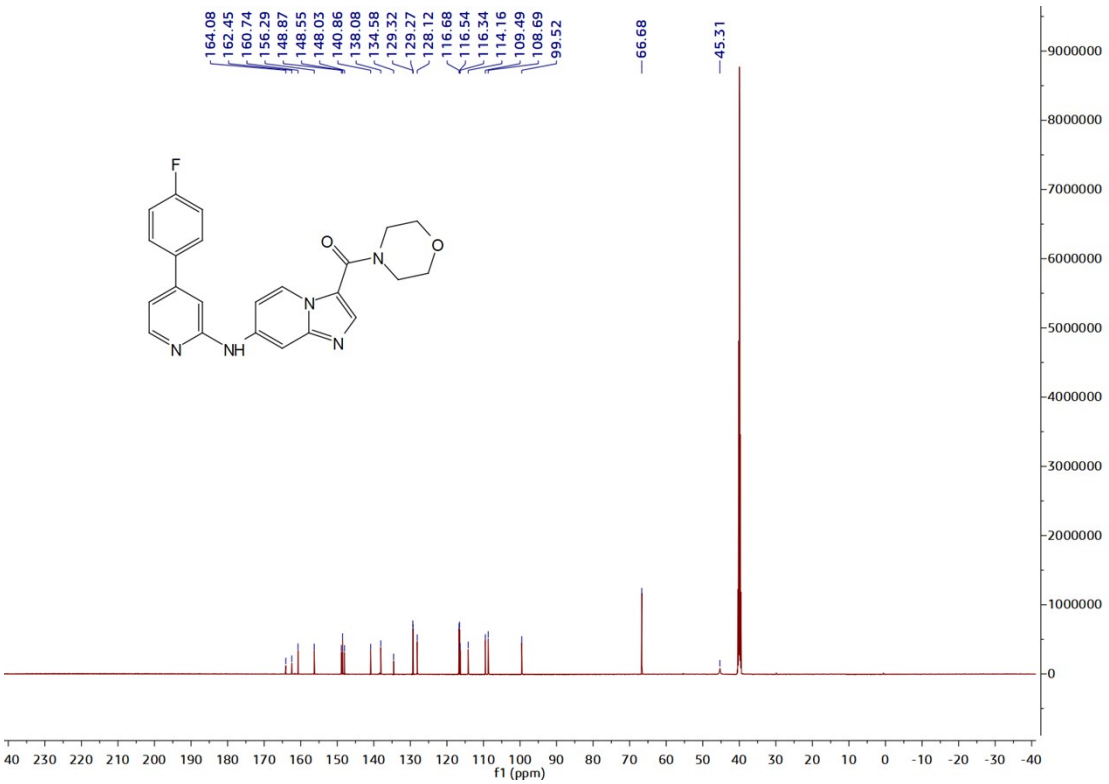


Figure S44.^{13}C NMR Spectrum of compound LA-4

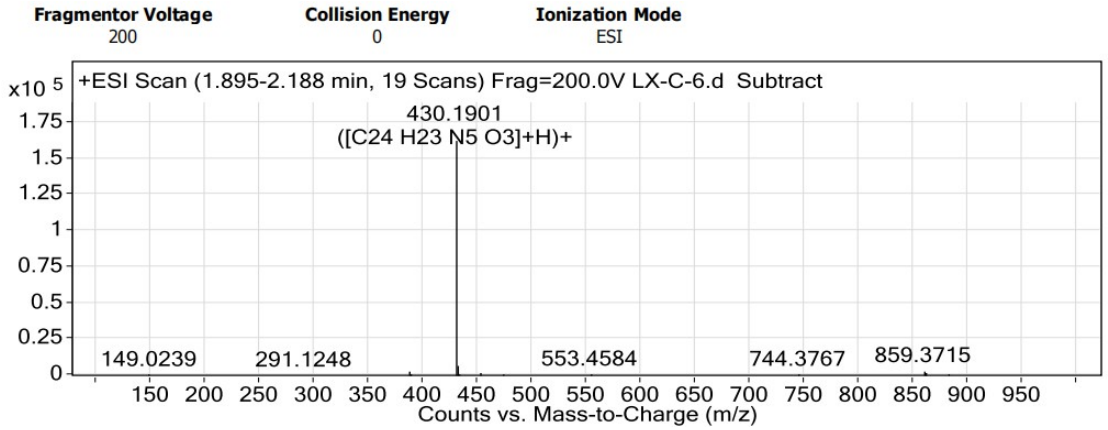


Figure S45.HRMS of compound LA-5

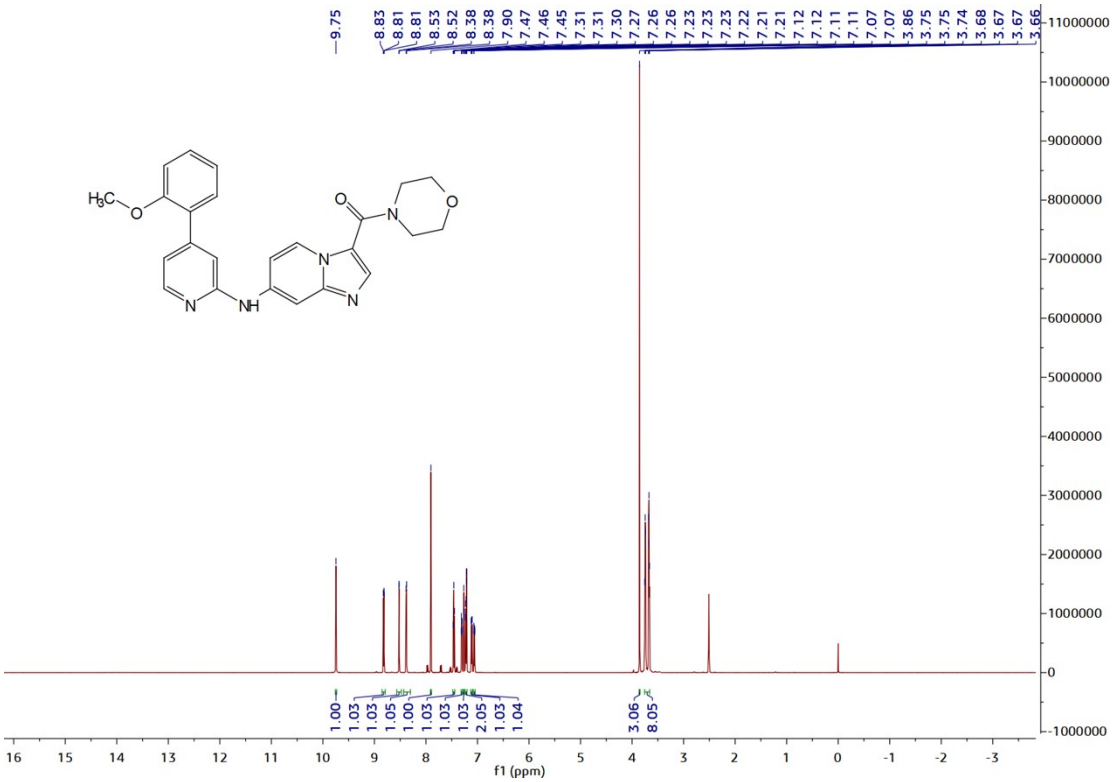


Figure S46.[1]H NMR Spectrum of compound LA-5

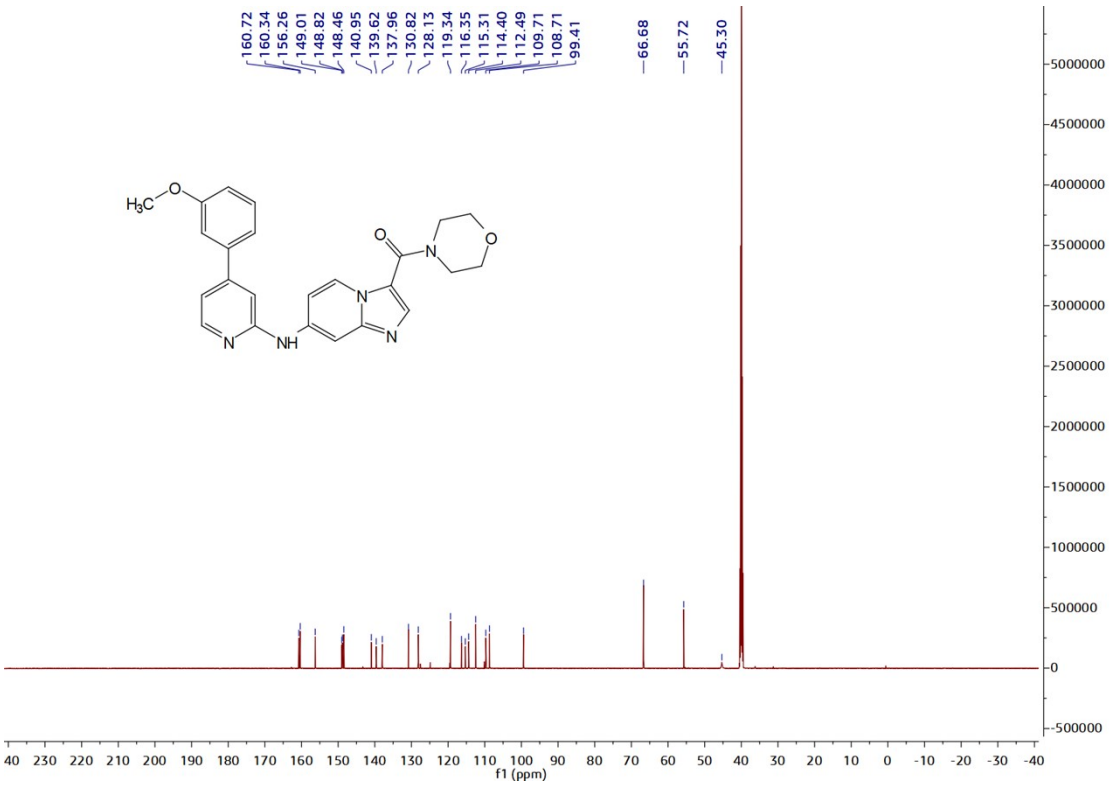


Figure S47.^{13}C NMR Spectrum of compound LA-5

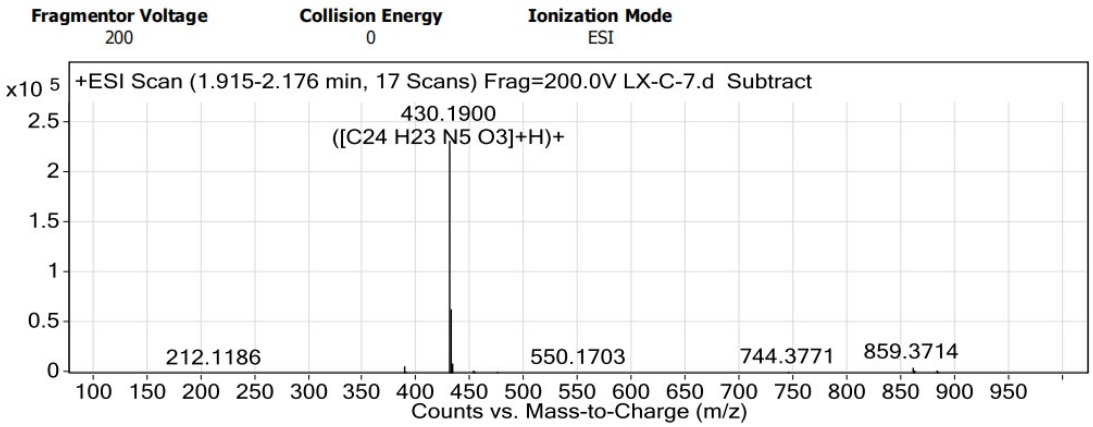


Figure S48.HRMS of compound LA-6

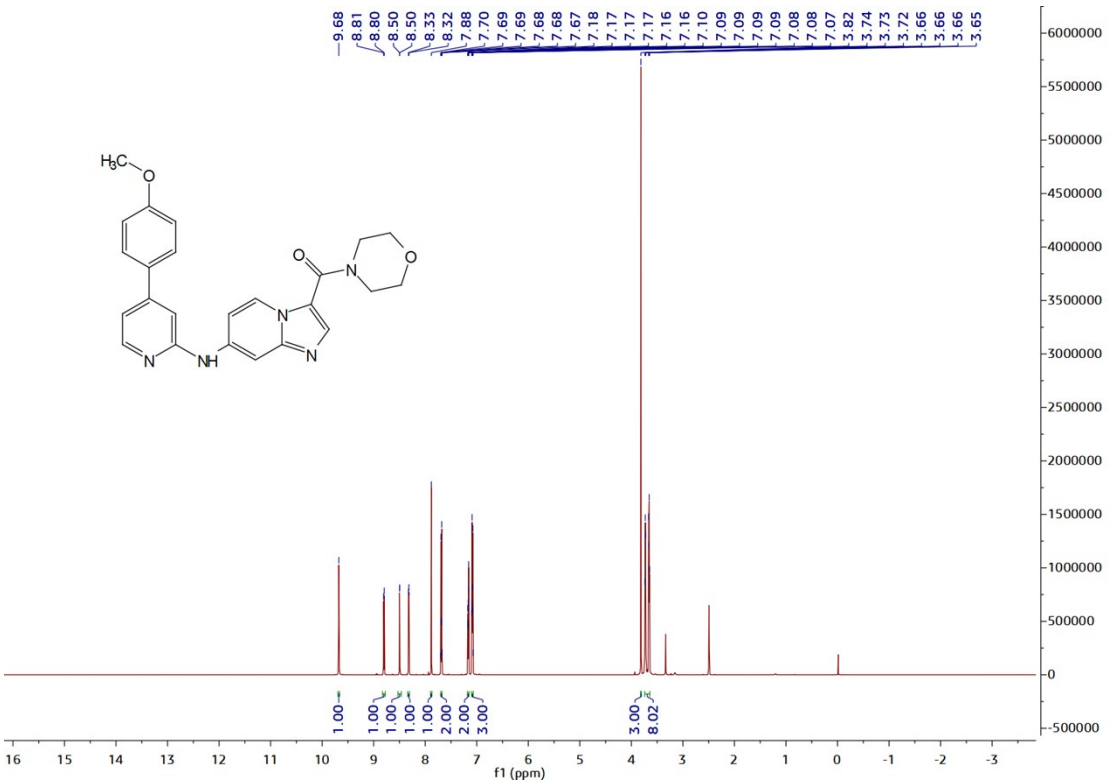


Figure S49.[1]H NMR Spectrum of compound LA-6

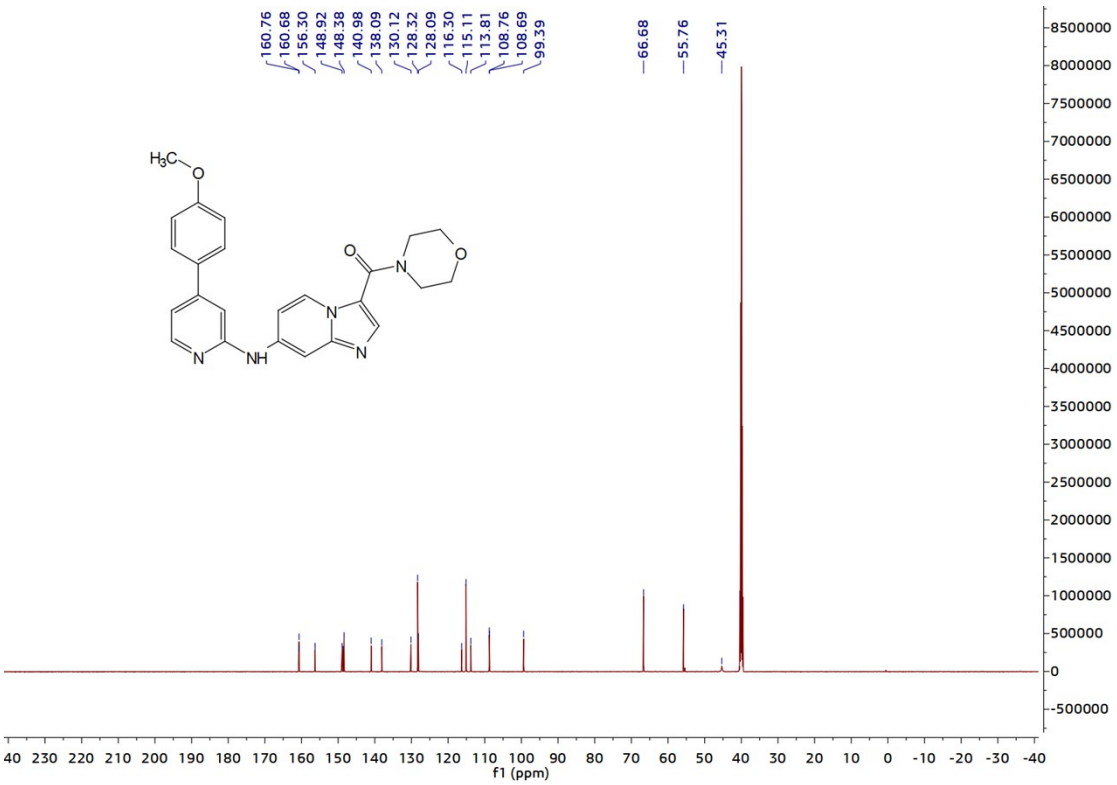


Figure S50.^{13}C NMR Spectrum of compound LA-6

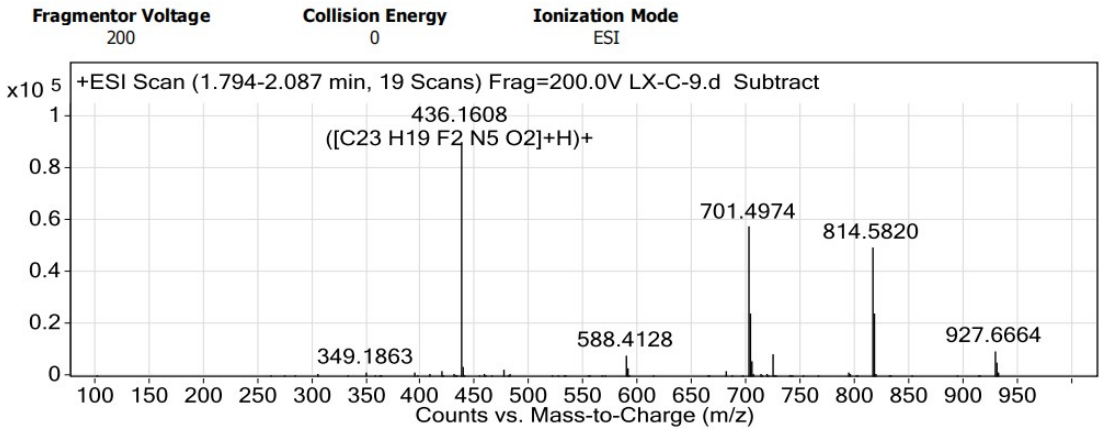


Figure S51. HRMS of compound LA-7

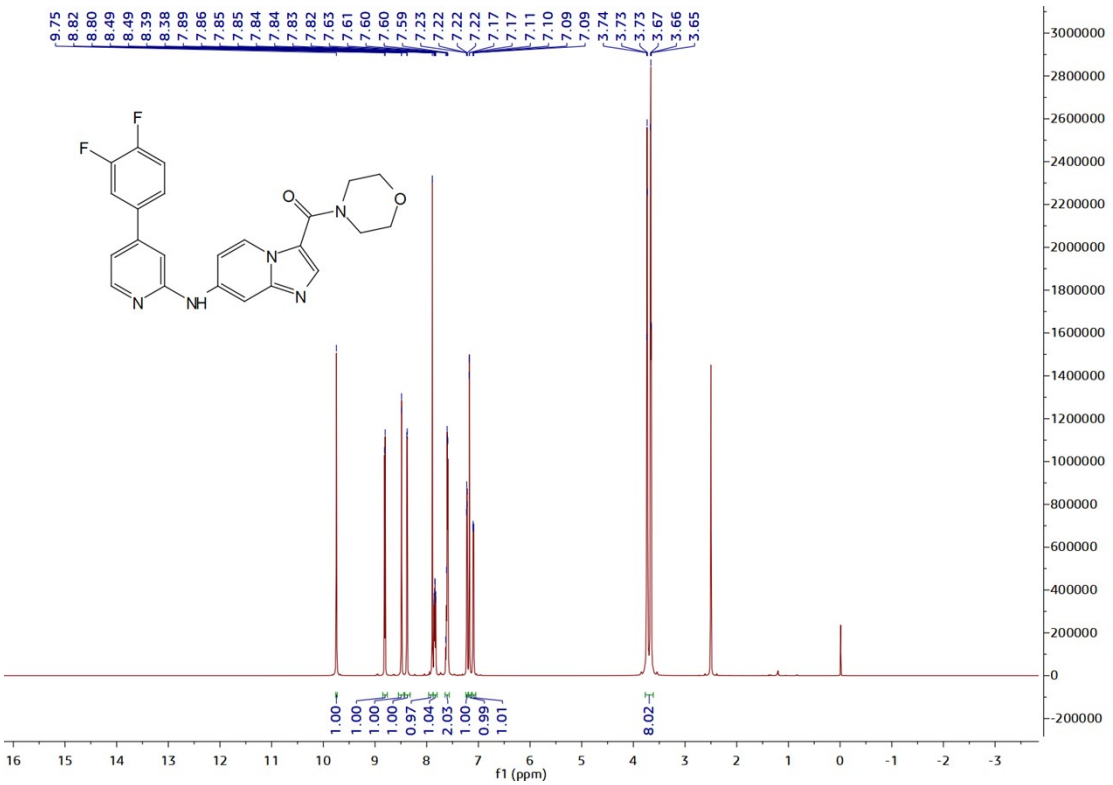


Figure S52. ^{1}H NMR Spectrum of compound LA-7

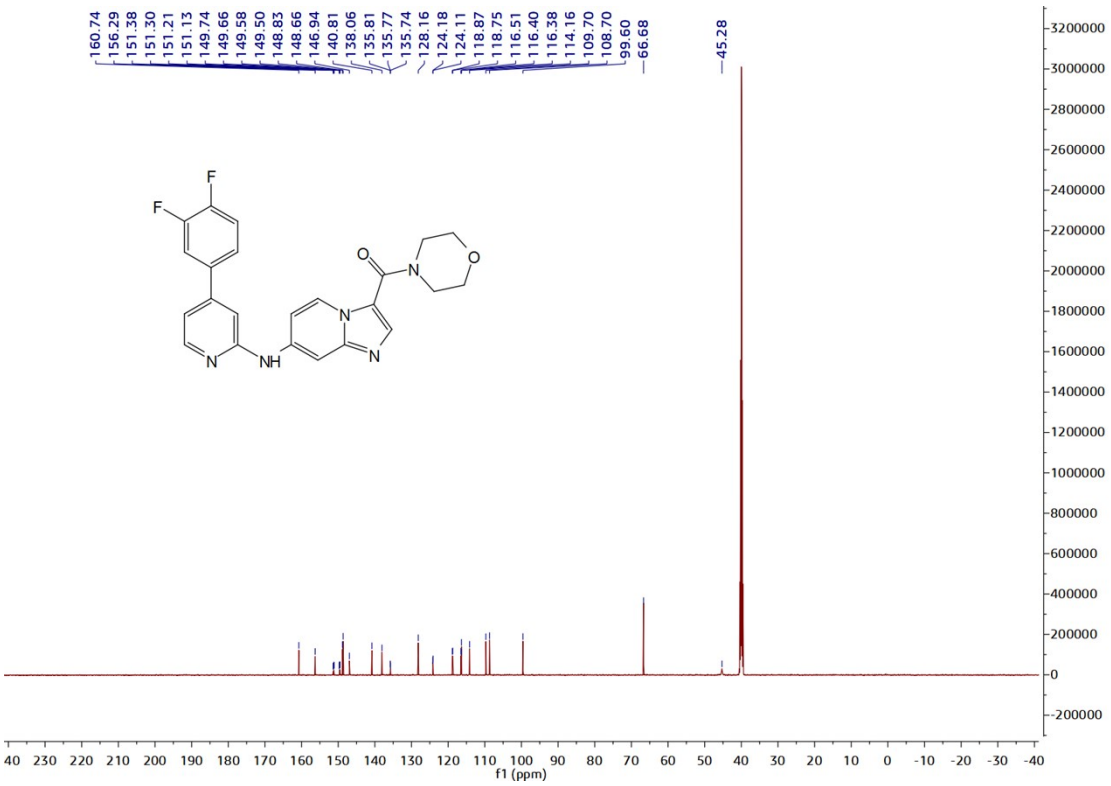


Figure S53. ^{13}C NMR Spectrum of compound LA-7

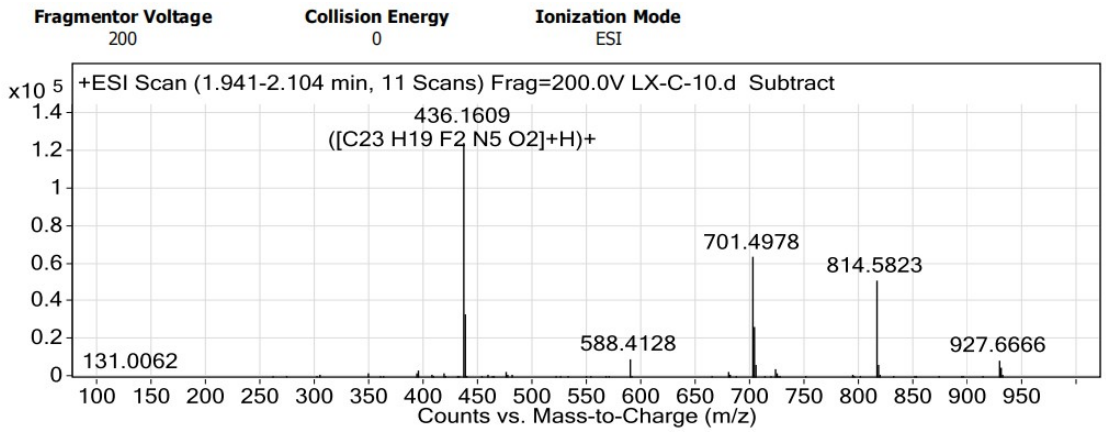


Figure S54. HRMS of compound LA-8

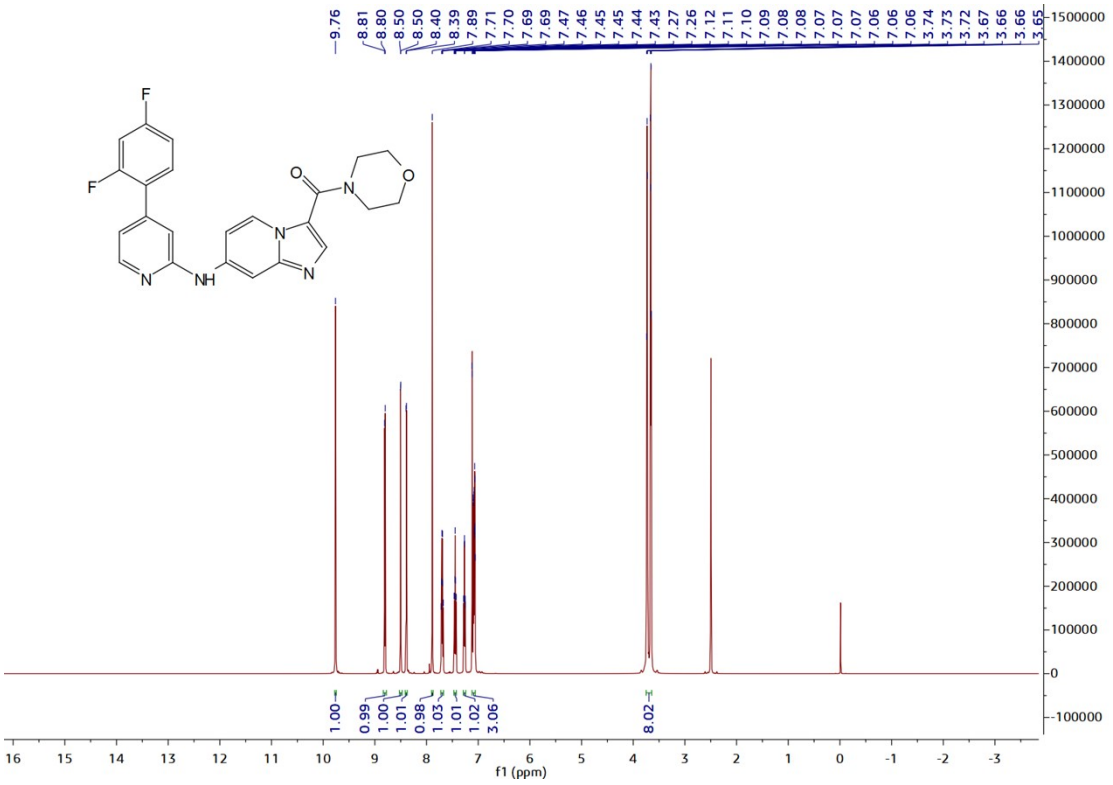


Figure S55. ^{1}H NMR Spectrum of compound LA-8

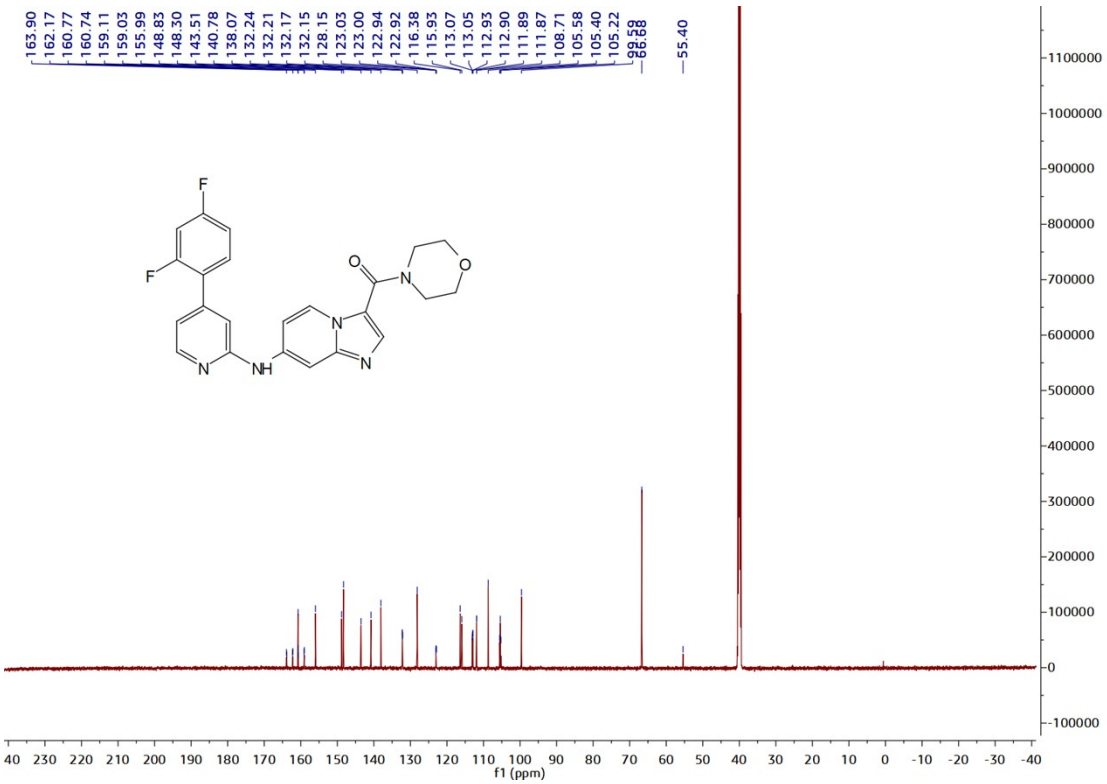


Figure S56. ^{13}C NMR Spectrum of compound LA-8

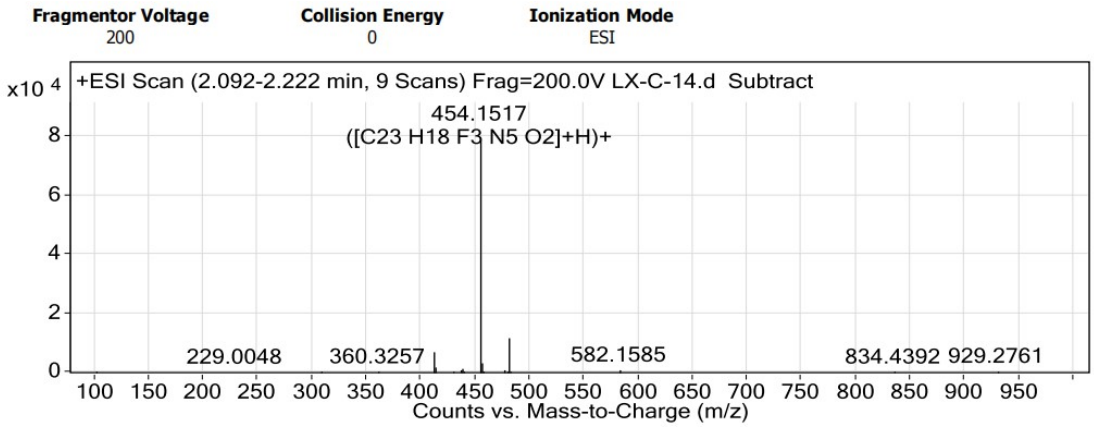


Figure S57. HRMS of compound LA-9

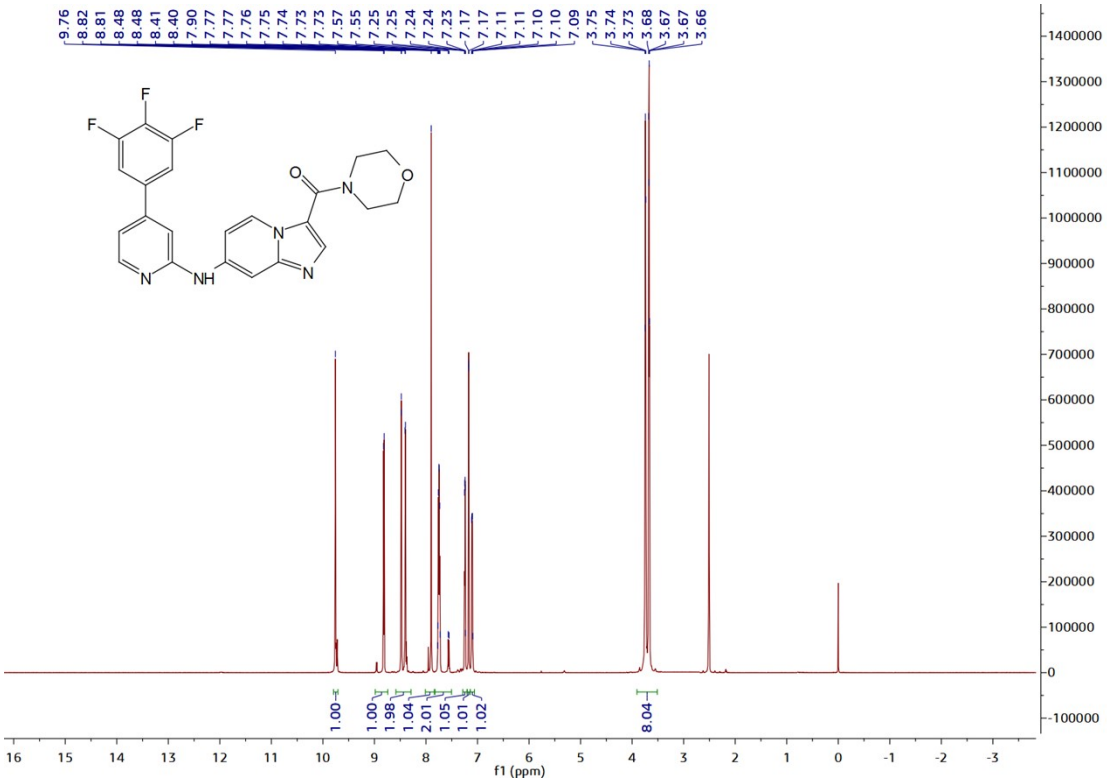


Figure S58. ^{1}H NMR Spectrum of compound LA-9

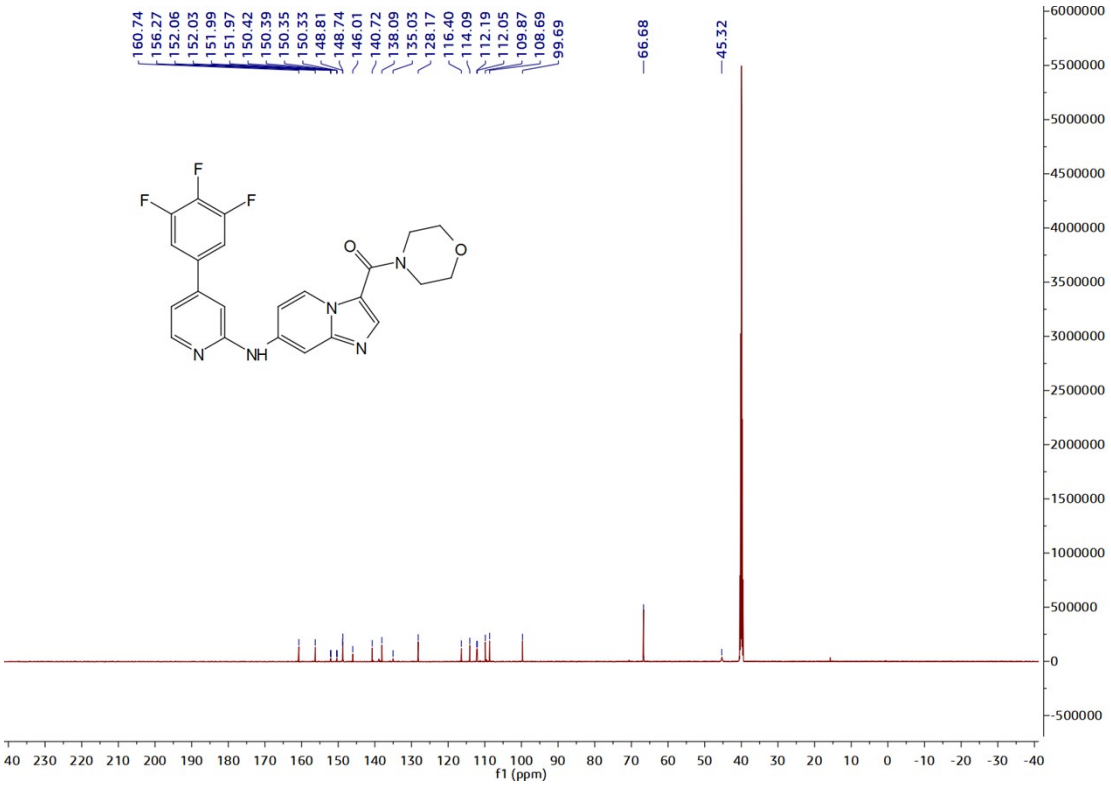


Figure S59. ^{13}C NMR Spectrum of compound LA-9

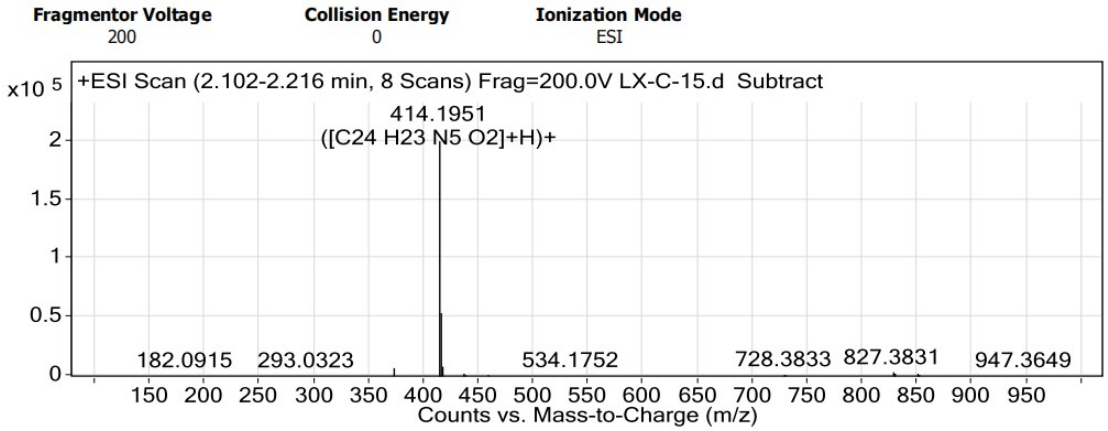


Figure S60. HRMS of compound LA-10

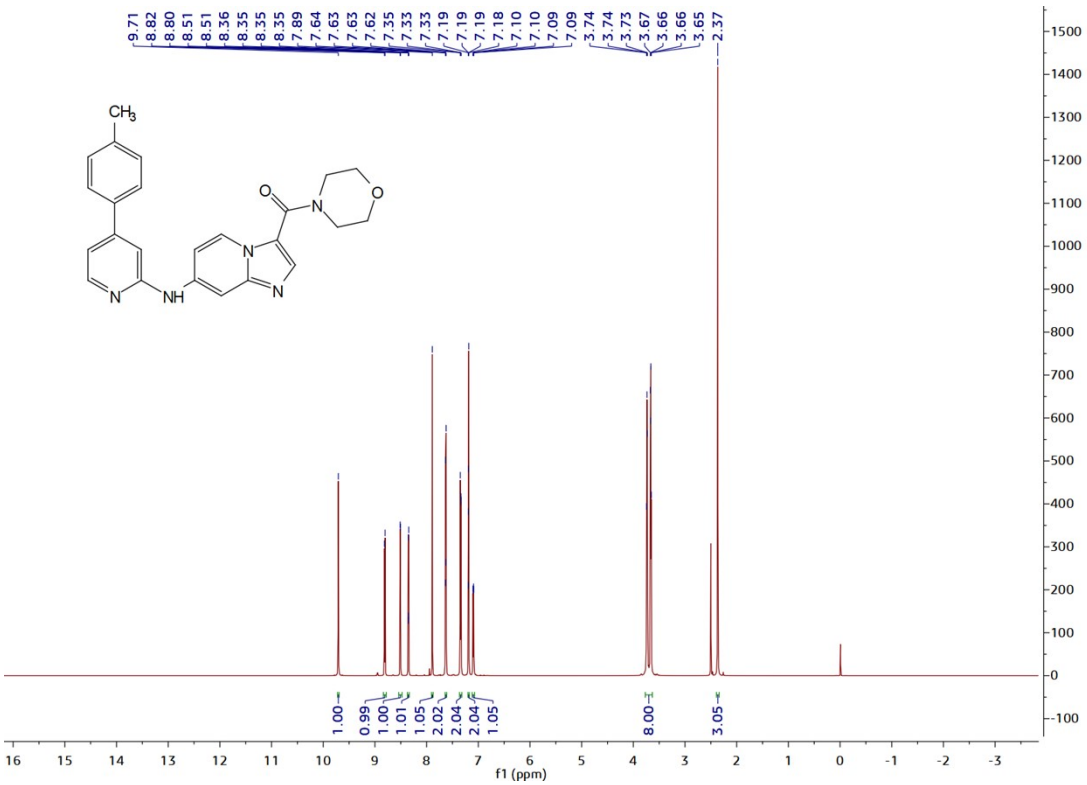


Figure S61. ^{1}H NMR Spectrum of compound LA-10

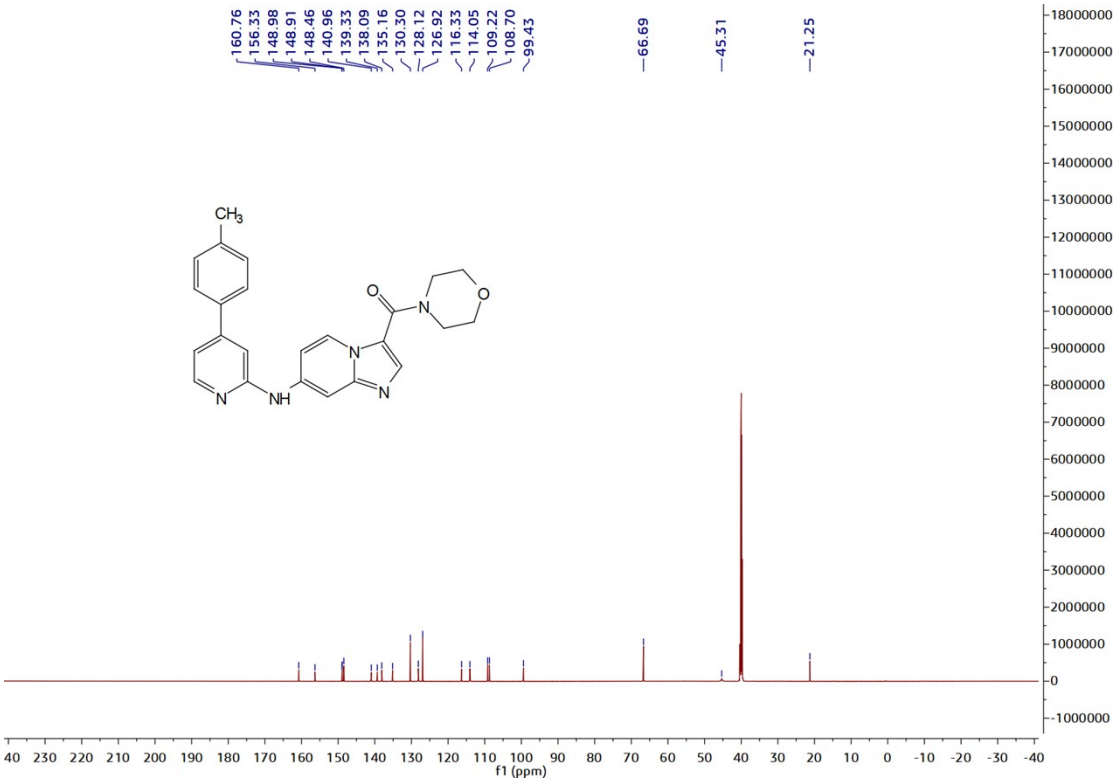


Figure S62. ^{13}C NMR Spectrum of compound LA-10

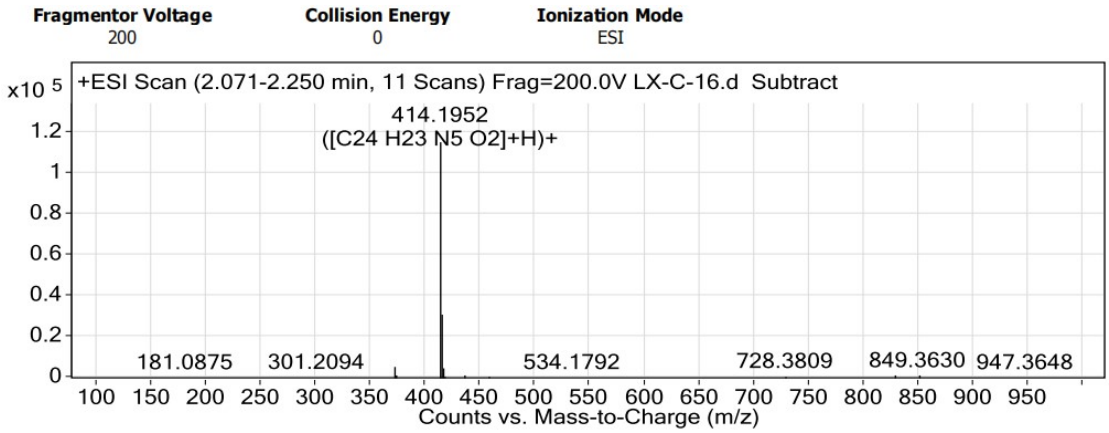


Figure S63. HRMS of compound LA-11

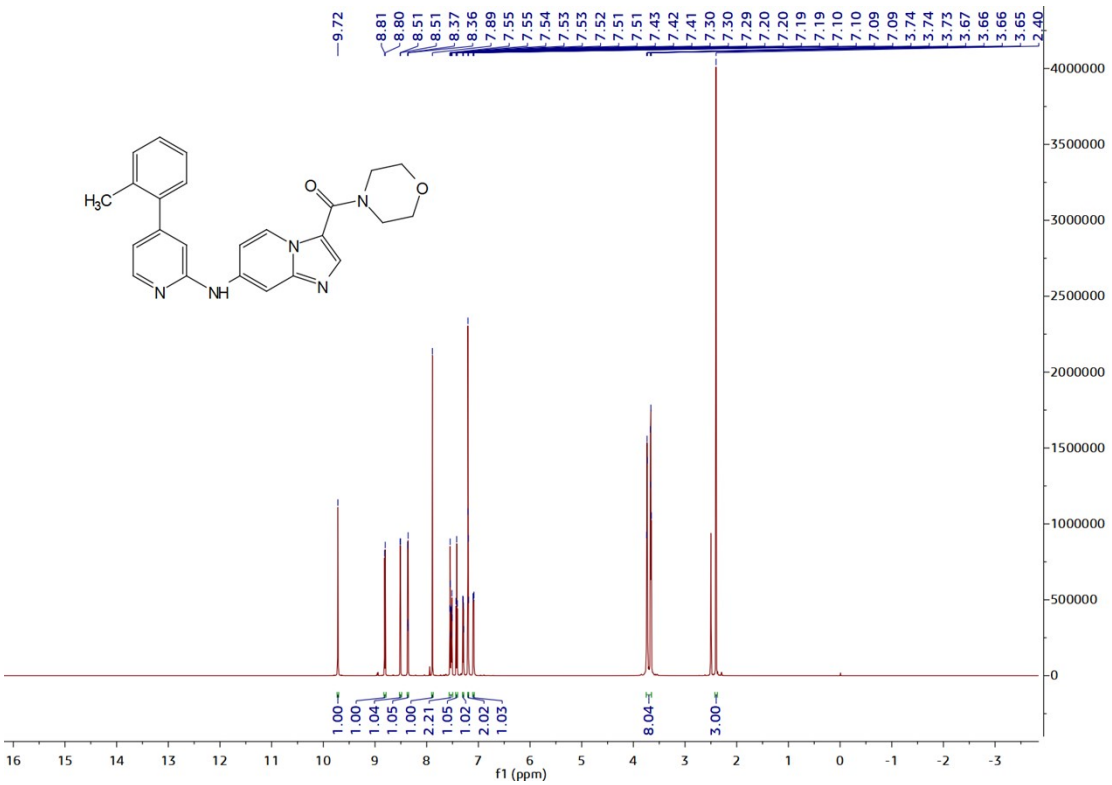


Figure S64. ^{1}H NMR Spectrum of compound LA-11

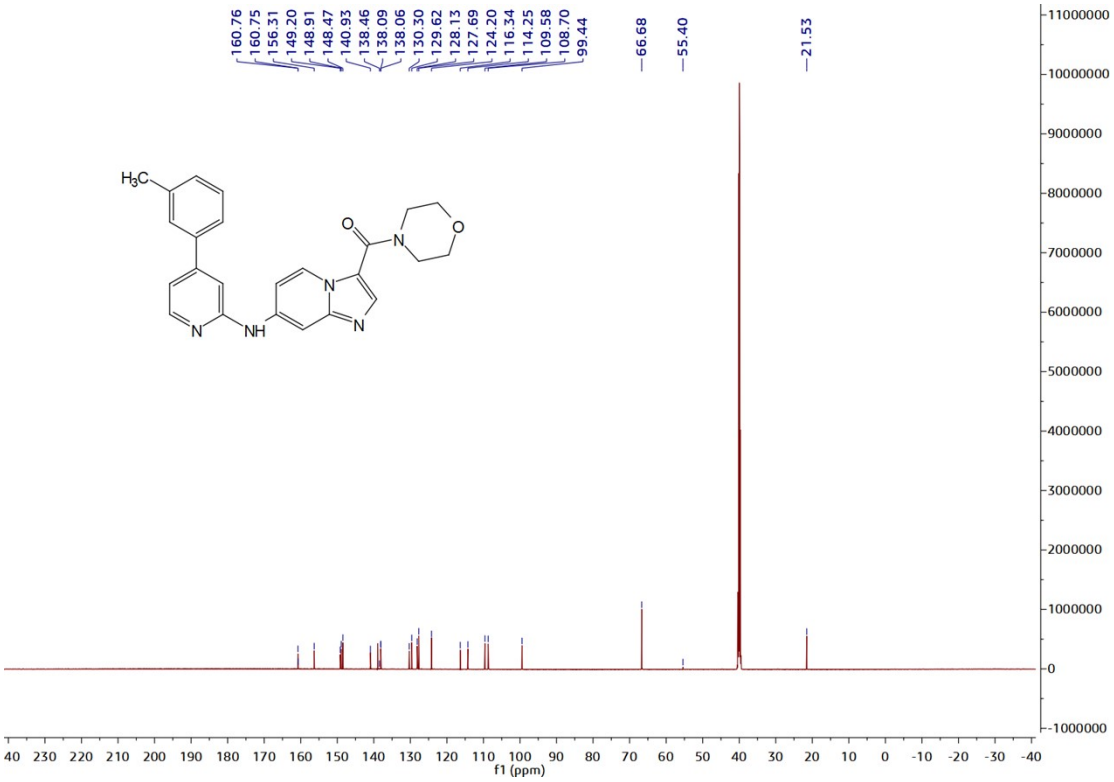


Figure S65. ^{13}C NMR Spectrum of compound LA-11

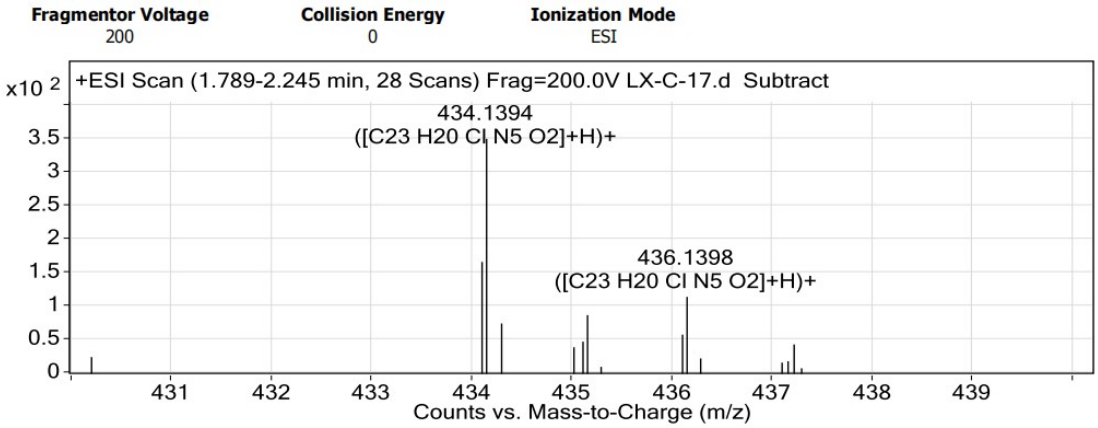


Figure S66. HRMS of compound LA-12

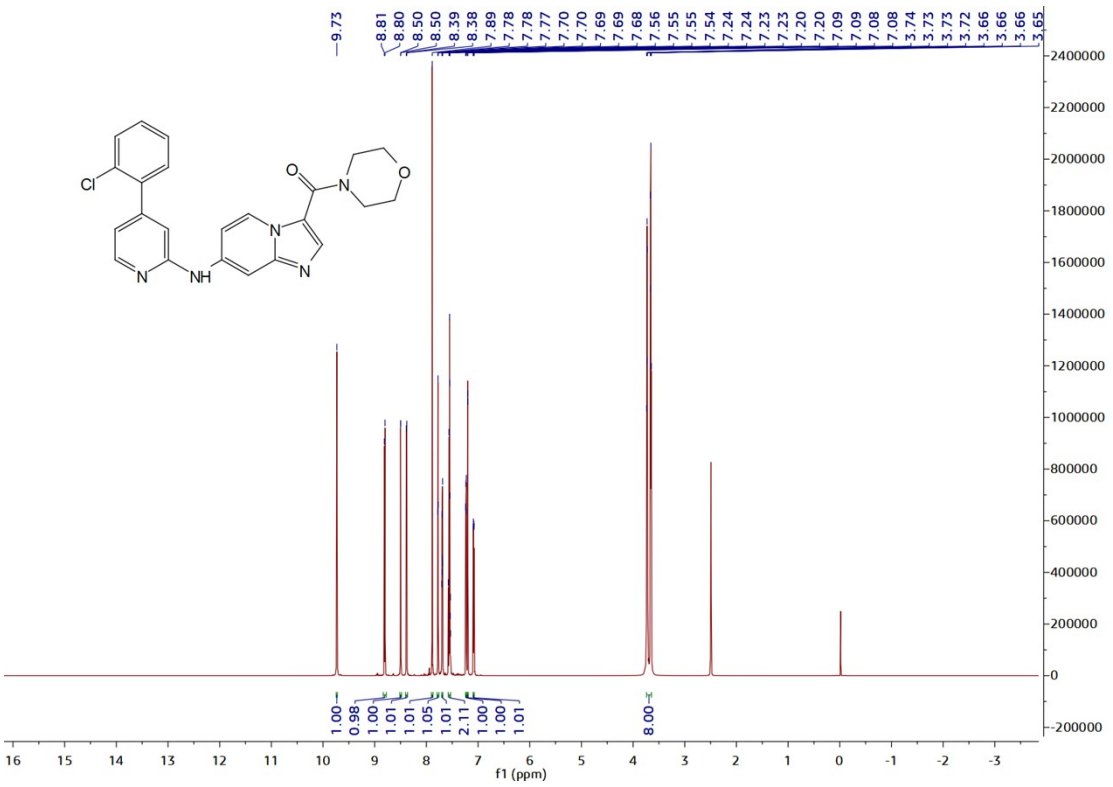


Figure S67. ^{1}H NMR Spectrum of compound LA-12

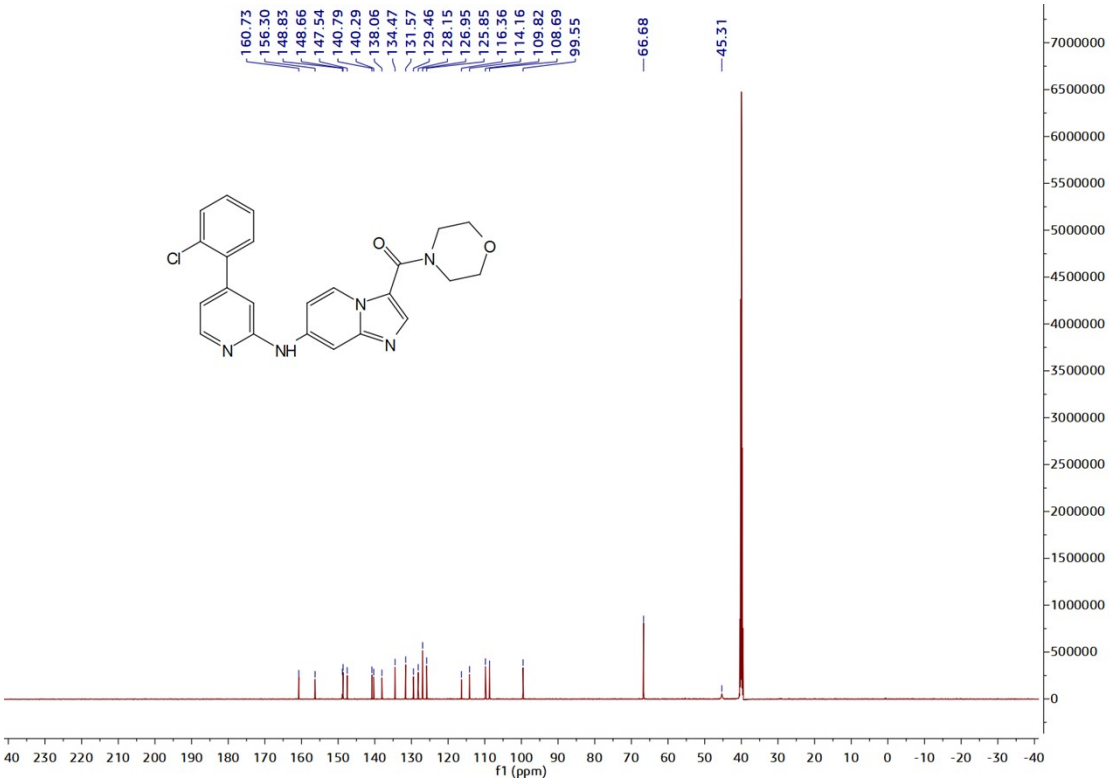


Figure S68. ^{13}C NMR Spectrum of compound LA-12

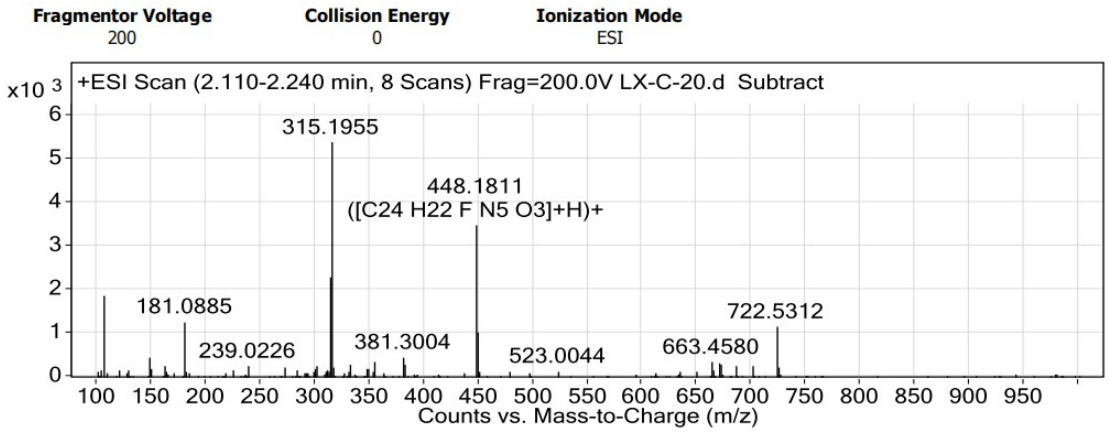


Figure S69. HRMS of compound LA-13

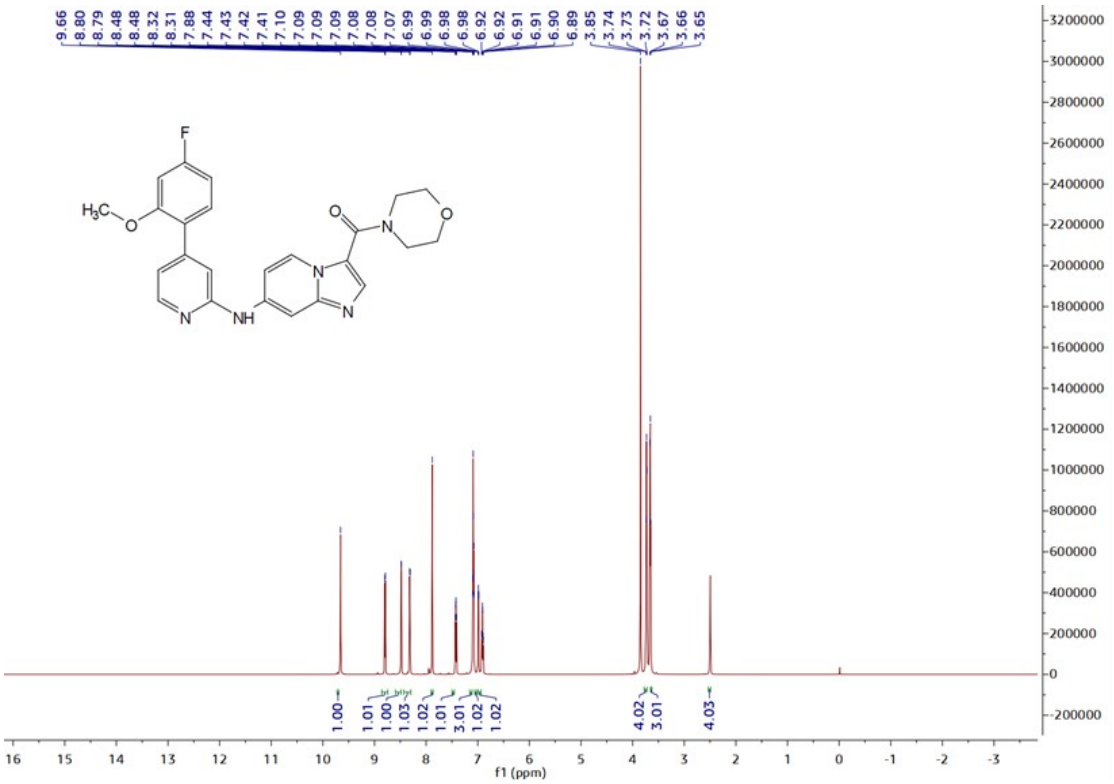


Figure S70. ^{1}H NMR Spectrum of compound LA-13

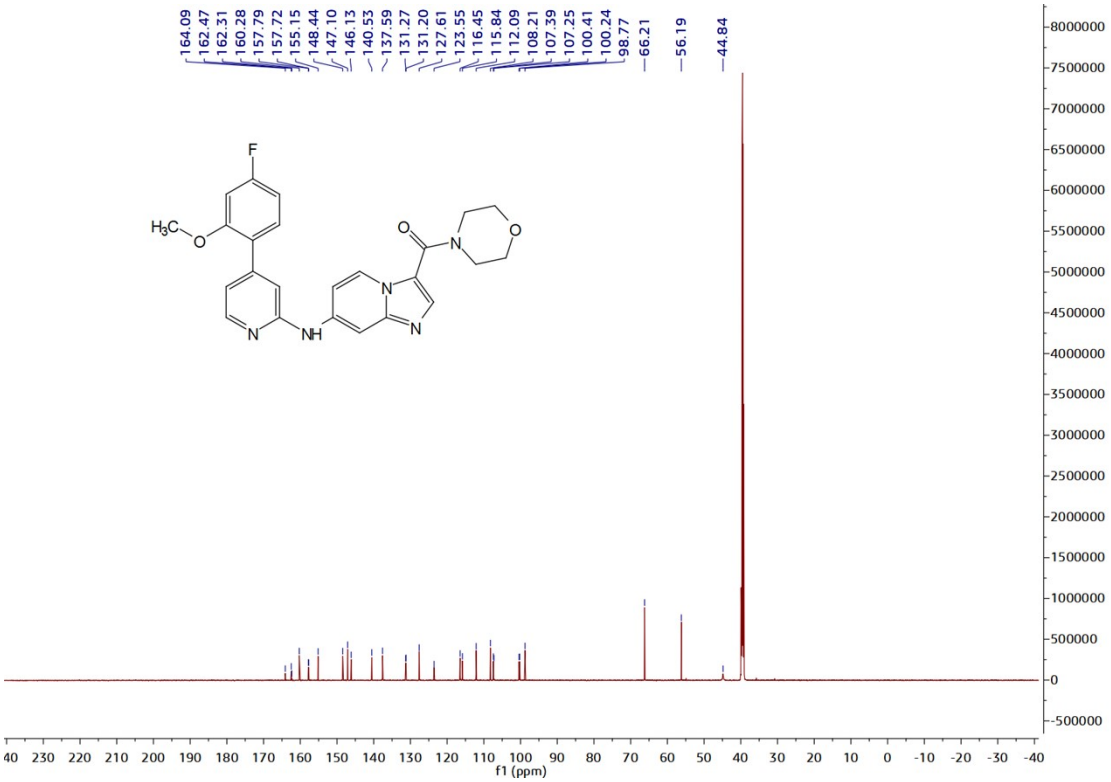


Figure S71. ^{13}C NMR Spectrum of compound LA-13

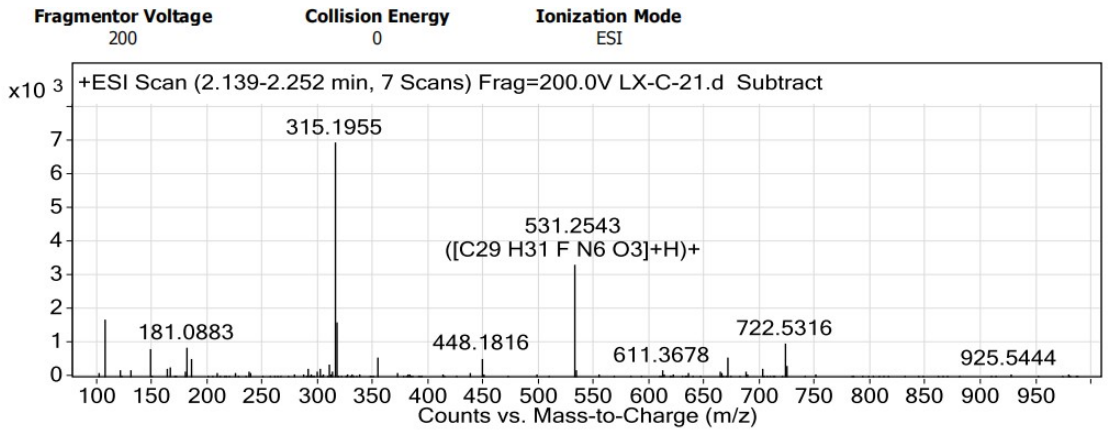


Figure S72. HRMS of compound LB-1

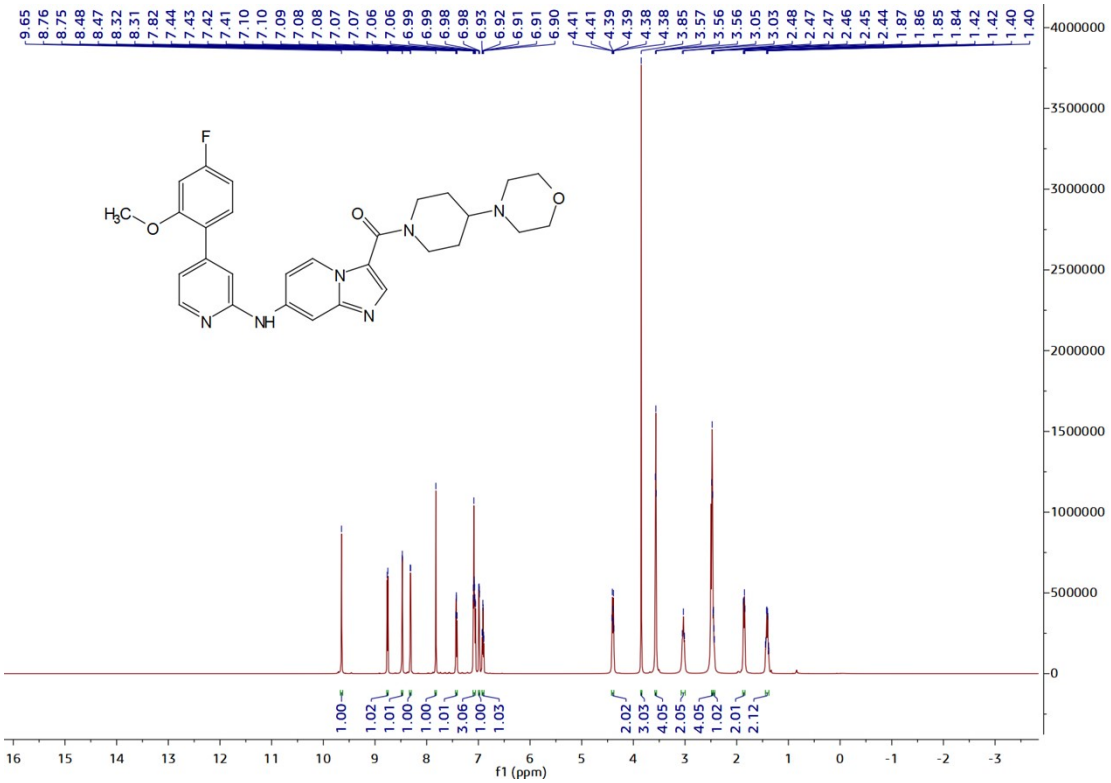


Figure S73. ^{1}H NMR Spectrum of compound LB-1

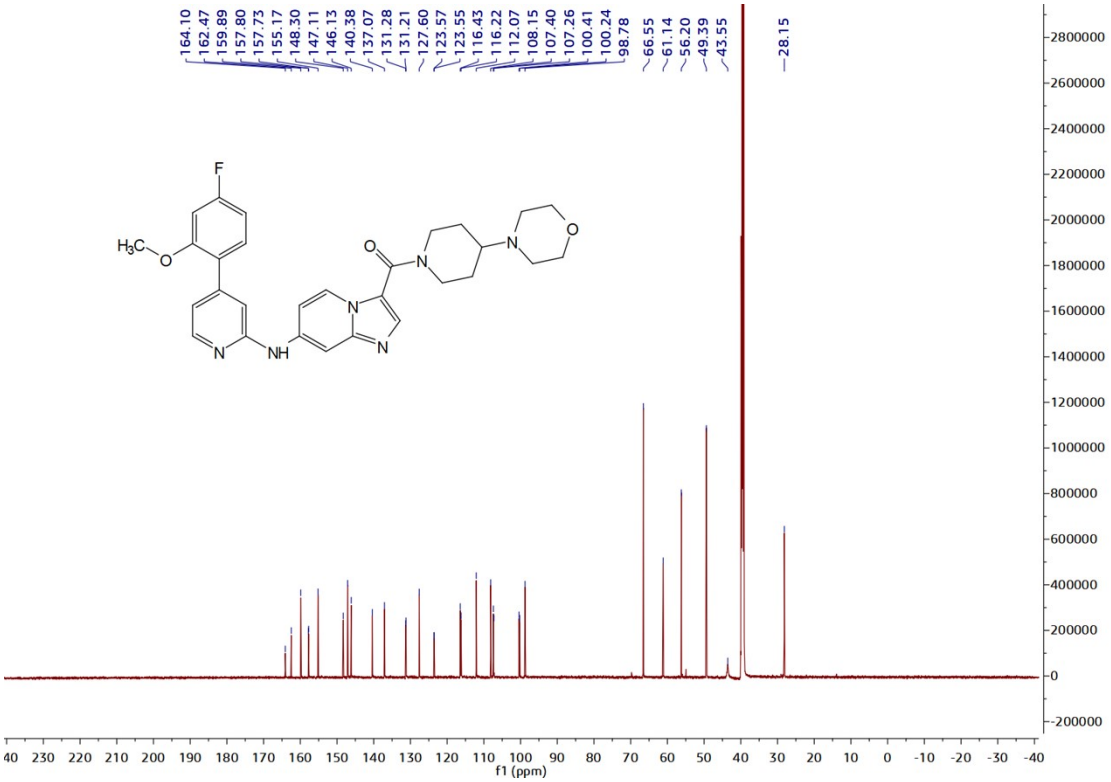


Figure S74. ^{13}C NMR Spectrum of compound LB-1

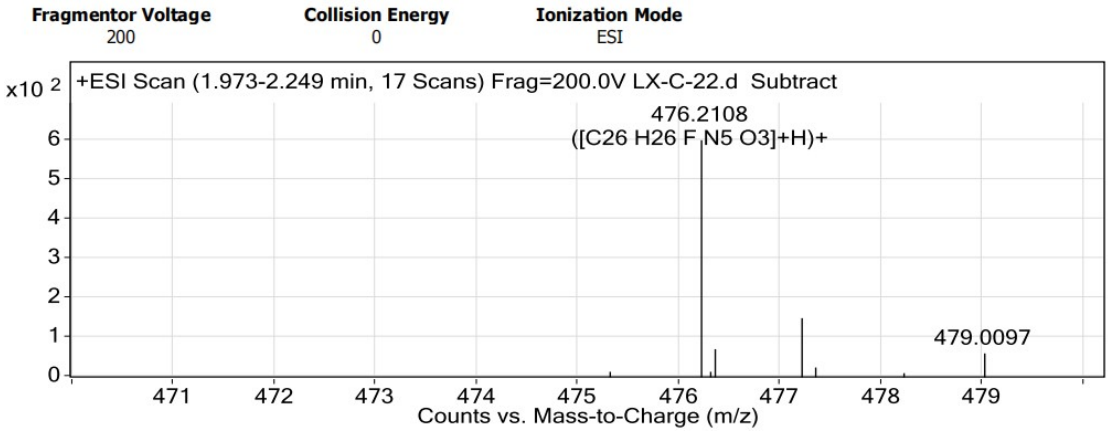


Figure S75. HRMS of compound LB-2

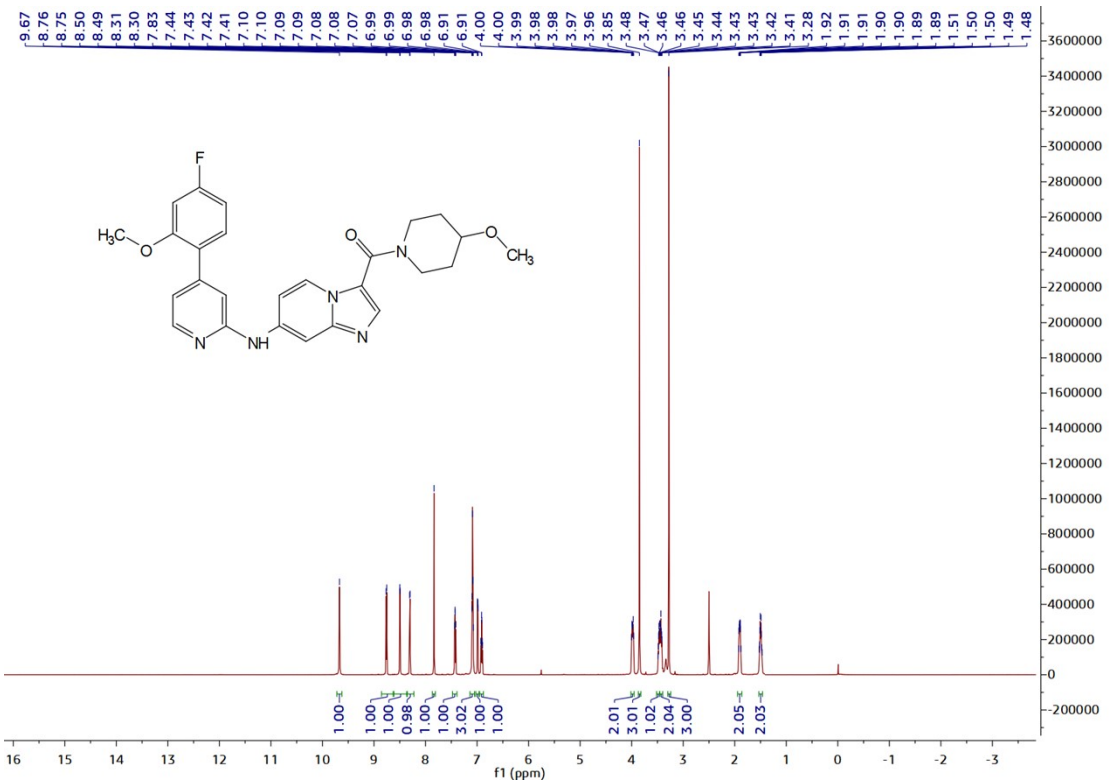


Figure S76. ^{1}H NMR Spectrum of compound LB-2

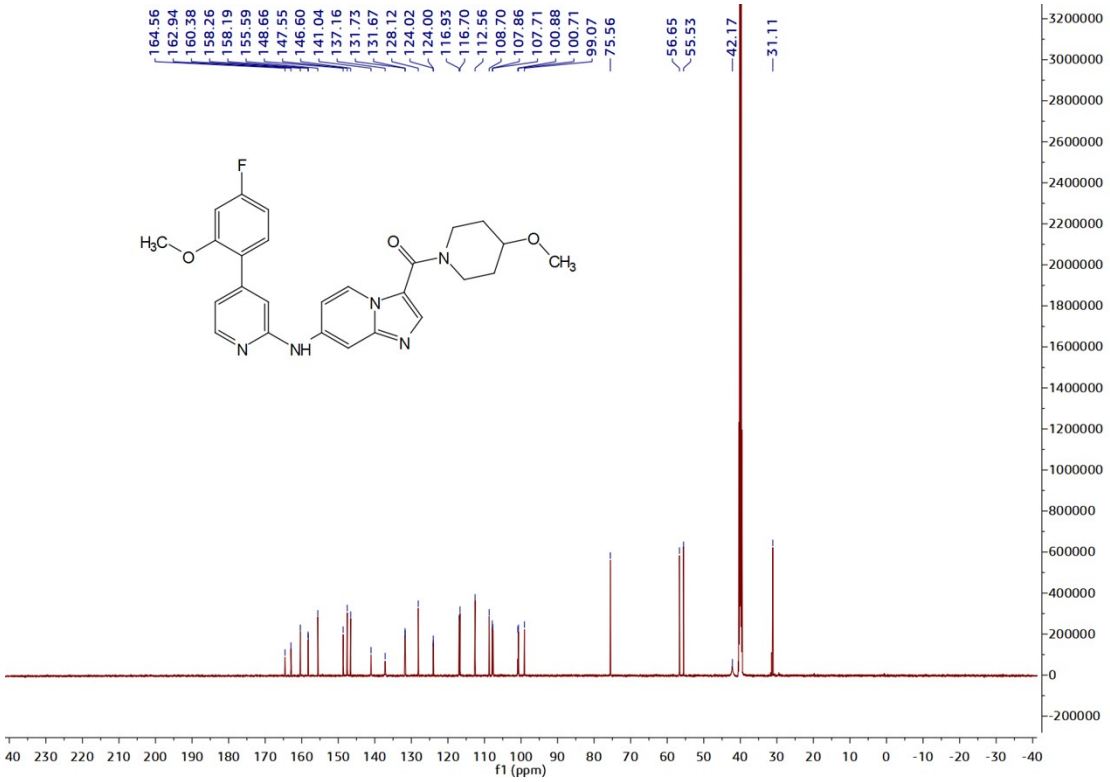


Figure S77. ^{13}C NMR Spectrum of compound LB-2

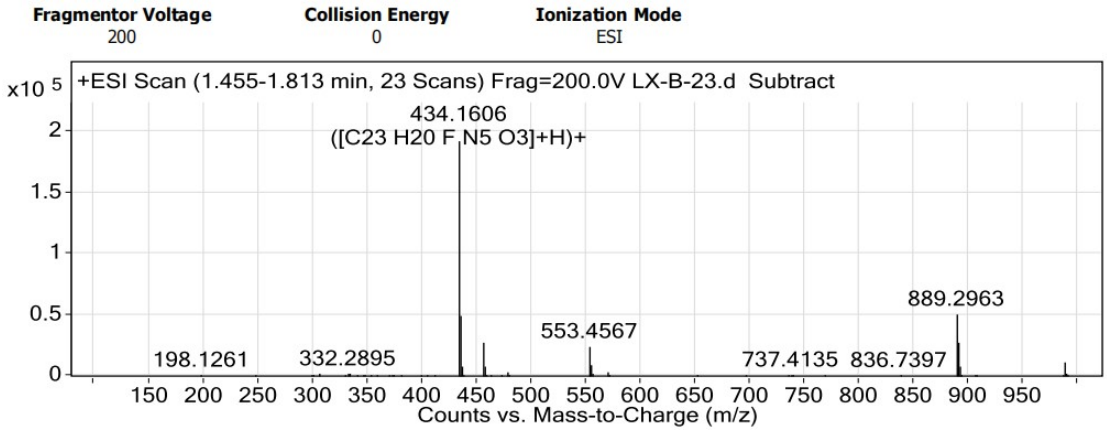


Figure S78. HRMS of compound LB-3

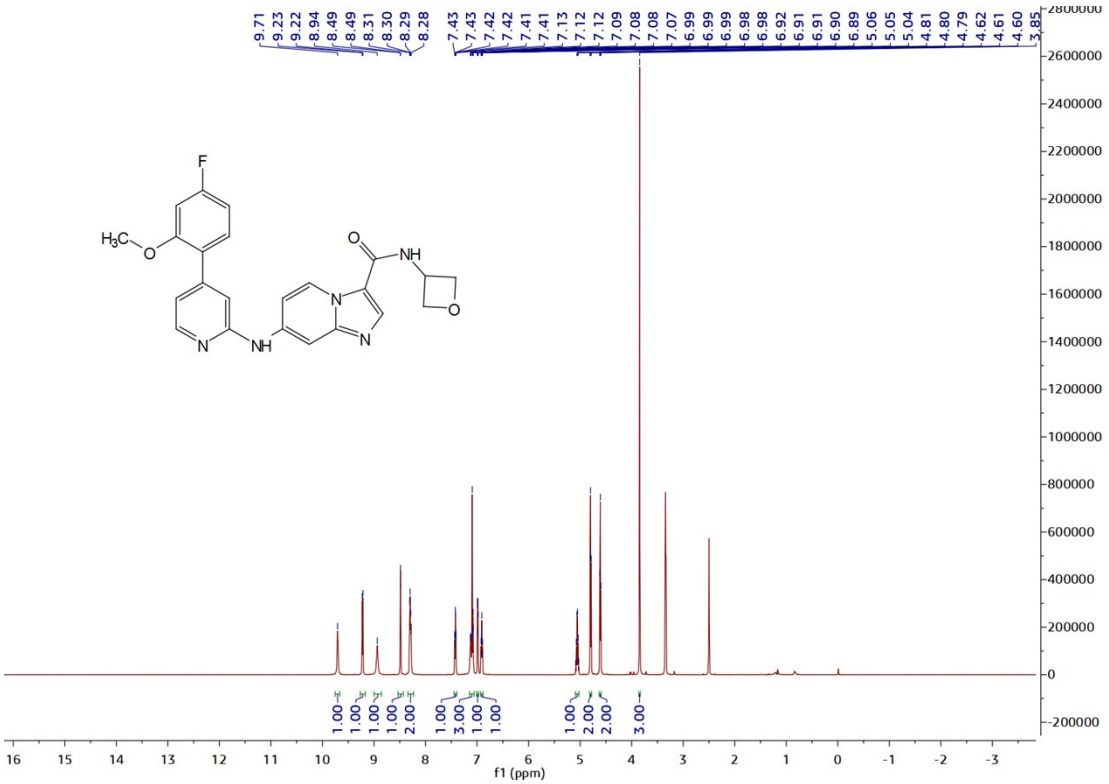


Figure S79. ^{1}H NMR Spectrum of compound LB-3

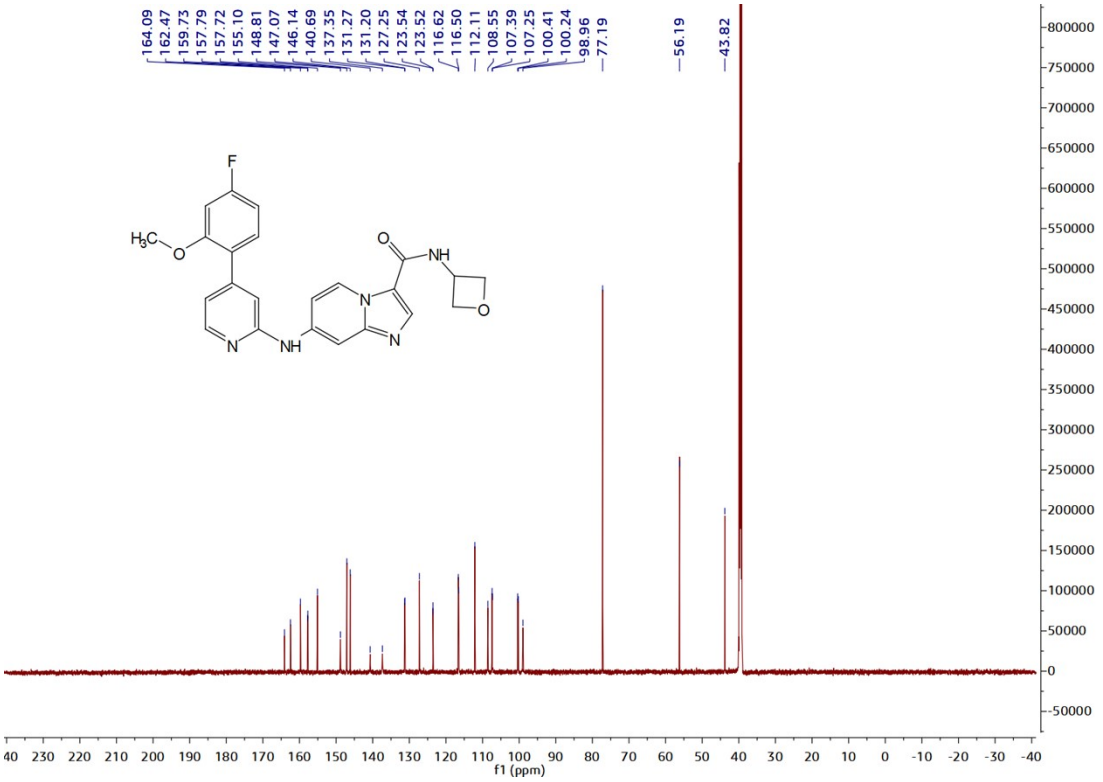


Figure S80. ^{13}C NMR Spectrum of compound LB-3

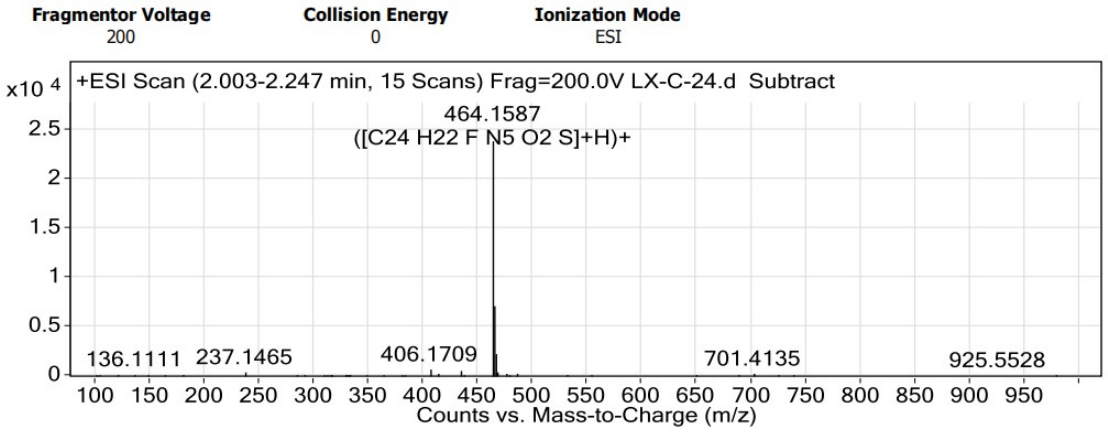


Figure S81. HRMS of compound LB-4

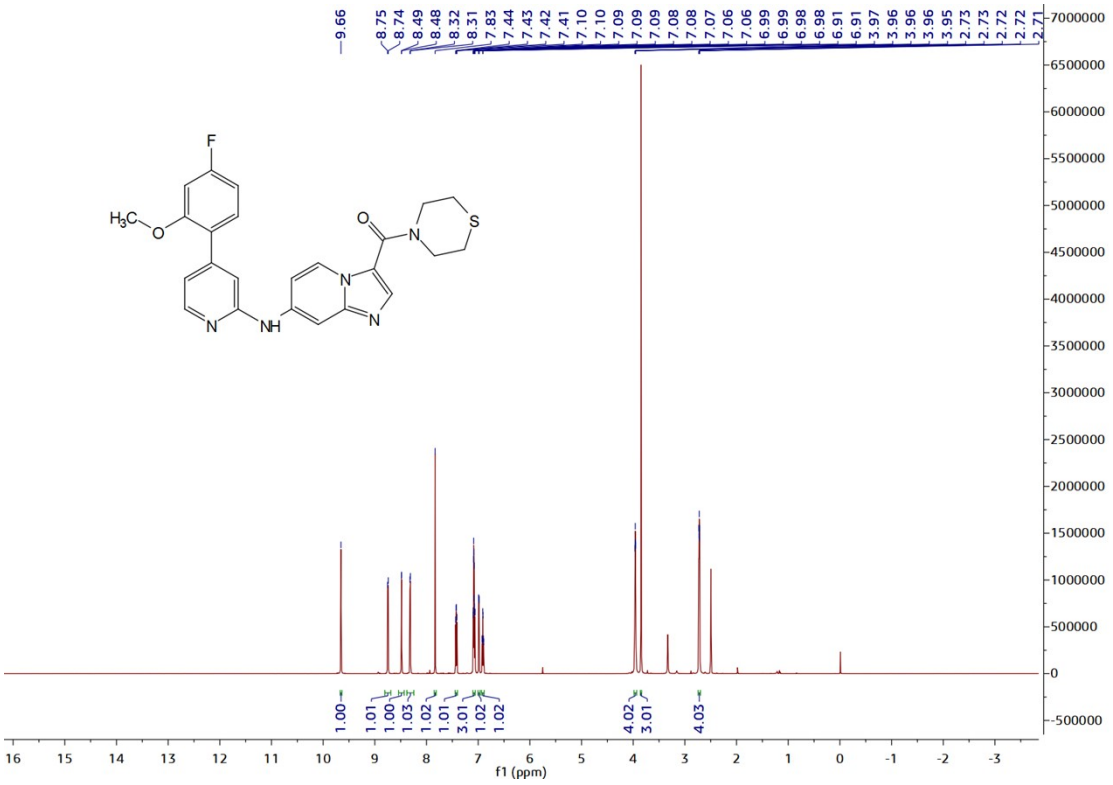


Figure S82. ^{1}H NMR Spectrum of compound LB-4

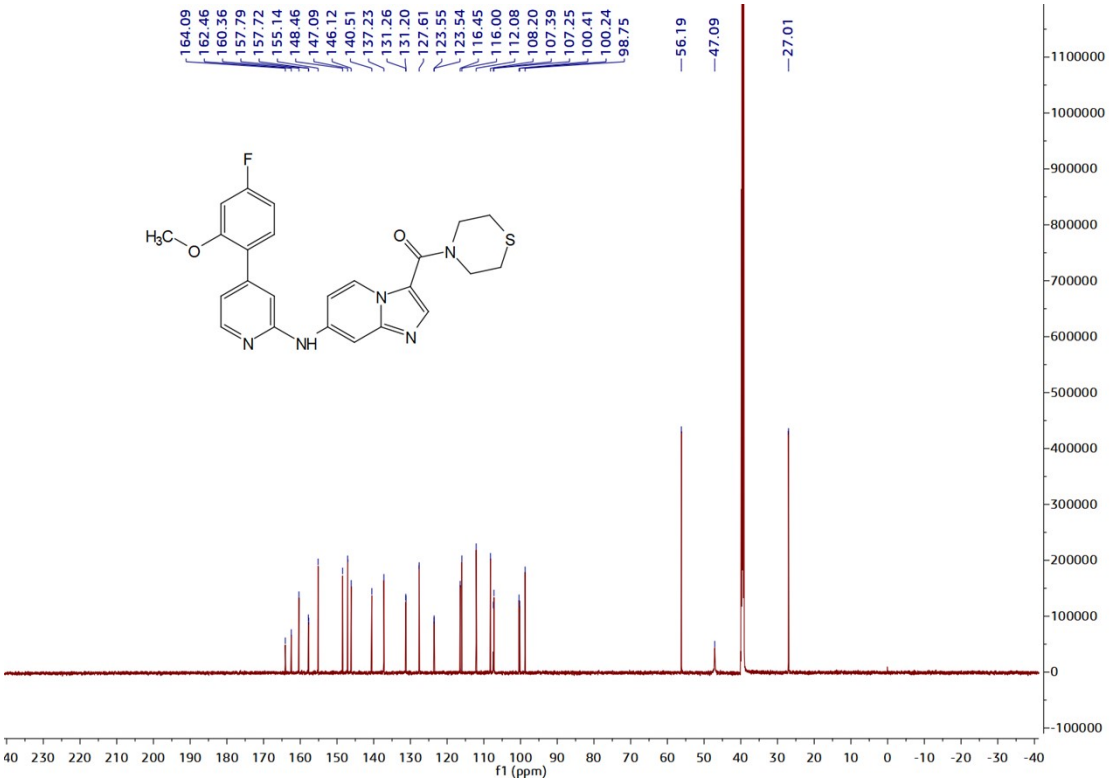


Figure S83. ^{13}C NMR Spectrum of compound LB-4

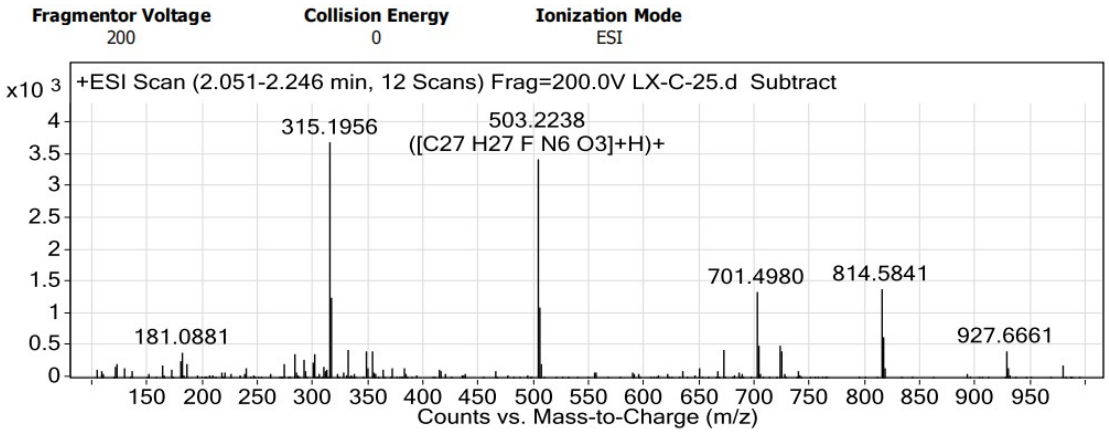


Figure S84. HRMS of compound LB-5

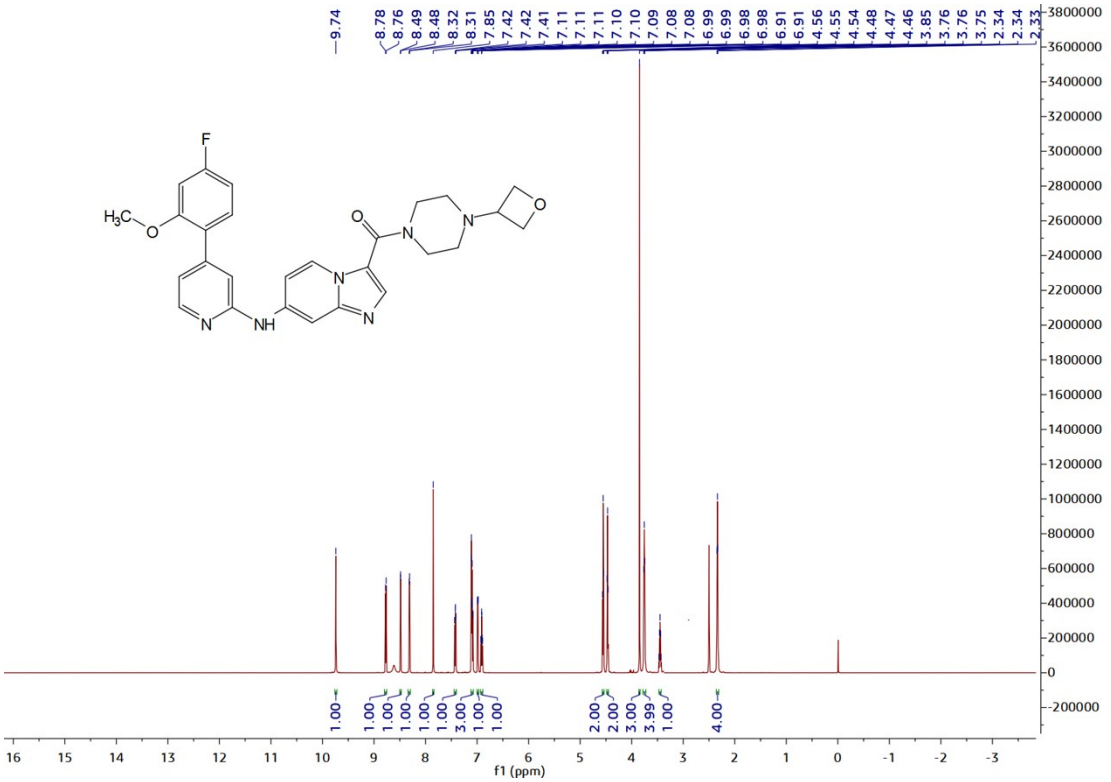


Figure S85. ^{1}H NMR Spectrum of compound LB-5

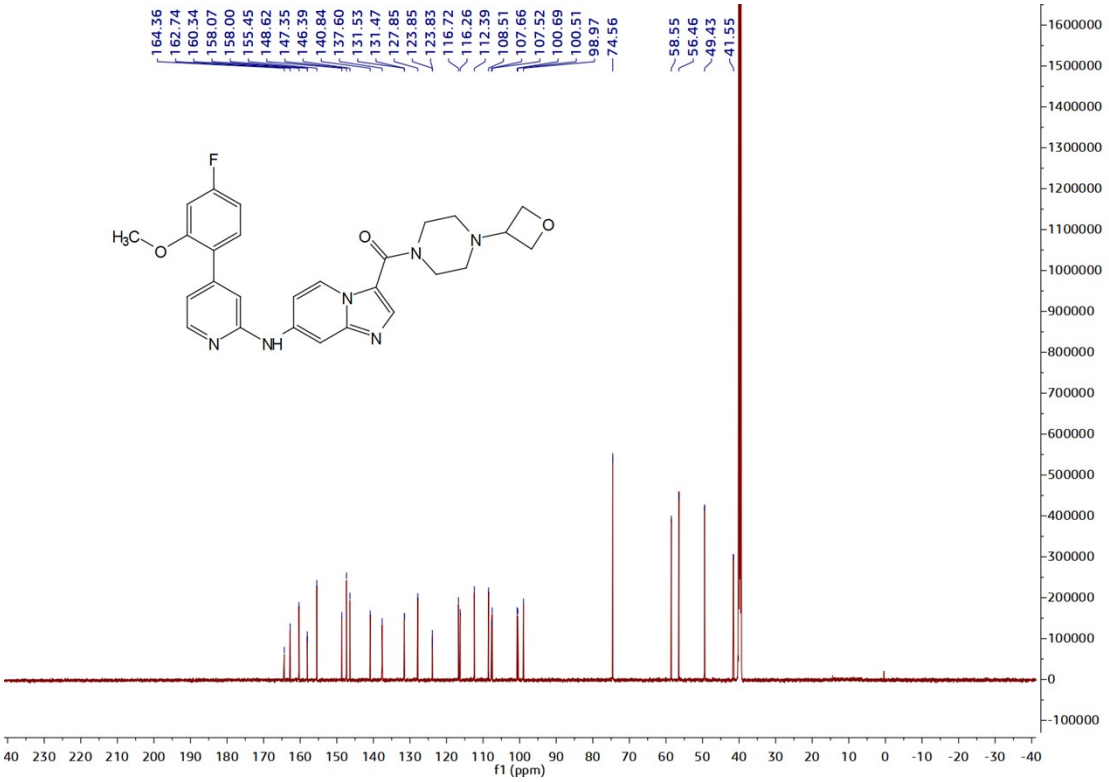


Figure S86. ^{13}C NMR Spectrum of compound LB-5

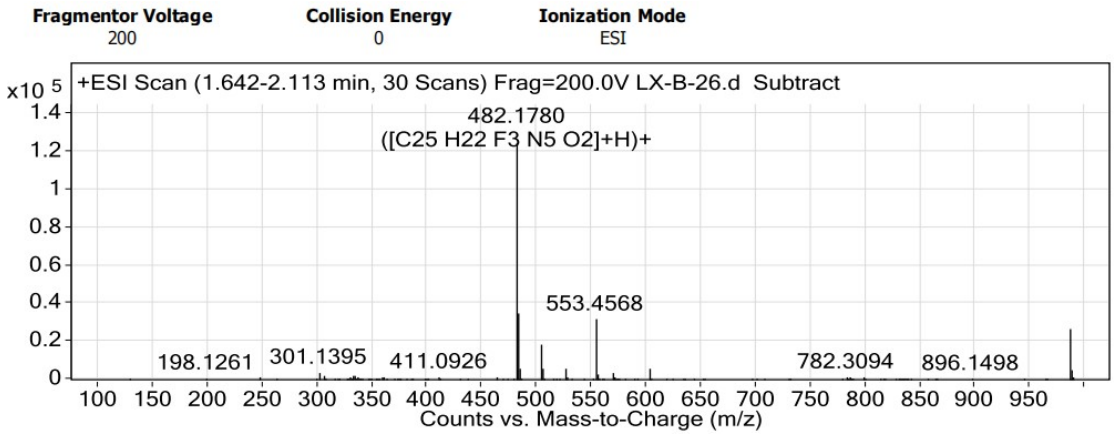


Figure S87. HRMS of compound LB-6

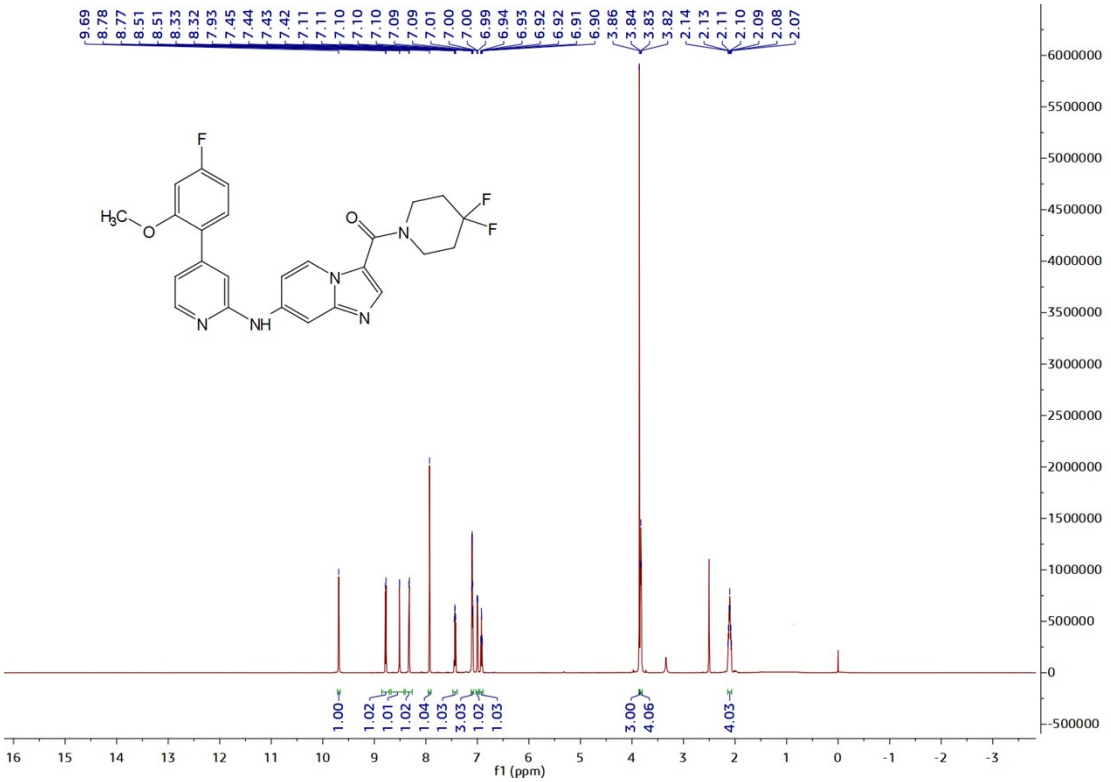


Figure S88. ^{1}H NMR Spectrum of compound LB-6

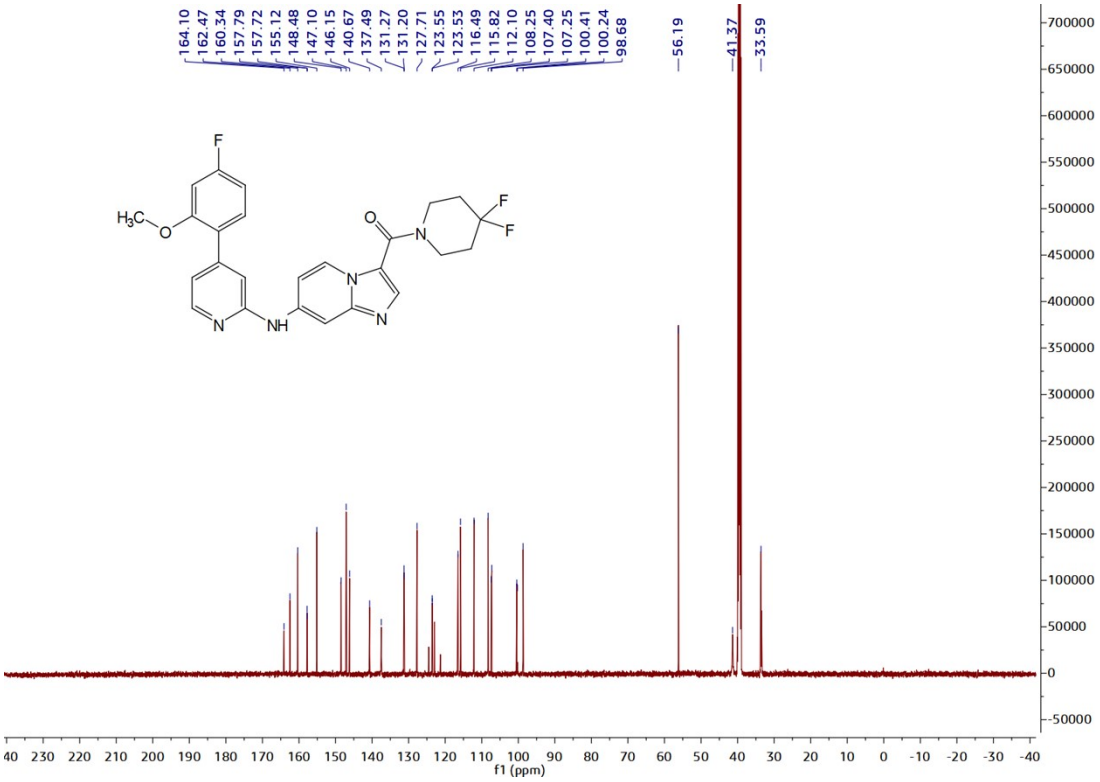


Figure S89. ^{13}C NMR Spectrum of compound LB-6

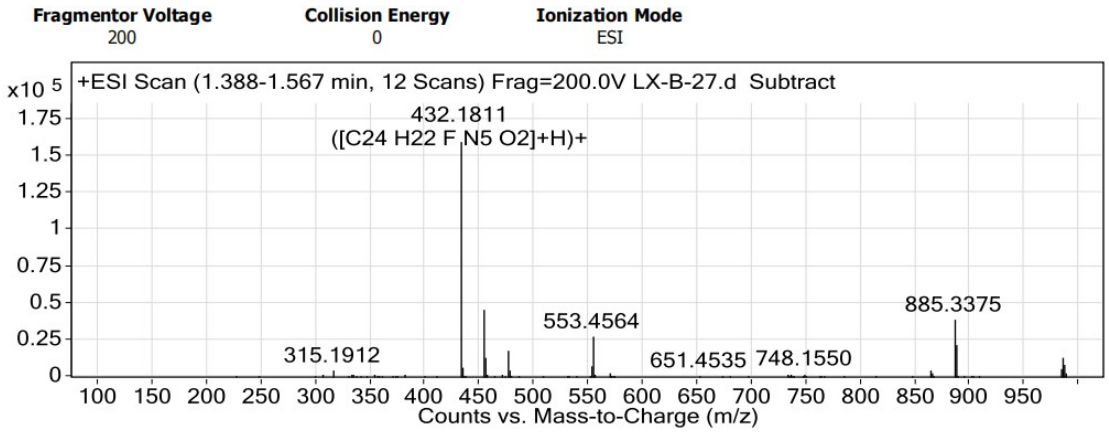


Figure S90. HRMS of compound LB-7

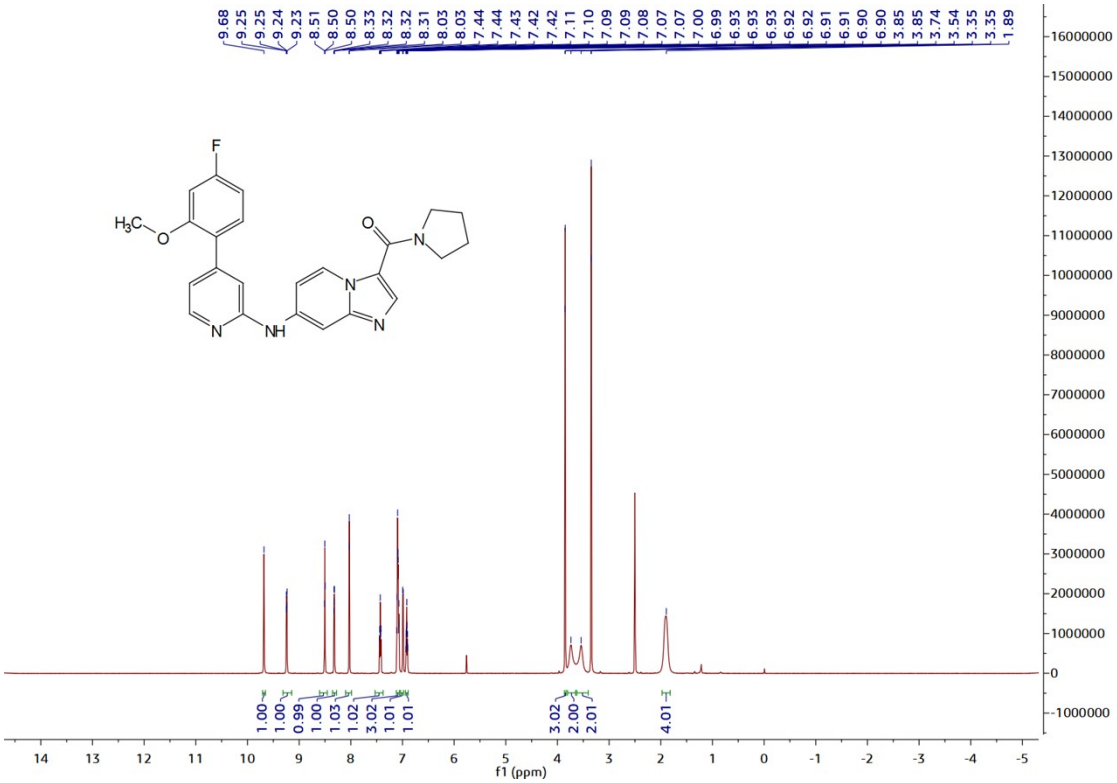


Figure S91. ^{1}H NMR Spectrum of compound LB-7

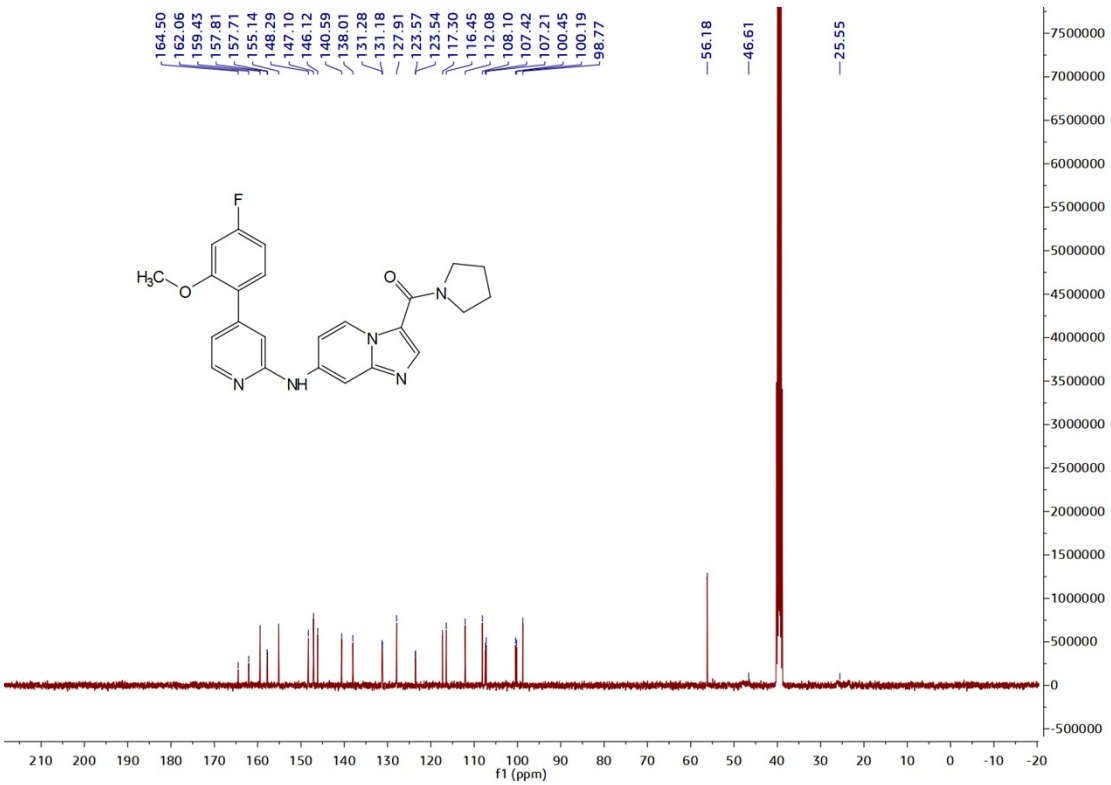


Figure S92. ^{13}C NMR Spectrum of compound LB-7

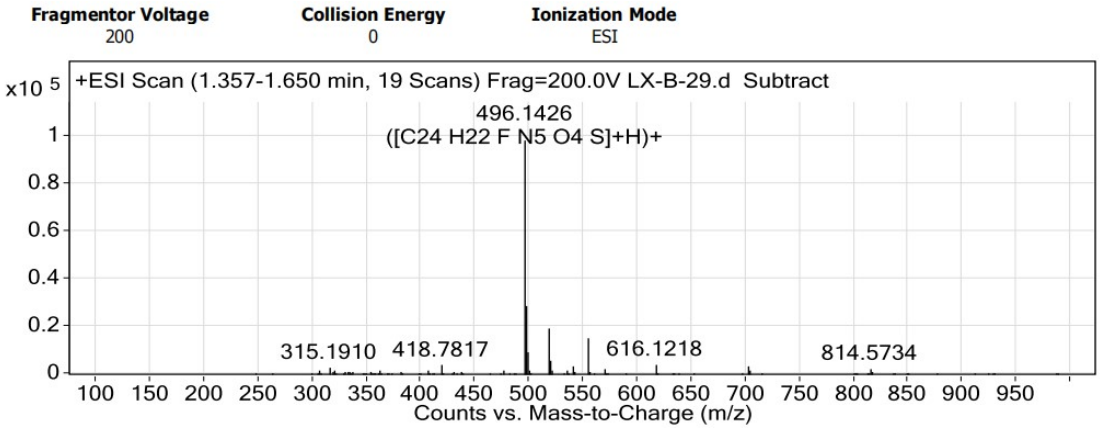


Figure S93. HRMS of compound LB-8

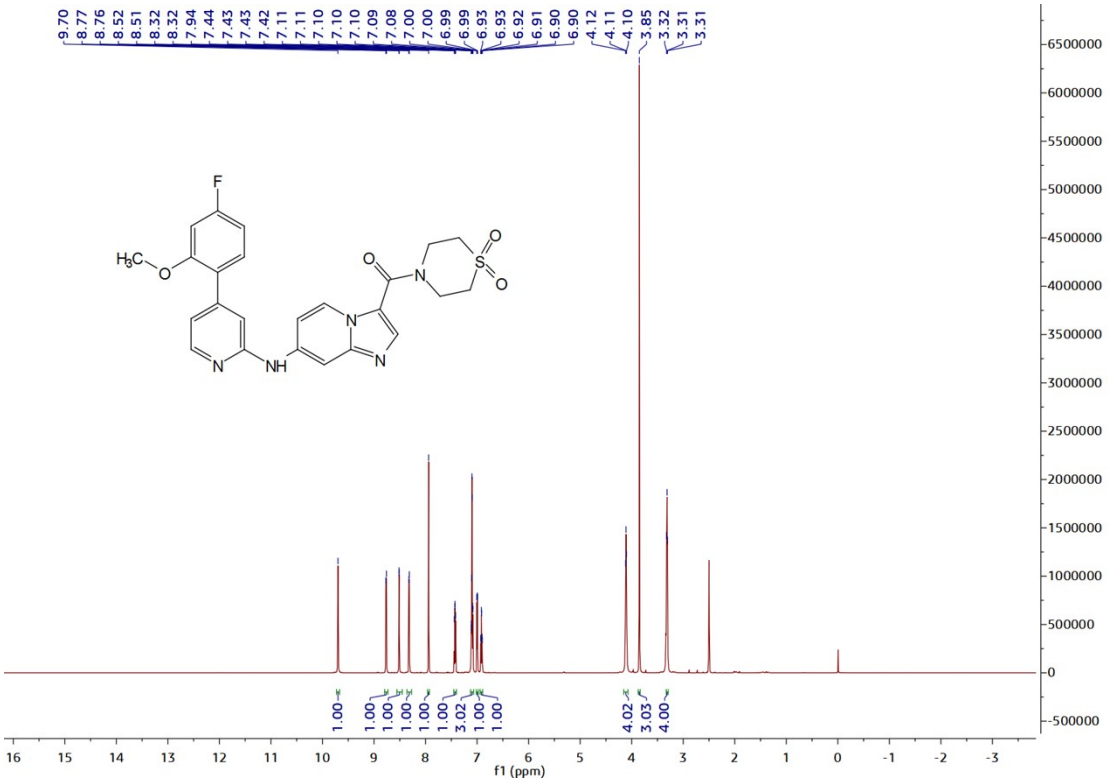


Figure S94. ^{1}H NMR Spectrum of compound LB-8

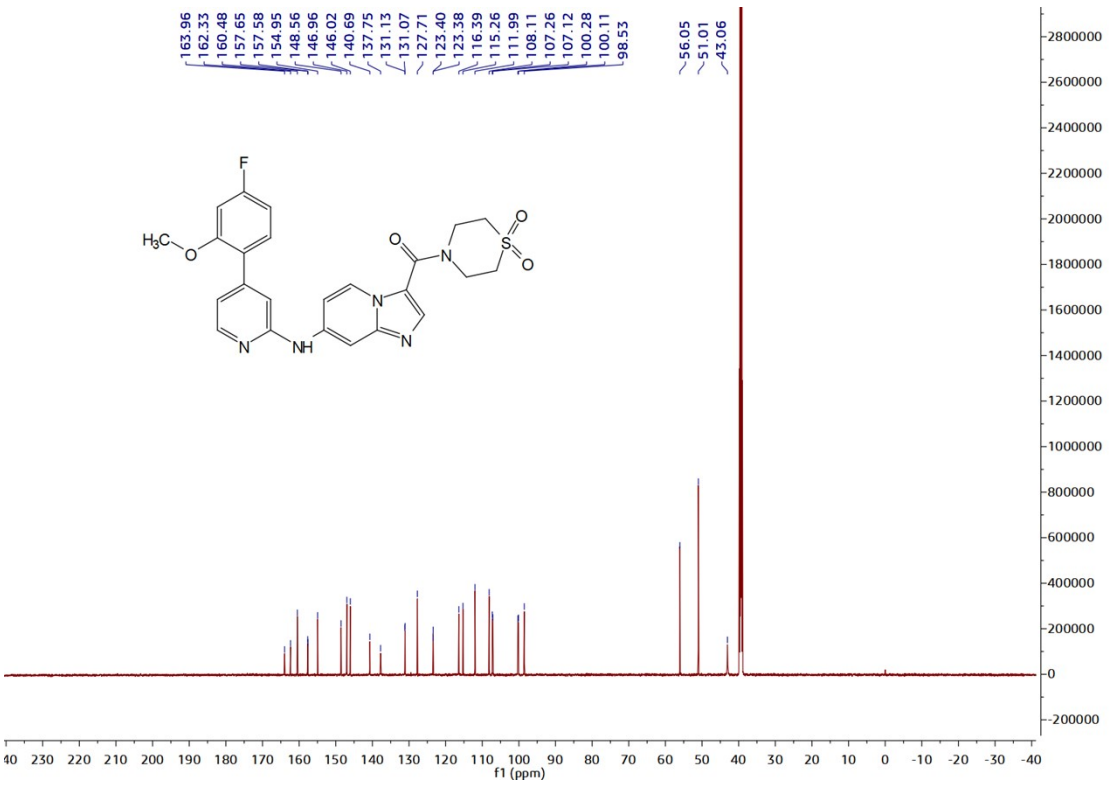


Figure S95. ^{13}C NMR Spectrum of compound LB-8

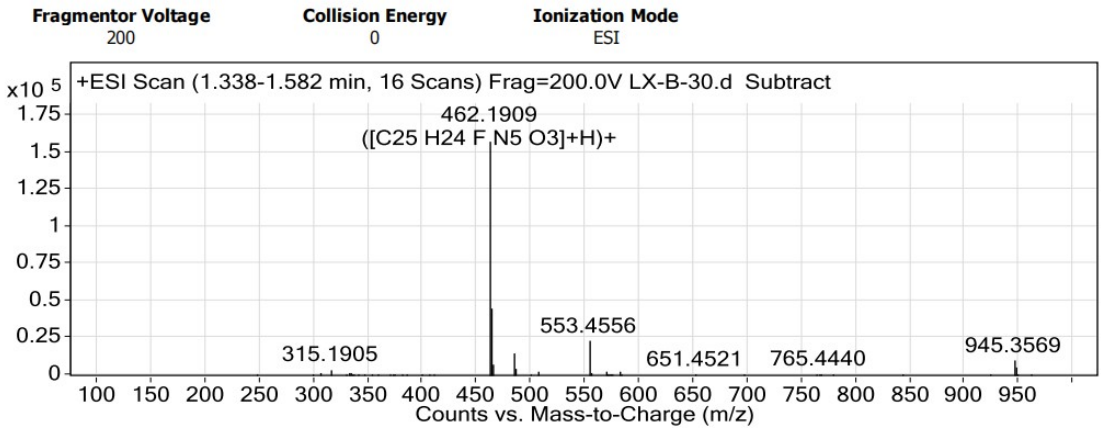


Figure S96. HRMS of compound LB-9

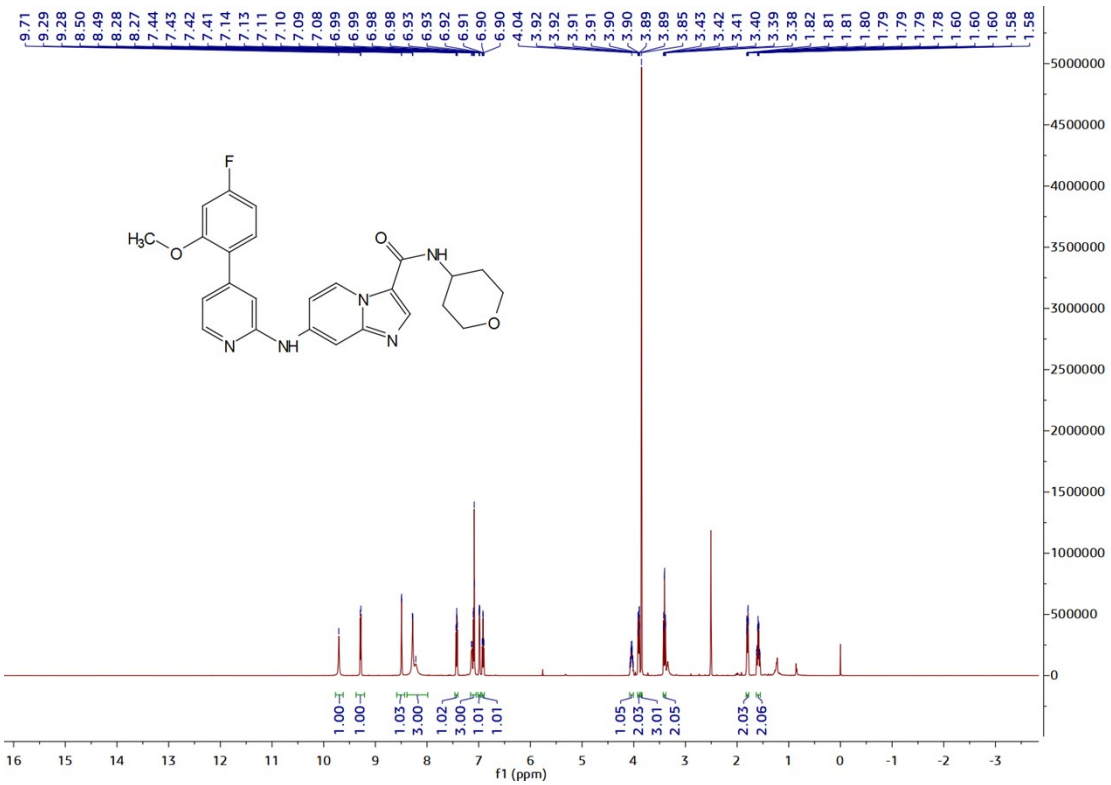


Figure S97. ^{1}H NMR Spectrum of compound LB-9

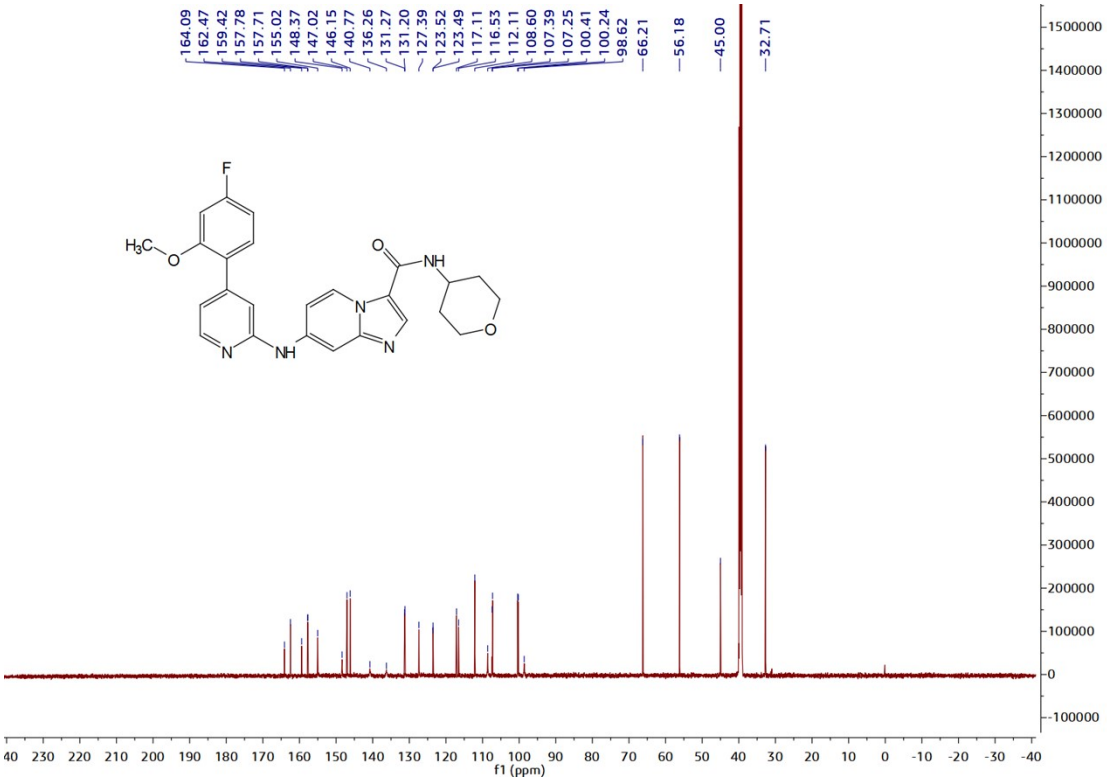


Figure S98. ^{13}C NMR Spectrum of compound LB-9

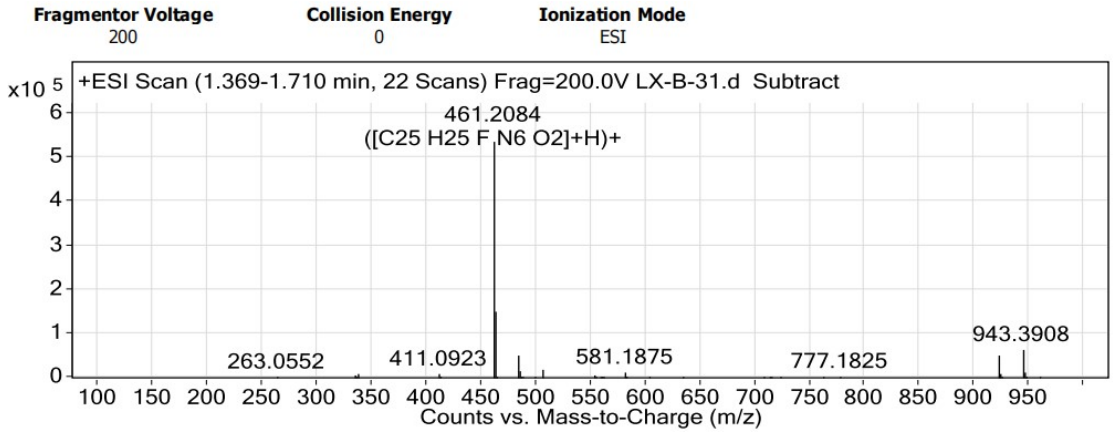


Figure S99. HRMS of compound LB-10

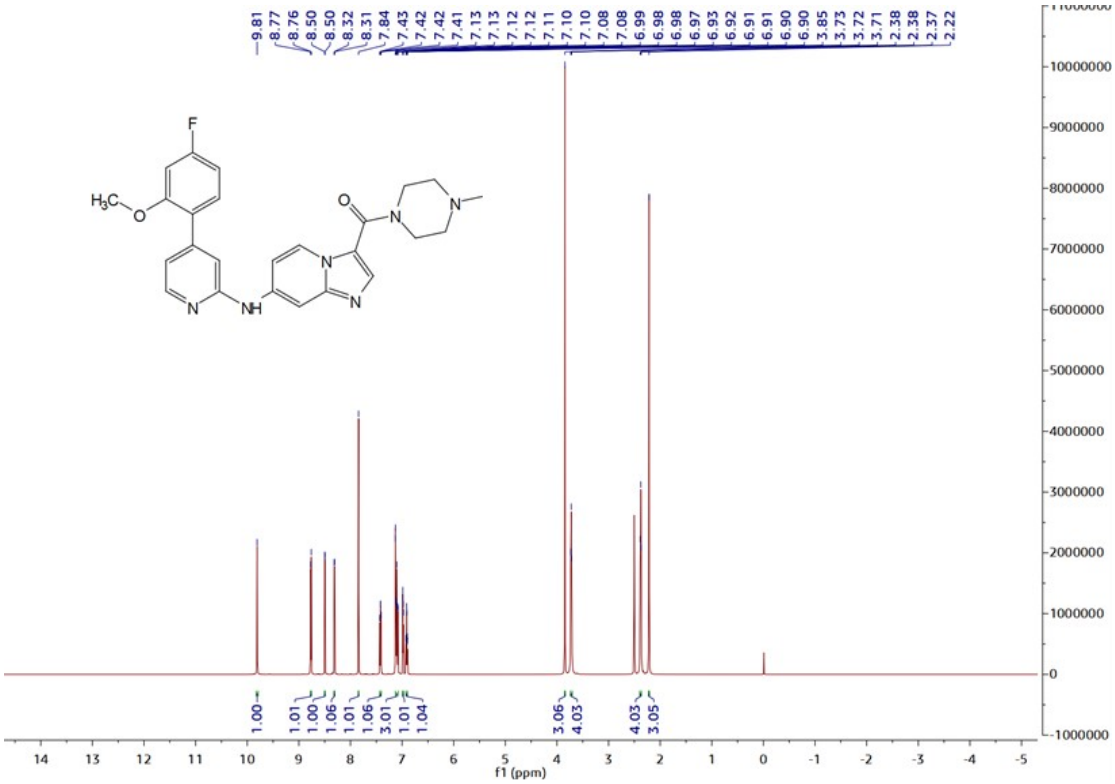


Figure S100. ^{1}H NMR Spectrum of compound LB-10

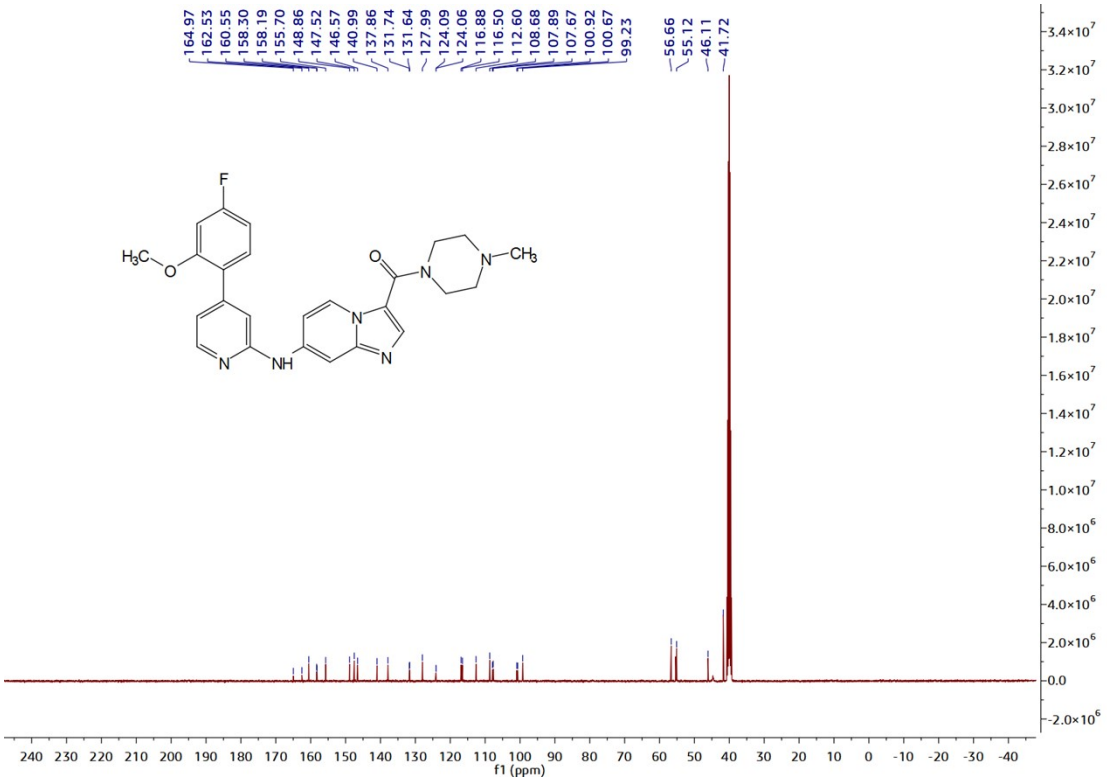


Figure S101. ^{13}C NMR Spectrum of compound LB-10

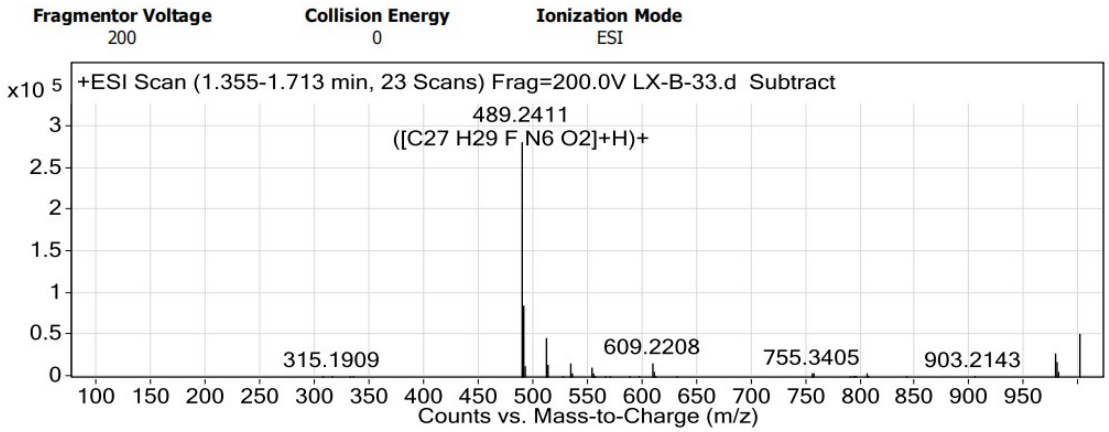


Figure S102. HRMS of compound LB-11

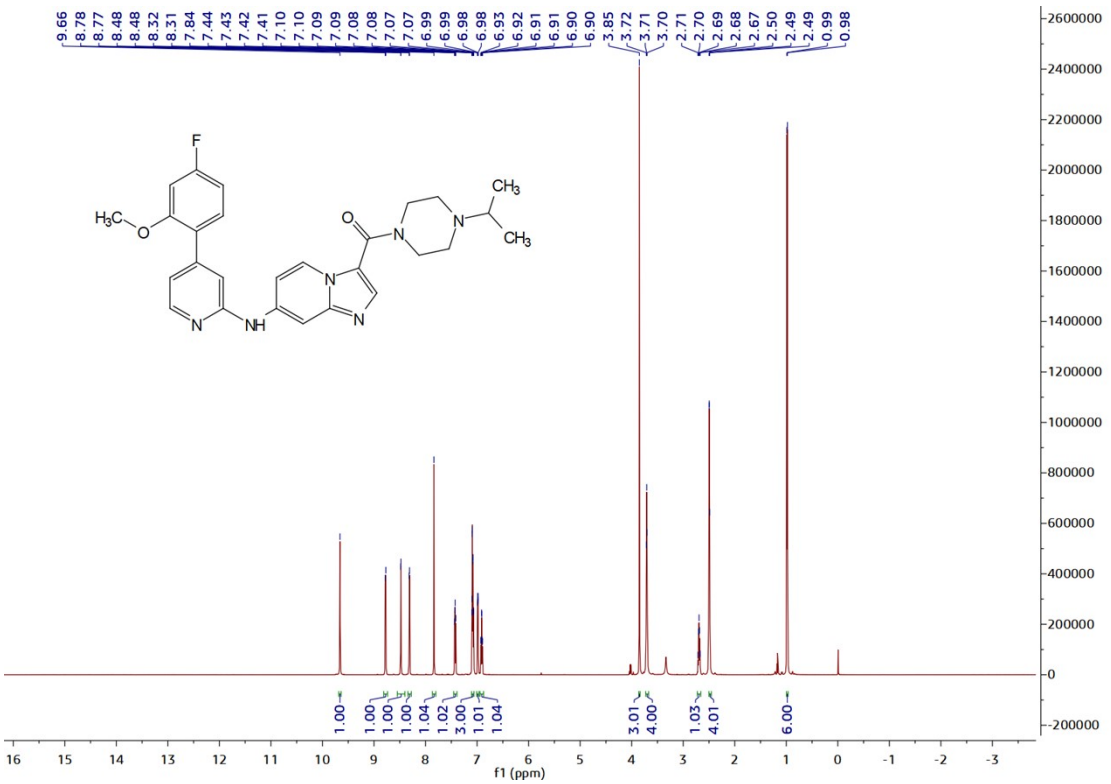


Figure S103. ^{1}H NMR Spectrum of compound LB-11

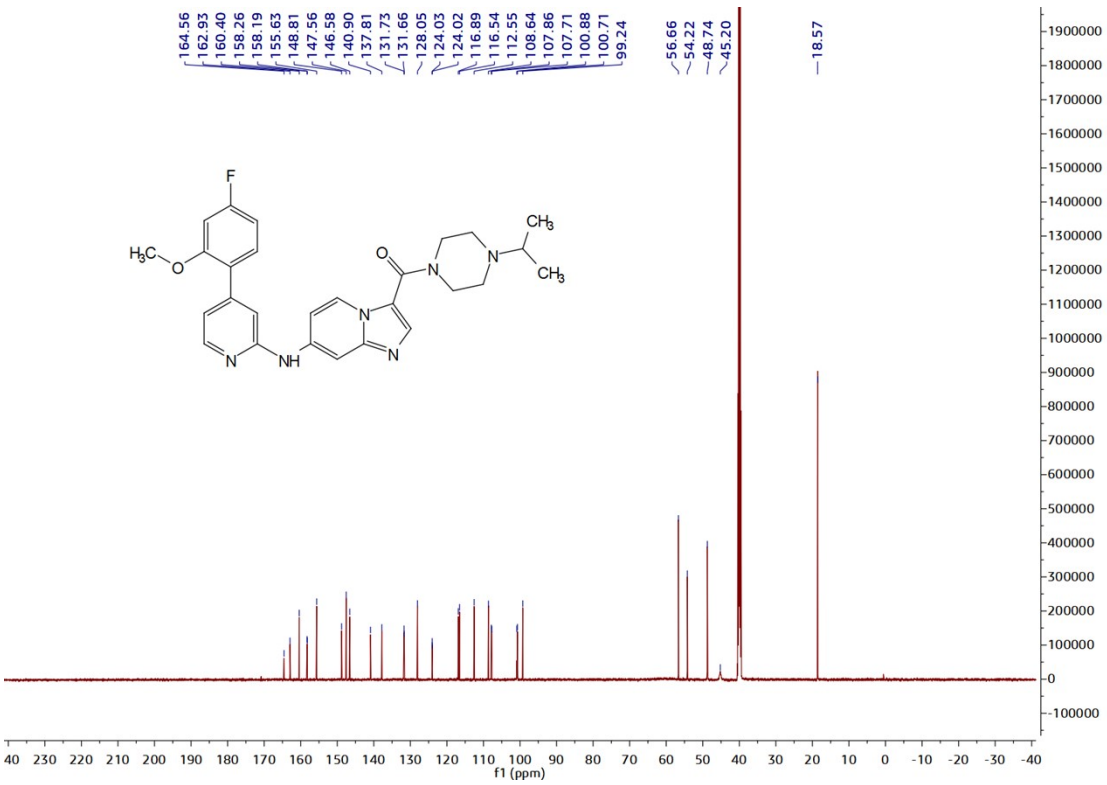


Figure S104. ^{13}C NMR Spectrum of compound LB-11

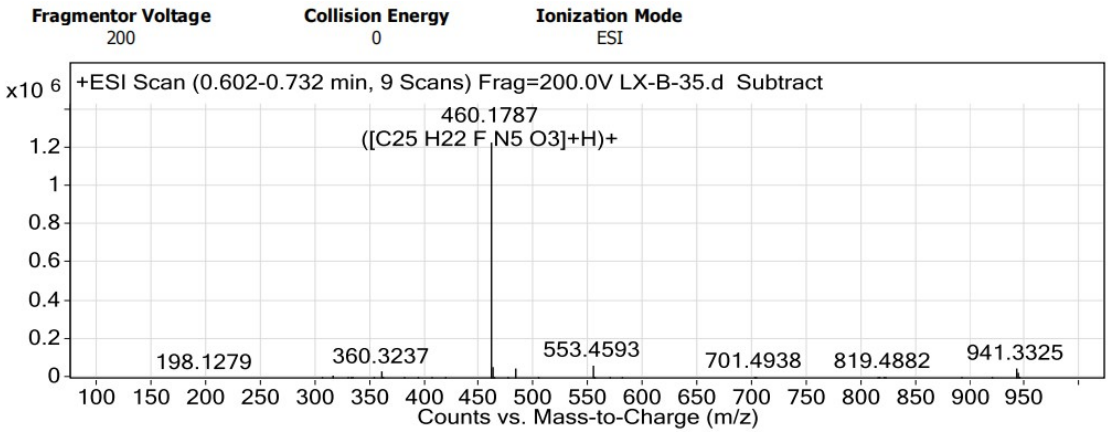


Figure S105. HRMS of compound LB-12

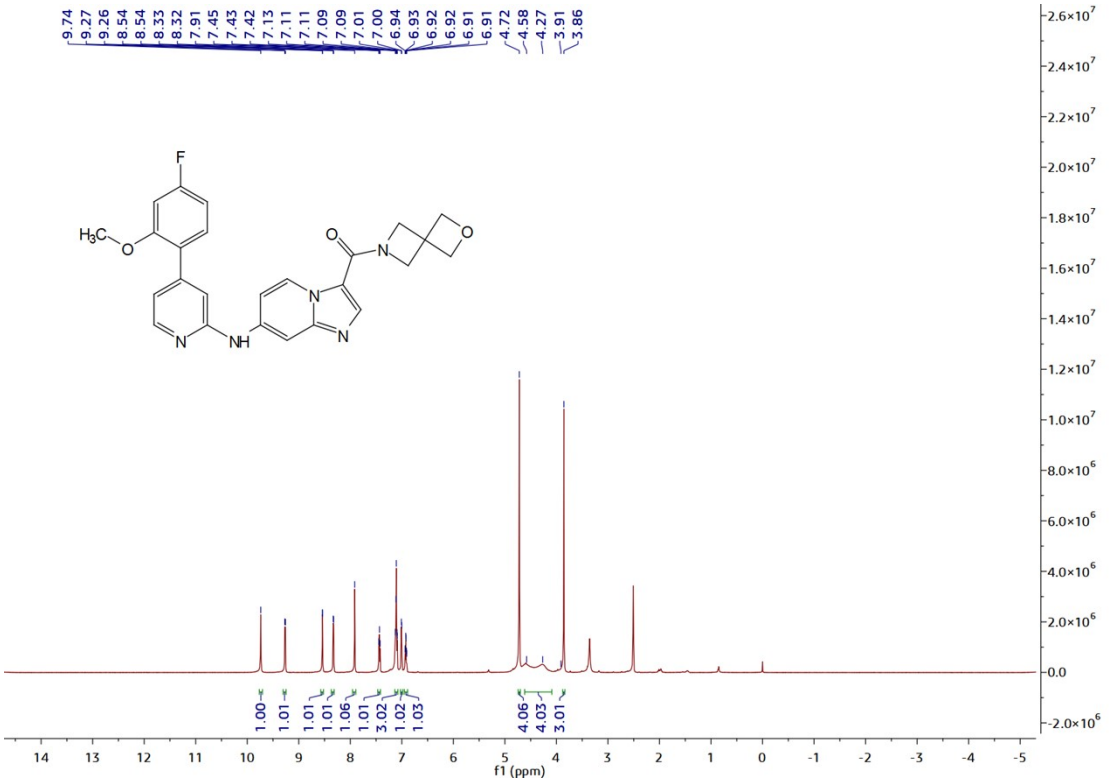


Figure S106. ^{1}H NMR Spectrum of compound LB-12

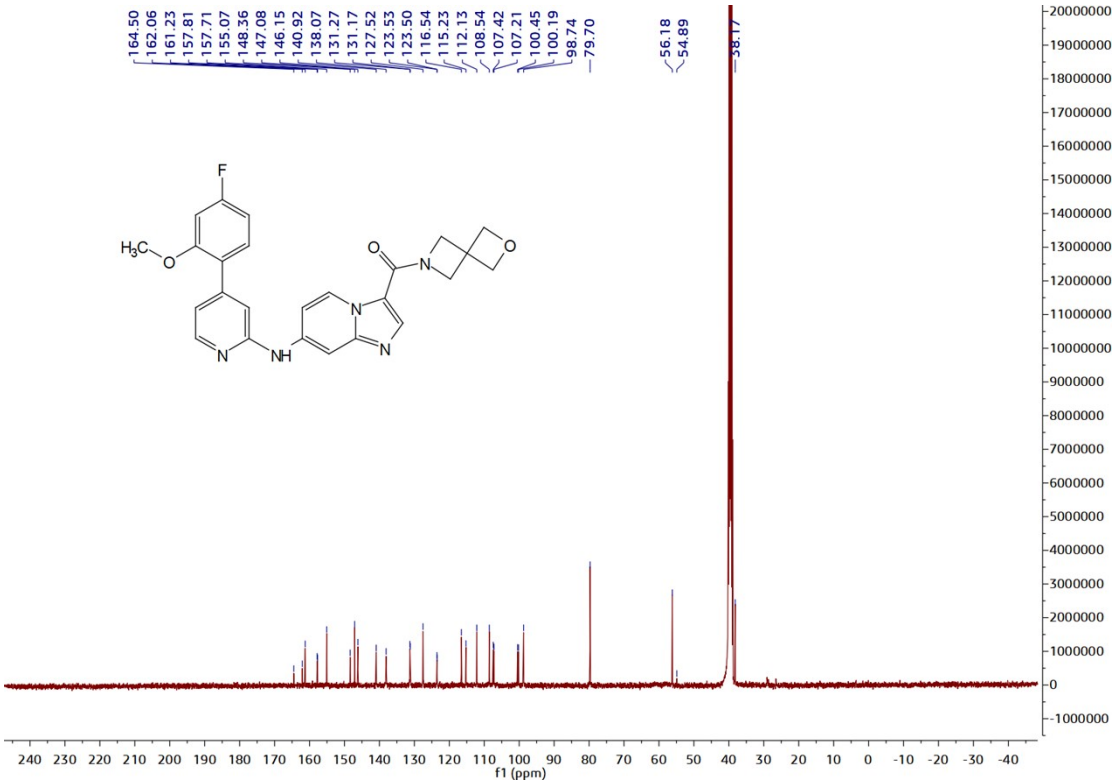
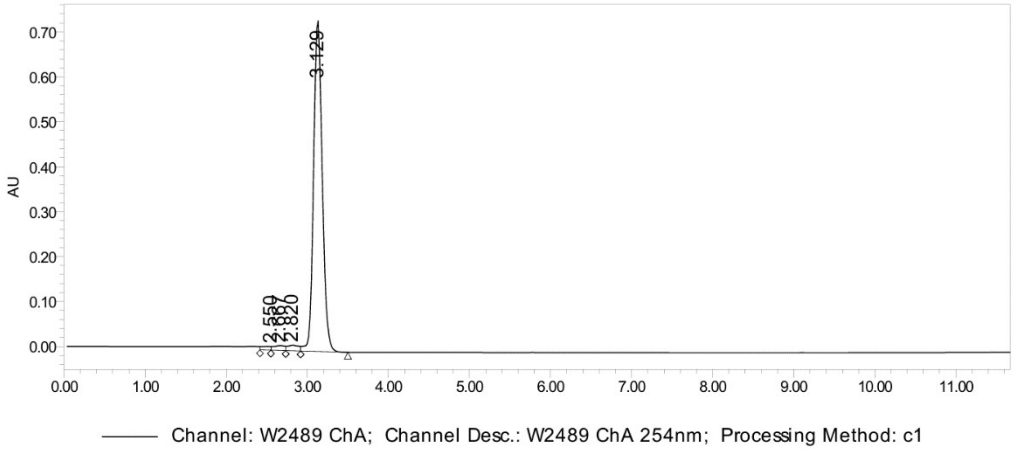


Figure S107. ^{13}C NMR Spectrum of compound LB-12



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		( )	(*)	%	( )
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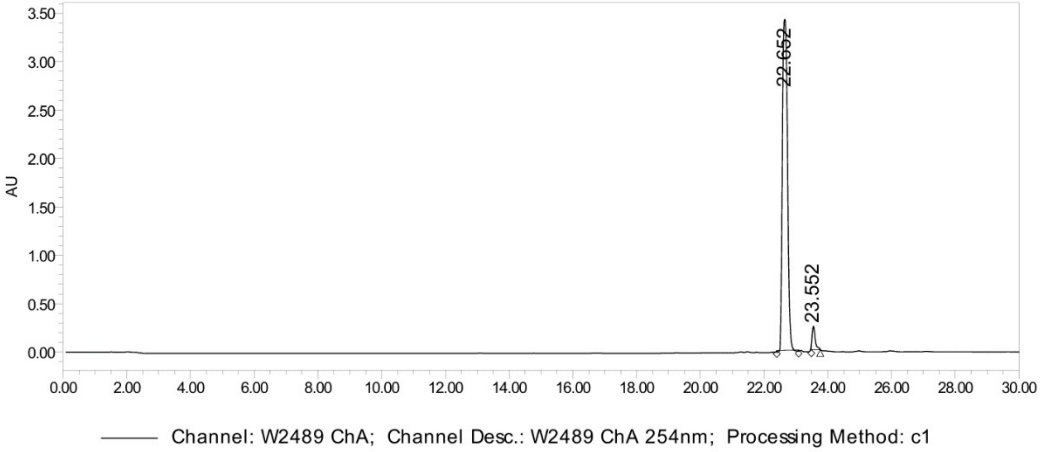
2	W2489 ChA 254nm	2.667	103503	1.80	10561
3	W2489 ChA 254nm	2.820	119896	2.08	12062
4	W2489 ChA 254nm	3.129	5470285	95.05	735815

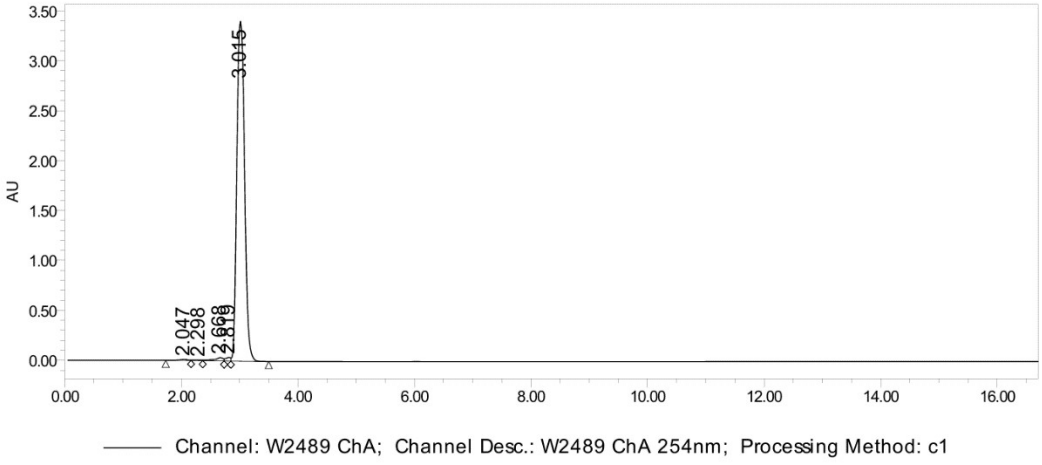
HPLC of compound LA-1



:

		( )	(*)	%	( )
1	W2489 ChA 254nm	22.652	38232112	95.98	3425754
2	W2489 ChA 254nm	23.552	1599763	4.02	242080

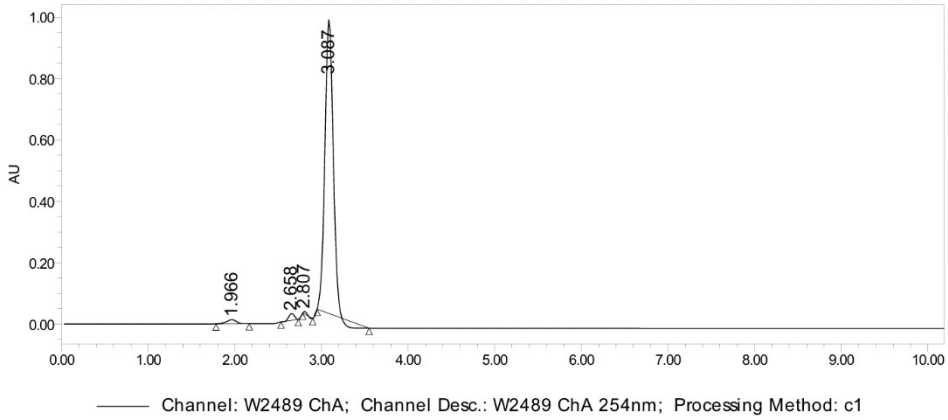
HPLC of compound LA-2



:

		( )	(*)	%	( )
1	W2489 ChA 254nm	2.047	107321	0.33	10928
2	W2489 ChA 254nm	2.298	70117	0.22	6453
3	W2489 ChA 254nm	2.668	354608	1.10	33203
4	W2489 ChA 254nm	2.819	210723	0.66	36321
5	W2489 ChA 254nm	3.015	31412391	97.69	3407225

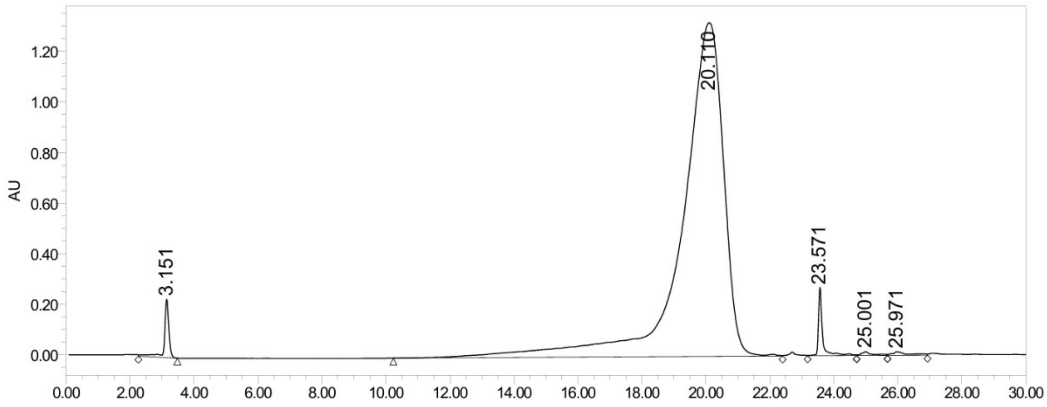
HPLC of compound LA-3



:

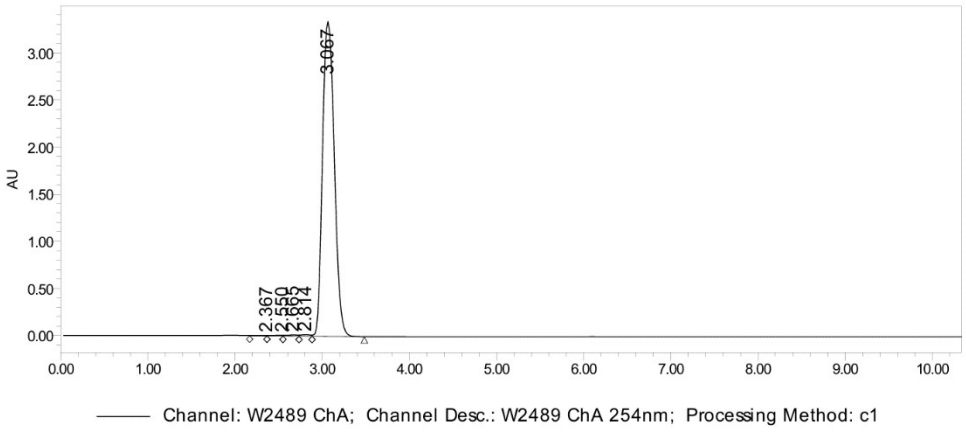
		( )	(*)	%	( )
1	W2489 ChA 254nm	1.966	100786	1.48	12777
2	W2489 ChA 254nm	2.658	101210	1.48	22050
3	W2489 ChA 254nm	2.807	29113	0.43	8260
4	W2489 ChA 254nm	3.087	6593679	96.61	954292

HPLC of compound LA-4



:

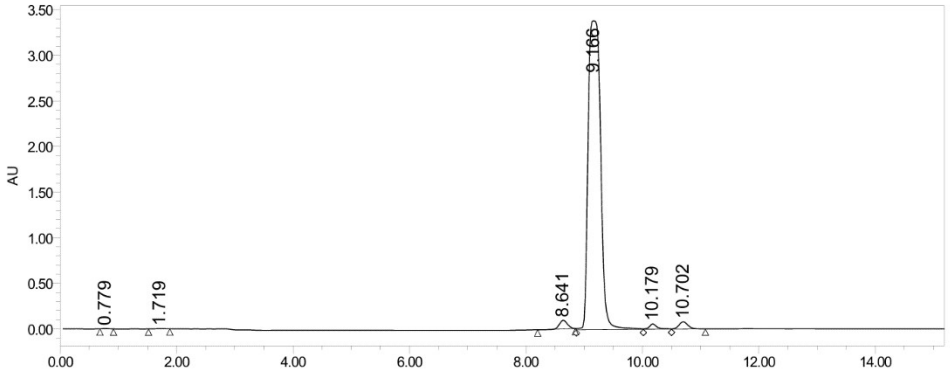
		( )	(*)	%	( )
1	W2489 ChA 254nm	3.151	2270687	1.84	230795
2	W2489 ChA 254nm	20.110	117800188	95.54	1318370
3	W2489 ChA 254nm	23.571	2408237	1.95	269749
4	W2489 ChA 254nm	25.001	348046	0.28	15174
5	W2489 ChA 254nm	25.971	467571	0.38	13764



:

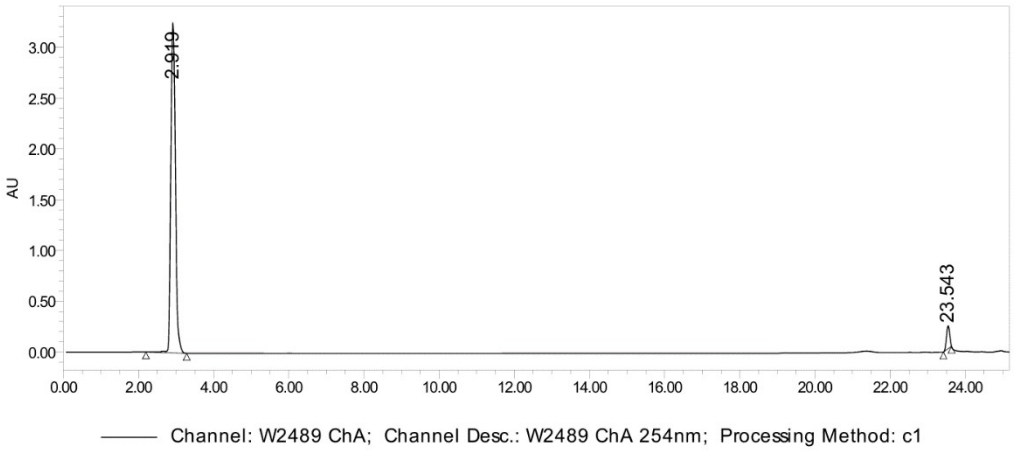
		( )	(*)	%	( )
1	W2489 ChA 254nm	2.367	57085	0.18	5404
2	W2489 ChA 254nm	2.550	71187	0.23	7557
3	W2489 ChA 254nm	2.665	118098	0.37	13155
4	W2489 ChA 254nm	2.814	126599	0.40	16036
5	W2489 ChA 254nm	3.067	31241707	98.82	3342487

HPLC of compound LA-5



:

		( )	(*)	%	( )
1	W2489 ChA 254nm	0.779	23280	0.04	3219
2	W2489 ChA 254nm	1.719	27302	0.05	2803
3	W2489 ChA 254nm	8.641	922427	1.76	95404
4	W2489 ChA 254nm	9.166	50019753	95.41	3385088
5	W2489 ChA 254nm	10.179	563992	1.08	59493
6	W2489 ChA 254nm	10.702	868078	1.66	80297

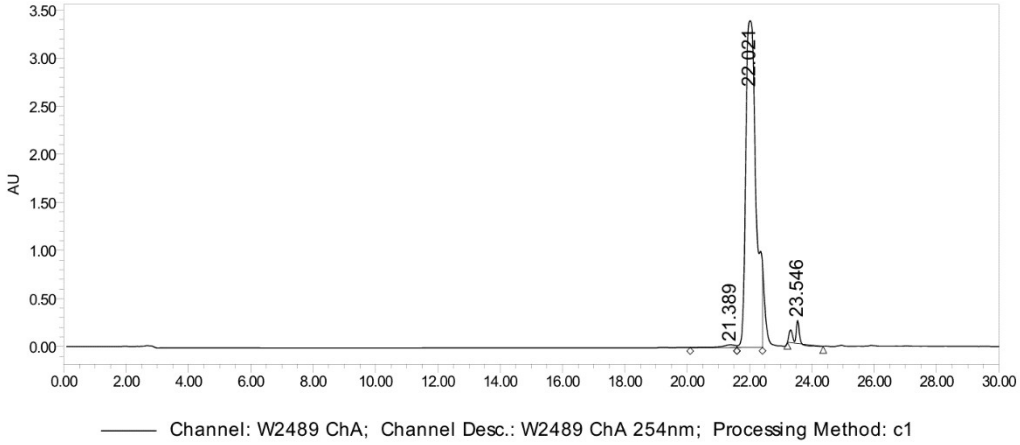


:

		( )	(*)	%	( )
1	W2489 ChA 254nm	2.919	27778407	95.50	3249808
2	W2489 ChA 254nm	23.543	1309077	4.50	226635

HPLC of compound LA-9

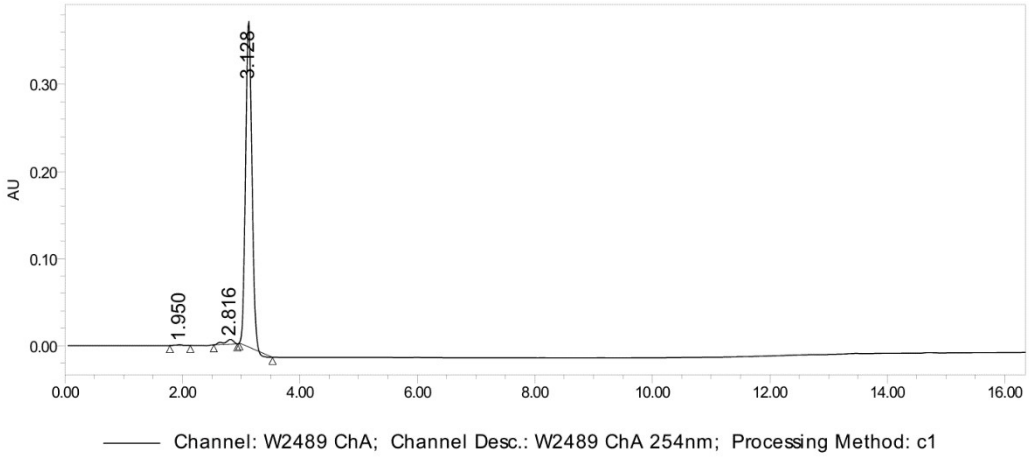
HPLC of compound LA-11



:

		( )	(*)	%	( )
1	W2489 ChA 254nm	21.389	831547	1.03	28250
2	W2489 ChA 254nm	22.021	76764001	95.40	3397950
3	W2489 ChA 254nm	23.546	2869442	3.57	239233

HPLC of compound LA-13



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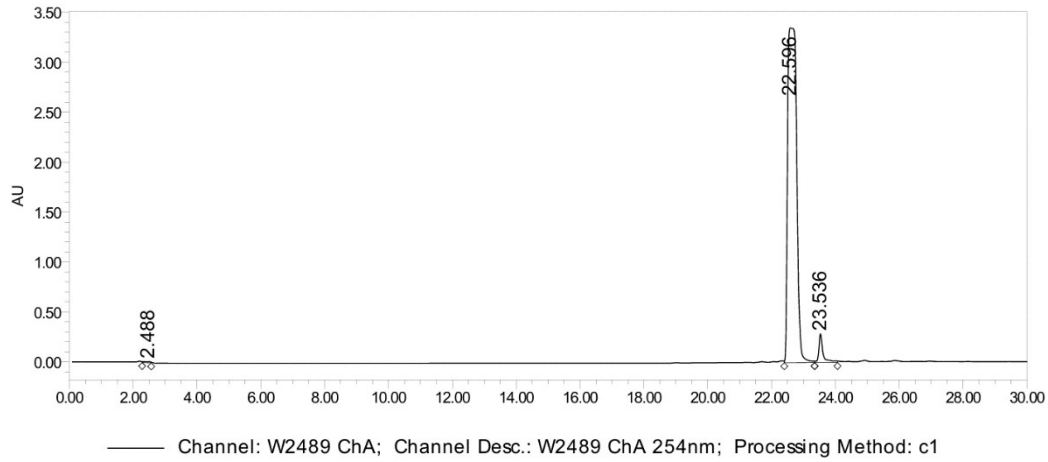
		( )	(*)	%	( )
1	W2489 ChA 254nm	1.950	7682	0.27	1071
2	W2489 ChA 254nm	2.816	58054	2.01	5517
3	W2489 ChA 254nm	3.128	2826930	97.73	374550

HPLC of compound LB-1



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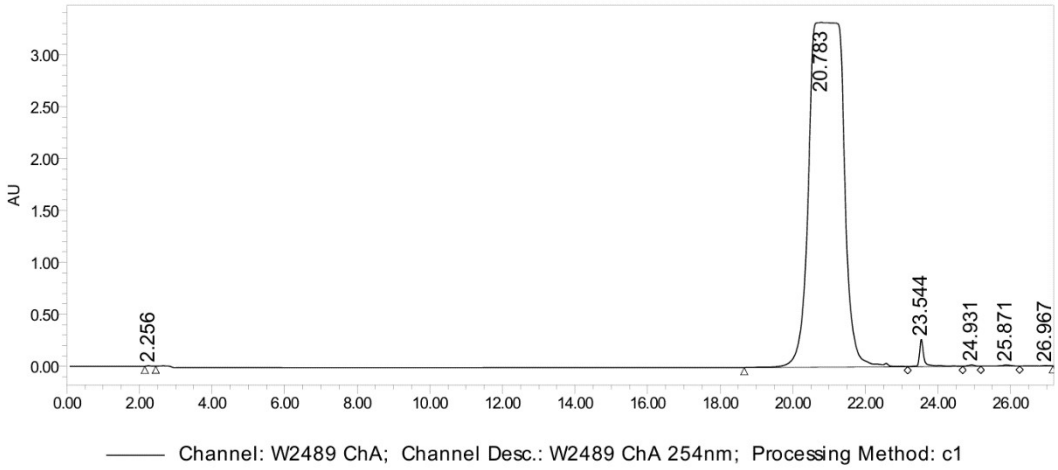
		( )	(*)	%	( )
1	W2489 ChA 254nm	2.488	168015	0.25	11519
2	W2489 ChA 254nm	22.596	65385853	95.90	3356116
3	W2489 ChA 254nm	23.536	2625861	3.85	288093

HPLC of compound LB-2



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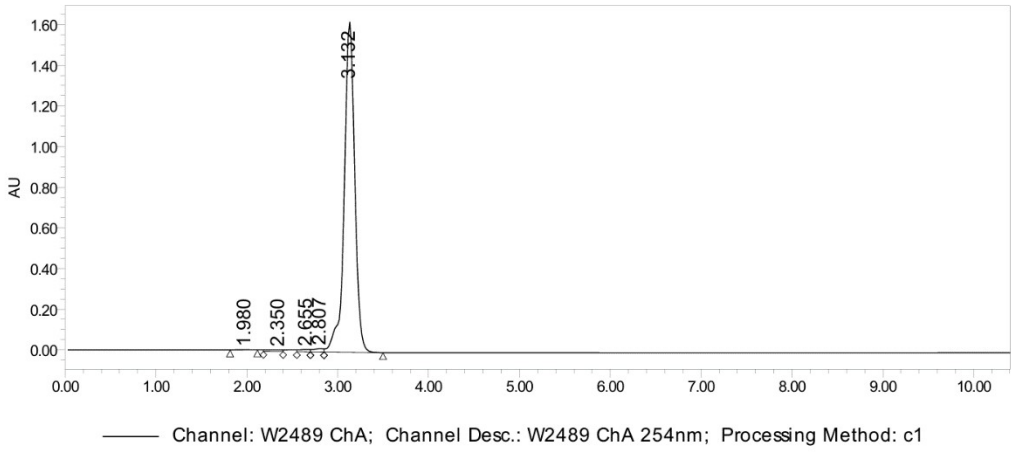
		( )	(*)	%	( )
1	W2489 ChA 254nm	2.256	29552	0.01	5039
2	W2489 ChA 254nm	20.783	218122222	98.69	3316567
3	W2489 ChA 254nm	23.544	2354464	1.07	262597
4	W2489 ChA 254nm	24.931	191716	0.09	13662
5	W2489 ChA 254nm	25.871	248625	0.11	11150
6	W2489 ChA 254nm	26.967	80315	0.04	3160

HPLC of compound LB-3



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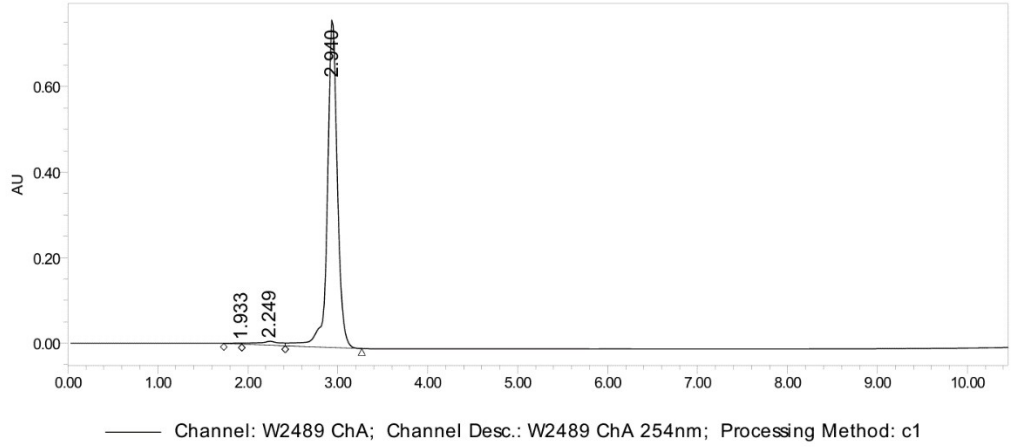
		( )	(*)	%	( )
1	W2489 ChA 254nm	1.980	8650	0.07	1107
2	W2489 ChA 254nm	2.350	99759	0.75	7975
3	W2489 ChA 254nm	2.655	95720	0.72	11784
4	W2489 ChA 254nm	2.807	127843	0.96	16314
5	W2489 ChA 254nm	3.132	12948352	97.50	1617524

HPLC of compound LB-4



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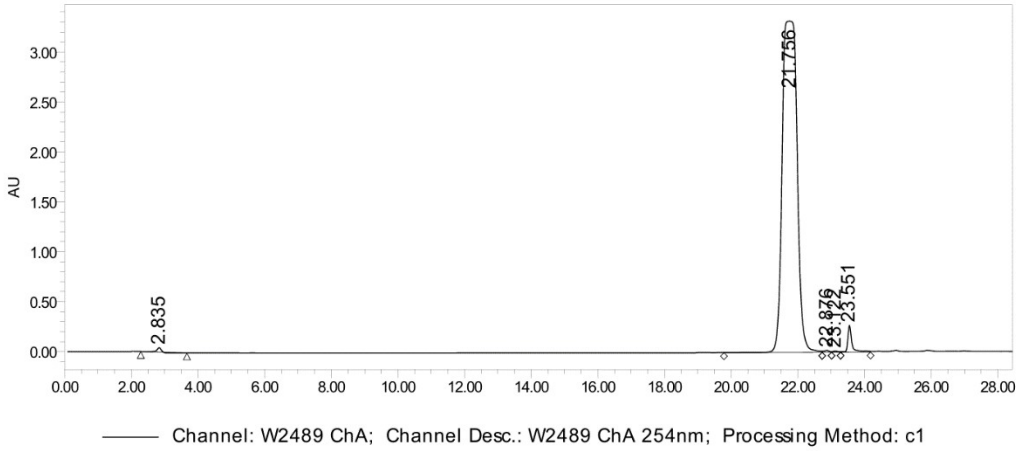
		( )	(*)	%	( )
1	W2489 ChA 254nm	1.933	20488	0.33	2547
2	W2489 ChA 254nm	2.249	164994	2.69	9315
3	W2489 ChA 254nm	2.940	5955968	96.98	768711

HPLC of compound LB-5



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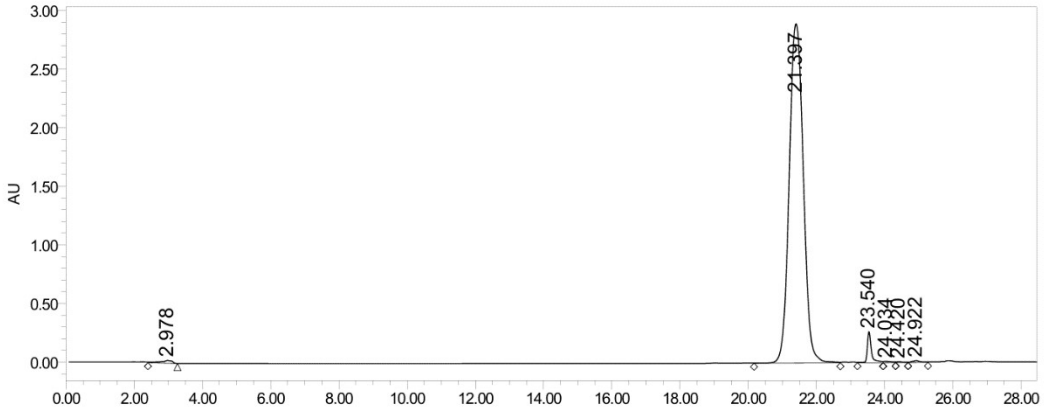
		( )	(*)	%	( )
1	W2489 ChA 254nm	2.835	547762	0.52	44101
2	W2489 ChA 254nm	21.756	102877137	96.96	3315555
3	W2489 ChA 254nm	22.876	214757	0.20	18714
4	W2489 ChA 254nm	23.122	132261	0.12	11694
5	W2489 ChA 254nm	23.551	2335103	2.20	267243

HPLC of compound LB-6



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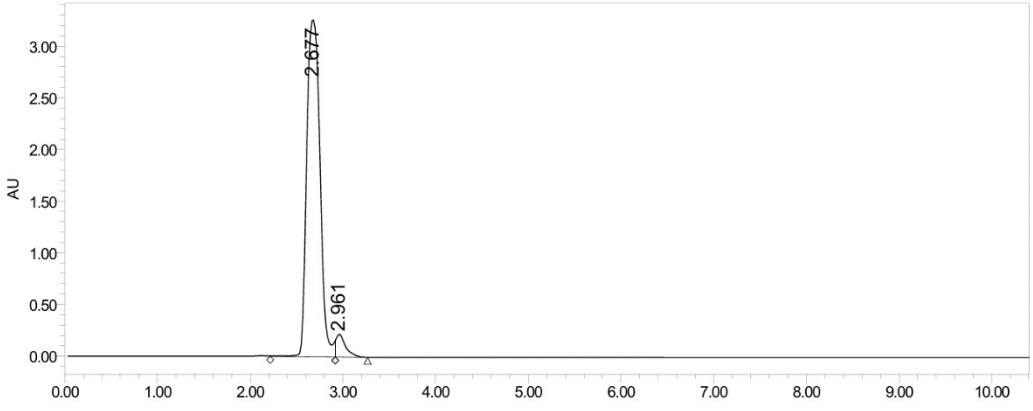
		( )	(*)	%	( )
1	W2489 ChA 254nm	2.978	597785	0.70	23849
2	W2489 ChA 254nm	21.397	82545498	96.07	2893850
3	W2489 ChA 254nm	23.540	2177330	2.53	265248
4	W2489 ChA 254nm	24.034	193202	0.22	11604
5	W2489 ChA 254nm	24.420	119720	0.14	7636
6	W2489 ChA 254nm	24.922	284704	0.33	15917

HPLC of compound LB-7



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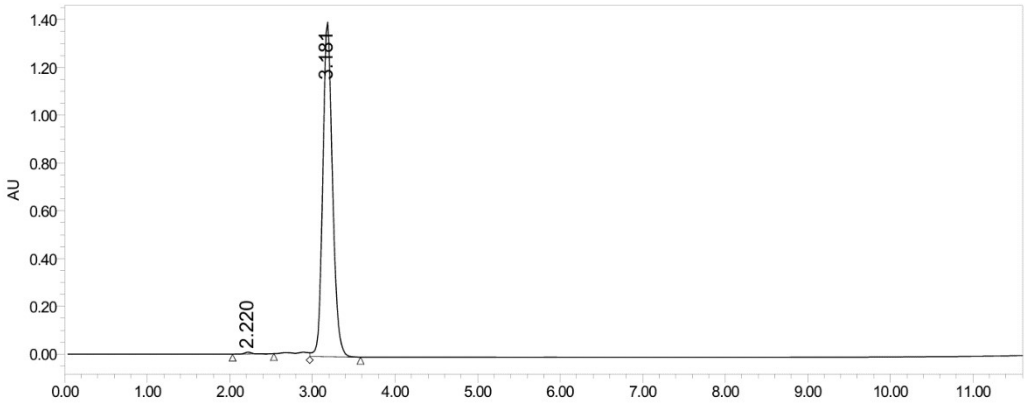
		( )	(*)	%	( )
1	W2489 ChA 254nm	2.677	32441609	95.35	3273115
2	W2489 ChA 254nm	2.961	1580489	4.65	217937

HPLC of compound LB-8



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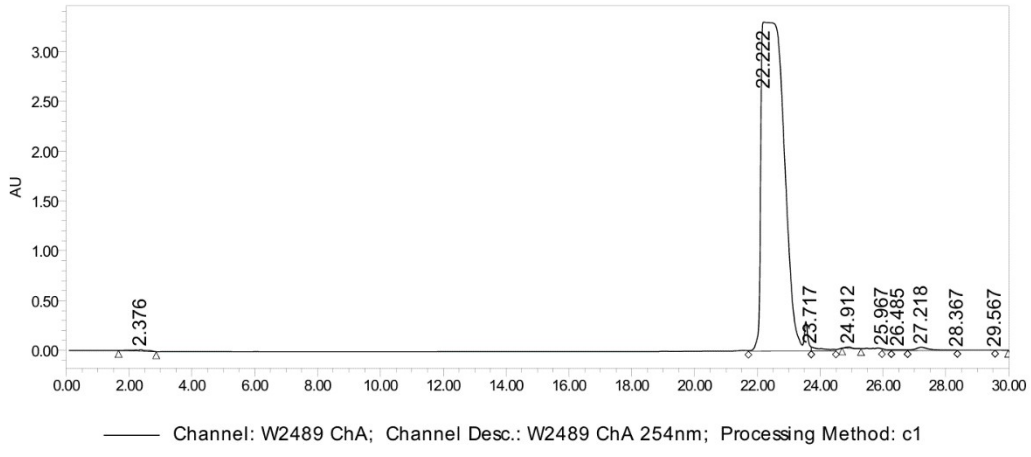
		( )	(*)	%	( )
1	W2489 ChA 254nm	2.220	43495	0.38	7214
2	W2489 ChA 254nm	3.181	11485661	99.62	1397973

HPLC of compound LB-9



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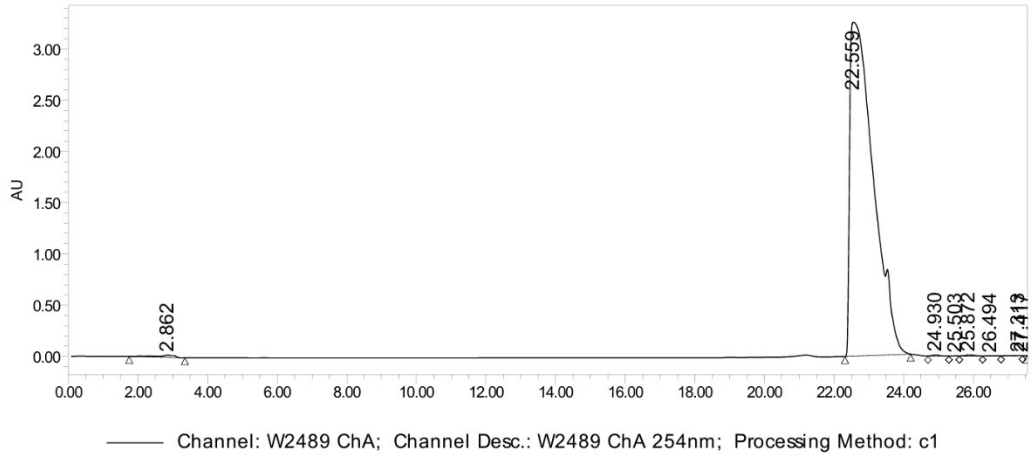
		( )	(*)	%	( )
1	W2489 ChA 254nm	2.376	457925	0.27	16211
2	W2489 ChA 254nm	22.222	167817751	97.97	3299366
3	W2489 ChA 254nm	23.717	1020242	0.60	40801
4	W2489 ChA 254nm	24.912	154757	0.09	12284
5	W2489 ChA 254nm	25.967	223766	0.13	20890
6	W2489 ChA 254nm	26.485	276829	0.16	9154
7	W2489 ChA 254nm	27.218	1182095	0.69	31962
8	W2489 ChA 254nm	28.367	156130	0.09	3391
9	W2489 ChA 254nm	29.567	10600	0.01	1061

HPLC of compound LB-10



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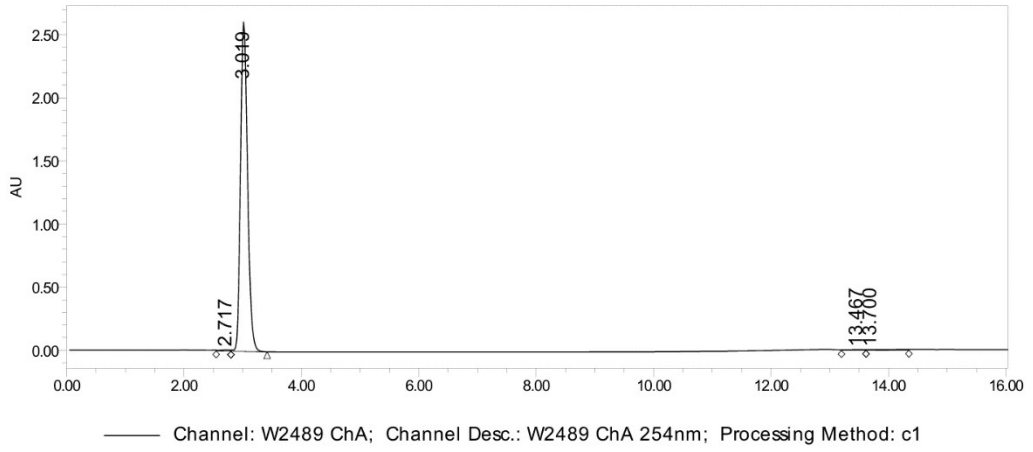
		( )	(*)	%	( )
1	W2489 ChA 254nm	2.862	717283	0.47	20740
2	W2489 ChA 254nm	22.559	152454168	99.04	3261190
3	W2489 ChA 254nm	24.930	261524	0.17	15340
4	W2489 ChA 254nm	25.503	60852	0.04	3637
5	W2489 ChA 254nm	25.872	266997	0.17	13322
6	W2489 ChA 254nm	26.494	58278	0.04	2149
7	W2489 ChA 254nm	27.313	110202	0.07	4064
8	W2489 ChA 254nm	27.417	6498	0.00	2104

HPLC of compound LB-11



:

		( )	(*)	%	( )
1	W2489 ChA 254nm	2.717	121543	0.55	10316
2	W2489 ChA 254nm	3.019	21692380	97.41	2606242
3	W2489 ChA 254nm	13.467	173711	0.78	7340
4	W2489 ChA 254nm	13.700	281909	1.27	6854

HPLC of compound LB-12

Pharmacological assay

Kinase Assays

The active CDK9/CyclinT1 kinase were purchased from Thermofisher and the kinase inhibition was detected by ADP-Glo Kinase Assay (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions. CDK9/CyclinT1, the assay was against the PDKtide substrate (KTFCGTPEYLAPEVRREPRILSEEEQEMFRDFDYIADWC) with a final concentration of 0.1 mg/mL. The final ATP concentrations of CDK9/CyclinT1 reactions was 10 μM. Maximum detection concentrations of tested compounds were 10 μM, 10 times gradient dilution. The kinase reaction of CDK9 lasted for 60 min in room temperature and finally detected by a microplate reader (PerkinElmer, Waltham, Ma, USA). The positive control AZD5438 (MedChemExpress), was diluted to 50mM in DMSO and stored at -20 °C before use.

Cell Cultures

As for cells used in this work, all the cell lines were purchased from the National Collection of Authenticated Cell Cultures, which were identified by STR. The serial numbers and conditions are as follows:

HCT116 (SCSP-5076, 37℃, 5 % CO_2, RPMI 1640 + 10 % FBS + 1 % P/S);

HT-29 (SCSP-5032, 37℃, 5 % CO_2, Mccoy`s 5A + 10 % FBS + 1 % P/S);

RKO (TCHu1116, 37℃, 5 % CO_2, MEM + 10 % FBS + 1 % P/S).

Cell cytotoxicity assay

Cytotoxicity of test compounds against different cell lines were evaluated using an CCK8 assay *in vitro*. Cells were seeded into 96-well plates at a density of 5×10^3 Cells per well and stabilized at 37℃ with 5% CO_2 for 24 h. Compounds were added to each well at various concentrations and then the cells were incubated for 48 h. The CCK8 solution (10 μL 0.5 mg/mL) was added to each well, and the cells were incubated for another 4 h. Then the absorbance of samples was measured at 450 nm. The GI_{50} values were calculated according to Logit method after getting the inhibitory rate.

Cell colony formation assay

HCT116 cells were seeded into 6-well plates with 2×10^3 cells/well at 37℃ for 24 h and then treated with compounds at various concentrations for 14 d. Then, cells were stained with crystal violet solution and clones' numbers were counted directly with naked eyes.

Annexin V/PI staining assay

Cells apoptosis was assessed using Annexin V/PI staining assay (C1052), which purchased from Beyotime Biotech Inc. HCT116 cells were seeded into 6-well plates for 24 h and then treated with compounds at various concentrations for 48 h. Then, cells were collected, washed with 500 μL annexin-binding buffer and stained with 5 μL annexin V-FITC and 5 μL PI for 15 min at 25℃. After that, the samples were analyzed by flow cytometry (BD Accuri C6 Plus, CA, USA).

ROS level assay

HCT116 cells were inoculated into 6-well plates with 3×10^5 cells/well, and then cultured for 24 h with 1.0 mL cell fluid per well. The cells were divided at various concentrations and treated with LB-1 for 48 h. Then, cells were collected, washed with 1.0 mL PBS each time, and the residual serum was washed. Dilute DCFH-DA with serum-free 1640 medium at a ratio of 1: 1000 to a final concentration of 10 μM for later use. Avoid light and add 1.0 mL DCFH-DA diluent to each well. Keep away from light and incubate in a 37 °C cell incubator for 20 min.

Serum-free 1640 culture medium was washed with 1 mL each time for three times to completely remove DCFH-DA that did not enter the cells. Add 1.0 mL of 0.25% trypsin without EDTA to each well to digest the cells in each well, stop the digestion, collect the cells in each well in an EP tube filled with corresponding culture solution, centrifuge at 2000 rpm for 5 min, discard the supernatant, and resuspend the cells with 0.5 mL PBS. After that, the samples were analyzed by flow cytometry (BD Accuri C6 Plus, CA, USA).

GreenNuc living cell Caspase 3 activity assay

HCT116 cells were seeded at a density of 3×10^5 cells per well and treated with various concentrations of compounds for 48 h. Subsequently, the cells were incubated with GreenNuc™ Caspase 3 substrate and finally analyzed by flow cytometry (BD Accuri C6 Plus, CA, USA).

Liver microsomal stability assay

The empty 'Incubation' plates T60 and NCF60 were pre-warmed for 10 min. The liver microsome (Human, Corning, Lot No.38295)) was diluted to 0.59 mg/mL as working solutions in 100 mM potassium phosphate buffer. The working solutions (445 μL) were transferred into pre-warmed 'Incubation' plates T60 and NCF60. Then, the two plates were shaken at 37°C for 10 min. After liver microsomes (54 μL) were transferred to another black plate, the compound working solution (5 μL) with a concentration of 100 μM was added and mixed three times thoroughly. To the black plate, NADPH solution (6 μL) (NADPH powder which purchased from Chem-Impex International) and quenching solution (180 μL) which contained 200 ng/mL tolbutamide and 200 ng/mL labetalol in acetonitrile were added sequentially. The buffer (50 μL) was added to the NCF60 plate. The mixture was mixed three times thoroughly, and then the plate was incubated at 37 °C for 60 min. 44 μL of NADPH cofactor was added to the T60 plate, and then the plate was incubated at 37 °C for 60 min as well. At 5, 15, 30, 45, and 60 min, 180 μL quenching solution was added to 'Quenching' plates, mixed once, and serially transferred 60 μL sample from T60 plate per time point to 'Quenching' plates. All sampling plates were shaken for 10 min, then centrifuged at 4000 rpm for 20 min, and supernatant (80 μL) was removed into pure water (240 μL) and mixed for 10 min by plate shaker. Each bioanalysis plate was sealed and shaken for 10 min prior to LC-MS/MS analysis.

Plasma protein binding assay

The pooled frozen human plasma was thawed in a water bath at 37 °C prior to the experiment. LB-1 to be tested was diluted into plasma preheated to 37℃, and the final concentration was 1 μM and the final concentration of warfarin in plasma was 2μM. Assemble the pretreated dialysis membrane into a dialysis plate, and add 120μL of the compound to be tested prepared by plasma to the administration surface of the osmotic membrane in each dialysis hole (warfarin prepared by 120 μL of plasma to the control group), and add 120 μL of 0.002% Tween -PB to the receiving surface (120μL of 0.002% Tween -PB to the control group), and take 20μL of the final concentration samples of the compound to be tested and the control compound to 96-hole sample plates. The remaining samples were incubated for 6 h at 37℃ with constant temperature oscillation. After 6 h incubation, 20 μL of dialysis receiving surface, administration surface and stability sample were taken to obtain sample B, sample A and 6 h stability sample. Add corresponding blank plasma or 0.002% Tween -PB into sample B, sample A, sample T0 and 6

h stable sample respectively, so that the volume ratio of plasma to buffer in each sample hole is 1:1. Add 300 μL precipitant containing internal standard into the sample hole, mix well and centrifuge. Add 150 μL of ultrapure water into the corresponding sample wells of a 96-well sample plate, take 150 μL of supernatant into the sample wells, mix well, and then conduct LC-MS(LC-MS-005) sample analysis.

Cytochrome P450 inhibition assay

Cytochrome P450 inhibition was evaluated in human liver microsomes (0.253 mg/mL) using five probe substrates (CYP1A2, CYP2D6, Quinidine; and CYP3A4, Ketoconazole.) in the presence of compounds (10 μM). After preincubation for 10 min at 37 °C, an NADPH-regenerating system was added. After the mixed system was incubated for 15 min at 37 °C, the reaction was stopped by adding 400 μL of cold stop solution (200 ng/mL tolbutamide and 200 ng/mL labetalol in acetonitrile). Then, the mixture was centrifuged, and the supernatant was analyzed by LC-MS/MS.

The human liver microsome was purchased from BIOIVT.

***In vivo* PK Study**

The SPF grade SD rats (Six in total, male, three for *p.o.* and three for *i.v.*) were purchased from Changchun Institute of Biological Products Co., Ltd. The animals were housed at a temperature of 20 ± 2 °C with a 12 h light-dark cycle and provided with a standard laboratory chow diet and *ad libitum* access to food and water besides fasted SD rats. The fasted SD rats removed food 10-14 hours before administration but drank freely and resumed feeding 4 hours after administration. All animals were sacrificed by cervical dislocation after isoflurane inhalation anesthesia. All animal experiments involved in this work had been approved by Experimental Animal Ethics Committee of Shenyang Pharmaceutical University. The specific files are presented in the email attachment. We have adhered to the ARRIVE guidelines in this work.

SD rats were received 2 mg/kg via intravenously injection (*i.v.*). The time points for blood sample collection were 0.08 (only for *iv*), 0.25, 0.5, 1, 2, 4, 8, and 24 h post dose (n = 3). For each point, 300 μL of collected blood was transferred into a new EP tube containing 10 μL of heparin as an anticoagulant and centrifuged at 4000 rpm for 10 min. Plasma (50 μL) was separated and transferred to another EP tube, and 200 μL of methanol containing predetermined IS was added to the plasma to precipitate plasma proteins. After being vortexed for 1 min, the mixture was centrifuged at 12,000 g for 10 min to finally obtain the supernatant for LC-MS/MS analysis.

SD rats were received 10 mg/kg via oral administration(*p.o.*). The time points for blood sample collection were 0.25 (only for *p.o*), 0.5 h，1 h，2 h，4 h，6 h，8 h，24 h post dose (n = 3). For each point, 300 μL of collected blood was transferred into a new EP tube containing 10 μL of heparin as an anticoagulant and centrifuged at 4000 rpm for 10 min. Plasma (50 μL) was separated and transferred to another EP tube, and 200 μL of methanol containing predetermined IS was added to the plasma to precipitate plasma proteins. After being vortexed for 1 min, the mixture was centrifuged at 12,000g for 10 min to finally obtain the supernatant for LC-MS/MS analysis.

The measures to reduce animal pain and injury were using ketamine or opioid therapy.

Molecular docking study

The CDK9 (PDB code: 4BCF) crystal structure was downloaded from protein data bank (https://www.rcsb.org/) and processed with the Protein Preparation Wizard in the Schr¨odinger

suite. The protein structures were adjusted and modified, followed by adding hydrogen atoms, deleting solvent water molecules, and defining right bonds orders using Prime. The protonation and tautomeric states of Asp, Lys, and His were assigned at pH 7.4 state. Afterward, all hydrogen atoms of CDK9 complexes were optimized with OPLS_2005 force field, which minimized and converged heavy atoms to an RMSD of 0.3. The selected inhibitors were prepared by using LigPrep from the Schr¨odinger suite with the OPLS_2005 force field. The structures of inhibitors were also adjusted and modified, followed by adding all hydrogen atoms, checking the bond order and atom types. Receptor grids were generated before docking with the active site determined by literature[1]. The prepared protein–ligand complex was imported into Glide 9.7, which was defined as the binding site. The size of the docking grid box was 20 Å × 20 Å × 20 Å. Based on the OPLS_2005 force field, the grid of CDK9 crystal structure was generated. The standard precision (SP) mode was set for docking studies without constrained binding to gain results.

Reference

1 Hole AJ, Baumli S, Shao H, et al. Comparative structural and functional studies of 4-(thiazol-5-yl)-2-(phenylamino) pyrimidine-5-carbonitrile CDK9 inhibitors suggest the basis for isotype selectivity. *J Med Chem.* 2013, 56, 3,660-70.