Supporting Information

for

Discovery of CMX990: A Potent SARS-CoV-2 3CL Protease Inhibitor Bearing a Novel Warhead

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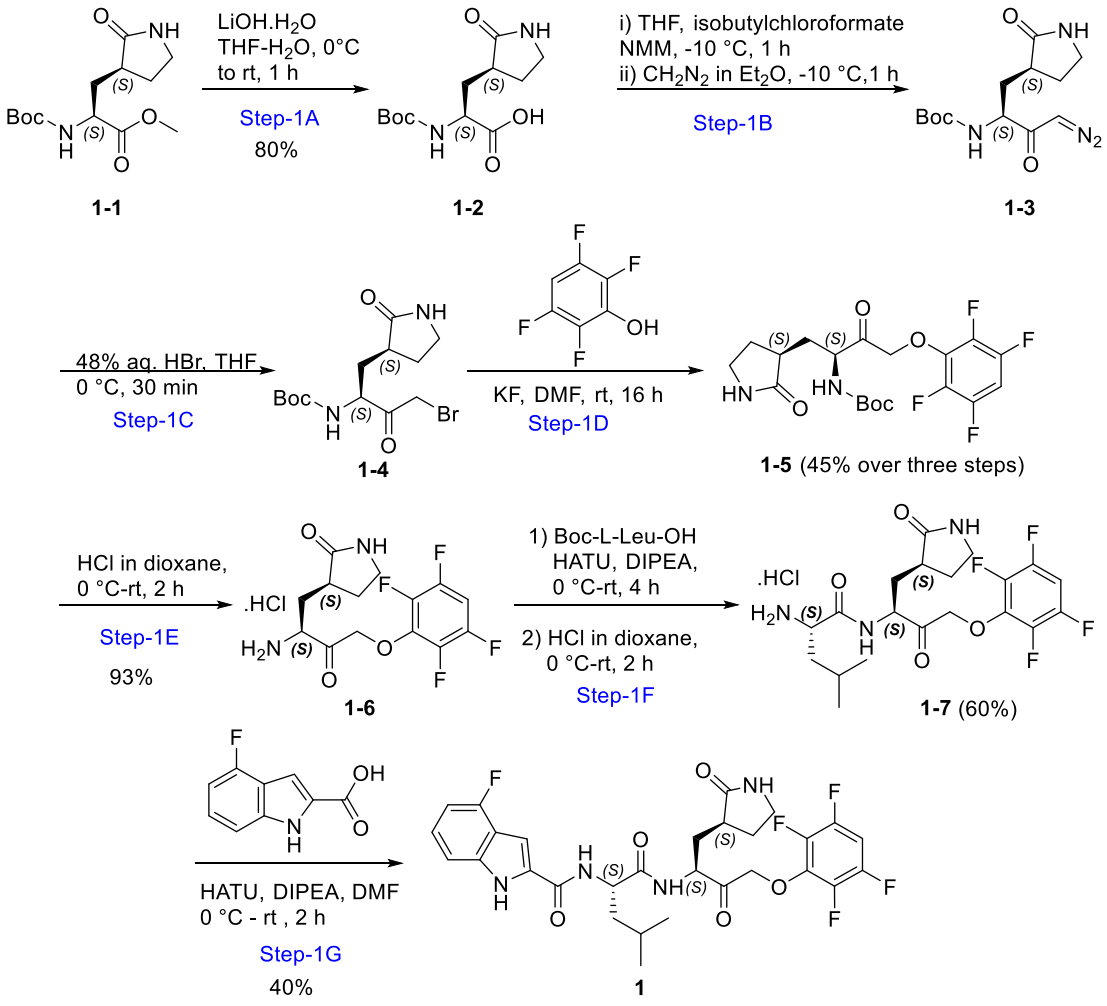
1. Experimental Procedures for Compounds 1-16

Materials and Methods

All reagents and solvents were purchased from commercial sources and used without further purification. Flash column chromatography was performed using silica gel (200−300 mesh). All reactions were monitored by TLC (pre-coated EMD silica gel 60 F254 TLC aluminum sheets and visualized with a UV lamp or appropriate stains) and/or LCMS (Waters Acquity UPLC system, 2 or 4 min run of a 10-90% mobile phase gradient of acetonitrile in water [+0.1% formic acid]). NMR spectra were obtained on Bruker AV400 or AV500 instruments, and data was analyzed using the MestReNova NMR software (Mestrelab Research S. L.). Chemical shifts (δ) are expressed in ppm and are internally referenced for ^{1}H NMR ($CHCl_3$ 7.26 ppm, DMSO-d_6 2.50 ppm) and ^{13}C NMR ($CDCl_3$ 77.16 ppm, DMSO-d_6 39.52 ppm). X-ray data was collected at room temperature on a Bruker D8 QUEST instrument with an IμS Mo microfocus source (λ = 0.7107 Å) and a PHOTON-III detector.

NMR spectra for the final proline containing derivatives commonly exist as rotamer populations in DMSO-d_6. The activated ketone in the novel warhead creates a transient reversible-equilibrium under acid modified (f.ex., TFA, formic acid) aqueous chromatography conditions, as represented by the appearance of a leading non-baseline separated second (minor) peak in the chromatogram. Occasionally, some compounds also show similar interactions/equilibrium in NMR, including interactions of the P2-proline nitrogen with the warhead ketone. The isolation of individual components of these non-baseline separated material, and re-injection results in the same equilibrium appearance. Hence, when doing chromatographic separations under reversed-phase conditions containing acidic modifiers, the entirety of this peak (leading minor-peak + the later-eluting major peak) should be isolated as a single peak. Alternatively, reversed-phase separation can be carried out without the presence of an acidic modifier, with a slight compromise on sharpness of the peak.

Synthesis of 4-fluoro-*N*-((*S*)-4-methyl-1-oxo-1-(((*S*)-3-oxo-1-((*S*)-2-oxopyrrolidin-3-yl)-4-(2,3,5,6-tetrafluorophenoxy)butan-2-yl)amino)pentan-2-yl)-1*H*-indole-2-carboxamide **(1)**



Step 1A: To a stirred solution of methyl (*S*)-2-((*tert*-butoxycarbonyl)amino)-3-((*S*)-2-oxopyrrolidin-3-yl)propanoate (**1-1**) (14.5 g, 50.64 mmol, 1.0 eq) in tetrahydrofuran (50 mL), lithium hydroxide monohydrate (2.55 g, 60.77 mmol, 1.2 eq) in water (10 mL) was added at 0 °C and the reaction mixture was stirred at 0 °C for 1 h. After completion, the reaction mixture was acidified with 2 N hydrochloric acid to pH~5 and extracted with ethyl acetate (3 × 300 mL). The combined organic layer was dried over anhydrous sodium sulphate and concentrated under reduced pressure to afford (*S*)-2-((*tert*-butoxycarbonyl)amino)-3-((*S*)-2-oxopyrrolidin-3-yl)propanoic acid (**1-2**, 11.0 g, 40.4 mmol, 80%) as a pale yellow gum. TLC system: EtOAc:pet-ether (7:3); R_f: 0.1.

Step 1B: To a stirred solution of (*S*)-2-((*tert*-butoxycarbonyl)amino)-3-((S)-2-oxopyrrolidin-3-yl)propanoic acid (**1-2**) (11.0 g, 36.72 mmol, 1.0 eq) in tetrahydrofuran (300 mL), *N*-methylmorpholine (4.83 g, 47.74 mmol, 1.3 eq) was added, then isobutyl chloroformate (10.18 g, 73.45 mmol, 2.0 eq) was added at -10 °C, and the reaction mixture was stirred at -10 °C for 1 h. After completion, the reaction mixture was filtered and washed with tetrahydrofuran (50 mL). Freshly prepared diazomethane in diethyl ether (prepared from 5.0 mol equivalent of diazald) was added to the filtrate at -10 °C, and the reaction mixture was stirred at room temperature for 1 h. After completion, the reaction mixture was diluted with water (100 mL) and extracted with ethyl acetate (3 × 300 mL). The combined organic layer was dried over anhydrous sodium sulphate and evaporated under reduced pressure to afford *tert*-butyl ((*S*)-4-diazo-3-oxo-1-((*S*)-2-oxopyrrolidin-3-yl)butan-2-yl)carbamate (**1-3**, 10.8 g, crude, ~36.44 mmol) as a yellow liquid. TLC system: EtOAc:pet-ether (7:3); R_f: 0.1.

Step 1C: To a stirred solution of *tert*-butyl ((*S*)-4-diazo-3-oxo-1-((*S*)-2-oxopyrrolidin-3-yl)butan-2-yl)carbamate (**1-3**, 10.8 g, 36.44 mmol, 1.0 eq) in tetrahydrofuran (300 mL) was added 48% aqueous hydrobromic acid (6.87 g, 43.73 mmol, 1.2 eq) dropwise at 0 °C and the mixture was stirred at 0°C for 30 min. After completion, the reaction mixture was basified with saturated sodium bicarbonate and extracted with ethyl acetate (3 × 150 mL). The combined organic layer was dried over anhydrous sodium sulphate and concentrated under reduced pressure to afford *tert*-butyl ((*S*)-4-bromo-3-oxo-1-((*S*)-2-oxopyrrolidin-3-yl)butan-2-yl)carbamate (**1-4**, 12.5 g, crude, ~35.79 mmol) as a yellow liquid. TLC system: EtOAc:pet-ether (7:3); R_f: 0.4.

Step 1D: To the mixture of *tert*-butyl ((*S*)-4-bromo-3-oxo-1-((*S*)-2-oxopyrrolidin-3-yl)butan-2-yl)carbamate (12.5 g, 35.79 mmol, 1.0 eq) and 2,3,5,6-tetrafluorophenol (5.94 g, 35.79 mmol, 1.0 eq) in dimethylformamide (50 mL), potassium fluoride (6.47 g, 111.33 mmol, 3.1 eq) was added, and the reaction mixture was stirred at room temperature for 24 h. After completion, the reaction mixture was diluted with water (100 mL) and extracted with ethyl acetate (3 × 300 mL). The combined organic layer was dried over anhydrous sodium sulfate and evaporated under reduced pressure to give crude product. The crude residue was then purified by silica gel (230-400 mesh) column chromatography using 40-60% ethyl acetate in pet-ether as a gradient to afford *tert*-butyl ((*S*)-3-oxo-1-((*R*)-2-oxopyrrolidin-3-yl)-4-(2,3,5,6-tetrafluorophenoxy)butan-

2-yl)carbamate (**1-5**, 7.1 g, 16.3 mmol, 45% over 3 steps) as a brown solid. TLC system: EtOAc:pet-ether (7:3); R_f: 0.5. LCMS *M/Z* 435.22 (M+1); ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.65-7.47 (m, 3H), 5.30-5.19 (m, 2H), 4.16-4.10 (m, 1H), 3.16-3.12 (m, 2H), 2.25-2.10 (m, 2H), 1.92-1.85 (m, 1H), 1.67-1.56 (m, 2H), 1.39 (s, 9H).

Step 1E: To a stirred solution of *tert*-butyl ((*S*)-3-oxo-1-((*S*)-2-oxopyrrolidin-3-yl)-4-(2,3,5,6-tetrafluorophenoxy)butan-2-yl)carbamate **(1-5)** (3.9 g, 8.66 mmol) in 1,4-dioxane (20 mL) was added 4 M HCl in 1,4-dioxane (8.66 mL, 34.64 mmol) at 0 °C and the resultant mixture was stirred at room temperature for 2 h. After completion, solvent was removed by under reduced pressure and the resulting crude product was washed with diethyl ether (2 × 30 mL) to afford (*S*)-3-((*S*)-2-amino-3-oxo-4-(2,3,5,6-tetrafluorophenoxy)butyl)pyrrolidin-2-one) hydrochloride (**1-6**, 3 g, 8.1 mmol, 93%) as a yellow oil. TLC system: MeOH: DCM (1:9); R_f: 0.2.

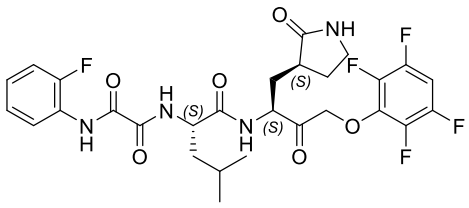
Step 1F: To a stirred solution of (*S*)-3-((S)-2-amino-3-oxo-4-(2,3,5,6-tetrafluorophenoxy)butyl)pyrrolidin-2-one (**1-6**, 3 g, 8.09 mmol) and (*tert*-butoxycarbonyl)-L-leucine (1.87 g, 8.09 mmol, 1.0 eq) in DMF (30 mL), HATU (4 g, 10.52 mmol, 1.3 eq) was added, followed by DIPEA (4.4 mL, 24.28 mmol, 3.0 eq) at 0°C, and the resultant mixture was stirred at room temperature for 4 h. After completion, cold water (40 mL) was added to the reaction mixture and extracted with EtOAc (3 × 50 mL). The combined organic layer was dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure to give crude product. The crude product was then purified by column chromatography over silica gel (230-400 mesh) using 45-50 % ethyl acetate in pet-ether as a gradient to afford *tert*-butyl ((*S*)-4-methyl-1-oxo-1-(((*S*)-3-oxo-1-((*S*)-2-oxopyrrolidin-3-yl)-4-(2,3,5,6-tetrafluorophenoxy)butan-2-yl)amino)pentan-2-yl)carbamate (3 g, 5.48 mmol, 68%) as an off-white solid. TLC system: EtOAc: pet-ether (7:3); R_f: 0.6.

To a stirred solution of *tert*-butyl ((*S*)-4-methyl-1-oxo-1-(((*S*)-3-oxo-1-((*S*)-2-oxopyrrolidin-3-yl)-4-(2,3,5,6-tetrafluorophenoxy)butan-2-yl)amino)pentan-2-yl)carbamate (0.2 g, 0.37 mmol) in 1,4-dioxane (20 mL) was added 4 M HCl in 1,4-dioxane (0.3 mL, 1.46 mmol) at 0 °C and the resultant mixture was stirred at room temperature for 2 h. After completion, solvent was removed under reduced pressure and the resulting crude residue was washed with diethyl ether (2 × 30 mL) to afford (S)-2-amino-4-methyl-*N*-((*S*)-3-oxo-1-((*S*)-2-oxopyrrolidin-3-yl)-4-

(2,3,5,6-tetrafluorophenoxy)butan-2-yl)pentanamide (**1-7**, 0.16 g, 0.33 mmol, 89%) as brown gummy solid. TLC system: EtOAc (100%); R_f: 0.1. LCMS [M+1]: 448.41.

Step 1G: To a solution of 4-fluoro-1*H*-indole-2-carboxylic acid (0.444 g, 2.48 mmol, 1.0 eq) in dry DMF (12 mL) at 0°C, the following was added: (*S*)-2-amino-4-methyl-*N*-((*S*)-3-oxo-1-((*S*)-2-oxopyrrolidin-3-yl)-4-(2,3,5,6-tetrafluorophenoxy)butan-2-yl)pentanamide hydrochloride (**1-7**, 1.2 g, 2.48 mmol, 1.0 eq), HATU (1.131 g, 2.98 mmol, 1.2 eq), and DIPEA (1.77 mL, 9.92 mmol, 4.0 eq). The resultant mixture was stirred at room temperature for 2 h. It was diluted with ethyl acetate (50 mL) and washed with saturated aqueous $NaHCO_3$ (30 mL), followed by water (30 mL) and brine (20 mL). The organic layer was dried over anhydrous sodium sulfate, filtered, and concentrated. The obtained residue was then purified by column chromatography over silica gel (230-400 mesh) using 50-55% EtOAc in pet-ether as an eluent) to afford 4-fluoro-*N*-((*S*)-4-methyl-1-oxo-1-(((*S*)-3-oxo-1-((*S*)-2-oxopyrrolidin-3-yl)-4-(2,3,5,6-tetrafluorophenoxy)butan-2-yl)amino)pentan-2-yl)-1*H*-indole-2-carboxamide (**1**, 0.6 g, 0.99 mmol, 40%) as an off-white solid. TLC system: EA: PE (70:30); R_f: 0.4. ^{1}H NMR (400 MHz, DMSO-*d6*) δ 11.90 (d, *J* = 8.9 Hz, 1H), 8.64-8.59 (m, 2H), 7.64 (s, 1H) 7.59-7.50 (m, 1H), 7.38 (d, *J* = 1.2 Hz, 1H), 7.26 (d, *J* = 8.2 Hz, 1H), 7.19-7.16 (m, 1H), 6.82 (dd, *J* = 10.8, 3.6 Hz, 1H), 5.3-5.18 (m, 2H), 4.52-4.46 (m, 2H), 3.14-3.07 (m, 2H), 2.35-1.95 (m, 3H), 1.78-1.52 (m, 5H), 0.94 (d, *J* = 6.8 Hz, 3H), 0.90 (d, *J* = 6.8 Hz, 3H); HRMS *m/z* calcd. for $C_{29}H_{30}F_5N_4O_5^+$ $[M+H]^+$ 609.2131, found 609.4000.

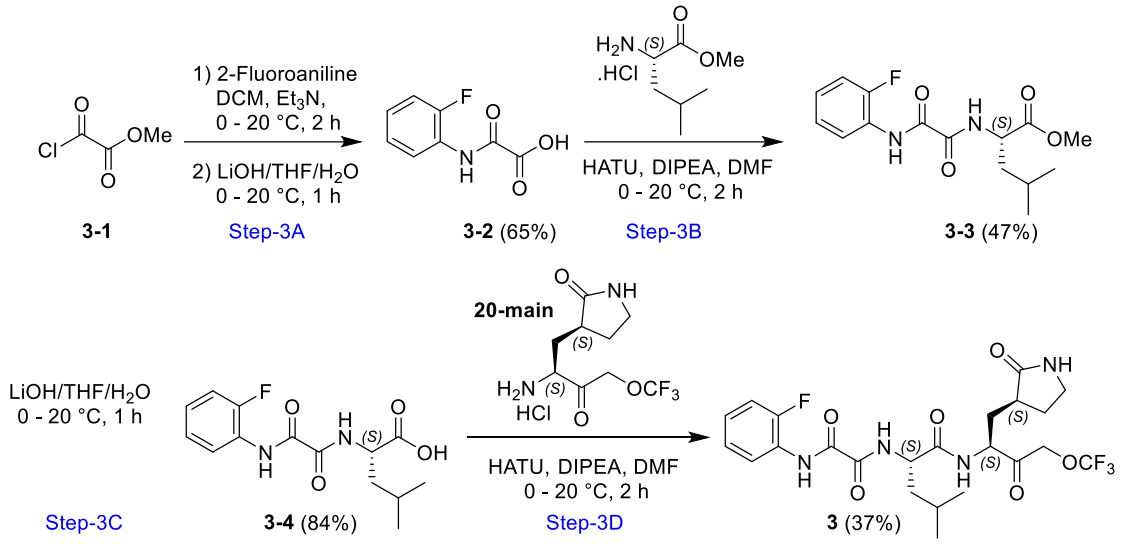
Synthesis of N^1-(2-fluorophenyl)-N^2-((*S*)-4-methyl-1-oxo-1-(((*S*)-3-oxo-1-((*S*)-2-oxopyrrolidin-3-yl)-4-(2,3,5,6-tetrafluorophenoxy)butan-2-yl)amino)pentan-2-yl)oxalamide **(2)**



Compound **2** (off-white solid) was synthesized according to the procedure outlined for **1**, using 2-((2-fluorophenyl)amino)-2-oxoacetic acid (CAS 84944-15-0, Combo-Blocks Inc. part# QZ-3085) as the P2 cap.

^{1}H NMR (400 MHz, DMSO-*d6*) δ 10.23 (s, 1H), 8.98 (d, *J* = 8.3 Hz, 1H), 8.56 (d, *J* = 7.9 Hz, 1H), 7.73 (td, *J* = 7.8, 1.9 Hz, 1H), 7.66 (br s, 1H), 7.61 – 7.51 (m, 1H), 7.39 – 7.18 (m, 3H), 5.25 (d, *J* = 17.7 Hz, 1H), 5.17 (d, *J* = 17.8 Hz, 1H), 4.52 – 4.44 (m, 1H), 4.42 – 4.32 (m, 1H), 3.20 – 3.06 (m, 2H), 2.31 – 2.22 (m, 1H), 2.16 – 2.05 (m, 1H), 2.02 – 1.92 (m, 1H), 1.81 – 1.72 (m, 1H), 1.70 – 1.43 (m, 4H), 0.93 – 0.86 (m, 6H); ^{19}F NMR (376 MHz, DMSO-d_6) δ -76.54, -125.96, -143.21 (dd, *J* = 22.6, 8.7 Hz), -159.38 (dd, *J* = 23.2, 9.1 Hz); HRMS *m/z* calcd. for $C_{28}H_{30}F_5N_4O_6^+$ $[M+H]^+$ 613.2080, found 613.4393.

Synthesis of N^1-(2-fluorophenyl)-N^2-((*S*)-4-methyl-1-oxo-1-(((*S*)-3-oxo-1-((*S*)-2-oxopyrrolidin-3-yl)-4-(trifluoromethoxy)butan-2-yl)amino)pentan-2-yl)oxalamide **(3)**



Step 3A: To a stirred solution of 2-fluoroaniline (3.0 g, 26.98 mmol, 1.0 eq) in DCM (30 mL) at 0 °C was added ethyl 2-chloro-2-oxoacetate (3.7 g, 28.33 mmol, 1.05 eq) and TEA (4.02 mL, 28.33 mmol, 1.05 eq) was added dropwise over 5 min, and the mixture was stirred at room temperature for 2 h. After completion, volatiles were removed through vacuum at 25 °C. The obtained crude product was dissolved in diethyl ether (100 mL) and filtered through a pad of celite washed with diethyl ether (5 × 50 mL). The collected filtrate was dried over Na_2SO_4, concentrated under reduced pressure to afford ethyl 2-((2-fluorophenyl)amino)-2-oxoacetate (4.0 g) as a colorless gummy liquid. TLC system: EtOAc: pet-ether (3:7); R_f: 0.6.

To a solution of 2-oxo-2-(phenylamino)acetate (4.0 g, 20.29 mmol, 1.0 eq) in THF (30.0 mL) at 0°C, was added LiOH.H_2O (1.0 g, 24.35 mmol, 1.2 eq) in water (10 mL). The resultant mixture

was stirred at room temperature for 1 h. After completion, the reaction mixture was acidified to pH ~4 using 5% aq. HCl (40 mL), and the aqueous layer was extracted with ethyl acetate (2 × 70 mL). The combined organic layer was dried over anhydrous sodium sulfate, filtered and concentrated to yield 2-((2-fluorophenyl)amino)-2-oxoacetic acid **(3-2)** (3.2 g, 17.4 mmol, 65% over two steps) as a colorless gummy liquid. TLC system: EtOAc: pet-ether (7:3); R_f: 0.1.

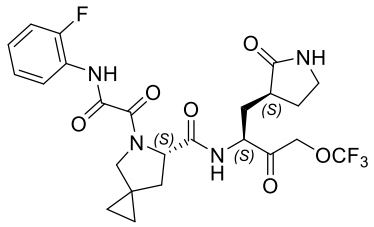
Step 3B: To a solution of 2-((2-fluorophenyl)amino)-2-oxoacetic acid **(3-2)** (5.0 g, 27.2 mmol, 1.0 eq) in anhydrous DMF (40 mL) the following was added: methyl *L*-leucinate hydrochloride (4.0 g, 27.2 mmol, 1.0 eq), HATU (13.47 g, 35.3 mmol, 1.3 eq), and DIPEA (14.2 mL, 81.6 mmol, 3.0 eq) at 0 °C. The resultant mixture was stirred at room temperature for 2 h. After completion, the reaction mixture was diluted with ethyl acetate (100 mL), washed with saturated aqueous $NaHCO_3$ (70 mL), followed by water (70 mL) and brine (40 mL). The organic layer was dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure to give crude product. The crude product was then purified by column chromatography over silica gel (230-400 mesh) (using 20-30% EtOAc in pet-ether as an eluent) to afford methyl (2-((2-fluorophenyl)amino)-2-oxoacetyl)-L-leucinate **3-3** (4.0 g, 12.8 mmol, 47%) as an off-white solid. TLC system: EtOAc: pet-ether (3:7); R_f: 0.5.

Step 3C: To a solution of methyl (2-((2-fluorophenyl)amino)-2-oxoacetyl)-L-leucinate **(3-3)** (4.0 g, 12.8 mmol, 1.0 eq) in THF (30.0 mL) at 0 °C, $LiOH.H_2O$ (0.65 g, 15.48 mmol, 1.2 eq) in water (7.0 mL) was added. The resultant mixture was stirred at room temperature for 1 h. After completion, the reaction mixture was acidified to pH ~4 using 5% aq. HCl (20 mL), and the aqueous layer was extracted with ethyl acetate (2 × 70 mL). The combined organic layer was dried over anhydrous sodium sulfate, filtered, and concentrated to yield (2-((2-fluorophenyl)amino)-2-oxoacetyl)-L-leucine **(3-4)** (3.2 g, 10.8 mmol, 84%) as a colorless gummy liquid. TLC system: EtOAc: pet-ether (7:3); R_f: 0.1. LC MS M/Z = 295.24 (M-H)- ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.81 (s, 1H), 10.30 (s, 1H), 9.09 (d, *J* = 8.3 Hz, 1H), 7.69-7.65 (m, 1H), 7.34-7.25 (m, 3H), 4.34-4.29 (m, 1H), 1.83 (t, *J* = 10.5 Hz, 1H), 1.59-1.56 (m, 2H), 0.91-0.87 (m, 6H).

Step 3D: To a stirred solution (2-((2-fluorophenyl)amino)-2-oxoacetyl)-D-leucine (**3-4**, 0.224 g, 0.757 mmol, 1.0 eq) and (*S*)-3-((*S*)-2-amino-3-oxo-4-(trifluoromethoxy)butyl)pyrrolidin-2-one hydrochloride (compound **20** in main manuscript, 0.22 g, 0.757 mmol, 1.0 eq) in DMF (4 mL),

HATU (0.374 g, 0.983 mmol, 1.3 eq) was added, followed by DIPEA (0.53 mL, 3.028 mmol, 4.0 eq) at 0 °C, and the mixture was stirred at room temperature for 2 h. After completion, water (25 mL) was added to the reaction mixture and extracted with ethyl acetate (3 × 25 mL). The combined organic layer was washed with brine (1 × 25 mL) dried over anhydrous sodium sulfate, concentrated under reduced pressure to give crude residue. The crude product was then purified by flash column chromatography over silica gel (230-400 mesh) to afford N^1-(2-fluorophenyl)-N^2-((*S*)-4-methyl-1-oxo-1-(((*S*)-3-oxo-1-((*S*)-2-oxopyrrolidin-3-yl)-4-(trifluoromethoxy)butan-2-yl)amino)pentan-2-yl)oxalamide **3** (0.15 g, 0.282 mmol, 37%) as an off-white solid. TLC system: EtOAc (100%); R_f: 0.2. Analytical Data: LC MS M/Z = 533.47 (M+1); ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.21 (s, 1H), 9.01 (d, *J* = 8.2 Hz, 1H), 8.58 (d, *J* = 7.6 Hz, 1H), 7.76-7.67 (m, 1H), 7.67 (s, 1H), 7.34-7.21 (m, 3H), 4.98 (q, *J* = 15.9 Hz, 2H), 4.42-4.36 (m, 2H), 3.17-3.12 (m, 2H), 2.49-2.26 (m, 2H), 2.10-2.06 (m, 1H), 1.97-1.90 (m, 1H), 1.96-1.54 (m, 5H), 0.90 (q, *J* = 5.6 Hz, 6H).

Synthesis of (*S*)-5-(2-((2-fluorophenyl)amino)-2-oxoacetyl)-*N*-((*S*)-3-oxo-1-((*S*)-2-oxopyrrolidin-3-yl)-4-(trifluoromethoxy)butan-2-yl)-5-azaspiro[2.4]heptane-6-carboxamide **(4)**



Compound **4** (white solid) was synthesized according to the procedure outlined (main manuscript) for **CMX990**.

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.21 (s, 0.7H), 10.09 (d, *J* = 1.6 Hz, 0.3H), 8.60 (t, *J* = 7.7 Hz, 1H), 7.87 (td, *J* = 7.6, 3.5 Hz, 0.4H), 7.71 – 7.53 (m, 1.6H), 7.40 – 7.06 (m, 3H), 5.23 – 4.86 (m, 3H), 4.56 (dd, *J* = 8.6, 4.4 Hz, 0.4H), 4.44 (ddd, *J* = 11.6, 7.9, 3.8 Hz, 0.4H), 4.35 (ddd, *J* = 11.5, 7.7, 3.6 Hz, 0.6H), 3.92 (d, *J* = 11.5 Hz, 0.4H), 3.82 (d, *J* = 11.5 Hz, 0.4H), 3.62 (d, *J* = 12.2 Hz, 0.6H), 3.22 – 3.00 (m, 1.6H), 2.89 (td, *J* = 9.3, 7.1 Hz, 0.8H), overlap at DMSO signal, 2.35 – 2.08

(m, 2H), 1.98 (ddd, *J* = 13.7, 11.6, 4.1 Hz, 2H), 1.86 – 1.44 (m, 3H), 0.73 – 0.42 (m, 4H); HRMS *m/z* calcd. for $C_{24}H_{27}F_4N_4O_6^+$ $[M+H]^+$ 543.1861, found 543.1788.

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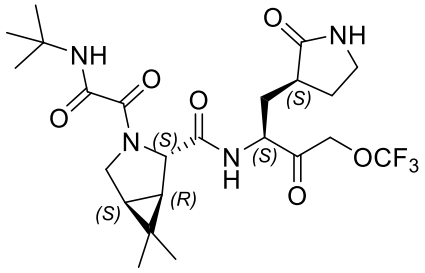
Synthesis of (1*R*,2*S*,5*S*)-3-(2-(*tert*-butylamino)-2-oxoacetyl)-6,6-dimethyl-*N*-((*S*)-3-oxo-1-((*S*)-2-oxopyrrolidin-3-yl)-4-(trifluoromethoxy)butan-2-yl)-3-azabicyclo[3.1.0]hexane-2-carboxamide **(5)**

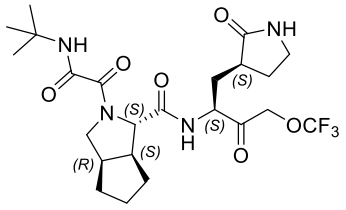


Compound **5** (white solid) was synthesized according to the procedure outlined (main manuscript) for **CMX990**.

^{1}H NMR (500 MHz, DMSO-d_6) δ 8.75 (d, *J* = 7.4 Hz, 0.3H), 8.65 (d, *J* = 7.7 Hz, 0.5H), 7.86 (s, 0.5H), 7.80 (s, 0.3H), 7.68 (s, 0.3H), 7.65 (s, 0.6H), 5.13 – 4.94 (m, 2H), 4.80 (s, 0.6H), 4.45 – 4.34 (m, 1H), 4.22 (s, 0.4H), 3.96 (dd, *J* = 12.0, 5.5 Hz, 0.4H), 3.83 (d, *J* = 12.1 Hz, 0.4H), 3.66 – 3.53 (m, 1.2H), 3.22 – 3.03 (m, 2H), 2.31 – 2.24 (m, 0.3H), 2.19 – 2.10 (m, 1H), 2.02 – 1.93 (m, 1H), 1.70 – 1.60 (m, 2H), 1.55 (dd, *J* = 7.7, 5.4 Hz, 0.4H), 1.48 (d, *J* = 7.6 Hz, 0.6H), 1.39 (dd, *J* = 7.7, 4.8 Hz,

0.6H), 1.33 (d, *J* = 7.7 Hz, 0.4H), 1.30 (overlap, 3H), 1.25 (s, 6H), 1.04 (s, 3H), 0.93 – 0.86 (m, 3H); HRMS *m/z* calcd. for $C_{23}H_{34}F_3N_4O_6^+$ $[M+H]^+$ 519.2425, found 519.8129.

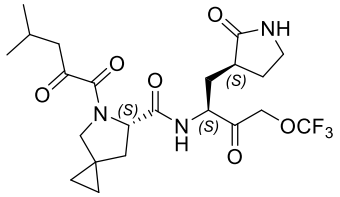
Synthesis of (1S,3a*R*,6a*S*)-2-(2-(*tert*-butylamino)-2-oxoacetyl)-*N*-((*S*)-3-oxo-1-((*S*)-2-oxopyrrolidin-3-yl)-4-(trifluoromethoxy)butan-2-yl)octahydrocyclopenta[*c*]pyrrole-1-carboxamide **(6)**



Compound **6** (white solid) was synthesized according to the procedure outlined (main manuscript) for **CMX990**.

1H NMR (499 MHz, DMSO-d_6) δ 8.67 (d, *J* = 7.5 Hz, 0.4H), 8.57 (d, *J* = 7.7 Hz, 0.6H), 7.91 (s, 0.6H), 7.83 (s, 0.4H), 7.66 (s, 0.4H), 7.63 (s, 0.6H), 5.13-4.90 (m, 2H), 4.70 (d, *J* = 2.3 Hz, 0.4H), 4.35 (tt, *J* = 7.6, 3.5 Hz, 1H), 4.12 (d, *J* = 4.1 Hz, 0.6H), 3.93 (dd, *J* = 11.8, 7.8 Hz, 0.6H), 3.68-3.57 (m, 1.4H), 3.48-3.35 (m, 1H), 3.24-2.98 (m, 2H), 2.76-2.63 (m, 1H), 2.35-2.20 (m, 1H), 2.13 (dt, *J* = 13.0, 7.6 Hz, 1H), 2.05-1.35 (m, 9H), 1.30 (s, 3.6H), 1.26 (s, 5.4H); HRMS *m/z* calcd. for $C_{23}H_{34}F_3N_4O_6^+$ $[M+H]^+$ 519.2425, found 519.0526.

Synthesis of (*S*)-5-(4-methyl-2-oxopentanoyl)-*N*-((*S*)-3-oxo-1-((*S*)-2-oxopyrrolidin-3-yl)-4-(trifluoromethoxy)butan-2-yl)-5-azaspiro[2.4]heptane-6-carboxamide **(7)**

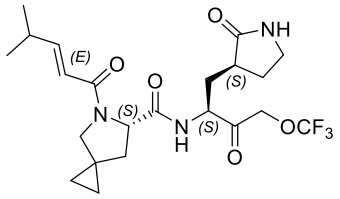


Compound **7** (white solid) was synthesized according to the procedure outlined (main manuscript) for **CMX990**.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.65 (d, *J* = 7.6 Hz, 0.2H), 8.53 (d, *J* = 7.9 Hz, 0.2H), 7.68 (d, *J* = 13.2 Hz, 0.6H), 5.11 – 4.55 (m, 2H), 4.50 – 4.31 (m, 1H), 4.24 – 3.82 (m, 0.4H), 3.68 – 3.40 (m, 1.6H), 3.22 – 3.05 (m, 2H), 2.81 – 2.61 (m, 2H), 2.42 – 1.89 (m, 5H), 1.87 – 1.74 (m, 1H), 1.73 –

1.36 (m, 3H), 0.98 – 0.79 (m, 6H), 0.70 – 0.39 (m, 4H); HRMS *m/z* calcd. for $C_{22}H_{31}F_3N_3O_6^+$ $[M+H]^+$ 490.2159, found 490.1619.

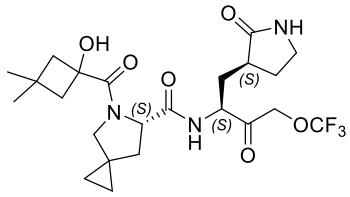
Synthesis of (*S*)-5-((*E*)-4-methylpent-2-enoyl)-*N*-((*S*)-3-oxo-1-((*S*)-2-oxopyrrolidin-3-yl)-4-(trifluoromethoxy)butan-2-yl)-5-azaspiro[2.4]heptane-6-carboxamide **(8)**



Compound **8** (white solid) was synthesized according to the procedure outlined (main manuscript) for **CMX990**.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.76 – 8.32 (m, 0.5H), 7.90 – 7.33 (m, 1H), 6.85 – 6.58 (m, 1H), 6.09 (dd, *J* = 15.1, 1.5 Hz, 1H), 5.91 (t, *J* = 14.1 Hz, 0.4H), 5.16 – 4.93 (m, 1H), 4.85 (d, *J* = 17.3 Hz, 0.5H), 4.76 – 4.61 (m, 0.4H), 4.55 – 4.29 (m, 1.5H), 3.97 – 3.69 (m, 1H), 3.64 – 3.44 (m, 2H), 3.22 – 2.95 (m, 2H), 2.27 – 2.05 (m, 3H), 2.03 – 1.91 (m, 1H), 1.83 – 1.45 (m, 3H), 1.08 – 0.91 (m, 6H), 0.63 – 0.41 (m, 4H); HRMS *m/z* calcd. for $C_{22}H_{31}F_3N_3O_5^+$ $[M+H]^+$ 474.2210, found 474.2299.

Synthesis of (*S*)-5-(1-hydroxy-3,3-dimethylcyclobutane-1-carbonyl)-*N*-((*S*)-3-oxo-1-((*S*)-2-oxopyrrolidin-3-yl)-4-(trifluoromethoxy)butan-2-yl)-5-azaspiro[2.4]heptane-6-carboxamide **(9)**

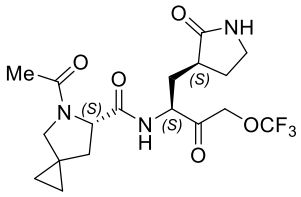


Compound **9** (white solid) was synthesized according to the procedure outlined (main manuscript) for **CMX990**.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.50 – 8.41 (m, 0.8H), 7.69 (s, 0.7H), 5.78 – 5.63 (m, 0.6H), 5.09 – 4.80 (m, 2H), 4.42 – 4.26 (m, 2H), 3.65 – 3.46 (m, 1.6H), 3.23 – 3.05 (m, 2.6H), 2.46 – 2.23 (m, 3H), 2.21 – 2.06 (m, 1H), 1.97 (qd, *J* = 8.5, 4.3 Hz, 2H), 1.90 – 1.53 (m, 5H), 1.20 – 1.11 (m, 3H),

0.98 (s, 0.5H), 0.93 (s, 3H), 0.64 – 0.35 (m, 4H); HRMS *m/z* calcd. for $C_{23}H_{33}F_3N_3O_6^+$ $[M+H]^+$ 504.2316, found 504.6798.

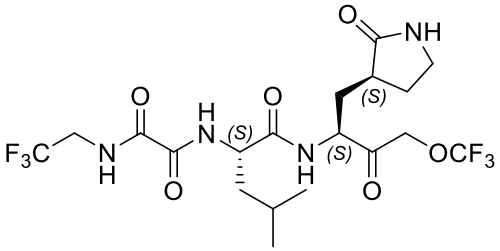
Synthesis of (*S*)-5-acetyl-*N*-((*S*)-3-oxo-1-((*S*)-2-oxopyrrolidin-3-yl)-4-(trifluoromethoxy)butan-2-yl)-5-azaspiro[2.4]heptane-6-carboxamide **(10)**



Compound **10** (waxy solid) was synthesized according to the procedure outlined (main manuscript) for **CMX990**, where simple acylation of P2-NH was done instead of a more elaborated *N*-cap.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.74 (d, *J* = 7.3 Hz, 0.3H), 8.46 (d, *J* = 7.8 Hz, 0.3H), 7.79 – 7.28 (m, 1H), 6.23 – 5.78 (m, 0.4H), 5.03 (d, *J* = 17.2 Hz, 1H), 4.84 (d, *J* = 17.4 Hz, 0.6H), 4.59 – 4.28 (m, 2H), 4.03 – 3.67 (m, 0.7H), 3.59 – 3.40 (m, 1.3H), 3.22 – 3.00 (m, 2H), 2.29 – 2.05 (m, 5H), 2.04 – 1.78 (m, 4H), 1.76 – 1.36 (m, 3H), 0.73 – 0.35 (m, 4H); HRMS *m/z* calcd. for $C_{18}H_{25}F_3N_3O_5^+$ $[M+H]^+$ 420.1741, found 420.5356.

Synthesis of N^1-((*S*)-4-methyl-1-oxo-1-(((*S*)-3-oxo-1-((*S*)-2-oxopyrrolidin-3-yl)-4-(trifluoromethoxy)butan-2-yl)amino)pentan-2-yl)-N^2-(2,2,2-trifluoroethyl)oxalamide **(11)**

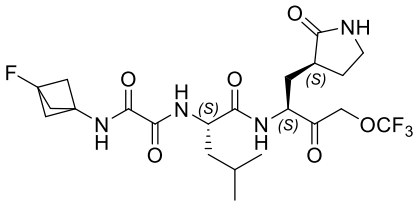


Compound **11** (off-white solid) was synthesized according to the procedure outlined for compound **3**.

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.38 (t, *J* = 6.7 Hz, 0.5H), 8.86 (d, *J* = 8.4 Hz, 0.5H), 8.59 (d, *J* = 7.6 Hz, 1H), 7.68 (s, 1H), 4.99 (d, *J* = 17.2 Hz, 1H), 4.94 (d, *J* = 17.1 Hz, 1H), 4.46 – 4.38 (m, 1H), 4.37 – 4.28 (m, 1H), 4.02 – 3.84 (m, 2H), 3.22 – 3.03 (m, 2H), 2.26 (tt, *J* = 10.0, 4.9 Hz, 1H), 2.15 – 2.03

(m, 1H), 2.01 – 1.90 (m, 1H), 1.78 – 1.47 (m, overlapped 5H), 0.89 (d, *J* = 6.1 Hz, 3H), 0.85 (d, *J* = 6.0 Hz, 3H); HRMS *m/z* calcd. for $C_{19}H_{27}F_6N_4O_6^+$ $[M+H]^+$ 521.1829, found 521.6230.

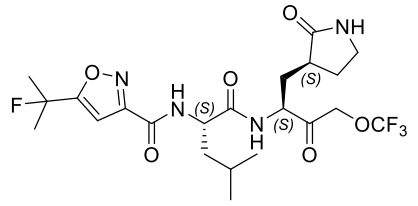
Synthesis of N^1-(3-fluorobicyclo[1.1.1]pentan-1-yl)-N^2-((*S*)-4-methyl-1-oxo-1-(((*S*)-3-oxo-1-((*S*)-2-oxopyrrolidin-3-yl)-4-(trifluoromethoxy)butan-2-yl)amino)pentan-2-yl)oxalamide **(12)**



Compound **12** (off-white solid) was synthesized according to the procedure outlined for compound **3**.

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.56 (s, 1H), 8.70 (d, *J* = 8.4 Hz, 1H), 8.52 (d, *J* = 7.7 Hz, 1H), 7.67 (s, 1H), 4.98 (d, *J* = 17.0 Hz, 1H), 4.92 (d, *J* = 17.1 Hz, 1H), 4.41 (ddd, *J* = 11.4, 7.7, 3.9 Hz, 1H), 4.30 (ddd, *J* = 10.2, 8.4, 3.7 Hz, 1H), 3.23 – 3.04 (m, 2H), 2.36 (d, *J* = 2.2 Hz, 6H), 2.25 (qd, *J* = 10.1, 4.0 Hz, 1H), 2.13 – 2.01 (m, 1H), 2.01 – 1.89 (m, 1H), 1.76 – 1.57 (m, 3H), 1.57 – 1.45 (m, 2H), 0.88 (d, *J* = 5.9 Hz, 3H), 0.84 (d, *J* = 5.7 Hz, 3H); HRMS *m/z* calcd. for $C_{22}H_{31}F_4N_4O_6^+$ $[M+H]^+$ 523.2174, found 523.4171.

Synthesis of 5-(2-fluoropropan-2-yl)-*N*-((*S*)-4-methyl-1-oxo-1-(((*S*)-3-oxo-1-((*S*)-2-oxopyrrolidin-3-yl)-4-(trifluoromethoxy)butan-2-yl)amino)pentan-2-yl)isoxazole-3-carboxamide **(13)**

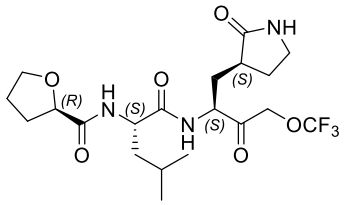


Compound **13** (off-white solid) was synthesized analogous to the procedure outlined for compound **3**.

^{1}H NMR (499 MHz, DMSO-d_6) δ 8.88 (d, *J* = 7.7 Hz, 1H), 8.59 (d, *J* = 7.7 Hz, 1H), 7.65 (s, 1H), 6.97 (d, *J* = 1.9 Hz, 1H), 5.00 (d, *J* = 17.0 Hz, 1H), 4.93 (d, *J* = 17.0 Hz, 1H), 4.49 – 4.37 (m, 2H), 3.20 – 3.06 (m, 2H), 2.32 – 2.04 (m, 2H), 1.97 (ddd, *J* = 13.9, 11.1, 4.2 Hz, 1H), 1.79 (s, 3H), 1.74 (s, 3H),

1.72 – 1.48 (m, 4H), 0.92 (d, *J* = 6.5 Hz, 3H), 0.88 (d, *J* = 6.5 Hz, 3H); HRMS *m/z* calcd. for $C_{22}H_{31}F_4N_4O_6^+$ $[M+H]^+$ 523.2174, found 523.1797.

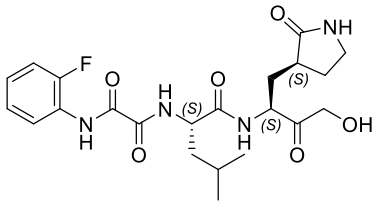
Synthesis of (*R*)-*N*-((*S*)-4-methyl-1-oxo-1-(((*S*)-3-oxo-1-((*S*)-2-oxopyrrolidin-3-yl)-4-(trifluoromethoxy)butan-2-yl)amino)pentan-2-yl)tetrahydrofuran-2-carboxamide **(14)**



Compound **14** (off-white solid) was synthesized analogous to the procedure outlined for compound **3**.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.57 (d, *J* = 7.4 Hz, 1H), 7.75 – 7.66 (m, 2H), 4.98 (s, 2H), 4.45 – 4.35 (m, 1H), 4.34 – 4.18 (m, 2H), 3.92 (dt, *J* = 7.7, 6.3 Hz, 1H), 3.77 (dt, *J* = 7.8, 6.3 Hz, 1H), 3.22 – 3.04 (m, 2H), 2.30 – 2.19 (m, 1H), 2.17 – 2.04 (m, 2H), 2.02 – 1.91 (m, 1H), 1.89 – 1.75 (m, 3H), 1.72 – 1.42 (m, 5H), 0.91 (d, *J* = 6.0 Hz, 3H), 0.86 (d, *J* = 6.1 Hz, 3H); HRMS *m/z* calcd. for $C_{20}H_{31}F_3N_3O_6^+$ $[M+H]^+$ 466.2159, found 466.3391.

Synthesis of N^1-(2-fluorophenyl)-N^2-((*S*)-1-(((*S*)-4-hydroxy-3-oxo-1-((*S*)-2-oxopyrrolidin-3-yl)butan-2-yl)amino)-4-methyl-1-oxopentan-2-yl)oxalamide **(15)**

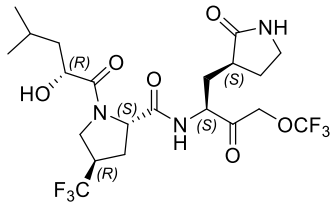


Compound **15** (off-white solid) was synthesized analogous to the procedure outlined for compound **3**, using the hydroxymethyl ketone warhead described in literature [1].

^{1}H NMR (499 MHz, DMSO-d_6) δ 10.27 (s, 1H), 8.87 (d, *J* = 8.6 Hz, 1H), 8.48 (d, *J* = 7.9 Hz, 1H), 7.70 (td, *J* = 7.9, 1.8 Hz, 1H), 7.64 (s, 1H), 7.39 – 7.19 (m, 3H), 5.10 (t, *J* = 5.9 Hz, 1H), 4.53 – 4.35 (m, 2H), 4.24 (dd, *J* = 18.7, 6.1 Hz, 1H), 4.14 (dd, *J* = 18.6, 5.9 Hz, 1H), 3.20 – 3.07 (m, 2H), 2.33 – 2.25 (m, 1H), 2.15 – 2.08 (m, 1H), 1.92 (ddd, *J* = 13.8, 11.3, 4.1 Hz, 1H), 1.80 – 1.49 (m, 5H), 1.30

– 1.22 (m, 1H), 0.91 (d, *J* = 6.2 Hz, 3H), 0.89 (d, *J* = 6.1 Hz, 3H); HRMS *m/z* calcd. for $C_{22}H_{30}FN_4O_6^+$ $[M+H]^+$ 465.2144, found 465.3612.

Synthesis of (2*S*,4*R*)-1-((*R*)-2-hydroxy-4-methylpentanoyl)-*N*-((*S*)-3-oxo-1-((*S*)-2-oxopyrrolidin-3-yl)-4-(trifluoromethoxy)butan-2-yl)-4-(trifluoromethyl)pyrrolidine-2-carboxamide **(16)**



Compound **16** was synthesized according to the procedure outlined (main manuscript) for **CMX990**.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.79 (d, *J* = 7.2 Hz, 0.3H), 8.68 (d, *J* = 7.7 Hz, 0.7H), 7.74 (s, 0.3H), 7.66 (s, 0.7H), 5.30 – 4.80 (m, 3H), 4.45 – 4.38 (m, 1H), 4.38 – 4.30 (m, 0.7H), 4.21 – 4.12 (m, 0.7H), 4.02 – 3.89 (m, 1H), 3.81 – 3.72 (m, 0.7H), 3.60 (d, *J* = 8.1 Hz, 0.5H), 3.21 – 3.05 (m, 2H), 2.62 – 2.54 (m, overlap to DMSO), 2.43 – 2.17 (m, 2H), 2.17 – 1.84 (m, 3H), 1.82 – 1.53 (m, 3H), 1.49 – 1.37 (m, 1H), 1.35 – 1.19 (m, 2H), 0.96 – 0.78 (m, 6H); HRMS *m/z* calcd. for $C_{21}H_{30}F_6N_3O_6^+$ $[M+H]^+$ 534.2033, found 534.3318.

2. CMX990 Single Crystal Structure Information

Empirical formula	$C_{22}H_{32}F_3N_3O_6$
Formula weight	491.50
Temperature	294(2) K
Wavelength	0.71073 Å
Crystal system	Monoclinic
Space group	$P2_1$
Unit cell dimensions	a = 6.26460(10) Å α = 90° b = 21.3342(4) Å β = 93.1118(7)° c = 9.3051(2) Å ɣ = 90°
Volume	1241.80(4) $Å^3$
Z	2
Density (calculated)	1.314 Mg/m^3
Absorption coefficient	0.110 mm^{-1}
F(000)	520
Crystal size	0.260 x 0.240 x 0.180 mm^3
Θ range for data collection	2.391 to 30.630°
Index ranges	-8<=h<=8, -30<=k<=30, -13<=l<=13
Reflections collected	45011
Independent reflections	7558 [R(int) = 0.0449]
Completeness to θ = 25.242°	98.3 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.7461 and 0.6922
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	7558 / 177 / 367
Goodness-of-fit on F^2	1.028
Final R indices [I>2σ(I)]	R1 = 0.0476, wR2 = 0.1196
R indices (all data)	R1 = 0.0672, wR2 = 0.1330
Absolute structure parameter	-0.19(18)
Largest diff. peak and hole	0.223 and -0.158 e.$Å^{-3}$
Measurement	Bruker D8 QUEST PHOTON-III Detector
Software Used	SHELXTL-PLUS

3. *In Vitro* Biology

The following viral strains were obtained through BEI Resources, NIAID, NIH: Isolate HCOV-19 USA-WA1/2020 (NR-52281), Isolate hCoV-19/USA/OR-OHSU-PHL00037/2021 (Alpha Variant, NR- 55461), Isolate HCOV-19/USA/PHC658/2021 (Delta Variant, NR-55691), isolate HCOV-19/USA/MD-HP20874/2021 (Omicron Variant, NR-56461), Human Coronavirus, OC43 (NR-52725), and Human Coronavirus, 229E (NR-52726). The SARS-CoV-2 assays were conducted as described previously [2]. HCoV-OC43 was propagated in HCT-8 cells (ATCC CCL-244) and compound activity was assayed in a high-content imaging assay after a 48 hr incubation using the mouse monoclonal antibody OC-43 strain clone 541-8F (Sigma Millipore MAB9012) and goat anti-mouse H+L conjugated Alexa 488 secondary (Thermo Fisher Scientific A11001). HCoV-229E was propagated in MRC-5 pd25 cells (Sigma cat # 05081101-1VL) and compound activity determined by assessing cell viability at 120 hr post-infection using CellTiter-Glo® (Promega No G7573). The uninfected cytotoxicity counter screens were incubated in parallel with the antiviral assays and cell viability assessed using CellTiter-Glo®.

SARS-CoV-2 M^{pro} Enzymatic Assay

Direct inhibition of the SARS-CoV-2 3CL protease was evaluated in a biochemical assay with 50 nM recombinant SARS-CoV-2 3CLpro and 15 µM fluorogenic substrate (Dabcyl-TSAVLQSGFRK-Glu(EDANS)) in a buffer of 50 mM Tris-HCl, pH 7.3, 1 mM EDTA, 0.01% BSA, and 0.001% Triton. SARS-CoV-2 3CLpro dissolved in 25 µL assay buffer was mixed with 12-point serial dilutions of compound. The mixture was incubated at 37°C for 30 min before addition of the substrate. The fluorescence signal at 350 nm (excitation)/490 nm (emission) was immediately measured every 2 min for 10 min with SpectraMax® M5 multi-mode microplate reader at 37°C. RFU at the 6th min of reactions with compound at various concentrations compared to the reaction with the lowest concentration were used to generate IC_{50} curves.

4. DMPK Assays

Microsomal stability and plasma protein binding studies

Studies were carried out at WuXi AppTec (Shanghai, China). For LC/MS/MS analysis, test and control compounds were quantified using peak area ratio of analyte and internal standard. The metabolic stability in liver microsomes was determined using the compound depletion approach and quantified by LC/MS/MS. Human, mouse, rat, dog, or cyno microsomes were used (BD Gentest). The rate and extent of metabolism was determined by the disappearance of the parent compound, allowing for the determination of in vitro half-life (t1/2), intrinsic clearance (Clint), and the extraction ratio (ER) in various species. Protein binding in plasma was determined using equilibrium dialysis. Compounds were tested at 2 µM in triplicate, and concentrations were quantified by LC/MS/MS.

Hepatocyte stability studies

Studies were carried out at Aragen Life Sciences (India). Stability of test article (1 µM) was monitored at 0, 15, 30, 60, 90, and 120 min in the presence of hepatocytes (source = Thermo Fisher Scientific, cell density = 0.4 million/mL), and the data (%Remaining, half-life, Clearance) generated in duplicate (analysis = LCMS/MS quantification of test article using an internal standard).

hERG manual patch clamp assay

Compounds were tested for effect on hERG potassium channels using the whole-cell patch clamp technique with a Multiclamp 700 patch-clamp amplifier (Molecular Devices, USA) at

WuXi AppTec (Shanghai, China). CHO cells stably expressing hERG potassium channels from Aviva Biosciences (San Diego, CA) were tested with compounds at five concentrations, in a three-fold serial dilution starting at 30 µM, compared to vehicle (negative) control and amitriptyline (WuXi AppTec, Shanghai, China) (positive) controls. Percentage of control (vehicle) values were calculated in duplicate for each concentration of drug, and data analysis was performed using Clampfit (V10.7, Molecular Deveices) and GraphPad Prism.

Mini-Ames

Mini-Ames study was done at WuXi AppTec (Shanghai, China) to evaluate the test article **CMX990**, for its ability to induce reverse mutations both in the presence and absence of S9 mix at the histidine locus in the genome of four strains of *Salmonella typhimurium* (TA98, TA100, TA1535, and TA97a) and at the tryptophan locus in the genome of *Escherichia coli* WP2 uvrA (pKM101). Assay was conducted in the presence and absence of S9 mix along with the concurrent negative/solvent control (DMSO) using six wells and positive controls using three wells. The tested dose levels in the mutagenicity assay with five tester strains in the presence and absence of S9 mix were 1.5, 4, 10, 25, 64, 160, 400, and 1000 µg per well, three wells per dose. The study was conducted using fresh cultures of the bacterial strains and fresh test article formulations.

***In vitro* micronucleus induction**

In vitro microwell micronucleus screening assay in Chinese hamster ovary cells (CHO-WBL, Merck Research Laboratories, USA) was carried out at WuXi AppTec (Shanghai, China). Clastogenicity/aneugenicity was measured by the extent of micronucleus formation with and

without exogenous metabolic activation (Aroclor 1254 induced rat liver S9, Molecular Toxicology (Boone, NC). Cultures of CHO-WBL cells in Microwell 8-well chamber slides (Thermo Fisher Scientific Inc.) were exposed in duplicate to multiple concentrations of test article as well as to positive (cyclophosphamide monohydrate, mitomycin C) and solvent controls. In the S9 activated test system (test article concentrations: 50, 100, 245, 491.5 µg/mL), exposure was for 3 h; in the non-activated test system (test article concentrations: 50, 100, 240, 260, 280, 300, 320, 340, 360, 380, 400, 420, 440, 460, 480 µg/mL), treatment was for 3 h and for 24 h. Cells were fixed and stained with acridine orange, and 2000 binucleated cells (1000 binucleated cells/culture) were scored for each test and control article concentration.

Direct and Time-Dependent Inhibition of Cytochrome P450 Enzymes in Human Liver Microsomes

CMX990 was tested (WuXi AppTec, China) over a test concentration range of 0.00545 to 50.0 µM in pooled human liver microsomes (0.1 mg protein/mL, mixed gender) for inhibition of human cytochrome (CYP) P450 isozymes (CYPs 1A2, 2B6, 2C8, 2C9, 2C19, 2D6 and 3A4). Assays (n=2) were performed in 0.1 M potassium phosphate buffer (pH 7.4), with a pre-incubation time of 0 and 30 minutes at 37°C with and without NADPH (1 mM). Bioanalysis was conducted by LC-MS/MS.

B/P ratio

The ratio of **CMX990** (incubation concentration = 1 µM) in whole blood over plasma ($K_{B/P}$), the respective drug concentrations in the erythrocytes to plasma ($K_{E/P}$), and % recovery in blood

were measured in assays (controls = diclofenac, chloroquine, chlorthalidone) conducted at WuXi AppTec, China.

***In vitro* safety profiling assays**

The Eurofins SafetyScreen44™ Panel (Cerep) was used to assess the off-target effects of **CMX990** at a single concentration of 10 µM. Electrophysiological assays (cardiac panel) were conducted to profile the compound for activities on the ion channel targets (voltage-Gated Sodium: HEK-Nav1.5, voltage-Gated Calcium: HEK-Cav1.2, voltage-Gated Potassium: CHO-hERG) using the QPatch electrophysiological platform at Eurofins Panlabs Inc.

In vitro peptidase selectivity panel was executed at Eurofins Panlabs Inc. against a panel of ~30 mammalian serine and cysteine peptidases to assess potential off-target pharmacology that might lead to toxicity. Test compound initially was measured against the specified enzymes at a single concentration (10 µM) to determine % inhibition relative to controls; assays were performed in duplicate. Follow-up IC_{50} analysis was conducted if the % inhibition at 10 µM was greater than 50% using 5 concentrations (10, 3, 1, 0.3, and 0.1 µM) of the test compound to determine IC_{50} if possible.

***In vivo* assays**

Animal Study: Animal Care and Compound Application.

Animal experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the respective study locations. Pharmacokinetic studies were conducted at Aragen (India), Calibr (San Diego, CA), or Pharmaron Inc. (China)

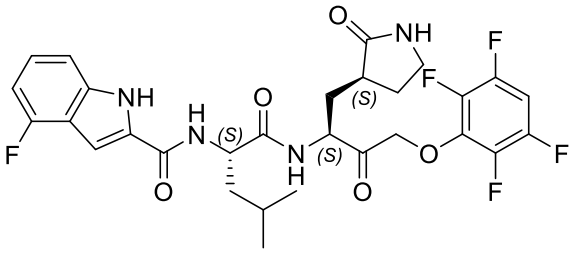
Pharmacokinetics in animals [CD1-mouse, SD-rat, Syrian hamster, beagle dog, or cynomolgus monkeys]

For all (IV or PO) reported pharmacokinetic studies in this manuscript, three fasted animals per study group were administered the test article as a solution in 50% PEG 300, 10% ethanol, and 40% saline. Blood samples were collected at 0.083, 0.5, 1, 3, 5, 8, and 24 hr post-doing for IV studies; and at 0.5, 1, 3, 5, 8, and 24 hr post-dosing for PO studies.

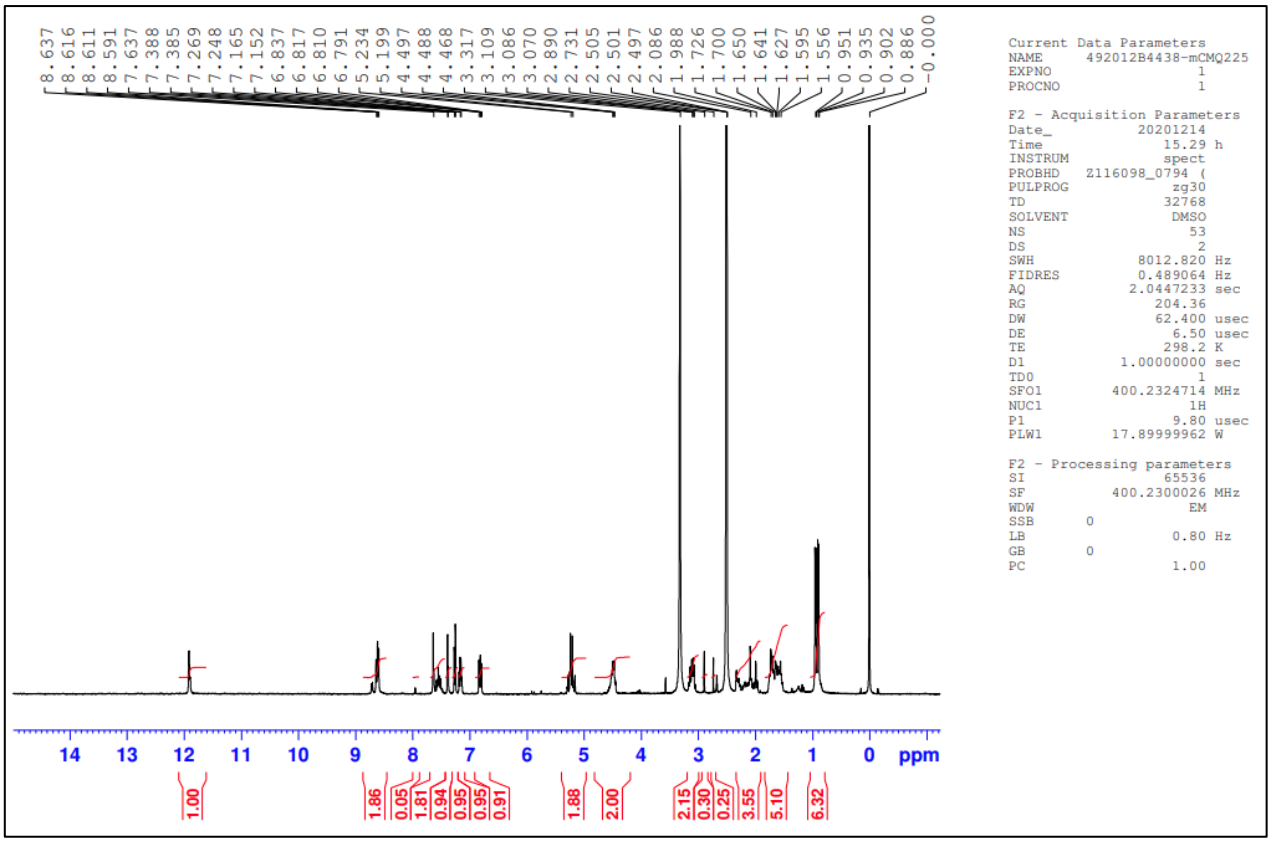
The blood samples were centrifuged to obtain the plasma, which was stored below -20°C until analysis. Plasma concentrations were determined by liquid chromatography/tandem mass spectrometry (LC−MS/MS). The PK parameters were determined by non-compartmental methods using WinNonLin (v6.1 or higher version, Certara Inc.).

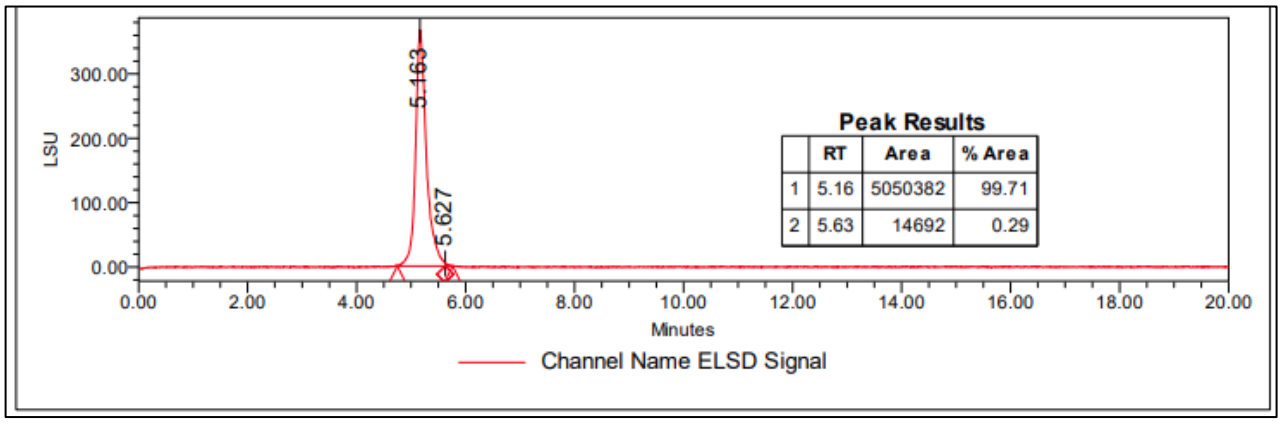
5. Characterization data for Compounds 1-16

Compound #1: ^{1}H NMR and HPLC Chromatogram

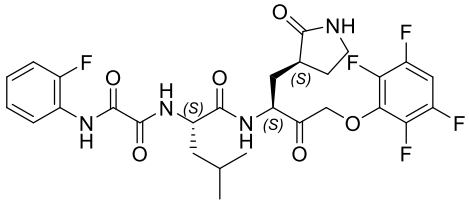


Chemical Formula: $C_{29}H_{29}F_5N_4O_5$
Exact Mass: 608.2058
Molecular Weight: 608.5660

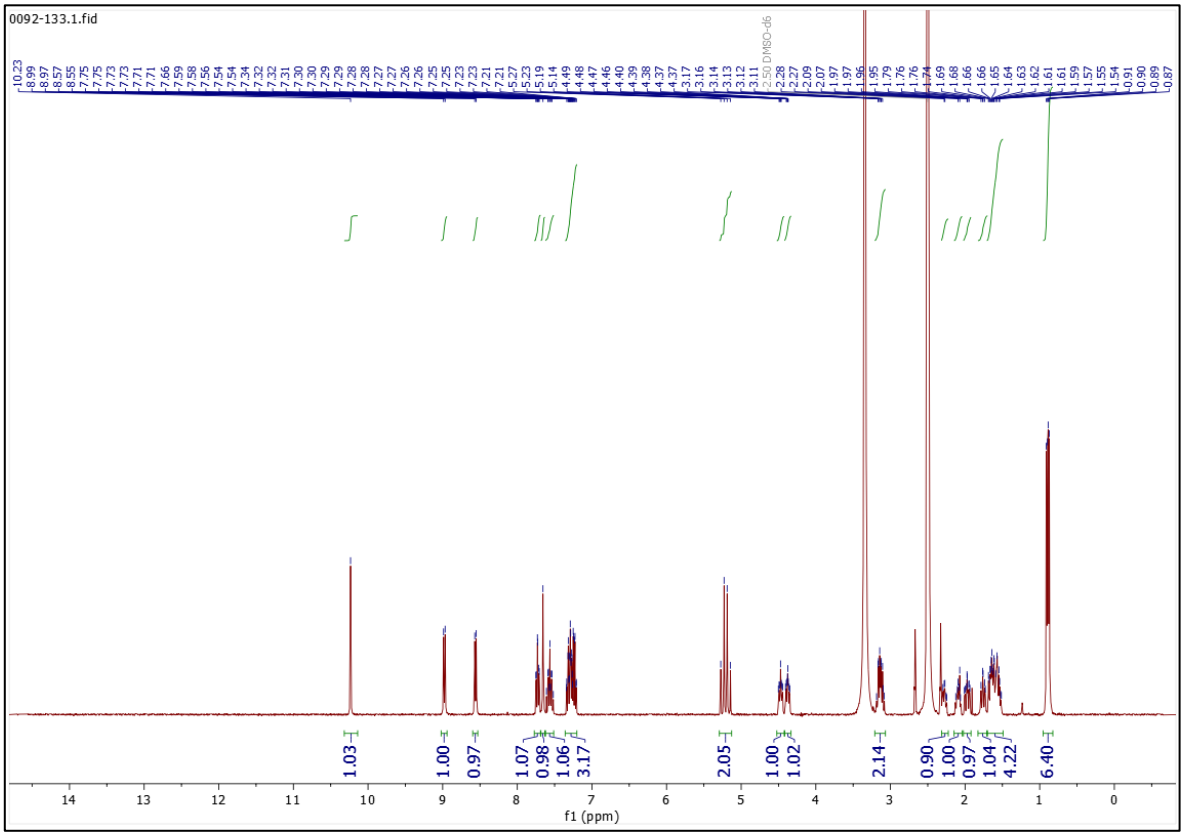


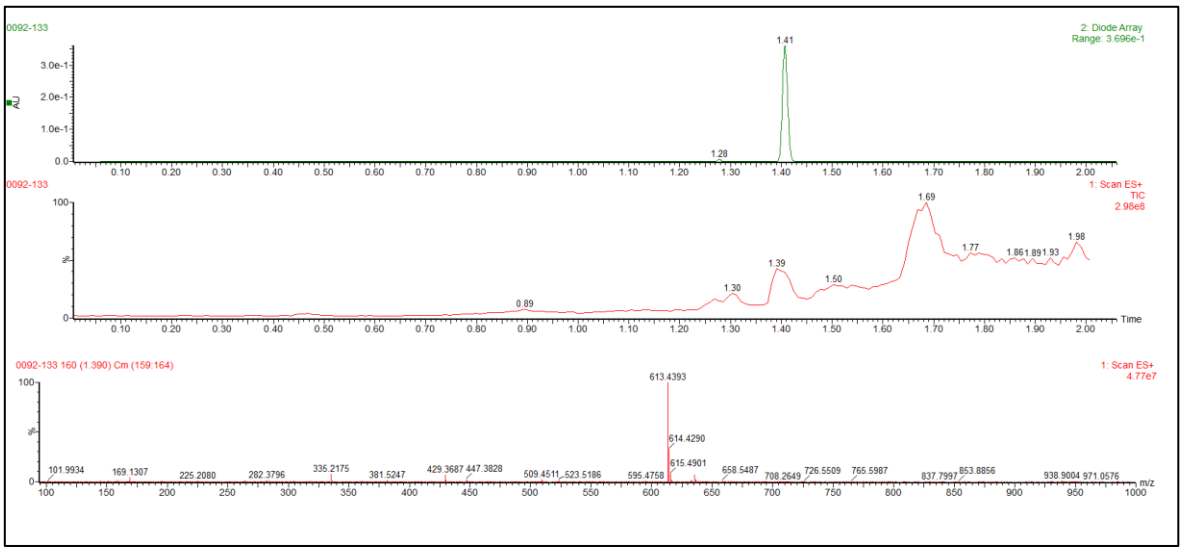


Compound #2: ^{1}H NMR and LCMS Spectra

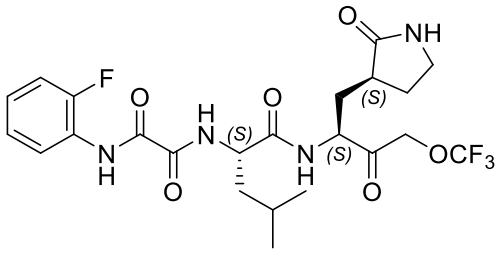


Chemical Formula: $C_{28}H_{29}F_5N_4O_6$
Exact Mass: 612.2007
Molecular Weight: 612.5540

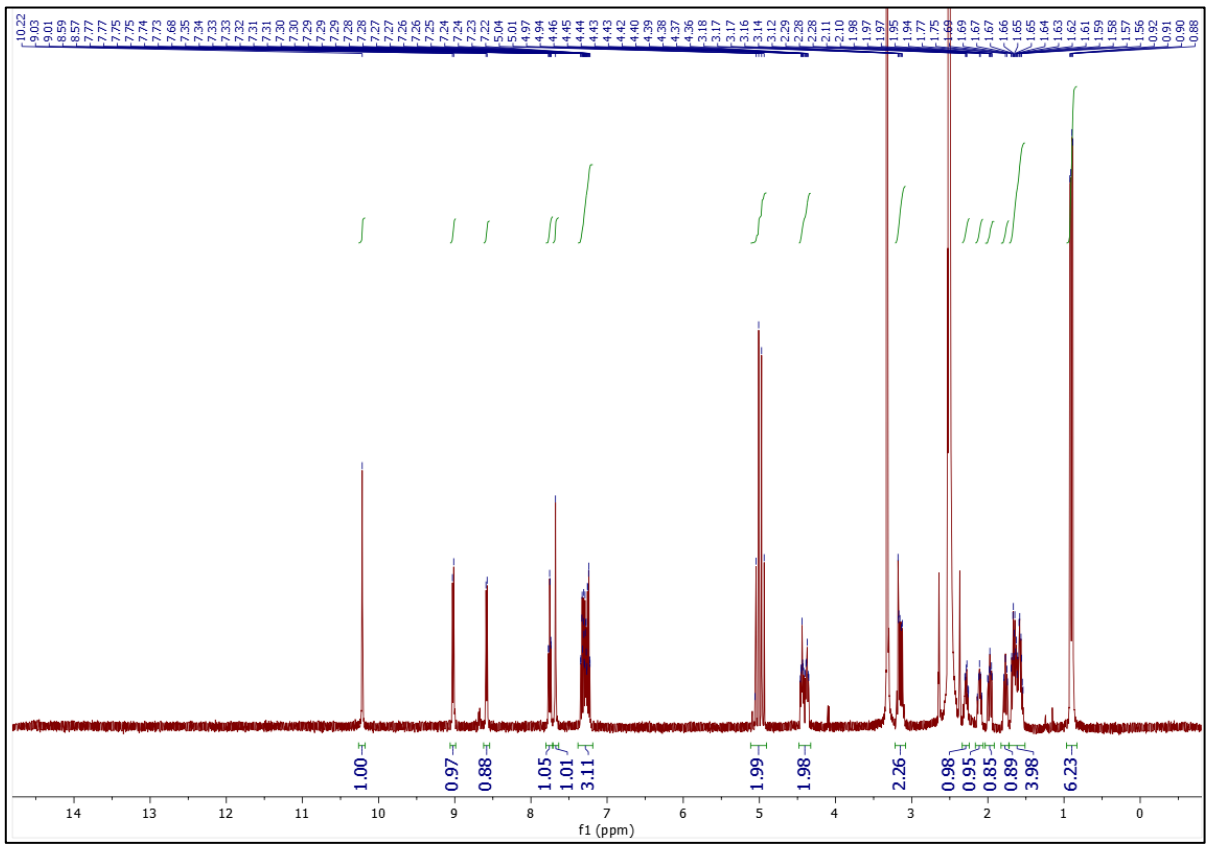


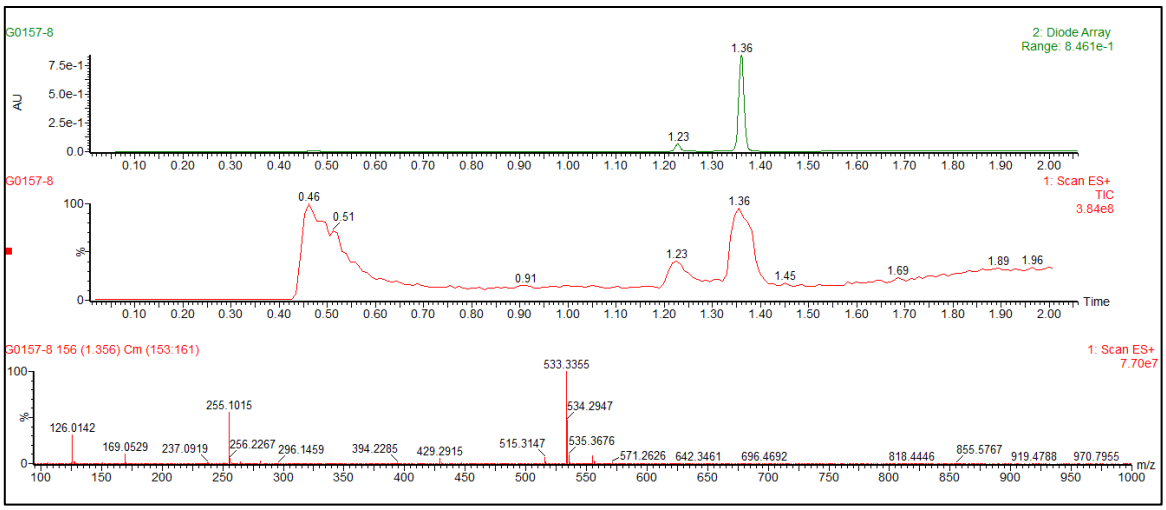


Compound #3: ^{1}H NMR and LCMS Spectra

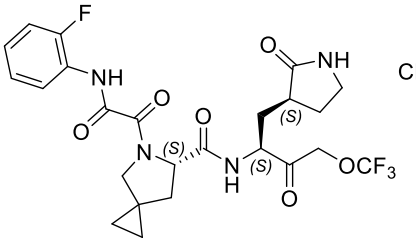


Chemical Formula: $C_{23}H_{28}F_4N_4O_6$
Exact Mass: 532.1945
Molecular Weight: 532.4926

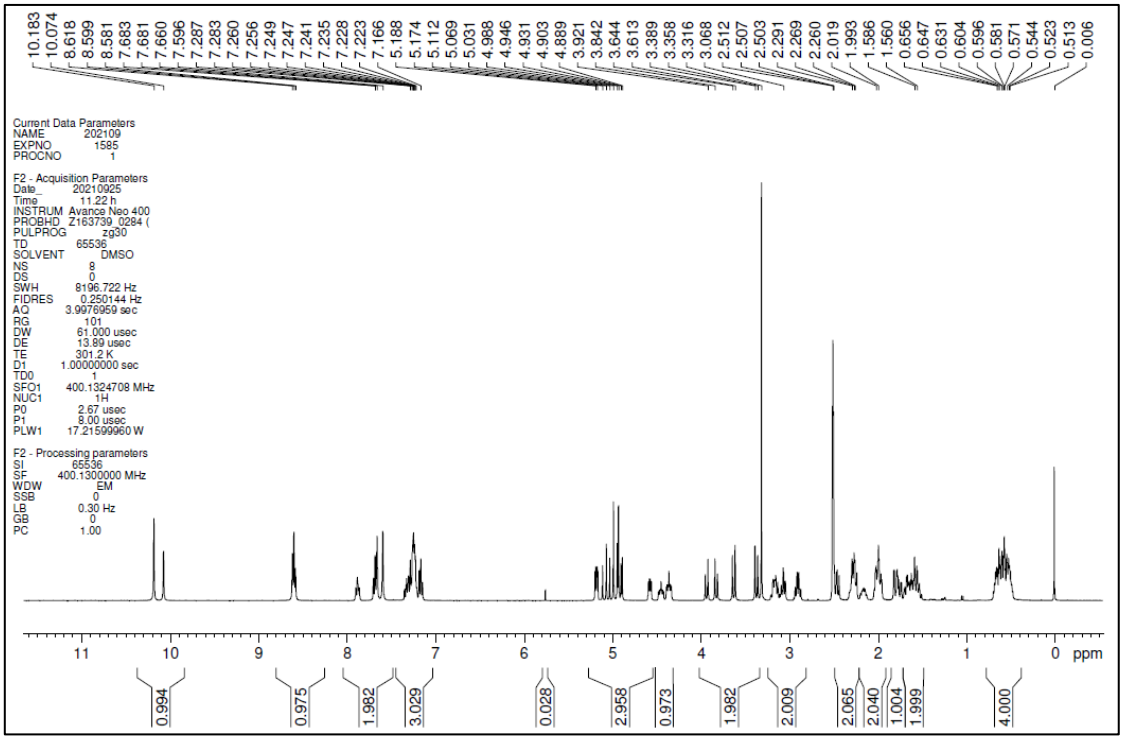


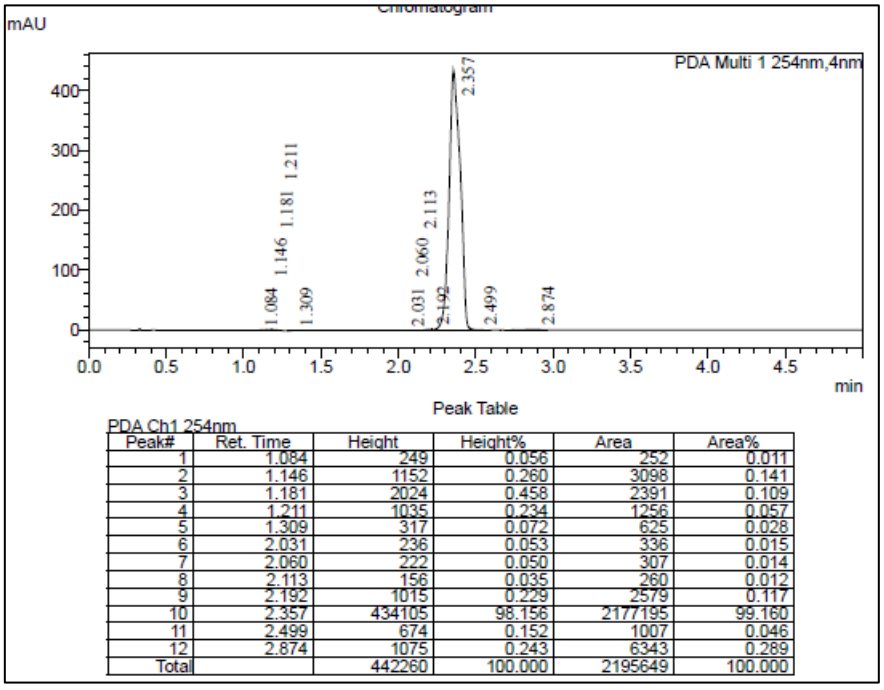


Compound #4: ^{1}H NMR and HPLC Chromatogram



Chemical Formula: $C_{24}H_{26}F_4N_4O_6$
Exact Mass: 542.1788
Molecular Weight: 542.4876



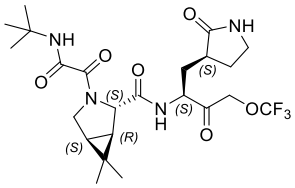


Peak Table

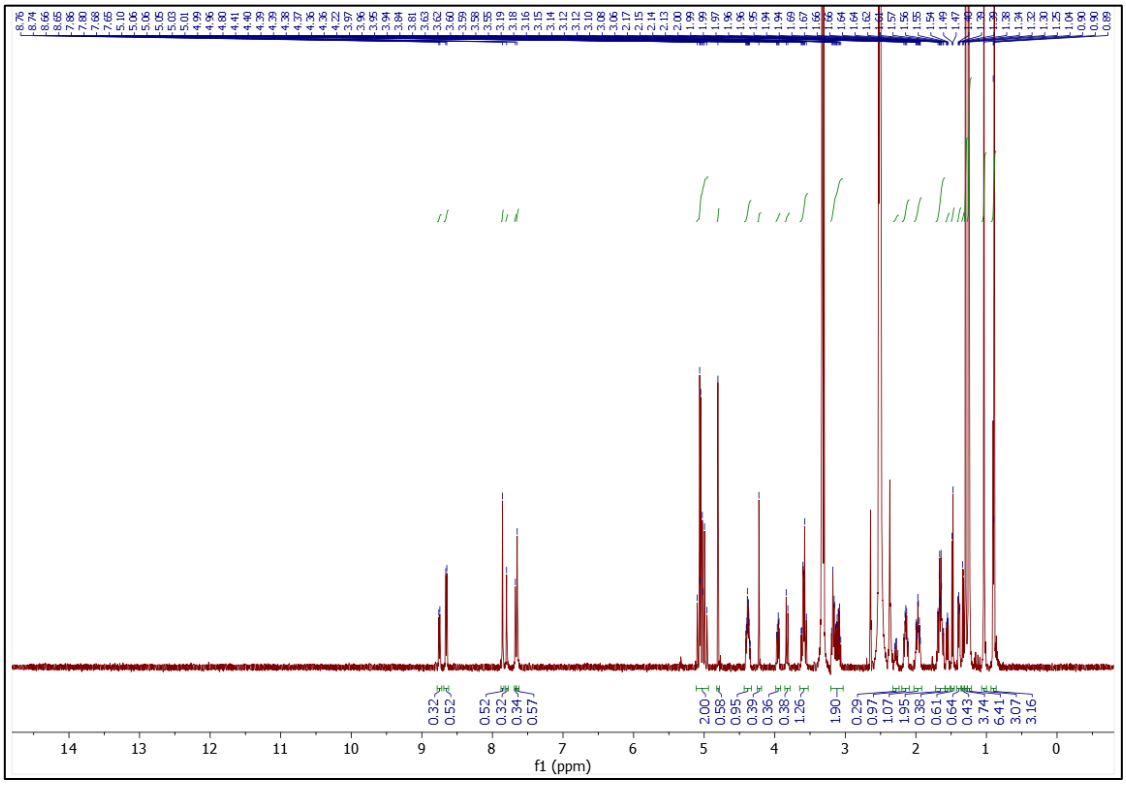
PDA Ch1 254nm

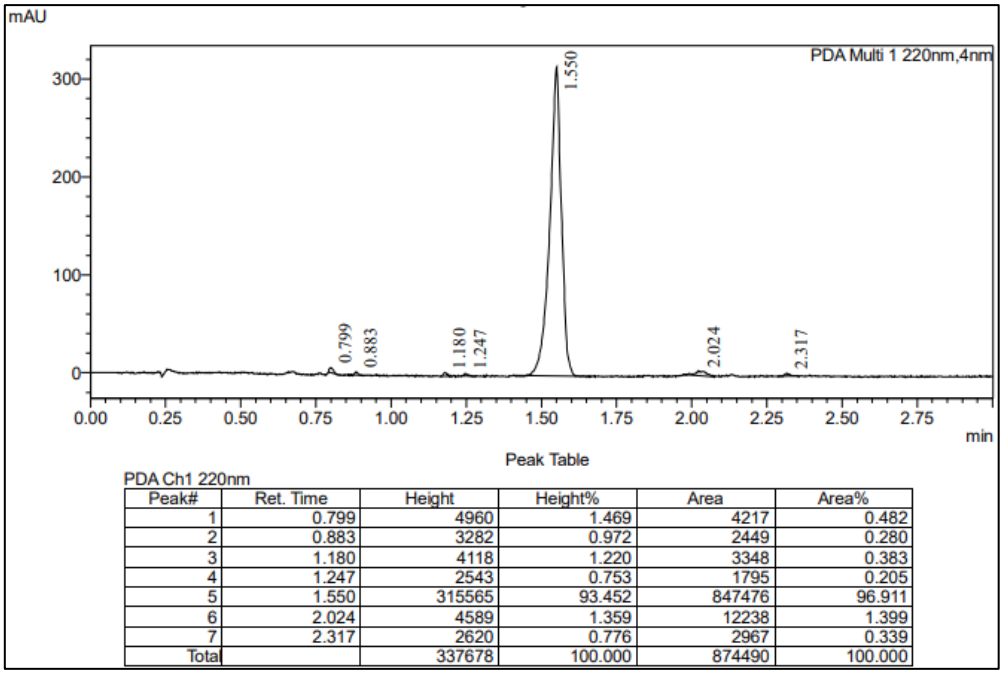
Peak#	Ret. Time	Height	Height%	Area	Area%
1	1.084	249	0.056	252	0.011
2	1.146	1152	0.260	3098	0.141
3	1.181	2024	0.458	2391	0.109
4	1.211	1035	0.234	1256	0.057
5	1.309	317	0.072	625	0.028
6	2.031	236	0.053	336	0.015
7	2.060	222	0.050	307	0.014
8	2.113	156	0.035	260	0.012
9	2.192	1015	0.229	2579	0.117
10	2.357	434105	98.156	2177195	99.160
11	2.499	674	0.152	1007	0.046
12	2.874	1075	0.243	6343	0.289
Total		442260	100.000	2195649	100.000

Compound #5: ^{1}H NMR and HPLC Chromatogram



Chemical Formula: $C_{23}H_{33}F_3N_4O_6$
Exact Mass: 518.2352
Molecular Weight: 518.5342



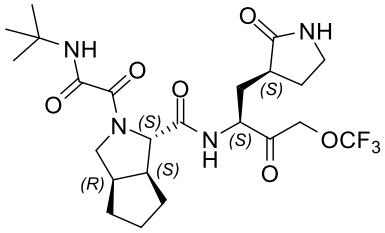


Peak Table

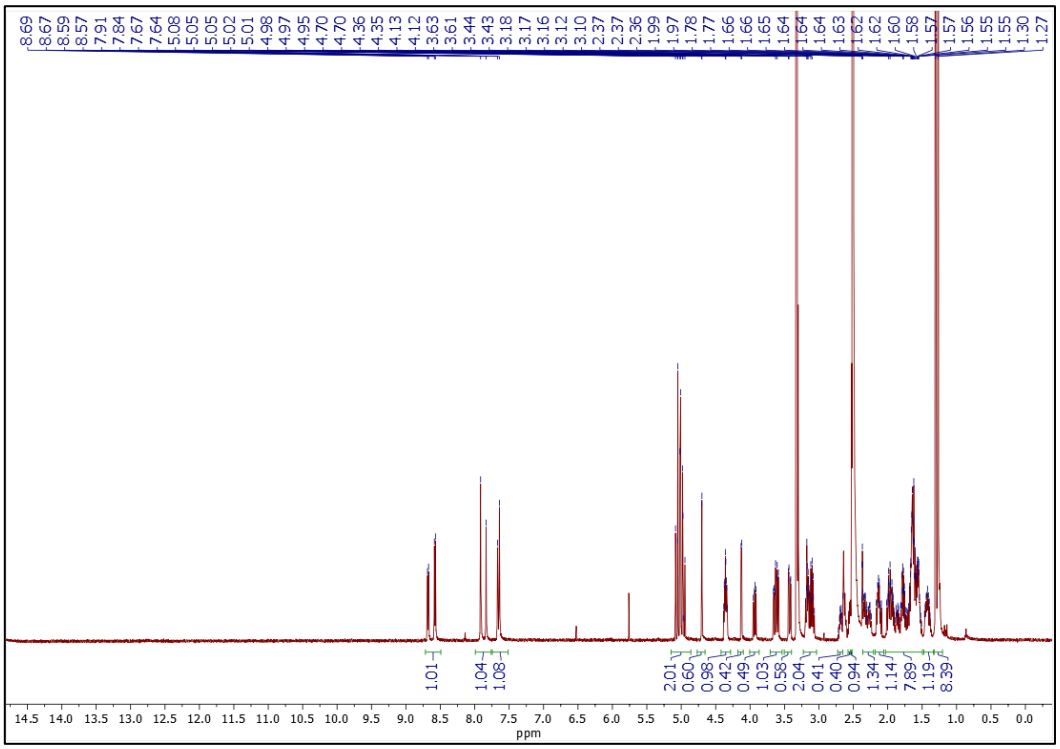
PDA Ch1 220nm

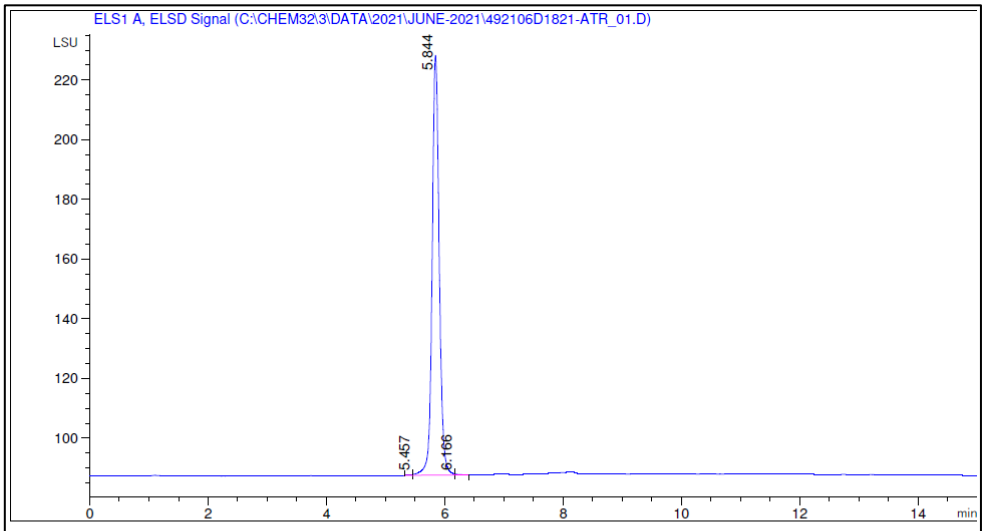
Peak#	Ret. Time	Height	Height%	Area	Area%
1	0.799	4960	1.469	4217	0.482
2	0.883	3282	0.972	2449	0.280
3	1.180	4118	1.220	3348	0.383
4	1.247	2543	0.753	1795	0.205
5	1.550	315565	93.452	847476	96.911
6	2.024	4589	1.359	12238	1.399
7	2.317	2620	0.776	2967	0.339
Total		337678	100.000	874490	100.000

Compound #6: ^{1}H NMR and HPLC Chromatogram



Chemical Formula: $C_{23}H_{33}F_3N_4O_6$
Exact Mass: 518.2352
Molecular Weight: 518.5342

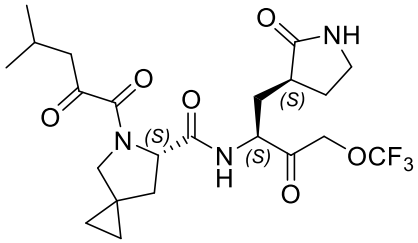




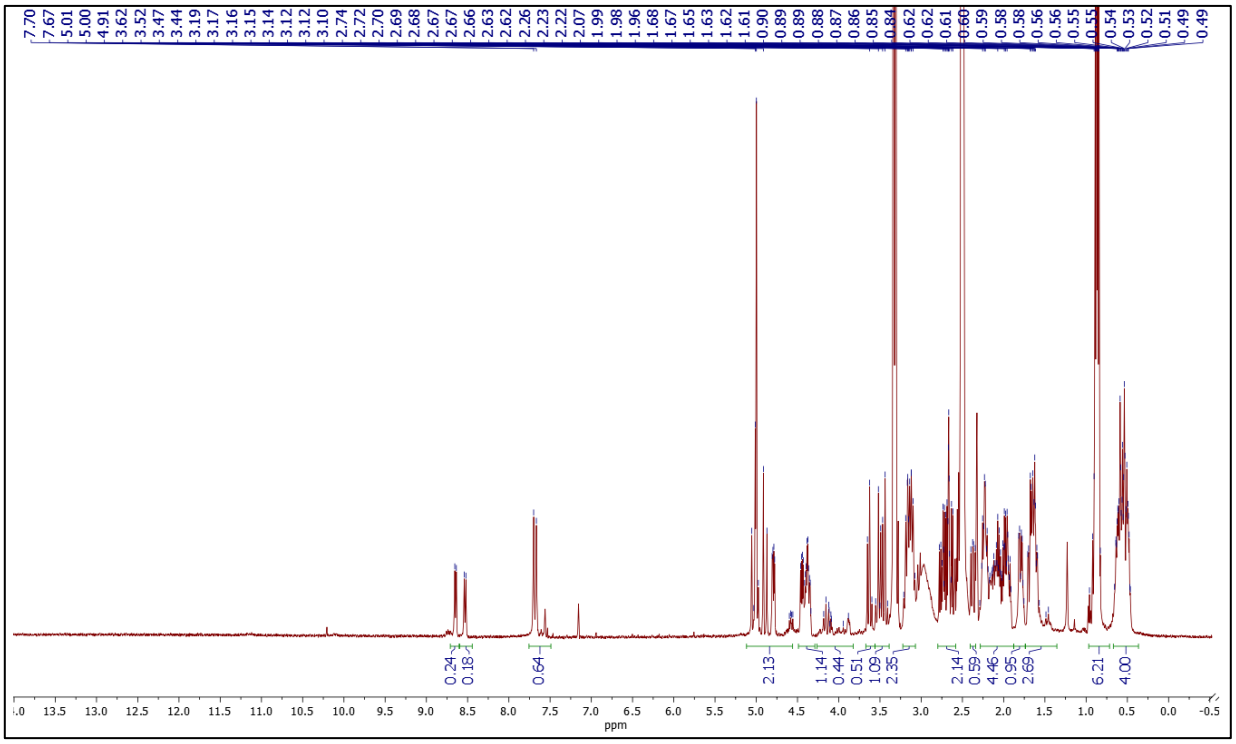
ELS1 A, ELSD Signal

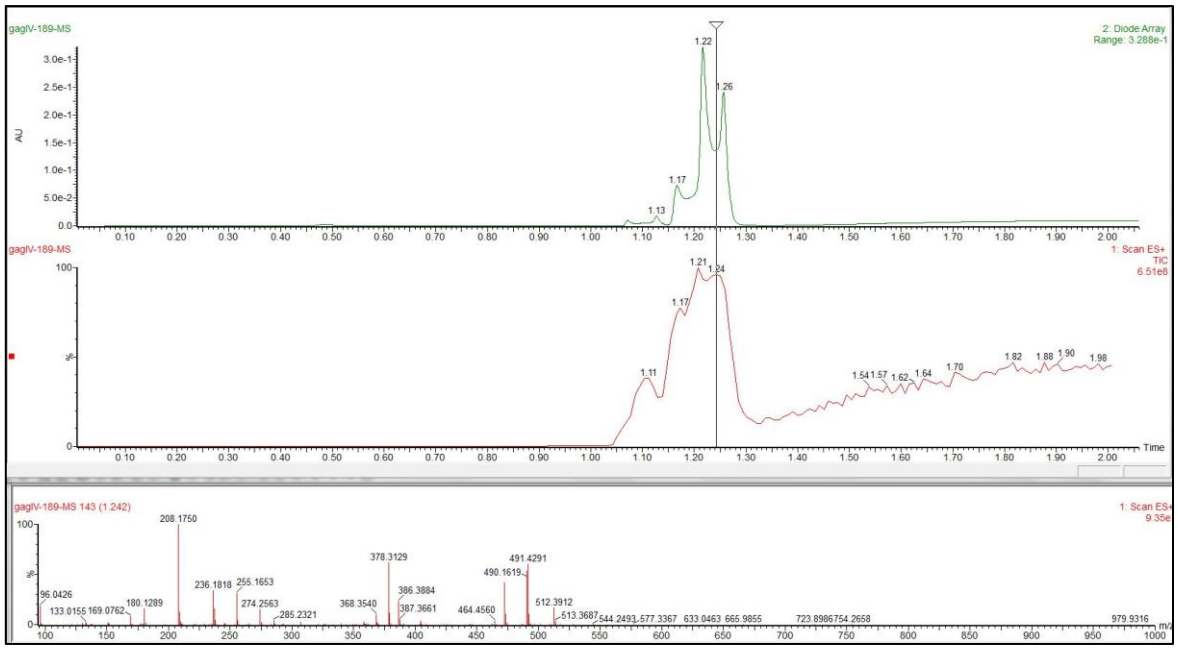
PEAK No	RT min	Area	Area %
1	5.457	0.944	0.080
2	5.844	1174.030	99.764
3	6.166	1.834	0.156

Compound #7: ^{1}H NMR and LCMS

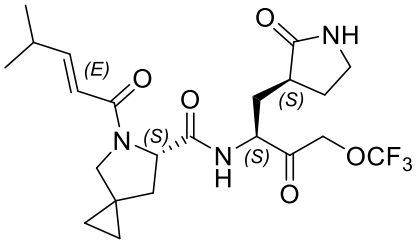


Chemical Formula: $C_{22}H_{30}F_3N_3O_6$
Exact Mass: 489.2087
Molecular Weight: 489.4922

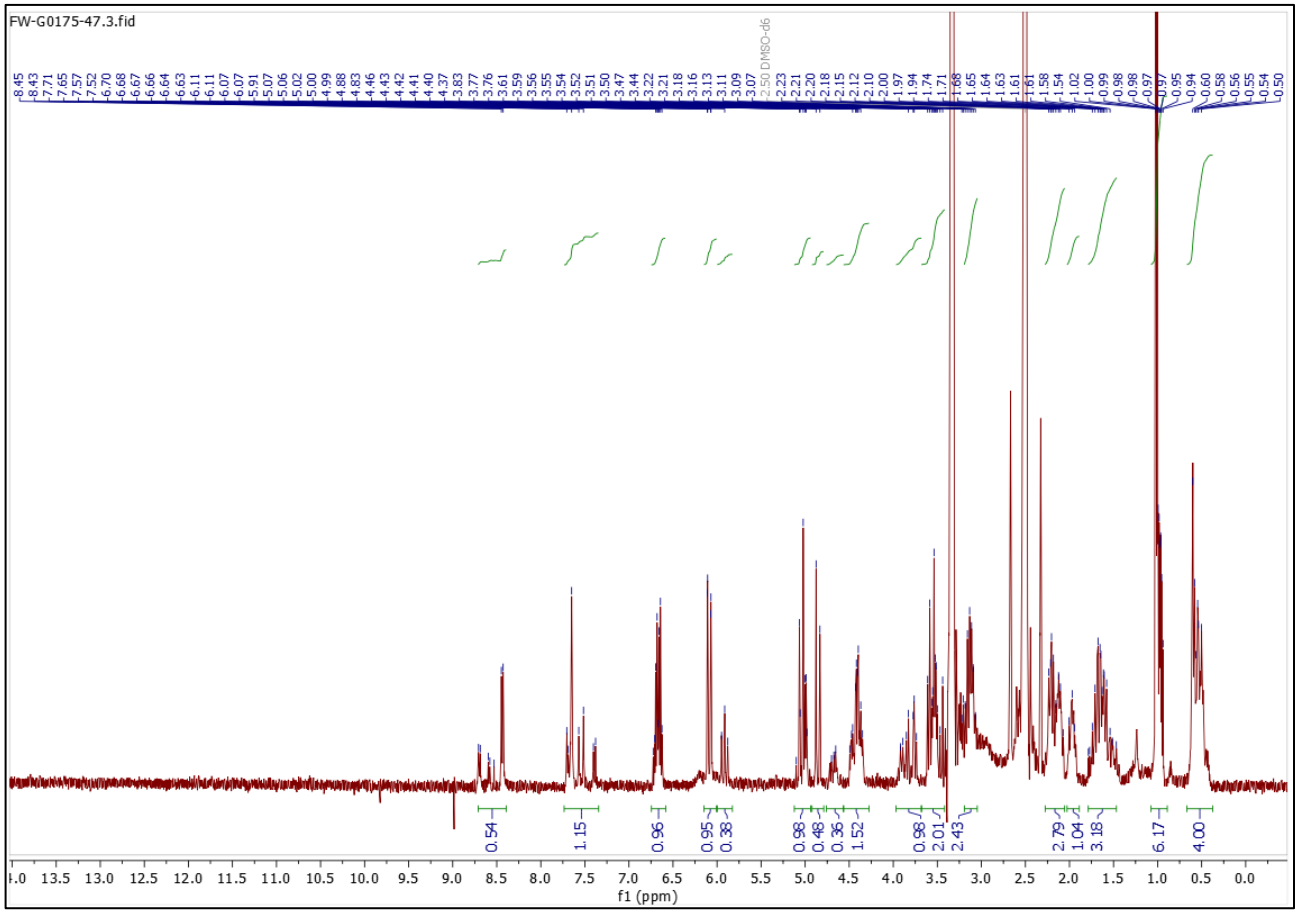


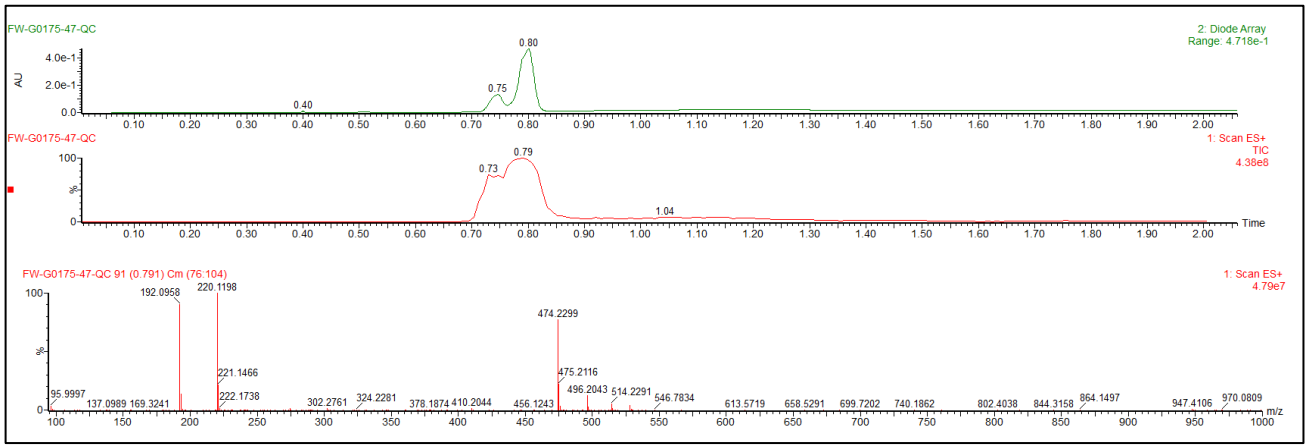


Compound #8: ^{1}H NMR and LCMS

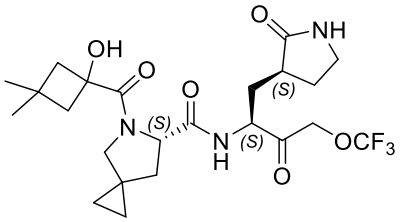


Chemical Formula: $C_{22}H_{30}F_3N_3O_5$
Exact Mass: 473.2138
Molecular Weight: 473.4932

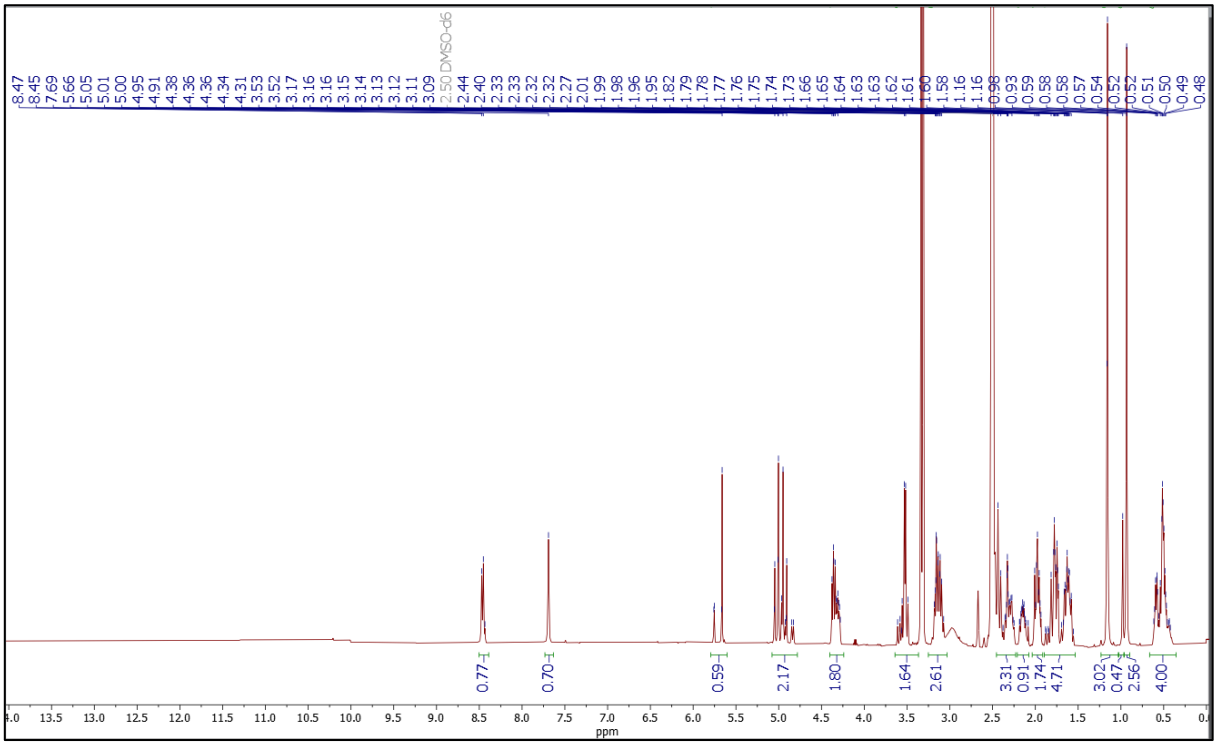


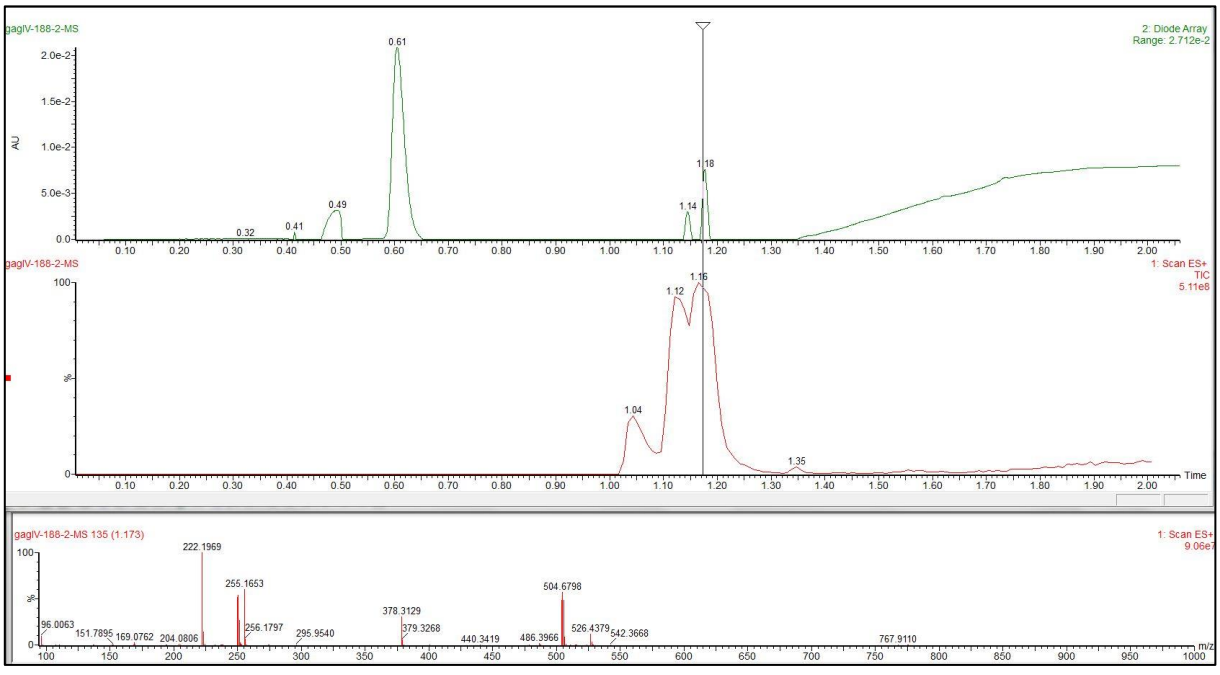


Compound #9: ^{1}H NMR and LCMS

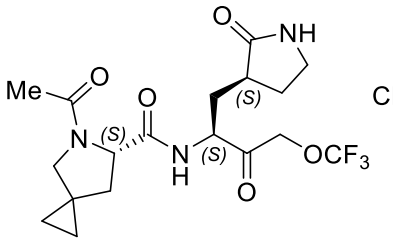


Chemical Formula: $C_{23}H_{32}F_3N_3O_6$
Exact Mass: 503.2243
Molecular Weight: 503.5192

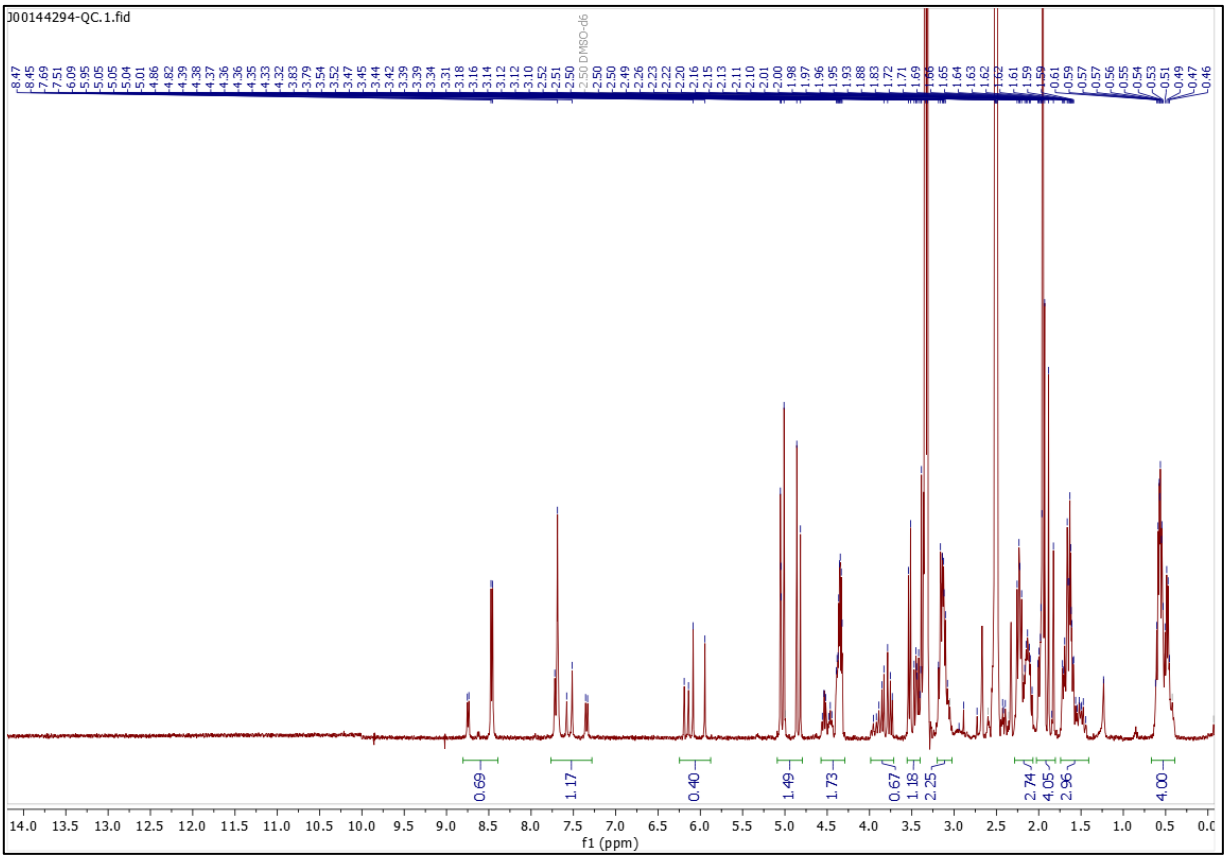


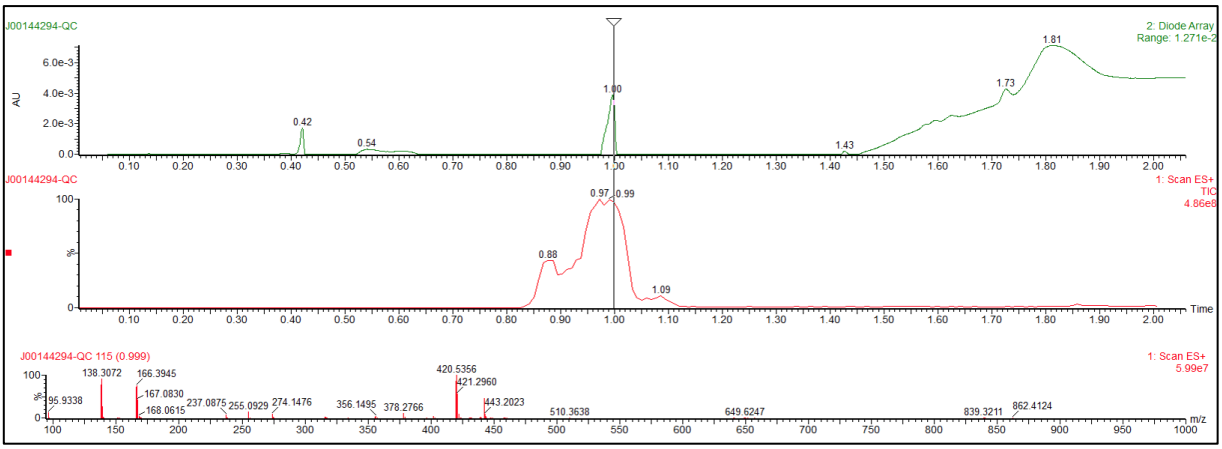


Compound #10: ^{1}H NMR and LCMS

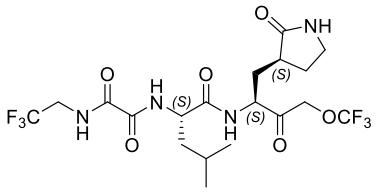


Chemical Formula: $C_{18}H_{24}F_3N_3O_5$
Exact Mass: 419.1668
Molecular Weight: 419.4012

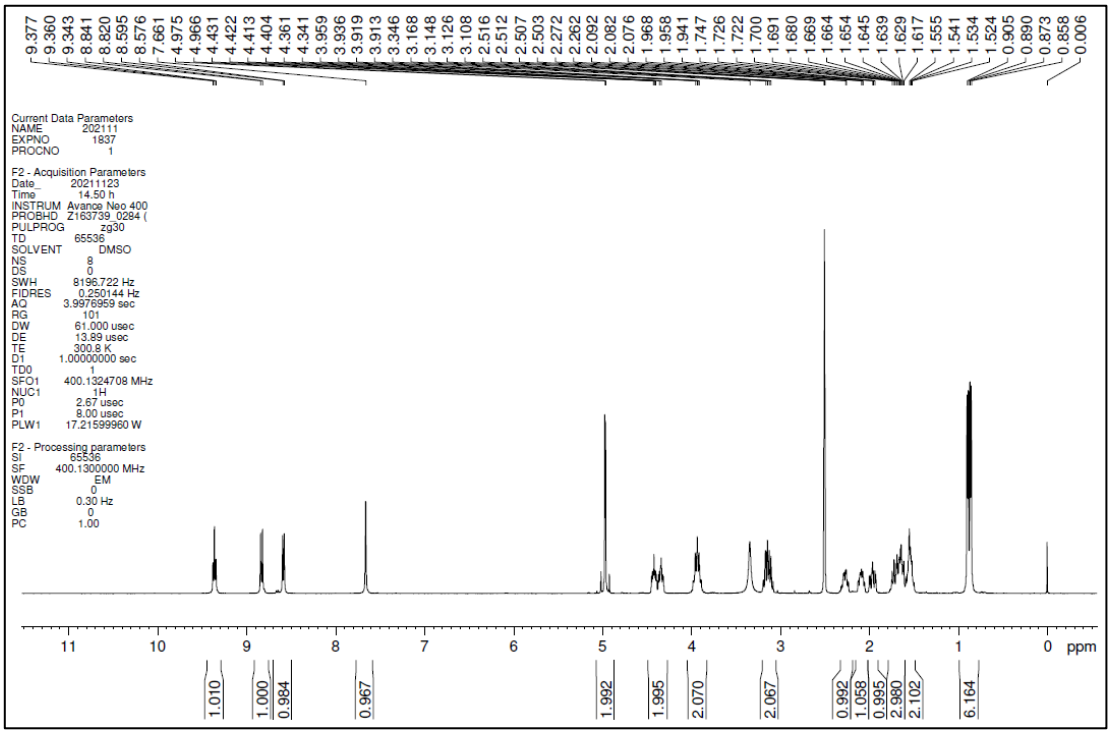


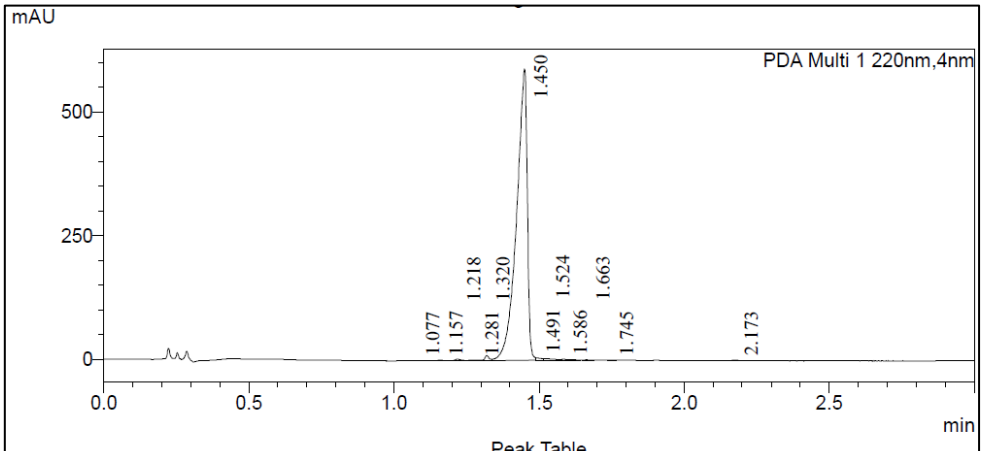


Compound #11: ^{1}H NMR and HPLC Chromatogram



Chemical Formula: $C_{19}H_{26}F_6N_4O_6$
Exact Mass: 520.1757
Molecular Weight: 520.4294



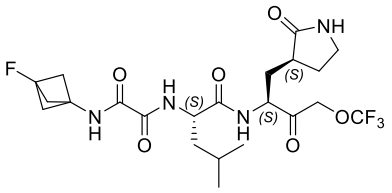


Peak Table

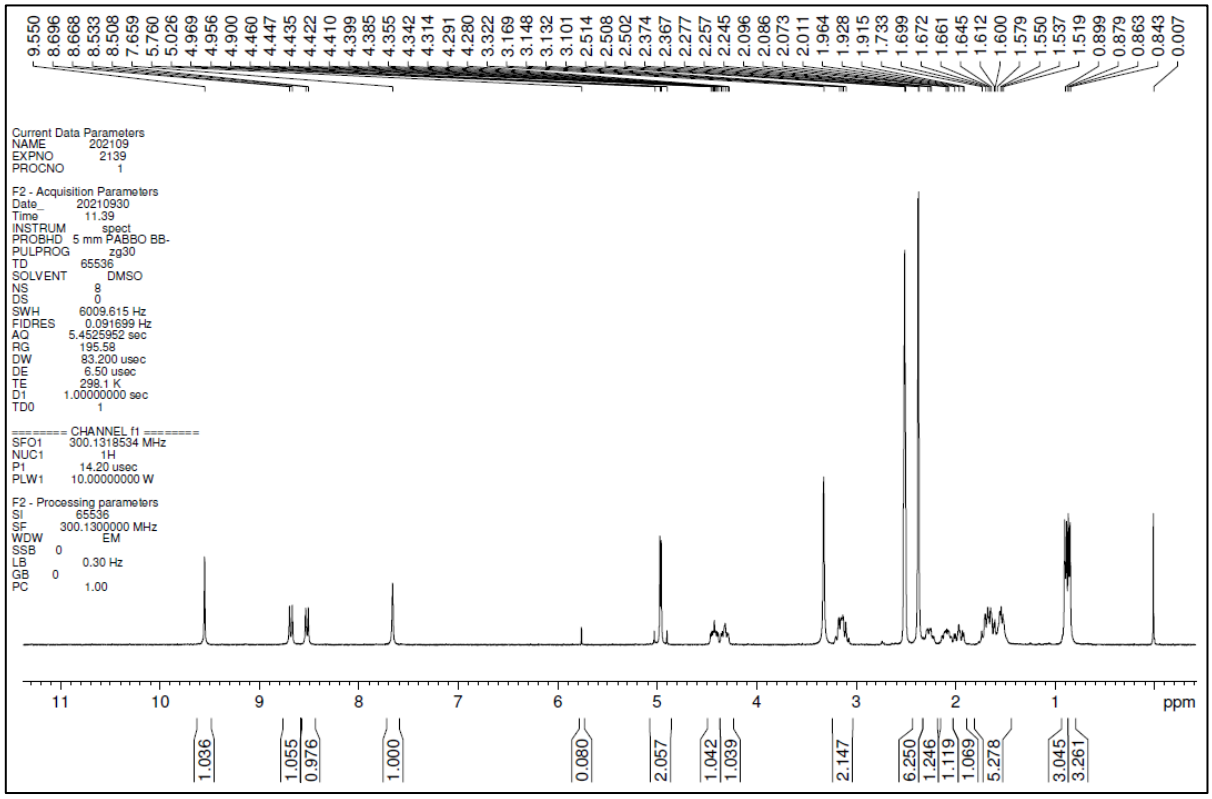
PDA Ch1 220nm

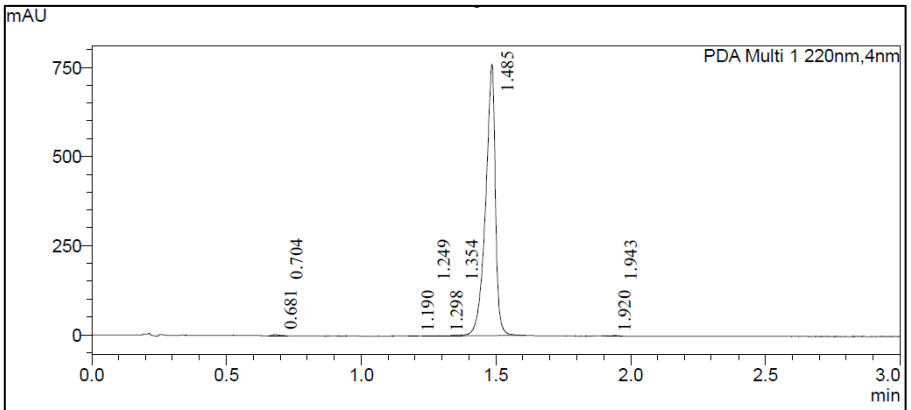
Peak#	Ret. Time	Height	Height%	Area	Area%
1	1.077	497	0.081	425	0.028
2	1.157	851	0.138	973	0.063
3	1.218	2742	0.446	3159	0.205
4	1.281	843	0.137	1346	0.087
5	1.320	10016	1.628	9899	0.643
6	1.450	588219	95.616	1504009	97.703
7	1.491	4883	0.794	6188	0.402
8	1.524	3308	0.538	6873	0.446
9	1.586	1509	0.245	4021	0.261
10	1.663	578	0.094	564	0.037
11	1.745	391	0.064	413	0.027
12	2.173	1355	0.220	1501	0.098
Total		615191	100.000	1539370	100.000

Compound #12: ^{1}H NMR and HPLC Chromatogram



Chemical Formula: $C_{22}H_{30}F_4N_4O_6$
Exact Mass: 522.2101
Molecular Weight: 522.4976



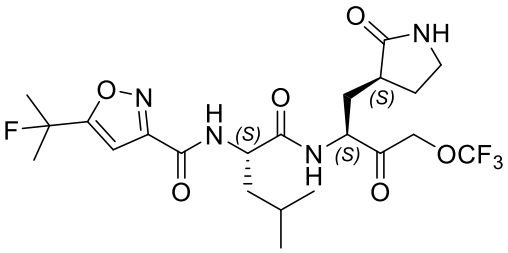


Peak Table

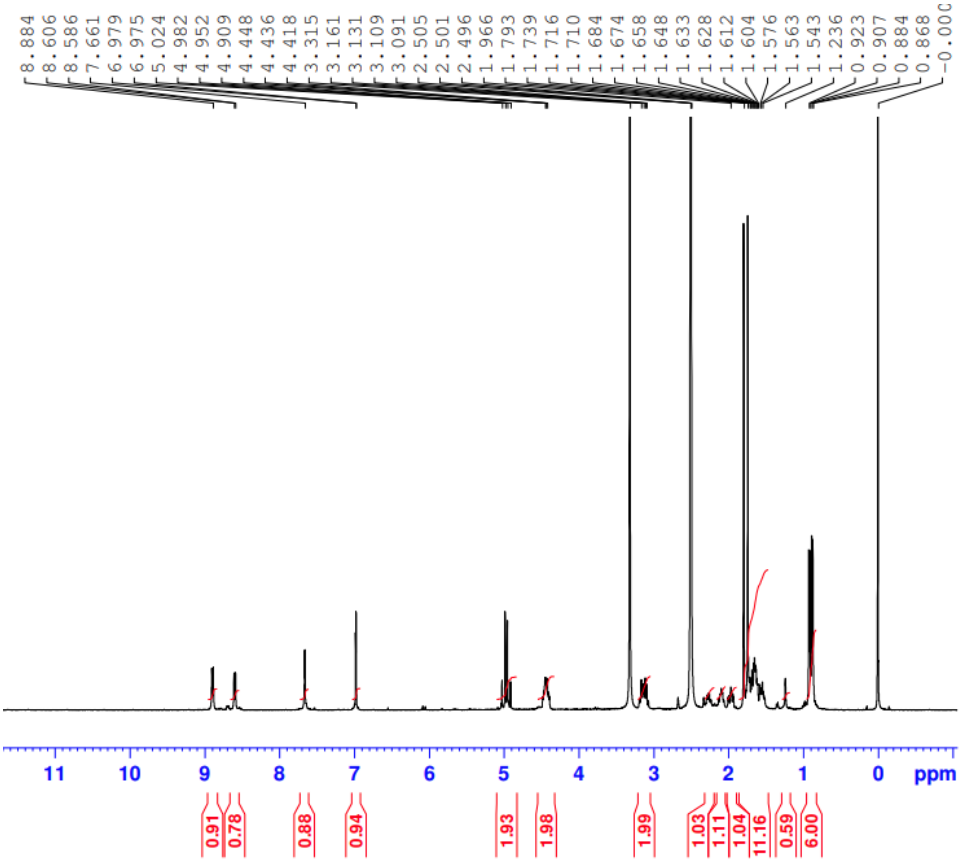
PDA Ch1 220nm

Peak#	Ret. Time	Height	Height%	Area	Area%
1	0.681	3947	0.511	4063	0.210
2	0.704	973	0.126	998	0.052
3	1.190	639	0.083	642	0.033
4	1.249	315	0.041	303	0.016
5	1.298	498	0.065	676	0.035
6	1.354	3005	0.389	6040	0.312
7	1.485	759697	98.447	1917909	99.159
8	1.920	801	0.104	981	0.051
9	1.943	1809	0.234	2556	0.132
Total		771684	100.000	1934168	100.000

Compound #13: ^{1}H NMR and HPLC Chromatogram



Chemical Formula: $C_{22}H_{30}F_4N_4O_6$
Exact Mass: 522.2101
Molecular Weight: 522.4976

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Current Data Parameters
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EXPNO                        1
PROCNO                       1

F2 - Acquisition Parameters
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Time                 16.13 h
INSTRUM               spect
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PULPROG                zg30
TD                    32768
SOLVENT                DMSO
NS                       64
DS                        2
SWH                8012.820 Hz
FIDRES             0.489064 Hz
AQ                 2.0447233 sec
RG                    204.36
DW                  62.400 usec
DE                   6.50 usec
TE                  298.2 K
D1                 1.00000000 sec
TD0                       1
SFO1            400.2324714 MHz
NUC1                      1H
P0                   3.33 usec
P1                  10.00 usec
PLW1            17.25300026 W

F2 - Processing parameters
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WDW                      EM
SSB                       0
LB                     0.80 Hz
GB                        0
PC                     1.00
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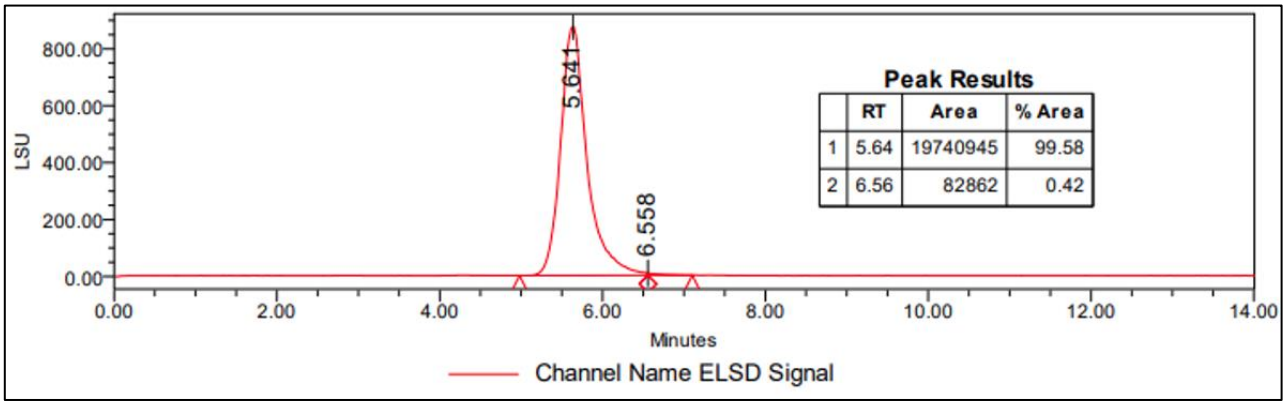
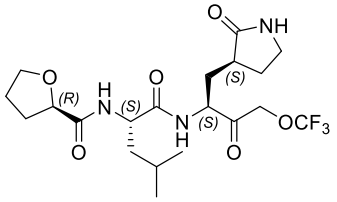


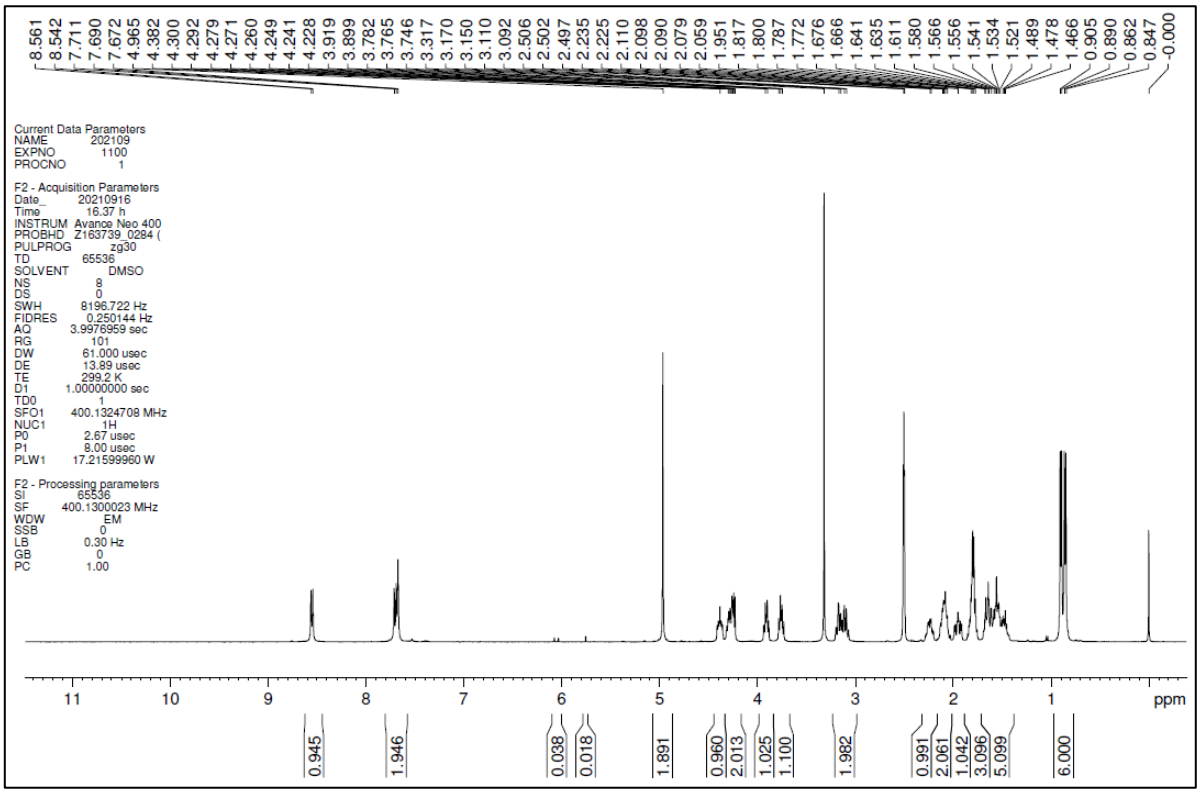
Peak Results

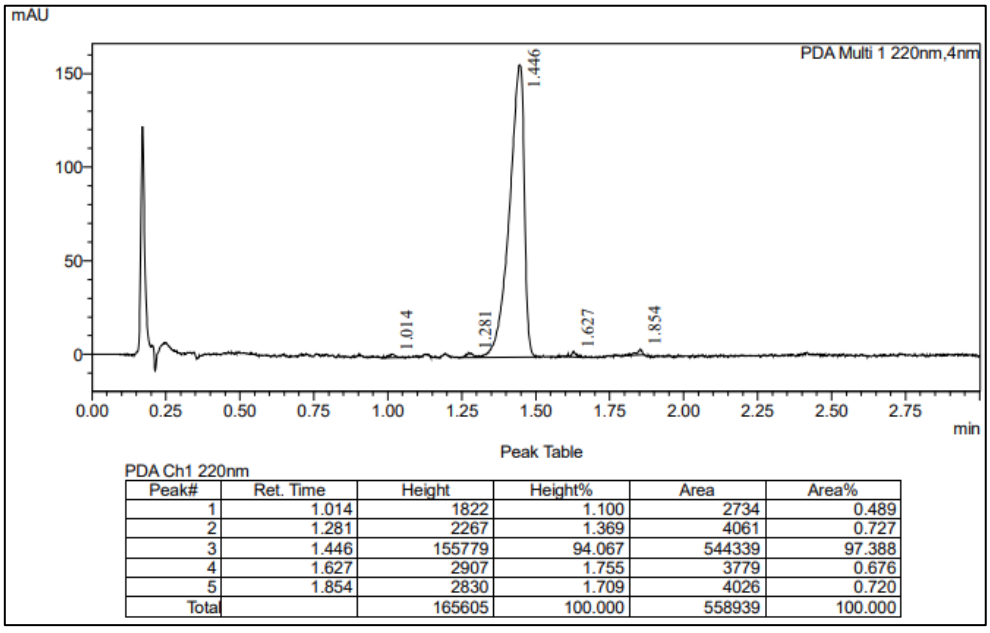
	RT	Area	% Area
1	5.64	19740945	99.58
2	6.56	82862	0.42

Compound #14: ^{1}H NMR and HPLC Chromatogram



Chemical Formula: $C_{20}H_{30}F_3N_3O_6$
Exact Mass: 465.2087
Molecular Weight: 465.4702



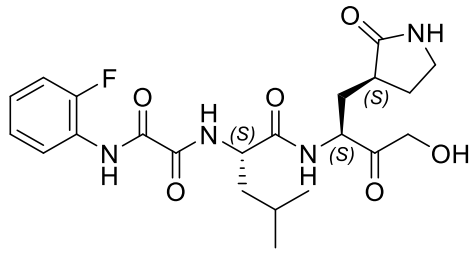


Peak Table

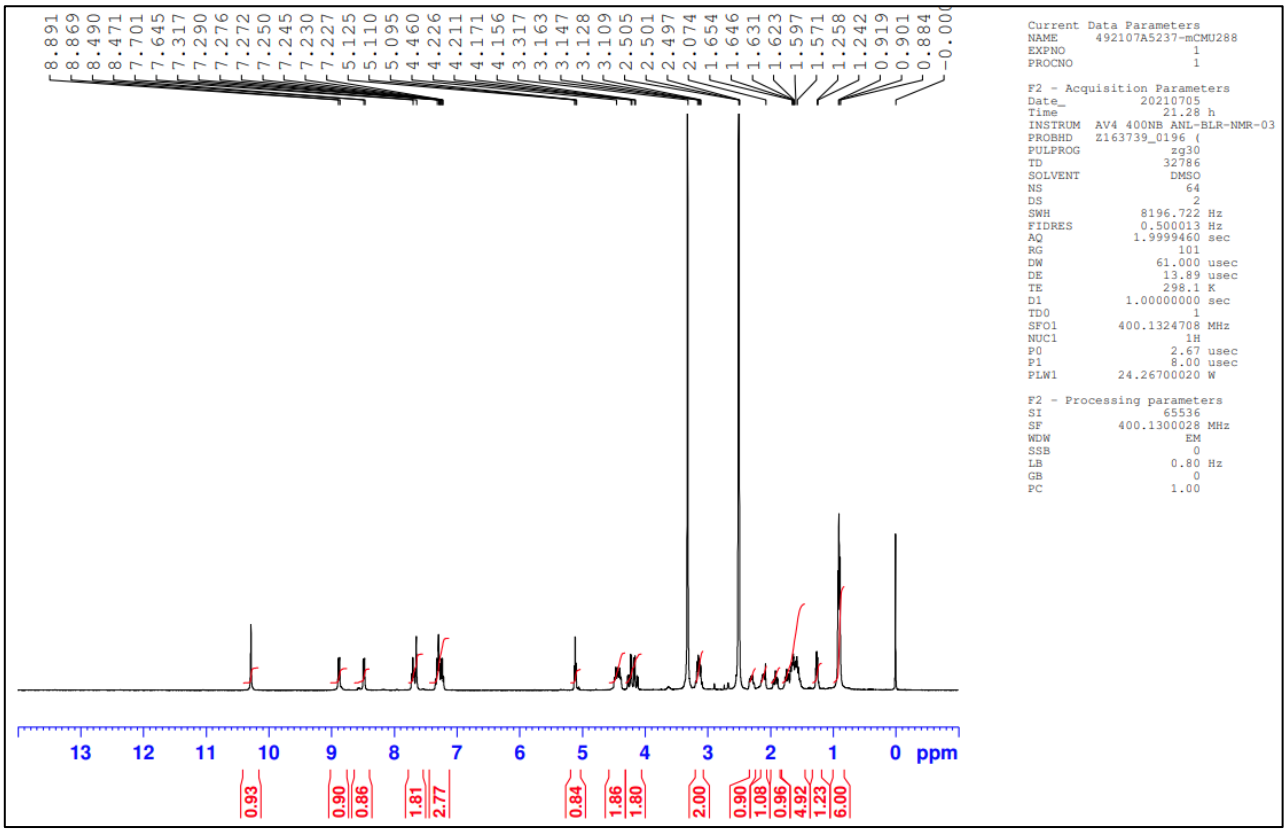
PDA Ch1 220nm

Peak#	Ret. Time	Height	Height%	Area	Area%
1	1.014	1822	1.100	2734	0.489
2	1.281	2267	1.369	4061	0.727
3	1.446	155779	94.067	544339	97.388
4	1.627	2907	1.755	3779	0.676
5	1.854	2830	1.709	4026	0.720
Total		165605	100.000	558939	100.000

Compound #15: ^{1}H NMR and LCMS



Chemical Formula: $C_{22}H_{29}FN_4O_6$
Exact Mass: 464.2071
Molecular Weight: 464.4944

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Current Data Parameters
NAME      492107A5237-mCMU288
EXPNO                      1
PROCNO                     1

F2 - Acquisition Parameters
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Time                 21.28 h
INSTRUM   AV4 400NB ANL-BLR-NMR-03
PROBHD    Z163739_0196 (
PULPROG              zg30
TD                  32786
SOLVENT              DMSO
NS                      64
DS                       2
SWH             8196.722 Hz
FIDRES             0.500013 Hz
AQ                1.9999460 sec
RG                     101
DW                  61.000 usec
DE                  13.89 usec
TE                  298.1 K
D1              1.00000000 sec
TD0                      1
SFO1           400.1324708 MHz
NUC1                   1H
P0                  2.67 usec
P1                  8.00 usec
PLW1           24.26700020 W

F2 - Processing parameters
SI                   65536
SF             400.1300028 MHz
WDW                    EM
SSB                     0
LB                   0.80 Hz
GB                      0
PC                   1.00
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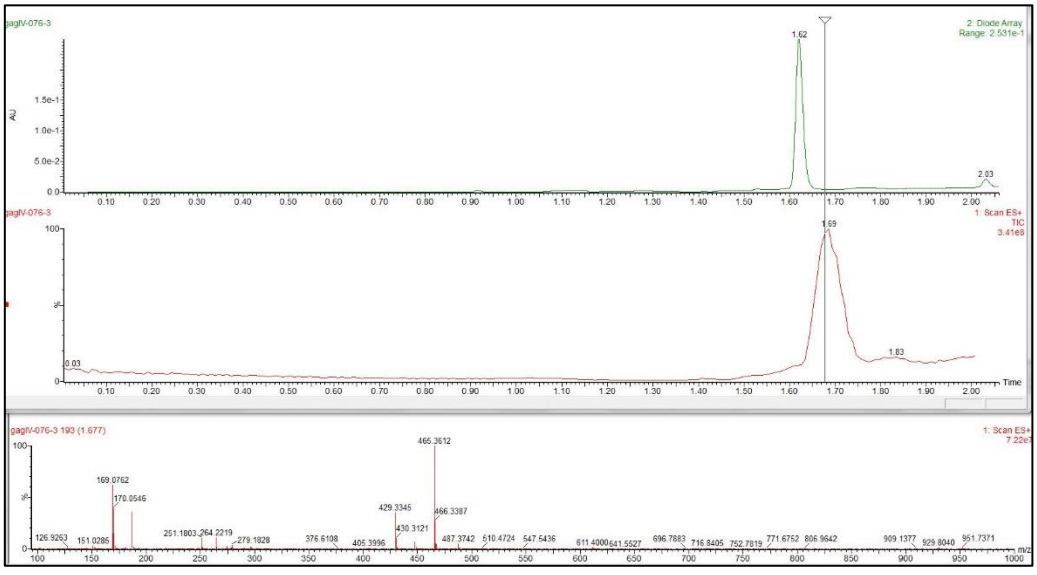
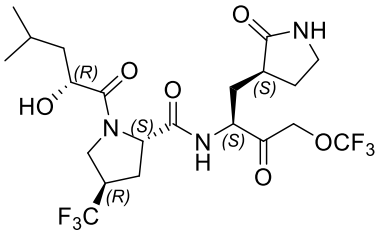
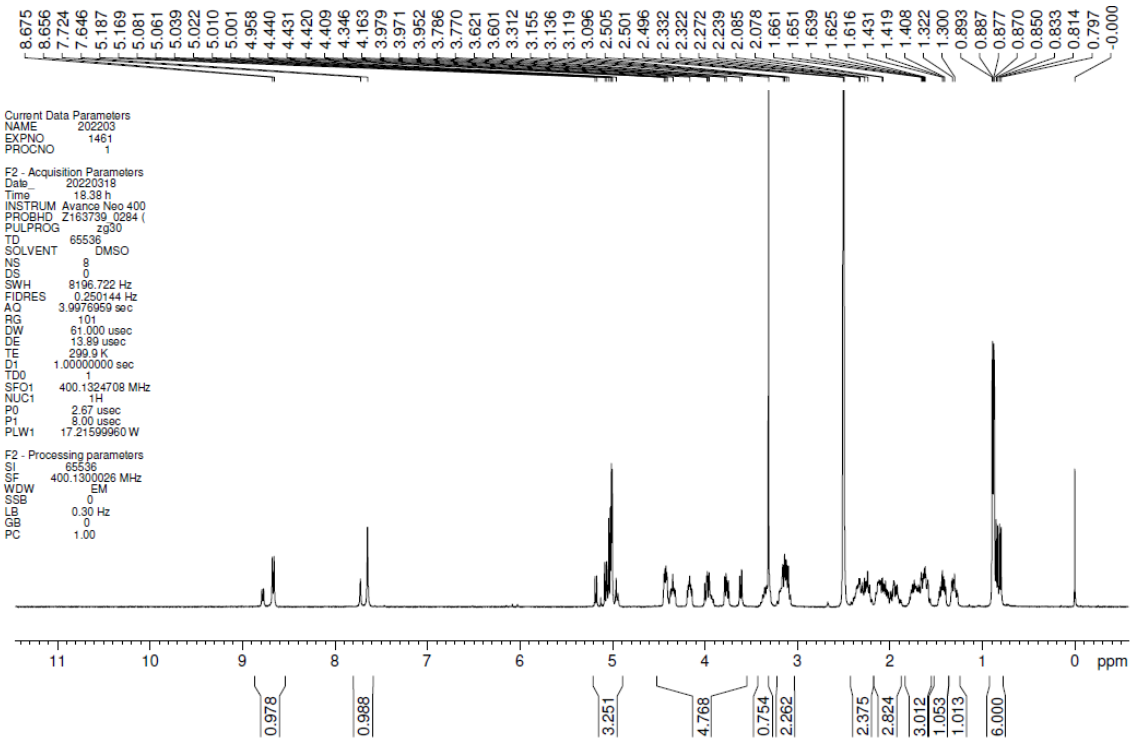
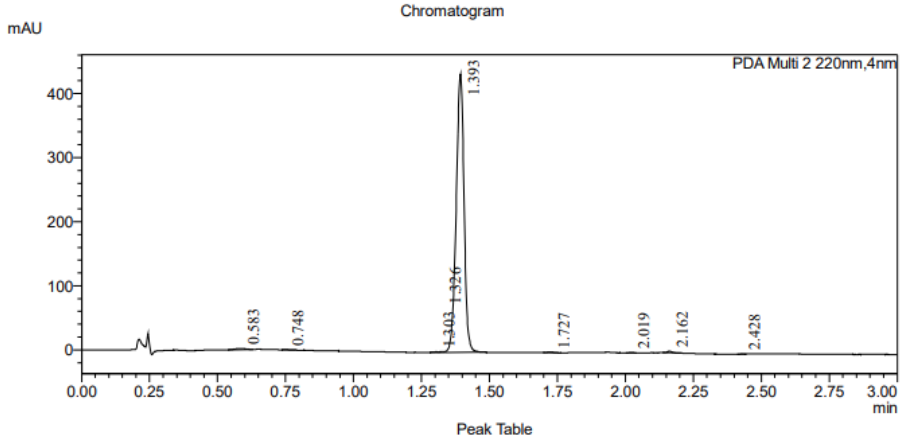


Compound #16: ^{1}H NMR and HPLC Chromatogram



Chemical Formula: $C_{21}H_{29}F_6N_3O_6$
Exact Mass: 533.1961
Molecular Weight: 533.4684



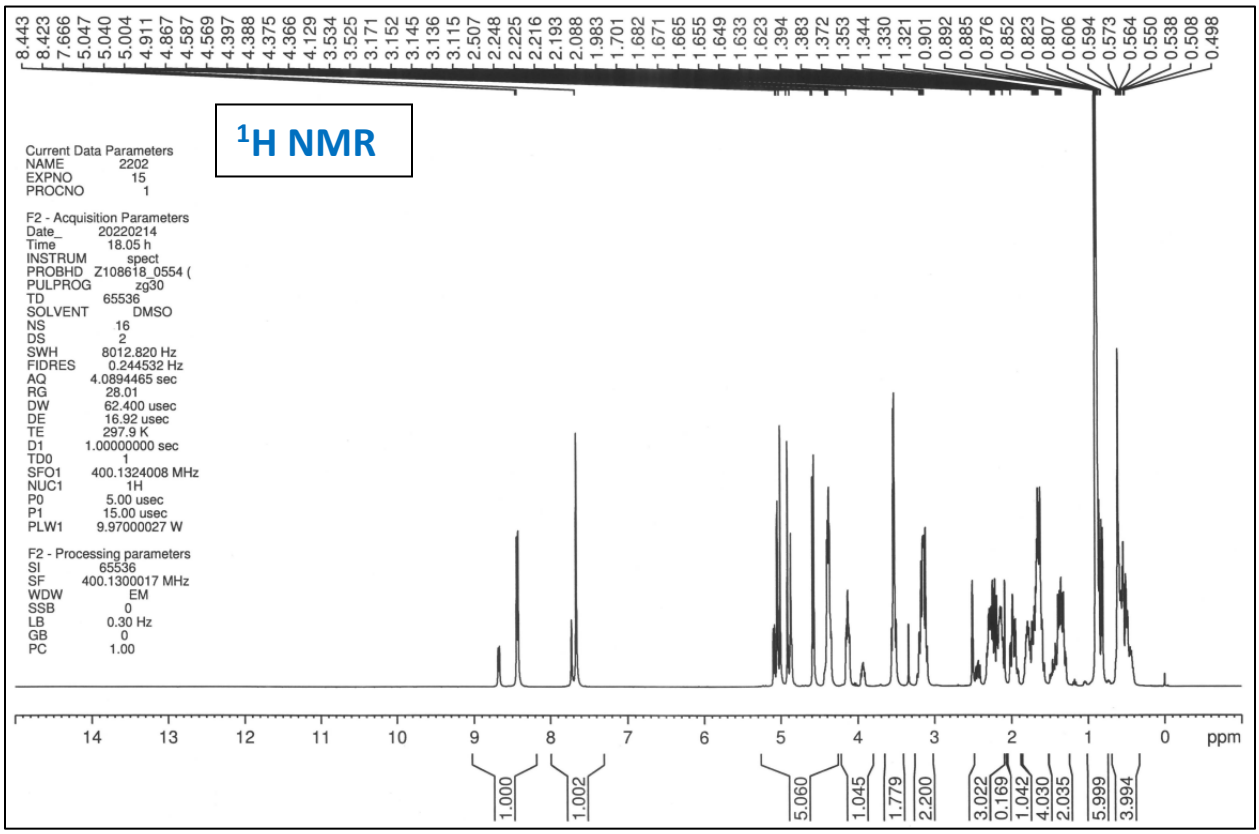


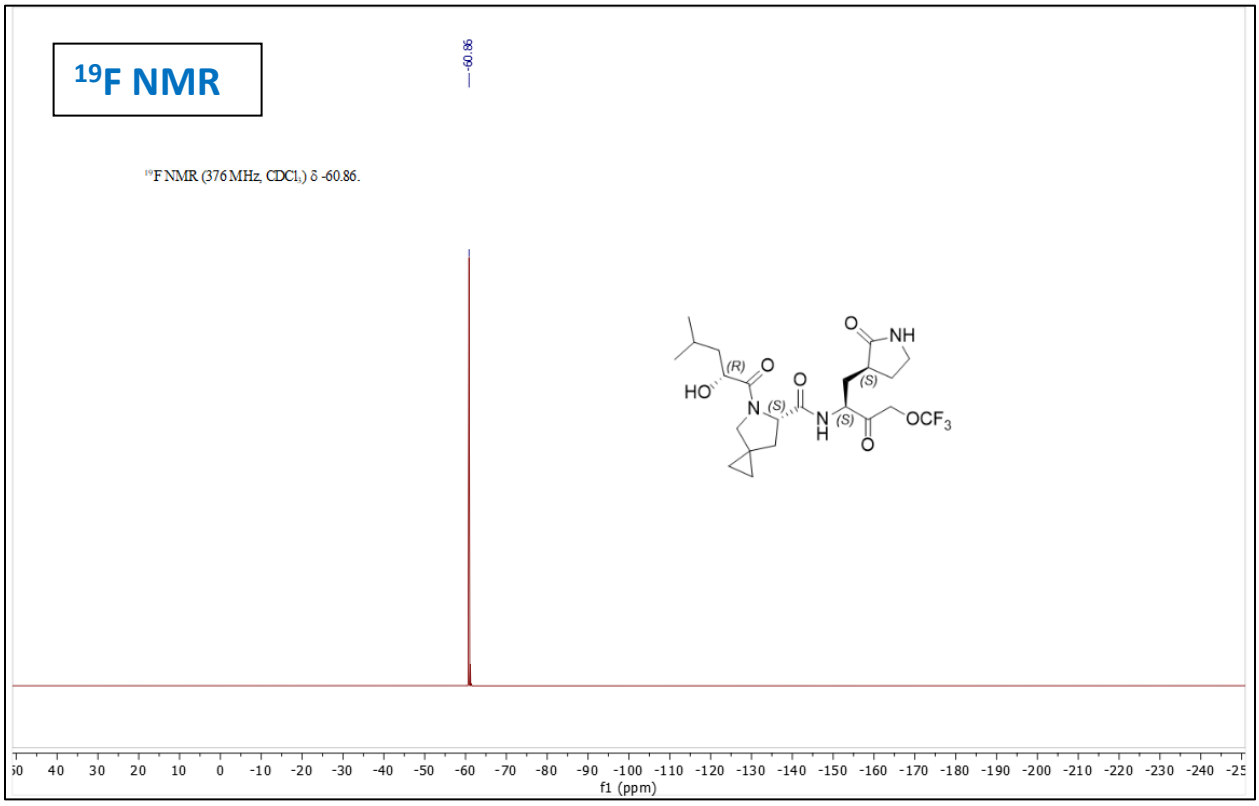
Peak Table

PDA Ch2 220nm

Peak#	Ret. Time	Height	Height%	Area	Area%
1	0.583	1704	0.384	4613	0.520
2	0.748	872	0.197	903	0.102
3	1.303	826	0.186	1019	0.115
4	1.326	1244	0.280	1226	0.138
5	1.393	434145	97.875	873015	98.490
6	1.727	1021	0.230	1529	0.173
7	2.019	663	0.149	741	0.084
8	2.162	2620	0.591	3047	0.344
9	2.428	475	0.107	305	0.034
Total		443570	100.000	886398	100.000

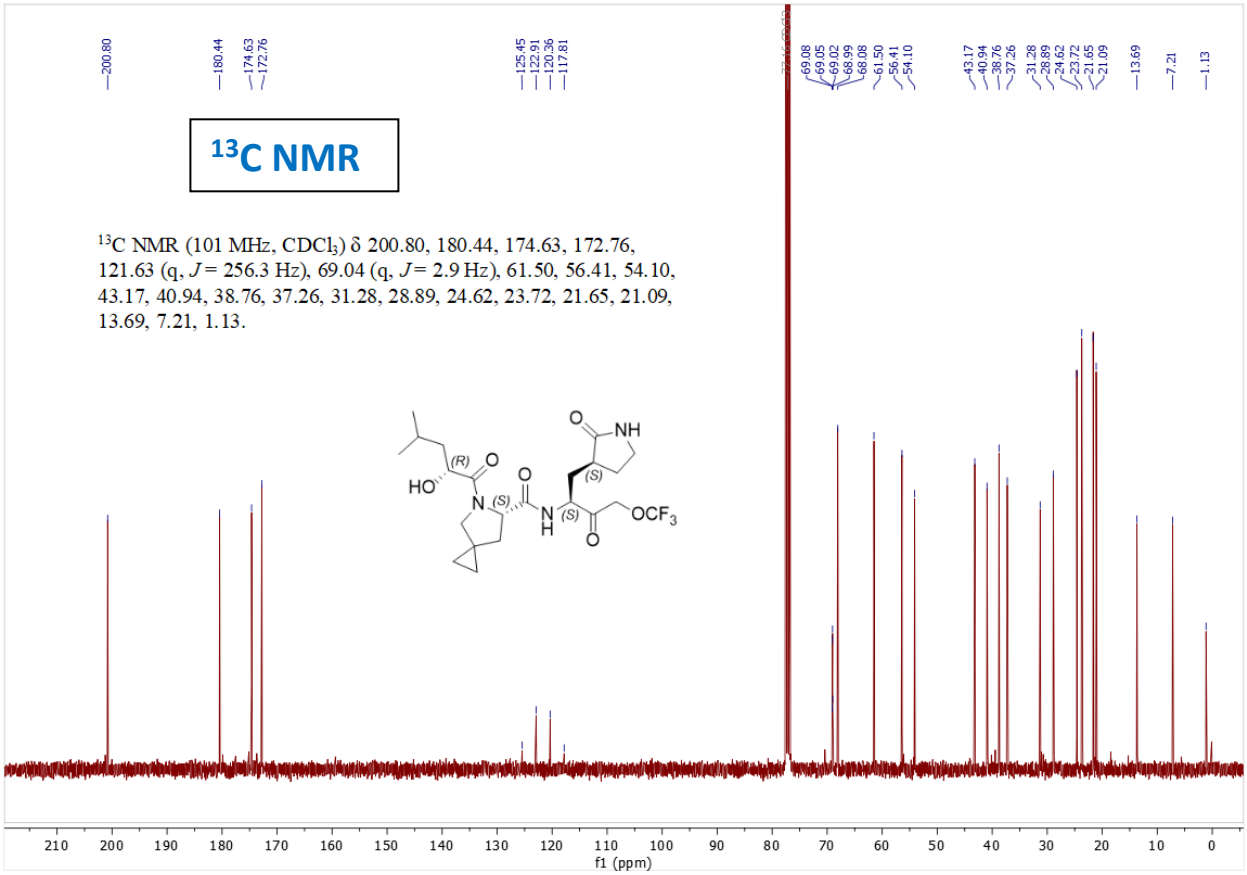
6. Spectral data for CMX990



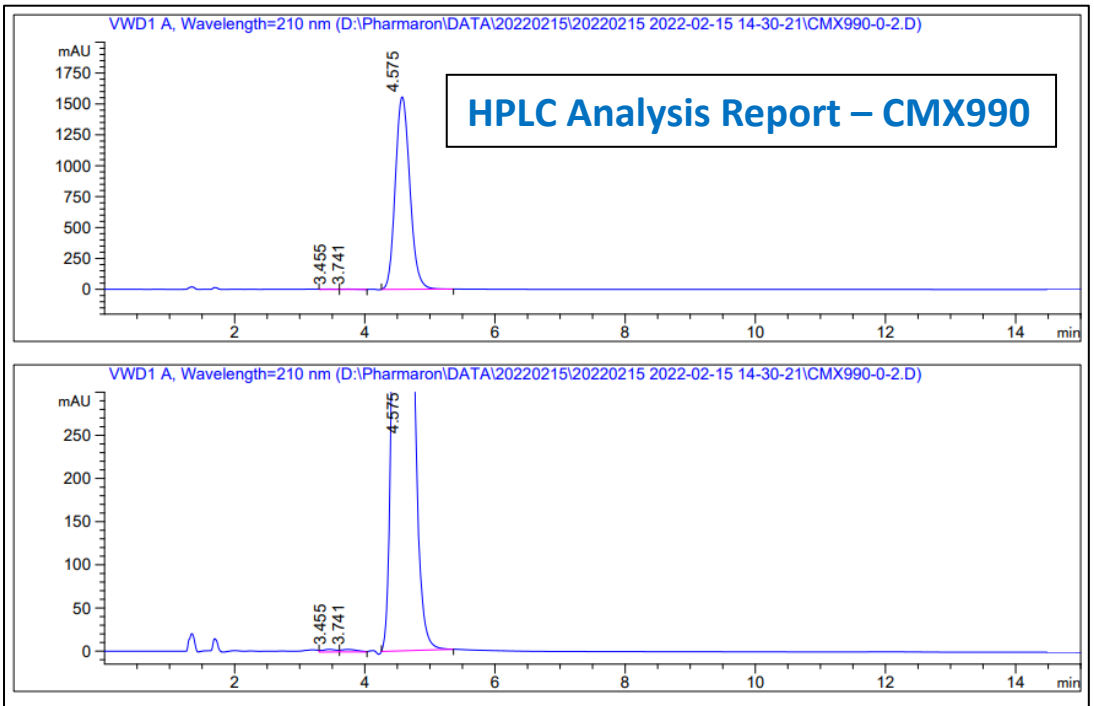


^{13}C NMR

^{13}C NMR (101 MHz, $CDCl_3$) δ 200.80, 180.44, 174.63, 172.76, 121.63 (q, J = 256.3 Hz), 69.04 (q, J = 2.9 Hz), 61.50, 56.41, 54.10, 43.17, 40.94, 38.76, 37.26, 31.28, 28.89, 24.62, 23.72, 21.65, 21.09, 13.69, 7.21, 1.13.

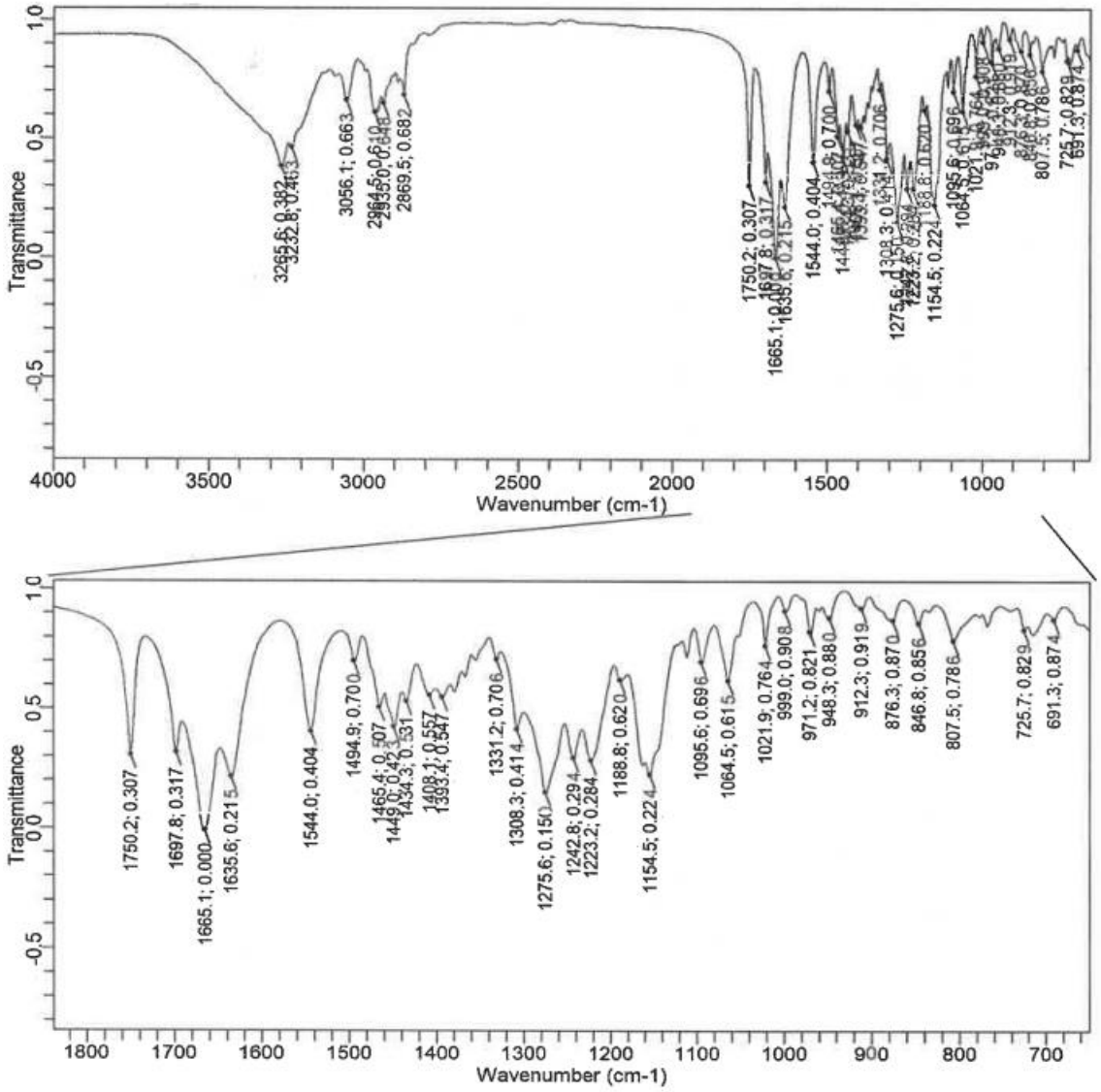


HPLC Analysis Report – CMX990



#	RT	Symmetry	Width	Height	Height %	Area	Area %
1	3.46	0.000	0.249	3.10	0.20	46.3548	0.1915
2	3.74	0.657	0.284	2.97	0.19	50.4810	0.2085
3	4.57	0.856	0.258	1557.36	99.61	2.4112e4	99.6000

IR Spectrum – CMX990



LCMS Conditions and Mass Spectrum – CMX990

Acquired by	: System Administrator
Sample Name	: LCMS18-PDM-CLR-CBR-SPC-2022-06B-0-1(EB2137259-022A1)1T
Injection Volume	: 0.8
Data File	: LCMS18-PDM-CLR-CBR-SPC-2022-06B-0-1(EB2137259-022A1)1T.lcd
Method File	: ACN-Water-0.05%TFA-5%B-1.5-2MIN(90-900).lcm
Date Acquired	: 2022/2/23 15:25:58
Comment	: Mobile phase A:Water+0.05%TFA Mobile phase B:Acetonitrile+0.05%TFA

Instrument Name	: Shimadzu LCMS-2020
<<Pump>>	
Mode	: Binary gradient
Pump A	: LC-20AD
Pump B	: LC-20AD
Total Flow	: 1.5000 mL/min
B Conc.	: 5.0 %
<<Oven>>	
Oven Temperature	: 40 C
<<PDA>>	
PDA Model	: SPD-M20A
Lamp	: D2
Start Wavelength	: 190 nm
End Wavelength	: 400 nm
<<Column>>	
Column Name	: HALO C18
Length	: 30 mm
Internal Diameter	: 3.0 mm
Description	: 2.7 um particles

<<Interface>>	
Interface	:ESI
DL Temperature	:250 C
Nebulizing Gas Frow	:1.50 L/min
Heat Block	:250 C
Drying Gas	:On
	15.00 L/min
<<MS Parameter>>	
Initial Valve Position	:-
--Segment 1 Event 1--	
Start Time	:0.00 min
End Time	:2.00 min
Acquisition Mode	:Scan
Polarity	:Positive
Event Time	:0.40 sec
Detector Voltage	:+1.45 kV
Threshold	:0
Start m/z	:90.00
End m/z	:900.00
Scan Speed	:2143 u/sec
Interface Volt.	:Use the Data in the Tuning File
DL Volt.	:Use the Data in the Tuning File
Qarray DC Voltage	:Use the Data in the Tuning File
Qarray DC Voltage	:Use the Data in the Tuning File

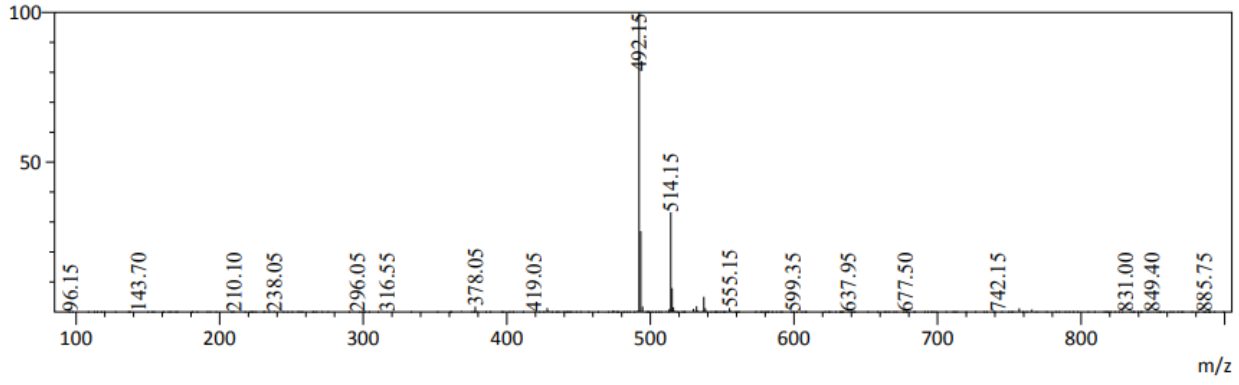
<<LC Time Program>>

Time	Module	Command	Value
0.01	Pumps	Pump B Conc.	5
1.20	Pumps	Pump B Conc.	100
1.80	Pumps	Pump B Conc.	100
1.83	Pumps	Pump B Conc.	5
2.00	Controller	Stop	

Mass Spectrum

Retention time: 0.977

Spectrum Mode:Single 0.977(153) Base Peak:492.15(6128739)
BG Mode:Averaged 0.950-1.043(149-163) Segment 1 - Event 1



References

1. Hoffman, R. L.; Kania, R. S.; Brothers, M. A.; Davies, J. F.; Ferre, R. A.; Gajiwala, K. S.; He, M.; Hogan, R. J.; Kozminski, K.; Li, L. Y.; Lockner, J. W.; Lou, J.; Marra, M. T.; Mitchell, L. J., Jr.; Murray, B. W.; Nieman, J. A.; Noell, S.; Planken, S. P.; Rowe, T.; Ryan, K.; Smith, G. J., 3rd; Solowiej, J. E.; Steppan, C. M.; Taggart, B., Discovery of Ketone-Based Covalent Inhibitors of Coronavirus 3CL Proteases for the Potential Therapeutic Treatment of COVID-19. *J Med Chem* **2020,** *63* (21), 12725-12747.

2. Bakowski, M. A.; Beutler, N.; Wolff, K. C.; Kirkpatrick, M. G.; Chen, E.; Nguyen, T. H.; Riva, L.; Shaabani, N.; Parren, M.; Ricketts, J.; Gupta, A. K.; Pan, K.; Kuo, P.; Fuller, M.; Garcia, E.; Teijaro, J. R.; Yang, L.; Sahoo, D.; Chi, V.; Huang, E.; Vargas, N.; Roberts, A. J.; Das, S.; Ghosh, P.; Woods, A. K.; Joseph, S. B.; Hull, M. V.; Schultz, P. G.; Burton, D. R.; Chatterjee, A. K.; McNamara, C. W.; Rogers, T. F., Drug repurposing screens identify chemical entities for the development of COVID-19 interventions. *Nat Commun* **2021,** *12* (1), 3309.