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Polyene-Resistant Mutants of *Aspergillus fennelliae*: Identification of Sterols

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Four mutants strains of *Aspergillus fennelliae* resistant to various polyene antibiotics have been analyzed for their sterol content. The mutant strains contained $\Delta^{7,22,24(28)}$-ergostatrien-3β-ol and $\Delta^{7,22}$-ergostadien-3β-ol as the major sterols, whereas the wild types contained ergosterol, indicating that the mutants contain metabolic blocks for C_5-C_6 dehydrogenation and C_{24}-C_{28} reduction in the biosynthesis of ergosterol. Revertant sectors arising from the mutant colonies contained either more $\Delta^{7,22}$-ergostadien-3β-ol than the parent mutant strain or showed the reappearance of ergosterol. The revertant sectors also showed lower resistance to amphotericin B and a higher rate of sporulation than the parent mutant strain. These results confirm our previous observations that the presence of ergosterol is directly related to the drug susceptibility and the normal rate of sporulation in this species.

The development of resistance to polyene antibiotics in fungi has been shown to coincide with alterations in the sterol content of the cells (1, 2, 5-7, 10-14).

In 1972, Parks et al. (12) identified one of the altered sterols from a nystatin-resistant mutant of *Saccharomyces cerevisiae* (11, 13) as $\Delta^{8,22}$-ergostadien-3β-ol. More recently, Fryberg et al. (5) and Barton et al. (2) identified several biosynthetic precursors of ergosterol as the major sterols in nystatin-resistant mutants of *S. cerevisiae*. A mutant of *S. cerevisiae* resistant to relatively low concentrations of nystatin was found to contain as a major sterol 5,6-dihydroergosterol, an immediate precursor of ergosterol. The mutants resistant to higher concentrations of nystatin, on the other hand, containing earlier precursors such as $\Delta^{7,22,24(28)}$-ergostatrien-3β-ol, $\Delta^{8,22,24(28)}$-ergostatrein -3β-ol, and fecosterol ($\Delta^{8,24(28)}$-ergostadien-3β-ol.

We have recently reported observations of polyene resistance and correlated sterol changes in the mutants of *Aspergillus fennelliae* (10). In this communication, we report the identification of the sterols accumulated in the mutants of *A. fennelliae*. Evidence supporting our previous hypothesis that ergosterol is one of the necessary components for the normal rate of asexual and sexual sporulation is also presented.

MATERIALS AND METHODS

Strains and cultural conditions. The polyene-resistant mutants, AF4-NS1, p-NS1, AF5-AB1, p-AB1, and wild-type strains of *A. fennelliae*, described elsewhere (10), were used in this experiment. The wild-type strains were maintained on a malt extract-agar medium, and the mutants were maintained on the same medium but containing 4 μg of amphotericin B per ml for AF5-AB1 and p-AB1 and 10 U of nystatin per ml for AF4-NS1 and p-NS1. For sterol extractions, the strains were cultured in malt extract-broth medium at 30 C on a gyrotory shaker (115 rpm) for 5 days. To isolate a reverse mutant, AF5-AB1 and p-AB1 were maintained also on drug-free malt extract-agar medium.

Sterol extraction and purification. Mycelial pads grown on malt extract medium were washed once in distilled water and saponified with 30% methanolic KOH at reflux for 3 h before the sterols were extracted with hexane (10). The hexane extracts were then washed with distilled water, and the solvent was evaporated under a stream of nitrogen. The isolation of sterols from the crude non-saponifiable hexane extracts (1 g) was carried out by adsorption-column chromatography on alumina (30 g). Fractions were eluted as follows: (i) petroleum ether-benzene (9:1, 100 ml, and 1:1, 100 ml); (ii) benzene-ether (9:1, 100 ml; 3:1, 100 ml; and 1:1, 100 ml); (iii) ether-methanol (19:1, 100 ml, and 4:1, 100 ml), and (iv) ethyl acetate-chloroform-methanol-acetic acid (13:13:13:1, 100 ml). Sterols in each fraction were detected by subsequent thin-layer chromatography. Most of the sterols were usually eluted with benzene containing 10% ether. Such sterol mixtures were then chromatographed on 5% $AgNO_3$-impregnated Silica Gel G thin-layer plates, using benzene-ethyl acetate (5:1) as the developing solvent. Bands of the separated materials were detected with the Liebermann-Burchard reagent (10) and scraped from the plate. The sterols were eluted with chloroform and recovered by evapo-

ration of the solvent at room temperature in a stream of nitrogen.

Ultraviolet (UV) spectra were measured on ethanolic solutions with a Cary model 14 spectrophotometer.

Nuclear magnetic resonance (NMR) spectra were measured on a Varian XL-100 modified for Fourier transform NMR spectroscopy by Digilabs Inc., using a 410H pulser. Spectra were obtained by using about 0.5 ml of a millimolar solution of compound in $CDCl_3$ by accumulating 5,000 scans with 8,000 data points and a repetition rate of 2 s, giving a theoretical resolving power of 0.5 Hz.

Gas-liquid chromatography (GLC) was carried out using a Glowall gas chromatograph containing a 6 foot by ¼ inch (ca. 182.9 by 0.64 cm) outer diameter column packed with 1% OV-17 on Supelcoport. This column was used isothermally at 230 C with a flame ionization detector.

Gas chromatography-mass spectrometry was effected by using an LKB-9000 with a 6 foot by ¼ inch outer diameter column containing 1% OV-17 on Supelcoport. This column was programmed from 230 C at 6 C/min. Mass spectra were measured at an ionizing voltage of 70 eV, a source temperature of 270 C, and a scan speed of approximately 10 s/decade in mass.

RESULTS

The predominant sterol of wild-type *A. fennelliae* is ergosterol. The mutants, on the other hand, produce significantly decreased amounts of this sterol in the strains AF4-NS1 and p-NS1 and none at all in the strains AF5-AB1 and p-AB1. However, all the mutants contain sterols related to ergosterol and presumably its biosynthetic precursors.

Two sterols of the mutants AF5-AB1 and p-AB1 were isolated by thin-layer chromatography on $AgNO_3$-impregnated silica gel plates after column chromatography; this preparative separation was not complete and UV and NMR spectra were measured on samples that were estimated by GLC to be about 90% pure.

The UV spectra of the two major sterols were measured and are shown in Fig. 1. One of the mutant sterols (A) has a maximum at 205 nm, whereas the other (B) has peaks at 225 and 232 nm. These spectra stand in contrast to that of ergosterol (e.g., from the wild type [C]), whose UV spectrum has maxima at 271, 282, and 293 nm. The UV spectra of the sterols from the mutants AF4-NS1 and p-NS1 indicated the presence of all three compounds, A, B, and C, although in different relative proportions.

The sterol C could be readily separated from A and B by thin-layer chromatography by virtue of its low R_f value (0.28). With R_f values of 0.33 and 0.34, respectively, however, A and B could not be completely separated from one another in this way.

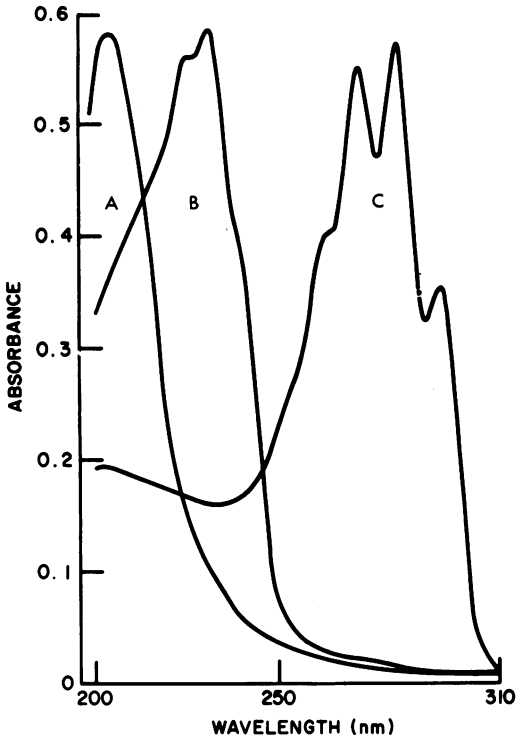


FIG. 1. *UV spectra of the two new sterols A and B and of ergosterol (sterol C).*

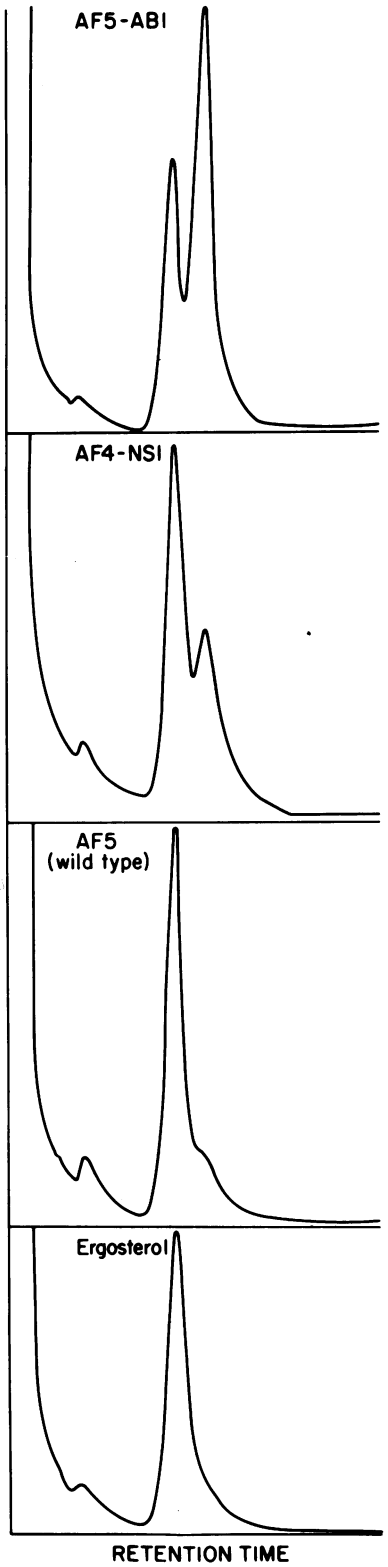
Analysis of the sterol components of the various mixtures was readily effected, however, by GLC (Fig. 2). The sterol mixture obtained from the mutant AF5-AB1 gave two major peaks. The first of these co-chromatographs with sterol A, and the second component co-chromatographs with sterol B. The UV spectrum of the mixture is consistent with its formulation as a mixture of sterols A and B.

Gas chromatography of the mixtures of sterols from the mutant AF4-NS1 also afforded two peaks. The minor peak co-chromatographs with sterol B, but the larger peak, which is eluted earlier, could be demonstrated to contain sterol A and ergosterol (sterol C).

Ergosterol is by far the major sterol from wild-type *A. fennelliae*, although traces of a different sterol with a slightly longer retention time were also detected in this strain.

The gas chromatograms of the sterol mixtures isolated from p-AB1 and p-NS1 were identical with those from AF5-AB1 and AF4-NS1, respectively.

Gas chromatography-mass spectrometry of the mixtures containing sterol A revealed its molecular weight to be 398, thus permitting its formulation as a dihydroergosterol. Its UV spectrum precluded the presence of a conjugated



diene system, and the intense ions at *m/e* 255 and 271 in its mass spectrum suggest that the side chain of sterol A must have a mass of 127 (i.e., 398 – 271) and, therefore, a formula of C_9H_{19}. One double bond of the sterol must therefore be in its side chain and the other must be in one of the rings.

One of the several compounds that fulfills these cirteria is $\Delta^{7,22}$-ergostadien-3β-ol (5,6-dihydroergosterol). An authentic sample of this compound was found to be inseparable from sterol A by GLC, and the mass spectrum of sterol A (Fig. 3) is identical to that of the authentic material.

The mass spectrum of sterol B establishes its molecular weight as 396; sterol B is therefore an isomer of ergosterol itself. The UV spectrum of sterol B has a maximum at 232 nm. This suggests that the compound must contain a conjugated diene system, almost certainly in the side chain, in view of the low wavelength of this absorption maximum. This conclusion is supported by the mass spectrum of sterol B (Fig. 3), in which the high intensity of the ion at *m/e* 271, which corresponds to loss from the molecular ion of a C_9H_{17} side chain, and the low intensity of the molecular ion itself suggest that sterol B has a $\Delta^{22,24(28)}$ structure. This is fully confirmed by the NMR spectrum of sterol B, which also permits the conclusion that the third double bond is in the B ring, at C_7–C_8. This NMR spectrum, obtained by Fourier transform techniques on about 1 mg of the compound, is identical to that published (3, 4) for $\Delta^{7,22,24(28)}$-ergostatrien-3β-ol (Table 1).

Neither of these sterols possesses a C_5–C_6 double bond, and this suggests that the enzyme system responsible for C_5–C_6 dehydrogenation is blocked totally in the mutants AF5-AB1 and p-AB1 and partially in the mutants AF4-NS1 and p-NS1. The mutants AF5-AB1 and p-AB1 are stable in that their sterol patterns and morphological abnormalities remain unchanged during prolonged maintenance on a drug-free medium. However, a morphologically distinct sector has occasionally emerged during the subculturing of the strains AF5-AB1 and p-AB1.

A strain AF5-AB1-R (Fig. 4), isolated from a sector formed by AF5-AB1, produced normal conidial heads only in the dry area of slant cultures, and the strain p-AB1-R isolated from a sector formed by p-AB1 produced conidial heads uniformly over the surface of the colony. The growth rate of AF5-AB1-R remained the

FIG. 2. *Gas-liquid chromatograms of the sterols from the mutants AF5-AB1, AF4-NS1, and wild-type AF5 and pure ergosterol.*

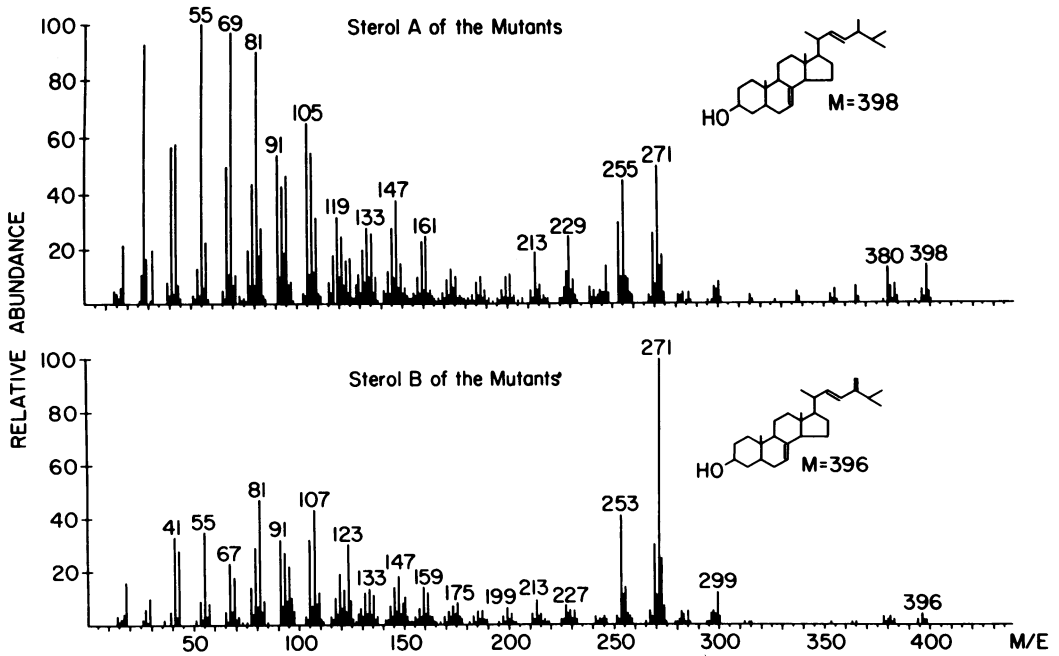


FIG. 3. *Mass spectra of the two sterols A and B from polyene-resistant mutants.*

TABLE 1. *NMR spectrum of* $\Delta^{7,22,24(28)}$*-ergostatrien-3β-ol*

Protons	Results from[a]	
	Fryberg et al. (5)	This work
C_{23}-H	5.92 (d, 16 Hz)	5.96 (d, 16 Hz)
C_{22}-H	5.52 (d of d, 8, 16 Hz)	5.60 (d of d, 8, 16 Hz)
C_7-H	5.14 (broad s)	5.19 (broad s)
C_{28}-H	4.80 (d, 2 Hz)	4.84 (d, 2 Hz)
C_{25}-H	2.48 (sept., 7 Hz)	2.50 (sept., 7 Hz)
C_{26}, C_{27} methyl groups	1.06 (d, 7 Hz)	1.03 (d, 7 Hz)
C_{19} methyl group	0.90 (s)	0.91 (s)
C_{18} methyl group	0.54 (s)	0.59 (s)

[a] Chemical shifts are quoted in parts per million measured downfield from the internal standard, tetramethylsilane. Abbreviations: d, doublet; s, singlet; sept., septet.

same as that of the parental mutant strains (AF5-AB1). The strain p-AB1-R, however, showed a slight increase in the growth rate as compared with that of p-AB1. Analysis by GLC (Fig. 4) of the sterol content of AF5-AB1-R showed an increase in the level of $\Delta^{7,22}$-ergostadien-3β-ol as compared with AF5-AB1, and the UV spectrum of the mixture of sterols isolated from p-AB1-R, also shown in Fig. 4, revealed the presence of appreciable amounts of ergosterol, in addition to sterols A and B.

The minimal inhibitory concentration of amphotericin B for AF5-AB1-R and p-AB1-R was measured as described in the previous paper (10) and was compared with the values obtained for AF5-AB1 and P-AB1. The minimal inhibitory concentration of amphotericin B for AF5-AB1 and p-AB1 was 64 μg/ml. The minimal inhibitory concentration of the drug for AF5-AB1-R and p-AB1-R, however, was 16 μg/ml and 2 μg/ml, respectively, indicating a fourfold and a 32-fold decrease as compared with the parental mutants AF5-AB1 and P-AB1. The strains AF5-AB1-R and p-AB1-R appear to result from the process of reverse mutation. Other markers, such as the mating type, remained the same as in the parental mutant strains.

The strain p-AB1 (*a* mating type) produces fertile cleistothecia when crossed with the oppo-

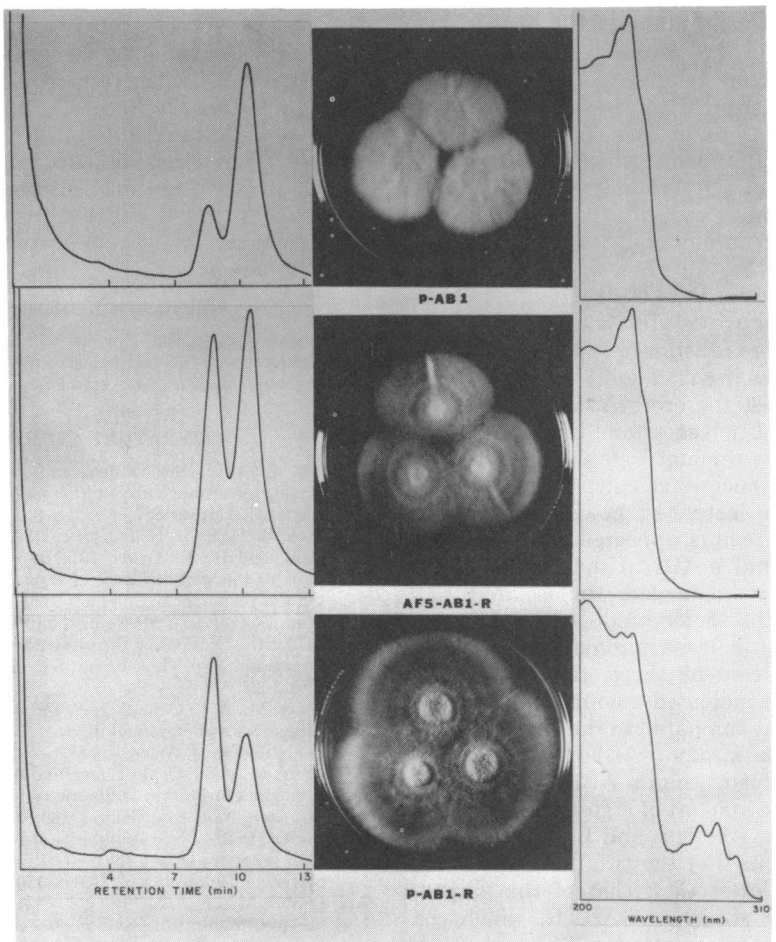


FIG. 4. *Gas-liquid chromatograms (left) and UV spectra (right) of the sterols and colony morphology (center) of p-AB1, AF5-AB1-R, and p-AB1-R.*

site mating strain of wild type and fails to produce any stage of sexual reproduction when paired with an opposite strain such as AF5-AB1 (*A* type), which lacks ergosterol (10). The revertant p-AB1-R (*a*) also produces fertile cleistothecia when crossed with *A* strain of the wild type, but, unlike p-AB1, it produces abortive cleistothecia when paired with AF5-AB1.

These results confirm our previous conclusion (10) that synthesis of ergosterol is directly related to the normal rate of asexual and sexual sporulation and the susceptibility of *A. fennelliae* to amphotericin B.

DISCUSSION

The fact that the susceptibility of fungi to polyene antibiotics can be diminished by alterations in the sterol content of the cells has been well documented in a number of publications (1, 2, 5–7, 10–14). The mutants AF5-AB1 and p-AB1 produce principally $\Delta^{7,22}$-ergostadien-3β-ol and $\Delta^{7,22,24(28)}$-ergostatrien-3β-ol. These mutants are clearly devoid of the $\Delta^{5,7}$ sterols, whereas the wild-type strains produce ergosterol as the major sterol. Thus, the enzyme system responsible for dehydrogenation of the C_5–C_6 bond appears to be absent in the mutant strains. To some extent, this is also true of the enzyme system required for the reduction of the C_{24}–C_{28} double bond.

A mutant of *S. cerevisiae*, designated *nys*-3 (11, 13), has been found to produce a number of C_5–C_6-saturated sterols such as $\Delta^{7,22}$-ergostadien-3β-ol, $\Delta^{7,22,24(28)}$-ergostatrien-3β-ol, $\Delta^{7,24(28)}$-ergostadien-3β-ol, $\Delta^{8,22}$ ergostadien-3β-ol, $\Delta^{8,22,24(28)}$-ergostatrien-3β-ol, and $\Delta^{8,24(28)}$-ergostadien-3β-ol (2, 5, 12). The

removal of the conjugated double bond system in the B-ring of the sterols seems to be one of the crucial factors related to the resistance of the strains towards the polyene antibiotics.

The observations by Hsuchen and Feingold (8, 9) that the amphotericin B- and nystatin-induced permeability changes in dipalmitoyllecithin liposomes were drastically suppressed by the incorporation of Δ^5 sterols and were unaffected by the incorporation of $\Delta^{5,7}$ sterols support this hypothesis. Furthermore, the mutants that produce Δ^8 sterols have been shown (2, 5) to be more resistant to the drugs than the mutants producing Δ^7 sterols in yeast. These findings suggest the order $\Delta^{5,7}$, Δ^7, Δ^8 for the effectiveness of polyene-sterol binding.

The mutants remained stable for at least 13 generations of successive culturing in drug-free media. In two instances, however, partial reverse mutant sectors appeared in the colonies of AF5-AB1 and p-AB1. Although the growth rate remained unchanged, the isolated AF5-AB1-R from the sector produced by AF5-AB1 showed a slight increase in conidiation. Analysis of the sterol content shows that AF5-AB1-R synthesizes an increased amount of $\Delta^{7,22}$-ergostadien-3β-ol as compared to the parental strain AF5-AB1. The strain p-AB1-R was found to grow slightly faster than p-AB1 and to contain an appreciable amount of ergosterol in addition to the two new sterols A and B. The resistance to amphotericin B of the two new isolates was considerably lower than that of the parental mutants. The strain AF5-AB1-R, which contained more $\Delta^{7,22}$-ergostadien-3β-ol than the parental strain, showed a fourfold decrease in resistance, whereas p-AB1-R, which contained only a fraction of the ergosterol, showed a 32-fold decrease in resistance as compared to the parental strain.

The fact that an increase in $\Delta^{7,22}$-ergostadien-3β-ol in AF5-AB1-R accompanied increased sporulation and susceptibility to the drug suggests that this sterol may be closer than $\Delta^{7,22,24(28)}$-ergostatrien-3β-ol to ergosterol on the biosynthetic pathway to the latter.

These observations, in turn, support the hypothesis of Fryberg et al. (5) that $\Delta^{7,22}$-ergostadien-3β-ol is the immediate biogenetic precursor of ergosterol in the sensitive wild types of yeast.

Although p-AB1-R failed to complete the sexual state when crossed with AF5-AB1, cleistothecia devoid of ascospores were produced in these pairs. The mutant p-AB1 does not exhibit any sign of sexual state when crossed with AF5-AB1. The amount of ergosterol produced by p-AB1-R, although sufficient for the formation of abortive cleistothecia, was apparently insufficient for the completion of a sexual state.

These observations indicate that ergosterol is directly involved not only in the susceptibility of the strains to polyene antibiotics but also in the normal rate of asexual and sexual sporulation in *A. fennelliae*.

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