5-(2-CARBOXYETHYL)-6-HYDROXY-7-METHOXYBENZOFURAN, A Fungal Metabolite of Xanthotoxin

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Key Word Index—Gibberella pulicaris; Fusarium sambucinum; xanthotoxin; xanthotoxol; fungal metabolism; phytoalexin; furanocoumarin.

Abstract—Tolerant strains of Gibberella pulicaris (anamorph: Fusarium sambucinum) metabolize the phytoalexin xanthotoxin to 5-(2-carboxyethyl)-6-hydroxy-7-methoxybenzofuran. This compound does not inhibit the growth of strains that are sensitive to xanthotoxin itself and all xanthotoxin-tolerant and xanthotoxin-sensitive strains tested were able to further degrade the metabolite. This is the first report of fungal detoxification of a furanocoumarin.

RESULTS AND DISCUSSION

Earlier, we investigated the effects of furanocoumarins on the growth and metabolism of strains of the trichothece toxin-producing fungi Gibberella pulicaris (Fr.) Sacco (anamorph: Fusarium sambucinum Fuckel) and Fusarium sporotrichioides Sherb. [1-3]. Xanthotoxin (Ia) is one of the most common furanocoumarins produced by plants and, among those tested, proved to be the most potent in affecting the growth and metabolism of these plant pathogenic fungi. In general, G. pulicaris strains that had been isolated from diseased plant tissue were tolerant of and able to metabolize xanthotoxin while those isolated from soil were restricted in growth and were unable to alter its structure [3]. As an extension of these studies, we now report the structure of 2b, an early metabolite of xanthotoxin formed by these fungi. This compound, earlier isolated in microgram quantities from the urine of a goat that had been fed [14C]-labelled xanthotoxin [4], had been tentatively characterized by GC-MS and by 1H NMR of that small sample. Although xanthotoxin metabolism has been extensively studied in other organisms [5], this is the first report of 2b as a fungal degradation product and, to our knowledge, the first report of fungal detoxification of a furanocoumarin.

INTRODUCTION

We first hypothesized that the fungal metabolite might be xanthotoxol (Ib) and that the process involved a demethylation step in a manner similar to the detoxification of the pterocarpan pisatin by Nectria haematococca [6]; conversion of xanthotoxin to xanthotoxol is a known metabolic pathway in mammals and birds [5]. However, neither tolerant nor sensitive strains of either fungal species metabolized exogenously applied xanthotoxol (data not shown); and it was not found in the growth media after xanthotoxin treatment. When [14C]-labelled xanthotoxin was incubated with a xanthotoxin-tolerant strain of G. pulicaris and the reaction studied over time, the label was systematically transferred to the aqueous phase of the system. Acidification of this aqueous phase returned all of the labelled material to the organic phase, suggesting that a free acid had been formed. In a larger-scale preparation with unlabelled xanthotoxin, methylation of the organic phase followed by TLC gave the methyl ester 2a, identified by its mass and 1H NMR spectra. Subsequently, about 10 mg of the metabolite 2b was recovered from xanthotoxin-amended agar plates of strain R-6380 (G. pulicaris) and an additional 30 mg was synthesized. The effects of 2b and xanthotoxin on selected xanthotoxin-tolerant and xanthotoxin-sensitive strains of G. pulicaris are shown in Table 1. Sensitive strains R-110, R-5920, R-8135 and R-5867 [3] are clearly inhibited by and unable to metabolize xanthotoxin while strain R-6380 is tolerant of and able to metabolize it completely. All strains tested are tolerant of and able to further metabolize 2b (as evidenced by its disappearance) with strain R-5867 showing some growth inhibition. Metabolic products of 2b were not characterized. Residual metabolite 2b was recovered from xanthotoxin-treated plates inoculated with strain R-6380. Although little is known about the enzymatic reduction of furanocoumarins, it is interesting to speculate on...
the mechanism of production of 2b [4]. Because all coumarins are extremely stable in the lactone form [7], it would seem unlikely that the ring is opened before reduction unless the 3,4-double bond is immediately isomerized to the trans geometry yielding a stable hydroxy acid. In the lactone form, the 3,4-double bonds of coumarins are always more difficult to chemically hydrolyze than are double bonds in a side chain or the non-nuclear double bond of a furanocoumarin [7]. If the fungus has overcome this difficulty, production of 2b would follow because hydrolytic opening of the lactone ring of a 3,4-dihydrocoumarin gives a hydroxy acid with little tendency to lactonize [7]. Therefore, fungal reduction of xanthotoxin probably proceeds by opening of the lactone ring followed by isomerization and reduction or by hydrogenation of the 3,4-double bond followed by, or concomitant with, opening of the lactone ring. In any case, conversion of xanthotoxin to metabolite 2b greatly reduces its fungal toxicity, as evidenced by the growth of xanthotoxin-sensitive strains in its presence. All strains tested were able to degrade 2b to unknown products. While the data in Table 1 were obtained only from strains of G. pulicaris, preliminary investigations of tolerant strains of F. sporotrichioides indicate that the same metabolite is formed.

**EXPERIMENTAL**

Xanthotoxin was purchased from Sigma. Xanthotoxinol and \[^{14}\text{C}\]labelled xanthotoxin were prepared by the method of ref. [8]. The specific activity of the labeled compound was 68 \(\mu\)Ci mmol\(^{-1}\). GC-MS and \(^1\)H NMR spectra were obtained and HPLC analyses were carried out as previously described [3, 10].

**Culture methods.** The strains of G. pulicaris used in this study were obtained and identified as previously described (R-6380 from *Solanum tuberosum* Germany; R110 from *Pinus* U.S.A.; R-8135 from soil, South Africa; R-5867 from soil, Australia; and R-5920 from *Pinus*, Australia) [3]. For long-term storage, strains were maintained on V-8 agar media (M-20) [9] at 4° and as lyophilized conidial suspensions in the Agricultural Research Service Collection, Peoria, Illinois. For each experiment, fresh transfers of each strain were grown on the same medium on an alternative 12 hr 25° light/20° dark schedule.

For fungitoxicity and metabolism studies, duplicate 35 \(\times\) 10 mm plastic Petri dishes containing 1 ml of medium and 1% DMSO with or without 200 \(\mu\)g of the test compounds were inoculated with plugs (2 mm diameter) cut from the growing margin of cultures less than 10-days-old and placed with the mycelial surface appressed to the agar surface at the edge of the plate. Plates were incubated for 7 days at 25° in the dark. The radius (from the inoculum plug to the growing margin) was measured daily for at least 7 days, or until fungal growth reached the opposite edge of the plate. Radial growth rates were approximately linear for all strains either in the presence or in the absence of test compounds.

For \[^{14}\text{C}\]xanthotoxin feeding experiments, conidia from strain R-6380 were used to inoculate 25-ml volumes of Czapek-Dox liquid medium (M-83) [9] in two 50-ml Erlenmeyer flasks. Cultures were incubated at 28° in the dark at 200 rpm. After 24 hr incubation, the mycelia were harvested, washed with sterile distilled H₂O and suspended (2 g wet wt) in 20 ml of 50 mM K-Pi, pH 6.5. The culture was then incubated at 25° in a water bath reciprocal shaker at 120 rpm. \[^{14}\text{C}\]Xanthotoxin (1 mg in 200 \(\mu\)l DMSO) was added to the culture and two 500 \(\mu\)l samples were taken at 0-, 1/2-, 1-, 2-, 3-, 4-, 8- and 24-hr intervals. Total radioactivity was determined by liquid scintillation counting of a 500 \(\mu\)l sample in 5 ml of Aqueous Counting Scintillant (Amer­sham) and was unchanged over the course of the experiment. Organic-phase soluble radioactivity was determined by liquid scintillation counting of a 500 \(\mu\)l sample in 5 ml of Organic Counting Scintillant (Amer­sham). Over the course of the experiment, organic-phase soluble radioactivity gradually decreased to less than 30% of the original activity. When a sample taken after 2-hr incubation was acidified by the addition of 500 \(\mu\)l of 3 M HCl to the counting vial in the presence of Organic Scintillant, counts increased from 75 to 100% of control.

**Extraction and preparation of 2a and 2b.** After the 7-day growth period, agar was removed from the assay plates, dried, and extracted with EtOAc. Residual xanthotoxin was quantitated by HPLC [10] of an aliquot of this solution. For production of 2b, plates containing a total of 60 mg xanthotoxin and inoculated with strain R-6380 were handled in the same manner. After extraction and solvent removal, the residue was taken up in Et₂O and esterified with CH₂N₂. Ester 2a (10 mg) was pu­

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Radial growth rate (mm/day)*</th>
<th>Recovery†</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Xanthotoxin</td>
</tr>
<tr>
<td>R-6380</td>
<td>6.5±0</td>
<td>5.2±0.2</td>
</tr>
<tr>
<td>R-110</td>
<td>5.5±0.5</td>
<td>0.4±0.1</td>
</tr>
<tr>
<td>R-8135</td>
<td>4.2±0.2</td>
<td>0.9±0.2</td>
</tr>
<tr>
<td>R-5867</td>
<td>6.5±0</td>
<td>0.4±0.2</td>
</tr>
<tr>
<td>R-5920</td>
<td>4.5±0.5</td>
<td>0.6±0</td>
</tr>
</tbody>
</table>

* Average and range of two trials on 1-ml agar plates amended with 200 \(\mu\)g of test compound in 1% v/v DMSO. Incubated seven days in the dark at 25°.

† As per cent of controls, measured by HPLC [10]. Average of two plates—essentially all 200 \(\mu\)g of xanthotoxin were recovered from the control plates.

‡ A total of ca 60 \(\mu\)g of metabolite recovered from these plates.
A fungal metabolite of xanthotoxin was refluxed 3 hr in 10% NaOMe in MeOH. After most of the solvent had been removed by evapn, the residue was partitioned between Et₂O and H₂O. The aq. layer was made to pH 4 with dilute HCl and then extracted with EtOAc. Following solvent removal, the residue was refluxed 1 hr in 0.5% H₂SO₄ in MeOH and the product was extracted with Et₂O. The ester was recovered by evapn and taken up in EtOH containing 5% hydrazine hydrate and was agitated for 8 hr by a stream of O₂. Solvent removal and extraction with EtOAc gave a product that consisted primarily of 2a and its unsaturated analogue. Final purification was accomplished by HPLC on a 9.4 × 250 mm Zorbax ODS-2 (Dupont) column eluted with MeCN−H₂O (1:1). Saponification in 5% NaOH−EtOH followed by acidification and extraction gave 30 mg of 2b.

REFERENCES