Inhibition of Polyketide Synthesis in *Alternaria alternata* by the Fatty Acid Synthesis Inhibitor Cerulenin

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The fatty acid synthase inhibitor cerulenin (50 to 100 μg/ml) inhibited production of the polyketide mycotoxins alternariol (AOH) and alternariol monomethyl ether (AME) by the mold *Alternaria alternata*. The results suggested that AOH synthesis was inhibited by a direct mechanism by cerulenin, whereas production of AME was probably limited by a shortage of the precursor AOH.

The antibiotic cerulenin has been shown to inhibit lipid biosynthesis by an irreversible inhibition of β-ketoacylacyl carrier protein synthase (condensing enzyme) of the fatty acid synthase (FAS) (2). FAS from a wide variety of organisms, including fungi and bacteria, has been found to be inhibited by cerulenin (14), and Funabashi et al. (4) suggested that cerulenin binds irreversibly to a cysteine residue at the active site of β-ketoacylacyl carrier protein synthase in yeast FAS. Cerulenin also inhibited sterol synthesis in yeast cells by inhibiting β-hydroxy-β-methylglutaryl coenzyme A synthase involved in mevalonate synthesis (13).

Polyketides include several mycotoxins, e.g., the toxic and carcinogenic aflatoxins, and also many antibiotics. Polyketides are synthesized by the condensation of simple carboxylic acids such as acetate and malonate in a way similar to the manner in which fatty acids are, but the reductions required during fatty acid synthesis are limited in polyketide formation (8). Synthesis of many polyketides is also inhibited by cerulenin, and it has been suggested that cerulenin inhibits the condensation step in polyketide synthesis as well (10–12, 15).

The most common polyketide mycotoxins produced by *Alternaria alternata* are alternariol (AOH) and alternariol monomethyl ether (AME). AOH is presumed to be the first polyketide formed (5), and AME is synthesized by S-adenosylmethionine-dependent methylation of AOH, catalyzed by an AOH-O-methyltransferase (AOH-MT) (18). The production of AOH and AME was strongly reduced when the fungus was grown in light (17). In light, however, the lipid content of the mycelia was 25% higher than that in darkness (7). The aim of the present work was to study the effect of the FAS inhibitor cerulenin on the synthesis of AOH and AME by *A. alternata*.

*A. alternata* (Fr.) Keissler, strain At 32, was used in this study. Strain preservation and fungal growth conditions have been described in detail by Haggblom and Niehaus (6).

Briefly, the fungus was grown as drop cultures on the bottom of plastic petri dishes. Each drop culture contained 0.25 ml of modified Czapek Dox medium (5) and 10³ spores. The petri dishes were sealed and placed in darkness at 20°C. Cerulenin (Sigma Chemical Co., St. Louis, Mo.) was added to a concentration of 50 or 100 μg/ml (0.22 or 0.45 mM, respectively) in 1% ethanol (final concentrations) to the cultures under aseptic conditions at 3 or 5 days after inoculation. The petri dishes were then sealed and reincubated at 20°C in darkness. Cultures were harvested 4 to 7 days after inoculation and frozen at −20°C until polyketide extraction. AOH and AME were quantified by thin-layer chromatography by the method of Häggblom and Unestam (7), but with toluene-dioxane-acetic acid (95:25:4; vol/vol/vol) as the developing solvent for the thin-layer chromatography plates. Mycelial dry weights were determined after the mycelia were dried for 4 h at 105°C.

For preparation of enzyme extracts, cultures of *A. alternata* were grown for 6 days in darkness at 20°C without any additions. Mycelia were harvested, washed with deionized water, frozen at −70°C, and immediately lyophilized. Lyophilized mycelia were stored at −20°C with a dessicant until enzyme extraction. Preparation of enzyme extracts was done as described by Orvehed et al. (16). Activity of AOH-MT was measured as described by Orvehed et al. (16), but without prior desalting of the extracts. FAS activity was measured spectrophotometrically by following the A₄₅₀ in a total volume of 300 μl (1). The assay mixture contained 50 μM acetyl coenzyme A, 70 μM malonyl coenzyme A, 0.2 mM NADPH, 0.9 mg of bovine serum albumin per ml (Sigma), and 50 to 100 μl of enzyme extract in 0.1 M sodium phosphate (pH 7.0), 1 mM EDTA, 1 mM β-mercaptoethanol, 0.2 mM phenylmethylsulfonylfluoride, and 20% glycerol at 25°C.

Cerulenin inhibition of FAS activity in vitro was studied by adding 50 or 100 μg of cerulenin per ml (0.22 and 0.45 mM, respectively) in 1% ethanol (final concentrations) to the enzyme extracts, which were then incubated for 1 h on ice. The activities of FAS and AOH-MT were measured before and after removal of low-molecular-weight compounds from the extracts by gel filtration on Sephadex G-25 (PD 10 column; Pharmacia, Uppsala, Sweden).

Cerulenin addition to drop cultures of *A. alternata* 3 days after inoculation, i.e., before the initiation of AOH and AME accumulation (16), resulted in a dose-dependent reduction of the polyketide content of the cultures, while the dry weight was only slightly affected by this addition (Fig. 1). In comparison with results of controls that received 1% ethanol, the addition of cerulenin to the cultures when polyketide synthesis had started (5 days) caused a rapid decrease in the AOH content. Further polyketide accumulation was inhibited by both cerulenin concentrations and was not restored during the remaining incubation period (Fig. 2).

The activity of FAS in vitro was not affected in controls with 1% ethanol compared with its activity in untreated extracts. Cerulenin, on the other hand, caused a decrease in FAS activity to approximately 30% of that of the controls.

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Days after inoculation

FIG. 1. Accumulation of AOH (a), AME (b), and dry weight (c) by drop cultures of A. alternata. Cerulenin (50 μg/ml [•] or 100 μg/ml [△]) or, as a control, 1% ethanol (○) was added after 3 days of growth (arrow). The results are the means of findings for eight mycelia in two separate experiments. For AOH and AME, the standard deviation is represented by bars.

The accumulation of AOH and AME by A. alternata was reduced by the addition of cerulenin to the growth medium before the onset of polyketide production even at concentrations that did not affect mycelial growth. This is in agreement with results showing cerulenin inhibition of the synthesis of most other polyketides, including macrolide antibiotics (11, 15), fungal melanin (10), and the fungal metabolite 6-methylsalicylic acid (12).

If cerulenin was added to A. alternata cultures during the polyketide synthetic phase, further polyketide accumulation was immediately inhibited. The rapid inhibition of AOH accumulation after cerulenin addition suggests that there was a direct effect of cerulenin on the synthesis of AOH.

TABLE 1. Effect of cerulenin on FAS and AOH-MT activities

<table>
<thead>
<tr>
<th>Addition to culture</th>
<th>Activity (%)</th>
<th>FAS</th>
<th>AOH-MT</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100^</td>
<td>100^</td>
<td></td>
</tr>
<tr>
<td>1% Ethanol</td>
<td>96 ± 19</td>
<td>116 ± 33</td>
<td></td>
</tr>
<tr>
<td>Cerulenin 50 μg/ml</td>
<td>35 ± 7</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>100 μg/ml</td>
<td>32 ± 12</td>
<td>106 ± 20</td>
<td></td>
</tr>
</tbody>
</table>

^ Values ± standard deviations were obtained from three (FAS) or two (AOH-MT) separate experiments.

^ FAS activity in extracts without any addition varied between 8.9 and 13.4 nmol min⁻¹ mg of protein⁻¹, and AOH-MT activity varied between 0.4 and 0.6 nmol min⁻¹ mg of protein⁻¹.

—, Not tested.
drop in AOH content during the first 12 h after cerulenin addition may have been due to a polyketide turnover which occurs during this period (9). Since no in vitro effect of cerulenin on the AME-forming enzyme AOH-MT was found, the AME accumulation, which was directly related to the AOH content of the cultures, was most likely reduced because of a shortage of the precursor AOH.

These results are in contrast with those obtained with Aspergillus parasiticus. The synthesis of the polyketide aflatoxins in A. parasiticus was greatly stimulated when cerulenin (100 µg/ml) was added to the growth medium (3), but the mechanism causing the stimulation is not known.

In the present work, 68% of in vitro activity of FAS was inhibited irreversibly by cerulenin (100 µg/ml). The activities of FAS and the polyketide synthase 6-methylsalicylic acid synthase from Penicillium urticae were 68 and 60% inhibited in vitro, respectively, by the same cerulenin concentration (12), whereas inhibition of the affected enzyme in yeast sterol synthesis required a much higher cerulenin concentration (13). Results in the present work support the view that cerulenin inhibits AOH synthesis in A. alternata, but the mechanism by which this occurs is still unknown, mainly because no in vitro assay for the enzyme involved in the synthesis of AOH is yet known (5, 19).

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REFERENCES