Biochem. J. (1985) 231, 609–615 (Printed in Great Britain)

Sterol biosynthesis *de novo* via cycloartenol by the soil amoeba *Acanthamoeba polyphaga*

Daniel RAEDERSTORFF and Michel ROHMER*
École Nationale Supérieure de Chimie de Mulhouse, 3 rue Alfred Werner, 68093 Mulhouse Cedex, France

The soil amoeba *Acanthamoeba polyphaga* is capable of synthesizing its sterols *de novo* from acetate. The major sterols are ergosterol and poriferasta-5,7,22-trienol. Furthermore C_{28} and C_{29} sterols of still unknown structure with an aromatic B-ring are also synthesized by the amoeba. The first cyclic sterol precursor is cycloartenol, which is the sterol precursor in all photosynthetic phyla. No trace of lanosterol, which is the sterol precursor in animals and fungi, could be detected. These results show that at least some of the biochemical processes of *Acanthamoeba polyphaga* might be phylogenetically related to those of unicellular algae. Addition of exogenous sterols to the culture medium does not influence the sterol biosynthesis and the sterol composition of the cells.

INTRODUCTION

Sterols are membrane reinforcers in most eukaryotic cells (Demel & de Kruyff, 1976), but it has been recently postulated that other polyterpenes, such as hopanoids or carotenoids, might play a similar role, for instance in prokaryotes (Rohmer *et al.*, 1979, 1984; Ourisson & Rohmer, 1982). This assertion has already received experimental support from artificial biomembrane models (Poralla *et al.*, 1980; Bisseret *et al.*, 1983; Benz *et al.*, 1983; Kannenberg *et al.*, 1983) or biological systems (Kannenberg & Poralla, 1982; Poralla *et al.*, 1984). In order to understand better the role of these polyterpenoids, we decided to study the membrane reinforcers of protozoa. These lower eukaryotes are very interesting from this point of view, and many questions concerning their membrane reinforcers are up to now unanswered. Are they capable of sterol biosynthesis *de novo*? Do they synthesize other types of membrane reinforcers? Are they capable of finding suitable reinforcers in their diet, and do they modify them? The ciliate *Tetrahymena pyriformis* synthesizes tetrahymanol, a hopanoid-like triterpene, and incorporates it mainly in its cytoplasmic membrane (Thompson *et al.*, 1971). When *Tetrahymena* is grown in the presence of sterols, tetrahymanol synthesis is fully inhibited, and the sterol is found in the membranes after slight modifications (Conner *et al.*, 1968; Ferguson *et al.*, 1975). This was the first proof of biological equivalence between two different classes of triterpenoids. Another ciliate, *Paramecium tetraurelia*, requires stigmasterol (Conner *et al.*, 1971). In other protozoa, such as the soil amoeba *Acanthamoeba castellanii* (Smith & Korn, 1968; Ulsamer *et al.*, 1969) or in the trypanosomid *Crithidia fasciculata* (Halevy, 1966), several $\Delta^{5,7}$-sterols were detected. Very few protozoa have been in the meantime thoroughly studied, mostly because it is difficult to obtain and maintain axenic strains and to grow these microbes in the large quantities required for chemical and biochemical analysis.

Acanthamoeba polyphaga is a common small free-living soil amoeba. Some *Acanthamoeba* species have attracted many investigators because of the cosmopolitan distribution of this genus, and the recent recognition that several strains are pathogenic to humans and animals (Stevens & O'Dell, 1973; Culbertson, 1971).

In a preliminary communication we have reported that cycloartenol is the sterol precursor in *Acanthamoeba polyphaga* (Raederstorff & Rohmer, 1984*a*). We describe now the isolation and full identification of all detected tetracyclic metabolites involved in the sterol-biosynthetic pathway.

EXPERIMENTAL

Culture of *Acanthamoeba polyphaga*

Axenic *Acanthamoeba polyphaga* strain 7418 (Derr-Harf *et al.*, 1978) was grown at 30 °C for 9 days on a rotatory shaker (100 rev./min) in 2-litre Erlenmeyer flasks containing an enriched proteose/peptone medium (500 ml) (Weisman & Korn, 1966). Cells (average yield 0.15–0.40 g dry wt./litre) from a 10-litre culture were harvested at the end of their exponential growth phase by centrifugation (500 *g*), suspended in a phosphate buffer [350 ml, pH 6.7, containing, per litre of water: KH_2PO_4, 0.9 g; K_2HPO_4, 1 g; $(NH_4)_2SO_4$, 1 g; $MgSO_4,7\,H_2O$, 0.25 g] and incubated for 50 min at 30 °C on a rotatory shaker with sodium [1-^{14}C]acetate (total radioactivity: 1 mCi, specific activity: 54 Ci/mol).

Extraction and separation of sterols and sterols precursors

All analytical procedures and the separation scheme were as described previously (Raederstorff & Rohmer, 1984*b*; Neunlist *et al.*, 1985). Radioactive bands were detected on t.l.c. plates by using a Berthold LB 2832 linear detector. Radioactivity measurements were made on a Packard Prias liquid-scintillation counter fitted with an external standard allowing quenching correction and determination of the results in c.p.m. and d.p.m.

The freeze-dried cells (1–4 g) were extracted twice under reflux with chloroform/methanol (2:1, v/v). The combined extracts were evaporated to dryness *in vacuo* and hydrolysed for 1 h under reflux with methanolic 6%

* To whom correspondence and reprint requests should be sent.

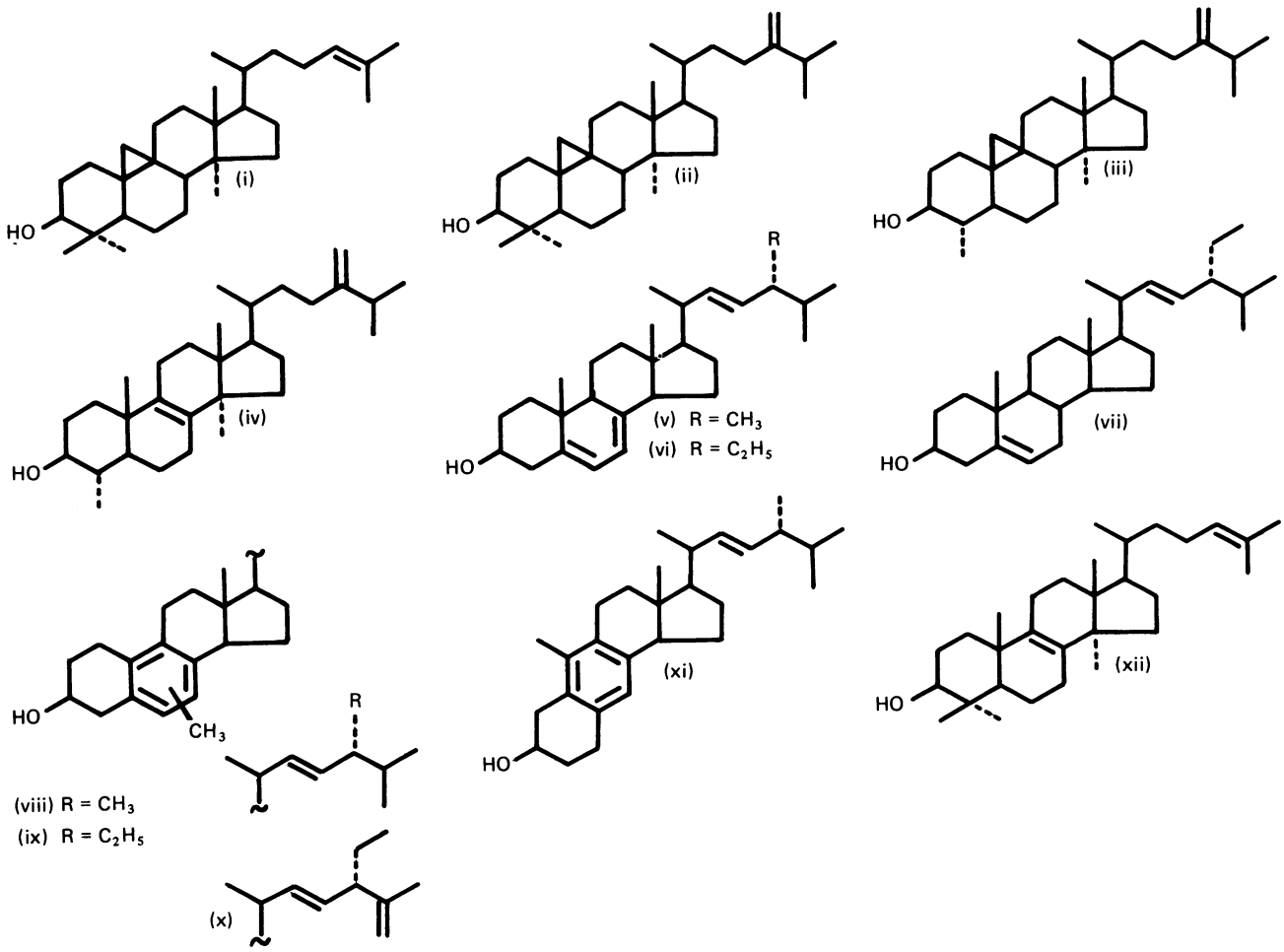

Fig. 1. Sterols and sterol precursors of *Acanthamoeba polyphaga*

(w/v) KOH (10 ml). After addition of water and hexane extraction, the non-saponifiable lipids were separated by t.l.c. with dichloromethane as solvent (two migrations) into 4,4-dimethylsterols (R_F 0.40), 4α-methylsterols (R_F 0.32), aromatic sterols (R_F 0.29) and sterols (R_F 0.22). All four fractions were separately acetylated, and their acetates were further purified by t.l.c. using cyclohexane/ethyl acetate (9:1, v/v; R_F 0.38) as eluent. The acetates of the three major sterols were separated one from another by t.l.c. (three migrations) on silica gel impregnated with $AgNO_3$ (Rohmer *et al.*, 1975), with cyclohexane/toluene (7:3, v/v), and gave the acetates of compound (VIII) (Fig. 1) and other minor sterols (R_F 0.54), a mixture of acetates of minor sterols (R_F 0.32) and a mixture of the acetates of compounds (V) and (VI) (Fig. 1) ($R_F = 0.16$). This last fraction was divided into three bands: the less polar one, containing the pure acetate of compound (VI), the most polar one the pure acetate of compound (V) and the middle one a mixture of both acetates. The sterol acetates from the fraction containing poriferasteryl acetate were further separated by t.l.c. on silica gel impregnated with $AgNO_3$, with ethanol-free chloroform as eluent, giving the pure acetate of compound (VII) (R_F 0.38) and minor sterol acetates.

The fraction containing the aromatic sterols was purified by t.l.c. with cyclohexane/ethyl acetate (9:1, v/v; two migrations) as eluent, giving aromatic sterols (R_F 0.14) and some 4α-methylsterols (R_F 0.20). Aromatic sterols were acetylated and further purified as described above. After t.l.c. on silica gel impregnated with $AgNO_3$ (cyclohexane/toluene, 7:3, v/v; three migrations), pure acetates of compound (IX) (R_F 0.30), compound (VIII) R_F 0.27) and compound (X) (R_F 0.10) were obtained.

Isolation and characterization of cycloartenol (I)

The acetates of the 4,4-dimethylsterols were separated by continuous t.l.c. on silica gel impregnated with $AgNO_3$ (Rohmer *et al.*, 1975), with cyclohexane/toluene (7:3, v/v) as eluent. Two fractions were obtained, corresponding to the acetates of cycloartenol (I) and/or lanosterol (XIII) (R_F 0.43) and 24-methylenecycloartanol (II) (R_F 0.21). In order to appreciate the radiochemical purity of cycloartenol (I) and to detect the possible presence of lanosterol (XII), cycloartenol and lanosterol were added as carrier to the chloroform/methanol extract. The separation procedure was carried out as described above and completed by one or both of the following procedures.

Procedure 1. After epoxidation with *p*-nitroperbenzoic acid, the monoepoxide of cycloartenyl acetate (R_F 0.51) was separated from the diepoxide of lanosteryl acetate (R_F 0.35) by t.l.c. (cyclohexane/ethyl acetate, 17:3, v/v) (Ponsinet & Ourisson, 1965). The biosynthetically

^{14}C-labelled cycloartenyl acetate monoepoxide was recrystallized from chloroform/dichloromethane to constant specific radioactivity (Table 3) after addition of ^{3}H-labelled cycloartenyl acetate monoepoxide synthesized by acetylation with [^{3}H]acetic anhydride and epoxidation of cycloartenol (Benveniste *et al.*, 1969).

Procedure 2. The acetates of cycloartenol (I) and lanosterol (XII) were separated by reverse-phase h.p.l.c. on a Nucleosil C_{18} column (30 mm × 3.9 mm) with methanol/water (94:6, v/v; 2 ml/min) as eluent and a Spectra Physics SP6040 refractometer as detector.

Physical properties of described products

P.m.r. spectra were recorded in (^{2}H)chloroform solution; chemical shifts are given in p.p.m. with reference to chloroform ($\delta = 7.265$). In mass-spectral data, relative abundance of ions above m/z 200 are given in parentheses.

Cycloartenol (I). Mass spectrum (g.l.c./m.s.) of the acetate of (I): m/z 468 (M^+,8), 453 (M^+ − Me,4), 408 (M^+ − AcOH,26), 393 (M^+ − AcOH − Me,23), 365 (10), 339 (9) 297 (8), 286 (ring-B fragmentation induced by cyclopropane ring, 14), 271 (8).

24-Methylenecycloartenol (II). Mass spectrum (g.l.c./m.s.) of the acetate of (II): m/z 482 (M^+,8), 467 (M^+ − Me,6), 439 (2), 422 (M^+ − AcOH,42), 407 (M^+ − AcOH − Me,31), 379 (15), 353 (8), 339 (4), 300 (ring-B fragmentation induced by cyclopropane ring, 16), 297 (15), 255 (5).

Cycloeucalenol (III). Mass spectrum (g.l.c./m.s.) of the acetate of (III): m/z 468 (M^+,6), 453 (M^+ − Me,8), 408 (M^+ − AcOH,64), 393 (M^+ − AcOH − Me,55), 365 (5), 353 (6), 300 (ring-B fragmentation induced by the cyclopropane ring, 9), 283 (19), 241 (8).

Obtusifoliol (IV). Mass spectrum (g.l.c./m.s.) of the acetate of (IV): m/z 468 (M^+,41), 453 (M^+ − Me,100), 425 (9), 408 (M^+ − AcOH,9), 393 (M^+ − AcOH − Me,61), 369 (11), 329 (4), 309 (18), 301 (10), 287 (28), 275 (13), 269 (9), 255 (8).

Ergosterol (V). P.m.r. (80 MHz) of the acetate of (V): δ (p.p.m.) 0.629 (3H,s,C-18), 0.828 (3H,d,*J* 7 Hz,C-27), 0.837 (3H,d,*J* 7 Hz,C-26), 0.915 (3H,d,*J* 7 Hz,C-28), 0.953 (3H,s,C-19), 1.035 (3H,d,*J* 7 Hz,C-21), 2.036 (3H,s,CH_3CO_2-), 4.68 (1H,m,3α-H), 5.25 (2H,m,22- and 23-H), 5.43 (1H,m,6-H), 5.60 (1H,m,7-H).

Mass spectrum of the acetate of (V): m/z 438 (M^+,9), 378 (M^+ − AcOH,100), 363 (M^+ − AcOH − Me,34), 337 (6), 253 (M^+ − AcOH − side chain,), 237 (8), 211 (10).

U.v. spectrum (in methanol): λ(nm) 293,282,271,263.

Ergost-8(14)-en-3β-yl acetate. Ergosteryl acetate (2 mg) dissolved in ethyl acetate (2 ml) containing 0.1 M-$HClO_4$ (120 μl) was stirred with Adam's catalyst under H_2 at room temperature and atmospheric pressure overnight. The catalyst was removed by filtration, and the hydrogenated sterol purified by t.l.c. and identified by p.m.r. (Rubinstein *et al.*, 1976). P.m.r. (200 MHz): δ(p.p.m.) 0.712 (3H,s,C-19), 0.787 (6H,d,*J* 7 Hz,C-27 and C-28), 0.844 (3H,s,C-18), 0.861 (3H,d,*J* 7 Hz,C-26), 0.939 (3H,d,*J* 7 Hz,C-21), 2.033 (3H,s,CH_3CO_2-), 4.72 (1H,m,3α-H).

Δ^7-Poriferasterol (VI). P.m.r. (80 MHz) spectrum of the acetate of (VI): δ (p.p.m.) 0.633 (3H,s,C-18), 0.794 (3H,d,*J* 6 Hz,C-27), 0.814 (3H,t,*J* 6 Hz,C-29), 0.851 (3H,d,*J* 6 Hz,C-26), 0.956 (3H,s,C-19), 1.048 (3H,d,*J* 7 Hz,C-21), 2.034 (3H,s,CH_3CO_2-), 4.69 (1H,m,3α-H), 5.10 (2H,m,22- and 23-H), 5.40 (1H,m,6-H), 5.55 (1H,m,7-H).

Mass spectrum (g.l.c./m.s.) of the acetate of (VI): m/z 452 (M^+,14), 392 (M^+ − AcOH,100), 377 (M^+ − AcOH − Me,37), 349 (3), 253 (M^+ − AcOH − side chain, 54), 237 (7), 211 (14).

U.v. spectrum (in methanol): λ (nm) 293,282,271,263.

Poriferast-8(14)-en-3β-yl acetate. Δ^7-Poriferasteryl acetate (2 mg) was hydrogenated in the same way as ergosteryl acetate, and the hydrogenation product identified by p.m.r. P.m.r. (200 MHz) spectrum: δ (p.p.m.) 0.712 (3H,s,C-19), 0.817 (3H,d,*J* 7 Hz,C-27), 0.835 (3H,d,*J* 7 Hz,C-26), 0.846 (3H,s,C-18), 0.861 (3H,t,*J* 7 Hz,C-29), 0.946 (3H,d,*J* 7 Hz,C-21), 2.033 (3H,s,CH_3CO_2-), 4.72 (1H,m,3α-H).

Poriferasterol (VII). P.m.r. (200 MHz) spectrum of the acetate of (VII): δ (p.p.m.) 0.695 (3H,s,C-18), 0.792 (3H,d,*J* 7 Hz,C-27), 0.809 (3H,t,*J* 7 Hz,C-29), 0.844 (3H,d,*J* 6 Hz,C-26), 1.021 (3H,s,C-19), 1.025 (3H,d,*J* 7 Hz,C-21), 2.038 (3H,s,CH_3CO_2-), 4.62 (1H,m,3 -H), 5.11 (2H,m,22- and 23-H), 5.36 (1 H,m,6-H).

Mass spectrum (g.l.c./m.s.) of the acetate of (VII): m/z 394 (M^+ − AcOH,100), 379 (M^+ − AcOH − Me,13), 351 (15), 281 (9), 255 (M^+ − AcOH − side chain, 47), 253 (19).

Aromatic sterol (VIII). P.m.r. (200 MHz) spectrum of the acetate of (VIII): δ (p.p.m.) 0.619 (3H,s,C-18) 0.841 (3H,d,*J* 6.5 Hz,C-27), 0.856 (3H,d,*J* 6.5 Hz,C-26), 0.939 (3H,d,*J* 6.5 Hz,C-28), 1.093 (3H,d,*J* 6.5 Hz,C-21), 2.080 (3H,s,CH_3CO_2-), 2.181 (3H,s,C-19 methyl group on aromatic ring), 2.68 (6H,m,benzylic protons), 3.01 (1H,dd,*J* 16.5 Hz, 5.5 Hz, 4α-H), 5.20 (1H,m,3α-H), 5.24 (2H,m,22- and 23-H), 6.74 (1H,s,aromatic proton).

Mass spectrum (g.l.c./m.s.) of the acetate of (VIII): m/z 376 (M^+ − AcOH,100), 374 (4), 251 (15), 235 (4), 224 (7), 209 (18), 197 (15), 195 (10), 157 (10), 155 (14).

U.v. spectrum (in methanol): λ (nm) 270; ($\epsilon = 1100$ litre·mol^{-1}·cm^{-1}).

Aromatic sterol (IX). P.m.r. (200 MHz) spectrum of the acetate of (IX): δ (p.p.m.) 0.625 (3H,s,C-18), 0.814 (3H,d,*J* 6.5 Hz,C-27), 0.843 (3H,t,*J* 7 Hz,C-29), 0.865 (3H,d,*J* 6.5 Hz,C-26), 1.107 (3H,d,*J* 6.5 Hz,C-21), 2.083 (3H,s,CH_3CO_2-), 2.184 (3H,s,C-19 methyl group on aromatic ring), 2.69 (6H,m,benzylic protons), 2.99 (1H,dd,*J* 16.5 Hz,5.5 Hz,benzylic 4α-H), 5.17 (3H,m,3α-,22- and 23-H), 6.74 (1H,s,aromatic proton).

Mass spectrum (g.l.c./m.s.) of the acetate of (IX): m/z 390 (M^+ − AcOH,100), 388 (6), 251 (15), 249 (4), 224 (12), 209 (19), 207 (4), 197 (13), 195 (12), 157 (10), 155 (15).

U.v. spectrum (in methanol): λ (nm) 270 ($\epsilon = 1100$ litre·mol^{-1}·cm^{-1}).

Table 1. Sterols and sterol precursors in *Acanthamoeba polyphaga*

Sterol	Relative amount (%, w/w)
4,4-Dimethylsterols	
Cycloartenol (I)	45
24-Methylenecycloartanol (II)	55
4α-Methylsterols	
Cycloeucalenol (III)	20
Obtusifoliol (IV)	60
Unidentified	20
Sterols	
Ergosterol (V)	42
Δ^7-Poriferasterol (VI)	48
Poriferasterol (VII)	5
Minor sterols	5
Aromatic sterols	
(VIII)	40
(IX)	50
(X)	5
Unidentified	5

Aromatic sterol (X). P.m.r. (200 MHz) spectrum of the acetate of (X): δ (p.p.m.) 0.616 (3H,s,C-18), 0.854 (3H,t,*J* 6.5 Hz,C-29), 1.090 (3H,d,*J* 6.5 Hz, C-21), 1.668 (3H,s,C-27), 2.079 (3H,s,CH_3CO_2-), 2.178 (3H,s,C-19 methyl group on aromatic ring), 2.68 (6H,m,benzylic protons), 2.98 (1H,dd,*J* 16.5 Hz,5.5 Hz,4α-H), 4.72 (2H,broad s,26-H), 5.20 (2H,m,22- and 23-H), 5.25 (2H,m,22- and 23-H), 6.74 (1H,s,aromatic protons).

Mass spectrum (g.l.c./m.s.) of the acetate of (X): *m/z* 388 (M^+ − AcOH,100), 366 (5), 278 (6), 251 (M^+ − side chain − AcOH,35), 249 (12), 224 (12), 209 (35), 207 (9).

1(10→6)*abeo*-Ergosta-5,7,9,33-tetraene (XI). This was synthesized as described by Bosworth *et al.* (1977). P.m.r. (200 MHz) spectrum of the acetate of (XI): δ (p.p.m.) 0.597 (3H,s,C-18), 0.838 (3H,d,*J* 6.5 Hz,C-27), 0.854 (3H,d,*J* 6.5 Hz,C-26), 0.937 (3H,d,*J* 7 Hz,C-28), 1.089 (3H,d,*J* 6.5 Hz,C-21), 2.059 (3H,s,CH_3CO_2-), 2.077 (3H,s,C-19 methyl group on aromatic ring), 2.72 (6H,m,benzylic protons), 3.03 (1H,dd,*J* 16.5 Hz,5.5 Hz,4-H), 5.23 (3H,m,3α-, 22- and 23-H), 6.66 (1H,s,aromatic proton).

U.v. spectrum (in methanol): λ (nm) 269 ($\epsilon = 500$ litre·mol^{-1}·cm^{-1}).

RESULTS

Sterols of *Acanthamoeba polyphaga*

Sterols (about 4–5 mg dry wt./g) were isolated from the non-saponifiable fraction of *A. polyphaga* without differentiation between free sterols and sterol esters. Three major sterols were isolated and identified as ergosterol (V), poriferasterol (VII) and 24β-ethylcholesta-5,7,22-trienol (VI) (Table 1). They are accompanied by several minor sterols, the structures of which have been tentatively assigned by g.l.c.–m.s. Many of them are accumulated when the cells are treated with sterol-biosynthesis inhibitors such as tridemorph and fenpropimorph (D. Raederstorff & M. Rohmer, unpublished work), but their full identification has yet to be reported.

The stereochemistry at C-24 of 24-alkylsterols is of interesting taxonomic value and was determined by p.m.r. spectroscopy for all three major sterols. The 24*R* (or 24β) configuration of acetate of poriferasterol (VII) was indicated by the chemical shifts of the signals of the C-21 and C-29 methyl groups appearing respectively as a doublet at 1.025 p.p.m. and a triplet at 0.808 p.p.m., whereas they appear at 1.017 and 0.799 in the spectrum of the 24*S* (or 24α) epimer (Rubinstein *et al.*, 1976; Chiu & Patterson, 1981). In the case of Δ^{22}-sterols it is difficult to determine unambiguously the C-24 configuration by p.m.r. spectroscopy. The acetates of (V) and (VI) were therefore hydrogenated and the configuration determined on the resulting $\Delta^{8(14)}$-sterols. The chemical shifts of the C-21 and C-27 methyl groups (0.939 and 0.787) of acetate of ergost-8(14)-enol resulting from the hydrogenation of acetate of (V) permitted a 24*S* (or 24β) assignment, those of the C-26 and C-29 methyl groups (0.835 and 0.861) of the acetate of stigast-8(14)-enol arising from the hydrogenations of acetate of (VI) permitted also a 24*S* (or 24β) assignment.

Sterol precursors

Only two 4,4-dimethylsterols, cycloartenol (I) and 24-methylenecycloartanol (II), could be detected by g.l.c.–m.s. and g.l.c. Their acetates were compared with authentic reference material, the mass spectra of the acetates of (I) and (II) showing the typical ring-B cleavage at *m/z* 286 or 300 induced by the cyclopropane ring (Audier *et al.*, 1966). No lanosterol or lanostenol could be detected, even by sensitive radiochemical methods (Tables 2 and 3).

The 4α-methylsterol fraction also contained only two major compounds, cycloeucalenol (III) and obtusifoliol (IV), identified by g.l.c. and g.l.c.–m.s. of their acetates. In this case also the mass spectrum of cycloeucalenyl acetate showed the typical fragmentation at *m/z* 300 of cyclopropylsterols (Audier *et al.*, 1966).

Aromatic sterols

A sterol fraction of unusual polarity was found between 4α-methylsterols and sterols. G.l.c.–m.s. showed immediately from the fragments corresponding to the loss of acetic acid (*m/z* 376 or 390) that the two major compounds were a C_{28} and a C_{29} sterol with four unsaturations. P.m.r. spectra of the acetates of (VII) and (X) showed a singlet corresponding to an aromatic proton ($\delta = 6.74$ p.p.m.) and a singlet corresponding to a methyl group on an aromatic ring ($\delta = 2.18$ p.p.m.). Furthermore the signals of seven benzylic protons were found at 2.69 p.p.m. (6H) and 3.01 p.p.m. (1H). Irradiation of the presumed 3α-H proton at 5.20 p.p.m. modified the signal of the benzylic proton at 3.01 p.p.m. into a doublet ($J = 16.5$ Hz), showing that the benzylic C-4α proton [the signal of which appears at lower field (3.01 p.p.m.) than the signal of the axial proton at C-4β (2.69 p.p.m.)] is only coupled with the C-4β proton with a large geminal coupling constant (16.5 Hz) and the axial C-3α proton with an axial/equatorial coupling constant (5.5 Hz). All these features are compatible with an aromatic B-ring. The chemical shift of the C-18 methyl group at 0.597 p.p.m. indicated a probable *trans* ring-C/D junction; in the case of *cis* junction the signal of this methyl group appears at 0.98 p.p.m. (Steele *et al.*, 1963). From the p.m.r. spectra it is also clear that the side-chain

Table 2. Sterol precursor and sterol labelling in *Acanthamoeba polyphaga* after incubation with [1-^{14}C]acetate (sp. radioactivity 54 Ci/mol)

Sterol or precursor	Radioactivity (d.p.m.)
Incubated [1-^{14}C]acetate*	2.23×10^9
4,4-Dimethylsterols	1.02×10^5
4α-Methylsterols	1.43×10^5
Sterols	1.02×10^6
Incubated [1-^{14}C]acetate*	8.8×10^8
4,4-Dimethylsteryl acetates	2.9×10^4
Lanosteryl and cycloartenyl acetates‡	2.2×10^4
Cycloartenyl acetate§	2.0×10^4
Lanosteryl acetate§	0
24-Methylenecycloartanyl acetate‡	5.3×10^3
Incubated [1-^{14}C]acetate†	8.8×10^8
Steryl acetates‡	1.4×10^5
Ergosteryl acetate‡	4.0×10^4
Δ^7-Poriferasteryl acetate‡	6.0×10^4
Poriferasteryl acetate‡	1.0×10^3
Aromatic sterols	3.5×10^4

* Labelling time 50 min.
† Labelling time 7 h.
‡ Isolated by t.l.c. on $AgNO_3$-impregnated silica gel.
§ Isolated by reverse-phase h.p.l.c.

structures of (VIII) and (IX) were identical with those of ergosterol (V) and Δ^7-poriferasterol (VI).

A third minor C_{29} aromatic sterol (X) possessing Δ^{22} and Δ^{25} double bonds in its side chain has been isolated. The signals of the C-21 methyl group (doublet at 1.090 p.p.m.), C-29 methyl group (triplet at 0.854 p.p.m.) and C-27 methyl group on double bond (singlet at 1.668 p.p.m.), the olefinic protons of the methylene group (broad singlet at 4.71 p.p.m.) and the two olefinic protons of the Δ^{22} double bond (multiplet at 5.25 p.p.m.) are in full accordance with the structure of a $\Delta^{22,25}$ side chain (Bolger *et al.*, 1970).

From all previous data two different structures with a phenanthrene (VIII)- or anthracene (XI)-derived skeleton are possible for the aromatic sterols (VIII), (IX) and (X) (Fig. 1). Direct comparison (by p.m.r., g.l.c. and u.v. spectroscopy) of the acetate of (VIII) isolated from *A. polyphaga* and the acetate of the anthrasterol (XI) synthesized by the method of Bosworth *et al.* (1977) showed that the two products are different. The structures already proposed by Korn *et al.* (1969) for (VIII) and (IX) on the basis of u.v. spectroscopy and low-field p.m.r. spectra of the total mixture are still in accordance with our high-field p.m.r. and g.l.c.–m.s. data obtained on the isolated compounds, but further work is required in order to determine their structures unambiguously.

Sterol biosynthesis

After incubation of *A. polyphaga* cells with [1-^{14}C]acetate, all polycyclic compounds involved in the sterol-biosynthetic pathway were labelled (Table 2), showing unambiguously that this amoeba is capable of sterol biosynthesis *de novo*. Furthermore, the labelling experiments confirmed the absence, in *A. polyphaga*, of lanosterol, which might have been present in amounts too low to be detected by g.l.c. because of a possible high turnover of a precursor. Indeed, after addition of carrier material, no significative radioactivity was detected in lanosteryl acetate isolated by reverse-phase h.p.l.c. or in the diepoxide of lanosteryl acetate, showing that no lanosterol was synthesized during the incubation (Table 3). Cycloartenyl acetate or its monoepoxide were, in contrast, labelled. Recrystallization to constant specific radioactivity of this monoepoxide showed clearly that the crystals retained all their radioactivity after the first recrystallization, and that the $^3H/^{14}C$ ratio remained constant in the crystals and the mother liquors during four recrystallizations (Table 3). We conclude that lanosterol is absent from *A. polyphaga* and that sterol biosynthesis follows the normal cycloartenol pathway of photosynthetic organisms (Benveniste *et al.*, 1966; Gibbons *et al.*, 1971; Goad, 1971).

DISCUSSION

Our labelling experiments have shown that *A. polyphaga* synthesizes its sterols *de novo*. Furthermore, this amoeba seems unable to utilize exogenous sterols. Indeed, the addition of cholesterol, stigmasterol or cyclolaudenol at a 25 μM concentration to the culture medium modified neither quantitatively nor qualitatively

Table 3. Recrystallization to constant specific radioactivity of the monoepoxide of cycloartenyl acetate and the diepoxide of lanosteryl acetate after incubation with [1-^{14}C]acetate in *Acanthamoeba polyphaga* (sp. radioactivity 54 Ci/mol, incubation time 50 min)

^3H-labelled carrier material was added to the ^{14}C-labelled biosynthetic mixture.

	^{14}C radioactivity (d.p.m)	$^3H/^{14}C$ ratio	
		Crystals	Mother liquor
Incubated [1-^{14}C]acetate	2.2×10^9		
4,4-Dimethylsteryl acetates	5.5×10^4		
Cycloartenyl and lanosteryl acetates	3.1×10^4	18.4	—
24-Methylenecycloartanyl acetate	1.1×10^4		
Diepoxide of lanosteryl acetate	0		
Monoepoxide of cycloartenyl acetate	2.4×10^4	18.6	—
First recrystallization	2.0×10^4	18.7	18.1
Second recrystallization	1.5×10^4	19.0	18.3
Third recrystallization	1.4×10^4	19.0	19.0
Fourth recrystallization	1.2×10^4	18.9	18.7

the sterol composition of the cells. This situation is completely different to that observed in ciliates: *Paramecium tetraurelia* utilizes stigmasterol from the culture medium (Conner *et al.*, 1971), and *Tetrahymena pyriformis* is capable of utilizing a variety of sterols after their conversion into $\Delta^{5,7,22}$-sterols (Mallory & Conner, 1970; Nes *et al.*, 1975) or even cycloartenol or cyclolaudenol, in this case without modification (D. Raederstorff & M. Rohmer, unpublished work). The presence in *A. polyphaga* of the $\Delta^{5,7,22}$-sterols (V) and (VI) as major sterols and of the curious aromatic sterols (VIII) and (IX) is in accordance with previous results obtained on *Acanthamoeba castellanii* and seems to indicate that these compounds are typical of this genus. The significance of these aromatic sterols is still unclear. As previously reported for *A. castellanii*, they appear in large amounts only in cell-free systems of *A. polyphaga*, the simultaneous disappearance of the $\Delta^{5,7}$-sterols indicating that the aromatic sterols probably arise from the modification of the major $\Delta^{5,7}$-sterols of the amoeba.

The presence of a plant biosynthetic pathway for the sterol biosynthesis in this protozoon and particularly the identification of cycloartenol as sterol precursor were fully unexpected. This implies the presence of at least three enzymic reactions that are typical of phytosterol biosynthesis: (i) the cyclization of squalene epoxide into cycloartenol (Goad, 1971; Heintz & Benveniste, 1970), (ii) the first methylation step occurring at the 4,4-dimethylsterol level and revealed by the presence of 24-methylenecycloartanol (II) (Wojciechowski *et al.*, 1973; Fonteneau *et al.*, 1977), and (iii) finally the isomerization of cycloeucalenol to obtusifoliol by a cyclopropane isomerase revealed by the presence of these two 4α-methylsterols (Heintz *et al.*, 1972; Heintz & Benveniste, 1974). From the point of view of sterol biosynthesis it is possible to divide all living eukaryotes into two categories: lanosterol is the sterol precursor in all non-photosynthetic phyla (animals and fungi), and cycloartenol in all photosynthetic phyla (plants and algae) (Goad, 1971, and references cited therein). Up to now no exceptions to this rule were known. Non-photosynthetic organisms synthesizing cycloartenol, such as *Astasia longa* for instance (Rohmer & Brandt, 1973), which is an apoplastide euglenophyte, could be in each case easily related to a photosynthetic phylum. The presence of a sterol metabolic pathway that is typical of plants or algae in a soil amoeba (Fig. 1) raises an interesting phylogenetic problem. Halevy & Finkelstein (1965) and Halevy *et al.* (1966) have already suggested, on the basis of biological and morphological criteria, that some soil amoebae might be derived from phytoflagellates. It is, for instance, known that some phytoflagellates have an amoeboid phase in their life cycle (Halevy & Finkelstein, 1965), whereas some amoebae like *Naegleria* have a flagellate phase (Kowit & Fulton, 1974). Furthermore, the stereochemistry at C-24 in the sterol side chains from *A. polyphaga* confirms a possible phylogenetic relation of this amoeba with some unicellular algae: this configuration is 24β, as in most algal sterols, but definitely different from the 24α configuration found in most sterols from higher plants (Nes, 1977).

In most classifications of protozoa, *Acanthamoeba* is placed in the same superclass, Rhizopoda (Levine *et al.*, 1980), as slime molds such as *Physarum polycephalum* and *Dictyostelium discoideum*, which synthesize their sterols from lanosterol (Lenfant *et al.*, 1970; Ellouz & Lenfant, 1969). Does this classification, adopted for the convenience of taxonomy, reflect phylogenetic relationship? Certainly not from the point of view of sterol biosynthesis; perhaps they are when one compares the homologies of 5S rRNA sequences: the amoeba *Acanthamoeba* and the true slime mold *Physarum* are by comparison of these sequences, relatively close, but both are only remotely related to the cellular slime mold *Dictyostelium* (Kumazaki *et al.*, 1983). This apparent contradiction needs further work on the biochemistry of protozoa in order to clarify the phylogenetic relationships of these microbes. Sterol biosynthesis might be a useful took for this purpose.

We thank Dr. C. Derr who supplied us with the *Acanthamoeba* strain and helped us to grow this micro-organism, Professor G. Ourisson for stimulating discussions, Dr. H. Strub and Mrs. E. Krempp for the p.m.r. measurements, and the Centre National de la Recherche Scientifique (Unité Associée 135) for financial support.

REFERENCES

Audier, H. E., Beugelmans, R. & Das, D. C. (1966) Tetrahedron Lett. 4631–4637
Benveniste, P., Hirth, L. & Ourisson, G. (1966) Phytochemistry **5**, 45–58
Benveniste, P., Hewlins, M. J. E. & Frittig, B. (1969) Eur. J. Biochem. **9**, 526–533
Benz, R., Hallman, D., Poralla, K. & Eibl, H. (1983) Chem. Phys. Lipids **34**, 7–24
Bisseret, P., Wolff, G., Albrecht, A.-M., Tanaka, T., Nakatani, Y. & Ourisson, G. (1983) Biochem. Biophys. Res. Commun. **110**, 320–324
Bolger, L. M., Rees, H. H., Ghisalberti, E. L., Goad, L. J. & Goodwin, T. W. (1970) Biochem. J. **118**, 197–200
Bosworth, N., Emke, A., Midgby, J. M., Moore, C. J., Whalley, W. S., Ferguson, G. & Marsh, W. C. (1977) J. Chem. Soc. Perkin I, 805–809
Chiu, P. L. & Patterson, G. W. (1981) Lipids **16**, 203–206
Conner, R. L., Landrey, J. R., Burns, C. H. & Mallory, F. B. (1968) J. Protozool. **15**, 600–605
Conner, R. L., Landrey, J. R., Kaneshiro, E. S. & van Wagtendonk, W. J. (1971) Biochim. Biophys. Acta **239**, 312–319
Culbertson, C. G. (1971) Annu. Rev. Microbiol. **25**, 231–254
Demel, R. A. & de Kruyff, B. (1976) Biochim. Biophys. Acta **457**, 109–132
Derr-Harf, C., Molet, B., Schreiber, J. & Kremer, M. (1978) Ann. Parasitol. Hum. Comp. **53**, 467–477
Ellouz, R. & Lenfant, M. (1969) Tetrahedron Lett. 609–612
Ferguson, K. A., Davis, F. M., Conner, R. L., Landrey, J. R. & Mallory, F. B. (1975) J. Biol. Chem. **250**, 6998–7005
Fonteneau, P., Hartmann-Bouillon, M. A. & Benveniste, P. (1977) Plant Sci. Lett. **10**, 147–155
Gibbons, G. F., Goad, L. J., Goodwin, T. W. & Nes, W. R. (1971) J. Biol. Chem. **246**, 3967–3976
Goad, L. J. (1971) Biochem. Soc. Symp. **29**, 45–77
Halevy, S. & Finkelstein, S. (1965) J. Protozool. **12**, 250–252
Halevy, S. (1966) Exp. Parasitol. **18**, 296–300
Halevy, S., Avivi, L. & Katan, H. (1966) J. Protozool. **13**, 480–483
Heintz, R. & Benveniste, P. (1970) Phytochemistry **9**, 1499–1503
Heintz, R., Benveniste, P. & Bimpson, T. (1972) Biochem. Biophys. Res. Commun. **46**, 766–772
Heintz, R. & Benveniste, P. (1974) J. Biol. Chem. **249**, 4267–4274
Kannenberg, E. & Poralla, K. (1982) Arch. Microbiol. **133**, 100–102
Kannenberg, E., Blume, A., McElhaney, R. N. & Poralla, K. (1983) Biochim. Biophys. Acta **733**, 111–116

Korn, E. D., Ulsamer, A. G., Weihing, R., Wetzel, M. G. & Wright, P. L. (1969) Biochim. Biophys. Acta **187**, 555–563
Kowit, J. D. & Fulton, C. (1974) J. Biol. Chem. **249**, 3638–3646
Kumazaki, T., Hori, H. & Osawa, S. (1983) J. Mol. Evol. **19**, 411–419
Lenfant, M., Lecompte, M. F. & Farugia, G. (1970) Phytochemistry **9**, 2529–2535
Levine, N. D., Corliss, J. O., Cox, F. E. G., Deroux, G., Grain, J., Honigberg, B. M., Leedale, G. F., Loeblich, A. R., Lorn, J., Lynn, D., Merinfeld, E. G., Page, F. C., Poljansky, G., Sprague, V., Vavra, J. & Wallace, F. G. (1980) J. Protozool. **27**, 37–58
Mallory, F. B. & Conner, R. L. (1970) Lipids **6**, 149–153
Nes, W. R., Alcaide, A., Landrey, J. R. & Conner, R. L. (1975) Lipids **10**, 140–144
Nes, W. R. (1977) Adv. Lipid Res. **15**, 233–324
Neunlist, S., Holst, O. & Rohmer, M. (1985) Eur. J. Biochem. **147**, 561–568
Ourisson, G. & Rohmer, M. (1982) Curr. Top. Membr. Transp. **17**, 153–182
Ponsinet, G. & Ourisson, G. (1965) Phytochemistry **4**, 799–811
Poralla, K., Kannenberg, E. & Blume, A. (1980) FEBS Lett. **113**, 107–110
Poralla, K., Härtner, T. & Kannenberg, E. (1984) FEMS Microbiol. Lett. **23**, 253–256

Received 14 May 1985/21 June 1985; accepted 1 July 1985

Raederstorff, D. & Rohmer, M. (1984*a*) C.R. Hebd. Seances Acad. Sci. **298II**, 631–634
Raederstorff, D. & Rohmer, M. (1984*b*) Phytochemistry **23**, 2835–2838
Rohmer, M. & Brandt, R. D. (1973) Eur. J. Biochem. **36**, 446–454
Rohmer, M., Ourisson, G., Benveniste, P. & Bimpson, T. (1975) Phytochemistry **14**, 727–730
Rohmer, M., Bouvier, P. & Ourisson, G. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 847–851
Rohmer, M., Bouvier-Navé, P. & Ourisson, G. (1984) J. Gen. Microbiol. **130**, 1137–1150
Rubinstein, I., Goad, L. J., Clague, A. D. H. & Mulheirn, L. J. (1976) Phytochemistry **15**, 195–200
Smith, F. R. & Korn, E. D. (1968) J. Lipid Res. **9**, 405–408
Steele, J. A., Cohen, L. A. & Mosettig, E. (1963) J. Am. Chem. Soc. **86**, 1134–1138
Stevens, A. R. & O'Dell, W. D. (1973) Proc. Soc. Exp. Biol. Med. **143**, 474–478
Thompson, A., Jr., Bamberry, J. R. & Nozawa, Y. (1971) Biochemistry **10**, 4441–4447
Ulsamer, A. G., Smith, F. R. & Korn, E. D. (1969) J. Cell Biol. **43**, 105–109
Weisman, R. A. & Korn, K. D. (1966) Biochim. Biophys. Acta **116**, 229–238
Wojciechowski, Z. A., Goad, L. J. & Goodwin, T. W. (1973) Biochem. J. **136**, 405–412