Supporting Information

Renal-Clearable Molecular Probe for Near-Infrared Fluorescence Imaging and Urinalysis of SARS-CoV-2

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1. General Information

All commercial reagents were purchased from reputable vendors and used without further purification, unless indicated otherwise. 2-chlorotrityl chloride polystyrene resin, Fmoc-Gln(Trt)-OH, Fmoc-Leu-OH, Fmoc-Tle-OH, Fmoc-Abu-OH, 1-hydroxybenzotriazole hydrate (HOBT), 3-[bis(dimethylamino)methyl]imium][3H-benzotriazol-1-oxide hexafluorophosphate (HBTU) were purchased from Sangon Biotech for SPPS synthesis. All reactions were sealed with septa through which a nitrogen atmosphere was introduced unless otherwise stated. All non-aqueous reactions were carried out under a nitrogen atmosphere in oven-dried glassware. Reactions were conducted in round-bottomed flasks containing Teflon-coated magnetic stir bars. Heating of reactions was accomplished with a silicon oil bath on top of a stirring hotplate equipped with an electronic contact thermometer to maintain the indicated temperatures. Reaction progress was monitored by TLC on pre-coated silica plates (Merck 60 F254 nm, 250 μm thickness) and spots were visualized by UV light or appropriate staining (e.g. phosphomolybdic acid stain (PMA), basic KMnO4). Flash column chromatography was carried out using 200 or 400 mesh silica gel. All 1H NMR and 13C NMR spectra were carried out on a Bruker ACF-400 MHz NMR spectrometer. Chemical shifts were reported in parts per million (ppm) relative to residual solvent peaks. 1H and 13C chemical shifts (δ) were referenced to TMS or residual solvent peaks (CDCl3 = 7.26 ppm and (CD3)2SO = 2.50 ppm) for 1H NMR. The following abbreviations were used for reporting 1H NMR spectra: chemical shift (δ ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublets, m = multiplet), coupling constant (Hz), integration. Electrospray ionization-mass spectrometry (ESI-MS) spectra were acquired on a Thermo Finnigan Polaris Q quadrupole ion trap mass spectrometer equipped with a standard ESI source. HPLC analyses were done on an Agilent 1260 system equipped with a G1311B pump, UV detector and an Agilent Zorbax SB-C18 RP (9.4 × 250 mm) column, with methanol and water as the eluent. UV/Vis spectra were measured on a Shimadzu UV-2450 spectrophotometer. Fluorescence measurements were performed on a Fluorolog 3-TCSPC spectrofluorometer (Horiba Jobin Yvon). In silico calculation of the partition coefficients (Log D at pH 7.4) was calculated using Marvin and JChem calculator plug-ins (ChemAxon, Hungary).

SARS-CoV-2 main protease (Cat. No. SAE0172) and γ-glutamyl transferase (GGT) was purchased from Sigma Aldrich. SARS-CoV-1 main protease, furin, urokinase (uPA) and caspase-3 were purchased from R&D Systems. Hepatitis C Virus (HCV) NS4A/NS3-3 protease was purchased from Sigma. Microspray aerosolizer for intratracheal administration in mice was purchased from PenWu, Bio Jane Trading Limited. Living mice imaging was acquired with IVIS Spectrum imaging system (PerkinElmer, Inc.). Blood samples were collected using heparinized capillary tubes (Paul Marienfeld, Germany). Urine samples were collected with metabolic cages (Lab Products Inc, USA).
2. Chemistry

2.1. Synthetic Scheme

Scheme S1. Synthetic routes for a) CyOH, b) P-HPβCD c) Cy-CD and d) SARS-CyCD.
2.2. Synthetic Procedures

CyOH, P-HPβCD and CyCD were synthesised according to reported protocols.1,2

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\begin{align*}
\text{Synthesis of SARS(Trt): Peptide N-Acetyl-Abu-Tle-Leu-Gln(Trt)-OH (1.40 g, 2.0 mmol) was}\\
synthesised by solid-phase peptide synthesis (SPPS).^1\text{H NMR (400 MHz, DMSO-}d_6) \delta: 12.54\\
(\text{s, 1H}), 8.60 (\text{s, 1H}), 8.03-7.96 (\text{m, 3H}), 7.62 (d, J = 9.44 Hz, 1H), 7.29-7.16 (m, 15H), 4.36\\
(q, J = 7.6 Hz, 1H), 4.29-4.23 (m, 2H), 4.17-4.11 (m, 1H), 2.41-2.27 (m, 2H), 2.00-1.90 (m, 1H), 1.84 (s, 3H), 1.73-1.58 (m, 6H), 1.07-1.01 (m, 1H), 1.00-0.84 (m, 17H).\ ^{13}\text{C NMR (100 MHz, DMSO-}d_6) \delta 173.59, 172.35, 171.90, 171.66, 170.19, 169.70, 162.78, 145.36, 128.98, 127.90, 126.77, 69.68, 60.03, 54.32, 52.03, 51.14, 41.28, 36.24, 34.69, 33.02, 31.24, 27.70, 27.09, 25.56, 24.53, 23.53, 22.89, 21.93, 10.58. \text{MS (ESI): m/z = 742.29 [M + H]^+}.\\
\end{align*}
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\begin{align*}
\text{Synthesis of SARS(Trt)-PABA: N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ, 197}\\
\text{mg, 0.8 mmol) was added to SARS(Trt) (300 mg, 0.4 mmol) dissolved in anhydrous DCM.}\\
The reaction mixture was stirred at room temperature for 20 min. Next, 4-aminobenzyl alcohol\\
(99 mg, 0.8 mmol) was added and the reaction was stirred at room temperature for 16 h. The}\\
\text{reaction mixture was concentrated under reduced pressure and triturated with ether (50 mL) thrice}\\
to obtain pure SARS(Trt)-PABA as a white solid (300 mg, 88%). ^1\text{H NMR (400 MHz, DMSO-}d_6) \delta: 9.91 (\text{s, 1H}), 8.64 (\text{s, 1H}), 8.04-7.95 (\text{m, 3H}), 7.63 (d, J = 9.24 Hz, 1H), 7.53 (d, J = 8.08 Hz, 2H), 7.28-7.16 (m, 18H), 4.43 (s, 2H), 4.36-4.23 (m, 4H), 2.37-2.33 (m, 2H), 2.01-2.00 (m, 1H), 1.83 (s, 3H), 1.57-1.44 (m, 6H), 0.93-0.80 (m, 18H). \ ^{13}\text{C NMR (100 MHz, DMSO-}d_6) \delta 172.27, 172.05, 171.79, 170.45, 170.38, 169.78, 145.36, 138.01, 137.93, 129.00, 127.90, 127.34, 126.77, 119.48, 69.71, 65.39, 63.10, 60.22, 55.33, 54.37, 53.80, 51.38, 40.98, 34.65, 33.10, 28.64, 27.11, 25.53, 24.62, 23.55, 23.42, 22.90, 21.85, 15.63, 10.60. \text{MS (ESI): m/z = 847.54 [M + H]^+}.\\
\end{align*}
\]

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**Synthesis of SARS(Trt)-Br:** SARS(Trt)-PABA (120 mg, 0.14 mmol) was dissolved in anhydrous THF and cooled in an ice bath. Next, PBr₃ (27 μL, 0.28 mmol) was added dropwise. The reaction mixture was left to stir at 0 °C for 2 h or until reaction was completed as indicated by TLC. Following which, the solvent was removed under reduced pressure. The resulting crude solids was dissolved in DCM (100 mL) and washed with saturated NaHCO₃ (50 mL x 3) and brine (50 mL x 1). The organic layer was dried was anhydrous Na₂SO₄ and concentrated under reduced pressure. The crude product was triturated with ether (10 mL x 2) to yield a powdery off-white solid. Crude SARS(Trt)-Br was used immediately in the next step without further purification.

**Synthesis of SARS(Trt)-Cy:** CyOH (12 mg, 0.025 mmol) was dissolved in 1 mL anhydrous THF/ACN (1:1), followed by the addition of K₂CO₃ (13.8 mg, 0.10 mmol). The reaction was stirred at room temperature for 10 minutes. SARS(Trt)-Br (91 mg, 0.10 mmol) was then added, and the reaction mixture was left to stir at 50 °C for 4 h. After completion, the resulting mixture was concentrated under reduced pressure. The resulting crude solids was dissolved in DCM (50 mL) and washed with water (25 mL x 2) and brine (25 mL x 1). The organic layer was dried was anhydrous Na₂SO₄ and concentrated under reduced pressure. Pure SARS(Trt)-Cy (10.0 mg, 30%) was then obtained after preparative HPLC purification. ¹H NMR (400 MHz, Methanol-d₄) δ: 8.75 (d, J = 16.0 Hz, 1H), 8.15 (d, J = 7.60 Hz, 1H), 7.75-7.73 (m, 1H), 7.70 (d, J = 8.00 Hz, 1H), 7.65 (d, J = 12.0 Hz, 2H), 7.55-7.54 (m, 2H), 7.48-7.40 (m, 5H), 7.26-7.23 (m, 15H), 7.07-7.04 (m, 2H), 6.51 (d, J = 16.0 Hz, 1H), 5.24 (s, 2H), 4.45-4.35 (m, 4H), 4.28-4.23 (m, 4H), 3.49-3.43 (m, 3H), 2.80-2.77 (m, 2H), 2.75-2.72 (m, 2H), 2.50-2.49 (m, 3H), 1.97 (s, 3H), 1.82 (s, 6H), 1.80-1.73 (m, 6H), 1.68-1.61 (m, 8H), 0.95-0.87 (m, 17H). ¹³C NMR (100 MHz, Methanol-d₄) δ 177.59, 173.28, 173.15, 172.81, 172.77, 172.27, 171.44, 170.55, 162.38, 161.99, 154.39, 145.78, 144.57, 142.05, 141.47, 138.22, 134.15, 132.22,
128.87, 128.65, 127.95, 127.35, 127.06, 126.43, 122.57, 119.98, 119.85, 115.96, 114.51, 112.36, 103.12, 101.38, 70.23, 70.17, 61.28, 53.60, 52.05, 50.64, 50.39, 44.13, 40.01, 33.74, 32.44, 29.37, 27.09, 27.07, 25.87, 25.56, 24.51, 24.46, 23.94, 23.65, 22.33, 22.11, 21.02, 20.44, 20.23, 13.01, 9.35. MS (ESI): m/z = 1295.65 [M]+.

Synthesis of SARS-Cy: SARS(Trt)-Cy (13 mg, 0.01 mmol) was dissolved in 1 mL DCM and cooled in an ice bath. Triethylsilane (25 µL, 2.5% v/v) was added, followed by TFA (200 µL, 20% v/v). The reaction was left to stir in an ice bath for 16 h. The reaction was monitored by analytical HPLC. Upon completion, the reaction was diluted with DCM (50 mL) and washed with saturated NaHCO₃ solution (25 mL x 3) and brine (25 mL x 1). The organic layer was dried with anhydrous Na₂SO₄ and concentrated under reduced pressure. Pure SARS-Cy (7.2 mg, 68%) was then obtained after preparative HPLC purification. 

\[ \text{H NMR (400 MHz, Methanol-d₄)} \delta: 8.78 \text{ (d, } J = 14.72 \text{ Hz, 1H)}, 8.23-8.18 \text{ (m, 2H)}, 7.74-7.69 \text{ (m, 4H)}, 7.65-7.63 \text{ (m, 1H)}, 7.57-7.44 \text{ (m, 7H)}, 7.10-7.06 \text{ (m, 2 H)}, 6.53 \text{ (d, } J = 14.8 \text{ Hz, 1H)}, 5.96-5.86 \text{ (m, 2H)}, 5.36 \text{ (t, } J = 4.64 \text{ Hz, 1H)}, 5.31-5.26 \text{ (m, 3H)}, 5.20 \text{ (dd, } J = 1.28 \text{ Hz, 10.48 Hz, 2H)}, 4.56-4.54 \text{ (m, 2H)}, 4.49-4.46 \text{ (m, 1H)}, 4.42-4.37 \text{ (m, 2H)}, 4.25-4.22 \text{ (m, 2H)}, 3.46 \text{ (t, } J = 6.5 \text{ Hz, 2H)}, 2.82-2.73 \text{ (m, 4H)}, 2.54-2.47 \text{ (m, 2H)}, 2.21 \text{ (t, } J = 8.0 \text{ Hz, 2H)}, 2.1-2.0 \text{ (m, 2H)}, 2.00 \text{ (s, 3H)}, 1.99-1.95 \text{ (m, 2H)}, 1.84 \text{ (d, } J = 2.0 \text{ Hz, 5H)}, 1.85-1.75 \text{ (m, 2H)}, 1.72-1.57 \text{ (m, 3H)}, 1.00-0.96 \text{ (m, 9H)}, 0.94-0.87 \text{ (m, 8H)}. \text{ C NMR (100 MHz, Methanol-d₄)} \delta 177.6275, 173.3062, 173.1101, 172.2337, 170.4758, 162.3822, 161.9813, 154.4020, 145.7650, 142.0564, 141.4598, 138.2226, 134.1084, 132.2178, 128.8447, 128.7012, 128.0307, 127.9603, 127.2451, 126.9818, 122.5178, 120.0715, 119.9645, 115.9419, 114.4215, 114.3063, 112.2939, 103.1012, 101.2972, 70.1755, 68.8361, 61.1539, 55.1961, 53.6366, 52.0928, 50.5805, 50.3936, 44.1057, 40.017, 33.8934, 33.8291, 31.1836, 29.3431, 28.9009, 28.6118, 27.0505, 25.8024, 25.5863, 25.4928, 24.5075, 24.4272, 23.6523, 22.0044, 20.9464, 20.4729, 20.2578, 9.2921. MS (ESI): m/z = 1053.57 [M]+.
Synthesis of compound SARS-CyCD: SARS-Cy (24 mg, 0.02 mmol) and P-HPβCD (40 mg, 0.025 mmol) was first dissolved in 3 mL DMSO/H2O (2:1). The reaction was purged with nitrogen gas for 5 mins. Sodium ascorbate (31.6 mg, 0.16 mmol) and CuSO$_4$·5H$_2$O (10.0 mg, 0.04 mmol) was next added. The reaction was purged with nitrogen gas for another 5 mins and allowed to stir at room temperature for 24 h. Upon completion, the reaction was dialysed with deionised water. Pure SARS-CyCD (20 mg, 33%) was then obtained after preparative HPLC purification. $^1$H NMR (400 MHz, DMSO-d$_6$) $\delta$: 9.95 (s, 1H), 8.03-7.98 (m, 4H), 7.66-7.45 (m, 5H), 7.27-7.23 (m, 4H), 7.11-7.01 (m, 2H), 6.78 (s, 2H), 5.89-5.71 (m, 8H), 5.54-5.46 (m, 3H), 5.10-5.02 (m, 8H), 4.83 (s, 7H), 4.68-4.34 (m, 19H), 4.36-4.14 (m, 10H), 3.75-3.62 (m, 61H), 2.13-2.12 (m, 2H), 1.84 (s, 6H), 1.63-1.60 (m, 2H), 1.50-1.45 (m, 2H), 1.02 (s, 18H), 0.90-0.81 (m, 17H). $^{13}$C NMR (100 MHz, DMSO-d$_6$) $\delta$: 174.02, 173.23, 172.33, 172.04, 170.74, 170.47, 169.83, 129.24, 128.95, 127.91, 126.78, 119.70, 105.89, 91.77, 88.35, 80.78, 77.50, 75.27, 73.73, 65.75, 65.51, 60.18, 58.37, 58.28, 58.03, 56.11, 54.36, 53.70, 51.41, 34.61, 31.85, 31.71, 30.86, 29.47, 29.13, 29.01, 28.26, 28.10, 27.05, 25.56, 25.43, 24.56, 23.50, 22.88, 22.53, 21.80, 20.30, 19.65, 14.39, 13.93, 10.58. MALDI-TOF MS found: 2,600–3,000.

3. Results & Discussion

3.1. In Vitro Tests

Preparation of stock solution. SARS-CyCD and CyCD was dissolved in DMSO to obtain a 5 mM stock solution. Enzyme stock solutions of SARS-CoV-2 M$_{pro}$, γ-glutamyl transferase (GGT), SARS-CoV-1 M$_{pro}$, Hepatitis C Virus (HCV) NS4A/NS3 protease, furin, and caspase-3 were prepared in distilled water.

UV and fluorescence measurements. SARS-CoV-2 M$_{pro}$ was incubated in freshly prepared TRIS buffer (20 mM TRIS, $p$H = 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM DTT) at 37 °C before 10 µM of SARS-CyCD (500 µM DMSO stock) was added. After 60 mins, UV/VIS and fluorescence measurements of the solution were performed using Shimadzu UV-2450 spectrophotometer and Fluorolog 3-TCSPC spectrofluorometer (Horiba Jobin Yvon),
respectively. Fluorescence images were acquired using the IVIS spectrum imaging system with excitation at 675 ± 10 nm and emission at 720 ± 10 nm and an acquisition time of 0.1 s.

**Enzyme kinetic studies.** Various concentrations of SARS-CyCD (1, 5, 10, 15, 20, 40, 80, 150 μM) were incubated with SARS-CoV-2 M\(^{\text{pro}}\) (250 nM) at 37 °C for 30 min in a 100 μL system of TRIS buffer (20 mM TRIS, pH = 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM DTT). After incubation, the mixture was injected into HPLC (methanol/water) for quantification analyses. The initial reaction velocity (μmol/s) was calculated, plotted against SARS-CyCD concentration, and fitted to a Michaelis-Menten curve. The kinetic parameters were calculated using Michaelis-Menten equation: \( V = V_{\text{max}} \times [S] \left( K_m + [S] \right) \), where \( V \) is initial velocity, and [S] is substrate concentration. The calculated parameters are as follows: \( V_{\text{max}} = 0.208 \, \mu\text{M min}^{-1} \), \( K_m = 11.45 \, \mu\text{M} \), \( k_{\text{cat}} = 0.014 \, \text{s}^{-1} \), \( k_{\text{cat}}/K_m = 12.2 \times 10^6 \, \text{M}^{-1} \text{s}^{-1} \).

**In vitro selectivity studies.** SARS-CyCD was incubated with the indicated enzymes including HCV NS4A/NS3 protease, urokinase, caspase 3, furin, GGT, SARS-CoV-1 M\(^{\text{pro}}\) and SARS-CoV-2 M\(^{\text{pro}}\) in TRIS buffer (20 mM TRIS, pH = 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM DTT) at 37 °C for 120 min. Fluorescence images were acquired using the IVIS spectrum imaging system with excitation at 675 ± 10 nm and emission at 720 ± 10 nm and an acquisition time of 0.1 s.

**3.2. In Vivo Imaging**

All animal experiments were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of the Nanyang Technological University-Institutional Animal Care and Use Committee (NTU-IACUC) and approved by the Institutional Animal Care and Use Committee (IACUC) for Animal Experiment, Singapore. Adult female Ncr nude mice (18 – 20 g) were housed in a temperature controlled (22 °C) room with 12 h dark light cycles (0700 h on and 1900 h off). The animals were provided ad libitum with food (Tecklad T.2918.CS Irradiated Rodent Diet purchased from Acre Engineering Pte Ltd) and water.

**Intratracheal administration.** 20 μL of SARS-CyCD (10 mg mL\(^{-1}\) in PBS) or SARS-CoV-2 M\(^{\text{pro}}\) (1 mg mL\(^{-1}\) in PBS) was loaded into the microspray aerosolizer (purchased from PenWu, Bio Jane Trading Limited.). Then, the tip of the aerosolizer was gently inserted down the trachea of the anesthetized mice, followed by intratracheal administration of aerosols into the lungs of mice.
**In vivo biodistribution studies.** Mice were i.t. injected with saline (control), SARS-CyCD or CyCD (2 μmol kg⁻¹ body weight) and imaged using the IVIS spectrum imaging system at 0, 20, 40, 60, 90, and 120 mins post-injection. The abdominal cavity and resected organs from mice were imaged after euthanization at 24 h post-injection. Fluorescence images were acquired using the IVIS spectrum imaging system with excitation at 675 ± 10 nm and emission at 720 ± 10 nm.

**Pharmacokinetics studies.** Mice were anesthetized by isoflurane anesthesia for the entire duration of the experiment. The end of the tail was cut for blood extraction. Blood was sampled in heparinized capillary tubes as a reference before injection. Mice were intratracheally (i.t) injected with SARS-CyCD or CyCD (2 μmol kg⁻¹ body weight) and blood was sampled at 1, 20, 40, 60, 80, 120, 180, and 360 min post-injection. Collected blood samples were stored in an ice box to prevent clotting before centrifugation at 4500 r.p.m for 15 min. SARS-CyCD and CyCD in the blood were quantified using the IVIS spectrum imaging system. Calibration curves were established using different concentrations of SARS-CyCD or CyCD in blank plasma. The percentage injected dose per gram (%ID/g) was calculated as: %ID/g = dose in plasma sample/injected dose/blood weight x100%. Quantification results were presented as an absorption phase followed by an elimination phase and plotted as a function of time. A bi-exponential decay curve to estimate elimination half life t₁/₂, which can be calculated as: t₁/₂ = ln(2)/slope of elimination phase.

**Renal clearance studies.** Mice were i.t injected with SARS-CyCD/CyCD (2 μmol kg⁻¹ body weight) and placed in metabolic cages. Urine was collected at 3, 6, 12, and 24 h post-injection, diluted in PBS and centrifuged at 4500 r.p.m. for 10 min and filtered by 0.22 μm syringe filter. SARS-CyCD in the urine was quantified using the IVIS spectrum imaging system and HPLC. The fluorescence spectra were measured for the urine samples. Mice were sacrificed and major organs were collected, homogenized in PBS buffer (10 mM, pH 7.4), and centrifuged at 4500 r.p.m for 15 min to remove insoluble components. The supernatant containing extracted molecules were taken for fluorescence measurements using the IVIS spectrum imaging system.

**Real-time in vivo NIRF imaging of SARS-CoV-2 Mpro in living mice.** Real-time NIRF imaging was conducted at t = 0, 10, 20, 30, 40, 60, 90, and 120 min after sequential i.t injection of SARS-CoV-2 Mpro (0.15 mg kg⁻¹ body weight), followed by SARS-CyCD (2 μmol kg⁻¹ body weight). Fluorescence images were acquired using the IVIS spectrum imaging system. NIRF
intensities of lungs and bladder in living mice were analyzed by the ROI analysis using the Living Image 4.3 Software.

**Urinalysis of SARS-CoV-2 M<sup>pro</sup> in living mice.** Urine was collected using metabolic cages from living mice after sequential i.t injection of SARS-CoV-2 M<sup>pro</sup> (0.15 mg kg<sup>-1</sup> body weight), followed by SARS-CyCD (2 μmol kg<sup>-1</sup> body weight) at 3, 6, 12, 24 h post i.t. injection. The collected urine samples were centrifuged at 4500 r.p.m. for 8 min, filtered by 0.22 μm syringe filter, and measured using the IVIS spectrum imaging system with excitation at 675 ± 10 nm and emission at 720 ± 10 nm.

**In vivo stability studies.** The collected urine in PBS (10 mM, pH 7.4) was measured on a fluorescence spectrophotometer, imaged by the IVIS spectrum imaging system, and analysed by HPLC.

**Statistics analysis.** The in vivo fluorescence intensities were quantified with ROI analysis using Living Image 4.3 Software. Data are mean ± standard deviation (S.D.) unless stated otherwise. Investigators were blinded to group allocation during experiments. Statistical differences between two groups were tested with a two-tailed Student’s t-test and more than three groups were determined by one-way analysis of variance followed by Tukey’s post hoc test. For all tests, P values less than 0.05 were considered statistically significant. *P < 0.05, **P < 0.01 and ***P < 0.001. All statistical calculations were performed using GraphPad Prism 6.0, including assumptions of tests used.

| Probe   | Log D value |
|---------|-------------|
| SARS-Cy | 3.68        |
| SARS-CyCD | -4.21   |
| CyCD    | -5.00       |

**Table S1.** LogD values of SARS-Cy, SARS-CyCD and CyCD.
**Figure S1.** Nonlinear regression analysis of cleavage rate $V$ ($\mu$M min$^{-1}$) of SARS-Cy/SARS-CyCD as a function of substrate concentration. Various concentrations of SARS-Cy/SARS-CyCD (1, 5, 10, 20, 40, 80, 150 $\mu$M) were incubated with SARS-CoV-2 M$^\text{pro}$ (250 nM) at 37 °C for 30 min in Tris buffer (20 mM, pH 7.4). After incubation, the mixture was measured by HPLC.

|                      | SARS-Cy | SARS-CyCD |
|----------------------|---------|-----------|
| Michaelis–Menten Constants ($K_m$) | 13.5 $\mu$M | 11.5 $\mu$M |
| Catalytic Rate Constants ($k_{cat}$) | 0.028 s$^{-1}$ | 0.014 s$^{-1}$ |
| Catalytic Efficiency ($k_{cat}/K_m$) | $20.7 \times 10^6$ M$^{-1}$s$^{-1}$ | $12.2 \times 10^6$ M$^{-1}$s$^{-1}$ |

**Figure S2.** a) Fluorescence images acquired with an IVIS spectrum imaging system of excreted CyCD and SARS-CyCD in the urine from living mice at different timepoints post i.t. injection of CyCD or SARS-CyCD (2 $\mu$mol kg$^{-1}$ body weight). b) Fluorescence spectra of excreted SARS-CyCD in the urine of living mice with SARS-CyCD in PBS as reference.
Figure S3. a) Dynamic NIRF intensities of lungs (dorsal) as a function of time in saline or SARS-CoV-2 M^pro treated living mice (0.15 mg kg^{-1} body weight), co-injected with SARS-CyCD (2 μmol kg^{-1} body weight). b) Fluorescence images of lungs collected from saline or SARS-CoV-2 M^pro treated mice (0.15 mg kg^{-1} body weight), co-injected with SARS-CyCD (2 μmol kg^{-1} body weight) after 24 h post i.t. injection. The values relative to the control groups. **p < 0.01 (n = 3).

Figure S4. Cell viability of NIH3T3 fibroblast cells after 24 h incubation with SARS-CyCD at different concentrations.
4. NMR, MS, HPLC and IR Spectra

Figure S5. $^1$H NMR spectrum of SARS(Trt) (1) in DMSO-$d_6$.

Figure S6. $^{13}$C NMR spectrum of SARS(Trt) (1) in DMSO-$d_6$. 
Figure S7. $^1$H NMR spectrum of SARS(Trt)-PABA (2) in DMSO-$d_6$.

Figure S8. $^{13}$C NMR spectrum of SARS(Trt)-PABA (2) in DMSO-$d_6$. 
Figure S9. $^1$H NMR spectrum of SARS(Trt)-Cy (4) in Methanol-$d_4$.

Figure S10. $^{13}$C NMR spectrum of SARS(Trt)-Cy (4) in Methanol-$d_4$. 
Figure S11. $^1$H NMR spectrum of SARS-Cy (5) in Methanol-$d_4$.

Figure S12. $^{13}$C NMR spectrum of SARS-Cy (5) in Methanol-$d_4$. 
Figure S13. $^1$H NMR spectrum of SARS-CyCD in DMSO-$d_6$.

Figure S14. $^{13}$C NMR spectrum of SARS-CyCD in DMSO-$d_6$. 
Figure S15. ESI-MS spectrum of SARS(Trt) (1).

Figure S16. ESI-MS spectrum of SARS(Trt)-PABA (2).
**Figure S17.** ESI-MS spectrum of SARS(Trt)-Cy (4).

**Figure S18.** ESI-MS spectrum of SARS-Cy (5).
Figure S19. MALDI-TOF spectra of P-HPβCD and SARS-CyCD. The mass range of SARS-CyCD is the sum of the molecular weight of one SARS-Cy fragment and one P-HPβCD.

Figure S20. HPLC profile of SARS(Trt)-Cy (4).
Figure S21. HPLC profile of SARS-Cy (5).

Figure S22. IR spectrum of SARS(Trt) (1).
Figure S23. IR spectrum of SARS(Trt)-PABA (2).

Figure S24. IR spectrum of SARS(Trt)-Cy (4).
**Figure S25.** IR spectrum of SARS-Cy (5).

**Figure S26.** IR spectrum of SARS-CyCD.
5. References

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