# Microbial Transformation of the *Trichoderma* Metabolite 6-*n*-Pentyl-2*H*-pyran-2-one

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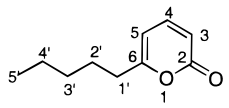
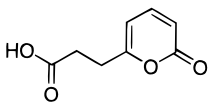
Biotransformation of the antifungal *Trichoderma* metabolite 6-*n*-pentyl-2*H*-pyran-2-one (6PAP) (**1**) by *Botrytis cinerea* generated the previously reported 3-(2-pyron-6-yl)propionic acid (**2**) and 5-(2-pyron-6-yl)pentanoic acid (**3**) and allowed the isolation and characterization of a previously tentatively assigned product as 5-(2-pyron-6-yl)pentan-2-ol (**5**), plus allowed isolation of a new transformation product identified as 5-(2-pyron-6-yl)pentanoic acid (**4**). The full NMR spectral assignments of these four compounds are presented here for the first time, including some corrections to assignments previously published for 6PAP. Information is also presented on the relative toxicity of 6PAP and its four biotransformation products to *B. cinerea*, which shows that the metabolism products have reduced toxicity to the pathogenic organism.

*Trichoderma* species and their metabolites are being studied 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**), which is toxic to many common plant pathogens, including *Botrytis cinerea*,[3] and which has also shown promise as a topical treatment for the control of rot in stored kiwifruit.[4] Antibiotic compounds produced by *Trichoderma* species contribute to their biocontrol function, although it is unclear whether this is a specific biological function of 6PAP.[5]

Recently, it was reported that liquid cultures of *B. cinerea* transformed 6PAP in high yield into at least three new metabolites oxygenated on the terminal carbons of the side chain, rather than the expected hydrolytic cleavage of the pyrone ring. Two acidic metabolites were identified as 3-(2-pyron-6-yl)propionic acid (**2**) and 5-(2-pyron-6-yl)pentanoic acid (**3**), while the third compound was tentatively identified as either 5-(2-pyron-6-yl)pentanol (**4**) or 5-(2-pyron-6-yl)pentan-2-ol (**5**) on the basis of its mass spectrum.[6]




**1**

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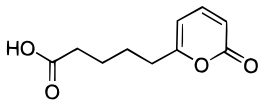


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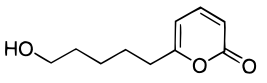
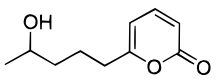



**4**




**5**

If **1** is to be used as a biocontrol agent, it will be necessary to measure its breakdown and metabolism on the surface of fruit. It is thus important to be able to determine all likely metabolites present. These would include metabolites produced both by the fruit and by any pathogenic organisms on the fruit. Access to oxygenated species such as those produced by *B. cinerea* may be useful in determining the effect of side-chain substitutents on the fungitoxicity of these compounds and could also have practical value as synthetic precursors for further chemical studies. The present study was undertaken to isolate and to fully characterize the major *B. cinerea* metabolites of 6PAP, including those that previously had been only partly characterized.

## Results and Discussion

The degradation of **1** dosed into actively growing cultures of *B. cinerea* in each of two media and concomitant appearance of metabolites, is presented in Figure 1. Metabolism occurred more rapidly in medium A, to give a mixture of mainly three metabolites, **2**, **3**, and **5**. An additional metabolite, **4**, was observed during the course of the experiment but was not evident on the final sampling date. In medium B metabolism occurred more slowly, to give principally metabolite **4** with smaller yields of metabolites **2**, **3**, and **5**. The total metabolite concentration at the end of the experiment accounted for 82% and 78% of added **1**, for media A and B, respectively. The standard deviation between replicates was less than 15% of the mean values shown in Figure 1.

After 29 days the added **1** had been completely consumed from medium A and mostly consumed from medium B. Replicates of each medium were combined and extracted to recover the metabolites. The acid and neutral extract fractions were then each assessed for the presence of pyrone-containing metabolites by HPLC prior to isolation of the metabolites by semipreparative HPLC.

The two acid metabolites were identified as **2** and **3** as previously reported.[6] These structures were confirmed by $^{1}$H- and $^{13}$C-NMR (Tables 1 and 2), and NMR spectral assignments made using a combination of DEPT, COSY, LR COSY, and HMQC experiments. The previously isolated alcohol metabolite was positively

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**Table 1.** $^1$H-NMR Assignments ($\delta$) [mult, $J$ (Hz)] for 6PAP (**1**)[10] and Related Metabolites

| position | **1**[a] | **2**[b] | **3**[b] | **4**[a] | **5**[a] |
|---|---|---|---|---|---|
| 3 | 6.07 (d, 9.4) | 6.18 (d, 9.3) | 6.17 (d, 9.3) | 6.13 (d, 9.4) | 6.15 (d, 9.4) |
| 4 | 7.21 (dd, 9.4, 6.6) | 7.46 (dd, 9.3, 6.7) | 7.46 (dd, 9.3, 6.6) | 7.24 (dd, 9.4, 6.5) | 7.25 (dd, 9.4, 6.5) |
| 5 | 5.92 (d, 6.6) | 6.24 (d, 6.7) | 6.22 (d, 6.6) | 5.96 (d, 6.5) | 5.98 (d, 6.5) |
| 6 | | | | | |
| 1′ | 2.41 (t, 7.6) | 2.83 (t, 7.2) | 2.56 (t, 7.0) | 2.49 (t, 7.5) | 2.51 (t, 7.6) |
| 2′ | 1.59 (m) | 2.67 (t, 7.2) | 1.66 (m) | 1.69 (m) | 1.76 (m) |
| 3′ | 1.22−1.30 (m) | | 1.71 (m) | 1.42 (m) | 1.48 (m) |
| 4′ | 1.22−1.30 (m) | | 2.34 (t, 6.9) | 1.58 (m) | 3.82 (m) |
| 5′ | 0.83 (m) | | | 3.63 (t, 6.4) | 1.20 (d, 6.2) |
| OH | | | | 1.81 (br s) | 1.60 (br s) |

[a] In $CDCl_3$. [b] In $CD_3OD$.

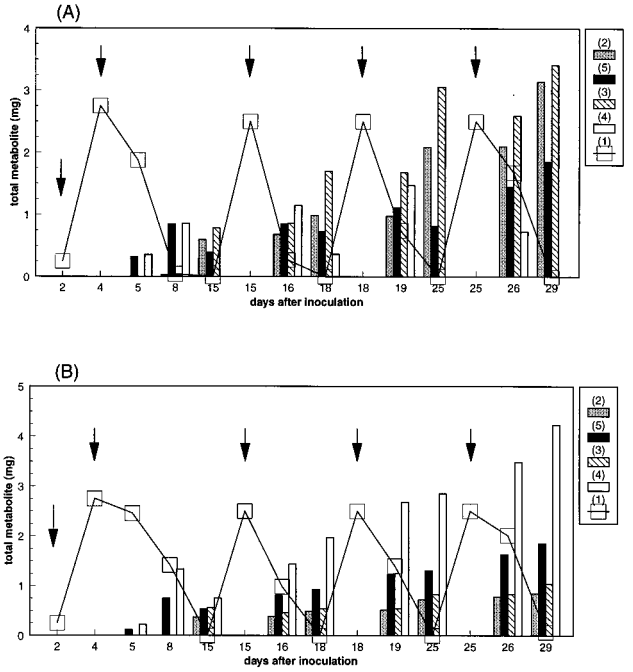


**Figure 1.** Degradation of **1** and appearance of metabolites **2**−**5** in actively growing *B. cinerea* cultures in (**A**) medium A, and (**B**) medium B. Arrows indicate additions of **1** to the culture medium. Points represent the means of 5 replicate determinations.

**Table 2.** $^{13}$C-NMR Assignments ($\delta$) (mult) for 6PAP (**1**)[10] and Related Metabolites

| position | **1**[a] | **2**[b] | **3**[b] | **4**[a] | **5**[a] |
|---|---|---|---|---|---|
| 2 | 162.9 (s) | 164.9 (s) | 165.2 (s) | 163.0 (s) | 162.9 (s) |
| 3 | 113.0 (s) | 113.9 (d) | 113.6 (d) | 113.2 (d) | 113.3 (d) |
| 4 | 143.8 (s) | 146.4 (d) | 146.5 (d) | 143.8 (d) | 143.7 (d) |
| 5 | 102.7 (s) | 104.7 (d) | 104.7 (d) | 102.8 (d) | 102.8 (d) |
| 6 | 166.8 (s) | 166.2 (s) | 167.6 (s) | 166.5 (s) | 166.3 (s) |
| 1′ | 33.8 (t) | 29.8 (t) | 34.0 (t) | 33.8 (t) | 33.7 (t) |
| 2′ | 26.6 (t) | 31.7 (t) | 25.3 (t) | 26.7 (t) | 23.2 (t) |
| 3′ | 31.1 (t) | 175.3 (s) | 27.4 (t) | 25.2 (t) | 38.3 (t) |
| 4′ | 22.3 (t) | | 34.5 (t) | 32.3 (t) | 67.7 (d) |
| 5′ | 13.9 (q) | | 177.2 (s) | 62.6 (t) | 23.7 (q) |

[a] In $CDCl_3$. [b] In $CD_3OD$.

identified as **5**, and a new metabolite, observed principally in the B-medium, was identified as **4**. Metabolite **5** had previously been characterized by mass spectral data only and had been tentatively proposed to be either **4** or **5**.[6] Both metabolites gave similar EIMS fragmentation patterns and had the same molecular ion ($M^+$) at $m/z$ 182. $^1$H- and $^{13}$C-NMR data for both alcohols are reported here for the first time (Tables 1 and 2). Full assignments were made using DEPT, COSY, HMQC, and HMBC gradient experiments. Table 3 illustrates the observed connectivities from 2D NMR plots for metabolite **4** as an example. A number of these assignments were inconsistent with those reported in the literature for **1**. $^1$H- and $^{13}$C-NMR assignments of **1** based on HMQC and HMBC gradient experiments are also shown in Tables 1 and 2 and are consistent with assignments made for the four biotransformation metabolites. These experiments indicated that earlier NMR data reported for **1** incorrectly assign C-2 as C-6, C-6 as C-2, C-2′ as C-3′, and C-3′ as C-2′.[3,7,8]

**Table 3.** Observed Connectivities from 2D NMR Plots for **4**

| position | $^{13}$C ppm | HMQC $^1$H−$^{13}$C | COSY $^1$H−$^1$H | HMBC long-range $^1$H−$^{13}$C |
|---|---|---|---|---|
| 2 | 163.0 (s) | | | |
| 3 | 113.2 (d) | 6.13 (d) | H-4 | C-2, C-5 |
| 4 | 143.8 (d) | 7.24 (dd) | H-3, H-5 | C-6, C-2, C-5 (w) |
| 5 | 102.8 (d) | 5.96 (d) | H-4 | C-6, C-3, C-1′, C-4 (w) |
| 6 | 166.5 (s) | | | |
| 1′ | 33.8 (t) | 2.49 (t) | H-2′ | C-5, C-6, C-2′, C-3′ |
| 2′ | 26.7 (t) | 1.69 (m) | H-1′, H-3′ | C-1′, C-3′, C-4′ |
| 3′ | 25.2 (t) | 1.42 (m) | H-2′, H-4′ | C-1′, C-2′, C-4′, C-5′ |
| 4′ | 32.3 (t) | 1.58 (m) | H-5′ | C-2′, C-3′, C-5′ |
| 5′ | 62.6 (t) | 3.63 (t) | H-4′ | C-4′, C-3′ |

The toxicity of **1** and biotransformation products **2**−**5** to *B. cinerea* was determined using germination experiments. Germination for the control was 100%, and there was no visible difference able to be measured between the control and any of the transformed metabolites at concentrations of up to 1000 $\mu$g/mL. Under the same conditions, compound **1**, at a concentration of 125 $\mu$g/mL, reduced germination to 18%, and inhibited it fully at 200 $\mu$g/mL.

These results confirm that *B. cinerea* has the ability to transform a potentially toxic secondary metabolite from a competitive fungus into products of reduced toxicity to itself. Preliminary studies with selected *Fusarium* species have shown that an isolate of *F. crookwellense* can metabolize **1** to form some of the same biotransformation products, thus this detoxification mechanism may exist in a number of fungal genera.

## Experimental Section

**General Experimental Procedures.** Analytical HPLC was performed on a Prodigy 5 ODS-2 column (4.6 × 150 mm) (Phenomenex) 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 20 min, then programmed using a linear solvent gradient to MeOH−1% HOAc (60:40) over 5 min, and held for 7 min before resetting to the original conditions. GC−MS was performed using an Ultra 2 column and a Kratos MS80RFA instrument (EI source 220 °C, 30 eV). NMR spectra, including 2D COSY spectra, HMBC, and HMQC experiments were obtained from $CD_3OD$ or $CDCl_3$ solutions

either at 300.13 MHz ($^1$H) and 75.47 MHz ($^{13}$C) using a Bruker AC-300 spectrometer or at 400.13 ($^1$H) and 100.61 MHz ($^{13}$C) using a Bruker Avance DRX-400 spectrometer. Chemical shifts are reported relative to TMS.

**Organism and Liquid Media.** *B. cinerea* was isolated from infected kiwifruit in 1991[9] by culturing at 22 °C on gels of potato dextrose agar (3% w/v) adjusted to pH 3.5 by addition of lactic acid. Isolates were maintained by reculturing annually at 0 °C on kiwifruit and reisolating. Conidia were harvested from 3-week-old cultures and used at the rate of 40,000/mL to inoculate liquid cultures. 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), and tartaric acid (2 g/L). To medium A was added casein hydrolysate (1 g/L) and to medium B histidine (1 g/L).

**Microbial Transformation of 6PAP (1) by *B. cinerea*.** Five 100-mL conical flasks, each containing 25 mL of medium A, and five 100-mL flasks, each containing 25 mL of medium B, were inoculated with *B. cinerea* as described above and incubated with gentle agitation at 20 °C in a temperature-controlled room programmed with a 16-h light cycle/day. Twenty-four hours after inoculation, an aqueous solution of **1** was added to each flask to give a concentration of 10 $\mu$g/mL. An additional 100 $\mu$g/mL was added on days 4, 15, 18, and 25. Subsamples (100 $\mu$L) were taken from each flask 24 h after each 100-$\mu$g/mL addition, at regular intervals thereafter, and immediately preceding a dose of **1**. Each subsample was diluted with 100 $\mu$L of MeOH, filtered to remove hyphal growth, and analyzed by HPLC for 6PAP and metabolites [retention times 4.6 min (**2**), 15.6 min (**5**), 16.3 min (**3**), 17.4 min (**4**), and 31.7 min (**1**)] with detection at 300 nm, a suitable wavelength for observation of pyrone-containing compounds.

**Extraction and Isolation.** After incubation for 29 days the replicates of each medium were combined and each resulting solution extracted with EtOAc (3 × 250 mL). These two EtOAc solutions were then washed with 0.1 M $NaHCO_3$ (pH 8.6, 125 mL) to back extract the acid metabolites and allow isolation of the neutral metabolites. The aqueous solution was then acidified to pH 2.2 with 0.1 M HCl (130 mL), and re-extracted with EtOAc to recover the acid metabolites. Semi-preparative HPLC of the acid fraction on the analytical column with MeOH−1% aqueous HOAc (1:4) yielded **2**, white needles, mp 85−86 °C, (9 mg) and **3**, a white powder, mp 72.5−74 °C, (12 mg). The neutral metabolites were separated on an Econosphere silica column (10 × 250 mm) (Alltech) using 2-propanol−hexane (1:7, 6 mL/min) as eluent to give **5**, a colorless oil (6 mg at 20.8 min) [EIMS *m/z* 182 [$M^+$] (9), 164 (16), 149 (3), 136 (25), 123 (58), 122 (26), 110 (22), 107 (19), 95 (100), 94 (26), 82 (19), 68 (17), 55 (10), 39 (16)], and **4**, a colorless oil (12 mg at 25.6 min): EIMS *m/z* 182 [$M^+$] (20), 164 (42), 149 (4), 136 (6), 123 (78), 122 (99), 110 (92), 107 (20), 95 (100), 94 (82), 82 (14), 68 (12), 55 (18), 45 (32), 39 (18).

**Toxicity Bioassay.** The toxicities to *B. cinerea* of 6PAP and its four biotransformation products were determined by observing the effect of the compounds on conidial germination. Medium A (100 $\mu$L) fortified with a range of concentrations of each compound [0 (control), 125, 250, 500, and 1000 $\mu$g/mL; 2 replicates at each concentration] was inoculated with a *B. cinerea* conidial suspension (10 $\mu$L) containing $4.9 \times 10^7$ spores/mL. The vials were gently agitated at 20 °C for 24 h, then the percentage of conidia with incipient or well-developed germ tubes was determined using a microscope/hemocytometer. (Differences measurable for 6PAP treatments only and above 125 $\mu$g/mL as stated in the text.)

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(10) The skeleton numbering system for $^1$H and $^{13}$C assignments (Tables 1 and 2) is shown for 6PAP. To facilitate comparison between **1** and its biotransformation products, the same numbering system has been used for metabolites **2**−**5**.

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