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Detection of the Ergosterol and Episterol Isomers Lichesterol and Fecosterol in Nystatin-Resistant Mutants of *Neurospora crassa*

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Wild-type Neurospora crassa *is completely inhibited by 5 ppm nystatin. Ultraviolet-induced mutants have been isolated that grow in the presence of 60 ppm of the antibiotic. Gas–liquid chromatographic, mass spectroscopic, and nuclear magnetic resonance analyses showed the wild-type sterols to be ergosterol (ergosta-5,7,22-trien-3β-ol) and episterol (ergosta-7,24(28)-dien-3β-ol) in a 3:1 ratio. The mutants contained lichesterol (ergosta-5,8,22-trien-3β-ol) and fecosterol (ergosta-8,24(28)-dien-3β-ol) in a 2:1 ratio, differing from the wild type only in the position of the B-ring unsaturation. A deficiency of an ergosta-8,24(28)-dien-3β-ol:ergosta-7,24(28)-dien-3β-ol isomerase is indicated.*

KEY WORDS: steroids; *Neurospora;* mutants; lichesterol; nystatin.

INTRODUCTION

Nystatin is a polyene antifungal antibiotic widely used in medical and veterinary practice to control monilial mycoses. The clinical and laboratory finding of nystatin-resistant strains (Woods, 1971; Athar and Winner, 1971) of some fungal pathogens has prompted a study of the mechanism of resistance. Nystatin is known to bind to the membrane sterols on *Neurospora* and yeasts, changing permeability characteristics and inhibiting growth (Kinsky, 1961, 1962; Lampen *et al.*, 1962). Resistant mutants have been isolated (Grindle, 1973), but to date no reports exist on the chemical analysis of the endogenous sterol fractions of wild-type or resistant strains of *Neurospora.*

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This report describes the triterpene and steroid contents of wild-type nystatin-sensitive (*nys*^{s}) and nystatin-resistant (*nys*^{r}) strains of *Neurospora crassa*.

MATERIALS AND METHODS

Isolation of Mutants

A compact ("ragged" isol No. B53, "crisp" isol No. B123) *nys*^{s} strain of *Neurospora crassa* suitable for mutant hunts (Maling, 1960) was obtained from the Fungal Genetics Stock Center, Arcata, California. The culture conditions, crossing, and ultraviolet irradiation procedures are described elsewhere (Goldie and Subden, 1973; Morris and Subden, 1974). One-tenth milliliter aliquots of irradiated conidial suspension (5×10^6 conidia/ml or 10^5 viable conidia) were plated on medium containing 10 ppm nystatin. Surviving colonies were streaked on Szybalski (Bryson and Szybalski, 1952) plate gradients to 100 ppm nystatin (mycostatin, Calbiochem). Clones from the most resistant isolates were then backcrossed to an albino strain (*al-3* isol No. RP 100) to obtain *nys*^{r} progeny with a wild-type growth habit. The *nys*^{r} progeny that were morphologically wild type were tested on race tubes (60 cm × 8 mm ID) containing 20 ppm nystatin. Resistant strains were retested on race tubes containing 40, 60, and 100 ppm nystatin. The most resistant progeny from each cross were then cultured in 12-liter batches of liquid medium for steroid analysis.

Heat or light decreases nystatin activity, so the nystatin was dissolved in propylene glycol, then added to the medium after autoclaving. Initially, the concentration was estimated from the 307-nm absorption maximum ($E^{1\%}_{1cm}$, 2.5) and the degree of denaturation from secondary peaks at 354 and 384 nm. These peaks were empirically found to be associated with loss of activity.

Steroid Extractions

Mycelia from 6-day-old 12-liter batches were harvested in cheesecloth, washed with distilled water, filtered on Whatman No. 1 paper, and then lyophilized. The lyophilized cells were macerated in a Waring blender with chloroform–methanol (2:1) and the mixture was refluxed for 2 hr. The mixture was filtered, the filtrate concentrated to dryness, and the resulting extract hydrolyzed with methanolic potassium hydroxide solution (10%) for 2 hr. The solution was diluted with water and the lipids were removed by ether extraction. The sterol fraction was purified from this fraction by preparative silica gel thin-layer chromatography with chloroform as the solvent. The sterol band was located by ultraviolet absorption and scraped from the plate, eluted with diethyl ether, concentrated, and weighed to give the free sterol fraction.

The lipid-extracted cells were then refluxed with methanolic potassium

hydroxide solution (10%) for 2 hr, and filtered on Whatman No. 1 paper. The solution was diluted with water and extracted with diethyl ether. The bound sterols were isolated from this extract as described above.

Analysis and Separation of Individual Sterols

The above free and bound sterol fractions were pooled and converted into their corresponding acetate derivatives by treatment with acetic anhydride–pyridine (1:2). The resulting acetates were analyzed by gas–liquid chromatography on 6-ft by $\frac{1}{4}$-inch glass column packed with 3% OV_{17} (Applied Science) at 250 C using helium as a carrier at 50 ml/min.

Acetyl derivatives were also separated and purified by repeated thin-layer chromatography on silica gel and alumina plates impregnated with 20% $AgNO_3$. The individual steryl acetates were further characterized by mass spectroscopy (Varian MAT-7-CH-7) and nuclear magnetic resonance spectroscopy (Varian 220-MHz spectrometer).

RESULTS

Ten mutants were isolated that would grow in the presence of 60 ppm nystatin.

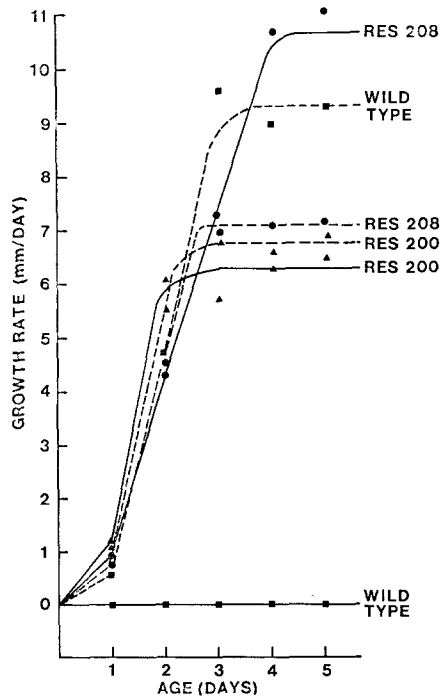

Fig. 1. Growth rates of three strains of *Neurospora crassa*. ■, *nys*^{s} 74-OR23-1A; ▲, *nys*^{r} RES 200-a; ●, *nys*^{r} RES 208-a. – – –, Minimal medium; ———, minimal medium plus 60 ppm nystatin from 60-cm long race tubes.

Table I. Percentage Composition of Chloroform–Methanol and Methanolic Potassium Hydroxide Extractable Sterols of *Neurospora crassa*

Sterol	Relative R_c[a]	Molecular ion (m/e)	Chloroform–methanol extract (mg/kg)[b]		Methanolic potassium hydroxide extract (mg/kg)	
			Wild type	Mutants	Wild type	Mutants
Ergosta-5,8,22-trien-3β-ol	4.05	438		4.4		0.30
Ergosta-5,7,22-trien-3β-ol	4.30	438	2.8		trace	
Ergosta-8,24(28)-dien-3β-ol	4.80	440		5.3		0.32
Ergosta-7,24(28)-dien-3β-ol	5.30	440	1.0		trace	

[a] GLC conditions are given in Materials and Methods; R_c refers to retention times of steryl acetates relative to 5α-cholestane.

[b] Milligrams of sterol per kilogram of freeze-dried mycelium.

Table II. Chemical Shift Data for the Methyl and Olefinic Proton Resonances (δ, ppm) of the Steryl Acetates Isolated from *Neurospora crassa*

Sterol	Methyl groups						Olefinic protons		
	C18	C19	C21	C26	C27	C28	C6/7	C22/23	C24/28
Ergosta-5,8,22-trien-3β-ol	0.67(*s*)	1.20(*s*)	1.02(*d*) (J = 6 Hz)	0.82(*d*) (J = 7 Hz)	0.84(*d*) (J = 7 Hz)	0.92(*d*) (J = 7 Hz)	5.44(*m*)	5.19(*m*)	
Ergosta-5,7,22-trien-3β-ol	0.63(*s*)	0.96(*d*)	1.02(*d*) (J = 6 Hz)	0.81(*d*) (J = 7 Hz)	0.83(*d*) (J = 7 Hz)	0.91(*d*) (J = 7 Hz)	5.35(*m*)	5.09(*m*)	
Ergosta-8,24(28)-dien-3β-ol	0.61(*s*)	0.96(*s*)	0.95(*d*) (J = 7 Hz)	1.03(*d*) (J = 7 Hz)	1.03(*d*) (J = 7 Hz)				4.68(*m*)
Ergosta-7,24(28)-dien-3β-ol	0.54(*s*)	0.81(*s*)	1.03(*d*) (J = 7 Hz)	1.03(*d*) (J = 7 Hz)	1.03(*d*) (J = 7 Hz)		5.15(*m*)		4.70(*m*)

Two of these *nys*^{r} strains (RES 200-a, RES 208-a) were morphologically indistinguishable from wild type (*nys*^{s}) and were chosen for further study. RES 208-a exhibited some nystatin enhancement inasmuch as its growth rate was consistently higher on low levels (20 ppm) of nystatin medium than on minimal medium (Fig. 1). A small amount of growth of wild type (74-OR23-1A) after day 5 occurred on race tubes containing 20 ppm nystatin, but this was attributed to nystatin inactivation rather than any resistance mechanism.

Crosses of *nys*^{r} (RES 208)A × *nys*^{r} (RES 200)a were infertile, so a *nys*^{r} (RES 208), *al-3* (RP 100)A + *nys*^{s}*tryp-2* (75001)A heterokaryon was used as protoperithecal parent and *nys*^{r} (RES 200)a as the conidial parent. All viable progeny from the *nys*^{r} (RES 208)A, *al-3* (RP 100)a (in the heterokaryon) × *nys*^{r} (RES 200)a were *nys*^{r}. A heterokaryon *nys*^{r} (RES 200)a + *nys*^{r} (RES 208), *al-3* (RP 100)a was also nystatin resistant.

A bioassay using an albino *nys*^{s} strain in the nystatin-containing medium from which a log phase *nys*^{r} culture had been removed showed that approximately one-third to one-half of the nystatin was still biologically active.

The total chloroform–methanol extractable lipid fraction for both the *nys*^{s} and *nys*^{r} varied between 9 and 10% of dry weight, some components being sterols 0.40–0.70%, triterpenes 0.03%, tetraterpenes 0.01%, and fatty acids 3.38%.

GLC analysis of wild-type steroids gave two major peaks, which were different from those found in the mutants. However, the mass spectrometric analysis gave identical molecular ion values but different fragmentation patterns for mutant and wild-type steroids. The results of the GLC and mass spectrometric analyses are collated in Table I. The 220-MHz nuclear magnetic resonance spectra of all four compounds are summarized in Table II. The data were consistent with the sterol structures indicated in the tables and identical to results which have previously been reported (Safe, 1973; Lenton *et al.*, 1973; Barton *et al.*, 1972).

DISCUSSION

It has been shown that one allelic series associated with nystatin resistance in *Neurospora* operates by producing B-ring isomers of ergosterol (ergosta-5,7, 22-trien-3β-ol) and episterol (ergosta-7,24(28)-dien-3β-ol), namely lichesterol (ergosta-5,8,22-trien-3β-ol) and fecosterol (ergosta-8,24(28)-dien-3β-ol). A $\Delta 8 \rightarrow \Delta 7$ isomerase deficiency in a steroid biosynthetic scheme according to Barton *et al.* (1973) satisfies the data (Fig. 2).

Previously it had been shown that nystatin inhibition proceeds by some physicochemical interaction with the membrane sterols of sensitive fungi (Gotleib *et al.*, 1958). An exogenous sterol such as cholesterol or ergosterol can nullify the nystatin effect by forming inactive *in vitro* steroid–antibiotic

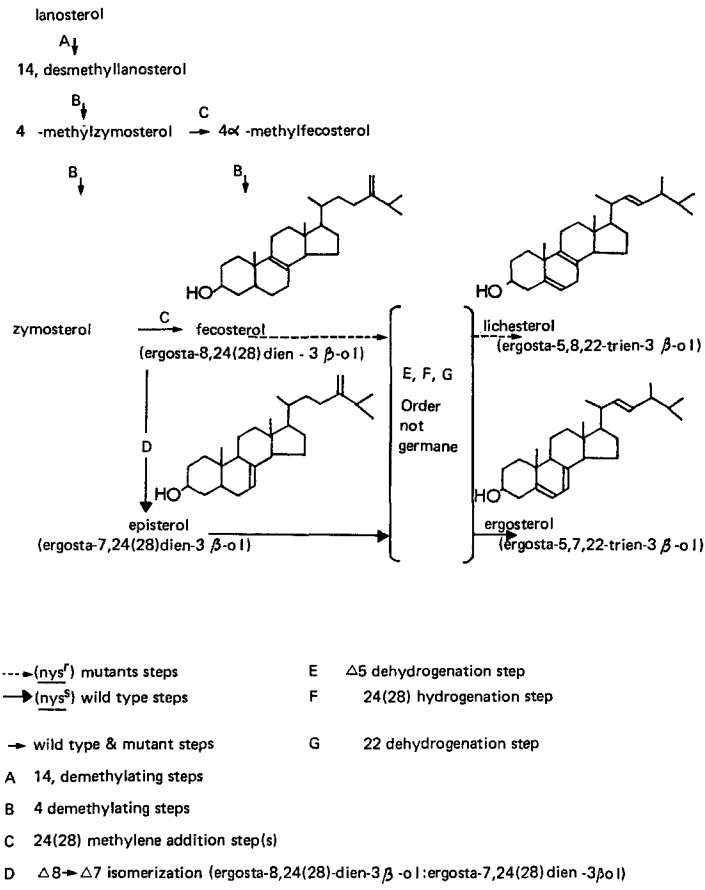

Fig. 2. Steroid biosynthetic scheme according to Barton *et al.* (1973) with added *nys*^{r} mutant sequences.

complexes (Lampen *et al.*, 1960). Because there was residual active nystatin in the medium from 4-day *nys*^{r} cultures, it can be assumed that the effect of the fecosterol–lichesterol isomers is endogenous and results in changes in the physicochemical properties of the membrane to preclude the inhibitory effects of nystatin.

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