Bioactive *p*-Terphenyl Derivatives from a *Cordyceps*-Colonizing Isolate of *Gliocladium* sp.

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Gliocladinins A (**1**) and B (**2**), two new *p*-terphenyl derivatives, have been isolated from solid cultures of an isolate of *Gliocladium* sp. that colonizes *Cordyceps sinensis*. The structures of these compounds were determined mainly by analysis of their NMR spectroscopic data. In standard disk assays, compounds **1** and **2** showed modest antimicrobial activity against *Staphylococcus aureus* (ATCC 6538). In addition, these compounds displayed inhibitory effects on the growth of two human tumor cell lines, HeLa and HCT116.

Several natural products containing the *p*-terphenyl moiety have been isolated from fungal sources. These compounds include 3,3″-dihydroxy-6′-desmethylterphenyllin, a potent antiinsectan natural product isolated from the sclerotia of *Penicillium raistrickii* Smith (Trichocomaceae) (NRRL 2039);[1] sarcodan, isolated from fruiting bodies of the basidiomycete *Sarcodon laevigatum*;[2] and the arenarins, cytotoxic metabolites against human tumor cell lines isolated from the sclerotia of *Aspergillus arenarius* (NRRL 5012).[3] *Cordyceps sinensis* (Berk.) Sacc. (later reclassified as *Hirsutella sinensis*),[4] also known as Chinese caterpillar fungus or "DongChong XiaCao" (summer-plant, winter-worm), has been widely used as a tonic and/or medicine for hundreds of years in the Orient. *Cordyceps* is a unique black, blade-shaped fungus found primarily at high altitude on the Qinghai-Tibetan plateau and endophytically parasitizes on dead caterpillars of the moth *Hepilus* spp. In late autumn, chemicals on the skin of the caterpillars interact with the fungal spores and release the fungal mycelia, which then infect the caterpillar. By early summer of the following year, the fungal infestation has killed the caterpillar and the fruiting body can be seen protruding from the caterpillar's head. The ongoing exploration of *C. sinensis* has shown that the species can produce many different bioactive compounds, and the medicinal benefits of *C. sinensis*have been demonstrated extensively.[5]

As part of our ongoing search for new bioactive natural products from unique fungal sources,[6] a subculture of an isolate of *Gliocladium* sp. (XZC04-CC-302), a fungus isolated from a sample of *C. sinensis* (Berk.) Sacc. that was collected in Linzhi, Tibet, People's Republic of China, was grown in solid-substrate fermentation culture. Its organic solvent extract displayed antibacterial activity against *Staphylococcus aureus* (ATCC 6538) and inhibitory effects on the growth of two human tumor cell lines, HeLa and HCT116. Bioassay-guided fractionation of this extract led to the isolation of two new *p*-terphenyl derivatives that have been named gliocladinins A (**1**) and B (**2**). Details of the isolation, structure elucidation, and biological activities of these compounds are reported here.

The molecular formula of gliocladinin A (**1**) was determined to be $C_{21}H_{22}O_6$ (11 degrees of unsaturation) by HRESIMS analysis [*m*/*z* 393.1309 $(M + Na)^+$; Δ −2.8 mmu], and this conclusion was supported by ^{1}H and ^{13}C NMR data (Table 1; due to sample limitation, the ^{13}C NMR data of **1** were extracted from its HMBC spectrum). Analysis of the ^{1}H, ^{13}C, and HMQC NMR data of **1** revealed the presence of three oxygenated methyl groups, one methylene unit, one oxymethine, one oxygenated sp^3 quaternary carbon, 14 aromatic or olefinic carbons (eight of which are protonated), and one ketone carbon. These data accounted for all but two exchangeable protons and required the compound to be tricyclic. The NMR data for **1** were characteristic of a terphenyl-type structure. Analysis of ^{1}H and ^{13}C NMR chemical shifts and the *J* values observed for eight aromatic protons led to the identification of two *para*-substituted aromatic rings. These assignments were further confirmed by relevant HMBC correlations. Three oxygenated methyl protons present in **1**showed HMBC correlations to C-4, C-6′, and C-4″, respectively, indicating that these three carbons bear methoxy groups. HMBC correlations of H_2-5′ with C-1′, C-4′, and C-6′, H-3′ with C-2′, and 3′-OH with C-2′ and C-4′ led to the completion of a polyoxygenated cyclohexenone moiety. HMBC correlations from H-2 and H-6 to C-1′ indicated that C-1 was directly attached to C-1′, while correlations from H-2″ and H-6″ to C-4′ led to the connection of C-1″ to C-4′. Key HMBC correlations from one exchangeable proton (δ_H 4.30) to C-2′ and C-4′ were observed, indicating that C-3′ carried a hydroxy group, and the remaining hydroxy group in **1** must be attached to the quaternary carbon C-4′. On the basis of these data, the planar structure of gliocladinin A was established as depicted in **1**.

The relative configuration of gliocladinin A (**1**) was assigned by analysis of NOESY data (Figure 1). NOESY correlation between

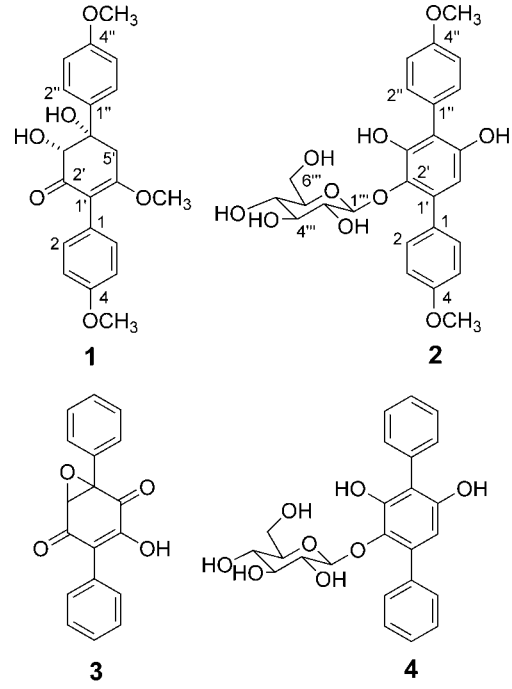

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Table 1. NMR Spectroscopic Data of Gliocladinin A (**1**) in $CDCl_3$

position	δ_H[a] (*J* in Hz)	δ_C[b,c] mult.	HMBC (H → C#)
1		124.5, qC	
2	7.21, d (8.4)	131.8, CH	4, 6, 1′
3	6.92, d (8.4)	113.7, CH	1, 4
4		159.1, qC	
4-OCH_3	3.82, s	55.5, CH_3	4
5	6.92, d (8.5)	113.7, CH	1, 4
6	7.21, d (8.5)	131.8, CH	2, 4, 1′
1′		116.0, qC	
2′		196.2, qC	
3′	4.80, s	75.3, CH	2′
3′-OH	4.30, br s		2′, 4′
4′		75.4, qC	
4′-OH	2.82, br s		
5′a	2.95, d (18)	39.8, CH_2	1′, 4′, 6′
5′b	3.17, d (18)		
6′		169.2, qC	
6′-OCH_3	3.74, s	56.9, CH_3	6′
1″		136.0, qC	
2″	7.55, d (8.4)	126.5, CH	4′, 4″, 6″
3″	6.98, d (8.4)	114.2, CH	1″, 4″
4″		159.2, qC	
4″-OCH_3	3.84, s	55.4, CH_3	4″
5″	6.98, d (8.4)	114.2, CH	1″, 4″
6″	7.55, d (8.4)	126.5, CH	4′, 2″, 4″

[a] Recorded at 400 MHz. [b] Recorded at 100 MHz. [c] Extracted from HMBC data.

H-3′ and H-5′a indicated that both protons adopted a pseudoaxial orientation with respect to the corresponding six-membered ring and *cis* to each other, while correlations of H-6″ with H-3′ and H-5′a were used to position the *p*-methoxyphenyl group attached to C-4′ in a pseudoequatorial orientation. On the basis of these data, the planar structure of gliocladinin A was established as shown in **1**.

The CD spectrum of **1** showed a positive Cotton effect at 238 ($\Delta\varepsilon$ +15.0) nm and a negative Cotton effect at 294 ($\Delta\varepsilon$ −9.50) nm. However, these Cotton effects could not be related to the absolute configuration at C-3′ and C-4′.

The molecular formula of gliocladinin B (**2**) was established as $C_{26}H_{28}O_{10}$ (13 degrees of unsaturation) by HRESIMS analysis [*m*/*z* 523.1570 $(M + Na)^+$; Δ −1.0 mmu] and NMR data (Table 2). Analysis of the ^1H and ^{13}C NMR data of **2** revealed the presence of two methoxy groups, three aromatic rings, and two phenolic protons, indicative of a typical terphenyl-type substructure. In addition, the ^1H and ^{13}C NMR resonances observed in the spectra of **2** were indicative of a glucose moiety. Further analysis of the NMR data of **2** established the identity of two *para*-substituted aromatic subunits with C-4 and C-4″ bearing methoxy groups. HMBC correlations from H-6′ to C-1, C-2′, C-4′, and C-5′, and two phenolic protons (δ_H 7.80 and 8.39, respectively) to C-4′, permitted identification of a pentasubstituted aromatic ring with C-3′ and C-5′ bearing hydroxy groups. HMBC correlations from H-2 and H-6 to C-1′ and from H-2″ and H-6″ to C-4′ led to the connection of C-1 to C-1′ and C-1″ to C-4′. An HMBC correlation from the anomeric proton H-1‴ to C-2′ located the glucose moiety at C-2′, therefore completing the planar structure of gliocladinin B as shown in **2**.

The glucose unit was connected to the terphenyl core via a β-linkage on the basis of downfield chemical shift of the anomeric carbon (δ_C 107.2) in the ^{13}C NMR spectrum of **2**, as well as the coupling constant of 7.8 Hz observed for the anomeric proton.[7] Upon acid hydrolysis of **2** with 6 N HCl, the liberated sugar was identified as D-glucose by comparison of its specific rotation with those of authentic samples of D-glucose and L-glucose.

In standard disk assays, gliocladinins A (**1**) and B (**2**) showed activity against *Staphylococcus aureus* (ATCC 6538) when tested at 100 μg/disk, affording zones of inhibition of 15 and 20 mm, respectively (ciprofloxacin: 35 mm zone of inhibition at 100 μg/disk). Gliocladinins A (**1**) and B (**2**) were evaluated further by broth dilution assay, and both displayed MIC values of greater than 100 μM (270 and 130 μM, respectively). However, none of these compounds showed antimicrobial activity against *Streptococcus mutans* (ATCC 25175), *Enterococcus faecalis* (ATCC 19433), and *Sarcina lutea* (CMCC B28001) or antifungal activity against *Geotrichum candidum* (AS2.498), *Candida albicans* (ATCC 10231), and *Aspergillus fumigatus* (ATCC 10894) at 100 μg/disk. Gliocladinins A (**1**) and B (**2**) were also evaluated against two human cancer cell lines: HeLa and HCT116. Compound **1** showed inhibitory effects on the growth of HeLa and HCT116 cells, with IC_{50} values of 54 and >270 μM, and compound **2** displayed IC_{50} values of 40 and 80–100 μM, respectively.

Most of the known *p*-terphenyl derivatives have been reported from fungi.[8] The initial steps in the biogenesis of *p*-terphenyls are the well-known reactions of primary metabolism that lead from shikimate to chorismate, and then to arylpyruvic acids.[8] The biosynthesis of gliocladinins A (**1**) and B (**2**) should proceed in a manner similar to that of other *p*-terphenyls. The core structure of gliocladinin A (**1**) is closely related to the known compound 2,3-epoxy-6-hydroxy-2,5-diphenyl-*p*-benzoquinone (**3**).[9] However, **1** differs from most known analogues by virtue of the presence of a pair of vicinal dihydroxy groups at C-2′ and C-3′, a methoxy at C-6′ instead of the 2,3-epoxy moiety, and a hydroxy group at C-6′ in addition to the presence of two methoxy groups at C-4 and C-4″. Gliocladinin B (**2**) possesses a common terphenyl-type core structure, but with a glucose unit attached via a β-linkage. The *p*-terphenyl β-glucoside is rare in the *p*-terphenyl class of compounds, with the only example of terfestatin A (**4**), which was isolated from *Streptomyces* sp. as an auxin-signaling inhibitor.[10] Gliocladinin B (**2**) differs from terfestatin A by having methoxy substituents at C-4 and C-4″.

Gliocladinins A (**1**) and B (**2**) are the first *p*-terphenyl derivatives to be reported from *Gliocladium* sp.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 241 polarimeter, and UV data were recorded on a Shimadzu Biospec-1601 spectrophotometer. The CD spectrum was recorded on a JASCO J-815 spectropolarimeter, using MeOH as solvent. ^1H and ^{13}C NMR data were acquired with Bruker Avance-300 and Avance-400 spectrometers using solvent resonances ($CDCl_3$ and acetone-d_6; δ_H 7.26/δ_C 77.0 and δ_H 2.09/δ_C 205.9, 30.6) as references. The HMQC and HMBC experiments were optimized for 145.0 and 8.0 Hz, respectively. ESIMS data were recorded on a Bruker Esquire 3000^{plus} spectrometer; HRESIMS data were obtained using a Bruker APEX III 7.0 T spectrometer.

Fungal Material. The culture of *Gliocladium* sp. (XZC04-CC-302) was isolated by Dr. Mu Wang from a sample of *C. sinensis* that was collected in Linzhi, Tibet, on March 1, 2004. The isolate was identified by Mr. Bingda Sun and assigned the accession number XZC04-CC-302 in Professor X. Liu's culture collection at the Institute of Microbiology, Chinese Academy of Sciences, Beijing. The isolate was subcultured on PDA slants at 25 °C for 5 days. Fermentation was carried out in six 500 mL Erlenmeyer flasks, each containing 50 g of rice. Distilled H_2O (50 mL) was added to each flask, and the contents were soaked overnight before autoclaving at 15 lb/in.^{2} for 30 min. The flasks were cooled to room temperature and inoculated with 3.0 mL of a hyphal cell suspension and incubated at 25 °C for 40 days.

Extraction and Isolation. The fermented rice substrate was extracted repeatedly with MEK (3 × 100 mL), and the organic solvent was evaporated to dryness under vacuum to afford 9.0 g of crude extract. The crude extract was partitioned between n-hexane/EtOAc/H_2O, and the EtOAc portion (2.0 g) was fractionated by Sephadex LH-20 column chromatography using MeOH as eluent. The active fraction (45 mg) was subsequently separated by Si gel column chromatography (2 × 13 cm) using $CHCl_3$–MeOH gradient elution. The fraction (15 mg) that was eluted with 1% MeOH was further separated by semipreparative reversed-phase HPLC (50% MeOH in H_2O over 5 min, 50%–90% over 30 min) on an Agilent Zorbax SB-C18 column (5 μm; 9.4 × 250

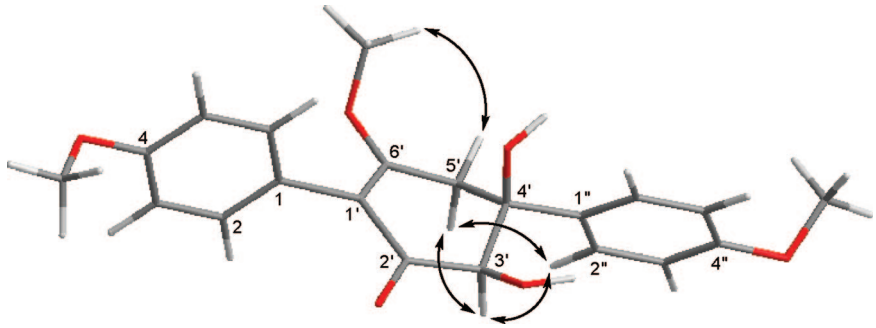

Figure 1. NOESY correlations used to establish the relative configuration of **1**.

Table 2. NMR Spectroscopic Data of Gliocladinin B (**2**) in Acetone-d_6

position	δ_H[a] (J in Hz)	δ_C[b] mult.	HMBC (H → C#)
1		131.2, qC	
2	7.51, d (8.5)	130.7, CH	4, 6, 1′
3	6.95, d (8.5)	113.1, CH	1, 5
4		159.0, qC	
4-OCH_3	3.83, s	54.6, CH_3	4
5	6.95, d (8.5)	113.1, CH	1, 3
6	7.51, d (8.5)	130.7, CH	2, 4, 1′
1′		134.4, qC	
2′		136.4, qC	
3′		148.7, qC	
3′-OH	8.39, br s		4′
4′		117.5, qC	
5′		151.8, qC	
5′-OH	7.80, br s		4′
6′	6.46, s	106.7, CH	1, 2′, 4′, 5′
1″		126.4, qC	
2″	7.41, d (8.4)	132.1, CH	4′, 4″, 6″
3″	6.98, d (8.4)	113.0, CH	1″, 4″, 5″
4″		158.6, qC	
4″-OCH_3	3.85, s	54.7, CH_3	4″
5″	6.98, d (8.4)	113.0, CH	1″, 3″, 4″
6″	7.41, d (8.4)	132.1, CH	4′, 2″, 4″,
1‴	4.39, d (7.8)	107.2, CH	2′
2‴	3.37, m	74.9, CH	
3‴	3.13, m	76.6, CH	
4‴	3.37, m	70.0, CH	
5‴	3.13, m	76.6, CH	
6‴a	3.37, m	61.7, CH_2	
6‴b	3.37, m		

[a] Recorded at 300 MHz. [b] Recorded at 75.5 MHz.

mm) at a flow rate of 2 mL/min to afford gliocladinins A (**1**; 2.0 mg) and B (**2**; 3.0 mg).

Gliocladinin A (1): white powder; $[\alpha]_D$ −10 (*c* 0.033 CH_3OH); UV (CH_3OH) λ_{max} 225 (ε 35 100), 274 (ε 16 400) nm; CD (*c* 1.0 × 10^{-4} M, MeOH) λ_{max} ($\Delta\varepsilon$) 238 (+15), 294 (−9.5) nm; ^{1}H NMR, ^{13}C NMR, and HMBC data, see Table 1; key NOESY correlations (acetone-d_6, 400 MHz) H-3′ ↔ H-5′a, H-2″/6″; H-5′a ↔ H-2″/6″, H-5′ ↔ 6′-OMe; HRESIMS *m*/*z* 393.1309 $[M + Na]^+$, calcd for $C_{21}H_{22}O_6Na$, 393.1311.

Gliocladinin B (2): pale yellow powder; $[\alpha]_D$ −20 (*c* 0.107 CH_3OH); UV (CH_3OH) λ_{max} 210 (ε 36 400), 272 (ε 34 600) nm; ^{1}H NMR, ^{13}C NMR, and HMBC data, see Table 2; HRESIMS *m*/*z* 523.1570 $[M + Na]^+$, calcd for $C_{26}H_{28}O_{10}Na$, 523.1580.

Determination of D-Glucose.[7,11] Gliocladinin B (**2**) (1.5 mg) in 300 μL of acetone was added to 700 μL of 6 N HCl in a hydrolysis tube and heated at 100 °C for 24 h. The reaction was then quenched with H_2O (3 mL) and extracted twice with 2 mL of EtOAc to remove the aglycone. The aqueous layer was subjected to optical rotation measurement. Identification of D-glucose in the aqueous layer was carried out by comparing the optical rotation of the liberated glucose ($[\alpha]_D$ +20) with those of authentic samples of D-glucose ($[\alpha]_D$ +100) and L-glucose ($[\alpha]_D$ −80).

Antimicrobial and Antifungal Bioassays. Antibacterial and antifungal bioassays were conducted according to a literature procedure.[12] The bacterial strains were grown on Mueller-Hinton agar, the yeasts *Candida albicans* (ATCC 10231) and *Geotrichum candidum* (AS2.498) were grown on Sabouraud dextrose agar, and the fungus *Aspergillus fumigatus* (ATCC 10894) was grown on potato dextrose agar. Test compounds were absorbed onto individual paper disks (6 mm diameter) at 100 μg/disk and placed on the surface of the agar. The assay plates were incubated at 25 °C for 48 h (at 37 °C for 24 h for antimicrobial activity) and examined for the presence of a zone of inhibition. The MIC values of gliocladinins A (**1**) and B (**2**) against *S. aureus* were determined using the broth dilution assay.[13]

MTT Assay. [14] In 96-well plates, each well was plated with 10^4 cells. After cell attachment overnight, the medium was removed, and each well was treated with 50 μL of medium containing 0.2% DMSO or appropriate concentration of test compounds (10 mg/mL as stock solution of a compound in DMSO and serial dilutions). Cells were treated at 37 °C for 4 h in a humidified incubator at 5% CO_2 first, and then the medium was changed to fresh Dulbecco's modified Eagle medium (DMEM). MTT (Sigma) was dissolved in serum free-medium or PBS at 0.5 mg/mL and sonicated briefly. In the dark, 50 μL of MTT/medium was added into each well after the medium was removed from the wells, and incubated at 37 °C for 3 h. Upon removal of MTT/medium, 100 μL of DMSO was added to each well and shaken at 60 rpm for 5 min to dissolve the precipitate. The assay plate was read at 540 nm using a microplate reader.

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra of gliocladinins A (**1**) and B (**2**). This material is available free of charge via the Internet at http://pubs.acs.org.

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