Inhibition of Ergosterol Biosynthesis by Etaconazole in *Ustilago maydis*

Edith Ebert, John Gaudin, Wolfgang Muecke, Klaus Ramsteiner, and Christian Vogel

Agricultural Division, CIBA-GEIGY Limited, CH-4002 Basel, Switzerland

Hermann Fuhrer

Central Research Laboratories, CIBA-GEIGY Limited, CH-4002 Basel, Switzerland

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 (14α-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), pyrimidines (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 biosynthesis only a few hours after incubation and at the same time various ergosterol intermediates accumulate. 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 morpholines (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 biosynthesis 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 treatment, respectively, the morphology of the hyphal tips was examined microscopically.

---

* 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 × 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 ^{1}H NMR spectrum was recorded with a Bruker HX-360 spectrometer in the FT mode, and the ^{13}C 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
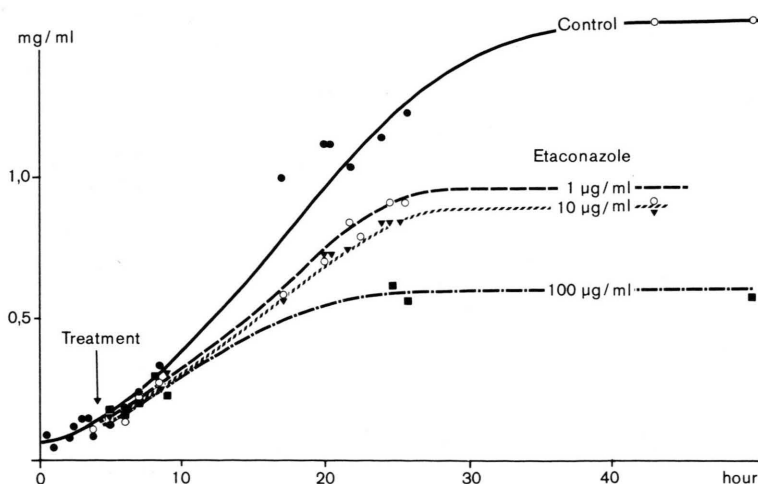

Fig. 1. The effect of etaconazole (1, 10, 100 μg/ml) on sporidial dry weight increase of *Ustilago maydis* liquid cultures.

Table I. Quantities of sterols detected in control and etaconazole (1, 10 and 100 µg/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).

| Com-pound	µg/100 mg dry weight of culture												
	Control				etaconazole								
					1 ppm				10 ppm				100 ppm
1	39.2	371.2	466.7	931.0	5.6	14.0	78.1	487.3	7.8	17.1	78.2	558.1	13.7
2	–	–	–	–	–	–	–	–	–	–	–	–	–
3	15.8	83.0	20.8	43.4	221.3	386.4	246.1	483.3	191.4	545.1	246.1	464.0	146.2
4	–	–	–	–	60.5	148.4	87.1	185.1	47.2	431.6	87.1	281.6	111.1
5	–	–	–	–	–	9.6	23.8	51.7	–	142.1	23.8	133.6	58.6
6	–	–	–	–	–	–	–	–	–	–	–	–	–
7	10.9	205.3	201.0	342.6	–	–	–	–	–	–	–	–	–
8	113.3	233.4	162.3	366.4	71.4	–	–	–	63.4	–	–	–	–
9	–	–	–	–	–	17.2	31.0	45.1	–	21.8	31.0	66.4	92.3
	4	13	20	44	4	13	20	44	4	13	20	44	44

Extraction of sterols h after treatment of culture

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

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.
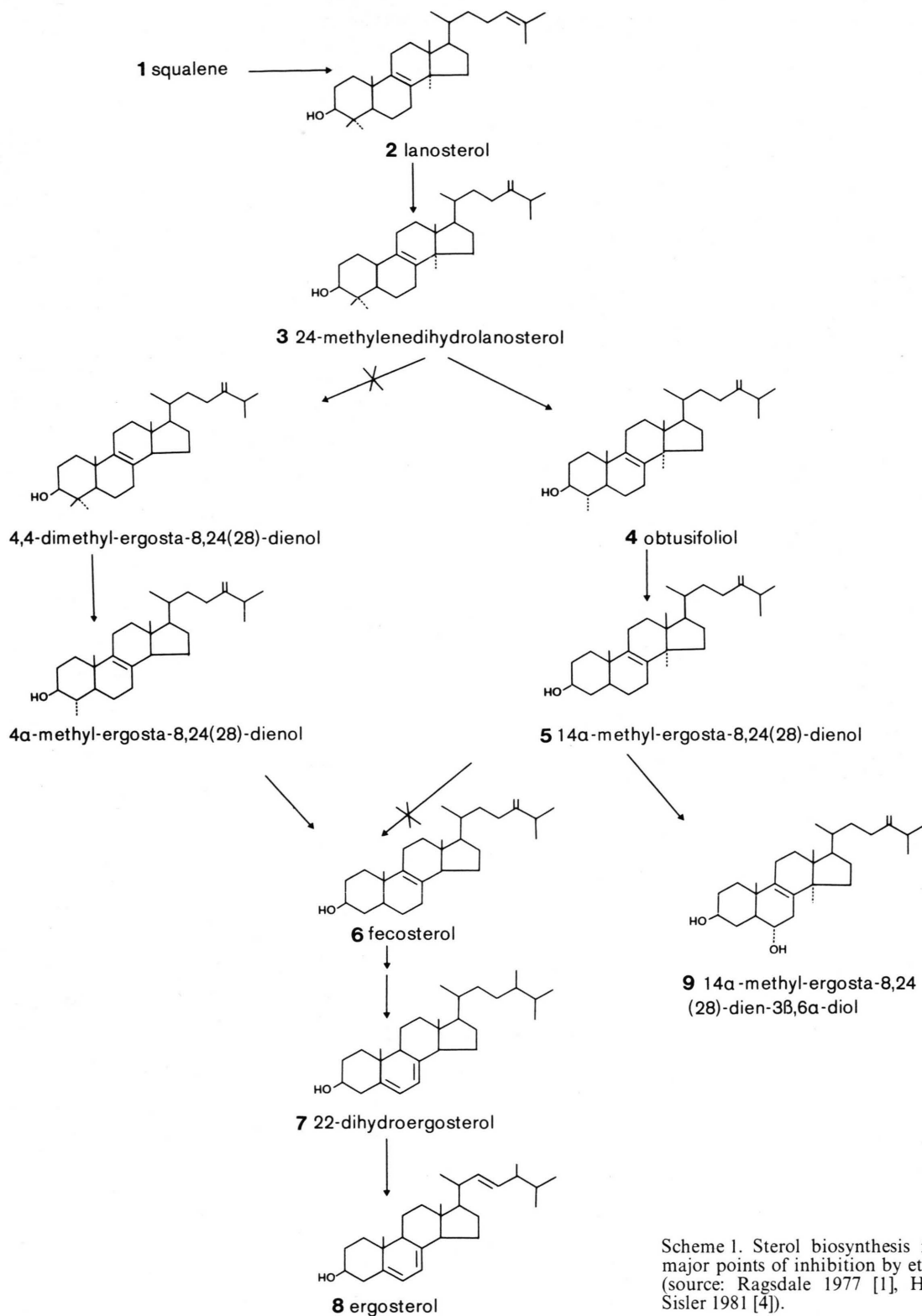
2. Effect of Etaconazole on sterol metabolism

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 14α-methyl-ergosta-8,24(28)-dienol (**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
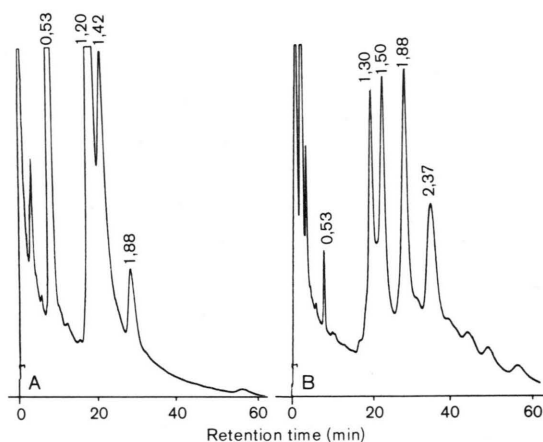
Ergosterol – Intermediates in *Ustilage maydis* (48 h)

Com-pound	Rel. retention time	Name
1	0.53	squalene
2		lanosterol
3	1.88	24-methylendihydrolanosterol
4	1.50	obtusifoliol
5	1.30	14α-methyl-8,24(28)ergostadienol
6		fecosterol
7	1.42	22-dihydroergosterol
8	1.20	ergosterol
9	2.37	14α-methyl-ergosta-8,24(28)-dien-3β,6α-diol

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).

1 squalene

2 lanosterol

3 24-methylenedihydrolanosterol

4,4-dimethyl-ergosta-8,24(28)-dienol

4 obtusifoliol

4α-methyl-ergosta-8,24(28)-dienol

5 14α-methyl-ergosta-8,24(28)-dienol

6 fecosterol

7 22-dihydroergosterol

8 ergosterol

9 14α-methyl-ergosta-8,24
(28)-dien-3ß,6α-diol

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 $M^{+\cdot}$ (m/z 428, $C_{29}H_{48}O_2$) very easily loses a CH_3 radical (m/z 413, perhaps preferentially from the allylic C-14 position), followed by the two hydroxyl groups as H_2O molecules (m/z 395 and 377). The most conspicuous fragment in the upper mass range m/z 329 ($C_{22}H_{33}O_2$) and its two dehydration products m/z 311 and 293 ($C_{22}H_{31}O$ and $C_{22}H_{29}$) are presumably due to a double loss of CH_3/C_6H_{12}. Thus the C_6H_{12} elimination (McLafferty rearrangement of the unsaturated side-chain) would be significant as an index for the position of the 24-methylene double link deduced from the NMR data (*cf.* Zaretskii [9]).

When reacted with BSA, the compound gives a bis(trimethylsilyl) derivative with $M^{+\cdot}$ at m/z 572, corresponding to the silylation of the two hydroxyl groups. A fragmentation sequence m/z 572 (M) → m/z 557 (M−CH_3) → m/z 467 (557−Me_3SiOH) → m/z 377 (467−Me_3SiOH) corresponds, at least formally, to the gradual $CH_3/H_2O/H_2O$ loss in the underivated compound. The above C_6H_{12}/CH_3 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
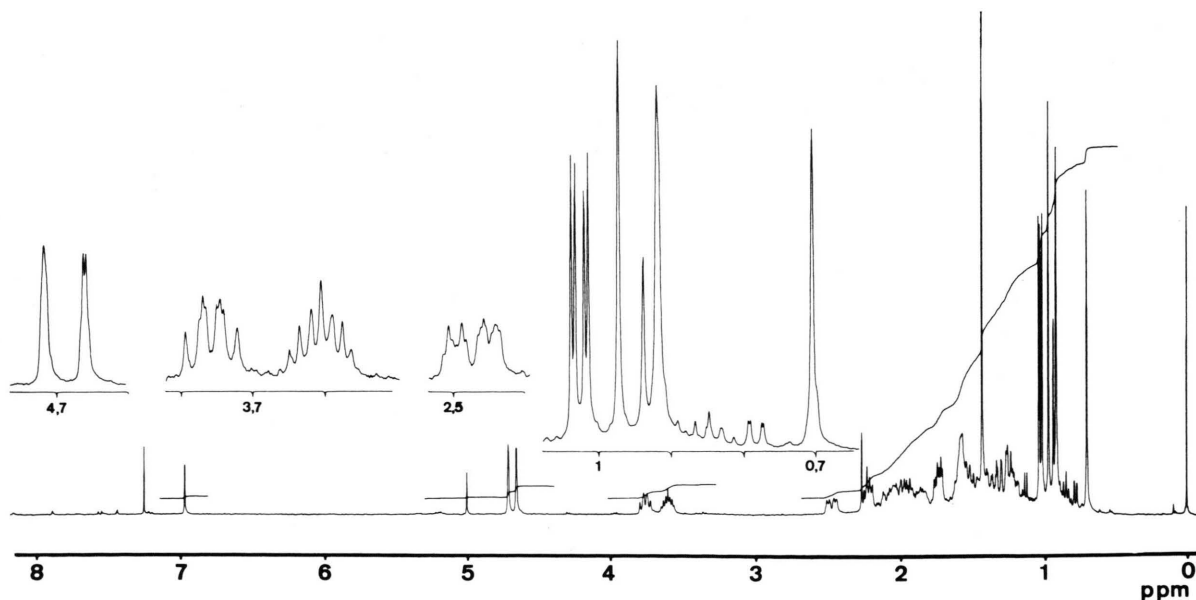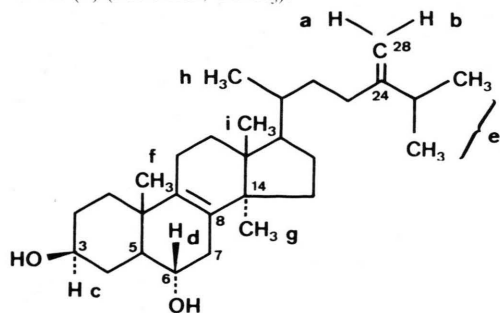

Fig. 3. ^1H NMR spectrum of the new sterol metabolite (360 MHz, $CDCl_3$).

Table II. Characteristic proton signals of the sterol metabolite (**9**) (360 MHz, CDCl₃).

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

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

¹³C	9	10	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	–

according to the empirical formula is still present but that gives no signals in the ¹H 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 ¹H 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.

¹³C NMR spectrum. Table III compares the ¹³C 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 cholestandiol **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.

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-

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 14α-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 14α-methyl-ergosta-8,24(28)-dien-3β,6α-diol (9) is metabolically derived from the known intermediate of ergosterol biosynthesis 14α-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.

[1] N. N. Ragsdale, Antifungal compounds, (M. R. Siegel and H. D. Sisler, eds.), Vol. II, p. 333, Marcel Dekker Inc., New York and Basel, 1977.
[2] T. Staub, F. Schwinn, and P. Urech, Phytopathology 69, 1046 (1979).
[3] P. Urech, J. F. Schwinn, J. Speich, and T. Staub, Proc. Brit. Crop Protection Conference – Pest and Diseases, 508 (1979).
[4] M. J. Henry and H. D. Sisler, Pestic. Sci. 12, 98 (1981).
[5] M. R. Siegel, Plant Disease 65, 986 (1981).
[6] T. Kato, M. Shoami, and Y. Kawase, J. Pestic. Sci. 5, 69 (1980).
[7] H. Buchenauer, Z. Pflanzenkrankheiten, Pflanzenschutz 84, 286 (1977).
[8] R. B. Holtz and L. C. Schisler, Lipids 7, 251 (1972).
[9] Ze'ev V. Zaretskii, Mass spectrometry of Steroids, p. 114, John Wiley & Sons, New York 1976.
[10] J. L. Sherald and H. D. Sisler, Pestic. Biochem. Physiol. 5, 477 (1975).
[11] T. Kato, S. Tanaka, M. Ueda, and Y. Kawase, Agr. Biol. Chem. 39, 169 (1976).
[12] H. Buchenauer, Pestic. Sci. 9, 507 (1978).
[13] J. D. Weete, Fungal lipids biochemistry, p. 175, Plenum Press, New York and London 1974.
[14] M. Akhtar and M. A. Parvez, Biochem. J. 108, 527 (1968).