Severe Acute Respiratory Syndrome Coronavirus Papain-like Novel Protease Inhibitors: Design, Synthesis, Protein–Ligand X-ray Structure and Biological Evaluation‡

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The design, synthesis, X-ray crystal structure, molecular modeling, and biological evaluation of a series of new generation SARS-CoV PLpro inhibitors are described. A new lead compound 3 (6577871) was identified via high-throughput screening of a diverse chemical library. Subsequently, we carried out lead optimization and structure−activity studies to provide a series of improved inhibitors that show potent PLpro inhibition and antiviral activity against SARS-CoV infected Vero E6 cells. Interestingly, the (S)-Me inhibitor 15h (enzyme IC₅₀ = 0.56 μM; antiviral EC₅₀ = 9.1 μM) and the corresponding (R)-Me 15g (IC₅₀ = 0.32 μM; antiviral EC₅₀ = 9.1 μM) are the most potent compounds in this series, with nearly equivalent enzymatic inhibition and antiviral activity. A protein−ligand X-ray structure of 15g-bound SARS-CoV PLpro and a corresponding model of 15h docked to PLpro provide intriguing molecular insight into the ligand-binding site interactions.

Introduction

Severe acute respiratory syndrome (SARS⁵) was first reported in Guangdong province, China, in November 2002.¹ SARS is a contagious respiratory illness with no effective treatment to date. SARS affected three continents, infecting more than 8000 individuals and causing nearly 800 deaths. Fortunately, the spread of SARS-CoV was contained after the initial outbreaks through public health measures. As it turned out, the etiological agent of SARS is a novel coronavirus, SARS-CoV.²³ There have been no known new cases of SARS since 2005. However, recent isolation of strains from zoonotic origins thought to be the reservoir for SARS-CoV raises the possibility of a reemergence of SARS and related ailments.⁴² Consequently, design and development of antivirals effective against SARS-CoV should be an important priority against future outbreaks.

Biochemical events critical to the viral replication revealed a number of important targets for therapeutic intervention of SARS.⁴⁶ Most notably, two cysteine proteases, a papain-like protease (PLpro) and a 3C-like protease (3CLpro), play a critical role in the virus-mediated RNA replication. Not surprisingly, numerous studies related to the development of SARS-CoV 3CLpro inhibitors have already been reported.⁸⁹ In contrast, very few inhibitor design efforts against SARS-CoV PLpro have been reported. We recently reported the discovery and design of a series of unprecedented noncovalent SARS-CoV PLpro inhibitors displaying antiviral activity against SARS-CoV with no associated cytotoxicity.¹⁰ Subsequently, a protein−ligand X-ray structure provided important molecular insights for further design and optimization of inhibitors.¹⁰ This initial work demonstrated that PLpro is a viable target for the development of anti-SARS therapeutics.

Besides viral peptide cleavage, recent structural and functional studies demonstrated that PLpro is involved in a number of other important biochemical events, such as deubiquitination, deISGylation, and involvement in the virus evasion from the innate immune response.¹¹¹² The homologous enzyme PLP2, from the human coronavirus 229E, has been shown to be critical to 229E viral replication.¹³ In addition, recent studies have shown that human deubiquitinating enzymes are potential anticancer drug-design targets. Thus, PLpro is a significant target for development of drugs against SARS and is a model for development of drugs against other deubiquitinating enzymes involved in human diseases. Recently, our primary screening of a library of 50,080 diverse, druglike compounds led to the identification of two compounds after lead validation. Both leads reproducibly inhibited PLpro in a dose dependent manner in the absence and presence of Triton-X. Subsequently, our optimization efforts of the most potent lead, 1 (7724772), containing a benzamide scaffold (IC₅₀ = 20.1 ± 1.1 μM) led to the design of novel PLpro inhibitor 2 and related derivatives that displayed antiviral activity against SARS-CoV. We recently reported a detailed study describing synthesis, biological studies, and X-ray structure of the protein−ligand complex of 2-bound PLpro.¹⁰ In our continuing studies toward the development of noncovalent/reversible PLpro inhibitors, we...
have now investigated the potential of the second and less potent lead that evolved from our high-throughput screening efforts. The second HTS lead, compound 3 (Figure 1), contains a piperidine carboxamide scaffold and exhibited an IC50 value of 59 μM. Our subsequent lead optimization efforts led to the design of potent inhibitor 15g (IC50 = 0.32 μM) which inhibited SARS-CoV viral replication in Vero cells with an EC50 value of 9.1 μM. The corresponding enantiomer 15h has shown slightly less potent enzyme inhibitory activity (IC50 = 0.56 μM) and similar antiviral potency. A protein–ligand X-ray structure of 15g-bound SARS-CoV PLpro was determined. Interestingly, this structure revealed a unique mode of binding with SARS-CoV PLpro and that key molecular interactions of inhibitor 15g are quite different from the active-site interactions with inhibitor 2. Herein we describe the design, synthesis, structure–activity studies, molecular modeling, protein–ligand X-ray structure, and biological evaluation of a series of novel and noncovalent inhibitors of SARS-CoV PLpro.

Chemistry

To ascertain the importance of the position of the methoxy substituent in lead inhibitor 3, we have synthesized the corresponding 2-methoxy and 3-methoxybenzyl derivatives. As shown in Scheme 1, Boc-piperidine-4-carboxylic acid 4 was coupled with 2- and 3-methoxybenzylamines 5a and 5b using N-(3-dimethylaminopropyl)-N0-ethylcarbodiimide hydrochloride (EDCI) and 1-hydroxybenzotriazole hydrate (HOBT) in the presence of N-methylmorpholine (NMM) in CH2Cl2 to provide coupling products 6a and 6b in 92% and 94% yield, respectively. Removal of Boc-group by exposure to trifluoroacetic acid (TFA) in CH2Cl2 at 0–23 °C for 6 h afforded the respective amine. Reductive amination of these amines with 1-naphthaldehyde using Na(OAc)3BH in the presence of acetic acid furnished inhibitors 7a and 7b in 70% and 71% yield, respectively.

For structure–activity studies and optimization of potency, we planned to synthesize derivatives of both 1- and 2-naphthylpiperidin-4-carboxylic acids and coupled them with various substituted benzylamine derivatives. The synthesis of substituted piperidine-4-carboxylic acids is shown in Scheme 2. Alkylation of dimethyl malonate 8 with commercially available 2-bromomethyl-1,3-dioxolane 9 in the presence of KO’Bu in DMSO at 23 °C afforded malonate derivative 10 as described previously.14 Deprotection of the ketal functionalities was carried out by treatment of 10 with 10% aqueous HCl in THF at 23 °C. The reaction was quenched with solid NaHCO3, and the resulting crude dialdehyde was used directly for the subsequent condensation reaction. Condensation of the dialdehyde with various
optically active (S)- and (R)-1-methyl-1-naphthylmethylamines, 1-methyl-2-naphthylmethylamine, 2-naphthylmethylamine, 1-naphthylmethylamine, and dimethyl-1-naphthylmethylamine \(11a-g\) in aqueous THF for 16 h afforded dihydropyridines \(12a-g\) in 39\%-62\% yield.\(^{15,16}\) Catalytic hydrogenation of dihydropyridines \(12a-f\) in ethylacetate at 23 °C provided various piperidine derivatives \(13a-f\) in 60\%-94\% yield.

The synthesis of various test inhibitors is shown in Scheme 3. Treatment of diesters \(13a-f\) with NaCN in DMF at reflux for 16 h provided methyl esters \(14a-g\) in 38\%-92\% yield. Dihydropyridine derivative \(12g\) was similarly converted to methyl ester \(14g\) in a two-step sequence. Saponification of \(14a-g\) with aqueous LiOH in a mixture (3:1:1) of THF, methanol, and water at 23 °C for 16 h afforded the corresponding carboxylic acids. Coupling of these resulting carboxylic acids with benzylamine derivatives \(5a-d\) utilizing EDCI in the presence of diisopropylethylamine as described above furnished various inhibitors \(15a-k\) in excellent yield (80\%-99\%).

To evaluate the effect of the corresponding piperazine derivatives, we sought to synthesize racemic piperazine derivative \(20\), and the synthesis is outlined in Scheme 4. Reductive amination\(^{17}\) of Boc-piperazine \(16\)\(^{18}\) with 1-acetonaphthone \(17\) using sodium cyanoborohydride in a mixture (50:1) of methanol and acetic acid at 23 °C for 48 h afforded \(18\) in 24\% yield. Removal of the Boc-group by treatment with trifluoroacetic acid in \(\text{CH}_2\text{Cl}_2\) at 23 °C for 2 h provided amine \(19\).\(^{19}\) Treatment of 4-methoxybenzylamine \(5c\) in the presence of \(N,N'\)-carbonyldiimidazole in \(\text{CH}_2\text{Cl}_2\) followed by addition of \(19\) and stirring of the resulting mixture at 23 °C for 4 h afforded piperazine derivative \(20\) in 90\% yield.

Results and Discussion

The second HTS lead \(3\) is considerably weaker than the first lead inhibitor \(1\), a benzamide derivative of 2-naphthylethylamine. To enhance activity, we first investigated the effect of 2-methoxy and 3-methoxy derivatives \(7a\) and \(7b\) on PLpro inhibitory activity. As shown in Table 1, 2-methoxy derivative \(7a\) showed a very poor inhibitory activity. The 3-methoxy...
and evaluated the corresponding unsubstituted derivatives in the 1- and 2-naphthylmethyl positions, we have synthesized 15g and 15h to examine the preference for a methyl group over a hydrogen at these positions. We have also examined the corresponding 2-methoxy derivative 15i and the 3-methoxy derivative 15j. These compounds exhibited potency comparable to the corresponding methoxy or benzamide derivatives. Interestingly, the 2-(R)-methoxy derivative 15k exhibited improvement of enzyme inhibitory activity with an IC50 value of 0.34 μM. The corresponding 3-methoxy derivative 15l showed comparable antiviral activity. Inhibitor 15l better enzyme activity than the (R)-methyl derivative 15m.

We next examined the effect of a piperazine ring in place of piperidine in 15n by preparing compound 15o. However, this piperazine derivative showed no activity against PLpro. Most likely, the piperazine derivative showed no activity against PLpro because of the structural constraints imposed by the carbon to nitrogen replacement on this ring. The new nitrogen is then attached to the amide group, forming a urea moiety. This urea moiety will tend to be planar, imposing a flexibility constraint. GOLD docking shows the amide to rotate ~90° away from the optimal hydrogen-bonding orientation (data not shown) of the other active compounds described here.

Our structure–activity studies established that both m-methoxy and p-methoxy derivatives (15b and 15c) are equally potent. Our preliminary modeling studies indicated that either methoxy oxygen (meta or para) is within proximity to form a hydrogen bond with the Gln-270 carboxamide side chain. On the basis of these possible interactions, we incorporated a benzodioxolane ring and examined its effect on inhibitory potency. As shown in Table 2, dioxolane derivative 15g exhibits potency comparable to the corresponding m- and p-derivatives 15b and 15c. The corresponding (S)-derivative 15h also shows comparable enzyme inhibitory activity. To examine the preference for a methyl group over a hydrogen at the 1- and 2-naphthylmethyl positions, we have synthesized and evaluated the corresponding unsubstituted derivatives 15i and 15j. As shown, both compounds displayed significant reduction in potency, indicating the importance of the methyl group. We have also examined the corresponding gem-dimethyl derivative 15k. Interestingly, this compound is inactive, indicating that both methyl groups cannot be accommodated by the PLpro active site.

Antiviral activities of selected PLpro inhibitors were determined, and the results are shown in Table 3. The compounds were assayed for their ability to rescue a Vero cell culture from SARS-CoV infection. The viability of virus-infected Vero E6 cells as a function of inhibitor concentration was measured relative to mock-infected cells using a luminescence assay. This protocol allows for the evaluation of both inhibitor efficacy and cytotoxicity. As can be observed from the data presented in Table 3, the original HTS lead (3) does not show any antiviral activity. However, all 2-, 3-, and 4-methoxy derivatives 15a–e show comparable antiviral activity. Inhibitor 15f with a 2-naphthyl substituent displayed no antiviral activity. While the (R)-methyl derivative 15g showed slightly better enzyme activity than the (S)-methyl derivative 15h, both inhibitors exhibited the same antiviral potency (EC50 = 9.1 μM). Interestingly, both dioxolane derivatives 15g and 15h showed antiviral activity approximately comparable to the activity of the corresponding methoxy or benzamide derivatives reported in our previous studies.

To obtain molecular insight into the ligand-binding site interactions, the X-ray crystal structure of 15g bound to PLpro was determined. Interestingly, the binding mode and key molecular interactions of inhibitor 15g are quite different than predicted and are different from the active-site interactions with the benzamide-derived inhibitors we previously reported. As shown in Figure 2, the inhibitor binds to a loop adjacent to the active site via a series of interactions including a hydrogen-bond formed between the carboxamide NH of the inhibitor and the backbone carbonyl of Tyr-269, with 15g wrapped around the β-turn. The 15g bound PLpro crystal structure also confirms the presence of a few structural water molecules conserved between the apo enzyme (PDB code

Table 2. Structure and Activity of Benzodioxolane Derivatives

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15a</td>
<td><img src="image1.png" alt="Structure" /></td>
<td>0.32 ± 0.01</td>
</tr>
<tr>
<td>15b</td>
<td><img src="image2.png" alt="Structure" /></td>
<td>0.56 ± 0.03</td>
</tr>
<tr>
<td>15c</td>
<td><img src="image3.png" alt="Structure" /></td>
<td>~45</td>
</tr>
<tr>
<td>15d</td>
<td><img src="image4.png" alt="Structure" /></td>
<td>~100</td>
</tr>
<tr>
<td>15e</td>
<td><img src="image5.png" alt="Structure" /></td>
<td>&gt;200</td>
</tr>
</tbody>
</table>

Table 3. Evaluation of Compounds as Inhibitors of SARS-CoV Replication in a Cell-Based Assay

<table>
<thead>
<tr>
<th>compd</th>
<th>IC50 (μM)</th>
<th>EC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>59.2 ± 7.8</td>
<td>NI</td>
</tr>
<tr>
<td>15a</td>
<td>1.21 ± 0.04</td>
<td>11.6 ± 0.6</td>
</tr>
<tr>
<td>15b</td>
<td>0.34 ± 0.01</td>
<td>9.7 ± 0.3</td>
</tr>
<tr>
<td>15c</td>
<td>0.34 ± 0.01</td>
<td>10.2 ± 0.5</td>
</tr>
<tr>
<td>15f</td>
<td>5.8 ± 0.1</td>
<td>&gt;25</td>
</tr>
<tr>
<td>15g</td>
<td>0.32 ± 0.01</td>
<td>9.1 ± 0.5</td>
</tr>
<tr>
<td>15h</td>
<td>0.56 ± 0.03</td>
<td>9.1 ± 0.3</td>
</tr>
</tbody>
</table>

Interestingly, the (S)-methyl derivative 15h showed slightly better enzyme activity than the (S)-methyl derivative 15h, both inhibitors exhibited the same antiviral potency (EC50 = 9.1 μM). Interestingly, both dioxolane derivatives 15g and 15h showed antiviral activity approximately comparable to the activity of the corresponding methoxy or benzamide derivatives reported in our previous studies.
2FE8) and inhibitor 2 bound PLpro (PDB code 3E9S). One of the conserved water molecules sits in the P5 pocket shown in Figure 2 as spheres between residues Asp-165, Asp-303, and Thr-302, preventing the inhibitor naphthyl rings from occupying this pocket. In the stereoimage of 15g-bound PLpro we also show two other water molecules near residue Leu-163 and Lys-158 that may prevent the benzodioxolane ring from flipping down toward Lys-158.

Figure 3 superimposes 15g and our previously developed inhibitor, 2,\textsuperscript{10} and demonstrates that the binding mode differs significantly between the two inhibitors. Interestingly, the turn region between Tyr-269 and Gln-270 also shows significant flexibility, particularly in the case of inhibitor 2 (PDB code 3E9S), where the peptide bond between Tyr-269 and Gln-270 flips by 180° to enable a hydrogen bond interaction between the backbone nitrogen of Tyr-269 and the carboxamide oxygen in inhibitor 2. The carboxamide nitrogen makes a hydrogen bond with the side chain carboxylate of Asp-165. The carboxamide nitrogen of inhibitor 15g (yellow) forms a hydrogen bond with the backbone carbonyl

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**Figure 2.** Stereorepresentation of 15g bound to PLpro, including the conserved waters adjacent to the binding site that may influence the binding conformation, as described in the text.

**Figure 3.** X-ray structure of inhibitor 15g-bound (yellow) PLpro (gray) (PDB code 3MJ5) superimposed on the X-ray structure of inhibitor 2-bound (cyan) PLpro (pink) (PDB code 3E9S).
Managing the potency of both enantiomers. However, the carbonyl oxygen of Tyr-269 in a similar fashion, thereby decreasing the freedom around the carbon atom and locks the compound in a conformation where one of the methyl groups exhibits a bumping collision with the side chain of Asp-165. One of the methyl groups in 15k shifts almost 1.2 Å toward residue Asp-165 when compared to the single methyl substitution (R)-Me in 15g, as can be seen in Figure 4B. It is important to note that the side chain of this Asp-165 is locked in its position by a hydrogen bond with the backbone NH of Arg-167. Hence, the gem-dimethyl substitution is not favorably accommodated in the active site because in order to fit the hydrophobic methyl group near the hydrophilic residue, the aspartic acid side chain would have to move out, thereby breaking structural hydrogen bonding with Arg-167.

This hypothesis is further validated by the GoldScore scoring function of GOLD, version 4.1, during the docking study. Compound 15k is heavily penalized because of an unfavorable internal energy term (−12 compared to about −6 for both 15g and 15h) which is a sum of the internal torsional strain and internal van der Waals energy terms of the ligand. Docking with flexible residues also suggests that the Gln-270 side chain may adopt conformations that might enable hydrogen bonding interactions with one of the 1,3 benzodioxolane oxygens in 15g and 15h (within 3 Å). However, all docked conformations generated for 15k show a loss of this hydrogen bonding interaction. The closest benzodioxolane oxygen of 15k is at least 4.8 Å away from the side chain of Gln-270 (not shown). Figure 4B highlights the potential bumping collision of one of the methyl groups of 15k with Asp-165, demonstrating that two methyl groups cannot be accommodated favorably at this position.

In our previous study, we discussed the SAR of the analogues of our first HTS hit 1 and the evolution of inhibitor 2 in great detail. In distinct contrast to the present work, that series of compounds is extremely sensitive to the enantiomeric form of the compound. From docking studies we concluded that the (R)-Me form was active whereas the (S)-Me was inactive because the (S)-Me conformation pushed the carbamide group of the inhibitor away from the backbone NH of Tyr-269, inhibiting hydrogen bond formation with the loop residue.

Figure 4. (A) Superposition of enantiomer 15h (blue) with the crystal structure of 15g-bound (yellow) PLpro. (B) Docked alignment of the gem-dimethyl substituted compound in the 15g-ligand removed PLpro crystal structure. The bumping collision of one of the methyl groups of the gem-dimethyl (magenta) 15k with the Asp-165 carboxylate is noted.

Modeling Studies

To understand the SAR of the analogues of HTS hit compound 3, we used computer modeling to explore the interactions of this series of inhibitors with PLpro. The activity of this series of compounds is independent of stereoisomerism in contrast to the series of compounds synthesized from the first HTS hit compound 1. GOLD redocking of inhibitor 15g into the PLpro crystal structure described above produces a heavy atom rmsd of 1.7 Å with the crystal structure conformation of 15g, indicating that docking satisfactorily reproduces the experimental structure. When the inhibitors 15g, 15h, and 15k are docked into the ligand removed 15g-bound PLpro crystal structure (with residues Tyr-269 and Gln-270 flagged as flexible), the internal strain scores of the compounds correlate very well with their enzymatic activities. The conserved overlapping water molecules observed in both chains A and B of the 15g-bound PLpro crystal structure were included for all docking studies. To investigate the structural basis of the potency insensitivity to the (R)-Me (15g) versus (S)-Me (15h) configuration, we show the docked model of inhibitor 15h superimposed on the crystal structure of 15g-bound PLpro in Figure 4A. From this model, we observe an inversion of the piperidine ring between the (R)-Me and (S)-Me binding modes that allows the naphthyl rings of both isomers to be accommodated in the active site in very similar orientations. The flexible piperidine ring also acts as a spacer group that enables the carbamide NH of both 15h and 15g to hydrogen-bond with the backbone carbonyl oxygen of Tyr-269 in a similar fashion, thereby retaining the potency of both enantiomers. However, the gem-dimethyl substitution in 15k decreases the freedom around the carbon atom and locks the compound in a conformation where
Experimental Section

Chemistry. 1H NMR and 13C NMR spectra were recorded on a Varian Oxford 300 and Bruker Avance 400 spectrometers. Optical rotations were recorded on a Perkin-Elmer 341 polarimeter. Anhydrous solvent was obtained as follows: CH2Cl2 by distillation from CaH2, THF by distillation from Na and benzophenone. All other solvents were reagent grade. Column chromatography was performed with Whatman 250-400 mesh silica gel under low pressure of 3–5 psi. TLC was carried out with E. Merck silica gel 60-F-254 plates. Purity of all test compounds was determined by HPLC and HPLC analysis in the different solvent systems. All test compounds showed ≥95% purity.

1-(tert-Butyloxy carbonyl)-4-(3-methoxybenzylation)carbonyl-piperidine (6b). To a solution of 1-(tert-butyloxy carbonyl)piperidine-4-carboxylic acid (344 mg, 1.5 mmol) in dry CH2Cl2 (5 mL), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC-6Cl) (287 mg, 1.5 mmol), 1-hydroxybenzotriazole hydrate (HOBT-H2O) (203 mg, 1.5 mmol), N-methylmorpholine (NMM) (0.16 mL, 1.5 mmol), and 3-methoxybenzylamine (0.13 mL, 1.5 mmol) were added successively at 23 °C under argon atmosphere, and the resulting reaction mixture was stirred for 5 h at the same temperature. The reaction mixture was quenched with aqueous NaOH solution and extracted with CH2Cl2. The organic layers were dried over anhydrous Na2SO4, quenched with aqueous NaOH solution and extracted with EtOAc. The organic layers were dried over anhydrous Na2SO4, and the aqueous layer was extracted several times with ethyl acetate. The combined organic layers were dried over anhydrous Na2SO4. The solvent was removed under reduced pressure to furnish the amine. To the crude amine in dry CH2Cl2 (5 mL), 1-naphthaldehyde (77 µL, 0.57 mmol), NaOAc/BH (121 mg, 0.57 mmol), and AcOH (33 µL, 0.57 mmol) were added successively at 23 °C, and the resulting mixture was stirred for 12 h at 23 °C. The reaction mixture was basified with 2 N NaOH and diluted with CH2Cl2 and H2O. The organic layer was separated and the aqueous layer extracted with CH2Cl2. The combined organic layers were dried over anhydrous Na2SO4. Solvent was removed under reduced pressure and the resulting residue was purified by column chromatography over silica gel (2% MeOH/CH2Cl2) to provide 1-[1-naphthylmethyl] -4-(3-methoxybenzylamino)carbonylpiperidine as a viscous liquid (79 mg, 71%). 1H NMR (400 MHz, CDCl3): δ 8.28 – 8.33 (m, 1H), 7.82 – 7.88 (m, 1H), 7.77 (dd, J = 2.2 and 7.1 Hz, 1H), 7.44 – 7.53 (m, 2H), 7.36 – 7.43 (m, 2H), 7.23 (t, J = 7.8 Hz, 1H), 6.77 – 6.86 (m, 3H), 5.79 (br, 1H), 4.40 (d, J = 5.7 Hz, 2H), 3.88 (s, 1H), 3.78 (s, 3H), 2.94 – 3.04 (m, 2H), 2.15 (tt, J = 4.2 and 11.4 Hz, 1H), 2.06 (dt, J = 2.7 and 11.3 Hz, 2H), 1.72 – 1.88 (m, 4H). 13C NMR (100 MHz, CDCl3): δ 174.9, 159.8, 139.9, 134.3, 133.8, 132.5, 129.7, 128.3, 127.8, 127.4, 125.7, 125.6, 125.0, 124.8, 119.9, 113.3, 112.9, 61.3, 55.3, 53.3, 43.6, 43.3, 29.1 (IR (neat): 3290, 2922, 1644, 1598, 1263 cm−1. MS (EI): m/z: 389 [M + H+].

1-[1-Naphthylmethyl]-4-[2-methoxybenzylamino]carbonyl-piperidine (7a). The title compound 7a was obtained as described for compound 7b in 70% yield (viscous liquid). 1H NMR (400 MHz, CDCl3): δ 8.30 (d, J = 7.9 Hz, 1H), 7.84 (d, J = 7.1 Hz, 1H), 7.77 (d, J = 7.1 Hz, 1H), 7.44 – 7.53 (m, 2H), 7.37 – 7.43 (m, 2H), 7.21 – 7.30 (m, 2H), 6.83 – 6.94 (m, 2H), 5.98 (brs, 1H), 4.43 (d, J = 5.6 Hz, 2H), 3.87 (s, 2H), 3.84 (s, 3H), 2.98 (d, J = 11.2 Hz, 2H), 2.01 – 2.20 (m, 1H), 1.68 – 1.84 (m, 4H). 13C NMR (100 MHz, CDCl3): δ 174.6, 157.5, 153.4, 133.8, 132.5, 129.8, 128.8, 128.3, 127.8, 127.2, 126.3, 125.7, 125.6, 125.1, 124.8, 120.7, 110.3, 61.3, 55.3, 53.4, 43.6, 39.3, 29.0. IR (neat): 3305, 1643, 1600, 1242 cm−1. MS (EI): m/z: 389 [M + H+]..

1-[1-(Naphthyl)ethyl]-4-[4-(bis(methoxycarbonyl)1,4-dihydropyridine (12a). A solution of malonate 10 (1.8 g, 5.92 mmol) in 35% hydrogen peroxide (15 mL) was stirred for 18 h at 23 °C. The solution was neutralized with powdered sodium hydrogen carbonate, and then 1-(Naphthylmethylformamide 11 (1.0 g, 5.84 mmol) in THF (5 mL) was added. After the mixture was stirred for 16 h at 23 °C, the aqueous layer was extracted with EtOAc and dried over Na2SO4. Removal of the solvent afforded the residue, which was purified by silica gel column chromatography to furnish compound 12a (1.1 g, 54%) as a colorless oil. Rf = 0.74 (hexane/EtOAc = 1:1). [α]D = −58 (c = 1, CHCl3). 1H NMR (300 MHz, CDCl3): δ 7.90 (d, 1H, J = 7.8 Hz), 7.84 (d, 1H, J = 7.8 Hz), 7.81 – 7.75 (m, 5H), 7.54 – 7.47 (m, 4H), 6.21 (d, 1H, J = 8.3 Hz, 5.16 (d, 1H, J = 8.6 Hz, 4.77 (d, 2H, J = 8.3 Hz, 3.69 Hz), 1.67 (d, 3H, J = 6.6 Hz). 13CNMR (75 MHz, CDCl3): δ 171.4, 136.2, 133.7, 130.8, 129.2, 128.7, 128.4, 126.3, 126.7, 123.7, 122.8, 95.3, 56.8, 54.0, 52.4, 19.4. IR (neat): 2951, 1736, 1249, 1069 cm−1. MS (EI): m/z: 352 [M−H−]. HRMS (EI, calef for C32H22NO3: 525.1549, found 525.1553.

1-[1-(Naphthyl)ethyl]-4-[4-(bis(methoxycarbonyl)1,4-dihydropyridine (12b). The title compound was obtained as described in compound 12a in 58% yield (colorless oil). Rf = 0.79 (hexane/ EtOAc = 1:1). [α]D = +32 (c = 1, CHCl3). 1H NMR (300 MHz, CDCl3): δ 7.84 – 7.78 (m, 3H), 7.66 (s, 1H), 7.49 – 7.43 (m, 2H), 7.33 (dd, 1H, J = 1.5 and 8.7 Hz), 6.21 (d, 2H, J = 8.3 Hz), 4.78 (d, 2H, J = 8.3 Hz), 4.59 (q, 1H, J = 6.9 Hz), 3.72 (s, 6H), 1.64 (d, 3H, J = 6.9 Hz). 13CNMR (75 MHz,
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1-[(2-Naphthyl)ethyl]-4,4-bis(methoxycarbonyl)-1,4-dihydropyridine (12c). The title compound was obtained as described in compound 12a in 4% yield (colorless oil). 

IR (neat): 2950, 1731, 1253, 1066 cm⁻¹. MS (EI): m/z 355 [M⁺]. HRMS (EI), calcd for C₂₆H₂₅NO₄ 355.1784, found 355.1781.

1-[(2-Naphthyl)ethyl]-4,4-bis(methoxycarbonyl)-1,4-dihydropyridine (12d). The title compound was obtained as described in compound 12a in 42% yield (colorless oil). Rf = 0.77 (hexane/EtOAc = 1:1). [α]D⁰ +24 (c 1, CHCl₃). MS (EI): m/z 355 [M⁺]. HRMS (EI), calcd for C₂₆H₂₅NO₄ 355.1784, found 355.1786.

1-[(1-Naphthyl)methyl]-4,4-bis(methoxycarbonyl)-1,4-dihydropyridine (12f). The title compound was obtained as described in compound 12a in 62% yield (colorless oil). Rf = 0.80 (hexane/EtOAc = 1:1). [α]D⁰ +27 (c 1, CHCl₃). MS (EI): m/z 356 [M⁺ + H⁺]. HRMS (EI), calcd for C₁₈H₁₆NO₂ 356.1862, found 356.1865.

1-[(1-Naphthyl)methyl]-4,4-bis(methoxycarbonyl)-1,4-dihydropyridine (13a). The title compound was obtained as described in compound 13a in 60% yield (colorless oil). Rf = 0.70 (hexane/EtOAc = 1:1). [α]D⁰ +26 (c 1, CHCl₃). MS (EI): m/z 355 [M⁺]. HRMS (EI), calcd for C₂₆H₂₅NO₄ 355.1784, found 355.1781.

1-[(1-Naphthyl)methyl]-4,4-bis(methoxycarbonyl) piperidine (13b). The title compound was obtained as described in compound 13a in 74% yield (colorless oil). 

IR (neat): 2950, 1732, 1254, 1072 cm⁻¹. MS (EI): m/z 356 [M⁺]. HRMS (EI), calcd for C₂₆H₂₅NO₄ 356.1825, found 356.1829.

MS (EI): m/z 388 [M + Na⁺]. HRMS (EI), calcd for C₂₆H₂₅NO₄Na 388.1525, found 388.1529.

1-[(1-Naphthyl)ethyl]-4,4-bis(methoxycarbonyl) piperidine (13c). The title compound was obtained as described in compound 13a in 94% yield (colorless oil). Rf = 0.48 (hexane/EtOAc = 1:1). [α]D⁰ +26 (c 1, CHCl₃). MS (EI): m/z 355 [M⁺]. HRMS (EI), calcd for C₂₆H₂₅NO₄ 355.1784, found 355.1786.

1-[(1-Naphthyl)ethyl]-4,4-bis(methoxycarbonyl) piperidine (13d). The title compound was obtained as described in compound 13a in 62% yield (colorless oil). Rf = 0.62 (hexane/EtOAc = 1:1). [α]D⁰ +24 (c 1, CHCl₃). MS (EI): m/z 356 [M⁺ + H⁺]. HRMS (EI), calcd for C₂₆H₂₅NO₄ 356.1862, found 356.1865.

1-[(1-Naphthyl)ethyl]-4,4-bis(methoxycarbonyl) piperidine (13e). The title compound was obtained as described in compound 13a in 60% yield (colorless oil). Rf = 0.70 (hexane/EtOAc = 1:1). [α]D⁰ +26 (c 1, CHCl₃). MS (EI): m/z 355 [M⁺]. HRMS (EI), calcd for C₂₆H₂₅NO₄ 355.1784, found 355.1781.
The title compound was obtained as described in compound 14a in 90% yield (colorless oil). \( R_4 = 0.47 \) (CH\(_2\)Cl\(_2\)/MeOH = 9:1). \[\delta^{1}D = \pm 0.16 \text{ c (1, CHCl\(_3\))} \]

The title compound was obtained as described in compound 14a in 90% yield (colorless oil). \( R_4 = 0.47 \) (CH\(_2\)Cl\(_2\)/MeOH = 9:1). \[\delta^{1}D = \pm 0.16 \text{ c (1, CHCl\(_3\))} \]

To a stirred solution of ester 14a (106 mg, 0.36 mmol) in THF/MeOH/H\(_2\)O (3:1) (8 mL) was added LiOH·H\(_2\)O (22.4 mg, 0.53 mmol) at 0°C, and the mixture was allowed to stir for 16 h at 23°C. The mixture was concentrated, and to it was added a saturated NaHCO\(_3\) solution. The mixture was extracted with Et\(_2\)O. The aqueous layer was adjusted to pH 2 with 10% HCl solution and extracted with EtOAc. The organic layers were dried over Na\(_2\)SO\(_4\), filtered, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to furnish compound 15a (143 mg, 99%) as a white amorphous solid. \( R_4 = 0.42 \) (CH\(_2\)Cl\(_2\)/MeOH = 9:1). \[\delta^{1}D = \pm 0.2 \text{ c (1, CHCl\(_3\))} \]

The title compound was obtained as described in compound 15a in 95% yield (white amorphous solid). \( R_4 = 0.49 \) (CH\(_2\)Cl\(_2\)/MeOH = 9:1). \[\delta^{1}D = \pm 0.2 \text{ c (1, CHCl\(_3\))} \]

The title compound was obtained as described in compound 15a in 95% yield (white amorphous solid). \( R_4 = 0.49 \) (CH\(_2\)Cl\(_2\)/MeOH = 9:1). \[\delta^{1}D = \pm 0.2 \text{ c (1, CHCl\(_3\))} \]

The title compound was obtained as described in compound 15a in 95% yield (white amorphous solid). \( R_4 = 0.49 \) (CH\(_2\)Cl\(_2\)/MeOH = 9:1). \[\delta^{1}D = \pm 0.2 \text{ c (1, CHCl\(_3\))} \]

The title compound was obtained as described in compound 15a in 95% yield (white amorphous solid). \( R_4 = 0.49 \) (CH\(_2\)Cl\(_2\)/MeOH = 9:1). \[\delta^{1}D = \pm 0.2 \text{ c (1, CHCl\(_3\))} \]

The title compound was obtained as described in compound 15a in 95% yield (white amorphous solid). \( R_4 = 0.49 \) (CH\(_2\)Cl\(_2\)/MeOH = 9:1). \[\delta^{1}D = \pm 0.2 \text{ c (1, CHCl\(_3\))} \]

The title compound was obtained as described in compound 15a in 95% yield (white amorphous solid). \( R_4 = 0.49 \) (CH\(_2\)Cl\(_2\)/MeOH = 9:1). \[\delta^{1}D = \pm 0.2 \text{ c (1, CHCl\(_3\))} \]
1. ([R]-1-(2-Naphthylmethyl)-4-(2-methoxybenzylamino)carbonylpiperidine (15a). The title compound was obtained as described in compound 15a in 98% yield (white amorphous solid). \( R_f = 0.47 (\text{CH}_2\text{Cl}_2/\text{MeOH} = 9:1). \) \( \text{HRMS} (\text{ESI}) \), calcd for C\text{25}H\text{30}N\text{2}O\text{3} 403.2022, found 403.2025.

2. ([1-Methyl-1-(naphthylmethyl)-4-(2-methylenedioxy)benzylamino]carbonylpiperidine (15b). The title compound was obtained as described in compound 15a in 90% yield (white amorphous solid). \( R_f = 0.51 (\text{hexane}/\text{EtOAc} = 1:1). \) \( \text{HRMS} (\text{ESI}) \), calcd for C\text{26}H\text{31}N\text{2}O\text{2} 403.2386, found 403.2386.

3. ([R]-1-(2-Naphthylmethyl)-4-(3-methoxybenzylamino)carbonylpiperidine (15f). The title compound was obtained as described in compound 15a in 83% yield (white amorphous solid). \( R_f = 0.37 (\text{CH}_2\text{Cl}_2/\text{MeOH} = 9:1). \) \( \text{HRMS} (\text{ESI}) \), calcd for C\text{26}H\text{31}N\text{2}O\text{2} 403.2386, found 403.2392.

4. ([1-R]-1-(1-Naphthylmethyl)-4-[3,4-methylenedioxy]benzylamino]carbonylpiperidine (15g). The title compound was obtained as described in compound 15a in 93% yield (white amorphous solid). \( R_f = 0.47 (\text{CH}_2\text{Cl}_2/\text{MeOH} = 9:1). \) \( \text{HRMS} (\text{ESI}) \), calcd for C\text{26}H\text{31}N\text{2}O\text{2} 403.2386, found 403.2387.

5. ([R]-1-(1-Naphthylmethyl)-4-[3,4-methylenedioxy]benzylamino]carbonylpiperidine (15h). The title compound was obtained as described in compound 15a in 80% yield (white amorphous solid). \( R_f = 0.56 (\text{CH}_2\text{Cl}_2/\text{MeOH} = 9:1). \) \( \text{HRMS} (\text{ESI}) \), calcd for C\text{26}H\text{31}N\text{2}O\text{2} 403.2387, found 403.2373.

6. ([1-Naphthylmethyl]-4-[3,4-methylenedioxy]benzylamino]carbonylpiperidine (15i). The title compound was obtained as described in compound 15a in 99% yield (white amorphous solid). \( R_f = 0.48 (\text{CH}_2\text{Cl}_2/\text{MeOH} = 9:1). \) \( \text{HRMS} (\text{ESI}) \), calcd for C\text{26}H\text{31}N\text{2}O\text{2} 403.2387, found 403202.

7. ([2-Naphthylmethyl]-4-[3,4-methylenedioxy]benzylamino]carbonylpiperidine (15j). The title compound was obtained as described in compound 15a in 88% yield (white amorphous solid). \( R_f = 0.42 (\text{CH}_2\text{Cl}_2/\text{MeOH} = 9:1). \) \( \text{HRMS} (\text{ESI}) \), calcd for C\text{26}H\text{31}N\text{2}O\text{2} 403.2387, found 403.2025.
were flash-frozen in liquid nitrogen and then transferred into a dry nitrogen stream at 100 K for X-ray data collection. The data set of the complex was collected at the Southeast Regional Collaborative Access Team (SER-CAT) beamline X29 at the Advanced Photon Source, Argonne National Laboratory. Data were processed and scaled using the HKL2000 program suite. Crystals belonged to the space group C2, with two monomers in the asymmetric unit. The inhibitor-complexed structure was solved to 2.63 Å by molecular replacement using the SARS-CoV PLpro apoenzyme structure (PDB entry 2FE8) as a search model in the AMoRe program of the CCP4 suite. Model manual rounds of positional and B-factor refinement and map building were performed using CNS. The structure was deposited under PDB code 3MJ5.

SARS-CoV Antiviral and PLpro Inhibition Assays. SARS-CoV antiviral assays and PLpro inhibition assays were performed as previously described.10

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Supporting Information Available: HPLC and HRMS data of inhibitors. This material is available free of charge via the Internet at http://pubs.acs.org.

References
