Supporting Information

Difluoromethyl-1,3,4-oxadiazoles are selective, mechanism-based, and essentially irreversible inhibitors of histone deacetylase 6

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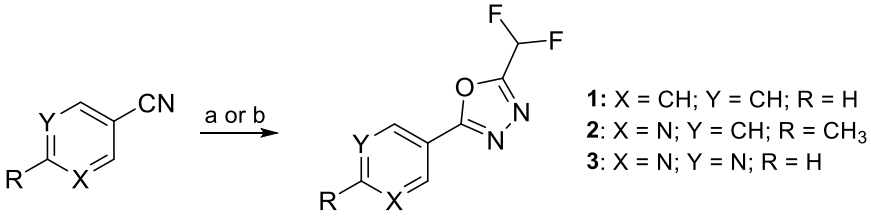
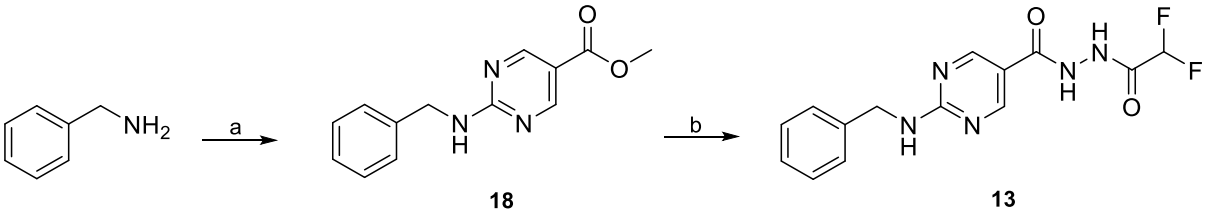
Content

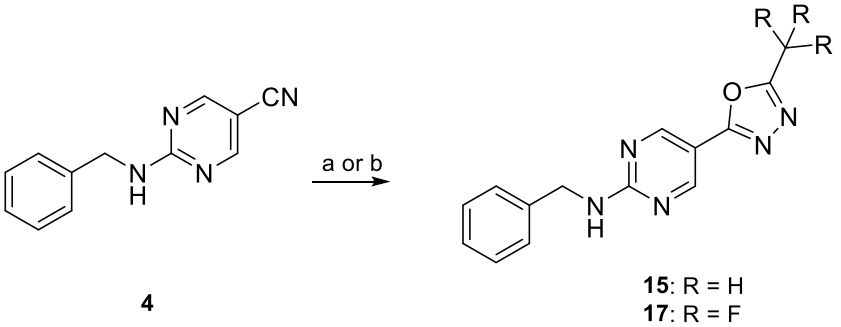
1 Supplementary Schemes, Figures, Equations and Tables



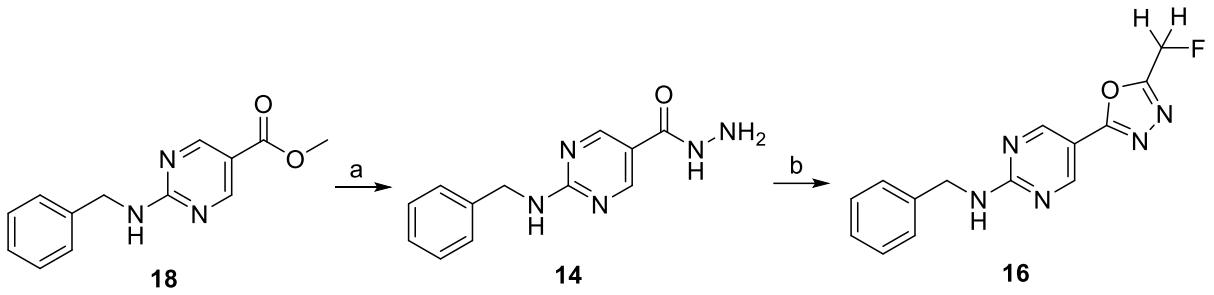
Scheme S1. Synthesis of HDAC6 inhibitor fragments. a) i: NaN_3, NH_4Cl, $LiCl \cdot H_2O$, DMF, 100 °C, 150 W, 24 h; ii: DFAA, DCM, rt., 24 h (**1**, **3**); b) i: NaN_3, NH_4Cl, LiCl, DMF, 100 °C, 18 h; ii: difluoroacetic anhydride (DFAA), toluene, 70 °C, 18 h (**2**).



Scheme S2. Synthesis of the acylhydrazide **13**. a) Methyl 2-chlorpyrimidine-5-carboxylate, DIPEA, EtOH, 90 °C, 18 h; b) i: hydrazine monohydrate, MeOH, 70 °C, 3 h; ii: DFAA, DMF, 70 °C, 1 h.



Scheme S3. Synthesis of the trifluoromethyl-1,3,4-oxadiazole (**17**) and methyl-1,3,4-oxadiazole (**15**) analogs. a) i: NaN_3, NH_4Cl, LiCl, DMF, 100 °C, 18 h; ii: trifluoroacetic anhydride, toluene, 70 °C, 18 h (**14**); b) i: NaN_3, NH_4Cl, LiCl, DMF, 100 °C, 18 h; ii: acetic anhydride, toluene, 70 °C, 18 h; iii: K_2CO_3, $MeOH/H_2O$ (**15**).



Scheme S4. Synthesis of hydrazide **14** and monofluoromethyl-1,3,4-oxadiazole **16**. a) hydrazine monohydrate, MeOH, 70 °C, 3 h; b) i: monofluroacetic acid, DMF, 70 °C, 3 h; ii: Burgess reagent, THF, 60 °C, 18 h.

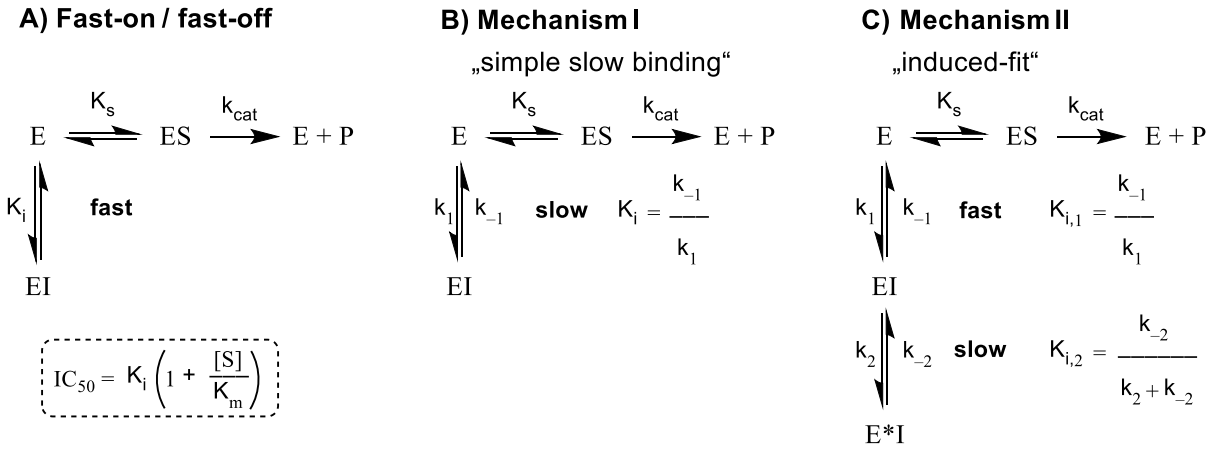


Figure S1. Representative examples of different kinetic mechanisms of enzyme inhibition, including the relationships between the respective association and dissociation rate constants (e. g., k_1 & k_{-1}) and the related equilibrium dissociation constant K_i. **A)** Fast-on/fast-off binding kinetics. For competitive fast-on/fast-off inhibitors the half maximum inhibitory concentration (IC_{50}) and the K_i are directly related by the Cheng-Prusoff equation[1]; **B)** slow-binding Mechanism I: single-step slow binding, k_1 & k_{-1} are inherently slow; **C)** slow-binding Mechanism II: two-step slow binding. Initially, inhibitor and enzyme form an encounter complex [EI] that subsequently slowly undergoes isomerization to a binary enzyme inhibitor complex [E*I].[2]

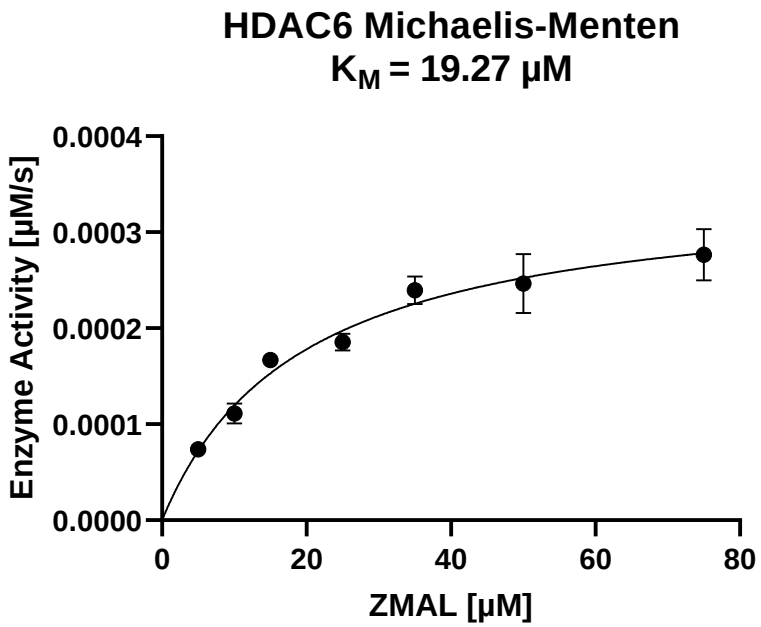
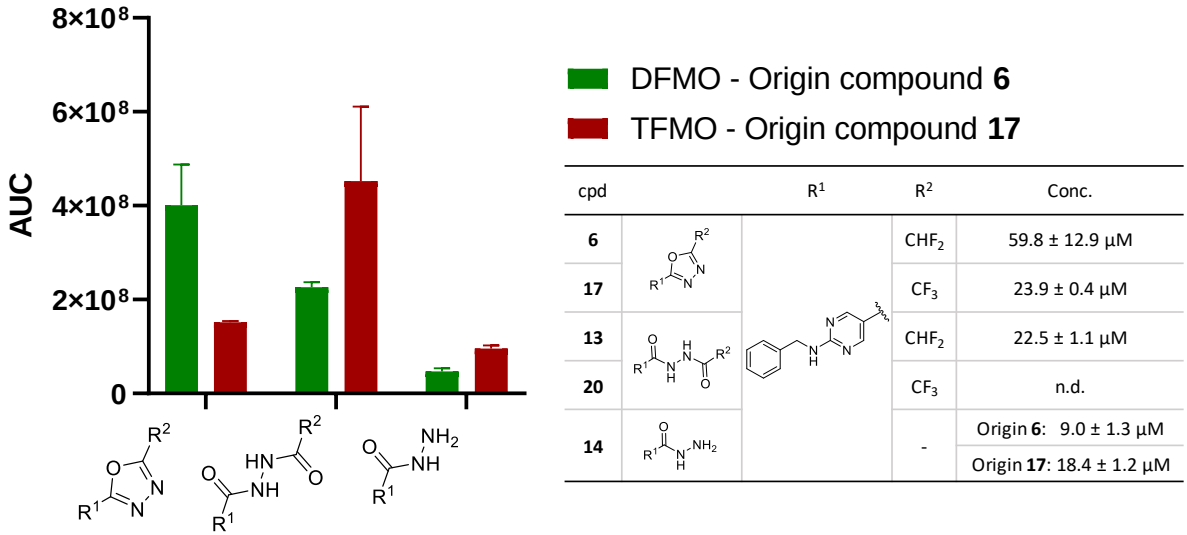


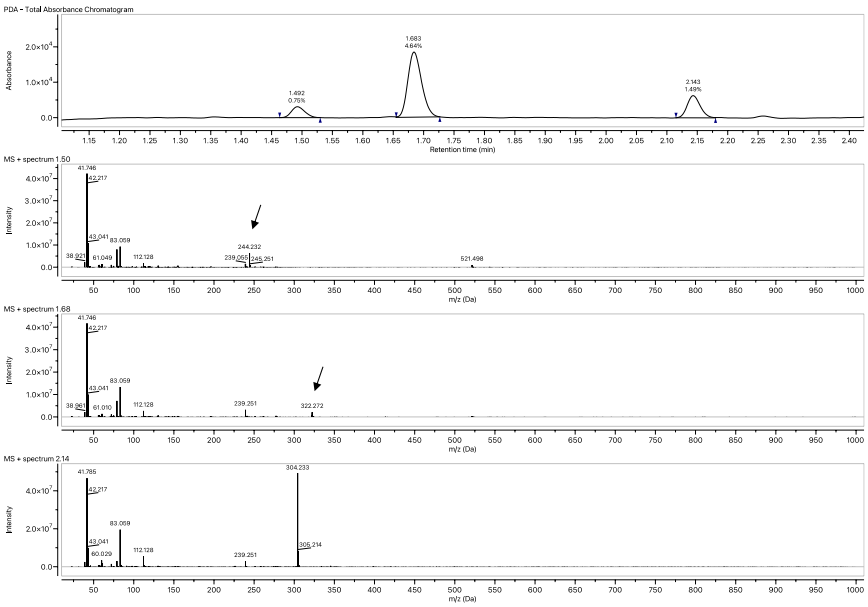
Figure S2. Michaelis-Menten constant K_M determination for HDAC6 using a series of substrate concentrations. Steady-state velocities [µM*s^{-1}] (mean ± SD) were plotted against the corresponding substrate concentrations [µM] and fitted to the Michaelis-Menten equation yielding the Michaelis-Menten constant: K_M HDAC6 = 19.27 µM. Experiment was performed in triplicates.

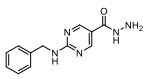


cpd		R¹	R²	Conc.
6			CHF_2	59.8 ± 12.9 µM
17			CF_3	23.9 ± 0.4 µM
13			CHF_2	22.5 ± 1.1 µM
20			CF_3	n.d.
14			-	Origin **6**: 9.0 ± 1.3 µM Origin **17**: 18.4 ± 1.2 µM

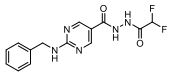
Figure S3. Quantified hydrolysis products from LC-UV-MS analysis after over night incubation of the respective compound (100 µM) with HDAC6. Experiments were performed in triplicates. DFMO: difluoromethyl-1,3,4-oxadiazole; TFMO: trifluoromethyl-1,3,4-oxadiazole. n.d.: not determined.

A

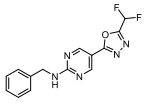




14: $[M+H]^+$: 244.28

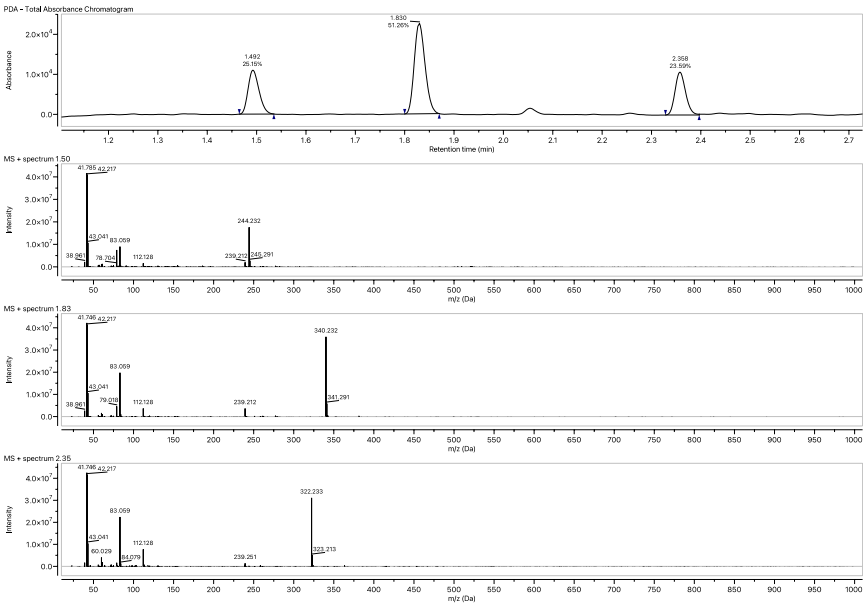


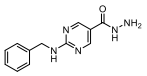
13: $[M+H]^+$: 322.30



6: $[M+H]^+$: 304.28

B





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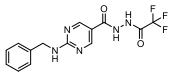

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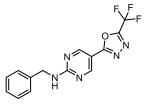
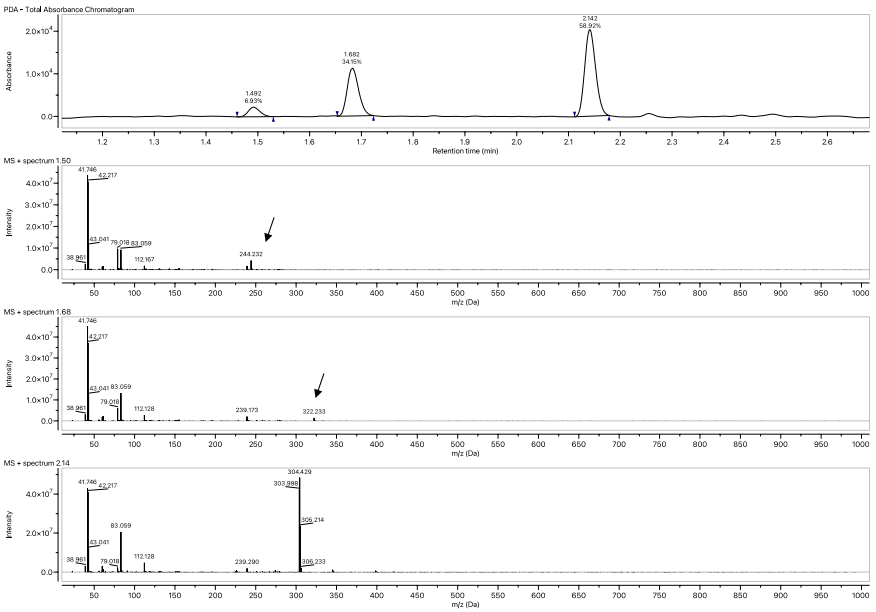
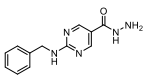


17: $[M+H]^+$: 322.27

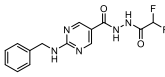
Figure S4. Representative UV and related mass traces from two independent LC-UV-MS experiments. **A**: Compound **6** was incubated with HDAC6 overnight; **B**: Compound **17** was incubated with HDAC6 overnight; x axis: retention time in mins (chromatogram), m/z ration (mass spectras), y axis: intensity in Absorbance Units (AU). Experiments were performed in triplicates.

A

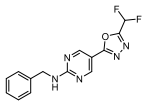




14: $[M+H]^+$: 244.28

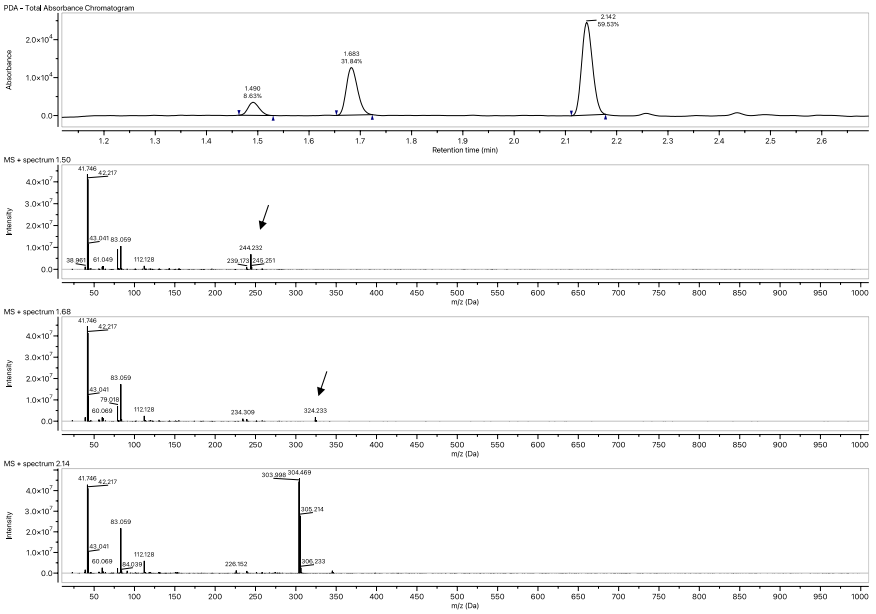


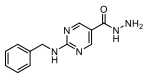
13: $[M+H]^+$: 322.30



6: $[M+H]^+$: 304.28

B





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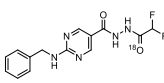

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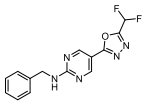
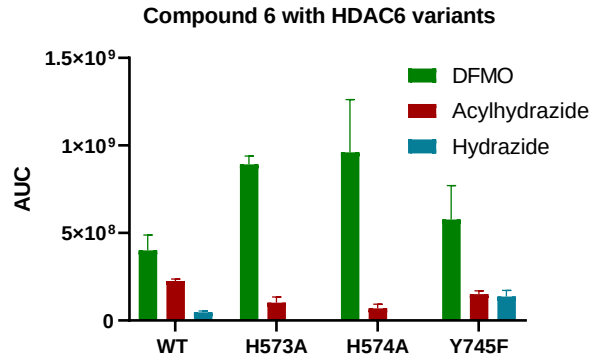


6: $[M+H]^+$: 304.28

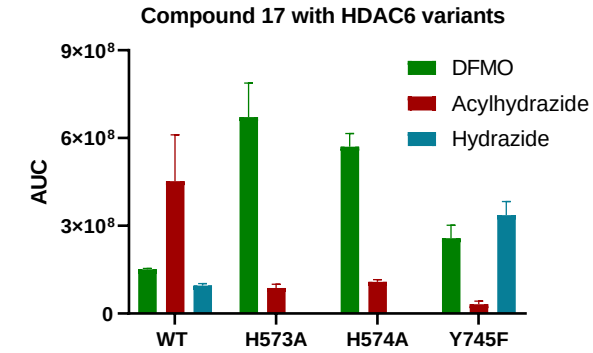
Figure S5. Representative UV and related mass traces of LC-UV-MS experiments. **A**: Compound **6** was incubated with HDAC6 overnight in $H_2{}^{16}O$ water; **B**: Compound **6** incubated with HDAC6 overnight in $H_2{}^{18}O$ water; x axis: retention time in mins (chromatogram), m/z ration (mass spectras), y axis: intensity in Absorbance Units (AU). Experiments were performed in triplicates.

A



HDAC6	Hydrolysis products from compound 6	
	Acylhydrazide **13**	Hydrazide **14**
WT	22.5 ± 1.11 µM	9.0 ± 1.3 µM
H573A	10.2 ± 3.2 µM	n.o.
H574A	6.9 ± 2.3 µM	n.o.
Y745F	14.9 ± 1.9 µM	26.2 ± 6.8 µM

B



HDAC6	Hydrolysis products from compound 17	
	Acylhydrazide **20**	Hydrazide **14**
WT	n.d.	18.6 µM ± 1.2 µM
H573A	n.d.	n.o.
H574A	n.d.	n.o.
Y745F	n.d.	64.4 ± 8.9 µM

Figure S6. Quantified hydrolysis products from LC-UV-MS analysis after over night incubation of the respective compound (100 µM) with various HDAC6 mutants (wild-type (WT), H573A, H574A, Y745F). Experiments were performed in triplicates. n.d.: not determined; n.o.: not observed.

$$[P] = v_{ss}t + \frac{v_{in} - v_{ss}}{k_{obs}}\left(1 - e^{-k_{obs}t}\right) \qquad \text{(Eq. 1)}$$

Equation 1. Time-dependent product formation for inhibitors showing slow-binding Mechanism I&II. [P]: amount of generated AMC; v_{ss}: steady-state velocity (product formation); t: time; v_{in}: initial velocity (product formation); k_{obs}: apparent first-order rate constant for the conversion from v_{in} to v_{ss}.

$$k_{obs} = k_{-1} + k_1\left(1 + \frac{[S]}{K_M}\right)[I] \qquad \text{(Eq. 2)}$$

Equation 2. The single-step slow-binding Mechanism I results in a linear relationship between k_{obs} and inhibitor concentration. k_{-1}: dissociation rate constant; k_1: association rate constant; [S]: substrate concentration; K_M: Michelis-Menten constant; [I]: inhibitor concentration.

$$k_{obs} = k_{-2} + \frac{k_2}{[I] + K_{i,1}\left(1 + \frac{[S]}{K_M}\right)}[I] \qquad \text{(Eq. 3)}$$

Equation 3. The two-step slow-binding Mechanism II results in a hyperbolic relationship between k_{obs} and inhibitor concentration. k_{-2}: secondary dissociation rate constant; k_2: secondary association rate constant; $K_{i,1}$: equilibrium dissociation constant of the enzyme inhibitor encounter complex [EI].

Table S1: Data collection and refinement statistics[a]

	HDAC6 CD2–**13** Complex
Space group	$P2_12_12_1$
a,b,c (Å)	74.60, 92.30, 96.60
α, β, γ (°)	90.00, 90.00, 90.00
R_{merge}[b]	0.210 (0.706)
R_{pim}[c]	0.084(0.289)
$CC_{1/2}$[d]	0.993(0.851)
Redundancy	1.9
Completeness (%)	99.5(94.3)
I/σ	7.3(2.5)
Refinement	
Resolution (Å)	36.216–2.00 (2.07–2.00)
No. reflections	90179 (8857)
R_{work}/R_{free}[e]	0.185/0.223 (0.228/0.0.266)
Number of Atoms[f]	
Protein	5469
Ligand	52
Solvent	424
Average B factors (Å^2)	
Protein	15
Ligand	20
Solvent	20
RMS Deviations	
Bond lengths (Å)	0.03
Bond angles (°)	1.4
Ramachandran Plot[g]	
Favored	97.01
Allowed	2.99
Outliers	0.00

[a]Values in parentheses refer to the highest-resolution shell of data.

[b]$R_{merge} = \sum_h\sum_i|I_{i,h} - \langle I\rangle_h|/\sum_h\sum_i I_{i,h}$, where $\langle I\rangle_h$ is the average intensity calculated for reflection *h* from *i* replicate measurements.

[c]$R_{p.i.m.} = (\sum_h(1/(N-1))^{1/2}\sum_i|I_{i,h} - \langle I\rangle_h|)/\sum_h\sum_i I_{i,h}$, where N is the number of reflections and $\langle I\rangle_h$ is the average intensity calculated for reflection *h* from replicate measurements.

[d]Pearson correlation coefficient between random half-datasets.

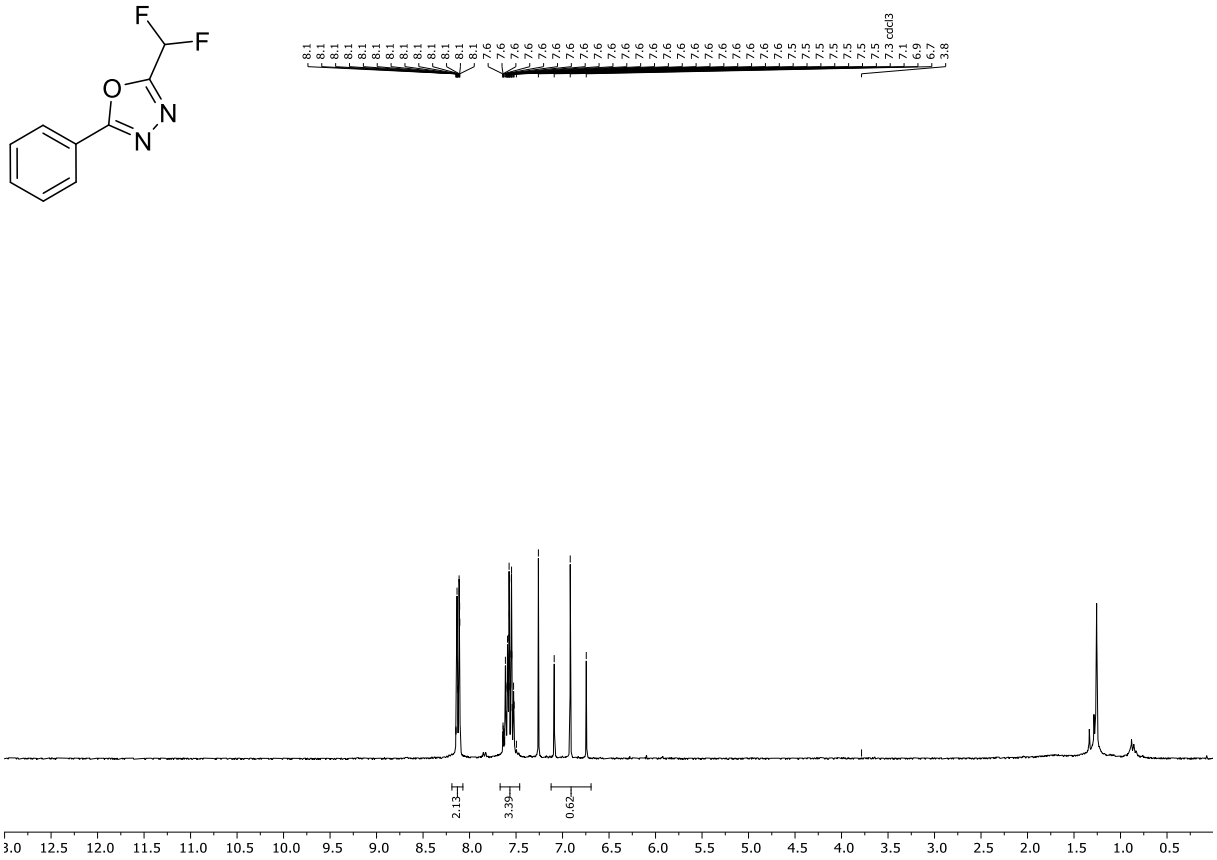
[e]$R_{work} = \sum||F_o| - |F_c||/\sum|F_o|$ for reflections contained in the working set. $|F_o|$ and $|F_c|$ are the observed and calculated structure factor amplitudes, respectively. R_{free} is calculated using the same expression for reflections contained in the test set held aside during refinement.

[f]Per asymmetric unit.

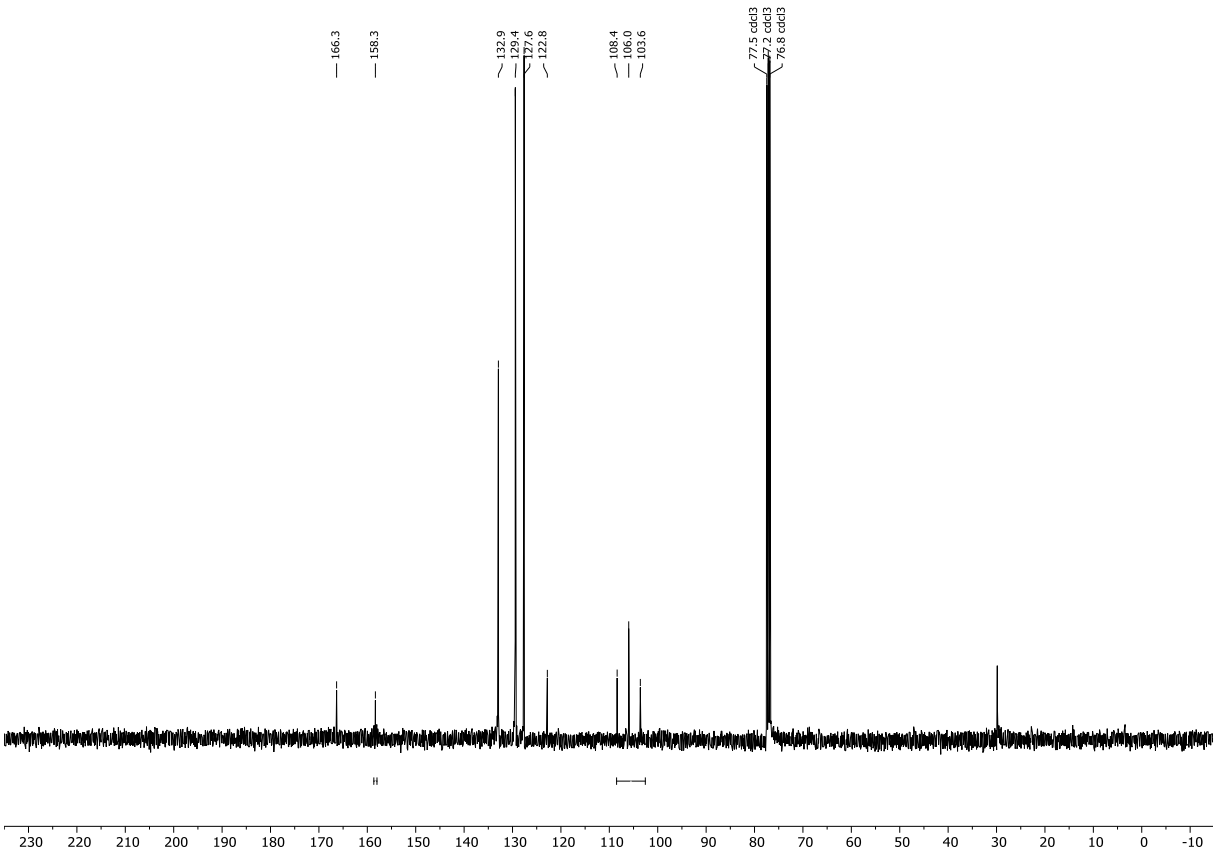
[g]Calculated with MolProbity.

2 NMR Data of synthesized compounds

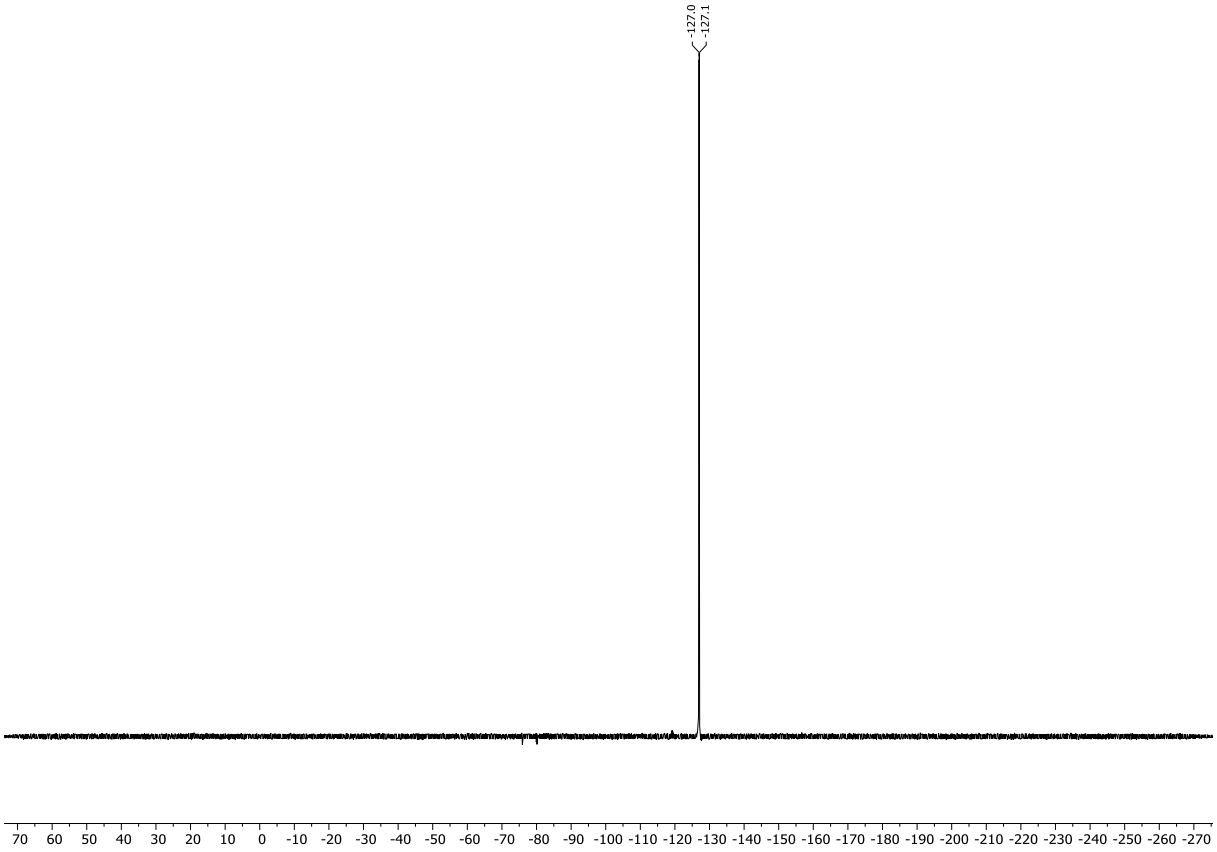
^{1}H NMR spectrum of **1** (300 MHz, $CDCl_3$)



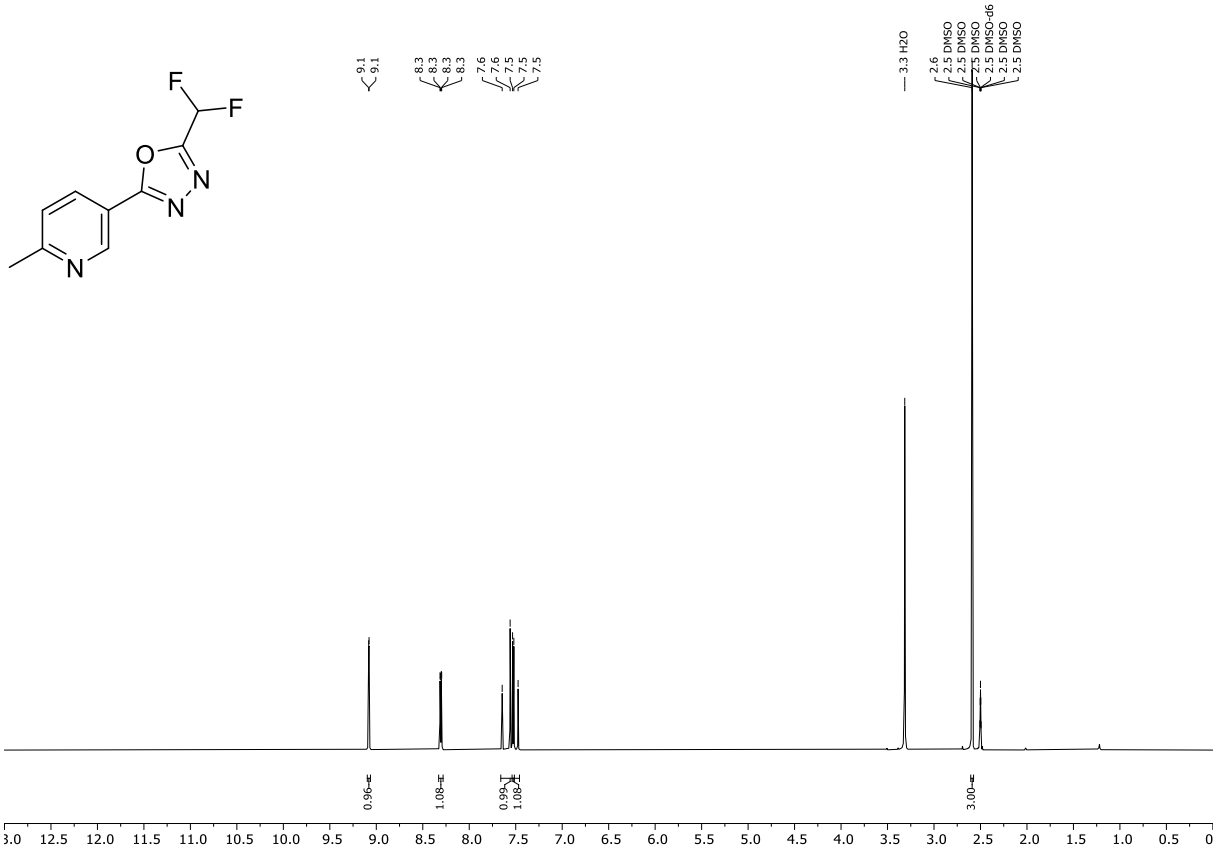
^{13}C NMR spectrum of **1** (101 MHz, $CDCl_3$)



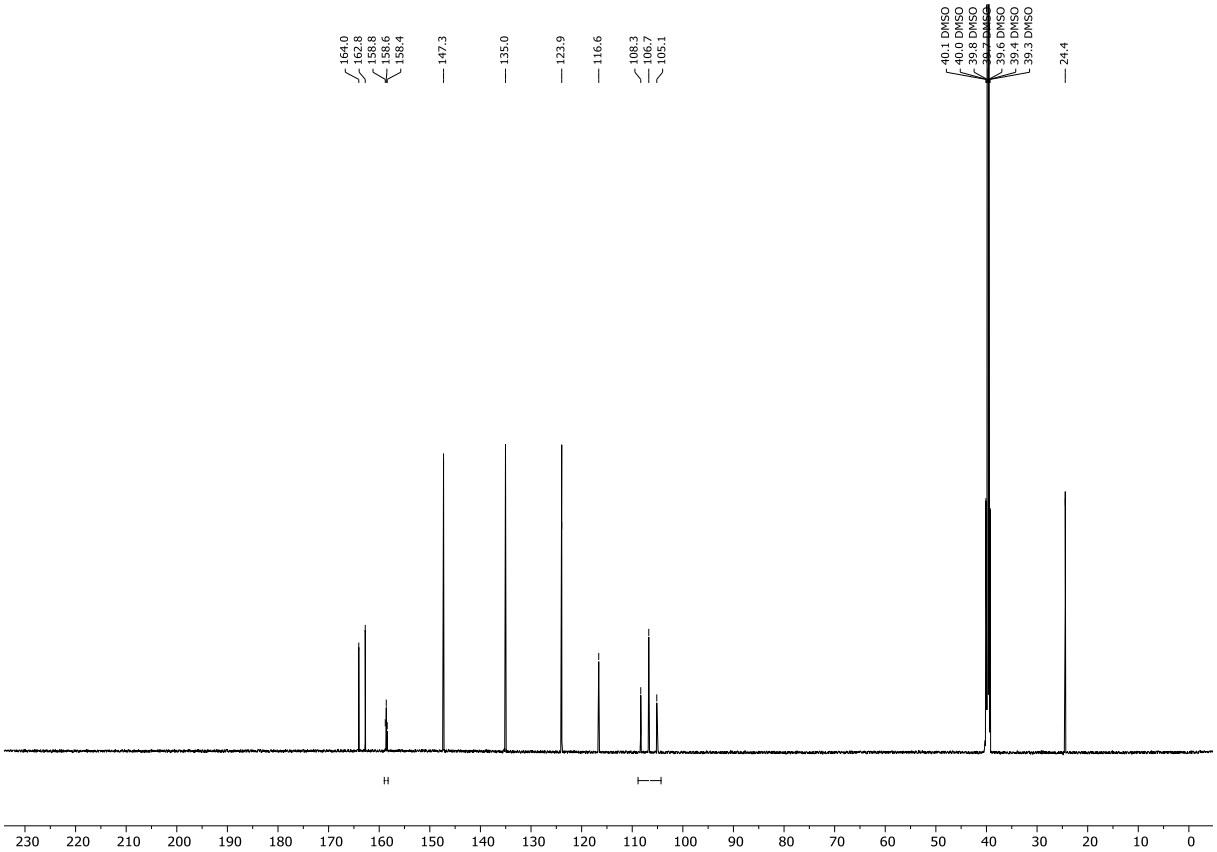
^{19}F NMR spectrum of **1** (377 MHz, $CDCl_3$)



^{1}H NMR spectrum of **2** (600 MHz, DMSO-d_6)

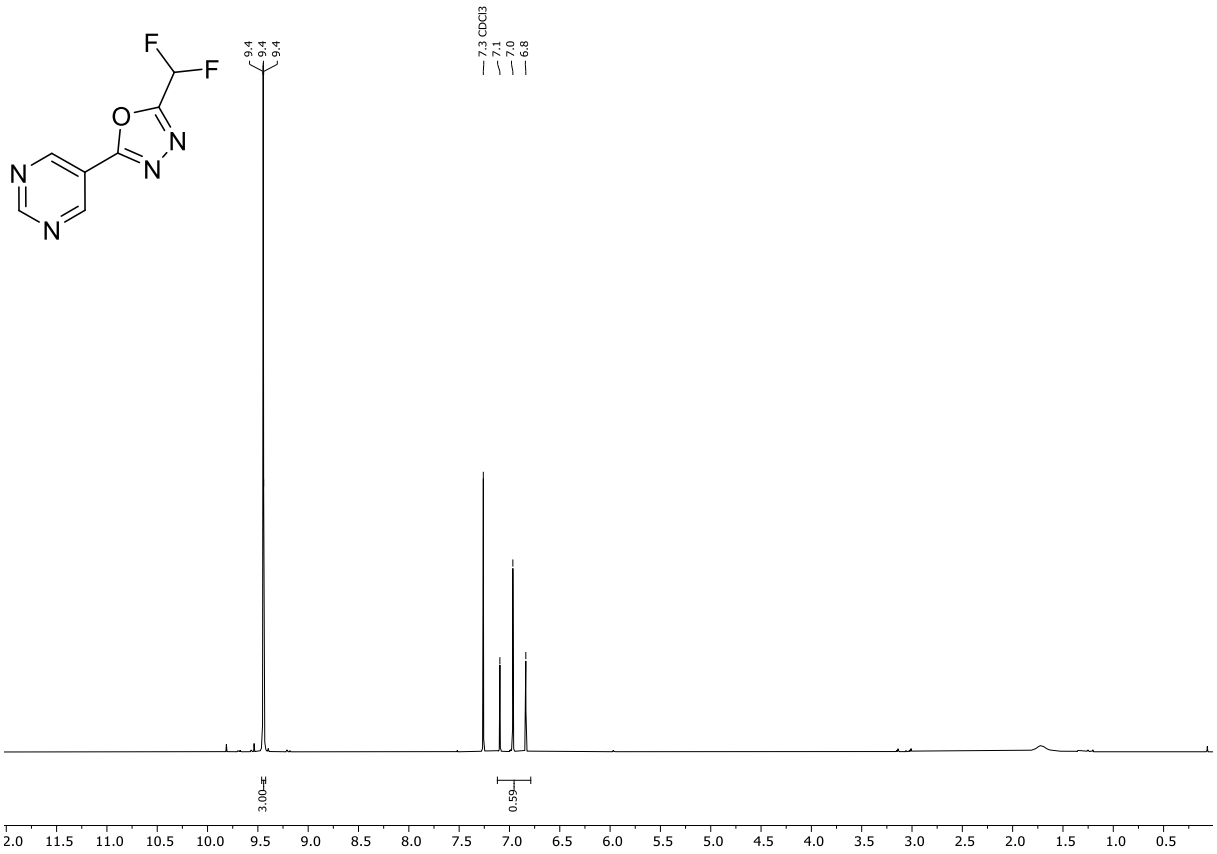


^{13}C NMR spectrum of **2** (151 MHz, DMSO-d_6)

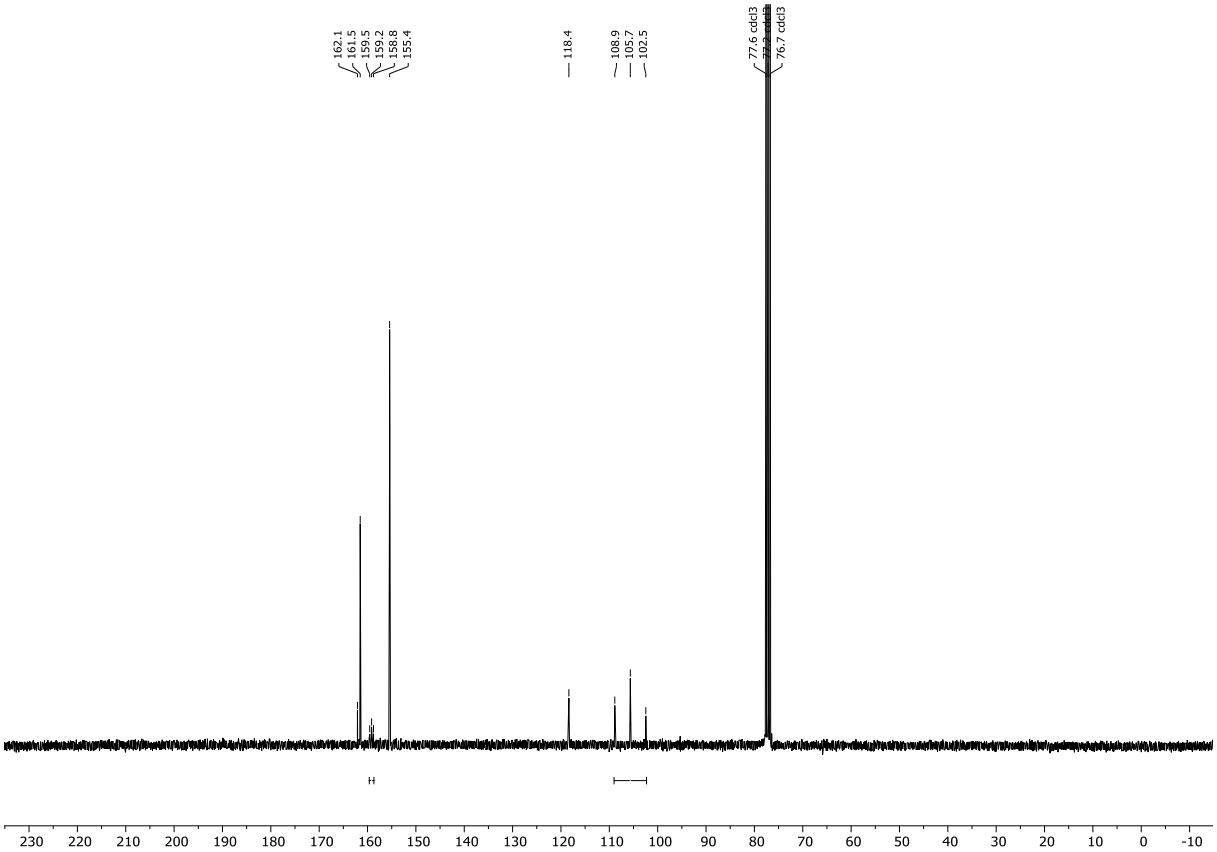


^{19}F NMR spectrum of **2** (565 MHz, DMSO-d_6)

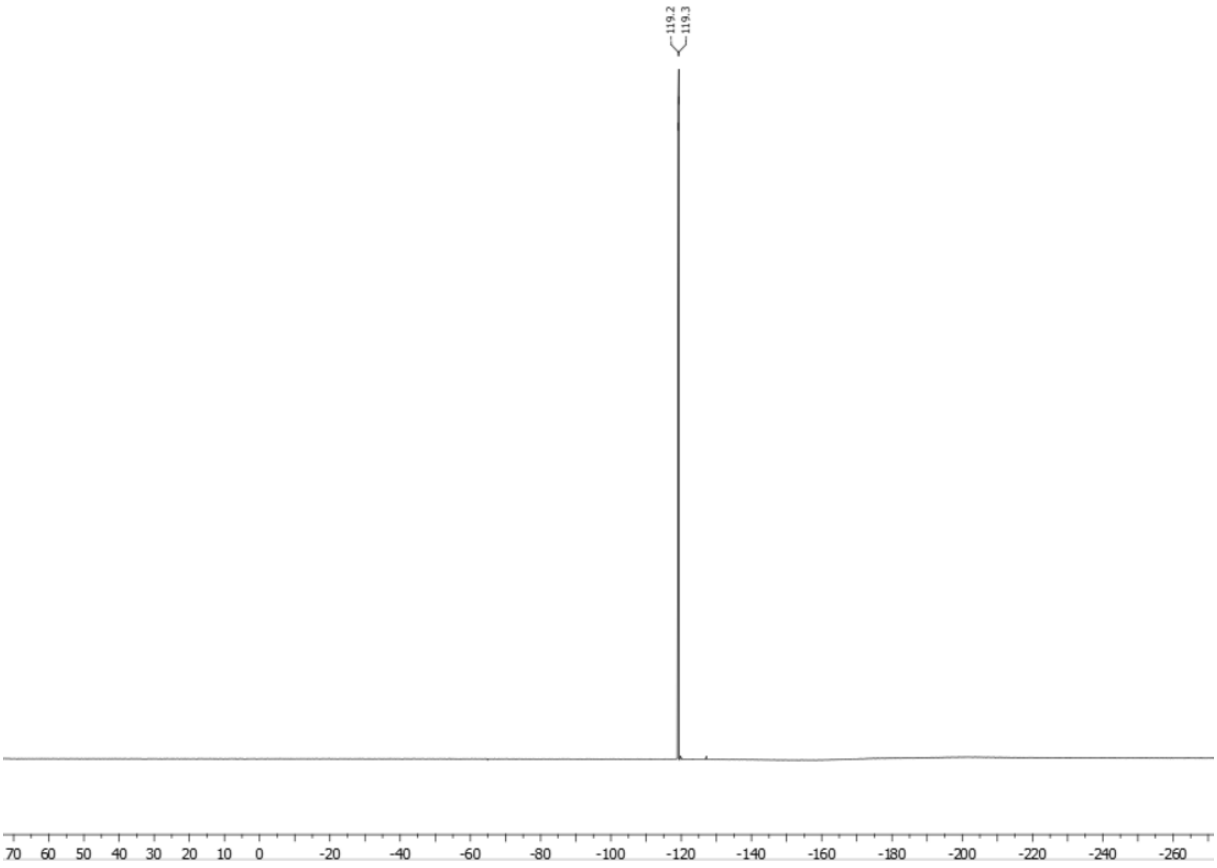
^{1}H NMR spectrum of **3** (400 MHz, $CDCl_3$)



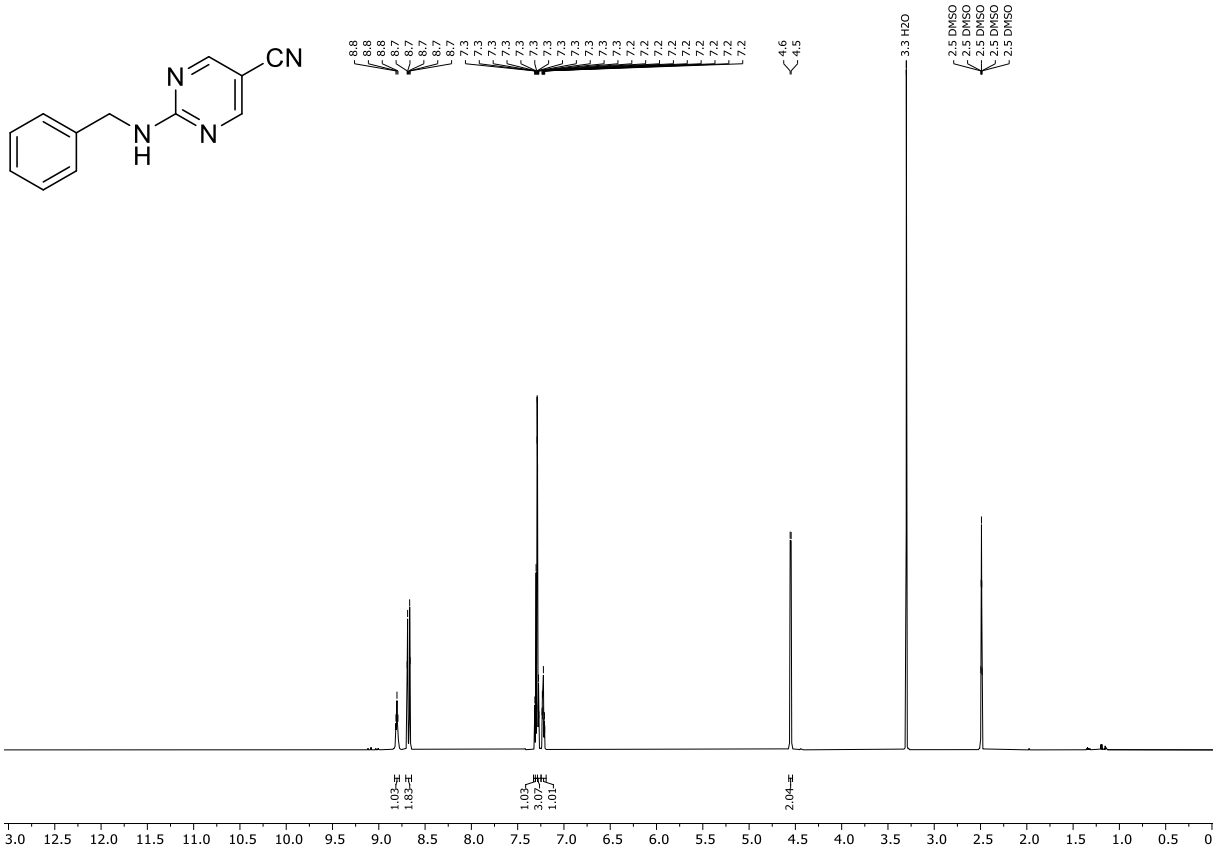
^{13}C NMR spectrum of **3** (75 MHz, $CDCl_3$)



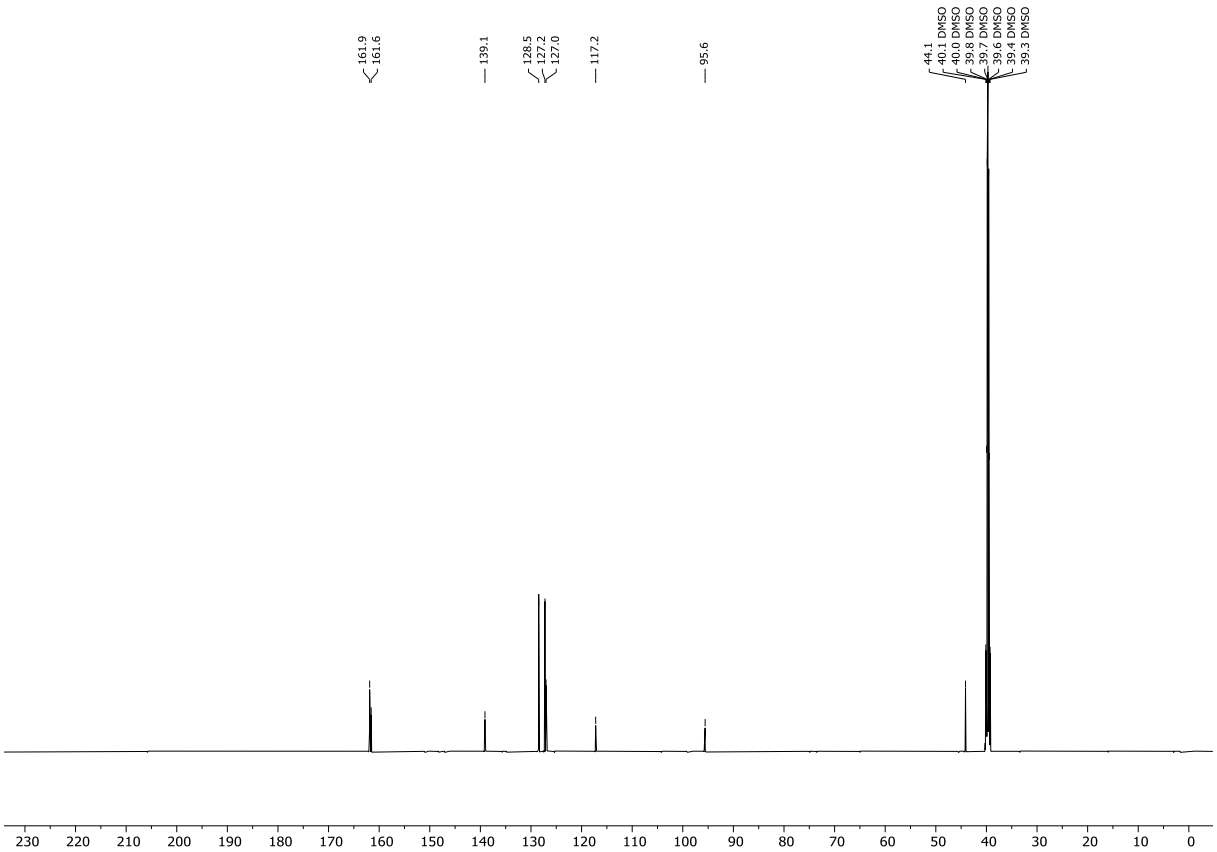
^{19}F NMR spectrum of **3** (377 MHz, $CDCl_3$)



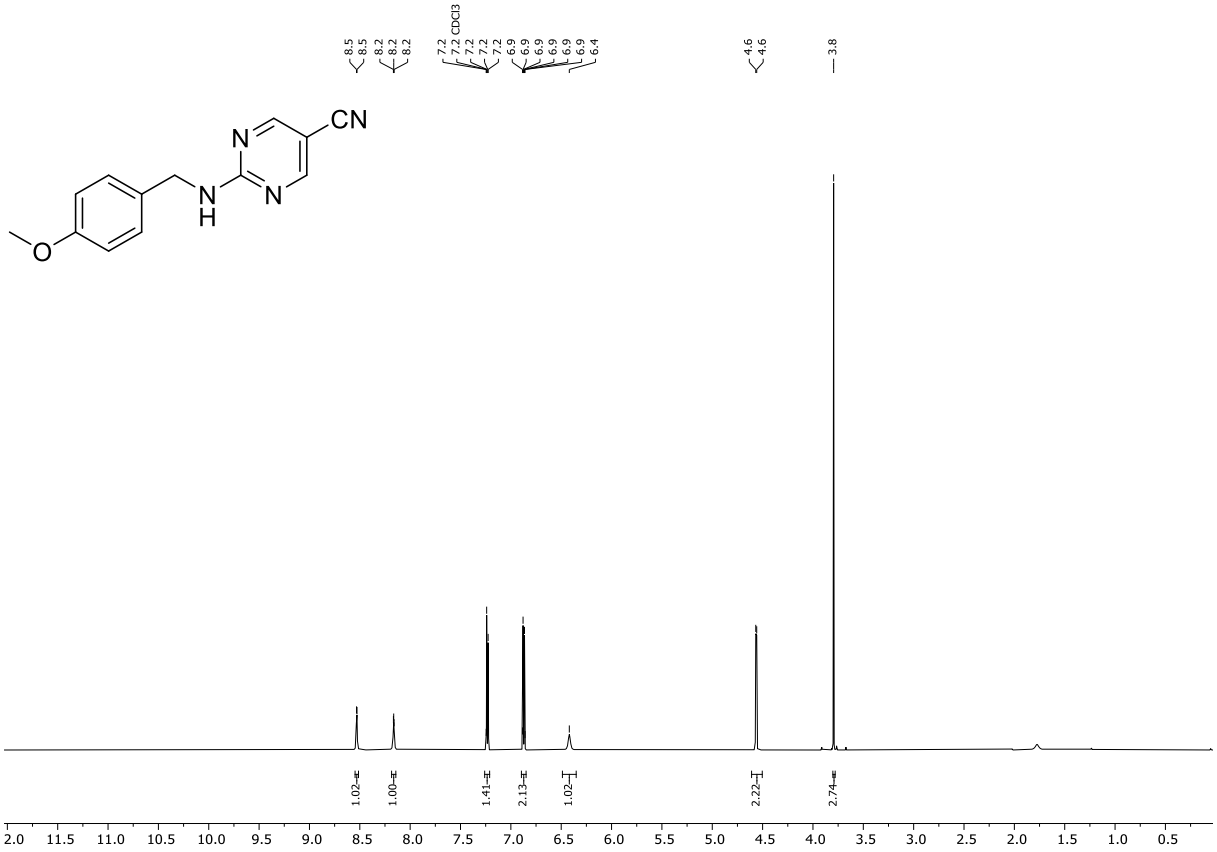
^{1}H NMR spectrum of **4** (600 MHz, DMSO-d_6)



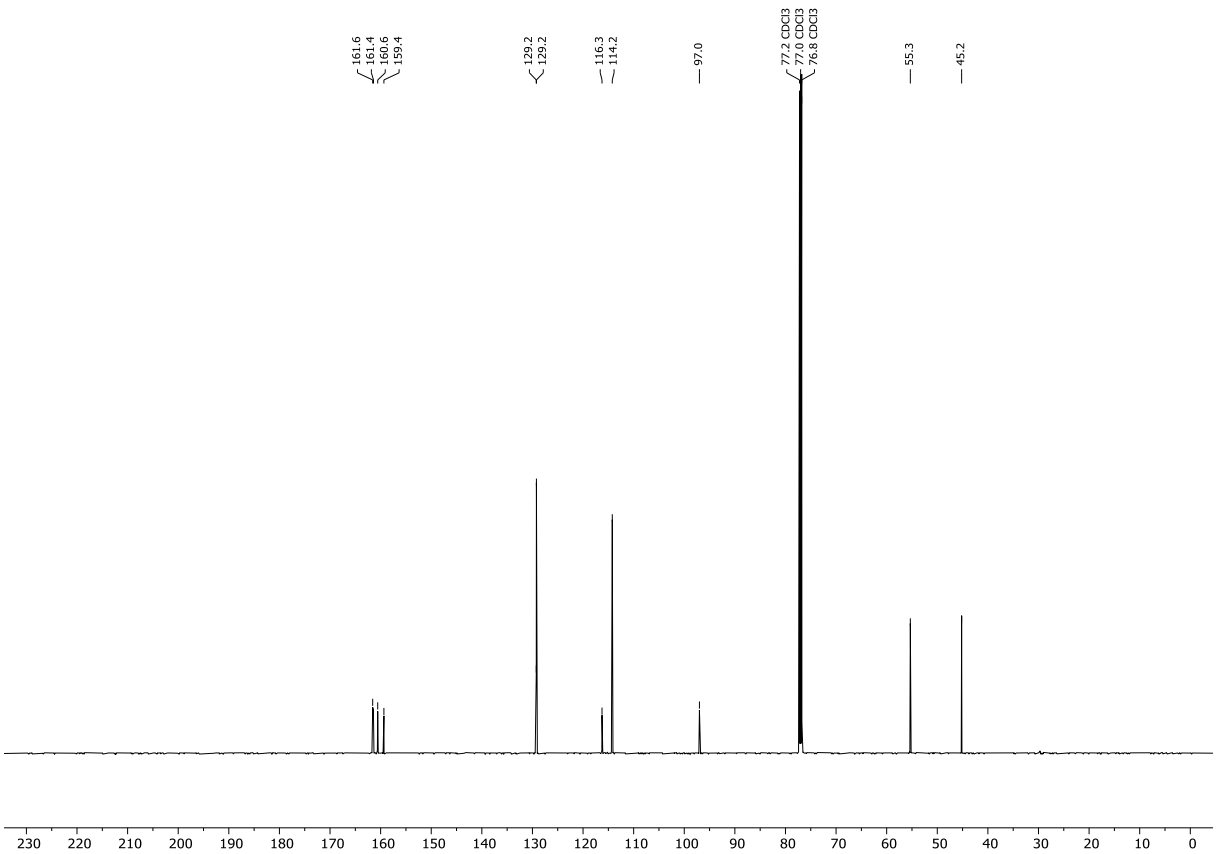
^{13}C NMR spectrum of **4** (151 MHz, DMSO-d_6)



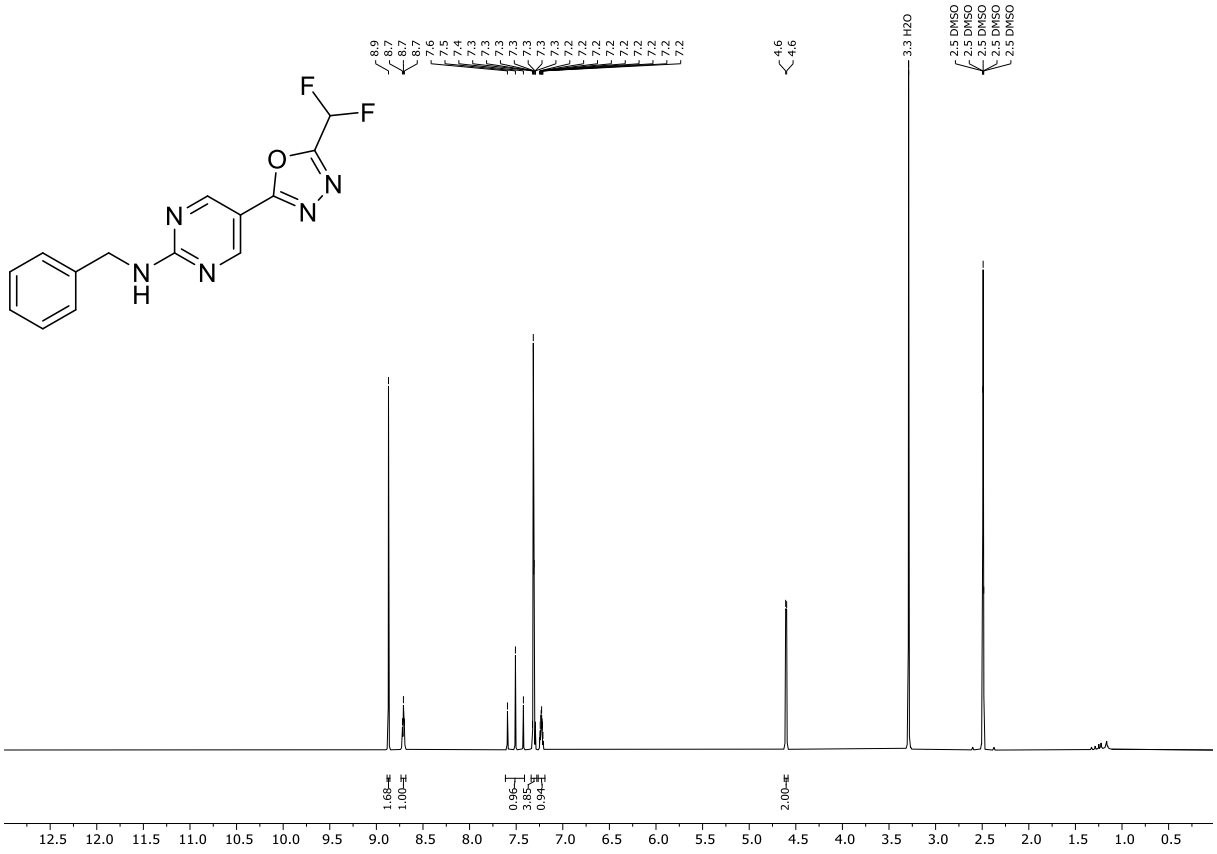
^{1}H NMR spectrum of **5** (600 MHz, $CDCl_3$)



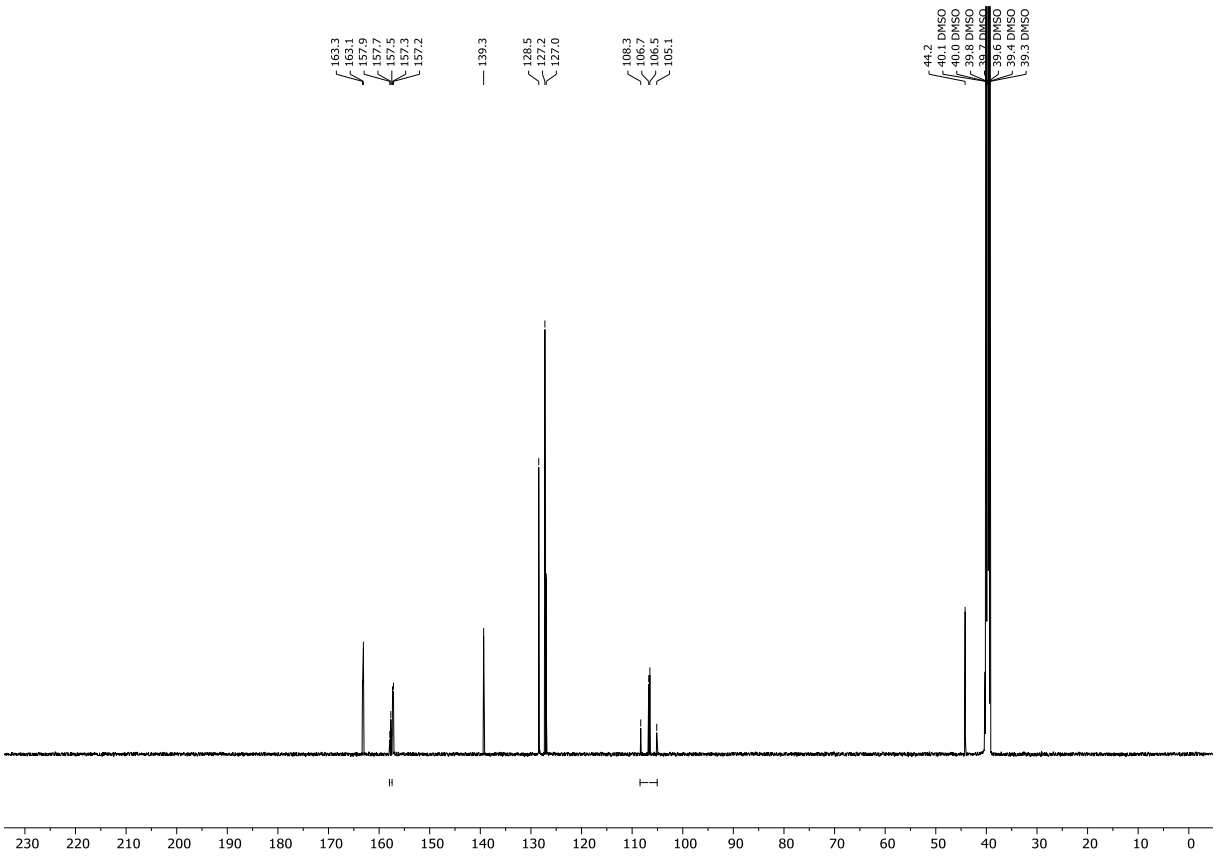
^{13}C NMR spectrum of **5** (151 MHz, $CDCl_3$)



^{1}H NMR spectrum of **6** (600 MHz, DMSO-d_6)

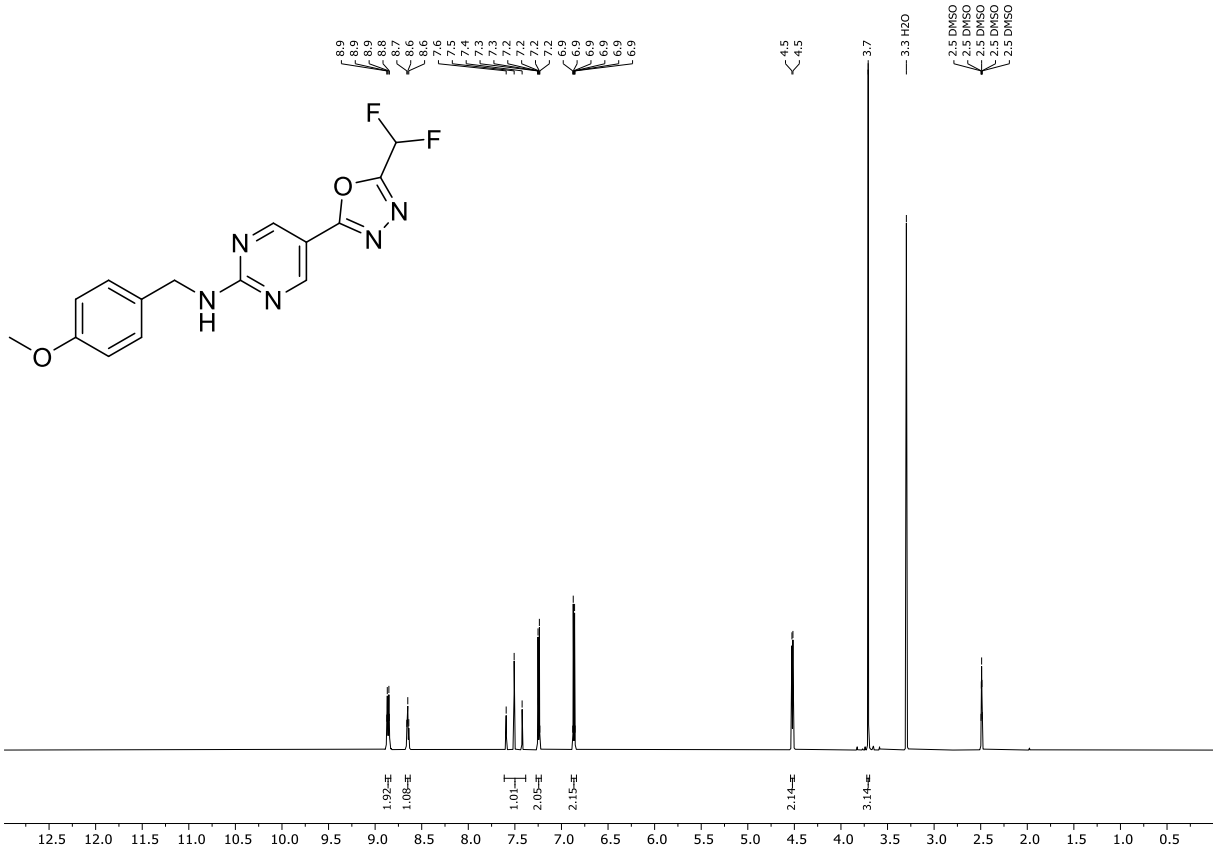


^{13}C NMR spectrum of **6** (151 MHz, DMSO-d_6)

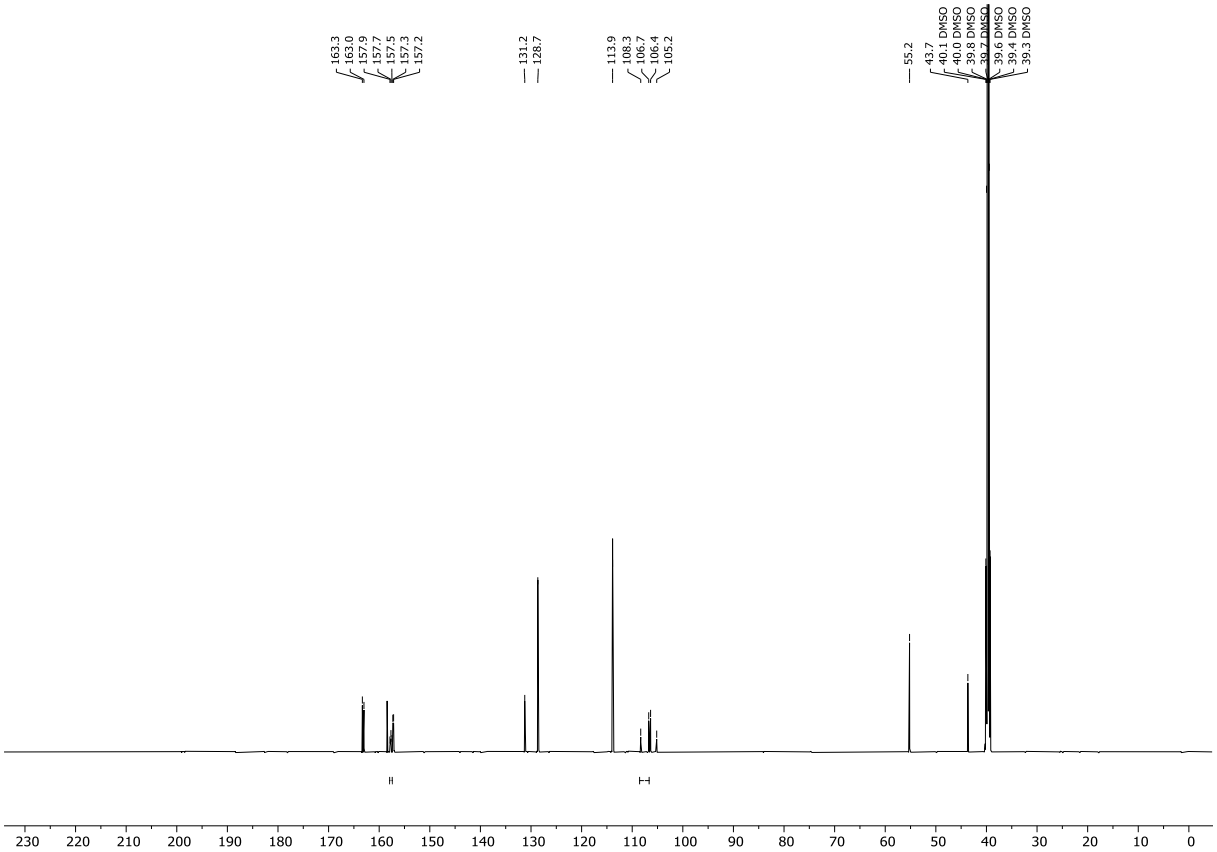


^{19}F NMR spectrum of **6** (565 MHz, DMSO-d_6)

^{1}H NMR spectrum of **7** (600 MHz, DMSO-d_6)



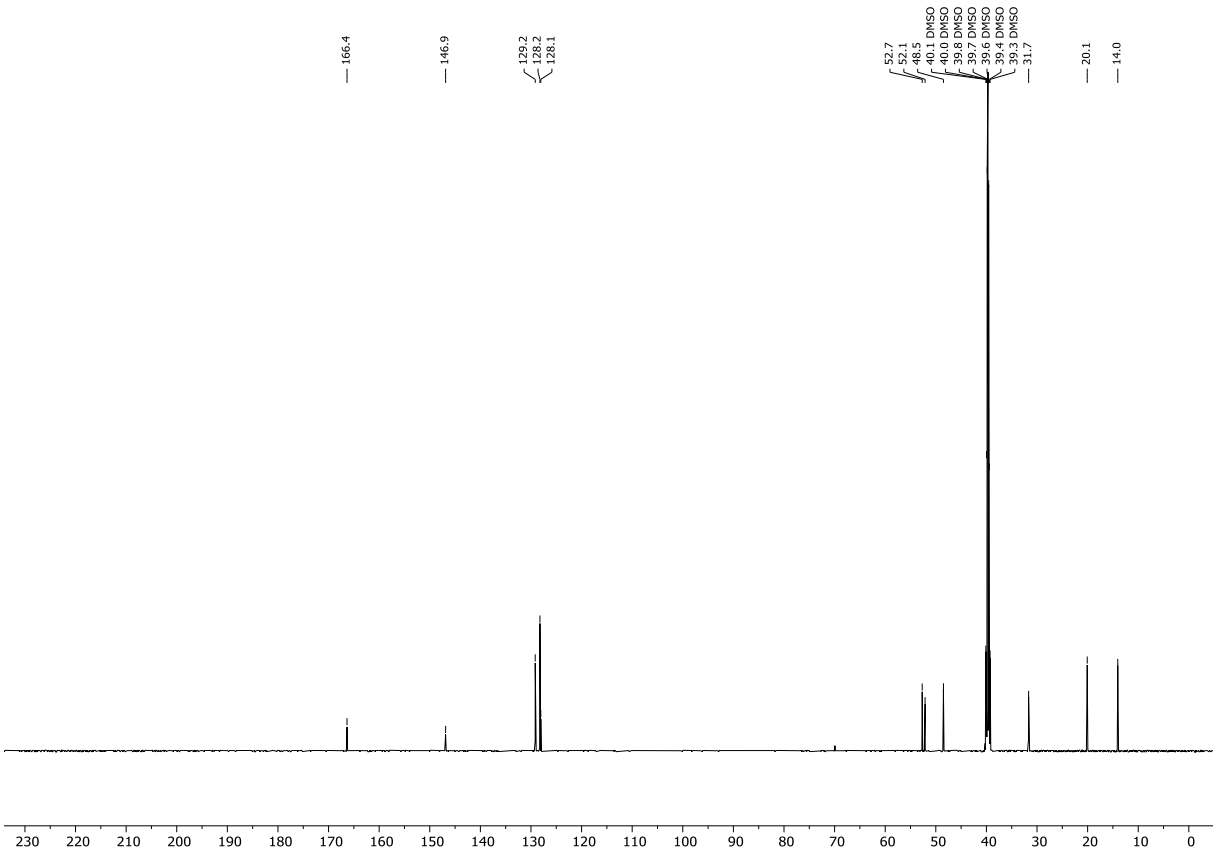
^{13}C NMR spectrum of **7** (151 MHz, DMSO-d_6)



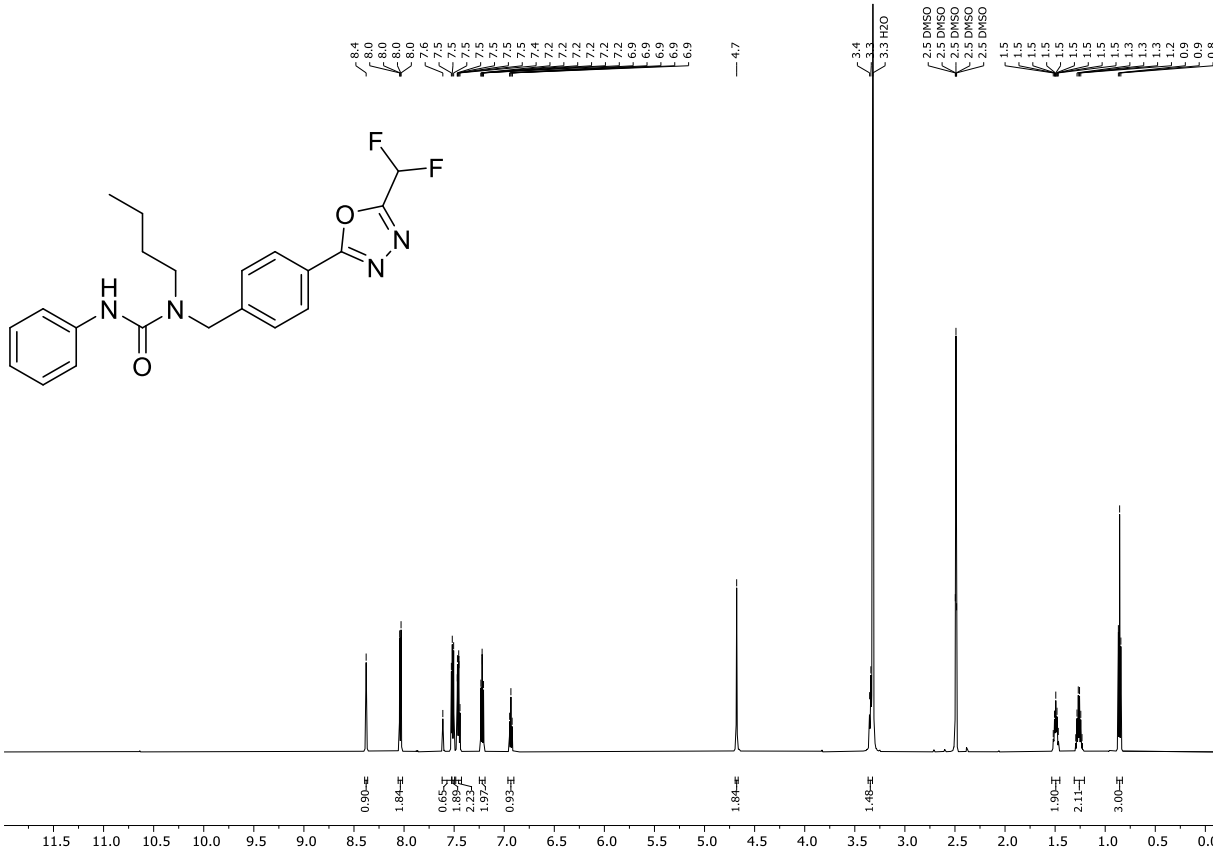
^{19}F NMR spectrum of **7** (565 MHz, DMSO-d_6)

^{1}H NMR spectrum of **8** (600 MHz, DMSO-d_6)

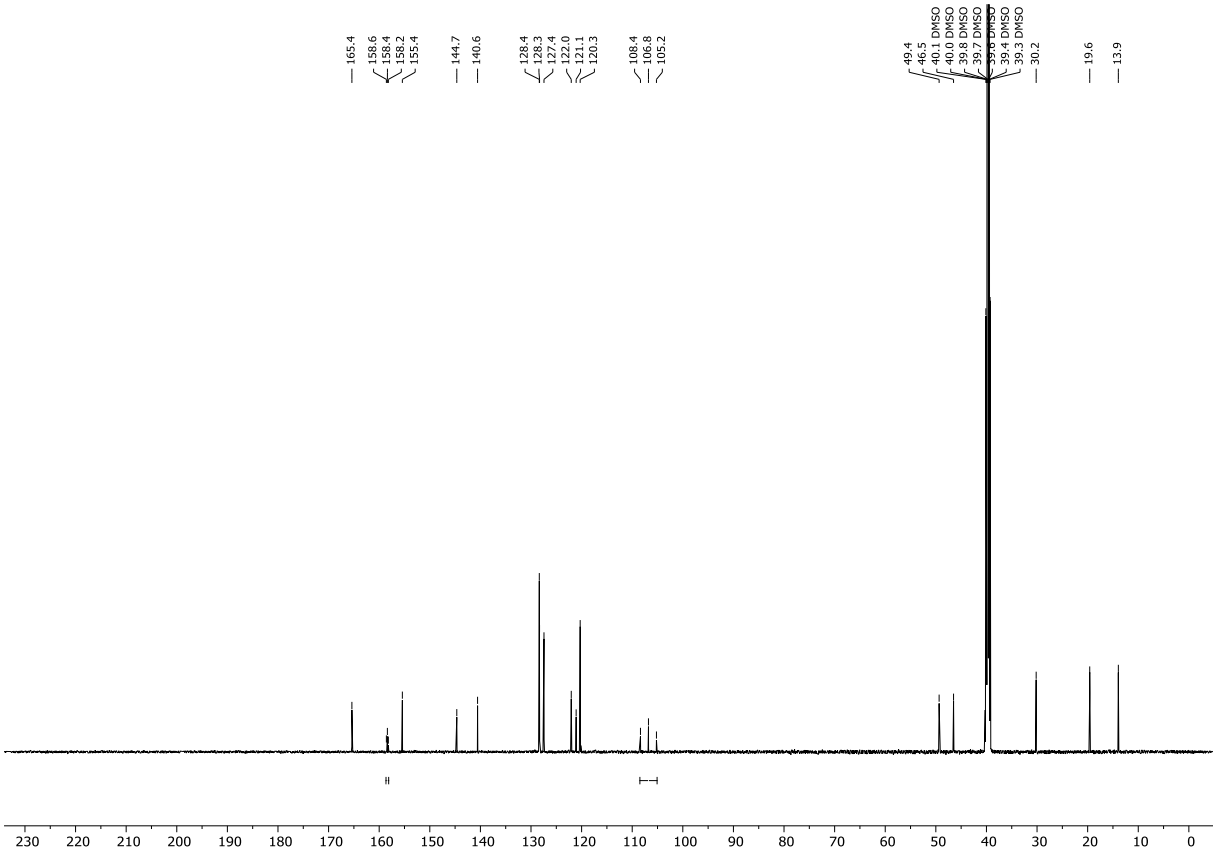
^{13}C NMR spectrum of **8** (151 MHz, DMSO-d_6)



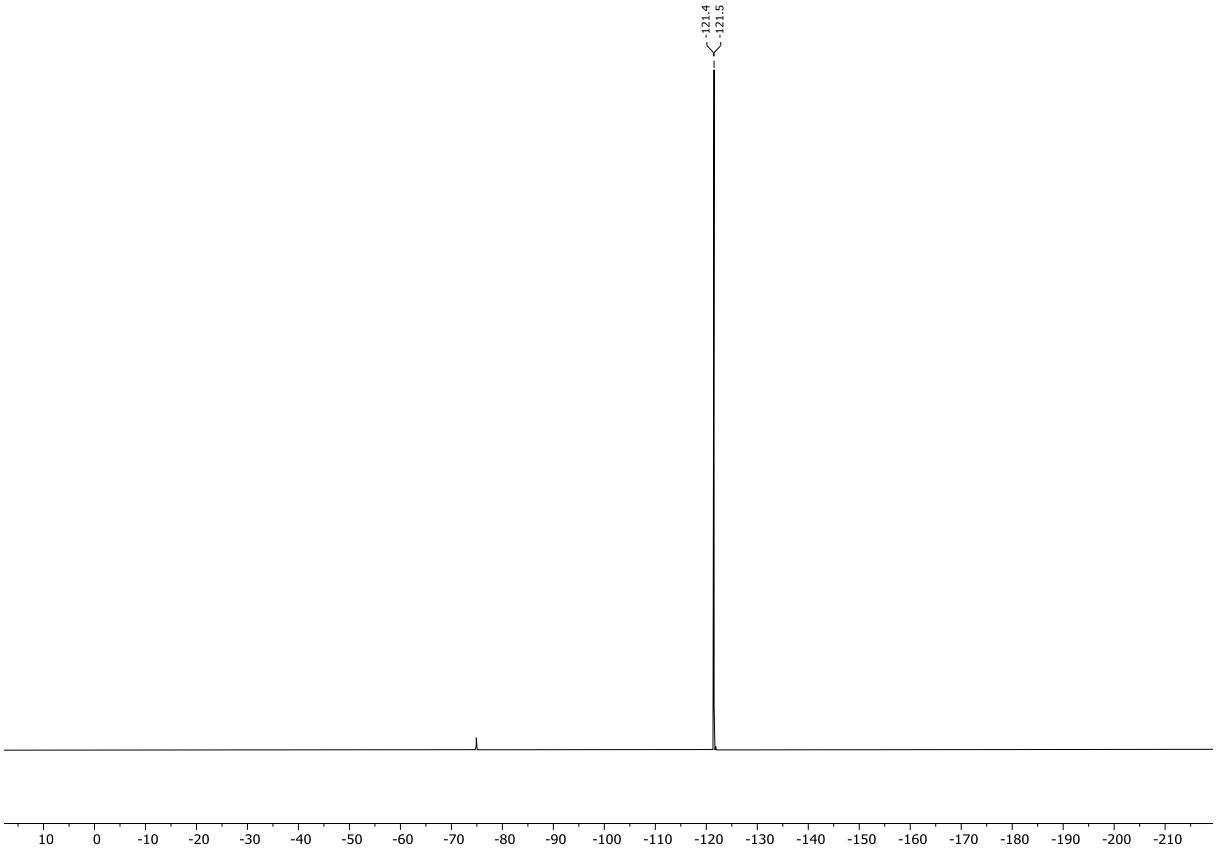
^{1}H NMR spectrum of **9** (600 MHz, DMSO-d_6)



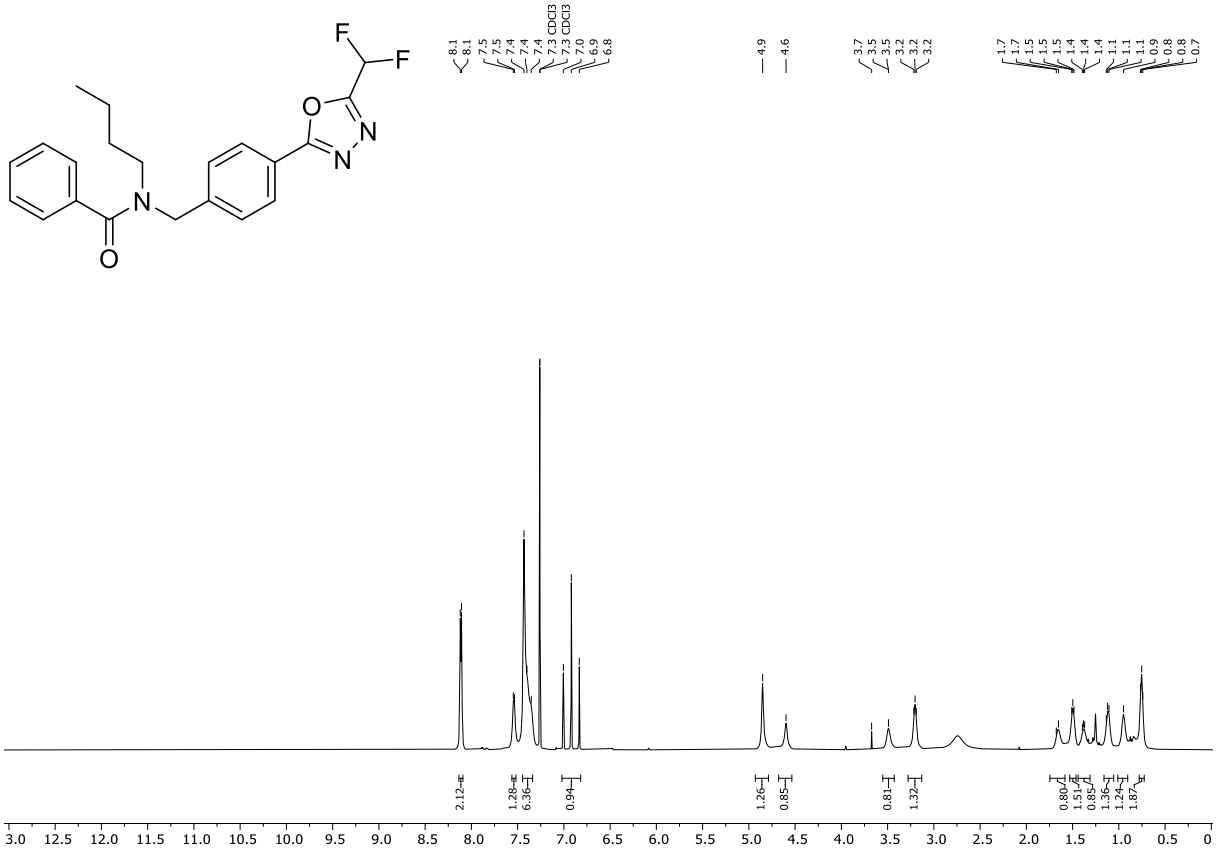
^{13}C NMR spectrum of **9** (151 MHz, DMSO-d_6)



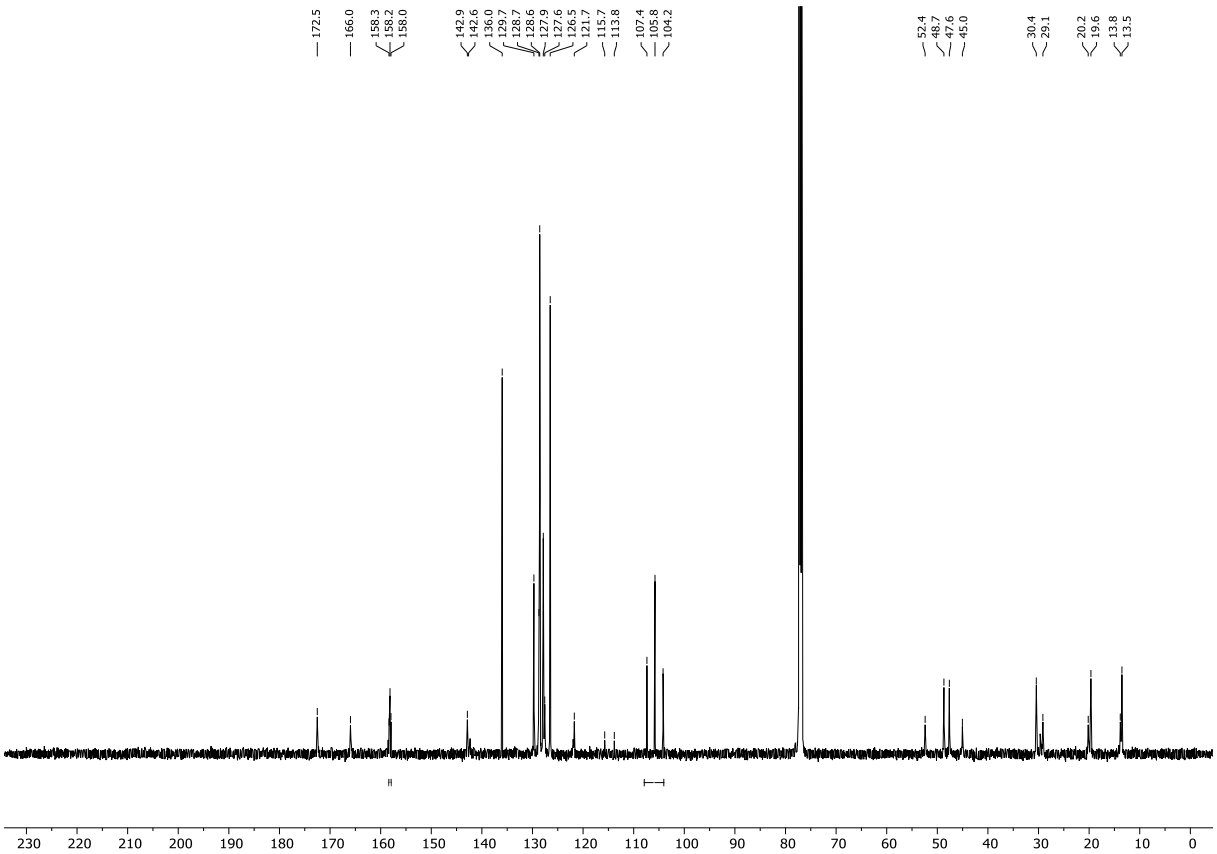
^{19}F NMR spectrum of **9** (565 MHz, DMSO-d_6)



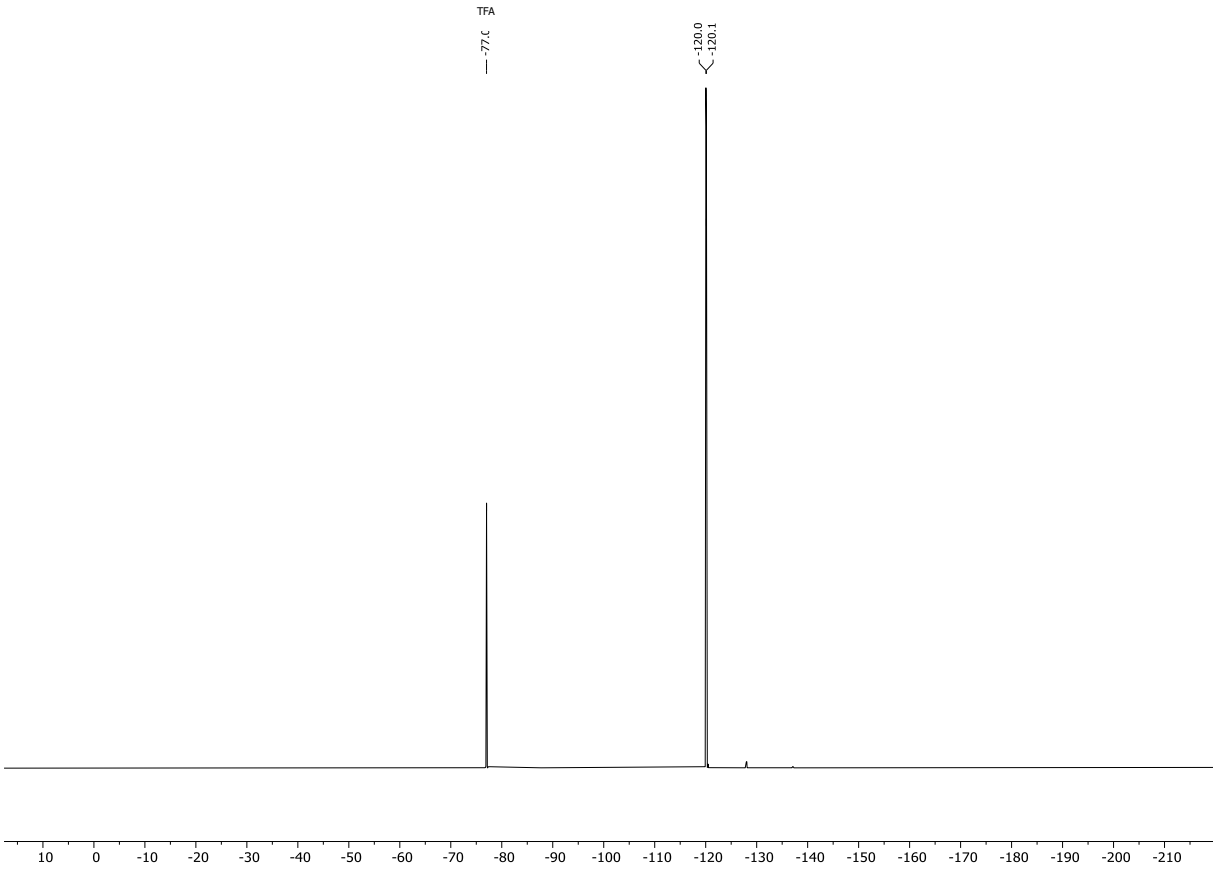
^{1}H NMR spectrum of **10** (600 MHz, $CDCl_3$)



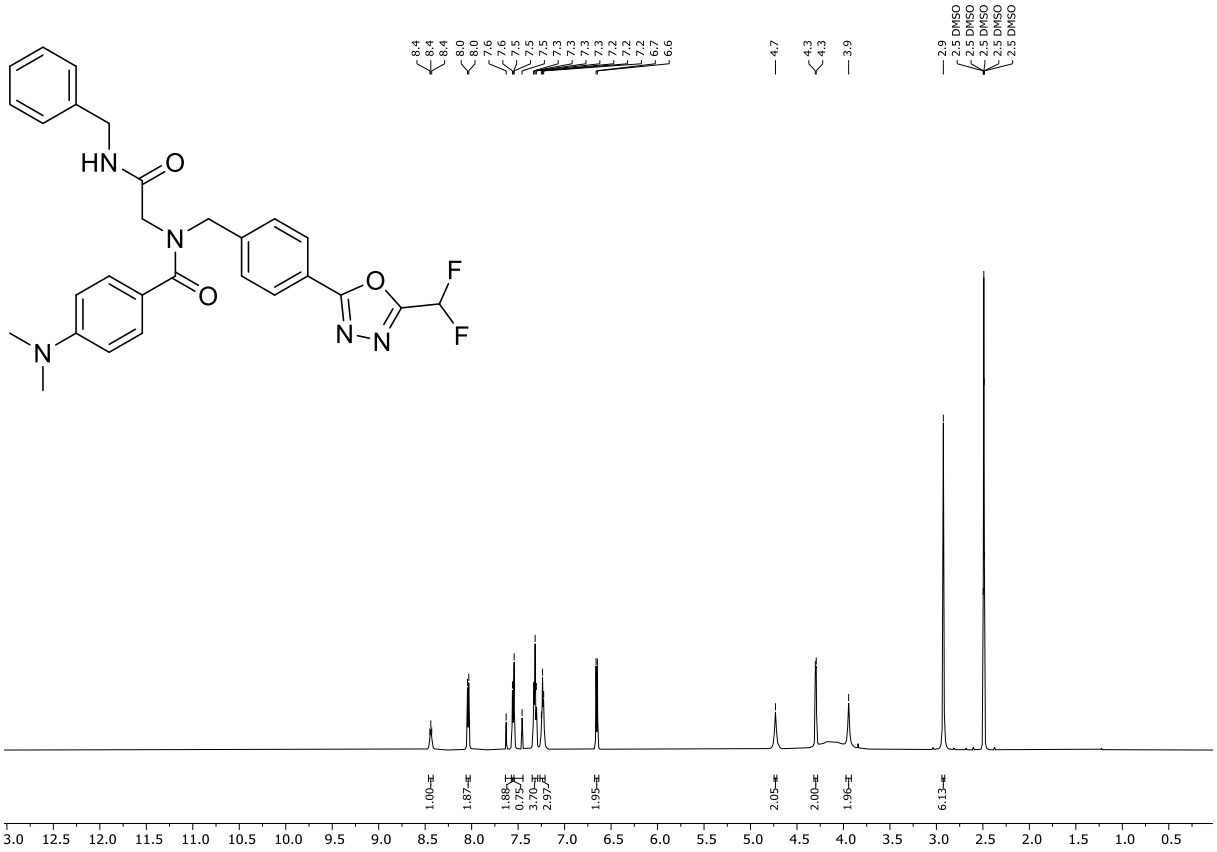
^{13}C NMR spectrum of **10** (151 MHz, $CDCl_3$)



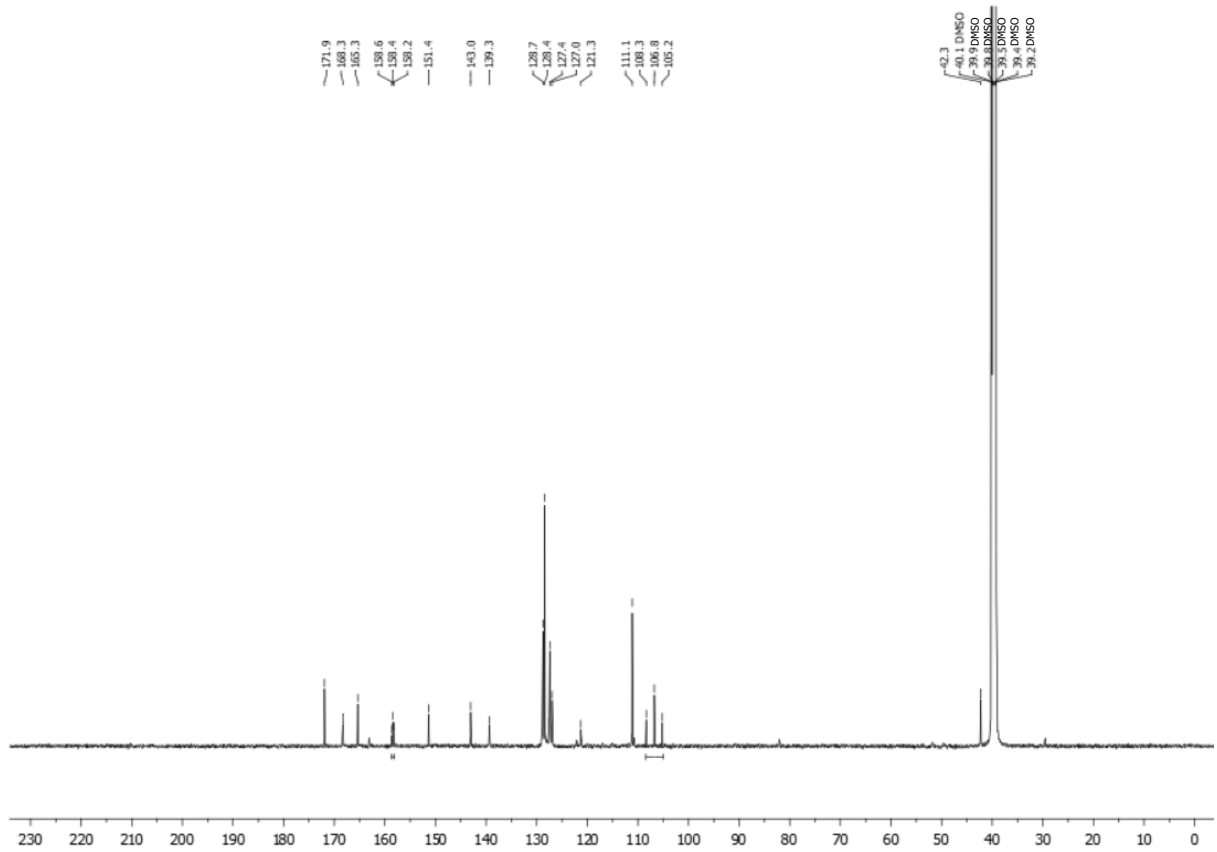
^{19}F NMR spectrum of **10** (565 MHz, $CDCl_3$)



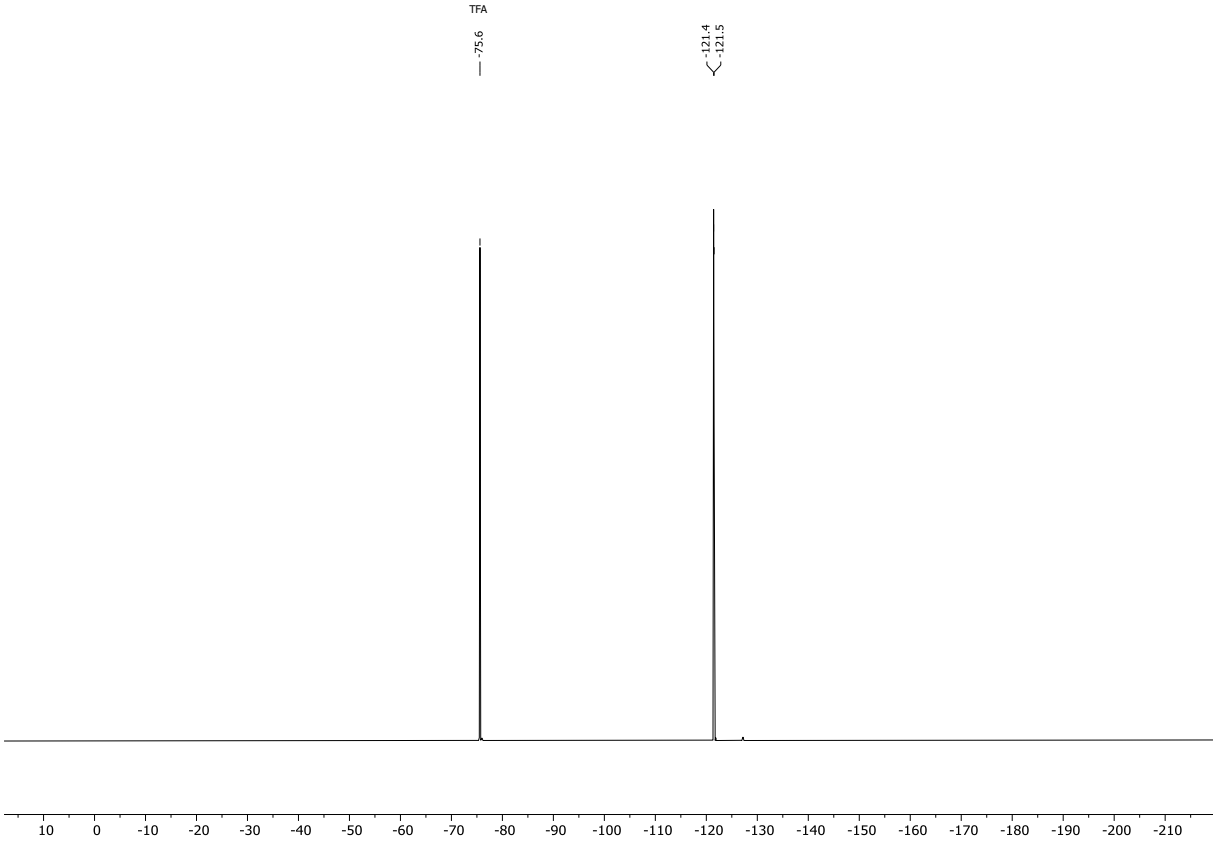
^{1}H NMR spectrum of **12** (600 MHz, DMSO-d_6)



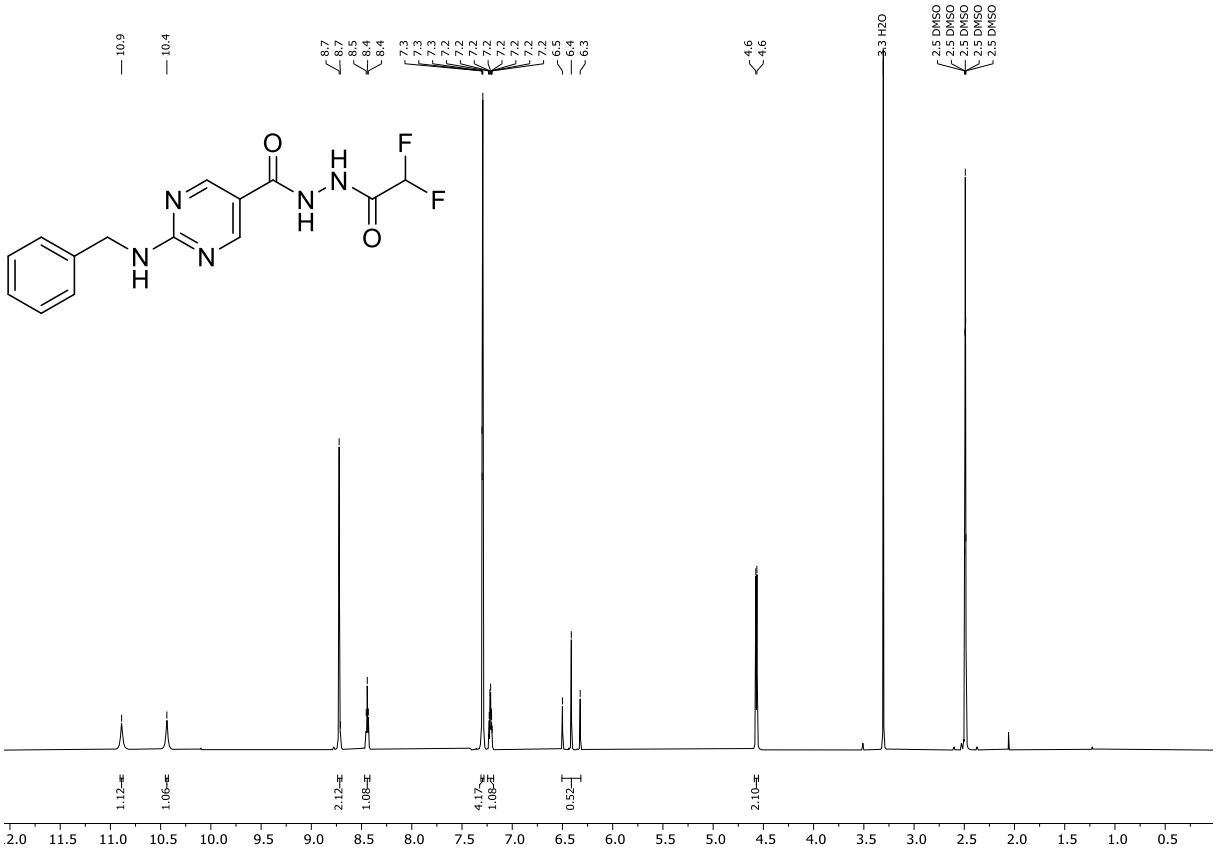
^{13}C NMR spectrum of **12** (151 MHz, DMSO-d_6)



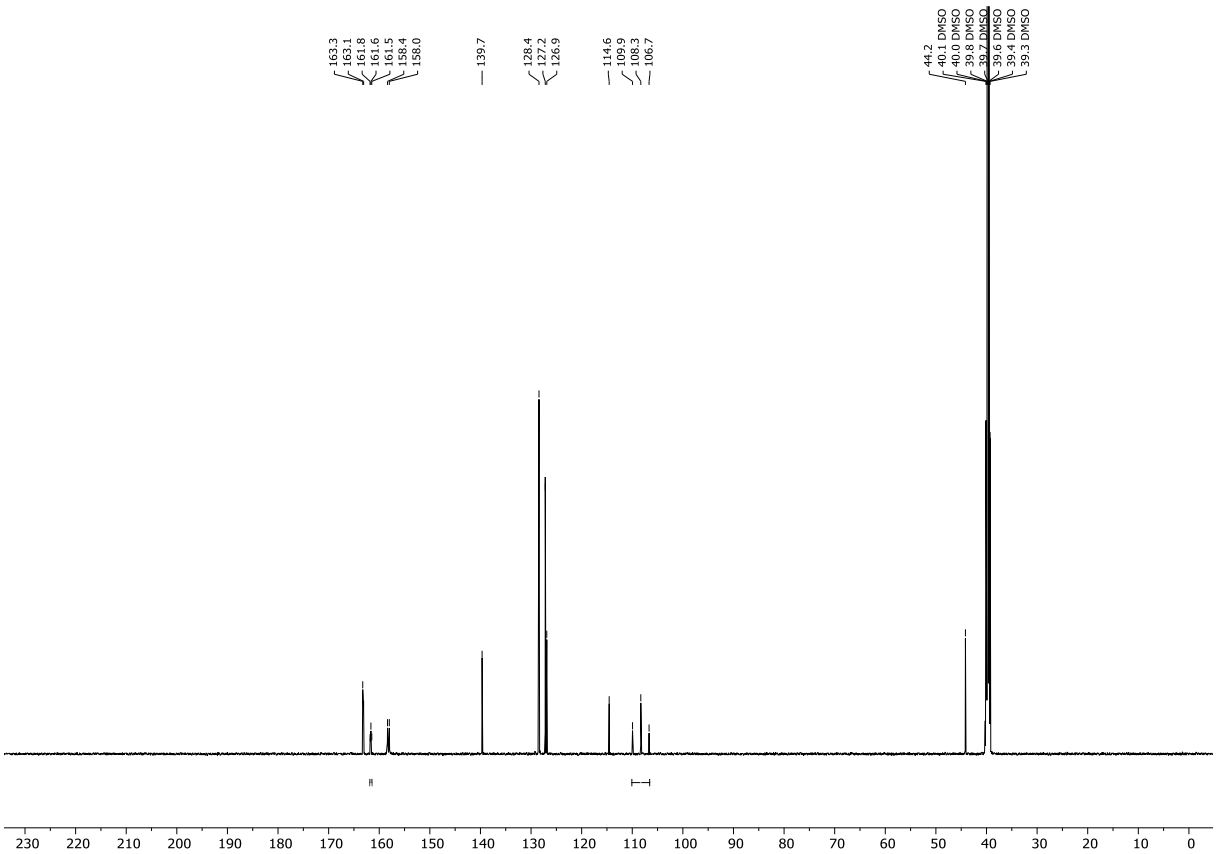
^{19}F NMR spectrum of **12** (565 MHz, DMSO-d_6)



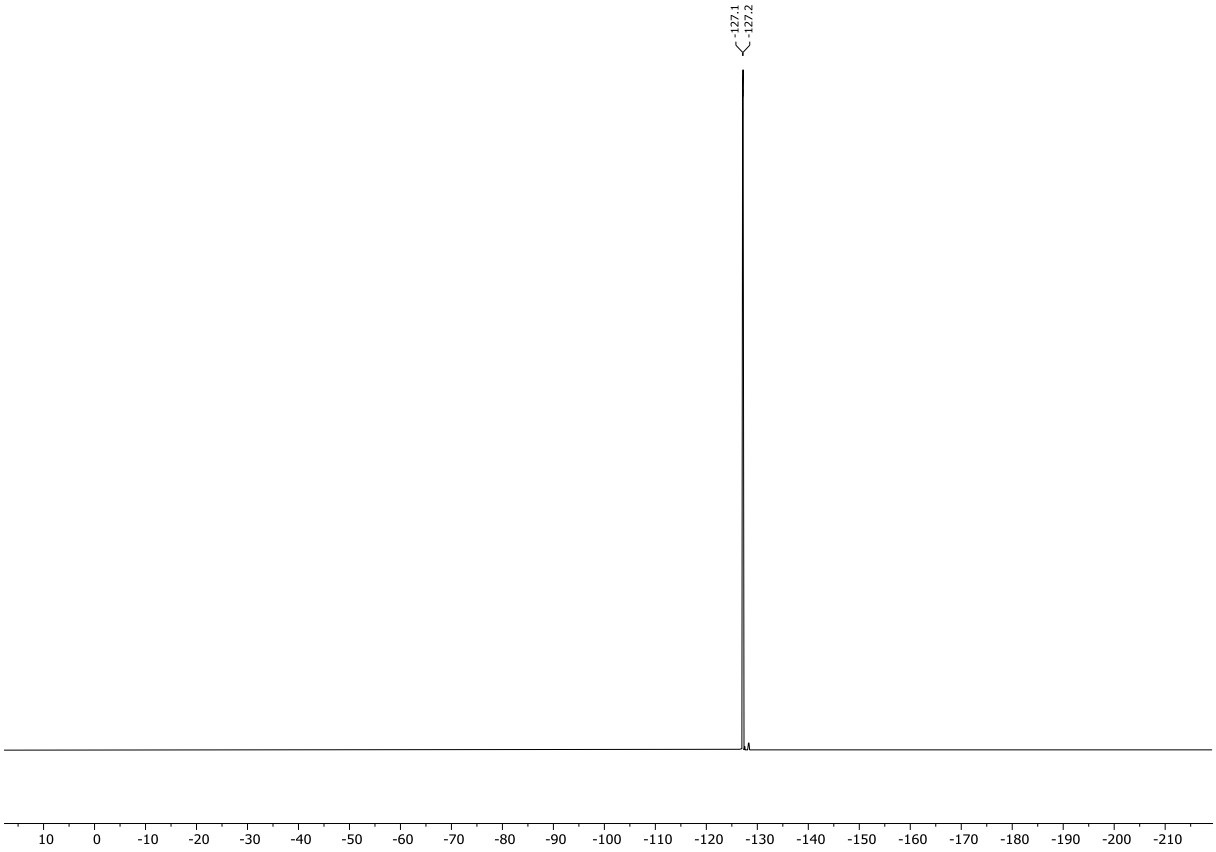
^{1}H NMR spectrum of **13** (600 MHz, DMSO-d_6)



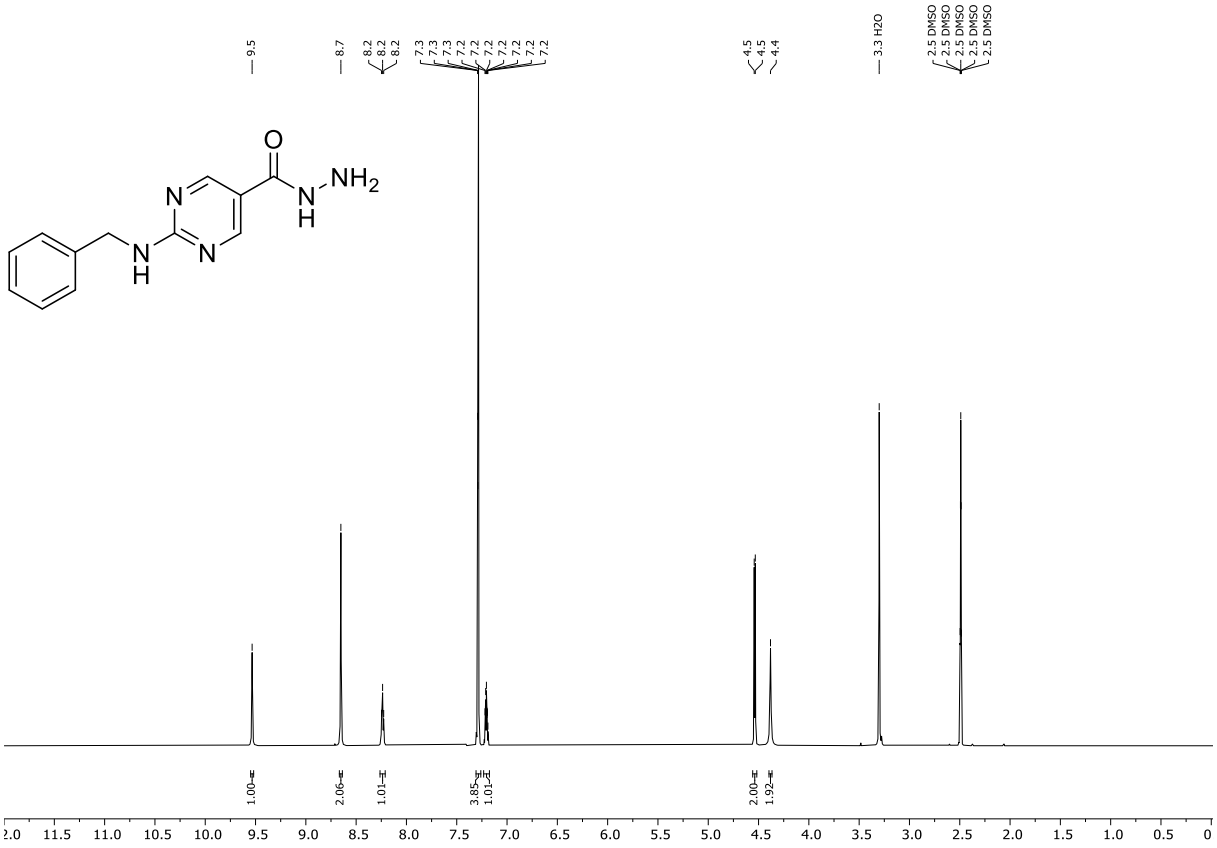
^{13}C NMR spectrum of **13** (151 MHz, DMSO-d_6)



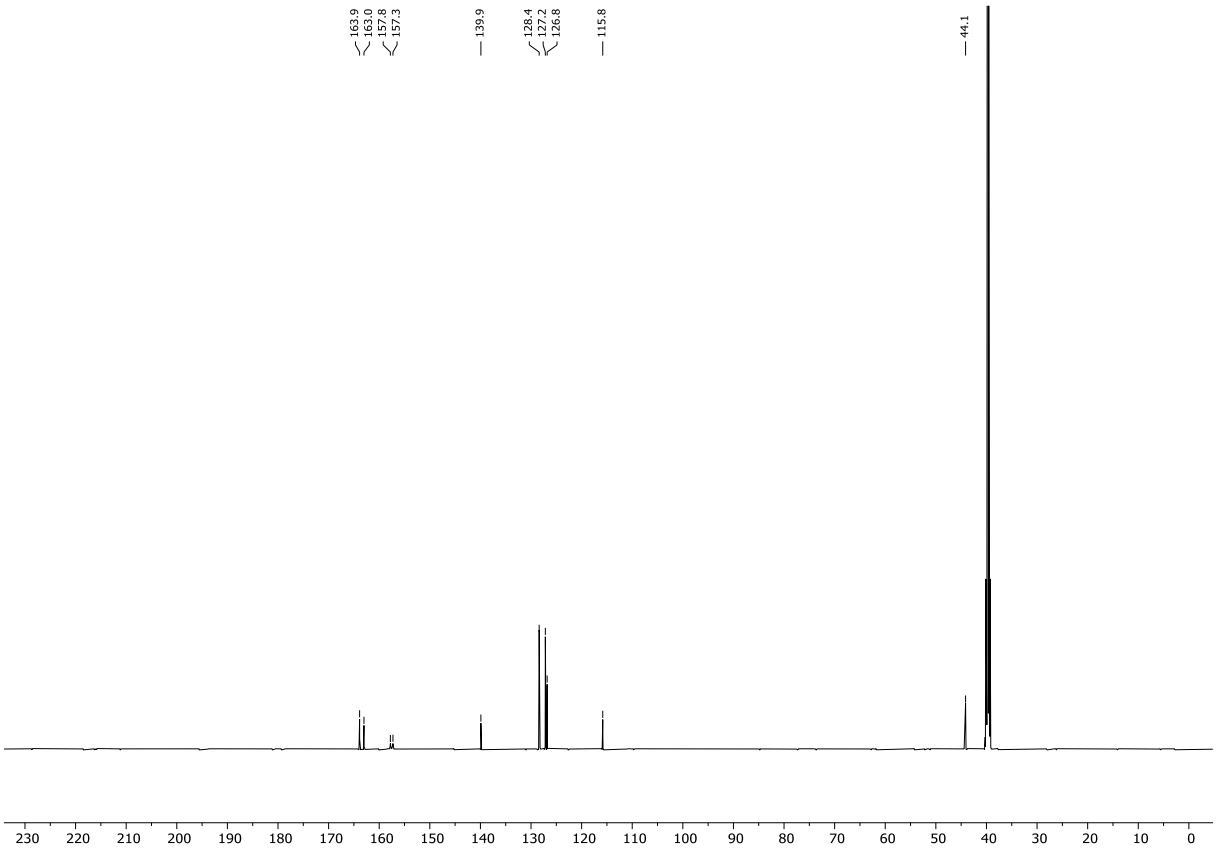
^{19}F NMR spectrum of **13** (565 MHz, DMSO-d_6)



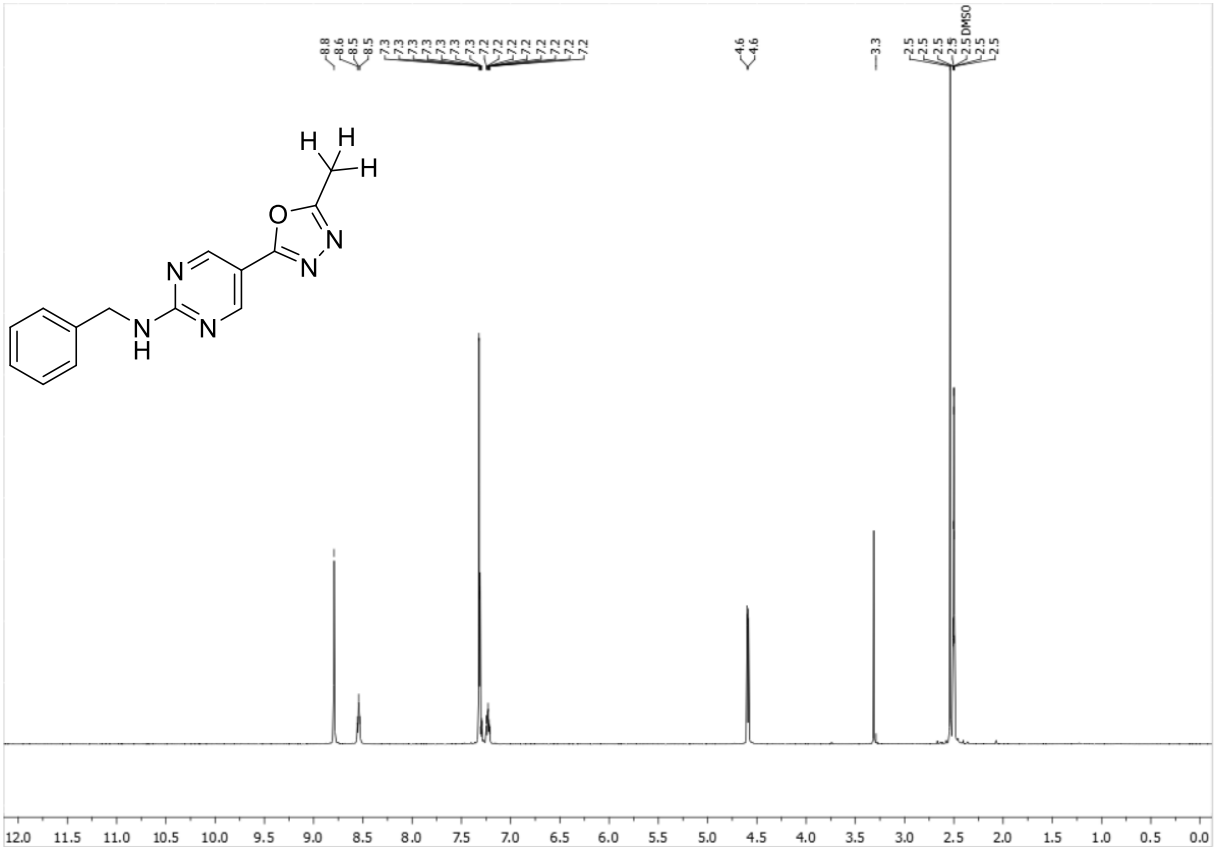
^{1}H NMR spectrum of **14** (600 MHz, DMSO-d_6)



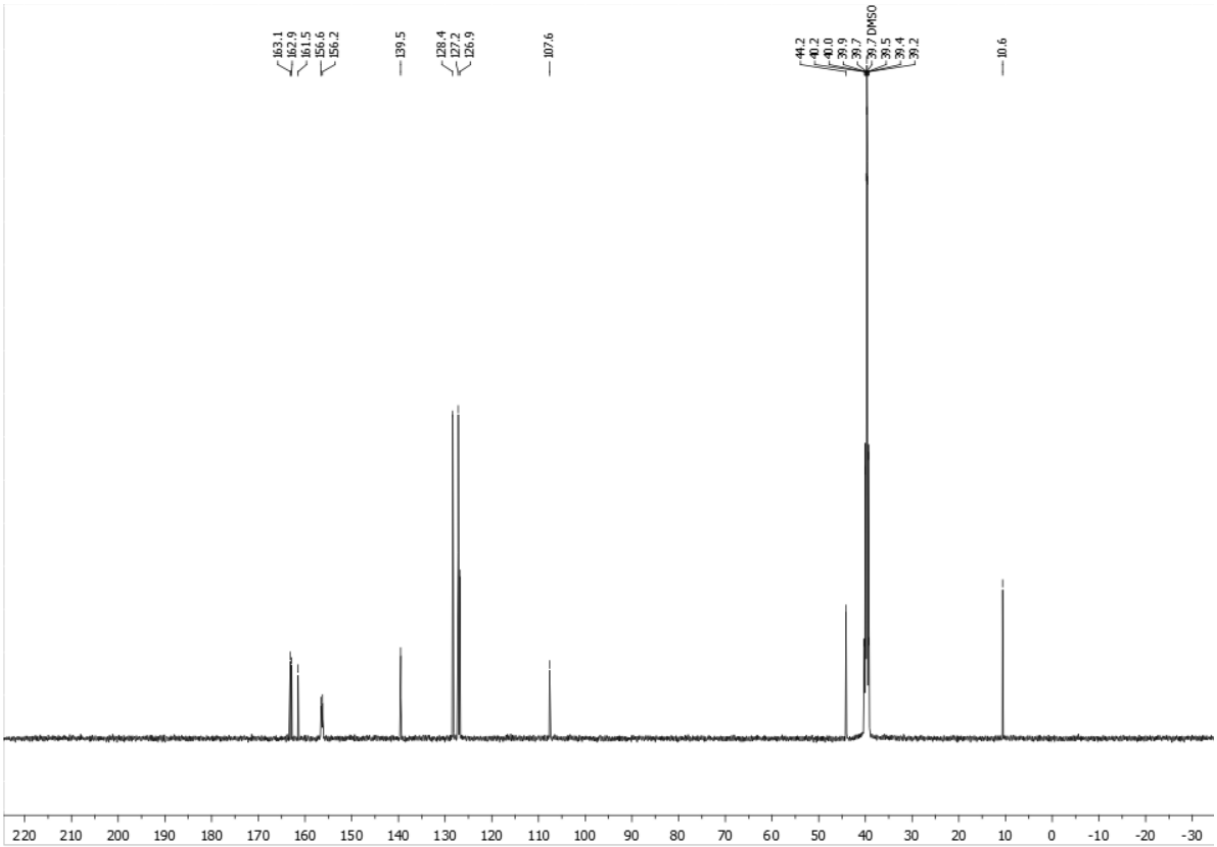
^{13}C NMR spectrum of **14** (151 MHz, DMSO-d_6)



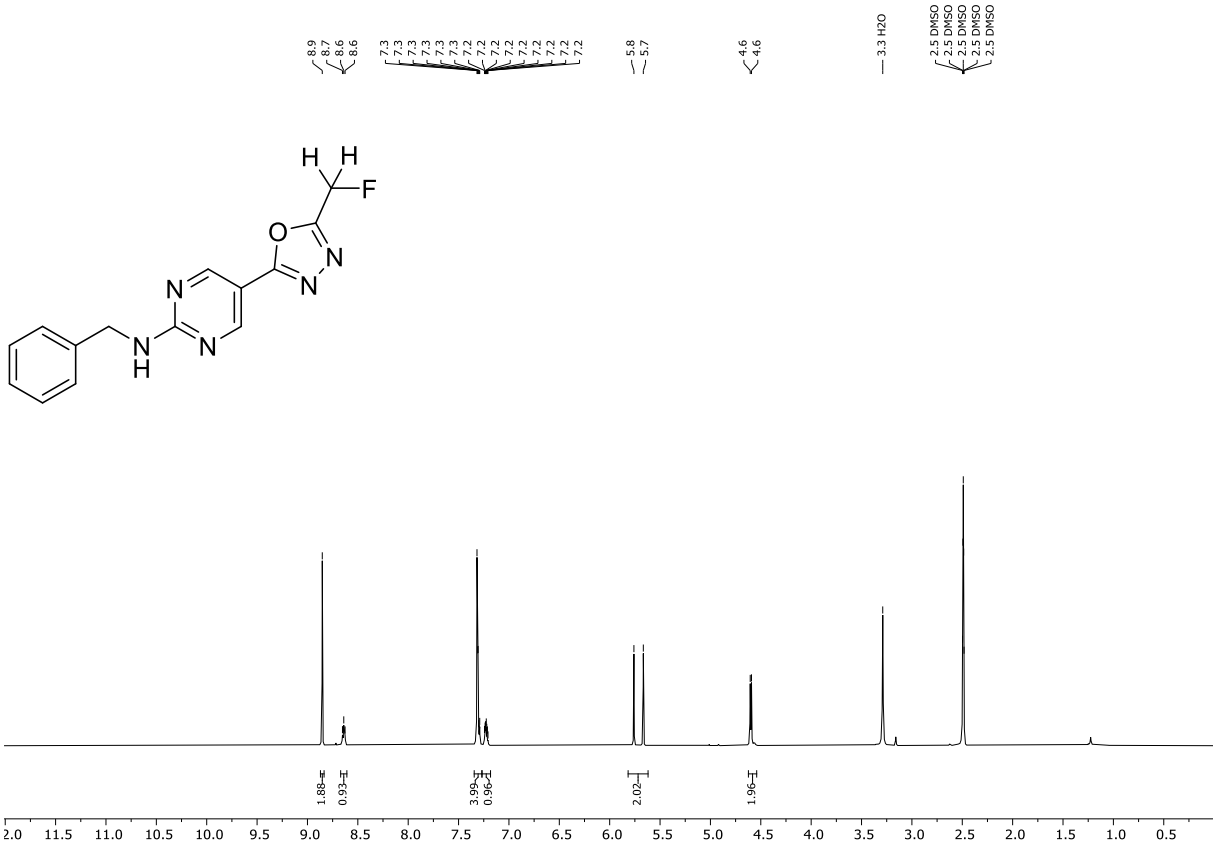
^{1}H NMR spectrum of **15** (600 MHz, DMSO-d_6)



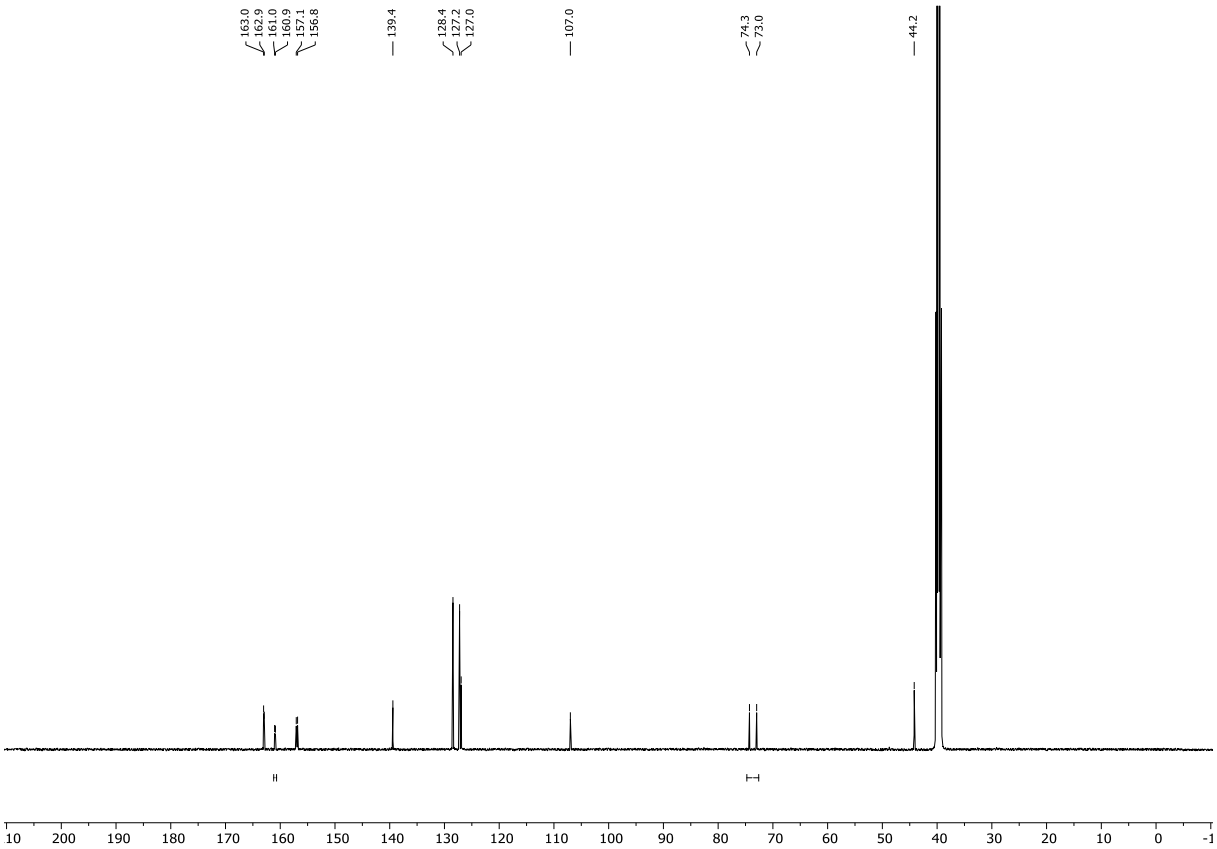
^{13}C NMR spectrum of **15** (126 MHz, DMSO-d_6)



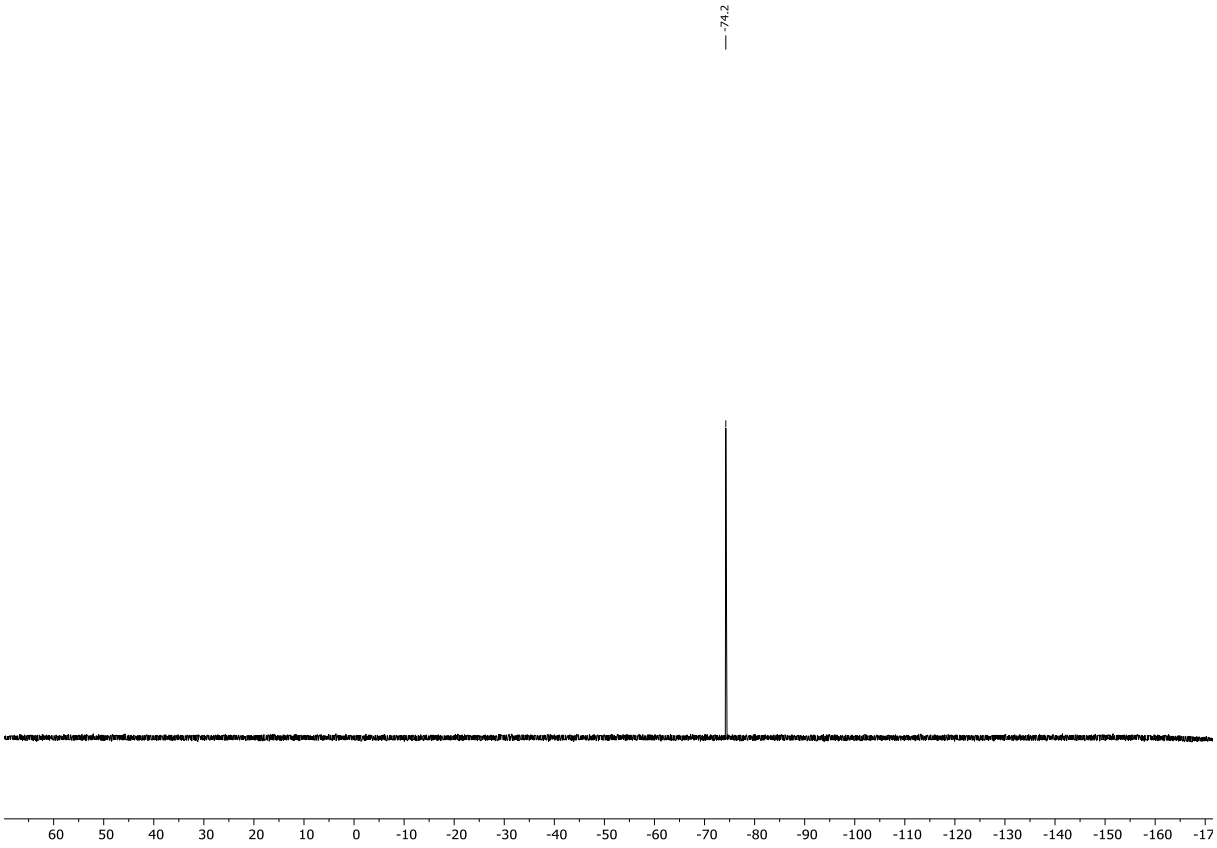
^{1}H NMR spectrum of **16** (500 MHz, DMSO-d_6)



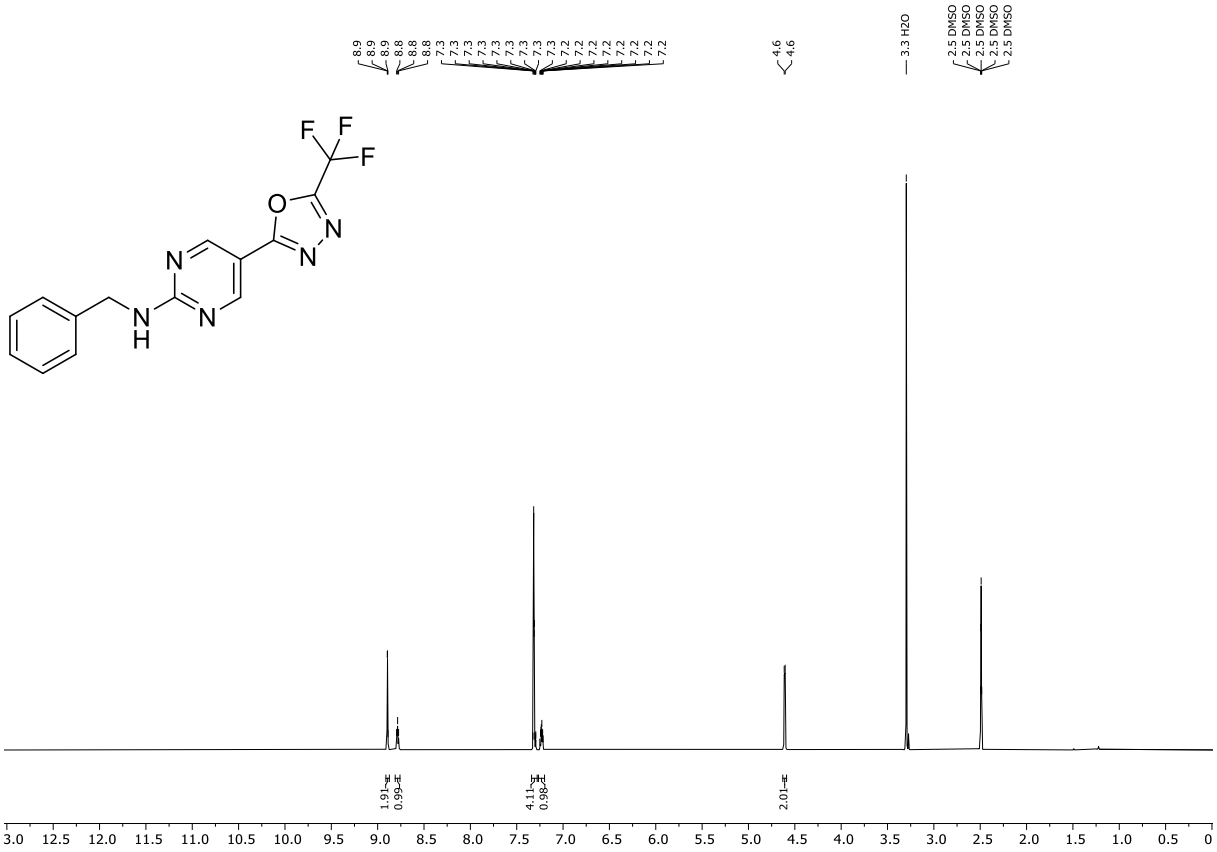
^{13}C NMR spectrum of **16** (126 MHz, DMSO-d_6)



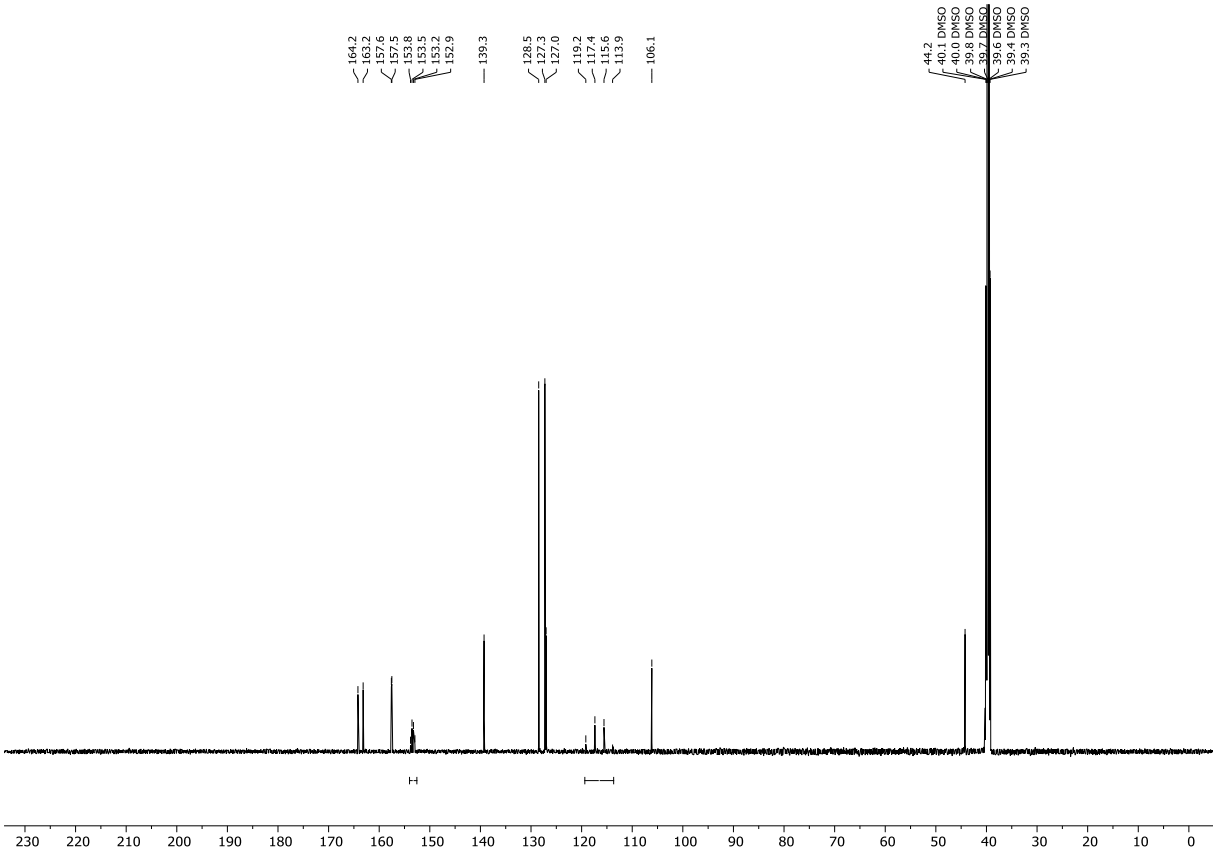
^{19}F NMR spectrum of **16** (471 MHz, DMSO-d_6)



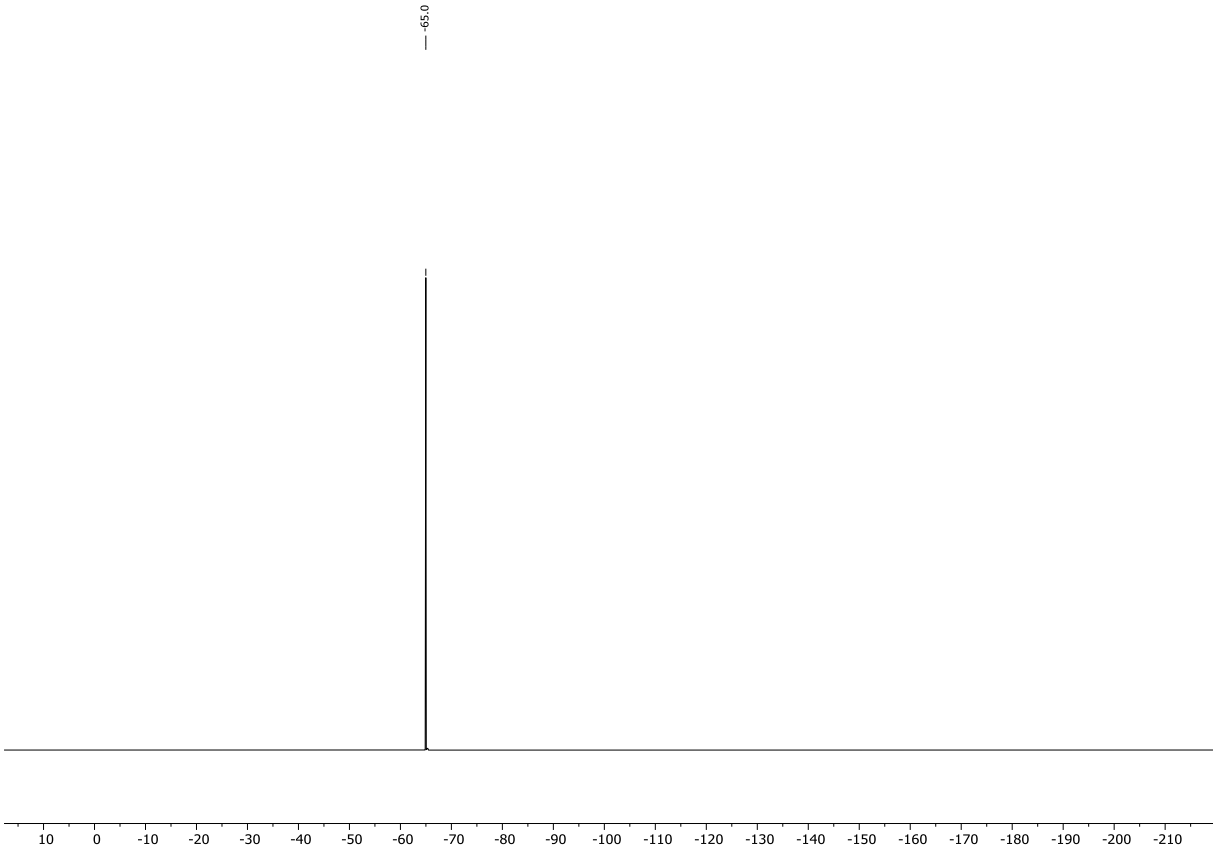
^{1}H NMR spectrum of **17** (600 MHz, DMSO-d_6)



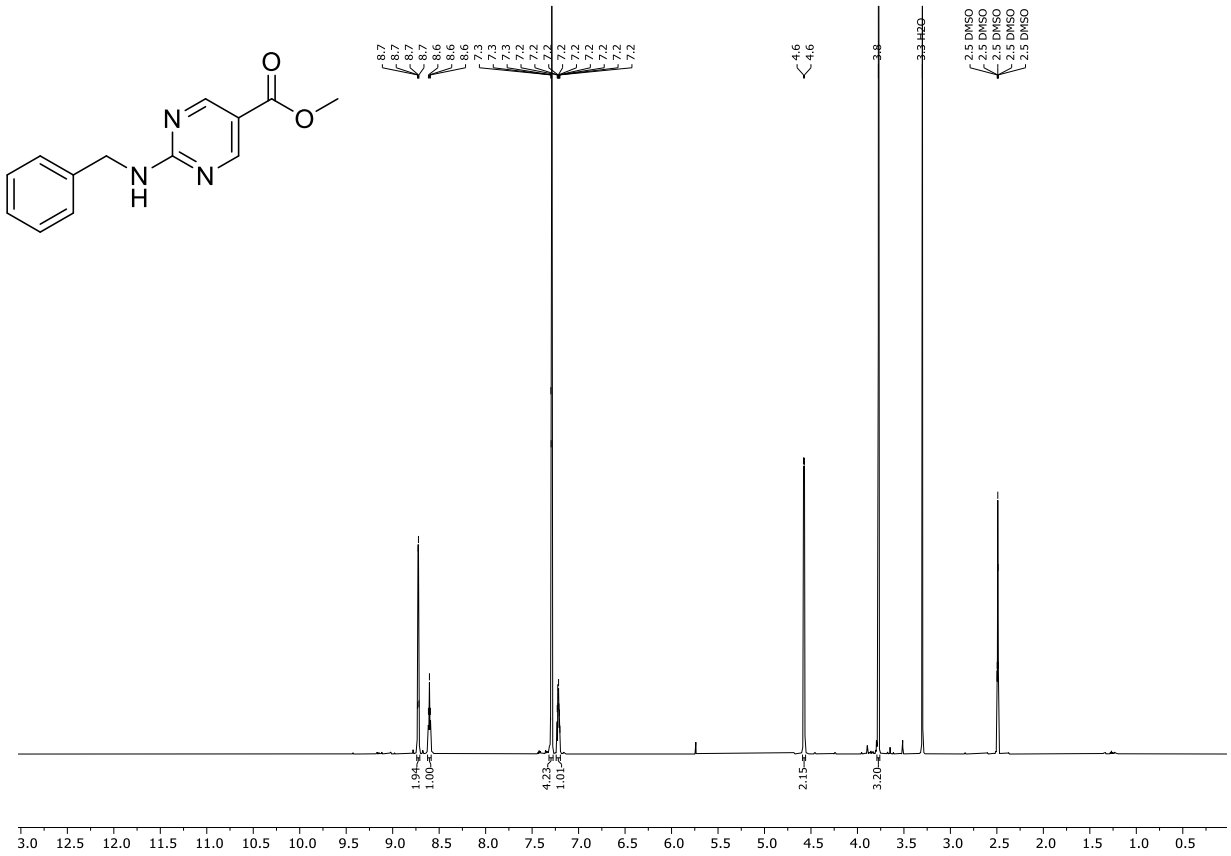
^{13}C NMR spectrum of **17** (151 MHz, DMSO-d_6)



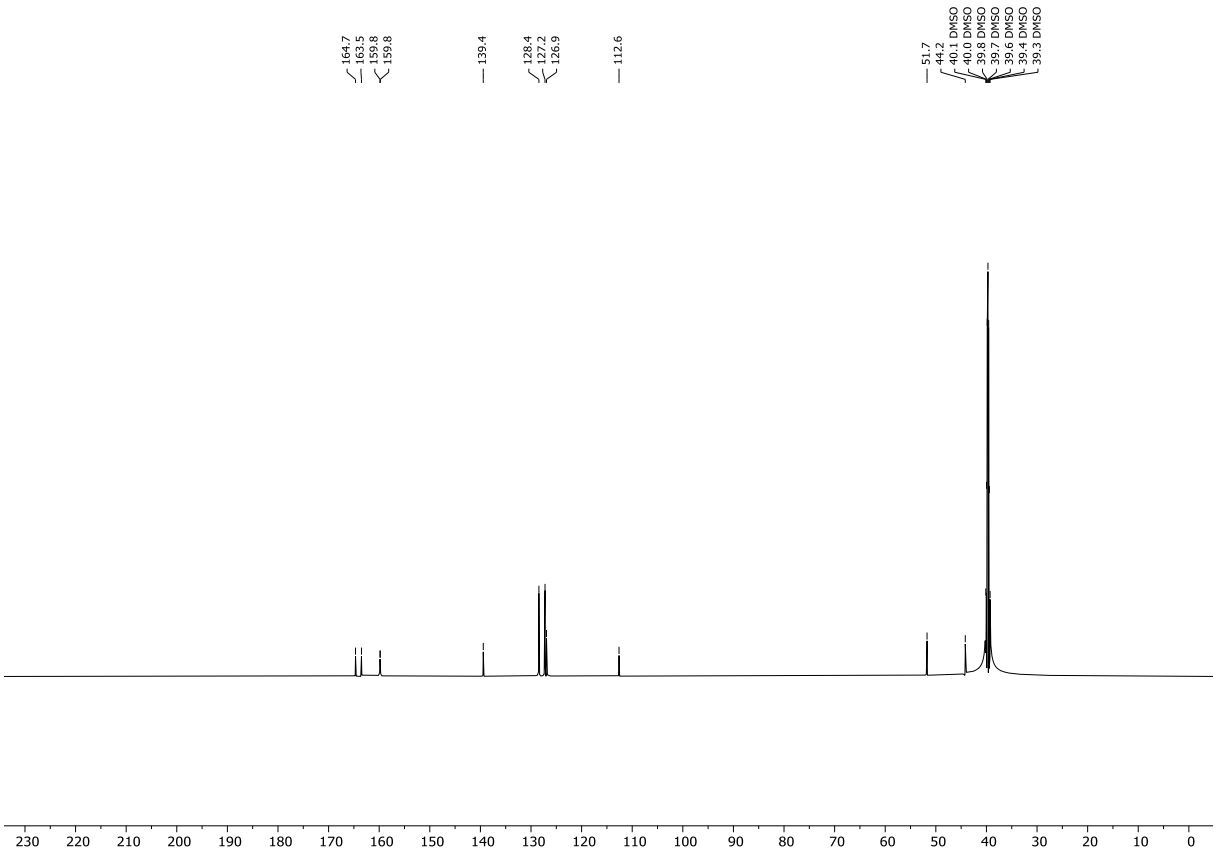
^{19}F NMR spectrum of **17** (565 MHz, DMSO-d_6)



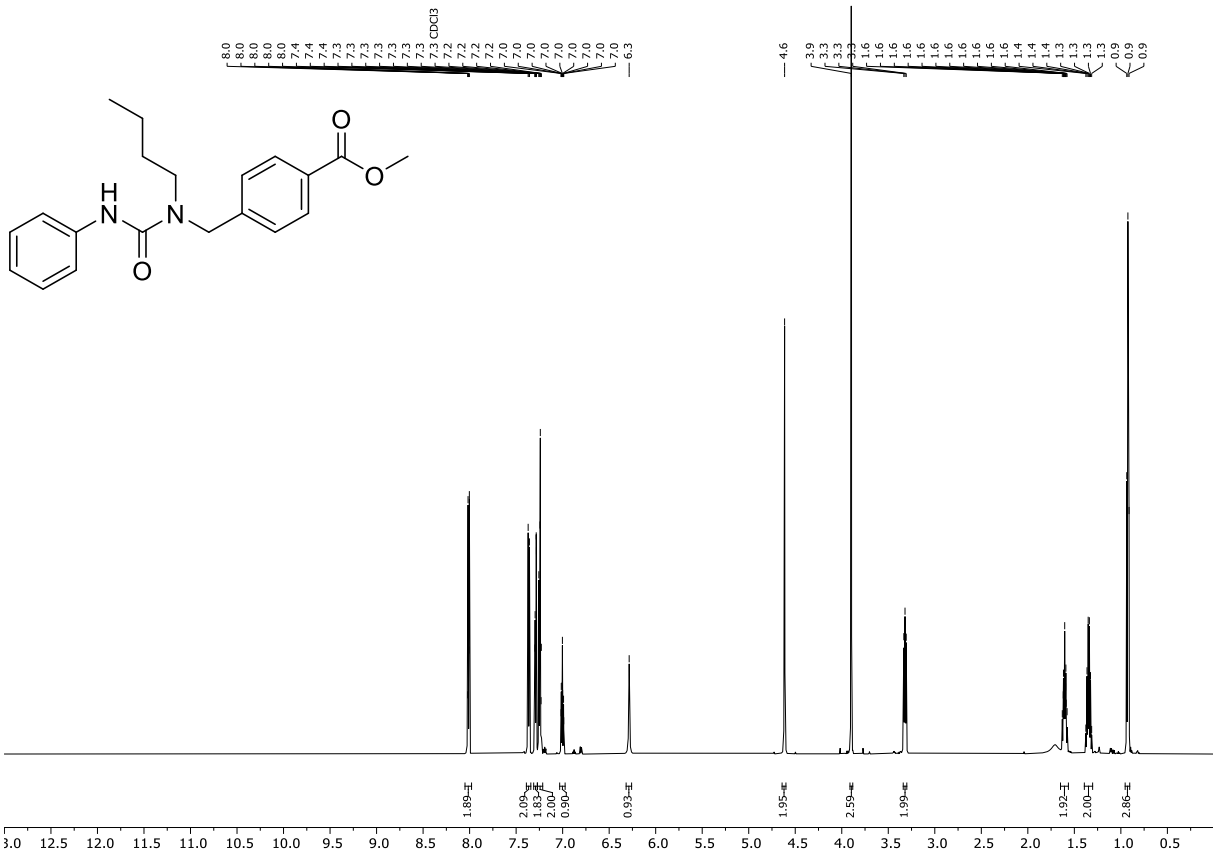
^{1}H NMR spectrum of **18** (600 MHz, DMSO-d_6)



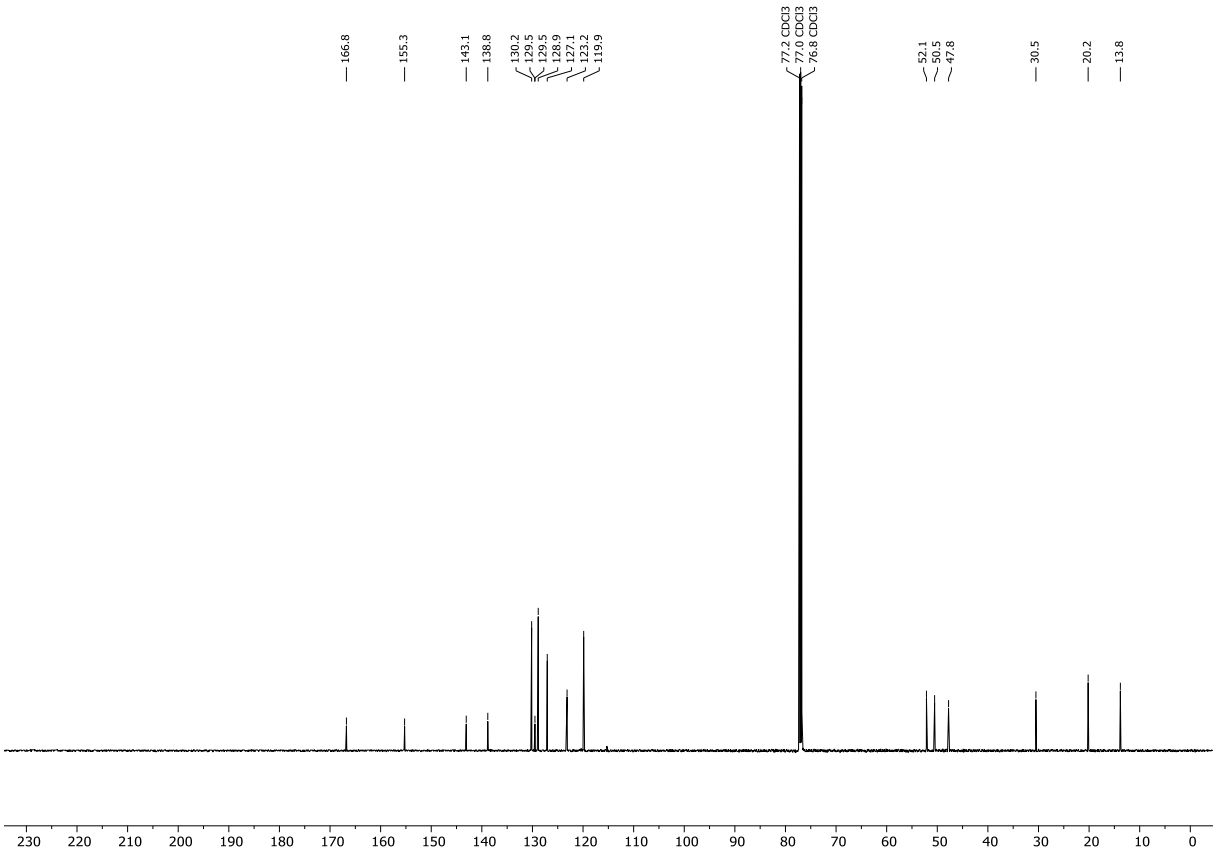
^{13}C NMR spectrum of **18** (151 MHz, DMSO-d_6)



^{1}H NMR spectrum of **19** (600 MHz, $CDCl_3$)

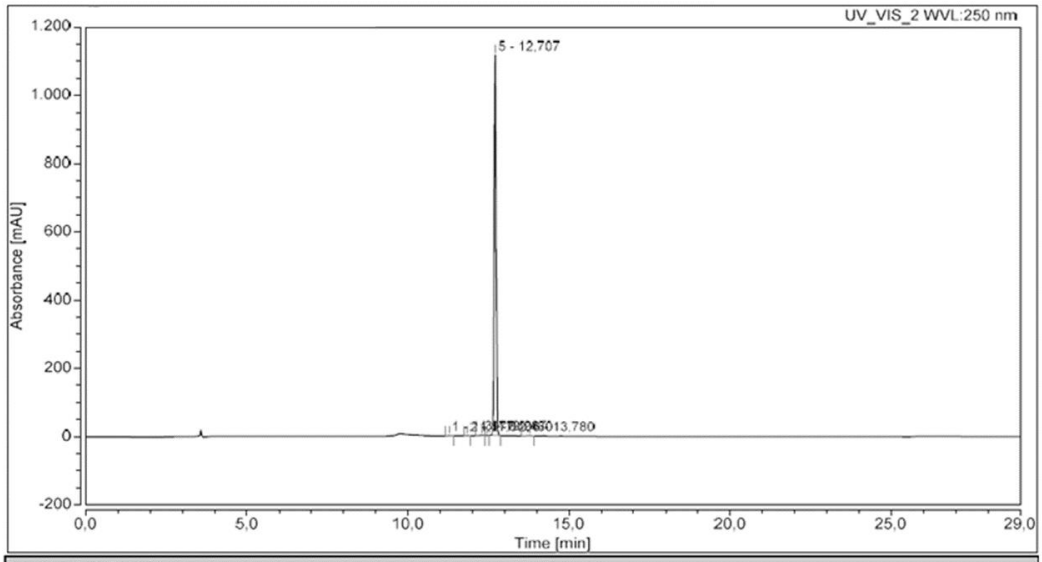


^{13}C NMR spectrum of **19** (151 MHz, $CDCl_3$)



3 HPLC Chromatograms

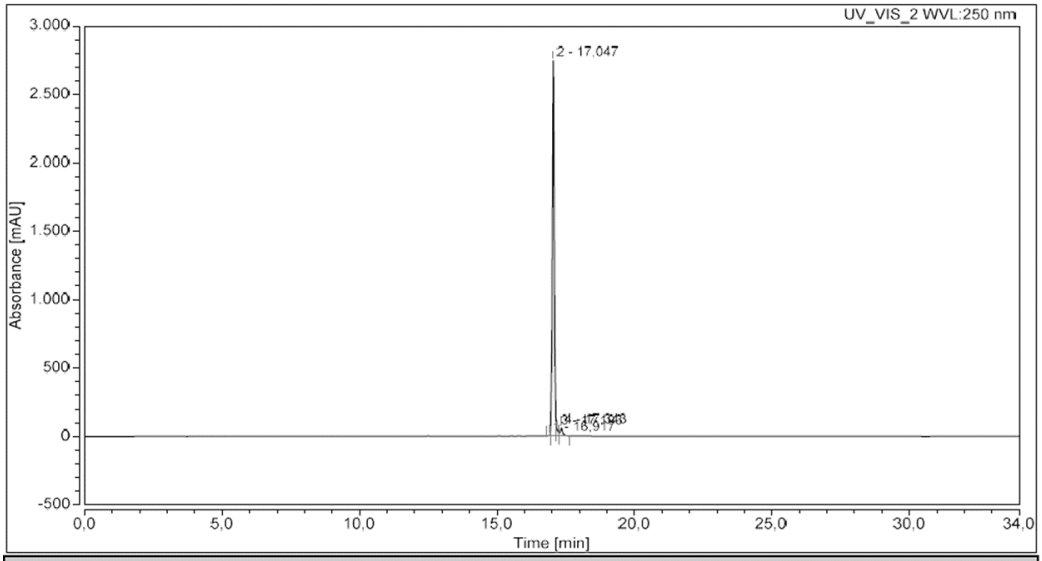
HPLC chromatogram of **6**.



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		11,277	0,104	1,336	0,14	0,12	n.a.
2		11,840	0,055	0,610	0,07	0,05	n.a.
3		12,287	0,197	2,193	0,26	0,20	n.a.
4		12,460	0,063	1,000	0,08	0,09	n.a.
5		12,707	74,825	1114,514	99,27	99,44	n.a.
6		13,780	0,127	1,122	0,17	0,10	n.a.
Total:			**75,372**	**1120,775**	**100,00**	**100,00**	

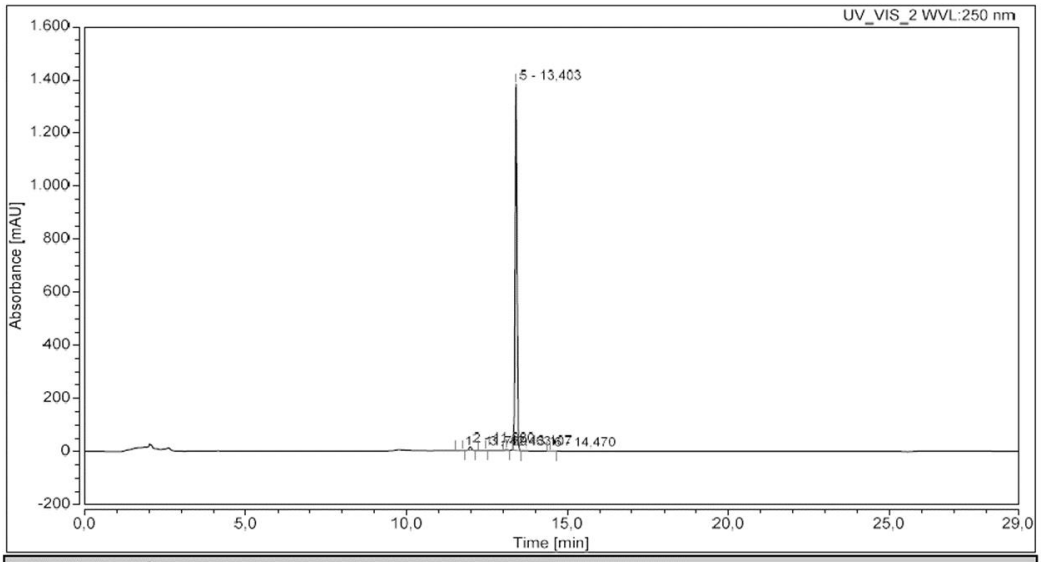
HPLC chromatogram of **9**.



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		16,917	0,383	6,653	0,16	0,23	n.a.
2		17,047	229,074	2744,890	95,72	95,88	n.a.
3		17,193	3,848	51,276	1,61	1,79	n.a.
4		17,343	5,999	60,125	2,51	2,10	n.a.
Total:			**239,304**	**2862,945**	**100,00**	**100,00**	

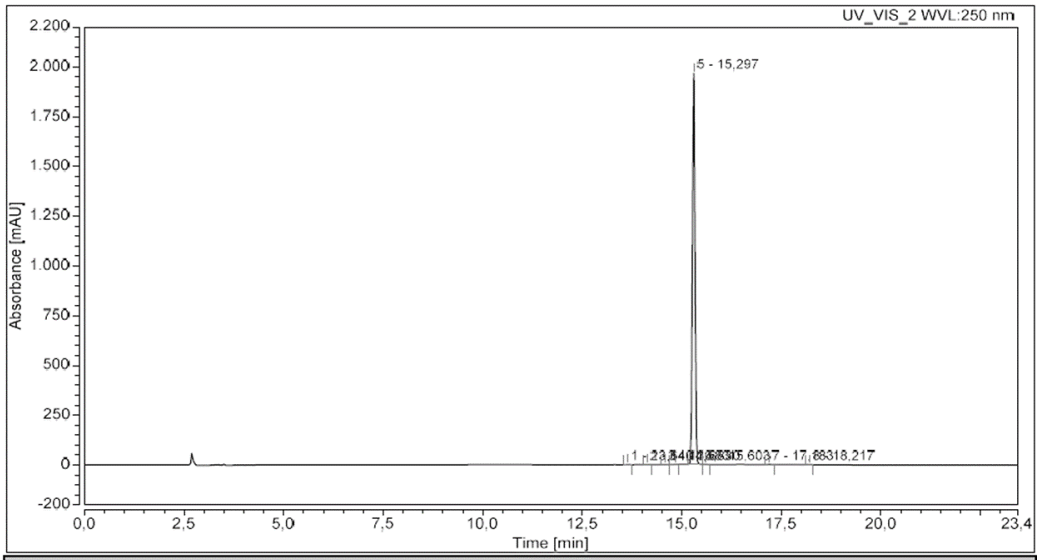
HPLC chromatogram of **10**.



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		11,730	0,188	1,976	0,20	0,14	n.a.
2		11,980	1,382	16,316	1,46	1,16	n.a.
3		12,463	0,056	0,399	0,06	0,03	n.a.
4		13,107	0,361	4,111	0,38	0,29	n.a.
5		13,403	92,416	1380,661	97,84	98,33	n.a.
6		14,470	0,051	0,618	0,05	0,04	n.a.
Total:			**94,454**	**1404,081**	**100,00**	**100,00**	

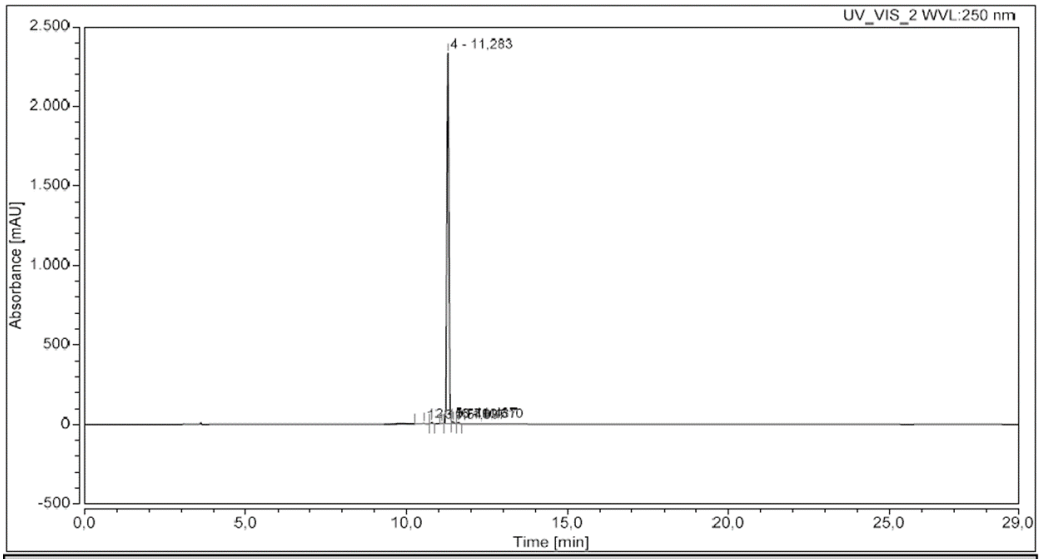
HPLC chromatogram of **12**.



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		13,640	0,109	1,534	0,07	0,08	n.a.
2		14,130	0,139	1,959	0,09	0,10	n.a.
3		14,583	0,060	0,785	0,04	0,04	n.a.
4		14,830	0,088	1,154	0,06	0,06	n.a.
5		15,297	150,362	1964,865	99,55	99,54	n.a.
6		15,603	0,083	1,056	0,05	0,05	n.a.
7		17,183	0,150	1,757	0,10	0,09	n.a.
8		18,217	0,052	0,787	0,03	0,04	n.a.
Total:			**151,042**	**1973,896**	**100,00**	**100,00**	

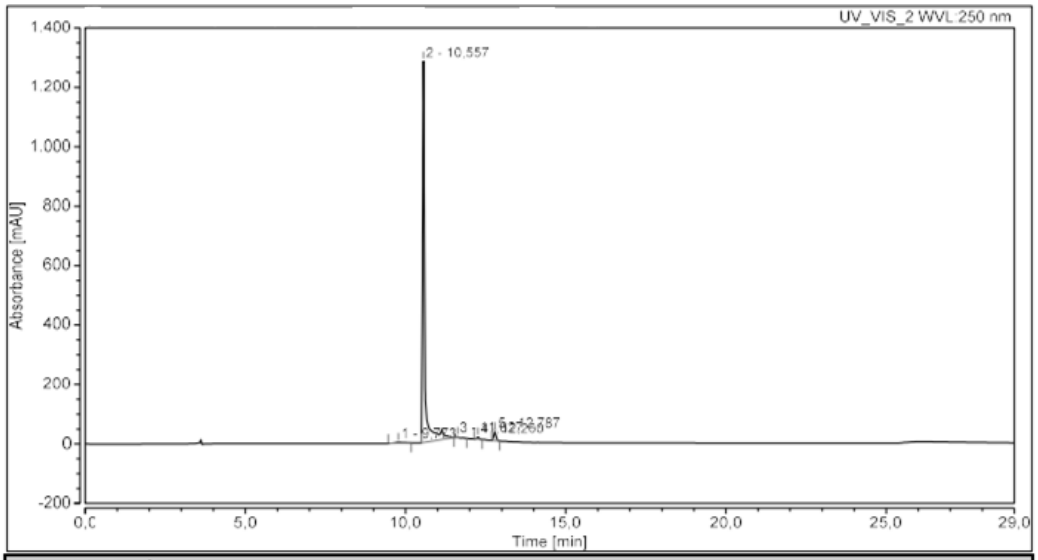
HPLC chromatogram of **13**.



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		10,540	0,760	6,097	0,45	0,26	n.a.
2		10,790	0,628	9,940	0,38	0,42	n.a.
3		11,097	0,229	3,264	0,14	0,14	n.a.
4		11,283	163,658	2332,505	97,92	98,18	n.a.
5		11,437	1,170	13,436	0,70	0,57	n.a.
6		11,610	0,689	10,437	0,41	0,44	n.a.
Total:			**167,134**	**2375,678**	**100,00**	**100,00**	

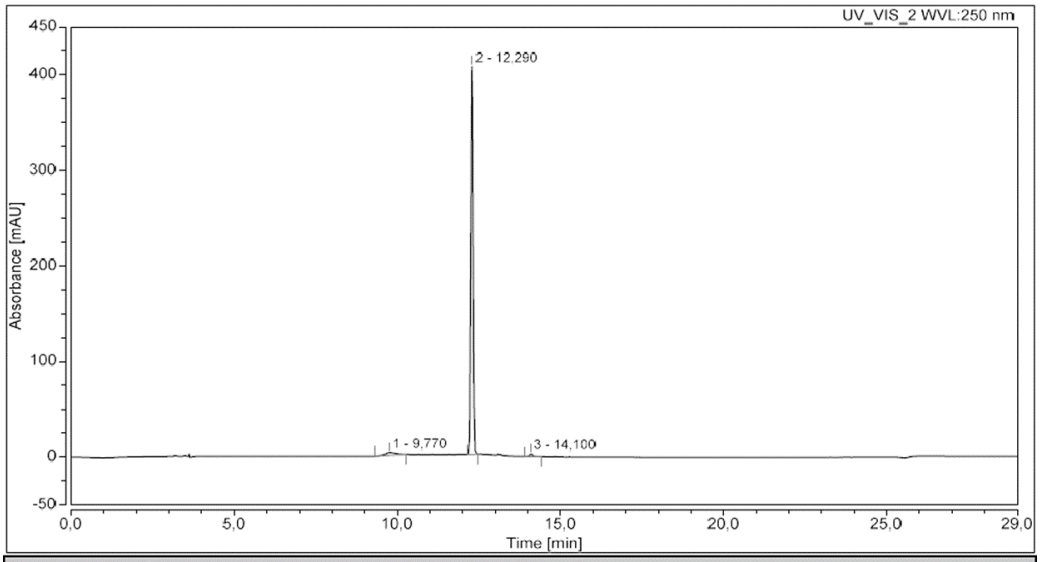
HPLC chromatogram of **14**.



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		9,773	0,904	2,965	0,87	0,22	n.a.
2		10,557	99,659	1283,046	95,85	96,64	n.a.
3		11,627	0,416	4,202	0,40	0,32	n.a.
4		12,260	0,582	7,570	0,56	0,57	n.a.
5		12,787	2,415	29,840	2,32	2,25	n.a.
Total:			**103,977**	**1327,622**	**100,00**	**100,00**	

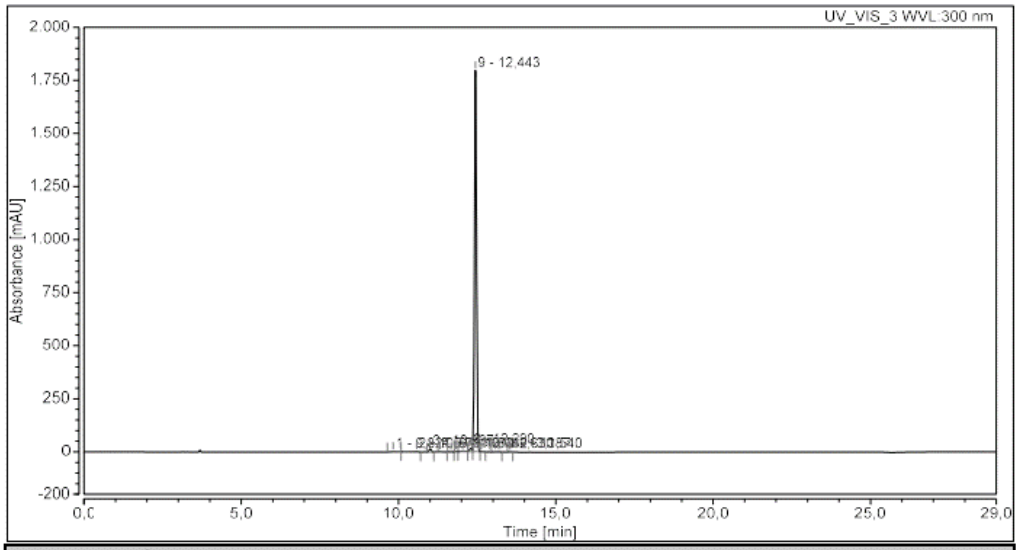
HPLC chromatogram of **15**.



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		9,770	1,128	2,972	3,40	0,72	n.a.
2		12,290	31,769	405,888	95,71	98,56	n.a.
3		14,100	0,295	2,959	0,89	0,72	n.a.
Total:			**33,191**	**411,819**	**100,00**	**100,00**	

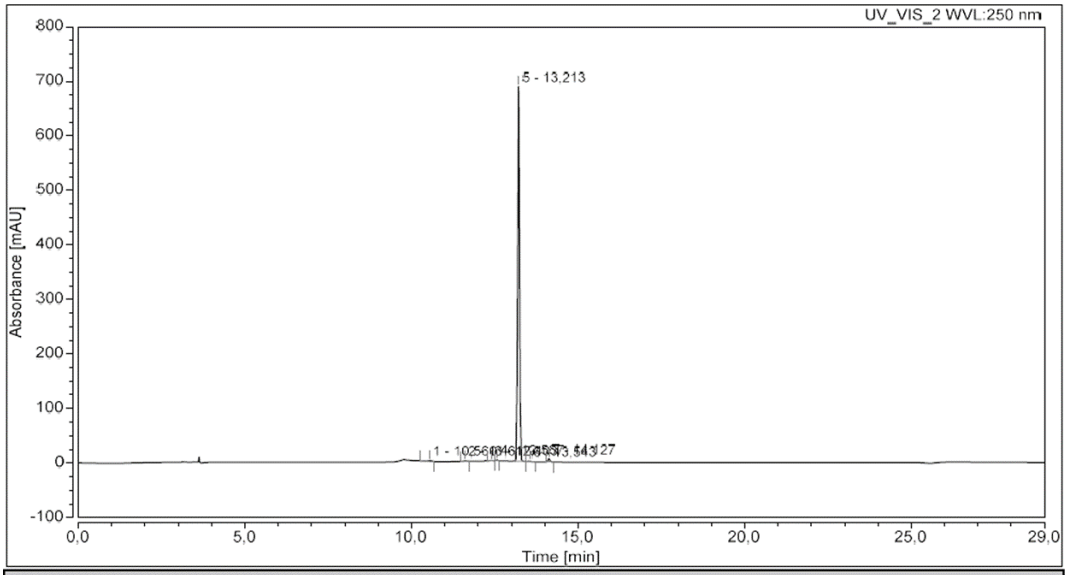
HPLC chromatogram of **16**.



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		9,827	0,241	1,164	0,18	0,06	n.a.
2		10,570	0,493	2,484	0,36	0,13	n.a.
3		10,997	1,181	16,517	0,87	0,90	n.a.
4		11,317	0,282	2,511	0,21	0,14	n.a.
5		11,670	0,056	0,587	0,04	0,03	n.a.
6		11,833	0,077	1,249	0,06	0,07	n.a.
7		12,040	0,506	3,387	0,37	0,18	n.a.
8		12,290	1,301	17,316	0,95	0,94	n.a.
9		12,443	132,078	1794,888	96,72	97,36	n.a.
10		12,630	0,075	0,679	0,06	0,04	n.a.
11		13,187	0,169	1,286	0,12	0,07	n.a.
12		13,540	0,097	1,498	0,07	0,08	n.a.
Total:			**136,557**	**1843,567**	**100,00**	**100,00**	

HPLC chromatogram of **17**.



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		10,560	0,283	2,011	0,63	0,29	n.a.
2		11,617	0,096	1,234	0,21	0,18	n.a.
3		12,403	0,079	0,833	0,18	0,12	n.a.
4		12,567	0,149	2,399	0,33	0,34	n.a.
5		13,213	43,425	687,496	97,30	97,94	n.a.
6		13,543	0,125	1,441	0,28	0,21	n.a.
7		14,127	0,473	6,510	1,06	0,93	n.a.
Total:			**44,629**	**701,925**	**100,00**	**100,00**	

4 References

(1) Yung-Chi, C.; Prusoff, W. H. Relationship between the Inhibition Constant (KI) and the Concentration of Inhibitor which causes 50 per cent Inhibition (I50) of an Enzymatic Reaction. *Biochem. Pharmacol.* **1973**, *22*, 3099–3108.

(2) Schäker-Hübner, L.; Haschemi, R.; Büch, T.; Kraft, F. B.; Brumme, B.; Schöler, A.; Jenke, R.; Meiler, J.; Aigner, A.; Bendas, G.; Hansen, F. K. Balancing Histone Deacetylase (HDAC) Inhibition and Drug-Likeness: Biological and Physicochemical Evaluation of Class I Selective HDAC Inhibitors. *ChemMedChem* **2022**, *17*, e202100755.