Molecular Networking-Guided Isolation of Cycloartane-type Triterpenoids from *Curculigo orchioides* and Their Inhibitory Effect on Nitric Oxide Production

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ABSTRACT: The MolNetEnhancer workflow was applied to molecular networking analysis of the CH$_2$Cl$_2$-soluble fraction of the rhizomes of *Curculigo orchioides*, which showed a potent inhibitory effect on the lipopolysaccharide (LPS)-induced nitric oxide production. Among the molecular network, clusters of cycloartane-type triterpenoids were classified using the ClassyFire module of MolNetEnhancer, and their structures were predicted by the in silico fragment analysis tool, Network Annotation Propagation (NAP). Using mass spectrometry (MS)-guided isolation methods, six cycloartane-type triterpenoids (1−6) were isolated, and their structures were elucidated based on the interpretation of NMR, HRESIMS, and single-crystal X-ray diffraction. Among the isolates, compounds 1 and 4, which have an α,β-unsaturated carbonyl moiety on the A-ring, exhibited significant inhibitory effects on LPS-induced nitric oxide production in RAW264.7 cells with IC$_{50}$ values of 12.4 and 11.8 μM, respectively.

INTRODUCTION

The rhizomes of *Curculigo orchioides* Gaertn. (Amaryllidaceae) have been used as a traditional folk medicine in Asia for the treatment of impotence, jaundice, asthma, decline in physical strength, and arthritis. In the previous phytochemical studies of the genus *Curculigo*, phenolic compounds including chlorophenolic glycosides, lignans, and triterpenoidal saponins have been reported as the main bioactive constituents. Various pharmacological investigations have revealed the broad biological activities of the extracts and isolates. The ethanol extract of *C. orchioides* and isolated phenolic compounds such as curculigoside A, curculigoside B, curculigine A, and curculigine D exhibited antiosteoporotic activity. In addition, cycloartane glycosides exhibited cytotoxic activity against HL-60 human promyelocytic leukemia cells. As part of an ongoing research program aimed at discovering new bioactive constituents in medicinal plants, the MeOH extract of the rhizomes of *C. orchioides* and the subsequent CH$_2$Cl$_2$-soluble fraction were found to possess inhibitory effect against the lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW264.7 cells (IC$_{50}$ = 27.7 and 20.0 μg/mL, respectively).

Liquid chromatography high-resolution mass spectrometry (LC-HRMS/MS)-based molecular networking analysis by the Global Natural Products Social (GNPS) molecular networking web platform efficiently organized and visualized the cluster of structurally related compounds. Its rapidly growing social library makes it possible to predict the structure of an extract before intensive chromatographic isolation and structure determination. In addition, a spectral library could be propagated using in silico analysis tools such as the Network Annotation Propagation (NAP). Recently, Nasir et al. applied a combination of molecular networking and LC-MS/MS profiling to annotate phenolic glycosides and norlignans in the rhizomes and leaf extract of *C. latifolia*. However, the molecular network of the CH$_2$Cl$_2$-soluble fraction of *C. orchioides* showed a different pattern, with only a few compounds matching the GNPS library.

In the present study, MolNetEnhancer, which integrates outputs from molecular networking, MS2LDA, in silico tools (NAP or DEREPLICATOR), and automated chemical classification through ClassyFire, was applied to the generated molecular networking analysis. The clusters of triterpenoids were classified by MolNetEnhancer, and the detailed structure
was predicted to be cycloartane by NAP. Through targeted isolation of these clusters, six cycloartane-type triterpenoids (1–6), including five new compounds, were isolated. Herein, we report the molecular networking-guided isolation and structure determination of compounds 1–6, as well as the structure–activity relationship (SAR) of their inhibitory effects against LPS-induced nitric oxide production in RAW264.7 cells.

RESULTS AND DISCUSSION

The LC-HRMS/MS data of the CH₂Cl₂-soluble fraction of C. orchioides were analyzed by classical molecular networking via the GNPS web platform (https://gnps.ucsd.edu). At first, only one node in cluster 1 matched with 1,2-dihydroxyheptadec-16-en-4-yl acetate in the GNPS spectrum library, but the spectrum similarity was not good, with a mass difference of 94.09 and cosine score of 0.75 (Figure 1A). Therefore, an in silico fragment analysis tool (NAP) combined with the structure

Figure 1. Molecular networking analysis of the CH₂Cl₂-soluble fraction of C. orchioides. (A) Spectrum match of the node of molecular networking with GNPS library. (B) Structures of top ranked NAP candidates using GNPS and SUPNAT library. (C) Automatic classification and visualization of each cluster by the MolNetEnhancer. The chemical class of the largest (the cluster filled with red color) clusters were revealed as triterpenoids. The singleton node was excluded in this figure.
library of GNPS and SUPER NATURAL 2 (SUPNAT) (Figure 1B) was used to conduct analysis. The results of classical molecular networking and NAP were integrated by MolNetEnhancer of the GNPS web platform, which automatically classified the chemical class of each cluster (Figure 1C). Among them, the largest cluster (cluster 1) revealed as that of triterpenoids and the result of NAP also predicted the structure of each node as various triterpenoids containing cycloartane types. Because many cycoartane-type saponins have been previously reported in C. ochroides, it was reasonable to select cycoartane-type triterpenoids among the predicted candidates. In addition, only a few aglycones were reported, and many of which are produced through hydrolysis from isolated saponins. Therefore, cluster 1 was expected to contain new cycloartane-type triterpenoids. Twelve subfractions from silica gel chromatography were tested for their inhibitory effects on NO production in LPS-induced RAW264.7 cells, and the defined subfraction COC9, containing triterpenoids, showed the most potent inhibitory effects (Figure S2). Therefore, this fraction was investigated.

Compound 1 was isolated as a white amorphous powder. Its molecular formula was assigned as C_{30}H_{48}O_{4} based on HRESIMS data (m/z 473.3624 [M + H]^+; calc 473.3625), indicating seven indices of hydrogen deficiency. The ^1H NMR data for 1 displayed two olefinic protons (δ_H 6.80, 5.96), three oxymethine protons (δ_H 4.57, 3.97, 3.39), four tertiary methyl (δ_H 1.10, 0.93, 0.91, 0.89), and three methyl protons (δ_H 1.10, 0.93 × 2) (Table 1). The ^13C NMR and HSQC spectra exhibited 30 carbon signals, including one carbonyl, two olefinic carbons, five quaternary carbons, seven methylenes, eight methines, and seven methyl groups (Table 2). The ^1H and ^13C NMR data of 1 were similar to those of 12α,16β-dihydroxycycoartane-3,24-dione, except for the presence of a double bond and an additional oxymethine

**Table 1.** ^1H NMR (400^a, 500^b, and 800^c MHz) Data for Compounds 1—6^d

| no. | 1^b | 2^b | 3^b | 4^b | 5^b | 6^b |
|-----|-----|-----|-----|-----|-----|-----|
| 1   | 6.80 (d, 10.1) | 6.80 (d, 10.1) | 1.77 (m) | 6.78 (d, 10.1) | 2.36 (m) | 1.58 (m) |
| 2   | 5.96 (d, 10.0) | 5.95 (d, 10.0) | 2.73 (m) | 5.95 (d, 10.1) | 2.76 (m) | 1.78 (m) |
| 3   | 2.14 (m) | 2.17 (m) | 1.68 (dd, 4.3, 12.5) | 2.07 (dd, 6.9, 10.2) | 1.72 (m) | 1.32 (m) |
| 6   | 1.67 (m) | 1.66 (m) | 1.45 (m) | 1.60 (m) | 1.56 (m) | 1.66 (m) |
| 7   | 1.47 (m) | 1.47 (m) | 1.38 (m) | 1.56 (m) | 1.40 (m) | 1.34 (m) |
| 8   | 1.79 (m) | 1.81 (dd, 5.1, 12.4) | 1.65 (dd, 4.3, 13.0) | 2.13 (dd, 3.9, 12.7) | 1.69 (m) | 1.49 (m) |
| 9   | 2.12 (m) | 2.11 (m) | 2.40 (m) | 1.96 (m) | 2.09 (m) | 1.96 (m) |
| 10  | 1.97 (m) | 1.99 (m) | 1.93 (m) | 1.54 (m) | 1.19 (m) | 1.76 (m) |
| 11  | 3.97 (dd, 5.6, 9.1) | 3.94 (dd, 5.6, 9.1) | 4.21 (m) | 1.65 (m) | 1.83 (m) | 3.87 (dd, 6.0, 9.6) |
| 12  | 2.06 (m) | 2.03 (m) | 2.27 (dd, 8.1, 12.9) | 2.01 (dd, 8.1, 13.1) | 2.02 (m) | 2.06 (dd, 8.1, 13.2) |
| 13  | 1.45 (m) | 1.50 (m) | 1.86 (m) | 1.46 (dd, 4.7, 13.1) | 1.47 (m) | 1.41 (dd, 4.2, 13.2) |
| 14  | 4.57 (dd, d, 2.8, 8.0, 11.6) | 4.64 (dd, 2.8, 7.7, 12.0) | 4.91 (dd, 2.8, 7.8, 12.9) | 4.47 (dd, 2.5, 7.5, 12.5) | 4.48 (dd, 2.9, 7.8, 12.0) | 4.56 (dd, 2.8, 7.5, 12.6) |
| 15  | 2.20 (dd, 8.0, 11.6) | 2.13 (m) | 2.91 (dd, 7.8, 11.0) | 1.89 (dd, 7.5, 11.0) | 1.89 (m) | 2.18 (dd, 7.5, 11.2) |
| 16  | 1.09 (s) | 1.08 (s) | 1.48 (s) | 1.19 (s) | 1.23 (s) | 1.08 (s) |
| 17  | 1.25 (m) | 1.25 (d, 4.6) | 0.65 (d, 4.0) | 1.32 (d, 4.6) | 0.83 (d, 4.2) | 0.55 (d, 4.3) |
| 18  | 0.95 (m) | 0.94 (d, 4.6) | 0.59 (d, 4.3) | 0.79 (d, 4.6) | 0.60 (d, 4.3) | 0.44 (d, 4.4) |
| 19  | 1.89 (m) | 1.24 (m) | 2.55 (m) | 2.33 (m) | 2.29 (m) | 1.87 (m) |
| 20  | 1.10 (d, 6.5) | 1.06 (d, 6.8) | 1.52 (d, 6.7) | 0.95 (d, 7.0) | 0.95 (d, 7.1) | 1.08 (d, 7.1) |
| 21  | 1.75 (m) | 2.07 (m) | 2.42 (m) | 4.06 (dt, 2.2, 10.1) | 4.06 (dt, 2.2, 9.8) | 2.36 (m) |
| 22  | 1.19 (m) | 1.08 (m) | 1.89 (m) | 1.75 (m) | 1.76 (m) | 1.86 (m) |
| 23  | 1.72 (m) | 2.77 (m) | 1.81 (m) | 1.68 (m) | 1.67 (m) | 1.72 (m) |
| 24  | 3.39 (m) | 3.68 (m) | 3.68 (m) | 3.63 (m) | 3.64 (m) | 3.39 (m) |
| 25  | 1.67 (m) | 2.64 (sept, 6.9) | 1.58 (m) | 1.84 (m) | 1.81 (m) | 1.65 (m) |
| 26  | 0.91 (d, 6.8) | 1.13 (d, 6.8) | 1.12 (d, 6.8) | 1.01 (d, 6.6) | 1.01 (d, 6.6) | 0.93 (d, 6.8) |
| 27  | 0.91 (d, 6.8) | 1.12 (d, 6.8) | 1.10 (d, 6.8) | 0.90 (d, 6.8) | 0.91 (d, 6.8) | 0.91 (d, 6.8) |
| 28  | 1.03 (s) | 1.04 (s) | 1.34 (s) | 0.92 (s) | 0.92 (s) | 0.99 (s) |
| 29  | 1.12 (s) | 1.12 (s) | 1.17 (s) | 1.11 (s) | 1.11 (s) | 0.98 (s) |
| 30  | 0.97 (s) | 0.97 (s) | 1.07 (s) | 0.97 (s) | 1.05 (s) | 0.81 (s) |

^aAssignments were based on COSY and HSQC experiments. Compound 3 was dissolved with pyridine-d_5 and others used CDCl_3.
instead of a carbonyl group. The position of the αβ-
unsaturated carbonyl group at the C-1/C-3 was determined
by the HMBC correlations from H-19 (δH 1.25 and 0.95) to
olefinic carbon (δC 153.9) and from CH3-29 and CH3-30 (δH
1.12 and 0.97) to a carbonyl carbon (δC 205.1). The proton
signals of H-19 (δH 1.25 and 0.95) of 1 were shifted downfield
compared with those of 3 (δH 0.65 and 0.59) because of the
anisotropic effect caused by the alkene group between C-1 and
C-2. Furthermore, through the HMBC correlations from CH3-18
(δH 1.09) to C-12 (δC 73.1) and from H2-15 (δH 2.06, 1.45)
and H-20 (δH 1.89) to C-16 (δC 72.2), and from H-25 (δH
1.67), CH3-26 (δH 0.93), and CH2-27 (δH 0.93) to C-24 (δC
78.7), the planar structure of 1 was determined to be 12,16,24-
trihydroxyxycloartane-1-en-3-one (Figure 2). The relative
configuration of 1 was deduced from the NOESY experiment.

The NOE correlations of H-19/30-CH3, H-6β, H-8, H-8/18-
CH3, and 18-CH3/H-20 revealed the β-axial orientations of H-
8, 18-CH3, and H-20. In addition, the NOE correlations
between H-16 and H-17 and 28-CH3 revealed their α-
orientations (Figure 2). Single-crystal X-ray diffraction analysis
with Cu Kα radiation [Flack parameter = 0.00(5) and Hooft
parameter = 0.02(4)] clearly corroborated the absolute
configuration of 1 (Figure 3). Thus, the structure of 1 was
determined to be (S)R,8S,9R,10S,12S,13R,14R,15S,16S,17R,20R,24S)-12,16,24-trihydroxyxycloartane-1-en-3-one and named curculigone A.

Compound 2 was obtained as a white amorphous powder.
The molecular formula of 2 was deduced from the HRESIMS
data as C30H46O4 (m/z 471.3467 [M + H]+; calcld 471.3469),
indicating eight indices of hydrogen deficiency. A comparison

The relative configuration of 1 was deduced from the NOESY experiment.
Figure 3. X-ray ORTEP plot for the molecular structure of compound 1.

The relative configuration of 2 was determined to be the same as that of 1 based on their similar NOE correlations (Figure 4). Furthermore, single-crystal X-ray diffraction analysis using Cu Kα radiation was performed on 2, and the Flack parameter = −0.14(8) and Hooft parameter = 0.07(4) permitted the absolute configuration (Figure 5). Thus, the structure of 2 was determined to be (5S,8S,9R,10S,12S,13R,14S,16S,17R,20R)-12,16-dihydroxyoctacarate-1-en-3,24-one and named curculigone B.

Compound 3 was obtained as a white amorphous powder. Its molecular formula was determined by the HRESIMS data 

\[ \text{C}_{30} \text{H}_{48} \text{O}_{4} \text{ (m/z) 497.3598 [M + Na], calcd 497.3601}, \]

indicating six indices of hydrogen deficiency, one less than that of 1. In the \(^1\text{H}\) NMR and \(^{13}\text{C}\) NMR data, the signals corresponding to the double bond disappeared, indicating the reduction of the double bond between C-1 and C-2 in 1 (Tables 1 and 2). The positions of the carbonyl group and three hydroxy groups were determined by the HMBC correlations of H-8 (δH 1.84), CH-30/C-24 (δC 71.73), H-19 (δH 1.32) and CH-26 (δC 163.31), whereas that of 1, the dihedral angle between H-20 and H-22 was -163.3°, whereas that of the model of 20S*,22R* was 69.4°. Thus, the configurations of 2 were elucidated by the ROESY experiment with the aid of computational 3D modeling after MM2 energy minimization (Figure 8). As with compounds 1–3, the relative configurations of H-8β, CH-18β, CH-30α, H-5α, and CH-28α were deduced from their ROESY correlations. In addition, the large coupling constant between H-17 and H-20 (J = 11.0 Hz), together with the ROESY correlation between CH-18β and H-20, clearly determined the β-configuration of H-20. The coupling constant between H-20 and H-22 exhibited a large value (J = 10.1 Hz), and two possible configurations were simulated using computational 3D modeling for the preferred match. In the case of the 3D model of 20S*,22R*, the dihedral angle between H-20 and H-22 was -163.3°, whereas that of the model of 20S*,22R* was 69.4°. Thus, the configurations of H-20 and H-22 were determined to be 20S*,22R* according to the Karplus equation (Figure 8A). Furthermore, in the computational model for the 22R*,24R* configuration, the distance between H-22 and H-24 was 3.83 Å, which is too far to show the NOE effect. For 22R*,24S*, the distance of H-22 and H-24 (2.66 Å) corresponded with the observed ROESY correlation between H-22 (δH 4.06) and H-24 (δH 3.63) (Figure 8B). The optical rotation of compound 4 was
measured as +3.5 (c 0.5, methanol), which has the same polarity as compounds 1–3. Therefore, the structure of 4 was determined as (5R,8S,9S,10S,13R,14S,16S,17R,20S,22R,24S)-16,22,24-trihydroxycycloartenol-1-en-3-one and named curculigone D. Compound 5 was obtained as a white amorphous powder and assigned the molecular formula C_{30}H_{46}O_8 based on its HRESIMS data (m/z 475.3777 [M + H]^+, calcd 475.3782) and ^{13}C NMR data. With one less hydrogen deficiency than 4, along with the disappearance of the olefinic moiety in ^1H and ^{13}C NMR data revealed the reduction of the double bond between C-1 and C-2 of 4 (Tables 1 and 2). The planar structure of 5 was determined to be 16,22,24-trihydroxycycloartenol-3-one by HMBC experiment. The large coupling constant between H-20 and H-22 (J = 9.8 Hz) matched well with the 3D modeling for 20^R,22^R,24^S (2.66 Å) rather than 22^R,24^R,20^S (3.83 Å) (Figure 9). The positive optical rotation value of +5.9 (c 0.5, methanol) confirmed the absolute configuration of 5. Therefore, the structure of 5 was determined as (5R,8S,9S,10R,13R,14S,16S,17R,20S,22R,24S)-16,22,24-trihydroxycycloartenol-3-one and named curculigone D.

Compound 6 was obtained as a white amorphous powder. Its molecular formula was determined by the HRESIMS data as C_{30}H_{42}O_8 (m/z 499.3757 [M + Na]^+; calcd 499.3758). The ^1H and ^{13}C NMR data of 6 resembled those of 3, except for the absence of the carbonyl group and the presence of an additional hydroxyl group (Tables 1 and 2). These data were consistent with those of (24S)-9,19-cyclolanostan-3β,12α,16β,24-tetrol, which was produced by the enzymatic hydrolysis of (24S)-12α,16β,24-trihydroxy-9,19-cyclolanostan-3β-hydroxyl-β-D-glucopyranoside. After structure determination, the clusters classified as triterpenoids by MolNetEnhancer were annotated according to their structures (Figure 10). The structures predicted by the NAP module with GNPS and the SUPNAT library were close with the actual structure, except for the position of double bond, and/or the number of hydroxyl group. The isolated compounds were mostly present as adducts of [M + H – H_2O]^+ or [M + H – 2H_2O]^+, whereas most of the predicted candidates were present as protonated or a sodium adduct in the spectrum library. This gap in the adducts may result in a difference between predicted and actual structures.

All compounds were tested for their inhibitory effects on LPS-induced NO production in RAW264.7 macrophages using aminoguanidine as a positive control (Table 3). The cytotoxicity of the compounds was examined using the MTT reagent, and none of the test compounds showed significant cytotoxicity at their effective concentrations for the inhibition of NO production (Figure S41, Supporting Information). A comparison of the IC_{50} values of compounds 1–6 suggested the α,β-unsaturated carbonyl moiety on the A-ring was critical for NO inhibition. The lack of an inhibitory effect on NO production in compound 2, despite having an α,β-unsaturated carbonyl moiety in the A-ring, could be due to the introduction of an additional carbonyl group at C-24.

In conclusion, the MolNetEnhancer module, coupled with the in silico fragment analysis tool NAP, was applied to the

Figure 6. Key HMBC, COSY, and NOESY correlations of compound 3.

Figure 7. X-ray ORTEP plot for the molecular structure of compound 3.
of C. orchioides may have potential for the treatment of inflammation-associated diseases and are worthy of further study for their mechanism of action.

## EXPERIMENTAL SECTION

**General Experimental Procedures.** The optical rotations were measured using a JASCO DIP-1000 polarimeter. UV and IR spectra were obtained using a JASCO UV-550 spectrophotometer and JASCO FT-IR 4100 spectrometer, respectively. NMR spectra were recorded on Bruker AVANCE 400, 500, and 800 MHz spectrometers using CDCl₃ and pyridine-d₅ as solvents. HR-ESI-MS and UPLC-MS/MS analyses were performed using an Orbitrap Exploris 120 mass spectrometer coupled with a Vanquish UHPLC system (ThermoFisher Scientific). Chromatographic elution was conducted on a YMC Triart C₁₈ (100 × 2.1 mm, 1.9 μm, 0.3 mL/min) column at a temperature of 30 °C, with a mobile phase of water +0.1% formic acid (A) and CH₃CN + 0.1% formic acid (B), and the gradient consisted of a linear gradient of 10—100% B (0–10 min). Mass detection was performed in the m/z range of 200–2000, and the resolution of the Orbitrap mass analyzer was fixed at 60,000 for the full MS scan and 15,000 for the data-dependent MS² scan. The following parameters were used during MS measurements: a spray voltage of 3.5/2.5 kV for the positive/negative modes, ion transfer tube temperature of 320 °C, HESI probe vaporizer temperature of 275 °C, RF lens 70 (%). Ultrapure nitrogen (>99.999%) was used as both sheath and auxiliary gas of the HESI probe and set to 50 and 15 arb, respectively. A normalized higher-energy collision dissociation energy of 30% was used for the collision of ions in the Orbitrap detector. MS/MS fragmentation was obtained by the data-dependent MSⁿ mode to obtain an MS³ spectrum of the four most intense ions, with a dynamic exclusion filter to exclude the repeated fragmentation of ions within 2.5 s after acquiring the MS² spectrum. Column chromatography was performed using a silica gel column (Merck, 70-230 mesh). MPLC was performed using a Biotage Isolera Prime chromatography system with Lichroprep RP-18 (Merck, 40–63 μm). Preparative HPLC was performed using a Waters HPLC system equipped with two Waters 515 pumps, a 2996 photodiode array detector, and a YMC J’sphere ODS H-80 column (4 μm, 150 × 20 mm, i.d., flow rate 6.0 mL/min). Thin layer chromatography was performed using precoated silica gel 60 F₂₅₄ (0.25 mm, Merck) plates, and spots were detected using a 10% vanillin-H₂SO₄ aqueous spray reagent.

**Plant Material.** Dried rhizomes of C. orchioides were purchased from the Kyungdong herbal market (Seoul, South Korea) in May 2021 and were identified by one of the authors (B.Y.H.). A voucher specimen (CBNU-2021-05-CO) was deposited at the Herbarium of the College of Pharmacy, Chungbuk National University, South Korea.

**Molecular Networking-Based Analysis.** LC-MS/MS data were uploaded to the GNPS (http://gnps.ucsd.edu) for classical molecular networking. The product ion and fragment ion mass tolerances were set to 0.02 Da. The edge between the nodes was created when they displayed cosine scores above 0.7 and more than six matched peaks. Furthermore, these edges were kept in the network if and only if each of the nodes appeared in each of the top 10 most similar nodes. The network was downloaded and visualized using Cytoscape 3.8.2,²² and the NAP tool in the GNPS web platform was used for in silico analysis of the molecular network. The following parameters were used for this purpose: 10 first candidates,
mass tolerance of 5 ppm, database of GNPS and SUPNAT2 (Supernatural II). After analysis, the resulting file was exported to Cytoscape, and the structures were visualized using the ChemViz2 plug-in. The workflow and results of classical molecular networking and NAP could be browsed on the following GNPS Web site links:

https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=e68dc185ec488972286f5e97f1b8
https://proteomics2.ucsd.edu/ProteoSAFe/status.jsp?task=d5b5890d96d4b4b85d42566c7e59f16

The MolNetEnhancer workflow was conducted via “Advanced Views-Experimental Views” tab of the result of classical networking with the job ID of NAP above. The result (https://proteomics2.ucsd.edu/ProteoSAFe/status.jsp?task=d5b5890d96d4b4b85d42566c7e59f16) was downloaded and imported to Cytoscape, and the color of the clusters was automatically filled upon the chemical subclass.

**Extraction and Isolation.** The dried rhizomes of *C. orchioides* (3.0 kg) were extracted with MeOH (3 × 18 L) by maceration for 3 days at room temperature (25 °C) and then filtered and evaporated under reduced pressure to obtain a MeOH extract. A suspension of the extract (150 g) in distilled water was partitioned sequentially with n-hexane (2 × 2 L), CH₂Cl₂ (2 × 2 L), EtOAc (2 × 2 L), and n-BuOH (2 × 2 L). The CH₂Cl₂-soluble fraction (8.3 g) was subjected to silica gel column chromatography and eluted using a CH₂Cl₂−MeOH gradient system (30:1 to 0:1) to yield 12 fractions (COC1−COC12). COC9 (2.5 g) was further separated by RP-MPLC and eluted with a MeOH−H₂O gradient system (20:80 to 100:0) to obtain 18 subfractions (COC9-1−COC9-18). COC9-12 (134.2 mg) was further purified by preparative HPLC (MeCN−H₂O, 57:43, isocratic) to yield (24S)-9,19-cyclolanostane-3β,12α,16β,24-tetrol (6) (tᵣ = 22.5 min, 2.1 mg), compound 1 (tᵣ = 27.1 min, 4.6 mg), and compound 2 (tᵣ = 49.0 min, 12.2 mg). COC9-13 (116.8 mg) was isolated by preparative HPLC (MeCN−H₂O, 55:45, isocratic) to obtain compounds 4 (tᵣ = 41.5 min, 1.6 mg) and 3 (tᵣ = 43.0 min, 7.2 mg). COC9-14 (80.4 mg) was purified using preparative HPLC (MeCN−H₂O, 65:35, isocratic) to yield compound 5 (tᵣ = 32.8 min, 2.5 mg).

**Curculigone A (1):** White amorphous powder; [α]D²⁵ +5.5 (c 1.0, MeOH); UV (MeOH) λ_max 270 nm; IR ν_max (film)

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Table 3. Inhibitory Effects of Compounds 1−6 against LPS-Induced NO Production in RAW264.7 Cells and Their Cytotoxicity

| compound | IC₅₀ (µM)ᵇ | CC₅₀ (µM)ᶜ |
|----------|------------|------------|
| 1        | 12.4 ± 0.9 | 76.7 ± 1.0 |
| 2        | >100       | >100       |
| 3        | >100       | >100       |
| 4        | 11.8 ± 1.2 | 66.9 ± 3.1 |
| 5        | 29.4 ± 0.2 | 89.1 ± 2.6 |
| 6        | 41.7 ± 0.7 | 54.8 ± 4.6 |

ᵇIC₅₀: 50% inhibitory concentration. ᵇIC₅₀: 50% cytotoxic concentration.

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Figure 10. Annotation of compounds 1−6 on the triterpenoid clusters.
3390, 2956, 2925, 2854, 1705, 1384, and 1102 cm⁻¹; 1H NMR (400 MHz, CDCl₃) and ¹³C NMR (100 MHz, CDCl₃), see Tables 1 and 2; HRESIMS m/z 473.3624 [M + H]^+ (calcd for C₀₉H₁₃O₄, 473.3625).

**Curculigone B (2):** White amorphous powder; [α]D° 25 +3.2 (c 0.5, MeOH); UV (MeOH) λmax 269.2 nm; IR νmax (film) 3398, 2920, 2915, 2856, 1711, 1705, 1384, and 1102 cm⁻¹; 1H NMR (400 MHz, CDCl₃) and ¹³C NMR (100 MHz, CDCl₃), see Tables 1 and 2; HRESIMS m/z 473.3467 [M + H]^+ (calcd for C₀₉H₁₃O₄, 473.3469).

**Curculigone C (3):** White amorphous powder; [α]D° 25 +14.3 (c 1.0, MeOH); UV (MeOH) λmax 210 nm; IR νmax (film) 3405, 2951, 2925, 2848, 1710, and 1036 cm⁻¹; 1H NMR (500 MHz, pyridine-d₅) and ¹³C NMR (125 MHz, pyridine-d₅), see Tables 1 and 2; HRESIMS m/z 497.3598 [M + Na]^+ (calcd for C₁₅H₂₁N₂O₄, 497.3601).

**Curculigone D (4):** White amorphous powder; [α]D° 25 +3.5 (c 0.5, MeOH); UV (MeOH) λmax 270 nm; IR νmax (film) 3393, 2947, 2922, 2858, 1704, and 1106 cm⁻¹; 1H NMR (800 MHz, CDCl₃) and ¹³C NMR (200 MHz, CDCl₃), see Tables 1 and 2; HRESIMS m/z 473.3623 [M + H]^+ (calcd for C₀₉H₁₃O₄, 473.3625).

**Curculigone E (5):** White amorphous powder; [α]D° 25 +5.9 (c 0.5, MeOH); UV (MeOH) λmax 210 nm; IR νmax (film) 3383, 2957, 2930, 2850, 1709, and 1098 cm⁻¹; 1H NMR (400 MHz, CDCl₃) and ¹³C NMR (100 MHz, CDCl₃), see Tables 1 and 2; HRESIMS m/z 475.3777 [M + H]^+ (calcd for C₀₉H₁₃O₄, 475.3782).

(24S)-9,19-Cyclooctanone-3β,12α,16β,24-tetrol (6): White amorphous powder; [α]D° +26.0 (c 1.0, MeOH); UV (MeOH) λmax 224 nm; IR νmax (film) 3365, 2957, 2925, 2848, and 1036 cm⁻¹; 1H NMR (400 MHz, CDCl₃) and ¹³C NMR (100 MHz, CDCl₃), see Tables 1 and 2; HRESIMS m/z 499.3757 [M + Na]^+ (calcd for C₁₅H₂₁N₂O₄, 499.3758).

**X-ray Crystallographic Analysis of Compounds 1–3.** Single crystals of compounds 1–3 were recrystallized by an evaporation method with MeOH and analyzed using a Bruker D8 Venture diffractometer equipped with a monochromatic fine-focus Cu Kα (λ = 1.54178 Å) radiation source. Data collection was carried out using a PHOTON 100 CMOS detector at 223(2) K using the APEX2 software (Bruker AXS Inc.). The crystal structure was refined by full-matrix least-squares refinement using the SHELXL-2014 computer program. Further analysis of the properties of the single crystals was performed using PLATON. Molecular graphics were computed using the Mercury 4.2 software. Crystallographic data for 1–3 were deposited in the Cambridge Crystallographic Data Centre (deposition numbers CCDC 2173493, CCDC 2173494, and CCDC 2173491. These data can be obtained free of charge at www.ccdc.cam.ac.uk.

**Crystal Data of Curculigone A (1):** Monoclinic crystal, C₀₉H₁₃O₄, Mᵣ = 490.70, size 0.155 × 0.070 × 0.055 mm³, a = 13.4533(4) Å, b = 7.5536(2) Å, c = 13.6495(3) Å, α = 90.00⁰, β = 92.5160(10)⁰, γ = 90.00⁰, V = 1385.74(7) Å³, T = 223(2) K, space group P2₁2₁2₁, Z = 2, μ = 0.613 mm⁻¹; 29287 collected reflections, 5803 independent reflections (Rint = 0.0275), R1 (all data) = 0.0445, wR2 (all data) = 0.1252. The absolute structure was determined by the Flack parameter x = 0.00(5) and Bijvoet pair analysis by Hooft method (Hooft y = 0.02(4), P2 (true) = 1.000, P3 (true) = 1.000, P3 (racemic twin) = 0.000).

**Crystal Data of Curculigone B (2):** Orthorhombic crystal, C₀₉H₁₆O₄, Mᵣ = 470.67, size 0.564 × 0.053 × 0.030 mm³, a = 7.9126(2) Å, b = 31.3030(7) Å, c = 31.6212(8) Å, α = 90.00⁰, β = 90.00⁰, γ = 90.00⁰, V = 7832.3(3) Å³, T = 223(2) K, space group P2₁2₁2₁, Z = 12, μ = 0.604 mm⁻¹; 91532 collected reflections, 16472 independent reflections (Rint = 0.0714), R1 (all data) = 0.0738, wR2 (all data) = 0.1569. The absolute structure was determined by the Flack parameter x = −0.14(8) and Bijvoet pair analysis by Hooft method (Hooft y = −0.16(7), P2 (true) = 1.000, P3 (true) = 1.000, P3 (racemic twin) = 0.000).

**Crystal Data of Curculigone C (3):** Monoclinic crystal, C₁₅H₂₁N₂O₄, Mᵣ = 506.74, size 0.600 × 0.097 × 0.088 mm³, a = 13.4720(15) Å, b = 7.5719(6) Å, c = 13.7835(9) Å, α = 90.00⁰, β = 91.428(6)⁰, γ = 90.00⁰, V = 1405.6(6) Å³, T = 223(2) K, space group P2₁2₁2₁, Z = 2, μ = 0.618 mm⁻¹; 25265 collected reflections, 5828 independent reflections (Rint = 0.0281), R1 (all data) = 0.0432, wR2 (all data) = 0.1242. The absolute structure was determined by the Flack parameter x = 0.08(5) and Bijvoet pair analysis by Hooft method (Hooft y = 0.07(4), P2 (true) = 1.000, P3 (true) = 1.000, P3 (racemic twin) = 0.000).

**Measurement of LPS-Induced Nitric Oxide Production and Cell Viability.** As an indicator of NO synthesis, the nitrite concentration in the supernatant of RAW264.7 cells was measured according to the Griess reaction following a previously reported protocol. Cell viability of the remaining cells was determined by a MTT (Sigma Chemical Co., St. Louis, MO)-based colorimetric assay.²³

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**ASSOCIATED CONTENT**

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c03243.

1D NMR, 2D NMR, and HRESIMS of compounds 1–6 (PDF)
X-ray crystallography data of 1 (CIF)
X-ray crystallography data of 2 (CIF)
X-ray crystallography data of 3 (CIF)

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