Supplementary Information

Cellular Engagement of the SARS-CoV-2 Macrodomain by GS-441524 and Enhanced In Vitro Inhibition by its Diphosphorylated Metabolite

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Synthesis of phosphorylated derivatives of GS-441524

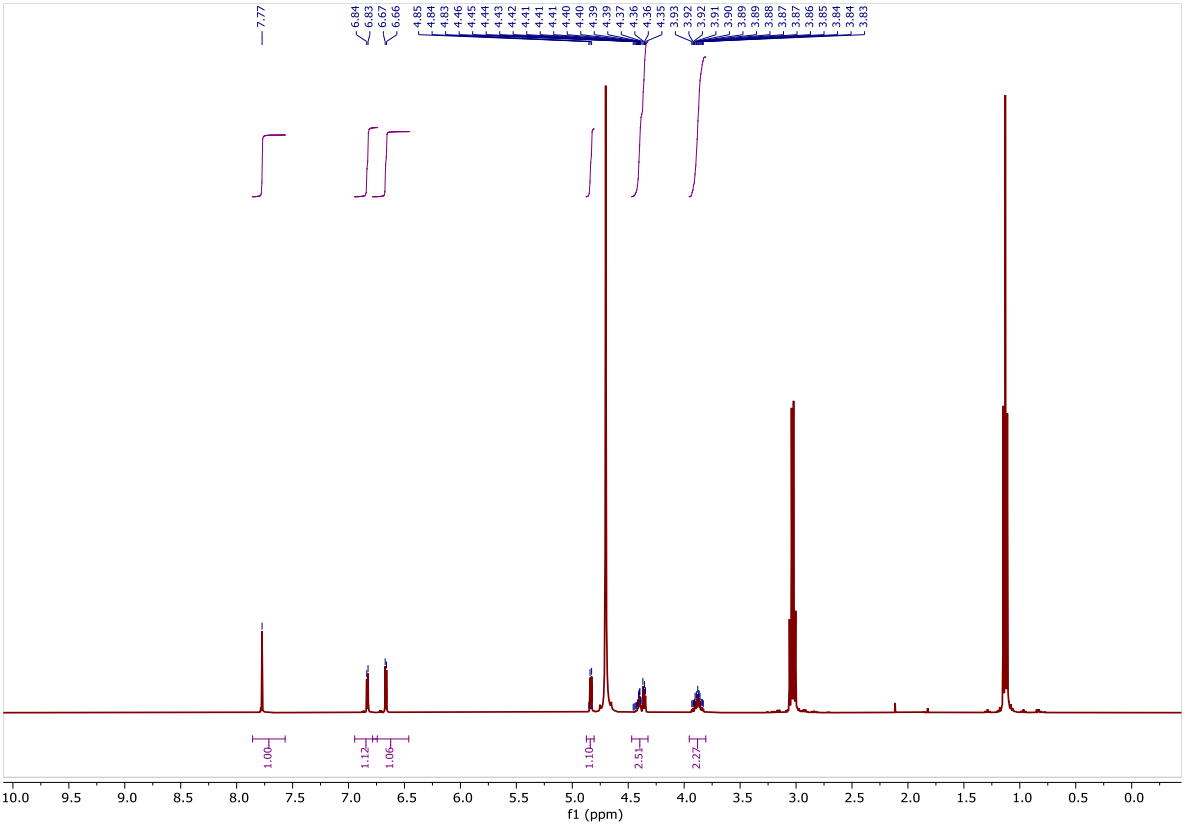
Reagents: Reagents and solvents were purchased from Fluorochem, Sigma–Aldrich and AlfaAesar. Trimethylphosphate and phosphorus(V)oxychloride were dried by distillation. CH_3CN was degassed under a positive stream of argon just before its use. Unless otherwise stated, all the reactions were performed under a positive atmosphere of argon by standard syringe, cannula and septa techniques. Reactions were monitored by thin-layer chromatography (TLC) using Merck silica gel 60 F254 plates and visualized by UV (254 nm). Purifications of nucleoside phosphates were performed using HPLC (Waters modular HPLC system) on a column packed with 10 µm C18 reversed phase (Phenomenex, Luna C18 (2) 100 Å). NMR spectra were measured on a Bruker AVANCE 400 in D_2O solutions at 25 °C. Chemical shifts (in ppm, δ scale) were referenced to the residual solvent signal in ^1H spectra. Coupling constants (*J*) are given in Hz. HPLC-MS analyses were performed on Shimadzu UFLC-MS-2020 system with ESI. Column: Acquity UPLC BEH C18 1.7 µm, 2.1x50 mm. Solvent A: H_2O + 0.1% HCOOH; Solvent B: CH_3CN + 0.1% COOH. Total flow 0.6 ml/min. Total time of the method 10 min. The mass spectrum was recorded in the range 100-1500 m/z both in positive and negative mode with event time 0.2 s. or in the range 350-1300 m/z in positive mode with event time 0.2 s.

GS-441524 monophosphate

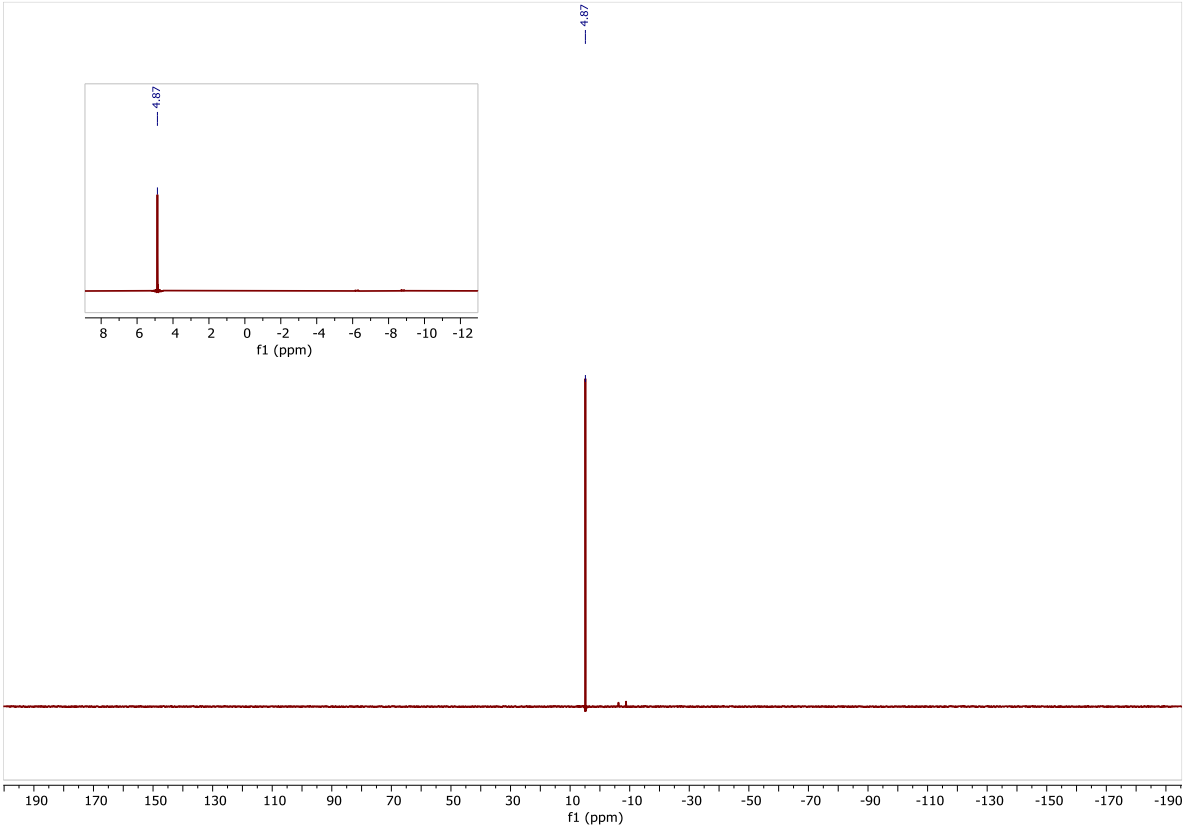
The commercially available nucleoside GS-441524 (CAS No.: 1191237-69-0, 10 mg, 0.034 mmol) was dried under vacuum for 2 hours at 50 °C. The solid was dissolved in dry trimethylphosphate (300 µL) and the resulting solution was cooled to 0 °C. Freshly distilled $POCl_3$ (1.2 equiv., 0.04 mmol, 6 mg, 4 µL) was added and the reaction mixture was stirred at 0 °C for 1 hour. The reaction was quenched by dropwise addition of cold 2M TEAB (1 mL). The mixture was concentrated on a rotary evaporator and the residue was co-evaporated several times with milli-Q water. The crude product was dissolved in water (ca 3 mL), filtered and purified by semi-preparative HPLC using a linear gradient of methanol (5→100%) in 0.1 M TEAB buffer. The appropriate fractions were combined and evaporated on a rotary evaporator. The viscous oil was co-evaporated three times with milli-Q water and the product was finally freeze-dried from water to obtain a white solid (11 mg, triethylammonium salt, 66% yield).

^1H NMR (400 MHz, D_2O) δ 7.77 (s, 1H), 6.83 (d, *J* = 4.7 Hz, 1H), 6.67 (d, *J* = 4.7 Hz, 1H), 4.83 (d, *J* = 5.6 Hz, 1H), 4.47 – 4.32 (m, 3H), 3.95 – 3.81 (m, 2H). ^{31}P NMR (162 MHz, D_2O) δ 4.87. MS (calcd. for $C_{12}H_{14}N_5O_7P$): 371.063; found $[M+H]^+$ 372.10; found $[M-H]^-$ 370.10.

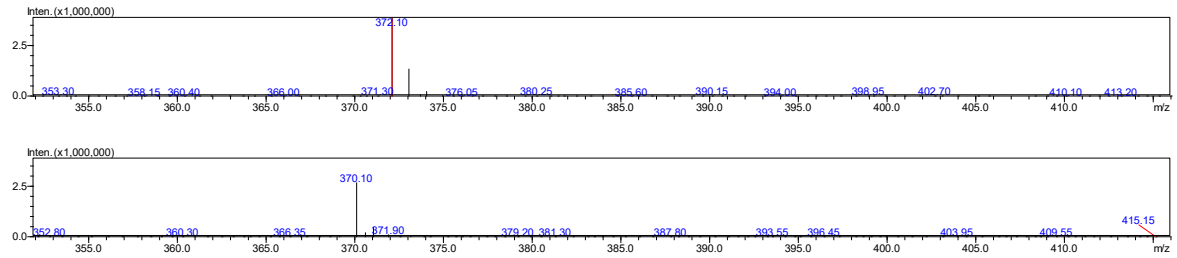
^{1}H NMR of GS-441524 monophosphate



^{31}P NMR of GS-441524 monophosphate



Mass analysis of GS-441524 monophosphate

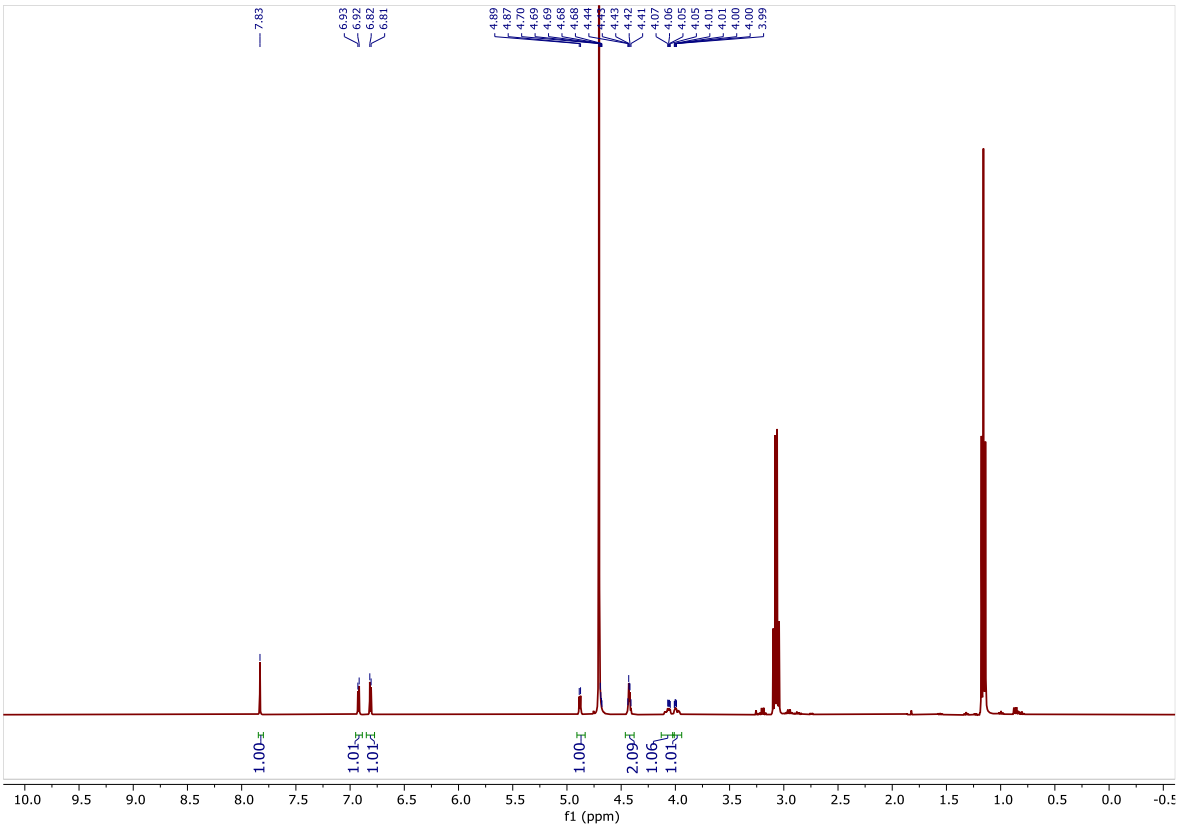


GS-441524 diphosphate

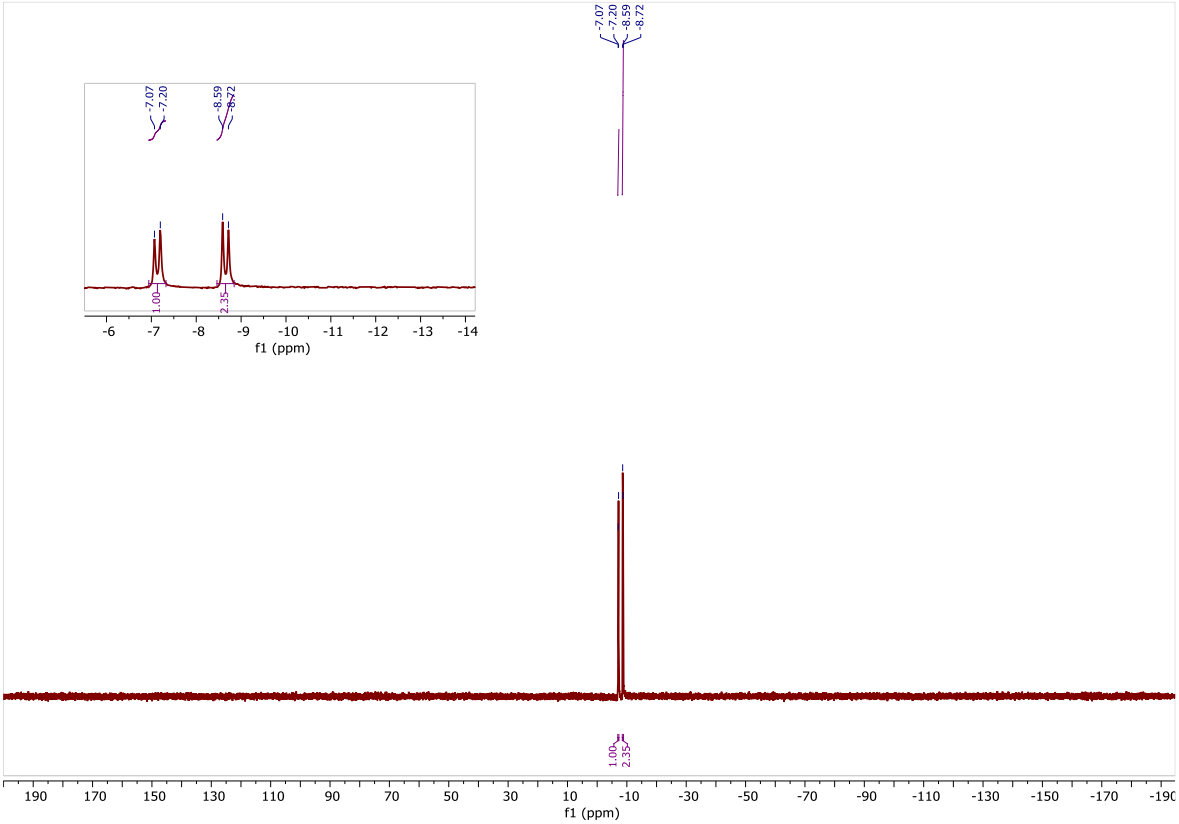
The commercially available nucleoside GS-441524 (CAS No.: 1191237-69-0, 50 mg, 0.172 mmol) was dried under vacuum for 2 hours at 50 °C. The solid was dissolved in dry trimethylphosphate (750 µL) and the resulting solution was cooled at 0 °C. Freshly distilled $POCl_3$ (1.2 equiv., 0.206 mmol, 32 mg, 19.3 µL) was added and the reaction mixture was stirred at 0 °C for 1 hour. Then, an ice-cold solution of tributylammonium orthophosphate (5 equiv., 0.86 mmol, 402 mg) and tributylamine (5 equiv., 0.86 mmol, 159 mg, 204 µL) in anhydrous acetonitrile (2 mL) was added dropwise to the reaction mixture at 0 °C. The reaction mixture was stirred for 1.5 hour at 0 °C and then quenched by dropwise addition of cold 2M TEAB (1 mL). The mixture was concentrated on a rotary evaporator and the residue was co-evaporated several times with milli-Q water. The crude product was dissolved in water (ca 3 mL), filtered and purified by semi-preparative HPLC using a linear gradient of methanol (5→100%) in 0.1 M TEAB buffer. The appropriate fractions were combined and evaporated on a rotary evaporator. The viscous oil was co-evaporated three times with milli-Q water and the product was finally freeze-dried from water to obtain a white solid (25 mg, triethylammonium salt, 23% yield).

^{1}H NMR (401 MHz, D_2O) δ 7.83 (s, 1H), 6.92 (d, *J* = 4.7 Hz, 1H), 6.81 (d, *J* = 4.8 Hz, 1H), 4.88 (d, *J* = 4.8 Hz, 1H), 4.42 (dd, *J* = 5.9, 2.5 Hz, 2H), 4.06 (dd, *J* = 6.3, 3.1 Hz, 1H), 4.03 – 3.94 (m, 1H). ^{31}P NMR (162 MHz, D_2O) δ -8.24 (d, *J* = 19.4 Hz), -8.88 (d, *J* = 19.9 Hz), -20.67 (t, *J* = 19.7 Hz). MS (calcd. for $C_{12}H_{15}N_5O_{10}P_2$): 451.029; found $[M+H]^+$ 452.05; found $[M-H]^-$ 449.95.

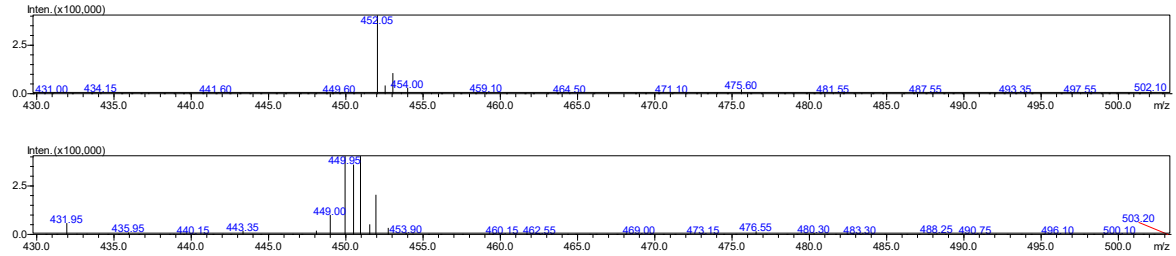
^{1}H NMR of GS-441524 diphosphate



^{31}P NMR of GS-441524 diphosphate



Mass analysis of GS-441524 diphosphate

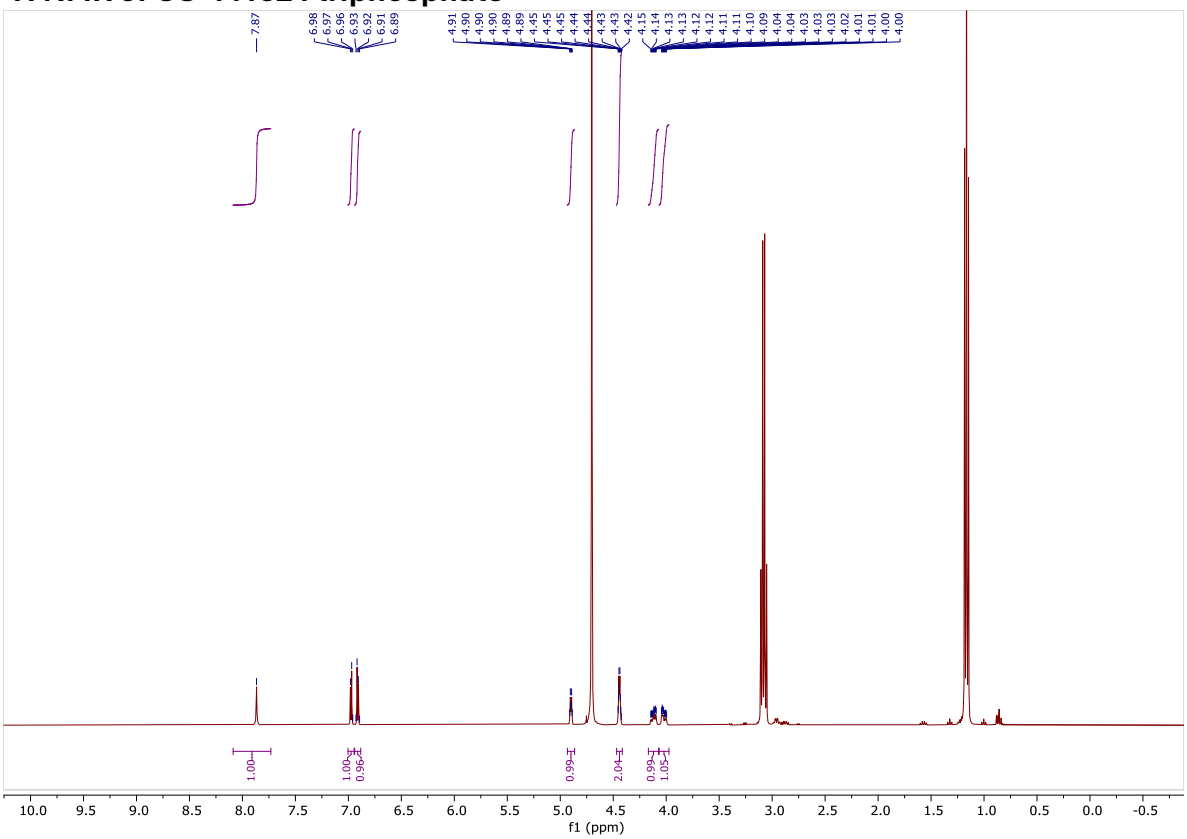


GS-441524 triphosphate

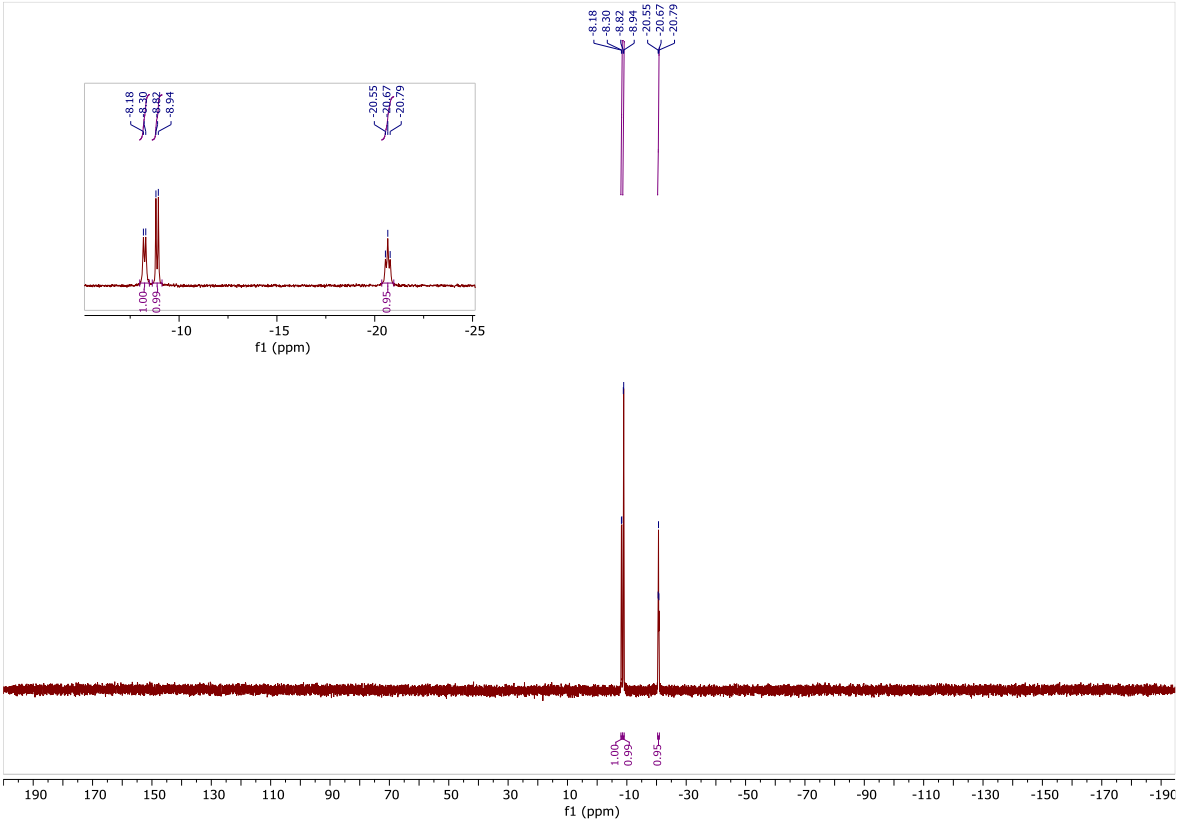
The commercially available nucleoside GS-441524 (CAS No.: 1191237-69-0, 50 mg, 0.172 mmol) was dried under vacuum for 2 hours at 50 °C. The solid was dissolved in dry trimethylphosphate (750 µL) and the resulting solution was cooled at 0 °C. Freshly distilled $POCl_3$ (1.2 equiv., 0.206 mmol, 32 mg, 19.3 µL) was added and the reaction mixture was stirred at 0 °C for 1 hour. Then, an ice-cold solution of tributylammonium pyrophosphate (5 equiv., 0.86 mmol, 471 mg) in anhydrous acetonitrile (6 mL) was added dropwise to the reaction mixture at 0 °C. The reaction mixture was stirred for 1 hour at 0 °C and then quenched by dropwise addition of cold 2M TEAB (1 mL). The mixture was concentrated on a rotary evaporator and the residue was co-evaporated several times with milli-Q water. The crude product was dissolved in water (ca 3 mL), filtered and purified by semi-preparative HPLC using a linear gradient of methanol (5→100%) in 0.1 M TEAB buffer. The appropriate fractions were combined and evaporated on a rotary evaporator. The viscous oil was co-evaporated three times with milli-Q water and the product was finally freeze-dried from water to obtain a white solid (83 mg, triethylammonium salt, 58% yield).

^{1}H NMR (401 MHz, Deuterium Oxide) δ 7.87 (s, 1H), 6.97 (d, *J* = 4.8 Hz, 1H), 6.91 (d, *J* = 4.7 Hz, 1H), 4.93 – 4.86 (m, 1H), 4.44 (dd, *J* = 3.4, 1.8 Hz, 2H), 4.17 – 4.07 (m, 1H), 4.07 – 3.97 (m, 1H). ^{31}P NMR (162 MHz, Deuterium Oxide) δ -8.24 (d, *J* = 19.4 Hz), -8.88 (d, *J* = 19.9 Hz), -20.67 (t, *J* = 19.7 Hz). MS (calcd. for $C_{12}H_{16}N_5O_{13}P_3$): 530.996; found $[M+H]^+$ 531.90; found $[M-H]^-$ 529.70.

^{1}H NMR of GS-441524 triphosphate



^{31}P NMR of GS-441524 triphosphate



Mass analysis of GS-441524 triphosphate

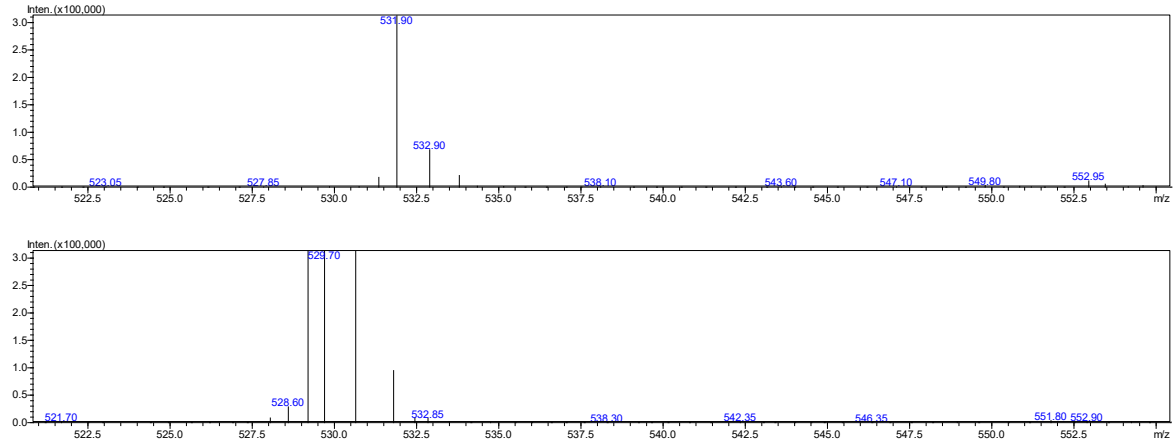


Figure S1

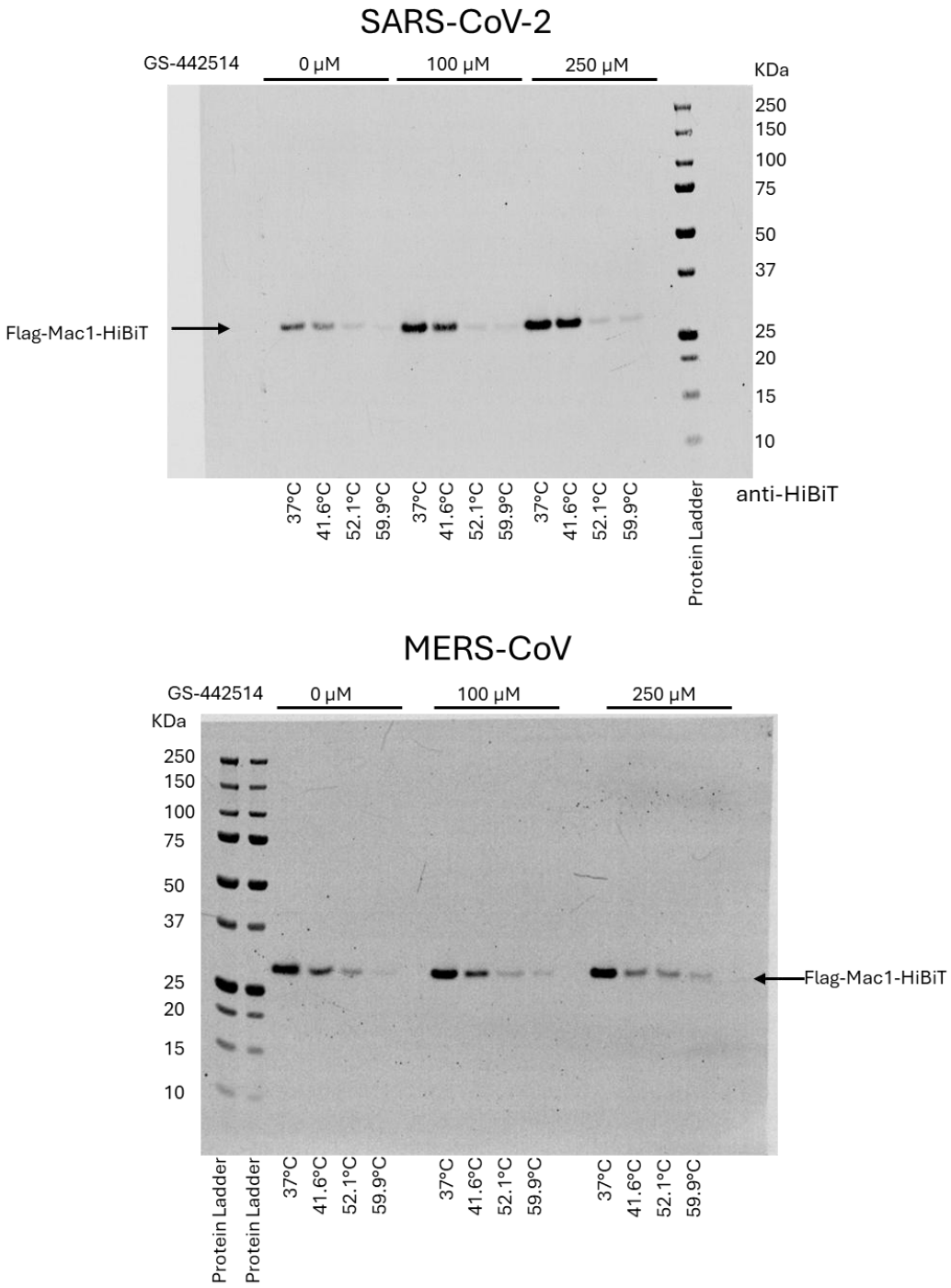


Figure S1: *Full-length blots for Figure 1E*. Western blot showing a dose-dependent stabilization of SARS-CoV-2 Mac1 but not MERS-CoV Mac1 at different temperatures using anti-HiBiT antibody.

Figure S2

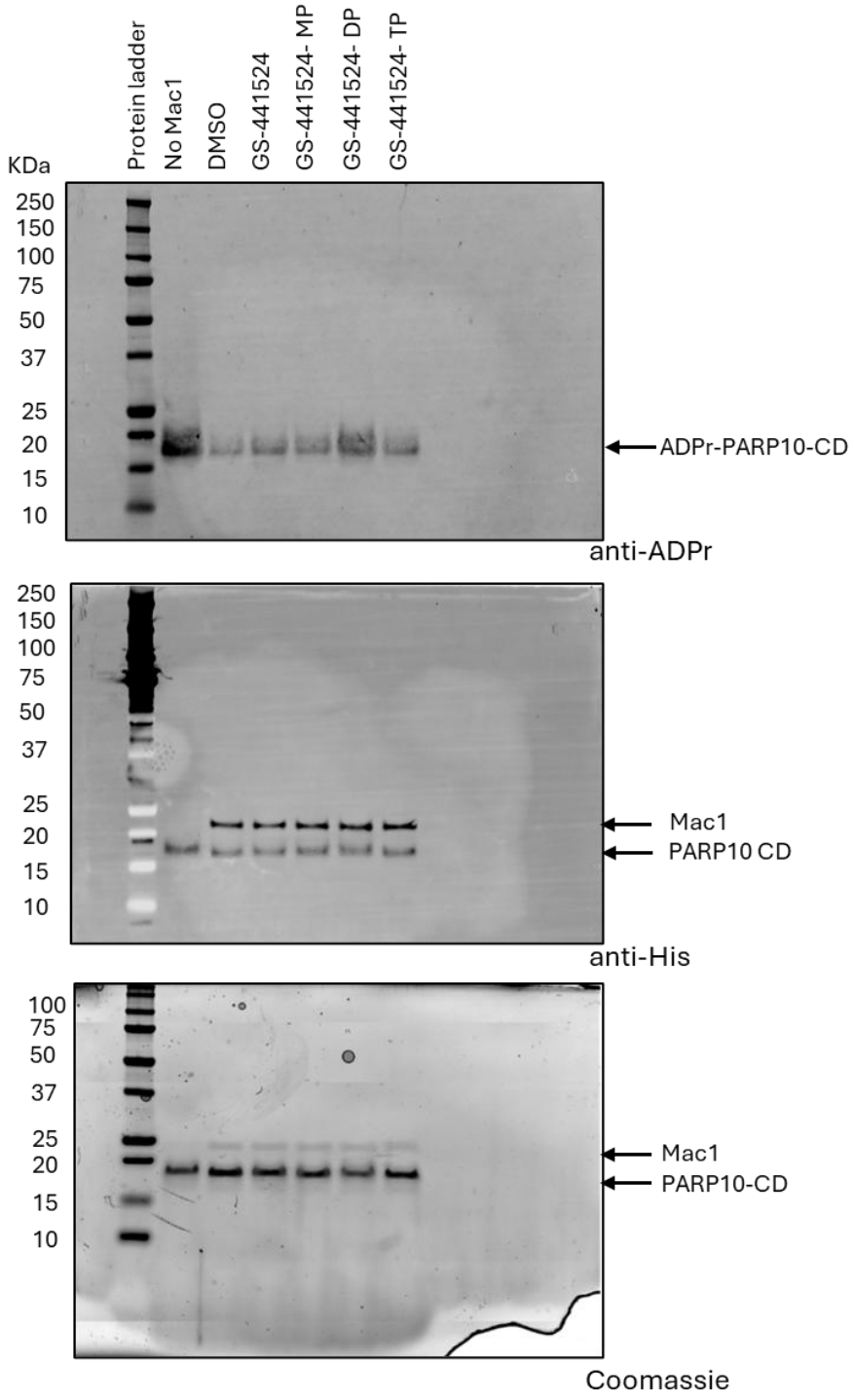
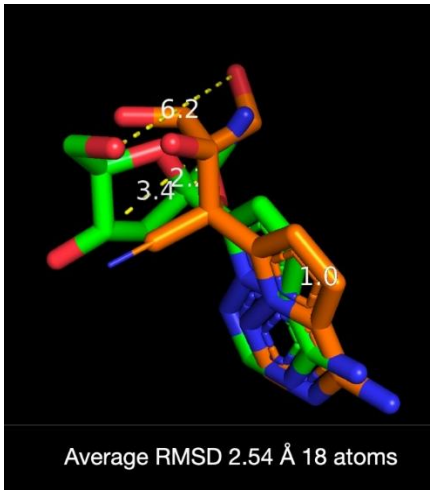

Protein ladder
No Mac1
DMSO
GS-441524
GS-441524- MP
GS-441524- DP
GS-441524- TP
KDa
250
150
100
75
50
37
25
20
15
10
ADPr-PARP10-CD
anti-ADPr
Mac1
PARP10 CD
anti-His
Mac1
PARP10-CD
Coomassie

Figure S2: *Full-length Gels and blots for Figure 2D.* Western blot showing inhibition of Mac1, indicated by increased ADP-ribosylated substrate signal upon treatment with GS-441524 or its phosphorylated derivatives compared with DMSO control. His-tagged PARP-10 ADP-ribosylated substrate and the His-tagged Mac1 used in the reaction are probed using anti-His antibody and monitored using Coomassie stain to show equal input amounts across different treatments.

Figure S3

A



B

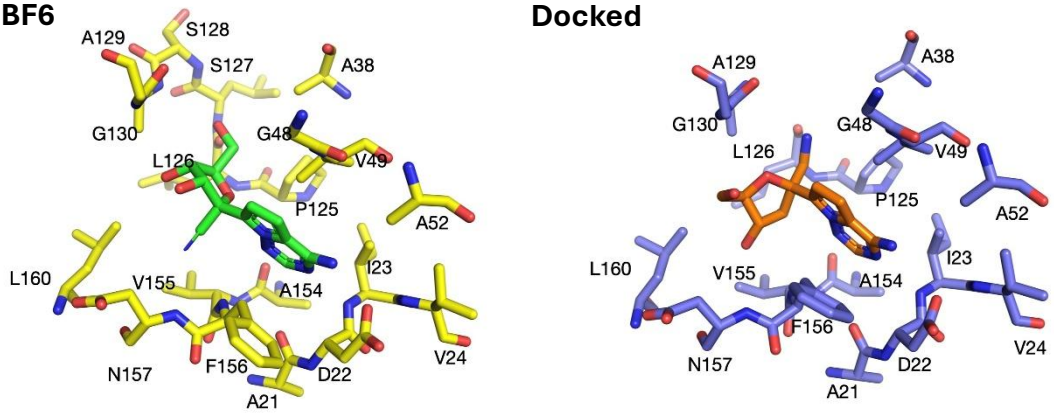


Figure S3: A. Comparison of docked GS-441524 with its crystal structure (PDB: 7BF6), demonstrating excellent agreement between the predicted and observed structures bound to SARS-CoV-2 Mac1. B. Comparison of network interactions of GS-441524 with the SAS-CoV-2 Mac1 in the crystal structure vs the docked pose. Residues within 5 Å of either the

docked ligand or the ligand in the crystal structure (7BF6) are shown. In the predicted structure, the ribose adopts a flipped conformation, positioning S128 and S127 approximately 6 Å away; consequently, these residues are not observed in the docked pose.