Biotransformation of the *Trichoderma* Metabolite 6-*n*-Pentyl-2*H*-pyran-2-one (6PAP) by Selected Fungal Isolates

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A variety of fungi were tested for their ability to transform the antifungal *Trichoderma* metabolite 6-*n*-pentyl-2*H*-pyran-2-one (6PAP) (**1**). Three *Penicillium* isolates, a *Sclerotinia* isolate, and a *Fusarium* isolate were all able to rapidly metabolize **1** and gave mixtures of isomers of monohydroxylated **1** and, in some cases, products resulting from further oxidation to carboxylic acids. Among these products were four previously unidentified metabolites (**6**, **7**, **8**, and **9**) which were isolated and characterized by NMR spectroscopy. *Sphaeropsis sapinea*, *Ophiostoma quercus*, *Ophiostoma piceae*, a *Verticillium* sp., and two additional *Fusarium* isolates were unable to metabolize **1** efficiently.

Trichoderma species and their metabolites have been the focus of considerable attention over recent years as potential biological control agents.[1,2] Among the most active of these metabolites is 6-*n*-pentyl-2*H*-pyran-2-one (6PAP) (**1**), a polyketide possessing a characteristic coconut-like aroma. Compound **1** is known to be toxic to a number of common plant pathogens and has been shown to be effective as a topical treatment for the control of *Botrytis cinerea* rot in stored kiwifruit.[3]

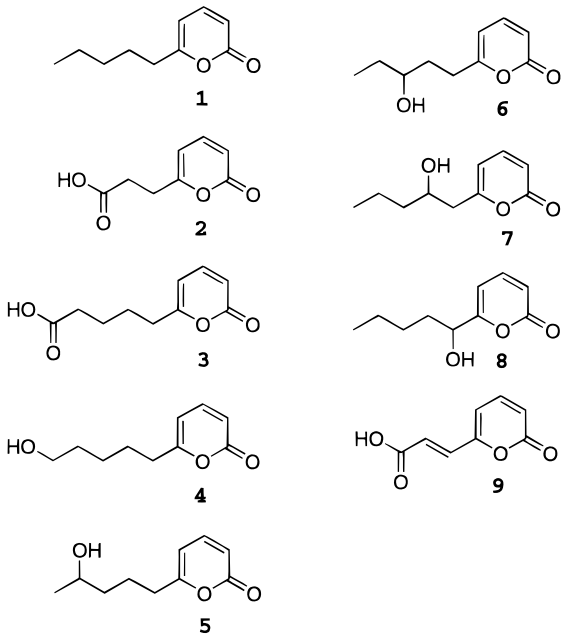
Recently, we reported that the pathogen *B. cinerea* was able to metabolize **1** into products of reduced toxicity to itself.[4] Liquid cultures of *B. cinerea* rapidly transformed **1** to give two acid metabolites, 3-(2-pyron-6-yl)propanoic acid (**2**) and 5-(2-pyron-6-yl)pentanoic acid (**3**), and two monohydroxylated metabolites, 5-(2-pyron-6-yl)pentan-1-ol (**4**) and 5-(2-pyron-6-yl)pentan-2-ol (**5**). The biological activity of the four metabolites relative to **1** was determined using spore germination assays, and all were found to be considerably less toxic to the pathogen fungus than **1**.

To fully study the mode of action of **1** as a fungal biocontrol agent, it is relevant to examine its metabolism by the targeted plant pathogens. To do this it is essential to identify and characterize the resulting metabolites. In this study we present data for the biotransformation of **1** by a range of fungal pathogen isolates.

Results and Discussion

Results for the degradation of **1** by a number of fungal isolates are indicated in Table 1. Three *Penicillium* isolates, a *Sclerotinia* isolate, and a *Fusarium* isolate rapidly metabolized **1**. The resulting transformation products consisted of mixtures of monohydroxylated isomers of **1**, while in some cases further oxidation to carboxylic acids occurred. *Sphaeropsis sapinea*, *Ophiostoma quercus*, *Ophiostoma piceae*, a *Verticillium* sp., and two additional *Fusarium* isolates showed a reduced ability to metabolize **1**. Results obtained for a *Botrytis cinerea* isolate from an earlier study[4] are also included in the table for comparison.

Biotransformation products retaining the pyrone ring, or otherwise having significant UV absorption (300 nm), were separated by preparative HPLC after extraction of the combined replicates of the *Penicillium* culture reactions. These materials were characterized and then used to identify the transformation products from the other fungal cultures using analytical HPLC. Compounds **2**−**5** were reported in earlier work,[4,5] but **6**−**9** are new transformation products. The structure of these new metabolites



was determined by NMR spectroscopy and HRMS. The position of the hydroxyl group (**6**−**8**) or other functional groups (**9**) on the side chain was confirmed by observed connectivities from COSY, HMQC, and HMBC gradient experiments and by comparison with NMR data from the previously isolated metabolites (**2**−**5**).

Depending on the fungal isolate used, oxidation by monohydroxylation occurred at some or all of the five positions on the pentyl side chain in **1**. Usually, **8** or **5** accumulated more than **4**, **7**, or **6**. However, the previous study using *B. cinerea*[4] showed that limiting the amount of nitrogen in the liquid culture media could change the ratio of these metabolites. Further oxidation to the carboxylic acids **2** and **3** was also observed with *B. cinerea*, and also occurred in significant quantities for the three *Penicillium* isolates and the *Sclerotinia* isolate. In the case of the *Fusarium crookwellense* isolate **3** occurred only as a minor component while **2** was not observed. The unsatu-

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Table 1. Biotransformation Products formed after Dosing Selected Fungal Isolates with **1**[a]

organism	% of **1** consumed	metabolites							
		4	**5**	**6**	**7**	**8**	**2**	**3**	**9**
P. expansium	98	nd[b]	+	+	+	+++	+++	+	+
P. digitatum	98	+	+	+	+	++	+++	+	+
P. italicum	85	+	+	+	+	++	+++	+	+
O. quercus	15	+	+	+	nd	+	nd	nd	nd
O. piceae	16	+	+	+	+	+	nd	nd	nd
S. sclerotiorum	100	nd[b]	nd[b]	nd	nd	nd	+++	++	+
F. graminearum	25	+	+	nd	nd	nd	nd	+	nd
F. culmorum	10	nd	nd	nd	+	+	nd	+	nd
F. crookwellense	63	+	+++	nd	+	+	nd	+	nd
B. cinerea	100	nd[b]	++	nd	nd	nd	+++	+++	nd
Verticillium sp.	7	+	+	+	+	+	nd	nd	nd
S. sapinea	24	+	+	nd	nd	+	+	+	+

[a] nd, not detectable; +, 0−10% of accumulated metabolite concentration; ++, 10−30% of accumulated metabolite concentration; +++, >30% of accumulated metabolite concentration. [b] Not detected at final extraction but present at earlier sampling dates.

rated acid, **9**, was also formed with the *Penicillium* isolates and with *S. sclerotiorum*, but only as a minor component.

It has been suggested that the production of hydroxylated metabolites is part of a fungal strategy to eliminate metabolites which would otherwise be toxic.[6] We have previously reported biological activity data for **1** and the transformation metabolites **2**−**5**.[4] In fungal germination assays all four metabolites were found to be considerably less toxic to the fungus than **1**. Results from other studies on the effects of sesquiterpenes in germination assays also suggest that increases in the hydrophilic properties of these compounds by hydroxylation or deacetylation cause a reduction in toxicity and antifungal activity.[7] Our studies show that a range of fungal pathogen isolates have the ability to metabolize **1** to form a number of hydroxylated biotransformation products. This detoxification mechanism seems to exist in a number of fungal genera, as the ability to oxidize compounds by hydroxylation is reported to be widespread among filamentous fungi.[8]

Experimental Section

General Experimental Procedures. Analytical HPLC was performed on a Prodigy 5 ODS-2 column (4.6 × 150 mm) (Phenomenex), oven temperature 35 °C, using a UV detector set at 300 nm. The initial mobile phase, MeOH−1% aqueous HOAc (20:80) at 1 mL/min, was held for 15 min, then programmed using a linear solvent gradient to MeOH−1% HOAc (60:40) over 5 min, and held for 8 min before resetting to the original conditions. NMR spectra, including two-dimensional COSY spectra, HMBC and HMQC experiments were obtained from CD_3OD or $CDCl_3$ solutions at either 300.13 MHz (^{1}H) and at 75.47 MHz (^{13}C) using a Bruker AC-300 spectrometer, or at 400.13 (^{1}H) and at 100.61 MHz (^{13}C) using a Bruker Avance DRX-400 spectrometer. Chemical shifts are reported relative to TMS.

Organism and Liquid Media. Fungal cultures of *Penicillium expansium*, *Penicillium digitatum*, *Penicillium italicum*, *O. quercus* (hardwood), *O. piceae* (conifer), *Sclerotinia sclerotiorum*, *Fusarium graminearum*, *Fusarium culmorum*, *F. crookwellense*, *B. cinerea*, *Verticillium* sp., and *S. sapinea*, formerly *Diplodia pinea*, were maintained on potato dextrose agar slopes at 20 °C. The liquid cultures consisted of a basal medium of glucose (10 g/L), thiamine HCl (0.1 g/L), Czapek's minerals (1 mL/L), tartaric acid (2 g/L), and casein hydrolysate (1 g/L).

Microbial Transformation of 6PAP (1) by Fungal Isolates. For each fungal isolate two 100 mL conical flasks, each containing 25 mL of the liquid medium, were inoculated with three agar core plugs (4 mm diameter) from the actively growing edge of the appropriate isolate and incubated with gentle agitation at 20 °C in a temperature-controlled room. An aqueous solution of **1** (Sigma-Aldrich Chemical Co., St. Louis, MO) was prepared and added to each flask 24 h after inoculation to give a concentration of 10 μg/mL. An additional 100 μg/mL was added on days 4, 12, 18, and 27. Subsamples (100 μL) were taken from each flask 24 h after each 100 μg/mL addition, at regular intervals thereafter, and immediately preceding a dose of **1**. Each subsample was diluted with 100 μL of MeOH, filtered to remove hyphal growth, and analyzed by HPLC for 6PAP and transformation products. The retention times for identified biotransformation products were 4.1 min (**2**), 6.3 min (**9**), 12.8 min (**3**), 13.2 min (**5**), 14.1 min (**4**), 16.6 min (**6**), 22.5 min (**7**), 25.3 min (**8**), and 28.9 min (**1**).

Extraction and Isolation. After incubation for 36 days, the three *Penicillium* isolates were combined and the resulting solution was extracted with ethyl acetate (3 × 100 mL). The ethyl acetate solution was then washed with 0.1 M $NaHCO_3$ (pH 8.6, 200 mL) to back-extract the acid metabolites and allow isolation of the neutral metabolites. The aqueous solution was then acidified to pH 1.9 with 0.1 M HCl (250 mL) and re-extracted with ethyl acetate to recover the acid metabolites. Semipreparative HPLC of the acid fraction on the analytical column with MeOH−1% aqueous HOAc (1:19) yielded **2**,[4] white needles mp 83−85 °C (11 mg), and compound **9** (1.8 mg). The neutral fraction was applied to a silica column (5 g), and the column was eluted stepwise with 10 mL each of hexane (S1), hexanes−ethyl acetate (19:1) (S2), hexanes−ethyl acetate (9:1) (S3), hexanes−ethyl acetate (4:1) (S4), hexanes−ethyl acetate (1:1) (S5), ethyl acetate (S6), MeOH−ethyl acetate (1:9) (S7), and MeOH (S8). The latter three fractions (S6−S8) contained the products, and these were separated by HPLC. Semipreparative HPLC of S6 on the analytical column with MeOH−H_2O (36:64) yielded compound **7** (0.5 mg at 8.2 min) and compound **8** (3.2 mg at 14.2 min). S7 was separated using the same conditions to give in addition to **7** (2.8 mg) and **8** (0.8 mg), an earlier eluting fraction (2.5−7 min) comprising two components. This fraction was further separated using MeOH−H_2O (1:4) as eluant, yielding compound **5**[4] (0.9 mg at 17.0 min) and compound **6** (1.6 mg at 21.6 min). HPLC of S8 with MeOH−H_2O (28:72) gave a mixture of early eluting minor unidentified components (3.3 mg) and **5** (0.4 mg at 8.2 min). Peaks corresponding to **3**, **4** and **5** were identified by coelution with authentic standards.

Compound 6, 5-(2-pyron-6-yl)pentan-3-ol: colorless oil; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.25 (1H, dd, J = 9.6, 6.5 Hz, H-4′), 6.16 (1H, d, J = 9.6 Hz, H-3′), 6.01 (1H, dd, J = 6.5, 0.8 Hz, H-5′), 3.58 (1H, m, H-3), 2.61 (2H, m, H-5), 1.95 (1H, m, H-4a), 1.70 (1H, m, H-4b), 1.53 (2H, m, H-2), 0.96 (3H, t, J = 7.4 Hz, H-1); HREIMS m/z 182.0941 (calcd for $C_{10}H_{14}O_3$, 182.0943).

Compound 7, 5-(2-pyron-6-yl)pentan-4-ol: colorless oil; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.27 (1H, dd, J = 9.4, 6.6 Hz, H-4′), 6.19 (1H, d, J = 9.4 Hz, H-3′), 6.07 (1H, d, J = 6.6 Hz, H-5′), 4.06 (1H, m, H-4), 2.67 (1H, dd, J = 14.6, 3.9 Hz, H-5a), 2.55 (1H, J = 14.6, 8.5 Hz, H-5b), 1.51 (2H, m, H-3), 1.15 (2H, m, H-2), 0.95 (3H, t, J = 6.9 Hz, H-1); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 164.0 (s, C-6′), 161.8 (s, C-2′), 144.3 (d, C-4′), 113.7 (d, C-3′), 104.3 (d, C-5′), 68.9 (d, C-4), 41.7 (t, C-5), 39.0 (t, C-3),

17.9 (t, C-2), 13.8 (q, C-1); HREIMS *m/z* 182.0943 (calcd for $C_{10}H_{14}O_3$, 182.0943).

Compound 8, 5-(2-pyron-6-yl)pentan-5-ol: colorless oil; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.32 (1H, dd, J = 9.4, 6.6 Hz, H-4′), 6.26 (1H, d, J = 6.6 Hz, H-5′), 6.20 (1H, d, J = 9.4 Hz, H-3′), 4.40 (1H, dd, J = 7.8, 7.7 Hz, H-5), 1.85 (1H, m, H-4a), 1.74 (1H, m, H-4b), 1.25−1.48 (4H, m, H-2,3), 0.91 (3H, t, J = 7.0 Hz, H-1); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 166.8 (s, C-6′), 162.0 (s, C-2′), 143.6 (d, C-4′), 114.3 (d, C-3′), 101.2 (d, C-5′), 71.0 (d, C-5), 35.1 (t, C-4), 27.3 (t, C-3), 22.4 (t, C-2), 14.0 (q, C-1); HREIMS *m/z* 182.0941 (calcd for $C_{10}H_{14}O_3$, 182.0943).

Compound 9,3-(2-pyron-6-yl)propenoic acid: white powder; mp 205−208 °C; ^{1}H NMR (CD_3OD, 400 MHz) δ 7.53 (1H, dd, J = 9.4, 6.6 Hz, H-4′), 7.11 (1H, d, J = 15.6 Hz, H-3), 6.63 (1H, d, J = 15.6 Hz, H-2), 6.55 (1H, dd, J = 6.6, 0.9 Hz, H-5′), 6.35 (1H, dd, J = 9.4, 0.9 Hz, H-3′); ^{13}C NMR (CD_3OD, 100 MHz) δ 169.3 (s, C-1), 161.9 (s, C-2′), 157.6 (s, C-6′), 144.3 (d, C-4′), 132.3 (d, C-3), 127.2 (d, C-2), 116.8 (d, C-3′), 108.8 (d, C-5′).

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References and Notes

(1) Ghisalberti, E. L.; Sivasithamparam, K. *Soil Biol. Biochem*. **1991**, *23*, 1011−1020.
(2) Cutler, H. G.; Hill, R. A. In *Biological Control of Postharvest Diseases Theory and Practice*; Wilson, C. L., Wisniewski, M. E., Eds.; U.S. Department of Agriculture: Kearneysville, WV, 1994; pp 135−152.
(3) Poole, P. R.; Whitaker, G.; Ward, B. G. *J. Sci. Food Agric.* **1998**, *77*, 81−86.
(4) Cooney, J. M.; Lauren, D. R.; Poole, P. R.; Whitaker, G. *J. Nat. Prod.* **1997**, *60*, 1242−1244.
(5) Poole, P. R.; Whitaker, G. *J. Agric. Food Chem*. **1997**, *45*, 249−252.
(6) Kinderlerer, J. L. *Int. Biodeterior. Biodegrad*. **1993**, *32*, 213−224.
(7) Sato, N.; Yoshizawa, Y.; Miyazaki, H.; Murai, A. *Ann. Phytopathol. Soc. Jpn*. **1985**, *51*, 494−497.
(8) Hammer, E.; Schauer, F. *Mycol. Res.* **1997**, *101*, 433−436.

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