Biotransformation of 6-Pentyl-2-pyrone by *Botrytis cinerea* in Liquid Cultures

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Metabolism of 6-pentyl-2-pyrone (6PP) added at rates up to 120 μg/mL to liquid *Botrytis cinerea* cultures produced the previously unreported compounds 3-(2-pyron-6-yl)propanoic acid and 5-(2-pyron-6-yl)pentanoic acid. Another metabolite was identified tentatively from its mass spectrum as either 5-(2-pyron-6-yl)pentanol or 5-(2-pyron-6-yl)pentan-2-ol. *B. cinerea* hyphae remained viable with 6PP at 200 μg/mL, but their growth was suppressed at this concentration. Pyrone-containing metabolites were not observed in all systems to which 6PP had been added, but were formed in both hyphal development and sporulating phases of growth. These metabolites were stable in the presence of actively growing *B. cinerea* cultures and did not appear to alter fungal growth. It is proposed that the changes to the alkyl side chain in the metabolites reduced their lipophilicity and ability to permeate cell membranes relative to the parent compound, thus reducing their toxicity to the fungus.

Keywords: *6-Pentyl-2-pyrone; 3-(2-pyron-6-yl)propanoic acid; 5-(2-pyron-6-yl)pentanoic acid; 5-(2-pyron-6-yl)pentanol; Botrytis cinerea; fungal metabolism; toxicity; biotransformation*

INTRODUCTION

6-Pentyl-2-pyrone (6PP) is toxic to common fungal plant pathogens including *Botrytis cinerea* (Claydon *et al.*, 1987). It is produced by certain species of *Trichoderma* fungi, where it is associated with antibiotic properties (Collins and Halim, 1972; Worasatit *et al.*, 1994). This toxicity has been related to the ability of 6PP to adsorb onto hydrophobic cell membranes (Scarselletti and Faull, 1994) and is analogous to the toxicity of the cucurbitacins to *B. cinerea* by laccase inhibition (Viterbo *et al.*, 1993), for which detailed structure–activity studies have been reported. Scarselletti and Faull (1994) proposed that 6PP did not have any single mode of toxicity, but affected diverse metabolic processes in sensitive fungi.

If growth is not fully suppressed, fungi will overgrow 6PP zones in plate culture (Claydon *et al.*, 1987), suggesting that 6PP is metabolized in these conditions. The content of cultures of 6PP-producing *Trichoderma* strains is known to decline in idiophasic growth (J. M. Cooney, personal communication, 1996), but the metabolites in these systems have not been characterized.

6PP has been proposed for use, either directly or as a product of *Trichoderma* biocontrol systems, for protection of crops such as kiwifruit and grape against *B. cinerea* rots (Cutler and Hill, 1994). The metabolism of 6PP by *B. cinerea* and the compounds formed have relevance to the mode of action and to possible mammalian toxicity.

MATERIALS AND METHODS

Materials. Purified water (MilliQ: Waters Corporation) was used. Methanol (HPLC grade), acetic acid [analytical reagent (AR) grade], ethyl acetate (AR), diethyl ether (AR), chloroform (AR), hexanes (AR), anhydrous $MgSO_4$, and polysorbate 80 (Tween 80) were supplied by BDH (NZ) Ltd. Glucose, casein hydrolysate, thiamine HCl, tartaric acid, lactic acid, deuterochloroform, deuterium oxide, and 6PP ("6-amyl-α-pyrone") were supplied by Sigma-Aldrich Chemical Company (St. Louis, MO). Potato dextrose agar (PDA) was obtained from Gibco (NZ) Ltd. *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was supplied by Lab Supply Pierce (NZ) Ltd. Diazomethane was prepared in diethyl ether solution by the addition of NaOH to *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (Diazald, Aldrich Chemical Company, Milwaukee WI), by the manufacturer's recommended procedure.

Chromatography. High-performance liquid chromatographic (HPLC) determination of 6PP and detection of metabolites was carried out with a system consisting of two pumps (Biorad) controlled by a Baseline (Waters Corporation)) operating system, with mixer, autosampler, column oven held at 30 °C, column (Zorbax ODS 250 × 4.6 mm i.d., 5 μm; Du Pont), and variable wavelength UV detection at 301 nm (Linear UVIS 200). The mobile phase was run at 1 mL/min. The initial mobile phase, A (900:100 methanol:10 acetic acid), was run for 2 min and then mixed in a linear gradient with B (200:10:790, methanol:acetic acid:water), progressing to 100% B over 8 min, and then holding for 7 min.

Gas chromatography-mass spectroscopy (GC-MS) was done with a Kratos MS80 RFA system fitted with a 30 m × 0.25-mm i.d. capillary column (PTE5 × 0.25 μm film; Supelco), and run in the electron-impact (EI) mode at 30 eV. A linear temperature ramp from 80 to 280 °C over 20 min was used.

Thin-layer chromatography (TLC) was done on glass-backed fluorescent silica gel plates (Merck 60CF254 20 × 20 × 0.1 cm) with chloroform:hexanes:acetic acid (7:2:1) as the mobile phase.

Nuclear Magnetic Resonance (NMR) Spectra. ^1H and ^{13}C NMR spectra were obtained with a Bruker DRX-400 instrument operated at 400.13 MHz for ^1H and 100.62 MHz for ^{13}C. Samples were dissolved in either D_2O or $CDCl_3$. Chemical shifts are reported relative to tetramethylsilane (TMS).

Liquid Culture of *Botrytis cinerea*. *B. cinerea* was isolated from infected kiwifruit in 1991 by culturing at 22 °C on gels of PDA (3% w/v) adjusted to pH 3.5 by addition of lactic acid. Isolates were maintained by reculturing annually at 0 °C in kiwifruit and reisolating on the PDA gels. Conidia were harvested from 3-week-old cultures and used at the rate

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40 000/mL to inoculate liquid cultures. The liquid cultures consisted of a base medium of glucose (10 g/L), casein hydrolysate (1 g/L), thiamine HCl (0.1 g/L), Czapek's minerals without nitrate or ammonium salts (1 mL/L), and tartaric acid (2 g/L) at pH 3.3. At various stages of growth, 6PP was added as a suspension in 1% (v/v) polysorbate 80:water. Untreated controls were also prepared. The cultures were incubated with gentle agitation at 22 °C in a 12-h lighting cycle. Under these conditions, ~75% of conidia swelled and developed germ tubes within 12 h.

Tolerance of *B. cinerea* Cultures to 6PP. To establish the tolerance of germinated *B. cinerea* to 6PP in liquid media, 6PP was added 24 h after inoculation to the cultures to give concentrations equivalent to 10, 25, 50, 120, 220, and 450 μg/mL. Twenty-five-milliliter volumes of culture in 100-mL conical flasks were used. Additional 6PP, equivalent to 120 or 213 μg/mL, was added after 7 days to the 120-μg/mL culture (the highest concentration exhibiting hyphal growth at that time), and after a further 3 days, 5-μL subsamples of the medium were plated on PDA gels. Growth in these cultures was monitored over 30 days.

Metabolite Production. For the initial studies, 6PP was added to give a concentration of 10 μg/mL, 24 h after inoculation as before. An additional 100 μg/mL was added after 72 h and again at 15 days, the stage when darkening of the hyphae and initial conidial formation on surface growth was first observed. After the 15-day addition, 2-mL subsamples were withdrawn at various intervals over 4 days, filtered through a sintered glass disc, and run on HPLC to monitor residual 6PP and the production of metabolites. Another subsample taken at 15 days was centrifuged at 0 °C (11000*g* for 10 min) to give a clear supernatant. 6PP (70 μg/mL) was added to this centrifugate and monitored for 6PP content over 1.5 h.

Metabolites were produced on a larger scale by treating 100 mL of cultures contained in 250-mL vessels with 6PP at 50 μg/mL at 2 days after inoculation and again after 21 days. The cultures were extracted after 45 days.

GC-MS Investigations of Pyrone Metabolites. Cultures from the initial metabolite production study were extracted 23 h after the 6PP addition into ethyl acetate, dried over anhydrous $MgSO_4$, evaporated under nitrogen to dryness, redissolved in a small quantity of ethyl acetate, and derivatized by adding diazomethane in diethyl ether solution. The concentrated extracts were analyzed by GC-MS.

Isolation and NMR Spectra of Metabolites. Two metabolites were separated from culture media for NMR investigations. For the first ("metabolite II"), cultures were filtered and passed through C-18 500-mg cartridges (Alltech) twice to remove unmetabolized 6PP and the less polar metabolites. The unretained material was extracted into ethyl acetate and was shown by HPLC to contain only one UV absorbing peak at 301 nm, with a retention time of 6.5 min. This material was dissolved in $CDCl_3$ to determine ^{13}C and ^{1}H NMR spectra.

For the second metabolite ("metabolite III"), culture media were extracted in ethyl acetate, dried over $MgSO_4$, and evaporated to dryness at 40 °C under reduced pressure. The residue was redissolved in a minimum volume of ethyl acetate and separated by TLC. The band visible under 254 nm light extending from 54 to 62% of the solvent front was scraped off and extracted three times into 2 mL of methanol. the combined extracts were filtered, evaporated to dryness under nitrogen, and dissolved in D_2O for determining the ^{1}H NMR spectrum. There was insufficient material for ^{13}C NMR.

Subsamples of the separated metabolites were treated with diazomethane and analyzed by GC-MS as before.

RESULTS

Tolerance of *B. cinerea* to 6PP. Visible *B. cinerea* hyphal growth occurred within 7 days for culture media to which 6PP up to 120 μg/mL had been added. No visible growth occurred over 30 days for cultures with 6PP at 220 μg/mL or greater, and at 7 days there was no significant reduction in 6PP content. Subsamples from the cultures taken 3 days after the second addition of 6PP at 7 days all grew on the PDA gel plates. At 30 days, fungal development had resumed for the 120-μg/mL addition, but not for the 213-μg/mL addition.

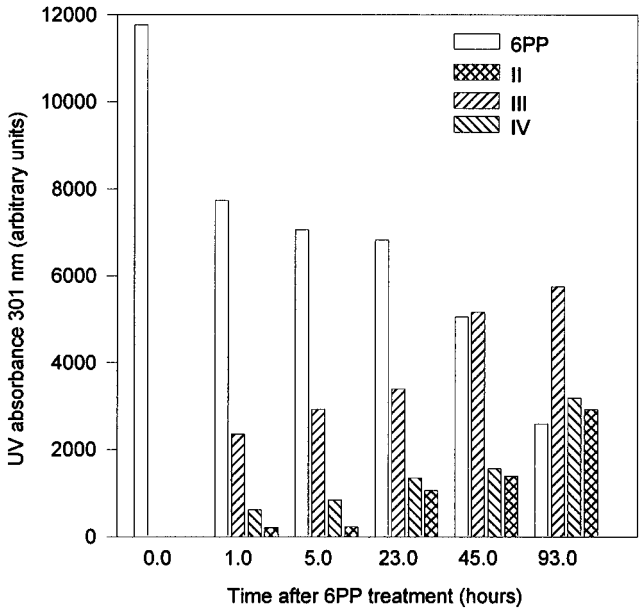

Figure 1. Rate of degradation of 6PP and appearance of pyrone-containing metabolites. 6PP was added to an actively growing *B. cinerea* culture previously treated with 6PP. Metabolite absorptions, determined by HPLC with uv detection, have been adjusted by subtracting the values recorded at the time of 6PP addition.

Metabolite Production. In the HPLC chromatograms, 6PP appeared at a retention time of 14.05 min. The minimum detectable concentration of 6PP in the media, limited by coextracted material, was 0.8 μg/mL. In the media for initial studies of metabolite production, the HPLC chromatograms showed that residual 6PP declined over the first 7 days to <8 μg/mL, or 7% of the amounts added, whereas new peaks, attributed to 6PP metabolites, appeared at 6.5, 11.2 and 11.6 min (the latter two not fully resolved). Initial breakdown of 6PP added at 15 days to the cultures was rapid with levels reducing to ~70% of initial levels within the first hour; later conversion was much slower, with a half-life in the order of 60 h; and after 196 h, only 1.5% of the original 6PP was present. The pyrone metabolites increased, maintaining similar proportions during depletion of the 6PP (Figure 1). The clear centrifugate also degraded 6PP, causing a 10% loss over 1.5 h, the only time measured.

In the larger scale production of metabolites, 6PP residues had declined to 88% of the original application at 10 days, with the appearance of a metabolite peak at a retention time of 10.68 min, absorbing at 301 nm with ~7% of the intensity of the original 6PP addition. After another day, residual 6PP declined to 70%, and the metabolite absorption was 28%. At 21 days, 6PP had declined to 1.4% and the 10.68-min metabolite was no longer present and was not investigated further. All three of the previously observed metabolites were present, with their total absorption equivalent to 26% of the original 6PP.

Mass and Nuclear Magnetic Resonance Spectra. The NMR spectra of metabolites II (^{1}H and ^{13}C) and III (^{1}H only) are listed in Table 1. The assignments of the spectral features were guided by the published spectra

Table 1. ^1H and ^{13}C NMR Chemical Shifts (δ) of Atoms in Metabolites II and III

position[c]	metabolite II[a]			metabolite III[b]	
	^{13}C	^1H		^1H	
2	163.30				
3	113.85	6.20 d	J = 9.6 Hz	6.30 d	J = 9.2 Hz
4	143.68	7.29 d, d	J = 6.8, 9.4 Hz	7.63 d, d	J = 6.8, 9.3 Hz
5	103.34	6.07 d	J = 6.8 Hz	6.39 d	J = 7.0 Hz
6	166.20				
1′	—[d]	2.84 t	J = 6.7 Hz	2.64 t	J = 7.2 Hz
2′	—[d]	2.75 t	J = 6.7 Hz		
3′	175.9			1.69 m	two overlapping multiplets
4′				2.31 t	J = 7.2 Hz

[a] Dissolved in $CDCl_3$. [b] Dissolved in D_2O. [c] Numbers 2, 3, 4, 5, and 6 refer to the pyrone ring carbon positions, and 1′, 2′, 3′, and 4′ refer to the side chain. [d] In the range 28−33; interfering peaks in this spectral region make assignment uncertain.

of 6PP (Cutler *et al.*, 1986) and by the proposed chemical structure. Four prominent peaks were observed in the GC-MS total ion chromatograms of the diazomethane-reacted culture extracts, and their principal ions are listed in Table 2. The peak at 19.32 min coincided with authentic 6PP (I). Attempts to generate mass spectra without derivatization or after reaction with MSTFA at 60 °C for 1 h were unsuccessful. The retention times and mass spectra of the compounds separated for NMR and reacted with diazomethane were the same as for the metabolites extracted directly from the culture media.

DISCUSSION

The mass spectra of 6PP and the metabolites (or their methyl esters) all showed ions m/z = 95, 81, and 68, that are observed in 6PP (Cutler *et al.*, 1986) and which are characteristic of the pyrone ring. The NMR spectra of metabolites II and III are consistent with those compounds being 3-(2-pyron-6-yl)propanoic acid and 5-(2-pyron-6-yl)pentanoic acid, respectively (Figure 2). The mass spectral fragment ions of the methyl ester of II would correspond to sequential elimination of OCH_3 (151), CO (123), and H (122) or C_2H_4 (95), and for the methyl ester of III, to CH_3OH (178) and CH_2CHCO (123) elimination.

Metabolite IV was not sufficiently separated by TLC for NMR spectra to be determined. The mass spectrum indicated that it is the alcohol corresponding to metabolite III [that is, 5-(2-pyron-6-yl)pentanol], with the observed fragment ions resulting from sequential elimination of H_2O (164) and $C_3H_5^+$ or C_3H_6 (123 or 122). However 5-(2-pyron-6-yl)pentan-2-ol would give a similar mass spectrum and is a possible structure.

Pyrones were not major metabolites under all culture conditions; for example, pyrones occurred in only trace amounts in the early stages of the larger scale cultures. The reasons for the differences in 6PP metabolism are not clear, but pyrone metabolites may be favored in later idiophasic growth when glucose becomes depleted. It is possible that prior exposure enhanced the ability of cultures to subsequently degrade 6PP, in the manner observed for gallic acid-induced extracellular laccase activity in *B. cinerea* (Marbach *et al.*, 1983), but this aspect was not investigated. Under favorable conditions, it would appear from the HPLC data that a significant proportion of the 6PP could be converted to these products, if it is assumed that the extinction coefficients at 301 nm for these compounds are similar.

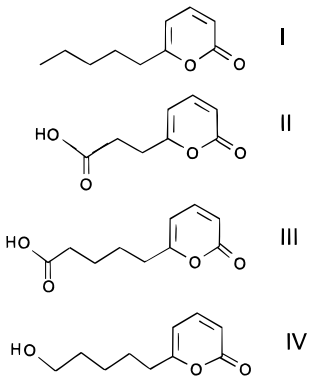

Figure 2. 6-Pentyl-2-pyrone (I) and proposed structures of its metabolites produced in *B. cinerea* cultures.

The moderately strong UV absorption at this wavelength is a consequence of electron delocalization over the pyrone ring, and indicates a degree of stabilization. 6PP is stable to acid or mild alkaline hydrolysis (it is not affected by 2 M HCl or CH_3COONa at 50 °C for 2 h, nor by prolonged contact with weak acids such as the culture medium used), but is degraded by bases such as 1 M NaOH or the silylation reagent MSTFA (unpublished results). The reaction with NaOH produces 2-heptanone, presumably by base hydrolysis of the lactone group, followed by hydration of the 5−6 C=C bond and retro-aldol cleavage. 2-Heptanone was not detected in ethyl acetate extracts of *B. cinerea* cultures.

The rapid initial decline of 6PP after addition to the cultures or to centrifugate at 15 days and slower later breakdown is consistent with an extracellular oxidation process limited by cofactors that are dependent on active metabolism of *B. cinerea* for replenishment. As the concentrations added were not lethal to the culture, some respiration must continue prior to depletion of 6PP and resumption of active hyphal growth. In the decay study, the metabolites maintained similar proportions to each other and increased during the period that 6PP was metabolized in the cultures, suggesting that they were stable products rather than being formed sequentially. The continued presence of these compounds after resumption of hyphal growth on depletion of 6PP is further evidence that they are stable products. The metabolite observed in early stages of the larger scale cultures may be a single precursor of the stable metabolites.

The metabolites did not prevent normal growth in *B. cinerea* cultures after 6PP was depleted, and they did not appear to be significantly metabolized in subsequent growth. This result implies that the changes to the pentyl side chain reduced the toxicity in *B. cinerea*. It has previously been proposed that absorbed 6PP could act by forming a water-impermeable barrier on hyphal cells (Scarselletti and Faull, 1994). Viterbo *et al.* (1993) proposed that laccase inhibition in *B. cinerea* by cucurbitacin D and I and derivatized forms was related to their relative solubilities in cell membranes, as this would determine the extent to which they could penetrate the cells. The proportion of the total 6PP that could be absorbed onto fungal structures would increase greatly as its concentration approached its water solubility (0.60 μg/mL; unpublished data). This relationship is consistent with both the increasing toxicity of increasing concentrations to *B. cinerea* and also the upper concentration limit observed in 6PP-producing *Trichoderma* cultures (Tekin *et al.*, 1995). As the observed metabolites of 6PP produced by *B. cinerea* are less

Table 2. Mass Spectra and Proposed Structures of Compounds Identified by GC-MS of Diazomethane-Reacted Extracts of *B. cinerea* Cultures Containing 6-Pentyl-2-pyrone

compound	assigned structure[a]	RT[b] (min)	molecular ion and principal peaks (relative intensities)
I	6-pentyl-2-pyrone	19.32	M^+: 166 (25); 138 (6), 110 (36), 95 (100), 81 (33)
II-Me	methyl 3-(2-pyron-6-yl)propanoate	11.56	M^+: 182 (33); 151 (33), 123 (49), 122 (77), 95 (100), 81 (17)
III-Me	methyl 5-(2-pyron-6-yl)pentanoate	13.27	M^+: 210 (16); 178 (34), 123 (100), 95 (81), 81 (11)
IV	5-(2-pyron-6-yl)pentanol	14.55	M^+: 182 (15); 164 (22), 123 (56), 122 (100), 110 (82), 95 (100), 94 (55)

[a] II-Me and III-Me are methyl esters resulting from the reaction of diazomethane with the free carboxylic acids. [b] RT, retention time.

lipophilic than 6PP, they would be less readily absorbed on fungal surfaces. This reduced lipophilicity would explain both their reduced toxicity and their stability in *B. cinerea* cultures.

It is considered that formation of secondary metabolites is a highly strain specific, late change in fungal evolution (Vining, 1990). The formation of metabolites often peaks at the transition to sporulating growth, perhaps fulfilling a temporary requirement to protect vulnerable transitional structures. The observed metabolites have not been reported previously. The (structurally) related compound 6-(penten-1-yl)-2-pyrone has been observed in *Trichoderma* cultures (Moss *et al.*, 1975), although it is not clear whether it is a *Trichoderma* secondary metabolite or whether it is formed as a degradation product of the 6PP formed in these cultures. 6PP has been reported to have antimicrobial properties (unpublished work cited by Claydon *et al.*, 1987). In *B. cinerea*, the 6PP alkyl side chain is evidently important in determining its toxicity, and the ability to oxidize this side chain may represent an adaptation in *B. cinerea* to competitive pressure from *Trichoderma* or other fungal species that produce secondary metabolites with similar structures.

ABBREVIATIONS USED

6PP, 6-pentyl-2-pyrone; MSTFA, *N*-methyl-*N*-trimethylsilyltrifluoroacetamide; PDA, potato dextrose agar; AR, analytical reagent grade; HPLC, high-performance liquid chromatography; UV, ultraviolet; EI, electron impact; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; GC-MS, gas chromatography−mass spectroscopy.

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