Supporting Information

A warhead substitution study on the coronavirus main protease inhibitor Nirmatrelvir.

Subramanyam Vankadara, Monique Danielle Dawson†, Jia Yi Fong†, Qin Yao Oh†, Qi An Ang, Boping Liu, Hong Yun Chang, Judice Koh, Xiaoying Koh, Qian Wen Tan, Joma Joy and Cheng San Brian Chia*

Experimental Drug Development Centre, 10 Biopolis Road, Chromos #08-01, Singapore 138670.

†These authors contributed equally.

*Corresponding author

Tel.: +65 64070348

E-mail: cschia@eddc.a-star.edu.sg (Cheng San Brian Chia)

ORCID ID: 0000-0002-1567-3983

Contents

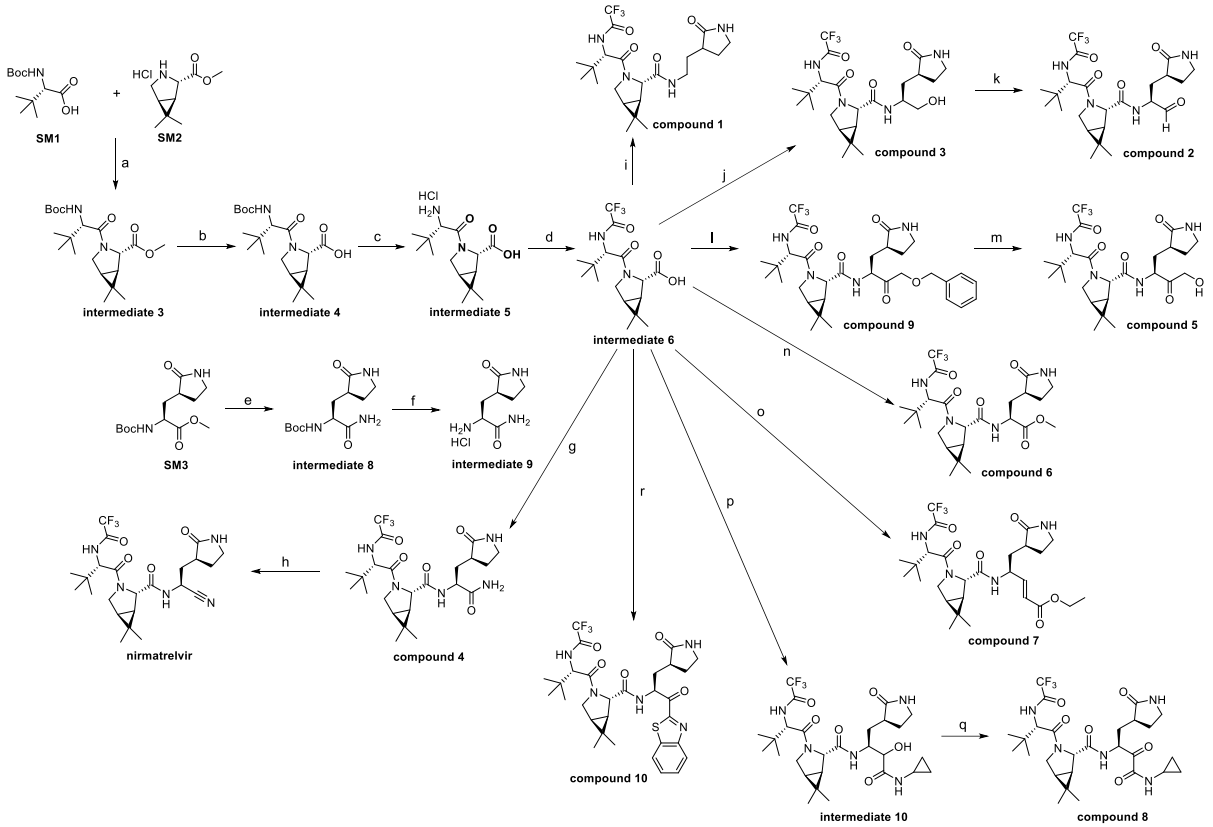
General chemistry

All reagents and solvents were purchased from commercial vendors (e.g. Merck/Sigma-Aldrich and Combi-Blocks) and were used without further purification. **SM1** and **SM2** were purchased from Combi-Blocks (#OR-1200 and #QM-6899 respectively). **SM3** was purchased from 1PlusChem (#1P00CAH9). Acetonitrile was purchased from VWR (Germany). Deuterated solvents were purchased from Cambridge Isotope Laboratories (USA). Flash chromatography was performed on an automated system (Teledyne ISCO Combiflash RF200) using silica column (RediSep RF Silica, 230–400 Mesh, 60 Å average pore size). Test compounds were purified using a reverse-phase C18 column (Waters X-Bridge OBD 5μ C18, 150 x 19 mm) on a high performance liquid chromatography (HPLC) system with an ultraviolet detector (Waters Auto Prep with 2489 UV detector and ACQUITY QDa mass detector). All test compounds were characterized by electrospray ionization high-resolution spectrometry (Thermo Fisher Scientific Q Exactive Quadrupole Orbitrap mass spectrometer). NMR spectra were recorded on a 400 MHz spectrometer (Bruker Ascend 400, Germany). Chemical shifts were expressed as δ (ppm) relative to the solvent peak.

Abbreviations

DABCYL	4-(dimethylaminoazo)benzene-4-carboxylic acid
DIPEA	N,N-diisopropylethylamine
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EDANS	5-((2-aminoethyl)amino)-naphthalene-1-sulfonic acid
EMEM	eagle's minimum essential medium
eq	equivalents
ESI	electrospray ionisation
FBS	fetal bovine serum
FRET	fluorescence resonance energy transfer
HATU	hexafluorophosphate azabenzotriazole tetramethyl uronium
HPLC	high performance liquid chromatography
IPTG	isopropyl β-D-1-thiogalactopyranoside
MS	mass spectrometry
NMR	nuclear magnetic resonance
RT	room temperature
SM	starting material
TB	Terrific broth
TFAA	trifluoroacetic anhydride
THF	tetrahydrofuran

Synthesis scheme of test compounds



Reaction schemes. a) HATU, DIPEA, DMF, 0 °C to RT, 1 h; b) LiOH (aq) THF, 0 °C to RT, 3 h; c) 4M HCl in 1,4-dioxane, 0 °C to RT, 6 h; d) TFAA, Et_3N, CH_2Cl_2, 0 °C to RT, 2 h; e) 7M NH_3 in CH_3OH, 50 °C, 5 h; f) 4M HCl in 1,4-dioxane, 0 °C to RT, 2 h; g) intermediate 9, HATU, DIPEA, DMF, 0 °C to RT, 0.5 h; h) Burgess reagent, CH_2Cl_2, 45 °C, 0.5 h; i) 3-(2-aminoethyl)pyrrolidin-2-one, HATU, DIPEA, DMF, 0 °C to RT, 0.5 h; j) (*S*)-3-(*S*)-2-amino-3-hydroxypropylpyrrolidin-2-one; HATU, DIPEA, DMF, 0 °C to RT, 0.5 h; k) Dess-Martin periodinane; DMF, 0 °C to RT, 1 h; l) (*S*)-3-(*S*)-2-amino-4-benzyloxy-3-oxobutyl pyrrolidin-2-one, HATU, DIPEA, DMF, 0 °C to RT, 0.5 h; m) H_2 (g), Pd/C, CH_3OH, RT, 18 h; n) methyl-(2*S*)-2-amino-3-[(3*S*)-2-oxopyrrolidin-3-yl]propanoate, HATU, DIPEA, DMF, 0 °C to RT, 0.5 h; o) ethyl-(*S*,*E*)-4-amino-5-(*S*-2-oxopyrrolidin-3-yl)pent-2-enoate, HATU, DIPEA, DMF, 0 °C to RT, 0.5 h; p) (3*S*)-3-amino-N-cyclopropyl-2-hydroxy-4-(*S*)-2-oxopyrrolidin-3-yl butanamide, HATU, DIPEA, DMF, 0 °C to RT, 0.5 h; q) Dess-Martin periodinane, DMF, 0 °C to RT, 3.5 h; r) (*S*)-3-(*S*)-2-amino-3-benzothiazol-2-yl-3-oxopropylpyrrolidin-2-one, HATU, DIPEA, DMF, 0 °C to RT, 0.5 h.

a) Synthesis of intermediate 3

(*S*)-2-(tert-butoxycarbonylamino)-3,3-dimethylbutanoic acid (**SM1**; 500 mg; 2.162 mmol; 1 eq.) and methyl(1*R*,2*S*,5*S*)-6,6-dimethyl-3-azabicyclo[3.1.0]hexane-2-carboxylate (**SM2**; 401 mg; 1.946 mmol; 0.9 eq.) were dissolved in anhydrous DMF (3 mL) in a 10 mL round-bottom flask. Next, DIPEA (1.13 mL; 10.809 mmol; 5 eq.) was added at 0 °C followed by HATU (905 mg; 2.378 mmol; 1.1 eq.). The mixture was stirred for 1 h. at RT under N_2 (g). Next, the reaction was diluted with ethyl acetate and washed with sat. $NaHCO_3$ (2x) followed by brine. The organic layer was collected, dried using Na_2SO_4 and concentrated *in vacuo*. The target was purified by flash chromatography (1−100% CH_3OH/CH_2Cl_2) to yield **intermediate 3** (515 mg; 62%). ESI-quadrupole-MS: *m/z* calc $C_{20}H_{35}N_2O_5$ ($M+H^+$) 383.26 found 383.20. No unexpected or unusually high safety hazards encountered during synthesis.

b) Synthesis of intermediate 4

Intermediate 3 (515 mg; 1.34 mmol; 1 eq.) was dissolved in THF (2 mL) in a 10 mL round-bottom flask. LiOH (97 mg; 4.02 mmol; 3 eq.) pre-dissolved in water (2 mL) was added at 0 °C and the mixture stirred for 3 h. at RT. The reaction mixture was quenched with sat. NH_4Cl solution (2 mL) at 0 °C and acidified to pH 3 with dropwise addition of 1M HCl. The target was extracted with CH_2Cl_2, washed with brine, dried over Na_2SO_4 and concentrated *in vacuo*. The target was dried overnight to yield **intermediate 4** as a white powder (427 mg; 86%). ESI-quadrupole-MS: *m/z* calc $C_{19}H_{33}N_2O_5$ ($M+H^+$) 369.24 found 369.20. No unexpected or unusually high safety hazards encountered during synthesis.

c) Synthesis of intermediate 5

Intermediate 4 (388 mg; 1.053 mmol; 1 eq.) was dissolved in 1,4-dioxane (0.5 mL) in a 25 mL round-bottom flask. 4M HCl in 1,4-dioxane (4 mL; 1.57 mmol; 15 eq.) was added at 0 °C and the reaction mixture stirred for 6 h. at RT. Next, the solvent was removed *in vacuo* and dried *in vacuo* for 18 h. to yield **intermediate 5** as a HCl salt (320 mg; 99%). ESI-quadrupole-MS: *m/z* calc $C_{14}H_{25}N_2O_3$ ($M+H^+$) 269.19 found 269.10. No unexpected or unusually high safety hazards encountered during synthesis.

d) Synthesis of intermediate 6

Intermediate 5 HCl salt (320 mg; 1.04 mmol; 1 eq.) was dissolved in anhydrous CH_2Cl_2 (10 mL) in a 25 mL round-bottom flask. Et_3N (0.45 mL; 3.12 mmol; 3 eq.) was added at 0 °C under N_2 (g). After 2 min., TFAA (0.22 mL; 1.5 mmol; 1.5 eq.) was added and the mixture stirred for 1.5 h. at RT under N_2 (g). About 30% of unreacted intermediate 5 was observed by LCMS. Additional triethylamine (80 μL; 0.551 mmol; 0.5 eq.) was added at 0 °C under N_2 (g). After 2 min., TFAA (39 μL; 0.276 mmol; 0.26 eq.) was added and the mixture stirred for 0.5 h. at RT. CH_2Cl_2 was removed *in vacuo* and the crude product was purified by preparative HPLC with 20 to 70% acetonitrile containing 0.1% formic acid and water containing 0.1% formic acid to yield **intermediate carboxylic acid 6** (252 mg; 66%) ESI-quadrupole -MS: *m/z* calc $C_{16}H_{24}F_3N_2O_4$ ($M+H^+$) 365.17 found 365.10. No unexpected or unusually high safety hazards encountered during synthesis.

e) Synthesis of intermediate 8

Methyl(*S*)-2-(*tert*-butoxycarbonylamino)-3-(*S*)-2-oxopyrrolidin-3-yl propanoate (**SM3**; 510 mg; 1.78 mmol; 1 eq.) was dissolved in 7M ammonia in CH_3OH solution (6.4 mL) at RT and the reaction mixture was stirred at 50 °C for 5 h. in a 20 mL thick glass vial. Next, the solvent was removed *in vacuo* and the crude target was purified by flash chromatography (1−100% CH_3OH/CH_2Cl_2) to yield **intermediate 8** (428 mg; 89%) APCI-MS: *m/z* calc $C_{12}H_{22}N_3O_4$ ($M+H^+$) 272.16 found 272.10. No unexpected or unusually high safety hazards encountered during synthesis.

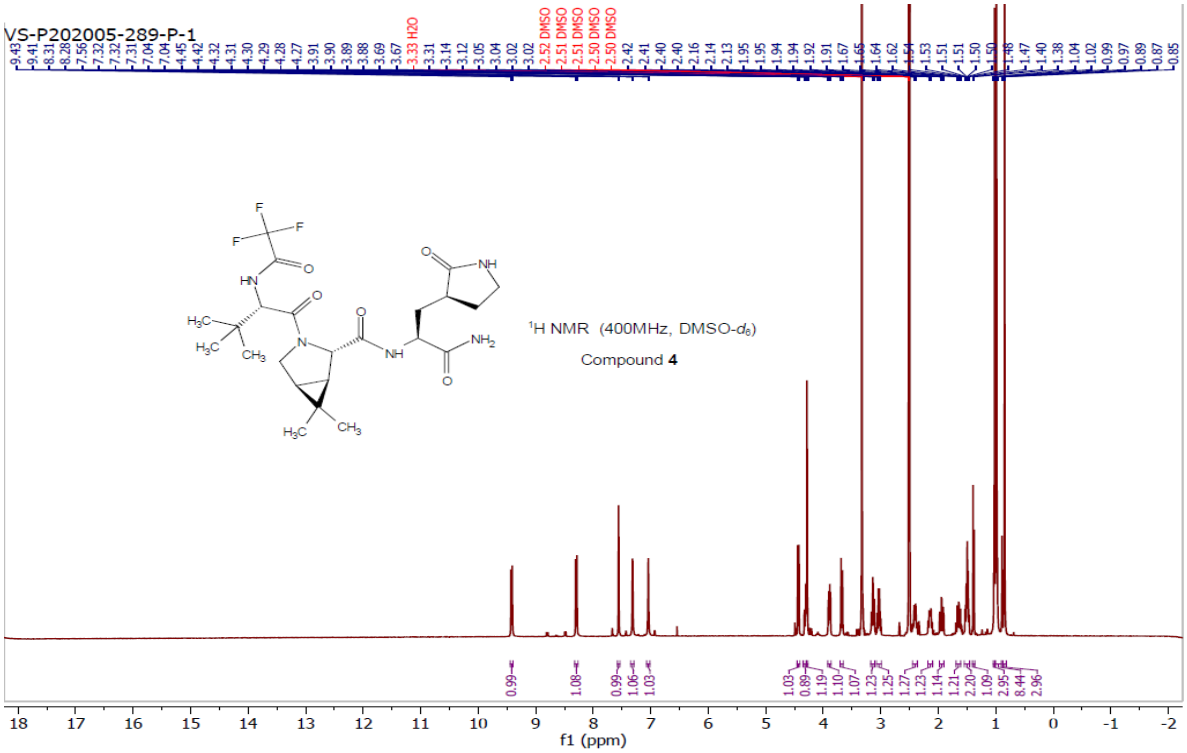
f) Synthesis of intermediate 9

Intermediate 8 (183 mg; 0.674 mmol; 1 eq.) was dissolved in 1,4-dioxane (0.4 mL) in a 10 mL round-bottom flask. 4M HCl in 1,4-dioxane (3.3 mL; 6.74 mmol; 20 eq.) was added to the mixture at 0 °C and stirred for 1 h. at RT. The solvent was removed *in vacuo* and the target dried *in vacuo* for 18 h. to yield **intermediate 9** as a HCl salt (138 mg; 99%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.33 (s, 3H), 7.98 (s, 2H), 7.60 (s, 1H), 3.89 – 3.74 (m, 1H), 3.28 – 3.09 (m, 2H), 2.57 – 2.51 (m, 1H, merged with solvent peak), 2.34 – 2.23 (m, 1H), 2.00 (ddd, *J* = 14.3, 9.2, 6.4 Hz, 1H), 1.83 – 1.63 (m, 2H).

Not detected by ESI-quadrupole-MS due to very low molecular weight (171.2). No unexpected or unusually high safety hazards encountered during synthesis.

g) Synthesis of compound 4

Intermediate carboxylic acid 6 (50 mg; 0.137 mmol; 1 eq.) and intermediate 9, HCl salt (35 mg; 0.169 mmol; 1.2 eq.) were dissolved in anhydrous DMF (4 mL) in a 10 mL round-bottom flask. DIPEA (120 μL; 0.69 mmol; 5 eq.) was added at 0 °C. After 1 min., HATU (58 mg; 0.15 mmol; 1.1 eq.) was added and the reaction mixture stirred for 0.5 h. at RT under N_2 (g). Next, the reaction was diluted with CH_2Cl_2, washed with sat. $NaHCO_3$ (2x), water and brine. The CH_2Cl_2 layer was dried with Na_2SO_4 and concentrated *in vacuo*. The crude target was purified by preparative HPLC with 20 to 70% acetonitrile containing 0.1% formic acid and water containing 0.1% formic acid to yield **compound 4** (54 mg; 76%) Spectral data: ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 9.42 (d, *J* = 8.6 Hz, 1H), 8.30 (d, *J* = 8.9 Hz, 1H), 7.56 (s, 1H), 7.32 (s, 1H), 7.04 (s, 1H), 4.43 (d, *J* = 8.6 Hz, 1H), 4.35 – 4.29 (m, 1H), 4.28 (s, 1H), 3.90 (dd, *J* = 10.3, 5.4 Hz, 1H), 3.68 (d, *J* = 10.4 Hz, 1H), 3.14 (t, *J* = 9.0 Hz, 1H), 3.08 – 2.98 (m, 1H), 2.46 – 2.36 (m, 1H), 2.19 – 2.09 (m, 1H), 1.99 – 1.90 (m, 1H), 1.71 – 1.59 (m, 1H), 1.55 – 1.45 (m, 2H), 1.39 (d, *J* = 7.6 Hz, 1H), 1.02 (s, 3H), 0.99 (s, 9H), 0.85 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 179.10, 173.99, 171.11, 167.68, 157.32 (q, *J* = 37.37 Hz), 116.34 (q, *J* = 288.86 Hz), 60.72, 58.58, 50.77, 48.18, 40.00, 37.78, 35.14, 34.57, 31.03, 27.87, 27.57, 26.75, 26.34, 19.06, 12.87 (21 of 23 peaks recorded due to P3 *t*-butyl). ^{19}F NMR (376 MHz, DMSO-d_6) δ -72.74. ESI-quadrupole-MS: *m/z* calc $C_{23}H_{35}F_3N_5O_5$ ($M+H^+$) 518.2590, found 518.2582. No unexpected or unusually high safety hazards encountered during synthesis.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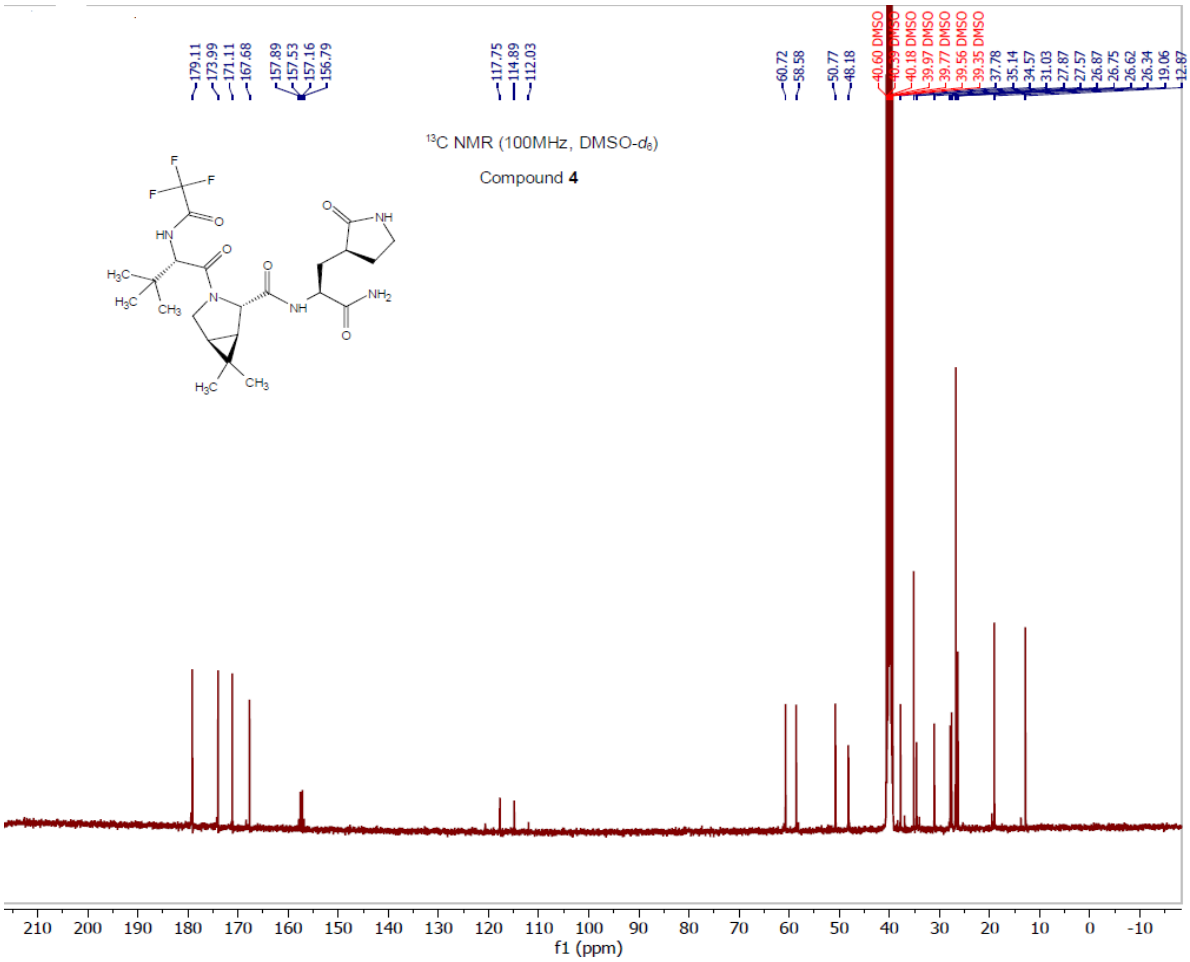
f1 (ppm)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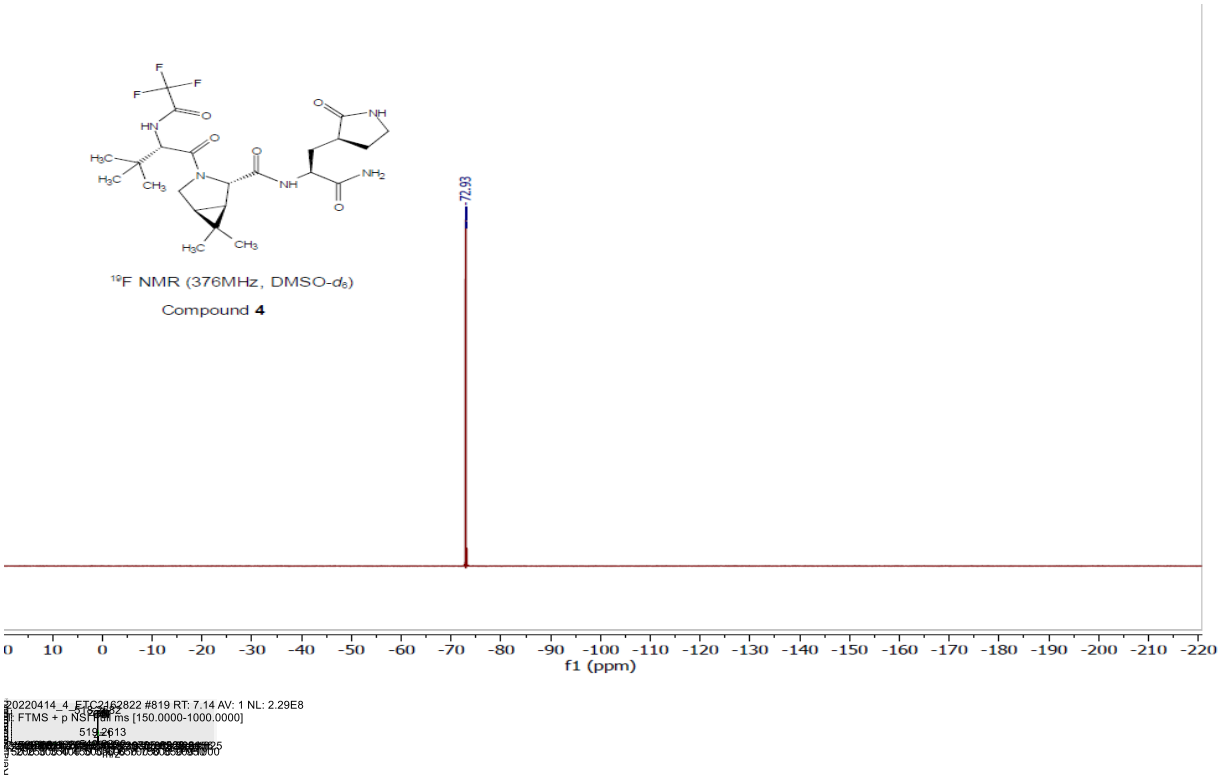
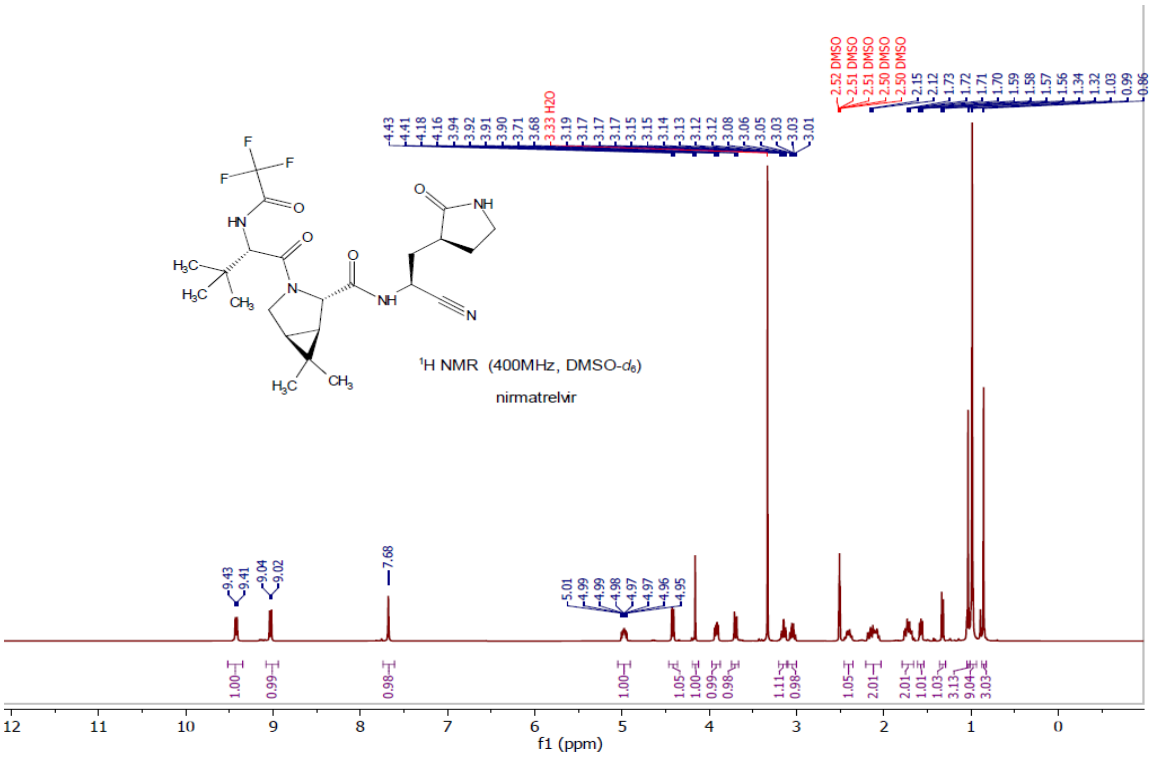
Compound **4**
f1 (ppm)

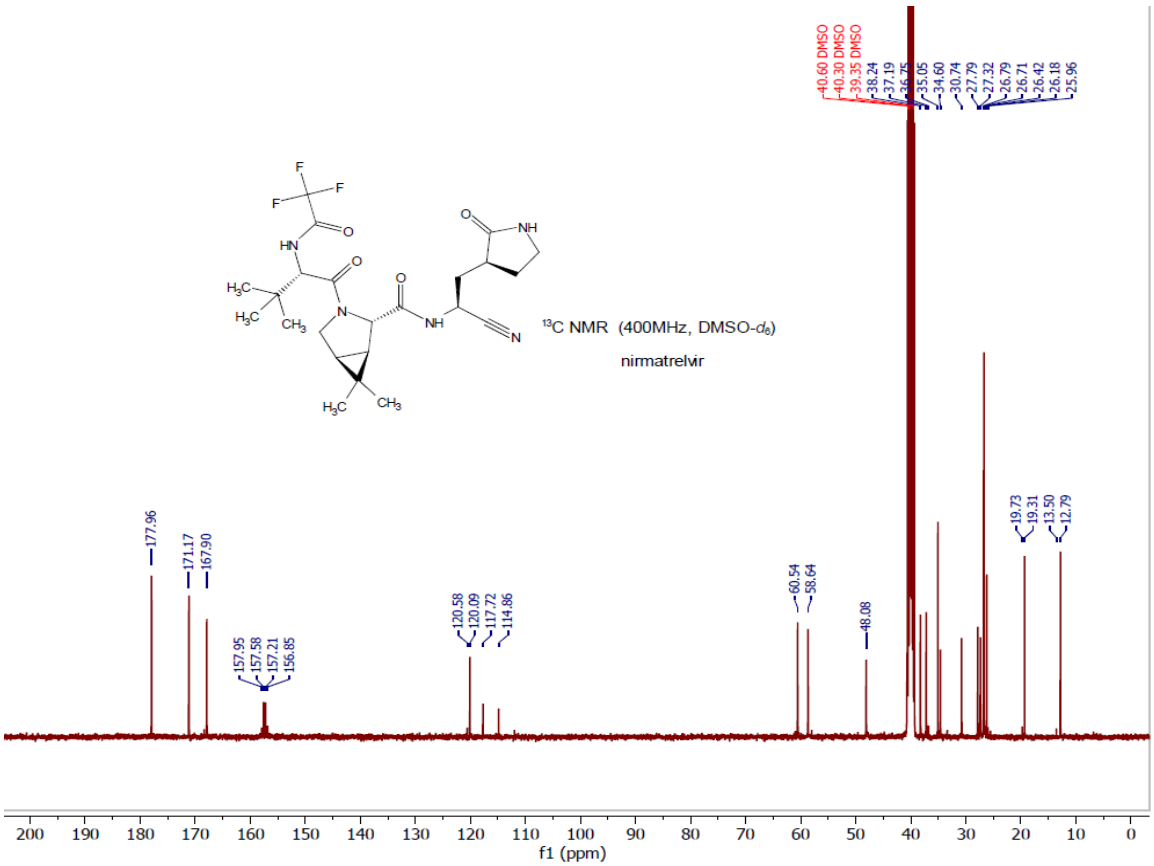


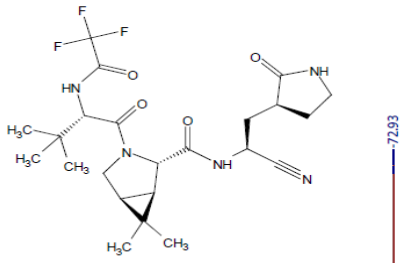
h) Synthesis of nirmatrelvir

Compound 4 (26 mg; 0.050 mmol; 1 eq.) was dissolved in anhydrous CH_2Cl_2 (5 mL) in a 10 mL two neck round-bottom flask equipped with a condenser. Burgess reagent (30 mg; 0.125 mmol; 2.5 eq.) was added at RT and heated to reflux for 0.5 h. The solvent was removed *in vacuo* and the crude target was purified by preparative HPLC with 20 to 70% acetonitrile containing 0.1% formic acid and water containing 0.1% formic acid to yield **nirmatrelvir** (16 mg, 64%). Spectral data: ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 9.48 – 9.33 (broad s, 1H), 9.02 (d, J = 8.54 Hz, 1H), 7.67 (s, 1H), 5.04 – 4.90 (m, 1H), 4.41 (d, J = 5.75 Hz, 1H), 4.15 (s, 1H), 3.96 – 3.86 (m, 1H), 3.69 (d, J = 10.46 Hz, 1H), 3.14 (t, J = 9.0 Hz, 1H), 3.08 – 2.99 (m, 1H), 2.44 – 2.36 (m, 1H), 2.19 – 2.03 (m, 2H), 1.77 – 1.66 (m, 2H), 1.60 – 1.54 (m, 1H), 1.32 (d, J = 7.67 Hz, 1H), 1.02 (s, 3H), 0.98 (s, 9H), 0.85 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 177.96, 171.17, 167.90, 157.4 (q, J = 36 Hz), 120.09 (CN), 116.3 (q, J = 288 Hz), 60.54, 58.64, 48.08, 40.90, 38.24, 37.19, 35.05, 34.60, 30.74, 27.79, 27.32, 26.71,

26.18, 19.31, 12.79 (21 of 23 peaks recorded due to P3 *t*-butyl; see manuscript reference 6 DOI: 10.1126/science.abl4784). ^{19}F NMR (376 MHz, DMSO-d_6) δ -72.93. ESI-quadrupole-MS: *m/z* calc $C_{23}H_{33}F_3N_5O_4$ ($M+H^+$) 500.2485, found 500.2476. No unexpected or unusually high safety hazards encountered during synthesis.

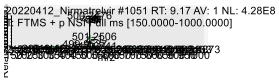






^{19}F NMR (400MHz, DMSO-d_6)

nirmatrelvir



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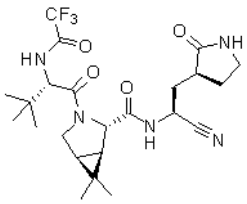

nirmatrelvir

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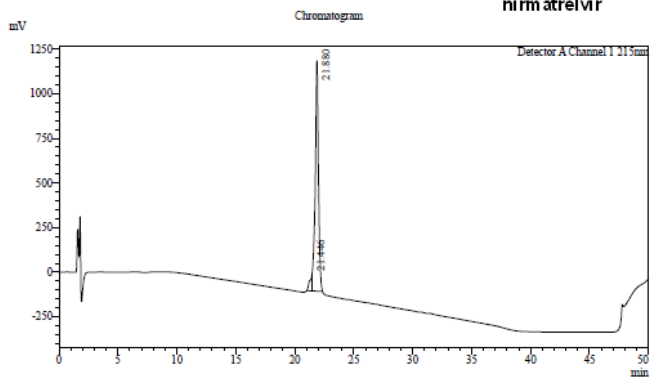

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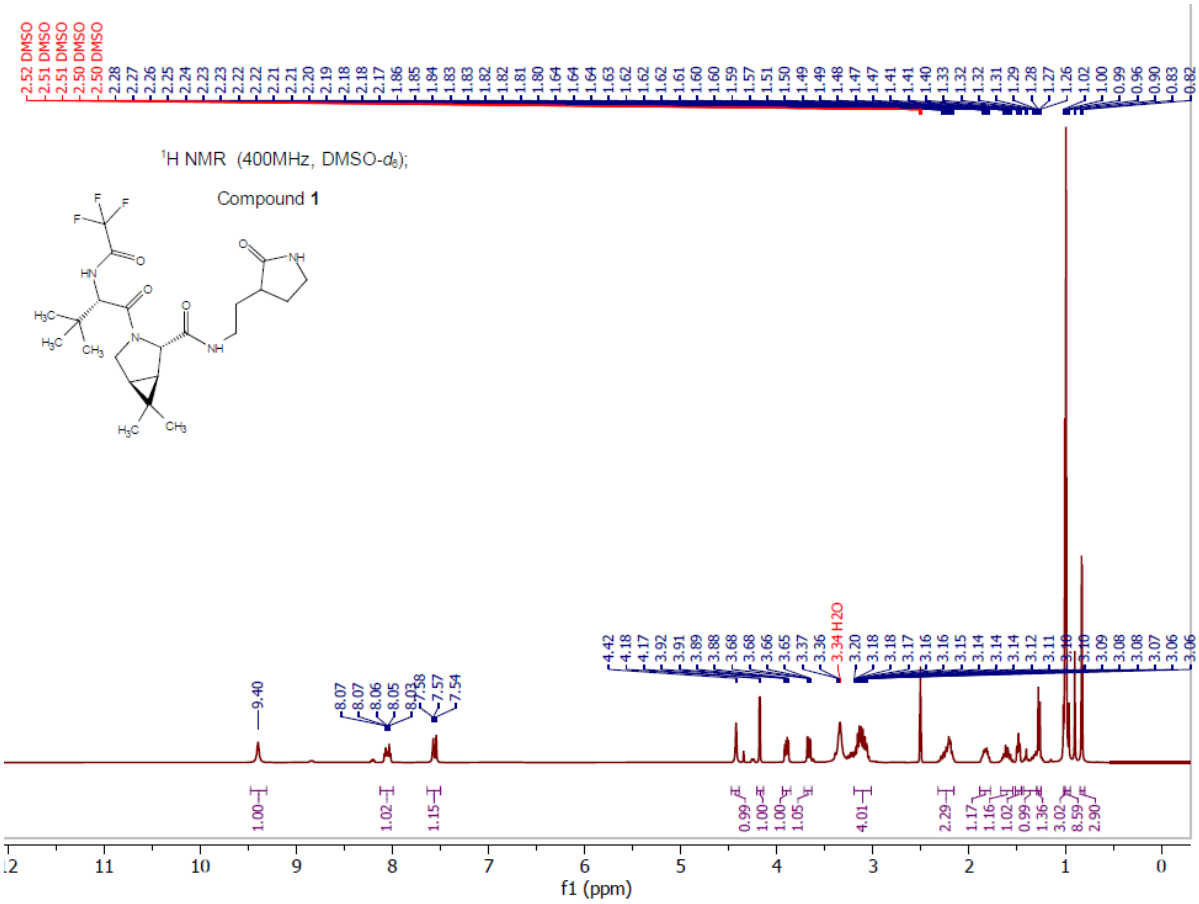
i) Synthesis of compound 1

Intermediate carboxylic acid 6 (35 mg; 0.096 mmol; 1 eq.) and 3-(2-aminoethyl)pyrrolidin-2-one HCl salt (16.6 mg; 0.10 mmol; 1.05 eq.) were dissolved in anhydrous DMF (1 mL) in a 10 mL round-bottom flask. DIPEA (85 μL; 0.48 mmol; 5 eq.) was added at 0 °C. After 1 min., HATU (41 mg; 0.105 mmol; 1.1 eq.) was added and the reaction mixture stirred for 0.5 h. at RT under N_2 (g). Next, the reaction was diluted with ethyl acetate, washed with sat. $NaHCO_3$ (2x), water and brine. The ethyl acetate layer was dried with Na_2SO_4 and concentrated *in vacuo*. The crude target was purified by preparative HPLC with 25 to 55% acetonitrile containing 0.1% formic acid and water containing 0.1% formic acid to yield **compound 1** (35 mg; 77%). Spectral data: ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 9.40 (s, 1H), 8.13 – 7.99 (m, 1H), 7.56 (d, *J* = 12.0 Hz, 1H), 4.42 (s, 1H), 4.18 (d, *J* = 3.5 Hz, 1H), 3.90 (dd, *J* = 10.3, 5.4 Hz, 1H), 3.67 (dd, *J* = 10.5, 2.5 Hz, 1H), 3.20 – 3.02 (m, 4H), 2.32 – 2.16 (m, 2H), 1.89 – 1.78 (m, 1H), 1.67 – 1.54 (m, 1H), 1.52 – 1.46 (m, 1H), 1.43 – 1.30 (m, 1H), 1.27 (d, *J* = 7.6 Hz, 1H), 1.00 (s, 3H), 0.99 (s, 9H), 0.83 (d, *J* = 3.2 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 179.18, 178.96, 170.96, 170.93, 167.60, 157.37 (q, *J* = 36.36 Hz), 116.34 (q, *J* = 287.85 Hz), 60.78, 60.75, 58.67, 58.64, 48.03, 47.97, 38.98, 38.42, 37.46, 36.98, 35.15, 34.03, 31.36, 31.27, 31.24, 31.09, 27.88, 27.42, 27.30, 26.85, 26.80, 26.37, 26.34, 19.07, 12.80, 12.78 (more than 22 peaks observed as compound is a diastereomer). ^{19}F NMR (376 MHz, DMSO-d_6) δ -72.95. ESI-quadrupole-MS: *m/z* calc $C_{22}H_{34}F_3N_4O_4$ ($M+H^+$) 475.2532, found 475.2526. No unexpected or unusually high safety hazards encountered during synthesis.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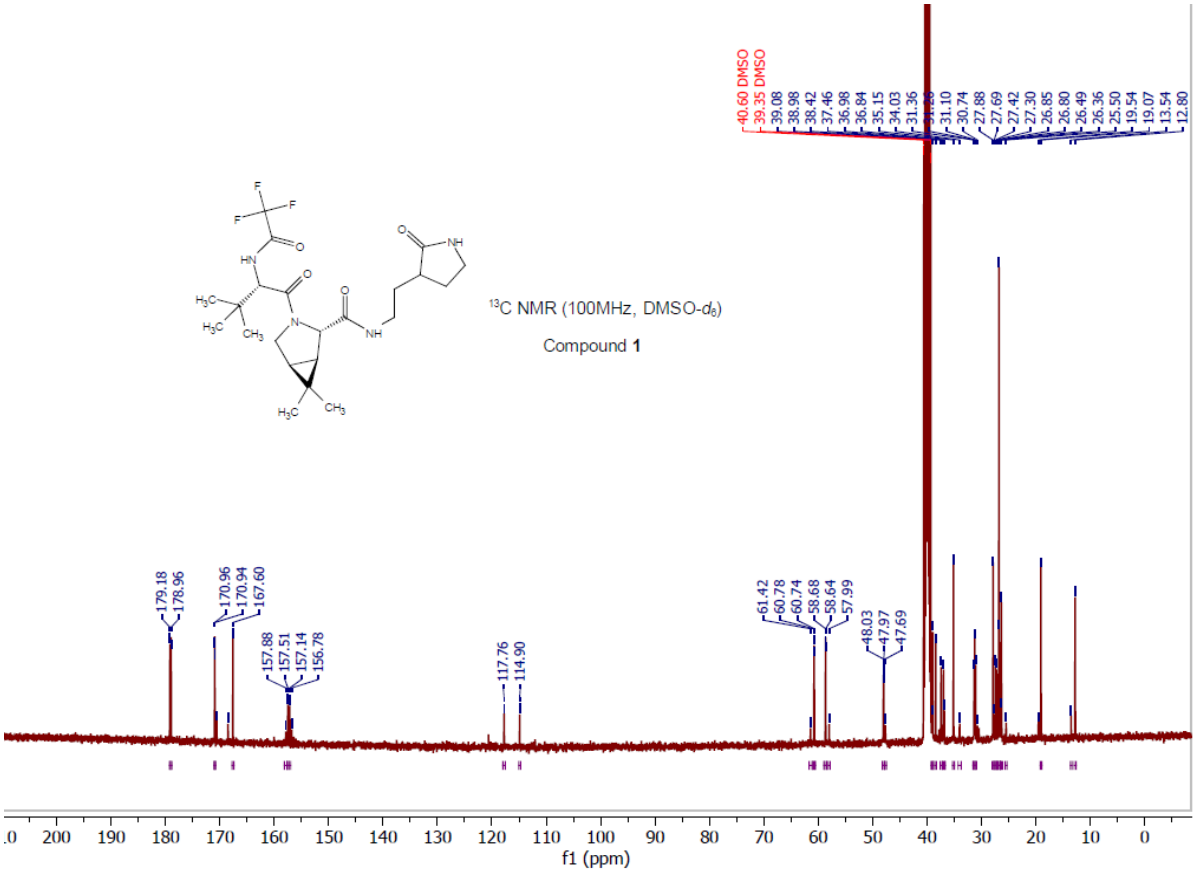
Compound **1**
f1 (ppm)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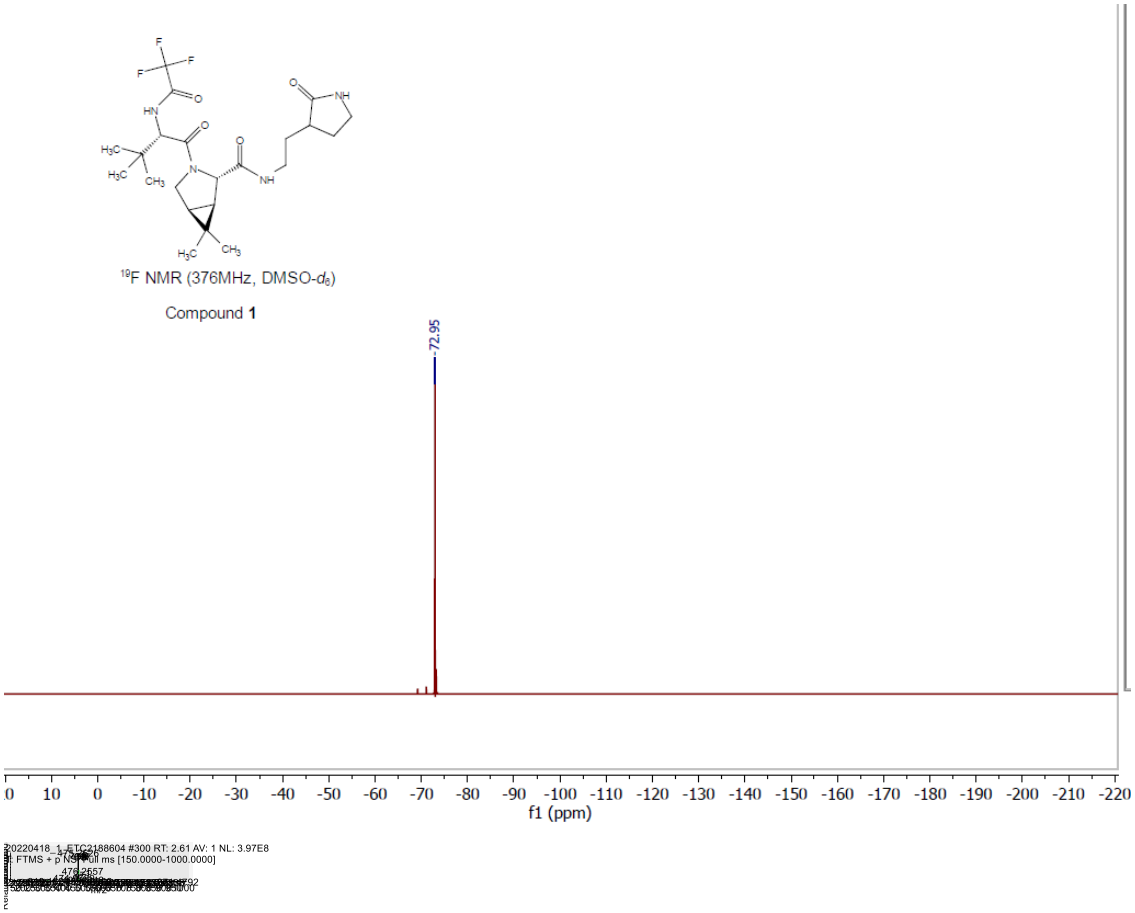
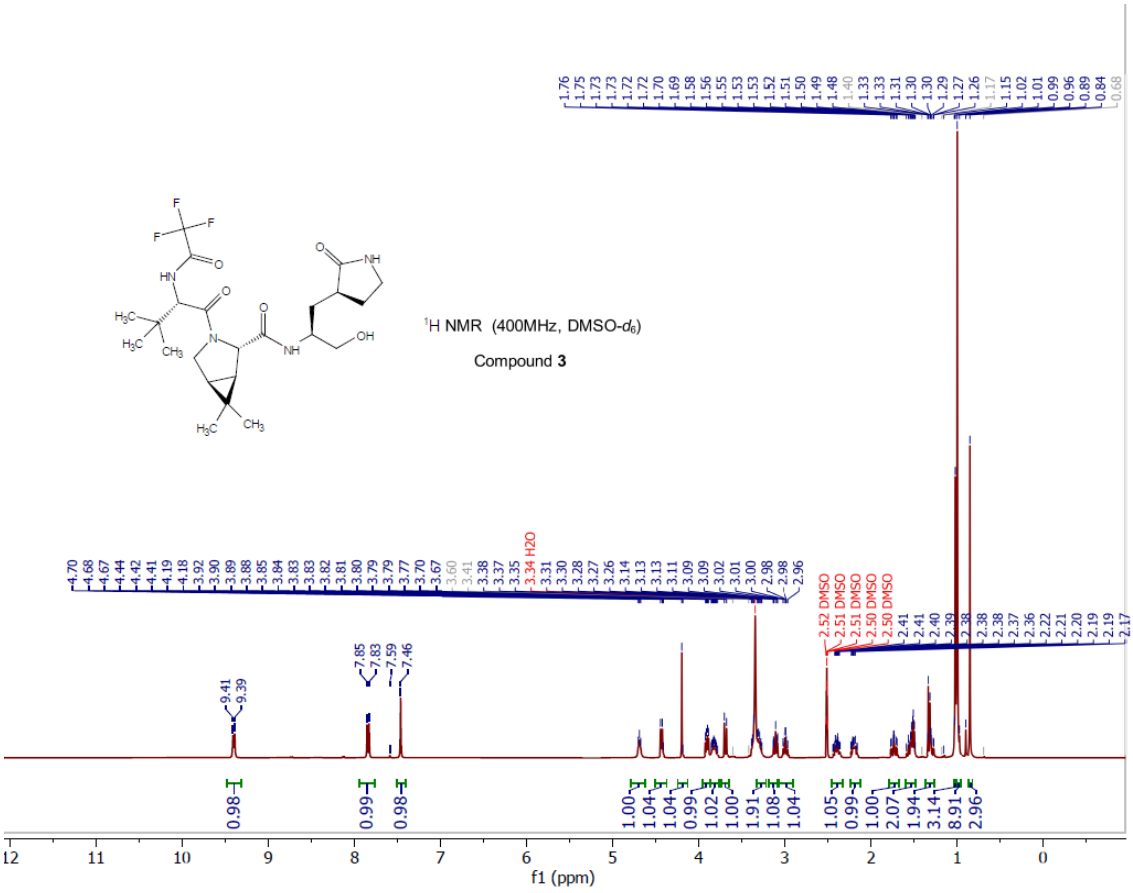
f1 (ppm)

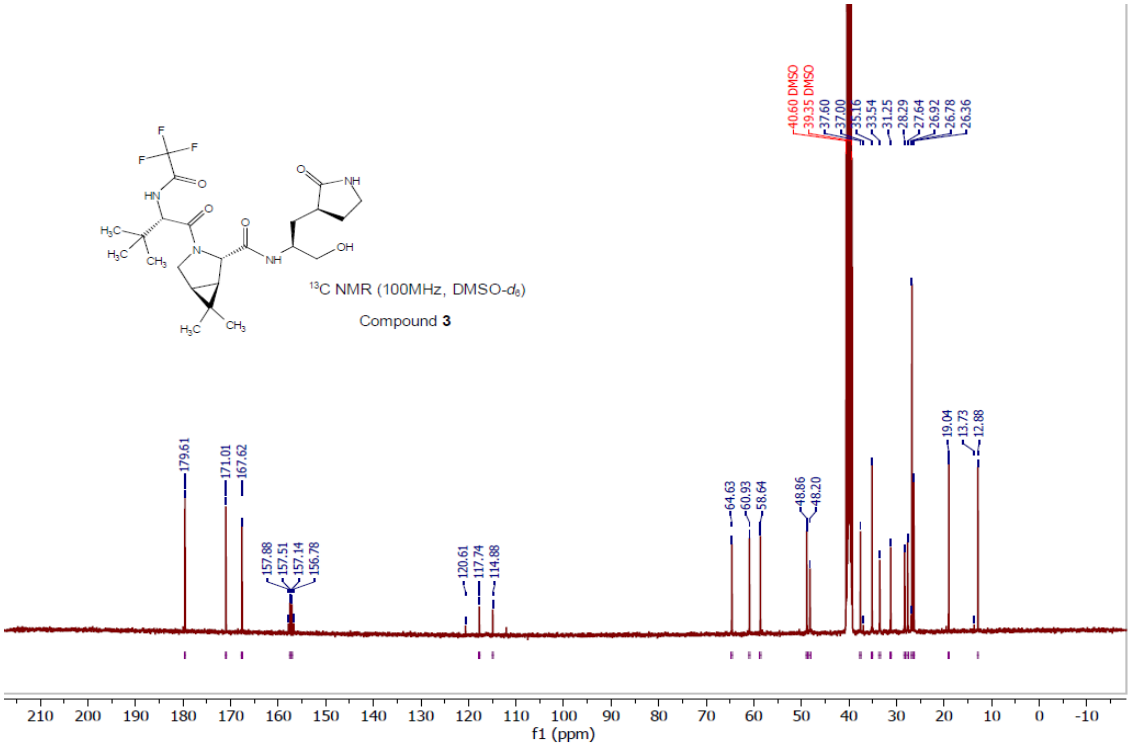


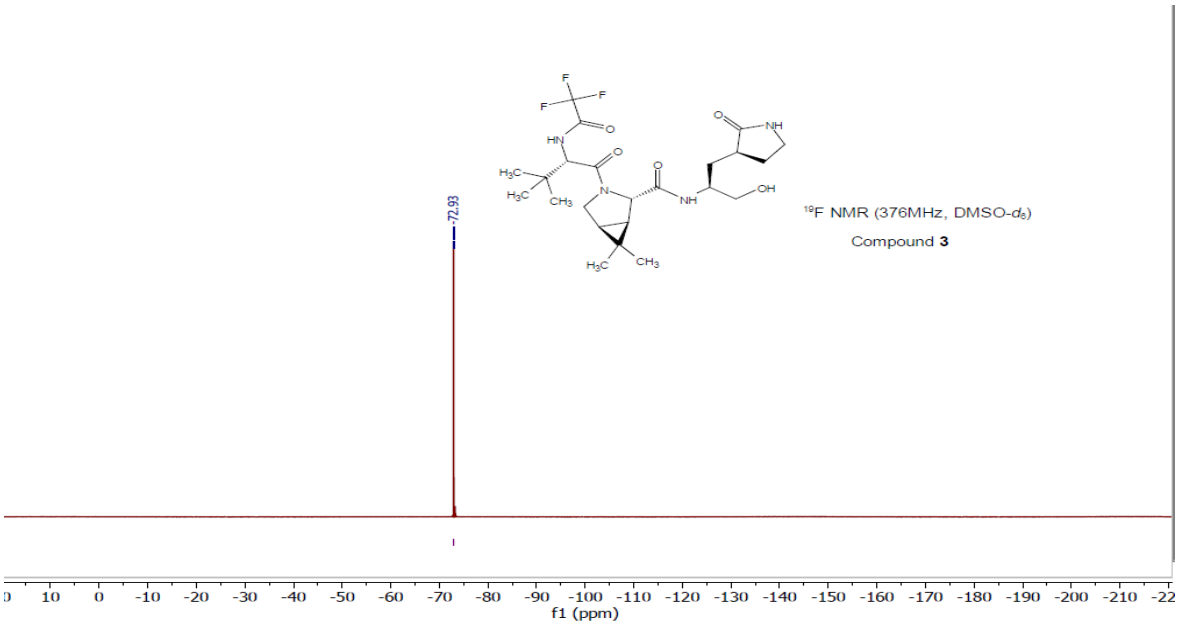
j) Synthesis of compound 3

Intermediate carboxylic acid 6 (30 mg; 0.082 mmol; 1 eq.) and (*S*)-3-(*S*)-2-amino-3-hydroxypropylpyrrolidin-2-one HCl salt (20 mg; 0.098 mmol; 1.2 eq.) were dissolved in anhydrous DMF (1 mL) in a 10 mL round-bottom flask. DIPEA (72 μL; 0.41 mmol; 5 eq.) was added at 0 °C. After 1 min., HATU (38 mg; 0.098 mmol; 1.2 eq.) was added and the reaction mixture stirred for 0.5 h. at RT under N_2 (g). Next, the reaction was diluted with ethyl acetate, washed with sat. $NaHCO_3$ (2x), water and brine. The ethyl acetate layer was dried with Na_2SO_4 and concentrated *in vacuo*. The crude target was purified by preparative HPLC with 25 to 55% acetonitrile containing 0.1% formic acid and water containing 0.1% formic acid to yield **compound 3** (33 mg; 80%). Spectral data: ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 9.40 (d, *J* = 8.1 Hz, 1H), 7.84 (d, *J* = 9.4 Hz, 1H), 7.46 (s, 1H), 4.68 (t, *J* = 5.6 Hz, 1H), 4.43 (d, *J* = 8.2 Hz, 1H), 4.19 (s, 1H),

3.90 (dd, *J* = 10.3, 5.4 Hz, 1H), 3.87 – 3.76 (m, 1H), 3.68 (d, *J* = 10.3 Hz, 1H), 3.32 – 3.22 (m, 2H), 3.16 – 3.06 (m, 1H), 3.05 – 2.93 (m, 1H), 2.46 – 2.33 (m, 1H), 2.24 – 2.12 (m, 1H), 1.73 (ddd, *J* = 13.6, 12.1, 3.3 Hz, 1H), 1.59 – 1.45 (m, 2H), 1.35 – 1.23 (m, 2H), 1.01 (s, 3H), 0.99 (s, 9H), 0.84 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 179.61, 171.01, 167.62, 157.35 (q, *J* = 37.3 Hz), 116.33 (q, *J* = 288.86 Hz), 64.63, 60.93, 58.64, 48.86, 48.20, 40.00, 37.60, 35.16, 33.54, 31.25, 28.29, 27.64, 26.78, 26.36, 19.04, 12.88 (21 of 23 peaks recorded due to P3 *t*-butyl). ^{19}F NMR (376 MHz, DMSO-d_6) δ -72.93. ESI-quadrupole-MS: *m/z* calc $C_{23}H_{36}F_3N_4O_5$ ($M+H^+$) 505.2638, found 505.2628. No unexpected or unusually high safety hazards encountered during synthesis.



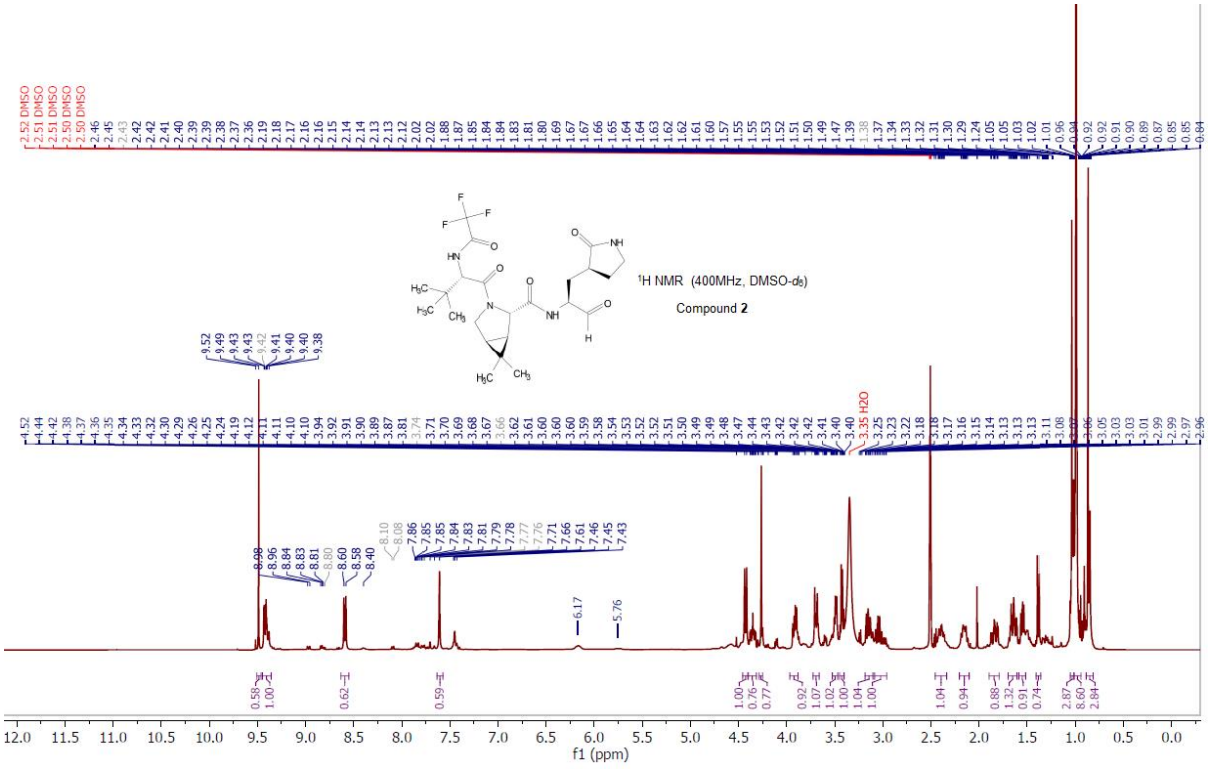


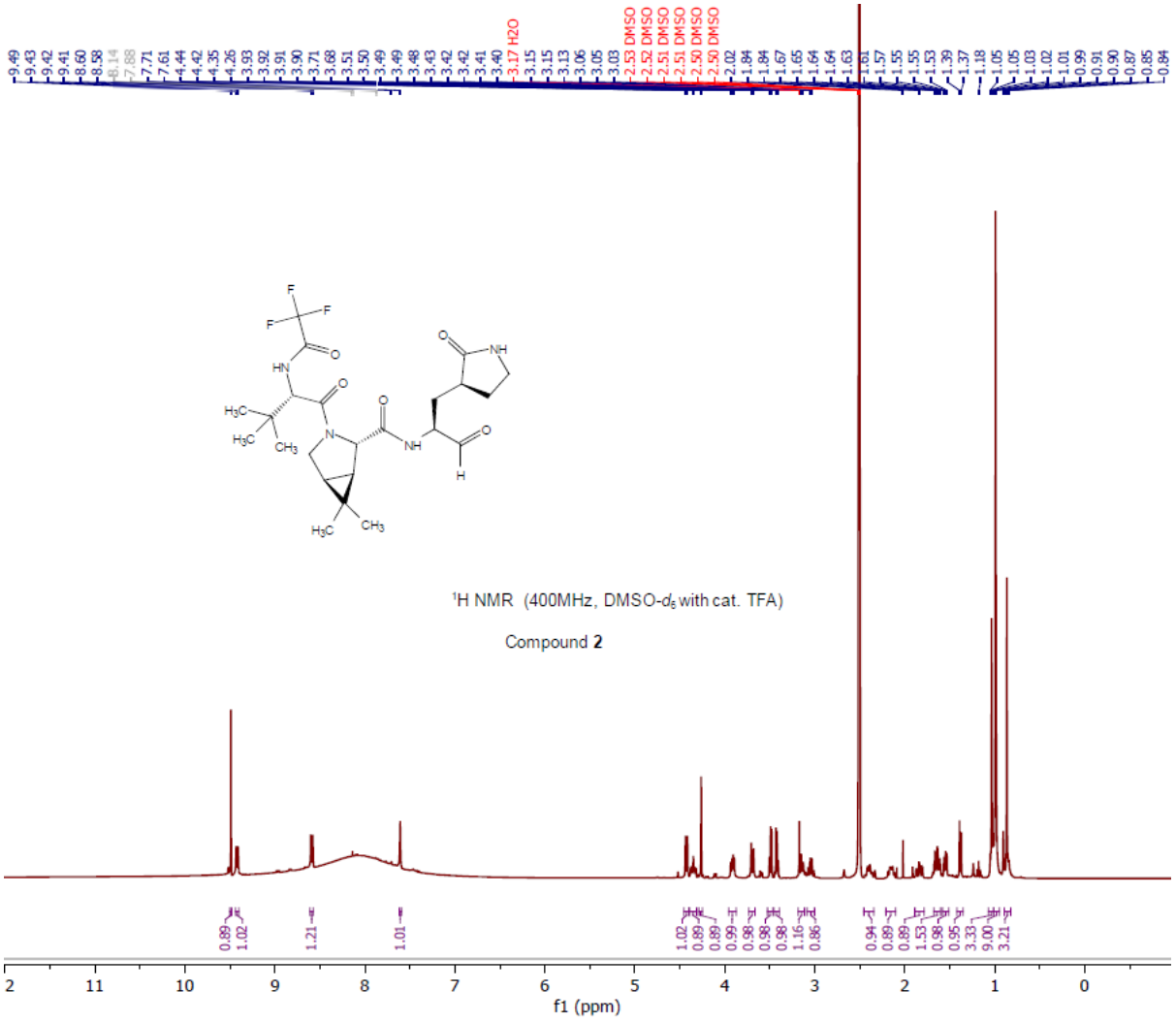


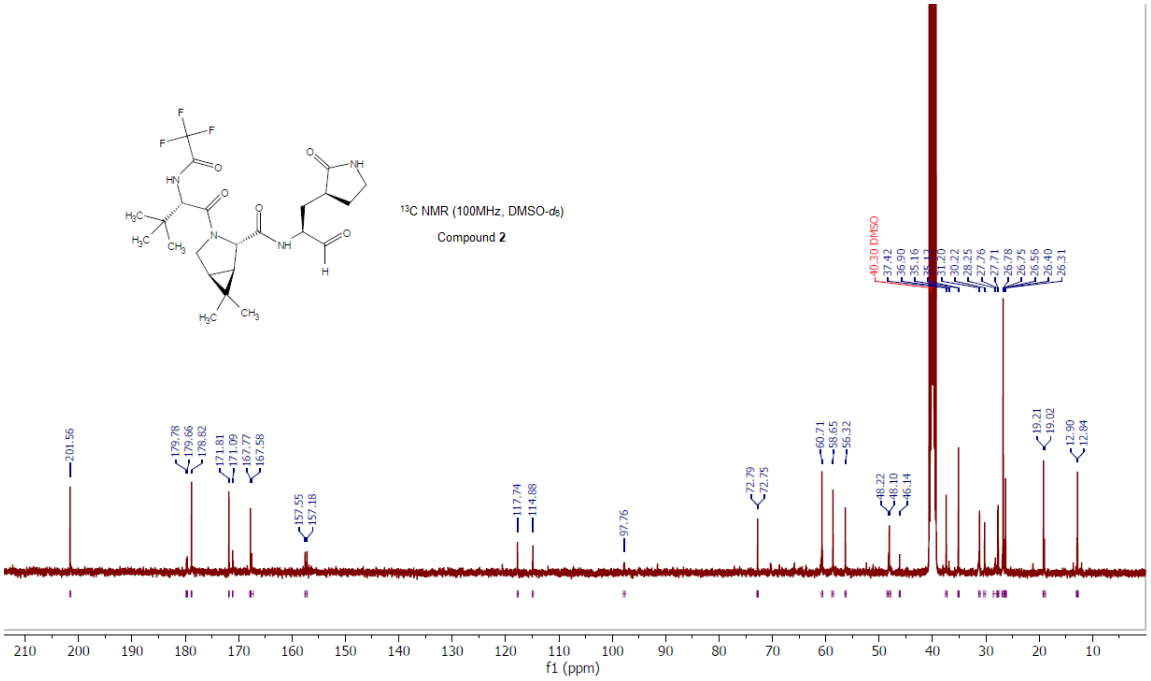
20220414_3_FTC218608 #823 RT: 7.18 AV: 1 NL: 7.94E8
T: FTMS + p NSI Full ms [150.0000-1000.0000]
508.2660

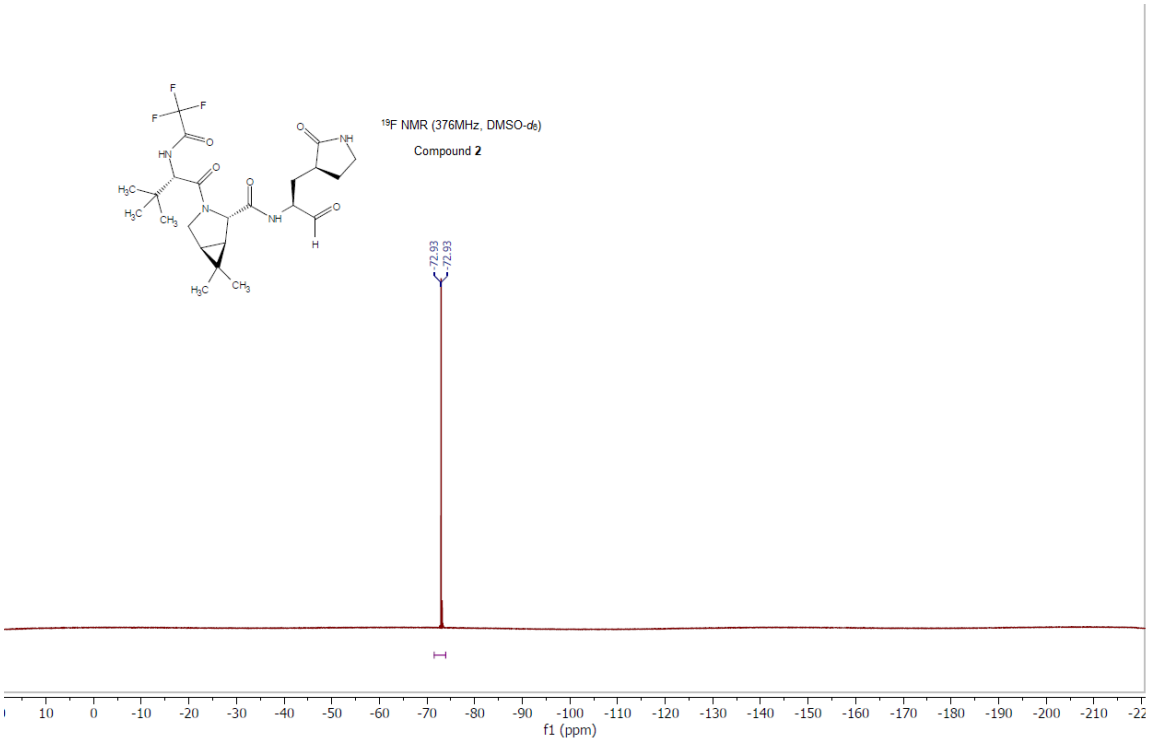
k) Synthesis of compound 2

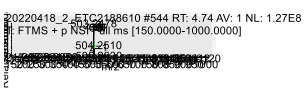
Compound 3 (15 mg; 0.030 mmol; 1 eq.) was dissolved in anhydrous DMF (0.6 mL) in a 10 mL round-bottom flask. Dess-Martin periodinane (20 mg; 0.048 mmol; 1.6 eq.) was added at 0 °C and the mixture stirred for 1 h. at room temperature under N_2 (g). Next, the reaction was diluted with ethyl acetate, quenched with saturated $NaHCO_3$ and saturated $Na_2S_2O_3$ solution (1:1 ratio) at 0 °C. The ethyl acetate layer was washed with sat. $NaHCO_3$ (2x) followed by 5% LiCl (aq) and brine. The ethyl acetate layer was collected and dried using Na_2SO_4 and concentrated *in vacuo*. The crude target was purified by preparative HPLC with 25 to 55% acetonitrile containing 0.1% formic acid and water containing 0.1% formic acid to yield **compound 2** (10 mg; 66%). Spectral data: ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 9.49 (s, 1H, CHO), 9.45 – 9.36 (m, 1H), 8.59 (d, *J* = 8.4 Hz, 1H), 7.61 (s, 1H), 4.43 (d, *J* = 8.5 Hz, 1H), 4.40 – 4.31 (m, 1H), 4.26 (s, 1H), 3.97 – 3.88 (m, 1H), 3.73 – 3.66 (m, 1H), 3.52 – 3.47 (m, 1H), 3.45 – 3.40 (m, 1H), 3.18 – 3.10 (m, 1H), 3.08 – 2.96 (m, 1H), 2.45 – 2.34 (m, 1H), 2.20 – 2.10 (m, 1H), 1.89 – 1.79 (m, 1H), 1.70 – 1.60 (m, 1H), 1.55 (dd, *J* = 7.7, 5.3 Hz, 1H), 1.38 (d, *J* = 7.7 Hz, 1H), 1.02 (s, 3H), 0.99 (s, 9H), 0.86 (s, 3H) [Note: compound exists in two forms, ~60% aldehyde, ~40% hydrate, making NMR interpretation difficult]. ^{13}C NMR (100 MHz, DMSO-d_6) δ 201.56, 171.81/171.09, 167.77/167.58, 157.32 (q, *J* = 37.37 Hz), 116.34 (q, *J* = 288.86 Hz), 72.79/72.75, 60.71, 58.65, 56.32, 48.22/48.10, 46.14, 37.42, 35.12, 31.20, 30.22, 28.25, 27.71/27.42, 26.78/26.75, 26.56/26.40, 19.21/19.02, 12.90/12.84 [Note: compound exists in two forms, ~60% aldehyde, ~40% hydrate, making NMR interpretation difficult]. ^{19}F NMR (376 MHz, DMSO-d_6) δ -72.93. ESI-quadrupole-MS: *m/z* calc $C_{23}H_{34}F_3N_4O_5$ ($M+H^+$) 503.2481, found 503.2478. No unexpected or unusually high safety hazards encountered during synthesis.


^{1}H NMR (400MHz, DMSO-d_6)
Compound **2**
f1 (ppm)


^{1}H NMR (400MHz, DMSO-d_6 with cat. TFA)
Compound **2**
f1 (ppm)

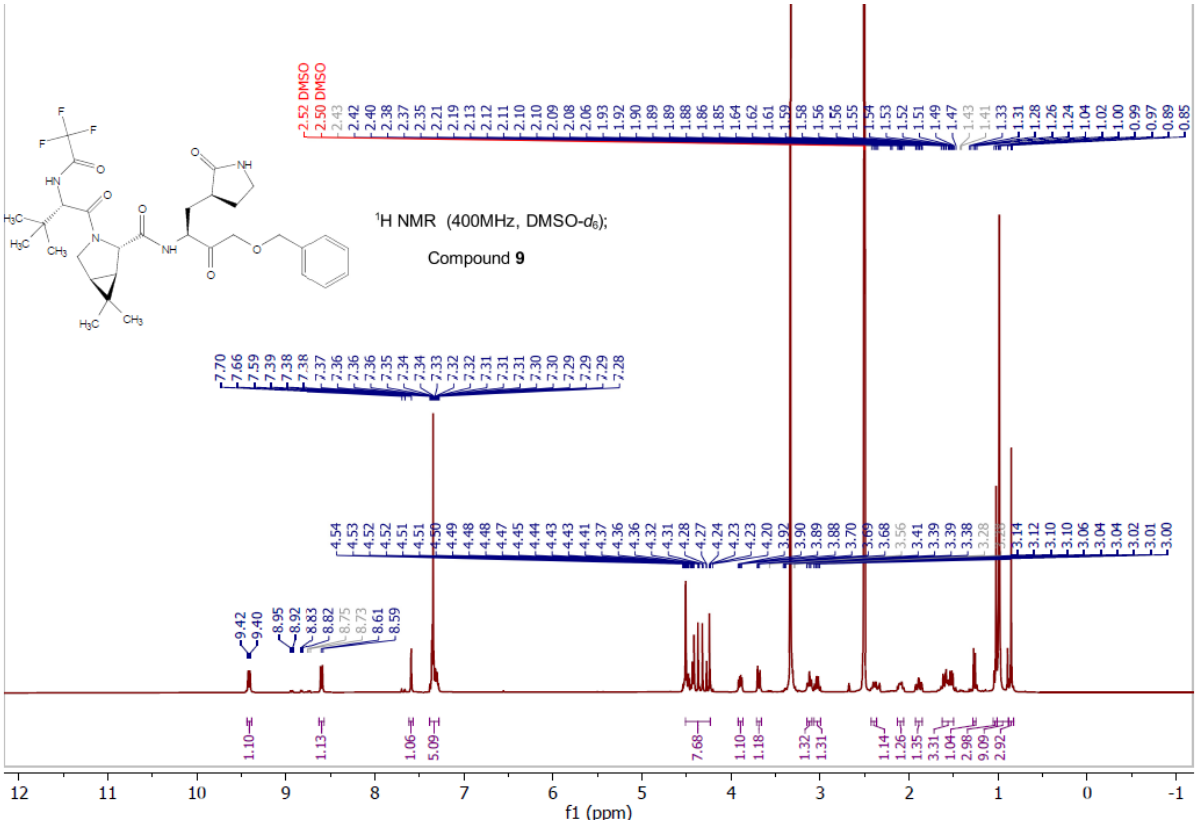

^{13}C NMR (100MHz, DMSO-d_6)
Compound **2**
201.56
179.78
179.66
178.82
171.81
171.09
167.77
167.58
157.55
157.18
117.74
114.88
97.76
72.79
72.75
60.71
59.65
56.32
48.22
48.10
46.14
40.30 DMSO
37.42
36.90
35.16
31.20
30.22
28.25
27.76
27.71
26.78
26.75
26.56
26.40
26.31
19.21
19.02
12.90
12.84
f1 (ppm)

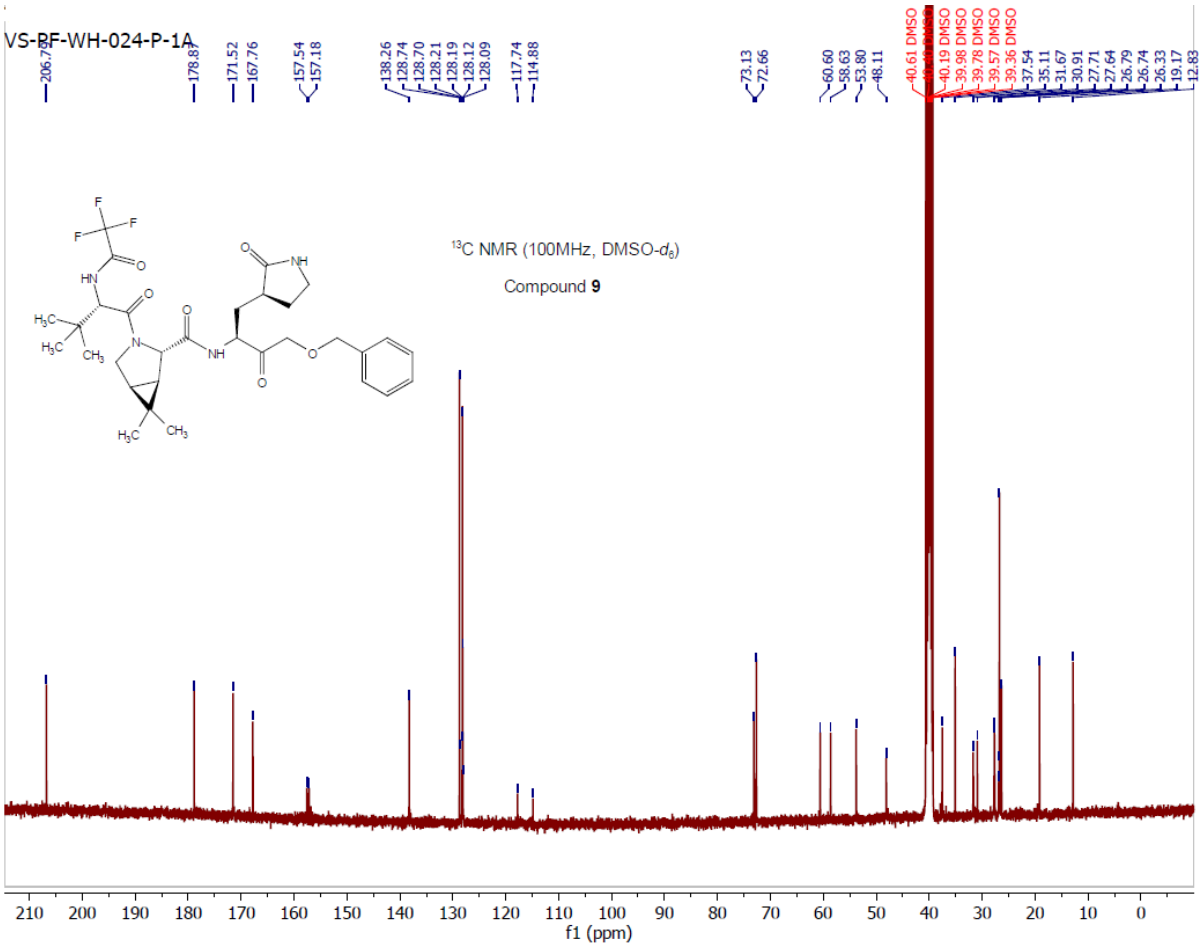

^{19}F NMR (376MHz, DMSO-d_6)
Compound **2**
-72.93
-72.93
f1 (ppm)

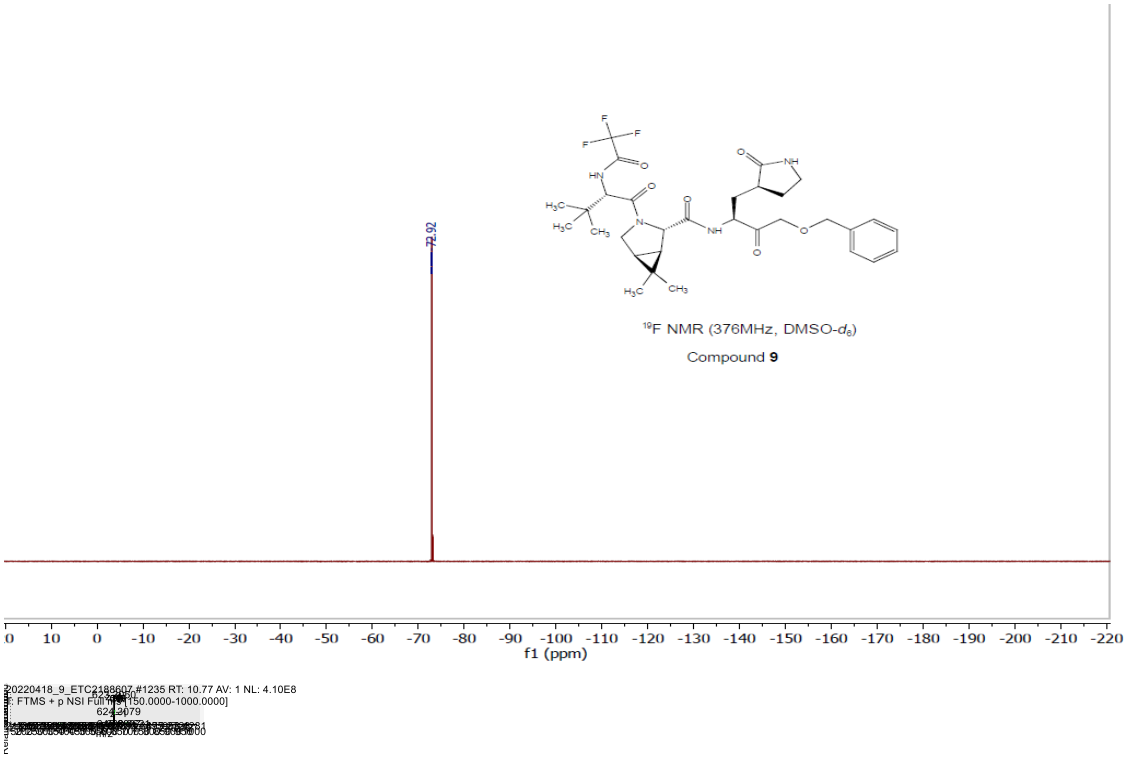

20220418_2_ETC2188610 #544 RT: 4.74 AV: 1 NL: 1.27E8
FTMS + p NSI Full ms [150.0000-1000.0000]
504.2610

l) Synthesis of compound 9

Intermediate carboxylic acid 6 (30 mg; 0.082 mmol; 1 eq.) and (*S*)-3-(*S*)-2-amino-4-benzyloxy-3-oxobutyl pyrrolidin-2-one HCl salt (34 mg; 0.107 mmol; 1.3 eq.) were dissolved in anhydrous DMF (1 mL) in a 10 mL round-bottom flask. DIPEA (72 μL; 0.41 mmol; 5 eq.) was added at 0 °C. After 1 min., HATU (38 mg; 0.098 mmol; 1.2 eq.) was added and the reaction mixture stirred for 0.5 h. at RT under N_2 (g). Next, the reaction was diluted with ethyl acetate, washed with sat. $NaHCO_3$ (2x), water and brine. The ethyl acetate layer was dried with Na_2SO_4 and concentrated *in vacuo*. The crude target was purified by preparative HPLC with 20 to 70% acetonitrile containing 0.1% formic acid and water containing 0.1% formic acid to yield **compound 9** (18 mg; 35%). Spectral data: ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 9.41 (d, *J* = 8.5 Hz, 1H), 8.60 (d, *J* = 8.5 Hz, 1H), 7.59 (s, 1H), 7.42 – 7.26 (m, 5H), 4.52 – 4.23 (m, 7H), 3.90 (dd, *J* = 10.3, 5.5 Hz, 1H), 3.69 (d, *J* = 10.4 Hz, 1H), 3.12 (t, *J* = 9.2 Hz, 1H), 3.07 – 2.99 (m, 1H), 2.43 – 2.36 (m, 1H), 2.14 – 2.05 (m, 1H), 1.94 – 1.84 (m, 1H), 1.65 – 1.50 (m, 3H), 1.27 (d, *J* = 7.7 Hz, 1H), 1.02 (s, 3H), 0.99 (s, 9H), 0.85 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 206.79, 178.87, 171.52, 167.77, 157.35 (q, *J* = 37.3 Hz), 138.26, 128.74, 128.21, 128.12, 116.33 (q, *J* = 288.86 Hz), 73.13, 72.66, 60.60, 58.63, 53.80, 48.11, 40.00, 37.54, 35.11, 31.67, 30.91, 27.71, 27.64, 26.74, 26.33, 19.16, 12.83 (27 of 31 peaks recorded due to P3 *t*-butyl and phenyl). ^{19}F NMR (376 MHz, DMSO-d_6) δ -72.92. ESI-quadrupole-MS: *m/z* calc $C_{31}H_{42}F_3N_4O_6$ ($M+H^+$) 623.3056, found 623.3050. No unexpected or unusually high safety hazards encountered during synthesis.


^{1}H NMR (400MHz, DMSO-d_6);
Compound **9**
f1 (ppm)

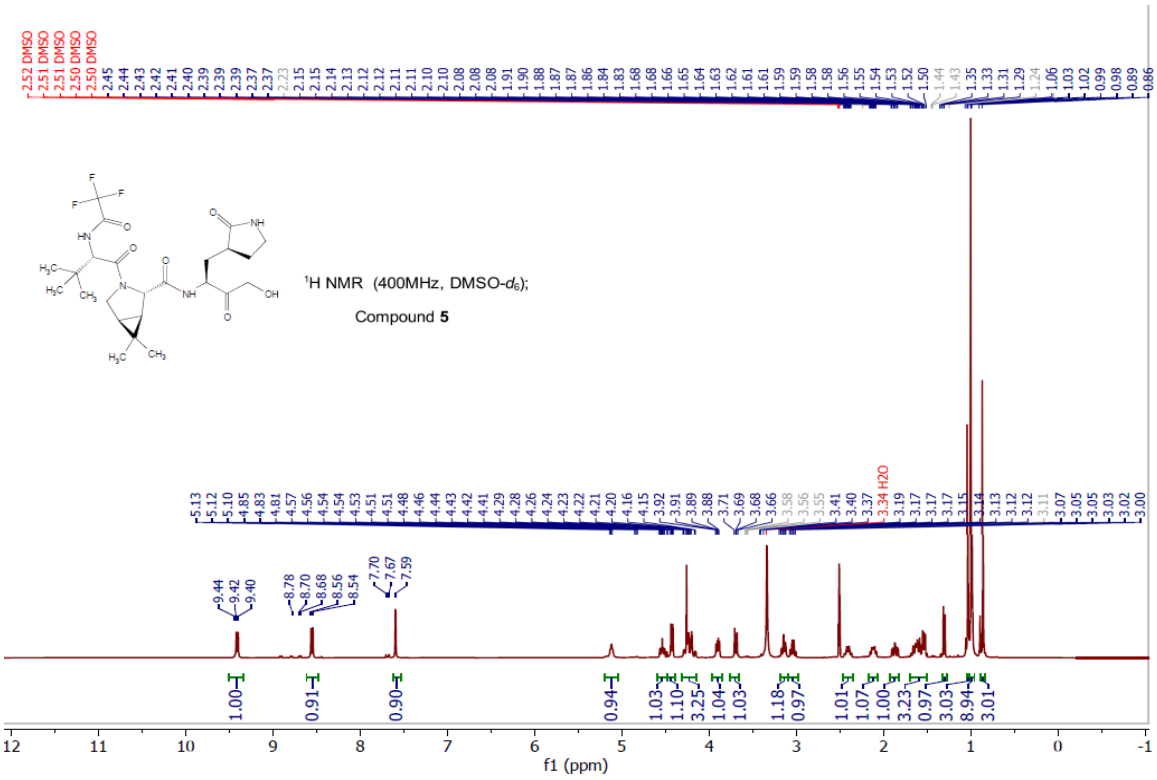

VS-RF-WH-024-P-1A
^{13}C NMR (100MHz, DMSO-d_6)
Compound **9**
f1 (ppm)

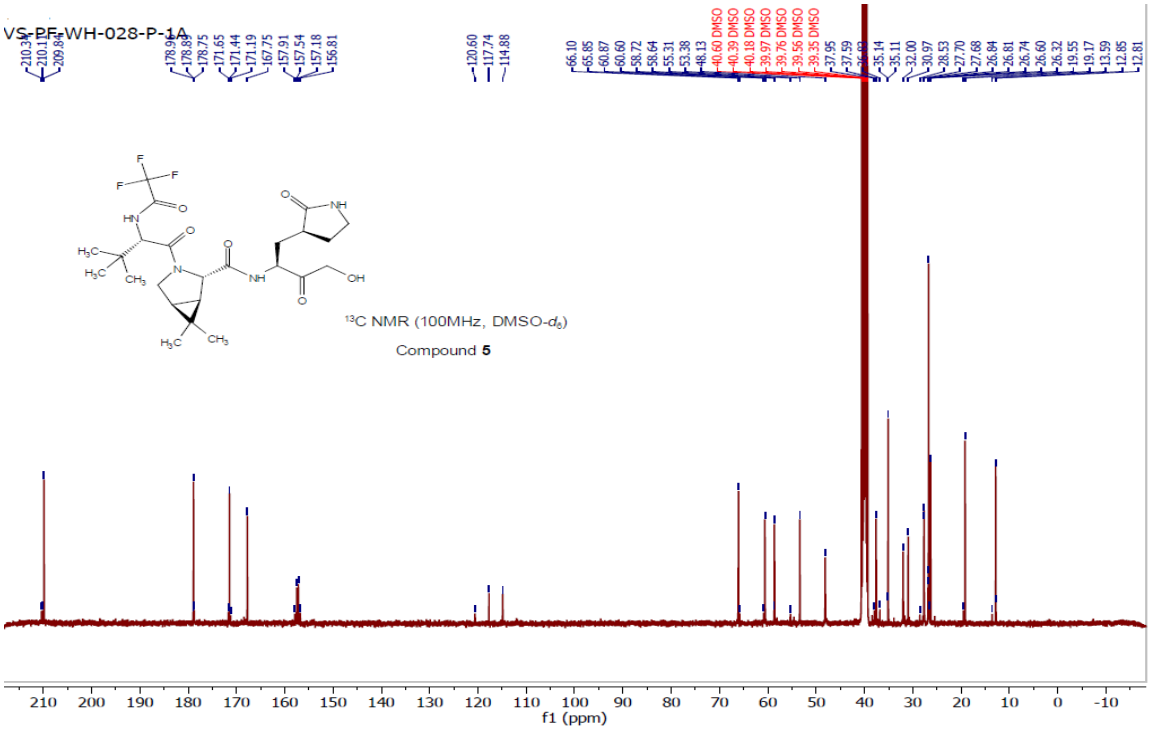


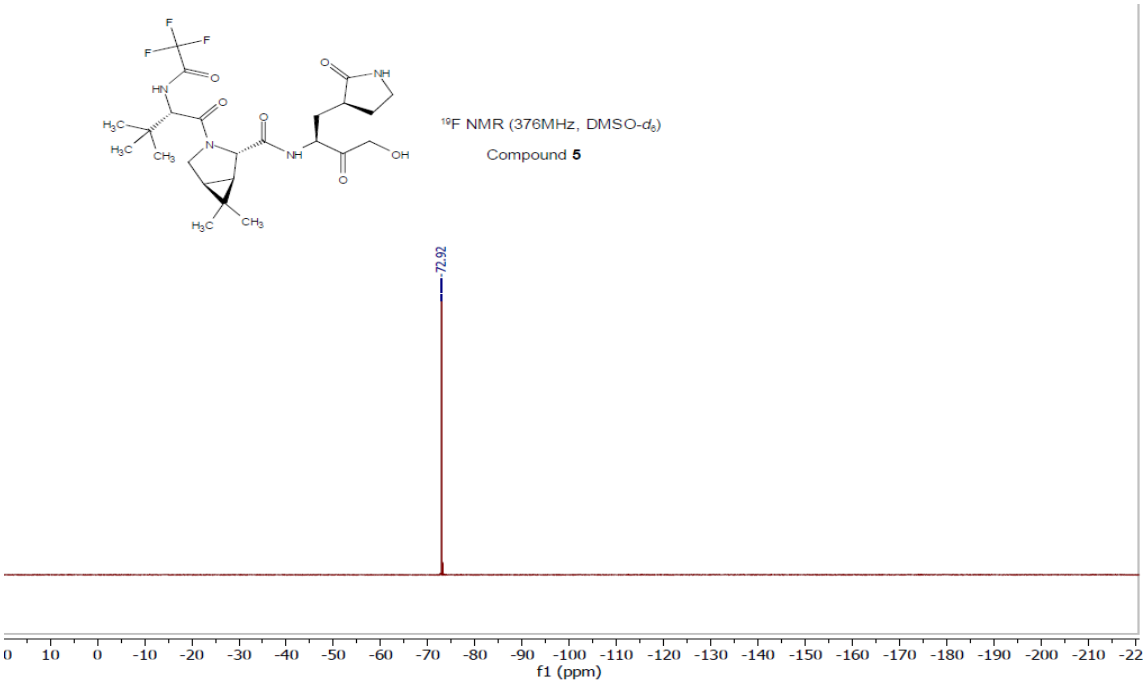
m) Synthesis of compound 5

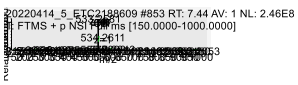
Compound 9 (14 mg; 0.022 mmol; 1 eq.) was dissolved in CH_3OH (2 mL) in a 10 mL round-bottom flask. Pd/C (3 mg; 10 wt. % loading) was added and the reaction mixture stirred for 18 h. at RT under H_2 (g) atmosphere using a balloon. Next, the reaction was diluted with CH_3OH, filtered through syringe filter and the filtrate was concentrated *in vacuo*. The crude target was purified by preparative HPLC with 20 to 70% acetonitrile containing 0.1% formic acid and water containing 0.1% formic acid to yield **compound 5** (8 mg; 68%). Spectral data: ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 9.41 (d, *J* = 8.4 Hz, 1H), 8.55 (d, *J* = 8.6 Hz, 1H), 7.59 (s, 1H), 5.20 – 5.05 (m, 1H), 4.54 (ddd, *J* = 12.0, 8.6, 3.4 Hz, 1H), 4.43 (d, *J* = 8.5 Hz, 1H), 4.32 – 4.13 (m, 3H), 3.90 (dd, *J* = 10.3, 5.5 Hz, 1H), 3.69 (d, *J* = 10.4 Hz, 1H), 3.19 – 3.09 (m, 1H), 3.09 – 2.97 (m, 1H), 2.47 – 2.35 (m, 1H), 2.18 – 2.06 (m, 1H), 1.87 (ddd, *J* = 13.7, 12.0, 3.7 Hz, 1H), 1.70 – 1.48 (m, 3H), 1.30 (d, *J* = 7.7 Hz, 1H), 1.03 (s, 3H), 0.99 (s, 9H), 0.86 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 209.84, 178.89, 171.44, 167.75, 157.32 (q, *J* = 36.36 Hz),

116.2 (q, J = 288.86 Hz), 66.10, 60.60, 58.64, 53.38, 48.13, 40.00, 37.59, 35.11, 32.00, 30.97, 27.67, 26.82, 26.74, 26.32, 19.17, 12.85 (22 of 24 peaks recorded due to P3 *t*-butyl). ^{19}F NMR (376 MHz, DMSO-d_6) δ -72.92. ESI-quadrupole-MS: *m/z* calc $C_{24}H_{36}F_3N_4O_6$ ($M+H^+$) 533.2587, found 533.2581. No unexpected or unusually high safety hazards encountered during synthesis.




VS-PF-WH-028-P-1A
210.34
210.15
209.84
178.96
178.89
178.75
171.65
171.44
171.19
167.75
157.91
157.54
157.18
156.81
120.60
117.74
114.88
66.10
65.85
60.87
60.60
58.72
58.64
55.31
53.38
48.13
40.60 DMSO
40.39 DMSO
40.18 DMSO
39.97 DMSO
39.76 DMSO
39.56 DMSO
39.35 DMSO
37.95
37.59
35.14
35.11
32.00
30.97
28.53
27.70
27.68
26.84
26.81
26.74
26.60
26.32
19.55
19.17
13.59
12.85
12.81
^{13}C NMR (100MHz, DMSO-d_6)
Compound 5
f1 (ppm)


^{19}F NMR (376MHz, DMSO-d_6)
Compound 5
72.92
f1 (ppm)


20220414_5_ETC2188609 #853 RT: 7.44 AV: 1 NL: 2.46E8
FTMS + p NSI Full ms [150.0000-1000.0000]
534.2611

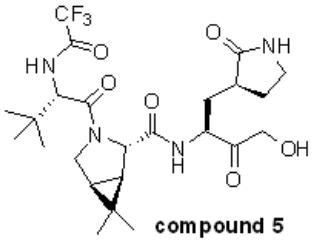
Column: Phenominex Luna 5 U 100mmX4.6mm C18(2) 100A

Mobile phase A: 0.1% TFA in Aqueous
Mobile phase B: Acetonitrile

<<LC Time Program>>

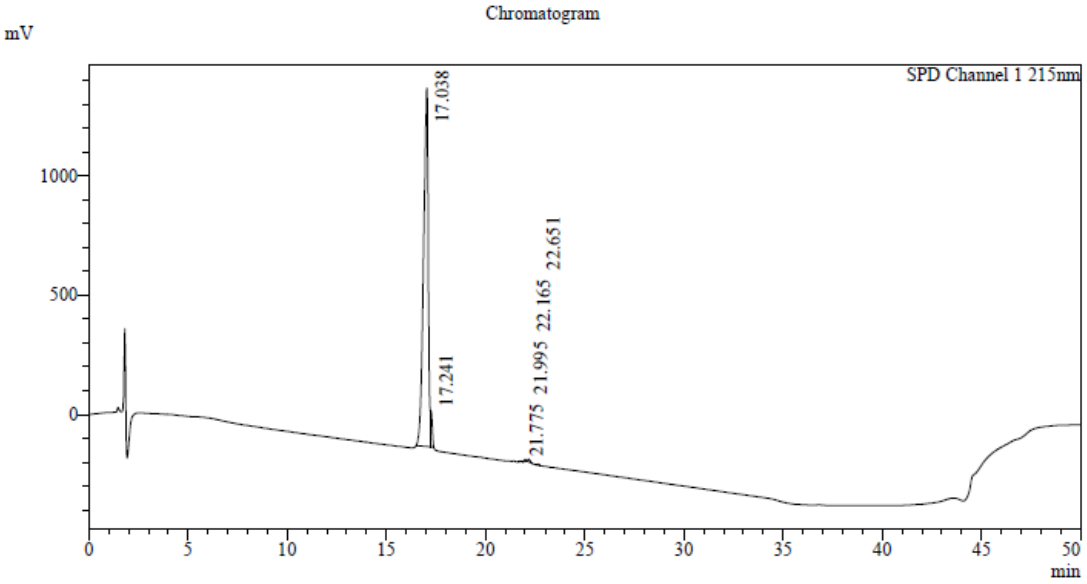
Time	Module	Command	Value
0.01	Pumps	B.Conc	5
15.00	Pumps	B.Conc	90
18.00	Pumps	B.Conc	90
18.01	Pumps	B.Conc	5
24.00	Pumps	B.Conc	5
24.01	Controller		Stop

Flow : 1 mL/min



compound 5

<Chromatogram>



Peak Table

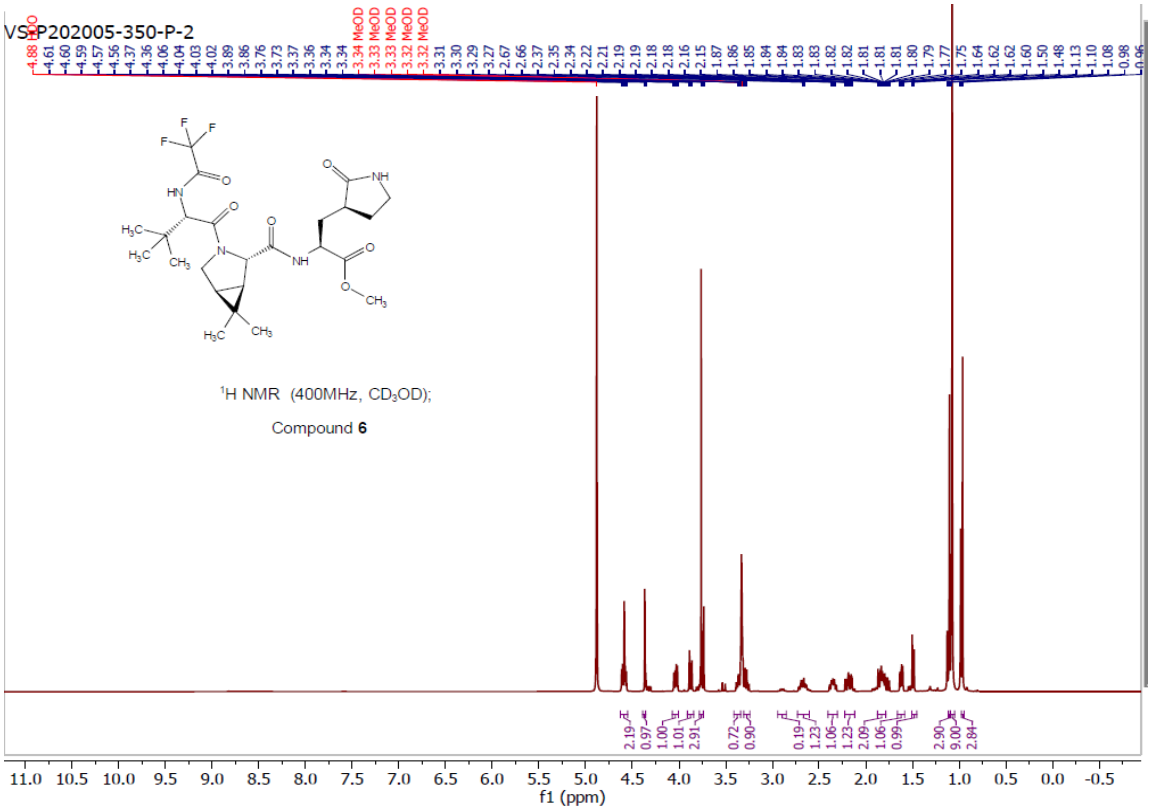
SPD Channel 1 215nm

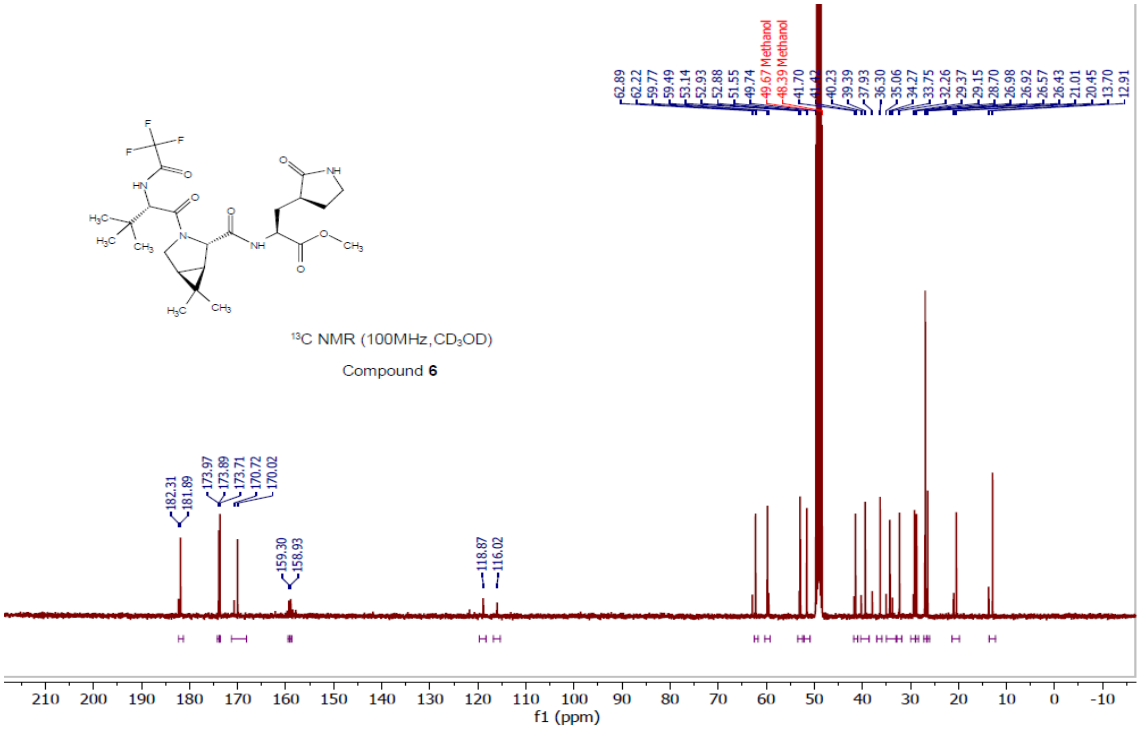
Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name	Area%
1	17.038	24582145	1502047	96.030		M		96.030
2	17.241	828899	150387	3.238		V M		3.238
3	21.775	26586	4700	0.104		M		0.104
4	21.995	43668	7557	0.171		M		0.171
5	22.165	90561	10917	0.354		M		0.354
6	22.651	26540	4369	0.104		M		0.104
Total		25598399	1679976					100.000

n) Synthesis of compound 6

Intermediate carboxylic acid 6 (30 mg; 0.082 mmol; 1 eq.) and methyl (2*S*)-2-amino-3-[(3*S*)-2-oxopyrrolidin-3-yl]propanoate HCl salt (19.5 mg; 0.086 mmol; 1.05 eq.) were dissolved in anhydrous DMF (1 mL) in a 10 mL round-bottom flask. DIPEA (73 μL; 0.412 mmol; 5 eq.) was added at 0 °C. After 1 min., HATU (35 mg; 0.091 mmol; 1.1 eq.) was added and the reaction mixture stirred for 0.5 h. at RT under N_2 (g). Next, the reaction was diluted with CH_2Cl_2, washed with sat. $NaHCO_3$ (2x), water and brine. The CH_2Cl_2 layer was dried with Na_2SO_4 and concentrated *in vacuo*. The crude target was purified by preparative HPLC with 40 to 70% acetonitrile containing 0.1% formic acid and water containing 0.1% formic acid to yield **compound 6** (19 mg; 43%). Spectral

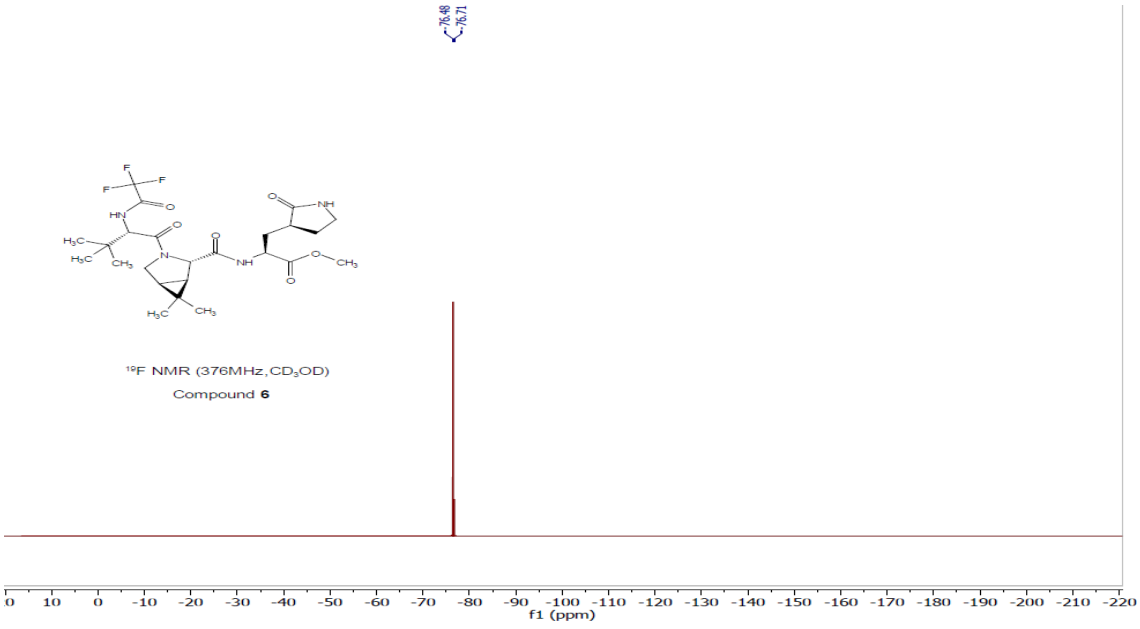
data: ^{1}H NMR (400 MHz, CD_3OD): δ (ppm) 4.64 – 4.54 (m, 2H), 4.37 (s, 1H), 4.04 (dd, *J* = 10.3, 5.5 Hz, 1H), 3.88 (d, *J* = 10.3 Hz, 1H), 3.76 (s, 3H), 3.42 – 3.35 (m, 1H), 3.31 – 3.24 (m, 1H), 2.73 – 2.61 (m, 1H), 2.40 – 2.30 (m, 1H), 2.24 – 2.11 (m, 1H), 1.88 – 1.78 (m, 2H), 1.62 (dd, *J* = 7.7, 5.3 Hz, 1H), 1.49 (d, *J* = 7.7 Hz, 1H), 1.10 (s, 3H), 1.08 (s, 9H), 0.96 (s, 3H) (Note: as CD_3OD solvent used, the 3 amide NH are not observed due to deuterium exchange). ^{13}C NMR (100 MHz, CD_3OD) δ 181.89, 173.89, 173.71, 170.02, 159.11 (q, *J* = 37.3 Hz, 117.48 (q, *J* = 287.85 Hz), 62.22, 59.77, 52.93, 51.55, 42.00, 41.42, 39.39, 36.30, 34.27, 32.26, 29.15, 28.70, 26.92, 26.43, 20.45, 12.91 (22 of 24 peaks recorded due to P3 *t*-butyl). ^{19}F NMR (376 MHz, CD_3OD) δ -76.48. ESI-quadrupole-MS: *m/z* calc $C_{24}H_{36}F_3N_4O_6$ ($M+H^+$) 533.2587, found 533.2580. No unexpected or unusually high safety hazards encountered during synthesis.





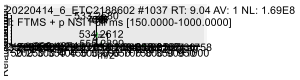
^{13}C NMR (100MHz, CD_3OD)

Compound **6**



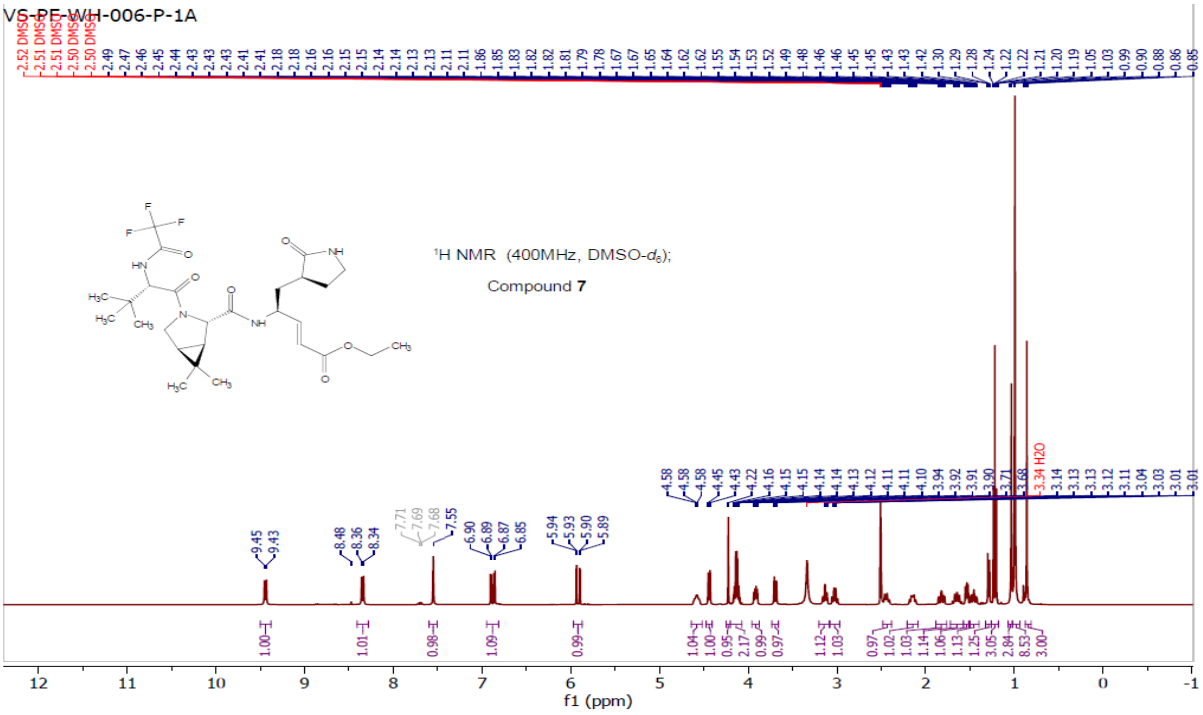
^{19}F NMR (376MHz, CD_3OD)

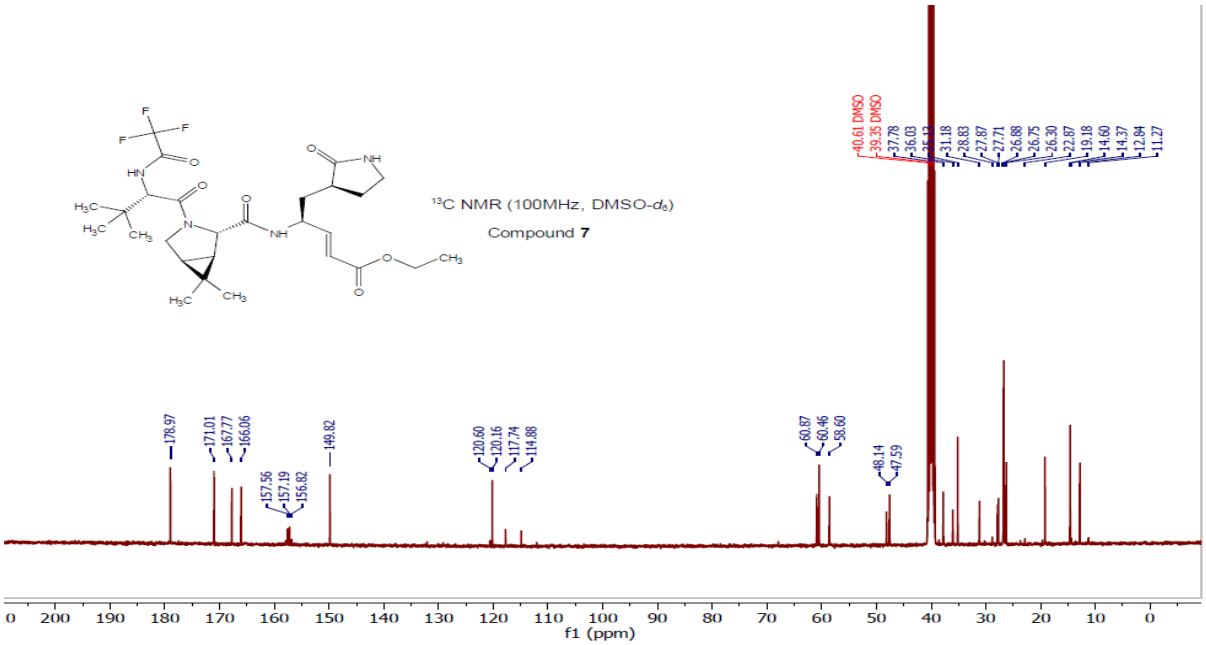
Compound **6**

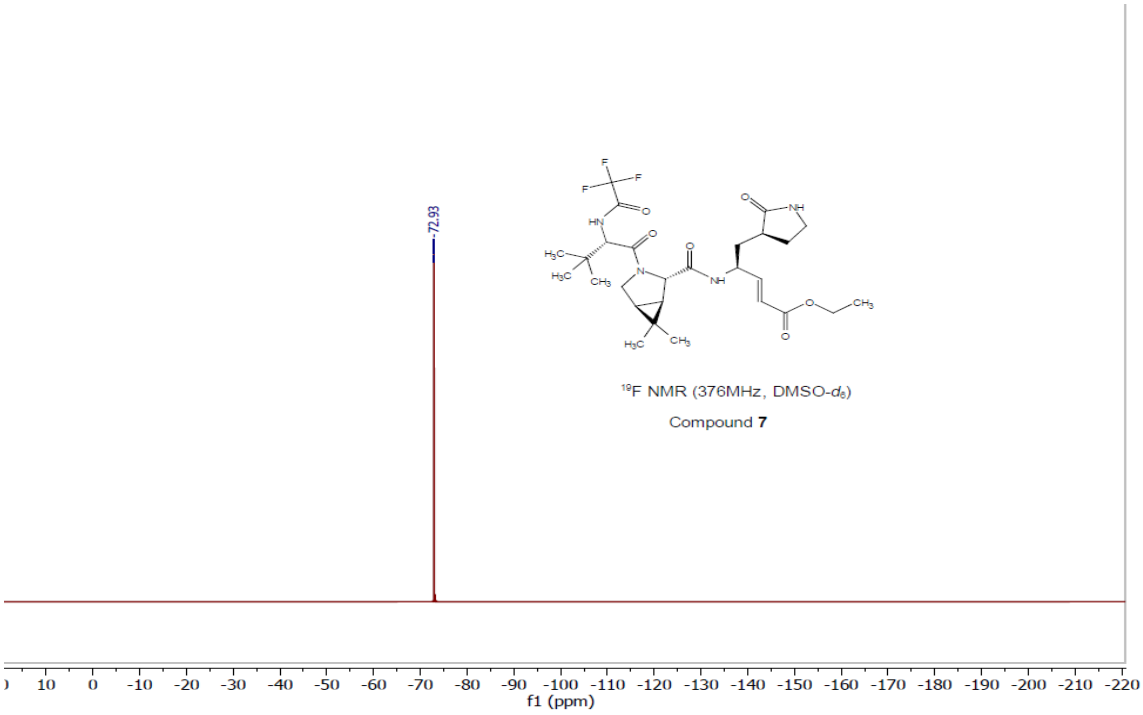


o) Synthesis of compound 7

Intermediate carboxylic acid 6 (35 mg; 0.096 mmol; 1 eq.) and ethyl-(*S*,*E*)-4-amino-5-(*S*-2-oxopyrrolidin-3-yl)pent-2-enoate HCl salt (28 mg; 0.105 mmol; 1.1 eq.) were dissolved in anhydrous DMF (1 mL) in a 10 mL round-bottom flask. DIPEA (84 μL; 0.48 mmol; 5 eq.) was added at 0 °C. After 1 min., HATU (41 mg; 0.105 mmol; 1.1 eq.) was added and the reaction mixture stirred for 0.5 h. at RT under N_2 (g). Next, the reaction was diluted with CH_2Cl_2, washed with sat. $NaHCO_3$ (2x), water and brine. The CH_2Cl_2 layer was dried with Na_2SO_4 and concentrated *in vacuo*. The crude target was purified by preparative HPLC with 40 to 70% acetonitrile containing 0.1% formic acid and water containing 0.1% formic acid to yield **compound 7** (25 mg; 46%). Spectral data: ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 9.44 (d, *J* = 8.5 Hz, 1H), 8.35 (d, *J* = 9.0 Hz, 1H), 7.55 (s, 1H), 6.88 (dd, *J* = 15.7, 4.9 Hz, 1H), 5.92 (dd, *J* = 15.7, 1.7 Hz, 1H), 4.65 – 4.51 (m, 1H), 4.44 (d, *J* = 8.6 Hz, 1H), 4.22 (s, 1H), 4.19 – 4.07 (m, 2H), 3.92 (dd, *J* = 10.3, 5.5 Hz, 1H), 3.69 (d, *J* = 10.4 Hz, 1H), 3.13 (t, *J* = 1.5 Hz, 1H), 3.09 – 2.96 (m, 1H), 2.48 – 2.39 (m, 1H), 2.21 – 2.08 (m, 1H), 1.82 (ddd, *J* = 13.4, 11.9, 3.5 Hz, 1H), 1.71 – 1.58 (m, 1H), 1.58 – 1.51 (m, 1H), 1.51 – 1.41 (m, 1H), 1.29 (d, *J* = 7.7 Hz, 1H), 1.22 (t, *J* = 7.1 Hz, 3H), 1.03 (s, 3H), 0.99 (s, 9H), 0.86 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 178.97, 171.01, 167.77, 166.06, 157.36 (q, *J* = 37.37 Hz), 149.83, 120.16, 116.35 (q, *J* = 288.86 Hz), 60.87, 60.46, 58.60, 48.14, 47.59, 40.00, 37.78, 36.03, 35.13, 31.18, 27.87, 27.71, 26.75, 26.30, 19.18, 14.60, 12.84 (25 of 27 peaks recorded due to P3 *t*-butyl). ^{19}F NMR (376 MHz, DMSO-d_6) δ -72.93. ESI-quadrupole-MS: *m/z* calc $C_{27}H_{40}F_3N_4O_6$ ($M+H^+$) 573.2900, found 573.2891. No unexpected or unusually high safety hazards encountered during synthesis.

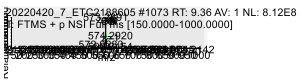

VS-PF-WH-006-P-1A
^{1}H NMR (400MHz, DMSO-d_6);
Compound 7
f1 (ppm)


^{13}C NMR (100MHz, DMSO-d_6)
Compound 7
f1 (ppm)



^{19}F NMR (376MHz, DMSO-d_6)

Compound **7**



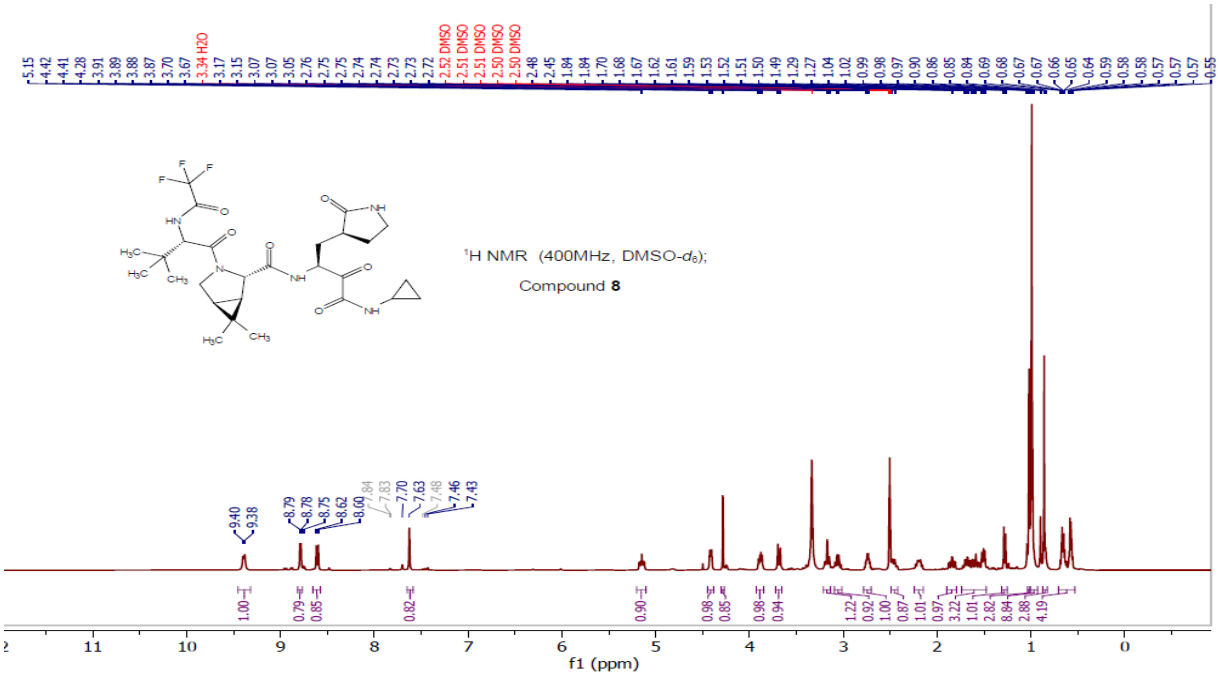
p) Synthesis of intermediate 10

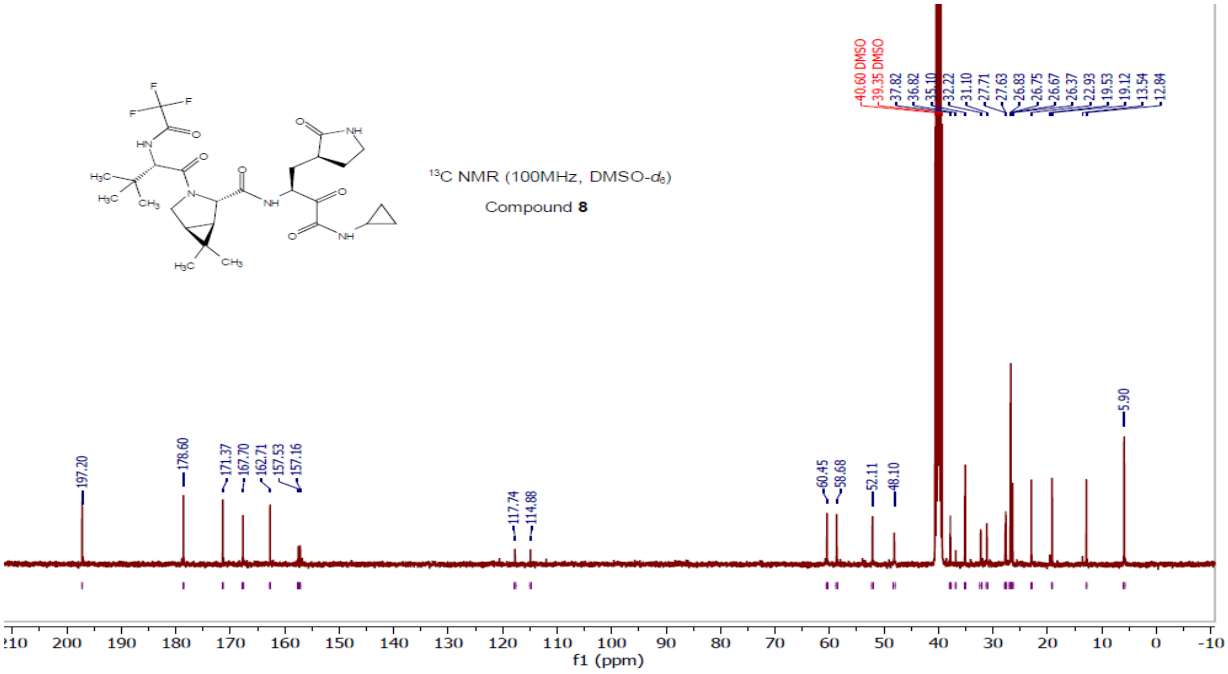
Intermediate carboxylic acid 6 (20 mg; 0.055 mmol; 1 eq.) and (3*S*)-3-amino-N-cyclopropyl-2-hydroxy-4-(*S*)-2-oxopyrrolidin-3-yl butanamide HCl salt (16.1 mg; 0.058 mmol; 1.05 eq.) were dissolved in anhydrous DMF (1 mL) in a 10 mL round-bottom flask. DIPEA (49 μL; 0.27 mmol; 5 eq.) was added at 0 °C. After 1 min., HATU (23 mg; 0.06 mmol; 1.1 eq.) was added and the reaction mixture stirred for 0.5 h. at RT under N_2 (g). Next, the reaction was diluted with ethyl acetate, washed with sat. $NaHCO_3$ (2x), water and brine. The ethyl acetate layer was dried with Na_2SO_4 and concentrated *in vacuo*. The crude target was purified by preparative HPLC with 20 to 70% acetonitrile containing 0.1% formic acid and water containing 0.1% formic acid to yield **intermediate 10** (15 mg; 46%). ESI-quadrupole-MS: *m/z* calc $C_{27}H_{41}F_3N_5O_6$ ($M+H^+$)

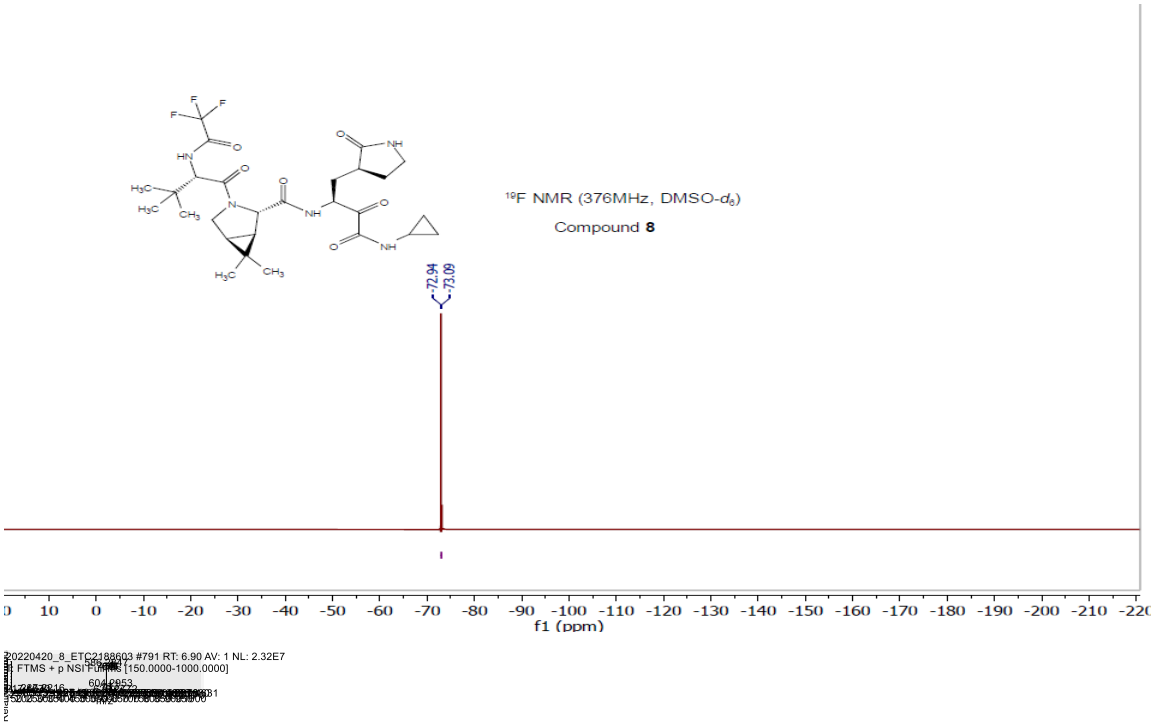
588.30 found 588.00. No unexpected or unusually high safety hazards encountered during synthesis.

q) Synthesis of compound 8

Intermediate 10 (15 mg; 0.026 mmol; 1 eq.) was dissolved in anhydrous DMF (1 mL) in a 10 mL round-bottom flask. Dess-Martin periodinane (16.2 mg; 0.038 mmol; 1.6 eq.) was added at 0 °C and the mixture stirred for 3.5 h. at RT under N_2 (g). Next, the reaction was diluted with ethyl acetate, quenched with sat.$NaHCO_3$ and sat.$Na_2S_2O_3$ solution (1:1 ratio) at 0 °C. The ethyl acetate layer was washed with sat. $NaHCO_3$ (2x) followed by 5% LiCl (aq) and brine. The ethyl acetate layer was collected and dried using Na_2SO_4 and concentrated *in vacuo*. The residue was purified by preparative HPLC with 20 to 70% acetonitrile containing 0.1% formic acid and water containing 0.1% formic acid to yield **compound 8** (12 mg; 79%). Spectral data: ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 9.39 (d, J = 7.6 Hz, 1H), 8.79 (d, J = 5.1 Hz, 1H), 8.61 (d, J = 8.2 Hz, 1H), 7.63 (s, 1H), 5.20 – 5.11 (m, 1H), 4.41 (d, J = 7.3 Hz, 1H), 4.28 (s, 1H), 3.89 (dd, J = 10.3, 5.5 Hz, 1H), 3.68 (d, J = 10.4 Hz, 1H), 3.21 – 3.14 (m, 1H), 3.10 – 3.02 (m, 1H), 2.78 – 2.70 (m, 1H), 2.49 – 2.42 (m, 1H), 2.24 – 2.15 (m, 1H), 1.84 (ddd, J = 13.5, 11.8, 3.7 Hz, 1H), 1.74 – 1.48 (m, 3H), 1.28 (d, J = 7.7 Hz, 1H),1.02 (s, 3H), 0.99 (s, 9H), 0.86 (s, 3H), 0.71 – 0.53 (m, 4H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 197.20, 178.60, 171.37, 167.70, 162.71, 157.37 (q, J = 37.3 Hz) 115.97 (q, J = 245.43 Hz), 60.45, 58.68, 52.11, 48.10, 40.00, 37.82, 36.82, 35.10, 32.22, 31.10, 27.71, 27.63, 26.75, 26.37, 22.93, 19.12, 12.84, 5.90 (25 of 27 peaks recorded due to P3 *t*-butyl). ^{19}F NMR (376 MHz, DMSO-d_6) δ -72.94. ESI-quadrupole-MS: *m/z* calc $C_{27}H_{39}F_3N_5O_6$ ($M+H^+$) 586.2852, found 586.2847. No unexpected or unusually high safety hazards encountered during synthesis.


^{1}H NMR (400MHz, DMSO-d_6);
Compound **8**
f1 (ppm)

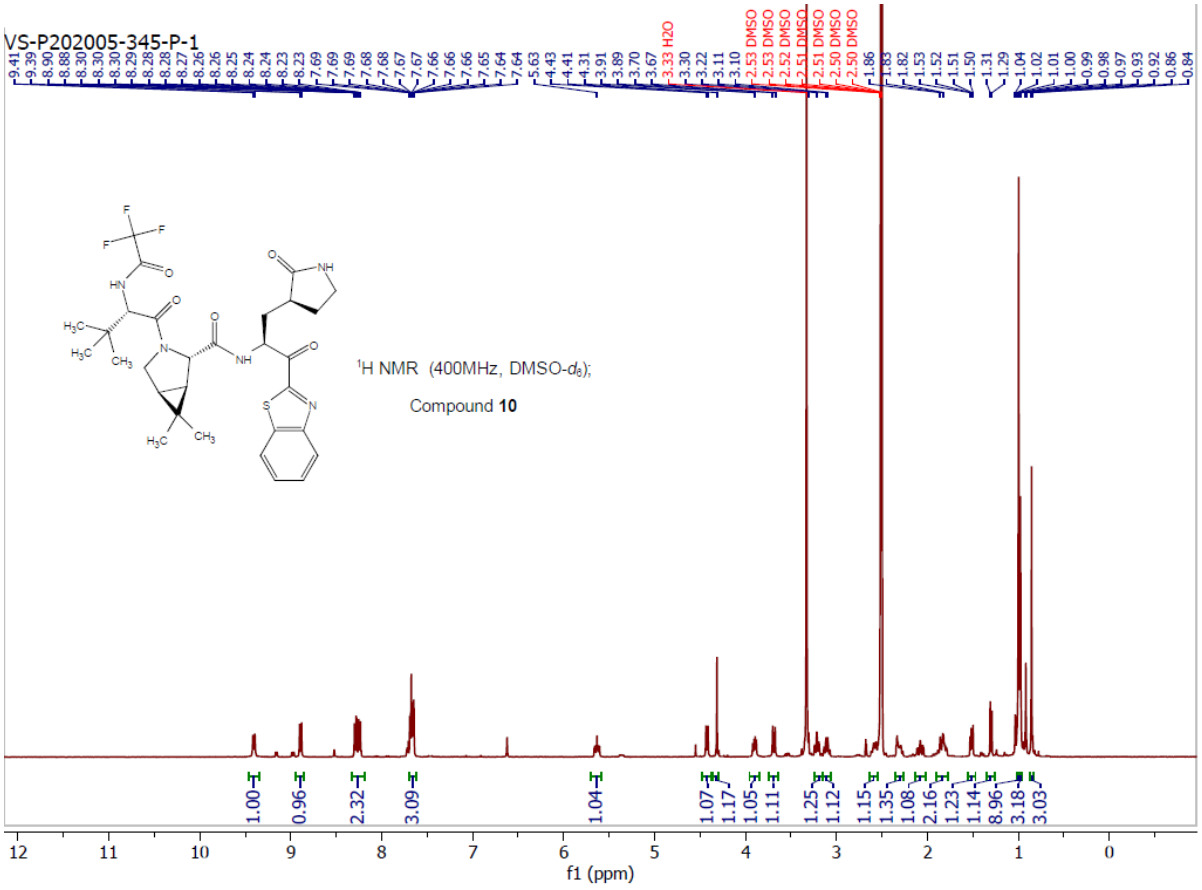

^{13}C NMR (100MHz, DMSO-d_6)
Compound **8**
f1 (ppm)

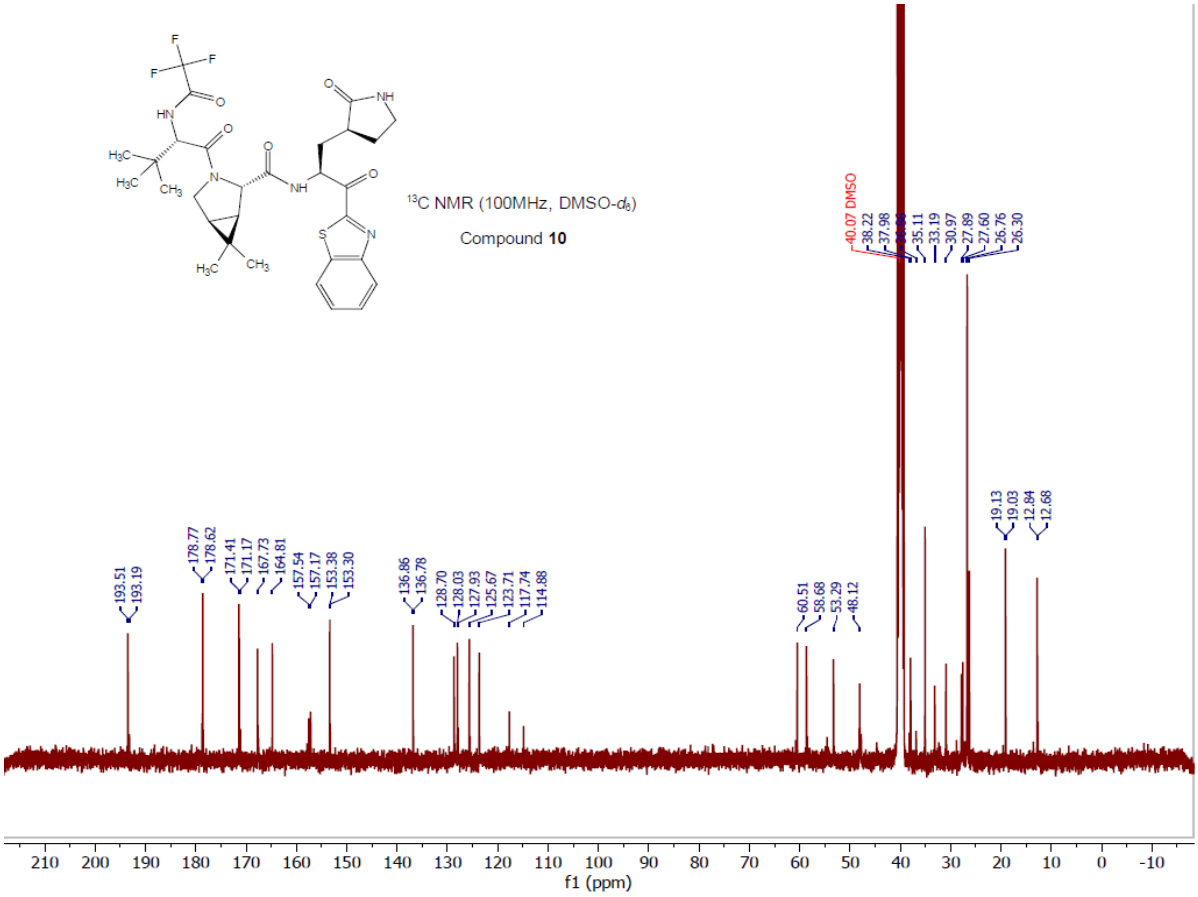


r) Synthesis of compound 10

Intermediate carboxylic acid 6 (20 mg; 0.055 mmol; 1 eq.) and (*S*)-3-(*S*)-2-amino-3-benzothiazol-2-yl-3-oxopropylpyrrolidin-2-one HCl salt (19 mg; 0.058 mmol; 1.05 eq.) were dissolved in anhydrous DMF (1 mL) in a 10 mL round-bottom flask. DIPEA (49 μL; 0.274 mmol; 5 eq.) was added at 0 °C. After 1 min., HATU (23 mg; 0.06 mmol; 1.1 eq.) was added and the reaction mixture stirred for 0.5 h. at RT under N_2 (g). Next, the reaction was diluted with CH_2Cl_2, washed with sat. $NaHCO_3$ (2x), water and brine. The CH_2Cl_2 layer was dried with Na_2SO_4 and concentrated *in vacuo*. The crude target was purified by preparative HPLC with 20 to 70% acetonitrile containing 0.1% formic acid and water containing 0.1% formic acid to yield **compound 10** (11 mg; 31%). Spectral data: ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 9.40 (d, *J* = 8.4 Hz, 1H), 8.89 (d, *J* = 8.1 Hz, 1H), 8.32 – 8.22 (m, 2H), 7.74 – 7.61 (m, 3H), 5.63 (ddd, *J* = 11.6, 8.1, 3.2 Hz, 1H), 4.42 (d, *J* = 8.3 Hz, 1H), 4.31 (s, 1H), 3.90 (dd, *J* = 10.3, 5.5 Hz, 1H), 3.69 (d, *J* = 10.5 Hz, 1H), 3.22 (t, *J* = 9.3 Hz, 1H), 3.18 – 3.07 (m, 1H), 2.63 – 2.55 (m, 1H), 2.32 – 2.26 (m, 1H), 2.14 – 2.03 (m, 1H), 1.90 – 1.77 (m, 2H), 1.51 (dd, *J* = 7.7, 5.4 Hz,

1H), 1.30 (d, *J* = 7.6 Hz, 1H), 1.00 (s, 9H), 0.98 (s, 3H), 0.86 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 193.51, 178.62, 171.41, 167.73, 164.82, 157.37 (q, *J* = 37.3 Hz), 153.38, 136.86, 128.70, 128.03, 125.67, 123.72, 116.36 (q, *J* = 288.86 Hz), 60.51, 58.68, 53.29, 48.12, 40.00, 38.22, 35.11, 33.19, 30.97, 27.89, 27.60, 26.76, 26.30, 19.13, 12.84 (28 of 30 peaks recorded due to P3 *t*-butyl). ^{19}F NMR (376 MHz, DMSO-d_6) δ -72.92. ESI-quadrupole-MS: *m/z* calc $C_{30}H_{37}F_3N_5O_5S$ ($M+H^+$) 636.2468, found 636.2460. No unexpected or unusually high safety hazards encountered during synthesis.




^{13}C NMR (100MHz, DMSO-d_6)
Compound **10**
40.07 DMSO
38.22
37.98
35.11
33.19
30.97
27.89
27.60
26.76
26.30
193.51
193.19
178.77
178.62
171.41
171.17
167.73
164.81
157.54
157.17
153.38
153.30
136.86
136.78
128.70
128.03
127.93
125.67
123.71
117.74
114.88
60.51
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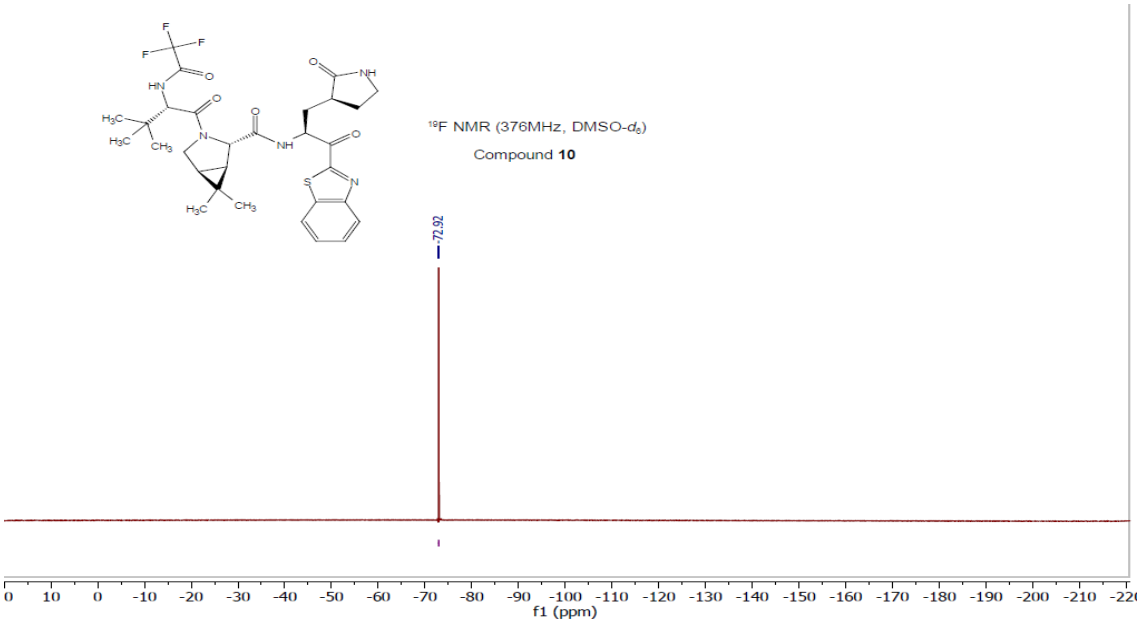
53.29
48.12
19.13
19.03
12.84
12.68
f1 (ppm)

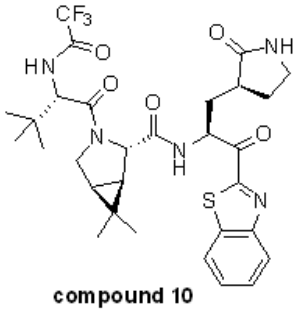

^{19}F NMR (376MHz, DMSO-d_6)
Compound **10**
-72.92
f1 (ppm)

Column: Phenominex Luna 5 U 100mmX4.6mm C18(2) 100A

Mobile phase A: 0.1% TFA in Aqueous
Mobile phase B: Acetonitrile

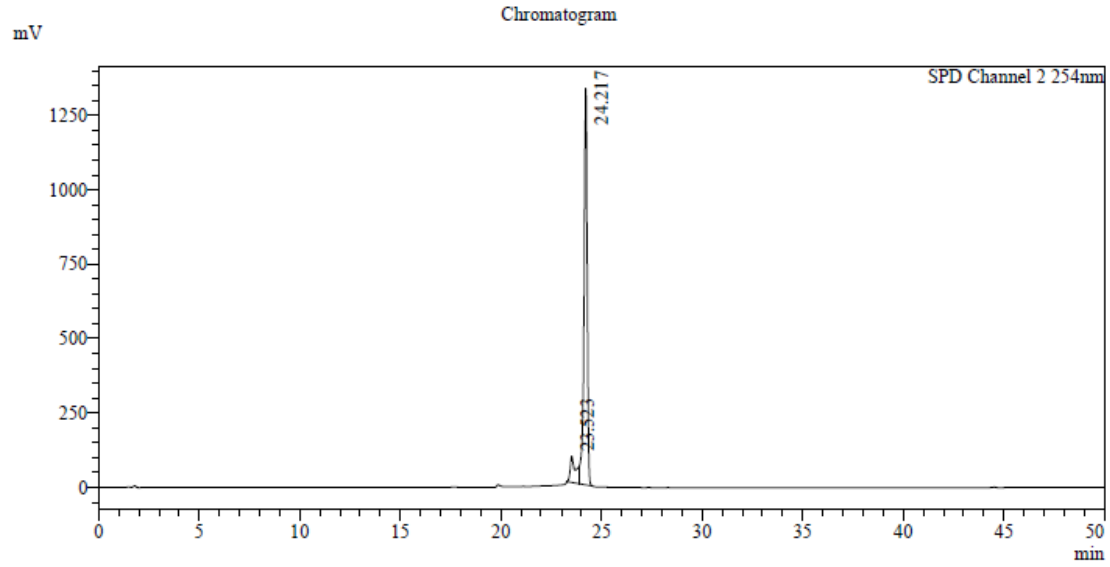
<<LC Time Program>>
Time Module Command Value
0.01 Pumps B.Conc 5
30.00 Pumps B.Conc 90
30.01 Pumps B.Conc 95
40.00 Pumps B.Conc 95
40.01 Pumps B.Conc 5

Flow : 1 mL/min



compound 10

<Chromatogram>



Peak Table

SPD Channel 2 254nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	23.523	1638726	88261	9.966		M	
2	24.217	14804492	1332670	90.034		V M	
Total		16443218	1420932				

Viral protease production

The full-length gene encoding SARS-CoV-2 3CLpro from strain BetaCoV/Wuhan/WIV04/ 2019 (Accession NC_045512) was synthesized and cloned into NdeI and XhoI site of pET29a(+) vector by Genscript (USA) as described previously (Ma, C. et al. *Cell Res*. **2020**, *30*, 678−692. https://doi.org/10.1038/s41422-020-0356-z). The codon optimized plasmid for *E. coli* expression was transformed into

competent BL21(DE3) cells. A single colony was picked to inoculate 10 mL of Terrific Broth (TB) supplemented with 50 mg/L kanamycin and grown at 37 °C with shaking at 200 rpm. The 10-mL inoculum was added to 1 L of TB with 50 mg/L kanamycin and grown to an optical density at 600 nm of 2.5. The culture was induced using 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and grown at 37 °C for an additional 2 hrs. The cell pellet was re-suspended in lysis buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 2mM dithiothreitol (DTT) and 10 µg/mL DNase I), and lysed by sonication (25% amplitude, 2 seconds on / 2 seconds off). Cell debris were removed by centrifugation at 39,191x g for 30 min at 4°C. The supernatant was loaded onto an equilibrated HisTrap HP column (Cytiva). The column was washed with wash buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 5 mM imidazole, 2 mM DTT) followed by another wash with 30 mM imidazole. Protein was eluted using 300 mM imidazole and further purified by size exclusion chromatography on HiLoad 16/600 Superdex 200 prep grade column (Cytiva) with 20mM Tris, pH 7.5, 100mM NaCl, 2mM DTT. Fractions from resulted peak were pooled and concentrated using centrifugal filter unit of 10,000-molecular-weight-cutoff. The purity and molecular weight of the protein was confirmed with SDS-PAGE and mass spectrometry (MS). A predominant peak at 34,863 Da was observed by MS which matched the calculated molecular weight of SARS-CoV-2 C-His-3CLpro without the N-terminal methionine residue. Similarly, the full-length gene encoding human coronavirus 229E (HCoV-229E, Accession X69721.1) was cloned in pET29a(+) with a C-terminus His tag. A similar expression and purification condition was followed for HCoV-229E except that the cells were transformed in Rosetta (DE3), cultured in Luria broth and after induction the cells were grown overnight at 18 °C. The molecular weight of 34,120 Da was observed using MS and matched with the calculated mass without the N-terminal methionine residue.

Biochemical FRET assay (IC_{50})

The FRET based protease assay is based on a published protocol (Kim, Y. et al. *J. Virol*. **2012**, *86*, 11754−11762. https://doi:10.1128/JVI.01348-12). The peptide substrate (DABCYL)KTSAVLQSGFRKM(Glu)(EDANS) was synthesized by Genscript (USA). Test compounds were 3-fold serially diluted in 100% DMSO to 15

concentrations, starting at 3.33 mM. 1.5 µL of the serially diluted compounds were transferred to a black 384 well assay plate (Cat. 781900, Greiner). 23.5 µL of 2.13X concentration of SARS-CoV-2 Chis-3CLpro enzyme prepared in assay buffer was added to the compounds and incubated for 30 mins at 25°C. 25 µL of 2X concentration of peptide substrate was added to the assay plate and incubated at 37°C for 1.5 h. The final assay contained 12.5 nM of enzyme, 6 µM substrate and 3% DMSO in assay buffer containing 50 mM HEPES at pH 7.5, 100 mM NaCl, and 0.01% Triton X-100 and 1 mM DTT. The starting test compound concentration started at 100 µM. The FRET signal was measured using an excitation wavelength of 340 nm (UV[TRF] 340/60 nm, Barcode 101), emission wavelength of 490 nm (DSPPsion 486/10 filter, Barcode 220) and Lance/DELFIA D400 single mirror (Barcode 412) on Envision plate reader (2104 EnVision Multilabel Plate Readers, Perkin Elmer). The dose-response curves were fitted with a variable slope using GraphPad Prism software (GraphPad, USA) to determine a compound's IC_{50}. Experiments were conducted in duplicates and IC_{50}s were determined from two independent experiments.

Cell-based HCoV 229E inhibition assay (EC_{50})

MRC5 human lung fibroblast cells were cultured in a 96-well plate, seeded at 10,000 cells per well in EMEM + 10% FBS overnight. The next day, the culture medium was removed from each well and HCoV 229E virus at an MOI of 0.01 were added to the cells in EMEM + 2% FBS. After 1 hour infection, virus inoculum was removed and test compounds were added to the cells. The compounds were titrated from 50 µM with 8-point, 5-fold serial dilution in EMEM + 2% FBS + 0.5% DMSO. CellTiter-Glo (Promega) was added to the cells after 4 days incubation to determine cell viability. Assay readout was performed using and luminescence measurement on a Tecan Spark plate reader. Experiments were performed with 4 biological replicates in technical triplicates. EC_{50} values were determined by nonlinear fit of luminescence readouts from virus-infected, compound-treated wells. Data analysis was performed using GraphPad Prism 8.