Insect Toxins from an Endophytic Fungus from Wintergreen

John A. Findlay,* Sentsetsa Buthelezi, Guoqiang Li, and Michelle Seveck

Department of Chemistry, University of New Brunswick, Fredericton, New Brunswick E3B 6E2, Canada

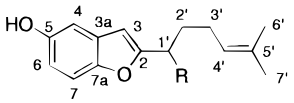
J. David Miller

Plant Research Centre, Agriculture Canada, Ottawa, Ontario K1A 0C6, Canada

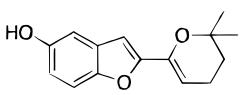
Received May 2, 1997[⊗]

Two new compounds, 5-hydroxy-2- (1′-oxo-5′-methyl-4′-hexenyl)benzofuran (**1**) and 5-hydroxy-2-(1′-hydroxy-5′-methyl-4′-hexenyl)benzofuran (**2**), have been isolated via bioassay-directed fractionation of culture extracts of an unidentified endophytic fungus obtained from wintergreen, *Gaultheria procumbens* L. Their structures have been deduced from spectral data and confirmed by synthesis. Both **1** and **2** show toxicity to spruce budworm (*Christoneura fumiferana* Clem.) cells, and **1** is also toxic to the larvae.

Earlier we reported the bioassay-directed isolation and structure elucidation of several insect toxins from a variety of endophytic fungi obtained from needles of balsam fir,[1,2] black spruce,[3] and eastern larch.[4] We have also examined a number of forest ground cover plant endophytes for the production of insect toxins and now report the discovery of two new 5-hydroxybenzofuran derivatives **1** and **2**, obtained by bioassay-directed fractionation of an extract of cultures of an endophyte from wintergreen, *Gaultheria procumbens* L., which are toxic to spruce budworm, *Christoneura fumiferana* Clem.



1 R=O
2 R=OH



3

Compound **1** was obtained as a yellow crystalline solid whose composition, $C_{15}H_{16}O_3$, was deduced from HRE-IMS and ^1H and ^{13}C NMR data. The latter spectrum shows the presence of 11 SP^2 carbons, five of which are protonated as revealed by DEPT analysis, and the remaining six quaternary carbons include one carbonyl (δ 191.7). In addition, two methyl and two methylene carbons are present. A $^1H-^1H$ COSY spectrum shows that a vinylic proton at δ 5.14 is coupled to both methyl groups as well as the methylene at δ 2.44, which in turn is coupled to the remaining methylene at δ 2.94; thus, the sequence $(CH_3)_2C{=}CHCH_2CH_2-$ was readily established. The remainder of the NMR data (see Table 1) is consistent only with a 2-acyl-5-hydroxybenzofuran moiety. Thus, on the basis of NMR data, we formulate this toxin as 5-hydroxy-2-(1′-oxo-5′-methyl-4′-hexenyl) benzofuran (**1**). The IR spectrum shows strong bands at ν_{max} 3366 (OH), 1659 (C=O), and 1548 (aromatic) cm^{-1}, and the EIMS base peak *m/z* 176 and fragment *m/z* 161 are readily accounted for via side chain cleavages at C2′−C3′ and C1′−C2′, respectively, thus confirming the functional groups and side chain features of **1**.

Compound **2**, obtained only in trace amounts as a yellowish oil, shows a similar ^1H NMR spectrum to that of **1** in the downfield region but differed by the presence of a methine multiplet at δ 4.76. By $^1H-^1H$ COSY experiments this signal was shown to be coupled to a methylene at δ 1.94, in turn coupled to another methylene at δ 2.11 that also correlated to a vinylic proton signal at δ 5.13. On the basis of these limited data we tentatively formulated the structure as **2** and embarked on a synthesis to provide corroboration.

Table 1. NMR Data[a,b] for 1−3 in $CDCl_3$

	1		**2**		**3**	
position	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C
2		150.8		160.2		153.9
3	7.37 d (0.7)	112.7	6.42 bs	102.6	6.62 s	101.1
3a		127.8		128.9		129.7
4	7.08 d (2.6)	107.2	6.86 bs	106.1	6.90 dd (0.5, 2.6)	106.0
5		152.5		151.5		151.5
6	7.02 dd (2.6, 9.1)	117.7	6.74 dd (2.6, 8.7)	112.7	6.72 dd (2.6, 8.7)	112.6
7	7.41 d (9.1)	113.1	7.22 (8.7)	111.5	7.24 d (8.7)	111.2
7a		153.5		149.7		149.7
1′		191.7	4.77 t (6.7)	68.0		143.0
2′	2.94 t (7.5)	39.1	1.95 dd (∼7.4)	35.4	5.56 t (4.1)	98.1
3′	2.44 q (∼7.4)	22.9	2.11 m	24.0	2.23 m	18.6
4′	5.14 m	122.5	5.13	123.2	1.69 t (6.7)	32.6
5′		133.2		128.9		
6′	1.61 d (0.5)	17.7	1.57 d (0.7)	17.7	1.31 s	26.3
7′	1.66 s	25.7	1.66 s	25.7	1.31 s	26.3
5-OH	5.85 (br)		5.6 br		4.65 br	
1′-OH			2.45 br			

[a] δ (ppm); J (H_z). [b] Assignments facilitated by HMQC, HMBC, and NOE data.

Condensation of 2-hydroxy-5-methoxybenzaldehyde with chloroacetonitrile in refluxing DMF containing K_2CO_3 afforded 2-cyano-5-methoxybenzofuran,[5] which was demethylated to 1-cyano-5-hydroxybenzofuran[6] via treatment with BBr_3 in CH_2Cl_2. Treatment of the latter with excess Grignard reagent prepared from commercially available 5-bromo-2-methyl-2-pentene and Mg in ether, followed by acidic workup, gave **1**. Reduction of **1** with $NaBH_4$ in CH_3OH gave **2** identical in ^1HNMR and TLC characteristics with the natural compound. It may be noted that prolonged exposure of **1** to acid results in its conversion to the tricylic ether **3**.

Benzofurans variously substituted at the 5-position have been reported from the Basidiomycete *Stereum subpileatum*, and the biosynthesis of 5-substituted benzofurans has been studied by Bu'Lock et al.,[7] who proposed a multistep sequence in which the C_2 and C_3 carbons of the benzofuran possibly derive from mevalonic acid via dimethylallyl pyrophosphate. Our dis-

[⊗] Abstract published in *Advance ACS Abstracts,* October 15, 1997.

S0163-3864(97)00222-X CCC: $14.00

covery of the 2-(5′-methyl-4′-hexenyl)benzofuran derivatives lends strong support for this biogenetic proposal in that the side chain as well as C_2 and C_3 of **1** and **2** could be derived from a geranyl pyrophosphate moiety with demethylation at 1′ the site of oxygenation of the side chain.

In our spruce budworm (*Christoneura fumiferana* Clem.) larval feeding bioassay[1] compound **1** resulted in 36% mortality when tested at a level of 0.8 μmol per insect, which indicates a substantially lower toxicity than that of azadirachtin.[8] Compound **1** was comparable to the control vomitoxin in our budworm cell toxicity assay,[1] while natural **2** was somewhat less active. Natural **2** was not obtained in sufficient quantity to measure specific rotation or conduct a larval assay. Synthetic racemic **2** did not display significant activity in the larval assay at the level (0.8 μmol/insect) tested.

Experimental Section

General Experimental Procedures. Melting points were taken with a Kofler hot-stage apparatus and are uncorrected. IR spectra were recorded as films on a Bruker IF-S25 spectrometer. PTLC was performed with precoated Si gel F254 (1 mm) plates unless otherwise indicated. NMR data were recorded in $CDCl_3$ on a Varian Unity 400 spectrometer, using the solvent as reference. All 2D spectra were recorded nonspinning at 25 °C. HRMS were recorded on a Kratos MS-50 instrument.

Fungal Strain. The endophyte strain #4GP4C2 was isolated from a wintergreen (*Gaultheria procumbens* L.) leaf collected at Acadia Forest Research Station near Fredericton, New Brunswick, in June 1985. Attempts to induce this isolate to sporulate in culture were unsuccessful. The isolate is preserved in liquid nitrogen and deposited in the Canadian Collection of Fungal Cultures (Centre for Land and Biological Research, Agriculture Canada, Ottawa, Ontario K1A 0C6) as DAOM 221611.

Extraction and Isolation. Endophyte strain #4GP4-C2 was fermented (10 L scale) according to our established protocols.[1] The culture filtrate was extracted with $CHCl_3$ (3 × 3 L) and the total extract evaporated to dryness at 20 °C under vacuum, yielding a crude extract (0.44 g) that was subjected to flash chromatography on silica gel using hexane (0.5 L) hexane−$CHCl_3$ (1:1), $CHCl_3$, $CHCl_3$−CH_3OH (1:1), and $CHCl_3$−CH_3-OH−H_2O (4:6:1) consecutively as eluants. From the $CHCl_3$−CH_3OH fractions, compounds **1** (4.4 mg) and **2** (0.8 mg) were obtained and further purified by PTLC using $CHCl_3$:*n*-C_6H_{14}:CH_3OH (14:4:1).

5-Hydroxy-2-(1′-oxo-5′-methyl-4′-hexenyl) benzofuran (1): yellow/brown solid; mp 56−57 °C; IR ν_{max} ($CHCl_3$) 3360, 1659, 1548 cm^{-1}; UV λ_{max} (MeOH) 204 (ϵ 22 000), 218 (ϵ 15 000), 292 (ϵ 22 000) 336 sh (ϵ 6300); HREIMS *m*/*z* $[M]^+$ 244.1103 (calcd for $C_{15}H_{16}O_3$ 244.1099); NMR data, see Table 1.

5-Hydroxy-2-(1′-hydroxy-5′-methyl-4′-hexenyl)-benzofuran (2): yellow brown amorphous solid; IR ν_{max} ($CHCl_3$) 3330, 1621, 1604, 1590; UV λ_{max} nm 208 (ϵ 21 000), 250 (ϵ 12 000), 296 (ϵ 3800); HREIMS *m*/*z* $[M]^+$ 246.1253 (calcd for $C_{15}H_{18}O_3$ 246.1256); NMR data, see Table 1.

Synthesis of 5-Hydroxy-2-(1′-oxo-5′-methyl-4′-hexenyl)benzofuran (1). To Mg turnings (311 mg) in ether (11 mL) and a small crystal of iodine was added 5-bromo-2-methyl-2-pentene (730 mg) and the mixture heated with a heat gun to initiate reflux. After 20 min, the cloudy solution was treated with 1-cyano-5-hydroxybenzofuran[5] (28 mg) in ether (4 mL) and the mixture stirred 18 h at rt. The reaction mixture was quenched with 2 N HCl (30 mL) and the aqueous layer separated and stirred 2 h at rt and then extracted with ether (3 × 20 mL). The dried (Mg_2SO_4) etheral extract gave a yellow oily residue (29 mg) that was purified by PTLC, giving **1** (15 mg, 33%), identical in physical and spectroscopic properties to natural **1**.

Reduction of 1 to 5-Hydroxy-2-(1′-hydroxy-5′-methyl-4′-hexenyl)benzofuran, (2). $NaBH_4$ (50 mg) was added to a solution of **1** (27 mg) in MeOH (5 mL), and the mixture was stirred at rt for 1 h and then evaporated to dryness in vacuo. The residue was dissolved in water (7 mL) and the pH adjusted to 3−4 before extraction with $CHCl_3$ (3 × 5 mL). The residue (28 mg) obtained after drying ($MgSO_4$) and evaporation was purified by PTLC (Kieselgel 60 F254, 0.5 mm) using $CHCl_3$/EtOAC (9:1), giving pure alcohol **2** (23 mg), 84%, R_f = 0.17): mp 82−84 °C; identical in spectroscopic properties with natural **2**.

Preparation of 3. When the Grignard complex in the above procedure for synthesis of **1** was decomposed by treatment with ice (50 mL) and concd HCl (30 mL) and heated 2 h at 70 °C the major product was **3**: yellow brown solid; mp 138−140; IR ν_{max} ($CHCl_3$) 3340, 1705, 1690, 1680 cm^{-1}; UV λ_{max} (MeOH) 206 (ϵ 2300), 280 (ϵ 18 700) 286 (ϵ 19 000), 306 (14 000); HREIMS *m*/*z* $[M]^+$ 244.1083 (calcd for $C_{15}H_{16}O_3$ 244.1099); NMR data, see Table 1.

Acknowledgment. Thanks are due to the Natural Sciences and Engineering Research Council of Canada and the Forestry Canada Green Plan (Research Network for the Discovery and Development of Natural Products for Integrated Forest Pest Management) for financial assistance and to Kathleen Edwards and Dan Drummond for assistance with bioassays and mass spectra, respectively.

References and Notes

(1) Calhoun, L. A.; Findlay, J. A.; Miller, J. D.; Whitney, N. J. *Mycol. Res.* **1992**, 96, 281−286.
(2) Findlay, J. A.; Li, G.; Penner, P. E.; Miller, J. D. *J. Nat. Prod.* **1995**, 58, 197−200.
(3) Findlay, J. A.; Buthelezi, S.; Lavoie, R.; Peña-Rodriguez, L.; Miller, J. D. *J. Nat. Prod.* **1995**, 58, 1759−1766.
(4) Findlay, J. A.; Li, G.; Johnson, J. A. *Can. J. Chem.* **1997**, *75*, 716−719.
(5) René, L.; Royer, R. *Bull. Soc. Chim.* **1971**, 4329−4331.
(6) René, L.; Buisson, J. P., Royer, R. *Bull. Soc. Chim.* **1974**, 475−476.
(7) Bu'Lock, J. D.; Kaye, B.; Hudson, A. T. *Chem. Commun.* **1967**, 814−815.
(8) Thomas, A. W.; Strunz, G. M.; Chiasson, M.; Chan, T. H. *Entomol. Exp. Appl.* **1992**, 62, 37−46.

NP970222J