Ergosterol and Lanosterol from *Aspergillus nidulans*

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Ergosterol was identified as the major free sterol of *Aspergillus nidulans* by thin-layer chromatography, alumina column chromatography, gas-liquid chromatography, high-performance liquid chromatography, UV spectroscopy, proton magnetic resonance spectroscopy and mass spectral analysis. Lanosterol, the initial cyclized precursor of ergosterol, was identified as a minor component of the free sterols. In the steryl ester material, however, lanosterol was usually more abundant than ergosterol, suggesting that the esters serve as storage compounds for the membrane sterol precursors.

INTRODUCTION

Aspergillus fennelliae (Kim & Kwon-Chung, 1974), *A. flavus* (Rambo & Bean, 1974), *A. fumigatus* (Sherald & Sisler, 1975), *A. parasiticus* (Rambo & Bean, 1974) and *A. niger* (Weete & Laseter, 1974; Barton & Bruun, 1951) contain ergosterol (24 β -methylcholesta-5,7,*trans*-22-trien-3 β -ol) as a major sterol component. However, in *A. oryzae* (Fujino & Ohnishi, 1979) no ergosterol was detected, but rather brassicasterol (24 β -methylcholesta-5,*trans*-22-dien-3 β -ol) was the major free sterol with episterol (24-methylcholesta-7,24(28)-dien-3 β -ol) as a secondary constituent. Episterol was the major esterified sterol in this organism. This suggests that the composition of the separate cellular sterol pools does not simply mirror the total composition. Similar quantitative dissimilarities between the free sterols and the steryl esters have been described for *Saccharomyces cerevisiae* (Bailey & Parks, 1975; Taylor & Parks, 1978), *Phycomyces blakesleeana* (Bartlett & Mercer, 1974) and *Neurospora crassa* (Pierce *et al.*, 1979). In general, it has been found that biosynthetic intermediates in the pathway to the ultimate membrane sterol are quantitatively more prominent in steryl esters than in the free sterols.

While *A. nidulans* has been a model for many biochemical, genetic, and developmental studies (Clutterbuck, 1977; Smith & Pateman, 1977), its sterol composition has not previously been explored. The known divergence within the aspergilli indicates the inappropriate nature of compositional generalization from another species. Because of the potential usefulness of *A. nidulans* in elucidation of the regulatory mechanisms involved in sterol biosynthesis, we have proceeded with this compositional analysis.

METHODS

Organism and growth conditions. *Aspergillus nidulans* FGSC-4 was grown in a defined glucose/salts medium described previously (Adler *et al.*, 1981). Three replicate culturings (experiments 1, 2, and 3 in Table 1) were grown (at different times) for 3 d in 2-litre conical flasks, each containing one litre of medium, on a rotary shaker (Lab-Line Orbit Shaker, Model 3590). In experiments 1 and 3, cultures were shaken at 100 rev. min⁻¹; in experiment 2 they were shaken at 50 rev. min⁻¹. Incubation temperature was 30 °C. Cells were collected by filtration through Miracloth (Chicopee Mills, Inc., New York, U.S.A.) as described previously (Gealt *et al.*, 1976) and frozen at -70 °C until used.

Sterol extraction. Frozen cells were lyophilized and then stored at -70°C until extraction. Soxhlet extraction with acetone was performed for 48 h. Samples were maintained in darkness during both lyophilization and extraction to avoid light-induced degradation of ergosterol. The acetone-solubilized material was dried by flash evaporation and the lipids were extracted from it using anhydrous diethyl ether; this step was required because non-lipid material was also removed from the cells by acetone. Sterols from the ether extract were further purified either by thin-layer chromatography (TLC) on silica gel 60 or by alumina column chromatography. The following TLC conditions were used: (a) hexane/ether/glacial acetic acid (85:15:0.5, by vol.) was used for general lipid separations; (b) benzene/ethyl acetate (9:1, v/v) was used for the separation of 4,4-desmethyl from 4,4-dimethyl free sterols; and (c) benzene/*n*-hexane (1:1, v/v) was used for separation of sterol esters and triglycerides. Lipid material was visualized after TLC by charring the plate which had been sprayed with phosphomolybdic acid (10%, w/v, in ethanol). Alternatively, visualization by long-wave UV was used to corroborate the location of various compounds with characteristic fluorescence (e.g. the violet fluorescence of ergosterol). Sterols and sterol esters were separated by alumina chromatography using an ether into hexane gradient increasing in 5% increments. Alumina (Al_2O_3 , Woelm Neutral, ICN Pharmaceutical) was hydrated with 8.0 or 8.7% water before use. Free and esterified sterols were located by absorbance at 282 nm, which is characteristic of ergosterol and other $\Delta^{5,7}$ sterols. Precise location of sterols eluted from the column was achieved by analytical TLC of individual fractions using solvent system (a) (above). TLC using solvent (c) confirmed the location of the sterol esters. To saponify sterol esters, the appropriate fractions from TLC or alumina chromatography were suspended in 5% (w/v) KOH in methanol/water (90:10, v/v) and refluxed for 1 to 2 h. Sterols were extracted with ether and neutralized by backwashing with water. The saponified material was stored at -70°C until analysis.

Analysis of sterols. Gas-liquid chromatography (GLC) was performed on XE-60 (1%) on Chromosorb Q, 100–200 mesh at 235°C using either a Perkin-Elmer gas chromatograph, Model 801, or a Hewlett Packard GLC, Model 5840. Both instruments used flame ionization detectors. All retention times are given relative to that of cholesterol [relative retention time (RRT) = 1.00]. High-performance liquid chromatography (HPLC) was performed on a μ -Bondapak C-18 column (Waters, Inc.) using 100% acetonitrile as the mobile phase. The effluent was analysed by UV absorption in an LDC Spectromonitor III (Laboratory Data Control). Retention is indicated by comparison of k' for the sample peak to k' of a cholesterol standard [$\alpha_c = (k'_{\text{sample}})/(k'_{\text{cholesterol}})$]. UV spectroscopy was performed on a Beckman spectrophotometer, model 24. Mass spectral (MS) analysis was performed on a Finnegan Series 4021 GLC-MS instrument with a Series 6000 data system, using direct probe for sample introduction. Proton magnetic resonance (PMR) spectra were obtained at 360 MHz at ambient temperature on a Bruker instrument, model WH-360, in CDCl_3 with $\text{Si}(\text{CH}_3)_4$ as internal standard, at the National Institutes of Health sponsored PMR regional facility at the University of Pennsylvania, Philadelphia, PA, U.S.A. Ergosterol (Sigma) was recrystallized from ethanol for analytical comparisons. Lanosterol was the gift of W. R. Nes (Drexel University).

RESULTS AND DISCUSSION

Ergosterol as the major free sterol

Approximately 0.5% of the fungal dry weight was found to be free (non-esterified) sterol. When this material was examined by GLC, only a single component, with a RRT of 1.36, was observed (Table 1, experiments 1 and 2). This retention was identical to that of authentic ergosterol. When free sterols were purified using the silica TLC method, which can separate the 4,4-desmethyl from the 4,4-dimethyl free sterols, the material which co-migrated with the ergosterol standard also showed only the GLC component with RRT = 1.36. Using HPLC, ergosterol ($\alpha_c = 0.73$) was the dominant peak, comprising at least 84% of the total free sterol. The *A. nidulans* ergosterol (from experiment 2) was purified by HPLC for further analysis. When it was examined by UV spectroscopy, λ_{max} values were at 270, 282 and 294 nm, indicating a sterol, like ergosterol, which contains a $\Delta^{5,7}$ conjugated bond system. No strong absorption at 240 nm, indicative of a component containing a $\Delta^{22,24(28)}$ conjugated bond system, or at 251 nm, indicative of a $\Delta^{8,14}$ bond system, was observed. The $\Delta^{22,24(28)}$ conjugated bonds are found in certain ergosterol precursors, e.g., 22(23)-dehydroepisterol and 24(28)-dehydroergosterol. The sterol material was further analysed by electron-impact MS. Fragments which were indicative of ergosterol included: M^+ , m/e of 396 (87%); $\text{M}^+ - \text{CH}_3$, $-\text{H}_2\text{O}$, 363 (100%); $\text{M}^+ - \text{side chain}$, 271 (8%); $\text{M}^+ - \text{side chain}$, $-\text{H}_2\text{O}$, 253 (62%); and $\text{M}^+ - \text{side chain}$, $-\text{C}_3\text{H}_6$, $-\text{H}_2\text{O}$, 211 (42%). Additional confirmation of our identification came from PMR, which showed identical chemical shifts in p.p.m., for both the *A. nidulans*

Table 1. GLC and HPLC migration of the sterols of *A. nidulans*

Expt	Separation technique	GLC			HPLC				
		RRT	Free sterol (%)	Steryl ester (%)	α_c	Free sterol (%)	Steryl ester (%)		
1	Alumina column				0.59	5	—		
					0.68	—	21		
		1.36	100	32	0.73	84	11		
		1.54	—	68	0.89	5	66		
					1.07	7	3		
2	Alumina column				0.59	1	ND		
		1.36	100	93	0.73	89			
		1.54	—	7	0.84	2			
					1.07	7			
3	Silica TLC	Desmethyl		Dimethyl		Desmethyl		Dimethyl	ND
					0.58	—	3		
		1.01	—	—	5	0.64	—	2	
		1.36	100	—	36	0.73	97	—	
						0.81	—	12	
						0.85	1	—	
		1.54	—	54	59	0.89	—	82	
		1.72	—	4	trace	1.03	2	—	
		1.89	—	41	trace				

ND, Not determined.

free sterol and authentic ergosterol: C-18(s), 0.63; C-19(s), 0.95; C-21(d), 1.04 ($J = 6$ Hz); C-26,27(d), 0.82 and 0.84 ($J = 6$ Hz); and C-28(d), 0.92 ($J = 7$ Hz). Especially indicative of ergosterol was the presence of a doublet for C-28, indicating an additional carbon on the sterol side chain, i.e. the methyl addition at C-24. The addition of a methylene at C-24, present in certain ergosterol precursors, e.g. episterol, shows no such peak in the methyl region of the spectrum. The location of C-21 on the spectrum was indicative of the presence of a Δ^{22} double bond in the side chain. These spectra are identical to those previously reported for ergosterol from other Ascomycete species (Adler *et al.*, 1977). We conclude that ergosterol was the major free sterol of *A. nidulans*, totalling 85 % of this sterol fraction.

Lanosterol as the major dimethyl sterol

Minor sterol components of *A. nidulans* were observed both by GLC and by HPLC. The HPLC technique was apparently more sensitive to minor components in the sterol sample, being able to resolve components which were masked by ergosterol in the GLC. Lanosterol (RRT = 1.54 on XE-60), which is known generally to be the initial dimethyl precursor of ergosterol in fungi, including *A. fumigatus* and *A. oryzae*, was thus a probable trace component of the free sterol fraction. A component with this RRT was found in the dimethyl fraction of our free sterols (Table 1, experiment 3). This peak corresponds to the HPLC component with $\alpha_c = 0.89$ found in both experiments 1 and 3. In experiment 2 lanosterol was undoubtedly eluted within the $\alpha_c = 0.84$ peak, the deviation in the retention probably being due to the presence of other components, e.g. the $\alpha_c = 0.81$ (dimethyl) and $\alpha_c = 0.85$ (desmethyl) components observed in experiment 3. Examination of the dimethyl sterol material from experiment 3 yielded a PMR spectrum which contained several peaks consistent with lanosterol as the major component: C-4 at 0.81 and 0.98 p.p.m., C-14 at 0.88 p.p.m., C-18 at 0.69 p.p.m., C-19 at 1.00 p.p.m., C-21 at 0.89 p.p.m., and C-26,27 at 1.67 p.p.m. While not totally confirmatory because of the weakness of the spectrum, when taken together with the TLC, GLC, and HPLC evidence, the presence of lanosterol as a free sterol seems certain.

Components other than ergosterol and lanosterol were observed in the sterol material purified from *A. nidulans*. The component with $\alpha_c = 0.81$, a dimethyl sterol observed in experiment 3, may correspond to the GLC peak with RRT = 1.89, based solely on their relative prominence in the respective analyses. Retention characteristics indicate that this component was both larger than ergosterol and more polar than lanosterol. Known ergosterol precursors in other organisms, such as 24-methylene lanosterol or 4,4-dimethyl fecosterol, would behave similarly to this unknown. Positive identification of this component, and others, awaits purification of larger amounts of material.

Steryl esters of A. nidulans

Quantitative differences were apparent from a comparison of the composition of the free sterol and steryl ester fractions from this fungus. The requirement for ergosterol as the major sterol appears to be restricted to the free sterols; in only one case (experiment 2) did ergosterol predominate in the esters. The lanosterol content was consistently higher in the esters than in the free sterols. The other components observed following saponification of the esters might represent various sterol intermediates being accumulated by *A. nidulans* as occurs in other fungi. The amount of ergosterol precursors in the steryl esters was seen to increase in *S. cerevisiae* as cells entered the stationary phase (Taylor & Parks, 1978).

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