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Cyathane diterpenoids from fruiting bodies of *Phellodon niger*

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Abstract: Four new cyathane-type diterpenoids, nigernins C–F (**1**–**4**), together with four known compounds, were isolated from the fruiting bodies of the basidiomycete *Phellodon niger*. The structures of these new compounds were established on the basis of spectroscopic analysis, including 1D and 2D NMR experiments. In addition, nigernin F (**4**) with an unusual 3,4-*seco* cyathane diterpenoid skeleton was found to occur in nature for the first time. It was suggested to be as an oxidation product of C-3-C-4 bond cleavage of nigernin E (**3**).

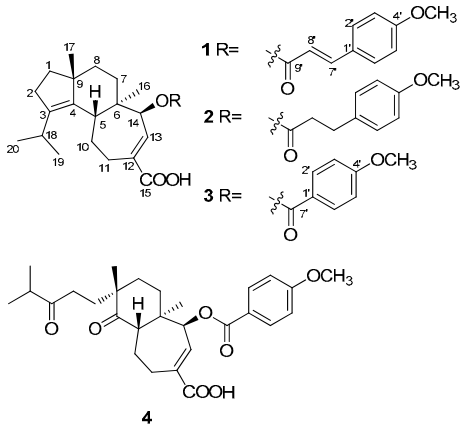
Keywords: cyathane, diterpenoid, nigernin, *Phellodon niger*

Introduction

Phellodon niger is an edible fungus belonging to the family Hydnaceae.[1] In our continuing search for novel and secondary metabolites from higher fungi of Yunnan province in China, we have previously isolated two new cyathane diterpenoids, nigernins A and B from this fungus.[2] Further research for the cyathane-type diterpenoids in the fruiting bodies of *P. niger* led to the isolation of four new cyathanes, nigernins C–F (**1**–**4**), along with four known compounds, sarcodonin δ,[3] 1,2-diacetoxy-3-(4′-hydroxyphenyl)-4,7,8-trihydroxy-dibenzofuran (Bl-V),[4] grifolic acid[5] and uridine.[6] Herein, we report the isolation and structure elucidation of the new compounds.

Results and Discussion

Compound **1** was isolated as white amorphous powder. The molecular formula was established to be $C_{30}H_{38}O_5$ based on HREIMS at *m/z* 478.2701 $[M]^+$ (calcd for $C_{30}H_{38}O_5$ $[M]^+$, 478.2719), indicating twelve degrees of unsaturation. The IR spectrum showed the presence of a hydroxy (3423 cm^{-1}) group, a benzene ring (1604, 1513, 1460 cm^{-1}) and two carbonyl (1713, 1690 cm^{-1}) groups. The ^1H NMR spectrum (Table 1) indicated the presence of four methyls [δ_H 0.88 (3H, s, H-16); 0.96 (6H, d, J = 6.7 Hz, H-19 and 20); 1.12 (3H, s, H-17)], a methoxyl group at δ_H 3.84 (3H, s, 4′-OCH_3), a 1′,4′-disubstituted benzene ring [δ_H 6.91 (2H, d, J = 8.7 Hz, H-3′ and 5′) and 7.49 (2H, d, J = 8.7 Hz, H-2′ and 6′)], a trans-double bond [δ_H 6.34 (1H, d, J = 15.9 Hz, H-8′) and 7.69 (1H, d, J = 15.9 Hz, H-7′)], an olefinic proton at δ_H 7.22 (1H, d, J = 7.1 Hz, H-13), and an oxymethine at δ_H 5.02 (1H, d, J = 7.1 Hz, H-14). The ^{13}C NMR spectrum of **1** (Table 1) revealed the presence of one *p*-methoxycinnamoyloxyl moiety [δ_C 55.4 (q),



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114.3 (d × 2), 115.1 (d), 127.0 (s), 129.8 (d × 2), 145.0 (d), 161.5 (s), 166.5 (s)]. The remaining 20 carbons were ascribable for four methyls, six methylenes, four methines, five quaternary carbons, and one carbonyl group. Comparison of NMR data of **1** with those for nigernin A, previously isolated from this fungus,[2] revealed the presence of the characteristic signals of a cyathane-type diterpenoid. The absence of a methylene resonance at δ_C 43.4 in the ^{13}C NMR spectrum of nigernin A, and the appearance of the signals at δ_C 77.8 and δ_H 5.02 (d, J = 7.1 Hz) in the NMR spectra of **1**, suggested the existence of an oxygenated methine attributable to C-14 in **1**. This was supported by the coupling constant (J = 7.1 Hz) of H-13 at δ_H 7.22, and the HMBC correlations from H-14 to C-5, C-12 and C-13, and from H-16 to C-14 (Figure

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1). In addition, the HMBC correlation from H-14 to C-9′ suggested that the *p*-methoxycinnamoyloxyl ester unit was linked to C-14. The ROESY correlations between H-5 and H-17, H-17 and H-8*β*, H-16 and H-8*α*, H-16 and H-14 were observed in **1**. It indicated H-14 to be *α*-oriented (Figure 2).

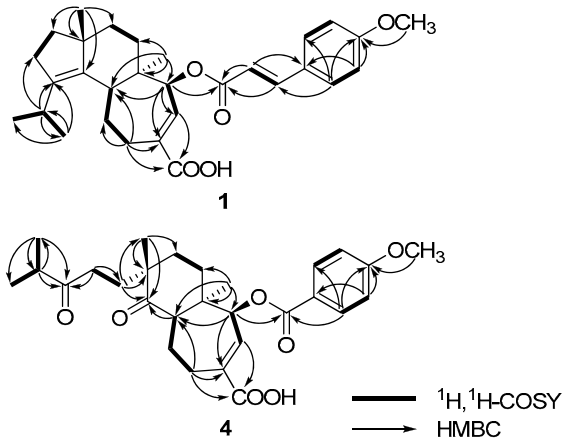

Figure 1. Key ^{1}H,^{1}H-COSY and HMBC correlations of **1** and **4**.

Thus, the structure of **1** was elucidated as 14*β*-(*p*-methoxycinnamoyloxyl)-cyatha-3,12-diene-15-oic acid, and named as nigernin C.

Compound **2** was obtained as white amorphous powder, giving the molecular formula $C_{30}H_{40}O_5$ by the HREIMS at *m/z* 480.2880 $[M]^+$ (calcd for $C_{30}H_{40}O_5$, $[M]^+$, 480.2876). The NMR spectral data of **2** (Table 1) were very similar to those of **1**, suggesting that **2** was also a cyathane diterpenoid. The key difference was the double bond in *p*-methoxycinnamoyloxyl unit of **1** replaced by two methylenes in **2**. This was confirmed by the HMBC spectrum, which showed correlations of H-7′ with C-1′, C-2′, C-6′, C-8′ and C-9′, and of H-8′ with C-1′, C-7′ and C-9′. In addition, correlation from δ_H 4.86 (1H, d, J = 7.2 Hz, H-14) to δ_C 172.3 (s, C-9′) was also observed in the HMBC spectra, indicating that the 3-(4-methoxyphenyl)propanoyloxyl ester unit was also linked to C-14 of the cyathane skeleton. The stereochemistry of **2** was in accordance with **1** by the analysis of the ROESY spectrum. Consequently, the structure of **2** was determined as 14*β*-(3-(4-methoxyphenyl)propanoyloxyl)-cyatha-3,12-diene-15-oic acid, and named as nigernin D.

Compound **3** was isolated as white amorphous powder. Its molecular formula was determined to be $C_{28}H_{36}O_5$ on the basis of molecular ion peak at *m/z* 452.2554 in the HREIMS (calcd for $C_{28}H_{36}O_5$ $[M]^+$, 452.2563), in combination with the ^{13}C NMR and DEPT spectra. The ^{1}H and ^{13}C NMR spectroscopic data of **3** (Table 1) were very similar to those of **1**, except for a *p*-methoxybenzoyloxyl group in **3** instead of the *p*-methoxycinnamoyloxyl group in **1**. The proton signals at δ_H 6.94 (2H, d, J = 8.9 Hz, H-3′ and 5′) and 8.04 (2H, d, J = 8.9 Hz, H-2′ and 6′) in the ^{1}H NMR spectrum, together with the ^{13}C-NMR signals at δ_C 55.5 (q), 113.8 (d × 2), 122.4 (s), 131.7 (d × 2), 163.5 (s), 165.5 (s) were determined readily as a *p*-methoxybenzoyloxyl unit. The location of the substituent and the stereochemistry of **3** were the same as those in **1** on the basis of analysis of HMBC and ROESY data. Therefore, compound **3** was identified as 14*β*-(*p*-methoxybenzoyloxyl)-cyatha-3,12-diene-15-oic acid, and named as nigernin E.

Compound **4** was obtained as white, amorphous powder and assigned the molecular formula $C_{28}H_{36}O_7$ as deduced by HREIMS (found *m/z* 484.2450 $[M]^+$, calcd for $C_{28}H_{36}O_7$ $[M]^+$, 484.2461). Comparison of the ^{1}H and ^{13}C NMR data of **4** (Table 1) with those of **3** indicated that their structures were similar. The main differences between these two compounds were the appearance of two keto carbonyl signals at δ_C 215.0 (s, C-3) and 215.5 (s, C-4) in **4** and the absence of two olefinic quaternary carbons [δ_C 140.4 (s, C-3) and 138.1(s, C-4)] in **3**. In addition, the EIMS of **4** with molecular ion peak $[M]^+$ at *m/z* 484 suggested more 32 mass units than that of **3**. On the basis of above evidence and the literature,[7] compound **4** should be a

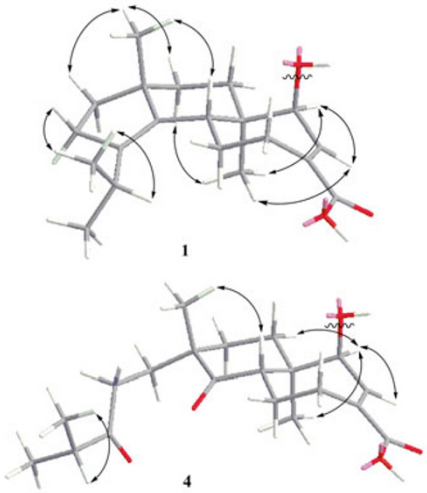

Figure 2. Key ROESY correlations of compounds **1** and **4**.

3,4-*seco* cyathane diterpenoid due to an oxidation cleavage of C-3-C-4 double bond of **3**. This was also confirmed by HMBC correlations (Figure 1) from H-2, H-18, H-19 and H-20 to C-3, and from H-5 and H-17 to C-4. The ROESY (Figure 2) correlations between H-5 and H-17 of **4** indicated that the methyl at C-9 is *β*. Therefore, the structure of **4** was determined to be 3,4-*seco* nigernin E, and named as nigernin F. To the best of our knowledge, this is the first report of the 3,4-*seco* cyathane skeleton from nature.

Experimental Section

General Experimental Procedures. Optical rotations were measured with a Horiba SEPA-300 polarimeter. UV spectra were recorded on a Shimadzu UV-2401PC spectrophotometer. IR spectra were obtained on a Bruker Tensor 27 spectrometer with KBr pellets. NMR spectra were recorded on a Bruker AV-400 or a DRX-500 spectrometer with TMS as an internal standard. EIMS and HREIMS were recorded on a VG Autospec-3000 mass spectrometer. Silica gel (200-300 mesh, Qingdao Marine Chemical Inc., China) and Sephadex LH-20 (Amersham Biosciences, Sweden) were used for column chromatography. HPLC was performed on an Agilent 1100 liquid chromatography system equipped with a Zorbax SB-C_{18} column (9.4 mm × 150 mm). TLC was performed on silica gel plates (GF254, Qingdao Marine Chemical Inc., China). The

spots on TLC were visualized by UV light (254/365 nm) and sprayed with 10% H_2SO_4 in ethanol, followed by heating.

Table 1. ^{1}H and ^{13}C NMR (400/100MHz) data of 1–4 in $CDCl_3$ (δ in ppm, J in Hz).

Pos.	1		2		3		4	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	1.59, m; 1.51, m	37.8, CH_2	1.56, m; 1.47, m[b]	37.7, CH_2	1.59, m; 1.50, m	37.8, CH_2	1.70, t (7.4)	32.2, CH_2
2	2.29, t (7,5)	28.5, CH_2	2.26, t (7.5)	28.5, CH_2	2.29 (t, 7.6)	28.6, CH_2	2.53, m; 2.45, m	35.4, CH_2
3		140.3, qC		140.3, qC		140.5, qC		215.0, qC
4		138.2, qC		138.1, qC		138.1, qC		215.5, qC
5	3.06, m	44.0, CH	2.91, m	43.8, CH	3.17, m	44.2, CH	3.46, m	51.6, CH
6		41.5, qC		41.2, qC		41.6, qC		43.9, qC
7	1.99, m	33.9, CH_2	1.77, td (13.4, 4.3)	33.8, CH_2	2.04, m	34.1, CH_2	2.38, m	32.2, CH_2
	1.18 br, d (13.5)		1.04, m		1.19 br, d (13.8)		1.37, d (13.5)	
8	1.55, m	36.5, CH_2	1.47, m[b]	36.4, CH_2	1.54, m	36.5, CH_2	1.78, m	33.1, CH_2
	1.42 br, d (13.5)		1.34 br, d (11.8)		1.40 br, d (12.5)		1.59, m	
9		49.2, qC		49.1, qC		49.2, qC		46.2, qC
10	1.98, m	26.2, CH_2	1.90, m	26.2, CH_2	2.00, m	26.4, CH_2	1.94 br, d (13.7)	21.0, CH_2
							1.50, m	
11	2.81, dt (15.5, 4.5)	25.7, CH_2	2.72 br, d (15.4)	25.6, CH_2	2.82 br, d (15.9)	25.8, CH_2	2.91, dd (15.9, 4.5)	24.2, CH_2
	2.57, m		2.34, m		2.56, m		2.39, m	
12		136.6, qC		136.8, qC		136.9, qC		137.7, qC
13	7.22, d (7.1)	141.6, CH	7.10, d (7.2)[c]	140.9, CH	7.25, d (7.3)	141.4, CH	7.22, d (7.3)	139.8, CH
14	5.02, d (7.1)	77.8, CH	4.86, d (7.2)	78.0, CH	5.09, d (7.3)	78.0, CH	5.15, d (7.3)	76.5, qC
15		172.5, qC		171.5, qC		172.3, qC		171.3, qC
16	0.88, s	16.5, CH_3	0.80, s	16.4, CH_3	0.90, s	16.5, CH_3	0.83, s	16.8, CH_3
17	1.12, s	24.3, CH_3	1.03, s	24.2, CH_3	1.11, s	24.3, CH_3	1.23, s	23.6, CH_3
18	3.00, m	26.8, CH	2.95, m	26.8, CH	3.01, m	26.8, CH	2.63, m	40.9, CH
19	0.96, d (6.7)	21.9, CH_3[a]	0.93, d (6.7)	21.9, CH_3[a]	0.96, d (6.7)	21.9, CH_3[a]	1.09, d (6.9)	18.3, CH_3
20	0.96, d (6.7)	21.7, CH_3[a]	0.93, d (6.7)	21.6, CH_3[a]	0.96, d (6.7)	21.7, CH_3[a]	1.09, d (6.9)	18.3, CH_3
1′		127.0, qC		132.2, qC		122.4, qC		121.9, qC
2′	7.49, d (8.7)	129.8, CH	7.10, d (6.7)[b]	129.2, CH	8.04, d (8.9)	131.7, CH	8.01, d (8.5)	131.6, CH
3′	6.91, d (8.7)	114.3, CH	6.81, d (6.7)	113.9, CH	6.94, d (8.9)	113.8, CH	6.95, d (8.5)	114.0, CH
4′		161.5, qC		158.1, qC		163.5, qC		163.7, qC
5′	6.91, d (8.7)	114.3, CH	6.81, d (6.7)	113.9, CH	6.94, d (8.9)	113.8, CH	6.95, d (8.5)	114.0, CH
6′	7.49, d (8.7)	129.8, CH	7.10, d (6.7)[b]	129.2, CH	8.04, d (8.9)	131.7, CH	8.01, d (8.5)	131.6, CH
7′	7.69, d (15.9)	145.0, CH	2.93, t (7.6)	30.3, CH_2		165.5, qC		165.2, qC
8′	6.34, d (15.9)	115.1, CH	2.66, t (7.6)	36.2, CH_2				
9′		166.5, qC		172.3, qC				
OCH_3	3.84, s	55.4, CH_3	3.78, s	55.2, CH_3	3.86, s	55.5, CH_3	3.87, s	55.5, CH_3

[a]Interchangeable assignments. [b]Overlapping resonances.

Fungal Material. The basidiomycete *P. niger* was collected at Wuding of Yunnan Province in August 2009, and identified by Prof. Zhu-Liang Yang, Kunming Institute of Botany. The voucher specimen was deposited at the Herbarium of Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and Isolation. The air-dried fruiting bodies (950 g) were extracted three times with $CHCl_3$/MeOH (1:1, v/v) at room temperature. After removal of the solvent by evaporation, the residue (98.0 g) was subjected to silica gel column eluted with a petroleum ether-acetone gradient system (1:0–1:1, v/v) to give fractions A–I. Fraction E was subjected to Sephadex LH-20 using $CHCl_3$-MeOH (1:1, v/v) to give 4 subfractions: E1–E4. Subfraction E3 was further purified by silica gel chromatography ($CHCl_3$/MeOH, 50:1) and preparative HPLC (CH_3CN/H_2O, 65:35) to obtain **2** (9.6 mg) and **3** (18.3 mg). Compound **4** (13.2 mg) was purified from subfraction E2 by preparative HPLC (CH_3CN/H_2O, 40:60). Fraction F was passed through Sephadex LH-20 using $CHCl_3$/MeOH (1:1, v/v) and repeated column chromatography over silica gel, and finally purified by preparative HPLC using a mobile phase of CH_3CN/H_2O (75:25 and 65:35) to afford **1** (16.2 mg) and grifolic acid (12.3 mg), respectively. Sarcodonin δ (17.0 mg) was purified from fraction G by repeated silica gel column chromatography. Fraction H was subjected to silica gel, Sephadex LH-20, and preparative HPLC to give 1,2-diacetoxy-3-(4′-hydroxyphenyl)-4,7,8-trihydroxy-dibenzofuran (5.4 mg). Fraction I was separated over silica gel eluted with $CHCl_3$/MeOH (10:1), and then further purified by preparative HPLC to af-

ford uridine (2.6 mg).

Nigernin C (1): white amorphous powder; $[\alpha]_D^{20}$ − 26.7 (*c* 0.27, MeOH); UV (MeOH) λ_{max} (log ε): 311 (4.09), 223 (3.97) nm; IR (KBr) ν_{max}: 3423, 2958, 2935, 2866, 1713, 1690, 1604, 1513, 1460, 1252, 1170, 999, 828 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; EIMS *m/z* 478 $[M]^+$; HREIMS *m/z* 478.2701 $[M]^+$ (calcd for $C_{30}H_{38}O_5$ $[M]^+$, 478.2719).

Nigernin D (2): white amorphous powder; $[\alpha]_D^{20}$ − 2.64 (*c* 0.24, MeOH); UV (MeOH) λ_{max} (log ε): 223 (3.85) nm; IR (KBr) ν_{max}: 3432, 2955, 2934, 2866, 1690, 1613, 1514, 1461, 1248 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; EIMS *m/z* 480 $[M]^+$; HREIMS *m/z* 480.2880 $[M]^+$ (calcd for $C_{30}H_{40}O_5$ $[M]^+$, 480.2876).

Nigernin E (3): white amorphous powder; $[\alpha]_D^{20}$ − 37.0 (*c* 0.21, MeOH); UV (MeOH) λ_{max} (log ε): 258 (3.94) nm; IR (KBr) ν_{max}: 3424, 2958, 2936, 2866, 1717, 1690, 1607, 1511, 1459, 1257, 1167, 1098 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; EIMS *m/z* 452 $[M]^+$; HREIMS *m/z* 452.2554 $[M]^+$ (calcd for $C_{28}H_{36}O_5$ $[M]^+$, 452.2563).

Nigernin F (4): white amorphous powder; $[\alpha]_D^{20}$ + 33.4 (*c* 0.21, MeOH); UV (MeOH) λ_{max} (log ε): 259 (4.01) nm; IR (KBr) ν_{max}: 3434, 2968, 2936, 2871, 1710, 1606, 1512, 1462, 1258, 1167, 1098 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; EIMS *m/z* 484 $[M]^+$; HREIMS *m/z* 484.2450 $[M]^+$ (calcd for $C_{28}H_{36}O_7$ $[M]^+$, 484.2461).

Cytotoxicity Assay. The cytotoxicity assay against C8166 cells (CC_{50}) was assessed using the MTT method and anti-HIV-1 activity was evaluated by the inhibition assay for the cytopathic effects of HIV-1 (EC_{50}).[19]

Electronic Supplementary Material

Supplementary material is available in the online version of this article at http://dx.doi.org/10.1007/s13659-011-0002-z and is accessible for authorized users.

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