Electronic Supplementary Material (ESI) for Chemical Science.

Azapeptide activity-based probes for the SARS-CoV-2 main protease enable visualization of inhibition in infected cells

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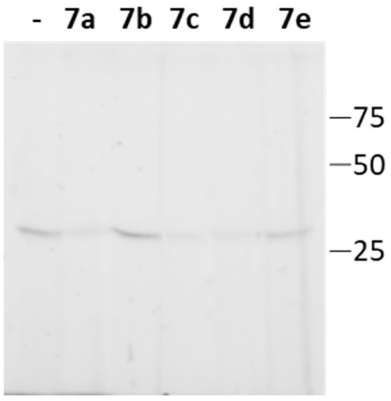


Figure S1. Screening of probes **7a-e** (10 μM) on purified SARS-CoV-2 M^{pro} in competition with fluorescent M^{pro}-reactive probe TAMRA-Abu-Tle-Leu-Gln-AOMK (5 μM). All probes except **7b** show inhibition.

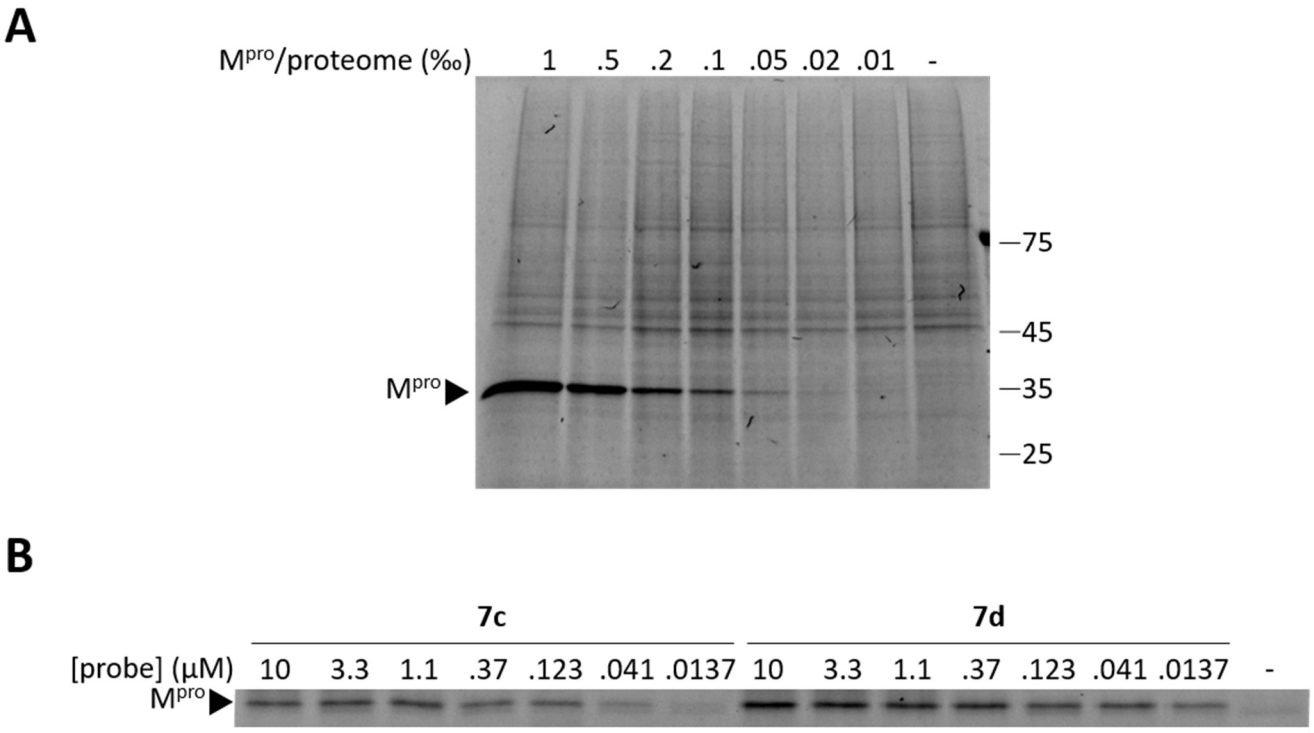


Figure S2. (A**)** Labeling of decreasing amounts of recombinant M^{pro}, spiked in HEK293T lysates (1 mg/mL total protein, pH 7.4) with probe **7d**. Probe-labeled M^{pro} can still be visualized up to a concentration of 0.05‰ of the total protein (= 50 ng/mL). (B) Labelling of 1 µg/mL recombinant M^{pro} (1 µg/mL, activity-adjusted concentration = 20 nM, pH 7.4) with decreasing probe concentrations of **8c** and **8d**. Probe-labeled M^{pro} can be detected with **8c** down to 41 nM and with **8d** down to the substoichiometric concentration of 13.7 nM.

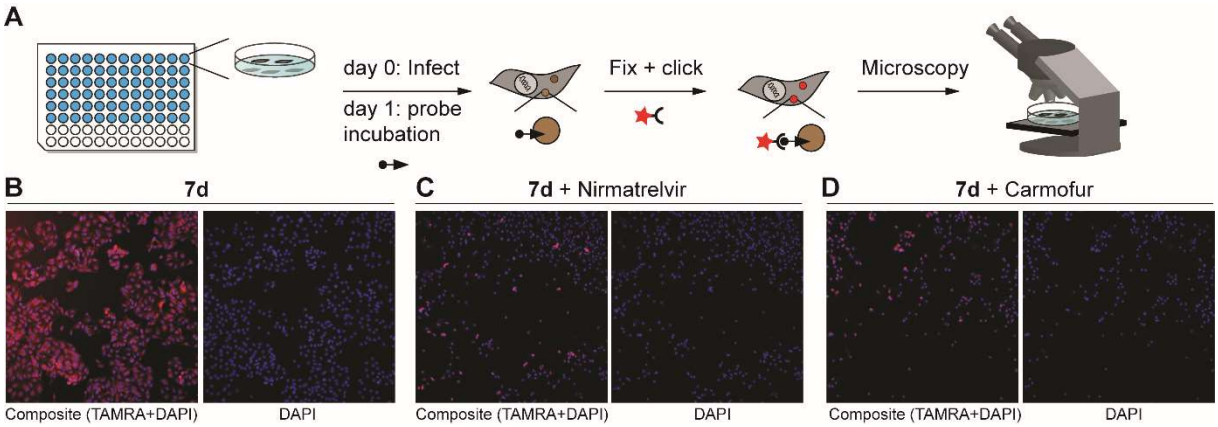


Figure S3. Fluorescence microscopy of virally infected cells, treated with or without competitor. (A) Summary of workflow. One day after infection of VeroE6 cells, probes were added to cells – with or without inhibitor pretreatment. Click chemistry with TAMRA-N_3 was performed after fixation, followed by fluorescent microscopy. (B-D) Treatment with probe **7d** (10 μM) results in red staining of infected cells, which can be attributed to M^{pro} activity, as M^{pro} inhibitors Nirmatrelvir (panel C; 5 μM) and Carmofur (panel D; 50 μM) dramatically reduce the signal by probe **7d**.

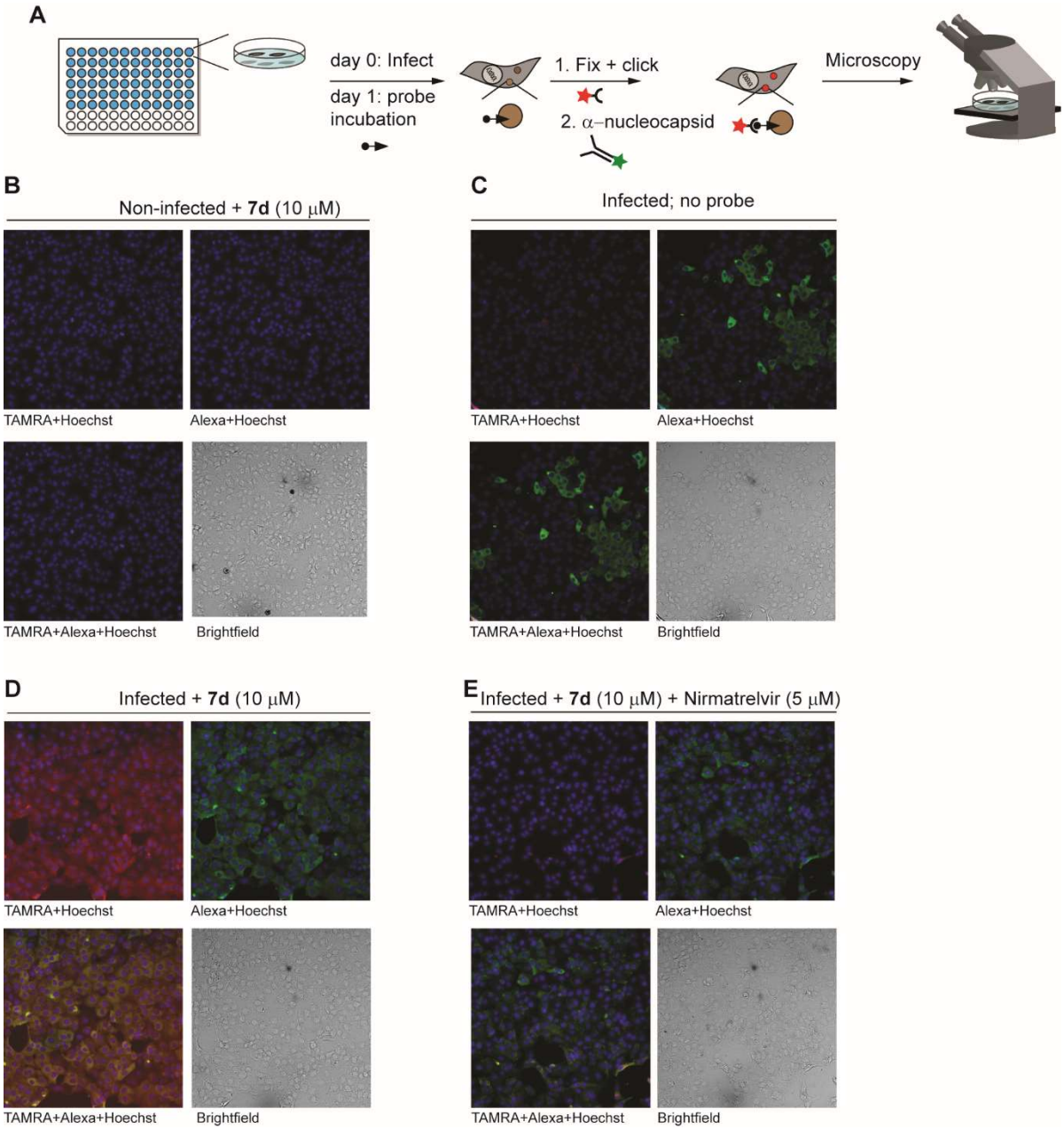


Figure S4. Overlay channels as well as brightfield microscopy imaging of the pictures presented in Figure 4 in the main text.

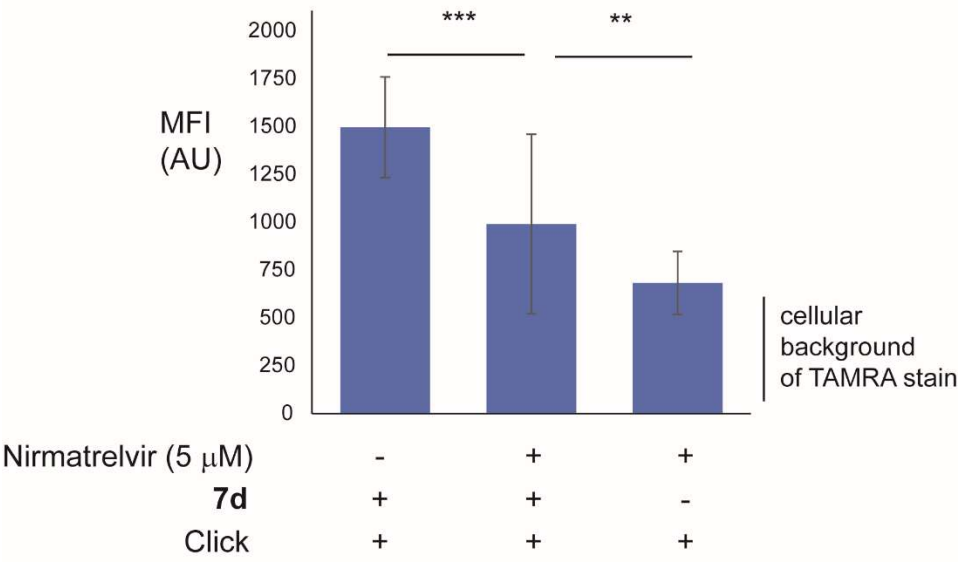


Figure S5. Quantification of cellular fluorescence after probe treatment (with or without Nirmatrelvir pretreatment). Note that cellular fluorescence is relativelty high, likely due to the low probe cell permeability and the high TAMRA-azide concentration during click chemistry. Future work may circumvent this by improving cell permeability (see conclusions) as well as making a probe that is directly attached to a fluorophore, which will lower total fluorophore background.

Experimental Procedures

General materials and methods

All starting materials for chemical reactions were purchased from commercial suppliers and used without purification. All solvents were of synthesis grade or higher. Progress of solution phase reactions was monitored by TLC on pre-coated 0.20 mm thick ALUGRAM® TLC sheets (silicagel-60 with UV indicator) using UV light and/or staining with cerium ammonium molybdate or ninhydrin followed by heating. Nuclear magnetic resonance spectra were recorded on a Bruker Ultrashield™ 300 or 600 MHz NMR Spectrometer. Chemical shifts are reported in ppm relative to the residual solvent peak. LC-MS spectra were recorded on a Prominence Ultra-fast Liquid Chromatography system (Shimadzu) using a Waters X-bridge 2.1 mm C18 column with a gradient of 5%-80% acetonitrile in water (with 0.1% formic acid) over 22 min. HPLC purification was performed on a Prominence Ultra-fast Liquid Chromatography system (Shimadzu) using a Waters X-bridge 150 mm C18 prep column with a gradient of acetonitrile in water (with 0.1% trifluoroacetic acid) over 32 min. Purity >95% of all final compounds was assessed by integration of the HPLC spectra.

1-Allyl 2-(*tert*-butyl) 1-(3-(*tert*-butoxy)-3-oxopropyl)hydrazine-1,2-dicarboxylate (3)

tert-Butyl carbazate (300 mg, 2.27 mmol) was dissolved in 4 mL dry DMF. DIEA (808 µL, 4.54 mmol) and *tert*-butyl 3-bromopropanoate (379 µL, 2.27 mmol) were added to this solution, which was heated to 40°C and stirred for 24 hours. Upon completion of the reaction (TLC: EtOAc/petroleum ether 3:7), the solvents were evaporated. The crude material was redissolved in 4 mL dry DMF and cooled to 0°C. DIEA (989 µL, 5.67 mmol) and Allyloxycarbonyl chloride (362 µL, 3.4 mmol) were added to the reaction mixture. The reaction mixture was allowed to warm to room temperature and stirred overnight. Upon completion of the reaction (TLC: EtOAc/petroleum ether 1:4), the reaction was diluted with 20 mL $NaHCO_3$ (sat. aq.) and extracted five times with 20 mL EtOAc. The organic fraction was dried over $MgSO_4$ and evaporate to dryness. The crude product was purified over column

chromatography (EtOAc/petroleum ether 1:4 to 1:3) to yield the title compound as a colorless oil. (204.53 mg, yield = 26.2%). ^{1}H NMR (300 MHz, $CDCl_3$, 298K): 5.35-5.30 (m, 1H), 4.67-4.58 (m, 2H), 3.82-3.75 (m, 2H), 2.55 (t, 2H, *J* = 6.5 Hz), 1.63-1.62 (m, 1H), 2.30-2.17 (m, 4H), 1.49-1.46 (m, 9H), 1.44 (s, 9H). LC-MS: m/z calcd. for $C_{16}H_{29}N_2O_6$ $[M+H]^+$ 345.20, found: 345.00.

2-Fmoc 1-allyl 1-(3-(*tert*-butoxy)-3-oxopropyl)hydrazine-1,2-dicarboxylate (4)

Compound **3** (120 mg, 348 µmol) was dissolved in 3 mL DCM, to which 3 mL TFA was added. The reaction was stirred for 30 minutes at room temperature. Upon completion of the reaction (EtOAc/petroleum ether 1:4), the solvents were evaporated and the crude material co-evaporated with toluene to remove residual TFA. The crude product was redissolved in 2 mL DCM and cooled to 0°C on ice. DIEA (125 µL, 700 µmol) and Fmoc-Cl (135 mg, 522 µmol) were added to the reaction mixture, after which the ice was removed. The round-bottom flask was equipped with a cooler and the reaction mixture was heated to 40°C and stirred overnight. Upon completion of the reaction (TLC: DCM/MeOH 20:1), the solvents were evaporated and the crude product purified over column chromatography (DCM/MeOH 1:0 -> 20:1) (98.67 mg, yield = 69.0%). ^{1}H NMR (300 MHz, $CDCl_3$, 298K): 7.76 (d, *J* = 7.7 Hz), 7.62–7.56 (m, 2H), 7.40 (t, 2H, *J* = 7.5 Hz), 7.31 (t, 2H, *J* = 7.4 Hz), 6.98-6.92 (m, 1H), 5.96-5.81 (m, 1H), 5.37-5.12 (m, 2H), 4.68-4.42 (m, 4H), 4.28-4.22 (m, 2H), 2.60-2.40 (m, 2H), 1.44 (s, 9H). LC-MS: m/z calcd. for $C_{22}H_{23}N_2O_6$ $[M+H]^+$ 411.15, found: 410.90.

Solid phase chemistry procedures

Coupling of compound 4 to Rink amide resin (5)

Fmoc protected Rink amide resin (244 mg, 180 µmol) (0.74 mmol active sites/g) was treated with piperidine/DMF 1/4 (2.5 mL) for 5 min to swell the resin and deprotect the Fmoc group. Next, the resin was washed three times with DMF. All following Fmoc deprotections were performed according to this procedure.

Compound **4** (85 mg, 207 µmol, 1.15 eq.) was coupled to the deprotected rink amide resin with HBTU (1.15 eq.) and DIPEA (3 eq.) in 1200 µL of DMF by incubating overnight at room temperature. After this and each following coupling, the resin was washed three times with DMF and three times with DCM.

Synthesis of the peptide backbone (6)

After deprotection of **5**, Fmoc-L-Leu-OH was coupled to the unprotected nitrogen on the resin by adding amino acid (15 eq.), HBTU (15 eq.) and DIPEA (30 eq.) in 1200 µL DMF and incubating overnight at room temperature. After deprotection of the N-terminus, subsequently Fmoc-L-Tle-OH and hexynoic acid were coupled to the resin by adding (amino)acid (3 eq.), HBTU (3 eq.) and DIEA (10 eq.) in 1200 µL DMF and incubating for 4h.

Warhead coupling

After synthesis peptide backbone **6**, the resins were divided into aliquots of 10 µmol for coupling of the warheads. First, the Alloc group was removed from the resins using general procedure 1.

*General procedure 1: Alloc deprotection of **6***. Alloc groups were removed by first washing the resin three times with dry DCM. Then, the resin was suspended in 1 mL dry DCM, to which $Pd(PPh_3)_4$ (0.25 eq.) and $PhSiH_3$ (24 eq.) were added under an Ar atmosphere. Reactions were incubated for 30 min, after which the solutions were drained and the resin was once more incubated with fresh deprotection solution. Afterwards, the resin was washed 3x with DCM.

Ethyl (2*S*,3*S*)-3-(1-(3-amino-3-oxopropyl)-2-(((*S*)-2-(hex-5-ynamido)-3,3-dimethylbutanoyl)-L-leucyl) hydrazine-1-carbonyl)oxirane-2-carboxylate (7a)

After deprotection of the resin using the general procedure, the resin (10 µmol active sites) was suspended in 600 µL dry DMF, to which DIEA (27 µL, 150 µmol), ethyl (2*S*,3*S*)-epoxysuccinate (16 mg, 100 µmol) and HBTU (38 mg, 100 µmol) were added. The reaction was incubated overnight at room temperature and checked for completion using LC/MS. After completion, the resin was washed three times with DCM. The probe was cleaved from the resin by using a 95:2.5:2.5 mixture of TFA:triisopropyl silane:water and purified using HPLC. The product was obtained as a transparent oil. (0.16 mg, yield = 2.8%) HRMS (MALDI-TOF): m/z calcd. for $C_{27}H_{43}N_5O_8Na$ $[M+Na]^+$ 588.3009, found: 588.3500.

N-((*S*)-1-(((*S*)-1-(2-(3-amino-3-oxopropyl)-2-(vinylsulfonyl)hydrazinyl)-4-methyl-1-oxopentan-2-yl)amino)-3,3-dimethyl-1-oxobutan-2-yl)hex-5-ynamide (7b)

After deprotection of the resin using the general procedure, the resin (10 µmol active sites) was suspended in 600 µL dry DCM, to which DIEA (27 µL, 150 µmol) and 2-chloroethane-1-sulfonyl chloride (11 µL, 100 µmol) were added. The reaction was incubated overnight at room temperature and checked for completion using LC/MS. After completion, the resin was washed three times with DCM. The probe was cleaved from the resin by using a 95:2.5:2.5 mixture of TFA:TIS:water and purified using HPLC. The product was obtained as a transparent oil. (0.04 mg, yield = 0.8%) HRMS (MALDI-TOF): m/z calcd. for $C_{23}H_{39}N_5O_6SNa$ $[M+Na]^+$ 536.2619, found: 536.2877.

Methyl (*E*)-4-(1-(3-amino-3-oxopropyl)-2-(((*S*)-2-(hex-5-ynamido)-3,3-dimethylbutanoyl)-L-leucyl)hydrazinyl)-4-oxobut-2-enoate (7c)

After deprotection of the resin using the general procedure, the resin (10 µmol active sites) was suspended in 600 µL dry DCM, to which DIEA (27 µL, 150 µmol), monomethyl fumarate (13 mg, 100 µmol) and HBTU (38 mg, 100 µmol) were added. The reaction was incubated overnight at room temperature and checked for completion using LC/MS. After completion, the resin was washed three times with DCM. The probe was cleaved from the resin by using a 95:2.5:2.5 mixture of TFA:TIS:water and purified using HPLC. The product was obtained as

a transparent oil. (0.33 mg, yield = 6.2%) HRMS (MALDI-TOF): m/z calcd. for $C_{26}H_{41}N_5O_7Na$ $[M+Na]^+$ 558.2904, found: 558.3372.

N-((*S*)-1-(((*S*)-1-(2-(3-amino-3-oxopropyl)-2-(2-chloroacetyl)hydrazinyl)-4-methyl-1-oxopentan-2-yl)amino)-3,3-dimethyl-1-oxobutan-2-yl)hex-5-ynamide (7d)

After deprotection of the resin using the general procedure, the resin (10 µmol active sites) was suspended in 600 µL dry DCM, to which DIEA (27 µL, 150 µmol) and chloroacetyl chloride (8 µL, 100 µmol) were added. The reaction was incubated overnight at room temperature and checked for completion using LC/MS. After completion, the resin was washed three times with DCM. The probe was cleaved from the resin by using a 95:2.5:2.5 mixture of TFA:TIS:water and purified using HPLC. The product was obtained as a transparent oil. (0.23 mg, yield = 4.6%) HRMS (MALDI-TOF): m/z calcd. for $C_{23}H_{39}N_5O_6SNa$ $[M+Na]^+$ 522.2459, found: 522.2564.

N-((*S*)-1-(((*S*)-1-(2-(3-amino-3-oxopropyl)-2-(2-chloro-5-nitrobenzoyl)hydrazinyl)-4-methyl-1-oxopentan-2-yl)amino)-3,3-dimethyl-1-oxobutan-2-yl)hex-5-ynamide (7e)

After deprotection of the resin using the general procedure, the resin (10 µmol active sites) was suspended in 600 µL dry DCM, to which DIEA (27 µL, 150 µmol), 2-chloro-5-nitrobenzoic acid (13 mg, 100 µmol) and HBTU (38 mg, 100 µmol) were added. The reaction was incubated overnight at room temperature and checked for completion using LC/MS. After completion, the resin was washed three times with DCM. The probe was cleaved from the resin by using a 95:2.5:2.5 mixture of TFA:TIS:water and purified using HPLC. The product was obtained as a white solid. (0.24 mg, yield = 4.0%) HRMS (MALDI-TOF): m/z calcd. for $C_{26}H_{42}N_5O_7$ $[M+Na]^+$ 606.2569, found: 606.1404.

Production of SARS-CoV-2 M^{pro}. The expression and purification of M^{pro} was performed as reported previously by Van De Plassche and Barniol-i-Xicota et al.[1]

Quikchange mutagenesis of SARS-CoV-2 M^{pro}

In order to generate the C145A active site mutant, two primers were designed with the following sequence: 5'-AAA AGC CCA CCG AAC CAG CGC TGC CAT TTA GGA AGC-3' and 5'-GCT TCC TTA ATG GCA GCG CTG GTT CGG TGG GCT TTT-3' (synthesized by Integrated DNA Technologies). A PCR mixture was made containing 37 ng of the pGEX-6p-1 vector containing WT SARS-CoV-2 M^{pro}, 125 ng of each primer, 200 μM dNTP (final concentration), 2U of HiFi DNA polymerase (VWR, Belgium), 1x HiFi buffer (VWR, Belgium) in a final volume of 50 μL. Next, 25 cycles were run in a thermocycler with 30 s melting at 95 °C, 60 s annealing at 55 °C and 6 min extension at 68 °C, with a final extension at the end of all cycles of 10 min. After the PCR, 1 μL of fast digest Dpn-I (Life Technologies) was added and incubated for 30 min at 37 °C and subsequently 5 min at 80 °C. Presence of the mutagenized plasmid was checked on an aliquot of the mixture using an agarose gel. The sample was frozen until transformation into chemocompetent *E. coli* strain DH5α. Mutagenized plasmid was isolated by miniprep and verified by DNA sequencing (LGC Genomics).

Initial activity-assessment of probes 7a-e

30 µL purified Mpro (16.67 ng/ µL) in HEPES buffer (50 mM HEPES,150 mM NaCl,1 mM DTT, pH 7.5) was incubated with 0.3 µL of a DMSO stock (1 mM) of the indicated probe (**7a-e**) or 0.3 µL DMSO for 30 min at 37 °C. Next, 0.3 µL of a DMSO stock (500 µM) of the reported fluorescent M^{pro}-reactive probe TAMRA-Abu-Tle-Leu-Gln-AOMK[1] was added to the samples, which were incubated for another 30 min at 37 °C. 4x Sample buffer was added to the samples, which were boiled at 95 °C for 2 min. Samples were loaded onto a 15% acrylamide gel and resolved with SDS-PAGE. The gel was read on a Typhoon FLA 9500 scanner with excitation at 532 nm and detection at 568 nm with the photomultiplier set at 900 V. Residual enzyme activity in the probe-treated samples was quantified by measuring the fluorescence using ImageJ software and comparing to the fluorescence measured for the DMSO-treated control sample.

Production of SARS-CoV-2 M^{pro} mutant C145A.

An overnight culture was prepared from a single colony of freshly transformed bacteria (Escherichia coli strain BL21 (DE3) for Mpro C145A). The overnight culture was diluted 200 times into LB media with 100 µg/mL Ampicillin kept at 37°C and 300 rpm. Once the OD_{600} = 0.7 the expression was induced with 0.5 mM IPTG, and shaken at 18°C for 18h. The cells were pelleted at 5500 rpm at 4°C for 15 min. Pelleted cells were lysed by 5 rounds of sonication (10 sec on, 10 sec off) on ice in lysis buffer (20 mM Tris, pH 7.8; 150 mM NaCl). The mixture was centrifuged at 100,000 × g, 60 min, 4°C to pellet the membrane fraction. The soluble fraction containing the inactive mutant of the SARS-CoV-2 main protease was incubated for 3 h at room temperature with active M^{pro} (0.67 mg/mL in assay buffer) in order to cleave the GST tag and generate the native N-termini of the M^{pro} C145A. After this time the whole was subjected to nickel-nitrilotriacetic acid beads (Qiagen; 1 mL beads per 1 L bacterial expression culture) for 1 h at room temperature. After this time the inactive protease was eluted by increasing imidazole washing steps (25 mM, 50 mM, 200 mM, and 500 mM final elution in 20 mM Tris-NaOH, pH 7.8, 150 mM NaCl). The elution fractions corresponding

to 200 mM imidazole were diluted to a final protein concentration of 1 mg/mL in order to avoid protein precipitation (concentration measured using a Bradford colorimetric assay). To those fractions PreScission protease was added to cleave the C-terminal His_6 tag, following the instructions of the commercial vendor (Sigma-Aldrich, ref GE27-0843-01). The protease mixture was transferred into a dialysis cassette 10 K cut-off and dialyzed against reaction buffer 1 (20 mM Tris, 150 mM NaCl, 1 mM DTT, 1mM EDTA, pH 7.8) or reaction buffer 2 (20 mM HEPES-NaOH, 150 mM NaCl, 1 mM DTT, 1mM EDTA, pH 7.8) for 22 h at 4°C to ensure cleavage completion. As a final step, the whole was incubated with GST-beads for 1h at room temperature. After that time the sample was spun down at 500g, 4°C, for 10 min and the supernatant collected, one wash with either reaction buffer 1 or 2 was performed in order to ensure complete elution of M^{pro} C145A. The combined fractions were then concentrated to half of their volume using an Amicon ultra 15 centrifugal filters (10 kDa, Merck Millipore) at 3000 rpm and 4°C.

Labeling limit of probes 7c and 7d on purified M^{pro}

Limit of probe detection was measured by using seven different compound concentrations of probes **7c** and **7d** in a 1:3 serial dilution, starting at a highest concentration of 10 μM. To this end, 30 μL of active M^{pro} (1 μg/mL) (50 mM HEPES, 150 mM NaCl, 1 mM DTT, pH 7.5) was incubated for 1 h at room temperature with 0.3 μL of a 100x stock solution of probe in DMSO or 0.3 μL DMSO. Next, 0.5 μL TAMRA-azide (1.5 mM in DMSO), 0.3 μL THPTA (5 mM in 4:1 t-BuOH: DMSO), 0.6 μL sodium ascorbate (50 mM in water) and 0.6 μL $CuSO_4$ (50 mM in water) were added to each sample and the samples were incubated 1 h at room temperature. Afterwards, 10 μL of 4x sample buffer was added to the samples, which were subsequently boiled at 95 °C for 2 minutes. Samples were resolved on an SDS-PAGE 12% acrylamide gel and visualized by in-gel fluorescence measuring on a Typhoon FLA 9500 scanner with excitation at 532 nm and detection at 568 nm with the photomultiplier set at 900 V .

Detection limit of M^{pro} in lysates by detection with probe 7d

Limit of enzyme detection was measured by spiking HEK 293T lysates (1 mg/mL) (50 mM HEPES, 150 mM NaCl, 1 mM DTT, pH 7.5) with a dilution series of seven concentrations of active M^{pro} ranging from 1 µg/mL to 10 ng/mL. A non-spiked sample was used as a negative control. 30 µL of each lysate was labeled with 0.3 µL of a 100 µM stock of probe **7d** (DMSO) for 1h. Next, 0.5 µL TAMRA-azide (1.5 mM in DMSO), 0.3 µL THPTA (5 mM in 4:1 t-BuOH: DMSO), 0.6 µL sodium ascorbate (50 mM in water) and 0.6 µL $CuSO_4$ (50 mM in water) were added to each sample and the samples were incubated 1 h at room temperature. Afterwards, 10 µL of 4x sample buffer was added to the samples, which were subsequently boiled at 95 °C for 2 minutes. Samples were resolved on an SDS-PAGE 12% acrylamide gel and visualized by in-gel fluorescence measuring on a Typhoon FLA 9500 scanner with excitation at 532 nm and detection at 568 nm with the photomultiplier set at 900 V.

Direct labelling of probe targets

30 µL of HEK 293T lysate (1 mg/mL) (50 mM HEPES, 150 mM NaCl, 1 mM DTT, pH 7.5) was incubated for 1 h with 0.3 µL of a 1 mM stock solution of probe **7a**, **7c**, **7d** or **7e** or with 0.3 µL DMSO. Next, 0.5 µL TAMRA-azide (1.5 mM in DMSO), 0.3 µL THPTA (5 mM in 4:1 t-BuOH: DMSO), 0.6 µL sodium ascorbate (50 mM in water) and 0.6 µL $CuSO_4$ (50 mM in water) were added to each sample and the samples were incubated 1 h at room temperature. Afterwards, 10 µL of 4x sample buffer was added to the samples, which were subsequently boiled at 95 °C for 2 minutes. Samples were resolved on an SDS-PAGE 12% acrylamide gel and visualized by in-gel fluorescence measuring on a Typhoon FLA 9500 scanner with excitation at 532 nm and detection at 568 nm with the photomultiplier set at 900 V.

IC_{50}^{App} determination on purified SARS-CoV-2 M^{pro} using a fluorogenic substrate

9.8 µL of a stock of purified M^{pro} (200 nM total enzyme) in HEPES buffer (50 mM HEPES,150 mM NaCl,1 mM DTT, pH 7.5) was loaded into the wells of a black, flat-bottomed 96-well plate. Each well was incubated with 0.2 µL of a DMSO stock of the indicated probe (**7a-e**) in a factor three dilution series ranging from 2.25 mM to 1 µM or 0.2 µL DMSO for 30 minutes at room temperature. Each probe concentration and control was set up three times. Next, 10 µL of a 200 µM stock of an Ac-Abu-Tle-Leu-Gln-MCA substrate in HEPES buffer (50 mM HEPES,150 mM NaCl,1 mM DTT, pH 7.5) was added to the wells, which were incubated for another hour at 37 °C. Fluorescence was measured at 355 nm excitation and 460 nm emission in a SpectraMax iD3 Multi-Mode Microplate Reader (Molecular Devices). The values measured were expressed as a percentage compared to a sample that was treated with DMSO instead of a DMSO stock of probe. IC_{50}^{App} values were calculated using GraphPad Prism® software after plotting the resulting percentages. Low IC_{50}^{App} values were obtained for probes **7a**, **7c** and **7d** using this method, for probe 7d, the value is potentially below the lower limit of this assay. IC_{50}^{App} were reassessed for these probes using the more sensitive approach described below.

IC_{50}^{App} determination on purified SARS-CoV-2 M^{pro} using a quenched fluorescent susbtrate

Compounds were seeded into assay-ready plates (Greiner 384 low volume 784900) using an Echo 555 acoustic dispenser, and DMSO was back-filled for a uniform concentration in assay plates (maximum 1%). Screening assays were performed in duplicate at 20 µM and 50 µM. Hits displaying more than 50% inhibition at 50 µM were confirmed by dose response assays. Reagents for M^{pro} assay reagents were dispensed into the assay plate in 10 µl volumes for a final of 20 µl. Final reaction concentrations were 20 mM HEPES, pH 7.3, 1mM TCEP, 50 mM NaCl, 0.01% Tween-20, 10% glycerol, 5 nM Mpro, 375 nM fluorogenic peptide substrate ([5-FAM]-AVLQSGFR-[Lys(Dabcyl)]-K-amide). Mpro was pre-incubated for 15 minutes at room temperature with compound before addition of substrate. Protease reaction

was measured continuously in a BMG Pherastar FS with a 480/520 ex/em filter set. Data analysis is done with CDD.

In silico docking

To minimize the number of rotatable bonds and to reduce the complexity of docking, only the warhead, P1 and P2 residue were taken for docking. The N-terminus of the P2 was capped with an acetyl and structures were energy minimized using a MMFF94s force field. We then used Autodocktools 1.5.7 to prepare the receptor (PDB code: 6LZE with the inhibitor and water molecules removed) and the ligand by adding polar hydrogens and calculating Gasteiger charges. We restricted the movement of the alpha nitrogen of the azapeptide to the coordinates of the alpha carbon of the corresponding inhibitor of the 6LZE structure by using a gridmap with a Gaussian energy well of 0.5 Å wide and an energy barrier height of 10,000 at coordinates -11.179, 15.857, 66.120. A gridmap with 32, 40 and 32 points in x, y, z directions were used with a spacing of 0.375 Å with gridcenter at -10.589, 11.789 and 67.067. Autogrid 4.2.6 was used for calculating gridmaps and Autodock 4.2.6 for docking.[2] The top 10 poses were manually inspected for P1 and P2 residue overlap with the original inhibitor and the one with highest overlap was chosen for measurement of the distance between the active site cysteine and the electrophilic warhead. Structures were visualized in PyMol.[3]

EC_{50} determination of probes 7a, 7c and 7d on SARS-CoV-2 infected VeroE6 cells

All SARS-CoV-2-related experimental work was performed in the certified, high-containment biosafety level-3 facilities of the Rega Institute at the KU Leuven. This assay was performed as described by Chiu et al.[4]

The SARS-CoV-2 isolate used in this study was the BetaCov/Belgium/GHB-03021/2020 (EPI ISL407976|2020-02-03), which was isolated from a Belgian patient returning from Wuhan in February 2020. The isolate was passaged 7 times on VeroE6 cells which introduced two series of amino acid deletions in the spike protein (PMID: 33203860).[5]

The infectious content of the virus stock was determined by titration on Vero E6 cells (MOI = 0.001). The SARS-CoV-2 antiviral assay is derived from the previously established SARS-CoV assay (PMID: 15961169).[6] In this assay, fluorescence of VeroE6-eGFP cells (provided by Dr. K. Andries J&JPRD; Beerse, Belgium) declines after infection with SARS-CoV-2 due to the cytopathogenic effect of the virus. In the presence of an antiviral compound, the cytopathogenicity is inhibited and the fluorescent signal maintained. The compounds were added in serial dilutions to the cells one day before infection with SARS- CoV-2. Four days after infection eGFP fluorescence was assessed with high content imaging. VeroE6 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco cat no 41965-039) supplemented with heat-inactivated 10% v/v fetal calf serum (FCS; Biowest) and 500 µg/mL Geneticin (Gibco cat no 10131-0275). and kept under 5% CO_2 at 37°C.

Stock solutions of the various compounds in DMSO (10 mM) were prepared. The test compounds were serially diluted in assay medium (DMEM supplemented with 2% v/v FCS). Diluted compounds were then mixed with SARS-CoV-2 (MOI = 0.001) at 20 TCID50/well and VeroE6-eGFP cells corresponding to a final density of 25,000 cells/well in 96-well µClear® plates (Greiner Bio-One). The plates were incubated in a humidified incubator at 37°C and 5% CO_2. At 4 days p.i., the wells were examined for eGFP expression using an argon laser-scanning microscope. The microscope settings were excitation at 488 nm and emission at 510 nm and the fluorescence images of the wells were converted into signal values. The results were expressed as EC_{50} values defined as the concentration of compound achieving 50% inhibition of the virus-reduced eGFP signals as compared to the untreated virus-infected control cells.

Measurement of CC_{50} in Huh7 cells

Toxicity of compounds in the absence of virus was evaluated in a standard MTS-assay on Huh7 cells, in which an MTS/Phenazine methosulphate (PMS) stock solution (2 mg/mL MTS (Promega,) and 46 µg/mL PMS (Sigma–Aldrich) in PBS at pH 6–6.5) was diluted 1/10 in MEM (Life Technologies, Gent, Belgium – cat no 21090-022). Medium was aspirated from

wells and 100 µL of MTS/PMS solution was added. After 1 h incubation at 37 °C absorbance was measured at 498 nm.

Imaging of active M^{pro} in SARS-CoV-2 infected VeroE6 cells using probe 7d

VeroE6 cells were seeded at 25000 cells/well in DMEM, supplemented with 2% heat-inactivated FCS on a µClear® 96 well plate (Greiner Bio-One). Cells were infected with with SARS-CoV-2 (BetaCov/Belgium/GHB-03021/2020) at MOI = 0.001 on the same day. 24 h post infection, the medium was aspirated and replaced by 100 µL of DMEM spiked with 0.1% DMSO or 0.1% of a DMSO stock of Carmofur (50 mM) or Nirmatrelvir (5 mM). The cells were incubated for 2h at 37°C, after which the media was aspirated. Next, the DMSO-treated sample was incubated with 100 µL of DMEM, spiked with 0.1% of a DMSO stock of **7d** (10 mM) and 0.1% of DMSO or 0.1% of a DMSO stock of Carmofur (50 mM) or Nirmatrelvir (5 mM). The cells were treated for 2h at 37°C, after which the media were aspirated and the cells washed once with PBS. Cells were fixated by treatment with 100 µL of a 3.7% formaldehyde solution for 15 minutes at room temperature. The fixation solution was next aspirated and the cells washed three times with PBS. Afterwards, cells were permeabilized by treatment with 100 µL of 0.05% Triton-X 100 in PBS for 3 minutes at room temperature. Next, the permeabilization solution was aspirated and the cells were washed three times with PBS. The visualization of probe-labeled enzymes was done by subjecting the permeabilized cells to 100 µL of a 0.1 M HEPES, 0.5 M NaCl, pH 7.5 click chemistry buffer with 1% of a TAMRA-N_3 stock (2 mM, DMSO), 1% of a THPTA stock (20 mM, DMSO), 1% of a $CuSO_4$ stock (100 mM, water) and 1% of a sodium ascorbate stock (1 M, water) for 1 hour at room temperature. Then, the click chemistry buffer was aspirated and the cells were washed three times with PBS. Next, the cells were incubated with a 1 µg/mL solution of Hoechst 33342 in PBS for 5 minutes at room temperature. Afterwards, the staining solution was aspirated and the cells were washed four times for 10 minutes with PBS under shaking. 100 µL PBS was

~~added to the stained cells, and DAPI and TAMRA images were taken with a ThermoFisher ArrayScan XTI.~~

Detection of Mpro in lysates of SARS-CoV-2 infected VeroE6 cells using probe 7d

VeroE6 cells were seeded at $2*10^6$ cells in DMEM, supplemented with 2% heat-inactivated FCS on a 6-well flat-bottom plate (Falcon) and at $6.6*10^7$ in a T150 flask (TPP). The cells on the plate were infected with with SARS-CoV-2 (BetaCov/Belgium/GHB-03021/2020) at MOI = 0.001 on the same day. 24 h post infection of the plated cells and at 90% confluency of the cells in the flask, the media were aspirated and 1 mL PBS was added to both the infected and the non-infected cells. The cells were detached by gently scraping the surface of the vessels and collected in Eppendorf tubes. Next, the cells were spun down using a benchtop centrifuge (500 g, 5 min, room temperature) and the supernatant was carefully removed. 200 µL of ice-cold lysis buffer (20 mM HEPES, 150 mM NaCl, 0.5% Triton X-100, pH 7.5) was added to the infected cell pellet and 400 µL of the same lysis buffer to the non-infected pellet. The cells were lysed by up-and-down pipetting for 30 s every 5 min over 30 min. Both lysates were spun down using a benchtop centrifuge (13000 rpm, 5 min, room temperature), after which the supernatants were collected in Eppendorf tubes. 150 µL of the infected lysate was incubated with 1.5 µL of a DMSO stock of **7d** (10 mM) for 1h, after which is was irradiated with UV light for another hour to inactivate any virus that may still be presend. It was subsequently snap-frozen in liquid nitrogen. Protein concentration were determined for both lysates using a BCA assay (0.51 mg/mL for infected lysate, 2.06 mg/mL for non-infected lysate), after which both lysates were diluted to a protein concentration of 0.50 mg/mL with storage buffer (20 mM HEPES, 150 mM NaCl, pH 7.5). 30 µL of non-infected lysate was spiked with SARS-CoV-2 Mpro to a final concentration of 2 µg/mL. This sample and a non-spiked 30 µL sample of the same lysate were treated with 0.3 µL of a 100 µM stock of probe **7d** (DMSO) for 1h. Next, 0.5 µL TAMRA-azide (1.5 mM in DMSO), 0.3 µL THPTA (5 mM in 4:1 t-BuOH: DMSO), 0.6 µL sodium ascorbate (50 mM in water) and 0.6 µL CuSO4 (50 mM in water) were added to the spiked sample, the non-spiked sample and 30 µL of the infected lysate and the samples were incubated 1 h at room temperature. Afterwards, 10 µL of 4x

sample buffer was added to the samples, which were subsequently boiled at 95 °C for 2 minutes. Samples were resolved on an SDS-PAGE 12% acrylamide gel and visualized by in-gel fluorescence measuring on a Typhoon FLA 9500 scanner with excitation at 532 nm and detection at 568 nm with the photomultiplier set at 900 V. Next, the proteins were transferred onto a nitrocellulose membrane. The membrane was blocked with 3% milk in PBST for 1h. The blocking solution was discarded and the membrane was washed with PBST, prior to overnight incubation with 10 mL rabbit anti-SARS-CoV-2 Mpro antibody (PA5116940, Thermo Scientific) (0.68 µg/mL in PBST). Afterwards, the antibody-solution was decanted and the membrane was washed four times with PBST. Next, the membrane was incubated with HRP-coated goat anti-rabbit IgG antibody (0.025 µg/mL in PBST) for 1h. Afterwards, the antibody-solution was decanted and the membrane was washed four times with PBST and incubated with SuperSignal West Pico Plus™ chemiluminescent substrate (Life Technologies) just prior to development using a BioRad ChemiDoc imaging system.

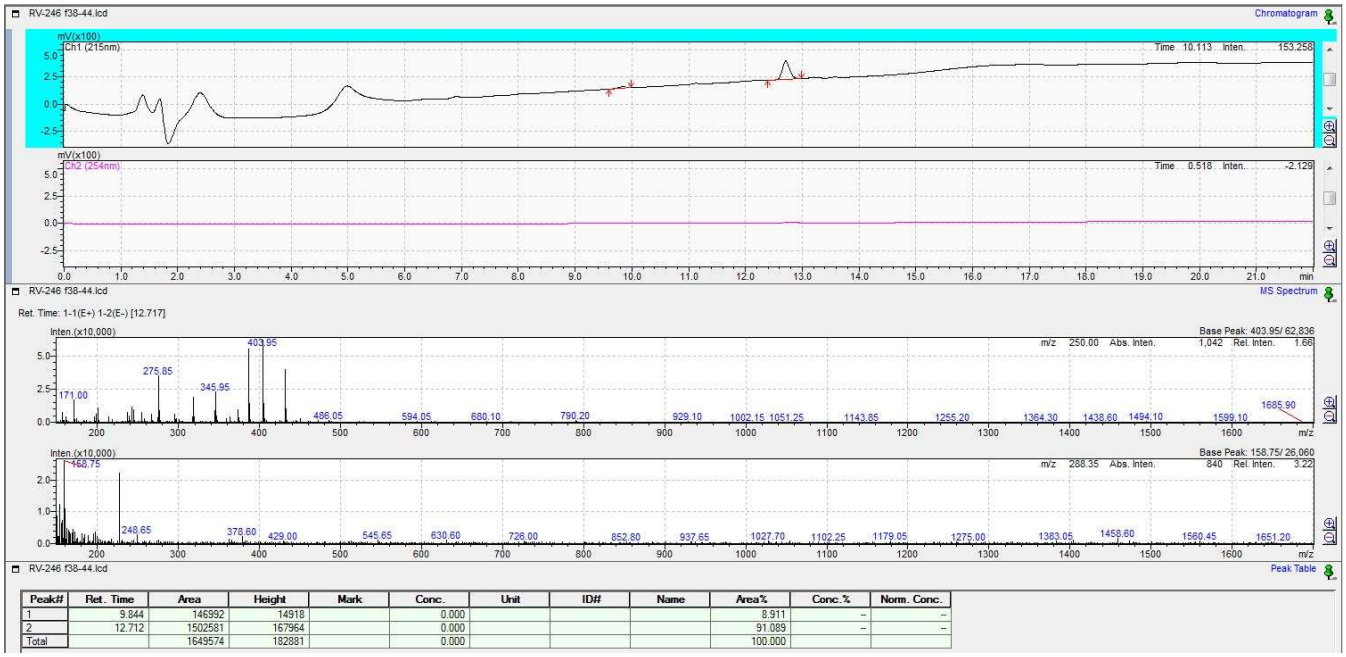
Fluorescence imaging of M^{pro} in infeceted cells using probe 7d

VeroE6 cells were seeded at 25000 cells/well in DMEM, supplemented with 2% heat-inactivated FCS, on a µClear® 96 well plate (Greiner Bio-One). Each of the following treatments was done in triplicates. All but one triplicate of cells were infected with SARS-CoV-2 (BetaCov/Belgium/GHB-03021/2020) at MOI = 0.001 on the same day. 24 h post infection, the media were aspirated and replaced by 100 µL of DMEM spiked with 0.1% DMSO or 0.1% of a DMSO stock of Nirmatrevir (5mM; two triplicates)). Non-infected cells were treated with 100 µL DMEM spiked with 0.1% DMSO. The cells were incubated for 2h at 37°C, after which the media were aspirated. One triplicate of Nirmatrelvir (5 µM) treated cells was incubated with 100 µL DMEM, spiked with 0.1% DMSO and 0.1% of a DMSO stock of Nirmatrelvir (5 mM). All other cells were treated with 100 µL DMEM, spiked with 0.1% of a DMSO stock of **7d** (10 mM) and 0.1% DMSO or 0.1% of the same Nirmatrelvir stock with which they were treated in the previous step. The cells were incubated for 2h at 37°C, after which the media were aspirated and the cells were washed once with PBS. Next, Cells were

fixated by treatment with 100 µL of a 3.7% formaldehyde solution for 15 minutes at room temperature. The fixation solution was next aspirated and the cells washed three times with PBS. Afterwards, cells were permeabilized by treatment with 100 µL of 0.05% Triton-X 100 in PBS for 3 minutes at room temperature. Next, the permeabilization solution was aspirated and the cells were washed three times with PBS. The visualization of probe-labeled enzymes was done by subjecting the permeabilized cells to 100 µL of a 0.1 M HEPES, 0.5 M NaCl, pH 7.5 click chemistry buffer with 1% of a TAMRA-azide stock (2 mM, DMSO), 1% of a THPTA stock (20 mM, DMSO), 1% of a $CuSO_4$ stock (100 mM, water) and 1% of a sodium ascorbate stock (1 M, water) for 1 hour at room temperature. Then, the click chemistry buffer was aspirated and the cells were washed three times with PBS. Optionally, the samples were immunostained. To this end, the cells were first treated with 100 µL 10% normal goat serum (NGS) in PBS for 40 min at room temperature to block non-specific binding sites. The blocking solution was removed and replaced with 100 µL of a 1/100 dilution of Rabbit anti-SARS-CoV-2 nucleocapsid antibody (GeneTex, GTX135357) in 10% NGS. Cells were treated for 1 h at room temperature, after which the primary antibody solution was removed and the cells were washed four times for 5 minutes with PBS. A solution of 1/500 Alexa Fluor™ 647 labeled goat anti-rabbit IgG (Invitrogen, A-21244) in 10% NGS was added to the cells, which were incubated for another 1 h at room temperature in the dark. The secondary antibody solution was removed and the cells were washed four times for 5 min with PBS in the dark. Next, the cells were incubated with a 1 µg/mL solution of Hoechst 33342 in PBS for 5 min at room temperature. Afterwards, the staining solution was aspirated and the cells were washed four times for 10 minutes with PBS. 100 µL PBS was added to the stained cells, and Brightfield, DAPI, AF647 and TAMRA images were taken of each well with a ThermoFisher ArrayScan XTI. For quantification with ImageJ, manual cell masks were generated (10 cells per replicate) as well as background from an area without cells. Mean fluorescence per cell was calculated using the ROI manager with subtraction of the plate fluorescence. Data are represented as average +/- standard deviation (N=30) and a two tailed t-test was performed to compare samples.

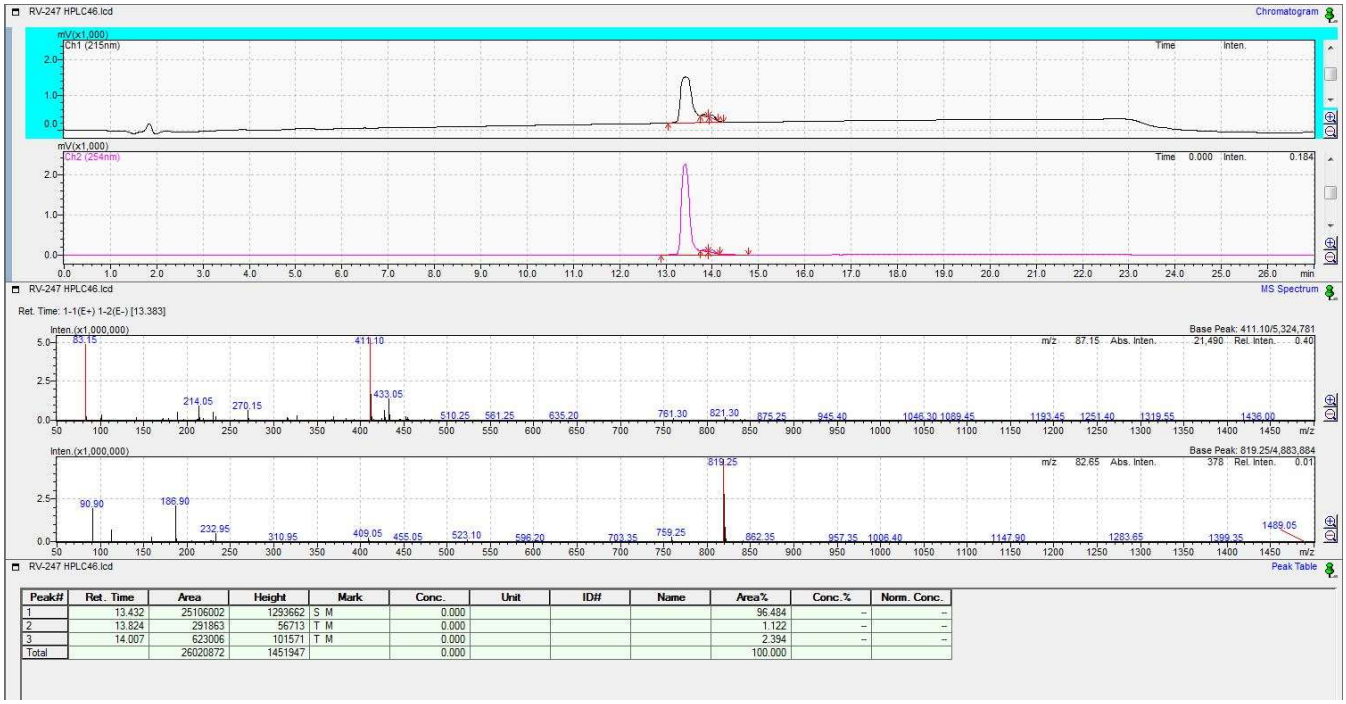
Copies of spectra and chromatograms

3



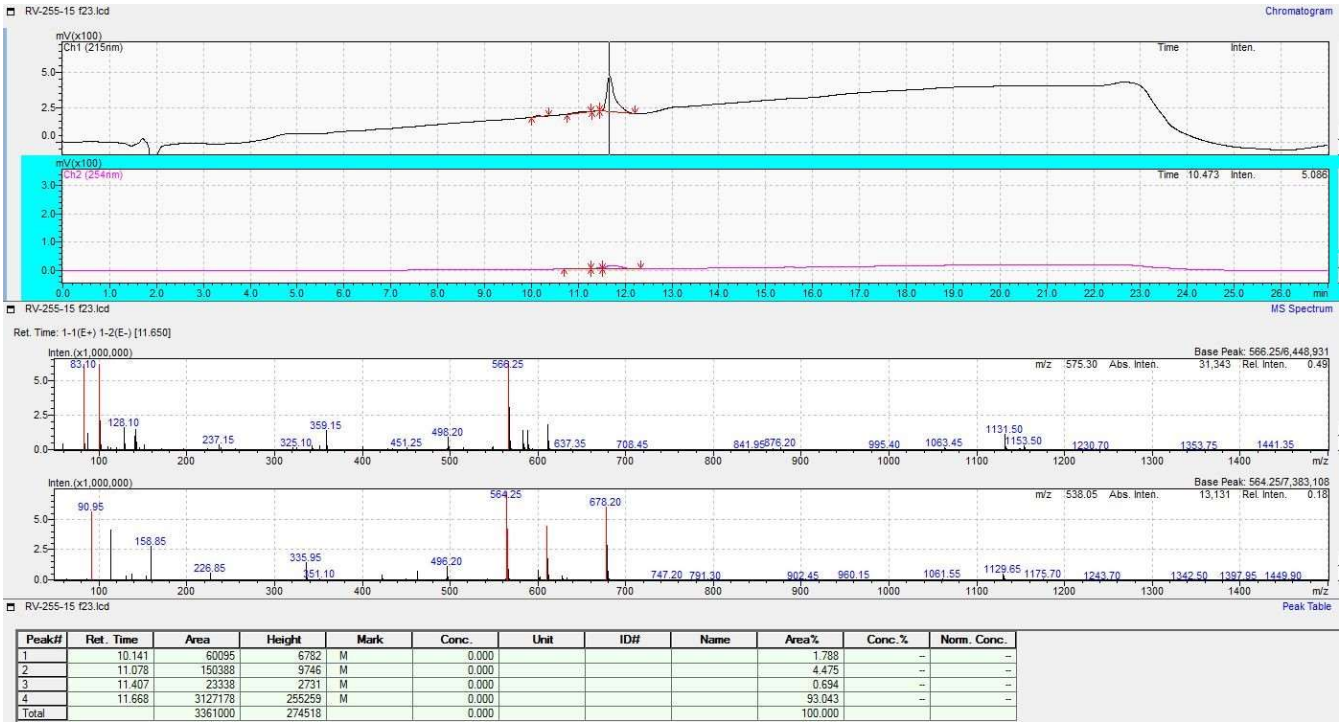
Peak#	Ret. Time	Area	Height	Mark	Conc.	Unit	ID#	Name	Area%	Conc.%	Norm. Conc.
1	9.844	146992	14918		0.000				8.911	--	--
2	12.712	1502581	167964		0.000				91.089	--	--
Total		1649574	182881		0.000				100.000		

4



Peak#	Ret. Time	Area	Height	Mark	Conc.	Unit	ID#	Name	Area%	Conc.%	Norm. Conc.
1	13.432	25106002	1293662	S M	0.000				96.484	--	--
2	13.824	291863	56713	T M	0.000				1.122	--	--
3	14.007	623006	101571	T M	0.000				2.394	--	--
Total		26020872	1451947		0.000				100.000		

7a



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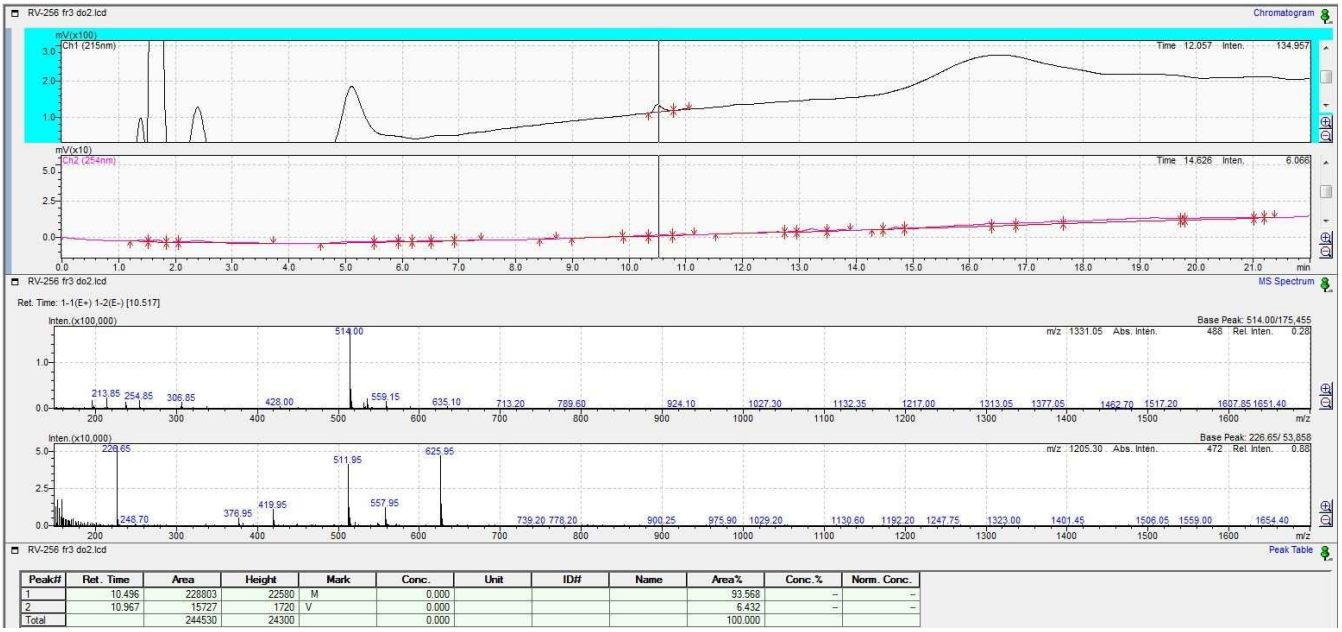
1	10.141	60095	6782	M	0.000				1.788	--	--
2	11.078	150388	9746	M	0.000				4.475	--	--
3	11.407	23338	2731	M	0.000				0.694	--	--
4	11.668	3127178	255259	M	0.000				93.043	--	--
Total		3361000	274518		0.000				100.000		

7b



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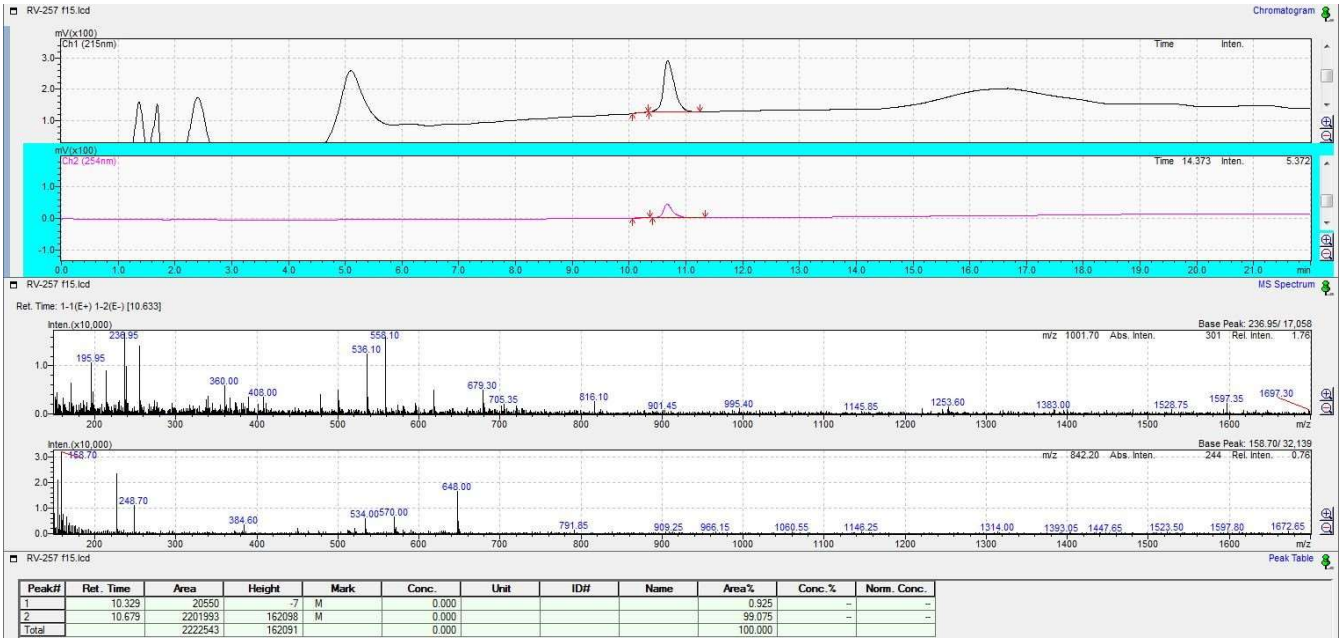
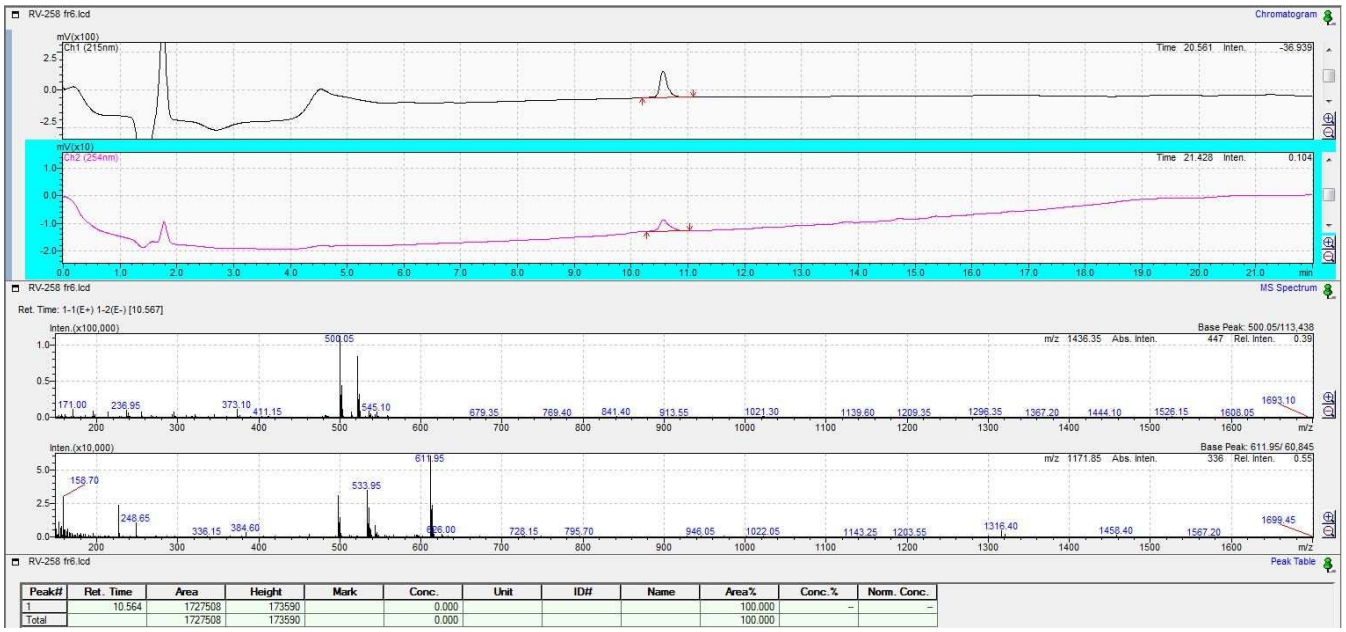
1	10.496	228803	22580	M	0.000				93.568	--	--
2	10.967	15727	1720	V	0.000				6.432	--	--
Total		244530	24300		0.000				100.000		

7c



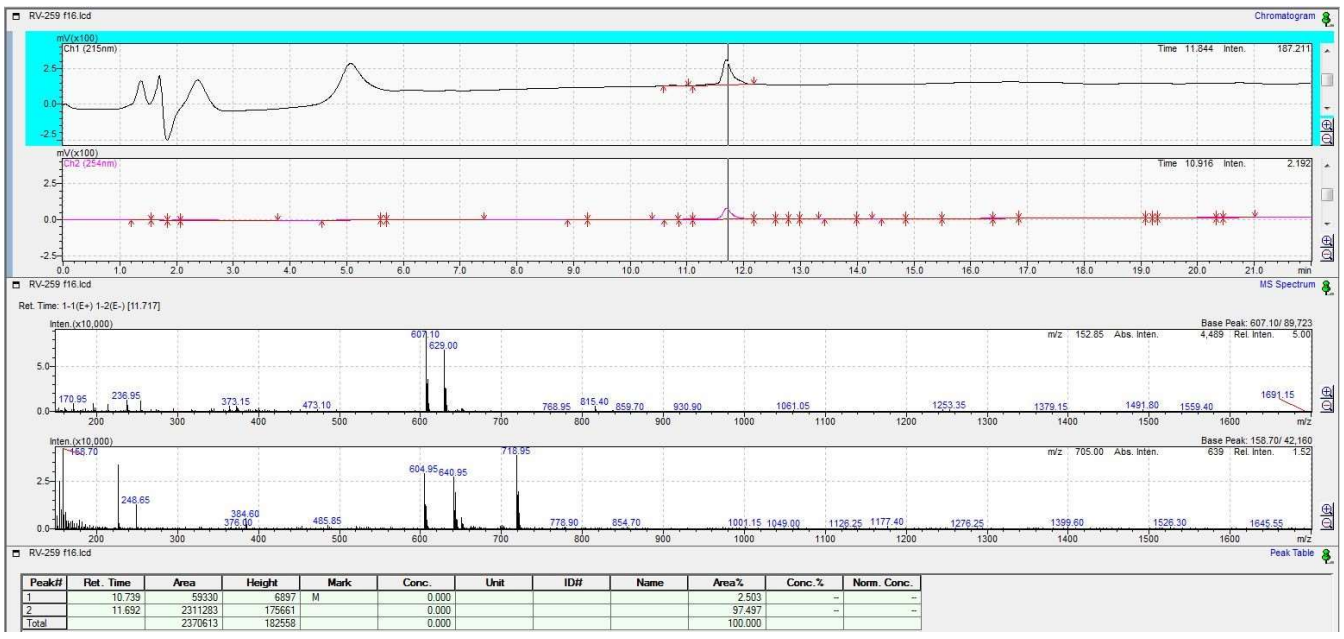
Peak#	Ret. Time	Area	Height	Mark	Conc.	Unit	ID#	Name	Area%	Conc.%	Norm. Conc.
1	10.329	20550	-7	M	0.000				0.925	--	--
2	10.679	2201993	162098	M	0.000				99.075	--	--
Total		2222543	162091		0.000				100.000		

7d.



Peak#	Ret. Time	Area	Height	Mark	Conc.	Unit	ID#	Name	Area%	Conc.%	Norm. Conc.
1	10.564	1727508	173590		0.000				100.000	--	--
Total		1727508	173590		0.000				100.000		

7e.



Peak#	Ret. Time	Area	Height	Mark	Conc.	Unit	ID#	Name	Area%	Conc.%	Norm. Conc.
1	10.739	59330	6897	M	0.000				2.503	--	--
2	11.692	2311283	175661		0.000				97.497	--	--
Total		2370613	182558		0.000				100.000		

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