Identification of a sterol mutant of *Neurospora crassa* deficient in $\Delta^{14,15}$ -reductase activity

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A mutant (*erg-3*) of *Neurospora crassa* resistant to the polyene antibiotic nystatin was compared with its sensitive, wild-type parent to detect differences in sterol composition using gas chromatography–mass spectrometry. The major sterol in wild-type mycelia, comprising 80% of the total, was ergosterol. The major sterols in mutant mycelia, comprising 86% of the total, were $\Delta^{8,14}$ -sterols. It is proposed that the nystatin-resistant strain is unable to synthesize ergosterol because it lacks $\Delta^{14,15}$ -reductase activity as a result of a mutation in the *erg-3* gene.

Introduction

The biosynthetic pathway of ergosterol, the major sterol of many fungal species, has been studied extensively (Mercer, 1984; Weete, 1989). Initial work, conducted mainly with the yeast *Saccharomyces cerevisiae*, identified numerous intermediates in the pathway by analysing mutants blocked at various stages of sterol biosynthesis (Fryberg *et al.*, 1974; Bard *et al.*, 1977; Trocha *et al.*, 1977). Yeast mutants (designated *erg*) collectively are defective in almost every major biosynthetic step after cyclization of squalene (Parks *et al.*, 1985); they include mutants defective in Δ^{8-7} isomerization (*erg2*), 5(6) desaturation (*erg3*), 24(28) hydrogenation (*erg4*), 22(23) desaturation (*erg5*), C-24 transmethylation (*erg6*) (Bard *et al.*, 1977) and C-14 demethylation (*erg11*) (Gollub *et al.*, 1974). Mutants of filamentous fungi blocked at similar steps in the sterol biosynthesis pathway have also been isolated. For example, Grindle & Farrow (1978) reported mutants of *Neurospora crassa* with gene defects affecting Δ^{8-7} isomerization (*erg-1*), 24(28) hydrogenation (*erg-2*) and C-24 transmethylation (*erg-4*).

The biosynthetic pathway of ergosterol includes a $\Delta^{8,14}$ -sterol intermediate formed as a result of the demethylation of lanosterol or 24-methylenedihydrolanosterol. The Δ^{14} double bond is then reduced to give the corresponding Δ^8 -sterol (Mercer, 1984). To date, no mutant with a block in the reduction of the Δ^{14} double bond has been reported in the literature. This paper identifies such a mutant of *N. crassa*; the mutant

(designated *erg-3*) was originally isolated by Grindle (1973, 1974) but the enzyme defect caused by the *erg-3* gene was not determined.

Methods

Sources of strains. The wild-type 74-OR8-1a was obtained from the Fungal Genetics Stock Center, University of Kansas Medical Center, Kansas 66103, USA. The mutant *erg-3* was derived from 74-OR8-1a by UV-mutagenesis (Grindle, 1974), and is available from the above Stock Center.

Growth of cultures. The wild-type and mutant were maintained on solid medium as described previously (Grindle, 1973). For sterol analysis, mycelium was grown to mid-exponential phase in liquid minimal medium (Vogel, 1964) at 26 °C on a rotary shaker (150 r.p.m.), harvested by filtration, and washed twice with distilled water.

Sterol extraction and derivatization. Mycelium (2 g) was acid-labilized by resuspending in 10 vols 0.1 M-HCl and heated at 100 °C for 20 min. The acid-labilized mycelium was harvested by centrifugation, washed twice with 10 ml of distilled water, and resuspended in 2 ml 60% (w/v) KOH in water, 3 ml methanol and 2 ml 0.5% (w/v) pyrogallol in methanol (Parks *et al.*, 1985). The mycelial suspension was homogenized with 0.5 g of glass beads (0.45–0.5 mm) in an MSE homogenizer for 4 × 10 s and saponified at 85 °C for 2 h. After cooling, the non-saponifiable lipids (sterols) were extracted with heptane (3 × 3 ml), dried over anhydrous sodium sulphate, and the heptane extract was analysed by UV-spectrophotometry at 210–300 nm. The heptane extract was evaporated to dryness with warm nitrogen and the residue dissolved in a mixture of anhydrous pyridine, hexamethyldisilazane and trimethylchlorosilane (7:2:1, by vol.). The mixture was left overnight at room-temperature, and the resulting trimethylsilyl (TMS) sterol derivatives were analysed by gas chromatography and gas chromatography–mass spectrometry.

Gas chromatography (GC) and gas chromatography–mass spectrometry (TC-MS). GC was performed on a Packard 437A gas chromatograph,

Abbreviation: TMS, trimethylsilyl.

Fig. 1. Deduced structures of sterols. A, ergosterol; B, episterol; C, obtusifoliol; D, 24-methylenedihydrolanosterol; E, ergosta-5,8,14,24(28)-tetraene-3 β -ol; G, ergosta-8,14,24(28)-trien-3 β -ol; H, ignosterol. (F, not shown.)

fitted with a flame ionization detector, using a DB5 fused silica capillary column (30 m $\times$ 0.32 mm i.d., film thickness 0.25 μ m). Analyses were conducted isothermally at 260 $^{\circ}$ C with an injection temperature of 300 $^{\circ}$ C. The carrier gas was hydrogen with a flow rate of 4 ml min $^{-1}$. Peak areas were determined using a Shimadzu Chromatopac C-R3A data processor. GC/MS was performed on a VG 20-250 quadrupole instrument coupled to a Hewlett-Packard 5890 gas chromatograph. TMS derivatives were separated on a BP5 fused silica capillary column (12.5 m $\times$ 0.33 mm i.d., 0.25 μ m film thickness; SGE) using a temperature programme of 230–260 $^{\circ}$ C increasing at 5 $^{\circ}$ C min $^{-1}$. The carrier gas was helium and the pressure at the column inlet was 10 p.s.i. A cold on-column injector (SGE OCI3) was utilized and the column was coupled directly to the ion source of the mass spectrometer; ion source temperature 200 $^{\circ}$ C, electron energy 70 eV and electron current 100 μ A. Identification of sterols was based on comparison with authentic standards and by reference to published data on mass spectra and relative retention times of known sterols.

Table 1. Relative retention times and quantities of TMS-sterols in wild-type and *erg-3* strains of *N. crassa*

Sterol*	Relative retention time†	Quantity‡	
		Strain . . .	
		Wild-type	<i>erg-3</i>
A	1.26	80	—
B	1.46	6	—
C	1.56	2	4
D	1.79	2	1
E	1.14	—	25
F	1.29	—	5
G	1.33	—	43
H	1.35	—	13
Other(s)	—	10	9

* Structures shown in Fig. 1.

† Relative to cholesterol TMS ether as internal standard.

‡ Expressed as area % of total sterols.

Results and Discussion

Ultraviolet spectrophotometry of sterol extracts from the mutant *erg-3* showed an absorption maximum at 250 nm and a shoulder at 245 nm, which confirmed the observations of Grindle & Farrow (1978). Such a spectrum is characteristic of $\Delta^{8,14}$ -sterols (Mercer, 1988). No absorption maxima at 293, 282 and 272 nm were apparent, indicating the absence of $\Delta^{5,7}$ -dien-sterols. In contrast, the absorption spectrum of the wild-type sterol extract showed the presence of $\Delta^{5,7}$ -dien-sterols.

The relative retention times of the major sterols and their abundance in terms of peak area (Table 1) were determined from chromatograms of TMS-derivatized sterol extracts. The structures of the major sterols were deduced from their mass spectra (Table 2), and are depicted in Fig. 1.

Mass spectral data confirmed that the major sterol (A) in the wild-type was ergosterol, comprising 80% of the total sterols. A minor sterol (B) was identified as ergosta-7,24(28)-dien-3 β -ol (episterol), by comparison with mass spectra from Osumi *et al.* (1978) and Howell *et al.* (1990). Trace amounts of sterols having retention times comparable with, and fragmentation patterns of, obtusifoliol (C) and 24-methylenedihydrolanosterol (D) as their TMS derivatives were also detected.

In contrast, there were no detectable amounts of ergosterol in the *erg-3* mutant. In conjunction with the UV-spectrophotometric data, the GC-MS data verified the presence of $\Delta^{8,14}$ -conjugated-dien-sterols. The TMS ethers of sterols E, F, G and H, comprising 86% of the total sterols, each have mass spectra typical of $\Delta^{8,14}$ -dien-

Table 2. Mass spectra of major sterols as their TMS ethers in wild-type and *erg-3* strains of *N. crassa*

Figures in parentheses are the abundances of the ions.

Fragmentation†	Sterol* . . .	A	B	C	D	E	F	G	H
[M] ⁺		468(12)	470(3)	498(20)	512(20)	466(12)	466(5)	468(30)	470(40)
[M-Me] ⁺		453(2)	455(8)	483(37)	497(28)	451(3)	—	453(28)	455(12)
[M-C ₃ H ₇] ⁺		—	—	—	—	—	—	425(10)	—
[M-ROH] ⁺		378(8)	—	—	—	376(3)	—	—	380(4)
[M-84] ⁺		—	386(10)	—	—	—	—	—	—
[M-Me-84] ⁺		—	—	—	—	—	—	369(20)	—
[M-ROH-Me] ⁺		363(65)	—	393(55)	407(95)	361(100)	361(38)	363(15)	365(75)
[M-SC] ⁺		—	—	—	—	—	—	343(7)	343(4)
[M-SC-H(2H)] ⁺		—	343(50)	—	—	340(10)	—	—	—
[M-131] ⁺		337(53)	—	—	—	—	—	—	—
[M-ROH-C ₃ H ₇] ⁺		—	—	—	—	—	—	335(16)	—
[M-ROH-Me-84] ⁺		—	—	309(15)	324(18)	277(8)	277(8)	279(12)	—
[M-SC-ROH] ⁺		253(18)	255(15)	—	—	—	—	253(9)	253(18)
[M-SC-ROH-H(2H)] ⁺		—	—	281(18)	—	250(38)	250(5)	251(14)	—
[M-SC-ROH-Me-(H)] ⁺		—	—	—	282(15)	235(30)	235(9)	237(28)	238(28)
[M-SC-ROH-C ₃ H ₆ -Me+(H)] ⁺		—	—	227(25)	241(35)	—	—	—	—
[M-SC-ROH-C ₃ H ₆] ⁺		211(25)	213(18)	241(26)	—	209(16)	207(12)	—	—

* Structures shown in Fig. 1.

† Me, 15 (loss of methyl); C₃H₇, 43 (loss of C-25 to C-27); ROH, 90 (loss of Me₃SiOH); 84 (loss of C-23 to C-28 in 24 methylene side-chain); SC (loss of side chain); 131 (loss of C-1 to C-3); C₃H₆, 42 (loss of C-15 to C-17).

sterol TMS derivatives showing characteristic fragments [M-SC-ROH-Me]⁺ and [M-Me-ROH]⁺ (where SC denotes the side-chain). Further, the TMS ethers of sterols G and H display fragments [M-SC-ROH]⁺ and [M-SC]⁺ which also are typical of $\Delta^{8,14}$ -sterol derivatives (Rahier & Benveniste, 1989) (Table 2). Sterol H (comprising 13% of total sterols) as its trimethylsilyl ether has a molecular mass ion of *m/z* 470, indicating that it is a C₂₈ dienol. The lack of a fragment corresponding to [M-SC-2H]⁺ indicates that the side chain is unsaturated, while the [M-SC-ROH-Me]⁺ fragment at *m/z* 238 shows that the mass of the side chain is 127. This information, in addition to the UV spectrum, and comparison with published data (Loeffler & Hayes, 1990), shows that sterol H is consistent with ergosta-8,14-dien-3 β -ol (ignosterol).

The predominant sterol G (43% of total sterols) in *erg-3* has an additional double bond as indicated by its trimethylsilyl ether molecular ion at *m/z* 468. The occurrence of fragments [M-84-Me]⁺ at *m/z* 369 and [M-84-ROH-Me]⁺ at *m/z* 279 indicates the presence of a 24(28) double bond in the side chain, these fragments arising due to allylic cleavage of the 22-23 bond by McLafferty-type rearrangement (Rahier & Benveniste, 1989). These data are consistent with sterol G being ergosta-8,14,24(28)-trien-3 β -ol (Girling *et al.*, 1988).

Sterol E, comprising 25% of the total sterols in *erg-3*, has a trimethylsilyl molecular ion of *m/z* 466 which indicates that the sterol is a tetraene. The occurrence of fragment [M-84-ROH-Me]⁺ at *m/z* 277 indicates the presence of a 24(28) double bond in the side-chain, but

the location of the fourth double bond is difficult to determine. The base peak [M-Me-ROH]⁺ at *m/z* 361 (100%) is characteristic of a $\Delta^{5,8}$ -sterol (Rahier & Benveniste, 1989), and so the fourth double bond could be located at C-5=6. Although not confirmed, sterol E is tentatively identified as ergosta-5,8,14,24(28)-tetraene-3 β -ol.

Sterol F (6% of total sterols in *erg-3*) has a molecular ion at the same *m/z* value as sterol E (*m/z* 466) and a similar fragmentation pattern. However, the signal was very small and it could not be characterized further. Minor peaks corresponding to obtusifoliol (4% of total sterols) and 24-methylenedihydrolanosterol (1% of total sterols) were also detected in the *erg-3* mutant.

Since most of the sterols isolated from the *erg-3* mutant are $\Delta^{8,14}$ -conjugated-diens, the mutant must be defective/deficient in $\Delta^{14,15}$ -reductase activity. Presumably, *erg-3* is the structural gene encoding this enzyme. To our knowledge, this is the first description in the literature of such a biochemical lesion in a fungal sterol mutant. It should be possible to confirm the absence of $\Delta^{14,15}$ -reductase activity in the mutant by measuring enzyme activity directly in cell-free preparations using the assay described by Bottema & Parks (1978). The defective $\Delta^{14,15}$ -reductase would appear to prevent Δ^{8-7} -isomerase activity, indicating the requirement of a saturated bond at C-14-15 for isomerase activity. However, both $\Delta^{5,6}$ -dehydrogenase and 24(28)-hydrogenase activities seem to be partially functional.

The defect at $\Delta^{14,15}$ -reductase mimics the action of certain sterol biosynthesis inhibitors such as

fenpropidin, 15-azasterol and azadecalins. These compounds inhibit $\Delta^{14,15}$ -reductase (Baloch & Mercer, 1987; Bottema & Parks, 1978; Taton *et al.*, 1989) and result in the accumulation of $\Delta^{8,14}$ -dien-sterols (Mercer, 1988; Taton *et al.*, 1989).

The $\Delta^{14,15}$ -reductase mutant (*erg-3*) could be of value in the analysis of sterol biosynthesis inhibition by morpholine and piperidine fungicides. Studies are in progress to determine the effect of tridemorph, fenpropimorph and fenpropidin on growth and sterol composition of *erg-3*. Molecular biology studies to clone the $\Delta^{14,15}$ -reductase gene by complementation of the *erg-3* mutant are also in progress.

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