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Steroids from the Myxobacterium *Nannocystis exedens*

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Squalene and three sterols were isolated from *Nannocystis exedens* (Myxobacterales). The sterol content was about 0·4% of the dry weight; squalene content was in the same range. The main component was identified as cholest-8(9)-en-3β-ol. [^{14}C]Mevalonate was readily incorporated into the sterols. The bacterium was not inhibited by nystatin or amphotericin B.

INTRODUCTION

During an investigation into the carotenoids of *Nannocystis exedens*, a ubiquitous soil bacterium (Reichenbach, 1970), substantial amounts of steroid-like lipids were discovered. In addition, a substance with the retention time of squalene appeared in the gas-liquid chromatogram. As steroids are quite unusual in prokaryotes, we decided to isolate and characterize these compounds.

METHODS

Strains and culture conditions. *Nannocystis exedens* strain Na e1(= *N. exedens* HR 1 = German Collection of Microorganisms DSM 71; type strain: Reichenbach, 1970) was used for the chemical investigation. The organism was cultivated in 1 litre Erlenmeyer flasks containing 300 ml PL liquid medium [consisting of Probion LS 600 (single cell protein; Hoechst), 1%; $MgSO_4.7H_2O$, 0·1%; and $CaCl_2.2H_2O$, 0·1%; pH 7·0, autoclaved]. The flasks were shaken at 30 °C at 160 r.p.m. From 8·7 l of culture, 235 g of wet cells were obtained, corresponding to about 60 g dry wt. Alternatively the organism was grown in MD1 medium [consisting of Casitone (Difco), 0·3%; $CaCl_2.2H_2O$, 0·05%; $MgSO_4.7H_2O$, 0·2%; cyanocobalamine, 0·5 mg l^{-1}; and trace elements solution; pH 7·2, autoclaved]. Cell yields were much lower in this medium. The incorporation experiments were performed with *N. exedens* strain Na e158 (= *N. exedens* HR 2), isolated in 1980 from field soil collected near Cairo, Egypt.

Isolation of steroids. The wet cell mass (235 g) was extracted three times with 250 ml acetone, and the aqueous acetone solution was extracted three times with 200 ml toluene. After removal of the toluene under vacuum, there remained 1·1 g of crude material. GLC of an aliquot, with cholesterol as a standard, showed that this material contained about 220 mg of steroids. The crude material was dissolved in CH_2Cl_2/methanol (1:1, v/v) and chromatographed on a column (850 × 35 mm) of Sephadex LH-20 with CH_2Cl_2/methanol as the eluant. There were 90 fractions of 15 ml each, and steroids could be demonstrated by TLC in fractions 48 to 65 (total residue 300 mg). This material was chromatographed on a silica gel column (Lobar column size B, self-packed with LiChrosorb Si60 silica gel, 10 µm particles; Merck) with hexane/acetone (6:4, v/v) as the eluant. GLC of the 170 fractions (1 ml each) showed the steroids to be in fractions 51 to 86 (total residue 210 mg).

Analytical chromatography. For TLC, silica gel on aluminium sheets (Merck) was used, with acetone/hexane (35:65, v/v) as the solvent, the steroids having an R_f of 0·6. The acetylated steroids were chromatographed on silica gel (Kieselgel 60 HF_{254}; Merck) impregnated with 10% (w/w) $AgNO_3$ and activated by heating at 110 °C for 30 min; using toluene/ethyl acetate (9:1, v/v), the R_f values of the sterol acetates were 0·44, 0·52 and 0·58. For detection the plates were sprayed with a solution of 2% (w/w) $SbCl_5$ in $CHCl_3$ and heated to 110 °C.

GLC was performed on a Hewlett-Packard 5730 chromatograph equipped with a glass column (2 m × 4 mm) containing 3% (w/w) OV-17 on Chromosorb W HP, 80 to 100 mesh. The carrier gas was helium (20 ml min^{-1}), the temperature was either programmed from 250 to 300 °C (8 °C min^{-1}) or held at 300 °C, and detection was by

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thermal conductivity. Under isothermal conditions the retention time for squalene was 1·2 min, and that for the sterols (one broad peak) 3·6 min. Alternatively, a Siemens Sichromat 1 chromatograph was used, equipped either with a quartz capillary (25 m × 25 mm) containing SP 2100, or with a quartz capillary (25 m × 35 mm) containing OV-1701. The carrier gas was again helium (0·8 ml min^{-1}) at a temperature of 280 °C. A flame ionization detector and a 1 :80 inlet splitting ratio was used. Combined GLC–mass spectroscopy was done on a Perkin-Elmer F 22 chromatograph equipped with a quartz capillary (15 m × 0·25 mm) containing DB 1–15 N (= SE 30, from Chrompack). The carrier gas was helium (0·8 ml min^{-1}) and the temperature was programmed from 150 to 300 °C (6 °C min^{-1}). The chromatograph was connected via an open coupling to an AEI MS 30 mass spectrometer (ionization energy 24 eV; resolution $M/\Delta M$ = 1000, i.e. 10% valley; scanning interval 6 s).

Acetylation of steroids. The steroid fraction (200 mg) was dissolved in 5 ml pyridine and treated with 5 ml acetic anhydride for 12 h at room temperature. The dry residue was chromatographed on a silica gel column, as above, with hexane/diethyl ether (8 :2, v/v) as the eluant, yielding 180 mg of acetates. By slow crystallization from methanol, 30 mg of the main product was obtained from the mixture, with a melting point of 123–124 °C.

NMR spectroscopy. A Bruker WH 400 spectrometer was used to record un-decoupled ^{13}C spectra with C^2HCl_3 as the solvent and tetramethylsilane as the internal standard.

Labelling studies. Strain Na e158 was grown in 150 ml PL medium in a 500 ml conical flask at 30 °C with shaking (160 r.p.m.). Immediately after inoculation (at 10%, v/v), 15 μCi of *N,N′*-dibenzylethylenediamine-di-DL-[2-^{14}C]mevalonate [Amersham, specific activity 53 μCi $mmol^{-1}$ (1·96 MBq $mmol^{-1}$) or 26·5 mCi $mmol^{-1}$ (980·5 MBq $mmol^{-1}$) with respect to free mevalonic acid] was added. The dark red culture was harvested by centrifugation after 47 h; the yield of wet mass (5·1 g) was kept frozen at − 20 °C until the steroids were extracted with acetone. At the beginning and at the end of the incubation period, samples (0·2 ml) were taken and their radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer. The total radioactivity (medium plus cells) was initially 2·4 × 10^7 c.p.m. and remained constant till the end. At harvest time, 0·5 ml samples were pipetted into 5 ml ice-cold 14% (w/v) TCA. After standing for 45 min in an ice-bath, the precipitate was collected on glass microfibre filters (Whatman GF/B), washed once with 5 ml cold 5% TCA and three times with 5 ml water. Total radioactivity in TCA-insoluble material was 1·3 × 10^6 c.p.m., or about 5% of the added radioactivity. Incorporation of ^{14}C into the steroids was determined by HPLC on a prepacked Hibar EC column (125 × 4 mm) of LiChrosorb RP-18, 5 μm particles (Merck) with the solvent system methanol/diethyl ether/water (78·5 :17 :4·5, by vol.). The chromatograph (Waters) was equipped with a UV-detector (210 nm; Kontron Uvikon 725) and a ^{14}C-detector (Packard Tri-Carb RAM) with a 100 μl flow-through cell.

Inhibition studies. Strain Na e158 was grown in 50 ml of either PL or MD1 medium in shake cultures at 30 °C. Immediately after inoculation, varying amounts of nystatin (Mycostatin; Serva) or amphotericin B (Calbiochem), both dissolved in methanol (10 mg in 20 ml), were added.

RESULTS

Cells of the myxobacterium *N. exedens* strain Na e1 were extracted with acetone. The crude extract was purified by Sephadex LH-20 and silica-gel chromatography, and the resulting material analysed by GLC. The gas chromatogram showed a strong peak with the same retention time as authentic squalene, from which our compound did not separate upon co-chromatography. In addition, there was a major and a minor peak in the region of cholesterol; however, both peaks differed slightly from cholesterol in their retention times. Combined GLC–mass spectroscopy gave one molecular ion at m/z 386 for the major peak, and two molecular ions at m/z 386 and m/z 384 for the minor peak, the latter obviously containing two components which were not separated by GLC. The mass spectrum of the main component was very similar to that of cholesterol (Wulfson *et al.*, 1964). There were, however, differences in the relative intensities of several peaks. The fragmentation pattern was too uncharacteristic to allow a deduction of the chemical structure; still, it seemed likely that the myxobacterial compounds differed from cholesterol merely in the position and, in case of m/z 384, in the number of double bonds. The squalene peak had its molecular ion at m/z 410 as expected.

As we did not succeed in separating the three sterols on thin layers of silica gel impregnated with $AgNO_3$ (Ikan & Cudzinovski, 1965), the mixture was acetylated. With the acetates we obtained three spots on silica gel/$AgNO_3$ (Copius-Peereboom & Beeks, 1965), but they were too close together to allow easy separation on a preparative scale. On GLC the acetates appeared as three separate peaks in the quantitative ratio of 72:18:10 (Fig. 1). The acetylated main component was purified by crystallization from methanol. The ^{13}C-NMR of the acetate of the main compound had signals at δ 11·3 (q), 17·7 (q), 18·8 (q), 21·4 (q), 22·6 (q), 22·8 (q), 23·8 (t),

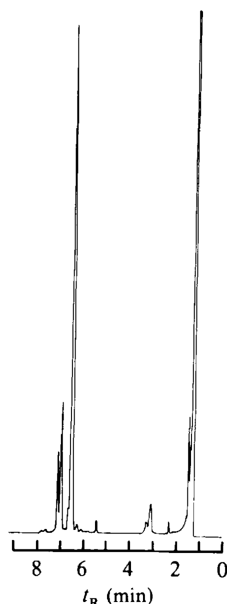

Fig. 1. Capillary GLC of the acetylated sterol fraction of *N. exedens*. The peaks of the three sterol acetates are between t_R 6·5 and 7·5. A sichromat with a quartz capillary (25 m × 0·35 mm) containing OV-1701 was used.

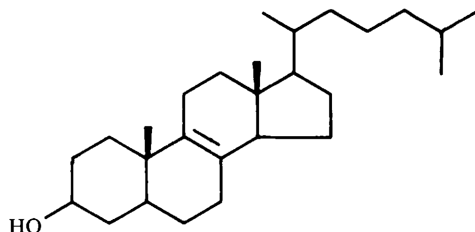

Fig. 2. Cholest-8(9)-en-3β-ol, the main sterol of *N. exedens*.

24·0 (t), 25·4 (t), 27·1 (t), 27·7 (t), 28·0 (d), 28·8 (t), 34·3 (t), 35·0 (t), 35·7 (s), 36·2 (t), 36·3 (d), 37·0 (t), 39·6 (t), 40·7 (d), 42·2 (s), 51·9 (d), 55·0 (d), 73·6 (d), 128·4 (s), 134·8 (s), 170·5 (s) (q = quartet, t = triplet, d = doublet, and s = singlet). This spectrum was identical with that of the acetate of cholest-8(9)-en-3β-ol (Tsuda & Schroepfer, 1979; Fig. 2). The melting point of our acetate (123 to 124 °C) was identical with published data (123·5 to 125 °C; Hylands *et al.*, 1977). The structure was also confirmed by X-ray analysis (Dr William S. Sheldrick, GBF, personal communication). The minor components were present in the mother liquor in too small quantities to allow an unequivocal structure elucidation. The quantities of steroid isolated from *N. exedens* were quite substantial, being about 0·4% of the total dry weight. The squalene content was in approximately the same range, but was found to vary considerably from experiment to experiment.

As steroids are ubiquitous in eukaryotes and may easily be introduced as impurities of medium components or in solvents, unequivocal proof for *de novo* synthesis of the isolated compounds by *N. exedens* was indispensable. Strain Na e1 was cultivated in two different media. Large-scale preparations were cultivated in PL medium because it permitted superior growth. The main component of this medium, Probion, is a single cell protein preparation produced by Hoechst from methanol-utilizing *Methylomonas clara* (Schlingmann *et al.*, 1982). Analysis of Probion LS 600 showed no trace of squalene or steroids; nor are steroids mentioned in the published lipid analyses of *M. clara* (Schlingmann *et al.*, 1982; Tiemeyer & Rambeck,

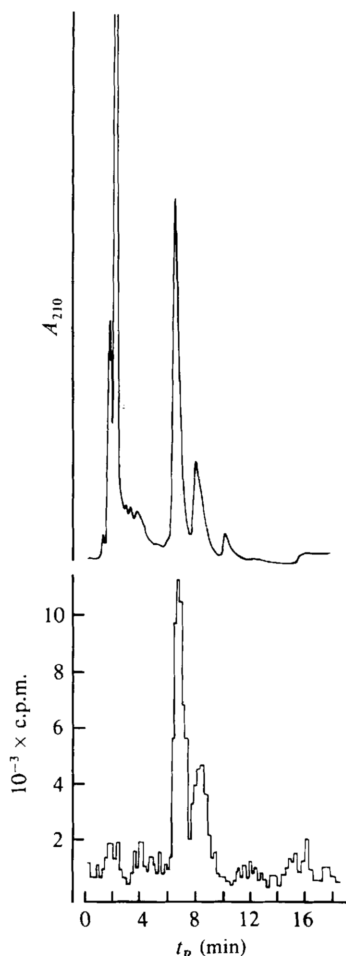

Fig. 3. HPLC of the sterol fraction of *N. exedens* grown in presence of [^{14}C]mevalonate. Upper trace: detection by UV absorbance with two sterol peaks at t_R 7 and 8. Lower trace: distribution of radioactivity along the same profile.

1982). Alternatively, Na e1 was cultivated in MD1 medium, based on steroid-free Casitone (Difco), giving a quantitatively different pattern of the same three steroids. When large quantities of acetone (Merck) were evaporated, the residue contained no steroids. To demonstrate *de novo* synthesis by use of tracer studies [^{14}C]mevalonate, the precursor of the carbon skeleton of squalene and the steroids, was added to cultures of strain Na e158, which has the same steroid pattern as Na e1. The radioactivity was incorporated efficiently into TCA-insoluble material (about 5% of the added radioactivity), and the three sterols became heavily and specifically labelled, as shown by analysis of the extract by HPLC combined with ^{14}C-detection (Fig. 3). The two sterol peaks contained more than 80% of the radioactivity of the sample.

The antifungal polyene antibiotics act by binding to membrane steroids (for a review, see Hamilton-Miller, 1974). To determine whether *N. exedens* was sensitive to these inhibitors, strain Na e158 was grown in two different media in the presence of either nystatin or amphotericin B at 5, 15 and 30 μg ml^{-1}. Only with amphotericin B at the exceedingly high concentration of 30 μg ml^{-1} was a slightly reduced pigmentation and a delay in culture development occasionally observed. When inocula of *N. exedens* on agar plates were dusted with nystatin or amphotericin B powder, the bacteria invariably grew out from between the

antibiotic particles, while growth of moulds under the same circumstances was completely inhibited. In fact we have used this method routinely to purify newly isolated *Nannocystis* strains from contaminating fungi.

DISCUSSION

Almost all eukaryotic cells contain steroids as constituents of their membranes, usually in the range of 25 to 30% of the total lipid (Razin, 1978). Filamentous fungi may contain between 0·1 and 4% of their dry matter as steroids, and yeasts occasionally have up to 10% (Weete, 1980). In contrast, steroids have been found in relatively few prokaryotes and then usually in very small quantities. Many mycoplasmas (genera *Mycoplasma* and *Spiroplasma*) grow only in presence of steroids, which they incorporate into their membranes (Smith & Lynn, 1958; Smith & Rothblat, 1962; Razin, 1978) and the same seems to be the case with several serum-dependent treponemas (Lemcke & Burrows, 1980). Steroids seem to be widely distributed among cyanobacteria, e.g. *Anacystis nidulans* and *Fremyella diplosiphon* (small quantities; Reitz & Hamilton, 1968), *Phormidium luridum* (0·01 to 0·02% of the dry weight; de Souza & Nes, 1968), *Spirulina maxima* (no quantities given; Martinez Nadal, 1971), and *Spirulina platensis*, *Calothrix* sp. and *Nostoc commune* (no quantities given; Paoletti *et al.*, 1976). Steroids have also been isolated from the new phototrophic prokaryote, *Prochloron* (no quantities given; Perry *et al.*, 1978). Steroids in trace amounts were detected in extracts from *Escherichia coli* (0·0004%; Schubert *et al.*, 1964) [a result not corroborated by other laboratories (Nes *et al.*, 1980)], in *Streptomyces olivaceus* (0·0035%; Schubert *et al.*, 1967) and in *Methylobacterium organophilum* (0·00003%; Patt & Hanson, 1978). Small to moderate quantities of steroids were found in *Azotobacter chroococcum* (0·01%; Schubert *et al.*, 1968), *Cellulomonas dehydrogenans* (0·03 to 0·05%; Weeks & Francesconi, 1978), and stable L-forms of *Staphylococcus aureus* (0·03 to 0·05%; Hayami *et al.*, 1979). A sterol has been found in *Corynebacterium simplex* grown on n-alkanes, but no data on structure or quantity are given (Yanagawa *et al.*, 1972). There is only one other bacterium that produces quantities of steroids comparable to those of *N. exedens* (0·4%), viz. *Methylococcus capsulatus* grown on methane (0·22%; Bird *et al.*, 1971).

The occurrence of squalene in prokaryotes is more common. The many bacteria that synthesize hopanoids (Rohmer *et al.*, 1979) must also contain squalene, the direct precursor for these triterpenes. Traces of squalene are reported from *Phormidium luridum* (de Souza & Nes, 1968), *Prochloron* (Perry *et al.*, 1978), *Rhodospirillum rubrum* and *Rhodomicrobium vannielii* (Han & Calvin, 1969; Bird *et al.*, 1971), and *Cellulomonas dehydrogenans* (0·002 to 0·005%; Weeks & Francesconi, 1978). More substantial quantities have been isolated from *Staphylococcus aureus* (0·01%, in addition to 0·08% dehydrosqualene; Suzue *et al.*, 1968), *Methylobacterium organophilum* (0·07%; Patt & Hanson, 1978), *Halobacterium cutirubrum* (0·15% including di- and tetrahydrosqualenes; Tornabene *et al.*, 1969; Kramer *et al.*, 1972), and *Methylococcus capsulatus* (0·55%; Bird *et al.*, 1971). *Nannocystis exedens* falls into the upper range (0·1 to 0·3%).

The determination of the structure of bacterial steroids has not always been unequivocal because of the small amounts available. In almost all cases more than one steroid was present, and without exception they were 3β-sterols. The cyanobacteria and *Prochloron* contained cholesterol and C24-methylated and C24-ethylated sterols and homologues with variations of double bond number and position. From *Methylococcus capsulatus*, 4-methyl and 4,4-dimethyl cholestenols with various combinations of double bonds in the 8(9) and 24 positions were isolated. *Methylobacterium organophilum* contained 4,4,14-trimethyl-cholesta-8(9),22,24-trienol, *Cellulomonas dehydrogenans* contained cholesterol and β-sitosterol, *Staphylococcus* L-forms and *Streptomyces olivaceus* contained cholesterol, and *Azotobacter chroococcum* contained several ergestenols and C4-methylated sterols. *Nannocystis exedens* with its cholest-8(9)-en-3β-ol fits well into this picture. Obviously, prokaryotes do not conform to the distinction between zoo-, phyto- and mycosterols (Weete, 1980).

De novo synthesis of squalene and steroids by prokaryotes has been demonstrated in several cases, usually by incorporation studies. Suzue *et al.* (1968), working with a *Staphylococcus in vitro* system, obtained [^{14}C]squalene from [^{14}C]farnesyl pyrophosphate. *Cellulomonas dehydrogenans*

incorporated radioactivity from [^{14}C]glucose into squalene and sterols (Weeks & Francesconi, 1978), stable L-forms of *Staphylococcus aureus* incorporated [^{14}C]acetate into cholesterol (Hayami *et al.*, 1979), and *Halobacterium cutirubrum* incorporated [^{14}C]acetate into squalene (Tornabene *et al.*, 1969). Squalene cyclase was isolated from *Methylococcus capsulatus* and studied *in vitro* (Rohmer *et al.*, 1979). On the other hand, Reitz & Hamilton (1968) could not achieve the incorporation of [^{14}C]acetate into steroids by cyanobacteria, although CO_2, phytol and fatty acids became labelled. In our case, the sterols became intensively labelled when [^{14}C]mevalonate was added to the culture, leaving no doubt about their origin.

The bacterial steroids, like those of eukaryotes, are probably located in the cellular membranes, although direct experimental evidence is lacking in most cases. Cholesterol-dependent and cholesterol-independent mycoplasmas incorporate exogenous cholesterol exclusively into their membranes (Smith & Rothblat, 1962; Bittman & Rottem, 1976; Razin, 1978). Gram-positive and Gram-negative bacteria may possibly behave like *Acholeplasma* and incorporate exogenous cholesterol into their membranes (the Gram-negative organisms preferentially into their cytoplasmic membranes) (Smith & Rothblat, 1962; Razin, 1975). Polyene antibiotics, like nystatin, amphotericin and filipin, act by binding to membrane sterols, and thus are usually non-toxic for prokaryotes. However, *Mycoplasma* is inhibited, and *Acholeplasma* becomes sensitive when grown in presence of cholesterol (for review, see Hamilton-Miller, 1974). Interestingly, *N. exedens* is totally resistant to nystatin and amphotericin B. Also several cyanobacteria were completely resistant to amphotericin A, and only *Phormidium* responded to high concentrations of nystatin (Hunter & McVeigh, 1961). These observations apparently do not support the idea of sterols as essential membrane components in these bacteria; however, it is possible that the antibiotics are simply kept from the cytoplasmic membrane by the barrier of the (sterol-free?) outer membrane.

Steroids seem to be present in most, perhaps all, *Nannocystis* strains. It remains to be seen whether different steroids are synthesized by different strains, and whether there are steroid producers among other myxobacteria and gliding bacteria.

Probion was a gift from the Hoechst company. Dr D. Claus, DSM, provided the soil sample from which strain Na e158 was isolated. The GLC–mass spectra and ^{13}C-NMR spectra were recorded by the Spectroscopy Department of the GBF. Miss Susanne Petersen, Miss Bettina Evert and Miss Barbara Witte provided careful technical assistance. We wish to thank all these persons for their cooperation.

REFERENCES

Bird, C. W., Lynch, J. M., Pirt, F. J., Reid, W. W., Brooks, C. J. W. & Middleditch, B. S. (1971). Steroids and squalene in *Methylococcus capsulatus* grown on methane. *Nature, London* **230**, 473–474.

Bittman, R. & Rottem, S. (1976). Distribution of cholesterol between the outer and inner halves of the lipid bilayer of *Mycoplasma* cell membranes. *Biochemical and Biophysical Research Communications* **71**, 318–324.

Copius-Peereboom, J. W. & Beeks, W. (1965). The analysis of mixtures of animal and vegetable fats. V. Separation of sterol acetates by thin-layer chromatography in reversed-phase systems and on silica gel-G silver nitrate layers. *Journal of Chromatography* **17**, 99–113.

Hamilton-Miller, J. M. T. (1974). Fungal sterols and the mode of action of the polyene antibiotics. *Advances in Applied Microbiology* **17**, 109–134.

Han, J. & Calvin, M. (1969). Hydrocarbon distribution of algae and bacteria, and microbiological activity in sediments. *Proceedings of the National Academy of Sciences of the United States of America* **64**, 436–443.

Hayami, M., Okabe, A., Sasai, K., Hayashi, H. & Kanemasa, Y. (1979). Presence and synthesis of cholesterol in stable staphylococcal L-forms. *Journal of Bacteriology* **140**, 859–863.

Hunter, E. O. & McVeigh, I. (1961). The effects of selected antibiotics on pure cultures of algae. *American Journal of Botany* **48**, 179–185.

Hylands, P. J., Midgley, J. M., Smith, C., Wallis, A. F. A. & Whalley, W. B. (1977). Unsaturated steroids. Part 5. Synthesis of 4α-methyl-cholest-8(9)-en-3β-ol. *Journal of the Chemical Society. Perkin Transactions I*, 817–819.

Ikan, R. & Cudzinovski, M. (1965). Separation of sterols and corresponding stanols on thin layers of silica impregnated with silver nitrate. *Journal of Chromatography* **18**, 422–423.

Kramer, J. K. G., Kushwaha, S. C. & Kates, M. (1972). Structure determination of the squalene, dihydrosqualene and tetrahydrosqualene in *Halobacterium cutirubrum*. *Biochimica et biophysica acta* **270**, 103–110.

Lemcke, R. M. & Burrows, M. R. (1980). Sterol requirement for the growth of *Treponema hyodysenteriae*. *Journal of General Microbiology* **116**, 539–543.

MARTINEZ NADAL, N. G. (1971). Sterols of *Spirulina maxima*. *Phytochemistry* **10**, 2537–2538.

NES, W. R., ADLER, J. H., FRASINEL, C., NES, W. D., YOUNG, M. & JOSEPH, J. M. (1980). The independence of photosynthesis and aerobiosis from sterol biosynthesis in bacteria. *Phytochemistry* **19**, 1439–1443.

PAOLETTI, C., PUSHPARAJ, B., FLORENZANO, G., CAPELLA, P. & LERCKER, G. (1976). Unsaponifiable matter of green and blue-green algal lipids as a factor of biochemical differentiation of their biomasses. II. Terpenic alcohol and sterol fractions. *Lipids* **11**, 266–271.

PATT, T. E. & HANSON, R. S. (1978). Intracytoplasmic membrane, phospholipid, and sterol content of *Methylobacterium organophilum* cells grown under different conditions. *Journal of Bacteriology* **134**, 636–644.

PERRY, G. J., GILLAN, F. T. & JOHNS, R. B. (1978). Lipid composition of a prochlorophyte. *Journal of Phycology* **14**, 369–371.

RAZIN, S. (1975). Cholesterol incorporation into bacterial membranes. *Journal of Bacteriology* **124**, 570–572.

RAZIN, S. (1978). The mycoplasmas. *Microbiological Reviews* **42**, 414–470.

REICHENBACH, H. (1970). *Nannocystis exedens* gen. nov., spec. nov., a new myxobacterium of the family Sorangiaceae. *Archiv für Mikrobiologie* **70**, 119–138.

REITZ, R. C. & HAMILTON, J. G. (1968). The isolation and identification of two sterols from two species of blue-green algae. *Comparative Biochemistry and Physiology* **25**, 401–416.

ROHMER, M., BOUVIER, P. & OURISSON, G. (1979). Molecular evolution of biomembranes: structural equivalents and phylogenetic precursors of sterols. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 847–851.

SCHLINGMANN, M., FAUST, U. & PRÄVE, P. (1982). Aufarbeitung und Isolierung funktioneller Bioproteine. In *Mikrobielle Proteingewinnung und Biotechnologie*, pp. 117–124. Edited by P. Präve, K. Schügerl & H. Zucker. Weinheim: Verlag Chemie.

SCHUBERT, K., ROSE, G., TUMMLER, R. & IKEKAWA, N. (1964). Sterine in *Escherichia coli*. *Hoppe Seyler's Zeitschrift für physiologische Chemie* **339**, 293–296.

SCHUBERT, K., ROSE, G. & HÖRHOLD, C. (1967). Cholesterin in *Streptomyces olivaceus*. *Biochimica et biophysica acta* **137**, 168–171.

SCHUBERT, K., ROSE, G., WACHTEL, H., HÖRHOLD, C. & IKEKAWA, N. (1968). Zum Vorkommen von Sterinen in Bakterien. *European Journal of Biochemistry* **5**, 246–251.

SMITH, P. F. & LYNN, R. J. (1958). Lipid requirements for the growth of pleuropneumonia-like organisms. *Journal of Bacteriology* **76**, 264–269.

SMITH, P. F. & ROTHBLAT, G. H. (1962). Comparison of lipid composition of pleuropneumonia-like and L-type organisms. *Journal of Bacteriology* **83**, 500–506.

DE SOUZA, N. J. & NES, W. R. (1968). Sterols: isolation from a blue-green alga. *Science* **162**, 363.

SUZUE, G., TSUKADA, K., NAKAI, C. & TANAKA, S. (1968). Presence of squalene in *Staphylococcus*. *Archives of Biochemistry and Biophysics* **123**, 644.

TIEMEYER, W. & RAMBECK, W. (1982). Analytische Charakterisierung von nicht-proteinartigen Begleitstoffen in einem SCP (*Methylomonas clara*). In *Mikrobielle Proteingewinnung und Biotechnologie*, pp. 183–188. Edited by P. Präve, K. Schügerl & H. Zucker. Weinheim: Verlag Chemie.

TORNABENE, T. G., KATES, M., GELPI, E. & ORO, J. (1969). Occurrence of squalene, di- and tetrahydrosqualenes, and vitamin MK_8 in an extremely halophilic bacterium, *Halobacterium cutirubrum*. *Journal of Lipid Research* **10**, 294–303.

TSUDA, M. & SCHROEPFER, G. J. (1979). Carbon-13 nuclear magnetic resonance studies of C_{27} sterol precursors of cholesterol. *Journal of Organic Chemistry* **44**, 1290–1293.

WEEKS, O. B. & FRANCESCONI, M. D. (1978). Occurrence of squalene and sterols in *Cellulomonas dehydrogenans* (Arnaudi 1942) comb. nov. Hester 1971. *Journal of Bacteriology* **136**, 614–624.

WEETE, J. D. (1980). *Lipid Biochemistry of Fungi and Other Organisms*. New York: Plenum Press.

WULFSON, N. S., ZARETSKII, V. J., ZAIKIN, V. G., SEGAL, G. M., TORGOV, I. V. & FRADKINA, T. P. (1964). Mass spectrometric location of the double bond in steroid systems. *Tetrahedron Letters*, 3015–3022.

YANAGAWA, S., FUJII, K., TANAKA, A. & FUKUI, S. (1972). Lipid composition and localization of 10-methyl branched-chain fatty acids in *Corynebacterium simplex* grown on n-alkanes. *Agricultural and Biological Chemistry* **36**, 2123–2128.