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STEROL ANALYSIS OF DMI-RESISTANT AND -SENSITIVE STRAINS OF *VENTURIA INAEQUALIS*

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Key Word Index—*Venturia inaequalis*; Pleosporaceae; apple scab; ergosterol biosynthesis; DMI-resistance; fenarimol; ergosta-5,7-dien-3β-ol; ergosta-7,24(24^1)-dien-3β-ol; ergost-7-en-3β-ol; eburicol.

Abstract—The sterol composition of *Venturia inaequalis* strains sensitive and resistant to sterol demethylation-inhibiting fungicides (DMI) were analysed. Without DMI treatment, both strains contained ergosta-5,7-dien-3β-ol, ergosterol, ergosta-7,24(24^1)-dien-3β-ol and ergost-7-en-3β-ol. Ergosta-5,7-dien-3β-ol was the main sterol in both strains. Treatment with fenarimol decreased the 4-desmethyl sterols and accumulated eburicol in both strains, indicating that this fungicide inhibits C14-demethylase of not only the sensitive strain but also the resistant strain. The accumulation of eburicol in the resistant strain occurred at higher concentration of fenarimol than in the sensitive strain.

INTRODUCTION

Sterol demethylation-inhibiting fungicides (DMI) have been used worldwide for the control of apple scab caused by *Venturia inaequalis* (Cooke) Winter. Although the development of DMI-resistance in the field has been slow, there have been several reports of the failure of DMI to control the disease [1, 2].

The mechanism of DMI-resistance has been studied intensively using laboratory-generated mutants of several fungi [3]. However, there have been few studies of the mechanism in field-resistant strains reported to date. In *V. inaequalis*, field-sensitive strains maintained on medium amended with fenarimol often showed reduced sensitivity to this fungicide [4]. Furthermore, on fungicide-free medium, fenarimol sensitivity of some, but not all, field-resistant strains increased [4]. These results are consistent with those of a previous report on instability of DMI (flusilazole)-sensitivity of this fungus [5]. Such phenotypic instability might be involved in the slow development of resistance to DMI in the field. To investigate the biochemical mechanism of DMI-resistance in *V. inaequalis*, we performed comparative analysis of the sterols of sensitive and resistant strains in the presence and absence of fenarimol.

RESULTS AND DISCUSSION

Effects of fenarimol on mycelial growth of DMI-sensitive and resistant strains

Inhibitory effects of fenarimol on mycelial growth of strain 133 (DMI-sensitive) and strain 179 (DMI-resistant) were estimated in shaking cultures. The EC_{50} values, calculated from the data shown in Fig. 1, were 3 nM for strain 133 and 280 nM for strain 179.

Sterol composition of DMI-sensitive and resistant strains

Nonsaponifiable lipids were extracted from untreated mycelia of DMI-sensitive and resistant strains, and the sterol components were analysed by HPLC with a YMC-ODS column. In both strains, the main peak in the chromatograms was **c** (R_t = 25.3 min), and additional peaks **a** (R_t = 21.6 min), **b** (R_t = 22.3 min), and **d** (R_t = 28.3 min) were also observed (Fig. 2A, B). The most distinctive difference between the two chromatograms was the size of peak **a**; this peak in the chromatogram of the resistant strain was about 14 times larger than that of the sensitive strain. This result suggested a correlation between DMI-resistance and the accumulation of the compound in peak **a** in mycelia. Therefore, we examined the sterol components of 10 field strains of *V. inaequalis* which differed in their levels of resistance to fenarimol. However, no correlation was detected between DMI-resistance and the size of peak **a** (data not shown). In all strains used, the main peak was **c**, suggesting that the component of peak **c** is probably a functional sterol in *V. inaequalis*. The component of peak **c** was identified as ergosta-5,7-dien-3β-ol as described below.

Isolation and identification of sterol components in untreated mycelia

Nonsaponifiable lipids extracted from untreated mycelia of strain 133 and strain 179 were separated by preparative TLC to obtain crude 4-desmethyl sterols.

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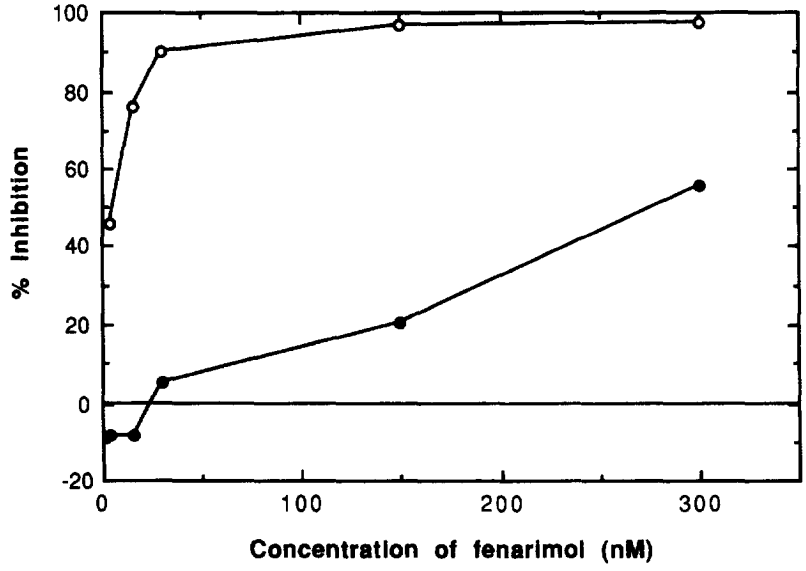


Fig. 1. Inhibitory effects of fenarimol on mycelial growth of *Venturia inaequalis*: DMI-sensitive strain 133 (○) and resistant strain 179 (●).

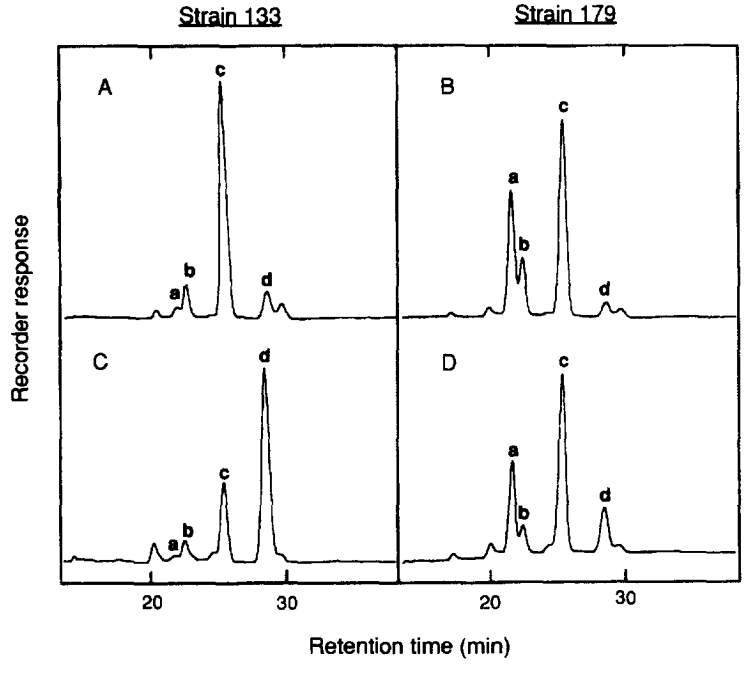
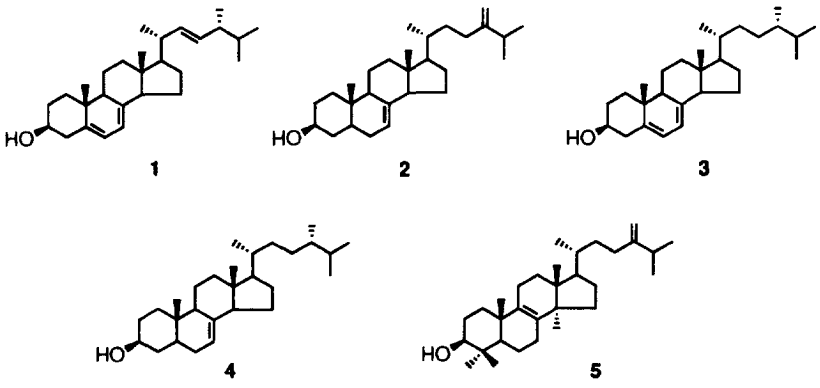


Fig. 2. HPLC chromatograms of nonsaponifiable lipids extracted from *Venturia inaequalis*: untreated (A) and 30 nM fenarimol-treated (C) mycelia of strain 133, and untreated (B) and 30 nM fenarimol-treated (D) mycelia of strain 179. Column, YMC-pack A-312 ODS 150 × 6 mm i.d.; mobile phase, MeOH; flow rate, 0.8 ml min^{-1}; detection, 210 nm.

Crude 4-methyl sterols were also obtained in trace amounts. The 4-desmethyl sterols were purified individually by preparative HPLC yielding ergosta-5,7-dien-3β-ol (**3**), ergost-7-en-3β-ol (**4**), together with a mixture of ergosterol (**1**) and ergosta-7,24(24^1)-dien-3β-ol (**2**). The compounds **1**, **2**, **3**, and **4** corresponded to peaks **a**, **b**, **c** and **d**, respectively. These compounds were characterized by EI-mass spectrometry and NMR as described below.

We could not separate compound **1** from compound **2** by preparative TLC and HPLC, because the R_f values of both compounds were identical and their retention times were similar (R_t = 21.6 min and 22.3 min, respectively). The retention time of compound **1** was identical



to that of authentic ergosterol suggesting that **1** might be ergosterol. The EI-mass spectrum of a mixture of compounds **1** and **2** showed fragment ions at *m/z* 398, 396, 383, 378, 363, 337, 314, 299 and 271. The fragment ions 396 $[M]^+$, 378 $[M - H_2O]^+$, 363 $[M - Me - H_2O]^+$, 337 $[M - C_3H_7O]^+$ and 271 $[M - sc]^+$ were identical to those of authentic ergosterol and from published data [6], indicating that the fragment ions are for compound **1** and 398 $[M]^+$, 383 $[M - Me]^+$, 314 $[M - C_6H_{12}]^+$ and 299 $[M - C_6H_{12} - Me]^+$ are for compound **2**. The fragment ions at 314 $[M - C_6H_{12}]^+$, and 299 $[M - C_6H_{12} - Me]^+$ of **2** characterize the 24-methylene side chain structure. The ^1H NMR spectrum of the mixture provided more useful structural information. Proton signals of **1** were assigned by comparison with authentic ergosterol and from published data [6], and those of **2** were assigned by subtraction of the signals of **1** from the complicated signals of the mixture (Table 1). The following signals in **1** and **2** were diagnostic for sterols with 5(6)-ene, 7(8)-ene, 22(23)-ene and 24(24^1)-ene structures. A double-doublet signal at δ 5.57, double-double-doublet signal at δ 2.47, and triplet signal at δ 2.28 of **1** indicated an olefinic C-6 proton and two C-4 protons. The double-double-doublet signal at δ 5.38 of **1** corresponded to olefinic C-7 proton. Signals at δ 5.15–5.26 of **1** indicated olefinic C-22 and C-23 protons. A doublet signal at δ 4.66 and a singlet signal at δ 4.72 indicated two olefinic C-24^1 protons of **2**. The multiplet signal at δ 5.16 of **2** corresponded to olefinic C-7 proton. Complicated ^{13}C NMR signals were obtained from the mixture. Signals of compound **1** corresponded to those of authentic ergosterol and from published data [7, 8]. Carbon signals of **2** were identified by subtraction of the signals of **1** from those of the mixture (Table 2). C-1 to C-19 of **2** were assigned by comparison with published data of ergosta-7,22-diene-3β-ol [9], and C-20 to C-28 of **2** were assigned by comparison with signals of authentic eburicol and from our published data [6]. From the data described above, compounds **1** and **2** were identified as ergosterol and ergosta-7,24(24^1)-dien-3β-ol, respectively.

The EI-mass spectrum of compound **3** gave a molecular ion at *m/z* 398. The fragment ion at *m/z* 339 $[M - C_3H_7O]^+$ suggested a B ring with a diene system [10]. The fragment ions at *m/z* 270 $[M - sc]^+$ and 252 $[M - sc - H_2O]^+$ indicated a saturated side-chain containing a 24-methyl substituent. The ^1H NMR spectrum of **3** is shown in Table 1. A double-doublet signal at δ 5.58, double-double-doublet signal at δ 2.47 and triplet signal at δ 2.28 indicate an olefinic C-6 proton and two C-4 protons. The double-double-doublet signal at δ 5.39 corresponded to olefinic C-7 proton. ^{13}C NMR spectral data of **3** are shown in Table 2. The signals were assigned using two-dimensional NMR (HSQC, HMBC and DQFC). Assignment of C-1 to C-19 of **3** corresponded to the signals of authentic ergosterol. These results indicate that compound **3** was ergosta-5,7-dien-3β-ol.

The EI-mass spectral fragment ion pattern of compound **4** was homologous to that of **3**, differing only by a shift of two atomic mass units. In the ^1H NMR spectrum of **4**, a multiplet signal at δ 5.16 corresponded to olefinic C-7 proton (Table 1). Two terminal methyl protons, 26-H and 27-H, showed magnetic nonequivalency and give well-resolved doublets at δ 0.79–0.86 (J = 6.8 Hz). Carbon signals of **4** were assigned using two-dimensional NMR (HSQC, HMBC and DQFC), indicating that the compound was ergost-7-en-3β-ol. The assignment of **4** was in agreement with published data [11].

A possible pathway of sterol biosynthesis in *V. inaequalis* is shown in Fig. 3. In the ergosterol biosynthetic pathway preferred by most fungi, the final step is the saturation of the 24,24^1-double bond [12]. However, *V. inaequalis* accumulated **1**–**4** in the untreated mycelia. This result indicates that saturation of the 24,24^1-double bond in *V. inaequalis* occurs after Δ^8–Δ^7 isomerization and is then followed by introduction of the 5,6- and 22,23-double bonds. The preferred route in *V. inaequalis* is therefore probably more like that in *Candida* spp. [12] than that in most fungi. However, the existence of a similar route in most fungi could not be excluded from our results.

Effects of fenarimol on sterol composition of DMI-sensitive and resistant strains

To examine the mechanism of DMI resistance, mycelia of DMI-sensitive and DMI-resistant strains grown in the presence of fenarimol were collected and the sterol components were analysed by HPLC with a YMC-ODS column. The relative amount of each sterol was determined by peak area measurement. After treatment with

Table 1. ^{1}H NMR (400 MHz) spectral data (in $CDCl_3$) of sterols isolated from mycelia of *Venturia inaequalis*

Compound	H-18	H-19	H-21	H-26 or	H-27	H-28	H-30	H-31	H-32	H-3α	H-4α	H-4β	H-6	H-7	Other protons
Ergosterol (**1**)	0.632 *s*	0.948 *s*	1.038 *d* $J = 6.6$	0.825 *d* $J = 6.6$	0.841 *d* $J = 6.6$	0.919 *d* $J = 6.8$				3.637 *m*	2.470 *ddd* $J = 14.2$, 4.5, 2.2	2.282 *t* $J = 11.9$	5.571 *dd* $J = 5.7$, 2.5	5.384 *ddd* $J = 5.7$, 2.7, 2.6	H-22 and H-23 5.145–5.259
Ergosta-7,24(24^1) dien-3β-ol(**2**)	0.541 *s*	0.798 *s*	0.955 *d* $J = 6.5$	1.023 *d* $J = 6.8$	1.029 *d* $J = 6.8$	4.715 *s* 4.658 *d* $J = 1.4$				3.595 *m*				5.162 *m*	H-25, 2.23, 1H, *m*
Ergosta-5,7-dien-3β-ol (**3**)	0.619 *s*	0.945 *s*	0.946 *d* $J = 6.6$	0.787 *d* $J = 6.8$	0.859 *d* $J = 6.8$	0.782 *d* $J = 6.9$				3.639 *m*	2.472 *ddd* $J = 14.2$, 5.2, 2.7	2.283 *t* $J = 12.0$	5.575 *dd* $J = 5.6$, 2.5	5.389 *ddd* $J = 5.6$, 3.0, 3.0	
Ergosta-7-en 3β-ol (**4**)	0.534 *s*	0.796 *s*	0.926 *d* $J = 6.3$	0.785 *d* $J = 6.9$	0.857 *d* $J = 6.8$	0.779 *d* $J = 6.8$				3.595 *m*				5.160 *m*	
Eburicol (**5**)	0.696 *s*	0.984 *s*	0.925 *d* $J = 6.3$	1.025 *d* $J = 6.9$	1.031 *d* $J = 6.8$	4.716 *s* 4.660 *d* $J = 1.4$	1.002 *s*	0.812 *s*	0.884 *s*	3.235 *dd* $J = 4.6$, 11.7					H-25, 2.23, 1H, *m*

Table 2. ^{13}C NMR (100 MHz) spectral data of sterols isolated from untreated or fenarimol-treated mycelia of *Venturia inaequalis**

C	1	2	3	4	5
1	38.39 (*t*)	37.16 (*t*)	38.38 (*t*)	37.16 (*t*)	35.61
2	32.01 (*t*)	31.50 (*t*)	32.00 (*t*)	31.51 (*t*)	27.88
3	70.48 (*d*)	71.07 (*d*)	70.48 (*d*)	71.08 (*d*)	78.99
4	40.82 (*t*)	38.01 (*t*)	40.79 (*t*)	38.03 (*t*)	38.89
5	139.80 (*s*)	40.27 (*d*)	139.76 (*s*)	40.28 (*d*)	50.44†
6	119.60 (*d*)	29.66 (*t*)	119.61 (*d*)	29.67 (*t*)	18.27
7	116.30 (*d*)	117.49 (*d*)	116.27 (*d*)	117.43 (*d*)	26.52
8	141.35 (*s*)	139.56 (*s*)	141.43 (*s*)	139.63 (*s*)	134.44‡
9	46.27 (*d*)	49.47 (*d*)	46.26 (*d*)	49.48 (*d*)	134.42‡
10	37.05 (*s*)	34.22 (*s*)	37.02 (*s*)	34.23 (*s*)	37.04
11	21.13 (*t*)	21.57 (*t*)	21.12 (*t*)	21.57 (*t*)	21.01
12	39.11 (*t*)	39.59 (*t*)	39.20 (*t*)	39.57 (*t*)	31.02
13	42.85 (*s*)	43.43 (*s*)	42.92 (*s*)	43.39 (*s*)	44.53
14	54.57 (*d*)	55.05 (*d*)	54.50 (*d*)	55.05 (*d*)	50.11
15	23.01 (*t*)	22.96 (*t*)	23.02 (*t*)	22.97 (*t*)	30.85
16	28.28 (*t*)	27.93 (*t*)	28.05 (*t*)	27.91 (*t*)	28.20
17	55.77 (*d*)	56.04 (*d*)	55.77 (*d*)	56.05 (*d*)	50.40†
18	12.06 (*q*)	11.85 (*q*)	11.81 (*q*)	11.84 (*q*)	15.76
19	16.29 (*q*)	13.03 (*q*)	16.28 (*q*)	13.03 (*q*)	19.14
20	40.41 (*d*)	36.19 (*d*)	36.54 (*d*)	36.63 (*d*)	36.48
21	21.10 (*q*)	18.84 (*q*)	19.02 (*q*)	19.03 (*q*)	18.71
22	135.57 (*d*)	34.67 (*t*)	33.67 (*t*)	33.69 (*t*)	35.02
23	132.00 (*d*)	31.09 (*t*)	30.66 (*t*)	30.71 (*t*)	31.29
24	42.83 (*d*)	156.86 (*s*)	39.08 (*d*)	39.10 (*d*)	156.91
25	33.10 (*d*)	33.82 (*d*)	31.49 (*d*)	31.49 (*d*)	33.82
26	19.94 (*q*)†	22.00 (*q*)†	20.50 (*q*)†	20.50 (*q*)†	21.99§
27	19.65 (*q*)†	21.87 (*q*)†	17.60 (*q*)†	17.61 (*q*)†	21.86§
28	17.60 (*q*)	105.96 (*t*)	15.44 (*q*)	15.45 (*q*)	105.94
29	—	—	—	—	—
30	—	—	—	—	27.97
31	—	—	—	—	15.40
32	—	—	—	—	24.26

*Multiplicity of each signal is shown in parentheses.
†‡§Assignments may be reversed in each vertical column.

fenarimol, sizes of peaks **a**, **b** and **c** decreased, while that of peak **d** increased in both strains (Fig. 2). By using a different ODS-column (develosil HG-5), peak **d** was shown to include two compounds (R_t = 11.3 min and 12.1 min). One of the compounds had a retention time (R_t = 12.1 min) identical to that of compound **4**, and another compound (R_t = 11.3 min) did not correspond with any of the compounds described above. The ratio, estimated by the peak areas, of **4** to the unknown compound in the 300 nM fenarimol-treated strain 133 was approximately 2:8, and that in the 300 nM fenarimol-treated strain 179 was approximately 1:9 (chromatograms are not shown). Thus, in fenarimol-treated mycelia, levels of 4-desmethyl sterols (**1**–**4**) decreased and the unknown compound was accumulated in both strains. The fenarimol concentrations which affected the sterol components in culture differed in both strains. The sterol distribution changed markedly at more than 30 nM fenarimol in strain 133 (DMI-sensitive) and at more than 300 nM in strain 179 (DMI-resistant) (Fig. 4).

Isolation and identification of the unknown compound detected in fenarimol-treated mycelia

Nonsaponifiable lipids extracted from fenarimol-treated mycelia were separated by preparative TLC to obtain crude 4-methyl sterols. The 4-methyl sterols were purified by preparative HPLC giving eburicol (**5**; 24-methylene-24,25-dihydrolanosterol) as the main sterol. The retention time of **5** on HPLC with a develosil-ODS column was identical to that of the unknown compound described above. This compound was characterized by NMR and mass spectroscopy. The EI-mass spectrum of **5** showed molecular ion at *m/z* 440. The fragment ions at *m/z* 341 $[M - Me - C_6H_{12}]^+$, and 323 $[M - Me - C_6H_{12} - H_2O]^+$ indicated a 24-methylene side-chain. The fragmentation pattern was identical to that from published data [13]. ^1H and ^{13}C NMR spectral data of **5** are shown in Tables 1 and 2. Both spectra corresponded to authentic eburicol.

In the presence of fenarimol, both strains accumulated eburicol (**5**; the substrate of the C14-demethylase) which

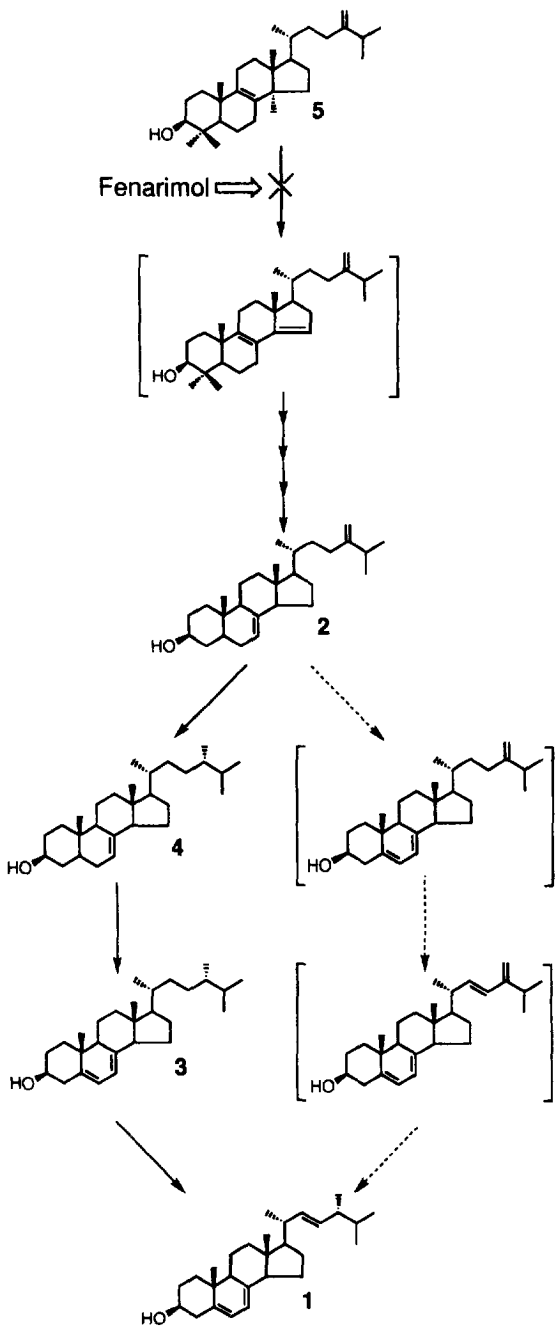


Fig. 3. Possible reaction sequences involved in the conversion of eburicol into ergosterol in *Venturia inaequalis*, and the inhibition by fenarimol: **1**, ergosterol; **2**, ergosta-7,24(24[1])-dien-3β-ol; **3**, ergosta-5,7-dien-3β-ol; **4**, ergost-7-en-3β-ol; **5**, eburicol. - - →, Reactions involved in the preferred pathway in most fungi; →, reactions involved in the preferred pathway in *Candida* spp. [12]. Sterols which were not detected in mycelia of *V. inaequalis* are shown in brackets.

was hardly observed in untreated cultures, and showed decreased levels of 4-desmethyl sterols, **1**–**4**. This result indicates that fenarimol acts as an inhibitor in the C14-demethylation step not only in DMI-sensitive but also in DMI-resistant *V. inaequalis* (Fig. 3). The sterol alteration occurred with fenarimol treatment at low concentration in the DMI-sensitive strain, and at high concentration in the resistant strain (Fig. 4). From these results, the following possible mechanisms of DMI resistance can be suggested: (1) decrease of DMI-sensitivity of C14-demethylase; (2) overproduction of C14-demethylase in the cells; (3) increased xenobiotic metabolization ability; and (4) alteration of selective permeability of cell membranes. Clearly, further research is needed to clarify the mechanism of DMI resistance.

EXPERIMENTAL

General. ^1H (400 MHz) and ^{13}C (100 MHz) NMR: $CDCl_3$ with TMS as int. standard. EI-MS: 70 eV. Prep. TLC: silica gel 60 F_{254} (0.5 mm). HPLC: YMC-pack A-312 ODS (S15) column (150 × 6 mm i.d.) or develosil ODS-HG-5 column (150 × 4.6 mm i.d.). Prep. HPLC: YMC pack SH-343-15 ODS (S15) column (250 × 20 mm i.d.).

Chemicals. Fenarimol was kindly supplied as pure active ingredients by Dow-Elanco Japan Ltd., Japan. Authentic ergosterol, ergosta-5,7,22-trien-3β-ol, was prepared from untreated mycelia of *Botrytis cinerea* [6]. Authentic eburicol, 4,4,14α,24-tetramethyl-5α-cholesta-8,24(24[1])-dien-3β-ol, was prepared from SSF-109, (*dl*)-*cis*-1-(4-chlorophenyl)-2-(1H-1,2,4-triazol-1-yl) cyclo-heptanol-treated mycelia of *B. cinerea* [6].

Organisms and growth conditions. Strain 133 (DMI-sensitive) and strain 179 (DMI-resistant) of *V. inaequalis* (Cooke) Winter were kindly supplied by Dr L. Parisi, INRA Angers, France. These strains were obtained by monoconidial isolation from apple trees naturally infected with scab. They were grown on potato-dextrose agar medium (DIFCO) for 45 days at 20°. Mycelial disks 4 mm diameter were cut from the margins of colonies and transferred to MY-broth medium (malt extract 3 g l^{-1}, yeast extract 3 g l^{-1}, peptone 5 g l^{-1}, dextrose 10 g l^{-1}) and then cultured for 1 month at 20°. Mycelia were collected by filtration and homogenized aseptically. Culture flasks containing MY-broth medium with or without ethanolic solns of fenarimol were inoculated with the mycelial homogenates and shaken for 7 days at 20°. Mycelia were collected by filtration, lyophilized and weighed.

Analysis of steroidal components in mycelia of V. inaequalis. The lyophilized mycelia (50 mg dry wt) were suspended in 10 ml of ethanolic KOH (10% KOH in 60% EtOH) using a Polytron homogenizer followed by reflux at 70° for 1 hr. The reaction mixture was extracted 2× with 10 ml of *n*-hexane. The organic layer was washed with H_2O, dried by passing through anhydrous Na_2SO_4, and evapd to yield nonsaponifiable lipids. This mixt. was then sepd by HPLC (MeOH, 0.8 ml min^{-1}; UV_{210}) and relative amounts of each sterol were determined by peak area measurement.

Isolation of steroidal components from mycelia of V. inaequalis. (1) Lyophilized untreated mycelia (20 g dry wt) were extracted with MeOH at ambient temp, and then with hot $CHCl_3$. The extract was saponified under

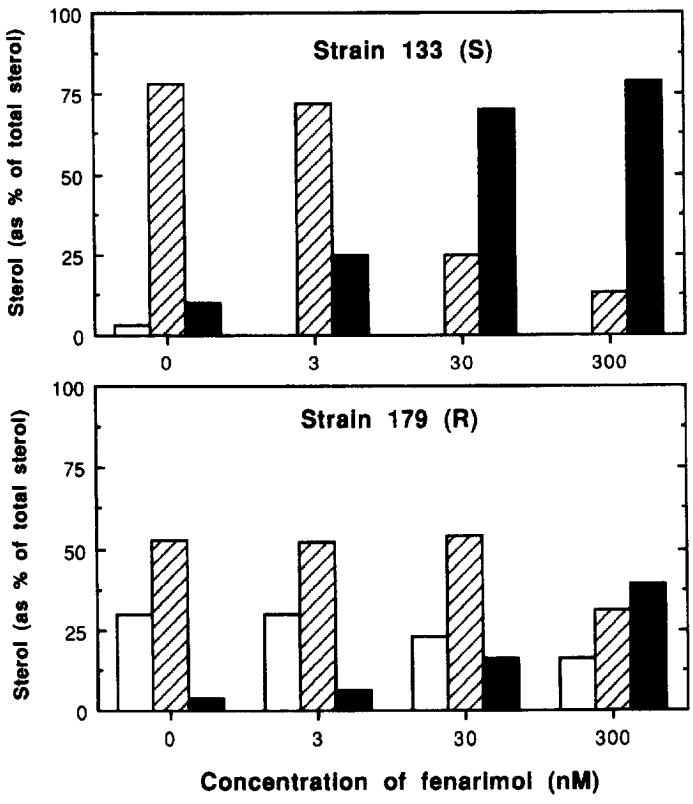


Fig. 4. Sterol distributions in untreated or fenarimol-treated mycelia of *Venturia inaequalis*: □, ergosterol (**1**); ■, ergosta-5,7-dien-3β-ol (**3**); ■, ergost-7-en-3β-ol and eburicol (**5**); S, DMI-sensitive; R, DMI-resistant. The relative amount of each sterol was determined by peak area measurement on HPLC chromatograms.

reflux at 70° for 1 hr with 350 ml of ethanolic KOH. The reaction mixture was extracted with *n*-hexane, and the organic layer was washed with distilled water, dried, and evaporated to yield nonsaponifiable lipids (400 mg). This was then chromatographed by prep. TLC (*n*-heptane–isopropyl ether–acetic acid, 60:40:4) and prep. HPLC (MeOH, 17 ml min^{-1}; UV_{210}) to give ergosta-5,7-dien-3β-ol (**3**) (28 mg), ergost-7-en-3β-ol (**4**) (5 mg), and a mixture of ergosterol (**1**) and ergosta-7,24(24[1])-dien-3β-ol (**2**) (20 mg). (2) Nonsaponifiable lipids were extracted from lyophilized fenarimol-treated mycelia (2 g dry wt). Prep. TLC and prep. HPLC of the lipids yielded eburicol (**5**) (1.5 mg).

Mixture of ergosterol (**1**) ((24R)- *ergosta*-5,7,22-*trien*-3β-*ol*) *and ergosta*- 7,24(24[1])-*dien*-3β-*ol* (**2**). EI-MS *m/z*: 398, 396, 383, 378, 363, 337, 314, 299, 271; ^{1}H NMR (Table 1); ^{13}C NMR (Table 2).

Ergosta-5,7-*dien*-3β-*ol* (**3**). EIMS *m/z* (rel. int.): 398 $[M]^+$ (68), 383 $[M - Me]^+$ (7), 380 $[M - H_2O]^+$ (32), 365 $[M - Me - H_2O]^+$ (100), 339 $[M - C_3H_7O]^+$ (47), 270 $[M - sc]^+$ (13), 252 $[M - H_2O - sc]^+$ (69); ^{1}H NMR (Table 1); ^{13}C NMR (Table 2).

Ergost-7-*en*-3β-*ol* (**4**). EIMS *m/z* (rel. int.): 400 $[M]^+$ (100), 385 $[M - Me]^+$ (32), 382 $[M - H_2O]^+$ (19), 367 $[M - Me - H_2O]^+$ (12), 272 $[M - sc]^+$ (21), 254 $[M - H_2O - sc]^+$ (81), 230 $[M - sc - C_3H_6]^+$ (21), 212 $[M - H_2O - sc - C_3H_6]^+$ (35); ^{1}H NMR (Table 1); ^{13}C NMR (Table 2).

Eburicol (**5**) (4,4,14α,24-*tetramethyl*-5α-*cholesta*-8, 24(24[1])-*dien*-3β-*ol*).

EI-MS *m/z* (rel. int.): 440 $[M]^+$ (35), 425 $[M - Me]^+$ (100), 407 $[M - Me - H_2O]^+$ (53), 397 $[M - C_3H_7]^+$ (9), 341 $[M - Me - C_6H_{12}]^+$ (17), 327 $[M - 113]^+$ (4), 323 $[M - Me - H_2O - C_6H_{12}]^+$ (13), 297 $[M - H_2O - sc]^+$ (2), 273 $[M - sc - C_3H_6]^+$ (11), 255 $[M - H_2O - sc - C_3H_6]^+$ (15); ^{1}H NMR (Table 1); ^{13}C NMR (Table 2).

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