Inhibition of Ergosterol Biosynthesis by Etaconazole in
Ustilago maydis

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The triazole fungicide etaconazole (CGA 64 251) interferes with the ergosterol biosynthesis in
Ustilago maydis by inhibiting the C-14 demethylation of the sterol nucleus. During the late log
growth phase of U. maydis a novel endogenous sterol metabolite (14β-methyl-ergosta-8,24(28)-
dien-3β,6α-diol) was discovered and analyzed, which accumulates under the influence of the
fungicide. The structure of this metabolite points to a hydroxylation-dehydration mechanism for
the introduction of the double bond at C-5 during the ergosterol biosynthesis.

Introduction

In the past decade a series of substances have
been discovered that inhibit the biosynthesis of
ergosterol. Some of them have been developed as
fungicides and introduced to practical application.
They include various structural classes, such as
piperazines (triforine), pyridines (S-1358), pyrimi-
dines (fenarimol, triarimol), triazoles (triadimefon),
and others [1]. More recently, further analogues of
one of these classes of compounds, namely the
triazoles, have been developed as plant protection
fungicides, such as etaconazole and propiconazole
[2, 3]. All these fungicides inhibit ergosterol biosyn-
thesis only a few hours after incubation and at the
same time various ergosterol intermediates accu-
mulate. Inhibition of C-14 demethylation in the
sterol nucleus by these fungicides and also by
etaconazole [4] is regarded as the mechanism of
action that accounts for their fungitoxicity (for
review see Siegel [5].

A further group of plant protecting fungicides
that interferes with ergosterol biosynthesis are mor-
pholines (tridemorph). They specifically inhibit the
C-8 double bond isomerization to position 7 [6].

In the case of the triazole fungicides in previous
studies the early log growth stage had always been
investigated, as well as the accumulation of the
various sterol intermediates of ergosterol biosyn-
thesis studied after an inhibition time of only a few
hours. It was of interest for us to follow up the
appearance and accumulation of the various sterol
intermediates of ergosterol biosynthesis throughout
the entire growth phase of the fungus under the
influence of etaconazole.

Methods

1. Culture methods

Sporidia of Ustilago maydis (DC) Cda. (CBS
132.08) were grown in a nutrient medium according
to the method described by Buchenauer [7]. The
initial concentration was 0.05 mg ml⁻¹ dry weight.
After 4 h, when the dry weight had reached
0.14 mg ml⁻¹, the fungicide dissolved in acetone
(0.5%) was added. The checks were treated with
0.5% acetone. The inhibitory action of etaconazole*
on the growth of the fungus was registered as a
growth curve recording dry weight determinations.
The values were obtained from three tests each with
3 replications. After 4, 13, 20, and 44 h after treat-
ment, respectively, the morphology of the hyphal tips
was examined microscopically.

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* BSI/ISO Common name proposed for the compound
CGA 64 251.
2. Sterol analysis by gas chromatography

The total lipids were extracted from the washed and lyophilized sporidia with chloroform and methanol (2:1 v/v) following the procedure of GC analysis described by Buchenauer [7]. In order to determine the relative retention time and to quantify the amount of the various sterol intermediates which were present, cholesterol and ergosterol, respectively, were used as standards.

3. Isolation and characterization of a new sterol metabolite

The sterol fraction (approx. 2000 mg) was evaporated to dryness and the residue extracted with di-i-propyl ether/MeOH (1/1). The extract (approx. 1000 mg) was chromatographed on silica gel. Chromatography was performed on a Lichroprep Si 60 column Merck, 40–63 μm (310 x 25 mm) with a flow of 1.5 ml/min.

The following eluents were subsequently used: a) di-i-propyl ether (fractions 1–3), b) lin. gradient → 20% MeOH in di-i-propyl ether (fractions 4–6), c) 20/80 MeOH/di-i-propyl ether (fractions 7–10). The raw product (fraction 9, 55 mg) was purified by HPLC on silica gel (2 ×) with MeOH/di-i-propyl ether (2/98) as the eluent. A HP 1084 B Liquid Chromatograph Hewlett Packard equipped with a Lichrosorb SI 60 column Merck, 5 μm (250 × 4.9 mm) was used. The flow was 1.0 ml/min. The raw product obtained (5.9 mg) was further purified by HPLC on silica gel (2 ×) with MeOH/di-i-propyl ether (3/97) as the eluent. The pure product (5.2 mg) was crystallized from hexane/di-i-propyl ether (95/5), m.p.: 167–168 °C. All separation procedures were monitored by gas-liquid chromatography as the metabolite lacks acceptable UV absorption.

4. Methods of structure analysis of the new sterol metabolite

MS spectra were recorded with a Varian MAT CH 7 (70 eV ionizing voltage, 250 °C ion source temperature, direct sample insertion) and a Finnigan 4000 mass spectrometer (GC-MS, column OV-17). The 1H NMR spectrum was recorded with a Bruker HX-360 spectrometer in the FT mode, and the 13C NMR spectrum with the same apparatus at 90.52 MHz. Deuterochloroform was used as the solvent, and the deuterium resonance of the solvent served as the lock signal.

Results

1. Effects of Etaconazole on mycelial growth and dry weight increases of culture

The dry weight increases in *Ustilago maydis* culture were observed throughout the entire log growth phase of the fungus culture. In our system (Fig. 1) the log growth phase of *U. maydis* in the control cultures is finished after about 30 h. The dry weight of the sporidia grown under the influence of the fungicide was, as expected, reduced.

Eight, 17, 24, and 48 h after the start of the experiment, microscopic examination revealed severe swelling of hyphal tips in the treated cultures as
referred to in the literature [4]. Sporidia treated with fungicide that were removed after cultivation during 24 and 48 h, respectively, and placed in a fresh nutrient medium showed further increases in growth and dry weight (data not shown). From this it is concluded that the hyphae are not killed and that the action of etaconazole is fungistatic.

2. Effect of Etaconazole on sterol metabolism

The appearance and the quantitative distribution of the sterol metabolites were investigated after 8, 17, 24, and 48 h, respectively, (Table I). Ergosterol (8) with the relative retention time $t_R$ of 1.2 was already sharply reduced after 4 h by the fungicide treatment, and at the later intervals could no longer be detected. The intermediate 7 to which the structure of 22-dihydroergosterol was tentatively assigned [8] (cf. Scheme 1) could no longer be detected at any of the test intervals. On the other hand, the various methylated intermediates of ergosterol biosynthesis accumulated as mentioned in the literature [1]. These are 24-methylenedihydrolanosterol (3), obtusifoliol (4), and 14a-methyl-ergosta-8,24(28)-dien-3β,6α-diol (5).

Our special attention was drawn to a peak at $t_R = 2.37$ indicating a new ergosterol metabolite (9), so far not mentioned or described in the literature (Fig. 2). It is not yet visible after 8 h, but is found at all the other intervals after all rates of treatment. The GC peak was particularly marked for cultures referred to in the literature [4]. Sporidia treated with fungicide that were removed after cultivation during 24 and 48 h, respectively, and placed in a fresh nutrient medium showed further increases in growth and dry weight (data not shown). From this it is concluded that the hyphae are not killed and that the action of etaconazole is fungistatic.

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**Table I.** Quantities of sterols detected in control and etaconazole (1, 10 and 100 ng/ml) treated mycelia of *Ustilago maydis* 4, 13, 20, and/or 44 h after treatment (corresponds to 8, 17, 24 and/or 48 h after start of the experiment).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Control</th>
<th>Etaconazole 1 ppm</th>
<th>Etaconazole 10 ppm</th>
<th>Etaconazole 100 ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>39.2</td>
<td>371.2</td>
<td>466.7</td>
<td>931.0</td>
</tr>
<tr>
<td>2</td>
<td>39.2</td>
<td>371.2</td>
<td>466.7</td>
<td>931.0</td>
</tr>
<tr>
<td>3</td>
<td>15.8</td>
<td>83.0</td>
<td>20.8</td>
<td>43.4</td>
</tr>
<tr>
<td>4</td>
<td>60.5</td>
<td>148.4</td>
<td>9.6</td>
<td>23.8</td>
</tr>
<tr>
<td>5</td>
<td>221.3</td>
<td>386.4</td>
<td>246.1</td>
<td>483.3</td>
</tr>
<tr>
<td>6</td>
<td>87.1</td>
<td>185.1</td>
<td>47.2</td>
<td>23.8</td>
</tr>
<tr>
<td>7</td>
<td>246.1</td>
<td>483.3</td>
<td>191.4</td>
<td>133.6</td>
</tr>
<tr>
<td>8</td>
<td>431.6</td>
<td>87.1</td>
<td>87.1</td>
<td>558.1</td>
</tr>
<tr>
<td>9</td>
<td>558.1</td>
<td>13.7</td>
<td>558.1</td>
<td>13.7</td>
</tr>
</tbody>
</table>

* Quantities (averaged from three experiments) are based on cholesterol as standard. — = not detected.

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**Fig. 2.** Gas chromatographic analysis of saponified sterols in sporidia of *Ustilago maydis* after an incubation time of 44 h: A) sterols of control sporidia (authentic cholesterol $t_R = 1.00$/ergosterol $t_R = 1.20$); B) sterols of sporidia treated with etaconazole (100 μg/ml).
Scheme 1. Sterol biosynthesis indicating major points of inhibition by etaconazole (source: Ragsdale 1977 [1], Henry and Sisler 1981 [4]).
that had been treated for 24 and 48 h with 100 ppm of the fungicide. The metabolite was isolated from these cultures and identified.

3. Determination of the structure of sterol metabolite 9

On the basis of spectroscopic investigations (MS and NMR) it was possible to establish the structure 9 for the metabolite: 14α-methyl-ergosta-8,24(28)-dien-3β,6α-diol. The following spectroscopic data justify this structure:

Mass spectrum. The mass spectrum of the compound agrees with the proposed structure. The molecule ion M⁺ (m/z 428, C₂₉H₄₈O₂) very easily loses a CH₃ radical (m/z 413, perhaps preferentially from the allylic C-14 position), followed by the two hydroxyl groups as H₂O molecules (m/z 395 and 377). The most conspicuous fragment in the upper mass range m/z 329 (C₂₂H₃₃O₂) and its two dehydration products m/z 311 and 293 (C₂₂H₃₁O and C₂₂H₂₉) are presumably due to a double loss of CH₃/CH₂ groups. A fragmentation sequence m/z 572 (M) → m/z 557 (M−CH₃) → m/z 467 (557−Me₃SiOH) → m/z 377 (467−Me₆SiOH) corresponds, at least formally, to the gradual CH₃/H₂O/H₂O loss in the underivatized compound. The above C₈H₁₂/CH₃ double loss has also a parallel in a weak peak at m/z 473.

¹H NMR spectrum. Fig. 3 shows the ¹H NMR spectrum of the sterol metabolite, and Table II gives the structurally relevant data and their assignment. The molecule shows three tertiary methyl groups, one secondary methyl group, one isopropyl group, one terminal methylene group and finally two secondary alcohols. The following can be said regarding the position of the structural elements in the molecule on the basis of the proton data: since the compound under investigation is a sort of precursor of ergosterol, the secondary methyl group, isopropyl group and the terminal methylene group belong most probably to the side-chain bound at C-17. On the other hand the three tertiary methyl groups belong to the basic steroid structure, whereby besides the two usual angular methyl groups at C-10 and C-13 the third methyl group can be assumed to be linked in an α-position at C-14 (by analogy with e.g. lanosterol 2). The splitting patterns of the methine proton signals due to the two secondary alcohols are grounds for concluding the positions 3β OH and 6α OH. Hence the double bond that

Fig. 3. ¹H NMR spectrum of the new sterol metabolite (360 MHz, CDCl₃).
Table II. Characteristic proton signals of the sterol metabolite (9) (360 MHz, CDCl$_3$).

<table>
<thead>
<tr>
<th>Signal</th>
<th>Chemical shift</th>
<th>Multiplicity (coupling constants in Hz)</th>
<th>Number of protons</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>4.71</td>
<td>doublet (dispersed)</td>
<td>1</td>
</tr>
<tr>
<td>b</td>
<td>4.63</td>
<td>doublet (1.5)</td>
<td>1</td>
</tr>
<tr>
<td>c</td>
<td>3.76</td>
<td>triplet of doublet (9; 6)</td>
<td>1</td>
</tr>
<tr>
<td>d</td>
<td>3.60</td>
<td>multiplet</td>
<td>1</td>
</tr>
<tr>
<td>e</td>
<td>1.03</td>
<td>double doublet (12; 12; 6; 6)</td>
<td>6</td>
</tr>
<tr>
<td>f</td>
<td>0.98</td>
<td>singlet</td>
<td>3</td>
</tr>
<tr>
<td>g</td>
<td>0.92</td>
<td>singlet</td>
<td>3</td>
</tr>
<tr>
<td>h</td>
<td>0.93</td>
<td>doublet (7)</td>
<td>3</td>
</tr>
<tr>
<td>i</td>
<td>0.705</td>
<td>singlet</td>
<td>3</td>
</tr>
</tbody>
</table>

According to the empirical formula is still present but that gives no signals in the $^1$H NMR spectrum must be situated between C-8 and C-9. The situation in the region of the C and D rings in the unknown compound is therefore the same as in e.g. lanosterol (2). The $^1$H NMR spectrum of a reference substance* with a terminal methylene double bond between C-24 and C-28 also exhibits the same vinyl proton resonances ($\delta = 4.71$ and $\delta = 4.63$ ppm) as found in the spectrum of the new sterol metabolite.

$^{13}$C NMR spectrum. Table III compares the $^{13}$C data of dihydrolanosterol (10) and cholestan-3β,6α-diol (11) with those of the unknown compound. From this comparison it can be seen that in the region of the C and D rings the sterol metabolite must have the same structure as lanosterol. The data of cholestan-3β,6α-diol further show that the compound under examination must also have a similar structure in the region of the A and B rings. If the data of the cholestadiol 11 are further corrected for the influence of the C-8, C-9 double bond, complete identity is established.

* The reference substance was euphorba-8,24(28)-dien-3,7,11-trion, for the supply of which our gratitude is expressed to Prof. O. Jeger, Swiss Federal Institute of Technology, Zürich.

Table III. $^{13}$C signals and their assignment of the sterol metabolite 9 and of the two reference substances dihydrolanosterol (10) and cholestan-3β,6α-diol (11).

| 1 | 35.3 | 35.6 | 38.8 |
| 2 | 31.1 | 27.7 | 32.9 |
| 3 | 71.1 | 79.0 | 69.5 |
| 4 | 36.0 | 38.9 | 37.3 |
| 5 | 47.7 | 50.4 | 53.4 |
| 6 | 67.6 | 19.1 | 71.7 |
| 7 | 34.9 | 26.5 | 43.5 |
| 8 | 133.0 | 134.4 | 35.4 |
| 9 | 133.6 | 134.4 | 55.1 |
| 10 | 33.8 | 37.0 | 34.2 |
| 11 | 21.7 | 21.0 | 22.3 |
| 12 | 30.3 | 31.0 | 40.9 |
| 13 | 44.5 | 44.5 | 43.3 |
| 14 | 49.6 | 49.8 | 57.4 |
| 15 | 31.1 | 30.8 | 24.9 |
| 16 | 24.3 | 24.3 | 29.1 |
| 17 | 50.2 | 50.5 | 57.4 |
| 18 | 15.7 | 15.8 | 13.0 |
| 19 | 18.3 | 18.3 | 14.4 |
| 20 | 36.4 | 36.5 | 36.8 |
| 21 | 18.7 | 18.7 | 19.7 |
| 22 | 30.7 | 36.5 | 37.3 |
| 23 | 30.9 | 24.1 | 24.9 |
| 24 | 156.8 | 39.5 | 40.5 |
| 25 | 32.0 | 28.0 | 28.5 |
| 26 | 22.0 | 22.5 | 23.4 |
| 27 | 21.8 | 22.8 | 23.4 |
| 28 | 105.9 | — | — |
| 29 | 28.1 | 28.2 | — |
| 30 | — | 27.9 | — |
| 31 | — | 15.4 | — |

Discussion

During the early log growth phase C-14 methyl sterols are accumulated in Ustilago maydis cultures treated with etaconazole. This indicates that the fungicidal mechanism is an inhibition of C-14 demethylation as described earlier by Henry and Sisler [4] for this class of fungicides. During the later log growth phase of the fungus a sterol metabolite is formed that had not been previously noticed for two methodical reasons.

In the methods used by previous authors who investigated the inhibition site of ergosterol bio-
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synthesis by chemicals [7, 10–12]. GC analysis was stopped after reaching a retention time of 2.0 (relative to ergosterol 1.2). Thus, the new metabolite could not be found since it appears only at a retention time of 2.37.

The second reason why this sterol metabolite was missed is that it occurs only in the later log growth phase (after 13 h or longer of incubation with the fungicide). Previous investigations were usually carried out by incubating only a few hours.

The intermediates of ergosterol biosynthesis that appear under the influence of etaconazole in U. maydis essentially fit the pattern of ergosterol biosynthesis in which Ragsdale [1] and Henry & Sisler [4] indicate the major points of inhibition by fungicides. Inhibition of C-14 demethylation leads to accumulation of the ergosterol intermediates 24-methylenedihydrolanosterol (3), obtusifoliol (4), and 14z-methyl-ergosta-8,24(28)-dienol (5). A further sterol intermediate, which occurs in the controls but is lacking in the sporidia treated with etaconazole, is an ergostadienol with a molecular weight of 398; MS indicates one hydroxyl group and two double bonds. Fragmentation of this compound corresponds to the mass spectrum proposed for 22-dihydroergosterol (7) [8].

We suggest that the new sterol compound 14z-methyl-ergosta-8,24(28)-dien-3β,6α-diol (9) is metabolically derived from the known intermediate of ergosterol biosynthesis 14z-methyl-ergosta-8,24(28)-dienol (5) since it is not found before the latter metabolite can be detected (see Table I). Again, the C-14 demethylation in 5 seems to be the crucial point that under the influence of the fungicide cannot be overcome, but at the same time, the formation of the diol 9 is favoured.

The generation of the conjugated diene system in ring B of ergosterol from precursors such as fecosterol (6) involves the isomerization of the C-8 double bond to the C-7 position and, in a second step, the introduction of the double bond at C-5 [13]. In particular, the mechanism of the second step remains unclear as no natural intermediates in this reaction could be found so far. It has been shown that in yeast the C-5 double bond formation in the ergosterol biosynthesis is oxygen-dependent [14]. Two basic mechanisms were proposed for the origin of this double bond:

1. a hydroxylation-dehydration mechanism which requires a hydroxylated intermediate, and
2. a dehydrogenation mechanism.

The structure of the new metabolite 9 with an α-hydroxyl group in the C-6 position of the sterol nucleus can be regarded as an indication that the introduction of the C-5 double bond takes place in accordance with the first mechanism proposed. Its formation under the influence of the fungicide can be understood in the sense that the intact C-14 methyl group in the natural intermediate 5 seems to prevent the migration of the C-8 double bond to the C-7 position. The enzymatic hydroxylation, however, is not inhibited, and, interestingly enough, it can take place at the C-6 α-position even in the absence of an activating allylic C-7 double bond.

Acknowledgements

We particularly thank Dr. W. Richter for recording and interpreting our MS spectra, Dr. B. Donzel for his preliminary work on HPLC separation of the new ergosterol metabolite, and Mr. B. Zussy for his technical assistance.