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Marine *Streptomyces*-Derived Lipids Inhibit SARS-CoV-2 3CLpro Through In Vitro and Predicted Multi-Site Binding Mechanisms

Doralyn S. Dalisay ^{1,2,3,*} , Jomari C. Mateo ², Jade Joshua R. Teodosio ² , Leighiara S. de Guzman ², Neaven Bon Joy M. Marcial ², Dion Paul C. Caspe ⁴, Lex Aliko P. Balida ² and Jamia Azdina Jamal ¹

¹ Centre for Drug and Herbal Development, Faculty of Pharmacy, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, Kuala Lumpur 50300, Malaysia; jamia@ukm.edu.my

² Center for Chemical Biology and Biotechnology, University of San Agustin, Iloilo City 5000, Philippines; jomarimateo@usa.edu.ph (J.C.M.); jjteodosio@usa.edu.ph (J.J.R.T.); leighiaradeguzman@usa.edu.ph (L.S.d.G.); nbjmarcial@usa.edu.ph (N.B.J.M.M.); labalida@usa.edu.ph (L.A.P.B.)

³ Department of Biology, University of San Agustin, Iloilo City 5000, Philippines

⁴ Magnetic Resonance Facility, University of San Agustin, Iloilo City 5000, Philippines; dpcaspe@usa.edu.ph

* Correspondence: ddalisay@usa.edu.ph; Tel.: +63-33-501-0350

Abstract

Background: The SARS-CoV-2 3CLpro is essential for viral replication and an attractive target for antiviral intervention. While most strategies target the catalytic site, recent studies suggest that the dimerization interface and cryptic allosteric pockets offer alternative mechanisms for inhibition. **Objective:** This study investigated lipid metabolites from the marine sediment-derived *Streptomyces* sp. DSD454^T as potential multi-site 3CLpro inhibitors. **Methods:** Metabolites were extracted from cultured biomass and characterized using LCMS-QTOF, MS/MS (LCMS-TQ), and ¹H NMR, with identities confirmed against authentic standards. 3CLpro inhibition was assessed using a FRET-based assay, and ligand–protein interactions were evaluated through molecular docking and MM/GBSA calculations. Lipid content and comparative lipidomic signatures were examined across bioactive *Streptomyces* strains through LCMS-TQ and BODIPYTM 493/503 staining. **Results:** Palmitoleic and linoleic acids were identified as major constituents and inhibited SARS-CoV-2 3CLpro with IC₅₀ values of 1.59 µg/mL (6.25 µM) and 5.29 µg/mL (18.88 µM). Molecular docking predicted that both fatty acids bind not only to the catalytic site but also to the dimerization interface and cryptic allosteric pocket. Additional lipids, including 9-heptadecenoic acid, linolenic acid, 9-HODE, and monoacylglycerols such as aggrecerides A–C and glyceryl-based lipids, showed similarly favorable multi-site binding profiles. *Streptomyces* sp. DSD454^T also exhibited substantial lipid accumulation (~63% of crude extract). Across bioactive *Streptomyces* strains, a conserved lipid signature correlated strongly with 3CLpro inhibition. **Conclusions:** This study highlights the potential of microbial lipids as promising scaffolds for developing catalytic and allosteric SARS-CoV-2 3CLpro inhibitors and underscore marine *Streptomyces* as a valuable source of structurally simple yet mechanistically versatile antiviral metabolites.

Keywords: SARS-CoV-2 3CLpro; marine-derived *Streptomyces*; lipids; allosteric inhibition; palmitoleic acid; linoleic acid; antiviral activity; molecular docking



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1. Introduction

The replication of the RNA genome plays a vital role in the life cycle of SARS-CoV-2 [1–3]. The core of this process involves translating viral RNA into two significant polyproteins, pp1a and pp1ab. These polyproteins require proteolytic cleavage to produce functional non-structural proteins (NSPs) that facilitate viral replication [4–7]. Two viral proteases are responsible for these cleavage events: the papain-like protease (PLpro) [8,9] and the 3-chymotrypsin-like protease (3CLpro), also known as the main protease (Mpro) [10,11]. PLpro cleaves the N-terminal region of polyproteins, producing nsps that play a critical role in immune evasion and the initial phases of viral replication [8,12]. PLpro exhibits similarities to human deubiquitinating enzymes [8,13]. In contrast, 3CLpro cleaves at 11 different locations on the polyproteins to free the nsps necessary for creating the replication-transcription complex (RTC), which is important for viral RNA production and the formation of new virions [6,11,14]. 3CLpro is composed of three distinct domains. Domains I and II (residues 1–184) form antiparallel β -barrels with a chymotrypsin-like fold, creating a substrate-binding site between them [11,15,16]. Domain III (residues 201–303) is made up of α -helices and is connected to Domain II by a flexible hinge loop (residues Gln189-Asp197) [15,17,18]. The protease is active only in its dimeric form, with each monomer contributing a catalytic dyad composed of Cys145 and His41 [19,20]. Previous studies have highlighted the importance of Domain III in promoting dimerization and maintaining enzymatic activity, suggesting it plays a more active role than merely providing structural support [17,21–23]. These domains offer multiple potential targets for therapeutic intervention, underscoring 3CLpro as a crucial element in the pursuit of COVID-19 therapies [23]. The catalytic site of 3CLpro is important for its proteolytic activity and has been a major target of drug discovery efforts. Cys145 and His41, which form a catalytic dyad at this position, cleave viral polyproteins at specific points (Leu-Gln-Ser-Ala-Gly) [19,24]. These sequences are exclusive to viral proteins and are absent in human proteases [16,24]. The precise nature of the catalytic site of 3CLpro makes it a suitable target for antiviral drugs, which minimizes the chances of unintended effects in humans [19,25,26]. Although the majority of antiviral approaches have focused on the catalytic domain of 3CLpro, recent findings indicate that the dimerization interface and hidden allosteric pockets play a significant role in enzymatic regulation. However, these areas have not been thoroughly investigated as potential drug targets [25,27–31]. Therefore, these sites act as alternative or supplementary drug targets, expanding the possibilities for inhibitor design beyond the conventional approach of blocking the active site.

With this expanded understanding of 3CLpro architecture and the regulatory roles of its non-catalytic regions, recent efforts have increasingly focused on leveraging these structural vulnerabilities for therapeutic applications. A variety of studies have highlighted inhibitors that focus on allosteric sites and dimer interfaces, impacting 3CLpro activity without engaging the catalytic cleft. A recent investigation revealed new peptidomimetic carboxamides (Figure 1) that were originally selected for the active site but instead engage with a validated allosteric pocket. This interaction modifies the balance of proteases, promoting the monomeric form and thereby hindering 3CLpro in infected cells [32]. A recent study also explored the use of repurposed drugs like minocycline, bexarotene, ledipasvir, and simeprevir (Figure 1), which engage with an allosteric site at the dimer interface. The results, derived from structural, biophysical, and crystallographic analyses, revealed that interactions in this region cause the movement of key helices in domain III, weaken the dimer, and initiate conformational alterations in residues vital for preserving the active conformation [33]. In addition, a computational analysis strongly supports the possibilities for advancing drug development in this area. A recent study developed derivatives of benzo[b]thiophene and benzo[b]furan (Figure 1) with the intention of targeting a pocket

located between domains II and III. This approach led to candidates anticipated to demonstrate dual affinity for the catalytic environment as well as the dimerization site [34]. Finally, a documented report provided an in-depth thermodynamic analysis of the dimer-monomer equilibrium, showing that fluorinated 1,2,4-oxadiazoles compounds (Figure 1) discovered via virtual screening can either destabilize or stabilize the dimer, resulting in corresponding changes in enzymatic activity [35]. All of these inhibitors are synthetic or repurposed molecules, underscoring the need to explore natural-product scaffolds as alternative sources of structural diversity capable of modulating these non-catalytic sites.

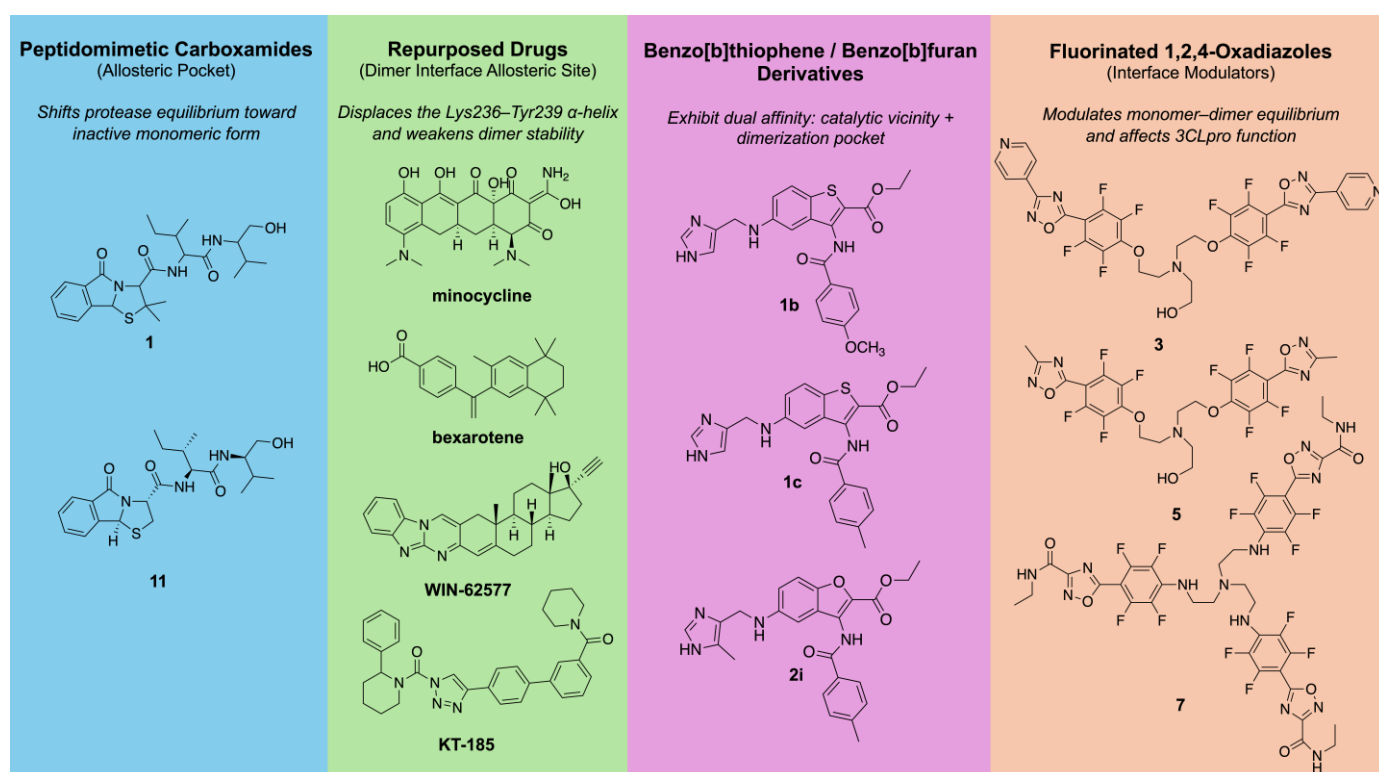


Figure 1. Structural classes of non-catalytic SARS-CoV-2 3CLpro inhibitors grouped according to binding site and mechanistic effect. Peptidomimetic carboxamides (blue panel) interact with an allosteric pocket located between Domains II and III and shift the protease equilibrium toward the inactive monomeric state (1, 11). Repurposed drugs such as minocycline, bexarotene, WIN-62577, and KT-185 (green panel), bind an allosteric site at the dimer interface, displacing the Lys236–Tyr239 α -helix and weakening dimer stability. Benzo[b]thiophene and benzo[b]furan derivatives (pink panel; compounds 1b, 1c, and 2i) exhibit dual-site affinity for the catalytic vicinity and the Domain II–III dimerization pocket. Fluorinated 1,2,4-oxadiazoles (peach panel; compounds 3, 5, and 7) act as interface modulators that alter the monomer–dimer equilibrium and impact 3CLpro proteolytic function. Note: All compound names and numbers in the figure are presented exactly as reported in the original papers cited [32–35].

Expanding on the recent insights into the multi-site susceptibility of 3CLpro, natural products present an intriguing source of structurally varied molecules that can take advantage of these mechanistic weaknesses. Natural products have historically served as rich sources of antiviral scaffolds, many of which engage with protein targets via both standard and atypical binding mechanisms. Microbial metabolites, especially those from *Streptomyces*, are notable for their chemodiversity, ecological adaptability, and evolutionary refinement for biological functions [36–43]. *Streptomyces* derived from marine sediments live in environments that are both competitive and chemically active, which leads to the production of polyketides, non-ribosomal peptides, and other bioactive compounds [44–47].

These include lipids and lipid-related compounds can influence enzyme activity through various interactions, both catalytic and non-catalytic. In our recently published study [48], we showed that marine sediments from the Philippines are an underexplored but highly productive source of metabolites that inhibit SARS-CoV-2 3CLpro. Using a combination of biochemical screening, metabolomics, and computational analysis, we discovered several antiviral candidate molecules sourced from marine-derived *Streptomyces*, which include lipid metabolites that potentially bind to multiple sites on 3CLpro.

In this study, we focused on *Streptomyces* sp. DSD454^T, a strain previously identified as one of the most bioactive isolates in our initial screening campaign [48]. The strain was characterized through morphological, biochemical, and phylogenetic analyses, followed by metabolomic profiling and bioassay-guided fractionation to isolate active compounds. These studies established *Streptomyces* sp. DSD454^T as a prolific producer of lipid-derived compounds. Among them, the unsaturated fatty acids palmitoleic acid and linoleic acid emerged as key bioactive constituents, each showing marked inhibition of SARS-CoV-2 3CLpro in enzymatic assays. To gain insight into their mode of action, structure-based molecular docking was performed, revealing potential binding at three functionally important regions of the protease: the catalytic site, the dimerization interface, and cryptic allosteric pockets that may be susceptible to inhibition by flexible, hydrophobic molecules. Additionally, fatty acids putatively identified as 9-heptadecenoic acid, linolenic acid, and 9-hydroxy-10,12-octadecadienoic acid (9-HODE), along with monoacylglycerols such as aggrecerides A-C and glyceryl-based lipids including 2,3-dihydroxypropyl octadeca-9,12-dienoate, 2,3-dihydroxypropyl hexadec-9-enoate, 2,3-dihydroxypropyl nonadeca-9,12-dienoate, glyceryl heptadecanoate, and glyceryl pentadecanoate, demonstrated favorable predicted binding affinities at both catalytic and non-catalytic regions of 3CLpro, indicating a potential multi-site mechanism of inhibition. Using metabolomics, biochemical inhibition assays, and computational modeling, we establish the antiviral potential of fatty acids and monoacylglycerols, and reveal marine sediment-derived *Streptomyces* as a rich yet underexplored source of lipid scaffolds targeting catalytic and allosteric sites in the coronavirus main protease for pharmaceutical development.

2. Results

2.1. Morphological and Biochemical Characterization of *Streptomyces* sp. DSD454^T

The marine sediment-derived *Streptomyces* sp. DSD454^T is phylogenetically identical to *S. enissocaesilis* (NR 115668.1) with 100% sequence identity [48]. It showed morphological characteristics of the genus *Streptomyces*. When grown on MM1 agar it formed colonies with wrinkled and rough surfaces. In addition, the strain also displayed concentric rings of spore chains. The variation in colony morphology, with some regions appearing smooth and others rough, may reflect different stages of growth and sporulation. Furthermore, the colonies exhibited a mixture of grey aerial and yellowish-brown substrate mycelium, with evident sporulation structures (Figure 2A) and brown diffusible pigment when grown in MM1 agar. The scanning electron microscope (SEM) image at 5000× magnification revealed a complex network of densely elongated, straight, cylindrical mycelia approximately 2 µm in diameter and 10 µm in length (Figure 2B). Furthermore, strain DSD454^T produced chains of cylindrical sporogenic structures, measuring approximately 1 µm in diameter and up to 15 µm in length, as shown in their collective spores (Figure 2C).

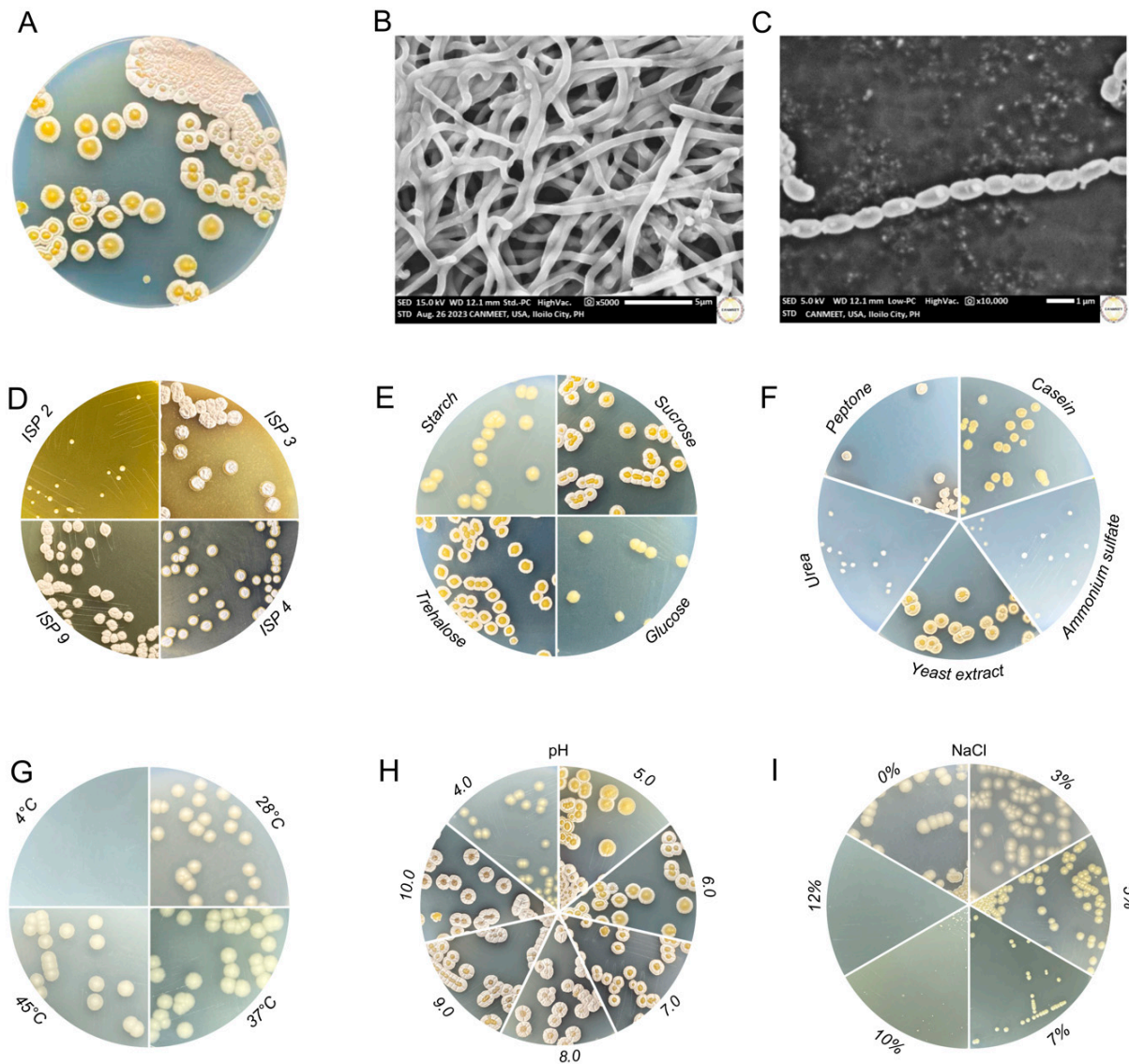


Figure 2. Morphological and physiological characterization of *Streptomyces* sp. DSD454^T. (A–C) Colony morphology and ultrastructure of *Streptomyces* sp. DSD454^T grown on MM1 agar and visualized by scanning electron microscopy (magnification = 5000× and 10,000×). (D–I) Growth of *Streptomyces* sp. DSD454^T under varying nutritional and environmental conditions, including different media (D), carbon sources (E), nitrogen sources (F), temperatures (G), pH levels (H), and NaCl concentrations (I).

Abundant aerial and substrate mycelial development was observed on ISP3, ISP4, and ISP9 media. In contrast, cultures grown on ISP2 exhibited reduced growth, with sparse or minimal formation of aerial and substrate mycelia. Diffusible pigment formation was not detected in any of the ISP media examined (Figure 2D and Table S1). All sugars examined were metabolized by *Streptomyces* sp. DSD454^T when provided as the sole carbon source. The colonies displayed a characteristic yellowish phenotype observed across all media tested (Figure 2E and Table S2).

Next, we examined how the strain develops colonies when different nitrogen sources are present. Poor aerial or substrate mycelium development was noted on conditions containing ammonium sulfate, urea, and peptone (Figure 2F and Table S3). Sparse aerial and substrate mycelial development was the outcome of growth on yeast extract. Cultures cultivated on casein, on the other hand, exhibited extensive aerial and substrate mycelial growth. In all of the nitrogen sources that were examined, no diffusible pigment was found.

To identify the ideal growing conditions for strain DSD454^T, the impact of temperature on growth was evaluated in addition to nitrogen usage. At 4 °C, as shown in Figure 2G and Table S4, no growth took place. The ample aerial and substrate mycelia were visible at 28 °C and 37 °C. This observation suggests that these temperatures were ideal for the development of the strain. At 45 °C, a noticeable decrease in development was observed. This includes visibility of scant mycelium, and no diffusible pigment was generated at any of the tested temperatures.

In order to evaluate the strain's tolerance to varying degrees of medium acidity or alkalinity, we next looked at how pH affected development. At pH 4, significant development was noted, as indicated in Figure 2H and Table S5. At pH 5–6, aerial and substrate mycelial development became noticeable, and it gradually improved from pH 7 to pH 10. In all measured pH range, diffuse pigment formation was not seen.

To assess salinity tolerance, the halotolerance of *Streptomyces* sp. strain DSD454^T was evaluated in media supplemented with increasing concentrations of NaCl (0–12% *w/v*). At 0–5% NaCl, robust development with an abundance of aerial and substrate mycelium was seen (Figure 2I and Table S6). Although colony density and coloration were decreased, there was still substantial mycelial growth at 7% NaCl. Growth declined sharply at 10% NaCl and was abolished at 12%, while no diffusible pigment formation occurred at any of the salt concentrations examined.

Finally, enzymatic activity profiling of strain DSD454^T was performed to determine its metabolic capabilities. Enzymatic assays (Table S7) showed broad activity across phosphatases, esterases, peptidases, and glycosidases, except for α -galactosidase, β -glucuronidase, and α -fucosidase, which were negative.

2.2. Phylogenetic Placement of *Streptomyces* sp. DSD454^T Based on Multilocus Sequence Analysis of Housekeeping Genes

The taxonomic position of *Streptomyces* sp. DSD454^T was inferred from a maximum-likelihood phylogeny constructed with concatenated sequences of the housekeeping genes *atpD*, *recA*, and *trpB* (Figure S1). The analysis included type strains of related *Streptomyces* species and was evaluated using bootstrap support values. According to our observations, strain DSD454^T does not belong to the clade of *S. vinaceusdrappus* or *S. rochei*. Instead, it shows a tight but distinct phylogenetic connection with *S. ennisocaesilis* DSD012^T by forming a sister lineage that is clearly separated from other members of the genus. The robustness of this phylogenetic split is highlighted by the strong bootstrap value (90%) that confirms the bifurcation between *Streptomyces* sp. DSD454^T and *S. ennisocaesilis*.

2.3. Biomass Production and Bioassay-Guided Purification of DSD454^T Extract for SARS-CoV-2 3CL^{pro} Inhibitors

A total of 50 L of MM1 molten agar was used to produce the biomass of strain DSD454^T. Ethyl acetate extraction from this 50 L biomass yielded 4.4 g of DSD454^T crude extract, which had an oily consistency. The crude extract was subjected to solid phase extraction, resulting in 3 g of methanol-soluble extract (coded as DSD454I). To further purify the DSD454I sample, preparative reversed-phase high-performance liquid chromatography was performed. The eluates were pooled based on their absorbance in the range of 190 to 790 nm, producing 25 distinct fractions (H1–H25) (Figure S2). Multiple retention times showed distinct peaks, with fractions H2, H7, H18, H20, H21, and H22 exhibiting strong PDA responses. Fractions H19–H25 contained most of the DSD454I extract eluates that were between 90 and 100% MeCN. The bulk material from fractions H19–H24, which displayed an oily consistency, yielded 5.6–22.3% (Table S8). Collectively, these fractions accounted for 63% of the crude extract.

A total of 25 HPLC fractions derived from *Streptomyces* sp. DSD454^T was evaluated for their ability to inhibit SARS-CoV-2 3CLpro using a FRET-based enzymatic assay. All experiments were conducted in three independent trials, each performed in triplicate. Due to observed solubility issues with mid-to-late eluting fractions (H10–H25) in assay buffer containing DTT, a modified sample preparation method was adopted. Each fraction was first prepared as a 50 mg/mL stock in DMSO. For testing, 0.5 µL of this stock was diluted into 99.5 µL of assay buffer with 1 mM DTT and vortexed immediately to prevent precipitation. This modification enabled consistent and soluble delivery of these samples for enzymatic testing.

The positive control, GC376 at 50 µg/mL (green bar), achieved 96% inhibition of 3CLpro activity, confirming the assay's validity as shown in Figure 3. In contrast, the negative control H₂O (red bar) showed negligible inhibition. At 10 µg/mL, the methanol-soluble extract DSD454I completely inhibited 3CLpro activity (100%, dark blue bar). Fractions H1B–H9, tested at 100 µg/mL in 0.1% DMSO, represented the early-eluting set and showed weak inhibition (<50%, white bars). Conversely, fractions H10–H25, tested at 50 µg/mL, displayed a broader range of activities. Moderate inhibition (50–60%) was observed in fractions H11B, H13B, and H14 (yellow bars), while stronger effects (80–90%) were seen in fractions H15, H17, H18, H19, and H20 (light blue bars). The most potent inhibition (>90%) was exhibited by fractions H21, H22, H23, and H24 (dark blue bars). Conversely, fraction H25 decreased activity, with inhibition falling below 50% (indicated by the white bar) (Figure 3). Minimal inhibition occurred in early fractions, moderate activity in mid-eluting samples, and the strongest effect in late fractions, with H21–H24 consistently producing >90% inhibition of SARS-CoV-2 3CLpro. These findings offer a targeted basis for chemical characterization and identifying the active constituents responsible for 3CLpro inhibition.

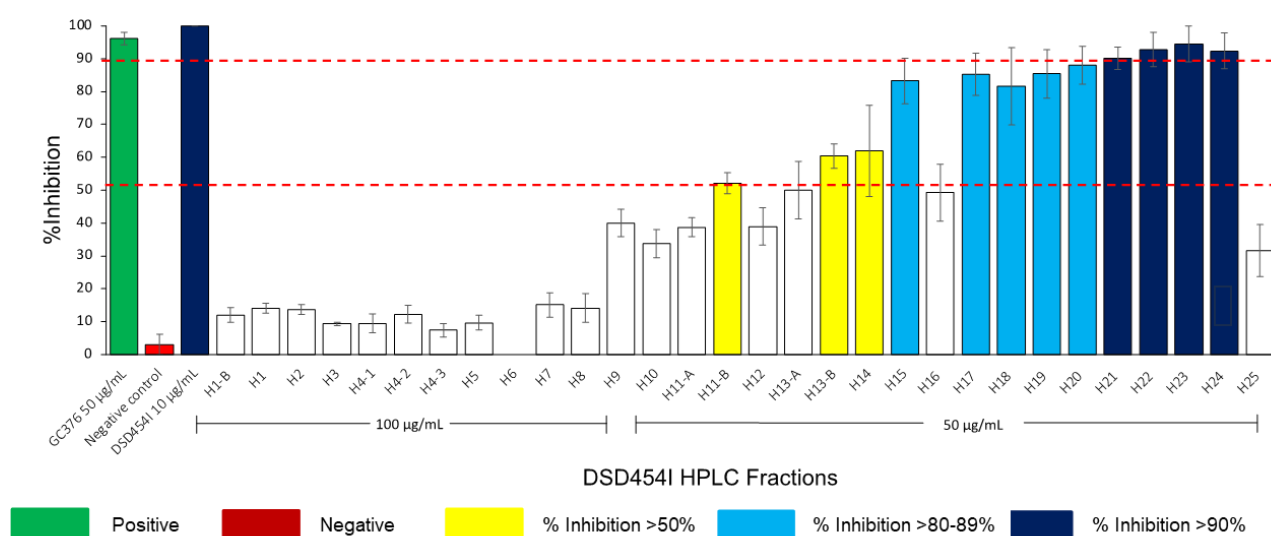


Figure 3. Inhibitory activity of *Streptomyces* sp. DSD454^T HPLC fractions against SARS-CoV-2 3CLpro. Fractions H1B–H25 obtained from HPLC fractionation of *Streptomyces* sp. DSD454^T extract were evaluated for their ability to inhibit SARS-CoV-2 3CLpro using a FRET-based enzymatic assay. A positive control GC376 (green) and a negative control H₂O (red) were included, alongside the crude extract DSD454I (dark blue). Fractions were color-coded by activity group for visualization. Error bars represent standard deviation across three independent experiments performed in triplicate. HPLC fractions with >50% and 90% inhibition (red broken line) of 3CLpro activity were identified as hits.

2.4. NMR Characterization of Bioactive HPLC Fractions Revealed Lipidic Components

Stacked ¹H NMR spectra are presented in Figure 4 for HPLC-purified fractions DSD454 H17 through H24, each exhibiting inhibitory activity against SARS-CoV-2 3CLpro, with

inhibition values ranging from 81.62% to 94.54%. We then examined the resonance signals for consistency and distinct chemical features indicative of lipid components. The terminal methyl (-CH₃) and methylene (-CH₂-) groups of long-chain fatty acid moieties were noted in fractions H17 to H19, which displayed prominent resonances that appeared in the aliphatic region (δ 0.80–1.50 ppm) (Figures S3–S5).

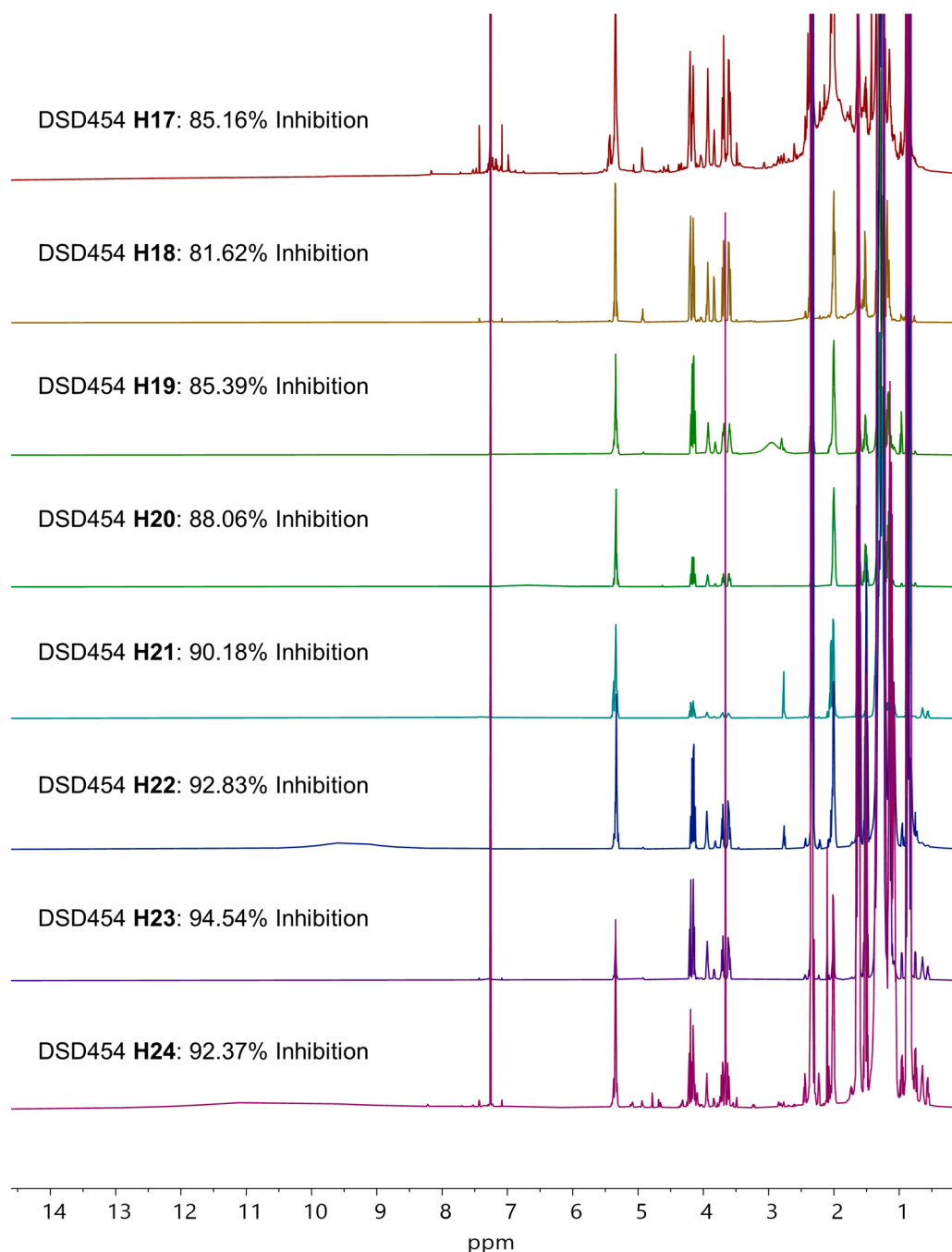


Figure 4. ¹H NMR spectral analysis of bioactive HPLC fractions DSD454 H17–H24. Stacked ¹H NMR spectra of HPLC-purified fractions DSD454 H17–H24 acquired in CDCl₃ at 298 K and 600 MHz on a Bruker Avance® NMR spectrometer (Bruker Corporation, Billerica, MA, USA) equipped with a 5 mm triple resonance cryoprobe. Spectra were analyzed for resonance features indicative of lipidic structures in fractions exhibiting SARS-CoV-2 3CLpro inhibitory activity.

Fractions DSD454 H20 through H24 exhibited enhanced signal intensity in the olefinic region (δ 5.20–5.50 ppm), attributable to protons on carbon-carbon double bonds within

unsaturated lipid chains. These peaks were particularly observed in DSD454 H20–H24, correlating with the highest inhibitory activities observed (88.06% to 94.54%) (Figures S6–S10).

There were no aromatic lipid compounds detected, as there were minimal or absent aromatic resonances (δ 6.80–7.50 ppm) among the HPLC fractions with 3CLPro activity. We also noted that fractions H21–H24 exhibit signatures of monoacylglycerols, as indicated by the glycerol backbone signals, typically found between δ 3.40–4.40 ppm (Figures S7–S10). Collectively, the ^1H NMR spectra among bioactive HPLC fractions suggest the presence of structurally related lipid compounds enriched in unsaturated aliphatic chains and likely involved in 3CLPro inhibition.

2.5. Dereplication-Guided Identification of Lipid-like Compounds in Bioactive HPLC Fractions of *Streptomyces* sp. DSD454I

The bioactive compounds present in four HPLC fractions (H21, H22, H23, and H24), all of which showed over 90% inhibition of SARS-CoV-2 3CLPro at a concentration of 50 $\mu\text{g/mL}$, were identified to link their 3CLPro activity. We performed the dereplication strategy by analyzing the MS and MS/MS data obtained from LCMS-TQ and high-resolution LCMS-QTOF. Additionally, ^1H NMR data were used to provide signature signals for compound identity and to prioritize structurally distinct compounds for additional characterization.

Due to its strong inhibitory activity and well resolved peak in the chromatogram, fraction H21 was selected for detailed analysis to initiate the dereplication process. The LCMS-TQ analysis performed at 190 nm revealed two key UV absorbance peaks that appeared between 9.5 and 10.2 min (Figure 5A). These corresponded to protonated molecular ions with m/z values of 255.25 $[\text{M}+\text{H}]^+$ and 281.25 $[\text{M}+\text{H}]^+$, respectively (Figures 5B and 5D). These masses were subjected to validation via LCMS-QTOF in negative ion mode, yielding deprotonated ions at m/z 253.2175 $[\text{M}-\text{H}]^-$ and 279.2327 $[\text{M}-\text{H}]^-$ (Figures S11 and S12). Accurate mass measurements corresponded to molecular formulas $\text{C}_{16}\text{H}_{30}\text{O}_2$ (double bond equivalent [DBE] = 2.0) and $\text{C}_{18}\text{H}_{32}\text{O}_2$ (DBE = 3.0), within acceptable mass error limits of <5 ppm (Table 1).

MS/MS-based dereplication using the GNPS platform identified the two compounds as palmitoleic acid **1** and linoleic acid **2** based on spectral similarity. These identifications were confirmed through direct comparison with authentic standards. Overlaying the UV and chromatographic profiles confirmed that the retention times and elution behaviors matched those of the standards for palmitoleic and linoleic acid (Figure 5A). Additionally, MS/MS fragmentation spectra showed diagnostic product ions consistent with fatty acid fragmentation patterns seen in the reference compounds. Compound **1** generated prominent fragments at m/z 237.15, 219.20, 163.05, and 135.05 with proposed formulas of $\text{C}_{16}\text{H}_{29}\text{O}^+$, $\text{C}_{16}\text{H}^+_{27}$, $\text{C}_{16}\text{H}^+_{19}$, and $\text{C}_{16}\text{H}_{29}\text{O}^+$ respectively (Figure 5C). Compound **2** displayed a similar fragmentation pattern, yielding ions at m/z 263.20 ($\text{C}_{18}\text{H}_{31}\text{O}^+$), 245.25 ($\text{C}_{18}\text{H}^+_{29}$), and 133.10 ($\text{C}_{10}\text{H}^+_{13}$) (Figure 5E).

Further structural validation was achieved through ^1H NMR spectroscopy of H21. The spectrum revealed vinylic proton signals in the δ 5.2–5.5 ppm range, supporting the presence of unsaturated aliphatic chains. Strong methylene signals appeared in the δ 0.9–2.8 ppm region, indicating extended saturated carbon chains (Figure S7). These spectral features align with the presence of mono- and polyunsaturated fatty acids. Quantitative analysis of the UV chromatograms indicated that Fraction H21 contains approximately 44% palmitoleic acid and 56% linoleic acid (0.8:1) based on peak area (Figure 5F). These findings confirm that H21 contains a mixture of structurally defined fatty acids **1** and **2**, which likely contribute to the observed antiviral activity. Based on this composition, the estimated amounts of compounds **1** and **2** in the total crude extract are approximately 2.46% and 3.13%, respectively.

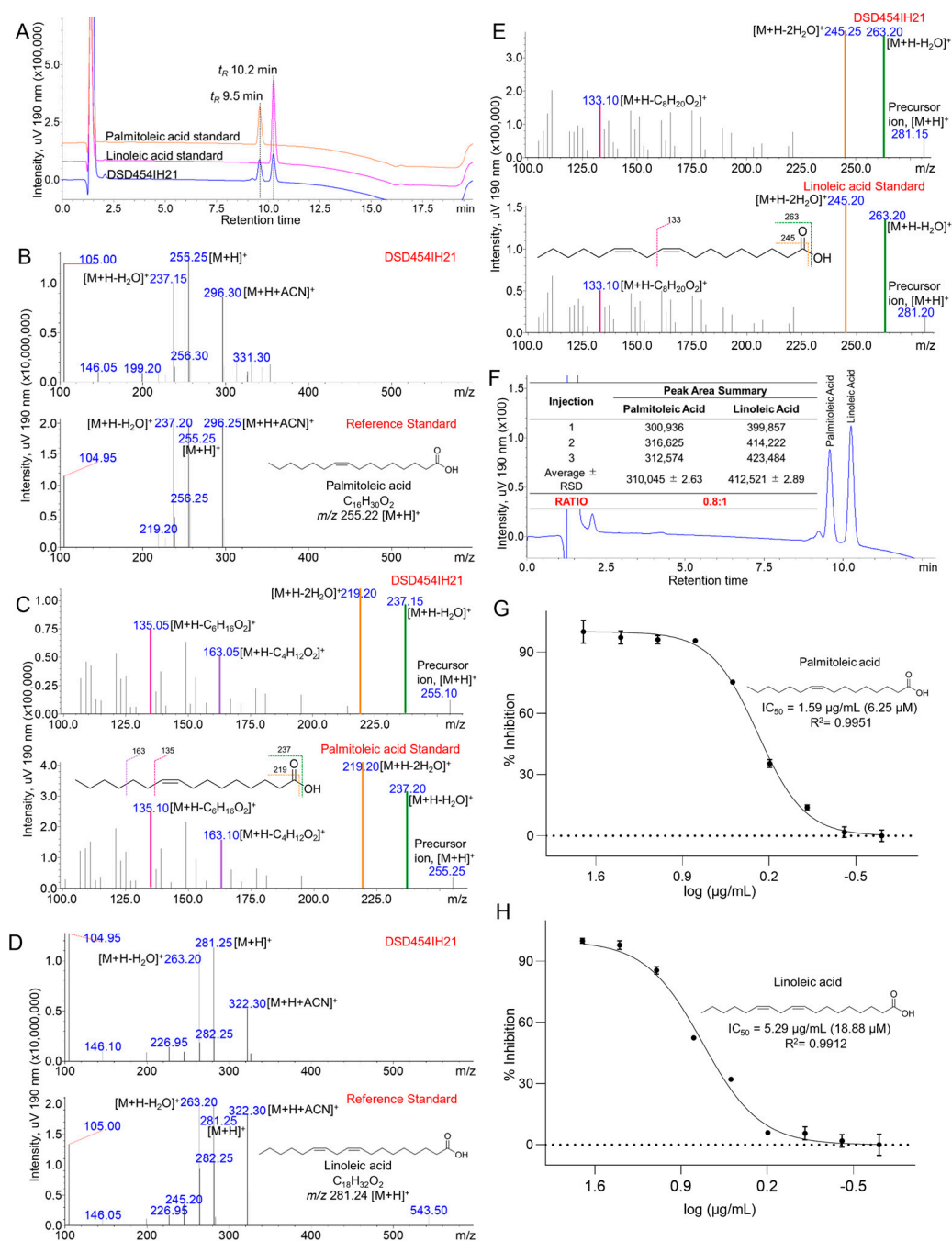


Figure 5. Chromatographic, mass spectrometric, and quantitative validation of palmitoleic acid **1** and linoleic acid **2** in bioactive Fraction H21. (A) Overlay of UV absorbance and chromatographic retention profiles of Fraction H21 with analytical standards of palmitoleic acid and linoleic acid. (B) MS of palmitoleic acid from Fraction H21 and reference standard. (C) MS/MS spectra of palmitoleic acid from Fraction H21 and reference standard. The fragmentation is color-coded according to their corresponding m/z peaks. (D) MS of linoleic acid from Fraction H21 and reference standard. (E) MS/MS spectra of linoleic acid from Fraction H21 and reference standard. The fragmentation is color-coded according to their corresponding m/z peaks. (F) Quantitative analysis of the UV chromatogram for Fraction H21 showing integrated peak areas corresponding to palmitoleic acid and linoleic acid based on relative abundance. (G) IC₅₀ inhibition of palmitoleic acid against SARS-CoV-2 3CLpro. (H) IC₅₀ inhibition of linoleic acid against SARS-CoV-2 3CLpro.

Table 1. Chemical Profiling of HPLC Fractions from *Streptomyces* sp. DSD454I sample: HRMS and MS/MS-Based Dereplication via LCMS-QTOF and LCMS-TQ Using Fragmentation Pattern Analysis and Database-Assisted Structural Annotation.

Compound No.	Compound Name	Fraction	HRMS m/z	Predicted Chemical Formula	Δ ppm Error	DBE	TQ-MS MS/MS Fragments
1	palmitoleic acid	H20, H21	253.2175 [M-H] [−]	C ₁₆ H ₃₀ O ₂	2.0	2.0	237.15, 219.20, 163.05, 135.05
2	linoleic acid	H21	279.2327 [M-H] [−]	C ₁₈ H ₃₂ O ₂	1.1	3.0	263.20, 245.25, 133.10
3	aggreceride B	H22	313.2736 [M+H-H ₂ O] ⁺	C ₁₉ H ₃₈ O ₄	−2.1	1.0	313.25, 257.25, 239.20, 109.10
4	9-heptadecenoic acid	H22	267.2329 [M-H] [−]	C ₁₇ H ₃₂ O ₂	3.0	2.0	251.25, 233.20, 209.15, 181.05, 135.10
5	aggreceride C	H23, H24	327.2888 [M+H-H ₂ O] ⁺	C ₂₀ H ₄₀ O ₄	−3.1	1.0	327.30, 271.20, 253.25, 109.10
6	2,3-dihydroxypropyl octadeca-9,12-dienoate	H23	355.2831 [M+H] ⁺	C ₂₁ H ₃₈ O ₄	−4.8	3.0	337.40, 293.50, 263.20
7	2,3-dihydroxypropyl nonadeca-9,12-dienoate	H24	369.2999 [M+H] ⁺	C ₂₂ H ₄₀ O ₄	−1.6	3.0	277.20, 253.40, 151.00, 137.00, 123.05, 109.10
8	2,3-dihydroxypropyl heptadec-9-enoate	H20	325.2739 [M+H-H ₂ O] ⁺	C ₂₀ H ₃₈ O ₄	−1.2	2.0	325.25, 269.20, 251.25, 233.20, 135.10, 121.10, 109.10
9	2,3-dihydroxypropyl hexadec-9-enoate	H17, H18, H19	311.2588 [M+H-H ₂ O] ⁺	C ₁₉ H ₃₆ O ₄	−1.3	2.0	311.25, 255.25, 237.20, 219.20, 135.10, 121.10, 109.10
10	aggreceride A	H19	299.2581 [M+H-H ₂ O] ⁺	C ₁₈ H ₃₆ O ₄	−1.7	1.0	299.25, 243.20, 137.15, 123.10, 111.10
11	linolenic acid	H19	277.2168 [M-H] [−]	C ₁₈ H ₃₀ O ₂	0.0	4.0	261.20, 223.10, 209.10, 187.15, 173.10, 149.15, 137.20, 123.15, 109.10
12	9-hydroxy-10,12-octadecadienoic acid	H17	279.2330 [M+H-H ₂ O] ⁺	C ₁₈ H ₃₂ O ₃	2.1	3.0	279.20, 261.20, 223.20, 149.10
13	2,3-dihydroxypropyl pentadec-9-enoate	H17	297.2434 [M+H-H ₂ O] ⁺	C ₁₈ H ₃₄ O ₄	1.3	2.0	297.20, 241.20, 223.20, 205.20, 149.10, 135.10, 121.10

Fraction H22 showed 92.83% inhibition of 3CLpro at 50 µg/mL. It eluted with 100% acetonitrile and appeared as a single UV-absorbance peak. LCMS-QTOF analysis revealed two components with m/z 313.2736 [M+H-H₂O]⁺ and 267.2329 [M-H][−] (Figures S13 and S14). LCMS-QTOF and MS/MS confirmed these as aggreceride B **3**, and 9-heptadecenoic acid **4**, respectively (Figure 6 and Table 1), with molecular formulas C₁₉H₃₈O₄ (DBE = 1.0) and C₁₇H₃₂O₂ (DBE = 2.0) (Figures S13, S14, S24 and S25). ¹H NMR spectra of H22 showed characteristic aliphatic (δ 0.7–2.8 ppm) and olefinic resonances (δ 5.2–5.5 ppm), supporting the presence of unsaturated fatty acid and monoacylglycerol structures (Figure S8).

Fraction H23 showed 94.54% inhibition. LCMS-TQ and QTOF analyses identified three major compounds (Figure 6, Table 1): an unannotated compound (m/z 357.35 [M+H]⁺, C₂₀H₄₀N₂O₃), aggreceride C **5** (m/z 327.2888 [M+H-H₂O]⁺, C₂₀H₄₀O₄) (Figure S15) and 2,3-dihydroxypropyl octadeca-9,12-dienoate **6** (m/z 355.2831 [M+H]⁺, C₂₁H₃₈O₄) (Figure S16). The MS/MS spectra and high-resolution mass data matched reported structures in public databases (Figures S26 and S27). The ¹H NMR spectra displayed vinylic signals for unsatu-

rated fatty acids (δ 5.2–5.5 ppm) and glycerol-related signals (δ 3.5–4.3 ppm), supporting the identification of compounds 5 and 6 (Figure S9).

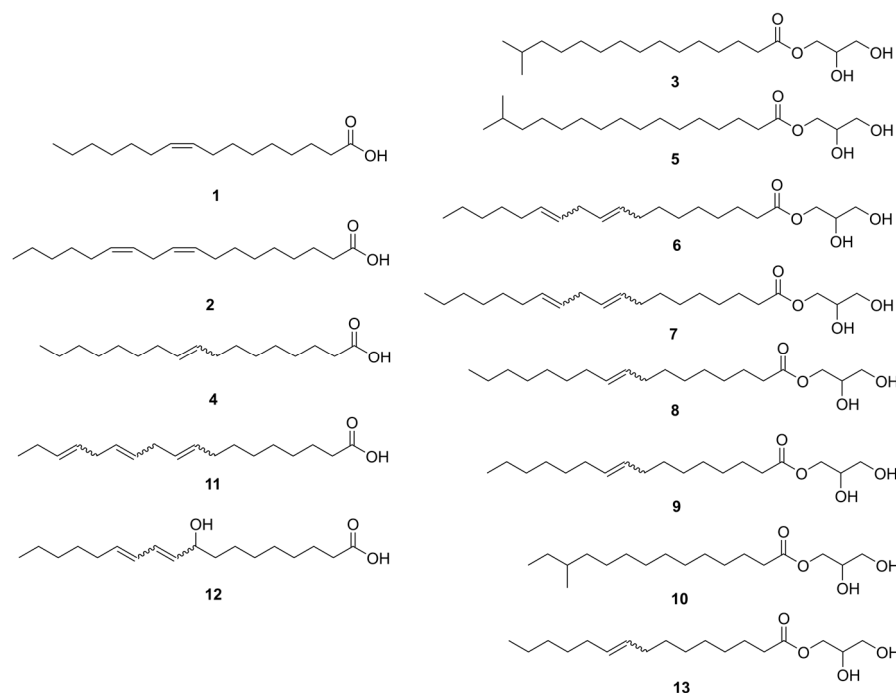


Figure 6. Lipidic compounds from *Streptomyces* sp. DSD454^T associated with SARS-CoV-2 3CLpro inhibition. A combined dereplication workflow integrating LCMS and MS/MS with triple quadrupole (LC-MS-TQ), high-resolution LC-MS-QTOF, and ¹H NMR spectroscopy was employed to identify compounds and prioritize structurally distinct lipidic compounds for further characterization.

Fraction H24 exhibited 92.37% inhibition. Despite weak UV absorbance, TIC analysis revealed two dominant peaks. LCMS-QTOF and MS/MS confirmed the presence of aggregeride C 5 and 2,3-dihydroxypropyl nonadeca-9,12-dienoate 7 (m/z 369.2999 [M+H]⁺), consistent with C₂₂H₄₀O₄ (DBE = 3.0) (Figure 6, Figures S17 and S28, Table 1). ¹H NMR analysis of H24 showed vinylic resonances (δ 5.2–5.5 ppm) attributed to the hydroxylated fatty acid ester, distinguishing it from the saturated monoacylglycerol (δ 3.5–4.3 ppm) (Figure S10).

Fraction H20 showed 88.06% inhibition. LCMS-TQ revealed two UV peaks, corresponding to 2,3-dihydroxypropyl heptadec-9-enoate 8 (C₂₀H₃₈O₄, m/z 325.2739 [M+H-H₂O]⁺, Figure 6 and Figure S18, Table 1), and compound 1. Compound 8 was identified based on its elemental composition and a diagnostic neutral loss of 74 Da in MS/MS, which corresponds to [glycerol-H₂O] (Table 1 and Figure S29). Compound 1 was confirmed by comparison to authentic standards. ¹H NMR supported the presence of aliphatic (δ 0.7–2.4 ppm) and vinylic proton environments (δ 5.3–5.4 ppm) (Figure S6).

Fraction H19 showed 85.39% inhibition of SARS-CoV-2 3CLpro at 50 μ g/mL. LCMS-QTOF analysis identified three main components: 2,3-dihydroxypropyl hexadec-9-enoate 9, m/z 311.2588 [M+H-H₂O]⁺, C₁₉H₃₆O₄ (Figure S19), aggregeride A 10 (m/z 299.2581 [M+H-H₂O]⁺, C₁₈H₃₆O₄) (Figure S20), and linolenic acid 11 (m/z 277.2168 [M-H][−], C₁₈H₃₀O₂) (Figure 6 and Figure S21, Table 1). Compounds 9 and 10 were characterized by their diagnostic neutral loss of 74 Da in MS/MS spectra, indicating the loss of a dehydrated glycerol group ([glycerol-H₂O]), which aligns with monoacylglycerol fragmentation patterns (Figure 6, Figures S30 and S31 and Table 1). Compound 11 was identified based on its high-resolution mass, accurate elemental composition, and a DBE of 4.0, indicative of a trienoic unsaturated fatty acid. The ¹H NMR spectrum of H19 displayed signals of

aliphatic protons (δ 0.8–2.9 ppm), along with a triplet at δ 2.8 ppm indicating the bis-allylic protons of compound **11**. Vinylic peaks at (δ 5.2–5.5 ppm) further support the identification of compounds **9** and **10** (Figure S5).

Fraction H17 showed 85.16% inhibition of SARS-CoV-2 3CLpro at a concentration of 50 $\mu\text{g/mL}$ and was found to contain three distinct compounds. High-resolution LCMS-QTOF analysis identified 9-HODE **12** (m/z 279.2330 $[\text{M}+\text{H}-\text{H}_2\text{O}]^+$, $\text{C}_{18}\text{H}_{32}\text{O}_3$) (Figure 6 and Figure S22, Table 1), 2,3-dihydroxypropyl hexadec-9-enoate **9** (m/z 311.2588 $[\text{M}+\text{H}-\text{H}_2\text{O}]^+$, $\text{C}_{19}\text{H}_{36}\text{O}_4$) (Figure 6 and Figure S19, Table 1), and 2,3-dihydroxypropyl pentadec-9-enoate **13** (m/z 297.2434, $[\text{M}+\text{H}-\text{H}_2\text{O}]^+$, $\text{C}_{18}\text{H}_{34}\text{O}_4$) (Figure 6 and Figure S23, Table 1). The MS/MS fragmentation spectrum of compound **12** matched that of 9-HODE, showing characteristic neutral loss of water and fragment ions typical of hydroxylated linoleic acid derivatives (Figure S33). Compounds **9** and **13** exhibited a diagnostic neutral loss of 74 Da, corresponding to the loss of a dehydrated glycerol moiety ($[\text{glycerol}-\text{H}_2\text{O}]$), which is a hallmark of monoacylglycerols (Figure 6, Figures S30 and S34, Table 1). The remaining fragment ions aligned with those of C16:1 and C15:1 fatty acids, respectively, confirming their structures as lipid-like compounds. The ^1H NMR spectrum showed vinylic peaks (δ 5.2–5.5 ppm) and glycerol-related signals (δ 3.5–4.4 ppm), confirming the identification of compounds **9**, **12**, and **13** (Figure S3).

Fraction H18 exhibited 81.62% inhibition. LCMS-QTOF identified 2,3-dihydroxypropyl hexadec-9-enoate **9** as the major component. The MS/MS spectra and high-resolution mass data were consistent with reported structures in public databases (Figure 6 and Figure S30, Table 1). ^1H NMR spectra revealed a monoacylglycerol structure, with esterification at the sn-1 position of glycerol (δ 3.5–4.3 ppm) and a palmitoleic acid side chain (δ 0.8–2.5 ppm) (Figure S4).

Collectively, these findings indicate that lipid-derived compounds, encompassing unsaturated fatty acids and monoacylglycerols, are broadly distributed across the bioactive fractions (H17–H24) and are likely key contributors to the observed inhibition of SARS-CoV-2 3CLpro.

2.6. Inhibitory Activities of Palmitoleic Acid **1** and Linoleic Acid **2**

We encountered difficulties in purification and isolating fatty acids and their derivatives in pure form from the DSD454^T strain extract. Therefore, pure compounds for additional analysis were obtained from suppliers. These compounds, including palmitoleic acid and linoleic acid, were tested against the 3CLpro inhibition assay. Their inhibitory efficacy against SARS-CoV-2-3CLpro was assessed using their IC_{50} values and matching correlation coefficients (R^2). Palmitoleic acid demonstrated a strong inhibitory effect with an IC_{50} of 1.59 $\mu\text{g/mL}$ (6.25 μM) and an R^2 value of 0.99, indicating a positive correlation between concentration and inhibition. Conversely, linoleic acid demonstrated an IC_{50} value of 5.29 $\mu\text{g/mL}$ (18.88 μM) and a correlation coefficient (R^2) of 0.99 (Figure 5G,H).

2.7. BODIPYTM 493/503 Staining Reveals Intracellular Lipid Distribution in *Streptomyces* Mycelia

We employed BODIPYTM 493/503 staining, a fluorescent lipophilic dye that selectively binds to neutral lipids, to check the presence and localization of intracellular lipids in *Streptomyces* sp. DSD454^T mycelia. Images of the same field of view were taken and analyzed in brightfield, fluorescence, and merged channel modes (Figure 7). The brightfield image (Figure 7A) showed a thick network of branching hyphae, typical of the filamentous shape of *Streptomyces*. In Figure 7B, a strong green BODIPYTM 493/503 signal was observed along the hyphae. The overlaid fluorescence and brightfield images (Figure 7C) showed that the lipid signal was in the same place as the mycelial structures.

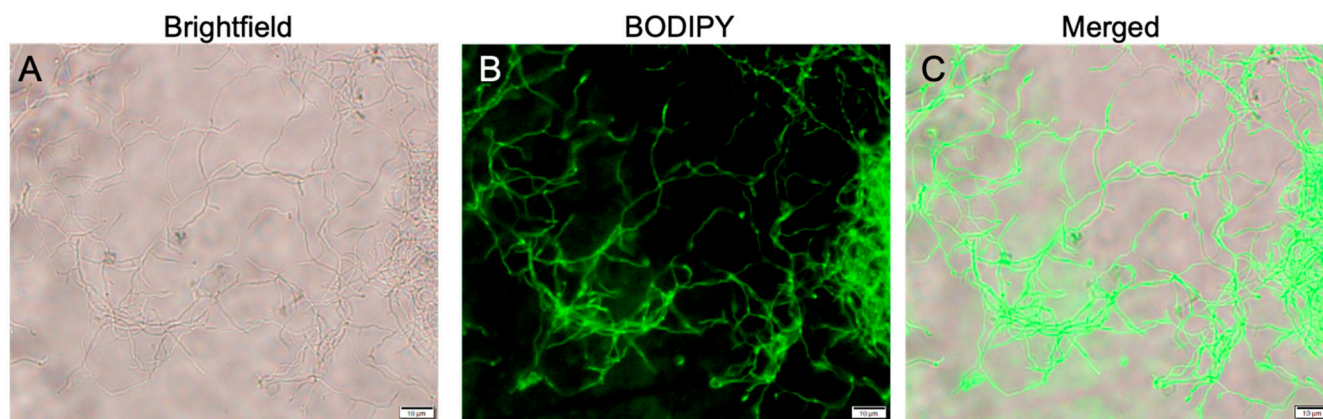


Figure 7. BODIPY™ 493/503 Staining Reveals Intracellular Lipid Localization in *Streptomyces* sp. DSD454^T Mycelia. Representative microscopy images showing lipid distribution in *Streptomyces* sp. DSD454^T mycelia stained with the lipophilic dye BODIPY™ 493/503. (A) Brightfield image showing the filamentous morphology and dense hyphal network characteristic of *Streptomyces* species. (B) Fluorescence image revealing intense green BODIPY™ 493/503 signal along the hyphae, indicating the presence of intracellular lipid-enriched regions in the mycelia. (C) Merged image of the brightfield and fluorescence channels, confirming the colocalization of lipid signal within the mycelial structures. Note: Scale bar represents 10 µm.

We observed that the BODIPY™ 493/503 fluorescence displayed intense spots but a heterogeneous distribution of lipid-rich compartments along the hyphae. This spatial distribution pattern indicates that lipid accumulation transpires throughout the mycelium rather than in designated subcellular regions. These microscopic observations are consistent with our HPLC and LCMS purification data, which indicated that approximately 63% of the total crude extract is composed of oily (lipid-based) material. Subsequent fractionation and chemical characterization of these materials have identified lipid compounds that most likely contribute to the SARS-CoV-2 3CLpro inhibitory activity.

2.8. Comparative Lipid Metabolite Profiling of 3CLpro Inhibitory *Streptomyces* Strains DSD149^T, DSD735^T, DSD2604^T, and DSD2893^T

During bioassay-guided preparative HPLC purification of the extract from *Streptomyces* sp. DSD454^T, late-eluting fractions enriched in lipophilic components, demonstrated over 80% inhibition of SARS-CoV-2 3CLpro. These fractions, previously characterized as containing fatty acids and their derivatives, served as a reference for evaluating chemical similarity across other 3CLpro bioactive *Streptomyces* strains [48]. We separated polar and lipid-soluble parts of crude extracts from *Streptomyces* sp. DSD149^T, *Streptomyces* sp. DSD735^T, *Streptomyces* sp. DSD2893^T, and *Streptomyces* sp. DSD2604^T was partitioned into methanol and hexane fractions. In vitro screening at 10 µg/mL revealed that all hexane fractions inhibited 3CLpro by over 90%, whereas methanol fractions exhibited less than 30% inhibition (Figure S35).

We used LCMS-TQ analysis described in the Materials and Methods Section 4.7 for the hexane fractions to compare the lipid profiles of different strains. The DSD454H extracts exhibited six UV peaks at 190 nm, with retention times corresponding to those noted in the late-eluting HPLC fractions DSD454IH17–H24, previously linked to 3CLpro inhibition (Figure 8A and Figure S2). There was no significant absorbance at 254 nm across all five *Streptomyces* strains (Figure 8). Strains DSD454^T and DSD149^T displayed nearly identical chromatographic profiles, with the six lipid-associated peaks eluting at equivalent retention times and comparable relative intensities (Figure 8B). The IC₅₀ values for both strains were also similar, at 2.43 µg/mL and 2.47 µg/mL, respectively [48]. On the other hand, DSD735^T, DSD2893^T, and DSD2604^T all showed the six lipid-related peaks, but

their intensities were very different. During the lipid-rich retention window (7.0–8.5 min), DSD735^T and DSD2604^T had lower peak intensities, while DSD2893^T had levels that were in the middle (Table S9). These three strains were associated with higher IC₅₀ values of 3.15 µg/mL, 3.92 µg/mL, and 4.73 µg/mL, respectively [48].

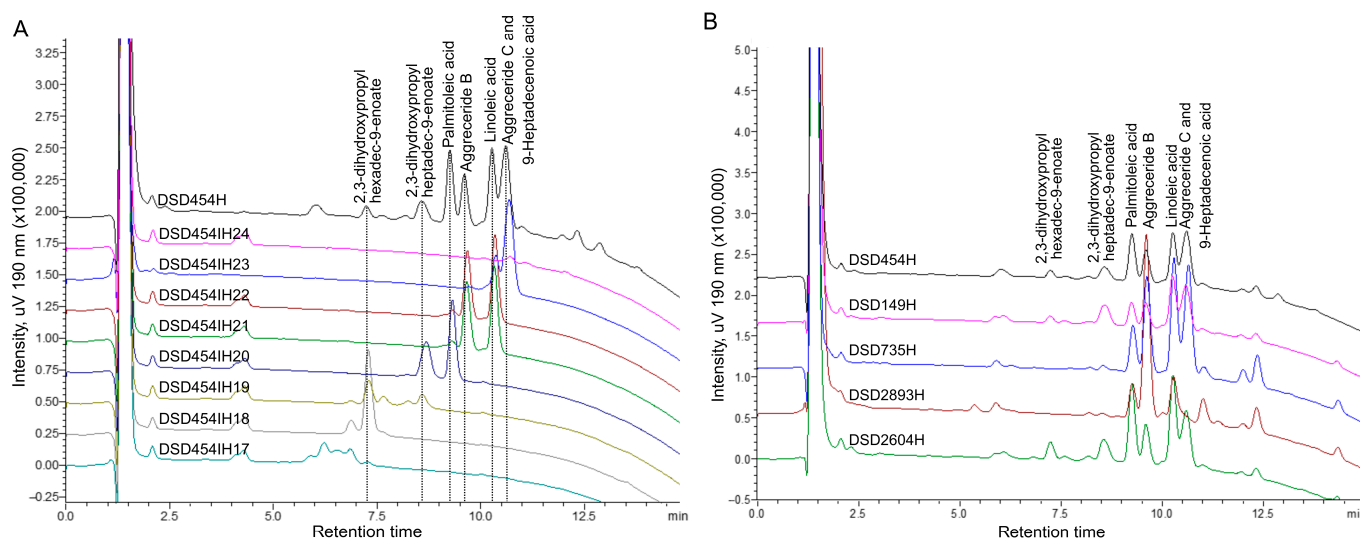


Figure 8. Comparative lipid metabolite profiling of SARS-CoV-2 3CLpro inhibitory *Streptomyces* strains. (A) LC-UV chromatogram overlay (190 nm) of the hexane fraction from *Streptomyces* sp. DSD454^T and its corresponding bioactive HPLC fractions (H17–H24). Chromatograms were acquired using the DSD454IH17–H24 method optimized for the detection of lipid compounds associated with 3CLpro inhibition. (B) LC-UV chromatogram overlays (190 nm) of hexane fractions from *Streptomyces* spp. DSD454^T, DSD149^T, DSD735^T, DSD2893^T, and DSD2604^T. All strains exhibited six conserved lipid-associated peaks, with DSD454^T and DSD149^T displaying nearly identical retention times and relative peak intensities.

We used both Pearson’s correlation coefficient (r) and Spearman’s rank correlation coefficient (ρ) to look at the relationship between compound potency and chromatographic features. We did this by comparing IC₅₀ values and peak areas (Peaks 1–6). Table S10 shows a summary of the correlation coefficients and their p -values. Palmitoleic acid **1** had the strongest positive linear correlation with IC₅₀ ($r = 0.71$, $p = 0.18$) and a moderate rank correlation ($\rho = 0.31$) among all the peaks. 2,3-dihydroxypropyl hexadec-9-enoate **9** exhibited a moderate linear correlation ($r = 0.42$, $p = 0.48$) and an insignificant rank correlation ($\rho = 0.05$). Aggregeride B **3** showed a moderate linear correlation ($r = 0.35$, $p = 0.56$) and the strongest rank correlation ($\rho = 0.60$). Linoleic acid **2** produced weak linear ($r = 0.27$, $p = 0.65$) and monotonic ($\rho = 0.20$) correlations. 2,3-dihydroxypropyl heptadec-9-enoate **8**, 9-heptadecenoic acid **4**, and aggregeride C **5** exhibited minimal to weak correlations in both analyses, with 2,3-dihydroxypropyl heptadec-9-enoate **8** showing $r = 0.02$ ($p = 0.97$), $\rho = 0.10$, and 9-heptadecenoic acid **4** and aggregeride C **5** showing $r = -0.21$ ($p = 0.74$), $\rho = 0.10$. No correlation attained statistical significance at the standard threshold of $p < 0.05$.

2.9. Molecular Docking Analysis of Palmitoleic Acid **1** and Linoleic Acid **2** with SARS-CoV-2 3CLpro

To examine the potential antiviral interactions of palmitoleic acid **1** and linoleic acid **2** with SARS-CoV-2 3CLpro, molecular docking studies were conducted, focusing on three functionally distinct sites on the protease: the catalytic site [6,11,15], the dimerization interface [24,27,29], and the cryptic site [25,28,30,49]. These sites were chosen because they are known to play important roles in enzymatic function and allosteric regulation. The active dyad and the substrate binding sites are necessary for the proteolytic processing of

viral polyproteins [24]. The dimerization site facilitates the active protease dimer formation, and the cryptic site is proposed to be a regulatory pocket that can change the shape and activity of the enzyme [28]. Alotaketol analogue 19, a sesterterpenoid with an alkyl chain from a marine sponge with bioactivity against SARS-CoV-2 Omicron [50], was used as a positive control. After docking simulations, we used the GB8 scoring function to calculate the free energy of structure-truncated MM/PB(GB)SA [51]. This function considers solvation effects and entropy, which makes it more accurate for estimating binding energy in dynamic or hydrophobic binding environments.

Alotaketol analogue 19 showed favorable and strong binding affinity at the catalytic site, as shown by its docking score of -6.5 kcal/mol and interactions with the residues Cys145, His41, Met49, and Glu166 with the GB8 rescoring binding free energy of -34.33 . Meanwhile, GC376, a peptide-based known inhibitor of 3CLpro showed the highest binding affinity (-7.5 kcal/mol) at the catalytic site by having a strong hydrogen bonding interactions towards the interacting residues at the 3CLpro's active site such as His41, Ser144, Cys145, His163, and His164 (Figure 9). It also exhibited hydrophobic interactions towards Met49, Gly143, Glu166, and Gln189 (Tables S11–S14). Compound 2 had a docking score of -4.7 kcal/mol and a GB8 value of -29.30 . It interacted with fewer important residues at the active site and did not interact with His41 or Ser144. Nonetheless, the Cys145 interaction through hydrogen bonding in compound 2 may be crucial and specific (Figure 9A) while compound 1 had a docking score of -4.5 kcal/mol, which interacted with active site residues, such as His41, Cys145, Met49, Ser144, His163, Met165, Glu166, and Gln189 and its GB8 value was -22.47 (Tables S14 and S15).

Alotaketol analogue 19 also had strong affinity at the dimerization interface, with a docking score of -7.9 kcal/mol and interactions between with Met6 (GB8: -30.83). Meanwhile, a peptide inhibitor GC376 showed similar binding of -7.9 kcal/mol and GB8 of -27.37 and have hydrophobic interaction to important residues of dimeric site such as Met6, Ser139, and Glu290. There were very few interactions between compounds 1 and 2 and the dimeric site. Compound 1 had a docking score of -6.5 kcal/mol and is bound to dimeric site interacting with Met6 and Glu290 (GB8: -29.54) through van der Waals forces (Tables S11–S14). Meanwhile, compound 2 had a docking score of -6.7 kcal/mol and GB8 score of -27.24 (Figure 9B and Table S15), interacting with Met6 through an alkyl group and with Arg298 through van der Waals forces (Tables S11–S14).

Alotaketol analogue 19 also binds to the cryptic site, with a docking score of -7.0 kcal/mol and van der Waals interactions with Tyr239 and Leu272, along with an alkyl interaction with Leu287 (GB8: -25.12). Positive control GC376 showed the highest binding affinity at the cryptic site of SARS-CoV-2 3CLpro showing -8.5 kcal/mol and GB8 value of -27.48 with hydrogen bonding to important residues to 3CLpro cryptic site such as Arg131, Leu287, and Asp289. Compound 1 has a docking score of -5.1 kcal/mol and interacts with Leu272, Leu287, Tyr239, Arg131, and Asp289. It also has a GB8 value of -29.90 (Figure 9A–C and Tables S11–S14). Meanwhile, with a docking score of -6.1 kcal/mol, compound 2 interacts with Leu272, Leu287, Asp289 and Arg131 (GB8: -29.18).

In the cryptic site, both fatty acids showed the same level of interaction across five amino acid residues (Arg131, Tyr239, Leu272, Leu287, and Asp289) (Figure 9C), as evidence in their binding energy showing no significant differences in this site (Figure 9D). The only difference is how these amino acids interact with each other. Compound 2 shows more hydrophobic (alkyl) interactions with Tyr239 compared to the van der Waals interaction of palmitoleic acid (Figure 9A) (Tables S11–S14). On the other hand, compound 1 exhibits a hydrogen bond with Arg131, signifying a generally more robust and specific interaction compared to the hydrophobic interactions (van der Waals) in compound 2.

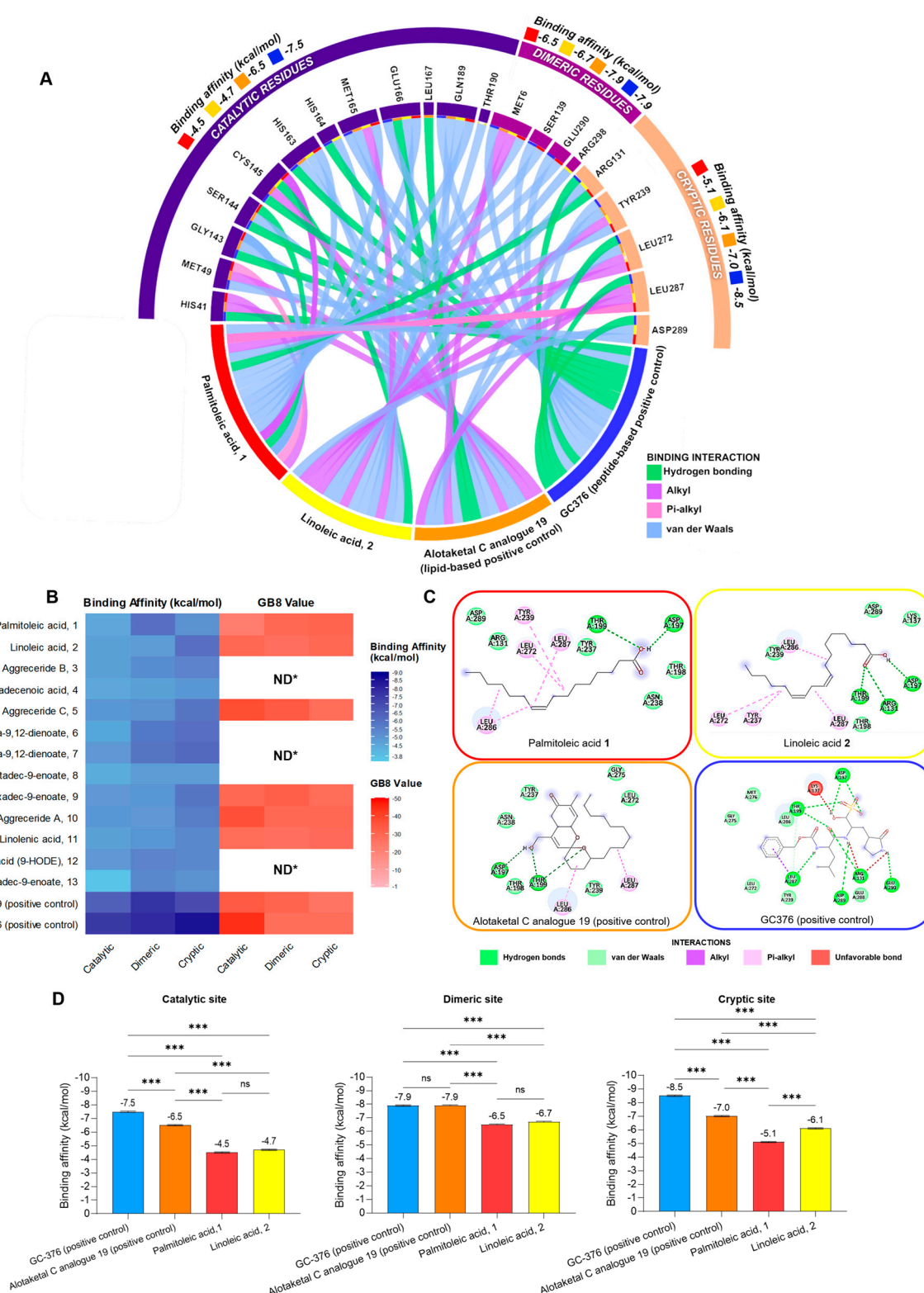


Figure 9. Molecular docking of lipid-based control Alotaketral C analogue 19, peptide-based GC376, palmitoleic acid 1, and linoleic acid 2 with SARS-CoV-2 3CLpro. (A) Circos plot illustrating the predicted molecular interactions of palmitoleic acid 1, linoleic acid 2, Alotaketral C analogue 19 (lipid-based positive control), and GC376 (peptide-based positive control), with key residues located at the catalytic, dimerization, and cryptic binding sites of SARS-CoV-2 3CLpro. Interacting residues are color-coded by interaction type (e.g., hydrogen bonds, van der Waals, π -alkyl), and domains are highlighted to distinguish site-specific binding patterns. (B) Heatmap showing the predicted binding affinities (kcal/mol) and GB8 values of the 13 lipid compounds across catalytic, dimeric, and cryptic sites. GB8 scores indicate binding site strength and selectivity. Compounds marked with ND* (Not

Determined) were excluded from GB8 analysis due to technical limitations of the fastDRH platform. (C) Molecular docking poses of palmitoleic acid **1**, linoleic acid **2**, lipid-based positive control alotaketral C analogue 19, and peptide-based positive control GC376 at the cryptic site of 3CLpro, highlighting key interactions with conserved residues such as Arg131, Tyr239, Leu272, Leu287, and Asp289. Interactions are visualized as hydrogen bonds, van der Waals forces, alkyl, and π -alkyl contacts. (D) Statistical comparisons of the binding affinity of palmitoleic acid **1**, linoleic acid **2**, lipid-based positive control alotaketral C analogue 19, and peptide-based positive control GC376. Note that (***) signifies that p value is <0.05 and considered as statistically significant. ns: no significance.

2.10. Comparative Binding Affinities of Palmitoleic Acid **1** and Linoleic Acid **2** Across the Catalytic, Dimeric, and Cryptic Sites of SARS-CoV-2 3CLpro

Statistical analysis was performed to confirm the consistency of measurements across replicates. The binding affinities of GC376 (peptide-based positive control), alotaketral C analogue 19 (lipid-based positive control), compound **1**, and **2** were compared across the catalytic, dimeric, and cryptic binding sites of 3CLpro (Figure 9D).

At the catalytic site, GC376 (-7.5 kcal/mol) showed the strongest binding, followed by alotaketral C analogue 19 (-6.5 kcal/mol). Compounds **1** and **2** displayed moderate binding energies (-4.5 and -4.7 kcal/mol, respectively). One-way ANOVA with Tukey's post hoc testing indicated significant differences between GC376 and both fatty acids ($p < 0.001$), whereas no significant difference was observed between compounds **1** and **2** ($p > 0.05$). At the dimeric site, GC376 and alotaketral C analogue 19 exhibited similar binding affinities (-7.9 kcal/mol). Compounds **1** and **2** again showed moderate binding (-6.5 and -6.7 kcal/mol), with no significant difference between them ($p > 0.999$) (Figure 9D).

GC376 remained the strongest binder (-8.5 kcal/mol) at the cryptic allosteric site, followed by alotaketral C analogue 19 (-7.0 kcal/mol). Compound **2** showed stronger binding (-6.1 kcal/mol) than compound **1** (-5.1 kcal/mol), supported by a statistically significant difference ($p < 0.001$). These results demonstrate consistent binding trends across the three binding regions, with GC376 and the reference analogue exhibiting the highest affinities and linoleic acid **2** showing comparatively stronger engagement at the cryptic allosteric site.

2.11. Predicted Multi-Site Binding of Other Fatty Acids and Monoglycerides to SARS-CoV-2 3CLpro

The analysis of molecular docking showed that the lipid compounds displayed varying binding interactions within the catalytic, dimeric, and cryptic regions of the SARS-CoV-2 3CLpro, as detailed in Tables S11–S13. In the study of 13 lipid compounds present in *Streptomyces* sp. DSD454^T extract, several lipids showed the ability to interact with residues found in multiple functional sites of the 3CLpro.

We also observed that at the catalytic site, all compound interacted with at least three key residues. Compounds **3** and **5** demonstrated the most significant interaction with 12 amino acid residues, including Cys145, His163, His164, Met165, and Glu166, which are crucial for the enzyme's activity. In a similar manner, compound **9** engaged with eleven catalytic residues. In contrast, compounds **4**, **6**, **11**, and **12** consistently established hydrogen bonds and van der Waals interactions with both polar residues, such as Glu166 and His163, as well as hydrophobic ones like Met165, indicating stable binding within the active site (Figures S36–S38).

The binding at the dimeric site, which influences dimerization and activation of proteases, was relatively weak. Most compounds only interacted with one or two residues. Despite this, compound **8** showed good interactions with Met6, Ser10, and Arg298, suggesting that they might help disrupt the stability of the dimer interface.

At the cryptic site, several compounds interacted with residues like Arg131, Tyr239, Leu272, Leu287, Glu288, and Asp289, which have been linked to allosteric modulation.

Compounds **5** and **6** displayed the most interactions at the cryptic sites. Each compound interacts with up to eight amino acid residues, including hydrogen bonding with Glu288 and hydrophobic interactions or alkyl interactions with Tyr239, Leu271, and Leu272. These interactions could either make inactive forms of the protease more stable or make it less flexible in terms of its shape.

In addition, a closer look at the GB8 scores showed that compounds **5** and **10** had strong predicted binding at the catalytic, dimeric, and cryptic sites. Their scores were similar to those of the positive control, alotaketral C analogue **19**, suggesting a good binding profile. On the other hand, compounds **9** and **11** had moderate GB8 values and were less likely to bind to the dimeric and cryptic pockets. The positive controls, lipid-based positive control alotaketral C analogue **19** and peptide-based positive control GC376, consistently exhibited elevated GB8 values at all three target sites, thereby reinforcing the dependability of the scoring methodology. It is important to note that only 6 out of the 13 compounds were successfully evaluated for GB8 values due to technical issues encountered with the fastDRH platform [51]. At the time this study was conducted, the fastDRH server was inaccessible online, which prevented further analysis. Consequently, the remaining compounds were marked as Not Determined (ND) (Figure 9B).

2.12. Predicted Physicochemical, Pharmacokinetic, and Toxicological Properties of Fatty Acids and Monoacylglycerides

All thirteen lipid-derived compounds complied with Lipinski's Rule of Five, indicating favorable oral drug-likeness (Table 2). The topological polar surface area (TPSA) values calculated were 37.30 Å² for free fatty acids, 57.53 Å² for hydroxylinoleic acid, and 66.76 Å² for monoacylglycerides and similar glyceride analogues.

The predicted Caco-2 permeability values were between −5.156 and −5.044 log units, which means that most compounds had very good permeability. Compounds **3** and **5** were different because they had lower permeability (−5.156 to −5.155 log units) (Table 2). The predicted oral bioavailability (F20) was highest for fatty acids **1**, **2**, and **11** (0.148–0.222), and with moderate bioavailability scores for F30 and F50. The other compounds, on the other hand, exhibited moderate to poor F20 bioavailability (0.440–0.897), F30 (0.604–0.971), and F50 (0.628–0.967) bioavailability. Plasma protein binding (PPB) was high for all compounds (96.8–99.1%), which means that they bind well to serum proteins.

Predictions of cytochrome P450 (CYP) interactions showed that compounds **2** and **4** were most likely to inhibit CYP2C9. Most free fatty acids had a substrate probability close to 1.000, but compounds **3**, **5**, **8**, **10**, and **13** had a very high predicted substrate affinity. Several monoacylglycerides (compounds **3**, **5**, **6**, **7**, **8**, **9**, **10** and **13**; 0.814–1.000) were likely to inhibit CYP3A4, while all of the compounds were likely to be good CYP3A4 substrates. The likelihood of inhibiting CYP2C8 was also consistently high (0.737–1.000) for all compounds. It was predicted that fatty acid amide hydrolase (FAAH) metabolism would be more effective for compounds **1** and **4** (0.480 and 0.662) (Table 2).

The predicted half-lives were usually short (0.354–0.950), and the clearance rates were between excellent and moderate. For free fatty acids, the rates were between 3.371 and 4.648 mL/min/kg, and for glyceride derivatives, they were up to 5.535 mL/min/kg (Table 2).

Toxicological predictions suggested low to moderate risk profiles. For most compounds, the Ames mutagenicity probabilities were less than or equal to 0.20. For linolenic acid **11** (0.551) and some monoacylglycerides (0.401–0.424), they were higher. Compound **4** (0.678) and some monoacylglycerides (0.554–0.584) had the highest chances of being toxic to humans' livers (Table 2). The chances of hERG inhibition were low (≤ 0.201). The predicted rat oral acute toxicity (LD₅₀) values for all 13 compounds were low, which means that these compounds are not very toxic.

Table 2. Predicted Physicochemical, Pharmacokinetic, and Toxicological Properties of Fatty Acids and Monoacylglycerols from *Streptomyces* sp. DSD454^T.

Com- pound No.	Compound Name	Physico- chemical Property	Medicinal Chemistry	Absorption			Distribution		Metabolism				Excretion				Toxicity			
				TPSA ^a	Lipinski Rule	Caco-2 Perme- ability	Bioavailability Score ^b			PPB ^c	CYP2C9 ^d		CYP3A4 ^d		CYP2C8 ^d	FAAH ^{e,*}	Half- Life (T _{1/2})	Clearance (CL _{plasma})	AMES ^f	Human Hepa- toxicity
		F _{20%}	F _{30%}				F _{50%}	Inhibitor	Substrate		Inhibitor	Substrate	Inhibitor							
1	Palmitoleic acid	37.30	Accepted	−5.085	0.183	0.636	0.381	98.30%	0.693	1.000	0.000	0.000	1.000	0.480	0.764	3.371	0.095	0.232	0.132	0.083
2	Linoleic acid	37.30	Accepted	−5.051	0.148	0.388	0.608	97.50%	0.713	1.000	0.001	0.000	1.000	0.256	0.502	3.964	0.107	0.138	0.159	0.033
3	Aggreceride B	66.76	Accepted	−5.156	0.523	0.828	0.920	97.30%	0.018	0.178	0.814	0.202	0.786	0.102	0.556	5.410	0.394	0.359	0.106	0.019
4	9- Heptadecenoic acid	37.30	Accepted	−5.075	0.535	0.927	0.693	99.10%	0.855	0.999	0.003	0.000	1.000	0.662	0.869	3.380	0.017	0.678	0.071	0.043
5	Aggreceride C	66.76	Accepted	−5.155	0.598	0.882	0.935	97.50%	0.018	0.190	0.814	0.192	0.803	0.105	0.590	5.344	0.359	0.362	0.122	0.018
6	2,3- dihydroxypropyl octadeca-9,12- dienoate	66.76	Accepted	−5.073	0.613	0.604	0.959	97.50%	0.020	1.000	0.999	0.000	1.000	0.248	0.449	5.535	0.424	0.086	0.185	0.005
7	2,3- dihydroxypropyl nonadeca-9,12- dienoate	66.76	Accepted	−5.044	0.809	0.874	0.967	97.60%	0.031	0.999	0.998	0.000	1.000	0.231	0.458	5.403	0.401	0.104	0.201	0.005
8	2,3- dihydroxypropyl heptadec-9- enoate	66.76	Accepted	−5.093	0.897	0.971	0.955	98.40%	0.110	0.030	1.000	0.000	1.000	0.105	0.660	4.843	0.095	0.554	0.084	0.007
9	2,3- dihydroxypropyl hexadec-9- enoate	66.76	Accepted	−5.061	0.704	0.812	0.878	97.80%	0.020	0.925	0.998	0.000	1.000	0.160	0.605	4.917	0.393	0.151	0.155	0.014
10	Aggreceride A	66.76	Accepted	−5.123	0.837	0.904	0.909	96.80%	0.025	0.136	0.777	0.067	0.737	0.112	0.599	5.134	0.365	0.319	0.086	0.017
11	Linolenic acid	37.30	Accepted	−5.054	0.222	0.497	0.441	98.20%	0.025	1.000	0.002	0.000	1.000	0.174	0.354	4.648	0.551	0.016	0.177	0.036
12	Hydroxylinoleic acid (9-HODE)	57.53	Accepted	−5.125	0.440	0.963	0.628	97.10%	0.322	1.000	0.000	0.000	0.999	0.098	0.950	3.905	0.202	0.567	0.094	0.104
13	2,3- dihydroxypropyl pentadec-9- enoate	66.76	Accepted	−5.075	0.585	0.781	0.952	98.10%	0.371	0.045	0.999	0.000	1.000	0.127	0.673	4.884	0.136	0.584	0.070	0.007

Legend (as labelled by Admetlab 3.0):

	Excellent
	Medium
	Poor

^a TPSA = Topological Polar Surface Area; ^b Bioavailability score F20%/F30%/F50% = Oral bioavailability at 20%, 30%, or 50%, respectively; ^c PPB = Plasma Protein Binding; ^d CYP2C9, CYP3A4, CYP2C8 = Major cytochrome P450 enzymes involved in hepatic metabolism; ^e FAAH = Fatty Acid Amide Hydrolase; ^f AMES = Ames test for mutagenicity; ^g hERG = Human Ether-à-go-go-Related Gene. * FAAH probability score predicted by SwissTarget Prediction.

3. Discussion

3.1. Morphological and Phylogenetic Characterization of *Streptomyces* sp. DSD454^T

The marine-derived actinobacterial isolate *Streptomyces* sp. DSD454^T, which shows complete 16S rRNA sequence similarity with *S. ennisocaesilis* [48], displayed unique morphological traits characteristic of the *Streptomyces* genus. When *Streptomyces* sp. DSD454^T is cultivated on MM1 agar, it produces a colony with concentric zones and a rough, wrinkled surface. This observation highlights the distinct life cycles of *Streptomyces* and suggests a complex process of mycelial development and morphogenesis [52]. The formation of a diffusible brown pigment and the existence of gray aerial mycelia and yellowish-brown substrate mycelia are consistent with pigmentation patterns associated with *Streptomyces* species' production of secondary metabolites [53–55].

The use of scanning electron microscopy provided additional confirmation of the complex structure of *Streptomyces* sp. DSD454^T, revealing a complex network of elongated, cylindrical hyphae. The observations suggest a complex sporulation system, likely influenced by known developmental gene clusters such as *whi* and *bld* [56–58]. This cellular differentiation is required for their survival in environments where nutrients can vary, which is often associated with the initiation of secondary metabolite production [57].

To determine the position of *Streptomyces* sp. DSD454^T within the phylogenetic tree, we constructed a maximum-likelihood tree using concatenated sequences from the housekeeping genes *atpD*, *recA*, and *trpB*. This approach is often employed to differentiate between closely related species in the genus *Streptomyces*, as single-gene phylogenies frequently lack the resolution required for precise identification. The analysis revealed that *Streptomyces* sp. DSD454^T and *S. ennisocaesilis* DSD012^T clustered closely together in a robust clade, which is supported by a bootstrap value of 90%. This positioning indicates a close evolutionary relationship between the two strains. *Streptomyces* sp. DSD454^T, in contrast, did not group with other closely related species, including *S. rochei* and *S. vinaceusdrappus*. This indicates that it occupies a distinctive position within the genus.

The strong bootstrap value reinforces the distinction between DSD454^T and *S. ennisocaesilis* DSD012^T, providing us with assurance that this separation is reliable. While these two strains share many similarities, the phylogenetic differences indicate that DSD454^T may represent a distinct taxon. To determine whether DSD454^T is a new species, further studies are needed, including whole-genome comparisons (such as ANI and dDDH) and an examination of its characteristics. The current MLSA data clearly show that *Streptomyces* sp. DSD454^T holds a unique place compared to known type strains, highlighting the need for further taxonomic evaluation.

3.2. Physiological and Biochemical Traits Suggest Ecological Adaptation

The differences in pigmentation observed in *Streptomyces* sp. DSD454^T across various culture media indicates that the nutrient composition and environmental stimuli closely influence the process of pigment formation [59–61]. The strain's physiological tolerance, as demonstrated in this study, highlights its ecological flexibility and its ability to flourish in harsh environments such as marine sediments. These observed physiological tolerances include salinity levels as high as 7% NaCl, pH levels ranging from 4 to 10, and temperatures between 28 and 45 °C, with an optimal temperature of 37 °C. These characteristics, influenced by its habitat in marine sediment, highlight how well-suited it is as a flexible microbial system for producing compounds under a range of fermentation conditions. The enzymatic profile also highlights the unique adaptations to specific ecological niches. The positive activity of alkaline phosphatase, leucine arylamidase, and *N*-acetyl-β-glucosaminidase suggest their significant involvement in the recycling of nutrients, particularly through processes like phosphorus turnover, proteolysis, and the breakdown

of chitin [62–64]. Moreover, activities of valine arylamidase, α -mannosidase, and esterase (C4), alongside the absence of α -galactosidase, β -glucuronidase, and α -fucosidase activities, indicate a metabolism that has been specially adapted to thrive in nutrient-poor marine sediments. This array of enzymes reflects the strategies used by marine sediment-derived actinobacteria, which depend on the gradual breakdown of organic matter, hydrocarbons, and chitin-based materials to obtain nutrients continuously [62–64].

3.3. Extraction and Fractionation Reveal Abundant Lipid Compounds

The crude extract from *Streptomyces* sp. DSD454^T exhibited fractions that significantly inhibited SARS-CoV-2 3CLpro after being fractionated by HPLC. The majority of these fractions occurred in the late-eluting regions of the chromatographic profile. The fractions that eluted at high concentrations of organic solvent, almost 100% acetonitrile, showed the highest levels of inhibitory activity, indicating the presence of hydrophobic compounds with limited solubility in water. This elution pattern reveals a major difference compared to traditional polar or peptide-based viral protease inhibitors, such as the positive control GC376 [15]. Hence, the chromatographic behavior observed indicates a unique category of non-polar, lipid-like secondary metabolites with inhibitory effects against 3CLpro. The increase in activity observed in this specific hydrophobic region suggests a related set of compounds, which may include long-chain fatty acid amides, glycerol-like lipids, or alkylated small molecules. Many of these compounds are recognized for their interactions with membrane-associated or hydrophobic targets, including viral proteases [65–68].

The assay protocol was modified to accurately assess the inhibitory activity of the hydrophobic and poorly soluble fractions, as outlined in Section 4.9. By employing this adjusted approach, we successfully reduced variability caused by solubility differences and attained accurate measurements of 3CLpro inhibition. Linking specific inhibitory profiles to their corresponding retention times allowed for a more reliable comparison of the bioactivity of the chromatographic fractions, thereby facilitating the dereplication process. The data obtained from this testing provide a valuable basis for annotating putative compounds present in the DSD454^T extract and prioritizing candidates for further compound characterization.

3.4. Metabolomic Profiling Reveals Diverse Lipids and Fatty Acid Derivatives

Dereplication of 3CLpro bioactive HPLC fractions (H15–H24) from *Streptomyces* sp. DSD454^T has identified a cluster of compounds with characteristics consistent with those of lipid-derived secondary metabolites. The two major groups included unsaturated fatty acids and monoacylglycerols. These compound classes are often linked to microbial lipid metabolism and ecological interactions between microorganisms [47,69–71]. Actinomycetes frequently biosynthesize these compound classes via Type II fatty acid synthase (FAS II) systems, which utilize various enzymes to accelerate specific steps in the elongation and modification of fatty acids. This provides the system with more flexibility and regulatory control than the eukaryotic Type I system, and it often yields complex, lipophilic structures with a wide range of bioactivities [69,72–75]. These compounds have been increasingly acknowledged in bacteria not only for their antimicrobial and cytotoxic attributes but also for their ecological functions in chemical defense and interspecies signaling [69,76–78]. The possible discovery of lipid-tail-modified compounds in these fractions highlights an unexplored chemical domain with potential for the development of antiviral drugs [79,80].

Important unsaturated fatty acids found include palmitoleic acid **1**, linoleic acid **2**, linolenic acid **11**, and 9-heptadecenoic acid **4**. This suggests that *Streptomyces* sp. DSD454^T may have specialized its metabolism to better handle external stress and a lack of nutrients during experimental fermentation. Across fractions H17–H24, several monoacylglycerols

were found next to their free fatty acids. There were 2,3-dihydroxypropyl hexadec-9-enoate **9**, 2,3-dihydroxypropyl pentadec-9-enoate **13**, and 2,3-dihydroxypropyl octadeca-9,12-dienoate **6**, as well as aggrecerides A **10**, B **3**, and C **5**, which are structurally similar [81,82]. These compounds likely originate from the acylation of glycerol by bifunctional acyltransferases attached to membranes [83]. Reports documented that these esterification reactions are often observed in *Streptomyces* during morphological differentiation or the stationary phase [52,84,85]. Monoacylglycerols and their free acid precursors are consistently identified, suggesting a controlled lipid biosynthesis network that could promote membrane fluidity in stressful situations [69,70,84,86]. In addition to standard fatty acids and monoacylglycerols, structurally altered lipids, such as oxidized derivatives like 9-HODE **12**, were also identified. These compounds likely originate from enzymatic tailoring reactions, including oxidation, hydroxylation, and *N*-acylation. These are biosynthetic strategies that are often encoded in gene clusters or operons that modify lipids [46,87,88].

3.5. BODIPYTM 493/503 Staining Reveals Lipid Accumulation Within Mycelia of *Streptomyces* sp. DSD454^T

We visualized the buildup of intracellular lipids in *Streptomyces* sp. DSD454^T using BODIPYTM 493/503 staining. The green fluorescence signals were observed throughout the mycelial network, with varying intensity spots indicating the existence of lipid-rich areas scattered within the hyphae. These distinct spatial distributions resemble lipid droplets or comparable storage structures in filamentous bacteria, where lipid reserves are utilized for growth, development, and metabolic adjustments [47,70,71,85,89].

The lipid accumulation in *Streptomyces* sp. DSD454^T, as shown by BODIPYTM 493/503 staining, corroborates our chemical and quantitative analyses. Approximately 63% of the total crude extract comprises oily and lipid-based components, including fatty acids and monoacylglycerols, as confirmed by HPLC, LCMS, and NMR profiling. The exact biosynthetic pathways for these compounds are still not fully understood; however, the lipid enrichment observed suggests that intracellular lipid reservoirs in *Streptomyces* may have roles that extend beyond mere passive storage. Rather, they could signify metabolically active centers that aid in the synthesis of specific lipids related to secondary metabolism and chemical defense. This corresponds with new perspectives that connect the arrangement within cells and the division of space with metabolic flow and the production of natural products in filamentous actinomycetes.

3.6. Comparative Lipidomics Across Bioactive *Streptomyces* Strains

In this work, we also investigated the chemical profiles of five bioactive *Streptomyces* strains with inhibitory activity against SARS-CoV-2 3CLpro to determine whether lipophilic compounds identified in *Streptomyces* sp. DSD454^T are consistently produced and whether specific chromatographic features correlate with compound potency.

Lipophilic compounds were detected in the hexane fractions of all strains, and six shared peaks were observed across chromatograms recorded at 190 nm. These peaks matched the retention times of active lipophilic compounds from *Streptomyces* sp. DSD454^T. The similarity in chromatographic profiles between DSD454^T and DSD149^T, especially in peak pattern and relative intensity, corresponded with their nearly identical IC₅₀ values (2.43 µg/mL and 2.47 µg/mL, respectively). This observation suggests a link between lipid content and antiviral activity. Although *Streptomyces* spp. DSD735^T, DSD2893^T, and DSD2604^T also displayed the same six lipid peaks, their lower intensities in the key retention window (7.0–8.5 min) coincided with higher IC₅₀ values. Hence, this indicates a possible relationship between the abundance and potency of lipids in the extract.

A correlation analysis was performed between chromatographic peak areas and IC_{50} values to further investigate these observations. Although none of the correlations were statistically significant ($p < 0.05$), likely due to the small sample size ($n = 5$), some trends were observed. Palmitoleic acid **1** showed the strongest positive linear correlation with IC_{50} and a moderate monotonic association, which means it may be linked to lower bioactivity. Aggregeride B **3** exhibited a moderate linear correlation and the most robust rank-based correlation, suggesting a possible nonlinear or threshold-based relationship. 2,3-dihydroxypropyl hexadec-9-enoate **9** exhibited moderate Pearson correlations and weak Spearman coefficients, indicating inconsistencies in its association with bioactivity. In both analyses, 2,3-dihydroxypropyl heptadec-9-enoate **8**, linoleic acid **2**, 9-heptadecenoic acid **4**, and aggregeride C **5** were weak or not at all correlated.

Although the results lack statistical significance, the observed patterns, particularly for palmitoleic acid **1** and aggregeride B **3**, suggest that specific lipophilic components may influence 3CLpro inhibitory activity. The presence of lipid peaks in all strains suggests that they represent a shared chemical characteristic among 3CLpro-active *Streptomyces* spp. However, the variance in intensity indicates that the efficacy of 3CLpro inhibition may be influenced by variations in lipid content or structural analogs. These results establish a foundation for subsequent study utilizing targeted isolation and extensive strain libraries to investigate the influence of lipophilic compounds on antiviral activity.

3.7. Confirmation of Palmitoleic Acid **1** and Linoleic Acid **2** and Their Inhibitory Activity Against 3CLpro

The metabolomic profiling of *Streptomyces* sp. DSD454^T exhibited many lipid-like compounds, as identified using high-resolution mass spectrometry (HRMS) and triple quadrupole mass spectrometry (TQMS). Among the various mass spectrometry signals associated with fatty acids and other hydrophobic compounds, only compounds **1** and **2** were unambiguously identified. Their structures were further validated using tandem MS/MS, NMR, and direct comparison with authentic reference standards.

The dereplication technique facilitated the first annotation of more lipid-like compounds; nevertheless, these identifications were solely dependent on mass-based spectrum matching and lacked independent validation. Consequently, the confidence in these proposed annotations was insufficient to justify their incorporation into subsequent in vitro biological experiments. Furthermore, our attempts to extract these compounds were unsuccessful, primarily due to their high lipophilicity and limited chromatographic resolution, as evidenced by the co-elution of lipid compounds. These technical restrictions made it hard to validate their structure and test their biological activity.

Consequently, only compounds **1** and **2**, whose identities were clearly verified, proceeded to in vitro biological testing. In these assays, we employed authentic standards, since the extraction of the native compounds from the sample was unreliable under our laboratory conditions, as previously outlined. Of the two compounds tested, compound **1** showed greater activity with an IC_{50} of 1.59 $\mu\text{g/mL}$ (6.25 μM), while compound **2** had an IC_{50} of 5.29 $\mu\text{g/mL}$ (18.88 μM). Although these findings are promising, additional validation in appropriate in vivo models is crucial to verify the antiviral effectiveness and pharmacodynamic properties of palmitoleic and linoleic acids.

The enhanced inhibitory activity of compound **1** against 3CLpro was somewhat surprising, considering that compound **2** (C18:2, ω -6), a well-known polyunsaturated fatty acid, has been thoroughly investigated for its anti-inflammatory and broad-spectrum antiviral effects [90–93]. Research has increasingly demonstrated that compound **2** interacts with a specific hydrophobic pocket in the spike (S) protein of SARS-CoV-2. This interaction stabilizes the protein in a “locked,” non-infectious state, thereby inhibiting its ability to engage with the ACE2 receptor [90,91,94]. Interestingly, molecular docking studies of long-

chain fatty acids revealed that those with extended carbon chains (C20–C24) in contrast to shorter-chain analogs like compound **2** (C18:2), show a consistently higher binding affinity for the fatty acid pocket in the “locked” conformation of the SARS-CoV-2 spike protein [95]. Reports also documented that compound **2** exhibits a strong interaction with SARS-CoV-2 RNA-dependent RNA polymerase (RdRp) through hydrophobic interactions with various residues. This interaction disrupts viral RNA synthesis and replication in both in vitro and in vivo models [96,97].

While earlier research has mainly concentrated on the interactions of fatty acids with the SARS-CoV-2 spike protein [90,91] and the RdRp protein [96], there are no existing reports detailing the direct inhibition of the 3CLpro through allosteric binding by fatty acids. Although compound **1** has been less explored compared to compound **2**, their structural resemblance indicates a possible common mechanism of action, likely involving allosteric modulation. The initial findings offer insights into the structure-activity relationships (SARs) of microbial-derived fatty acids, specifically regarding the impact of mono-versus polyunsaturation on binding affinity at the cryptic and dimeric regions of 3CLpro.

To better interpret the biochemical activity of the two fatty acids in the context of their predicted binding preferences, we integrated the experimental inhibition data with the computational docking results. Our findings enabled us to evaluate the experimentally observed inhibition of compounds **1** and **2** in relation to their predicted binding characteristics with 3CLpro. This comparison was carried out by examining the relationship between their biochemical inhibition and the binding affinities obtained through docking, supported by statistical cross-validation. Compound **1** exhibited markedly stronger biochemical inhibition, showing approximately three-fold greater potency than compound **2** ($IC_{50} = 6.25 \mu M$ vs. $18.88 \mu M$). In contrast, docking analysis showed that compound **2** consistently displayed higher predicted binding affinity across the catalytic, dimeric, and cryptic sites of 3CLpro. This difference is expected, as docking evaluates static ligand–protein interactions within defined pockets, whereas IC_{50} values reflect inhibition under assay conditions influenced by solvation, molecular flexibility, and hydrophobic interactions. Statistical cross-validation further confirmed that the experimental and computational datasets were internally consistent, showing low variance across docking replicates and stable IC_{50} estimates across assay replicates. Taken together, these findings show that compound **1** demonstrates greater functional inhibition in vitro, whereas compound **2** exhibits stronger predicted affinity for non-catalytic pockets, highlighting the complementary nature of their interaction with 3CLpro.

3.8. Predicted Multi-Site Interactions of Natural Lipids from Marine *Streptomyces* with SARS-CoV-2 3CLpro

Molecular docking studies were conducted to examine the interaction between compounds **1** and **2** with SARS-CoV-2 3CLpro and to determine their binding properties, gaining insight into how molecular differences may influence their biological activity. While much of current inhibitor design focuses on the catalytic site [6,11,15], growing evidence suggests that dimerization and cryptic pockets could serve as effective and potentially superior targets [24,25,27–29]. The dimerization site is essential for the assembly of the catalytically active 3CLpro dimer. This is governed by key amino acid residues such as Arg4, Met6, Ser10, Gly11, Glu14, Asn28, Ser139, Phe140, Ser147, Glu166, Glu290, and Arg298 [27,29]. Perturbation of the dimeric junction significantly compromises 3CLpro activity, as indicated by previous studies [27,29]. Meanwhile, the cryptic site, often hidden in the apo state, can be uncovered or activated upon ligand binding, offering a way to disrupt protease structure or limit substrate access [28,98]. Important residues at these sites include Lys5, Met6, Pro108, Gly109, Arg131, Trp218, Phe219, Tyr239, Glu240, Leu271,

Leu272, Leu287, Glu88, Asp289, Glu290, Arg298, Gln299, and Val303 [25]. Molecular docking analysis shows that both **1** and **2** interact with key residues.

The results of molecular docking experiments, which include catalytic, dimeric, and cryptic sites, along with binding affinity, IC_{50} values, and GB8 energy scores, indicate a multi-site inhibition mechanism for compounds **1** and **2**. Compound **2** binds to the catalytic site by forming a hydrogen bond with Cys145, a residue crucial for the protease's nucleophilic cleavage process. This specific interaction may disrupt the enzyme's ability to catalyze reactions effectively. In contrast, compound **1** binds to the catalytic site by interacting with a wider range of residues, including His41, Ser144, and Glu166, mainly through van der Waals, hydrogen bonding, and alkyl interactions. Notably, compound **2** does not directly interact with Ser144 at the 3CLpro catalytic site. This absence of interaction suggests that compound **2** may be less effective at interfering with catalysis, which aligns with its higher IC_{50} value, as directly observed.

Both compounds exhibited a comparatively weak dimeric interface interaction, which is necessary for the enzymatic dimer of 3CLpro to function. Compound **1** interacted with Met6 and Glu290 through van der Waals forces, whereas compound **2** interacted with both Met6 (through alkyl forces) and Arg298 (through van der Waals forces). A lower GB8 score at the dimeric area for **1** (-29.54 compared to -27.24 for **2**) indicates that these connections are weak, although they might make the dimer less stable.

At the cryptic site, both fatty acids interact with hydrophobic residues, such as Leu272 and Leu287. Interestingly, compound **1** interacts with Arg131 through a hydrogen bond, which may contribute to a stabilizing effect that promotes an inactive state of the enzyme. Meanwhile, compound **2** interacts with Tyr239 and Leu287 through alkyl contacts, as well as with other hydrophobic residues Arg131 and Asp289 through van der Waals interactions. This indicates a chemically distinct but functionally similar approach to allosteric modulation. Both compounds exhibit comparable GB8 values at this site (-29.18 for compound **2** compared to -29.90 for compound **1**), indicating that the cryptic site plays a crucial role in the overall binding energy and could serve as a secondary inhibitory hotspot.

We further gain insights into how compounds **1** and **2** interact with SARS-CoV-2 3CLpro by utilizing the fastDRH webserver. This platform integrates conventional molecular docking with MM/PB(GB)SA-based rescoring and per-residue energy analysis [51]. Instead of relying solely on docking scores, we focused on GB8 rescoring values to more accurately reflect actual binding affinities. GB8 stands out from traditional docking functions by integrating solvation effects, entropy, and the flexibility of both ligands and receptors. This makes it particularly effective for assessing interactions at non-classical or dynamic binding sites, including dimerization and cryptic pockets [51].

Fatty acids exhibit structural flexibility and hydrophobic characteristics, and their interactions with proteins often engage adaptable or allosteric surfaces. Consequently, the GB8 rescoring proved to be particularly useful in revealing the greater inhibitory activity of compound **1** ($IC_{50} = 6.25 \mu M$) compared to compound **2** ($IC_{50} = 18.88 \mu M$), despite compound **1** showing somewhat lower or similar binding affinities across sites (ranging from -4.5 to -6.5 kcal/mol) compared to compound **2** (-4.7 to -6.7 kcal/mol). The observed difference becomes clear when examining the GB8 scores, which provide a broader assessment of binding stability by considering solvation and conformational changes. Notably, compound **1** had higher GB8 scores at the cryptic site (-29.90) and the dimeric site (-29.54). These scores show that the binding site is more stable in terms of its shape. This observation may facilitate enhanced inhibition of 3CLpro. Moreover, these results suggest that the dimerization and cryptic sites may serve as more accessible and energetically favorable targets for lipophilic inhibitors compared to the highly conserved active site.

The fact that other lipid compounds, including monoacylglycerols and aggrecerides, can bind to three different sites, suggests that they could be useful for a wide range of therapeutic uses. Because of their amphipathic property, they can interact with both the enzyme's polar and hydrophobic regions. This property may facilitate various types of inhibition, including competitive and non-competitive interactions. This twofold approach enhances their effectiveness against viruses and may reduce the chances of mutations related to resistance developing at a single binding site.

3.9. The Safety and Pharmacokinetic Effects of Predicted ADMET Profiles

The fatty acids and monoacylglycerides derived from *Streptomyces* sp. DSD454^T lipids showed good drug-likeness because they all followed Lipinski's Rule of Five. Additionally, all had TPSA values suitable for passive absorption. For most compounds, the predicted Caco-2 permeability was very good. However, compounds **3** and **5** had slightly lower values. Some fatty acids (compounds **1**, **2**, and **11**) exhibited the highest oral bioavailability, whereas monoacylglycerides typically required higher predicted exposure thresholds.

The high plasma protein binding (96.8–99.1%) of the compounds indicates prolonged systemic retention; however, it constrains the free drug fraction, thereby increasing the likelihood of displacement interactions. Moreover, the predicted CYP inhibition profiles indicate a risk of metabolic interactions, particularly with CYP2C9 (compounds **2** and **4**) and CYP2C8 (all compounds). There is also significant CYP3A4 metabolism. FAAH metabolism of compounds **1** and **4** may yield supplementary bioactive compounds.

The short-predicted half-lives and moderate to high clearance values suggest that strategies for formulation or structure are needed to increase exposure. Toxicological predictions were generally positive, indicating low mutagenicity, hERG inhibition, and acute toxicity. However, certain compounds have shown increased possibilities for hepatotoxicity and mutagenicity, requiring further safety assessment.

3.10. Limitations of the Study and Future Directions

Despite the promising results of this study, it is essential to consider the specific limitations when interpreting them. Initially, this research did not evaluate viral inhibition within a SARS-CoV-2 context; rather, we established our IC₅₀ values through an in vitro FRET bioassay model instead of a specific viral infection assay. Secondly, although our in vitro findings indicated distinct inhibitory patterns, we did not conduct direct target validation on SARS-CoV-2 to verify interaction with 3CLpro, and the possibility of off-target effects remains unexcluded. Third, traditional docking and scoring techniques are fundamentally constrained, as they frequently overlook protein flexibility, ligand-induced conformational changes, and solvent-mediated interactions that significantly affect binding affinity. To capture these dynamic characteristics, complementary in silico methodologies, such as molecular dynamics simulations, are essential. Lastly, our study was confined to fatty acids and monoacylglycerols due to purifying challenges that hindered a thorough profile of the lipidome of *Streptomyces* sp. DSD454^T.

These results offer several avenues for future research. Initially, it is reasonable to confirm docking predictions using surface plasmon resonance or isothermal titration calorimetry. Moreover, enhancing lipid purification methods, such as chemical modification, is needed to retrieve the more difficult-to-isolate lipid elements from the extract. Further investigation is needed to functionally evaluate the additional lipid compounds in this study. Cell-based in vitro and in vivo testing of these compounds is important for validating the biological significance of docking predictions and establishing structure-activity relationships. On the other hand, ADMET profiles indicate that these natural lipids have development potential, provided that metabolic stability and the risks of CYP-mediated

interactions are considered in future optimization and validation studies. Ultimately, evaluating palmitoleic and linoleic acids in live SARS-CoV-2 assays, whether individually or in combination with antivirals like remdesivir, may yield direct insights into their therapeutic potential.

4. Materials and Methods

4.1. Morphological and Biochemical Characterization of *Streptomyces* sp. DSD454^T

The morphological features of *Streptomyces* sp. DSD454^T was observed based on growth rate, substrate, and mycelial features on enriched marine medium 1 agar (MM1) [99–102]. The mycelia and aerial spores were visualized using a scanning electron microscope (JEOL JCM-7000 NeoScope, Tokyo, Japan). The cells were centrifuged and pelleted, then fixed using 2.5% glutaraldehyde for 2 h and subsequently incubated at 4 °C. They were washed twice with PBS and sequentially dehydrated using 30%, 50%, 85%, 95%, and absolute ethanol. The dehydrated cells were air-dried before mounting on a carbon tape, and then placed in an aluminum stub. Both mycelia and spores were then sputter-coated with gold for 30 s. The morphology was observed at 5000× magnification in high-vacuum mode with standard resolution.

The physiological characterization of *Streptomyces* sp. DSD454^T was carried out by examining growth at various temperatures, pH levels, and salt concentrations. The *Streptomyces* sp. DSD454^T was investigated for its temperature tolerance by incubating at 4 °C, 28 °C, 37 °C, and 45 °C for 7 days. Tolerance to pH was determined at levels ranging from pH 4.0 to 10.0, at 1 pH unit intervals. Additionally, salt tolerance was assessed using the previously described method [99]. The utilization of carbon sources was determined using glucose (1% w/v), sucrose (1% w/v), starch (1% w/v), and trehalose (1% w/v) as well as nitrogen sources such as peptone (0.2% w/v), yeast extract (0.2% w/v), and casein (0.2% w/v), for organic nitrogen sources, and ammonium sulfate (0.2% w/v) and urea (0.2% w/v) for inorganic nitrogen sources. Furthermore, its physiological characteristics were evaluated by cultivating the cells in International *Streptomyces* Project (ISP) media (ISP2, ISP3, ISP4, and ISP9) and incubating them at 28 °C for 7 days. Additionally, the enzymatic and biochemical characteristics of *Streptomyces* sp. DSD454^T was analyzed using API ZYM kit (bioMérieux, Marcy-l'Étoile, France) using previously established method [103].

4.2. Multilocus Sequence Analysis (MLSA) and Phylogenetic Tree Construction

Multilocus sequence analysis (MLSA) was performed following established methodologies [101,103]. Three conserved housekeeping genes were selected for amplification: *atpD* (ATP synthase F1 β -subunit) using primers *atpDF* (5'-GTCGGCGACTTCACCAAGGGCAAGGTGTTCAACACC-3') and *atpDR* (5'-GTGAAGTCTTGCGACGTGGGTGTTCTGGGACAGGAA-3'); *recA* (recombinase A) using primers *recAF* (5'-CCGCRCTCGCA CAGATTGAACGSCAATTC-3') and *recAR* (5'-GCSAGGTCGGGGTTGTCCTTSAGGAAGTTGCG-3'); and *trpB* (tryptophan synthase β -subunit) using primers *trpBF* (5'-GCGCGAGGACCTGAACCACACCGGCTCACACAAGATCAACA-3') and *trpBR* (5'-TCGATGGCCGGGATGATGCCCTCGGTGCGCGACAGCAGGC-3'), following the primer sets described by Sabido et al. [101]. PCR amplifications were carried out in 20 μ L reaction volumes containing 10 μ L SYBR[®] SuperMix, 2 μ L each of forward and reverse primers (10 mM), 2 μ L nuclease-free water, and 4 μ L DNA template (20 ng/ μ L). The thermocycling program consisted of an initial denaturation at 98 °C for 3 min; 40 cycles of 98 °C for 10 s, 60 °C for 10 s, and 72 °C for 60 s; followed by a final extension at 72 °C for 5 min, conducted on a BIOER LineGene 9600 Real-Time Thermal Cycler (Hangzhou, China). PCR products were purified using the QIAquick[®] PCR Cleanup Kit (Qiagen Ltd., Hilden, Germany) according to the manufacturer's instructions. Sequencing was performed by 1st BASE (Apical

Scientific Sdn. Bhd., Seri Kembangan, Malaysia). Resulting sequences were concatenated to generate composite MLSA sequences. Multiple sequence alignments were conducted using MUSCLE in MEGA v11.0 (Pennsylvania State University, PA, USA) [104]. Phylogenetic relationships were inferred using the Kimura two-parameter model [105], with nodal support assessed through 1000 bootstrap replicates. All gene sequences generated in this study have been deposited in the GenBank database under accession numbers PV771072, PV771073, and PV771074.

4.3. Large-Scale Cultivation and Crude Extraction of Secondary Metabolites from *Streptomyces* sp. DSD454^T

The large-scale cultivation of *Streptomyces* sp. DSD454^T as a source of bioactive 3CLpro secondary metabolites was followed using the previously described method [101,103].

4.4. Solid Phase Extraction (SPE) via Flash Column Chromatography

To remove highly polar media components from the crude extract of *Streptomyces* sp. DSD454^T, a large-scale SPE was conducted using flash column chromatography (Biotage Isolera One, Uppsala, Sweden). A total of 1.5 g of crude extract was dissolved in 1 mL of methanol (MeOH) and applied to a C18 SPE sample disc. The sample disc was dried under vacuum and subsequently inserted into a 30 g C18 flash chromatography cartridge (Biotage® Sfär, 100 Å, 30 µm) for further processing. H₂O and MeOH served as the mobile phases, which were pumped at a rate of 25 mL/min. The cartridge was preconditioned with 3 column volumes (CV) of MeOH followed by 3 CVs of H₂O. Elution with H₂O was performed for 3 CVs, followed by elution with 3 CVs of MeOH. The MeOH eluate was dried in vacuo at 35 °C and was coded as DSD454I. A total of 3.08 g of DSD454I (77% yield) was obtained from 4.0 g of crude extract.

4.5. Purification of DSD454I via Preparatory High-Performance Liquid Chromatography (Prep-HPLC)

To isolate the SARS-CoV-2 3CLpro inhibitors, the compounds obtained from DSD454I were purified using reversed-phase HPLC. A Waters® preparatory HPLC system (Waters Corporation, Manchester, UK) equipped with a Waters® 2998 photodiode array (PDA), Waters® Acquity QDa, quaternary chromatographic pump (Waters® 2535 Quaternary pump), manual injector, a make-up pump (Waters® 515 make-up pump), and a splitter that divides the outlet flow from the column to the detectors and fraction collector (Waters® Fraction Collector III) at a ratio of 1:1000 was utilized. The QDa is supplied with a constant flow of N₂ gas at 100 psi. Data were acquired at 190–700 nm in the PDA and 100–1200 *m/z* in the QDa. Purification was conducted on a Waters® Xbridge Prep C18 column (5 µm, 19 × 150 mm) using H₂O/MeCN as the mobile phase, pumped as follows: 80:20 H₂O/MeCN (0 to 4.24 min), linear gradient elution to 100% MeCN (4.24 to 43.24 min), 100% MeCN (43.24 to 51.66 min), reverting to 80:20 H₂O/MeCN (51.66 to 54.66 min), and equilibration at 80:20 H₂O/MeCN (54.66 to 60.00 min). The DSD454I sample was dissolved in MeOH to create a 762 mg/mL solution. The sample was then injected at 90 µL/injection, and fraction collection was performed every min. The fractions were pooled based on the PDA signal to obtain 25 DSD454I HPLC fractions.

4.6. High-Resolution Electrospray Ionization Mass Spectrometry

The chemical profile of the HPLC fractions was investigated using high resolution mass spectrometry. MS^E data acquisition (low energy, 0 eV; high energy, ramp 25 to 75 eV) in centroid mode was performed on a Waters Synapt® XS QToF mass spectrometer, coupled with a Waters Acquity® UPLC Class I and an electrospray ionization (ESI) source (Waters Corporation, Milford, MA, USA). The mass spectrometer was calibrated in both

positive and negative polarity in resolution mode using sodium formate (NaHCOO) as calibrant. MS^E data were acquired over a mass ion range of 100 to 1500 Da, with a scan time set at 0.15 s. Separation of compounds was conducted in a C18 column (Waters Acquity[®] UPLC BEH, 2.1 × 50 mm, 1.7 μm) held at 30 °C. The mobile phase consisted of H₂O/MeCN (Solvent A/Solvent B) with 0.1% HCOOH pumped at 0.3 mL/min as follows: 20% B (0 to 0.5 min), 20% to 100% B (0.5 to 5.5 min), 100% B (5.5 to 7.5 min), 100% to 20% B (7.5 to 8.0 min), and 20% B (8.0 to 8.5 min). Compounds eluting at different retention times were subjected to a capillary voltage of 3 kV (positive) or 1.5 kV (negative), with a 100 °C source temperature, a 500 L/h desolvation gas (N₂) flow, and a 250 °C desolvation temperature. A lockspray mass correction was performed using leucine-enkephalin (m/z 556.2771 [M+H]⁺, m/z 554.2615 [M-H][−]). Acquired MS^E data were processed using MassLynx[®] software version 4.2 (Waters Corporation, Milford, MA, USA). Dereplication using accurate mass measurements and MS/MS spectral matches from databases such as Global Natural Products Social Molecular Networking (GNPS) [106], PubChem [107], StreptomeDB [108], and CFM-ID [109] was performed.

4.7. LCMS-TQ Chemical Profiling of HPLC Fractions

HPLC fractions that showed >80% SARS-CoV-2 3CLPro inhibition were investigated using LCMS-TQ. Samples were dissolved in MeOH to create a 2 mg/mL stock solution and then diluted 20-fold with MeOH to create a 100 ng/μL solution. The working solutions were injected into the LCMS system (Shimadzu LCMS 8045[®], Kyoto, Japan) at 3 μL (300 ng). A Phenomenex Synergi[®] (Torrance, CA, USA) C18 column (4 μm, 80 Å, 100 × 2 mm) held at 40 °C was used as the stationary phase, and H₂O and MeCN with 0.1% HCOOH served as the mobile phase. The mobile phase was pumped at 0.25 mL/min as follows: 1.5 min at 65% MeCN, 1.5 min to 14.0 min gradient elution from 65% to 100% MeCN, 14.0 min to 17.0 min at 100% MeCN, 17.0 min to 19.0 min from 100% to 65%, and 19.0 min to 20.0 min at 65% MeCN. Data were acquired at UV 254 and 190 nm. The optimized MS parameters were used in the experiment: 2 L/min nebulizing gas, 10 L/min heating gas, 10 L/min drying gas, 300 °C interface temperature, 250 °C desolvation line temperature, and 400 °C heating block temperature. MS data were acquired over a mass-to-charge (m/z) range of 100–1500 and were visualized using LabSolutions[®] software version 5.114 (Shimadzu). MS/MS analyses of precursor m/z were performed at 15–40 eV. Dereplication of MS/MS data and spectral matches was carried out using the GNPS [106] and CFM-ID [109].

4.8. Nuclear Magnetic Resonance Spectroscopy

The ¹H NMR spectral data of HPLC fractions were recorded in CDCl₃ (99.8%) at 298 K and 600 MHz on Bruker Avance[®] NMR (Bruker Corporation, Billerica, MA, USA) spectrometer equipped with 5 mm triple resonance cryoprobe with ¹H/¹⁹F, ¹³C, and ¹⁵N direct detection. The operating frequency for ¹H nuclei was at 600 MHz, and a standard Bruker pulse program was used for all the performed ¹H NMR experiments. The 1D- NMR spectra were calibrated based on chloroform residual peak (i.e., 1H at 7.26 ppm). TopSpin[®] Software version 4.1.4 was used to acquire the 1D- and 2D-NMR data, while both TopSpin[®] Software version 4.1.4 and MestreNova[®] software version 14.2.2 were used to perform post-processing on the spectral data.

4.9. Fluorescence Resonance Energy Transfer (FRET) Assay for SARS-CoV-2 3CLpro Inhibition and IC₅₀ Determination

SARS-CoV-2 3CLpro assay kits containing the 3CLpro assay buffer, GC376 (a well-established 3CLpro inhibitor used as the positive control) [15,110,111], dithiothreitol (DTT), and the fluorogenic 3CLpro substrate were employed in this experiment. The substrate was an internally quenched 14-mer peptide (KTS AVLQSGFRKME) labeled with DABCYL

at the N-terminus and EDANS at the C-terminus. Inhibition of protease activity results in a measurable fluorescence signal, which was quantified using a fluorescence resonance energy transfer (FRET)-based detection system [10]. For assay setup, 30 μ L of diluted 3CLpro was dispensed into wells designated for the positive control, negative control, and test inhibitors. This was followed by the addition of 10 μ L of the appropriate vehicles, GC376, or sample solutions. For blank controls, 30 μ L of 1 mM DTT was added to blank well, while 10 μ L of inhibitor buffers were added to blank wells. Plates were incubated at room temperature with gentle agitation for 30 min. The reaction was initiated by adding 10 μ L of the fluorogenic substrate to each well under dark conditions. Final assay conditions consisted of 10 μ g/mL sample, 0.1% DMSO, and 100 μ M GC376. The 96-well plates were maintained in the dark at room temperature for 8 h. All experiments were performed in duplicate. Fluorescence intensity was recorded using a BMG Labtech CLARIOstar (BMG LabTech, Ortenberg, Germany) microplate reader at 360 nm excitation and 460 nm emission. Readings from blank were subtracted from GC376 and vehicle values, while readings from blank were subtracted from sample and vehicle values. The 3CLpro inhibitory activity of the HPLC fractions was determined following the manufacturer's protocol with minor modifications, as previously reported. IC₅₀ values were calculated using the same workflow, in which percent inhibition (mean $\pm$ SEM) was analyzed through nonlinear regression of dose–response curves using GraphPad Prism v9.0 (GraphPad Software, San Diego, CA, USA) [48].

4.10. Assessment of Lipophilic Compounds in 3CLpro Active *Streptomyces* Strains

To assess the distribution of these lipophilic compounds across other bioactive *Streptomyces* strains, crude extracts from *Streptomyces* spp. DSD149^T, DSD735^T, DSD2893^T, and DSD2604^T were prepared in the same manner as strain DSD454^T and partitioned using methanol and hexane (1:1, *v/v*) to separate polar and lipid-soluble components. The hexane and methanol fractions were concentrated under reduced pressure and stored at -20°C until use. In vitro screening of the hexane and methanol fractions was conducted to assess the inhibition of SARS-CoV-2 3CLpro. Each fraction was tested at a final concentration of 10 μ g/mL using a fluorometric substrate-based enzymatic assay as described in Section 4.9. Inhibition percentages were calculated relative to untreated control wells.

Chemical profiling of bioactive fractions was performed by LCMS-TQ using the analytical method established for DSD454IH17–H24 (see Section 4.7). Comparative analysis focused on the retention window of 5.0–11.0 min, corresponding to the lipid-rich region identified in *Streptomyces* sp. DSD454^T.

To evaluate the relationship between chromatographic peak areas and extract potency against SARS-CoV-2 3CLpro, Pearson and Spearman correlation analyses were performed using IC₅₀ values and the corresponding LC-UV peak intensities for each *Streptomyces* strain. Peak areas were derived from LC chromatograms recorded at 190 nm, and IC₅₀ values of extracts were obtained from in vitro 3CLpro inhibition assays conducted at 10 μ g/mL [48].

Pearson's correlation coefficient (*r*) was calculated to assess the strength and direction of linear relationships between IC₅₀ values and individual peak areas. Spearman's rank correlation coefficient (ρ) was employed to evaluate monotonic relationships by comparing the ranked values of IC₅₀ and peak intensities. Correlation coefficients and their associated two-tailed *p*-values were computed in Excel. Statistical significance was established at *p* < 0.05. Both correlation metrics were applied to the same set of five strains and seven chromatographic peaks. The correlation results were tabulated and ranked by the magnitude of their coefficients.

4.11. BODIPYTM 493/503 Staining of *Streptomyces* for Intracellular Lipid Visualization

Intracellular triacylglycerol lipids were visualized using the fluorescent dye BODIPYTM 493/503 (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene), obtained from Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA), following a modified protocol of Aprilliana et al. [71]. *Streptomyces* sp. DSD454^T were initially cultured in MM1 dextrose broth consisting of 10 g of dextrose monohydrate, 4 g of yeast extract, and 2 g of peptone dissolved in 1 L of artificial seawater and incubated for 3 days at room temperature using a shaking incubator. Post-incubation, cells were harvested by centrifugation at 3000 rpm for 5 min. The resulting cell pellets were washed twice with 1 mL phosphate-buffered saline (PBS, pH 7.0). The washed cell pellets were transferred into 1.5 mL Eppendorf tubes and resuspended in 100 μ L of 2 μ M BODIPYTM 493/503 staining solution in DMSO. The cell suspension was incubated for 30 min on ice in the dark. After staining, the cells were washed twice with PBS. The supernatant was discarded, and the stained cells were finally resuspended in 50 μ L of PBS. An 8 μ L aliquot of the cell suspension was dispensed onto a glass microscope slide. Fluorescent imaging was performed using Olympus IX-83 fluorescence microscope (Olympus, Tokyo, Japan) under the green channel and fluorophore specific for BODIPYTM 493/503, allowing visualization of lipid bodies within the cells.

4.12. Molecular Docking Experiment

The ligand used in this study was optimized and uploaded into AutoDock Tools for molecular docking. The optimized ligand structure was saved in PDBQT format. The protein structure was prepared using AutoDock Tools v1.5.7. Initial preparation involved deleting water molecules from the protein structure. Hydrogen atoms (polar only) were added to the protein structure. Kollman charges were assigned to the protein and the protein structure was saved as a PDBQT file. Regions of interest for ligand binding were determined based on the study by Jiménez-Avalos et al. [25] with minor modifications. Three binding sites were defined: Catalytic/Active (AS) site with grid dimensions set to 35 $\times$ 35 $\times$ 35 Å and coordinates at −15.117, 14.564, and 67.870; Dimeric Site (DS) with grid dimensions set at 35 $\times$ 35 $\times$ 35 Å and 1.738, −3.380, 4.457 as coordinates; and Cryptic Site (CS) with grid dimensions set to 35 $\times$ 35 $\times$ 35 Å and coordinates at 9.104, 12.126, −6.685.

These coordinates were selected to comprehensively cover the active and cryptic regions within the protein's structure that are most likely to participate in ligand binding. To ensure chemically accurate protonation of the protein target prior to molecular docking, the SARS-CoV-2 3CLpro structure (PDB ID: 6YB7) was processed using H++ web-based platform [112], that predicts the pKa values of ionizable residues and assigns their corresponding protonation states at the specified pH. In this study, the pH was set to 7.4 to reflect the physiological conditions under which the protease is active. Under these parameters, H++ automatically adds missing hydrogen atoms and optimizes the hydrogen-bonding network, including appropriate amino acid tautomers, thereby generating a structurally consistent protein model suitable for reliable docking analysis.

The grid spacing was set to 0.5 Å. Molecular docking calculations were performed using AutoDock Vina v1.5.7 via Microsoft Windows 10 command prompt. The ligand pose with root-mean square deviation (RMSD) of <2.0 Å was then subjected for post-docking analysis to further evaluate the receptor-ligand interactions using BIOVIA Discovery Studio v.2024.

To refine docking results and improve binding affinity estimation, we employed an integrated structure-truncated Molecular Mechanics/Poisson–Boltzmann or Generalized Born Surface Area (MM/PB(GB)SA) rescoring approach, as implemented in the fastDRH webserver [51]. Using the protein-ligand complexes generated via molecular docking using AutoDock Vina v1.5.7, the top-ranked pose was selected for subsequent rescoring.

To improve computational efficiency, the protein structure was truncated to retain only residues within a 12 Å radius of the ligand, preserving the key interactions within the binding site while significantly reducing system size. Molecular mechanics energies were calculated using the AMBER ff14SB force field for proteins. Ligand parameters were assigned using the General AMBER Force Field (GAFF), with partial charges derived via the AM1-BCC method. Binding free energy calculations were performed using the MM/PB(GB)SA method [51], which includes gas-phase molecular mechanics energy and solvation free energy. The latter was calculated using either the Poisson-Boltzmann (PB) or Generalized Born (GB) model for the polar component, and the solvent-accessible surface area (SASA) model for the non-polar component. Entropic contributions were not included. Ligands were rescored based on their computed binding free energies. A known peptide-based SARS-CoV-2 3CLpro inhibitor, peptide-based GC376, and lipid-based alotaketral C analogue 19, was used as the positive control in all docking experiments.

4.13. ADMET Prediction Using ADMETlab 3.0

The absorption, distribution, metabolism, excretion, and toxicity (ADMET) profiles of selected natural lipids were predicted using ADMETlab version 3.0 (<https://admetlab3.scbdd.com/>) [113]. The chemical structures of 13 lipid compounds including fatty acids and monoglycerides were drawn or imported as SMILES strings and uploaded to the platform. The Fatty Acid Amide Hydrolase (FAAH) probability score was predicted by SwissTarget Prediction [114]. Toxicity predictions encompassed AMES mutagenicity, hepatotoxicity, hERG channel inhibition, and rat oral acute toxicity (LD50). All predictions were conducted under default settings, and results were used to inform the prioritization of lipid compounds for in vitro and in vivo validation.

5. Conclusions

This research emphasizes the promise of sediment-derived *Streptomyces* as a valuable source of natural lipids that exhibit antiviral properties. Palmitoleic and linoleic acids were clearly identified and showed strong inhibition of SARS-CoV-2 3CLpro in their purified states. Additional compounds, such as 9-heptadecenoic acid, linolenic acid, 9-HODE, and various monoacylglycerols including aggrecerides A-C, 2,3-dihydroxypropyl octadeca-9,12-dienoate, and 2,3-dihydroxypropyl hexadec-9-enoate, were tentatively identified using HRMS, tandem MS, NMR, and dereplication analyses.

Molecular docking and MM/GBSA-based rescoring demonstrated that these lipid compounds engage with both the catalytic site of 3CLpro and the dimerization interface, as well as a hidden allosteric pocket. The observed multi-site binding behavior indicates a potential non-competitive or allosteric inhibition mechanism, presenting a viable approach to address the challenges linked to traditional active-site inhibitors.

Our findings indicate that naturally occurring lipids, especially those from marine *Streptomyces*, could be promising candidates for the development of a new class of 3CLpro inhibitors. Future research will concentrate on confirming the antiviral effectiveness of these compounds using appropriate in vivo cell and animal models. Additionally, it will investigate their formulation potential to enhance pharmacokinetic properties, improve bioavailability, and facilitate therapeutic use. The studies conducted are crucial for converting lipids derived from microbes into potential antiviral drug candidates.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ph19020294/s1>, Table S1. Growth of *Streptomyces* sp. DSD454^T on ISP media. Table S2. Carbon source utilization profile of *Streptomyces* sp. DSD454^T. Table S3. Nitrogen source utilization profile of *Streptomyces* sp. DSD454^T. Table S4. Temperature-dependent growth of *Streptomyces* sp. DSD454^T. Table S5. pH-dependent growth of *Streptomyces*

sp. DSD454^T. Table S6. Salt tolerance of *Streptomyces* sp. DSD454^T. Table S7. Enzymatic activity profile of *Streptomyces* sp. DSD454^T. Table S8. Yield distribution of HPLC fractions from DSD454I extract. Table S9. Comparative lipid metabolite profiling of SARS-CoV-2 3CLpro inhibitory *Streptomyces* strains. Table S10. Correlation analysis between UV peak areas and 3CLpro inhibitory activity. Table S11. Summary of interacting atoms of lipid metabolites with critical amino acids in the catalytic site of SARS-CoV-2 3CLpro as described in the study of Goyal (2020) [24]. Table S12. Summary of interacting atoms of lipid metabolites with amino acids in the dimeric site of SARS-CoV-2 3CLpro as described in the study of Goyal (2020) [24] and Jimenez-Avalos et al. (2021) [25]. Table S13. Summary of interacting atoms of lipid metabolites with amino acids in the cryptic site of SARS-CoV-2 3CLpro as described in the study of Jimenez-Avalos et al. (2021) [25]. Table S14. Summary of the interaction of palmitoleic acid **1**, linoleic acid **2**, lipid-based positive control Alotaketol C analogue **19**, and peptide-based GC376 against the important interacting amino acid residues. Table S15. Summary of binding affinity (kcal/mol) and GB8 value of 13 fatty acids against the catalytic, dimeric, and cryptic site of SARS-CoV-2 3CLpro. Figure S1. Phylogenetic placement of *Streptomyces* sp. DSD454^T. Figure S2. PDA chromatogram of DSD454I fractions (H1–H25). Figure S3. ¹H NMR spectrum of bioactive HPLC fraction H17 showing 2,3-dihydroxypropyl hexadec-9-enoate **9**, 9-hydroxy-10,12-octadecadienoic acid **12**, and 2,3-dihydroxypropyl pentadec-9-enoate **13** resonance signals (600 MHz, CDCl₃, 298 K). Figure S4. ¹H NMR spectrum of bioactive HPLC fraction H18 showing monoacylglycerol profile with palmitoleic acid side chain resonance signals (600 MHz, CDCl₃, 298 K). Figure S5. ¹H NMR spectrum of bioactive HPLC fraction H19 showing signatures of 2,3-dihydroxypropyl hexadec-9-enoate **9**, aggregeride A **10**, and linolenic acid **11** resonance signals (600 MHz, CDCl₃, 298 K). Figure S6. ¹H NMR spectrum of bioactive HPLC fraction H20 showing 2,3-dihydroxypropyl heptadec-9-enoate **8** and palmitoleic acid resonance signals (600 MHz, CDCl₃, 298 K). Figure S7. ¹H NMR spectra of bioactive HPLC fraction H21 showing palmitoleic acid **1** and linoleic acid **2** resonance signals (600 MHz, CDCl₃, 298 K). Figure S8. ¹H NMR spectrum of bioactive HPLC fraction H22 showing aggregeride B **3** and 9-heptadecenoic acid **4** resonance signals (600 MHz, CDCl₃, 298 K). Figure S9. ¹H NMR spectrum of bioactive HPLC fraction H23 showing aggregeride C **5** and 2,3-dihydroxypropyl octadeca-9,12-dienoate **6** resonance signals (600 MHz, CDCl₃, 298 K). Figure S10. ¹H NMR spectrum of bioactive HPLC fraction H24 showing hydroxylated fatty acid esters resonance signals (600 MHz, CDCl₃, 298 K). Figure S11. High Resolution Mass Spectrum (HRMS) of Palmitoleic acid **1** at m/z 253.2175 [M-H][−] in DSD454IH20. Figure S12. High Resolution Mass Spectrum (HRMS) of Linoleic acid **2** at m/z 279.2327 [M-H][−] in DSD454IH21. Figure S13. High Resolution Mass Spectrum (HRMS) of Aggregeride B **3** at m/z 313.2736 [M+H-H₂O]⁺ in DSD454IH22. Figure S14. High Resolution Mass Spectrum (HRMS) of 9-Heptadecenoic acid **4** at m/z 267.2329 [M-H][−] in DSD454IH22. Figure S15. High Resolution Mass Spectrum (HRMS) of Aggregeride C **5** at m/z 327.2888 [M+H-H₂O]⁺ in DSD454IH23. Figure S16. High Resolution Mass Spectrum (HRMS) of 2,3-dihydroxypropyl octadeca-9,12-dienoate **6** at m/z 355.2831 [M+H]⁺ in DSD454IH23. Figure S17. High Resolution Mass Spectrum (HRMS) of 2,3-dihydroxypropyl nonadeca-9,12-dienoate **7** at m/z 369.2999 [M+H]⁺ in DSD454IH24. Figure S18. High Resolution Mass Spectrum (HRMS) of 2,3-dihydroxypropyl heptadec-9-enoate **8** at m/z 325.2739 [M+H-H₂O]⁺ in DSD454IH20. Figure S19. High Resolution Mass Spectrum (HRMS) of 2,3-dihydroxypropyl hexadec-9-enoate **9** at m/z 311.2588 [M+H-H₂O]⁺ in DSD454IH17. Figure S20. High Resolution Mass Spectrum (HRMS) of Aggregeride A **10** at m/z 299.2581 [M+H-H₂O]⁺ in DSD454IH19. Figure S21. High Resolution Mass Spectrum (HRMS) of Linolenic acid **11** at m/z 277.2168 [M-H][−] in DSD454IH19. Figure S22. High Resolution Mass Spectrum (HRMS) of 9-HODE **12** at m/z 279.2330 [M+H-H₂O]⁺ in DSD454IH17. Figure S23. High Resolution Mass Spectrum (HRMS) of 2,3-dihydroxypropyl pentadec-9-enoate **13** at m/z 297.2434 [M+H-H₂O]⁺ in DSD454IH17. Figure S24. MS/MS fragmentation spectrum with proposed fragment structure of aggregeride B **3** at m/z 331.30 [M+H]⁺. Figure S25. MS/MS fragmentation spectrum with proposed fragment structure of 9-heptadecenoic acid **4** at m/z 269.30 [M+H]⁺. Figure S26. MS/MS fragmentation spectrum with proposed fragment structure of aggregeride C **5** at m/z 345.35 [M+H]⁺. Figure S27. MS/MS fragmentation spectrum with proposed fragment structure of 2,3-dihydroxypropyl octadeca-9,12-dienoate **6** at m/z 355.35 [M+H]⁺. Figure S28. MS/MS fragmen-

tation spectrum with proposed fragment structure of 2,3-dihydroxypropyl nonadeca-9,12-dienoate **7** at m/z 369.30 $[M+H]^+$. Figure S29. MS/MS fragmentation spectrum with proposed fragment structure of 2,3-dihydroxypropyl heptadec-9-enoate **8** at m/z 343.25 $[M+H]^+$. Figure S30. MS/MS fragmentation spectrum with proposed fragment structure of 2,3-dihydroxypropyl hexadec-9-enoate **9** at m/z 329.25 $[M+H]^+$. Figure S31. MS/MS fragmentation spectrum with proposed fragment structure of aggregeride A **10** at m/z 317.30 $[M+H]^+$. Figure S32. MS/MS fragmentation spectrum with proposed fragment structure of linolenic acid **11** at m/z 279.25 $[M+H]^+$. Figure S33. MS/MS fragmentation spectrum with proposed fragment structure of 9-hydroxy-10,12-octadecadienoic acid **12** at m/z 297.25 $[M+H]^+$. Figure S34. MS/MS fragmentation spectrum with proposed fragment structure of 2,3-dihydroxypropyl pentadec-9-enoate **13** at m/z 315.25 $[M+H]^+$. Figure S35. SARS-CoV-2 CLpro inhibition of hexane-soluble metabolites from DSD149^T, DSD735^T, DSD2893^T, and DSD2604^T. Figure S36. Binding Interactions of lipid metabolites on SARS-CoV-2 3CLpro catalytic site. Figure S37. Binding Interactions of lipid metabolites on SARS-CoV-2 3CLpro dimeric site. Figure S38. Binding Interactions of lipid metabolites on SARS-CoV-2 3CLpro cryptic site.

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Abbreviations

3CLpro	3-Chymotrypsin-like protease (main protease of SARS-CoV-2)
ADMET	Absorption, Distribution, Metabolism, Excretion, and Toxicity
BODIPY TM 493/503	Boron-dipyrromethene (fluorescent dye)
COVID-19	Coronavirus disease 2019
CYP	Cytochrome P450
FRET	Förster Resonance Energy Transfer
GBSA	Generalized Born Surface Area
HRMS	High-Resolution Mass Spectrometry
IC ₅₀	Half-maximal inhibitory concentration
LC–MS	Liquid Chromatography–Mass Spectrometry
LC–MS/MS	Liquid Chromatography–Tandem Mass Spectrometry
MM/GBSA	Molecular Mechanics/Generalized Born Surface Area
NMR	Nuclear Magnetic Resonance
PDB	Protein Data Bank
RdRp	RNA-dependent RNA polymerase
TPSA	Topological Polar Surface Area

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