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Benzophenones from an Endophytic Fungus, *Graphiopsis chlorocephala*, from *Paeonia lactiflora* Cultivated in the Presence of an NAD$^+$-Dependent HDAC Inhibitor

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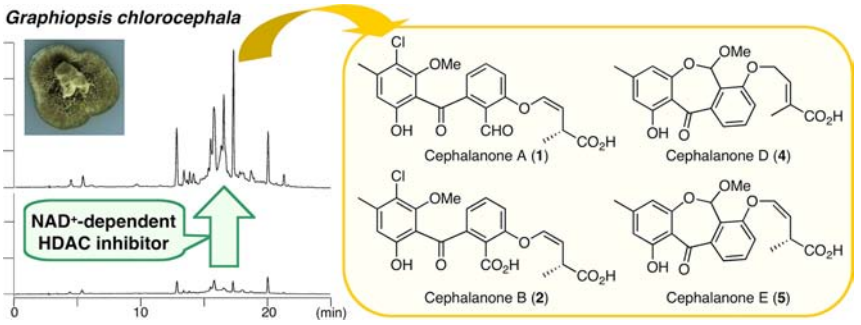
ABSTRACT



Graphiopsis chlorocephala **was separated from the surface-sterilized healthy leaves of** ***Paeonia lactiflora*** **(Paeoniaceae) and cultivated with nicotinamide (an NAD$^+$-dependent HDAC inhibitor). The culture conditions significantly enhanced secondary metabolite production in the fungus and led to the isolation of a structurally diverse set of new benzophenones, cephalanones A–F (1–6), and a known 2-(2,6-dihydroxy-4-methylbenzoyl)-6-hydroxybenzoic acid (7). The structures of 1–6 were determined from NMR data, single crystal X-ray diffraction, and chemical transformations.**

The epigenetic manipulation of fungal gene expression by small molecule DNA methyl transferase and/or histone deacetylase (HDAC) inhibitors influences secondary metabolism in the fungus and is an appropriate method for exploring novel fungal metabolites prepared through cryptic biosynthetic pathways.[1] To date, the technique has been applied to only a limited number of fungi, and a handful of new natural products have been isolated.[2] In an effort to develop the method further and obtain a variety of fungal secondary metabolites, we applied small molecule epigenetic modifiers, particularly suberoyl bis-hydroxamic acid (a Zn(II)-type HDAC inhibitor), to entomopathogenic fungi and successfully isolated numerous novel natural products containing unprecedented carbon skeletal compounds.[3]

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(1) (a) Shwab, E. K.; Bok, J. W.; Tribus, M.; Galehr, J.; Graessle, S.; Keller, N. P. *Eukaryot. Cell* **2007**, *6*, 1656–1664. (b) Cichewicz, R, H. *Nat. Prod. Rep.* **2010**, *27*, 11–22.

(2) (a) Williams, R. B.; Henrikson, J. C.; Hoover, A. R.; Lee, A. E.; Cichewicz, R. H. *Org. Biomol. Chem.* **2008**, *7*, 1895–1897. (b) Henrikson, J. C.; Hoover, A. R.; Joyner, P. M.; Cichewicz, R. H. *Org. Biomol. Chem.* **2009**, *7*, 435–438. (c) Wang, X.; Sena Filho, J. G.; Hoover, A. R.; King, J. B.; Ellis, T. K.; Powell, D. R.; Cichewicz, R. H. *J. Nat. Prod.* **2010**, *73*, 942–948. (d) Vervoort, H. C.; Draškovic, M.; Crews, P. *Org. Lett.* **2011**, *13*, 410–413.

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Our recent study provided the first evidence that an NAD^+-dependent HDAC inhibitor, nicotinamide, could enhance secondary metabolite production in *Chaetomium mollipilium* by increasing the histone acetylation levels. In this way, we obtained a variety of unique C_{13}-polyketides,[4] demonstrating that chemical epigenetic manipulation techniques involving the application of HADC inhibitors could promote the generation of a variety of new compounds from attenuated or silent biosynthetic pathways in fungi. The type of fungi that will respond to the method by displaying altered metabolite production has yet to be characterized.

Recent studies have revealed that certain endophytic fungi produce medicinally important compounds identical to those contained in the host plants, such as paclitaxel (*Taxus brevifolia*),[5] podophyllotoxin (*Podophyllum peltatum*),[6] camptothecin (*Camptotheca acuminata*),[7] hypericin and emodin (*Hypericum perforatum*),[8] ginkolide B (*Ginkgo biloba*),[9] and piperin (*Piper longum* L.).[10] These findings would indicate crosstalk between the endophytic fungi and the host plants on the genetic level, bearing peculiar biosynthetic pathways. The application of a chemical epigenetic method to endophytic fungi may plausibly lead to novel bioactive compounds[11] as well as new analogs of the bioactive plant constituents. In our search for the endophytic fungi of medicinal plants, we separated *Graphiopsis chlorocephala* from the surface-sterilized healthy leaves of *Paeonia lactiflora* (Paeoniaceae).

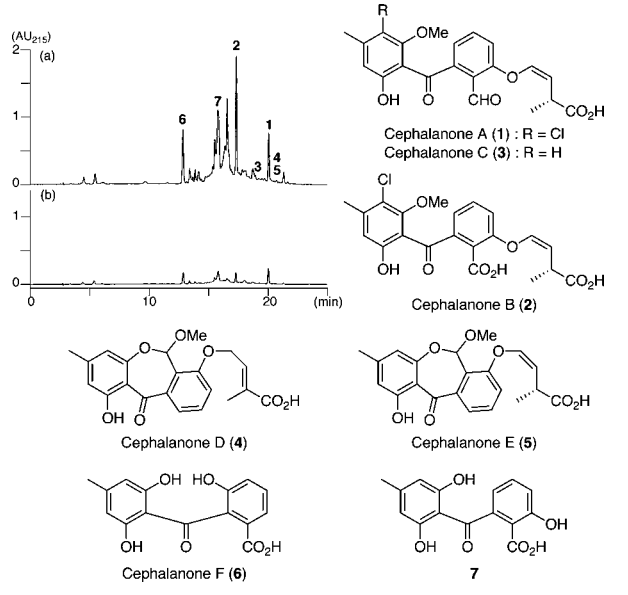


Figure 1. HPLC profiles of the EtOAc extracts of *G. chlorocephala* cultivated with (a) nicotinamide 10 μM and (b) control as detected by UV absorption at 215 nm, and structures of **1**–**7**.

This medicinal plant is one of the most important materials in oriental medicine, and its principal bioactive constituent, paeoniflorin, is widely used in the treatment of cerebral ischemia, epilepsy, and neurodegenerative disorders, such as Alzheimer's disease and Parkinson's disease.[12] We attempted to induce the synthesis of novel secondary metabolites and paeoniflorin analogs by cultivating the endophytic fungus *G. chlorocephala* in the presence of HADC inhibitors and found that cultivation with nicotinamide (10 μM) yielded a notable enrichment in secondary metabolite production (Figure 1), leading to the isolation of six new benzophenones, cephalanones A–F (**1**–**6**) and 2-(2,6-dihydroxy-4-methylbenzoyl)-6-hydroxybenzoic acid (**7**). Among these, cephalanones A (**1**) and B (**2**) were characterized as uncommon chlorinated benzophenones. Here, we elucidate the structures of the new compounds based on spectroscopic data, X-ray crystallography, and chemical conversion techniques.

G. chlorocephala was cultivated in potato dextrose broth (PDB) with 10 μM nicotinamide for 14 days at 25 °C. The culture medium (7.5 L) was extracted twice with ethyl acetate. The ethyl acetate extract (1.4 g) was separated by Sephadex LH-20, silica gel column chromatography, and reversed-phase HPLC to afford cephalanones A (**1**, 54.1 mg), B (**2**, 109.7 mg), C (**3**, 1.9 mg), D (**4**, 2.2 mg), E (**5**, 4.5 mg), F (**6**, 2.1 mg), and 2-(2,6-dihydroxy-4-methylbenzoyl)-6-hydroxybenzoic acid (**7**, 39.7 mg) (Figure 1).

The HRFABMS of cephalanone A (**1**) at m/z 419.0896 $[M + H]^+$ (calcd: m/z 419.0892) showed its molecular formula to be $C_{21}H_{19}O_7Cl$ (12 degrees of unsaturation). The IR spectrum (1707, 1682, and 1627 cm^{-1}) indicated the presence of three types of unsaturated carbonyl groups, which was further confirmed by the presence of resonances at δ 200.7 (C-8), 188.3 (C-1), and 179.7 (C-4′) in the ^{13}C NMR spectrum. Additionally, the ^{13}C NMR and DEPT spectra revealed the presence of eight sp^2 quaternary, six sp^2 tertiary, one methine, one methoxy methyl, and two methyl carbons (Table S1). The ^1H NMR spectrum displayed signals due to a 1,2,3-trisubstituted benzene ring [δ 7.60 (t, J = 8.0 Hz, H-5), 7.14 (d, J = 8.0 Hz, H-4), 6.97 (d, J = 8.0 Hz, H-6)] and a pentasubstituted benzene ring [δ 6.77 (s, H-11)] (Table S1). The sequential ^1H–^1H COSY

(3) (a) Asai, T.; Chung, Y. M.; Sakurai, H.; Ozeki, T.; Chang, F. R.; Yamashita, K.; Oshima, Y. *Org. Lett.* **2012**, *14*, 513–515. (b) Asai, T.; Yamamoto, T.; Oshima, Y. *Org. Lett.* **2012**, *14*, 2006–2009.

(4) Asai, T.; Morita, S.; Shirata, N.; Taniguchi, T.; Monde, K.; Sakurai, H.; Ozeki, T.; Oshima, Y. *Org. Lett.* **2012**, *14*, 5456–5459.

(5) (a) Stierle, A.; Strobel, G.; Stierle, D. *Science* **1993**, *260*, 214–216. (b) Soliman, S. S. M.; Tsao, R.; Raizada, M. N. *J. Nat. Prod.* **2011**, *74*, 2497–2504.

(6) Eyberger, A. L.; Dondapati, R.; Porter, J. R. *J. Nat. Prod.* **2006**, *69*, 1121–1124.

(7) (a) Kusari, S.; Zühlke, S.; Spiteller, M. *J. Nat. Prod.* **2009**, *72*, 2–7. (b) Shweta, S.; Zuehlke, S.; Ramesha, B. T.; Priti, V.; Kumar, P. M.; Ravikanth, G.; Spiteller, M.; Vasudeva, R.; Shaanker, R. U. *Phytochemistry* **2010**, *71*, 117–122.

(8) Kusari, S.; Lamshöft, M.; Zühlke, S.; Spiteller, M. *J. Nat. Prod.* **2008**, *71*, 159–162.

(9) Cui, Y.; Yi, D.; Bai, X.; Sun, B.; Zhao, Y.; Zhang, Y. *Fitoterapia* **2012**, *83*, 913–920.

(10) Verma, V. C.; Lobkovsky, E.; Gang, A. C.; Singh, S. K.; Prakash, S. *J. Antibiot.* **2011**, *64*, 427–431.

(11) (a) Asai, T.; Luo, D.; Yamashita, K.; Oshima, Y. *Org. Lett.* **2013**, *15*, 1020–1023. (b) Chen, H.; Awakawa, T.; Sun, J.; Wakimoto, T.; Abe, I. *Nat. Prod. Bioprospect.* **2013**, *3*, 20–23. (c) Sun, J.; Awakawa, T.; Noguchi, H.; Abe, I. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 6397–6400.

(12) Cao, B. Y.; Yang, Y. P.; Luo, W. F.; Mao, C. J.; Han, R.; Sun, X.; Cheng, J.; Liu, C. F. *J. Ethnopharmacol.* **2010**, *131*, 122–129.

correlations through H-1′ to H_3-5′ and the HMBC correlations of H_3-5′/C-2′, C-3′, C-4′ indicated the structure of the oxidized prenyl moiety. The prenyl moiety was linked to C-3 by an enol ether bond, as indicated by the C−H long-range correlation between H-1′ and C-3 (Figure 2). The substitution pattern, including the aldehyde (C-1) and ketone group (C-8) on the 1,2,3-trisubstituted benzene ring, was elucidated by the HMBC correlations of H-1/C-2, C-7; H-4/C-2; H-5/C-3, C-7; H-6/C-2, C-8. The molecular formula of **1** indicated the presence of a chlorine atom on the pentasubstituted benzene ring. The ^{1}H NMR spectrum revealed a downfield shifted exchangeable proton at δ 12.40 (Table S1), indicating the presence of a C-10 hydroxyl group hydrogen bonded to the C-8 carbonyl. The HMBC correlations among H_3-15/C-11, C-12, C-13; H-11/C-9, C-10, C-13; OMe/C-14 indicated the structure of the C-13 chlorinated pentasubstituted benzene ring (Figure 2).

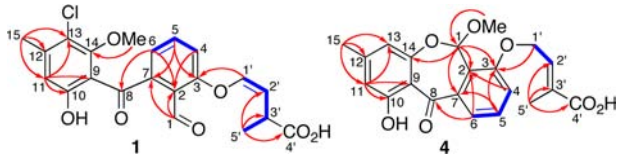
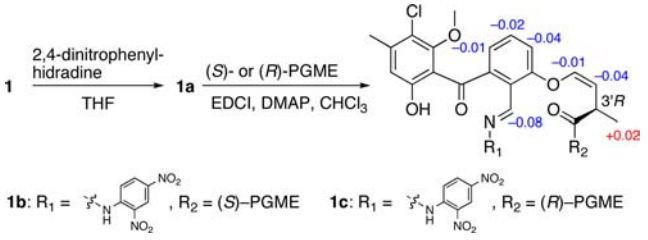


Figure 2. Key ^{1}H−^{1}H COSY (blue bold line) and HMBC (red arrow) correlations of **1** and **4**.

The *cis* geometry of the C-1′/C-2′ double bond was deduced from the small J value (6.0 Hz) (Table S1). After the selective protection of the aldehyde as a 2,4-dinitrophenylhydrazone (**1a**), the carboxylic acid was converted to an (*S*)- or (*R*)-phenylglycine methyl ester (**1b**, **1c**) and the PGME method was applied.[13] The values of $\Delta\delta$ (**1b**−**1c**) allowed us to identify the molecule as having a 3′*R* configuration (Scheme 1).

Scheme 1. Conversion of **1** to **1b** and **1c** *via* **1a**, and the $\Delta\delta$(**1b**−**1c**) Values



The ^{1}H and ^{13}C NMR spectra of cephalanone B (**2**), $C_{21}H_{19}O_8Cl$ [HRFABMS: m/z 435.0819 $[M + H]^+$ (calcd: m/z 435.0841)], agreed well with those of **1**, except that **2** had an α,β-unsaturated carboxylic acid [δ_C 170.4 (C-1)] in place of the aldehyde in **1** (Table S1). This observation suggested that the C-1 aldehyde in **1** was oxidized to the carboxylic acid in **2**. Analysis of the COSY and HMBC spectra confirmed the structure of **2**.

Cephalanone C (**3**) was expected to be a dechlorinated analog of **1** based on the molecular formula, $C_{21}H_{20}O_7$ [HRFABMS: m/z 385.1289 $[M + H]^+$ (calcd: m/z 385.1282)], and the UV and IR spectra. The ^{1}H and ^{13}C NMR spectra resembled those of **1** except for the presence of an additional aromatic proton [δ 6.05 (brs, H-13)] (Table S1). Moreover, the aromatic proton correlated with both H_3-15 and OMe in the 1D NOE experiment, indicating that the chlorine atom at C-13 in **1** was replaced by a proton in **3**.

The HRESIMS of cephalanone D (**4**) at m/z 383.1139 $[M - H]^-$ (calcd: m/z 383.1136) suggested that the molecular formula was $C_{21}H_{20}O_7$ (12 degrees of unsaturation). The strong IR absorptions (1695 and 1635 cm^{-1}) indicated the presence of two types of α,β-unsaturated carbonyl groups. The ^{13}C NMR and DEPT spectra implied the presence of one keto carbonyl, one carboxyl, eight sp^2 quaternary, six sp^2 tertiary, one acetal, one oxy methylene, and two methyl carbons (Table S2). The presence of two carbonyl and 14 sp^2 carbons revealed a three-ring system in the molecule. As in **1**, three proton signals [δ 7.52 (d, J = 7.8 Hz, H-6), 7.42 (t, J = 7.8 Hz, H-5), 7.06 (d, J = 7.8 Hz, H-4)] assignable to a 1,2,3-trisubstituted benzene ring were observed in the ^{1}H NMR spectrum. In addition, two *meta*-coupled aromatic protons [δ 6.52 (brs, H-11), 6.39 (brs, H-13)] indicated the presence of a 1,2,3,5-tetrasubstituted benzene ring (Table S2). The ^{1}H−^{1}H COSY correlations between H_2-1′/H-2′ and the HMBC correlations of H_3-5′/C-2′, C-3′, C-4′ indicated an oxidized prenyl unit structure with an α,β-unsaturated carboxylic acid (C-1′−C-5′). The HMBC correlations of H-1/C-2, C-3, C-7; H-4/C-2; H-5/C-3, C-7; H-6/C-2, C-8; H_2-1′/C-3 showed that C-2, C-3, and C-7 on the trisubstituted benzene ring were linked to the C-1 acetal, C-1′, *via* an ether bond, and a C-8 keto carbonyl, respectively. The correlation involving OMe/C-1 indicated that the methoxy group was positioned on C-1 (Figure 2). As in **1**, the presence of a downfield-shifted exchangeable proton at δ 13.00 (10-OH) in the ^{1}H NMR spectrum (Table S2) was consistent with the presence of a phenolic hydroxy group at C-10 and a C-8−C-9−C-10 linkage. The structure of the tetrasubstituted benzene ring (C-9−C-14) was determined based on the HMBC correlations of H_3-15/C-11, C-12, C-13; H-11/C-9, C-10; H-13/C-9, C-14. Finally, the C-1−O−C-14 bonding configuration was elucidated from the H-1/C-14 long-range correlation (Figure 2). The geometry of the C-2′/C-3′ trisubstituted double bond was determined to be *E* by X-ray crystallography (Figure 3). 1*S*-**4** and 1*R*-**4** were present as an enantiomeric pair, as indicated by the complementary hydrogen bonding between the carboxylic acids, observed in the X-ray crystallographic analysis (Figure S1).

The ^{1}H and ^{13}C NMR spectra of cephalanone E (**5**), $C_{21}H_{20}O_7$ [(−)HRESIMS at m/z 383.1137 $[M - H]^-$ (calcd: m/z 383.1136)], agreed well with the corresponding spectra of **4**, except for the signals assignable to C-1′−C-5′ (Table S2). The signals due to C-1′−C-5′ agreed well with

(13) Yabuuchi, T.; Kusumi, T. *J. Org. Chem.* **2000**, *65*, 397–404.

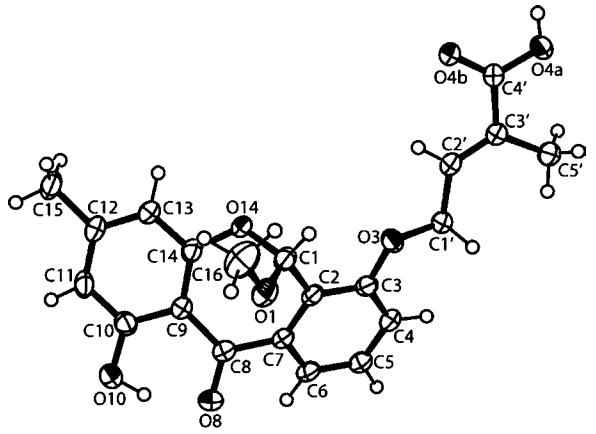


Figure 3. ORTEP stereodiagram of **4**.

those observed in **1** but not with those of **4** (Tables S1 and S2), suggesting that **5** possessed the same oxidized prenyl moiety as **1**.

The absolute stereochemistries of **2**, **3**, and **5** at C-3′ were also asummed to be *R* based on their biosynthetic relationship with **1**.

Cephalanone F (**6**) possessed the same molecular formula, $C_{21}H_{20}O_7$ [HRESIMS: *m*/*z* 311.0518 $[M + Na]^+$ (calcd: *m*/*z* 311.0526)], as 2-(2,6-dihydroxy-4-methylbenzoyl)-6-hydroxybenzoic acid (**7**). The UV and IR spectra were identical to those of **7**. The similarities of the ^1H and ^{13}C NMR spectra to the corresponding spectra of **7** suggested that **6** possessed a benzophenone core composed of 1,2,3-trisubstituted and *C*2 symmetrical 1,2,3,5-tetrasubstituted benzene rings (Table S3); however, the ^1H and ^{13}C chemical shifts of the signals on the trisubstituted benzene differed from those of **7**. The HMBC correlations of H-3/C-1, C-7; H-4/C-2, C-6; H-5/C-7 revealed the substitutions on C-2 [carboxylic acid (C-1)], C-6 (hydroxy group), and C-7 [ketone (C-8)] (Figure S2). Thus the structure of **6** was determined to be 2-(2,6-dihydroxy-4-methylbenzoyl)-3-hydroxybenzoic acid and was named cephalanone F.

In this study, we demonstrated that the addition of nicotinamide, an NAD^+-dependent HDAC inhibitor, to the culture medium of the endophytic *G. chlorocephala*, which was isolated from the surface-sterilized healthy leaves of *P. lactiflora*, significantly stimulated its benzophenone production. Although a paeoniflorin analog could not be obtained from the culture medium, chemical manipulation of the fungal epigenetics using an NAD^+-dependent HDAC inhibitor permitted the isolation of a variety of new benzophenones, including the uncommon chlorinated compounds, cephalanones A (**1**) and B (**2**). This result provides the first evidence that NAD^+-dependent HDAC inhibitors are useful for the discovery of new natural products from endophytic fungi. This study also expands the applications of chemical epigenetic methods.

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Supporting Information Available. Experimental methods, full spectroscopic data and NMR spectra of new compounds, and the cif file for cephalanone D (**4**). This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.