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Article

Cyclohumulanoid Sesquiterpenes Induced by the Noncompetitive Coculture of *Phellinus orientoasiaticus* and *Xylodon flaviporus*

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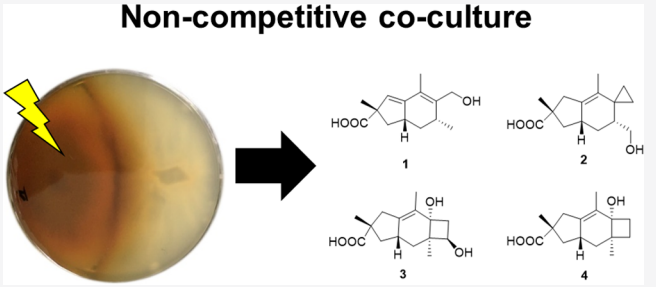
ABSTRACT: Microbial cocultivation has been applied as a strategy to induce the biosynthesis of specialized metabolites. However, most previous studies have focused on competitive interactions between test strains. During our LC-MS-based chemical screening of randomized cocultures of Basidiomycetous fungi, we discovered that the coculture of *Phellinus orientoasiaticus* (Hymenochaetaceae) and *Xylodon flaviporus* (Schizoporaceae) induces multiple metabolites, although they did not show any competitive morphology. Targeted isolation yielded three new sesquiterpenes (**1**−**3**) along with five known analogues (**4**−**8**). The structures of the isolates were determined by MS and NMR experiments as well as electronic circular dichroism analysis. LC-MS analysis suggested that cyclohumulanoids of illudane-, sterpurane-, and tremulane-type scaffolds (**1**−**7**) were produced by *P. orientoasiaticus*, whereas a drimane-type sesquiterpene (**8**) was produced by *X. flaviporus*. None of the isolates exhibited antifungal activity or cytotoxicity, and compounds **1**−**7** exhibited NO production of LPS-treated RAW276.4 cells in a range of 15.9% to 38.0% at 100 μM.



Fungal specialized metabolites have been an important resource for drug discovery. Basidiomycota is the second largest division of the kingdom Fungi, comprising approximately 35 000 species, and includes mushroom-forming fungi. Basidiomycetes have been overlooked as a resource for natural product discovery, especially compared to the Ascomycota, the largest division, which contains the genera *Aspergillus*, *Penicillium*, and *Fusarium*.[1] This may be due to challenges in genomic approaches and biomass acquisition, as highlighted previously.[2,3] However, previous studies on medicinal and poisonous mushrooms revealed the richness of bioactive terpenoids in Basidiomycetes,[4−6] which was supported by the recent massive genome mining analysis of publicly available fungal genomes.[7]

Our previous studies demonstrated that LC-MS/MS-based untargeted metabolomics with mycelial cultures can efficiently reveal the uncharted chemical diversity of mushroom-forming fungi.[8] However, the study also revealed limitations of the culture-based approach, showing that a large part of chemical diversity is lost in the culture extract, compared to the wild fruiting bodies. This issue is not new to natural product chemists. Since the complete genome sequences of *Streptomyces coelicolor* and *S. avermitilis* were assembled,[9,10] it has been known that many components of microbial biosynthetic pathways are not activated in common lab cultivation environments. Activation of silent biosynthetic pathways has become a key solution for microbial natural product discovery, and many strategies have been introduced,[11] including microbial cocultivation. This is achieved by inducing chemical interactions with competing species.[12] Previous studies showed that microbial cocultures expand the structural diversity acquired from microorganisms and/or increase the quantity of biosynthesized metabolites.[13] As a follow-up study of our investigation on specialized metabolites of Basidiomycetes,[8] we performed cocultures of fungal strains to reveal how their metabolomes change. Due to the lack of knowledge on the ecological interactions between Basidiomycetous fungi occurring in nature, 40 species from two orders, Hymenochaetales and Polyporales (Table S1, Supporting Information), were randomly paired, to create 43 coculture plates. Among the plates, some exhibited antagonistic interactions between the two strains, while others did not show any growth inhibitory interfaces (Figure S1, Supporting Information); previous coculture studies tend to focus on the antagonistic phenotype because it implies the presence of antimicrobial metabolites[14,15] whose biosynthesis is induced by the encounter. However, chemical interactions between species can occur in a nonantagonistic manner, although they are not easily

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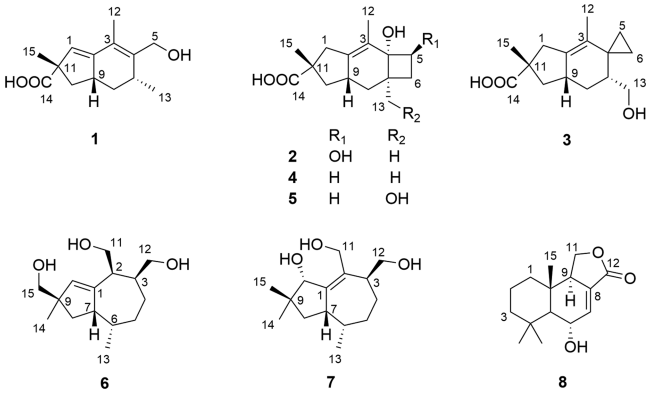
recognized by visual inspection. Thus, we analyzed all the plates with LC-MS/MS to determine if there was any change in the metabolome even for plates without antagonistic interactions. One of the nonantagonistic coculture plates, which consisted of *Phellinus orientoasiaticus* (Hymenochaetaceae) and *Xylodon flaviporus* (Schizoporaceae), exhibited the induction of many specialized metabolites not observed in axenic cultures of either species. Here we describe the targeted isolation and structural elucidation of sesquiterpenes produced under these conditions.



RESULTS AND DISCUSSION

On the coculture plate, *P. orientoasiaticus* and *X. flaviporus* encountered each other 10 days after inoculation and formed a brown-colored border on day 14 (Figure 1A). The LC-MS/MS analysis on a methanolic extract revealed that several metabolites were present at the borderline of the two fungi. Furthermore, most of them were not biosynthesized in axenic cultures (Figure 1B). These metabolites were not observed on day 10, which indicated that they were induced by the encounter between the cocultured strains (Figure S2, Supporting Information). Most induced metabolites were

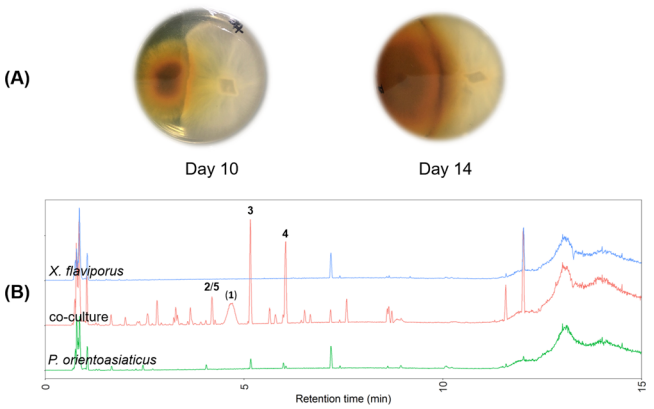


Figure 1. (A) Coculture plates of *P. orientoasiaticus* (left) and *X. flaviporus* (right), taken at 10 and 14 days postinoculation. (B) LC-MS base peak ion (BPI) chromatograms of the extracts of 14-day axenic and cocultures of *P. orientoasiaticus* and *X. flaviporus* (negative ion mode). The major chromatographic peaks isolated in this study are denoted with their corresponding compound numbers. Compounds **2** and **5** coeluted. The ion intensity for **1** was smaller than the one shown here; it coeluted with isobaric unidentified compounds. Compounds **6**–**8** were not detected in the negative ion mode.

deduced to be sesquiterpenes, because their molecular formula contained 15 carbons. Preliminary identification by analysis through the Global Natural Products Social Molecular Networking (GNPS)[16] did not provide any clues about the structures, mainly due to the scarcity of fragment ions, which suggested the rigidity of chemical structures.

To obtain abundant biomass for targeted isolation of induced metabolites, *P. orientoasiaticus* and *X. flaviporus* were cultured separately in liquid medium for 5 days and then mixed and incubated for a further 28 days. This was extracted using EtOAc, and the LC-MS analysis showed that the metabolome was similar to that obtained from solid medium (Figure S3, Supporting Information). Some induced compounds were detected in the axenic liquid cultures but with low ion intensities, supporting that coculture has accelerated the biosynthesis of these compounds (Figure S4, Supporting Information). Targeted isolation of the induced metabolites yielded eight sesquiterpenes (**1**–**8**). The planar structures of known compounds **4**–**8** were identified to be sterpuric acid (**4**), 13-hydroxysterpuric acid (**5**), tremul-1(10)-ene-11,12,14-triol (**6**), tremulene-10,11,12-triol (**7**), and 6-hydroxycinnamolide (**8**) by comparison of their NMR data with those reported in the literature.[17−20]

Compound **1** was suggested to be a nor-sesquiterpene derivative based on its molecular formula of $C_{14}H_{20}O_3$ indicated by HRESIMS and the occurrence of 14 carbon resonances in the ^{13}C NMR spectrum. The similarity between the ^{1}H and ^{13}C NMR spectra of **1** and those of **4**, especially the presence of three methyl groups [δ_H 1.89 (d, J = 2.0, H-12), 1.29 (s, H-15), and 1.15 (d, J = 7.0, H-13)] and one carboxylic acid group (δ_C 180.1, C-14) (Table 1), indicated that compound **1** is a nor-sterpurane derivative. The ^{1}H and ^{13}C NMR spectra of **1** also showed the presence of an olefinic methine (δ_C/δ_H 127.8/5.64) and three olefinic tertiary carbons (δ_C 146.8, 141.1, and 128.1). The HMBC correlations from H-15 to C-1 (δ_C 127.8), C-11 (δ_C 54.1) and C-14, from H-1 (δ_H 5.64) to C-2 (δ_C 146.8) and C-11, and from H-12 to C-2, C-3, and C-4 (δ_C 128.1) placed the conjugated diene group from C-1 to C-4. The HMBC correlations from the oxygenated methylene group [δ_H 4.32 (1H, d, J = 11.8) and 4.21 (1H, d, J = 11.8), H-5s] to C-3, C-4, and C-7 (δ_C 34.9) and from H-13 to C-4, C-7, and C-8 (δ_C 41.4) confirmed the planar structure of **1** and the absence of C-6, compared to the common structures of sterpurane-type sesquiterpenes (Figure 2A). A NOESY experiment showed NOE correlations between H-7 and H-9 and between H-9 and H-15, suggesting that these protons are placed in the same orientation from the plane of the ring (Figure 2B). Thus, compound **1** was identified as (7*R**,9*R**,11*S**)-6-nor-4-hydroxy-1,3(4)-distepuren-14-oic acid.

Compound **2** had the molecular formula $C_{15}H_{22}O_4$ indicated by HRESIMS. The ^{1}H and ^{13}C NMR data of **2** (Table 1) were similar to those of **4**, except for the presence of an oxygenated methine group (δ_C/δ_H 73.9/3.78). The $^{1}H-^{1}H$ COSY correlation between the oxygenated methine (H-5) and H-6 (δ_H 1.94 and 1.29) suggested that the cyclobutane of **2** is hydroxylated, and the HMBC correlations from H-6s to C-7 (δ_C 43.4), C-8 (δ_C 37.4), and C-13 (δ_C 25.4) and from H-5 to C-3 (δ_C 128.6) confirmed the position of the substitution as C-5. The NOESY NMR spectrum of **2** showed an NOE correlation between H-9 (δ_H 2.49) and H-15 (δ_H 1.32) but did not show any correlation between H-9 and H-13 (δ_H 1.21), which suggested the relative configuration of 7*R**,9*R**,11*S**.

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J. Nat. Prod. 2022, 85, 511−518

Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data of Compounds 1−3 in CD_3OD (500/125 MHz)

	1		2		3	
position	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
1α	127.8, CH	5.64, s	42.4, CH_2	2.86, d (17.4)	42.7, CH_2	2.80, d (16.8)
1β				2.24, d (17.4)		2.15, m
2	146.8, C		140.6, C		137.7, C	
3	141.1, C		128.6, C		128.0, C	
4	128.1, C		74.7, C		23.1, C	
5	59.8, CH_2	4.32, d (11.8)	73.9, CH	3.78, dd (6.8, 1.6)	9.1, CH_2	0.65, m
		4.21, d (11.8)				
6α			34.6, CH_2	1.29, dd (13.0, 1.6)	7.7, CH_2	0.55, m
6β				1.94, dd (13.0, 6.8)		
7	34.9, CH	2.59, br s	43.4, C		43.3, CH	1.82, m
8α	41.4, CH_2	1.04, m	37.4, CH_2	0.80, t-like	33.3, CH_2	1.02, dd (22.7, 11.2)
8β		2.06, m		1.56, dd (12.8, 5.7)		2.15, m
9	42.5, CH	2.77, m	37.7, CH	2.49, br s	40.9, CH	2.58, br s
10α	44.0, CH_2	1.90, m	45.4, CH_2	1.68, t-like	45.5, CH_2	1.60, t-like
10β		2.04, m		1.88, dd (11.9, 6.8)		1.82, m
11	54.1, C		48.1, C		48.5, C	
12	14.4, CH_3	1.89, d (2.0)	13.2, CH_3	1.63, d (1.1)	12.9, CH_3	1.26, m
13	20.5, CH_3	1.15, d (7.0)	25.4, CH_3	1.21, s	64.5, CH_2	3.34, dd (10.6, 4.3)
						3.12, dd (10.6, 7.9)
14	180.1, C		182.1, C		182.4, C	
15	24.2, CH_3	1.29, s	25.5, CH_3	1.32, s	25.5, CH_3	1.27, s

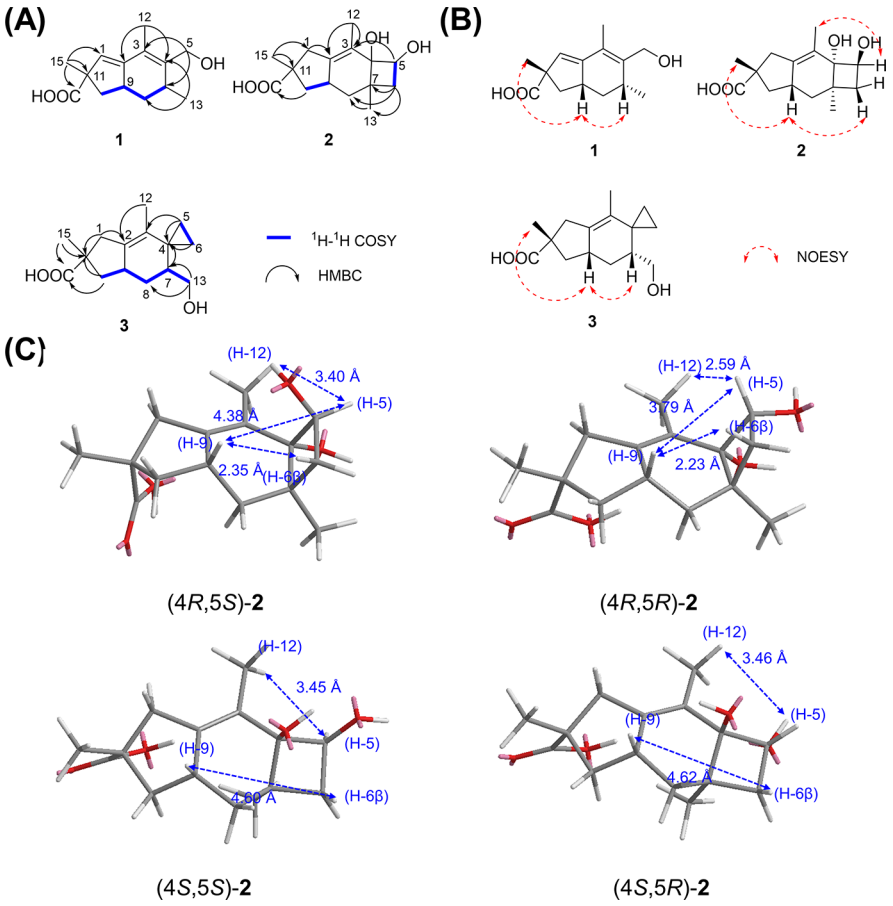

Figure 2. (A) Key ^{1}H−^{1}H COSY and HMBC correlations of compounds 1−3. (B) Key NOE correlations of 1−3. (C) MM2 energy-minimized 3D structural models of possible stereoisomers of 2. Estimated distances between H-5, H-9, H-6β, and H-12 are shown.

The relative configuration at C-4 was deduced to be 4R*, because the previous X-ray crystallographic analysis of 4 revealed the *cis*-fusion between the four- and six-membered rings of sterpurane-type sesquiterpenes.[17] This assumption was further supported by the NOESY experiment. The NOE correlation signal between H-9 (δ_H 2.49) and H-6a (δ_H 1.94)

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J. Nat. Prod. 2022, 85, 511−518

was observed (Figure 2B), and MM2 energy-minimized 3D structural models of possible isomers of **2** showed that this NOE correlation could be observed in the 4*R** isomer (Figure 2C). The relative configuration at C-5 was deduced from the NOESY spectrum. H-5 exhibited an NOE correlation with H-12 but not with H-9, which suggested that H-5 and H-9 were placed in a different direction from the plane of the fused ring system (Figure 2C). Therefore, the relative configuration of **2** was determined to be 4*R**,5*S**,7*R**,9*R**,11*S**.

The HRESIMS spectrum of **3** exhibited an HRESIMS peak at *m*/*z* 249.1487 $[M - H]^-$, indicating a molecular formula of $C_{15}H_{22}O_3$. In the ^{1}H NMR spectrum, compound **3** exhibited upfield shifted resonances of two methylene groups at δ_H 0.65 (m, H-5) and 0.55 (m, H-6), indicating compound **3** is an illudane-type sesquiterpene containing a cyclopropane (Table 1). The ^{1}H and ^{13}C NMR spectra of **3** were similar to those of sulphureuine A,[21] but an oxygenated methine group (δ_C/δ_H 64.5/3.34 and 3.12) was observed in place of one of the methyl groups. These protons exhibited HMBC correlations with C-4, C-7, and C-8, suggesting that compound **3** is a 13-hydroxylated analogue of sulphureuine A (Figure 2A). The relative configuration of **3** was identified based on NOE correlations between H-9 and H-15 and between H-9 and H-7 (Figure 2B). Although compound **3** is a 13-hydroxy derivative of sulphureuine A, we chose not to use the sulphureuine series naming for **3**, because it is an illudane-type and not a drimane-type sesquiterpene.[21] Thus, we named compound **3** 13-hydroxy-7-deoxypaneolilludinic acid.

The absolute configurations of **1**−**5** were deduced to be identical because they were assumed to be congeners.[22,23] Previously reported sterpurane-type sesquiterpenoids are known to have a 9*R*-configuration,[24,25] and dextrorotation of **4** confirmed the 9*R*-configuration.[17] It is known that (+)-sterpurene, which is a 9*R* derivative, showed a positive Cotton effect at 205 nm in electronic circular dichroism (ECD) experiments,[25] and ECD spectra of **2**, **4**, and **5** showed the same pattern (Figure S30, Supporting Information). A recent density functional theory (DFT)-based calculation of the ECD spectrum of tricosterpurol supports this conclusion.[26] Thus, the absolute configuration of **2** was identified as 4*S*,5*R*,7*R*,9*R*,11*S*. For the absolute configurations of compounds **1** and **3**, we performed DFT-based ECD calculations and compared the results with the experimental spectra. As shown in Figure 3, the Boltzmann-averaged computed ECD spectra of the 7*R*,9*R*,11*S*-isomer of **1** and 7*R*,9*R*,11*R*-isomer of **3** were similar to the experimental spectra, although there were wavelength shifts of Cotton effects. As the 9*R*-configurations were identical to those of congeners **2**, **4**, and **5**, we determined the absolute configurations of **1** and **3** as shown. The absolute configurations of compounds **6**−**8** were identified as (2*S*,3*S*,6*S*,7*S*,9*R*)-tremul-1(10)-ene-11,12,14-triol (**6**), (3*S*,6*S*,7*S*,10*S*)-tremulene-10,11,12-triol (**7**), and 6α-hydroxycinnamolide (**8**), by comparison of their optical rotation to literature values.[17−20]

To confirm whether the biosynthesis of the isolated compounds was induced by cocultivation, we compared the chemical profiles of the EtOAc extracts of the 15-day fermentation broths acquired by co- and axenic cultures. Compounds **1**, **2**, and **5** were exclusively detected in the coculture broth, while compounds **3**, **4**, **6**, and **7** were also detected in the axenic culture of *P. orientoasiaticus* (Figure 4 and Figure S5, Supporting Information). From these results, it can be deduced that *P. orientoasiaticus* is the producer strain of

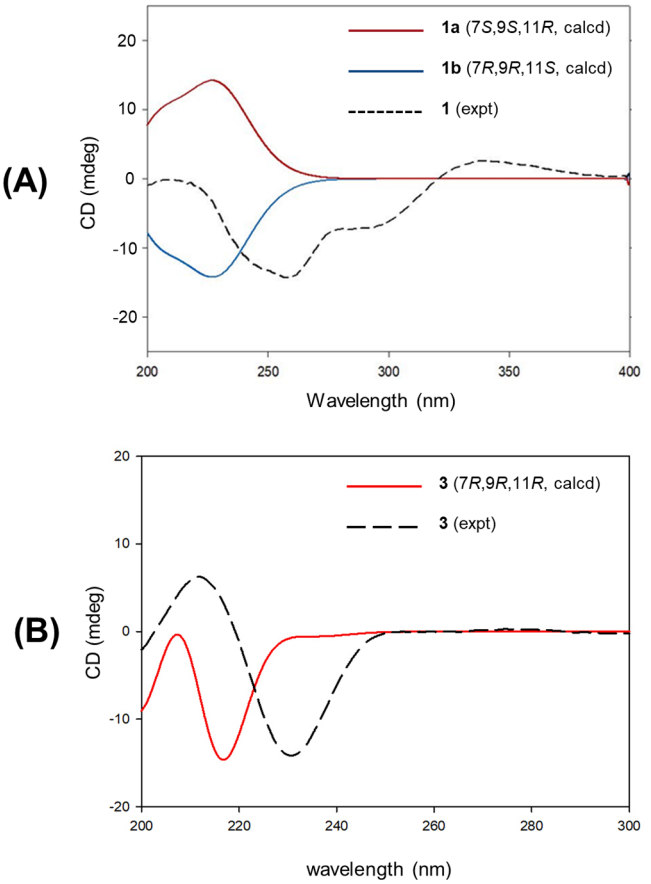

Figure 3. Experimental and calculated ECD spectra of compounds **1** (A) and **3** (B).

cyclohumulanoid sesquiterpenes. Although these compounds were produced in the case of axenic culture, cocultivation significantly increased the quantity of compounds **1**−**7**. However, as mentioned above, no sign of competitive interactions was observed in the cocultivation plate, which suggests that these compounds are not defensive metabolites. This was further confirmed by the fact that none of the isolates **1**−**8** exhibited antifungal activity in the disk diffusion assay performed against *Aspergillus flavus* and *A. fumigatus*. The LC-MS analysis on the coculture plates of *P. orientoasiaticus* with *A. flavus* and *A. fumigatus* showed that compounds **3**, **4**, and **7** were induced in the coculture with *A. flavus*, while the coculture with *A. fumigatus* exhibited compounds **4**, **6**, and **7** (Figures S6 and S7, Supporting Information). This result suggested that compounds **1**, **2**, and **5** may not have been biosynthesized from *P. orientoasiaticus* alone. Previous studies revealed that biotransformation is another important mechanism enriching the chemical diversity in fungal cocultures.[27,28] We suppose that compounds **1**, **2**, and **5** might be products of bioconversion by *X. flaviporus* as a part of the chemical interactions between *P. orientoasiaticus* and *X. flaviporus*; however, further experiments are required to prove this hypothesis. Compound **8** was detected in the axenic culture broth of *X. flaviporus*, which shows *X. flaviporus* is the producer of this compound. The production of compound **8** did not increase by cocultivation.

As many structurally similar compounds, especially illudin S, are known as cytotoxic agents,[29] we tested the cytotoxicity of the isolated compounds. However, none of the isolates

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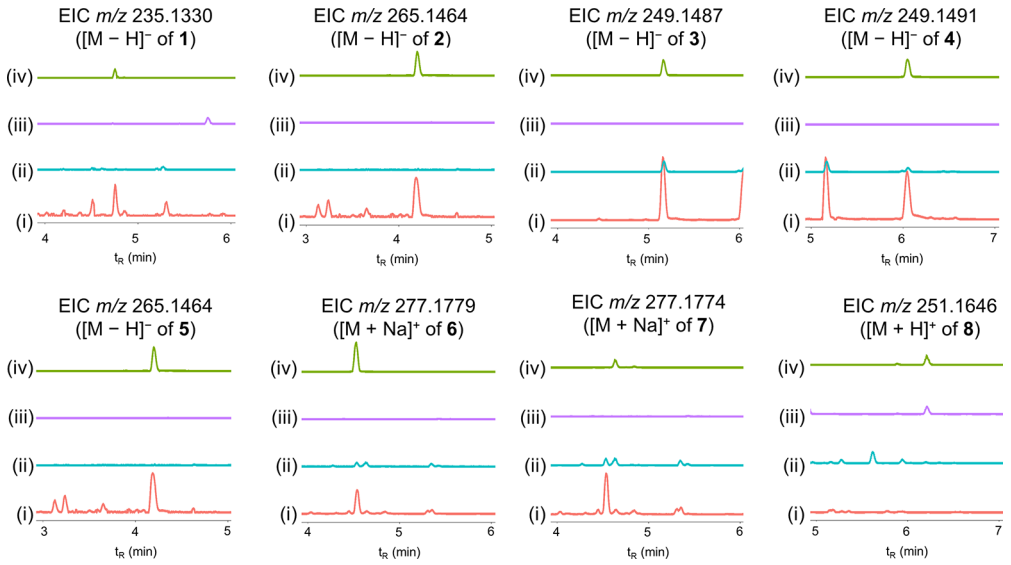

Figure 4. LC-MS extracted-ion chromatograms (EIC) with the indicated *m/z* values for compounds **1**−**8** visualized the relative abundances of the metabolites in the extracts of 15-day fermentation broths of (i) the coculture and (ii) the axenic culture of *P. orientoasiaticus* and (iii) *X. flaviporus*. (iv) Chromatogram of the purified compound. As compounds **2** and **5** have identical molecular formulas and retention times, the relative abundance of these compounds in (i), (ii), and (iii) was further compared using an alternative chromatographic condition (Figure S5, Supporting Information).

exhibited cytotoxicity against colorectal adenocarcinoma Caco-2 cell lines. As the isolates did not exhibit any cytotoxicity, we applied them to screenings for protective activities. As illudane- and sterpurane-type sesquiterpenes from *Cryptomarasmius aucubae* inhibited lipopolysaccharide (LPS)-induced NO production in BV-2 cells,[30] we evaluated anti-inflammatory activity of compounds **1**−**7** using an LPS-induced NO inhibition assay using the RAW264.7 cell line (compound **8** was excluded due to the amount available). Compounds **1**−**7** inhibited the LPS-stimulated NO production in a concentration-dependent manner and showed no prominent cytotoxicity (Figure 5). Compounds **1**, **2**, and **6** showed the highest inhibition of NO production by 34.8%, 36.4%, and 38.0% compared to the LPS-treated control at 100 μM.

Metabolites induced in the noncompetitive cocultures are relatively difficult to prioritize because of the lack of morphological signals; however, this work showed that untargeted metabolomics applying LC-MS can be an effective tool for detecting the induction of specialized metabolites.

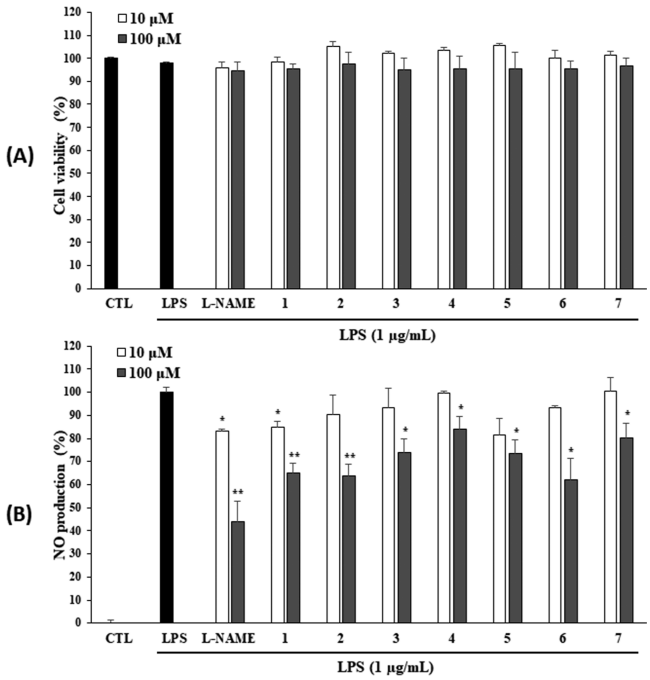

Figure 5. Effects of compounds **1**−**7** on cell viability (A) and NO production (B) of RAW264.7 cells treated with 1 μg/mL LPS for 20 h. Data are represented as the means ± standard deviations (SD) of three replicates. *$p < 0.05$, **$p < 0.01$, vs LPS-treated group. L-Nitroarginine methyl ester (L-NAME) was used as a positive control.

EXPERIMENTAL SECTION

General Experimental Procedures. The optical rotation experiments were conducted using a Jasco P-200 digital polarimeter (Jasco, Tokyo, Japan) with a sodium lamp (589 nm) using a 10 cm cell at 20 °C. UV and ECD spectra were recorded on a Jasco J-1500 spectropolarimeter (Jasco, Tokyo, Japan) and a Chirascan CD spectrometer (Applied Photophysics, Surrey, UK). NMR spectra were obtained using a Bruker Avance III HD 500 spectrometer (Bruker BioSpin, Billerica, MA, USA). Preparative HPLC was performed using a Waters 600 HPLC system equipped with a Spursil C_{18} EP column (250 × 10.0 mm, 5 μm, Dikma Technologies, Foothill Ranch, CA, USA) or a Hector-M C_{18} column (250 × 21.2 mm or 250 × 10 mm, 5 μm, RS Tech, Daejeon, Korea). The solvents used for LC-MS and preparative HPLC were purchased from J. T. Baker (Avantor, Phillipsburg, NJ, USA). Deuterated solvents for NMR analyses were purchased from Cambridge Isotope Laboratories (Cambridge, MA, USA).

Fungal Materials. *Phellinus orientoasiaticus* L.W. Zhou & Y.C. Dai (KMRB19040316) and *Xylodon flaviporus* (Berk. & M.A. Curtis ex Cooke) Ryvarden (KMRB19080638) were obtained from the Korea Mushroom Resource Bank at Seoul National University and maintained on potato dextrose agar (PDA; KisanBio Co., Ltd., Seoul, Korea) at 27 °C. The full list of the 40 fungal species involved in the initial randomized coculture screening is given in Table S1, Supporting Information. Solid culture-based cocultivation screening was performed according to the procedure described in a previous study.[31] Two 5 mm agar plugs from each fungal species were inoculated on opposite sides of a Petri dish containing PDA, and the

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J. Nat. Prod. 2022, 85, 511−518

plates were incubated at 27 °C for 21 days. Liquid culture-based coculture was performed to prepare the biomass for targeted isolation. Agar plugs from axenic plate cultures were inoculated into Erlenmeyer flasks containing 250 mL of potato dextrose broth (PDB) and maintained for 5 days as seed cultures. Each seed culture flask of *P. orientoasiaticus* was mixed with a flask of *X. flaviporus* to give a coculture flask containing 250 mL of media. Coculturing fermentation was carried out for 28 days at 27 °C on a rotary shaker (130 rpm).

LC-MS Analysis. For the solid plates, each Petri dish was extracted twice with 40 mL of MeOH to yield the crude extract. For the liquid culture flasks, 5 mL of broth was collected and dried from each flask. The collected extracts were prepared for LC-MS analyses by dissolution in 50% MeOH−H_2O at 1 mg/mL concentration and then filtered through a PTFE syringe filter (Altoss, Sejong, Korea). LC-MS analyses were performed on a Waters Acquity I-Class UPLC system (Waters Co., Milford, MA, USA) linked to a Waters VION IMS QTOF mass spectrometer (Waters MS Technologies, Manchester, UK) equipped with an electrospray ionization interface (ESI). Chromatographic separation was performed with a Waters Acquity UPLC BEH C_{18} (100 × 2.1 mm, 1.7 μm) column eluted with a mixture of 0.1% formic acid in H_2O (A) and MeCN (B). The mobile phase was pumped in a flow rate of 0.3 mL/min, with a linear gradient of 10−100% B (0.2−12.0 min) followed by a 3 min washing with 100% B and 3 min reconditioning with 10% B. The samples (2.0 μL of injection volume) were analyzed in MS^E data-independent acquisition mode. The low collision energy for the detection of the precursor ions was set to 6 eV, while the high collision energy for fragmentation was 20−40 eV. Raw LC-MS data were deposited in the MassIVE (https://massive.ucsd.edu) with the accession no. MSV000088271.

Targeted Isolation of Induced Metabolites. Broth from 20 coculturing fermentation flasks was filtered through cotton, extracted with EtOAc, and then dried to obtain 926 mg of extract. The crude extract was subjected to preparative HPLC (Hector-M C_{18} column, 10 mL/min, MeCN−H_2O, 30:70 → 100:0) to obtain 10 fractions, A1−A10. A3 was further separated by preparative HPLC (Spursil C_{18} EP column, 4 mL/min, MeCN−H_2O, 30:70 → 80:20) to yield compound **3** (20.4 mg, t_R = 17.5 min). A4 was subjected to preparative HPLC (Hector-M C_{18} column, 4 mL/min, MeCN−H_2O, 50:50 isocratic) and yielded compounds **4** (10.4 mg, t_R = 8.4 min) and **8** (1.2 mg, t_R = 9.6 min). Ten additional flasks were fermented for 20 days, and 782.5 mg of extract was obtained using the aforementioned method. Preparative HPLC (Hector-M C_{18} column, 10 mL/min, MeCN−H_2O, 30:70 → 100:0) was applied to fractionate the second batch of extract into fractions B1−B7. B3 was further separated into subfractions B3a−B3e by preparative HPLC (Hector-M C_{18} column, 4 mL/min, MeCN−H_2O, 30:70 → 60:40). B3a was fractionated using preparative HPLC (Spursil C_{18} EP column, 4 mL/min, MeCN−H_2O, 37:63 → 40:60) to yield compounds **5** (5.5 mg, t_R = 8.2 min) and **2** (3.8 mg, t_R = 11.0 min). Compound **6** (6.2 mg, t_R = 5.2 min) was obtained by preparative HPLC (Spursil C_{18} EP column, 4 mL/min, MeCN−H_2O, 45:55 → 50:50) separation of B3b. B3c was isolated by preparative HPLC (Spursil C_{18} EP column, 4 mL/min, MeCN−H_2O, 40:60 → 50:50) to yield compound **7** (4.2 mg, t_R = 7.0 min). Compound **1** (2.2 mg, t_R = 9.4 min) was purified from Bd by preparative HPLC (Spursil C_{18} EP column, 4 mL/min, MeCN−H_2O, 50:50 isocratic).

*6-Nor-4-hydroxy-1,3(4)-distepuren-14-oic acid (**1**):* amorphous oil, $C_{14}H_{20}O_3$; $[\alpha]_D^{20}$ −28.36 (*c* 0.025, MeOH); UV λ_{max} (log ε) 258 (sh) (0.31) nm; ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 341 (+2.58), 281 (sh) (−7.26), 258 (−14.31) nm; ^{1}H NMR and ^{13}C NMR data, see Table 1; HRESIMS *m*/*z* 235.1330 $[M - H]^-$ (calcd for $C_{14}H_{19}O_3$, 235.1340).

*6-Hydroxysterpuric acid (**2**):* yellow powder, $C_{15}H_{22}O_4$; $[\alpha]_D^{20}$ +45.60 (*c* 0.025, MeOH); UV λ_{max} (log ε) 246 (0.24) nm, ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 322 (+1.73), 246 (−5.12), 200 (+2.53) nm; ^{1}H NMR and ^{13}C NMR data, see Table 1; HRESIMS *m*/*z* 265.1464 $[M - H]^-$ (calcd for $C_{15}H_{21}O_4$, 265.1453).

*13-Hydroxy-7-deoxypaneolilludinic acid (**3**):* amorphous powder, $C_{15}H_{22}O_3$; $[\alpha]_D^{20}$ −85.96 (*c* 0.025, MeOH); UV λ_{max} (log ε) 205 (1.71) nm; ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 350 (+0.19), 230 (−15.90), 213 (+11.31) nm; ^{1}H NMR and ^{13}C NMR data, see Table 1; HRESIMS *m*/*z* 249.1487 $[M - H]^-$ (calcd for $C_{15}H_{21}O_3$, 249.1496).

*Sterpuric acid (**4**):* white needles, $C_{15}H_{22}O_3$; $[\alpha]_D^{20}$ +47.40 (*c* 0.01, MeOH); UV λ_{max} (log ε) 202 (0.85) nm; ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 270 (+0.32), 226 (−0.73), 204 (+45.23) nm; ^{1}H NMR and ^{13}C NMR data, see Table S2, Supporting Information; HRESIMS *m*/*z* 249.1491 $[M - H]^-$ (calcd for $C_{15}H_{21}O_3$, 249.1496).

*13-Hydroxysterpuric acid (**5**):* yellow powder, $C_{16}H_{24}O_3$; $[\alpha]_D^{20}$ +48.24 (*c* 0.025, MeOH); UV λ_{max} (log ε) 200 (0.28) nm; ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 227 (−0.62), 204 (+3.66) nm; ^{1}H NMR and ^{13}C NMR data, see Table S2, Supporting Information; HRESIMS *m*/*z* 265.1464 $[M - H]^-$ (calcd for $C_{15}H_{21}O_4$, 265.1453).

*(2S,3S,6S,7S,9R)-Tremul-1(10)-ene-11,12,14-triol (**6**):* amorphous oil, $C_{15}H_{26}O_3$; $[\alpha]_D^{20}$ −59.82 (*c* 0.025, MeOH); ^{1}H NMR and ^{13}C NMR data, see Table S2, Supporting Information; HRESIMS *m*/*z* 277.1779 $[M + Na]^+$ (calcd for $C_{15}H_{26}NaO_3$, 277.1774).

*(3S,6S,7S,10S)-Tremulene-10,11,12-triol (**7**):* amorphous oil, $C_{15}H_{26}O_3$; $[\alpha]_D^{20}$ +30.42 (*c* 0.025, MeOH); ^{1}H NMR and ^{13}C NMR data, see Table S2, Supporting Information; HRESIMS *m*/*z* 277.1774 $[M + Na]^+$ (calcd for $C_{15}H_{26}NaO_3$, 277.1774).

*6α-Hydroxycinnamolide (**8**):* amorphous powder, $C_{15}H_{22}O_3$; $[\alpha]_D^{20}$ +28.20 (*c* 0.01, MeOH); ^{1}H NMR and ^{13}C NMR data, see Table S2, Supporting Information; HRESIMS *m*/*z* 251.1646 $[M + H]^+$ (calcd for $C_{15}H_{23}O_3$, 251.1642).

Original NMR FIDs are available at 10.5281/zenodo.5606393.

DFT-Based ECD Calculations for Compounds 1 and 3. Conformational searches were performed using MMFF44 calculations with search limits of 10 kJ/mol in the MacroModel software (version 2019-3, Schrödinger LLC) for compound **1** or 1.0 kcal/mol in Conflex 7 (Conflex Corp., Tokyo, Japan) for **3**. Geometry optimizations of the calculated conformers and their free energy calculations were performed using Tmolex 4.4 and Turbomole software (COSMOLogic GmbH & Co. kg, Germany) with the DFT settings of B3-LYP/6-31+G(d,p) (**1**) or B3-LYP/def-SVP (**3**). The computed ECD spectra of all optimized conformers were calculated by time-dependent DFT (TDDFT) using B3-LYP/6-31+G(d,p) (**1**) or B3-LYP/def-TZVP (**3**) and averaged by the Boltzmann distribution.

Evaluation of Antifungal Activity. Antifungal activity against *Aspergillus flavus* and *A. fumigatus* was tested using the disk diffusion assay. Paper disks (diameter, 5 mm) were soaked with MeOH solutions of the isolated compounds and dried to contain 50 or 100 μg of the compounds. The prepared disks were placed on PDA plates, and the test strains were inoculated and cultured for 5 days to evaluate whether the compounds caused any growth inhibition zone. Disks containing 10 and 20 μg amphotericin B were used as positive controls.

Cytotoxicity Assay Using the Caco2 Human Colorectal Cancer Cell Line. Caco2 cells, acquired from the Korean Cell Line Bank (KCLB, Seoul, Korea), were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (HyClone, Logan, UT, USA). Cells were maintained in an incubator at 37 °C with 5% CO_2. Viability of Caco2 cells was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Cells were seeded in 96-well plates at a density of 2.5 × 10^3 cells/well and incubated for 12 h, then were left untreated or were treated with the isolates (dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100 mM and diluted with H_2O to make the final concentrations from 12.5 to 100 μM) and incubated for 48 h followed by incubating with MTT for 4 h. DMSO was added to cells after removing medium from the wells. Absorbance was measured at 570 nm using a microplate reader (BioTek, Winooski, VT, USA).

Assay for Cell Viability and NO Production of the LPS-Induced RAW264.7 Cell Line. The mouse macrophage RAW 264.7 cells were acquired from the KCLB. The cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2 as monolayers in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin−streptomycin solution (10 000 U/mL). After preincubation

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J. Nat. Prod. 2022, 85, 511−518

(24 h) at a density of 1×10^5 cells/well in 96-well plates, cells were treated with each compound at final concentrations of 10 or 100 μM in serum-free culture medium and then were stimulated with LPS (1 μg/mL) for 20 h. The supernatant was harvested, and equal volumes of Griess reagent A [0.1% (w/v) *N*-(1-naphthyl)ethylenediamine in H_2O] and B [1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid] were added. After incubation at rt for 10 min, the absorbance was measured at 550 nm using a microplate reader. The cultured cells were incubated with MTT (5 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 4 h. After removal of supernatant and addition of 100 μL of DMSO (Sigma-Aldrich), the absorbance was measured at 570 nm.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jnatprod.1c01022.

Species list and picture of the randomized coculture; LC-MS chromatograms of coculture experiments, NMR data of compounds **4**−**8**, raw NMR and MS spectra of compounds **1**−**3**, and ECD spectra of compounds **2**, **4**, and **5** (PDF)

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Notes

The authors declare no competing financial interest.

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■ DEDICATION

Dedicated to Dr. William H. Gerwick, University of California at San Diego, for his pioneering work on bioactive natural products.

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