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6*H*-Dibenzo[*b,d*]pyran-6-one derivatives from the cultured lichen mycobionts of *Graphis* spp. and their biosynthetic origin

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Abstract

The spore-derived mycobionts of the lichen *Graphis prunicola, G. cognata* and *G. scripta* were cultivated on a malt–yeast extract medium supplemented with 10% sucrose and their metabolites were investigated. Graphislactones A–D were isolated from the cultures of *G. prunicola*, while alternariol and graphislactones A and C were isolated from those of *G. cognata*. From the cultured mycobionts of *G. scripta*, a new 6*H*-dibenzo[*b,d*]pyran-6-one derivative, graphislactone E with graphislactones A and C was obtained. On the other hand, cultivation of the mycobionts of *G. prunicola* on a malt–yeast extract medium supplemented with 2.5% sucrose and 0.25% sodium acetate produced two new metabolites, graphislactones E and F. Their structures were determined by spectroscopic methods. The biogenetic origin of the carbon skeleton in both compounds was verified by administering sodium [1-^{13}C]-acetate and sodium [1,2-$^{13}C_2$]-acetate.

Keywords: *Graphis prunicola*; *G. cognata*; *G. scripta*; Lichen; Isolated mycobiont; 6*H*-Dibenzo[*b,d*]pyran-6-one; Graphislactones; Biogenesis

1. Introduction

Lichens are symbiotic organisms comprised of a fungus, mycobiont, and a photosynthetic partner, photobiont, which may be a cyanobacterium and/or green alga. They produce diverse secondary metabolites, some of which show a wide range of potentially useful biological activities (Yamamoto, 1991; Huneck, 1999). Our recent studies demonstrated that cultures of lichen mycobionts have an ability under osmotically stressed conditions to produce substances which have never been detected in the lichenized state but are structurally related to fungal metabolites (Tanahashi et al., 1997). Thus, cultures of lichen mycobionts could be good tools for investigating the potential ability of the fungal partner in secondary metabolism. In the course of our studies on cultured lichen mycobionts of *Graphis* species (Tanahashi et al., 2000; Takenaka et al., 2000), we cultivated mycobionts of the lichen *G. prunicola* Vain., *G. cognata* Müll. Arg. and *G. scripta* (L.) Ach., and isolated from their cultures several 6*H*-dibenzo[*b,d*]pyran-6-one derivatives, two of which were new. In this paper, we report the structure determination and biosynthesis of these compounds.

2. Results and discussion

Graphis prunicola, *G. cognata* and *G. scripta* (Graphidaceae) were collected in Florida, USA, Ehime, Japan, and Sumik, Slovenia, respectively, and their polyspore-derived mycobionts were cultured on a conventional malt-yeast extract medium supplemented with 10% sucrose at 18 °C in the dark. After cultivation for 9–13 months, the colonies and agar medium were harvested and extracted with cold Et_2O or Me_2CO.

Fractionation of the extract from cultured mycobionts of *G. prunicola* by preparative TLC afforded four compounds, **1**, **2**, **3** and **4**. These compounds were identified as graphislactones A–D from their spectral data and direct comparison with authentic samples, which had previously been isolated from cultured mycobionts of *G. scripta* (L.) Ach. var. *pulverulenta* Ach (Tanahashi et al., 1997). They were also characterized as their acetates **1a**, **2a**, **3a** and **4a**. The results confirmed the pre-

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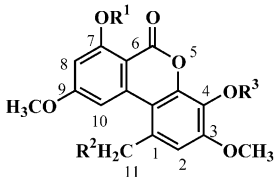
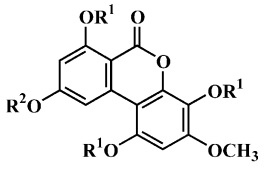
liminary screening where graphislactones A–C (**1**–**3**) were detected by TLC in the cultured mycobionts of *G. prunicola* (Hamada et al., 1997).

From the cultured mycobionts of *G. cognata,* a phenolic compound **5** was isolated along with graphislactones A (**1**) and C (**3**). Its spectral features allowed identification of **5** as the mycotoxin alternariol (Raistrick et al., 1953; Stinson et al., 1986). This is the first instance that alternariol is isolated from cultured mycobionts of lichen.

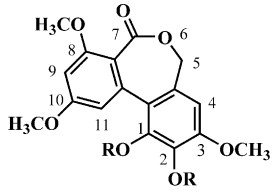
From the cultured mycobionts of *G. scripta*, a new phenolic compound **6** was isolated together with graphislactones A (**1**) and C (**3**). The HR-EIMS of **6** established a molecualr formula of $C_{15}H_{12}O_7$. Its ^{1}H NMR spectrum exhibited signals for two methoxyl groups at δ 3.90 and 3.92 (each *s*), a pair of *meta*-coupled aromatic protons at δ 6.75 and 7.82 (each *d*, $J = 2.5$ Hz), and an aromatic proton at δ 6.85 (*br s*). These ^{1}H NMR spectral features of **6** resembled those of **1** except for the absence of a methyl signal (Tanahashi et al., 1997). Conventional acetylation of **6** afforded triacetate **6a**, indicating the presence of three phenolic hydroxyl groups in **6**. These findings suggested that the methyl group at C-1 was replaced by a hydroxyl group in **6**. The placement of methoxyl groups at C-3 and C-9 was substantiated by the NOESY spectrum of **6a**, which showed cross-peaks between (1) H-2 (δ 6.85) and OMe (δ 3.90), (2) H-8 (δ 6.75) and OAc (δ 2.43), (3) H-8 and OMe (δ 3.92), and (4) H-10 (δ 7.82) and OAc (δ 2.46). Furthermore, the HMBC correlations from H-10 to C-9 and C-10b and from OMe (δ 3.92) to C-9 were fully consistent with structure **6a**. Thus, the structure of the isolated compound was elucidated as shown and compound **6** was designated as graphislactone E (Scheme 1).



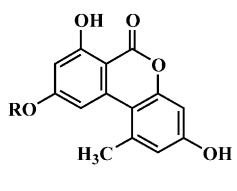
1: $R^1 = R^2 = R^3 = H$
2: $R^1 = CH_3$, $R^2 = R^3 = H$
3: $R^1 = R^3 = H$, $R^2 = OH$
1a: $R^1 = R^3 = Ac$, $R^2 = H$
2a: $R^1 = CH_3$, $R^2 = H$, $R^3 = Ac$
3a: $R^1 = R^3 = Ac$, $R^2 = OAc$



6: $R^1 = H$, $R^2 = CH_3$
8: $R^1 = R^2 = H$
6a: $R^1 = Ac$, $R^2 = CH_3$
8a: $R^1 = R^2 = Ac$



4: R = H
4a: R = Ac



5: R = H
7: $R = CH_3$

Scheme 1.

The structural similarity of graphislactones to alternariol (**5**) suggested that graphislactones could be postulated to be biosynthesized via alternariol (**5**) and alternariol methyl ether (**7**). The proposal was supported by the fact that we have isolated alternariol (**5**) together with graphislactones A (**1**) and C (**3**) from the cultured mycobionts of *G. cognata*. The biosynthesis of arternariol (**5**) has already been studied. The possible pathway via norlichexanthone was ruled out and it was proven that alternariol (**5**) could be biosynthesized by simple cyclization and aromatization of a polyketide precursor (Darenbrock and Simpson, 1987). To prove the incorporation pattern of acetate units into graphislactones, administration experiments with ^{13}C-labelled acetates were conducted. Administration of 0.01–0.05% sodium acetate to the mycobionts of *G. prunicola* cultivated on a malt–yeast extract medium supplemented with 10% sucrose resulted in no significant incorporation, while administration of 0.5–1.0% sodium acetate damaged the cultured cells. When the mycobionts were cultivated on a malt–yeast extract medium supplemented with 2.5% sucrose and 0.25% sodium acetate, the metabolic characteristics of the cultures changed significantly; i.e. two metabolites **6** and **8** were the main products instead of graphislactones A–D (**1**–**4**).

These two compounds **6** and **8** were unstable and showed no acetyl signal in their ^{1}H NMR spectra, therefore the structures were determined as their acetates. One compound was identical with graphislactone E triacetate (**6a**). The EI mass spectrum of another compound **8a** exhibited a molecular ion peak at *m*/*z* 458. Its ^{1}H NMR spectrum exhibited signals for a methoxyl group at δ 3.91, four acetoxyl groups at δ 2.33, 2.35, 2.43 and 2.46, a pair of *meta*-coupled aromatic protons at δ 6.99 and 8.33 (each *d*, $J = 2.0$ Hz), and an aromatic proton at δ 6.86 (*br s*). These ^{1}H NMR spectral features of **8a** were closely similar to those of **6a** except for the absence of a methoxyl group and presence of an additional acetyl group in **8a**. Comparison of their ^{13}C NMR spectral data and detailed NMR studies including NOESY and HMBC experiments demonstrated that the methoxyl group at C-9 was replaced by an acetoxyl group in **8a**. Accordingly, the new compound **8** was formulated as shown and named graphislactone F.

Administration of [1-^{13}C]-acetate and [1,2-$^{13}C_2$]-acetate resulted in moderate incorporation into graphislactones E (**6**) and F (**8**). The ^{13}C NMR spectra of graphislactone E triacetate (**6a**) and graphislactone F tetraacetate (**8a**) showed that C-1 carbon of acetate was incorporated into the carbons at C-1, 3, 4a, 6, 7, 9 and 10a in graphislactones E (**6**) and F (**8**). The carbon pairs originating from intact acetate units were determined by 2D INADEQUATE experiments with **6a** and **8a** isolated from the cultured mycobionts incubated with

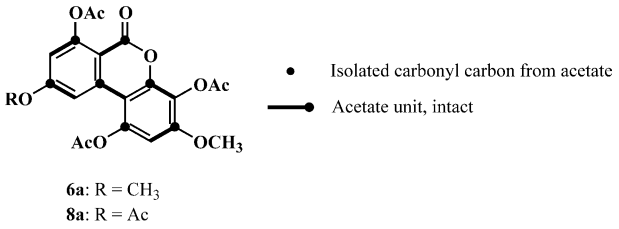


Fig. 1. Labelling pattern of graphisactone E triacetate (**6a**) and graphislactone F tetraacetate (**8a**) derived from [1,2-$^{13}C_2$]-acetate.

[1,2-$^{13}C_2$]-acetate (Fig. 1). Thus, it was shown that acetate units were incorporated into graphislactones E (**6**) and F (**8**) in the same manner as in alternariol (**5**) (Stinson et al., 1986). Alternariol (**5**) could suffer oxidative demethylation at C-1, hydroxylation at C-4 and *O*-methylation to lead graphislactones E (**6**) and F (**8**). On the other hand, alternariol (**5**) could be transformed to graphislactones A–D (**1**–**4**) without demethylation (Fig. 2).

6*H*-Dibenzo[*b*,*d*]pyran-6-one derivatives, as represented by alternariol (**5**), have so far been isolated from fungus *Alternaria tenuis* but not from the thallus of lichens. However, the present studies demonstrated that production of graphislactones is not restricted to a certain strain of cultured mycobionts and that graphislactones might be biosynthesized via alternariol (**5**). It might be postulated that the dormant fungal metabolism was induced in the cultures of the isolated mycobionts. From the view-point of evolution, the origin of isolated mycobionts might be the same as that of free-living fungi, for example, *Alternaria*, and they gained different lifestyles and were separated in the evolutionary process (Lutzoni et al., 2001). In the symbiotic state with photobionts, the mycobionts might have conserved the original metabolic ability of ancient mycobionts. These metabolic pathways might be normally supressed by any action of the photobiont, but expressed in the isolated mycobiont.

3. Experimental

3.1. General

Melting points were measured on a Yanaco micro melting point apparatus and are uncorrected. The UV spectra were recorded on a Shimadzu UV-240 spectrophotometer and the IR spectra on a Shimadzu FTIR-8200 infrared spectrophotometer. HR-EIMS were obtained with a Hitachi M-4100 mass spectrometer. The NMR experiments were performed with Varian VXR-500, Varian Gemini-300 and Varian Gemini-200 spectrometers, with tetramethylsilane as internal standard. TLC was performed on precoated Kieselgel 60F254 plates (Merck) and spots were visualized under UV light.

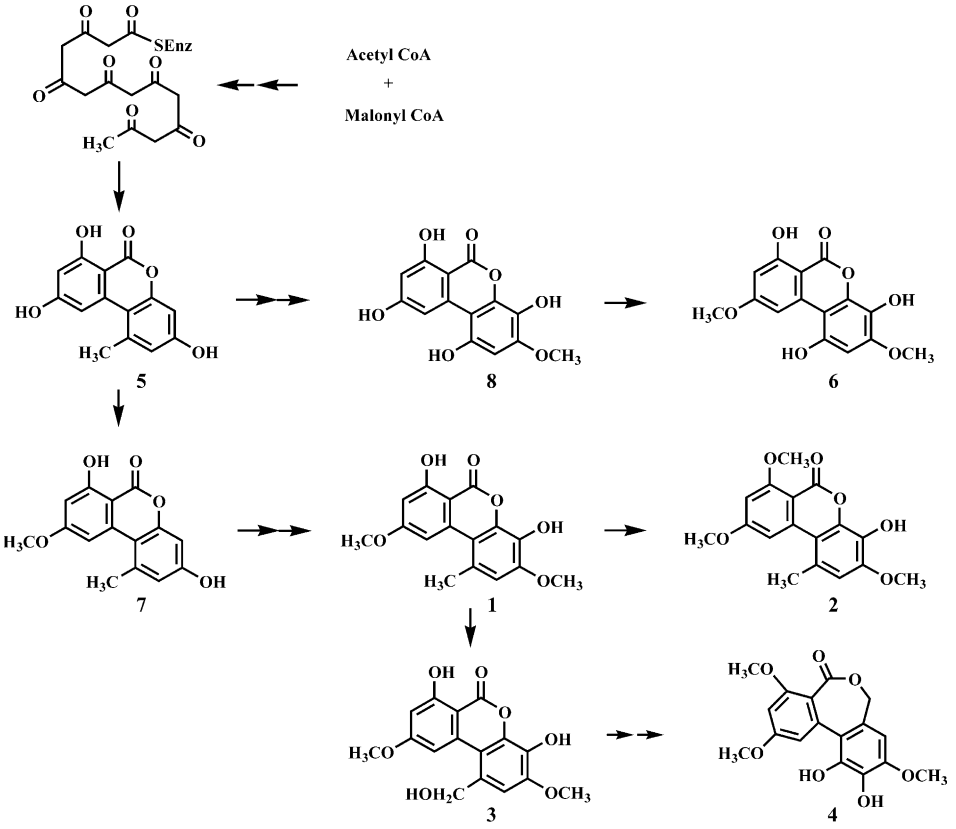


Fig. 2. Biogenetic sequence for alternariol and graphislactones A–F.

3.2. Plant material

Specimens of *Graphis prunicola* were collected from the bark of trees in Florida, USA (10 m alt.) in 1996. The voucher specimen (No. NH9592553) was identified by Dr. B. Ryan, Arizona State University, USA and deposited at Osaka City Institute of Public Health and Environmental Sciences. Mycobionts of *G. prunicola* were obtained from the spores discharged from apothecia of a thallus, and were cultivated in 32 test tubes containing modified MY10 medium (malt extract 10 g, yeast extract 4 g, sucrose 100 g, agar 15 g, H_2O 1 l, pH 7) at 18 °C in the dark. After cultivation for 9 months, the colonies and slants with crystals were harvested.

Specimens of *G. cognata* and *G. scripta* were collected from the bark of trees in Mikawa, Kamiukena-gun, Ehime Prefecture, Japan (700 m alt.) in 1994 and from the bark of trees in Sumik, Slovenia (900 m alt.) in 1996, respectively. The voucher specimens were identified by Professor M. Nakanishi of Hiroshima University, Japan, and were deposited at Osaka City Institute of Public Health and Environmental Sciences with the registration No. NH940223 (*G. cognata*) and No. NH9682844 (*G. scripta*). Mycobionts of *G. cognata* and *G. scripta* were cultivated on the same medium described earlier at 18 °C in the dark for 10 and 13 months, respectively.

3.3. Extraction and isolation

3.3.1. Graphis prunicola

The harvested colonies (dry wt. 13.7 g) were continuously extracted with Et_2O. The Et_2O extract (113.1 mg) was repeatedly subjected to preparative TLC with $CHCl_3$–MeOH (9:1) and AcOEt–C_6H_6–EtOH (4:1:0.5) to give **1** (33.9 mg), **2** (3.2 mg), **3** (6.0 mg), and **4** (3.2 mg). Conventional acetylation of **1**–**4** gave **1a**–**4a**, respectively.

Graphislactone A diacetate (**1a**): ^{1}H NMR ($CDCl_3$): δ 2.33 (3H, *br s*, 1-Me), 2.43, 2.46 (each 3H, *s*, 2 × OAc), 3.91, 3.92 (each 3H, *s*, 2 × OMe), 7.82 (1H, *d*, J = 2.4 Hz, H-8), 6.85 (1H, *br s*, H-2), 7.82 (1H, *d*, J = 2.4 Hz, H-10). HR-EIMS *m*/*z*: calc. for $C_{20}H_{18}O_8$ $[M]^+$: 386.1002. Found: 386.1010.

Graphislactone B acetate (**2a**): ^{1}H NMR ($CDCl_3$): δ 2.43 (3H, *s*, 7-OAc), 2.83 (3H, *br s*, 1-Me), 3.91, 3.95, 4.00 (each 3H, *s*, 3 × OMe), 6.55 (1H, *d*, J = 2.1 Hz, H-8), 6.74 (1H, *br s*, H-2), 7.25 (1H, *d*, J = 2.1 Hz, H-10). HR-EIMS *m*/*z*: calc. for $C_{19}H_{18}O_7$ $[M]^+$: 358.1053. Found: 358.1058.

Graphislactone C triacetate (**3a**): ^{1}H NMR ($CDCl_3$): δ 2.18 (3H, *s*, 1-CH_2OAc), 2.41, 2.43 (each 3H, *s*, 2 × OAc), 3.94, 3.95 (each 3H, *s*, 2 × OMe), 5.46 (2H, *br s*, CH_2), 6.79 (1H, *d*, J = 2.4 Hz, H-8), 7.12 (1H, *br s*, H-2), 7.53 (1H, *d*, J = 2.4 Hz, H-10). HR-EIMS *m*/*z*: calc. for $C_{22}H_{20}O_{10}$ $[M]^+$: 444.1057. Found: 444.1056.

Graphislactone D diacetate (**4a**): ^{1}H NMR ($CDCl_3$): δ 2.18, 2.31 (each 3H, *s*, 2 × OAc), 3.82, 3.89 × 2 (each 3H, *s*, 3 × OMe), 4.81, 4.96 (each 1H, *d*, J = 12.5 Hz, H_2-5), 6.55 (1H, *d*, J = 2.4 Hz, H-9), 6.59 (1H, *d*, J = 2.4 Hz, H-11), 6.96 (1H, *br s*, H-4). HR-EIMS *m*/*z*: calc. for $C_{21}H_{20}O_9$ $[M]^+$: 416.1107. Found: 416.1097.

3.3.2. Graphis cognata

The harvested colonies (fr. wt. 39.6 g) were extracted with acetone at room temp., and the combined extracts were concentrated under reduced pressure to give a residue (3.8 g). The residue was purified by a combination of a CC on silica gel with $CHCl_3$–MeOH and prep. TLC with $CHCl_3$–MeOH (9:1), toluene–AcOH (20:3), or C_6H_6–dioxane–AcOH (36:9:1) giving rise to **1** (13.6 mg), **3** (2.6 mg), and **5** (14.0 mg). Compounds **1** and **3** were identified as graphislactones A and C, respectively.

Alternariol (**5**): Colorless crystalline solid, mp. > 300° ($CHCl_3$). UV λ_{max}^{MeOH} nm (log ϵ): 211*sh* (4.33), 256 (4.63), 289 (3.97), 300 (4.00), 333 (4.00), 340 (4.01). ^{13}C NMR as in ref. (Stinson et al., 1986). HR-EIMS *m*/*z*: calc. for $C_{14}H_{10}O_5$ $[M]^+$: 258.0525. Found: 258.0556.

3.3.3. Graphis scripta

The harvested colonies (dry wt. 15.9 g) were continuously extracted with acetone. The acetone extract (78.0 mg) was repeatedly subjected to prep. TLC with toluene–acetone (4:1) and prep. HPLC (μBondasphere 5 μC18–100 Å, H_2O–CH_3CN, 1:1) to give graphislactone A (**1**) (2.7 mg), graphislactone C (**3**) (6.3 mg) and graphislactone E (**6**) (13.6 mg).

Graphislactone E (**6**): Colorless crystalline solid, UV λ_{max}^{MeOH} nm (log ϵ): 226, 260, 288 *sh*, 298 *sh*, 339. ^{1}H NMR (CD_3OD): δ 3.90 (3H, *s*, 3-OMe), 3.92 (3H, *s*, 9-OMe), 6.46 (1H, *d*, J = 2.5 Hz, H-8), 6.53 (1H, *br s*, H-2), 8.16 (1H, *d*, J = 2.5 Hz, H-10). ^{13}C NMR (CD_3OD): δ 56.1, 56.6 (3-OMe, 9-OMe), 92.6 (C-2), 99.4 (C-10b), 100.4 (C-10), 102.3 (C-6a), 104.8 (C-8), 131.9 (C-4), 138.7 (C-10a), 145.9 (C-4a), 146.5 (C-1), 150.9 (C-3), 165.4 (C-9), 167.1 (C-6), 168.4 (C-7). HR-EIMS *m*/*z*: calc. for $C_{15}H_{12}O_7$ $[M]^+$: 304.0583. Found: 304.0601.

3.4. Acetylation of *6*

Compound **6** (6.2 mg) was acetylated with Ac_2O–pyridine (each 0.1 ml) and the crude acetate was purified by prep. TLC ($CHCl_3$) and prep. HPLC (μBondasphere 5 μC18-100Å, H_2O–CH_3CN, 1:1) to yield graphislactone E triacetate (**6a**) (5.4 mg). **6a**: Colorless crystalline solid, mp 199–200 °C (MeOH). UV λ_{max}^{MeOH} nm (log ϵ): 225 (4.31), 257 (4.60), 286.5 (3.98), 300 (3.98), 322.5 (3.91). IR ν_{max}^{KBr} cm^{-1}: 1774, 1736, 1611, 1562. ^{1}H NMR ($CDCl_3$): δ 2.33 (3H, *s*, 4-OAc), 2.43 (3H, *s*, 7-OAc), 2.46 (3H, *s*, 1-OAc), 3.90 (3H, *s*, 3-OMe), 3.92 (3H, *s*, 9-OMe), 6.75 (1H, *d*, J = 2.5 Hz,

H-8), 6.85 (1H, *br s*, H-2), 7.82 (1H, *d*, $J=2.5$ Hz, H-10). ^{13}C NMR ($CDCl_3$): δ 20.2 (4-CO$\underline{C}H_3$), 20.8 (1-CO$\underline{C}H_3$), 21.2 (7-CO$\underline{C}H_3$), 55.8, 56.5 (3-OMe, 9-OMe), 99.1 (C-2), 105.1 (C-10b), 106.6 (C-6a), 107.7 (C-10), 109.5 (C-8), 129.8 (C-4), 136.8 (C-10a), 141.0 (C-4a), 150.4 (C-1), 153.4 (C-3), 154.8 (C-7), 156.9 (C-6), 164.9 (C-9), 167.0 (1-$\underline{C}OCH_3$), 167.6 (4-$\underline{C}OCH_3$), 169.5 (7-$\underline{C}OCH_3$). HMBC correlations: H-2→C-1/C-3/C-4/C-10b, 3-OMe→C-3, H-8→C-6a/C-7/C-8, 9-OMe→C-9, H-10→C-6a/C-8/C-9/C-10b. HR-EIMS *m*/*z*: calc. for $C_{21}H_{18}O_{10}$ $[M]^+$: 430.0900. Found: 430.0918.

3.5. Administration experiments

Cultured mycobionts of *G. prunicola* were transferred to 8 test tubes containing modified MY medium (malt extract 10 g, yeast extract 4 g, sucrose 25 g, agar 15 g, H_2O 1l, pH 7) supplemented with sodium [1-^{13}C]-acetate (25 mg /tube). The cultures were grown at 18 °C in the dark for 6 months. The colonies (dry wt. 1.99 g) were harvested and successively extracted with Et_2O and acetone. The Et_2O extract (24.7 mg) and acetone extract (91.5 mg) were purified by prep. TLC with toluene–acetone (9:1), to give crude **6** (9.6 mg) and a mixture of **6** and **8** (24.8 mg). Acetylation of **6** in Ac_2O–pyridine yielded **6a** (4.9 mg). The mixture of **6** and **8** was acetylated and purified by prep. TLC ($CHCl_3$) and prep. HPLC (H_2O–CH_3CN, 1:1) to afford **6a** (1.6 mg) and **8a** (8.1 mg). **6a**: ^1H NMR ($CDCl_3$): δ 2.34 (3H, *s*, 4-OAc), 2.43 (3H, *s*, 7-OAc), 2.46 (3H, *s*, 1-OAc), 3.91 (3H, *s*, 3-OMe), 3.92 (3H, *s*, 9-OMe), 6.75 (1H, *d*, $J=2.5$ Hz, H-8), 6.85 (1H, *br s*, H-2), 7.82 (1H, *d*, $J=2.5$ Hz, H-10). EIMS *m*/*z*: (%): 430 $[M]^+$ (6.8), 388 (16.3), 346 (17.6), 304 (100). **8a**: Colorless crystalline solid, ^{1}HNMR ($CDCl_3$): δ 2.33 (3H, *s*, 1-OAc), 2.35 (3H, *s*, 7-OAc), 2.43 (3H, *s*, 9-OAc), 2.46 (3H, *s*, 4-OAc), 3.91 (3H, *s*, 3-OMe), 6.86 (1H, *br s*, H-2), 6.99 (1H, *d*, $J=2.0$ Hz, H-8), 8.33 (1H, *d*, $J=2.0$ Hz, H-10). ^{13}C NMR ($CDCl_3$): δ 20.2, 20.6, 21.1, 21.4 (4 × CO$\underline{C}H_3$), 56.5 (3-OMe), 100.0 (C-2), 104.9 (C-10b), 110.8 (C-6a), 114.8 (C-10), 116.5 (C-8), 130.1 (C-4), 136.6 (C-10a), 141.1 (C-4a), 150.2 (C-1), 153.71 (C-3), 153.74 (C-7), 155.7 (C-9), 156.5 (C-6), 167.1 (4-$\underline{C}OCH_3$), 167.5 (1-$\underline{C}OCH_3$), 167.7 (7-$\underline{C}OCH_3$), 169.2 (9-$\underline{C}OCH_3$). HMBC correlations: H-2→C-1/C-3/C-4/C-10b, 3-OMe→C-3, H-8→C-6a/C-7/C-8/C-9, H-10→C-6a/C-8/C-9/C-10b. EIMS *m*/*z* (%): 458 $[M]^+$ (6.0), 416 (18.1), 374 (30.8), 332 (100), 290 (83.6).

Administration of sodium [1,2-$^{13}C_2$]-acetate to the cultures (seven test tubes) was performed in the same way as for [1-^{13}C]-CH_3COONa. The colonies (dry wt. 1.72 g) were worked-up as earlier to afford **6a** (9.6 mg) and **8a** (5.2 mg).

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