Target-Based Virtual Screening and LC/MS-Guided Isolation Procedure for Identifying Phloroglucinol-Terpenoid Inhibitors of SARS-CoV-2

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ABSTRACT: The coronavirus disease 2019 (COVID-19) pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has led to more than 5 million deaths worldwide to date. Due to the limited therapeutic options so far available, target-based virtual screening with LC/MS support was applied to identify the novel and high-content compounds 1–4 with inhibitory effects on SARS-CoV-2 in Vero E6 cells from the plant Dryopteris wallichiana. These compounds were also evaluated against SARS-CoV-2 in Calu-3 cells and showed unambiguous inhibitory activity. The inhibition assay of targets showed that compounds 3 and 4 mainly inhibited SARS-CoV-2 3CLpro, with effective $K_i$ values. Through docking and molecular dynamics modeling, the binding site is described, providing a comprehensive understanding of 3CLpro and interactions for 3, including hydrogen bonds, hydrophobic bonds, and the spatial occupation of the B ring. Compounds 3 and 4 represent new, potential lead compounds for the development of anti-SARS-CoV-2 drugs. This study has led to the development of a target-based virtual screening method for exploring the potency of natural products and for identifying natural bioactive compounds for possible COVID-19 treatment.

The recently described SARS-CoV-2, the causative agent of the coronavirus disease 2019 (COVID-19) pandemic, has caused over 320 million cases of infection and more than 5 million deaths thus far, leading to worldwide economic and social disruption.1–10 There are only a few effective antiviral drugs available for the prophylactic or therapeutic treatment of the highly contagious SARS-CoV-2 in humans. Also, a few pre-existing agents and drug candidates, such as remdesivir (RDV), have been used as treatment regimens against COVID-19. An RDV prodrug, a nucleoside-analogue inhibitor, impairs viral infection by targeting RNA-dependent RNA polymerase (RdRp). RDV potently inhibits SARS-CoV-2 replication at EC$_{50}$ values of 0.77–3.68 μM.5,6 Recent reports of the compassionate use of RDV were reported in 68% of patients with severe COVID-19.2,5,6 With different mechanisms, inhibitors or blockers such as birmifosbuvir (AT-527) and PF-07321332, have been developed for structure-based drug discovery.7–10 Birmifosbuvir, an orally administered double prodrug of a guanosine nucleotide analogue, has potent antiviral activity by targeting both RdRp and the Nidovirus RdRp-associated nucleotidyltransferase (NiRAN) in vitro, including SARS-CoV-1, SARS-CoV-2, and hepatitis C virus. This prodrug is currently in phase II clinical trials for SARS-CoV-2 inhibition, and phase III clinical trials are expected to be completed very soon.11,12 PF-07321332, designed by Pfizer, is a second-generation, orally available SARS-CoV-2-3CL protease (3CLpro) inhibitor and has been evaluated in a phase I clinical trial study (NCT04756531) for safety. Phase II/III clinical trials (NCT05011513 and NCT05047601) to evaluate safety and efficacy are currently in progress, and combination therapy with low-dose ritonavir is expected to help 3CLpro remain active in the body for longer periods.13,14 The conclusions of phase trials are pending and will determine whether useful agents such as birmifosbuvir, plitidepsin, and PF-07321332 are effective in the treatment of COVID-19.12 Thus far, target-specific drugs are still expected to be exploited for SARS-CoV-2.

Natural products and their derivatives have played an important role in the new drug development process, and nearly 50% of these products are FDA-approved drugs.15 Natural products represent a good source of bioactive molecules.
Figure 1. Target-based virtual screening used for natural product development against SARS-CoV-2. (A) Possible targets for new drug development (2′-O-methyltransferase (2′OMT, PDB: 6W7S), 3CL protease (3CLpro, PDB: 6M2N), nonstructural proteins 3 (NSP3, PDB: 6W6Y), nonstructural proteins 15 (NSP15, PDB: 6WXC), nonstructural proteins 1 (NSP1, PDB: 7K3N), helicase (Hel, PDB: 6JYT), nonstructural proteins 15 (NSP15, PDB: 6WXC), RNA-dependent RNA polymerase (RdRp, PDB: 7B3C), spike glycoprotein (SGP, PDB: 6VXX), spike-RBD (RBD, PDB: 7B14), transmembrane protease serine 2 (TMPRSS2, PDB: 7MEQ), nucleocapsid (Npro, PDB: 6WZQ), cathepsin B (CTSB, PDB: 3AI8), angiotensin-converting enzyme 2 (ACE2, PDB: 6CS2)). (B) Compound database of Dryopteris species. (C) Predicted target network of target-based virtual screening. (D) Predicted molecular fragment and molecular mass. (E) LC/MS analysis of the diethyl ether fraction from D. wallichiana. Acquity Zorbax SB-C18 column (4.6 mm × 250 mm, 5 μm) at a flow rate of 1.0 mL/min; gradient elution starting at 5% MeOH in water, ramping to 100% in 100 min, then to 100% MeOH with 0.5% HCOOH.

Figure 2. (A) Chemical structures. (B) Key HMBC, COSY, and NOE correlations of 1−4.
and are characterized by extensive structural diversity.\textsuperscript{16,17} In an effort to combat SARS-CoV-2, medicinal chemists have developed different and practicable strategies for new drugs. Target-based virtual screening involves docking many molecules to potential multifocal targets by a virtual screening procedure. The key docking scores and the number of directed edges were used to guide the selection of potentially active molecules. This approach is easy to implement, and many new medicinal functions of natural products can be exploited (Figure 1).\textsuperscript{18} This strategy was used to screen natural products of Dryopteris species (Table S1, Supporting Information) to obtain molecules that may have a blocking or inhibitory effect on coronavirus. The results of the virtual screening showed that compounds 1BIV-10 and 1BIV-11 have the characteristics of good docking scores and multiple directed edges (Figure S1, Supporting Information). When predicted active molecules (1BIV-10 and 1BIV-11) were found in the compound library of Dryopteris species, fragment-based potential molecules were obtained by LC/MS and HPLC methods. According to these structures, molecular masses of 510 and 718 Da were found to have similar fragments. In addition, the investigation of compounds with unusual terpenoid moieties attached to acylphloroglucinol residues from Dryopteris species is of interest. The next step was to isolate and identify target compounds by HPLC, LC/MS, NMR, and X-ray crystallographic techniques. LC/MS-guided isolation of compounds from Dryopteris wallichiana (Spreng.) Hylander (Dryopteridaceae) afforded two new acylphloroglucinols, wallichins E and F (1 and 2, Figure 2A), along with the previously known compounds wallichins C (3) and D (4).\textsuperscript{19}

### RESULTS AND DISCUSSION

#### Structural Identification

Table 1. ¹H (600 MHz) and ¹³C (150 MHz) NMR Spectroscopic Data of Compounds 1 and 2 (δ in ppm and J in Hz)

| No. | δ_H type | δ_C mult. (J in Hz) | HMBC | Δ_C type | δ_H mult. (J in Hz) | HMBC |
|-----|----------|---------------------|------|----------|---------------------|------|
| 1   | 189.1 C  |                     |      | 189.1 C  |                     |      |
| 2   | 108.3 C  |                     |      | 108.3 C  |                     |      |
| 3   | 174.9 C  |                     |      | 175.0 C  |                     |      |
| 4   | 47.8 C   |                     |      | 47.8 C   |                     |      |
| 5   | 196.8 C  |                     |      | 197.0 C  |                     |      |
| 6   | 108.3 C  |                     |      | 108.3 C  |                     |      |
| 7   | 17.9 CH₂ | 2.34−2.45, m       | 14°  | 18.0 CH₁ | 2.35−2.47, m       | 14°  |
| 8   | 25.2 CH₃ | 1.34, s             | 3, 5 | 25.2 CH₁ | 1.34, s             | 3, 5 |
| 9   | 24.9 CH₃ | 1.30, s             | 3, 5 | 24.8 CH₁ | 1.30, s             | 3, 5 |
| 10  | 200.6 C  |                     |      | 200.7 C  |                     |      |
| 11  | 27.8 CH₂ | 2.51, s             | 10   | 27.9 CH₁ | 2.51, s             | 10   |
| 12  | 19.2 CH₂ |                     |      | 19.2 CH₁ |                     |      |
| 13  | 18.1 CH₁ | 1.81, m             |      | 19.3 CH₁ |                     |      |
| 14  | 47.8 C   |                     |      | 38.7 CH₂ |                     |      |
| 15  | 50.4 CH  |                     |      | 50.4 CH  |                     |      |
| 16  | 27.3 CH₂ |                     |      | 27.3 CH₂ |                     |      |
| 17  | 38.4 CH₂ |                     |      | 38.4 CH₂ |                     |      |
| 18  | 149.9 C  |                     |      | 149.9 C  |                     |      |
| 19  | 58.0 CH  |                     |      | 58.0 CH  |                     |      |
| 20  | 39.6 C   |                     |      | 39.6 C   |                     |      |
| 21  | 2.29−2.37, m |           | 5, 6, 8, 9, 17° | 2.29−2.37, m | 5, 6, 8, 9, 17° |
| 22  | 4.16 td (12.8, 4.3) |  | 5, 7, 8° | 4.16 td (12.8, 4.3) | 5, 7, 8° |
| 23  | 1.38 m   |                     | 8, 10° | 1.38 m   |                     | 8, 10° |
| 24  | 2.00−2.06, m |           | 5, 6, 8, 9, 17° | 2.00−2.06, m | 5, 6, 8, 9, 17° |
| 25  | 5.04 CH  |                     |      | 5.04 CH  |                     |      |
| 26  | 23.1 CH₂ |                     | 8, 9, 12, 15° | 23.1 CH₂ | 2.16, ddd (16.2, 10.8, 6.4) 8, 9, 12, 15° |
| 27  | 129.4 CH₁ | 5.48 t (6.4)        | 5, 9, 11, 14, 16° | 129.4 CH₁ | 5.48 t (6.4) 5, 9, 11, 14, 16° |
| 28  | 133.2 C  |                     |      | 133.2 C  |                     |      |
| 29  | 84.0 CH  |                     |      | 84.0 CH  |                     |      |
| 30  | 25.9 CH₂ |                     | 8, 9, 12, 13° | 25.9 CH₂ | 4.52, m 7, 12, 13°, 15°, 16° |
| 31  | 12.3 CH₁ |                     | 12, 13, 14° | 12.3 CH₁ | 1.81−1.98, m, 2H ND² 12, 13, 14° |
| 32  | 108.3 CH₂ |                     | 7, 8, 9° | 108.3 CH₂ | 4.52, m 7, 8, 9° |
| 33  | 180.0 CH₁ |                     |      | 180.0 CH₁ |                     |      |
| 34  | 17.1 CH₁ |                     | 3, 4, 5° | 17.1 CH₁ | 1.14, s 3, 4, 5° |
| 35  | 15.0 CH₁ |                     | 1, 5, 9, 10° | 15.0 CH₁ | 0.79, s 1, 5, 9, 10° |
| 36  | OH-S     |                     |      | OH-S     | 19.02, brs 19.02, brs |

*Overlapping signals. **ND: not determined due to overlap with other signals or solvent signals.
signals. The $^1$H NMR spectrum of 1 in acetone-$d_6$ displayed a singlet at a very low field ($\delta_H$ 19.02 ppm, –OH), which was assigned to an enolizable $\beta$-triketo carbonyl group. The $^1$H NMR signals of the gem-dimethyl group at 1.30 and 1.34 ppm (3H each, s) were attributed to the presence of an acylphloroglucinol acid ring system. Two doublets resonating at 2.34–2.45 ppm were observed in the $^1$H NMR spectrum of 1, indicating a methylene bridge, and its $^{13}$C NMR chemical shift (17.9 ppm) was consistent with the CH$_2$ moiety being connected to a terpenoid unit and an acylphloroglucinol moiety. The HMBC spectrum of 1 showed cross-peaks between H-11/C-10 (Figure 2B), as well as between H-14″/C-7′, supporting the occurrence of an acetyl moiety (108.3 ppm, C-6) on the A ring and the linkage position of the terpenoid moiety. To clarify the absolute configuration of 1 (Figure 2A), the geometries of the R and S configurations in gas were optimized initially at the density functional theory (DFT)-B3LYP/6-31G(d) level. The experimental ECD spectrum of (+)-1 displayed negative Cotton effects at 200–223 nm and positive CEs at 223–374 nm. The computed ECD spectrum at the 14″S configuration matched well with the experimental ECD spectrum of 1 (Figure 3A). The $n \rightarrow \pi^*$ electronic transitions from S0 of the acylphloroglucinol moiety to S3 of the olefin group (Figure 3C) afforded a rotatory strength $\Delta \varepsilon = +0.8$ at 304 nm, which was in agreement with the weak positive CEs $\Delta \varepsilon = +1.5$ in the experimental ECD spectrum. The other positive rotatory strength at electronic transitions between 304 and 350 nm was dominated by other $\pi \rightarrow \pi^*$ characters of the acylphloroglucinol group (>90%)}

Figure 3. (A, B) Comparison of the calculated ECD spectra for 4″S,5″S,9″R,10″S,14″S and 4″S,5″S,9″R,10″S,14″R at the TD-DFT-B3LYP/6-31G(d) level with the experimental spectra of compounds 1 and 2 in MeOH (the red trace indicates the gas phase; the blue trace indicates the PCM in MeOH). (C) Natural transition orbitals (NTOs) of the most stable conformer involved in each transition computed at the TD-DFT-B3LYP/6-31G(d) level.
contributions), which was consistent with the experimental positive CEs in the ECD spectrum. The negative rotatory band \( (\Delta \varepsilon = -3.8) \) at 210 nm was in accordance with the experimental negative CE \( \Delta \varepsilon = -2.8 \) in the ECD spectrum. The negative rotatory band \( (\Delta \varepsilon = -3.8) \) at 210 nm was in accordance with the experimental negative CE \( \Delta \varepsilon = -2.8 \) in the ECD spectrum. The configuration of C-14″ in 1 could be identified from the comparison results of the ECD spectrum using TD-DFT at the B3LYP/6-31G(d) level in the gas model and PCM. Single-crystal X-ray diffraction analysis confirmed this (Figure 4 and Figure S2, Supporting Information) using Cu Kα radiation, which allowed for assignment of the absolute configuration of (+)-1 (wallichin E) as 4″S,5″S,9″R,10″S,14″S.

Figure 4. X-ray diagram of 1 (14″S), showing a keto absolute configuration and a pyran skeleton at the C ring. The bond length of 1.2368 Å at the C-5/O-5 positions is less than 1.2938 Å at the C-1/O-1 positions; thus the double bonds could be assigned to the C-5/O-5 positions.

Compound 2 (wallichin F) gave the same molecular formula, \( C_{31}H_{42}O_{6} \), as compound 1. Both the \(^1\)H and \(^13\)C NMR spectroscopic data of 2 were closely comparable to those of 1, but key differences were displayed at C-12″, C13″, and C-14″ (Table 1). Thus, it was evident that 1 and 2 are isomers. The \([\alpha]_{D}^2\) value of 2 was found to be +9.5, while the corresponding value for 1 was +61.8. To clarify the diastereomeric nature of 1, the ECD spectra of 2 were calculated, and the absolute configuration at the C-14″ position was established as R by analysis of the computed ECD data (Figure 3B). Therefore, the structure of (+)-2 was assigned as 4″S,5″S,9″R,10″S,14″R.

Inhibition of SARS-CoV-2 and 3CLpro. The signals of mAU detected at 280 nm indicated that it contains two major compounds (3 and 4) at a ratio of approximately 1.2:1, with retention times of 3 and 4 at 11.8 and 15.2 min, respectively (Figure S3A, Supporting Information). The SARS-CoV-2 inhibitory activities of the isolated compounds were evaluated in vitro in Vero E6 cells. Separate line charts of the inhibitory percentage at a final concentration of 10 μM are shown in Figure 5A. Among these compounds, the mixed compounds 3 and 4 presented a high inhibition rate of 87.7%, and 2 had a 51.7% inhibition rate. Thus the mixed compounds 3 and 4 were used to test further activities. The natural content ratio (1.2:1) of the mixture showed an inhibitory activity of 6.8 μM EC\(_{50}\) (Figure 5B). Mixed compounds 3 and 4 and pure 3 and 4 displayed inhibitory activities against SARS-CoV-2, with EC\(_{50}\) values from 4.5 to 12.1 μM in Vero E6 cells. Figure 5B shows not only that the natural ratio of 3 and 4 maintained SARS-CoV-2 inhibition activity but also that purified compound 3 exhibited improved inhibitory activity toward SARS-CoV-2 at the cellular level.

Figure 5. Activities of 1–4 against SARS-CoV-2. (A) The % inhibition of SARS-CoV-2 was measured at a final concentration of 10 μM (\( n = 3 \), independent experiments). (B) Vero E6 cells at an MOI of 0.01 were infected with SARS-CoV-2 and treated with a series of concentrations of the indicated antivirals for 24 h p.i. (\( n = 3 \)). (C) Calu-3 cells were infected with SARS-CoV-2 at an MOI of 0.05 and treated with different doses of the indicated antivirals for 24 h p.i. (\( n = 3 \)). (D) Representative inhibition curves for baicalein, a mixture of compounds 3 and 4, and pure 3 and 4 against 3CLpro (\( n = 3 \)). (E–H) Affinities of mixed 3 and 4, pure 3 and 4, and baicalein for 3CLpro analyzed using an SPR assay.
When comparing the EC50 values of 3 and 4, there were slight differences due to the chirality at C-14″ R or C-14″ S. The in vitro cytotoxicity of the compounds was tested using a CCK-8 assay. The resulting CC50 values of compounds in Vero E6 cells were over 140 μM (Figure S3B, Supporting Information), indicating low cytotoxicity. The corresponding selectivity index (SI = CC50/EC50) values were >27, >35, and >11 for a mixture of 3 and 4 and pure 3 and 4, respectively. To validate the activity mechanism, Vero E6 cells were infected with both VSV-delG/VSV and VSV-delG/SARS-CoV-2 pseudoviruses at a multiplicity of infection (MOI) of 0.01 in different concentrations of a mixture of 3 and 4 and pure 3 and 4 between 50 and 100 μM (Figure S3C and D, Supporting Information). An inhibition rate below 50% at 100 μM showed that these compounds are not able to block SARS-CoV-2-infected cells. The results indicated that compounds 3 and 4 may act as SARS-CoV-2 inhibitors. It was then investigated as to whether the cell-type-dependent differences in entry inhibition translated into differential

| compound(s) | ligand binding sites | Kd (μM) | IC50 (μM, mean ± SD) | EC50 in Vero E6 cells (μM, mean ± SD) | ClogPb |
|-------------|---------------------|---------|----------------------|---------------------------------------|--------|
| 3+4         |                     | 12.0    | 5.6 ± 0.8            | 6.8 ± 1.6                             | 5.41   |
| 3           | PHE140, ASN142, MET165, GLU166, ARG188, GLN192 | 16.6    | 7.5 ± 2.1            | 4.5 ± 1.7                             | 5.41   |
| 4           | THR24, THR25, HIS41, SER46, MET165, PRO168, ASP187, ARG188, GLN192 | 14.3    | 8.1 ± 2.0            | 12.1 ± 2.3                            | 5.41   |
| baicalein   | HIS41, MET49, LEU141, CY5145, GLU166 | 31.3    | 5.1 ± 1.2            | 3.19                                  |

Molecular docking was performed with AutoDock4.2 to create an interaction model and identify the essential amino acid residues of 3CLpro. The binding sites of baicalein were determined from the PDB file (6M2N). The octanol–water partition coefficient (ClogP) was obtained using the ALOGPS 2.1 program.
inhibition of authentic SARS-CoV-2. The EC50 values of pure 3 and 4 were examined in Calu-3 cells infected with SARS-CoV-2 at an MOI of 0.05. The test results showed that 3 and 4 suppressed SARS-CoV-2 with EC50 values of 20.2 and 30.0 μM, respectively (Figure 5C). Furthermore, a molecular dynamics simulation revealed anisotropic behaviors with ligand RMSDs during 0–15 ns. The RMSD values of 3CLpro varied greatly with ligand RMSDs during 0–15 ns. To explain this phenomenon, principal component analysis (PCA) was performed with the MODE-TASK script. The main differences in the MD trajectory were classified into two clusters including the red zone at 100 ns and the blue zone at 10 ns (Figure 6G). The representative structures are aligned in Figure 6H. The differences were attributed to loose loop regions of 3CLpro allowing for the side structure to be more flexible. These simulation results provide the interaction details together of 3CLpro inhibited by 3 and an interpretation for the potent activity determined for compound 3 against SARS-CoV-2.

### EXPERIMENTAL SECTION

**General Experimental Procedures.** The melting point was measured on an X-4 micro melting point apparatus. Optical rotations were determined using a Rudolph Autopol III polarimeter (Rudolph Research Analytical, Hackettstown, NJ, USA). UV spectra were recorded with a Shimadzu UV-2401A spectrophotometer. The Fourier transform infrared (FT-IR) spectra were recorded using a Thermo Scientific Nicolet iS10 FT-IR spectrometer. The ECD data were recorded on a Bruker AV-600 (1H: 150 MHz, 13C: 60 MHz) NMR spectrometer. HRESIMS data and analytical LC/MS data were obtained on an Agilent 6540 Q-TOF mass spectrometer.

**Plant Material.** Rhizomes of *D. wallachiana* were collected in November 2019 from Fu Gong County of Yunnan Province, People’s Republic of China, and identified by Dr. Zheng-Yu Zuo. A voucher specimen (KUN 151997) was deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

**Extraction and Isolation.** The chopped, air-dried rhizomes of *D. wallachiana* (~4700 g) were extracted for 2 weeks at 40 °C with EtOAc, and the resulting extract (~125 g) was subjected to silica gel chromatography to afford two fractions, 1 (~31 g) and 2 (~17 g). Fraction 1 was treated by column chromatography (CC) on a Sephadex LH-20 column and further purified by HPLC [UltraspHERE Si (10 mm × 250 mm, 5 μm)] using n-hexane–EtOAc (95:5) together with HOAc (0.5%), 2 mL/min, to obtain 3 (320 mg, ~0.5% yield) and 4 (260 mg, ~0.4% yield). Fraction 2 was reprocessed by HPLC [Hyperil BDS C18 (10 mm × 250 mm, 5 μm), H2O–MeOH–isopropanol (30:35:35) with 0.5% HCOOH, 2.0 mL/min] to purify 1 and 2. Fraction B also yielded 1 (45 mg, ~0.075% yield) and 2 (10 mg, ~0.016% yield).

**Wallilchin E (1):** Yellow gum; mp 240–247 °C; [α]23D +61.8 (c 0.5, MeOH); UV (MeOH) λmax (log ε) 203 (4.03), 226 (3.82), 242 (3.82), 276 (3.64), 325 (3.78) nm; IR (ATR) νmax 3080, 2931, 2866, 1693, 1658, 1621, 1569, 1525, 1471 cm−1; ECD ([c 5.5 × 10−4 M, MeOH] λmax (Δε) 209 (~2.78), 240 (~1.98), 322 (~1.96); [1H NMR (600 MHz, acetone-d6) and 13C NMR (150 MHz, acetone-d6), Table 1; HRESIMS m/z [M + H]+ 511.3054 (calcd for C31H43O6)]

**Wallilchin F (2):** Brown gum; [α]23D +9.5 (c 0.4, MeOH); UV (MeOH) λmax (log ε) 203 (4.05), 227 (3.83), 242 (3.85), 276 (3.67), 326 (3.83) nm; IR (ATR) νmax 3080, 2931, 2865, 1694, 1657, 1620, 1569, 1524, 1471 cm−1; ECD ([c 7.8 × 10−4 M, MeOH] λmax (Δε) 199 (2.43), 229 (~1.83), 247 (~0.72), 301 (~0.73), 318 (~1.14); [1H NMR (600 MHz, acetone-d6) and 13C NMR (150 MHz, acetone-d6), Table 1; HRESIMS m/z [M + H]+ 511.3060 (calcd for C31H43O6)]

**Crystal Structure Analysis of Compound 1.** Crystals of 1 were obtained from CH3Cl–CH3OH (1:1) at 4 °C, which was analyzed by X-ray diffraction with Cu Kα radiation (Figure S2, Supporting Information). The intensity data were collected at 100 K on a Bruker APEX DUO diffractometer equipped with an APEX II CCD using Cu Kα radiation. Cell refinement and data reduction were performed with Bruker SAINT. Structures were resolved by the program and full-matrix least-squares calculations. All non-hydrogen atoms were refined anisotropically, and all hydrogen atoms were fixed at the calculated positions. Crystallographic data were deposited at the Cambridge Crystallographic Data Center (number: CCDC 2106340).

**Crystal Data of 1.** C31H43O6 M = 510.64, a = 6.1780(2) Å, b = 7.9180(3) Å, c = 14.8892(6) Å, α = 93.3094(10)°, β = 96.4600(10)°, γ = 125.1980(10)°, V = 713.11(6) Å3, T = 100(2) K, space group P1, Z = 1,
μ(Cu Kα) = 0.655 mm⁻¹, 21 537 reflections measured, 5432 independent reflections (Rint = 0.0532). The final R1 values were 0.0548 (I > 2σ(I)). The final wR2(F2) values were 0.1417 (I > 2σ(I)). The final R1 values were 0.0555 (all data). The final wR2(F2) values were 0.1434 (all data). The goodness of fit on F² was 1.016. The absolute configuration was determined by the Flack parameter s = -0.01 (10).

Molecular Modeling for 3CLpro and Compound 3. The 3CLpro structure was docked with 3 and 4 via the AutoDock 4.2 software package. The conversion of 3 and 4 from 2D to 3D structures was carried out using Open Babel GUI software. Processing of 3CL protease (3CLpro, PDB entry 6M2N) was performed with AutoDock Tools (ADT). Water molecules and original ligands were removed using the edit module. Gasteiger charges and polar hydrogen atoms were assigned to the 3D structure, and the genetic algorithm (GA) run was selected as 50 to obtain the conformations. The center coordinate was obtained from the original ligand, and a docking grid with a size of 60 × 60 × 60 was used. Compound 3 was then docked into the active site of 3CLpro to obtain a complex structure. The structure was subjected to MD simulation using GROMACS 5.0. The antechamber tool was used to generate the restricted electrostatic point charge (RESP) of 3 after calculation by Gaussian 09 at the HF/6-31G(d,p) level. A force field of 3 was taken from the general AMBER force field (G9SB). Compound 3 and the TIP3P water model were added with a boundary 2.0 Å away from 3CLpro atoms. The whole system was neutralized by the counterions, and then the steepest descent algorithm was used to minimize within a force tolerance of 10 kJ/mol. Consequently, the systems with position restraints were equilibrated to 310 K on the 3CLpro and ligand atom. The pressure and temperature were controlled by a Parrinello restraints were equilibrated to 310 K on the 3CLpro and ligand atom. The pressure and temperature were controlled by a Parrinello-Rahman barostat (2 bar) with τp = 2.0 ps and a compressibility of 4.5 × 10⁻⁵ bar and the V-rescale method with τv = 0.1 ps, respectively. Long-range electrostatic interactions were involved within a 1.2 nm cutoff using the particle-mesh Ewald (PME) algorithm. Constraining the bond lengths and angles of hydrogen bonds was applied by the linus algorithm. The initial 1.0 ns MD simulation of NPT was position restrained during the equilibration period. Canonical 100 ns MD simulation runs were performed for protein–ligand complexes. The time step and the saved coordinates were set to 2.0 fs and 1.0 ps. 3CLpro–ligand interaction files were taken from the PLIP online tool (https://plip-tool.biotec.tu-dresden.de/plip-web/plip/index) and visualized using PyMOL.

3CLpro Enzyme Activity Inhibition Test. The 3CLpro inhibitory activity of the compounds was determined by a FRET protease assay. MCA-AVLQGSR-Lys(Dnp)-Lys-NH₂ solution was used as the fluorogenic substrate (Genscript). According to previous reports, a fluorescent value of the tested compounds (3CLpro, compound 3, and 4) was used and baicalein at different concentrations (50, 40, 30, 15, 7.5, 3.75, and 1.875 μM) were injected for 120 s at a flow rate of 40 μL/min. A cleaning solution of PBS with 50% DMSO was used to regenerate the CMS chip. All response values were collected at 25 °C. Biacore Evaluation Software 2.0 was used to generate Kd values.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jnatprod.1c00805. HPLC traces of 1–4 (PDF) X-ray crystallographic data of 1 (CIF) Compound database, the protocol of target-based virtual screening, structural identification of compounds 3 and 4, the natural content, cytotoxicity and pseudovirus activities of compounds 3 and 4, affinities of the mixed 3 and 4 and pure 3 and 4 for RBD analyzed by using an SPR assay, NMR spectra of 3 and 4 in Table S3, comparison of the calculated ECD spectra for (−)-3 and (+)-4, and UV, ECD, OR, HRESIMS, and NMR spectra of 1–4 (PDF)

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Notes
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