A New Lead for Nonpeptidic Active-Site-Directed Inhibitors of the Severe Acute Respiratory Syndrome Coronavirus Main Protease Discovered by a Combination of Screening and Docking Methods

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The coronavirus main protease, $\text{M}^{\text{pro}}$, is considered to be a major target for drugs suitable for combating coronavirus infections including severe acute respiratory syndrome (SARS). An HPLC-based screening of electrophilic compounds that was performed to identify potential $\text{M}^{\text{pro}}$ inhibitors revealed etacrynic acid tert-butylamide (6a) as an effective nonpeptidic inhibitor.

Docking studies suggested a binding mode in which the phenyl ring acts as a spacer bridging the inhibitor’s activated double bond and its hydrophobic tert-butyl moiety. The latter is supposed to fit into the S4 pocket of the target protease. Furthermore, these studies revealed etacrynic acid amide (6b) as a promising lead for nonpeptidic active-site-directed $\text{M}^{\text{pro}}$ inhibitors.

In a fluorimetric enzyme assay using a novel fluorescence resonance energy transfer (FRET) pair labeled substrate, compound 6b showed a $K_i$ value of 35.3 μM. Since the novel lead compound does not target the S1′, S1, and S2 subsites of the enzyme’s substrate-binding pockets, there is room for improvement that underlines the lead character of compound 6b.

Introduction

Coronaviruses are important pathogens that mainly cause respiratory and enteric disease in humans, livestock, and domestic animals. For example, transmissible gastroenteritis virus (TGEV), a group I coronavirus, causes diarrhea in young piglets, and SARS-CoV, a group II coronavirus, causes severe acute respiratory syndrome (SARS). It is generally believed that SARS-CoV only very recently crossed the species barrier from an unknown animal reservoir to humans, thus causing the severe SARS epidemic in 2002-2003 with more than 800 deaths worldwide. With genome sizes of about 30 kilobases, coronaviruses are the largest plus-strand RNA viruses currently known. In their life cycles, coronaviruses employ several unique mechanisms and enzymes that discriminate them from all other RNA viruses. Following receptor-mediated entry into the host cell, the genome RNA is translated to produce two large replicase polyproteins that are autocatalytically cleaved by two or three viral proteases. The key enzyme in this process is the coronavirus main protease, $\text{M}^{\text{pro}}$, a cysteine protease featuring a two-β-barrel fold, which cleaves the replicase polyproteins at as many as 11 conserved sites. Because of its essential role in proteolytic processing, the $\text{M}^{\text{pro}}$ is considered to be an attractive target for new antiviral drugs against SARS and other coronavirus infections.

A number of potential inhibitors have been proposed employing molecular modeling and virtual screening techniques. However, the inhibitory potency of these compounds has not yet been verified. Only a small number of potent protease inhibitors were identified by screening assays thus far. These include an HIV protease inhibitor identified by a broad cell-based screening, two nonpeptidic heterocyclic compounds that were identified by a broad protease inhibition screening, a 3-quinolinecarboxylic acid ester and an imidazolidine containing inhibitors, and boronic acid derivatives that are supposed to target serine residues of the protease. Most of these studies used commercially available compound libraries for their screening assays. With the exception of a peptidyl chloromethyl ketone and the peptidomimetic inhibitor AG-7088, none of the compounds identified to date were developed and predicted to target the active site cysteine residue. This lack of active-site-directed lead structures motivated the search for new leads with proven active-site-directed activity.

The scrutinized compounds contain electrophilic building blocks (aziridine, epoxide, Michael system) that are known to react with nucleophilic amino acids within the active site of proteases. Derivatives of etacrynic acid (5a) (Scheme 1), which is a well-known diuretic drug and which also contains an activated double bond, were chosen as potential nonpeptidic leads owing to their activity toward the cysteine protease papain. The properties of these compounds as new nonpeptidic SARS-CoV $\text{M}^{\text{pro}}$ inhibitors are presented herein.
Results and Discussion

Syntheses. Etacrynic acid (5a) and its analogues and ester derivatives were prepared according to a previously described pathway (Scheme 1, Table 1). Anisoles (1) (X, Y = H, Cl, CH₃) were acylated with acid chlorides (R¹) by Friedel–Crafts acylation. Dependent on the acylation conditions, the acylated phenols (2, 9) or anisoles (7) were obtained. The phenols (2, 9) were alkylated to yield phenoxy acetic acid esters (3, 10). These compounds and the anisoles (7) were subjected to the Mannich reaction with TMDM to yield compound series 4, 8, and 11, containing the desired Michael system. Aldol condensation of phenoxy acetic acid esters (3) with HCHO yielded the acids (5). Aminolysis of the active ester of etacrynic acid (5a) gave the amides (6).

Enzyme Inhibition and Docking. First, a comprehensive screening was performed with an HPLC-based assay using VSYGSTLQjAGLRKMA for TGEV Mpro and VSVNSTLQjSGLRKMA for SARS-CoV Mpro as substrates (Table 2). The most promising inhibitor, etacrynic acid tert-butylamide (6a), was identified by the screening (Figure 1, >80% inhibition of both enzymes at 100 μM).

This derivative is one of a series of etacrynic acid amides that were developed as new nonpeptidic cysteine protease inhibitors containing a Michael system as the electrophilic fragment. Only the amide 6a showed inhibition of the coronaviral Mpro compounds, whereas the acid 5a is only weakly active in the HPLC assay (approximately 20% inhibition at 100 μM, Table 2). To better understand the relevant interactions between this inhibitor and the SARS-CoV Mpro, docking experiments using FlexX were carried out. The binding site was extracted from the recently published structure of an enzyme inhibitor complex of SARS Mpro (PDB code: 1UK4).

In the best docking pose (Figure 2) compound 6a is spanning across the binding pockets with the tert-butyl group located in the hydrophobic S4 pocket, the dichlorobenzyl moiety located close to S3, and the terminal ethyl group at the Michael system pointing away from the active site (Figure 2). Hydrogen bonds can be formed between the ligand and amino acids His163, Glu166, and Gln189 (Figure 3). The docking suggests that the reactive center of the inhibitor, namely, the activated double bond, is located

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**Scheme 1.** Synthesis of Etacrynic Acid (5a, X = Y = Cl, R¹ = Et) and Derivatives

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**Figure 1.** HPLC profiles of proteolytic reactions for determination of enzymatic activity of SARS-CoV Mpro and of inhibition by 6a ([I] = 100 μM): E, enzyme (1.4 μg in a final volume of 30 μL); S, substrate VSVNSTLQjSGLRKMA, [S] = 0.50 mM; P¹ and P², hydrolysis products. The enzyme was incubated for 30 min with the inhibitor followed by an incubation period of 60 min with the substrate. After the incubation, a 2% TFA solution was added and the mixture was separated on an HPLC system (Zorbax SB-Aq RP-C18 column with a linear gradient from 5% to 90% acetonitrile in water containing 0.1% TFA). Peaks were detected at 215 nm.
in proximity to the sulfur of Cys145 (3.40 Å), pinpointing that these compounds target the active-site.

Figure 3. Proposed binding mode of 6a. Hydrogen bonds are formed between the ligand and amino acids His163, Glu166, and Gln189 of the enzyme. The docking suggests that the reactive center of the inhibitor, namely, the activated double bond, is located in proximity to the sulfur of Cys145, pinpointing that these compound targets the active site.

Figure 4. Proposed binding mode of 6b. This compound is predicted to form hydrogen bonds with Gln189, Glu166, Thr190, and Gln192 via its terminal amino group. The carbonyl group of the Michael system interacts with Gly143. The reactive double bond is still close to the sulfur of Cys145.

Table 1. Etacrynic Acid Derivatives Tested for SARS-CoV Mpro Inhibition

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Table 2. Inhibition of TGEV Mpro and SARS-CoV Mpro by Etacrynic Acid Derivatives As Obtained in the HPLC-Based Assay

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<tr>
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* (+) 10–50% inhibition; (++) 50–80% inhibition; (+++) > 80% inhibition.

active site and because it was thought to be synthetically accessible. To double-check whether this manually derived modification actually results in an improved interaction, compound 6b was automatically docked into the enzyme. It was predicted to form the same hydrogen bonds with Gln189 and Glu166 as compound 6a. In addition to that, compound 6b also has the potential to form two additional hydrogen bonds to Thr190 and Gln192 via its terminal amino group (see Figure 4).

Because of the additional hydrogen bonds, the molecule slightly turns around within the active site so that the carbonyl group of the Michael system is no longer
in the proximity of His163 but instead interacts with Gly143. However, the reactive double bond is still close to the sulfur of Cys145 (3.48 Å). Docking of a few more not yet synthesized compounds with amines and alkyl chains and aliphatic rings of various length and complexity did not result in as good docking scores and as plausible binding modes as those of compound 6b. Hence, it was decided to synthesize the latter compound to verify the predictions derived from docking.

Compound 6b was synthesized via aminolysis of the active ester of etacrynic acid with R-methylalanine amide. HPLC assays on SARS-CoV Mpro with the above-mentioned peptide substrate showed the inhibitors (6a and 6b) to be equipotent between 20 and 100 μM (Table 2). Since the HPLC assay is time-consuming and intricate and only allows for crude differentiation between the inhibitors, we performed fluorimetric assays at 100 μM inhibitor concentrations using a fluorescence resonance energy transfer (FRET) pair labeled substrate. The substrate o-aminobenzoic acid Ser-Val-Thr-Leu-Gln-J-Ser-Gly-(o-NO2)Tyr-Arg(Mts)-OH, which was chosen according to previously described assays,24,45 was synthesized by automated solid-phase peptide synthesis. In contrast to the substrate described in ref 24, we omitted deprotection of the arginine side chain and used o-nitro-Tyr (3-nitro-Tyr)45 instead of m-nitro-Tyr as the fluorescence acceptor.46 These modifications do not decrease the affinity to the enzymes but in contrast enhance it (TGEV Mpro $K_m = 85.5 \mu M$, SARS-CoV Mpro $K_m = 190 \mu M$ (Figure 5)); the reference value for o-aminobenzoic acid Ser-Val-Thr-Leu-Gln-J-Ser-Gly-(m-NO2)Tyr-Arg-OH on SARS-CoV Mpro is $K_m = 820 \pm 130 \mu M$).24

To check the validity of the docking results, we tested several other etacrylic acid derivatives in addition to amide 6b in the fluorimetric assay (Figures 6 and 7, Table 3). Some of these derivatives have already also been tested on the CAC1 model cysteine protease papain (Table 3).34 Among these derivatives, the most potent SARS-CoV Mpro inhibitor is indeed amide 6b (Figures 6 and 7), which corroborates the predicted binding of the α-methylalanine amide residue into the S4 pocket. In addition, the results clearly show that at least one chloro substituent at the phenyl moiety is necessary for SARS-CoV Mpro inhibition (Figure 6). Compounds with an unsubstituted phenyl ring or a methyl substituent (4b–d) are inactive at 100 μM. This also applies to the dimethyl substituted phenoxy acetic acid 5b. With respect to this latter finding, the inhibition of the coronaviral cysteine protease follows a different trend than the inhibition of papain for which a quite good $K_i$
value of 33 μM was obtained for 5b (Table 3). In contrast to this, the other derivatives listed in Table 3 exhibit the same order of inhibition potency for SARS-CoV Mpro and for papain.

Concerning the possible diuretic activity of etacrynic acid derivatives, it is quite promising that only the amides and esters display SARS-CoV Mpro inhibition. Diuretic activity of etacrynic acid is connected to the free acid group because of active transport of the drug to the site of diuretic action (the luminal membrane of the cells of the thick ascending loop) via the organic acid transport mechanism. Thus, hydrolytically stable amides or esters should be void of diuretic side effects.

**Summary**

A comprehensive HPLC-based screening with various protease inhibitors containing electrophilic moieties revealed nonpeptidic etacrynic acid tert-butyl amide (6a) as the most promising lead for new inhibitors of coronaviral main proteases. Docking experiments with 6a and other etacrynic acid amides led to the development of 6b, which exhibits slightly improved affinity to SARS-CoV Mpro. To the best of our knowledge, this compound is the first nonpeptidic inhibitor targeting the active site cysteine residue of the SARS-CoV Mpro. Recently and independently of our work a paper concerning the development of inhibitors derived from AG-7088 was published. According to the predicted binding mode, the phenyl moiety of the etacrynic acid derivatives serves as a spacer that links the active site and the S4 pocket. The fact that there are no interactions with other subsites known to contribute to the Mpro substrate binding (S1′, S1, and S2) might explain the not yet optimal inhibitory potency (6b: $K_i = 35.3 \mu M$) and the missing selectivity of this inhibitor class. Interactions with these binding sites could be achieved with additional substituents attached to the phenyl ring that might lead to superior activity and improved selectivity compared to CAC1 proteases. Our future efforts will therefore concentrate on variations of the substituents at the phenyl ring of 6b.

**Experimental Section**

**General Information.** Melting points were determined in open capillary on a melting point apparatus, model 530, from Büchi, Switzerland. CHN analyses were determined with a CHNS-932, Leco. HR-ESI mass spectra were recorded on a Finnigan MAT 90, Thermo Electron GmbH, Germany, at 70 eV ionization energy. HR-ESI-MS spectra were recorded on a FT-ICR APEX II from Bruker, Germany. ESI mass spectra were recorded on an Agilent 1100 ion trap equipped with an HPLC system from Agilent. NMR spectra were recorded on an Avance 400 MHz spectrometer from Bruker Biospin GmbH, Germany (solvent, CDCl₃. 1H NMR, 400.13 MHz; 13C NMR, 100.61 MHz). IR spectra were recorded on a PharmalyzIR FT-IR spectrometer from BioRad. The $\alpha$ values were determined on a 241 polarimeter from Perkin-Elmer. The refractive indices were determined on an apparatus from ATG GmbH, Germany. Hydrostatic column chromatography was performed with silica gel from Merck (silica gel 60, 0.063–0.2 mm or 70–230 mesh).

All solvents were purified and dried prior to use according to standard literature procedures.

**Protein Expression and Purification.** The TGEV Mpro was expressed in *Escherichia coli* and purified to near homogeneity using amylose agarose (New England Biolabs), phenyl Sepharose HP (Pharmacia), and Superdex 75 (Pharmacia) columns using previously described protocols. The purified enzyme was stored at −70 °C in buffer containing 12 mM Tris-HCl, pH 7.4, 120 mM NaCl, 0.1 mM EDTA, and 1 mM DTT. To express the SARS-CoV Mpro, the coding sequence of amino acid residues from 3236 to 3544 of SARS-CoV (strain Frankfurt 1) polyprotein 1a was amplified by RT-PCR from poly(A) RNA isolated from SARS-CoV-infected Vero E6 cells using oligo-
nucleotides JZ468 (5′-TCTGCTTCTGCAAGTGTTTGGAGAATGTGATACCC-3′) and JZ429N (5′-AAGAATCTTCAATGTGTTGTGTGGTAACACAGGATGTCTGCAACATC-3′). The PCR product was 5′-phosphorylated using T4 polynucleotide kinase, digested with EcoRI and ligated with the resultant plasmid, pMal-SARS-CoV-Mpro-His, to the T4 polynucleotide kinase, digested with EcoRI and ligated with CAACATC-3′ sequence reconstituted the natural N-terminal Mpro sequence SAVLQ, that precede the SARS-CoV Mpro sequence in the viral replicase polyprotein. This N-terminal extension of the Mpro sequence recomplemented the natural N-terminal Mpro autoprocessing site (SAVLQ/SGFRK) and thus resulted in a very efficient intracellular autocatalytic release of the Mpro N-terminus (H2N-SAVLQ/SGFRK-...) from the MBP fusion protein. This generated the authentic Mpro N-terminus, which is known to be required for the full enzymatic activities of coronavirus main proteases.11,12 At its C-terminus, the Mpro domain was extended by six consecutive His residues, allowing for affinity chromatography purification of the protein on Ni-NTA agarose columns. The pMal-SARS-CoV-Mpro-His plasmid DNA was used to transform competent E. coli TB1 cells. Following induction of expression with 1 mM isopropyl β-D-thiogalactopyranoside, the cells were grown at 25°C for 3 h. Cell lysates were prepared as described previously,36 and the His-tagged SARS-CoV Mpro was purified on Ni-NTA agarose (Qiagen) and Superdex 75 (Pharmacia) columns. The purified protein was concentrated using Centricon YM-3 filter units (Millipore) and then the respective substrate for 30 min (TGEV Mpro) or 60 min (SARS-CoV Mpro), either 0.44 mM VSYGSTLQAGLRKMA was purchased from Bachem. The peptide substrate VSVNSTLQ was recrystallized.

**HPLC Assays.** The peptide substrate VSYGSTLQAGLRKMA was purchased from Jerini, Berlin, Germany, and VSVNSTLQAGLRKMA was purchased from Bachem. The assays were performed using a 20 mM Tris-HCl buffer at pH 7.5 containing 15% DMSO (final concentration), 200 mM NaCl, 1 mM DTT, and 1 mM EDTA. An amount of 1.4 μM of enzyme (TGEV Mpro or SARS-CoV Mpro), either 0.44 mM VSYGSTLQAGLRKMA,36 as substrate for TGEV Mpro or 0.50 mM VSVNSTLQAGLRKMA,36,37 as substrate for SARS-CoV Mpro and 20, 50, or 100 μM inhibitor were used in a final volume of 30 μL.36 Stock solutions of substrates and inhibitors were prepared in DMSO and diluted with assay buffer, and enzymes were dissolved in buffer. The enzymes were incubated 30 min with each inhibitor followed by an incubation period with the respective substrate for 30 min (TGEV Mpro) or 60 min (SARS-CoV Mpro). After the incubation, a 2% TFA solution was added and the mixture was separated on a Varian ProStar HPLC system using an Agilent Zorbax SB-Aq RP-C18 column with a linear gradient from 5% to 90% acetonitrile in water (flow: 1 mL min⁻¹) containing 0.1% TFA. The solution is detected at 215 nm. Calculations of inhibition were performed by comparing the peak integrals of the two hydrolysis products (P1 and P2, relative to the corresponding signal areas in the absence of inhibitor. All assays were done in triplicate.

**Fluorimetric Enzyme Assays.** The fluorimetric enzyme assays were performed on a Cary Eclipse fluorescence spectrophotometer from Varian, Darmstadt, Germany, using a microplate reader (excitation 325 nm, emission 425 nm). For the inhibition assays 96-well microplates from Nunc GmbH, Wiesbaden, Germany, were used. Assays were performed at 25°C in a 20 mM Tris-HCl buffer, pH 7.5, containing 0.1 mM EDTA, 1 mM DTT, 200 mM NaCl, and 12.5% DMSO (final concentration) in a total volume of 200 μL. The final substrate concentration for inhibition assays was 50 μM, and the final enzyme concentration was 4.25 μg mL⁻¹. Inhibitors were used at 100 μM final concentration for preliminary screening (results shown in Figure 6) or at concentrations between 10 and 140 μM for determination of Ki values (results shown in Figure 7 and Table 3).

For determination of Ki values, the substrate was used in concentrations between 50 and 300 μM (SARS-CoV Mpro) and between 20 and 100 μM (TGEV Mpro). Values were corrected for the inner filter effect according to ref 24 and 50.

Fluorescence increase was measured over a period of 10 min for Kd determination and 20 min for inhibition assays. Substrate and inhibitor stock solutions were prepared in DMSO and were diluted with assay buffer, and enzymes were dissolved in buffer.

**Synthesis of the FRET Pair Labeled Substrate.** The substrate was synthesized by solid-phase peptide synthesis on a Milligen 9050 PepSynthesizer using standard Fmoc protocol. Synthesis was performed in DMF/DCM (60:40) with DIC/HOBt activation starting from Fmoc-Arg(Mts)-Wang-resin. The substrate was cleaved from the resin by treatment with TFA/DCM (1:1). The product substrate as TFA salt was purified and characterized by HPLC (see Supporting Information) and LC–MS. ESI-MS calcd for C35H62N2O4Si: [M – H]: 1357.48. Found: 1357.0.

**Syntheses of Inhibitors.** The syntheses of compounds 4a-d, 5a, 5b, 6a,c–f–h,k are described in ref 34.

**General Methods for Syntheses of Etacrylic Acid Derivatives 4b,c,e,f, 8a–c, and 11a.** Method A: Friedel–Crafts Acylation to Compounds 2 and 9. Amounts of 1 equiv of anisole and 1.5 equiv of acid chloride are dissolved under N2 atmosphere in 50–100 mL of absolute CH2Cl2 and the mixture is cooled to 0–5 °C. An amount of 1.5 equiv of AlCl3 is added within 30 min, and the mixture is stirred for 2–3 h. A total of 75–100 mL of CH2Cl2 is removed by distillation and is substituted by the same amount of CH2Cl2. This procedure is repeated twice. An additional amount of 1.5 equiv of AlCl3 is added, and the mixture is heated under reflux for 2.5–3 h. The mixture is poured on ice and acidified with concentrated HCl to pH 1. Tartaric acid is added for the complexation of aluminum until the solution is cleared up. The solution is extracted with EtOEt, and the organic layer is washed with water and KOH solution (10%). The organic layer is dried with Na2SO4, filtered off, and the crude product obtained after removal of the solvent is recrystallized.

**Method B: Syntheses of Phenoxyacetic Acid Esters (3).** An amount of 1 equiv of phenol is dissolved in THF under N2 atmosphere. Then 1 equiv of K-tart-butylate and a catalytic amount (0.1 equiv) of KI are added. The mixture is heated at 60 °C, and an amount of 1.1–2.0 equiv of ethyl bromoacetate is added slowly. The mixture is stirred for 30–60 min, poured into water, acidified to pH 1 with concentrated HCl, and extracted with EtOEt. The organic layer is washed with 10% KOH, water, and brine and dried with Na2SO4. After filtration, the solvent is removed in vacuo and the remaining residue is recrystallized or distilled.

**Method C: Mannich Reaction with TMDM To Produce 4.** Amounts of 1 equiv of phenoxy acetic acid ester derivative, 20 equiv of TMDM, and 20 equiv of Ac2O are mixed. The mixture is heated under reflux at 85 °C. The reaction is followed by 1H NMR spectroscopy. After completion of the reaction, the mixture is dissolved in 200 mL of DCM. Saturated K2CO3 solution is added until gas evolution stops. The mixture is filtered through Celite, and the filtrate is dried with Na2SO4. After filtration, the solvent is removed in vacuo. The product is purified by column chromatography.

The syntheses of etacrylic acid amides 6b, 6d, 6e, 6i,53 and 6l were performed starting from etacrylic acid (5a) and the corresponding amines as described earlier.54

**Ethyl [3-Methyl-4-(2-methylacryloyl)phenoxyacetaoyl]acetate (4b). Synthesis of Ethyl [3-Methyl-4-propionylphenoxy]acetate (3b).** Method B is used. Starting from 13.7 g (83.4 mmol) of 1-(4-hydroxy-2-methylphenyl)propene-1-one (2b),54 9.23 g (84 mmol) K-tart-butyrate, and 20.9 g (125.1 mmol) of ethyl bromoacetate in 75 mL of THF, an amount of 10.75 g (43.0 mmol, 52%) of ethyl-(3-methyl-4-propionylphenoxy)acetate (3b) is obtained as a colorless solid; mp 33–34 °C (ethanol). Anal. (C17H13O4) C, H, N. 1H NMR (400.13 MHz,
Ethyl [2-Chloro-4-(2-methylacryloyl)phenoxy]acetate (4e). Synthesis of Ethyl [2-Chloro-4-propionylphenoxy]acetate (3e). Method B is used. Starting from 13.85 g (75 mmol) of 1-(3-chloro-4-hydroxyphenyl)propane-1-one (2e),56 8.42 g (75 mmol) of K-tert-butylate, and 18.78 g (122.5 mmol) of ethyl bromoacetate in 75 mL of THF, an amount of 13.0 g (48.02 mmol, 64%) of ethyl [2-chloro-4-propionylphenoxy]acetate (3e) is obtained as a colorless solid, mp 125-126°C (ethanol). Anal. (C₉H₇ClO₂) C, H. %; H NMR (400 MHz, CDCl₃, 300 K, TMS): δ 1.20 (3H, t, J = 7.33 Hz, H₃C-CH₂-), 1.29 (3H, t, J = 7.20 Hz, H₃C-CH₂-O), 2.93 (2H, q, J = 7.33 Hz, H₃C-CH₂-CO), 4.27 (2H, q, J = 7.20 Hz, H₃C-CH₂-O), 4.76 (2H, s, O-CH₂-CH₂-O), 6.84 (1H, d, J = 8.58, amorphous Hα), 7.83 (1H, d, dd, J = 2.02 Hz, J = 8.59, amorphous Hα), 8.02 (1H, d, J = 2.02 Hz, amorphous Hα). 1C NMR (100.61 MHz, CDCl₃, 300 K, TMS): δ 12.83 (H₃C-CH₂-CO), 14.13 (H₃C-CH₂-O), 31.55 (H₃C-CH₂-CH₃), 61.74 (H₃C-CH₂-O), 65.96 (O-CH₂-CH₂-O), 112.52 (amorphous C₆H₅), 123.49 (arom q₆C₂ or q₅C₃), 123.03 (amorphous C₆H₅), 130.73 (arom C₆H₅), 131.51 (arom q₆C₂ or q₅C₃), 156.96 (arom q₇C₂), 167.70 (O-CH₂-CH₂-O), 198.32 (Ar-CO).

Synthesis of Ethyl [2-Chloro-4-(2-methylenobutyryl)phenoxy]acetate (4f). Synthesis of Ethyl (4-Butyryl-3-methylphenyl)acetate (3f). Method B is used. Starting from 1.35 g (5 mmol) of ethyl (2-chloro-4-propionylphenoxy)acetate (3e), 766 mg (7.5 mmol) of TMDM, and 5.4 g (52.5 mmol) of acetic anhydride, an amount of 225 mg (0.8 mmol, 16%) of 4f was obtained as a colorless solid, mp 55-56°C (cyclohexane). Anal. (C₁₆H₁₄O₂C₂H₃) C, H. %; H NMR (400.19 MHz, CDCl₃, 300 K, TMS) δ 1.30 (3H, t, J = 7.34 Hz, H₃C-CH₂-), 1.36 (3H, t, J = 7.34 Hz, H₃C-CH₂-), 1.00 (3H, t, J = 7.34 Hz, H₃C-CH₂-), 4.29 (2H, q, J = 7.16 Hz, -CH₂-CH₂-), 4.77 (2H, s, O-CH₂-CH₂-O), 5.17 (1H, s, =C-H), 5.87 (1H, s, =C-H), 6.84 (1H, d, J = 8.35 Hz, Ar-H), 7.66 (1H, dd, J = 2.15 Hz, J = 8.83 Hz, Ar-H), 7.85 (1H, d, J = 2.15 Hz, Ar-H). 1C NMR (100.61 MHz, CDCl₃, 300 K, TMS): δ 14.12 (C₂H₆-C₂H₄), 18.71 (CH₃), 60.85 (CH₃-C₂H₄), 123.13 (amorphous C₆H₅), 123.13 (amorphous C₆H₅), 127.39 (CH₃-C₂H₄), 128.59 (CH₃-C₂H₄), 131.74 (amorphous C₆H₅), 145.46 (H₃C-C₂H₄), 155.26 (arom q₇C₂), 167.73 (O-CH₂-CH₂-O), 195.76 (CO-Ar).

Ethyl [2-Chloro-4-(2-methylenobutyryl)phenoxy]acetate (4g). Synthesis of Ethyl (4-Butyryl-2-chlorophenoxy)acetate (3f). Method B is used. Starting from 9.93 g (50 mmol) of 1-(3-chloro-4-hydroxyphenyl)butane-1-one (2f),57 5.61 g (50 mmol) of K-tert-butylate, 9.18 g (55 mmol) of ethyl bromoacetate in 75 mL of THF, an amount of 10.37 g (36.42 mmol, 73%) of the target compound is obtained as colorless solid, mp 97-98°C (ethanol). Anal. (C₁₆H₁₄ClO₂) C, H, N %; H NMR (400.42 MHz, CDCl₃, 300 K, TMS) δ 1.00 (3H, t, J = 7.34 Hz, H₃C-CH₂-), 1.75 (2H, sex, J = 7.33 Hz, 7.46 Hz, H₃C-CH₂-C₂H₄), 2.88 (2H, q, J = 7.33 Hz, H₃C-CH₂-C₂H₄), 4.77 (2H, s, O-CH₂-CH₂-O), 6.84 (1H, d, J = 8.59 Hz, amorphous Hα), 7.83 (1H, dd, d, J = 2.27 Hz, J = 8.59, amorphous Hα), 8.02 (1H, d, d, J = 2.27 Hz, amorphous Hα). 1C NMR (100.61 MHz, CDCl₃, 300 K, TMS): δ 13.86 (H₃C-CH₂-C₄H₄), 14.14 (H₃C-CH₂-C₂H₄), 17.79 (H₃C-CH₂-C₂H₄), 40.25 (H₃C-CH₂-C₂H₄), 61.74 (H₃C-CH₂-C₂H₄), 65.97 (O-CH₂-CH₂-O), 112.52 (amorphous C₆H₅), 123.49 (arom q₆C₂ or q₅C₃), 128.11 (amorphous C₆H₅), 130.80 (amorphous C₆H₅), 131.71 (amorphous q₆C₂ or q₅C₃), 156.96 (arom q₇C₂), 167.71 (O-CH₂-CH₂-O), 197.88 (Ar-CO).

Synthesis of Ethyl [2-Chloro-4-(2-methylenobutyryl)phenoxy]acetate (4f). Method C is used. Starting from 2.85 g (10 mmol) of ethyl-(4-butyryl-2-chlorophenoxy)acetate (3f), 9.53 g (93.23 mmol) of TMDM, and 10.8 g (105.8 mmol) of acetic anhydride, an amount of 990 mg (3.34 mmol, 33%) of 4f is obtained as a colorless liquid. nD²⁰ = 1.5248. Ethyl 4-Chloro-2-hydroxy-3-methylphenylacetate (4f). Method B is used. Starting from 1.35 g (5 mmol) of ethyl-(4-butyryl-2-chlorophenoxy)acetate (3f), 766 mg (7.5 mmol) of TMDM, and 5.4 g (52.5 mmol) of acetic anhydride, an amount of 225 mg (0.8 mmol, 16%) of 4f was obtained as a colorless solid, mp 55-56°C (cyclohexane). Anal. (C₁₆H₁₄ClO₂) C, H, N %; H NMR (400.19 MHz, CDCl₃, 300 K, TMS) δ 14.12 (C₂H₆-C₂H₄), 18.71 (CH₃), 60.85 (CH₃-C₂H₄), 123.13 (amorphous C₆H₅), 123.13 (amorphous C₆H₅), 127.39 (CH₃-C₂H₄), 128.59 (CH₃-C₂H₄), 131.74 (amorphous C₆H₅), 145.46 (H₃C-C₂H₄), 155.26 (arom q₇C₂), 167.73 (O-CH₂-CH₂-O), 195.76 (CO-Ar).
25.42 (H2C=CH2−C=C), 61.69 (O−CH2−CH2), 65.94 (O−CH2−CO), 112.33 (arom C6H1), 123.13 (C=CH2 + aron C6H1, C=Cl), 129.64 (arom C6H1, CH3), 136.02 (arom C6H1, C=Cl), 149.38 (C=CH2), 165.64 (aron C6H1), 167.71 (O−CH2−C=O), 195.98 (Ar C=O).

2-[2-(2,3-Dichloro-4-(2-methylethlyureyl)phenoxy)-acetylamino]-3-methylpropionin Acid Carboxamide (6b). Colorless solid, mp 157−158 °C (ethanol acetate). Anal. (C81H, C19Cl2N3O6)·H2O: C 58.81, H 4.20, N 12.00. Found: C 58.85, H 4.25, N 12.30. 1H NMR (400.13 MHz, CDCl3, 300 K, TMS): δ 7.33 Hz, H2C=CH2−C=O, 4.28 (2H, q, J = 7.33 Hz, H2C=CH2−C=O, 4.70 (2H, s, O−CH2−CO), 5.60 (1H, s, C=CH2), 5.97 (1H, s, C=CH2), 6.93 (1H, d, J = 8.34 Hz, aron H2), 7.18 (1H, t, J = 7.58 Hz, aron H2, aniline), 7.22 (1H, d, J = 8.34 Hz, aron H2), 0.16 (s, CH3), 0.80 (t, J = 7.58 Hz, aron H2, aniline), 0.82 (d, J = 8.34 Hz, aron H2), 0.16 (s, CH3).

N-Benzyl-2-[2,3-dichloro-4-(2-methylethlyureyl)phenoxy]acetamidem (6i). Colorless solid, mp 90−92 °C (cyclohexane). Anal. (C81H, C19Cl2N3O6)·H2O: C 58.81, H 4.20, N 12.00. Found: C 58.85, H 4.25, N 12.30. 1H NMR (400.13 MHz, CDCl3, 300 K, TMS): δ 7.33 Hz, H2C=CH2−C=O, 4.28 (2H, q, J = 7.33 Hz, H2C=CH2−C=O, 4.70 (2H, s, O−CH2−CO), 5.60 (1H, s, C=CH2), 5.97 (1H, s, C=CH2), 6.93 (1H, d, J = 8.34 Hz, aron H2), 7.18 (1H, t, J = 7.58 Hz, aron H2, aniline), 7.22 (1H, d, J = 8.34 Hz, aron H2), 0.16 (s, CH3), 0.80 (t, J = 7.58 Hz, aron H2, aniline), 0.82 (d, J = 8.34 Hz, aron H2), 0.16 (s, CH3).

Benzyl 2-(2,3-Dichloro-4-(2-methylethlyureyl)phenoxy)acetamidem (6i). Colorless solid, mp 157−158 °C (ethanol acetate). Anal. (C81H, C19Cl2N3O6)·H2O: C 58.81, H 4.20, N 12.00. Found: C 58.85, H 4.25, N 12.30. 1H NMR (400.13 MHz, CDCl3, 300 K, TMS): δ 7.33 Hz, H2C=CH2−C=O, 4.28 (2H, q, J = 7.33 Hz, H2C=CH2−C=O, 4.70 (2H, s, O−CH2−CO), 5.60 (1H, s, C=CH2), 5.97 (1H, s, C=CH2), 6.93 (1H, d, J = 8.34 Hz, aron H2), 7.18 (1H, t, J = 7.58 Hz, aron H2, aniline), 7.22 (1H, d, J = 8.34 Hz, aron H2), 0.16 (s, CH3), 0.80 (t, J = 7.58 Hz, aron H2, aniline), 0.82 (d, J = 8.34 Hz, aron H2), 0.16 (s, CH3).

N-Benzyl-2-[2,3-dichloro-4-(2-methylethlyureyl)phenoxy]acetamidem (6i). Colorless solid, mp 90−92 °C (cyclohexane). Anal. (C81H, C19Cl2N3O6)·H2O: C 58.81, H 4.20, N 12.00. Found: C 58.85, H 4.25, N 12.30. 1H NMR (400.13 MHz, CDCl3, 300 K, TMS): δ 7.33 Hz, H2C=CH2−C=O, 4.28 (2H, q, J = 7.33 Hz, H2C=CH2−C=O, 4.70 (2H, s, O−CH2−CO), 5.60 (1H, s, C=CH2), 5.97 (1H, s, C=CH2), 6.93 (1H, d, J = 8.34 Hz, aron H2), 7.18 (1H, t, J = 7.58 Hz, aron H2, aniline), 7.22 (1H, d, J = 8.34 Hz, aron H2), 0.16 (s, CH3), 0.80 (t, J = 7.58 Hz, aron H2, aniline), 0.82 (d, J = 8.34 Hz, aron H2), 0.16 (s, CH3).

Benzyl 2-(2,3-Dichloro-4-(2-methylethlyureyl)phenoxy)acetamidem (6i). Colorless solid, mp 157−158 °C (ethanol acetate). Anal. (C81H, C19Cl2N3O6)·H2O: C 58.81, H 4.20, N 12.00. Found: C 58.85, H 4.25, N 12.30. 1H NMR (400.13 MHz, CDCl3, 300 K, TMS): δ 7.33 Hz, H2C=CH2−C=O, 4.28 (2H, q, J = 7.33 Hz, H2C=CH2−C=O, 4.70 (2H, s, O−CH2−CO), 5.60 (1H, s, C=CH2), 5.97 (1H, s, C=CH2), 6.93 (1H, d, J = 8.34 Hz, aron H2), 7.18 (1H, t, J = 7.58 Hz, aron H2, aniline), 7.22 (1H, d, J = 8.34 Hz, aron H2), 0.16 (s, CH3), 0.80 (t, J = 7.58 Hz, aron H2, aniline), 0.82 (d, J = 8.34 Hz, aron H2), 0.16 (s, CH3).

Benzyl 2-(2,3-Dichloro-4-(2-methylethlyureyl)phenoxy)acetamidem (6i). Colorless solid, mp 157−158 °C (ethanol acetate). Anal. (C81H, C19Cl2N3O6)·H2O: C 58.81, H 4.20, N 12.00. Found: C 58.85, H 4.25, N 12.30. 1H NMR (400.13 MHz, CDCl3, 300 K, TMS): δ 7.33 Hz, H2C=CH2−C=O, 4.28 (2H, q, J = 7.33 Hz, H2C=CH2−C=O, 4.70 (2H, s, O−CH2−CO), 5.60 (1H, s, C=CH2), 5.97 (1H, s, C=CH2), 6.93 (1H, d, J = 8.34 Hz, aron H2), 7.18 (1H, t, J = 7.58 Hz, aron H2, aniline), 7.22 (1H, d, J = 8.34 Hz, aron H2), 0.16 (s, CH3), 0.80 (t, J = 7.58 Hz, aron H2, aniline), 0.82 (d, J = 8.34 Hz, aron H2), 0.16 (s, CH3).
is obtained as yellow crystals after purification by column chromatography, mp 84–85 °C (ethanol). Anal. (C<sub>12</sub>H<sub>10</sub>CIN<sub>2</sub>O<sub>4</sub>) C, H, N. H<sup>1</sup>NMR (400.13 MHz, CDCl<sub>3</sub>, 300 K, TMS): δ 2.11 (2H, quin, J = 7.71 Hz, J = 7.08 Hz, -CH<sub>2</sub>-CH<sub>2</sub>-CO-), 2.82 (2H, t, J = 7.71 Hz, CH<sub>2</sub>-CH<sub>2</sub>-CH=CH<sub>2</sub>-CO), 2.94 (2H, t, J = 7.08 Hz, -CH<sub>2</sub>-CO), 3.97 (3H, s, O-CH<sub>3</sub>), 6.96 (1H, d, J = 8.79 Hz, arom H<sub>1</sub>), 7.37 (2H, d, J = 8.72 Hz, arom H<sub>2</sub> + H<sub>3</sub>), 7.80 (1H, dd, J = 8.99 Hz, arom H<sub>0</sub>), 7.96 (1H, d, J = 8.72 Hz, arom H<sub>2</sub> + H<sub>3</sub>).<sup>1</sup>C NMR (100.61 MHz, CDCl<sub>3</sub>, 300 K): δ 25.13 (CH<sub>3</sub>-CH<sub>2</sub>-CO), 35.00 (CH<sub>2</sub>-CH<sub>2</sub>-CH=CH<sub>2</sub>-CO), 36.98 (-CH<sub>2</sub>-CO), 56.37 (O-CH<sub>3</sub>), 111.31 (arom C<sub>5</sub>, CH), 122.92 (arom C<sub>6</sub>, C<sub>Cl</sub>, C-Cl), 123.71 (arom C<sub>4</sub> + C<sub>6</sub>, CH), 128.35 (arom C<sub>7</sub>, CH), 130.31 (arom C<sub>8</sub>, CH), 130.39 (arom C<sub>7</sub>), 146.50 (arom C<sub>9</sub>), 149.54 (arom C<sub>10</sub>), 158.80 (arom C<sub>11</sub>), 196.94 (O=CO).

**Synthesis of 1-(3-Chloro-4-methoxyphenyl)-2-methylene-4-(4-nitrophenyl)butanone-1-oxide (8c).** Method C is used. Starting from 1.67 g (5 mmol) of 1-(3-chloro-4-methoxyphenyl)-ene-4-(4-nitrophenyl)butane-1-one (7c), 4.46 g (40.9 mmol) of TMDM, and 5.4 g (52.9 mmol) of acetic anhydride, the crude product was purified by column chromatography on silica gel 60 (CHCl<sub>3</sub>). Yield 789 mg (2.28 mmol, 46%), yellow crystals, mp 86 °C (ethanol). Anal. (C<sub>23</sub>H<sub>17</sub>Cl<sub>2</sub>N<sub>2</sub>O<sub>4</sub>) C, H, N. H<sup>1</sup>NMR (400.13 MHz, CDCl<sub>3</sub>, 300 K, TMS): δ 2.82 (2H, t, J = 7.64 Hz, Ar-CH<sub>2</sub>-CH=CH=CH<sub>2</sub>-CO), 2.95 (2H, t, J = 7.64 Hz, Ar-CH<sub>2</sub>-CH=CH-CH<sub>2</sub>-CO), 3.98 (3H, s, O-CH<sub>3</sub>), 7.34 (2H, d, J = 8.72 Hz, arom H<sub>2</sub>, CH), 128.35 (arom C<sub>5</sub>, CH), 129.24 (arom C<sub>6</sub> + C<sub>8</sub>, CH), 130.31 (arom C<sub>7</sub>, CH), 130.39 (arom C<sub>7</sub>), 146.50 (arom C<sub>9</sub>), 149.54 (arom C<sub>10</sub>), 158.80 (arom C<sub>11</sub>), 196.94 (O=CO).

**Enzyme Preparation for Docking Procedure.** The starting conformation for the docking procedure was the crystal structure of in complex with Cbz-Val-Asn-Ser-Thr-Leu-Gln-CMK (PDB code: 1UKA).<sup>15</sup> Owing to the covalent binding mode of the coocrystallized ligand Cbz-Val-Asn-Ser-Thr-Leu-Gln-CMK, the active center of SARS-CoV M<sup>pro</sup> as found in the crystal structure is very tight. Hence, prior to docking, the covalent bond between ligand and receptor was eliminated, the ligands’ chloromethyl ketone substructure was rebuilt, and water molecules were deleted. Then the enzyme was relaxed using SYBYL 6.9 (hot region around the ligand, 6 Å; interesting region, 12 Å; 5000 steps; Tripos force field).<sup>62</sup>

Since the water molecule in subpocket S2 (HOH16) is deeply buried, the relaxation was also done with this water molecule kept in place. However, no obvious differences in the resulting geometries were observed in both structures. Put differently, for both enzyme conformations, this part of S2 is without reach for a potential ligand; therefore, the conformation obtained with all water molecules deleted was used for docking.

**Active Site Definition.** Using the SETREF directive within Flexx, the active site was defined as the complete atomic coordinates found within 8 Å of the heavy atoms of the coocrystallized ligand. For interpretation purposes, only those docking poses located within this active site were considered.

**Ligand Preparation.** All compounds were sketched in 2D and then converted to 3D structures using SYBYL 6.9. Since Flexx is working in torsional space only (i.e., bond lengths and angles are kept constant), a geometry optimization of the ligands (500 steps, Gasteiger–Huckel charges, dielectric constant of 4.0, Tripos force field) was performed.

**Docking Procedure.** Flexx was used with standard settings. Base fragment selection was done automatically, and for base fragment placement, the triangle matching strategy was applied. For each ligand, a maximum of 100 placements were stored; however, only those ligands where the position was within the active site were considered.

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**Appendix**

**Abbreviations.** Amino acids are (S)-configured unless otherwise indicated; either the one- or the three-letter code is used. AG-7088, (E)-(S)-4-((2R,5S)-2-(4-fluorobenzyl)-6-methyl-5-[(5-methylisoxazole-3-carbonyl)amino]-4-oxoheptanoylamino)-5-((S)-2-oxypyrrolidin-3-ylpent-2-enic acid ethyl ester; DCC, dicyclohexylcarbodiimide; CMK, chloromethyl ketone; DCM, dichloromethane; DIC, 1,3-diisopropylcarbodiimide; DMP, dimethylformamide; DMSO, dimethyl sulfoxide; DPFA, diphenylphosphorazidate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EEDQ, 1-ethylcarbonyl-2-ethoxy-1,2-dihydroquinoline; FRET, fluorescence resonance energy transfer; HOBt, 1-hydroxybenzotriazole; HOSuc, N-hydroxysuccinimide; M<sup>pro</sup>, main protease; Mts, 2,4,6-trimethylbenzenesulfonil (mesitylenesulfonyl); SARS-CoV, severe acute respiratory syn-
drome coronavirus; TEA, triethylamine; TFA, trifluoroacetic acid; TGEV, transmissible gastroenteritis virus; THF, tetrahydrofuran; TDM, N,N,N',N'-tetramethyldiaminomethane.

Supporting Information Available: IR data and data confirming the purity of the target compounds (HPLC, HRMS, combustion analyses). This material is available free of charge via the Internet at http://pubs.acs.org.

References


(15) AG-7088 (rupintrivir) contains a Michael system (vinyl-Gln mimetic) as electrophile. It is in clinical development as an inhibitor of 3C protease of human rhinovirus. It is reported to exhibit low affinity to the SARS-CoV protease.17


(24) The first peptide substrate VSYGSSLQ<sub>4</sub>GLRLKMA represents an H<sub>2</sub>O<sub>2</sub>-229E M<sub>229E</sub> cleavage site.19 The second peptide VSNOSSLQR<sub>3</sub>GERLKMA was also a substrate of SARS-CoV M<sub>229E</sub> and for this reason, is new being sold by Bachem as an SARS-CoV M<sub>229E</sub> substrate (although it does not represent a cognate SARS-CoV M<sub>229E</sub> cleavage site). The lower enzyme activity against this substrate is reflected in the higher incubation time used in the HPLC-based assays.


(26) Supporting Information Available: IR data and data confirming the purity of the target compounds (HPLC, HRMS, combustion analyses). This material is available free of charge via the Internet at http://pubs.acs.org.
(40) In some of the docking poses the studied compounds were not located within the binding site but were docked to the surface of the protein. Consequently, the “best” docking pose in this study was defined as the pose with the most favorable docking score for compounds that were located inside the binding pocket. For all virtual compounds (i.e., those that were not readily synthesized when the docking study was carried out) a further constraint was added. Since the activated double bond of compound 6a was predicted to be in proximity to the active site Cys145 and since it was the working hypothesis that this double bond reacts with Cys145, only those docking poses were scrutinized where the double bond was close to Cys145.


(42) Adding the van der Waals radii of the involved heavy atoms sulfur and carbon results in 3.4 Å. Hence, when only noncovalent interactions are modeled, the two atoms cannot move closer because clashes are penalized by the scoring function of the docking tool.

(43) Glu166 C=O ↔ H–N: 1.66 Å, angle NHO 159.5. Gln189 N–H ↔ O=C: 2.03 Å, angle NHO 170.2.

(44) Thr190 C=O ↔ H–N: 1.70 Å, angle NHO 147.6. Gln192 N–H ↔ O=C: 2.03 Å, angle NHO 147.6.


(46) The descriptors ortho and meta are used with respect to the phenolic hydroxyl group.


(51) Since no time-dependent inhibition was observed with the tested compounds, only K_i values could be determined. Dialysis assays with the CAC1 cysteine protease cathepsin B showed the inhibitors to act reversibly (unpublished results).


(60) Feniai, G.; Recknow, W. A. Procede de photographie en couleurs et nouveaux produits pour sa mise en oeuvre (Procedure for color photography and new products for its implementation). Patent BE 560859, 1957 (Eastman Kodak Co.).


(62) SYBYL, version 6.9; Tripos Inc. (1699 South Hanley Road, St. Louis, MO 63144), 2001.