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.\textsuperscript{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.\textsuperscript{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 \(\delta\),\textsuperscript{3} 1,2-diacetoxy-3-(4′-hydroxyphenyl)-4,7,8-trihydroxy-dibenzo-furan (Bl-V),\textsuperscript{4} grifolic acid\textsuperscript{5} and uridine.\textsuperscript{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 \(C_{38}H_{38}O_{3}\) based on HREIMS at \(m/z\) 478.2701 [M]\textsuperscript{+} (calcd for \(C_{38}H_{38}O_{3}\) [M]\textsuperscript{+}, 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 \(^1\)H NMR spectrum (Table 1) indicated the presence of four methyls [\(\delta_H 0.88\) (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 \(\delta_H 3.84\) (3H, s, 4′-OCH\(_3\)), a 1′,4′-disubstituted benzene ring [\(\delta_H 6.91\) (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 [\(\delta_H 6.34\) (1H, d, \(J = 15.9\) Hz, H-8′) and 7.69 (1H, d, \(J = 15.9\) Hz, H-7′)], an olefinic proton at \(\delta_H 7.22\) (1H, d, \(J = 7.1\) Hz, H-13), and an oxymethine at \(\delta_H 5.02\) (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 [\(\delta_C 55.4\) (q), 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,\textsuperscript{7} revealed the presence of the characteristic signals of a cyathane-type diterpenoid. The absence of a methylene resonance at \(\delta_C 43.4\) in the \(^{13}\)C NMR spectrum of nigernin A, and the appearance of the signals at \(\delta_C 77.8\) and \(\delta_H 5.02\) (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 \(\delta_H 7.22\), 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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Optical rotations were +28° and 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-8β, H-16 and H-8γ, H-16 and H-14 were observed in 1. It indicated H-14 to be α-oriented (Figure 2).

Thus, the structure of 1 was elucidated as 14β-(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 C₂₀H₂₂O₆ by the HREIMS at m/z 480.2880 [M⁺] (calcd for C₂₀H₂₂O₆ [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 of H-8′ with C-1′, C-7′ and C-9′. In addition, correlation from δ, 4.86 (1H, d, J = 7.2 Hz, H-14) to δC 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 14β-(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 C₂₃H₂₄O₈ on the basis of molecular ion peak at m/z 452.2554 in the HREIMS (calcd for C₂₃H₂₄O₈ [M⁺], 452.2563), in combination with the ¹³C NMR and DEPT spectra. The ¹H and ¹³C NMR spectroscopic data of 3 (Table 1) were very similar to those of 1, except for a p-methoxybenzoyloxyl group in 3 instead of a p-methoxycinnamoyloxyl group in 1. The proton signals at δH 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 ¹H NMR spectrum, together with the ¹³C-NMR signals at δC 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 14β-(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 C₂₅H₂₆O₇ as deduced by HREIMS (found m/z 484.2450 [M⁺], calcd for C₂₅H₂₆O₇ [M⁺], 484.2461). Comparison of the ¹H and ¹³C 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 δC 215.0 (s, C-3) and 215.5 (s, C-4) in 4 and the absence of two olefinic quaternary carbons [δC 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, compound 4 should be a 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-C₁₈ 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% H2SO4 in ethanol, followed by heating.

subfractions: E1–E4. Subfraction E3 was further purified by silica gel chromatography (CHCl3:MeOH, 50:1) and preparative HPLC (CH3CN/H2O, 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 (CH3CN/H2O, 40:60). Fraction F was passed through Sephadex LH-20 using CHCl3:MeOH (1:1, v/v) and repeated column chromatography over silica gel, and finally purified by preparative HPLC using a mobile phase of CH3CN/H2O (75:25 and 65:35) to afford 1 (16.2 mg) and grrifolic 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-dibenzo[b,e]furan (5.4 mg). Fraction I was separated over silica gel eluted with CHCl3:MeOH (10:1), and then further purified by preparative HPLC to af-

Table 1. 1H and 13C NMR (400/100MHz) data of 1–4 in CDCl3 (δ in ppm, J in Hz).

| Pos. | δH | δC | δH | δC | δH | δC | δH | δC |
|------|----|----|----|----|----|----|----|----|
| 1    | 1.59, m; 1.51, m | 37.8, CH2 | 1.56, m; 1.47, m | 37.7, CH2 | 1.59, m; 1.50, m | 37.8, CH2 | 1.70, t (7.4) | 32.2, CH2 |
| 2    | 2.29, t (7.5) | 28.5, CH2 | 2.26, t (7.5) | 28.5, CH2 | 2.29 (t, 7.6) | 28.6, CH2 | 2.53, m; 2.45, m | 35.4, CH2 |
| 3    | 140.3, qC | | 140.3, qC | | 140.5, qC | | | |
| 4    | 138.2, qC | | 138.1, qC | | 138.1, 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 | | | |
| 7    | 1.99, m | 33.9, CH2 | 1.77, td (13.4, 4.3) | 33.8, CH2 | 2.04, m | 34.1, CH2 | 2.38, m | 32.2, CH2 |
| 8    | 1.18 br, d (13.5) | 21.04, m | | 1.19 br, d (13.8) | 1.37, d (13.5) | | |
| 9    | 1.55, m | 36.5, CH2 | 1.47, m8 | 36.4, CH2 | 1.54, m | 36.5, CH2 | 1.78, m | 33.1, CH2 |
| 10   | 1.42 br, d (13.5) | 36.5, CH2 | 1.34 br, d (11.8) | 1.40 br, d (12.5) | | | |
| 11   | 1.98, m | 26.2, CH2 | 1.90, m | 26.2, CH2 | 2.00, m | 26.4, CH2 | 1.94 br, d (13.7) | 21.0, CH2 |
| 12   | 2.81, dt (15.5, 4.5) | 25.7, CH2 | 2.72 br, d (15.4) | 25.6, CH2 | 2.82 br, d (15.9) | 25.8, CH2 | 2.91, dd (15.9, 4.5) | 24.2, CH2 |
| 13   | 2.57, m | 2.34, m | | | | | | |
| 14   | 136.6, qC | | 136.8, qC | | 136.9, qC | | | 137.7, qC |
| 15   | 7.22, d (7.1) | 141.6, CH | 7.10, d (7.2) | 140.9, CH | 7.25, d (7.3) | 141.4, CH | 7.22, d (7.3) | 139.8, CH |
| 16   | 50.2, 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 |
| 17   | 172.5, qC | | 171.5, qC | | 172.3, qC | | | |
| 18   | 0.88, s | 16.5, CH2 | 0.80, s | | 16.4, CH2 | 0.90, s | 16.5, CH2 | 0.83, s |
| 19   | 1.12, s | 24.3, CH3 | 1.03, s | | 24.2, CH3 | 1.11, s | 24.3, CH3 | 1.23, s |
| 20   | 3.00, m | 26.8, CH2 | 2.95, m | | 26.8, CH2 | 3.01, m | 26.8, CH2 | 2.63, m |
| 21   | 0.96, d (6.7) | 21.9, CH3a | 0.93, d (6.7) | 21.9, CH3a | 0.96, d (6.7) | 21.9, CH3a | 1.09, d (6.9) | 18.3, CH3 |
| 22   | 0.96, d (6.7) | 21.7, CH3a | 0.93, d (6.7) | 21.6, CH3a | 0.96, d (6.7) | 21.7, CH3a | 1.09, d (6.9) | 18.3, CH3 |
| 23   | 127.0, qC | | 132.2, qC | | 122.4, qC | | | |
| 24   | 7.49, d (8.7) | 129.8, CH | 7.10, d (8.7) | 129.2, CH | 8.04, d (8.9) | 131.7, CH | 8.01, d (8.5) | 131.6, CH |
| 25   | 6.91, d (8.7) | 114.3, CH2 | 6.81, d (8.7) | 113.9, CH2 | 6.94, d (8.9) | 113.8, CH2 | 6.95, d (8.5) | 114.0, CH |
| 26   | 161.5, qC | | 158.1, qC | | 163.5, qC | | | |
| 27   | 6.91, d (8.7) | 114.3, CH2 | 6.81, d (8.7) | 113.9, CH2 | 6.94, d (8.9) | 113.8, CH2 | 6.95, d (8.5) | 114.0, CH |
| 28   | 7.49, d (8.7) | 129.8, CH | 7.10, d (6.7) | 129.2, CH | 8.04, d (8.9) | 131.7, CH | 8.01, d (8.5) | 131.6, CH |
| 29   | 7.69, d (15.9) | 145.0, CH | 2.93, t (7.6) | 30.3, CH2 | | | |
| 30   | 6.34, d (15.9) | 115.1, CH2 | 2.66, t (7.6) | 36.2, CH2 | | | |
| 31   | 166.5, qC | | 172.3, qC | | | | | |

*Interchangeable assignments. †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, Kunning Institute of Botany. The voucher specimen was deposited at the Herbarium of Kunning Institute of Botany, Chinese Academy of Sciences.

Extraction and Isolation. The air-dried fruiting bodies (950 g) were extracted three times with CHCl3: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 (1.0:1.1:1.1, v/v/v) to give fractions A–I. Fraction E was subjected to Sephadex LH-20 using CHCl3:MeOH (1:1, v/v) to give 4.
ford uridine (2.6 mg).

**Nigernin C (1):** white amorphous powder; $[\alpha]_{D}^{20} = 26.7$ (c 0.27, MeOH); UV (MeOH) $\lambda_{max}$ (log $\epsilon$): 311 (4.09), 223 (3.97) nm; IR (KBr) $\nu_{max}$: 3423, 2958, 2935, 2866, 1713, 1690, 1604, 1513, 1460, 1252, 1170, 999, 828 cm$^{-1}$; $^1$H 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) $\lambda_{max}$ (log $\epsilon$): 223 (3.85) nm; IR (KBr) $\nu_{max}$: 3432, 2955, 2934, 2866, 1690, 1613, 1514, 1461, 1248 cm$^{-1}$; $^1$H 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) $\lambda_{max}$ (log $\epsilon$): 258 (3.94) nm; IR (KBr) $\nu_{max}$: 3424, 2958, 2936, 2866, 1717, 1690, 1607, 1511, 1459, 1257, 1167, 1098 cm$^{-1}$; $^1$H 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) $\lambda_{max}$ (log $\epsilon$): 259 (4.01) nm; IR (KBr) $\nu_{max}$: 3434, 2968, 2936, 2871, 1710, 1606, 1512, 1462, 1258, 1167, 1098 cm$^{-1}$; $^1$H 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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