Design, synthesis, biological evaluation and X-ray structural studies of potent human dihydroorotate dehydrogenase inhibitors based on hydroxylated azole scaffolds

Supporting Information

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# Both authors contributed equally to this work.

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Table S1. Comparison of docking scores and experimental activity on *h*DHODH.

Compound	**Glide Score (Kcal/mol)**	***h*DHODH IC_{50} ± SE (µM)**
BQN	-14.81	0.0018±0.0003
A771726	-9.21	0.388±0.064
1	-11.34	0.599±0.074
2	-10.91	0.289±0.017
3	-12.52	0.044±0.005
4	-13.40	0.016±0.001
5	-13.30	0.041±0.007
6	-12.67	0.045±0.013
6a	-12.09	0.853±0.140
6b	-10.56	6.0±1.9
7	-13.59	0.018±0.001
8	-12.52	0.108±0.010
9	-13.55	0.036±0.004

2D - NMR of compound 18.

Compound **18** was checked with 2D NMR experiments to confirm the position of the NCH_3 in the pyrazolic ring.

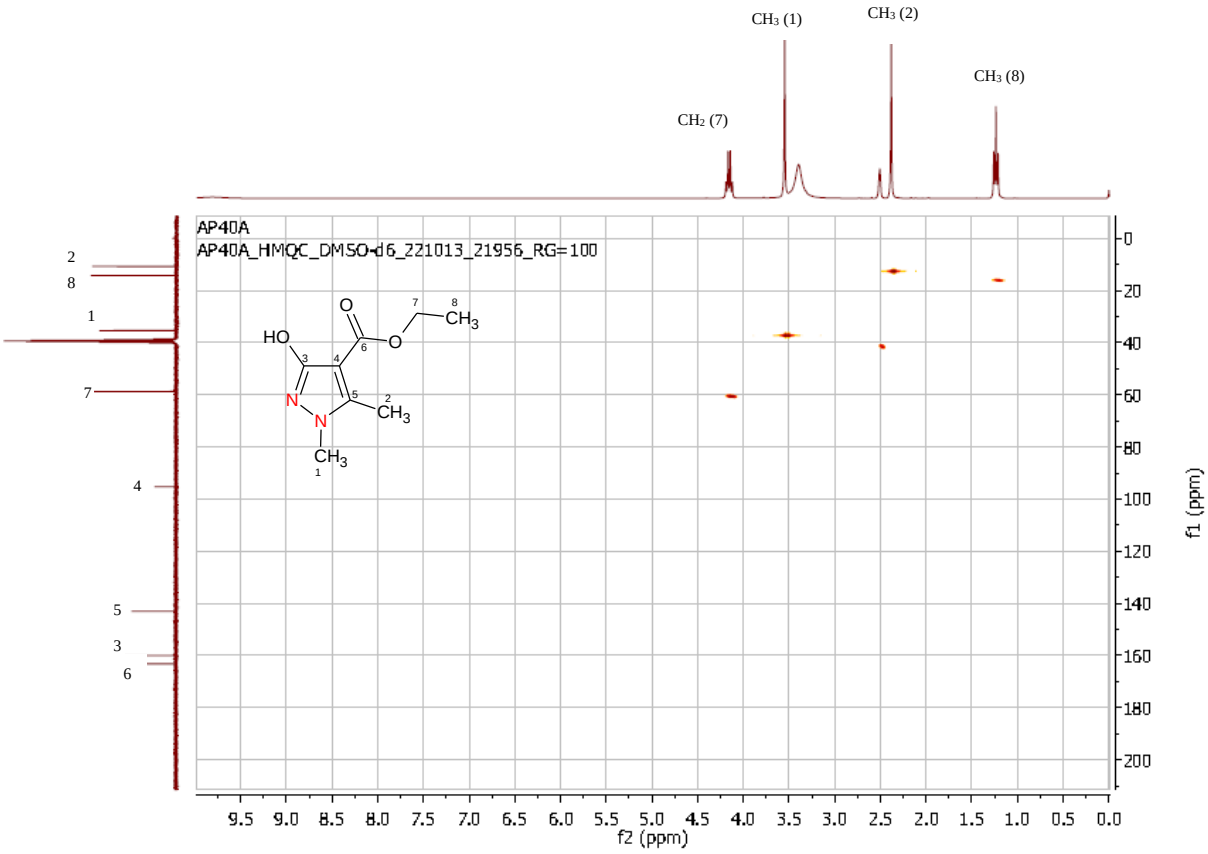


Figure S1. HMQC (Heteronuclear Multiple-Quantum Correlation) spectrum of and atom numbering of compound **18**. ^{1}H and ^{13}C assignments are reported directly on the spectrum.

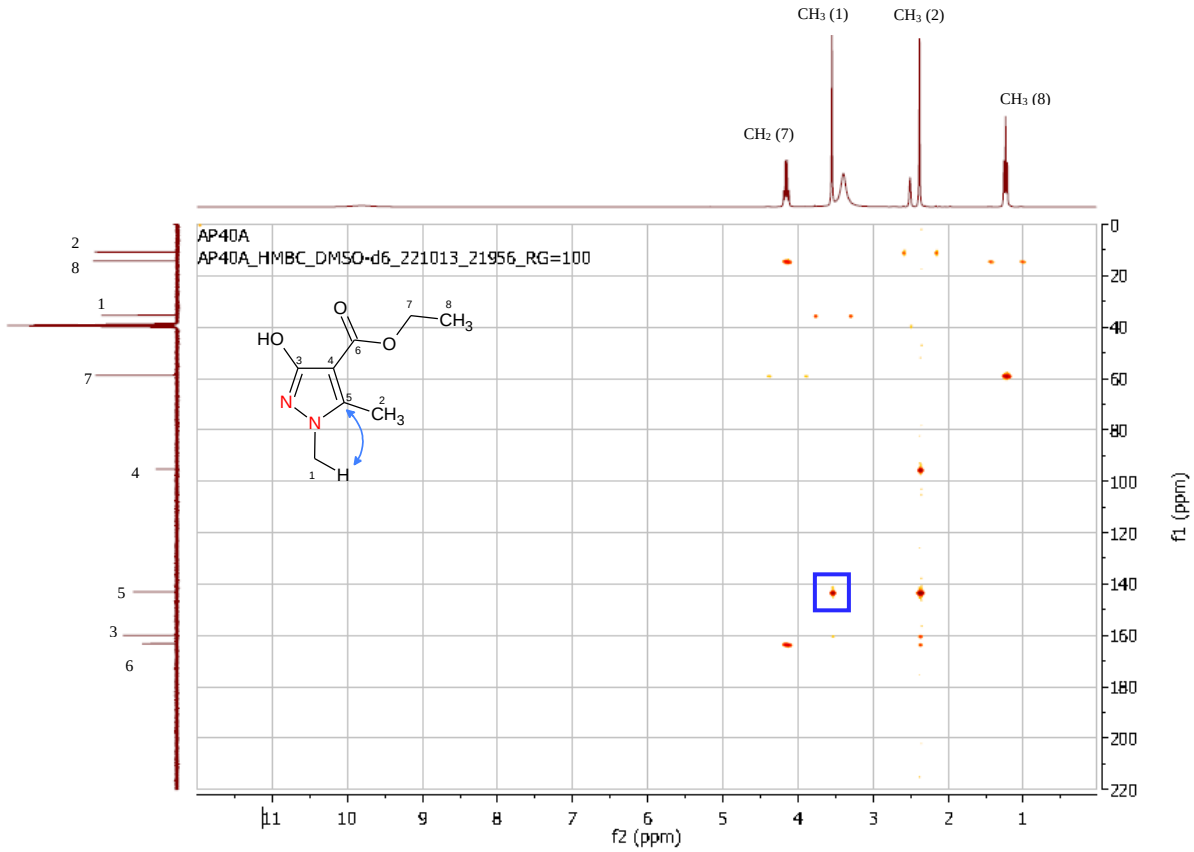


Figure S2. HMBC (Heteronuclear Multiple Bond Correlation) spectrum of compound **18**. ^{3}J correlation between ^{1}H-1 and ^{13}C-5 is highlighted by a blue box**.**

Solubility assay at pH 7.4 for compounds 4 -7, compared with brequinar and teriflunomide.

Each solid compound (1mg) was added to 1 mL of Phosphate Buffer Solution (10mM with 10 mM KCl, pH 7.4). The samples were shaken with an orbital shaker at 25°C for 24 h. These suspensions were filtered through a PES 0.2 μm filter (Agilent) and the solutions were chromatographically analyzed. Quantitative analysis was performed by HPLC-UV system (Waters) equipped with an autosampler of 10 μL injection volume (Waters 717 plus), a binary HPLC pump (Waters 1525 EF) and a photodiode array detector (Waters 2996). LC analysis was performed using *Phenomenex Kinetex Biphenyl Column* (4.6x150, 5 μm, 100 Å). Analysis were carried out at a flow rate of 1 mL/min using gradient elution with eluent A being trifluoroacetic acid (TFA) 0.1% in water, and B TFA 0.1% in MeOH for brequinar, teriflunomide and compounds **4**, **6** and **7**. The analysis started with 50% of eluent B and gradient profile used was: (time min, %B) 10.0, 80%; 22.4, 100%; 34.9, 100%. For compound **5** eluent A was TFA 0.1% in water and eluent B acetonitrile, gradient profile was: (time, %B): 0, 50%; 7.5, 50%; 22.4, 100%; 32.4, 100%. Quantification of the single compound was made through relative calibration curve obtained analyzing standard solutions in MeOH. The solubility is expressed as μM concentration of saturated solution.

Stability assay of compounds 4 - 7, compared with brequinar and teriflunomide, in cell test conditions.

To verify the stability of compounds in cell test conditions 100 μM of each compound was incubated for 72h at 37°C on Jurkat cells. At the end of incubation cells were lysed with MeOH 1:2 (v/v) ratio, kept on ice for 5 min and centrifuged at 13,000 x g for 5 min at 4°C. Supernatants were filtered through a PES 0.2 μm filter (Agilent) and the solutions were chromatographically analyzed as described above. The results were compared with solutions suitably prepared in cell culture medium and immediately added with MeOH. The results are expressed as % of unmodified parent compound at 72 h.

Table S2. Solubility in PBS buffer and stability in cell culture medium after 72 h for compounds **4**-**7**.

	μM in PBS buffer	% compound at 72h
brequinar	359	98
teriflunomide	2692	101
4	1694	80
5	< 0.1*	94
6	711	88
7	48	99

*Solubility in PBS with 1% DMSO: 7 μM. Solubility in cell medium with 1% DMSO > 100 μM (UV checked).

Table S3: X-ray data collection and refinement statistics.

	Compound **4**	Compound **5**	Compound **6**
Resolution range[a] (Å)	29.85 - 1.85 (1.91 - 1.85)	30.43 - 1.95 (2.02 - 1.95)	26.29 - 1.75 (1.81 - 1.75)
Space group	P 3_221	P 3_221	P 3_221
Unit cell parameters (Å) a=b c	 91.19 122.64	 90.85 122.92	 91.07 122.52
Total reflections	471,097 (63,322)	404,893 (57,732)	1,109,261 (154,920)
Unique reflections	50,880 (4,991)	43,350 (4,310)	59,768 (5,913)
Multiplicity	9.3 (3.1)	9.3 (9.0)	18.5 (18.0)
Completeness (%)	100.00 (100.00)	100.00 (100.00)	100.00 (100.00)
Mean $I/\sigma(I)$	9.37 (2.93)	9.19 (2.88)	8.9 (3.70)
Wilson B-factor (Å^2)	15.05	15.83	11.12
R-merge[b]	0.17 (0.69)	0.20 (0.75)	0.26 (0.79)
Rpim[c]	0.06 (0.24)	0.06 (0.26)	0.06 (0.19)
Reflections used in refinement	50,874 (4,991)	43,345 (4,310)	59,765 (5,913)
Reflections used for R-free	2,522 (242)	2,100 (198)	2,910 (313)
R_{factor}/R_{free} (%)[d]	16.18 / 18.93	16.72 / 20.16	16.79 / 19.74
RMSD from ideal values			
Bond angles (°)	1.26	1.37	1.33
Bond distances (Å)	0.017	0.017	0.017
Number of non-hydrogen atoms	3,076	2,997	3,072
Macromolecules	2,708	2,684	2,692
Ligands	80	80	79
Protein residues	352	348	350
Ramachandran favored (%)	97	97	98
Ramachandran allowed (%)	2.9	2.9	2.3
Ramachandran outliers (%)	0	0	0
Rotamer outliers (%)	0.34	0.69	0.35
Clashscore	0.72	1.8	0.54
Average B-factor (Å)	15.73	16.42	12.88
Protein	14.84	15.72	11.94
FMN	6.6	5.9	4.6
ORO	8.3	8.8	7.0
Inhibitor	17.1	34.2	13.3
Solvent	25.1	24.13	22.14

[a] Number in parentheses indicate the outer-resolution shell.

[b] $R_{\text{merge}} = \sum_{\text{hkl}} \sum_i |I_i\,(\text{hkl}) - \langle I(\text{hkl}) \rangle| / \sum_{\text{hkl}} \sum_i I_i\,(\text{hkl})$.
[c] $R_{\text{p.i.m.}} = \sum_{\text{hkl}} [1/(N-1)]^{1/2} \sum_i |I_i\,(\text{hkl}) - \langle I(\text{hkl}) \rangle| / \sum_{\text{hkl}} \sum_i I_i\,(\text{hkl})$.
[d] $R_{factor}/R_{free} = \sum_{hkl} ||F_o| - |F_c|| / \sum_{hkl} |F_o|$, where F_o and F_c are the observed and calculated structure factors, respectively.

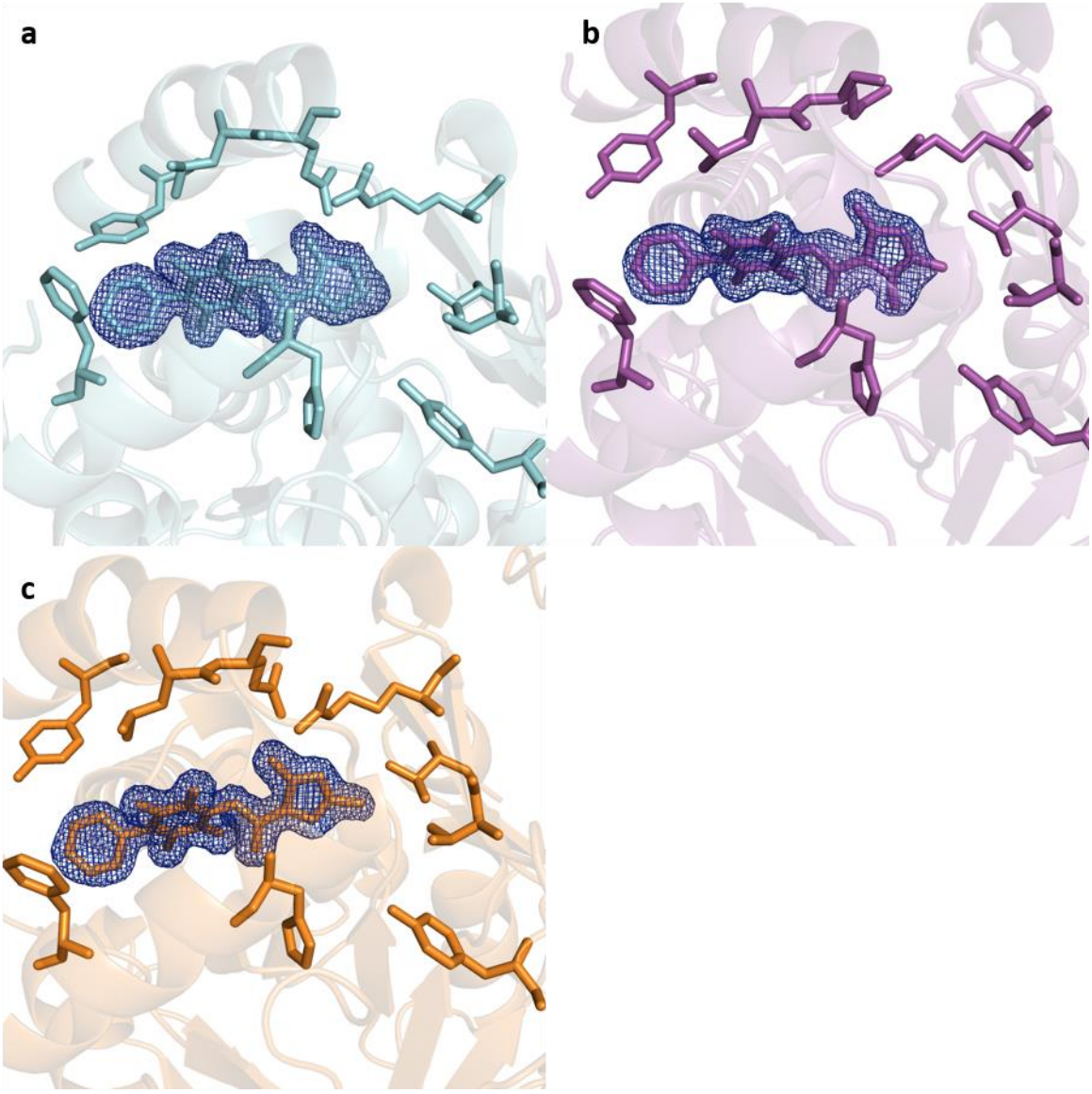
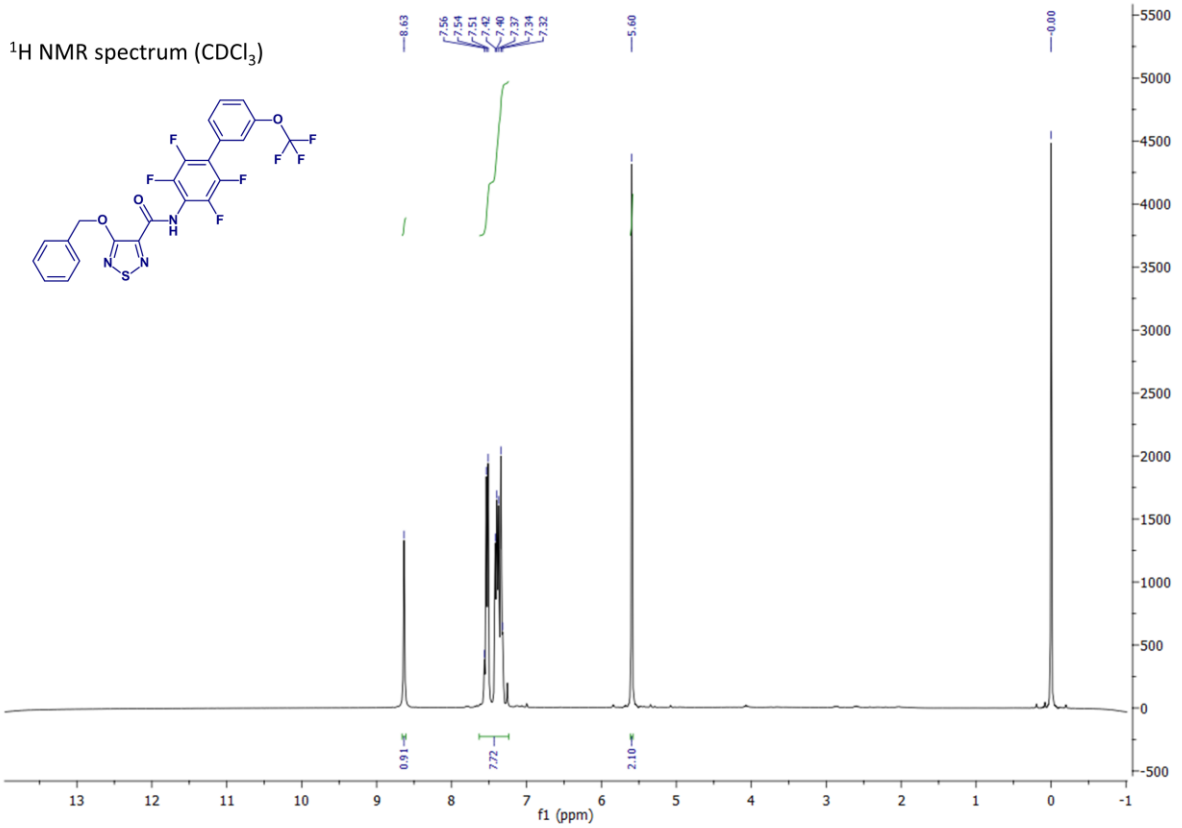


Figure S3. . Binding of compounds (a) **4**, (b) **5** and (c) **6** in the ubiquinone binding site of *h*DHODH. The $2F_{obs}$- F_{calc} maps are computed before the compounds were modeled. The $2F_{obs}$- F_{calc} is contoured at 1.0 s. The figure was made using PyMOL (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC, http://www.pymol.org).

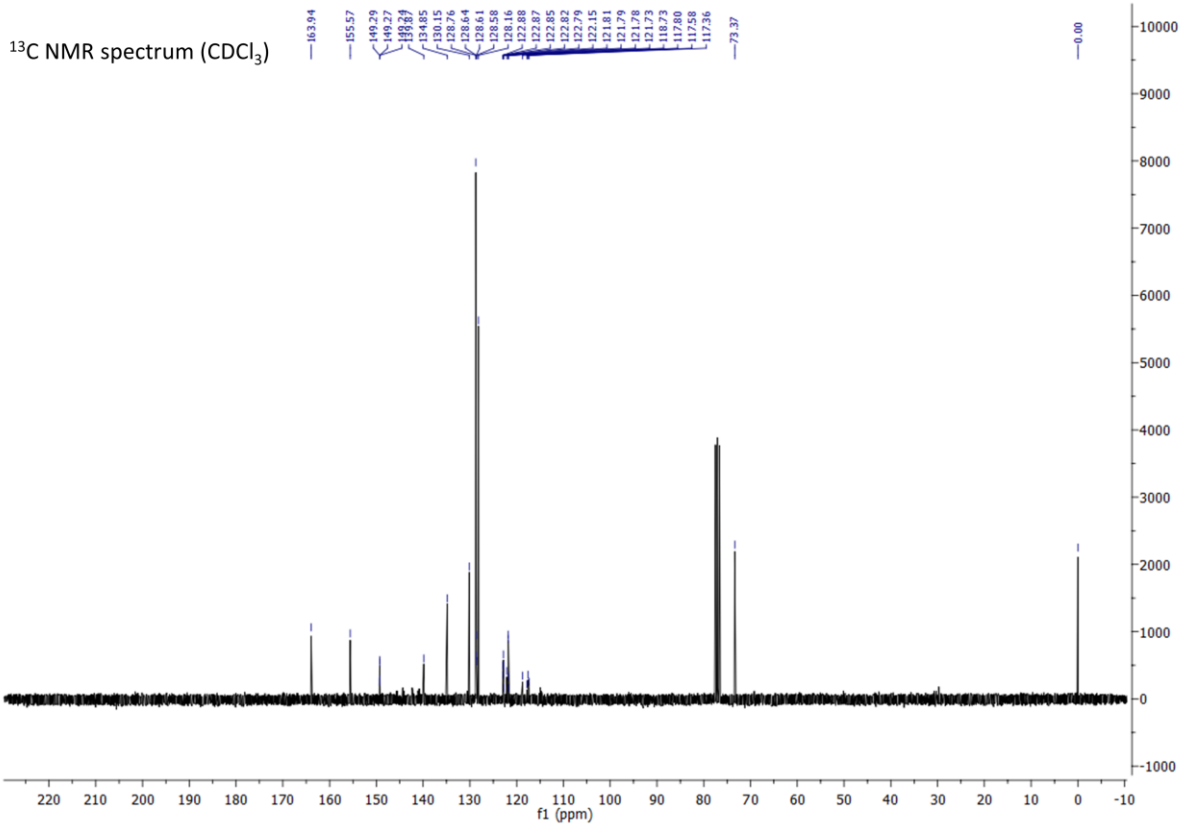
^{1}H-, ^{13}C-NMR and IR of representative compounds

4-Benzyloxy-N-[2,3,5,6-tetrafluoro-3'-(trifluoromethoxy)[1,1'-biphenyl]-4-yl]-1,2,5-thiadiazole-3-carboxamide (16)

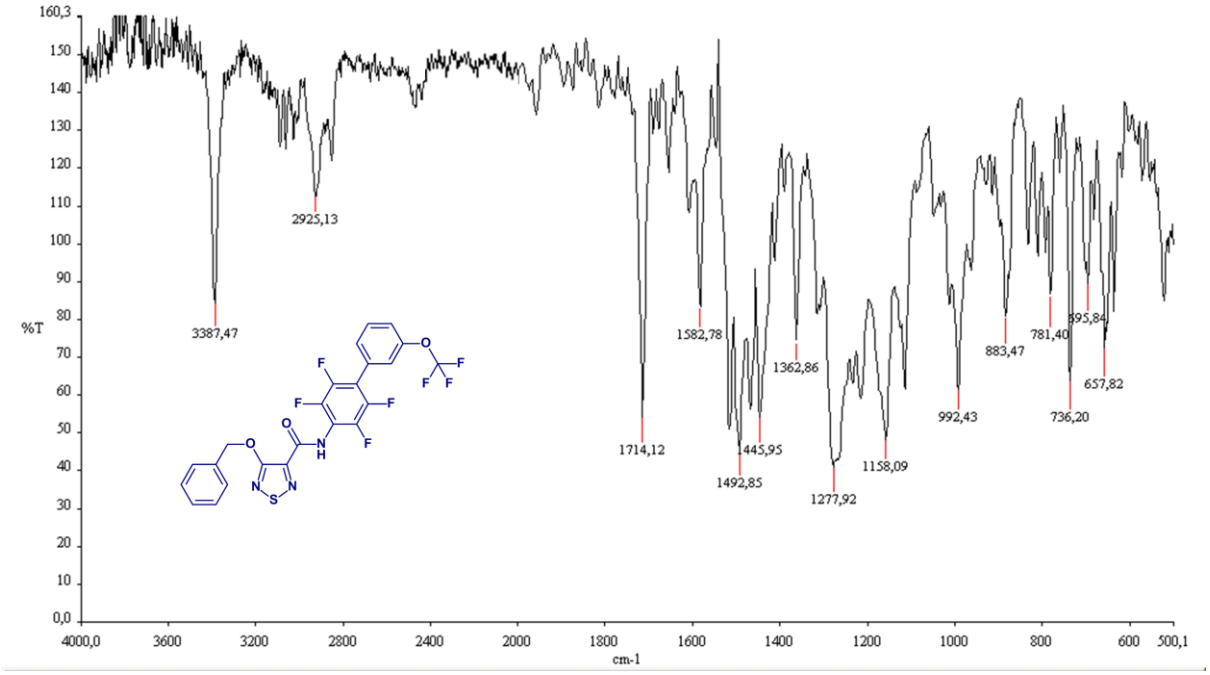
^{1}H NMR spectrum ($CDCl_3$)



^{13}C NMR spectrum ($CDCl_3$)

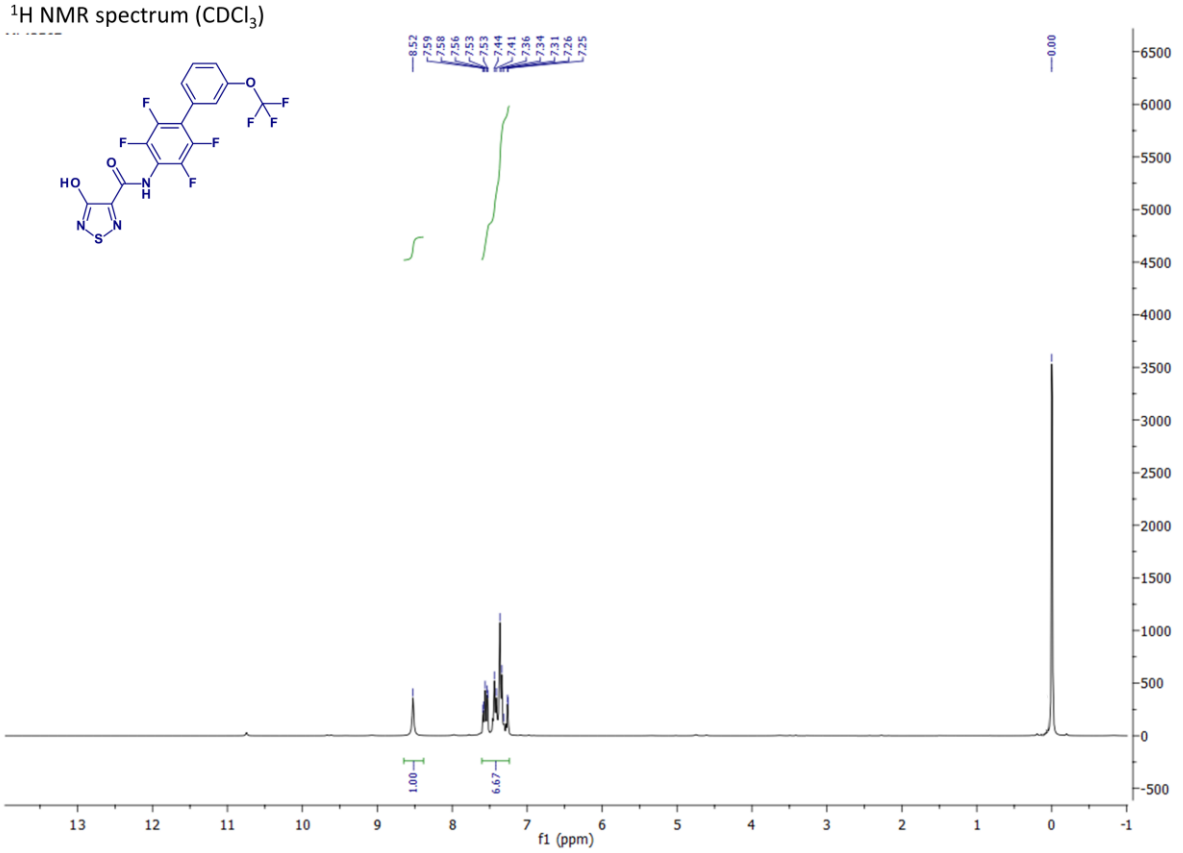


IR (KBr) spectrum

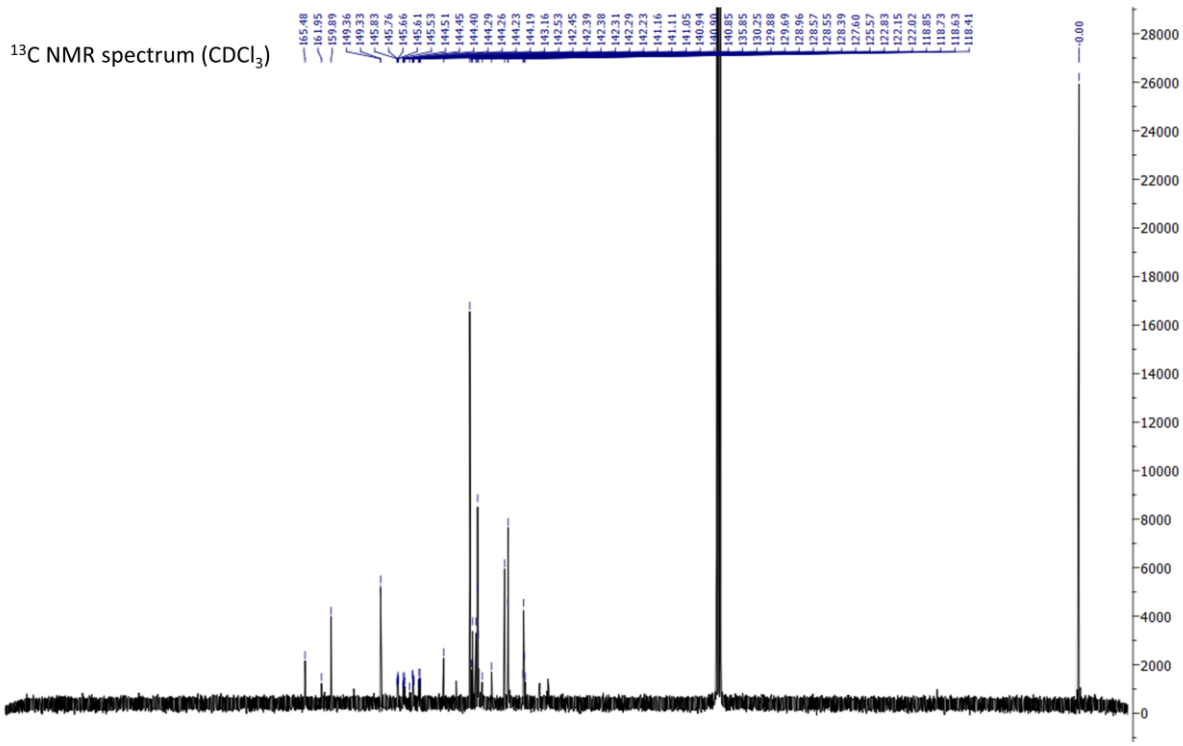


4-Hydroxy-N-[2,3,5,6-tetrafluoro-3'-(trifluoromethoxy)[1,1'-biphenyl]-4-yl]-1,2,5-thiadiazole-3-carboxamide (3)

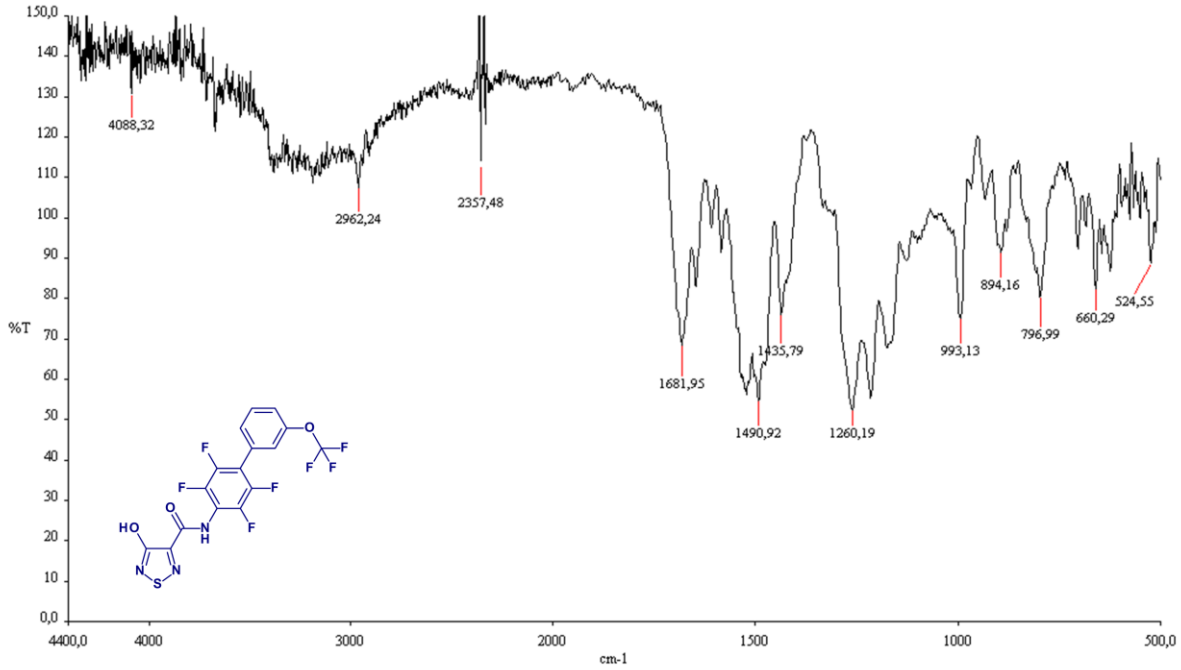
^{1}H NMR spectrum ($CDCl_3$)



^{13}C NMR spectrum ($CDCl_3$)

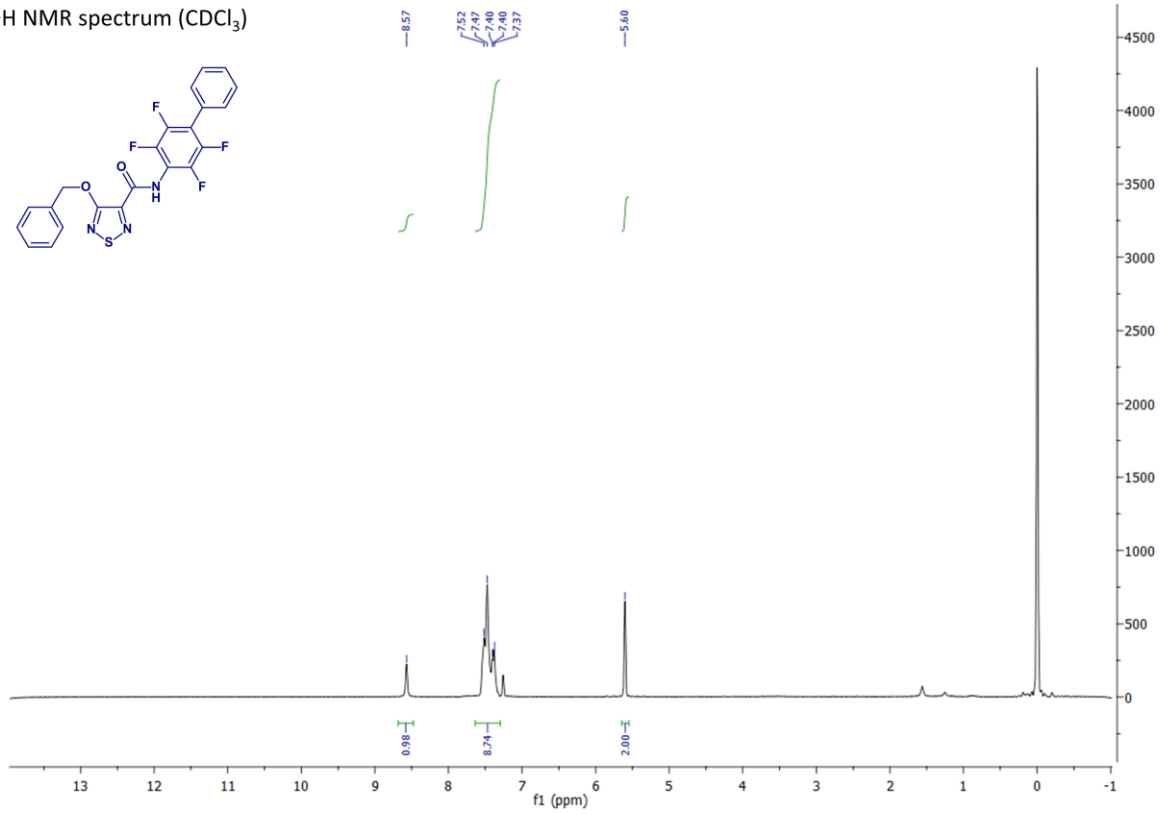


IR (KBr) spectrum

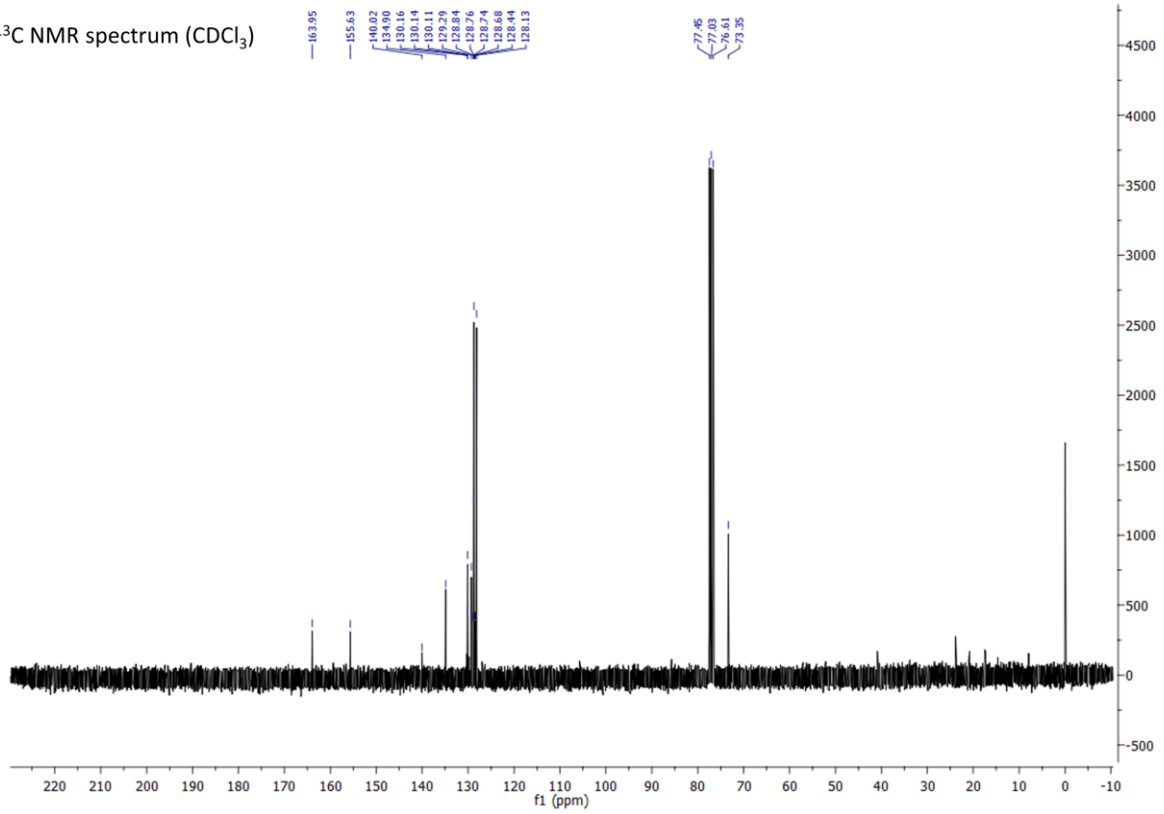


4-Benzyloxy-N-(2,3,5,6-tetrafluoro[1,1'-biphenyl]-4-yl)-1,2,5-thiadiazole-3-carboxamide (17)

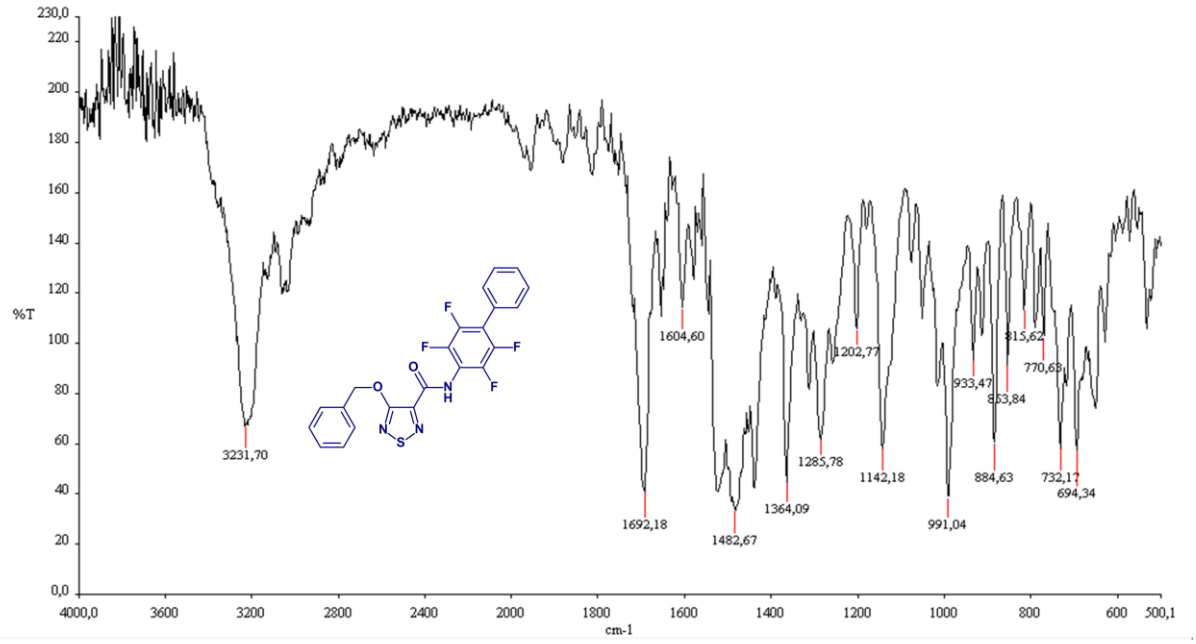
^{1}H NMR spectrum ($CDCl_3$)



^{13}C NMR spectrum ($CDCl_3$)

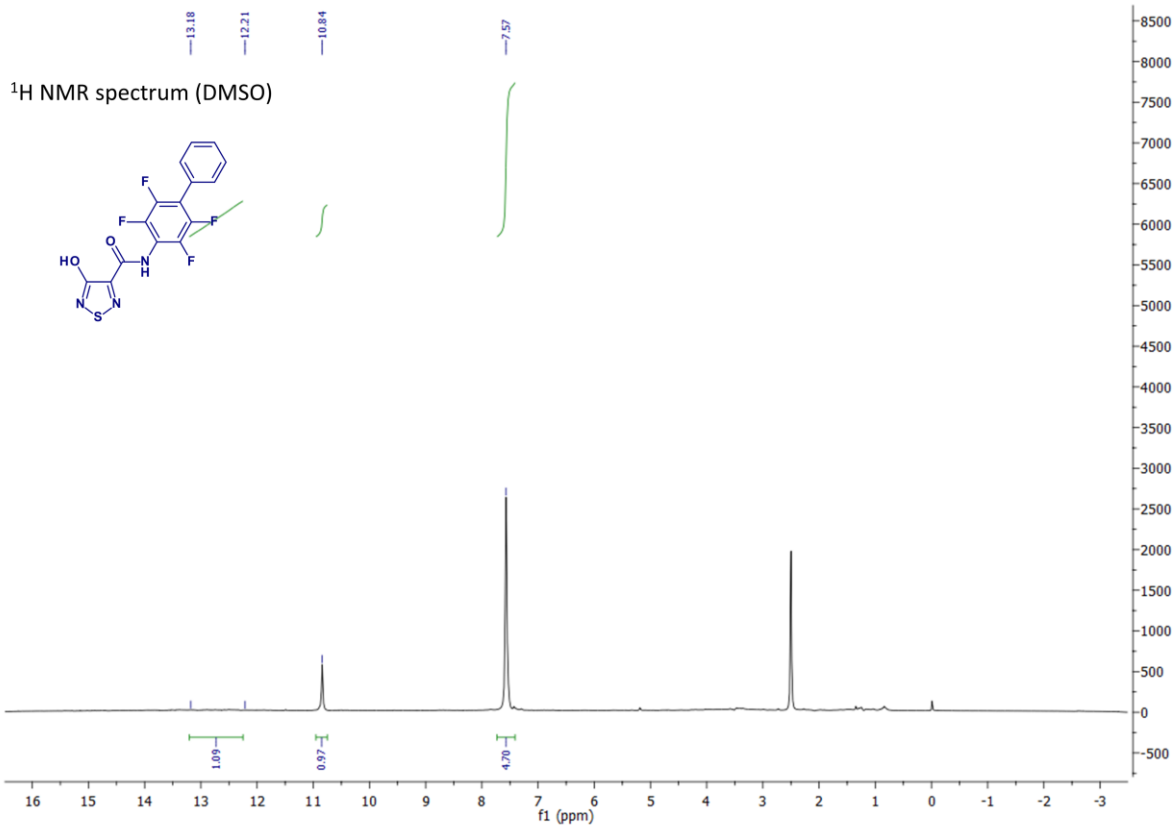


IR (KBr) spectrum

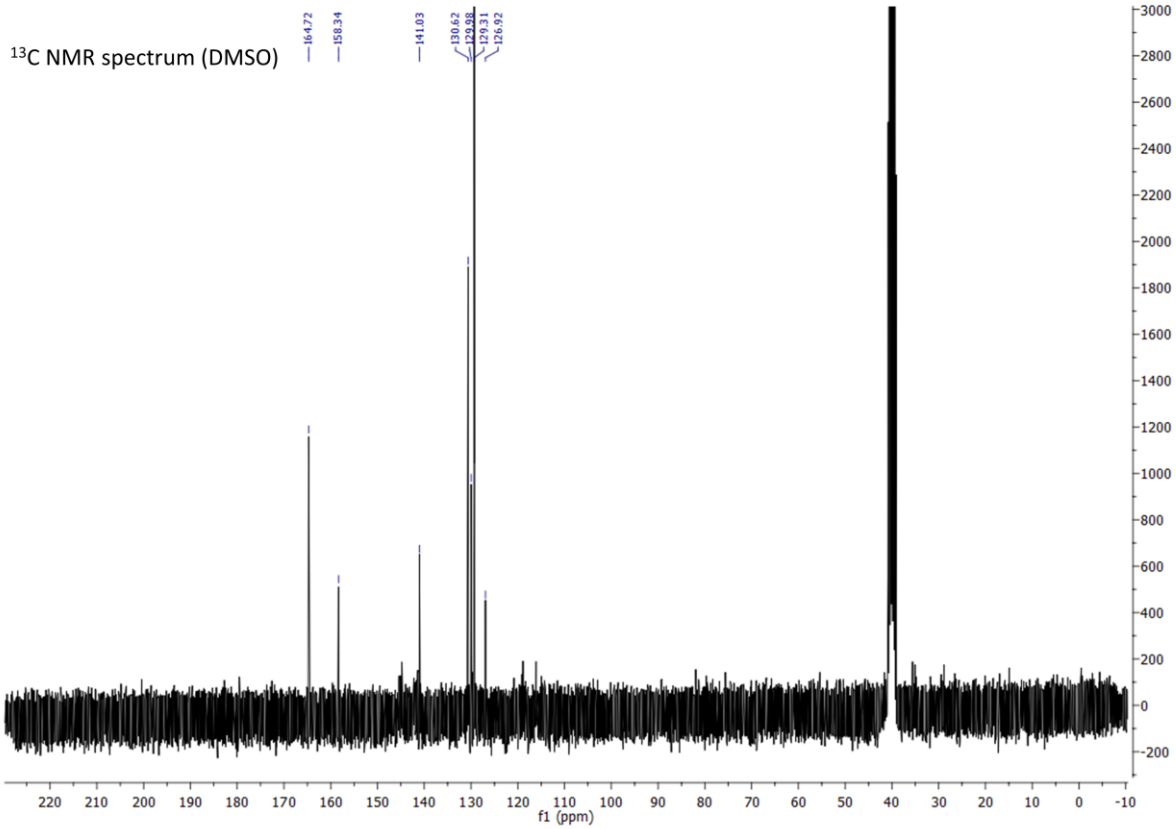


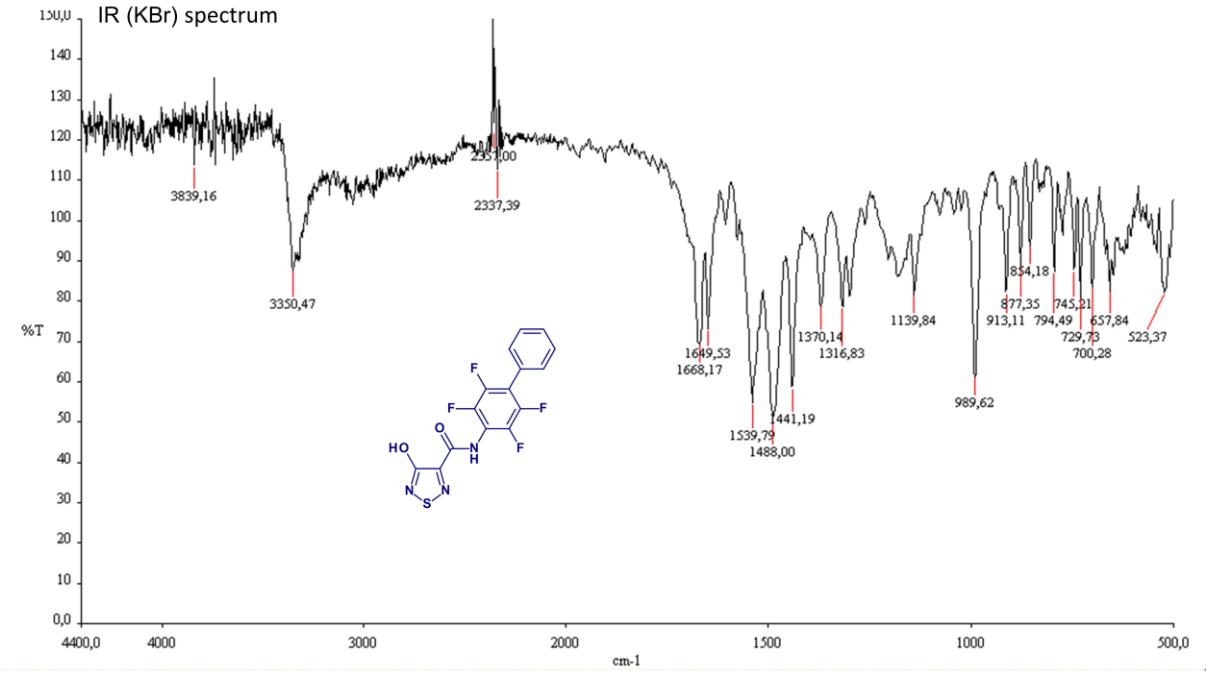
4-Hydroxy-N-(2,3,5,6-tetrafluoro[1,1'-biphenyl]-4-yl)-1,2,5-thiadiazole-3-carboxamide (4)

^{1}H NMR spectrum (DMSO)



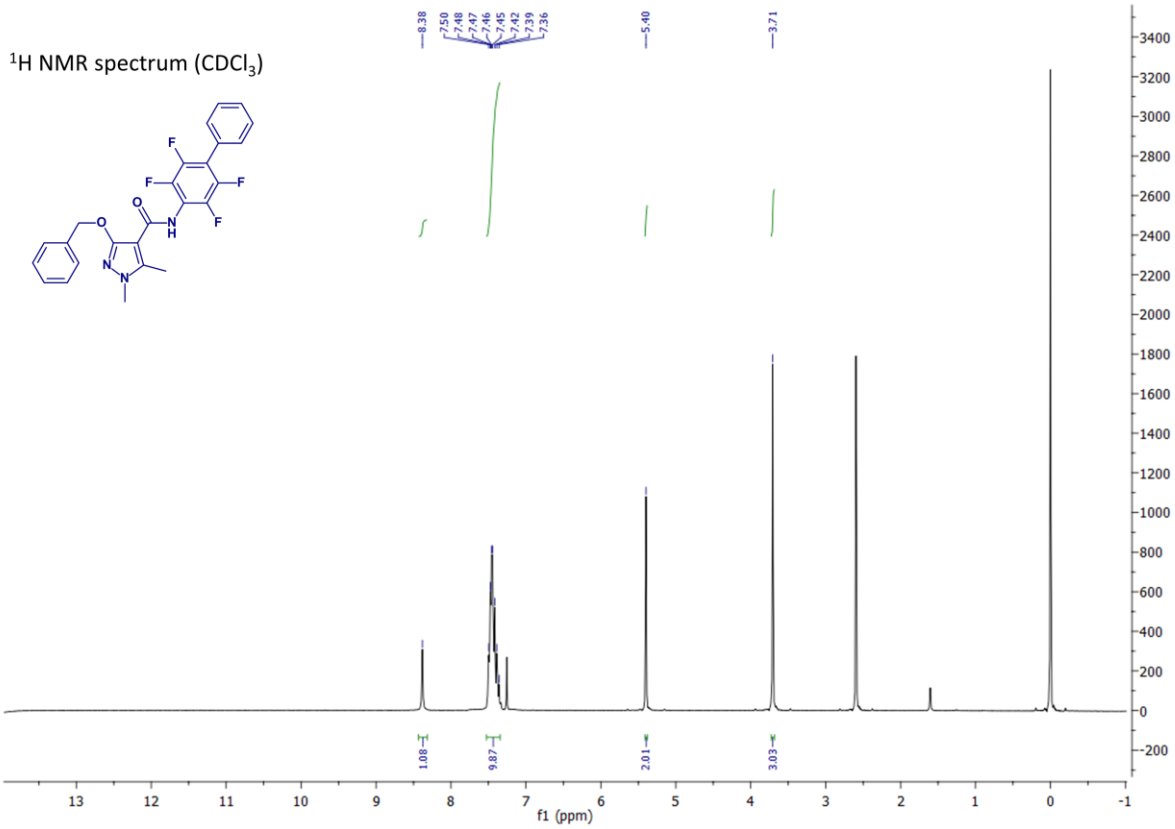
^{13}C NMR spectrum (DMSO)



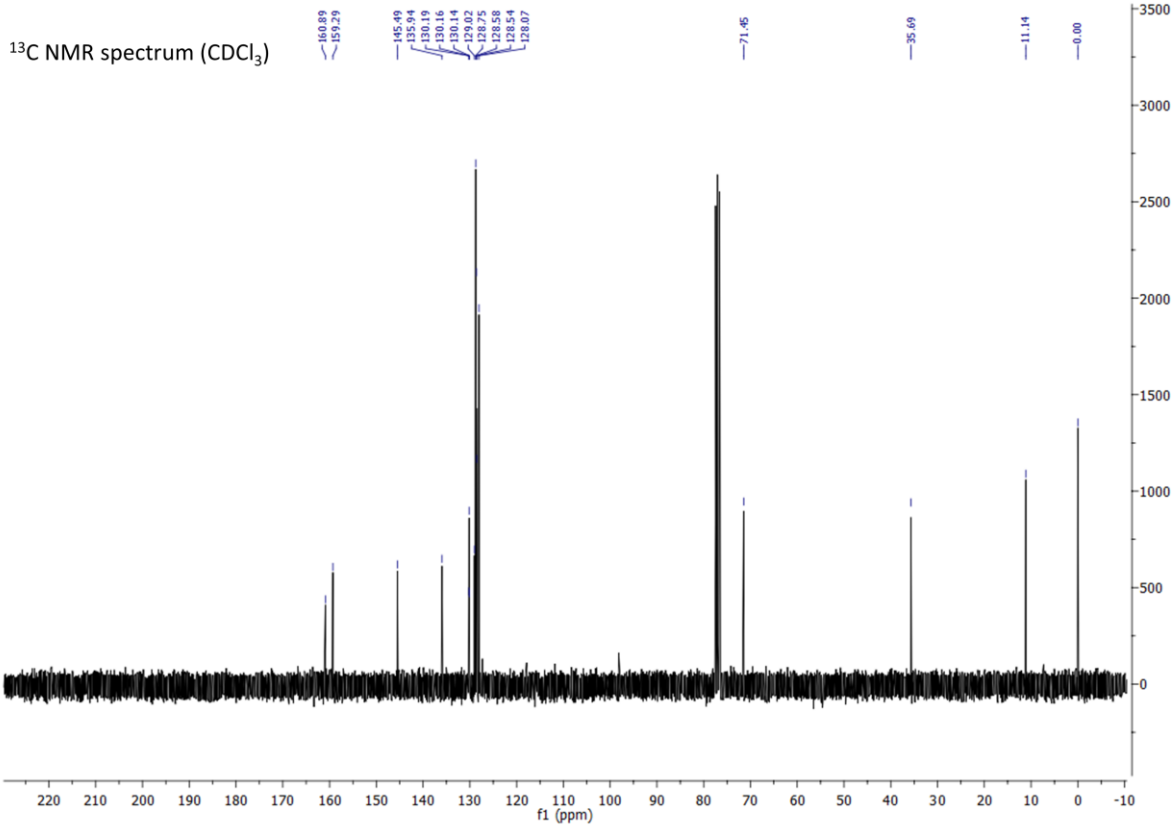

IR (KBr) spectrum
%T
cm-1
3839,16
3350,47
2337,39
1649,53
1668,17
1539,79
1488,00
1441,19
1370,14
1316,83
1139,84
989,62
913,11
877,35
854,18
794,49
745,21
729,73
700,28
657,84
523,37
HO
O
N
H
F
F
F
F
N
S
N

3-Benzyloxy-1,5-dimethyl-N-(2,3,5,6-tetrafluoro[1,1'-biphenyl]-4-yl)-1H-pyrazole-4-carboxamide (22)

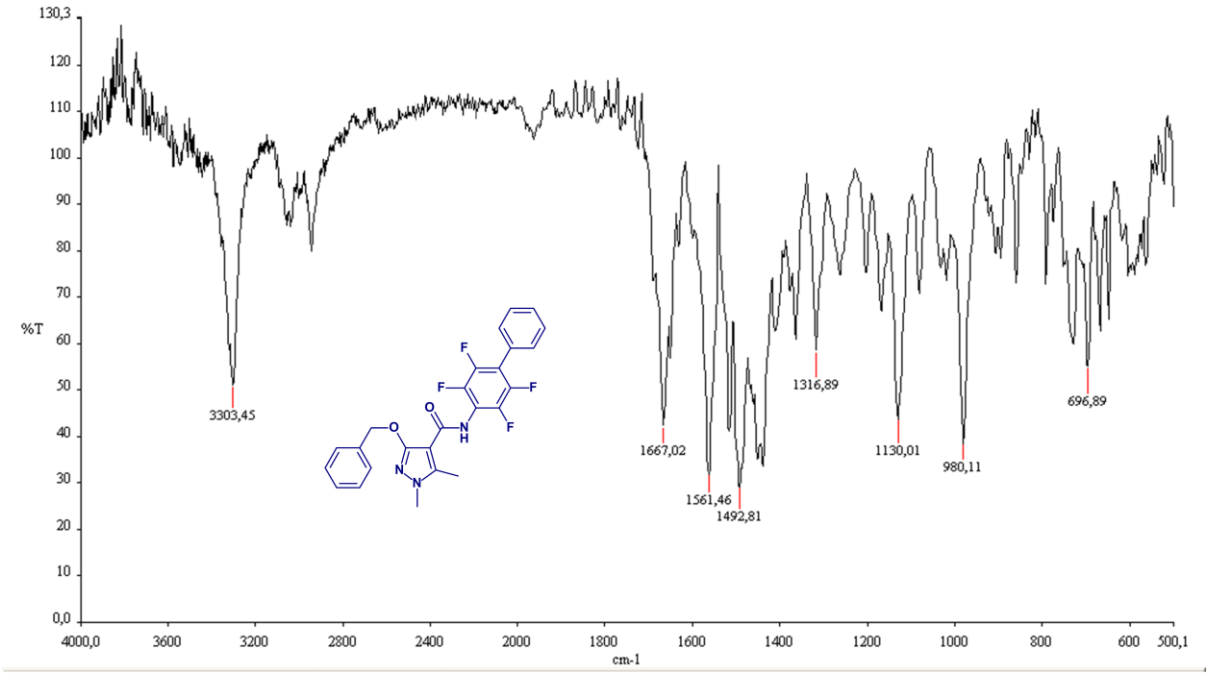
^{1}H NMR spectrum ($CDCl_3$)



^{13}C NMR spectrum ($CDCl_3$)

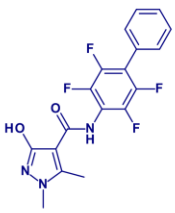


IR (KBr) spectrum

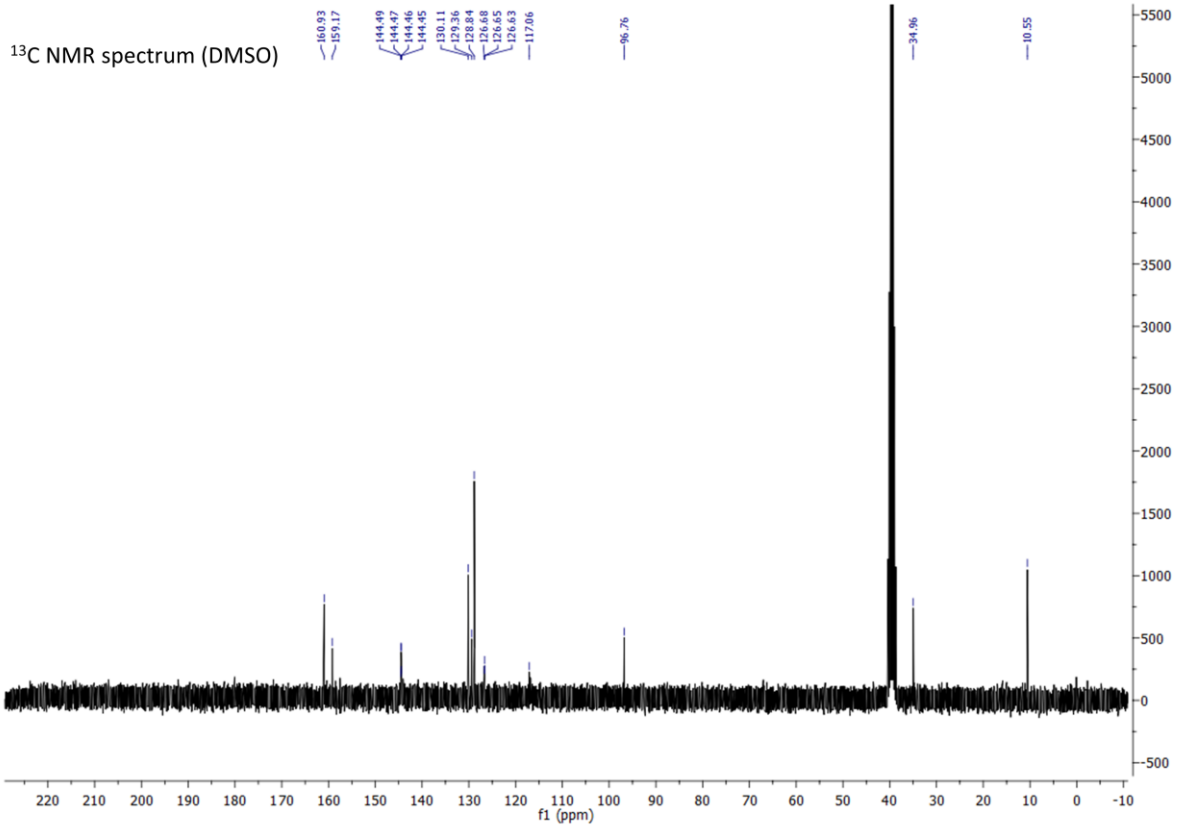


3-Hydroxy-1,5-dimethyl-N-(2,3,5,6-tetrafluoro[1,1'-biphenyl]-4-yl)-1H-pyrazole-4-carboxamide (5)

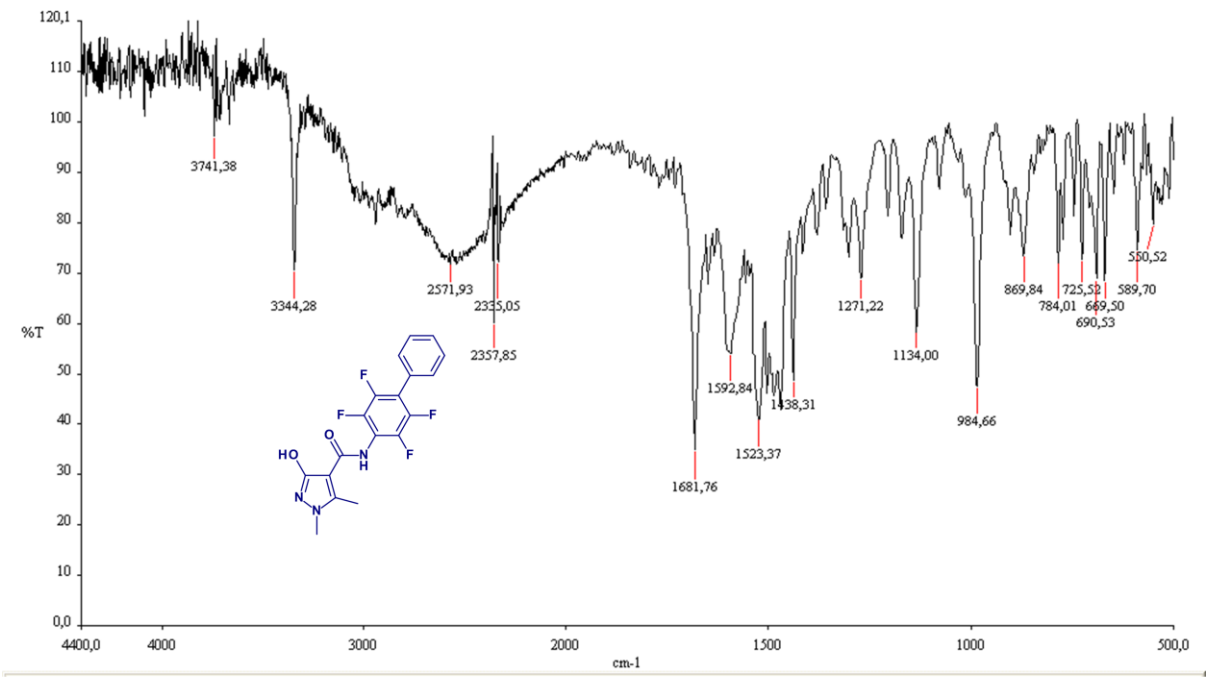
^{1}H NMR spectrum (DMSO)



^{13}C NMR spectrum (DMSO)

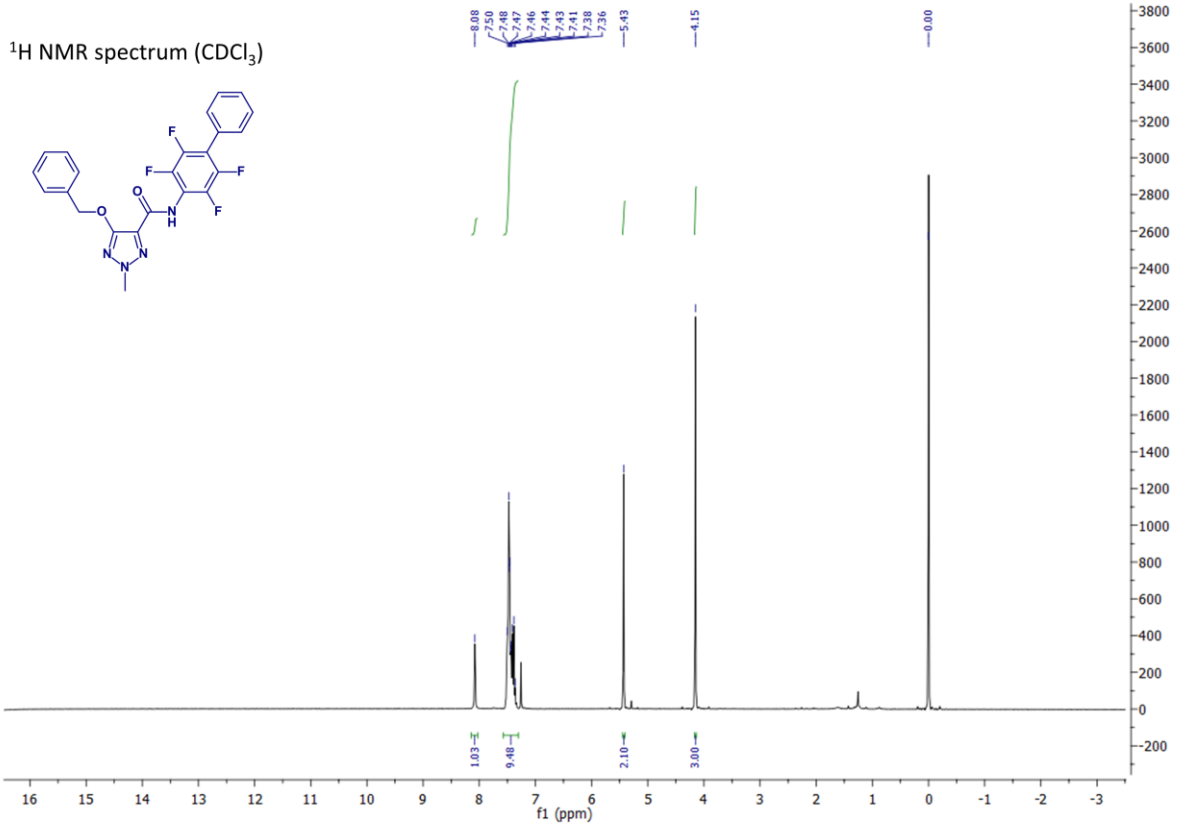


IR (KBr) spectrum

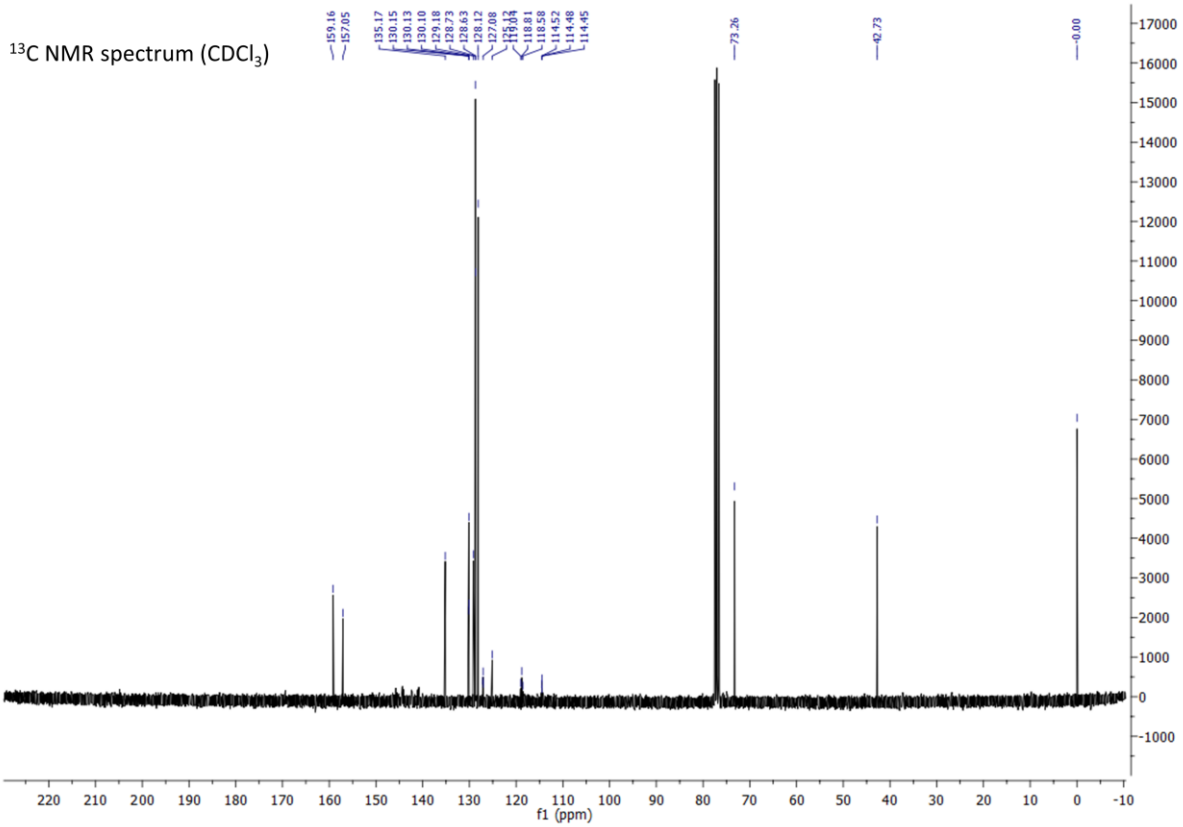


5-Benzyloxy-2-methyl-N-(2,3,5,6-tetrafluoro[1,1'-biphenyl]-4-yl)-2H-1,2,3-triazole-4-carboxamide (31)

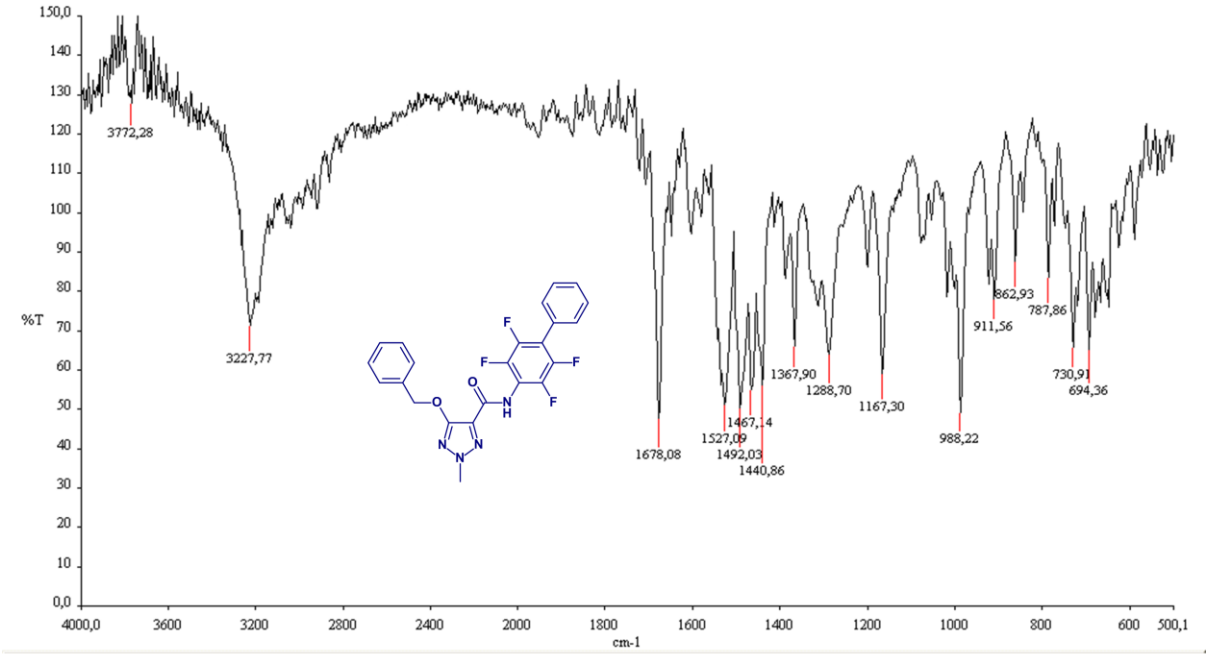
^{1}H NMR spectrum ($CDCl_3$)



^{13}C NMR spectrum ($CDCl_3$)

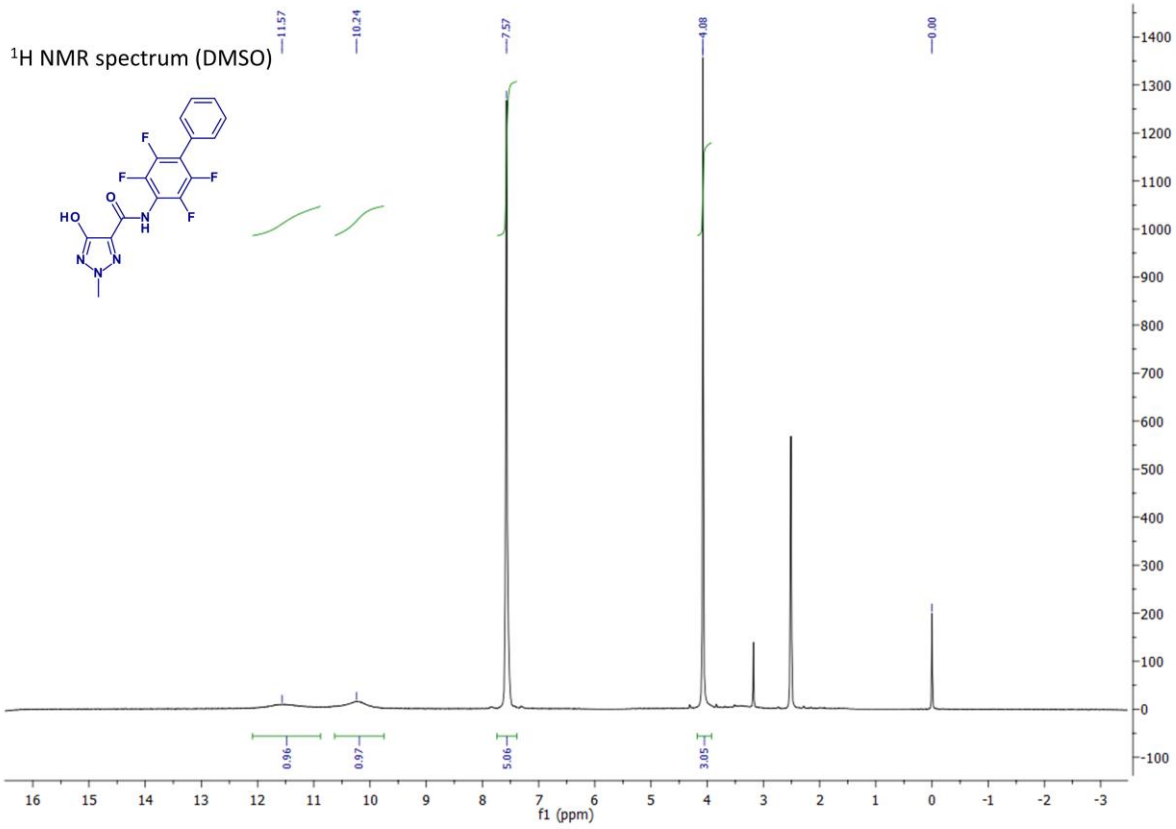


IR (KBr) spectrum

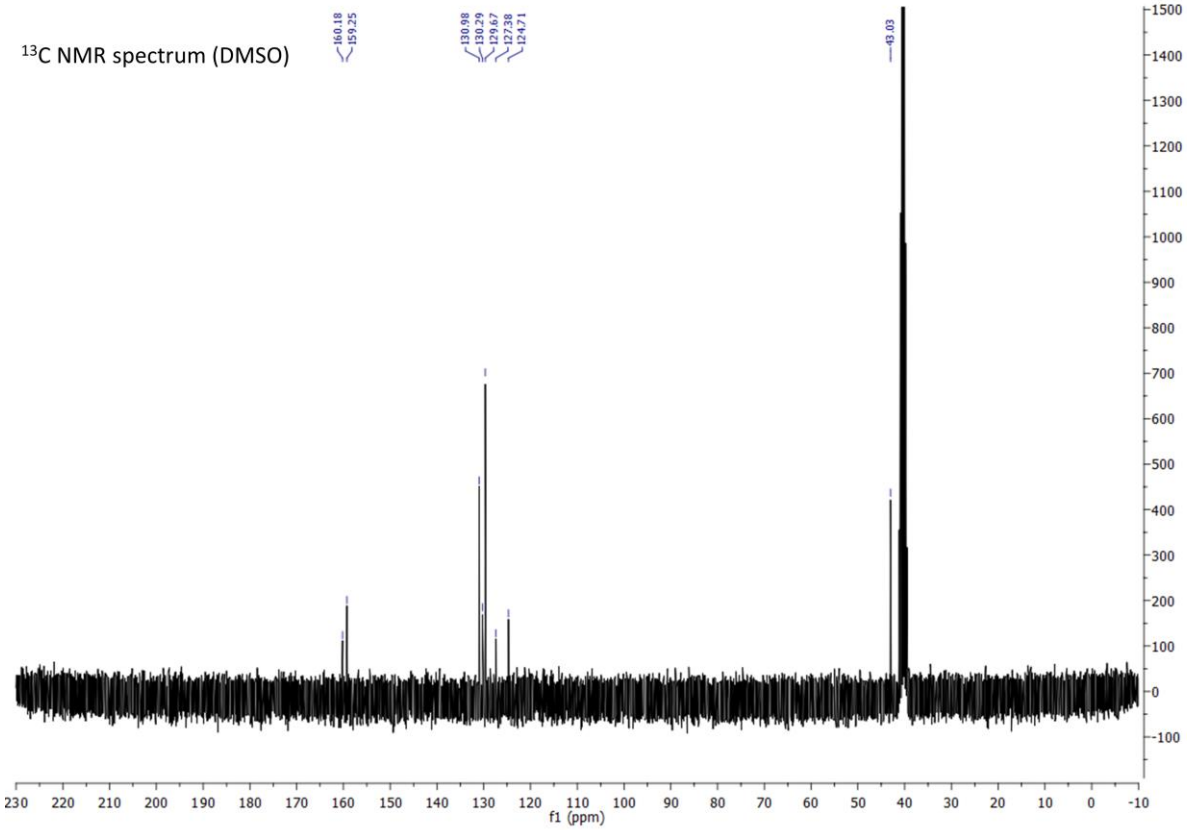


5-Hydroxy-2-methyl-N-(2,3,5,6-tetrafluoro[1,1'-biphenyl]-4-yl)-2H-1,2,3-triazole-4-carboxamide (6)

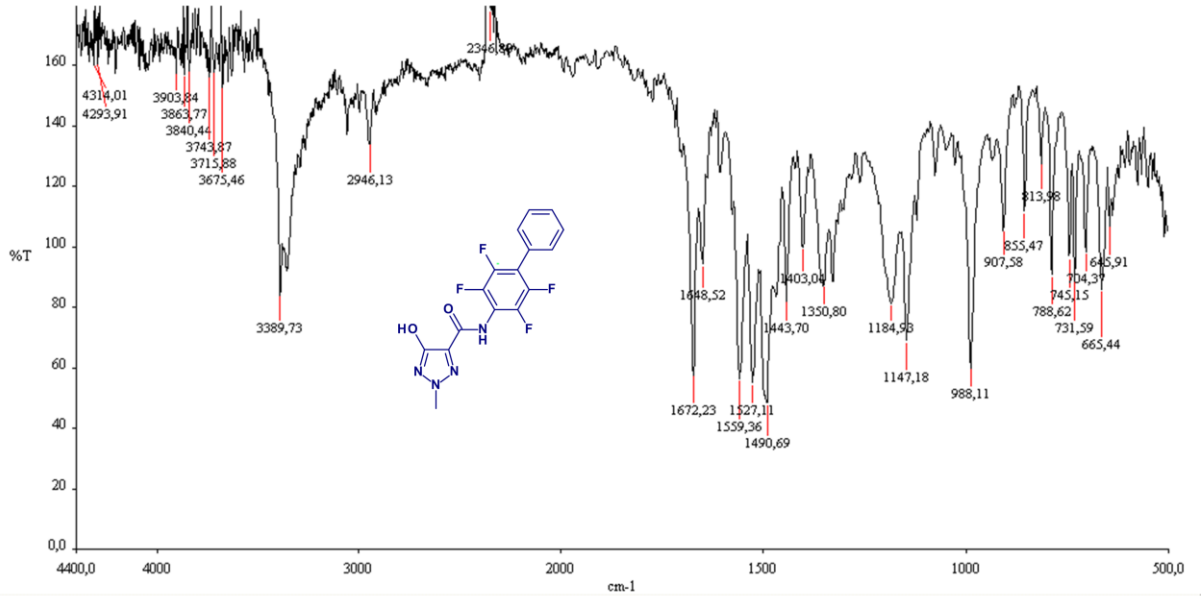
^{1}H NMR spectrum (DMSO)



^{13}C NMR spectrum (DMSO)

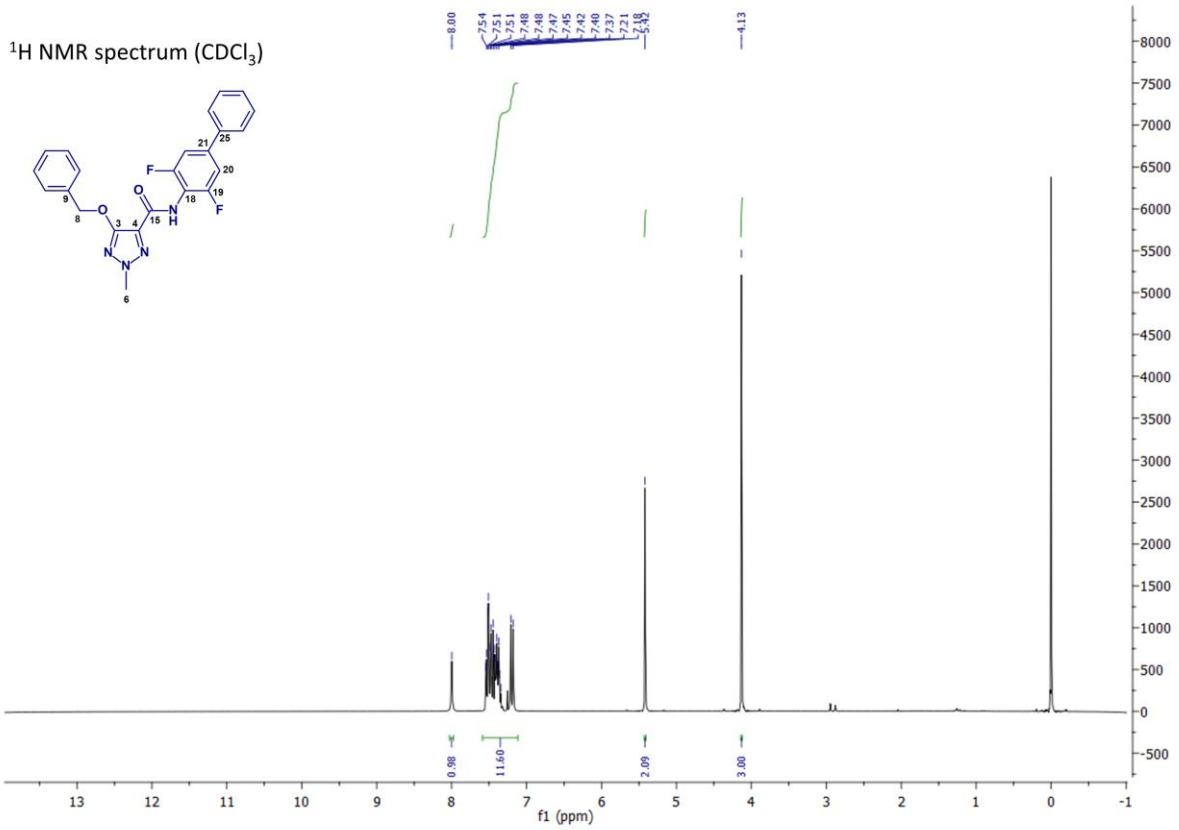


IR (KBr) spectrum

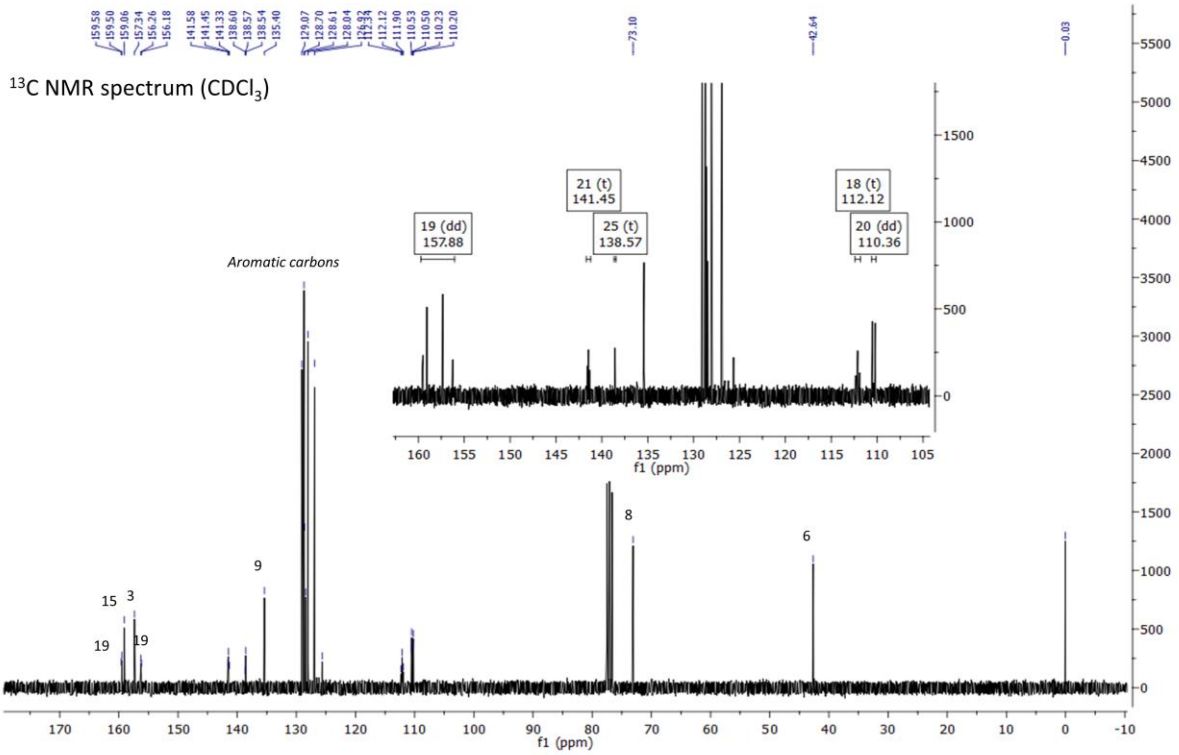


5-(Benzyloxy)-N-(3,5-difluoro[1,1'-biphenyl]-4-yl)-2-methyl-2H-1,2,3-triazole-4-carboxamide (32)

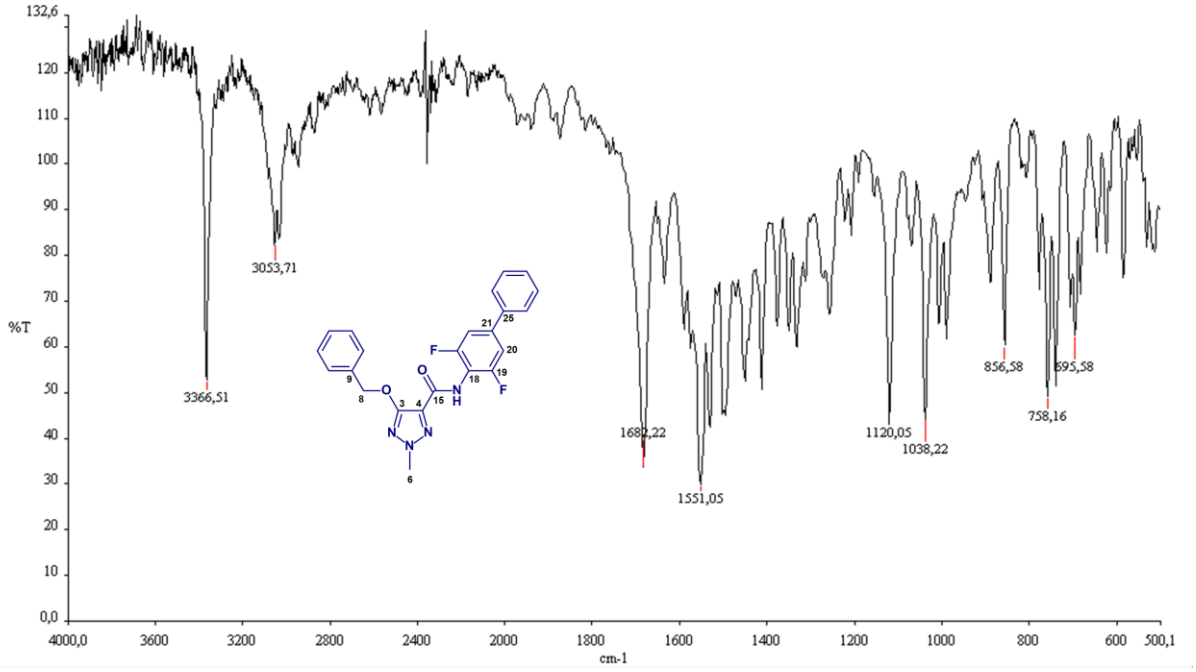
^{1}H NMR spectrum ($CDCl_3$)



^{13}C NMR spectrum ($CDCl_3$)

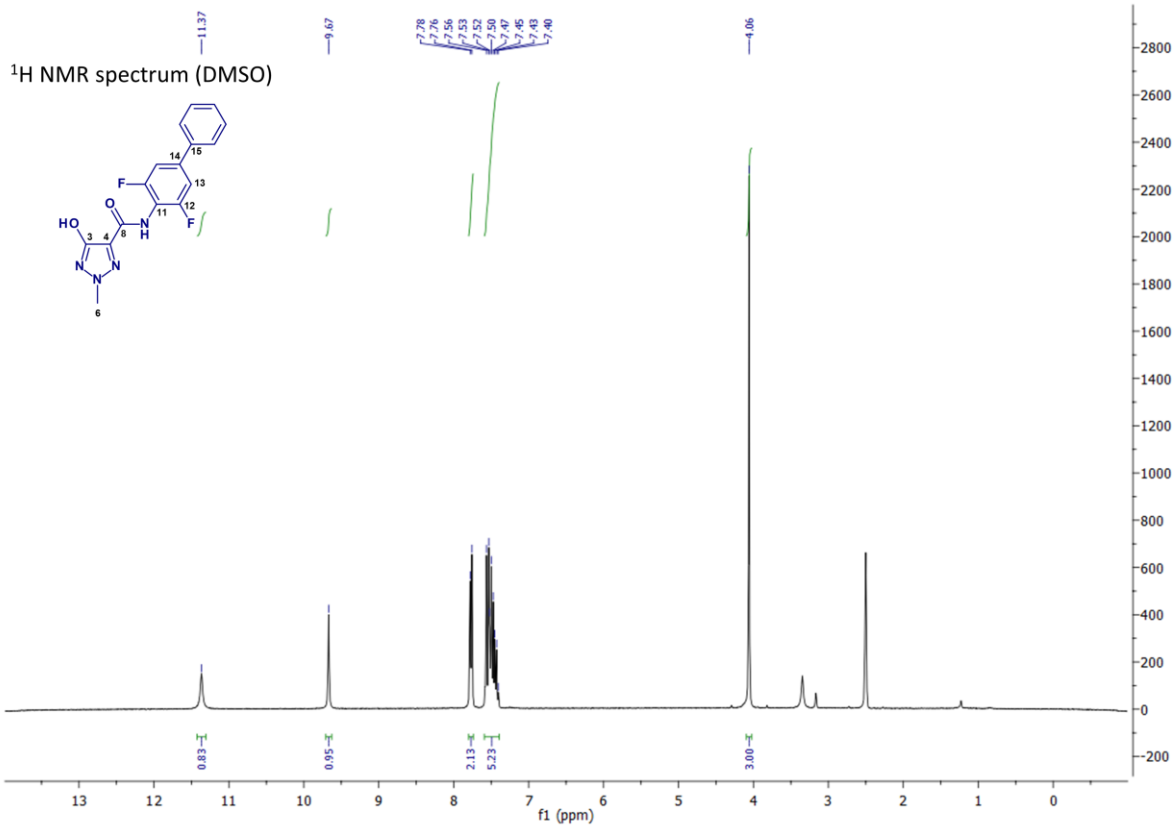


IR (KBr) spectrum

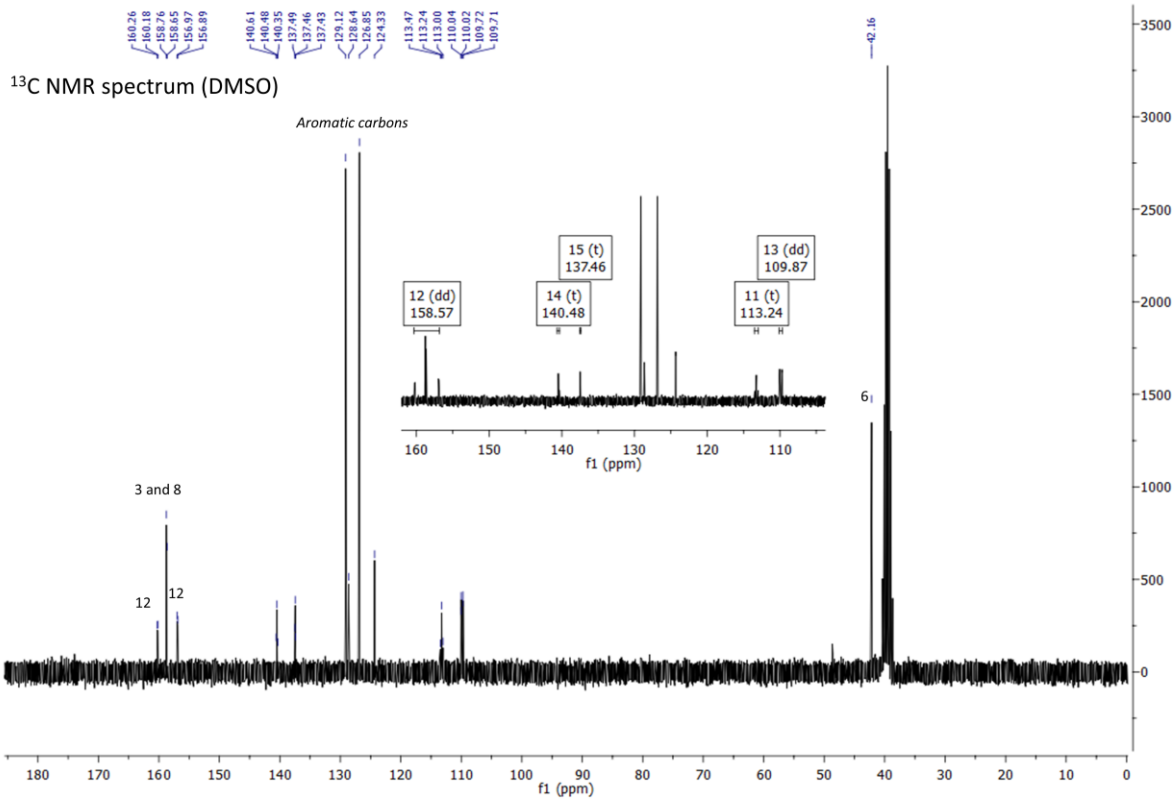


N-(3,5-Difluoro[1,1'-biphenyl]-4-yl)-5-hydroxy-2-methyl-2H-1,2,3-triazole-4-carboxamide (6a)

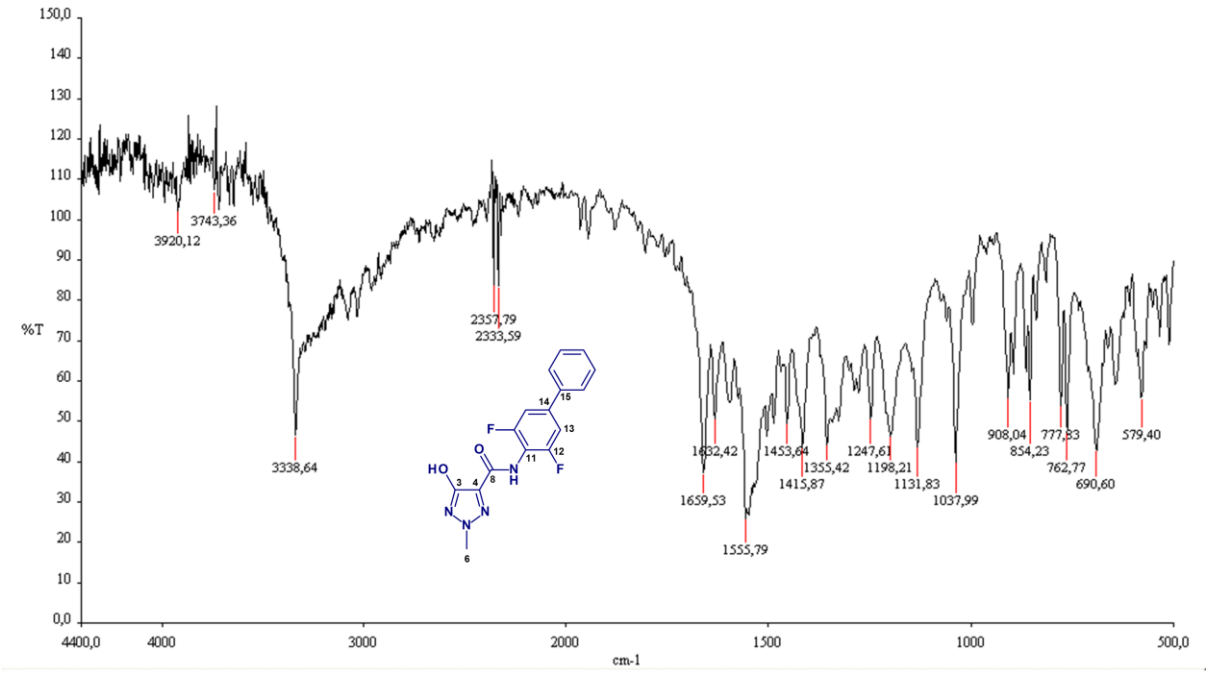
^{1}H NMR spectrum (DMSO)



^{13}C NMR spectrum (DMSO)

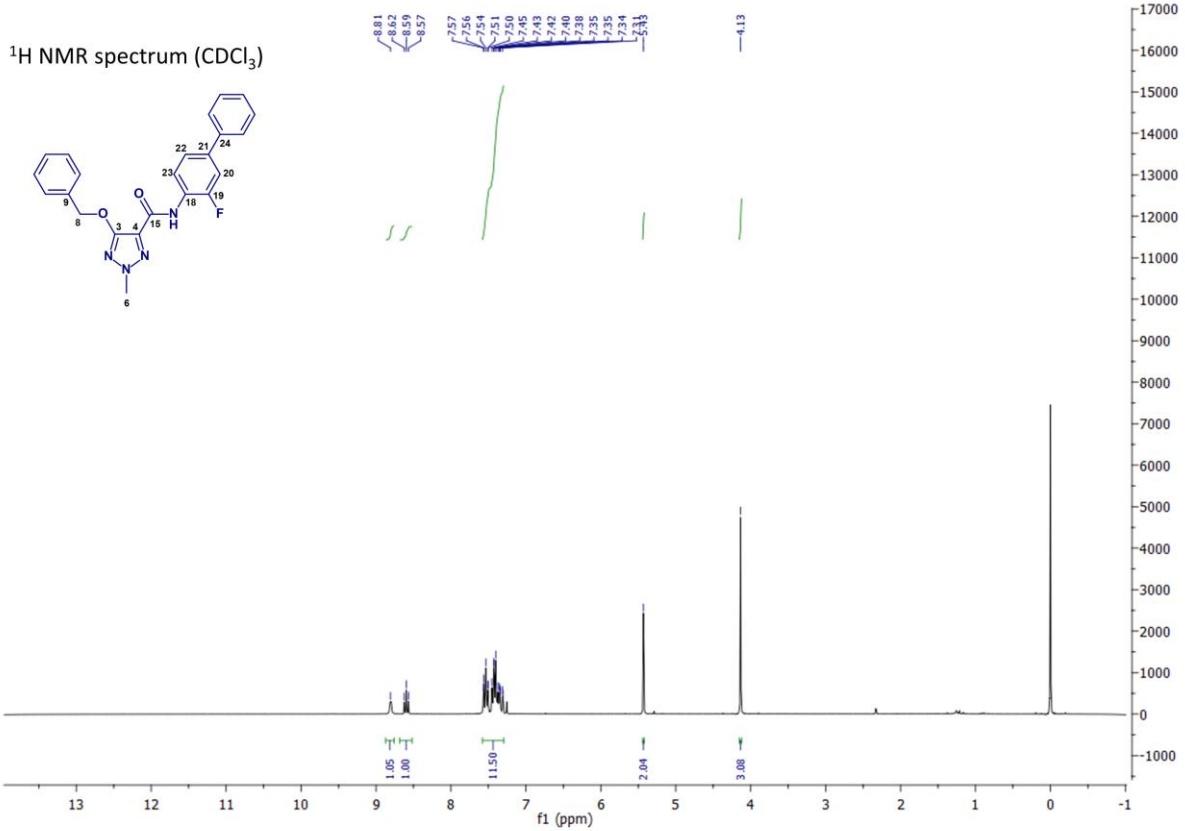


IR (KBr) spectrum

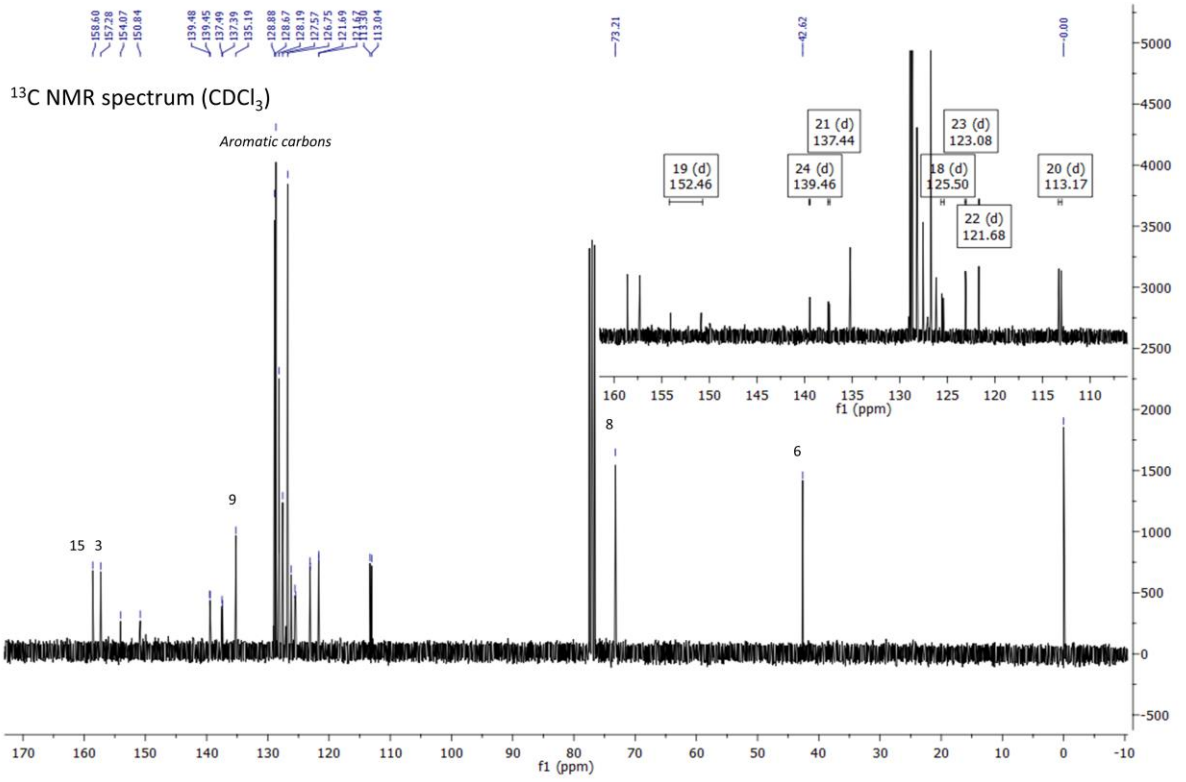


5-(Benzyloxy)-N-(3-fluoro[1,1'-biphenyl]-4-yl)-2-methyl-2H-1,2,3-triazole-4-carboxamide (33)

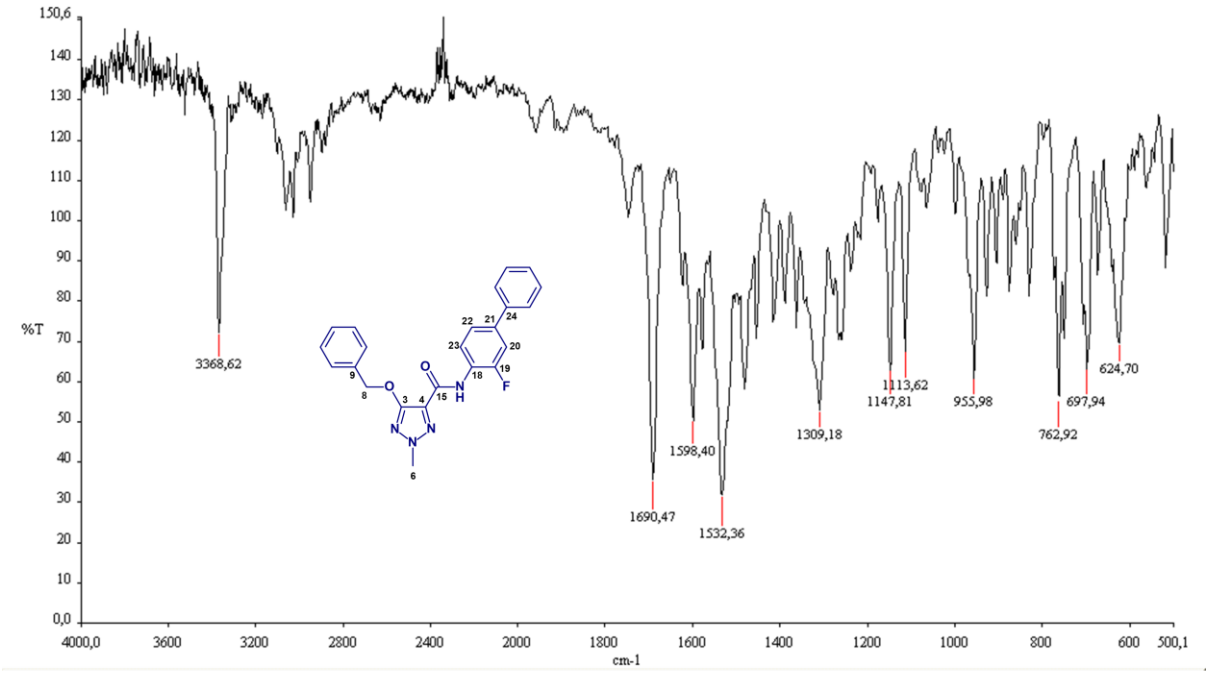
^{1}H NMR spectrum ($CDCl_3$)



^{13}C NMR spectrum ($CDCl_3$)

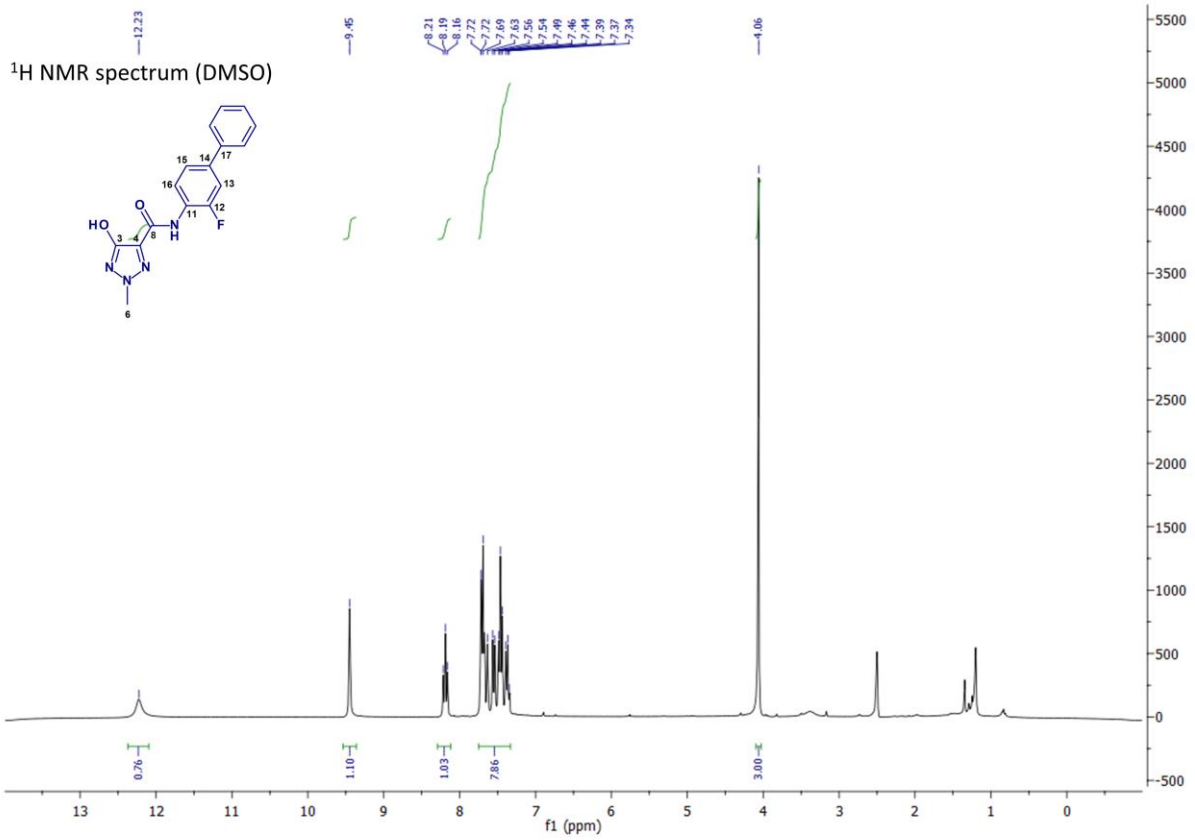


IR (KBr) spectrum

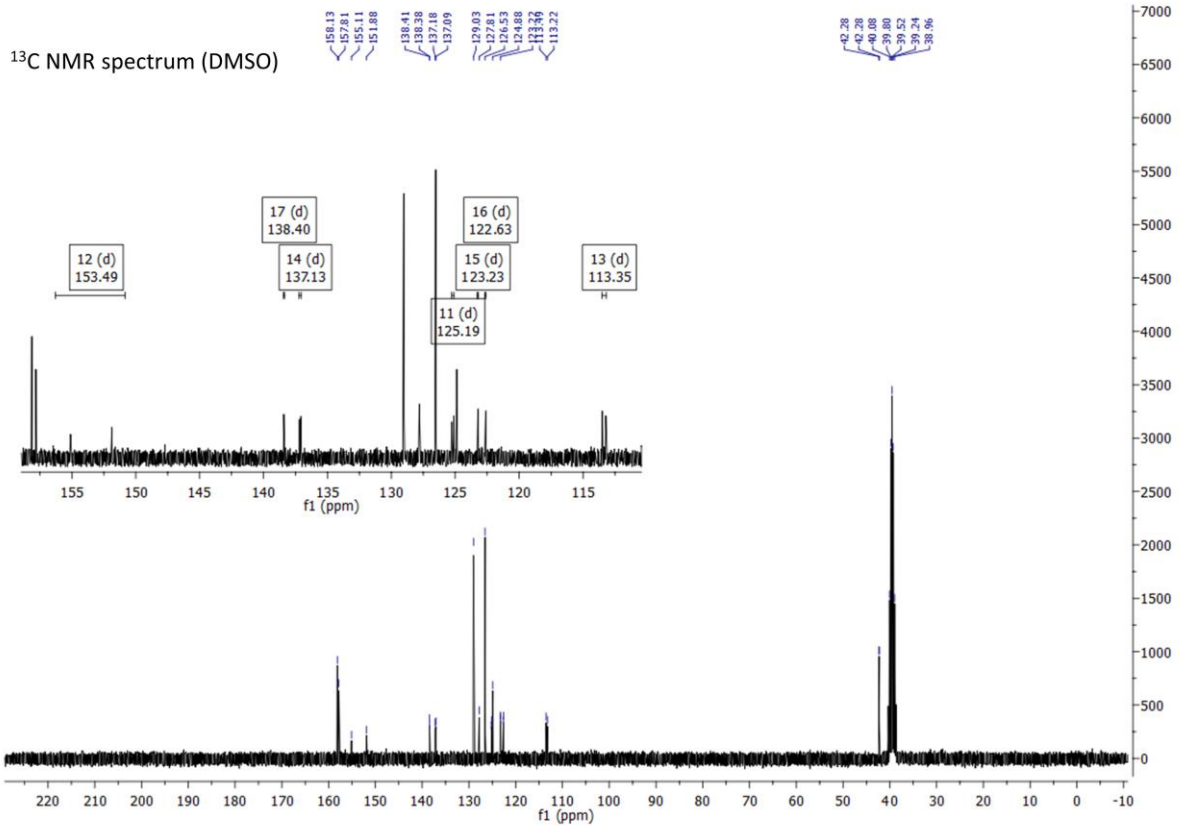


5-(Benzyloxy)-N-(3-fluoro[1,1'-biphenyl]-4-yl)-2-methyl-2H-1,2,3-triazole-4-carboxamide (6b)

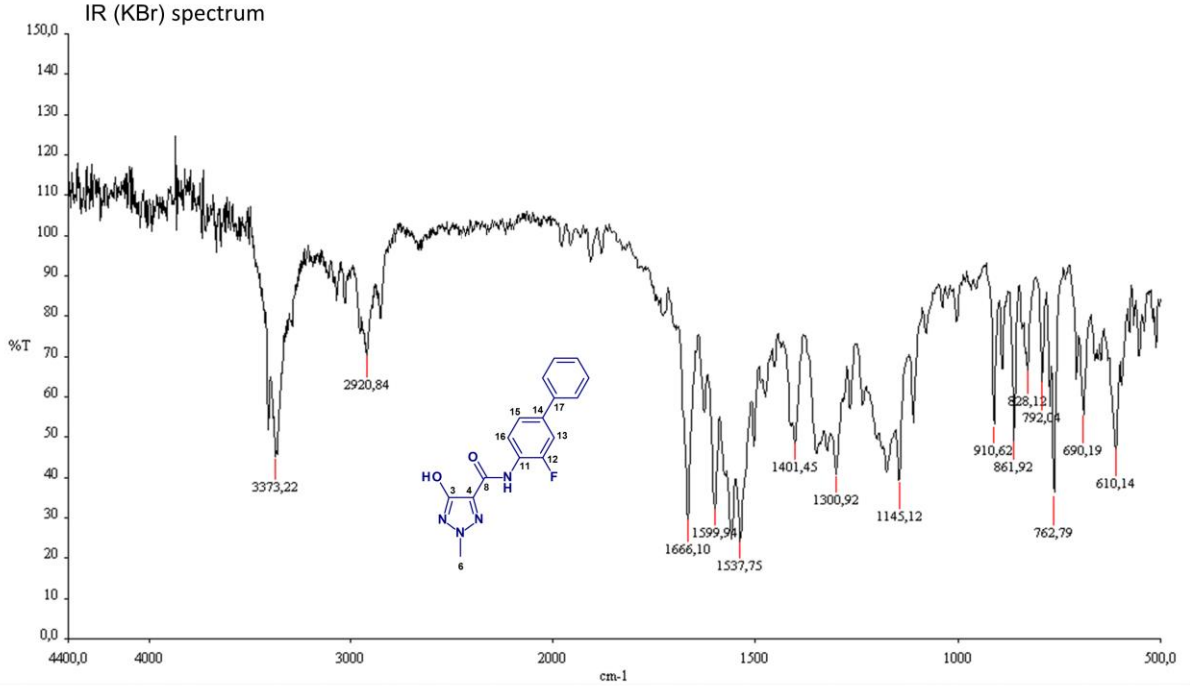
^{1}H NMR spectrum (DMSO)



^{13}C NMR spectrum (DMSO)

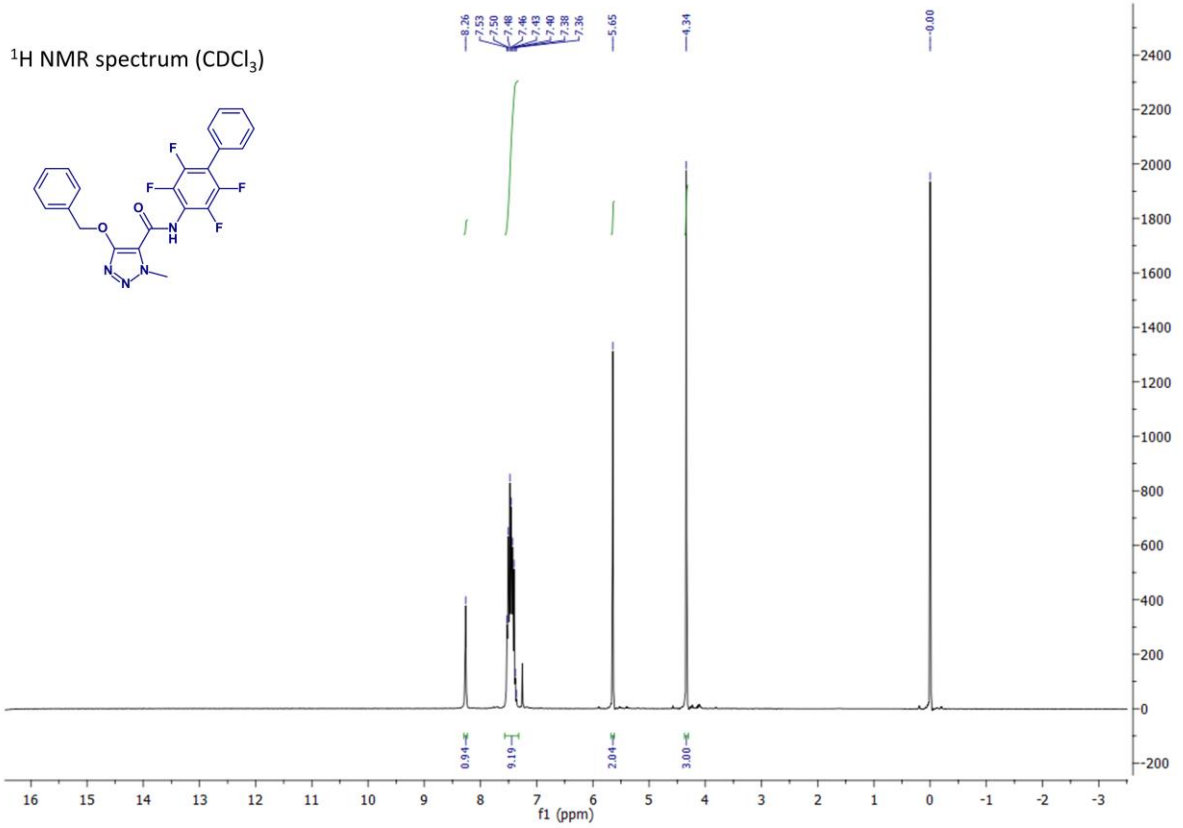


IR (KBr) spectrum

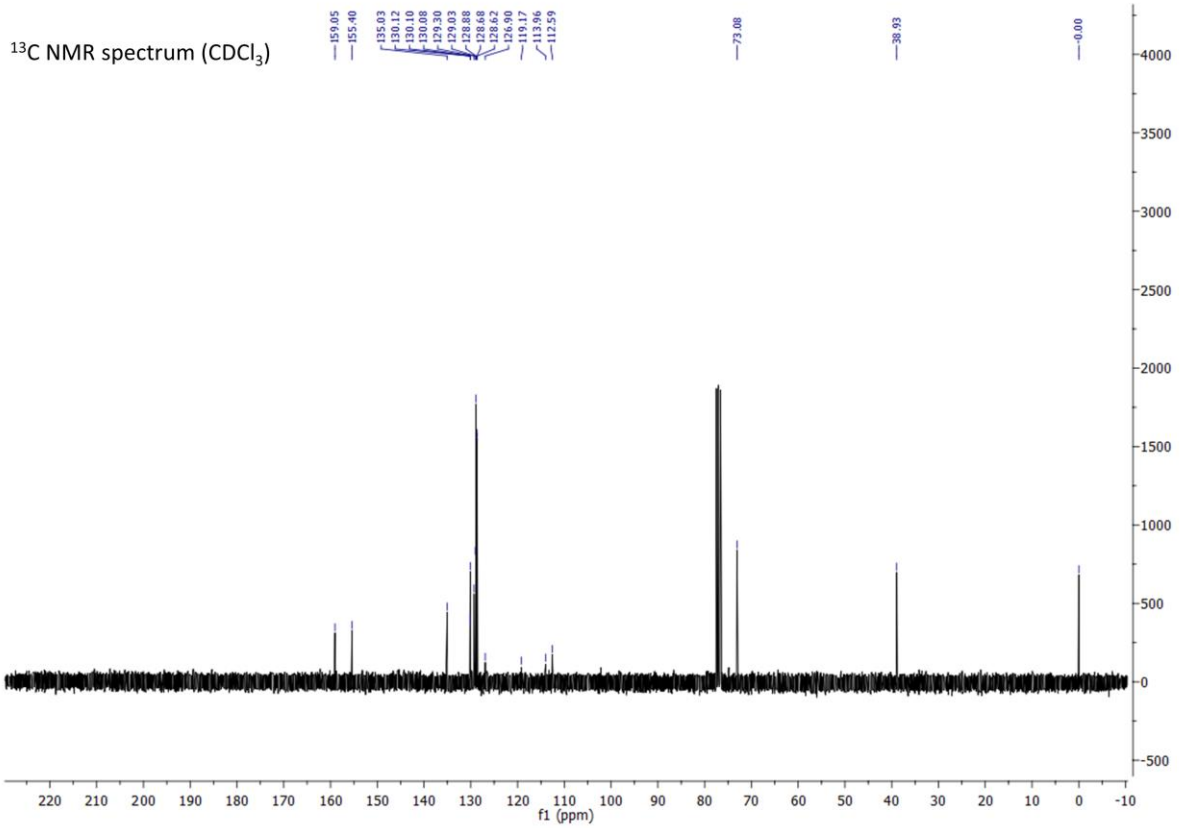


4-Benzyloxy-1-methyl-N-(2,3,5,6-tetrafluoro[1,1'-biphenyl]-4-yl)-1H-1,2,3-triazole-5-carboxamide (34)

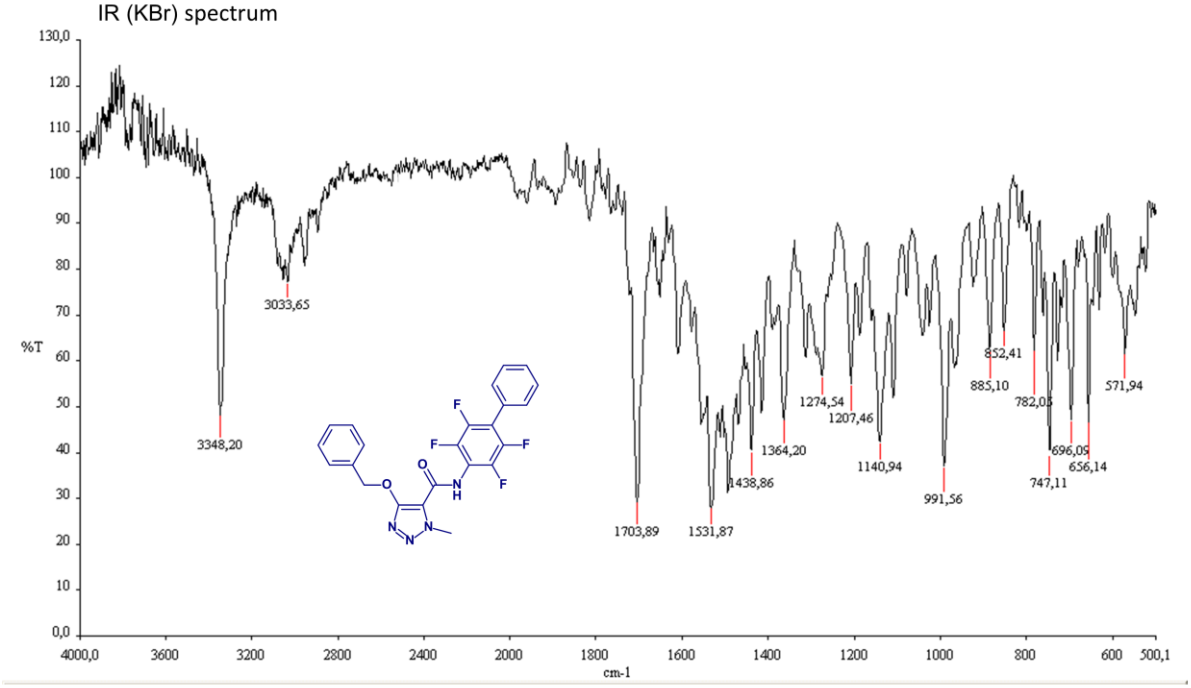
^{1}H NMR spectrum ($CDCl_3$)



^{13}C NMR spectrum ($CDCl_3$)

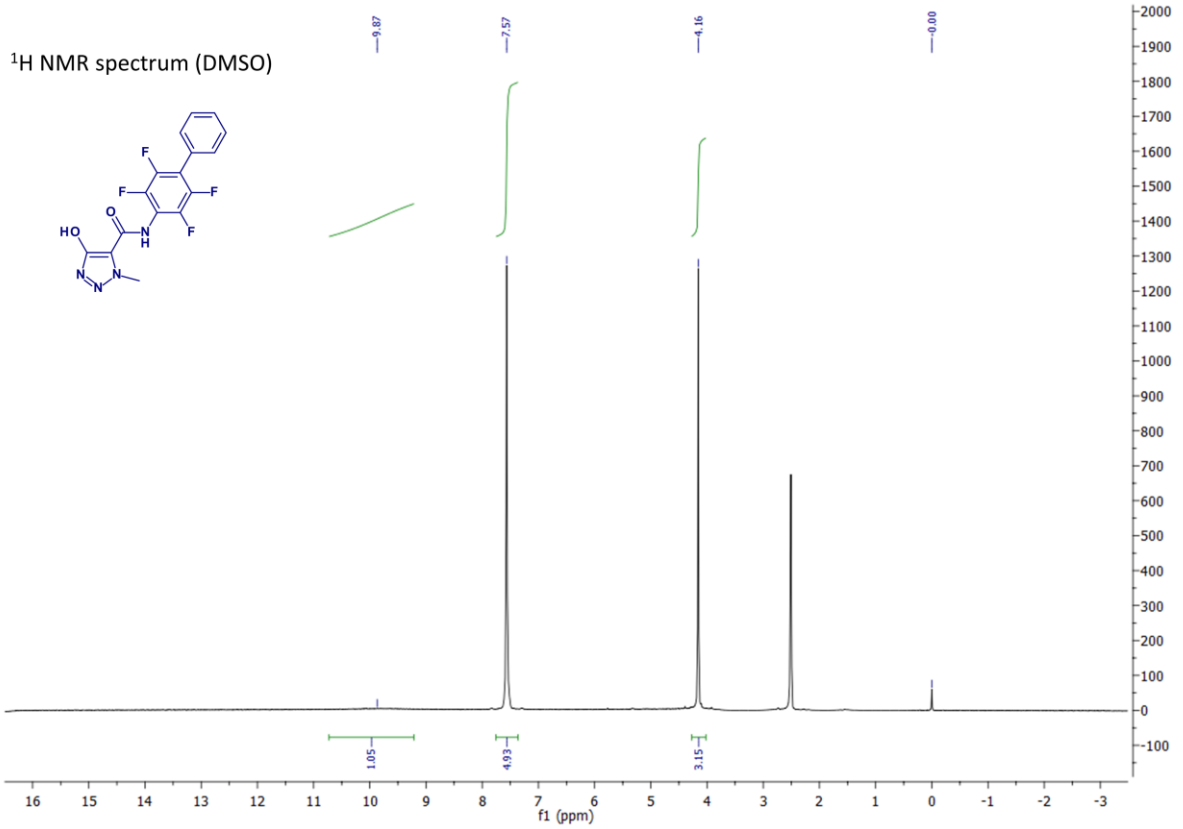


IR (KBr) spectrum

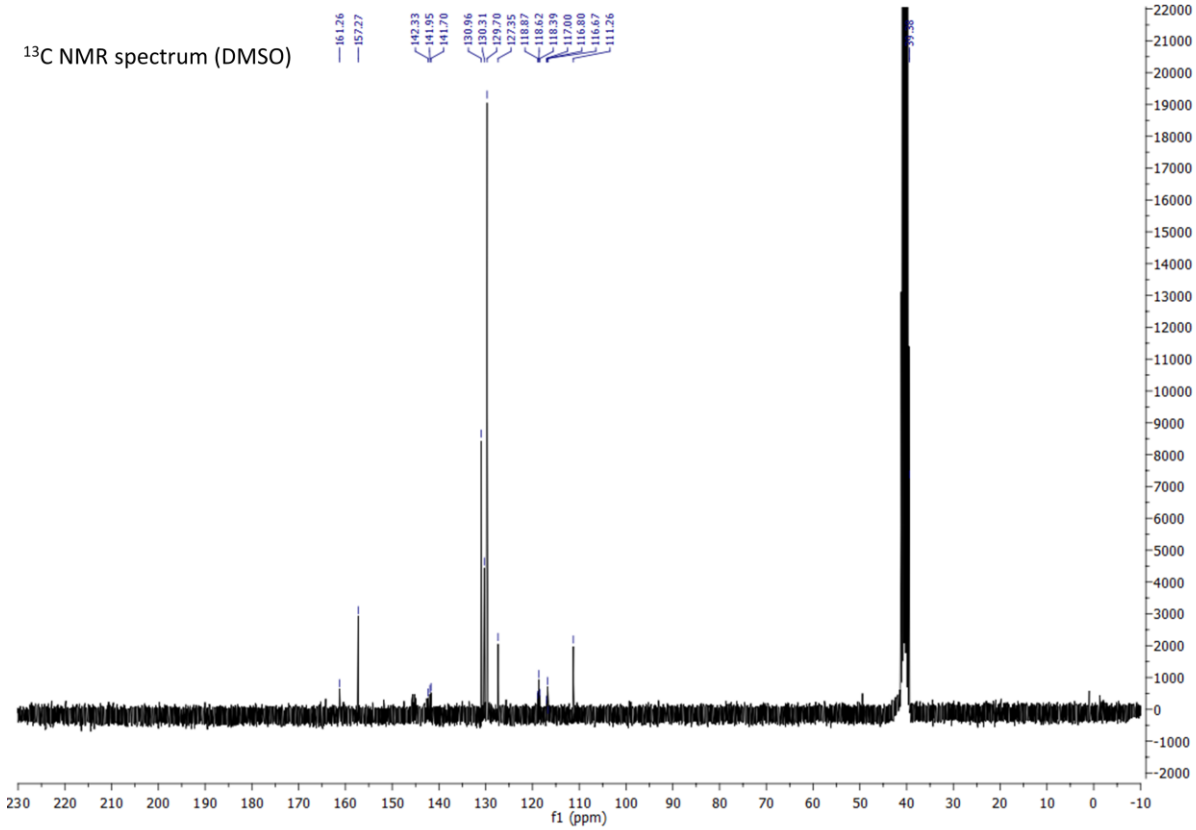


4-Hydroxy-1-methyl-N-(2,3,5,6-tetrafluoro[1,1'-biphenyl]-4-yl)-1H-1,2,3-triazole-5-carboxamide (7)

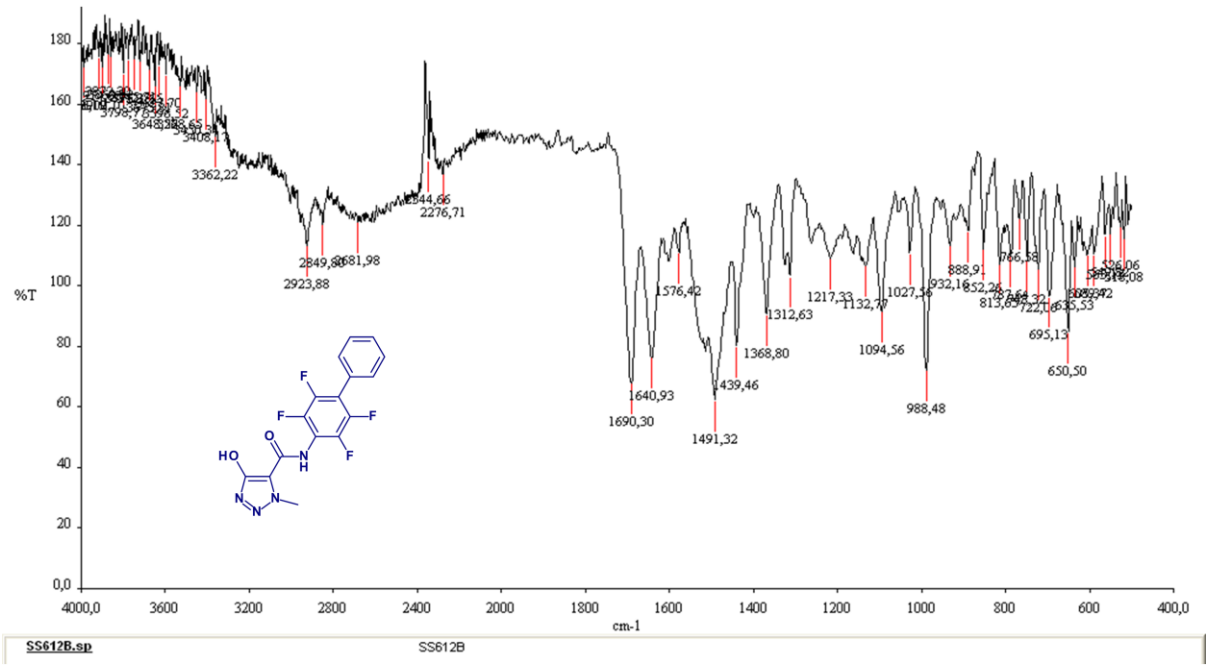
^{1}H NMR spectrum (DMSO)



^{13}C NMR spectrum (DMSO)

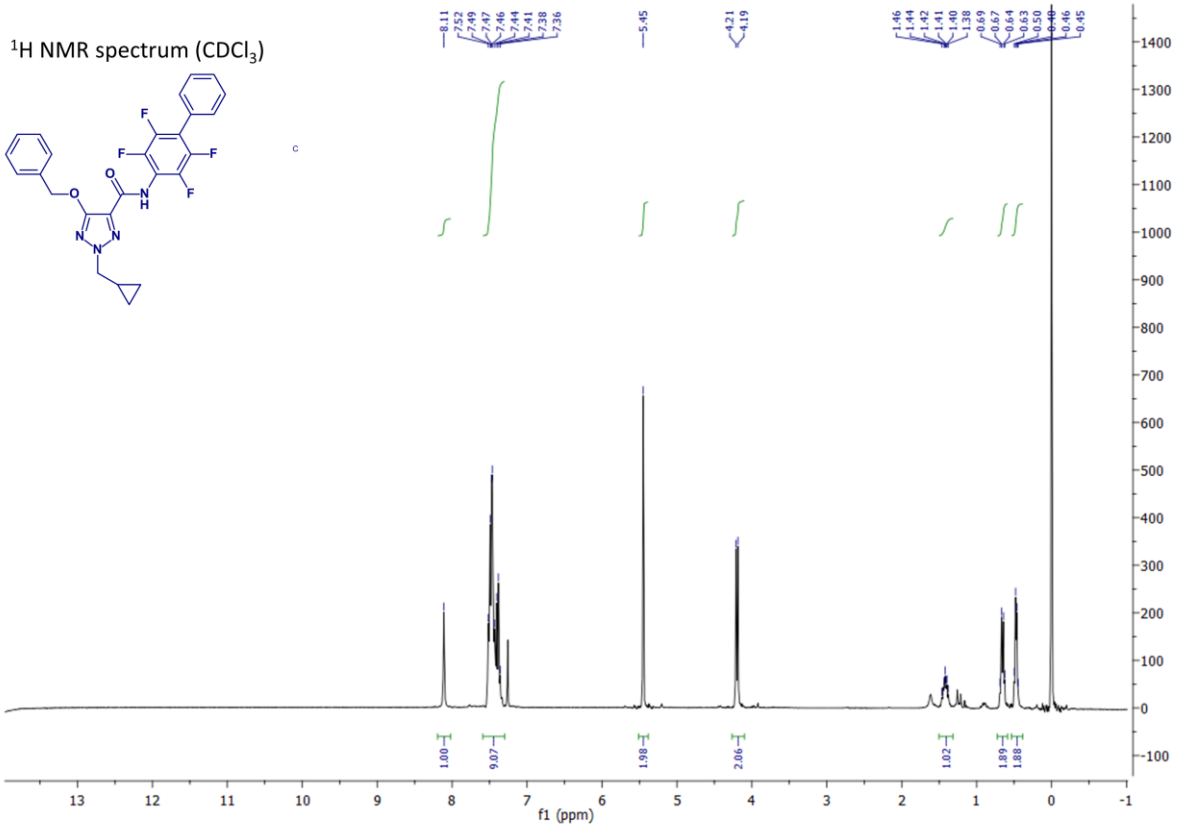


IR (KBr) spectrum

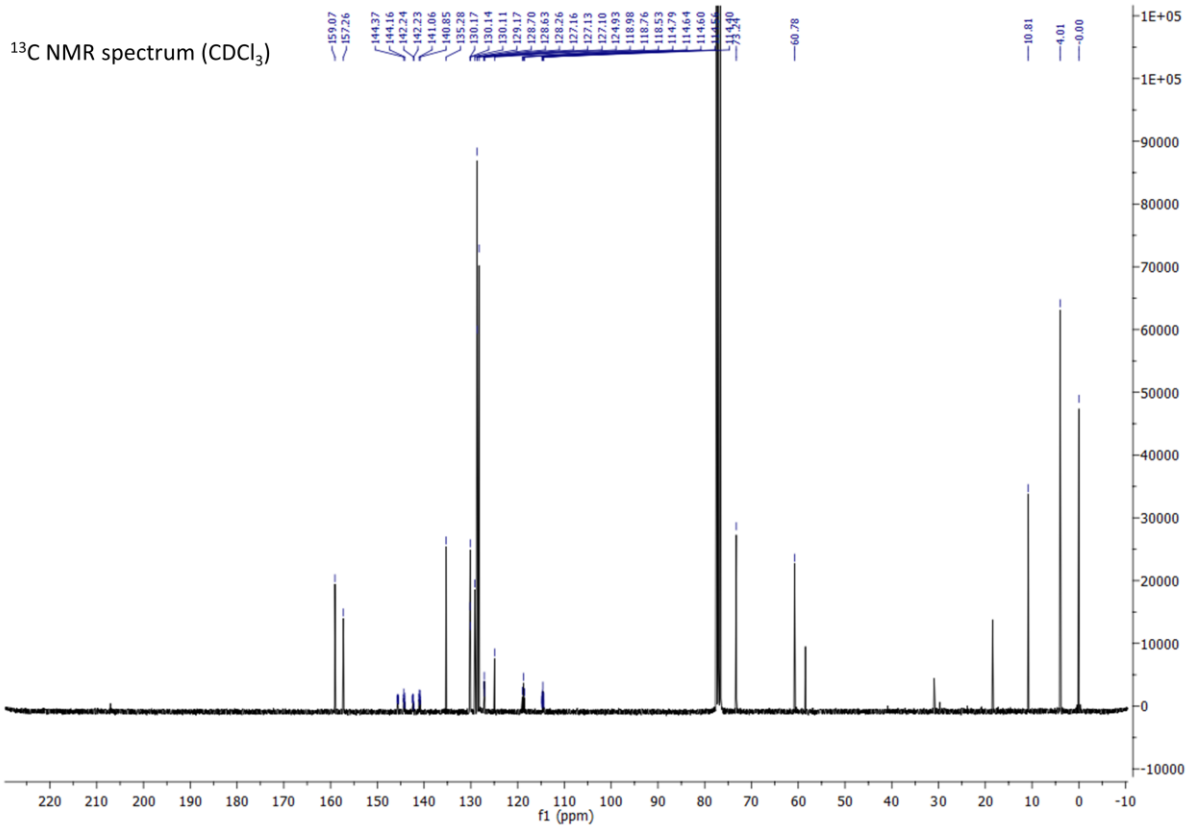


5-Benzyloxy-2-(cyclopropylmethyl)-N-(2,3,5,6-tetrafluoro[1,1'-biphenyl]-4-yl)-2H-1,2,3-triazole-4-carboxamide (35)

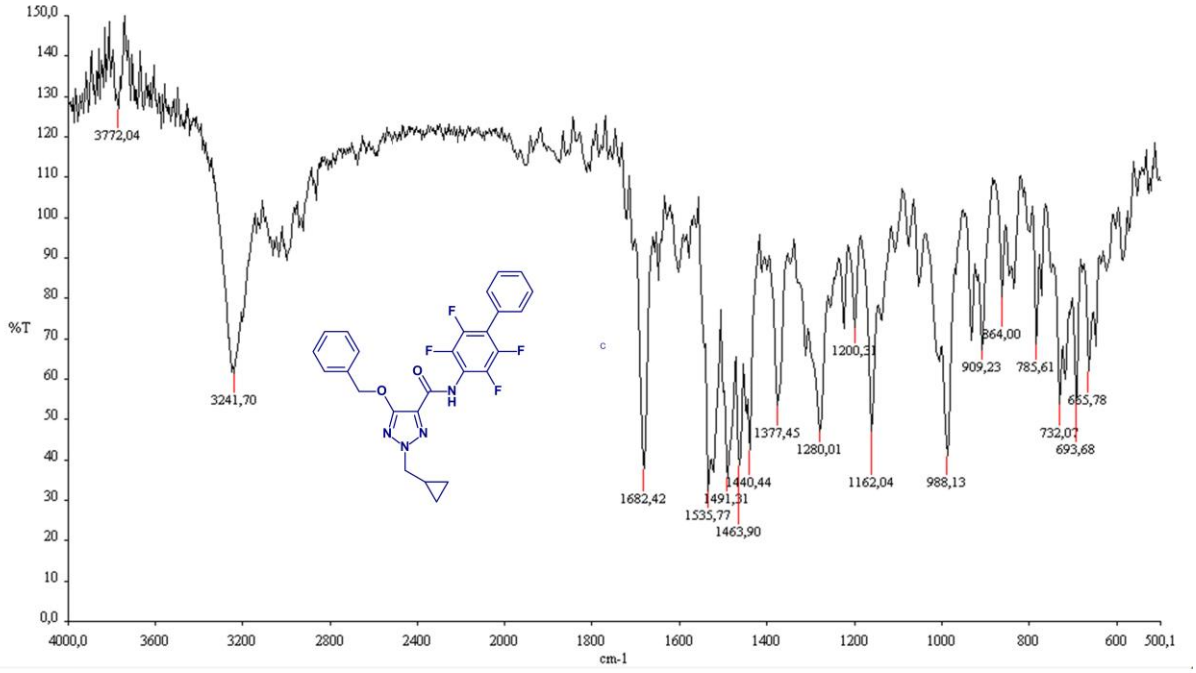
^{1}H NMR spectrum ($CDCl_3$)



^{13}C NMR spectrum ($CDCl_3$)

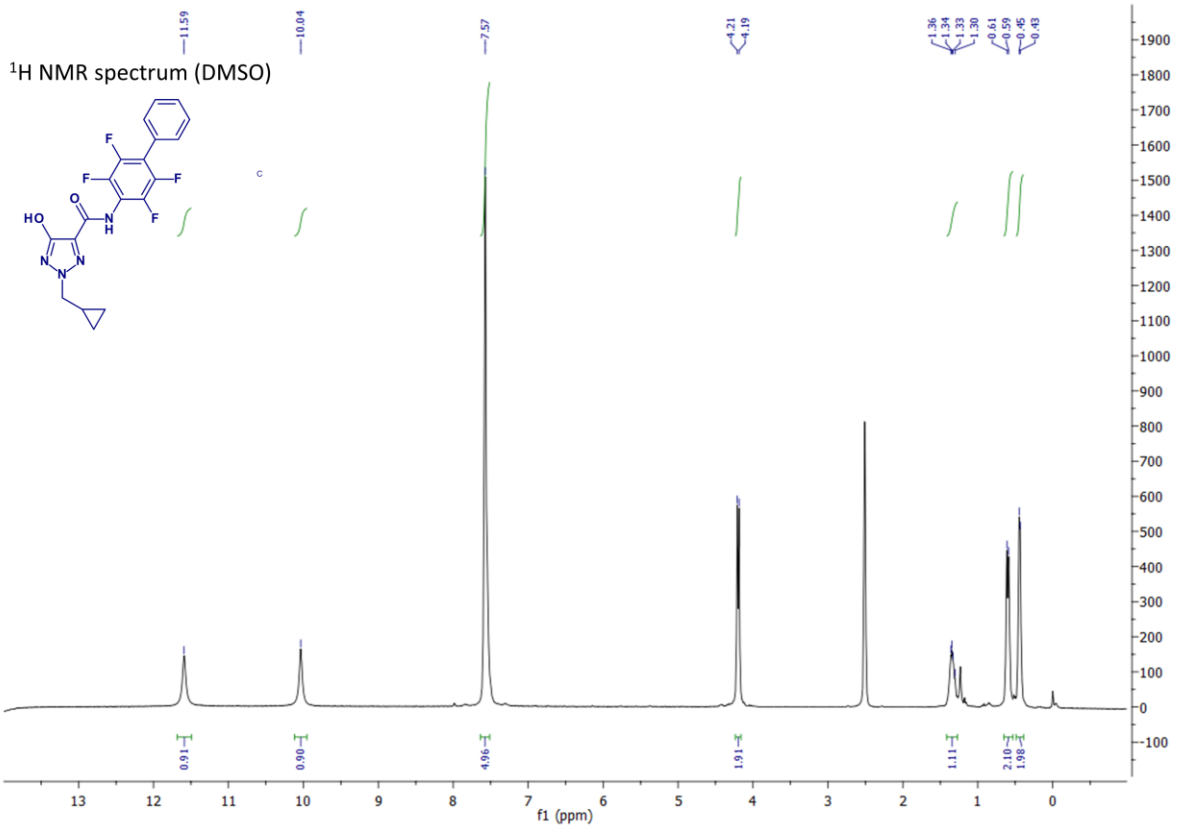


IR (KBr) spectrum

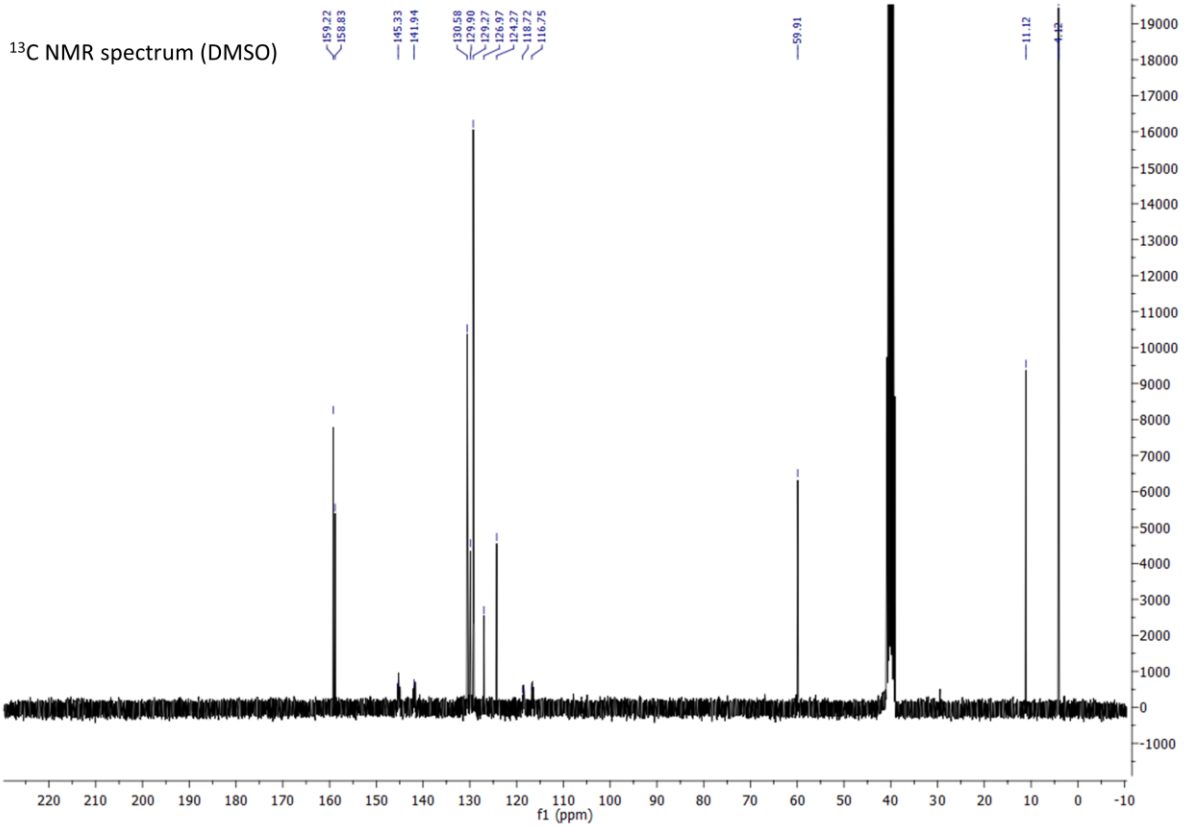


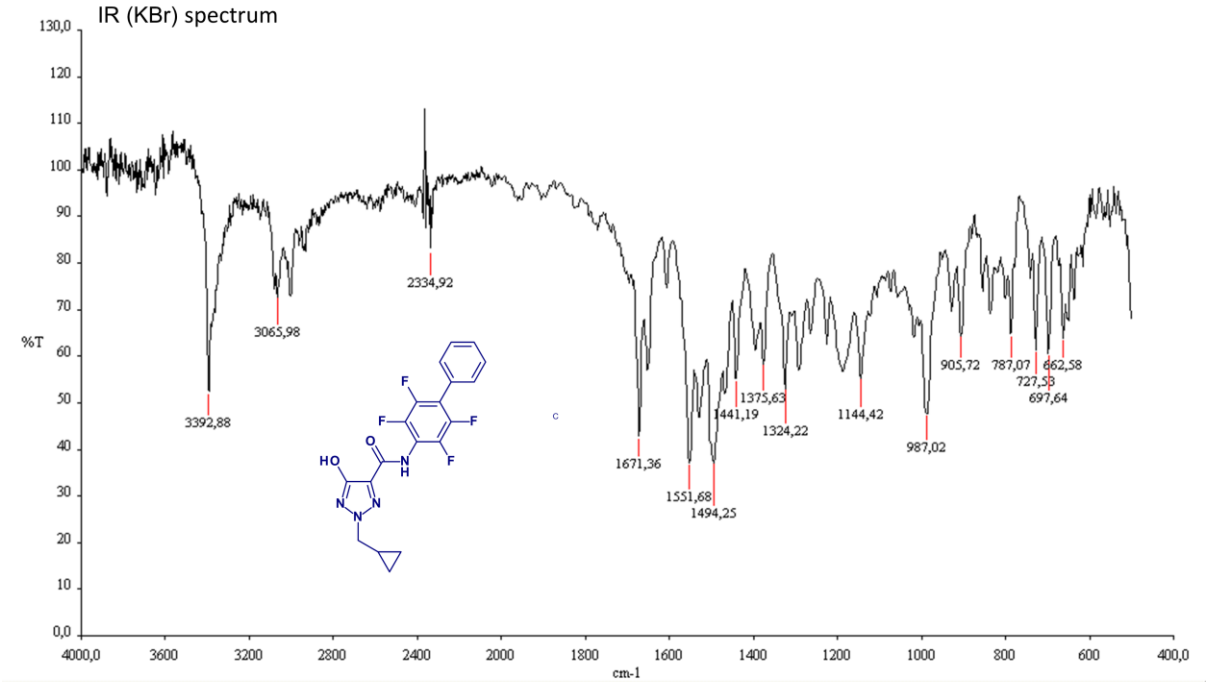
2-(Cyclopropylmethyl)-5-hydroxy-N-(2,3,5,6-tetrafluoro[1,1'-biphenyl]-4-yl)-2H-1,2,3-triazole-4-carboxamide (8)

^{1}H NMR spectrum (DMSO)



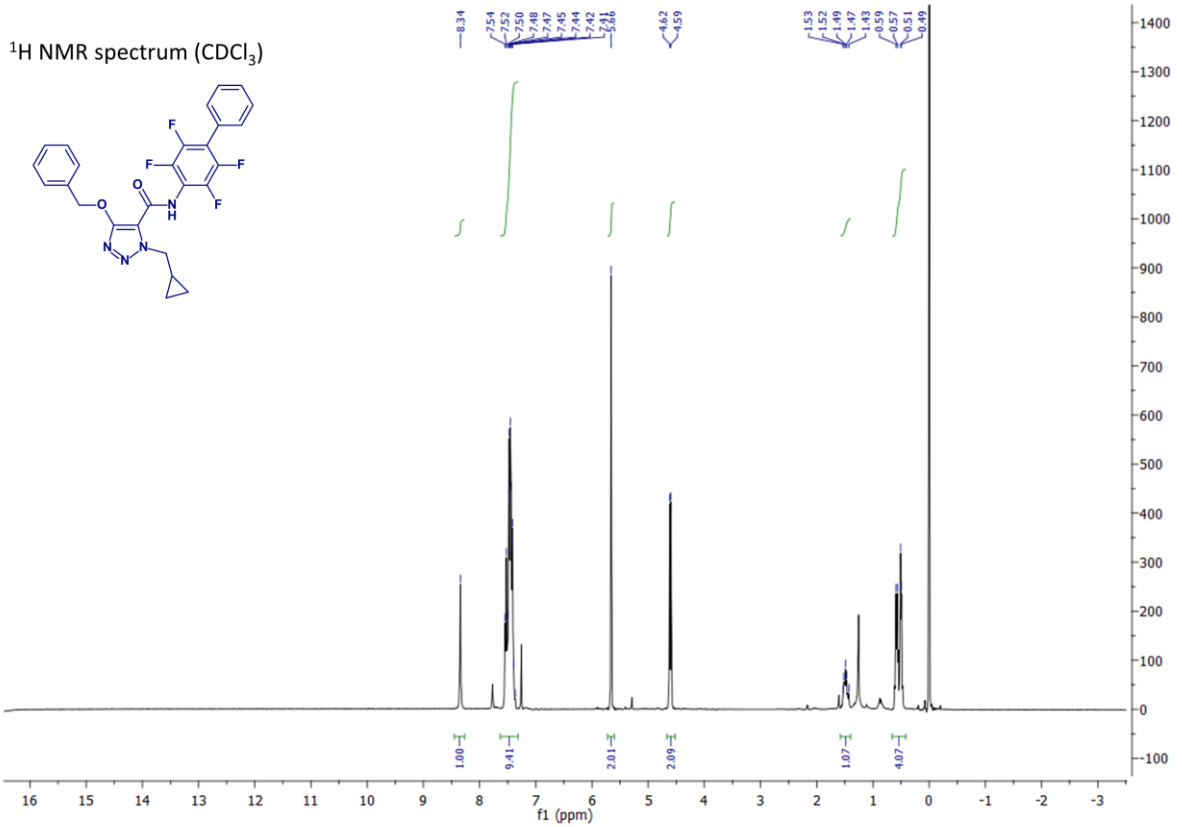
^{13}C NMR spectrum (DMSO)



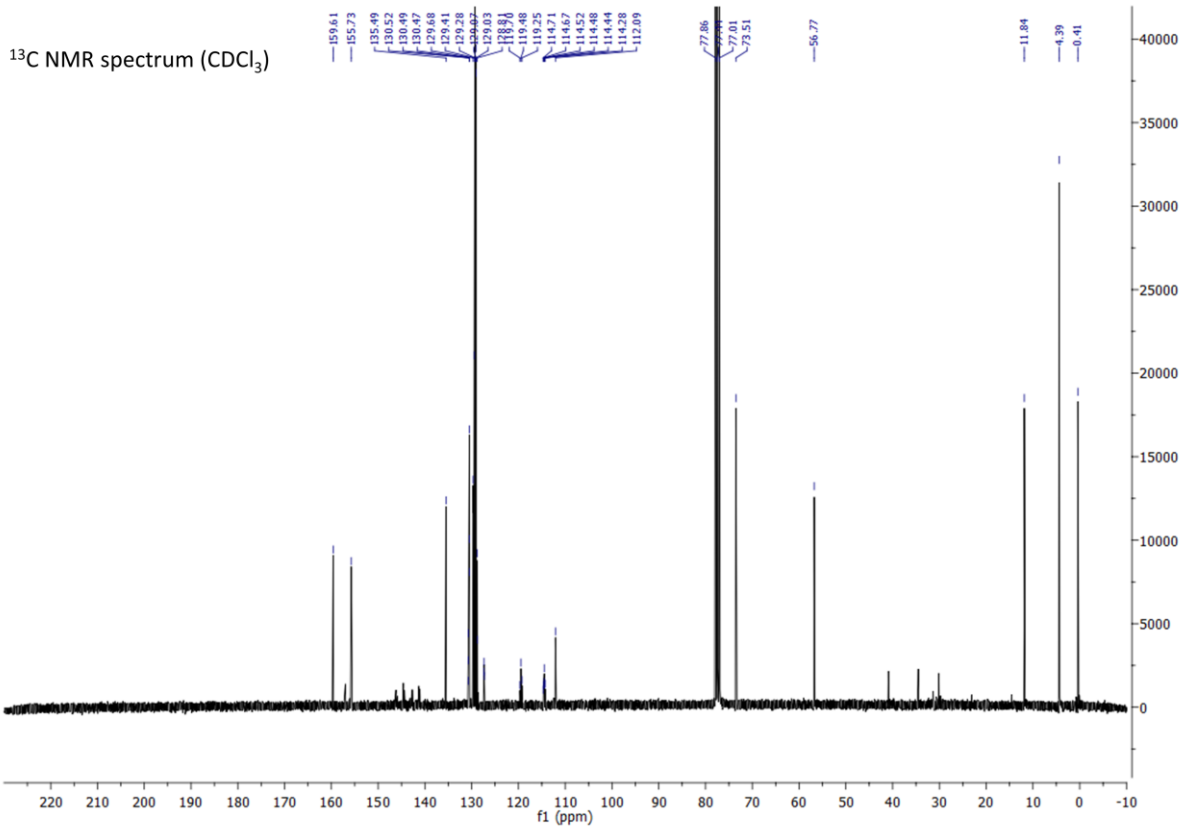

IR (KBr) spectrum
%T
cm-1
3392,88
3065,98
2334,92
1671,36
1551,68
1494,25
1441,19
1375,63
1324,22
1144,42
987,02
905,72
787,07
727,53
697,64
662,58
HO
O
N
H
F

4-Benzyloxy-1-(cyclopropylmethyl)-N-(2,3,5,6-tetrafluoro[1,1'-biphenyl]-4-yl)-1H-1,2,3-triazole-4-carboxamide (36)

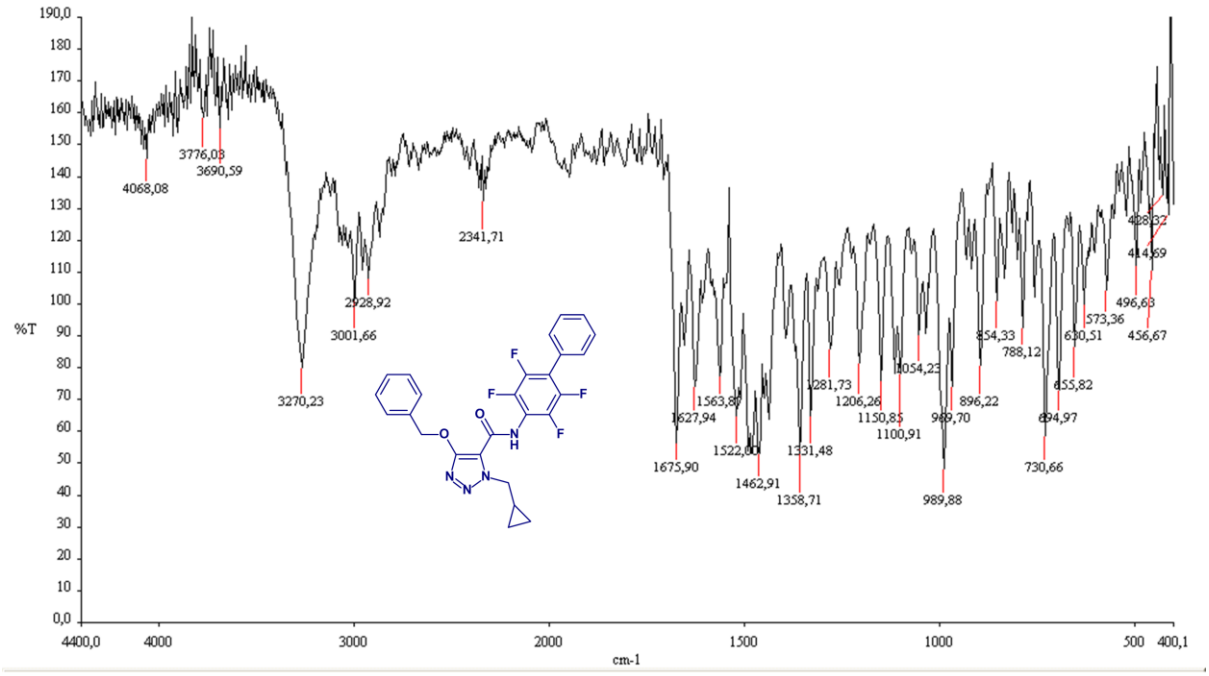
^{1}H NMR spectrum ($CDCl_3$)



^{13}C NMR spectrum ($CDCl_3$)

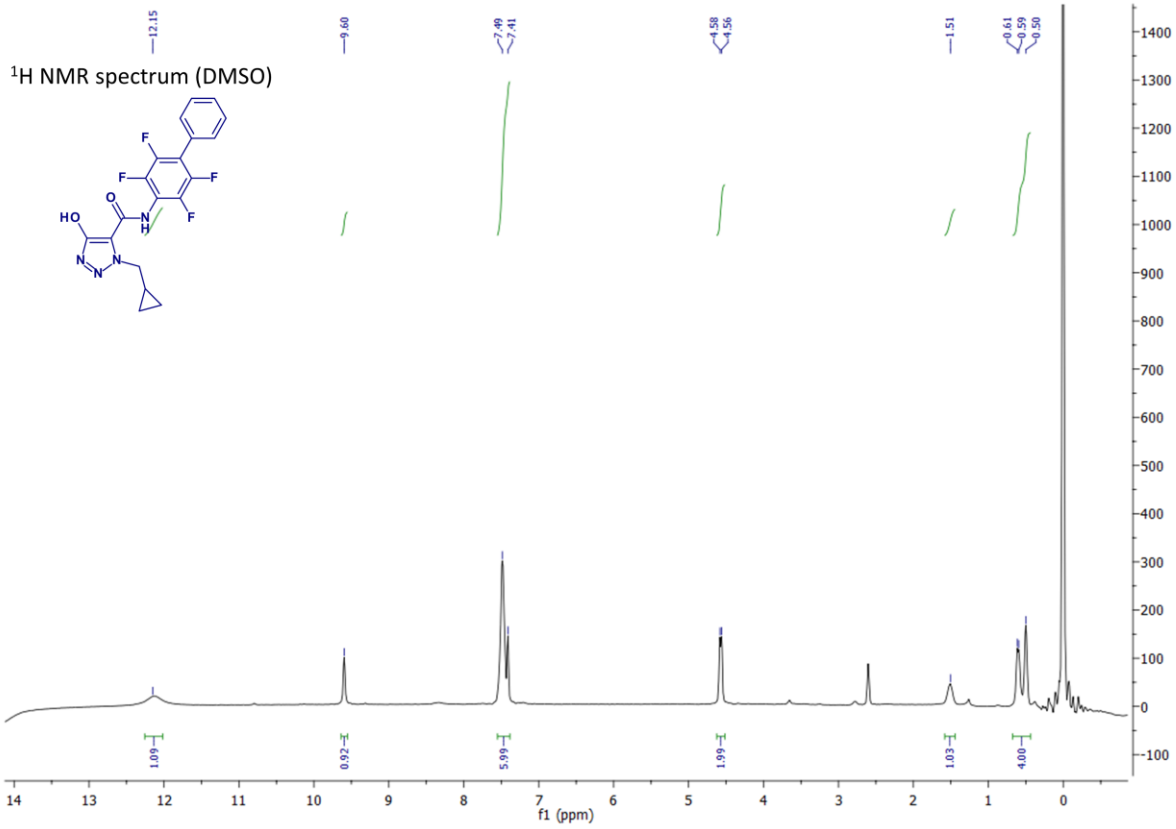


IR (KBr) spectrum

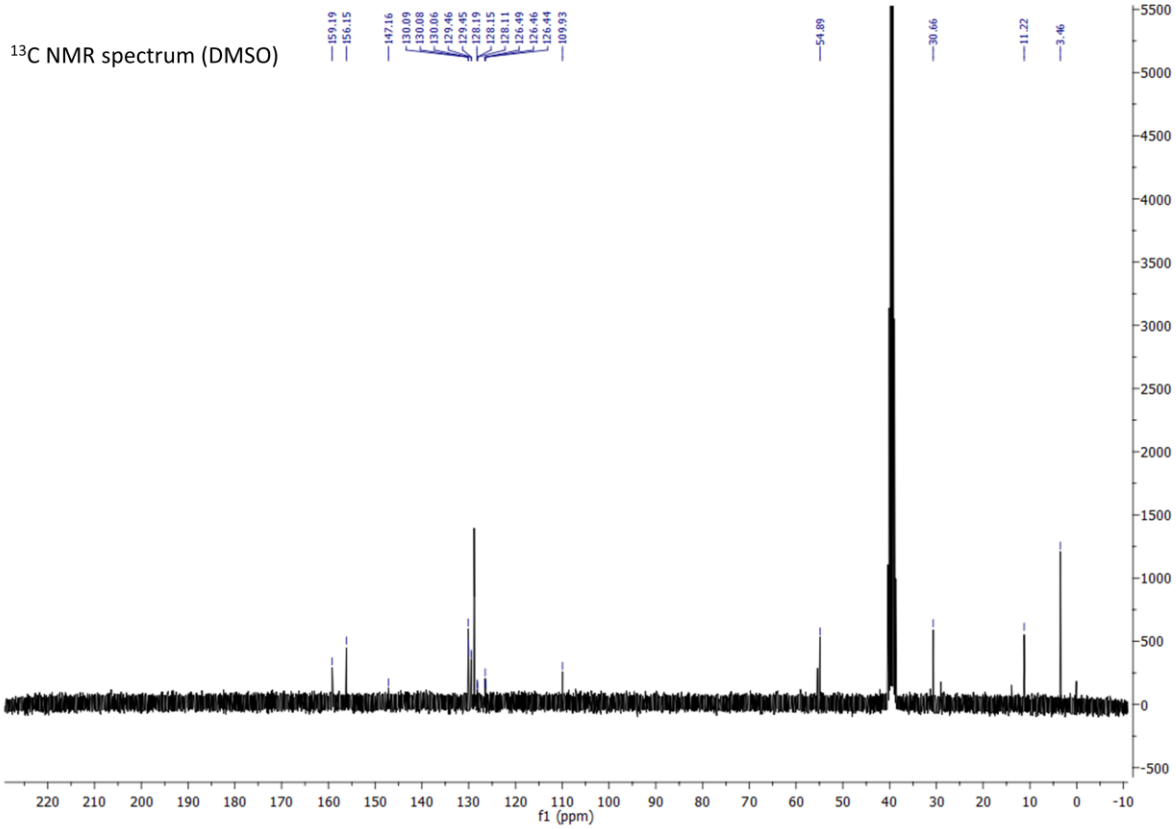


1-(Cyclopropylmethyl)-4-hydroxy-N-(2,3,5,6-tetrafluoro[1,1'-biphenyl]-4-yl)-1H-1,2,3-triazole-5-carboxamide (9)

^{1}H NMR spectrum (DMSO)



^{13}C NMR spectrum (DMSO)



IR (KBr) spectrum