Characterization of Polyketide Metabolites from Foliar Endophytes of *Picea glauca*

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A collection of 250 foliar endophytes of *Picea glauca* (white spruce) yielded several isolates that produced metabolites toxic to *Choristoneura fumiferana* (spruce budworm). Three of these strains were selected for further study based on their ability to be cultured and produce secondary metabolites under laboratory conditions. The culture filtrate of each was extracted and analyzed by LC-MS and LC-NMR, and the major metabolites were isolated and characterized. Structures were elucidated by spectroscopic analyses including 2D NMR and HRMS and by comparison to literature data. In some cases the extract was methylated in order to facilitate separation, but the original natural structure was determined by comparing the NMR data of the isolated methylated product with that of the stop-flow NMR of the underivatized extract (i.e., **2a**, **2b**, and **4**). Two of these metabolites, **1** and **2a**, are new structures, **3** and **4** are reported here for the first time as fungal metabolites, and **5**−**10** as known fungal metabolites from other species. Tyrosol (**10**) was the only common metabolite found in all three extracts but did not account for the observed toxicity to *C. fumiferana*.

Foliar fungal endophytes of conifers may fulfill several ecological roles including limiting needle herbivory.[1] Over the past two decades, we have identified a number of anti-insectan metabolites produced by these fungi.[2,3] Needles of trees colonized with these endophytes have been shown to contain the toxins. These toxins in turn are responsible for reducing the growth rate of *Choristoneura fumiferana* larvae (spruce budworm). The presence of the endophyte and its toxin is thought to increase the development time of larvae, put the populations out of reproductive synchrony, and expose them to a greater risk of death from parasitoides, pathogens, and predation. This is thought to decrease the risk of an epidemic.[4,5] For one endophyte, a species of *Phialocephala*, we have demonstrated the growth and persistence of the fungus and its toxin in trees for more than 8 years under nursery and field conditions.[6,7] In the present study, 250 foliar endophytes were isolated from the needles of superior *Picea glauca* (white spruce) trees located in northeastern North America. A number of these strains were randomly selected to search for new toxin-producing strains. Here we report on three strains from this group in terms of toxicity to *C. fumiferana*, taxonomy, and major secondary metabolite production.

Results and Discussion

Three strains were of interest with respect to their ability to grow in liquid culture, their substantial secondary metabolite production, their homologous DNA sequences to fungal endophytes, and their measurable toxicity to *C. fumiferana*. The first strain, CBS 120381, was isolated from the needles of *P. glauca* in St. George, NB, Canada. At the time of publication there are few available DNA sequences for related fungi. The data indicate that it is in the Xylariaceae and close but not identical to *Nemania serpens* (98% ITS sequence similarity).[8,9] CBS 120379 and CBS 120380 were both isolated from the needles of different *P. glauca* trees located in Sussex, NB, Canada. The sequencing data indicate that both are most closely related to unidentified fungal species isolated from black spruce in Quebec, Canada. Both are species of *Lophodermium* (94% and 98% ITS similarity, respectively), with CBS 120379 most similar to fungi in the Rhytistimataceae.[10,11]

The individual concentrated EtOAc extracts of the filtrates from 1 L fermentations of these strains were used to test for toxicity to *C. fumiferana* larvae. After 1 week on a diet containing the individual extracts, the insects were weighed and their head capsule width was measured. The results of the diet incorporating bioassays for CBS 120381 showed significant reductions in both weight ($p < 0.001$) and head capsule size ($p = 0.076$) when compared to controls. Similar results were also found for the other *P. glauca* strains: CBS 120379 weight ($p = 0.010$), head capsule size ($p < 0.001$); CBS 120380 weight ($p = 0.090$), head capsule size ($p = 0.055$). Disc diffusion tests for all three strains showed similar 1−1.5 mm zones of inhibition to *Saccharomyces cerevisae* when compared to controls.

In order to identify the toxic components, the crude EtOAc extract of each strain was first screened by LC-MS and LC-NMR and then the major components were purified by column chromatography and/or preparative TLC (PTLC). In some cases the extract was methylated with diazomethane in order to facilitate separation. Structures were elucidated primarily by analysis of MS and NMR data, and confirmations were made by comparison with published data. CBS 120381 yielded five metabolites, **5**−**7**, **9**, and **10**, CBS 120379 yielded **1**, **8**, and **10**, and CBS 120380 yielded **2a**, **3**, **4**, and **10**. Compounds **2b** and **4** were isolated post-treatment of the extract with diazomethane and therefore theoretically represent methylated versions of the natural products. However, the original natural product structures have been confirmed (i.e., **2a** and **4**) by comparison of the data for the isolated metabolites with the LC-MS and LC-NMR data from the pretreatment extract screening.

The xanthone-derived compound **1** was isolated as a white solid, whose composition $C_{16}H_{12}O_6$ was deduced by HRMS and ^{13}C and ^{1}H NMR data analyses (Table 1). Broad IR absorptions at 3363, 1738, and 1644 cm^{-1} indicated hydroxy and carbonyl groups, respectively. The ^{1}H NMR spectrum of **1** displayed two downfield D_2O-exchangeable singlets at δ 12.39 and 7.75 attributed to the phenolic protons at C-8 and C-2, respectively. Four aromatic protons consisting of a one-proton singlet at δ 7.38 (H-4) and three vicinal protons as an AMX spin system at δ 7.57 (t, $J = 8.0$ Hz, H-6), 6.90 (d, $J = 8.1$ Hz, H-5), and 6.79 (d, $J = 8.0$ Hz, H-7) were also present. Additionally, a methoxy singlet at δ 3.99 and an aromatic methyl singlet at δ 2.43 were observed. These ^{1}H NMR chemical shifts were similar to that of mycoxanthone (however, disubstituted in ring C), previously isolated from *Mycosphaerella rosigena*.[12] They are also comparable with reported data typical of the xanthone skeleton[13] and xanthone analogues reported from the endophytic fungus *Xylaria* sp. FRR 5657,[14] the macrofungus *Xylaria* sp.,[15] and *Leptographium wageneri*.[16] The ^{13}C NMR spectrum of **1**

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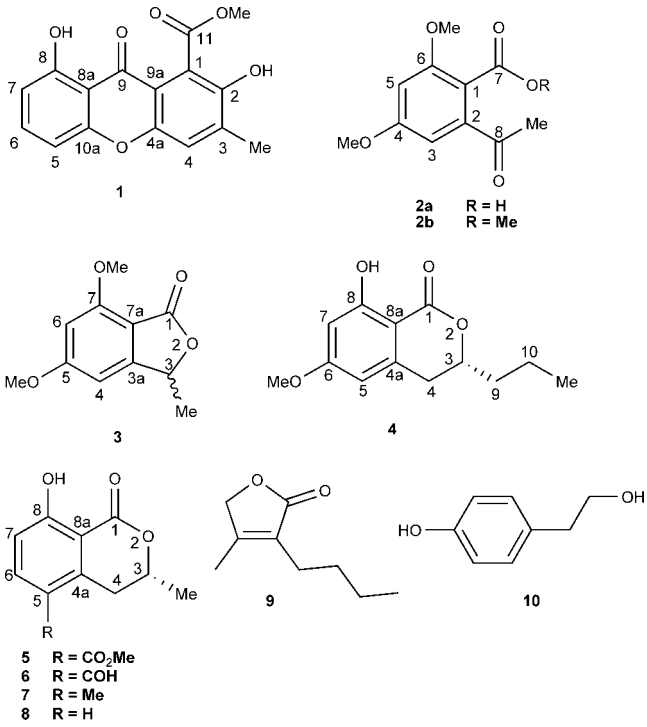
Table 1. NMR Data for Compounds **1** and **2b** (500 MHz, ^{1}H; 700 MHz, ^{13}C $CDCl_3$)[a]

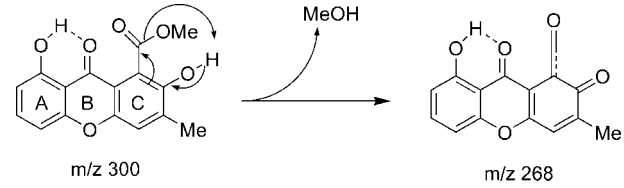
	1			**2b**		
position	δ_H (*J*, Hz)	δ_C	HMBC	δ_H (*J*, Hz)	δ_C	HMBC
1		130.8			115.6	
2		151.9			138.6	
2-OH	7.75 br s		C2, C3			
3		137.2		6.83 d (2.1)	105.7	C1, C4, C5, C8
3-Me	2.43 s	17.0	C2, C3, C4			
4	7.38 s	122.5	C2, C4a, C9a		161.5	
4a		150.7				
4-OMe				3.88 s	55.7	C4
5	6.90 d (8.1)	106.8	C7, C8a, C10a	6.62 d (2.1)	101.7	C1, C3, C4, C6
6	7.57 t (8.0)	136.3	C8, C10a		158.4	
6-OMe				3.85 s	56.3	C6
7	6.79 d (8.0)	110.4	C5, C8, C8a		168.1	
7-OMe				3.90 s	52.6	C7
8		161.4			198.2	
8a		109.3				
8-OH	12.39 s		C7, C8, C8a			
8-Me				2.53 s	28.7	C8, C2
9		180.4				
9a		116.5				
10a		155.5				
11		169.7				
11-OMe	3.99 s	53.1	C11			

[a] Note: The data for **2a** are reported for the diazomethane-treated version of the metabolite (**2b**).



revealed a xanthone carbon at δ 180.4, a polyaromatic system with 12 signals resonating at δ 106.8−161.4, a methyl at δ 17.0, and a carboxymethoxy moiety resonating at δ 53.1 (OCH_3) and 169.7 (C═O). The HSQC spectrum facilitated the assignment of the protonated carbons (Table 1). The strong HMBC correlations observed from 8-OH to δ 110.4 (C-7), 161.4 (C-8), and 109.3 (C-8a); from H-7 to δ 106.8 (C-5), 161.4 (C-8), and 109.3 (C-8a); from H-6 to δ 161.4 (C-8) and 155.5 (C-10a); and from H-5 to δ 110.4 (C-7), 109.3 (C-8a), and 155.5 (C-10a) established the structure of ring A. Diagnostic HMBC correlations observed between 2-OH and carbon resonances at δ 151.9 (C-2) and 137.2 (C-3); H-4 and carbon resonances at δ 151.9 (C-2), 116.5 (C-9a), and 150.7 (C-4a); and the methyl group and carbon resonances at δ 151.9 (C-2), 137.2 (C-3), and 122.5 (C-4) were consistent with the substitution pattern in ring C (Table 1). The positioning of the phenolic OH at C-2 was further supported by the base peak observed

Scheme 1. Dominant Loss of MeOH That Is Highly Characteristic of Salicylate Esters



at *m/z* 268 [M^+ − MeOH], a dominant loss that is characteristic of salicylate esters, requiring the OH to be adjacent to the methyl ester substituent (Scheme 1) as previously illustrated for a related compound where the phenolic methyl group was located in ring A.[17] The C-2 hydroxy group in this structure is also biosynthetically favored.[18] On the basis of this data compound **1** was characterized as 2,8-dihydroxy-3-methyl-9-oxoxanthene-1-carboxylic acid methyl ester. This is the first report of this structure, although related structures have been previously reported as fungal metabolites (see above).

Compound **2b** was obtained as a colorless liquid following treatment with diazomethane, in an attempt to improve compound separation. The formula was determined to be $C_{12}H_{14}O_5$ (HRMS), and the observed strong IR absorption at 1724 cm^{-1} confirmed the presence of ester groups. The ^{1}H NMR spectrum for **2b** contained two aromatic *meta*-coupled protons at δ 6.83 (J = 2.1 Hz, H-3) and 6.62 (J = 2.1 Hz, H-5), three methoxy singlets at δ 3.90, 3.88, and 3.85, and one methyl singlet at δ 2.53. The ^{13}C NMR data for **2b** revealed a ketone (δ 198.2) and an ester carbon (δ 168.1) assigned to C-8 and C-7, respectively. A tetrasubstituted benzene ring system was evident from the six aromatic carbons, of which two were methines [δ 105.7 (C-3) and 101.7 (C-5)] and four were quarternary [δ 115.6 (C-1), 138.5 (C-2), 161.5 (C-4), and 158.4 (C-6)]. The high-field chemical shifts for C-4 and C-6 indicated that they were both oxygenated aromatic carbons. The presence of three methoxy groups (δ 56.3, 55.7, 52.3) and one methyl (δ 28.7) was also confirmed. The protonated carbons were assigned on the basis of HSQC correlations, and the connectivity was confirmed by the analysis of key HMBC correlations (Table 1). Both of the aromatic protons H-3 and H-5 showed cross-peaks to C-1 and C-4 as well as to each other, suggesting that the methoxy ester substituent was located at C-1, while one methoxy group was at C-4. H-3 exhibited further correlations to C-8 (ketone), and the

Table 2. ^{1}H (500 MHz, $CDCl_3$) and ^{13}C NMR (700 MHz) Data for Compounds **3**, **4**, and **5**

	3		**4**		**5**	
position	δ_H (*J*, Hz)	δ_C	δ_H (*J*, Hz)	δ_C	δ_H (J, Hz)	δ_C
1		168.4		169.8		170.3
2						
3	5.36 q (6.7)	76.2	4.51 m	78.9	4.67 m	75.2
3a		156.3				
4	6.38 d (1.8)	98.7	2.86 dd (16.2, 11.0)	36.8	3.03 dd (18.0, 11.8)	32.6
			2.81 dd (16.2, 4.4)		3.87 dd (18.0, 3.2)	
4a				141.0		143.6
5		166.7	6.34 d (2.3)	101.8		118.7
6	6.40 d (1.8)	97.2		165.7	8.11 d (9.0)	138.3
7		159.6	6.23 d (2.3)	99.4	6.92 d (9.0)	116.4
7a		106.6				
8				164.5		165.7
8a				106.2		109.1
8-OH			11.22 s		11.81 s	
OMe	3.93 s	56.0	3.80 s	55.5	3.86 s	51.7
7-OMe	3.87 s	55.9				
CO_2Me						166.4
Me	1.56 d (6.7)	20.5	0.99 t (7.5)	13.8	1.54 d (6.4)	20.4
9-CH_2			1.55 m	33.2		
			1.48 m			
10-CH_2			1.84 m	18.1		
			1.66 m			

cross-peaks between the acetoxy methyl (8-Me) and C-2 established the connection of the acetoxy substituent to C-2. H-5 was further correlated to C-6, and the cross-peak between C-6 and the second aromatic methoxy (6-OMe) was consistent with the proposed structure for 2-acetyl-4,6-dimethoxybenzoic acid methyl ester (**2b**). Although **2a** was not formally isolated, its structure was deduced from the isolation and characterization of the methylated compound **2b**. LC-MS data for **2a** (from the untreated extract) provided a mass of 224, corresponding to a difference of 14 mass units from **2b** (isolated compound). This mass difference of 14 shows that only one functionality, the free acid, was methylated, corresponding to a loss of one proton and the addition of a methyl function. Further concrete evidence is provided by the LC-NMR data for **2a**, which clearly show two aromatic protons, one methyl, and only two OMe functions vs the three OMe's observed in the isolated compound **2b**. Thus the structure of the natural product **2a** was determined to be 2-acetyl-4,6-dimethoxybenzoic acid.

Compound **3** was isolated as a white solid; HRMS determined its formula to be $C_{11}H_{12}O_4$. The ^{1}H NMR resonances showed 2 OMe functions at δ 3.87 and 3.93, two *meta*-coupled aromatic protons at δ 6.38 (J = 1.8 Hz, H-4) and 6.40 (J = 1.8 Hz, H-6), and a secondary hydroxy function at δ 5.36 with an adjacent methyl at δ 1.56. The data agree with the literature values and are consistent with the structure for 5,7-dimethoxy-3-methylpthalide.[19,20] The ^{13}C chemical shift assignments are reported for the first time (Table 2). Compound **3** was previously reported as a synthetic product in the synthesis of key intermediates for mycophenolic acid,[20] in the selective nuclear lithiation of aromatic compounds,[21] and in the synthesis of pretetramides.[19] It is of unknown toxicity and is reported here for the first time as a fungal metabolite. The configuration at C-3 was not reported.

Compound **4** was isolated post-treatment of the extract with diazomethane as a white solid. HRMS analysis provided a formula of $C_{13}H_{16}O_4$. The ^{1}H NMR spectrum was in agreement with the published data for 3,4-dihydro-8-hydroxy-6-methoxy-(3*R*)-propyliso-coumarin.[22,23] The chirality at the C-3 position was determined to be *R* ($[\alpha]_D$ negative) by comparison to the published data for both the *R* and *S* synthetic compounds.[23] The ^{13}C NMR chemical shift assignments are reported for the first time (Table 2). LC-MS data on the pretreated extract for **4** determined the mass to be 236, which is identical to the mass of the isolated compound. The LC-NMR analysis on the pretreated extract also shows an identical ^{1}H NMR spectrum to that of the isolated compound, including the presence of the OMe function at C-6 prior to treatment with diazomethane. This evidence proves that treatment of the extract with diazomethane had no effect on the isolated compound **4**, which is identical to the natural product produced by the endophyte. Compound **4** was previously isolated from the roots of *Solidago multiradiata*[22] with no reported configuration at C-3 and was later synthesized as both enantiomers.[23] It is reported here for the first time as a fungal metabolite and is of unknown toxicity.

The known biosynthetically related metabolites **5**−**7** were also isolated during this investigation. (3*R*)-5-Carbomethoxymellein (**5**) was identified by comparison of the ^{1}H NMR and MS data acquired with reported values.[24] The ^{13}C NMR chemical shift assignments are reported for the first time (Table 2). Compound **5** was originally isolated as the major metabolite from *Hypoxylon mammatum,* a species related to our isolate according to the sequencing data.[24] The (3*S*)-enantiomer has also been isolated from *Tubercularia* sp., an endophytic fungus of *Taxus mairei*.[25,26] (3*R*)-5-Formylmellein (**6**) was first discovered in wood samples infested by fungi during storage.[27] Recently it was isolated from a culture of *Nodulisporium* sp. The ^{1}H NMR resonances observed here were consistent with literature values.[28] (3*R*)-5-Methylmellein (**7**) first appeared in the literature as the main phytotoxic metabolite of *Fusicoccum amygdale*.[29] It has been reported from several endophytic fungi such as *Phomopsis* sp. from *Adenocarpus foliolosus,*[30] 3893# in mangrove[31] and 1893# in marine-derived mangrove.[32] Additionally, it was reported to be produced by *Cephalosporium* sp. AL031,[33] *Biscogniauxia mediterranea,*[34] and *Cytospora eucalypticola*.[35] It also occurs as one of the constituents from the heartwood of the Fijian species *Euphorbia fidjiana*. The ^{1}H NMR data for **7** matched the previously reported literature.[36] According to Whalley and Edwards[37] *Nummulariella marginata* produces **6** and **7** as major secondary metabolites. Both compounds have also been detected from *Nummularia discreta*[24] and from the roots of *Rhus javanica* L. var. *roxburghiana*.[38]

Compound **8** was isolated as a white solid with the formula $C_8H_{10}O_2$ determined by HRMS. The ^{1}H NMR chemical shifts of **8** were in agreement with the literature values for (3*R*)-mellein.[28,39–42] Compound **8** was originally isolated from *Aspergillus melleus*[43] and later was found to be produced by an array of fungal species.[44] It was also reported from endophytic fungi such as *Pezicula livida, Plectophomella* sp., *Cryptosporiopsis malicoticis,* and *Geniculospo-rium* sp.[40,45] Mellein also occurs in plants,[46] in insects as part of their defense secretions,[47] and as a trail pheromone component in

the hindgut of *Lasius fuliginosus*.[48] It has been reported to be strongly fungicidal, herbicidal, and algicidal.[49]

The mellein-related metabolites **4**−**8** exhibited diagnostic features in their ^{1}H NMR spectra, which readily allow for their assignment and characterization. The appearance of a one-proton singlet at δ 11−12 is deshielded due to hydrogen bonding of this OH proton with the C=O group at C-1, and a one-proton multiplet at δ 4.5−4.7 is ascribed to H-3. Additionally, a methyl doublet was also observed at δ 1.5 ($J \approx 6$ Hz) for compounds **5**−**8**. The (3*R*)-absolute configuration is known to be common to this class of compounds[2,39] and was confirmed by measuring the optical rotation of **5**. The same configuration was assumed for **5**−**8** by virtue of similar NMR data. In particular, H-4 has large couplings to H-3 ($J_{3,4} = 11-12$ Hz), in which H-3 and H-4 are *trans*-diaxial.

Compound **9** was isolated as a light orange liquid with the formula $C_9H_{14}O_2$, determined by HRMS. The NMR data agreed with published data for 3-butyl-4-methyl-5*H*-furan-2-one.[50−52] Compound **9** was originally isolated from the fungus *Hypoxylon serpens,* which is closely related to our isolate.[50] It has also appeared in some natural product syntheses.[51−53] Compound **9** is of unknown toxicity but was isolated as the major metabolite of CBS 120381.

Compound **10** was isolated as a white solid. HRMS determined its formula to be $C_8H_{10}O_2$. The ^{1}H NMR data agreed with the recently reported literature data for tyrosol.[54,55] It is produced by a large variety of organisms including from *Xylaria longipes*[56] and from the stomata of *Epichloe typhina*.[57]

Metabolites **1**−**10** represent the major compounds produced by the three selected isolates, all of which showed similar toxicity to *C. fumiferana* and yeast cells. Of these compounds, **1** and **2a** are new metabolites albeit structurally related to known metabolites. Compound **3** is reported for the first time as a natural product, **4** has been previously reported as a plant metabolite, and **5**−**10** are known fungal metabolites. The only common metabolite produced by the three strains studied was tyrosol.

Metabolites **1**−**9** all have a polyketide biosynthetic origin, while tyrosol is derived from tyrosine. Compound **2a** appears to be derived from orsellinic acid and is a possible precursor to **3**, through reduction at C-8 and subsequent cyclization and elimination of water. Compounds **4**−**8** are all 3,4-dihydroxyisocoumarin derivatives of pentaketide origin.

Preliminary toxicity testing for tyrosol using the Hep3B cell line (ATCC# HB-8064) indicated that it was not a potent cytotoxin (data not shown). The toxicity of compounds **1**, **2a**, **3**, **4**, and **9** is unknown. Mellein (**8**) has been shown to be strongly fungicidal, herbicidal, and algicidal.[49] Since **4**−**7** are structurally similar to mellein, with each possessing the isocoumarin ring system, it is likely that these will also exhibit some toxicity.

In addition to the rugulosin-producing endophyte DAOM 229536,[4,5] the vermiculin-producing endophyte (DAOM 229535) was also shown to affect larval growth on needles.[4] The effect of these new strains *in planta* requires similar testing, which is now underway. However, it appears likely that they would follow the same pattern as with the other two species. Elucidation of the secondary metabolites from these relatively poorly characterized or novel species of fungal endophytes provides new information that contributes to their polyphasic taxonomy. This information is required in order to achieve our goal of providing tolerance of Acadian forests to spruce budworm.

Experimental Section

General Experimental Procedures. Optical rotation was measured on a Perkin-Elmer 141 polarimeter. Initial HPLC separation and preliminary screening of the extracts was performed using a Phenomenex Synergi Max-RP column (250 mm × 4.6 mm) and a CH_3CN-H_2O gradient suitable for both subsequent MS and NMR analysis. NMR data were acquired on either a Bruker Avance 400 with 5 mm autotuning broadband probe with Z gradient, a Bruker ARX 500 MHz with a 3 mm inverse probe, or a Bruker AVANCE 700 MHz with a cryogenic (TCI) probehead (Health Canada, Ottawa). LC-NMR was performed using a Varian 9010 HPLC system with UV detector attached to a Varian 600 MHz INOVA spectrometer equipped with a 5 mm broadband cryoprobe with LC-NMR insert (60 μL active volume). Spectra were acquired in stop-flow mode. HRMS data were obtained on a Kratos Concept instrument (University of Ottawa, Mass Spectrometry Centre).

Fungal Strains. The fungal strains CBS (Centraalbureau voor Schimmelcultures) 120379 and CBS 120380 were isolated from *Picea glauca* (white spruce) needles near Sussex, New Brunswick, Canada. CBS 120381 was isolated from the needles of a *P. glauca* tree in St. George, New Brunswick, Canada. These strains were selected via a screening process from a much larger collection of white and red spruce endophytes (~2000) isolated from mature spruce tree needles collected in Maine, U.S.A., and New Brunswick and Nova Scotia, Canada.

Toxicity Testing. Disc diffusion assays[58] were employed for preliminary antifungal testing. The dried extracts (50 mg) were dissolved in 1 mL of CH_2Cl_2, and 10 μL was pipetted onto each small filter under aseptic conditions and left overnight to allow for solvent evaporation. The discs were then carefully placed onto malt extract agar plates that were inoculated with a lawn of *Saccharomyces cerevisae*. The plates were incubated at 25 °C and the resulting inhibition zones measured. Each extract was also tested in bioassays using disease-free *C. fumiferana* (purchased from Natural Resources Canada, Sault Ste. Marie, Ontario). The extract of each endophyte culture was dissolved in CH_2Cl_2 and incorporated into the synthetic diet at 400 μg/g and allowed to evaporate overnight. Ten second-instar *C. fumiferana* larvae were added to each of 3 cups used per extract. They were held in a growth chamber at 25 °C and 55% relative humidity with 16 h light/day until the insects reached the fifth instar as determined by head capsule size (approximately 1 week).[59] Each insect was then weighed and its head capsule width was determined. Statistical analysis was performed using ANOVA, Fisher's LSD on each with rugulosin as a positive control and CH_2Cl_2 as a negative control (SYSTAT v 10.2; San Jose, CA).[7]

Sequencing. DNA was extracted from fungal cultures using an UltraClean DNA isolation kit (MO BIO Laboratories 12224-250). PCR and sequencing was done by Laboratory Services, University of Guelph, Guelph, Ontario. The PCR primers used were ITS4 (5′ TCC TCC GCT TAT TGA TAT GC 3′) and ITS1F (5′ CTT GGT CAT TTA GAG GAA GTA A 3′).[60] The PCR fragments were subsequently sequenced then aligned using MAFFT[61] and compared to each other and the sequences of known fungi with phylogenetic analysis using parsimony (PAUP).[62] Individual sequences were also BLAST searched using the BLASTN algorithm against the NCBI nucleotide collection database to search for homologous sequences.

Fermentation, Extraction, and Isolation. All strains were stored on 2% MEA (malt extract agar, Difco) slants at 5 °C and fermented in groups of 5 Glaxo bottles each containing 1 L of 2% ME medium for 3 months at 20 °C. The cultures were then harvested and filtered, the mycelium was frozen for later use, and the filtrate was extracted with 2 × EtOAc and dried by rotary evaporation. The extracts were weighed and first screened by LC-MS using electrospray ionization in both positive and negative ion mode. Each extract was then screened using LC-NMR in stop-flow mode where ^{1}H NMR data were acquired for each major peak to evaluate the endophyte's secondary metabolite production.

CBS 120380. The crude EtOAc extract (40 mg) from the filtrate culture was subjected to PTLC eluting with 10% MeOH−$CHCl_3$ (100 mL). Extraction of the main UV-active band with 100% EtOAc (50 mL) furnished 5,7-dimethoxy-3-methylpthalide (**3**) as a white solid (0.7 mg).

Purification of a second crude filtrate extract (70 mg) was performed by column chromatography on Si gel eluting first with hexanes−$CHCl_3$ mixtures (0−100%, 100 mL aliquots), followed by MeOH−$CHCl_3$ solvent gradient (0−15%, 100 mL aliquots). The major fraction was again subjected to PTLC using multiple elutions [MeOH−$CHCl_3$ 1%, 3%, and 5%, 100 mL aliquots] to afford tyrosol (**10**) (1 mg).

In an attempt to isolate the polar compounds, another crude filtrate extract (110 mg) was methylated with diazomethane prior to purification. The extract was stirred at 0 °C in CH_2Cl_2 (20 mL) with five drops of MEOH to aid solubility. Excess yellow ether diazomethane solution was added, and stirring continued for 1 h at 0 °C before it was warmed to rt [diazomethane solution was prepared by adding nitrosomethylurea (200 mg) to a solution of 20% KOH (2 mL) and Et_2O (6 mL) at 0 °C].

The reaction mixture was washed with H_2O (2 × 5 mL) and the solvent evaporated *in vacuo* before it was purified by Si gel chromatography, eluting sequentially with mixtures of EtOAc−hexanes (0−100%, 100 mL portions) and MeOH−$CHCl_3$ solvent gradients (5 and 10%, 100 mL portions). The two most promising fractions by TLC were subjected to further purification. Fraction 1 was applied to a small Si gel column eluting with EtOAc−hexanes solvent gradient (0−30%, 10 mL each) to afford 3,4-dihydro-8-hydroxy-6-methoxy-(3*R*)-propylisocoumarin (**4**) (1 mg). Multiple elution of fraction 2 by PTLC (15%, 30%, 35% EtOAc−hexanes, 100 mL each) followed by Si gel column chromatography [100% hexanes, 10%, 20%, 30% EtOAc−hexanes (20 mL each)] provided 2-acetyl-4,6-dimethoxybenzoic acid methyl ester (**2b**) (1 mg).

CBS 120379. The crude EtOAc (74 mg) from the culture filtrate was subjected to Si gel chromatography eluting consecutively with hexanes−$CHCl_3$ (0−100%, 100 mL each) and MeOH−$CHCl_3$ solvent gradients (1−10%, 100 mL each). The major fraction was further purified twice by PTLC, first using 30% EtOAc−hexanes (100 mL) as eluants followed by eluting successively with 50% $CHCl_3$−hexanes, 100% $CHCl_3$, and 1% MeOH−$CHCl_3$ (100 mL each), to afford 2,8-dihydroxy-3-methyl-9-oxoxanthene-1-carboxylic acid methyl ester (**1**) as a white solid (1 mg).

Another crude filtrate extract (20 mg) was fractionated by PTLC, eluting first with 5% MeOH−$CHCl_3$ followed by 10% MeOH−$CHCl_3$ solvent systems (100 mL each). The main fraction was further purified by PTLC eluting with 15% EtOAc−hexanes and then 30% EtOAc−hexanes (100 mL each), to afford (3*R*)-mellein (**8**) (0.5 mg).

Fractionation of the crude filtrate extract (50 mg) was also performed by column chromatography over Si gel eluting successively with EtOAc−hexanes (0−20%, 50 mL aliquots) and MeOH−$CHCl_3$ solvent gradients (0−15%, 50 mL aliquots). The main fraction was separated by PTLC using 10% MeOH−$CHCl_3$ (100 mL) as the eluant, providing tyrosol (**10**) (1 mg).

CBS 120381. The crude filtrate extract (590 mg) was purified by column chromatography on Si gel eluting successively with EtOAc−hexanes solvent gradient (0−100% EtOAc, 100 mL portions) to afford 3-butyl-4-methyl-5*H*-furan-2-one (**9**) as a light orange liquid (165.8 mg) and a mixture of compounds **5**−**7** (25 mg). Separation of **5**−**7** was achieved by PTLC eluting first with 10% EtOAc−hexanes followed by 15% EtOAc−hexanes (100 mL each). The UV-active bands were extracted with 100% EtOAc to provide (3*R*)-5-carbomethoxymellein (**5**) (3.5 mg), 5-formylmellein (**6**) (2.8 mg), and 5-methylmellein (**7**) (3 mg). Further purification of the polar fractions was carried out by PTLC using 10% MeOH−$CHCl_3$ (100 mL) as eluant, to yield tyrosol (**10**) (2 mg). Purification of the crude cell extract (260 mg) was performed on a Si gel column using hexanes−$CHCl_3$ and MeOH−$CHCl_3$ solvent gradients as eluants (0−100% and 1−10%, respectively, 100 mL each) to afford 3-butyl-4-methyl-5*H*-furan-2-one (**9**) (5 mg).

2,8-Dihydroxy-3-methyl-9-oxoxanthene-1-carboxylic acid methyl ester (1): ^1H and ^{13}C NMR see Table 1; EIMS *m/z* 300.1 $[M]^+$ (21), 269.0 (21), 268.0 (100), 161.9 (9), 149.0 (18), 142.9 (7), 57.1 (11), 55.1 (8), 51.0 (7), 43.1 (11), 39.0 (10); HRMS *m/z* 300.0662 (calcd for $C_{16}H_{12}O_6$, 300.0634).

2-Acetyl-4,6-dimethoxybenzoic acid (2a); (methyl ester) (2b): ^1H and ^{13}C NMR see Table 1; EIMS *m/z* 238.1 $[M]^+$ (37), 223.1 (92), 208.1 (18), 207.1 (100), 165.1 (5), 161.9 (9), 142.9 (7), 106.0 (6), 63.0 (5); HRMS *m/z* 238.0854 (calcd for $C_{12}H_{14}O_5$, 238.0841).

5,7-Dimethoxy-3-methylpthalide (3): ^1H and ^{13}C NMR see Table 2; EIMS *m/z* 208.1 $[M]^+$ (62), 207.1 (18), 194.1 (12), 193.1 (100), 190.1 (38), 165.1 (58), 162.1 (31), 161.1 (16), 135.1 (29), 43 (34); HRMS *m/z* 208.0725 (calcd for $C_{11}H_{12}O_4$, 208.0736).

3,4-Dihydro-8-hydroxy-6-methoxy-(3*R*)-propylisocoumarin (4): $[\alpha]^{24}_D$ −30 (*c* 1, $CHCl_3$); ^1H and ^{13}C NMR see Table 2; EIMS *m/z* 236.1 $[M]^+$ (83), 220.1 (34), 193.1 (42), 178.1 (57), 177.1 (33), 176.0 (34), 165.1 (48), 164.0 (65), 152 (100), 150.1 (36), 137.1 (34); HRMS *m/z* 236.1059 (calcd for $C_{13}H_{16}O_4$, 236.1049).

(3*R*)-5-Carbomethoxymellein. 5-Carbomethyoxy-3,4-dihydro-8-hydroxy-(3*R*)-methylisocoumarin (**5**): $[\alpha]^{24}_D$ −151 (*c* 1, $CHCl_3$); ^1H and ^{13}C NMR see Table 2; EIMS *m/z* 236 $[M]^+$ (33), 207 (16), 61 (15), 45 (15), 43 (100); HRMS *m/z* 236.0676 (calcd for $C_{12}H_{12}O_5$, 236.0665).

(3*R*)-5-Formylmellein. 3,4-Dihydro-5-formyl-8-hydroxy-(3*R*)-methylisocoumarin (**6**): ^1H NMR ($CDCl_3$, 400 MHz) δ 11.94 (s, 1H, OH), 10.01 (s, CHO), 7.92 (d, J = 8.8 Hz, 1H), 7.06 (d, J = 8.8 Hz, 1H), 4.70 (m, 1H), 3.92 (dd, J = 17.6 Hz, J = 3.2 Hz, 1H), 3.02 (dd, J = 17.7 Hz, J = 11.9 Hz, 1H), 1.58 (d, J = 6.4 Hz, 3H); EIMS *m/z* 206 $[M]^+$ (100), 205 (16), 191 (20), 190 (16), 177 (23), 173 (26), 163 (48), 136 (45), 136 (45), 135 (18), 134 (30), 77 (17), 55 (15), 39 (17); HRMS *m/z* 206.0566 (calcd for $C_{11}H_{10}O_4$, 206.0579).

(3*R*)-5-Methylmellein. 3,4-Dihydro-(3*R*),5-dimethyl-8-hydroxyisocoumarin (**7**): ^1H NMR ($CDCl_3$, 400 MHz) δ 11.02 (s, 1H, OH), 7.31 (d, J = 8.5 Hz, 1H), 6.84 (d, J = 8.5 Hz, 1H), 4.70 (m, 1H), 2.97 (dd, J = 16.7 Hz, J = 3.4 Hz, 1H), 2.74 (dd, J = 16.6 Hz, J = 11.5 Hz, 1H), 2.21 (s, 3H), 1.57 (d, J = 6.1 Hz); EIMS *m/z* 192 $[M]^+$ (100), 174 (17), 163 (25), 148 (19); HRMS *m/z* 192.0793 (calcd for $C_{11}H_{12}O_3$, 192.0787).

(3*R*)-Mellein. 3,4-Dihydro-(3*R*)-methyl-8-hydroxyisocoumarin (**8**): ^1H NMR ($CDCl_3$, 400 MHz) δ 11.04 (s, OH), 7.41 (dd, J = 8.2 Hz, J = 7.6 Hz, 1H), 6.89 (d, J = 8.4 Hz, 1H), 6.69 (dd, J = 7.4 Hz, J = 1.0 Hz, 1H), 4.74 (sextet, J = 6.4 Hz, 1H), 2.93 (d, J = 6.8 Hz, 2H), 1.53 (d, J = 6.0 Hz, 3H); EIMS *m/z* 178 $[M]^+$ (59), 160 (20), 149 (15), 134 (37), 61 (19), 45 (16), 43 (100), 29 (16); HRMS *m/z* 178.0638 (calcd for $C_{10}H_{10}O_3$, 178.0630).

3-Butyl-4-methyl-5*H*-furan-2-one (9): ^1H NMR ($CDCl_3$, 400 MHz) δ 4.58 (s, 2H), 2.22 (t, J = 7.6 Hz, 2H), 1.97 (s, 3H), 1.43 (quintet, J = 7.5, 2H), 1.28 (sextet, J = 7.5 Hz, 2H), 0.87 (t, J = 7.4 Hz, 3H); EIMS *m/z* 154 $[M]^+$ (26), 139 (13), 125 (13), 112 (100), 55 (28), 41 (13); HRMS *m/z* 154.1003 (calcd for $C_9H_{14}O_2$, 154.0994).

Tyrosol. *p*-Hydroxyphenethyl alcohol (**10**): ^1H NMR ($CDCl_3$, 400 MHz) δ 7.08 (d, J = 8.4 Hz, 2H), 6.77 (d, J = 8.8 Hz, 2H), 3.81 (t, J = 6.4 Hz, 2H), 2.79 (t, J = 6.6 Hz, 2H); EIMS *m/z* 138 $[M]^+$ (25), 124 (16), 107 (100), 91 (10), 77 (14), 69 (11), 55 (14), 43 (22), 41 (17); HRMS *m/z* 138.0681 (calcd for $C_8H_{10}O_2$, 138.0681).

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