Bioactive Polyketides From the Potato Endophytic Fungus Aspergillus carneus

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Abstract
A previously undescribed polyketide (1) and 3 known analogs (2-4) were obtained from cultures of the potato endophytic fungus Aspergillus carneus. The structures were elucidated on the basis of extensive nuclear magnetic resonance spectroscopic data. The absolute configuration of 1 was further determined by electronic circular dichroism and optical rotation calculations. Compounds 1-4 showed moderate antifungal activity against plant pathogens. Compounds 1, 2, and 4 inhibited nitric oxide production in lipopolysaccharide-stimulated RAW264.7 cells, with half-maximal inhibitory concentration values of 13.36, 30.16, and 51.47 µM, respectively. Compound 4 showed effective antioxidant activity.

Keywords
Aspergillus carneus, polyketide, structural elucidation, bioactivity, endophytic fungus

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Potato is the fourth most widely grown food crop worldwide.¹ In recent years, the area of potato planting in China has steadily increased. However, the occurrence of potato pests and diseases has also been increasing yearly.² The yield and quality of potatoes are often limited by plant diseases triggered by pathogens, such as Phytophthora infestans, Rhizoctonia solani, and Fusarium oxysporum.³,4 In particular, the infamous P. infestans, a persistent and severe threat to potato production globally, causes the disease known as late blight, has spread quickly. This pathogen can quickly lead to potato plant death and, in turn, causes the disease known as late blight, has spread quickly. This pathogen can quickly lead to potato plant death and, in turn, the large-scale reduction of production and even crop failure. Additionally, P. infestans causes tuber rot, affects the quality of potatoes, and results in huge economic losses to the potato industry. Therefore, new and effective natural fungicides are urgently required.

Aspergillus carneus, a fungus in the Discellaceae family, is widely distributed in nature. Many metabolites have been reported from this fungus, such as quinazolinones, anthraquinone, polyketide, alkaloids, sesquiterpenoids, benzene ring derivatives, glycosides, and dipeptides. Furthermore, these metabolites show excellent biological, including antifungal, cytotoxic, and antioxidative activities.⁵⁻¹² Our primary bioassay suggested that the crude extract of the culture broth of A. carneus has a mild inhibitory effect on the pathogen F. oxysporum. Therefore, a chemical study of this extract was carried out. As a result, a new polyketide, aspergillolide (1), and 3 known analogs (2-4) were obtained (Figure 1).¹³ All compounds were evaluated for their antipathogenic activities against 3 plant pathogenic strains. Additionally, their anti-nitric oxide (NO) production, antioxidative, and cytotoxic effects were evaluated.

Results and Discussion
Aspergillolide (1) was obtained as a light yellow oil. The molecular formula, C₁₈H₁₆O₅, was determined by high resolution-electrospray ionization-mass spectrometry (HR-ESI-MS) from the ion at m/z 313.10685 [M + H]+ (calcd. for C₁₈H₁₇O₅ 313.10760), which indicates 11 degrees of unsaturation. The infrared (IR) spectrum showed the presence of hydroxy (3367 cm⁻¹), carbonyl (1660 cm⁻¹), and phenyl (1454 cm⁻¹) groups. The ¹H nuclear magnetic resonance (NMR) spectrum (Supplemental Table S1) showed 2 pairs of resonances, typical for para-disubstituted phenyl groups, at δH 7.36 and 6.86 (2H, d, J = 8.7 Hz) and δH 6.99 and 6.73 (2H, d, J = 8.4 Hz). Additionally, a singlet for a methine at δH 3.52 (1H, s), 2 doublets for a methylene group at δH 3.95 and 3.62 (1H, d, J = 15.2 Hz), and a methoxy group at δH 3.52 (3H, s) were identified. The ¹³C NMR
spectrum and distortionless enhancement by polarization transfer data revealed the presence of 18 carbon resonances ascribable to 1 methoxyl, 1 methylene, 1 methine, 14 olefinic carbons, and 1 carbonyl carbon. These data, along with the ultraviolet absorptions at 210, 225, and 285 nm, indicate that compound 1 possesses a butenolide skeleton with a similar structure to that of microperfururanone (3), a known compound isolated in this study. The key difference was that 2 phenyl groups were substituted by an OH group at C-4 and C-4'. Furthermore, the OH at C-4 was replaced by an OMe group, as supported by the heteronuclear multiple bond correlation from δH 3.52 (3H, s, H3-6) to δC 104.1 (d, C-4) (Figure 2). The detailed analysis of 2-dimensional (2D) NMR data suggests that the other parts of 1 were the same as those of 3. Therefore, the planar structure of 1 was identified as 4,4'-dihydroxy-microperfururanone.

To establish the absolute configuration, electronic circular dichroism (ECD) calculations were performed on the optical rotation and CD curve of 1. The calculated ECD data of 1a matched well with the experimental curve (Supplemental Figure S1). Additionally, the optical rotation value of compound 1 was similar to that of compound 3 (+3.6°). The calculated specific rotation (+363.6° for (4R)-1; −366.4° for (4S)-1) also suggests that the absolute configuration of 1 is (4R)-aspergillolide (Supplemental Figure S4 and S5). Three other known compounds were identified as (±)-asperteretal D (2), microperfururanone (3), and butyrolactone I (4) by comparison of their NMR spectroscopic data with those reported in the literature.

Compounds 1-4 were assessed for their inhibitory activities against 3 potato pathogens, P. infestans (late blight), R. solani (black scurf), and F. oxysporum (blast). All compounds showed inhibitory activities against F. oxysporum; compound 3 showed the most significant inhibitory activity against F. oxysporum compared with the positive control. Compounds 3 and 4 showed weak inhibitory activities against P. infestans and R. solani (Supplemental Table S6).

In addition to their antipathogenic activity, all compounds were evaluated for their anti-NO production and cytotoxic and antioxidative effects. Compound 2 showed potent inhibition, with a half-maximal inhibitory concentration (IC50) value of 13.36 µM, whereas compounds 1 and 4 displayed moderate activities, with IC50 values ranging from 30.16 to 51.47 µM (Supplemental Table S7). Compound 2 exhibited inhibitory activities against the growth of cancer cell lines MCF-7 (IC50 = 6.86 µM) and A549 (IC50 = 10.07 µM) (Supplemental Table S8). Compound 4 showed powerful antioxidant activity (Supplemental Table S9). According to the literature, butyrolactone II shows moderate antioxidant activity. Comparing the structures of compound 4 and butyrolactone II, the C-3" isopentenyl group was not the key factor of the activity and, comparing compounds 2, 4, and asperteretal E, the substitution pattern of the 5-membered lactone ring, as well as the methoxycarbonyl group at the C-4 position, maybe crucial factors for the antioxidant activity (Figure 3).
Conclusion

One new and 3 known polyketides were isolated from the potato endophytic fungus *A. carneus*. These compounds exhibit widespread biological, including antipathogenic, anti-NO production, antioxidative, and cytotoxic activities. Therefore, *A. carneus* may be a potential resource for the development of new natural fungicides.

Experimental Section

General Experimental Procedures

Optical rotations (OR) were recorded on a Rudolph AUTOPOL IV polarimeter, ultraviolet (UV) spectra on a UH5300 UV VIS Double Beam Spectrophotometer, IR spectra on an IR Tracer-100 Fourier Transform Infrared Spectrophotometer, and 1D and 2D spectra on a Bruker Avance III 600 MHz spectrophotometer with TMS as an internal standard. CD spectra were recorded with an Applied Photophysics Chirascan-Plus CD Spectrometer, and HR-ESI-MS on an ultra high-performance liquid chromatography (HPLC) system and a Q Exactive HF Mass Spectrometer. Medium-pressure liquid chromatography (MPLC) was performed on a Biotage Isolera Onsystem with an RP-18 column. Preparative HPLC was performed on an Agilent 1260 liquid chromatography system (Agilent Technologies, Santa Clara, CA, USA) with a Zorbax SB-C18 (5 µm, 4.6 × 250 mm) column, a Daicel chiral column (AS-H, 5 µm, 4.6 × 250 mm), and a diode array detector. Fractions were monitored by thin-layer chromatography (Qingdao Marine Chemical Inc., China), and compounds were visualized by heating silica gel plates after spraying with 10% sulfuric acid solution.

Fungal Material

The fungus L03 was isolated from fresh and healthy potato tissue collected from Lincang, Yunnan Province, China, in 2012. The strain was identified as *A. carneus* by 18S rDNA-seq...
and deposited at South-Central University for Nationalities, China. The sequence data for this strain had been submitted to the DDBJ/EMBL/GenBank with accession No. KX437770.1.

**Extraction and Isolation**

The fermentation broth was extracted 6 times with ethyl acetate to give a crude extract (236 g). The latter was submitted to silica gel (200-300 mesh) column chromatography (CC) using a chloroform-methanol (MeOH) gradient (from 1:0 to 0:1) to yield 7 fractions (A-G). Fraction C (20.3 g) was fractionated by preparative MPLC over an RP C18 column with a gradient flow of MeOH-water (H2O) from 30% to 100% to obtain 10 subfractions (C1-C10). Fraction C3 (1.7 g) was further purified by Sephadex LH-20 chromatography with methanol to afford 7 subfractions (C7-1 to C7-7). Subsequently, semipreparative HPLC using a C18 column afforded compound 3 (5 mg, 18% MeCN in H2O, 4 mL/min). Fraction C4 (4.6 g) was further fractionated by silica gel CC (light petroleum-acetone, 20:1-0:1) to afford 6 subfractions (C4-1 to C4-10). Fraction C4-3 (0.7 g) was separated by preparative HPLC to give compounds 1 (12.5 mg) and 2 (6 mg, 12%-17% acetonitrile [MeCN] in H2O, 4 mL/min). Fraction D (30.7 g) was treated by preparative MPLC using an RP C18 column with a gradient flow of MeOH-H2O from 20% to 100% to obtain 8 subfractions (D1-D8). Fraction D5 (2.3 g) was further separated by silica gel (light petroleum-acetone, 30:1-0:1) and Sephadex LH-20 (MeOH) chromatography. Subsequent purification by semipreparative HPLC afforded 4 (4.6 mg, 10%-23% MeCN in H2O, 4 mL/min).

Aspergillicolide (I): slight yellow oil; [α]D21.6 = 23.1 (c 0.5, MeOH); UV (MeOH): 210 (3.48), 225 (3.40), 285 (3.10); IR (potassium bromide): 3392, 1651, 1454, 1413 cm−1; 1H-NMR (600 MHz, methanol-d4) and 13C-NMR (150 MHz, methanol-d4): see Supplemental Table S1; HR-ESI-MS (pos.): 313.10685 [M + H]+ (calcd. for C18H17O5, 313.10760).

**Computation Methods**

**ECD calculation.** A conformation search based on molecular mechanics with Merck Molecular Force Fields performed for 1a and 1b gave the 2 stable conformers, respectively (Supplemental Figure S2 and S3). These conformers were further optimized by the density functional theory method at the B3LYP/6-311G(2d,p) level using the Gaussian 16 program package. The ECDs were calculated using density functional theory (TDDFT) at B3LYP/6-311+G(2d, p) level in methanol with a PCM model. The calculated ECD curves were all generated using SpecDis 1.71.

**Specific optical rotation calculation.** A conformation search based on molecular mechanics with MMFF force fields performed for 1a and 1b gave the 2 stable conformers, respectively (Supplemental Figure S2 and S3). These were further optimized by the density functional theory method at the B3LYP/6-311G(2d,p) level by the Gaussian 16 program package. The specific optical rotation calculations were made at the B3LYP/6-311+G(2d,p) level in MeOH with a PCM model. The calculated specific optical rotation data of these conformers were averaged according to the Boltzmann distribution theory and their relative Gibbs free energy (Supplemental Table S4 and S5).

**Antifungal Assay**

Compounds 1-4 were subjected to minimal inhibitory concentration tests against 3 phytopathogenic fungi, P. infestans, R. solani, and F. oxysporum, on potato dextrose agar (PDA) medium using a 2-fold serial dilution in microplate wells over the range of 6.25-200 μg/mL. To this end, into each of the first column of a 96-well plate was placed 95 μL of PDA along with 5 μL of fungus liquid as a control, at the same time, 95 μL of PDA and 5 μL of fungal solution were placed in the third to sixth columns of the 96-well plate and mixed well. Into each of the second column of the 96-well plate was placed 186 μL of PDA, 10 μL of fungus solution, and 4 μL of the sample. After mixing well, 100 μL was removed and added to the third column of the 96-well plate. After mixing, 100 μL was added to the fourth column, and so on, to perform a decreasing concentration dilution. The final 100 μL was discarded. Observations of the plates were made after 24 hours of incubation at 26 °C in order to acquire the minimum inhibitory concentrations with no growth in the well taken as that value. Three replicates were maintained to confirm the antifungal activity.

**NO Production Inhibitory Assay**

RAW264.7 cells were used for the anti-inflammatory assay, cultured in Dulbecco’s Modified Eagle’s medium (DMEM) (HiClone, USA) with 10% fetal bovine serum (FBS; PAN, Germany), 100 U/mL penicillin, and 100 g/mL streptomycin in a humidified incubator (5% carbon dioxide [CO2], 37 °C). RAW264.7 cells (5 × 105 cells/mL) were seeded into a 96-well plate for 2 hours. After 12 hours of incubation, they were treated with various concentrations of test compounds (10-40 mM) for 2 hours, then incubated with or without lipopolysaccharide (500 ng/mL) for 24 hours. The medium was removed, and 100 μL/well of 3-(4,5-dimethylthiazol-2-yI)-2,5-diphenyltetrazolium bromide (MTT) solution (0.5 mg/mL) was added. The plate was incubated subsequently for 4 hours at 37 °C. Formazan crystals in each well were dissolved with 150 μL dimethyl sulfoxide (DMSO). Finally, the absorbance was measured at 570 nm on a microplate reader (TECAN, Germany). Cell viability was expressed as the percentage of the control, which was set to 100%. A Griess reagent kit (Promega, USA) was used to collect the cell supernatants for measurement of NO production in the supernatants. The absorbance was measured at 540 nm using a microplate reader.

**Antioxidant Assay**

DPPH radical scavenging activity. Each of the 4 polyketide compounds (0.1 mL) was separately mixed with 3.9 mL of...
methanolic solution containing 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals (6 × 10^5 mol/L). The mixture was shaken vigorously and reacted for 30 minutes, shaded from light (until stable absorbance values were obtained). The reduction of the DPPH radical was determined using a spectrophotometer to read the absorbance at 517 nm; vitamin C was used as a positive control.

ABTS radical scavenging activity. 2,2’-Azobis (2-amipropionate) dihydrochloride (Sigma-Aldrich, Co.) (7 mM) was mixed with 2.45 mM 2,2’-Azino-di-[3-ethylbenthiazoline sulfonate] (ABTS) and reacted for 16 hours at 23 °C. Then, 50 µL sample and 100 µL of ABTS solution were reacted at 23 °C for 20 minutes after adding to a 96-well plate, and the absorbance was measured at 734 nm; vitamin C was used as a positive control.

Cytotoxicity Assay

Human breast carcinoma cells (MCF-7) and human lung cancer cells (A549) were used in the cytotoxicity assay. The cells were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C in a humidified incubator with 5% CO_2. The toxicity of compounds 1-4 to the cell lines MCF-7 and A549 were assessed using the MTT assay. Cells in the logarithmic growth phase were inoculated in 96-well plates (1 × 10^5 cells/well) and cultured. Next, the cells were treated with compounds at concentrations of 5, 3, 2, 1, 0.5, and 0.25 µM for 24 hours. Next, removing the supernatant carefully, 0.5 mg/mL MTT was added to each well and incubated for 4 hours. Subsequently, the medium was discarded and 100 µL of DMSO was added to each well. The absorbance was recorded at 568 nm by using a microplate reader. Cisplatin was used as a positive control; its IC_{50} value was 9.16 µM.

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Declaration of Conflicting Interests

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Supplemental Material

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