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Secondary metabolites from anti-insect extracts of endophytic fungi isolated from *Picea rubens*

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ABSTRACT

The extracts of a selection of 150 foliar fungal endophytes isolated from *Picea rubens* (red spruce) needles were screened by LC–MS and assayed for toxicity. Three of these strains that were toxic to the forest pest *Choristoneura fumiferana* (eastern spruce budworm) in dietary bioassays were selected for further study. Their culture extracts were analyzed by LC–NMR spectroscopy, and the major metabolites were isolated by LC–MS–SPE or PTLC/column chromatography and characterized. The structures were elucidated by spectroscopic analyses including 2D NMR, HRMS and by comparison to literature data. Compounds **1** and **5**–**7** are hitherto unknown whereas compounds **2** and **3** are natural products described for the first time. Compound **4** is reported for the first time as a fungal metabolite and **8**–**9** were identified as known fungal metabolites in genera.

1. Introduction

Fungal endophytes colonize internal tissues of plants without resulting in overt damage while the tissue is alive. These have been studied from a variety of perspectives including; for the discovery of new bioactive compounds (Zhang et al., 2006) as well as the origin of plant secondary metabolites (Wink, 2008). However, most effort so far has been placed on their effects on animal production from the consumption of cool season grasses. Toxic metabolites produced by endophytic fungi in these grasses greatly lessen the damage resulting from herbivorous insects that consume such grasses. These fungi are vertically transmitted through the seed (Clay and Schardl, 2002). All but one of 17 field and greenhouse studies comparing the productivity of fescue infected with endophytes versus endophyte free fescue, found an increase in relative productivity for the infected fescue (Clay, 1997). The average increase was 30%; however under conditions of greatest biotic and abiotic stress, this increase can be much larger.

We have shown that some strains of foliar endophytes isolated from conifers produce anti-insect compounds (Findlay et al., 2003; Sumarah et al., 2008a; Sumarah and Miller, 2009). Unlike the cool season fescue endophytes discussed above, these fungi are horizontally transmitted to emergent seedlings in *Picea glauca* (white spruce; Miller et al., 2009). Infections of trees by one such endophyte, *Phialocephala scopiformis* reduces the growth of *Choristoneura fumiferana* (Miller et al., 2002, 2008; Sumarah et al., 2008b). Another economically-important conifer in northeastern North America is *Picea rubens* (red spruce). We isolated ca. 2000 *P. rubens* fungal endophytes from nine areas across New Brunswick, Nova Scotia and Maine. The sampling was from superior *P. rubens* which we hypothesized would ensure representative collection of the endophytes and metabolite diversity from this area. A systematic selection of 150 strains from the collection was grouped on the basis of morphotype and ITS sequence. Fermentations of each were made according to established procedures and their extracts were tested for dietary toxicity to *C. fumiferana* and analyzed by LC–MS and LC–NMR (Sumarah et al., 2008a). Sixteen of these strains that grew well in liquid culture showed toxicity to *C. fumiferana*. Here we present the characterization of the major secondary metabolites from culture of three of these strains with potential for use in forest protection.

2. Results and discussion

2.1. Fermentation and screening

From the 150 strains of *P. rubens*, endophytes that were grown in 1 L fermentations, three were selected for further study and

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deposited at either CBS (Centraalbureau voor Schimmelcultures) or DAOM (Department of Agriculture, Ottawa, Mycology). The results of the ITS sequencing determined that DAOM 239833 is conspecific with the morphospecies *Dwayaangam colodena*. This species was previously reported from streams in Québec boreal and mixed-wood forests as a new aquatic hyphomycete (Sokolski et al., 2006). It was not possible to classify CBS 121942 or CBS 121944 based on their ITS sequence data as only poor matches were observed. All three of the fungal endophytes studied are either poorly- or un-described species.

2.2. Dietary bioassays

The concentrated EtOAc filtrate extract from each of the three isolates was tested for toxicity to *C. fumiferana* larvae (Sumarah et al., 2008a). After one week on diet containing the individual extracts, the insects were weighed and their head capsule width measured. The results of the dietary bioassays showed significant reduction compared to controls for CBS 121942 (weight, $P = 0.015$), CBS 121944 (weight, $P = 0.008$) and DAOM 239833 (head capsule width, $P = 0.019$). The extract from CBS 121942 was also antifungal to *Saccharomyces cerevisae* in disc diffusion tests (Sumarah et al., 2008a), where 2 mm zones of inhibition were observed that were comparable to the known antifungal metabolite nystatin (10 μL of 0.25 mg/mL).

2.3. Isolation and structure determination

In order to identify the major components, the crude EtOAc extract of each strain was first screened by LC–MS and LC–NMR spectroscopy and then either purified by column chromatography and/or preparatory TLC (PTLC) or isolated directly from crude extracts by LC–MS–SPE. Structures were elucidated primarily by analysis of HRMS and NMR data and in the case of the known metabolites, confirmed by comparison with published data. CBS 121942 yielded a polyketide metabolite **1** and two biosynthetically related compounds **2** and **3**; CBS 121944 yielded two sesquiterpenes **4** and **5**; and DAOM 239833 yielded the four biosynthetically related maleic anhydrides **6**–**9** (see Fig. 1).

Compound **1** was isolated as a white powder with the formula $C_{14}H_{18}O_5$ determined by HRMS; this was indicative of six double bond equivalents. The ^1H and ^{13}C NMR spectroscopic data for **1** are shown in Table 1. NMR data are reported for both the isolated compound in $CDCl_3$ and on the LC–NMR peak in CD_3CN/D_2O. The differences in multiplicities reported in the two data sets are the result of poor line shape resolution in the LC–NMR experiment due to the different solvent system required for chromatographic separation. The structure of **1** was deduced from analysis of the NMR data which showed that this compound was related to the phytotoxic metabolite seiricuprolide (Ballio et al., 1988), with a rearrangement of the substituents. The ^1H NMR spectrum showed four olefinic CH peaks, indicative of two separate disubstituted HC=CH double bonds; two double doublets at δ 5.95 (H-9) and 6.90 (H-10) in a *trans* orientation ($J_{9,10} = 15.6$ Hz) and two doublets of double doublets at δ 5.84 (H-12) and 5.25 (H-13) in a *cis* orientation ($J_{12,13} = 12.0$ Hz). A multiplet was observed at δ 5.15 (H-11) consistent with a secondary alcohol. Two adjacent oxygenated CH peaks were evident at δ 3.65 (H-14) and 3.51 (H-1) with chemical shifts and J values indicative of an epoxide ring ($J_{1,14} = 1.8$ Hz); and three adjacent aliphatic CH_2 groups (H-3, H-4 and H-5) as evident from COSY. The last piece of evidence observed in the ^1H spectrum was the CH_3 doublet at δ 1.20 ($J = 6.0$ Hz) coupling with a CH group at δ 4.84 (*m*). The HSQC data provided correlation of these ^1H chemical shifts with their corresponding carbons. The two epoxide ring carbons (C-14 and C-1) were assigned to the signals at δ 55.6 and 58.9, respectively. From the ^{13}C NMR spectroscopic data, two

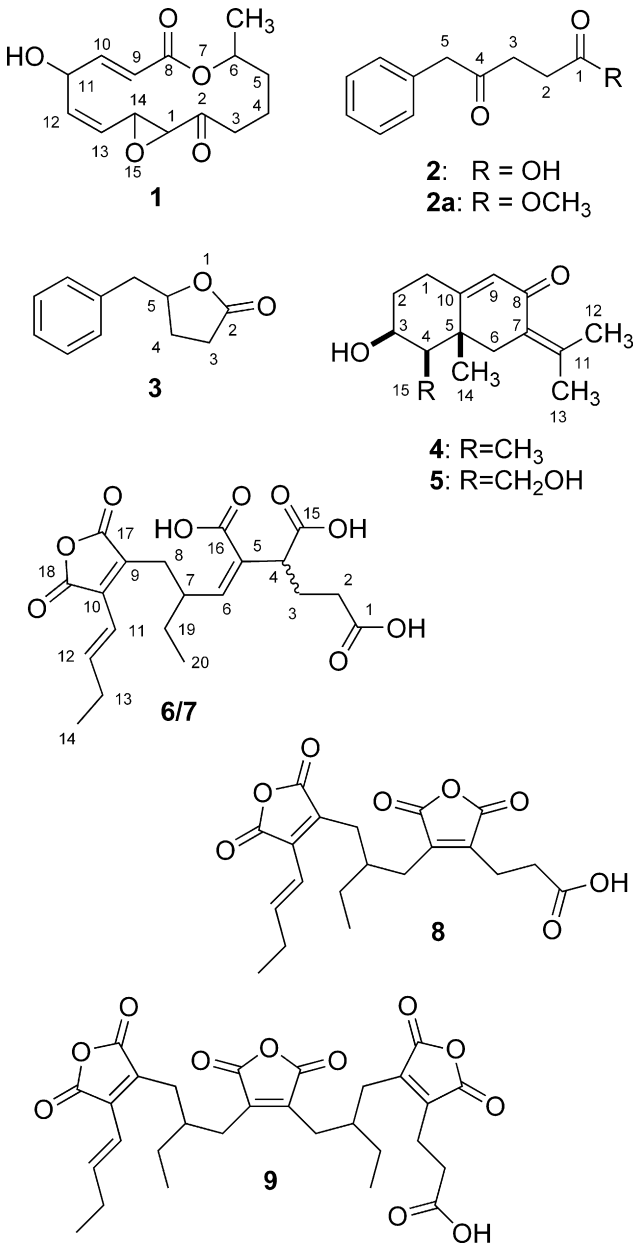

Fig. 1. Compounds **1**–**9** with numbering schemes used for NMR assignment.

C=Os were observed, one at δ 206.9 (C-2) indicative of a ketone and one at 165 ppm (C-8) indicative of an ester linkage. The HMBC data provided the adjacencies of the substituents at C-6 and C-11 and fixed the ketone at C-2. The presence of the two C=Cs, an epoxide ring and two C=Os, indicated that the entire molecule required only one ring closure to satisfy the six double bond equivalents. Compound **1** is similar to seiricuprolide but differs by a rearrangement of the relative location of the epoxide ring and the C-12, C-13 double bond, as well as possessing a ketone at C-2 instead of a hydroxyl function. The relative configuration of the substituents at C-11, C-6 and the C-1,14 epoxide ring was not determined, due to the small amount of material isolated. This would require either a total synthesis or a crystal structure as was the case for seiricuprolide (Ballio et al., 1988; Bartolucci et al., 1992). Compound **1** has a similar structural backbone to the known anti-insect (Findlay et al., 2003) and antifungal metabolite vermiculin (Utermark and Karlovsky, 2007) but its insect toxicity is unknown.

Table 1
^{1}H and ^{13}C NMR spectroscopic data for compound **1**. (A) $CDCl_3$ (400 MHz), (B) LC–NMR CD_3CN/D_2O (600 MHz).

Position	(A)		(B)		(A&B)
	δ_H	δ_C	δ_H	δ_C	HMBC
1	3.51 *d* (1.8)	58.9	3.46 *d* (2.0)	59.8	2
2		206.9		207.0	
3	2.64 *ddd* (19.5, 7.2, 2.6) 2.57 *ddd* (19.5, 7.2, 2.6)	38.5	2.59 *ddd* (19.3, 9.0, 3.8) 2.53 *ddd* (19.3, 9.0, 3.8)	39.4	2, 4, 5
4	1.82 *m* 1.60 *m*	18.7	1.79 *m* 1.54 *m*	19.3	2, 3, 5, 6
5	1.70 *m*	32.5	1.67 *m*	33.2	3, 4, 6, CH_3
6	4.84 *m*	72.5	4.80 *m*	74.2	8, CH_3
7					
8		165.1		167.1	
9	5.95 *dd* (15.6, 1.2)	122.5	5.89 *d* (15.8)	121.9	8, 10, 11
10	6.90 *dd* (15.6, 6.3)	146.0	6.85 *dd* (15.8, 6.4)	148.5	8, 9, 11, 12
11	5.15 *m*	69.4	5.10 *m*	69.2	
12	5.84 *ddd* (12.0, 3.6, 1.2)	136.7	5.80 *dd* (11.9, 3.5)	138.3	10
13	5.25 *ddd* (12.0, 7.2, 2.4)	127.2	5.15 *m*	127.3	11
14	3.65 *dd* (7.2, 1.8)	55.6	3.62 *d* (7.2)	57.0	12, 13, 1, 2
CH_3	1.20 *d* (6.0)	20.7	1.16 *d* (6.3)	21.0	5, 6

Note: Differences in multiplicity observed between A and B are the result of the solvent interactions during LC–NMR analysis.

Compounds **2** and **3** were also isolated as white powders from CBS 121942. The formula for **2** was determined to be $C_{11}H_{12}O_3$ by HRMS. The ^{1}H NMR spectroscopic data indicated a benzyl functionality at δ 7.27 (*m*, 5H) and 3.74 (*s*, 2H), and two coupled CH_2s adjacent to carbonyl groups at δ 2.75 (*t*, J = 6.4 Hz, 2H) and 2.60 (*t*, J = 6.4 Hz, 2H). The initial ^{13}C data indicated 10 of the expected 11 carbons including a single acidic carbonyl function at 176.4 ppm. The ketone at C-4 was only observed after treatment of the compound with diazomethane to convert the acid into a methyl ester **2a** thus allowing for the observation of the C-4 carbon signal at δ 207.1. The NMR spectroscopic data for compound **2** were in agreement with the known compound 4-oxo-5-phenyl-pentanoic acid (Ketterer et al., 2006), which was described as an intermediate in the synthesis of enantiomerically pure tricyclic benzomorphan analogues. Compound **2** is reported here for the first time as a natural product.

The HRMS for compound **3** determined the formula as $C_{11}H_{12}O_2$ indicating a loss of oxygen from **2**. The ^{1}H NMR spectroscopic data for compound **3** shows: five aromatic protons in a *mono*-substituted benzene ring at δ 7.28 (*m*, 5H), as was present in **2**; a CH adjacent to an ester function at δ 4.74 (*quintet*, J = 6.7 Hz, 1H); a diastereotopic CH_2 at δ 3.08 (*dd*, J = 14.0, 6.0 Hz, 1H), and 2.93 (*dd*, J = 14.0, 6.3 Hz, 1H); and two adjacent CH_2s at δ 2.42 (*m*, 2H), 2.24 (*m*, 1H) and 1.96 (*m*, 1H). The ^{13}C data were used to assign chemical shifts of all of the 11 carbon atoms expected from the HRMS. The NMR data for compound **3** were also in good agreement with that reported for 5-benzyl-dihydro-furan-2-one, which is a compound in the above synthesis of **2** (Ketterer et al., 2006). Compound **3** is also a new natural product and the co-occurrence of these compounds suggests a biosynthetic relationship where **2** would be a precursor of **3** through formation of a lactone ring.

The structurally-related metabolites **4** and **5** were both isolated as white powders from CBS 121944. The formula of each was determined by HRMS to be $C_{15}H_{22}O_2$ and $C_{15}H_{22}O_3$, respectively. The ^{1}H NMR spectroscopic data for compounds **4** and **5** are very similar except that the C-15 methyl doublet at δ 0.82 (*d*, J = 7.1 Hz, 3H) in **4** is replaced by a hydroxy methylene at δ 3.40 (*dd*, J = 10.5 Hz, 4 Hz, 1H) and 3.62 (*dd*, J = 10.5 Hz, 4 Hz, 1H), in compound **5** indicative of an oxidation (Table 2). The ^{1}H NMR spectrum of both compounds had characteristic C=CH peaks at δ ~5.9, *gem*-dimethyl peaks at δ ~1.54 and ~2.26 and an additional methyl singlet at δ 1.1–1.2. They also contain two adjacent CH_2 groups and an additional isolated CH_2 in the case of **5** as evident from COSY. The 3-OH peaks in both compounds and the 15-OH peak in compound **5** were not observed. The assignment of the peak at δ ~1.55 to the C-13 methyl was based on the observation of an NOE to the H-6β proton at δ = 2.77. No NOE was observed for the C-12 methyl at δ = 2.26. The assignment of all 15 carbon chemical shifts, including the C=O at δ ~191 was accomplished by analysis of the HMQC and HMBC data. The data for compound **4** were in close agreement with that of isopetasol (Sugama et al., 1983). Compound **5** was determined to be similar with an oxidation of the methyl at C-15 to a primary alcohol. Careful examination of the NOE data for **4** established that the relative configuration at C-3 was opposite from that reported for isopetasol (Sugama et al., 1983). Specifically, the observation of an NOE between the C-14 methyl and H-6β at δ 2.77 determined that methyl function to be β. A similar NOE between the C-15 methyl and both the C-14 methyl and the H-6β determined C-15 to also be β. The observation of an NOE between H-4 and H-6α as well as H-3 determined both H-4 and H-3 as α. The NOE data for compound **5** also showed the same configuration at C-3 as for compound **4**. The structure of **4** was subsequently determined to be 3-epiisopetasol previously isolated from the plant *Senecio nebrodensis* (Barrero et al., 1991) and described in synthetic studies (Bohlmann and Otto, 1982; Yamakawa et al., 1974, 1979). Compound **5** was determined to be the new structure 15-hydroxy-3-epiisopetasol. The configuration of the secondary alcohol at C-3 was similar to related sesquiterpenes isolated from a different fungal species (Sørensen et al., 2007).

Compounds **6**–**9** were isolated from DAOM 239833 using LC–MS–SPE and characterized by NMR following elution of the compounds from the SPE cartridges with CD_3CN. The isolated metabolites were first analyzed by LC–MS in both ESI+ and ESI− mode to determine the molecular formula of each. In spite of a difference in retention time compounds **6** (the more polar metabolite) and **7** have identical mass spectra and very similar NMR spectra suggesting that they are stereoisomers in a 3:1 ratio. In ESI+ mode both are characterized by the 431 [M+Na], 391 [M+H−H_2O], 373 [M+H−2(H_2O)], 426 [M+NH_4] and 839 [2M+Na], the molecular

Table 2
^{1}H and ^{13}C NMR spectroscopic data for compound **5** in C_6D_6 at 600 (^{1}H) and 150 (^{13}C) MHz.

Position	δ_H	δ_C
1	2.59 *ddt* (14.5, 5.3, 2) 1.70 *ddd* (14.5, 4, 2)	26.8
2	1.50 *ddt* (13.8, 5.1, 2) 1.17 *m*	33.8
3	3.88 *m*	67.7
4	0.95 *m*	52.0
5		41.4
6	2.77 *d* (13.2) 1.90 *d* (13.2)	42.5
7		128.6
8		190.7
9	5.91 *d* (2)	126.9
10		166.5
11		142.5
12	2.26 *d* (2)	22.8
13	1.54 *d* (2)	22.0
14	1.21 *s*	20.8
15	3.62 *dd* (10.5, 4) 3.40 *dd* (10.5, 4)	61.1

ion of 408.141 (409M+H) is not observed. In ESI− mode a large 407 [M−H] peak was observed confirming the mass of 408, which gives a molecular formula of $C_{20}H_{24}O_9$ for both, indicating nine double bond equivalents. In the individual ^1H spectra (Table 3) of compounds **6** and **7**, 21 of the 24 expected protons from the HRMS are observed, including; three olefinic CH protons comprised of one trisubstituted C=CH proton (H-6) at δ 5.67 (*d*, *J* = 10.0 Hz, 1H), and one disubstituted CH=CH protons (H-11 and H-12) at δ 6.30 (*dt*, *J* = 16.0 Hz, 2.0 Hz, 1H) and at δ 7.09 (*dt*, *J* = 16.0, 7.0 Hz, 1H), respectively; two CH_3 triplets at δ 3.2 and 3.23 ppm indicating the presence of CH_2–CH_3s; two CHs next to a C=C or C=O at ~3.2 ppm; and numerous CH_2s at δ 1.5–2.6. The spin systems of the five CH_2 groups and their adjacent functionalities were identified from the COSY spectrum. The HMQC spectrum established 12 ^1H–^{13}C direct bond correlations observed in this compound. The HMBC spectrum helped to determine chemical shifts of 5 of the 8 of the quaternary carbons, leaving three carbonyls (C-16, C-17 and C-18) that were not observed by any 2D NMR method. Direct ^{13}C NMR showed resonances at δ 168.1, 167.1 and 165.8 assigned to these respective carbonyl groups. Out of the five carbonyls, three must be free acids (C-1, C-15 and C-16) in order to satisfy the molecular formula and the NMR spectroscopic data (Table 3). The number of C=O functions in addition to the double bonds observed leaves only one ring function to satisfy the nine double bond equivalents. These NMR data, in conjunction with the adjacencies determined by analysis of the HMBC, determined the structures of **6** and **7** as stereoisomers. From a full set of specific NOEs, the orientation of H-6 was determined to be *trans* to the C-16 acid function on C-5 in both compounds. An NOE was observed between H-6 and H-4 as well as H-3 suggests that C-15, C-16 was stabilized by hydrogen bonding. From the structure of **6**, there are only two possible stereochemical centres, namely C-4 and C-7. The spectra of **6** and **7** are virtually identical, except for slight differences in the chemical shifts of protons H-7 and H-4. However, the C-7 shift remains identical, as do the ^{13}C and ^1H shifts of positions 8 and 19, indicating the difference is likely the orientation of the substituents at C-4. But the assignment of the absolute configuration remains elusive.

Compounds **8** and **9** were also analyzed in both ESI+ and ESI− mode, compound **8** was characterized in ESI+ mode by the 413 [M+Na], 391 [M+H], 408 [M+NH_4] and 803 [2M+Na]. In the ESI− mode, a large 345 [M−H−CH_3CHO] peak is observed as well as a 779 [2M−H] to confirm the mass of 390.1308 which gives a molecular formula of $C_{20}H_{22}O_8$. Compound **9** was characterized in ESI+ mode by the 557 [M+H], 574 [M+NH_4] and 579 [M+Na]. In the ESI− mode, a 555 [M−H] peak is observed as well as a 511 [M−H−CH_3CHO] to confirm the mass of 556.1945 which gives a molecular formula of $C_{29}H_{32}O_{11}$. The full set of ^1H, ^{13}C and 2D NMR spectroscopic data were acquired as before for compounds **8** and **9**. This information, along with the molecular formula and a search of the Antibase metabolite database, allowed for the determination of the compounds to be the known fungal metabolites, cordyanhydrides A and B, respectively (Isaka et al., 2000; Yamanishi et al., 2000). Isaka et al. (2000) reported the isolation of **8** and **9** from *Cordyceps pseudomilitaris*, a known insect pathogenic fungus. While metabolites **6** and **7** are new structures, the similarity to **8** and subsequently **9** suggests that one or both are biosynthetic precursors. The occurrence of stereoisomers as fungal metabolites is not new; one example was the characterization of the *R* and *S* 3-OH epimers of 3,13-dihydroxy-11-epiapotrichothec-9-ene observed in several *Fusarium* species (Greenhalgh et al., 1989).

Metabolites **1**–**9** represent the major compounds produced by the three *P. rubens* endophytes studied, all of which showed toxicity to *C. fumiferana* and for the extract that contained compounds **1**–**3**, to yeast cells. The three strains were selected from a screening process that provided a representation of geographic, taxonomic and secondary metabolite diversity. Of these compounds **1** and **5**–**7** are new structures, albeit related to known metabolites. Compounds **2** and **3** are reported as natural products for the first time, **4** is reported as a fungal metabolite for the first time and the remaining metabolites have been previously reported as fungal metabolites but from other species. Unlike the observation of tyrosol in several *P. glauca* strains (Sumarah et al., 2008a), no common metabolite was observed in the *P. rubens* strains discussed here. However, the clear similarities between tyrosol and **2** and **3** suggest similar biosynthetic pathways are employed by these species from *Picea* trees. Metabolite **1** is a polyketide and compounds **4** and **5** are sesquiterpenes, derived biosynthetically from the condensation of three C_5 units of mevalonic acid. Compounds **6**–**9** are nonadrides bearing two or three maleic anhydride moieties derived from either two or three nine carbon units. The variety in chemical structure of metabolites (**1**–**9**) from these extracts is in contrast to our previous study of toxic *P. glauca* endophytes, where the major of metabolites were all of polyketide origin (Sumarah et al., 2008a). Two additional *P. rubens* strains reported elsewhere (Sumarah and Miller, 2009) produced a number of isocoumarin derivatives of ramulosin that are known to be toxic to *C. fumiferana*. Sumarah and Miller (2009) also reported the isolation of 5-hydroxymethyl-2-furaldehyde, which is known to be toxic to *Drosophila melanogaster* and the antifungal compound *p*-hydroxyphenylacetic acid which is biosynthetically related to tyrosol and compounds **2** and **3** from *P. rubens*.

However, in spite of these structural differences, the compounds are unified in that the extracts of these strains all showed toxicity to *C. fumiferana* larvae at 400 μg/g synthetic diet based on reductions in either head capsule size or weight gain. This effect plus the antifungal activity of the extract from CBS 121942 could be explained by its production of compound **1** which is structurally similar to other known fungal metabolites. The extract for CBS 121944 significantly reduced the weight of insects in the dietary bioassays. This effect can be attributed to the presence of both compounds **4** and **5**. These metabolites are biosynthetically similar to other known insect toxins such as PR toxin and derivatives isolated from the *Penicillium* species (Sørensen et al., 2007). The biological activity of maleic anhydrides was reviewed by Chen et al.

Table 3
^1H and ^{13}C NMR spectroscopic data for compounds **6** and **7** in CD_3CN at 600 (^1H) and 175 (^{13}C) MHz.

Position	6		7	
	δ_H	δ_C	δ_H	δ_C
1		174.2		174.2
2	2.24 *m*	31.7	2.22 *m*	31.8
3	2.06 *m*	26.5	2.03 *m*	26.0
	1.79 *m*		1.76 *m*	
4	3.20 *dd* (7.5)	49.3	3.26 *dd* (7.5)	48.9
5		132.2		132.4
6	5.67 *d* (10.0)	146.6	5.70 *d* (10.0)	145.8
7	3.23 *m*	41.2	3.20 *m*	41.2
8	2.62 *dd* (14.0, 4.0)	29.2	2.60 *dd* (13.0, 5.0)	29.3
	2.48 *dd* (13.0, 10.0)		2.49 *dd* (13.0, 10.0)	
9		138.8		138.8
10		138.3		138.3
11	6.30 *dt* (16.0, 2.0)	117.7	6.31 *dt* (16.0, 2.0)	117.6
12	7.09 *dt* (16.0, 7.0)	148.8	7.12 *dt* (16.0, 6.5)	149.1
13	2.28 *m*	27.7	2.29 *m*	27.7
14	1.07 *t* (7.5)	12.8	1.08 *t* (7.5)	12.7
15		174.1		173.9
16		168.1		168.2
17		167.3		167.2
18		165.8		165.8
19	1.59 *m*	29.0	1.59 *m*	29.0
	1.38 *m*		1.39 *m*	
20	0.87 *t* (7.5)	12.0	0.86 *t* (7.5)	12.0

(2007), where it was shown that numerous members of this group are inhibitors of protein phosphatase and have herbicidal, antifungal, and antibacterial activity. The two maleic anhydrides **8** and **9** were previously isolated from an insect pathogen (Isaka et al., 2000), and their effects on *C. fumerferana* are likely attributable to their presence in the extracts tested.

In field experiments using trees, we have shown that *P. glauca* infected with *P. scopiformis* disrupted the growth of *C. fumiferana*. The effect was attributable to the presence of the toxin (Miller et al., 2008). Similar experiments are in progress with the strains discussed in this report. The effect of these new strains and their toxins *in planta* is the next step to understanding the nature of the interaction between these endophytes and the tree and their potential in forest protection.

2.4. Conclusions

The screening of 150 fungal endophytes of *P. rubens* resulted in a number extracts that showed toxicity to the forest pest *C. fumiferana* in dietary bioassays. Isolation of the major metabolites from three of these strains has led to the discovery of four new compounds (**1** and **5**–**7**); two new natural products (**2** and **3**) previously reported as synthetic products; compound **4** is reported as fungal metabolite for the first time and **8**–**9** were identified as known fungal metabolites from new species.

3. Experimental

3.1. General

Initial HPLC 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 (0–100% CH_3CN over 20 min). NMR spectroscopic data were acquired on either a Bruker Avance 400 MHz spectrometer with 5 mm broadband probe with Z gradient, a Bruker ARX 500 MHz spectrometer with a 3 mm broadband inverse probe or a Bruker AVANCE 700 MHz with a cryogenic (TCI) probehead (Health Canada, Ottawa). LC–NMR was performed in stop-flow mode using a Varian 9010 HPLC system with UV detector and a Varian Inova 600 MHz spectrometer equipped with a 5 mm HCN PFG Cold Probe with LC–NMR flow-cell insert (60 μL active volume). The LC–MS–SPE system consisted of an Agilent 1100 series HPLC, with an Accurate ICP-04-20 1:20 flow-splitter by LC Packings, Bruker Esquire 4000 ion trap mass spectrometer, and a Bruker/Spark Prospekt II LC–SPE–NMR interface module with N_2-blanketing of the SPE compartment, all managed by Bruker HyStar/Esquire Control software. A flow rate of 0.75 mL/min was used and a post-column makeup flow of H_2O (1.25 mL/min) was added to reduce the solvent strength for trapping on Spark Hysphere Resin GP 10 × 2 mm SPE cartridges. The samples were filtered through 0.45 μm GHP Acrodisc (13 mm) syringe filters prior to injections. SPE fractions were eluted into 3 mm NMR tubes with 160 μL CD_3CN and NMR data were acquired using the Varian Inova 600 MHz instrument. HRMS (EI) data was obtained on a Kratos Concept instrument (University of Ottawa, Mass Spectrometry Centre).

3.2. Fungal strains

The fungal strain CBS 121942 was isolated from a *P. rubens* originating in eastern Nova Scotia; CBS 121944, and DAOM 239833 were isolated from different *P. rubens* originating in western Nova Scotia. The GPS coordinates from each tree sample were recorded such that trees could be re-sampled if needed. When suitable analytical methods are developed for the analysis of the metabolites in needles, this will be attempted. These strains were selected via a screening process from a much larger collection of greater than 2000 *P. rubens* endophytes isolated from mature needles located in eastern Canada and Maine, USA.

3.3. Toxicity testing

Laboratory grown *C. fumiferana* were tested in dietary bioassays against the EtOAc extract from the fermentations of each strain. The extract of was dissolved in CH_2Cl_2 and incorporated into the synthetic diet (400 μg/g), respectively, as previously described (Sumarah et al., 2008a). Additionally, disc diffusion assays (Vincent and Vincent, 1944) were employed for preliminary antifungal testing using *S. cerevisae* for CBS 121942 with nystatin used as a positive control (Sumarah et al., 2008a).

3.4. Sequencing

DNA was extracted from fungal cultures using an UltraClean DNA Isolation Kit (MO BIO Laboratories 12224-250). PCR and sequencing was performed by Laboratory Services, University of Guelph, Ontario. Data were treated as previously described for endophytes from *P. glauca* (Sumarah et al., 2008a).

3.5. Fermentation, extraction and isolation

All strains were stored on 2% MEA (malt extract agar, Difco) slants at 5 °C and fermented on a 3 L scale in Glaxo bottles containing 1 L of 2% ME medium for 3 months (20 °C). The cultures were then harvested, filtered, extracted with 2 × equal volumes of EtOAc and dried by rotary evaporation. The extracts were weighed and initially 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 ^1H NMR data were acquired for each major peak to evaluate the secondary metabolite production of the endophyte.

3.6. CBS 121942

Fractionation of the crude filtrate extract (160 mg) was performed by Si gel column chromatography (CC) eluting first with EtOAc–hexanes solvent gradient (0–70%, 100 mL aliquots) then MeOH–$CHCl_3$ solvent gradient (0–20%, 50 mL aliquots) to provide two major fractions. The first major fraction (10 mg) was re-purified by PTLC eluting with MeOH–$CHCl_3$ (5:95) to afford compound **1** (1 mg). Fraction 2 (40 mg) was re-applied to a Si gel column eluting sequentially with $CHCl_3$–hexanes solvent gradient (0–100%, 100 mL aliquots) followed by MeOH–$CHCl_3$ solvent gradient (0–20%), yielding compound **2** (7 mg) and **3** (1 mg). A 3 mg portion of compound **2** was methylated with diazomethane to confirm the presence of the acid group (Sumarah et al., 2008a). It was stirred at 0 °C in CH_2Cl_2 (20 mL), before excess ether diazomethane solution was added and stirring continued for 1 h at 0 °C before it was warmed to r.t. [diazomethane solution was prepared by adding nitrosomethylurea (100 mg) to a solution of 20% KOH (2 mL) and diethyl ether (5 mL) at 0 °C]. The reaction mixture was washed with water (2 × 5 mL) and the solvent evaporated *in vacuo*. The crude product was purified by PTLC using 35% EtOAc–hexanes (100 mL) as eluants, to furnish compound **2a**, the methylated version, used for the purposes of solving the structure of **2**.

3.7. CBS 121944

The crude filtrate extract (80 mg) was dissolved in CH_3CN (1 mL) and injected (10 μL) for separation and isolation using LC–MS–SPE. The peaks of interest were collected on the SPE cartridges

by monitoring the UV and MS spectral data for each. Compounds **4** and **5** were isolated by LC–MS–SPE using a triple trap for each. The SPE cartridges were dried for 30 min with a constant flow of N_2. The compounds were eluted from the SPE cartridges directly into 3 mm NMR tubes with 160 μL of CD_3CN. The solvent was eventually changed by evaporation in the NMR tubes using N_2 and reconstitution with 160 μL of C_6D_6 for optimized NMR.

3.8. DAOM 239833

The crude filtrate extract (80 mg) was also analyzed using LC–MS–SPE in triple trap mode with emphasis on the major peaks in the chromatogram. Separation and isolated was achieved as described above. The four non-polar compounds **6–9** were all trapped, isolated and collected using this method. The isolated peaks were then transferred directly from the SPE cartridges into 3 mm NMR tubes using CD_3CN and NMR data were obtained at 600 MHz.

3.9. Compound **1**

(9E,12Z)11-Hydroxy-6-methyl-7,15-dioxabicyclo[12.1.0]pentadeca-9,12-diene-2,8-dione: ^{1}H NMR (400 MHz, $CDCl_3$, 600 MHz, CH_3CN/D_2O) and ^{13}C NMR (100 MHz, $CDCl_3$, 150 MHz, CH_3CN/D_2O) see Table 1; EIMS *m/z* 266 $[M]^+$ (11), 180 (14), 115 (83), 85 (43), 68 (86), 41 (100); HRMS *m/z* 266.1153 (calc. for $[C_{14}H_{18}O_5]$, 266.1154).

3.10. Compound **5**

15-Hydroxy-3-epiisopetasol: ^{1}H NMR (600 MHz, CH_3CN) and ^{13}C NMR (150 MHz, CH_3CN) see Table 2; HRMS *m/z* 250.1548 (calc. for $[C_{15}H_{22}O_3]$, 250.1569).

3.11. Compound **6**

7-(4-But-1-enyl-2,5-dioxo-2,5-dihydro-furan-3-ylmethyl)-4,5-dicarboxy-non-5-enoic acid: ^{1}H NMR (600 MHz, C_6D_6) and ^{13}C NMR (175 MHz, C_6D_6) see Table 3; HRMS *m/z* 408.1415 (calc. for $[C_{20}H_{24}O_9]$, 408.1420).

3.12. Compound **7**

*7-(4-But-1-enyl-2,5-dioxo-2,5-dihydro-furan-3-ylmethyl)-4,5-dicarboxy-non-5-enoic acid (**7**):* ^{1}H NMR (600 MHz, C_6D_6) and ^{13}C NMR (175 MHz, C_6D_6) see Table 3; HRMS *m/z* 408.1411 (calc. for $[C_{20}H_{24}O_9]$, 408.1420).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2010.01.015.

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