Identification of a Dual Inhibitor of Secreted Phospholipase A<sub>2</sub> (GIIA sPLA<sub>2</sub>) and SARS-CoV-2 Main Protease

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Abstract: The development of novel agents to combat COVID-19 is of high importance. SARS-CoV-2 main protease (M<sub>pro</sub>) is a highly attractive target for the development of novel antivirals and a variety of inhibitors have already been developed. Accumulating evidence on the pathobiology of COVID-19 has shown that lipids and lipid metabolizing enzymes are critically involved in the severity of the infection. The purpose of the present study was to identify an inhibitor able to simultaneously inhibit both SARS-CoV-2 M<sub>pro</sub> and phospholipase A<sub>2</sub> (PLA<sub>2</sub>), an enzyme which plays a significant role in inflammatory diseases. Evaluating several PLA<sub>2</sub> inhibitors, we demonstrate that the previously known potent inhibitor of Group IIA secretory PLA<sub>2</sub>, GK241, may also weakly inhibit SARS-CoV-2 M<sub>pro</sub>. Molecular mechanics docking and molecular dynamics calculations shed light on the interactions between GK241 and SARS-CoV-2 M<sub>pro</sub>. 2-Oxoamide GK241 may represent a lead molecular structure for the development of dual PLA<sub>2</sub> and SARS-CoV-2 M<sub>pro</sub> inhibitors.

Keywords: COVID-19; inhibitors; main protease; 2-oxoamides; phospholipase A<sub>2</sub>; SARS-CoV-2

1. Introduction

With more than 450 million cases of infected people and 6 million casualties globally, the discovery of efficient agents to treat COVID-19, which is caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), is an unmet need [1]. Pioneering studies showed that enzymes, such as RNA-dependent RNA polymerase (RdRp) and SARS-CoV-2 main protease (M<sub>pro</sub>), are attractive targets for the development of novel antiviral agents [2,3].

Studies aiming to understand the pathobiology of COVID-19 have also demonstrated the involvement of lipids and lipid metabolizing enzymes in this potentially lethal infection [4,5]. Phospholipases A<sub>2</sub> (PLA<sub>2</sub>s) are enzymes which catalyze the hydrolysis of membrane glycerophospholipids, releasing free fatty acids (FFAs) and lysophospholipids and initiating arachidonic acid (AA) cascade and promotion of inflammation [6–8]. Proteomics studies on SARS-CoV-2 infected cells have revealed alterations of proteins linked to the inflammatory response due to the viral infection [9]. The expression of two PLA<sub>2</sub>s, namely cytosolic PLA<sub>2</sub> (GIVA cPLA<sub>2</sub>) and secreted PLA<sub>2</sub> (GIIA sPLA<sub>2</sub>) was notably differentiated after 24 h of infection [9]. Plasma metabolomic and lipidomic studies associated
with COVID-19 showed elevated levels of FFAs and reduction in phosphatidylcholines (PCs), which indicated increased enzymatic activity of PLA2s [10]. Large-scale plasma analysis has revealed that lipids are strongly involved in the response to infection [11]. The concentrations of oleic acid (OA, C18:1) and AA (C20:4) were directly correlated to the severity of the disease in COVID-19 patients who required admission to an intensive care unit [11]. Most recently, an independent cohort study has demonstrated elevated levels of GIIA sPLA2 in the plasma of deceased patients in comparison to patients with severe or mild COVID-19, indicating that GIIA sPLA2 is associated with increased mortality due to COVID-19 [12]. This study highlights the importance of GIIA sPLA2, establishing this enzyme as a factor that leads to severe COVID-19 morbidity and mortality, and suggesting it as a therapeutic target to prevent COVID-19 mortality.

Previously described data [4,5,9–12], as well as data reported in recent review articles summarizing the role of PLA2s in inflammatory diseases [13,14], prompted us to explore if we could identify a small-molecule inhibitor able to simultaneously inhibit PLA2 and SARS-CoV-2 Mpro. It would be advantageous, if we could target two enzymes with a dual inhibitor able to simultaneously block virus replication by inhibiting SARS-CoV-2 Mpro and regulate the inflammatory response by inhibiting PLA2. In the present study, we focus on 2-oxoamide (also known as α-ketoamide) small molecules as appropriate agents to inhibit both PLA2 and SARS-CoV-2 Mpro and we describe the first dual inhibitor of GIIA sPLA2 and SARS-CoV-2 Mpro.

2. Results

2.1. Design of Inhibitors

In 2020, Hilgenfeld and coworkers demonstrated that 2-oxoamides are potent inhibitors of SARS-CoV-2 Mpro [15] and reported the X-ray structure of SARS-CoV-2 Mpro in complex with the 2-oxoamide inhibitor 1 (Figure 1) [15]. SARS-CoV-2 Mpro is a cysteine protease and its key cysteine residue may attack small-molecule inhibitors containing either a reactive carbonyl group or a Michael acceptor functionality [15–21]. In previous years, we have designed and synthesized a variety of 2-oxoamides as inhibitors of PLA2s. More specifically, we have developed 2-oxoamides (2), which are based on non-natural δ- or γ-amino acids and selectively inhibit GIVA cPLA2 [22,23], while 2-oxoamides (3) based on natural α-amino acids selectively inhibit GIIA sPLA2 [24].

To explore if known inhibitors of PLA2s may inhibit SARS-CoV-2 Mpro, we selected three 2-oxoamides previously developed by us, which are selective inhibitors of either GIVA cPLA2 (AX109 and AX074) [22,23] or GIIA sPLA2 (GK241) [24], and pentafluoroethyl ketone GK187, which selectively inhibits calcium-independent GIVA iPLA2 [25]. Their structures are shown in Table 1. Since our initial in vitro studies have shown that GIIA sPLA2 inhibitor GK241 inhibits SARS-CoV-2 Mpro, we have also synthesized several GK241 analogs for SAR studies.
**Table 1.** In Vitro inhibition of SARS-CoV-2 M<sup>PRO</sup> by known PLA<sub>2</sub> inhibitors.

| Entry | Code | Structure | Inhibition Rate (%) (40 μM) | Inhibition Rate (%) (100 μM) |
|-------|------|-----------|-----------------------------|-------------------------------|
| 1     | AX109| ![Structure AX109](image) | 20.38 ± 2.75 | 32.11 ± 5.44 |
| 2     | AX074| ![Structure AX074](image) | 47.83 ± 3.27 | 50.52 ± 2.63 |
| 3     | GK241| ![Structure GK241](image) | 76.90 ± 0.41 | 97.33 ± 1.15 |
| 4     | GK187| ![Structure GK187](image) | 26.72 ± 3.50 | 32.62 ± 4.67 |

* [SARS-CoV-2 M<sup>PRO</sup>]: 0.5 μM, buffer: 20 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.3.

2.2. Synthesis

2-Oxamides 7a–g were synthesized in two steps from long chain α-hydroxycarboxylic acids 4a,b and amines 5a–g (see Scheme 1 below). The coupling was carried out employing 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) as the coupling agent in the presence of 1-hydroxybenzotriazole (HOBt) and was followed by an oxidation reaction using Dess–Martin periodinane (Scheme 1) [26].

![Scheme 1. Synthesis of 2-oxamides from 2-hydroxycarboxylic acids 4a,b and amines 5a–g.](image)

(a) EDC·HCl, HOBt, Et<sub>3</sub>N, dry CH<sub>2</sub>Cl<sub>2</sub>, 1 h at 0 °C, 16 h at r.t.; (b) Dess–Martin periodinane, dry CH<sub>2</sub>Cl<sub>2</sub>, 1 h, r.t.
tert-Butyl esters 7a and 7f,g were deprotected by treatment with trifluoroacetic acid (TFA) to provide the corresponding carboxylic acids 8a–c (Scheme 2).

Scheme 2. Removal of tert-butyl ester group. (a) 50% TFA in CH2Cl2 (0.5 M), 3 h, r.t.

Amine 5b was synthesized from carbobenzoxy-L-valinol by protection of the hydroxyl group, using tert-butyldimethylsilyl chloride, as described in [27], and then removal of the Cbz group. Amine 5c was synthesized from tert-butyloxycarbonyl-L-valinol (9) by protection of the hydroxyl group, followed by removal of the Boc group (Scheme 3).

Scheme 3. Synthesis of amine 5c. (a) BnBr, NaH, dry DMF, 16 h, 0 °C to r.t.; (b) 4N HCl/MeOH, 2 h, r.t.

2.3. Inhibition of SARS-CoV-2 M<sup>pro</sup> by 2-Oxoamide PLA2 Inhibitors and Analogs

The inhibitory potency of the known PLA2 inhibitors and all of the new 2-oxoamides synthesized against SARS-CoV-2 M<sup>pro</sup> was assessed by determining the extent of enzyme inhibition (% inhibition); the results are summarized in Tables 1 and 2. In these experiments, 40 μM or 100 μM of 2-oxoamide, 0.5 μM of SARS-CoV-2 M<sup>pro</sup>, and 10 μM of the fluorescence resonance energy transfer (FRET) substrate Dabcyl-KTSAVLQ↓SGFRKM-E(Edans)-NH2 in 20 mM tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.3), 100 mM NaCl, 1 mM ethylene diamine tetra-acetic acid (EDTA), were used.
As shown in Table 1, 2-oxoamide GIVA cPLA₂ inhibitors AX109 and AX074 weakly inhibited SARS-CoV-2 M\textsuperscript{pro} at concentrations of 40 μM and 100 μM (entries 1 and 2, Table 1), but none of them higher than 50% even at 100 μM. Interestingly, the 2-oxoamide GIIA sPLA₂ inhibitor GK241 inhibited SARS-CoV-2 M\textsuperscript{pro} almost completely at 100 μM and by 76.9% at 40 μM (entry 3, Table 1). Pentafluoroethyl ketone GK187, which is a selective and potent GVIA iPLA₂ inhibitor, showed a very weak effect even at 100 μM. Overall, the selective GIIA sPLA₂ inhibitor GK241 was found to inhibit SARS-CoV-2 M\textsuperscript{pro}. The IC\textsubscript{50} value for GK241, determined by the inhibition curve, was found to be 24 μM.

The results for the in vitro inhibition of SARS-CoV-2 M\textsuperscript{pro} by the analogs of GK241 are summarized in Table 2. Conversion of the free carboxyl to the corresponding amide 7d or to a hydroxymethyl-protected group 7c, 7b resulted in abolishment of the inhibitory potency (entries 2–4, Table 2), indicating that a free carboxyl group was necessary for the inhibition. When the long chain of GK241 was replaced by a shorter one (reduction

### Table 2. In Vitro inhibition of SARS-CoV-2 M\textsuperscript{pro} by GK241 analogs.

| Entry | Code | Structure | Inhibition Rate (%) \textsuperscript{a} (40 μM) |
|-------|------|-----------|-----------------------------------------------|
| 1     | 7a   |           |                                               |
| 2     | 7b   |           |                                               |
| 3     | 7c   |           |                                               |
| 4     | 7d   |           |                                               |
| 5     | 7e   |           |                                               |
| 6     | 7f   |           | 3.83 ± 1.34                                   |
| 7     | 8a   |           | 65.49 ± 2.79                                  |
| 8     | 8b   |           | 6.21 ± 5.24                                   |
| 9     | 8d   |           |                                               |

\textsuperscript{a} [SARS-CoV-2 M\textsuperscript{pro}]: 0.5 μM, buffer: 20 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.3. \textsuperscript{b} Enhanced the enzymatic activity. \textsuperscript{c} No inhibition.
by four carbon atoms), the inhibitory potency of 8d on SARS-CoV-2 MPpro was abolished (entry 9, Table 2).

When the valine residue of GK241 was replaced by alanine, 8a (entry 7, Table 2) was found to inhibit SARS-CoV-2 MPpro by 65.49% at 40 µM, while the conversion of the free carboxyl group to an ester (7a, entry 1, Table 2) again led to the abolishment of the inhibitory potency. The derivatives 8b and 7f, based on an Ala-Ala dipeptide, presented almost no activity (entries 8 and 6, Table 2). Finally, compound 7e, based on a glutamine surrogate, did not present any activity (entry 5, Table 2).

2.4. Molecular Mechanics Docking and Molecular Dynamics Calculations

To obtain a better insight into the interactions between SARS-CoV-2 MPpro and the most active compound GK241, we applied molecular mechanics docking and molecular dynamics (MD) calculations. More specifically, to simulate the specific interaction, compound GK241 was subjected to covalent docking calculations as implemented in the Maestro Schroedinger suite by creating a covalent bond between Cys145 and the 2-carbonyl carbon of the 2-oxoamide moiety. The procedure transformed carbonyl to an sp3 carbon atom and the adjacent oxygen to a hydroxyl group. The crystal structure of SARS-CoV-2 MPpro protein PDB 6Y2F [15] was adequately prepared for simulations by adding the two missing flexible residues E47 and D48, as this loop was near the binding cavity and initial calculations showed that it could interact with the ligand. Moreover, initial calculations showed that the formation of the covalent bond could result in both R and S configurations for the 2-carbon atom, which is not surprising, since there are examples in the literature reporting inhibitors that may lead to both R and S configurations [28]. Thus, docking calculations were finally performed to collect 100 structures for each 2-carbon atom configuration. These structures were further ranked according to the ligand binding energy calculated using the MM-GBSA approach, ranging between −58.69 to −10.66 kcal/mol. Common structural characteristic of the generated structures for both the R and S configuration groups was the tendency to orient the long aliphatic chain to S2, S3, S4 protease clefts (Figure 2A,B), while differences were mainly observed in the conformation of the covalent bond formatted between Cys145 and the 2-oxoamide carbonyl, as expressed by the dihedral angle Cβcys145-Scys145-C2-O2. The lowest energy structures of up to 3 kcal relative binding energy were grouped according to common conformational characteristics of the ligand; representative structures are shown in Figure 2C and Figure S1 (Supplementary Materials). The resulting docking structures appeared to sample the available conformational space by orienting the valine moiety carboxylate (structures 3 and 5) or isopropyl (structures 2 and 4) to the S1 cavity, while in structures 1 and 6 the –OH group or the initial part of the aliphatic chain were oriented in this cleft. Another interesting structural characteristic was that only structures 2, 5 and 7 interacted with H41 specifically through the –OH group.

These seven selected structures were further subjected to 50 nsec MD calculations to assess the stability of the protein-ligand interaction. The calculated binding energies and mean RMSD for both protein and ligand are summarized in Table S1 (Supplementary Materials) along with major interactions between the ligand and specific protein residues. Visual inspection of the MD trajectories and conformational flexibility of both the protein and the ligand showed that, in most of the structures, the long aliphatic chain had the tendency to widely explore the conformational space, probably inducing protein conformational changes. The RMSF calculated for the protein Cα showed that the loop between residues 45–50, in particular, exhibited increased flexibility (Figure S2, Supplementary Materials) which appeared to be related to interactions with part of the long aliphatic chain. A relative flexibility was also observed for residues 185–192, specifically in the case of structure 1. Among the seven structures, the most stable during MD simulations was found to be structure 7, and, thus, MD simulations were extended to 150 nsec to validate the initial observation (Figure 2D). In structure 7, the 2-carbon atom exhibited an S configuration and both the –OH and amide moieties aligned, in general, very well with the ketoamide crystal structure reported by Hilgenfeld and coworkers [15] (Figure 2A,B). The –OH group
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showed that the loop between residues 45–50, in particular, exhibited increased flexibility (Figure S2, Supplementary Materials) which appeared to be related to interactions with protein residues. (D) RMSD for the protein (blue) and the ligand (magenta), as well as protein residue RMSF during the 150 nsec MD simulation.

Figure 2. Structural characteristics of SARS-CoV-2 Mpro.GK241 interaction as provided by docking and MD simulations. (A) GK241 occupying the main cavity of the enzyme active site. (B) Comparison of structure 7 with crystal structure PDB 6Y2F. The formation of the covalent bond produces exactly the same configuration, and the amide bond has similar orientation, while the long aliphatic chain occupies part of the S2 and S3 and S4 cavities. (C) Interactions of structure 7 with different protein residues. (D) RMSD for the protein (blue) and the ligand (magenta), as well as protein residue RMSF during the 150 nsec MD simulation.

In order to further rationalize the experimental results, the derivatives 8d and 8a were subjected to covalent docking following the same procedure as above, resulting in 100 structures for each of the R and S 2-carbon configurations. The calculated MM-GBSA interaction energies were higher in both cases than those observed for GK241, ranging between −50.91 and −8.35 kcal/mol for 8d and −56.99 and −25.98 kcal/mol for 8a. For comparison with GK241, we selected structures, similar to structure 7, specifically having an S configuration and forming an H bond between the −OH and residue H41, which are presented in Figure S3 (Supplementary Materials) along with the crystal structure and structure 7 for comparison. Concerning the derivative 8d, a major difference observed was that the 10-carbon-atom aliphatic chain was always oriented differently covering part of the S1 and S2 cavities, as shown in Figure S3A (Supplementary Materials). This major difference in the conformation of the aliphatic side-chain reflected a major difference in the MM-GBSA calculated binding energy of ~4 kcal compared to GK241 structure 7 and was in agreement with the experimental results showing no activity at 40 µM concentration. On
the other hand, derivative 8a showed major similarities with GK241 as far as the aliphatic chain was concerned, mainly occupying the same part of the enzyme active site. However, the alanine moiety of 8a occupied the S1 cavity differently to GK241 as the N-\text{C}^{\alpha}_{\text{ala}} bond adopted a different conformation compared to GK241, resulting in a different orientation of the methyl group of the alanine moiety compared to the corresponding valine isopropyl. These differences can explain the small differences observed in the experimental activity of these derivatives.

3. Discussion

\textit{PLA}_2s are a superfamily of enzymes [6–8] which are involved in almost any inflammatory disease [6–8,13,14,29]. In humans, three \textit{PLA}_2 types, represented by GIIA s\textit{PLA}_2, GIVA c\textit{PLA}_2 and GVIA i\textit{PLA}_2, are of high medicinal interest and have been targets for the development of small-molecule synthetic inhibitors [13]. Among the various classes of synthetic inhibitors, 2-oxoamides constitute a class of compounds whose members can selectively inhibit either GIVA c\textit{PLA}_2 or GIIA s\textit{PLA}_2. The results of the present study showed that the selective 2-oxamide inhibitors of GIVA c\textit{PLA}_2 AX109 and AX074 [22,23] did not exhibit any appreciable inhibition of SARS-CoV-2 M\textsuperscript{pro}. Similarly, the selective pentafluoroethyl ketone inhibitor of GVIA i\textit{PLA}_2 GK187 [25] did not show appreciable inhibition of SARS-CoV-2 M\textsuperscript{pro}. On the contrary, the potent 2-oxamide inhibitor of GIIA s\textit{PLA}_2 (IC\textsubscript{50} 143 nM) [24] was found to inhibit SARS-CoV-2 M\textsuperscript{pro} with an IC\textsubscript{50} value of 24 \textmu M. Given that GIIA s\textit{PLA}_2 has most recently been recognized as a factor contributing to the severity and mortality of COVID-19 [12,30], this finding is of high importance.

Peptide and peptide-mimetic 2-oxoamides have been identified as potent inhibitors of SARS-CoV-2 M\textsuperscript{pro} and their interaction with the catalytic site of the cysteine protease SARS-CoV-2 M\textsuperscript{pro} has been defined by determining the X-ray structure of the enzyme-inhibitor complex [15]. Inhibitors of SARS-CoV-2 M\textsuperscript{pro} have attracted high interest as candidate antiviral drugs [31,32], and, recently, the inhibitor PF-07321332 (nirmatrelvir) has received emergency approval by the Food and Drug Administration (FDA).

Inflammation is a critical factor in COVID-19 [5,33], and, consequently, agents able to combat virus replication, and, at the same time, regulate inflammation, could offer a new approach for the treatment of COVID-19. Since GIIA s\textit{PLA}_2 is associated with increased mortality by COVID-19 [12], and lipid mediators arising from the activity of \textit{PLA}_2 have been correlated with severe SARS-CoV-2 infection in humans [34], a therapeutic compound able to simultaneously inhibit both SARS-CoV-2 M\textsuperscript{pro} and GIIA s\textit{PLA}_2 would be of great value, as it would significantly reduce the risk of COVID-19 mortality. For the first time, a dual inhibitor of GIIA s\textit{PLA}_2 and SARS-CoV-2 M\textsuperscript{pro} is identified. GK241 shows weak inhibitory activity against SARS-CoV-2 M\textsuperscript{pro} compared to other known 2-oxoamide SARS-CoV-2 M\textsuperscript{pro} inhibitors; however, it may represent a basis for the development of a new class of potent dual-action inhibitors.

4. Materials and Methods

4.1. General Chemistry Methods

Forced-flow chromatography on Merck\textsuperscript{®} (Merck, Darmstadt, Germany) Kieselgel 60 F\textsubscript{254} 230–400 mesh was used for the purification of the products, while aluminum-backed silica plates (0.2 mm, 60 F\textsubscript{254}) were used for thin-layer chromatography (TLC). The visualization of the developed chromatograms was performed by fluorescence quenching using phosphomolybdic acid, ninhydrin or potassium permanganate stains. The melting points were determined on a Buchi\textsuperscript{®} 530 apparatus (Buchi, Flawil, Switzerland) and were uncorrected. Specific rotations were measured on an AA-65 series (Optical Activity Ltd., Bury, UK) polarimeter. \textsuperscript{1}H and \textsuperscript{13}C NMR spectra were recorded on a Varian\textsuperscript{®} Mercury (Varian, Palo Alto, CA, USA) (200 MHz and 50 MHz, respectively) or a Bruker Avance Neo (Bruker, Faellanden, Switzerland) (400 MHz and 100 MHz, respectively) and were internally referenced to residual solvent signals. The data for \textsuperscript{1}H NMR are reported as follows: chemical shift (δ ppm), multiplicity (s = singlet, d = doublet, t = triplet, qu = quintet,
4.2. General Procedure for the Coupling of α-Hydroxycarboxylic Acids 4a,b with Amines 5a–g

To a stirred solution of amine 5a–g (1.0 mmol), cooled to 0 °C, triethylamine (2.2 mmol, 0.31 mL), EDC·HCl (1.1 mmol, 211 mg), HOBr (1 mmol, 135 mg) and α-hydroxycarboxylic acid 4a,b (1.0 mmol) were added consecutively. After stirring for 1 h at 0 °C and for 16 h at room temperature (r.t.), the solvent was removed under reduced pressure. The residue was diluted in EtOAc and washed with brine (10 mL), an aqueous solution of 1N HCl (10 mL), brine (10 mL), an aqueous solution of 5% NaHCO₃ (10 mL) and brine (10 mL), consecutively. The organic layer was dried over Na₂SO₄ and concentrated under reduced pressure. The resulting α-hydroxamide 6a–g was further purified (if necessary) by flash chromatography eluting with the appropriate mixture of EtOAc:petroleum ether (40–60 °C).

4.2.1. (2S)-tert-Butyl 2-((2-hydroxyhexadecanamido)propanoate (6a)

Yield 85%; White solid; mp: 56–57 °C; Diastereoisomer 1: 1H NMR (200 MHz, CDCl₃): δ = 7.23–7.16 (m, 1H, NH), 4.40–4.23 (m, 2H, CH, OH), 4.02–3.97 (m, 1H, CH), 1.67–1.07 (m, 38H, 13 × CH₂, 4 × CH₃), 0.76 (t, J = 5.9 Hz, 3H, CH₃CH₂), 13C NMR (50 MHz, CDCl₃): δ = 174.3, 172.4, 82.0, 71.9, 48.2, 34.7, 31.9, 29.7, 29.64, 29.58, 29.5, 29.3, 27.9, 25.0, 22.5, 18.4, 14.1; Diastereoisomer 2: 1H NMR (200 MHz, CDCl₃): δ = 7.11 (d, J = 7.6 Hz, 1H, NH), 4.43 (dt, J = 14.5, 7.2 Hz, 1H, CH), 4.10–4.04 (m, 1H, CH), 3.40 (br s, 1H, OH), 1.78–1.12 (m, 38H, 13 × CH₂, 4 × CH₃), 0.85 (t, J = 6.4 Hz, 3H, CH₃CH₂); 13C NMR (50 MHz, CDCl₃): δ = 173.9, 172.2, 82.2, 72.1, 48.4, 34.9, 32.0, 29.79, 29.75, 29.7, 29.6, 29.53, 29.46, 28.0, 25.1, 22.8, 18.6, 14.2; HRMS (ESI) [M + Na⁺] m/z: 422.3243; (calculated for [C₂₃H₄₅N₃NaO₄]⁺ 422.3241).

4.2.2. N-((S)-1-((tert-Butyldimethylsilyl)oxy)-3-methylbutan-2-yl)-2-hydroxyhexadecanamide (6b)

Yield 80%; Colorless solid of low melting point; 1H NMR (200 MHz, CDCl₃): δ = 6.81–6.73 (m, 1H, NH), 4.11–4.04 (m, 1H, CH), 3.76–3.40 (m, 5H, CH₂O, 2 × CH, OH), 1.94–1.18 (m, 26H, 13 × CH₂, 0.93–0.74 (m, 18H, 6 × CH₃), 0.02 (s, 6H, 2 × SiCH₃); 13C NMR (50 MHz, CDCl₃): δ = 173.9, 173.7, 72.2, 72.0, 62.9, 55.4, 35.3, 35.3, 32.0, 29.81, 29.77, 29.7, 29.6, 29.5, 28.9, 25.9, 25.2, 25.0, 22.8, 19.7, 19.0, 18.3, 14.2, −5.5; HRMS (ESI) [M + Na⁺] m/z: 494.4000; (calculated for [C₂₇H₅₇N₃NaO₄Si]⁺ 494.4000).

4.2.3. N-((S)-1-(Benzyloxy)-3-methylbutan-2-yl)-2-hydroxyhexadecanamide (6c)

Yield 77%; White solid; mp: 63–64 °C; 1H NMR (200 MHz, CDCl₃): δ = 7.38–7.18 (m, 5H, 5 × ArH), 6.80–6.68 (m, 1H, NH), 4.54–4.41 (m, 2H, CH₂Ar), 4.12–3.99 (m, 1H, CHO), 3.91–3.79 (m, 1H, CHNH), 3.61–3.32 (m, 3H, CH, CH₂O), 2.09–1.13 (m, 26H, 13 × CH₂, 0.93–0.84 (m, 9H, 3 × CH₃); 13C NMR (50 MHz, CDCl₃): δ = 173.99, 173.96, 138.15, 138.10, 128.5, 127.9, 127.81, 127.76, 73.3, 73.2, 72.0, 70.21, 70.18, 54.0, 53.9, 35.2, 32.0, 29.81, 29.77, 29.72, 29.69, 29.6, 29.5, 29.3, 25.1, 25.0, 22.8, 19.6, 19.0, 14.2; HRMS (ESI) [M + H⁺] m/z: 448.3785; (calculated for [C₂₉H₃₉NO₃]⁺ 448.3785).

4.2.4. N-((S)-1-(Butylamino)-3-methyl-1-oxobutan-2-yl)-2-hydroxyhexadecanamide (6d)

Yield 76%; White solid; mp: 95–97 °C; 1H NMR (200 MHz, CDCl₃): δ = 7.53 (d, J = 9.3 Hz, 1H, NHCH), 7.19 (t, J = 5.8 Hz, 0.5H, NHCH₂), 7.02 (t, J = 5.7 Hz, 0.5H, NHCH₂), 4.97 (d, J = 4.9 Hz, 0.5H, CH), 4.50 (d, J = 5.3 Hz, 0.5H, CH), 4.32–4.20 (m, 1H, CH), 4.17–4.04 (m, 1H, CH), 3.36–3.01 (m, 3H, NHCH₂OH), 2.17–2.00 (m, 2H, CH₂H), 1.87–1.12 (m, 28H, 14 × CH₂), 0.95–0.82 (m, 12H, 4 × CH₃); 13C NMR (50 MHz, CDCl₃): δ = 175.03, 174.96, 171.8, 171.6, 72.3, 58.7, 58.4, 39.42, 39.38, 35.1, 32.0, 31.5, 31.1, 29.83, 29.78, 29.5, 25.3, 25.2, 22.8, 20.23, 20.20, 19.4, 18.7, 18.6, 14.2, 13.8; HRMS (ESI) [M + Na⁺] m/z: 449.3715; (calculated for [C₂₅H₃₀N₂NaO₃]⁺ 449.3714).

m = triplet, and br s = broad signal), coupling constant, integration and peak assignment. The data for 13C NMR are reported in terms of the chemical shift (δ ppm). High-resolution mass spectrometry (HRMS) spectra were recorded on a Bruker® Maxis Impact QTOF (Bruker Daltonics, Bremen, Germany) spectrometer.
4.2.5. (2S)-Methyl 2-(2-hydroxyhexadecanamido)-3-(2-oxopyrrolidin-3-yl)propanoate (6e)

Yield 57%; Yellow oil; 1H NMR (200 MHz, CDCl3): δ = 8.45 (d, J = 7.8 Hz, 0.17H, NH), 8.27 (d, J = 8.6 Hz, 0.17H, NH), 7.97 (d, J = 7.4 Hz, 0.33H, NH), 7.84 (d, J = 7.9 Hz, 0.33H, NH), 6.69 (s, 0.17H, NH), 6.59 (s, 0.17H, NH), 6.54 (s, 0.33H, NH), 6.46 (s, 0.33H, NH), 4.64–4.49 (m, 1H, CH), 4.14–4.04 (m, 1H, CH), 3.71 (s, 3H, OCH3), 3.51–3.40 (m, 1H, CHF), 3.36–3.28 (m, 1H, CHF2), 2.54–2.04 (m, 4H, CH2, CH, OH), 1.94–1.53 (m, 4H, 2 × CH2), 1.49–0.94 (m, 24H, 12 × CH2), 0.85 (t, J = 5.9 Hz, 3H, CH3); HRMS (ESI) [M + Na]+ m/z: 463.3139; (calculated for [C23H44N2NaO3]+: 463.3142).

4.2.6. (2S)-tert-Butyl 2-((2S)-2-(2-hydroxyhexadecanamido)propanamido)propanoate (6f)

Yield 75%; White solid; mp: 65–66 °C; 1H NMR (200 MHz, CDCl3): δ = 7.41 (d, J = 7.9 Hz, 1H, NH), 7.28–7.23 (m, 1H, NH), 4.65–4.51 (m, 1H, CH), 4.42–4.28 (m, 1H, CH), 4.18–4.03 (m, 2H, CH, OH), 2.01–1.10 (m, 41H, 13 × CH2, 5 × CH3), 0.84 (t, J = 6.5 Hz, 3H, CH2CH3); 13C NMR (50 MHz, CDCl3): δ = 174.7, 174.6, 172.3, 172.1, 171.9, 171.8, 81.98, 81.95, 72.1, 72.0, 48.9, 48.4, 34.9, 32.0, 29.8, 29.74, 29.66, 29.4, 28.0, 25.1, 22.8, 18.8, 18.6, 18.2, 18.1, 14.2; HRMS (ESI) [M + Na]+ m/z: 493.3612; (calculated for [C28H50N2NaO3]+: 493.3612).

4.2.7. (2S)-tert-Butyl 2-(2-hydroxydodecanamido)-3-methylbutanolate (6g)

Yield 87%; White solid of low melting point; 1H NMR (400 MHz, CDCl3): δ = 6.94 (d, J = 9.3 Hz, 0.5H, NH), 6.76 (d, J = 9.4 Hz, 0.5H, NH), 4.45 (dd, J = 9.1, 4.5 Hz, 1H, CH), 4.14 (dd, J = 7.8, 3.8 Hz, 1H, CH), 2.24–2.14 (m, 1H, CH(CH3)2), 1.87–1.79 (m, 1H, CHCH(OH)), 1.69–1.59 (m, 1H, CH(CH=CH(OH)), 1.47–1.25 (m, 25H, 8 × CH2, 3 × CH3), 0.98–0.86 (m, 9H, 3 × CH3); 13C NMR (100 MHz, CDCl3): δ = 173.94, 173.89, 171.3, 171.1, 82.2, 72.5, 72.1, 57.3, 57.1, 35.3, 35.1, 32.0, 31.6, 31.5, 29.72, 29.70, 29.65, 29.52, 29.46, 28.2, 25.1, 25.0, 22.8, 19.12, 19.07, 17.8, 17.7, 14.2; HRMS (ESI) [M + Na]+ m/z: 394.2928; (calculated for [C21H34NaO4]+: 394.2928).

4.3. General Procedure for the Oxidation of Hydroxyamides 6a–g to Oxoamides 7a–g

To a stirred solution of α-hydroxy-amides (6a–g) (1 mmol) in dry CH2Cl2 (0.2 M), under an inert argon atmosphere, Dess–Martin periodinane (1.3 mmol, 551 mg) was added. After stirring for 1 h, the solvent was removed under reduced pressure and EtO2 (30 mL) was added. The organic layer was washed with a saturated solution of aqueous NaHCO3 (20 mL) containing Na2S2O3 (1.5 g, 9.5 mmol), H2O (20 mL), and dried over Na2SO4. After removal of the organic solvent under reduced pressure, the residue was purified by flash chromatography eluting with the appropriate mixture of EtOAc:petroleum ether (40–60 °C).

4.3.1. (S)-tert-Butyl 2-(2-oxohexadecanamido)propanoate (7a)

Yield 87%; White solid; mp: 44–45 °C; [α]D = +5 (c = 1 in CHCl3); 1H NMR (400 MHz, CDCl3): δ = 7.42 (d, J = 7.5 Hz, 1H, NH), 4.35 (qu, J = 7.2 Hz, 1H, CHNH), 2.83 (t, J = 7.3 Hz, 2H, CH2CO), 1.57–1.50 (m, 2H, CH2CH2CO), 1.41 (s, 9H, 3 × C(CH3)3), 1.35 (d, J = 7.1 Hz, 3H, CHCH3J), 1.27–1.09 (m, 22H, 11 × CH2), 0.81 (t, J = 6.5 Hz, 3H, CH2CH3); 13C NMR (100 MHz, CDCl3): δ = 198.5, 171.1, 159.6, 82.2, 48.6, 36.7, 31.9, 29.70, 29.69, 29.67, 29.6, 29.5, 29.38, 29.35, 29.1, 27.9, 23.2, 22.7, 18.2, 14.1; HRMS (ESI) [M + Na]+ m/z: 420.3084; (calculated for [C23H34NaO4]+: 420.3084).

4.3.2. (S)-N-(1-(tert-Butyldimethylsiloxyl)-3-methylbutan-2-yl)-2-oxohexadecanamide (7b)

Yield 89%; Colorless oil; [α]D = −19 (c = 1 in CHCl3); 1H NMR (400 MHz, CDCl3): δ = 7.12 (d, J = 9.6 Hz, 1H, NH), 3.73 (dd, J = 10.2, 3.3 Hz, 1H, CHHO), 3.64 (dd, J = 13.3, 7.4, 3.6 Hz, 1H, CHNH), 3.56 (dd, J = 10.2, 3.9 Hz, 1H, CHHO), 2.89 (t, J = 7.4 Hz, 2H, CH2CO), 1.99–1.87 (m, 1H, CHCHNH), 1.62–1.55 (m, 2H, CH2CH2CO), 1.34–1.18 (m, 22H, 11 × CH2), 0.93 (d, J = 6.8 Hz, 3H, CH2CH3), 0.89–0.84 (m, 15H, 2 × CH3CH3, 3 × CH3C), 0.01 (d, J = 1.9 Hz, 6H, 2 × CH3Si); 13C NMR (100 MHz, CDCl3): δ = 199.6, 160.0, 62.7, 56.1, 36.9,
4.3.3. (S)-N-(1-Benzoyloxy)-3-methylbutan-2-yl)-2-oxohexadecanamide (7c)

Yield 93%; White solid; mp: 47-48 °C; [α]D = -34 (c = 1 in CHCl3); 1H NMR (400 MHz, CDCl3): δ = 7.37-7.28 (m, 5H, 5 × ArH), 7.17 (d, J = 9.7 Hz, 1H, NH), 4.51 (d and d, J = 12.1 Hz, 2H, CH2Ph), 3.87-3.80 (m, 1H, CH(NH)), 3.61 (dd, J = 9.7, 4.2 Hz, 1H, CHHO), 3.46 (dd, J = 9.7, 4.0 Hz, 1H, CHHO), 3.00-2.86 (m, 2H, CH2CO), 2.05-1.97 (m, 1H, CHCHNH), 1.66-1.59 (m, 2H, CH2CH2CO), 1.39-1.20 (m, 22H, 11 × CH2), 0.96-0.89 (m, 9H, 2 × CH3CH2CO, CH2CH2CO); 13C NMR (100 MHz, CDCl3): δ = 199.5, 160.1, 138.0, 128.5, 127.7, 73.3, 69.7, 54.6, 36.9, 32.0, 29.8, 29.75, 29.73, 29.68, 29.5, 29.43, 29.36, 29.2, 23.3, 22.8, 19.5, 18.9, 14.2; HRMS (ESI) [M + Na]+ m/z: 468.3448; (calculated for [C25H55NNaO3] +: 468.3448).

4.3.4. (S)-N-(1-Butylamino)-3-methyl-1-oxobutan-2-yl)-2-oxohexadecanamide (7d)

Yield 89%; White solid; mp: 78-79 °C; [α]D = -20 (c = 1 in CHCl3); 1H NMR (400 MHz, CDCl3): δ = 7.56 (d, J = 9.2 Hz, 1H, NHCO), 6.52 (br s, 1H, NHCH2), 4.18-4.14 (m, 1H, CH(NH)), 3.33-3.25 (m, 1H, CHCHNH), 3.18-3.10 (m, 1H, CHCHNH), 2.91-2.76 (m, 2H, CH2CO), 2.17-2.07 (m, 1H, CHCHNH), 1.61-1.53 (m, 2H, CH2), 1.49-1.42 (m, 2H, CH2), 1.34-1.13 (m, 24H, 12 × CH2), 0.94-0.82 (m, 12H, 4 × CH2); 13C NMR (100 MHz, CDCl3): δ = 198.3, 170.1, 160.3, 59.0, 39.4, 36.9, 32.0, 31.7, 31.3, 29.74, 29.73, 29.71, 29.66, 29.5, 29.4, 29.2, 23.2, 22.7, 20.2, 19.3, 18.4, 14.2, 13.8; HRMS (ESI) [M + Na]+ m/z: 447.3557; (calculated for [C23H48N2NaO3] +: 447.3557).

4.3.5. (2S)-Methyl 2-(2-oxohexadecanamido)-(3-(2-oxopyrrolidin-3-yl)propanoate (7e)

Yield 57%; White solid; mp: 59-61 °C; [α]D = -3 (c = 1 in CHCl3); 1H NMR (400 MHz, CDCl3): δ = 8.91 (d, J = 7.8 Hz, 0.25H, NH), 8.19 (d, J = 7.9 Hz, 0.75H, NH), 7.07 (s, 0.25H, NH), 6.88 (s, 0.75H, NH), 4.66-4.61 (m, 0.25H, NHCH), 4.54-4.48 (m, 0.75H, NHCH), 3.72 (s, 3H, CH3), 3.37-3.27 (m, 2H, NHCH2), 2.85 (t, J = 7.4 Hz, 2H, CH2CO), 2.50-2.14 (m, 3H, CH2, CH), 2.02-1.78 (m, 2H, CH2), 1.60-1.52 (m, 2H, CH2), 1.32-1.18 (m, 22H, 11 × CH2), 0.84 (t, J = 6.9 Hz, 3H, CH3CH2), 13C NMR (100 MHz, CDCl3): δ = 198.4, 198.2, 179.9, 179.6, 171.5, 171.4, 160.7, 160.6, 52.7, 52.6, 51.5, 51.3, 50.8, 40.6, 38.5, 38.4, 37.0, 36.9, 33.4, 32.6, 32.0, 29.74, 29.72, 29.70, 29.66, 29.52, 29.51, 29.4, 28.5, 28.4, 23.21, 23.18, 22.7, 14.2; HRMS (ESI) [M + Na]+ m/z: 461.2990; (calculated for [C24H42N2NaO5] +: 461.2986).

4.3.6. (S)-tert-Butyl 2-((S)-2-(2-oxohexadecanamido)propanamido)propanoate (7f)

Yield 82%; White solid; mp: 92-94 °C; [α]D = -12 (c = 1 in CHCl3); 1H NMR (400 MHz, CDCl3): δ = 7.58 (d, J = 7.3 Hz, 1H, NHCOOC), 6.82-6.71 (m, 1H, NHCHCOOC), 4.49 (qu, J = 7.1 Hz, 1H, CHNHCOOC), 4.41 (qu, J = 7.2 Hz, 1H, CHCOOC), 2.86 (t, J = 7.2 Hz, 2H, CH2CO), 1.60-1.53 (m, 2H, CH2CH2CO), 1.43 (s, 9H, 3 × C3H3), 1.41 (d, J = 7.0 Hz, 3H, CH3CH(NH)COOC), 1.33 (d, J = 7.2 Hz, 3H, CH3CHCOOC), 1.29-1.16 (m, 22H, 11 × CH2), 0.84 (t, J = 6.7 Hz, 3H, CH3CH2), 13C NMR (100 MHz, CDCl3): δ = 198.4, 171.9, 170.7, 160.0, 82.2, 48.9, 36.9, 32.0, 29.8, 29.74, 29.71, 29.66, 29.5, 29.4, 28.1, 23.2, 22.8, 18.54, 18.46, 14.2; HRMS (ESI) [M + Na]+ m/z: 491.3455; (calculated for [C26H48N2NaO5] +: 491.3455).

4.3.7. (S)-tert-Butyl 3-methyl-2-(2-oxodecanamidobutanoate (7g)

Yield 67%; Colorless oil; [α]D = +14 (c = 1 in CHCl3); 1H NMR (400 MHz, CDCl3): δ = 7.34 (d, J = 9.2 Hz, 1H, NH), 4.33 (dd, J = 9.1, 4.6 Hz, 1H, CHNH), 2.86 (t, J = 7.4 Hz, 2H, CH2CO), 2.23-2.12 (m, 1H, CHCHNH), 1.61-1.53 (m, 2H, CH2CH2CO), 1.44 (s, 9H, 3 × C3H3), 1.32-1.15 (m, 14H, 7 × CH2), 0.90 (dd, J = 7.0, 5.8 Hz, 6H, 2 × CH2CH3), 0.84 (t, J = 6.9 Hz, 3H, CH3CH2), 13C NMR (100 MHz, CDCl3): δ = 198.7, 170.1, 160.1, 82.4, 57.6, 36.8, 32.0, 31.6, 29.6, 29.5, 29.39, 29.36, 29.1, 28.1, 23.3, 22.7, 19.0, 17.6, 14.2; HRMS (ESI) [M + Na]+ m/z: 392.2769; (calculated for [C21H39NNaO4] +: 392.2771).
4.4. General Procedure of Deprotection of tert-Butyl Esters to Carboxylic Acids 8a–c

To a stirred solution of tert-butyl ester 7a,b,g (1 mmol) in dry CH₂Cl₂ (1 mL), TFA (1 mL) was added and the reaction mixture was left stirring for 3 hrs. After removal of the solvent, the residue was diluted in diethyl ether and precipitation by petroleum ether (40–60 °C) and filtration afforded the desired product.

4.4.1. (S)-2-(2-Oxohexadecanamido)propionic Acid (8a)

Yield 96%; White solid; mp: 101–103 °C; [α]D = +13 (c = 1 in CHCl₃); ¹H NMR (400 MHz, CDCl₃): δ = 8.88 (br s, 1H, COOH), 7.42 (d, J = 7.6 Hz, 1H, NH), 4.58 (qu, J = 7.3 Hz, 1H, CHCH₃), 2.90 (t, J = 7.3 Hz, 2H, CH₂CO₂), 1.65–1.16 (m, 2H, CH₂CH₂CH₃), 1.52 (d, J = 7.2 Hz, 3H, CH₃CH₂), 1.36–1.20 (m, 2H, 11 × CH₂), 0.87 (t, J = 6.6 Hz, 3H, CH₂CH₃); ¹³C NMR (100 MHz, CDCl₃): δ = 198.4, 176.8, 160.0, 48.1, 36.8, 32.0, 29.77, 29.75, 29.7, 29.54, 29.45, 29.4, 29.1, 23.2, 22.8, 17.8, 14.2; HRMS (ESI) [M-H]⁻ m/z: 340.2492; (calculated for [C₁₀H₁₄N₄O₄]⁻ m/z: 340.2493).

4.4.2. (S)-(S)-2-(2-Oxohexadecanamido)propanamido)propanic Acid (8b)

Yield 84%; White solid; mp: 157–158 °C; [α]D = +8 (c = 1 in DMF); ¹H NMR (400 MHz, DMSO-d₆): δ = 12.52 (br s, 1H, COOH), 7.39 (d, J = 9.1 Hz, 1H, NHCOO), 8.25 (d, J = 7.3 Hz, 1H, NHCO), 4.32 (qu, J = 7.3 Hz, 1H, CHCOOH), 2.36–2.23 (m, 1H, CHCH₃), 1.65–1.56 (m, 2H, CH₂CH₂CH₃), 1.30–1.20 (m, 28H, 11 × CH₂, 2 × CH₂CH₃), 0.86 (t, J = 6.8 Hz, 3H, CH₂CH₂CH₃); ¹³C NMR (100 MHz, DMSO-d₆): δ = 198.8, 173.8, 171.1, 160.6, 47.9, 47.5, 36.5, 31.3, 29.03, 29.02, 29.00, 28.99, 28.96, 28.84, 28.78, 28.7, 28.4, 22.7, 22.1, 18.0, 17.1, 13.9; HRMS (ESI) [M-H]⁻ m/z: 411.2864; (calculated for [C₂₂H₃₀N₂O₆]⁻ m/z: 411.2864).

4.4.3. (S)-3-Methyl-2-(2-oxododecanamido)butanoic Acid (8c)

Yield 59%; White solid; mp: 53–54 °C; [α]D = +6 (c = 1 in CHCl₃); ¹H NMR (400 MHz, CDCl₃): δ = 10.59 (s, 1H, COOH), 7.39 (d, J = 9.1 Hz, 1H, NH), 4.57–4.44 (m, 1H, CHNH), 2.89 (t, J = 7.5 Hz, 2H, CH₂CO₂), 2.36–2.23 (m, 1H, CH(CH₃)₂), 1.65–1.52 (m, 2H, CH₂CH₂CH₃), 1.36–1.16 (m, 14H, 7 × CH₂), 1.03–0.79 (m, 9H, 3 × CH₃); ¹³C NMR (100 MHz, CDCl₃): δ = 198.5, 176.1, 160.3, 57.2, 36.9, 32.0, 31.2, 29.6, 29.5, 29.43, 29.40, 29.31, 23.3, 22.8, 19.1, 17.6, 14.2; HRMS (ESI) [M-H]⁻ m/z: 312.2176; (calculated for [C₂₁H₂₉N₂O₄]⁻ m/z: 312.2180).

4.5. (S)-1-((tert-Butyldimethylsilyl)oxy)-3-methylbutan-2-amine (5b)

To a solution of benzyl (S)-(1-((tert-butyldimethylsilyl)oxy)-3-methylbutan-2-yl)carbamate (1.0 mmol, 353 mg) in MeOH (10 mL), 10% Pd/C (0.05 mmol, 53 mg) was added, and the reaction was left stirring under H₂ for 16 hrs. Upon completion, the reaction mixture was filtered through celite, and the solvent was evaporated under reduced pressure to afford the desired product. Yield 96%; Pale yellow solid of low melting point; [α]D = +4 (c = 1 in CHCl₃); ¹H NMR (200 MHz, CDCl₃): δ = 3.65 (dd, J = 9.9, 4.2 Hz, 1H, CHH), 3.43 (dd, J = 9.9, 7.2 Hz, 1H, CHH), 3.01 (br s, 2H, NH₂), 2.66–2.57 (m, 1H, CHNH₂), 1.77–1.61 (m, 1H, CH(CH₃)₂), 1.04–0.71 (m, 15H, 5 × CH₂), 0.03 (s, 6H, 2 × CH₃Si); HRMS (ESI) [M + H]⁺ m/z: 218.1934; (calculated for [C₁₁H₂₈NOSi]+ m/z: 218.1935).

4.6. (S)-tert-Butyl (1-(benzoxyl)-3-methylbutan-2-yl)carbamate (10)

To a flame-dried flask, under argon, NaH 60% (1.3 mmol, 52 mg) and dry N,N-dimethylformamide (DMF) (1.3 mL) were added. A solution of alcohol 9 (1.0 mmol, 203 mg) in dry DMF (0.7 mL) was added dropwise at 0 °C. The reaction mixture was stirred for 30 min at 0 °C and then benzyl bromide (1.1 mmol, 0.13 mL) was added dropwise. The reaction mixture was left stirring for 16 hrs at room temperature. Upon completion, the reaction mixture was quenched with a saturated aqueous solution of NH₄Cl (1 mL), H₂O (3 mL) was added, the aqueous layer was extracted with ethyl acetate (2 × 10 mL) and the combined organic layers were washed with H₂O (15 mL). The organic layer was collected, and after drying over Na₂SO₄, the solvent was removed under reduced pressure. The product was purified by flash chromatography eluting with a mixture of EtOAc:petroleum
ether (40–60 °C)–5:95. Yield 47%; Colorless oil; [α]D = −20 (c = 1 in CHCl3); 1H NMR (200 MHz, CDCl3): δ = 7.44–7.13 (m, 5H, 5 × ArH), 4.78 (d, J = 7.9 Hz, 1H, NH), 4.56–4.42 (m, 2H, CH2Ph), 3.61–3.37 (m, 3H, CH2CH, CHNH), 1.99–1.82 (m, 1H, CH(CH3)2), 1.45 (s, 9H, C(CH3)3), 0.92 (d, J = 6.8 Hz, 3H, CH3), 0.91 (d, J = 6.8 Hz, 3H, CH3); 13C NMR (50 MHz, CDCl3): δ = 155.9, 138.3, 128.4, 127.62, 127.58, 78.9, 73.1, 70.5, 55.5, 29.6, 28.4, 19.6, 18.7; HRMS (ESI) [M + H]+ m/z: 294.2064; (calculated for [C17H28NO3]+ 294.2064).

4.7. (S)-1-(Benzyloxy)-3-methylbutan-2-aminium chloride (5c)

N-Boc-Protected amine 10 (1.0 mmol, 294 mg) was stirred for 2 hrs with a 4N solution of HCl in MeOH (50.0 mmol, 12.5 mL). Upon completion of the reaction, Et2O was added, and the solvents were removed under reduced pressure. The latter was repeated until complete removal of HCl, to afford the desired product. Yield 100%; White solid; mp: 115–118 °C; [α]D = +20 (c = 1 in MeOH); 1H NMR (200 MHz, CD3OD): δ = 7.41–7.25 (m, 5H, 5 × ArH), 4.65–4.52 (m, 2H, CH2Ph), 3.73–3.56 (m, 2H, CH2CH), 3.16–3.08 (m, 1H, CHNH2), 2.11–1.93 (m, 1H, CH(CH3)2), 1.03 (d, J = 6.8 Hz, 3H, CH3), 0.97 (d, J = 6.8 Hz, 3H, CH3); HRMS (ESI) [M + H]+ m/z: 194.1535; (calculated for [C12H20NO]+ 194.1539).

4.8. Enzyme Assay

The enzyme inhibition assay was performed as previously described [15]. A buffer composed of 20 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.3 was used for the enzyme inhibition assay. For the determination of the inhibition rate, 0.5 µM of SARS-CoV-2 Mpro was incubated with 40 µM or 100 µM of 2-oxoamide in the buffer at 37 °C for 10 min. The FRET substrate was then added to each well at a final concentration of 10 µM and a final total volume of 50 µL, to initiate the reaction. The GraphPad Prism 6.0 software (GraphPad) was used for the calculation of % inhibition rate. Measurements of the inhibition rate for the compounds were performed in triplicate and are presented as mean ± SD.

4.9. Covalent Docking Calculations

4.9.1. Protein Preparation

Docking calculations were performed using the SARS-CoV-2 MPpro crystallographic structure in complex with the covalent α-ketoamide inhibitor 13b (PDB ID: 6Y2F) [15]. Preparation and minimization of MPpro, using the Protein Preparation Wizard tool within the Maestro Schrodinger suite, were performed to ensure structural correctness. Hydrogen addition, bond orders and steric clashes correction, water molecules and HetAtoms deletion, charge optimization and restrained minimization supported by the OPLS3 force field were achieved [35]. Moreover, addition of the missing residues E47 and D48 was performed using the Crosslink Proteins tool, in the Maestro Schrodinger suite. The inhibitor GK241 and analogs 8a and 8d were prepared for docking using the LigPrep tool, in the Maestro Schrodinger suite [36].

4.9.2. Covalent Docking

Covalent docking is a multiple step process, that is designed upon Schrodinger’s Glide and Prime, capable to determine ligands activity against a protein target taking into account both non-covalent interactions and covalent bond formation. Covalent docking calculations were carried out using the Covalent Docking application, implemented in Maestro Schrodinger suite. Initially, pose selection was carried out using non-covalent docking simulations (Glide) and positional constraints. Specifically, ligand docking was performed in a mutated binding site. The reactive residue was transformed to alanine and the ligand warhead (the ligand moiety able to form covalent bond) was docked closely to the catalytic residue avoiding unfavorable clashes. Subsequently, the mutation was reversed, and receptor sampling was performed. Covalent bond formation was achieved based on geometric criteria and structural optimization. The following step involved both minimization of protein–ligand complexes in vacuum, and clustering of the optimized poses. This early selection was used as a basis for the further minimization, scoring and
ranking of covalent docking poses using the Prime VSGB2.0 energy model. Finally, an additional scoring function generated the affinity score that represents an average value of both the pre-reaction and post-reaction Glide Scores assessing the overall covalent docking procedure. Simultaneously, MM-GBSA energy property calculations for the structure of the receptor, the ligand and the protein-ligand complex were carried out for every docking pose [37]. In this case, Cys145 was identified as the reactive residue in Mpro and nucleophilic addition to a double bond as the reaction type. MM-GBSA scoring was selected and an output of 100 poses per ligand reaction site was achieved [37]. A covalent bond was formed between the reactive residue, Cys145 and the C2 carbonyl of the inhibitor. Subsequently, Mpro–ligand complexes with thiohemiketal formation between ligand the 2-oxo group and the catalytic Cys145 were selected.

4.10. Molecular Dynamics Simulations

MD simulations were performed using Desmond software, which offers a simple setup. Each calculation comprised an eight-step, automated workflow, divided into two categories. A seven-step protocol enabling the system to be minimized and equilibrated was followed by simulation during the last step of the process. This workflow process allowed for the study of protein–ligand interactions and their conformational variations over time and evaluation of the effects of water molecules in the complex [38]. Initially, the System Builder tool in Desmond was used for the preparation of the complexes. TIP3P was selected as a solvent model, OPLS_2005 was assigned as the force field and the system was embedded in a triclinic shaped box. The volume of the box was minimized, and the negative charges were neutralized by the addition of Na+ ions and 0.15 M of salt was added. Subsequently, MD simulations of 50 ns were performed in Desmond2020-1 using an NPT ensemble. A Nose–Hoover chain thermostat and Martyna–Tobias–Klein barostat were applied to maintain the temperature and pressure constant at 300 Kelvin and 1.01325 bar, respectively.

5. Conclusions

In this paper, we present our results on the identification of a synthetic 2-oxoamide inhibitor, which is a known potent inhibitor of GIIA sPLA2 (IC50 143 nM) [24], and, at the same time, can weakly inhibit SARS-CoV-2 Mpro (IC50 24 µM). The known 2-oxoamides AX109 and AX074, which selectively inhibit GIVA cPLA2, as well as pentafluoroethyl ketone GK187, which is a selective and potent inhibitor of GIVIA iPLA2, did not exhibit any appreciable inhibition of SARS-CoV-2 Mpro. The free carboxyl group and the long chain of the inhibitor GK241 were necessary for the inhibition of SARS-CoV-2 Mpro. Since the role of GIIA sPLA2 in mortality from COVID-19 has recently been recognized [12], the development of dual inhibitors of GIIA sPLA2 and SARS-CoV-2 Mpro appears to represent an attractive strategy for the development of novel agents to treat COVID-19. 2-Oxoamide GK241 may provide a lead structure for the development of such dual inhibitors.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/ph15080961/s1: Table S1. Structural details of structures 1–7 concerning the configuration of 2-carbon and the dihedral angle CβC145–SC145–C2–O2 related to the covalent bond formatted between Cys145 and 2-oxoamide moiety. Molecular dynamics RMSDs for the protein and the ligand along with MM-GBSA binding energy; Figure S1. Structural details for the Mpro–GK241 interactions in structures 1–6 resulting from covalent docking calculations; Figure S2. Protein residues root mean square fluctuation during the 50 nsec MD simulation showing local changes in the protein chain. Figure S3. Structures of 8d (A) in magenta and 8a (B) in blue bound to Mpro in comparison with GK241 (C) in green and crystal structure PDB 6Y2F (D) in khaki. The side-chain in derivative 8d adopts a curved conformation exploring part of S1 and S2 protease cavities. 1H NMR and 13C NMR spectra of the compounds synthesized.

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review and editing, G.K. and R.H.; supervision, G.K. and R.H. All authors have read and agreed to the published version of the manuscript.

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**Abbreviations**

| Acronym | Description |
|---------|-------------|
| AA      | arachidonic acid |
| DMF     | N,N-dimethylformamide |
| EDC·HCl | 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride |
| EDTA    | ethylene diamine tetra-acetic acid |
| FDA     | Food and Drug Administration |
| FFAs    | free fatty acids |
| FRET    | fluorescence resonance energy transfer |
| GIIA sPLA₂ | secreted PLA₂ |
| GIVA cPLA₂ | cytosolic PLA₂ |
| GIVIA iPLA₂ | calcium-independent PLA₂ |
| HOBt    | 1-hydroxybenzotriazole |
| HRMS    | high-resolution mass spectrometry |
| Mpro    | main protease |
| MD      | molecular dynamics |
| OA      | oleic acid |
| PCs     | phosphatidylcholines |
| PLA₂    | phospholipase A₂ |
| RdRp    | RNA-dependent RNA polymerase |
| r.t.    | room temperature |
| SARS-CoV-2 | severe acute respiratory syndrome coronavirus-2 |
| TFA     | trifluoroacetic acid |
| THF     | tetrahydrofuran |
| TLC     | thin-layer chromatography |
| Tris    | tris(hydroxymethyl)aminomethane |

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