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Antifungal metabolites from fungal endophytes of *Pinus strobus*

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ABSTRACT

The extracts of five foliar fungal endophytes isolated from *Pinus strobus* (eastern white pine) that showed antifungal activity in disc diffusion assays were selected for further study. From these strains, the aliphatic polyketide compound **1** and three related sesquiterpenes **2–4** were isolated and characterized. Compound **2** is reported for the first time as a natural product and the *E/Z* conformational isomers **3** and **4** were hitherto unknown. Additionally, the three known macrolides; pyrenophorol (**5**), dihydropyrenophorin (**6**), and pyrenophorin (**7**) were isolated and identified. Their structures were elucidated by spectroscopic analyses including 2D NMR, HRMS and by comparison to literature data where available. The isolated compounds **1**, **2,** and **5** were antifungal against both the rust *Microbotryum violaceum* and *Saccharomyces cerevisae*.

1. Introduction

Extracts of foliar fungal endophytes of *Picea glauca* and *P. rubens* (white and red spruce) have been shown to be antifungal and toxic to insects when incorporated into diets (Sumarah et al., 2008b, 2010; Sumarah and Miller, 2009). It has been demonstrated that seedlings can be inoculated with these endophytes and that their metabolites accumulate in the needles, providing the tree with tolerance against spruce budworm (Miller et al., 2002, 2008; Sumarah et al., 2008a). *Cronartium ribicola* (white pine blister rust) has affected *Pinus monticola* (western white pine) to the point where it is no longer a viable commercial species in many regions of British Columbia, Canada. Natural populations of *Pinus* are highly susceptible to this disease. After these experiments were initiated, various species of *Pinus* were shown to harbor endophytes (Ganley et al., 2008). Endophytes were isolated from the needles of superior *Pinus strobus* (eastern white pine) trees originating in New Brunswick, Nova Scotia, Quebec and Prince Edward Island, Canada (Sumarah and Miller, 2009). Strains were cultured using established procedures and the extract from each endophyte was tested for antifungal activity to *Saccharomyces cerevisae*. Strains that exhibited toxicity were analyzed by LC-HRMS and the major metabolites were isolated by LC-MS-SPE. Characterization by NMR and MS of the major metabolites from five of these endophytes is reported here.

2. Results and discussion

2.1. Screening

Five strains were selected from the original 35 isolated from superior *P. strobus* trees. These were selected based on the antifungal activity of crude extracts and preliminary LC-HRMS analysis. The results of the DNA sequencing for all five strains show that they are all species of *Lophodermium* (Deckert et al., 2002); sequences are deposited in Genbank (JF706648–JF706652). Morphotypes were selected by the visual appearance in culture. Further classification of the strains was not possible because they did not sporulate in culture and possibly are un-described taxa.

2.2. Toxicity

Initial antifungal testing showed that extracts from all five endophytes were antifungal to *S. cerevisae*. The results of the Oxford disc assay using the isolated compounds confirmed these observations for both *S. cerevisae* and *Microbotryum violaceum* (data not shown). A quantitative test protocol based on cell density using OD measurements at 600 nm in 96 well microplates was employed. Statistically-significant reductions in cell density were observed for compounds **1**, **2,** and **5** at 24 h against *S. cerevisae* (ANOVA, $p < 0.000$). The Minimum Inhibitory Concentration (MIC) for

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nystatin and compound **5** was 4 μM and for compounds **1** and **2**, 2 μM. In the assay using *M. violaceum* at 48 h, the three compounds were statistically significant (ANOVA, $p < 0.000$) as compared to controls, with compound **2** being the most antifungal based on results of the response at 24 h. In the assay used, the MIC for nystatin was 2 μM, 3 μM for compound **2** and 4 μM for compound **1** (see Fig. 1).

2.3. Isolation and structure determination

Following initial screening, the extracts were analyzed using LC-MS-SPE/NMR, where a number of the major metabolites were isolated on GP resin cartridges. The compounds were then transferred directly using 160 μL of deuterated solvent into 3 mm NMR tubes for acquisition of 1D and 2D NMR spectroscopic data. Structures were elucidated primarily by analysis of HRMS and NMR data, as well as by comparison with literature data where available. The five strains studied produced an aliphatic polyketide metabolite **1**, three related sesquiterpenes **2–4** and three known macrolides **5–7** (see Table 1).

Compound **1** was assigned the formula $C_{12}H_{14}O_6$ based on HRMS analysis. It was isolated as the major metabolite from CBS 127938 and CBS 127942. All 14 protons were identified in the ^{1}H NMR spectrum (see Table 2), with one in the very downfield region at δ 9.57 (*s*), three protons in the unsaturated region δ 8.39 (*dd*, J = 11.8/0.8), δ 7.23 (*dd*, J = 11.4/11.8), and δ 6.22 (*dd*, J = 11.4/0.8) that through coupling analysis were shown to originate from two conjugated double bonds, one protons from an oxygen-bound CH-group δ 6.15 (*s*), a methoxy group at δ 3.76 (*s*), as well as two other methyl groups, at δ 2.11 and 2.08, appearing as singlets. Analysis of HSQC and HMBC data indicated the presence of several carbonyl moieties; an aldehyde, a ketone and two esters. On the basis of the HMBC data, especially the correlations from H-6 to C-4, C-5, C-7, C-9, and C-11, as well as NOE correlations from H-4 to H-11 and H-3 to H-6, the structure for compound **1** was determined.

Compound **2** was assigned the formula $C_{15}H_{22}O_3$ on the basis of HRMS, and was isolated from CBS 127938, CBS 127940, and CBS

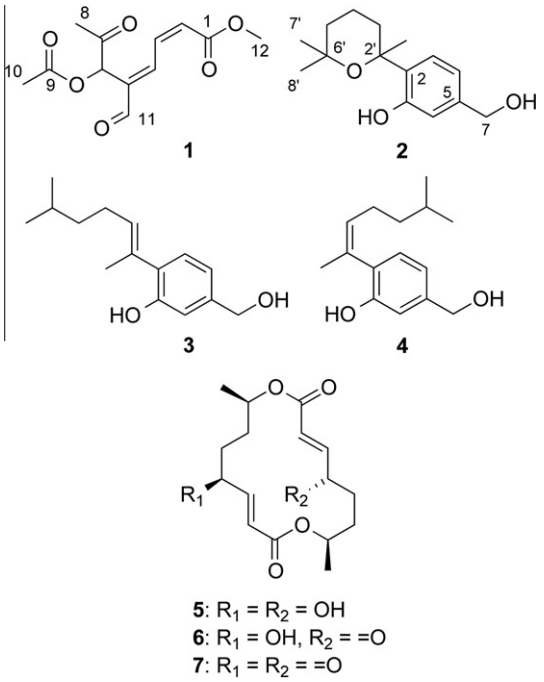

Fig. 1. Compounds **1–7** with numbering schemes used for NMR assignment.

Table 1
Metabolite production from strains studied.

Strain	1	2	3	4	5	6	7
CBS 127939					++	++	++
CBS 127938	++	+	+		+		
CBS 127940		+	+	+			
CBS 127942	++	+	+	+	+		
CBS 127941		+	+	+	+		+

(+), <2 mg/L; (++), >5 mg/L.

Table 2
^{1}H (700 MHz) and ^{13}C (176 MHz) NMR spectroscopic data for compound **1** in CD_3CN.

Position	δ_C	δ_H, (J in Hz)	HMBC$_{H\rightarrow C}$	NOE
1	166.9			3
2	128.5	6.22 (*dd*, $J_{2,3}$ = 11.4, $J_{2,4}$ = 0.8)	1, 4	
3	136.6	7.23 (*dd*, $J_{3,2}$ = 11.4, $J_{3,4}$ = 11.8)	1, 4, 5	2, 6
4	146.8	8.39 (*dd*, $J_{4,3}$ = 11.8, $J_{4,2}$ = 0.8)	2, 6, 11	11
5	140.6			
6	72.4	6.15 (*s*)	4, 5, 7, 9, 11	3, 8
7	202.9			
8	26.7	2.11 (*s*)	6, 7	6
9	170.4			
10	21.1	2.08 (*s*)	9	
11	194.4	9.57 (*s*)	4, 5, 6	4
12	52.5	3.76 (*s*)	1, 2	

127942. The ^{1}H NMR spectroscopic data (see Table 3) shows signals for three aromatic protons at δ 7.07 (*d*, J = 7.9 Hz), δ 6.78 (*dd*, J = 7.9 and 1.6 Hz), and δ 6.73 (*d*, J = 7.9 Hz), suggesting the presence of a 1,2,4-trisubstituted aromatic ring in the molecule. Additionally, three singlet methyl groups at δ 1.43, 1.23, and 0.93; four methylene groups, three as multiplets, and giving rise to several signals due to diastereotopic nature of the protons (δ 2.43, 1.73, 1.68, 1.62, and 1.52), and one as a doublet (δ 4.48, J = 5.9 Hz) were observed. Furthermore, two single protons were observed, at δ 9.06 (*s*) and 3.10 (*t*, J = 5.9 Hz). HSQC and HMBC spectra contained resonances for 15 carbon atoms, of which five were quarternary. Three of these were substituted aromatic carbons (δ 157.5, 142.8, and 130.8), with the down-field signal originating from an oxygen-bearing carbon. Two other quarternary carbons at δ 78.5 and 75.3, respectively, were also identified as oxygen-bearing carbon atoms. Furthermore, resonances from three aromatic CH carbons were present (δ 126.1, 118.8, and 116.5), as well as four CH_2 carbons (δ 64.5, 37.8, 34.8, and 17.6), with the signal at δ 64.5 confirming the presence of a CH_2–OH group. Three additional resonances (δ 32.6, 32.0, and 25.7) corresponded to the three methyl groups detected in the ^{1}H NMR spectrum. Analysis of the HSQC spectrum indicated that the protons at δ 3.10 and 9.06 were not directly attached to a carbon atom, which led to the assignment of these as an aliphatic alcohol, and a phenol respectively. The position of substituents on the aromatic ring was assigned based on HMBC correlations, as H-7 has correlations to C-4, C-5, and C-6, attaching this substituent to C-5. The chemical shift value of C-1 places the phenol at this position, as well as the HMBC correlations to C-2′ from H-3, and to C-2 from H-9′. The presence of diastereotopic protons was established by analysis of HSQC data, and the skeleton of the heterocyclic ring was determined based on COSY and HMBC correlations. The position of the methyl groups was revealed through HMBC correlations from H-7′ and H-8′ to C-5′, C-6′ and between methyl groups. H-9′ showed HMBC correlations to C-2, C-2′, and C-3′, attaching this group to C-2′. Compound **2** is a new natural product, but has previously been reported as an intermediate in a synthesis of sydowic acid (Serra, 2000). The reported OR of $[\alpha]_D^{20}$ = +40 (*c* 2.2, $CHCl_3$) did not agree with our data that showed zero rotation. Further chiral LC-HRMS analysis