Supporting information

Discovery of S-217622, a Non-Covalent Oral SARS-CoV-2 3CL Protease Inhibitor Clinical Candidate for Treating COVID-19

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†These authors contributed equally to this work.

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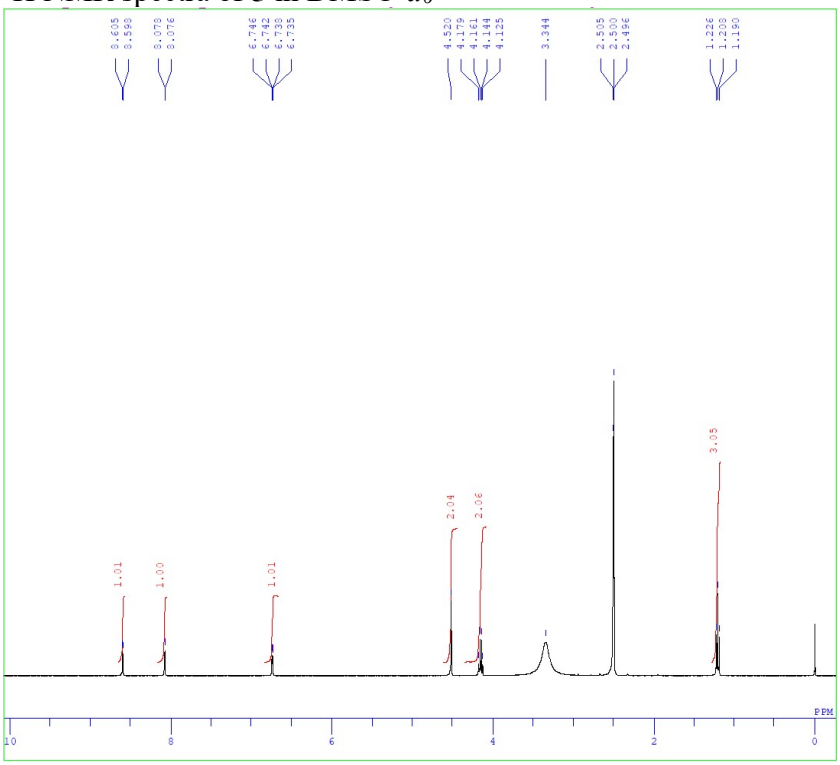

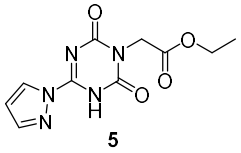

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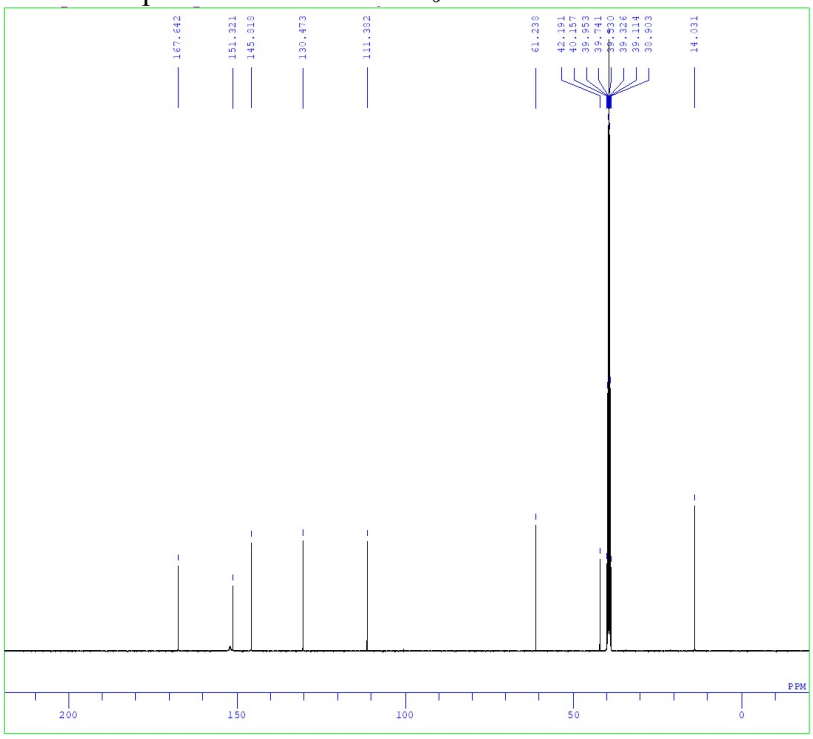
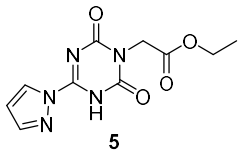
^{13}C NMR spectra of **5** in DMSO-d_6





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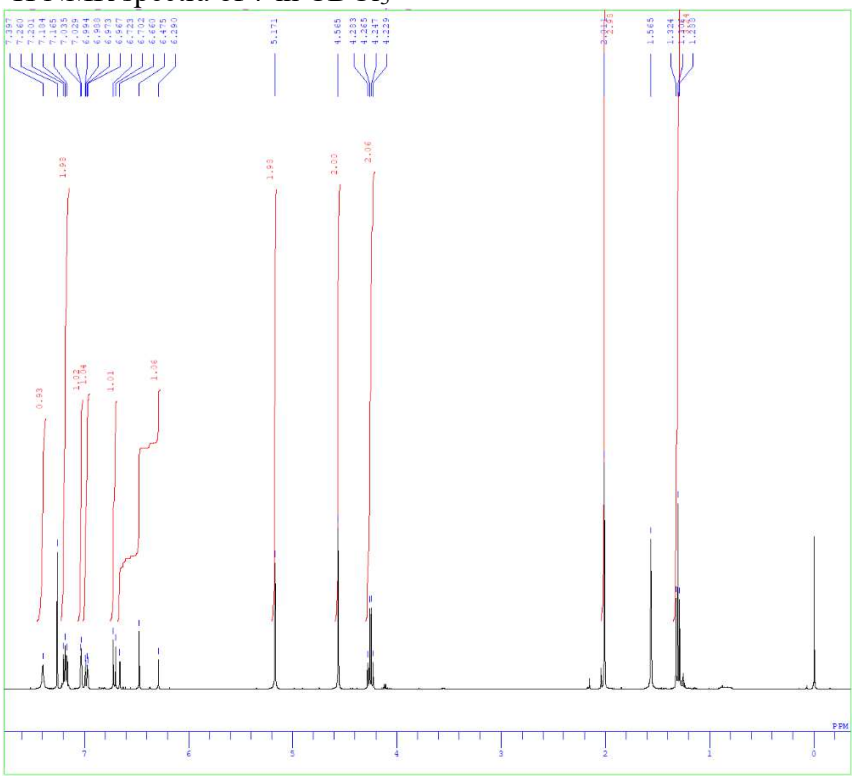
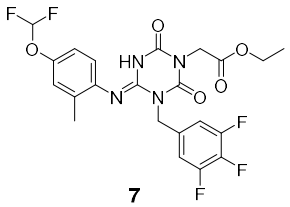
^{1}H NMR spectra of **7** in $CDCl_3$





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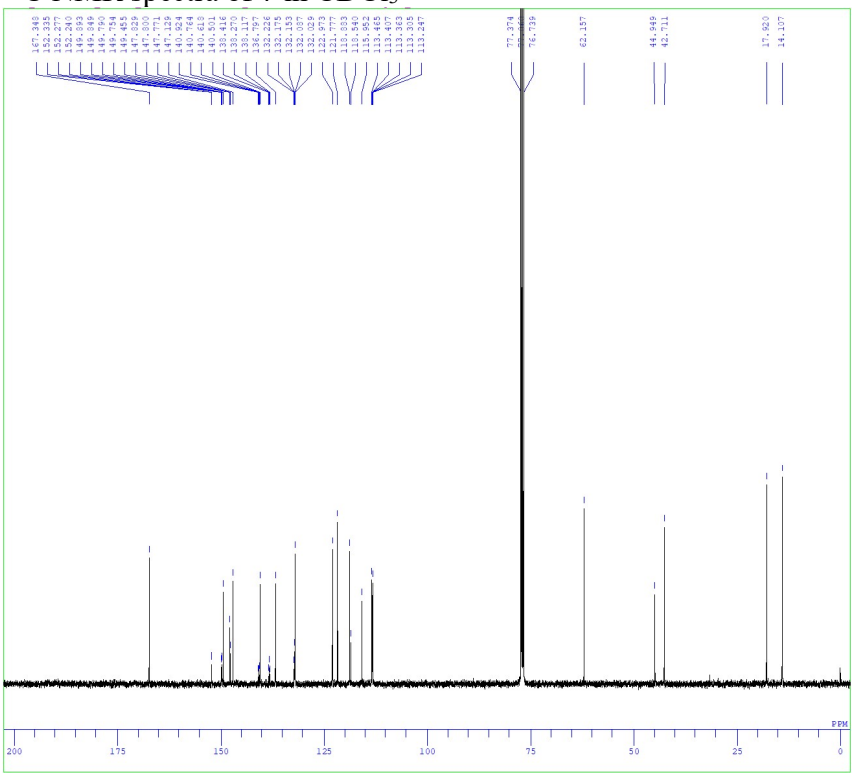
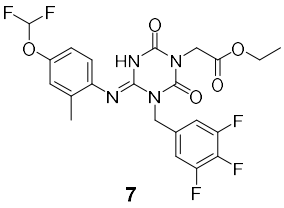
^{13}C NMR spectra of **7** in $CDCl_3$





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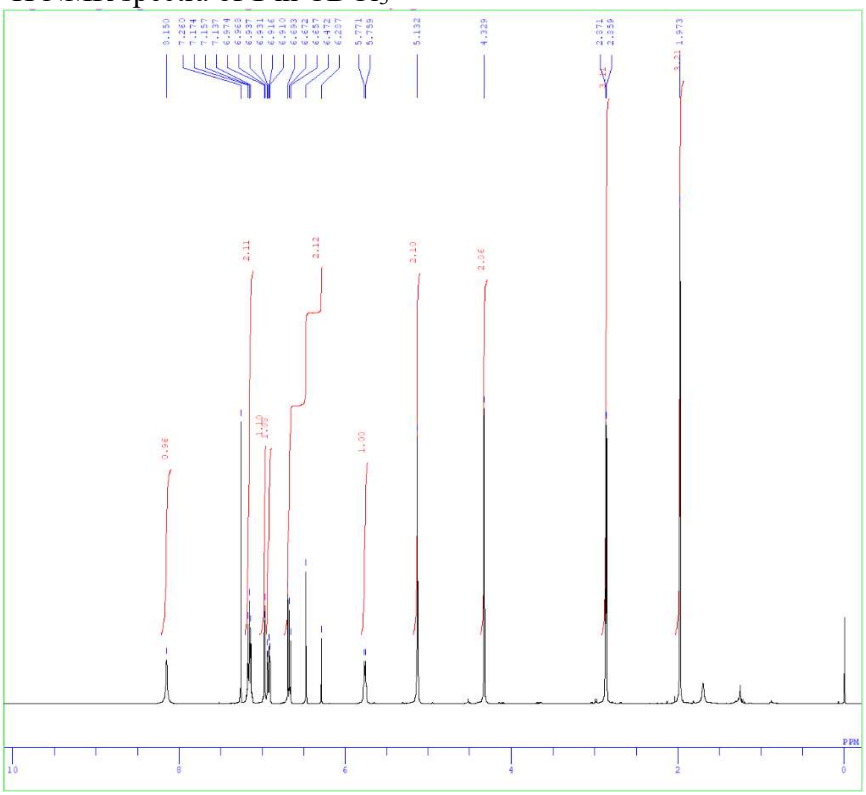

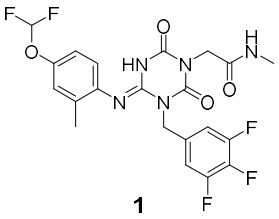

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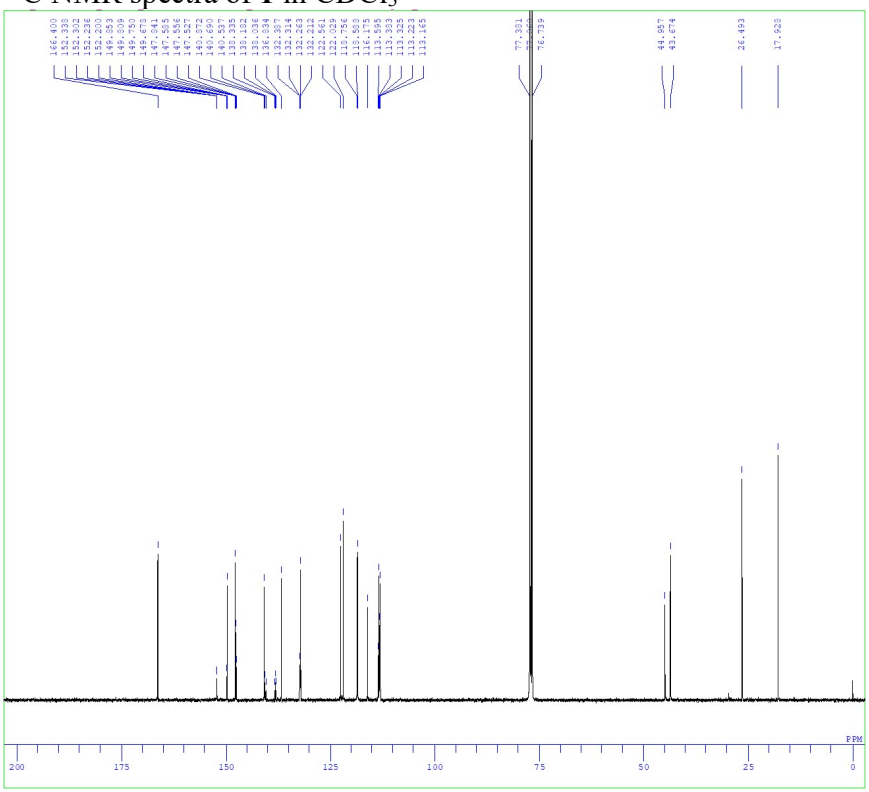
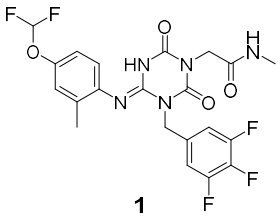
^{13}C NMR spectra of **1** in $CDCl_3$





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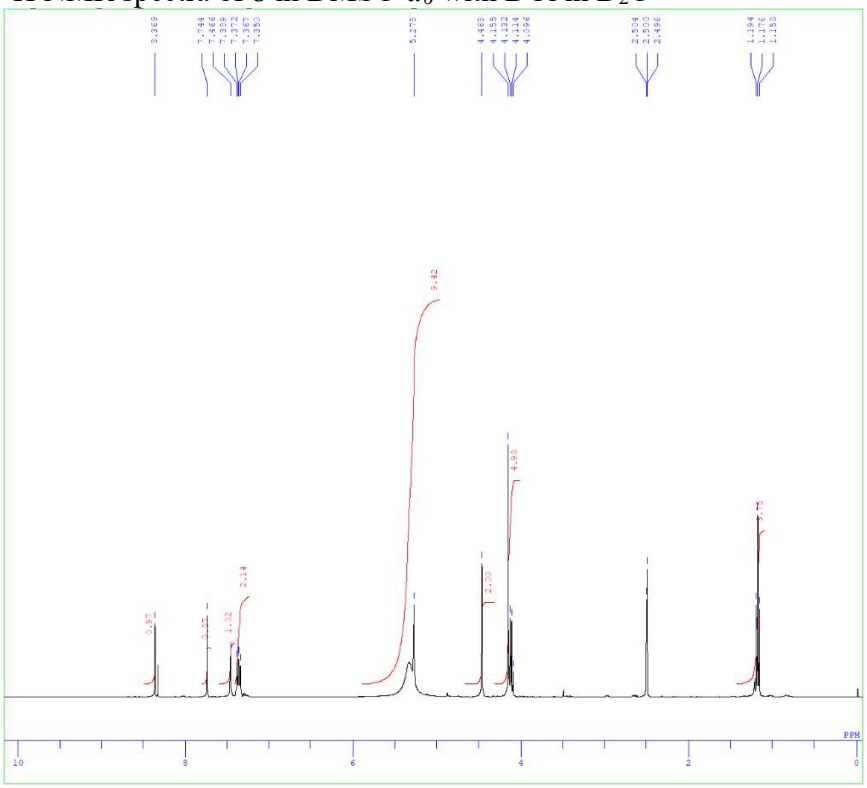
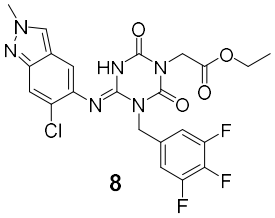
^{1}H NMR spectra of **8** in DMSO-d_6 with DCl in D_2O





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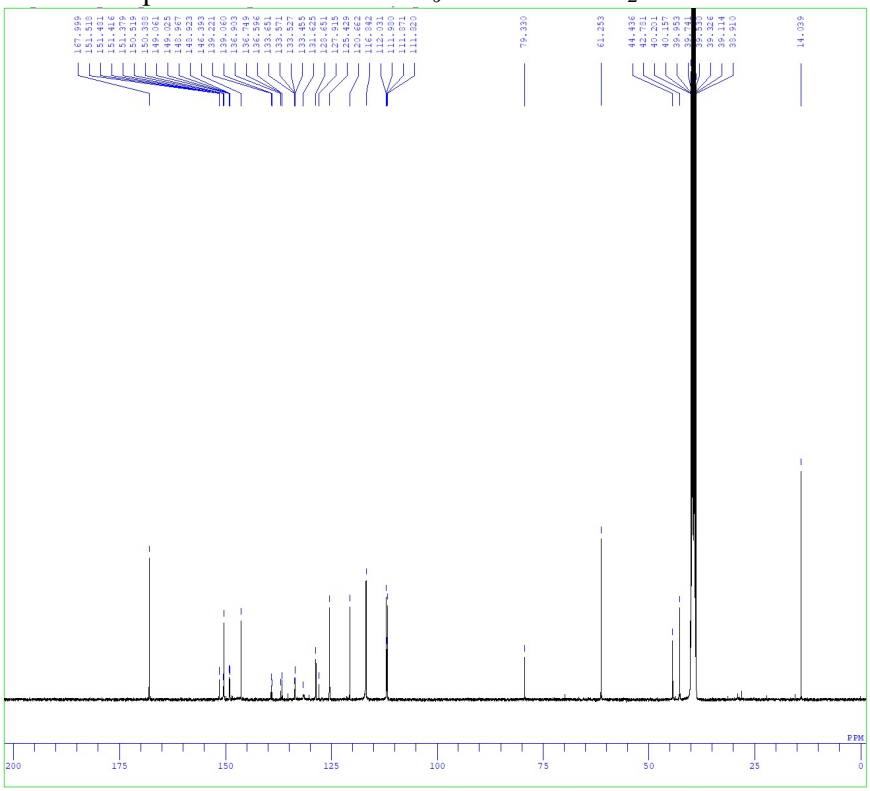
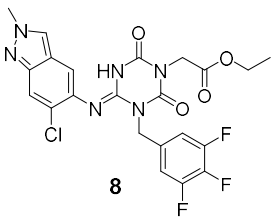
^{13}C NMR spectra of **8** in DMSO-d_6 with DCl in D_2O





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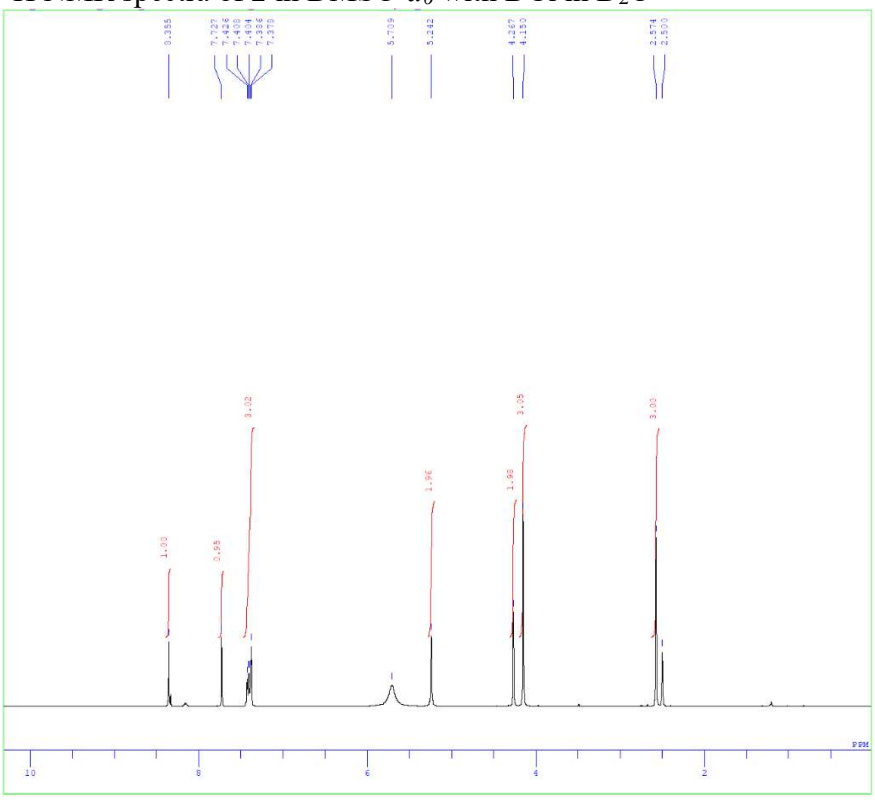
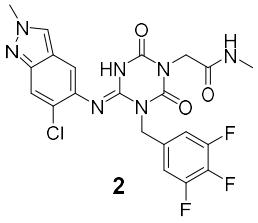
^{1}H NMR spectra of **2** in DMSO-d_6 with DCl in D_2O





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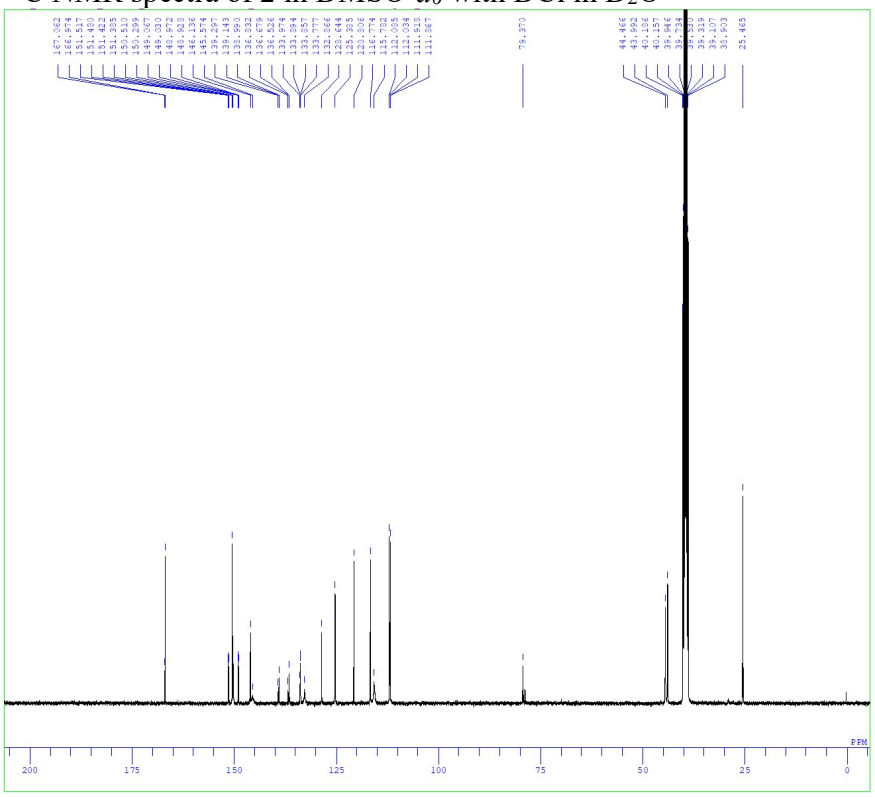

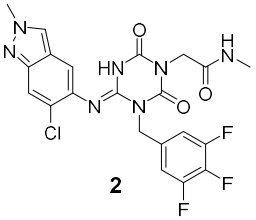

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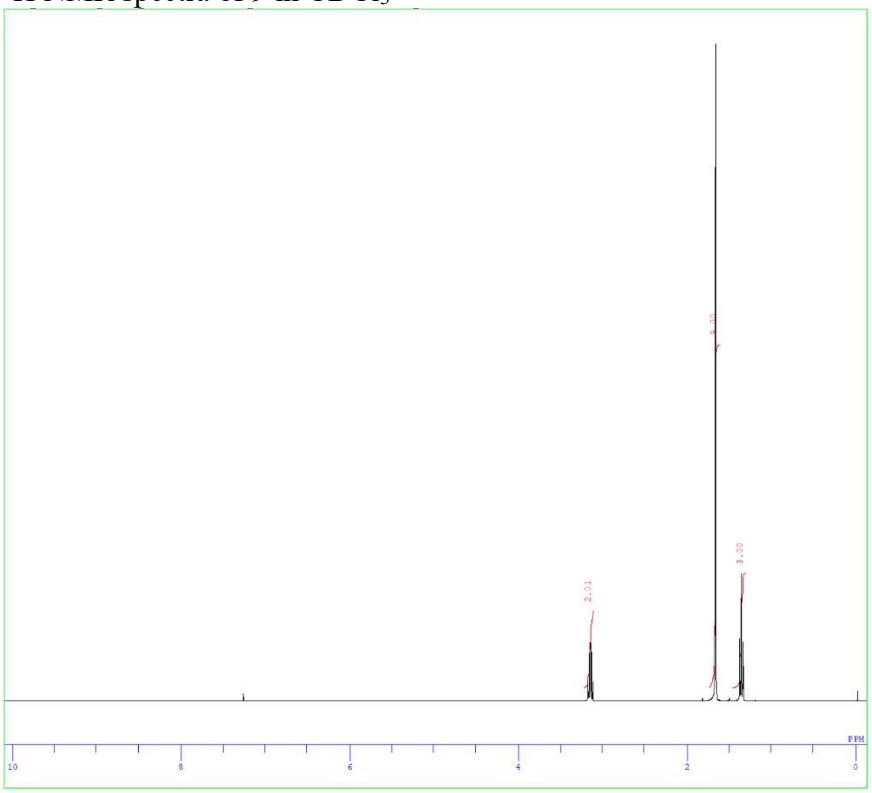

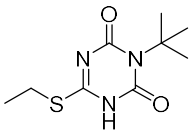

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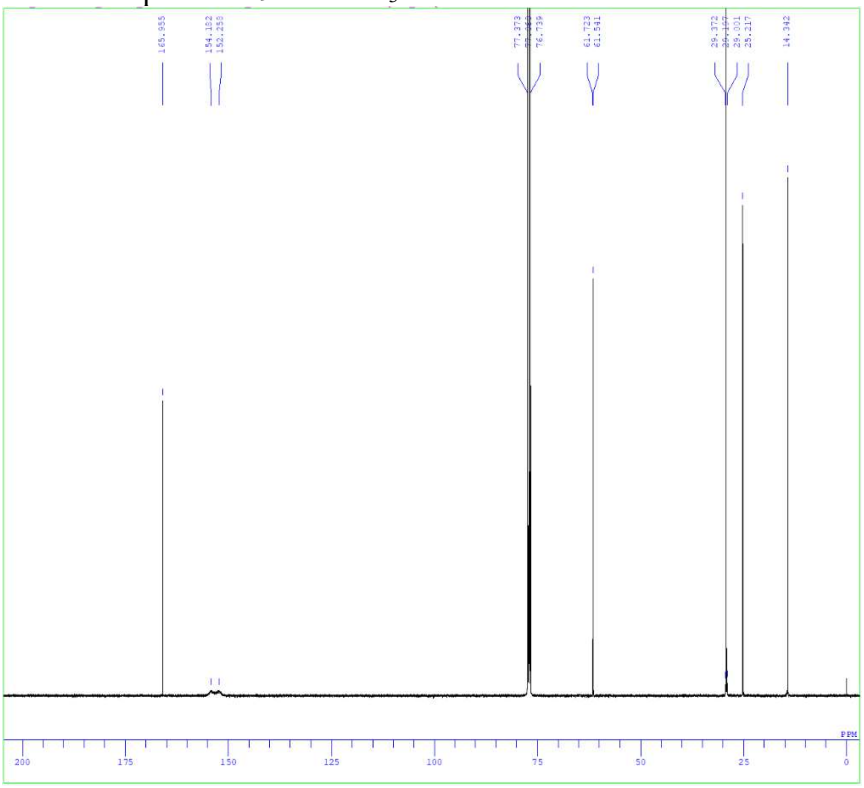
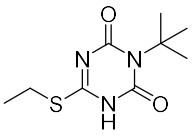
^{13}C NMR spectra of **9** in $CDCl_3$





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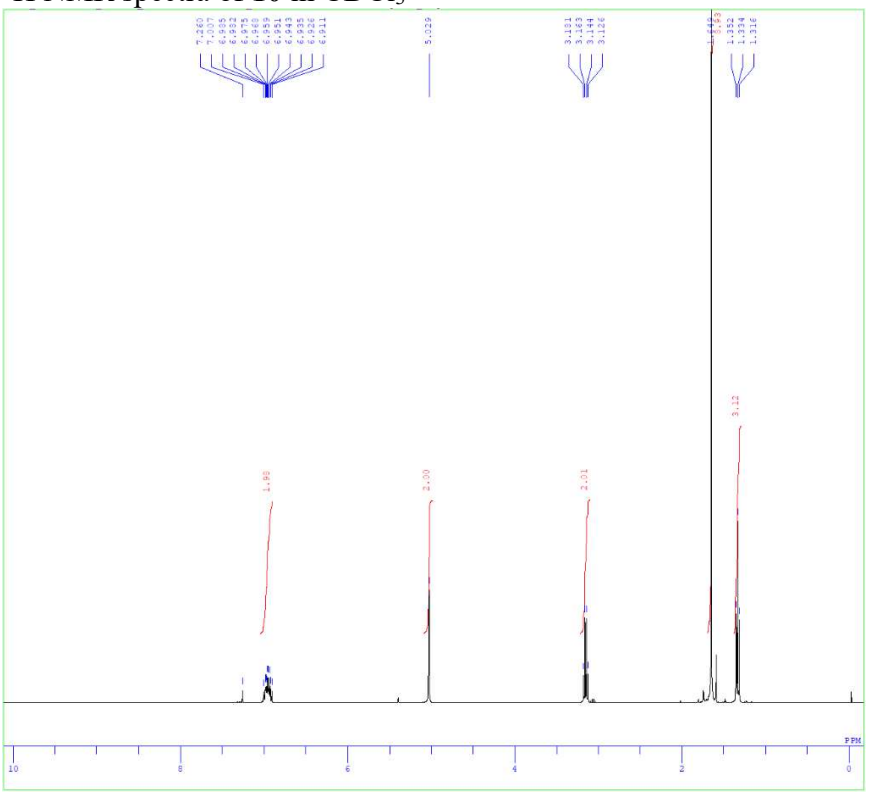

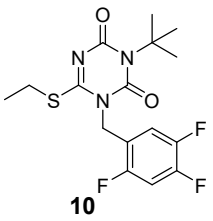

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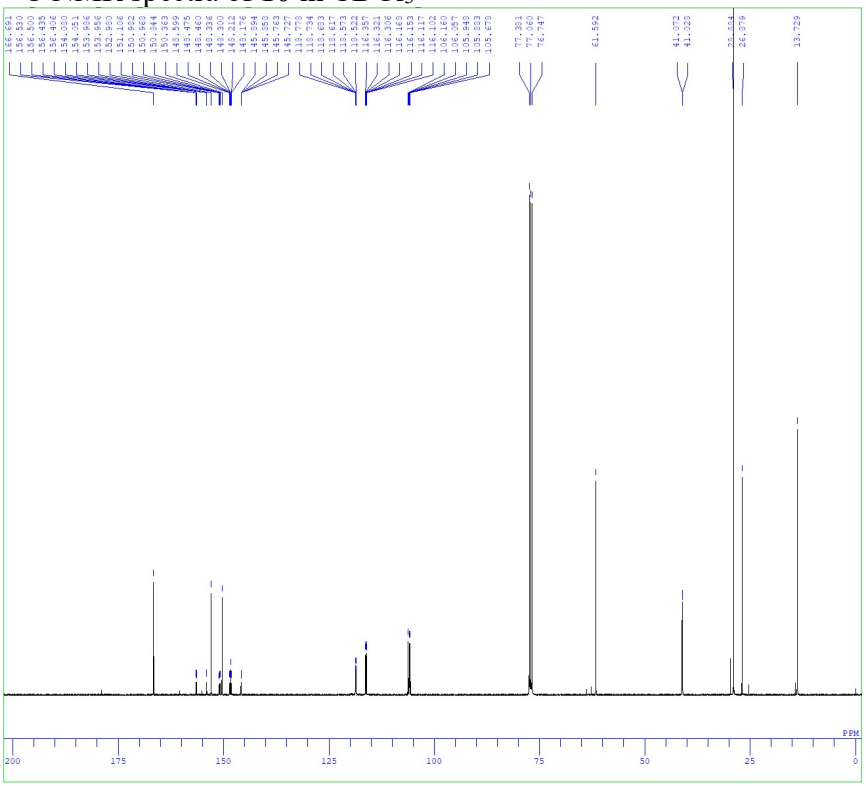
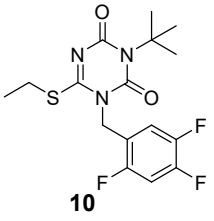
^{13}C NMR spectra of **10** in $CDCl_3$





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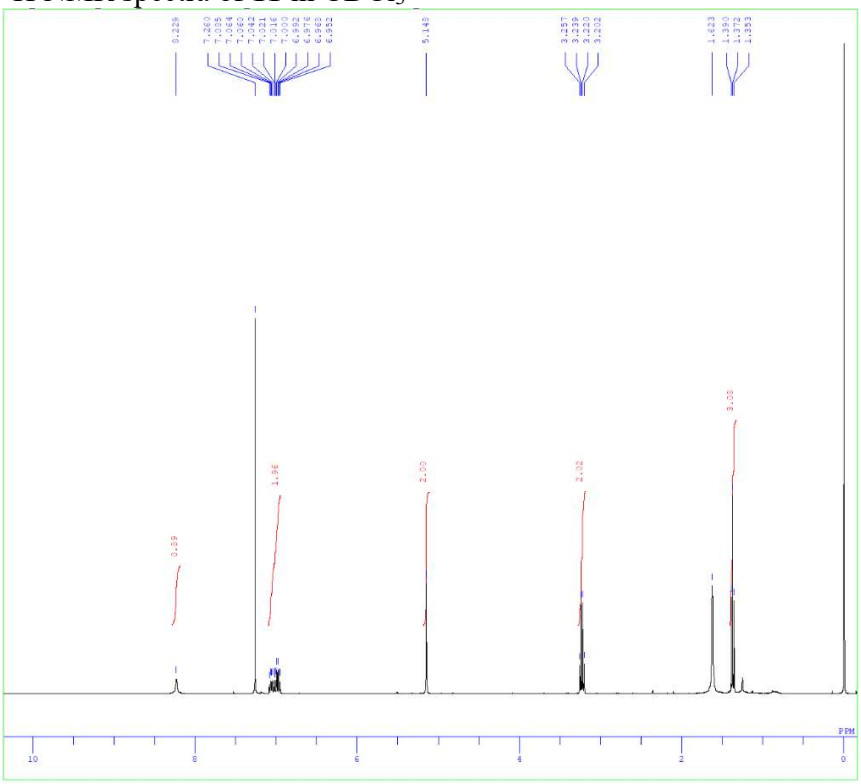
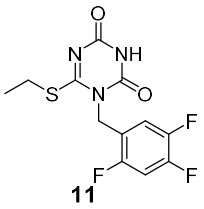
^{1}H NMR spectra of **11** in $CDCl_3$





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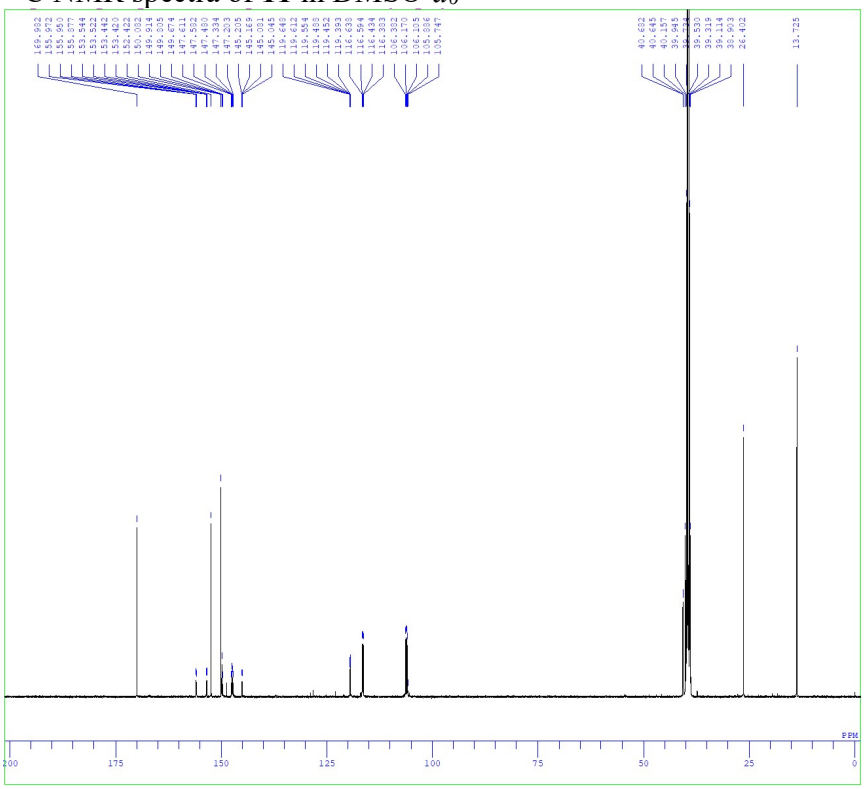
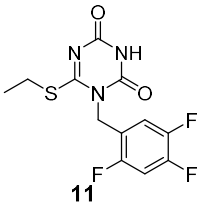
^{13}C NMR spectra of **11** in DMSO-d_6





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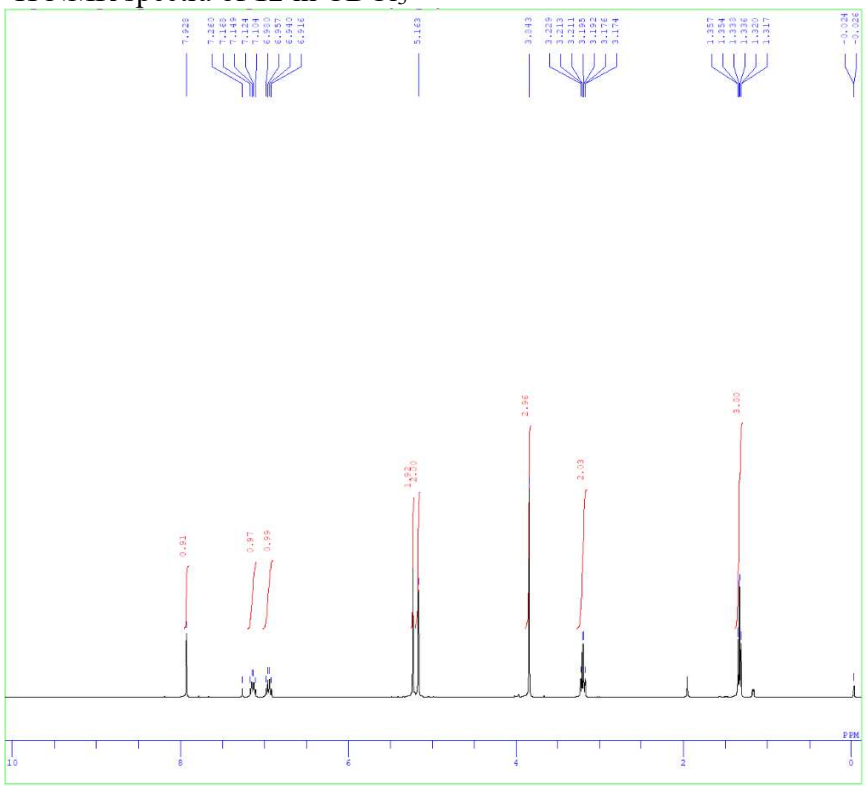
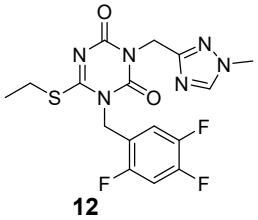
^{1}H NMR spectra of **12** in $CDCl_3$





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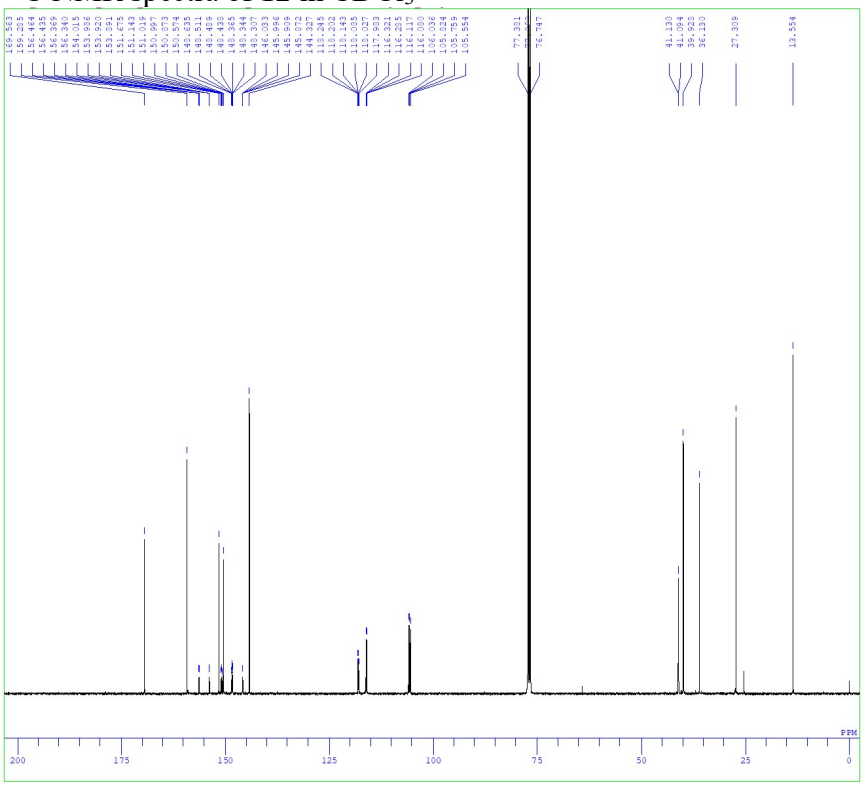

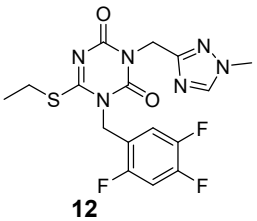

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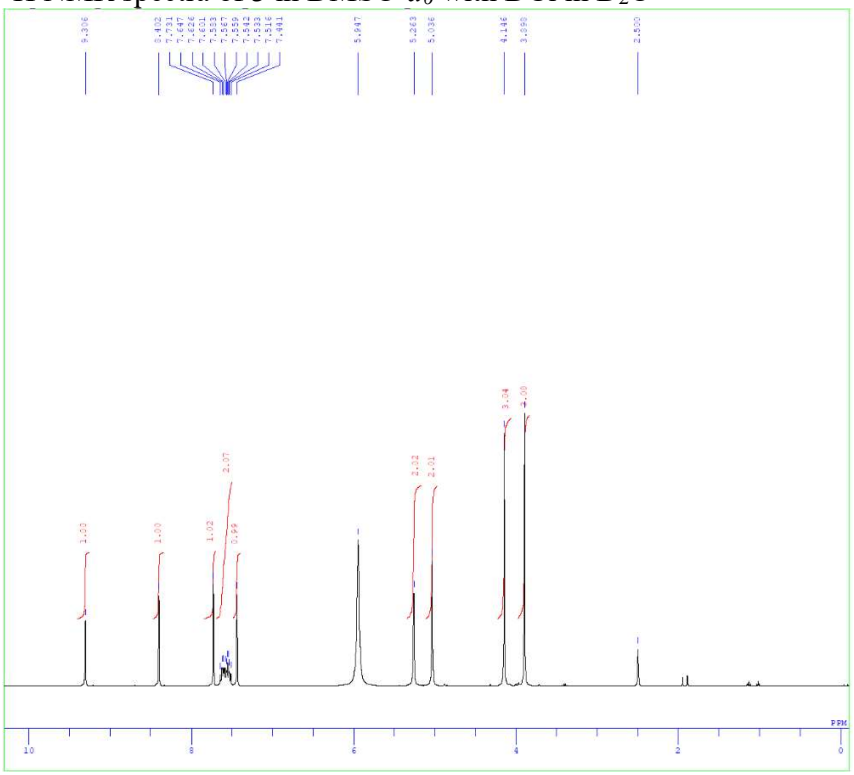
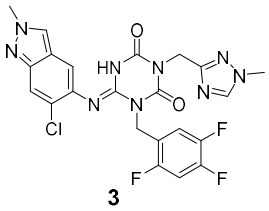
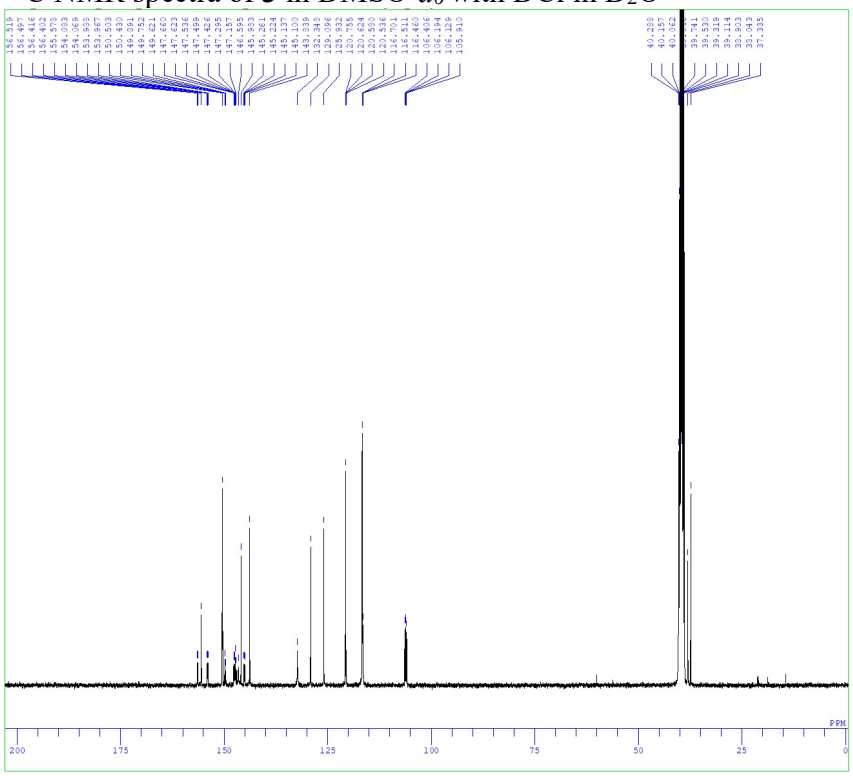
^{1}H NMR spectra of **3** in DMSO-d_6 with DCl in D_2O

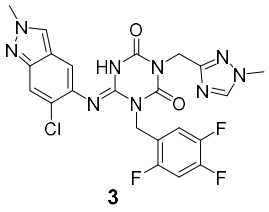




3

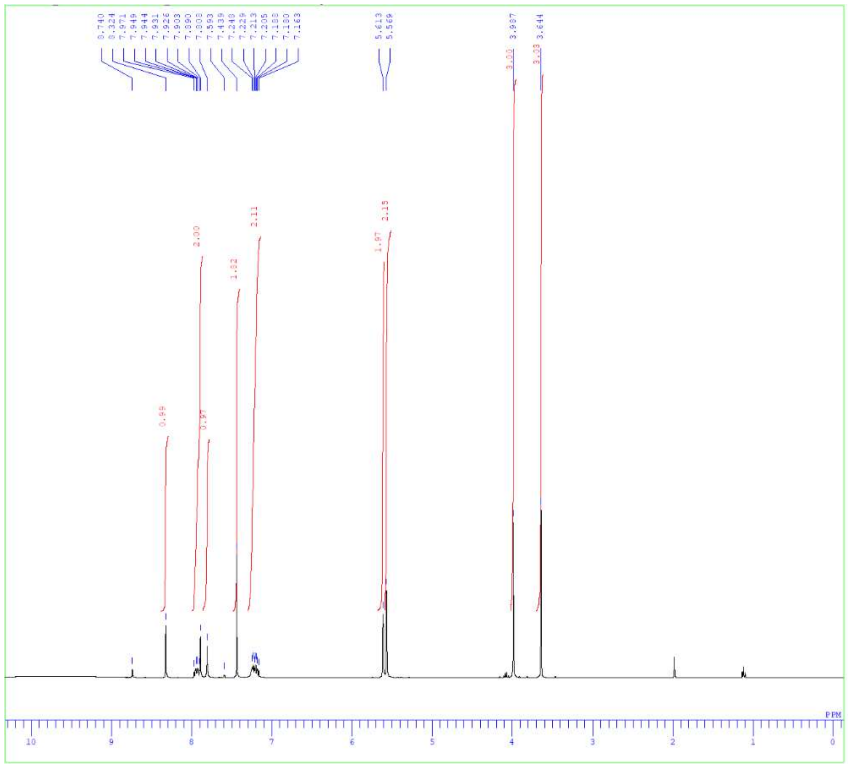
^{13}C NMR spectra of **3** in DMSO-d_6 with DCl in D_2O

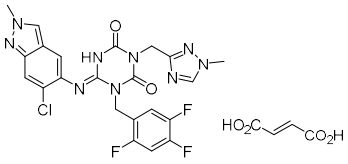




3

^{1}H NMR spectra of compound **3 (S-217622)** fumaric acid co-crystal in pyridine-d_5





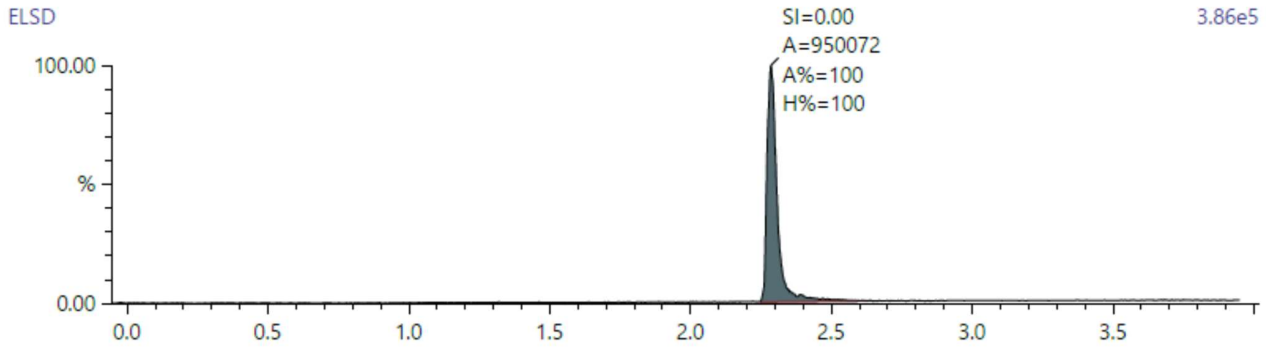
3 (fumaric acid co-crystal)

HPLC traces of compound 1-3

Procedure

Analytical liquid chromatography/mass spectroscopy (LC/MS) was performed on a Shimadzu Shim-pack XR-ODS (C18, 2.2 µm, 3.0 × 50 mm, linear gradient from 10% to 100% B over 3 min, then 100% B for 1 min [A = water + 0.1% formic acid, B = MeCN + 0.1% formic acid], flow rate: 1.6 mL/min) using a Shimadzu UFLC system equipped with a LCMS-2020 mass spectrometer, LC-20AD binary gradient module, SPD-M20A photodiode array detector (detection at 254 nm), and SIL-20AC sample manager.

Compound **1**



Pk#	RT	Area	Area %	Height	Height %
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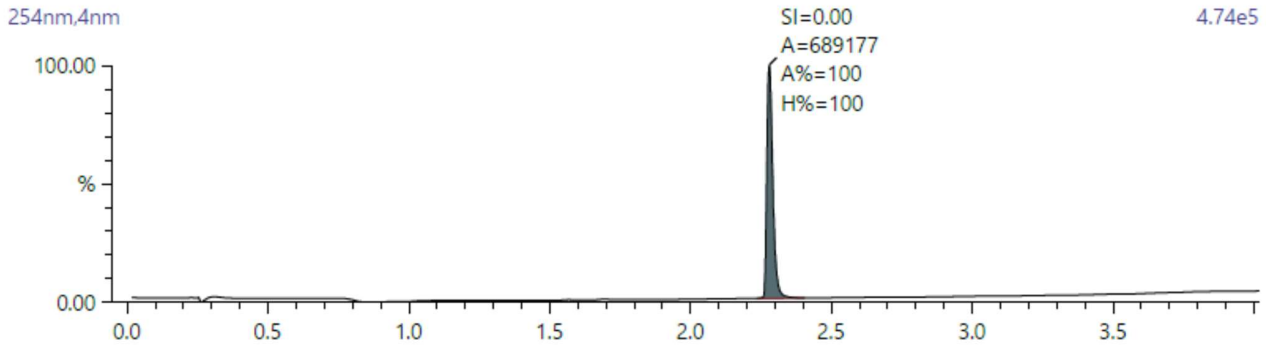
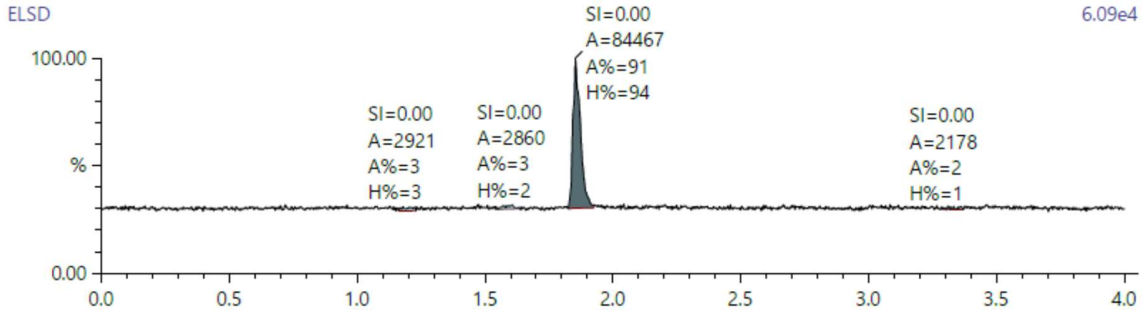
Totals:		**950,072**	**100.000**	**376,089**	**100.000**

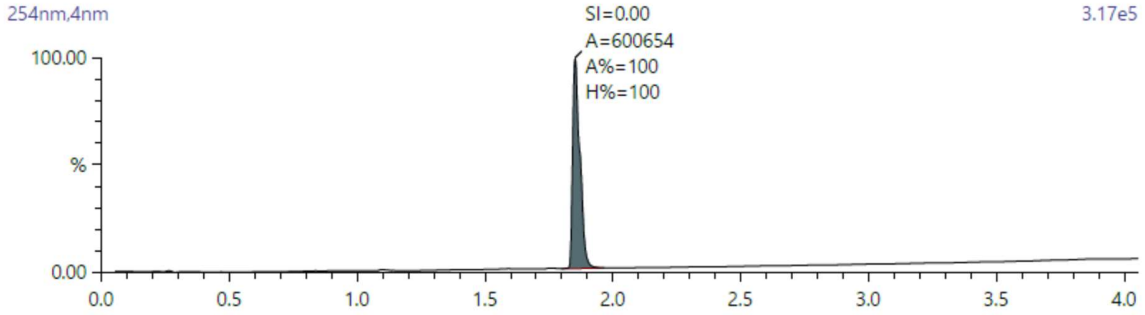


Pk#	RT	Area	Area %	Height	Height %
6	2.279	689,177	100.000	474,219	100.000
Totals:		**689,177**	**100.000**	**474,219**	**100.000**

Compound **2**



Pk#	RT	Area	Area %	Height	Height %
1	1.180	2,921	3.161	1,204	2.665
2	1.607	2,860	3.094	1,126	2.492
3	1.854	84,467	91.389	42,478	94.042
4	3.297	2,178	2.356	362	0.801
Totals:		**92,426**	**100.000**	**45,169**	**100.000**



Pk#	RT	Area	Area %	Height	Height %
3	1.853	600,654	100.000	312,342	100.000
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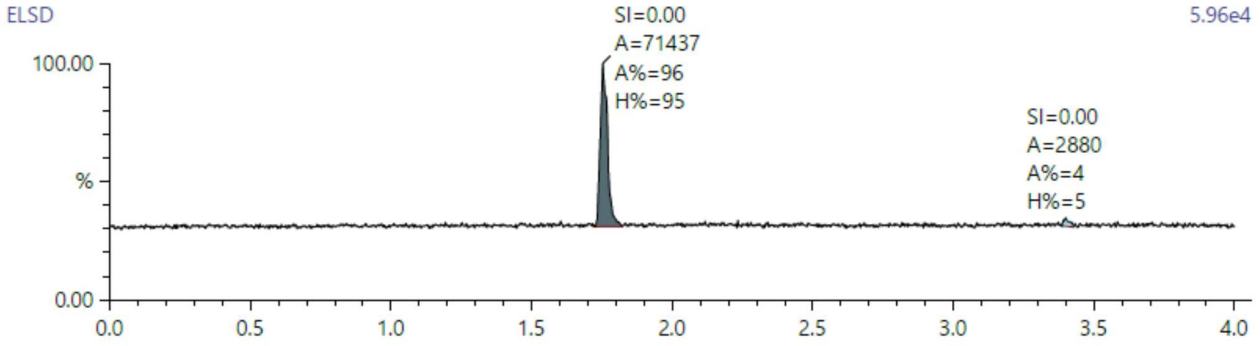
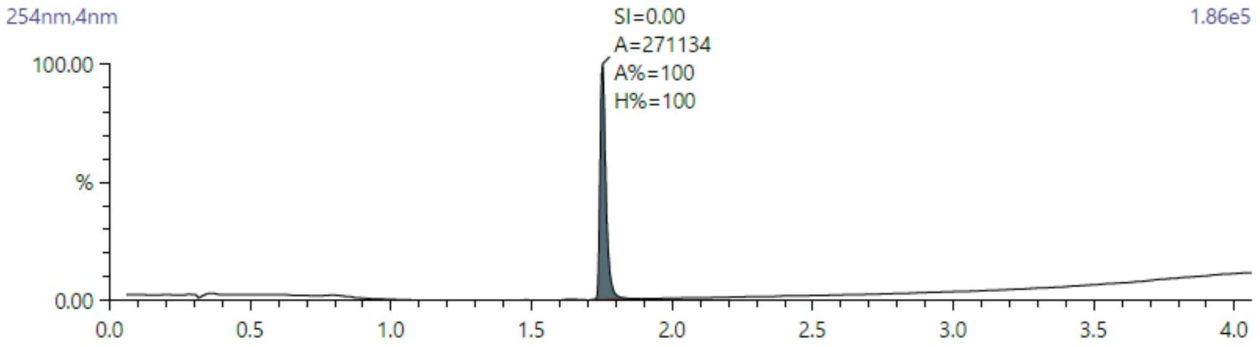
Compound **3** (free form)



Pk#	RT	Area	Area %	Height	Height %
1	1.754	71,437	96.124	41,034	95.111
2	3.399	2,880	3.876	2,109	4.889
Totals:		**74,317**	**100.000**	**43,144**	**100.000**



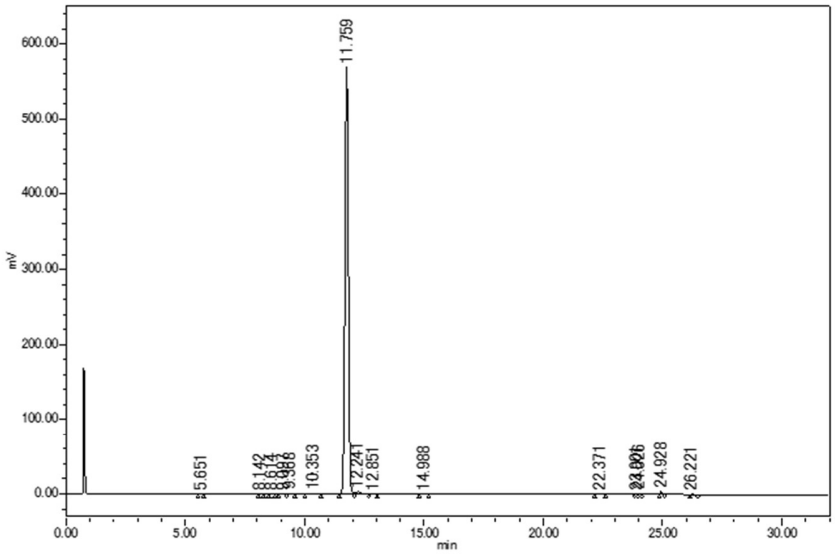
Pk#	RT	Area	Area %	Height	Height %
1	1.752	271,134	100.000	189,771	100.000
Totals:		**271,134**	**100.000**	**189,771**	**100.000**

HPLC chromatogram of 3 (S-217622) fumaric acid co-crystal

Analytical liquid chromatography of S-217622 fumaric acid co-crystal was performed on a ACQUITY UPLC BEH C18 Column (1.7 µm, 2.1 mm I.D.x100 mm, gradient from 10% to 100% B [A = 10 mmol/L $HCOONH_4$ /H_2O, B = MeCN], flow rate: 0.3 mL/min) using a Shimadzu Nexera system equipped with LC-20AD binary gradient module and SPD-20AV detector (detection at 255 nm).

Time-program for gradient elution

Time (min)	Mobile Phase B (%)
0	10
3	30
20	30
26	90
32	90
32.01	10
42	Stop



Retention time (min)	Peak area (µV·sec)	Peak height (µV)	% area
5.7	1179	320	0.02
8.1	1119	212	0.02
8.6	1316	219	0.02
9.1	1095	132	0.02
9.4	6948	987	0.12
10.4	14968	881	0.26
11.8	5559146	569193	98.21
12.2	45115	3668	0.80
12.9	1613	151	0.03
15.0	1242	102	0.02
22.4	4185	364	0.07
23.9	1721	436	0.03
24.0	4907	1408	0.09
24.9	13517	4116	0.24
26.2	2513	410	0.04

Experimental Procedures for *in vitro* safety.

Human ether-a-go-go-related gene inhibition assay

To evaluate an electrocardiogram QT interval prolongation of S-217622, effects on delayed rectifier K+ current (IKr) were evaluated using Chinese hamster ovary (CHO) cells expressing the human ether-a-go-go-related gene channel. The study was conducted in compliance with good laboratory practice regulations.

CHO cells were retained at a membrane potential of −80 mV with a whole-cell patch-clamp system (EPC-10 amplifier/PatchMaster v2.8 software, HEKA Co., Ltd.), and IKr was elicited via repolarization pulse at −40 mV for 2 sec after a depolarization pulse at +20 mV for 1 sec. S-217622 (fumaric acid co-crystal) was dissolved in DMSO and diluted 200-fold with external solution to prepare an objective concentration. The S-217622 concentration levels were 10, 30 and 100 μM. E-4031 at 0.1 μM and the vehicle (0.5% DMSO) were applied as positive and negative controls, respectively.

From the recorded IKr, an absolute value of the tail peak current was measured based on the current value at the resting membrane potential using the whole-cell patch-clamp method. The percentages of the preapplication values in the test substance and control groups were compensated by the mean value of the percentage of the preapplication value in the negative-control group, and the compensated suppression rates were calculated.

Patch-clamp solutions were as follows. Internal solution: KCL: 130mmol/L, $MgCl_2$: 1 mmol/L, MgATP: 5 mmol/L, EGTA: 5 mmol/L, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid): 10 mmol/L, pH=7.2. External solution: NaCl: 137 mmol/L, KCl: 4 mmol/L, $CaCl_2$: 1.8 mmol/L, $MgCl_2$: 1 mmol/L, glucose: 10 mmol/L, HEPES: 10 mmol/L, pH=7.4.

***In vitro* micronucleus test.** To evaluate the clastogenic potential of S-217622, an *in vitro* micronucleus test was conducted using TK6 cells (human lymphoblast-derived) in a short-term treatment with and without a metabolic activation system (S9 mix) and continuous treatment without S9 mix. S9 mix containing 9,000 g of liver supernatant fraction was prepared from Sprague-Dawley rats treated with phenobarbital and 5,6-benzofravone. The study was conducted in compliance with good laboratory practice regulations.

A TK6 cell suspension was used in the absence of metabolic activation in the 3-h and 24-h treatment groups or in the presence of metabolic activation in the 3-h treatment group. Suspensions were mixed with S-217622 (fumaric acid co-crystal) in DMSO solution with S9 mix in the presence of metabolic activation

in the 4-h treatment group, then incubated at 37°C. The negative-control (DMSO) and positive-control (mitomycin C, cyclophosphamide monohydrate, or colchicine) substances were prepared concurrently. After the end of the short-term treatment, cells were washed and then incubated in fresh culture medium for 21 h. After incubation, the cells were counted to evaluate cytotoxicity, and the *in vitro* micronucleus test was conducted with S-217622 (fumaric acid co-crystal) at doses of 150–250 μg/mL for short-term treatment and 75–125 μg/mL for continuous treatment based on cytotoxicity. The nucleic acid was stained with acridine orange, and the micronuclear frequency of the specimens was observed under a fluorescence microscope. The test was considered positive when a significant and dose-dependent increase was noted in the number of cells with micronuclei in the test-substance groups compared with the negative-control group under any treatment condition.

Ames test. To evaluate the mutagenic potential of S-217622, a bacterial reverse-mutation test was conducted via the preincubation method using five bacterial strains, including *Salmonella typhimurium* (TA98, TA100, TA1535, and TA1537) and *Escherichia coli* (WP2*uvrA*) in the presence or absence of a metabolic activation system (S9 mix). S9 mix containing 9,000 g of liver supernatant fraction was prepared from Sprague-Dawley rats treated with phenobarbital and 5,6-benzofravone. The study was conducted in compliance with good laboratory practice regulations.

S-217622 (fumaric acid co-crystal, DMSO solution) was mixed with S9 mix in the presence of metabolic activation or phosphate buffer in the absence of metabolic activation and 0.1 mL of test strain suspension (1×10^9 cells/mL or greater) and incubated at 37°C for 20 min. Then, the mixture with a layer of soft agar containing histidine and biotin or tryptophan was overlaid on minimal glucose agar plates. The negative-control (DMSO) and positive-control (4-nitroquinoline 1-oxide, sodium azide, 9-aminoacridine hydrochloride monohydrate, or 2-aminoanthracene) substances were prepared concurrently. The mutation test was conducted with S-217622 (fumaric acid co-crystal) at 156–5000 μg/plate in TA98, TA100, TA1535, and WP2*uvrA* and at 39.1–5000 μg/plate in TA1537. After incubation at 37°C for 48 h, the revertant colonies were counted and evaluated by comparing them with the negative-control group. The test was considered positive when the number of revertant colonies was concentration-dependently increased and twofold or greater increased over the number of colonies of the negative-control group.

***In vitro* 3T3.** To evaluate the phototoxicity potential of S-217622, an *in vitro* phototoxicity study was conducted with cultured mammalian cells. The study was conducted in compliance with good laboratory

practice regulations. A fibroblastic cell line derived from BALB/c mice (BALB/3T3 cells) was cultured in 96-well plates and treated with S-217622 (fumaric acid co-crystal) for 1 h followed by UV-A (5 J/cm^2) and UV-B (68.7 mJ/cm^2) irradiation. For the comparator, no irradiation was conducted. The phototoxicity test was performed at 0.781–100 μg/mL. A vehicle (DMSO)-treated group and a chlorpromazine hydrochloride-treated group were set as the negative and positive controls, respectively. Cell viability was determined by neutral-red extraction from cells (measurement of absorbance at 540 nm). When the IC_{50} could be determined for both the irradiated and nonirradiated plates, the result was determined from the PIF. When the IC_{50} could not be determined for either the irradiated or nonirradiated plates, the result was determined from the MPE. The judgment criteria are shown below.

No phototoxicity: PIF < 5 or MPE < 0.15
Phototoxicity: 5 ≤ PIF or 0.15 ≤ MPE

Table S1. The calculated IC_{50} values for S-217622 (fumaric acid co-crystal) to SARS-CoV-2 3CL protease activity in each experiment.

Experiment	IC_{50} (μM)
Exp. No.1	0.0122
Exp. No.2	0.0130
Exp. No.3	0.0143
Mean	0.0132
Standard deviation	0.0011

Table S2. EC_{50} values of S-217622 (fumaric acid co-crystal) and remdesivir on cytopathic effects in SARS-CoV-2 3CL-infected VeroE6/TMPRSS2 cells. [a]standard deviation. The mean and SD were calculated from three independent experiments.

Strains	Pango Lineage	EC_{50} (μM)			
		S-217622		Remdesivir	
		Mean	SD[a]	Mean	SD[a]
hCoV-19/Japan/TY/WK-521/2020	A	0.37	0.060	1.9	0.14
hCoV-19/Japan/QK002/2020	B.1.1.7	0.33	0.050	0.87	0.027
hCoV-19/Japan/QHN001/2020	B.1.1.7	0.31	0.070	0.97	0.14
hCoV-19/Japan/QHN002/2020	B.1.1.7	0.46	0.044	0.99	0.18
hCoV-19/Japan/TY7-501/2021	P.1	0.50	0.048	2.1	0.39
hCoV-19/Japan/TY7-503/2021	P.1	0.43	0.00085	1.0	0.16
hCoV-19/Japan/TY8-612/2021	B.1.351	0.40	0.048	1.2	0.30
hCoV-19/Japan/TY11-927-P1/2021	B.1.617.2	0.41	0.014	1.6	0.22
hCoV-19/Japan/TY38-873/2021	B.1.1.529	0.29	0.054	1.1	0.28

Table S3. CC_{50} values of S-217622 (fumaric acid co-crystal) and remdesivir against VeroE6/TMPRSS2 cells. [a]standard deviation. The mean and SD were calculated from three independent experiments.

Substance	CC_{50} (μM)				
	Exp.1	Exp.2	Exp.3	Mean	SD[a]
S-217622	>100	>100	>100	>100	-
Remdesivir	>100	>100	>100	>100	-

Table S4. *In vitro* safety profiles of S-217622 (fumaric acid co-crystal)

***In vitro* safety assays**	**Results**
hERG inhibition assay	>100 μM
Bacterial reverse mutation test (Ames test)	Negative
Micronucleus test	Negative
3T3 assay	No Phototoxicity

Table S5. Diffraction data and refinement statistics for SARS-CoV-2 3CL^{pro} complexed with compound **1** and compound **3** (**S-217622**).

[a]

	3CL^{pro} – Compound 1	**3CL^{pro} – Compound 3 (S-217622)**
PDB code	7VTH	7VU6
Data collection		
Space group	P21	P21
Cell dimensions		
a, b, c (Å)	44.41 54.27 114.50	55.47 99.23 58.88
α, β, γ (°)	90.00 99.42 90.00	90.00 108.05 90.00
Wavelength (Å)	1.54178	1.54178
Resolution (Å)	28.24 – 2.00(2.05 -2.00)	28.68 - 1.80(1.84 -1.80)
Completeness (%)	99.5 (98.9)	99.8(100.0)
R_{merge} *(%)*[a,b]	9.0(27.7)	6.0(42.9)
I/σ(I) [a]	8.3(3.3)	20.5(2.6)
Refinement		
Resolution (Å)	112.952 – 2.001	55.986-1.800
No. of reflections	34366	52687
R_{work}/R_{free}	0.2050/0.2568	0.2241/0.2790
B factor (Å2)		
Protein	16.2	17.8
Ligand	27.4	14.3
Water	22.2	25.3
R.m.s deviations		
Bond length (Å)	0.0128	0.0175
Bond angles (°)	1.576	1.796
Ramachandran plot (%)		
Favored	97.2	97.5
Allowed	2.1	2.2
Outliers	0.7	0.3

Values in parentheses are for the highest resolution shell.

[b] $R_{merge} = \Sigma| I - \langle I \rangle |/\Sigma I$, where I is the intensity of observation I and $\langle I \rangle$ is the mean intensity of the reflection.