Journal of Natural Products
Vol. 58, No. 11, pp. 1759-1766, November 1995

BIOACTIVE ISOCOUMARINS AND RELATED METABOLITES FROM CONIFER ENDOPHYTES

JOHN A. FINDLAY,* SENTSETSA BUTHELEZI, RICO LAVOIE, LUIS PEÑA-RODRIGUEZ,

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

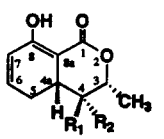
and J. DAVID MILLER

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

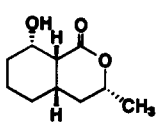
ABSTRACT.—Six new [**1–4**, **7**, **9**] and two known [**6** and **8**] metabolites have been isolated from culture filtrates of conifer endophyte strains of *Conoplea elegantula*. Their structures have been determined by spectroscopic means. Compound **1** was toxic to spruce budworm cells, and both **1** and **3** were toxic to spruce budworm larvae.

In a continuing study directed at the discovery of insect toxins from woody plant endophytic fungi, we have encountered several isocoumarin-producing endophyte strains, identified as *Canoplea elegantula* (Cooke) M.B. Ellis, whose culture filtrates show toxicity to spruce budworm (*Choristoneura fumiferana* Clem.) cells and larvae (1). In each case several isocoumarins related to ramulosin [**5**] or mellein were isolated and bioassayed. Compounds **1** and **3** were both toxic to spruce budworm larvae and **1** was also toxic to budworm cells. Compound **6**, (3*R*,4a*S*,6*R*)-6-hydroxyramulosin, has been isolated previously from *Pestalotia ramulosa* (2), while (3*R*,4*S*)-4-hydroxymellein [**8**] has been found in the fungus *Septoria nodorum* Berk (3). Because **6** and **8** isolated in our work were identical in spectroscopic characteristics and sign of rotation with the above, we represent these and their structurally related optically active co-metabolites **1–4** and **7** with the absolute configurations shown, on the assumption that the C-3 absolute configuration is common to all. Less certain is the absolute configuration of **9**, which appears to be a rearranged norpentaketide possibly related at C-3 to the other metabolites isolated.

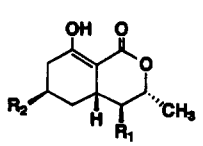
The ^{13}C- and DEPT nmr spectra of **1** showed the presence of three non-protonated and two protonated sp^2 carbons, together with one methyl, one methylene, and two oxygenated methines. The 400 MHz ^{1}H-nmr spectrum showed a methyl doublet at δ 1.44 (J=6.2 Hz) coupled to a methine signal at δ 4.14 (J=6.2 Hz, J=9.5 Hz), in turn coupled



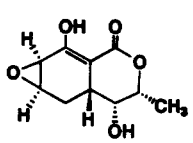
1 R_1=OH, R_2=H
2 R_1=H, R_2=OH



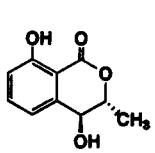
3



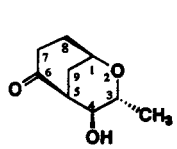
4 R_1=OH, R_2=H
5 R_1,R_2=H
6 R_1=H, R_2=OH



7



8



9

TABLE 1. ^{1}H- and ^{13}C-Nmr Chemical Shift ($CDCl_3$) Data for Compounds **1–4**, **7**, and **9**.

Position	1			2		3		
	^{1}H[a]	J (Hz)	^{13}C[b]	^{1}H[a]	J (Hz)	^{1}H[a]	J (Hz)	^{13}C[b]
1			169.2					175.0
3	4.14 dq	9.5,6.2	78.2	4.68 dq	2.0,7.2	4.40 m		73.4
4	3.42 dd	ca. 9.5	74.3	3.70 dd	2.0,3.0	α 2.24 m		36.6
						β 1.22 m		
4a	2.74 m		37.4	3.10 ddd	3.0,7.0,17.0	2.15 m		31.9
5	α 2.0 dddd	3.0,3.0,17.0,17.0	27.6	α 2.55 dddd	2.8,2.8,17.6,17.0	1.61 m		33.0
	β 2.65 m			β 2.20 m		0.94 m		
6	6.49 ddd	9.9,10.0,3.0	140.4	6.52 m		1.72 m		23.6
						1.24 m		
7	6.06 dd	9.9,3.0	124.4	6.05 dd	2.8,9.6	1.88 m		30.9
						1.72 m		
8			170.9			3.62 m		70.2
8a			90.5			2.96 t	5.9	44.2
Me-3	1.44 d	6.2	17.8	1.33 d	6.8	1.34 d	6.2	20.7
OH-8....	12.80 s			12.97 s				
9								

[a]Assignments assisted by COSY spectra.
[b]Assignments assisted by HETCOR spectra.
[c]Double doublet $J_{3,4} \approx J_{4,4a}$.
[d]Signals too weak to discern.

to a methine at δ 3.42 (J=ca. 9.5 Hz, J=ca. 9.5 Hz). These features are consistent with the C-3/C-4 moiety of **1** in which H-3/H-4 are trans-diaxial. Full ^{13}C- and ^{1}H-nmr chemical shift assignments are provided in Table 1 and the latter were facilitated by a COSY spectrum (Figure 1) which, in conjunction with a HETCOR experiment, provided connectivity confirmation from C-3 through C-7. The presence of an enolized β-keto lactone was substantiated by the characteristic ^{1}H-nmr signal for OH-8 at δ 12.8 (s) and ir absorptions at 1642 and 1608 cm^{-1} (4). The uv spectrum showed a λ max at 298 μm (ε 12,390) consistent with the dienone system in structure **1**.

The eims of **1** displayed a prominent molecular ion at *m/z* 196 plus major ions at *m/z* 139 and 121 (base peak) corresponding to losses of C_3H_5O and $C_3H_7O_2$, respectively, resulting from cleavage at the C-2–C-3 and C-4–C-4a bonds followed by dehydration. Thus, we conclude that compound **1** is (3*R*,4*S*,4a*R*)-4,8-dihydroxy-3-methyl-3,4,4a,5-tetrahydro-1H-2-benzopyran-1-one.

The eims of **2** was very similar to that of **1** showing a prominent molecular ion at *m/z* 196 plus major fragments at *m/z* 139 ($M^+-C_3H_5O$) and 121 (base peak, $M^+-C_3H_5O-H_2O$). The ^{1}H-nmr spectra of **2** and **1** were very similar, but showed some significant differences in chemical shifts and/or coupling constants for signals assigned to H-4, H-4a, H-5α, and H-5β (see Table 1). In particular H-4, which has large couplings to H-3 and H-4a in **1** due to trans-diaxial relationships, appeared as a double doublet at δ 3.70 ($J_{3,4}$=2.0 Hz, $J_{4,4a}$=3.0 Hz) in **2**. Thus, OH-4 is α in **2**. The signal for H-5α in **2** was very similar in splitting pattern to its counterpart in compound **1**. Thus, the configuration at H-4a is common to both molecules and both displayed unusually large $J_{4a,5\alpha}$ coupling constants of 17–18 Hz. Compound **2** is thus the C-4 epimer of **1**, namely, (3*R*,4*R*,4a*R*)-4,8-dihydroxy-3-methyl-3,4,4a,5-tetrahydro-1H-2-benzopyran-1-one.

The ^{13}C-nmr and DEPT spectra of **3** indicated a total of ten carbons comprising one carbonyl, one methyl, four methylenes, and four methines, two of which (δ 70.2, 73.4 ppm) are oxygenated. Ir data supported the presence of γ-

TABLE 1. Continued

4			7			9		
^1H[a]	*J* (Hz)	^{13}C[b]	^1H[a]	*J* (Hz)	^{13}C[b]	^1H[a]	*J* (Hz)	^{13}C[b]
		170.7			[d]	4.33 m		69.4
4.14 dq	6.2,9.2	78.8	4.61 q	2.0,6.8	79.8	3.30 dq	5.6,9.7	68.7
3.16 t[c]	ca. 9.8	73.6	3.72 m		66.9	3.42 dd	4.2,9.7	74.3
2.36 m		40.0	2.72 ddd	2.8,5.8,12.0	31.9			
2.36 m		26.0	α 2.22 ddd	3.1,5.8,13.6	29.7	2.52 m		34.2
1.08 m			β 1.98 ddd	12.0,13.6				
1.97 m		20.5	3.72 m		54.9			209.5
1.60 m								
1.97 m		28.9	3.50 d	4.2	49.8	2.32 dd[a]	6.0,17.6	
2.20 m						2.92 dq[b]	1.6,17.6	40.3
		176.2			[d]	2.45 dd[a]	4.6,17.8	
						2.73 dq[b]	2.2,17.8	47.0
		94.4			90.2			
1.43 d	6.2	18.4	1.34 d	6.8		1.21 d	5.6	18.8
13.62 s			13.19 s					
						1.94 ddd[a]	1.6,3.6,13.7	31.6
						2.09 m[b]		

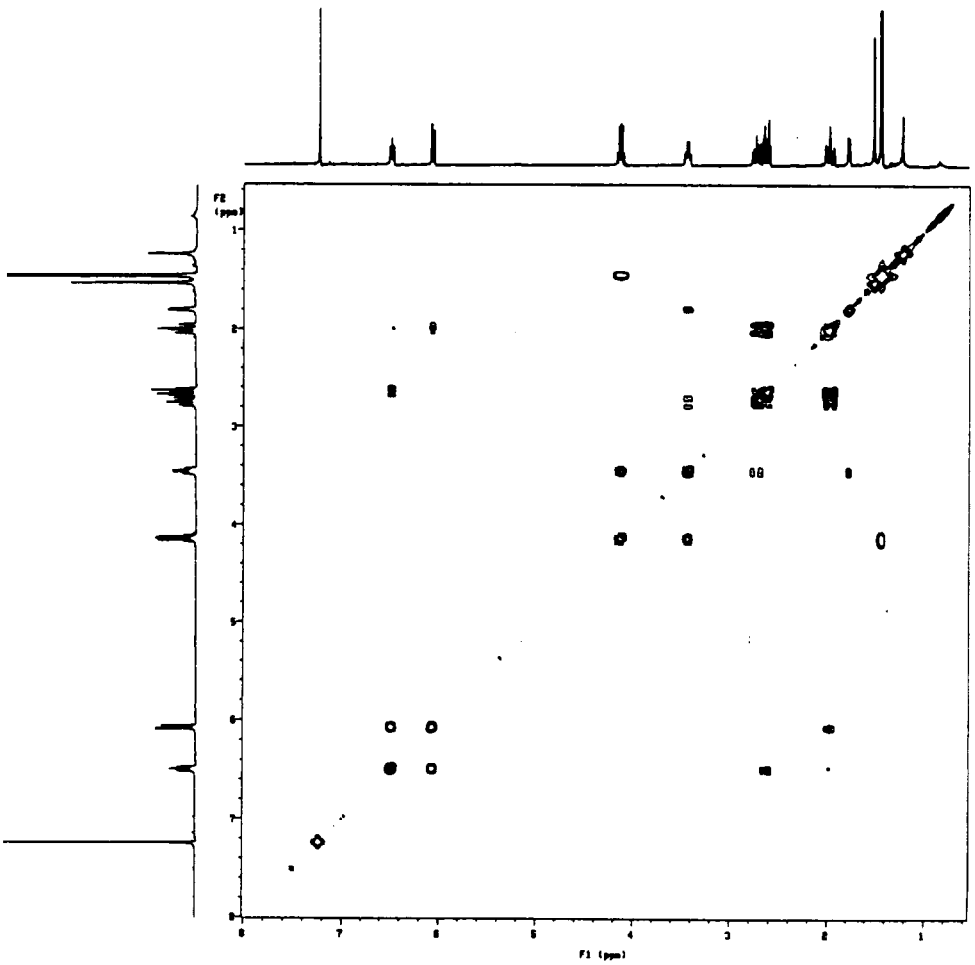


FIGURE 1. COSY 90 spectrum of **1**.

lactone (1735 cm^{-1}) and hydroxyl (3504 cm^{-1}, br) functions. From the 400 MHz ^{1}H-nmr, COSY, and HETCOR spectra, it was possible to assign completely the ^{1}H- and ^{13}C-nmr chemical shifts (see Table 1) in terms of structure **3**. In particular, the dispersion of signals and cross-peaks in the COSY spectrum (Figure 2) afforded unambiguous connectivity information from H-3 through H-8. The stereochemistry was implied from the ^{1}H-nmr signal for H-8a, a double doublet which appeared as a broadened triplet due to couplings of the same order (ca. 6 Hz) with H-8 and H-4a indicating a cis ring junction and an α OH at C-8. The stereochemistry was confirmed by nOe difference spectroscopy which showed significant enhancements between H-3/H-4β (4.1%), H-4β/H-4a (20.6%), H-4a/H-8a (4.2%), H-8a/H-7 (3.5%), and H-8a/H-3 (8.7%).

The eims of **3** gave no molecular ion but a base peak at *m/z* 113 corresponding to $M^+ - C_4H_7O$, which is readily explained via the mechanism depicted in Scheme 1. Thus, we have assigned the structure (3*R*,4a*S*,8*S*,8a*R*)-8-hydroxy-3-methyl-3,4,4a,5,6,7,8,8a-octahydro-1H-2-benzopyran-1-one to compound **3**.

The hreims of **4** showed a molecular ion consistent with the elemental composition $C_{10}H_{14}O_4$ and major ions at *m/z* 141 ($M^+ - C_3H_5O$) and *m/z* 123 ($141 - H_2O$), indicating a ms fragmentation pattern parallel with those of **1** and

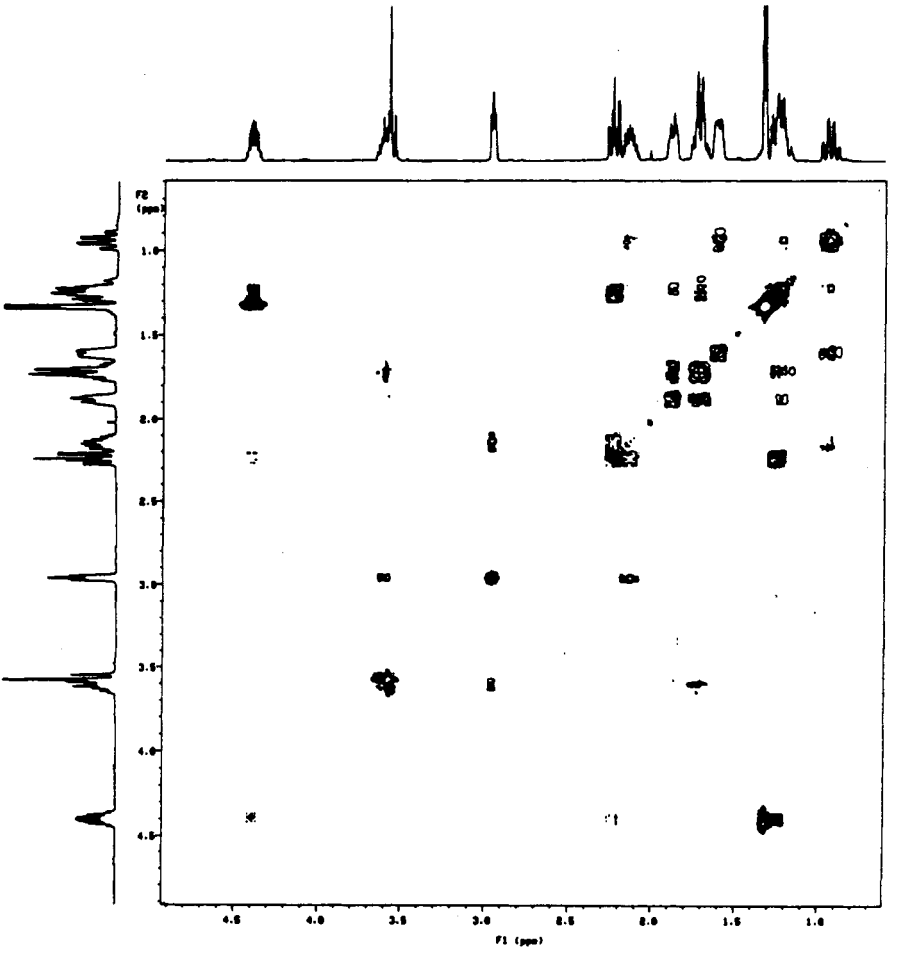
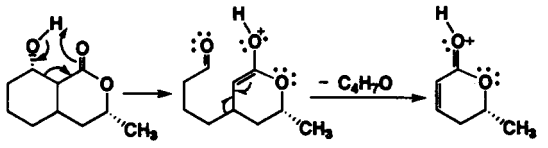


FIGURE 2. COSY 90 spectrum of **3**.



3

SCHEME 1. Mass spectral fragmentation of **3**.

2. Analysis of the ^{1}H, COSY, ^{13}C, and HETCOR nmr data (see Table 1) showed **4** to be a 4-hydroxyramulosin derivative. From the coupling constants, $J_{3,4}$=9.2 Hz, $J_{4,4a}$=ca. 9.8 Hz, it was evident that the stereochemistry at C-3, C-4, and C-4a is the same as that in **1**. Thus we have formulated **4** as (3*R*,4*S*,6*R*)-3,4,4a,5,6,7-hexahydro-4,8-dihydroxy-3-methyl-1H-2-benzopyran-1-one.

The composition of **7** ($C_{10}H_{12}O_5$) was established by hreims which, in addition to a strong molecular ion, displayed major fragments at *m/z* 194 (M^+-H_2O), 155 ($M^+-C_3H_5O$), 137 (*m/z* 155$-H_2O$), and 121 (*m/z* 137$-O$), consistent with its formulation. The location of the epoxide at C-6/C-7 was indicated by the ^{1}H-nmr signal for H-7 (δ 3.50, d, J=4.2 Hz) which showed coupling only to H-6. The β-orientation of the epoxide was indicated by the apparent minimal coupling of H-6 with H-5β. Inspection of Dreiding models showed that for the α-epoxide, the dihedral angle between H-6 and H-5β would be ca. 0° and, consequently, a large coupling would be expected. The stereochemistry at C-3, C-4, and C-4a is the same as that in **2** in view of the small couplings ($J_{3,4}$=2.0 Hz and $J_{4,4a}$=ca. 3 Hz). Complete ^{1}H- and ^{13}C-nmr chemical shift assignments for **7** are listed in Table 1.

The elemental composition ($C_9H_{14}O_3$) of optically active **9** was revealed by hreims which also displayed a prominent ion at *m/z* 113 ($M^+-C_3H_5O$), indicative of the presence of a β-hydroxy-α-methyl ether moiety as in **1**, **2**, **4**, and **7**. The ^{13}C-nmr spectrum and DEPT analysis indicated the presence of one sp^2 carbon plus one methyl, three methylenes, and three oxygenated methines. The ir spectrum showed hydroxyl (3420 cm^{-1}) and ketonic (1698 cm^{-1}) absorptions indicative of intramolecular hydrogen bonding of a cyclohexanone. A COSY spectrum, together with a ^{1}H-^{1}H decoupling experiment, afforded complete connectivity information for the protonated carbons as in structure **9** which, together with HETCOR and ^{1}H-nmr spectra, permitted complete chemical shift assignments (see Table 1). The relative stereochemistry of **9** was deduced from coupling constant data, in particular $J_{3,4}$=9.7 Hz and $J_{4,5}$=4.2 Hz, which were consistent only with trans-diaxial protons at C-3/C-4. In addition, nOe data (Figure 3) fully supported this stereochemistry and indicated a boat conformation for the cyclohexanone ring in view of the significant nOe between H-3 and H-8b and the lack thereof between H-3 and H-7b.

Fragmented pentaketides containing fewer than ten carbons are well-known and their biogenesis has been studied in some cases. For example, terrein, from *Aspergillus terreus*, has been shown to be derived in part by loss of the carboxyl

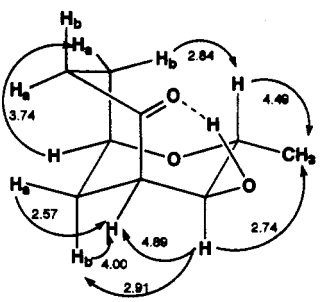
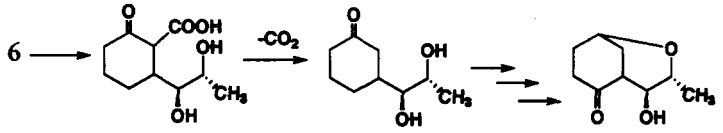


FIGURE 3. Percent nOe enhancements by difference nmr spectroscopy for **9**.

carbon from a 3,4-dihydroxy-3-methylisocoumarin precursor (5). This suggests that **9** might arise via decarboxylation of a hydroxylated β-keto acid derived from a precursor like **6** followed by ether formation/oxygen function modification as outlined in Scheme 2. Thus, we have formulated compound **1** as (1*S*,3*R*,4*S*,5*S*)-4-hydroxy-3-methyl-2-oxobicyclo[3.3.1]non-6-one, assuming that C-3 relates in absolute configuration to C-3 of (−)-6-hydroxymellein, a co-metabolite in the endophyte strains producing **9**.



9

SCHEME 2. Outline of possible biogenetic pathway for **9**.

Both **1** and **3** showed significant toxicity toward spruce budworm larvae in a feeding assay in which test compounds were incorporated into an artificial diet and fed to second-instar larvae whose development/survival was monitored over 14 days (see Table 2). Compounds **4**, **6**, and **8** were inactive at comparable concentrations and compounds **2**, **7**, and **9** were not assayed because of insufficient material. Compound **1** also showed toxicity toward spruce budworm cells comparable to that of vomitoxin (6), while **3**, **4**, **8**, and **9** were inactive at comparable concentrations. The toxicity to budworm larvae appears to be substantially lower than that of azadirachtin, in view of the studies of Thomas *et al.* (7) who determined an LD_{50} value of 0.9–0.105 micrograms for ingestion of azadirachtin by 6th-instar spruce budworm larvae.

To date, most naturally occurring 8-hydroxy-3-methyl-3,4-dihydroisocoumarins isolated from fungi are aromatic mellein derivatives, while relatively few ramulosin-related compounds are known. Many of these metabolites have been reported to exhibit a variety of biological activities (8,9) including phytotoxicity (3,9) but we are not aware of any previous reports of toxicity to insects.

The endophyte *Canoplea elegantula* responsible for producing the aforementioned metabolites was first described from conifer needles collected in the United States (10). It was also reported from needles of balsam fir (*Abies balsamea*) near Quebec City, Canada (11).

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—High-resolution mass spectra were recorded on a Kratos MS-50 instrument. Optical rotations were

TABLE 2. Effects of **1** and **3** on Spruce Budworm Larvae in Feeding Assay.[a]

Diet treatment (quantity)	Surviving larvae in instar					Survival % (total insects)
	2	3	4	5	6	
Control			21			84 (25)
Crude extract (19 mg)	1	4	8	2		60 (25)
1 (5.6 mg)	1	4	9	2		64 (25)
3 (13.7 mg)		1	14	2		68 (25)

[a]For details of larval assay, see Calhoun *et al.* (1).
[b]Quantity of additive incorporated into diet and offered to total number of insects (25).

measured on a Perkin-Elmer 241 polarimeter. Mps were taken with a Kofler hot-stage apparatus and are uncorrected. The ir spectra were recorded as films on a Bruker IF-S25 spectrometer. Prep. tlc was performed with precoated Si gel F_{254} (1-mm) plates. Nmr data were recorded in $CDCl_3$ on a Varian Unity 400 spectrometer, using the solvent as reference.

FUNGAL STRAINS.—The fungal strains 6BS10K1 and 7BS37C1 were isolated from black spruce (*Picea mariana* BSP) needles obtained from trees in Parc Ashuapmushuan and Parc des Laurentides, Quebec, Canada in August 1991. The endophytes were cultured as described previously (5). After filtration, the mycelia were freeze-dried and the media frozen and kept in cold storage until used.

EXTRACTION AND ISOLATION.—Endophyte strains 7BS37C1 and 6BS10K1 were each fermented on a ca. 3-liter scale using 250-ml Erlenmeyer flasks according to our established protocols. The culture filtrates were each extracted with EtOAc (ca. 3 liters) by stirring for 4 h. The extracts were concentrated *in vacuo* (<30°) and examined by analytical tlc and ^{1}H-nmr spectroscopy which showed the extracts to be virtually identical in composition, and they were combined to afford 775 mg of a crude extract. Fractionation of 750 mg of this extract by flash chromatography on Si gel (Merck 60 F_{254}, 230–400 mesh), eluting first with *n*-C_6H_{12}-$CH_3COOC_2H_5$-CH_3OH (65:34:1) (2.5 liters) then (55:44:1) (1.5 liters), to provide **8** (4.7 mg), **4** (245.6 mg), **1** (23 mg), **3** (8.4 mg), **6** (15.6 mg), and **9** (16.4 mg). Further purification was carried out in some cases by prep. tlc on Si gel, using $CHCl_3$-*n*-C_6H_{12}-CH_3OH (15:5:1) for **1**, **3**, and **9**, and $CHCl_3$-C_6H_{12}-CH_3OH (7:2:1) for **6**. Further quantities of **4**, **6**, **7**, **8** and **9** plus **2** (5.4 mg) and **7** (2.5 mg) were obtained by parallel chromatographic separation of combined extracts (723 mg) from several small-scale (ca. 750 ml) fermentations of isolates from black spruce needles, which by tlc and ^{1}H- nmr comparison showed a common metabolite profile. Thus, **2** and **7** were purified from Si gel flash chromatographic fractions by prep. tlc with *n*-C_6H_{12}-$CH_3COOC_2H_5$-CH_3OH.

(3R,4S,4aR)-4,8-Dihydroxy-3-methyl-3,4,4a,5-tetrahydro-1H-2-benzopyran-1-one [**1**].—White flakes; mp 120–123°; $[\alpha]^{20}D$ +164.0° (c=0.014, $CHCl_3$); ir ν max 3416, 1642, 1680 cm^{-1}; uv (EtOH) λ max 295 (ϵ 12,400) nm; eims (70 eV) *m/z* $[M]^+$ 196 (23), 139 (52), 121 (100).

(3R,4R,4aR)-4,8-Dihydroxy-3-methyl-3,4,4a,5-tetrahydro-1H-2-benzopyran-1-one [**2**].—Amorphous; eims (70 eV) *m/z* $[M]^+$ 196 (20), 139 (49), 121 (100).

(3R,4aS,8S,8aR)-8-Hydroxy-3-methyl-3,4,4a,5,6,7,8,8a-octahydro-1H-2-benzopyran-1-one [**3**].—Yellow oil; $[\alpha^{20}D$ −244° (c=0.007, $CHCl_3$); ir ν max 3504, 1735 cm^{-1}; eims (70 eV) *m/z* $[M+H]^+$ 185 (1), 166 (1), 156 (29), 113 (100).

(3R,4S,6R)-3,4,4a,5,6,7-Hexahydro-4,8-dihydroxy-3-methyl-1H-2-benzopyran-1-one [**4**].—White needles; mp 140–143°; $[\alpha]^{20}D$ +16.7° (c=0.001, $CHCl_3$); ir (dry film) ν max 3344, 1631, 1458 cm^{-1}; uv (EtOH) λ max 269 (ϵ 22416) nm; hreims (70 eV) *m/z* $[M]^+$ 198.0871 (calcd for $C_{10}H_{14}O_4$, 198.0892) (24), 165 (3), 141 (44), 123 (100).

(3R,6R)-3,4,4a,5,6,7-Hexahydro-6,8-dihydroxy-3-methyl-1H-2-benzopyran-1-one [**6**].—Clear needles; mp 130–134° (lit. 132–133°); $[\alpha]^{20}D$ +40.8° (c=0.0009, $CHCl_3$) (lit. +91.6°, c=1, MeOH) (2); ir (film) ν max 3444, 1637, 1603 cm^{-1}; uv (EtOH) λ max 265 (ϵ14722) nm; hreims (70 eV) *m/z* $[M]^+$ 198.0883 (45) (calcd for $C_{10}H_{14}O_4$ 198.0892), 180 (100), 162 (34), 139 (36), 126 (20).

(3R,4R,4aR,6R)-4,8-Dihydroxy-6,7-epoxy-3,4,4a,5,6,7-hexahydro-1H-2-benzopyran-1-one [**7**].—Colorless oil; $[\alpha]D$ +47.3° (c=0.0003, $CHCl_3$); hreims (70 eV) *m/z* 212.0676 $[M]^+$ (38) (calcd for $C_{10}H_{12}O_5$, 212.0685), 195 (10), 177 (17), 155 (23), 149 (76), 143 (24), 137 (100).

(3R,4S)-3,4-Dihydro-4,8-dihydroxy-3-methyl-1H-2-benzopyran-1-one [**8**].—White needles; mp 129–132° [lit. (3) 131–132°]; $[\alpha]D$ −11° (c=0.0038, $CHCl_3$) (lit. −29°, MeOH) (3); hreims (70 eV) *m/z* 194.0583 $[M]^+$ (60) (calcd for $C_{10}H_{10}O_4$ 194.0579), 150 (91), 121 (100).

4-Hydroxy-3-methyl-2-oxabicyclo[3.3.1]non-6-one [**9**].—White crystals; mp 91–92°; $[\alpha]D$ +55.3° (c=0.0163, $CHCl_3$); ir (film) ν max 3420, 1698 cm^{-1}; hreims *m/z* 170.0938 $[M]^+$ (47) (calcd for $C_9H_{14}O_3$ 170.0944), 152 (6), 126 (33), 97 (47).

ACKNOWLEDGMENTS

Thanks are due to the National 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 Tammy Mercer, Dan Drummond, and Martin Frenette for assistance with bioassays, mass spectra, and preliminary isolation, respectively.

LITERATURE CITED

1. L.A. Calhoun, J.A. Findlay, J.D. Miller, and N.J. Whitney, *Mycol. Res.*, **96**, 281 (1992).
2. S.W. Tanenbaum, S.C. Agarwal, T. Williams, and R.G. Pitcher, *Tetrahedron Lett.*, 2377 (1970).
3. M. Devys, M. Barbier, J.-F. Bousquet, and A. Kollmann, *Z. Naturforsch.*, **47c**, 779 (1992).

4. J.A. Findlay, J.M. Matsoukas, and J. Krepinsky, *Can. J. Chem.*, **54**, 3419 (1976).
5. R.A. Hill, R.H. Carter, and J. Staunton, *Chem. Commun.*, 380 (1975).
6. C.L. Clark, J.D. Miller, and N.J. Whitney, *Mycol. Res.*, **93**, 508 (1989).
7. A.W. Thomas, G.M. Strunz, M. Chiasson, and T.H. Chan, *Entomol. Exp. Appl.*, **62**, 37 (1992).
8. W.B. Turner and D.C. Aldridge, "Fungal Metabolites II." Academic Press, London, 1983, pp. 82–109.
9. R.F. Struck, M.C. Thorpe, W.C. Coburn, Jr., and Y.F. Shealy, *Tetrahedron Lett.*, 1589 (1967).
10. M.B. Ellis, "Dematiaceous Hyphomycetes." Commonwealth Agricultural Bureau, Kew, Richmond, Surrey, UK, 1971.
11. L.E. Petrini, O. Petrini, and G. Laflamme, *Phytoprotection*, **70**, 97 (1989).

Received 7 April 1995