7-Dehydrostigmasterol and ergosterol: the major sterols of an amoeba

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ABSTRACT The sterol fraction of *Acanthamoeba* (Neff) contains 60% 7-dehydrostigmasterol and 40% ergosterol. The sterols were characterized by infrared and ultraviolet spectroscopy, gas chromatography, specific reactions, and mass spectral analysis. Sterols constitute 5% of the lipids and 15% of the neutral lipids in this species.

KEY WORDS sterols · amoebae · *Acanthamoeba* (sp.) · ergosterol · 7-dehydrostigmasterol · taxonomy

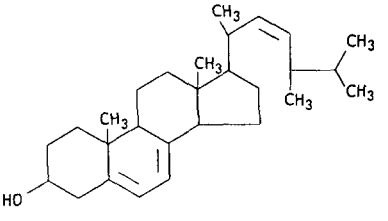
ANALYSIS OF THE FATTY ACID composition of protists has provided a useful tool for investigating taxonomic and phylogenetic relationships (1, 2). To select one example, the two soil amoebae, *Acanthamoeba* (Neff) and *Hartmannella*, and the true slime mold amoeba, *Physarum polycephalum*, have identical fatty compositions (1, 3, 4) whereas the cellular slime mold amoeba, *Dictyostelium discoideum*, synthesizes completely unrelated unsaturated fatty acids (5, 6). The protozoa have also proved useful in establishing biosynthetic and metabolic relationships among the polyunsaturated fatty acids (7–9). Similar benefits might be expected to accrue from investigations of the nature of the protozoal sterols. We now report that the two major sterols of *Acanthamoeba* (Neff) are 7-dehydrostigmasterol and ergosterol (Fig. 1).

EXPERIMENTAL PROCEDURES AND RESULTS

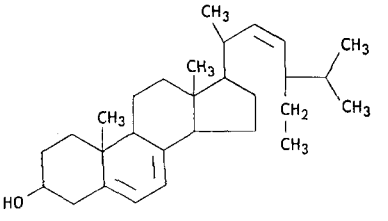
Isolation of the Sterols

Amoebae were grown in 1-liter shaking cultures in a proteose peptone–glucose medium as described previously (10). The cells were harvested after 6–8 days, at which time they contained about 5% cysts. The amoebae were washed with 0.015 M phosphate, pH 6.7, and extracted with 20 volumes of chloroform–methanol 2:1. The lipid extract was taken to dryness on a rotary evaporator and the lipids were dissolved in heptane. The total lipids were fractionated on a column of silicic acid (Unisil, 100–200 mesh, Clarkson Chemical Company, Inc., Williamsport, Pa.) with a load of 500 μg of lipid phosphorus for each g of silicic acid. Neutral lipids were eluted with the following solutions of diethyl ether in heptane (20 ml of each per g of silicic acid): 1%, 4%, 8%, 25%, and 100%. Phospholipids were then eluted with 100% methanol. Total lipids, neutral lipids, and phospholipids were estimated by weight.

Each neutral lipid fraction was analyzed by thin-layer chromatography on 0.2 mm thick Silica Gel G (Brinkmann Instruments Inc., Westbury, N.Y.) with petroleum ether–diethyl ether–glacial acetic acid 80:20:1.



ERGOSTEROL



7-DEHYDROSTIGMASTEROL

FIG. 1. Formulae of ergosterol and 7-dehydrostigmasterol.

Abbreviation: GLC, gas–liquid chromatography.

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Compounds were made visible by exposure to iodine vapor and identified by comparison with standards. Sterols were present in the fraction eluted with 25% ether.

This fraction was also analyzed by GLC on a 6 ft column of 1% SE-30 (methylpolysiloxy gum, Applied Science Laboratories Inc., State College, Pa.) at a temperature of either 225 or 250°C and an inlet pressure of 10 psi of argon. Two major peaks and several minor ones, with shorter retention times, were observed. After recrystallization of the sterols from methanol only the two major compounds remained. The faster-moving compound had a retention time identical with that of ergosterol and the slower compound had a retention time less than that of stigmasterol.

An amount of fresh-growth medium equal to that on which the amoebae were grown was lyophilized and the residue was subjected to the same procedures. No sterols were found.

Identification of the Sterols

The IR spectrum of the mixed amoeba sterols was strikingly similar to that of standard ergosterol in the "fingerprint" region of 800–1350 cm^{-1}. The absorption band at 970 cm^{-1} indicated the presence of a *trans* double bond in the sterol side chain. Spectra were obtained as fused KBr pellets with a Beckman IR7 spectrophotometer.

Small amounts of the individual sterols (about 100 μg) were separated by preparative GLC in 1% SE-30 and collected on silicone-treated Chromosorb W (Johns-Mansville) in glass cartridges. The sterols were eluted with 5 ml of diethyl ether. Both sterols had the same UV absorption spectrum as ergosterol (double major peak at 274 and 282 mμ, minor peak at 290 mμ, shoulder at 262 mμ). The peak at 282 mμ is indicative of a conjugated diene in ring B (11).

The mixed sterols (500 μg in 3 ml of ethanol–acetone 1:1) were quantitatively precipitated by the addition of 1 ml of 0.5% digitonin in 80% ethanol (12). Recovery of sterols was measured by the Liebermann–Burchard reaction (12). Formation of digitonides indicates the presence of a 3β-hydroxyl group. Both sterols were fast-reacting in the modified Liebermann–Burchard reaction (12); this fast reaction is characteristic of 5,7-diene sterols. The rate of reaction was determined by measuring the increase in absorbance at 620 mμ in a Cary recording spectrophotometer.

By these criteria the compound with lower GLC retention time was ergosterol. The second sterol differed from ergosterol only in its retention time, which suggests that the sterol nucleus was the same as ergosterol but that the side chain might be longer.

Identifications were established by mass spectral analysis. The sterol emerging earlier showed a parent peak at m/e 396 and a fragmentation pattern identical with that of ergosterol (Fig. 2 and reference 13). The slower-moving sterol showed a parent peak at m/e 410 (Fig. 3), consistent with its being a homologue of ergosterol with an extra methylene group in the side chain. The mass spectrum of this compound was almost identical with that of synthetic 7-dehydrostigmasterol (14). Several peaks were mass-matched on an AEI mass spectrometer.

		Calcd.	*Found*
M	$C_{29}H_{46}O$	410.355	410.356
M − CH_5O	$C_{28}H_{41}$	377.321	377.321
M − C_3H_7O	$C_{26}H_{39}$	351.305	351.3075
M − $C_{10}H_{19}$	$C_{19}H_{27}$	271.206	271.2045

The principal peaks of the mass spectrum of 7-dehydrostigmasterol can be explained by the same fragmentation pattern (Fig. 4) as has been proposed for ergosterol (13). The identity of the mass peaks for the two sterols after removal of the side chain (m/e 271 and 253) confirms that the sterols differ only in their side chains.

The identification of the two amoeba sterols as ergosterol and 7-dehydrostigmasterol was supported by

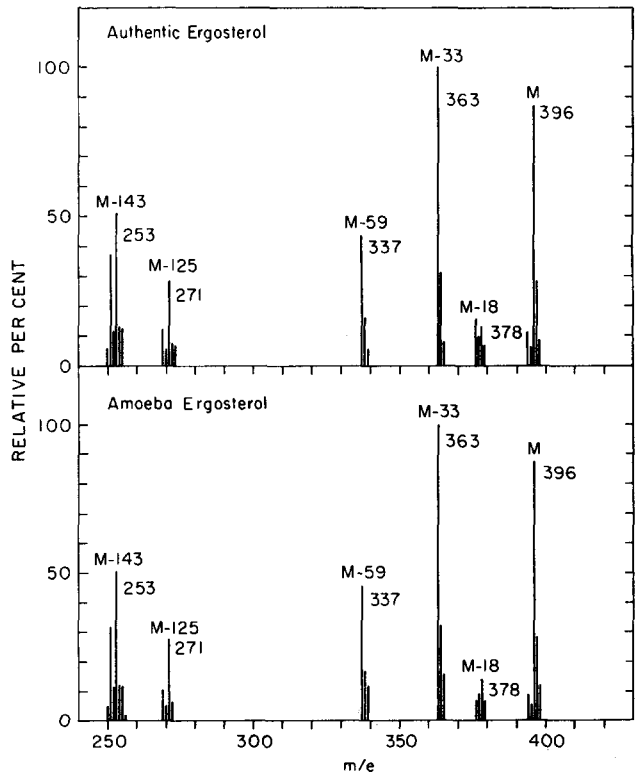

FIG. 2. Mass spectra of amoeba ergosterol and authentic ergosterol. An LKB 9000 gas chromatograph–mass spectrometer was used. The gas-chromatographic inlet contained 1% SE-30. Chromatographic conditions were essentially as described in the text. The ion source was at 290°C, the energy of the ionizing beam was 70 ev, and the accelerating voltage was 3.5 kv.

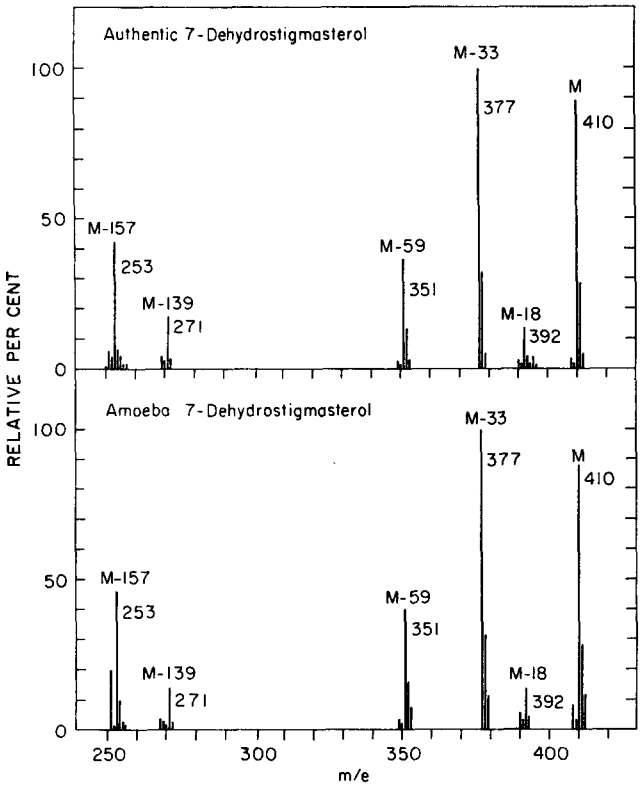

FIG. 3. Mass spectra of amoeba 7-dehydrostigmasterol and standard 7-dehydrostigmasterol. Experimental conditions were as described for Fig. 2. The standard was a generous gift from Dr. O. Wintersteiner (14).

comparison of the gas-chromatographic retention times of the two amoeba sterols and their trimethylsilyl ethers with those of appropriate standards in two different systems (Table 1).

Degradation of the Sterol Side Chain

The mixed sterols (25 mg) were allowed to react with 250 mg of OsO_4 in a solution of 25 ml of diethyl ether and 1.25 ml of pyridine for 4 days under nitrogen at room temperature (15). The precipitate was collected and refluxed with a solution of Na_2SO_3 to convert the osmates to polyglycols. The reaction mixture was evaporated to dryness and the polyglycols were extracted with 95% ethanol. Glycols were then cleaved with a 10% excess of periodic acid for 30 min at room temperature. The resultant aldehydes were converted to 2,3-dinitrophenylhydrazones, and the precipitate was collected by centrifugation and washed twice with cold water. The hydrazones were dissolved in benzene and applied to a column which contained 1 g of alumina previously dried at 130° C for 2 days. The hydrazones were eluted with 20 ml of benzene, evaporated to dryness, and dissolved in ethyl acetate. The products were then analyzed by GLC on a 6 ft × 1/4 inch column of 3% OV-17 (phenyl methyl siloxane) on Gas-Chrom Q at 200°C with an input pressure of 15 psi of argon.

TABLE 1 IDENTIFICATION OF AMOEBA STEROLS AND THEIR TRIMETHYLSILYL ETHERS BY GLC

	3% OV-17*		1% SE-30†	
	Alcohol	Ether	Alcohol	Ether
Amoeba ergosterol	0.80‡	0.62	0.60	0.78
Standard ergosterol	0.80	0.62	0.60	0.78
Amoeba 7-dehydrostigmasterol	1.00§	0.77	0.82	1.00¶
Standard 7-dehydrostigmasterol	1.00§	0.77	0.82	1.00¶

* 250°C, 15 psi of argon, Gas-Chrom P, column 6 ft × 1/4 inch.
† 225°C, 15 psi of argon, Gas-Chrom P, column 6 ft × 1/4 inch.
‡ Retention times relative to that of the slowest compound (7-dehydrostigmasterol on SE-30 and trimethylsilyl 7-dehydrostigmasterol on OV-17).
§ 15.4 min.
¶ 17.8 min.

The chromatographic pattern of the products obtained from the mixed sterols was identical with that obtained from standard ergosterol except for one additional peak. One of the common peaks had the retention time (19.5 min) of the 2,4-dinitrophenylhydrazone of 2,3-dimethyl butyraldehyde, the cleavage product expected from ergosterol. The additional peak observed in the products from the amoeba sterols was identical in retention time (23.3 min) with the major product obtained from standard stigmasterol. The mass spectrum of this derivative had a parent peak at m/e 294 (and the loss of an isopropyl group), as expected for the 2,4-dinitrophenylhydrazone of 2-ethyl-3-methyl butyraldehyde (2-isopropyl butyraldehyde). This is the expected product upon cleavage of the side chain of 7-dehydrostigmasterol.

The presence of two double bonds in ring B leads to a variety of reaction products, and, therefore, the two compounds discussed above account for only about 10% of the combined area of the chromatographic peaks.

Concentration of the Sterols

Amoeboid cells contain 0.5 mg of ergosterol and 0.7 mg of 7-dehydrostigmasterol per 10^8 cells as measured by GLC. Encysted cells (16) contain 0.3 mg and 0.4 mg per 10^8 cells, respectively. The two sterols account for approximately 5% of the total lipids (15% of the neutral lipids) of amoeboid cells and 3% of the total lipid (6% of the neutral lipid) of encysted cells. Sterol esters were detected by thin-layer chromatography of the 1% ether eluate of the silicic acid column but these account for less than 2.5% of the total sterols as measured by the Liebermann–Burchard reaction.

DISCUSSION

7-Dehydrostigmasterol had previously been found only in certain bivalves (17). It is probably of wide occurrence

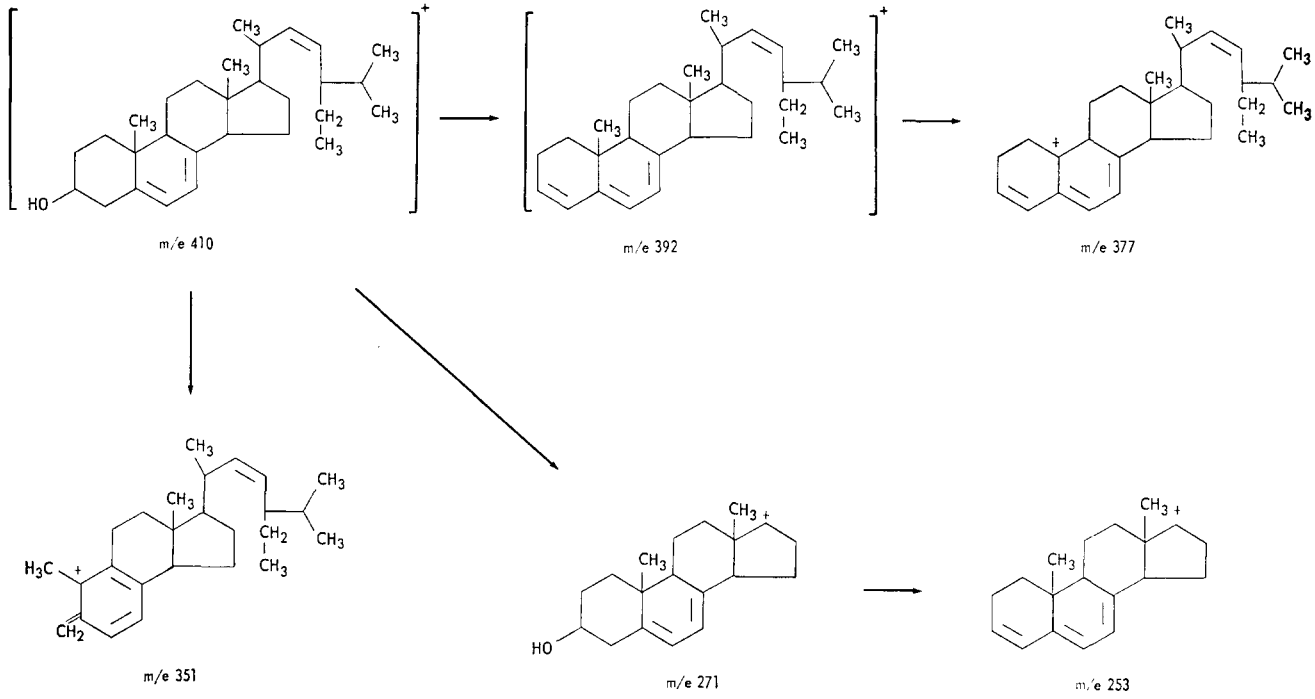

FIG. 4. Fragmentation scheme for 7-dehydrostigmasterol. The structure for the ion m/e 351 is one of several possibilities.

in protists, however, since a sterol with apparently identical retention time has been observed in the amoebae *Hartmannella rhysodes* and *Mayorella palestinensis* (18) and the phytoflagellates *Ochromonas danica* and *Euglena gracilis* (19), which also contain ergosterol. The cellular slime mold *Dictyostelium discoideum* synthesizes 22-stigmaster-3β-ol, a related compound in which the double bonds in the B ring are reduced (20).

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