Mycopathologia **127**: 19–27, 1994.

Secondary metabolites of *Penicillium bilaii* strain PB-50

M.E. Savard,[1] J.D. Miller,[1] L.A. Blais,[1] K.A. Seifert[2] & R.A. Samson[3]
[1]*Plant Research Centre, and* [2] *Centre for Land and Biological Resources Research, Agriculture Canada, Ottawa, Ontario, Canada;* [3]*Centraalbureau voor Schimmelcultures, Baarn, The Netherlands*

Received 9 September 1992; accepted in revised form 7 April 1994

Abstract. A phosphate-solubilizing strain of *Penicillium bilaii* was tested for the production of gliotoxin and other toxic compounds. The strain was fermented under five different conditions to allow the expression of various metabolites, including gliotoxin. These included Czapek-yeast extract medium under both shaken and still conditions as well as Czapek-yeast extract/malt extract/peptone medium and sucrose/glycerol medium in shake flasks. In addition, culture filtrate from an industrial fermentation of the fungus was examined. No gliotoxin was produced in any of the media. No other expected *P. bilaii* metabolites were found. Three compounds were identified in all samples: dibutyl phthalate, 1-(4-hydroxy-phenyl)ethanone and 4-hydroxy-3,6-dimethyl-2H-pyran-2-one. The production of other metabolites was dependent on the culture conditions. Two hyalodendrin derivatives were found in some fermentations and two related compounds were tentatively identified. None of the compounds found have been reported as toxic. The identity of the culture was confirmed by comparison with the ex-type culture of *P. bilaii*.

Key words: Gliotoxin, Hyalodendrin, *Penicillium bilaii*, PB-50

Introduction

The large scale application of living fungi to soil, for example, as mycoherbicides, or as biological controls of insects or plant pathogens, has generated concerns about the safety of such practices. Many fungi produce potent toxins that can affect worker safety, the environment and food quality [1]. A strain of *Penicillium bilaii* (ATCC 22348), isolated from Alberta soil, has been shown to solubilize phosphate and a number of inorganic micronutrients [2]. The fungus can be used as an inoculant to increase the health, growth rates and yields of a number of crop plants [3–5]. This has enabled the commercialization of *P. bilaii* (ATCC 22348) as a microbial inoculant (Canadian patents 1,306,566 and 1,308,270; US patent 5,026,417) under the tradenames 'PB-50' and 'PROVIDE' by Philom Bios Inc., Saskatoon, Saskatchewan. PB-50 is the first non-rhizobial inoculant to receive registration for commercial use as a supplement under the Canadian Fertilizers Act (Reg. Nos. 900025A, 920064A).

Penicillium species are known to produce a number of mycotoxins, including the agriculturally-important toxins ochratoxin A, citrinin and roquefortine. Numerous other toxic metabolites are produced and some have been used as taxonomic aids [6, 7]. In the case of *Penicillium bilaii*, little information on its metabolites was available. However, some strains identified as *P. bilaii* had been reported to produce gliotoxin (Frisvad, unpublished data).

Gliotoxin is an antibiotic produced by several species of *Penicillium* and *Aspergillus fumigatus* [8]. In the case of the latter species, the production of gliotoxin is considered to be involved in the pathology of aspergillosis. Gliotoxin is potently immunosuppressive [9].

The US Environmental Protection Agency has released draft guidelines for regulation of microbial productions of biotechnology under the Toxic Substances Control Act. In the case of filamentous fungi, the guidelines require high standards of taxonomic certainty and certification that a strain does not produce toxins (40 CFR parts 700, 720, 721, 723 and 725). This work attempts to provide an approach to meeting these requirements by determining the structures of metabolites produced in fermentations of the commercial PB-50 strain (i.e., *P. bilaii* ATCC 22348) and whether it could produce gliotoxin. To achieve this goal, the strain was grown under culture conditions providing a variety of different nutrient and oxygen tensions, including those conditions reported to facilitate the production of gliotoxin. In principle,

such an approach allows a more complete expression of the metabolites of a given strain [7]. The identification of the strain PB50 was confirmed by comparison with the ex-type culture of *P. bilaii* (CBS 221.66), isolated from soil in the former USSR.

Materials and methods

Microorganism. The commercially used PB-50 culture was obtained from Dr. Dan Polonenko, Philom Bios Ltd and grown on 2% Malt Extract Agar (MEA) slants for 7–10 days at 25 °C. The taxonomic characters of the strain were studied using the methods of Pitt [10]. Cultures were grown for 7 days on MEA, Czapek Yeast Agar (CYA) and 25% Glycerol Nitrate Agar (G25N) at 25 °C with 12 hours light, 12 hours dark, and on CYA at 5 and 31 °C in darkness; the CYA was emended with 1 ml/l of a trace element solution consisting of $CuSO_4.5H_2O$ (0.5 g) and $ZnSO_4.7H_2O$ (1.0 g) in 100 ml distilled water [11].

Fermentation. Inoculum for fermentation studies was prepared by macerating the culture in 50 ml sterile distilled water. Aliquots (2.5 ml) of the resulting suspension were added to 250 ml Erlenmeyer flasks containing 50 ml of inoculation medium (CZ-met) made up of ultrapure water (1 liter), NH_4Cl (3 g), $FeSO_4.7H_2O$ (0.2 g), $MgSO_4.7H_2O$ (2 g), KH_2PO_4 (2 g), peptone (2 g), yeast extract (2 g), malt extract (2 g; all Difco), and glucose (20 g). After 48 h of incubation in the dark at 28 °C on a rotary shaker (220 rpm, 3.81 cm throw), the suspension was macerated and employed in production fermentations as follows:

(1) An aliquot (5% v/v) was dispensed into each of 14 250-ml-Erlenmeyer flasks each containing 50 ml of Czapek medium plus 10 g/l yeast extract (CZ-YE) [12]. These were placed on the shaker as above for 10 days.

(2) Glaxo bottles containing 1 liter of CZ-YE were inoculated as above and incubated for 14 days at 28 °C.

(3) Ten 250-ml-Erlenmeyer flasks containing 50 ml CZ-met medium were inoculated and incubated on the shaker as above for 10 days.

(4) Eight 250-ml-Erlenmeyer flasks containing 50 ml of ultrapure water (1 liter), $(NH_4)_2HPO_4$ (1 g), KH_2PO_4 (3 g), $MgSO_4.7H_2O$ (0.2 g), NaCl (5 g), sucrose (40 g), and glycerol (10 g). These were inoculated and incubated as above for 10 days.

In addition, a culture filtrate (2l) from a commercial PB-50 spore-production fermentation run was provided by Philom Bios Ltd.

Extraction. All culture filtrates were treated in the same way: extraction with 2 × 1/3 vol. of chloroform, drying of the extract over anhydrous sodium sulfate and concentration of the extract. A reference to the extraction of gliotoxin used 2 × 10% vol. of $CHCl_3$ [13]. Yields of crude extract:

CZ-YE, shaken:	21.3 mg	(30.4 mg/l)	(A)
CZ-YE, still:	238.9 mg	(39.8 mg/l)	(B)
CZ-Met, shaken:	11.8 mg	(23.6 mg/l)	(C)
suc/gly, shaken:	7.6 mg	(19.0 mg/l)	(D)
Philom Bios:	485.0 mg	(242.5 mg/l)	(E)

Instruments. ^1H and ^{13}C NMR spectra were obtained with a Bruker AM500 spectrometer. Chemical shifts were referenced to residual $CHCl_3$ at 7.24 ppm and $CDCl_3$ at 77.0 ppm for ^1H and ^{13}C spectra, respectively, and reported relative to TMS. Mass spectra were obtained on a Finnigan MAT 4500 GC/MS system operating in the electron impact mode. DB-5 fused silica columns [15–20 m × 0.32 mm (i.d.), 0.25 μm film] were used with He as a carrier gas at 10 psi. The GC was programmed from 120 to 280 °C at 15 °C/min.

Isolation and characterization. Each crude extract was first analysed by GC/MS. A standard of gliotoxin (provided by A. Taylor of the National Research Council of Canada) was also analysed in this way. As little as 20 ng gliotoxin/μl of solution could be easily detected by GC/MS, corresponding to 50 ppb for a 400 ml culture filtrate typically concentrated to 1 ml for GC/MS analysis. No gliotoxin was ever found in the culture filtrates. GC/MS: m/z 244(22), 242 (100), 226(20), 214(85), 213(55).

The extracts were chromatographed by HPLC (Varian Vista 5500) on a CSC 10 μm 300 × 10 mm CN column with isopropanol/hexane (5/95, v/v) and the effluents monitored at 220 nm. Gravity column chromatography on silica gel was unsatisfactory because of very low recoveries of material.

The compounds isolated were analysed by NMR and GC/MS as above. For other compounds, not obtained in sufficient quantities for NMR analysis,

only the mass spectra from the GC/MS analysis of the crude extracts are reported. The mass spectral and NMR data obtained for the secondary metabolites isolated follow:

2,4-Dihydroxy-3,6-dimethylbenzaldehyde, **2:** GC/MS: m/z 166(M+)(59), 165(100), 137(23), 120(29), 109(22), 91(42), 83(42), 79(33), 77(32), 69(21), 65(33), 63(28), 55(63), 53(47), 51(54), 43(42), 41(59), 38(43).^{1}H NMR (δ, ppm)(CD_2Cl_2): 12.67 (s, 4-0H (phenolic)), 10.08 (s, HC=0, aldehyde), 6.22 (s, H-5), 2.49 (s, 3-CH_3), 2.05 (s, 6-CH_3). ^{13}C NMR (δ, ppm) ($CDCl_3$): 193.00 (C=0, aldehyde), 164.12 (C-4), 161.06 (C-2), 141.35 (C-6), 113.28 (C-1), 109.92 (C-5), 108.86 (C-3), 17.93 (6-CH_3), 6.77 (3-CH_3).

Dibutyl phthalate, **3:** GC/MS: m/z 278(M^+), 223, 205, 149(100), 119. ^{1}H NMR (δ, ppm): 7.7, 7.5 (mAB, H-3 and H-6, H-4 and H-5), 4.3 (t, 8-CH_2), 1.8 (m, 9-CH_2), 1.4 (m, 10-CH_2), 0.9 (t, 11-CH_3).

Bisdethiodi(methylthio)hyalodendrin, **4:** GC/MS: m/z 323(1) (M-$CH_2$0H)$^+$, 307(95) (M-SCH_3)$^+$, 276(5) (M-SCH_3-$CH_2$0H)$^+$, 260(40) (M-2SCH_3)$^+$, 243(8), 231(85), 215(40), 188(15), 173(11), 155(13), 132(45), 116(12), 91(100), 72(16). ^{1}H NMR (CD_2Cl_2): 7.25 (d, H-12 and H-16, J = 5.2 Hz; H-14, d, J = 2.0 Hz); 7.09 (d of d, H-13 and H-15, J = 7.1, 2.0 Hz); 3.74 (d, AB, H-7, J = 11.8 Hz); 3.66 (d, AB, H-10, J = 13.9 Hz); 3.25 (s, 9-NCH_3); 3.13 (d, AB, H-10, J = 13.9 Hz); 3.02 (d, AB, H-7, J = 11.8 Hz); 2.98 (s, 8-NCH_3); 2.26 (s, 18-SCH_3); 2.09 (s, 17-SCH_3). ^{13}C NMR (CD_2Cl_2): 166.0 (C-5), 165.6 (C-2), 134.9 (C-11), 130.4 (C-12 and C-16), 129.0 (C-13 and C-15), 128.1 (C-14), 64.8 (C-7), 42.8 (C-10), 31.2 (C-9), 29.6(C-8), 14.5 (C-18), 13.5 (C-17)

Bisdethiodehydroxymethyldi(methylthio)hyalodendrin, **5:** GC/MS: m/z 277(67)(M-SCH_3)$^+$, 249(5)(M-SCH_3-CO)$^+$, 230(22)(M-2SCH3)$^+$, 202(54)(M-2SCH_3-CO)$^+$, 175(8), 158(13), 132(21), 125(15), 117(20), 91(100).

1-(4-Hydroxyphenyl)ethanone, **6:** GC/MS: m/z 136(31)(M^+), 121(100), 93(33), 65(32), 63(12), 43(33), 39(51) ^{1}H NMR ($CDCl_3$): 7.9 (d, H-2 and H-6); 6.8 (d, H-3 and H-5); 2.18 (s, 8-CH_3).

4-Hydroxy-3,6-dimethyl-2H-pyran-2-one, **7:** GC/MS: m/z 140(M^+)(42), 112(29), 111(35), 97(18), 85(61), 69(50), 56(38), 43(100), 40(22), 38(14). ^{1}H NMR ($CDCl_3$): 5.8 (s, H-5), 2.53 (s, 3-CH_3), 1.92 (s, 6-CH_3).

8: GC/MS: m/z 266(M^+)(42), 248(22), 237(7), 221(22), 219(18), 191(85), 189(43), 175(68), 165(27), 147(53), 115(61), 102(31), 92(73), 91(100), 77(56). High Resolution MS: calc. for $C_{12}H_{14}N_2O_3S$: 266.0725, calc. for $C_{12}H_{14}N_2O_5$: 266.0902, found: 266.0950. ^{1}H NMR (CD_2Cl_2):7.2–7.5 (m); 4.10 (d, 1H, J = 15.2 Hz); 3.81(d, 1H, J = 15.2 Hz); 3.5–3.7 (m). ^{1}H NMR ($CDCl_3$): 7.2–7.5 (m), 3.73 (t, 2H, J = 4.5 Hz), 3.67 (s, 2H), 3.61 (t, 2H, J = 4.5 Hz).

9: GC/MS: m/z 265(6), 264(M^+)(43), 246(4), 237 (5), 236(20), 219(3), 208(16), 207(10), 191(28), 160(18), 79(39), 77(100). High Resolution MS: calc. for $C_{12}H_{12}N_2O_3S$: 264.0568, calc. for $C_{12}H_{12}N_2O_5$: 264.0746, found: 264.0725. ^{1}H NMR (CD_2Cl_2): 7.34–7.52, 6.08 (s); 5.94(s); 3.73 (d of d); 3.67 (s); 3.61 (d of d). ^{13}C NMR ($CDCl_3$): 129.7, 129.2, 129.0, 128.7, 128.6, 126.0, 96.0, 77.9, 72.4, 70.4, 61.7.

10: GC/MS: m/z 173(100), 111(21), 99(44), 86(15), 83(48), 70(17), 56(22), 55 (100), 45(45), 42(36), 41(30), 39(18). ^{1}H NMR ($CDCl_3$):5.80 (s); 2.19 (s); 1.93 (s); 1.24 (bs).

11: GC/MS: m/z 182(4), 181(3), 140(100), 139(40), 121(12), 111(10), 94(10), 83(7), 77(3), 65(12), 54(20), 43(67).

12: GC/MS: m/z 209(63), 208(100), 180(18), 163(12), 136(70), 108(30), 96(12), 69(50).

Description of organism

Penicillium bilaii Khalabuda, Not. Syst. Crypt. Inst. Bot. Acad. Sci. URSS 6: 165. 1950 (Fig. 4).

Colonies on CYA at 25 °C 24–30 mm diam after 7 days, wrinkled with both radial and concentric folds, velutinous with floccose patches, conidia abundantly produced over entire colony except marginal 1-2 mm, Greyish Turquoise (24E5[1]) in mass; aerial mycelium white, villose, mostly visible at margin; exudate clear, in small to medium size droplets, superficial or somewhat embedded; soluble pigment yellow, colouring

[1] Colours in capital letters and corresponding codes refer to Kornerup & Wanscher [14].

most of agar; reverse orange (5B8). Colonies on MEA at 25 °C 24–28 mm diam, plane, of medium density, velutinous to somewhat lanose, conidial production moderate to dense over most of colony, Blueish Grey (23BC2-4) to Dark Turquoise (24F5) in mass; aerial mycelium sparse to abundant, white; exudate not produced; soluble pigment yellow; reverse Yellow (3A6) to Greyish Yellow (2B6). Colonies on G25N at 25 °C 12-14 mm diam, wrinkled, dense, velutinous, conidia abundantly produced over entire colony except about 1 mm at the margin, Dull Green in mass (28E4 in centre, 25D3 at margin) tending to be slightly blueish near the margin; aerial mycelium sparse, white, mostly visible at the margins; exudate absent; soluble pigment not visible; reverse white with red spots. Colonies on CYA at 5 °C sterile, 4–5 mm diam, convex, with white, lanose aerial mycelium; exudate absent; soluble pigment visible in agar as very faint yellow zone; reverse light yellow. Colonies on CYA at 37 °C sterile, restricted, 5–12 mm diam, wrinkled, with white, pubescent to felty aerial mycelium; exudate absent; soluble pigment visible in the agar as a faint yellow zone surrounded by a faint brown halo; reverse light brown, heavily wrinkled.

Conidiophores on MEA monoverticillate (Figs. 4a, c), arising from agar surface and from aerial mycelium. Stipes 30–70 μm long, 2–2.5 μm wide, smooth walled, mostly vesiculate, with the vesicle globose to ellipsoidal, 3–5 μm wide. Phialides in whorls of 5–12, 7–9 μm long, ampulliform, 1.5–2.5 μm wide at the base, 2.5-3 μm wide at the broadest part, and about 1 μm wide at the conidiogenous aperture, periclinal thickening not seen. Conidia (Figs. 4b, d) 2.5-3(-4) μm diam, globose, with minutely roughened walls, forming columns.

The strain PB-50 and the ex-type culture of *P. bilaii* (CBS 221.66) showed identical colony morphologies and microscopic characters (Fig. 4).

Results and discussion

Each crude extract was analyzed first by GC/MS. The fermentations showed four or five major compounds in slightly different ratios (see Fig. 1). The GC analysis of the extract of the Philom Bios production medium culture filtrate contained large concentrations of isopropanol oligomers. These probably came from anti-foaming or emulsifying agents. The large concentration of these compounds explains the very high yield of crude extract obtained for this culture.

The two CZ-YE cultures, A and B, yielded the same metabolites, but in different proportions, as determined by GC/MS. The four strongest peaks were due to 2,4-dihydroxy-3,6-dimethyl-benzaldehyde, **2**, dibutyl phthalate, **3**, a common plasticizer and preservative, bisdethiodi(methylthio)hyalodendrin, **4**, an epidithiodiketo-piperazine fungal metabolite first isolated from *Hyalodendron* sp. [15], and what is thought to be an artifactual decomposition product of **4**, the deshydroxymethyl analog **5**.

The structure of compound **2** was suggested by a mass spectral library search and confirmed by NMR spectra and comparison with literature data [16]. Search of a mass spectral library also yielded the structure **3**. The presence of a strong ion at 149 a.m.u. is typical of phthalates and NMR confirmed the presence of butyl esters. This common plasticizer may have been incorporated during processing.

The EI mass spectrum of **4** did not show a molecular ion at m/z 354; however, a weak fragment ion corresponding to loss of a primary alcohol group occurred at m/z 323, and a strong ion at m/z 307 indicated the loss of an S-CH3 group. Confirmation of this structure was obtained by comparison of MS and ^{1}H NMR data with that published by Strunz et al. [15], and by assignment of the ^{13}C NMR spectrum. This compound has no antifungal activity [17].

Although **5** appeared to be a major component of the CZ-YE culture extracts, it was impossible to isolate. Chromatographical separation of the components of the extracts always yielded what appeared to be a mixture of **4** and **5** by GC/MS. However, when an NMR spectrum of this fraction was obtained, it showed only one compound, the known **4**. The broadness of the GC/MS peak for **5** is another indication that this compound is produced by decomposition of **4** on the GC column. As for the structure of **5**, its fragmentation pattern was very similar to that of **4** with all the major peaks shifted by 30 a.m.u., suggesting the loss of the 7-CH_2O unit. The lack of a 72 a.m.u. fragment as for the $N(CH_3)CCH_2OH$ fragment of **4** supports this theory [15]. A molecular weight of 324 a.m.u. for **5** (30 less than **4**) is the same as for hyalodendrin; however, comparison of the mass spectra of **5** and hyalodendrin [18] showed them to be significantly different.

The GC trace of the extract from cultures grown in CZ-Met medium (C, in Fig. 1) showed five major peaks, with the largest peak iden-

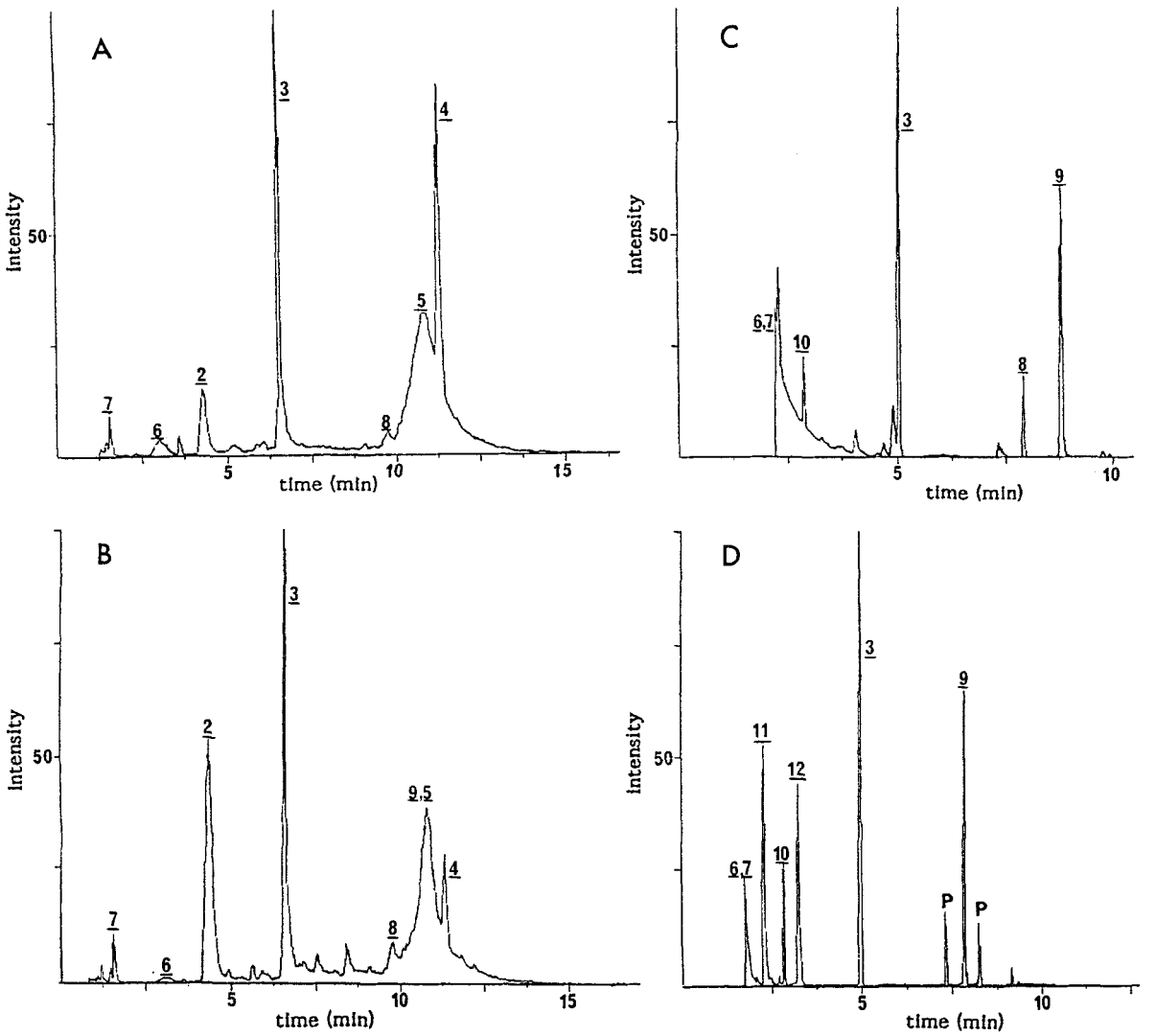


Fig. 1. GC/MS traces of crude extracts from the four PB-50 cultures. A: CZ-YE shaken culture, GC conditions: 20 m. DB5-Q column, 120–280 °C at 15 °C/min.; B: CZ-YE still culture, GC conditions as for A; C: CZ-Met shaken culture, GC conditions: 15 m. DB5-M column, 120–280 °C at 15 °C/min.; D: sucrose/glycerol shaken culture, GC conditions same as for C. Numbers refer to compounds in text and in Fig. 2. P: phthalates.

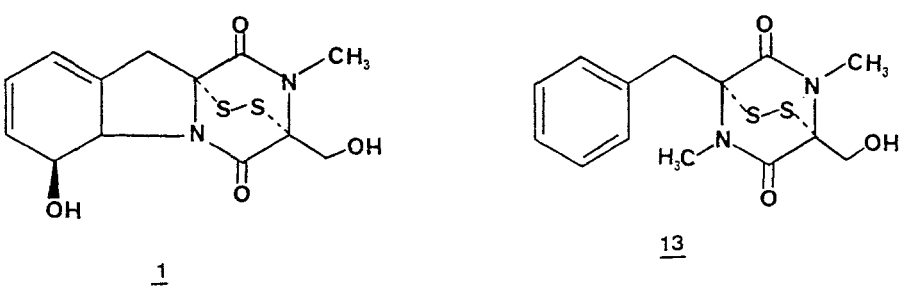
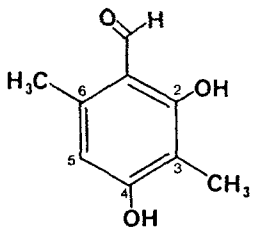
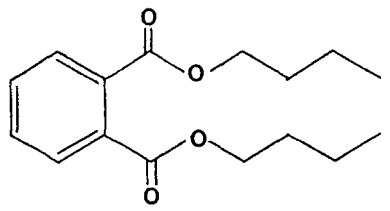


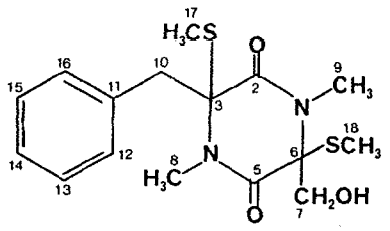
Fig. 2. Structures of gliotoxin, **1** and hyalodendrin, **13**, the two most likely toxins to be found in the PB-50 cultures.



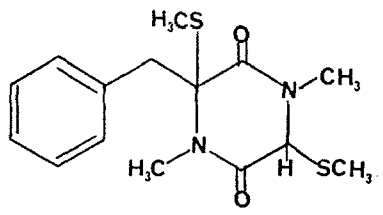
2



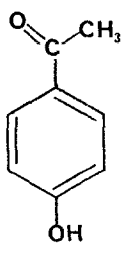
3



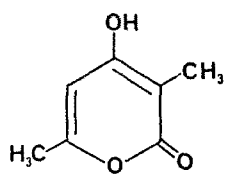
4



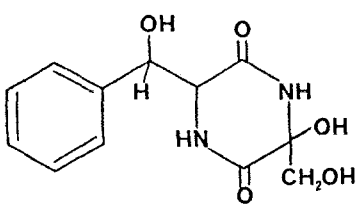
5



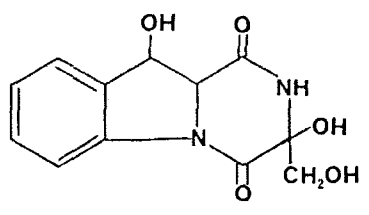
6



7



8



9

Fig. 3. Compounds identified in the PB-50 cultures: 2,4-dihydroxy-3,6-dimethylbenzaldehyde, **2**; dibutylphthalate, **3**; bisdethio-di(methylthio)hyalodendrin, **4**, and its decomposition product, **5**; 1-(4-hydroxyphenyl)ethanone, **6**; 4-hydroxy-3,6-dimethyl-2H-pyran-2-one **7**. The compounds **8** and **9** were also tentatively identified.

tified as dibutyl phthalate, **3**. HPLC separation of the extract resulted in the isolation of three pure compounds. Compounds **6** and **7** were identified by their mass spectra and from the ^{1}H NMR data as 1-(4-hydroxyphenyl)ethanone (p-hydroxy-acetophenone) and 4-hydroxy-3,6-dimethyl-2H-pyran-2-one, respectively. Confirmation of these structures was obtained by comparison with literature spectra (19). These minor compounds were found in the extracts of all cultures.

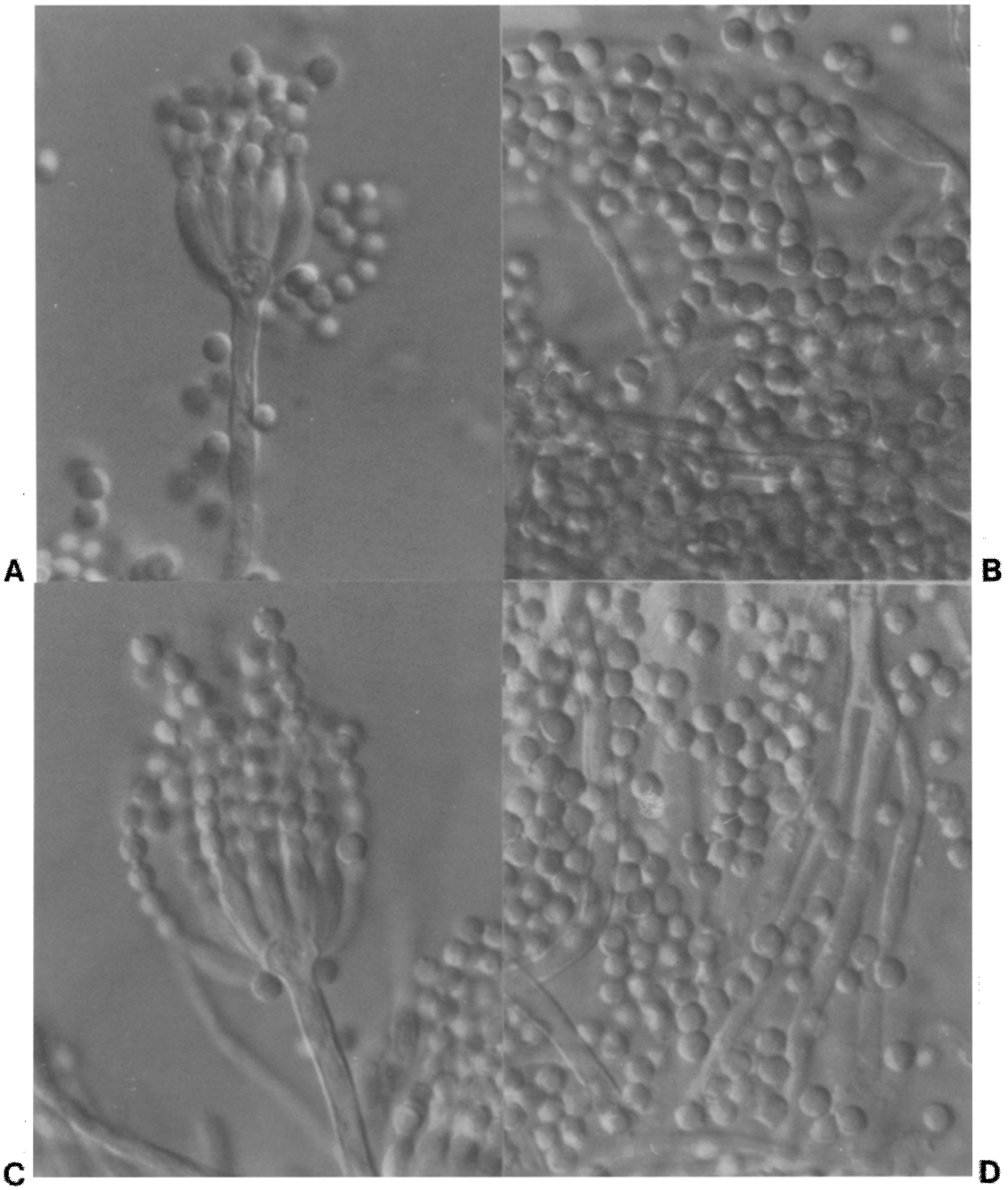


Fig. 4. Conidiophores and conidia of *Penicillium bilaii*. A, B. Ex-type strain of *P. bilaii*, CBS 226.66. C, D. Strain PB-50. All figures × 1750.

The mass spectrum of compound **8** showed a molecular ion at m/z 266, with a strong ion at m/z 91, suggesting a benzyl group. An ion at m/z 175 corresponded to the loss of this benzyl group from the parent compound. Loss of H_2O from the parent compound as indicated by the ion at m/z 248, suggested the presence of an alcohol. High resolution MS on the molecular ion showed that **8** did

not contain any sulfur atom. The ^1H NMR spectrum of this fraction showed resonances corresponding to aromaticity at 7.2–7.6 ppm, which integrated for five protons. In $CDCl_3$, the ^1H NMR spectrum showed a pair of triplets bracketing a singlet at 3.67 ppm, all three systems integrating for two protons each. In CD_2Cl_2 however, two doublets and a broad multiplet appeared. The large difference in coupling constants between the $CDCl_3$ triplets and the CD_2Cl_2 doublets (4.5 Hz *vs* 15.2 Hz) led to the conclusion that these signals belonged to different protons. The CD_2Cl_2 doublets belong to a methylene group (CH_2OH) where the two hydrogens are held in different environments, possibly by H-bonding of a hydroxyl group to a carbonyl oxygen, while in $CDCl_3$, this CH_2OH is freely rotating and the methylene signals are averaged into a singlet. This structural feature is compatible with a hydroxymethylpiperazinedione structure such as in the hyalodendrin-type compounds. There was an insufficient amount of this compound for a more complete characterization.

Comparison of the ^1H NMR spectra of **8** and **9** suggested that they were analogs, with a molecular weight of 264 for **9**. Unlike **8**, the MS of **9** lacked a peak at m/z = 91. There was a strong fragment ion at m/z 77, which indicated the presence of an aromatic ring albeit not as a benzyl group. The ion at m/z 246 corresponded to loss of water from the parent compound, suggesting the presence of an alcohol. An ion at m/z 236 corresponded to loss of 28 amu (C=O or CH_2-N or C-NH_2). The ^1H NMR spectrum for this compound was very similar to that of **8** with aromatic protons at 7.2–7.6 ppm, and two triplets bracketing a singlet around 3.8 ppm. However, the coupling pattern of the aromatic protons was slightly different and the triplets were not as symmetrical as in **8**. Only ten carbons were visible in the ^{13}C NMR spectrum, six of which were aromatic (126.0–129.7 ppm). Other resonances occurred at 96.0 ppm, 72.4 ppm, 70.4 ppm, and 61.7 ppm (CH_2OH). Not all carbons were visible due to the small amount of sample available. It was assumed that the missing carbons were carbonyls.

The molecular formula of **9** was determined by high resolution MS to be $C_{12}H_{12}N_2O_5$. By analogy to **4**, the formula $C_{12}H_{12}N_2O_3S$ had been expected, resulting from the loss of one sulfur atom and all methyl groups. Similarly, the molecular formula of **8** was determined to be $C_{12}H_{14}N_2O_5$. Assuming the carbons missing from the ^{13}C spectra to be carbonyls, the structures shown in Fig. 3 for **8** and **9** are proposed. A more complete characterization will require more material.

The mass spectrum of compound **10** showed a strong ion at m/z 173, with smaller ions at m/z 186, 188 and 216. Not enough of this compound was isolated to obtain NMR spectra. Similarly, not enough of the other two peaks, **11** and **12**, seen in Fig. 1D could be isolated for characterization. Their mass spectra did not match any library spectra or provide enough information for identification.

Extraction of the culture filtrate provided by Philom Bios yielded mainly a mixture of isopropanol oligomers. Since these compounds masked any other ones, extraction or chromatography was not practical. Instead, the GC/MS data for the extract was scanned for the presence of major ions belonging to gliotoxin and the compounds identified from the other culture conditions. None of the major compounds were found. The only positive identifications were made for **6** and two phthalates. The particular phthalates could not be identified.

According to the International Code of Botanical Nomenclature, the application of a fungal name is based on a designated, dried type specimen, from which a representative culture, known as the ex-type culture, may have been derived. Pitt [10] included the ex-type culture of *Penicillium lilacinoechinulatum* Abe ex G. Smith and several other soil isolates in his standardized description of *P. bilaii*. Therefore, his description of *P. bilaii* was broadened to fit all the characteristics of the aberrant isolates. We have demonstrated that the strain PB-50 is the same species as the ex-type culture of *P. bilaii*. It seems likely that the gliotoxin-producing strains may ultimately be shown to belong to another species.

Conclusion

No gliotoxin or other known toxins were found in the PB-50 cultures (detection limit established with a gliotoxin standard at 50 ppb). However, three hyalodendrin derivatives were isolated. The main component of the mixture was positively identified as bisdethiodi(methylthio)-hyalodendrin **4**. Two related compounds occurred in small amounts: **8** and **9**. All three of these compounds are biosynthetically related to gliotoxin, which is a hydroxylated cyclic analog of hyalodendrin. However, all of the isolated compounds lack the disulfide bridge where the biological activity of hyalodendrin and gliotoxin resides. Other

components include dibutyl phthalate (**3**) (major component which could have been introduced during processing) and three minor components: 2,4-dihydroxy-3,6-dimethylbenzaldehyde (**2**), 1-(4-hydroxyphenyl)-ethanone (**6**) and 4-hydroxy-3,6-dimethyl-2H-pyran-2-one (**7**). Three very minor compounds remain unidentified (**10**, **11**, **12**). However, it has been ascertained that they are not gliotoxin or hyalodendrin.

References

1. Miller JD. Mycology, mycologists and biotechnology. In: Hawksworth DL, ed. Frontiers in Mycology. Regensburg: CAB International, 1991: 225–40.
2. Kucey RMN. Increased phosphorus uptake by wheat and field beans inoculated with a phosphorus-solubilizing *Penicillium bilaii* strain and with vesicular-arbuscular mycorrhizal fungi. Appl Environ Microbiol 1987; 53: 2699–703.
3. Downey J, Van Kessel C. Dual inoculation of *Pisum sativum* with *Rhizobium leguminosarum* and *Penicillium bilaii*. Biol Fertil Soils 1990; 10: 194–96.
4. Kucey RMN. Effect of *Penicillium bilaii* on the solubility and uptake of P and micronutrients from soil by wheat. Can J Soil Sci 1988; 68: 261–70.
5. Kucey RMN, Leggett ME. Increased yields and phosphorus uptake by Westar canola (*Brassica napus* L.) inoculated with a phosphate-solubilizing isolate of *Penicillium bilaii*. Can J Soil Sci 1989; 69: 425–32.
6. Frisvad JC, Filtenborg O. Terverticillate penicillia: chemotaxonomy and mycotoxin production. Mycologia 1989; 89: 837–61.
7. Miller JD, Greenhalgh R, Wang Y, Lu M. Trichothecene chemotypes of three *Fusarium* species. Mycologia 1991; 83: 121–30.
8. Sankhala RH. Metabolic products of *Penicillium restrictum*. Indian J Exp Biol 1968; 6: 57–58.
9. Mullbacher A, Eichner RD. Immunosuppression in vitro by a metabolite of a human pathogenic fungus. Proc Natl Acad Sci USA 1984; 81: 3835–37.
10. Pitt, JI. The genus *Penicillium* and its teleomorphic states *Eupenicillium* and *Talaromyces*. London: Academic Press, 1979.
11. Samson RA, Van Reenen-Hoekstra ES. Introduction to foodborne fungi. Baarn: Centraalbureau voor Schimmelcultures, 1988.
12. Booth C. Fungal culture medium. In: Booth C, ed. Methods in microbiology, Vol. 4. New York: Academic Press, 1971: 49–94.
13. Johnson JR, Bruce WF, Dutcher JD. Gliotoxin, the antibiotic principle of *Gliocadium fimbriatum*, I Production, physical and biological properties. J Am Chem Soc 1943; 65: 2005–2009.
14. Kornerup A, Wanscher JH. Methuen handbook of colour, 3rd edition. London: Eyre Methuen, 1978.
15. Strunz GM, Heissner CJ, Kakushima M, Stillwell MA. Metabolites of *Hyalodendron* sp.: Bisdethiodi (methylthio) hyalodendrin. Can J Chem 1974; 52: 325–326.
16. Bruun T. The isomeric *m*-methoxytrimethylphenols and related compounds. Acta Chem Scand 1971; 25: 2837–51.
17. Stillwell MA, Magasi LP, Strunz GM. Production, isolation and antimicrobial activity of hyalodendrin, a new antibiotic produced by a species of *Hyalodendron*. Can J Microbiol 1974; 20: 759–64.
18. Strunz GM, Kakushima M, Stillwell MA, Heissner CJ. Hyalodendrin: A new fungitoxic epidithiodioxopiperazine produced by a *Hyalodendron* species. J Chem Soc Perkin I 1973; 2600–602.
19. Aldrich Library of NMR Spectra, 2nd ed, Vol. 2, 34C, 1983.

Address for correspondence: Dr Marc E. Savard, Plant Research Centre, Agriculture Canada, Ontario, Canada K1A 0C6
Phone: (613) 995 3700; Fax: (613) 992 7909